CARDIOVASCULAR BENEFITS OF OKRA IN LOW DENSITY LIPOPROTEIN RECEPTOR KNOCKOUT MICE

By

Rokiatou Kone Berethe

A Thesis
Submitted to the Faculty of Graduate Studies of
The University of Manitoba
in Partial Fulfillment of the Requirements of the Degree of

MASTER OF SCIENCE

Department of Food and Human Nutritional Sciences
Faculty of Agricultural and Food Science
University of Manitoba
Winnipeg, Manitoba, Canada

ABSTRACT

Atherosclerotic cardiovascular diseases (CVDs) are the main non-communicable conditions worldwide. Okra (*Abelmoschus esculentus L. Moench*) is commonly used in many countries including Sub-Saharan Africa. Although the plant is highly valued for its potential health benefits, its cardiovascular benefits are not scientifically well investigated. In an attempt to investigate its cardiovascular benefits, twenty-five male mice were randomly assigned to 3 groups with 2 different diets, chow diet (control (LDLr-KO), n=6 and C57BL/6, n=10) and okra powder diet (LDLr-KO, n=9) at 20% (w/w) for 20 weeks. All diets contained 0.06% (w/w) dietary cholesterol. The incorporation of okra powder in the diet did not increase the total phenolic content of the diet. At baseline and during the experimental course, body weight, food consumption, and plasma lipid levels were measured regularly. At the end of the study, atherosclerotic lesion development was assessed in the aortic roots using light microscopy techniques. The food intake was not significantly different in any experimental groups. However, supplementation of okra changed the weight gain rate, and the plasma total cholesterol and triglycerides levels declined significantly in the okra diet group during the study course. These changes were accompanied by slight reductions in the extent of atherosclerosis (0.17±0.05 vs 0.25±0.05 mm^2 with $p>0.05$) as compared to controls. Also, there was a significant increase in the plasma level of IL-10 in the okra-treated animals, as compared to that in the control group. These findings may indicate that the anti-hyperlipidemic effects of okra may be mediated through anti-inflammatory mechanisms. More research is needed to warrant confirmation of such suggestions.

ACKNOWLEDGMENTS

I would like to express my gratitude to the University of Manitoba for letting me fulfill my dream of being a student here. I would also like to thank the Department of Food and Human Nutritional Sciences for giving me the opportunity to write an MSc thesis. I am also thankful to the Human Ecology Building at University of Manitoba, Canadian Centre for Agri-Food Research in Health and Medicine, St-Boniface Hospital Albrechtsen Research Centre for providing the great facilities to conduct this research.

Most of all, I would like to express my deepest appreciation to my advisor, Dr Moghadasian, who has the attitude and the substance of a genius: he continually and convincingly conveyed a spirit of adventure regarding research and excitement regarding teaching. Without his guidance and persistent help, this dissertation would not have been possible.

I would like to thank my committee members, Dr. Michael Eskin and Dr. Thomas Netticadan for your assistance and suggestions throughout my project. I also would like to acknowledge Dr. Rotimi Aluko and Dr. Monisola A. Alashi who allowed me to do part of my research and learn many advanced laboratory skills in Dr Aluko's laboratory.

Special thanks to Dr Kabo Masisi, who assisted me from the beginning to the end of this project.

My sincere gratitude goes to Dr Olaiya and Dr. Adewale Michael Esan from the University of Ibadan who provided me the sample for the experiment.

I also owed a great debt of gratitude to Dr Mohamed Sangare for supporting me with data analysis.

My deepest gratitude goes to the faculty, support staff, and students of the Department of Food and Human Nutritional Sciences, Canadian Center for Agri-food Research in Health and Medicine (CCARM) and St-Boniface Hospital Albrechtsen Research Centre for supporting me both on and off the water. I am grateful to Khuong Le, Hibah Khawar, Ramandeep Kaur, Comfort Ajibola, Olayinka Olarewaju. I also wish to thank the summer students (Breanne Semenko, Meagan Mamchur).

Finally, I would like to thank my family: my parents, my brothers and sisters, my nephews, and my nieces for supporting me spiritually throughout my life.

DEDICATION

To Allah (SWT) for letting me live to see this thesis through.

To my Dad (Missa Kone) and late Mom (Diouraba Diallo) who believed in me and taught me to be honest and always to strive for the best. You have been a source of encouragement and inspiration to me throughout my life.

To my husband (Mody Berethe) who has been a constant source of support and encouragement during the challenges of motherhood and graduate studies.

To my children (Thierno, Mama Soukho, Missa, Hawa, Diah) who have been affected in every way possible by this quest.

With your love, you all made me stronger, better and more fulfilled than I could have ever imagined.

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LIST OF ABBREVIATIONS

AD: Alzheimer's disease

AICR: American Institute for Cancer Research

AIDS: Acquired immunodeficiency syndrome

AOAC: Association of Official Analytical Chemists

ApoE-/- : Apolipoprotein E deficient

CAT : Catalase

CE: Cholesterol esterase

CHAP: Chicago Health and Aging Study

CO: Cholesterol oxidase

CVDs: Cardiovascular diseases

DASH: Dietary Approaches to Stop Hypertension

DDQ: 2,3-dichloro-5,6-dicyanobenzoquinone

DNA: Deoxyribonucleic acid

FAO: Food and Agriculture Organization of the United Nations

GAE: Gallic acid equivalent

GK: Glycerol kinase

GPO: Glycerol phosphate oxidase

GPx: Glutathione peroxidase

HDL: High-density lipoprotein

HNE: Hydroxynonenal

HO-1: Hemeoxygenase-1

HPLC: High-performance liquid chromatography

IAA: Indole acetic acid and salt treatments

IL-1 : Interleukin 1

IL-1β : Interleukin 1 beta

LDL: Low density lipoprotein

LDL receptor knockout (LDL-r-KO)

LPS: Lipopolysaccharide

MDA: Malondialdehyde

MPO: Myeloperoxidase

NAD(P)H oxidases (Nox): Nicotinamide adenine dinucleotide phosphate oxidase

NF-kB: Nuclear factor-kappa B

NIHORT: National Horticultural Research Institute

NO: Nitric oxide

OP: Okra powder

OPA: Okra pod accession

OxLDL: Oxidized LDL

PCSK9: Proprotein convertase subtilisin/kexin type 9

TC: Total Cholesterol

TG: Triglycerides

WHO: World Health Organization

1 Introduction

Cardiovascular diseases (CVDs) remain the leading global cause of death. According to the World Health Organization (WHO) fact sheet of Cardiovascular diseases (1), CVDs refers to a group of disorders of the heart and blood vessels, which include coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis and, pulmonary embolism. The WHO has estimated that over 17.3 million people die every year from CVDs, which represents 31% of all deaths in the world, a number that is expected to grow to >23.6 million by 2030 (2, 3). In Canada, every hour, 12 adults aged 20 years or older die from heart diseases including CVDs (4). Economists project that the cost of not investing in CVDs prevention and treatment could amount to as much as $ 47 trillion worldwide in the next 25 years (5). There are many risk factors associated with CVDs such as obesity, hypertension, dyslipidemia, smoking, etc. The primary underlying cause of CVDs is believed to be atherosclerosis(6).

Atherosclerosis is characterized by the accumulation of fatty deposits in macrophages in large and medium-sized arteries. Atherosclerosis lesions contain multiple oxidized lipids derived from lipids in low-density lipoproteins (LDL) (6). The biological properties of the lipids in mildly oxidized LDL have been shown to be different from those induced by lipid in highly oxidized LDL. It is now clear that one mechanism whereby cells *in vitro* render LDL a substrate for the scavenger pathway is via oxidation of LDL lipids and resulting in modification of apolipoprotein B-100 (7) These observations form the basis for the oxidative modification hypothesis of atherosclerosis, in which LDL transverses the sub-endothelial space of the lesion-prone arterial site. Oxidized low-density lipoprotein (Ox-LDL) had also been shown to stimulate proliferation of smooth muscle cells (8) and to be immunogenic by eliciting the production of auto-antibodies and the formation of immune complexes that can also facilitate macrophage internalization. That is why in the past 2 decades, numerous scientists have been intensively focused on targeting oxidative inflammation for the primary and secondary prevention of atherosclerosis. Various drugs such as statins, antihypertensive and anticoagulants have been used to control risk factors of atherosclerosis. They have been found to be effective and successful in preventing the disease. However, the diet has always been considered by the National Cholesterol Education Program and the American Heart Association as the first line of intervention to reduce risk factors of CVDs (9). Recently, several epidemiological studies reported that medicinal plants are associated with the reduction of morbidity and mortality rates

from chronic diseases(9, 10). Plants have been shown to be rich in phytochemicals including flavonoids (11, 12) .

Current findings support the hypothesis that oxidative stress plays a significant role in the initiation and progression of atherosclerosis(13). OxLDL is believed to generate a vicious cycle of inflammation oxidation within the intima and thus initiate atherosclerosis through foam cell formation(14, 15). This indicates that it is crucial to regulate oxidative stress and inhibit the inflammatory responses to prevent the development of atherosclerosis.

Studies have shown that hyperlipidemia and hyperglycemia are associated with increased oxidative damage which affects antioxidant status and lipoprotein levels (16). Lipid lowering vegetables or fruits may reduce harmful blood lipids especially after meals consumption added to their antioxidant properties (17, 18). The antioxidant effect of numerous plants is mainly due to their high flavonoid content (19, 20). Consequently, they may prevent or reverse atherosclerosis and vascular endothelium damage. Nonetheless, some mechanisms by which the anti-inflammatory effects of flavonoids may be implicated in the protection toward cardiovascular disease are the downregulation of inflammatory mediators production such as tumor necrosis factor-alpha (TNF-α) at endothelial level (21, 22).

Okra (*Abelmoschus esculentus L. Moench*) is a tropical vegetable or fruit belonging to the Mallow family. It is easy to be cultivated and grows well in tropical, subtropical, and warm temperate regions of the world. Immature okra pods are consumed in most areas of the world (23). It plays an important role in the human diet and is a good source of carbohydrates, minerals, vitamins, protein, fat, fiber, phytochemicals and is also a source of dietary medicine (24). Research has shown that okra may contribute to the upregulation of antioxidant status as well as a promising chemo-preventive agent due to its high content in phenolic compounds. Okra seeds and pods are thought to be rich in phenolic compounds mainly flavonoids such as catechin oligomers, quercetin derivatives and flavanol derivatives (25). Current findings suggest that a higher intake of flavonoids may be protective against cardiovascular diseases through their antioxidant, anti-platelet, anti-inflammatory effects. They also increase HDL level, reduce LDL level, and so improve the endothelial function (14, 15, 26).

Previous studies reported that okra polysaccharides possess antidiabetic and antihyperlipidemic roles in C57BL/6 mice (27, 28), anticomplementary and hypoglycemic activity in normal mice (29). In addition, Okada *et al.*(30) reported that okra effectively decreased TNF-α levels in 3T3-L1 adipocytes, indicating that okra may play a role in the regulation of glucose and lipid metabolism. However, information on cholesterol lowering and

anti-inflammatory effects of okra in experimental animals with atherosclerosis and dyslipidemia are not scientifically well documented. We hypothesized that adequate intakes of okra reduce cardiovascular risks through alterations in cholesterol, triglycerides, and inflammatory pathways in LDL receptor knockout (LDL-r-KO) mice. The LDL-r-KO mouse model closely resembles the disease in humans and has been frequently used by our group and others to assess the impact of dietary agents on atherosclerosis (31, 32).

2 Literature review

2.1 Atherosclerosis

Atherosclerosis is a chronic inflammation affecting the innermost layer of arteries (33, 34). Atherosclerosis is described as the association between fatty degeneration and vessel hardening (35). It is characterized by the accumulation of fat accompanied by some macrophages, lipids, smooth muscle cells, collagen, cell debris, calcium in the subintima which invade the arterial lumen (36). The process affects large and medium sized arteries. The fatty deposits are also called atheromatous plaque (37). The deposit of these plaques begins with the build up of small cholesterol crystals in the inner layer and its underlying smooth muscle. Furthermore, the evolution of the plaques continues by the inflammatory proliferation of fibrous tissues and the surrounding smooth muscle which will make the intimal surface to bulge into the lumen and subsequently reduce the blood flow. Then the connective tissue production by fibroblasts and deposition of calcium in the lesion cause stiffening of the arteries. At the end, there will be the formation of a thrombus which will result in the sudden blockage of blood flow (38, 39).

2.1.1 Risk factors

It has been stated that nine out of ten Canadians live at least with one risk factor of CVDs (40). The risk factors accelerate the process of oxidation and inflammation which contribute to atherosclerosis. Epidemiological studies suggest that an interaction between genetic, health behaviour and environmental factors predispose to atherosclerosis. The major factors are classified as non-modifiable and modifiable. Non-modifiable factors include genetics, ethnicity, gender, age. The modifiable factors which can be managed according to literature comprise dyslipidemia, diabetes, smoking, sedentary lifestyle, poor diet, high blood pressure,

unhealthy diet, stress (41). These factors could be modified by pharmacological products or lifestyle behavior (40).

2.1.1.1 Lipids

Research on the relationship between cholesterol and arteriosclerosis started centuries ago. In the 1840s, Vogel (42) first discovered the buildup of cholesterol in the artery. However, in 1904, the German anatomopathologist Felix Jacob Marchand (43) was credited for coining the term "atherosclerosis", in German "atherosklerose". He determined the hardening of the artery by the "gruel-like" fatty substance buildup inside and that the obstructive process in the artery is caused by the fatty streak. However, it took a lot of trials for the scientists to pinpoint the causal relationship between cholesterol and atherosclerosis. In 1908, Ignastowski investigate unintentionally the first exclusively nutrition investigation toward experimental atherosclerosis (42, 44). He hypothesized that "feeding rabbits meat would lead to pathologic changes typical of the aging process." He fed meat to inherently vegetarian adult rabbits. The accidental finding from these trials was that atherosclerotic lesions were developed in the animals due to meat consumption. Because those results were a paradox, it took him as well as other scientists many years to clarify the question. In 1910, Dr. N. Stuckey under the supervision of Dr. Ignastowski induced atherosclerosis by feeding adults rabbits with meat and newly weaned rabbits with egg yolk and milk (42). Both groups developed atherosclerosis. However, another group which was fed with egg white did not develop any lesion. Based on these results, investigators concluded that the risk factors were probably meat and egg yolk. A few years later, in 1912, Sobolev (45) investigated meat and egg yolk to determine which factor could lead to atherosclerosis. The findings oriented all research toward fat and cholesterol shadowing trials on dietary protein. In order to pursue the tendency, Sobolev decided to conduct new series of experiments. They fed rabbits with fish oil, sunflower oil, and phospholipid. The team found out that none of those animals developed atherosclerosis. The current understanding and clinical logic of lipid hypothesis belong to the impeccable investigation performed by Anitschkow and Dr. Chalatow in 1913 (46). They pursued the research by feeding pure cholesterol to rabbits for an extended period of time. Between weeks 4 and 8, rabbits started developing typical atheromatous lesions. Throughout this and subsequent experiments, Dr. Anitschkow and the rest of the team concluded that "There can be no atherosclerosis without cholesterol" (47).

2.1.1.2 Genetic

Genes are a crucial factor which makes a subtle contribution in a person's susceptibly to develop certain diseases such as atherosclerosis. Atherosclerosis is a condition which involves both multiple genetic and environmental factors. Epidemiological studies suggest that some genes predispose to atherosclerosis (48, 49). For example, Reschen *et al.* (50) demonstrated that the genetic locus containing the phosphatase and actin regulator PHACTR1 is an important gene expressed in atherosclerotic lesions. The atherogenic stimuli in macrophages as well as endothelial cells regulate the atherosclerosis candidate gene which is like the one for inflammatory stimulus. Another research conducted by Marenberg *et al.* (51) assessed the risk of death from atherosclerosis in pairs of monozygotic and dizygotic twins which had a family history of premature atherosclerosis. The age at which one died of coronary disease was identified as a main independent variable to anticipate the death of the other twin from coronary atherosclerosis. Data from 26 years of follow-up were used. Results depict that at an early age, death from atherosclerosis is influenced by genetic factors in both male and female.

2.1.2 Inflammation and atherosclerosis

Inflammation is a biological process that represents the body's primordial detection and alarm in response to stimuli arising from foreign toxins, damaged cells, microbial pathogens and irritants (52). In atherosclerosis, LDL retained in the intima undergoes oxidative modification (34). The OxLDL which is considered now as a harmful stimulus contributes to a reaction of immune and vascular systems (14). The immune produce white blood cells which destroy the harmful stimulus and the vascular system transport leukocyte into cells (53). When the immune system through leukocytes (monocytes and T lymphocytes) and endothelial cells fail to counteract or destroy the OxLDL, the further immune response is triggered which results from the artery becoming inflamed (54). The OxLDL are taken up by dendritic cells, macrophages, smooth muscles cells and form lipid-laden foam cells. Expression of adhesion molecules, growth factors, chemokines, proinflammatory cytokines (TNF-α, IL-1 and interferon-gamma) and other mediators of inflammation also will further elaborate this reaction in the artery wall (55). This condition added to other factors including hypoxia, reactive oxygen species (ROS), nitric oxide (NO) in surplus, the inflammation within the atherosclerotic lesion will evolve to atheroprogression (56).

2.1.3 Animal model for Atherosclerotic CVD

Overall, the best animal model for human atherosclerosis research must have similar anatomy and pathophysiology as humans (60). Also, this animal species should have the potential to be manipulated through genetic or pharmacological techniques to obtain findings that can be applied to humans (61). Furthermore, it must be available, affordable, and easy to handle. In particular, it must be able to develop and share the topography of lesions stages with humans starting from the initial fatty streak stage, the fibrous plaque stage to the complicated lesion stage (57). Despite efforts that have been made to develop ideal animal models that replicate human atherosclerosis or lipoprotein profile, each of these models has its advantages and limitations. Numerous studies have mostly used mice and rabbits followed by pigs and non-human primates (58, 59). The mouse is the predominant species used in atherosclerosis research for decades. Some of the advantages include low-cost maintenance, rapid breeding, well known genetic background and easy genetic manipulation, capacity to develop atherogenesis in a short time frame (58, 59). LDLr-KO mice and Apolipoprotein E deficient (ApoE-/-) are both commonly used in atherosclerosis investigation (60). ApoE3-Leiden and PCSK9-AAV mice are also occasionally used (57). Nevertheless, the small size and certain physiological parameters revealed some limitations. Another factor is that man and mice do not respond to dietary cholesterol in the same manner. Mouse carries most plasma cholesterol in HDL particles. Man absorbs 50% of dietary cholesterol which is covalently bound to LDL cholesterol. This might justify why wild-type mice (C57BL/6J) do not develop atherosclerosis but human does (57). LDLr-KO mice fed with a western diet result in strongly elevated lipoprotein profile with a high probability to develop rapidly larger and more advanced atherosclerotic lesions (61). According to Veseli *et al.* (57) In LDLr-KO mice, the absence of LDL receptor does not have any impact on inflammation which implies that atherosclerotic plaque development in this mouse model depends on elevated plasma lipid levels. Moreover, this mice model shares a similar lipid profile which mimics human familial hypercholesterolaemia condition (62, 63). Furthermore, the arterial lesions in human family hypercholesterolemia look like the lesions in aortic valves and aortic roots of these animals (59) .

2.2 Oxidative stress

Oxidative stress is generated when the balance between antioxidant defenses and free radicals such as ROS is disrupted in favor of the latter. This phenomenon occurs when there is an

exhaustion of antioxidants and/or a gathering of ROS (64). When ROS accumulate in the human body, cells may endeavor to counterbalance oxidation effects and the redox process, by activating or silencing gene encoding defensive enzymes, by activating some stress-induced transcription factors and by producing anti-inflammatory and proinflammatory cytokines (19). When the redox state is not at an optimal level, it can result in cell damage which is demonstrated by a change in Deoxyribonucleic acid (DNA) structure and an alteration of lipid and protein function (65, 66) . Multiple studies suggested that oxidative stress plays a significant role in the pathogenesis of many acute and chronic diseases including cardiovascular diseases, neurodegenerative diseases, diabetes, cancers, aging, immune disorders, etc. (67-70). To prevent diseases, cell viability, proliferation, activation as well as organ function must be restored. Under physiological conditions, antioxidants which are either generated endogenously or externally by our diet, provide health benefits through direct reduction of oxidative stress (71). In the body, antioxidants act to prevent oxidative injury and cell death by scavenging reactive oxygen species, giving hydrogen compound away, destroying singlet oxygen, and terminating lipid peroxidation or chelating metal ion (72) .

