

**ANTI-ATHEROGENIC EFFECTS OF DIETARY SUPPLEMENTATION  
OF KGENGWE (CITRULLUS LANATUS) SEED POWDER IN LOW  
DENSITY LIPOPROTEIN RECEPTOR KNOCK-OUT MICE**

By

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## ABSTRACT

Kgenwge melon (*Citrullus lanatus*) is an indigenous food crop of sub-Saharan Africa and various other tropical countries, commonly utilized as food and/or snack. Current reports evidenced lower mortality rates due to coronary artery diseases in such nations. Only a few studies have demonstrated anti-inflammatory properties of the conventional watermelon consumption through improved lipid profile in humans and animal models. Therefore, the aim of this thesis was to explore the anti-atherogenic potential of Kgenwge seed powder (KSP) and its possible mechanisms of action to exhibit such effects in low-density lipoprotein receptor knockout (LDL-r-KO) mice fed with an atherogenic (0.06% (w/w) cholesterol) chow. Twenty male LDL-r-KO mice were divided into two groups; control (n=10, fed with atherogenic diet) and treated (n=10, fed with an atherogenic diet supplemented with 10% (w/w) KSP) for a period of 20 weeks. During the experimental course, animal body weight gain, food intake, and plasma lipid levels were measured and compared between both the experimental groups. Unlike the previous reports, the present study did not show any significant changes in the plasma lipid levels between the groups. However, KSP treated group showed a significant ( $p < 0.05$ ) decrease of the atherosclerotic lesion size in the aortic roots as compared to that in the controls. Thus, the plasma samples of mice were further assessed to investigate any substantial alterations in the plasma cytokine levels, oxylipin profile, and plasma and fecal metabolites. The anti-atherogenic potential of KSP was attributed to significant ( $p < 0.05$ ) and beneficial variations in the plasma inflammatory markers, oxylipins, plasma and fecal metabolites in KSP treated mice in comparison to controls. These preliminary set of data indicate the cardioprotective properties of this fruit's seeds which are presumed to be mediated through changes in the inflammatory pathways.

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## DEDICATION

*To my loving parents, my elder brother and my younger sister  
for their unconditional love and support.*

## FOREWORD

The Manuscript style has been followed to produce this thesis. Two manuscripts are included in the thesis bookended by Chapter I (*introduction and literature review*) and Chapter IV (*general discussion and conclusion*). The preparation of both the manuscripts are completed using the format of the journals in which the manuscripts are/intended to be published. Manuscript in Chapter I is published in the “Applied Physiology, Nutrition and Metabolism” journal and addressed the anti-atherogenic potential of KSP consumption in LDL-r-KO mice for a period of twenty weeks. Chapter II consists of the second manuscript which deals with the investigation of the possible mechanisms of action of anti-atherogenic effects of KSP supplementation in the experimental mice through the analysis of plasma oxylipins profile and plasma and fecal metabolites. This manuscript is being further developed for peer-reviewed publication. The bridging statements are added before Chapter II and III in order to deliver consistency which rationally link both the chapters together. Lastly, the final Chapter IV includes the overall conclusions, strengths and limitations, future directions, and implications in the field of nutrition.

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## LIST OF ABBREVIATIONS

<b>AHA</b>	American heart association
<b>APO</b>	Apolipoprotein
<b>BCAA</b>	Branched chain amino acid
<b>COX</b>	Cyclooxygenase
<b>CRP</b>	C-reactive protein
<b>CSF</b>	Colony stimulating factor
<b>CVD</b>	Cardiovascular diseases
<b>CYP</b>	Cytochrome P
<b>DC</b>	Dendritic cells
<b>DHA</b>	Docosahexaenoic acid
<b>EpODE</b>	Epoxyoctadecadienoic acid
<b>EpOME</b>	Epoxyoctadecenoic acid
<b>EPO</b>	Erythropoietin
<b>GC</b>	Gas chromatography
<b>GM</b>	Granulocyte-macrophage
<b>GRO</b>	Growth regulated oncogenes
<b>HC</b>	High cholesterol
<b>HDL</b>	High density lipoprotein
<b>HEPE</b>	Hydroxyeicosapentaenoic acid
<b>HETE</b>	Hydroxyeicosatetraenoic acid
<b>HMG-CoA</b>	3-hydroxy-3-methylglutaryl-co-enzyme A

<b>HODE</b>	Hydroxyoctadecadienoic acid
<b>HOME</b>	Hydroxyoctadecenoic acid
<b>HPLC</b>	High performance liquid chromatography
<b>IFN</b>	Interferon
<b>IL</b>	Interleukin
<b>KO</b>	Knock out
<b>KSP</b>	Kgengwe seed powder
<b>LDL</b>	Low density lipoprotein
<b>LDLR</b>	Low density lipoprotein receptor
<b>LOX</b>	Lipoxygenase
<b>MCP</b>	Monocyte chemoattractant protein
<b>MIP</b>	Macrophage inflammatory protein
<b>MS</b>	Mass spectrometry
<b>MUFA</b>	Monounsaturated fatty acid
<b>ND</b>	Not detectable
<b>NO</b>	Nitric oxide
<b>NOS</b>	Nitric oxide synthase
<b>NS</b>	Not significant
<b>PGE</b>	Prostaglandin E
<b>PPAR</b>	Peroxisome proliferator-activated receptors
<b>PUFA</b>	Polyunsaturated fatty acid
<b>SFA</b>	Saturated fatty acid
<b>TC</b>	Total cholesterol

<b>TG</b>	Triglycerides
<b>TNF</b>	Tumor necrosis factor
<b>VEGF</b>	Vascular endothelial growth factor
<b>WHO</b>	World health organization



# **Chapter I**

## **INTRODUCTION AND LITERATURE REVIEW**

### **1.1 Introduction**

Cardiovascular diseases (CVD) are one of the most common non-communicable ailments, accountable for approximately 17.8 billion global deaths in 2017, of which low-income and middle-income countries were responsible for more than three fourth of worldwide mortality (Roth et al., 2015). It has been suggested that the risk of premature mortality due to CVD to be decreased by 82% through lifestyle modifications (Stampfer, Hu, Manson, Rimm, & Willett, 2000). Mere improvements in the dietary patterns reduce the risk of CVD by 60% (Steffen et al., 2003). World Health Organization (WHO) has recorded that the number of premature deaths due to CVD will be raised to approximately 25,000,000 deaths by 2030, with an increase in the total economic cost of about 1 trillion dollars (World Health Organization, 2017). The major etiological factors involved in the pathogenesis of CVD could be diabetes, obesity, dyslipidemia, and hypertension. Diet and lifestyle have always been recognized as a substantial risk factor for cardiac health (Eckel et al., 2014). Regular consumption of foods rich in antioxidants, dietary fiber, omega-3 fatty acids, plant sterols, and other functional ingredients are associated with the reduction of atherosclerosis in both human and animal models (Benjamin et al., 2017).

Previous studies have reported that the consumption of whole grains and fruits are associated inversely with the incidence of CVD (Dahl & Stewart, 2015). Many studies have found that the inflammation is the integral and plausible factor in the progression of atherogenic disease (Benjamin et al., 2017; Hu, 2003). Increases in the consumption of omega-3 fatty acids,

particularly in prevention of CVD, has been suggested in most of the studies, while a particular ratio of omega 6:omega-3 fatty acids has not been well defined (Simopoulos, 2002). Populations consuming plant-based diets particularly rich in fruits and vegetables experience lower incidence of cardiovascular morbidities (Kim et al., 2019). The statistical data with respect to global and regional mortality rates of CVD has reported that sub Saharan African regions showed a decreased burden of ischemic heart diseases and lower death rate in comparison to the developed nations (Roth et al., 2018). While the lower burden of CVD in sub Saharan African countries is low, there is no evidence of any particular food stuff in their diet promoting such effects.

It has been demonstrated that wild watermelon is considered as an edible but underutilized crop in Africa (Biswas, Ghosal, Chattopadhyay, & Datta, 2017). The populations among those regions consume either whole fruit and/or it's seeds as a staple food product to fulfill their nutritional needs (Mujaju, 2011). The leftover plant products from the wild watermelon crops are used as animal feeding (Chivenge, Mabhaudhi, Modi, & Mafongoya, 2015). This wild watermelon is well adapted to the local land conditions which are mostly drought prone regions and thus, this crop is not only a nutritional or dietary support to the indigenous populations but also helps to quench thirst for both humans and animals (Modi & Mabhaudhi, 2013). Apart from south African regions such as western Kalahari and Sahara deserts, this wild watermelon is also cultivated in various tropical regions worldwide including East, West, and middle Asia, Japan, China, and Mediterranean Africa (Cheikhoussef et al., 2017; Komane, Vermaak, Kamatou, Summers, & Viljoen, 2017; Vermaak, Kamatou, Komane-Mofokeng, Viljoen, & Beckett, 2011).

In addition to the established health benefits of plant-based foods and dietary agents (Kim et al., 2019), there may be a number of other foods that their health properties are only

recognised in their regional populations. One of such plant products is seeds from Kgengwe (*Citrullus lanatus*), wild watermelon, which is native to some countries of sub Saharan Africa. Because of same botanical order, both conventional watermelon and Kgengwe belong to the same species (Mujaju, 2011). Only few studies have mentioned the cardioprotective effects of the whole watermelon fruit (Aminu et al., 2018; Hong, Beidler, Hooshmand, Figueroa, & Kern, 2018; Poduri, Rateri, Saha, Saha, & Daugherty, 2013). Few previous reports have recognized the conventional watermelon as a rich source of citrulline and linoleic acid (Hayashi et al., 2005; Rimando & Perkins-Veazie, 2005). Existing findings support the hypothesis that the consumption of watermelon improves plasma lipid profile and arterial pressure including anti-inflammatory and endothelium dependent vaso-relaxation/ aortic vascular remodeling effects of citrulline administration in animal models, and thereby, reduce the risk of cardiovascular morbidities (Figueroa, Sanchez-Gonzalez, Perkins-Veazie, & Arjmandi, 2011; Hong et al., 2018; Poduri et al., 2013). To our best knowledge, only one study has established the anti atherogenic effects of *Citrullus lanatus* (2% extract) in LDL-receptor null mice (Poduri et al., 2013). Other studies have reported the anti-atherogenic and anti-inflammatory effects of *Citrullus lanatus* in various animal models and human subjects (Abd El-Razek & Sadeek, 2011; Aminu et al., 2018; Hong et al., 2018, 2015; Madhavi, 2012). No previous studies have shown the direct cardioprotective effects of wild watermelon seeds in either animals or humans. Current literature lacks scientific reports of health promoting properties of KSP products.

## **1.2 Literature review**

### **1.2.1 Cardiovascular diseases**

CVD are the collection of disorders which involve both blood vessels and heart including coronary artery disease, cerebrovascular disease, peripheral arterial disease, congenital heart disease, and pulmonary embolism and deep vein thrombosis. Acute events such as stroke and heart attack occur due to blood flow blockage in heart and/ or brain, primarily because of the formation of fatty streaks on the inner arterial walls of the blood vessels (World Health Organization, 2017). According to the American Heart Association and the National Institutes of Health- 2019 statistics update, CVD has remained the global health burden and primary root of mortality worldwide (Benjamin et al., 2019). Interactions among genetic predisposition, environmental conditions, and health behaviors act as fuel in cardiovascular events. The chief modifiable risk factors of CVD include high cholesterol, hypertension, sedentary lifestyle, poor dietary habits, obesity, diabetes, smoking, and stress (Buttar, Li, & Ravi, 2005). Data from Global Burden of Disease Study has suggested that modifiable risk factors of CVD could attribute to approximately 90% of stroke risk (Feigin et al., 2016). The American Heart Association's 2020 impact goals revealed that 20% reduction of deaths attributed to stroke and CVD, need an emphasis on the acute CVD events treatment and secondary prevention through improving and/ or controlling the risk factors and health behaviors (Benjamin et al., 2019).

### **1.2.2 Atherosclerosis**

Atherosclerosis is a chronic, progressive, fibroproliferative, immunoinflammatory, multifocal, and smoldering disease of medium to large sized arteries due to accumulation of lipids and fibrous rudiments (Lusis, 2000). By far, atherosclerosis is the foremost underlying

etiological factor of carotid artery disease, peripheral arterial disease, and coronary artery disease. Major life-threatening events such as stroke and acute coronary artery syndrome may happen when thrombosis superimpose a ruptured and eroded atherosclerotic plaque (Naghavi et al., 2003; Spagnoli et al., 2004). Major players in harbouring of advanced atherosclerotic lesions involve intimal smooth muscle cells, leukocytes, and endothelial cells which place brain, myocardium, and other body organs at high risk (Falk, 2006).

### **1.2.3 Cellular components involved in plaque formation**

Endothelial cells, intimal smooth muscle cells, and leukocytes are the major cellular components involved in the formation and development of atherosclerotic plaque (Falk, 2006).

#### **1.2.3.1 Endothelial cells**

An intact but dysfunctional endothelium aggravates the formation of atherosclerotic lesions in lesion prone area (Libby, 2002). Later, de-endothelialized areas cover the advanced lesions where endothelial cells may then disappear with or without attracting platelets to the uncovered subendothelial tissue (Davies, Woolf, Rowles, & Pepper, 1988).

According to the magnitude and concentration, plasma lipoprotein particles exude through the leaky and malfunctioning endothelium into subendothelial area. Potential atherogenic lipoprotein particles retain in the subendothelial space where they get oxidized and turn out to be proinflammatory, cytotoxic, and proatherogenic (Libby, 2002). Though the mechanism of atherogenic conversion of lipoprotein molecules is still unfamiliar but the oxidation of low-density lipoproteins (LDL) could be mediated by 15-lipoxygenase, nitric oxide synthase (NOS), and/ or myeloperoxidase (Glass & Witztum, 2001).

### **1.2.3.2 Smooth muscle cells**

Only few endothelial cells, T-cells, and macrophages get involved in the development of fatty streak which is characterized by the formation of early and asymptomatic foam lesions (Falk, 2006). In atherosclerotic disease progression, the immunomodulatory response is mostly driven by the fibroproliferative response of smooth muscles cells. Over the years, if atherosclerotic provocations persist then it turns out into ischemia where blood flow is reduced, and lumen is lost (Kragel, Reddy, Wittes, & Roberts, 1989). Though the smooth muscle cells are an important connective tissue producing units in both normal and diseased intima conditions (Schwartz, Virmani, & Rosenfeld, 2000). These cells function in repairing and protection of intima, but the dysfunctional cells are mostly unfavorable because of plaque rupture. The rupture sites lack smooth muscle cells is still unknown, but the apoptotic cell death could play an essential role in rupturing the plaque (Geng & Libby, 2002; Kolodgie et al., 2003).

### **1.2.3.3 Leukocytes**

In atherogenesis, focal recruitment of the monocytes is one of the earliest cellular responses, with lesser extent to T-cells (Hansson, 2005; Libby, 2002). These cellular responses seem to be accountable for atherosclerotic disease progression. Plasma cells and B lymphocytes are rarely involved in the intimal plaque but may abundantly seen in the adventitia close to the advanced intimal injury (Houtkamp, De Boer, Van Der Loos, Van Der Wal, & Becker, 2001). During acute ischemic events, mast cells may be involved occurring both in intima and adventitia (Kaartinen et al., 1998) whereas neutrophils are recruited mostly in thrombosed coronary plaques as result of plaque rupture (Naruko et al., 2002). Blood borne cells do not only rely on the endothelial adhesion for disease progression, but trans-endothelial migration is

equally vital for attacking the lesion. For this, chemotactic cytokines, also known as chemokines, are important to present. Experimental studies have suggested that monocyte chemoattractant protein-1 (MCP-1) and oxidized LDL are essential atherogenic chemoattractant (Libby, 2002). Both monocytes and T-cells (but not B-lymphocytes and neutrophils) are recruited by MCP-1. Macrophages, smooth muscle cells, and endothelial cells exaggerate the over expression of MCP-1 in atherosclerosis. Cytokine such as interleukin-8 may promote monocyte trafficking (Glass & Witztum, 2001).

Monocytes further get differentiated into macrophages and centralize the atherosclerotic lipoproteins within intima with the help of scavenger receptors in experimental atherosclerosis. The hallmark of both early and late stages of atherosclerotic lesions is indicated with the occurrence of abundant amounts of cholesteryl esters in the lipid-loaded macrophages (Glass & Witztum, 2001). With unceasing supply of atherogenic lipoproteins, the macrophages engulf until they perish whereas scavenger receptors are not down regulated because of cellular cholesterol buildup. Apoptosis or/ and necrosis contribute to the death of macrophages and lead to the formation of destabilized and soft lipid-rich core in the plaque. Under certain conditions (high HDL and low LDL), macrophages may contract while releasing cellular cholesterol with the help of membrane transporters to extracellular HDL, mostly during the initial step of reverse cholesterol transport system (Glass & Witztum, 2001; Lewis & Rader, 2005; Nissen et al., 2003).

Therefore, immune activation is a continuous process in atherosclerotic lesion progression (Hansson, 2005). Albeit the immunomodulation favors the development of this disease, but involvement of lymphocytes is not always needed in the atherosclerosis. A plenty of candidate particles in the lesion including modified LDL, microbial agents, heat shock proteins etc. are responsible for immune activation. Of these, the most widespread data suggest the role of

oxidized LDL recognized by T-cells in atherosclerotic plaque formation (Hansson, 2005; Nilsson, Hansson, & Shah, 2005).

#### **1.2.4 Inflammation and cytokines**

The functioning of immune system plays a vital role in the activation of an acute inflammatory response during there times of acute injury and infection including CVD (Pearson et al., 2003). This acute inflammatory response further attracts immune mediators and leukocytes to either the injury site or infectious region (Deng, Lyon, Bergin, Caligiuri, & Hsueh, 2016). Furthermore, during the state of physical inactivity, obesity, stress, and poor dietary habits, there is a promotion of inflammatory stimulus and these factors exaggerate to make it more chronic in nature (Iantorno et al., 2014). Many chronic diseases are associated with the increased risk of inflammation including CVD, diabetes, and cancer. The quality of diet has been recognized as one of the powerful environmental influencers to inhibit the state of chronic systemic inflammation (Ahluwalia, Andreeva, Kesse-Guyot, & Hercberg, 2013). The consumption of fruits, vegetables, whole grains, spices, herbs, and lean meat proteins including fish is linked with lower burden of systemic inflammatory state. On the other hand, Western diets loaded with high amount of saturated fats, simple carbohydrates, processed and fried red meats stimulate a pro-inflammatory status inside the body (Ahluwalia et al., 2013).

