THE ANTI-INFLAMMATORY EFFECTS OF WHEAT GERM OIL ON LIPOPOLYSACCHARIDE-ACTIVATED HUMAN MONOCYTIC THP-1 CELLS

By

SAWANYA JANTHACHOTIKUN

Bachelor of Science in Food Technology

Prince of Songkla University

Songkhla, Thailand

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Thesis Approved:

Dr. Edralin Lucas

Thesis Adviser

Dr. Barbara Stoecker

Dr. Brenda Smith

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Abstract: Heart disease is the leading cause of death, and the underlying pathological feature is atherosclerosis. Atherosclerosis is an inflammatory disease involving immune cells with macrophages as key agents in the disease progression by secreting cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-10. Dietary bioactive components are being investigated for their health benefits including anti-inflammatory properties. Wheat germ oil (WGO) is rich in omega-3 fatty acid, vitamin E and sterols which have been reported to have anti-inflammatory properties. The present study investigated the anti-inflammatory effects of four WGOs (WGO 1, WGO 2, WGO 3 and WGO 4) prepared using different extraction methods on cytokine production in lipopolysaccharide (LPS)-activated human monocytic THP-1 cells. THP-1 cells were treated with low or high dose (0.25 and 1.5×10^{-3} %) of each WGOs in combination with LPS (1 µg/mL) for 6 h. None of the WGOs tested induced cell death or had negative effects on cell proliferation. At high dose, all WGOs significantly reduced IL-6 ($P = 0.0351$) but only WGO 1 - 3 significantly decreased TNF-α production. Additionally, both doses of WGO 3 and WGO 4 increased IL-10 production, an anti-inflammatory cytokine. High doses of WGOs also significantly down-regulated TNF-α and IL-6 gene expression. Moreover, WGO 3 and 4 at high dose increased IL-10 gene expression. These findings suggest that WGOs affect both pro- and anti-inflammatory cytokines involved in the pathogenesis of atherosclerosis and that the method of extraction influences its anti-inflammatory properties.

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CHAPTER I

INTRODUCTION

Heart disease is the leading cause of death in both men and women in the U.S. (Centers for Disease Control and Prevention (CDC), 2015). Each year, about 610,000 Americans die from heart disease which accounts for one in every four deaths (CDC, 2015). The overall costs of heart disease, both direct and indirect costs, are more than $320.1 billion each year (CDC, 2015). Coronary heart disease (CHD) is the most common type of heart disease which kills more than 385,000 people annually and costing $108.9 billion each year in the U.S (Murphy, Xu, & Kochanek, 2012). CHD is a consequence of the buildup of plaque in coronary arteries, a condition called atherosclerosis (CDC, 2015).

Atherosclerosis is a chronic progressive condition that continuously narrows and hardens the arteries throughout the body. When atherosclerosis affects the coronary arteries, it causes CHD (Smith Jr et al., 2006). Atherosclerosis develops over many years before any clinical symptoms are observed and can occur at different ages in different populations. It is estimated that 1.7 % or 4.6 million Americans have atherosclerosis (Smith Jr et al., 2006). Atherosclerosis can slowly develop throughout the life span, with inflammation playing an important role in the initiation and progression of the disease (Libby, Ridker, & Maseri, 2002). From its early to advanced stages, the atherogenic process involves a series of immune cells (e.g., monocytes, macrophages, T cells), inflammatory proteins (e.g., cytokines, chemokines), and inflammatory

responses from vascular cells (i.e., endothelium expression of adhesion molecules) (Plutzky, 2001). Under normal conditions, leukocytes, such as monocytes and T cells, adhere poorly to the normal endothelium. However, continuous elevation of low density lipoprotein (LDL)-cholesterol in the blood cause it to be up taken into the endothelium and become oxidized, where the inflammation in atherosclerosis begins (Packard & Libby, 2008).

Oxidized LDL particles, adipokines and pro-inflammatory cytokines stimulate endothelial cells to express on their surface adhesion molecules, such as P- and E-selectin, intercellular adhesion molecules (ICAM) and vascular cell adhesion molecule (VCAM), which act as receptors of monocytes and T-cells (Kaperonis, Liapis, Kakisis, Dimitroulis, & Papavassiliou, 2006). These molecules recruit monocytes circulating in the blood to attach to the vessel wall. Monocytes then migrate into the intima and begin to differentiate into macrophages which start to express scavenger receptors on their surface to increase oxidized LDL uptake. LDL-loaded macrophages then become foam cells which up-regulate the inflammatory response through the secretion of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, and interleukin (IL)-1 and -6 (Packard & Libby, 2008). Retention of macrophages along with activated T-cells and endothelial cells heighten the production of pro-inflammatory cytokines, resulting in chronic inflammation and development of atherosclerosis (Plutzky, 2001).

TNF-α is an important pro-inflammatory cytokine that plays a key role in the progression of inflammation in atherosclerosis (Libby et al., 2002). It inhibits lipoprotein lipase which is needed to lower systemic LDL levels and elevated triglyceride, leading to more LDL particles taken up into the endothelium (Hansson, 2001). TNF-α also stimulates the expression of adhesion molecules (ICAM-1) in endothelial cells, resulting in increased monocyte migration and foam cell formation (Hansson, 2001). The expression of other pro-inflammation cytokines, including IL-6, in atherosclerosis is also induced by TNF-α, leading to chronic inflammation (Z. Wang, Castresana, & Newman, 2001). The production and gene expression of TNF-α are found

to be controlled by nuclear factor-kappa B (NF-κB), the transcription factor (Z. Wang et al., 2001). The important roles of TNF-α in atherosclerosis make it a therapeutic target with a focus on inhibition of the NF-κB pathway.

IL-6 is another pro-inflammatory cytokine secreted by macrophages that plays a vital role in atherosclerosis (Libby et al., 2002). It stimulates production of hepatic C-reactive protein (CRP), an inflammatory marker, which is an integrator for several inflammatory stimuli and mediates phagocytosis, resulting in promotion of inflammation (Ait-Oufella, Taleb, Mallat, & Tedgui, 2011). Administration of IL-6 to apolipoprotein E (apoE) knockout (KO) mice, a model of atherosclerosis, significantly increased atherosclerotic lesions, indicating a vital role of IL-6 in plaque development (Huber, Sakkinen, Conze, Hardin, & Tracy, 1999). Therefore, suppression of IL-6 activity may lead to reduction of inflammation in atherosclerosis.

The inflammatory response appears to be suppressed by anti-inflammatory cytokines (Mallat, Heymes, et al., 1999). In atherosclerosis, IL-10 is an anti-inflammatory cytokine secreted mainly by monocytes/macrophages in order to suppress the pro-inflammatory cytokines, including TNF-α and IL-6 (Mallat, Heymes, et al., 1999; Stenvinkel et al., 2005). IL-10 was found to inhibit NF-κB activation which is required for production and gene expression of pro-inflammatory cytokines involved in atherosclerosis (Monaco et al., 2004). It also decreases chemotactic factors, such as IL-8, which attract more leukocytes to the inflammatory site (Stenvinkel et al., 2005). Therefore, increased IL-10 production may be another therapeutic target to suppress inflammation in atherosclerosis.

The involvement of inflammation via the action of cytokines secreted by monocytes/macrophages in atherosclerosis has spurred interest in reducing the inflammatory response for atherosclerosis prevention and treatment. Additionally, there is also an increased

interest in macrophages because they not only act as lipid scavenger cells, but also as immunocompetent cells with a profound impact on the make-up of atherosclerotic plaques.

Various options are available to delay the progression and treat atherosclerosis, including pharmacological options, lifestyle changes, and surgical procedures (Taylor, Sullenberger, Lee, Lee, & Grace, 2004). Pharmaceutical options such as statins, warfarin and angiotensin-converting enzyme (ACE) inhibitors, are available that can slow the progression of atherosclerosis by lowering cholesterol level, blood pressure or thrombus, respectively (X.-Q. Zhao et al., 2004). Surgical procedures used to treat atherosclerosis include angioplasty and coronary artery bypass grafting (Hobson et al., 2008). Side effects for the use of drugs include stomach upset, joint pain, liver damage, and headache, and surgical procedures are generally very costly (X.-Q. Zhao et al., 2004). Lifestyle changes, including switching to healthy eating, becoming more physically active, maintaining a healthy weight, managing stress and smoking cessation, play a key role in reducing risk factors for atherosclerosis (Dod et al., 2010).

Diet plays a key role in the progression of atherosclerosis (Glueck, 1979). High intake of simple carbohydrates and saturated fat are atherogenic, and contribute to the risk for obesity, a risk factor for atherosclerosis (Glueck, 1979). However, plant-based diets with plenty of fruits, vegetables, beans and whole grains have been shown to reduce inflammation (Watzl, 2008). Plant-based diets are rich in bioactive compounds, including polyphenols and antioxidants, which have been demonstrated to have anti-inflammatory effects (Watzl, 2008). Therefore, dietary modifications, such as increased consumption of a plant-based diet, are subject to increasing research for their potential to delay the development of atherosclerosis.

Polyphenols and antioxidants with anti-inflammatory activities are found in a variety of plants, including whole grains (Kris-Etherton et al., 2004). Wheat is a grain that is widely consumed and is known as a good source of polyunsaturated fatty acids, vitamins, minerals, and

phenolic compounds (Atwell, 2001). Although whole wheat contains significant amounts of nutrients and bioactive compounds, the most nutrient-dense part of the wheat plant is the germ (Brandolini & Hidalgo, 2012). Wheat germ is removed from the wheat during refining process and is an important by-product of flour production (Brandolini & Hidalgo, 2012). In the past, the utilization of wheat germ was limited, and it was typically used to feed animals (Mahmoud, Mohdaly, & Elneairy, 2015). Wheat germ has a high amount of oil which is rich in bioactive compounds, including omega-3 fatty acid (alpha-linolenic acid, ALA), tocopherols, policosanols and sterols (Eisenmenger & Dunford, 2008; Mahmoud et al., 2015). This fact has increased researchers' interest to investigate the potential health benefits of wheat germ oil (WGO), including its anti-inflammatory properties.

Individual components (i.e., omega-3 fatty acids, vitamin E, policosanols and sterols) found in WGO have been reported to have a variety of pharmacological effects, including anti-inflammatory properties (Consolazio, Matoush, Nelson, Isaac, & Hursh, 1964; Cureton & Pohnoorf, 1955; Hussein, Abdel-Aal, & Elghwab; Khalifa, Khalil, Barakat, & Hassan, 2011; Watzl, 2008; Yuldasheva, Ul'chenko, & Glushenkova, 2010). ALA (found in high concentrations in WGO) was reported to suppress the production and gene expression of pro-inflammatory cytokines (TNFα, IL-1β and IL-6) (Zhao et al., 2005) and eicosanoids (Mori & Beilin, 2004). The most bioactive form of tocopherols, α-tocopherol, was also found to have an anti-inflammatory properties by inhibiting phospholipase A_2 and suppressing production of TNF-α and IL-6 in differentiated THP-1 cells (Chandra et al., 2002; Guo, Zingg, Meydani, & Azzi, 2007). WGO is also known as a rich source of policosanols (Eisenmenger & Dunford, 2008). Policosanols, a mixture of aliphatic alcohols, are known for their cholesterol-lowering effects and have been used to prevent atherosclerosis by reducing total LDL cholesterol levels (Eisenmenger & Dunford, 2008; Marinangeli, Jones, Kassis, & Eskin, 2010). Additionally, policosanols have been shown to reduce atheroma formation by decreasing platelet aggregation and foam cell

formation in animals models of cardiovascular disease (Varady, Wang, & Jones, 2003). Although there are no studies that have reported the anti-inflammatory property of policosanols, their potential to reduce LDL cholesterol and foam cell formation may indirectly reflect its ability to reduce inflammation. Dietary phytosterols have also been reported to have an anti-inflammatory effect by decreasing TNF-α and IL-6 and increasing IL-10 in apoE KO mice (Nashed, Yeganeh, HayGlass, & Moghadasian, 2005). These findings of anti-inflammatory properties exhibited by bioactive compounds found in WGO may reflect the potential of WGO as an anti-inflammatory agent.

Extraction methods of WGO have been reported to influence the composition and amount of bioactive compounds (Eisenmenger & Dunford, 2008; Piras et al., 2009; Taniguchi, Tsuji, Shibata, & Kobayashi, 1985). WGO processed by solvent extraction methods using supercritical carbon dioxide (SC-CO_2), has been reported to have higher α-tocopherol content than that of hexane extracted WGO (Eisenmenger & Dunford, 2008; Shao, Sun, & Ying, 2008). However, extraction methods and refining have no effect on fatty acid compositions (Eisenmenger & Dunford, 2008; Shao et al., 2008). Oil refining was also shown to decrease amounts of tocopherols significantly during deodorization (T. Wang & Johnson, 2001). The studies discussed above demonstrate that processing influenced the content of bioactive compounds of WGO and in turn, its pharmacological properties.

Even though there are studies showing that the individual components (i.e. ALA, α-tocopherol and policosanols) of WGO have anti-inflammatory activities, there is limited research investigating anti-inflammatory effects of WGO itself. Additionally, how WGO has been processed can influence the amount of these bioactive compounds. Therefore, this study will investigate the anti-inflammatory effect of WGOs prepared using different extraction methods.

Wheat is a primary crop grown in Oklahoma (Oklahoma Wheat Commission (OWC), 2015). Each year, Oklahoma farmers grow approximately 6 million acres of winter wheat, making it the most important crop ("Wheat Facts,"n.d.). In 2003, Oklahoma ranked second nationally in the production of winter wheat which was worth $443 million to the Oklahoma economy ("Wheat Facts," n.d.). Since wheat germ is a major by-product of the flour milling industry with limited use, research investigating the anti-inflammatory properties of WGOs will provide wheat growers another potential market for their product.

The ***specific aims*** of this study are to (a) determine the anti-inflammatory effect of WGOs extracted using different processes and (b) compare the dose-dependent effects of the WGOs in inhibiting pro-inflammatory and increasing anti-inflammatory genes and cytokine production in LPS-stimulated THP-1 cells. We ***hypothesize*** that high dose of WGO extracted with supercritical CO_2 will exhibit the most potent anti-inflammatory effects. This study capitalized on an *in vitro* approach using THP-1 monocytes to investigate the effects of four differentially extracted WGOs.

There are a few limitations of this study:

1) This study used an *in vitro* approach. Therefore, the findings may not be able to apply directly to *in vivo* models. Animal and clinical research is needed to confirm the findings of this study.

2) Although the literature supports the anti-inflammatory properties of bioactive compounds found in WGO, we cannot identify which of those compound(s) is responsible for the anti-inflammatory effect of WGO observed in this study. Other components present in WGO that are not reported in the literature may possibly also exert anti-inflammatory effects.

3) Human monocytic THP-1 cells are suspension cells. During the assessment of cell proliferation (using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay), we cannot aspirate WGO at the end of treatment period without losing a significant amount of

cells. Therefore, WGOs were incubated with the MTT reagents which may affect the results of this assay.

4) In this study, the high dose of WGOs was prepared from a stock solution (dissolved in ethanol) to give a final ethanol (vehicle) concentration of 0.01%, the highest concentration of ethanol that will not cause cytotoxicity. Control was also prepared to have the same ethanol concentration as the high dose WGO. The low dose WGOs was also prepared from the same stock solution and had a lower concentration of ethanol. We did not prepare another control with the same concentration of ethanol as the low dose WGO. Therefore, all comparisons were done with the control containing 0.01% ethanol which may not be the most accurate when comparing the effects of low dose WGO.

CHAPTER II

REVIEW OF LITERATURE

Many studies have investigated the anti-inflammatory properties of nutrients and non-nutritive bioactive constituents found in plant-based diets. Although the literature covers a wide variety of food components showing such properties, this review will discuss literature relevant to the present study. This review of literature is divided into six sections: 1) heart disease statistics, 2) pathophysiology of atherosclerosis, 3) treatment options for atherosclerosis, 4) atherosclerosis and diet, 5) wheat, and 6) bioactive compounds in WGO and their anti-inflammatory properties.