2.2.1 Oxidative stress on cellular components

2.2.1.1 Deoxyribonucleic acid (DNA)

Excessive production of ROS could lead to a change of DNA structure in many ways, which involves the formation of single-strand breaks and double-strand breaks in the DNA chain and/or modification in sugar-bound or purine (77). On condition that this is not repaired, DNA oxidation results in damage of nitrogenous bases and deoxyribose, which induces DNA mutations, losses or translocation, and formation of DNA-protein cross-links (78). Alterations of DNA accumulate with aging and are relevant in many disease states including atherosclerosis diseases (79). In recent years, the correlation between DNA damage, premature vascular aging, and development as well as the progression of atherosclerosis has been thoroughly investigated. Vascular function is specifically affected when there is genomic imbalance within the cell (80). Genomic instability leads to cell-division cycle arrest, programmed cell death, premature senescence in coronary artery endothelial cells (80). In this way, continuous exposure to risk factors such as diabetes mellitus, dyslipidemia causing overproduction of ROS and reactive nitrogen species (RNS) triggers DNA damage within plaque and vascular aging is seen as a consequence of this abnormality (81). However, the study conducted by Fetterman et al. (80)

agreed partially about the interaction between DNA damage, vascular function, and atherosclerosis. They found out that there is an association between mitochondrial DNA damage in peripheral blood mononuclear cells with microvascular pulsatility. Nonetheless, they didn't find any correlation with the vasodilator function which is not consistent with prior research showing abnormalities in both functions (82).

2.2.1.2 Effects of Oxidative Stress on lipids

Lipids are the most involved group of macromolecules that are targeted by oxidative stress. In the first place, lipid peroxidation was studied by food chemists as a mechanism describing the deterioration caused to dietary oils and fats (73). Nonetheless, other scientists believed that lipid peroxidation was the repercussion of toxic substances which provide reactive oxygen species (from activation of nicotinamide adenine dinucleotide phosphate oxidase (NAD(P)H oxidase), NO uncoupling, and mitochondria), disrupt the movement within the cell membrane and induce a cellular damage (74). Lipid peroxidation is considered as one of the most extensively studied consequences of ROS attack and remains of potential importance in vascular damage (75). Lipid peroxide results in the oxidation of lipids containing carbon-carbon double bonds such as polyunsaturated fatty acids (PUFAs) by a free radical (76). PUFA residues enclosed in the phospholipids of cell membrane are the primarily decomposed substances (77). One of the most atherogenic lipoprotein particles, the LDL particle carries in overall 2200 cholesterol molecules (free and esterified). The number of fatty acids in the various lipid classes is on average 2600 which are partly composed by polyunsaturated fatty acids (PUFAS), specifically linoleic acid, arachidonic acid, and docosahexaenoic acid (78). Consequently, this implies that LDL in addition to being rich in cholesterol also contains PUFAs that are tremendously at risk of lipid peroxidation (78). However, in normal condition, PUFAs are well shielded by considerable amounts of antioxidants. Of the antioxidants found in LDL, alpha tocopherol occupies the most prominent place. Seven molecules of alpha-tocopherol are present in every particle of LDL (78). These findings led to the hypothesis that denaturation or one-of-a-kind chemical reactions that affect LDL are the chief culprit in atherosclerosis (79).

Final products of peroxidation such as isoprostanes, malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) are able to inactivate many cellular proteins by forming protein cross-linkages (76). Moreover, these mediators are involved in the pathophysiology of a wide range of inflammatory diseases (76). Isoprostanes are products of peroxidation of arachidonic acid (80). Measuring levels of isoprostanes in body fluids and tissue specimen is usually seen as the most

meticulous way to assess oxidative stress in vivo (81). Increased levels of isoprostanes have been widely considered as biomarkers of lipid peroxidation (80) and also as a risk factor for coronary atherosclerosis(78), scleroderma (82), Alzheimer disease (83) and asthma (84).

Malondialdehyde is the principal and the most popular studied product of PUFA peroxidation in the cells through enzymatic and non enzymatic processes (73). This aldehyde is a highly reactive and toxic molecule and has been widely used for many years as the most reliable marker that determines lipid peroxidation in clinical situations (85). Its interaction with the DNA bases as well as proteins generate Schiff-base adducts which trigger inflammatory processes initiating activation of the complement system in atherosclerosis (86). Many researchers have reported that MDA may act by affecting upstream signaling kinases and subsequently inhibits the redox signaling messenger causing cytotoxicity (86). The cellular toxicity is expressed by an exaggerated cell proliferation or cellular death and inhibits gene expression (87).

Among final products obtained from the oxidative breakdown of LDL which are behind its proatherogenic effects, 4-HNE is the most intensively studied. In addition to its physiological and protective role as a signaling molecule and regulator of gene expression, reports identified 4-HNE's cytotoxic and inhibitor of gene expression functions (88). This property of 4-HNE is supposed to cause a great number of inflammatory and degenerative diseases including diabetes mellitus (89, 90), atherosclerosis (91) , cancer (92), Alzheimer's and Parkinson's diseases (93), etc. Greater levels of 4-HNE are consistently detected in OxLDL (78) and fibrotic plaque in humans' plasma (94) which explains its probable connection with the pathogenesis of atherosclerosis. This atherogenic aldehyde stimulates the formation of smooth muscles cells and macrophages which are the two key cell types involved in the underlying mechanism of chronic inflammatory illustrated by excessive fibrogenesis (95).

2.2.1.3 Effects of oxidative stress on proteins

Oxidative stress affects proteins through the denaturation of their cellular structure, the transformation of amino acid residues and prosthetic group enzymes, the disintegration of the peptide chain and aggregation of proteins, which lead to the alteration or loss of their biological function (96). In more details, these modifications could be achieved through direct or indirect processes. The direct process involves oxidation of critical amino acid side chains with metals ion and hydrogen peroxide which leads to the production of semialdehyde amino acids (97, 98). Most of these changes may alter enzyme activity and happen with cysteine, arginine, proline and lysine residues (99-101). Moreover, they could modify the transcriptional activity if the oxidant

groups are located inside the cistrome of transcription factors (102, 103). Another direct reaction could be conformational rearrangement between or inside the molecule(101, 104). For example, the building of disulfide bridges between or within proteins could lead to a damage in protein activity or function. The oxidation of protein by metal ions could alter the protein membrane which will result in the disintegration process (105). The hydroxyl radical-mediated oxidation of lipids provides indirect reaction causing lipid membrane damage and loss of cell membrane integrity. The end-products of the lipids peroxidation such as aldehydes will in turn alter protein membrane probably by changing ion channel and the functions of cell surface receptors (105, 106). The consequence of both direct and indirect reactions is influence of gene transcription process on the control of inflammatory factors, on the enhancement of vascular cell proliferation and on cellular death (107-109). In addition to its atherogenic effect, the oxidation of proteins is correlated with other pathogenic states such as diabetes, Parkinson disease, Alzheimer's disease, rheumatoid arthritis, etc. (110).

2.2.2 Vegetables and their health benefits

Vegetables are important sources of fiber, vitamins, minerals, and phytochemicals which are essential for the human body to function properly. They have a high nutritional density, due to their high nutrient content and low caloric intake. Multiple studies have shown the correlation between high consumption of vegetables and lower incidence and mortality rate due to chronic diseases such as cardiovascular diseases, a certain form of cancer, immune dysfunction as well as neurodegenerative diseases.

2.2.2.1 Cardiovascular diseases

Numerous human cohort and case–control studies depicted a strong association between vegetable consumption and a reduction of the rate of cardiovascular events. In the European Prospective investigation into Cancer and Nutrition cohort, consumption of more than 8 servings of vegetables per day is associated with a 22% reduction in the risk of death from ischemic heart disease compared to consumption of less than 3 servings per day (111). In prospective studies, there is a reduction of the risk of coronary events of about 14-20% and 18% between extreme vegetable consumption, and a 31% and 10% decrease in strokes, with Nurses' Health Study and professionals' health Study (112, 113). Another approach shows a 4% reduction in the risk of coronary events per additional daily serving of vegetables 5% of the relative risk of stroke (114). An intervention study of 11,000 coronary patients shows that recurrences decrease by 40-45% in

patients who ate vegetables more than once a day compared to those who never or rarely ate them (114).

Numerous studies have shown the effects of vitamins in regulating the mechanisms of oxidation, migration and cellular apoptosis, as well as in the processes of thrombosis and vascular lipid accumulation (115). Two main hypotheses involve vitamins in the pathophysiological processes of atherosclerosis and its complications. According to the first, LDL oxidation promotes the formation and development of atheroma plaque by stimulating the activation of monocytes in the subendothelial space of the vessels (116). The corollary is that the absorption of antioxidant vitamins from vegetables could inhibit the oxidation of LDL and consequently the development of atheroma plaque. The second stems from the role of homocysteine in atherothrombosis (117). A decrease in plasma homocysteine concentrations, induced by vitamins B6, B9 and B12 in green vegetables, could thus reduce the risk of thrombotic events.

Vegetables provide about 28% of the total polyphenol intake (118) which is estimated at about 1g per day or 10 times the intake of vitamin C and 100 times that of vitamin E. A recent compilation of prospective studies (119) highlights a likely protective effect of greater flavonoid intake of cardiovascular disease. But there was no association between flavonoid intake and cancer protection. At present, it is not possible to establish recommended daily intakes for polyphenols. The protection of polyphenols from cardiovascular disease has appeared to be mainly related to their antioxidant effects (particularly the protective effect on lipoprotein peroxidation). It has been shown in many studies that polyphenols neutralize free radicals, which are extremely reactive and harmful to our biomolecules (proteins, lipids, DNA). At the plasma level, the biological effect of polyphenols does not appear to be due to a direct antiradical effect because their bioavailability is low (120, 121). On the other hand, polyphenols can modulate the expression or activity of molecules involved in the atherosclerotic process, for example, decrease the production of pro-inflammatory factors, and stimulate the production of anti-inflammatory factors. They are also able to reduce other risk factors for cardiovascular disease, such as hyperlipemia. However, an antiradical effect of polyphenols is still possible in the digestive tract, where they are largely the majority during digestion (122). They could then act by limiting the deleterious effects of the pro-oxidant substances present in the meal and protect other dietary antioxidants from degradation. Polyphenols would certainly have a synergy or complementarity role with other antioxidants (vitamins C and E, carotenoids) in preventing cardiovascular risk.

2.2.2.2 Hypertension

Multiple analysis indicates that vegetable consumption might be beneficial for the prevention and management of hypertension. The Dietary Approaches to Stop Hypertension (DASH) study revealed that vegetable consumption leads to a hypotensive effect in moderately hypertensive subjects (123). Additionally, Gupta *et al.* demonstrated that the adoption of a diet rich in vegetables by normotensive or hypertensive individuals lowers blood pressure (124).

2.2.2.3 Hypercholesterolemia

Many vegetable components have proven cholesterol-lowering properties. Fiber is a digestion-resistant plant compound that promotes the excretion of cholesterol in feces by altering bile physiology and the entero-hepatic cycle of cholesterol (125). A meta-analysis of controlled clinical trials showed respectively a 0.25 mmol/l and 0.30 mmol/l decrease in LDL-cholesterol and total cholesterol relative to control for the consumption of 3 grams or more of soluble fiber per day (126). Other lipid balance parameters are not affected, suggesting a specific effect on LDL-cholesterol and total cholesterol metabolism. Phytosterols (sitosterol, sitostanol, camposterol and campostanol) are chemical constituents of plants (127). They inhibit the intestinal absorption of dietary cholesterol through direct competition mechanisms (127). Under controlled experimental conditions, ingestion of large amounts of phytosterols (2 g/d) decreases plasma levels of LDL-cholesterol in humans (128). The effects of vegetable consumption on plasma LDL-cholesterol have been reported in several studies(129-131).

2.2.2.4 Obesity

Obesity is a risk factor for diabetes and ischemic heart disease, which results from an imbalance between energy intake and expenditure. Fruits and vegetables are a food energy source of reduced density due to their limited fat content) and a high-water content and non-digestible fiber. At equal weight, they provide fewer calories than other foods. In the general context of energy abundance that characterizes the eating habits of Western societies, a high consumption of vegetables, as a substitute for denser foods, could contribute to the decrease in daily caloric intake (132) and weight loss (133). Short investigations seem to confirm these hypotheses. These controlled experiments showed that consumption of low-energy foods was associated with a decrease in daily caloric intakes (134). The results indicate that the feeling of satiety is controlled by the weight of the food ingested.

2.2.2.5 Diabetes

Increased consumption of vegetables has been associated with a decreased risk of developing diabetes in women in the National Health and Nutrition Examination Survey (135). Such an association has not been found in men. The risk is reduced by almost 40% in women who consume at least 2-3 servings vegetables per day (RR: 0.65, CI 95%: 0.420.88) compared to those who do not. In the same cohort, fruit and vegetable consumption was shown to be lower in people with metabolic syndrome. In these subjects, plasma concentrations of various micronutrients (vitamins C, carotene, lutein/zeaxanthin) are significantly lower than those of subjects with no metabolic syndrome (135)). More recently, Lindstrom et al. (136) report that higher fiber intake (corresponding to a diet richer in whole grains, fruits and vegetables) combined with lower intake of fats, especially saturated, leads to higher decreased weight and reduced the risk of developing type II diabetes in obese individuals.

2.2.2.6 Neurodegenerative diseases

A prospective study found a decrease in the risk of cognitive decline in women who consumed a lot of leafy green vegetables, rich in lutein and zeaxanthin, both xanthophyll and carotenoids, but also folates (137) . These results are found in the Chicago Health and Aging Study (CHAP), which found a slower cognitive decline in older adults who consumed at least 4 servings of vegetables per day, mostly leafy green vegetables (138). Data in animals corroborate these hypotheses. Supplementation of the diet of older rats with extracts from spinach delayed age-related neural and cognitive deficits (139, 140). The effect of spinach has been attributed by these authors to their antioxidant and anti-inflammatory drugs, but also to the direct effect of the flavonoids they contain on cellular signaling that improves brain plasticity in the hippocampus. The antioxidants (vitamin C, carotenoids, flavonoids) contained in some vegetables could therefore help protect the brain from oxidative stress and prevent the occurrence of degenerative brain disease.

2.2.2.7 Cancer

Vegetables have a protective effect against certain cancers, in many prospective investigations, and case-control studies. Various expert committees synthesize the literature and analyze evidence, the most recent being from the World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR), published in 2007 (141). It affirms the protective role of vegetables on colon cancer and breast cancer. Other studies showed the

association between the increased consumption of vegetables and a reduced risk of rectum, prostate and breast cancer (142, 143) The protective effects of vegetables are dependent on the intake of polyphenols (144), vitamin C, vitamins B9, B6, B12 (145) that improve antioxidant defenses, limit tumor promotion or progression (inhibition of angiogenesis) (146). Vegetables have an indirect protective effect, through their limiting effect on overweight and obesity, which are recognized factors of colon, kidney, esophagus, breast (postmenopausal) and endometrial cancers (143).

2.3 Okra

2.3.1 Okra composition and its phytochemicals

Okra fruits are widely consumed in different countries in the world because of its importance in human diet (various uses of the fresh leaves, seeds, buds, pods, flowers and stems) and also the fact that the plant grows easily in tropical, sub-tropical and warm temperate regions (147). Moreover, the production of okra fruits is known as the most affordable and accessible source of macro and micronutrients in many developing countries (148, 149). Various parts of the *abelmoschus esculentus* plant have been considered with a wide range of health benefits such as cholesterol lowering effect, antiseptic, antistress, diuretic, anti-diabetic, antimicrobial, antioxidant, etc. (24, 150-153). Okra fruit is a perishable vegetable due to its high moisture content (contains about 85% water (154)).

2.3.1.1 Proximate

The main compounds of the distinct parts of okra have been scientifically documented. Gemede *et al.* (155) investigated the proximate content of eight indigenous okra pod accessions grown in Ethiopia according to the Association of Official Analytical Chemists (AOAC). The fresh okra pods were sliced equally in multiple portions. Then, sliced pods were dried using two techniques, sun-dried followed by oven-dried at 45 degrees Celsius. In addition, the dried products were ground separately into powder before being submitted to analysis. The results revealed that there is a variation in proximate content between the different varieties of okra pods. The proximate content ranges on a dry weight basis are as follow: moisture 9.69–13.33%, crude protein 10.25–26.16%, crude fat 0.56–2.49%, crude fiber 11.97–29.93%, crude ash 5.37–11.30%, utilizable carbohydrate 36.66–50.97%, and gross energy 197.26–245.55 kcal/100 g.

Depending on the variety studied, quantitative analysis of okra compound might show different results. Adelakun *et al.* (24) directly roasted the seeds of one accession of Nigerian okra in an oven at 160°C for 10–60 mins but they didn't sun-dried them first like the experiment mentioned above. They found the highest yield of the compound's protein (42.14–38.10%), fat (31.04–17.22%), ash (4.06–3.42%), fiber (3.45–3.60%) and sugar (8.82–8.65%). The mechanism behind the variation in the proximate content of different pods is not clearly reported. It may be related to genetic factors or processing time.

2.3.1.2 Polyphenolic compounds

Many biological and epidemiological studies have reported that increased consumption of fruits and vegetable reduces the risk of the development of certain diseases due to their antioxidant properties (25). Antioxidant properties of fruits and vegetables are attributed to the presence of nutrients such as vitamins C and E, carotenoids, polyphenols and fiber (156, 157). There is evidence that during the past few decades, scientific interest in the polyphenolic content of a plant has considerably increased with an option of potential use as nutraceuticals for the treatment of diseases such as AIDS, cardiovascular diseases, diabetes, cancer, bacterial infection, neurodegenerative diseases (158, 159). Phenolic compounds are generally referred to as naturally occurring compounds which have multiple biological activities (160). They are also seen as the most abundant products from secondary metabolites of a plant and are usually associated with the defense against ultraviolet radiation and attack by pathogens (156, 157). Like other vegetables, phenolic compounds are considered the main bioactive components in okra fruits. A recent study showed that okra is a rich phenolic source because of the existence of isoquercitrin, protocatechuic acid, quercetin-3-O-gentiobioside, quercetin, and rutin, while isoquercitrin and quercetin-3-O-gentiobioside were detected as the main phenolic compounds (161).

2.3.2 Okra processing

To limit or stop the progression of enzymatic and microbial activities and for better preservation, Graham *et al.* (162) enounced that taking okra fruits through some primary processing might be the most suitable procedure. Sun-drying is the most common mean used to preserve the nutritional content of the crops because of its availability (163). AOAC reported that depending on the method, heat treatment could affect the "bioavailability of micronutrients and cause the loss of minerals or the destruction of vitamins" of some vegetables at too high temperatures (164). Kouassi *et al.* (163) studied the proximate chemical composition of two

varieties of okra dried using two different methods: electric drying and sun drying. Different samples of each variety of okra were separately exposed to the sun for 3 weeks and in an electric oven at a temperature of 60°C for 48 hours. After the drying period, the products have been ground using a mortar before being analyzed. The results showed that both drying methods increased the levels of proximate including carbohydrates and proteins with an interesting energy value. Nonetheless this energy value increases more when dried by an electric oven. However, there is no trace of polyphenol in both drying methods.

Another efficient processing way to preserve the nutritional value of okra fruits is to grind the dried okra fruits into fine particles. This method is also very effective when determining phytochemical properties of a powder (150). In extraction techniques, fine particles have remarkable physical characteristics such as solubility and dispersibility (165). To illustrate this point of view, Chen *et al.* (150) evaluated the physicochemical properties of okra powder and its effect on cholesterol adsorption. Different particle sizes of okra powder were used. The authors suggested that decreasing okra powder particle size significantly increases total flavonoids extraction as well as improves the adsorption of total cholesterol.

