EFFECT OF COCOA POWDER ON BIOMARKERS OF INFLAMMATION AND SOME BIOCHEMICAL PARAMETERS IN POLOXAMER 407 INDUCED HYPERLIPIDAEMIC MALE WISTAR RATS

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NOVEMBER, 2021

DECLARATION

I declare that the work in this dissertation entitled: "Effect of Cocoa Powder on Biomarkers of Inflammation and some Biochemical Parameters of Poloxamer 407 Induced Hyperlipidaemia in Male Wistar Rats" has been performed by me in the Department of Human Physiology. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

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CERTIFICATION

The dissertation entitled "EFFECT OF COCOA POWDER ON BIOMARKERS OF INFLAMMATION AND SOME BIOCHEMICAL PARAMETERS OF POLOXAMER 407 INDUCED HYPERLIPIDAEMIA IN MALE WISTAR RATS" by Lami DANMALLAM meets the regulations governing the award of the degree of Master of Science in Human Physiology of the Ahmadu Bello University Zaria, and is approved for its' contribution to knowledge and literacy presentation

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DEDICATION

This work is dedicated to my parents, Late Mr John Danmallam and Mrs Eunice John Danmallam

ABSTRACT

Cardiovascular diseases are the leading cause of death globally. Hyperlipidaemia and inflammation usually lead to cardiovascular diseases. A substance that can reduce hyperlipidaemia and inflammation will in turn reduce cardiovascular diseases. The aim of the study is to evaluate the effect of cocoa powder on inflammatory biomarkers and some biochemical parameters in poloxamer 407 induced hyperlipidaemic male Wistar rats. Thirty male Wistar rats divided into 6 groups with 5 rats in each group were used for the study. The rats were divided into: control (Iml distilled water), poloxamer 407 group (500mg/kg), poloxamer 407+ atorvastatin group (20mg/kg), poloxamer 407 + 250mg/kg of cocoa powder, poloxamer 407 + 500mg/kg of cocoa powder and poloxamer 407 + 1000mg/kg of cocoa powder. Poloxamer 407 was administered to induce hyperlipidaemia in the rats. At the end of the experiment, the serum was collected to assay for lipid profile (total cholesterol, triglycerides, low density lipoprotein and high density lipoprotein), biomarkers of lipid peroxidation (malondialdehyde) and oxidative stress (superoxide dismutase, glutathione peroxidase and catalase activity). Heart homogenate was used to assay for biomarkers of inflammation (tumour necrosis factor-α and interleukin-10) and coronary arteries were harvested and used for histology. Cocoa powder significantly (p<0.05) lowered serum levels of total cholesterol in P407+ATV (1043.82±1.49 mg/dL), P407+250 CP (269.79 ±12.05 mg/dL), P407+500 CP (256.69 ± 7.12 mg/dL) and P407+1000 CP (151.83 ± 12.79 mg/dL) when compared to P407 (1551.67 ± 129.05 mg/dL). Cocoa powder significantly (p<0.05) lowered serum levels of triglycerides in P407+ATV (52.70 ± 7.86mg/dL), P407+250 CP (213.51 ± 19.51 mg/dL), P407+500 CP (159.62 ± 15.25 mg/dL) and P407+1000 CP (55.93 ± 2.89mg/dL) when compared to P407 (294.66 ± 54.73 mg/dL). Cocoa powder significantly (p<0.05) lowered serum levels of low density lipoprotein in P407+ATV (132.44 ± 18.72

mg/dL), P407+250 CP (227.90 ± 15.17 mg/dL), P407+500 CP (165.92 ± 18.68 mg/dL) and P407+1000 CP (153.17 ± 4.12 mg/dL) when compared to P407 (1124 ± 22.07). Cocoa powder significantly (p<0.05) lowered serum levels of malondialdehyde in P407+500 CP (28.08 ± 2.71 nmol/L) and P407+1000 CP (25.92 ± 2.28 nmol/L) when compared to P407 (45.38 ± 4.90 nmol/L). Cocoa powder significantly increased serum activities of superoxide dismutase P407+ATV (27.04 ± 1.84 U/ml), P407+250 CP (30.04 ± 3.64 U/ml), P407+500 CP (30.96 ± 3.75 U/ml) and P407+1000 CP (40.08 ± 2.75 U/ml) when compared to P407 (15.10 ± 0.84 U/ml). Cocoa powder significantly increased serum activity of glutathione peroxidase in P407+500 CP (31.70 ± 2.46nmol/min/ml) and P407+1000 CP (32.90 ± 1.43 nmol/min/ml) when compared to P407 (23.40 ± 1.63 nmol/min/ml). Interleukin-10 level in heart homogenate were significantly (p<0.05) higher in P407+1000 (919.88 ± 57.99 pg/ml) when compared to P407+ATV (656± 23.50 pg/ml). Histological examination showed that cocoa powder decreased adipocyte infiltration. In conclusion, cocoa power had a significant effect on hyperlipidaemia, oxidative stress and atherosclerosis. Further study should be carried to determine the effect of the individual components of cocoa powder

Table of Contents

Title page i
Declaration iv
Certification v
Acknowledgements vi
Dedication vii
List of Figures xiii
List of Tables xiv
List of Plates xv
Abbrevations xvi
Abstract viii

1.0 INTRODUCTION 1
1.1 Statement of Research Problem 3
1.2 Justification of Study 4
1.3 Aim and Objectives 4
1.4 Research Hypothesis 6

2.0 LITERATURE REVIEW 7
2.1 Cocoa Overview 7
2.2 Health Effects of Cocoa 12
2.2.1 Antioxidant potential 12
2.2.2 Cardioprotection 13
2.2.3 Chemo-protection 17
2.2.4 Neuroprotection 18
2.3 Poplyphenols 19
2.4 Hyperlipidaemia 23
2.4.1 Atherosclerosis as a risk factor 23
2.4.2 Classification of hyperlipidaemia 24
2.4.3 Cocoa and lipid profile 25
2.5 Oxidative Stress 25
2.5.1 Free radicals 29
2.5.2 Oxidative stress and CVDs 31

2.5.3 Cocoa powder and oxidative stress 32

2.6 Inflammation 32
2.6.1 Mechanism of inflammation 33
2.6.2 Cocoa powder and inflammation 37
2.7 Poloxamer 407 and hyperlipidaemia..37

3.0 MATERIALS AND METHODS 39
3.1 Chemicals/Materials 39
3.2 Toxicity Study...39
3.3 Methodology 40
3.3.1 Proximate analysis of cocoa powder..40
3.3.2 Drug preparation 42
3.3.3 Ethical Approval 42
3.3.4 Experimental Animals 43
3.3.5 Induction of Hyperlipidaemia 43
3.3.6 Experimental Design..43
3.3.7 Collection and Preparation of Serum Samples for Biochemical Assays 44
3.3.8 Determination of Lipid Profile..45
3.3.9 Estimation of biomarkers of oxidative stress 46
3.3.10 Estimation of biomarkers of inflammation 49
3.3.11 Histology of the coronary artery 50
3.4 Statistical analysis...51

4.0 RESULTS 52
4.1 Proximate Analysis of Acute Toxicity Test of Cocoa Powder 52
4.2 Lipid Profile...55
4.3 Oxidative Stress Parameters...63
4.4 Biomarkers of inflammation...65
4.5 Result of cocoa powder on histopathological changes of coronary arteries 69

5.0 DISCUSSION 81

6.0 CONCLUSION AND RECOMMENDATION 86
6.1 Conclusion 86
6.2 Recommendation.. 87
6.3 Contributions to knowledge 87
REFERENCES.. 89
APPENDIX... 108

List of Figures

Figure 2.1: **Theobroma cacao (cocoa tree) 9**

Figure 2.2; **Cocoa pods containing cocoa beans...10**

Figure2.3; **Cocoa powder 11**

Figure 2.4; **Classes of phytochemicals 20**

Figure 2.5; **General concept of oxidative stress 27**

Figure 2.6; **Oxidative stress drivers and metabolic consequences on adipose tissue 28**

Figure 2.7; **Different reactive oxygen species sources 30**

Figure 2.8; **Mechanism of inflammation 36**

Figure 4.1; **Effect of cocoa powder on serum total cholesterol..................................56**

Figure 4.2; **Effect of cocoa powder on serum triglyceride..58**

Figure 4.3; **Effect of cocoa powder on serum Low density lipoprotein.....................60**

Figure 4.4; **Effect of cocoa powder on serum high density lipoprotein.....................62**

Figure 4.5; **Effect of cocoa powder on serum tumour necrosis factor-α....................66**

Figure 4.6; **Effect of cocoa powder on serum interleukin-10.....................................68**

List of Tables

Table 4.1; Proximate analysis of cocoa powder 53

Table 4.2; LD_{50} (lethal dose) of cocoa powder..54

Table 4:3; Effect of cocoa powder on serum biomarkers of oxidative stress and lipid peroxidation 64

List of Plates

Plate I: Photomicrograph of the coronary artery of the normal control 70

Plate II: Photomicrograph of the coronary artery of negative control 72

Plate III: Photomicrograph of the coronary artery of the group induced with hyperlipidaemia and treated with 20 mg/kg of atorvastatin 74

Plate IV: Photomicrograph of the coronary artery of the group induced with hyperlipidaemia and treated with 250 mg/kg of cocoa powder..........................76

Plate V: Photomicrograph of the coronary artery of the group induced with hyperlipidaemia and treated with 500 mg/kg of cocoa powder..........................78

Plate VI: Photomicrograph of the coronary artery of the group induced with hyperlipidaemia and treated with 1000mg/kg of cocoa powder..80

Abbreviations

ABTS; (2,2-azino-bis (3-ethylbenzthiazoline-6-sulphoric acid))

ADP; Adenosine diphosphate

Ag; Antigen

AKT; Protein kinase B

AMPK; 5' adenosine monophosphate-activated protein kinase

ANOVA; Analysis of variance

AOAC; Association of official agricultural chemists

ATP; Automated tissue processor

ATV; Atorvastatin

B cells; Bursa cells

cAMP/PKA; Cyclic adenosine monophosphate/protein kinase-A

CAT; Catalase

CD36; Cluster of differentiation-36

CP; Cocoa powder

CPE; Cocoa powder extract

Cu^{+}; Copper ion

CVDs; Cardiovascular diseases

CYP74AI; Cholesterol 7 alpha-hydroxylase

DAMP; Damage-associated molecular patterns

DC; Dendritic cells

DNA; Deoxyribonucleic acid

DPPH; 2,2-diphenyl-1-picrylhydrazyl

DPX; Distyrene plasticizer xylene

ELISA; Enzyme-linked immunosorbent assay

eNOS; Endothelial nitric oxide

ERK; Extracellular signal regulated kinase

Fe^{2+}; Iron ion

GPx; Glutathione peroxidase

H&E; Hematoxylin and eosin

H_2O_2; Hydrogen peroxide

HDL; High density lipoprotein

HNO_3; Nitrous acid

HO-1; Heme oxygenase

HO_2; Hydroperoxyl

HoFH; Heterogygous familial hypercholesterolaemia

HoFH; Homogygous familial hypercholesrolaemia

HRP; Horseradish peroxidase

ICAM-1; Intercellular adhesion molecule

IFN-γ; Interferon-gamma

IKB; Inhibitor of nuclear factor kappa B

IKK; Inhibitor of kappa B kinase

IL-10; Interleukin-10

LDL; Low density lipoprotein

MAPK; Mitogen-activated protein kinase

MCP-1; monocyte chemoattractant protein-1

MDA; Malondialdehyde

MEK; Methyl ethyl ketone

MHC; Major histocompatibility complex

mRNA; Messenger ribonucleic acid

My88; Myeloid differentiation primary response 88

M_φ; Macrophage

N_2O_3; Dinitrogen trioxide

N_2O_4; Dinitrogen tetroxide

NADPH; Nicotinamide adenine dinucleotide phosphate

NC; Normal control

NF-KB; Nuclear factor kappa-B

NLR; Neutrophil-lymphocyte ratio

NO; Nitric oxide

NO_2; Nitrogendioxide

NO_2Cl; Nitryl chloride

NOS; Nitric oxide synthase

NOX2; Nicotinamide adenine dinucleotide phosphate oxidase-2

Nrf2; Nuclear factor-erythroid factor 2-related factor 2

O_2-; Superoxide

OH^-; hydroxyl

OHdG; 8, Hydroxy-2'-deoxyguanosine

ONOO; Peroxynitrite

ONOOH; Peroxynitrous acid

oxLDL; Oxidized LDL

P13K; Phosphatidylinositol 3-kinase

P407; Poloxamer 407

PAMP; Pathogen-associated molecular patterns

Q3GA; Quercetin-3-glucuronide

RAW 264.7; Ralph, Raschke and Watson cells

RNA; Ribonucleic acid

RNS; Reactive nitrogen species

RO; Alkoxyl

RO_2; Peroxyl

ROONO; alkyl peroxynitrite

ROS; Reactive oxygen species

SABC; Strept avidin biotin-peroxidase complex

SEM; Standard error of mean

SIRT-1; Sirtuin-1

SOD; Superoxide dismutase

SR-A; Scavenger receptor-A

T cells; Thymus cells

TBARS; Thiobarbituric acid reactive substances

TC; Total cholesterol

TCR; T-cell receptor

TG; Triglycerids

TGF; Transforming growth factor-beta

TH0; T-helper type 0

TLRs; Toll-like receptors

TMB; Tetramethylbenzidine

TNF-α; Tumour necrosis factor-α

Treg; Regulatory T-cell

VCAM-1; Vascular cell adhesion molecule 1

CHAPTER ONE

1.0 INTRODUCTION

Cardiovascular disease is the leading cause of death globally (Mc Namara *et al*., 2019). Atherosclerosis, the underlying syndrome of cardiovascular disease, is a major pathological condition involving lipid metabolism, inflammation, innate and adaptive immunity (Davinelli *et al*., 2018). Clinical studies have demonstrated that atherosclerosis may generate series of cardiovascular events including coronary heart disease and stroke (Brian *et al*., 2017;Libby *et al*., 2018)).

Hyperlipidaemia, an abnormally high level of any or all lipids or lipoproteins in the blood is becoming a major health problem in the world (Mao *et al*., 2013). Hyperlipidaemia is divided into primary and secondary hyperlipidaemia. Primary hyperlipidaemia is usually due to genetic causes, such as mutation in a receptor protein, while secondary hyperlipidaemia is caused by an underlying cause like diabetes, nephritic syndrome, chronic alcoholism and hypothyroidism (Leila *et al*., 2018). Cigarette smoking, obesity, high-fat diet and an inactive lifestyle can lead to hyperlipidaemia (Sameer *et al*., 2011). In hyperlipidaemia, the lipoproteins deposit in the walls of arteries arising from aorta, restricting the blood supply to the heart. This phenomenon is known as atherosclerosis. Higher deposition of lipoproteins completely blocks the blood supply to the heart, thus leading to myocardial infarction (Niharika, 2017). Hyperlipidaemia is related to increased oxidative stress, thus causing significant production of oxygen free radicals which may lead to oxidative modifications in low density lipoproteins that in turn leads to the initiation and progression of atherosclerosis and associated cardiovascular diseases (Guo *et al*., 2019).

Atherogenesis begins when excess low density lipoproteins accumulate in sub-endothelial space and are oxidatively modified, and are taken up selectively by macrophages and monocytes (Ryan *et al*., 2016). Atherosclerosis has two fundamental hallmarks: lipid accumulation and inflammation. One of the most important causes for the initiation and progression of atherosclerosis is a persistent increase in circulating low density lipoprotein levels. In this respect, macrophages play a very important role by increasing accumulation of lipids in blood vessels, leading to inflammation and plaque formation (Zuliani *et al*., 2013). Low density lipoprotein accumulates within the intimal space and subsequently undergoes modifications such as oxidation, thus converting low density lipoprotein to oxidized low density lipoprotein, which acts as a major immunogen. Modified low density lipoprotein serves as a chemo-attractant for monocytes and macrophages. Low density lipoprotein oxidation appears to be a fundamental event in the development of atherosclerotic lesion and in the initiation of the inflammatory cascade. Intracellular inflammatory pathways play a major role in the development of atherosclerosis (Papapanagiotou *et al*., 2015), with different transcription factors involved including nuclear factor-kappa B (Welty, 2013), activator protein 1 (Lue *et al*., 2011) and early growth response (Albrecht *et al*., 2010).

Oxidative stress is a risk factor of cardiovascular disease, at physiological level, reactive oxygen species function as signalling molecules that regulate a wide range of processes in the cardiovascular system and they also contribute to the maintenance of cardiovascular homeostasis (Pignatelli *et al*., 2018). However, excessive reactive oxygen species generation plays a pivotal role in the genesis, progression and clinical consequences of cardiovascular diseases (Gábor *et al*., 2014). Research has shown that cocoa powder has nearly twice the antioxidant properties of red wine, and up to three times the antioxidants found in green tea (Ottavani *et al*., 2002). Cocoa powder also contains many minerals, including magnesium,

iron, chromium, vitamin C and zinc (Lee *et al*., 2003). Cocoa powder is an unsweetened powder which is obtained by grinding cocoa beans and pressing out the butter resulting in cocoa powder with low-fat content. The source of the cocoa bean is the cocoa tree, *Theobroma cacao* (Miller *et al*., 2008). Cocoa powder is used to make chocolate, chocolate syrup and chocolate confectionaries (Ismara *et al*., 2017). Cocoa powder is rich in polyphenols, such as catechin, quercetin (including its glucoside), clovamide, deoxyclovamide and procyanidin (Carrie, 2017). Some epidemiologic studies show that high intake of cocoa powder confers a benefit on cardiovascular outcome (Matsumoto, 2018). Cocoa powder is able to control blood pressure positively by the antioxidant present in cocoa which promotes the production of nitric oxide (Gonzàlez-Barrio *et al*., 2020). This substance is essential in relaxing blood vessels so that the heart is not forced to work too hard. Cocoa powder has been shown to have the ability to prevent potential fatal blood clot forming and causing stroke or heart attacks (Kade *et al*, 2015).

1.1 Statement of Research Problem

In the United States atherosclerotic cardiovascular disease is the leading cause of death (Johnson *et al*., 2014). Furthermore, approximately 28.5 million American adults have total cholesterol levels that exceed 240mg/dl, placing them at high risk of atherosclerotic cardiovascular disease (Virani *et al*., 2020). The prevalence of hypertension among urban and rural dwellers was 30.6% and 24.6% respectively. It has been projected that by 2030, 39.1 million cases of hypertension will be recorded among people aged at least 20 years in Nigeria (Adeloye *et al*., 2015). Hyperlipidaemia is a major risk factor for cardiovascular disease (ischaemic heart disease and stroke), which is a leading cause of death in the world. It accounts for more than 17.3 million deaths per year, a number expected to grow to more than

23.6 million by 2030 (Niharika, 2017). In the south-westhern part of Nigeria, there is 23.1% prevalent rate of cardiovascular diseases (Akinbade *et al*., 2020).

The prevalence of hypercholesterolaemia in Nigeria is 38% with prevalence in women slightly higher (42%) compared with men (38%). The prevalence is highest in the south-south and lowest in the south-west and north-east (Adeloye *et al*., 2020).

1.2 Justification of Study

Nigeria is a producer of cocoa, yet there is some level of ignorance about the numerous health benefits of cocoa powder. Many people are bedevilled by debilitating cardiovascular diseases. The need for a natural product that can be used in the management of these diseases cannot be overemphasized. Atorvastatin is a drug for hyperlipidaemia and it has numerous side effects, hence the need for research into natural hyperlipidaemic substances cannot be overemphasized

Cocoa powder supplementation might be a regimen for healthy living, hence the need for in-depth and diverse studies into its effects and mechanism of action. It is important to evaluate whether cocoa powder has a positive effect on lipid levels and oxidative stress which has been implicated in cardiovascular diseases.