Inflammation is rooted with atherosclerosis during the early observation and promotion of CVD (Ross, 1999). The formation of atherosclerotic plaque is characterized by a unique microenvironment and is promoted by repeated inflammatory and reparative actions, which is amplified and quickly initiated by various inflammatory mediators (Libby, 2002). Ross and colleagues pioneered in describing the principle mechanisms in the development of



atherosclerotic plaque, including the recruitment of smooth muscle cell proliferation, transendothelial macrophages, and their migration towards the inner vascular wall, and participation of lymphocytes (Ross, 1999).

Cytokines are a group of a low molecular weight proteins with over 100 recognized till present. Cytokines are differentiated into several subgroups including the interleukins, chemokines, colony stimulating factors, tumour necrosis factors, growth factors, and interferons. During the formation of atherosclerotic plaques, various cytokines are expressed and involved in the progression of CVD and further capable of producing more cytokines in response. These cytokines can be principally classified as pro- or anti- atherogenic through their beneficial roles, some of which are not as clear or established previously.

Various key cytokines in the different stages of the progression of atherosclerotic disease play a crucial role in either promotion or inhibition of inflammation (Iantorno et al., 2014). Many previous studies used the animal model systems such as apolipoprotein E deficient (apo E-KO) mice and the LDL receptor null (LDL-r-KO) mice in order to understand the advancement of the molecular stages of atherosclerosis and the role of different cytokines in disease progression (Getz & Reardon, 2012; Zadelaar et al., 2007). These mouse models act as an excellent experimental animals because of the spontaneous formation of atherosclerotic lesions when fed on high fat, Western- type diet or high cholesterol chow (Getz & Reardon, 2012). It should always be considered that the extrapolation of outcomes from such animal models can be differently translated to humans because of the differences between human and animal species, including various inflammatory responses and lipoprotein metabolism (Libby, Lichtman, & Hansson, 2013; Libby, Ridker, & Hansson, 2011). Role of various cytokines in atherosclerosis using animal models are summarized in **Table 1.1**.

**Table 1.1 Key role of different cytokines in atherosclerosis**

<b>Cytokine</b>	<b>Mouse model</b>	<b>Effect on Atherosclerosis</b>	<b>Outcome</b>	<b>Reference</b>
IL-1 $\alpha$	LDL-r-KO and ApoE-/-	Macrophage derived IL-1 $\alpha$ enhances atherosclerosis in LDL-r-KO mice and targeted IL-1 $\alpha$ immunization decreases plaque formation in ApoE-/- mice.	Harmful	(Kamari et al., 2011; Tissot et al., 2013)
IL-1 $\beta$	ApoE-/-	Deficiency of IL-1 $\beta$ inversely associated with atherosclerosis but unstable plaque development.	Controversial	(Chamberlain et al., 2009)
IL-2	ApoE-/-	Enhanced atherosclerosis through IL-2 injection therapy.	Harmful	(Upadhyay, Mooteri, Peckham, & Pai, 2004)
IL-4	ApoE-/- and LDL-r-KO	No involvement in the atherosclerosis irrespective of ailment induction.	Neutral	(King, Szilvassy, & Daugherty, 2002)
IL-6	ApoE-/- and LDL-r-KO	Injection of IL-6 in ApoE-/- mice enhanced the lesion development and pro inflammatory cytokines. However, deficiency of IL-6 enhanced atherosclerotic plaques.	Controversial	(Schieffer et al., 2004; Zhang et al., 2012)

IL-10	LDL-r-KO and ApoE <sup>-/-</sup>	Overexpression of IL-10 attenuated advanced lesions, inflammation, and oxidative stress in atherosclerosis.	Beneficial	(Pinderski et al., 2002; Pinderski Oslund et al., 1999)
IL-12	LDL-r-KO and ApoE <sup>-/-</sup>	IL-12 functioning blockage through vaccination showed anti-atherogenic properties through increased collagen content and smooth muscle cells. IL-12 deficiency in ApoE <sup>-/-</sup> decreased lesion size.	Harmful	(Davenport & Tipping, 2003; Hauer et al., 2005)
IL-13	LDL-r-KO	Deficiency of IL-13 promotes atherosclerosis and lesion collagen content.	Beneficial	(Cardilo-Reis et al., 2012)
IL-15	LDL-r-KO and C57BL/6	Aggravates lesion development in LDL-r-KO mice and thickens intima followed by carotid artery injury in C57BL/6 mice.	Harmful	(Cercek et al., 2006; van Es et al., 2011)
IL-17	LDL-r-KO and ApoE <sup>-/-</sup>	Acts as pro-atherogenic through recruitment and feedback mechanism of aortic myeloid cell in LDL-r-KO mice while its deficiency in ApoE <sup>-/-</sup> model has neutral effect on atherosclerosis	Controversial	(Butcher, Gjurich, Phillips, & Galkina, 2012; Tang, 2019)

burden but promotes systemic inflammation.

IL-19	LDL-r-KO	Cytokine administration enhanced Th2 polarization and decreased gene expression of pro-inflammatory markers.	Beneficial	(Ellison et al., 2013)
IL-27	LDL-r-KO	Deficiency of cytokine or its receptors attenuates activation of macrophages, modified LDL uptake, and pro-inflammatory cytokine recruitment.	Harmful	(Hirase et al., 2013; Koltsova et al., 2012)
IL-33	ApoE-/-	IL-33 administration reduced IFN- $\gamma$ concentrations and foam cell formation in atherosclerosis.	Beneficial	(Miller et al., 2008)
GM-CSF	LDL-r-KO and ApoE-/-	GM-CSF deficiency promoted advances plaque development through apoptosis susceptibility of macrophages in LDL-r-KO mice. Another study reported GM-CSF administration in ApoE-/- model promotes atherogenesis.	Controversial	(Ditiatkovski, Toh, & Bobik, 2006; Liu et al., 2006)
IFN- $\gamma$	LDL-r-KO and ApoE-/-	Cytokine deficiency attenuates the lesion development in both	Harmful	(Niwa et al., 2004; Whitman,

		ApoE <sup>-/-</sup> and LDL-r-KO mice through increased collagen concentrations and reduced lipid accumulation.		Ravisankar, & Daugherty, 2002)
TNF- $\alpha$	LDL-r-KO and ApoE <sup>-/-</sup>	Cytokine deficiency decreased expression of several pro-inflammatory marker levels in ApoE <sup>-/-</sup> mice. Similarly, cytokine deficient bone marrow transplantation reduced atherosclerosis in LDL-r-KO model.	Harmful	(Br��n��n et al., 2004; Xanthoulea et al., 2008)

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IL= interleukin; GM-CSF= granulocyte-macrophage colony stimulating factor; IFN- $\gamma$ =

interferon gamma; TNF- $\alpha$ = tumor necrosis factor-alpha; LDL-r-KO= low density lipoprotein receptor knock out; ApoE<sup>-/-</sup>= apolipoprotein E knock out

### 1.2.5 Role of polyunsaturated fatty acid metabolites in CVD

Apart from other risk markers of CVD, the metabolites of polyunsaturated fatty acids (PUFA) are considered as early diagnostic tools in the therapeutic treatment of the inflammatory diseases such as atherosclerosis (Pearson et al., 2003). Oxylipins are known as the oxygenated products of PUFA that can regulate the vascular response in inflammation and coronary heart diseases (Melissa Gabbs, Leng, Devassy, Monirujjaman, & Aukema, 2015; Nishimaki & Seki, 1999; Norris & Dennis, 2014). The biological functions of PUFA metabolites are linked to a number of inflammatory diseases and are still being elucidated (Tourdot, Ahmed, & Holinstat, 2014). Interconnected and overlapping role of oxylipins in the pathological states such as

hyperlipidemia, thrombosis, diabetes, and hypertension have been linked in the several studies showing the various signaling pathways of oxylipins (Gleim, Stitham, Tang, Martin, & Hwa, 2012).

Oxylipins are short lived but potent products therefore they are regulated closely and exert their effects in paracrine and autocrine system (Tourdot et al., 2014). Free PUFA in various tissues are either mono or deoxygenated through the action of cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome (CYP) P-450 enzymes (Massey & Nicolaou, 2013). These enzymes further distinguish the oxylipins into distinct classes depending upon the intakes of dietary n-3 and n-6 PUFA (Massey & Nicolaou, 2013). COX enzyme isoforms convert arachidonic acid into prostaglandins and thromboxanes. Similarly, these enzymes can form hydroxy metabolites such as 11-HETE from arachidonic acid and 9-HODE from linoleic acid (Thuresson, Lakkides, & Smith, 2000). On the other hand, LOX can help in the production of leukotrienes, lipoxins, protectins, and eoxins. Furthermore, LOX enzymes also catalyze arachidonic acid to form HETEs (Askari et al., 2014).

Linoleic acid metabolism through COX and LOX enzymes includes the formation of the epoxy products, EpOMEs, which on hydration produce DiHOMEs (Askari et al., 2014). The hydroxy metabolites of linoleic acid are HODEs which are produced with the action of LOX (Tourdot et al., 2014). All these metabolites together take part in the different pathways suggesting the multifaced roles of oxylipins in CVD (Luria et al., 2007). The supplementation of omega-3 and omega-6 PUFA exhibit a variety of health roles against the regulation of inflammatory mediators including eicosanoids, cytokines, and reactive oxygen species (Calder, 2004; Sacks & Campos, 2006). The metabolites of linoleic acid such as 13-HODE not only

involved in the relaxation of smooth muscle cells but has also demonstrated proinflammatory activities in several experimental animal models (Hattori et al., 2008; Obinata & Izumi, 2009).

Additionally, prostaglandins are a distinct group belonging to the family of prostanoids through arachidonic acid metabolism. Most of these prostaglandins such as PGE2 show proinflammatory effects and found to regulate the expression of inflammatory markers such as IL-10 (Sha, Brüne, & Weigert, 2012; Tam, 2013). Similarly, the attenuation of inflammation has been seen with PGD2 in the experimental models of colitis (Tam, 2013). COX plays a major role in the signaling of prostaglandins and inversely related to the progression of inflammatory and CVD events which has been suggested in high blood pressure human subjects (Caligiuri et al., 2017, 2016). On the other hand, thromboxanes associated with aggregation and vessel constriction in the endothelial walls (Cheng et al., 2002). A positive association have been reported in thromboxanes and high blood pressure and multiple cardiovascular events in other studies (Caligiuri et al., 2017, 2016). Therefore, the regulation of both proinflammatory and inflammatory mechanisms act as a key role of various oxylipins and are necessary for the attenuation of cardiovascular events.

## **1.2.6 Kgengwe melon (*Citrullus lanatus*)**

### **1.2.6.1 Botanical characteristics**

Kgengwe fruit (*Citrullus lanatus* or *C. lanatus*), belongs to Cucurbitaceae family, a source of various micronutrients, proteins, fats, and carbohydrates which are packed in its pulp, skin, and seeds. The scientific name *Citrullus lanatus* has been derived from both Greek and Latin words. The part *Citrullus* came from Greek word means fruit and *lanatus*, is a Latin word, refers to being wooly or small hairs on the stem and pulp of the plant (Paris, 2015). According to

Jeffrey (1978), Kgengwe (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) is an annual herb with long stems of up to 10m lying or creeping on the ground, with curly tendrils. Leaves are 5-20 by 3-19 cm, and hairy, usually deeply palmate with 3-5 lobes, on 2-19 cm long petioles (Mujaju, 2011). The fruits of the wild Kalahari melon are small and round, whereas the cultivated forms are large oblong fruits. They vary in colour from pale yellow or light green (wild form) to dark green (cultivars), and with or without stripes; the pulp varies from yellow or green (wild forms) to dark red (cultivars). The flesh of the fruit is pale greenish yellow to reddish pink in modern cultivars and numerous seeds are embedded in the fruit pulp, they are usually brown to black (Mcgregor, 2012; Mujaju, 2011). Basically, it is a vine like flowering plant which is like watermelon (*Citrullus vulgaris*) but the species is a representative of *Citrullus ecirrhosus*. Therefore, it is a tendril-less South African endemic species, commonly known as citron or Tsamma melon (Erhirhie & Ekene, 2013).

**Botanical Description** (Erhirhie & Ekene, 2013)

Kingdom: Plantae

Phylum: Embryophyta

Class: Dicotyledoneae

Order: Cucurbitales

Family: Cucurbitaceae

Genus: *Citrullus*

Species: *C. lanatus*



#### **1.2.6.2 Origin and food value of wild watermelon**

In early history, indigenous crops such as grains, fruits, and vegetables played an important role in the South African countries as a source of food security (Modi, 2003). Apart from their early past, indigenous crops are still a significant part of the rural farming system to fulfil the nutritional needs (Mabaya, Jackson, Ruethling, Carter, & Castle, 2014). Indigenous watermelon, also known as Tsamma melon or Kgengwe, is a unique plant of the Kalahari desert and other water arid regions of Africa due to its drought tolerant capacity (Modi & Mabhaudhi, 2013). This plant has been an important source of both food and water to the local people and livestock because of its low production cost and easy growing capacity (Mabhaudhi, Chimonyo, & Modi, 2017). The literature evidence the origin of wild watermelon in Kalahari desert, Sahara desert, and South Africa where the diversity of this melon species grow (Çürük, Sermenli, Mavi, & Evrendilek, 2004; Mujaju, 2011). This wild watermelon species (Kgengwe) is also largely distributed in the tropical and sub-tropical countries including the warm temperate zones around the world (Mabaya et al., 2014). Wild watermelon is considered as an edible species where the whole fruit including its leaves, flesh, rind, and seeds are cooked and consumed by the indigenous populations (Perkins-Veazie, Davis, & Collins, 2012). This wild *Citrullus lanatus* is featured as a traditional crop in the conventional farming and production system of Africa.

Previous literature has demonstrated the early use of this wild fruit seeds in the diet of Eastern Cape culture where the whole fruits and its seed are mixed with maize meal or rice to cook into a traditional porridge dish (Bhat, Rubuluza, & Jäger, 2002). Apart from its use a food source, wild *Citrullus lanatus* is also intercropped with the other crops such as maize to control weeds (Çürük et al., 2004) or grow in combination with the Sorghum crop fields in drought prone regions (Modi & Mabhaudhi, 2013). Kgengwe fruit is used in the preservation for making

jams and jellies due to its high pectin concentration, and exclusively used in processing and pickling (Laghetti & Hammer, 2007).

Not only the whole fruit, but the seeds of this watermelon are also of economic importance. Wild *Citrullus lanatus* seeds are a rich source of both fats and proteins; and are widely eaten as raw or roasted snacks, used in cooking as an oil seed or added to the other foodstuff/dishes (Alka, Anamika, & Ranu, 2018). The oil meal cake of these seeds after oil extraction are used as a feed for domestic animals. Apart from cooking, *Citrullus lanatus* seeds have pharmaceutical uses as well in the cosmetic industry (Vermaak et al., 2011). Being a reliable form of food and water for indigenous populations, wild *Citrullus lanatus* also has approximately more than one year of shelf life. That is why, wild watermelon is a versatile fruit because of its harsh climate tolerant capacity and its multi-facet properties for both humans and livestock; it can play a significant role in the global food security with a potential of commercialization (Zulu & Modi, 2010). Thus, wild watermelon may prove to be a crucial alternative food crop in the high temperature zones in the context of global climatic disruptions as well.

### **1.2.7 Conventional watermelon, L-citrulline, and nitric oxide**

Watermelon is known to be high in free water and a rich source of L-citrulline which consequently raise the plasma L-citrulline and L-arginine concentrations (Flam, Eichler, & Solomonson, 2007). L-citrulline is also a precursor of endogenous L-arginine which gets quickly available to produce NO (Collins et al., 2007; Mandel, Levy, Izkovitch, & Korman, 2005). The production of NO through the extracellular L-arginine availability acts as a rate limiting factor (Flam et al., 2007). The essential role of citrulline-NO cycle in the L-arginine and NO production

is comparable to the L-arginine oral supplementation (Flam et al., 2007). Additionally, the production of NO through the oral supplementation of L-citrulline and L-arginine occur via endogenous NOS (Figuroa, Wong, Hooshmand, & Sanchez-Gonzalez, 2013). A previous study has compared L arginine availability during the supplementation of watermelon juice in Zucker diabetic fatty rats and found the increased serum levels of L-arginine, NO, nitrite and nitrate, and other oxidation products in the experimental animals (Wu et al., 2007).

Wu et al. concluded that the production of NO through L- arginine synthesis exhibited through L-citrulline present in the watermelon juice (Wu et al., 2007). Another study testified that six week watermelon supplementation in the middle aged pre-hypertensive subjects may have beneficial effects on their pulse pressure, decreased blood pressure, and reduced risk of atherosclerosis (Figuroa et al., 2013). Only a few studies have reported the anti-atherogenic activities and anti-inflammatory properties of *Citrullus lanatus* in mouse models (Poduri et al., 2013). Poduri et al. demonstrated the anti-atherogenic properties of conventional watermelon extract consumption in LDL-r-KO mice due to the presence of citrulline (Poduri et al. 2013).

The previous study stated a significant decrease in the plasma lipid profile in citrulline treated mice (Poduri et al. 2013). Vaso-relaxation and aortic vascular remodeling effects of citrulline administration have been observed in other animal models (Hayashi et al., 2005; Iantorno et al., 2014). *Citrullus lanatus* fed mice also shown reduced concentrations of plasma TC and LDL concentrations with improved plasma inflammatory markers (Hong et al., 2015). Improved endothelial stiffness and arterial functioning through the significant increase in NO levels have been evinced in both animal studies and human subjects consuming *Citrullus lanatus* (Altaş, Kizil, Kizil, Ketani, & Haris, 2011; Figuroa et al., 2011).

### **1.2.8 Bioactive properties of Citrullus lanatus seeds**

Various *in vivo* and *in vitro* studies on Citrullus lanatus seed oil demonstrated anti-inflammatory action in rats and human red blood cell membranes. The significant decrease in the inflammation was seen after three hours in rat paw edema models when fed with Citrullus lanatus seed oil at various concentrations ranging from 100-500 µg/ml and the potential of Citrullus lanatus oil was comparable to standard Diclofenac (10 mg/kg) (Madhavi, 2012). Additionally, researcher have also shown the hepatoprotective activities of Citrullus lanatus seed oil in rats (125 mg and 250 mg oil) for 10 days and found significant reductions in the serum hepatic enzymes responsible for liver damage (Madhavi, Vakati, & Rahman, 2012).

