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## Anti-Inflammatory and Anti-Oxidative Properties of Dried Plum Polyphenols in RAW264.7 Macrophage Cells

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ANTI-INFLAMMATORY AND ANTI-OXIDATIVE PROPERTIES OF DRIED PLUM  
POLYPHENOLS IN RAW264.7 MACROPHAGE CELLS

By

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## NOMENCLATURE

BCA	Bicinchoninic acid assay
COX	Cyclooxygenase
CRP	C reactive protein
EPA	Eicosapentanoic acid
GAE	Gallic acid equivalent
IF $\gamma$	Interferon gamma
IL-1 $\beta$	Interleukin-1 beta
iNOS	Inducive nitric oxide synthase
LPS	Lipopolysaccharide
MDA	Malondialdehyde
NF $\kappa$ B	Nuclear factor kaapa B
NO	Nitric oxide
NSAIDs	Nonsteroidal antiinflammatory drugs
PBS	Phosphate buffer saline
PG	Prostaglandins
PKC	Protein kinase C
PPAR- $\gamma$	Peroxisome proliferator activated receptor- $\gamma$
PVDF	Polyvinylidene diflouride
RANKL	Receptor activator of NF $\kappa$ B ligand
SDS	Sodium dodecyl sulphate
TLR	Toll like receptors
TNF- $\alpha$	Tumor necrosis alpha



## ABSTRACT

This thesis presents the anti-inflammatory and anti-oxidative properties of dried plum polyphenols in RAW264.7 macrophages. We hypothesize that dried plum polyphenols may benefit in diseases of chronic inflammatory origin. Chronic inflammation is characterized by accumulation of macrophages at injury site and macrophages start the inflammatory cascade. Polyphenols have been found to possess strong anti-inflammatory and anti-oxidant properties. To substantiate this assumption, we tested the chlorogenic acid rich dried plum polyphenols using RAW264.7 macrophage cells. Macrophages express nitric oxide (NO) and cyclooxygenase-2 (COX-2) dependent prostaglandins under chronic inflammatory conditions. RAW264.7 macrophage were stimulated with 1  $\mu\text{g/ml}$  of bacterial lipopolysaccharide (LPS) to induce inflammation and treated with different doses of polyphenols (0, 0.1, 1, 10, 100 and 1000  $\mu\text{g/ml}$ ), for 12 hrs. Polyphenols at a dose of 1000  $\mu\text{g/ml}$  of medium was able to significantly reduce the NO production by 43% compared with LPS stimulated cells only. Moreover, LPS (10 ng/ml) induced expression of COX-2 was significantly reduced by 100 and 1000  $\mu\text{g/ml}$  polyphenols. To investigate the anti-oxidant activity of polyphenols, macrophage cells were stimulated with 100  $\mu\text{g/ml}$  of  $\text{FeSO}_4$  + 1mM/ml of  $\text{H}_2\text{O}_2$  to induce lipid peroxidation. Polyphenols at a dose of 1000  $\mu\text{g/ml}$  showed 32% reduction in malondialdehyde production. Conclusively, our study indicated that dried plum polyphenols are potent anti-inflammatory and anti-oxidative agents and can have implications in prevention of diseases of chronic inflammatory origin including bone loss.

## CHAPTER 1

### INTRODUCTION

Inflammation is the oldest defense and healing mechanism against tissue injuries. Collectively, inflammation is characterized by redness, heat, swelling and pain which are the result of host tissue challenged by microbial toxins, tissue injury or traumatic conditions (1). Inflammation can be acute or chronic (1). Acute inflammation evades early but chronic inflammation persists for a long time and may cause a plethora of diseases including osteoarthritis, asthma, atherosclerosis, inflammatory bowel disease, psoriasis, cancer, Crohn's disease and ankylosing spondylitis (2). According to Global Information Inc. (3), the cost of anti-inflammatory drugs in year 2005 was \$31.1 billion and this number is expected to reach \$47.8 billion in 2010. Approximately, 60 million Americans use the non steroidal anti-inflammatory drugs (NSAIDs) annually to treat inflammation related diseases and especially rheumatological disorders and arthritis (4). However, NSAIDs like aspirin have been reported to induce gastrointestinal (GI) discomfort due to their cyclooxygenase-1 (COX-1) inhibitory effects, which are implicated in GI protection (5). The distinction between COX-1 and COX-2 leads to the development of selective COX-2 inhibitors to prevent the GI side effects of NSAIDs. COX-2 is an inducible enzyme and responds to hormones, cytokines, stress, growth factors and found increased in diseases of chronic inflammation whereas COX-1 is a constitutive enzyme and coupled to COX-2 in physiologic conditions (6). However, certain selective COX-2 inhibitor NSAIDs have been found to increase cardiovascular risk (7) because selective COX-2 inhibitors prevent formation of prostanoids which are essential in vasodilatation and as a result the COX-1 mediated thromboxane remains unaffected. Thromboxanes and prostanoids have antagonistic effects and disturbance in their balance poses the risk of cardiovascular diseases (8). To reduce the GI damage, proton pump inhibitor therapy has been introduced in the treatment (9). Proton pumps are the proteins which are responsible for secreting  $H^+$  directly in the stomach. However, studies have shown that use of proton pump inhibitors in NSAIDs related GI damage, is associated with the risk factor of *Clostridium difficile* linked diarrhea (10). Moreover,

some researchers also have found acute renal failure as a result of NSAIDs use (11). NSAIDs inhibit the production of renal prostaglandins and negatively affect glomerular filtration rate and salt excretion (12). In this scenario, a sustainable cure is required for the treatment of inflammation related diseases.

Currently, research has shown the anti-inflammatory properties of several plant based bioactive compounds (13) but the exact mechanism of action is not known (14). These plant based compounds have been shown to have antioxidant activity (15). In a study by Hooshmand et al. (18), genistein a bioactive compound in soybeans, was found to reduce the levels of proinflammatory markers in chondrocytes. Recently, effect of dried plum polyphenols has been observed on treatment of colon cancer (16) and bone loss (17). These beneficial effects of dried plum polyphenols in part can be due to their anti-inflammatory property. However, no study has examined the anti-inflammatory properties of dried plum and its mechanism of action.

The purpose of the present study was to determine whether dried plum polyphenols inhibit the synthesis of pro-inflammatory molecules and oxidative stress *in vitro*. Once, the mechanism of action at cellular level is known, that knowledge can be applied in formulation of new treatments, nutritional therapy design and pharmaceutical development for treatment and prevention of diseases of inflammatory origin.

**Hypothesis:** Dried plum polyphenols have potent anti-inflammatory and anti-oxidative properties. To test this hypothesis, we have the following two specific aims:

**Specific Aim-1:** To investigate the extent to which dried plum polyphenols suppress the production of pro-inflammatory molecules in RAW 264.7 macrophage cells, stimulated by lipopolysaccharide (LPS). To achieve this aim the production of inflammatory markers such as nitric oxide (NO) and COX-2 were tested in cells using Griess Reagent assay and western blot analysis, respectively.

**Specific Aim-2:** To examine whether dried plum polyphenols suppress the production of oxidative stress markers using RAW 264.7 macrophage cells stimulated by H<sub>2</sub>O<sub>2</sub>+ FeSO<sub>4</sub>. To achieve this aim Malondialdehyde (MDA) level, a sensitive oxidative stress

marker, was measured in cell lysates using Thiobarbituric Acid Reactive Substances (TBARS) kit.

## CHAPTER 2

### REVIEW OF LITERATURE

#### **2.1. Acute Inflammation:**

Inflammation is an essential response of the immune system to restore homeostasis after injury to any tissue. Within the seconds of tissue injury, the symptoms of inflammation start with dilation of blood vessels, increase in blood flow, increase in capillary permeability and neutrophil migration into interstitial spaces. These activities produce the classical symptoms of redness, swelling, heat and pain (1). The cells at injury site produce a variety of inflammatory mediators such as prostaglandins, leukotrienes and histamines (19). These mediators bind to the endothelial cell receptors and cause vasodilation and diapedesis (19). Prostaglandins are the pro-inflammatory molecules generated from the arachidonic acid by the action of COX-2 enzyme and impart role in vasodilation and blood flow (20). The capillaries surrounding basement membrane also rearrange themselves, in response to inflammatory mediators to facilitate the filtration of plasma macromolecules to the juxtaposed tissue. At the injury site, smooth muscles inside blood vessels contracts and the blood flow becomes slow through the smaller capillaries (21). As a result, more leukocytes can adhere to the capillary walls. Moreover, the space between endothelial cells in smaller blood vessels increases due to the contraction of endothelial cells. This process increases the diameter of blood vessels and called vasodilation (22). The endothelial cells on inner walls of blood capillaries express adhesion molecules. These adhesion molecules attach to the integrins present on leukocytes (23). This process results in flattening and filtration of leukocytes into surrounding tissue and called extravasation or diapedesis of leukocytes. Neutrophils are the characteristic immune cells found in majority at the site of injury in acute inflammation (24). These cells are phagocytic in nature and can ingest microorganisms or particles and generate reactive oxygen species and hydrolytic enzymes to destroy them (25). The cells at afflicted sites produce interleukin-8, interferon gamma and complementary proteins and monocytic chemotactic proteins in response to injury, which

generate a chemotaxic movement of neutrophils towards injury site (26;27). Moreover, degranulation of neutrophils occurs at the site which release lactoferrin, cathelicidin, bactericidal increasing proteins, defensins, cathapsins and gelatinases to destroy the foreign particle or antigen and expedite the inflammatory process (24).