1.3 Aim and Objectives

1.3.1 Aim

The aim of this study was to evaluate the effect of cocoa powder on lipid profile, some biomarkers of inflammation and biomarkers of oxidative stress in poloxamer 407 induced hyperlipidaemic male Wistar rats

1.3.2 Specific objectives

Specific objectives of the study were to evaluate the following parameters in male Wistar rats:

1. The effect of cocoa powder on lipid profile

i. Total cholesterol (TC)

ii Triglycerides (TG)

iii Low density lipoprotein (LDL)

iv. High density lipoprotein (HDL)

2. The effect of cocoa powder on biomarkers of oxidative stress

i. Malondialdehyde (MDA) concentration

ii. Superoxide dismutase (SOD) activity

iii. Glutathione peroxidase (GPx) activity

iv. Catalase activity

3. The effect of cocoa powder on biomarker of inflammation

i. Tumour necrosis factor-α

ii Interleukin-10

4. Evaluation of histology of coronary artery

1.4 Research Hypothesis

Cocoa powder has no effect on biomarkers of inflammation and some biochemical parameters in poloxamer 407 induced hyperlipidaemic male Wistar rats

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Cocoa Overview

The cocoa bean is the dried and fully fermented seed of *Theobroma cacao* and it contains the cocoa solids which is a mixture of non-fat substance and cocoa butter (the fat). Cocoa beans are used to produce chocolate. The diploid tropical fruit crop species (2n=2x=20), *Theobroma cacao*, is an economically important agricultural commodity for millions of individuals worldwide. The scientific name means food for the gods in greek. The crop is cultivated by about 6 million farmers globally, and the livelihood of more than 40 million people depend on cocoa (Beg *et al*., 2017). Cocoa originated from the Amazonian basin and it is presently cultivated in many regions of the humid tropics (Nieves-Orduña*et al*., 2021). It is common in West Africa and Ivory Coast is the largest producer of cocoa.

One of the major cash crops in Nigeria is cocoa powder (Tenkap and Balogun, 2020). Cocoa and its derivatives are major dietary sources of antioxidants due to their high phenolic content (Oracz and Żyżelewicz, 2020). Numerous dietary intervention studies in humans and animals indicate that the flavonol-rich foods and beverages exert cardio-protective effects with respect to vascular function and platelet reactivity (Carl *et al*., 2005). An epidemiological long term study reported that cocoa has lowering effect on cardiovascular mortality in elderly men (Buijsse *et al*., 2006) and postmenopausal women (Mink *et al*., 2007). Flavonol- rich cocoa consumption has been reported to improve endothelial function (Sun *et al*., 2019) and reduce incidence of atherosclerotic diseases (McCullough *et al*., 2006). Polyphenols are products of plants' secondary metabolism, they are not required for plant development and growth but are essential for plant communication and defence (Iriti and Faoro, 2009).

Flavonoids are commonly found in edible plants and are diphenylpropanes, which include family members such as flavones, isoflavones, anthocyanins, flavanols, chalcones, catechins. Polyphenolic flavonoids have the ability to scavenge superoxide, hydroxyl and peroxyl free radicals and are therefore potent antioxidants (Guven*et al*., 2019).

There are three main varieties of cocoa, forastero, criollo and trinitario. Forastero is the most frequently used variety, comprising 80% to 90% of the world cocoa production. Forastero has high disease resistance and high yielding nature compared to criollo and is cheaper (Rusconi and Conti, 2010). Criollo is rarer and considered a delicacy due to their superior flavour. They represent 5% to 10% of world cocoa production. They have increased susceptibility to pest and disease and low vigour (Rusconi and Conti, 2010). Trinitario is a hybrid produced from crosses between criollo and forastero varieties.

Figure 2.1; Cocoa tree (Theobroma cacao) showing its fruits (Ronse and Louis, 2010)

Figure 2.2; Cocoa pods containing cocoa beans (name; cacao, family; malvaceae) (Elsie, 2013)

Figure 2.3; Cocoa powder (Elsie, 2013)

2.2 Health Benefits of Cocoa

The polyphenols are the major contributors to the numerous health benefits of cocoa (Zzaman *et al*., 2014 ; Bellesia and Tagliazucchi, 2014). Cocoa seed is a rich source of polyphenolic antioxidants and it has been documented that cocoa-based products supply a greater proportion of the dietary intake of phenolic antioxidants than do other sources of antioxidants like berries, soya beans, green tea and wine (Lee *et al*., 2003). All the antioxidant properties of cocoa, especially the high flavonoid content, are now of great interest to humans due to its profound effect on health. Interestingly, the claim that cocoa polyphenols could prevent cancer or slow down the progression of cancer has received increased attention (Martin *et al*., 2013). Also flavonoids extracted from cocoa have been shown to play a pivotal role in mediating innate and acquired immunity (Ramiro-Puig and Castell, 2009) and also have been shown to effect diet induced obesity and insulin resistance (Dorenkott *et al*., 2014). Emerging reports support the claim that cocoa flavonoids may serve as cardio-protective agents. It has been speculated that these compounds modulate mediators of inflammation (Keen *et al*., 2005). Cocoa flavonoid and procyanidins (Liu *et al*., 2015; Bowser *et al*., 2017) have been reported to decrease platelet aggregation via increasing concentration of epicatechin and catechin in the plasma (Keen *et al*., 2005; Murphy *et al*., 2003).

Cocoa, due to its high polyphenolic content, has attracted increased attention from nutritional and pharmacological view points. Cocoa powder shows promising antioxidant, cardio-protective, neuro-protective and chemo-protective potentials (Andújar *et al*., 2012).

2.2.1 Antioxidant potential

The polyphenols contained in cocoa powder have strong antioxidant properties. The antioxidant capacity of cocoa beans per serving is higher than that of red wine or black or

green tea. (Jolić *et al*., 2011), and the total polyphenolic and flavonoid content is about 611mg gallic acid equivalents and 564mg epicatechin equivalents respectively (Jahurul *et al*., 2013). *In vitro* experimental studies have shown that cocoa polyphenols scavenge reactive oxygen species, such as, (2,2-azino-bis (3-ethylbenzthiazoline-6-sulphoric acid, ABTS)), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and superoxide radicals, hypochlorite and peroxynitrate anions, also inhibits lipid peroxidation and chelate free pro-oxidant metal irons (Fe^{2+} ,Cu^{+}) (Andújar *et al*., 2012). Cocoa flavanols inhibit ultraviolent C-induced oxidative DNA damage, being as potent as vitamin C, α-tocopherol and glutathione in this function. A decrease of DNA and glutathione oxidation has been reported in rats with chronic supplementation of diet with 2% cocoa. In humans, it has been reported that flavanol-rich cocoa products increase the total plasma antioxidant capacity and dose-dependently reduce the concentration of plasma thiobarbituric acid reactive substances (Keen *et al*., 2005). It was reported in a randomized crossover study in obese adults at risk of insulin resistance, that the consumption of cocoa beverages providing an average dose of 400mg flavanols per day for 5 days, lowered the levels of total 8-isoprostanes, markers of *in vivo* lipid peroxidation and the plasma concentration of C-reactive protein, a biomarker of inflammation (Stote *et al*., 2012). Conversely, other authors did not detect changes in biomarkers of oxidative stress in healthy individuals (Arranz *et al*., 2013).

2.2.2 Cardio-protection

Many epidemiological studies have shown that several cardiac disease conditions like stroke, heart failure, coronary artery disease and cardiovascular disease-related mortality have reduced due to intake of cocoa powder (Crozier and Hurst, 2014). It was reported cocoa intake is associated with a 45% to 50% lower risk of cardiovascular death in prospective analysis (Buijsse *et al*., 2006). The cardio-protective capacity of cocoa has been attributed to

an improvement in metabolic and anti-inflammatory effects, modulation of blood pressure, inhibition of platelet activation and aggregation, endothelial function and antioxidant status (Kothe *et al*., 2013).

2.2.2.1 Effect on blood pressure

Several studies carried out on spontaneously hypertensive rats have shown that single oral administration of cocoa powder with high content of procyanidins decreases blood pressure. Also, long term soluble cocoa fibre intake was shown to reduce the development of hypertension in spontaneously hypertensive rats (Cienfuegos-Jovellanos *et al*., 2009; Sánchez *et al*., 2010). Several epidemiological studies (intervention duration varies from 2 weeks to 18 weeks and daily intake of flavonoids ranges from 30mg to 1000mg), have shown that cocoa intake has antihypertensive effects, lowering systolic, diastolic and mean blood pressure in normotensive subjects. Cocoa also has antihypertensive effect in prehypertensive and hypertensive patients (Arranz *et al*., 2013 ; Grassi and Ferri, 2014). The consumption of cocoa-based beverages with high flavanol concentration attenuates the blood pressure response to exercise. A meta-analysis of 10 randomized controlled trials has shown that the short term consumption of polyphenol-rich cocoa products is linked to statistically significant reductions of systolic (-4.5mmHg) and diastolic (-2.5mmHg) blood pressure (Desch *et al*., 2010). In hypertensive individuals, such values may be considered to be of clinical relevance in the context in which it has been estimated that a reduction of 5mmHg in systolic blood pressure has been shown to decrease the risk of cardiovascular disease by approximately 20% over 5 years (Grassi and Ferri, 2014).

The mechanism of action of cocoa which gives it its antihypertensive property is by the improvement in nitric oxide (NO) bioavailability, a molecule which plays a vital role in the

maintenance of vascular homeostasis. In an experiment it was reported that cocoa procyanidins could stimulate endothelial-dependent vasodilation mediated through activation of NO synthase (NOS) and subsequent NO production (Karim *et al*., 2000).

2.2.2.2 Effect on endothelial function

Endothelial dysfunction is directly linked with early stages of atherosclerosis and other cardiovascular diseases (CVDs) (Allgrove and Davison, 2014). It involves the ability to regulate vascular tone and alterations in the endothelium-derived regulatory mediators (Keen *et al*., 2005). Cocoa powder intake improves the flow-mediated dilatation of the brachial artery, a clinical marker of endothelial function (Crozier and Hurst, 2014). It has been reported that ingestion of cocoa flavanols leads to a dose-dependent increase in flow-mediated dilatation, a dose of 616 mg of flavanols causes half-maximal flow mediated dilatation response (Allgrove and Davison, 2014). Flavanols ameliorate the endothelial dysfunction by modulating the endothelial cell eicosanoid system pathway and lowering the oxidative damage (Keen *et al*., 2005). Flavonoids inhibit 5-lipoxygenase, decrease the level of vasoconstrictor leukotrienes and increase the level of vasodilating prostacyclins.

2.2.2.3 Metabolic effect

The oxidation of LDL is a vital step in the development of atherosclerosis, because oxidized LDL become more susceptible to be up taken by the arterial wall-resident macrophages. Studies have shown that the administration of polyphenol-rich cocoa powder increases the resistance of LDL to oxidation and reduces atherosclerotic plaque formation in hypercholesterolemic rabbits (Kurosawa *et al*., 2005). Experiments carried out on human subjects have shown that daily intake of cocoa powder (≥ 13g/d) reduces LDL levels and LDL's susceptibility to oxidation and increases HDL levels (Allgrove and Davison, 2014;

Grassi and Ferri, 2014). Also, cocoa consumption ameliorates lipid profile in hypercholesterolemic humans and dark chocolate intake reduces the serum LDL in hypertensive patients (Arranz *et al*., 2013). Despite the fact that cocoa is rich in fats content, it does not seem to have undesirable effect on lipid metabolism (Grassi and Ferri, 2014). The effect that cocoa has on LDL oxidation has been attributed to the free radical scavenging properties, metal ion chelating ability, or changes in the LDL surface that promote a reduction in LDL oxidative susceptibility (Allgrove and Davison, 2014).

2.2.2.4 Anti-inflammatory effect

Chronic inflammation is strongly associated with atherosclerosis. Several studies have shown that cocoa polyphenols may modulate cytokines involved in the inflammation response. It has been reported that moderate intake of products is linked with anti-inflammatory effect, thus the subjects who consumed 20 g of dark chocolate every days had lower serum C-reactive protein than the non-consumers or high consumers (Allgrove and Davison, 2014). Cocoa exhibits anti-inflammatory effects via reduction of the secretion of the pro-inflammatory cytokines and increase in production of the anti-inflammatory cytokines. Catechins have the ability to suppress the endothelial production of IL-8, a cytokine which plays an important role in the onset and development of atherosclerosis. Cocoa B-type procyanidins and catechins can inhibit the nuclear factor kappa-B (NF-KB)-driven gene transcription. Apart from the regulation of the expression of the gene involved in inflammation, cell proliferation and survival, NF-KB regulates gene transcription of cytokines involved in atherosclerosis progression (Keen *et al*., 2005).

2.2.2.5 Antiplatelet activity

Platelet dysfunction is implicated in the development of peripheral arterial occlusion, stroke, acute myocardial infarction and other atherosclerotic diseases. *In vivo* and *in vitro* studies have shown that cocoa polyphenol possess antithrombotic effects, decreasing platelet activation, aggregation and adhesion. The intake of flavanol-rich cocoa beverages is associated with a significant anti-platelet activity which can be achieved with doses of 600 mg and 900 mg of flavanols (Schramm *et al*., 2001). Eicosanoid metabolism within platelets and changes in plasma leukotriene-prostacyclin ratio, membrane fluidity, membrane receptor function and intracellular signalling could be responsible for the anti-platelet activity of cocoa powder. Also, the ability of cocoa polyphenol to reduce the ADP-induced expression of the activated confirmation of glycoprotein IIb/IIIa surface proteins might also be involved (Andújar *et al*., 2012;Grassi and Ferri, 2014). The latter are platelet receptors that bind adhesive proteins and promote aggregation (Scarborough *et al*., 1999).

2.2.3 Chemo-protection

The chemo-preventive potential of cocoa and cocoa polyphenols has been investigated in many *in vivo* and *in vitro* studies. Cell cultures studies were conducted on different cancer cell line, and it was observed that cocoa extracts and cocoa polyphenols possess antioxidant, pro-apoptotic, anti-proliferative, anti-angiogenetic and anti metastatic effects (Martin *et al*., 2013).

Cocoa polyphenols were found to be involved in the suppression of cell proliferation by blocking the cell cycle at G1/S or G2/M phases, modulating the activities of some cell-cycle regulatory proteins and inhibiting type II topoisomerases (Carnésecchi *et al*., 2002).

2.2.4 Neuro-protection

Conducted experiments suggest that cocoa polyphenols have neuro-protective, neuro-modulatory and neuro-rescue activities that are beneficial against aging and neuro-degenerative diseases. Cocoa polyphenols cross the blood-brain barrier, increase cerebral blood flow and stimulate the brain perfusion, reduce cerebral oedema, promote neuronal function and different kinds of visual and cognitive tasks. (McShea *et al*., 2008; Nehling, 2013).

Mechanism via which cocoa polyphenols act is by directly interacting with cellular and molecular signalling cascades, primarily with MEK, ERK and P13K pathways, particularly in the brain regions associated with learning and memory. Also, due to increased NO production with vasodilating and anti-inflammatory effects, they stimulate angiogenesis and central and peripheral blood flow (Sokolov *et al*., 2013).

A lot of human studies have shown that dietary flavanol-rich cocoa intake was linked with an increase in cerebral blood flow velocity (Sorond *et al*., 2008), improvement of cognition, learning memory and mood (Letenneur *et al*., 2007), vision (Huber *et al*., 2006), amelioration of the cognitive decline due to aging or Alzheimer's disease (Desideri *et al*., 2012). Single acute appropriate dose or subchronic administration of cocoa flavanols determines immediate effects on behaviour and cognition, while chronic administration causes long-lasting effects (Sokolov *et al*., 2013). Other possible beneficial effects of flavanol-rich cocoa include immune function modulation, skin health/photoprotective properties (Visiolo *et al*., 2012; Jahurul *et al*., 2013; Kothe *et al*., 2013) and anti-cariogenic activity (Ferrazzano *et al*., 2009).

2.3 Polyphenols

They are the largest category of phytochemicals and act as powerful antioxidants because of their multiple hydroxyl groups. Polyphenols are divided into three classes: flavonoids, phenolics and phenolic acids (Pietta, 2000)

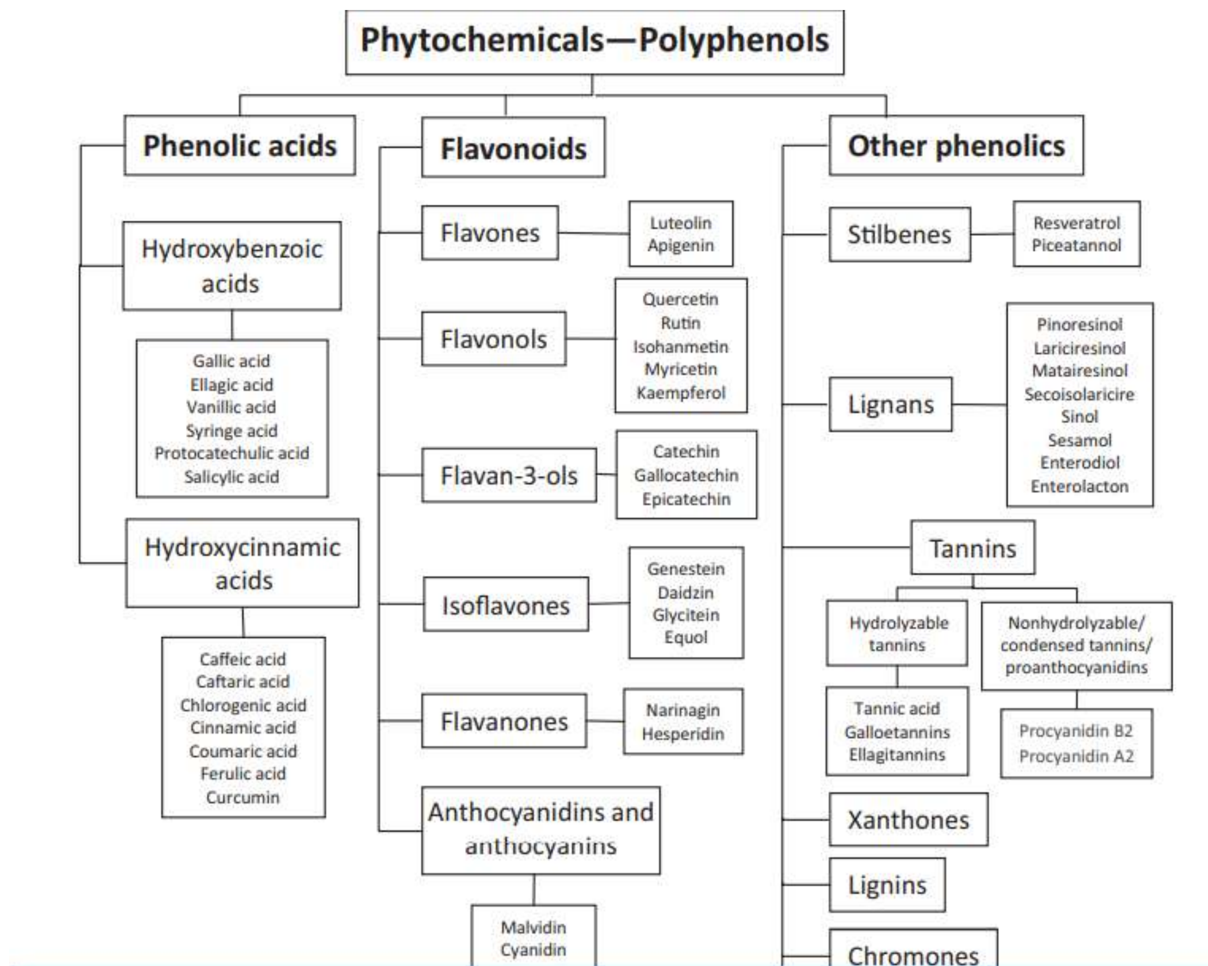

Figure 2.4; Classes of phytochemicals (Martinez et al., 2017)

2.3.1. Mechanism of Action of Flavonoids on Lipids

Cathechins are flavonoids and epigallocatechin gallate is the most abundant compound (Cione *et al*., 2019). Epigallocatechin gallate is a catechin conjugated with gallate acid. Two or more aromatic rings of polyphenols have at least one hydroxyl group linked by a carbon bridge that is the major source of electron donor and efficiently scavenges for reactive oxygen species (singlet oxygen). Cocoa is the richest source of epigallocatechin (Cione *et al*., 2019). Catechins possess a poor bioavailability which is due to their rapid degradation under physiological conditions and their low absorption in the intestinal tract by passive diffusion (Murakami, 2014). Mechanism of action involves inhibition of lipid absorption in the intestine by interfering with the micelle formation, emulsification, hydrolysis, solubilisation and inhibition of squalene oxidase, a key enzyme in the hepatic cholesterol biosynthesis (Abe *et al*., 2000;Koo and Noh, 2007). Apart from the cholesterol lowering potential, cathechins have been described to have a triglyceride lowering effect (Snoussi *et al*., 2014). This is done via inhibition of hepatic lipogenesis and more specifically, the inhibition of SREBP-1 (Shrestha *et al*., 2009).