Another study has suggested the anti-ulcer activity of watermelon seeds in albino Wistar rats where the methanolic seed extract showed a reduction in dose dependent manner with a maximum potential at 800mg/kg (Bhardwaj, Kumar, Dabasa, & Alam, 2012). Apart from anti-inflammatory activities, the ethanolic, hexane, and chloroformic extracts of Citrullus lanatus seeds also presented antimicrobial activities against bacteria such as Escherichia coli, Staphylococcus aureus, Bacillus subtilis, and Pseudomonas aureginosa (Hassan, Sirat, Yagi, Koko, & Abdelwahab, 2011). Similarly, another study demonstrated that out of acetic, methanolic, and chloroformic extract of Citrullus lanatus seeds, the maximum antioxidant potential exhibited in methanolic extract of watermelon seeds (Singh Gill, Sood, Muthuraman, Bali, & Dev Sharma, 2011).

### **1.2.9 Nutritional composition of Citrullus lanatus**

Watermelon is produced in the diverse forms and can be grown in the wild conditions. Its cultivation history is long back over 4000 years ago in Africa and brought by Spanish people to

different parts of America (Erhirhie & Ekene, 2013). Watermelon can be used for various dishes, salads, snack, and dessert in different countries. Watermelon juice drinks are common in tropical countries containing low calories watermelon food (Alka et al., 2018). The brown flat seeds of *Citrullus lanatus* exhibit a distinct nutty taste upon roasting and has better nutritional profile than the flesh and rind of watermelon (Biswas et al., 2017). The seeds of watermelon place a major importance in the oil and pharmaceutical industries. Being a rich source of protein and fat, watermelon seeds are used in the enhancement of various nutrition products (Moaddabdoost Baboli & Safe Kordi, 2010). *Citrullus lanatus* contains about 92% of water and 6% of simple sugars by weight. Like many other fruits belonging to Cucurbitaceae family, it is a very good source of vitamins and minerals (Alka et al., 2018). Watermelon fruit with red or pink flesh contains a good amount of lycopene which is a known natural antioxidant (Mandel et al., 2005). The nutritional composition of dried watermelon seed without its outer shell per 100 g and edible mature fruit of watermelon per 100 g are shown in **Table 1.2** and **Table 1.3** respectively.

**Table 1.2 Nutritional composition of dried watermelon seeds\***

<b>Component</b>	<b>Amount per 100 g</b>
Water	5.1 g
Energy	557 kcal
Protein	28.3 g
Fat	47.4 g
Carbohydrate	15.3 g
Calcium	54 mg
Phosphorus	755 mg
Iron	7.3 mg
Thiamin	0.19 mg
Riboflavin	0.15 mg
Niacin	3.55 mg
Folate	58 µg

\*Adapted from Erhirhie and Ekene, 2013

**Table 1.3 Nutritional composition of edible watermelon fruit\***

<b>Component</b>	<b>Amount per 100 g</b>
Water	91.5 g
Energy	32 kcal
Protein	0.6 g
Fat	0.4 g
Carbohydrate	7.2 g
Dietary fibre	0.4 g
Calcium	8 mg
Phosphorus	9 mg
Iron	0.17 mg
Thiamin	0.08 mg
Riboflavin	0.02 mg
Niacin	0.2 mg
Folate	2 µg
Ascorbic acid	9.6 mg

\*Adapted from Erhirhie and Ekene, 2013

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## STUDY RATIONALE

Inclusion of fruits and vegetables in the daily diet is believed to reduce the risk of CVD. The cardioprotective effects of several dietary antioxidants, fibre, and n-3 PUFA have also been elucidated. There are hundreds of such indigenous fruits whose functional properties are unknown or very less recognised among only regional communities. Kgengwe is such kind of fruit and is typically a wild watermelon, also known as Tsamma or citron melon, cultivated mostly in the desert areas of sub-Saharan Africa and is mostly consumed as a part of staple diet. Indigenous people in Africa consume this fruit as whole and/ or its seeds where traditionally, seeds are roasted and cooked as porridges (Mabaya, Jackson, Ruethling, Carter, & Castle, 2014; Vermaak et al., 2011). Current literature showed the lower prevalence of cardiovascular morbidities in sub-Saharan African countries where the farming and utilization of Kgengwe fruit in their traditional food system is common as compared to that in the high income developed countries (Roth et al., 2015). Few animal and human studies have demonstrated the consumption of cultivated watermelon and reduced inflammation (Poduri et al., 2013). To the best of our knowledge, very little is known about the anti-atherogenic properties of this wild fruit in relation to CVD in such regions. Only few studies have established the citrulline as the functional ingredient of *Citrullus lanatus* to exhibit lipid lowering properties in animals and humans (Altaş, Kizil, Kizil, Ketani, & Haris, 2011; Figueroa et al., 2011). However, the particular mechanisms of action to display anti-atherogenic effects due to the consumption of wild watermelon are still unknown and not fully understood. Therefore, this study was proposed to generate evidence of the anti-atherogenic properties of Kgengwe (*Citrullus lanatus*) seeds and to deduce the mechanisms of action of such effects in the experimental mouse models prone to atherosclerosis and dyslipidemia.



## **STUDY HYPOTHESIS AND OBJECTIVES**

### **Hypothesis**

Consumption of the experimental diets supplemented with 10%(w/w) of KSP will show anti-atherogenic potential in LDL-r-KO mice.

### **Objectives**

The purpose of this study was to examine the cardioprotective effects of sub-Saharan African wild melon, Kgengwe, seeds powder in LDL-r-KO mice. The specific objectives are as follows:

1. To investigate the anti-atherogenic properties of KSP in LDL-r-KO mice.
2. To identify potential mechanisms of action of KSP in experimental animals.
3. To assess the effects of KSP consumption on plasma and fecal metabolites of experimental mice.

## **BRIDGING STATEMENT TO CHAPTER II**

The primary objective of this thesis was to investigate the potential of consuming Kgengwe (*Citrullus lanatus*) seed supplemented atherogenic diets on CVD risk markers such as plasma total cholesterol (TC) and triglyceride (TG) levels and plasma cytokines concentrations in LDL-r-KO mice. To understand this, the following chapter includes a journal article containing an elaborated animal study protocol, methodology, and primary findings. Results of the histological and morphometrical examination of the aortic roots to measure the atherosclerotic lesion size development in KSP treated mice and controls are presented. Nevertheless, these preliminary results suspect KSP as a potential cardioprotective food to lower the risk of CVD through a significant decrease in the atherosclerotic lesion size.

## Chapter II

### **ANTI-ATHEROGENIC PROPERTIES OF KGENGWE (*CITRULLUS LANATUS*) SEED POWDER IN LOW DENSITY LIPOPROTEIN RECEPTOR KNOCKOUT MICE ARE MEDIATED THROUGH BENEFICIAL ALTERATIONS IN INFLAMMATORY PATHWAYS**

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## 2.1 Abstract

Kgengwe fruits are commonly consumed in Sub-Saharan countries. Recent reports indicated low coronary artery disease rates in those regions. To investigate anti atherogenic properties and potential mechanisms of action of Kgengwe seed powder (KSP), male low-density lipoprotein receptor knockout (LDL-r-KO) mice were fed with an atherogenic diet supplemented with (treated, n=10) or without (controls, n=10) 10% (w/w) KSP for 20 weeks. Proximate analysis revealed that KSP contained 38% fiber and 15% lipids. KSP supplementation was not associated with significant changes in body weight gain rate, food intake, and plasma lipid levels. However, the average atherosclerotic lesion size in the aortic roots in the KSP-treated group was 58% smaller than that in the control group (0.26 vs 0.11 mm<sup>2</sup>, p<0.05). This strong anti-atherogenic effect was associated with significant increases in the average plasma levels of certain cytokines such as IL-10 (6 vs 13, pg/mL, p<0.05), GM-CSF (0.1 vs 0.2, pg/mL, p<0.05), and EPO (7 vs 16, pg/mL, p<0.05) along with reductions in the average levels of plasma MCP-1 (19 vs 14, pg/mL, p<0.05) and MIP-2 (28 vs 13, pg/m, p<0.05). Except for relatively high levels of saturated fatty acids, KSP possesses balanced nutrient compositions with strong anti-atherogenic properties which may be mediated through alterations in inflammatory pathways. Additional studies warrant confirmation and mechanism(s) of action of such effects.

### **Novelty bullets**

1. Kgengwe seeds prevent atherogenesis in LDL-r-KO mice
2. Kgengwe seeds increase circulating levels of IL-10 and EPO
3. No reduction in plasma TC levels

**Keywords:** *Citrullus lanatus; LDL-r-KO-mice; Atherosclerosis; Inflammation; Lipids; Cytokines*

## 2.2 Introduction

One of the leading causes of morbidity and mortality in both developed and developing countries is CVD. World Health Organization (WHO) has estimated that the number of CVD mortality will be raised to approximately 25 million deaths by 2030, with a total economic cost of about \$1.2 trillion (Chong, Macdonald, & Lovegrove, 2010; World Health Organization, 2017). The major reasons for the positive trends in increasing prevalence of CVD could be obesity, diabetes, dyslipidemia, and hypertension (Benjamin et al., 2017). Diet and lifestyle have been recognized as a substantial and controllable risk factor for CVD (Hong, Beidler, Hooshmand, Figueroa, & Kern, 2018). Regular and adequate consumption of foods rich in dietary fiber, antioxidants, n-3 fatty acids, plant sterols, and other functional ingredients is associated with reduced CVD risk factors (Dupasquier et al., 2007; Varshney & Budoff, 2016). Inflammation has been considered as an integral and plausible factor in atherogenesis (Iantorno et al. 2014). Among many dietary agents, dietary n-6 and n-3 fatty acids contribute to the production of a number of metabolites with known inflammatory or anti-inflammatory properties (Simopoulos 2002). Overall, n-3 fatty acids can result in the production of anti-inflammatory biomarkers, while n-6 fatty acid products have pro-inflammatory activities. Therefore, it is suggested that a high ratio of dietary n-6:n-3 fatty acid may contribute to an inflammatory state, with an adverse effect on organ function (Hamazaki and Okuyama 2003). This is because these fatty acids compete for enzymes COX and LOX (Simopoulos, 2002).

In addition to established health benefits of certain foods and dietary agents, there may be hundreds of other foods that their health benefits are only known to regional populations. One of such plant products is seeds from Kgengwe fruits cultivated in the hot climatic parts of East, West, and Middle Asia, Mediterranean Africa, Japan, and China (Cheikhoussef et al., 2017;

Komane, Vermaak, Kamatou, Summers, & Viljoen, 2017; Vermaak, Kamatou, Komane-Mofokeng, Viljoen, & Beckett, 2011). Lower incidence of CVD has been reported in populations consuming plant-based diets rich in fruits and vegetables (Kim et al., 2019). Global and regional mortality from CVD suggested that sub-Saharan African countries experienced lesser burden of coronary heart diseases and lower mortality as compared to high income developed countries (Roth et al., 2015). Wild watermelon (*Citrullus lanatus*) has been considered as an edible but underutilized and neglected crop species in Africa. In sub-Saharan African regions, the whole fruits and seeds are commonly consumed as a staple food and/or snack (Mabaya, Jackson, Ruethling, Carter, & Castle, 2014; Vermaak et al., 2011). Other parts of the plant are used for farm animal feeding. Unlike other staple foods such as wheat, rice, and maize, this wild melon species is well adapted to local land conditions especially water scarcity (Chivenge, Mabhaudhi, Modi, & Mafongoya, 2015).

There is a long history of wild watermelon cultivation in Africa as staple food (rind, seed, and flesh) (Chivenge et al., 2015); the seeds of this wild fruit are rich in proteins (Moaddabdoost Baboli & Safe Kordi, 2010) and lipids (Achu, Fokou, Tchiégang, Fotso, & Tchouanguep, 2005). The flesh of Kgengwe melon is a rich source of water for humans and livestock in Kalahari desert. Previous literature has demonstrated the anti-ulcerative activity of methanolic extract of *Citrullus lanatus* seeds in different ulcer models of rats (Bhardwaj, Kumar, Dabasa, & Alam, 2012; Singh Gill, Sood, Muthuraman, Bali, & Dev Sharma, 2011). Another *in-vitro* study has suggested the anti-microbial potential of hexane, chloroformic, and ethanolic extracts of fruits and seeds of wild *Citrullus lanatus* var. *citroides* against different kinds of bacteria (Hassan, Sirat, Yagi, Koko, & Abdelwahab, 2011). Only a few studies have suggested the anti-atherogenic and anti-inflammatory potential of conventional watermelon in animal models (Poduri, Rateri,

Saha, Saha, & Daugherty, 2013). Previous studies have recognized conventional watermelon as a rich source of n-6 linoleic acid and citrulline (Biswas, Ghosal, Chattopadhyay, & Datta, 2017). We speculated that high levels of bioactive compounds such as linoleic acid and dietary fiber along with other phytochemicals in the seeds of Kgengwe fruits (*Citrullus lanatus*) could beneficially alter lipid metabolism and inflammatory responses resulting in the prevention of atherosclerosis.

Based on the aforementioned studies and our own experiences with other similar food products (Dupasquier et al., 2007; Surendiran et al., 2013), we assumed that it would be reasonable to hypothesize that regular consumption of KSP will prevent atherogenesis. Furthermore, this effect is presumed to be mediated through an anti-inflammatory mechanism. Thus, the aim of this study was to investigate potential cardiovascular benefits of KSP in an established animal model, namely low-density lipoprotein (LDL) receptor knockout (LDL-r-KO) mice over twenty weeks. Our specific objectives include the i) evaluation of lipid lowering and anti-atherosclerotic properties of KSP, and ii) investigation of the effect of long term consumption of KSP on inflammatory status.

## **2.3 Materials and Methods**

### **2.3.1 Experimental diets**

KSP was collected from Khutse in the Kalahari Desert in Botswana and provided by Dr. Kobue-Lekalake, Botswana University of Agriculture and Natural Resources. Samples of KSP were used for proximate analysis and fatty acid profiling, before being added to the experimental diets. Proximate analysis of KSP was carried out by Central Testing Laboratory (Lab #447592,



Winnipeg, MB) (**Table 2.1**). The study diets were made using Mouse Diet 9F 5020 (Ren's Feed and Supplies Limited, ON, Canada) with or without 10% (w/w) KSP per our standard methods (Surendiran et al., 2013). **Table 2.2** summarizes the nutritional composition of normal mouse chow. All the experimental diets were supplemented with 0.06% (w/w) dietary cholesterol (Surendiran et al. 2013).

### **2.3.2 Lipid extraction and fatty acid analysis of KSP**

Lipid extraction of KSP was carried out according to the method of Folch et al. (1957) with slight modification. KSP sample was homogenized in 10 mL of 0.025% of calcium chloride ( $\text{CaCl}_2$ ) followed by extraction with 30 mL of chloroform: methanol (2:1, v/v). The suspension was vortexed at room temperature for 1 min and left for 1 hr to separate into three layers. The top two layers were discarded. The bottom layer containing lipids was collected and dried at 55°C on heating block using an evaporating unit and nitrogen gas.

For methylation, dry lipid was dissolved in Toluene and vortex to mix, followed by addition of 3N Methanolic-HCl and vortexing. This mixture was incubated at 80°C for 1 hr to facilitate the addition of methyl group onto the fatty acid. The methylated lipid samples were separated on a Varian WCOT Fused Silica CP-SELECT FAME column (100 m  $\times$  0.25 mm diameter and 0.25  $\mu\text{m}$  film thickness; Varian Canada Inc., Mississauga, Ontario) using a Varian 450 GC with FID (Flame ionization Detector). A mixture of methylated fatty acid standard, (code # GLC-463, purchased from Nu-check (Nu-chek Prep Inc., nu-chekprep.com) was also run at the same condition. The initial column temperature was operated at 130°C for 2 min, followed by raising the temperature to 175°C at 25°C/min. After 25 min, the temperature rose again to 240°C at 3°C/min and held for 10 min. Total run time was 60.47 min, and samples were run with a 20:1

split ratio and column flow of 0.8 ml/min, injector temperature 270°C and detector temperature 290°C; hydrogen was used as the carrier gas as described previously (M. Moghadasian, Moghadasian, Le, Hydamaka, & Zahradka, 2015).

For identification, each individual methylated fatty acid at the end of the column run was detected by FID (Flame ionization Detector) and represented as peak chromatogram. Each fatty acid was identified based on the retention time compared to that of the standard. Their concentrations were calculated by Variant Compass software and express as area percent of the total of all peak area and stated as previously (Riediger et al., 2009, 2008).

### **2.3.3 Animal experimentation**

The animal protocol was approved by the Animal Care Committee at the University of Manitoba, Winnipeg, Canada. Twenty male LDL-r-KO mice (4 weeks old) were purchased from the Jackson Laboratory, Bar Harbor, ME, U.S.A. The experimentation on mice was conducted in Animal Facility at St. Boniface Hospital Research Centre (Winnipeg, MB, Canada). All mice were fed with regular mouse chow for initial first week of quarantine period. The experimental animals were kept in a temperature regulated room adjusted at 22°C to 24°C. The light and dark cycle of the rooms were equally divided to 12:12 hr.

The mice were divided into two groups of control (n=10) and treated (n=10), matched for their body weight and plasma total cholesterol (TC) levels after a week of adaptation period as previously described (Moghadasian, 2006). The control group received the mouse chow supplemented with 0.06% (w/w) cholesterol, and the treated group with the same diet containing 10% (w/w) KSP. The study period was 20 weeks. The mice were kept in pairs in conventional cages. The experimental diets were made on monthly basis and stored in dark bags and closed

vessels in a cold room at 4°C temperature until used. Weekly scheduled diets were provided to mice. The ad-libitum food and water were accessible to all the animals. The food pellets left in the cages were discarded.

Body weight and food intake were recorded weekly. Blood samples were taken at the baseline and every 4 weeks from the jugular vein of approximately 12-hr fasting mice under light anesthesia and used for lipid measurements. At the end of the study period, the mice were killed using CO<sub>2</sub> gas followed by cardiac puncture; final blood sample was taken from the heart and the heart was removed and processed for histological examinations. **Fig. 2.1** summarizes experimental design.