Acute inflammation is a very useful process in maintaining tissue homeostasis. It activates blood platelets (28), provide antibodies (29), and facilitate adhesion of microorganisms to phagocytic cells (30). Lysozymes, cathelidins and defensins secreted by neutrophils degrade peptidoglycan, cleave peptides and can neutralize LPS, create pores in cytoplasmic membranes of bacteria, respectively (31;32). Moreover it compensates for the increased demand of nutrients at the injury site and deprives the microbes from iron by providing transferrin (33;34). Phagocytic activities in acute inflammation increase the infiltration of leukocytes and lymphocytes, which kills the infected and antigen loaded cells (35;36).

## **2.2. Resolution of Acute Inflammation:**

In chronic inflammation, neutrophils are the primary granulocytes that accumulate at injury site. Removal of accumulated neutrophils is a necessary step in resolution phase of inflammation. Resident macrophages release interleukin-1 (IL-1) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) to stimulate natural killer (NK) cells and T lymphocytes to release interferon gamma (IF $\gamma$ ). IF $\gamma$  binds back to macrophages and cause the release of fibroblast growth factor (FGF) to start tissue remodeling (37;38). The endothelial and fibroblast cells proliferate and make a network of new capillaries. Collagen is deposited into the injury site to make connective scar tissue to close the exposed or open area. Lipid derived mediators play an important role in resolution of inflammation (39). During inflammation, platelet and leukocyte interaction leads to the formation of lipoxins A<sub>4</sub> and B<sub>4</sub> (40). These lipoxins are the signals of resolution of inflammation as they inhibit the granulocyte recruitment from the post capillary venules (40). At this stage, prostaglandins activate the transcription of 15-lipoxygenase, which catalyzs the formation of lipoxins from arachidonic acid instead of prostaglandins (41). Moreover, eicosanoids form E and D series resolvins and protectins which play role in inflammation resolution (42). At the same time, IL-6 promotes apoptosis of polymorphonuclear cells (PMN) and macrophages engulf the apoptotic PMN. Additionally, at this stage Lipoxin A<sub>4</sub> stimulates macrophages

to release IL-10 and transforming growth factor-  $\beta$  (TGF- $\beta$ ) which may help in fibrosis (43). After clearing the debris, macrophages leave the inflammation site and go to nearest lymph nodes (44).

### **2.3 Chronic Inflammation:**

A successful resolution of acute inflammation does not provide much opportunity to transform into chronic inflammation. However, failure of endogenous pro-resolving mediators, incomplete neutralization of inflammatory stimulus, or persistent attack of stimuli leads to chronic inflammation (38). Chronic inflammation can be characterized by increased angiogenesis (45), fibrogenesis (46), monocyte infiltration (47) and necrosis (48). Angiogenesis is the process of formation of new blood vessels from existing one. However, when this process becomes uncontrolled it may lead to chronic inflammation. Many cells are known to produce angiogenic factors such as fibroblast, mast cells and macrophages (49). Macrophages are the potent source of the angiogenic factors and cytokines which are capable to start the angiogenesis (50). A number of mediators such as granulocyte colony stimulating factor (G-CSF), granulocyte monocyte colony stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), basal fibroblast growth factor (bFGF), TGF- $\beta$ , TNF- $\alpha$ , prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), NO, IL-1, IL-6, IL-8 and platelet activating factor act directly or indirectly on endothelial cells and promote angiogenesis (45). TGF- $\beta$  produced from macrophages acts on fibroblasts and initiate fibrosis (51). Thrombin is produced in the coagulation process and considered as a pro-inflammatory molecule (52). It activates endothelial cells to produce IL-6 and monocyte chemoattractant protein-1 (MCP-1) and is considered a link between acute to chronic inflammation transformation (52). MCP-1 then attracts inflammatory monocytes to endothelial cells and after diapedesis they migrate to specific tissue and transform to macrophages, for further inflammatory action (53). In chronic inflammation neutrophils are replaced by monocytes which become tissue macrophages. Macrophages can be activated by lymphocytes, immune complexes and complementary proteins (54). The activated macrophages release hydrolytic enzymes, cytolytic C3a and C3b proteins, thromboplastin and polyamine oxidase for lysis of tumor cells (55). Batista et al. (56) demonstrated that activated macrophages express higher amounts of pro-inflammatory molecules such as IL-1 $\beta$ , IL-6, TNF- $\alpha$ , hydrogen peroxide and increased phagocytic and

chemotactic capacity in *in vitro* chronic inflammation model (56). Macrophages secrete TGF- $\beta$  for fibrogenesis; however, it also modulates MCP-1 level and causes more attraction of inflammatory monocyte from blood stream to inflammation site (56A). In the presence of persistent antigen stimuli, activated macrophages start the destruction of normal tissues by these activities and causes diseases of chronic inflammatory origin (55).

#### **2.4. Biomarkers of Inflammation:**

NO and COX-2 are considered important biomarkers of inflammation in *in vitro* models. NO is a key molecule in pathophysiology of inflammatory reactions and can induce various pro-inflammatory cytokines such as IL-1, IL-6, IL-8, TNF- $\alpha$  in various leukocytes and lymphocytes. Christiane et al. (57) showed that NO stimulates neutrophils to produce IL-8, which promotes the chemotaxis of PMN cells in a dose dependant manner. *In vitro* cell culture study proved that NO up-regulates TNF- $\alpha$  in U937 human peripheral blood mononuclear cells (58). Induction of NO during persistent inflammation causes DNA damage and may cause cancer in some cases (59). NO also causes increased expression of a reliable and important marker of inflammation, COX-2 (252). COX-2 is an inducible enzyme which acts on arachidonate (a lipid mediator) and produces pro-inflammatory PGs. PGE<sub>2</sub> is a potent molecule in inflammation and induces the production of various pro-inflammatory cytokines such as IL-6 and IL-1 (60). Portanova et al. (61) used anti-PGE<sub>2</sub> monoclonal antibody 2B5 in carragenan induced paw inflammation model of rats. Pretreatment of this antibody prevents the symptoms of edema and hyperalgesia in afflicted paws. Moreover, reduction in mRNA level of IL-6 was observed in afflicted tissue. Anderson et al. (62) demonstrated that adjuvant induced arthritis in rats causes increase in local COX-2 mRNA expression. Inhibition of COX-2 by selective COX-2 inhibitor causes decrease in COX-2 mRNA, IL-1 and IL-6 mRNA level in serum. Moreover, it also decreases inflammatory cell infiltrate in synovium. These studies showed an indirect impact of COX-2 expression in inflammation.

In addition, C-reactive protein (CRP) is also considered as a good serum marker of inflammation and synthesized mainly by hepatic cells (63) and adipocytes (64). The main function of CRP is to bind the foreign particles and damaged cells to make easier for phagocytosis (65). NO and COX-2 stimulate the production of IL-6 in various cells.



IL-6 acts on hepatic cells to produce CRP in the serum (66). Increased CRP in serum is associated with various diseases such as atherosclerosis (67), osteoarthritis (68) and cancer (69). Some lipid peroxidation products such as malondialdehyde have been also used in literature as inflammatory markers (77). During inflammation, NO combines with ionic oxygen and forms peroxynitrites. Peroxynitrites then react with lipids in cell membranes and stimulates the production of molecules which causes DNA damage or up-stream regulation of pro-inflammatory molecules by acting at transcription level (78).

## **2.5. Inflammation Related Chronic Diseases:**

If not properly resolved, acute inflammation leads to chronic inflammation. In chronic inflammation, the continuous efforts of immune system to recover from injury, results in change of surrounding tissue physiology and destruction of nearby cells. Release of cytokines and chemokines in blood stream may pose systemic responses and results a plethora of diseases such as cancer, atherosclerosis, arthritis, Alzheimer's disease, osteoporosis, diabetes and obesity.