On the other hand, further processing may not be beneficial for all parts of okra pods. Different genotypes of okra seeds were investigated by Graham *et al.* (162). After harvest, seeds of each variety of okra were stored in a freezer after removal from the pod. Then, they were sun-dried for 3 days before being roasted for 3 min at a temperature of 200°C. This procedure was followed by the milling process. Subsequently, they divided the okra powder into two portions, one to defat and the other non defatted before submitting them for total phenol content analysis. The results indicated that roasted undefatted okra powder has the highest total phenol content than the defatted sample. Nonetheless defatting process has a contrary effect on the protein content of a variety of Nigerian *Abelmoschus esculentus*. Oyelade *et al.* (166) confirmed that defatted okra seed flour samples contains a higher level of protein (40% to 55%) compared to the fat sample (22% to 34%). This implies that advanced processing may affect the phytochemical contents of okra seeds depending on the compounds. Another study evaluated the effect of pre-treatment on the mineral composition of a different variety of Nigerian okra seeds (167). Adelakun *et al.*(167) divided okra seeds into 4 portions to analyse their mineral content for the sake of widening the usage of the seeds. The authors soaked the first portion of fresh okra seeds in water for 6-48 h before drying in a cabinet at 45 ±2°C for 48 hours. The second portion which has been blanched followed different steps: steeped in distilled water for 24 h, drained, steamed for 10-60 min, dried at 45 ±2°C or 24 hours. The third sample have been malted and were also

steeped for 24 hours, allowed to germinate for 1 to 5 days with constant wetting then dried at 45 ±2°C for 48 hours. The last samples were oven roasted at 160°C for 10-20 min. After all samples have been separately milled to flour. The results of the analysis showed that soaking lowers the level of all minerals assessed and depends on the time. Blanching lowers also all mineral composition except Magnesium. However, the amount of Ca, Na, Zn and Mn are enhanced during Malting process while P, K, Mg and Fe are reduced. Roasting process enhances all the mineral content excluding Phosphorus and Magnesium. This implies that most processing methods except roasting result in leaching losses of mineral.

2.3.3 Health Benefits of Okra

A recent study done on the pharmacokinetics and bioavailability of okra highlighted that okra is a therapeutic agent against various chronic diseases including cardiovascular disease (168). Because of its high fiber content, okra adjusts blood fat levels, as illustrated by a 2005 Canadian clinical study involving 34 hyperlipidemic subjects (169). Another study published in 2014 by the Phytotherapy research journal revealed that okra has clearly a beneficial effect against hypercholesterolemia and dyslipidemia and promotes bile acid excretion (170, 171). The authors suggest that eating okra regularly could therefore help improve cardiovascular health and reduce the risk of certain diseases such as atherosclerosis. Okra along with its various parts are all beneficial for human health. In Bangladesh, researchers depict that okra extract may have the ability to reduce enzyme activities that have been associated with hypertension and other chronic diseases (172). A recent study shows that okra seed supplementation on serum lipid profile of hypercholesterolemia induced rat produces a significant reduction of serum LDL cholesterol. The present study confirms that okra seed powder is effective for lipid lowering (173). Additionally, okra seed oil consumption for a period of 30 days induced hypercholesterolemia shows an inhibition in cholesterol, and triglyceride levels in Sprague Dawley rats. Therefore, okra seed oil has the potential to ameliorate hypercholesterolemia. (174)

Zhang *et al.* indicated that dietary okra fruit powder consumption is a novel strategy which causes strong inhibition of body weight gain and liver fat accumulation therefore, prevents obesity, metabolic syndrome in high fat diet-induced obese mice (175). Another study reveals that okra seed supplementation for a period of 42 days produces significant body weight reduction in serum lipid profile of hypercholesterolemia induced rat (173). Additionally, a previous systematic review highlighted the potential efficacy of different parts of okra including polyphenolic compounds especially oligomeric catechins and flavonol derivatives such as

quercetin in metabolic syndrome (176). Low in energy and source of fiber, okra therefore occupies a prominent place in the slimming diet.

When consumed raw, cooked and sometimes in dehydrated form, okra ensures the proper function of the digestive system by regulating intestinal transit (177) and also by improving the absorption of water through its mucilage fibers in an unbalanced environment. This ability to absorb water will bring a significant feeling of satiety and therefore an appetite suppressant effect that might be helpful in weight control (177). As a medicinal plant, *Abelmoschus esculentus* has various bioactive components and is widely used worldwide as a traditional medicine for the treatment of other diseases such as ulcers and gastritis. *Abelmoschus esculentus* is used as a mucilaginous food additive against Helicobacter pylori (178, 179). An *in vivo* study conducted by Ortaç *et al.*(180) indicates that okra inhibits ulcer formation, significantly decreases edema, hemorrhage and inflammation scores in adult Sprague Dawley male albino rats therefore could reduce the gastric ulcer as seen from biochemical and histopathological results.

The high fiber content of okra can also prevent colon cancer. Fiber reduces the risk of colon and rectal cancer and removes toxins from the intestines. Antioxidants in okra, especially quercetin, also contribute to cancer prevention by helping to neutralize free radicals. In 2010, Vayssade *et al.* (181) found that the pectin extracted from the okra reduce the proliferation of skin cancer cells, particularly by stopping their cycle which induced their apoptosis. A Brazilian study published in 2014 identified and purified a lectin of okra, a protein that binds specifically and reversibly to certain carbohydrates. According to this study, this lectin presents in vitro anticancer activity on a strain of human breast cancer cells (MCF7) (182). The high content of total flavonoids of okra flowers prove that okra has a natural antioxidant effect and exerts significant antitumor efficacy on the inhibition of colorectal cancer cell proliferation and metastasis as well as tumor growth in vivo (183).

Because of its high content of antioxidants (polyphenols and flavonoids) (24, 152, 184-190) and its high content of vitamin C (16.3 mg/100 g), okra has potential anti-inflammatory properties (191, 192) . A study conducted by Freitas *et al.* on okra showed an anti-inflammatory activity (191). The authors evaluated *A. esculentus* lectin (AEL) efficacy in reducing zymosan-induced temporomandibular joint inflammatory hypernociception in rats along with the mechanism of action through which it exerts anti-inflammatory activity. Animals were pre-treated with AEL (0.01, 0.1 or 1 mg/kg) before zymosan (Zy) injection in the temporomandibular joint (TMJ) to determine anti-inflammatory activity. Also, animals were pre-treated with ZnPP-IX (3 mg/kg), a specific HO-1 inhibitor, or aminoguanidine (30 mg/kg), a

selective iNOS inhibitor, before lectin administration to evaluate the potential effect of the hemeoxygenase-1 (HO-1) and the NO pathways on AEL efficiency. Multiple procedures were applied: inflammatory hypernociception test, leukocyte count, myeloperoxidase (MPO) activity, vascular permeability determination, histopathological and immunohistochemistry analysis (TNF-α, IL-1β, HO-1) of TMJ tissue as well as the dosage of (TNF-α, IL-1β, HO-1). The results showed that *abelmoschus esculentus* lectin increased inflammatory nociceptive threshold, reduced leukocyte influx along with MPO activity, leukocyte influx into the synovial membrane, and Evans Blue extravasation. It promoted HO-1 overexpression whilst decreased TNF-α and IL-1β expression in the TMJ tissue. The lectin reduced TNF-α and IL-1β levels in TMJ tissue and trigeminal ganglion. These findings suggest that AEL efficacy depends on TNF-α/IL-1β inhibition and HO-1 pathway integrity. Mairue *et al.* (193) also investigate the inhibitory effects of okra (*Abelmoschus esculentus* Linn.) extract on the production of pro-inflammatory cytokines in lipopolysaccharide (LPS)-stimulated BV2 microglia. Non-cytotoxic quantity of okra at concentrations of 50, 100 and 200 μg/mL were used in this study. BV2 cells were cultured and treated with LPS in the presence or absence of okra at the concentrations indicated above. TNF-α, IL-1β, phosphorylation levels of nuclear factor-kappa B (NF-kB) p65 and Akt were determined. The treated BV2 cells with okra concentrations of 50, 100 and 200 μg/mL significantly suppressed LPS-induced NO compared to untreated cells. There was also a significant decrease in the production of TNF-α and IL-1β in okra-treated BV2 microglia cells. The level of LPS induced NF-kB p65 phosphorylation was significantly decreased by okra treatment. In addition, okra inhibited LPS-induced Akt phosphorylation, which is an upstream molecule of NF-kB. To sum up, Okra exerts anti-inflammatory effects in LPS-stimulated BV2 microglial cells by suppressing Akt-mediated NF-κB pathway. This suggests that okra might be a valuable agent for the treatment of anti-neuroinflammatory diseases mediated by microglial cells (193).

Okra is made up of a good amount of vitamin C, known for its antioxidant power and to strengthen the immune system by promoting the formation of white blood cells in the body (194). Several scientific studies have also demonstrated the immunomodulatory activity of okra (195-197). Traditional holistic medicine since centuries directly associates okra plant and its parts to anti-diabetic, regulation of glucose and lipid metabolism effects. Recent studies show that okra can help prevent and treat diabetes (28, 198-203). More specifically, it helps reduce and stabilize blood sugar (170, 204). Fan *et al.* (205) showed that *abelmoschus esculentus* can reduce blood glucose and lipid levels in an obese mouse model (205). Another study carried out

on obese diabetic rats induced with streptozotocin, also confirms that supplementing the diet with okra powder for 30 days significantly reduces the levels of glucose, cholesterol and blood triglycerides (206). This treatment is also beneficial in mitigating the damage to the pancreatic tissue of these diabetic rats. Chinese researchers have purified a polysaccharide fraction of an aqueous extract of okra pod containing more specifically rhamnogalacturonane (207). The latter is an extraordinarily complex pectic polysaccharide (sugar) found in the primary wall of plants but whose functions are largely unknown. The authors of the study published in Food Chemistry in 2018 showed that this purified fraction of rhamnogalacturonane, at a dose of 200 mg/kg, exerts a hypoglycemic effect on a group of mice made diabetic by streptozotocin. As part of a healthy diet, okra could therefore prove to be a useful choice for regulating lipid and sugar levels (178). An in vitro investigation of The abscisic acid extracted from Okra showed a hypoglycemic effect (208). Morady *et al.* (209) investigated the effect of okra consumption on lipid profiles and glycemic indices of serum in Type 2 diabetic (T2D) patients. The findings showed that 8 weeks okra consumption resulted in a significant decrease in fasting plasma glucose and can elicit improvements in lipid profile among T2D patients.

Researchers have discovered a "neuroprotective" activity of okra, which could lead to the testing of new symptoms' treatments for diseases like Alzheimer's, dementia or Parkinson's disease (193). A Thai study published in 2011, in Neurochemistry International., showed that okra reverses cognitive deficits (memory and learning), in vitro, in an animal model. Okra extract not only reverses these cognitive deficits, but it also protects morphological changes in hippocampal neurons (210). Consistent, okra improved spatial memory in an animal model of scopolamine-induced cognitive impairment (152). In another scientific study, the researchers revealed that okra polysaccharides could in particular stimulate dendritic cells in rats in vitro (197). Under these conditions, okra would therefore have enormous potential to stimulate memory and promote neuronal survival. Earlier, a study suggested that a high-fat diet can aggravate the metabolic disorder in Alzheimer's disease (AD) mice and okra polysaccharides can significantly reverse the metabolic disorder induced by high-fat diet and improve the cognitive impairment of the AD mice (211).

Okra is a vegetable crop that are consumed in nearly all parts of the world. Previous studies have reported that okra along with its various parts have antioxidant potential (212). To illustrate this, a recent study revealed that the antioxidant capacity of okra may be due to the large amounts of polyphenols (213). A study elaborated that okra methanol extract contains a large amount of polyphenols such as quercetin-3-O-gentiobioside, quercetin-3-O-glucoside

(isoquercitrin), rutin, a quercetin derivative, protocatechuic acid, and a catechin derivative, has a very strong antioxidant effect therefore can reduce the influence of toxicity induced by lead acetate in mice kidney (214). Okra could also help fight infections because of its antioxidant content, especially vitamin C (179, 215, 216). The results of a study carried out on okra seeds have shown antibacterial activity against several bacterial strains: Salmonella, Listeria which are the cause of severe food poisoning and digestive problems (215). Okra is traditionally used on the skin to treat psoriasis, eczema and other skin conditions. This plant would also help to delay the skin aging process due to its antioxidant power and therefore, to maintain a young and dynamic looking skin (217).

3 Rationale for the study

Based on growing evidence, there are strong links between high consumption of vegetables and reduced risk of cardiovascular diseases. Such benefits of vegetables are attributed to their phytochemicals such as polyphenols, fiber, etc. which possess antioxidant and anti-inflammatory properties.

Abelmoschus esculentus or okra is used for ages as an edible vegetable in many countries. Currently, components of okra also have elevated levels of antioxidant and anti-inflammatory activities. According to multiple studies, it is believed that various parts of the plant are employed in the treatment or prevention of chronic diseases. However, the scientific literature does not provide in-depth details on the cardiovascular benefits of okra fruits. Therefore, understanding the anti-atherogenic benefits of okra might support selection of okra for inclusion in healthy diets and as functional food ingredients in new products. Therefore, the present study aims to investigate and evaluate the effectiveness of *abelmoschus esculentus* on reducing cardiovascular risk factors in LDLr-KO mice.

4 Study hypothesis and objectives

4.1 Hypothesis

- Chow diet enriched with okra powder (20% w/w) and cholesterol (0.06% w/w) will reduce atherosclerosis risks in LDLr-KO mice.
- Okra powder reduces atherosclerosis in LDL r KO mice; this effect is mediated through a beneficial impact on lipids levels and inflammatory pathway.

4.2 Objectives

This study aims to:

- Investigate the effect of okra powder on plasma lipid profile in LDLr-KO mice.
- Investigate the impact of okra powder on inflammatory markers in LDLr-KO mice.
- To study if such changes result in the prevention of atherosclerotic lesions in this animal model.

5 Materials and Methods

5.1 Okra preparation

Okra seeds genotype LD88 used for this study were obtained from the genetic resources' laboratory (Product Development Programme), National Horticultural Research Institute (NIHORT), Ibadan, Nigeria. The seeds were grown at the National Horticultural Research Institute, Ibadan, Nigeria (NIHORT) in 2016. Seeds were sterilized with 1% (w/v) sodium hypochlorite for 15 min and washed thoroughly with distilled water. The seeds were then pretreated with indole acetic acid (0.4 mM). Okra plants were grown in polyethylene bags in the soil without NaCl (control) and under salinization levels corresponding to the osmotic potential of NaCl solution of 50, 100, 150, or 200 mM. Saline solutions were added to the soil in such a way that the soil solution acquired the assigned salinization levels at field capacity. The experiment was conducted in a screen house at the National Horticultural Research Institute, Ibadan, Nigeria (NIHORT) and each pot contained 10 kg of clay soil. There was a total of 105 pots (3 treatments x 5 levels x 3 replicates) used as follows: (I) Control and salt treatments (reference group); (II) Indole acetic acid and salt treatments (IAA treatment); (III) Salicylic acid and salt treatments (SA treatment). Plants were irrigated with normal tap water on a weekly basis to achieve soil water field capacity for four weeks. Plant fruits were harvested after 45 days, and air dried to constant mass and grounded to coarse to fine powder then packed in polyethylene plastic bags. 1000 g of fruit powdered of okra seeds treated with 0.4 mM of indole acetic acid were shipped from the University of Ibadan in Nigeria to St Boniface Hospital Research Centre in Winnipeg, Canada. Upon reception, the package was stored at -4°C before analysis and diet preparation.

5.2 Okra proximate analysis

Proximate analysis of okra was conducted by Central Testing Laboratory (Lab #447592, 49 Winnipeg, MB). Moisture content of the okra powder was determined according to "Moisture analyzed AOAC 930.15" and "Moisture Received AOAC 922.02". The crude protein was determined by a modification of AOAC using sub-components 990.03. The crude fiber and the crude fat were quantified using AOCS using sub-components respectively Ba6a-05 and Am 5-04. The ash content was determined using AOAC 923.03.

5.3 Experimental animals

In the current study, nineteen LDLr-KO mice (4 weeks old) and six C57BL/6 mice (4-week-old), were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed in groups of two or three in conventional mouse cages in a room with controlled temperature of (24±1ºC) and a 12:12-h light-dark cycle. They had *ad libitum* access to food and water. After consuming standard mouse chow for 1 week, blood samples were collected through jugular vein under light anesthesia after 4 hours fasting and baseline plasma total cholesterol was measured. The LDLr-KO mice were divided into two groups (n = 10 control; n = 9 treated) with similar mean plasma total cholesterol concentrations and body weight as previously described (218, 219). Six C57BL/6 mice were also used as control (C57BL/6). The length of the experiment was 20 weeks. The experimental diets were provided to the animals on a weekly schedule. The leftover food was discarded. The food was made as needed, and stored in dark bags, and closed containers in a cold room (at 4 °C) until used. During the experiment, we collected blood samples at 3-week intervals from the jugular vein in lightly anesthetized animals. Body weight and food intake were recorded every week. Fecal samples were collected during the last week of the study. Collected fecal materials were stored at -80 °C. At the end of the experiment, mice were sacrificed using carbon dioxide followed by collection of final blood samples through cardiac puncture, per our standard procedures (Dupasquier *et al.(220)*). Various internal tissues including heart, aorta, spleen, abdominal fat, liver, and kidneys were collected, weighed, and stored at -80 °C until analysis. Parts of the tissues were fixed in 10% buffered formalin and sectioned for histological examinations.

5.4 Experimental design and diets

The diets were designed based on AIN-93G semi-Purified Rodent Diet (Diet #110700) with slight modification. All the diets were prepared in our laboratory using Mouse Diet 9F 5020 (Ren's Feed and Supplies Limited, ON, Canada). To prevent oxidation, all diets were stored in airtight plastic bags and closed containers in a cold room at 0-4°C during the entire course of the study.

The mice were fed with one of the following diets for 20 weeks: (a) mouse chow diet 9F supplemented with 0.06% (w/w) cholesterol and used as the control diet; the control diet was supplemented with 20% (w/w) okra powder. The diet was prepared with a mixture of chow diet, okra powder (for treated diet), cholesterol and distilled water. The doughs were then sliced into pellets on multiple trays and placed in an oven for drying at approximatively 40-45 °C for 48 hours. The food was made according to the stock, and stored in dark bags, and closed containers in a cold room (at 4 °C) until used.

Table 1. Macronutrient composition of the mouse diet 9F (chow diet) (%)

Components	**Mouse diet 9F (%)**
Protein	20.5
Fat	9.0
Crude Fiber	2.7
Ash	4.8
Non-fiber carbohydrate	39.74

5.5 Ethics statement

Animal protocols and schedules, including animal housing, care, diets and animal experimental procedures, data collection and personnel, were approved by the Ethic for Guide and Care and Use of Laboratory Animals of the University of Manitoba Committee (Winnipeg, MB, Canada) under protocol number: 13-053/2-AC10879 (amended in 2016).

5.6 Data collection

5.6.1 Food intake and body weight

Every week, body weights of the animals were measured. The 24-hour food intake was estimated every 4 weeks. The food given and that remaining in each cage were weighed to calculate the food consumption.

5.6.2 Blood sampling and tissue collection

All mice were fasted for approximately 4 hours prior to blood collection during the study. Approximately a maximum of 0.6% of mice circulating blood volume (~150 μL) was collected by certified animal technicians at the R. O. Burrell Lab, St Boniface Albrechtsen Research Centre. Blood samples were drawn through the jugular vein of lightly anesthetized animals during the study (at baseline, week 4, 8. 12, 16 and 20) using heparinized syringes. At the end of the study (week 24), mice were euthanized using carbon dioxide gas and final blood samples were collected via cardiac puncture. The blood samples were kept on ice during blood collection and thereafter centrifuged at 5000 rpm for 15 min at 4 °C using Centrifuge 5804 R (Eppendorf Canada, Mississauga, ON, Canada) to obtain plasma samples. Aliquots of plasma were stored at -20 °C until analysis. The heart, liver, kidney, spleen, aorta, and abdominal fat tissue samples from each mouse were collected, weighed, and stored at -80 °C until analysis. For histological examination, tissues were fixed in 10% buffered formalin and stored until analysis.

5.6.3 Plasma Total cholesterol (TC) and Triglycerides

Plasma was used for the determination of total cholesterol (TC) and triglycerides (TG) using standard enzymatic kits (Sekisui Diagnostics, Charlottetown, PE, Canada) following the manufacturer's instructions.

For TC assay, cholesterol esters are hydrolyzed to free cholesterol by cholesterol esterase (CE). The free cholesterol is then oxidized by cholesterol oxidase (CO) to cholest-4-ene-3-one with the simultaneous production of hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide produces couples with 4-aminoantipyrine and p-hydroxybenzoate, to produce a chromogen. The intensity of the color measured is directly proportional to the amount of total cholesterol in the sample. Briefly, 250 μL of TC reagent was added to 3 μL of each standard and plasma samples pre-pipetted on a microplate reader. The readings were taken at a maximum absorbance of 505 nm after 20 min of incubation at room temperature using a microplate reader (autoreader EL311; Bio-tek instruments).

For TG assay, plasma triglycerides are hydrolyzed to glycerol and free fatty acids by lipase. In the presence of ATP and glycerol kinase (GK), the glycerol is phosphorylated to glycerol-1-phosphate. The glycerol-1-phosphate is then oxidized by glycerol phosphate oxidase (GPO) to yield hydrogen peroxidase (H_2O_2). H_2O_2 coupled with p-chlorophenol and 4-aminoantipyrine to give rise to a red quinoneimine complex, in which the intensity of the color is proportional to the concentration of triglycerides in the sample. The procedures of plasma triglyceride determination were similar with that of plasma cholesterol assay, except 300 µL of reagent was added to each 5 µL of standards and plasma samples and the readings were taken at 520 nm. All samples were measured in duplicate to obtain mean values.