I. Heart disease statistics

Heart disease is the leading cause of death in the U.S. (CDC, 2015). Every year, about 610,000 people die of heart disease, which accounts for 1 in every 4 deaths (CDC, 2015). Heart disease is also the leading cause of death in most racial and ethnic populations in the U.S, including African Americans, American Indians, Asians, Hispanics, and Whites (Go et al., 2013). In the U.S., the highest death rate due to heart disease was found in the South while the lowest rate was in the West during 2008-2010 (CDC, 2015). Coronary heart disease (CHD) is the most common type of heart disease and kills more than 385,000 people annually in the U.S (Go et al., 2013). The incidence rate of CHD tends to increase with age. The greatest prevalence was observed among individual ≥ 65 years old, followed by those aged 45-64 years (Berry et al., 2012). The CHD prevalence among ethnicities differs with highest rate in American Indians,

followed by African American, Whites, Hispanics, and Asians (Heron, 2012). CHD is a consequence of atherosclerosis, the hardening of coronary arteries (CDC, 2015). Atherosclerosis, also known as atherosclerotic cardiovascular disease (ASCVD), is considered a serious health condition which leads to other causes of CVD death. It is estimated that costs associated with atherosclerosis will rise from $84.8 billion to $202 billion between 2015 and 2030 (Fleg et al., 2013).

Atherosclerosis is a slow, complex disease that can start in childhood and continuously progresses over time. The extent and severity of atherosclerosis in youth can predict risk of clinically manifested CHD. Strong et al. (1999) investigated atherosclerosis severity in US adolescents and young adults. The authors reported that in the youngest group (15-19 years), intimal lesions were observed in all aortas and most of the right coronary arteries and the highest prevalence were found in the oldest age group (30-34 years) (Strong et al., 1999). These results demonstrated that the onset of atherosclerosis begins in childhood or adolescence. Age consistently produced the strongest association with atherosclerosis. According to Webber and colleagues (2012), subjects with atherosclerosis were about 5 years older than those without this condition. Also, subjects aged 40 years old and older had the higher prevalence of disease (45.9 %) as compared with those aged 24 years old and younger (6.6 %) (Webber, Seguin, Burnett, Clark, & Otto, 2012).

II. Pathophysiology of atherosclerosis

Atherosclerosis is the underlying cause of CHD. Understanding atherosclerosis provides ideas for strategies to prevent or reverse this disease. Atherosclerosis may begin in the early stage of life (before age 10) with thin, fatty streaks present in the intima of coronary arteries (McGill et al., 2000). In the early stage of the disease, fatty streaks do not block blood flow; however, these fatty streaks develop into thickened and hardened fibrous plaques over time (McGill et al., 2000).

Atherosclerosis occurs when LDL particles in the blood enter the arterial intima and become oxidized. These oxidized LDL cause an inflammatory condition within the intima (Insull Jr, 2009). On their surface, endothelial cells begin to express adhesion molecules (selectins, intercellular adhesion molecules (ICAMs), and vascular cell adhesion molecule (VCAMs)) which serve as receptors for integrins and glycoconjugates on the surface of monocytes and T-cells (Insull Jr, 2009). VCAM-1 then binds monocytes and T lymphocytes. After their attachment to the endothelium, monocytes and T-cells migrate into the intima, with the help of several chemoattractant molecules, including monocyte chemoattractant protein-1 (MCP-1) which is responsible for monocytes migration (Kaperonis et al., 2006). Monocytes migrate into the inflamed site and become macrophages. Monocyte-derived macrophages serve as scavenger cells and producers of cytokines, including TNF-α and IL-6, which are responsible for the progression of atherosclerosis (Linton & Fazio, 2003). Macrophages take up oxidized LDL and become foam cells, which in turn further promote inflammation by secreting pro-inflammatory mediators which up-regulate adhesion molecules on the surface of endothelial cells (Plutzky, 2004).

Accumulation of foam cells leads to the formation of fatty streaks which then expand into the vessel wall (Libby et al., 2002). In the fatty-streak lesion, foam cells secrete several molecules, including cytokines (i.e. TNF-α and IL-6) that can stimulate the migration and proliferation of smooth muscle cells (SMC) in the intima (Libby, Ridker, & Hansson, 2011). In response to inflammatory stimulation, SMCs then release enzymes to degrade elastin and collagen and secrete factors to recruit more monocytes (Libby et al., 2011). The macrophage-lipid contents of the plaque also produce pro-inflammatory mediators (Libby et al., 2011). Pro-inflammatory cytokines, including IL-1, TNF-α and CRP stimulate the expression of adhesion molecules, which regulate the attachment of the monocytes to the endothelium (Libby et al., 2011). Gradually, an advanced atherosclerotic lesion, called plaque, is formed, with the

characteristics of lipid and necrotic core covered by a thin fibrous cap (Libby et al., 2011). Apoptosis of SMC is responsible for calcification which causes the plaques to become hardened and inflexible which reduces the ability of the artery to increase blood flow when needed (Libby et al., 2011). This process continuously repeats, resulting in the development of plaque in the coronary arteries (Insull Jr, 2009). As the plaques grow in the artery opening, the blood is squeezed through a smaller gap, resulting in increased pressure to the narrowed lumen. This causes damage to the caps covering the plaque, resulting in plaque rupture which causes thrombosis, a formation of a blood clot which can completely block the artery (Insull Jr, 2009).

A. Role of TNF-α in atherosclerosis

TNF-α secreted from monocytes/macrophages is a "multifunctional pro-inflammatory cytokine" that plays an important role in the pathogenesis of atherosclerosis due to its varied roles in the inflammatory process (Kleinbongard, Heusch, & Schulz, 2010). The secretion of TNF-α from monocytes/macrophages is stimulated by oxidized-LDL as well as interferon gamma (IFN-γ), a pro-inflammatory cytokine produced by activated T-cells, in a dose-dependent manner. Once released, TNF-α induces the expression of acyl-CoA-cholesterol transferase 1 through NF-κB activation to increase the uptake of LDL cholesterol by differentiating monocytes, resulting in the accumulation of foam cells or fatty streaks (Kleinbongard et al., 2010). TNF-α was also found to up-regulate production and gene expression of scavenger receptors for oxidized LDL, a scavenger-receptor A (SRA) and lectin-like oxidized LDL receptor-1, through NF-κB signaling in differentiated THP-1 cells (Hashizume & Mihara, 2012).

TNF-α was also reported to be involved in plaque development. Ohta et al. (2005) investigated the role of TNF-α in apolipoprotein E (apo E) KO and TNF-α/apoE double KO mice. The authors found that the area of atherosclerotic plaque in the aortic luminal surface and the aortic sinus of apoE/TNF-α double KO mice was significantly smaller than that of apoE KO mice while the serum cholesterol levels in both groups were similar (Ohta et al., 2005). The gene

expressions of adhesion molecules (ICAM-1, VCAM-1 and MCP-1) and SRA, a receptor recognizing oxidized-LDL on macrophages, were found to be higher in apoE KO mice than in apoE/TNF-α double KO mice. These findings demonstrated that TNF-α plays an atherogenic role by up-regulating monocytes infiltration and uptake of oxidized-LDL by macrophages (Ohta et al., 2005). Xiao and colleagues (2009) reported similar results in that fatty streak lesions in apoE KO mice were more prominent than in apoE/TNF-α double KO mice. Other atherogenic factors, IL-1β, MCP-1 and NF-κB, were also found to be reduced in apoE/TNF-α double KO mice (Xiao et al., 2009). TNF-α also continuously stimulates inflammatory responses by increasing the production of IL-6 and CRP as well as monocyte infiltration by up-regulating the adhesion molecules (ICAM-1 and VCAM-1) (Bruunsgaard, Skinhøj, Pedersen, Schroll, & Pedersen, 2000). These findings demonstrated that TNF-α plays a key role in the progression of atherosclerosis and should be considered a target of prevention and treatment for atherosclerosis.

B. Role of IL-6 in atherosclerosis

IL-6 is produced by a variety of cell types, including monocytes/macrophages (Schuett, Luchtefeld, Grothusen, Grote, & Schieffer, 2009). Under normal condition, the level of IL-6 is relatively low; however, during an inflammatory stage of atherosclerosis, the expression of IL-6 is increased in monocytes and macrophages through the induction of several stimuli, including TNF-α (Schuett et al., 2009). A study in rabbits reported that in the atherosclerotic arteries, IL-6 gene expression was 40 fold higher than in non-atherosclerotic arteries, indicating the involvement of IL-6 in atherosclerosis (Seino et al., 1994). IL-6 plays a vital role in regulation of the acute phase response and is a major mediator of hepatic CRP, an inflammatory marker (Song & Schindler, 2004). IL-6 was also found to be associated with foam cell formation by increasing the expression of scavenger receptors and adhesion molecule (Hashizume & Mihara, 2012). An increase of production and gene expression of scavenger-receptor A (SRA), a receptor for

oxidized-LDL, was found in IL-6 stimulated THP-1 macrophages, indicating the role of IL-6 in foam cell formation (Hashizume & Mihara, 2012).

C. Role of IL-10 in atherosclerosis

IL-10 is an anti-inflammatory cytokine secreted mainly by macrophages to attenuate inflammatory responses and is found in early and advanced stages of atherosclerosis (Mallat, Besnard, et al., 1999). Mallat et al. (1999) investigated the protective role of IL-10 in atherosclerosis. The authors reported that in IL-10 KO mice fed atherogenic diets, lipid accumulation was 3 fold higher than that of wild-type mice. However, injection of murine IL-10 into IL-10 KO mice decreased the atherosclerotic lesion by 60%, indicating the positive role of IL-10 in atherosclerosis (Mallat, Besnard, et al., 1999). Caligiuri et al. (2003) observed similar results in apoE/IL-10 double KO mice. LDL-cholesterol and markers of coagulation were higher in apoE/IL-10 double KO mice than those of apoE KO mice, indicating that the absence of IL-10 leads to the progression of atherosclerosis (Caligiuri et al., 2003). IL-10 also down-regulates the activity of pro-inflammatory cytokines, including TNF-α. In an *in vitro* study, IL-10 was found to inhibit human aortic vascular smooth muscle cell proliferation stimulated by TNF-α (Selzman et al., 1998). Waehre et al. (2002) reported that TNF-α production and gene expression in subjects with angina were higher than those of healthy subjects. Also, incubation of IL-10 with peripheral blood mononuclear cells (PBMC) derived from subjects with angina inhibited TNF-α secretion (Caligiuri et al., 2003). These findings demonstrated that IL-10 exhibits anti-inflammatory effects by inhibiting the expression of pro-inflammatory cytokines, especially TNF-α, and reducing LDL-cholesterol accumulation.

Pro- and anti-inflammatory cytokines produced by monocytes/macrophages play an important role in the progression of atherosclerosis. Therapeutic agents focused on reducing pro-inflammatory and increasing anti-inflammatory cytokines may serve as potential alternatives for atherosclerosis prevention and treatment.

III. Treatment options for atherosclerosis

There are several treatments available that help slow the progression of atherosclerosis, including pharmacological options, lifestyle changes and surgical procedures. Lipid-lowering therapy is one therapeutic strategy to delay atherosclerotic plaque formation. Nissen et al. (2006) reported that a very high-dose statin therapy using rosuvastatin reduced LDL cholesterol by 53.2% and increased HDL cholesterol by 14.7% compared to baseline, resulting in significant reversion of atherosclerosis (Nissen, Nicholls, Sipahi, & et al., 2006). Similarly, an intensive lipid-lowering drug, atorvastatin, was found to be more effective than pravastatin, a moderate lipid lowering drug, in decreasing the development of atherosclerosis (Nissen, Tuzcu, Schoenhagen, & et al., 2004). The difference in response may be related to the greater reduction in atherogenic lipoproteins and CRP with atorvastatin compared to pravastatin.

Because atherosclerosis is also an inflammatory disease, new anti-inflammatory therapies attract great interest. Steffen et al. (2005) found that low dose oral intake of Δ-9 tetrahydrocannabinol (THC), a derivative of cannabinoids, significantly inhibited the progression of atherosclerosis by diminishing the proliferation capacity and decreasing interferon-γ secretion of lymphoid cells isolated from atherosclerotic plaques. THC also suppressed macrophage chemotaxis, one of key steps in the progression of atherosclerosis (Steffens et al., 2005). Gene therapy also showed potential to reduce atherogenesis. According to Ni et al. (2001), the use of anti–monocyte chemoattractant protein-1(MCP-1) gene therapy reduced atherosclerosis. The expression of MCP-1, a monocyte chemotactic factor, is increased in atherosclerotic lesions. The use of an N-terminal deletion mutant of MCP-1 to block MCP-1– mediated monocyte chemotaxis, was reported to reduce atherosclerosis (Ni et al., 2001).

Hormone replacement therapy has been thought to be another treatment for atherosclerosis in postmenopausal women. However, Hodis et al. (2003) reported that administration of 17 β-estradiol, the endogenous estrogen molecule, either alone or with

medroxyprogesterone acetate had no significant effect on the development of atherosclerosis in postmenopausal women (Hodis et al., 2003). Another study also found that estrogen either with or without antioxidant vitamin supplement (vitamin E and vitamin C) did not provide benefits for treating atherosclerosis (Waters et al., 2002).

Lifestyle changes are considered a potential strategy to reduce and slow the progression of atherosclerosis. A study by Dod et al. (2010) demonstrated that 12 weeks of intensive lifestyle changes delayed atherosclerosis by reducing endothelial dysfunction and inflammatory makers. Their findings also showed that intensive lifestyle changes improved flow-mediated dilation and decreased CRP and IL-6, resulting in the improvement of atherosclerosis (Dod et al., 2010). Ornish and colleagues (2013) also reported reversion of atherosclerosis occurred after 5 years of intensive lifestyle intervention. The average percent diameter stenosis decreased by 1.75% after 1 year and by 3.1% after 5 years with intensive lifestyle intervention, whereas the control group increased by 2.3% and by 11.8% during the same period (Ornish et al., 2013).

Although lifestyle change and drugs are usually the treatment of choice, sometimes a more aggressive approach, such as surgical procedure, is needed for advanced stages of atherosclerosis. The common surgeries used to treat atherosclerosis include balloon angioplasty, stenting and coronary artery bypass surgery (Hobson et al., 2008). In angioplasty, a balloon-tipped catheter is used to open narrowed or blocked arteries by inserting the catheter into the blocked artery, resulting in improved blood flow (Hobson et al., 2008). Angioplasty is frequently followed by stenting, a process where a tube is placed in the artery to keep the lumen open after a successful dilatation (Hobson et al., 2008). If angioplasty with stenting is not successful in reducing the progression of atherosclerosis, coronary bypass procedures may be required. Arteries or veins from other areas in the body are used to bypass the coronary arteries, resulting in improved blood flow to the heart (Hobson et al., 2008).

Although many treatments are available for atherosclerosis, cost, compliance, and adverse effects are issues associated with these therapeutic options. Therefore, researchers are always looking for alternative approaches in delaying or preventing the development of atherosclerosis.

IV. Atherosclerosis and diet

Diet plays a key role in the incidence of atherosclerosis (Glueck, 1979). Saturated fat and cholesterol are known to be the cause of fatty streak and foam cell formations, the early stages of atherosclerosis (Glueck, 1979). In murine models of atherosclerosis, lipid or cholesterol-rich diets are frequently used to induce atherosclerotic lesions (Getz & Reardon, 2006). Cholesterol is a known pro-atherogenic molecule and a plasma level of at least 300 mg/mL is needed to induce atherosclerosis (Getz & Reardon, 2006). It was found that postmenopausal women with a low fat consumption had the lower rate of atherosclerosis progression (Mozaffarian, Rimm, & Herrington, 2004). However, high glycemic index carbohydrates were reported to have a positive correlation with atherosclerotic progression (Mozaffarian et al., 2004).