#### **2.3.4 Body weight, organ weight, and food intake of the mice**

Weekly body weight of the mice was recorded using an electronic weighing scale. Various internal organs (including liver, kidney, spleen, and heart), and abdominal fat were inspected and collected at autopsy, weighed, and stored appropriately for further analyses. Weekly food intake was estimated by subtracting the remaining food in the cage at the end of the week from the food provided at the beginning of the week. Further calculations were performed to estimate the amount of food consumed by each mouse per day. The cross checking of cages was also carried out in order to detect any hidden pellets of diet in the cage to avoid any error in the calculated total food consumed.

#### **2.3.5 Estimation of plasma cholesterol and triglyceride levels**

Plasma samples were used for determination of TC and TG concentrations using our standard enzymatic procedures (M. H. Moghadasian, 2006).

### **2.3.6 Analysis of plasma cytokine concentrations**

Plasma samples from the final blood collection were used for the analysis of plasma cytokine intensity, using Meso Scale Discovery U-PLEX Mouse multiplex assay panel (Meso Scale Diagnostics, Rockville, Maryland 20850-3173, USA). A group of 35 biomarkers of inflammation was used. These assays consisted of chemokines (interferon gamma-induced protein-10 (IP-10), growth regulated oncogenes (KC/GRO), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory proteins (MIP-1 $\alpha$ , MIP-1 $\beta$ , MIP 2, and MIP-3 $\alpha$ )), T cell combo (granulocyte-macrophage colony stimulating factor (GM-CSF), interferon gamma (IFN- $\gamma$ ), interleukins (IL-2, IL-4, IL-9, IL-10, IL-13, IL-17A, IL-17E/IL 25, IL-17F, IL-21, IL-22), and tumor necrosis factor-alpha (TNF- $\alpha$ )), TH1/TH2 Combo (IL-1 $\beta$ , IL-5, and IL-12p70A), TH17 Combo 1 (IL-17C, IL-23, and IL-33), TH17 Combo 2 (IL-6, erythropoietin (EPO), IL-27p28/IL-30, vascular endothelial growth factor A (VEGF-A), IL-15, IL-16, and IL-17A/F). Briefly, the biotinylated antibodies for the specific linkers were coupled and then U-plex plate coating was performed for plasma samples (n=5; 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. The intensity of 35- Plex biomarkers was analyzed using MSD Workbench 3.0 software for reading standard curves involving a 4-parameter logistic fit (Magnusson et al., 2012).

### **2.3.7 Measurement of atherosclerotic lesion size**

At the end of the study, base parts of the heart samples were collected and fixed in 10% buffered formalin and processed for histological examination. Aortic root sections were cut and mounted; the sections were stained with hematoxylin and eosin (H&E). Ten, 5- $\mu$ m sections representing

the whole length of aortic root were utilized for morphological and morphometrical examinations of atherosclerotic lesions. Atherosclerotic lesion size was calculated using light microscopy techniques and Image ProPlus digitizing system (Media Cybernetics Inc., Rockville, MD 20850 USA).

### **2.3.8 Statistical Analysis**

Data are presented as mean  $\pm$  standard deviations (SD). Student's *t* test was performed for comparison between the control and treated groups, using Microsoft Excel 2016 Statistical package for Windows 2010, version 1809. *P* < 0.05 was considered as statistically significant difference between the two groups of the mice.

## **2.4 Results**

### **2.4.1 Kgengwe seed powder proximate and fatty acid composition**

KSP proximate composition revealed that approximately 40% of dry matter in KSP was fiber. Proteins and fats each constitute approximately 15% of KSP, and the non-fiber carbohydrate constitutes for 25% of dry matter. Approximately 63% of total fatty acids of KSP was n-6 fatty acids predominantly linoleic acid, while total n-3 fatty acids counted for only 0.15%. The amounts of saturated fatty acids and monounsaturated fatty acids were approximately 21% and 14%, respectively. Summarized and detailed fatty acid profile of KSP is provided in **Table 2.3**

### **2.4.2 Body weight, organ weight, abdominal fat, and food intake of the mice**

A steady increase in the body weight was observed in both control and treated groups over the period of twenty weeks (**Table 3**). On average, each mouse consumed approximately 3g diet per

day and gained 0.77g of body weight per week. The results showed that the mean body weight, organ weight, abdominal fat, and food intake were comparable between the control and treated mice (**Table 2.4**).

#### **2.4.3 Plasma lipid levels**

Consistent with our previous observations, an addition of 0.06% (w/w) cholesterol led to a prominent rise in the total plasma cholesterol levels in all animals (Surendiran et al., 2013). The plasma TC levels almost doubled at week 20 as compared to the baseline data in both control and treated groups. The levels of plasma TC were comparable between the two groups of control and treated mice throughout the study period. The KSP treated group had approximately 10% lower plasma TG levels as compared with those in the control group at the end of the study. However, plasma TG concentrations were statistically similar between the two groups of animals. Plasma lipid levels are shown in **Table 2.4**.

#### **2.4.4 Plasma concentrations of cytokines**

We measured an array of plasma inflammatory markers at week 20 of the study. As compared with the control animals, the KSP-treated mice showed a statistically significant higher concentration of the following inflammatory markers: interleukins (IL-10, IL-16, IL-17, IL-22, and IL-27p28/IL-30), macrophage inflammatory protein-3 alpha (MIP-3 $\alpha$ ), erythropoietin (EPO), granulocyte-macrophage colony-stimulating factor (GM-CSF), and keratinocyte chemoattractant/ growth regulated oncogene (KC/GRO). On the other hand, the levels of monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein- 2 (MIP-2) were reduced in the plasma of KSP-treated mice as compared to those in the control group

(Table 2.5). Other inflammatory markers such as INF- $\gamma$ , and TNF- $\alpha$  levels were comparable between the two groups of mice.

#### 2.4.5 Atherosclerotic lesion size

Atherosclerotic lesion size in the aortic roots of the mice was measured at the end of the study. Animals ingesting KSP showed a significant decrease in the atherosclerotic lesion size as compared to that in the control mice ( $0.26 \pm 0.05$  vs  $0.11 \pm 0.02$ , mm<sup>2</sup>,  $p < 0.05$ ). As shown on Panel A, Fig. 2.2, the atherosclerotic lesion size in the KSP-treated group was reduced by 58% as compared with that in the control group. Furthermore, these lesions were much less advanced in the KSP-treated mice, while they appeared lipid rich in the control group. Panel B, Fig. 2.2 illustrates representative photomicrographs of aortic roots of the control group and KSP-treated animals; arrows point to the advanced lipid-rich atherosclerotic lesions in the control group, while circle shows minimal atherosclerotic lesions in the KSP-treated group.

### 2.5 Discussion

Kgengwe fruits and seeds are commonly consumed as a staple food or snack in several African countries. However, literature provides limited or no scientific reports on its cardioprotective effects and potential functionality. We previously reported an association between anti-atherogenic properties of phytosterols and modulations of inflammatory pathways in apo E-KO mice (Nashed, Yeganeh, HayGlass, & Moghadasian, 2005). In particular, we reported an increase in the levels of IL-10 in animals treated with phytosterols. Other studies have also reported such association in other experimental models (Poduri et al., 2013). These studies

prompted us to investigate whether observed anti-atherogenic properties of KSP are associated with increased levels of IL-10 and perhaps other beneficial alterations in inflammatory pathways.

KSP is a rich source of dietary fiber, as the proximate analysis revealed approximately 40% of dry matter to be fiber which could be considered as a potential functional anti-atherogenic component in KSP. The Academy of Nutrition and Dietetics extensively reported the health benefits of dietary fiber in daily diet to reduce the risk of metabolic disorders and CVD (Dahl & Stewart, 2015). One of such benefits could be LDL-cholesterol-lowering properties (Anderson, Hanna, Peng, & Kryscio, 2000). Many studies have shown that appropriate amounts of dietary fiber may reduce LDL-cholesterol levels by approximately 5% (Surampudi, Enkhmaa, Anuurad, & Berglund, 2016). However, we did not find any significant reduction in the lipid profile in KSP treated group as compared to the control group. This lack of cholesterol-lowering effect could be related to different types of dietary fiber. In this study, we did not identify the ratio of viscous to non-viscous fiber in KSP. Another field of interest would be investigation of how such high dietary fiber food products may impact gut microbiome. Recent studies have reported a link between alterations in gut microbiome and prevention of atherogenesis in LDL-r-KO mice (M. H. Moghadasian et al., 2019). Thus, it can be speculated that, at least in part, anti-atherogenic activities of KSP may also be linked to beneficial alterations in gut microbiome and/or their metabolic products. Such mechanistic studies are among our future plans.

We utilized KSP (10% w/w) dose based on a previous study (Dupasquier et al., 2007). The closest study to the present investigation that we could find was the one which utilized the conventional watermelon products such as seed oil or extract in animal models (Madhavi, 2012; Poduri et al., 2013). While these studies focused on the role of citrulline as a potential agent possessing anti-atherogenic effects, we are reporting a strong link between alterations in



inflammatory markers and anti-atherogenic properties of KSP. This is particularly interesting observation, because KSP is almost void of n-3, but very rich in n-6 fatty acids. The impact of high intake of n-6 fatty acids on health status has been a matter of discussion over the past decades. In particular, cardiovascular benefits of dietary n-6 fatty acids have been controversial. For example, a comprehensive review of cohort studies representing a total of 310,602 subjects and a total of 12,479 coronary heart events showed an inverse and dose-dependent relationship between linoleic acid consumption and coronary heart disease (Farvid et al., 2014). Despite such findings, many health authorities and research groups suggested a decrease in dietary n-6 fatty acid intakes along with an increase in n-3 fatty acid intakes to achieve a ratio of 2-4:1 for n-6:n-3 fatty acid intakes (Hamazaki & Okuyama, 2003; Simopoulos, 2002). One major reason behind such recommendation could be that the n-6 fatty acids can compete with n-3 fatty acids in LOX and COX pathways, resulting in a general inflammatory status (Simopoulos, 2002). In this regard, Ramsden and co-workers reported that replacement of saturated fat with linoleic acid was associated with an increased all-cause death rate (Ramsden et al., 2013).

The present study provides evidence that in this experimental setting high intake of n-6 fatty acid was associated with prevention of atherosclerotic disease along with increased levels of some anti-inflammatory biomarkers such as IL-10. Another study reported anti-atherogenic properties of n-6 PUFA diets was associated with higher levels of aortic catalase (Penumetcha, Song, Merchant, & Parthasarathy, 2012). It should be mentioned that fatty acid metabolites including prostaglandins, leukotrienes, and related biomarkers play a crucial role in cardiovascular function. For this reason, we are currently investigating the association between reduced atherosclerosis and the levels of approximately 100 fatty acid metabolites in this animal model.

Another functional ingredient of KSP could be oleic acid. Previous studies have reported cardiovascular benefits of n-6 linoleic acid and oleic acid in both animals and humans (Sacks & Campos, 2006). In this regard, Perdomo and co-workers reported that oleic acid could improve cardiovascular function and prevent early and late cellular atherosclerotic processes primarily through the prevention of inflammatory responses induced by palmitate or TNF $\alpha$  (Perdomo et al., 2015). Similarly, another review article critically assessed the importance of the role of oleic acid in health and disease (Carrillo, Cavia, & Alonso-Torre, 2012), highlighting the potential health benefits of oleic acid against disorders with inflammatory backgrounds such as atherosclerosis.

One of the main mechanisms for the anti-atherogenic effects of diets is believed to be reductions in plasma cholesterol levels. Although the current study reports a significant 58% reduction in the atherosclerotic lesion size in KSP-treated animals as compared with that in the controls, this effect was not associated with a significant reduction in plasma TC levels over the 20 weeks of the experiment. Furthermore, morphological examinations of the aortic roots revealed a very early stage of atherogenesis, suggesting prevention of atherosclerotic lesion development in the KSP-treated mice, while such lesions were advanced and lipid rich in the control group. High levels of saturated fatty acids in KSP may be responsible for a lack of TC-lowering effect. Furthermore, one may suggest that high intakes of saturated fat might also increase the levels of high density lipoprotein (HDL) cholesterol, and hence contributed to its anti-atherogenic properties. Although inadequate amounts of plasma samples did not allow to perform lipoprotein profiling in this study, an investigation on HDL cholesterol metabolism under such experimental conditions will be carried out in our future studies.

Similar to our observations, Poduri et al. reported the anti-atherogenic effects of citrulline from *Citrullus lanatus* in the same animal model (Poduri et al., 2013). Both Kgengwe and *Citrullus lanatus* (cultivated watermelon) are from the same botanical order. Therefore, they may possess similar phytochemicals for prevention of diet-induced atherosclerosis. Unlike the present study, the previous report showed a small but statistically significant reduction in plasma total and LDL cholesterol concentrations in citrulline (from *Citrullus lanatus*) -treated LDL-r-KO mice (Poduri et al., 2013). Other studies suggested additional biological properties, including anti-inflammatory and endothelium dependent vaso-relaxation/ aortic vascular remodeling effects of citrulline administration in animal models (Hayashi et al., 2005; Iantorno et al., 2014). In contrast to the present KSP study, other studies reported significant reductions in plasma TC and intermediate/ LDL concentrations in experimental mice (Hong et al., 2015; Poduri et al., 2013).

Inflammation has long been recognized as a risk factor in the progression of atherosclerosis; in fact atherosclerosis is recognized as an inflammatory disease (Iantorno et al., 2014). We extended our studies to investigate possible anti-inflammatory mechanisms for KSP. As mentioned above, our results show an increased level of anti-inflammatory IL-10 in KSP-fed mice. IL-10 is a type of cytokine with its homo dimer structure that forms a complex with its receptors IL-10R1/IL-10R2. Basically, it targets macrophages, monocytes T cells, B cells, Natural Killer (NK) cells, mast cells, and granulocytes with its immunosuppressive function through antigen presenting cells. This cytokine also impacts the production of immunoglobulins E (IgE) and G (IgG) by B cells. This mechanism induced a systemic anti-inflammatory response, (Akdis et al., 2011) resulting in prevention of atherosclerosis (Pinderski Oslund et al., 1999).

Poduri and colleagues have also reported a significant decrease in the systemic inflammation due to profound increase in the plasma concentrations of IL-10 with evidence of decreased

atherosclerotic lesion size in the aortic arch and thoracic aorta of LDL-r-KO mice (Poduri et al., 2013). In line with our data, another study has demonstrated that IL-10 overexpression by bone marrow transplantation decreased the atherosclerotic lesion size in LDL-r-KO mice, when fed with an atherogenic diet (Pinderski et al., 2002). Another cytokine related to IL-10 family is IL-22, originating from active T cells. IL-22 has risen almost double in KSP-treated group as compared to controls. In one study, it is reported that the gene delivery of IL-22 was associated with an increased production of IL-10 and IL-10 mRNA expression in draining lymph nodes of BALB/c mice (Nakagome et al., 2011).

EPO is also considered as an anti-atherogenic plasma biomarker (Lu et al., 2010). We recently reported that long-time wild rice consumption was associated with a significant decrease in atherogenesis and a significant 109% increase in plasma EPO concentrations in LDL-r-KO mice (Moghadasian et al., 2019). It is interesting that the current study also reports an increase of more than 200 times in the average plasma concentrations of EPO in KSP-treated mice as compared to that in the control animals (6.5 vs 15.8, pg/mL,  $p < 0.05$ ). It is suggested that EPO may regulate intracellular lipid accumulation which was demonstrated by decreased cellular levels of cholesterol and TG in macrophages, most likely mediated through liver X receptor (Lu et al., 2010).

Nonetheless, both the cell proliferation and recruitment of the monocytes equally contribute to the inception of atherosclerotic lesions. In our study, nearly two-fold increase in the concentration of GM-CSF was observed in the KSP treated mice as compared to the control group. Among proliferating cells, dendritic cells (DC) form most of the intimal cells in early atherosclerotic lesions and GM-CSF markedly increased DC production in the lesion development (Zhu, Chen, Jongstra-Bilen, & Cybulsky, 2009). A further study complementing

the present study showed about 20-50% decrease in the atherosclerotic lesion size with an increase in GM-CSF levels in hyperlipidemic LDL-r-KO mice (Haghighat, Weiss, Whalin, Cowan, & Taylor, 2007). A case-controlled study showed a strong negative association between plasma M-CSF and MCP-1 levels and newly established coronary events (Schiopu et al., 2016). This study reported that the subjects with increased macrophage colony stimulating factor (M-CSF) and lower MCP-1 levels were at low risk of developing CVD. However, we detected an increased concentration of GM-CSF with a simultaneous and significant decrease in the plasma levels of MCP-1 in KSP-treated animals as compared to those in the control group.

Although we present strong anti-atherosclerotic effects of a non-conventional food product in a commonly used animal model, we were not able to establish exact mechanisms of action. One of our study limitations could be its inability to address any potential sex-related variations in anti-atherogenic activities. As we have observed in our previous study (Surendiran et al. 2013), another limitation could be related to a lack of established dose response relationship in this animal model and its relation to potential human use. Lastly, we have not been able to extend our studies to establish mechanisms for important findings such as a lack of cholesterol-lowering effects. All of these pitfalls need to be addressed in our future studies.

## **2.6 Conclusions**

In conclusion, our findings suggest that Kgengwe seeds at 10% (w/w) supplementation into animal diet show cardioprotective properties over the 20 weeks of experimental course in LDL-r-KO mice. These effects have not been mediated through reductions in conventional risk factors such as plasma cholesterol or TG levels. Further tests suggested that Kgengwe seeds might prevent progression of atherosclerosis through favorable alterations in the systemic inflammatory

markers. Of particular interest are increases in the plasma levels of IL-10, GM-CSF, and EPO and decreases in plasma concentrations of MCP-1 and MIP-2. Additional studies in this and other animal models are recommended to better understand the mechanisms of anti-atherogenic properties of Kgengwe seeds.

### **Conflict of Interest**

The authors have no conflict of interest to report.

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**Table 2.1 Proximate composition of Kgengwe seed powder**

<b>Parameters</b>	<b>Amount (%)</b>
Dry Matter	94.72
Crude Fiber	38.44
Non-Fiber Carbs	25.38
Fat	14.97
Crude protein	14.18
Moisture	5.28
Ash	1.75

**Table 2.2 The nutrient and energy composition (%) of the mouse diet**

<b>Parameters</b>	<b>%</b>
<i>Constitutes</i>	
Protein	21.6
Lipids	9.0
Starch	39.4
Sugars	1.78
Crude fibre	2.2
Ash	5.0
<i>Energy (% Kcal)</i>	
Protein	26.0
Fat	24.4
Carbohydrates	49.6
<b>Total energy</b>	<b>100</b>

Physiological fuel value for protein, fat, and carbohydrates is 4,9,4 kcal/g, respectively (Mouse Diet 9F 5020 (Ren's Feed and Supplies Limited, ON, Canada)).

