EFFECTS OF *CINNAMOMUM ZEYLANICUM* ON ATHEROGENESIS

by

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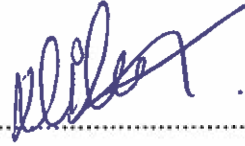

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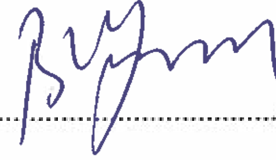

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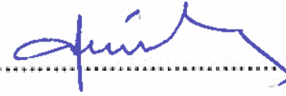

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ABSTRACT

EFFECTS OF *CINNAMOMUM ZEYLANICUM* ON ATHEROGENESIS

Cinnamomum zeylanicum which is the true cinnamon belongs to *Lauraceae* family. Bark of the *Cinnamomum zeylanicum* mainly consists of cinnamaldehyde which is the active compound. Active substances of *Cinnamomum zeylanicum* extract were found effective on reducing the blood glucose and lipid metabolism which are directly related to atherosclerosis and cardiovascular diseases. Atherosclerosis is a chronic inflammatory disease and primary cause of cardiovascular diseases. Our project seeks to assess the effect of *Cinnamomum zeylanicum* water extract on Nf-κB and $sPLA_2$ in relation to atherogenesis in primary isolated rat aortic vascular smooth muscle (VSM) cells. In parallel to these studies animal experiments were performed to observe the effect of cinnamon on high cholesterol diet fed Wistar Hannover rats.

The results of *in vitro* studies indicated that, 50 µg/ml oxidized lipoprotein (ox-LDL) stimulates VSM cell proliferation in 24 hours and the co-treatment of 50 µg/ml ox-LDL cinnamon extract containing 1 µg/ml cinnamaldehyde and pure *trans*-cinnamaldehyde (1µg/ml) were not effective on the inhibition of VSM cell proliferation. Although ox-LDL had a proliferative effect on VSM cell, it diminished the active NF-κB levels. Moreover the treatment of cinnamaldehyde and cinnamon extract in the presence of ox-LDL (50µg/ml) led to a decrease in the active $sPLA_2$ amount. Animals were fed with high cholesterol diet with or without of 300 mg/kg and 600 mg/kg cinnamon extract did not show a significant difference in the plasma cholesterol HDL, LDL, VLDL, triglyceride levels. Therefore, it is not possible to conclude whether cinnamon extract is efficient on reducing the progression of atherogenesis.

ÖZET

*CINNAMOMUM ZEYLANICUM'*UN ATEROGENEZE OLAN ETKİSİ

Gerçek tarçın olarak bilinen *Cinnamomum zeylanicum*, Sri Lanka'da yetişen Lauraceae familyasına ait bir bitki türüdür. *Cinnamomum zeylanicum'*un kabuk kısmı aktif maddesi olan sinnamaldehit içermektedir. Sinnamaldehitin kardiyovasküler hastalıklarla ve aterosklerozla bağlantısı olduğu bilinen yüksek kan şekerini düşürdüğü ve lipid metabolizmasını etkilediği bilinmektedir. Ateroskleroz kronik enflamatuar bir hastalık olup kardiyovasküler hastalıkların birincil nedenlerinden biridir. Bu projede, *Cinnamomum zeylanicum*'un ateroskleroz üzerine olan etkisi, sıçan aortundan izole edilen vasküler düz kas (VDK) hücrelerinde, Nf-κB ve $sPLA_2$ arasındaki etkileşime bakılarak tespit edilmeye çalışılmıştır. Bu çalışmalara paralel olarak tarçının etkisi Wistar Hannover türü sıçanlarda incelenmiş, bu hayvanlara yüksek kolesterol (%45 kkal) içeren yem verilerek ateroskleroz oluşumunu başlatmak amaçlanmıştır.

Deney ortamında yapılan çalışmalarda 50 µg/ml ok-DYL'nin (oksitlenmiş düşük yoğunluklu lipoprotein) 24 saatte VDK hücrelerini çoğalmaya teşvik ettiği gözlemlenmiştir. Bununla beraber ok-DYL ile birlikte 1µg/ml sinnamaldehit içeren tarçın ekstraktının ve saf *trans*-sinnamaldehitin hücrelere verilmesi sonucunda VDK hücrelerinin çoğalması azaltan bir etki görülmemiştir. Ok-DYL nin VDK hücrelerini çoğalttığı bilinmesine rağmen aktif Nf-κB düzeyinde azalmaya sebep olmuştur. Bununla birlikte ok-DYL varlığında sinnamaldehit ve tarçın ekstraktının aktif $sPLA_2$ miktarını azalttığı belirlenmiştir. Hayvanlarda ise yüksek kolesterol diyeti varlığında 300 mg/kg ve 600 mg/kg tarçın ile beslenen ve sadece yüksek kolesterol diyeti ile beslenen hayvanlardaki total kolesterol, YYL (yüksek yoğunluklu lipoprotein), DYL (düşük yoğunluklu lipoprotein), ÇDYL (çok düşük yoğunluklu lipoprotein) seviyelerinde belirgin bir değişim saptanmamıştır. Bu sebepten dolayı tarçının ateroskleroz oluşumunu yavaşlatıcı veya engelleyici bir etkisi olduğu belirlenmemiştir.

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LIST OF SYMBOLS / ABBREVIATIONS

Apo-E	Apolipoprotein E
Apo-B	Apolipoprotein-B
CVD	Cardiovascular diseases
EMSA	Electro Mobility Shift Assay
HDL	High density lipoprotein
LDL	Low density lipoprotein
Mm LDL	Minimally modified low density lipoprotein
Ox-LDL	Oxidized low density lipoprotein
PLA	Phospholipase A
RNA	Ribonucleic acid
VSM	Vascular smooth muscle
VLDL	Very low density lipoprotein
WHO	World Health Organization

1. INTRODUCTION

1.1. CARDIOVASCULAR DISEASES AND ATHEROSCLEROSIS

According to the record of World Health Organization (WHO) atherosclerosis, is a major factor for the development of cardiovascular diseases (CVD) including coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis, pulmonary embolism [1]. Atherosclerosis is also known to be the principal cause of death in Western societies and Turkey [2, 3]. There are two types of risk factors which can induce the formation of atherosclerosis; first group is non-modifiable factors such as age, gender, genetic, ethnicity and the other group is the modifiable factors including hypertension, obesity, a high lipid diet and tobacco consumption which are mostly related with the life style choices and eating disorders [4]. According to Turkey Republic Ministry of Health records, the cumulative rate of mortality in CVDs is much more common in male population than in female population (Tables 1.1.-1.2). As a result, male population is more susceptible to CVDs and atherosclerosis than female population.

Table 1.1. Existing and Prospective Mortality rate of CVDs in Male Population in Turkey by years [5]

YEARS	AGE	0-4	5-14	15-29	30-44	45-59	60-69	70+	**TOTAL**
	2000	579	956	2145	6781	19697	24171	48057	**102386**
	2010	879	836	1912	7886	29235	29619	64333	**134700**
	2020	734	598	1499	8418	39931	47070	77414	**175663**
	2030	626	410	1136	8058	49152	66473	109713	**235567**

Table.1.2. Existing and Prospective Mortality rate of CVDs in Female Population in Turkey by years [5]

	AGE	0-4	5-14	15-29	30-44	45-59	60-69	70+	**TOTAL**
YEARS	2000	406	426	987	4049	11569	18934	66701	**103071**
	2010	518	327	628	4220	12820	17888	87010	**123411**
	2020	393	210	425	3987	13031	20963	105289	**144297**
	2030	314	128	277	3345	11396	21832	143237	**180530**

Pathogenesis of atherosclerosis can be classified in general by two important conditions; lipid accumulation and inflammation both of which lead to the accumulation of cholesterol-rich lipid plagues in the arterial tunica intima (the innermost layer of an artery wall located between the endothelium and the tunica media; Figure 1) [6]. These situations are distinctive factors that separate atherosclerosis from other chronic diseases. The other issues that can contribute and trigger the formation of CVDs are hypertension, diabetes, hyperlipidemia and the development of atherosclerotic plaque though inflammatory responses [7].

Several lipoproteins are responsible from carrying serum cholesterol. These lipoproteins are classified as; chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). Cylomicrons is responsible from the transporting of dietary lipids and the rest of the other lipoproteins operate in the transport of endogenous lipids [8]. Liver synthesize, triglyceride-rich VLDL containing apolipoprotein-B (apo-B) and apolipoprotein E (apo-E) whose function is carrying the fatty acids to adipose tissue and muscles and the rest of the VLDL remnants are metabolized to LDL particles in the liver. The most of the serum cholesterol is transported by LDL in the human body. Increasing levels of LDL in circulating blood is related with the initiation of atherogenesis within arteries [4, 6].

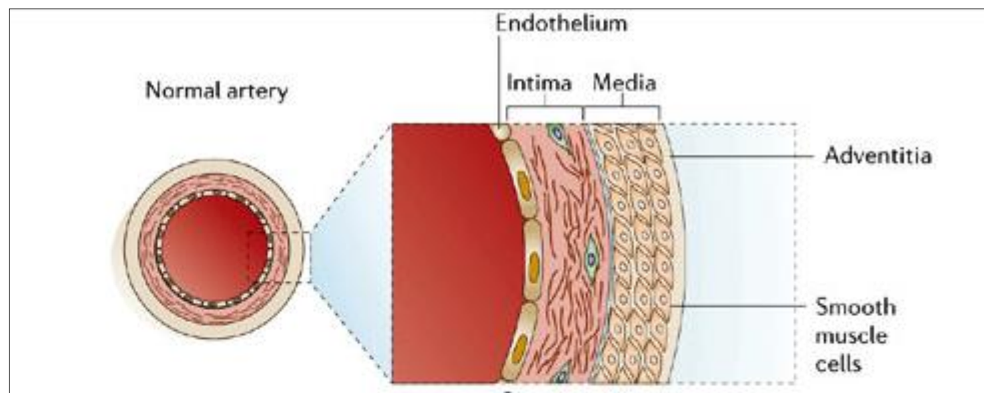

Figure 1.1. Schematic structure of a blood vessel. (a) Tunica adventitia, the outer layer, mainly composed of collagen fibers with some nerve fibers and lymphatic vessels (b) Tunica media consists of elastic fibers and smooth muscle cells. (c) Tunica intima, the innermost layer, consists of basement membrane, internal elastic lamina [9]

The importance of changes in LDL such as modifications and oxidations in the development of CVD, drive the scientist to investigate the alterations in the structure of LDL during the progression of disease. LDL is formed by a hydrophobic core of polyunsaturated fatty acids and esterified cholesterol molecules surrounded by a shell of phospholipids, unesterified cholesterol and apolipoprotein-B [10]. According to the density LDL can be classified mainly in three groups; (i) large LDL which are LDL1 and LDL2 with d=1.032 g/ml density; (ii) intermediate LDL called LDL 3 with d=1.030-1.040 g/ml density; and (iii) small dense LDL which are LDL 4 and LDL 5 with 1.040-1.065 g/ml density [11]. With respect to the size of the diameters LDL can be grouped into two subclasses; subclass pattern A are the LDL particles that have a diameter larger than 25 nanometer; subclass B are the LDL particles which have a diameter less than 25 nm. In the characterization of subclass pattern B, it was seen that the small dense LDL particles are more dominant rather than the other type of LDL molecules. Small dense LDL can penetrate more easily through the tunica intima (subendothelial space) and also more prone to the oxidation than the other type of LDLs, hence constitute the major risk group for the cardiovascular diseases and atherosclerosis [3, 12, 13].

1.2. PATHOGENESIS OF ATHEROGENESIS

Formation of the atherosclerotic plaque is called atherogenesis and characterized by five interrelated phase. The main step of atherogenesis is the infiltration of LDL from blood vessel to the subendothelial space and the physical trapment of LDL particles in the tunica intima. The next step starts with the endothelial dysfunction which could be the result of endothelial injury or endothelial stress according to "response to injury hypothesis'' [14] and carries on with the extravasion of monocytes into the intima in response to the inflammatory signals and subsequent monocyte differentiation into macrophages. Increasing levels of LDL together with the cytokines released by the trapped macrophages in the tunica intima not only leads to the adhesion of the circulating monocytes to the arterial endothelial cells and but also induces the transmigration of monocytes to the subendothelial space. Moreover, the increased LDL levels accelerate the infiltration of LDL from the arterial space to the subendothelial space [15]. Once native LDL is trapped in the subendothelial space it undergoes several modifications including oxidation [3]. Many components in LDL such as phospholipids, cholesterol, unsaturated fatty acids, apolipoprotein B can undergo oxidation reactions. In the early phase of atherogenesis mild oxidation of LDL creates minimally modified LDL (mmLDL) in the subendothelial space [16]. MmLDL is a key factor in the activation of monocyte recruitment. It stimulates the circulating blood monocytes and induces their migration towards the luminal endothelial surface.

''Monocyte recruitment'' is a multistep molecular mechanism consisting of; initial attachment of rolling cells, arrest and migration through cell to cell borders and transmigration to the subendothelial space [17]. In the first step adhesion proteins of the selectin family are responsible for the tethering and rolling of monocytes among the endothelial monolayer [18, 19]. On the luminal face of activated endothelial cells P-selectin and E-selectin proteins are expressed while monocytes express the corresponding glycoprotein ligands for these receptors. However, the interactions between these selectins and their ligands are not wholly responsible for the adhesion of monocytes. The attachment also depends on the commitment of integrin ß1 and ß2 on the monocyte surface to this molecular cascade [17, 20-22]. The interactions of integrins with their ligands intracellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecules (VCAM-1) on

the endothelial cells provide a strong anchorage for the monocytes to the luminal face of endothelial cells. In normal conditions monocytes do not have an affinity for these endothelial cell surface molecule ligands, however, when the endothelial cells are activated by mmLDL or receive a chemokine signal such as MCP-1, they are induced to express P-selectin, E-selectin, ICAM-1 and VCAM-1 driving the monocyte adhesion to the endothelial cell surface [23, 24]. Increased expression of these adhesion molecules indicates that there is an endothelial dysfunction at the particular arterial site bringing about the risk for the development of atherosclerotic injury [25-28**]**. During the formation of atherosclerotic lesions monocytes produce many chemotactic molecules such as monocyte chemoattractant protein-1 (MCP-1) and its receptor CCR-2 (Chemokine receptor-2), macrophage colony stimulating factor (M-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF), migratory inflammatory protein (MIP), tumor necrosis factor (TNF)-α, transforming growth factor–ß (TGF-ß), and the chemokines RANTES and fractalkine (CX3CL1). In addition, these monocytes are activated by various inflammatory molecules such as interleukin (IL)-1-6-8-10-12 and (TNF-α) that are produced by the intima cells in response to the lipoprotein infiltration [29-31]. After adhesion of monocytes, the next step is the migration of monocytes into the subendothelial space. In this step PECAM-1 (platelet adhesion molecule-1 is expressed on monocytes and leading to the extravasion of monocytes) as well as JAM-A (junctional adhesion molecule-A, expressed on endothelial cells) and CD99 (is expressed on most type of leucocytes) molecules which are abundant at the endothelial cell-cell junctions play an important role [17]

While PECAM-1 and CD99 forms the hemophilic interactions, CD-99 on monocytes interacts with CD-99 on endothelium and PECAM-1 on the monocytes behaves in the same manner. The relocation of JAM-A and PECAM-1 to the apical surface on the inflamed endothelium provide a strong anchorage of monocytes and give a direction on the migration of cells through the subendothelial space [32].

Table 1.3. The list of endothelial cell adhesion molecules playing an important role in leukocyte recruitment [17]

Molecule	Cell Expression	Ligand/Counter receptor	Role in Cascade
E-selectin	EC	Sialyl Lewis^x, L-selectin, carbohydrates	Rolling
P-selectin	Pla, EC, Mac	PSGL-1, L-selectin, CD43, sulfo sLe^x	Rolling
L-selectin	Leu	PSGL-1, P-, E-selectin (carbohydrates)	Rolling
VCAM-1	EC, SMC,	VLA-4 (α4ß1 integrin); α4ß7 integrin	Arrest, Rolling
ICAM-1	EC, SMC, Leu	LFA-1, Mac-1, p150 (ß2 integrins)	Arrest, Migration
ICAM-2	EC	LFA-1	Arrest, Migration
JAM-A	EC, Leu ,Pla, Ep	JAM-A	Signaling, Adhesion
JAM-B	EC ,Pla	JAM-C, VLA-4	Adhesion
JAM-C	EC, Pla ,Ep	JAM-B, Mac-1	Signaling, Adhesion
PECAM-1	EC,Pla,Leu	PECAM-1,	Signaling, Adhesion
CD99	EC, Leu	CD99	Adhesion
PVR/CD155	EC, Ep	DNAM-1 (CD226)	Adhesion

After the recruitment and infiltration of monocytes through the subendothelial space, cells are stimulated with M-CSF to proliferate and differentiate into macrophages. This differentiation is characterized by the formation of primary and secondary lysosomes, and vacuoles and by the increased level of cytoplasm containing the vesicles. Once matured, macrophages modify the mmLDL to more a oxidized form [33].