Increased ICAM-1 and VCAM-1 expression were observed in human endothelial cells incubated with HDL derived from healthy subjects consuming diets rich in coconut oil (high in saturated fat) but decreased in those that ate diets rich in safflower oil (high in PUFA) (Nicholls et al., 2006). The authors concluded that this effect is likely due to the fatty acid composition of the oils; with diets high in saturated fat suppressing the anti-inflammatory effect of HDL (Nicholls et al., 2006). These findings demonstrated that dietary fatty acids might influence atherogenesis.

Clinical studies have demonstrated that increased consumption of plant-based diets improved cardiovascular risk profile. Erkkila et al. (2005) investigated the relationship between the development of atherosclerosis and the consumption of cereal fiber and whole grains in

postmenopausal women with coronary artery disease. The study showed that higher intakes of cereal fiber (> 6 servings of whole grains per week) were related to decreased coronary artery diameter (Erkkilä, Herrington, Mozaffarian, & Lichtenstein, 2005). Also, higher intake of cereal fiber or whole-grain foods appeared to reduce the stenosis progression. These findings showed that higher intakes of diets rich in cereal fiber and whole grain were able to delay the progression of atherosclerosis in postmenopausal women (Erkkilä et al., 2005).

Brown et al. (1999) found that in a meta-analysis of 67 controlled trials, various soluble fibers significantly reduced total and LDL cholesterol. Soluble fiber (2–10 g/d) was significantly related to the reduction of total cholesterol and LDL cholesterol (Brown, Rosner, Willett, & Sacks, 1999). However, triacylglycerols and HDL cholesterol were not significantly influenced by soluble fibers. Also, the effects of soluble fibers from oat, psyllium, or pectin, on plasma lipids were similar (Brown et al., 1999).

Wolk et al. (1999) established the Nurse's Health Study, a cohort study of 68,782 US women aged 37-64 years old, to investigate the effect of the long-term intake (10 years) of dietary fiber on CHD risk. The authors found that high fiber intake, especially fiber from cereals, reduced the risk for CHD (Wolk et al., 1999). Moreover, the Los Angeles Atherosclerosis Study reported that intima-media thickness (IMT) progression was inversely associated with the intakes of viscous fiber and pectin (Wu et al., 2003). The authors concluded that viscous fiber, especially pectin, consumption may serve as a therapeutic agent for atherosclerosis via its ability to decrease IMT progression (Wu et al., 2003).

The findings discussed above demonstrate that diets high in saturated fat and cholesterol increased the development of atherosclerosis while plant-based diets and diets rich in oil have protective effects against the progression of atherosclerosis.

Edible seeds, their oils and atherosclerosis

Inflammation plays a key role in the progression of atherosclerosis (Libby et al., 2002). Therapeutic agents targeting inflammation have been thought to suppress the development of this disease. Plant-based diets have been studied for their health benefits and have been reported to have a variety of pharmacological properties, including anti-inflammatory effects (Watzl, 2008). Edible seeds, including legumes, beans, grains, and nuts, as well as their oils are foods that have been reported to exhibit anti-inflammatory properties due to their high content of bioactive compounds (Watzl, 2008).

Camago et al. (2010) found that breakfast rich in virgin olive oil containing high phenolic compounds (398 ppm) decreased expression of several genes involved in inflammation, including TNF-α and IL-6, in peripheral blood mononuclear cells derived from subjects with metabolic syndrome. The authors suggested that this effect was due to the inhibition of NF-κB activation which reduced the transcription of the inflammatory cytokines (Camargo et al., 2010).

Few animal studies also reported the anti-atherogenic and anti-inflammatory effects of edible seeds and their oils (Dupasquier et al., 2007; Lucas et al., 2011; Lucas et al., 2004; Patade et al., 2008). Lucas et al. (2004) investigated the effect of three different doses of flaxseed (7.5, 15 and 22.5% for 120 days) on atherosclerotic risk factors in ovariectomized (ovx) hamsters. All three doses of flaxseed prevented the increase of total plasma cholesterol as well as area of fatty streak lesions induced by ovariectomy (Lucas et al., 2004). To examine whether flaxseed and its oil have similar cholesterol-lowering effects, Lucas and colleagues (2011) compared the effect of flaxseed (15% w/w) with flaxseed oil (adjusted to the same amount of oil found in 15% flaxseed) using the same study model. Only flaxseed significantly inhibited the increase in serum total cholesterol (12%) in ovx hamsters while serum triglyceride and liver lipids were not altered (Lucas et al., 2011). These findings were in agreement with the findings of a clinical study that supplemented Native American postmenopausal women with 30 g of flaxseed with and without

oat bran fiber for 3 months (Patade et al. 2008). Supplementation of flaxseed both with and without oat bran fiber significantly decreased LDL-cholesterol and total cholesterol while HDL-cholesterol and triglyceride levels were not changed (Patade et al., 2008).

Supplementation of 10 % ground flaxseed (rich in ALA) in cholesterol-rich diet significantly increased plasma ALA levels and decreased IL-6 and VCAM-1, inflammatory markers, in LDL-receptor KO mice (Dupasquier et al., 2007). Regular diet with flaxseed did not alter plasma cholesterol; however, when incorporated in a cholesterol-rich diet, flaxseed prevented the increase in plasma cholesterol, resulting in reduction of atherosclerosis in aortas of mice (Dupasquier et al., 2007). The authors attributed these effects to ALA found in flaxseed. It has been reported that the antiatherogenic effects of ALA is through its ability to suppress pro-inflammatory cytokines and adhesion molecules which reduces macrophage infiltration and proliferation of atherosclerotic lesions (Dupasquier et al., 2007).

The anti-inflammatory properties of vegetable oils have also been investigated in several clinical studies. Gillingham et al. evaluated the effects of canola oil (high in oleic acid) and a mixture of canola and flaxseed oil supplementation for 28 days on lipid profiles and inflammatory markers in hypercholesterolaemic participants. Both canola oil and mixed oil diets significantly decreased LDL cholesterol and total cholesterol levels as well as LDL:HDL ratio; with mixed oil diet being more potent (Gillingham, Gustafson, Han, Jassal, & Jones, 2011). None of the assessed inflammatory markers, except E-selectin, and intima-media thickness were affected by treatment. Mixed oil diet reduced E-selectin concentration; however, this diet also reduced HDL-cholesterol (Gillingham et al., 2011). These findings demonstrated that diets rich in vegetable oils may delay atherosclerotic lesion formation through its cholesterol-lowering effects. Incorporating vegetable oils rich in omega-3 fatty acid will provide additional benefits due to their anti-inflammatory effects.

Zhao et al. (2004) found that walnuts, rich in linoleic acid (LA) and ALA, and flaxseed oil (rich in ALA) supplementation for 6 weeks in hypercholesterolaemic subjects reduced pro-inflammatory markers involved in atherosclerosis. Both types of diets also reduced serum total cholesterol, LDL- and HDL-cholesterol and triglycerides (G. Zhao et al., 2004). The authors concluded that diets containing walnuts, low in saturated fat and cholesterol and rich in PUFA, have lipid and cholesterol-lowering effects. Diet high in ALA (flaxseed oil) decreased atherosclerosis risk by its anti-inflammatory effect beyond its lipid-lowering effects (G. Zhao et al., 2004).

Papageorgiou and colleagues (2011) investigated the acute effects of several dietary oils on adhesion molecules and TNF-α in healthy subjects. Participants were randomly assigned to receive extra virgin olive oil, soybean oil, corn oil, or cod liver oil (50 mL). Olive oil, soybean oil and cod liver oil significantly decreased TNF-α 3 h after oil consumption while corn oil had no significant effect (Papageorgiou et al., 2011). All types of oils reduced ICAM-1, and changes in TNF-α level were associated with changes in ICAM-1 but not VCAM-1. These results suggested that different types of oils may exert different effects on intracellular pathways, and that TNF-α may regulate the expression of ICAM-1(Papageorgiou et al., 2011). These effects may be influenced by the high omega-3 fatty acid content (ALA, DHA or EPA) in these oils (Papageorgiou et al., 2011).

El-Marasy et al. (2012) investigated the effect of WGO on memory impairment in rats. The authors found that WGO supplementation (170 mg/kg) for 14 days reversed memory impairment induced by scopolamine. WGO decreased TNF-α level and increased glutathione in the brain(El-Marasy, El-Shenawy, El-Khatib, El-Shabrawy, & Kenawy, 2012). The authors suggested that the cognitive effect of WGO was likely due to a decrease in TNF-α in the brain and this was attributed to the high content of Vitamin E in the oil (El-Marasy et al., 2012).

Findings discussed above demonstrated that several vegetable oils exhibit pharmacological effects, including anti-inflammatory properties which make it possible for them to serve as therapeutic agents for atherosclerosis.

V. Wheat

Wheat is one of the most widely consumed cereal grain with the third-highest production worldwide (Statista, 2015). In 2014-2015, the U.S. was ranked as the fifth leading wheat producer worldwide (Statista, 2015). Wheat grain contains endosperm, bran, and germ, which account for approximately 82%, 15%, and 3% of the grain, respectively (Atwell, 2001). Wheat grain is approximately 75% carbohydrate, and therefore, the importance of carbohydrate takes precedence over vitamins, minerals, and phytochemicals (Atwell, 2001). The most concentrated proteins found in wheat are gliadins and glutenins, and lipids are a very minor component, only consisting of about 1-3% (Atwell, 2001).

A. Oklahoma wheat

Wheat is a primary crop grown in Oklahoma and is very important to the state's economy (OWC, 2015). In 2003, Oklahoma ranked second nationally in the production of hard red winter wheat and became first in 2013 (Hunger et al., 2014). Wheat was worth $443 million to the Oklahoma economy in 2003 (OWC, 2015). Oklahoma has nearly 6 million acres land planted with wheat ("Wheat Facts," n.d.). Most Oklahoma farmers grow hard red winter wheat in the fall, and most important wheat varieties grown in Oklahoma are Jagger, Endurance and Overley (OWC, 2015).

B. Wheat germ

Wheat germ is the embryo or sprouting section of the seed and is about 2.5 % of the kernel weight (Mahmoud et al., 2015). It is an important by-product of the flour milling industry because the fat content of wheat germ limits the keeping quality of the flour (Atwell, 2001). In

the past, wheat germ was typically used to feed animals (Mahmoud et al., 2015). However, the germ has recently been sold as a supplement to add to food products to improve the nutritional value, flavor and texture (Brandolini & Hidalgo, 2012). Wheat germ has a high content of oil that is rich in essential fatty acids and contains vitamins B_1, B_2, B_6, and other biologically active compounds (Yuldasheva et al., 2010). Due to its high content of oil, wheat germ is used to produce WGO.

C. Wheat germ oil (WGO)

WGO is a product with a very high nutritional value. It is an excellent source of alpha-linolenic acid (ALA) (omega-3 fatty acid), α-tocopherol (vitamin E), and other bioactive compounds, including policosanols and sterols (Eisenmenger & Dunford, 2008; Yuldasheva et al., 2010). Typically, WGO contains 58% linoleic acid, 9% alpha-linolenic acid (ALA), 0.2% vitamin E, and 0.01% octacosanol(Niu, Jiang, Pan, & Pang, 2013). The anti-inflammatory property of WGO may be attributed to ALA, tocopherols (vitamin E), policosanols and sterols (Eisenmenger & Dunford, 2008; Nashed et al., 2005; Rallidis et al., 2003; Yuldasheva et al., 2010).

D. WGO and extraction methods

There are several extraction methods used to prepare WGO, both mechanical (e.g. screw pressed) and chemical processes (e.g. supercritical carbon dioxide, SC-CO_2). These processes may affect the amount of bioactive compounds (Eisenmenger & Dunford, 2008; Febrianto & Yang, 2011).

Mechanical pressing methods have been used to extract edible oil for a very long time before the introduction of solvent extraction (Matthäus, 2008). A screw press is a conventional method used to extract oil from raw materials such as nuts and seed (Matthäus, 2008). The raw materials are continuously fed into the machine under high pressure. During pressing, the raw

material can be heated up which causes a significant loss of nutrients, especially tocopherol and phytosterol (Matthäus, 2008). Mechanical processing also provides a low yield of oil, leaving a significant amount of it in the wet mass (Matthäus, 2008).

To improve on these disadvantages, solvent extraction has been developed as an alternative to conventional methods (Piras et al., 2009). SC-CO_2 is an extraction solvent that has been used widely because it is inexpensive, nontoxic, nonflammable, easily separated from the extract, and preserves nutrient (Piras et al., 2009). Hexane is also a common extraction solvent; however, when it is separated from the wet mass by steaming, this extraction method causes oxidation and degradation of heat-sensitive components (Piras et al., 2009).

Eisenmenger and Dunford (2008) compared bioactive compounds in differentially extracted WGOs. They found that SC-CO_2 extracted WGO had a higher amount of α-tocopherol than that of hexane extracted WGO (Eisenmenger & Dunford, 2008). However, the extraction methods had no effect on fatty acid compositions of the oils. Subcritical butane (SB) extraction is a novel extraction method used to extract oil from wheat germ. Xu et al. (2015) compared bioactive compounds in WGOs extracted by three different solvent extractions, SC-CO_2, hexane and SB extractions. The results showed that there was no difference in oil yield and fatty acid contents with the three extraction methods. However, the phytosterol content of SB extracted WGO was highest followed by SC-CO_2 extracted and hexane extracted WGOs (Xu, Han, Zhou, Wu, & Ding, 2015). SC-CO_2 extracted WGO has the highest α-tocopherol content followed by SB extracted and hexane extracted WGOs. The authors concluded that SB extraction may be more effective to retain WGO bioactive compounds compared with hexane extraction but not SC-CO_2 extraction (Xu et al., 2015). Another study also observed similar results that SC-CO_2 extracted WGO had higher α-tocopherol content than that of hexane extracted WGO (Molero G mez & Mart nez de la Ossa, 2000). However, Taniguchi et al. (1985) reported that the contents of α- and β-tocopherol of SC-CO_2 extracted WGO were similar to that of hexane extracted WGO.

Oil refining was also reported to affect tocopherol content of WGO. For example, reduction of α-, β- and total tocopherol was observed during deodorization (T. Wang & Johnson, 2001). These findings demonstrated that extraction methods may influence the amount of bioactive compounds found in WGO.

VI. Bioactive compounds in WGO and their anti-inflammatory properties

A. Alpha-linolenic acid (ALA)

Omega-3 fatty acids have been reported to have anti-inflammatory properties, and WGO is one of the richest plant sources of ALA (Eisenmenger & Dunford, 2008). In 2004, Zhao and colleagues examined the anti-inflammatory effect of dietary ALA in hypercholesterolemic men and women. Participants were fed diets low in saturated fat and cholesterol and supplemented with either ALA (ALA diet) or linoleic acid (LA diet). Both ALA and LA diets decreased CRP, VCAM-1, and E-selectin; however, ALA diet had a more favorable effect. Both ALA and LA diets showed similar effects in reducing serum total cholesterol, LDL cholesterol and triglycerides in the subjects (G. Zhao et al., 2004). ALA decreased CVD risk and atherosclerosis by inhibiting vascular inflammation beyond its lipid-lowering effects (G. Zhao et al., 2004).

To further investigate the anti-inflammatory properties of omega-3 fatty acids, Zhao et al. (2005) compared the anti-inflammatory effects of linoleic acid (LA), ALA, and DHA to that of palmitic acid (PA), in lipopolysaccharide (LPS)-stimulated human monocytic THP-1 cells. They found that after treatment with LA, ALA, and DHA, the secretion of pro-inflammatory cytokines, including IL-6, IL-1β, and TNF-α, were significantly decreased compared to those treated with PA (Zhao et al., 2005). ALA and DHA had stronger effects among all the treatments. Moreover, LA, ALA, and DHA decreased IL-6, IL-1β, and TNF-α gene expression and NF-κB DNA binding activity, whereas peroxisome proliferator-activated receptor-γ (PPARγ) DNA-binding activity was increased (Zhao et al., 2005). The authors concluded that the anti-inflammatory

effects of omega-3 fatty acids were in part, due to the inhibition of NF-κB which activates PPARγ and reduces cytokine release (Zhao et al., 2005).