### **2.5.1. Cancer:**

In 2008, the estimated new cases of all cancers were 1,437,180 and estimated mortality due to cancer was 565,650 with an economic impact of \$219.2 billion (79). Cancer is the result of uncontrolled growth of cells which invade the surrounding tissues and impair the function of the tissues. The carcinogenic cells may metastasize in other tissues and alter their functions as well. The process of carcinogenesis is generally divided into three phases consisting of initiation, promotion and progression. Inflammation can have an impact on all of the three phases. In 1863, Rudolph Virchow (80) found leukocytes in tumors and proposed that it portrays the chronic inflammation as cancer origin. In chronic inflammation, inflammatory cells produce NO. The released NO in the milieu cause initiation of carcinogenic process by causing mutagenic changes such as alteration in DNA base pairs, breakage of DNA strands, enhance expression of proto-oncogenes and degradation of tumor suppressor genes (81). Graziewicz et al. (82) showed that DNA ligases (DNA repair enzymes) exposed to reactive nitrogen species were not active in repair of single strand interruptions in double stranded substrate. Reactive nitrite species not only causes mutations in DNA but also alter RNA, proteins and initiate tumorigenesis (83). Gallo et al. (84), observed the role of NO in angiogenesis

and tumor promotion in head and neck cancer. Tumor specimens and human squamous carcinoma A-431 cells were found to express more angiogenesis when supplied with NO on as compared to non NO group (84). An increased rate of angiogenesis leads to neoplastic tissue formation. NO also act on preformed tumor cells and cause tumor cell progression and metastasis by inducing tumor cell proliferation, migration and invasion (85). Chronic inflammation mediates the onset of cancer either from viral or bacterial infections or from hazardous chemical exposure and epigenetic changes. Cancer due to *Helicobacter Pylori* is a well established model of infection induced gastric cancer (86). *H. pylori* binds to epithelial cells and stimulate immune response containing interferon- $\gamma$ , TNF- $\alpha$  and IL-12 and other pro-inflammatory cytokines, growth regulated oncogene- $\alpha$ , macrophage inflammatory protein-1  $\alpha$  and monocyte chemotactic protein through activation of transcription factor NF $\kappa$ B (87). The bacteria persist in the intestine for decades and source the antigens consistently. The immune system increase chronic inflammatory response which cause cellular damage and increased cell turnover and finally induce carcinogenesis. At cellular levels, accumulation of leukocyte increase release of reactive nitrogen and oxygen species which upregulate the expression of COX-2 enzyme (86). Expression of COX-2 is pivotal in chronic inflammation as it catalyze the formation of prostaglandins. Evidences shows that over-expression of COX-2 in colon carcinoma cells causes endothelial cell migration and angiogenesis by the production of PGs and proangiogenic factors (88;89). In hepatocellular carcinoma, COX-2 has been found to increase angiogenesis via the pathway of vascular endothelial growth factor (VEGF) (90). Human cervical carcinoma study showed that increased expression of COX-2 is related to increased tumor growth and metastasis through the expression of VEGF expression (91).

### **2.5.2. Atherosclerosis:**

The origin of atherosclerosis includes onset of chronic inflammation due to endothelial dysfunction and sensitization of immune system. Various factors such as oxidative stress, psychological stress, cytokines, infections, toxins, smoke and dust participate in production of predominantly two kinds of auto-antigens by endothelial cells (93;94). The first one is heat shock proteins (HSP) and second one is oxidized low density lipoprotein (oxLDL). Above mentioned factors modulate the pivotal homeostatic

activities of endothelial cells by increased production of reactive oxygen species (ROS) at mitochondrial level and reduced activity of NO which ultimately change the permeability, inflammatory and vasodilatory properties of endothelial cells which is known as endothelial dysfunction (95). Increased oxidative stress causes the production of HSP60 from both outside and inside of the endothelial cells (96). Production of HSP is the natural response to the stress as these proteins help in configuration of structural and functional proteins (97). Now these proteins become the target of innate immune cells as they mimic some pathogenic HSPs (98). Billack et al. (99) showed that treatment of macrophages and endothelial cells with HSP60, increases COX-2 and iNOS expression in both kinds of cells. This process starts the recruitment of inflammatory cells and initiates inflammation. The second auto-antigen in cardiovascular diseases is oxLDL. LDL is an important molecule in the cholesterol transportation to the peripheral tissues for proper cell function and high density lipoprotein (HDL) carries back the used and disintegrated cholesterol molecule to the liver (100). Local inflammation precedes the LDL retention in intercellular spaces. During inflammation TGF- $\beta$  is produced by the surrounding cells and this causes the increased production of proteoglycans (101). Proteoglycans have negatively charged sulfate groups which bind to the positively charged apo B-100 group of LDL (102). The surrounding enzymes and pro-inflammatory milieu cause oxidation of LDL molecule and this oxidation release lipid peroxides, which activate the inflammatory reactions in nearby cells (103). To substantiate this theory, Norata et al. (104) demonstrated that presence of oxidized LDL in human endothelial cells significantly increases the COX-2 expression. In a double blind study, Chenevard et al. (105) showed that use of celecoxib (selective COX-2 inhibitor) in a dose of 200 mg per day improved endothelium dependant vasodilation and reduced the high sensitivity CRP level in 14 male patients ( Age 63-69 years) afflicted with severe coronary artery disease.

### **2.5.3. Arthritis:**

Arthritis is a joint disease of inflammatory origin characterized by synovial inflammation, synovial hyperplasia, irregular responses of humoral cells and joint pain (18). The destruction of hyaline articular cartilage and modulation of underneath bone which emerges as osteophyte, increase the thickness of subchondral bone and cause stiffness and pain (106). Inflammation has been found to be a primary cause of

osteoarthritis (107). Benito et al., (108), found significant increase in angiogenesis, VEGF, TNF- $\alpha$ , IL-1 $\beta$ , NFkB and COX-2 expression in synovial tissue of patients with early osteoarthritis. In-vitro studies have proved that chondrocytes increase the expression of COX-2 and iNOS and up-regulate the synthesis of PGE<sub>2</sub> and NO when stimulated by pro-inflammatory molecule IL-1 $\beta$  (109;110). However, the real cause of cytokine generation remains unknown but research has revealed that obesity, bone density, gender, nutrient status and genetics may help in cartilage destruction (111).

#### **2.5.4. Alzheimer's Disease (AD):**

Alzheimer's is a brain disease which affects the parts of the brain that control memory, thought and language and generally occur after the age of 60 years (113). Research has suggested that oxidative stress is the leading cause of Alzheimer's disease. In aging populations, decreased copper absorption and increased exit of intracellular copper, makes it unavailable to anti-oxidant enzymes such as superoxide dismutase which are dependent upon copper for proper function (114). Eventually, this process increases the oxidative load of the cell leading to oxidation of membrane lipids and activation of pro-inflammatory molecules. Increased oxidative stress causes formation of amyloid plaques and neurofibrillary tangles by oxidation of polypeptides (115). The formation of amyloid plaques and neurofibrillary tangles in brain tissue leads to formation of clumps. The nerve cells in the area of clumps die and impair neuronal conductivity (115). Highly insoluble and proteolysis resistant fibrils are the main cause of initiation of chronic inflammation inside brain tissue (116). The amyloid plaques and dying cells act as antigenic signals and stimulate the immune cells to release inflammatory cytokines. Chromogranin A is a protein secreted by the neurons in brain and causes activation of iNOS and production of NO in microglial (tissue specific macrophages) cells (117). Microglia and neurons react to the inflammatory antigens and produce the inflammatory cytokines (118). Once these cells are activated they release IL-1 which causes to release IL-6 and both of these interleukins stimulate the release of macrophage colony stimulating factor (M-CSF) (118). After while the inflammatory monocytes and macrophages are recruited to the afflicted area and inflammatory cascade starts. A study on 1285 patients (Age 62-85 year) showed that there is a positive correlation between acute inflammation markers such as CRP, and IL-6 and

neurovascular diseases in older patients afflicted with cognitive decline (119). Fujimi et al. (120) compared the hippocampal tissue (obtained by autopsy) from AD and non-demented subjects and found an increased expression of COX-2 in hippocampus of AD patients as compared to non-demented subjects.

#### **2.5.5. Other diseases of chronic inflammation origin:**

In addition, there are other chronic diseases with chronic inflammatory origin. Chronic inflammation is associated with insulin resistance and obesity. Glucose and fat intake induces low grade inflammation possibly by increasing oxidative stress in leukocytes and monocytes (127). In obesity, higher lipids deposition in fat cells increases their size. Hypertrophic adipocytes produce IL-6, TNF- $\alpha$  and monocyte chemoattractant protein (MCP-1) (128). These inflammatory molecules cause the endothelium to up-regulate the production of inflammatory molecules to recruit monocytes (128). The production of cytokines activates the NF $\kappa$ B (129) signaling pathway inside hepatic cells which make the cells resistant to insulin (130).