**Table 2.3 Detailed fatty acid composition of Kgengwe seed powder**

<b>Fatty acid</b>	<b>%</b>
Lauric acid (C12:0)	0.1735
Myristic acid (C14:0)	0.106
Myristoleic acid (C14:1)	0.0055
Palmitic acid (C16:0)	12.2595
Palmitoleic acid (C16:1)	0.096
Stearic acid (C18:0)	8.4565
Oleic acid (C18:1n9)	14.028
Linoleic acid (C18:2n6)	62.653
Alpha-Linolenic acid (C18:3n3)	0.149
Arachidic acid (C20:0)	0.2995
Gondoic acid (C20:1n9)	0.0635
Eicosadienoic acid (C20:2)	0.007
Eicosatrienoic acid (C20:3n3)	0.001
Arachidonic acid (C20:4n6)	0
Eicosapentaenoic acid (C20:5n-3)	0.001
Docosadienoic acid (C22:2n6)	0.118
Docosapentaenoic acid (C22:5n3)	0.0005
Docosahexaenoic acid (C22:6n3)	0.003
Total n-3 PUFA	0.15
Total n-6 PUFA	62.65
Total SFA	21.30



Total MUFA	14.19
Total PUFA	62.81
Sum	98.30
Others	1.70
Total Fat	100.00

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SFA, Saturated Fatty acid;

MUFA, Monounsaturated Fatty Acid;

PUFA, Polyunsaturated Fatty Acid

**Table 2.4 Effects of experimental diets on body weight, food consumption, and plasma total cholesterol and triglyceride levels over the experimental course of 20 weeks**

Parameters	Week 0		Week 4		Week 12		Week 20		<i>P</i> - value
	Control	KSP-treated	Control	KSP-treated	Control	KSP-treated	Control	KSP-treated	
Body weight (g)	18.3 ± 1.3	18.4 ± 1.4	23.9 ± 2.2	23.6 ± 2.2	30.8 ± 2.4	30.9 ± 3.5	30.8 ± 2.4	31.7 ± 3.7	NS
Food intake (g)	3.2 ± 0.4	3.4 ± 0.4	3.5 ± 0.2	3.8 ± 0.3	3.0 ± 0.4	3.5 ± 0.5	2.9 ± 0.3	3.5 ± 0.9	NS
Plasma total cholesterol (mg/dl)	120 ± 16	121 ± 15	133 ± 24	121 ± 15	165.8 ± 40.7	132.5 ± 22.1	242 ± 35	267 ± 52	NS
Plasma triglycerides (mg/dl)	128.9 ± 32.8	113.0 ± 32.0	60.7 ± 15.4	63.8 ± 14.7	61.8 ± 11.9	45.8 ± 8.6	101.1 ± 23.5	90.5 ± 19.6	NS

Values are means (± SD) for both treatment groups (Control, n=10; KSP treated, n=10), NS= Not significant.

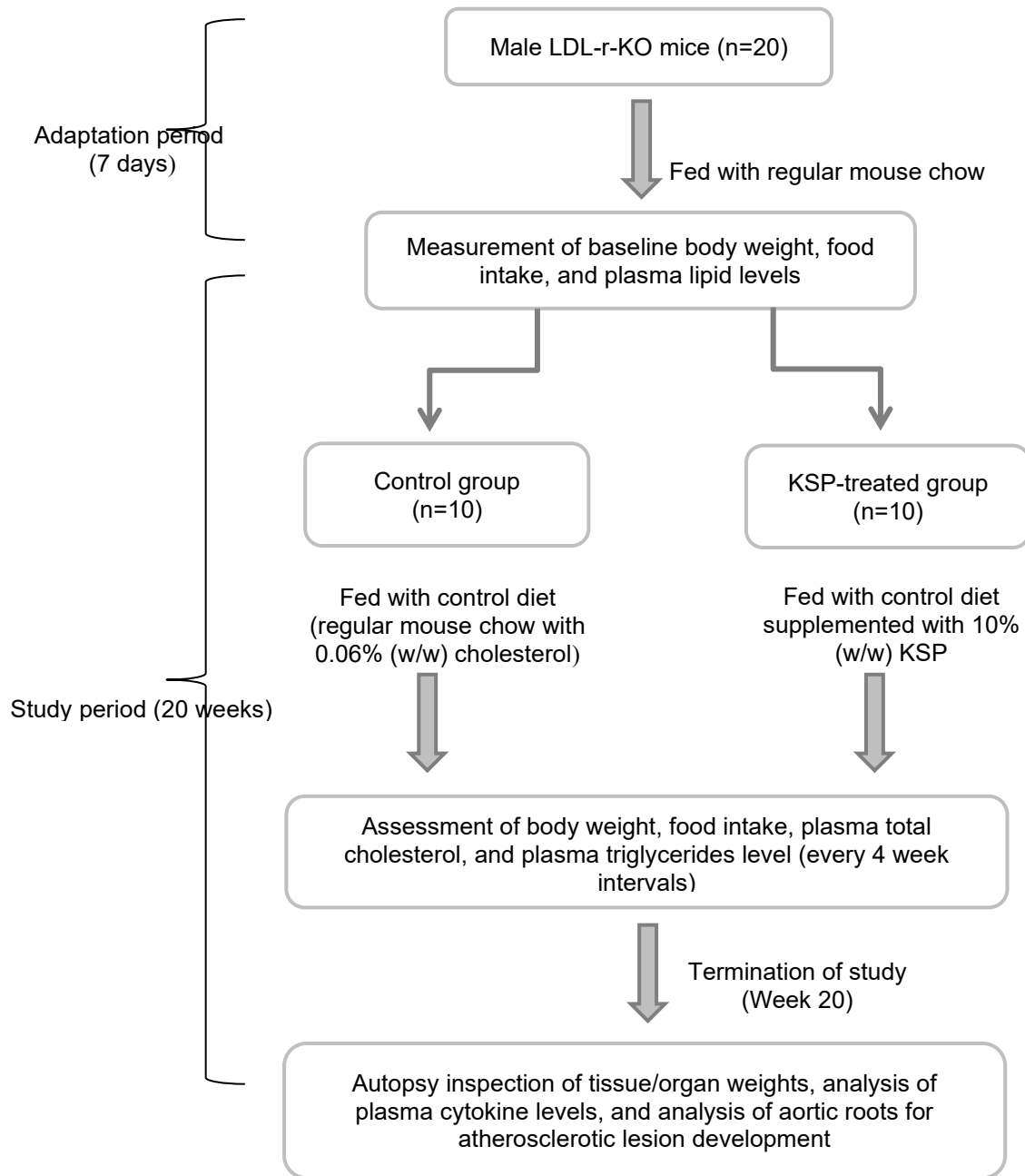
**Table 2.5 Effect of experimental diets on plasma inflammatory biomarkers at week 20 of the study**

Plasma cytokines levels (pg/mL)	Control (n=5)	KSP-treated (n=5)	<i>P</i> -value
IP-10	37.8 ± 2.7	36.7 ± 4.4	0.30
MCP-1	19.5 ± 2.3	14.1 ± 2.7	0.02
IL-9	ND	ND	ND
MIP-1 $\alpha$	2.1 ± 1.0	3.3 ± 2.2	0.55
MIP-1 $\beta$	16.5 ± 2.3	18.2 ± 1.3	0.19
MIP-2	28.4 ± 2.2	13 ± 4.3	0.00
MIP-3 $\alpha$	22.2 ± 4.7	306.7 ± 135.7	0.01
IL-17A/F	ND	ND	ND
IL-12 p40	1042.6 ± 168.9	1057.6 ± 252.9	0.91
IL-16	645.8 ± 14.4	934.2 ± 93.9	0.00
IL-17	0.3 ± 0.1	0.9 ± 0.2	0.01
IL-17C	2.3 ± 1.2	2.9 ± 0.0	0.77
IL-17E	0.2 ± 0.0	0.2 ± 0.1	0.92
IL-21	ND	ND	ND
IL-22	5.0 ± 1.2	10.9 ± 1.6	0.00
IL-10	6.0 ± 1.87	13.0 ± 1.6	0.00
IL-17F	ND	ND	ND
IL-15	19.2 ± 9.0	28.6 ± 6.7	0.36
IL-13	ND	ND	ND
IL-23	ND	ND	ND
IL-27p28/IL-30	2.2 ± 0.1	3.8 ± 0.7	0.02
IL-33	1.7 ± 0.8	1.4 ± 0.5	0.74

IL-4	0.1 ± 0.0	ND	ND
VEGF	6.1 ± 0.6	6.4 ± 0.4	0.37
EPO	6.7 ± 2.7	15.8 ± 4.5	0.00
GM-CSF	0.1 ± 0.0	0.2 ± 0.0	0.01
IFN- $\gamma$	0.4 ± 0.1	0.6 ± 0.2	0.13
IL-5	1.6 ± 0.3	2.2 ± 1.0	0.25
IL-1 $\beta$	ND	ND	ND
IL-6	7.2 ± 2.2	4.9 ± 1.2	0.55
IL-2	ND	ND	ND
IL-12p70	16.1 ± 1.6	16.3 ± 3.8	0.96
KC/GRO	68.6 ± 15.9	92.4 ± 5.7	0.01
TNF- $\alpha$	6.8 ± 0.7	6.2 ± 2.2	0.59

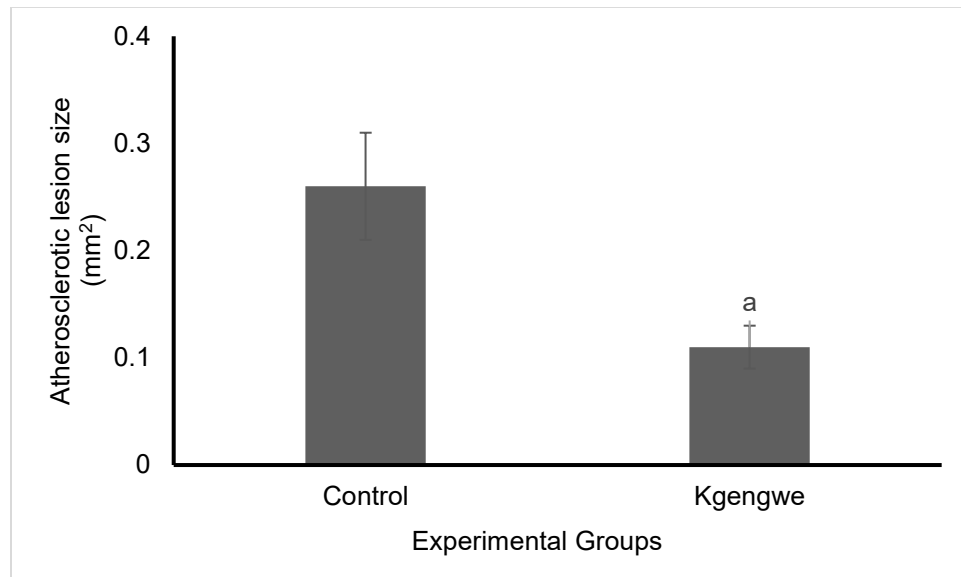
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Values are means  $\pm$  SD for both experimental groups (Control, n=5; KSP treated, n=5, 2 mice plasma were pooled per each n), (p <0.05 by students t-test significantly differed from control diet); ND= not detectable.

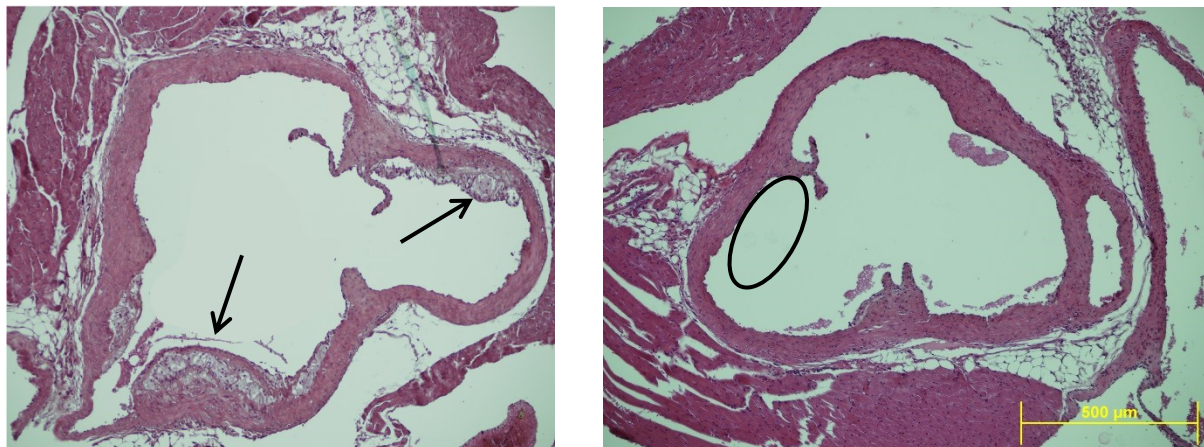


**Figure 2.1 Experimental design**

**A**



**B**



**Figure 2.2 Effects of Kgengwe seed powder (KSP) diet on atherosclerotic lesion size in aortic roots of male LDL-r-KO mice measured at termination of the study (control, n=10; KSP-treated, n=10).**

**Panel A** shows mean  $\pm$  SD for the atherosclerotic lesion size; **Panel B** depicts representative photomicrographs of aortic roots of control (left image) and KSP-treated mice (right image). Arrows point towards well established atherosclerotic lesions in the control group; encircled portions show limited atherosclerotic lesion development in the KSP-treated group. <sup>a</sup> $p < 0.05$  significantly different from control group

### **BRIDGING STATEMENT TO CHAPTER III**

The secondary objective of this thesis is to identify the possible mechanism of action of anti-atherosclerotic properties of KSP in LDL-r-KO mice. A significant decrease in the atherosclerotic lesion size in the aortic roots of KSP fed mice was determined in the preceding chapter. Interestingly, no significant changes in the conventional CVD risk markers such as plasma TC and TG levels were observed in the treated mice in comparison to that in controls at the end of the study. Therefore, it is crucial to address the other possible mechanisms of action through which KSP prevented atherogenesis. The following chapter includes a manuscript principally focused on analysis of the alterations in the plasma and fecal metabolites, and plasma oxylipin levels in relation to anti-atherogenic effect of KSP diets. The significant changes in the plasma and fecal metabolites and plasma oxylipin levels in KSP treated mice as compared to that in the controls demonstrate the multi-facet mechanistic approach of KSP in the significant reduction of atherosclerotic lesion size and increase in the anti-inflammatory cytokines through cross regulatory pathways.

## Chapter III

### **ANTI-ATHEROGENIC EFFECTS OF CITRULLUS LANATUS SEED POWDER MAY BE MEDIATED THROUGH INCREASES IN PGE2 INDUCED IL-10 AND L-CITRULLINE IN LDL-R-KO MICE**

**(To be further developed for submission)**

#### **3.1 Abstract**

Our previous study (Kaur et al., 2020) demonstrated that Kgengwe seed powder (KSP) supplementation (10% w/w) showed significant alterations in plasma cytokine levels, particularly IL-10, and decreased the atherosclerotic lesion size (58%) in the aortic roots of atherogenic diet fed low density lipoprotein knock out (LDL-r-KO) male mice for a study period of 20 weeks. The present study is focused on assessing the mechanistic action of the anti-atherosclerotic potential of KSP in these animal models. This study provides the insight into the interplay of plasma cytokines and oxylipins mediated decrease in the atherosclerotic lesion size in KSP treated mice as compared to that of controls. Notably, among plasma and fecal metabolites levels at week 20, plasma concentrations of citrulline and arginine increased significantly ( $p < 0.05$ ) in the KSP treated mice as compared to that in controls. While, the concentrations of fecal citrulline levels also increased significantly ( $p < 0.05$ ) in the KSP treated mice in comparison to controls at week 20. On the other hand, out of 91 oxylipins, PGE2, 13-HODE, 9,10,13-triHOME, 9,10 EpOME, 12,13 EpOME, and 15,16 EpODE raised significantly ( $p < 0.05$  by Mann–Whitney U test) by approximately 1.0 log<sub>2</sub> folds in KSP treated group. Plasma IL-10 levels and PGE2 levels showed a significant linear positive correlation in the experimental mice. A major finding of this study was increased levels of plasma citrulline, arginine, and PGE2 which supported the hypothesis that not plasma lipid levels but improved



inflammatory status through increased IL-10 concentrations in *Citrullus lanatus* or KSP supplemented mice presented reduced atherosclerotic lesion size. These findings indicate that wild *Citrullus lanatus* seeds may prove to be an atheroprotective functional food.

**Keywords:** *Citrullus lanatus*; *LDL-r-KO-mice*; *Cytokines*; *Atherosclerosis*; *Prostaglandins*; *Citrulline*

### 3.2 Introduction

Despite significant advancements in the medical field, CVD remained a prominent root of the morbidity and mortality in both developed and developing nations. Higher prevalence of diabetes, metabolic syndrome, and obesity along with cigarette smoking and sedentary lifestyles always fuel to the onset of cardiovascular events (Benjamin et al., 2017). Fact sheet of the World Health Organization (WHO, 2017) has reported an increase in the global premature deaths to be approximately 25 million by 2025. The 2013-2020 global non-communicable diseases action plan targets to control this mortality rate through directly focus on the worldwide CVD burden as two of their comprehensive strategies (WHO, 2017). One of such dietary approach in controlling cardiovascular disorders could be achieved through consumption of *Citrullus lanatus* (Hong et al., 2015; Poduri, Rateri, Saha, Saha, & Daugherty, 2013). The consumption of *Citrullus lanatus* through increase of nitric oxide (NO), leading to the improvements in the arterial dysfunction and endothelial stiffness as well as hepatic lipid peroxidation in both animal and human studies (Altaş, Kizil, Kizil, Ketani, & Haris, 2011; Figueroa, Sanchez-Gonzalez, Perkins-Veazie, & Arjmandi, 2011). Conventional watermelon is known for its high L-citrulline content (0.9-4mg/kg of fresh whole fruit) (Perkins-Veazie, Davis, & Collins, 2012), identified for its effects on the release of endogenous NO (Figueroa et al., 2011; Rimando & Perkins-Veazie, 2005; Wu Guoyao et al., 2007).