1.2.1 Foam cell formation

Monocyte derived macrophages act in response to the chemokine signals in order to metabolize the modified lipids in the microenvironment of subendothelial space [34]. Oxidation of LDL produce more negatively charged lipoprotein particles that is not recognized by the classical LDL receptors, but by the scavenger receptors on macrophage surface. In the tunica intima, macrophages take up the oxidized LDL into their cytoplasmic

vesicles, thereby increase their lipid content and become large foam cells [35] by internalizing, degrading ox-LDL into cholesterol particles which results in the formation of free cholesterol and cholesterol esters [36]. Free cholesterol can be esterified by the cholesterol acyl CoA:aceyltransferase-1 (ACAT-1) and accumulates as cholesterol ester droplets in the cytoplasm of macrophages. Interestingly the increasing levels of intracellular cholesterol do not affect the expression of scavenger receptors therefore this lipid accumulation continues until macrophages turn into lipid-laden foam cells [37, 38].

As LDL is prone to the toxic oxidation by macrophages, in the further step of atherogenesis its accumulation leads to apoptotic death of the foam cells [39].

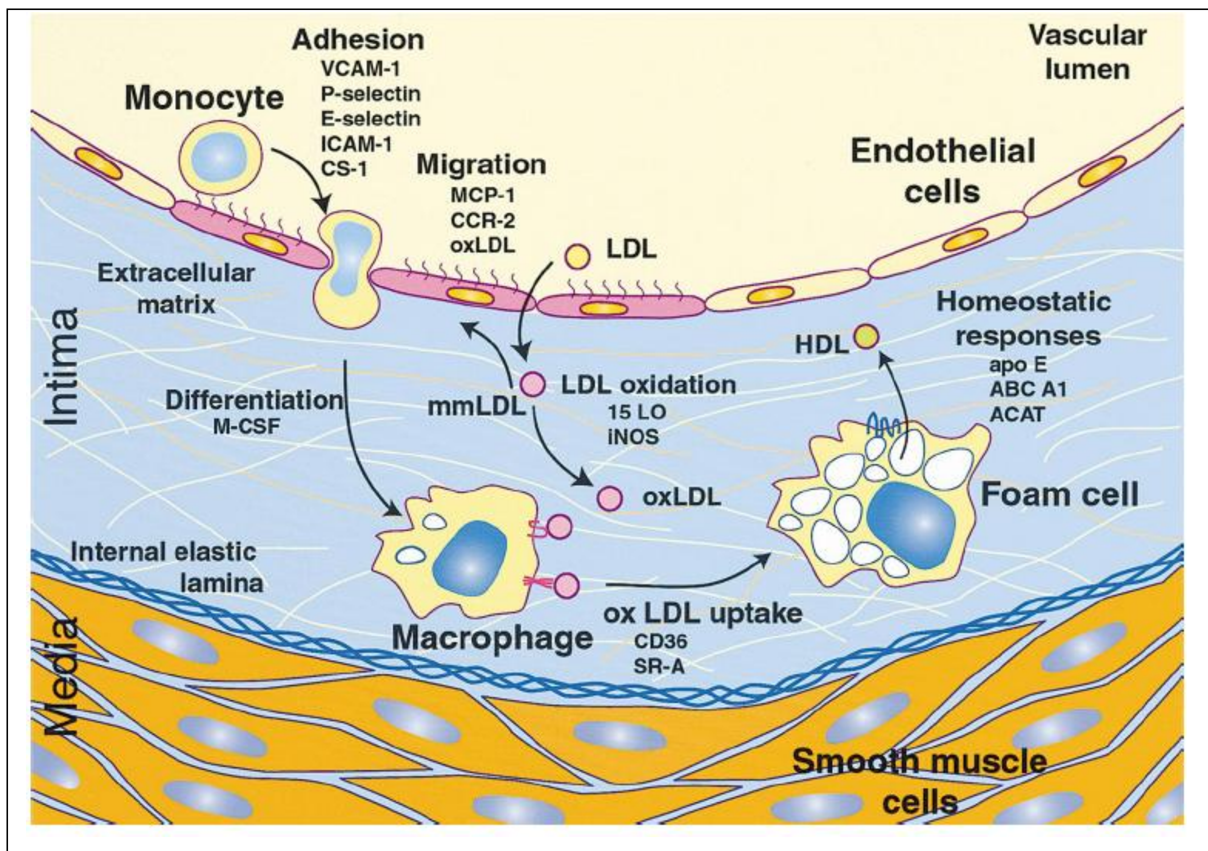

Figure 1.2. Schematic view of foam cell formation. Infiltration of LDL molecule into subendothelial space leading to LDL oxidation, progressing from minimally modified LDL to extensively oxidized LDL. Induction of mmLDL and inflammatory molecules trigger endothelial cells to express cell adhesion molecules (VCAM-1, E-selectin, P-selectin, ICAM-1) to attach the monocytes on endothelial surface and penetrate (MCP-1, CCR-2) them through the subendothelial space. Infiltrated monocytes differentiate into

macrophages to uptake ox-LDL by scanvenger receptors (CD36, SR-A) that leads to the foam cell formation. Esterification of ox-LDL cholesterol causes the formation of lipid droplets that are more soluble forms and can be exported to HDL receptors by cholesterol transporters (ABCA1) [14]

Upon the inflammatory provocation, TNF-α stimulates foam cell formation by upregulating the receptors on macrophages to uptake modified lipoproteins while INF-γ inhibits cholesterol efflux from macrophages [40, 41, 42, 43]. Although several proteins are included in the process of LDL uptake, scavenger receptor A (SR-A) and CD36 proteins are the most important ones [44]. Other scavenger receptors such as LOX-1 (lectin-like oxidized LDL receptor), CD-68, SR-phosphatidlyserine and oxidized lipoprotein (SR-PSOX) are also capable of binding to the ox-LDL but their role in atherogenesis is still not clarified [45]. In addition, SR-BI (scavenger receptor class-B type I) which belongs to CD36 family and ABCA1 (ATP-binding cassette transporter A-I) molecules are also known to take part in the lipid accumulation of the macrophages. [46, 47]

1.2.2. Atherosclerotic Lesion Progression

Following the fatty streak formation, the lesion assumes a more complex structure and leads to the disease progression that is characterized by the vascular smooth muscle cell (VSMC) migration and proliferation from the tunica media to the subendothelial space (tunica intima) [6, 48] and matrix deposition. Platelet derived growth factor (PDGF) synthesized by the macrophages due to induction with ox-LDL, stimulates the proliferation and migration of VSM cells from the tunica media to the intima over the fatty streaks. VSM cells then synthesize the extracellular matrix collagen resulting in the formation of fibrotic atheromatous plaque [49, 50].

This fibrous cap formation is a representation of the healing mechanism against the formation of injury and the process is reversible at this time point. The recruitment, migration, and proliferation of VSM cells on the fatty streak are considered to be beneficial as the ultimate aim is to stabilize the plaque by increasing collagen synthesis on the lesion side (Figure 3). However, VSM cells gradually accumulate cholesterol in their cytoplasm

and become foam cells which results in the dysfunction and death of these cells. The formation of SMC derived-foam cells is detrimental as it renders the plaque vulnerable to rupture [51].

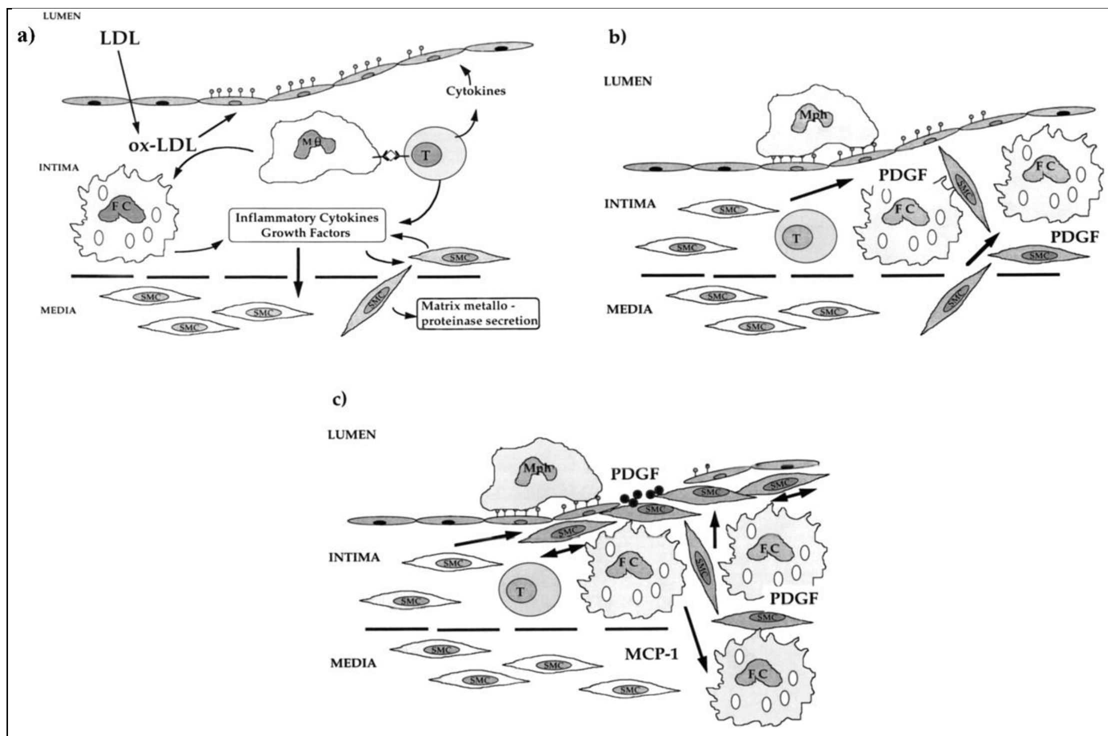

Figure 1.3. Scheme of cell migration in plaque morphogenesis. (a) LDL infiltration, monocyte recruitment and movement through subendothelial space followed by LDL oxidation inducing the production of MCP-1 which stimulates the macrophage-derived foam cell formation. (b) Macrophage-derived PDGF stimulates the migration of VSM cells from tunica media to intima through and around the foam cells. (c) Formation of fibrous cap by further migration and proliferation of VSM cells that is induced by PDGF (Mph=monocyte/macrophage) [52]

The formation of necrotic core, the further stage in advanced atherosclerotic lesions, is still unclear but it was suggested that necrotic core is formed by the death of macrophage–derived foam cells that are trapped within the lesion by the formation of overlaying fibrous matrix due to hypoxia or the cytotoxic effects of oxidized lipids or cytokines. When macrophages become necrotic they excrete all their stored lipid and proteolytic enzymes into the extracellular space leading to the formation of a large pool of

lipid and cell debris [53]. Macrophages also capable of degrading the collagen which reduces to the strength of the fibrous cap, making it weak and susceptible to the rupture [54, 55]. The released oxidized LDL and insoluble lipid from necrotic macrophages leads to the formation of the 'Gruel' characteristic of advanced lesion [56].

The next stage plaque rapture, initiates the coagulation by the recruitment of platelets from the vascular lumen and causes the formation of thrombus It is still unclear whether the calcification in the tunica intima, which is a morphological marker for the advanced atherosclerotic lesion, is a destabilizing or stabilizing factor for the atherosclerotic plague. However accumulating evidence suggests that minimally oxidized LDL together with TNF-α secreted by IFN- γ activated macrophages act as a regulator of bone formation and induce VSM cells to accumulate calcium crystals within their cytoplasm [57, 58]. When VSM cells undergo apoptosis, deposited calcium minerals were excreted in the tunica intima leading to the calcification of atherosclerotic plaque in advanced lesion of atherosclerosis [58].

1.2.3. NF- κB, a mediator in Atherosclerotic lesion

Transcription factors direct the gene expression by virtue of their specific binding to the promoter and enhancer sites of the relevant genes [59, 60]. Several genes are that responsible from the immune responses is regulated mainly by Nf-κB/Rel A family transcription factors. Five types of Nf- κB/Rel A family were discovered in mammals; **Rel A** (p65), **Rel B, p50/p105** (NF-κB1), **p52/p100** (NF-κB2) and **c-Rel**, comprising the same conserved Rel homology domain of 300 amino acid that functions in the DNA binding, dimerization, nuclear localization and inhibitor binding [59, 61, 63].

In the inactivated state Nf-κB (which is composed of p50 and p65) is located in the cytosol bound to its inhibitor protein IkB (mainly IkB-α) [61]. Upon activation by the upstream signaling pathways such as inflammatory cytokines, reactive oxygen intermediates and microorganisms, specific serine residues of IkB- α is phosphorylated by IκB kinases leading to release of IkB-α from Nf-κB, and subsequent ubiquitinationNo longer hindered by IkB-α Nf-κB can then translocate to the nucleus and find its nuclear target on the DNA [63, 64]. Importance of Nf-κB nuclear targets in the development of

atherosclerosis was first shown by Brand et al in 1996 by using immunohistochemical and immunofloresence analysis [65].

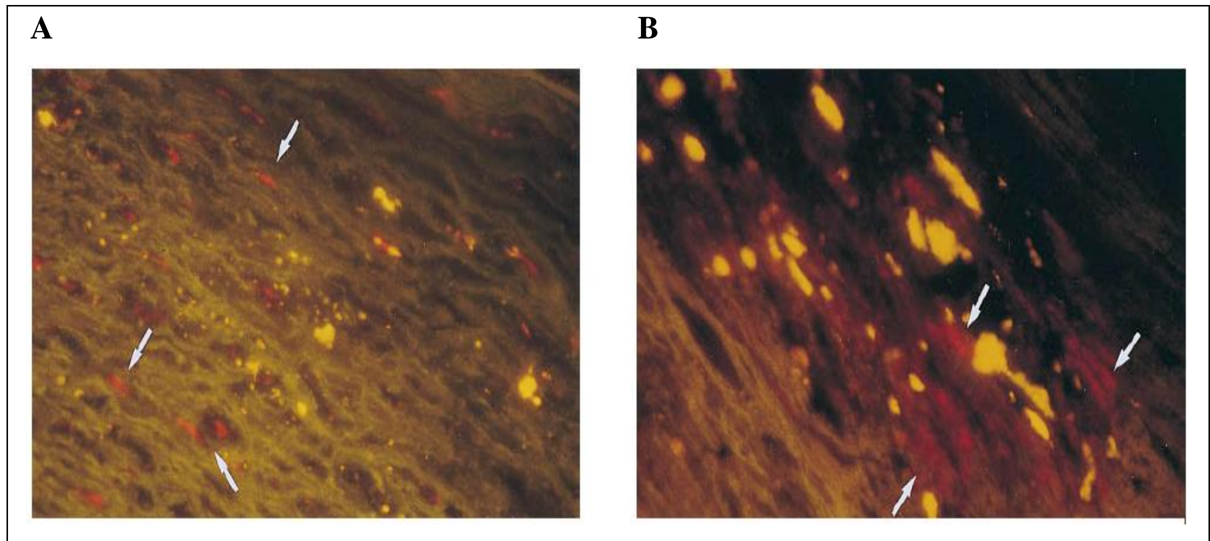

Figure 1.4. Presence of activated Nf-κB in advanced stage of atherogenesis. (A) Activated NF-κB transcription factor in the fibrotic thickened tunica intima (x150). (B) Activated NF-κB in the atheromatous region of an advanced atherosclerotic region (x160). Nf-κB transcription factor was detected with Cy3 (red fluorescence) dye (white arrows) by using α -p65 mAb (monoclonal antibody) which recognizes an epitope on the p65 subunit of Nf-κB protein [65]

In these experiments Nf-κB was detected in the regions of tunica media and tunica intima as well as in the cellular components of these regions such as VSMC, macrophages and endothelial cells. Therefore the importance Nf-κB transcription factor in the formation of atherogenesis has been under investigation since that time.

The cell adhesion molecules including ICAM, VCAM, selectins, integrins and cytokines (i.e. MCP-1) that takes the centre stage during the molecular events leading to monocyte recruitment from the vascular lumen to subendothelial space is expressionally controlled by the Nf-κB activation [66, 67]. Nf-κB transcription factor is activated by the modified LDL in the early stage of atherogenesis and provides the appropriate signal for the development of endothelial cell dysfunction. Further activation of Nf-κB is provided by M-CSF that differentiates monocytes to macrophages by triggering the expression of

scavenger receptors to pick up modified LDLs [68]. In addition, the synthesis of pro-inflammatory molecules such as TNF- α, IL-1 by macrophages during the manifestation of disease induces the expression of Nf-κB in VSM cells. Nf-κB activation occurs in many steps of atherogenesis, which makes this activator molecule an attractive pharmaceutical target for drug discovery [69].

1.3. PHOSPHOLIPASE A_2

Phospholipase A_2 , is an enzyme that is responsible from the hydrolysis of the sn-2 ester bonds in the glyceroacyl phospholipids within the lipoproteins and cell membranes to produce free fatty acids which is known as arachidonic acid (AA), and lyso-phospholipids [70]. Arachidonic acid is the precursor of the eicosanoid family of potent inflammatory mediators such as prostaglandins, thromboxanes, leukotrienes and lipoxins which stimulates the initiation of inflammatory response [71]. Mammalian PLA_2s constitute a superfamily that can be divided into four distinct groups including small, secreted PLAs (sPLA), large intracellular PLAs (cPLAs), calcium-independent PLAs (iPLAs), and PAF acetylhydrolases (PAF-AHs) [72, 73]. In vivo and in vitro studies are pointed to the biological actions of PLA_2 in the arterial wall and indicated that secretory form of PLA_2 can hydrolyze high density lipoproteins (HDL) and low Density Lipoproteins (LDL) by generating a decreased anti atherogenic HDL and atherogenic LDL [74]. Increasing levels of modified LDLs also increasing the retention of modified LDL to extracellular matrix (ECM) by PLA_2 (Figure 1.5) [75]. $sPLA_2$-modified apoB-100 containing lipoproteins are prone to undergo further enzymatic and non-enzymatic modifications which consequently can induce the accumulation of the intracellular lipids in the macrophages and strong attachment to the extracellular matrix proteoglycans. Furthermore, $sPLA_2$ stimulates the aggregation and fusion of the matrix-bound lipoproteins (Figure1.5) [76, 77].