Osteoporosis is another disease considered as of inflammatory origin. Osteoporosis is a diseases of bones in which bone resorption by osteoclasts exceeds bone formation and may cause weakening of bones. Osteoclasts are of hematopoietic origin in macrophage lineage (121). Mounting evidences are available of chronic inflammation as a cause of osteoporosis (122). Changhai et al. (123) observed the reverse relationship between circulating pro-inflammatory markers such as IL-6, TNF- $\alpha$  and bone mineral density in older adults (Age 52-78 years, 48% female). Evidences showed that there is positive relationship between oral inflammation and osteoporosis as well (124). Inflammatory molecules upregulate the production of receptor activator for NF $\kappa$ B ligand (RANKL) which binds to RANK and cause differentiation and activation of osteoclast (126).

#### **2.6. Anti-inflammatory Drugs:**

A number of drugs have been developed to cure the diseases of chronic inflammation origin. These drugs can be divided into two groups; steroidal anti-inflammatory drugs and non-steroidal anti-inflammatory drugs (NSAIDs). Steroids are the chemical compounds released by the adrenal gland and have anti-inflammatory action by different mechanisms. As an example, glucocorticoids are the steroidal hormones

which bind to the cell receptors and cause anti-inflammatory reaction. Glucocorticoid receptors are bound with heat shock proteins in cytoplasm. After activation receptors translocate to the nucleus and modulate transcriptional activities (131). Glucocorticoids enhance the expression of nearly 130 genes which include the anti-inflammation, phagocytosis, anti-oxidative stress and suppress the pro-inflammatory genes (132;133). In addition, glucocorticoids may express non genomic pathways by restricting ATP consuming activities and these effects are much more rapid than genomic effects (134). Corticosteroids, another type of steroid hormones, inhibit the activity of phospholipase A<sub>2</sub> and diminish the production of arachidonic acid upon activation of cells by pro-inflammatory molecules (135). Prostaglandins and leukotriens are thus inhibited by corticosteroids through the action of phospholipase A<sub>2</sub> (136). However, a number of side effects are revealed as a result of glucocorticoid use in inflammatory diseases. Glucocorticoids enhance glucose levels by degrading proteins and modulating fatty acid metabolism partly. This catabolic interference by corticosteroids leads to tissue remodeling, osteoporosis, insulin resistance and diabetes (137). Long term use of glucacorticoids increases the apoptosis of hypertrophic chondrocytes in growth plate which reduces the longitudinal growth of bones (138). The second category of anti-inflammatory drugs is non-steroidal anti-inflammatory (NSAIDs) drugs. They show their effect by inhibiting the action of cyclooxygenases instead of phospholipase A<sub>2</sub> and do not prevent the activity of lipoxygenase (135). The drugs such as aspirin inhibit both the activity of COX -1 and 2 but not lipoxygenase. COX-1 is a constitutive enzyme and expressed in physiological conditions while COX-2 is inducible and expressed in inflammatory condition to synthesize the prostaglandins (139). However, inhibition of COX-1 cause gastrointestinal discomfort as this enzyme is implicated in intestinal protection (140). To reduce the gastrointestinal damage, proton pump inhibitor therapy has been introduced in the treatment (9). However, the use of proton pump inhibitors is associated with the risk factor of *Clostridium difficile* linked diarrhea (10). Moreover, some researchers have found acute renal failure as a result of NSAIDs use (11). NSAIDs inhibit the production of renal prostaglandins and negatively affect glomerular filtration rate and salt excretion (12). Hence, synthetic anti-inflammatory drugs are more associated with negative effects rather than positive effects.

## **2.7. Life style and dietary approaches to modify the burden of chronic diseases:**

The chariot of chronic diseases is on the way and going ahead with higher pace than expected. As Benjamin Franklin once said “An ounce of prevention is worth a pound of cure” sounds competitive in case of chronic diseases. Various life style and dietary modifications has been proposed to alleviate the misery of chronic diseases. Regular exercise is one of them and found to prevent chronic inflammation (141). A study by Earl S. (142) on more than 13,000 participants  $\geq 20$  years, showed a decreased serum CRP level after light, moderate and vigorous physical activity. During exercise, contraction of muscle produces acute phase IL-6 which stimulate the production of anti-inflammatory cytokines such as Interleukin-1 receptor antagonist (IL-1ra) and IL-10 in plasma (143). Muscle contraction has also been shown to inhibit TNF- $\alpha$  production in mononuclear immune cells (144). Exercise also prevents the muscle fatigue in cancer patients (145). Physical activity or exercise also may help in alleviating depression/psychological stress, mood swings anxiety and stress responsivity (146) and prevent insulin resistance and metabolic disorder caused by inflammation originated from psychological stress (147). Some other lifestyle modifications such as avoiding smoking, tobacco chewing and alcohol intake can have a positive impact on immune system. Smoking is a great risk factor in atherosclerosis and lung diseases. Kangavari et al. (148) showed that macrophage immunoreactivity and matrix metalloproteinase activity increased significantly in the carotid endarterectomy specimens of smoker compared to non-smoker subjects. Cigarette smoking is associated with asthma by airway inflammation (149). Sputum cell counts, IL-8, eosinophilic anionic protein, inflammatory neutrophil count was found to be significantly higher in smokers compared to non-smokers and depict increased inflammation (149). In a six week smoking cessation study, a considerable improvement in lung function and reduction in sputum neutrophil count was observed compared to smokers who didn't cease to smoke (150). Alcohol consumption is a debatable subject. Imhof et al. (151) showed that moderate alcohol consumption in healthy subjects (2,275 men and 2,186 women, 25 to 74 years) reduces the serum biomarkers of inflammation such as CRP, where as another study suggested that heavy consumption of alcohol causes metabolic syndrome and diseases such as diabetes, coronary heart diseases, stroke and peripheral arterial disease (152).

In addition to life style modifications, alterations in dietary approaches may prevent chronic diseases of inflammatory origin. Researchers have suggested various dietary modifications such as intake of polyunsaturated fatty acids, diets rich in fruits and vegetables and avoidance of refined grains may help in reduction of inflammation. Omega-3 fatty acids in diet show anti-inflammatory activity and inhibit the production of pro-inflammatory molecules such as TNF- $\alpha$ , IL-1, IL-2 and adhesion molecules in mononuclear immune cells and vascular endothelial cells respectively (153). Moreover, substitution of saturated fats with non-hydrogenated unsaturated fats in diet reduces the risk of cardiovascular diseases and diabetes (154). A recent study suggests that eicosapentanoic acid (EPA) and docosaheaxanoic acid (DHA) act through proxisome proliferator activated receptors- $\gamma$  (PPAR-  $\gamma$ ) and inhibit the synthesis of pro-inflammatory molecules (155). Mozaffarian et al. (156) demonstrated significant increase in serum soluble TNF- $\alpha$  receptors, IL-6 and CRP in 823 healthy women after taking trans-fatty acid rich diet for four years. Vegetarian diets with sufficient fiber content are considered as anti-inflammatory diets as well. Research suggested that long term vegetarian diet (5-55 years) significantly improve the antioxidant status by increasing plasma ascorbic acid content and lower the triacylglycerol, uric acid and high sensitivity serum CRP in fasting plasma of thirty vegetarians (mean age 44.2 $\pm$ 9 years) (157). An epidemiological study showed that diets rich in fiber decrease the serum CRP significantly in 3920 participants ( $\geq$ 20 years old) (158). Nuts in vegetarian diets are a rich source of monounsaturated and polyunsaturated fatty acids and high arginine content (159). Arginine is a substrate for NO production in the arteries which considered as a vasodilator and consumption of nuts has shown to lowers the plasma CRP and IL-6 (160). However, avoidance of refined grains is strongly encouraged in the healthy diets because they lose most of the vitamins, minerals, essential fatty acids and fiber during processing. Hyperglycemic diets are also not considered as healthy ingredients to anti-inflammatory effects because they are found to increase serum IL-6 and TNF- $\alpha$  levels (161;162). Fruits and vegetables are good source of fibers, vitamins, minerals and several bioactive compounds. Diets rich in fruits and vegetables have been shown to significantly reduce inflammatory biomarker such as CRP levels in both men and women (445 Hispanic and 154 non Hispanic elders,  $\geq$  60



years) (163). Overall, a whole diet and life style modifications have a potential to prevent the escalating diseases of chronic inflammation.