Kgengwe, also known as Tsamma melon, wild melon or wild *Citrullus lanatus*, is cultivated in Sub-Saharan countries, plus some regions of Japan, China, and Middle Asia. However, the ethno-pharmaceutical and health properties of this wild-type fruit is limited (Cheikhyyoussef et al., 2017; Komane, Vermaak, Kamatou, Summers, & Viljoen, 2017; Vermaak, Kamatou, Komane-Mofokeng, Viljoen, & Beckett, 2011). To present, there are only few studies

suggesting the direct cardioprotective effects of *Citrullus lanatus* in animal models (Hong, Beidler, Hooshmand, Figueroa, & Kern, 2018; Hong et al., 2015; Poduri et al., 2013). Very recently, we reported the anti-atherogenic properties of wild watermelon seeds, Kgengwe seed powder (KSP), supplemented in cholesterol rich diet fed low density lipoprotein knock out (LDL-r-KO) mice (Kaur et al., 2020). Our data show that the fatty acid profile of seeds of *Citrullus lanatus* are rich in n-6 polyunsaturated fatty acids (PUFA) especially linoleic acid but contained negligible levels of n-3 PUFA (linolenic acid). Therefore, we speculated that the high amount of linoleic acid or its biologically produced metabolites could play a major in its anti-atherogenic properties. One of such metabolites could be oxylipins which can be produced by enzymatic reactions, including COX (cyclooxygenase), LOX (lipoxygenase), and cytochrome P450 in different cells (Melissa Gabbs, Leng, Devassy, Monirujjaman, & Aukema, 2015). Oxylipins could play a role in either pro- and/or anti-inflammatory fashions (Dennis & Norris, 2015; Groeger et al., 2010). While atherosclerosis is known as an inflammatory disease, several studies reported benefits of anti-inflammatory markers in prevention of the disease (Iantorno et al., 2014; Sacks & Campos, 2006). In an effort to explore the potential mechanisms of action, we decided to determine oxylipin profiles and thereby define any association between KSP-prevented atherosclerosis and plasma and fecal metabolites.

### **3.3 Materials and Methods**

#### **3.3.1 Experimental animals**

The biological samples used in this study were collected from our previous mouse study (Chapter 3, Kaur et al., 2020). Briefly, the experimentation was conducted on twenty LDL-r-KO mice (4 weeks old, male) obtained from the Jackson's laboratory (Bar Harbor, ME, U.S.A). The

animal experimentation was carried out in the Animal Facility at St. Boniface Hospital Albrechtsen Research Centre (Winnipeg, MB, CA). All mice were kept in pairs in a temperature-controlled facility where the chambers were regulated between 22°C to 24°C. The conventional mice cages were kept in 12:12hr light and dark cycle. During the first initial week of quarantine, all animals were provided with the normal mouse chow and ad-libitum water. After one-week span of adaptation, baseline blood samples were collected following approximately 12 hr fasting. The blood was drawn from jugular vein using light anesthesia. Based on the estimated body weights and baseline plasma TC levels, mice were divided into control (n=10) and treated groups (n=10) as described previously (Moghadasian, 2006). The Animal Care Committee at the University of Manitoba (Winnipeg, Canada) approved the animal study protocol.

### **3.3.2 Study diets**

Mouse Diet 9F 5020 (Ren's Feed and Supplies Limited, ON, Canada) was used to make experimental diets. The treated group diet was supplemented with 10% (w/w) wild *Citrullus lanatus* seeds powder supplied by Dr. Kobue-Lekalake (Botswana University of Agriculture and Natural Resources) obtained from Khutse (Kalahari Desert, Botswana). The study was carried out for a period of 20 weeks. The experimental diets were contained 0.06% (w/w) dietary cholesterol to make them atherogenic according to our previous studies (Surendiran et al., 2013).

### **3.3.3 Sample collection and analysis**

The diet samples and KSP were analysed for their nutrient composition and fatty acid profile respectively as previously stated (Kaur et al., 2020). The general animal study procedures included the assessment of body weight gain (using electronic weighing scale), average food intake, and the inspection of changes in the plasma TC and TG levels using standard enzymatic

kits and lab procedures at 4 weeks intervals as per our recent study (Kaur et al., 2020). Plasma samples of all the experimental mice were also analysed for 35 different cytokines concentrations using Meso Scale Discovery U-PLEX Mouse multiplex assay panel (Meso Scale Diagnostics, Rockville, Maryland 20850-3173, USA) as mentioned in our recent studies (Kaur et al., 2020; Moghadasian et al., 2019).

### **3.3.4 Plasma oxylipin profile analysis**

Plasma samples of control and treated mice were collected at week 20 and stored at -80°C till analysis. Because of inadequate plasma volume, plasma samples of two experimental mice in both groups were pooled (n=5, each group). High performance liquid chromatography mass spectrometry (HPLC-MS) (QTRAP 6500; Sciex, ON, Canada) multiple-reaction monitoring (MRM) was used to analyse the plasma oxylipin profile as described by Caligiuri and co-authors (Caligiuri et al., 2014). A total of 167 oxylipins were analysed in the profile. Briefly, plasma samples (90µL) were added with 10µL of antioxidant mixture (0.2 mg/mL butylated hydroxytoluene, 0.2 mg/mL ethylenediaminetetraacetic acid, 2mg/mL triphenylphosphonium, 2 mg/mL indomethacin in a solution of 2:1:1 methanol:methanol:water) and 100 µL of deuterated internal standards (Cayman Chemical, An Arbor, MI). Solid phase extraction (SPE) of the plasma samples was run on Strata-X SPE columns (Phenomenex, Torrance, CA). Before loading the samples on the columns, the plasma mixture was regulated to pH<3 and SPE columns were eluted with 3mL of pH 3 water and methanol (3mL). All the samples were finally rinsed with 10% methanol solution (pH 3) before adding into the columns. Lastly, hexane (1mL) were injected in all the columns to vacuum down the residual materials. The eluted samples were then dried and resuspended in the water/acetonitrile/acetic acid (70/30/0.2, v/v/v) mobile phase for

further HPLC-MS analysis. Oxylipins were quantified using the stable isotope dilution method (Hall & Murphy, 1998).

### **3.3.5 Plasma and fecal metabolites**

Plasma samples of the experimental animals from week 20 were analysed for metabolomics profile using liquid chromatography (LC)- mass spectrometry (MS/MS) methods as stated by Kervezee et al. (Kervezee, Cermakian, & Boivin, 2019). Plasma (100µL, n=5) samples (200mg, n=5) were pooled because of inadequate plasma and stored at -80°C prior to analysis. The Metabolomics Innovation Centre (TMIC) (University of Alberta, Edmonton, CA) performed the full panel plasma metabolites analysis. Briefly, a targeted metabolomics approach was utilized to examine the samples with a custom combination of direct injection MS/MS with a reverse-phase LC-MS/MS and an ABI 4000 Q-Trap (Applied Biosystems/MDS Sciex) mass spectrometer. A total of 125 endogenous metabolites were included in the full panel; targeted for identification and quantification using isotope-labeled internal standards through the analytes derivatization and extraction. The targeted metabolites were detected with selective mass-spectrometric method using MRM pairs as described previously (Moghadasian et al., 2019).

### **3.3.6 Atherosclerotic lesion measurement**

Aortae sections were cut from the beginning and processed for examining atherosclerotic lesions through morphological evaluation (Moghadasian et al., 2016). Hematoxylin and eosin (H&E) dye was used for the staining of the aortic root sections. Semiquantitative investigation of atherosclerotic lesions in the control and treated mice was carried out using light microscopy methods as described previously (Kaur et al., 2020).

### 3.3.7 Statistical analysis

All the data are presented as mean  $\pm$  standard deviation (SD). Mann-Whitney U test (Wilcoxon rank-sum test) as non-parametric test and Student's *t*-test (parametric test) were performed to analyse the significant differences between control and treated groups. Microsoft Office Excel (365, Microsoft, Redmond, USA) and the comprehensive 'R' Archive Network statistics software (3.6.1 version, <https://www.r-project.org/>) with 'wilcox.test' function were used to compute the *p*-value between the experimental groups; where  $p < 0.05$  (based on the degrees of freedom) was considered as statistically significant between the control and treated groups. Association between the atherosclerotic lesion size and other risk markers in both the groups were analysed using Pearson's correlation 'CORREL' function in Microsoft Office Excel (365, Microsoft, Redmond, USA).

## 3.4 Results

### 3.4.1 Plasma and fecal metabolites

An array of 125 plasma and fecal metabolites were analyzed using LC-MS/MS in control and treated animals. To enhance the confidence interval of the smaller sample size of plasma metabolites ( $n=5$ ), Mann-Whitney U test was applied. Only significantly differed plasma and fecal metabolites values of treated group in comparison to that in the control group were considered to focus on results to get a narrower view of the metabolites profile. Out of 27 differentially significant plasma metabolites, plasma citrulline and arginine levels were increased by 37.6% and 54.7% respectively in KSP treated group as compared to those in the control group. Other plasma amino acid metabolites including leucine, tryptophan, tyrosine, alpha-

Ketoglutaric acid, and proline nearly doubled in the treated groups as compared to those in the controls. Other than the above mentioned, plasma beta-hydroxybutyric acid up surged significantly in the treated group (145.7%) as compared to the control group. In KSP treated group, plasma short chain fatty acids including propionic, indole-acetic and pyruvic acids increased significantly in comparison to those in the control group. On the other hand, the levels of succinic acid decreased by 82.6% in the treated group. The fecal metabolites such as short chain fatty acids decreased significantly ( $p < 0.05$  using Mann–Whitney U test) in KSP treated group as compared that in controls. However, the concentrations of citrulline excretion in fecal matter was found significantly higher (136%) in KSP group in comparison to the controls. These data are summarized in **Tables 3.1 and 3.2**.

### 3.4.2 Plasma oxylipin profile

Out of 167 plasma oxylipin panel, a total of 91 oxylipins were quantified. **Table 3.3** summarizes the significant plasma oxylipin levels in the experimental animals at week 20 of the study period. Plasma oxylipins including prostaglandin E2 (PGE2), 15-keto-prostaglandin E2 (15k PGE2), 13-hydroxyoctadecadienoic acid (13-HODE), 9,10,13- trihydroxyoctadecenoic acid (9,10,13 triHOME), 9,10-epoxyoctadecenoic acid (9,10 EpOME), 12,13-epoxyoctadecenoic acid (12,13 EpOME), and 15,16-epoxyoctadecadienoic acid (15,16 EpODE) increased significantly ( $p < 0.05$  by Mann–Whitney U test) in KSP treated group by approximately 1.0 log<sub>2</sub> fold change ranging from 0.7 to 2.0 log<sub>2</sub> fold changes as compared to those in the control group. On the other hand, 12-hydroxyeicosapentaenoic acid (12-HEPE), 20-hydroxyeicosatetraenoic acid (20-HETE), and docosahexaenoic acid (DHA) exhibited 1.0, 0.7, and 0.4 folds decrease respectively in KSP treated mice (significant at  $p < 0.05$  using Mann–Whitney U test) as compared to those in the



control mice (**Fig. 3.1**). Remaining oxylipins were either under lower levels of detectable limit or comparable between both the experimental groups.

### **3.4.3 Association between atherosclerotic lesion size, IL-10, and PGE2**

The decrease of atherosclerotic lesion size clearly evidenced the anti-atherosclerotic properties of the KSP in experimental models as demonstrated previously (Kaur et al., 2020). Interestingly, plasma IL-10 levels displayed a significantly ( $p < 0.05$ ) strong, positive, and linear relationship with plasma PGE2 levels in the experimental mice ( $r = 0.93$ ) (**Fig. 3.2**). However, the atherosclerotic lesion size in the aortic roots showed a non-significant but moderately negative correlation with plasma IL-10 levels in both the groups ( $r = -0.66$ ,  $p > 0.05$ ) (**Fig. 3.3**). Furthermore, **Fig. 3.4** depicts a significant ( $p < 0.05$ ) and strong negative linear relationship of plasma citrulline levels with atherosclerotic lesion size in the animals ( $r = -0.84$ ).

## **3.5 Discussion**

The present study unveils an array of significant alterations in the plasma cytokine levels, plasma metabolites, and oxylipin profile in response to an anti-atherogenic diet, KSP, in LDL-r-KO mice over a period of 20 weeks. Atherogenesis is far beyond from just the deposition of lipids in the arterial walls and considered as one of the major sources of inflammation in the body (Ross, 1999). Previous studies have reported the anti-inflammatory properties of watermelon powder in Sprague-Dawley rats fed with atherogenic chow (Hong et al., 2015). Conventional watermelon and Kgengwe (wild watermelon) belong to the same botanical order and thus, it is presumed that both possess similar or nearly similar biological properties. Dyslipidemia is one of the prominent risk factors of CVD (Chong, Macdonald, & Lovegrove, 2010). Unlike other studies showing

cardioprotective effects of *Citrullus lanatus* in different animal models through improved lipid profile (Hong et al., 2018; Poduri et al., 2013), our results did not indicate any beneficial effects of KSP on plasma cholesterol levels as stated in the previous study (Kaur et al., 2020).

Nonetheless, the atherosclerotic lesion size in the aortic roots of the atherogenic diets fed KSP treated group was significantly lowered than that of the control group at the end of the study period which was linked to the significant increase in the plasma anti-inflammatory marker IL-10 in the treated group (Kaur et al., 2020).

The plasma metabolites assay of the experimental mice evinced a significant increase in the plasma citrulline levels in KSP treated mice as compared to the control mice. Being a rich source of L-citrulline, consumption of watermelon juice and/ or watermelon supplemented diets raised plasma L-arginine levels in both animal and human studies (Abd El-Razek & Sadeek, 2011; Collins et al., 2007). Similarly, another study found beneficial changes in the arterial systolic and diastolic pressure in heart failure patients after the oral ingestion of L-citrulline for two months. It has also been established that the consumption of watermelon decreased arterial stiffness and aortic blood pressure in prehypertensive and hypertensive humans (Figuroa et al., 2011; Figuroa, Wong, Hooshmand, & Sanchez-Gonzalez, 2013). L-arginine, produced from its precursor L-citrulline present in *Citrullus lanatus*, plays a crucial role in the production of endothelial NO (Fu et al., 2005). Our results of mice plasma metabolites indicated a significant increase of arginine levels in KSP treated mice as compared to that of the control mice. Similar findings have been reported by others as well (Abd El-Razek & Sadeek, 2011; Collins et al., 2007). Inflammation is a robust patron to the onset of arterial stiffness, atherogenesis, and consequently CVD (Iantorno et al., 2014). Previous literature has reported the reduction of inflammation by downregulating the rat hepatic COX-2 expression and upregulating the gene

expression of endothelial nitric oxide synthase (eNOS) (Hong et al., 2018, 2015). Furthermore, it was suggested that NO production due to higher expression of eNOS in watermelon fed Sprague-Dawley rats is better alternative to L-arginine supplementation (Hong et al., 2018). Adjacent to the findings from previous studies, the present study also attempted to analyse the inflammatory status of the experimental mice through investigating the plasma cytokines levels (Kaur et al., 2020) and plasma oxylipin profile. Among plasma cytokines, IL-10 has been investigated widely and is known for its anti-inflammatory properties (Akdis et al., 2011) and thus, overexpression of plasma IL-10 involves in reversing atherogenesis (Pinderski et al., 2002). A similar study utilizing the same animal model has also reported the significant increase in the plasma IL-10 levels and decrease in the plasma MCP-1 concentrations after feeding watermelon extract supplemented diets (Poduri et al., 2013). Parallel to these findings, our previous study (Kaur et al., 2020) also shown a significant rise in IL-10 concentrations and a concurrent dip in the pro-inflammatory cytokine, MCP-1, levels in KSP treated mice as compared to those in the controls.

As both pro-inflammatory status and inflammation involve the inception of atherogenesis, significant alterations in other plasma cytokines including EPO, GM-CSF, and MCP-1 also involve in diminishing the atherosclerotic cardiac events in animal and human studies (Lu et al., 2010; Schioppa et al., 2016). Apart from lipids, plasma amino acids also play an important role in both humans and animal models suggesting their pro- and/or anti-atherogenic effects in the development of macrophage foam cells (Rom & Aviram, 2017). Both leucine and isoleucine, branched chain amino acids (BCAA), have shown a significant increase of nearly one-fold in KSP treated mice as compared to that in the control mice. A previous study has independently correlated the plasma levels of BCAA with cardiac events and dyslipidemia in 472 Chinese subjects (Yang et al., 2014). On the other hand, BCAA also ameliorate the cardiac

health in heart failure Dahl salt-sensitive rat models (Tanada et al., 2015). Likewise, the anti-atherogenic potential of the supplementation of leucine in apoE-KO mice models has been demonstrated through decrease in serum MCP-1 levels and reduction in the atherosclerotic lesion size (Zhao et al., 2016). A significant increase in the plasma tryptophan (102%) and kynurenine (88.5%) levels in KSP treated mice as compared to that of the controls may suggest the inverse relation with MCP-1 levels in KSP-treated mice as reported in another study where one of the metabolites of L-tryptophan, 3-hydroxyanthranilic acid formed through kynurenine pathway, inhibits the production of MCP-1 levels associated with the atherogenic inflammation in endothelial cells inside human umbilical vein (Pae et al., 2006).

KSP is a rich source of dietary linoleic acid and almost devoid of linolenic acid as described previously (Kaur et al., 2020). It has also been established that linoleic acid, mostly contends with linolenic acid for COX and LOX enzymes and their pathways and thus, generate an inflammatory response in the body (Simopoulos, 2002). Consumption of dietary omega-6 fatty acids in relation to the heart health has always remained controversial. However, linoleic acid consumption has derived a dose-dependent inverse relationship in 310,602 individuals and 12,479 coronary heart events studied in a comprehensive systemic cohort review (Farvid et al., 2014). In this regard, the present study extended further to analyse the plasma oxylipins derived from linoleic acid rich KSP in the experimental mice. Interestingly, PGE<sub>2</sub>, an extensively explored prostaglandin, was found to be significantly higher (1.2 folds) in the KSP treated mice as compared to that in the controls. Though the principle metabolites of arachidonic acid are prostanoids including the bioactive form, PGE<sub>2</sub>, produced in the presence of COX isoforms c. In the absence of other PUFA, the intake of linoleic acid acts as precursor of PGE<sub>2</sub> and increases its production in the tissue (Namazi 2004). The endogenous production of arachidonic acid and

PGE2 from the supplementation of linoleic acid has been reported in cystic fibrosis patients (Zaman et al. 2010). Likewise, another study (Sammon 1999) reported the increased levels of plasma PGE2 in rats fed with diets abundant in linoleic acid. The higher production of PGE2 in the salivary and gingival glands has been demonstrated with high linoleic acid consumption (Sammon 1999).