Most of the studies have indicated that $sPLA_2$ exists in many stages of atherosclerosis rather than $cPLA_2$ which is also present in atherosclerotic progression but not as much as $sPLA_2$ [78]. $sPLA_2$ contains a histidine/arginine catalytic domain which is the active center of enzyme and Ca^{+2} loop that is essential for the function of enzymes. Ten members of $sPLA_2$ identified up today are; group IB, IIA, IIC, IID, IIE, IIF, III, V, X, and XII [79].In the progression of atherosclerotic lesion, the expression of group-IIA is mainly

attributed to the SMCs of tunica media when stimulated by the IFN- γ which increases the expression of $sPLA_2$-IIA mRNA and protein secretion 2 to 6 fold. According to these evidences, PLA_2 is potentially high risk factor during atherogenesis [80, 81].

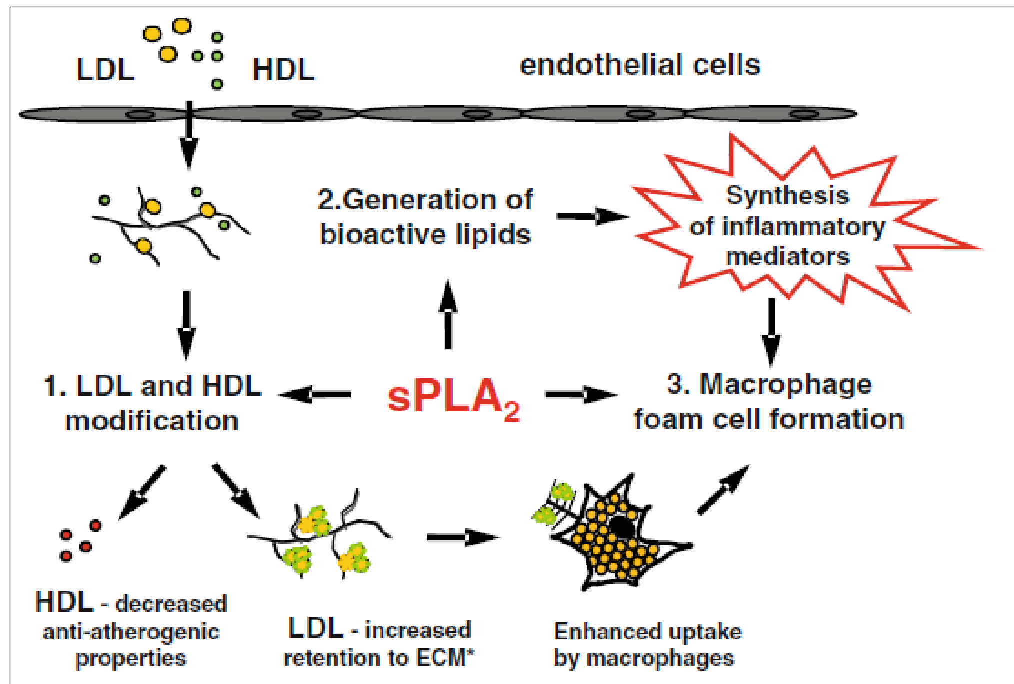

Figure 1.5. Functions of $sPLA_2$ in the atherogenesis. 1. Leading to LDL and HDL influx into subendothelial space, $sPLA_2$ modifiy these proteins by hydrolyzing. The modified HDL loses its anti-atherogenic effect also modified LDL prone to more oxidative reactions.2. $sPLA_2$ generates bioactive lipid mediators, including free fatty acids and lysophospholipids that trigger the inflammatory responses and synthesize more inflammatory mediators to activate macrophages.3. Macrophages uptake ox-LDL and become foam cells [82]

1.4. CINNAMON

Cinnamons belong to the Lauraceae family and consist of 250 different species that could be found in India, China, and Australia widely. The most favorite cinnamon species are *Cinnamomum zeylanicum* and *Cinnamon cassia* which are originated from different countries [83]. *Cinnamon cassia* is also known as Chinese cinnamon and taken its name from where it grows, China. Ceylon cinnamon which is known as the true cinnamon grows

in Sri Lanka and its scientific name is *Cinnamomum zeylanicum*. Cinnamon barks and leaves of these species are the sources of cinnamon oil which is mainly used in the scientific experiments [84, 85].

The most remarkable difference between these species is the amount of coumarin compound they possess [86]. *In vivo* experiments reported hepatoxic effects of coumarin on rats and using large amounts of coumarin was banned by Federal Institute for risk Assessment Berlin, Germany, a decision that was also confirmed by European Food Safety Authority [87]. The part of the plant which was powdered to use in experiments is important due to its ingredients. Cinnamon bark oil which is obtained from the inner bark and cinnamon leaf oil are steam distillation products of *Cinnamomum zeylanicum*. Most of the chemical constituents of the essential oils are monoterpenes, sesquiterpenes and related oxygen derivatives of these two types of compounds [88]. The main constitute of the cinnamon bark oil is cinnamaldehyde, while cinnamon leaf oil comprises not only cinnamaldehyde but also eugenol as detected by the gas chromatography and mass spectrometry analysis. Cinnamon bark oil consists of cineole (< 3%, w/v), linalool (1-6 %, w/v), ß-caryophyllen (1-4%), safrole (<0.5%, w/v), trans-cinnamic aldehyde (55-75%, w/v), eugenol (<7.5%, w/w), coumarin (<0.05%, w/v), trnas-2-methoxycinnamaldehde (0,1-1%, w/v), and benzyl benzoate (< 1 %, w/v).

Constituents of the cinnamon leaf are the same but their percentages are different. Especially cinnamaldehyde amount of the leaf is much lower than the bark. Cinnamon leaf consist of cineole (<1%, w/v), linalool (1.5-3.5%, w/v), ß-caryophyllene (1.5-7.0%, w/v), safrole (<3% , w/v), trans-cinnamic aldehyde which is the stable form of cinnamaldehyde (<3.0%, w/v), eugenol (70-85%, w/v), coumarin (<1%, w/v), trans-2-methoxycinnamaldehde (01-1%, w/v), and benzyl benzoatel (<1%, w/v) [89].

Aqueous *Cinnamon cassia* extract was found effective on lowering the lipid metabolism, glucose uptake, glycogen synthesis and increases the phosphorylation of the insulin receptors and triggers the insulin molecular cascade [90, 91]. Also the main component of cinnamon bark oil, cinnamaldehyde obtained from cinnamon zeylanicum was found effective on the blood glucose metabolism [92]. Moreover, the promotion of

wound healing, anti-inflammatory and anti-microbial effects of cinnamon was reported [93-95].

1.5. ANIMAL MODELS FOR ATHEROSCLEROSIS

Choosing the ideal animal model is important to mimic the progression of atherogenesis as same as in the human lesions. Because of the multifunctional nature of the cardiovascular disease it is nearly impossible to find the suitable model for these experiments. Larger animal models such as primates, swine, dog, pig are unfavorable due to the ethical concerns and financial problems [96].

Rabbits have been used as atherosclerotic model animals. This herbivore is not genetically prone to atherosclerosis so high cholesterol diet or vascular lesion induction is necessary to generate an atherosclerotic lesion. It is possible to increase their cholesterol level even with a mild cholesterol supplementation [97].

Rats which are also have been used in animal experiments since they were developed at Wistar Institute in 1920s and enormously preferred by the scientist due to their genetic similarity to humans. The normal rat is resistant to the atherosclerotic progression. Different types of models such as balloon injury of carotid artery that leads to atherosclerotic injury which initiates the lesion progression, diet-induced animal model in which animals are fed with a high cholesterol western type diet, and genetically modified animals that lacks specific genes that are responsible in the prevention of the atherogenesis are used as in vivo models of atherosclerosis [98-102].

Mouse models have been used widely however normal mice are short-lived and resistant to atherosclerosis development. Transgenic models with the deletion of apoB , apoE or LDLR eliminated these disadvantages. To accelerate the formation atherogenesis high fat cholesterol diets are used rather than chow diet as the daily food intake of animals [103-105].

1.6. AIM OF THE STUDY

The aim of the study is to enlighten the molecular cascade of early atherogenesis stage with emphasizing on the correlation between $sPLA_2$ activation, and Nf-κB activation which are assumed as important factors during the progression of disease. We are also offering an alternative therapy with *Cinnamomum zeylanicum* as a protective factor during the development of early atherogenesis.

In vivo studies have shown that active substances of different cinnamon types have a insulin–mimicking effect and improve the glucose uptake by activating insulin receptor kinase activity, autophosphorylation of the insulin receptor and glycogen synthase activity [90]. It was clearly observed that there is a noteworthy effect of cinnamon on the diabetes and lipid mechanism which are directly related to cardiovascular diseases and atherosclerosis. In addition, cinnamon has shown to possess an antioxidant effect therefore may have a protective role in the early stages of atherogenesis by preventing the oxidation of LDL [106]. However *in vitro* and *in vivo* studies attributing the antioxidant effect to cinnamon have been performed with *Cinnamon cassia* which contains cytotoxic coumarin substance. The present project utilizes *Cinnamomum zeylanicum* which has negligible amount of coumarin and has not been studied as anti-atherogenic agent yet [86].

In the light of previous experiments, this is the first study focusing on the effect of the *Cinnamomum zeylanicum* on early atherogenesis by analyzing the activation of Nf-κB along with $sPLA_2$ in ox-LDL induced primary VSM cells which were isolated from male rat aorta for in vitro studies. *In vivo* studies were performed on male Wistar Hanover rats by inducing the formation of atherosclerosis with high cholesterol diet for four weeks. At the end of the experiment animals were sacrificed and total blood was collected to measure the effect of cinnamon extract on the levels of total cholesterol, LDL, HDL, VLDL, triglyceride.

2. MATERIALS

2.1. INSTRUMENTS

The instruments used in this study are as follows:

- Laminar flow cabinet (ESCO Labculture Class II Biohazard Safety Cabinet 2A, Singapore)
- CO_2 incubator (Nuaire NU5510/E/G, USA)
- Centrifuge (Hettich mikro 22R and SİGMA 2-5 centrifuge, Germany)
- Vortex (Stuart SA8, UK)
- pH meter (Hanna instruments PH211, Germany)
- Mini-PROTEAN Tetra Cell Electrophoresis System (Bio-Rad 165-8001, USA)
- Mini Trans-Blot Cell Blotting System (Bio-Rad 170-3935, USA)
- Chemiluminescence imaging system (DNR Bioimaging Systems MF-ChemiBIS 3.2, Israel)
- -80 °C freezer (Thermo Forma -86 C ULT Freezer, USA)
- ELISA plate reader (Bio-Tek EL x 800, USA)
- CHRIST A 2-4 LD freezed-drier (UK)
- Buchi Rotary Evaporator (Italy)
- HPLC (Agilent 1290 Infinity LC, USA)

2.2. EQUIPMENTS

The laboratory equipments used in this study are as follows:

- Serological pipettes 25, 10, 5, 2 ml (Grenier-Bio or Axygen, USA)
- Micro pipettes 1000, 200, 100, 10, 2.5 µl (Thermo Scientific, USA)
- Polypropylene centrifuge tubes, 50 ml, 15 ml, 2 ml, 1 ml, 0.5 ml (Isolab, Germany)
- Cell culture flasks, T-25, T-75, T-150 and cell culture plates, 6-well, 96-well, (TPP Switzerland or Grenier-Bio, Germany)

- Cryovials (TPP, Switzerland)

2.3. CHEMICALS

- Dulbecco's Modified Eagle's Medium - high glucose (Sigma D6429.27 or Gibco 41966, USA)
- Fetal Bovine Serum (FBS) Cell culture tested (Sigma F9665, Germany)
- Penicillin-streptomycin (Thermo Scientific SV30010 or Biochrom A2213, Germany)
- Trypsin-EDTA (Biochrom L2153, Germany)
- Dimethyl sulfoxide (Santa Cruz sc-202581, USA)
- Dulbecco's Phosphate Buffered Saline (DPBS) (PAN Biotech P04-53500, Germany)
- Absolute Ethanol (AppliChem A3678, Germany)
- 2-propanol (AppliChem A3928, Germany)
- Ethylenediaminetetraacetic acid (EDTA) (Merck 108418)
- Ammonium persulfate (Sigma A3678, USA)
- N,N,N′,N′-Tetramethylethylenediamine (TEMED) (Sigma T7024, USA)
- Methanol 99% (Sigma 34885, USA)
- Collagenase Type II prepared from *Clostridium histolyticum* (Gibco 17101-015, USA)
- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Multicell 600-032-EG, Canada)
- Ethylenediaminetetraacetic acid (EDTA) (Merck K40173218 946, Germany)
- Ethylene glycol tetraacetic acid (EGTA) (Fluka 03779, USA)
- Dithiothreitol (DTT) (Applichem A1101, USA)
- Sodium Fluoride (NaF) (Riedel-de Haen 01148, Germany)
- Sodium orthovanadate (Na_3VO_4) (Sigma S6508, USA)
- Phenylmethanesulfonylfluoride (PMSF) (Sigma 78830, USA)
- Beta-Glycerophosphate (Sigma G6376, USA)
- Protease Inhibitor (PI) 1% (v/v) (Sigma P8340, USA)
- Igepal CA-630 (Sigma I8896, USA)

2.4. KITS AND SOLUTIONS

- WST-1 Cell proliferation Kit (Roche, USA)
- Human oxidized Low Density Lipoprotein (Kalen, USA)
- Correlate-Enzyme Assay Secretary Phospholipase A2 Kit (Assay Design,907-002,USA)
- LightShift Chemiluminescent EMSA Kit (Thermo Scientific, 20148, Germany)
- Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific, 89880, Germany)

2.5 ANTIBODIES

- Mouse monoclonal anti-actin α-SMC (Smooth muscle cell) antibody (Sigma, A 5228, Germany)
- Anti-mouse IgG-FITC (Sigma F6257, USA)

2.6. PLANT MATERIAL

- Cinnamon Sticks (DRUERA, Sri Lanka)

3. METHODS

3.1. METHODS IN CELL CULTURE

3.1.1. Isolation of vascular smooth muscle cell from rat aorta

Vascular smooth muscle cells were isolated from 8 weeks old Wistar Hannover male rat. The rat was anesthetized with 30% chlorohydrate (v/v) (1.5 ml per animal). The midline of abdomen was incised and the thorax was opened to expose the heart and lungs. Right atrium was notched to release the blood at the same time with perfusion. Perfusion was applied from the left apex of ventricle for three times with the isotonic chloride which contained heparin (1000 unit/ml) until the isotonic chloride was poured out from the right atrium which was notched at the beginning. Aorta was removed from the rat and dissected from the aortic arch through the abdominal aorta. Aorta was immersed in Dulbecco's Modified Medium (DMEM) containing 20% Fetal Bovine Serum (FBS) (v/v) and 1000 unit/ml Heparin to remove the adipose and connective tissue easier. The tissue was then sterilized within the serum free DMEM containing 10% Penicillin/Streptomycin (v/v) (1000 U/ml Penicillin and 1000 μg/ml Streptomycin) for 5 minutes in the tissue culture dish. Aorta was washed with the serum free DMEM three times and then transferred to a sterile tissue culture dish to be cut into small pieces using combination of forceps and a sharp scalpel. The aorta pieces were incubated in DMEM containing Collagenase Type II (2mg/ml, prepared from *Clostridium histolyticum*) for 45 minutes at 37°C incubator. After incubation, aorta pieces were washed with DMEM containing 20% FBS (v/v) then centrifuged at 300 x g for 5 minutes with complete DMEM (High glucose Dulbecco's Modified Medium with 10% FBS (v/v), 100 U/ml Penicillin and 1000μg/ml Streptomycin) for three times in 50 ml falcon tubes. This step is necessary to remove endothelial cells completely.

The aorta pieces were minced into smaller pieces in a culture dish and each piece were placed on a well of 6-well tissue culture plate. Sterile cover slips were pressed-placed onto the aorta pieces and 5ml of growth medium were added into each well. The culture was placed into the humidified atmosphere at 37°C, 5% (v/v) CO_2, 95% (v/v) air for 10

days. After 10 days, endothelial cell contamination was not observed and only VSM cells were migrated from aorta pieces to culture plate. Cells were harvested as described below Section 3.1.2.

All cells were cultured in a humidified atmosphere at 37°C, 5% CO_2 (v/v), 95% air (v/v) and Dulbecco's Modified Medium (DMEM) containing 10% FBS (v/v) and 100 U/ml Penicillin and 1000 µg/ml Streptomycin was used as the growth medium.

3.1.2. Cell Passaging

Cell passaging was performed when VSM cells reached to 70% confluency. The cell monolayer was rinsed with Phosphate Buffer Saline (PBS, pH 7.4) before treated with the 0.25% trypsin-EDTA (v/v) solution. Cells were incubated in an incubator for 3 minutes then collected in DMEM containing 10% FBS (v/v) to inactivate the trypsin and centrifuged at 300 x g for 5 minutes. Cells were resuspended in the growth medium and seeded in tissue culture flasks.

3.1.3. Determination of Cell Number

Cell were dislodged from cell culture plates and collected in falcon tube as described above (Section 3.1.2) and resuspended in the growth medium. 10 µl aliquots of the cell suspension were put in BrightLine hemocytometer. The middle area of hemocytometer was determined as the cell counting area by using the inverted light microscope (Nikon Eclipse TS100). The cell number per milliliter was calculated according to the formula ''counted number of cells x dilution factor/ mm^2 x chamber depth''.