A study by Ren and Chung (2007) showed that ALA exhibits an inhibitory effect on the production of nitric oxide (NO), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and TNF-α gene expressions induced by LPS in the murine macrophages cell line, RAW 264.7. This inhibitory effect of ALA is believed to be due to the inhibition of NF-κB activity (Ren & Chung, 2007). Similar results were also observed using a colitis model (Hassan et al., 2010). ALA-rich diet significantly increased eicosapentaenoic (EPA) acid and DHA compared with the colitic group not given ALA diet. ALA also decreased colon inflammation by lowering NF-κB activation and TNF-α gene expression and secretion (Hassan et al., 2010). They concluded that an ALA-rich formula is beneficial to colitis rats by inhibiting inflammatory stress (Hassan et al., 2010)

Rallidis et al. (2003) investigated the effect of ALA on inflammatory markers in dyslipidemic patients. Seventy six male dyslipidemic patients were randomly assigned to consume either 15 ml linseed oil (rich in ALA, n-3) or safflower oil (rich in LA, n-6) every day. After 3 months, dietary supplementation with ALA significantly decreased CRP, serum amyloid A (SAA) and IL-6 levels, but LA did not affect these parameters (Rallidis et al., 2003).

B. Tocopherols

Tocopherols have also been reported to have anti-inflammatory properties both *in vitro* and *in vivo*. Devaraj and colleagues have done several studies investigating the anti-inflammatory effects of α-tocopherol. Their first study investigated the effects of RRR-α-tocopherol supplementation on inflammatory markers and monocyte function. They reported that supplementation of α-tocopherol (1200 IU/day) for 8 weeks influenced monocyte function by decreasing monocyte-endothelial cell adhesion, lipid oxidation, superoxide (O2–), hydrogen

peroxide and pro-atherogenic cytokine (IL-1β) release after LPS-stimulation (S Devaraj, Li, & Jialal, 1996). The reduction in superoxide and lipid oxidation by α-tocopherol seemed to be mediated by inhibition of protein kinase C (PKC) activity which plays a vital role in superoxide secretion and LDL oxidation by activated monocytes (S Devaraj et al., 1996). These findings suggested that supplementation with α-tocopherol has a potential to reduce inflammation.

Consistent with the previous study, another clinical study by Devaraj and Jialal (2000) showed that in type 2 diabetic participants with and without macrovascular disease, α-tocopherol supplementation (1200 IU/day for 3 months) significantly down-regulated O2–, IL-1β, and TNF-α production, and reduced monocyte attachment to endothelial cells. In addition, α-tocopherol supplementation also decreased the expression of adhesion molecules (ICAM-1, VCAM-1, and E-selectin) (Sridevi Devaraj & Jialal, 2000). These findings suggested that α-tocopherol can be used as a strategy aimed at reducing risk of CVD in individulas with diabetes.

Schwenke et al. (2002) investigated the effect of α-tocopherol supplementation on plasma cholesterol and cholesterol deposition in arterial plaques of rabbits fed atherogenic diets. They found that rabbits fed a diet with high levels of dietary α-tocopherol (2,500 IU α -tocopherol/kg diet) generally had lower total plasma cholesterol concentrations than rabbits fed low doses of α-tocopherol (25 IU /kg diet) (Schwenke, Rudel, Sorci-Thomas, & Thomas, 2002). The authors concluded that high levels of α-tocopherol appeared to exhibit the most potent effect when in combination with polyunsaturated fat in the diet.

C. Policosanols

Policosanol is a combination of aliphatic alcohols whose major form is octacosanol (Irmak, Dunford, & Milligan, 2006). WGO contains high amount of policosanols, especially in the form of octacosanol (Eisenmenger & Dunford, 2008). A few studies reported that octacosanol, the main component of policosanols, has anti-inflammatory effects (Consolazio et

al., 1964). Carbajal et al. (1998) reported that oral administration of D-002, a product isolated from beeswax containing octacosanol (16.9%), to rats (100 and 200 mg/kg) resulted in the decrease of exudate volume in carrageenan-induced pleuritic inflammation. The authors concluded that this was likely due to a significant reduction of leukotriene B4 (LTB4) in the exudate (Carbajal, Arruzazabala, Valdes, & Más, 1998). This result suggested that D-002 containing octacosanol is an anti-inflammatory agent which is most likely mediated by a reduction in LTB4 levels.

Oliveira et al. (2012) investigated the anti-inflammatory effects of octacosanol isolated from the leaves of Sabicea grisea var. grisea, an African plant used for fever and malaria treatment. The authors found that supplementation of octacosanol (1 and 10 mg/kg) significantly decreased total leukocyte count and neutrophil influx, as well as TNF-α production in mice with carrageenan-induced pleurisy (Oliveira et al., 2012). The mechanism responsible for the anti-inflammatory effects of octacosanol appears to be partly associated with inhibition of pathways for pro-inflammatory cytokine production (Oliveira et al., 2012).

D. Sterols

Phytosterols are plant origin steroidal compounds with similar structure to cholesterol (Phillips, Ruggio, & Ashraf-Khorassani, 2005). WGO is a rich source of the phytosterols, sitosterol and campesterol (Eisenmenger & Dunford, 2008). Phytosterols have been known to decrease cardiovascular disease risk due to their cholesterol-lowering effects (Nashed et al., 2005). However, there are also reports of the anti-inflammatory properties of phytosterols (Loizou, Lekakis, Chrousos, & Moutsatsou, 2010; Nashed et al., 2005; Navarro, De Las Heras, & Villar, 2001). Nash et al. (2005) investigated the anti-atherogenic effects of dietary phytosterol (2%) supplementation in apoE-KO mice for 14 weeks. Phytosterol-enriched diet decreased plasma cholesterol as well as the production of pro-inflammatory cytokines, TNF-α and IL-6 (Nashed et al., 2005). Supplementation with phytosterols also increased IL-10, an anti-

inflammatory cytokine. The reduction of plasma cholesterol and atherosclerotic lesions were also observed in the phytosterol-rich diet group (Nashed et al., 2005). The authors concluded that phytosterol exhibited anti-inflammatory effects by inhibiting pro- and increasing anti-inflammatory cytokine production and may serve as a potential intervention for atherosclerosis.

Loizou et al. (2010) examined the effect of β-sitosterol (0.1 – 200 μM) on ICAM-1 and VCAM-1 expression and monocytes (U937 cells) attachment in human aortic endothelial cells (HAECs) stimulated with TNF-α. Pretreatment of HAECs with β-sitosterol before TNF-α stimulation significantly down-regulated VCAM-1 and ICAM-1 as well as the attachment of U937 cells to TNF-α-stimulated HAEC (Loizou, Lekakis, Chrousos, & Moutsatsou, 2010). β-sitosterol treatment also decreased NF-κB phosphorylation, resulting in the suppression of NF-κB. These results were consistent with the findings of Bustos and colleagues (2008) in that β-sitosterol suppressed ICAM-1 expression, resulting in reduction of infiltration and attachment of human monocytic THP-1 cells to human umbilical vein endothelial cells (HUVECs) stimulated by oxidized LDL (Bustos, Duffau, Pacheco, & Ulloa, 2008).

Navarro et al. (2001) investigated the anti-inflammatory effects of a sterol fraction (7.6 % compesterol, 28.4 % stigmasterol and 61.1 % β-sitosterol) derived from the extract of Sideritis foetens CLEM, a local plant from Spain. Oral administration of the sterol fraction (30 and 60 mg/kg) reduced carrageenan paw edema in mice and suppressed ear edema induced by 12-O-tetradecanoylphorbol acetate (Navarro, De Las Heras, & Villar, 2001).

In a clinical trial, 3-week supplementation of a combination of 1.4 g sunola oil (rich in omega-3 fatty acid) and plant sterols (2 g) decreased CRP, TNF-α and IL-6 and increased adiponectin in hyperlipidemic subjects (Micallef & Garg, 2009). These finding showed that plant sterols in combination with omega-3 fatty acid may exert more potent effects than being by itself in inhibiting pro-inflammatory markers involved in the development of atherosclerosis.

Similarly, supplementation of plant sterol (1 g) for 8 weeks decreased CRP level as well as triglyceride and LDL-cholesterol in healthy subjects (Sridevi Devaraj, Autret, & Jialal, 2006).

From the review of literature, numerous studies have demonstrated that isolated bioactive components in WGO exhibit anti-inflammatory properties. However, whether WGO itself has anti-inflammatory effects still needs to be answered.

CHAPTER III

METHODOLOGY

Cell culture and wheat germ oil (WGO)

Human monocytic THP-1 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in a T-75 flask containing 25 mL RPMI-1640 media (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO), and 0.05 mM 2-mercaptoethanol (Gibco, Grand Island, NY) in an incubator containing 5% CO_2 at 37^oC. Since THP-1 cells change their characteristics over time, several studies used the cells at low passage numbers (5 – 12) to prevent changes in cell morphology (An et al., 2009; Equils et al., 2003; M. M. Oliveira, Charlab, & Pessolani, 2001; Tietze & Blömeke, 2008). Therefore, THP-1 cells grown between passage numbers 8 – 10 were used for the experiments.

Four wheat germ oil (WGO) samples were kindly provided by Dr. Nurhan Dunford (Department of Biosystems and Agricultural Engineering, Oklahoma State University, Stillwater, OK). WGO sources and extraction methods are shown in **Table 1**. Fatty acid, tocopherol, policosanol, and sterol composition of WGOs were analyzed by Dr. Dunford's group and shown in **Tables 2 – 4**

Table 1 Sources and extraction methods of WGOs used in this study

WGO sample	Source and extraction method
WGO 1	Screw pressed WGO prepared by Dr. Dunford's group
WGO 2	Supercritical carbon dioxide (CO_2) extracted WGO after centrifugation
WGO 3	Commercially refined WGO from Vitamin, Inc., Chicago, IL
WGO 4	Crude WGO commercially extracted with hexane by Vitamin, Inc., Chicago, IL

Table 2 Fatty acid composition of WGOs used in this study

WGO sample	Fatty acid composition of WGOs % of total fatty acid							
	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1
WGO 1	18.12±0.07	0.18±0.01	0.73±0.01	16.13±0.08	54.82±0.19	4.83±0.02	0.18±0.01	1.66±0.01
WGO 2	16.81±0.13	0.17±0.01	0.68±0.01	14.41±0.03	56.16±0.01	8.25±0.01	0.15±0.01	1.39±0.01
WGO 3	16.59±0.01	0.17±0.01	0.81±0.01	15.51±0.01	56.59±0.04	6.57±0.01	0.18±0.01	1.52±0.01
WGO 4	16.59±0.09	0.17±0.01	0.68±0.01	14.99±0.01	57.25±0.06	6.18±0.01	0.16±0.01	1.49±0.01

Table 3 Tocopherol content of WGOs used in this study

WGO sample	Tocopherol content of WGO (mg/g)			
	α-tocopherol	β-tocopherol	γ-tocopherol	δ-tocopherol
WGO 1	2.68 ± 0.19	1.14 ± 0.10	Not Detected	
WGO 2	2.81 ± 0.16	1.01 ± 0.03		
WGO 3	1.08 ± 0.11	0.93 ± 0.01		
WGO 4	1.03 ± 0.13	0.99 ± 0.01		

Table 4 Sterol and policosanol content of WGOs used in this study

WGO sample	Sterol content of WGO (mg/g)			Policosanol content of WGO (mg/g)				
	campesterol	stigmasterol	sitosterol	docosanol	tricosanol	hexacosanol	heptacosanol	octacosanol
WGO1	4.90 ± 0.16	0.12 ± 0.02	9.76 ± 0.19	Not Detected				
WGO2	4.89 ± 0.25	0.11 ± 0.01	11.73 ± 0.48					
WGO3	5.67 ± 0.21	0.19 ± 0.01	14.20 ± 0.23					
WGO4	6.43 ± 0.58	0.21 ± 0.02	15.99 ± 1.36					

Preparation of WGO stock and working solutions for cell culture studies

Preliminary studies involved determining the appropriate WGO and LPS concentrations to use in this study. For preliminary studies, commercial WGO (WGOC) from VIOBIN (Monticello, IL) was used. To determine the maximum volume of WGO that will dissolve in 100% ethanol, different volumes (25, 20, 15 and 10 μl) of WGOC were mixed with 100% ethanol. With the volume of WGOC used, 15 μl was the highest volume that was miscible in 1 mL of 100% ethanol and this was used as the stock solution (1.5 % WGO). To make the highest concentration of working solution, 1 μl of stock of WGO was added to 999 μl of RPMI-1640 media (1.5 x 10^{-3}% WGO), and this concentration of WGO had ethanol concentration below 0.1% (v/v).

Determination of appropriate WGO and lipopolysaccharide (LPS) concentrations

To determine appropriate WGO or LPS concentrations that will not cause cytotoxicity, cell viability and cell proliferation assay were used. For these analyses, THP-1 cells were seeded at a density of 1 x 10^5 cells/well and incubated with different concentrations of WGOC (0, 0.25, 0.75 and 1.5 x 10^{-3}%) or LPS (*Escherichia coli* LPS, 055:B5, Sigma Chemical Co., St. Louis, MO) (0, 10, 100 and 1000 ng/mL) in triplicate in a flat-bottom 96-well plate. The microplate was incubated for 6, 12, 24 or 48 h and cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This assay measures cell proliferation rate

based on the reduction of the yellow tetrazolium MTT by dehydrogenase enzymes in mitochondria of active cells to generate intracellular purple formazan which can be detected by spectrophotometric methods (Trevigen, 2014). MTT cell proliferation assay kit was obtained from Trevigen (Gaithersburg, MD). For this assay, 10 µl of MTT reagent was added to each well at the end of treatment period and the plate was left in the incubator for 4 h. When the purple precipitate was clearly visible, 100 µl detergent reagent was added to all wells and the plate was incubated overnight in the dark at room temperature. Absorbance was measured at 570 nm using a Synergy HT: multi-detection microplate reader (BioTek, Winooski, Vermont). Absorbance of treated cells was compared to control cells (incubated with ethanol for WGO or media alone for LPS), and cell proliferation was expressed as the percentage of control.

Cell viability was assessed by trypan blue staining. The principle of this method is that live cells do not absorb a viability dye (trypan blue), while dead cells do (Sigma-Aldrich, 2014). Cell suspension (10 µL) was mixed gently with trypan blue and allowed to stand for 5 minutes. Live cells and dead cells (stained cells) were counted using a hemocytometer and cell viability was expressed as a percentage of total cells.

Assessment of cytokine production in culture supernatants by enzyme-linked immunosorbent assay (ELISA)

Based on the literature and preliminary experiments, 6 h incubation period and LPS concentration of 1000 ng/mL were used in this study. The concentrations of TNF-α, IL-6 and IL-10 in cell culture supernatant were measured using ELISAs. This method detects the cytokines using an enzyme linked to an antibody or antigen as detection marker. THP-1 cells were seeded at a density of 1 x 10^6 cells/ml and incubated with LPS (1000 ng/mL) along with different concentrations of WGOs (0, 0.25 and 1.5 x 10^{-3}%) in a 6-well plate (2 mL/well) for 6 h. Supernatants were then collected to measure the concentrations of TNF-α, IL-6 and IL-10 using commercially available ELISA kits (R&D Systems, Minneapolis, MN).

For ELISA, assay diluent (100 μL) was added to each well of a primary antibody coated 96-well ELISA plate followed by culture supernatants or cytokine standards in duplicate (100 μL) and incubated for 2 h at room temperature. The plate was then washed and incubated with human high sensitive conjugate (200 μL) at room temperature for 2 h. After washing, substrate solution (50 μL) was added to each well and incubated for 1h and amplifier solution (50 μL) was then added to each well followed by 30 min incubation. Colored products were generated at this step, indicating a positive reaction. Stop solution was then added after incubation and the plate was read within 30 min using Synergy HT: multi-detection microplate reader (BioTek, Winooski, Vermont) set at 490 nm with wavelength correction set to 650 nm. The concentrations of cytokines (pg/mL) were calculated by comparing the absorbance of the samples with those of the standard curve generated for each assay.