Moreover, the improved production of PGE2 exaggerate Type 2 immune system characterized with overexpression of IL-10 in the inflammatory and autoimmune disorders (Sha et al. 2012; Nayeem 2018). In a previous study, PGE2 derived from COX-2, an isomeric and inducible form of COX, has been reported as a potent inducer of endogenous IL-10 in bone marrow derived dendritic cells (Harizi, Juzan, Pitard, Moreau, & Gualde, 2002). Similarly, another study has reported the similar results where PGE2 in combination with lipopolysaccharide promoted the expression of macrophage IL-10 through protein kinase A-dependent action (MacKenzie et al., 2013). In line with these studies, the current study also demonstrated the statistically significant and synchronised increase of PGE2 and an anti-inflammatory cytokine, IL-10, levels. Furthermore, the present study suggests the positive, strong, and significant correlation of PGE2 and IL-10 in both experimental animal groups.

The alterations in plasma oxylipins also revealed significantly higher concentrations of 9,10,13 triHOME (308% increase) in KSP treated group as compared to that in the controls. In agreement, other authors reported around 2-3 times increase in the plasma 9,10,13 triHOME levels in high dietary linoleic acid fed rats (Taha et al., 2018). Furthermore, 9,10,13 triHOME, metabolite of linoleic acid, is also assumed to be involved in the prostaglandin synthesis. One of the enzymatic stable oxidation products or metabolites of linoleic acid, 13-HODE, is reported to display anti-atherogenic properties through inhibiting the adhesion of platelets to the

endothelium (Wittwer & Hersberger, 2007). The vascular activation and endothelium integrity are maintained through the production of 13-HODE during inflammatory stages (Buchanan, Haas, Lagarde, & Guichardant, 1985; Szklenar, Kalkowski, Stangl, Lorenz, & Rühl, 2013). 13-HODE also acts as a surface ligand of peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) and peroxisome proliferator-activated receptor alpha (PPAR  $\alpha$ ) produced in macrophages (Schild et al., 2002). Additionally, PPAR $\gamma$  transcription factor is involved in the negative regulation of macrophage activation through the inhibition of a potent platelet aggregation molecule, thromboxane, and decreases the expression of pro-inflammatory cytokines (Ricote, Li, Willson, Kelly, & Glass, 1998). Szczukso et al., has suggested the role of 13-HODE in the clearance of the lipid debris from the vascular walls in early atherosclerosis (Szczuko et al., 2020). In agreement with the previous studies, our results also report 13-HODE levels to be significantly elevated by 0.8 folds in KSP treated mice as compared to that in the controls. This alteration in 13-HODE levels seems to be beneficial in the pro-atherosclerotic stages.

NO has a prominent function of vasodilation inside the body which participates in lipolysis in adipose tissues and promotes fatty acid oxidation (Jobgen et al., 2009; Jobgen, Fried, Fu, Meininger, & Wu, 2006). Moreover, NO is also known as a potent antioxidant which helps in reducing the inflammation and thereby, retards atherogenesis in hypercholesterolemic mice (Napoli et al., 2002). While the cross regulatory synthesis pathways of COX-2 and NOS are still controversial, a recent comprehensive review has evidenced the beneficial role of NO in hypertensive, hypercholesterolemic, and diabetic humans and in animal models (Förstermann, Xia, & Li, 2017). Few murine studies have suggested that the higher levels of macrophage COX-2 enzyme, a potent precursor of PGE<sub>2</sub>, is a stimulator of atherogenesis (Hui et al., 2010). Nevertheless, COX-2 in endogenous tissues and vascular smooth muscle cells is suggested to be

atheroprotective in mouse models through biosynthesis of prostacyclin (Tang et al., 2014), while other studies also demonstrate the independent production of prostacyclin without the involvement of COX-2 (Kirkby et al., 2014). The interactive role of COX-2 and NOS pathways is mostly studied in the inflamed cells. The literature shows a diverge and vice-versa cross-talk of both COX and NOS and their regulatory feedback actions upon each other. NO has been suggested to be an initiator of COX-2 synthesis in macrophage cells (Salvemini et al., 1993). Other investigators have found NO as an inhibitor of prostaglandin synthesis in lipopolysaccharide induced macrophages (Habib et al., 1997). Therefore, NO exerts a prominent role in the production and inhibition of constitutive (COX-1) and inducible (COX-2) forms of COX in macrophages among murine models (Clancy et al., 2000). On the other hand, the regulation of proinflammatory cytokines is important by means of which PGE2 cross regulates the NO synthesis via IL10 (Harizi & Gualde, 2006).

Taken together, a double-edged mechanism of action can be established where linoleic acid being a precursor of PGE2 induces the production of IL-10 to promote anti-inflammatory status in KSP treated LDL-r-KO mice bodies; on the other hand, the high amount of plasma citrulline levels in the KSP treated animals, being a precursor of arginine, is presumed to produce endothelial NO and vasodilation in the treated group as compared to those in the control group. Both of these proposed mechanistic approaches justify the significant decrease in the atherosclerotic lesion size in the aortic roots of KSP treated mice as compared to that in the controls (**Fig. 3.5**). The pooling of samples can be considered as one of the limitations of this study. However, the objective of this study was to observe the average changes in the control and treated animals due to the supplementation of KSP in the daily diet. Additionally, this novel study would direct the future studies to analyse the gut microbiome of KSP fed mice, to

investigate the dose dependent relationship between normal and LDL-r-KO mice, to analyse endogenous NO levels in animal models fed with KSP, and to explore the other possible mechanisms of action of KSP supplementation in both genders of mice under different experimental conditions.

### **3.6 Conclusion**

In a nutshell, this study demonstrates that the presence of significantly higher plasma levels of citrulline and PGE2 is presumed to upregulate the endothelial NO production in KSP treated LDL-r-KO mice as compared to those in the control mice. Nonetheless, the significant increase in the plasma IL-10 levels in KSP fed group as compared to that in the controls reduce the inflammatory response and pose anti-atherogenic effects. Both of these proposed mechanisms show *Citrullus lanatus* (Kgengwe) as a novel functional food with extensive cardioprotective properties. However, due to limited plasma volume, the present study could not determine the eNOS expression to generate the exact action of PGE2 and L-arginine in relation to NO production, which will be addressed in our future studies. Our results indicate a significant decrease in the atherosclerotic lesion size in the aortic roots of the treated mice as compared to those in the controls through significant alterations in plasma cytokines, plasma and metabolites, and plasma oxylipin levels. Therefore, 10% (w/w) supplementation of KSP in animal diets prove to be beneficial and suggest *Citrullus lanatus* as an alternate therapeutic agent to lower the CVD risk.



### 3.7 References

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**Table 3.1 Effects of experimental diets on plasma metabolite concentrations at week 20 of the study**

Metabolites (uM)	Experimental Groups		Changes	
	Control (n=5)	KSP-treated (n=5)	fold	%
<i>Amino acids</i>				
Creatinine	7.2 ± 1.5	10.6 ± 2.6*	0.6	↑ 48.3
Glycine	267.4 ± 40.6	354.8 ± 74.5*	0.4	↑ 32.7
Proline	98.1 ± 29.3	200.2 ± 61*	1.0	↑ 104.1
Valine	221.2 ± 15.1	410.4 ± 96.7*	0.9	↑ 85.5
Threonine	126.3 ± 27	236.8 ± 48.9*	0.9	↑ 87.5
Leucine	198 ± 23	412.8 ± 115.8*	1.1	↑ 108.5
Isoleucine	78.9 ± 6.3	146.6 ± 38.1*	0.9	↑ 85.9
Asparagine	31.8 ± 7.4	57.2 ± 13*	0.8	↑ 79.8
Methionine	66.9 ± 11.9	124.8 ± 51.3*	0.9	↑ 86.6
Phenylalanine	81.7 ± 4.9	115.8 ± 27.7*	0.5	↑ 41.7
Arginine	105.2 ± 11.6	162.8 ± 21.3*	0.6	↑ 54.7
Citrulline	63.1 ± 10.7	86.8 ± 6.7*	0.5	↑ 37.6
Tyrosine	94.7 ± 18.5	185.4 ± 106.8*	1.0	↑ 95.7
Tryptophan	46.8 ± 4.9	95 ± 24.2*	1.0	↑ 102.9
Kynurenine	0.8 ± 0.2	1.5 ± 0.4*	0.9	↑ 88.5
Ornithine	57.2 ± 14.9	113.3 ± 38.5*	1.0	↑ 98.1
Lysine	231.4 ± 45.3	323 ± 39.6*	0.5	↑ 39.6
Trimethylamine N-oxide	3.3 ± 0.5	4.8 ± 0.6*	0.5	↑ 45.7

beta-Hydroxybutyric acid	146.4 ± 17.2	359.8 ± 43.2*	1.3	↑ 145.7
alpha-Ketoglutaric acid	26.5 ± 9.4	51.7 ± 5.4*	1.0	↑ 95.1
<b><i>Short-chain fatty acids</i></b>				
Propionic acid	2.5 ± 0.7	4.4 ± 0.8*	0.8	↑ 79.6
Succinic acid	87.2 ± 17.8	15.2 ± 3.7*	-2.5	↓ 82.6
Pyruvic acid	125.3 ± 44.7	188 ± 49.2*	0.6	↑ 50.1
Indole acetic acid	0.2 ± 0.1	0.4 ± 0.1*	0.9	↑ 87.7
<b><i>Phospholipids</i></b>				
LYSOC16:0	219.1 ± 29.2	280 ± 39.4*	0.4	↑ 27.8
LYSOC18:2	151.6 ± 18	209 ± 29.3*	0.5	↑ 37.8
LYSOC18:0	118.4 ± 11.1	152.8 ± 22.8*	0.4	↑ 29

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Values are means ± SD for both treatment groups (Control, n=5; KSP treated, n=5), 2 mice plasma were pooled for each n; \*, p < 0.05 as compared with controls (using the Mann-Whitney test), ↑: Increase; ↓: Decrease.

**Table 3.2 Fecal metabolomics assay of the experimental groups at week 20**

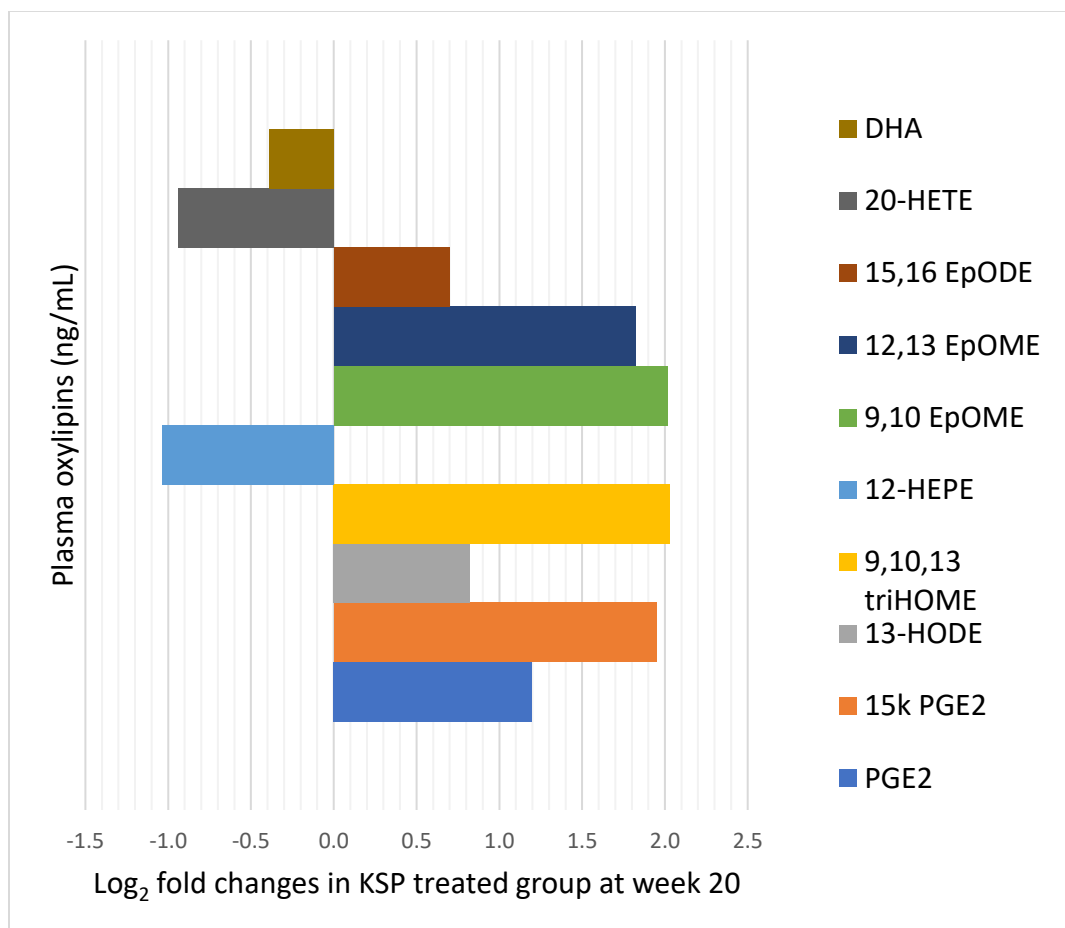
Metabolites (uM)	Experimental Groups		Changes	
	Control (n=5)	KSP-treated (n=5)	fold	%
<i>Amino acids</i>				
Citrulline	75.6 ± 20.3	178.8 ± 85.6*	1.2	↑ 136.5
<i>Short-chain fatty acids</i>				
Butyric acid	149.2 ± 2.3	140 ± 5.6*	-0.1	↓ 6.2
Propionic acid	918.2 ± 85.4	757.8 ± 50.9*	-0.3	↓ 17.5
Propionylcarnitine (C3)	0.11 ± 0.04	0.06 ± 0.004*	-0.8	↓ 43.3
Butenylcarnitine (C4:1)	0.05 ± 0	0.04 ± 0.01*	-0.4	↓ 26.4
Tiglylcarnitine (C5:1)	0.06 ± 0.01	0.05 ± 0.004*	-0.4	↓ 23
Caprylic acid (C8)	0.04 ± 0.04	0.01 ± 0.004*	-1.6	↓ 66.3
<i>Long-chain fatty acids</i>				
Hydroxyhexadecenoylcarnitine (C16:1OH)	0.02 ± 0.001	0.01 ± 0.001*	-0.5	↓ 29.8
Hydroxyhexadecanoylcarnitine (C16OH)	0.024 ± 0.004	0.016 ± 0.001*	-0.6	↓ 32.2
Vaccenic acid (C18:1)	0.02 ± 0.001	0.016 ± 0.002*	-0.2	↓ 15.4
Linoleic acid (C18:2)	0.015 ± 0.002	0.012 ± 0.002*	-0.3	↓ 21.3

Values are means ± SD for both treatment groups (Control, n=5; KSP treated, n=5), 2 mice plasma were pooled for each n; \*, p < 0.05 as compared with controls (using the Mann-Whitney test), ↑: Increase; ↓: Decrease.

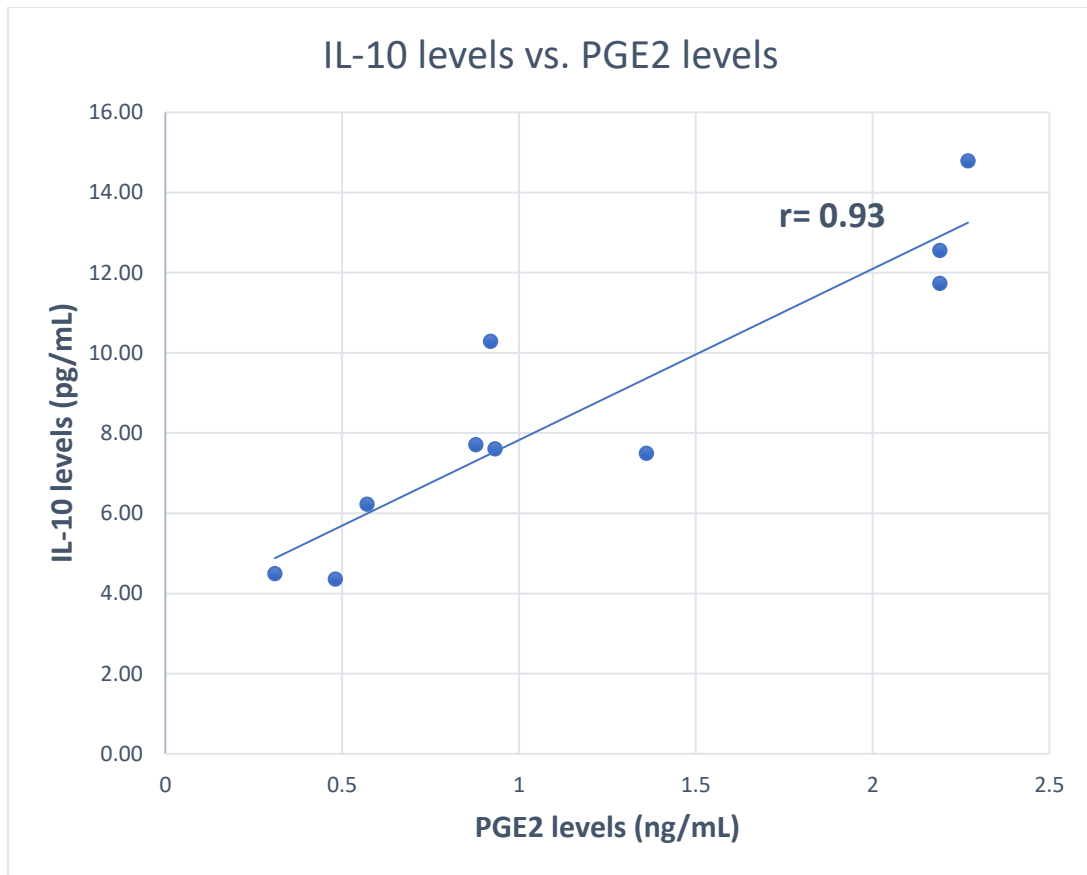
**Table 3.3 Plasma oxylipin profile of control and treated groups at week 20**