3.1.4. Cryopreservation of Cells

Cells were trypsinized and counted as described above (Section 3.1.2). Counted cells were centrifuged at 300 x g for 5 minutes. Density of cell number per one cryovial was determined as $1x10^6$ cells/mL and cells were resuspended in 1 milliliter freezing mixture containing 10% DMSO (Dimethyl sulfoxide) (v/v) and 90% FBS (v/v). Cells were then

frozen at -80°C but for long-term storage they were transferred to a liquid nitrogen container**.**

3.1.5. Thawing cells from storage

Stock cells were taken from the liquid container and quickly warmed up to 37°C. The cell suspension was collected into a sterile falcon tube and 5ml growth media was added drop wise onto cell suspension with continuous mixing to avoid killing the cells due to high increase in the osmotic pressure. The cell suspension was then centrifuged at 300 x g for 5 minutes to remove the DMSO in the freezing mix. Cell pellet was resuspended in growth medium and transferred into a tissue culture plate. The medium was then changed in the next 12-24 hours to remove the rest of the DMSO which is a toxic substance. Cells were passaged at least once before doing the experiments.

3.2. CELL VIABILITY ASSAY

Viability in VSM cells treated with different concentrations of FBS, ox-LDL, Cinnamaldehyde and cinnamon extract were detected using WST-1 cell viability assay. WST-1(4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1.3-benzene disulfonate) is a colorimetric assay which is used for the non radioactive quantification of cell proliferation, cell viability and cell toxicity. The assay procedure is based on the tetrazolium salts which are cleaved to formazan molecule by the mitochondrial enzymes. Increasing number of viable cells lead to the increasing mitochondrial dehydrogenase activity. This increment of enzyme activity follows the increasing number of formazan dye in viable cells which is related with the number of metabolically active cells.

In order to analyze the *in vitro* effect of *Cinnamon zeylanicum*, VSM cells were trypsinized and counted as 100.000 cells/ml and seeded on 96 well plate in DMEM containing 0.1% FBS (v/v). After overnight incubation, cells were washed once with PBS, pH 7.4 and pre-treated with 29.27 mg/ml cinnamon extract which comprise 1µg/ml active compound of cinnamaldehyde. The cinnamon extract was dissolved in DMEM containing 0.1% FBS (v/v) then filtered with 0.22 µm filter before adding on the 96 well plate. The amount of cinnamaldehyde in cinnamon extract was determined using HPLC analysis as

described in, Methods section 3.5. The following day, 29.27 mg/ml cinnamon extract was prepared freshly and cells were co-treated with different concentrations of ox-LDL and 29.27 mg/ml cinnamon extract and incubated for 24 hours. Cinnamaldehyde was dissolved in DMSO and diluted in DMEM containing 0.1% FBS (v/v) and final volume was arranged as 1µg/ml. VSM cells were co treated with cinnamaldehyde in the presence of increasing concentrations of ox-LDL.

To determine the cell viability, 5 µl from WST-1 solution and 45 µl from growth medium were mixed together and 50 µl of final volume was added to the each well of 96 well tissue culture plate. After addition of WST-1, cells were incubated in dark at 37°C for one hour. The absorbance of each sample was measured at 450 nm with 630 nm reference wavelength with an ELISA plate reader.

The cell viability obtained for the VSM cells which has received no treatment (i.e. ox-LDL, cinnamon extract. or cinnamaldehyde) was accepted as the control and represented 100% cell viability. The values obtained for different treatments were normalized against the control in all experiments.

3.3. IMMUNOFLORESCENT STAINING OF VSM CELLS

Cells were trypsinized and counted (as described above in Section 3.1.2) and seeded onto cover slip which was located inside the wells of a 6 well tissue culture plate at 300.000 density with sufficient growth media. Following a 24 hour incubation, cells were washed once with PBS, pH 7.4 and fixed with 3% paraformaldehyde (w/v) (in PBS, pH 7.4) for 15 minutes at room temperature. Fixative was removed and cells were gently washed with PBS, pH 7.4 for three times. At this step fixative must be removed completely. Cells were then permeablized with 1ml of 0.5% Triton X-100 (v/v) in PBS pH 7.4, for 10 minutes at room temperature. Triton X-100 is a detergent solution that is used to open the pores to the cell membrane and provides the permeabilization of the cells. After this step cell monolayer was washed three times with PBS, pH 7.4. Cover slip was placed into a new petri plate containing filter paper moistened with PBS, pH 7.4 to get rid of the remaining Triton-X-100. Cell monolayer was then blocked with the blocking buffer consisting of 3% bovine serum albumin (w/v) in PBS, pH 7.4 for 30 minutes at room

temperature. After the blocking step, cells were incubated with the mouse monoclonal anti-actin α-SMC (Smooth muscle cell) antibody diluted 1:200 in the blocking buffer for 1 hour at 37°C. Cells were then washed three times with PBS, pH 7.4 after primary antibody treatment. Cover slips were placed into a new petri plate containing PBS, pH 7.4 moistened filter paper. Samples were incubated with the FITC-labeled secondary antibody, diluted 1:200 in the blocking buffer for 1 hour at 37°C. Following this step cover slips were washed three times with PBS, pH 7.4 and mounted with the florescent mounting media containing DAPI (4', 6-diamidino-2-phenylindole), a florescent stain which can strongly bind to DNA. The cover slip was then placed on slides with the cell monolayer facing down into florescent mounting media. Slides were observed under florescent microscope Nikon Eclipse TS100 using 10X objective in 490 nm excitation and 515 nm barrier filter**.**

3.4. PREPARATION OF *CINNAMOMUM ZEYLANICUM* EXTRACT

Cinnamomum zeylanicum barks were purchased from DRUERA company, Shirelanka. Sticks were grounded in an industrial grade blender until they assumed powder form. 1000 mL hot distilled water was added to 100 gram of powder cinnamon and extraction was carried out with Buchi Rotary Evaporator at 60°C and 60 rpm for three hours. Extraction of cinnamon was filtered with a coarse filter paper (Macherey-Nagel, 751/75/20, Germany). Filtered extraction was freezed at -20°C and then lyophilized with CHRIST A 2-4 LD freezed-drier. 1100 gram of cinnamon barks were used during the experiment and the yield was around 5% (w/w).

3.5. DETERMINATION OF CINNAMALDEHYDE IN THE EXTRACT OF *CINNAMOMUM ZEYLANICUM* WITH HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

Agilent 1290 Infinity Liquid Chromatography series equipped with binary pump and fitted with a Zorbax SB-C18 Solvent Saver Plus analytical column (3.0 x 75 mm ; 3,5 µm particle size) was used to detect *trans*-cinnamaldehyde amount in water extract. The colon temperature was 45°C and injection volume was 5µl per sample. Detection was carried out using a 1290 Infinity Diode Array Detector variable wavelength detector at wavelength of

293 nm. The gradient mobile phase was consisted of (A) 1% acetic acid and (B) absolute methanol, with the flow rate of 1.5 ml /min. The elution programme was linear and carried out as shown in Table 3.1 Initiation condition was determined as 70% Mobile Phase A, 30% Mobile Phase B .

Table 3.1. Time table of Mobil Phases of HPLC protocol

Time Table (min)	Mobile Phase A (%)	Mobile Phase B (%)
0.1	70	30
2.5	60	40
3	60	40
3.1	70	30
4.0	Flow and pressure was stabilized for the next injection	

The compounds were quantified using 1290 Infinity ChemStation programme. The column was equilibrated with 70% Mobile Phase A 30% Mobile Phase B for 2 minutes prior to each analysis. Samples were dissolved in 25% of 1% acetic acid (v/v) and 75% of 100% Methanol (v/v) and filtered with 0.45µm filter before using in application.

3.6. MEASUREMENT OF PHOSPHOLIPASE A_2 WITH CORRELATE ASSAY ENZYME KIT

The activity of $sPLA_2$ in the cell media was detected using a purchased from Assay Designs. The kit uses a specific substrate for $sPLA_2$ that is converted into a sulfhydryl molecule. The presence of the sulfhydryl product is detected calorimetrically by using Ellman’s reagent, 5, 5'-Dithio-bis (2-nitrobenzoic acid) (DTNB), which forms a yellow colored product with the sulfhydryl formed. The amount of $sPLA_2$ in the sample is determined through the standard curve obtained by using the standard $sPLA_2$ supplied in

the kit. Before starting to the experiment all reagents were warmed at room temperature at least 30 minutes and $sPLA_2$ substrate vial was reconstituted with 3 ml of dH_2O. Reaction buffer which act as a blank, 50 µl of samples, and the standards of $sPLA_2$ were added to the microtiter plate in duplicate.100µl of reaction buffer and 50 µl of substrate buffer were added onto samples, respectively. Plate was then sealed with plate sealer and incubated at 37°C for 30 minutes. After incubation 25 µl of stop reagent and 25 µl of color reagent were added onto samples correspondingly. Samples were then mixed well by pipeting and incubated at room temperature for 10 minutes. The absorbance of each sample was measured at 405 nm wavelength in a ELISA plate reader.

3.7. ELECTRO MOBILITY SHIFT ASSAY (EMSA)

3.7.1. Nuclear Protein extraction

Cells were trypsinized and centrifuged at 300 x g for 10minutes. Pellets were dried and 200 ul of hypotonic solution containing 10 mM HEPES (w/v) (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 0,1 mM EDTA (Ethylenediamine tetra acetic acid) , 0,1 mM EGTA (w/v) (Ethylene glycol tetra acetic acid), 0,1 mM DTT (w/v) (Dithiothreitol) , 50 mM NaF (w/v) (Sodium fluoride), 1 mM Na_3VO_4 (w/v) (Sodium orthovanadate), 1% PMSF (v/v) (Phenylmethylsulfonyl fluoride), 30 mM B-glycerophosphate , %1 protease inhibitor cocktail (v/v) (Santa Cruz), distilled water was added and mixed by vortexing .Samples were incubated on ice for then minutes After incubation 20 ul of 10% Igepal (v/v) was added on samples and mixed by vortexing ,final concentration of Igepal was 1% in samples ,they were incubated again on ice for 20 minutes. Samples were centrifuged at 450 x g for 2.5 minutes to precipitate the cell membrane. Supernatant were transferred into a new tube without disturbing the pellet and then centrifuged at 4480 x g for 3 minutes to precipitate nuclei. Supernatant was removed completely which contains cytosolic fraction and 25 µl of hypertonic solution includes ; 20 mM HEPES (w/v), 420 mM NaCl (w/v) (Sodium chloride), 1 mM EDTA (w/v), 1mM DTT (w/v) , 50 mM NaF (w/v) , 1 mM Na_3VO_4 (w/v), 1% PMSF (v/v) (Phenylmethylsulfonyl fluoride), 30 mM B-glycerophosphate, %1 protease inhibitor cocktail (v/v) (Santa Cruz), distilled water , was added on samples and kept on ice for 30 minutes by vortexing in every 5 minutes. Samples

were centrifuged at 4480 x g for 10 minutes to remove nuclear membrane. Supernatant which is the nuclear fraction was removed carefully into a new tube and pass onto protein content assay.

3.7.2. Protein content assay

Pierce Comassie Protein Assay Kit-Microplate Procedure was used to measure the protein content of nuclear protein extracts. 2 mg/ml BSA solution was used as a standart and serial dilutions from BSA were prepared at a working range of 0.1-1.5 mg/ml. 5 µl of each BSA solution and 1 µl unknown protein sample were added into wells of a microtiter plate and incubated with 250 µl of Comassie reagent at room temperature for 10 minutes. The absorbance was measured at 590 nm using an ELISA plate reader. The standard curve graph was prepared using absorbance values of BSA standard samples versus their concentration to calculate the protein content of samples. Nuclear proteins were stored at -80°C.

3.7.3. Biotin 3’ End Labeling

The probe used in electrophoretic mobility shift assay (EMSA) was forward 5'- ACA ATC AGT TGA GGG GAC TTT CCC AGG CAA-3’and reverse 5’- TTG CCT GGG AAA GTC CCC TCA ACT GAT TGT-3' which was labeled using Biotin 3’ End DNA Labeling kit (Pierce). Labelling mixture was composed of 1 µl of 20 U/µl TdT (Termal deoxynucleotidly transferase) enzyme, 2 µl of 5X TdT Reaction buffer, and 7 µl of Dnase/Rnase free water. The reaction was prepared as shown in Table 3.2;

Table 3.2. Biotin 3’ End labeling reaction reagents

Component	Volume (µl)	Final Concentration
Dnase/Rnase Free Water	27	---
5X TdT Reaction Buffer	10	1X
Unlabelled Control Oligo	3	100 nM
Biotin-11-dUTP(5µM)	5	0.5µM
Diluted TdT (2 U/µl)	5	0.2 U/µl
Total Volume	50	---

This reaction was carried out both for the reverse and forward Nf-κB oligo primers. The reaction mix was incubated at 37°C for 30 minutes. To stop the reaction 2.5µl of 0.2M EDTA was used. To extract the TdT enzyme 50µl chloroform: isoamly alcohol (24:1) was added followed by vortexing the mixture. Samples were centrifuged for 2 minutes at 13,000xg in a microcentrifuge to separate the phases. Equal amounts of aqueous top phases was mixed together and incubated for 1 hour at 37°C water bath for the annealing reaction.

3.7.4. Electro mobility Shift Assay (EMSA) Gel Electrophoresis

EMSA is based on the binding of Nf-κB transcription factor to a labeled specific oligonucleotide probe which possesses the DNA binding sequence recognized by Nf-κB. The binding of Nf-κB to the labeled probe retard the migration of the probe through a nondenaturating gel. In other words, when this transcription factor protein binds to the dsDNA probe, the probe migrates slower than non-bound probe in a polyacrylamide gel. 12% (v/v) bottom gel and 6% (v/v) natural nondenaturating polyacrylamide gel in 0.5X TBE were used to characterize protein/DNA complexes. Top gel and bottom gel was prepared according to the Table 3.3.

Table 3.3. The compositions of polyacrylamide gels with different percentages

	Final % (w/v) acrylamide concentration	
Stock solutions:	6 %	12 %
30% (w(v) acrylamide/ 0.8% bisacrylamide	5 ml	9.4 ml
5X TBE(450 mM Tris (w/v), 450 mM Boric acid(w/v),10 mM EDTA (w/v), pH 8.3	2.5 ml	2.5 ml
dH_2O	17.3 ml	12.9 ml
10% APS (Ammonium per sulfate) (w/v)	175µl	175 µl
TEMED Tetramethylethylenediamine) (v/v)	8.75 µl	8.75 µl

The gel must be pre- run for 10 minutes at 70 volt then 90 volt for 60 minutes with 0.5X TBE on ice. Binding reaction was performed during the pre-run according to the manufacture's recommendations (Pierce LightShift Chemiluminescent EMSA kit,).

Table 3.4. Preparation of binding reaction mixture

Components	Final amount	Reaction volume
10 X Binding Buffer	1X	2 µl
100mM $MgCl_2$	5 mM	1 µl
1% Non–idet P40 (v/v)	0.05 %	1 µl
100 mg/ml BSA	2.5 mg/ml	0.5 µl
100µM Poly (dI·dC)	2.5 µM	0.5 µl
Total		5 µl

Binding reaction was prepared for each sample. 5 µl of binding reaction mixture was added on the nuclear extracts that contained 1.8 µg protein in 15µl hypertonic solution and

incubated at room temperature for 10 minutes. After incubation, 1µl of labeled probe at 3 pmol/µl concentration (labeling procedure was described in section 3.7.3) was added onto nuclear extract samples and incubated at room temperature for 90 minutes for binding reaction to take place. The control reaction was prepared according to Table 3.5., 30 minutes before the end of the binding reaction. Control reaction was performed to visualize the position of unlabelled free probe and did not contain any nuclear protein.

Table 3.5 Components of binding reaction and final reaction volume

Components	Reaction volume
Dnase/Rnase free water	11 µl
10X Binding Buffer	2 µl
50 % Glycerol	1 µl
100 mM $MgCl_2$	1 µl
1% Non-idet P40	1 µl
1µg/ml Poly dI·Dc	1 µl
EBNA Extract	1 µl
Biotin-EBNA Control DNA	2 µl
Total	20 µl

5µl of 5X Loading Buffer was added onto the samples and the control reaction. 20 µl of each sample was loaded onto the native polyacrylamide gel. Samples were electrophoresed at a constant 90 volts for 10 minutes to 120 volt until the bromophenol blue dye migrated approximately 3/4 down the length of the gel.

3.7.5. Transferring Binding Reactions to Nylon Membrane

Positive charged nylon membrane was equilibrated with 0.5X TBE at least for 10 minutes. Sandwich of gel and nylon membrane was prepared on black side of the cassette, which is the negative side, as follows; sponge bad, filter paper, gel, positive charged nylon membrane, filter paper, sponge respectively. Cassette was then inserted into the blotting apparatus by facing the gel side to the cathode electrode and the membrane side to the anode electrode. Transfer was performed at a constant 200 mA for 2 hours in 0,5X TBE

which was cooled to 10°C before the use. After the transfer was completed, membrane was placed with the bromophenol blue side up on a dry paper towel and cross-linked at 120 mJ/cm^2 at UV light cross-linker for 45-60 seconds.