2.3.2 Mechanism of Action of Flavonoids as Regards Antioxidant Enzymes

Quercetin-3-glucuronide (Q3GA) is a quercetin metabolite that is taken up by the human macrophages in the intima. It converts them to methylated derivatives that suppress the gene expression of scavenger receptor SR-A and CD36 (Kawai *et al*., 2008). *In vivo*, the antioxidant potential of quercetin has been expressed as lower levels of urinary isoprostane F2 and plasma MDA, that could be due to the ability to scavenge reactive oxygen species, chelate metal ions, reduces xanthase oxidase activity and to inhibit the MAPK pathway (Min *et al*., 2019). Quercetin increases eNOS activity in a dose-dependent manner via

phosphorylation on Ser1179 by cAMP/PKA pathway (Li *et al*., 2012). The antioxidant potential of quercetin is also reflected in the decrease of the oxidation levels of LDL (Bhaskar *et al*., 2016). Quercetin also reduces activities of SIRT-1 and AMPK, upregulates HO-1 and decreases the expression of oxLDL-induced NOX2 and NOX4 in humans (Hung *et al*., 2015).

Epigallocatechin gallate diminishes the effects of oxidative stress by inducing HO-1 expression in enterochromaffin cells exposed to H_2O_2 through activation of AKT and nuclear factor-erythroid 2-related factor 2 (Nrf_2) (Wu *et al*., 2006). Many other catechins exert antioxidant properties expressed as prevention of *in vitro* LDL oxidation in humans (Suzuki *et al*., 2016). Catechins scavenge peroxyl, hydroxyl and superoxide radicals, singlet oxygen and lipid peroxides, NO and peroxynitrate radicals (Ludwig *et al*., 2004).

2.3.3 Mechanism of action of flavonoids as regards tumour necrosis factor-α and Interleukin-10

Quercetin-3-glucuronide (Q3GA) a quercetin metabolite is taken up by the human macrophages in the intima and converted to methylated derivatives that suppress the gene expression of scavenger receptor SR-A and CD36 (Kawai *et al*., 2008). *In vivo*, the antioxidant potential of quercetin has been expressed as lower levels of urinary isoprostane F2 and plasma MDA, that could be due to the ability to scavenge reactive oxygen species, chelate metal ions, reduce xanthase oxidase activity and to inhibit the MAPK pathway (Min *et al*., 2019). Quercetin increases eNOS activity in a dose-dependent manner via phosphorylation on Ser1179 by cAMP/PKA pathway (Li *et al*., 2012).

2.4 Hyperlipidaemia

Hyperlipidaemia is a condition characterized by an elevation in any or all lipid profile and/or lipoproteins in the blood. Dyslipidaemia (which refers to abnormal levels of lipid in the blood) can also be described as elevated triglycerides, total cholesterol or low levels of HDL. Hyperlipidaemia is the major precursor of lipid related ailments such as atherosclerosis, coronary artery disease and is also involved in sudden death syndrome (Amit *et al*., 2011). The major cause of hyperlipidaemia includes changes in lifestyle habits (poor diet that is, diet with fat intake greater than 40% of total calories, saturated fat intake greater than 10% of total calories and cholesterol intake greater than 300mg per day). Other causes of hyperlipidaemia include the presence of treatable medical conditions such as obesity and lack of physical activity.

Atherosclerotic CVDs have the highest mortality rate globally, with ishaemic heart disease being one of the leading causes of death worldwide (Barquera *et al*., 2015). Atherosclerotic CVDs represent 28.8% of the global all-cause mortality. Experts have predicted that CVDs especially atherosclerosis will become the leading cause of death by 2020 (Barquera *et al*., 2015).

2.4.1 Atherosclerosis as a risk factor

Atherosclerosis, an inflammatory disease, is associated with lipid and metabolic abnormalities. Atherosclerosis is associated with alterations in the arteries. Atherosclerotic plaques are initiated by fatty streak or initial lesion. These initial lesions arise from localized increase in lipid content of lipoprotein and, in particular, of the portion that pertains to the LDL. These lipoproteins get attached to constituents of the extracellular matrix in the intima layer of the arteries and thereby increasing the lipid-rich particles within the arterial wall. These lipoprotein particles in the extracellular space of the intima may undergo oxidative

modification, forming oxidized lipoproteins that support a pathogenic role in atherogenesis (Moore *et al*., 2013). Oxidized LDL is toxic to the vascular network, whereas HDL acts as an antioxidant (Balagopal *et al*., 2011).

Reduced level of HDL is an important risk factor for CVDs, because of the reverse cholesterol transport that is mediated by this HDL, which provides an independent pathway for lipid removal, away from the atheroma formation (Barquera *et al*., 2015).

2.4.2 Classification of hyperlipidaemia

Hyperlipidaemia is grouped into two types viz; primary hyperlipidaemia and secondary hyperlipidaemia

2.4.2.1 Primary Hyperlipidaemia

Primary hyperlipidaemia is also known as familial hypercholesterolaemia. It is an autosomal dominant trait, involving a mutation of the LDL-receptor gene on chromosome19, which can often be identified by elevated levels of umbilical cord blood cholesterol (Krasi *et al*., 2019). Familial hypercholesterolaemia could either be homogygous familial hypercholesterolaemia (HoFH) or heteroxygous familial hypercholesterolaemia (HeFH).

2.4.2.2 Secondary Hyperlipidaemia

It results from another underlying disorder that leads to change in plasma lipid and lipoprotein metabolism. The most prevalent secondary cause of hyperlipidaemia is obesity (Chait and Brunzell, 1990).

2.4.3 Cocoa and Lipid Profile

Several studies have shown improvement in lipid profile after administration of cocoa powder. In a study carried out in Taiwan, using hamsters, it was discovered that administration of a combination of cocoa, coffee, green tea and garcinia reduced serum lipid content (triglycerides, total cholesterol and LDL) and hepatic lipid content (triglycerides and cholesterol) with dose dependent effect. In addition there was an increase in excretion of faecal lipids (bile salts) (Chih-Wei *et al.*, 2015). Another research carried out on mice showed that cocoa powder intake at both low and high doses, reduced plasma levels of total cholesterol and LDL. Also, atherosclerotic lesions were reduced in treated group when compared to control. An experiment was conducted on guinea pigs using unsweetened natural cocoa powder. It was reported that plasma HDL levels increased while LDL levels decreased.

2.5 Oxidative Stress

Oxidative stress refers to the imbalance between generation and clearance of oxidants. Oxidative stress has been illustrated in Figure 2.5. Oxidants include reactive free radicals and radicals including reactive oxygen species (ROS) and reactive nitrogen species (RNS) which cause deleterious effects in several organs (Matsubara *et al.*, 2015). ROS include superoxide (O_2-), hydroxyl (OH-), peroxyl (RO_2), alkoxyl (RO), hydroperoxyl (HO_2), hydrogen peroxide (H_2O_2), singlet oxygen and ozone. RNS are composed of nitric oxide (NO), nitrogendioxide (NO_2), nitrous acid (HNO_3), dinitrogen tetroxide (N_2O_4), dinitrogen trioxide (N_2O_3), peroxynitrite (ONOO), peroxynitrous acid (ONOOH), alkyl peroxynitrite (ROONO) and nitryl chloride (NO_2Cl).

Oxidizing agents can be produced by both endogenous sources (inflammatory cells, fibroblast, endothelial cells, epithelial cells, respiratory chain, xanthine and NADPH oxidase) and exogenous sources (cigarette smoking, exogenous toxins, pollution, radiation, carcinogens and drugs) (Choi *et al*., 2014; Nomura*et al*., 2014; Nourazarian *et al*., 2014).

In normal physiological conditions, oxidants are removed through antioxidant defence mechanism. If oxidants are incompletely cleared by antioxidants, oxidants will cause oxidative stress. Oxidative stress, whether mild or severe, might cause damage to cellular macro-components namely lipid, protein, sugar and DNA (Nikolaidis *et al*., 2012). Therefore, the ingestion of antioxidants would prevent such damage and in addition augment performance (Gomez-Cabrera *et al*., 2012).

(A) Normal condition

(B) Oxidative stress

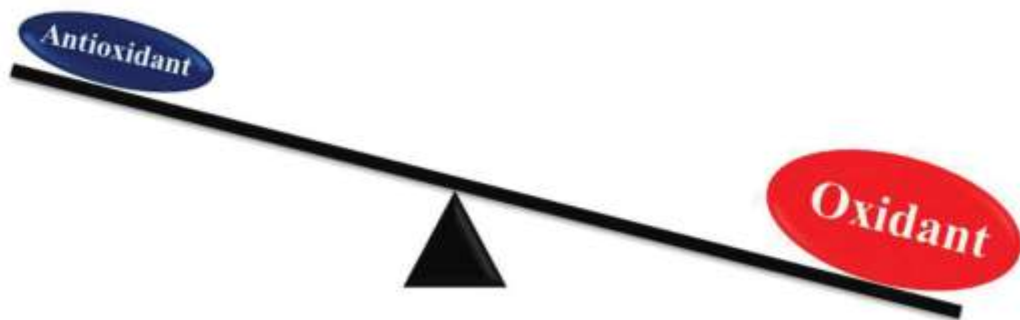

Figure 2.5: General concept of oxidative stress (A) Normal condition is indicated in the balance between oxidant production and antioxidant defence system and (B) Oxidative stress is demonstrated as the imbalance between generation and clearance of oxidant (Palipoch and Koomhin, 2015)

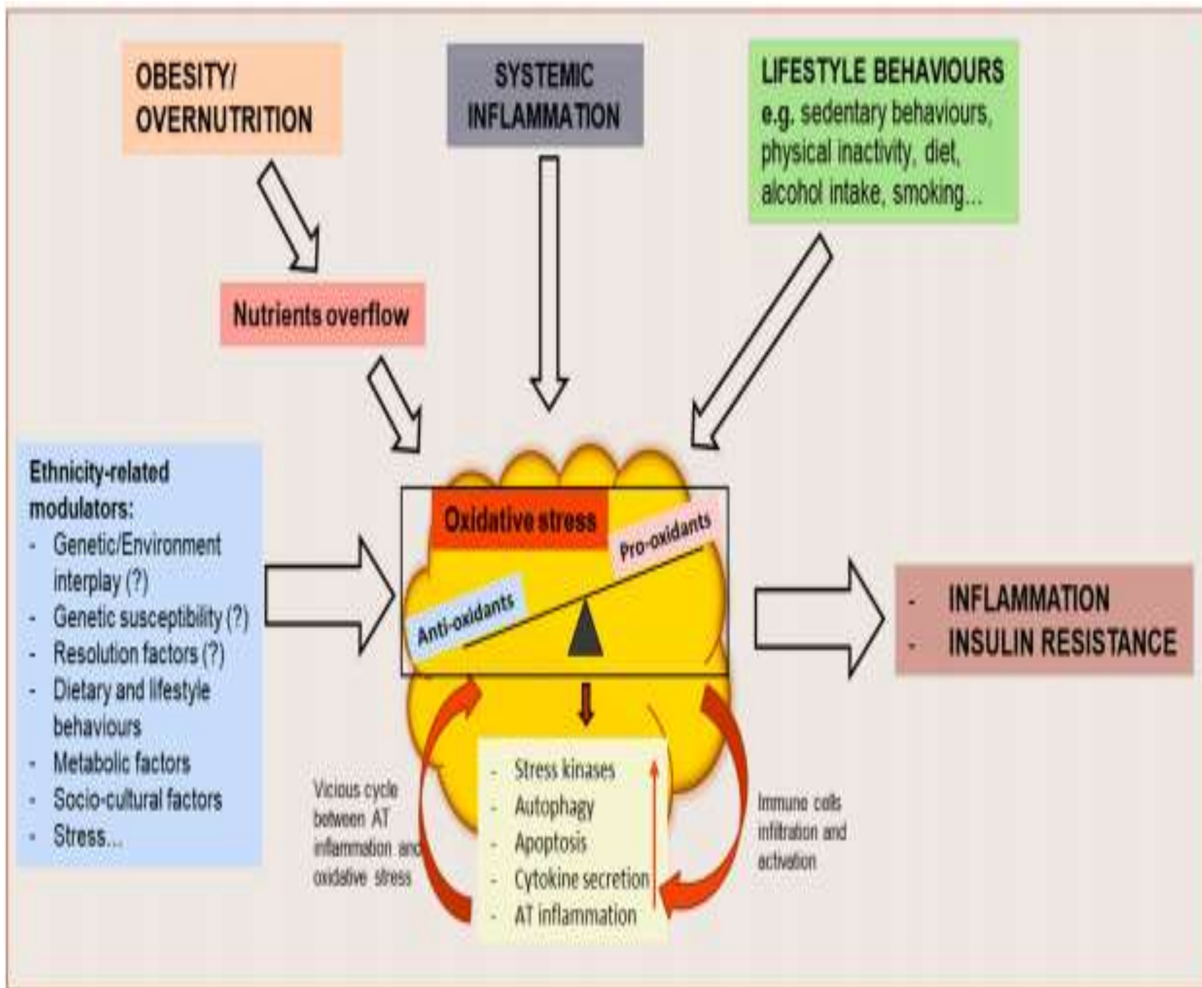

Figure 2.6: Oxidative stress drivers and metabolic consequences on adipose tissue (AT) (Nono Nankam *et al*., 2021)

Figure 2.6 shows that obesity may lead to nutrients overflow to adipose tissue, thus resulting in adipocyte hypertrophy and adipose tissue hypoxia which could lead to oxidative stress in tissue.

2.5.1 Free radicals

Free radicals are heterogeneous group of molecules that are characterized by a free valence electron in their outer atom orbital. Due to this unpaired electron, free radicals react with other molecules. Their biological half-life is very short in biological solutions, including cellular surroundings (Valko *et al*., 2007).

2.5.1.1 Negative effects of free radicals

High concentration of free radicals leads to a strong oxidative stress in cells, for instance, in monocytes and neutrophils of the immune system. Even low levels of ROS can cause cell damage by chemical inactivation of molecules such as DNA by base damage and single-strand breakage. Free radicals might also bring the cellular homeostasis to a state that is out of balance. Also, imbalance of ROS metabolism is associated with CVDs like stroke and heart attack. Free radicals are perpetrators of cell damage, aging and even cancer, while antioxidants on the other hand are seen as defence against these threats (Margaritis and Rousseau, 2008).

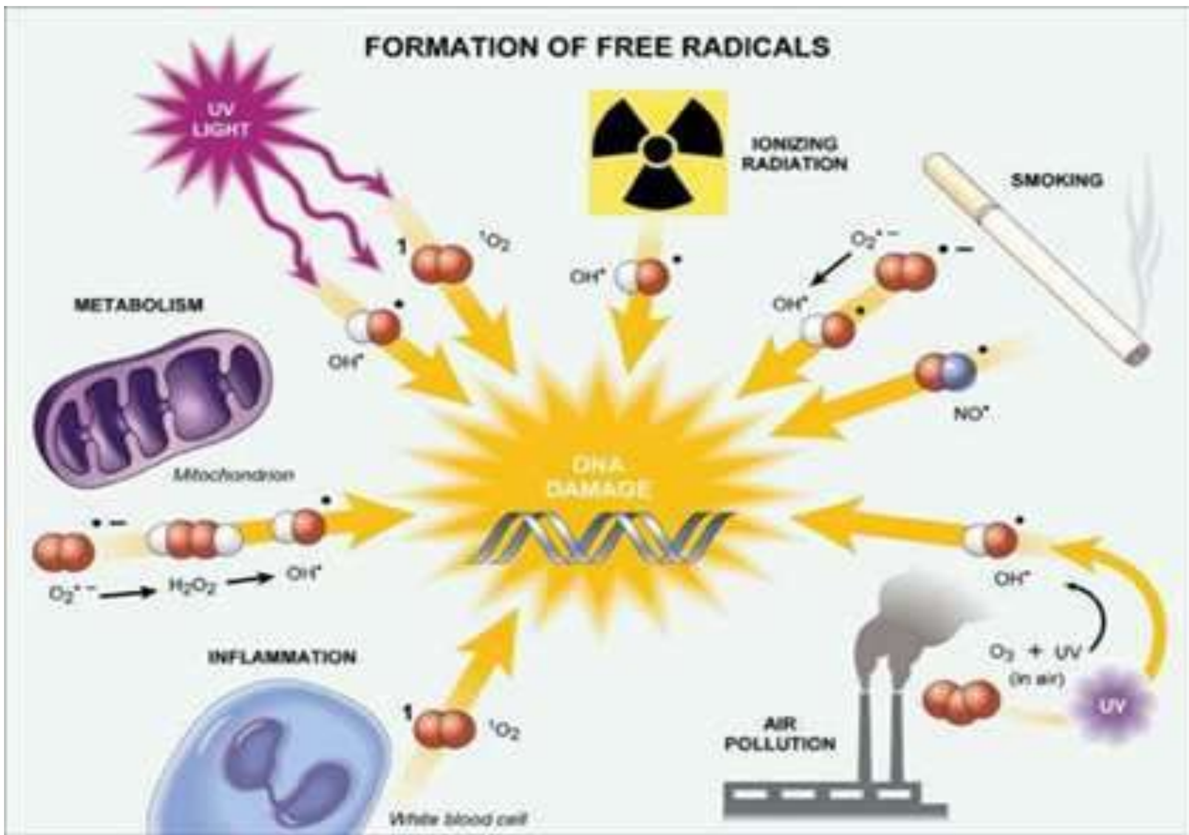

Figure 2.7; Different ROS sources (Han *et al*., 2001).

2.5.1.2 Lipid peroxidation

Oxidative stress can cause lipid peroxidation, causing different arrangements in the membrane lipid bilayer. This leads to inactivation of the membrane-bound receptors and enzymes and causes increase in tissue permeability (Giugliano *et al*., 1996). Lipid peroxidation leads to type II familial hypercholesterolaemia. Malondialdehyde and unsaturated aldehydes which are products of lipid peroxidation, have the ability to inactivate many cellular proteins by forming cross-linkages (Esterbauer *et al*., 1984). This causes depletion of intracellular glutathione, induces peroxide production (Uchida *et al*., 1999), activates epidermal growth factor receptor (Suc *et al*., 1998) and induces fibronectin production (Tsukagoshi *et al*., 2002).

2.5.2 Oxidative stress and CVDs

Several CVDs result from complications of atherosclerosis. Atherosclerosis which is a multi-factorial disease refers to the build-up of plaque (fats and cholesterol) in arterial walls. These build up has the potential to restrict blood flow to the heart or any other organ. Hypertension, hyperlipidaemia, diabetes and cigarette smoking are some risk factors of atherosclerosis. Previous studies showed that oxidative stress plays a vital role in the pathogenesis of the disease. Generation of ROS and oxidation of LDL play the key role in the oxidative signalling pathway to vascular inflammation from the generation of fatty streak development to plaque rupture (Cipollone *et al*., 2007). 8, Hydroxy-2'-deoxyguanosine (8-OHdG), an oxidative DNA damage biomarker, was found to be elevated in aorta fragments taken from patients suffering from severe atherosclerotic lesions (De Flora *et al*., 1997).

2.5.3 Cocoa powder and oxidative stress

A research carried out to evaluate the long term protective effect of epicatechin and cocoa phenolic extract on HepG2-cultured cells which are subjected to oxidative stress. Epicatechin increased glutathione peroxidase and glutathione reductase activities when compared to control cells (Cordero-Herrera *et al*., 2015). Also, young Zucker diabetic fatty rats were fed with cocoa-rich diet and it was discovered that activities of antioxidant enzymes; glutathione peroxidase and glutathione reductase, were increased in the renal cortex (Àlvarez-Cilleros *et al*., 2019).