## **2.8. Selected phytochemicals/ Nutraceuticals/ Functional Foods with anti-inflammatory properties:**

### **2.8.1. Isoflavones:**

Isoflavones are natural compounds found in plants and classified as phytoestrogens as they have similar chemical structure to mammalian estrogens (164). Both *in vivo* and *in vitro* studies have been conducted to prove the anti-inflammatory activities of isoflavones. Jun et al. (166) showed that isoflavones from kudzu such as biochanin-A, genistein, diadzeine and formononetin decrease NO production in LPS stimulated RAW264.7 macrophage cells by 62, 56, 39 and 33%, respectively. Yen et al. (167) also found that soybean isoflavones, genistein and daidzein in a concentration of 25-200  $\mu$  mol/L inhibit peroxynitrite mediated DNA damage in RAW264.7 macrophage cells by inhibiting NO production. Moreover, they demonstrated that genistein and diadzeine prevent the inactivation of antioxidant enzymes such as glutathione peroxidase, glutathione reductase and catalase in sodium nitroprusside stimulated RAW264.7 macrophage cells. Another *in vivo* study by the same group indicated that isoflavones from soyabean (4.0 mg/kg weight) for one week, reduced the NO production in serum of LPS (10 mg/kg weight) stimulated rats (167).

### **2.8.2. Vitamin E:**

Another famous nutrient with anti-inflammatory properties is vitamin E. Leary et al. (168) observed a significant reduction in COX-2 expression in IL-1 $\beta$  stimulated human adenocarcinoma cells treated with 10  $\mu$  mol/L  $\gamma$  tocopherols. All types of vitamin E such as  $\alpha$ ,  $\beta$  and  $\gamma$  tocopherols have shown anti-inflammatory effects but  $\gamma$ - tocopherols expressed highest activity of 56% (168). Vitamin E has been proposed more activity in inflammation caused by aging. Cell culture studies of macrophages from old and young mice showed that vitamin E suppress the LPS induced COX-2 expression, more significantly in macrophages derived from old mice compared to young mice and also inhibited age associated production of PGE<sub>2</sub> (169). Wu et al. (170) demonstrated that vitamin E act through the action on protein kinase C (PKC). Vitamin E prevents the

phosphorylation of PKC and makes it unavailable to act on transcription factors which ultimately stop the expression of inflammatory genes (170).

### **2.8.3 Curcumin:**

Curcumin is a yellow colored phenolic compound found in rhizomes of plant called Turmeric (*Curcuma longa*) and considered as a potent anti-inflammatory nutraceutical. Mounting evidences are present to show the anti-inflammatory activities of curcumin in different diseases (171). Jin et al. (172) showed that curcumin at a dose of 20 µg/ml inhibit the production of NO, PGE<sub>2</sub>, COX-2 mRNA and iNOS mRNA in LPS stimulated (0.5 µg/ml) BV2 microglial cells. Chen et al. (173) demonstrated that curcumin inhibit the synthesis of IL-1β and TNF-α in LPS stimulated RAW264.7 macrophage cells in a dose dependant manner. They also found that curcumin inhibit the expression of NFκB in LPS stimulated macrophages. Moreover, Brouet and Ohshima (174) demonstrated the anti-inflammatory properties of curcumin in LPS and IFN-γ stimulated macrophage cells.

### **2.8.4. Onions:**

Onion has been found to have a potent anti-inflammatory effect (175) as it contains a bioactive polyphenols called quercetin (176). Wang et al. (175) conducted *in vitro* experiment by using LPS stimulated RAW264.7 macrophages and found that treatment of cells significantly reduced the expression of NO and iNOS mRNA, COX-2 and NFκB expression as compared to only LPS treated cells. Dias et al. (177) demonstrated that quercetin reduces the expression of iNOS and NFκB in streptozotocin induced inflammation of rat liver. Furthermore, it was found that one gram dried onion per day for 4 weeks increased the bone mineral content by 17.7% in male rats and in ovariectomized female rats 1500 mg dried onion per day decreased the bone resorption by 25% (197). Later, it was discovered that compound responsible for decrease in resorption was gamma-L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide (198).

### **2.8.5. Prunes:**

Out of various species of plum, European plum (*Prunus domestica*) is commonly cultivated in United States. Due to its high flavinoids, mineral, sugar, vitamins and fiber content, European plum has been found to be used in remedies of many chronic diseases such as heart disease and cancer (179). In a breakthrough study, Arjmandi et al. (180)

found increased markers of bone formation in postmenopausal women who consumed dried plum as compared to non dried plum group. Deyhim et al. (195) found that dried plum in a dose of 5% was able to reverse the femoral and tibial bone loss in osteopenic ovariectomized rats. Moreover, Franklin et al. (196) showed that dried plum (5-25%) for 90 days, down regulate the gene expression of receptor activator of NFkB ligand (RANKL) in tibia of ovariectomized rats. These studies suggest that the dried plum may prevent the bone loss by acting as anti-inflammatory agent. Moreover, dried plum is a rich source of polyphenols such as chlorogenic acid, neochlorogenic acid, cryptochlorogenic acid, caffeic acid and cinnamic acid (15). Chlorogenic acid has been found to possess anti-inflammatory properties (184). Hence we propose that dried plum polyphenols may possess anti-inflammatory properties. If this hypothesis is found true, dried plum may have several implications in prevention of diseases of chronic inflammation origin.

## CHAPTER 3

### MATERIALS AND METHODS

#### 3.1. Polyphenol extraction:

Polyphenols were extracted according to a method described by Kim and Lee (181). Dried plum was chopped and powdered. Ten gram of dried plum powder was mixed with 100 ml of 80% (v/v) ethanol and sonicated while purging nitrogen gas and continuously mixed at room temperature in subdued light. The sonicated mixture was filtered through Whatman filter paper no-2 using vacuum suction. The filter cake remained on Whatman filter paper was rinsed with 50 ml absolute ethyl alcohol and sonicated again in 100 ml of 80% (v/v) ethanol and filtered through Whatman filter paper no-2. This process was repeated three times to extract all the polyphenols from plant cells. The filtrate was taken into a 1000 ml round bottom flask in a rotary evaporator. Ethanol was evaporated at 62<sup>o</sup>C until the volume was reduced to 40-50 ml. The sample was taken out, freeze dried and stored in -20<sup>o</sup>C freezer.

#### 3.2. Polyphenol Estimation:

Total polyphenols were estimated by Folin's Ciocalteu's reagent (Sigma, St Louis, MO) using gallic acid (TCI America, Portland, OR) as a standard, described by Kim et al. (182). Briefly, 0.1 g of freeze dried plum polyphenols was taken and mixed with 5 ml deionized water and vortexed. Half milliliter of this polyphenolic solution was taken out in separate test tube and mixed with 0.5 ml of Folin Ciocalteu's reagent. After 5 min, 5 ml of 7% Na<sub>2</sub>CO<sub>3</sub> solution was added and the solution was immediately diluted to a volume of 12.5 ml by adding 2 ml of deionized water to each test tube. The solution was incubated for 90 minutes at room temperature and absorbance was measured at 750 nm using spectrophotometer. The polyphenolic sample was compared with gallic acid standard curve and content was expressed as gallic acid equivalent (GAE).

#### 3.3. Cell Viability:

RAW264.7 macrophage cells (ATCC, Manassas, VA) were grown in Dulbecco Modified Eagle's Medium (DMEM; ATCC; Manassas, VA) supplemented with 10%

fetal bovine serum (FBS; ATCC; Manassas, VA) and 1% penicillin. At 80% confluence, cells were harvested and counted by hemacytometer (Thomas Scientific, Horsham, PA). Cells were mixed thoroughly in media to obtain a final concentration of  $10^5$  cells/ml of media. The cells were seeded at 20,000 cells/well, in 200  $\mu$ L of media in 96 well cell culture plates. After 24 hours the cells were doubled and the media was replaced with treatment of 0, 0.1, 1, 10, 100, 1000 and 10, 000  $\mu$ g/ml of dried plum polyphenols and 1  $\mu$ g/ml LPS. Cells were analyzed for viability by Cell Titer- Blue (Promega, Madison, WI) after 24 hours. Twenty  $\mu$ l of reagent was added to each well and shaken gently for 10 seconds and incubated at 37<sup>o</sup>C for 3 hours. Absorbance was recorded at 573 nm and plotted against concentration of the test compound.