3.7.6. Detection of Biotin-labeled DNA by Chemiluminescence

Blocking buffer and 4X wash buffer was slowly warmed to 37-50°C in a water bath until all particles were dissolved. The membrane was blocked with 10 ml of blocking buffer containing 100 µg/ml BSA for 15 minutes with gentle shaking at room temperature. For the detection of the membrane bound biotin labeled probe-protein complexes, membrane was incubated 30 minutes with gentle shaking at room temperature in the blocking buffer containing 33 µl of stabilized streptavidin-horseradish peroxidase conjugate at 1:300 dilution. After incubation, membrane was rinsed briefly with 12 ml of 1X Wash buffer and then washed with 1X Wash buffer solution four times in 15 ml for five minutes with gentle shaking at room temperature. In the each washing step membrane was transferred into a new tray. Following the washing steps, nylon membrane was transferred to the substrate equilibration buffer which was stored at 4°C and incubated for 5 minutes again with gentle shaking at room temperature. Meanwhile, the substrate working solution was prepared by adding 6 ml luminol/enhancer solution to stable peroxidase solution. Membrane was transferred to a new tray and incubated with the detection mix for 5 minutes at room temperature. It is important to distribute the detection mix equal to the each part of the membrane. After incubation, detection mix was discarded and the membranes were wrapped with stretch film and chemiluminescent signal was detected via Image J chemiluminescence imaging system.

3.8. ANIMAL EXPERIMENTS

8 weeks old Wistar Hannover male rats weighing about 200–250 g, were bred in individual cases at YUDETAM, Centre for Animal Health Studies at Yeditepe University. All the animals were kept and maintained under standard laboratory conditions; 20-24 °C of temperature, 55% humidity and expose to 24 hours of day light. Animals were divided into three groups; Control, High fat diet, High fat diet with cinnamon. Control group was fed with chow diet containing daily supplements; high fat diet group were fed a western

diet containing 45% kcal high fat cholesterol pellets and high fat diet with Cinnamon group were fed with the high cholesterol pellets and cinnamon extract for four weeks . Water soluble cinnamon extract was added to animal's drinking water daily. Before the start of the experiment, daily water consumption of animals was observed for one week and average of that week was taken as a daily consumption water volume. Daily intake of cinnamon extract was determined as 300 mg/kg and 600 mg/kg. Drinking water of animals was prepared due to the observation for the whole week consumption. Consumption of the water, amount of the pellets and weight of the animals was measured each day for statistical analysis. At the end of the experiments animals were sacrificed and the whole blood in the body collected in Z Serum Sep Clot Activator tubes and EDTA tubes for total cholesterol, HDL, LDL, VLDL, triglyceride analysis which was performed in Biochemistry Laboratories at Yeditepe University.

4. RESULTS AND DISCUSSION

4.1. CHARACTERIZATION OF RAT VASCULAR SMOOTH MUSCLE CELLS

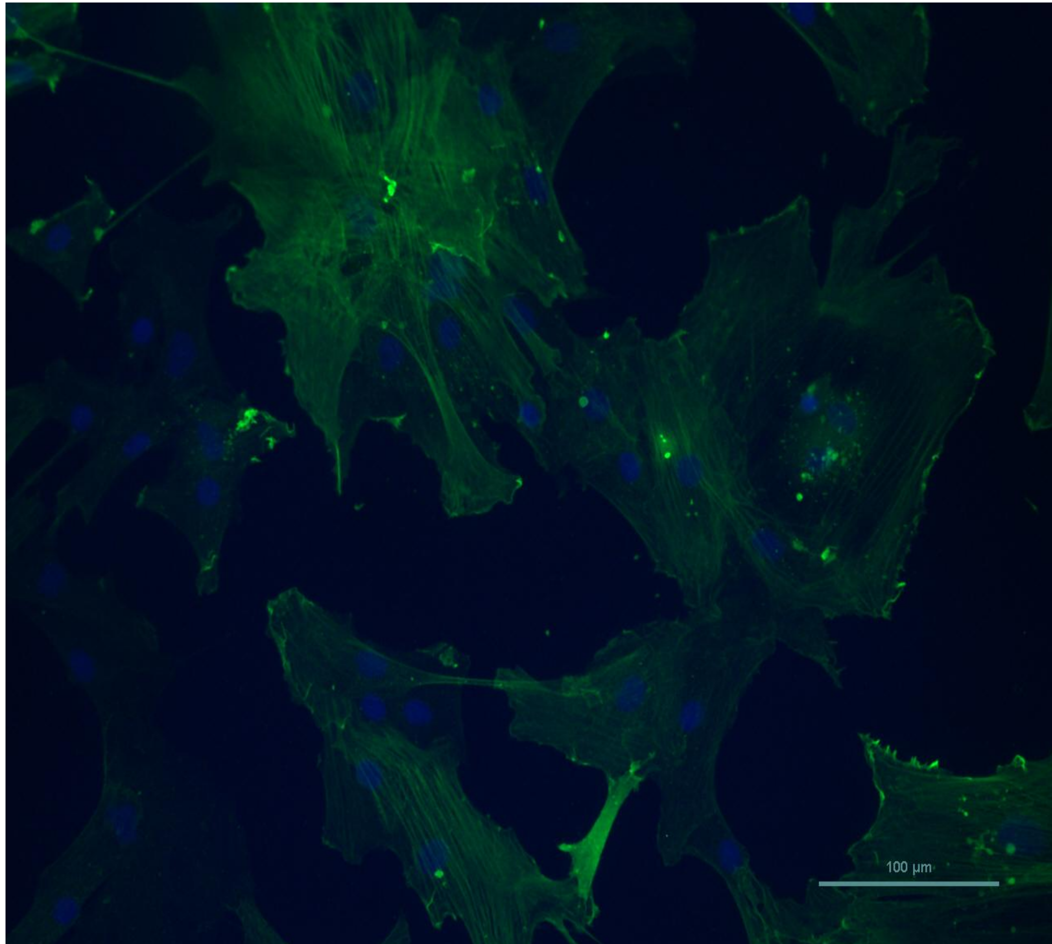

Figure 4.1. Immunoflorescent staining of VSM cells isolated from the male rat aorta (x10). Immunofluorescent staining was visualized using florescent microscope using 10X objective. The bar Shows 100 μm.

In order to characterize the cells isolated from the rat aorta as the vascular smooth muscle cells, a vascular smooth muscle cell marker α –actin was used. α-actin smooth muscle cell antibody is able to recognize exclusively α -smooth muscle actin which is highly expressed in VSM cells and widely in use for the characterization of this cell type. To visualize the presence of α –actin, VSM cells were seeded on coverslips and probed

with α -SMA and the nucleus of cells were stained with DAPI as described in Section 3.3. Figure 4.1 shows that the isolated cells were strongly stained with α –SMA confirming their vascular smooth muscle nature.

4.2. EFFECTS OF FBS ON VSM CELL PROLIFERATION

To investigate the distinct effect of the cinnamon extract, cinnamaldehyde and ox-LDL, it was important to minimize the proliferative effect of FBS in the growth media that also contains sPLA enzyme which could give false positive results during experiments.

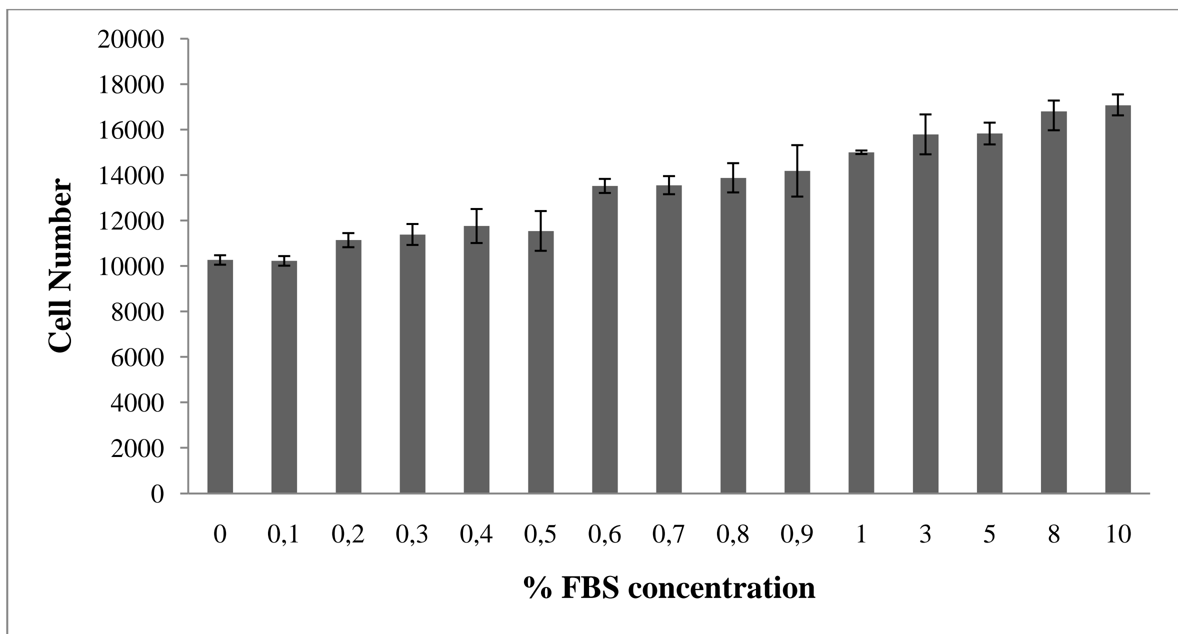

Figure 4.2. Effects of FBS treatment on proliferation of VSM cells were measured by WST-1 assay as described in Section 3.2. Data represents mean values ± S.D. per sample at least from three separate experiments performed in quadruplicate.

Although VSM cells are located at the endothelial wall in quiescent contractile situation, in cell culture conditions these cells were in the proliferative phenotype. In order to mimic the *in vivo* conditions in the blood vessel wall, VSM cells need to be kept in the quiescent state during the experiments by culturing these cells in low serum containing media. In addition, serum contains $sPLA_2$ therefore it is critical that VSM cells were cultured in the growth media containing the minimum percentage of serum to keep the

background signal as low as possible during $sPLA_2$ assays. To determine the minimum serum concentration that sustains the cells alive at the quiescent state, cell were seeded on a 96 well plate (10.000 cells/well) and incubated in media containing various concentration of serum, ranging from 0-10% (v/v). Following 24 hour incubation, cells viability was measured using the WST-1 assay (Figure 4.2). In order to express the absorbance values in means of cell number, a standard plate was prepared by seeding 2500, 5000, 7500, 10,000, 12,500 and 15,000 cells per well of a 96 well plate overnight. The absorbance values for the standard plate was obtained using the WST-1 assay at 18 hours which is the time was set as the time needed for VSM cells to adhere on the tissue culture plastic. In other words, cells seeded to the standard plate reflected the true cell number as there was not enough time for their proliferation before taking the measurement using WST-1 assay. As can be seen from the Figure 4.2, cells started to proliferate in the media containing serum more than 0.5% (v/v), therefore VSM cells were needed to be kept in growth media containing less than 0.5% serum (v/v). Data from WST-1 experiments suggests that the optimum concentration of serum for the VSM cells was 0.1% FBS (v/v) (Figure 4.2).

4.3. DETERMINATION OF THE PROLIFERATIVE EFFECT OX-LDL ON VSM CELLS

In order to analyze the minimum concentration of ox-LDL that would lead to cell proliferation, VSM cells were incubated with increasing concentrations of minimally ox-LDL (Kalen Biomedical) in DMEM containing 0.1% FBS (v/v). Cell viability was measured using the WST-1 assay as described in the Section 3.2. Figure 4.2 shows the effect of ox-LDL treatment on VSM cells after 24 hours.

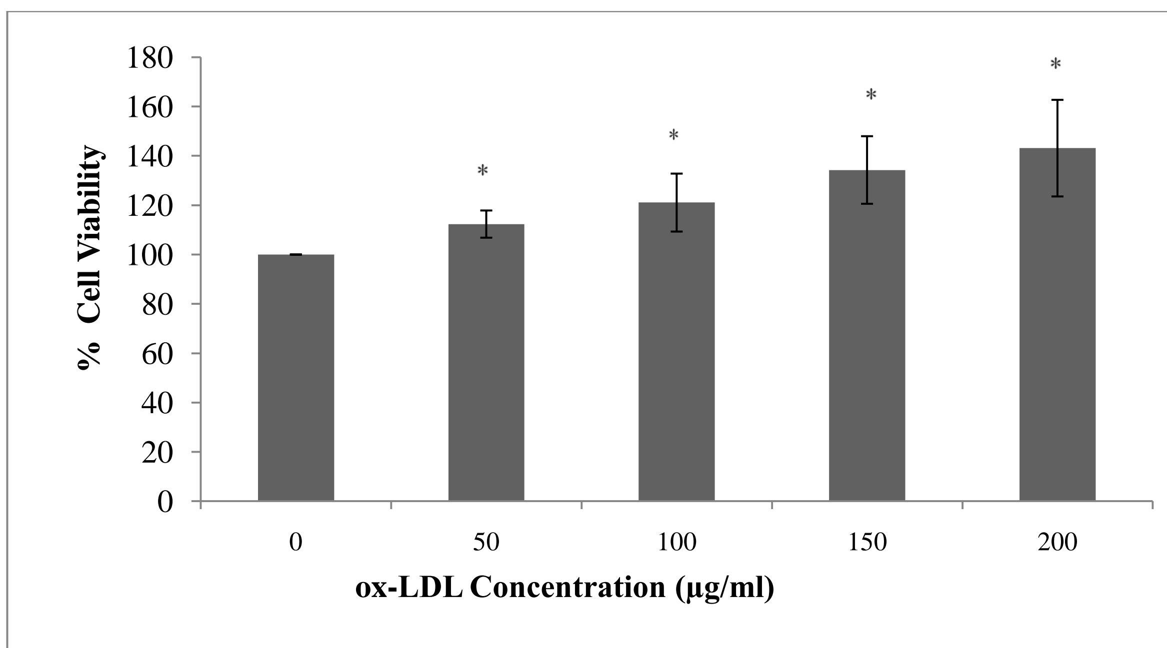

Figure 4.3**.** Effects of ox-LDL on VSM cells viability. Each column data corresponds to the proliferation levels of VSM cells for each concentration of ox-LDL± S.D., which was performed at least three times. Statistic analyses of all experiments were calculated with MedCal statistical software program.* symbol shows statistical difference between each coloum.

Results demonstrated that ox-LDL caused a statistically significant 12% increase in the cell proliferation at 50 µg/ml of ox-LDL ($p < 0.05$). At 100 µg/ml of ox-LDL, VSM cells showed a 21% increase in the cell proliferation. At 150 and 200 µg/ml concentrations of ox-LDL, a 40% increase in cell proliferation was measured for the VSM cells.

4.4. QUANTIFICATION OF *TRANS*-CINNAMALDEHYDE BY USING HPLC

To measure the active compound *trans*-cinnamaldehyde amount in the water soluble *Cinnamomum zeylanicum* extract, HPLC method was used as described in the Section 3.5. To display the uniform distribution of cinnamon extract, six different amount of extract 10.02 mg, 12.19 mg, 9.94 mg, 10.07 mg, 13.39 mg, 10.44 mg were used in the experiment, respectively.Chromotography results of cinnamon extract were shown below.