2.6 Inflammation

Inflammation is a pervasive form of defence that is broadly defined as a non-specific response to tissue malfunction and is activated by both innate and adaptive immune systems to fight against pathogenic intruders. Inflammation normally begins in a localised area, but depending on the severity of the infection/wound, it can spread rapidly to the periphery. This systemic response is triggered by pro-inflammatory cytokines, especially IL-1, IL-6 and TNF-α, which are released in the circulation and activate fever and sickness behaviours in the brain as well as acute phase protein secretion from the liver. (Sorci and Faivre, 2009). A unique feature of inflammation in relation to other facets of anti-parasite defences is that there is damage to self (Raberg *et al*., 1998; Graham *et al*., 2005).

Inflammation can also be defined as a biological reaction to a disrupted tissue homeostasis (Medzhitov, 2008). At its basic level it is a tissue destroying process that involves the recruitment of blood-derived products, like plasma proteins, leukocytes and fluid, into perturbed tissue. This migration is aided by alterations in the local vasculature that causes vasodilation, increased blood flow and vascular permeability. Infection by microbial invaders

is the major culprit that promotes inflammatory responses. However, injury or trauma (in the absence of parasitic infection) and exposure to foreign particles, pollutants and irritants, are also major triggers of inflammatory responses (Medzhitov, 2008), suggesting that this response likely evolved as a general adaptation for coping with damaged or malfunctioning tissue (Matzinger, 2002).

2.6.1 Mechanism of Inflammation

Inflammation involves a tightly regulated cascade of immunological, physiological and behavioural processes that are formed by soluble immune signalling molecules called cytokines. This complex process involves inflammatory cells: neutrophils, macrophages and lymphocytes (Abdulkhaleq *et al*., 2018).

The first stage of the inflammatory cascade involves recognition of damage or infection (Figure 2.8b). This is achieved by the detection of pathogen-associated molecular patterns (PAMPs), which are specifically targeted towards general motifs of molecules expressed by pathogens that are essential for pathogen survival, alarmins or damage-associated molecular patterns (DAMPs) are endogenous molecules which signal necrosis or damage and are also detected by innate immunity. An advantage of recognizing these signals is that inadvertent targeting of host cells and tissues is minimised. Unlike adaptive immunity, the innate immune system does not have the ability to distinguish among different strains of pathogens and whether or not such strains are virulent (harmful to the host). Many damage signals are recognized by germ-like encoded receptors, such as transmembrane toll-like receptors (TLRs) and intracellular nucleotide binding domain and leucine-rich-repeat containing receptors (Lange *et al*., 2001; Roach *et al*., 2005; Proell *et al*., 2008). Once recognition of ligands occurs, TLRs activate common signalling pathways that culminate in the activation of

NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cell; Figure 2.8c). This transcription factor is found in virtually all cell types and remains in an inactive state bound to an inhibitor protein, IkB (Ghosh *et al*., 1998). Upon transduction of the signal, NK-kB is released from IkB and translocates to the nucleus where transcription is upregulated through binding to target genes. Activation of NFkB does not require new protein synthesis, which allows for a rapid response. The NFkB signalling system is ancient, but there is phylogenetic evidence that regulation of immune function by this pathway in vertebrates evolved independently from invertebrate immune mechanism (Friedman and Hughes, 2002). Intracellular NLRs respond to increasing numbers of DAMPs that alert the immune system to cell injury and provide a proximate pathway for sensing exposure to possible toxins or pollutants in the environment. (Wang *et al*., 2020)

In the third stage of the inflammatory cascade, transcription and translation of genes are involved. This is the inducible expression of pro-inflammatory cytokines (Figure 2.8d). In combination with chemokines (attractants) and various co-stimulatory molecules, these soluble proteins facilitate the recruitment of effector cells (Figure 2.8e) such as monocytes and neutrophils to the site of disturbance. Neutrophils form a cytotoxic environment by releasing noxious chemicals from cytoplasmic granules (a process called degranulation). Rapid release of these chemicals requires consumption of both glucose and oxygen, known as respiratory burst. Poisonous chemicals released include highly ROS and RNS and various proteinases. These substances are destructive to both pathogens and hosts and essentially induce liquefaction of surrounding tissue to starve off microbial metastasis (Dickson and Zhou, 2020). These mechanisms are thus major contributors to host collateral damage. The net effect of these interactions culminates in the stereotypical cardinal signs of local

inflammation: heat, swelling, redness, pain and loss of function. The effector functions of inflammation are further regulated by the adaptive immune system (Figure2.8f).

The last stage of inflammation is its resolution (Figure 2.8g), which is critical for limiting collateral damage to the host (Serhan and Savill, 2005). After the first few hours of inflammation, a coordinated program of resolution is set into motion by tissue-resident and recruited macrophages. During acute inflammation, these cells produce pro-inflammatory prostaglandins and leukotrienes but rapidly switch to lipoxins, which block further neutrophil recruitment and instead favour enhanced infiltration of monocyte important for wound healing.

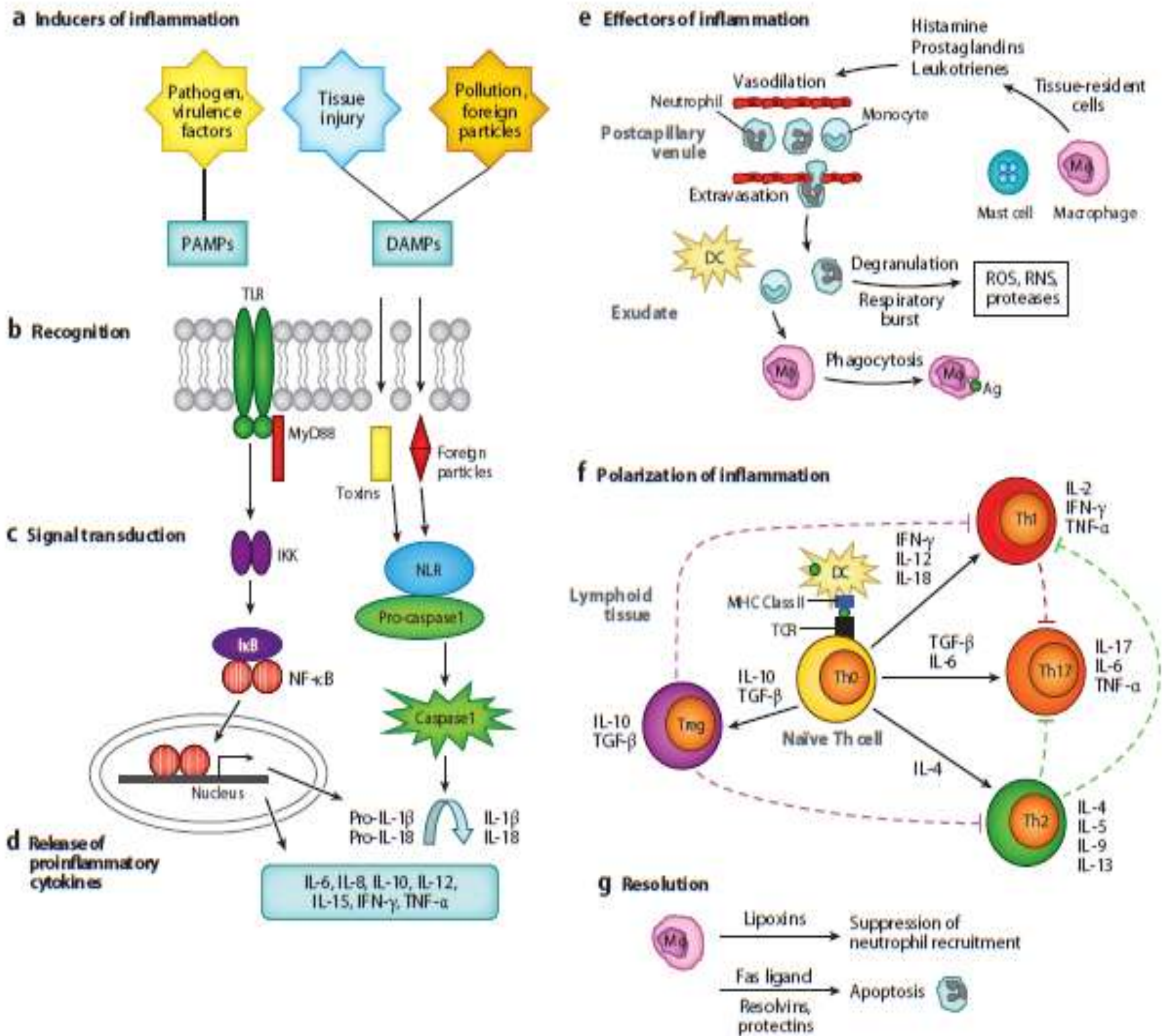

Figure 2.8 (a-g): Mechanism of inflammation (Noah *et al*., 2012)

2.6.2 Cocoa powder and inflammation

In an experiment using hamsters which were fed with a combined extract of cocoa, coffee, green tea and garcinia, it was reported that TNF-α levels improved. The treatment lowered serum TNF-α level by 27.45%-32.3%, when compared to the high-fat diet group (Chih-Wei *et al*., 2015). Also, to evaluate the anti-inflammatory effect of cocoa, a non-randomized, controlled, crossover, free-living study was carried out in healthy and moderately hypercholesterolemic volunteers. IL-10 levels increased and TNF-α value decreased (Martinez-Lopez *et al*., 2014). In another study, the effect of cocoa polyphenolic extract (CPE) on macrophage alternative M2 phenotype was investigated. The secretion of pro-inflammatory and anti-inflammatory cytokines were determined and compared to the control basal level in untreated cells. CPE significantly decreased macrophage response to M1 activation through the reduction of the secretion of TNF-α in M1 phenotype by up to 20% and increase secretion of IL-10 by up to 47% when compared to untreated cells (Dugo *et al*., 2017).

2.7 Poloxamer 407 and Hyperlipidaemia

Poloxamer 407 is a non-ionic surfactant block copolymer consisting of polyoxyethylene and polyoxypropylene units. It has bio-compatibility and potential to deliver different drugs for a variety of disease states (Johnston *et al*., 1992), and is a barrier for preventing post-surgical adhesions (Steinleitner *et al*., 1991). It has an unusual thermo-reversible property. It is liquid at room temperature while it self-assembles into micelles then aggregate into a gel at body temperature. These temperature micellization and gelation properties have led to the widespread use of poloxamer 407 in some personal care products like mouthwashes, deodorants and skin care products and also as an excipient in a variety of pharmaceutical

preparations (Dumortier *et al*., 2006). Johnston showed that one intramuscular and intraperitoneal injection of poloxamer 407 leads to dose-dependent hyperlipidaemia in rats, increasing plasma triacylglycerol more than 60 fold and cholesterol about 8 fold and since then has been a growing model in different hyperlipideamic studies (Johnston *et al*., 1992).

Poloxamer 407 augments levels of serum lipoproteins via its action at various levels in lipid metabolism, largely by inhibiting lipoprotein lipase, which facilitates the hydrolysis of triglycerides (Johnston and Palmer, 1993). Johnston *et al* investigated the effect of poloxamer 407 on lipoprotein lipase activity and found out that after 3 hours of intraperitoneal injection in rats the activity of the enzyme decreased by 95% compared to a normal saline treated control group (Johnston and Palmer, 1993). Also, poloxamer 407 causes indirect stimulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase, which is involved in cholesterol biosynthesis (Wout *et al*., 1992; Blonder *et al*., 1999).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Chemicals/Materials

The following were used in this study; distilled water, phosphate buffer, normal buffered formalin, ketamine hydrochloride, diazepam, poloxamer 407 (Sigma-Aldrich Germany), atorvastatin (Lipitor), cocoa powder (Oluji), 6 plastic cages (13cm X 6cm X 6cm), dissecting kit, plain tubes, mortar and pestle, centrifuge, microplate reader RT-2100C, ELISA kits for TNF-α and IL-10 (Wuhan Fine Biotech Co., Ltd., Wuhan, China), incubator, leitz microscope Germany, L 2508.

3.2 Toxicity Study

The median lethal dose (LD_{50}) of cocoa powder was carried out according to Lorke's method (Lorke 1983). This was done in two phases using 12 rats. In the first phase, rats were divided into three groups of three rats each, and were treated with 10mg, 100mg and 1000mg of cocoa powder respectively per kg body weight orally. The rats were observed for 24 hours for signs of toxicity, including death. After the first phase there were no signs of toxicity in the rats so the second phase commenced. Three rats were divided into three groups of one rat each, and were treated with 1600mg, 2900mg and 5000mg of cocoa powder, respectively per kg body weight. The rats were observed for 24 hours for signs of toxicity, including death. After the second phase there were no signs of toxicity and there was no mortality. The LD_{50} was then calculated from the results of the second phase as the square root of the product of the lowest lethal dose and the highest non-lethal dose. LD_{50} was greater than 5000. The dose was calculated as the 5th, 10th and 20th percent of the LD_{50}

3.3 Methodology

3.3.1 Proximate analysis of cocoa powder

i Antioxidants

Antioxidants were measured by hydrogen peroxide scavenging activity. Scavenging activity of hydrogen peroxide by cocoa powder was estimated using the method of Ruch *et al.*, (1989). Cocoa powder (4ml) prepared in distilled water at various concentration was mixed with 0.6 ml of 4mM H_2O_2 solution prepared in phosphate buffer (0.1M pH 7.4) and incubated for 10 minutes. The absorbance of the solution was taken at 230 nm against blank solution containing cocoa powder without H_2O_2. The amount of H_2O_2 radical inhibited by cocoa powder was calculated using the following equation;

H_2O_2-radical scavenging activity = {(Abscontrol – Abssample) / (Abscontrol)}x100

Where; Abscontrol is the absorbance of H_2O_2 radical + methanol; Abssample is the absorbance of H_2O_2 radical + cocoa powder sample or standard (Ruch *et al.*, 1989).

ii Standard ascorbic acid

Standard ascorbic acid was measured by hydrogen peroxide scavenging activity. Scavenging activity of hydrogen peroxide by cocoa powder was estimated using the method of Ruch *et al.*, (1989). Cocoa powder (4 ml) prepared in distilled water at various concentration was mixed with 0.6 ml of 4 mM H_2O_2 solution prepared in phosphate buffer (0.1M pH 7.4) and incubated for 10 minutes. The absorbance of the solution was taken at 230 nm against blank solution containing cocoa powder without H_2O_2. The amount of H_2O_2 radical inhibited by cocoa powder was calculated using the following equation;

H_2O_2 radical scavenging activity = {(Abscontrol – Abssample) / (Abscontrol)}x100

Where; Abscontrol is the absorbance of H_2O_2 radical + methanol; Abssample is the absorbance of H_2O_2 radical + cocoa powder sample or standard (Ruch *et al.*, 1989).

iii Lipids

Lipids content in cocoa powder was measured using soxhlet extraction method. 10 g of sample was continuously extracted with petroleum ether in a soxhlet apparatus for 6 hours. The petroleum ether was evaporated to dryness and the residual lipid was weighed. The total lipid was calculated in relation to the weight of the sample (Brain and Turner, 1975).

iv Ash value

2 g of the sample was placed into a nickel crucible and burnt into ash on a Bunsen burner flame until completely ashed. The weight of the ash was recorded and the percentage ash was calculated (British Pharmacopeia, 1993).

v Moisture

3g of the sample was weighed into a crucible placed in an oven at 105 °C and heated to constant weight. The weight of moisture lost was determined by evaporated moisture (British pharmacopeia, 1993).

vi Alkaloids

About 5g of sample was weighed into a 250ml beaker and 200ml of 10% acetic acid in ethanol was added and the mixture was covered and allowed to stand for 4 hours. It was filtered and the filtrate was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the sample until precipitation was complete. The precipitate was collected and washed with dilute ammonium hydroxide solution and filtered, dried and weighed. (Harbone, 1973).

vii Flavonoids

About 10 g of the sample was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through a whatman filter paper with

number 42. The filtrate was later transferred into a crucible and evaporated to dryness over a water bath and weighed to a constant weight. (Boham and Kocipai 1994).

viii Saponins

Saponins content was determined by using the method of Obadoni and Ochuko (2001).

ix Tannins

About 500mg (0.5g) of sample was weighed into a 50ml plastic bottle and 50 ml of distilled water was added and shaken for 1 hour on a mechanical shaker. The solution was filtered into a 50 ml volumetric flask and made up to the mark, then 5 ml of the filtrate was pipette out into a test tube and mixed with 2 ml of 0.1M $FeCl_3$ in 0.1 M HCl and 0.008M potassium ferrocyanide. The absorbance was measured at 720 nm within 10 minutes. (Van-Buren and Robinson 1981).

x Protein

Titration method was used to measure protein content (AOAC, 1990)

3.3.2 Drug preparation

Exactly 1 g of cocoa powder was dissolved in 10ml of distilled water. The stock concentration was obtained by calculating the amount of the powder in 1ml of water and it was found to be 100mg/ml. The mixture was stirred until there was no lump.

3.3.3 Ethical approval

Ethical approval was obtained from Committee on Animal Use and Care, Ahmadu Bello University Zaria. The approval number is ABUCAUC/2021/094.

3.3.4 Experimental animals

Thirty (30) male Wistar rats which weighed 130 g-150 g were procured from the Animal House of the Department of Human Physiology, Ahmadu Bello University, Zaria. The animals were kept in well aerated plastic laboratory cages in the animal house of the Department of Human Physiology, Ahmadu Bello University, Zaria. They were kept under temperature of 20 °C -22 °C, humidity of 30 %-60 % and 12/12 hour light/dark cycle. The rats were given access to standard animal feed and drinking water *ad libitum* during the experimental period

3.3.5 Induction of hyperlipidaemia

Poloxamer 407 (Sigma Aldrich, Schnelldorf, Germany) was used to induce hyperlipidaemia. Administration of poloxamer 407 was at a dose of 500mg/kg of body weight, intraperitoneally, on alternate days for 4 weeks. This was done because the hyperlipidaemic effect of poloxamer 407 lasts for about 72 hours after which the lipid levels in blood revert back to normal levels. Prior to the administration, poloxamer 407 was dissolved in distilled water (Johnston and Palmer 1993;Zuberu *et al*., 2017).

3.3.6 Experimental design

Animal Grouping

Thirty (30) male Wistar rats were divided into 6 groups. Each group consisted of 5 Wistar rats (n=5) which were treated for four weeks as follows:

Group 1: Control group (were given standard rat feed and water *ad libitum*)

Group 2: hyperlipidaemia (untreated)

Group 3: hyperlipidaemia + 20 mg/kg of atorvastatin (standard drug) (Zuberu *et al*., 2017)

Group 4: hyperlipidaemia+250 mg/kg cocoa powder orally once daily

Group 5: hyperlipidaemia+500 mg/kg cocoa powder orally once daily

Group 6: hyperlipidaemia+1000 mg/kg cocoa powder orally once daily

3.3.7 Collection and preparation of serum samples for biochemical assays

After the treatment period, the animals were sacrificed after mild anaesthesia with intraperitoneal injection of 75 mg/kg of ketamine hydrochloride and diazepam at 5 mg/kg. Blood sample was drawn from the heart via cardiac puncture and stored in plain bottles for biochemical analysis. The serum was separated by centrifugation at 3000 xg for 5 minutes. The serum collected was taken to the laboratory for determination of lipid profile, biomarkers of oxidative stress and lipid peroxidation. The abdomen was excised and the skin rolled back using forceps to expose the heart. The heart and coronary arteries were removed and rinsed in ice cold saline solution (isotonic) to remove blood cells and was then weighed. Each heart was divided into two and the portion with the coronary artery attached to it was fixed in neutral buffered formalin which was obtained from the Department of Pathology, Ahmadu Bello University Teaching Hospital, Shika-Zaria. After fixing for 48 hours it was processed with automated tissue processor (ATP) TP 1020 Leica made in Austria/Germany. The remaining portion of each heart was homogenized using a ceramic homogenizer in 9 volume of 0.05 mM potassium phosphate buffer solution of pH 7.4. The homogenates were then centrifuged at 6000 xg for 15 minutes and the supernatant was used for the analysis of biomarkers of inflammation (TNF-α and IL-10).

3.3.8 Determination of lipid profile

3.3.8.1 Determination of serum total cholesterol

The serum level of total cholesterol was quantified after enzymatic hydrolysis and oxidation of the sample as described by the method of Stein (1987). Briefly, 1000 µl of the reagent was added to each of the sample and standard. This was incubated for 10 minutes at 20-25 ^{0}C after mixing, and the absorbance of the sample (A sample) and standard (A standard) were measured against the reagent blank within 30 minutes at 546 nm. The value of total cholesterol present in the serum was expressed in mg/dl.