#### **3.4. Nitric Oxide:**

Twenty thousand cells in 200  $\mu$ L of media per well were seeded using 96 well. After 24 hours media was replaced with dried plum polyphenols (0, 0.1, 1, 10, 100, 1000  $\mu$ g/ml) and LPS (1  $\mu$ g/ml). After 12 hours NO was measured in the media by Griess Reagent system (Promega, Madison, WI) according to the instructions given by manufacturer. Briefly, 50  $\mu$ L of media was withdrawn from each sample and mixed with 50  $\mu$ L sulfanilamide solution and incubated for 10 minutes protected from light. 50  $\mu$ L of 1- Naphthyl ethylenediamene dihydrochloride (NED) solution was added into each sample and incubated for 10 minutes (light protected). Absorbance was measured at 550 nm and data were plotted against known standards.

#### **3.5. Western Blot:**

COX-2 protein was measured by western blot method as described by Hooshmand et al. (18). Macrophage cells ( $5 \times 10^5$ ) were seeded in 6 well plates to 80% confluency and treated with dried plum polyphenols in a dose of 0, 10, 100 and 1000  $\mu$ g/ml and after one hour with LPS (10  $\mu$ g/ml). After 6 hrs of treatment, cells were washed in ice cold phosphate buffer saline (PBS) and then harvested. Cells were centrifuged at  $1000 \times g$  for 1 min at 4<sup>o</sup>C and the supernatant was removed. 200  $\mu$ l of PBS in 0.1% TritonX-100 was added to cells and sonicated at 20% amplitude, 3 seconds pulse for 3-4 times. Cells were centrifuged at  $3000 \times g$  for 10 minutes at 4<sup>o</sup>C. The supernatant was removed and the cell pallet was discarded. The protein content was determined in each sample by bicinchoninic acid assay (BCA; Fisher Scientific; Rockland, IL) for the

equal loading of protein in gel. Briefly, 20  $\mu$ l of sample was added in each well and then 200  $\mu$ l solution of BCA and cuprous ion (49:1) was added to each sample. The micro-well plate was shaken gently for 1 minute and incubated in dark for 30 minutes and absorbance was measured at 570 nm. Twenty  $\mu$ g of protein from each sample was taken by adjusting with autoclaved water and thereafter 12  $\mu$ l of loading buffer (0.125 mol/L Tris, 4% sodium dodecyl sulphate, 20% glycerol, 10% 2-mercaptoethanol, 0.003% bromophenol blue pH 6.8) was added to each sample. The sample was heated in boiling water for 5 minutes and finally sample was vortexed. 12% sodium dodecyl sulphate gel was prepared and samples were loaded into gel and protein was separated for 2.5 hours (100 V) in mini gel electrophoretic cell (Bio-Rad Laboratories, Hercules, CA). The separated protein was transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA) for 30 minutes at 15 V in Trans Blot SD Semi Dry Transfer Cell (Bio-Rad Laboratories, Hercules, CA). The membrane was cut into two pieces according to molecular weight of standard protein. PVDF membrane was blocked with TBS-Tween (50 m mol/L Tris, 150 m mol/L NaCl, 0.2% Tween-20, pH 7.5) and 5% non fat milk powder for 1 hour. The primary antibodies of COX-2 and  $\beta$ -actin (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the membranes in 1:500 dilutions and incubated over night in blocking buffer. After several times washing the membrane in TBS Tween, the secondary anti-body (Santa Cruz Biotechnology, Santa Cruz, CA) were added in a dilution of 1:1500 for 2 hours. Membranes were treated with equal volumes of horse radish peroxide buffer and horse radish peroxidase enhancer (BioRad Laboratories, Hercules, CA). The bands were developed and intensity of bands was calculated using versadoc imaging system from biorad.

### **3.6. Malondialdehyde Measurement:**

Malondialdehyde was measured by colorimetric microplate assay for lipid peroxidation (Oxford Biomedical Research, MI). Briefly, macrophages were grown in 75cm<sup>2</sup> flasks at 80% confluency and treated with 1000  $\mu$ g/ml of dried plum polyphenols. After one hour 100  $\mu$  mol/L FeSO<sub>4</sub> and 1 m mol/L H<sub>2</sub>O<sub>2</sub> were added to the culture and kept for 4 hours incubation. Cells were washed with PBS, scrapped and mixed at  $1.5 \times 10^7$  cells per ml of PBS and sonicated to homogenize. The homogenate was centrifuged at 3000  $\times$ g at 4<sup>o</sup>C for 10 minutes to obtain a protein rich supernatant. The protein

concentration was estimated with BCA assay as described earlier. Equal amount (80  $\mu\text{g}$ ) of protein in 140  $\mu\text{L}$  of sample was taken and mixed with 455  $\mu\text{L}$  of diluted reagent R1 (N-methyl-2-phenylindole in acetonitrile and ferric iron in methanol in 3:1 ratio) and 105  $\mu\text{L}$  37% HCL (12N HCL) provided by the manufactures and incubated for 1 hour at 45<sup>o</sup>C. After 1hour, absorbance was measured at 586 nm using microplate reader (Biotek, Winooski, VT) and malondialdehyde concentration was estimated by comparing with malondialdehyde standard curve.

## CHAPTER 4

### RESULTS

#### 4.1. Total Polyphenolic Content:

Total phenolic content of ethanolic extraction of dried plum was  $450.60 \pm 16.55$  mg GAE (Gallic acid equivalent)/100g of dried plum powder.

#### 4.2. Cell Viability:

Treatment with LPS ( $1 \mu\text{g/ml}$ ) and dried plum polyphenols in doses of 0.1, 1, 10, 100 and  $1000 \mu\text{g/ml}$  did not affect the viability of macrophages (Figure 1). Dried plum polyphenols in a dose of  $10,000 \mu\text{g/ml}$  decrease the cell viability significantly. Data are presented in the form of absorbance at 573 nm.

#### 4.3. Nitric Oxide Production:

Nitric oxide level in cell culture supernatant tended to increase as a result of LPS treatment (Figure 2). Dried plum polyphenols were effective in reducing NO (43%) at a dose of  $1000 \mu\text{g/ml}$  in comparison with LPS treated control cells. Other doses had no effect.

#### 4.4. COX-2 Expression:

LPS ( $10 \text{ ng/ml}$ ) significantly increased the protein level of COX-2 in macrophages while dried plum polyphenols significantly ( $p < 0.05$ ) decrease the protein level of COX-2 by 33% in a dose of  $100 \mu\text{g/ml}$  (Figure 3). The  $1000 \mu\text{g/ml}$  dose tended to decrease the COX-2 expression further but that was not significantly different from  $100 \mu\text{g/ml}$  dried plum polyphenols dose.

#### 4.5. Malondialdehyde Production:

$\text{FeSO}_4$  ( $100 \mu\text{M}$ ) and  $\text{H}_2\text{O}_2$  ( $1000 \mu\text{M}$ ) significantly increased the malondialdehyde level in cells, whereas dried plum polyphenols in a dose of  $1000 \mu\text{g/ml}$  decrease the malondialdehyde level by 30% (Figure 4).



## CHAPTER 5

### DISCUSSION

The findings of the present study reveal that dried plum polyphenols suppress the production of NO and COX -2 and also lowers malondialdehyde, a marker of lipid peroxidation, in LPS and FeSO<sub>4</sub>+H<sub>2</sub>O<sub>2</sub> stimulated macrophages. These findings support the earlier study by Bu et al. (183) where it was found that ethanol extracted dried plum polyphenols decrease NO production in LPS induced RAW264.7 macrophage cells. We tested dried plum polyphenols in doses of 0.1, 1, 10, 100, 1000 and 10,000 µg/ml using macrophage cells and found that only the dose of 10,000 µg/ml reduced the cell viability. The higher dose of polyphenols may have effects on pH of the media and can have adverse effect on cell viability. The 1000 µg/ml dose was most effective in reducing NO production. Similarly, dried plum polyphenols were able to reduce the expression of COX-2 in dose of 100 µg/ml. The 1000 µg/ml dose further reduced expression of COX-2 but that was not significantly different from 100 µg/ml dose. However, Bu et al. (183) found that doses of 20 and 30 µg/ml dried plum polyphenols are effective in reducing expression of inflammatory markers. In our study different effective doses were observed in reduction of NO and COX-2. The exact mechanism of action of these compounds is not known. But it is known that dried plum polyphenols are a mixture of mainly chlorogenic acid, caffeic acid and cinnamic acid (15). Out of these polyphenols, it is possible that one polyphenols lowers the NO production in higher concentration and other polyphenols reduces the expression of COX-2 in lower amount. These differences may arise due to structural and spatial arrangement of both polyphenols and target molecules. Studies showed the anti-inflammatory effects of these polyphenols separately. Lee et al. (184) showed that chlorogenic acid inhibits NO production completely in LPS induced RAW264.7 macrophage cells in a dose of 40 µ mol/ml (184). Moreover, 2-hydroxycinnamaldehyde (a derivative of cinnamic acid) isolated from bark of *Cinnamomum cassia*, was found to completely inhibit NO production at a dose of 40 µ mol/ml, in LPS stimulated macrophage cells (187). It was also revealed that 2-