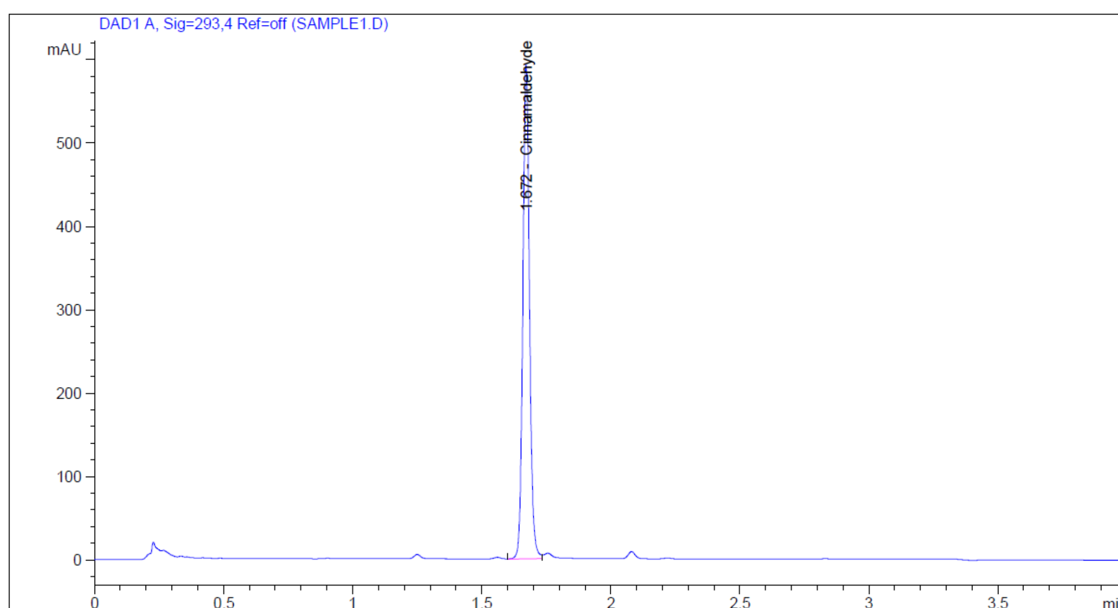

Figure 4.4. *Trans*-cinnamaldehyde amounts of 10.02 mg *Cinnamomum zeylanicum* extract

Table 4.1. Retention time (experiment time) and total amount of cinnamaldehyde per mg (for 10.02 mg)

Retention time (min)	**Type**	**Area (mAU*s)**	**Amt/Area**	**Amount mg/g extract**	**Name**
1.672	VV	1057.21448	3.32304e-5	35.06138	Cinnamaldehyde
Totals:				**35.06138**	

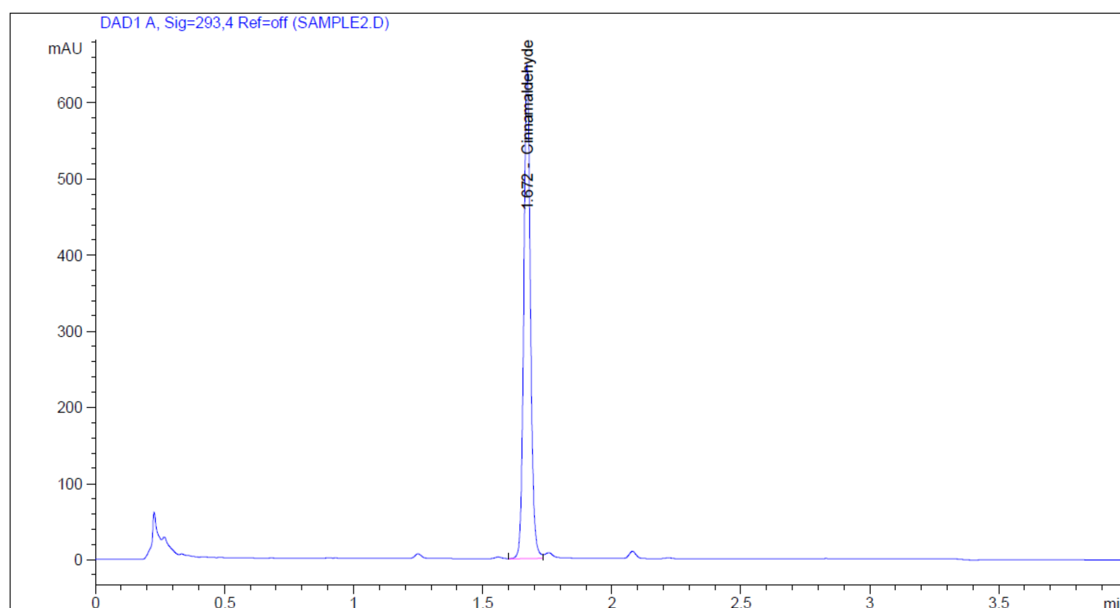

Figure 4.5. *Trans*-cinnamaldehyde amounts of 12.19 mg *Cinnamomum zeylanicum* extract

Table 4.2. Retention time (experiment time) and total amount of cinnamaldehyde per mg (for 12.19 mg)

Retention time (min)	**Type**	**Area (mAU*s)**	**Amt/Area**	**Amount mg/g extract**	**Name**
1,672	VV	1158.96863	3.32878e-5	31.64675	Cinnamaldehyde
Totals:				**31.64675**	

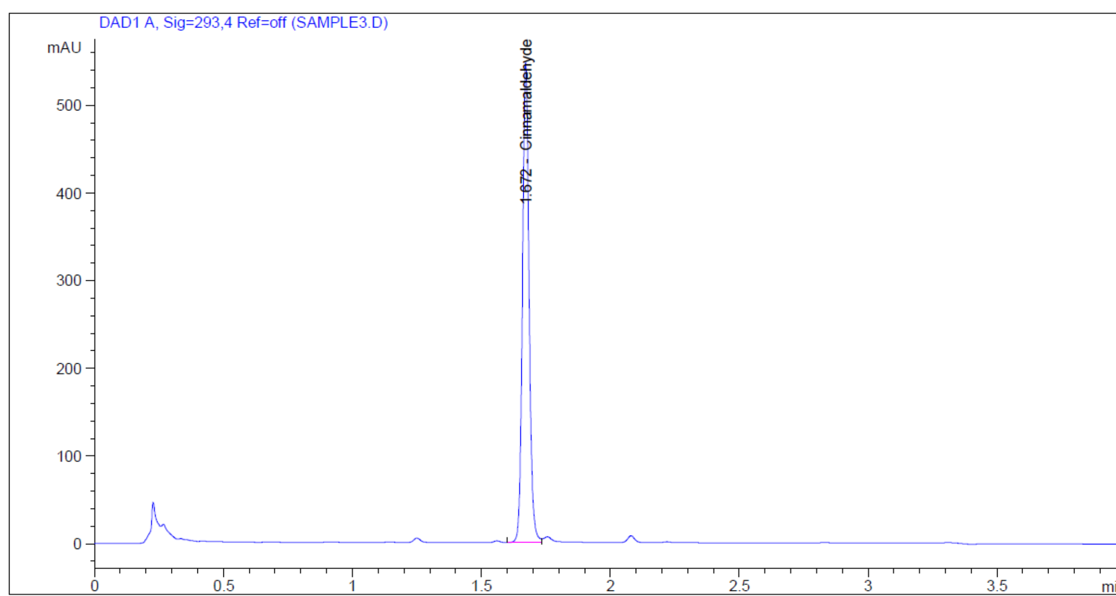

Figure 4.6. *Trans*-cinnamaldehyde amounts of 9.94 mg *Cinnamomum zeylanicum* extract

Table 4.3. Retention time (experiment time) and total amount of cinnamaldehyde per mg (for 9.94 mg)

Retention time (min)	Type	Area (mAU*s)	Amt/Area	Amount mg/g extract	Name
1.672	VV	977.38739	3.31770e-5	32.62231	Cinnamaldehyde
Totals:				**32.62231**	

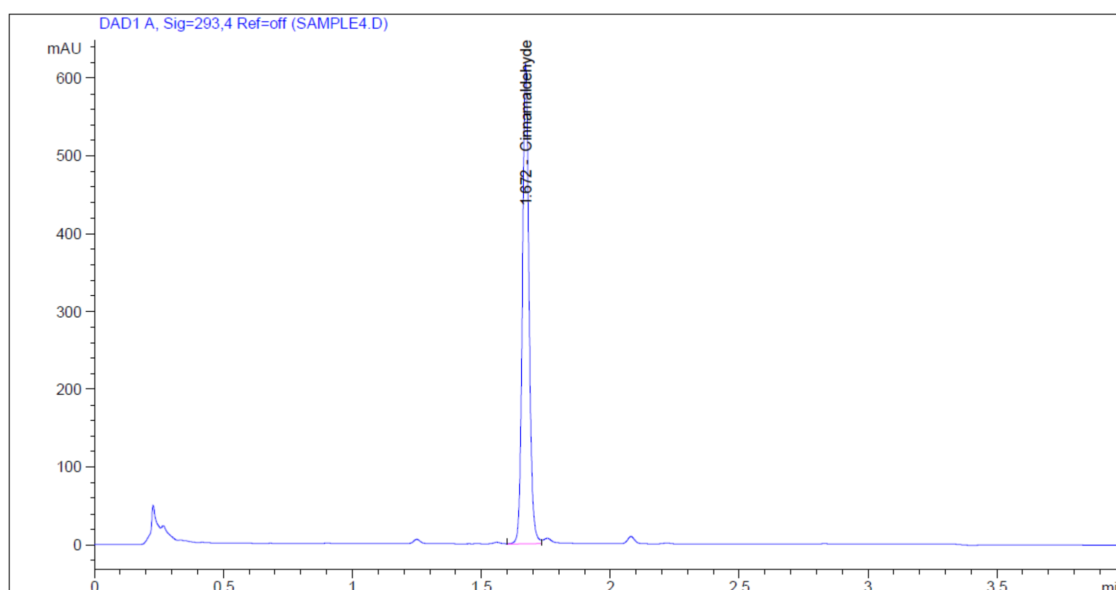

Figure 4.7. *Trans*-cinnamaldehyd*e* amounts of 10.07 mg *Cinnamomum zeylanicum* extract

Table 4.4. Retention time (experiment time) and total amount of cinnamaldehyde per mg (for 10.07 mg)

Retention time (min)	**Type**	**Area (mAU*s)**	**Amt/Area**	**Amount mg/g extract**	**Name**
1.672	VV	1102.18433	3.32571e-5	36.40065	Cinnamaldehyde
Totals:				**36.40065**	

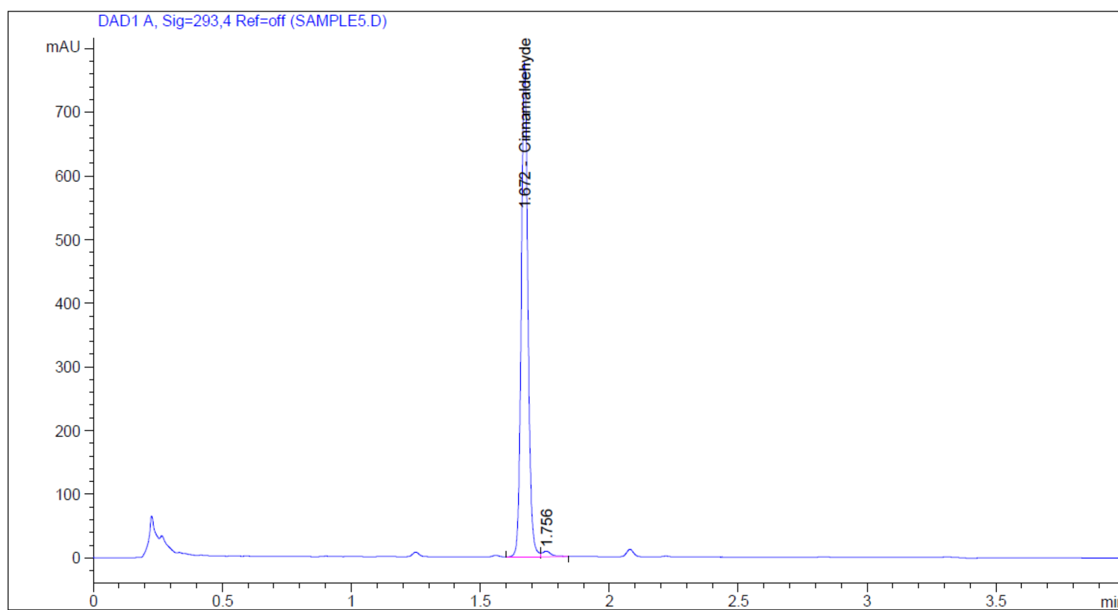

Figure 4.8. *Trans*-cinnemaldehyde amounts of 13.39 mg *Cinnamomum zeylanicum* extract

Table 4.5. Retention time (experiment time) and total amount of cinnamaldehyde per mg (for 13.39 mg)

Retention time (min)	**Type**	**Area (mAU*s)**	**Amt/Area**	**Amount mg/g extract**	**Name**
1,672	VV	1389.2262	3.33866e-5	34.63866	Cinnamaldehyde
Totals:				**34.63866**	

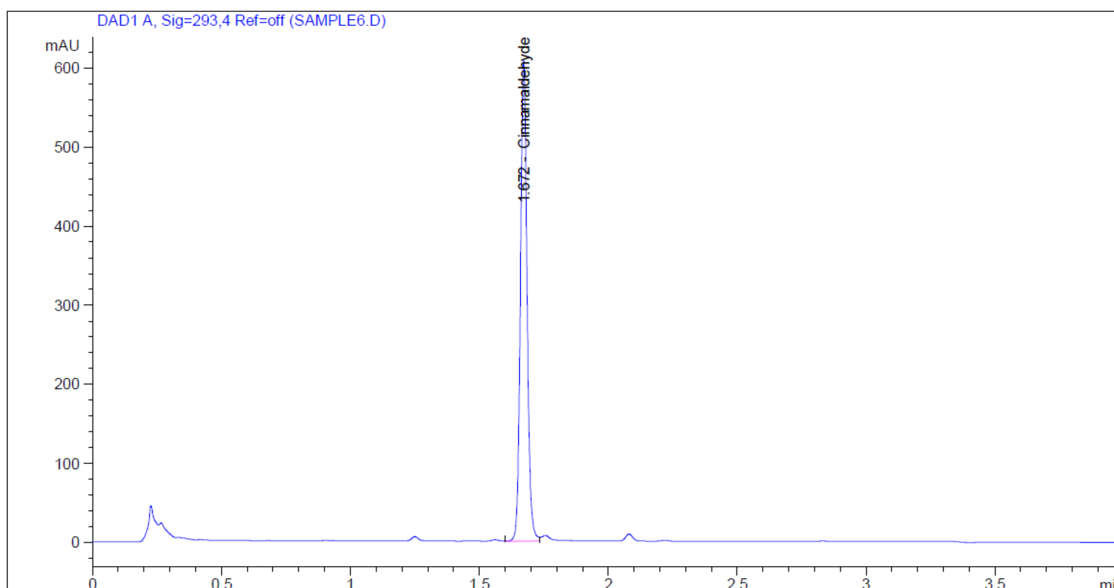

Figure 4.9. *Trans*-cinnemaldehyde amounts of 10.44 mg *Cinnamomum zeylanicum* extract

Table 4.6. Retention time (experiment time) and total amount of cinnamaldehyde per mg (for 10.44 mg)

Retention time(min)	**Type**	**Area (mAU*s)**	**Amt/Area**	**Amount mg/g extract**	**Name**
1,672	VV	1087.32935	3.32485e-5	34.62825	Cinnamaldehyde
Totals:				**34.62825**	

Table 4.7. Calculation the amount of each sample

Compund : Cinnamaldehyde (Signal: DADI 1, Sig=293,4 Ref=off)							
Run #	Type	RetTime (min)	Amount mg/g extract	Area (mAu*s)	Height mAU	Width (min)	Synm.
1	VV	1.672	35.06138	1057.21448	592.05975	0.0273	0.89
2	VV	1.672	31.64675	1158.96863	649.10663	0.0273	0.89
3	VV	1.672	32.62231	977.38739	546.91516	0.0273	0.89
4	VV	1.672	36.40065	1102.18433	616.97778	0.0273	0.89
5	VV	1.672	34.63866	1389.22632	776.71161	0.0274	0.89
6	VV	1.672	34.62825	1087.32935	607.69519	0.0274	0.89
Mean:		1.672	34.16633	1128.71841	631.57769	0.0273	0.89
S.D:		8.25e-5	1.72986	140.86195	78.59270	1.82e-5	8e-4
RSD:		4.93e-5	5.06304	12.47981	12.44387	0.0664	0.09
95% CI		8.65e-5	1.81537	147.82553	82.47797	1.91e-5	9e-4

As a result, 34.16633 mg cinnamaldehyde was found in 1 gram of Cinnamon extract.

To obtain the standard curve, *trans-* cinnamaldehyde (in 99% purity, Sigma) was used as a standard control compound.1.05 mg of *trans-cinnamaldehyde* was dissolved with the 25 % (v/v) of 1 % (v/v) acetic acid, 75 % of 100 % HPLC grade methanol in 1ml and filtered with 0,45µm filter. Serial dilutions were listed in Table 4.7.

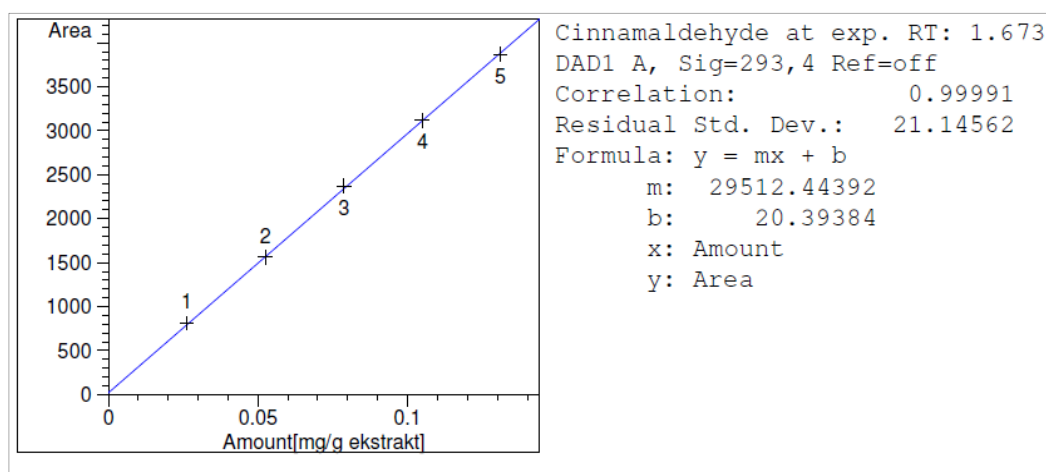

Figure 4.10. Standard curve

4.5. *IN VITRO* ATHEROGENETIC EFFECT of CINNAMON EXTRACT and CINNAMALDEHYDE

Given that in atherogenic conditions, VSM cells proliferate and give rise to vascular remodeling, in this study, the effect Cinnamon extract, Cinnamaldehyde on the ox-LDL induced proliferation of VSM cells was analyzed. After the pre-incubation of cells with cinnamon extract and cinnamaldehyde as described in Section 3.2, the effect of ox-LDL in the presence of cinnamon extract, cinnamaldehyde was measured with the WST-1 cell proliferation assay.

For each experiment, cinnamon extract was prepared freshly to avoid the oxidation reactions which can results in decreased effect of the active compound. Results from the Figure 4.11 shows that cinnamon extract co-treatment with increasing amounts of ox-LDL did not lead to a significant decrease in cell proliferation ($p > 0.05$) However, ox-LDL led to a statistically significant 15% increase in the proliferation of the VSM cells ($p < 0.05$) . Similarly, at concentration of 100 to 200 μg/ml of ox-LDL a significant 30% increased was observed in the VSM cell number regardless of the cinnamon co-treatment ($p < 0.05$).