5.6.4 Blood glucose level

A drop of blood was collected from the tail vain prior to anesthesia and blood glucose levels were measured using a glucose meter (ELITE XL, Bayer HealthCare, Mishawaka, IN).

5.6.5 Histological examination

Specimens of liver tissues and the entire heart was collected at the sacrifice and fixed in 10% buffered formalin. The top half of the heart, from the aortic arch and liver tissue were dehydrated with a series of gradual increasing concentrations of 70%, 80%, 95% and 100% ethanol (each for 1 hour) to completely remove the water. When dried, the samples were incubated twice in 100% xylene for 1 hour each to completely remove the alcohol and were spread in an open space to completely clear the xylene. The dried tissues were placed in TRUFLOW™ tissue cassettes (Fisher Scientific, Ottawa, ON, Canada) and fixed by submerging in 100% pre-melted paraffin wax and incubated for 3 hours at 70 °C using a Barnstead electrothermal paraffin section mounting bath (Thermo Scientific, Conroe, TX, USA). The samples were removed from cassettes and molded into tissue blocks using metal mold and fresh pre-melted paraffin wax and were placed on ice to obtain blocks. To obtain a single cell section, tissue blocks were segmented at 5 µm thickness using Shandon Finesse 325 microtome (Thermo Scientific Inc, Philadelphia, PA, USA). As soon as a good section was obtained from the microtome, the section was placed into a water bath (60 °C) and was fished onto the labelled microscope slide. Six sections were randomly selected from the liver tissue and at different levels of the aortic sinuses of each mouse to air dry overnight to allow good binding of the paraffin wax and to ensure a good staining. During the staining process, dried slides were

incubated at 65 °C on a heating block (IsoTemp. 125D, Fisher Scientific, Pittsburgh, PA, USA) for 20 min. This heating process aimed to de-paraffinize sections and to adhere the section onto the slide, then the slides were submerging in 100% xylene for 10 min to completely clear the paraffin, then 100% and 95% ethanol also for 10 minutes each to clear the xylene. After that, the slides were rinsed briefly (~ 5 times) with distilled water to ensure the complete removal of alcohol. Subsequently, the slides were stained with Harris hematoxylin solution (nucleus stain) for 10 min and washed in running tap water (~ 5 times), then incubate briefly with 2% sodium bicarbonate ($NaHCO_3$) and followed by rinsing immediately with tap water. The slides were then counterstained with alcoholic eosin for 30 seconds and rinse with several exchanges of tap water, followed by soaking successively in 95% alcohol, 100% ethanol and 100% xylene (each for 5 min). Finally, the slide will be permanent mounted with permount solution (Fisher Scientific catalogue #SP15-500) and let them air dry overnight. Images from six H&E stained sections representing the whole aortic root length and from liver sections were used for morphologically and morphometrically analysis (221). Total atherosclerotic lesions size from the aortic root sections were quantify using Image Pro-Plus digitizing software.

5.6.6 Quantification of total phenol content

Folin Ciocalteu method assay modified was used to determine the total phenolic contents of okra and chow diet by UV spectrophotometer (222). This method is the simplest method available for the measurement of phenolic content in foods (223). About 200 mg of dried okra and chow diet were each mixed with 50% aqueous methanol, soaked for 1 hour, centrifuged at a speed of 3800 rpm for 10 mins at a temperature of 4°C and filtered for measuring total phenolic content. An external calibration curve of gallic acid was used as standard for the determination of phenolic compound. Gallic acid stock solution with 1 mg/mL in 50% aqueous methanol was used for the preparation of the following dilution series: 25, 50, 75, 100, 125, 150, 200 and 250 µg/mL. Blank (50% of aqueous methanol) and different gallic concentrations? (250 µL) were added to 10 mL volumetric flask, containing 3.5 mL of DDQ (2,3-dichloro-5,6-dicyanobenzoquinone). The mixture was then mixed with 250 µL Folin-Ciocalteu reagent and 500 µL of 10% sodium carbonate. The reaction was incubated for 1 hour at room temperature. Samples were then measured by spectrophotometer (Cary 50 spectrophotometer, with Cary WinUV version software, Varian, Australia at 760 nm). The total phenolic contents of each sample are reported as milligram of gallic acid equivalent (mg GAE/g of dry mass).

For plasma samples, 450 µL of 1% acetic acid were added to 50 µL of plasma, vortexed and centrifuged. The resulting stock was used to determine the total phenol content following the Folin Ciocalteu method with light modification mentioned above.

As we did not have enough liver sample to proceed this experiment, we grouped the mice two by two based on approximative TC levels. 500 mg of liver was crushed with a mortar to obtain a paste. We added 1% of acetic acid to the liver paste, leave it aside for 1 hour to hydrate. After we centrifuged the mixture at a speed of 3800 rpm for 10 min at a temperature of 4°C and filtered for the extraction of total phenolic content using the Folin Ciocalteu method modified.

5.6.7 Quantification and identification of polyphenol compounds

Twenty µL of each sample from the stock solution (except the plasma that was used in pure) were evaluated following a method described by Lacopini *et al.* (224) and Ojeil *et al.* (225). High-performance liquid chromatography (Waters 2695) with a photodiode array detector (PDA) (Waters 996) and autosampler (Waters 717 plus) (Waters, Milford, MA, USA) were used for quantification and identification of individual phenolic compound. The phenolic compounds were separated using a 150 mm x 4.6 mm, 5 pm RP 18 analytical column (Gemini, Phenomenex, USA) (Phenomenex, Torrance, CA, USA), protected by a C18 guard column (4 × 3.0 mm, Phenomenex, Torrance, CA, USA). The mobile phase, as modified by Doshi (226), was operated by the analytical column at 30 °C, each sample was injected by autosampler and eluted with a gradient system consisting of a combination of solvents: (A): 1%acetic acid, (B): MeOH, (C) and (D): water. The flow rate was set at 1 mL/min. The gradient was programmed as follows: 35 min, A: 80% and B: 20%; 50 min, A: 70% and B: 30%; 60 min, A: 40% and B: 60%; 75 min, A:70% and B: 30% Chromatograms obtained at 280 and 320 (DAD K2800, KNAUER, Germany) were compared with those obtained on standard retention time of known phenolic compounds from Sigma-Aldrich: quercetin (65.48), Gallic acid (8.41), rutin (61.16), myricetin (62.69), catechin (29.69), caffeic (63.34).

5.6.8 Analysis of plasma cytokine intensity

Plasma samples from the final blood collection were used for the analysis of plasma cytokine intensity using Meso Scale Discovery U-PLEX multiplex assay kit panel (Meso Scale Diagnostics, Rockville, Maryland 20850-3173, USA). The intensity for IL-2, IL-4, IL-9, IL-10, IL-13, IL-21, IL-22, INF-γ, MIP-3α, and TNF-α were detected using T-cell combo plates. The biotinylated antibodies for the specific linkers were coupled and then U-plex plate coating was

performed on the pooled plasma samples (n=4; blood samples of every 2 mice were pooled to have enough plasma quantity). Lastly, detection antibody solution was added into each well of plate and the prepared MSD plate was read on the SI2400 Imager device. The intensity of 10-Plex biomarkers was detected using MSD Workbench 3.0 software to analyze standard curves involving a 4- parameter logistic fit (Magnusson et al., 2012). (Details in appendix 2.)

5.7 Statistical analysis

Non-parametric test of Wilcoxon rank-sum was used to compare okra, control and C57BL/6 groups means. Also, for each variable in our sample (body weight, blood glucose, total cholesterol and triglycerides), we compared their mean values from the 3 groups (okra, control and C57BL/6), by using the krukas-wallis test at a significance level of $p<0.05$. Statistical analyses were performed using STATA version 14 software (StataCorp, College Station, Texas, USA).

6 Results

6.1 Proximate analysis

Table 2. Proximate composition of okra (dry weigh basis)

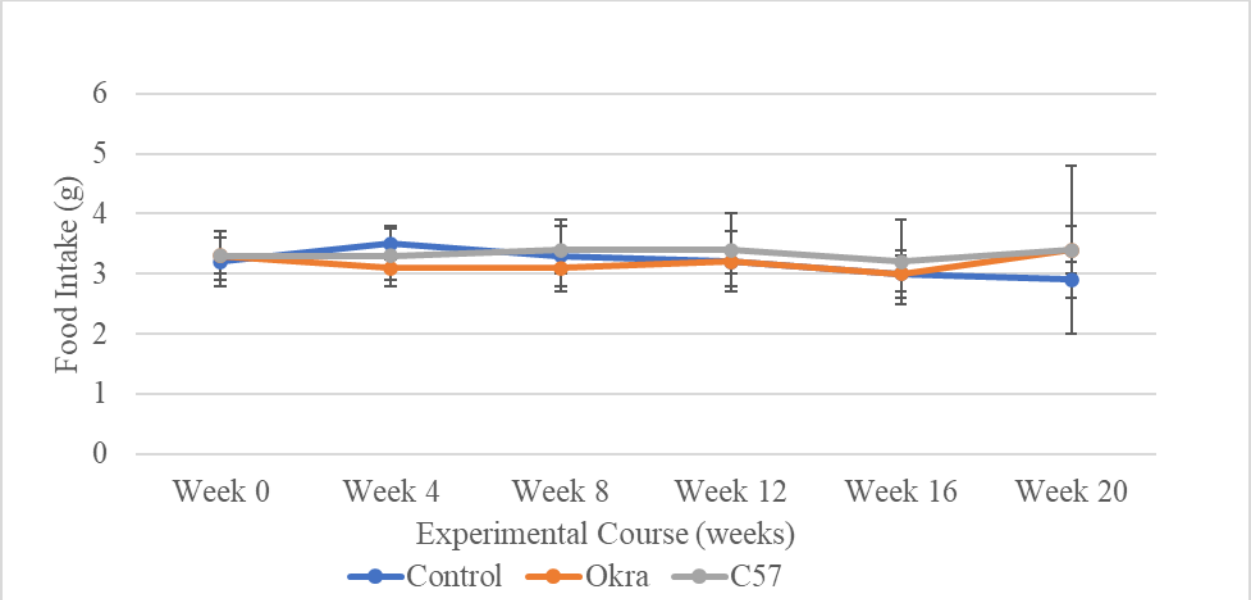
Proximate composition of okra g/100g (dry weight basis)	**Average value**
Moisture (%)	11.06
Crude Protein (%)	20.02
Crude Fibre (%)	53.15
Fat (%)	1.4
Ash (%)	10.64
Non fiber carbohydrates (%)	3.72

Data are expressed as mean of replicate determinations (n = 2).

6.2 Food Intake, body weight and tissue weight in mice

The average food intake among the groups was not statistically significant in the experimental groups (**Figure 1.**). A steady body weight gain was observed in all the mice during the experimental feeding period (**Table 3.**). Moreover, as shown in **Figure 2.,** final body weight of the okra group in this study significantly differs from the other groups ($p=0.021<0.05$). Despite similar dietary energy intake, the abdominal fat mass as well as liver weight of control and okra groups are statistically different ($p= 0.001$ and $p=0.005$, respectively) from the C57BL/6 group. In addition, kidney weight of okra group is different from the other groups ($p<0.005$). Our results showed no statistically significant differences in the other tissue weights including the heart and spleen among the experimental groups.

Figure 1. Food intake of LDLr-KO mice up to 20 weeks of experimental diet treatments



Data are presented as means ± standard error

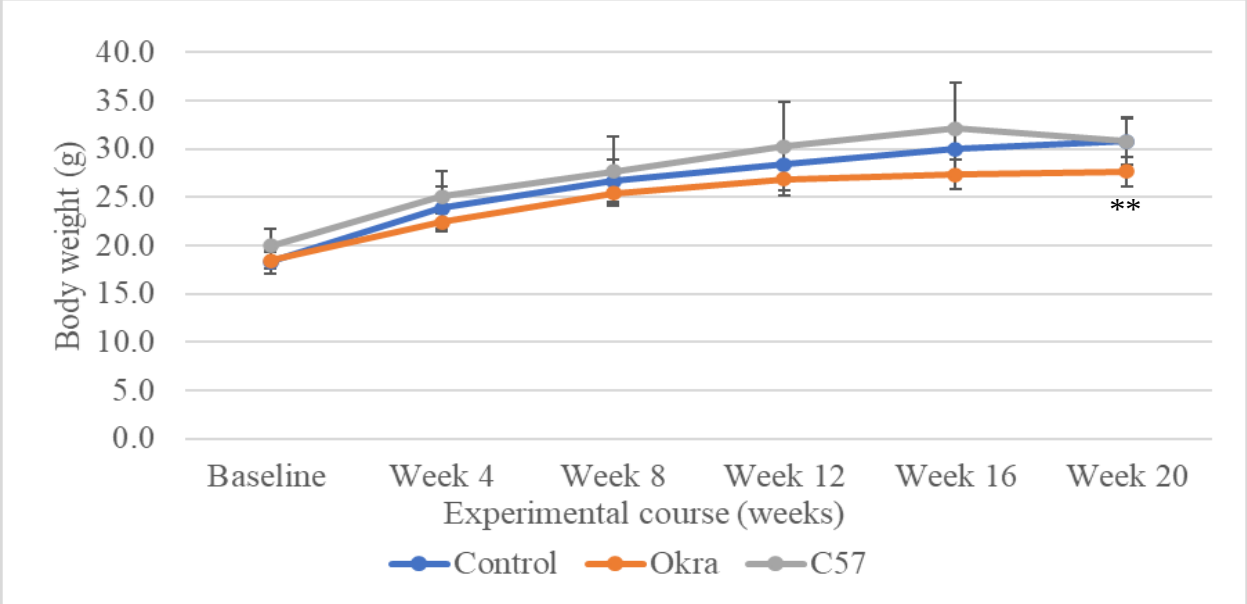
Table 3. Effects of diets on final body weight and tissue weight in the mice

	Weight (g)		
	Control	**Okra**	**C57BL/6**
Final Body weight	30.8±2.4	27.7±1.5**	33.2±5.3
Liver	1.37 ± 0 .15**	1.35±0.09**	1.77±0.38
Kidney	0.51 ± 0 .25	0.33 ± 0 .02**	0.46 ± 0 .03
Spleen	0.08 ± 0 .01	0.08 ± 0 .01	0.09 ± 0 .01
Heart	0.15 ± 0 .03	0.15 ± 0 .02	0.20 ±0.02
Abdominal fat	0.71 ± 0 .30**	0.69 ± 0 .37**	1.75 ± 0 .66

***p<0.05; **p<0.005**

Values are mean±standard error for each treatment group (control, n=10; okra, n=9; C57BL/6, n=6). The symbol (*) in row indicates that the values differ significantly at p< 0.05.

Figure 2. Effects of the experimental diets on body weights of LDLr-KO mice during the experimental course in all groups



****p<0.005**

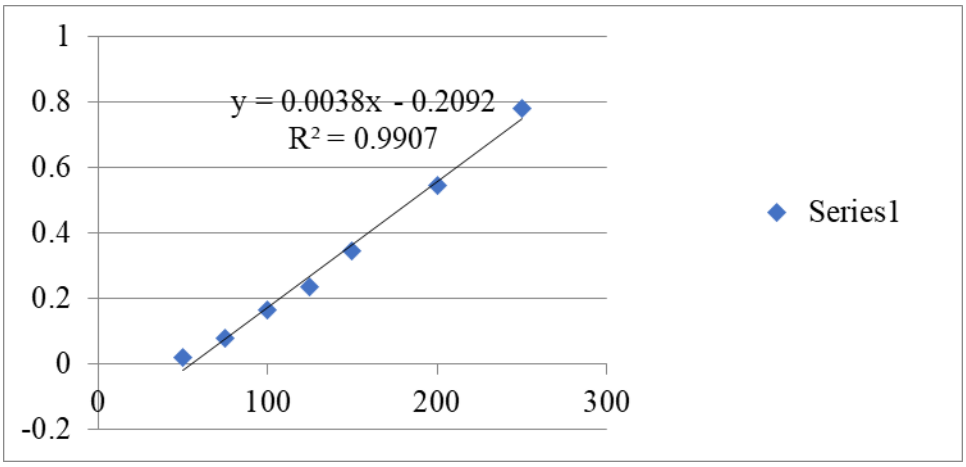
Data are presented as mean ± standard error for each treatment group.

6.3 Quantification of Total Phenol contents

6.3.1 Experimental diets

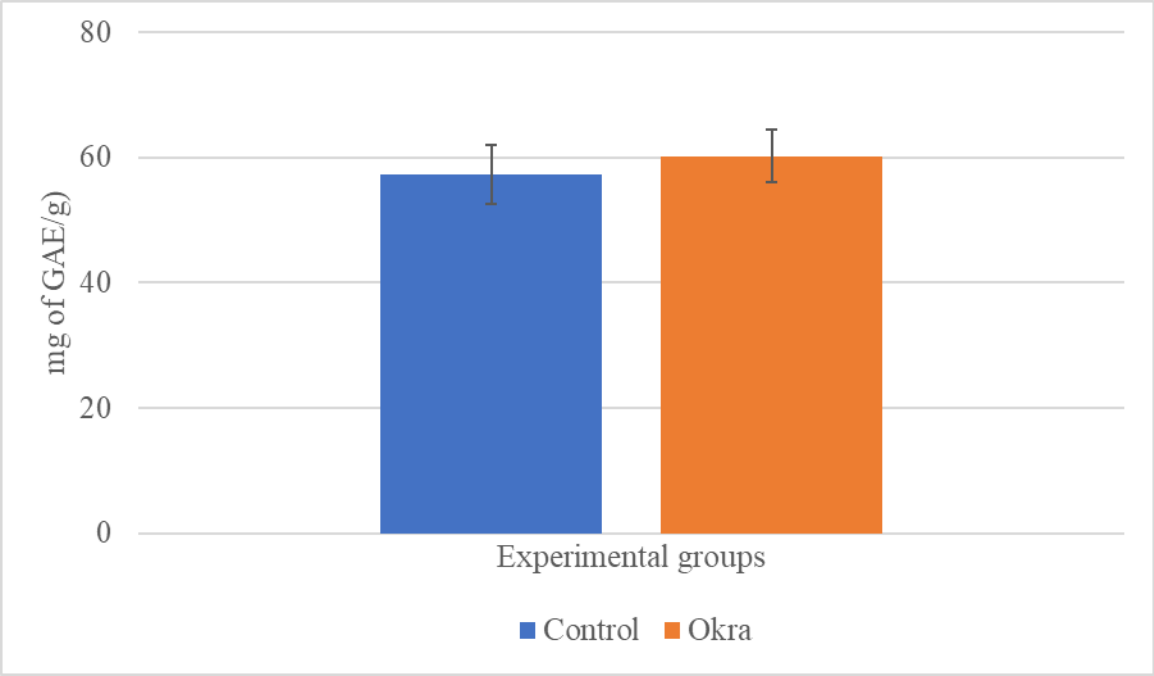
The total phenolic content of the experimental diets was determined using the Folin-Ciocalteu assay by constructing a standard curve with gallic acid (GA) (**Figure 3.**). taking into consideration the relationship between absorbance and concentration. The standard calibration curve equation was linear with the equation y= 0.0038x-0.2092 where R^2=0.9907.

Figure 3. Standard calibration curve of Gallic acid



Total phenolic contents (TPC) of the two diets are shown in **Figure 4.** It shows that the amount of TPC in control diet (57.35±4.75 mg GAE/g) is similar to the TPC in the okra diet (60.25±4.19 mg GAE/g).

Figure 4. Total phenolic contents in the food sample expressed in terms of Gallic acid equivalent (mg of GAE/g of dried food sample).