Total Cholesterol concentration = A sample /A standard x 196.86 mg/dl.

3.3.8.2 Determination of serum triglyceride

The serum triglyceride level was determined after enzymatic hydrolysis of the sample with lipases as described by Tietz (1990). Also, 1000 µl of the reagent was added to each sample and standard. This was incubated for 10 minutes at 20-25 °C after mixing, and the absorbance of the sample (A sample) and standard (A standard) was measured against the reagent blank within 30 minutes at 546 nm. The value of triglyceride present in the serum was expressed in mg/dl.

Triglyceride concentration = A sample/A standard x 194.0 mg/dL.

3.3.8.3 Determination of serum high-density lipoprotein cholesterol

The serum level of high-density lipoprotein cholesterol (HDL-C) was measured using the method described by Wacnic and Albers (1978). Low density lipoproteins (LDL and VLDL) and chylomicron fractions in the sample were precipitated quantitatively by addition of phosphotungstic acid in the presence of magnesium ions. The mixture was allowed to stand

for 10 minutes at 20-25 °C, and centrifuged for 10 minutes at 4000 xg. The supernatant represented the HDL-C fraction. The cholesterol concentration in the HDL fraction, which remained in the supernatant, was determined. The value of HDL-C was expressed in mg/dL.

3.3.8.4 Determination of serum low-density lipoprotein cholesterol

The serum level of LDL-C was measured according to the protocol of Friedewald *et al*., (1972) using the equation below;

LDL-C = TC - (TGL/5 + HDL-C). The value was expressed in mg/dL.

3.3.9 Estimation of biomarkers of oxidative stress

3.3.9.1 Estimation of lipid peroxidation (malondialdehyde)

Lipid peroxidation was estimated in the serum and it was carried out spectrophotometrically as thiobarbituric acid reactive substances (TBARS) (Tsikas, 2017). A principal component of TBARS is malondialdehyde (MDA), a product of lipid peroxidation. The assay for malondialdehyde was carried out using malondialdehyde ELISA kit according to the manufacturer's instruction. The assay employed the competitive inhibition enzyme immunoassay technique. A monoclonal antibody specific for MDA was pre-coated onto a microplate. A competitive inhibition reaction was launched between biotin labelled MDA and unlabelled MDA/samples with the pre-coated antibody specific for MDA. It was subjected to incubation, after which the unbound conjugate was washed off. Next, avidin was conjugated to Horseradish Peroxidase (HRP) and added to each microplate well and incubated. The amount of bound HRP conjugate was taken to be inversely proportional to the concentration of MDA in the sample. Substrate solution was added and it caused development of colour.

The intensity of colour developed was measured using a microplate reader and the reading was taken to be inversely proportional to the concentration of MDA in the sample.

3.3.9.2 Assay of superoxide dismutase (SOD) activity

The assay for the serum superoxide dismutase activity was carried out using rat superoxide dismutase (SOD) ELISA kit according to manufacturer's instruction. This assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for SOD was pre-coated onto a microplate. Standards and samples were pipetted into the wells, gently mixed, covered with a closure plate membrane and incubated for 30 minutes at 37 ºC. This caused any SOD present to be bound by the immobilized antibody. It was later on removed from the incubator, the wells were washed with a prepared 'wash solution'; the microplate was inverted and thereafter blot-dried by hitting onto an absorbent paper to remove the moisture. An enzyme-linked monoclonal antibody specific for SOD was added to the wells, sealed and, again incubated for 30 minutes at 37 ºC. It was washed to remove any unbound antibody-enzyme reagent, by adding substrate solutions (tetramethylbenzidine and hydrogen peroxide) to the wells and a colour developed in proportion to the amount of SOD bound in the initial step. This was covered and incubated for 15 minutes at 37 ºC. Colour which was developed was stopped by addition of 'stop solution' and the intensity of the color was then measured with a microplate reader. (Misra and Fridovich(1972).

3.3.9.3 Assay of glutathione peroxidase (GPx) activity:

The assay for the serum glutathione peroxidase activity was carried out using rat glutathione peroxidase (GPx) ELISA kit according to manufacturer's instruction. This assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for GPx was pre-coated onto a microplate. Standards and samples were pipetted into the

wells, gently mixed, covered with a closure plate membrane and incubated for 30 minutes at 37 ºC. This caused any GPx present to be bound by the immobilized antibody. After which it was removed from the incubator, and the wells were washed with a prepared 'wash solution'; the microplate was inverted and thereafter blot-dried by hitting onto an absorbent paper to remove the moisture. An enzyme-linked monoclonal antibody specific for GPx was added to the wells, sealed and, again incubated for 30 minutes at 37 ºC after which it was washed and blot-dried to remove any unbound antibody-enzyme reagent. Substrate solutions (tetramethylbenzidine and hydrogen peroxide) were added to the wells and a colour developed in proportion to the amount of GPx bound in the initial step. This was then covered and incubated for 15 minutes at 37 ºC. The colour development was stopped by addition of 'stop solution' and the intensity of the colour was measured with a microplate reader. (Rotruck *et al*., 1973)

3.3.9.4 Assay of catalase (CAT) activity

The assay for the serum catalase activity was carried out using rat catalase (CAT) ELISA kit according to manufacturer's instruction. A monoclonal antibody specific for CAT was pre-coated onto a microplate. Standards and samples were pipetted into the wells, gently mixed, covered with a closure plate membrane and was incubated for 30 minutes at 37 ºC. This allowed any CAT present to be bound by the immobilised antibody. It was later removed from the incubator and the wells were washed with a prepared 'wash solution' the microplate was inverted and thereafter blot-dried by hitting onto an absorbent paper to remove the moisture. An enzyme-linked monoclonal antibody specific for CAT was added to the wells, sealed and again incubated for 30 minutes at 37 ºC. Following a wash to remove any unbound antibody-enzyme reagent, substrate solutions (tetramethylbenzidine and hydrogen peroxide) were added to the wells and a colour developed in proportion to the amount of CAT bound in

the initial step. This was covered and incubated for 15 minutes at 37 ºC. The colour development was stopped by addition of 'stop solution' and the intensity of the colour was measured with a microplate reader. (Nelson and kiesow 1972).

3.3.10 Estimation of biomarkers of inflammation

3.3.10.1 Determination of TNF-α

Assay for TNF-α was carried out using rat TNF-α ELISA kit (according to manufacturer's instructions). The rat TNF-α ELISA kit which was purchased from Fine Test China. Plates were washed twice before adding standard, sample and control wells. 100 µL of standard or sample was added to each well and incubated for 90 minutes at 37 °C. Plates were aspirated and washed twice and then 100 µL of biotin-labeled antibody working solution was added to each well and incubated for 60 minutes at 37 °C. Plates were aspirated and washed twice. 100 µL SABC working solution was added into each well and incubated for 30 minutes at 37°C. Plates were aspirated and washed 5 times. 90 µL of TMB substrate was added and incubated for 15-30 minutes at 37 °C. 50 µL of stop solution was added and reading was immediately taken at 450 nm. (Barath *et al*., 1990).

3.3.10.2 Determination of interleukin-10

Assay for IL-10 was carried out using rat IL-10 ELISA kit (according to manufacturer's instructions). The rat IL-10 ELISA kit was purchased from Fine Test China. Plates were washed twice before adding standard, sample and control wells. 100 µL of standard or sample was added to each well and incubated for 90 minutes at 37°C. Plates were aspirated and washed twice and then 100 µL of biotin-labelled antibody working solution was added to each well and incubated for 60 minutes at 37 °C. Plates were aspirated and washed twice. 100 µL SABC working solution was added into each well and incubated for 30 minutes at 37

°C. Plates were aspirated and washed 5 times. TMB (90 µL) substrate was added and incubated for 15-30 minutes at 37 °C. Exactly 50 µL of stop solution was added and reading was immediately taken at 450 nm. (Barath *et al*., 1990).

3.3.11 Histology of the coronary artery

Sections of the coronary artery were fixed in neutral buffered formalin obtained from the Department of Pathology, Ahmadu Bello University Teaching Hospital, Shika-Zaria. After fixing for 48h it was processed with automated tissue processor (ATP) TP 1020 Leica made in Austria/Germany with the following regimens:

I. 70% ethanol – 2 hours – 1 change
II. 80% ethanol – 2 hours – 1 change
III. 100% ethanol - 2 hours – 1 change
IV. 100% ethanol - 2 hours – 1 change
V. 100% ethanol - 2 hours – 1 change
VI. 100% ethanol - 2 hours – 1 change
VII. 100% ethanol - 2 hours – 1 change
VIII. 100% ethanol - 2 hours – 1 change
IX. Toulene ---------------------1 hour
X. Toulene ---------------------1 hour
XI. Wax set at 60°C -----------1 hour
XII. Wax set at 60°C -----------1 hour

The section was embedded using plastic embedding devices Biotics Sweden and stainless moulds on the automatic embedding machine Leica, Austria. It was trimmed and sectioned on Leica rotator microtome after which it was stained with H&E technique.

H&E method

I. It was dewaxed in xylene for 2 changes and 2 minutes each
II. It was hydrated in descending grades of ethanol; 100%, 80% and 70% and then taken to water
III. It was stained in hematoxylin for 10 minutes
IV. It was washed in water
V. It was differentiated in 1% acid alcohol and was controlled microscopically
VI. It was washed in water
VII. It was blued in alkaline solution and washed with tap water for 2 minutes
VIII. It was washed in water
IX. It was counter-stained with eosin for 60 seconds
X. It was dehydrated in ascending grades of alcohol; 70%, 80% and 100%
XI. It was cleared in xylene in 2 changes for 2 minutes each
XII. It was mounted with synthetic mountant distyrene plasticizer xylene (DPX)
XIII. Results; Nuclei – blue, red blood cell – red and cytoplasm – pink (Suryalakshmi *et al*., 2016).

3.4 Statistical analyses

Data obtained from the study were expressed as mean ± SEM. Data was analyzed using one-way Analysis of Variance (ANOVA) and *Tukey's post hoc* test was used to compare value across groups. Values of $P<0.05$ were considered significantly using SPSS version 23.

CHAPTER FOUR

4.0 RESULTS

4.1 Proximate Analysis of Cocoa Powder

Table 4.1 one shows that the brand Oluji cocoa powder used for the study contained: 4.2 % of lipids, 8.7 % of moisture, 7.1 % of flavonoids, 0.65 % of saponins, 0.81 % of tannins, 1.5 % of ash value, 38.49 % of carbohydrates, 37.25 % of protein, and. 1.3 % of crude fibre

Table 4.2 represents the LD_{50} that was calculated according to the method by Lorke (1983) and it was found to be greater than 5000 due to the observation of no mortality.

Table 4.1: Proximate analysis of cocoa powder

Compound	Quantity (%)
Lipids	5.2
Ash value	1.5
Moisture	9.7
Flavonoids	8.1
Saponins	0.65
Tannins	0.81
Proteins	40.25
Carbohydrates	42.05
Crude fibre	1.3

Table 4.2: LD_{50} (lethal dose) of cocoa powder

Phase one

Groups	Dose (mg/kg of bw)	Number of deaths/Number of rats
Group 1	10	0/3
Group 2	100	0/3
Group 3	1000	0/3

Phase two

Groups	Dose (mg/kg of bw)	Number of deaths/Number of rats
Group 1	1600	0/1
Group 2	2900	0/1
Group 3	5000	0/1

4.2 Lipid Profile

In Figure 4.1, serum total cholesterol of P407 (1551.67±129.80 mg/dL) and P407+ATV (1043.82±11.49 mg/dL) were significantly ($p<0.05$) higher when compared to NC (48.93±2.49 mg/dL). Serum levels of total cholesterol in P407+ATV (1043.82±11.49 mg/dL), P407+250 CP (269.79±12.05 mg/dL), P407+ 500 CP (256.69±7.12 mg/dL) and P407+1000 CP (151.83±12.79 mg/dL) were significantly ($p<0.05$) lower when compared to P407 (1551.67±129.80 mg/dL). Also, serum total cholesterol levels were significantly ($p<0.05$) lower in P407+250 CP (269.79±12.05 mg/dL), P407+500 CP (256.69±7.12 mg/dL) and P407+1000 CP (151.83±12.7 9mg/dL) when compared to P407+ATV (1043.82±11.49 mg/dL).

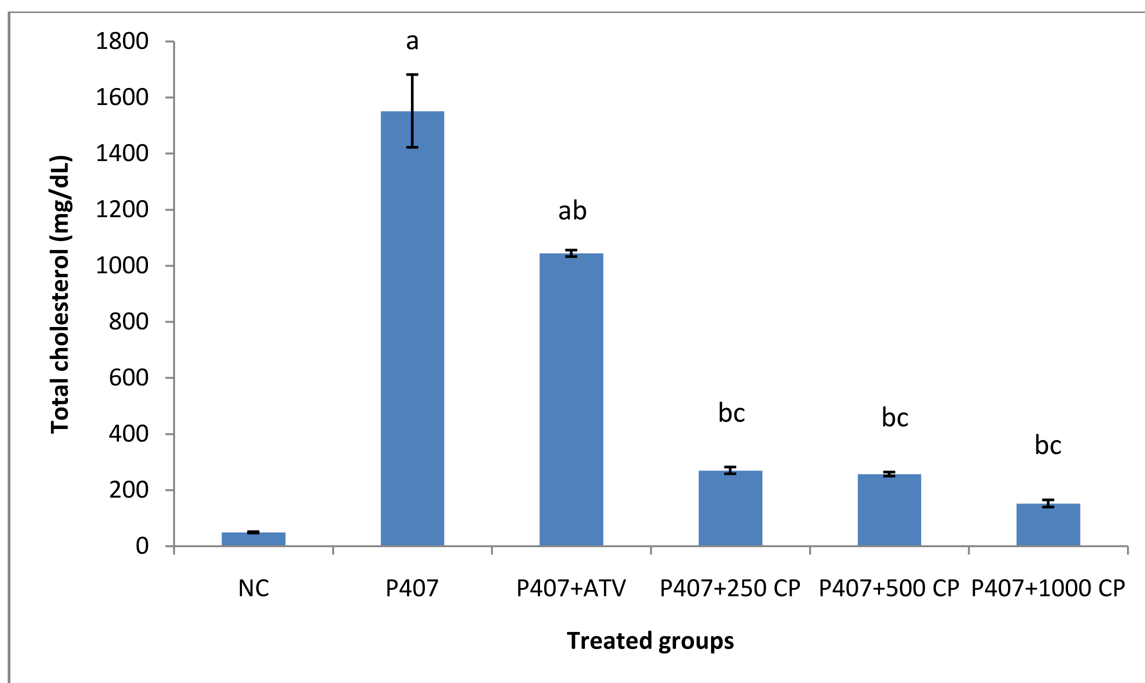

Figure 4.1; Effect of cocoa powder on total cholesterol

Results are presented as mean ± standard error of the mean. a= significant difference at $p \leq 0.05$ when compared to NC (normal control), b= significant difference at $p \leq 0.05$ when compared to P407 (poloxamer 407), c= significant difference at $p \leq 0.05$ when compared to P407+ATV (poloxamer 407+ atorvastatin) CP= Cocoa powder. One way ANOVA was used for analysis and *Tukey's post hoc* test was used to compare values across groups

In Figure 4.2, serum triglyceride was significantly ($p<0.05$) higher in P407 group (294.66±54.03 mg/dL), P407+250 CP (213.51±19.51 mg/dL) and P407+500 CP (159.62±15.25 mg/dL) when compared to NC (31.17±4.43 mg/dL). Serum triglyceride levels in P407+ATV (52.70±7.86 mg/dL), P407+ 500 CP (159.62±15.25 mg/dL) and P407+1000 CP (55.93±2.89 mg/dL) were significantly ($p<0.05$) lower when compared to P407 (294.66±54.03 mg/dL), while serum triglyceride level was significantly ($p<0.05$) higher in P407+250 CP (213.51±19.51 mg/dL) when compared to P407+ATV (52.70±7.86 mg/dL).

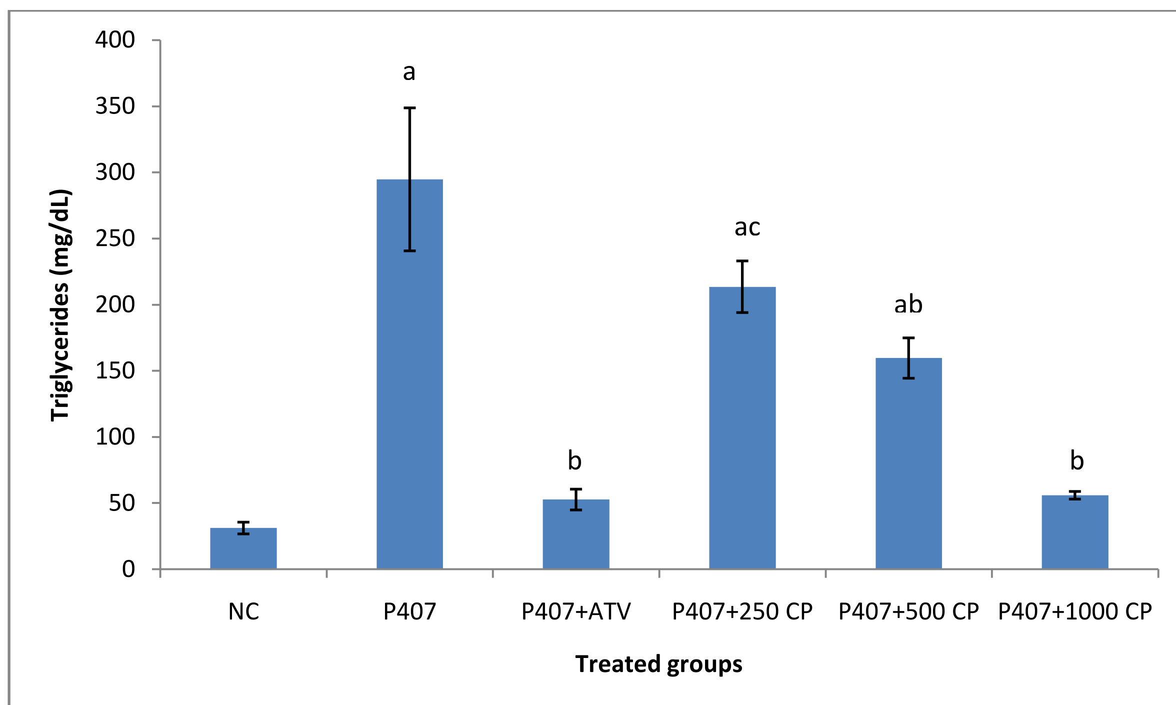

Figure 4.2; Effect of cocoa powder on triglyceride

Results are presented as mean ± standard error of the mean. a= significant difference at $p \leq 0.05$ when compared to NC (normal control), b= significant difference at $p \leq 0.05$ when compared to P407 (poloxamer 407), c= significant difference at $p \leq 0.05$ when compared to P407+ATV (poloxamer 407+ atorvastatin) CP= Cocoa powder. One way ANOVA was used for analysis and *Tukey's post hoc* test was used to compare values across groups.

In Figure 4.3, serum LDL was significantly ($p<0.05$) higher in P407 (1124.20±22.07 mg/dL), P407+ATV (132.44±18.72 mg/dL), P407+250 CP (227.90±15.17 mg/dL), P407+500 CP (165.92±18.68 mg/dL) and P407+1000 CP (153.17±4.12 mg/dL) when compared to NC (19.35±3.34 mg/dL). Serum levels of LDL were significantly ($p<0.05$) lower in P407+ATV (132.44±18.72 mg/dL), P407+250 CP (227.90±15.17 mg/dL), P407+500 CP (165.92±18.638 mg/dL) and P407+1000 CP (153.17±4.12 mg/dL), when compared to P407 (1124.20±22.07 mg/dL) and serum LDL level was significantly ($p<0.05$) higher in P407+250 CP (227.90±15.17 mg/dL), when compared to P407+ATV (132.44±18.72 mg/dL) was observed.