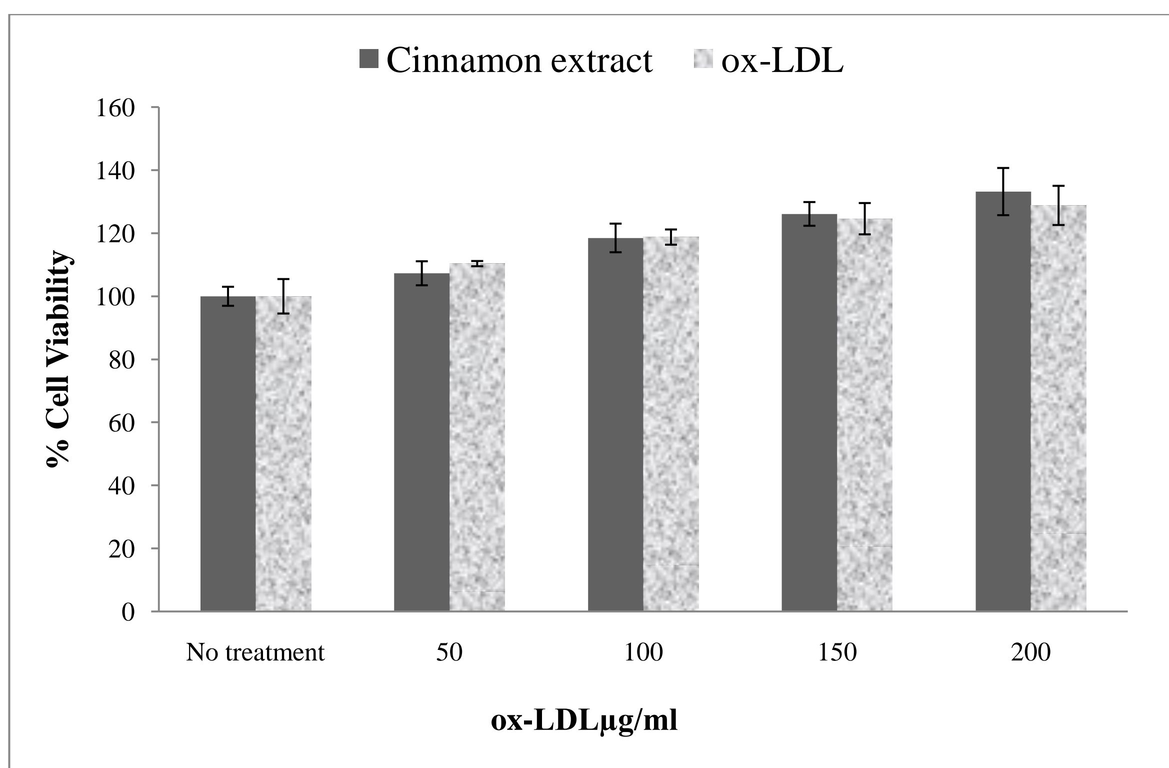

Figure 4.11. Effect of the cinnamon extract on the ox-LDL induced VSM cells. Cells were co treated with different concentrations of ox-LDL (μg/ml) and 29.27 mg of cinnamon extract, which contains 1 μg/ml cinnamaldehyde. Each column data is expressed as the mean percentage of control values± S.D. from three separate experiments performed in quadruple.

In order to observe whether pure *trans*-cinnamaldehyde would have a inhibitor effect on the proliferation of VSM cells induced by the treatments of ox-LDL, cells were pre-treated with 1 μg/ml of *trans*-cinnamaldehyde and exposed to increasing concentrations of ox-LDL. On this basis, ox-LDL induced cells were co-treated with pure *trans*-cinnamaldehyde.

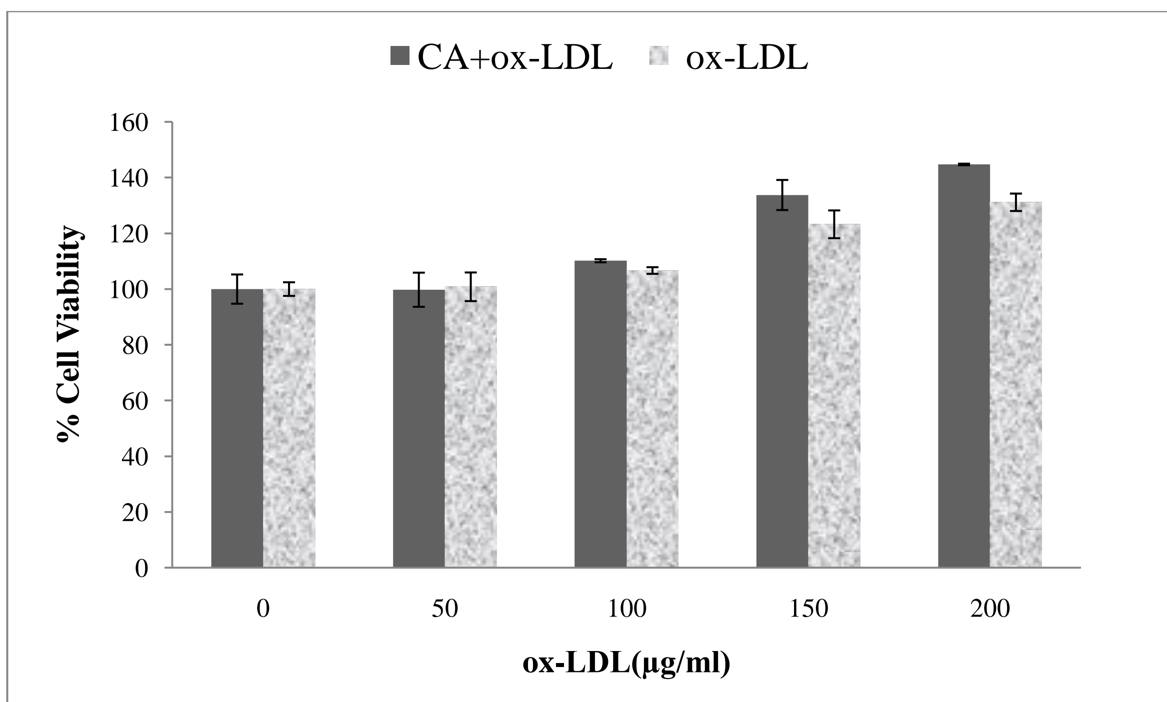

Figure 4.12. Effect of *trans*-cinnemaldeyhde on the ox-LDL induced VSM cells. Cells were co-treated with different concentrations of ox-LDL (µg/ml) and pure *trans*-cinnemaldeyhde (Sigma). Each column data is expressed as the mean percentage of control values± S.D. of three separate experiments performed in quadruple.

According to the results obtained from three experiments (Figure 4.12), cinnamaldehyde and cinnamon extract did not have any inhibitory effect on the proliferation of ox-LDL induced VSM cells ($p > 0.05$).

4.6. DETECTION OF $sPLA_2$

In order to determine the effect of ox-LDL cinnamaldehyde and cinnamon extract on the $sPLA_2$ activation, VSM cells were incubated with ox-LDL, cinnamaldehyde and cinnamon extract for 24 hours and each sample's medium were collected and $sPLA_2$ amount was determined using the Correlate Enzyme Assay kit.

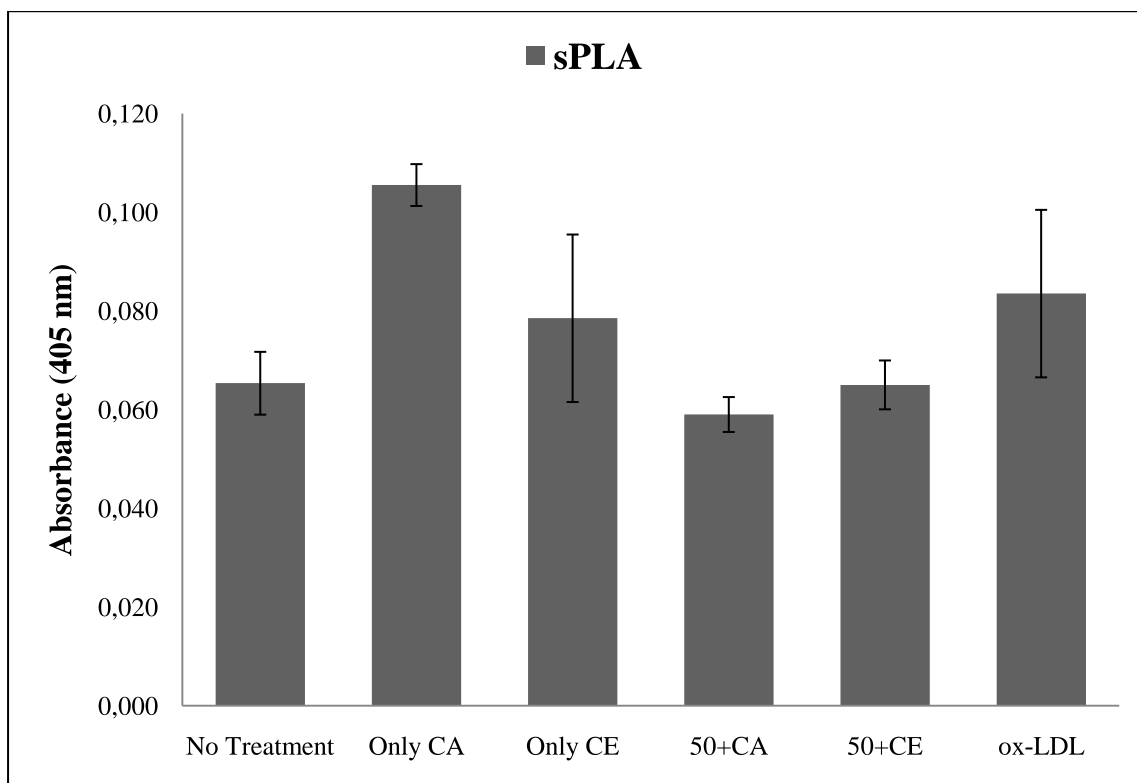

Figure 4.13. $sPLA_2$ amounts was measured with Correlate Assay Enzyme Kit as described in section 3.6. Each column data corresponds to the mean absorbance values at 405 nm ± S.D. which was performed in triplicate.

Ox-LDL induced VSM cells exhibited 1.28 fold increased amount of $sPLA_2$ in comparison to the control sample (no treatment) (Figure 4.13). The treatment of cinnamaldehyde and cinnamon extract alone led to a 1.61 and 1.20 fold increase in the $sPLA_2$ amount in VSM cells, respectively. Furthermore, co–treatment of VSM cells with cinnamaldehyde or cinnamon extract in the presence of ox-LDL led to a 1.1 fold reduction in $sPLA_2$ levels, when compared to the sPLA2 levels obtained for VSM cells treated with ox-LDL alone.

4.7. NF- κB ACTIVATION OF VSM CELLS

In order to analyze Nf-κB activation, VSM cells were cultured on 60 cm^2 culture dish (1x 10^6cells/dish) and treated with ox-LDL, cinnamon extract, and cinnamaldehyde. Nuclear fractions were isolated from VSM cells as described in the Section 3.7.1. Nuclear proteins (5μg) were hybridized with the DNA probe, separated in a non-denaturating

PAGE and then transferred to a positively charged nylon membrane as described in the Section 3.7. Nuclear proteins were probed with Streptavidin Peroxidase and detected with the chemiluminescent nucleic acid detection module as described in 3.7.6**.**

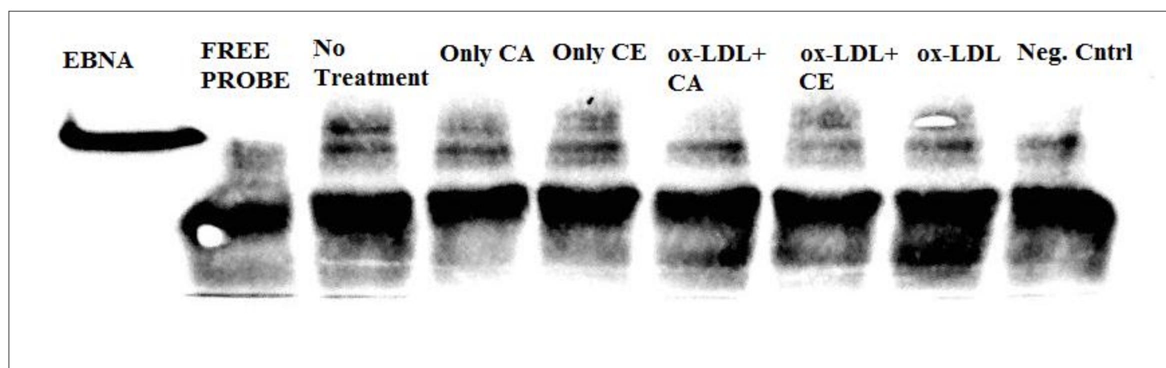

Figure 4.14. NF-κB activation in VSM cells were analyzed by EMSA as described in Section 3.7. Epstein-Barr Nuclear Antigen (EBNA) Extract (lane 1), free biotin labeled (lane 2) NF-κB promoter probe, and unlabelled NF-κB promoter probe in 100 fold excess (lane 9) was added as a control. Representative EMSA blot shows the activity levels of NFκB in the nuclear extracts of non-treated cells (lane 3) , cinnamaldehyde treated cells (lane 4), cinnamon extract treated cells (lane 5), ox-LDL(50 µg/ml) induced cinnamaldehyde treated cells (lane 6), ox-LDL (50 µg/ml) induced cinnamon extract treated cells (lane 7) and ox-LDL induced (50 µg/ml) cells (lane 8).

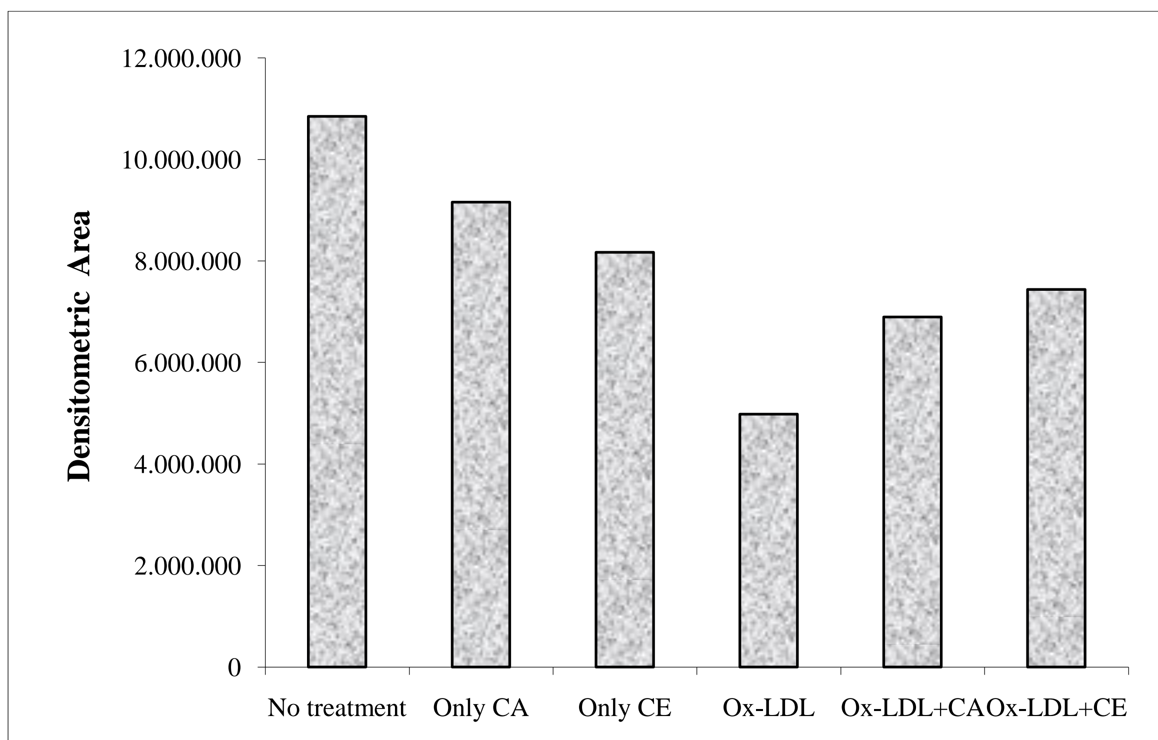

Figure 4.15. Densitometric quantification of active NF-κB nuclear protein levels were calculated with Image J Program. Each column corresponds to the density of the bands representing the amount of probe bound to the active NF-κB protein.

Although ox-LDL treatment of VSM cells resulted in an increase in the cell proliferation, it led to a decrease in the active NF-κB levels. VSM cells without any treatment showed high levels of active NF-κB and cinnamon extract and cinnamaldehyde treatment caused a decrease in these active NF-κB levels.

4.8. ANIMAL EXPERIMENTS

To elucidate the in vivo effect of cinnamon extract on the cholesterol metabolism, Wistar Hannover rats were fed with 45% (w/w) cholesterol diet for 4 weeks in the presence or absence of 300 mg/kg of cinnamon extract. Following the 4 week period, animals were weighed and scarified and the blood was collected for the determination of HDL, LDL, VLDL, total cholesterol, and triglyceride.