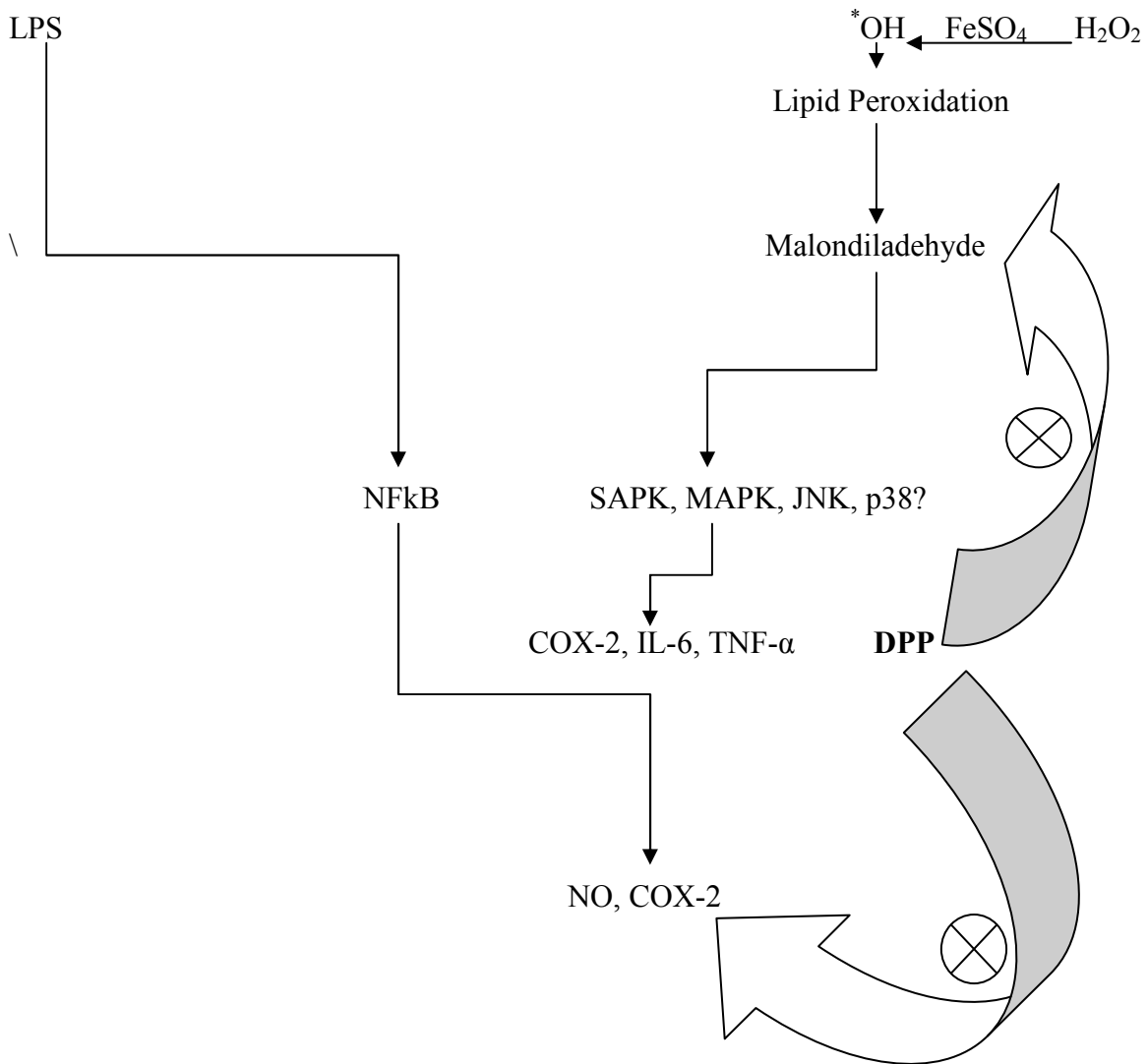
hydroxycinnamaldehyde significantly inhibits iNOS, COX-2 and NFκB expression in LPS stimulated macrophage (187). Michaluart et al. (188) showed that caffeic acid phenyl ester (derived from honeybee hive) inhibit PGE<sub>2</sub> production in rat carrageenan air pouch model of inflammation by inhibiting COX-2 expression dose dependently from 10 to 100 mg/kg weight of rat. Hitherto, the exact mechanism of action of polyphenols on cells is not known. However, it is suggested that polyphenols may exert their anti-inflammatory effects through inhibiting NFκB activation. NFκB is a transcription factor located at the cytoplasm and stimulated by degradation of NFκB heterodimer called IκB. Inflammatory agents degrade IκB and release NFκB molecule to move freely into nucleus, which causes expression of several inflammatory mediators (185). Inflammatory agents such as LPS stimulate NFκB through toll like receptors (TLR) (340;341). Polyphenols have been found to modulate the TLR derived inflammatory molecules. Youn et al. (186) showed that resveratrol (polyphenolic compound found mainly in grapes) inhibit NFκB activation by inhibition of TLR 3 and TLR 4 in LPS induced macrophages. Evidences showed that dried plum polyphenols inhibit NFκB activation in macrophages cells (183). However, these studies were limited to a single inflammatory agent, LPS. In general, in human body a gamut of inflammatory agents are present and their behavior and mechanism of action may be different. Hence, further studies are needed to investigate the effectiveness of plum polyphenols in different diseases.

Moreover, our study showed that dried plum polyphenols can reduce the oxidative stress in macrophage cells. Oxidation of membrane lipids can have impact on lipid peroxidation products such as 4-hydroxy-2-nonenal, have been shown to up-regulate inflammation by stimulation of COX-2 (189). Zarrouki et al. (190) showed that lipid peroxidation end product 4-hydroxy alkenals increase COX-2 expression by increasing phosphorylation of p38 MAP kinase in 3T3-L1 adipose cells. Moreover, melondialdehyde is a well known product of lipid peroxidation and form adducts with other protein molecules and impairs their function (191). Chen et al. (192) showed that melondialdehyde produced in ethanol stimulated rat liver cells form adducts with the purified enzyme cytochrome c oxidase from mitochondria and inhibit the protein activity. We tested the melondialdehyde production in RAW264.7 macrophage cells stimulated by FeSO<sub>4</sub> and H<sub>2</sub>O<sub>2</sub>. Dried plum polyphenols inhibited the melondialdehyde production by

30%. In a similar study, Leonard et al. (193) found that Essiac Tea (An herbal product containing phenolic compounds) significantly inhibits malondialdehyde production in FeSO<sub>4</sub> and H<sub>2</sub>O<sub>2</sub> stimulated RAW264.7 macrophages. Moreover, Ozer et al. (194) demonstrated that caffeic acid phenyl ester (a polyphenolic derivative) inhibited malondialdehyde production significantly in ischemic repurfused myocardium of rats in a dose of 50 μ mol/Kg weight of rat. These studies suggest that polyphenols may possess anti-oxidative properties.

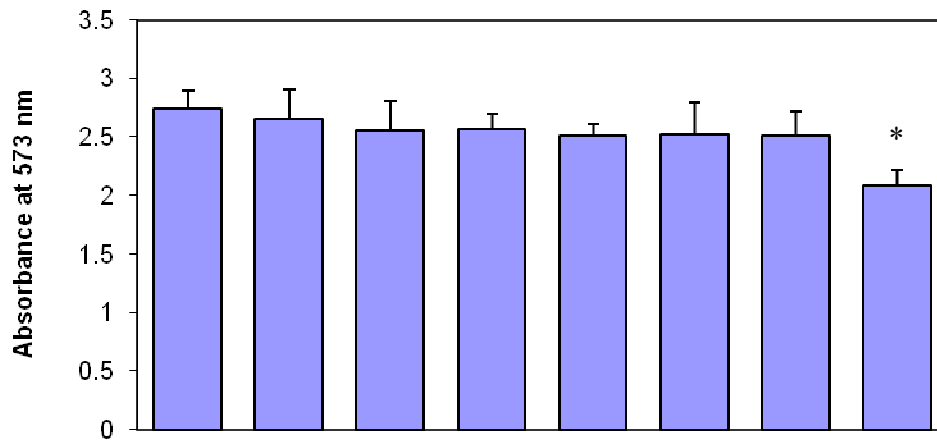
Our study indicated that dried plum polyphenols may act as potent anti-inflammatory agents as they inhibit expression of COX-2 and NO in LPS stimulated RAW264.7 macrophage and inhibit malondialdehyde production in FeSO<sub>4</sub> and H<sub>2</sub>O<sub>2</sub> stimulated RAW264.7 macrophages. Because, when we will use specific inflammatory agent or antigen specific to particular disease, effect of dried plum polyphenols can be stated more clearly regarding that disease. Moreover, being a cell culture study the doses cannot be applied to the *in vivo* studies and it gives a rough idea of mechanism of action of dried plum polyphenols.