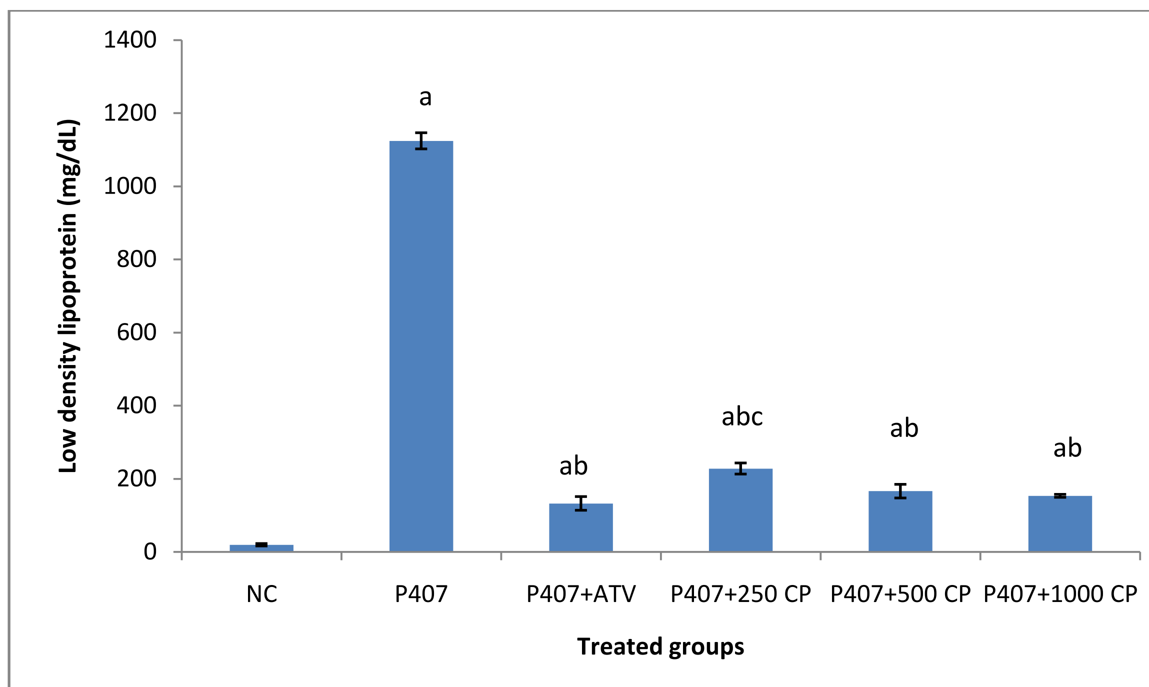

Figure 4.3; Effect of cocoa powder on low-density lipoprotein

Results are presented as mean ± standard error of the mean. a= significant difference at $p \le 0.05$ when compared to NC (normal control), b= significant difference at $p \le 0.05$ when compared to P407 (poloxamer 407), c= significant difference at $p \le 0.05$ when compared to P407+ATV (poloxamer 407+ atorvastatin) CP= Cocoa powder. One way ANOVA was used for analysis and *Tukey's post hoc* test was used to compare values across groups

In Figure 4.4 serum HDL levels were significantly $p<0.05$ higher in P407 (210.78±18.29 mg/dL) and P407+ATV (181.45±21.81 mg/dL) when compared to NC (23.68±1.48 mg/dL). Also, HDL levels were significantly ($p<0.05$) lower in P407+250 CP (41.01±7.70 mg/dL), P407+500 CP (17.80±2.38 mg/dL) and P407+1000 CP (9.95±1.46 mg/dL), when compared to P407 (210.78±18.29 mg/dL) and P407+ATV (181.45±21.81 mg/dL).

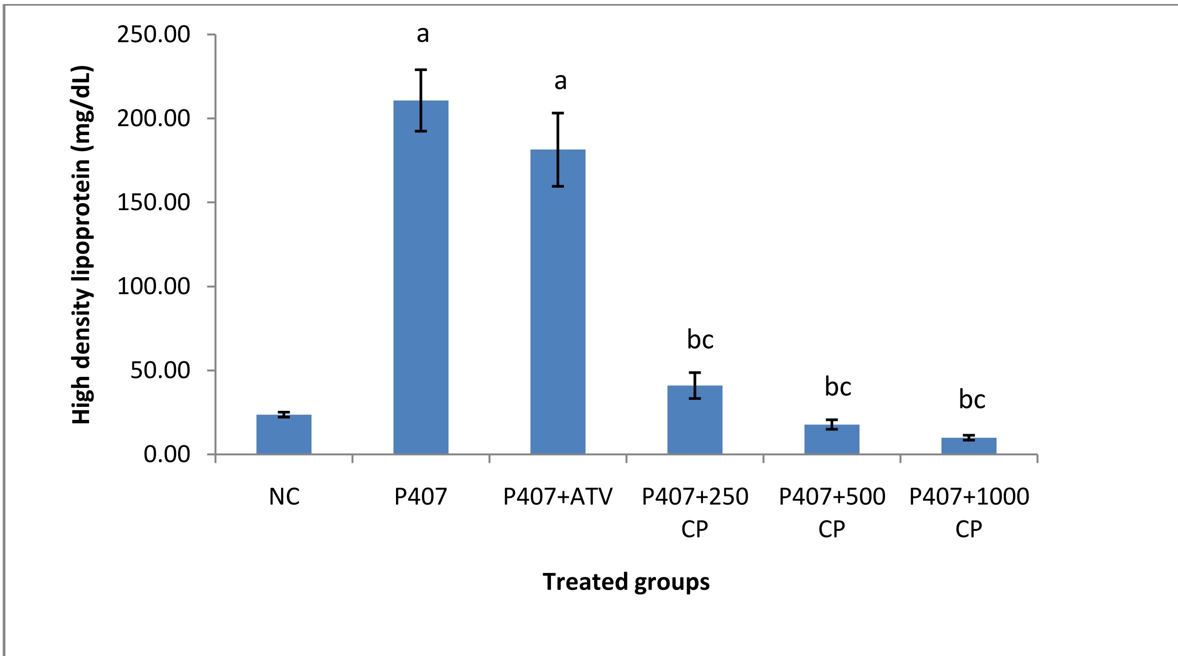

Figure 4.4; Effect of cocoa powder on high-density lipoprotein

Results were presented as mean ± standard error of the mean. a= significant difference at $p \leq 0.05$ when compared to NC (normal control), b= significant difference at $p \leq 0.05$ when compared to P407 (poloxamer 407), c= significant difference at $p \leq 0.05$ when compared to P407+ATV (poloxamer 407+ atorvastatin) CP= Cocoa powder. One way ANOVA was used for analysis and *Tukey's post hoc* test was used to compare values across groups

4.3 Oxidative Stress Parameters

The levels of serum MDA levels were significantly ($p<0.05$) higher in P407 (45.38±4.90 nmol/L), P407+ATV (43.15±2.95 nmol/L) and P407+250 CP (41.22±0.75 nmol/L) when compared to NC (18.72±0.98 nmol/L). When compared to P407 (45.38±4.90 nmol/ml), MDA was significantly ($p<0.05$) lower MDA in P407+500 CP (28.08±2.71 nmol/ml) and P407+1000 CP (25.92±2.28 nmol/ml), while P407+500 CP (28.08±2.71 nmol/ml) and P407+1000 CP (25.92±2.28 nmol/ml) were significantly ($p<0.05$) lower when compared to +P407+ATV (43.15±2.95 nmol/ml).

Serum activities of SOD in P407 (15.10±0.84 U/ml) were significantly ($p<0.05$) lower when compared to NC (33.94±1.94 U/ml). SOD activities in P407+ATV (27.04±1.84 U/ml), P407+250 CP (30.04±3.64 U/ml), P407+500 CP (30.96±3.75 U/ml) and P407+1000 CP (40.08±2.75 U/ml) were significantly ($p<0.05$) higher when compared to P407 (15.10±0.84 U/ml) and SOD activities in P407+1000 CP (40.08±2.75 U/ml) were significantly ($p<0.05$) higher when compared to P407+ATV (27.04±1.84 U/ml).
Serum GPx activities was significantly ($p<0.05$) higher in P407+500 CP (31.70±2.46 nmol/min/ml) and P407+1000 CP (32.90±1.43 nmol/min/ml) when compared to P407 (23.40±1.63 nmol/min/ml).

There was no statistical significance across the groups in CAT levels in the serum.

Table 4.3: Effect of Cocoa Powder on Serum Biomarkers of Oxidative Stress and Lipid Peroxidation

Treatment	MDA (nmol/L)	SOD (U/ml)	GPx (nmol/min/ml)	CAT (nmol/min/ml)
Normal control	18.72±0.98	33.94±1.94	26.80±2.10	17.40±2.73
P407	45.38±4.90[a]	15.10±0.84[a]	23.40±1.63	12.52±2.35
P407+ATV	43.15±2.95[a]	27.04±1.84[b]	27.40±2.20	12.60±1.21
P407+250mg/kg CP	41.22±0.75[a]	30.04±3.64[b]	30.70±0.75	13.22±1.08
P407+500mg/kg CP	28.08±2.71[bc]	30.96±3.75[b]	31.70±2.46[b]	15.86±1.47
P407+1000mg/kg CP	25.92±2.28[bc]	40.08±2.75[bc]	32.90±1.43[b]	10.44±0.39

a= significant difference at $p \leq 0.05$ when compared to NC (normal control), b= significant difference at $p \leq 0.05$ when compared to P407 (poloxamer 407), c= significant difference at $p \leq 0.05$ when compared to P407+ATV (poloxamer 407+ atorvastatin) CP= Cocoa powder. ATV= atorvastatin, SOD= superoxide dismutase, MDA= malondialdehyde, GPx= glutathione peroxidase and CAT= catalase

4.4 Biomarkers of Inflammation

In Figure 4.5, there was a decrease in TNF-α in heart homogenate in P407+ATV (235.74±23.52 pg/ml), P407+500 (246.53±12.09 pg/ml) and P407+1000 (260.96±18.55 pg/ml) when compared to NC (306.62±21.72 pg/ml) and P407 (313.62±25.38 pg/ml). The decrease was not statistically significant. There was an increase in TNF-α in heart homogenate in P407+250 (321.38±19.05 pg/ml), P407+500 (246.53±12.09 pg/ml) and P407+1000 (260.96±18.55 pg/ml) when compared to P407+ATV (235.74±23.52 pg/ml). The increase was not statistically significant.

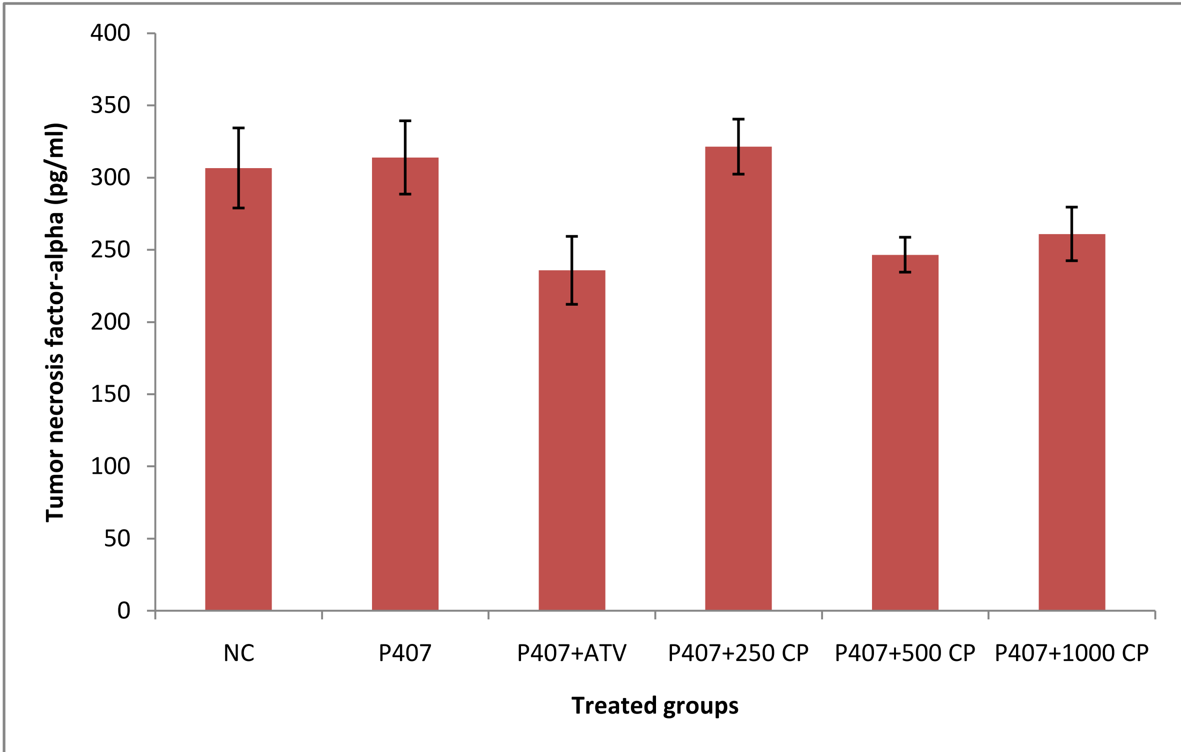

Figure 4.5; Effect of cocoa powder on tumour necrosis factor-α

Results were presented as mean ± standard error of the mean. ATV= atorvastatin CP= Cocoa powder

In figure 4.6 IL-10 levels were significantly ($p<0.05$) lower in P407 (876.42±15.52 pg/ml), P407+ATV (656.54±23.50 pg/ml), P407+250 CP (769.83±40.52 pg/ml), P407+500 CP (718.30±41.39 pg/ml) and P407+1000 CP (919.88±57.99 pg/ml) when compared to NC (1113.14±63.70 pg/ml). The levels of serum IL-10 was significantly ($p<0.05$) lower in P407+ATV (656.54±23.50 pg/ml) when compared to P407 (876.42±15.52 pg/ml) and it was statistically significantly ($p<0.05$) higher in P407+1000 CP (919.88±57.99 pg/ml) when compared to P407+ATV (656.54±23.50 pg/ml).

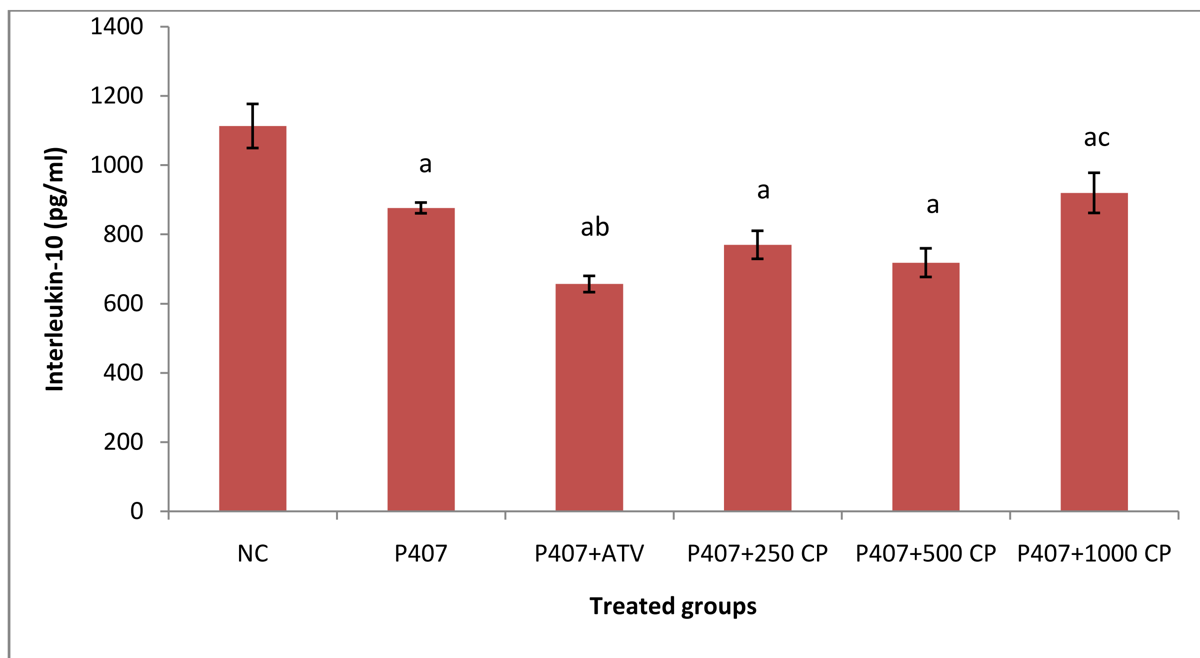

Figure 4.6; Effect of cocoa powder on interleukin-10

Results were presented as mean ± standard error of the mean. a= significant difference at $p \leq 0.05$ when compared to NC (normal control), b= significant difference at $p \leq 0.05$ when compared to P407 (poloxamer 407), c= significant difference at $p \leq 0.05$ when compared to P407+ATV (poloxamer 407+ atorvastatin) CP= Cocoa powder

4.5 Result of Cocoa Powder on Histopathological Changes of Coronary Arteries

Plate I shows normal *tunica intima*, *tunica media*, *tunica adventitia* of blood vessels with blood in the lumen and normal spindle-shaped cylindrical nuclei

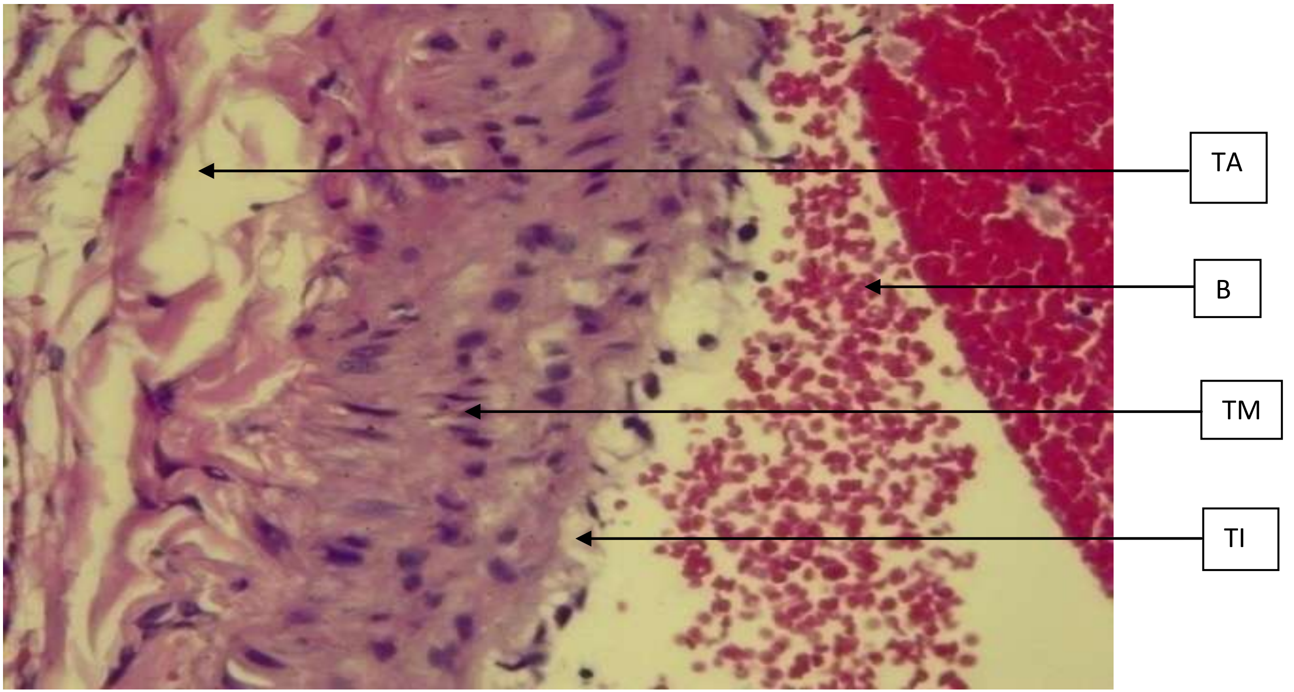

Plate I: Photomicrograph of cross-section of coronary artery in normal control group. *Tunica adventitia* (TA), *tunica media* (TM), *tunica intima* (TI) and blood (b). H&E, (Mag x 300)