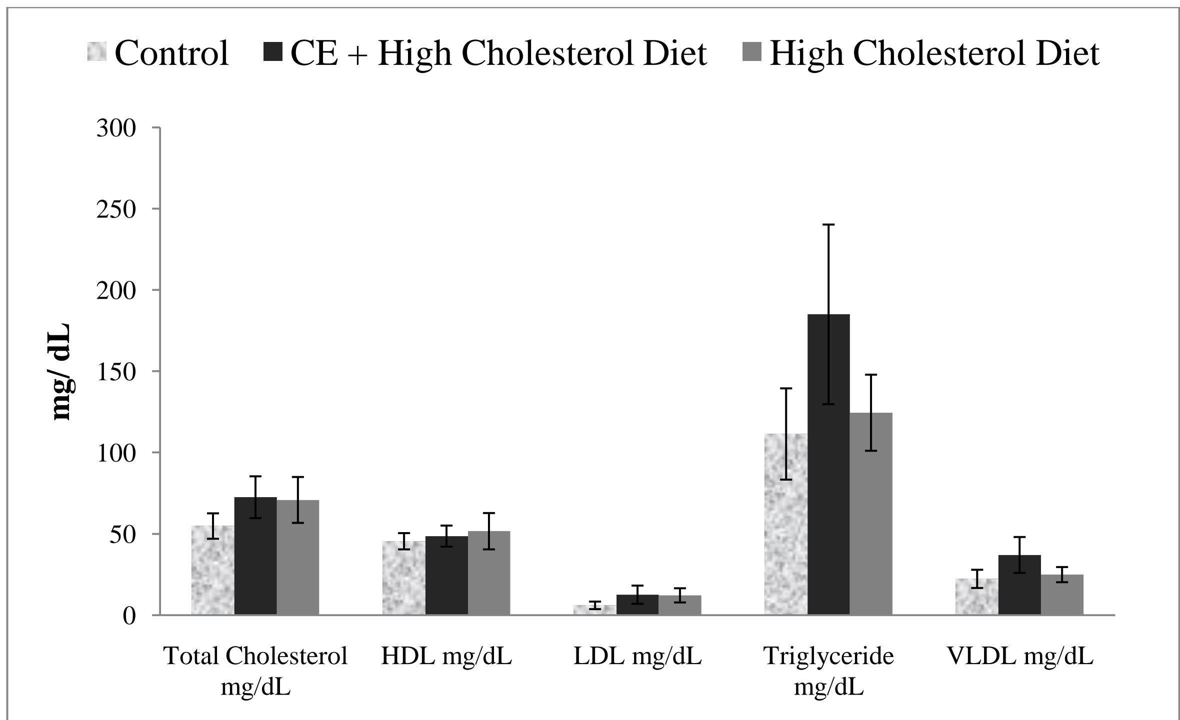

Figure 4.16. Biochemical analysis of blood obtained from the animals. Group 1 (Control Animals) were fed with chow diet, Group 2 (CE +High Cholesterol Diet) were fed with high cholesterol diet and cinnamon extract (300 mg/kg),Group 3 (High Cholesterol Diet) were fed with high cholesterol diet for 30 days.

The results showed that HDL, LDL, VLDL, total cholesterol, and triglyceride content of the serum from the three experimental groups were not significantly different than each other (Figure 4.16) ($p > 0.05$). In order to understand whether cinnamon extract has any effect on the cholesterol metabolism due to low dose of application, the animal experiments were repeated with a higher dose of cinnamon extract, 600 mg/kg.

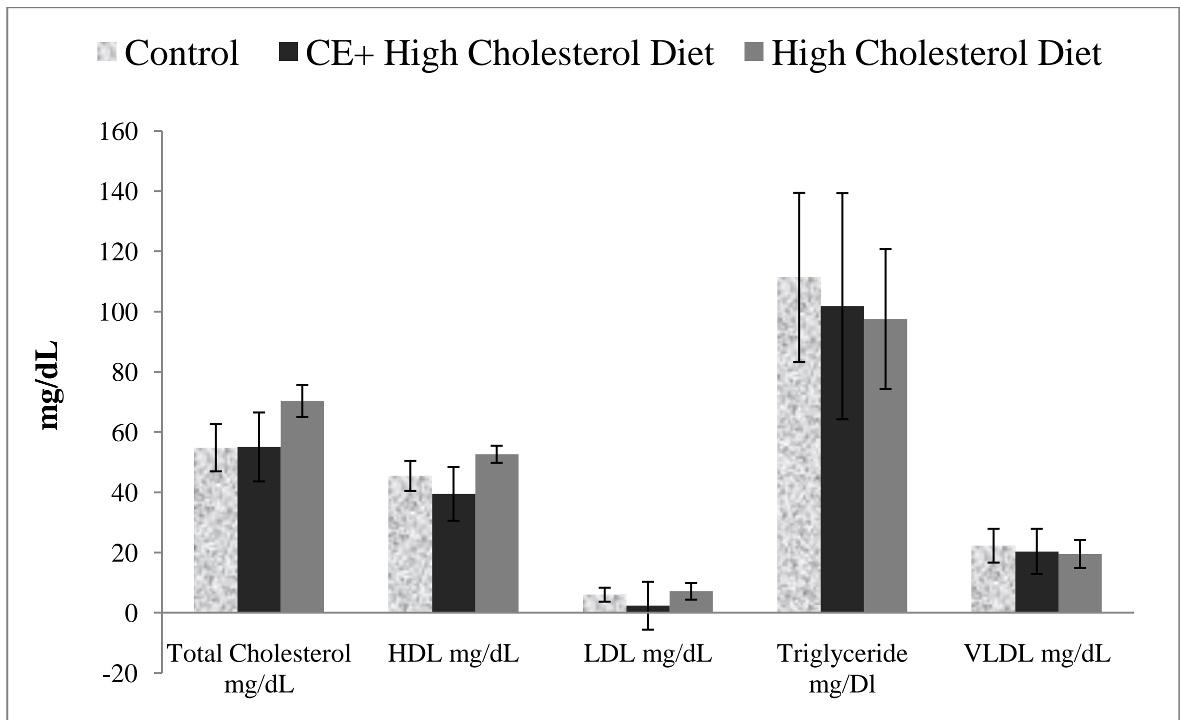

Figure 4.17. Biochemical analysis of blood obtained from the animals. Group 1 (Control Animals) were fed with chow diet, Group 2 (CE +High Cholesterol Diet) were fed with high cholesterol diet and cinnamon extract (600 mg/kg), Group 3 (High Cholesterol Diet) were fed with high cholesterol diet for 30 days.

Figure 4.17 shows that increased of cinnamon extract dose did not lead to a change in the serum HDL, LDL, VLDL, total cholesterol, and triglyceride levels.

When the weight of the animals were investigated in each group, it was seen that the high cholesterol diet fed animals were given cinnamon extract, a significant reduction in the weight gain of the animals in both cinnamon extract doses was achieved (Table 4.10 and Table 4.11).

Table 4.8. Weight change analysis of animals (Cinnamon extract 300 mg/kg)

Cinnamon extract 300 mg/kg	Number of the Animals(#)	Avarage of Group weight (g) at day =0	Avarage of Group weight (g) at day =30	Avarage gained weight (g)
Group 1 High Cholesterol+ Cinnamon Extract (300 mg/kg)	5	209.12	300.73	91.61
Group 2 High Cholesterol Diet	5	201.6	325.98	124.38
Group 3 Chow Diet	5	191.36	291,15	99.79

Table 4.9. Weight change analysis of animals (Cinnamon extract 600 mg/kg)

Cinnamon extract 600 mg/kg	Number of the Animals(#)	Avarage of Group weight (g) at day =0	Avarage of Group weight (g) at day =30	Avarage gained weight (g)
Group 1 High Cholesterol+ Cinnamon Extract (600 mg/kg)	6	238.79	355.9	117.11
Group 2 High Cholesterol Fat Diet	6	236.9	360.15	123.25

5. DISSCUSSION

Atherosclerosis begins in the childhood and develops slowly as a chronic inflammatory disease over decades. It is the hallmark of cardiovascular diseases and also one of the leading causes of death in the world population [107]. Many chemical drug treatments have currently being used to decrease the high levels of serum cholesterol, LDL, triglyceride in addition to the medical applications that are still the provided therapy to recover the injured arteries and thrombosis [108]. Besides these drug treatments and clinical applications, there could be an alternative therapy with herbal plants to eliminate the development of early atherogenesis which have fewer side effects than clinical therapies.

Early studies reported that Cinnamomum species have a functionality on the insulin-lipid metabolism. Active compounds of Cinnamon could mimic the biological effects of insulin by reducing the excess amount of glycogen in blood, resulting in a decrease in the blood glucose, LDL, total cholesterol and triglyceride levels [90]. According to recent reports, the effect of *Cinnamomum zeylanicum* which contains the lowest amount of toxic coumarin compound among the other Cinnamon types has a potential as a protective herbal agent to prevent the formation of atherogenesis in diabetic patients [86].

In normal arteries VSM cells are in quiescent contractile state and responsible from the regulation of blood vessel diameter and blood flow [109,110]. After the injury of arterial endothelial cells, macrophages and platelets release some mediators such as growth factors and cytokines which initiate the inflammation progression in arteries. These growth factors and cytokines induce VSM cells to change into active state from the quiescent contractile state [111, 112]. Dedifferentiated phenotypes of VSM cells have increased their capacity to synthesize ECM matrix to recover the injury side [113, 114]. This induced migratory and proliferative phenotype of VSM cell is present during the pathogenic vascular remodeling and responsible for the narrowing the diameter of vessel, which later causes the thrombosis [115].

In the light of these evidences to investigate the performance of *Cinnamon zeylanicum* on the formation of early atherogenesis our experiments was designed both in *in vitro* and *in vivo* conditions. Our study is the first study that investigates the effect of *Cinnamomum zeylanicum* water extract on the formation of atherosclerosis by measuring the change in $sPLA_2$ and active NF-kB level. For this purpose, vascular smooth muscle cells were used as *in vitro* model and non-obese, non-diabetic Wistar Hannover rats were used as animal model.

One of the active compounds of cinnamon, cinnamaldehyde was found to be unstable in blood stream, and its biological half-life was only 1.7 hour. Different amount of cinnamaldehyde were given to F344 rats but to mimic the *in vivo* conditions the maximum blood concentrations of CNMA were stabilized in 1 microgram/ml [116]. According to the HPLC results the 34,16663 mg cinnamaldehyde was found in 1 gram of cinnamon extract. Therefore, to obtain 1 microgram cinnamaldehyde in 1ml growth media 29.27 mg/ml cinnamon extract was used in the cell culture experiments.In agreement with Singh *et al.* , the amount of the *trans*-cinnamaldehyde is approximately 75% (v/v) of essential cinnamon bark oil .

According to the molecular mechanism of atherogenesis, VSM cell type was used as a model since they have a pivotal role in the formation of atherogenesis. These primary cells were isolated form male rat Wistar Hannover and the gender of the animal was determined according to the statistics of atherosclerosis in the Turkish population [5].

To mimic the atherogenesis lesion progression, VSM cells were brought into quiescent contractile state using very low FBS amount in DMEM growth media which is determined as 0.1% FBS (v/v). This low amount of FBS did not effect on the viability of VSM cells (Figure 4.2).

VSM cells were induced with different concentrations of ox-LDL which is an initiator agent in the early atherogenesis. Although, most of the studies were shown that concentration of ox-LDL at 100 μg/ml and more, were toxic on VSM cells [118-120] in our experiments no toxic effect of ox-LDL at any concentration on VSM cells was detected after 24 hours incubation. In agreement with the study performed by Lin *et al.,* [120] ox-

LDL treatment led to an increase in the proliferation of VSM cells at all concentrations. .Oxidation of LDL can vary from experiment to experiment depending on the starting material of LDL which is not reproducible and not reliable [120]. To stabilize the effect of ox-LDL, a commercially available ox-LDL product from Kalen biomedical was used in the project. According to the early stage mechanism of atherogenesis ox-LDL did not have any toxic function but had an inducible effect on VSM cell proliferation. Only at the end of the early stage of atherosclerosis ox-LDL became toxic after oxidation by the macrophages [6]. In this manner, the results from the project supports the *in vitro* findings in that in short time period such as 24 hours minimally ox-LDL showed proliferative effect on the VSM cells (Figure 4.3). Although the mechanisim of atherogenesis is quite complex it is possible to mimic the in vivo conditions for the early atherogenesis by using minimally ox-LDL in vitro [39].

In order to determine the effect of cinnamon extracts on the setting of atherosclerosis, VSM cells induced with 50 µg/ml ox-LDL were co-treated together with 29.27 mg/ml cinnamon extract. At 24 hours, cinnamon extract was not showed any significant reduction in the proliferation of VSM cells. Moreover pure cinnamaldehyde at 1µg/ml concentration (Sigma) was also shown to be ineffective on the mm ox-LDL induced VSMC proliferation. These data shows that cinnamaldehyde was not effective on the early set of atherogenesis. Ox-LDL (50 µg/ml) induced VSM cells did not lose their proliferation capacity with the pre-treatment and co-tratment of cinnamon extract.

The Phospholipase A_2 (PLA_2) hydrolysis of the *sn-2* ester bound of glycerophospholipids present in lipoproteins and cell membrane and generates nonesterified fatty acids (NEFAs) and lysophospholipids. Despite the other types of $PLA_{2,}$ most of the studies point out that $sPLA_2$ (secretory Phospholipase A_2) has a pivotal role in the formation of atherosclerosis [78, 80].

$sPLA_2$ can hydrolyses the apoliporoteinB-100 containing lipoproteins and these lipoproteins sustain further oxidation and enzymatic modification [77**,** 122, 123] of what . $sPLA_2$ modification of LDL results in the conformational changes in apoB-100 that increase apoB binding to the proteoglycans in the extracellular matrix. This hydrolyzing reaction might also be responsible for the lipid accumulation in the vessel walls by

decreasing the anti-atherogenic functions of HDL [74]. Moreover, generation of arachidonic acid via $sPLA_2$ hydrolysis results in the synthesis of eicosanoids, including prostaglandins, leukotrienes and thromboxanes. These molecules can trigger a variety of proinflammatory actions that lead to atherogenesis [78].

Based on these evidences to find out the effect of cinnamon zeylanicum on $sPLA_2$, ox-LDL (50 μg/ml) induced VSM cells were co-treated with the cinnamon extract (29,27 mg/ml) and cinnamaldehyde(1 μg/ml). Media of the samples were collected and subjected to analysis of $sPLA_2$ activity. Results showed that with respect to non treated sample, ox-LDL treated VSM cells showed increased $sPLA_2$ activation; however cinnamon extract and cinnamaldehyde co treated cells led to a decrease in the $sPLA_2$ activation levels.

The initiation of atherogenesis starts with the expression of cell adhesion molecules (ICAM-1, VCAM-1), cytokines and recruitment of monocytes which are under control of NF-κB. This transcriptional factor can be activated by the end products of lipid peroxidation such as oxidized LDL and proinflammatory cytokines in VSM cells [124, 125] As both $sPLA_2$ and NF-κB activation trigger the inflammatory response, we were expecting to have a parallel increase in $sPLA_2$ and NF-κB activation. However, basal levels of active NF-κB was found to be higher in the control VSM cells in comparison to ox-LDL, and/or the cinnamon extract / cinnamaldehyde treated samples, although ox-LDL induced VSM cells showed an increase $sPLA_2$ activation. Furthermore, other studies indicated that copper-oxidized LDL activates another transcription factor AP-1 (activator protein–1) instead of NF-κB. According to this data NF-κB could be decreased because of the AP-1 in ox-LDL induced VSM cells [126].

In animal studies high cholesterol diet induced rats are widely used in the atherosclerosis *in vivo* experiments. It is easier to handle the rats instead of mice and it is much cheaper to induce atherosclerosis with diet rather than producing transgenic mice. Wistar Hannover rats were induced by a high cholesterol diet containing 45% (w/w) kcal fat (Western diet) fat to trigger the atherosclerosis formation [98, 101]. Taking other animal studies as an example, our animal experiments were also performed in a 4 week-time period [127]. However rats did not exhibit increased serum levels of LDL, HDL, VLDL or total cholesterol after high cholesterol diet in 4 weeks period. In other words,

there were no significant differences in total cholesterol, LDL, HDL, VLDL, triglyceride between control group and high fat cholesterol diet group. Therefore it is not possible to say that cinnamon extract was effective or ineffective on reducing the total cholesterol, LDL, HDL, VLDL, triglyceride. Interestingly, supplementing the high fat cholesterol diet with cinnamon extract group led to a decrease in the weight of the animals suggesting that cinnamon might cause an increase the rate of metabolism.

6. CONCLUSION

It is evident that 50 μg/ml ox-LDL is enough to induce VSM cells to proliferate which is seen in the formation of atherogenesis. Although it is indicated that cinnamon is effective on the glucose and lipid metabolism, it was not give strong evidences to say that it is also effective on the early atherogenesis. Results showed that cinnamon extract and cinnamaldehyde treatment reduced the active amount $sPLA_2$ while the ox-LDL treatment led to an increase in the $sPLA_2$ levels. NF-κB did not give the expected results but the effect of the AP-1 protein should be considered. Although the literature was taken as an example, atherosclerosis was not successfully induced in the given time period in the chosen rat strain. In addition to that, giving the cinnamon extract via drinking water might cause oxidation of the active compound. Therefore cinnamon extract could be including in the feed of the animals.

7. FUTURE DIRECTIONS

In future *in vitro* studies ox-LDL duration could be increased from 24 hours than 72 hours. In addition, AP-1 transcription factor must be investigated in parallel with NF-κB. In animal experiments time period of inducing the animals with high cholesterol diet must be increased and ground cinnamon could be included in the feed of the animals as a pellet or a cake as the cinnamon extract is prone to oxidation.

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