Determination of cytokine gene expression by real-time polymerase chain reaction (RT-PCR)

THP-1 cells were seeded at the density of 1 x 10^6 cells/ml in a 6-well plate (2 mL/well) and incubated with and without LPS (1000 ng/mL) along with different concentrations of WGOs (0, 0.25 and 1.5 x 10^{-3}%) for 6 h. After 6 h incubation, the supernatants were taken off and cells were lysed with 800 μL of STAT-60 solution (Tel-Test Inc., TX) followed by addition of 150 μL of chloroform (Sigma-Aldrich, St. Louis, MO). The homogenate was shaken vigorously and centrifuged at 12,000 x *g* at 4°C for 15 min. After centrifugation, the colorless upper aqueous phase containing RNA was separated from the red lower phenol chloroform phase. The aqueous (upper clear) phase was carefully transferred to a new nuclease-free tube followed by addition of ice-cold isopropanol (Sigma-Aldrich, St. Louis, MO) and then incubated at -80°C overnight. After incubation, the samples were centrifuged at 12,000 x *g* at 4°C for 30 minutes and the supernatant was then discarded. Nuclease-free 75% ethanol was added to each sample to rinse the RNA pellet and centrifuged at 12,000 x *g* at 4°C for 10 minutes. Ethanol was removed and the RNA pellet was air-dried for 5 min. The RNA pellet was then resuspended in 10 μL

diethylpyrocarbonate (DEPC) H_2O and the concentrations and purity were measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). RNA quality was determined from the absorbance ratios at 260/230 nm and 260/280 nm ≥ 1.7. Samples were stored at -80°C until further analyses.

RT-PCR is used to detect gene expression by amplifying a specific sequence of DNA of target genes using short, sequence-specific oligosaccharides or primers added to the reaction (Valasek & Repa, 2005). The quantification of amplification of DNA is measured using fluorescent probes, and the results are related to the amount of original target sequence (Valasek & Repa, 2005). To synthesize complementary DNA (cDNA), DNase master mix was prepared [containing DNase mix (Roche, Indianapolis, IN), 25 mM $MgCl_2$ (Roche, Indianapolis, IN), 10x PCR buffer (Roche, Indianapolis, IN), and DEPC-H_2O], added to the RNA sample, and then incubated in a thermocycler (Biometra, Goettingen, Germany) at 37ºC for 30 min followed by heat inactivation of the enzyme at 75ºC for 10 min. Reverse transcription was done by adding Superscript II RTase (Invitrogen, Carlsbad, CA), 5x buffer (Invitrogen, Carlsbad, CA), dNTP mix (Promega, Madison, WI), 0.1 M DTT (Invitrogenv Carlsbad, CA), 0.8 mg/mL pdN6 (Roche Indianapolis, IN), and DEPC-H_2O to each sample and then incubated in the thermocycler at 25ºC for 10 min, 42ºC for 50 min, and 72ºC for 10 min and then a 4 º C soak cycle.

For performing RT-PCR, SYBR Green chemistry (Applied Biosystems, Foster City, CA) was used and the primers for each gene were obtained from literature. Primer validation was performed by analysis of template titration and dissociation curves (Valasek & Repa, 2005). Gene expression of pro- (TNF-α and IL-6) and anti-inflammatory (IL-10) cytokines were measured. Cyclophilin was used as the reference gene. Sequences of primers for cytokine and reference genes are showed in **Table 5**. The detection of RNA amplification was done by real-time quantitative PCR using an ABI Fast 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA). The reaction was done at 50°C for 2 min, 95°C for 10 min (denaturation), 95°C

for 15 sec (40 cycles of denaturation). Comparative cycle number at threshold (CT) method was used to analyze all RT-PCR results. The data were presented as relative mRNA expression of cytokines compared to control.

Table 5 Oligonucleotide sequences of primers used for real-time PCR

Gene name	Symbol	Primers	Nucleotide sequence (from 5' to 3')
Cyclophilin (Chanput, Mes, Vreeburg, Savelkoul, & Wichers, 2010)	Cyclo	Forward Reverse	TGCCATCGCCAAGGAGTAG TGCACAGACGGTCACTCAAA
Tumor Necrosis Factor Alpha (Chanput, Mes, Vreeburg, Savelkoul, & Wichers, 2010)	TNF-α	Forward Reverse	CTGCTGCACTTTGGAGTGAT AGATGATCTGACTGCCTGGG
Interleukin–6 (Folkard et al., 2014)	IL-6	Forward Reverse	CTCTTCAGAACGAATTGACAAACAAAT ATGTTACTCTTGTTACATGTCTTCTTTCTC
Interleukin–10 (Chanput, Mes, Vreeburg, Savelkoul, & Wichers, 2010)	IL-10	Forward Reverse	GTGATGCCCCAAGCTGAGA CACGGCCTTGCTCTTGTTTT

Statistical analyses

Data were analyzed using 2 x 4 factorial design using the Statistical Analysis System (SAS) version 9.3 (Cary, NC). Analyses of variance (ANOVA) and least square means were calculated using the general linear model (GLM) procedure. If only two groups were being compared, a student's t-test was used to determine the difference between means of two groups. Differences were considered significant at $P < 0.05$. Data were presented as lsmeans ± standard error (SE).

CHAPTER IV

RESULTS

Cell proliferation by MTT assay

The cytotoxic effects of different concentrations of WGO and treatment durations on human monocytic THP-1 cells were assessed by MTT assay. For these preliminary experiments, cells were treated with commercial wheat germ oil (WGOC) at 0, 0.25, 0.75, and 1.5 x 10^{-3} % for 6, 12, 24 and 48 h. Commercial wheat germ oil was used because the WGO formulation from Dr. Nurhan Dunford was not yet available when these preliminary experiments were done. Cell proliferation was shown as a percentage of control (untreated cells). When looking at the effect of concentration on cell proliferation (regardless of incubation time), there was no significant difference observed among all concentrations of WGOC used (P = 0.1344). There was also no significant differences among the treatment duration tested (P = 0.1419) **(Table 6)**. These indicated that there was no cytotoxicity effect of WGO on THP-1 cells. Therefore, the concentrations at 0.25 and 1.5 x 10^{-3}% WGO were used as a low and high dose of each WGO treatment for the further experiments.

Effects of LPS concentrations and treatment duration on cell proliferation

To determine appropriate LPS concentration and treatment duration, THP-1 cells were incubated with different concentrations (0, 10, 100 and 1000 ng/mL) of LPS for 6, 12, 24 and 48

h. There was a significant difference (P<0.0001) among the treatment duration tested (**Table 7**). Incubating THP-1 cells with LPS for 12 h had the highest cell proliferation followed by 6 h and 24 h (**Table 7**). Treatment duration for 48 h had the lowest cell proliferation. Although treatment duration of 12 h had the highest cell proliferation, we decided to use the 6 h treatment period based on literature indicating that gene expression and production of TNF-α is highest during this time period (Pérez-Pérez, Shepherd, Morrow, & Blaser, 1995; Zhao et al., 2005). Additionally, treatment duration of 6 h still has cell proliferative effect greater that 95% relative to the control.

There was no significant difference in cell proliferation among the four LPS concentrations used (**Table 7**). However, a number of studies investigating pro- (TNF-α and IL-6) and anti-inflammatory (IL-10) cytokine production and gene expression in THP-1 cells used LPS concentration at 1000 ng/mL and found that LPS at this concentration strongly induced cytokine production and gene expression (Chanput et al., 2010; Weldon, Mullen, Loscher, Hurley, & Roche, 2007; Zhao et al., 2005). Also, LPS at 10 ng/mL was reported to stimulate TNF-α production; however, this concentration was not sufficient to induce IL-6 and IL-10 secretion in THP-1 cells (Schildberger, Rossmanith, Eichhorn, Strassl, & Weber, 2013). Therefore, LPS concentration of 1000 ng/mL was used in this study.

Once LPS concentration and treatment duration were established (LPS at 1000 ng/mL for 6 h), cell viability and proliferation as well as cytokine production and gene expression were assessed under these conditions. Viability (P = 0.1010) and proliferation (P = 0.0823) were not statistically different between cells treated with and without LPS (**Table 8**). As expected, LPS significantly increased cytokine production (**Table 8**). IL-10, IL-6, and TNF-α increased by 17, 30, and more than 1000-fold, respectively with LPS stimulation at 1000 ng/mL for 6 h (**Table 8**). Additionally, gene expression of all cytokines was significantly up-regulated by LPS activation. Gene expression of IL-10, TNF-α and IL-6 increased by 23, 37 and 118 fold, respectively, relative to inactivated cells (**Figure 1**). These findings demonstrated that LPS concentration at

1000 ng/mL and treatment period for 6 h were sufficient to stimulate cytokine production and gene expression in human monocytic THP-1 cells.

Effects of WGO types on cell viability and proliferation by trypan blue staining and MTT assay

THP-1 cells were treated with three different concentrations (0, 0.25 and 1.5 x 10^{-3} %) of the four types of WGOs (WGO 1, WGO 2, WGO 3 and WGO 4) together with LPS (1000 ng/mL) for 6 h. At the end of the treatment period, cell viability and proliferation were measured by trypan blue staining and MTT assay, respectively. Overall, there were no significant differences in cell viability and proliferation among the four types and doses of WGO **(Table 9).** When comparing the effect of WGO types at both low and high dose, there were also no significant differences in cell viability and proliferation **(Figures 2-3)**.

TNF-α, IL-6 and IL-10 production by ELISA

To determine the effect of WGOs on cytokine production, THP-1 cells were incubated with four WGOs with LPS (1000 ng/mL) for 6 h. The supernatant was collected at the end of treatment period for analysis of cytokines. The pro- (TNF-α and IL-6) and anti- (IL-10) inflammatory cytokine production were assessed by ELISA. There was significant difference among the type of WGOs (P = 0.0045) and treatment doses (P = 0.0264) on TNF-α production **(Table 10)**. WGO 4 had the highest TNF-α production while the other three WGOs were statistically similar and lower than WGO 4 **(Table 10)**. High dose of WGOs was more potent than low dose to reduce TNF-α production **(Table 10)**. All the four WGOs at low dose had no effect on TNF-α production **(Figure 4)**. However, at high dose, WGO 4 had the highest TNF-α production while WGO 1, WGO 2 and WGO 3 had lower production and statistically similar to the control **(Figure 4)**.

All WGOs had similar effects on IL-6 production while higher dose had lower IL-6 compared to low dose (P <0.0001) **(Table 10)**. Like TNF-α, there were differences on IL-6

production at high dose of WGO (P = 0.0351) but not at the low dose **(Figure 5)**. All WGO types suppressed IL-6 production compared to the control **(Figure 5)**.

For the production of the anti-inflammatory cytokine, IL-10, there were significant differences among WGO types (P < 0.0001) but not doses used (P = 0.0993). WGO 3 was the most potent in increasing IL-10 while WGO 1 was the lowest **(Table 10)**. Both doses of the different WGO types showed a similar and significant effect on IL-10 production with WGO 3 being the most potent followed by WGO 4 **(Figure 6)**.

TNF-α, IL-6 and IL-10 gene expression by real-time (RT)-PCR

Pro- and anti-inflammatory cytokine gene expression in WGO treated THP-1 cells was assessed by RT-PCR. After 6 h of treatment, the cells were collected and RNA was extracted to measure gene expression of TNF-α, IL-6 and IL-10. All WGOs and both doses had no effects on the relative mRNA expression of TNF- α **(Table 11)**. None of WGO type at the low dose had significant effect on TNF- α gene expression **(Figure 7)**; however, WGO 1, WGO 2 and WGO 3 at the high dose significantly down-regulated TNF- α gene expression while WGO 4 had an intermediate effect **(Figure 7)**.

All WGO types and doses had similar effects on the relative mRNA expression of IL-6 **(Table 11)**. None of WGOs at the low dose had significant effects on IL-6 gene expression **(Figure 8)**; however, at high dose, all WGOs significantly reduced the relative gene expression of IL-6, with WGO 2 being the most effective **(Figure 8)**.

There were no significant differences in the anti-inflammatory cytokine, IL-10, gene expression among the four WGOs (**Table 11**). At the low dose, all WGOs had no effect on IL-10 gene expression compared to control **(Figure 9)**. However, the high dose of WGO 3 and WGO 4 significantly increased (P = 0.0020) IL-10 gene expression compared to the control and WGO 1 and WGO 2 **(Figure 9)**.

Table 6 Effects of WGO concentrations and treatment duration on human monocytic THP-1 cell proliferation[1,2]

Treatment duration (hours)	**Proliferation (% control)[2]**
6	94.86 ± 4.56
12	83.23 ± 4.30
24	96.68 ± 4.30
48	89.41 ± 4.30
WGO concentration (x 10^{-3} %)	
0	100.00 ± 4.30
0.25	88.90 ± 4.56
0.75	86.60 ± 4.30
1.5	88.68 ± 4.30
P values	
Time	0.1419
Concentration	0.1344
Time*Concentration	0.9762

[1]Cell proliferation was determined by MTT assay (Trevigen, Gaithersburg, MD). Values are presented as lsmean ± SE. Treatment was done in triplicate and the experiment was repeated two times.

[2]Control is THP-1 cells incubated with vehicle (ethanol equivalent to the highest WGO concentration) at each incubation time point and considered to have 100 % proliferation. The absorbance of LPS-stimulated cells was compared with those of control and presented as the percentage of control.

Table 7 Effect of lipopolysaccharide (LPS) concentrations and treatment duration on human monocytic THP-1 cell proliferation[1,2]

Treatment duration (hours)	**Proliferation (% control)[2]**
6	96.71 ± 2.31[b]
12	103.61 ± 2.31[a]
24	94.40 ± 2.31[b]
48	83.27 ± 2.31[c]
LPS concentration (ng/mL)	
0	100.00 ± 2.31
10	93.27 ± 2.31
100	91.30 ± 2.31
1000	93.42 ± 2.31
P values	
Time	<0.0001
Concentration	0.0630
Time*Concentration	0.0007

[1]Cell proliferation was determined by MTT assay (Trevigen, Gaithersburg, MD). Values are presented as lsmean ± SE. Treatment was done in triplicate and the experiment was repeated two times. Values that do not share the same letter were significantly different from each other ($P < 0.05$).

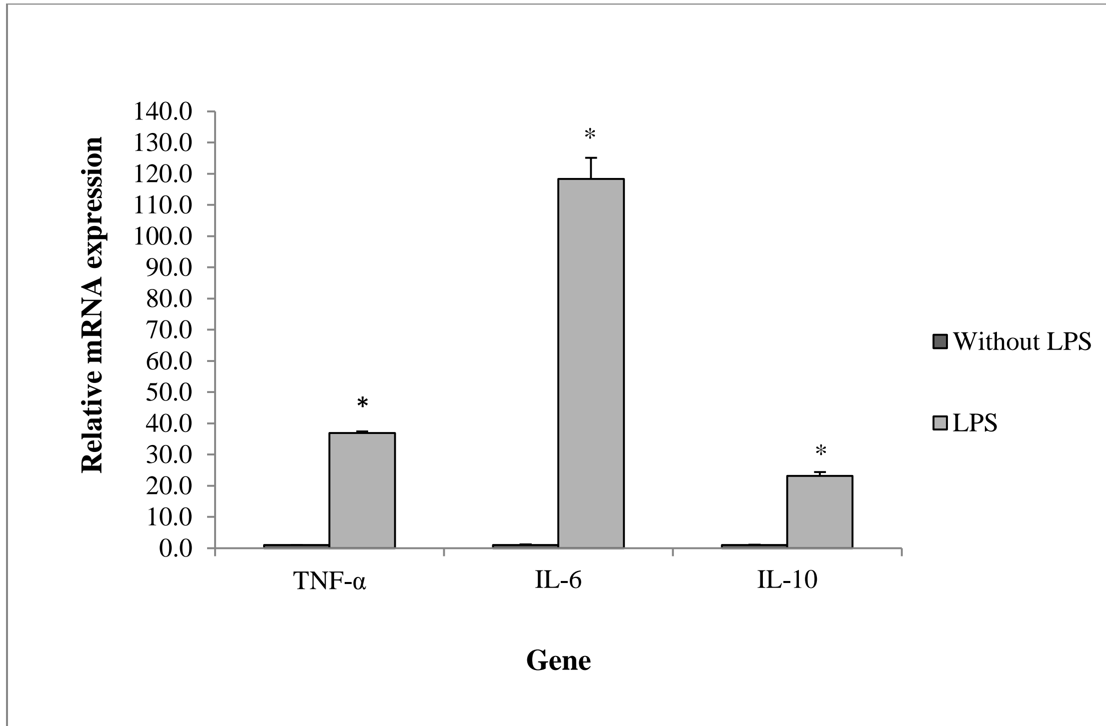
[2]Control is THP-1 cells incubated without LPS and considered to have 100 % proliferation. The absorbance of LPS-stimulated cells was compared with control and presented as the percentage of control.