## FIGURES



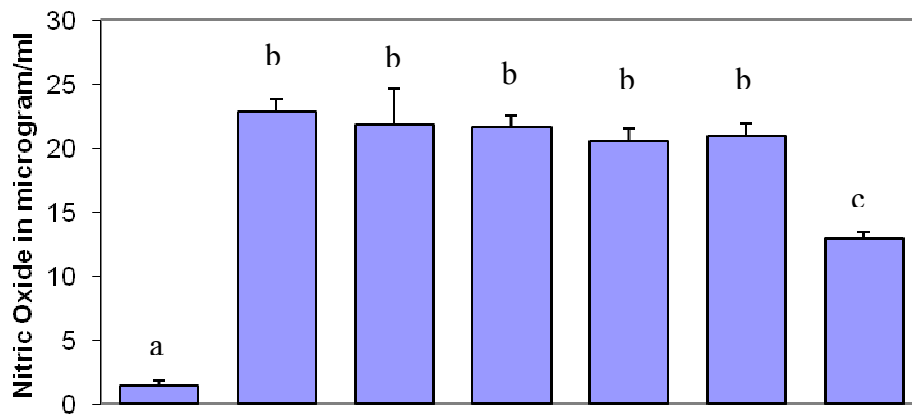
(SAPK: Stress activated protein kinases; MAPK: Mitogen activated protein kinases; JNK: c-Jun N-terminal kinases)

**Figure 1.** Schematic representation of the relationship between LPS, oxidative stress and possible role of dried plum polyphenols (DPP) in inflammatory processes.



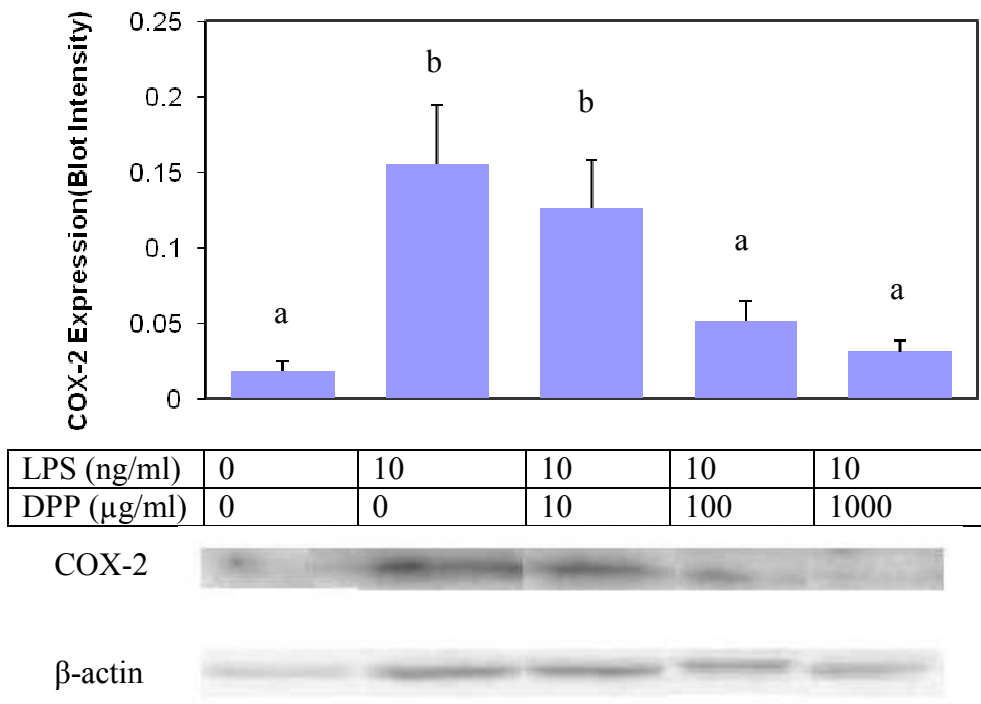
LPS (µg/ml)	0	1	1	1	1	1	1	1
DPP (µg/ml)	0	0	0.1	1	10	100	1000	10000

**Figure 2.** Represents cell viability which was measured by resazurin method.  
 Increased absorbance shows increase in viability  
 DPP; dried plum polyphenols, LPS; lipopolysaccharide  
 \*Denote significant difference as compared with all the other treatment  
 ( $p \leq 0.05$ )  
 Bar represents mean  $\pm$  standard deviation (SD)  
 n= 4 replications per treatment



LPS (μg/ml)	0	1	1	1	1	1	1
DPP (μg/ml)	0	0	0.1	1	10	100	1000

**Figure 3.** Represents nitric oxide level in culture supernatant measured via Griess reagent.  
 DPP; dried plum polyphenols, LPS; lipopolysaccharide  
 Bars that don't share the same superscript letters are significantly different from each other ( $p \leq 0.05$ )  
 Bar represents mean  $\pm$  standard deviation (SD)  
 n= 4 replications per treatment



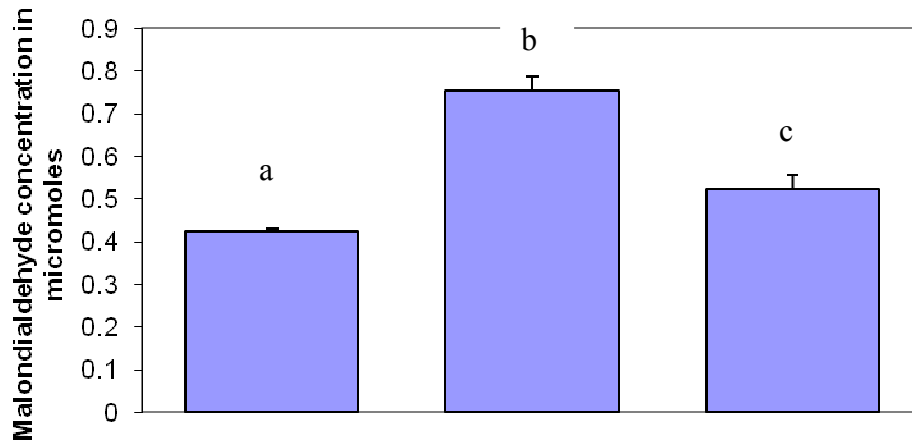
**Figure 4.** Represents protein level of COX-2 in cytosolic fraction of macrophages which was measured via western blot method.

DPP; dried plum polyphenols, LPS; lipopolysaccharide

Bars that don't share the same superscript letters are significantly different from each other ( $p \leq 0.05$ )

Bars represent mean of COX-2/  $\beta$ -actin  $\pm$  standard deviation (SD)

n= 3 replications per treatment



FeSO <sub>4</sub> (μM)	0	100	100
H <sub>2</sub> O <sub>2</sub> (μM)	0	1000	1000
DPP(1000μg/ml)	0	0	1000

**Figure 5.** Represents malondialdehyde concentration in cytosolic fraction of macrophages which was measured via TBARS kit.  
 DPP; dried plum polyphenols  
 Bars that don't share the same superscript letters are significantly different from each other ( $p \leq 0.05$ )  
 Bar represent mean  $\pm$  standard deviation (SD)  
 n= 3 replications per treatment



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## **BIOGRAPHICAL SKETCH**

Ajay Kumar

Ajay Kumar was born on September 5, 1979 in Hansi, India. He is the son of Thandi Ram and Bhetari Devi and younger brother of Sunil Kumar. He attended PCSD High School in Hansi, Haryana, India and graduated in March 1994. He earned his Bachelor of Science degree from CCS Haryana Agricultural University, Hisar, India on July 1, 2002. He also received Master of Science in Food Science and Technology from CCS Haryana Agricultural University, Hisar, India on December 31, 2004. He began his graduate work at Florida State University during the fall semester of 2007 to pursue a Master's degree in Nutritional Sciences. During his graduate studies he had the privilege of working on anti-inflammatory and anti-oxidative properties of dried plum polyphenols in RAW264.7 cells, which became the basis of his thesis. During this period he worked as a graduate research assistant under the guidance of Dr. Bahram Arjmandi. He also worked as a Quality Assurance Officer at Reliance Industries Ltd, India. He will graduate from Florida State University in August, 2009.