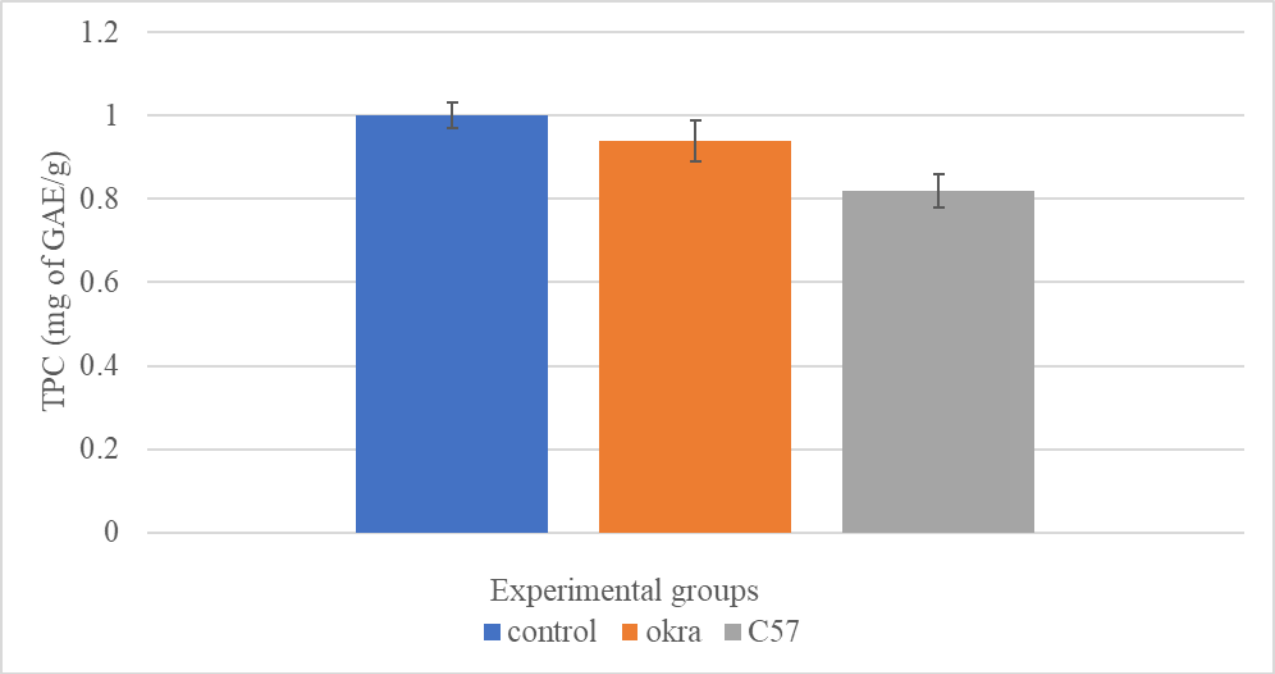
Data are presented as mean ± standard error.

6.3.2 Biological samples

6.3.2.1 Liver

As shown on **Figure 5.**, liver total phenolic contents were similar among three different groups of mice. However, non-significant higher total phenolic contents were detected in liver samples of control mice (1.001±0.03 mg of GAE/g) compared to okra fed mice (0.94±0.03 mg of GAE/g) and the C57BL/6 wild-type mice (0.82±0.03 mg of GAE/g).

Figure 5. TPC in liver samples of experimental animals expressed in terms of Gallic acid equivalent (mg of GAE/g of liver).

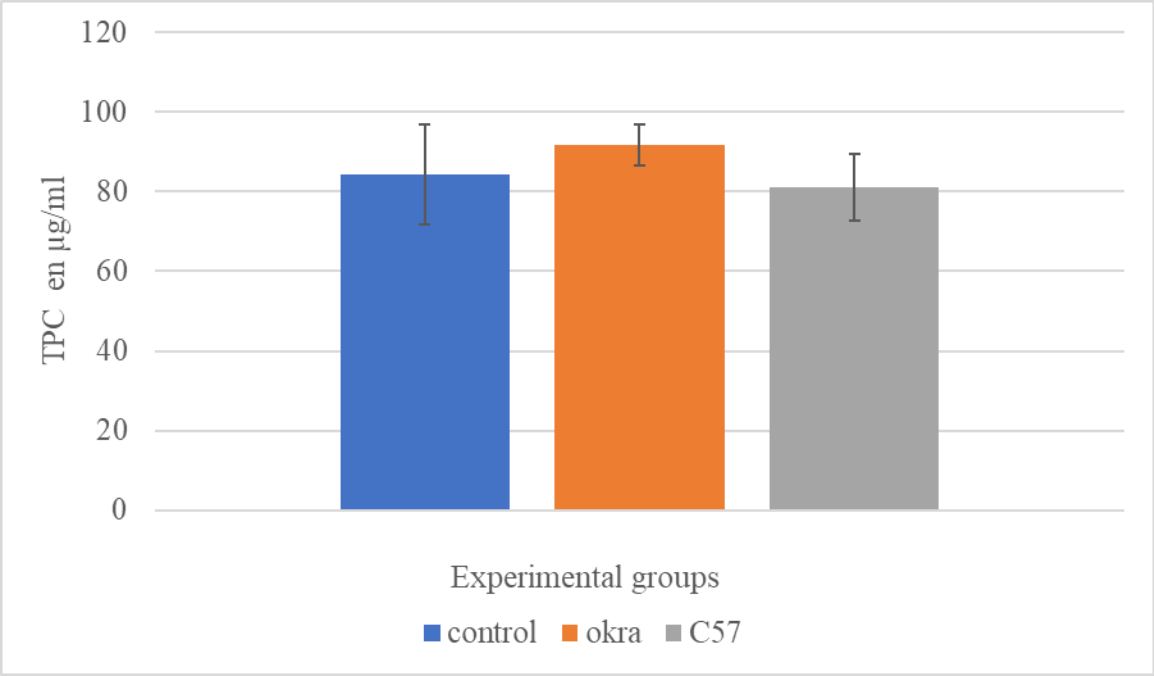


Data are presented as mean ± standard error (control, n=10; okra, n=9; C57BL/6, n=6).

6.3.2.2 Plasma

As shown on **Figure 6.**, plasma total phenolic contents were not significantly different among the 3 groups, control (84.43±12.6), C57BL/6 (81.04±8.33) and okra diet (91.79±5.18)

Figure 6. Total phenolic contents in plasma samples of experimental animals expressed in terms of gallic acid equivalent (ug of GAE/ml of plasma sample)



P*<0.05

Data are presented as mean ± standard error (control, n=10; okra, n=9; C57BL/6, n=6).

6.4 Individual phenolic compounds

Figure 7. Standard calibration curve of caffeic acid

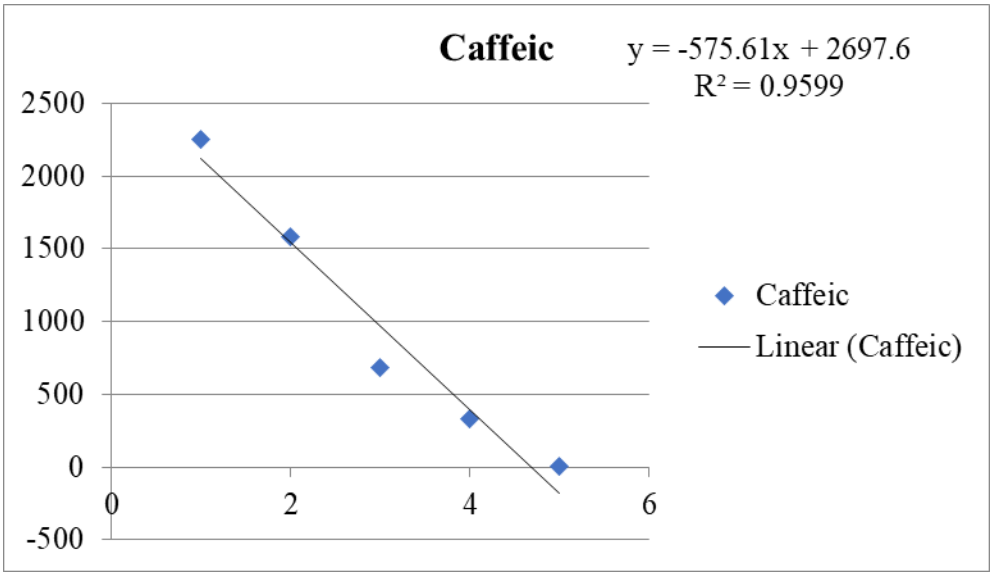


Figure 8. Standard calibration curve of myricetin

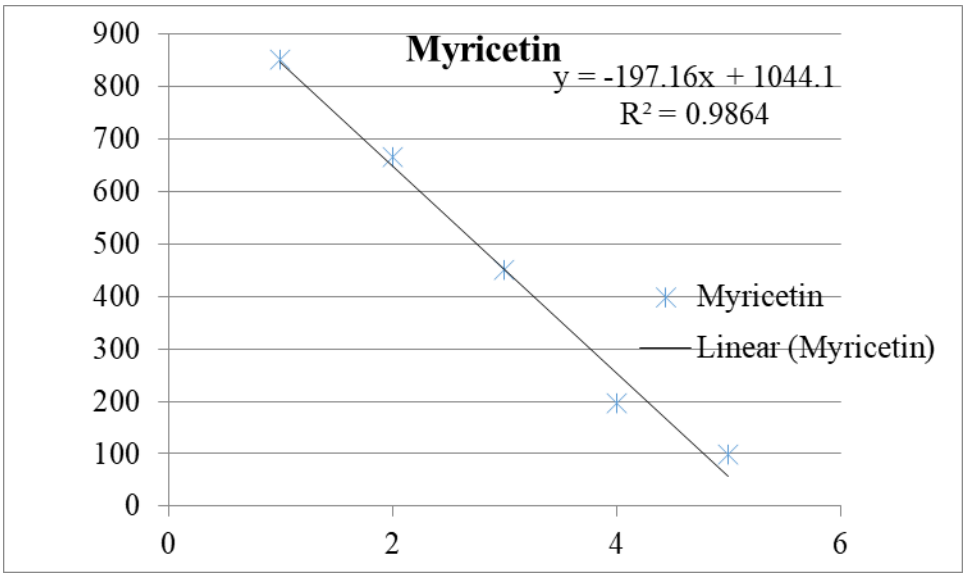


Figure 9. Standard calibration curve of rutin

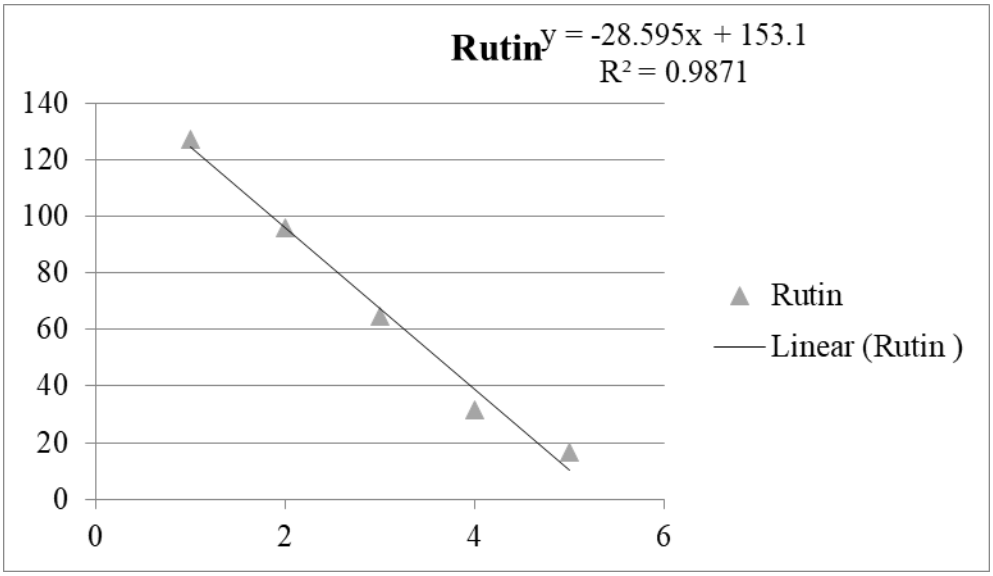


Figure 10. Standard calibration curve of quercetin

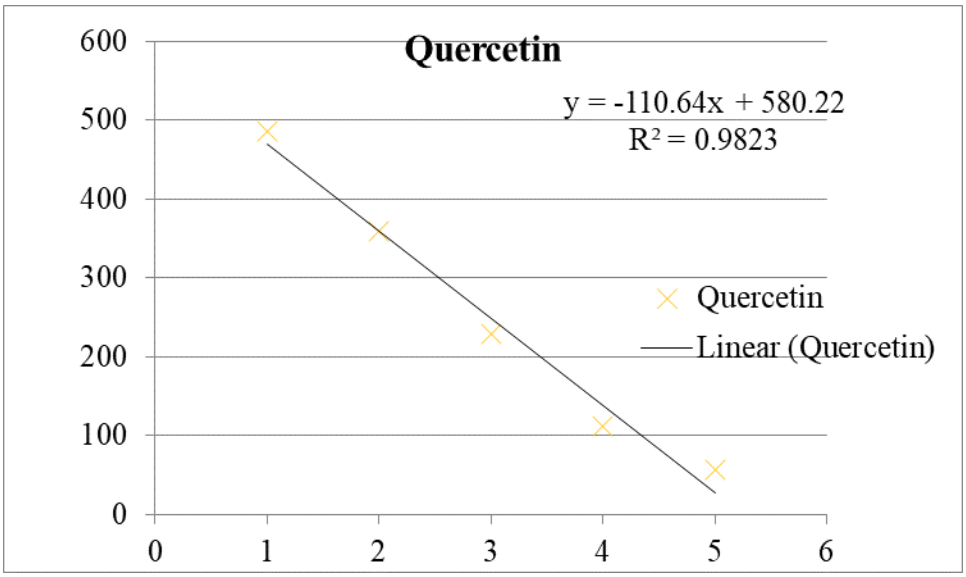
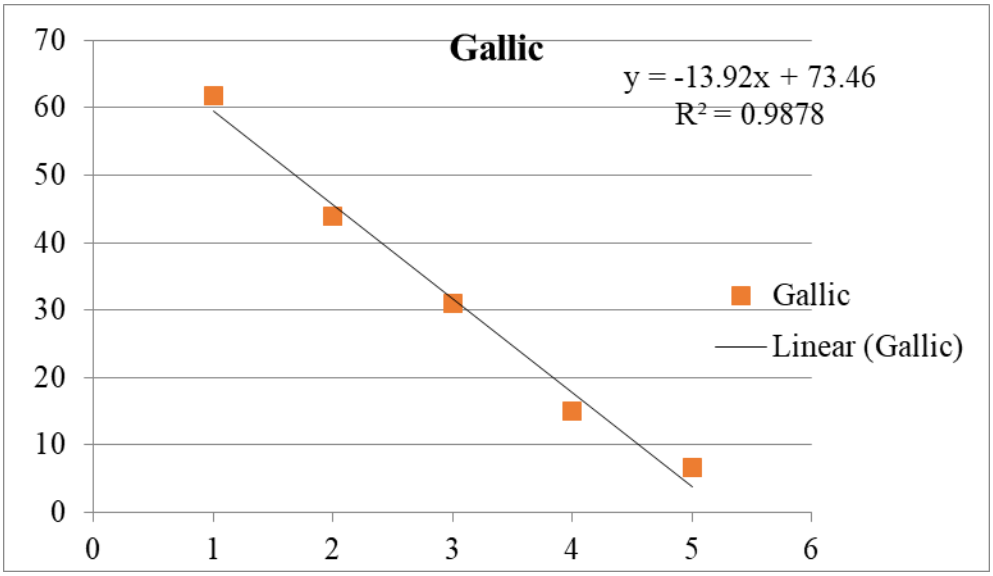


Figure 11. Standard calibration curve of Gallic acid



6.4.1 Concentrations of individual polyphenolic compounds

The concentrations of individual polyphenolic compounds were estimated from their retention time equivalent to standards (gallic acid: 8.69; rutin: 61.25; myricetin: 62.87; caffeic acid: 63.21; quercetin: 65.47). The percentage of individual polyphenol compounds in 10 mg of control diet according to standards used was 4.63±0.59% of gallic acid, 5.35±0.007 of rutin, 4.68±1.6E-05 of caffeic acid, 5.29±0of myricetin, 5.24±5.11of quercetin (**Table 4.)**. The composition of okra diet in phenolic compounds is like the control diet: 4.85±0.02 of gallic acid,

5.34±0.0008 of rutin, 4.68±1.23 of caffeic acid, 5.29±0.0002 of myricetin, 5.24±6.39 of quercetin.

Table 4. Individual phenolic compounds (flavonoids) detected in experimental diets

Compounds	Percentage (%) in 10 mg of Control diet	Percentage (%) in 10 mg of Okra diet
Gallic acid	4.63±0.59	4.85±0.02
Rutin	5.35±0.007	5.34±0.0008
Caffeic acid	4.68±1.6	4.68±1.23
Myricetin	5.29±0	5.29±0.0002
Quercetin	5.24±5.11	5.24±6.39

Data are presented as mean ± standard error

6.4.2 Biological samples

6.4.2.1 Liver

Table 5. shows representative results for Individual phenolic compounds detected in the mice liver. The number of individual compounds is similar among all experimental groups.

Table 5. Individual phenolic compounds detected in mice liver according to our standards

Samples	Gallic acid	Rutin	Caffeic acid	Myricetin	Quercetin
Control	5.26±0.03	0.25±0.01	4.68±8.98	5.29±0.005	5.24±3.22
Okra	5.28±0.0003	0.27±5.69	4.68±1.08	5.29±0.01	5.24±3.37
C57	5.28±0.001	0.26±1.31	4.69±4.02	5.29±0.002	5.24±6.8

Data are presented as mean ± standard error (control, n=10; okra, n=9; C57BL/6, n=6).

6.4.2.2 Plasma

The **Table 6.** shows representative results for individual phenolic compounds detected in the mice plasma. The number of individual compounds is similar among all experimental groups.

Table 6. Individual phenolic compounds detected in mice plasma according to our standards

Samples	Gallic acid	Rutin	Caffeic acid	Myricetin	Quercetin
Control	5.28±0.0001	0.26±0.0002	4.68±2.30E-06	5.29±0.001	5.24±7.95E-05
Okra	5.27±0.0004	0.26±0.002	4.68±5.69E-06	5.29±0.004	5.24±9.94E-05
C57	5.27±0.0007	0.26±1.23E-05	4.68±1.83E-05	5.28±0.01	5.24±2.66E-05

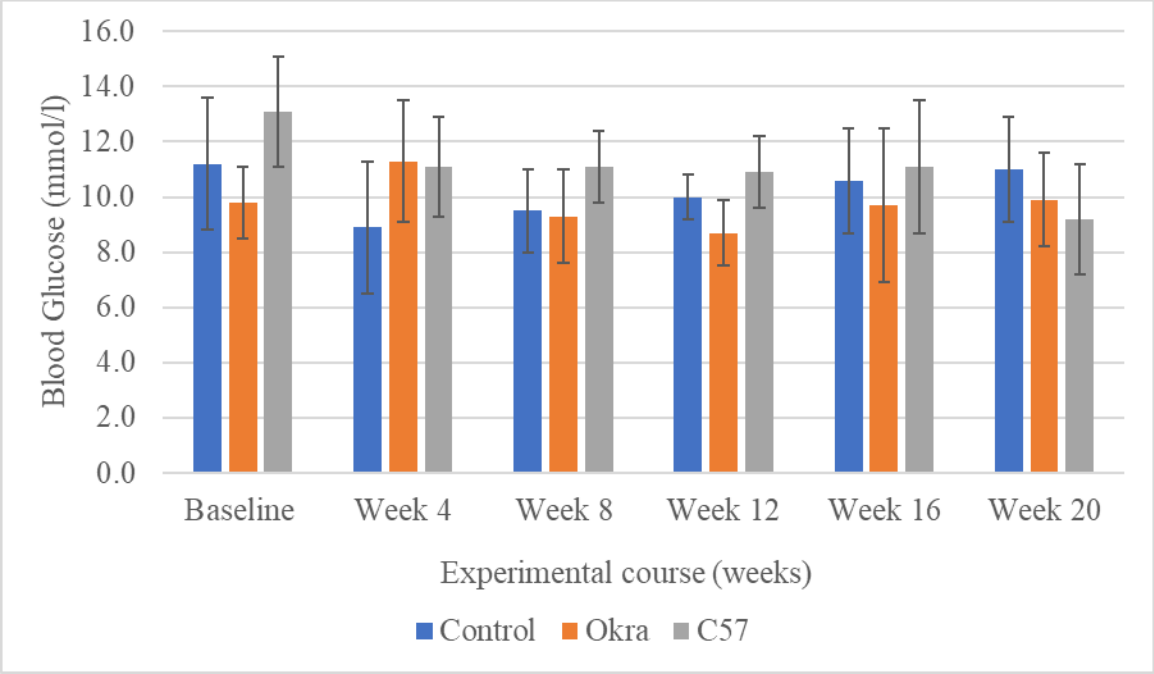
Data are presented as mean ± standard error (control, n=10; okra, n=9; C57BL/6, n=6).