Table 8 Cytokine production, cell viability and proliferation of THP-1 cells incubated with LPS (1000 ng/mL) for 6 h[1]

Measurement	Without LPS	With LPS	P value
Cell viability (%)	96.00 ± 1.00	93.33 ± 0.67	0.1010
Cell proliferation (% control)	100.00 ± 0.01	95.75 ± 0.01	0.0823
TNF-α (pg/mL)	3.87 ± 1.60	4858.70 ± 144.78	<.0001
IL-6 (pg/mL)	Not detected	30.18 ± 4.48	0.0005
IL-10 (pg/mL)	1.41 ± 0.58	24.56 ± 0.55	0.0001

[1]Cell viability and proliferation were assessed by trypan blue staining and MTT assay (Trevigen, Gaithersburg, MD), respectively. TNF-α, IL-6 and IL-10 production was measured by ELISA (R&D Systems, Minneapolis, MN). Values are lsmean ± SE. Treatment was done in triplicate and the experiment was repeated two times. $P < 0.05$ is considered significantly different.

Figure 1 Pro- and anti-inflammatory cytokine gene expression in THP-1 cells incubated with and without LPS at1000 ng/mL for 6 h[1]



[1]Cytokine gene expression was assessed by RT-PCR. Treatment was done in triplicate and the experiment was repeated two times. Bars represent lsmean ± SE. mRNA expression of LPS-stimulated THP-1 cells was presented relative to those of without LPS.

*indicates statistically ($P < 0.05$) significantly different to that without LPS.

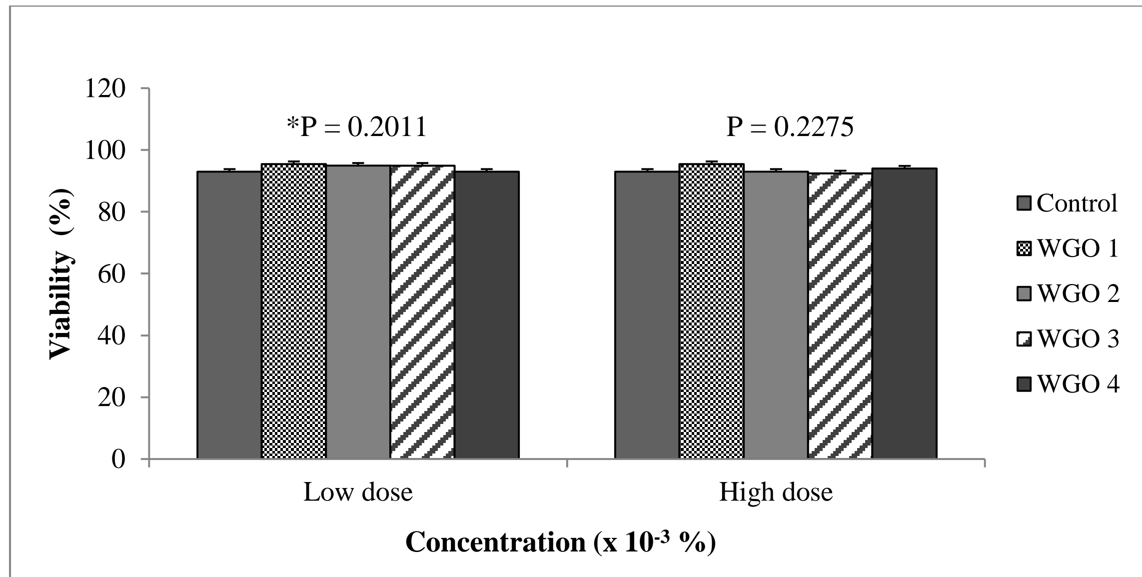
Table 9 Effects of WGO types and concentrations on viability and proliferation of LPS-stimulated human monocytic THP-1 cells[1,2]

WGO types	**Cell viability (%)**	**Cell proliferation (% control[2])**
WGO 1	95.50 ± 0.54	105.40 ± 3.19
WGO 2	94.00 ± 0.54	102.23 ± 3.19
WGO 3	93.75 ± 0.54	103.05 ± 3.19
WGO 4	94.00 ± 0.54	96.63 ± 3.19
WGO concentration (x 10^{-3} %)		
0.25	94.63 ± 0.43	104.44 ± 2.25
1.5	93.75 ± 0.43	99.22 ± 2.25
P values		
Type	0.1710	0.2688
Concentration	0.0728	0.1093
Type*Concentration	0.2975	0.1338

[1]Cell viability and proliferation were assessed by trypan blue staining and MTT assay (Trevigen, Gaithersburg, MD), respectively. Values are lsmeans ± SE. Treatment was done in triplicate and the experiment was performed two times. Values that do not share the same letter are significantly different ($P < 0.05$). WGO 1 = screw pressed, WGO 2 = supercritical CO_2 extracted after centrifugation, WGO 3 = commercially refined, WGO 4 = commercially hexane extracted wheat germ oil.

[2]Control is THP-1 cells incubated with vehicle (ethanol equivalent to the 1.5 x 10^{-3} % WGO concentration) and LPS and considered to have 100 % proliferation. The absorbance of treated cells was compared with those of control and presented as the percentage of control.

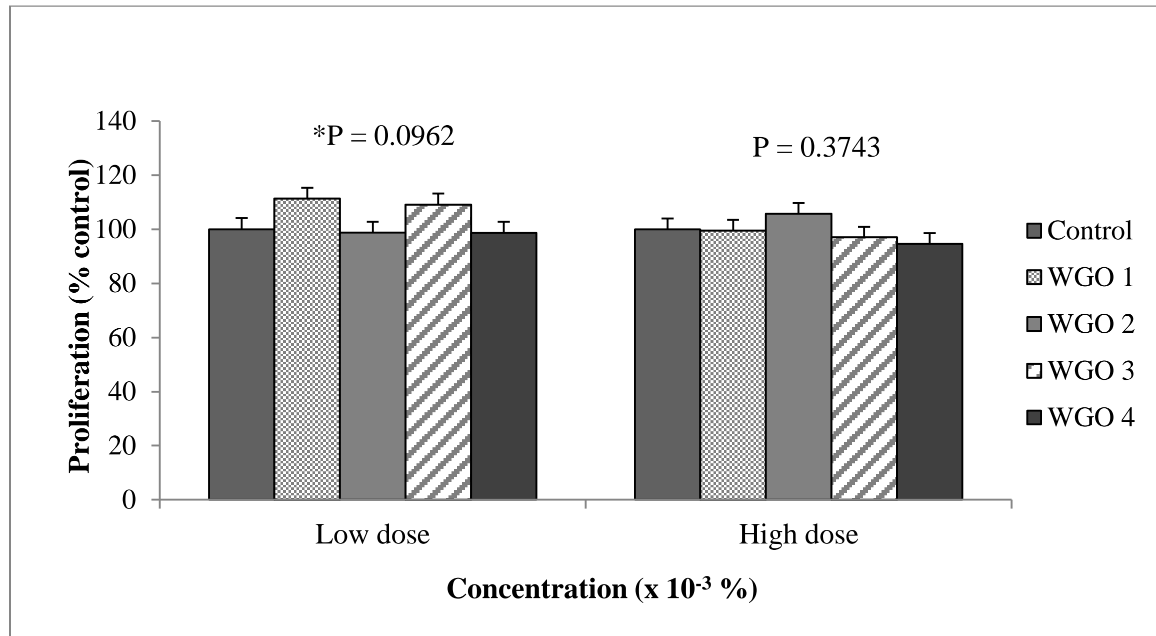
Figure 2 Effects of low and high dose of the different WGO types on cell viability of LPS-stimulated human monocytic THP-1 cells [1]



[1]Cell viability was measured by trypan blue staining. Treatment was done in triplicate and the experiment was performed two times. Bars represent lsmean ± SE. Low dose WGO = 0.25 x 10^{-3} % and high dose WGO = 1.5 x 10^{-3} %. WGO 1 = screw pressed, WGO 2 = supercritical CO_2 extracted after centrifugation, WGO 3 = commercially refined, WGO 4 = commercially hexane extracted wheat germ oil.

[*]Control is THP-1 cells treated with vehicle (ethanol equivalent to the high dose WGO concentration) and LPS.

Figure 3 Effects of low and high dose of the different WGO types on cell proliferation of LPS-stimulated human monocytic THP-1 cells [1]



[1]Cell proliferation was measured by MTT assay (Trevigen, Gaithersburg, MD). Treatment was done in triplicate and the experiment was performed two times. Bars represent lsmean ± SE. Low dose WGO = 0.25 x 10^{-3} % and high dose WGO = 1.5 x 10^{-3} %. WGO 1 = screw pressed, WGO 2 = supercritical CO_2 extracted after centrifugation, WGO 3 = commercially refined, WGO 4 = commercially hexane extracted wheat germ oil.

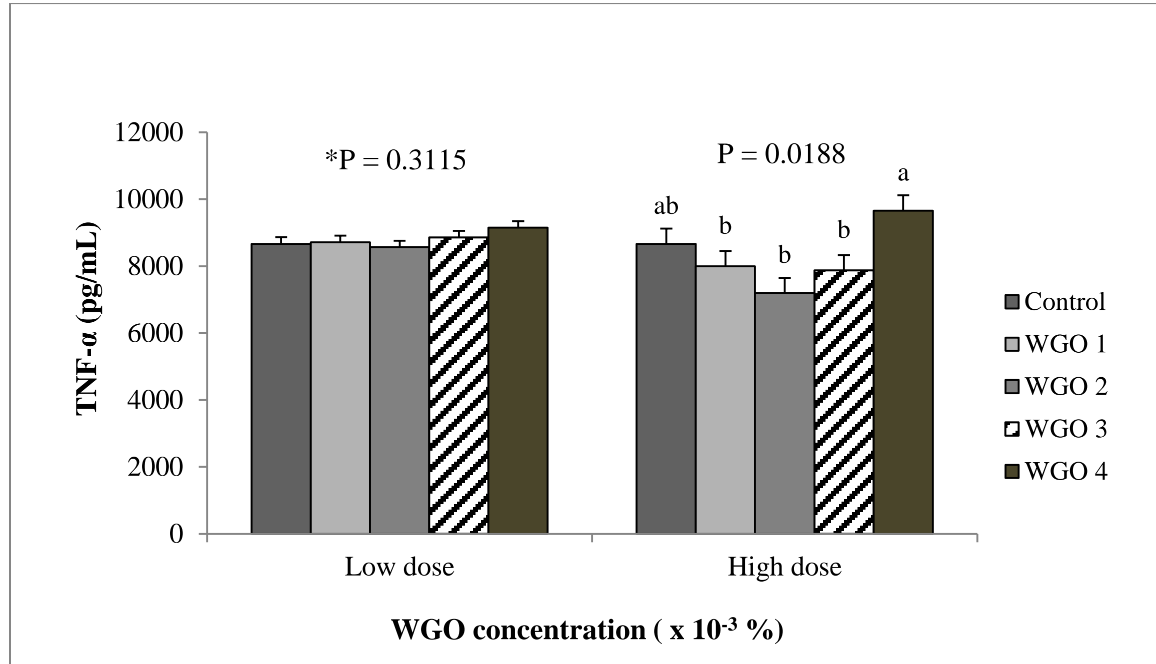
*Control is THP-1 cells treated with vehicle (ethanol equivalent to the high dose WGO concentration) and LPS and considered to have 100 % proliferation. The absorbance of treated cells was compared with control and presented as % of control.

Table 10 Effects of WGO types and concentrations on cytokine production in LPS-stimulated human monocytic THP-1 cells[1]

WGO types	**TNF-α (pg/mL)**	**IL-6 (pg/mL)**	**IL-10 (pg/mL)**
WGO 1	8354.71 ± 270.70[b]	23.16 ± 1.21	20.56 ± 0.88[c]
WGO 2	7883.39 ± 270.70[b]	24.84 ± 1.21	23.09 ± 0.88[bc]
WGO 3	8366.63 ± 270.70[b]	24.45 ± 1.21	27.28 ± 0.88[a]
WGO 4	9405.65 ± 270.70[a]	22.73 ± 1.21	25.58 ± 0.88[ab]
WGO concentration (x 10^{-3} %)			
0.25	8822.93 ± 165.90*	26.72 ± 1.47*	23.37 ± 0.80
1.5	8182.26 ± 165.90	20.87 ± 1.47	24.88 ± 0.80
P values			
Type	0.0045	0.5633	<0.0001
Concentration	0.0264	<0.0001	0.0993
Type*Concentration	0.1084	0.6344	0.3182

[1] Pro- and anti-inflammatory cytokine gene expression was assessed by ELISA (R&D Systems, Minneapolis, MN). Treatment was done in triplicate and the experiment was performed two times. Values are lsmeans ± SE. For each cytokine, values in WGO types that do not share the same letter are significantly different from each other ($P < 0.05$). *indicates significant difference between the two WGO concentrations. WGO 1 = screw pressed WGO, WGO 2 = supercritical CO_2 extracted after centrifugation, WGO 3 = commercially refined, WGO 4 = commercially hexane extracted wheat germ oil.

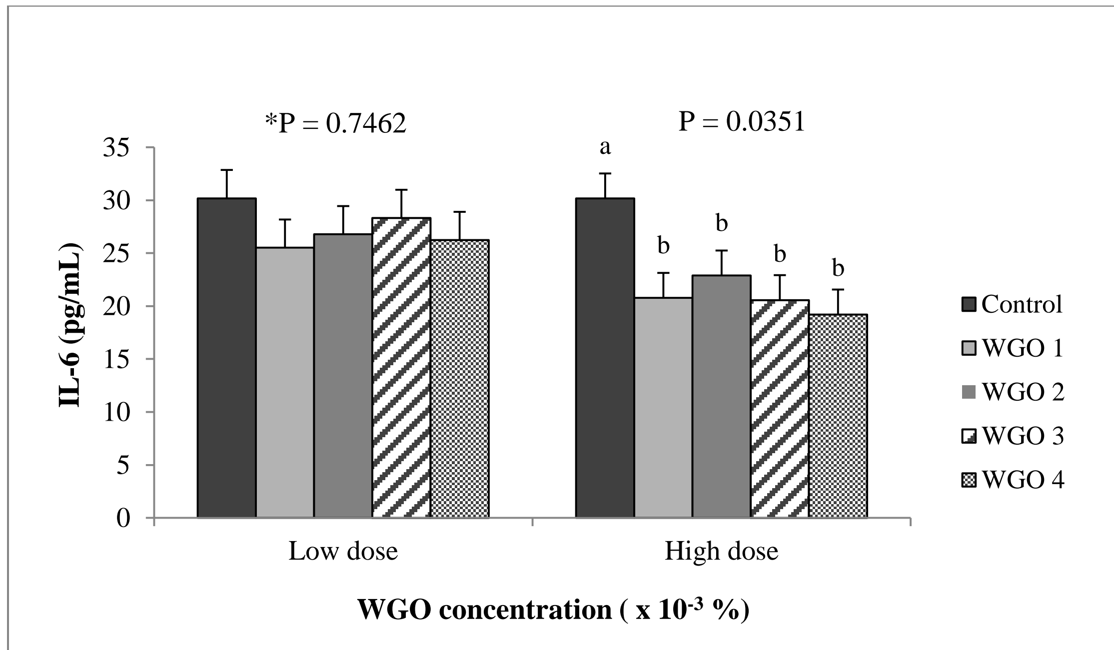
Figure 4 Effects of WGOs at low and high dose on TNF-α production in LPS-stimulated human monocytic THP-1 cells [1]



[1]TNF-α production was measured by ELISA (R&D Systems, Minneapolis, MN). Treatment was done in triplicate and the experiment was performed two times. Bars represent lsmean ± SE; bars that do not share the same letter are significantly different ($P < 0.05$) from each other. Low dose WGO = 0.25×10^{-3} % and high dose WGO = 1.5×10^{-3} %. WGO 1 = screw pressed, WGO 2 = supercritical CO_2 extracted after centrifugation, WGO 3 = commercially refined, WGO 4 = commercially hexane extracted wheat germ oil.