<b>Experimental Groups</b>			
<b>Metabolites (uM)</b>	<b>Control (n=5)</b>	<b>KSP-treated (n=5)</b>	<b>Changes (%)</b>
PGE2	0.709 ± 0.2	1.624 ± 0.6*	↑ 129.1
15k PGE2	0.642 ± 0.4	2.478 ± 0.9*	↑ 286.0
13-HODE	69.827 ± 47.4	123.58 ± 32.6*	↑ 77.0
9,10,13 triHOME	2.786 ± 2.3	11.366 ± 1.8*	↑ 308.0
12-HEPE	295.858 ± 98.3	144.027 ± 28.5*	↓ 51.3
9,10 EpOME	0.099 ± 0.1	0.4004 ± 0.1*	↑ 304.4
12,13 EpOME	0.723 ± 0.6	2.557 ± 0.8*	↑ 253.7
15,16 EpODE	5.537 ± 2.5	8.99 ± 1.5*	↑ 62.4
20-HETE	0.4003 ± 0.09	0.209 ± 0.009*	↓ 47.8
DHA	5215.36 ± 587.3	3982.37 ± 522.7*	↓ 23.6

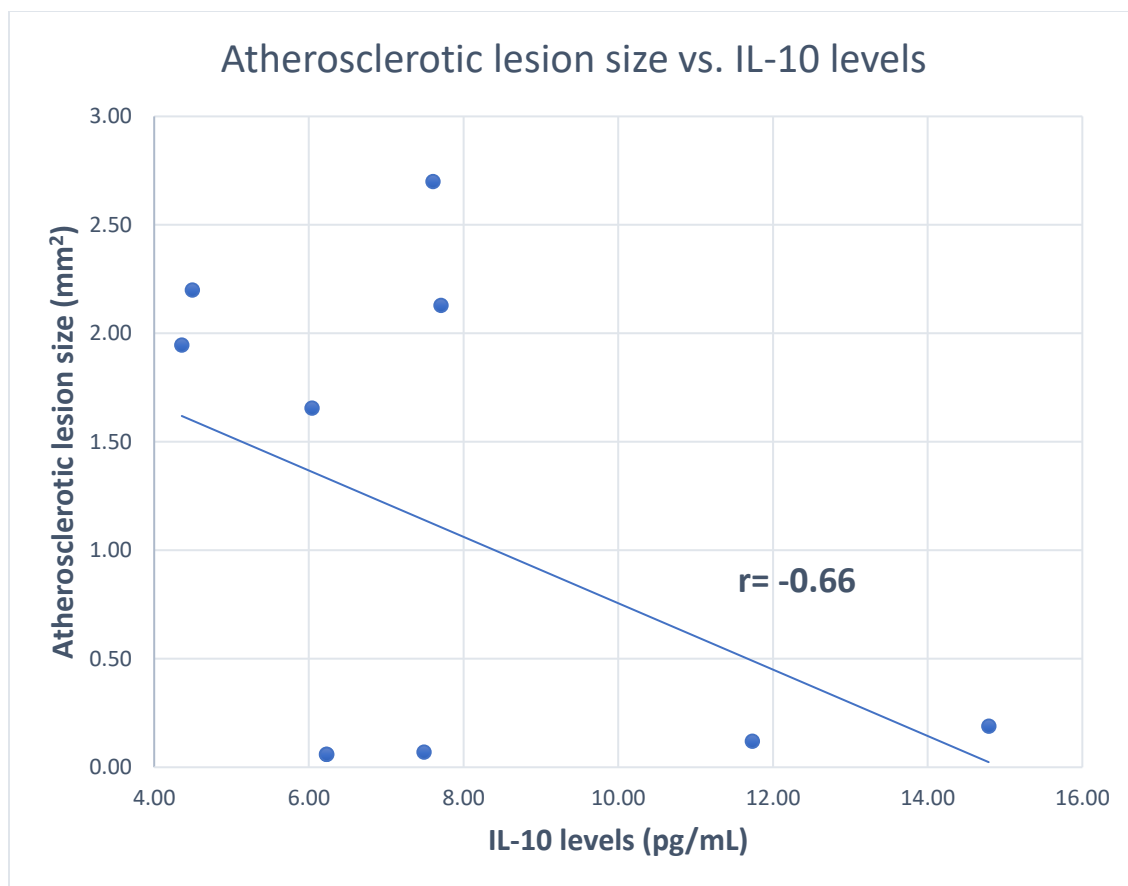
Values are means ± SD for both treatment groups (Control, n=5; KSP treated, n=5), 2 mice plasma were pooled for each n; \*, p < 0.05 as compared with controls (using the Mann-Whitney test), ↑: Increase; ↓: Decrease.



**Figure 3.1 Plasma oxylipins log<sub>2</sub> fold changes in KSP treated group at week 20**

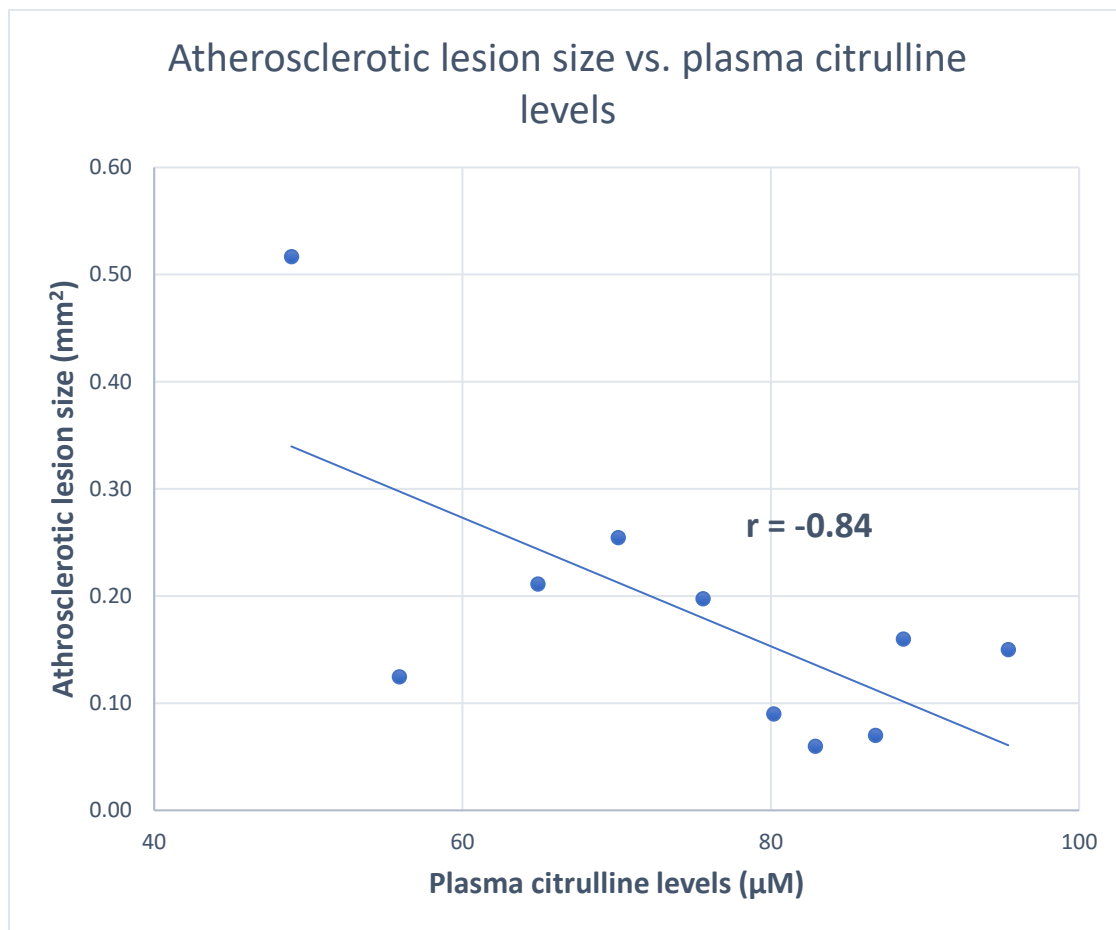


**Figure 3.2 Relation between plasma IL-10 and PGE2 levels**

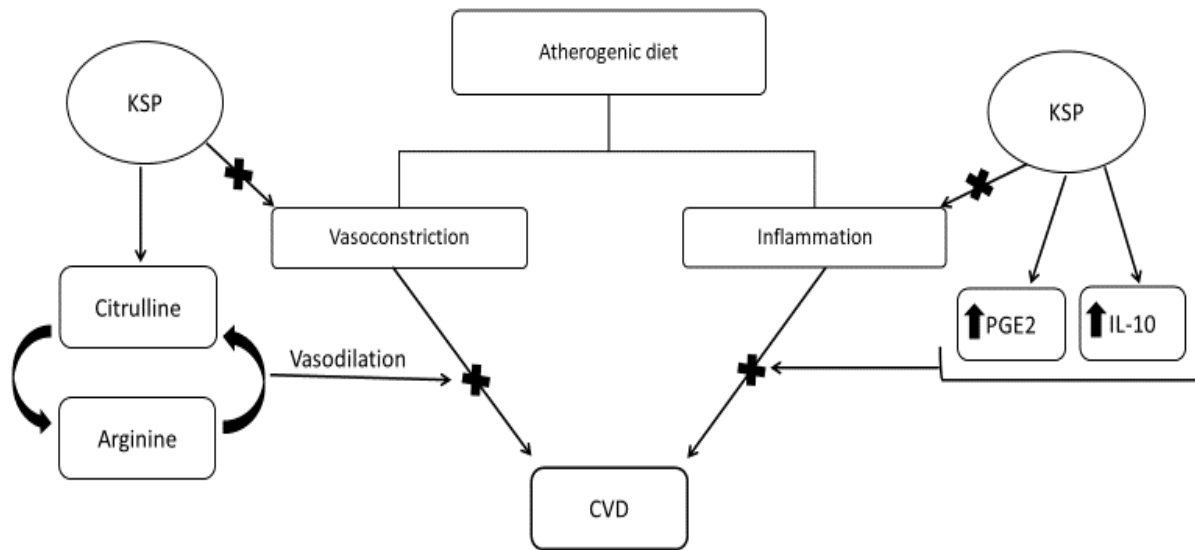


**Figure 3.3 Relation between atherosclerotic lesion size and plasma IL-10 levels in experimental mice at week 20**





**Figure 3.4 Relation between atherosclerotic lesion size and plasma citrulline levels in experimental mice at week 20**



**Figure 3.5 Possible mechanism of action through interplay of citrulline, IL-10, and PGE2**

## Chapter IV

### GENERAL DISCUSSION AND CONCLUSION

#### 4.1 General discussion and conclusion

Inflammation is a key mechanism in the progression of atherosclerotic disease (Golia et al., 2014). Accumulated scientific literature reports controversial statement to justify the specific ratio of omega-3: omega-6 PUFA (Simopoulos, 2002). The present study demonstrated KSP to be rich in linoleic acid and almost void of linolenic acid. Data from Chapter II revealed that KSP supplementation showed anti-atherogenic effects in LDL-r-KO mice evincing a significant ( $p < 0.05$ ) decrease (58%) in the size of atherosclerotic lesion in the aortic roots of treated group as compared to that in the control group. However, unlike the previous reports, the present study did not show any significant changes in the conventional risk markers of CVD such as plasma TC levels. Other parameters of experimental mice including body weight, food intake, and organ weight did not display any significant alterations between the groups. It is also suggested that the significant ( $p < 0.05$ ) increase in the plasma cytokines such as IL-10 exhibited the anti-inflammatory action in treated mice and prevented the development of the atherosclerotic lesion in the aortic roots. Albeit not a direct evidence, the beneficial significant alterations in the plasma cytokine levels is reported as the possible mechanism of action to deduce such effects. Nevertheless, it is critical to understand the association between higher levels of linoleic acid in KSP and anti-atherosclerotic effects.

Previous studies on conventional watermelon also concluded that citrulline content present in the Citrulline lanatus improved the lipid profile and inflammation through the

availability of endogenous NO in the experimental animals (Hong, Beidler, Hooshmand, Figueroa, & Kern, 2018; Mandel, Levy, Izkovitch, & Korman, 2005). Furthermore, it is suggested that the metabolites of linoleic acid such as 13-HODE and 9,10,13 triHOME promoted the inhibition of platelets to the endothelium in inflammatory state (Melissa Gabbs, Leng, Devassy, Monirujjaman, & Aukema, 2015), and therefore, support the clearance of lipids from the vessels (Szczuko et al., 2020). Data from Chapter III concluded that plasma metabolites such as citrulline, arginine, and other plasma amino acids were significantly ( $p < 0.05$ ) higher in the treated group as compared to that in controls. Moreover, the plasma oxylipins including PGE<sub>2</sub>, 13-HODE and 9,10,13 triHOME revealed approximately one-fold upsurge in KSP treated mice in comparison to the controls. Therefore, it is presumed that in the absence of other PUFA, linoleic acid in the KSP supplemented diets promoted the alterations in the plasma oxylipin levels, which further exhibited altered plasma inflammatory concentrations and barred the progression of atherosclerosis. On the other hand, KSP being a rich source of citrulline, significantly ( $p < 0.05$ ) improved plasma levels of NO precursor, arginine, in treated mice which may contribute to the atheroprotective effects of *Citrullus lanatus*. Furthermore, the preliminary data from these studies support the desirable cardioprotective effect of KSP supplementation in the study animal diets through decreased atherosclerotic lesion size and improved inflammatory status. Hence, Kgengwe melon seeds may prove to be an alternate sustainable food both in developing and developed countries.

## 4.2 Strengths and limitations

### Strengths

#### a) Study time period

The chief strength of this study is the length of 20 weeks experimental period; which was satisfactory to analyze the proposed risk factors and effect of the experimental diets on the animal models. The extended study period also allowed to determine the long-term effects of the consumption of Kgengwe seeds supplemented diets.

#### b) Animal model

LDL-r-KO mice is the most widely used animal models to study atherosclerosis and dyslipidemia (Getz & Reardon, 2012). Because the genetic disorder, familial hypercholesterolemia, and lipoprotein profile of this specific animal model imitate with that of humans (Zadelaar et al., 2007), so this mouse model is an excellent model for experimental atherosclerosis research.

#### c) Plasma cytokines, oxylipins, and plasma and fecal metabolites analysis

The insignificant changes in the plasma TC and TG levels in KSP treated group betrothed this study to explore other inflammatory markers and fatty acid metabolites. The mechanistic approach of KSP in the experimental mice is deduced through the significant beneficial alterations in the plasma cytokine levels, plasma oxylipin concentrations, and plasma and fecal metabolite levels. The significant changes in these markers in KSP treated mice founded the base of this study and directed to explore the anti-atherogenic action of KSP supplementation other than just focusing on the conventional CVD risk markers such as plasma lipid profile.

## Limitations

### a) Gender differences

Sex differences of mice can also be well-thought-out to mention while inferring the anti-atherogenic properties in mice. The current study was designed such to examine the anti-atherosclerotic properties of KSP in male LDL-r-KO mice, whereas, the female mice are less susceptible to develop severe atherosclerotic lesion than male mice as studied previously (Bentzon & Falk, 2010). Furthermore, the endogenous NOS expression in female mice are higher than male mice (Just & DeLorey, 2017) and estrogen hormone in females enhances the NOS expression and NO production in endogenous cells (Kleinert et al., 1998), which make female mice lesser prone to CVD risk.

### b) Animal sample size

Though the total number of mice in the present study is twenty and distributed  $n=10$  in each group namely, control and KSP treated group. The sample size of the animal models chosen limited the collection of the total amount of the plasma due to which the plasma samples were pooled in the analysis of plasma cytokines, metabolites, and oxylipins.

### c) Absence of wild type controls

The presence of C57BL/6 wild type mice as controls could eliminate the genetic variations in the present study analysis which might happened due to LDL-r-KO mouse models.

### 4.3 Future research

Future studies on the consumption of Kgengwe melon and its anti-atherogenic effects through underlying mechanisms of actions in different animal models are warranted. The potential area of research could be the investigation of the metabolism of L-citrulline from Kgengwe flesh, rind, and seeds and to analyse the cardioprotective properties of the whole melon consumption. Other functional components of Kgengwe melon can also be analysed including the presence of dietary fibre and different polyphenols to propose other health effects. Other than the aforementioned, both genders of the animal model should be included in the future studies to examine the KSP supplementation effects in daily diet.

Furthermore, the dose-response studies are also beneficial to determine the functional dose of the KSP supplementation to justify its anti-atherogenic effects. Nevertheless, the KSP should also be investigated for the presence of any toxins of its origin and its safe consumption in animal models before introducing this novel food into clinical setting. Determination of high sensitivity C-reactive protein (hs-CRP) and plasma COX concentrations may also be an essential step to confirm the inflammatory status of the body in relation to atherogenesis. This study did not present any significant changes in the plasma TC and TG levels in LDL-r-KO mice while displaying significantly lower atherosclerotic lesion size in the aortic roots of the treated mice as compared to that in the controls, thus, the full lipid profile including the HDL and LDL-cholesterol levels, and HMG-CoA reductase activities may be examined in the imminent studies.

Despite of high concentrations of linoleic acid, the nutritional composition of *Citrullus lanatus* seeds revealed high amounts of crude fiber (40%). Therefore, it would be interesting to recognise the alterations in the gut microbiome and its effects on the fecal and plasma

metabolites due to Kgengwe seeds consumption to better understand the significant changes in CVD risk markers. Lastly, the investigation of the parent component of the prostaglandins would reason the altered oxylin profile especially, this would cross confirm the overexpression of IL-10 levels. Exploration of the significant changes in the levels of other plasma cytokines such as GM-CSF, EPO, and MCP-1 in different animal models and clinical studies may provide confirmatory justification of atheroprotective potential of the novel Kgengwe melon.



#### **4.4 Implications in the field of nutrition**

Major findings of this study may contribute to the body of the research of this unexplored fruit on the cardiovascular risk markers. Results obtained from this study would also provide additional evidence for the anti-atherogenic properties of Kgengwe seeds. Moreover, this study would also promote the supplementation of wild watermelon seeds in the daily diet as a functional food, and the development of an effective food product from this novel wild watermelon. Lastly, the data may encourage the growth of this affordable crop both in the developing and developed countries. Being a cheap and abundant food source, Kgengwe would not only fulfill the nutritional needs of the indigenous populations but positively impact the health and agricultural sectors worldwide.

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