6.5 Biochemistry

6.5.1 Blood glucose

Fasting plasma glucose of the mice was measured using a commercial spectrophotometric kit. At week 20, there was no statistical difference in fasting blood glucose levels among all the experimental groups (p=0.16) (**Figure 12**.).

Figure 12. Blood glucose measurement of mice up to 20 weeks on experimental diets

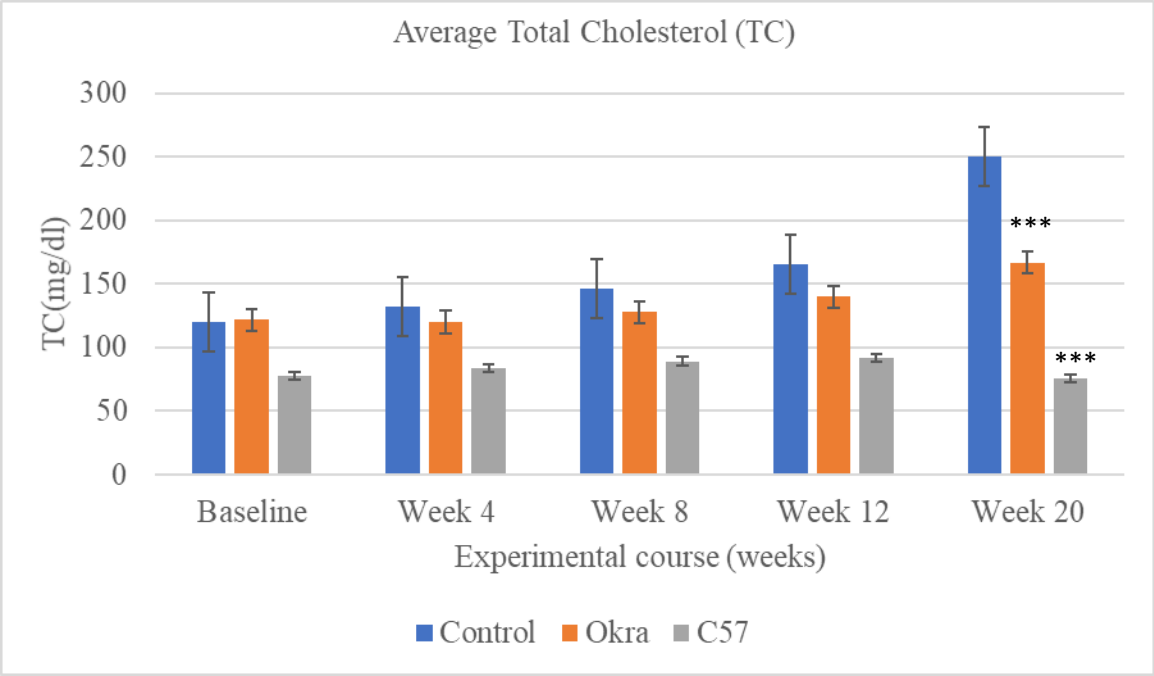


Data are presented as mean ± standard error (control, n=10; okra, n=9; C57BL/6, n=6).

6.5.2 Total Cholesterol (TC)

From the baseline to week 12, C57BL/6 shows a significant decrease in TC level compared to the 2 other groups. At the end of study, our results showed that LDLr-KO mice fed with okra diet and wild type mice C57BL/6 fed with control diet have significantly lower plasma total cholesterol level (167mg/dl and 75.5 mg/dL, respectively) than LDLr-knockout mice fed with control diet (250.4 mg/dL) as presented in **Figure 13.** with p=0.0001.

Figure 13. Total Cholesterol measurements of the mice up to 20 weeks on experimental diets.



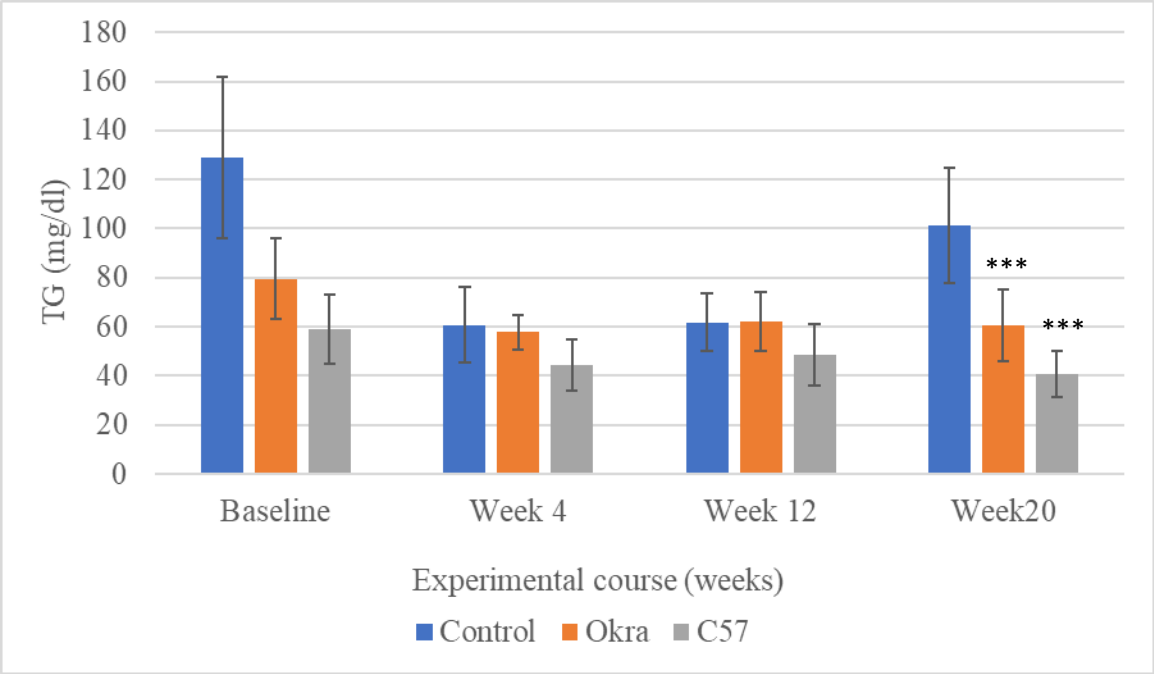
p*<0.0001**

Data are presented as mean ± standard error (control, n=10; okra, n=9; C57BL/6, n=6).

6.5.3 Triglycerides (TG)

Plasma triglycerides levels were comparable among all experimental groups (**Figure 14.**). The TG level at baseline from the control group was significantly higher compared to the 2 other groups (p<0.005). However, at week 4 and week 12, the 3 groups had similar TG levels. At week 20, LDLr-knockout mice fed with okra diet as well as C57BL/6 fed with control diet have again significantly lower plasma triglyceride concentrations (60.7 mg/dl and 40.6 mg/dl) compared to LDLr-knockout mice fed with control diet (101.1mg/dl) with p=0.0014

Figure 14. Plasma triglyceride concentrations over the experimental course



*****p <0.0001**

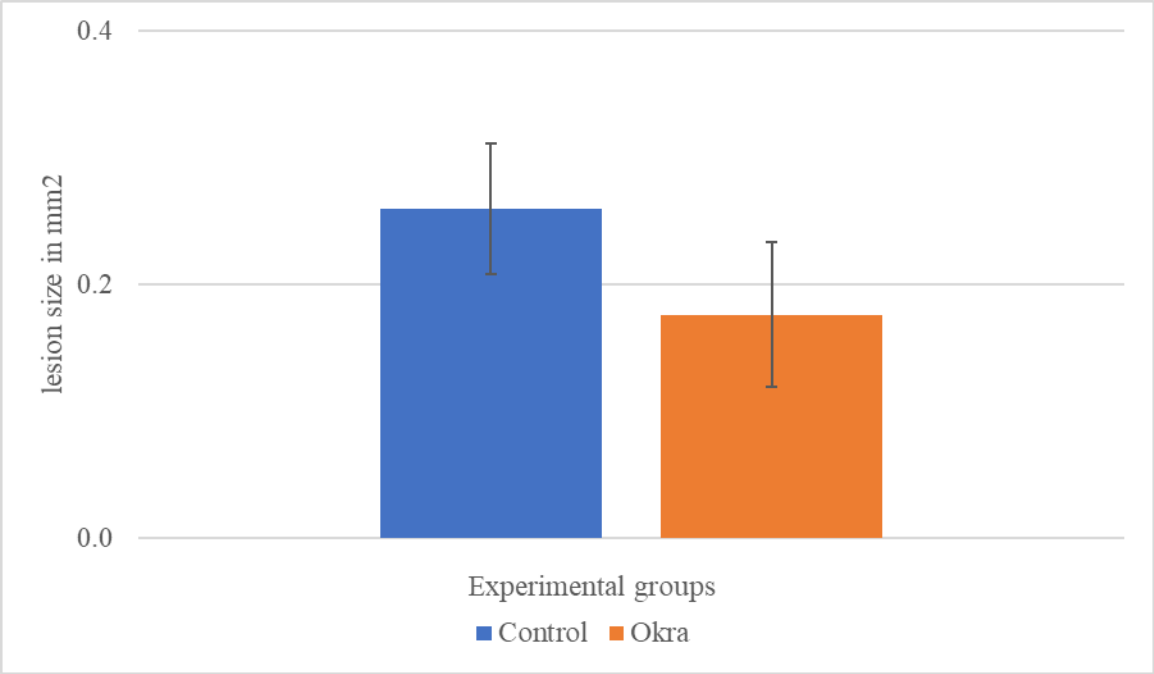
Data are mean +/- standard error.

6.6 Atherosclerotic lesion size

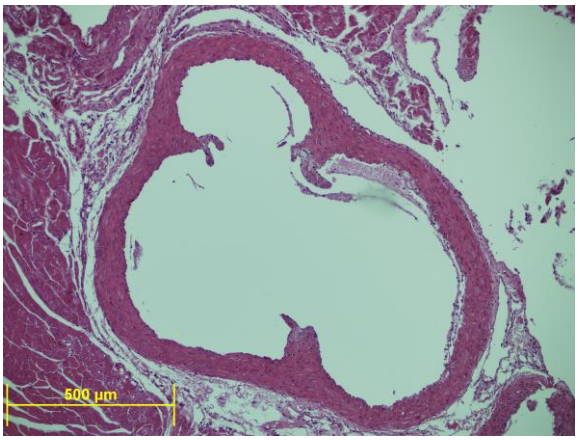
Figure 15. shows only a limited lesion formation in the aortic roots. There is a slight reduction in the extent of atherosclerosis lesion in okra treated mice as compared to controls (0.17±0.05 vs 0.25±0.05 mm^2 with $p>0.05$). However, no trace of lesion has been seen in the wild type C57BL/6 mice. Induction of our atherogenic diet didn't induce a significant atheromatic lesion in our experimental groups.

Figure 16. shows a representative photomicrograph of aortic roots of the 3 experimental groups.

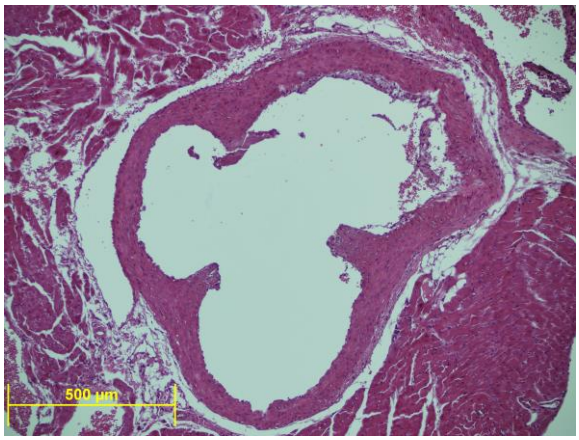
Figure 15. Extent of aortic atherosclerotic lesions following 20 weeks of dietary treatment.



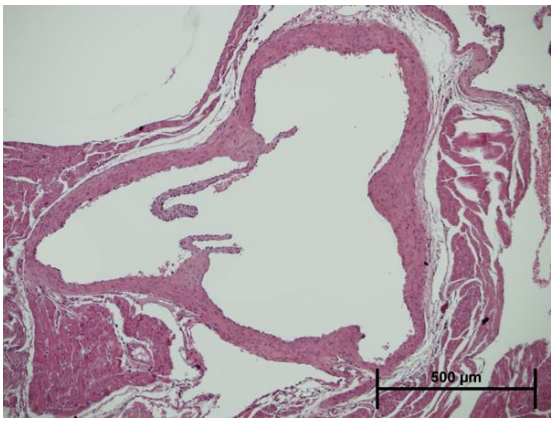
The lesion area was measured as the percentage of aortic luminal area covered by atherosclerotic lesions. Values are means ± SE (n = 4).



A



B



C

Figure 16. Histopathology of aortic roots from experimental mice.

Control group (Panel A); Okra group (Panel B); C57BL/6 (Panel C).

6.7 Inflammatory markers

Ten cytokines were measured by Meso Scale, IL-2, IL-4, IL-9, IL-10, IL-13, IL-21, IL-22, IFN-γ, MIP-3α (Mouse), TNF-α (Mouse). These cytokines have been chosen since their regulation is affected in a cardiovascular disease context. The effects of experimental diets on the inflammatory markers are shown below. Other markers such as IL10, IL-22, IFN-γ, MIP-3α (Mouse) and TNF-α (Mouse) were comparable. IL10 is the only marker which shows significant increase in the plasma of LDLr-knockout fed with okra diet compared to LDLr-knockout mice fed with the control diet ($p<0.005$).

Table 7. Effect of experimental diets on inflammatory markers

Cytokines	Control	Okra
IL-2	Below the detectable level	Below the detectable level
IL-4	Below the detectable level	Below the detectable level
IL-9	Below the detectable level	Below the detectable level
IL-10	4.32±1.77	9.34±2.19**
IL-13	Below the detectable level	Below the detectable level
IL-21	Below the detectable level	Below the detectable level
IL-22	3.09±1.39	2.20±1.64
IFN-γ	0.37±0.10	0.51±0.19
MIP-3α (Mouse)	23.34±6.20	57.10±34.14
TNF-α (Mouse)	5.59±1.42	3.63±1.51

P < 0.01.**

Values are mean ±standard error

7 Discussion

7.1 Proximate

Proximate analysis involves the determination of the major components of food as moisture, ash, crude fat, crude protein, crude fiber, and carbohydrate (227, 228). In this study, okra fruits powder (*Abelmoschus esculentus*), grown in south-western Nigeria, were analyzed. Moisture content determination is an integral part of the proximate composition analysis of food. As fresh okra pods vary considerably in water content, moisture contents were calculated on a dry weight basis, which allows a greater consistency of data. The moisture content in our okra sample was (11.06%) which agrees with the findings of Gemede et al. (155) which ranged between 9.69–13.33%. Our values are higher than the results of the three varieties of okra investigated by Kouassi et al. (229) (Zatta 7.85%; Abouakouassikro 6.90%; Sinzibo 7.10%). Moreover, our results fall within the standard range of 0-12% recommended by the Food and Agriculture Organization of the United Nations (FAO) (230). Consequently, *Abelmoschus esculentus* could be classified as a moderately hygroscopic substance with values between 9% and 15% moisture. The low moisture food is considered as low risk in terms of microbial

contamination therefore, it increases the shelf life of the product. Our data shows high percentage of fiber (56.46%) of okra. Observational studies have shown that dietary crude fiber intake is associated with decreased risk of cardiovascular diseases (125). It is inversely associated with risk of type 2 diabetes, coronary heart disease, myocardial infarction, congestive heart failure, and cardiovascular mortality (231, 232). Dietary crude fiber has important hypocholesterolemic effects and may reduce risk for coronary artery disease (233). Adetuyi *et al.* (234) reported that the crude fiber content of okra pod ranges from 10.15% to 11.63% which is much lower than the crude fiber obtained in this study (53.15%). The fiber content of the 2 okra varieties investigated by Kouassi *et al.* (229) were also lower than our studied sample (7.83% for the Baoule variety and 09.07 96% for the Dioula variety). In this regard, the consumption of *abelmoschus esculentus* should be considered to reduce the risk of CVD in humans. Regarding the protein content of okra fruit as shown, the values of the investigated sample (21.26%) were almost comparable with the finding of Gemede *et al.* (20.65%) (155) while higher than the value reported by Adetuyi *et al.* (234) (13.61–16.27%) and Nwachukwu *et al.* (235) (2.57%). The results obtained are slightly lower than the crude protein content of okra pod reported by Ogungbenle *et al.* (236) (23.4%), Soares *et al.* (237) (22.14%) and Huang (238) (22.98%). Our okra can be considered a high protein vegetable when compared with Cowpea, Catjang, Yardlong bean and Black-eyed pea (3.1–3.6%) (239). As shown by Effiong *et al.* (240), to be a good source of protein a vegetable is required to have at least 12% of their calorific value from the protein. This implies that okra fruit can serve as good source of protein. Studies suggest that high percentage of protein from vegetables is associated with a greater decrease in both total and LDL cholesterol especially in individuals with high fasting cholesterol concentrations which may contribute to their protective role against coronary heart disease (241, 242). The non-fiber carbohydrate content value presented in this study were lower than those reported by Nahri et al. (243). Recent randomized controlled trials document that low-carbohydrate diets not only decrease body weight but also improve cardiovascular risk factors (244, 245). Our sample could be considered as low source of non-fiber carbohydrate (3.72%) when compared with the content of some conventional sources like cereals with 72– 90% (246). In our finding, the fat content (1.4%) is higher than the value reported by Nwachukwu *et al.* (0.18%) (235) whereas lower than the value reported by Soares *et al.* (14.01%) (247). Dietary fats increase the palatability of food by absorbing and retaining flavors (248). Excess consumption of fat have been implicated in cardiovascular diseases such as atherosclerosis, stroke, whereas a diet supplying 1–2% of the

caloric requirement of the body from fat is sufficient to human beings (249) In this regard, the consumption of *abelmoschus esculentus* should be considered to reduce the risk of CVD in humans. The analysis of ash content in foods is simply the burning away of organic content and leaving the nutritionally important inorganic mineral contents present in the food material (250, 251). It is important because the amount of minerals can determine physiochemical properties of foods, as well as retard the growth of microorganisms. Our results depict that the sample contains high ash content (11.3%) which indicates that the okra would provide essential valuable and useful minerals needed for body development. The mean of ash content in this result (11.3%) is slightly higher than the findings of Adetuyi *et al.* (7.19–9.63 %) (234) whereas lower than the finding of Gemede *et al.* (OPA#6=11.30%) (155). The difference between our sample and other samples might be explained by geographical site and climatic conditions where the fruits were cultivated and harvested or to the investigated part of the plant as well as the degree of maturity of the okra fruit.

7.2 Food Intake, body weight and tissue weight in mice

Obesity contributes significantly to the overall problem of the onset of chronic metabolic diseases since it is a risk factor that can trigger many conditions such as CVD, diabetes, high blood pressure, stroke, pulmonary embolisms, certain cancers, etc. Evidence from numerous studies suggests an inverse relation between the consumption of okra and prevalence of obesity. Outcomes obtained from Nur Rahayuningsih *et al.*(252) concluded that lower body weight gain was consistently observed in white male rats who were fed with high fat diet combined with okra extract whereas consumption of only high fat diet contributed to greater weight gain in the control group. Our results show that the average food intake is equivalent between the three groups throughout our study, therefore, we conclude that the appetites of the mice were unaffected by okra treatment. Our results are in agreement with previous experimental data suggesting that extract of okra roots (28) and okra polysaccharide (202) don't significantly impact the food intake among the experimental groups. In our study, despite the same consumption, cholesterol diet caused elevated body weight in C57BL/6 and control group mice, but supplementation with okra fruit powder attenuated the weight gain in okra group mice. This result suggests that the reduction in the body weight of mice by okra powder supplementation was not through the inhibition of food intake. In this respect, Fan *et al.* (202). found that okra polysaccharide inhibits fat accumulation in C57BL/6 mice. However, Sabitha *et al.* (200)

showed that administration of mucilage from okra peel powder and okra seed powder in diabetic rats showed significant increase in body weight compared to the control group. The authors suggested that this action may be due to the preventive effect of the okra peel and seeds on structural protein degradation. However, another study shows that extract of okra roots doesn't decrease the body weight of the treated mice (28).