[*]Control is THP-1 cells treated with vehicle (ethanol equivalent to the high dose WGO concentration) and LPS.

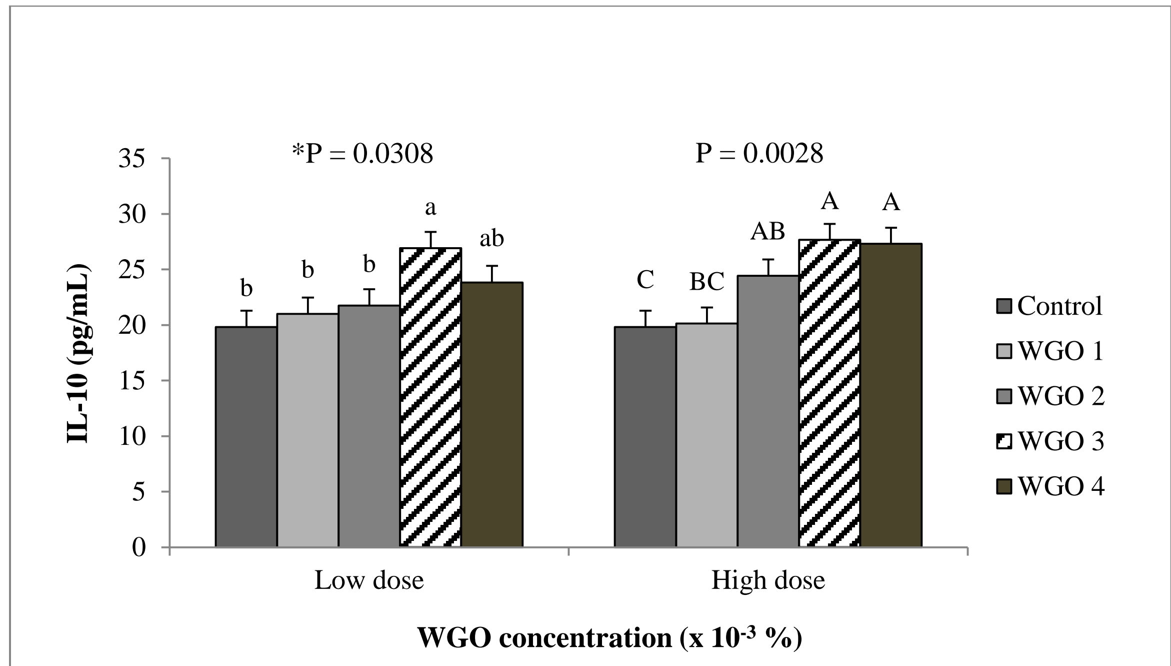
Figure 5 Effects of WGOs at low and high dose on IL-6 production in LPS-stimulated human monocytic THP-1 cells [1]



[1]IL-6 production was measured by ELISA (R&D Systems, Minneapolis, MN). Treatment was done in triplicate and the experiment was performed two times. Bars represent lsmean ± SE; bars that do not share the same letter are significantly different ($P < 0.05$) from each other. Low dose WGO = 0.25×10^{-3} % and high dose WGO = 1.5×10^{-3} %. WGO 1 = screw pressed, WGO 2 = supercritical CO_2 extracted after centrifugation, WGO 3 = commercially refined, WGO 4 = commercially hexane extracted wheat germ oil.

[*]Control is THP-1 cells treated with vehicle (ethanol equivalent to the high dose WGO concentration) and LPS.

Figure 6 Effects of WGOs at low and high dose on IL-10 production in LPS-stimulated human monocytic THP-1 cells [1]



[1]IL-10 production was measured by ELISA (R&D Systems, Minneapolis, MN). Treatment was done in triplicate and the experiment was performed two times. Bars represent lsmean ± SE; bars that do not share the same letter are significantly different ($P < 0.05$) from each other (small and capital letters are comparisons between the low and high dose, respectively). Low dose WGO = 0.25×10^{-3} % and high dose WGO = 1.5×10^{-3} %. WGO 1 = screw pressed, WGO 2 = supercritical CO_2 extracted after centrifugation, WGO 3 = commercially refined, WGO 4 = commercially hexane extracted wheat germ oil.

[*]Control is THP-1 cells treated with vehicle (ethanol equivalent to the high dose WGO concentration) and LPS.

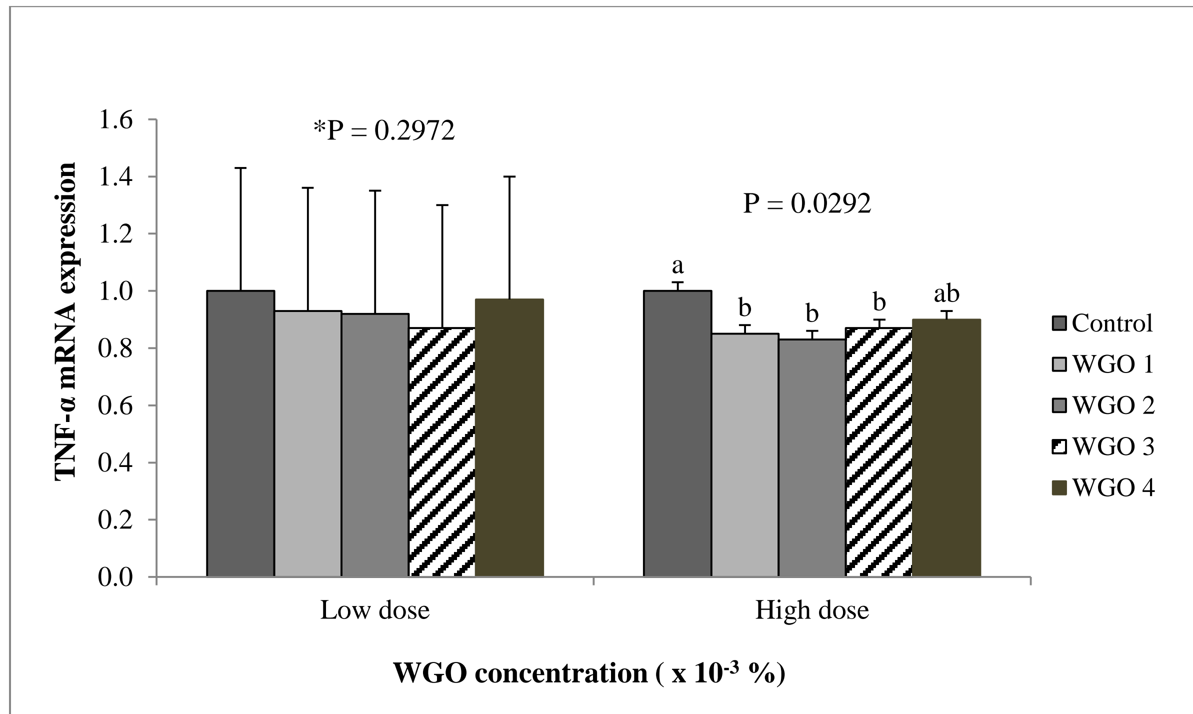
Table 11 Effects of WGO types and concentrations on gene expression of cytokines in LPS-stimulated human monocytic THP-1 cells[1,2]

WGO types	Relative mRNA expression[2]		
	TNF-α	**IL-6**	**IL-10**
WGO 1	0.89 ± 0.03	0.82 ± 0.15	0.95 ± 0.05[B]
WGO 2	0.87 ± 0.03	0.77 ± 0.15	1.17 ± 0.05[A]
WGO 3	0.87 ± 0.03	0.85 ± 0.15	1.33 ± 0.05[A]
WGO 4	0.93 ± 0.03	0.92 ± 0.15	1.26 ± 0.05[A]
WGO concentration (x 10^{-3} %)			
0.25	0.92 ± 0.02	0.88 ± 0.07	1.23 ± 0.05
1.5	0.86 ± 0.02	0.81 ± 0.07	1.12 ± 0.05
P values			
Type	0.5157	0.8135	0.0520
Concentration	0.1021	0.5281	0.2201
Type*Concentration	0.7723	0.8816	0.2631

[1] Pro- and anti-inflammatory cytokine gene expression was measured by RT-PCR. Values are lsmeans ± SE. Treatment was done in triplicate and the experiment was performed two times. For each cytokine, values in types and concentrations that do not share the same letter are significantly different from each other ($P < 0.05$). WGO 1 = screw pressed, WGO 2 = supercritical CO_2 extracted after centrifugation, WGO 3 = commercially refined, WGO 4 = commercially hexane extracted wheat germ oil.

[2]Control is cytokine gene expression of THP -1 cells treated with vehicle (ethanol equivalent to the high dose WGO concentration) and LPS. Each cytokine mRNA expression of WGO treated THP-1 cells was presented relative to control.

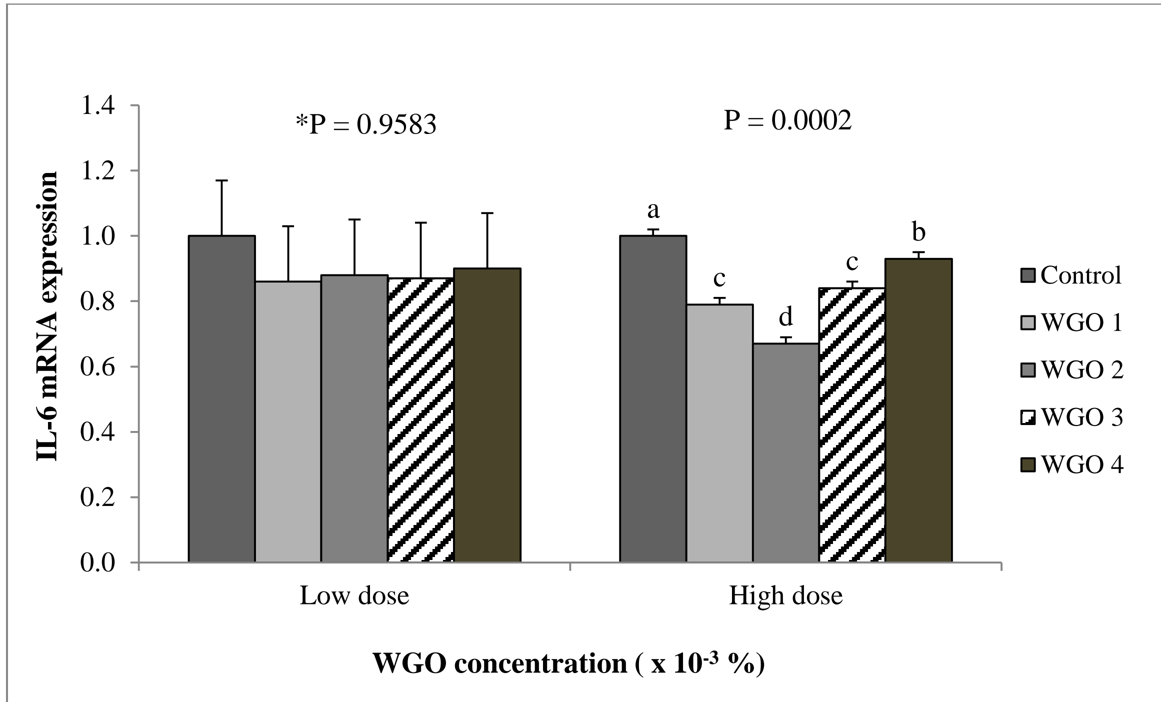
Figure 7 Effects of WGO types at low and high dose on TNF-α gene expression in LPS-stimulated human monocytic THP-1 cells[1]



[1]TNF-α gene expression was assessed by RT-PCR. Treatment was done in triplicate and the experiment was performed two times. Values are lsmean ± SE. Bars that do not share the same letter are significantly different from each other ($P < 0.05$). Low dose WGO = 0.25×10^{-3} % and high dose WGO = 1.5×10^{-3} %. WGO 1 = screw pressed, WGO 2 = supercritical CO_2 extracted after centrifugation, WGO 3 = commercially refined, WGO 4 = commercially hexane extracted wheat germ oil.

*Control is THP-1 cells treated with vehicle (ethanol equivalent to the high dose WGO concentration) and LPS. Each cytokine mRNA expression of WGO treated THP-1 cells was presented relative to control.

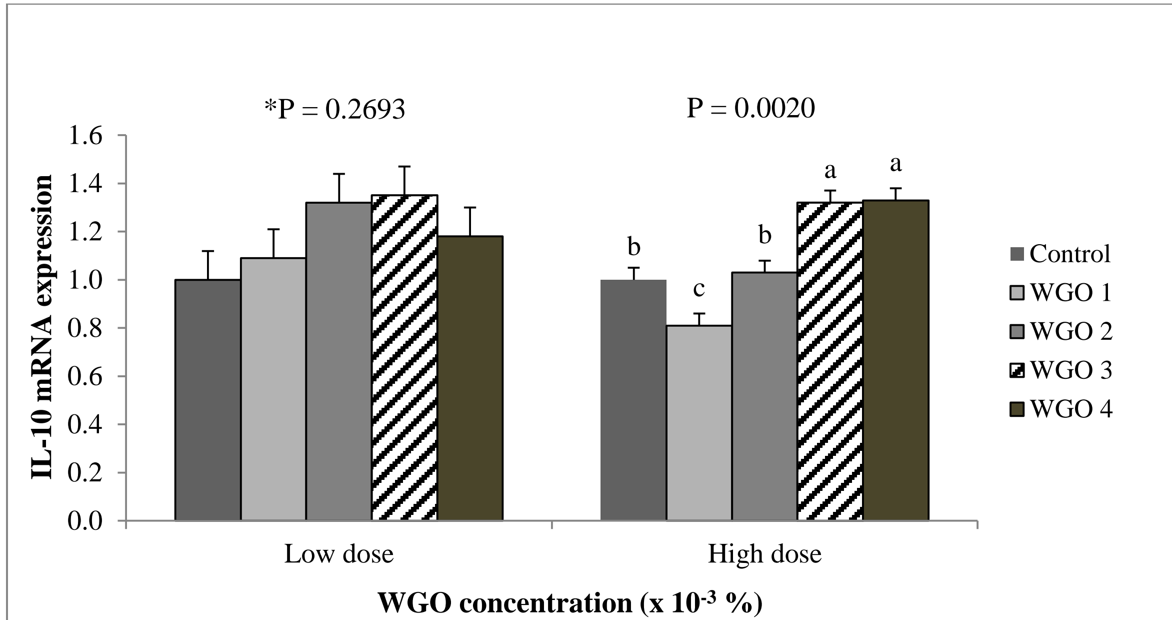
Figure 8 Effects of WGO types at low and high dose on IL-6 gene expression in LPS-stimulated human monocytic THP-1 cells[1]



[1]IL-6 gene expression was assessed by RT-PCR. Treatment was done in triplicate and the experiment was performed two times. Values are lsmean ± SE. Bars that do not share the same letter are significantly different from each other ($P < 0.05$). Low dose WGO = 0.25 x 10^{-3} % and high dose WGO = 1.5 x 10^{-3} %. WGO 1 = screw pressed, WGO 2 = supercritical CO_2 extracted after centrifugation, WGO 3 = commercially refined, WGO 4 = commercially hexane extracted wheat germ oil.

[*]Control is THP-1 cells treated with vehicle (ethanol equivalent to the high dose WGO concentration) and LPS. Each cytokine mRNA expression of WGO treated THP-1 cells was presented relative to control.

Figure 9 Effects of WGO types at low and high dose on IL-10 gene expression in LPS-stimulated human monocytic THP-1 cells[1]



[1]IL-10 gene expression was assessed by RT-PCR. Treatment was done in triplicate and the experiment was performed two times. Values are lsmean ± SE. Bars that do not share the same letter are significantly different from each other ($P < 0.05$). Low dose WGO = 0.25 x 10^{-3} % and high dose WGO = 1.5 x 10^{-3} %. WGO 1 = screw pressed WGO, WGO 2 = supercritical CO_2 extracted after centrifugation, WGO 3 = commercially refined, WGO 4 = commercially hexane extracted wheat germ oil.

*Control is THP-1 cells treated with vehicle (ethanol equivalent to the high dose WGO concentration) and LPS. Each cytokine mRNA expression of WGO treated THP-1 cells was presented relative to control.

CHAPTER V

DISCUSSION

The objectives of this study were to (a) determine the anti-inflammatory effects of wheat germ oils (WGOs) extracted using different processes and (b) compare the dose-dependent effects of the WGOs in inhibiting pro-inflammatory and increasing anti-inflammatory genes and cytokine production in LPS-stimulated THP-1 cells. The hypothesis of this study was that high dose of WGO extracted with supercritical CO_2 will exhibit the most potent anti-inflammatory effects.

WGO was chosen for this study because wheat is a the most important grain grown in Oklahoma and wheat germ, a main by-product of flour milling , is relatively high in nutrients (Brandolini & Hidalgo, 2012). The oil extracted from wheat germ (WGO) also contains several bioactive compounds which have been reported to have a variety of pharmacological properties (Micallef & Garg, 2009; Yang et al., 2010; Zhao et al., 2007; Zhao et al., 2005). ALA and α-tocopherol found in WGO have been reported to have a variety of health benefits, including anti-inflammatory properties (Sridevi Devaraj & Jialal, 2000; Rallidis et al., 2003; Yoshikawa et al., 1998; Zhao et al., 2005). WGO also contains significant amount of policosanols, a combination of aliphatic alcohols which have been reported to lower LDL cholesterol and improve physical performance in human (Cureton & Pohnoorf, 1955; Gouni-Berthold & Berthold, 2002; Irmak & Dunford, 2005; Más et al., 2001). WGO also contains a range of sterols, mainly β-sitosterol and

campesterol, which have been reported to exhibit anti-inflammatory properties (Barnes, 1982; Micallef & Garg, 2009; Navarro et al., 2001). Therefore, exploring the anti-inflammatory of WGO would add value to and support this by-product as an option for chronic inflammatory diseases, including atherosclerosis.

Methods used for extracting the oil may affect the amount and kind of bioactive compounds in WGO. A study by Taniguchi and colleagues found that α- and β-tocopherol in WGO extracted using supercritical carbon dioxide (SC-CO_2) were similar to those of hexane extracted WGO (Taniguchi et al., 1985). Other studies reported that SC-CO_2 extracted WGO had higher tocopherols than that of hexane extracted WGO; however, ALA contents in both WGOs were similar (Molero G mez & Mart nez de la Ossa, 2000). Oil refining process, especially during deodorization, was found to reduce a significant amount of nutritional components in oil, especially tocopherol content (T. Wang & Johnson, 2001). Therefore, it is important to investigate the effects of processing on these bioactive compounds.

In this study, four WGOs processed differently: screw pressed (WGO 1) supercritical extraction with CO_2 (WGO 2), refined (WGO 3) and hexane extracted (WGO 4) were used to determine their anti-inflammatory properties in LPS-stimulated monocytic THP-1 cells. As shown from the composition of these four WGO samples (Tables 2 - 4), processing influenced the concentrations of fatty acids, tocopherols, and sterols. Although WGO has been reported to be a rich source of policosanol (Eisenmenger & Dunford, 2008), there was no policosanol detected in all WGO samples used in this study. Therefore, we focused on ALA, tocopherol and sterol contents of WGO since most research has reported anti-inflammatory effects of these three molecules (Sridevi Devaraj & Jialal, 2005; Navarro et al., 2001; Xie et al., 2011; Yoshikawa et al., 1998; Zhao et al., 2005; G. Zhao et al., 2004).

Our initial experiments involved determining the cytotoxic effects of these WGOs. We found that the types and concentrations of WGOs used in the study have no effects on cell viability and proliferation. These data was consistent with the findings of Zhao and colleagues showing no cytotoxic effects of different doses ALA on THP-1 cells (Zhao et al., 2005). Another study found that different plant oils high in ALA, including olive, canola and flaxseed oils, did not reduce cell proliferation of the aggressive murine melanoma cell line B16-BL6 after 48 h incubation (Buckner, 2014).

The main focus of this study was to compare the effects of the types and doses of WGOs on gene expression and production of pro- and anti-inflammatory cytokines in LPS-stimulated THP-1 cells. TNF-α, a pro-inflammatory cytokine produced by monocytes and activated macrophages and play a vital role in the development of atherosclerosis, was assessed in this study (Al Batran, Al-Bayaty, Al-Obaidi, Hussain, & Mulok, 2014). It is known as "a master inflammatory cytokine" due to its ability to stimulate a number of inflammatory gene including IL-1β, MCP-1, ICAM-1, and VCAM-1 in several cell types, including macrophages (Al Batran et al., 2014). TNF-α also stimulates angiogenesis, smooth muscle cell proliferation (Ait-Oufella et al., 2011), and secretion of IL-6 from monocytes (Haddy et al., 2003). IL-6 appears to regulate hepatic CRP synthesis, resulting in increased inflammation (Stenvinkel, Barany, Heimbürger, Pecoits-Filho, & Lindholm, 2002). Our results showed that the high dose of all WGOs types, except WGO 4, significantly decreased TNF-α production. WGO 2 which contains the highest amount of ALA and α-tocopherol was the most effective. All WGOs at high dose was also found to reduce IL-6 secretion. Our findings are in agreement with what is reported in the literature. Daily oral administration of WGO (270 mg/kg) in mice for 21 days before injection of LPS (200 mg/kg) prevented the increase in plasma TNF-α and IL-6 and lipid peroxidation of liver cell membrane (Hussein, Abdel-Aal, & Elghwab, 2014). Substituting walnuts (37 g) and walnut oil (15 g), good sources of ALA like WGO, for half of fat found in an average American diet

reduced IL-6, IL-1β, and TNF-α production after six week intervention in hypercholesterolemic men and women (Zhao et al., 2007). El-Marasy et al. (2012) also found that pretreatment with WGO (170 mg/kg) for 14 days reversed scopolamine-induced memory impairment by reducing TNF-α level and increased glutathione in the brain of mice.

In our study, both doses of WGOs were sufficient to up-regulate the production of the anti-inflammatory cytokine IL-10. Both WGO 3 and 4 which contain high concentrations of sterols were potent in increasing IL-10 production. IL-10 is produced mainly by monocytes and macrophages and has been reported to potentially down-regulate pro-inflammatory cytokines, including TNF-α and IL-6, but the secretion of IL-10 usually follows that of pro-inflammatory cytokines (Schieffer et al., 2004; Stenvinkel et al., 2005). Phytosterols, rich in WGO, have been reported to have an anti-inflammatory effect (Jiang & Wang, 2005). Diet rich in phytosterols (2 %, w/w) was found to reduce atherosclerotic lesions in apoE KO mice due to its ability to decrease production of IL-6 and TNF-a, and increase levels of IL-10 in LPS-stimulated spleen cells derived from the mice (Nash et al., 2005). Phytosterols were also reported to increase IL-10 level and suppressed IL-1β in colon tissue of mice with colitis (Vitor et al., 2009). The potent effect of WGO 2 (SC-CO_2 extracted) in inhibiting TNF-α and IL-6 production compared to the other WGO types may be due to its higher ALA and α-tocopherol content. The high content of sterols in WGO 3(refined) and WGO 4 (hexane extracted) may play a role on their effectiveness in increasing IL-10 secretion. However, this statement is speculative at this point and needs to be further investigated. Our findings demonstrated that processing can affect the anti-inflammatory properties of WGO. However, we cannot determine which of bioactive compound(s) in WGO is responsible for this anti-inflammatory effect.

Most studies investigating the anti-inflammatory properties of plant oils have attributed it to the ALA, tocopherol, and sterol contents (Micallef & Garg, 2009; Yang et al., 2010; Zhao et al., 2007; Zhao et al., 2005). A study investigating the anti-inflammatory properties of different

doses (0-100 μM) of ALA found that pretreatment of THP-1 cells with ALA before LPS stimulation, inhibited TNF-α, IL-6 and IL-1β production and down-regulated gene expression of these cytokines in a dose-dependent manner (Zhao et al., 2005). This anti-inflammatory activity of ALA is due to inhibition of NF- κB DNA binding activity which is needed for the induction of IL-6, IL-1β, and TNF-α gene expression. ALA also up-regulated peroxisome-proliferator activated receptor ϒ (PPARϒ) activity which inhibits production and gene expression of IL-6, IL-1β, and TNF-α in monocytes (Zhao et al., 2005). Supplementation of pure α-tocopherol (1200 IU/d) for 3 months significantly decreased plasma CRP and monocyte's release of IL-6 in type 2 diabetes (Devaraj & Jialal, 2000).

The anti-inflammatory effects of plant oils were also demonstrated in several studies (El-Marasy, El-Shenawy, El-Khatib, El-Shabrawy, & Kenawy, 2012; Jiménez-Gómez et al., 2009; Masterjohn, 2007; Papageorgiou et al., 2011). Like WGO, flaxseed oil (53.3 g/100 g) is an excellent source of ALA (Simopoulos, 2002) and was reported to attenuate serum TNF-α in IL-10 knockout (KO) mice with an intestinal inflammation, an animal model of human inflammatory bowel disease (IBD) (Cohen, Moore, & Ward, 2005). Higher supplementation dose of ALA from ground flaxseed (10% w/w) in cholesterol-rich diet was reported to be more potent than the lower dose (5 and 1% w/w) in decreasing IL-6 and VCAM-1 expression in aortic tissues of LDL receptor knockout mice (Dupasquier et al., 2007). A study by Rallidis and colleagues demonstrated that linseed (flaxseed) oil (a rich source of ALA) supplementation (15 mL) three times per day for three months, significantly decreased CRP and IL-6 levels in patients with dyslipidemia (Rallidis et al., 2003). The authors suggested that these effects are likely be due to high ALA content of linseed oil which decreased arachidonic acid metabolites, prostaglandin E_2 and leukotriene B_4, which reduce IL-6 release.

In addition to cytokine production, we also investigated the effects of WGO on cytokine gene expression. At high dose, all types of WGO reduced TNF-α gene expression except WGO 4

which has an intermediate effect. All WGOs types at high dose significantly down-regulated IL-6 gene expression; with WGO 2 exhibiting the most potent effect. The concentration of WGOs has an effect on IL-10 gene expression. High dose of WGO 3 and WGO 4 were the most effective in increasing IL-10 gene expression. The effects of doses and WGO types on gene expression of the cytokines are consistent with TNF-α, IL-6 and IL-10 found in the cell media. Other plant oils have been shown to affect gene expression of cytokine involved in the development of atherosclerosis. The consumption of breakfast containing virgin olive oil high in phenol content (398 ppm) was found to down-regulate expression of pro-inflammatory genes in peripheral blood mononuclear cells derived from participants with metabolic syndrome (Camargo et al., 2010). Supplementation of flaxseed oil (5%) with or without 3.3% ALA ester of plant sterol for 18 weeks, reduced TNF-α and IL-6 protein in plasma and mRNA expression in liver, aorta, and monocytes of apoE-KO mice while IL-10 was not affected (Han et al., 2015).

Our findings on the effects of WGO on cytokine gene expression are also consistent to those investigating individual compounds that are also present in WGO. For example, Zhao and colleagues (2005) reported that pretreatment of THP-1 cells with different doses of ALA dose-dependently down-regulated TNF-α and IL-6 gene expression. Devaraj et al. (2005) reported that 3-month supplementation of high dose of α-tocopherol (1200 IU/day), significantly decreased TNF-α secretion and mRNA in LPS-activated monocytes isolated from healthy subjects. This effect is likely due to the ability of α-tocopherol to inhibit NF-κB and the lipoxygenase pathway, a key pathway involved in TNF-α production (Devaraj & Jialal, 2005). α-tocopherol was also able to suppress protein and mRNA of levels of IL-6 in THP-1 derived macrophages stimulated by ritonavir, a drug for HIV-infection (Guo et al., 2007).

In summary, high dose WGOs were able to inhibit pro-inflammatory cytokine (TNF-α and IL-6) production and gene expression in LPS-stimulated human monocytic THP-1 cells; with WGO extracted using SC-CO_2 being the most potent. Both doses of WGOs up-regulated

production and gene expression of the anti-inflammatory cytokine, IL-10; with refined and hexane extracted WGOs being the most potent. Our findings demonstrate that dose and WGO processing affects cytokine production and gene expression.

There were few limitations of our study. Since THP-1 cells are suspension cells; we were not able to aspirate the treatment at the end of incubation period without losing a significant amount of cells. Therefore, the treatment was incubated together with reagents while assessing cell proliferation with the MTT assay. However, we also assessed cell viability using trypan blue staining and the findings of these two assays were in agreement. Additionally, this study used an *in vitro* approach. Although we used an immortalized monocyte cell line derived from human as a study model to closely mimic characteristics of monocytes in human body, the findings from this study should be interpreted carefully and cannot apply directly to humans. *In vivo* studies are needed to support our findings. However, the dose of WGO to be used in an *in vivo* study also needs to be determined. The bioactive compounds in WGO responsible for this anti-inflammatory effect also need to be explored. For example, comparing the anti-inflammatory effect of ALA, tocopherols and sterols individually or in combination to that of WGO. Finally, the final concentration of ethanol in the control was similar to that in high dose but not low dose. This may affect the accuracy of the results when comparing low dose WGO with the control.

In this study, our experimental design utilized WGO incubated together with LPS. Our findings demonstrate the anti-inflammatory effects of WGO during inflammation. Experimental design that pre-treats the cell with WGO prior to LPS stimulation would demonstrate protective effect against inflammation. Whether WGO can also reverse inflammatory response needs to be assessed. Finally, there are several dietary oils being reported to have anti-inflammatory activities, such as flaxseed oil, walnut oil and olive oil (Brunelleschi et al., 2007; Camargo et al., 2010; Jiménez-Gómez et al., 2009; Park, Park, Hayek, Reinhart, & Chew, 2011) Further studies comparing the anti-inflammatory properties of WGO with these dietary oils could also be done.

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APPENDICES

VITA

Sawanya Janthachotikun

Candidate for the Degree of

Master of Science

Thesis: THE ANTI-INFLAMMATORY EFFECTS OF WHEAT GERM OIL ON LIPOPOLYSACCHARIDE-ACTIVATED HUMAN MONOCYTIC THP-1 CELLS

Major Field: Nutritional Sciences

Biographical:

Education:

Completed the requirements for the Master of Science in Nutritional Sciences at Oklahoma State University, Stillwater, Oklahoma in May, 2015.

Experience:
Research and teaching assistant in Nutritional Sciences at Oklahoma State University

Professional Memberships:
Student member of American Society for Nutrition
Student member of Academy of Nutrition and Dietetics
Student member of Oklahoma Academy of Nutrition and Dietetics
Student member of The Honor Society of Phi Kappa Phi
Student member of The Golden Key International Honor Society