In Plate II, which is P407, there was adipocyte infiltration and slight collagen destruction in the *tunica adventitia* and slightly inflamed nuclei in the *tunica media*

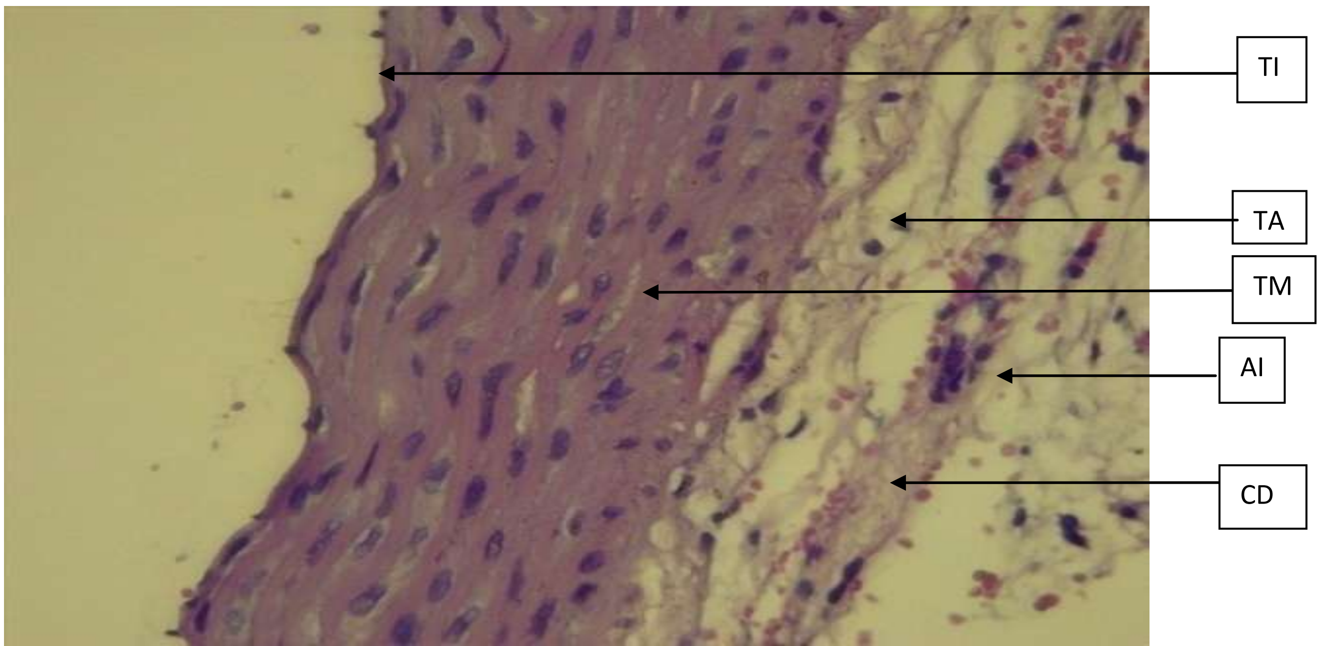

Plate II: Photomicrograph of cross-section of coronary artery in P407. *Tunica adventitia* (TA), *tunica media* (TM), *tunica intima* (TI), Collagen destruction (CD) and adipocytes infiltration (AI), H&E (Mag x 300)

Plate III shows inflamed nuclei in *tunica media* and adipocyte infiltration in *tunica adventitia*.

This micrograph is for P407+ATV

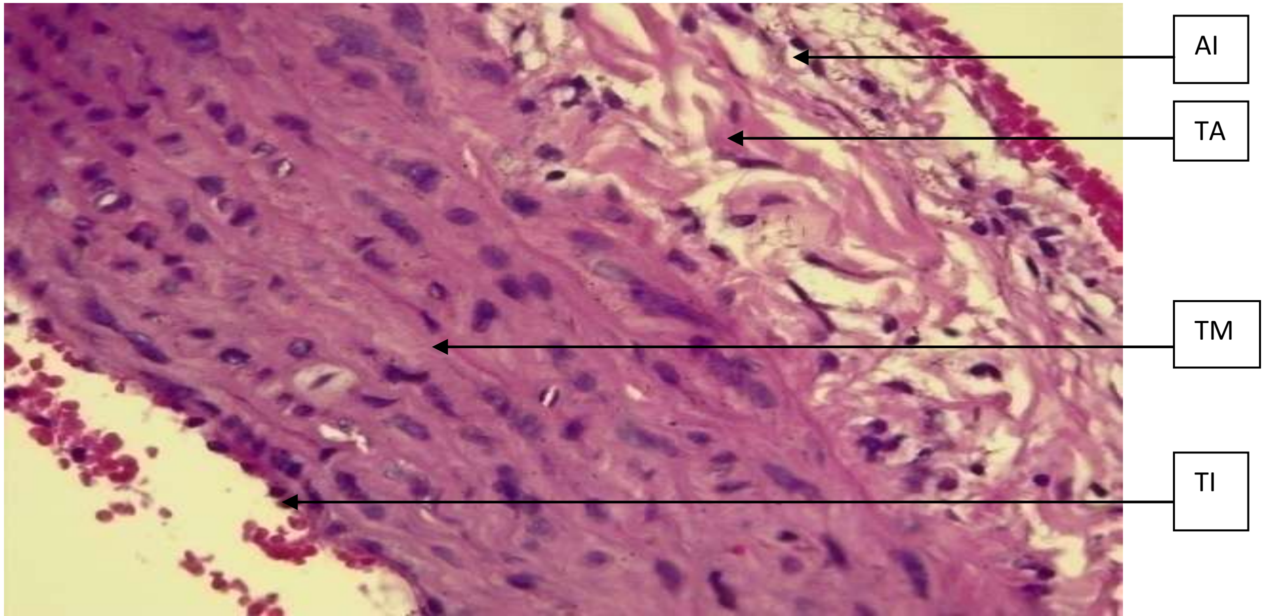

Plate III: Photomicrograph of cross-section of coronary artery in P407+ATV. *Tunica adventitia* (TA), *tunica media* (TM), *tunica intima* (TI) and adipocytes infiltration (AI), H&E (Mag x 300)

In plate IV belonging to P407+250 CP there was inflamed nuclei in *tunica media* and adipocyte infiltration and collagen destruction in *tunica adventitia.* The adipocyte infiltration was less compared to P407

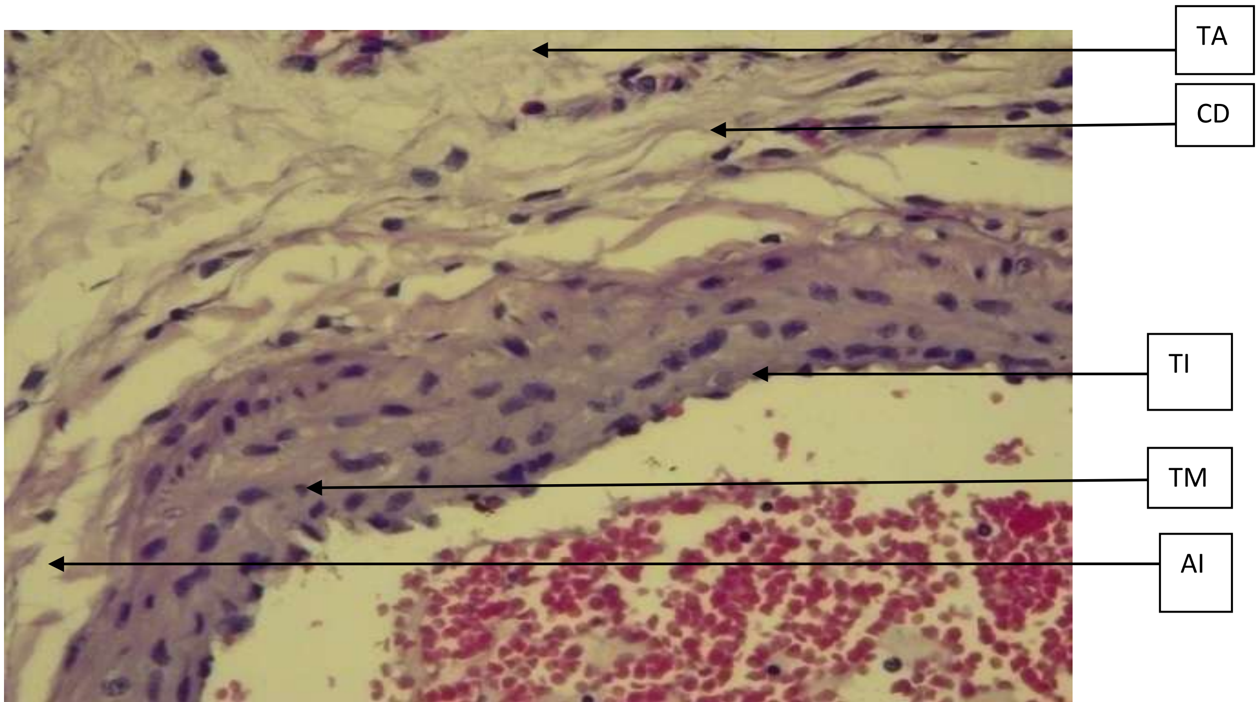

Plate IV: Photomicrograph of cross-section of coronary artery in P407+250 CP. *Tunica adventiti*a (TA), *tunica media* (TM), *tunica intima* (TI) collagen destruction (CD), and adipocytes infiltration (AI), H&E (Mag x 300)

Plate V reveals widened width of *tunica media* and enlarged nuclei in it, adipocyte infiltration in the *tunica adventitia* and lumen of the vessel in P407+500 CP

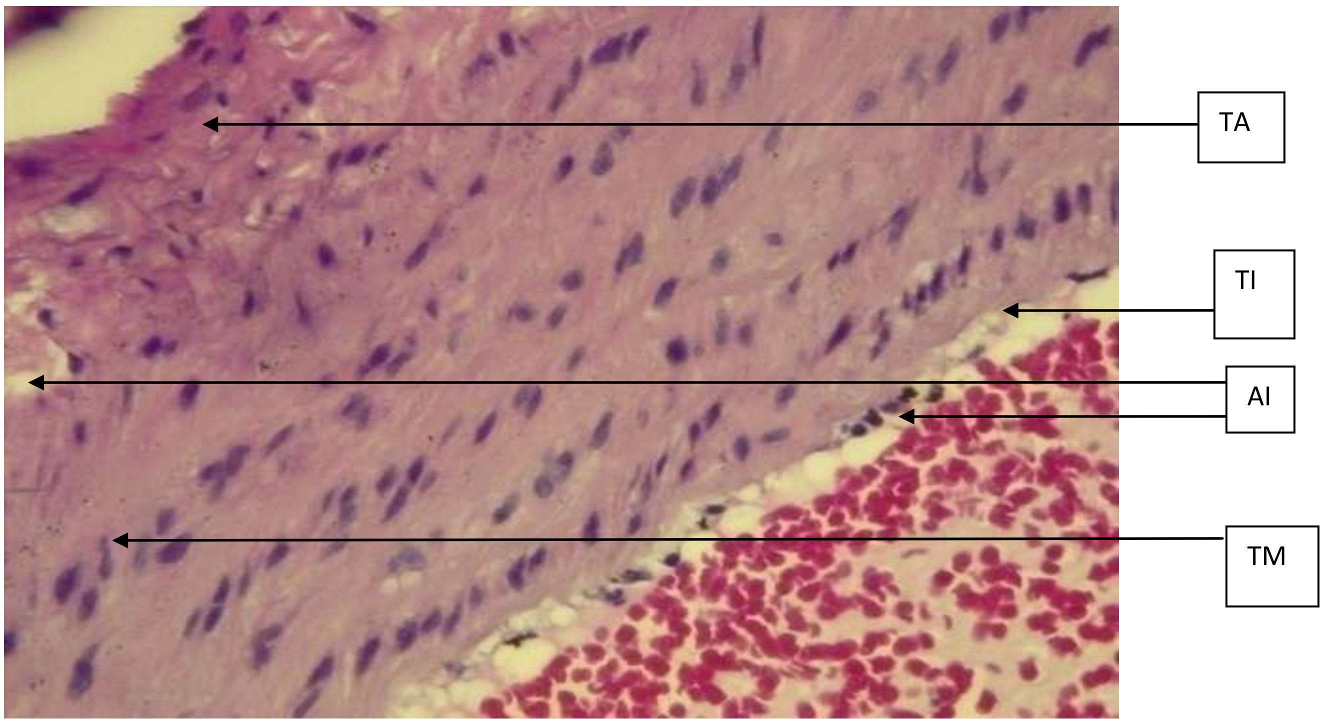

Plate v: Photomicrograph of the cross-section of coronary artery in P407+500 CP. *Tunica adventitia* (TA), *tunica media* (TM), *tunica intima* (TI) collagen destruction (CD), and adipocytes infiltration (AI), H&E (Mag x 300)

There is adipocyte infiltration in *tunica adventitia* and shrinkage of nuclei in the *tunica media* in plate VI which is for P407+1000mg/kg CP.

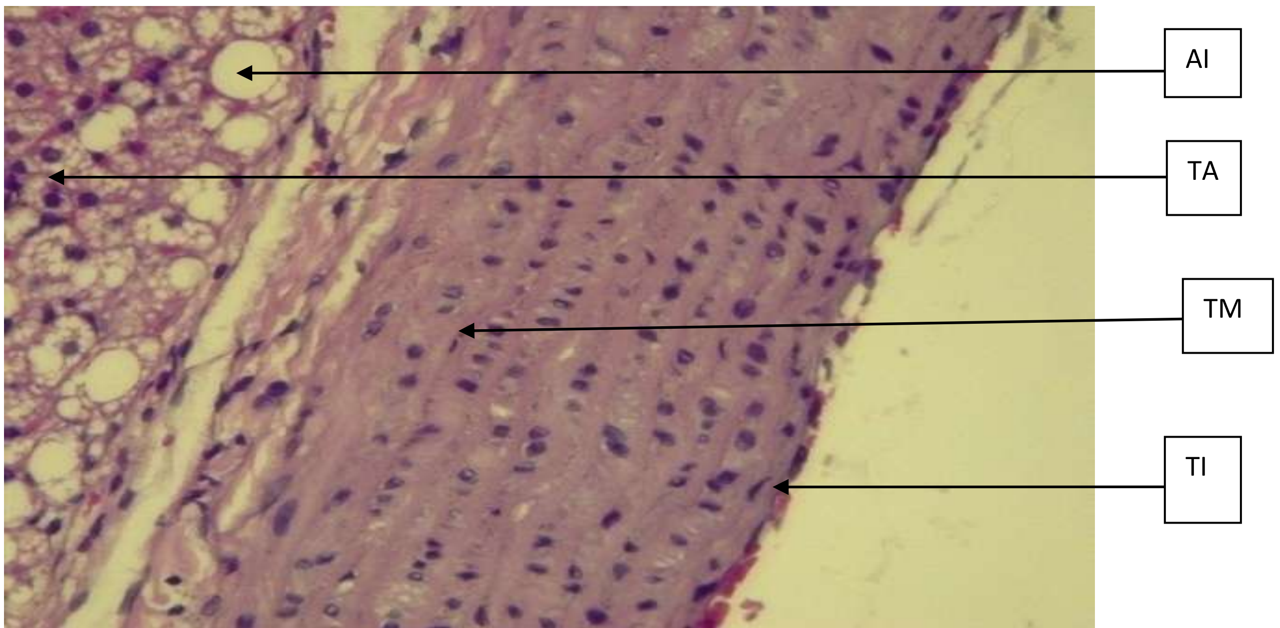

Plate VI: Photomicrograph of the cross-section of coronary artery in P407+1000 CP. *Tunica adventitia* (TA), *tunica media* (TM), *tunica intima* (TI) and adipocyte infiltration (AI), H&E (Mag x 300)

CHAPTER FIVE

5.0 DISCUSSION

Phytochemicals are biologically active compounds from plants which possess the ability to regulate physiological and pathological processes with beneficial effects on human health (Sobhani *et al*., 2020). In the present study, poloxamer 407 was administered to induce hyperlipidaemia. Poloxamer 407 induced hyperlipidaemia by inhibition of triglyceride degredation by directly inhibiting capillary lipoprotein lipase responsible for plasma triglyceride hydrolysis. (Wu *et al*., 2019).

The present study showed the presence of flavonoids, saponins, tannins, carbohydrates, proteins, lipids, ash, moisture and crude fibre. Flavonoids have been reported to inhibit LDL oxidation by 75% (Anosike and Cajetan, 2015). Some isoflavones (a type of flavonoid) increases resistance to LDL oxidation. Also, saponins, a phytochemical, lowers lipids by forming insoluble complexes with cholesterol or their bile salt precursor, thus making them unavailable for absorption (Navarro del Hierro *et al*., 2018). Also, flavonoids have been reported to stimulate the conversion of cholesterol to bile acids in the liver of rabbits fed with high fat diet and inhibit lipid absorption in the intestine (Abe *et al*., 2000; Koo and Noh, 2007). A study carried out by Dali *et al*., (2019) showed that flavonoids improved lipid profile in hypercholesterolemic rats (Dali *et al*., 2019). The protein component of cocoa powder might have played a role in the reduction of lipids because the peptides contained in protein hydrolysates (due to their hydrophobicity) posses high binding ability with bile acids and greatly reduces cholesterol levels by restricting reabsorption of bile salts in the ileum (Iwami *et al*., 1986) and the jejunum (Nagaoka *et al*., 1997); inhibiting the micelles solubility

of cholesterol absorption. This is caused by the direct interaction of the cholesterol mixed micelles and protein peptide hydrosylates with the bound phospholipids in the jejunal epithelia (Nagaoka *et al*., 1997).

The total cholesterol, triglyceride and LDL lowering effect of cocoa powder in the present study could be attributed to oxidative modification of LDL which is the major cause of atherosclerosis and atherosclerotic complications like coronary heart disease and this is highly linked to peroxyl radical formation (Brown and Goldstein, 1983;Frankel and Meyer, 2000). The radical-mediated oxidative chain reaction is a possible mechanism involved in LDL-oxidation. LDL-oxidation is a complex process with multiple steps involving both lipid and protein fractions via various mechanisms (Marnett, 2000). The standard drug atorvastatin, used in this study is reported to inhibit HMG CoA reductase, a rate limiting enzyme in the biosynthesis of cholesterol. Sheneni *et al*., (2018) postulated that atorvastatin might not decrease triglyceride concentration by activating lipoprotein lipase, the cocoa powder could have reduced triglyceride by either activating endothelium-bound lipoprotein lipase which hydrolyses the triglyceride into fatty acid and hence decreases triglyceride levels or by inhibiting lipolysis so that fatty acids are not converted to triglyceride (Shenani *et al* 2018).

Poloxamer 407 induced hyperlipidaemia resulted in elevation of serum MDA, an indication of oxidative stress. However, cocoa powder attenuated lipid peroxidation possibly via flavonoids causing increased enzyme activities which may suggest a greater capacity to clear oxygen free radicals, thus lowering MDA (Iskender *et al*., 2016). Saponins have been linked to decreased lipid peroxidation and elevated SOD and GPx activities. Also, saponins have

antiradical activity against OH radicals and O_2- radical scavenging activity *in vitro* (Li *et al*., 2010). Decroix *et al*., (2018) reported lowered levels of MDA in male cyclist subjected to exercise that were given 1765mg of cocoa flavonols (Decroix *et al*., 2018). MDA is produced by lipid decomposition of peroxide, thus MDA levels are usually used as marker of lipid peroxidation and reacts as free radicals are generated (Pillai *et al*., 2005).