C57BL/6 mice show the highest body weight at week 20 (33.2 g) which was expected because this type is more susceptible to diet-induced obesity (253). The Framingham Heart Study as well as other studies confirmed that weight gain exacerbates all elements of the cardiovascular risk factors, including dyslipidemia, hypertension, insulin-resistant glucose intolerance, left-ventricular hypertrophy, hyperuricemia, and elevated fibrinogen (254). In this study consumption of okra diets significantly reduced abdominal fat mass in okra treated LDLr-KO mice (0.71 ± 0 .30g) compared to the C57BL/6 group (1.75 ± 0 .66g). There is also a reduction of kidney fat in okra diet group (0.33 ± 0 .02 g) compared to the control (0.51 ± 0 .25g) and C57BL/6 (0.46 ± 0 .03 g) groups. Growing body of evidence report relations of visceral fat accumulation with a higher prevalence of impaired fasting glucose, diabetes, insulin resistance, hypertension, dyslipidemia, coronary artery disease, inflammation, and multiple risk factor clustering (255-261). Our results showed no statistically significant differences in other tissue weights including heart and spleen among the experimental groups.

7.3 Total phenol content of diet, liver, plasma

Many epidemiological studies have shown that phenolic compounds found in a wide variety of fruits and vegetables provide antioxidant, immunomodulatory and vasodilatory properties activities, thus reduce the risk of developing cardiovascular diseases (262). The beneficial impact of polyphenols on cardiovascular diseases is diverse, but critical to their beneficial impact is their role in countering chronic and acute inflammation (37, 52). In the present study, total phenol content of 2 foods samples were evaluated. The contents of total phenol content (**Figure 4.**) were similar in both diets. However, the TPC value reported by Gemede *et al.* (212) in the mucilage of the pods of an okra accession are lower than our findings (49.93 mg GAE/g) TPC. Adetuyi *et al.* (234) in 2014 also revealed the presence of phenolic compounds in okra seeds. The phenol content of the okra pods analyzed by the authors ranged from 185 mg GAE /100 g to 1460 mg GAE / 100 g sample which 3-foldand plus higher than the TPC value of our sample. The reason of this discrepancy observed may be due to the

geographical location and climatic conditions of the harvest environment or the investigated organ of the plant. Solvent used in the polyphenol extraction may also explain the different results. Indeed, it is difficult to compare the values of our yields with other studies, because the yield is relative and seems to be linked to the genetic properties of the plants, their geographical origin as well as the stage of the fruits before harvest and the conditions extraction (263). Incorporation of okra fruit in the mice okra diet also distinctly boosted the total phenolic content of this diet group. However, the high total phenol content of our okra diet does not reflect either on the plasma nor the liver TPC value.

7.4 Flavonoids contents of diets, liver, plasma

Research on the impact of polyphenols on oxidation, inflammation and cardiovascular risks has primarily concentrated on the flavonoid class. Several *in vitro*, *in vivo* and clinical trials have shown that foods rich in flavonoids such as cereal grains, fruits and vegetables modify endothelial formation of nitric oxide in isolated blood vessels and improve endothelial function in experimental models of cardiovascular disease (264, 265). Studies suggested that the antioxidant and anti-inflammatory properties of okra is mainly due to its numerous flavonoid compounds (24, 266, 267). Previous studies have shown that okra fruits are mainly composed of quercetin, isoquercitrin, rutin, quercetin-3-O-gentiobioside, hydroxycinnamic derivatives, and catechin derivatives (25, 186, 187, 268). Therefore, a total of 5 flavonoid compounds, including quercetin, rutin, myricetin, caffeic acid and gallic acid, were selected and investigated in our okra diet as well as our control diet. Our study revealed that all our diets contain approximatively the same amount of the flavonoid compound tested (**Table 4.**). It is difficult to compare our results to others because there is no data available on the flavonoid compound of okra mixed with chow diet and chow diet alone.

In the plasma and liver samples of our experimental animals, the percentage of our flavonoid standards are the same in each group. However, the lower amount of rutin compared to the other compounds is explained by its low bioavailability which is partly caused by its limited membrane permeability, poor stability and low aqueous solubility (269). The constrained bioavailability of rutin is also supported by its metabolization by gut microflora to other products after ingestion which conduct to very little absorption(270).

7.5 Blood glucose

In this study, the high fat diet did not exhibit significant difference between ($p = 0.16$) the experimental groups. Our results (**Figure 12.**) are not in agreement with the findings of Fan *et al.*(28, 202) which revealed that the extract of okra and okra polysaccharide could decrease fasting blood glucose levels and improve glucose tolerance in high fat induced mice compared to the control group. Sabitha *et al*. (200) also showed that administration of *Abelmoschus esculentus* seed and peel powder at 100 and 200 mg/kg dose in diabetic rats showed significant reduction in blood glucose level compared to diabetic control rats (200).

7.6 TC and TG

Hyperlipidemia is a major health problem that has risen steeply in incidence worldwide. It is well known that this disease has been widely implicated in atherosclerosis and atherosclerosis related conditions like, ischemic cerebrovascular disease, coronary heart disease (CHD), peripheral vascular disease and pancreatitis (271). Thus, many people would benefit by reducing blood cholesterol levels. Non-pharmacological measures like dietary restriction and exercise may help in lowering blood cholesterol (171). In the present study, the effects of okra powder on circulating plasma lipid levels were investigated. Okra diet significantly reduced plasma total cholesterol (167 mg/dL) and triglycerides (60.7 mg/dL) levels on okra treated mice compared to control (respectively 250.4 mg/dL and 101.1 mg/dL). Consistent with previous observations, okra diet exhibit plasma total cholesterol and triglycerides lowering effects on the experimental animals. Wang *et al.* (170) suggested that mice fed with hyperlipidemic diet supplemented by 1 or 2% okra powder (OP) for eight weeks exhibited decreased serum total cholesterol and triglycerides levels. Our results are also consistent with the findings of Liao *et al.* (188) who assessed the impacts of a novel polysaccharide isolated from OP in diabetic mice fed with a high-fat diet for eight weeks. At the end of the study, they found out that OP significantly alleviated the symptoms with improved lipid profile. Interestingly, extracts from okra total plant and fruit studied by Ngoc *et al.* (171) remarkably reduced the cholesterol and triglyceride levels in the plasma of hyperlipidemic mice. In the same trend, Adewale *et al.* (272) proved that ethanol extracts of okra at a dose of 200 and 400 mg/kg (AWOB) significantly lowered the TGs and cholesterol levels in Triton-Induced Hyperlipidemia Rats. Vindika *et al.* (273) showed that the crude water extract and the water fraction of okra fruit was successful in reducing plasma triglycerides and cholesterol levels of hypercholesterolemic rats. Another study

showed the antihyperlipidemic property of okra peel and seed powder in streptozotocin induced diabetic rats (200). The authors used only the extract from the mucilage of the okra fruits ensuring that the other functional compounds contained in seeds and peel are not included in the experimental diet. Maybe if the whole fruits were used, this will enhance the lipid lowering property of their treatment diet. Those findings along with our study proved that all different parts of okra have lipid lowering properties. As a result, we could add okra to the list of dietary medicine for hyperlipidemia. Various mechanisms have been proposed for anti-hyperlipidemic action of *A. esculentus*. Wang *et al.* (170) suggested that the abundant dietary fiber contained in okra might contribute to its hypolipidemic activity by binding to bile acids, consequently lowering TC through interfering with bile acids reabsorption. Although we did not examine this possibility, it is a reasonable hypothesis and would be consistent with our data. Wang's theory is deeply explained by an in-vitro study which proved that the okra skin extract exhibited cholesterol-lowering activities by inhibiting pancreatic cholesterol esterase activity, reducing the efficacy of cholesterol micellization, and binding to bile acids, which may result in delayed cholesterol absorption and increase cholesterol excretion(274). However, Adewale *et al.* (272) pointed out that the reduction of TC in triton-X induced hyperlipidemic rats by the *A. esculentus* extract was associated with a decrease of its LDL fraction, which is the target of several hypolipidemic drugs. Their result suggests that cholesterol-lowering activity of the herb extract can be a result from the rapid catabolism of LDL-C through its hepatic receptors for final elimination in the form of bile acids. Another possible explanation is that the hypolipidemic activity of okra might be mediated likely by upregulation of cholesterol degradation through cholesterol 7α-hydroxylase and by inhibition of lipogenesis through sterol regulatory element-binding protein 1c and fatty acid synthase (two key modulators of fatty acid and cholesterol biosynthesis) (170). Furthermore, Ngoc *et al.* (171) suggested that antihyperlipidemic effect of extracts from total plant and fruit of A. esculentus could be due to interfering with cholesterol biosynthesis. Additionally, Vindika *et al.* (273) mentioned that the lipid lowering property of okra bears polar groups since the activity was found in the water fraction. This is supported by Uraku *et al.* (275) who demonstrated that the crude water extract of okra fruit had shown elevated total bilirubin levels in blood of diabetic albino rats. Therefore, the authors concluded that low blood cholesterol level and other lipid parameters are due to catabolism of cholesterol into bilirubin. Moreover, Fan *et al.* (28) reported that the lipid lowering effect of okra's extract appeared to be through the inhibition of the expression of nuclear hormone

receptor PPARy which control the expression of various genes that are important for lipid homeostasis. The mechanism of action of hypolipidemic effect of okra remains controversial, that is why further scientific investigation are needed.

Interestingly, the plasma lipid levels achieved in C57BL/6 mice were much lower than those achieved in the other murine models throughout the entire study. This was expected even though both strains are standards used for hyperlipidemia study when fed a high cholesterol diet, LDLr-knockout mice are more susceptible than C57BL/6 to hyperlipidemia. The reason is that the genetic background and the lipoprotein composition of C57BL/6 and LDL receptor deficient mice are different.

The results of our study have relevance for humans to consider supplementation of okra powder in the diet. Okra supplementation may be beneficial for people suffering from or at risk of developing atherosclerosis by reducing aggravating factors such as dyslipidemia. Further studies should be done to determine the clinical influence of okra. It is important therefore, to discuss the relevance of our dietary intervention to humans.

7.7 Atherosclerotic lesion formation

Accumulations of fatty substances and cholesterol are thought to be a major cause of injury to the endothelium and underlying smooth muscle cells, recruiting macrophages-derived foam cells which are present in all stages of atherosclerosis and are believed to play an important role in both the initiation and progression of atherosclerotic lesions (37). Low-density lipoproteins (LDLs) are the main carrier of cholesterol in human plasma and their oxidation plays an important role in the early atherogenic process. The impact of antioxidants such as natural phenolic compounds inhibiting LDL oxidation has been confirmed in several studies. Reduced levels of LDL oxidation are associated with reduced levels of inflammatory state which result in prevention of advance atherosclerotic lesion (276). Okra is rich in phenolic antioxidants, and therefore, the phenolic antioxidants of okra are thought to produce health benefits through their antioxidant properties (217).

Despite the greater duration of the dietary intervention, there was a slight reduction in atherosclerotic lesion in okra treated mice compared to the control group. However, the complete absence of lesions in the aortic roots of the mice was somewhat expected as it appears that the induction of persistent hypercholesterolemia to levels >300 mg/dL is required for the development of experimental atherosclerosis in the mouse (277). In our study, no groups

exceeded this amount. This is not in agreement with previous studies which have demonstrated that LDLr knockout mice show dramatically elevated cholesterol levels and prominent lesions all over the aorta after 8 months on the Paigen diet (278) (279), supporting their utility as promising models for investigating the pathogenesis of atherosclerosis. The atherosclerotic lesion formed in the aortic roots of okra group is lower than control groups, in spite of substantial increases in total cholesterol concentrations in control mice which may show that phenolic compounds of okra don't influence the atherosclerotic lesion formation. The mice in the current study were followed for only 20 weeks on the cholesterol diet. It will be important to follow these mice for longer periods to determine whether control group will develop frank atherosclerotic lesions compared to okra and C57BL6 groups.

7.8 Inflammatory markers

Epidemiological and anatomopathological studies carried out in humans, combined with in vitro studies, using human cells, have provided evidence that atherosclerosis is a chronic inflammatory disease of large and medium-sized arteries. It has been shown that the formation of atherosclerotic plaque involves several stages: activation of the arterial endothelium by oxidized LDL, attraction and then diapedesis of monocytes and T lymphocytes circulating in the intima, the production of pro and anti-inflammatory cytokines, the production of matrix proteases and finally the induction of apoptosis of the different cell types leading to the formation of a necrotic lipid nucleus. In our study, we selected and measured, IL-2, IL-4, IL-9, IL-10, IL-13, IL-21, IL-22, IFN-γ, MIP-3α (Mouse), TNF-α (Mouse) since their regulation is affected in a cardiovascular disease context. Markers such as IL10, IL-22, IFN-γ, MIP-3α (Mouse) and TNF-α (Mouse) were comparable. Although we perceived changes in the other markers. IL10 showed significant increase in the plasma of LDLr-knockout fed with okra diet compared to LDLr-knockout mice fed with the control diet. IL10 is present in human atherosclerotic plaques and its local expression is inversely correlated with inflammation and cell death. In other words, plaques rich in IL10 are characterized by a lesser accumulation of inflammatory cells and apoptotic cells. Mice invalidated for the IL10 gene, put on a high-fat diet, develop more atherosclerotic plaques than control mice and these lesions are characterized by a greater infiltration of inflammatory cells such as T lymphocytes (280, 281). In our study, okra diet shows a pro-inflammatory effect on the treated mice. This is in agreement with other studies. Lectin of okra at 0.01, 0.1, and 1 mg/kg (i.v.) induced an inflammatory effect in mice (n=6-10). In another recent study, the

ethanol extract of okra (500, 250 or 100 mg/kg [p.o.]) was found to reduce inflammatory response (scores) in ethanol-induced acute gastric mucosal injury Wistar rats (n = 7) (180). Hydrolyzed okra extract and its polysaccharides (25–100 μg/mL) in rat bone marrow hematopoietic cells increased in mean hemoglobin content class II and CD80/86 expression levels, whereas reduced in endocytosis activity (282). In that study, an increase in the secretion of interleukin (IL)-12 and interferon-gamma, whereas a decrease in IL-10 production was also seen. Crude polysaccharides of the herb (25–100 mg/kg) significantly increased cell proliferation, nitric oxide production, inducible nitric oxide synthase expression, and tumor necrosis factor-α, IFN-γ, and IL-10 secretion in RAW264.7 cells (195). These findings show the potential of *A. esculentus* and its parts, as well as their derived components, on anti-inflammatory effects.

8 Summary of main findings and conclusion

Whole okra fruit powder shows high protein, high fiber, low fat, and high polyphenol contents in this study. Incorporation of okra powder in animal diet increases but not significantly its total phenolic contents compared to control diet. Moreover, okra powder attenuated the weight gain in the treated group. In addition, the okra diet beneficially modified plasma lipid profile, mainly through reducing circulating plasma total cholesterol and triglyceride levels. However, the glucose content was not influenced by the consumption of okra. There was a slight reduction in atherosclerotic lesion in okra treated mice compared to the control group indicating a minimal effect of long-term okra diet consumption on the atherosclerotic lesions. Improved lipid profile was in an agreement with the inflammatory process in the okra-treated group. Therefore, okra is shown to demonstrate higher potential health benefits compared to the chow diet. These health benefits are probably due to the synergy of its functional components and phytochemical compounds. In summary, long-term consumption of okra has desirable protective roles in improving plasma lipid profile and inflammatory markers in LDLr-KO mice. Supplementing a diet with okra powder may potentially lower cardiovascular risk through its hypocholesterolemia and pro-inflammatory effects.

9 Strengths and limitations

A considerable strength of this study is the duration of the experimental course, 20 weeks, which was enough to note effects of both treatment and time on each risk factors studied. The

prolonged experimental time also allowed us to measure the long-term effect and safety profile of treatment diets. Moreover, we used LDLr-KO mice as our experimental animals in the present study. LDLr-knockout mouse is one of the most extensively used animal models of dyslipidemia and atherosclerosis (283) . The plasma lipoprotein profile of this mice model is closer to the human situation; therefore, it is the best option when studying altered cholesterol metabolism. In addition, we were able to compare our treatment group to the wild type C57BL/6 mice which act as a control in cardiovascular risk factors measured, and also allow us to detect the difference between normal animals and our treated groups. Our study, like many others, has potential limitation. There were insufficient blood samples as well as liver amount for the measurement of total phenol content.

10 Recommendations for Future Studies

The findings of this research showed improved anti-hyperlipidemic and anti-inflammatory status of okra, therefore, they might be the potential alternative functional food ingredients to okra. However, further research is needed to assess the effects of okra on cardiovascular risk factors and the underlying mechanisms of actions will be appreciated. The fecal cholesterol excretion will be a big step to understand the hypocholesterolemia property of okra. Also, other lipid profiles such as high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) and very-low-density lipoprotein cholesterol (VLDL-C) and fecal cholesterol excretion should be investigated. It may be beneficial that in the future study, we determine the SOD, CAT and GPx to understand the mechanism underlying the potential anti-oxidative effect of the okra diet. Furthermore, other functional components of okra, such as crude fiber, and crude protein should be examined to identify their potential health benefits. This information will provide a new understanding of the mechanism of paradoxical effects of okra and may lead to the development of a better therapy for the treatment/prevention of atherosclerosis. All the results taken together can further encourage examining the potential clinical outcome of okra in patients with cardiovascular risk factors, and oxidative stress-related diseases.

Appendices

Appendix 1

Animal Care & Veterinary Services

UNIVERSITY OF MANITOBA | Research Ethics and Compliance
Office of the Vice-President (Research and International)

16 November 2016

TO: Dr. Mohammed Moghadasian
Department of Human Nutritional Sciences

FROM: Dr. B. MacNeil, Chair, Bannatyne Campus Animal Care Committee

RE: **Amendment to Protocol 13-053/1/2 (AC10879)**

Please be advised that the amendment as documented in your Application for Amendment (attached), to the above noted protocol, has been approved.

BMN/ck

Copy: Dr. R. Aitken, Director, R.O. Burrell Lab

Attach.

umanitoba.ca/research

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UNIVERSITY OF MANITOBA

Application For Amendment To Animal Use Protocol

UNIVERSITY OF MANITOBA
NOV / 8 2016
Research Ethics & Compliance
Animal Care

NOTE: An amendment may be used for minor changes in numbers of animals; addition and/or deletion of species; and minor modifications to procedures on live animals. Changes requiring full protocol submission include substantial changes in procedures previously described in an active protocol, addition of new procedures not before described in an active protocol, large changes in the number of animals.

For more detailed information pertaining to amendment versus full protocol submission, see link to Guideline 002
http://umanitoba.ca/research/orec/animal_care/animalcare_compliance_guidelines.html

13-053/2	December 12, 2016	35298	(UM Project # can be found using My Research Tools) http://umanitoba.ca/research/ors/mrt-faq.html
AC10879 Protocol #	Expiry Date	UM Project #	

PRINCIPAL INVESTIGATOR AND EMERGENCY CONTACT

Principal Investigator:

Moghadasian	Mohammed	MM	[redacted]	
Surname	First Name	Initial	Telephone	Fax
Professor	[redacted]	[redacted]		
Academic Appointment	Department/Faculty	Email Address		
[redacted]	[redacted]	[redacted]	[redacted]	
Office Address	City	Province	Postal Code	

An Investigate of antioxidant properties and Beneficial of Lipid Profile Effects of the Northern Wild Rice, Saskatoon Berry extract, Sea buckthorn Berry and Okra fruit in experimental animals

Project Title

1 REASON FOR AMENDMENT REQUEST

- [x] Personnel Changes: Addition or Deletion of Personnel, change in Competency Level and/or Change/Addition of duties/ procedures assigned to existing personnel
- [ ] Transfer of Protocol to Different PI
- [ ] Change in Animal Numbers (addition/deletion)
- [ ] Change in Species, Strain, Line, genotype, age and/or sex of animals
- [ ] Change in anesthetics and/or analgesics
- [ ] Change in drugs or compounds given to animals
- [ ] Change in Procedures and/or Addition of New Procedure(s)
- [ ] Change in Funding and/or Title of Project
- [ ] Other (Please Indicate):

2 PERSONNEL CHANGES

(Indicate name of personnel, place an "x" in the applicable box in column and follow instructions given in column heading)
(Click +/- to add or delete rows to the table)

Name	Addition *	Deletion **	Change in Competency Level ***	Change/Addition of duties/procedures assigned to existing personnel ***
Rokiatou Kone Berethe	X			

* For new additions, please complete section 2a below; ** Nothing else required, *** Submit a revised Schedule 1

2a Is the new person handling live animals | Yes | If yes, a Schedule 1 must be completed.

If the new person is not handling live animals, what is their role? **** Other (provide details)

**** Completion of a Schedule 1 is not required. However, these individuals must complete the Animal User Training Course. If this has not been completed please self register at http://umanitoba.ca/research/orec/ethics/animalcare_education_training.html

Note:
1) Where protocols have an associated Schedule 10A Risk Assessment or SBRC Schedule 10, addition of personnel will require a Schedule 10B to be submitted with this amendment.

2) Where protocols have an associated Schedule 4 Humane Endpoints, addition of personnel who are conducting endpoint monitoring will require a Schedule 4A to be submitted with this amendment.

http://umanitoba.ca/research/orec/animal_care/animalcare_forms.html

3 TRANSFER OF PROTOCOL TO DIFFERENT PRINCIPLE INVESTIGATOR (PI)

Indicate below the name of the PI now assigned to this protocol and the date this becomes effective. This PI assumes all responsibility for oversight of procedures, personnel, etc. **Submit a Schedule 1 and 10B (if applicable) for the new PI.**

Name of New PI _____ Effective date of transfer _____

Signature of New PI _____ Telephone _____ Fax _____

Academic Appointment _____ Department/Faculty _____ Email Address _____

Office Address _____ City _____ Province _____ Postal Code _____

Is the new person handling live animals | | If yes – A Schedule 1 must be completed. ** = Completion of a Schedule 1 is not required. However, these individuals must complete the Animal User Training Course. If this has not been completed please self register at: http://umanitoba.ca/research/orec/ethics/animalcare_education_training.html

If the new person is not handling live animals, what is their role? ** Other (provide details)

4 CHANGE IN NUMBERS OR TYPES OF ANIMALS REQUIRED

a) Complete the table below if you are:

- removing a currently approved species, strain/line/genotype or age/weight group from the protocol
- adding a new species, strain/line/genotype or age/weight group to the protocol
- requesting an increase in numbers for an already approved species, strain/line/genotype or age/weight group on the protocol

Note: Requests for different age groups of the same species, strain/line/genotype should be made separately. (example: Neonatal rats versus adult rats, weaned pigs versus feeder pigs, SCID mice versus CD1 mice) **Use the approved strain names as issued on your original approval letter and/or in subsequent amendment forms.**

Species (as per CCAC definition)	Strain/breed/Line/Genotype/Common Name	Sex	Age or Weight

Number currently approved "0" if new	Additional number being requested	Number to be removed (if applicable)	Total

b) Below, justify the animals requested in the table. Address the following in your explanation:

- how the change in number or type of animals relates to the current objectives of the protocol;
- provide information on experimental and control groups (including number of animals per group) by briefly indicating what procedures all the requested animals will undergo. New procedures must also be stated in section 7.
- expected failure rates for the procedures and impact on number of animals requested;
- what statistical calculations were used to arrive at number requested;
- if using animals to provide tissues for in vitro work, give the expected product yield from each animal.

5 CHANGES IN ANESTHETIC AND/OR ANALGESIC DRUGS

a) Complete the following table

Additional/New Anesthetics or Analgesics Requested	Dose and route of administration

b) Indicate the reasons for the changes as it relates to protocol objectives, animal welfare improvement, etc.

6 CHANGES TO DRUGS OR OTHER COMPOUNDS GIVEN TO ANIMALS (TEST AGENTS, ANTIBIOTICS, ETC.)

a) Complete the following table

Additional Agents Requested	Dose and Route of administration

b) Indicate the reasons for the changes as it relates to protocol objectives, animal welfare improvement, etc.

c) Please specify any expected side effects that may result from each of these changes.

7 CHANGE IN PROCEDURES AND/OR ADDITION OF NEW PROCEDURES

Note: If major procedural changes are to be made in this project, a new protocol must be submitted. Consult the Clinical Veterinarians or the Chair of the ACC for help in deciding whether your changes are minor or major. Alternatively, see link to Guideline 002 http://umanitoba.ca/research/orec/animal_care/animalcare_compliance_guidelines.html

a) Describe and justify any procedure changes and/or additions of new procedures to the protocol. Indicate what current study objective this change aims to address.

8 CHANGE IN FUNDING/MERIT AND/OR TITLE OF PROJECT

Source/Agency	Status of Funding	Was the project described in this protocol (including animal use) included in the proposal that was approved for funding?	Status of Merit Review

* If not included or if scientific merit review is required, please see the process for obtaining scientific merit review http://umanitoba.ca/research/orec/animal_care/animalcare_compliance_guidelines.html

Indicate revised project title if applicable.

9 SCHEDULES

Changes made to the initially approved protocol may result in the need to review and revise a number of previously approved Schedules or necessitate completing a new Schedule. If this amendment changes the information contained in a previously approved Schedule, a new Schedule containing the applicable initially approved information and the new information (as a result of this amendment) must be submitted.

On the list below, check off revised/new Schedules required and attach to this amendment. If you are unsure whether you need to submit a new/revised Schedule with this amendment, consult with the applicable Veterinarian for your Institution. (Clinical Veterinarians at U of M, Dr. Randy Aitken at SBRC).

	Yes	No
Schedule 1, Personnel Complete a Schedule 1 for all personnel as identified in Block 2 who are using live animals.	☑	☐
Schedule 2, Anesthesia, Sedation, Chemical Restraint When anesthesia, sedation, and/or chemical restraint agents are being used. If anesthesia immediately precedes euthanasia, a Schedule 2 is not required.	☐	☐
Schedule 2B, Use of a Neuromuscular Blocking Agent (NMB)	☐	☐

Schedule 3, Surgery When surgical procedures are being performed (both recovery and non-recovery).	☐	☐
Schedule 4, Humane Endpoints Required for C, D or E category of invasiveness experiments. The schedule will ask you to provide a description of conditions that may cause distress/discomfort, how they will be identified and what will be done to alleviate them.	☐	☐
Schedule 4A, Humane Endpoints - Adding New Personnel for the purposes of endpoint monitoring To be completed when new personnel are being added to the protocol (via an amendment) who will be involved with endpoint monitoring.	☐	☐
Schedule 5, Physical Restraint For restricted housing, e.g. metabolism crates/cages, or any restraint not normally part of regular husbandry practices and longer or more severe than normally required for examination, injection or a single blood collection in conscious animals. Completion of this schedule is not required for cattle restrained in a head gate/squeeze chute for surgical procedure.	☐	☐
Schedule 6, Nutrient and/or Diet Modifications For any alteration to the diet in which (a) specific nutrients are added or removed from the diet; (b) feedstuffs not normally fed are being used; (c) physical form of the diet is changed significantly from the usual form.	☐	☐
Schedule 7, Behavioural Experiments If the project involves behavioural manipulation, shock, negative reinforcement, punishment, removal of feed or water for behavioural reasons, predator/prey relationships, or sensory deprivation.	☐	☐
Schedule 8, Environmental Manipulation If the project involves environmental manipulation or imposes any potential adverse environmental effect. (Examples: changes in atmospheric gases, temperature, exposure to noxious gases, etc.)	☐	☐
Schedule 9, Teaching When the main purpose of animal use is education, including courses, workshops, demonstrations, etc.	☐	☐
Schedule 10A, Risk Assessment (U of M) To be completed when research compounds or agents specific to the project cause acute or chronic injury including but not limited to carcinogenicity, reproductive toxicity, heritable genetic damage, teratogenicity, embryotoxicity, mutagenicity, irritation, sensitization, fetal effects, or any other negative effects due to chronic or acute exposure. It must also be used when research agents are biohazardous, radioactive. If the research includes physical hazards not normally encountered in animal research such as working at heights or confined spaces. Refer to page 1 of the schedule for additional information.	☐	☐
Schedule 10.2A To be completed when hazard information for the second and subsequent agents included in the submission which meet the criteria for a Schedule 10A.	☐	☐
Schedule 10B Risk Assessment - Adding New Personnel To be completed when new personnel are being added to the protocol (via an amendment) where an associated Schedule 10A was submitted and approved with the initial protocol.	☐	☐
Schedule 10F Safe Work Practice 001 Formaldehyde Perfusion of Animals To be used when formaldehyde perfusion is the only hazardous agent used in the context of the research. If other hazardous agents are included include formaldehyde in the list of agents and reference Safe Work Practice 001.	☐	☐
Schedule 10, Risk Assessment (SBRC) To be completed if any of the administered agents of the protocol meet the criteria described on the schedule instructions.	☐	☐
Schedule 11, Field Study Where animal use is in whole or in part conducted in the field and/or the project involves capture or release of animals in the wild.	☐	☐
Schedule 12, Common Procedures To provide more detail for common procedures including blood and/or tissue collection prior to euthanasia (including tail snips and ear punches), fecal and ingesta collections; individual marking; administration of compounds via injection, oral administration (gavage or via feed or water); catheter placement, physiological measurements such as blood pressure and ultrasound, etc., removal of all feed and/or water, indwelling osmotic pumps, etc. NOTE: Injectable anesthetic and euthanasia agents do not need to be listed here.	☐	☐
Schedule 13, Genetically Engineered Laboratory Animals (including establishment of a breeding colony) If using any genetically modified animal including transgenic, knockout, knock-in, knock-down, etc. A Schedule 13 must be submitted for each genetically modified animal model.	☐	☐
Schedule 14, Offsite Housing If the project involves the use of animals on non-university property, excluding SBHRC and CancerCare Manitoba.	☐	☐
Schedule 15, Establishment and Maintenance of Breeding Colonies for Non-Genetically Manipulated Animals To provide details regarding the establishment and maintenance of an in-house breeding colony of laboratory animals which are neither "Livestock' nor genetically engineered (for example: rare species/strains which are not available commercially).	☐	☐
Schedule 15B, Establishment and/or Maintenance of Livestock Breeding Herds or Flocks (dairy, swine, poultry) To provide more detail about herd/flock management personnel, animal information and numbers used and produced	☐	☐

DECLARATION

The signature of the principal investigator below indicates agreement to all terms and conditions applied to the original protocol and this amendment. No other changes can be made to this protocol without further approved amendments or submission and approval of a new protocol to cover them.

[redacted] 7/11/2016

Principal Investigator — Date

Protocol Approved By:

[redacted] Nov 15/16

Chair, Animal Care Committee — Date

[redacted] Nov 8/16

Clinical Veterinarian — Date

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September 2016

CONFIDENTIAL

UNIVERSITY OF MANITOBA

Schedule 1, Personnel

1 Personal Infomation

Complete a separate Schedule 1 for each person working with live animals.

Berethe	Rokiatou	Rokiatou
Surname	First Name	Name Normally Used

Graduate Student [redacted]

Academic Position — E-mail address (Must be a UM email address)

[redacted]

Office phone number — Lab phone number

2 Procedures

Indicate all procedures this person will perform on animals **in this protocol**. Please be specific, e.g. if euthanasia is being performed, indicate method for each species (if more than one species is being utilized in the protocol). (Click +/- to add or delete rows to the table)

Name of Procedure	Competency Level *	If novice, indicate name of expert team member **
Handling	Novice	Khuong Le
Feeding	Novice	Khuong Le
Feces and terminal tissue collection	Novice	Khuong Le

* Competency Level Definitions (note: This should be determined by the principal investigator or the expert team member).
Novice: Not able to perform the procedure competently without help from a proficient team member. Requires the physical presence of an expert team member at all times when the procedure is being conducted. **Competent:** Competent to perform the procedure independently without the need to have an expert team member in attendance. **Expert:** Possesses mastery of procedure. Furthermore, the expert must possess the ability to teach the procedure correctly to others. For all but the most simple procedures, this category requires at least one year experience with it. ** Ensure a Schedule 1 is completed for this individual and that they are listed in Block 3

3 Ethics Training

a. Was ethics training obtained at the U of M — Yes

b. If YES, Veterinary Services will generate a training report and attach it with the protocol.

c. If NO, was ethics training obtained from another Canadian institution

d. If YES to 'c' above, please complete the table below and provide documentation from the applicable institution.
(Click +/- to add or delete rows to the table)

Institution	Year	Month

Note: Ethics training must have been received from a Canadian institution with CCAC GAP status.

4 Wet Lab Training

a. Was wet lab training obtained at the U of M: Yes

b. If YES, Veterinary Services will generate a training report and attach it with the protocol.

c. If NO to 'a' above, list all wet labs pertaining to the species/procedures in this protocol which were obtained at a site other than the U of M and provide documentation. (Click +/- to add or delete rows to the table)

Wet Lab Name	Institution	
Mouse wet lab	University of Manitoba	
Country	Year	Month
Canada	2016	May

Schedule 1 -Training Report

Rokiatou Kone Berethe

CCARM [redacted]

Wet Lab Training Received To Date	Species	Certification Date
IP Injection	Mice	May, 2016
Wet Lab: Introduction	Mice	May, 2016

Ethics Course	Species	Certification Date
AUTC: Biomedical - Acute & Chronic		December, 2015

Appendix 2

How to perform a Mesoscale Multiplex Assay

Kit was purchased from MSD; you can purchase premade antibody (analytes or biomarkers) panel or do your own choosing panel. Kit comes complete with all needed reagent for a complete successful assay:

Components: (provided with the kit)

1. One plate 96 well black plate with barcode and pre-coat with primary antibody linker, stored at 4^0 C.
2. A set of color-coded cap secondary linker related to the biomarker, number and description depend to your choosing. In our case we chose **10 biomarkers (analytes)** for cardiovascular diseases, stored at 4^0 C.
3. A set of small rectangular paper box store Biotinylated antibody (white cap) correspond to the color-coded cap secondary linker and a set of yellow cap detection reagent, stored at 4^0 C.
4. One bottle of stop solution (stored at 4^0 C).
5. Two bottles' diluents 41 & 45 remove from shipping container and stored at -20^0 C.
6. One bottle of Read T buffer, store at room temperature.
7. Three glasses vial of lyophlyzed

Materials needed: (NOT provided with the kit)

1. A 96-well plate shaker capable 300-700 rpm shaking is required for optimal assay performance.
2. Phosphate-buffered saline (PBS) + 0.05% Tween-20 for washing steps
3. Adhesive plate seals.
4. Eppendorf tubes or polypropylene plates (for diluting samples and calibrators).
5. Falcon tubes (15 mL and 50 mL).
6. Single channel pipettors that can accurately dispense 5-1000 µL.
7. Multichannel pipettors that can accurately dispense 25µL and 150 µL.
8. Pipette tips (with filter if possible).
9. Reagent Reservoirs (made of polypropylene or PVC)
10. Distilled or milli-Q water.
11. If available - automated plate washer (optional).

Note: Remove the two bottles of diluents 41 & 45 and put them at 4^0 C a day before the assay to thaw them out. At the day of the assay bring everything out to room temperature including thawing your samples. After your samples thaw, place them on ice.

Next day, Assay set up:

Step 1 Create Individual U-Plex Coupled antibody solution

1. Open the first small rectangular paper box to remove the first Biotinylated antibody (white cap), be careful, rule of thumb does one antibody at a time and from left to right and according to the color-coded secondary antibody linker.
2. Remove 200 µl from this white cap tube and add the correspondent color coded antibody linker, already has premeasured at 300 µl REMENBER LEFT TO RIGHT AND ONE AT ATIME, a total of 500 µl. Mix by vortexing (DO NOT SHAKE) and incubate at room temperature for 30 minutes.
3. Next Add 200 µl of the stop solution to the 500 µl created in the step 2 above and incubate at room temperature for 30 minutes. We now have a total of 700 µl solution.

Step 2 Prepare multiplex coating solution and coat the U-Plex plate

1. Combine 600 µl of each U-Plex coupled antibody, we have 10 antibodies coupled solution for a total of 6000 µl or 6 ml into a 15 ml polypropylene, mix by vortexing. DO NOT USE POLYSTYRENE TUBE AND DO NOT COMBINE ANTIBODY SHARE THE SAME LINKER. IF THE COMBINE VOLUME LESS THAN 6 ML, THEN BRING THE VOLUME UP TO 6 ML WITH THE STOP SOLUTION.
2. From the 6 ml solution in step 1 add 50 µl to each well of the assay plate using plastic media reservoir and multichannel pipettor.
3. Seal the plate with plate sealer and incubate at room on the orbital shaker setting at 500 rpm for 1 hour.
4. Ten minutes before the step 3 incubation end, prepare the calibrator (Standard) as following image.

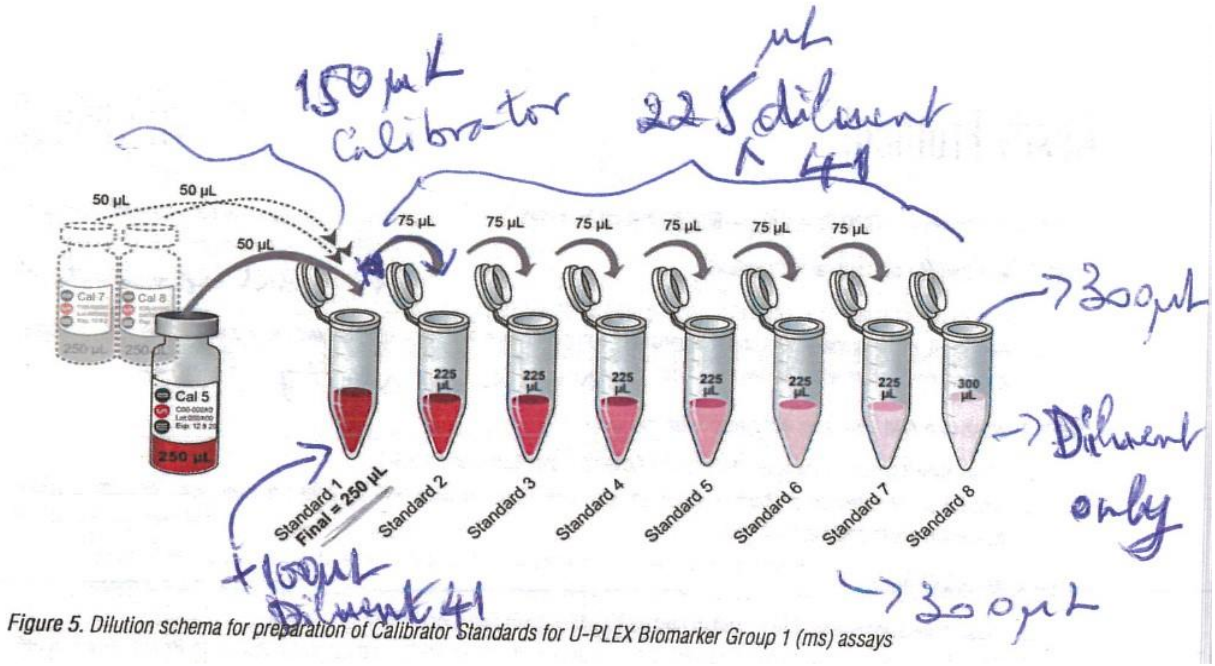

Figure 5. Dilution schema for preparation of Calibrator Standards for U-PLEX Biomarker Group 1 (ms) assays

5. When the step 3 incubation end, invert plate and with one quick straight down motion to empty the entire content of the multiplex coating.

6. Wash plate 3X with 150 µl / well 1X PBS – Tween 20 (0.05%), tap plate dry at the third wash critical for the next step.

Step 3 Assay

1. Add 25 µl of Diluent 41 to each well using multichannel pipette and reagent reservoir.

2. Then add 25 µl calibrator and sample appropriately as per your own Xcel sheet set up template. Each well now should have 50 µl total.

3. Seal the plate with plate sealer and incubate at room on the orbital shaker setting at 500 rpm for 1 hour.

4. Ten minutes before the step 3 end, prepare the Detection Antibody Solution as follow, take 60 µl from each of the yellow cap tube in the small square paper box and pool together in the 15 ml polypropylene tube, in our case 60 µl X 10 analytes = 600 µl + 5.4 ml of diluent 45 to bring the final volume to 6 ml solution.

5. At the end of step 3 incubation, invert plate and with one quick straight down motion to empty the entire content of samples and callibrator.

6. Wash plate 3X with 150 µl / well 1X PBS – Tween 20 (0.05%), tap plate dry at the third wash critical for the next step.

7. Add 50 µl of Detection Antibody Solution prepared in step 4 to each well using multichannel pipette and reagent reservoir.

8. Seal the plate with plate sealer and incubate at room on the orbital shaker setting at 500 rpm for 1 hour.

9. Ten minutes before the step 8 end, prepare the Read Buffer T solution as follow, take 10 ml of Read Buffer T (4X) add 10 ml of de-ionized water to make a 20 ml 2X Read Buffer T.

10. At the end of step 8 incubation, invert plate and with one quick straight down motion to empty the entire content of samples and callibrator.

11. Wash plate 3X with 150 µl / well 1X PBS – Tween 20 (0.05%), tap plate dry at the third wash, critical for the next step and make sure there are absolutely no bubbles. This could cause interfering when reading plate with the **MSD sector Imager.**

12. Add 150 µl of Read Buffer T Solution prepared in step 9 to each well using multichannel pipette and reagent reservoir. Read and analyzed the plate with the software on the MSD instrument.

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