There was an increase in SOD which is in line with Wu *et al*., (2020) that reported increase SOD activities in high-fat diet induced hyperlipidaemic rats. After treatment, cocoa powder performed better than atorvastatin in elevating SOD level, as SOD is considered the first line of defence against oxidative injury (Wang *et al*., 2018). Hyperlipidaemia eventually results in lower levels of SOD and GPx which may cause chain lipid peroxidation (Morin *et al*., 2015). The result obtained in SOD and GPx might have been due to flavonoids which seem to provide a protective effect by increasing the activity of antioxidant enzymes, which prepares cells to deal with generation of reactive oxygen species in the presence of stressors and this helps to minimize the oxidative stress induced damage (Cordero-Herrera *et al*., 2015). One of the main causes of atherosclerosis is LDL-modification and phenolic antioxidants are suggested to acts as inhibitors of LDL modification through free radical scavenging and hydrogen ion donation (Frankel and Meyer 2000). Flavonoids exert their anti-inflammatory and antioxidant effects through the activation of Nuclear factor-erythroid 2-related factor 2 (Nrf2), which induces the antioxidant enzymes transcription and inhibits Nuclear factor-kappa B (Nf-Kb) which is a key transcription factor in inflammatory responses (Mauro and Ilaria, 2016). Sànchez-Sànchez reported that polyphenol enriched cocoa extract activated SOD enzyme activity in embryos and in adult fish tissue by regulating expression of genes implicated in oxidative stress (Sànchez-Sànchez *et al*., 2018). However, the findings in this

study contradicts those recorded by Martins *et al*., 2020 in which flavonoids decrease glutathione peroxidase in human endothelial cells that were induced with oxidative stress (Martins *et al*., 2020).

The increase in IL-10 which was observed could be due to flavonoids and saponins. The outcome in this study is in line with Sunil *et al*., 2021 that reported an upregulation in IL-10 levels in lipopolysaccharide stimulated RAW 264.7 cells caused by flavonoids (Sunil *et al*., 2021). Flavonoids manifest anti-atherosclerotic effects in part due to its anti-inflammatory properties. Flavonoids reduce expression of VCAM-1, ICAM-1, E-selectin or MCP-1 in cultures of human enterochromaffin cells exposed to different pro-inflammatory stimuli (Bhaskar and Helen, 2016;Calabriso *et al*., 2016;Vera *et al*., 2018). Saponins upregulate the mRNA expression levels of IL-10 (Uttra *et al*., 2018). Following the induction of hyperlipidaemia, the microenvironment is dominated by pro-inflammatory immune cells (e.g neutrophils and M1 macrophages) (Yang and Hu, 2018) which release pro-inflammatory cytokines (eg TNF-α). This facilitates the migration and proliferation of dormant satellite cells (Peterson *et al*., 2011). As M1 transitions to M2, they secrete anti-inflammatory cytokines (eg IL-10), which reduces inflammation (Yang and Hu, 2018). The increase in pro-inflammatory cytokine may reflect a protein-facilitated eradication of inflammation.

Increased levels of IL-10 levels might be associated with the change of microbiota composition induced by crude fibre in cocoa powder (Shang *et al*., 2019). Cytokines direct inflammatory response and a balance between pro-inflammatory and anti-inflammatory cytokines are vital for protection against cardiovascular diseases. Pro-inflammatory cytokines initiate inflammatory response and have negative effects on the intestinal integrity and

epithelial function (Al-Sadi *et al*., 2009). In contrast, anti-inflammatory cytokines are a series of immune-regulatory molecules that control the pro-inflammatory cytokine response (Opal and DePalo, 2000). Most pro-inflammatory cytokines cause pathological opening of tight junction barrier thus increasing intestinal epithelial permeability, thereby facilitating movement of lipids into the interstitial fluid which will increase blood levels of cholesterol. IL-10 has an important barrier protective effect (Al-Sadi *et al*., 2009).

The low and moderate doses gave the best protection against fat deposition in the wall of the coronary artery in comparison to the highest dose, thus suggesting that the beneficial effect of cocoa is not dependent on a high dose. Flavonoids affected the histology of the coronary artery since they possess the ability to lower plasma lipids which is associated with the consequential reduction of lipid peroxides levels and aortic atherosclerotic plaque areas in apoE-deficient mice (Naveed *et al*., 2018). Decrease of atherosclerotic lesions is also due to the decreased inflammatory stress measured as decreased IL-1 (Lu *et al*., 2017). This was also seen in a study carried out by Zhou *et al*., (2018) in which flavonoids alleviated histopathological changes in the aorta of rats (Zhou *et al*., 2018). Also, Guan *et al*., (2016) reported that diet consisting of 0.2% and 2% cocoa powder reduced atherosclerosis in mice by the reduction of serum cholesterol, especially LDL, thereby inhibiting the development of atherosclerosis through lipid-lowering effect and also by modulation of intestinal inflammation (Guan *et al*., 2016).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

In conclusion, cocoa powder significantly decreased serum levels of total cholesterol, triglycerides and LDL in hyperlipidaemia, hence may have cardio protective properties and decrease the risk of cardiovascular diseases. Cocoa powder significantly decreased total cholesterol, triglyceride and LDL in hyperlipidaemia, but it did not restore the lipid levels to normal levels as was observed in the normal control. Cocoa powder significantly decreased plasma levels of MDA while it significantly increased SOD and GPx, thus indicating that cocoa powder reduces oxidative stress. Cocoa powder significantly decreased MDA levels in hyperlipidaemia but it did not restore MDA levels to normal levels as that observed in normal control. It also increased plasma level of SOD in hyperlipidaemia in group 6 (poloxamer 407+1000mg CP) to even higher levels than that seen in normal control. Cocoa powder significantly increased plasma levels of IL-10 at the highest dose of 1000mg/kg, in comparison to positive control thus indicating that cocoa powder combats inflammation positively. The hypolipidaemic and antioxidant effect of cocoa powder may be attributable to flavonoids and saponins contained in cocoa powder. The low and moderate dose of cocoa powder reduced atherosclerosis more than the highest dose.

6.2 Recommendation

This study has the following recommendation:

i. Cocoa powder might be considered as a hypolipidaemic agent and as an oxidative stress lowering agent

ii. Studies on long-term effect of cocoa powder administration should be done

iii. Further study should be carried out on the individual effect of each compound that is constituted in cocoa powder

6.3 Contributions to knowledge

i. At doses of 250 mg/kg, 500 mg/kg and 1000 mg/kg cocoa powder significantly ($p<0.05$) decreased serum levels of total cholesterol (269.79 ± 12.05 mg/dL, 256.69 ± 7.12 mg/dL and 151.83 ± 12.79 mg/dL). At doses of 500 mg/kg and 1000 mg/kg triglycerides serum levels were significantly ($p<0.05$) decreased (159.62±15.25 mg/dL and 55.93 ± 2.89 mg/dL) and LDL serum levels were significantly ($p<0.05$) decreased (227.90 ± 15.17 mg/dL, 165.92 ± 18.68 mg/dL and 153.17 ± 4.12 mg/dL). The decrease was in comparison to P407 group (1551.67 ± 129.80 mg/dL) (F = 129.55, 18.57, 689.83)

ii Cocoa powder significantly ($p<0.05$) decreased serum levels of MDA at doses of 500 mg/kg (28.08 ± 2.71 nmol/L) and 1000 mg/kg (25.92 ± 2.29 nmol/L) when compared to P407 (45.38 ± 4.91 nmol/L) (F = 15.37).

iii. At doses of 250 mg/kg, 500 mg/kg and 1000 mg/kg (30.04 ± 3.64 U/ml, 30.96 ± 3.75 U/ml and 40.08 ± 2.75 U/ml). Cocoa powder significantly ($p<0.05$) increased serum activities of SOD when compared to P407 (15.10 ± 0.84 U/ml) (F = 9.76)

iv. Cocoa powder significantly ($p<0.05$) increased serum activities of GPx at doses 500 mg/kg and 1000 mg/kg (31.70 ± 2.46 nmol/min/ml and 32.90 ± 1.43 nmol/min/ml) when compared to P407 group (23.40 ± 1.63 nmol/min/ml) ($F = 3.73$)

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APPENDIX

RAW VALUES

GROUPS	PARAMETERS									
	CHOL	**TRIG**	**LDL**	**HDL**	**MDA**	**SOD**	**GPx**	**CAT**	**TNF-α**	**IL-10**
Control	47.06	29.35	25.33	17.86	17	40.2	27.5	11.6	243.7	1077.23
Control	47.06	36.16	13.03	25	20.6	35.4	22.5	21.7	352.83	981.68
Control	58.82	25.81	29.37	24.29	19	30.9	34.5	22.9	384.45	1265.71
Control	46.01	45.16	15	26.12	21	34.2	25.5	10	250.1	980.56
Control	45.72	19.35	14.04	25.14	16	29	24	20.8	302	1260.51
Poloxamer	1200	387.1	1176.15	246.43	31.6	18.3	27	18.5	385.4	863.87
Poloxamer	1441.18	387	1060.19	223.57	35.6	14.6	26	11.8	365.1	899.21
Poloxamer	1417.15	374.19	1128.52	140.29	52.5	15.1	20	17.3	273.89	820.68
Poloxamer	1900	168	1090	226.45	51.6	13.5	19	7	280	900.01
Poloxamer	1800	157	1166.16	217.16	55.6	14	25	8	265	898.31
P+ATV	1064.71	41.15	91.5	189.29	47.5	29.1	27	11	239.18	698.95
P+ATV	1005.88	37.24	169.4	171.43	48.9	29	36	15	196.39	689.17
P+ATV	1029.41	81.31	185.16	235.71	47.3	31.5	25	11	283.12	696.34
P+ATV	1054	56.64	109.01	205.87	35	24.	24	16	290	598.14
P+ATV	1065.12	47.16	107.12	105.14	37	24	25	10	170.03	600.1
P+250 CP	223.53	219.35	199.73	60.71	41.7	37.5	30.5	15.5	363.05	926.7
P+250 CP	288.24	283.87	285.06	25	40.6	35	30.5	11	309.01	764.4
P+250 CP	270.49	193.55	225.61	21.43	43.7	20.4	33.5	12.6	264.84	727.75
P+250CP	278	203.47	224.1	53	39.1	35.3	29	11	365	700.16
P+250 CP	288.67	167.33	205	44.9	41	22	30	16	305	730.14

P+500 CP	261.76	183.87	240.05	10.71	35	24.4	35	15.1	264.84	804.97
P+500 CP	279.41	195.16	154.21	25	25.5	21.7	25.5	19.6	242.23	693.72
P+500 CP	245.06	109.35	145.29	14.29	26	29.5	26	12.6	272.47	819.37
P+500 CP	238.46	165.53	140.06	24	33.5	40.1	35	13	203.11	673.01
P+500 CP	258.76	144.18	150	15	20.4	39.1	37	19	250.21	600.45
P+1000 CP	156.47	51.61	165.46	7.14	30.5	37.3	37.5	10.6	204.47	819.67
P+1000 CP	125.88	66.77	141.57	7.14	23.4	31	30.5	10.7	259.63	926.7
P+1000 CP	188.24	55.81	158.82	10.71	18.5	41	31	9.6	321.68	1126.96
P+1000 CP	120.46	50.45	151	15.07	26.7	45	35	9.6	260	925.6
P+1000 CP	168.12	55	149	9.9	30.5	46.1	30.5	11.7	259	800.45

Chol-cholesterol, trig- triglyceride, LDL-low density lipoprotein, HDL-high density lipoprotein, MDA-malondialdehyde SOD-superoxide dismutase, GPx- glutathione peroxidase, CAT- catalase, TNF-α-tumour necrosis factor, IL-10-interleukin-10, P-poloxamer 407, ATV-atorvastatin, CP- cocoa powder

STATISTCAL ANALYSIS

TOTAL CHOLESTEROL

GROUPS	MEAN	STD. ERROR OF MEAN
NC	48.9340	2.48633
P	1551.6660	129.80313
P+ATV	1043.8240	11.49088
P+250 CP	269.7860	12.05143
P+500 CP	256.6900	7.11961
P+1000 CP	151.8340	12.78683
TOTAL	553.7890	104.23919

TRIGLYCERIDE

GROUPS	MEAN	STD. ERROR OF MEAN
NC	31.1660	4.42930
P	294.6580	54.03234
P+ATV	52.7000	7.86499
P+250 CP	213.5140	19.51465
P+500 CP	159.6180	15.25049
P+1000 CP	55.9280	2.89031
TOTAL	134.5973	20.13721

LOW DENSITY LIPOPROTEIN

GROUPS	MEAN	STD. ERROR OF MEAN
NC	19.3540	3.34082
P	1124.2040	22.07377
P+ATV	132.4380	18.72377
P+250 CP	227.9000	15.17384
P+500 CP	165.9220	18.68176
P+1000 CP	153.1700	4.12083
TOTAL	303.8313	69.33919

HIGH DENSITY LIPOPROTEIN

GROUPS	MEAN	STD. ERROR OF MEAN
NC	23.6820	1.48445
P	210.7800	18.28879
P+ATV	181.4480	21.81265
P+250 CP	41.0080	7.70285
P+500 CP	17.8000	2.83468
P+1000 CP	9.9520	1.46025
TOTAL	80.7783	15.97178

MALONDIALDEHYDE

GROUPS	MEAN	STD. ERROR OF MEAN
NC	18.7200	0.97898
P	45.3800	4.89575
P+ATV	43.1460	2.94787
P+250 CP	41.2200	0.75193
P+500 CP	28.0800	2.71319
P+1000 CP	25.9200	2.28000
TOTAL	33.7443	2.12493

SUPEROXIDE DISMUTASE

GROUPS	MEAN	STD. ERROR OF MEAN
NC	33.9400	1.93690
P	15.1000	0.84439
P+ATV	27.0400	1.84190
P+250 CP	30.0400	3.64343
P+500 CP	30.9600	3.74641
P+1000 CP	40.0800	2.75198
TOTAL	29.5267	1.72614

GLUTATHIONE PEROXIDASE

GROUPS	MEAN	STD. ERROR OF MEAN
NC	26.8000	2.09523
P	23.4000	1.63095
P+ATV	27.4000	2.20454
P+250 CP	30.7000	0.75166
P+500 CP	31.7000	2.45764
P+1000 CP	32.9000	1.42653
TOTAL	28.8167	0.91648

CATALASE

GROUPS	MEAN	STD. ERROR OF MEAN
NC	17.4000	2.72672
P	12.5200	2.34551
P+ATV	12.6000	1.20830
P+250 CP	13.2200	1.07629
P+500 CP	15.8600	1.47024
P+1000 CP	10.4400	0.39319
TOTAL	13.6733	0.77049

TUMOUR NECROSIS FACTOR-α

GROUPS	MEAN	STD. ERROR OF MEAN
NC	306.6160	21.71972
P	313.8960	25.38123
P+ATV	235.7440	23.52347
P+250 CP	321.3800	19.04887
P+500 CP	246.5720	12.09434
P+1000 CP	260.9560	18.55340
TOTAL	280.8607	10.25007

INTERLEUKIN-10

GROUPS	MEAN	STD. ERROR OF MEAN
NC	1113.1380	63.69625
P	876.4160	15.52336
P+ATV	656.5400	23.49830
P+250 CP	769.8300	40.52013
P+500 CP	718.3040	41.39494
P+1000 CP	919.8760	57.99436
TOTAL	842.3507	32.33671

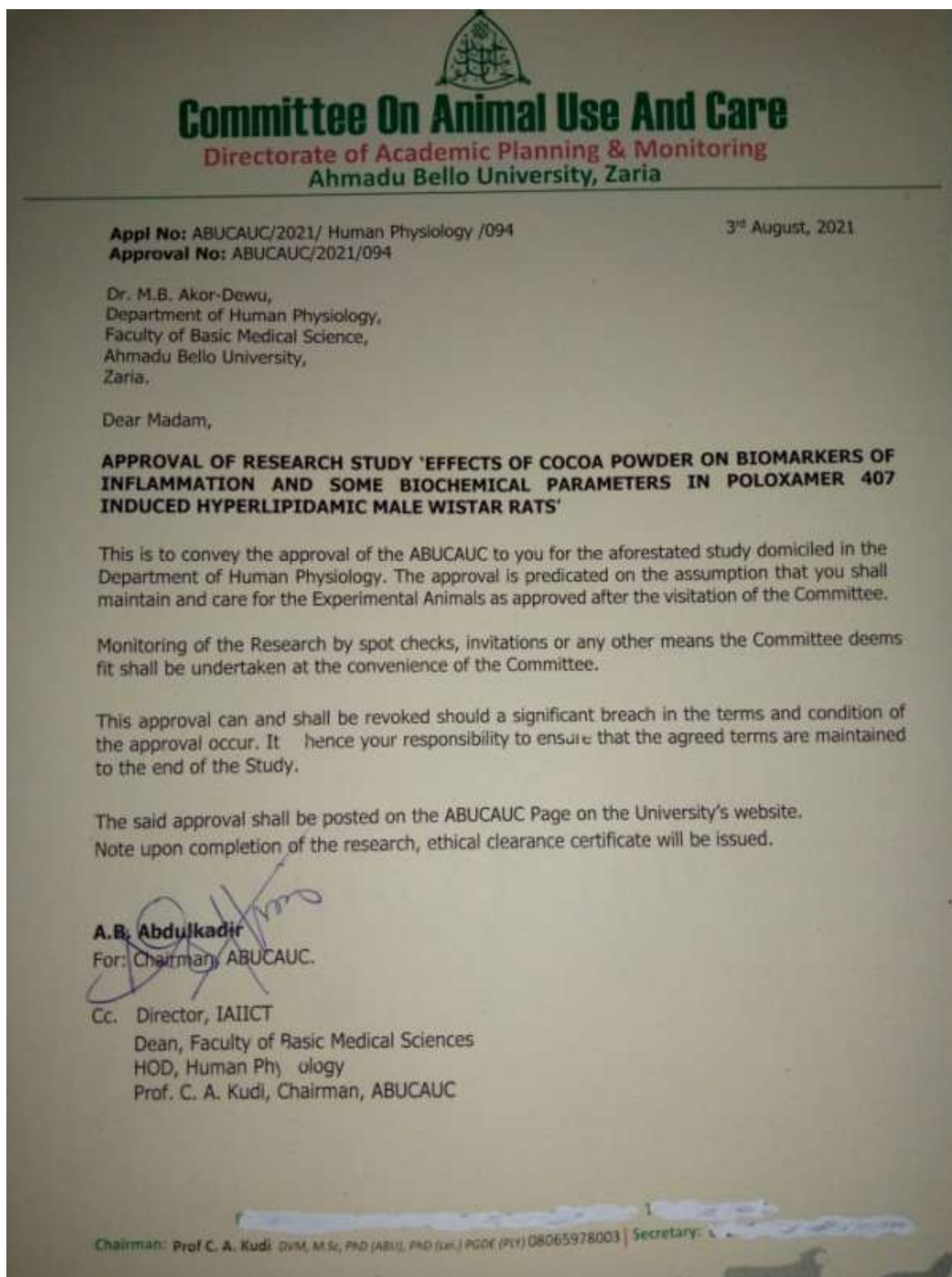
NC- normal control

P- poloxamer 407

ATV- atorvastatin

CP- cocoa powder

ETHICAL CLEARANCE



Committee On Animal Use And Care

Directorate of Academic Planning & Monitoring

Ahmadu Bello University, Zaria

Appl No: ABUCAUC/2021/ Human Physiology /094

Approval No: ABUCAUC/2021/094

3rd August, 2021

Dr. M.B. Akor-Dewu,
Department of Human Physiology,
Faculty of Basic Medical Science,
Ahmadu Bello University,
Zaria.

Dear Madam,

APPROVAL OF RESEARCH STUDY 'EFFECTS OF COCOA POWDER ON BIOMARKERS OF INFLAMMATION AND SOME BIOCHEMICAL PARAMETERS IN POLOXAMER 407 INDUCED HYPERLIPIDAMIC MALE WISTAR RATS'

This is to convey the approval of the ABUCAUC to you for the aforestated study domiciled in the Department of Human Physiology. The approval is predicated on the assumption that you shall maintain and care for the Experimental Animals as approved after the visitation of the Committee.

Monitoring of the Research by spot checks, invitations or any other means the Committee deems fit shall be undertaken at the convenience of the Committee.

This approval can and shall be revoked should a significant breach in the terms and condition of the approval occur. It hence your responsibility to ensure that the agreed terms are maintained to the end of the Study.

The said approval shall be posted on the ABUCAUC Page on the University's website.
Note upon completion of the research, ethical clearance certificate will be issued.

A.B. Abdulkadir
For: Chairman, ABUCAUC.

Cc. Director, IAIICT
Dean, Faculty of Basic Medical Sciences
HOD, Human Physiology
Prof. C. A. Kudi, Chairman, ABUCAUC

Chairman: **Prof C. A. Kudi** 08065978003 | Secretary: