STUDY ON THE ANTIATHEROSCLEROTIC EFFECTS OF *DESMODIUM* SPECIES WITH SPECIAL REFERENCE TO *DESMODIUM GYRANS* DC

THESIS SUBMITTED TO

UNIVERSITY OF CALICUT

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

(FACULTY OF SCIENCE)

VIPIN P. SIVARAM

UNDER THE GUIDANCE OF

DR. JOSE PADIKKALA

AMALA CANCER RESEARCH CENTRE

THRISSUR 680 555, KERALA, INDIA APRIL

2016

DECLARATION

I hereby declare that this thesis entitled **"**Study on the antiatherosclerotic effects of *Desmodium* species with special reference to *Desmodium gyrans* DC**"** is a bonafied record of research work carried out by me during the course of research under the guidance of Dr. Jose Padikkala. The thesis has been subjected to plagiarism checking and has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any university or society.

Place: Thrissur. Signature of candidate

Date: 09.03.2017 Vipin P. Sivaram

Dedicated to my parents,

wife and son

CERTIFICATE

This is to certify that the thesis entitled "Study of the antiatherosclerotic effects of *Desmodium* species with special reference to *Desmodium gyrans* DC" is an authentic account of work carried out by Mr. Vipin P Sivaram under my supervision and guidance and no part of this has been presented for any other degree, fellowship or other similar title in any other university or society. The work has been subjected to plagiarism checking.

Place: Amalanagar, Thrissur.

Date: 09.03.2017

Dr. Jose Padikkala
Supervising guide
Professor, Dept. of Biochemistry,
Amala Cancer Research Centre,
Thrissur.

CERTIFICATE

This is to certify that all the corrections/suggestions recommended by the adjudicators in the PhD thesis of Mr. Vipin P. Sivaram, Registration Order No: CDC/B3/479/Ph.D/2010 dated01.07.2011, entitled 'Study on The Antiatherosclerotic Effects of *Desmodium* Species with Special Reference to *Desmodium Gyrans* DC' have been duly incorporated. Also certify that I personally verified the thesis and have ensured the same.

Date: 09.03.2017

Dr. Jose Padikkala

Research guide

ACKNOWLEDGEMENT

I express my deep sense of gratitude and indebtedness to

- ***Dr. Jose Padikkala****, research supervising guide and Professor, Dept. of Biochemistry, Amala Cancer Research Centre, for his invaluable guidance, critical suggestions, constant support and for being the embodiment of encouragement throughout the course of this work and preparation of the thesis. He deserves a special mention of appreciation for the freedom given to express and execute ideas in the course of the pursuance of my cherished goal of completing research work.*
- ***Dr. Ramadasan Kuttan****, Research Director, Amala Cancer Research Centre, for providing me the facilities of Research Centre and for giving me valuable advices, critical comments and genuine encouragement throughout the course of my study.*
- *Padma Bhushan* ***Rev. Fr Gabriel*** *CMI****,*** *founder Director;* ***Rev. Fr. George Pius****,* ***Rev. Fr. Paul Achandy*** *CMI****,*** *former Directors; and* ***Rev. Fr. Francis Kurissery*** *CMI, Director, Amala Institute of Medical Sciences and Research Centre for officially permitting me to do research work in this esteemed Research Institution.*
- ***Dr. Girija Kuttan,*** *Professor;* ***Dr. TD Babu,*** *Associate Professor and* ***Dr. Achuthan C Raghavamenon****, Associate Professor and supervising guides in Amala Cancer Research Centre, for their supportive involvements and inspirational encouragements at various stages of my study, especially during the preparation of thesis.*
- ***Dr. Praveenlal****, former Principal, Govt. Medical College, Thrissur and* ***Dr. Pushpalatha M****, HOD, Dept. of Biochemistry, Govt. Medical College, Thrissur, for giving me official consent to accomplish my dream of pursuing PhD.*

- *All my senior and junior colleagues in Amala Cancer Research Centre especially **Dr. Adil Nafar**, **Dr. Jaimsha Rani, Dr. Fijesh Vijayan, Dr. Sudha Soman, Mrs. Seema Menon, Mrs. Lincy Lawrence** and **Mrs. Indu MS** for their earnest co-operation and judicious support extended to me. Together we made a buoyant team and supported each other, contributing greatly in achieving our personal goals. **Mrs. Johannah Natinga Mulakkal**, research assistant, Amala Cancer Research Centre, for the sincere and dedicated assistance at various stages for the successful completion of my studies.*
- *Office staff, **Mrs. Hemalatha** and **Mrs. Preetha C G** and animal house caretakers **Mrs. Thankamani** and **Mrs. Sumathy,** for all the help and support they extended to me.*
- *The group of fellow research colleagues belonging to various Departments in Amala Cancer Research Centre the names of all of whom pass through my mind. I individually thank each and every one of you for the emotional support and positive criticism at various stages of the work and for the comfortable environs provided at the work place.*
- *My mother **Vijayalekshmi Sivaram,** wife **Beena,** son **Nayan Anjan** and brother **Vimal** for being the epitomes of sacrifice, inspiration and constant encouragement and support throughout the period of my research work.*

……..And with utmost sincerity, I thank God almighty for playing the invisible role in my life, by passing his blessings at right times whenever I needed them most.

Vipin Sivaram

ABSTRACT

ABSTRACT

Desmodium gyrans belonging to the family Fabaceae, is an important medicinal plant in Ayurveda and Sidda sytems, for the treatment of skin disorders, rheumatoid arthritis, diabetes, heart ailments and also as an antidote to snake bite. Wound healing property of this plant is well known. However scientific reports on its pharmacological properties are scanty. In the present study, dried powder of entire plant was used and extracted (soxhlet extraction) with 70% methanol in water. Phytochemical, GC-MS and TLC analyses of the solvent free extract revealed the presence of hexadecanoic acid, 2,3, dihydro benzofuran, phytol, 2,6-dihexadecanoate, ascorbic acid, dexamethazone, 9,12,15-Octadecatrienoic acid which are having hypolipidemic, antioxidant, anti-inflammatory effects. The extract contained approximately 56 ± 2.89 μ g of GA eq/ g total phenols and 76.5 ± 2.12 μ g of QE eq/ g flavanoids. Acute and sub-acute toxicity studies conducted using Wistar rats of either sex showed the non toxicity of the extract even after oral administration of 2500 mg/ kg body weight of the animals. Therefore the LD_{50} value was estimated to be above 2500 mg/ kg body weight.

When the antioxidant activities of the extract was tested using in vitro assays, the IC_{50} values determined were 12.6, 4.0, 13.5, and 1.95 and 74.0 µg/ ml respectively for scavenging superoxide, DPPH, Hydroxyl and $ABTS^{+}$ radicals and inhibiting lipid peroxidation. Oral administration of the extract (100 and 250 mg/kg b.wt) could inhibit carrageenan induced acute inflammatory reactions in mice paw by 13.02 and 17.39%, and formalin induced chronic inflammation by 13.9 and 31.71%. The results thus suggested excellent antioxidant and anti-inflammatory activity of the extract.

Since ischemic cardiomyopathy is involved in atherosclerotic vascular disease and *D. gyrans* has been credited with cardiotonic properties in traditional medicine, a study was conducted to validate its cardioprotective effect under conditions of doxorubicin (DOX) induced cardiomyopathy and isoproterenol (ISO) induced myocardial ischemia in Sprague Dawley rats. Amelioration of superoxide dismutase (SOD), glutathione peroxidase (GPx) activities and reduced glutathione (GSH) levels were observed in extract treated (100 and 250 mg/ kg b. wt. of the animal, orally) ISO and DOX challenged group of animals. Improved activity of SOD (98%), GSH (50.9%)

and GPx (34%) were observed in ISO challenged animals with treatment of extract, while in DOX challenged group, the improvement was 28.9, 9.0 and 4.6% respectively for these parameters. Mitochondrial damage induced by ISO was studied and the extract effectively rendered protection to mitochondria as evidenced by the improved mitochondrial antioxidant enzyme activities. Cardiac marker enzyme (CPK lowered by 50%, LDH by 31% and AST by 55% in ISO treated groups and CPK lowered by 18%, LDH by 23% and AST by 25% in DOX treated group in comparison to respective controls) activities, ECG patterns and histopathology results also showed significant protection offered by *D. gyrans* to the ISO induced myocardial changes. These results suggest that the extract significantly protects against ISO induced myocardial infarction and DOX induced cardiomyopathy.

Antithrombotic and anticoagulant properties of the extract were tested by *in vivo* and *in vitro* experiments. Clotting time and prothrombin time were determined in plasma collected from extract treated (100 and 250 mg/ kg b. wt of the animal orally for a continuous period of one month) male Sprague Dawley rats. In this experimentheparin treated (injected through tail vein at a dose of 250 U/ kg b. wt of the animal for one day) standard group of rats had a significant delay in blood clotting time with an average increase of clotting time by 0.46 minutes when compared to the untreated group of rats. Clotting time of the extract treated rats showed a delay in clotting time of 0.16 minutes in higher dose which was 1.35% increase and statistically insignificant. In prothrombin time, the standard group of animals treated with heparin showed a delay in coagulation by 28% when compared to the normal group of animals. The treatment with the extract gave a delay in coagulation by 17.3% at higher dose.

Normal plasma re-calcification time of PRP was 3.15 minutes. The standard tube in which heparin was added showed no clotting of plasma even after a prolonged time period. The tubes in which different concentrations of the extract (150 and 250 μg/ ml) were added showed a delay of 0.14 minutes and 0.18 minutes respectively when compared to the normal.

In the platelet aggregation study, there was marked aggregation of platelet rich plasma (PRP) exposed to collagen, which was ameliorated up on addition ofthe extract in two different concentrations of 100 and 250 μg/ml. Collagen induced aggregation of PRP was brought down significantly as indicated by the change in the

optical densities. The lipid peroxidation estimated by the level of MDA formed, was significantly increased by 78.19% in the collagen treated control tubes in comparison to the normal untreated PRP. Exposure of the PRP to extract brought down MDA level by 19.2 and 29.94% in lower and higher doses (100 and 250 μg/ ml) respectively, when compared to the control.

Based on the results of the antioxidant, anti-inflammatory and antithrombotic efficacy, hypolipidemic effect of the extract was assessed in high fat diet (HFD) fed male Wistar rats. Oral administration of the extract at 100 and 250 mg/ kg of b. wt. of male Wistar rat for a period of 90 days simultaneously with HFD, produced lowering of serum total cholesterol by 9.2 and 9.8% and triglycerides by 30.8 and 40.1% compared to the untreated control group. An enhancement of HDL (20.0 and 28.33%) was observed in the lower and higher dose of the extract in comparison to the control. The HDL values were 72 and 77 mg/ dl, with the treatment of lower and higher doses of the extract compared to 56.4 mg/ dl in normal group. In the current study, it was also observed that the extract is able to enhance the activity of lipoprotein lipase enzyme activity, which is involved in the catabolism of triglycerides. Serum collected from the lower and higher doses of the extract (100 mg and 250 mg/ kg b. wt. of *D. gyrans* extract orally fed for a continuous period of 14 days) treated animals showed an enhanced liberation of glycerol from substrate used, namely hyperlipemic serum by 35.7 and 69% respectively, indicating an enhanced LPL activity in the treated groups. These results thus indicate the hypolipidemic efficacy of the extract.

Finally, to evaluate the antiatherosclerotic efficacy of the extract, New Zealand white rabbits were fed with high fat diet (HFD), and simultaneously orally fed with 250 mg/ kg b. wt. of the extract for 90 days. The results showed elevation of HDL cholesterol (elevated from 22 mg/ dl in normal group to 126 mg/ dl in the *D. gyrans* group) and improvement in atherogenic index (5.79 in the treated group in comparison to 16.3 in the control) in the extract treated animals, indicating its effectiveness against atherogenesis. Serum level of Apolipoprotein A-I and paraoxonase enzyme activity were found elevated in the extract administered group. Atherogenic fatty lesions which were evident in the HFD fed controls, significantly reduced with simultaneous treatment of the extract. There was also reduction in fat deposition in liver tissues of the extract treated group as evidenced from tissue lipid analysis.

In conclusion, the overall study indicates that 70% methanol in water extract of *D. gyrans* possesses antiatherosclerotic efficacy. The elevation in functional HDL cholesterol and its associated molecules suggest that the observed efficacy may possibly be due to enhanced cholesterol clearing through reverse cholesterol transport (RCT) which needs further study. The finding also suggests the therapeutic potential of *D. gyrans* against vascular intimal damage and atherogenesis and in turn validates the use of the plant in traditional medicine.

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ABTS	2', 2-azino-bis-3-ethyl benzthiazoline 6-sulphonic acid
ADP	Adenosine diphosphate
ALT	Alanine transaminase
Apo – A1	Apolipoprotein-A1
CHD	Coronary heart disease
COX	Cycloxygenase
DPPH	2, 2-diphenyl-1-picryl hydrazyl
DOX	Doxorubicine
GPx	Glutathione peroxidase
GSH	Glutathione reduced
HDL	High density lipoprotein
HFD	High fat diet
HMG-CoA	Hydroxymethylglutaryl CoA
hs-CRP	High sensitive C reactive protein
ISO	Isoproterenol
LDL	Low density lipoprotein
MDA	Malondialdehyde
MDG/DG	Methanol extract of *Desmodium gyrans*
RCT	Reverse cholesterol transport
PRP	Platelet rich plasma
PTT	Prothrombin time
SOD	Superoxide dismutase
SMC	Smooth muscle cells
The Extract	70% methanol in water extract of *Desmodium gyrans*
VCAM	Vascular cell adhesion molecule
ICAM	Intercellular adhesion molecule
MCP	Monocyte chemoattractant protein
MCSF	Macrophage colony stimulating factor
TNF	Tumour necrosis factor

Chapter 1

Introduction

and

Review of Literature

1.1. INTRODUCTION

Cardiovascular diseases (CVD) are a class of abnormalities related to heart and blood vessels. The most hazardous of them is coronary artery diseases (CAD), such as angina and myocardial infarction. Cerebrovascular disease (cerebral stroke), rheumatic heart disease, peripheral artery disease, cardiomyopathy and congenital heart disease also come under the class of cardiovascular diseases (Shanthi *et al*., 2011). Among these CAD, Stroke and peripheral vascular disease results from atherosclerosis. Free radical injury to endothelium often has been indicted in the initiation and progression of atherosclerosis. Further, atherosclerosis is fundamentally regarded as a lipid disorder, which involves both retentions of unsafe lipids as well as their oxidation. Persons with dyslipidemia are believed to be at the highest risk. Atherogenesis which starts with endothelial dysfunction and advances to rupture of atherosclerotic plaques, involves inflammation at various stages of their evolution. Hence all these aspects of atherosclerosis like the role of free radicals, the participation of inflammation and lipid derangement have been dealt in the current study.

Medicinal plants are increasingly finding their application in pharmaceutical, cosmetic, agricultural and food industry. The employment of the medicinal herbs for curative purposes has been well accepted in the history of civilizations (Elvin *et al*., 2001; Cravotto *et al*., 2010). With the commencement of medical research on herbal plants, it was established that plants have active principles, which are responsible for therapeutic action shown by them (Briskin *et al*., 2000). It is now well understood that plants have the capacity to produce a wide variety of chemical compounds that facilitate their effects on the human body through processes identical to those of drugs used in modern medicine. It has to be particularly noted that before the commencement of synthetic era, man was entirely reliant on medicinal herbs for prevention and cure of diseases. Many of the modern drugs presently preferred by medical practitioners have evolved from medicinal plants. This includes the popularly known aspirin, digitalis, quinine, strychnine, digoxin, atropine, morphine vincristine and

many more others (Gardner *et al*., 2007). With the enhanced efforts in scientific studies in herbal medicine, the crisis of drug resistance in treatment can also be solved. The use of herbal drugs is becoming more attractive in recent years as scientific substantiation of their efficacy has become more widely acceptable. With advent of the concept of antioxidants, herbal study is vastly influencing research resulting in the revelation of a number of herbal products having remarkable antioxidant action (Raza *et al*., 2000; Liao *et al*., 2008). This is especially significant in that oxidative stress has been blamed for several pathological conditions including cardiovascular diseases, cancer, neurological disorders, diabetes as well as ageing (Dhalla *et al*., 2000; Sayre *et al*., 2001; Jenner, 2003; Dalle-Donne *et al*., 2006).

The current work studies the antiatherosclerotic effects of Desmodium species of plants. The study concentrates on antiatherosclerotic effect of *Desmodium gyrans* owing to the pronounced cardioprotective effect believed to be shown by the plant in comparison to the other members of Desmodium (Figure 1.1.2). Various members belonging to genus Desmodium have already been scientifically screened and verified for their anti-inflammatory, anti oxidant and other biological properties validating their use in traditional medicine.

The most closely related species namely *Desmodium gangeticum* has proven hypolipidemic, cardio protective properties and give protection against cardiac reperfusion injury (Kurian *et al*., 2005). Ayurvedic preparations such as dasamoolaristham, dasamoolaharithiki and dasamoola ghrutham contain *Desmodium gangeticum* as important constituent and are used in inflammatory conditions and digestive tract disorders. Another species, *Desmodium triflorum* is used in eye diseases, spleen complaints, stomach trouble and diarrhoea. Leaves of this plant are laxative; and used in dysentery and applied to wounds (Lai *et al*., 2009). Roots are considered carminative, diuretic and used in cough and asthma (Yusuf *et al.* 2009). Woks done in *Desmodium triflorum* have revealed it's potent antioxidant and anti-proliferative properties (Kawshik *et al*., 2005; Lai *et al*., 2010). *Desmodium adscendens* is used in the treatment of asthma.

The plant is also used in the treatment of inflammation of ovary (Ampofo *et al*., 1977; Addy and Awumey, 1984; Addy and Burka, 1988).

Desmodium gyrans is used in Indian traditional and folk medicine since its leaves have diuretic, febrifugal and tonic properties. The roots are used as a remedy for asthma, cough, as anti-dysenteric and as emollient. It is believed to have cardioprotective and wound healing properties for which it is commonly used in traditional medicine (Gopalakrishnan and Rajameena, 2012).

Desmodium gyrans has a long history of use in Chinese traditional medicine to treat various ailments (Ma X *et al*., 2011). In view of the plant's traditional use in protection of heart, *Desmodium gyrans* was subject of study on its antiatherosclerotic effects. Apart from protection rendered by the plant against cardiotoxicity, all the aspects of atherosclerosis like the role of free radicals, the participation of inflammation and lipid derangement have been dealt in the currents study.

1.2. ATHEROSCLEROSIS

Atherosclerosis is the principal cause of fatality around the world and according to recent statistics the disease claims more lives than all types of cancers together (Lusis, 2000; Gaziano *et al*., 2008). World Health Organization predicts that economic prosperity and ensuing unhealthy lifestyle have the prospect of making the present scenario getting rampant (Kmietowicz *et al*., 2002).

Atherosclerosis is characterised by the buildup of modified cholesterol in large and medium sized arteries leading to a proliferation of certain distinct cells within the arterial wall that steadily invade the vessel lumen and slow down blood flow (Glass *et al*., 2001; Moreno *et al*., 2004). This process may take several decades in the life time of a person, mostly quite undetected until when a turbulent force from blood flow results in an atherosclerotic lesion becoming disrupted. This eventually leads to rupture of plaque followed by thrombosis resulting in reduced oxygen supply to key organs such as the heart and brain.

The necrosis of heart and brain that occurs as a result of reduced blood flow is termed myocardial infarction and stroke, respectively. These are also referred to as coronary artery disease and cerebrovascular disease, collectively coming under the medical condition described as cardiovascular disease (Ross *et al*., 1999).

1.2.1. ATHEROSCLEROTIC PROCESS

Atherosclerosis disease onset start early in the child hood and involves interplay of blood elements with endothelial surface of arterial wall. The general anatomy of mammalian arteries shows three layers

1.2.1.1. ***Tunica intima*** - Tunica intima forms the innermost layer of arteries and veins. In arteries this layer is composed of an elastic membrane lining and smooth single layered endothelium made of special type of epithelial tissue. The endothelium is the monolayer of epithelial cells coating the lumen of the arteries and structurally and metabolically it is strategically positioned, separating the vascular wall from the circulation and the blood constituents (Hill *et al*., 2003)

1.2.1.2. ***Tunica media*** - This is the middle layer of the walls of arteries and veins. It is composed of smooth muscle cells and elastic fibres. This layer is comparatively thicker in arteries than in veins. .

1.2.1.3. Tunica adventitia - The tougher outer covering of arteries and veins. It is mainly formed of connective tissue as well as collagen and elastic fibres. These fibres are rather flexible and allow the arteries and veins to extend to endure pressure that is exerted on the walls by flow of blood. .

Vascular endothelium is prone to injury due to a variety of reasons including accumulation of oxidised LDL particles, free radicals, and elevated levels of inflammatory molecules or chronic infections (by certain organisms like chlamydia pneumonia). Most of the known risk factors of atherosclerosis such as hypertention, cigarette smoking, hyperglycemia etc, contribute to the disease pathology by generating pro-oxidant and pro- inflammatory mediators (Munzel *et al*., 2008; Suessenbacher *et al*., 2011). Endothelium is having its own defense and repair mechanisms and immune cells such as T-lymphocytes, monocytes and platelets are attracted to the injured

site (Flammer *et al*., 2012; Eren *et al*., 2013). But when these defense mechanisms fail, the endothelium becomes permeable and the lymphocytes and monocytes drift into the intimal layer and a sequence of reactions follow attracting LDL particles to the site (Reis *et al*., 2001).

The monocytes at the sub endothelial space usually become macrophages that actively engulf oxidatively modified LDL through their scavenger receptors (Figure 1.2). Lipid accumulated macrophages undergo death due to apoptosis (Gokce *et al*., 2011). A mass of lipid laden macrophages thus become foam cells. During this process activated macrophages release several growth factors and inflammatory molecules. Growth factors sucha as platelet derived growth factors (PDGF) are strong inducers of smooth muscle cells (SMCs) and they start to migrate from the tunica media to the intimal layer covering the foam cells. Foam cells along with SMCs form an atheromatous buildup called the fatty streak (Davignon *et al*., 2004). It is interesting to note that the development of atherogenesis is potentially reversible at this phase.

At this stage, the macrophages and monocytes involved in the initial events begin to perish resulting in the formation of a necrotic core around which a fibrous cap consisting of smooth muscle and collagen is formed. The fibrous cap and lesions continue to enlarge into a final atheromatous plaque. More leucocytes and lipid fragments manage to enter the lesions through the most vulnerable sites on the plaque.

While the atheroma increases in size, the wall of the artery spreads out into the arterial lumen due to the presence of the elastic tissue migrated from tunica media. Angiogenesis or formation of minute blood vessels begins to sustain the plaque. The plaque begins to swell further into the vessel lumen as arterial wall is unable to withstand the pressure. The progression of plaque at this stage is like an ulcerous mass.

Continuous remodeling processes also take place and as a result, the presence of the atheromatous plaque is not detected easily at a sufficiently early stage. Hence the process carries on till there is thinning of the fibrous cap accompanied by fissuring of the endothelial surface. As a final event of plaque development, it may rupture at several sites but an attempt at maintaining a balance between rupture and repair is also

followed. When the rate of rupturing exceeds the rate of repair, the contents consisting of lipid fragments and cellular debris are released into the vessel lumen. The small vessels in the plaque also collapse and get exposed to coagulation factors on the endothelial surface resulting in thrombus formation (Figure 1.3). When the thrombus formed is significantly large, arterial luminal obstruction occurs resulting in myocardial infarction or stroke (Libby *et al*., 2002).

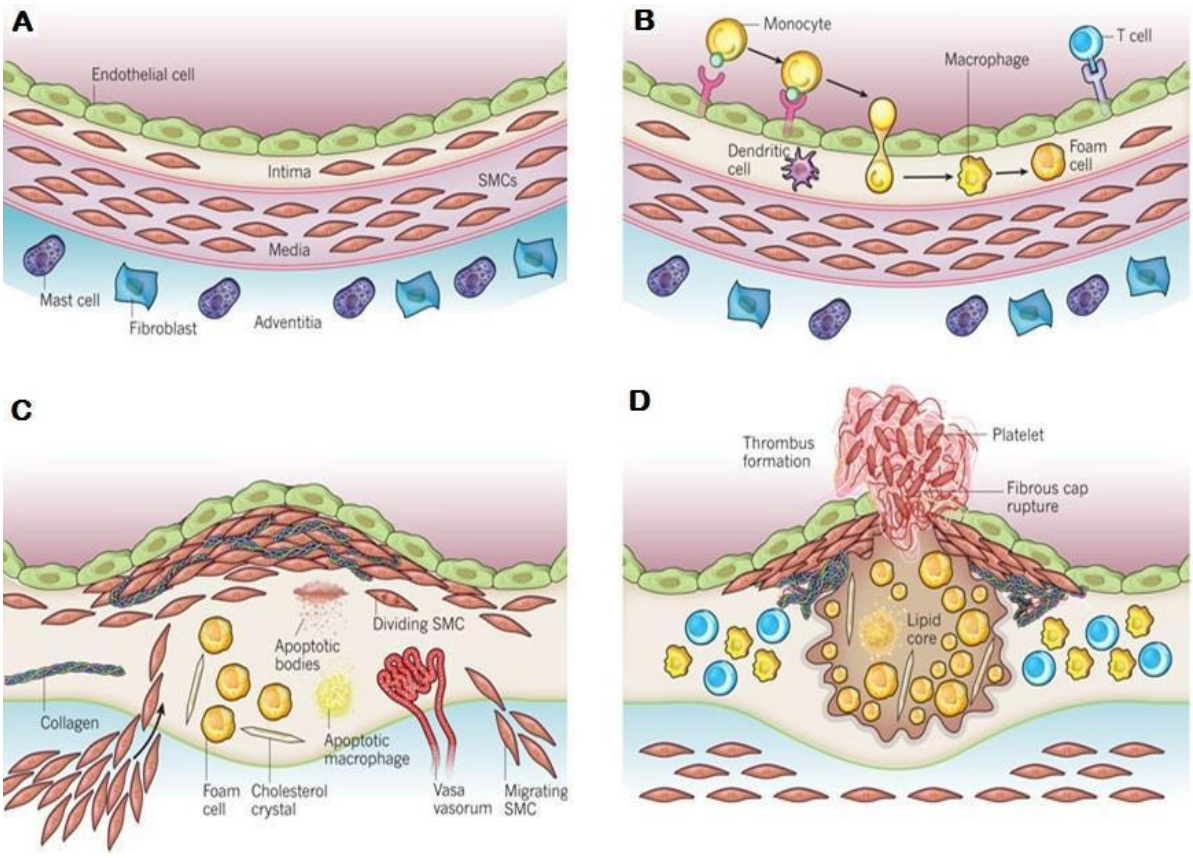


Illustration of different stages of atherosclerotic process

1.2.2. Morphology of atherosclerotic lesions.

As atherosclerosis exhibits as plaques, the morphology of lesions may go through six major stages that reflect the early, developing, and mature stages of the clinical conditions.

1.2.2.1. Type I. Artery with lesion-prone sites, adaptive thickening of the intima is the earliest histological modifications.

***1.2.2.2. Type* II.** Macrophages gather lipids to give rise to foam cells and lesions accumulate foam cells and other necrotic cells to give a fatty streak.

1.2.2.3. Type III. Pre atheroma: Continued foam cell formation and macrophage necrosis can produce lesions that contain small extracellular pools of lipid.

1.2.2.4. Type IV. Atheroma: Lesions are distinct by a thin tissue separation of the lipid core from the arterial lumen.

1.2.2.5. Type V. Fibro atheroma: Lesions exhibit a fibrous thickening in its structural appearance and is known as the fibrous cap.

1.2.2.6. Type VI. Complicated lesions: Mature lesions exhibit a structural design which are further complex and characterised by calcified fibrous areas and visible ulceration. This finally culminates in plaque rupture and accompanying thrombosis and hematoma. (Stary *et al*. 1995)

These types of lesions are often associated with symptoms of arterial embolisation. It was once thought that end-organ damage and infarction are due to gradual advancement of these lesions, but we now know the processes involved in precipitating heart attack and stroke are considerably more complex.

1.3. THEORIES OF ATHEROGENESIS

Three distinct hypothesis of development of atherogenesis have been emerged and currently under active investigation.

i. The response to injury hypothesis (Richard *et al*., 2002; Bonetti *et al*., 2003)
ii. The response to retention hypothesis (Kevin *et al*., 1995)
iii. Oxidative modification hypothesis (Witztum *et al*., 2001; Nakajima *et al*., 2006)

1.3.1. The response to endothelial injury hypothesis: The proposed initial step is endothelial denudation leading to a number of compensatory responses that alter the normal vascular homeostatic properties. For example injuries enhance endothelial adhesiveness

for leukocytes and platelets and alter the local vascular anticoagulant nature to a procoagulant one. Recruited leukocytes and platelets then releases cytokines, vasoactive agents and growth factors that promote an inflammatory response that is characterised by SMC migration into intima and their proliferation.

1.3.2. The response to LDL retention hypothesis: This says that lipoprotein retention is the inciting event for atherosclerosis. Experiments have shown that within 2 hours of LDL injection, into rabbits, arterial retention of LDL and its micro-aggregation can be observed. Apo B 100, the protein associated with LDL is retained within the arterial wall in close association with arterial proteoglycans. In addition to proteoglycans binding lipolytic and lysosomal enzymes like LPL enhance adherence of LDL which is independent of their own enzymatic activities. Once retained within arterial wall, LDL forms aggregates leading to macrophage assimilation and SMC foam cell formation.

1.3.3. Oxidative modification hypothesis: LDL in its native form is not atherogenic, but when modified by oxidatively or acetylated, LDL is readily internalised by macrophages through scavenger receptor pathway. Apo B100 is modified and this renders LDL succeptible to macrophage uptake producing cholesterol laden foam cells. It is this foam cell that forms the nidus of developing atherosclerotic lesion. Oxidised LDL induces production of macrophage chemotactic protein (MCP), which is chemotactic for monocytes and T-lymphocytes and stimulates vascular smooth muscle cells (SMC) proliferation.

1.4. RISK FACTORS

The most effective means of preventing arterial thrombosis and resulting complications is to prevent atherogenesis. A number of cardiovascular risk factors have been identified with respect to the underlying pathology of atherosclerosis, of which some are modifiable and the others are unmodifiable. There are too many proven risk factors that are strongly and consistently associated with atherosclerosis in a mode that is independent of other factors. Hypercholesterolemia, hypertension, tobacco usage, obesity, physical inactivity, age, family history, diabetes mellitus and male gender are a few of them. The first five of these risk factors are potentially modifiable, and there is evidence that their reversal reduces the complications of cardiovascular events.

1.4.1. Age

Age is among the central risk factors for predicting incidence of cardiovascular disease. The average risk of developing cardiovascular disease for an individual aged 60-64 year rises around seven times in comparison to a 30- to 34-year-old male (Wilson *et al*., 1997). Many studies all over the world have stated age as an overriding risk factor for occurrence of cardiovascular disease.

1.4.2. Gender

Numerous studies have revealed that male gender exhibit additional risk for cardiovascular disease compared with age matched women (Barrett-Connor *et al*., 1991). Although it is believed that estrogens safeguard women from cardiovascular disease since the condition is found to accelerate in women after menopause. However, this assumption has not been substantiated, as the treatment of postmenopausal women with estrogen has not helped reduce the frequency of cardiovascular disease in them (Hulley *et al*., 1998). The fact that women enjoy relatively higher concentrations of high-density lipoprotein (HDL) cholesterol than same age men may be a cause for the apparent protection delivered to them.

1.4.3. Hypercholesterolemia

A high level of Low density lipoprotein (LDL) cholesterol in the blood has been cited as the principal cause of injury to artery and vascular smooth muscle cells (SMCs) (Brown *et al*., 1997; Ross *et al*., 1999; Wu *et al*., 2005; Libby, 2006). Hereditary factors that cause defects in the genes that code for LDL-receptors or abnormalities in the regulatory mechanisms of LDL-receptors can lead to familial hypercholesterolemia. An unhealthy fatty diet habit can also result in hypercholesterolemia (Young and Libby, 2007). Hypercholesterolemia act to initiate the inflammatory response by causing expression of mononuclear leukocyte recruiting mechanisms.

Mediators of inflammation like Vascular cell adhesion molecules (VCAM), Tomour necrosis factor- α (TNF-α), Interleukin-1 (IL-1) and Macrophage colony

stimulating factor (MCSF) are found to accelerate binding of LDL to vascular endothelium and SMC by increasing the expression of genes that encode LDL receptors on it (Libby, 2006; Young and Libby, 2007). It is also established that high levels of oxidised LDL can inhibit endothelial cell vasodilator function and stimulate cytokine and growth factor production (Parhami *et al*., 1993).

High density lipoprotein cholesterol (HDL) or "good" cholesterol inhibits oxidative modification of LDL and blocks the pro-inflammatory and cytotoxic effects of oxidised LDL (Bobkova *et al*., 2003; Thompson *et al*., 2004). Oxidation of cholesterol characteristically occurs in unhealthy vessel wall and plays a major part in foam cell formation. Human carotid and coronary arteries are enriched in oxidised LDL and, significantly, unstable plaques appear to be specially enriched in oxidised LDL. In recent times, an increasing number of studies have evaluated the role and effects of oxidised LDL in preclinical atherosclerosis, endothelial dysfunction and response to statins.

Increased oxidised LDL cholesterol levels are associated with increased arterial thickness and impaired flow-mediated vasodilatation in asymptomatic subjects. Plasma oxidised LDL levels closely correlate with the incidence of coronary artery disease (CAD). Further, oxidised LDL is found to recruit circulating monocytes and T lymphocytes through chemotactic means (Quinn *et al.*, 1987; McMurray *et al*., 1993) and also retain them in the arterial wall, inhibit the capacity of resident macrophages to leave intimal layer, cause loss of endothelial integrity due to toxic effect and up regulate synthesis of inflammatory modulators like Macrophage chemotactic protein-1 (MCP - 1) and MCSF (Cushing *et al*., 1990; Rajavashisth *et al*., 1990). The last mentioned effect in turn leads to continued oxidation of more and more of LDL itself, thus giving rise to what is described as a **"**vicious cycle**"**.

Oxidised LDL contributes to movement of smooth muscle cells by enhancing the expression of platelet-derived growth factor (PDGF) by endothelial cells, macrophages and smooth muscle cells themselves, (Stiko-Rahm *et al*., 1992; Kohno *et al*., 1998; Kim *et al*., 2009). In the same way expression of basic fibroblast growth

factor (bFGF) also is stimulated by oxidised LDL again helping proliferation of smooth muscle cells (Lindner *et al*., 1991).

Oxidised LDL enhances the procoagulant activity of endothelium by a variety of mechanisms. Induction of the release of coagulation tissue factor (TF) by endothelial cells and smooth muscle cells (Frei, 1991; Pinn *et al*., 2000), stimulation of coagulation by reducing thrombomodulin transcription suppression of protein C activation (Wilson *et al*., 1998) and modulation of tissue factor pathway inhibitor being some of the substantiated modes (Ishii *et al*., 1996;Petit *et al*.,1999).

Oxidised LDL may even serve as a convincing biomarker since it may provide a relation between lipoprotein disorders and inflammation. An LDL particle is formed of 1600 molecules of cholesterol ester and 170 molecules of triglycerides, 700 molecules of phospholipid and 600 molecules of cholesterol (Gotto *et al*., 1986, Mertens *et al*., 2001) with one apoB-100 molecule surrounding the outer layer. About half of the fatty acids in LDL are polyunsaturated fatty acids (PUFAs). Obviously all these molecules are prone to oxidation either by enzymatic or nonenzymatic means. The formation of oxidised LDL cholesterol differ depending on whether it is the apolipoprotein part or the lipid part that is oxidised.

Although *in vivo* mechanism of LDL oxidation has not been understood properly, there are several mechanisms upon which numerous *in vitro* studies have been done. Metal ions bound to heme and ceruloplasmin are capable of initiating LDL oxidation (Smith *et al*., 1992; Lamb *et al* 1999, Stadler *et al*., 2004) by decomposition to hydroperoxides (ROO.), which are in turn converted to reactive aldehydes like malondialdehyde. 15-Lipoxygenase released from endothelial cells and macrophages converts polyunsaturated fatty acids to its hydroperoxides (Parthasarathy *et al*., 1989; Sendobry *et al.*, 1997). Myeloperoxidase released from activated macrophages give rise to free radicals including hypochlorous acid (HOCl), (Heinecke *et al*., 1999; Podrez *et al*., 2000,) which in turn are found to cause lipid and apoprotein peroxidation. Fatty acid part of LDL is prone to oxidation especially when there is the presence of linoleic acid, hydroxyoctadecaenoic acid, arachidonic acid (Suarna *et al*., 1995, Carpenter *et al*.,

1995; Carr *et al*., 2000) which can be rather easily be oxidised or hydroxylated. Cholesterol in LDL is also vulnerable to oxidation due to unsaturation between 5^{th} and 6^{th} carbon atom thus giving rise to products like oxysterol and hydroxyl cholesterol (Mattsson *et al*., 1996). The major lipid detected in atherosclerotic lesions namely phospholipids can also seen oxidised. In addition to these lipids, the protein part also is modified, chiefly Apo B which is oxidatively transformed (Heinecke *et al*., 1999) by chlorination or nitration and made to react with lipid peroxidation products. Oxidised Apo B functions as epitope for recognition by scavenger receptors on macrophages.

1.4.4. Hyperlipidemia

Hyperlipidemia is a state of enhanced level of various lipids in blood. Apart from the classic LDL and high density lipoprotein (HDL) cholesterol, other lipids like small dense LDL cholesterol, lipoprotein-a ($Lp_{(a)}$), and lipoprotein-associated phospholipase A2 (Lp-PLA2) also comes under this category. Lp (a) is a unique lipoprotein, similar to LDL cholesterol except for additional unique glycoprotein apolipoprotein, which is similar to plasminogen. The association of Lp (a) with CAD and its ability to act as a biomarker of risk appear to be strongest in patients with hypercholesterolemia and, in particular, in young patients with premature atherosclerosis. In this regard, increased levels of Lp (a) in plasma independently predict the presence of symptomatic and angiographically determined CAD, particularly in patients with elevated LDL cholesterol levels (Danesh *et al*., 2000). (Boffa *et al*., 2004; Koschinsky *et al*., 2005). An elevated level of Lp (a) is a risk factor for atherosclerosis and coronary heart disease since it is found to promote inflammation (Boffa *et al*., 2004). Its activity appears to be complemented by high levels of homocysteine, elevated LDL (Sotiriou *et al*., 2006) and in diabetes (Colwell *et al*., 2000). Lp (a) acts as a ligand that binds to β-integrin Mac-1; this augments the recruitment of adhesion molecules and their migration into the endothelium. Interacting with Mac-1, Lp (a) can induce transcription factors that controls pro-inflammatory genes like kappaB and NFκB.

1.4.5. Lp-PLA2

Lp-PLA2 is a 50-kD enzyme associated with LDL. The enzyme belongs to the family of A2 phospholipases and is produced mainly by macrophages, monocytes, mast cells, and T lymphocytes. Lp-PLA2 has pro-inflammatory properties as also it hydrolyses oxidised phospholipids to give lysophosphatidylcholine and free oxidised fattyacids. Oxidised LDL particles are rich in lysophosphatidylcholine, and one of the reasons of oxidised LDL becoming atherogenic has been attributed to this high lysophosphatidyl choline content.

Several epidemiological studies have reported Lp-PLA2 to be a predictor of CAD (Oei *et al*., 2005), although controversy still exists as to its independence from assisting in the function of LDL cholesterol. The relationship of Lp-PLA2 to LDL cholesterol has been supported by several studies presenting comparable decreases in Lp-PLA2 and LDL cholesterol levels in response to lipid-lowering agents (Eisaf *et al*., 2003).

1.4.6. Calcification in atherosclerotic plaques

Calcium mineral deposits are frequently seen associated with atherosclerotic plaques and predict a higher risk of myocardial infarction and death. Atherosclerotic lesions frequently become calcified and plaque structure, composition and stability can be found altered as per the severity of calcification. The calcification process can begin early in the life and can get more and more intricate as the disease advances and more complex lesions develop. The presence of calcium deposits in coronary arteries indicate the occurrence of atheromatous plaque, but an absence of coronary calcium deposition may not indicate an absence of plaque (Detrano *et al*., 2000)

Calcification is initiated among vascular smooth muscle cells, located in the muscle cells adjacent to atheromas and advances to the surface of atheromatous plaques. Later when these cells die, deposited calcium is accumulated and crystallised. It is believed that this process contributes to the rupture of plaques; although latest research on calcified plaques reports a mixed opinion on the role of calcification in

plaque rupture. Nevertheless, vascular calcification is constantly considered as a risk factor, in association with the other general risk factors of atherosclerosis (Iribarren *et al*., 2000).

Recent research suggests a different view for the process of calcification. Previously considered passive and degenerative, vascular calcification is now accepted as a pathobiological process resembling many features of embryonic bone formation (Demer *et al*., 2008). Findings suggest that atherosclerotic calcification is a controlled and regulated process similar to bone formation and occurs together with other phases of atherosclerotic progression (Lewis *et al*., 1996). Osteocalcin, which is Gla (gamma carboxyglutamate) containing protein, is assumed to have key role in the establishment of coronary calcification (Speer *et al*., 2002). Another protein namely, osteopontin, known to be involved in mineralization of bone, together with its mRNA, also have been detected in calcified atherosclerotic lesions. Potent factors for osteoblastic differentiation like the mRNA for bone morphogenetic protein-2a have also been reported. Cells harbouring these proteins may be the ones involved in initiation and progression of calcification (Karwowski *et al*., 2012). With the advent of these findings, the concept of considering calcification as a simple passive process of precipitation of calcium phosphate crystals was changed. It was replaced by the idea that atherosclerotic calcification is an organised and regulated process comparable to bone formation (Tintut *et al*., 2002; Yip *et al*., 2009).

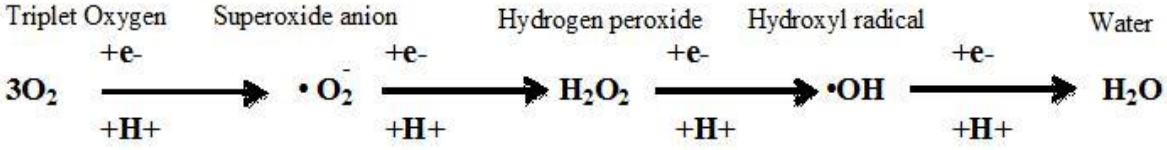
Calcification is seen more often in advanced lesions but it may also occur in in earlier lesions even though in small amounts. Plaques with calcium deposition are found to be larger during histopathological studies. A direct relation of arterial mineralisation to the possibility of plaque rupture is not clearly understood, although the degree of coronary calcium shows a direct relation with the severity of atherosclerosis in different individuals. Further research is needed to interpret the relation of coronary calcification to the pathogenesis of both atherosclerosis and plaque rupture (Huang *et al*., 2001). It is assumed that the vessel is rendered less susceptible to rupture when there is extensive calcification at later stages of atherosclerosis, whereas early or intermediate stage calcification may enhance vulnerability of plaques. This is

why it is stated that calcification alone cannot be considered as an ideal diagnostic marker for plaque rupture in atherosclerotic individuals.

1.4.7. Free radicals and oxidative stress

All atoms are said to be most stable in their ground state. An atom achieves this stable configuration when every electron in its outermost orbit has a complementary electron that spins in the opposite direction. Free radicals are termed so since they have atom (eg: Oxygen, Nitrogen) with an at least one unpaired electron in its outermost shell (Halliwell *et al*., 2007), and they are capable of independent existence. Free radicals are certain unstable molecules with short life and react with other molecules to attain a stable configuration (Figure 1.4). When a covalent bond between two molecules is broken and one electron remain with each atom, free radical generation can take place (Karlsson *et al*., 1997).

Reactive oxygen species (ROS) are highly reactive ions and belongs to the class of free radicals containing oxygen molecules in them and they are usually the byproducts of the regular metabolism of oxygen (Figure 1.5).



Schematic illustration of production of ROS

1.4.7.1. *The chief modes of generation of free radicals*

A. Endogenous causes of production

Free radicals are produced intracellularly through multiple mechanisms like immune cell activation, inflammation, mental stress, excessive exercise, ischemia, infection, aging etc.

i. Free radicals are produced as by product of cellular respiration. This occurs when a leakage of activated oxygen occurs from mitochondria during the final stage of oxidative phosphorylation.

ii. Free radicals are formed as byproducts by enzyme systems (NADPH oxidase, myeloperoxidases, xanthine oxidase etc.) in phagocytic cells, neutrophils and macrophage. The enhanced production of ROS by phagocytic

cells is preceded by an increased consumption of oxygen by these cells (Babior *et al*., 1973), which is called **"**the respiratory burst**"**. The non-phagocytic cells also generate ROS by a similar method.

B. Exogenous causes of production

i. An exposure to ionizing radiation can produce free radicals.

ii. Pollutants like herbicides and pesticides and cigarette smoking can propel synthesis of free radicals

iii. Lifestyle and food habits can also be associated with production of free radicals.

1.4.7.2. ***Examples for free radicals***

i. Superoxide ($\cdot O_2^-$)

ii. Hydroxyl radical ($\cdot$ OH)

iii. Alkoxy radical (RO•)

iv. Peroxy radicals (ROO•)

v. Peroxynitrite radical (ONOO-)

vi. Nitric oxide ($NO^{\bullet}$)

vii. Nitrogen dioxide ($NO_2^{\bullet}$)

viii. Lipid peroxyl ($LOO^{\bullet}$)

1.4.7.3. ***Examples for non- radical oxidants***

i. Singlet oxygen ($^1O_2$)

ii. Dinitrogen trioxide (N_2O_3)

iii. Lipid peroxide (LOOH)

iv. Nitrous acid (HNO_2)

v. Ozone (O_3)

vi. Hypochlorous acid (HOCl)

vii. Hydrogen peroxide (H_2O_2)

viii. Organic hydroperoxide (ROOH)

1.4.7.4. *Function and purpose*

Free radicals have some critical functions to be performed in cells, some of them are

i. Essential for production of hormones like thyroxine.

ii. Produced to destroy some bacteria and other engulfed pathogens as a mode of defense.

iii. Necessary for normal cell functions and signal transduction mechanisms.

1.4.7.5. *Free radicals in disease.*

Free radicals generated by usual methods turn harmful when adequate anti-oxidant defenses systems are absent. A balance between free radical generation and its adequate removal or inactivation is obligatory. When free radicals are produced in huge amounts, it cannot be satisfactorily controlled and that is when the balance is affected leading to cellular damage (Bahorun *et al*., 2006). A phenomenon termed oxidative stress can be established that can seriously alter the cell membranes and other cellular structures such as proteins, lipids, lipoproteins, and nucleic acids (Willcox *et al*., 2004; Pacher *et al*., 2007). Unwarranted free radical activation has been responsible in the manifestation of several disease (Genestra *et al*., 2007) processes like,

i. Inflammation (Valko *et al*., 2007)

ii. Carcinogenesis (Halliwell *et al*., 2007)

iii. Parkinson's disease and Alzheimer's disease (Pohanka *et al*., 2013)

v. Atherosclerosis (Ramond *et al*., 2011)

vi. Degenerative neurologic disease (Singh *et al*., 2004)

vii. Reperfusion injury

1.4.7.6. *Mechanism of ROS induced peroxidation of cellular membranes*

Cellular membranes and low-density lipoproteins are rich in fatty acids, especially polyunsaturated fatty acids (PUFAs) (Dekkers *et al*., 1996). PUFA is found to contribute to the fluidity of cellular membranes. Free radical chooses to take electrons from fatty acids of lipid bilayer of a cell, thus setting off a free radical attack on the cell. This is termed as lipid peroxidation. ROS is known to target the carbon-carbon unsaturated double bond of PUFAs. The bonding between carbon and hydrogen is weak, owing to the presence of C-C double bond in its vicinity. This enables easy dissociation of the hydrogen by a free radical. A free radical steals the single electron from the hydrogen associated with the carbon at the double bond leaving the carbon with an unpaired electron, turning it to a free radical. The membrane molecule attempts to stabilize the carbon-centered free radical by molecular rearrangement and the newly organised molecule is called a conjugated diene (CD). The conjugated diene can reacts with molecular oxygen to form a proxy radical, which in turn may steals an electron from another fatty acid (Figure 1.6). This process is called propagation which can attain the form of a continuous chain reaction (Halliwell *et al*., 1985)

1.4.7.7. *Oxidative stress in atherosclerosis: Role of oxygen derived free radicals*

Oxygen is absolutely indispensable for the life of aerobic organism but it may turn out to be toxic if supplied at higher concentrations. Dioxygen in its ground state is comparatively nonreactive but its partial reduction leads to generation of active oxygen species (AOS) such as singlet oxygen and super oxide radical anion (Miller *et al*., 1990).This eventually gives rise to oxidative stress which is basically the adverse effect of oxidant radicals on physiological functions of the body. Free oxygen radicals play fundamental role in the etiology of quite a few diseases like arthritis, cancer, and atherosclerosis. The oxidative damage to DNA is found to play vital role in aging

process and the presence of intracellular oxygen also can be responsible in initiating a chain of unintended reactions at the cellular level, and these reactions injure critical cell biomolecules (Kovacic *et al*., 2001; Valko *et al*., 2001). Hence, these radicals are hazardous and generate oxidative stress in both animals and plants, although plants and other organism have wide range of in built mechanisms to counter these free radical interventions. Many endogenous and nutritional compounds like superoxide dismutase, ferritin, transferrin, cereruloplasmin, tocopherol, carotene and ascorbic acid have antioxidant and free radical scavenging properties. Small amounts of reactive oxygen species are frequently formed in the body in the cell membranes and close to the cellular organelles. They are found act where they are generated and hence, they can damage most cell structures and biomolecules including membrane lipids, proteins, enzymes and nucleic acids. Cell has mechanisms to produce the small amounts of oxidants normally during metabolic reaction as also reactive species are formed in controlled amounts by neutrophil on exposure to microbes. This defense mechanism is beneficial to the body in that they play a part in destroying the microbes. Excess of oxidants, however, is what turns out to be harmful. All vital organs are under constant threat of oxidants and other free radicals, especially H_2O_2 (Dalle-Donne *et al*., 2006). Antioxidants can act by scavenging reactive oxygen species, by inhibiting their generation (e.g. by controlling activation of phagocytes), by binding transition metal ions and checking formation of OH and disintegration of lipid hydroperoxides, by repairing damage (e.g. α-repairing peroxyl radicals and thus preventing the chain reaction of lipid peroxidation) or combination any of these scheme (Dhalla *et al*., 2000; Sayre *et al.*, 2001; Jenner *et al*., 2003).

The available evidence from experimental and clinical studies suggests that oxidative stress is implicated in atherosclerotic diseases. It is well understood that atherosclerosis is characterised by a state of amplified oxidative stress characterised by lipid and protein oxidation in the vascular wall (Figure 1.7). Reactive oxygen species (ROS) are key mediators of signaling pathways that trigger inflammation in atherogenesis. The process starts from the initial endothelial injury and LDL oxidation, through lesion progression, to eventual plaque rupture. The probability for cellular

damage is initiated when the imbalance between oxidants and antioxidants moves in favour of oxidants. The potential oxidants may belong to two categories.

1.4.7.8. *Non-radical oxidants*

Nonradical oxidants that are devoid of the unpaired electron carried by the free radicals also are found to cause cellular damage especially to vessel walls. Some of the examples are hydrogen peroxide, hypochlorous acid, ozone, oxoperoxonitrate, peroxynitrous acid, alkyl peroxy nitrites and dinitrogen trioxide. Hydrogen peroxide is most dangerous of them since it can easily give rise to more potent free radical namely hydroxyl radical by multiple ways. Nonradical oxidants are found to react preferentially with protein molecules rather than lipids and hydrogen peroxide reacts with heme proteins (hemoglobin and cytochrome) generating ferryl heme which inturn initiate a chain reaction.

Many enzymes have been found responsible for ROS release. They include nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, myeloperoxidase (MPO), xanthine oxidase (XO), lipoxygenase (LO), nitric oxide synthase (NOS). ROS production also occurs from dysfunctional mitochondrial respiratory chain due to leakage of protons in the intermitochondrial membrane during terminal oxidation of electron.

The ROS-induced oxidative stress in cardiac and vascular myocytes has been associated with cardiovascular tissue injury (Dhalla *et al*., 2000). ROS-induced oxidative stress has role in various cardiovascular complications such as atherosclerosis, cardiomyopathies, ischemic heart disease, hypertension and congestive heart failure (Kukreja and Hess, 1992). The major causes of oxidative stress in cardiovascular system include:

(i) Xanthine oxidoreductase (XOR), (ii) NAD(P)H oxidase, (iii) NOS, (iv) The mitochondrial cytochromes and (v) hemoglobin (Berry and Hare, 2004; Hare and Stamler, 2005).

Oxidative stress is linked with enhanced formation of ROS that alters phospholipids and proteins causing oxidation and peroxidation of thiol groups (Molavi

and Mehta, 2004). The attacks by ROS lead to variation in membrane permeability, membrane lipid bilayer disruption and functional alteration of a range of cellular proteins. Besides protein and lipid damage, defects in myocyte function due to greater oxidative stress are considered to be associated with the action of ROS on subcellular organelles (Funda *et al*., 2008). Creatine kinase activity of rat cardiac mitochondria was found to decrease upon exposure to xanthine oxidase or hydrogen peroxide (Hayashi *et al*., 1998). Mitochondria of heart tissue treated with ROS showed decreased Ca^{2+} membrane transport. It may be concluded that oxidative stress may modify the activities of diverse subcellular structures, proteins, and lipids and thus altering myocyte function (Valko *et al*., 2007).

Insufficient synthesis of ATP in ischemic heart may also harm Ca^{2+} management mechanisms in the sarcolemmal and sarcoplasmic reticular membranes and thus result in Ca^{2+} overload. Reperfusion of ischemic heart may also raise the uptake of extracellular Ca^{2+} into the myocardium and form another factor contributing to Ca^{2+} overload. Intracellular accumulation of Ca^{2+} seems to be a common factor that prompts neointimal hyperplasia that leads to the occurrence of atherosclerosis,

vasoconstriction for the development of hypertension, myocardial cell damage seen in ischemia-reperfusion, and cardiac hypertrophy during heart failure (Valko *et al*., 2007).

Increased amounts of superoxide radical and hydrogen peroxide have been detected in hypertensive patients, and so the etiology of ROS-induced oxidative stress in the pathogenesis of hypertension is well-recognised (Romero and Reckelhoff, 1999). Superoxide encourages cell proliferation while hydrogen peroxide stimulates apoptosis and activates protein kinase C, indicating a function for protein kinase C in ROS-mediated vascular disease. ROS-induced oxidative stress in hypertensive patients is accompanied by lowered levels of antioxidants such as Vitamin E, GSH, and SOD, which are the prominent scavengers of free radicals (Li and Forsterman, 2000).

Oxidative damages in the arterial wall can lead to the atherosclerosis when the oxidants succeed in dominating antioxidants. Therefore, it is important to consider the environment of available antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase, transferases thiol-disulfide oxidoreductases and peroxiredoxins. The growing evidence from data of experimental

and clinical studies suggests that oxidative stress is implicated in atherosclerotic diseases. However, a better knowledge of mechanisms involved in ROS production and signaling pathways in vascular pathophysiology is essential for effective pharmacological interventions of vascular diseases.

1.4.8. Role of antioxidants

When the balance between oxidants and antioxidants is upset oxidative damage can be the result. When occur within arterial wall, this can give way to atherosclerosis. However, cells are having their own systems for protection which help keep the antioxidant levels always on the superior side (Wu *et al*., 2009). There are enzymatic and nonenzymtic antioxidants. Copper-Zinc dependent superoxide dismutase and Manganese dependent superoxide dismutase are there which catalyse dismutation of cytosolic and mitochondrial superoxide anions respectively. Catalase and glutathione peroxidase are responsible for nutralisation of hydrogen peroxide. Other antioxidant enzymes include glutathione reductase, thioredoxin, thioredoxin reductase and heme oxygenase. Nonenzymatic antioxidants comprise vitamin C (ascorbic acid), vitamin E (tocopherol) and ubiquinol.

1.4.8.1. *Superoxide dismutase*

Superoxide dismutase are enzymes that catalyse the change of superoxide (O_2-) radical intomolecular oxygen (O_2) or hydrogen peroxide (H_2O_2). Superoxide is a very potent free radical produced as a result of oxygen metabolism which can cause diverse cell damage. SOD is an important enzyme member of antioxidant defense systems in nearly all living cells. It was Irwin Fridowich and Joe and MC Cord who discovered the catalytic activity of superoxide dismutase in 1968 (McCord and Fridiwich, 1969). There are at least three major families of superoxide dismutase which are classified on the basis of protein confirmation and the metal cofactor present in it. The Cu and Zn dependent SOD, which binds both copper and zinc; Fe and Mn dependent, which bind either iron or manganese, and finally, the Ni dependent, which binds nickel.

Three forms of superoxide dismutase are present in humans, in all other mammals, and most chordates. In higher plants, SOD isozymes have been

localized in different cell compartments such as mitochondria and peroxisomes, cytosol, chloroplasts and apoplast (Corpas *et al*., 2001; Corpas et al., 2008). In plants, superoxide dismutase enzymes (SODs) act as antioxidants in protecting cellular components from oxidative modifications by reactive oxygen species (ROS) (Alscher *et al*., 2002).

Besides being the prime antioixidant molecule, SOD has powerful antinflammatory activity also. SOD is found to be highly effective in experimental management of chronic inflammation in colitis. Administration of SOD brings down generation of reactive oxygen species and there by reduces oxidative stress. As a consequence endothelial activation is inhibited and downregulation of factors that manage adhesion molecule expression and leukocyte-endothelial interactions is brought about. Therefore, it is suggested that such antioxidants may be employed as central molecules in new therapies for the treatment of various disease (Marberger *et al*., 1974; McGinness *et al*., 1978; Segui and Gironella, 2004; Wilcox *et al*., 2010).

1.4.8.2. *Glutathione*

Glutathione (GSH) is an important antioxidant in plants and animals, safeguarding vital cellular components against challenges caused by reactive oxygen species including free radicals, peroxides, lipid peroxides and heavy metals (Pompella *et al*., 2003). Glutathione is a tripeptide with a gamma peptide linkage between the carboxyl grup of the glutamate residue and the amino group of cycteine. Glutathione is the most important endogenous antioxidant produced by the cells, directly involved in the neutralisation of free radicals and reactive oxygen species. It also participates in the maintenance of other antioxidants such as vitamins C and E in their reduced state which is their active form. (Hughes *et al*., 1964)

Glutathione can alter between its reduced (GSH) and oxidized (GSSG) states. In the reduced glutathione, the thiol group of cysteine is able to donate a reducing equivalent ($H^+ + e^-$) to other unstable molecules, such as free radicals and reactive oxygen species. While donating its electron, glutathione becomes reactive and readily reacts with another such reactive glutathione to form glutathione disulfide (GSSG). The Regeneration of reduced GSH from GSSG can be mediated by the enzyme glutathione reductase (GSR) in presence of NADPH (Couto *et al*., 2013). In a given biological

system, the ratio of reduced glutathione to oxidized glutathione is often used as an evaluating measure of cellular toxicity status (Pastore *et al*., 2003).

1.4.8.3. *Glutathione peroxidase*

Glutathione peroxidase (GPx) is a family of enzymes having peroxidase activity. The main biological role of GPx is to guard organisms from oxidative damage. The antioxidant role of glutathione peroxidase is carried out by way of mediating the reduction of lipid hydroperoxides to their corresponding alcohols and by reduction of hydrogen peroxide to water (Muller *et al*., 2007).

The reaction that glutathione peroxidase catalyses is,

$$2GSH + H_2O_2 \rightarrow \mathbf{GS}\text{–}SG + 2H_2O$$

GSH represents reduced glutathione, and GS–SG represents the oxidized form, glutathione disulfide. The key enzyme Glutathione reductase then reduces the oxidized glutathione back to GSH.

$$GS\text{–}SG + NADPH + H^+ \rightarrow \beta\ GSH + NADP^+$$

1.4.8.4. *Catalase*

Catalase is an antioxidant enzyme found in all living organisms that are exposed to oxygen. It catalyses the breakdown of hydrogen peroxide to water and oxygen (Chelikani et al., 2004). It is a very important enzyme in defending the cell from oxidative damage caused by ROS. Catalase is a fast enzyme and has one of the highest turnover numbers among enzymes. It can catalyse the conversion of approximately 5 million hydrogen peroxide molecules to water and oxygen per second (Goodsell *et al*., 2007).

Catalase consists of four polypeptide chains of around 500 amino acids each forming a tetramer (Boon *et al.*, 2007). It contains four porphyrin heme (iron) groups which enable the enzyme to react with the hydrogen peroxide. Catalase is seen located in cellular organelle called the peroxisome (Alberts *et al*., 2003). The optimum pH for human catalase is around 7.0 (Maehly *et al*., 1954).

The reaction of mediated by catalase in the breakdown of hydrogen peroxide,

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

Hydrogen peroxide is a dangerous product of several normal metabolic processes and to avoid damage to cells and tissues, it must be rapidly changed into other substances. Hydrogen peroxide is mostly converted into less-reactive gaseous oxygen and water molecules as given in the above reaction (Gaetani *et al*., 1996).

1.4.9. Hypertension

Hypertension is manifested when systolic blood pressure exceeds 140 mmHg or diastolic blood pressure exceeds 90 mmHg (Rutan *et al*., 1989). There seems to be a definite linear relation between elevation of blood pressure and the greater incidence of atherosclerotic vascular disease (MacMahon *et al*., 1990). This is supported by numerous studies indicating that heart attack and stroke are considerably reduced in hypertensive patients who are on antihypertensive treatment (Heart Protection Study Collaborative Group 2002).

Hypertension is considered as a major risk factor of cardiovascular irregularities. Angiotensin II (AII) which is released in hypertensive individuals can act as a powerful vasoconstrictor and plays a significant role in atherosclerosis by enhancing proliferation of SMCs (Ballou *et al*., 2002). Angiotensin II hastens inflammation by facilitating smooth muscle lipooxygenase activity, which eventually yields oxidised LDL (Abu-Soud *et al*., 2000). Angiotensin II along with VCAM-1 also promotes expression of cytokines like IL-6 and MCP-1 which serve as initiators of inflammation. Hypertension in animal models is found to be accompanied with endothelial leukocyte adhesion, macrophage buildup, smooth muscle cell migration and proliferation, and intimal thickening. Apparently, all these consequences of a constantly elevated blood pressure contribute to the speeding up of atherosclerosis.

Hypertensive reactions cause generation of free radicals (superoxide anion and hydroxyl radicals) in the plasma that in turn both decrease nitrogen oxide synthesis and increase leukocyte adhesion (Baranano *et al*., 2002).

1.4.10. Diabetes

The risk of atherosclerosis is multiplied in diabetic patients. Due to the raised level of glucose in conditions of chronic hyperglycaemia, lipoproteins undergo glycation. The glycated lipids are derived to form advanced glycosylation end product (AGE) which can be detected by AGE receptors present on macrophages (Gleissner *et al*., 2007; Martindale *et al*., 2002). Glycated lipoproteins step up the activation of pro-inflammatory cytokines in the arterial endothelium (Renard *et al*., 2004; Libby, 2002). Insulin has a negative effect on uptake of LDL by hepatocyte LDL receptor whereas glucocorticoids have the opposite effect. Although the precise mechanism of insulin action is not clearly understood, this may explain hypercholesterolemia associated with uncontrolled diabetes (Krone *et al*., 1988; Duvillard *et al*., 2003; Vikramadithyan *et al*., 2005).

Insulin resistance in patients with type 2 diabetes leads to hypertriglyceridemia and dyslipidemia, which are characterised by low HDL levels and high very low density lipoprotein (VLDL) and LDL levels. In such diabetic patients, NO activity is weakened which means that the normal endothelial functioning is upset.

1.4.11. Tobacco smoking

There is now substantial approval that smoking is related to coronary artery disease, as cessation of smoking lowers the future threat of the disease. The actually possibility of heart attack in ex-smokers matches that of nonsmokers in about two years. Cigarette smoking promotes atherosclerosis and intensifies the activity of blood clotting factors, such as fibrinogen. In addition, nicotine raises blood pressure, and carbon monoxide present in cigarette smoke reduces the amount of oxygen that blood can carry. Nicotine and carbon monoxide contents elicit damaging effects on arteries by causing them to lose their normal behaviour that set the stage for plaque development. Atherogenesis is initiated with endothelial injury which can occur as a result of direct physical damage caused by tobacco products.

The free radicals generated from smoking directly results in oxidative stress and increased oxidation of LDL which triggers the recruitment of monocytes and T cells to the intima, the events which lead to formation of macrophages and other processes that

promote atherosclerosis. Regular cigarette smoking results in sustained high levels of circulating non-esterified fatty acids that also attract inflammatory response (Young and Libby, 2007).

1.4.12. Obesity and lifestyle

Obesity is excess body fat especially around the waistline. Obese people will have a poor body mass index (BMI). High intake of saturated fat which may raise total and LDL cholesterol and especially the intake of trans fats which raise total and LDL cholesterol while lowering HDL cholesterol (Mitchell *et al*., 2007), have become part of the changed food habits and consequently lead to obesity. Consistent high consumption of carbohydrate rich diet also is found to contribute to excess body fat.

Framingham Heart study conducted in the United States has persistently revealed that increasing prevalence of obesity is accompanied by increasing rates of CHD (Wilson *et al*., 1997; Kim *et al*., 2009). It has also been suggest that obesity is a risk factor for CHD independently of the other atherogenic risk factors (Eckel *et al*., 1998; Van Gaal *et al*., 2006). Obesity is found as a contributing cause for these standard risk factors and because of this reason obesity could be a more significant cause of atherosclerotic disease than an individual risk factor.

One of these is insulin resistance and hyperinsulinemia having a causative link with CHD. (Haffner *et al*., 1999, Smiley *et al*., 2001), Obese subjects typically exhibit a proinflammatory state that may predispose them to acute coronary syndromes, characterised by elevations of serum high-sensitivity C-reactive protein (hs-CRP). High levels of hs-CRP give rise to elevated cytokine production that may render otherwise stable atherosclerotic plaques vulnerable to plaque rupture (Chambers *et al*., 2001; Morrow *et al*., 2001). Another consequence of obesity is the increased release of plasminogen activator inhibitor-1 (PAI-1) from excess adipose tissue (Mavri et al., 2001; Mertens *et al*., 2001) which favors a prothrombotic state. This may promote atherogenesis and also enhance the dimensions of thrombosis in case of coronary plaque rupture.

Western dietary habits with consumption of diet having high fat and cholesterol has already been established as a leading cause for dyslipidemia, obesity, and

cardiovascular problems including atherosclerosis (Woo *et al*., 1999; Dwyer *et al*., 2001; Relling *et al*., 2006). These food habits coupled with sedentary lifestyle are being increasingly followed in developing countries, which has consistently enhanced the prevalence of these health problems on a global level. Excessive alcohol use leads to an increase in blood pressure, and increases the risk for heart disease. It also increases blood levels of triglycerides which contribute to atherosclerosis. Lack of regular physical activity can make people vulnerable to cardiovascular abnormalities.

Physical inactivity and sedentary lifestyle has been stated as an independent risk factor for cardiovascular disease. (Fletcher *et al*., 1992; Soufi *et al*., 2006) An inverse relationship between physical fitness and cardiovascular abnormalities has similarly been reported. (Manson *et al*., 1991; Paffenbarger *et al*., 1986) Studies have shown that lifestyle modification with enhancement in physical activity as a program is effective in improving cardiorespiratory fitness, and blood pressure (Dunn *et al*., 1999). Increases in level of physical activity are associated with reduction in persons already distressed by CHD, which suggest that unfit individuals can improve their risk profile by beginning a fitness-enhancing program (Whaley et al., 1995) this is supported by studies which show that taking up moderately vigorous exercise resulted in a substantial reduction in mortality. (Paffenbarger *et al*., 1993; Wannamethee *et al*., 1998; Schroeder *et al*., 2002)

The mechanisms by which physical activity mitigate CHD risk have not been satisfactorily elucidated. A higher fitness index can affect the other cardiac risk factors also in a positive way, including changes in lipid and lipoprotein metabolism (Relling *et al*., 2006). Individuals who regularly exercise retain lipoprotein profiles consistent with a low risk of CHD. Their HDL cholesterol level has been found to be higher than untrained individuals as well as triglyceride concentrations lower. Further, a dose dependent relationship between the extent of exercise performed and HDL cholesterol concentration is also been reported. (Durstine *et al*., 1987)

Coronary blood flow is enhanced by physical exercise which results in increased shear stress on the endothelial surface. Endothelial cells respond to transitory increases in shear stress by releasing vasodilator compounds like prostacyclin and NO. Persistent increases in shear stress elicit a response in endothelial cells that is

manifested, in part, by increased production of endothelial NO synthase. Regular physical exercise has been shown to improve endothelial function in experimental animals too (Goto *et al*., 1993; Higashi *et al*., 1999; Gill *et al*., 2007)

Lipid and lipoprotein metabolism along with alteration in endothelial function are the major contributing factors that benefit individuals who keep themselves fit by regular exercise. While it is known that there is potential for exercise to influence lipoprotein metabolism and endothelial function, the information about the mechanisms responsible for these changes has not been clearly known.

1.4.13. Infections

Certain chronic type of infections that take place away from the vascular system like gingivitis, prostatitis and bronchitis are found to induce atherogenesis (Muhlestein *et al*., 2003). These intracellular infections enhance the synthesis of cytokines that initiate inflammation. Thus, these infections increase the rate of development of atherosclerotic lesion. Complement activation and inflammation can be detected in atherosclerotic plaques subsequent to herpesvirus, cytomegalovirus or *Chlamydia pneumoniae* infection (Yasojima *et al*., 2001). Presence of *Chlamydia pneumoniae* has been detected in atherosclerotic plaques isolated from humans. The endothelial cells and SMCs can release pro inflammatory molecules in response to synthesis of toxic lipopolysaccharides and heat shock proteins by microorganisms like *Chlamydia pneumoniae*. These pro inflammatory molecules in turn facilitate the invasion of leukocytes into endothelial intima, which as already described, facilitate atherogenesis.

1.4.14. Role of Inflammation in atherosclerosis

Emerging research works have drawn attention to immune response and inflammation as critical factors that supplement the onset and progression of atherosclerosis. Recent studies has thrown more light on inflammatory responses during atherosclerosis giving convincing evidences to suggest that inflammation is the vital contributor to atherogenesis (Methe *et al*., 2007). Atherosclerotic lesions express comparative inflammatory responses as those found in typical inflammatory and

autoimmune diseases such as rheumatoid arthritis. Results from clinical trials have identified various biomarkers of inflammation such as C-reactive protein and Interleukin-18 to be associated with atherosclerosis. (Boamponsem *et al*., 2011)

Enhanced oxidative stress together with reduced Nitric Oxide activity- stimulates production of a number of cytokines such as interleukins, TNFα, MCP 1 or interferon, eventually attracting monocytes. Induction of adhesion molecules such as VCAM or ICAM promotes migration and differentiation of monocytes in the vessel wall. Inflammatory process generally promotes accumulation of macrophages and production of cytokines through mediation of different components.

1.4.14.1. *Inflammatory molecules*

A number of inflammatory molecules are found to play mediatory roles in the initiation as well as progression of atheromatous streaks. The effect of these mediators is diverse and comprises mitogenesis, intracellular matrix proliferation, foam cell development and angiogenesis. Studies conducted with such models in which expression of these inflammatory mediators have been **"**knocked-out**"** support a role for these mediators in the pathogenesis of atherosclerosis. During the beginning stage of the endothelial damage, endothelium is induced to express leukocyte adhesion molecules vascular cell adhesion molecule-1(VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and P-selectins (Libby, 2002; Zipes *et al*., 2005). The endothelium produces certain chemo attractants like monocyte chemoattractant protein-1 (MCP-1) which enable monocytes to migrate into the arterial wall (Ross *et al*., 1999; Zipes *et al*., 2005; Bobryshev *et al*., 2006).

It has been observed that genetically modified mice which do not express either VCAM-1 or MCP-1 are unable to develop atherosclerosis even if exposed to experimental atherogenic conditions. (Libby *et al*., 2002). T-cells are also taken up into the endothelium by binding to VCAM-1, which contributes to atherogenesis (Szmitko, 2003; Robertson *et al*., 2006). T-cells respond to inflammatory environment by the way of production of -interferon (IFN) and tumour necrosis factor (TNF) (Libby, 2002; Galkina *et al*., 2007). These inflammatory molecules further stimulate

macrophages, vascular endothelium and SMCs to propagate the inflammatory response (Ross, 1999). Macrophage colony stimulating factor (MCSF) enables the conversion of monocytes into macrophages which starts to accumulate oxidised LDL. (Bobkova and Poledne, 2003; Swirski *et al*., 2007). MCSF facilitates the expression a rich source of inflammatory activators such as cytokines (IFN- **,** lymphotoxin, TNF-α), chemokines, eicosanoids and platelet activating factor. This is followed by generation of reactive oxygen species (ROS) like superoxide anion leading to the damage of molecules or membrane structure of the artery (Libby P, 2002; Martindale *et al*., 2002).

As atherosclerosis sets in, continuous migration and proliferation of SMC occur followed by the thickening and subsequent dilation of arterial wall and calcification. This leads to further hardening of the plaque (Young and Libby, 2007). The phase is influenced by Platelet derived growth factor (PDGF) secreted from activated macrophages which help migration and proliferation of SMCs from the tunica media into the intima. Death of SMC complicates the atherosclerosis plaque.

Thinning and rupture of fibrous cap is the risk involved in advanced atheromatous plaques which is influenced by a collection of inflammatory mediators including CD40/CD40L, matrix metalloproteinases (MMP) and pro-inflammatory cytokines (Szmitko *et al*., 2003). MMP is a group of protease enzymes that cleave gelatins and type IV collagen component of basement membranes. In the presence of oxidized LDL, ROS, TNF-α and IL-1 (interleukin-1) within the endothelium, MMP is released from foam cells and SMCs. Atherosclerotic plaques become weak by its proteolytic activity and cytokines (TNF-α, IL-I), oxidised LDL and CD40L are involved in making the fibrous cap thin by enabling over expression of MMP. This stress created by inflammatory molecules results in disruption of the atherosclerotic plaque which can trigger thrombosis and possibly lead to acute myocardial infarction (Mach *et al*., 1998; Szmitko 2003).

IL-1, TNF-α, and CD40L are found to mediate expression of tissue factor, von Willebrand factor and subendothelial collagen which accelerate thrombin formation and

(Szmitko *et al*., 2003; Zirlik *et al*., 2007) consequently, platelets are activated and clump together to form aggregates.

1.4.14.2. *C-Reactive Protein (CRP)*

Due to the active participation of inflammatory molecules in pathogenesis of atherosclerosis, clinical interest has focused on the introduction and development of inflammatory risk markers for atherosclerosis. Principal molecules having highest priority among them are C reactive protein (CRP) and fibrinogen. Investigational works support the pro inflammatory and pro thrombotic effects these markers and the pathogenesis of atherosclerosis; there has been developing evidence to propose that CRP, usually produced from liver as an acute phase protein, is a very significant biomarker of inflammation (Ridker *et al*., 2005; Bisoendial *et al*., 2007). CRP is important in predicting atherosclerosis and as such function as valuable tools in the diagnosis and prognosis of atherosclerosis (Yasojima *et al*., 2001; Auer *et al*., 2002).

During acute coronary ischemia, levels of hs-CRP are predictive of a high risk of vascular events even when troponin levels are not detectable. This suggests that inflammation is associated with a vulnerable plaque even in the absence of detectable myocardial necrosis (Liuzzo *et al*., 1994). Increased levels of CRP may render otherwise stable atherosclerotic plaques vulnerable to rupture by diverse effects manifested through different types of mediator molecules (Morrow *et al*., 2000; Blake *et al*., 2001)

Synthesis of CRP is induced in the liver by IL-6 (Chi *et al*., 2001). Studies on the expression levels of CRP in atherosclerotic plaque revealed that CRP mRNA levels of plaque tissues were more than 10 times higher than that of normal artery (Pai *et al*., 2004). It was also discovered that there was an amplification of complement protein levels in plaque tissues; CRP stimulates complement activation so as to manage an inflammatory response (Yasojima *et al*., 2001). Detection of up regulated levels of CRP mRNA in macrophages and cells further supports its role. These findings backup the proposition that CRP may play a direct role in promoting the inflammatory elements of atherosclerosis.

CRP performs by several other ways too; it down regulates endothelial nitrogen oxide synthase (eNOS) expression and thereby inhibits nitrogen oxide release into the endothelium (Abrams *et al*., 1997; Verma *et al*., 2002), enhances the release of cell adhesion molecules (VCAM-1and ICAM), chemoattractants (MCP-1), enhance IL-8 and MMP-1 activity, aids migration of SMCs as well as support LDL uptake by macrophage. Endothelium responds to intensified levels of CRP by increasing the release of NFkB that in turn initiates the release of cytokines that facilitates cell apoptosis (Hirschfield *et al*., 2005). CRP is found to stimulate even processes like angiogenesis. The chief cause of elevated hs-CRP seems to be the persistent and subclinical chronic inflammation leading to athosclerosis.

1.4.14.3. *Interleukin-18*

Interleukin-18 (IL-18) is a cytokine synthesised by macrophages which binds to its receptor molecules expressed on lymphocytes (T-helper1), endothelial cells, SMCs, and macrophages (Chan *et al*., 1998). Cytokines like IL-1, TNF-**α and IL**-6 up regulate IL-18 gene expression in macrophages. Subsequently, leukocyte adhesion is increased as more IL-18 are synthesized which in turn reinforces VCAM-1 and ICAM-1 levels. IL-18 can lower plaque stability since it can increase the expression of MMP. The quantities of IL-18 detected in patients with myocardial infarction were greater than in normal patients (Blankenberg *et al*., 2000).

1.4.15. Hereditary factors

As in the case of many other conditions, heart disease can also run in the families. Genetic predisposition is found to enhance chances of cardio vascular risks in certain families. Familial hypercholesterolemia, hyperlipoproteinemia, high blood pressure and other vascular conditions may make a person susceptible to cardiovascular problems. The risk for cardiac problems can escalate even more when hereditary factors are combined with unhealthy lifestyle adoptions, such as smoking cigarettes and having poor diet.

1.5. MOLECULES INVOLVED IN COMBATING ATHEROGENESIS

1.5.1. High Density Lipoproteins

HDLs are biosynthesised in liver and small intestine, as primarily protein discoid particles. These newly formed HDLs are primarily composed of apoproteins like apoA-I, apoC-I, apoC-II and apoE and nearly devoid of cholesterol and cholesteryl esters. Actually, a major role of HDLs is to function as circulating stores of apoC-I, apoC-II and apoE. The most abundant protein in HDLs is ApoA-I constituting over 70% of the total protein content of HDL. Other than apoproteins, HDLs transport numerous enzymes that take part in anti-oxidant activities which include glutathione peroxidase 1 (GPx), paraoxonase 1 (PON1) and platelet activating factor acetyl hydrolase (PAF-AH, also called lipoprotein-associated phospholipase A_2, Lp-PLA_2). Additional enzymes found associated with HDLs are lecithin: cholesterol acyltransferase (LCAT) and cholesterol ester transfer protein (CETP) having specific roles. As many as 75 different types of proteins have been detected to be associated with the subclasses of circulating HDLs. The biological functionality of HDL is determined by the proteins, apoproteins, enzymes, and lysosphingolipids carried by these lipoprotein molecules.

1.5.1.1. Biological functions of HDL

HDL masterminds the clearance of cholesterol from systemic circulation through the process of reverse cholesterol transport (RCT). Various epidemiological and clinical studies over the past years have confirmed a direct correlation between the circulating levels of HDL cholesterol and a decline in the possibility for atherosclerosis and coronary heart disease (Wierzbicki *et al*., 2005). Individuals having HDL level above 50 mg/dL are many time less likely to have CHD than those with levels below 35-40 mg/dL. If HDL is not present in adequate amounts or does not function properly there is accumulation of cholesterol promoting the development of atherosclerosis.

In addition to its vital role in cholesterol homeostasis, HDL also positively influences proper endothelial function and has antithrombotic, anti-inflammatory, and

antioxidant activities. (Toth *et al*., 2007). By *in vitro* and *in vivo* assays, it has been possible to quantitatively asses the anti-inflammatory properties, as well as the antioxidant functions of HDL.

One of the chief functions of HDL is protection rendered to LDL against its oxidation. Oxidised LDL is formed in a series of three steps. The first step is the hydroperoxide seeding of LDL with the metabolic products of linoleic acid and arachidonic acid. The second step is subendothelial trapping of LDL and the buildup of additional reactive oxygen species in engulfed LDL. LDL phospholipids undergo nonenzymatic oxidation in the third step. This finally results in the formation of specific oxidised lipids that further accomplish its role in atherogenesis. (Navab, *et al*., 2000)

The actions of HDL-associated enzymes (paraoxonase and platelet-activating factor-acetylhydrolase) and apoprotein constituents are found to be significant in inhibition of LDL oxidation by HDL (Aviram *et al*., 1998; Marathe *et al*., 2003; Barter *et al*., 2007). The confirmed antioxidant activities of HDL are believed to support in this role. Oxidised LDL is proatherogenic, contributing to endothelial dysfunction and vascular inflammatory activity in the subendothelial space (Galle *et al*., 2006), which ultimately give rise to atherosclerosis.

Cell-free assays have been employed to measure the efficiency of HDLs to prevent the generation of oxidised phospholipids in LDLs as well as to determine the ability of HDLs to degrade oxidised phospholipids that are previously formed. Further, HDLs have been shown to hinder monocyte chemotactic response in presence of oxidised LDL.

Endothelial production of nitric oxide is vital in the functioning of blood vessel and HDL has been shown to oppose some of the defects associated with endothelial dysfunction which are caused by reduced nitric oxide production and upregulated expression of adhesion molecules like vascular cell adhesion molecule-1, intercellular adhesion molecule-1. (Xia *et al.*, 1999; Nofer *et al*., 2004) HDL also promotes

endothelial cell proliferation and migration and inhibits the apoptosis (antiapoptotic) of these cells, thereby helping to maintain intimal health. (Nofer *et al*., 2009)

Through a variety of mechanisms, HDL brings down platelet aggregation, thus possibly bringing down the risk for thrombus formation in vulnerable plaques (Nofer *et al*., 1998). The atheroprotective nature of HDL is not determined by the cholesterol in the HDL fraction, rather, it is the functional molecules carried by HDL which are actually responsible or capable of driving RCT as well as rendering beneficial effects along arterial walls that regulate the degree of protection from atherogenesis. Thus, there is preference for treatment modalities aimed at raising HDL levels in prevention of atherosclerosis and CHD. Current therapies are found to elevate HDL only by modest levels. Both the statins and the fibrates have only been shown to enhance HDL by around 5%–20%. Niacin is more efficient in elevating HDL, but is poorly tolerated in many patients. Therefore, mainstream as well as alternative approaches intended at increasing HDL levels are being actively pursued.

1.5.1.2. HDL and reverse cholesterol transport

Cholesterol that is synthesised in extra hepatic tissues or assimilated from circulating lipoproteins is returned to the liver for eventual excretion in a process called reverse cholesterol transport (RCT)(Fielding *et al*., 1995). This is the process by which the HDL particles which move through the circulation, extract free cholesterol from less-dense particles throughout the circulatory network, thereby decreasing the overall level of total cholesterol. HDL-C is considered "good cholesterol" due to the physiologic function it executes in reverse cholesterol transport. Nonhepatic peripheral cells acquire cholesterol through uptake of lipoproteins and also through bio synthesis. However, except for steroidogenic tissues that convert cholesterol to steroid hormones the rest of the cells are unable to catabolise it. The physiological need for reverse cholesterol transport is clear in that unless this unwanted free cholesterol is not removed, it can even be toxic to the cells harbouring them.

The HDL particle is actually a complex consisting of several apolipoproteins and other protein components like the enzyme paraoxonase together with various lipids.

The outer coat of the HDL particle is amphipathic and is formed of free cholesterol, apolipoproteins (eg, apoA-I, apoA-II, apoC, apoE), and phospholipids. The inner hydrophobic core of the HDL particle is rich in cholesteryl esters and retains a small amount of triglycerides.

The efflux of cholesterol is mediated by HDL through the following steps:

The primary step in reverse cholesterol transport is the efflux of cholesterol from cell membranes to acceptor molecules in the interstitial fluid. Two models have been suggested with regard to the transfer of cholesterol from plasma membrane to acceptor molecules.

The first is the *aqueous diffusion model*, in which cholesterol molecules spontaneously get released from cell membranes and are then integrated into acceptor particles after crossing the intervening aqueous space by diffusion (Johnson *et al*., 1991). Phospholipid vesicles, phospholipid-albumin complexes, and triglyceride-phospholipid emulsions are capable of efficiently removing cholesterol from cells by means of this mechanism. This model does not involve interaction with specific cell receptors.

The second model involves *the direct interaction of HDL* with cell surface binding sites, apparently by way of apoA-I through interaction with ATP- binding cassette transporter protein-A1 (ABCA-1). This triggers an intracellular signal leading to translocation of cholesterol from intracellular sites to the plasma membrane (Ho *et al*., 1980; Oram *et al*., 1996).The physiological acceptor for cholesterol in vivo is the nascent HDL particles, which are discoidal pre- -migrating complexes of phospholipid and apoA-I along with other amphipathic apoproteins, such as apo CI, apo CII and apoE. Cholesterol that is transported to nascent HDL particles is esterified by the enzyme lecithin-cholesterol acyl transferase (LCAT) to cholesteryl esters, and since they are hydrophobic, move into the interior of the HDL particles. As a result of cholesterol efflux, nascent pre- -HDL is converted to the α-migrating spherical mature HDL found in plasma. HDL can remove cholesterol from tissues through interaction with ATP- binding cassette transporter protein G1 (ABCG-1), which accounts for

approximately 20% of the removed cholesterol. Phospholipid transfer protein (PLTP) is another molecule capable of facilitating formation of nascent HDL particles or the HDL_2, which is similar to pre-beta-HDL, and can act as cholesterol acceptors (Figure 1.8).

The enzyme lecithin-cholesterol acyltransferase (LCAT) converts free cholesterol with the HDL to cholesteryl ester, in so doing moving the esterified cholesterol hydrophobic interior of the HDL particle, causing it to swell into a round, mature HDL particle. LCAT is vital for normal HDL metabolism, because in its absence, generation of mature HDL particles with normal esterified cholesterol core do not take place. Experiments performed with LCAT-deficient mice and humans have shown extremely low HDL-C levels and low apoA-I levels due to rapid catabolism of apoA-I.

Alpha-HDL, the mature HDL_3 containing free cholesterol and a cholesteryl ester core, is then returned to the liver through either of the below mentioned pathways.

First, through interactions of HDL with liver cell scavenger receptor class B-I (SR-BI), cholesterol is selectively taken up by the liver. HDL cholesteryl esters are delivered to the liver by a non-endocytotic process. This results in an HDL particle of reduced size and cholesteryl ester load (Pittman *et al*., 1987; Tall *et al*., 1993).

Second, the enzyme cholesteryl ester transfer protein (CETP) mediates the removal of cholesteryl esters from mature HDL and its transfer to apoB-containing lipoproteins in exchange for triglycerides. Cholesterol within apoB-containing lipoproteins is then taken up by the liver through interaction with liver cell LDL receptors (Spady *et al*., 1998).

As a result, around half the cholesterol is recycled through the CETP pathway and the rest gets recycled via reverse cholesterol transport. The final step in the reverse cholesterol transport pathway is excretion of the assimilated cholesterol from the liver into bile, either directly or after conversion to bile salts.

Some of the processes explained above are subject to additional levels of regulatory mechanisms from other proteins, including hormones and receptors. It has been substantiated that the peroxisomal proliferator activated receptor–alpha (PPAR-alpha) enhances transcription of the apoA-I gene. Moreover, liver X receptors (LXR) and retinoid X receptors (RXR) in turn control transcription of the gene for ABCA1, the receptor that is active in the formation and maturation of HDL.

It can be observed that each of the proteins and receptors emphasised here signifies as potential tools or points of intervention to elevate HDL level which eventually will promote reverse cholesterol transport.

1.5.2. Apo lipoprotein AI

Various evidences show that apoA-I is a major anti-atherogenic and anti-oxidant factor in HDL and has critical role in the HDL-mediated reverse cholesterol transport. ApoA-I is synthesised by the liver and can interact with cell membrane molecular complex known as the ATP-binding cassette transporter-1 (ABCA-1) in the hepatocytes as well as on macrophages, which is vital in RCT as described in earlier sections. It has been verified that mice overexpressing apoA-I have improved macrophage RCT (Zhang *et al*., 2003) and that mice deficient in apoA-I have markedly reduced macrophage RCT (Moore *et al*., 2005*).* Introducing pro-apoA-I into humans was demonstrated to increase biliary cholesterol and fecal sterol elimination, indicative of an enhanced reverse cholesterol transport.

Normal HDL with its, apolipoprotein AI (apoAI), obstruct all three steps in the formation of oxidised LDL (Navab *et al*., 2000). Studies done with pretreatment of LDL with apoAI has rendered LDL resistant to oxidation and reduced its chemotactic activity. ApoAI also makes LDL resistant to *in vivo* oxidation. It removes 13(S)-hydroxyperoxy octadecadienoic acid and 15(S)-hydroxyperoxy eicosatetraenoic acid from LDL which are found to enhance the nonenzymatic oxidation of both 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine and cholesterol linoleate of LDL. This action of apoAI prevents the formation of biologically aggressive oxidised phospholipids. Specific methionine residues (Met112 and Met148) of apoA-I have been

revealed to directly lower cholesterol ester hydroperoxide and phosphatidylcholine hydroperoxide content. Several epidemiological studies have shown an inverse correlation between coronary artery disease and the plasma apo A1 concentration (Buring *et al*., 1992). Statins could raise HDL levels, (Vega *et al*., 1998) by mediation through elevated synthesis and secretion of apoA-I. (Bonn *et al*., 2002). Simvastatin has been reported to escalate the plasma apo A1 level, particularly in patients with nonfamilial hyperlipoproteinemia type II (Homma *et al*., 2003). The plasma concentration of apo A1 is lower in smokers than in non-smokers (Dullaart *et al*., 1994), and physical exertion is reported to elevate the plasma level of apo A1 (Rashidlamir *et al*., 2001).

1.5.3. Paraoxonases

Paraoxonases are a family of enzymes that functionally hydrolyse organophosphates. Paraoxonase (PON) is synthesised in the liver and is one of the important antiatherogenic enzymes carried by HDL. PON has been demonstrated to shield against LDL against *in vitro* oxidation, an important process in atherogenesis. PON possesses anti-oxidant properties and it prevents the oxidation of LDL (Durrington *et al*., 2001; Marchegiani Marchegiani *et al*., 2008; Soran et al., 2009; Ganesh *et al*., 2012). Evidences suggest that the antioxidant nature of PON has direct role in making HDL functional and inhibiting LDL oxidation (Siddiqua et al., 2010). PON has been shown to enhance cholesterol efflux from macrophages in association with apo AI by promoting binding mediated by ABCA1. Importance of PON in preventing atherosclerosis has been validated in mice deficient in the enzyme. Human clinical studies have reported a higher level of PON activity associated with a lower frequency of major cardiovascular abnormalities (Ferretti *et al*., 2004). PON can efficiently hydrolyse lipid peroxides, hydroperoxides, cholesterol linoleate and hydrogen peroxide (Mackness *et al*., 1993; Watson *et al*., 1995; Aviram *et al*., 1998; Jaouad *et al*., 2003). The anti-inflammatory and antioxidant action of PON complements anti-atherosclerotic function of the protein. This is evident from the observation that other pathological conditions associated with inflammation and

oxidative stress, such as rheumatoid arthritis and Alzheimer disease, are regularly linked with reduced PON activity (Shih *et al*., 1998; Beltowski *et al*., 2003).

Mice lacking PON develop significantly larger atherosclerotic lesions in their aortas in comparison to their wild types (Shih *et al*., 2000), and mice in which PON is experimentally overexpressed exhibit atheroprotection in that they develop significantly lower degree of aortic lesions and decreased amounts of inflammatory chemokines like monocyte chemotactic protein (Durrington *et al*., 2001). In humans, PON activity has been demonstrated as an independent risk factor for CHD (Mackness *et al*., 2003).

1.5.4. HMG CoA reductase inhibitors: Statins

Statins are a class of drugs used to lower cholesterol levels by inhibiting the enzyme HMG-CoA reductase, the key enzyme involved in the production of cholesterol in liver. Around 70 percent of total cholesterol in the body is synthesised de novo. Statins have been successfully used to prevent cardiovascular disease and mortality in high-risk individuals. It has been shown that statins are very effective for treatment during the early stages of CVD and in those with potential for CVD (Schonbeck *et al*., 2004; Taylor *et al*., 2014).

Statins show properties beyond hypolipidemic activity in countering atherosclerosis. (Nissen *et al*., 2006). Prevention of cardiovascular disease is supposed to be effected through three more proposed mechanisms namely improved endothelial function, modulation of inflammatory responses and maintenance of plaque stability (Furberg *et al*., 1999; Martin *et al*., 2001) .

Statins primarily act by competitively inhibiting HMG-CoA reductase, the first committed enzyme of the cholesterol biosynthetic pathway. The product of HMG-CoA reductase namely mevalonate in turn can give rise to molecules such as isoprenoids, which influence cell proliferation processes. In this way, inhibition of the HMG CoA reductase not only affects the cholesterol biosynthesis but also controls vascular intimal cell proliferation taking place during atherogenesis.

In addition, it has been revealed that statins directly activate endothelial NO synthase (Eto *et al*., 2006), contributing to endothelial preservation. Besides improving vasodilation, statins also brings down serum CRP level (Jialal *et al*., 2001) and has antithrombotic effect also since it act on the coagulation system influencing the endothelial structure favourably (Dimitrios *et al*., 2009). Statins prompt release of anti-atherogenic cytokines such as IL-4 and IL-10 and ease macrophage expression of proatherogenic cytokines like IL-6 and TNF-α (Schonbeck *et al*., 2004). Expression of another atherogenic mediator namely MCP-1, involved in LDL oxidation also is found to be reduced with statins.

Therefore, statins have multitude effects leading to protection and cure of patients with hypercholesterolemia. However, there are some proven side effects for statin treatment including muscle pain, increased possibility of diabetes mellitus, and abnormalities in liver function. There are apprehensions on widespread use of statins because of some severe adverse effects, mainly muscle damage (Bellosta *et al*., 2012).

1.5.5. Niacin

Niacin, which is water-soluble vitamin (B3) has been in use as a lipid-lowering agent at higher doses with good effects for a long time. Niacin containing procedures are among the few treatment models that have demonstrated reduction in atherosclerotic progression or have even induced regression (Brown *et al*., 2001; Whitney *et al*., 2005).

Niacin inhibits the production of Apo B, increases VLDL catabolism mediated through the activity of the enzyme lipoprotein lipase, and is believed to alter LDL particle size distribution from predominantly small dense particles to larger, more buoyant, less atherogenic LDL particles (Rosenson *et al*., 2009). Atherogenesis is accelerated by small, dense LDL particles in comparison to larger, more buoyant particles. *In vitro* investigations suggest that niacin has a useful effect on SRB-I facilitated cholesterol efflux and on ABCA-1facilitated cholesterol efflux from macrophages both of which can elevate HDL and contribute to reverse cholesterol transport (Morgan *et al*., 2010). Evidences also suggest that the beneficial effects of

niacin may be controlled in part by its effects on acute atherosclerotic inflammation. (Monetecucco *et al*., 2010; Vaccari *et al*., 2010; Wu *et al*., 2010). In the cases of primary hyperlipidemia and mixed dyslipidemia, niacin increases HDL and lowers total cholesterol, LDL, apo B, and triglyceride levels (Maccubbin et al., 2008). Niacin inhibits hepatic diacylglycerol acyltransferase, thus in turn inhibiting hepatic triglyceride synthesis and very low density lipoprotein secretion (Pejic *et al*., 2006; Rosenson *et al*., 2009). Niacin possesses antioxidant and antiinflammatory effects that help them to be antiatherosclerotic. Niacin is believed to activate nuclear transcription factors such peroxisome proliferator activator receptor gamma (Meyers *et al*., 2004).

Despite its reported effects and multidimensional benefits, use of niacin may be suboptimal in actual clinical use, in part because many patients find it difficult to tolerate. Niacin is having contraindications in patients with active hepatic problems, peptic ulcer or arterial bleeding.

1.5.6. Fibrates

Fibrates are a widely and effectively used class of lipid-modifying agent that bring about a substantial decrease in plasma triglycerides and is proved to be associated with a reasonable decrease in LDL cholesterol and an upsurge in HDL cholesterol concentrations. Recent investigations indicate that the properties of fibrates are facilitated through transcriptional modifications of genes encoding for proteins that regulate lipoprotein metabolism (Staels *et al*., 1997). Fibrates induce specific transcription factors coming under the nuclear hormone receptor superfamily, namely peroxisome proliferator-activated receptors (PPARs). The PPAR-a form controls fibrate action on HDL cholesterol levels by transcriptional activation of the synthesis of apoA-I and apoA-II, the major HDL apolipoproteins (Vu-Dac *et al*., 1994; Berthou *et al*., 1996). Functional PPARs have been identified in the regulatory regions of the human lipoprotein lipase, apoC-III, apoA-II, and apoA-Igenes (Hertz *et al*., 1995; Vu-Dac *et al*., 1995). Fibrates decrease hepatic apoC-III synthesis, enhance lipoprotein lipase mediated lipolysis through PPAR induction. Fibrates effectively reduce VLDL production by encouraging cellular fatty acid uptake and promoting its conversion to

acyl-CoA derivatives, through the -oxidation pathway. This is also helped by a reduction in fatty acid and triglyceride synthesis. In short, changes in HDL apolipoprotein expression is the reason for the effect of fibrates on HDL metabolism and its hypotriglyceridemic effect is due to the enhanced catabolism of triglyceride rich particles and reduced secretion of VLDL. The triglyceride lowering activity of fibrates is best described by the increased induction of the enzyme lipoprotein lipase (Schoonjans *et al*., 1996) and the reduction of apolipoprotein C-III gene expression in hepatocytes, thus resulting in enhanced lipolysis of triglyceride rich particles and ensuing clearance of its remnants from plasma.

1.5.8. ACE-inhibitors and angiotensin blockers

Angiotensin-converting enzyme (ACE) along with angiotensin II has been found associated with atherosclerotic plaques (Diet *et al*., 1997). It has also been proven that expression of angiotensin II is linked with expression of inflammatory cytokines such as interleukin 6 . In effect, the renin angiotensin system intensifies the progression of inflammation in the vessel wall. Evidences explain that infusion of ACE inhibitors significantly improve coronary endothelial vasodilator dysfunction (Mancini et al., 1996) and increases myocardial blood circulation (Schneider *et al*., 1999). ACE inhibition has favorable effects on fibrinolysis and the inflammatory process (Soejima *et al*., 1999; Napoleone *et al*., 2000). Studies have (Yusuf *et al*., 2000) demonstrated that subsequent to administration of the ACE inhibitor, cardiovascular events were reduced by more than 20%. Similarly, angiotensin II receptor blockers are also capable of improving endothelial dysfunction as substantiated by studies in which decrease in strokes have been observed in hypertensive patients treated with losartan, an angiotensin blocker (Dahlof *et al*., 2002; Lindholm *et al*., 2002). Nevertheless, so far direct evaluation is not available to illustrate whether angiotensin receptor blockers are as effective as ACE inhibitors to avert cardiovascular events.

1.5.9. Peroxisome proliferator-activated receptors (PPARs)

The mounting information regarding the molecular basis of pathogenesis of atherosclerosis has led to the development of specific target molecules for anti-

atherosclerotic treatment. Peroxisome proliferator-activated receptors (PPARs) (Moreno *et al*., 2004) have emerged as a leading one among them. Peroxisome proliferator-activated receptor alpha (PPAR- **α**), also referred to as NR1C1 (nuclear receptor subfamily 1, group C1), is a nuclear hormone receptor protein that is encoded by the PPAR gene in humans (Sher *et al*., 1993). Along with peroxisome proliferator-activated receptor delta and peroxisome proliferator-activated receptor gamma, PPAR-**α** form an important member of the subfamily of peroxisome proliferator-activated receptors. It has been recognized as the nuclear receptor for various classes of hepatocarcinogens in rodents that causes proliferation of peroxisomes (Kersten *et al*., 1990). PPAR- **α**, being a transcription factor is found to be a major regulator of lipid metabolism in liver (Kersten *et al*., 2014). Apart from this, PPAR- **α** is believed to have potential for acting as an antiatherogenic agent because of diverse properties credited to it. PPAR- **α** has been found to modify the expression of Apo AI, ApoAII, ApoCIII genes by functioning as transcription factors which attach to specific sites on their genes. Expression of Apo AI and ApoAII genes are enhanced (Malmendier *et al*., 1985, Mellies *et al*., 1987) at the same time expression of Apo C III is repressed (Staels *et al*., 1995). Inhibition of adhesion molecules like VCAM-1, enhanced endothelial NO release, prevention of foam cell formation, and reduced uptake of glycated LDL and triglyceride-rich remnant lipoproteins are some of the most significant endothelial linked functions of PPAR-α that contribute to antiatherogenesis (Staels *et al*., 1995). Endothelial cells, smooth muscle-cells, macrophages, and T cells found within the atherosclerotic lesion, are capable of expression of PPAR-**α**. Reduction of angiogenesis, inhibition of smooth muscle cell (SMC) migration, upregulation of the interleukin-1 receptor antagonist and elevation of reverse cholesterol transport have also been reported as effects of PPAR-α activation (Mellies *et al*., 1987). It has also been demonstrated that treatment with Glitazone reduces CRP, MMP-9 and TNF-α serum levels, possibly through PPAR-α activation (Haffner *et al*., 2002, Marx *et al*., 2003).

Activation of PPAR-**α** promotes uptake and catabolism of fatty acids by induction of genes involved in fatty acid transport and oxidation. (Sher *et al*., 1993; Kersten *et al*., 1999) PPAR-**α** inturn is activated through ligand binding and synthetic ligands like fibrate drugs, used to treat hyperlipidemia, facilitate in this function. Fatty

acids such as arachidonic acid as well as members of polyunsaturated fatty acids and various fatty acid-derived compounds such as the 15-Hydroxyicosatetraenoic acid family of arachidonic acid metabolites act as endogenous ligands. (Kersten *et al*., 2014)

1.6. ANTITHROMBOTIC AND ANTICOAGULANT ACTIVITY

1.6.1. Effect of antithrombosis in inhibiting atherosclerosis

Anticoagulants in current clinical use include the vitamin K antagonists such as warfarin and heparin, and parenterally administrated direct thrombin inhibitors. Warfarin can be administrated orally; however, its major drawbacks include the necessity for monitoring because of a narrow therapeutic window and large inter-and intra-individual variability in response to dose, a slow onset of action, and extensive drug interactions. Heparins have a rapid onset of action, but must be administered parenterally. Despite recent developments, there is still an unmet need for safe, oral anticoagulants for therapeutic use (Hirsh *et al*., 2005). Factor Xa has emerged as particularly promising target for effective anticoagulation pathways. FXa catalyses the conversion of prothrombin to thrombin. One molecule of activated FX, results in the generation of more than 1000 thrombin molecules. Thus, inhibiting FXa may block this upregulation of thrombin generation, thereby diminishing thrombin-mediated activation of coagulation. Recent research has been paying attention to the identification of small molecule FXa inhibitors with good oral bioavailability and predictable pharmacokinetics. An oral, direct FXa inhibitor that does not require routine coagulation monitoring would offer significant advantages over current modes of therapeutic interventions (Perzborn *et al*., 2005)

1.6.2. Blood coagulation system and platelet aggregation

Blood coagulation is the process by which the nature of blood changes from liquid state to the state of gel, potentially resulting in hemostasis or prevention of bleeding. Coagulation involves activation, adhesion and aggregation of platelets along with formation and maturation of fibrin thread. Disorders of coagulation can result in

either bleeding (hemorrhage) or obstructive clotting called thrombosis (Basson *et al*., 2001). Mechanism of coagulation involves both a cellular phase involving platelets and a phase involving coagulation factors. Coagulation begins instantaneously when an injury to the blood vessel damages the endothelial lining of blood vessel. Contact of blood on the space under the endothelium sets off two processes. Alterations in platelet behaviour and the exposure of sub endothelial tissue factor to plasma Factor VII , which finally lead to fibrin formation Primary hemostasis is the first phase with platelets instantly forming a plug at the site of injury. The secondary hemostasis occurs simultaneously with additional coagulation factors apart from Factor VII, responding in a complex cascade manner to form fibrin strands, which allow strengthening of the platelet plug (Jackson *et al*., 2007).

When the endothelium is damaged, the underlying collagen is exposed to circulating platelets, which immediately bind to collagen directly through collagen specific glycoprotein Ia/IIa surface receptors. This adhesion is strengthened further by a factor called von Willebrand Factor (vWF), which is released both from the endothelium and from platelets. vWF forms further connections between the platelet's glycoprotein Ib/IX/V and the collagen fibrils (Maria *et al*., 2010). This arrangement of platelet to the extracellular matrix supports collagen interaction with platelet glycoprotein VI. Binding of collagen to glycoprotein VI triggers a signalling cascade that result in activation of platelet integrins, and the activated integrins inturn mediate tight binding of platelets to extracellular matrix. This process adhere platelets to the site of injury. Activation platelets release the contents of stored granules into the blood plasma. The granules include ADP, serotonin, PAF, vWF, platelet Factor IV, and TXA2, which, in turn, activate additional platelets. The granules contents activate a Gq-linked protein receptor cascade, resulting in increased cascade, resulting in enhanced calcium concentration in platelet's cytosol. The calcium activated protein kinase C, which in turn, activates phospholipase A2 (PLA2). PLA2 then modifies the integrin membrane glycoprotein IIb/IIIa aid in aggregation of adjacent platelets (completing primary hemostasis) (David *et al*., 2009).

The coagulation cascade of secondary hemostasis has two pathways both of which lead to fibrin formation. These are the contact activation pathway also known as intrinsic pathway, and the tissue factor pathway also known as extrinsic pathway. The pathways are a series of reactions, in which an inactive protease together with its glycoprotein co-factor are activated to become active form which in turn catalyse the next reaction in the cascade, finally resulting in fibrin cross links. The coagulation factors are generally zymogen forms of serine proteases, which proceed by cleaving downstream proteins. There are some exceptions like, FVIII and FV which are glycoproteins, and factor XIII which is a transglutaminase. These zymogens circulate as inactive coagulation factors. The coagulation cascade is classically divided into three stages. Both the tissue factor pathway and the contact activation pathway, activate the **"**final common pathway**"** of Factor X (Alban *et al*., 2008).

1.6.2.1. ***Extrinsic pathway for Initiating Clotting:*** The extrinsic pathway for initiating the generation of prothrombin activator begins with a traumatised vascular wall or traumatised extra vascular tissues that make contact with the blood. This lead to the following steps,

1. Release of tissue factor: Injured tissue release a complex of several factors called tissue factors or tissue thromboplastin. This factor consists especially of phospholipids from the tissue membranes and a lipoprotein complex that function mainly as a proteolytic enzyme.

2. Activation of Factor X: Role of Factor VII and tissue factor- the lipoprotein complex of tissue factor combine with blood coagulation Factor VII and, in the presence of calcium ions, acts enzymatically on Factor Xa.

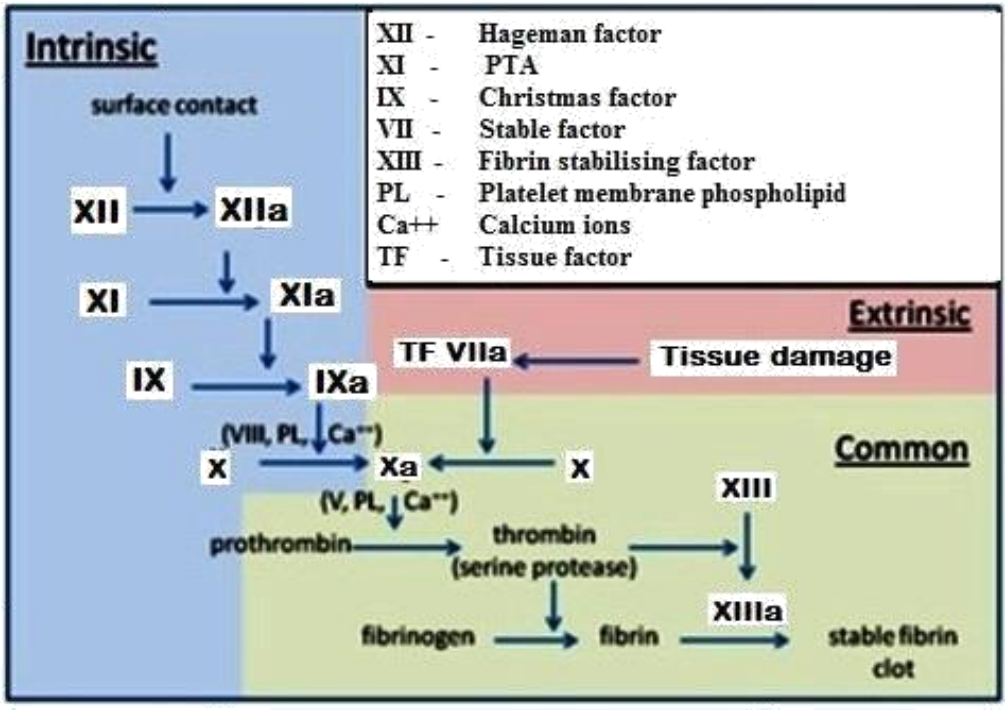


Illustration of Blood coagulation cascade pathways

3. Effect of activated Factor X (Xa) to give rise to prothrombin: The activated Factor X combines directly with tissue phospholipids that are seen as part of tissue factor or with additional phospholipids released by platelets as well as with Factor V to form prothrombin activator. Immediately, in the presence of Ca++, this act on prothrombin to form a thrombin, and the progression of coagulation proceeds as already explained. Initially, Factor V is inactive as a member of prothrombin activator complex, but as clotting begins and thrombin is released, thrombin activates Factor V since it is having a proteolytic property to activate Factor V. Thus, in the prothrombin activator complex, activated Factor X is the genuine protease that causes splitting of prothrombin to form thrombin; activated Factor V steps up this protease activity, and platelet phospholipids act as a molecule that further accelerates the process. Thrombin plays the positive feedback role of acting through Factor V, to stimulate the entire process once it commences (Shahriyary and Yazdanparast, 2007)

1.6.2.2. ***Intrinsic pathway for initiating clotting****:* The alternate mechanism for initiating formation of prothrombin activator complex, and subsequently for initiating

clotting, begins with trauma to the blood or exposure of the blood to vascular wall collagen. A series of cascading reactions is followed:

1. Blood trauma causes two events. One is activation of Factor XII and second is release of platelet phospholipids. Trauma to the blood or exposure of the blood to vascular wall collagen modifies two important clotting factors in the blood, namely Factor XII and platelets. When Factor XII is stressed, for instance by coming into contact with collagen or with a wettable surface such as glass, it assumes a new molecular design that converts it into a proteolytic enzyme known as **"**activated Factor XII**"**. Simultaneously, the platelets also get damaged because of adherence to either collagen or a wettable surface, resulting in release of platelet phospholipids that contain the lipoprotein called platelet factor 3, which also play a part in ensuing clotting reactions.

2. Activation of Factor XI: When the Factor XII is activated; it acts on Factor XI to activate this factor as well, which is the second step in intrinsic pathway. This process is assisted by HMW kininogen and is accelerated by prekallikrein.

3. Activation of Factor IX: The activated Factor XI then enzymatically acts on Factor IX to activate it.

4. Activation of Factor X and role of Factor VIII: The activated IX, acting in association with activated Factor VIII, platelet phospholipid and factor 3 from the traumatized platelets, activates Factor X. This step is slowed down during deficiency of either Factor VIII or platelets factors. Factor VIII is also called antihemophilic factor since its absence lead to classic haemophilia.

5. Role of activated Factor X in forming prothrombin activator: This process in the intrinsic pathway is the same as the last step in the extrinsic pathway of coagulation. Activated Factor X combines with Factor V and platelet or tissue phospholipid to form the prothrombin activator complex. This complex in turn immediately initiates the cleavage of prothrombin to form thrombin, thus completing the final clotting process (Arthur and Jhon, 2006).

1.6.2.3. ***Final common pathway****:* The classification of coagulation into two pathways is not natural; the concept originated from laboratory tests in which clotting times were measured after the clotting was initiated by glass (intrinsic) or by thromboplastin (a mix of tissue factor and phospholipid). In fact, thrombin is present already when platelets are making the plug. Thrombin has wide variety of function, in addition to the conversion of fibrinogen to fibrin. It is the most important platelet activator and besides that, it activates Factors VIII, V and also their inhibitor protein C in the presence of thrombomodulin. Thrombin also activates Factor XIII, which form covalent bonds that interlock the fibrin polymers.

Subsequent to activation by the coagulation pathways, the coagulation cascade is sustained in a prothrombotic state by the continued activation of FVIII and FIX to form the tenase complex. This is maintained until it is down-regulated by the anticoagulant pathway (Pallister and Watson, 2010).

1.6.2.4. Other factors in coagulation cascade pathways

i. Calcium and platelet membrane phospholipid are required for the tenase and prothrombinase complexes to function.

ii. Vitamin K acts as an essential factor to a hepatic enzyme gamma-glutamyl carboxylase that inserts a carboxyl group to glutamic acid residues on coagulation factors II, VII, IX and X, as well as Protein S, Protein C and Protein Z. During this process Vitamin K itself gets oxidised. An additional enzyme, Vitamin K epoxide reductase (VKORC) reduces the oxidised vitamin K back to its active form. VKORC is pharmacologically significant as a target of anticoagulant drugs like warfarin and related coumarins such as acenocoumarol, phenprocoumon, and dicumarol. The strategy of these drugs is to create a deficiency of reduced vitamin K, thereby affecting the normal functioning of coagulation factors.

1.6.2.5. Regulators in coagulation cascade pathways

i. Protein C is a major physiological innate anticoagulant. It is a vitamin K-dependent serine protease that is activated by thrombin into activated protein C (APC). Protein C

is activated in a series that begins with Protein C and thrombin binding to a cell surface protein namely thrombomodulin. Thrombomodulin binding activates Protein C. The activated Protein C, together with protein S and phospholipid, degrades FVa and FVIIIa. Quantitative or qualitative deficiency may lead to thrombophilia which is a tendency to develop thrombosis.

ii. Antithrombin is a serine protease inhibitor (serpin) that inactivate the serine protease enzymes namely thrombin, FIXa, FXa, FXIa and FXIIa. Antithrombin is persistently active, but its adhesion to these is increased in the presence of heparan sulfate or by the administration of heparin, an anticoagulant. Inborn or acquired deficiency of antithrombin lead to thrombophilia, a tendency to develop thrombosis.

iii. Plasmin, another regulator of coagulation is generated by proteolytic cleavage of a plasma protein synthesised in the liver namely plasminogen. Plasmin cleaves fibrin into fibrin degradation products thereby inhibiting fibrin network formation.

iv. Prostacyclin (PGI2) released by endothelium activates protein-linked receptors on platelet, which in turn, activates the enzyme adenylyl cyclase. This enzyme synthesises cAMP which inhibits the release of granules from platelets that would lead to activation of further platelets and the coagulation cascade (Giangrande *et al*., 2003).

Anticoagulants and anti-platelet agent are amongst the most regularly used medications during symptoms of thrombosis. Antiplatelet agents include aspirin, dipyridamole, ticlopidine, clopidogrel, ticagrelor and prasugrel. These are used even during angioplasty. Among the anticoagulants, warfarin and heparin constitute the most frequently used medications. Warfarin has an effect on the vitamin K-dependent clotting factors (II, VII, IX, X), protein C and protein S, whereas heparin and related compounds augment the action of antithrombin on thrombin and Factor Xa (Lan and Alvin, 2005).

1.6.3. Antithrombotic-anticoagulant agents and their mode of action

1.6.3.1. *Warfarin*: Warfarin is an anticoagulant generally employed in the prevention of thrombosis and thromboembolism. Warfarin is a derivative of dicoumarol, a 4-

hydroxycoumarin-derived mycotoxin anticoagulant initially discovered in contaminated sweet clover-based animal feeds (Rubin *et al*., 2008).

Warfarin is a drug usually referred to as a **"**blood thinner**"**. However this is a misnomer, since it does not reduce the viscosity of blood. Warfarin stalls the vitamin K-dependent production of biologically active form of the calcium-dependent clotting factors II, VII, IX and X, along with the regulatory factors namely, Protein C, Protein S and Protein Z. Warfarin downregulates epoxide reductase, thereby reducing available vitamin K and vitamin K hydroquinone in the tissues. This reduces the carboxylation action of the glutamyl carboxylase. As a result, the coagulation factors are no longer carboxylated at some of their glutamic acid residues, and become biologically inactive. Even though coagulation factors are synthesised, they have reduced functionality due to deficiency of carboxylation. These factors are jointly referred to as Protein Formed in Vitamin K Absence (PIVKAs). The final outcome of Warfarin administration is diminished blood coagulation in the patient (Liu *et al*., 2000; Ansell *et al*., 2008).

1.6.3.2. *Heparin*: Heparin, a highly sulfated glycosaminoglycan, is extensively used anticoagulant, and has the uppermost negative charge density among identified biological molecules. It is also employed to give an inner anticoagulant coating on various devices such as glassware and renal dialysis machines. Heparin and its derivatives are effective at checking deep vein thrombosis and pulmonary emboli in patients, but no evidence indicates any one is more efficient than the other in preventing mortality. Heparin binds to the enzyme inhibitor AT III, causing a conformational alteration that result in its activation through an increase in the flexibility of its reactive site loop. The activated AT III then inactivates thrombin and other proteases that are mediators in blood clotting. The rate of inactivation of these proteases by AT III can increase by 1000 times due to the binding of heparin (Marcello *et al*., 2005).

Antithrombin III binds to a specific pentasaccharide sulfate residue within the heparin polymer. This results in modification of its conformation in turn leading to inhibition of Factor Xa. As for thrombin inhibition, thrombin must directly attach to the heparin polymer at a site proximal to the pentasaccharide. The highly negative charge

density of heparin adds to its very strong electrostatic binding with thrombin. The development of ternary complex involving AT III, thrombin, and heparin eventually causes the inactivation of thrombin. In this function, heparin's activity against thrombin is size-dependent, with the ternary complex requiring at least 18 saccharide units for successful formation. In contrast, inhibition of factor Xa, requires merely the pentasaccharide binding site. This size difference has led to the development of Low-Molecular-Weight Heparin (LMWHs) and, more recently, to fondaparinux as pharmaceutical anticoagulant drugs. LMWHs and fondaparinux are intended for inhibition of factor Xa activity rather than antithrombin activity, with the aim of facilitating a smoother regulation of coagulation and a better therapeutic index. The chemical constitution is almost matching that of AT III binding pentasaccharide sequence found within polymeric heparin and heparan sulfate. The effects of heparin are calculated in the lab by the prothrombin time test, which measures the time taken for the blood plasma to clot (Hemker and Beguin, 1991).

1.6.4. Studies on antithrombotic and anticoagulant effect of medicinal plant

Pulmonary emboli, deep vein thrombosis, cardiac strokes and myocardial infarction are thromboembolic disorders. Thromboembolic disorders are the major reason for morbidity and mortality throughout the world. Anticoagulants take part in a critical role as agents for the prevention and treatment of thromboembolic disorders. Phytochemicals in plants may serve as an alternative source for the development of new anticoagulant agents. A number of medicinal plants have been evaluated for their antithrombotic and anticoagulant properties. There have been some significant research findings as illustrated below.

Careya arborea Roxb belongs to the family Lecythidaceae is found in several places of the world. The bark of this plant is traditionally used in treatment of tumors, bronchitis, astringents, skin diseases and as antidote to snake venom. Stem bark of the plant mainly possess pharmacological properties like antidiarrheal, analgesic, hepato-protective, antitumor, antileishmanial, antimicrobial and antioxidant activities (Kumar *et al*., 2010). The methanolic extract of bark of *Careya arborea* exhibited anticoagulant

activities when compared with the standard warfarin. The extract prolonged the time taken for blood clotting and there was a significant enhancement in the activated partial thromboplastin time, prothrombin time and thrombin time measurements.

Melastoma malabathricum Linn. belong to family Melastomataceae contains amides, triterpenoids, flavonoids, alkaloids and tannins. This plant has been reported for antidiarrhoeal, antibacterial, wound healing activities and the aqueous leaf extract has been used for its antinociceptive, anti-inflammatory, antipyretic, gastroprotective effects and antioxidant properties (Sirat *et al*., 2010). The aqueous leaf extract possesses potent anticoagulant property as shown by *in vitro* results. Activated partial thromboplastin time (aPTT) of plasma samples spiked with different concentrations of the leaf extract was markedly prolonged in a concentration-dependent manner. The aqueous leaf extract has been proved to influence the intrinsic pathway of the coagulation by causing clotting factor deficiency.

Gloriosa superba Linn. belonging to the family Lilaceae has gloriosine and colchicines as two phytochemical constituents and is used for treatment of gout and rheumatism. It possesses analgesic, anti-inflammatory, antitumour properties, and is used in treatment of snakebite (Jain *et al*., 2010). It is also used in respiratory complications, skin diseases and in familial Mediterranean fever (FMF). *Gloriosa superba Linn*. Extract has been verified for its anticoagulant properties and it is known to inhibit thrombin induced clotting.

Bauhinia forficata is a medicinal plant belongs to family Leguminosae and mainly contains flavonols, flavonoids, glycosides, kaempferitrin, astragalin, -sitosterol, organic acids, quercitrosides, rhmanose and saponins. *Bauhinia forficata* leaves are employed in Brazilian folk medicine as cure for diabetes, as a diuretic for kidney and urinary disorders, as a blood cleanser and for high cholesterol (Cunha *et al*., 2010). It also has anticoagulant, antifibrinogenolytic properties. Aqueous extract from aerial parts of *Bauhinia forficata* is a source of natural inhibitors of serine proteases which play role in preventing blood clotting induced by snake venom (Souza *et al*., 2009).

Jatropha curcas L. (Euphorbiaceae), is commonly grown in rural areas in India and is traditionally used for the treatment of sciatica, dropsy, paralysis, rheumatism, dysentery, diarrhoea, and certain skin diseases. The pharmacological activities it shows are analgesic, anti-inflammatory, antidiarrhoeal, heptoprotective, and anti-diabetic properties. Anticoagulant activity of the latex of *Jatropha curcas* was demonstrated with the diluted latex prolonging the clotting time (Upadhye *et al*., 2000).

Synclisia scabrida (family Menispermaceae) aqueous and ethanol extract significantly prolonged the prothrombin time (PT) of normal plasma, which implies that both extracts of *Synclisia scabrida* have anticoagulant properties (Iroegbu *et al*., 2005).

1.7. HERBAL MEDICATION

Medicinal plants have been the source for preventive and curative treatment for atherosclerosis over much of human history. Modern medicine considers herbalism as a form of alternate medicine. Eventhough the practice of herbalism sometimes lack support gathered using the scientific methods, the scientific community has made some recent significant strides in this direction. This can be seen from the revealed antitumour, hypolipidemic, anti-thrombotic, immunomodulatory, organoprotective and other properties of various medicinal plants. Modern medicine makes use of many plant-derived compounds as the basis for pharmaceutical drugs, applying modern standards of research. Many of the pharmaceuticals available today have a history of traditional use. This includes reserpine, vincristine, opium, quinine, digitalis, aspirin among many others. Around 7,000 medical preparations in the modern pharmacology are derived from plants. Medicinally active compounds isolated from plants have been found to show a positive correlation between their modern therapeutic use and their employment in traditional medicine (Asgary *et al*., 2006; Kim *et al*., 2009). It is not surprising that the search for herbal remedies as a mode of prevention and treatment for cancer and cardiovascular diseases, the two leading causes of mortality, is assuming pace in recent times. The future of medicinal plants research is highly promising in that millions of plants all over the world are yet to be verified for their medicinal properties and the results can be vital in the treatment of illnesses (Fabricant *et al*., 2001).

1.7.1. Phytochemicals in medicinal plants

Man and other animals depend on plants directly or indirectly for all their needs related to primary metabolites, naturally the dependence on secondary metabolites on plants is quite explicable. Phytochemicals from plants mostly follow similar mode of action as modern conventional drugs, which enable them to be equally effective also (Ameenah *et al*., 2006).

The use of herbal drugs against various diseases is drawing considerable importance these days. Various plants have been employed in Indian traditional clinical practice and as ingredients in Ayurvedic and Unani preparations. There has been a recent upsurge in research in this area after scientific authentication that they are effective for the therapeutic conditions to which they have usually been employed (Fabio *et al*., 2007). Pharmacognosy is the branch of modern medicine about study of medicines from plant sources. It is the selected active ingredients of plants that are used in modern medicine instead of the whole plants. These phytochemicals may be as such synthesised or altered to form pharmaceuticals.

Plants synthesise an array of organic compounds that are categorised as primary and secondary metabolites although a clearly defined boundary between the two groups does not exist. Primary metabolites are compounds having critical roles in regular metabolic processes like respiration, photosynthesis, growth and development. The biomolecules like carbohydrates, lipids, nucleotides, amino acids and organic acids belong to this group. Secondary metabolites are phytochemicals, which are produced and accumulate in remarkably high concentrations in some plants. These secondary metabolites are products which complement in the normal growth and development of plants but are not obligatory for survival of the plant. In this way, secondary metabolism complements primary metabolism and play a part in keeping plants healthy.

1.7.1.1 *General functions of secondary metabolites*

In general, the secondary metabolites have key role in protecting plants from herbivores and microbial infection. They have roles in pollination and seed-dispersal, act as allelopathic agents, UV protectants and signal molecules in nitrogen-fixation, besides being used as dyes, fibres, glues, oils, waxes, flavouring agents and perfumes. But their most important use for mankind appears to be as potential sources of new natural drugs and antibiotics. In recent years, the role of secondary metabolites as herbal drugs has become an increasingly important area of bio-medical research. Accumulating evidence suggest that their intake can give protection against cancers and many chronic diseases, including cardiovascular disease and Type II diabetes. The additive effect of phytochemicals or synergy is an important hypothesis in herbal pharmacognosy. In other words, there is a compounding effect between the components in a plant when compared to their individual properties which have been believed to ultimately contribute to its medicinal effects (Sharma *et al*., 1997; Heinrich *et al*., 2003; Venturi *et al*., 2007).

1.7.2. Types of secondary metabolites

A well-structured system of classification of secondary metabolites is not available. Classification is mostly based on their biosynthetic origins, upon which secondary metabolites are divided into four major groups.

i. Phenolics including flavonoids and polyphenolic compounds
ii. Sulphur-containing compounds
iii. Terpenoids
iv. Alkaloids

1.7.2.1. Phenolic compounds

Phenolic compounds are characterized by presence of at least one aromatic ring with one or more hydroxyl groups attached to it. More than 8000 phenolic structures have been reported which are widely distributed throughout the plant kingdom. Phenolic compounds range from the simplest, low-molecular weight, solitary aromatic-

ringed compounds to large and complex tannins and derived polyphenols. Phenolics are classified based on the number and arrangement of their carbon atoms.

Phenolics can be classified into two groups: *The flavonoids and the non-flavonoids.*

i. Flavonoids

Flavonoids are polyphenolics having fifteen carbons in it, with two aromatic rings connected by a three-carbon bridge. They are the most abundant of the phenolics found widespread the plant kingdom. They are present in high amounts in the epidermis of leaves and the skin of fruits and have essential roles as secondary metabolites. In plants, flavonoids are involved in such diverse processes as UV protection, pigmentation, stimulation of nitrogen-fixing nodules and disease resistance (Koes *et al.* 1994; Pierpoint, 2000). In higher plants, flavonoids act as chemical messengers, physiological regulators, and cell cycle inhibitors in addition to having inhibitory activity against organisms that cause plant diseases.

Consumables with a high flavonoid content include onions, blueberries and other berries, green tea, black tea, bananas, citrus fruits, Ginkgo biloba, red wine and dark chocolate (Hertog *et al*. 1992; Del Rio *et al.* 2004; Gu *et al.* 2004).

Flavonoids have been proved to have a wide range of biological and pharmacological activities which include anti-microbial, anti-allergic, anti-inflammatory, antioxidant, anti-cancer, cardio-protective (Kimura *et al*., 1985; Soleas *et al*., 1997) and anti-diarrheal activities.

ii. Non-flavonoids

The most important of the non-flavonoids are the phenolic acids, most notably gallic acid, tannins, C6–C3 hydroxycinammates and stilbenes. Notable sources in food include berries, beer, tea, olive oil, coffee, chocolate or cocoa, pomegranates, fruits, algae like spirulina, mushrooms and vegetables, which are potentially significant for supplying certain natural phenols.

1.7.2.2. Sulphur-containing compounds

The two major sources of sulphur-containing plant compounds in normal diet include,

i. Those derived from the glucosinolate–myrosinase (substrate–enzyme) system found in vegetables such as cabbages and broccoli (Manabe *et al.*1998; Lancaster *et al.* 2000; Fahey *et al.* 2001)

ii. Those derived from the alliin-alliinase system found within allium crops such as garlic and onion (Manabe *et al.* 1998; Lancaster *et al.* 2000).

Biological activity of sulphur-containing compounds

Sulphur-containing compounds provide the characteristic hot and pungent flavours of many of our salad crops and contribute to flavour components of cooked vegetables. Studies suggest that they have anti-inflammatory (Keiss *et al.* 2003) and antioxidant activity (Higuchi *et al.* 2003). They have been proved to have antiproliferative properties (Yu *et al.*1998; Chen *et al.* 2002; Fimognari *et al.* 2002; Misiewicz *et al.* 2003). These compounds may also protect against atherolosclerosis and other inflammatory diseases.

1.7.2.3. Terpenes

The terpenes or isoprenoids are one of the most miscellaneous classes of metabolites consisting over 30000 members of plant origin, encompassing flavours and fragrances, antibiotics, plant and animal hormones and membrane lipids (Dewick, 2001; Buckingham, 2004). In addition, terpenoids are structural and functional parts of a number of bioactive natural products. Some of such structures include phytol side chain of the chlorophylls, diterpenoid skeleton of the anti-cancer drug Taxol and the core structural unit of tetrahydrocannabinol (the major bioactive component of marijuana).

Terpenes are derived from isopentenyl pyrophosphate (IPPP) and dimethyl allyl pyrophosphate (DMAPP). These two building blocks are condensed together in a

sequential manner by the action of enzymes called prenyltransferases (Adam *et al.* 2002; Eisenreich *et al*. 2004). The products include geranyl-farnesyl and geranyl-geranyl pyrophosphates, squalene and phytoene which are the direct precursors of the major families of terpenes.

Terpenes and terpenoids are the primary constituents of the essential oils of many types of plants and flowers. Essential oils are used widely as natural flavor additives for food, as fragrances in perfumery and in traditional and alternative medicines (Castleman *et al*., 2003; Heinrich, 2003). It has been proved to have antibacterial property too (Kalemba and Kunicka, 2003).

Terpenes may be classified based on the number of isoprene units in the molecule; a prefix in the name indicates the number of terpene units, like monoterpenes, diterpenes and so on. Examples of important terpenes include prenol, isovaleric acid, geraniol, terpineol, farnesol, squalene, lycopene, gamma-carotenes and beta-carotenes (Fraser and Bramley, 2004).

1.7.2.4. Alkaloids

Alkaloids are a collection of low molecular weight, nitrogen-containing compounds derived from amino acid precursors and found in about one fifth of plant species. Due to their potential biochemical activities, a good number of the approximately 12,000 identified alkaloids have been employed as pharmaceuticals, stimulants and narcotics (Wink, 1998). Plant-derived alkaloids presently in clinical use comprise the analgesics morphine and codeine, the muscle relaxants, tubocurarine, papaverine the anti-neoplastic agent vinblastine and the gout suppressant colchicine. Other well-known alkaloids of plant origin include nicotine, caffeine and cocaine, and the synthetic *O*-diacetylated morphine derivative heroin. It was the isolation of morphine in 1806 which lead to the further research in alkaloids (Facchini *et al*., 2001). Currently many of the alkaloids are well known for their pharmacological effects and are used as medications and as herbal drugs.

1.7.3. *Applications phytochemicals in medicine*

Medicinal use of alkaloid-containing plants has a long history, and, thus, when the first alkaloids were isolated in the 19th century, it straightaway found application in clinical practice. Many alkaloids are now used in medicine, usually in the form of salts, which include the following:

The well-known central nervous stimulant strychnine is alkaloid. The anti-neoplastic agents vincristine and vinblastine from the Madagascar periwinkle (*Catharanthus roseus*) is another example. The significance of *C. roseus* as a source of anti-cancer medication has encouraged exhaustive research into the biochemistry of alkaloid biosynthesis in this plant. Hindus have used the Indian snakeroot (*Rauwolfia serpentaria*) for centuries as a febrifuge, and as an antidote to poisonous snakebites. The anti-malarial drug quinine is obtained from the stem and root bark of *Cinchona* species. Alkaloid caffeine was discovered in coffee (*Coffea arabica)* and tea (*Camellia sinensis*).

Alkaloid	***Action***
Atropine	Anticholinergic
Caffeine	Stimulant
Codeine	Cough medicine, Analgesic
Colchicine	A microtubule disrupter, Remedy for gout
Emetine	Antiprotozoal agent
Morphine	Analgesic
Nicotine	Stimulant
Quinine	Antipyretics, Antimalarial
Reserpine	Antihypertensive
Tubocurarine	Muscle relaxant
Vinblastine, Vincristine	Antitumor

1.8. *DESMODIUM* SPECIES OF PLANTS

Various members belonging to genus Desmodium have been scientifically screened and validated for their traditional use. A number of plants belonging to Desmodium have been elaborately employed as medicinal plants for their anti-inflammatory, anti oxidant and cardioprotective properties (Figure 1.9).

1.8.1. *Desmodium gangeticum* L.

Desmodium gangeticum is a small shrub of tropical region and is called 'shalparni' in Sanskrit. It has been widely used in Indian system of medicine as a bitter tonic, febrifuge, digestive, anticatarrhal, antiemetic, in inflammatory conditions of chest and various other inflammatory conditions caused by 'vata' disorders (Chopra *et al*., 2003). The aqueous extract of *D. gangeticum* has been reported to show severe antiwrithing activity, moderate central nervous system (CNS) depressant activity as well as antileishmanial activity (Jabbar *et al*., 2001;). Gangetin, a pterocarpnoid phytochemical isolated from *D. gangeticum* has been shown to have anti-inflammatory and analgesic properties (Ghosh *et al*., 1981). Alkaloids of this species showed anticholinesterase, smooth muscle stimulant, CNS stimulant and depressant responses on experimental administartion. Analytical studies on the species showed the presence of alkaloids, pterocarpnoid, flavone and isoflavanoid glycosides (Purushothman *et al*.,1971; Ghosal *et al*., 1971; Govindarajan *et al*., 2003). Due to its varied medicinal values its root has been used as one of the components of "Dasamoolarishta" and "Dasamoola kwaath" in Ayurvedic medicine. It should also be noted that *D. gangeticum* has proven hypolipidemic, cardio protective properties and give protection against cardiac reperfusion injury (Kurian *et al*., 2005). Ayurvedic preparations such as Dasamoolaristham, dasamoolaharithiki, dasamoola ghrutham contain *Desmodium gangeticum* DC as important constituent and are used in inflammatory conditions and other digestive tract disorders.

1.8.2. *Desmodium triflorum* DC.

Desmodium triflorum DC is a herb with branched stems. It is a creeper and forms a dense mat over soil. *D. triflorum* is used in eye diseases, spleen complaints, stomach trouble and diarrhoea. Leaves are laxative; and used in dysentery and applied to wounds (Lai *et al*., 2009). Roots are considered carminative, diuretic and used in cough and asthma. Woks done in Des. *triflorum* have revealed potent antioxidant and anti-proliferative properties and such efficacy is mainly attributed to its polyphenolic compounds (Kawshik *et al*., 2005; Lai *et al* .,2010). Phytochemical analysis has shown that leaves contain-phenylamine, tyramine, indole-3-acetic acid, hypaphorine, trigonelline, N, N-dimethyl tryptophan methyl ester and stachydrine. Roots contain 3, 4-di-hydroxy phenylethyl trimethyl ammonium cation, betaine and choline (Ghosal *et al*., 1971). Root also has hypaphorine, an alkaloid (Rastogi and Mehrotra, 1993; Ghani *et al*., 2003).

1.8.3. *Desmodium adscendens* DC.

Desmodium adscendens is used in the treatment of asthma (Ampofo *et al*., 1977; Addy and Awumey, 1984; Addy and Burka, 1988; Addy *et al*., 1995). The plant is used in the treatment of inflammation of ovary (Guarin *et al*., 1996). It has been proposed that the mode of action of the plant is the reduction of the release of histamine (Addy and Awumey, 1984) as also the inhibition of the cycloxygenase and lipoxygenase enzymes (Addy and Burka, 1988).

1.8.4. *Desmodium gyrans* DC.

Desmodium gyrans DC is an annual herb belonging to family Fabaceae. The plant is also known as **"**The Indian telegraphic plant**"** since it makes automatic movements of its leaves and because of this nature, it is also cited as one of the ten most curious plants in the world (Figure 1.10). *D. gyrans* is used in Indian traditional and folk medicine since its leaves have diuretic, febrifugal and tonic properties and roots are used as a remedy for asthma, cough, as anti dysenteric and as emollient. It is believed to have cardioprotective properties and remarkable wound healing effect for

which it is commonly used in traditional medicine (Gopalakrishnan and Rajameena, 2012). *D. gyrans* has a long history of use in Chinese traditional medicine to treat various ailments (Ma X *et al*., 2011). Presence of eighteen phytochemicals has been identified in *D. gyrans* leaves by GC-MS analysis. The major among them are 4,5-Dihydro-2-amino-4-(2,4-dichlorophenyl)-7-methyl-5-oxo-pyrano(3,2-c) pyran-3-carbonitrile (59.07%), pentaborane (13.86%), Pentanedinitrile (6.63%) and 3-chloro-1-butyne (4.82%) (Gopalakrishnan and Rajameena, 2012). The present study is designed to make a probe into the traditionally assumed medicinal effects of *D. gyrans* which have to be made known to the world especially since it has remained a 'virgin plant' and not been the subject of much research works.

Chapter 2

Materials and Methods

2.1. MATERIALS

2.1.1. Plant Materials

2.1.2. Distribution

2.1.3. Morphology

2.1.4. Preparation of the extract

2.1.5. Animals

2.1.5.1. *Approval of Institutional Animal Ethics Committee*

2.2. METHODS

2.2.1. Phytochemical Analysis

2.2.1.1. *Alkaloids: Dragendorff's test*

2.2.1.2. *Phytosterols: Libermann and Barchards test*

2.2.1.3. *Tannins: $FeCl_3$ test*

2.2.1.4. *Terpenoids: Salkowski test*

2.2.1.5. *Flavonoids: NaOH test*

2.2.1.6. *Phenolic compounds: Folin Ciocalteu test*

2.2.2. Determination of total flavonoid content

2.2.3. Determination of total phenolic content

2.2.4. Thin Layer Chromatography (TLC)

2.2.5. GC-MS analysis

2.2.6. Acute and sub-acute oral toxicity study and calculation of LD_{50} value

2.2.6.1. *Acute oral toxicity study*

2.2.10.4. *Studies on cardiac tissue*

2.2.10.5. *Tissue preparation for mitochondrial isolation*

2.2.11. Preparation of high fat diet

2.2.12. Protocol for hypolipidemic study on rats

2.2.13. Protocol for anti-atherogenic study on rabbits

2.2.14. Biochemical analysis

2.2.14.1. *Estimation of cholesterol*

2.2.14.2. *Estimation of HDL Cholesterol*

2.2.14.4. *Estimation of Triglycerides*

2.2.14.5. *Measurement of Apolipoprotein A-I and serum hs-CRP*

2.2.14.6. *Measurement of Serum paraoxonase activity*

2.2.14.7. *Estimation of HMG-CoA reductase enzyme*

2.2.14.7.1. *Reagents preparation for HMG-CoA reductase enzyme measurement*

2.2.14.7.2. *Procedure for estimation*:

2.2.15. Studies on liver morphology and histopathology

2.2.16. Morphology of oil Red O stained aorta

2.2.17. Lipoprotein lipase activity

2.2.17.1. *Reagent for incubation*

2.2.17.2. *Reagents for glycerol determination*

2.2.17.3. *Incubation procedure*

2.2.17.4. *Glycerol determination*

2.2.18. Protocol for lipid clearing experiment

2.2.19. Estimation of liver tissue lipids

2.2.20. Statistical analysis

2.1. MATERIALS

2.1.1. Plant materials

Binomial: *Desmodium gyrans* DC

Family: Fabaceae

Common names: Thozhukanni, Ramanamapacha

2.1.2. Distribution

Desmodium gyrans is distributed throughout in India particularly in south India and mainly survive in partially shaded regions of forest area. It is also seen in East Asian countries and Australia.

2.1.3. Morphology

The plant is a perennial shrub which grows to a length of 4-5 feet. The leaves are simple with 6-8 cms length and are provided with smaller leaflets at their base which automatically moves. Flowering occurs during October-November months with purple coloured flowers. Fruit is a pod.

2.1.4. Preparation of the extract

Desmodium gyrans was collected from Peechi forest area and identification was done by Dr. Sasidharan, Taxonomist, Kerala Forest Research Institute, Peechi, Kerala, India. A voucher specimen was deposited in the herbarium of Amala Cancer Research Centre (ACRH No.36/11). Sun dried and powdered entire plant was used for making extract in 70% methanol in water by using soxhlet apparatus. The extract was dried using vacuum desiccator and the yield of solvent free extract was 12% (w/w). It was re-suspended in double distilled water and used for further studies on animals.

2.1.5. Animals

New Zealand male white rabbits (1.75-2.0 kg) used for anti-atherogenic experiment and male Wistar rats (150-200 g) used for study on hypolipidemic effect

were procured from Small Animal Breeding Station (SABS), Kerala Veterinary and Animal Sciences University, Mannuthy, Kerala, India. The animals were acclimatized for a period of 14 days in uniform hygienic well ventilated cages under standard environmental setting.

Male Sprague Dawley rats (200 g) were obtained from the Small Animal Breeding Station (SABS), Kerala Veterinary and Animal Sciences University, Mannuthy, Kerala, India. The animals were maintained under standardised environmental condition (22-28 ºC, 60-70% relative humidity, 12 hr dark/ light cycle) and fed with standard rat feed (Sai Durga Feeds, Bangalore, India) and water *ad libitum.*

Balb/c mice (20-25 g) used for study were procured from SABS, Kerala Veterinary and Animal Sciences University, Mannuthy, Kerala, India. The animals were acclimatised for a period of 14 days in uniform hygienic well ventilated cages under standard environmental setting.

2.1.5.1. *Approval of institutional animal ethics committee*

All experiments in the study were carried out with prior approval from institutional animal ethics committee (IAEC/ACRC/2012-01) and strictly adhering to the guidelines of the committee for the purpose of control and supervision of experiments on animals (CPCSEA) constituted by the animal welfare division of Government of India.

2.2. METHODS

2.2.1. Phytochemical analysis

Phytochemical analysis was done using standard procedures described below. The whole plant extract made as per the procedure given in Ch 2.1.4, was used for the purpose.

2.2.1.1. *Alkaloids: Dragendorff's test* (Waldi, 1965)

Procedure: 50 mg of solvent free extract is dissolved in 5 ml dil. HCl and filtered. To a

few ml of filtrate add 2 ml of dragendorff's reagent. Appearance of prominent yellow colour confirms the presence of alkaloids

Reagent preparation: 5.2 g of Bismuth Carbonate and 4 g of Sodium Iodide are boiled for a few minutes with 25 ml Glacial Acetic Acid. After 12 hrs, the precipitated Sodium Acetate crystals are filtered off using sintered glass funnel. 20 ml of this solution is mixed with 80 ml ethyl acetate and 500 microliters distilled water and stored in amber coloured bottle. To prepare working solution, 10 ml of stock is mixed with 20 ml acetic acid and made upto 100 ml with distilled water

2.2.1.2. ***Phytosterols: Libermann and Barchards test*** (Joshi *et al*., 2013)

Procedure: 50 mg of extract was mixed with 2 ml acetic anhydride. The mixture is boiled ant then cooled and to it added 2 drops of concentrated H_2SO_4 along the sides of the test tube. Appearance of an array of that change from violet to blue to green indicates the presence of phytosterol

2.2.1.3. ***Tannins: $FeCl_3$ test*** (Mace, 1963)

Procedure: To 50 ml of extract added 5 ml distilled water and 2 drops of 5% $FeCl_3$ solution. Appearance of dark green colour indicates the presence of phenolic compounds.

2.2.1.4. ***Terpenoids: Salkowski test*** (Joshi *et al*., 2013)

Procedure: To 2 ml of extract dissolved in distilled water, 0.5 ml chloroform and 1.5 ml Con. H_2SO_4. A yellow ring that formed at the interphase, which turned reddish brown after 2 minutes confirms the presence of Terpenoids

2.2.1.5. ***Flavonoids: NaOH test*** (Odebiyi *et al*., 1978)

Procedure: To 1 ml of extract dissolved in water, 10 drops of 10% NaOH were added. Appearance of yellow colour indicates the presence of flavonoids. The yellow colour formed disappears upon addition of 3 ml of 10% HCl

2.2.1.6. ***Phenolic compounds: Folin Ciocalteu test*** (Singleton and Rossi, 1965)

Procedure: Phenolic compounds of sample can be detected by Folin Ciocalteu reagent. A portion of the extract is mixed with a few drops of diluted Folin Ciocalteu reagent and aqueous sodium carbonate solution. The mixture is allowed to stand for 10 min. Formation of gray colour indicates the presence of Phenolic group.

2.2.2. Determination of total flavonoid content

Colorimetric aluminum chloride method was used for determination of total flavonoid content (Nabavi *et al.,* 2008; Ebrahimzaded *et al.,* 2009). Exactly 0.5 ml solution of plant extract was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water, and kept at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm using spectrophotometer. Total flavonoid contents were calculated from a calibration curve using quercetin as standard. Quercetin solutions at serial concentrations 12.5, 25, 37.5, 50, 62.5, 75, 87.5 and100 mg/ ml in methanol were prepared. These standards were run using the same procedure and standard curve was prepared. Total flavonoid values are expressed in terms of quercetin equivalent.

2.2.3. Determination of total phenolic content

Total phenolic compound contents were determined by the Folin-Ciocalteau method (Ebrahimzaded *et al.,* 2008; Nabavi *et al.,* 2008). Exactly 0.5 ml of extract was added to5 ml pre diluted Folin Ciocalteu reagent (1:10 diluted with distilled water) ,mixed for 5 min and then4 ml aqueous Na2CO3 (1 M) was added. The mixture was allowed to stand for 15 min and the phenols were determined by colorimetrically at 765 nm. The standard curve was prepared using 0, 50, 100, 150, 200mg/ ml solutions of Gallic acid in methanol and water solvent (50:50, v/v). Total phenol values are expressed in terms of Gallic acid equivalent.

2.2.4. Thin layer chromatography (TLC)

TLC is a type of adsorption chromatography used to detect the presence of phytochemicals, like alkaloids, flavonoids, terpenoids and phenolic compounds. It is a semi quantitative method.

Principle: Principle is Similar to other chromatographic methods, and separation depends on the relative affinity of compounds towards either stationary or mobile phase. The compounds with higher affinity to stationary phase move slowly while the others move faster. The components in the mixture are separated and their character is identified by suitable detection techniques.

Procedure:

i. A thin line was drawn on the suitably cut TLC plate using a pencil at the bottom of the plate to apply the sample.

ii. Samples solution was applied on the line in using a capillary tube.

iii. The solvent mixture namely diethyl ether and toluene, taken in equal proportions (1:1) was poured into the TLC chamber up to a level of few centimeters from the bottom. A solvent moistened filter paper was placed on the inner wall of the chamber to maintain equal humidity.

iv. The TLC plate prepared with sample spot was placed in TLC chamber with the applied sample above the liquid level. The chamber is closed with a lid.

v. Sufficient time for the migration and development of spots after which the plate was removed and allowed to dry. The bands were observed visually and then exposed to Iodine

2.2.5. GC-MS analysis

The GC-MS analysis of the extract was done with Varian GC-MS –Saturn 2200. The inert gas helium (99.9995%) was used as carrier gas, at flow rate of 1.0 ml/min, Split ratio 20:1; sample size, 1μL injected using the splitless injection procedure; fused capillary silica columnVF5 ms (30 m × 0.25 mm × 0.25 μm). Temperatures: injector: 250˚C, detector: 300˚C, column: 100˚C, 10˚C min−1, 270˚C
(20 min). The overall GC running time was at 63 min.

The MS was performed at 70 eV. The MS scan parameters included a mass range of m/z 40-600, a scan interval of 0.5 s, a scan speed of 2000 amu s−1, and a

detector voltage of 1.0 kV. Identification of constituent compounds was conducted using the database of NIST libraries. Mass spectrum of individual unknown compounds was compared with the known compounds stockpiled in software database libraries. The name, molecular weight and structure of the components of the test materials were determined.

2.2.6. Acute and sub-acute oral toxicity study and calculation of LD_{50} value

2.2.6.1. *Acute oral toxicity study*

In acute toxicity study, *D. gyrans* extract at the dose of 500, 1000 and 2500 mg per kg body weight were orally administered as single dose to male and female Wistar rats, comprising of 6 animals in each group, having body weight ranging from 150-180 grams,. The rats were observed for 14 days for any toxic symptoms such as weakness, loss of appetite, difficulty in movement, response to noise and mortality. Blood was collected from each group and renal and liver function tests were performed in serum. Acute toxicity study was used to determine LD_{50} of *D. gyrans* extract (Lipnick *et al*., 1995).

2.2.6.2. *Sub-acute oral toxicity study*

In sub-acute toxicity study, two doses of *D. gyrans* extract at 150 and 250 mg per kg body weight were given as sub-lethal quantity to male and female Wistar rats with body weight from 150 -180 grams in each group for a continuous period of 28 days. Sub-acute toxicity study was used to determine effect of extract on biochemical parameters of tissues. The body weights of all the rats were documented on a weekly basis throughout the period of experiment. On completion of 28 days of extract treatment the rats were fasted for 8 hours and anaesthetised in chloroform vapour and blood samples were collected by cardiac puncture and subjected to Biochemical analyses. Liver and kidney were collected and subjected to histopathology studies.

2.2.7. Estimation of *in vitro* antioxidant activity

The antioxidant properties of *D. gyrans* were analysed by determining the scavenging effect of the generation of free radicals such as superoxide, DPPH, hydroxyl, ABTS radicals and inhibition of lipid peroxidation in various *in vitro* assay system.

2.2.7.1. *Superoxide radical scavenging activity*

Principle: The superoxide scavenging activity was determined by method of Mc Cord and Fredovich (1969), the Nitro Blue Tetrazolium (NBT) reduction method, which depends on superoxide radical generation by riboflavin upon induction by light. The superoxide radical reduced NBT to formazan complex that is blue coloured, measurable at 560 nm.

Procedure: Different concentrations of the extract as 0, 50, 100, 150, 200, 250, and 300 µg/ ml were added to the reaction mixture containing 0.003 mg NaCN in 0.1 M EDTA (200 µl each); 1.5 mM NBT (100 µl); 0.12 mM riboflavin (50 µl) and 0.6 M SOD buffer (pH 7.8) in to make a final volume of 3 ml. The tubes containing the reaction mixture were illuminated with incandescent lamp, continuously for 20 minutes. The optical density was measured at 530 nm before and after illumination. The optical density of control and extract treated samples were measured and effect of the test material to inhibit superoxide generation was evaluated by comparison.

2.2.7.2. *DPPH scavenging activity*

Principle: The extract of the plant was tested for their scavenging potential against the stable free radical DPPH (2, 2-diphenyl-1-picryl hydrazyl) following the method of Aquino *et al.*, (2001). In this radical form, DPPH has absorption maxima at 515 nm, which gradually disappeared on its reduction. Scavenging of DPPH radical by antioxidant compound will decrease the colour intensity, which can be compared with the control to get the percentage inhibition.

Procedure: Different concentrations of *D. gyrans* extract in the serial range of 0, 50,100,150, 200, 250 and 300 µg/ ml were added to 0.375 ml of freshly prepared DPPH solution in methanol. The volume was made up to 2 ml with methanol. After 20 minutes, the absorbance was measured at 515 nm. The percentage inhibition was calculated and concentration needed for 50% inhibition was found out.

2.2.7.3. *Hydroxyl radical scavenging activity*

Principle: The H_2O_2 scavenging ability of the extract of the plant was determined according to the method of Elizabeth and Rao (1990).The hydroxyl radical attacks

epoxy ribose, which eventually results in formation of thiobarbituric acid reacting substances (TBARS), which is estimated using a spectrometer at 530 nm. The scavenging activity of extract on hydroxyl radical (OH^-) was measured by the Thiobarbituric acid reacting substance (TBARS) method. The scavenging activity was measured by studying the competition for scavenging by deoxyribose and test compounds for hydroxyl radical generated by Fe^{3+} /Ascorbate/ H_2O_2 system (Fenton reaction formation). The hydroxyl radical attacks deoxyribose eventually resulting in the thiobarbituric acid reacting substances (Elizabeth and Rao, 1990).

Procedure: The reaction system contained deoxyribose (2.8 mM), $Fecl_3$ (0.1 mM), KH_2PO_4. KOH buffer (20 mM; pH 7.4) and from 10 µg to 1000 µg/ ml of the test material in a final volume of 1ml. the reaction mixture was incubated for 37^oC for one hour. The scavenging activity of hydroxyl radical was expressed.

2.2.7.4. *ABTS radical scavenging activity*

Principle: The antioxidant effect of the plant extracts was studied by their ability to scavenge the free radical ABTS (2', 2-azino-bis-3-ethyl benzthiazoline 6-sulphonic acid) by the method of Shirwaikar *et al.,* (2006). A ferryl myoglobin radical is formed from metmyoglobin and hydrogen peroxide. The ferryl myoglobin radical can oxidize ABTS (2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) to generate a radical cation, $ABTS^+$, which is green in colour and can be measured at 734 nm. Antioxidants suppress this reaction by electron donation radical scavenging and by inhibiting the formation of the coloured ABTS radical. The concentration of antioxidant in the test sample is inversely proportional to the ABTS radical formation. The radical scavenging activity of methanol extract of *D. gyrans* was determined using ferrrylmyoglobin/ABTS protocol of Rice Evans and Miller (Rice-Evan and Miller, 1994) with some minor modification.

Procedure: Briefly the reaction mixture (2 ml total volume) contained final concentration in a mixture ABTS (150 µM), Mb iii (2.5 µM), extract + PBS (to make up to 2 ml). The reaction was initiated by the addition of H_2O_2 (75 µM) and absorbance of ABTS radical scavenging activity extract was read at 734 nm and calculated the

result from the slopes of reaction curves obtained by plotting the absorbance against time.

2.2.7.5. *Inhibition of lipid peroxidation*

Principle: An *in vitro* model of Wistar rat liver slices were used for the induction of lipid peroxidation with H_2O_2 as an oxidant by method of Bishayee and Balasubramonian (1971). Malondialdehyde (MDA) produced during peroxidation can react with thiobarbituric acid (TBA) reagent to form a pink coloured product which has absorption maximum 532 nm. Lipid peroxidation was induced in rat liver homogenate by method of Bishayee and Balasubramonian (1971) and inhibition in the presence of extract estimated by TBA substance by the method of Ohkawa *et al.,* (1979).

Procedure: Different concentrations of the extract were incubated with 0.2 ml of rat liver homogenate (25%) containing 150 m M KCl, tris buffer (0.2 M, pH 7), ascorbic acid (0.3 Mm) and ferrous ion (0.8 Mm) in a total volume of 0.5 ml for one hour at 37 ^{o}C. After incubation, 0.4 ml of the reaction aliquote was treated with 0.2 ml SDS (8%), 1.5 ml TBA (0.8%) and 1.5 ml acetic acid (20%, pH 3.5). The total volume was made up to 4 ml by adding distilled water and kept in water bath at 100 ^{o}C for one hour. After cooling, 1 ml distilled water and 5 ml butanol: pyridine mixture (15:1, v/v) were added. After vigorous shaking, the tubes were centrifuged and the upper layer containing the chromophore was measured at 532 nm. The lipid peroxidation inhibition was calculated.

2.2.8. Anti-inflammatory activity

For assessing the anti–inflammatory activity, carrageenan induced paw edema (Acute Inflammation) and formalin induced paw edema (Chronic Inflammation) models were employed.

2.2.8.1. *Carrageenan induced paw edema: Protocol*

Female Balb/c mice were divided into five groups comprising five animals in each group. In all groups, acute inflammation was induced by sub plantar injection of

0.02 ml freshly prepared 1% suspension of carrageenan in right hind paw of mice. Group I with carrageenan alone served as positive control and group II was administered with diclofenac (10 mg/ kg body weight) intraperitoneally as standard reference drug. Group III and group IV were treated with *D. gyrans* extract (100 and 250 mg/ kg body weight) orally for five consecutive days. On the fifth day, acute inflammation was induced by sub plantar injection of freshly prepared carrageenan on right hind paw in the entire group. The inflammation was measured using vernier calipers one hour before and for 5 hours after carrageenan injection on an hourly basis.

2.2.8.2. *Formalin induced paw edema: Protocol*

Female balb/c mice were divided into five groups (5 animals/ group). Group I was kept as control and group II was treated with standard drug diclofenac (10 mg/ kg b.wt). Group III and group IV were treated with different concentration of *D. gyrans* extract (100 and 250 mg/ kg body weight) orally for five consecutive days. On the first day, chronic inflammation was induced by sub plantar injection of freshly prepared formalin (0.02 ml of 2% formalin) on the right hind paw in all groups. The inflammation was measured using vernier calipers before and after injection of formalin and for six consecutive days. Increase in thickness as a measure of inflammatory edema calculated using the formula of, $Pt\text{-}P_0$ where P_o is the initial thickness prior to induction. Percent inhibition of inflammation is calculated by the formula P_c - $P_t/P_c \times 100$. Where P_t is the increase in thickness of the treated. P_c is the control. Graph is also plotted using mean and SD calculated.

2.2.9. Antithrombotic and anticoagulant activity

2.2.9.1. *Study groups and sampling for anticoagulant study*

The animals were divided into three groups, normal, standard and extract treated group consisting of 6 male Sprague Dawley rats in each group. The standard group received heparin injected through tail vein at a dose of 250 U/ kg b. wt. of the animal for one day. The extract treated groups received *D. gyrans* extract at a dose of 100 and 250 mg/ kg b. wt. of the animal orally for a continuous period of fourteen days.

2.2.9.2. *Prothrombin time (PTT)*

Principle: The arrest of bleeding depends upon formation of primary platelet plug formed on site of injury followed by the formation of a stable fibrin network. Formation of this clot involves the sequential interaction of a series of plasma proteins and the interaction of these complexes with blood platelet and components released from tissues. Tissue thromboplastin, in presence of calcium initiates the extrinsic pathway of coagulation, which comprises plasma coagulation factors VII, X, V, prothrombin and fibrinogen. During oral anticoagulant therapy specific blood coagulation factors are depressed. Prothrombin time determination is the preferred method for screening anddiagnosis of congenital deficiency of factors II, V, VII and X. PTT is also used for monitoring of patients on oral anticoagulant therapy and as a liver function test.

Procedure: Plasma collected from normal male Sprague Dawley rat 0.1 ml is taken in a glass test tube and incubated at 37 ^{o}C for 5 minutes. Liquiplastin 0.2 ml is mixed with plasma and time is noted instantly. Formation of a visually detectable solid plasma clot occurs inside glass tube within a specified period of time. The time required for clot formation is noted in seconds.The experiment is repeated using heparin treated standard plasma and plasma from *D. gyrans* extract treated group of animals.Tissue thromboplastin in the presence of calcium initiates the extrinsic pathway of coagulation mechanism. When liquiplastin reagent is added to normal plasma, the clotting mechanism is initiated. PTT would be prolonged if there is a deficiency or delay of blood coagulation factor activity in the extrinsic pathway of the coagulation mechanism.

2.2.9.3. *Clotting time*

Principle: Clotting time is used as a screening test to monitor all phases of intrinsic coagulation system and to monitor heparin therapy. The coagulation time of whole blood is the time required for blood to clot under normal standard conditions.

Procedure: Blood was collected from normal rat through the tail vein and was transferred directly to capillary tube. The end of the capillary tube was broken at

regular intervals and clotting time was noted when a fibrin thread appears between broken pieces of capillary tubes. Time taken for clot to appear is noted. Clotting time was also assayed on rats injected with standard dose heparin injected through tail vein as well as on rats fed with *D. gyrans* extract.

2.2.9.4. *Plasma recalcification time*

Principle: Plasma re-calcification time was determined following the addition of M/ 100 $CaCl_2$ solution to the previously warmed plasma at 37 ºC. Platelet rich plasma (PRP) was prepared by centrifugation (1000 rpm × 5 min) of blood collected from normal donors.

Procedure: In tubes marked control, standard and extract treated, 400 μ l of PRP was taken, and these tubes were incubated for 1 minute at 37 ^{o}C. For determining normal plasma recalcification time, 20 μ l of saline was added to one of these tubes followed by 200 μ l of M/100 $CaCl_2$ and a stopwatch was started immediately. The time taken for formation of a firm plasma clot was noted. The experiment was repeated by adding 20

μ l of heparin in tube marked standard, and 20 μ l of *D. gyrans* extract in two concentrations of 100 and 250 μ g in place of saline used in normal control. The procedure was repeated in triplicate to determine the respective plasma re-calcification time. The values were noted in each of the tubes and were compared with the value of the normal control.

2.2.9.5. *Platelet aggregation study- collagen induced*

Principle: Platelet rich plasma (PRP) was prepared by centrifugation (1000 rpm × 5 min) of blood collected from normal donors. Platelet aggregation can be done using specific agents that directly induce aggregation or cause platelets to release endogenous ADP, or both. Platelet aggregation can be induced in vitro using thrombin, ADP, arachidonic acid, epinephrine or collagen and different mediators can be studied for their inhibition of platelet aggregation (Lan *et al*., 2005)**.**

Procedure: In a glass cuvette, 0.45 ml of PRP was taken and incubated with 50 µl of saline. The cuvette was incubated at 37 ºC for 5 minutes without disturbing the content. Platelet aggregation was initiated by adding 1 μ g/ ml of collagen. Aggregation was recorded for every minute continuously for 5 min using spectrophotometer at 340 nm. Aggregation results in a lowering of optical density of the contents in the tube. Decreases in optical density were recorded and a graph was plotted against time taken in minutes. The procedure was repeated substituting 50 μ l of *D. gyrans* extract in place of saline, in two concentrations of 150 and 250 μ g/ ml. The results obtained using normal saline and plant extract as test material were compared.

2.2.9.6. *Lipid peroxidation*

Principle: Malondialdehyde (MDA) produced during peroxidation of membrane lipids by cycloxygenase react with thiobarbituric acid (TBA) reagent to form a pink coloured product which has an absorption maximum at 532 nm. The assay is calibrated with 1,1,3,3, tetramethoxypropane, which on hydrolysis produces malondialdehyde. The results are expressed in terms of the amount of malondialdehyde produced during the reaction.

Procedure: PRP sample (0.1 ml) in Tris buffer was added to a reaction mixture containing KCl (0.1 ml), ascorbic acid (0.1 ml), ferrous ammonium sulphate (0.1 ml) and Tris buffer (0.1 ml). Final volume was 0.5 ml. The reaction mixture was incubated for 1 hr at 37 ºC. To 400 μ l of this reaction mixture added 0.2 ml SDS, 1.5 ml of acetic acid and 1.5 ml TBA and incubated for 1 hr at 95 ºC. After incubation, the reaction mixture was cooled and added 1 ml distilled water. To this mixture 5 ml of butanol-pyridine mixture (15:1, v/v) was added, mixed thoroughly and centrifuged at 3000 rpm for 10 minutes. Absorbance of upper layer containing the chromophore was measured at 532 nm against pyridine-butanol mixture. In control sample, 0.1 ml of PRP sample was added to a reaction mixture containing KCl (0.1 ml), ascorbic acid (0.1 ml), ferrous ammonium sulphate (0.1 ml) and Tris buffer (0.05 ml). In addition 0.05 ml collagen was also added (Instead of 1 ml of Tris buffer added in normal sample tube, 0.05 ml of Tris buffer and 0.05 ml of collagen were added in the control sample tube). In test solution 0.05 ml of drug extract of two different concentrations of 100 and 250 μ g/ ml were added in reaction mixture, in place of Tris buffer and 0.05 ml collagen was also

added in each tube. All the procedures were done in triplicate. The amount of MDA formed was expressed as n mol/ mg protein.

2.2.10. Cardioprotective study

2.2. 10.1. *Doxorubicin induced cardiomyopathy in rats: Protocol*

The study was conducted using male Sprague Dawley rats (150-200 g). Animals were randomly divided into groups I, II, III, IV and V of six each. Group I was maintained as normal and given saline intraperitonial injection from 8^{th} day of experiment. Group IV and V received *D. gyrans* extract orally at a dose of 100 and 250 mg/ kg b. wt. of the animal respectively, for a period 15 days and group III received standard probucol orally at a dose of 30 mg/ kg b. wt. of the animal for the same period of time. Group II was designated as positive control and groups II, III, IV and V received doxorubicin as intraperitonial injection on alternate days from 8^{th} day onwards to give a cumulative dose of 24 mg/ kg b. wt. of the animal. At the end of the experiment all overnight fasted animals were sacrificed, blood and heart were collected and properly processed for further analysis.

2.2. 10.2. *Isoproterenol induced myocardial infarction in rats: Protocol*

The study was conducted using male Sprague dawley rats (150-200 g). Animals were randomly divided into groups I, II, III, IV and V of six each. Group I was maintained as normal. Group IV and V received *D. gyrans* extract at a dose of 100 and 250 mg/ kg b. wt. of the animal respectively, for a period 21 days and group III received standard probucol at a dose of 30 mg per kg body weight of the animal for the same period. Group II was designated as positive control and groups II, III, IV and V received isoproterenol intra peritoneal injection on the 20^{th} and 21^{st} days to give cumulative dose of 85 mg/ kg b. wt. of the animal. At the end of the experiment all overnight fasted animals were sacrificed, blood and heart were collected and properly processed for further analysis.

2.2. 10.3. *Biochemical analysis*

Serum was used for analysis of cardiac enzymes namely creatine phosphokinase, lactate dehydrogenase and aspartate aminotransaminase using standard

commercial kits in fully automated analyzer EM 360 of Transasia Biomedicals. Liver and renal functions were also studied.

Estimation of creatine phosphokinase (CPK)

Principle: UV kinetic estimation of CPK was done based on the following reactions (Di. Witt *et al*., 1982)

Creatine phosphate + ADP $\xrightarrow{CPK}$ Creatine + ATP

ATP+ D Glucose $\xrightarrow{HK}$ G-6-P + ADP

G-6-P + $NADP^+$ $\xrightarrow{G\text{-}6\text{-}PDH}$ D-Glucose 6-P + NADP + H^+

HK- Hexokinase; G-6 PDH- Glucose 6 phosphate dehydrogenase

Estimation of lactate dehydrogenase (LDH)

Principle: UV kinetic estimation of LDH was done based on the following reactions (Wei Bhaar *et al*., 1975)

Pyruvate + NADH + H^+ $\xrightarrow{LDH}$ L-Lactate + NAD^+

Estimation of SGOT(AST)

Principle: UV kinetic estimation of SGOT was done based on the following reactions (Thefeld *et al*., 1974)

L- Asparate + alpha - ketoglutarate $\xrightarrow{AST}$ Oxaloacetate + L-Glutamate.

Oxaloacetate + NADH + H^+ $\xrightarrow{MDH}$ L- Malate + NAD^+

AST: Aspartate aminotransferase.
MDH : Malate dehydrogenase.

Estimation of SGPT(ALT)

Principle: UV kinetic estimation of SGPT was done based on the following reactions (Thefeld *et al*., 1974)

L-Alanine + alpha-ketogutarate $\xrightarrow{SGPT}$ Pyruvate + L-Glutamate

Pyruvate + NADH+ H^+ $\xrightarrow{LDH}$ L-Lactate + NAD^+

ALT – Alanine aminotranferase
LDH - Lactate dehydrogenase

Estimation of urea

Principle: Estimation of urea was done by GLDH method on the basis of the following reactions (Kassirer *et al*., 1971)

Urea + H_2O $\xrightarrow{Urease}$ $2NH_3 + CO_2$

$2\ NH_3 + 2\text{- ketoglutarate} + 2NADH \xrightarrow{GLDH} \text{L-Glutamate} + 2NAD^+ + 2H_2O$

Estimation of creatinine

Estimation of craetinine was done by Jaffe kinetic method on the basis of the following principle (Di. Witt *et al*., 1982).

Principle: Picric acid in an alkaline medium reacts with creatinine to form an orange coloured complex with the alkaline picrate. Intensity of the colour formed during the fixed time is directly proportional to the amount of creatinine present in the sample.

Creatinine + Alkaline Picrate ⟶ Orange Coloured Complex

2.2. 10.4. *Studies on cardiac tissue*

Fragments of heart were placed in 10% buffered formaldehyde. Later they were embedded in paraffin, cut and stained with hematoxylin and eosin stain and observed under optical microscope for histopathological findings. For antioxidant assay, heart tissue was removed and weighed and 3 ml of 0.1M Tris buffer was added per 1g of the tissue and homogenised. Both enzymatic and non-enzymatic antioxidants as well as lipid peroxidation assays were carried out using the tissue.

2.2.10.5. *Tissue preparation for mitochondrial isolation*

The heart was removed, washed in ice cold isotonic saline, dried using filter paper, weighed and homogenized in 0.25 M sucrose phosphate buffer at 4 °C. Sucrose phosphate buffer was prepared by dissolving 17.12 g sucrose in 200 ml of 0.1 M phosphate buffer adjusted to pH 7.0. The homogenate was centrifuged for 10 min at 600 rpm at 4 °C, nuclear and cytoskeleton fractions were discarded. The supernatant was centrifuged for 20 min at 12000 rpm at 4 ^{o}C to pellet mitochondria (Johnson, 1967). The mitochondria were re-suspended in phosphate buffer to a known volume of 2 ml. Vortexed and centrifuged again at 12,000 rpm for 20 min at 4 °C. The procedure was repeated and finally the mitochondrial pellet was suspended in 1 ml phosphate buffer and 0.5 ml Tween 80 and homogenized mildly to break the mitochondrial membrane. The resulting mitochondrial homogenate was used for assay of SOD, GPx, GSH and lipid peroxidation (Sudheesh *et al*., 2009).

2.2.17. Lipoprotein lipase (LPL) activity

The lipoprotein lipase releasing activity of the extract was determined by the method of Korn (1962). Triglyceride rich plasma was used as substrate. It was made to react with

plasma (as LPL enzyme source) of extract administered rats and the glycerol liberated durig experiment was compared with that of the plasma of normal and control rats.

2.2.17.1. ***Reagent for incubation***

0.5 M $(NH_4)_2$ SO_4
20% serum albumin (Bovine fraction V, Armours): adjusted to pH 8.5 with NaOH
Substrate: Serum from hypertriglyceridemic persons was used having a concentration of 400 mg of triglyceride per 100 ml.
Enzyme: Normal plasma, post heparin plasma and plasma from animals treated with *D. gyrans* extract was employed as enzyme source.

2.2.17.2. ***Reagents for glycerol determination:***

0.05 M Sodium periodate: 11.4 g of $HIO_4.2H_2O$ was dissolved in 900 ml of water, neutralised to methyl red with 1N NaOH and water was added to give a final volume of 1 Litre.

0.5 M Sodium arsenite: 22.5 g of NaOH and 50 g of As_2O_3 were dissolved in 1 Litre of water

1 N H_2SO_4
Chromotropic acid: 1 g of chromotropic acid was dissolved in 100 ml of water and added to 400 ml of sulphuric acid. The reagent was kept cooled throughout the mixing procedure. This reagent should always be prepared fresh.

2.2.17.3. ***Incubation procedure****:* Mixed 0.4 ml of albumin, 0.1 ml of $(NH_4)_2$ SO_4, 0.1 ml of substrate in a series of tubes. To the tubes thus arranged added 0.1 to 0.4 ml of enzyme followed by sufficient quantity of water to make a final volume of 1 ml and incubated at 37 ºC for 90 minutes, at the same time removing aliquots of 0.05 ml at 30 minutes intervals. The reagent was kept cold when mixed and the first sample was measured and taken as initial sample, before keeping the tubes for incubation. The transferred aliquots were kept in a 10 ml conical tip centrifuge tube containing 0.1 ml of 1N H_2SO_4. These can be kept at room temperature for several hours or frozen indefinitely before glycerol determination is performed.

2.2.17.4. ***Glycerol determination***: Added 0.1 ml each of periodate to the transferred aliquots kept in 10 ml conical tip centrifuge tubes containing 0.1 ml of 1 N H_2SO_4,

mixed well and let stand at room temperature for exactly 5 minutes. Then added 0.1 ml of arsenite, mixed well and let stand at room temperature for at least ten minutes. Added 9 ml of chromotropic acid mixed by inversion and placed in boiling water bath for 30 min. It has been found convenient to cap the tubes with a marble. Cooled and adjusted the volume to 10 ml with water and read the optical density at 570 nm. The assay was standardised against a glycerol solution of known molarity. The standard range is from 0.005 to 0.1 micromole of glycerol.

Glycerol standard: Glycerol of known concentration was used as standard for assaying unknown quantity of glycerol liberated in the aliquot tubes. 100 mg of glycerol was accurately weighed and dissolved in 100 ml of distilled water to give 100 mg/ 100 ml of glycerol. This stock glycerol solution was diluted to give a series of glycerol concentrations of 1 mg, 2 mg, 4 mg, 8 mg, 10 mg and 12 mg. 0.050 ml of these standard glycerol solutions were pipette out and the procedure followed for glycerol determination was performed. A standard curve was prepared to find out the concentration of glycerol in test solutions.

2.2.18. Lipid clearing experiment: Protocol

New Zealand male white rabbits (1-1.5 kg) used for the experiment, were divided into 4 groups of 6 each. The first group was kept as normal, second was given a single dose of 1mg/ kg b.wt of heparin (5000 IU/ ml) and used as standard. The third and fourth groups were orally fed with 100 and 250 mg/ kg b.wt. of *D. gyrans* extract respectively for a continuous period of 14 days. At the end of the experiment, blood were drawn from peripheral ear vein of all groups and centrifuged (3000 rpm × 5 min) to separate plasma and used for assay. The lipoprotein lipase releasing activity of the extract was determined by the method of Korn (1962) as explained above. The glycerol liberated was compared with that of normal and control samples.

2.2.11. Preparation of high fat diet

Standard High Fat Diet (HFD) was made using Bengal gram, sucrose, milk powder, raw cholesterol, hydrogenated fat, salt mineral mixture and egg yolk in the amounts mentioned in table 7.1, which formed nutrients approximately in the following proportions: 50%

carbohydrates, 25% proteins and 15% fats (Seethanathan *et al*., 1970). Bengal gram was made into flour and mixed with all other constituents and consistency was regulated by adding minimum amount of sterilized water. Sodium taurocholate was added to HFD to enhance emulsification process.The mixture was made into pellets mechanically and dried in oven at 40°C for three days. The perfectly dried pellets were kept closed in containers to avoid fungal infections and used within one week. HFD was given to selected group of animals at the rate of 100 g/ kg body weight along with oral feeding of cholesterol at 250 mg/kg body weight on alternate days for a period of three months.

2.2.12. Hypolipidemic study on rats: Protocol

The study was conducted using male Wistar rats (150-200 g). Animals were divided into five groups A, B, C, D and E of six each. Group A was fed normal rat diet and maintained as normal group. Groups B, C, D and E received HFD at the rate of 100 g/ kg b. wt. of the animal along with oral feeding of 250 mg/ kg b. wt. of cholesterol dissolved in 5 ml coconut oil on alternate days (Li X *et al*., 2016; Suresh et al., 2017) for a period of 3 months. Among these, group B was treated as control (HFD alone) and group C received standard drug atorvastatin (5 mg/ kg b. wt. /alternate day). *D. gyrans* extract was administered in groups D and E in doses of 100 and 200 mg/ kg b. wt. by oral intubation on alternate days for a period of 3 months and water was given to all groups *ad libitum*. At the end of the experiment, all overnight fasted animals were sacrificed and lipid profile was estimated using commercially available standard kits.

2.2.13. Anti-atherogenic study on rabbits: Protocol

Anti-atherogenic study was conducted using male New Zealand white rabbits (1.75-2.0 kg). Animals were divided into four groups A, B, C and D of six each. Group A was maintained as normal. Groups B, C and D received HFD at the rate of 100 g/ kg b. wt. of the animal along with oral feeding of 250 mg/ kg b. wt. of cholesterol dissolved in 5 ml coconut oil on alternate days (Li X *et al*., 2016; Suresh et al., 2017) for 3 months. Among these, group B was treated as control (HFD alone) and *D. gyrans* extract was administered in group D (250 mg/ kg b. wt. /alternate day) by oral intubation for a period of 3 months. Group C received standard drug probucol (10 mg/ kg b. wt. /alternate day) for 3 months and water was given to all groups *ad libitum*. Probucol is a drug having both antioxidant and hypolipidemic properties which render it cardioprotective activity. Its anti-atherogenic

effect on hypercholesterolemic rabbits has also been demonstrated (Oshima *et al*., 1998; Witting *et al*., 2000). At the end of the experiment, all overnight fasted animals were sacrificed under ether anaesthesia; blood, liver as well as heart with attached aorta extending to the iliac bifurcation were collected and used for the study.

2.2.14. Biochemical analysis

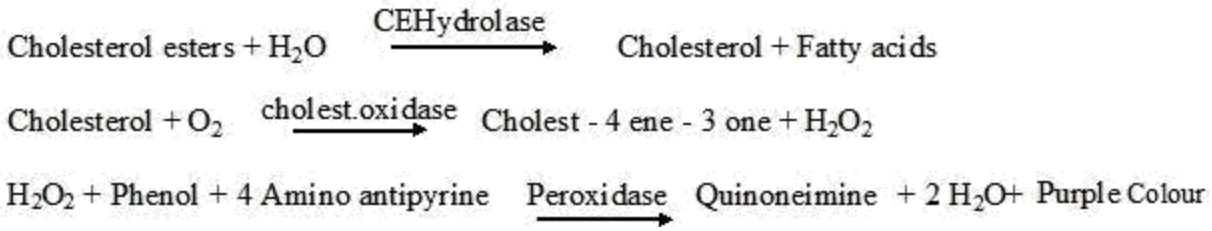
Serum was used for analysis of total cholesterol, triglycerides and lipoproteins using standard commercial kits in fully automated analyser EM 360 of Transasia biomedicals. Liver and renal functions were also analysed. Estimation of serum malondialdehyde (MDA) was done by Valipasha and Sadasivudu's procedure.

2.2.14.1. *Estimation of cholesterol*

Cholesterol was estimated by cholesterol oxidase - peroxidase method using the diagnostic kit purchased from Agappe diagnostics (Allain *et al*., 1974). The principle behind CHOD-POD method is illustrated below

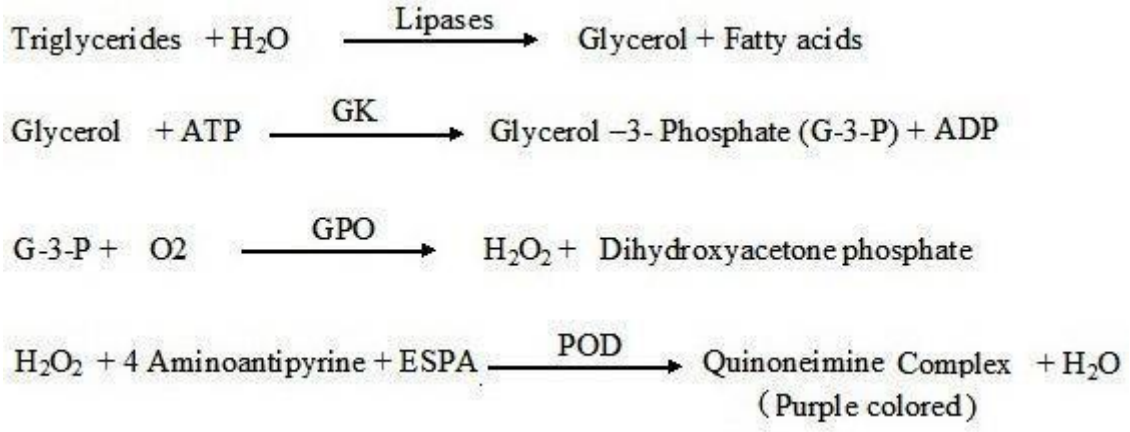
2.2.14.2. *Estimation of HDL cholesterol*

HDL Cholesterol was measured by direct measurement without precipitation. Non HDL fractions in the serum sample were masked by use of antibodies or complexing agents like cyclodextrin and the cholesterol fraction which is left in the solution was estimated by CHOD-POD method as illustrated above



2.2.14.3. ***Estimation of triglycerides***

Triglycerides in the sample was estimated by GOP- POD method using the diagnostic kit purchased from Agappe diagnostics, (Jacobs *et al*., 1960) the principle of which is illustrated below



GK- Glycerol kinase; GPO- Glycerol peroxidase; POD- Peroxidase
ESPA - Sodium N-ethyl-N-(3-sulfopropyl) m-anisidine

2.2.14.4. ***Measurement of apolipoprotein A-I and serum hs-CRP***

Apolipoprotein A-I and serum hs-CRP were determined by quantitative turbidimetric immunoassay based on principle of agglutination reaction using turbidimetric reagent kit provided by Tulip chemicals.

Determination of Apo A-I: The Apo A-I assay is an immunoturbidimetric procedure that measures increasing sample turbidity caused by the formation of insoluble immune complexes when antibody to ApoA-I is added to the sample (Velez et al., 1999). Sample containing ApoA-I is incubated with a buffer and a sample blank determination is performed prior to the addition of ApoA-I antibody. In the presence of an appropriate antibody in excess, the ApoA-I concentration is measured as a function of turbidity.

Determination of hs-CRP: Latex particles coated with antibody specific to human CRP aggregate in the presence of CRP from the sample forming immune complexes (Singer et al., 1956). The immune complexes cause an increase in light scattering which is proportional to the concentration of CRP in the serum. The light scattering is measured by

reading turbidity (absorbance) at 570 nm. The CRP concentration is determined from a calibration curve developed from CRP standards of known concentration.

2.2.14.5. ***Measurement of serum paraoxonase activity***

Serum paraoxonase activity was measured by comparison of the kinetic rate of change of absorbance of reactions. The assay tube contained 750 μL of Tris buffer, 50 μL of serum (1:2 dilutions) and 200 μL of 6 mmol/L paraoxon and the formation of *p*-nitrophenol was followed measuring its rate of change of absorbance at 405 nm for 5 min (Tomas *et al*., 2000). Paraoxon was purchased from Sigma Aldrich.

2.2.14.6. ***Estimation of HMG-CoA reductase enzyme***

Indirect measurement of hydroxylmethylglutaryl-CoA reductase (HMG-CoA reductase) enzyme which catalyses the committed step in cholesterol biosynthesis was done by method described by Rao and Ramakrishnan (1975). 3-hydroxy 3-methyl glutaryl CoA (HMG-CoA) and mevalonic acid concentrations in liver tissue homogenate were measured by the absorbance and the ratio between them was calculated. The ratio indicates the activity of the enzyme in the given sample. HMG-CoA reductase catalyses the reaction of converting 3-hydroxy 3-methyl glutaryl CoA to mevalonic acid, and any measured increase in the ratio indicate an enhanced concentration or accumulation of 3-hydroxy 3-methyl glutaryl CoA. A high ratio indicates a lower activity of the enzyme in the sample and so a reduced biosynthesis of cholesterol. On the other hand any decrease in the ratio indicates a high rate of biosynthesis of cholesterol.

2.2.14.6.1. ***Reagents preparation for HMG-CoA reductase enzyme measurement***

Hydroxyl amine hydrochloride reagent, 2 mol/ litre: In two different pH for estimation of HMG-CoA and mevalonate separately.

i. Hydroxyl amine hydrochloride reagent for mevalonate, pH 2.1

   Mix equal volume of hydroxylamine hydrochloride and distilled water freshly before use and adjust pH to 2.1

ii. Hydroxyl amine hydrochloride reagent for HMG-CoA, pH 5.5

Mix equal volume of hydroxylamine hydrochloride and sodium hydroxide freshly before use and adjust pH to 5.5

iii. Ferric chloride reagent: dissolve 5.2 g of trichloro acetic acid and 10 g of ferric chloride in 100 ml of 0.65 mol/ litre hydrochloric acid

iv. Dilute perchloric acid: 50 ml of concentrated perchloric acid was diluted to 100 ml using distilled water

2.2.14.6.2. *Procedure for estimation*:

HMG-CoA measurement: Mix equal volume of fresh 10% tissue homogenate and diluted perchloric acid. Allowed to stand for 5 minutes and centrifuged at 1000 rpm for 10 mnts. Treated 1ml of filtrate with 0.5 ml of freshly made hydroxyl amine reagent. Hydroxylamine of pH 5.5 is used for HMG CoA. Mixed well, and after 5 mnts added 1.5 ml of ferric chloride reagent and mixed again. HMG CoA forms hydroxaminic acid and form complexes with ferric salt. Incubated for 10 mnts and readings are taken at 540 nm against reagent blank. Mevalonate interferes with this assay at very low pH only.

Mevalonate measurement: Mix equal volume of fresh 10% tissue homogenate and diluted perchloric acid. Allowed to stand for 5 minutes and centrifuged at 1000 rpm for 10 mnts. Treated 1ml of filtrate with 0.5 ml of freshly made hydroxyl amine reagent. Hydroxylamine of pH 2.1 is used for HMG CoA. At this pH, the lactone form of mevalonate readily reacts with hydroxylamine to form hydroxamate. Mixed well, and after 5 mnts added 1.5 ml of ferric chloride reagent and mixed again. Incubated for 10 mnts and readings are taken at 540 nm against reagent blank.

2.2.15. Studies on liver and heart morphology and histopathology

Visual observations of liver and heart were made and photographs were taken. Fragments of liver and cardiac tissues were placed in 10% buffered formaline, which was prepared by adding 50 ml of 37% formaldehyde to 450 ml of distilled water into which 3.25 g dibasic sodium phosphate and 2 g monobasic sodium phosphate were added. 24 hours later tissue were embedded in paraffin, cut and stained with

haematoxylin and eosin (H and E) stain and observed under microscope. For antioxidant assay on liver tissue, 1 g of liver was weighed and 3 ml of 0.1 M Tris buffer was added to it and homogenised using homogeniser equipment and both enzymatic and non-enzymatic antioxidant assays as well as lipid peroxidation were performed.

2.2.16. Morphology of oil red O stained aorta

Oil red O (Sigma Aldrich) in the powdered form was dissolved in isopropyl alcohol. The excised aorta was cleaned using saline, cut opened with sterile surgical blade and treated with 10% buffered formalin and then washed with 60% isopropyl alcohol. The aorta was stained with oil red O and fatty streaks which appeared red were photographed.

2.2.19. Estimation of liver tissue lipids (Janet *et al*., 1978)

One gram of liver tissue was extracted with 2 ml of chloroform: methanol solvent mixture taken in the ratio 2:1. The extract was centrifuged for 30 mnts at 10,000 rpm. The supernatant was transferred to test tube taking care to avoid pellet. The supernatant is then exposed to 50 °C for 2 hrs to remove traces of chloroform and assayed for total cholesterol by CHOD-POD method of estimation of cholesterol.

2.2.19. Statistical analysis

The invitro assays were carried out three times with duplicate tubes and values were the average of six readings.All values in the study are represented as the mean ± standard deviation (SD) of six animals per group. The data were subjected to one way analysis of variance (ANOVA) and comparison was made by *Dunnet post hoc* test, turkey - Kramer multiple comparisons, using Graphpad instat version 3.5 software. Difference between groups of animals were considered to be significant if the $p < 0.01$ (denoted by a or c) and $p < 0.05$ (denoted by b or d),. Statistical difference of $p > 0.05$ was considered as not significant (ns).

Chapter 3

Phytochemical analysis, Antioxidant and Anti-inflammatory Effects of Desmodium gyrans DC

3. Phytochemical analysis, Antioxidant and Anti-inflammatory Effects

3. Phytochemical analysis, Antioxidant and Anti-inflammatory Effects

3.1. INTRODUCTION

Desmodium gyrans DC has been used in traditional medicine as a cardioprotective herbal plant and there is not much scientific literature supporting the medicinal use of the plant (Gopalakrishnan and Rajameena, 2012). The current research work which probes the antiatherosclerotic effects of the plant is also targeted to assess the properties that contribute to it. The basic study for detecting the presence of major phytochemicals which may contribute to the medicinal properties was conducted. The preliminary study is aimed to perform phytochemical screening and evaluate the antioxidant and anti-inflammatory effects of *Desmodium gyrans.*

The medicinal importance of plants comes from the presence of phytochemicals belonging to different classes. Phytochemicals detected or isolated in medicinal plants has beneficial effects on long-term well-being when used up by humans, and can be used to successfully treat human diseases. At least 12,000 such compounds have been isolated or known so far, which accounts for less than 10% of the total compounds that are actually existing (Lai et al., 2004). Secondary metabolites like phenolic compounds, glycosides, alkaloids etc., which are produced by plants is found to exert physiological and therapeutic effects. The constituents responsible for medicinal property in plants are generally these secondary metabolites. A systematic phytochemical analysis of a crude drug involves identification and documentation of secondary metabolites formed as a product of regular plant metabolism. In phytochemical analysis, the plant material, either as a whole or as parts, is subjected to phytochemical screening for the discovery of plant constituents. Taking into consideration these facts, the present study was designed to scrutinize the occurrence of various phytochemicals in *Desmodium gyrans* extracts.

Despite the fact that the apparent benefits and resultant demand of herbal drugs are on the rise, there is also a growing concern associated with the safety of using them

(Ertekin *et al*., 2005; Y. Saidu *et al*., 2010). Not much of the herbal products in the market have gone through the standardised procedures for assessment of their health hazards they may cause. This is especially significant in that in most of these products, the main principles which impart the assumed medicinal effect are not clearly known (Monika *et al*., 2013). Toxicity testing is done to reveal some of the threats that may be related to the use of herbs, which may help avoid potential harmful effects when they are employed as medicine especially in sensitive populations. Toxicity testing is important in that the procedure may help detect monitor the drug recovery process as also to set the safe dosage limits. The present study was aimed to determine LD50 and acute and sub-acute toxicity as per Organization for Economic Cooperation and Development (OECD) guidelines (Abrar *et al*., 2013).

There is now extensive biochemical, physiological and pharmacological substantiation to confirm relation between free radicals and cardiovascular tissue injury. Evidences suggest that disease conditions are directly or indirectly related to oxidative damage and that they have common means of molecular and cellular damage. Reactive oxygen or oxidant species (ROS) take part in normal cell signaling as mediators that control vascular function (Maulik *et al*., 2002). In the vascular wall, ROS are formed by all layers of blood vessel, including endothelium, smooth muscle, and adventitia (Lassegue *et al*., 2003). The loss of control of free-radical generation right from the mitochondrion can cause cardiovascular diseases through multiple mechanisms. One of the main concepts of free radical mediated onset of cardiovascular disease is endothelial dysfunction, which leads to the disruption of vascular wall microenvironment regulation (Shinyashiki *et al*., 2004). Oxidative stress also leads to oxidation of LDL (ox-LDL), which will be more easily uptaken by macrophages when compared to non-oxidized lipoproteins (Sanjib *et al*., 2011). Macrophages and smooth muscle cells in atherosclerotic vessels have been proven to be the main sources of oxidative substances and ROS (Antoniades *et al*., 2007).

Metabolic reactions taking place in our cells generate reactive oxygen species which are capable of critically damaging biomolecular structures (Pinn *et al*., 2000). In recent times one of the areas which have attracted a great deal of attention is

antioxidant research and management of diseases arising as a result of free radical stress. Although free radicals like superoxide radicals, hydrogen peroxide and highly reactive hydroxyl radicals and others are important in processes like phagocytosis, cell signaling and energy production,(Maryam *et al*., 2009) these can also lead to a variety of acute and chronic diseases including diabetes, cardiovascular disorders, cancer and a number of inflammatory diseases (Pham-Huy *et al*., 2008). The presence of safe and bioactive natural antioxidants which can act as scavengers of free radicals raises a substantial interest in deploying medicinal plants in curing of these diseases.

Many medicinal plants having antioxidant properties have been reported to have anti-inflammatory activity also (Surh *et al*., 2008, Vinegar *et al*., 1987). The carrageenan-induced paw edema in rat is most widely used for the screening of promising anti-inflammatory agents. Carrageenan is the phlogistic agent of choice for testing anti-inflammatory drugs as it is not known to be antigenic and is devoid of apparent systemic effects. Formalin induced paw edema in rats is widely used for screening potential anti-inflammatory agents. Formalin induced chronic inflammation and edema is also biphasic with an early neurogenic component involving release of bradykinin being followed by a later tissue-mediated response involving release of histamine, 5-hydroxytryptamine and prostaglandin.

For assessing the anti-inflammatory activity, carrageenan induced paw edema (acute inflammation) and formalin induced paw edema (chronic inflammation) model were employed. Inflammation is involved at all stages of atherosclerosis and is detected as a component of all forms of plaques (Ross 1999; Hansson *et al*., 2005). Moreover, a topographic correlation among an inflammatory infiltrate, plaque rupture and thrombosis has also been established (VanDer *et al*., 1994). Proinflammatory mediators for instance, interleukins and cytokines induce vascular cell growth and atherogenesis (Hansson *et al*., 2005), while inhibition of their inflammatory pathways reduce cell growth and atherosclerosis (Mach *et al*., 2004; Hansson *et al*., 2006). Because of these reasons atherosclerosis is viewed a vascular inflammatory process and the **"**infiltrative character**"** of atherosclerotic lesions have been noticed in cholesterol-fed animals

(Anitschkow *et al*., 1933). Hence screening of the extract for its anti-inflammatory properties is of significant in finding out the contribution that it make.

3.2. MATERIALS AND METHODS

3.2.1. *Preliminary phytochemical screening*

As reported in table 1, whole plant of *D. gyrans* was extracted with different solvents such as petroleum ether, toluene, chloroform, ethyl acetate, acetone, methanol and water and the extracts were separately analysed for the the presence of phytochemical constituents like phytosterols, terpenoids, flavonoids, phenols, saponins, alkaloids and tannins. Methanolic extract of *D. gyrans* showed the presence of alkaloids, sterols, terpenoids, tannins, flavonoids and phenolic compounds. Ethyl acetate extract, chloroform extract and acetone extract also showed the presence phytochemical constituents. The result suggested methanol as the most efficient solvent for extraction of phytochemical constituents from *D. gyrans* whole plant, when compared to other selected solvents. Petroleum ether extract provided the least number of extractable phytochemicals.

3.2.2. ***Phytochemical Analysis*** (chapter 2, section 2.2.1.)

The following phytochemical were analysed using *D. gyrans* extract (chapter 2, section 2.2.1.).

i. Alkaloids : Dragendorff's test
ii. Phytosterols: Libermann and Barchards test
iii. Tannins: $FeCl_3$ test
iv. Terpenoids: Salkowski test
v. Flavonoids: NaOH test
vi. Phenolic compounds: Folin Ciocalteu test

3.2.3. ***Preparation of the extract***

Subsequent to the results of extraction using different solvents, for further experiments *D. gyrans* extract was made in 70% methanol in water from powdered and dried entire

plant using soxhlet apparatus (Methods and materials chapter 2.1.4). For analytical purpose 1 milligram of the solvent free extract was dissolved in 1 ml of distilled water taking care not to leave any quantity of it undissolved.

3. 2.4. Determination of total flavonoid content

Colorimetric aluminum chloride method was used for determination of total flavonoid content (Nabavi *et al.,* 2008; Ebrahimzaded *et al.,* 2009). Exactly 0.5 ml (1 mg/ ml) solution of plant extract was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminumchloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water, and kept at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm using spectrophotometer. Total flavonoid contents were calculated from a calibration curve using quercetin as standard. Quercetin solutions at serial concentrations 12.5, 25, 37.5, 50, 62.5, 75, 87.5 and100 mg/ ml in methanol were prepared. These standards were run using the same procedure and standard curve was prepared. Total flavonoid values are expressed in terms of quercetin equivalent (QE).

3. 2.5. Determination of total phenolic content

Total phenolic compound contents were determined by the Folin-Ciocalteau method (Ebrahimzaded *et al.,* 2008; Nabavi *et al.,* 2008). Exactly 0.5 ml of extract (1 mg/ ml) was added to 5 ml pre diluted Folin Ciocalteu reagent (1:10 diluted with distilled water), mixed for 5 min and then 4 ml aqueous Na2CO3 (1 M) was added. The mixture was allowed to stand for 15 min and the phenols were determined by colorimetrically at 765 nm. Thestandard curve was prepared using 0, 50, 100, 150, 200 mg/ ml solutions of Gallic acid in methanol and water solvent (50:50, v/v). Total phenol values are expressed in terms of Gallic acid equivalent (GA).

3. 2.6. Thin layer chromatography (chapter 2, section 2.2.4.)

TLC is a type of adsorption chromatography used to detect the presence of phytochemicals, like alkaloids, flavonoids, terpenoids and phenolic compounds. It is a semi quantitative method. Principle is similar to other chromatographic methods, and

separation depends on the relative affinity of compounds towards either stationary or mobile phase. The compounds with higher affinity to stationary phase move slowly while the others move faster. The components in the mixture are separated and their character is identified by suitable detection techniques.

3. 2.7. GC-MS analysis

The GC-MS analysis of the extract was done with Varian GC-MS – Saturn 2200. The inert gas helium (99.9995%) was used as carrier gas, at flow rate of 1.0 ml/min, Split ratio 20:1; sample size, 1μL injected using the splitless injection procedure; fused capillary silica columnVF5 ms (30 m × 0.25 mm × 0.25 μm). Temperatures: injector: 250˚C, detector: 300˚C, column: 100˚C, 10˚C min−1, 270˚C (20 min). The overall GC running time was at 63 min.

The MS was performed at 70 eV. The MS scan parameters included a mass range of m/z 40-600, a scan interval of 0.5 s, a scan speed of 2000 amu s−1, and a detector voltage of 1.0 kV. Identification of constituent compounds was conducted using the database of NIST libraries. Mass spectrum of individual unknown compounds was compared with the known compounds stockpiled in software database libraries. The name, molecular weight and structure of the components of the test materials were determined.

3.2.8. *Acute toxicity study* (chapter 2, section 2.2.5.1.)

In acute toxicity study, *Des. gyrans* extract at the dose of 500, 1000 and 2500 mg per kg body weight were orally administered as single dose to test group male and female Wistar rats, comprising of 6 animals with body weight from 150 -180 grams in each group (Table 3.3) to determine immediate toxic effect (Kulkarni , 1993; Lipnick *et al*., 1995).

3.2.9. *Sub-acute toxicity study* (chapter 2, section 2.2.5.2.)

In sub-acute toxicity study, two doses of drug 100 and 250 mg per kg body weight were given as sub-lethal quantity to male and female Wistar rats with body weight from 150 -180 grams in each group (Table 3.4), for a continuous period of 28

days. Sub-acute toxicity study was used to determine effect of drug on Biochemical parameters of tissues (Abrar *et al*., 2013).

3.2.10. *Assessment of body weight*

Body weight individual male and female rats belonging to different groups were documented before the administration of drug on 1st day of the study and thereafter on the 7^{th}, 14^{th}, 21^{st} and 28^{th} days of the experiment. Alterations in the body weight were recorded and compared with that of the normal animals.

3.2.11. *Haematological profile*

Blood was analysed for parameters such as heamoglobin, RBC and Platelet using automated Haematology analyser. Total white blood cells (WBC) were measured. Differential count was done for which slide was prepared with Leishman's stain.

3.2.12. *Determination of biochemical parameters*

Biochemical parameters including serum total protein, serum liver specific enzymes namely aspartate aminotranferase (AST) and alanine aminotransferase and alkaline phosphatase were determined using commercial colourimetric kits provided by Transasia Biochemical Ltd. Blood urea and serum creatinine was estimated as a measure of monitoring injuries to kidney.

3.2.13. *Histopathological study*

After sacrificing the rats, portions of the liver and kidney tissues were collected for histopathological studies. The tissues were washed in normal saline and fixed immediately in 10% formalin for a period of at least 24 h, dehydrated with alcohol, embedded in paraffin, cut and stained with haematoxylin-eosin dye for photomicroscopic observation. The microscopic features of the organs of male and female rats were observed under magnification of 400 X and compared with the normal group.

3.2.14. Study of *in vitro* antioxidant activity

The antioxidant properties of 70% methanol in water extract of *D. gyrans* were analysed by determining the scavenging effect of the generation of free radicals such as superoxide, DPPH, hydroxyl, ABTS radicals and inhibition of lipid peroxidation in various *in vitro* assay system.

3.2.15. *Carrageenan induced paw edema: Acute anti- inflammatory study*

Female Balb/c mice were divided into five groups comprising five animals in each group. In all groups, acute inflammation was induced by sub plantar injection of 0.02 ml freshly prepared 1% suspension of carrageenan in right hind paw of mice. Group I with carrageenan alone served as positive control and group II was administered with diclofenac (10 mg/ kg b.wt) intraperitoneally as standard reference drug. Group III and group IV were treated with *D. gyrans* (100 and 250 mg/kg b.wt) orally for five consecutive days. On the fifth day, acute inflammation was induced by sub plantar injection of freshly prepared carrageenan on right hind paw in the entire group. The inflammation was measured using vernier calipers one hour before and for 5 hours after carrageenan injection on an hourly basis.

3.2.16. *Formalin induced paw edema: Chronic anti- inflammatory study*

Female balb/c mice were divided into five groups (5 animals/ group). Group I was kept as control and group II was treated with standard drug diclofenac (10 mg/ kg b.wt). Group III and group IV were treated with different concentration of *D. gyrans* (100 and 250 mg/ kg b.wt) orally for five consecutive days. On the first day, chronic inflammation was induced by sub plantar injection of freshly prepared formalin (0.02 ml of 2% formalin) on the right hind paw in all groups. The inflammation was measured using vernier calipers before and after injection of formalin and for six consecutive days. Increase in thickness as a measure of inflammatory edema calculated using the formula of, $Pt\text{-}P_0$ where P_o is the initial thickness prior to induction. Percent inhibition of inflammation is calculated by the formula $P_c - P_t/P_c \times 100$. Where P_t is the

increase in thickness of the treated. P_c is the control. Graph is also plotted using mean and SD calculated.

3. 3. RESULTS

Qualitative phytochemical analysis of *D. gyrans* extracts in different solvents showed the presence of phytochemicals like alkaloids, phytosterols, flavonoids, terpenes, tannins and phenolic compounds (Table 3.1). From the table it can be seen that during phytochemical analysis, presences of highest number of phytochemicals were detected in the extract prepared using methanol and water.

Results of of total flavonoid and total phenolic content analysis

Sl No:	Phytochemical anlysed	Result
1	Total Flavonoid content μ g of QE eq./ g extract	76.5 ± 2.12
2	Total Phenol content μ g of GA eq./ g extract	56 ± 2.89

The developed TLC plate was observed to detect the bands formed. Twelve bands were detected upon visual observation. More bands were made visible upon exposure to iodine vapour. The plate was then exposed to UVlight in a UV illumination chamber, which detected the presence of more than 20 bands in the TLC plate (Figure 3.1.1).

GC MS analysis done using 70% methanol in water extract of *D. gyrans* revealed the presence of a number of phytochemicals as shown by the chromatogram of compounds (Figure 3.1.2). The volatile compounds were dominated by the presence of phenolic derivatives (Table 3.2).

Acute administration of *D. Gyrans* did not show any decrease in the body weight of adult male and female Wistar rats used in this study. There was no observed weakness, alterations in food consumption or difficulty in movement. There was no mortality in animals and so LD_{50} value could not be calculated.

Sub-acute administration of *D. Gyrans* did not produce any decrease in body weight (Figure 3.2.1) or any change in the weight of the organs such as liver, kidney, spleen and brain. No statistically significant change was observed in haematological parameters in the animals such as total WBC, RBC, Platelet, haemoglobin as well as in the differential count as well as in hepatic parameters such as ALT, AST and ALP. Renal function tests such as urea and creatinine which were also similar to that of normal group of animals (Tables 3.5, 3.6 and 3.7). Histopathological study done on tissue sections showed that liver tissue was unaffected with normal portal triads and central venous system. Hepatocytes showed normal morphology. Sinusoidal spaces and Kupffer cells appeared normal (Figure 3.2.2). Section of Kidney showed normal glomerular structure. Appearance of cells was normal. Renal tubules showed normal morphology (Figure 3.2.3).

In vitro analysis of *D. gyrans* extract revealed the antioxidant potential of *D. gyrans* (Figure 3.3.1). It showed the superoxide scavenging activity of different concentrations of extract and IC_{50} value for this extract was found to be 12.6 μg/ ml. The IC_{50} value of the extract in scavenging the generated DPPH and hydroxyl radical were found to be 4.0 μg/ ml and 13.5 μg/ ml respectively. The dose dependent activity of the extract in scavenging ABTS radicals was given by IC50 value of 1.95 μg/ ml. The efficiency in inhibiting lipid peroxidation was shown by an IC50 value of 74 μg/ ml (Table 3.8). All these values were compared with that of a standard antioxidant namely vitamin C.

Both acute and chronic anti-inflammatory studies were performed in hind paw of Balb/c mice. The percentage inhibitions were found to be 13.02 and 17.39 in acute study (Tables 3.9 and 3.10) and 13.9 and 31.71in chronic anti-inflammatory experiments (Table 3.11). The results showed that 70% methanol extract of DG has excellent antioxidant activity and anti-inflammatory activity, especially during chronic inflammation.

3. 4. DISCUSSION

Qualitative phytochemical analysis of *Desmodium gyrans* extracts in different solvents showed presence of alkaloids, phytosterols, tannins, steroids, terpenoids and flavonoids. The fractions extracted using methanol and water gave highest amount of the phytochemicals. So a 70% methanol in water extract of the plant was used for all further experiments.

GC-MS analysis showed the presence of phytochemicals active in inhibiting inflammation thus validating the anti-inflammatory activity of *D. gyrans* extract. Some of the fragmented compounds revealed in GC-MS like, Hexadecanoic acid, 2,3,dihydro benzofuran, (Donald *et al*.,1976) phytol, (Camila *et al* ., 2013; Renan *et al*., 2014) 2,6-dihexadecanoate, ascorbic acid, dexamethazone, 9,12,15-Octadecatrienoic acid are having hypolipidemic, antioxidant, anti-inflammatory effects. Hexadecanoic acid inhibit inflammation by way of inhibiting phospholipase A(2) responsible for ester bond hydrolysis of membrane phospholipids and consequent release of fatty acids which is believed to initiate inflammation (Aparna *et al*., 2013). 9, 12, 15-Octadecatrienoic acid may reduce cardiovascular risk through a variety of biologic mechanisms, including regulation of platelet function, inflammation and endothelial cell function (Mozaffarian *et al*., 2005).

The acute and sub-acute toxicity studies done on rats revealed that *Desmodium gyrans* extract at a high dose or on a prolonged administration do not cause any deleterious effects on experimental animals. No behavioural changes or mortality was witnessed during the course of the experiment. Abnormal changes in body weight were not noted and a regular weight gain was recorded during sub-acute study. A normal food and water intake of animals indicated a regular metabolic status. Hematological parameters as well as markers of liver and renal dysfunctions were normal and did not show any irregular patterns of values, indicating normal performance of vital organs.

The outcome of the current study showed that the LD_{50} value of *Desmodium gyrans* extract was greater than 2500 mg/ kg body wt., which may be accepted as safe as per OECD guidelines. The current study on *Desmodium gyrans* extract did not reveal

any apparent toxicity on experimental rats and hence can be safely used for oral administration in further investigations involving animals.

The results revealed antioxidant efficacy of 70% methanolic extract of *D. gyrans* in scavenging free radicals generated in antioxidant assays and in inhibition of lipid peroxidation. Superoxide is generated as an unwanted byproduct of mitochondrial respiration as well as by activity of several cellular enzymes. The oxygen radical may have several effects either directly or through generation of other free radicals all of which lead to oxidative damages, endothelial dysfunction and altered gene transcription. Reactive Oxygen Species (ROS) are reported to be involved in diabetes, hypertension, atherosclerosis, heart failure and cancer. (Li *et al*., 2004)

The percentage inhibitions in anti-inflammatory study were found to be 13.02 and 17.39 in acute anti-inflammatory experiment and 13.9 and 31.71 in chronic anti-inflammatory experiment as summarised (Figures 3.3.2 and 3.3.3). Inflammation is basically a protective mechanism of the body and is considered as the second line of defense in the functioning of immune system. But inflammation can become harmful when it turns hypersensitive. Inflammations can be acute or chronic depending on the severity and duration of the development. The significant antioxidant activity of the extract observed in the current study can be considered contributing to its anti-inflammatory property also.

Carrageenan-induced edema is mediated through the release of prostaglandin and slow reacting substances of inflammatory response which peak at 3 hrs (Hosseinzadeh *et al*., 2000; Zakaria *et al*., 2001). Development of edema upon administration of carrageenan in the rat paw is a biphasic event (Somchit *et al*., 2008). In the initial phase (0-2.5 hrs after carrageenan injection, release of mediators namely histamine, serotonin and kinins occur (Daud *et al*., 2006). In the second phase increased production of inducible cyclooxygenase (COX) leads to increased synthesis of prostaglandins followed by marked increase in cellular infiltration and liberation of acute inflammatory mediators such as myeloperoxidase and cytokines (IL-1β, IL-6, TNF-α) at the inflammatory site. The subsequent release of reactive oxygen species

(ROS) and reactive nitrogen species, mediate the exaggerated inflammatory response (Panthong *et al*., 2004; Shivani *et al*., 2013). The prostaglandin, particularly contribute to increased blood flow through a vasodilatation in this phase. The second phase is found to be sensitive to most of the clinically effective anti-inflammatory drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs) (DiRosa *et al*., 1971).

The level of hs-CRP and nitric oxide are found reduced by the treatment with *D. gyrans* extract in acute anti-inflammatory study. C- reactive protein is considered as a causative agent for inflammatory response, besides being an inflammatory marker. So the observed reduction in CRP can be considered indicative of the anti-inflammatory properties of the extract. Inflammatory response is aggravated by activated macrophages which release NO, a powerful chemical having cytotoxic effects (Krol *et al*., 1995). Despite the fact that NO is considered as a molecule which protects endothelium from injuries with its effects on vasodilation, NO is having a harmful role of getting converted into peroxynitrates which in turn cause various chemical reactions in biological structures. These include nitration of tyrosine residues in proteins, lipid peroxidation, interference during mitochondrial transport and oxidation of biological thiol molecules (Maeda *et al*., 1998). *D. gyrans* extract is able to closely regulate the release of NO, leading to reduction of inflammatory reactions (Beasley *et al*., 1991). The anti-inflammatory activity of the plant extract can be credited to the potential to slow down the mediators of inflammatory reactions, especially the production of substances involved in the second phase of acute inflammatory response in addition to free radical scavenging.

Chronic inflammation has significant role in promoting chronic diseases like cardiovascular disease, diabetes and cancer and suppressing chronic inflammation may contribute in preventing them. Formalin induced paw edema in rats is widely used for screening potential anti-inflammatory agents. Formalin induced chronic inflammation also is biphasic with an early neurogenic component involving release of bradykinin being followed by a later tissue-mediated response involving release of histamine, 5-hydroxytryptamine and prostaglandin. *D. gyrans* extract may be imparting an inhibitory

role in these processes rendering protection against chronic inflammation (Figures 3.3.4 and 3.3.5).

The deployment of *D. gyrans* in asthma, cough, as emollient and in wound healing is justified since the study verifies antioxidant and anti-inflammatory properties of the extract. The reason for successful traditional use of *D. gyrans* in heart ailments is possibly due to its anti-inflammatory properties which assist in inhibiting atherogenesis since atherosclerosis is considered also as an inflammatory disorder (Meng *et al*., 2006; Vipin *et al*., 2014).

3.5. CONCLUSION

The medicinal properties of *Desmodium gyrans* can be credited to the presence of phytochemicals in it which resulted in the distinctive antioxidant properties and anti inflammatory activities revealed in the study. These findings validate the use of this plant in traditional medicine.

Chapter 4

Study of Antithrombotic and Anticoagulant Properties of Desmodium gyrans DC

4. Study of Antithrombotic and Anticoagulant Properties of *Desmodium gyrans* DC extract

4.1. INTRODUCTION

4.2. METHODS AND MATERIALS

4.2.1. Preparation of plant extract

4.2.2. Animals

4.2.3. Study groups and sampling

4.2.4. Clotting time

4.2.5. Prothrombin time (PTT)

4.2.6. Plasma re-calcification time

4.2.7. Platelet aggregation study - collagen induced

4.2.8. Lipid peroxidation

4.3. RESULTS

4.3.1. Clotting time

4.3.2. Prothrombin time

4.3.4. Plasma re-calcification time

4.3.5. Platelet aggregation study

4.3.6. Lipid peroxidation

4.4. DISCUSSION

4.5. CONCLUSION

4.1. INTRODUCTION

Thrombosis is the pathological development of blood clots and these clots subsequently may break free and become mobile. Thrombosis typically occurs during myocardial infarction as a result of atherosclerotic plaque rupture (Glass *et al*., 2001). Antithrombotic agent is any medication that prevents clot formation or dissolves already formed clots. They are used therapeutically for primary and secondary prevention or treatment of acute thrombus (Junichiro *et al*., 2005). Anticoagulant agents also prevent formation of internal clotting in the vessels either by affecting the availability or by disturbing the activation of blood coagulation factors and platelets. Anticoagulants and anti-platelet agents are amongst the most commonly used medications that inhibit activation of clotting factors which eventually prevents venous thrombosis (Yamada *et al*., 2004). Another major clinical objective of antithrombotic treatment, notably in patients undergoing coronary surgery, is to avoid further incidence of thrombosis (Yves *et al*., 2000). Anticoagulant drugs are also being regularly used following angioplasty to prevent recurrence.

A number of medicinal plants have been scientifically evaluated for their antithrombotic and anticoagulant properties. There have been some highly promising findings, *Careya arborea, Bauhinia forficata, Gloriosa superba* and *Jatropha curcas* being a few among them (Shahriyary *et al*., 2007; Surjeet *et al*., 2011). *Desmodium gyrans* DC, found in Kerala forests belong to the family Fabaceae. *D. gyrans* is popularly used in traditional and folk medicine since its leaves have diuretic, febrifugal and tonic properties. Its roots are used in Indian medicine as a remedy for asthma, coughs, as anti-dysenteric and as emollient. It has got a remarkable wound healing effect also. It has got significant antioxidant and anti-inflammatory property also which has already been evaluated during the current study. The objective of the study was to evaluate the antithrombotic and anticoagulant potential of methanolic extract of *D.gyrans*.

4.2. METHODS AND MATERIALS

4.2.1. Preparation of plant extract

The dried entire plant was powdered and subjected to extraction with 70% methanol in water using a soxhlet apparatus. The methanol extract of *D. gyrans* was filtered, concentrated and evaporated to dryness and the dried extract was dissolved again in distilled water and was used for the study.

4.2.2. Animals

Male Sprague Dawley rats (200 gm) were obtained from the Small Animal Breeding Station (SABS), Kerala Veterinary and Animal Sciences University, Mannuthy, Kerala, India. The animals were kept under standardised environmental conditions (22-30 ^{o}C, 60-70% relative humidity, 12 hr dark/light cycle) and fed with standard rat feed (Kerala Feeds, Thrissur, India) and water was provided *ad libitum.*

Animal experiments conducted during the study had prior permission from Institutional Animal Ethics Committee (IAEC) and followed the guideline of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division, Government of India.

4.2.3. Study groups and sampling

The animals were divided into four groups, normal, standard and Des. gyrans extract treated group I and II consisting of 6 male Sprague Dawley rats in each group. The standard group received heparin injected through tail vein at a dose of 250 U/ kg b.wt of the animal for one day. The extract treated groups received *D. gyrans* extract at a dose of 100 and 250 mg/ kg b.wt of the animal orally for a continuous period of one month.

4.2.4. Clotting time

Clotting time is used as a screening test to monitor all phases of intrinsic coagulation system and to monitor heparin therapy (Kinra *et al*., 2009). The coagulation time of whole blood is the time required for blood to clot under normal standard

conditions. Blood was collected from normal rat through the tail vein and was transferred directly to capillary tube. The end of the capillary tube was broken at regular intervals and clotting time was noted instantly when a fibrin thread appears between broken pieces of capillary tubes. Time taken for clot to appear was noted. Blood clotting time was also assayed in groups of animals injected with standard heparin as well as on rats orally fed with *D. gyrans* extract.

4.2.5. Prothrombin time (PTT)

Prothrombin time determination is the preferred method for screening and diagnosis of congenital deficiency of factors II, V, VII and X. PTT is also used for monitoring of patients on oral anticoagulant therapy and as a liver function test.

Plasma collected from normal male Sprague Dawley rat (0.1 ml) was taken in a glass test tube and incubated at 37 ºC for 5 minutes. Liquiplastin (0.2 ml) was mixed with plasma and time was noted instantly. Formation of a visually detectable solid plasma clot occurs inside glass tube within a specified period of time. The time in seconds required for clot formation was noted. The experiment was repeated using heparin treated standard plasma and plasma from extract treated group of animals. Tissue thromboplastin in the presence of calcium initiates the extrinsic pathway of coagulation mechanism. When liquiplastin reagent was added to normal plasma, the clotting mechanism was initiated. PTT would be prolonged if there is a deficiency or delay of blood coagulation factor activity in the extrinsic pathway of the coagulation mechanism.

4.2.6. Plasma re-calcification time

Plasma re-calcification time was determined following the addition of M/100 $CaCl_2$ solution to the previously warmed plasma at 37 ºC**.** The values were noted in each of the tubes and were compared with the value of the normal control (Materials and methods 2.2.9.4.).

4.2.7. Platelet aggregation study - collagen induced

Platelet rich plasma (PRP) was prepared by centrifugation (1000 rpm × 5 min) of blood collected from normal donors. Platelet aggregation was done as described in materials and methods (Materials and methods 2.2.9.5.).

4.2.8. Lipid peroxidation

Malondialdehyde (MDA) produced during peroxidation of membrane lipids by cycloxygenase react with thiobarbituric acid (TBA) reagent to form a pink coloured product which has an absorption maximum at 532 nm. The assay is calibrated with 1,1,3,3, tetramethoxypropane, which on hydrolysis produces malondialdehyde. The results are expressed in terms of the amount of malondialdehyde produced during the reaction (Materials and methods 2.2.9.6.)

4.3. RESULTS

4.3.1. Clotting time

Heparin treated standard group of rats gave a significant delay in blood clotting time in clotting experiment with an average increase of clotting time by 0.46 minutes when compared to the untreated group of rats (Table 4.1). Clotting time of *D. gyrans* extract high dose treated rats showed a delay in clotting time of 0.16 minutes in higher dose which was 2.60% increase.

4.3.2. Prothrombin time

The standard group of animals treated with heparin showed a delay in coagulation by 28% when compared to the normal group of animals (Table 4.2).The treatment with *D. gyrans* extract gave a delay in coagulation by 17.3% in higher dose.

4.3.4. Plasma re-calcification time

Normal plasma re-calcification time of PRP was 3.15 minutes. The standard tubes in which heparin were added showed no clotting of plasma even after a prolonged time period. The tubes in which different concentrations of *D. gyrans* extract (150 and

250 μg) were added showed a delay of 0.14 minutes and 0.18 minutes respectively when compared to the normal (Table 4.3).

4.3.5. Platelet aggregation study

From the graph of platelet aggregation study, it can be seen that there was marked aggregation of PRP with usage of collagen, which was ameliorated in dose dependent manner up on treatment with *D. gyrans* extract in two different concentrations of 100 and 250 μ g (Table 4.4). Collagen induced aggregation of PRP was brought down significantly as indicated by the change in the optical densities.

4.3.6. Lipid peroxidation

The value of lipid peroxidation was significantly increased by 78.19% in the control in comparison to the normal in the experiment (Table 4.5). On treatment with *D. gyrans* extract, lipid peroxidation was brought down by 19.2 and 29.94% in lower and higher dose respectively, when compared to the control.

4.4. DISCUSSION

The results of *in vitro* anti-platelet aggregation study revealed the property of *D. gyrans* extract to reduce clumping of platelets in platelet rich plasma. Some of the proven anti-platelet aggregation agents like aspirin are having an effect of even irreversibly reducing platelet count in the blood. But unlike aspirin, *D. gyrans* extract is not having such a property of irreversibly reducing platelet count as assessed from the platelet count analysis done during the study.

Phytochemical screening of extract of *D. gyrans* revealed the presence of phenolic compounds and flavonoids. These secondary metabolites are known to have various biological activities of which can be credited to the medicinal properties of *D. gyrans*. The antioxidant and anti-inflammatory properties of the extract have already been proved. Inhibition of cyclooxygenase (COX)-1, is a proven mechanism to prevent inflammatory reaction. Since the extract is able to exhibit a notable antioxidant activity together with anti-inflammatory effect, the property of inhibiting platelet aggregation

can be attributed to these proven findings. It has been shown that the anti-platelet activities of herbal extracts are often related to their polyphenol and antioxidant activities (Mattiello *et al*., 2009). Although a study on the exact mode of action is still has to be done, any of the mechanisms or a combination of them, as illustrated above, can be attributed as the possible means by which *D. gyrans* extract brings about inhibition of platelet aggregation as revealed in the present study.

The property to act as anti-platelet aggregation agent may be due to anyone of the mode of action of anti-platelet aggregation pathways (Figure 4.1) which has to be investigated. The mode of action of an anti-platelet aggregation agent can be classified into four types, namely COX-1 inhibition, ADP antagonistic action, phosphodiesterase inhibition or GP IIb/IIIa antagonistic action (Toth *et al*., 2013).

The arrest of bleeding depends upon formation of primary platelet plug formed on site of injury followed by the formation of a stable fibrin network. Formation of this clot involves the sequential interaction of a series of plasma proteins and the interaction of these complexes with blood platelet and components released from tissues. Tissue thromboplastin, in presence of calcium initiates the extrinsic pathway of coagulation, which comprises plasma coagulation factors VII, X, V, Prothrombin and Fibrinogen (Giangrande *et al*., 2003). During oral anticoagulant therapy specific blood coagulation factors are depressed.

Anticoagulant action of *D. gyrans* was verified through blood clotting time and plasma re-calcification experiments. Clotting of blood is delayed by a number of agents in blood which are having different modes of action. Most of the agents depend on the inhibition of coagulation factors by one method or the other. Activation of antithrombin III is the mechanism of action of heparin and other anticoagulant drugs like Dalteparin, Lepirudin, Enoxaparin and Fondaparinux (Messmore *et al*., 1993; Hirsh *et al*., 2004; Smythe *et al*., 2005).

Another regularly used anticoagulant agent namely Warfarin inhibits vitamin K reductase, resulting in exhaustion of the reduced form of vitamin K (vitamin KH_2). (Ansell *et al*., 2004, Rubin *et al*., 2008). Whether the synergic action of the phytochemicals present in the extract is able to influence the activation of

antithrombins, like the way heparin operates is to be investigated. Another prospect is of inhibiting gamma-glutamyl carboxylase that adds a carboxyl group to glutamic acid residues on factors II, VII, IX and X for activating these factors or inhibit Vitamin K which is an essential factor for activation of gamma-glutamyl carboxylase. Anticoagulant action of *D. gyrans* thus opens up scope for further study on its mechanism of action against coagulation.

Collagen is found to induce lipid peroxidation by inducing the arachidonate metabolic pathway. Increased lipid peroxidation may weaken the quasi-fluid nature of cell membrane. This inturn can impair the normal membrane structure and receptor functioning of cells. In the study lipid peroxidation has been found to be reversed by the action of extract. The extract could inhibit the process of lipid peroxidation induced by collagen on platelets. The extract was also able to significantly bring down lipid peroxidation in *in vitro* experiment of lipid peroxidation done in rat liver homogenate.

4.5. CONCLUSION

The study recommends *D. gyrans* extract as an effective anticoagulant and antithrombotic agent. This could possibly contribute to the perceived cardioprotective and antiathrosclerotic properties that *D. gyrans* possess.

Chapter 5

Protective Effect of Desmodium Gyrans DC against Doxorubicin Induced Cardiomyopathy and Isoproterenol Induced Myocardial Infarction

5. Protective effect of *Desmodium Gyrans* DC against doxorubicin induced Cardiomyopathy and isoproterenol induced myocardial infarction

5.1. INTRODUCTION

5.2. MATERIALS AND METHODS

5.2.1. *Animals*

5.2.2. *Protocol for doxorubicin induced cardiomyopathy in rats*

5.2.3. *Protocol for isoproterenol induced myocardial infarction in rats*

5.2.4. *ECG recording*

5.2.5. *Biochemical analysis*

5.2.6. *Studies on cardiac tissue*

5.3. RESULTS

5.1.3.1. *Cardiac tissue assays*

5.1.3.2. *Mitochondrial antioxidant assay*

5.1.3.3. *Serum analysis*

5.1.3.4. *Histopathological findings*

5.1.3.5. *ECG results*

5.4. DISCUSSION

5.5. CONCLUSION

5. Protective effect of *Desmodium Gyrans* DC extract against doxorubicin Induced cardiomyopathy and isoproterenol induced myocardial infarction

5.1. INTRODUCTION

Doxorubicin (DOX), which is an anthracyclin antibiotic used in treatment of a variety of cancers, is found to cause cardiotoxicity on overdose. The chronic cardiotoxicity is dose dependent and causes irreversible myocardial damage, resulting in dilated cardiomyopathy with fatal congestive heart failure (Von Hoff *et al*., 1979). The molecular mechanism involved in inducing myocardial damage by this anti-neoplastic agent is believed to be different from its therapeutic mode of action. Although the exact mechanisms involved remains to be further elucidated, alterations in calcium and iron homeostasis, mitochondrial dysfunction, inhibition of DNA replication by modifying DNA, direct membrane damage by lipid peroxidation have all been illustrated as causes (Doroshow *et al*., 1991; Xu *et al*., 2001). However, these events have been believed to be set off by oxidative stress through free radical generation. Hence the cause of doxorubicin cardiotoxicity is multifactorial, but the main cause of doxorubicin-induced cardiotoxicity can be said to be due to the formation of reactive oxygen species, which ultimately results in myocytes apoptosis (Horenstein *et al*., 2000). Doxorubicin and its metabolites are capable of producing superoxide anion which gets converted into hydrogen peroxide and then a more reactive hydroxyl radical which may affect cellular components and cell organelle like mitochondria and sarcoplasmic reticulum. The marked protective effect of herbal plant extracts like *Gingko biloba* and polyherbal extracts like, **"**CardiPro**"** against doxorubicin-induced cardiotoxicity in experimental animals have been reported previously (Naidu *et al*., 2002).

Isoproterenol (ISO), a beta adrenergic agonist and synthetic catecholamine is capable of releasing cytotoxic free radicals through redox cycling of its metabolites (Upaganlawar *et al*., 2011). In high doses, it alters myocardial membrane permeability

which leads to loss of functions and depletion of energy reserves of cardiac muscle cells and induces pathological, physiological and morphological alterations, which are comparable to those taking place in human myocardial infarction. It is an acute state of necrosis of the myocardium which is associated with elevated cardiac marker enzymes, lipid peroxidation and left ventricular dysfunction (Gupta *et al*., 2004; Rajadurai *et al*., 2006; Gayathri *et al*., 2011). Free radical mediated tissue injury leading to disruption of mitochondrial structure and intracellular calcium overload are other consequences. Hence high dose ISO induced myocardial infarction is used as a standard model to study the beneficial effect of many natural products on cardiac dysfunction (Wexler *et al*., 1978; Thippeswamy *et al*., 2009).

The present study is designed to make a probe into the cardioprotective potential of *D. gyrans* extract based on the biochemical and histopathological findings after inducing cardiomyopathy and myocardial infarction in experimental models of rats. Rats were pretreated with *D. gyrans* extract and cardiomyopathy was induced using doxorubicin and myocardial infarction was induced using isoproterenol .

The focus of the study is based on the traditionally assumed cardioprotective effect of the *Desmodium gyrans* (Gopalakrishnan and Rajameena, 2012). *D. gyrans* has a long history of use in Chinese traditional medicine to treat various ailments (Ma *et al*., 2011) including cardiac diseases. Closely related species *Desmodium gangeticum* has proven hypolipidemic, cardioprotective properties and gives protection against cardiac reperfusion injury (Kurian *et al*., 2005). The presence of safe and bioactive natural antioxidants raises a substantial interest in deploying these medicinal plants in cardiovascular and other diseases (Warrier *et al*, 1995).

5. 2. MATERIALS AND METHODS

5. 2.1. *Animals*

Sprague Dawley rats (150-200 gms), used for study were procured from Kerala Veterinary and Animal Sciences University, Mannuthy, Kerala, India. The animals were acclimatised for a period of 14 days in uniform hygienic well ventilated cages under standard environmental setting. Water was given to all groups *ad libitum*.

5.2. 2. *Protocol for doxorubicin induced cardiomyopathy in rats*

The study was conducted using male Sprague Dawley rats (150gms-200gms). Animals were randomly divided into groups I, II, III, IV and V of six each. Group I was maintained as normal and given saline intraperitonial injection from 8^{th} day of experiment. Group IV and V received *D. gyrans* extract orally at a dose of 100 and 250 mg/kg b. wt. of the animal respectively, for a period 15 days and group III received standard probucol orally at a dose of 30 mg/kg b. wt. of the animal for the same period of time. Group II was designated as positive control and groups II, III, IV and V received doxorubicin as intraperitonial injection on alternate days from 8^{th} day onwards to give a cumulative dose of 24 mg/kg b. wt. of the animal. At the end of the experiment all overnight fasted animals were sacrificed, blood and heart were collected and properly processed for further analysis.

5.2.3. *Protocol for Isoproterenol induced myocardial infarction in rats*

The study was conducted using male Sprague Dawley rats (150-200 gms). Animals were randomly divided into groups I, II, III, IV and V of six each. Group I was maintained as normal. Group IV and V received *D. gyrans* extract at a dose of 100 and 250 mg/ kg b. wt. of the animal respectively, for a period 21 days and group III received standard probucol at a dose of 30 mg per kg body weight of the animal for the same period. Group II was designated as positive control and groups II, III IV and V received isoproterenol intra peritoneal injection on the 20^{th} and 21^{st} days to give cumulative dose of 85 mg/kg b. wt. of the animal. At the end of the experiment, all overnight fasted animals were sacrificed; blood and heart were collected and properly processed for further analysis.

5.2.4. *ECG recording*

ECG of all the groups was recorded using Cardiart 800 automatic electrocardiograph after mildly sedating the rats using Ketamine 80 mg/ kg b.wt of animals through intraperitonial route.

5.2.5. *Biochemical analysis*

Serum was used for analysis of cardiac enzymes namely creatine phosphokinase (CPK), lactate dehydrogenase (LDH) and aspartate aminotransaminase (AST) using standard commercial kits in fully automated analyzer EM 360 of Transasia Biomedicals. Liver and renal functions were also studied.

5.2.6. *Studies on cardiac tissue*

Fragments of heart were placed in 10% buffered formaldehyde. Later they were embedded in paraffin, cut and stained with hematoxylin and eosin stain and observed under optical microscope at 400 X magnifications for histopathological findings. For antioxidant assay, 1gm of heart tissue was weighed and 3ml of 0.1M Tris buffer was added to it and homogenised. Both enzymatic and non-enzymatic antioxidants as well as lipid peroxidation assays were carried out using the tissue. Antioxidant assay on mitochondrial fraction isolated from cardiac tissue was also performed.

5. 3. RESULTS

5. 3.1. *Cardiac tissue assays*

The results of antioxidant assay on cardiac tissue homogenates from DOX positive control group of animals reveals a significant decline of antioxidants and increased lipid peroxidation (Figures 5.1.1 - 5.1.4). A significant amelioration of SOD, GSH and GPx was seen in both the lower and higher dose category of *D. gyrans* extract treated DOX group. In higher dose *D. gyrans* extract treated DOX group, SOD, GSH and GPx were improved by 28.9%, 15.6%, and 4.6%. Inhibition of lipid peroxidation in *D. gyrans* extract treated DOX group was 28.2% in comparison to the control.

The results of antioxidant assay on cardiac tissue homogenates from ISO positive control group of animals reveals a significant decline of antioxidants and increased lipid peroxidation (Figures 5.2.1 - 5.2.4). A significant amelioration of SOD, GSH and GPx was seen in both the lower and higher dose category of *D. gyrans* extract treated ISO group. In *D. gyrans* extract treated ISO group, SOD level was improved by

98%, GSH by 50.9%, and Gpx by 34%, using higher dose. This result is also seen repeated in lipid peroxidation assay. A significant 47.1% reduction in inhibition of lipid peroxidation was observed in *D. gyrans* extract treated ISO group in comparison to the control group of animals.

5. 3.2. *Mitochondrial antioxidant assay*

Mitochondrial antioxidant assay done on cardiac tissue of DOX treated positive control group showed substantial damage to mitochondria in the form of reduced antioxidant content and enhanced lipid peroxidation. But the *D. gyrans* extract treated group showed no significant improvement.

Mitochondrial damage induced by ISO was quiet evident from the results of ISO positive control group which were effectively ameliorated in the *D. gyrans* extract treated group (Figures 5.2.5-5.2.8). On treatment with the higher dose of *D. gyrans* extract, SOD level was enhanced by 20%, GSH by 18.8%, Gpx by 31% and LPO was improved by 31%.

5.3. 3. *Serum analysis*

The cardiac marker enzymes CPK, AST (SGOT) and LDH which were increased in serum with DOX treatment were restored to improved levels indicating a definite effect of the extract (Figures 5.1.5 - 5.1.7). In the higher dose of *D. gyrans* extract treated DOX group CPK was lowered by 18%, LDH by 23% and AST by 25%.

The cardiac marker enzymes in serum CPK, AST (SGOT) and LDH which were increased with ISO and DOX treatment were restored to improved levels indicating a definite effect of the extract (Figures 5.2.9 - 5.2.11). In higher dose *D. gyrans* extract treated ISO group CPK was lowered by 50%, LDH by 31% and AST by 55% in comparison to control.

5. 3.4. *Histopathological findings*

DOX on high dose cause pronounced neutrophil infiltration and myocardial tissue damage which were showed improvement with extract treatment (Figure 5.1.8).

Free radicals and reactive oxygen species have been implicated as a cause of a number of diseases including those affecting cardiac functioning.

ISO overdose lead to chronic necrotic degeneration of myocardial fibres, appearance of inflammatory cells, mononuclear cell infiltration, vacuolated appearance due to deposition of oil globules and endocardial damage (Lehr *et al*., 1972). These were significantly alleviated upon *D. gyrans* extract treatment (Figure 5.2.12). Free radicals and reactive oxygen species have been implicated as a cause of a number of diseases including those affecting cardiac functioning. An excessive occurrence of free radicals including superoxide radicals, hydroxyl radicals and hydrogen peroxide has been observed in failing myocardium (Madhesh *et al*., 2012). The heart is more susceptible to free radical-induced damage, because it has relatively low antioxidant enzymes such as super oxide dismutase and catalase (Myers *et al*., 1977).

5. 3.5. *ECG results*

DOX induction of cardiomyopathy showed ST depression and alterations in ventricular repolarisation phase in control animals (Figure 5.1.9).

In the electrocardiogram, ST segment elevation was the notable change with ISO induced MI in the control group of animals. Among the extract treated groups ISO induced rats showed a significant reinstatement to normal ECG pattern (Figure 5.2.13).

5. 4. DISCUSSION

An increase in myocardial lipid peroxidation and a decrease of antioxidant enzymes have been demonstrated in doxorubicin-treated mice. Antioxidants have been reported to have beneficial effects against DOX induced cardiotoxicity in mice and rats (Iliskovic *et al*., 1995; Liu *et al*., 2002). Probucol, a lipid lowering drug and an antioxidant, protects animals from DOX induced cardio toxicity (Demerdash *et al*., 2003). In the present study, *D. gyrans* extract renders a moderate protection to cardiac tissue against damages induced by treatment of rats with doxorubicin. The molecular mechanism involved in inducing myocardial damage by DOX is still contentious and appears to be different from its therapeutic mode of action. Alterations in calcium and iron homeostasis, mitochondrial dysfunction, inhibition of DNA replication by

modifying DNA, direct membrane damage by lipid peroxidation have all been illustrated as causes of DOX induced cardiomyopathy. *D. gyrans* extract is having antioxidant properties but it may not be effective enough to counter the other molecular mechanisms involved in the establishment of pathological symptoms of cardiomyopathy.

An excessive occurrence of free radicals including superoxide radicals, hydroxyl radicals and hydrogen peroxide has been observed in failing myocardium (Madhesh *et al*., 2012). The heart is more susceptible to free radical-induced damage, because it has relatively low antioxidant enzymes such as super oxide dismutase and catalase (Myers *et al*., 1977).

It has been shown that some of the known antioxidant molecules like Vitamin E, ascorbic acid and selenium are having limited effect in protecting heart tissue against DOX induced cardiomyopathy (Van Vleet *et al*., 1980; Paulo *et al*., 2004). Hence the role of antioxidants in protecting cardiac tissue against DOX is still controversial (Wahab *et al*., 2000, Acker *et al*., 2001; Ferreira *et al*., 2007). DOX undergoes reduction to its dihydrometabolic derivative namely doxorubicinol, which has been shown to be more cardio-toxic than DOX itself (Boucek Jr *et al*., 1987; Olson *et al*., 1990). Cytosolic enzymes like carbonyl reductases and aldoketoreductases are found to catalyse the steps involved in the conversion of doxorubicin to doxorubicinol. The agents which can act as inhibitors of these enzymes are shown to be cardioprotective (Forrest and Gonzalez, 2000). Further, antioxidants which exhibit the property of iron chelation are found to be better cardioprotective agents since such molecules inhibit redox cycling of iron between Fe^{2+} and Fe^{3+} oxidation states. It is this redox cycling which actually lead to generation of hydroxyl radical and so such antioxidants can be efficient in inhibiting even the formation of free radicals (Schroeder *et al*., 2002; Simunek *et al*., 2005; Sterba *et al*., 2006). Even though *D. gyrans* is having proven antioxidant property, it may not be enough to meet the requirements of protecting cardiac tissue against DOX induced cardiotoxicity. The pytochemicals available in the plant extract may be unable to inhibit the processes like doxorubicinol formation and free radical generation through iron chelation, which can be cited as the reason for the inconsistency in some of the parameters considered in the current study.

Isoproterenol, a β-adrenergic receptor agonist causes severe oxidative stress to the myocardium resulting in an infarct like necrosis of heart muscle. The isoproterenol induced myocardial infarction in rats is an accepted model to evaluate myocardial dysfunctions and to study the efficacy of natural and synthetic cardioprotective agents (Rathore *et al*., 1998). The results in the current study showed the ameliorating effect of *D. gyrans* extract on myocardial infarction induced by ISO. The cardiac marker enzymes CPK, LDH and AST, which were increased with ISO treatment were brought down to improved levels indicating a definite effect of the extract. Biochemical changes in ISO induced MI include drastic alteration in enzymatic and non-enzymatic antioxidant levels in cardiac tissue and cardiac mitochondrial fraction. Mitochondrial damage induced by ISO was quiet evident from the results of ISO positive control group which were effectively ameliorated in the *D. gyrans* extract treated rats. Histopathological changes induced by ISO overdose comprise chronic necrotic degeneration of myocardial fibres, mononuclear cell infiltration, detection of inflammatory cells, vacuolated appearance due to deposition of oil globules and endocardial damage (Jignasa *et al*., 2012). Myocardial changes are highest in subendocardial region of left ventricle and interventricular septum. *D. gyrans* extract seems to have imparted protection to cardiac tissue against most of these myocardial tissue damages in a significant manner.

The results of antioxidant assay performed on cardiac tissue homogenate and mitochondrial fraction from DOX and ISO study reveals the same with a significant decline of antioxidants and increased lipid peroxidation in positive control animals. To be noted is the more significant amelioration of SOD, GPx and GSH in the *D. gyrans* extract treated ISO group when compared to corresponding DOX treated rats. This is also repeated in lipid peroxidation assay.

The results in the current study showed the ameliorating effect of *D. gyrans* extract on myocardial infarction induced by ISO when compared to DOX induced cardiomyopathy. A primarily free radical mediated tissue injury leading to disruption of mitochondrial structure and intracellular calcium overload are the proven consequences of ISO overdose and these molecular mechanisms are different from the mechanisms involved in pathological manifestation of cardiomyopathy. Hence the involvement of *D. gyrans* extract in free radical scavenging, leading to interference of the above

mentioned molecular mechanisms of ISO toxicity can be the reason for its protective effect against myocardial infarction.

D. gyrans is successfully engaged in a number of diseases in traditional medicine and its use in heart ailments is one of the accepted means of herbal medication. Its closest species with which it shares phytochemicals namely *Desmodium gangeticum* is used in Dasamoola preparations and is a subject of much research works linked to cardioprotective role. *D. gyrans* shares much of its major phytochemicals with *Desmodium gangeticum* in that both contain phenolic and flavanoid compounds, derivatives of dimethyl tryptamine, phenethylamine, hypaphorine and choline. *D. gyrans* has also been verified for antioxidant properties (Gopalakrishnan and Rajameena, 2012), which may explain its protective effect on cardiac tissue.

5.5. CONCLUSION

The study done with the objective of finding how *D. gyrans* becomes 'good for heart' lead to the conclusion that it is effective against myocardial infarction whereas it shows reasonable protection against cardiomyopathy. The outcome of study validates the use of the plant in traditional medicine as a cardioprotective agent.

Chapter 6

Hypolipidemic and Lipid Clearing Effect of Desmodium gyrans DC

6. Hypolipidemic and Lipid Clearing Effect of *Desmodium gyrans* DC

6.1. INTRODUCTION

6.2. MATERIALS AND METHODS

6.2.1. Preparation of the extract

6.2.2. Animals

6.2.3. Preparation of high fat diet

6.2.4. Protocol for lipoprotein lipase assay

6.2.5. Reagents for incubation

6.2.6. Reagents for glycerol determination

6.2.7. Incubation procedure

6.2.8. Glycerol determination

6.2.9. Protocol for hypolipidemic study on rats

6.2.10. Protocol for lipid clearing experiment

6.2.11. Determination of Biochemical parameters

6.3. RESULTS

6.4. DISCUSSION

6.5. CONCLUSION

6.1. INTRODUCTION

Hyperlipidemia is the single most important risk factor in the commencement and development of atherogenesis. An altered lipoprotein metabolism and deranged lipid structure may lead to conditions such as coronary heart disease, ischemic cerebrovascular disease and peripheral vascular disease. Hyperlipidemia also plays indirect part in inducing the production of oxygen free radicals from polymorphonuclear leukocytes and monocytes (Crowther *et al*., 2005). Customary medical treatment of dyslipidemia using statins and other available medications are proved to have adverse effects (Anna *et al*., 2005). LDL oxidation is generally considered as a critical modification in the initiation of atherosclerosis (Grundy *et al*., 2002). But, none of the currently available treatments are aimed at prevention of endogenous oxidation of LDL Cholesterol. Significantly, there is a need for formulation of effective drugs which are more efficient in bringing down LDL Cholesterol level and checking the formation ox-LDL. Such pharmacological agents have to be devoid of side effects too (Thompson *et al*., 2004). At the present time, there is an increasing attention toward the potential health benefits of medicinal plants. A few indigenous Indian medicinal plants have been scientifically verified and found to be significant in management of hyperlipidemia (Julie *et al*., 2001; Halim *et al*., 2002; Patil *et al*., 2011).

Triglycerides are the most abundant fatty molecules in humans. The correlation between elevated levels of triglycerides and atherosclerosis, even in the absence of hypercholesterolemia, is believed to be predisposary to cardiovascular disease (Hokanson *et al*., 1996; Nordestgaard *et al*., 2014). Independent connection between elevated triglyceride (TG) levels, cardiovascular risk and mortality had been controversial (Schulte *et al*., 1999). Since elevated triglyceride levels are normally associated with corresponding alterations in high density lipoprotein (HDL), low density lipoprotein (LDL) and other lipoproteins, obviously, an isolated study on the effect of hypertriglyceridemia on cardiovascular problems could not be made for a long time (Sarwar *et al*., 2007).

Hypertriglyceridemic states are associated with increased VLDL production and delayed VLDL clearance from circulation. Hypertriglyceridemia is proved to commonly lead to decline in HDL and increase in atherogenic small dense LDL levels (Baigent *et al*., 2005; Alexander *et al*., 2014). Studies have verified that TG may act as causal risk factors for atherogenesis by methods, such as excessive release of free fatty acids (FFA), production of pro-inflammatory cytokines, fibrinogen and coagulation factors (Alexander *et al*., 2014). Latest studies have stated that hypertriglyceridemia may be involved in the progress of atherosclerosis by inducing thrombophilia. A consistently elevated TG in blood reduces HDL and enhances small dense LDL in blood, and by this means induce the deposition of cholesterol on the vascular wall. Simultaneously, the disease causes thrombotic formation and stimulates pathological actions of the vascular wall to spread atherosclerosis. So hypertriglyceridemia is considered as a very serious risk factor for atherosclerosis, especially when it happens in patients with hypercholesterolemia or reduced HDL (Yamada *et al*., 2001). There is evidence for the therapeutic value of lowering triglycerides and a promising view that reducing triglyceride-rich lipoproteins can be often crucial in atherogenesis (Durrington *et al*., 1998; Gianturco *et al*., 1998; Beatriz *et al*., 2011)

Lipoprotein lipase (LPL) enzyme belongs to the lipase gene family, which comprises pancreatic lipase, hepatic lipase, and endothelial lipase. LPL hydrolyses triglycerides in lipoproteins, for instance those found in chylomicrons and very low-density lipoproteins (VLDL) converting them to free fatty acids and monoacylglycerol molecules. LPL encourage the cellular uptake of chylomicron remnants, cholesterol-rich lipoproteins and fatty acids (Ma Y *et al*., 1994; Rinninger *et al*., 1998; Mead *et al*., 2002).

LPL uses ApoC- II as a cofactor (Braun *et al*., 1992; Wong *et al*., 2002). LPL attaches to the endothelial cells in capillaries through the protein glycosylphosphatidyl inositol HDL-binding protein 1 (GPIHBP1) and heparin sulfated proteoglycans.

LPL is a homodimer, and functions as triglyceride hydrolase and ligand for receptor-mediated cellular uptake of lipoprotein. The enzyme catalyses the conversion

of VLDL to IDL and then to LDL. LPL deficiency result in type I hyperlipoproteinemia and many other disorders of lipoprotein metabolism (Yost *et al*., 1998). LPL activity influence HDL cholesterol levels in different ways. When LPL mediate hydrolysis of TG-rich lipoproteins, surface lipids and apolipoproteins are transported to HDL (Patsch *et al*., 1978). The cholesterol in HDL that is swapped for TG is regulated by the amount of VLDL in bloodstream. As plasma TG level is decreased by the activity of LPL, cholesteryl ester transfer protein (CETP) - mediated HDL cholesterol reduction also is brought down. By changing the core lipid structure of HDL, the catabolic rate of apoA-I in HDL is altered. When HDL becomes enriched with triglycerides, the triglycerides can be more rapidly removed resulting in production of smaller apoA-I which are lower in lipid content (Newnham *et al*., 1992; Liang *et al*., 1994).

HDL that contain higher amount of TG are better substrates for HTCL. Apo-I which are poorer in lipid are more quickly removed from circulation. This mechanism leads to the improved fractional catabolic rates and low HDL levels have been observed in persons with hypertriglyceridemia (Brinton *et al*., 1991; Horowitz *et al*., 1993,). Correspondingly, an increased apoA-I catabolic rate is seen during LPL inhibition (Goldberg *et al*., 1990). In most humans, LPL activity is found to be positively correlated with HDL levels (Blades *et al*., 1993; Tornvall *et al*., 1995).

6.2. METHODS AND MATERIALS

6.2.1. Preparation of the extract

The plant was collected from Peechi forest area and identification was done by Dr. Sasidharan, Taxonomist, Kerala Forest Research Institute, Peechi, Kerala, India. Extract was made in 70% methanol from powdered and dried entire plant using soxhlet apparatus.

6.2.2. Animals

Male Wistar rats (150-200 g) used for study on hypolipidemic effect were procured from Small Animal Breeding Station (SABS), Kerala Veterinary and Animal

Sciences University, Mannuthy, Kerala, India. The animals were acclimatised for a period of 14 days in uniform hygienic well ventilated cages under standard environmental setting.

Male New Zealand male white rabbits (1 kg-1.5 kg) used for study on lipoprotein lipase activity was procured from Small Animal Breeding Station (SABS), Kerala Veterinary and Animal Sciences University, Mannuthy, Kerala, India. The animals were acclimatised for a period of 14 days in uniform hygienic well ventilated cages under standard environmental setting.

6.2.3. Preparation of high fat diet

Standard High Fat Diet (HFD) was made using Bengal gram, sucrose, milk powder, raw cholesterol, hydrogenated fat, salt mineral mixture and egg yolk in the amounts mentioned in table 7.1, which formed nutrients approximately in the following proportions: 55% carbohydrates, 25% proteins and 15% fats (Seethanathan *et al*., 1970). Bengal gram was made into flour and mixed with all other constituents and consistency was regulated by adding minimum amount of sterilized water. The mixture was made into pellets mechanically and dried in oven at 40°C for three days. The perfectly dried pellets were kept closed in containers to avoid fungal infections and used within one week. HFD was given to selected group of animals at the rate of 100 g/ kg body weight along with oral feeding of cholesterol at 250 mg/kg body weight on alternate days for a period of three months.

6.2.4. Protocol for lipoprotein lipase assay

Serum with high concentration of triglycerides was used as substrate for assaying the activity of lipoprotein lipase in test sample. Heparin which can release LPL from vascular endothelial cells was used as standard.

6.2.5. ***Reagent for incubation*** (chapter 2, section 2.2.15.1.)

0.5 M $(NH_4)_2$ SO_4

20% serum albumin

Substrate: Serum from hypertriglyceridemic persons was used having a concentration of 300-400 mg of triglyceride per 100 ml.

Enzyme: Normal plasma, post heparin plasma or plasma from animals treated with *D. gyrans* extract was employed as enzyme source.

6.2.6. ***Reagents for glycerol determination*** (chapter 2, section 2.2.15.2.)

0.05 M Sodium periodate

0.5 M Sodium arsenite

1 N H_2SO_4

Chromotropic acid

6.2.7. ***Incubation procedure***

Mixed 0.4 ml of albumin, 0.1 ml of $(NH_4)_2$ SO_4, 0.1 ml of substrate in a series of tubes. To the tubes thus arranged added 0.1 to 0.4 ml of enzyme followed by sufficient quantity of water to make a final volume of 1 ml and incubated at 37 ºC for 90 minutes, at the same time removing aliquots of 0.05 ml at 30 minutes intervals. The reagent was kept cold when mixed and the first sample was measured and taken as initial sample, before keeping the tubes for incubation. The transferred aliquots were kept in a 10 ml conical tip centrifuge tube containing 0.1 ml of 1N H_2SO_4. These can be kept at room temperature for several hours or frozen indefinitely before glycerol determination is performed.

6.2.8. ***Glycerol determination*** (chapter 2, section 2.2.15.4.)

Added 0.1 ml each of periodate to the transferred aliquots kept in 10 ml conical tip centrifuge tubes containing 0.1 ml of 1 N H_2SO_4, mixed well and incubated for 5 minutes. Then added 0.1 ml of arsenite, mixed well and incubated for 10 minutes. Added 9 ml of chromotropic acid mixed and placed in boiling water bath for 30 min. Cooled and adjusted the volume to 10 ml with water and read the optical density at 570 nm. The assay was standardised against a glycerol solution of known molarity. The standard range is from 0.005 to 0.1 micromole of glycerol.

Glycerol standard: Glycerol of known concentration, 100 mg/ 100 ml of glycerol was used as standard for assaying unknown quantity of glycerol liberated in the aliquot tubes. A standard curve was prepared to find out the concentration of glycerol in test solutions.

6.2.9. Protocol for hypolipidemic study on rats

The study was conducted using male Wistar rats (150-200 g). Animals were divided into five groups A, B, C, D and E of six each. Group A was fed normal rat diet and maintained as normal group. Groups B, C, D and E received HFD at the rate of 100 gm/kg b. wt. of the animal along with oral feeding of 250 mg/ kg b. wt. of cholesterol dissolved in 5 ml coconut oil on alternate days for a period of 3 months (Li X *et al*., 2016; Suresh et al., 2017). Among these, group B was the control and received no treatment (HFD alone) and group C received standard drug atorvastatin (5 mg/ kg b. wt. /alternate day). *D. gyrans* was administered in groups D and E in doses of 100 and 250 mg/ kg b.wt by oral intubation on alternate days for a period of 3 months and water was given to all groups *ad libitum*. At the end of the experiment, all animals fasted overnight and were sacrificed and lipid profile was estimated using commercially available standard kits.

6.2.10. Protocol for lipid clearing experiment

Male New Zealand male white rabbits (1 kg-1.5 kg) used for anti-atherogenic experiment, were divided into 4 groups of 6 each. The first group was kept as normal, second was given a single dose of 1mg/ kg b. wt. of heparin (5000 IU/ ml) and used as standard. The third and fourth groups were orally fed with 100 mg and 250 mg/ kg b. wt. of *D. gyrans* extract respectively for a continuous period of 14 days. At the end of the experiment, blood was drawn from peripheral ear vein of all groups and centrifuged (3000 rpm × 5min) to separate plasma and used for assay. The lipoprotein lipase releasing activity of the extract was determined by the method of Korn (1962) as explained above. The glycerol liberated was compared with that of normal and control samples.

6.2.11. Determination of Biochemical parameters (chapter 2, section 2.2.3.)

Total cholesterol, triglycerides, HDL cholesterol and LDL cholesterol were determined using commercially available Colourimetric reagent kits provided by Transasis Biomedicals Ltd.

6.3. RESULTS

In the current hypolipidemic study done in Wistar rats, upon treatment with methanol extract of *D. gyrans* a reduction in serum triglyceride levels were observed to be 30.8 and 40.1% in lower and higher doses respectively. Correspondingly, there was a reduction in total serum cholesterol level by 9.2 and 9.8% and LDL level by 19.11 and 29.32%, when compared to the control group. A significant enhancement of HDL was observed by 20.0 and 28.33% in the extract treated lower and higher groups in comparison to the control (Table 6.1). The elevation of HDL was significant in *D. gyrans* treated lower and higher dose groups with values of 72 and 77 mg/ dl, respectively compared to 56.4 mg/ dl in normal group of animals.

From the current study, it was observed that *D. gyrans* extract is able to enhance the activity of LPL in rabbits. The measurement of glycerol liberated is by the extract treated serum (Table 6.2). The lower and higher dose of the extract showed an enhanced liberation of glycerol from substrate used namely hyperlipemic serum by 35.7 and 69% respectively.

6.4. DISCUSSION

The current hypolipidemic study done on rats has showed a significance lowering of triglyceride in the extract treated group in comparison to control group. Together cholesterol level and LDL levels have also been brought down. Particularly noteworthy is the enhancement in HDL level in the treated groups.

Although the effect of hypertriglyceridemia on cardiovascular risk has long been a topic of argument, substantial data supporting the prognostic significance of this

lipid fraction has been obtained during the recent years only. Studies have found elevated TG level to be a possible independent risk factor for cardiovascular disease (Hokanson *et al*., 1996). Data from studies have shown a significant association between hypertriglyceridemia and CHD risk that is independent of total cholesterol, LDL-cholesterol or HDL-cholesterol levels (Assmann *et al*., 1996; Yarnell *et al*., 2001). Significantly, no controlled trials have confirmed the benefits of triglyceride lowering alone on clinical or cardiovascular outcomes, possibly because existing triglyceride-lowering medications also influence other lipid and lipoprotein levels and compositions.

Studies have shown that a reduction in TG will be beneficial only if there is a corresponding reduction in LDL values. This is because the breakdown product of TG namely fatty acids tend to contribute to the formation of LDL. From the values obtained, we can see that there is not only a reduction in LDL levels but HDL has also been significantly elevated. This shows that whatever fatty acid that is formed from breakdown of TG is being efficiently carried by HDL cholesterol for disposal to the liver. The results in the current study showed a lowering of total cholesterol also, which again can contribute to atheroprotection in the individual.

As per present awareness, TGs are not directly atherogenic but can be an important biomarker of CVD risk (Beatriz *et al*., 2011), because of their association with pro-atherogenic remnant particles. Apo C-III is a proinflammatory, proatherogenic protein that forms part of several classes of the plasma lipoproteins along with TG (Kamagate *et al*., 2008; Ooi *et al*., 2008; Zheng *et al*., 2010). Triglyceride-rich lipoproteins (TRLs) like VLDL and VLDL remnants, as well as chylomicron remnants function to promote atherogenesis independently of LDL (Beatriz *et al*., 2011).

TRLs and their remnants are involved in proatherogenic activities which include enhanced recruitment and attachment of monocytes to the endothelium. This is through the enhanced activity of endothelial and macrophage derived inflammatory proteins such as interleukin (IL)-1β, tumor necrosis factor-α (TNF-α), intercellular adhesion molecule-1, monocyte chemotactic protein-1, and matrix metalloproteinase-3
(Van *et al*., 2000); Together with this, an amplified expression of adhesion molecules also occurs which is implicated in endothelial dysfunction that marks the early signs of atherosclerosis (Kawakami *et al*., 2009; Wang *et al*., 2009). TRLs are also found to suppress the atheroprotective and anti-inflammatory effects of HDL by blocking cholesterol efflux from monocytes and macrophages (Palmer *et al*., 2004; Botham *et al*., 2007; Patel *et al*., 2009).

Lipoprotein lipase (LPL) hydrolyses triglycerides present in chylomicrons and VLDL, it catalyses the cleavage of the *sn*-1 and *sn*-3 ester bonds. It possesses also phospholipase activity much more than its triacylglycerol hydrolase activity. The phospholipase activity is necessary for opening the phospholipid case and to get to the hydrophobic interior of the lipoprotein particles. Lipoprotein lipase is involved in

catabolism and transport of triglycerides. Thus, it has a major role in the disposal of excess lipids from body. Lipoprotein lipase is distributed in many tissues, including striated muscle, adipose tissue, lactating mammary gland and heart. Parenchymal cells within the tissue synthesise the enzyme and it is secreted after posttranslational modifications. Tissue insulin is found to increases LPL activity in the adipose by transcriptional and posttranslational mechanisms. On the other hand, LPL activities in skeletal muscle and heart are elevated in starvation in comparison to its activity in the well-fed state. Physical work out enhances LPL activity in skeletal muscle, while hormone prolactin induces it in lactating mammary gland.

LPL deficiency is found to lower of the clearance of chylomicrons from blood. This may result in an elevated fasting plasma triglyceride level. The elevated plasma TG level, in general is reckoned as a risk factor for atherosclerosis and for other cardiovascular diseases. LPL does not circulate in blood, but is attached on the capillary endothelial cells. In the current study heparin administration prior to blood collection was used for securing the release of LPL from capillary wall. Heparin does not act as physiologic cofactor or activator for the enzyme, but is necessary for the release and subsequent activity of the enzyme. *D. gyrans* extract enhances the activity of LPL in a similar manner. This is significant especially since an enhanced LPL activity is critical

in bringing down high triglyceride level which otherwise may leads to reduction in HDL and increase in atherogenic small dense LDL levels. So the extract which enhances LPL activity together with its proven HDL enhancing activity can be of significant in safeguarding against atherosclerosis and other cardiovascular complications.

The current study has revealed hypolipidemic property of methanol extract of

D. gyrans by way of reducing triglycerides, total cholesterol and LDL fraction of cholesterol, at the same time showing an elevation in HDL fraction. There are a few established agents used in treatment of hyperlipidemia which have got such a range of effects. Statins which are more known as a hypocholesterolemic drug, also lower TG levels in plasma (Stein *et al*., 1998) and reduce concentrations of apo C-III (Sacks *et al*., 2002). Its TG-lowering effect is higher when the baseline TG level is greater. The most pronounced effect of fibrates (another of hypolipidemic drug) is to lower plasma TG (Manninen *et al*., 1992; Scott *et al*., 2009; Ginsberg *et al*., 2010). Though the most noted and potent effects of niacin is raising HDL-C levels, it also lowers TGs (Robins *et al*.,

2001; Kamanna *et al*., 2008;).Omega-3 polyunsaturated fatty acids (PUFAs) have dose-dependent TG-lowering effects resulting from a variety of mechanisms including decreased VLDL secretion and improved TG clearance from VLDL (Harris *et al*., 2008).

6.5. CONCLUSION

It can be concluded that extract of *D. gyrans* is having a remarkable potential of being considered as one of the safest medication for reducing triglycerides as well as cholesterol levels. The induction of lipoprotein lipase enzyme as revealed in the current study can be cited as the reason for its property of lowering triglycerides.

Chapter 7

Study on the Antiatherosclerotic Effect of Desmodium gyrans DC

7. Study on the Antiatherosclerotic Effect of *Desmodium gyrans* DC extract

7.1. INTRODUCTION

7.2. MATERIALS AND METHODS

7.2.1. Animals

7.2.2. Preparation of high fat diet

7.2.3. Protocol for anti-atherogenic study on rabbits

7.2.4. Biochemical analysis

7.2.5. Histopathology studies on liver tissue

7.2.6. Morphology of oil red O stained aorta

7.3. RESULTS

7.3.1. Effect of *Desmodium gyrans* on lipid profile of rabbits

7.3.2. Effect of *Desmodium gyrans* on other serum parameters

7.3.3. Antioxidant assay and lipid peroxidation assay in rabbit liver homogenate

7.3.4. Measurement of HMG-CoA reductase in rabbit liver homogenate

7.3.5. Effect of *Desmodium gyrans* on aortal fatty lesions

7.3.6. Liver morphological appearance

7.3.7. Liver histopathology study

7.4. DISCUSSION

7.5. CONCLUSION

7.1. INTRODUCTION

Atherosclerosis is a chronic, progressive, inflammatory disease with a long asymptomatic phase. Subclinical atherosclerosis eventually leads to cardiovascular dysfunctions such as myocardial infarction, unstable angina pectoris and cardiac arrest (Toth *et al*., 2008). Coronary heart disease (CHD) resulting from progressive atherosclerosis remains the most common cause of mortality in our population (Glass *et al*., 2001). Atherosclerosis is basically a lipid disorder in which the sub-endothelial layer of arteries gets deposited by oxidized low density lipoproteins (LDL). Increased intracellular generation of reactive oxygen species (ROS) has been proposed as a mechanism of tissue injury on the arterial endothelium which leads to a variety of pathological processes like inflammation, ischaemia and thrombosis (Stocker *et al*., 2004; Madamanchi *et al*., 2005; Grassi *et a*l., 2009). Atherogenesis involves other events also like formation of fatty streak which lead to atheromatous plaque that is facilitated by a number of dynamic factors like cell adhesion molecules VCAM-1, ICAM-1 and others like E-selectin, IL 1β, IL 8, tumour necrosis factor alpha (TNF α), platelet derived Growth factor (PGDF), macrophage colony stimulating factor (MCF) and macrophage chemotactic protein (Yangsoo *et al*., 1994; Rallidis *et al*., 2004).

Even though treatment modalities are available to address the problem of atherogenesis and related cardiac abnormalities, a search into indigenous drugs with a long descended heritage of traditional use is of supreme significance. India is rich in medicinal plants that usually find their way to several Ayurvedic preparations and the relative absence of side effects gives these herbal products a special demand (Ostad *et al*., 2001), the reason why people depends on such herbal remedies.

Desmodium gyrans DC which is an annual herb belonging to family Fabaceae. *D. gyrans* is used in Indian traditional and folk medicine since its leaves have diuretic, febrifugal and tonic properties and roots are used as a remedy for asthma, cough, as anti dysenteric and as emollient. It is believed to have cardio-tonic properties and remarkable wound healing effect for which it is commonly used in traditional medicine (Gopalakrishnan and Rajameena, 2012). *D. gyrans* has a long history of use in Chinese

traditional medicine to treat various ailments (Ma X *et al*., 2011). Phytochemical screening and pharmacognostical studies done in the plant have revealed the presence of medicinally significant phytochemicals (Gopalakrishnan and Rajameena, 2014). The antioxidant, anti-inflammatory and antithrombotic properties of *D. gyrans* have been proven in the current study. The *D. gyrans* extract renders significant cardioprotection in experimental animals. It should also be noted that a closely related species, *Desmodium gangeticum* has proven hypolipidemic, cardio protective properties and give protection against cardiac reperfusion injury. Male New Zealand white rabbits were chosen for antiatherosclerotic study since rabbit has been proven to be the best animal to do such studies (Jain *et al*., 2007). Rabbit quickly responds to a constant dose of high fat diet and atheromatous plaque development is faster in rabbits in comparison to other experimental animals (Amalia, 2004). The present study is designed to make a probe into the traditionally assumed medicinal property of *D. gyrans* as cardiotonic herbal plant which has to be made known to the world especially since it has remained a 'virgin plant' and not been the subject of much research works.

7.2. MATERIALS AND METHODS

7.2.1. Animals

Male New Zealand white rabbits (1.75-2.0 kg) used for anti-atherogenic experiment was procured from Small Animal Breeding Station (SABS), Kerala Veterinary and Animal Sciences University, Mannuthy, Kerala, India. The animals were acclimatized for a period of 14 days in uniform hygienic well ventilated cages under standard environmental setting.

7.2.2. Preparation of high fat diet

Standard High Fat Diet (HFD) was made using Bengal gram, sucrose, milk powder, raw cholesterol, hydrogenated fat, salt mineral mixture and egg yolk in the amounts mentioned in table 7.1, which formed nutrients approximately in the following proportions: 55% carbohydrates, 25% proteins and 15% fats (Seethanathan *et al*., 1970). Bengal gram was made into flour and mixed with all other constituents and consistency was regulated by adding minimum amount of sterilized water. The mixture was made into pellets mechanically and dried in oven at 40°C for three days. The perfectly dried pellets were kept closed in containers to avoid fungal infections and used within one week. HFD was given to selected group of animals at the rate of 100 g/ kg body weight along with

oral feeding of cholesterol at 250 mg/kg body weight on alternate days for a period of three months.

7.2.3. Protocol for anti-atherogenic study on rabbits

Anti-atherogenic study was conducted using male New Zealand white rabbits (1.75-2.0 kg). Animals were divided into four groups A, B, C and D of six each. Group A was maintained as normal. Groups B, C and D received HFD at the rate of 100 gm/ kg b. wt. of the animal along with oral feeding of 250 mg/ kg b. wt. of cholesterol dissolved in 5 ml coconut oil on alternate days for 3 months. Among these, group B was treated as control (HFD alone) and *D. gyrans* was administered in group D (200 mg/ kg b. wt. / alternate day) by oral intubation for a period of 3 months (Li X *et al*., 2016; Suresh et al., 2017). Group C received standard drug probucol (10 mg/ kg b. wt. /alternate day) for 3 months and water was given to all groups *ad libitum*. Probucol is a drug having both antioxidant and hypolipidemic properties which render it cardioprotective activity. Its anti-atherogenic effect on hypercholesterolemic rabbits has also been demonstrated (Witting *et al*., 2000; Oshima *et al*., 1998). At the end of the experiment, all overnight fasted animals were sacrificed under ether anaesthesia; blood, liver as well as heart with attached aorta extending to the iliac bifurcation were collected and used for the study.

7.2.4. Biochemical analysis (chapter 2, section 2.2.13.)

Serum was used for analysis of total cholesterol, triglycerides and lipoproteins using standard commercial kits in fully automated analyser EM 360 of Transasia Biomedicals. Liver and renal functions were also analysed. Estimation of serum malondialdehyde (MDA) was done by Valipasha and Sadasivudu's procedure. Apolipoprotein A-I and serum hs-CRP were determined by quantitative turbidimetric immunoassay based on principle of agglutination reaction. Serum paraoxonase activity was measured by comparison of the kinetic rate of change of absorbance of reactions. The assay tube contained 750 μL of Tris buffer, 50 μL of serum (1:2 dilutions) and 200 μL of 6 mmol/ L paraoxon and the formation of *p*-nitrophenol was followed measuring its rate of change of absorbance at 405 nm for 5 min (Tomas *et al*., 2000).

7.2.5. Histopathology studies on liver tissue

Visual observations of liver were made and photographs were taken. Fragments of liver were placed in 10% buffered formaldehyde and 24 hours later they were embedded in paraffin, cut and stained with haematoxylin and eosin (H and E) stain and observed under microscope.

For antioxidant assay on liver tissue, 1 g of liver was weighed and 3 ml of 0.1 M Tris buffer was added to it and homogenised using homogeniser equipment and both enzymatic and non-enzymatic antioxidant assays as well as lipid peroxidation were performed. Indirect measurement of hydroxylmethylglutaryl-CoA reductase (HMG-CoA reductase) enzyme which catalyses the committed step in cholesterol biosynthesis was done by method described by Rao and Ramakrishnan (1975).

7.2.6. Morphology of oil red O stained aorta

Oil red O (Sigma Aldrich) in the powdered form was dissolved in isopropyl alcohol. The excised aorta was cleaned using saline, cut opened and treated with 10% buffered formalin and then washed with 60% isopropyl alcohol. The aorta was stained with oil Red O and fatty streaks which appeared red were photographed.

7.3. RESULTS

7.3.1. Effect of *Desmodium gyrans* extract on lipid profile of rabbits

After 90 days of treatment, serum levels of total cholesterol, triglycerides and LDL-cholesterol were enhanced in the control group when compared to those of the normal group (Table 7.2). Concurrent application of *D. gyrans* caused a significant decrease in lipid values along with a remarkable elevation in the HDL-cholesterol level when compared to that of normal and control groups. The HDL value was elevated from 22 mg/ dl in normal group to 126 mg/ dl in the *D. gyrans* group, along with 21.6% lowering of total cholesterol in *D. gyrans* in comparison to control group. The LDL content lowered by 25.3% in *D. gyrans* compared with control group. The probucol treated group also showed similar hypolipidemic effect but HDL-cholesterol was raised

only to 69 mg/ dl. Moreover atherogenic index which is the ratio of total cholesterol to HDL indicating risk of cardiac abnormalities was lower in *D. gyrans* treated group (5.79) when compared to control (16.3) and probucol treated group (11.44).

7.3.2. Effect of *Desmodium gyrans* extract on serum parameters

Significant enhancement in serum level of Apo A-I fraction and paraoxonase activity was observed in *D. gyrans* treated group (Table 7.3). The Apo A-I was elevated from 31 mg/ dl in normal to 301 mg/ dl in *D. gyrans* treated group while paraoxonase activity enhanced by 27% in *D. gyrans* group. The control animals showed a decrease by 2.76% in paraoxonase activity compared to normal. The probucol treated group also showed moderate elevation of both of these parameters in comparison with the control group. The inflammatory marker, hs-CRP was elevated in the control group to 1.212 mg/ dl from 0.505 mg/ dl in normal indicating possible inflammatory state, but *D. gyrans* and probucol groups brought down the levels to 0.87 and 0.85 mg/ dl, respectively. Lipid peroxidation as shown by the level of MDA in serum was found to be 47.44% lesser in the *D. gyrans* group when compared to control group which gave a significantly high value of MDA in serum.

7.3.3. Antioxidant assay and lipid peroxidation assay in rabbit liver homogenate

Under normal conditions, free radical damage is prevented by antioxidant enzymes directly interacting with ROS (Figure 7.1), shows the activity of SOD, GPx, GSH and levels of lipid peroxidation, in liver tissue obtained from rabbits of different experiment groups. It can be seen from the results that *D. gyrans* enhanced SOD, GPx and GSH levels in liver by 25.07, 10.4 and 23% respectively and reduced lipid peroxidation by 34.03% in comparison to control group.

7.3.4. Measurement of HMG-CoA reductase in rabbit liver homogenate

HMG-CoA reductase activity was measured to assess the inhibitory effect of *D. gyrans* on biosynthesis of cholesterol. The activity of HMG-CoA reductase indirectly measured from the ratio of serum HMG-CoA to mevalonate in different groups under study (Figure 7.2). It can be seen that the ratio is raised in control, *D. gyrans* and standard

groups alike indicating that conversion of HMG-CoA to mevalonate was not favoured and HMG-CoA was accumulated. This revealed a lesser activity of HMG-CoA reductase in all HFD fed animals, which in turn indicated a lowered biosynthesis of cholesterol in all the HFD treated groups when compared to that of normal group.

7.3.5. Effect of *Desmodium gyrans* extract on aortal fatty lesions

Aortal fatty lesions were observed to find out the anti-atherogenic effect of *Des. gyrans*. Control group (C and D) shows artery wall thickened with atheromatous fatty lesions (Figure 7.3) as a result of the accumulation of fatty materials such as cholesterol, promoted by low-density lipoproteins (LDL). It can be compared to normal group (A and B). In the *D. gyrans* treated group (G and H) considerable reduction of fatty lesions can be observed and result of the *D. gyrans* treated group is better when compared to that of probucol treated group (E and F)

7.3.6. Liver morphological appearance

Fatty deposition on liver tissue was studied. In the study, control group was slightly yellow in color and greasy in appearance since it was congested with fat (Figure 7.4). Fatty liver appearance is due to the deposition of fats like triglycerides and cholesterol in liver. Marked decrease in fatty deposition was shown by *D. gyrans* treated group. The morphological appearance of *D. gyrans* group was better when compared to the standard group.

7.3.7. Liver histopathology study

Microscopic photographs of liver tissue stained with hematoxylin and eosin stain (Figure 7.5) shows liver cells filled with multiple fat droplets in the control group (B) that push the nucleus to the periphery of the cell, giving characteristic signet ring appearance (macro vesicular fatty change). These vesicles are well delineated and optically empty because fats dissolve during tissue processing. A reduced fatty appearance was detected in *D. gyrans* treated group (D).

7.4. DISCUSSION

In the current study, oral administration of *D. gyrans* extract, reduced hyperlipidemia and associated arterial fatty streaks in New Zealand white rabbits fed HFD for a period of 90 days. Improved antioxidants in their liver and decreased lipid peroxides in serum were also observed. Increased level of functional HDL and decreased hs-CRP level in the *D. gyrans* extract treated group signifies the anti-atherogenic potential of *D.gyrans*.

In HFD fed Wistar rats, *D. gyrans* extract treatment showed moderately lowered total cholesterol and significantly enhanced HDL cholesterol. Many studies have shown oxidation of LDL as the main cause of atherogenesis and HDL is proven to directly guard LDL against oxidative changes. It has also been reported that HDL could shield endothelium against dysfunctions by preventing cytotoxic effects of LDL (Rosenblat *et al*., 2011) and also prevent uptake of LDL by macrophages. HDL is proven to have antioxidant, anti-inflammatory and anti-thrombotic properties (Barter *et al*., 2004), but it is believed that reverse cholesterol transport (RCT) is the main atheroprotective mechanism of HDL (Remaley et al., 2006; Ueda *et al*., 2007). Hence a significant elevation in HDL level in the *D. gyrans* extract treated group can be considered as one of the factors that can contribute to the cardioprotective effect of the *D. gyrans* extract. However an enhancement of HDL may not be significant unless it is functional in its anti-atherogenic role.

The protective effect of HDL is attributed to its paraoxonase enzyme activity and Apo A-I content (Fuhrman *et al*., 2005; Laura *et al*., 2010;), both of which have been significantly enhanced by treatment with *D. gyrans* extract in HFD fed New Zealand white rabbits. This confirms that the raised HDL is functional. Several studies have shown that paraoxonase level is tightly linked with protective role of HDL (Durrington *et al*., 2011) and contributes to the protection conferred by HDL against LDL oxidation (Rotllan *et al*., 2008). The importance of paraoxonase in preventing atherosclerosis has been shown in paraoxonase deficient mice. Associated with HDL, Apo A-I protein enhances reverse cholesterol transport from tissues to the liver for excretion. Apo A-I is seen occupying the outer layer of the HDL particles and constitutes about 70% of the protein content of HDL.

Apo A-I has been shown to bind cholesterol *in vitro* and *in vivo*, the mechanism by which it ensures the uptake of LDL as the HDL particles move through blood circulation. It is also shown that supplementation with HDL or Apo-A-I into humans can increase RCT (Ohashi *et al*., 2005). Apo A-I is a cofactor for lecithin cholesterol acyl transferase (LCAT) which helps formation of plasma cholesteryl esters. Apo A-I can remove oxidized phospholipids from oxidized LDL and specific methionine residues on it have been shown to reduce cholesterol ester hydroperoxides and phophatidyl choline hydroperoxides. Apo A-I isolated as a prostacyclin (PG I2) stabilizing factor is known to have an anti-clotting effect also (Yui *et al*., 1988). In the present study *D. gyrans* extract treated group animals have shown both a significant elevation in functional HDL and an elevation in molecules that contribute to the functionality of HDL. Atherogenic index which is the ratio of total cholesterol to HDL indicating risk of cardiac abnormalities was very low in *D. gyrans* extract treated group when compared to control and probucol treated groups. Clinically there exists an inverse relation between circulating levels of HDL and the incidence of atherosclerosis and this relation has been proven to be even independent of LDL levels (Franceschini *et al*., 2001). There has been a growing interest in finding out new therapeutic interventions to enhance HDL level in addition to the strategies already available like administration of statins, fibrates, niacin, Apo-AI and CETP inhibitors. Hence enhancement of functional HDL level by treatment with *D. gyrans* extract comes as the most significant finding of the study.

Liver tissue HMG CoA reductase enzyme activity assessment reveals that there is reduction in cholesterol biosynthesis indicated by the insufficient synthesis of this rate limiting enzyme. But a fall in cholesterol biosynthesis is observed in all HFD fed groups and this may be due to a feedback mechanism working on all animals which reduce biosynthesis with feeding of HFD. So the reduced activity of HMG CoA reductase cannot be credited to the activity of *D. gyrans* extract. But results show that reduction of total cholesterol has resulted with treatment of *D. gyrans* extract when compared to the control and this point to another mechanism working on *D. gyrans* extract group alone which can be HDL driven RCT. Hence enhanced level of functional HDL cholesterol is assumed to play the important role in bringing down cholesterol level in *D. gyrans* extract treated animals.

The fatty liver buildup as a result of excess cholesterol and triglycerides is well pronounced in the control group both in morphological appearance and histopathological examinations. Diffused fat accumulation with greasy yellowish appearance of liver presents morphological fatty liver which was detected in control group. In histopathology study, the liver showed plenty of vacuolation in the control group, which is a sign of fat deposition over liver tissue. But in the *D. gyrans* extract treated group the symptoms have been significantly ameliorated. This indicates that as a consequence of RCT, a comparatively enhanced turnover of lipids might have occurred which led to the lesser deposition of fat in the liver of *D. gyrans* extract treated group.

Atherosclerosis is considered as an inflammatory disease (Ross *et al*., 1999; Ridker *et al*., 2004) and studies have shown that pro-inflammatory factors can contribute to atherogenesis by hindering RCT and cholesterol efflux (Libby *et al*., 2002). In the current study, the level of inflammation as revealed by inflammatory marker hs-CRP is higher in control group when compared to the *D. gyrans* extract treated group. This result is in agreement with the variations in atheromatous plaques observed in the oil red O stained aorta of these groups of animals. The aortal fatty deposition and ensuing inflammatory appearance have been ameliorated in the *D. gyrans* extract treated group. Apart from the HDL enhancing effect and ensuing RCT, intervention of phytochemicals of the extract with any one of the molecules involved in T-cell activation, smooth-muscle migration, foam cell formation, and platelet adherence and aggregation may be sufficient to reduce inflammation which eventually helps to retard the process of atherogenesis. This opens up possibility for further studies on the mechanisms of action of the extract.

7.5. CONCLUSION

D. gyrans extract is having hypolipidemic activity and a very significant effect on elevating functional HDL cholesterol level and reducing aortal fatty accumulation in rabbits. It reduced inflammatory changes on aorta, improved the activity of liver antioxidant enzymes and is especially effective in inhibiting lipid peroxidation. It is giving reasonable protection to the liver against fatty changes by way of controlling lipid levels. *D. gyrans* extract may possibly enhance reverse cholesterol transport since it has an effect in elevating functional HDL cholesterol and its associated molecules. The study reveals a

promising anti-atherogenic efficacy of *Desmodium gyrans* and validates its use in traditional medicine as cardiotonic.

Summary and Conclusions

D.gyrans extract is having a rich variety of phytochemicals including alkaloids, phenolic compound, flavonoids and terpene compounds which are usually associated with medicinal properties. The medicinal properties of *D. gyrans* can be credited to the presence of these phytochemicals in it which resulted in the distinctive antioxidant properties and anti inflammatory activities revealed in the study. These findings validate the use of this plant in traditional medicine.

The study recommends *D. gyrans* extract as an effective anticoagulant and antithrombotic agent. This could possibly contribute to the perceived cardioprotective and antiathrosclerotic properties that *D. gyrans* possess.

D. gyrans showed significant cardioprotective properties.The study done with the objective of finding how *D. gyrans* becomes 'good for heart' lead to the conclusion that it is effective against ISO induced myocardial infarction whereas it shows reasonable protection against DOX induced cardiomyopathy.

D. gyrans gave protection to the liver against fatty changes by way of controlling lipid levels in experimental animals. It can be concluded that extract of *D. gyrans* is having a remarkable potential of being considered as one of the safest medication for reducing triglycerides as well as cholesterol levels. The induction of lipoprotein lipase enzyme as revealed in the current study can be cited as the reason for its property of lowering serum triglycerides.

D. gyrans extract is having hypolipidemic activity and a very significant effect on elevating functional HDL cholesterol level and reducing aortal fatty accumulation in rabbits. It reduced inflammatory changes on aorta, improved the activity of liver antioxidant enzymes and is especially effective in inhibiting lipid peroxidation. It is giving protection to the liver against fatty changes by way of controlling lipid levels.

D. gyrans extract may possibly enhance reverse cholesterol transport since it has an effect in elevating functional HDL cholesterol and its associated molecules. The study thus revealed a promising hypolipidemic and anti-atherogenic efficacy of *Desmodium gyrans* and validates its use in traditional medicine as cardiotonic.

REFERENCES

Abrams J. Role of endothelial dysfunction in coronary artery disease. *Am J Cardiol*; 1997, 79, 2-9.

Abrar H M, Manjusha Sexena, Mohd Yousuf Malla. An acute oral toxicity study of methanolic extract from *Tridex procumbens* in Sprague Dawley's Rats as per OECD guidelines 423. *Asian J Plant Sci Res*; 2013, 3, 16-20.

Abu-Soud H M, Hazen S L. Nitric oxide is a physiological substrate for mammalian peroxidases. *J Biol Chem*; 2000, 275, 524-532.

Acker FAV, Hulshof J W, Haenen G R, Menge W M, Vijgh WJV, Bast A. New synthetic flavonoids as potent protectors against doxorubicin-induced cardiotoxicity. *Free Rad Bio Med*; 2001, 31, 31-37.

Adam P, Hecht S, Eisenreich W G. Biosynthesis of terpenes: studies on 1-hydroxy-2-methyl-2-(*E)*-butenyl 4-diphosphate reductase. *Proc Natl Acad Sci*; 2002, 99, 108-113.

Addy M E, Awumey E M. Effects of the Extract of Desmodium adscendens on Anaphylaxis. *J Ethnopharmacol*; 1984, 11, 282-292.

Addy M E, Burka J F. Effects of *Desmodium adscendens* fractions on antigen-and arachidonic acid-induced contractions of guinea pig airways. *Canadian J Physiol Pharmacol*; 1988, 66, 820-825.

Addy M E. An extract of *Desmodium adscendens* activates cyclooxygenase and increases prostaglandin synthesis by ram seminal vesicle microsomes. *Phytother Res*; 1995, 9, 287-293.

Alban S. Pharmacological strategies for inhibition of thrombin activity. *Curr Pharm Design*; 2008, 14, 1152-1175.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Molecular Biology of the Cell (4th ed.), Garland Science publishers, New York. 2002.

Alexander T, Robert K, Enrique Z F. Hypertriglyceridemia: a too long unfairly neglected major cardiovascular risk factor. *Cardiovascular Diabetology*; 2014, 13, 159-169.

Alscher R G, Erturk N, Heath L S. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *J Exp Bot*; 2002, 53, 1331-1345.

Amalia E Y. The laboratory rabbit: an animal model of atherosclerosis research. *Laboratory Animals*; 2004, 38, 246-256.

Ameenah G F. Medicinal plants: Traditions of yesterday and drugs of tomorrow. *Curr Med Chem*; 2006, 11, 1451-1460.

Ampofo O. Plants that heal. *World Health*; 1977, *26*, 28-34

Anitschkow. Experimental arteriosclerosis in animals, in Cowdry EV (2nd ed). Arteriosclerosis: A Survey of the Problem, Macmillan, New York, 1933.

Anna J W, Jerzy B. Adverse effects of statins. *Intl J Pharmacol*; 2005, 1, 210-225.

Ansell J, Hirsh J, Hylek E. Pharmacology and management of the vitamin K antagonists: American College of Chest Physicians evidence-based clinical practice guidelines (8th Edition). *Chest*; 2008, 133, 160-198.

Ansell J, Hirsh J, Poller L, Bussey H, Jacobson A, Hylek E. The pharmacology and management of the vitamin K antagonists: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy. *Chest*; 2004, 126, 204-233.

Antoniades C, Tousoulis D, Stefanadis C. Effect of endothelial nitric oxide synthase gene polymorphisms on oxidative stress, inflammatory status and coronary atherosclerosis: an example of transient phenotype. *J Am Coll Cardiol*; 2007, 49, 1226-234.

Aparna V, Dileep K V, Mandal P K, Karthe P, Sadasivan C, Haridas M. Anti-inflammatory property of n-hexadecanoic acid: structural evidence and kinetic assessment. *Chem Biol Drug Design*; 2012, 80, 434-439.

Aquino R, Morelli S, Lauri M R, Abdo S, Saija A, Tomaino. Phenolic constituents and antioxidant activity of an extract of *Anthurium versticular* leaves. *J National Production*; 2001, 64, 1019-1023.

Arthur C G, John E H. Text Book of Medical Physiology. *Elsevier Saunders*; 2006, 36, 457-468.

Asgary S, Jafari Dinani N, Madani H, Mahzoni P, Naderi G H. Effect of *Glycyrrhiza glabra* extract on aorta wall atherosclerotic lesion in hypercholesterolemic rabbits. *Pak J Nutr*; 2006, 5, 313-317.

Assmann G, Schulte H, Von Eckardstein A. Hypertriglyceridemia and elevated lipoprotein (a) are risk factors for major coronary events in middle-aged men. *Am J Cardiol*; 1996, 77, 1179-1184.

Auer J, Berent R, Lassnig E, Eber B. C-reactive protein coronary artery disease. *Jpn Heart J*; 2002, 43, 607-619.

Aviram M, Hardak E, Vaya J, Mahmood S, Milo S, Hoffmann A, Billicke S, Draganov D, Rosenblat M. Human serum Paraoxonases (PON1) Q and R selectively decrease lipid peroxides in human coronary and carotid atherosclerotic lesions: PON1 esterase and peroxide-like activities. *Circulation*; 2000, 101, 2510-2517.

Aviram M, Rosenblat M, Bisgaier C L, Newton R S, Primo-Parmo S L, La Du B N. Paraoxonase inhibits high-density lipoprotein oxidation and preserves its functions. A possible peroxidative role for paraoxonase. *J Clin Invest*; 1998, 101, 1581-1590.

Babior B M, Kipnes R S, Curnutte J T. The Productions by leukocytes of superoxide; a Potential Bactericidal Agent. *J Clin Invest*; 1973, 52, 741-757.

Bahorun T, Soobrattee M A, Luximon-Ramma V, Aruoma O I. Free radicals and antioxidants in cardiovascular health and disease. *Internet J Med Update*; 2006, 1, 1-17.

Baigent C, Keech A, Kearney P M. Cholesterol treatment trialists collaborators. efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. *Lancet*; 2005, 366, 1267-1278.

Balabhaskar R, Vijayalakshmi K. Preliminary phytochemical and pharmacognostic analysis of *Bauhinia tomentosa*. *J Chem Pharma Res*; 2015, 7, 271-277.

Ballou D P, Zhao Y, Brandish P E, Marletta M A. Revisiting the kinetics of nitric oxide (NO) binding to soluble guanylate cyclase: the simple NO-binding model is incorrect. *Proc Natl Acad Sci*; 2002, 99, 12097-12101.

Baranano D E, Rao M, Ferris C D, Snyder S H. Biliverdin reductase: a major physiologic cytoprotectant. *Proc Natl Acad Sci*; 2002, 99, 16093-16098.

Barrett-Connor E, Bush T L. Estrogen and coronary heart disease in women. *JAMA*; 1991, 265, 1861-1867.

Barter P J, Nicholls S, Anantharamaiah G M, Navab M, Fogelman A M. Anti-inflammatory Properties of HDL. *Circulation Research*; 2004, 95, 764-772.

Barter P J, Puranik R, Rye K A. New insights into the role of HDL as an anti-inflammatory agent in the prevention of cardiovascular disease. *Curr Cardiol Rep*; 2007, 9, 493-498.

Basson M D, Panzini L, Palmer R H. Effect of nabumetone and aspirin on colonic mucosal bleeding time. *Aliment Pharmacol Ther*; 2001, 15, 539-554

Beasley D, Schwartz J H, Brenner B M. Interleukin-1 induces prolonged L arginine dependent cyclic guanosine monophosphate and nitrite production in rat vascular smooth muscle cells. *J clin investi*; 1991, 87, 602-603.

Beatriz G, Frank M. Sacks. The Role of Triglycerides in Atherosclerosis. *Curr Cardiol Rep*; 2011, 13(6), 544-552.

Beisiegel U, Weber W, Bengtsson-Olivecrona G. Lipoprotein lipase enhances the binding of chylomicrons to low density lipoprotein receptor-related protein. *Proc Natl Acad Sci;* 1991, 88, 8342-8346.

Bellosta S, Corsini A. Statin drug interactions and related adverse reactions. *Expert Opin Drug Saf*; 2012, 11, 933-946.

Beltowski J, Wojcicka G, Jamroz A, Leptons decreases plasma paraoxonase 1 (PON1) activity and induces oxidative stress: the possible novel mechanism for proatherogenic effect of chronic hyperleptinemia. *Atherosclerosis*; 2003, 170, 21-29.

Benzie I F F, Strain J J. The ferric reducing ability of plasma as a measure of antioxidant power. *Anal Biochem*; 1996, 239, 70-76.

Berry C E, Hare J M. Xanthine oxidoreductase in the cardiovascular system: Molecular mechanisms and pathophysiologic implications. *J Physiol*; 2004, 555, 589-606.

Berthou L, Duverger N, Emmanuel F, Langouet S, Auwerx J, Guillouzo A, Fruchart J C, Rubin E, Denefle P, Staels B, Branellec D. Opposite regulation of human versus mouse apolipoprotein A-I by fibrates in human apo A-I transgenic mice. *J Clin Invest*; 1996, 97, 408-416.

Bishayee S, Balasubramanyam A S. Assay of lipid peroxide formation. *J Neurochem*; 1971, 18, 909-915.

Bisoendial R J, Kastelein J J, Stroes E S. C-reactive protein and atherogenesis: from fatty streak to clinical event. *Atherosclerosis*; 2007, 195, 10-18.

Blades B, Vega G L, Grundy S M. Activities of lipoprotein lipase and hepatic triglyceride lipase in postheparin plasma of patients with low concentrations of HDL cholesterol. *Artm'oscler Thromb*; 1993, 13, 1227-1235.

Blake G J, Ridker P M. Novel clinical markers of vascular wall inflammation. *Circ Res*; 2001, 89, 763-771.

Blankenberg S, Tiret L, Bickel C. Interleukin-18 is a strong predictor of cardiovascular death in stable and unstable angina.*Circulation*; 2002, 106, 24-30.

Boamponsem A G, Boamponsem L K. The Role of Inflammation in Atherosclerosis. *Adv in Applied Sci Res*; 2011, 2, 194-207.

Bobkova D, Poledne R. Lipid metabolism in atherogenesis. *Cesk Fysiol*; 2003, 52, 34-41.

Bobryshev Y V. Monocyte recruitment and foam cell formation in atherosclerosis. *Micron*; 2006, 37, 208-22.

Boffa M B, Marcovina S M, Koschinsky M L. Lipoprotein(a) as a risk factor for atherosclerosis and thrombosis: mechanistic insights from animal models.*Clin Biochem*; 2004, 37, 333-343.

Bonetti P O, Lerman L O, Lerman A. Endothelial dysfunc tion- a marker of atherosclerotic risk. *Arterioscler Thromb Vasc Biol*; 2003, 23, 168-175.

Bonn V, Cheung R C, Chen B, Taghibiglou C, Van Iderstine S C, Adlei K. Simvastatin, an HMG-CoA reductase inhibitor, induces the synthesis and secretion of apolipoprotein AI cells in HepG2 cells and primary hamster hepatocytes. *Atherosclerosis*; 2002, 163, 59-*68.*

Boon E M, Downs A, Marcey D. Catalase: H_2O_2: H_2O_2 Oxidoreductase. *Catalase Structural Tutorial Text*. Retrieved 2007-02-11.

Botham K M, Moore E H, De Pascale C. The induction of macrophage foam cell formation by chylomicron remnants. *Biochem Soc Trans*; 2007, 35, 454-458.

Boucek Jr. R J, Olson R D, Brenner D E, Ogunbunmi E M, Inui M, Fleishner S. The major metabolite of doxorubicin is a potent inhibitor of membrane-associated ion pumps. *J BiolChem*; 1987, 262, 15851-15856.

Braun J E, Severson D L. Regulation of the synthesis, processing and translocation of lipoprotein lipase. *Biochem J*; 1992, 287, 337-47.

Brinton E A, Eisenberg S, Breslow J L. Increased apoA-I and apoA-I1 fractional catabolic rate in patients with low high density lipoprotein-cholesterol levels with or without hypertriglyceridemia. *J Clin Invest*; 1991, 87, 536-544.

Briskin D P. Medicinal plants and phytomedicines. linking plant Biochemistry and Physiology to human health. *Plant Physio*; 2000, 124, 507-514.

Brown B G, Zhao X Q, Bardsley J. Secondary prevention of heart disease amongst patients with lipid abnormalities. Practice and trends in the United States. *J Intl Med*; 1997, 241-283.

Brown B G, Zhao X Q, Chait A. Simvastatin and niacin, antioxidant vitamins, or the combination for the prevention of coronary disease. *N Engl J Med*; 2001, 345, 1583-1592.

Buckingham J. Dictionary of Natural Products. Version 9.2 on CD-ROM. Chapman & Hall/CRC Press, London, New York, 2004.

Buring J E, O'Connor G T, Goldhaber S Z, Rosner B, Herbert P N, Blum C B, Breslow J L, Hennekens C H. Decreased HDL2 and HDL3 cholesterol, Apo A-I and Apo A-II, and increased risk of myocardial infarction. *Circulation*; 1992, 85, 22-29.

Caligiuri G, Rudling M, Ollivier V, Jacob M P, Michel J B. Interleukin-10 deficiency increases atherosclerosis, thrombosis, and low-density lipoproteins in apolipoprotein E knockout mice. *Mol Med*; 2003, 9, 10-17.

Camila C, Mirian S S, Vanine G M, Luciana M C, Reinaldo N A. Antinociceptive and antioxidant activities of phytol *in vivo* and *in vitro* models. *Neuroscience J*; 2013, 1, 1-9.

Carmela F, Michael N, Rossano C, Renato I, Giorgio C F, Patrizia H. Growth inhibition, cell-cycle arrest and apoptosis in human T-cell leukemia by the isothiocyanate sulforaphane. *Carcinogensis*; 2002, 23, 581-586.

Carpenter K L, Taylor S E, Van der Veen C, Williamson B K, Ballantine J A, Mitchinson M J. Lipids and oxidised lipids in human atherosclerotic lesions at different stages of development. *Biochim Biophys Acta*; 1995, 1256, 141-150.

Carr A C, McCall M R, Frei B. Oxidation of LDL by myeloperoxidase and reactive nitrogen species:reaction pathways and antioxidant protection. *Atreriosclero Throm Vascu Biol*; 2000, 20, 1716-1723.

Castleman M. The New Healing Herbs. Hinkler Books Pty Ltd., Dingley, Australia, 2003.

Chan A C. Vitamin E and atherosclerosis. *J Nutr*; 1998, 128, 1593-1596.

Chelikani P, Fita I, Loewen P C. Diversity of structures and properties among catalases. *Cell Mol Life Sc*; 2004, 61, 192-208.

Chen Y R, Han J, Kori R. Phenylethyl isothiocyanate induce apoptotic signaling via suppressing phosphatase activity against c-Jun N-terminal kinase. *J Biol Chem*; 2002, 277, 39334-39342.

Chi L, Li Y, Stehno-Bittel L. *J Interferon Cytokine Res*; 2001, 21, 231-239.

Chopra R N, Nayar S L, Chopra I C. Glossary of Indian Medicinal plants (1st ed.), Compounds in Allium species for human low density lipoprotein oxidation in vitro. *J Agric Food Chem*; 2003, 51, 7208-7214.

Corpas F J, Barroso J B, del Rio L A. Peroxisomes as a source of reactive oxygen species and nitric oxide signal molecules in plant cells.*Trends Plant Sci*; 2001, 6, 145-50.

Corpas F J, Fernandez Ocana A, Carreras A, Valderrama R, Luque F, Esteban F J, Rodriguez-Serrano M, Chaki M, Pedrajas J R, Sandalio L M, del Rio L A, Barroso J B. The expression of different superoxide dismutase forms is cell-type dependent in olive (*Olea europaea* L.) leaves. *Plant Cell Physiol*; 2006, 47, 984-994.

Couto Narciso, Malys Naglis, Gaskell Simon, Barber Jill. Partition and Turnover of Glutathione Reductase from Saccharomyces cerevisiae: a Proteomic Approach. *J Proteome Res*; 2013, 12, 2885-2894.

Cravotto G, Boffa L, Genzini L, Garella D. Phytotherapeutics: an evaluation of the potential of 1000 plants. *J Clin Pharm Ther*; 2010, 35, 11-48.

Crowther M A. Pathogenesis of atherosclerosis. *Am Soc Hematol*; 2005, 1, 436-445.

Cunha A M, Menon S, Menon R, Couto A G, Burger C, Biavatti M W. Hypoglycemic activity of dried extracts of *Bauhinia forficata* Link. *Phytomedicine*; 2010, 17, 37-41.

Cushing S D, Berliner J A, Valente A J, Territo M C, Navab M, Parhami F, Gerrity R, Schwartz C J, Fogelman A M. Minimally modified low density lipoprotein induces monocyte chemotactic protein (MCP-1) in human endothelial and smooth muscle cells. *Proc Natl Acad Sci*; 1990, 87, 5134-5138.

Dahlof B, Devereux R B, Kjeldsen S E. Cardiovascular morbidity and mortality in the Losartan Intervention For Endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol. *Lancet*; 2002, 359, 995-1003.

Dalle-Donne I, Rossi R, Colombo R, Giustarini D and Milzani A. Biomarkers of oxidative damage in human disease. *Clin Chem*; 2006, 52, 601-623.

Danesh J, Collins R, Peto R. Lipoprotein (a) and coronary artery disease: metaanalysis of prospective studies. *Circulation*; 2000, 102, 1082-1085.

Daud A, Habib N, Riera S. Anti-inflammatory, anti-nociceptive and antipyretic effects of extracts of *Phrygilanthus acutifolius* flowers. *J Ethnopharmacol*; 2006, 108, 198-203.

David L, Nigel K, Michael M, Denise O. Practical Hemostasis and Thrombosis (2nd ed), Wiley-Blackwell publishing Ltd., 2009.

Davignon J, Ganz P. Role of endothelial dysfunction in atherosclerosis. *Circulation*; 2004, 15, 109-115.

Dekkers J C, Van Doornen L J, Kemper H C. The Role of Antioxidant Vitamins and Enzymes in the Prevention of Exercise-Induced Muscle Damage. *Sports Med*; 1996, 21, 213-238.

Del Rio D, Stewart A J, Mullen W. HPLC-MS*n* analysis of phenolic compounds and purine alkaloids in green and black tea. *J Agric Food Chem*; 2004, 52, 2807-2815.

Demer L L, Tintut Y. Vascular calcification: pathobiology of a multifaceted disease. *Circulation*; 2008, 117, 2938-2948.

Demerdash, Ali A A, Ahmed MS, Osman AM. New aspects in probucol cardioprotection against doxorubicin induced cardio toxicity. *Cancer Chemothpharm*; 2003, 52, 411-416.

Detrano R C, Doherty M, Davies M, Stary H C. *Curr Probl Cardiol*; 2000, 25, 374-402.

Dewick P M. The mevalonate and deoxyxylulose phosphate pathways: terpenoids and steroids. In P M. Dewick (2nd ed.), Medicinal Natural Products: A Biosynthetic Approach, John Wiley & Sons Ltd., Chichester, 2001.

Dfeudis F V, *Ginkgo biloba* extract (Egb 761): Pharmacological activities and clinical applications. Elsevier editions, Scientifiques, 1991.

Dhalla N S, Temsah R M, Netticadan T. Role of oxidative stress in cardiovascular diseases. *J Hypertens*; 2000, 18, 655-673.

Diet F, Pratt R E, Berry G J. Increased accumulation of tissue ACE in human atherosclerotic coronary artery disease. *Circulation*; 1997, 94, 2756-2767.

Dimitrios K, Dimitrios C. Pleiotropic vasoprotective effects of statins: The chicken or the egg. *Drug Des Devel Ther*; 2009, 3, 191-204.

DiRosa, M, Giroud J P, Willoughby D A. Studies of the acute inflammatory response induced in rats in different sites by carrageenan and turpentine. *J Pathol*; 1971, 104, 15-29.

Donald T W, Howard A N, Guiragos K P, William L, Shankar K S. Comparative antilipidemic effects of various ethyl 5-substituted benzofuran, 2, 3-dihydrobenzofuran, and 3(2H)-benzofuranone-2-carboxylate analogs of clofibrate in a triton hyperlipidemic rat model. *Lipids*; 1976, 11, 384-391.

Doroshow J H. Doxorubicin-induced cardiac toxicity. *N Eng J Med*; 1991, 324, 843-845.

Dullaart R P, Hoogenberg K, Dikkeschei B D, Van Tol A. Higher plasma lipid transfer protein activities and unfavorable lipoprotein changes in cigarette-smoking men. *Arterioscler Thromb*; 1994, 14, 1581-1585.

Durrington P N, Mackness B, Mackness M I. Paraoxonase and atherosclerosis. *Arterioscler Thromb Vasc Biol*; 2001, 21, 473-480.

Durrington P N. Triglycerides are more important in atherosclerosis than epidemiology has suggested. *Atherosclerosis*; 1998, 141, 1, 57-62.

Durstine J L, Pate R R, Sparling P B, Wilson G E, Senn M D, Bartoli W P. Lipid, lipoprotein, and iron status of elite women distance runners. *Int J Sports Med*; 1987, 8, 119-123.

Duvillard L, Florentin E, Lizard G, Petit J M, Galland F, Monier S, Gambert P, Verges B. Cell surface expression of LDL receptor is decreased in type 2 diabetic patients and is normalized by insulin therapy. *Diabetes Care*; 2003, 26, 1540-1544.

Dwyer J H, Navab M, Dwyer K M, Hassan K, Sun P, Shircore A, Hama-Levy S, Hough G, Wang X, Drake T, Merz C N, Fogelman A M. Oxygenated carotenoid lutein and progression of early atherosclerosis: the Los Angeles atherosclerosis study. *Circulation*; 2001, 103, 2922-2927.

Ebrahimzadeh M A, Pourmorad F and Bekhradnia A R. Iron chelating activity screening, phenol and flavonoid content of some medicinal plants from Iran. *Afr J Biotechnol*; 2008, 32, 43-49.

Eckel R H, Krauss R M. American Heart Association call to action: obesity as a major risk factor for coronary heart disease: AHA Nutrition Committee. *Circulation*; 1998, 97, 2099-2100.

Eisenreich W, Bacher A, Arigoni D, Rohdich F. Biosynthesis of isoprenoids *via* the non-mevalonate pathway. *Cell Mol Life Sci*; 2004, 61, 1401-1426.

Elizabeth K, Rao M N A. Oxygen radical scavenging activity of curcumin. *Int J Pharmaceut*; 1990, 58, 237-240.

Elvin-Lewis M. Should we be concerned about herbal remedies. *J Ethnopharmacol* ; 2001, 75, 141-164.

Eren E, Yilmaz N, Aydin O. Functionally Defective High-Density Lipoprotein and Paraoxonase: A Couple for Endothelial Dysfunction in Atherosclerosis. *Cholesterol*; 2013, 1, 2-10.

Ertekin V, Selimoglu M A, Altinkaynak S A combination of unusual presentations of *Datura stramonium* intoxication in a child: rhabdomyolysis and fulminant hepatitius. *J Emerg Med*; 2005, 28, 227-228.

Eto M, Rathgeb L, Cosentino F, Kozai T, Luscher T F. Statins blunt thrombin-induced down-regulation of endothelial nitric oxide synthase expression in human endothelial cells. *J Cardiovasc Pharmacol*; 2006, 47, 663-667.

Fabio , Luigi G. Herbal medicine today: clinical and research issues. *Evid Based Complement Alternat Med*; 2007, 4, 37-40.

Fabricant D S, Farnsworth N R. The value of plants used in traditional medicine for drug discovery. *Environ. Health Perspect*; 2001, 109, 69-75.

Facchini P J. Alkaloid biosynthesis in plants: Biochemistry, cell biology, molecular regulation, and metabolic engineering applications. *Annu Rev Plant Physio Plant Mol Biol*; 2001, 52, 29-66.

Fahey J W, Zalcmann A T, Talalay P. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry*; 2001, 56(1), 45-51.

Ferreira A A, Yeum K J, Matsubara L S, Matsubara B B, Correa C R. Doxorubicin as an antioxidant: Maintenance of myocardial levels of lycopene under doxorubicin treatment. *Free Rad Bio Med*; 2007, 43, 740-751.

Ferretti G, Bacchetti T, Busni D, Rabini R A, Curatola G. Protective effect of paraoxonase activity in high-density lipoproteins against erythrocyte membranes peroxidation: a comparison between healthy subjects and type 1 diabetic patients. *J Clin Endocrinol Metab*; 2004, 89, 2957-2962.

Fielding C J, Fielding P E. Molecular physiology of reverse cholesterol transport. *J Lipid Res;* 1995, 36, 211-228.

Fimognari C, Nusse M, Cesari R, Iori R, Cantelli-Forti G, Hrelia P. Growth inhibition, cell-cycle arrest and apoptosis in human T-cell leukemia by the isothiocyanate sulforaphane. *Carcinogenesis*; 2002, 23, 581-586.

Flammer A J, Anderson T, Celermajer D S, Creager M A, Deanfield J, Ganz P, Hamburg N M, Luscher T F, Shechter M, Taddei S, Vita J A, Lerman A. The assessment of endothelial function: from research into clinical practice. *Circulation*; 2012, 126, 753-767.

Fletcher G F, Blair S N, Blumenthal J, Caspersen C, Chaitman B, Epstein S. Statement on exercise-Council on Clinical Cardiology, American Heart association. *Circulation*; 1992, 86, 340-344.

Forrest G L, Gonzalez B. Carbonyl reductase. *Chem. Biol. Interact*; 2000, 129, 21-40.

Franceschini G. Epidemiologic evidence for high-density lipoprotein cholesterol as a risk factor for coronary artery disease. *Am J Cardio*; 2001, 88, 9-13.

Fraser P D, Bramley P M. The biosynthesis and nutritional uses of carotenoids. *Prog Lipid Res*; 2004, 43, 228-265.

Frei B, Gaziano J M. Content of antioxidants, preformed lipid hydroperoxides, and cholesterol as predictors of the susceptibility of human LDL to metal ion-dependent and - independent oxidation. *J Lipid Res*; 1993, 34, 2135-2145.

Frei B. Ascorbic acid protects lipids in human plasma and low-density lipoprotein against oxidative damage. *Am J Clin Nutr*; 1991, 54, 1113-1118.

Fuhrman B, Gantman A, Aviram M. Paraoxonase 1 (PON1) deficiency in mice is associated with reduced expression of macrophage SR-BI and consequently the loss of HDL cytoprotection against apoptosis. *Atherosclerosis*; 2010, 211, 61-68.

Funda S P, Hakan G. The role of free radicals in ethiopathogenesis of diseases. *Adv in Mol Biol*; 2008, 1, 1-9.

Gaetani G F, Ferraris A M, Rolfo M, Mangerini R, Arena S, Kirkman H N. Predominant role of catalase in the disposal of hydrogen peroxide within human erythrocytes. *Blood*; 1996, 87, 1595-1599.

Gagne S E. A common truncation variant of lipoprotein lipase (Ser447X) confers protection against coronary heart disease: the Framingham Offspring Study. *Clin Genet*; 1999, 55, 450-454.

Galkina E, Kadl A, Sanders J, Varghese D, Sarembock I J, Ley K. Lymphocyte recruitment into the aortic wall before and during development of atherosclerosis is partially L-selectin dependent. *J Exp Med*; 2006, 203, 1273-1282.

Galkina E, Ley K. Leukocyte influx in atherosclerosis. *Curr Drug Targets*; 2007, 8, 1239-1248.

Galle J, Hansen-Hagge T, Wanner C, Seibold S. Impact of oxidised low density lipoprotein on vascular cells. *Atherosclerosis*; 2006, 185, 219-226.

Ganesh D G, Rahul Zine, Mukund R M. High sensitivity c-reactive protein, paraoxonase-1 and high Density lipoprotein cholesterol in myocardial infarction, *Int J Biomed Adv Res*; 2012, 3, 637-639.

Gardner C D, Lawson L D, Block E, Chatterjee L M, Kiazand A, Balise R R, Kraemer H C. Effect of Raw Garlic vs Commercial Garlic Supplements on Plasma Lipid Concentrations in Adults with Moderate Hypercholesterolemia: A Randomized Clinical Trial. *Arch Inter Med*; 2007, 167, 346-53.

Gayathri, Karthiga, Brindha, Selvaraj. Role of simvastatin in the reduction of oxidative stress in isoproterenol induced myocardial infarction in rats. *Drug Inven Today*; 2011, 3, 235-238.

Gaziano T A, Gaziano M J. In Harrisonis Principles of Internal Medicine (17^{th} ed.), McGraw-Hill, New York, 2008.

Genestra M. Oxyl radicals, redox-sensitive signalling cascades and antioxidants. *Review Cell Signal*; 2007, 19, 1807-1819.

Ghani A. Medicinal plants of Bangladesh with chemical constituents and uses (2nd ed.), Asiatic Society of Bangladesh, Dhaka, 2003.

Ghosal R, Srivastava S, Banerjee P K, Dutta S K. Alkaloids of *Desmodium triflorum. Phytochemistry*; 1971, 10, 3313-3314

Ghosh D, Anandkumar A. Anti-inflammatory and analgesic activities of gangetin, a pterocarpenoid from Desmodium gangeticum. *Indian J Pharmacol*; 1981, 15, 391-402.

Giangrande P F. Six character in search of author the history of the nomenclature of coagulation factors. *Br J Haematol*; 2003,121, 703-712.

Gianturco S H, Ramprasad M P, Song R. Apolipoprotein B-48 or its apolipoprotein B-100 equivalent mediates the binding of triglyceride rich lipoproteins to their unique human monocyte macrophage receptor. *Arterioscler Thromb Vasc Biol*; 1998, 18, 968-976.

Gill J M, Al-Mamari A, Ferrell W R, Cleland S J, Perry C G, Sattar N. Effect of prior moderate exercise on postprandial metabolism in men with type 2 diabetes: heterogeneity of responses. *Atherosclerosis*; 2007, 194, 134-143.

Ginsberg H N, Elam M B, Lovato L C. Effects of combination lipid therapy in type 2 diabetes mellitus. The ACCORD Study Group. *N Engl J Med*; 2010, 362, 1563-1574.

Glass C K, Witztum J L. Atherosclerosis- the road ahead. *Cell*; 2001, 104, 503-516.

Gleissner C A, Galkina E, Nadler J L, Ley K. Mechanisms by which diabetes increases cardiovascular disease. *Drug Discov Today*; 2007, 4, 131-140.

Gokce N. Clinical assessment of endothelial function: ready for prime time. *Circ Cardiovasc Imaging*; 2011, 4, 348-350.

Goldberg I J, Blaner W S, Vanni T M, Moukides M, Ramakrishnan R. Role of lipoprotein lipase in the regulation of high density lipoprotein apolipoprotein metabolism. *J Clin Invest*; 1990, 86, 463-473

Goodsell D S. Catalase. *Molecule of the Month*. RCSB Protein Data Bank. Retrieved 2007-02-11.

Gopalakrishnan S, Rajameena R. GC-MS analysis of some bioactive constituents of *Desmodium gyrans* DC. *Int Res J Pharmacy*; 2012, 3, 271-274.

Gopalakrishnan S, Rajameena R. Pharmacognostical and phytochemical studies on *Desmodium gyrans* DC. *WJPPS*; 2014, 3, 658-668.

Goto C, Higashi Y, Kimura M, Noma K, Hara K, Nakagawa. The effect of different intensities of exercise on endothelium-dependent vasodilation in humans: role of endothelium-dependent nitric oxide and oxidative stress. *Circulation*; 1993, 108, 530-535.

Gotto A M Jr., Pownall H J, Havel R J. Introduction to the plasma lipoproteins. *Methods Enzymol*; 1986, 128, 33-41.

Govindarajan R, Rastogi S, Vijayakumar M. Studies on the antioxidant activities of Desmodium gangeticum. *Biol Pharma Bull*; 2003, 26, 1423-1427.

Grassi D, Desideri G, Croce G, Tiberti S, Aggiob A, Ferri C. Flavonoids, vascular function and cardiovascular protection. *Curr Pharmaceut Des*; 2009, 15, 1072-1084.

Green L C, Wagner D A, Glogowski J, Skipper P L, Wishnok J S, Tannenbaum S R. Analysis of nitrate, nitrite, and (15N) nitrate in biological samples. *Anal Biochem*; 1982, 126, 131-138.

Grundy S M, Cleeman J I, Merz C N. *JAMA*; 2004, 28, 2486-2497.

Gu L, Kelm M A, Hammerstone J F. Concentrations of proanthocyanidins in common foods and estimates of consumption. *J Nutr*; 2004, 134, 613-617.

Gupta S K, Mohanty I, Talwar K K, Dinda A, Joshi S, Bansal P, Saxena A, Arya D S. Cardio protection from ischemia and reperfusion injury by *Withania somnifera*: a hemodynamic, biochemical and histopathological assessment. *Mol Cell Biochem*; 2004, 260, 39-47.

Haffner S M. Epidemiology of insulin resistance and its relation to coronary artery disease. *Am J Cardiol*; 1999, 84, 11-14.

Halim EM. Hypoglycemic, hypolipidemic and antioxidant properties of combination of curcumin from *Curcuma longa*, linn, and partially purified product from *Abroma augusta*, linn. in streptozotocin induced diabetes. *Ind J Clin Biochem*; 2002, 17, 33-43.

Halliwell B, Barry. Oxidative stress and cancer: have we moved forward? *Biochem J*; 2007, 401, 1-11.

Halliwell B, Gutteridge J C. The chemistry of oxygen radicals and other oxygen-derived species. In: Free Radicals in Biology and Medicine (3rd ed.), Oxford University Press, New York, 1985.

Hansson G K, Libby P. The immune response in atherosclerosis: a double-edged sword. *Nat Rev Immunol*; 2006, 6, 508-519.

Hansson G K. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med*; 2005, 352, 1685-1695.

Hare J M, Stamler J S. NO redox disequilibrium in the failing heart and cardiovascular system. *J Clin Invest*; 2005, 115, 509-517.

Harris W S, Miller M, Tighe A P. Omega-3 fatty acids and coronary heart disease risk: clinical and mechanistic perspectives. *Atherosclerosis*; 2008, 197, 12-24.

Heinecke J W. Mass spectrometric quantification of amino acid oxidation products in proteins: insights into pathways that promote LDL oxidation in the human artery wall. *FASEB J*; 1999, 13, 1113-1120.

Heinrich M. Ethnobotany and natural products: the search for new molecules, new treatments of old diseases or a better understanding of indigenous cultures? *Curr Top Med Chem*; 2003, 3, 141-154.

Hemker H C, Beguin S. Mode of action of heparin and related drugs. *Semin Thromb Hemost*; *1991*, 17, 29-34.

Hertog M L, Hollman P H, Katan M B. Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in The Netherlands. *J Agric Food Chem*; 1992, 40, 2379-2383.

Hertz R, Bishara Shieban J, Bar Tana J. Mode of action of peroxisome proliferators as hypolipidemic drugs: suppression of apolipoprotein C-III. *J Biol Chem*; 1995, 270, 470-475.

Higashi Y, Sasaki S, Kurisu S, Yoshimizu A, Sasaki N, Matsuura H. Regular aerobic exercise augments endothelium-dependent vascular relaxation in normotensive as well as hypertensive subjects: role of endothelium-derived nitric oxide. *Circulation*; 1999, 100, 1194-1202.

Hill J M, Zalos G, Halcox J P, Schenke W H, Waclawiw M A, Quyyumi A A, Finkel T. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med*; 2003, 348, 593-600.

Hirschfield G M, Gallimore J R, Kahan M C. Transgenic human C-reactiveprotein is not proatherogenic in apolipoprotein E-deficient mice. *Proc Natl Acad Sci USA*; 2005, 102: 8309-8314.

Hirsh J, Fuster V, Ansell J, Halperin J. American Heart Association American College of Cardiology Foundation guide to warfarin therapy. *J Am Coll Cardiol*; 2003, 41, 1633-1652.

Hirsh J, Martin O D, Weitz J I. New anticoagulants. *Blood*; 2005, 105, 453-463.

Hirsh J, Raschke R. Heparin and low-molecular-weight heparin. The Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy. *Chest*; 2004, 126, 188-203.

Ho Y K, Brown M S, Goldstein J L. Hydrolysis and excretion of cytoplasmic cholesteryl esters by macrophages: stimulation by high density lipoprotein and other agents. *J Lipid Res*; 1980, 21, 391-398.

Hokanson J E, Austin M A. Plasma triglyceride level is a risk factor for cardiovascular disease independent of high-density lipoprotein cholesterol level: a meta-analysis of population-based prospective studies. *J Cardiovasc Risk*; 1996, 3, 213-219.

Horenstein MS, Vander Heide R S, L Ecuyer T J. Molecular basis of anthracycline-induced cardiotoxicity and its prevention. *Mol Genet Metab*; 2000, 71, 436-444.

Horowitz B S, Goldberg I J, Merab J, Ramakrishnan V R, Ginsberg H N. Increased plasma and renal clearance of an exchangeable pool of apolipoprotein A-I in subjects with low levels of high density lipoprotein cholesterol. *Clin Invest;* 1993, 91, 1743-1752.

Hosseinzadeh H, Ramezani M, Salmani G. Antinociceptive, anti-inflammatory and acute toxicity effects of *Zataria multiflora* Boiss extracts in mice and rats. *J Ethanopharmacol*; 2000, 73, 379-385.

Huang H, Virmani R, Younis H, Burke A P, Kamm R D, Lee RT. The impact of calcification on the biomechanical stability of atherosclerotic plaques. *Circulation*; 2001, 103, 1051-1056.

Hughes R E. Reduction of dehydroascorbic acid by animal tissues. *Nature*; 1964, 203, 1068-1069.

Hulley S, Grady D, Bush T, Furberg C, Herrington D, Riggs B, Vittinghoff E. Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. *JAMA*; 1998, 280, 605-613.

Iliskovic S N, Hill M, Chow D A, Singal P K. Probucol protects against adriamycin cardiomyopathy without interfering with its antitumor effect. *Circulation*; 1995, 91, 10-15.

Iribarren C, Sidney S, Sternfeld B, Browner W S. Calcification of the aortic arch: risk factors and association with coronary heart disease, stroke, and peripheral vascular disease. *JAMA*; 2000, 283, 2810-2815.

Iroegbu C U, Okoli S. In vitro antibacterial activity of *Synclisia scabrida* whole root extracts. *Afr J Biotechnol*; 2005, 4, 946-952.

Ishii H, Kizaki K, Horie S, Kazama M. Oxidised low density lipoprotein reduces thrombomodulin transcription in cultured human endothelial cells through degradation of the lipoprotein in lysosomes. *J Biol Chem*; 1996, 271, 8458-8465.

Jabbar S, Khan M T, Choudhuri M S. The effects of aqueous extracts of *Desmodium gangeticum* DC. (Leguminosae) on the central nervous system. *Pharmazie*; 2001, 56, 506-508.

Jackson S P. The growing complexity of platelet aggregation. *Blood*; 2007, 109, 5087-5095.

Jain A P, Suryavanshi S. *Gloriosa superba* Linn. - A Pharmacological Review. *Int J Pharma Res Develop*; 2010, 2, 24-30.

Jain G C, Jhalani S, Agarwal S, Jain K. Hypolipidemic and antiatherosclerotic effect of *Leptadenia pyrotechnica* extract in cholesterol fed rabbits. *Asian J Exp Sci*; 2007, 21, 115-122.

Jaouad L, Milochevitch C, Khalil A. PON1 paraoxonase activity is reduced during HDL oxidation and is an indicator of HDL antioxidant capacity. *Free Rad Res*; 2003, 37, 77-83.

Jason M R Gill, Ali Al Mamari, William R Ferrell, Stephen J Cleland, Chris J Packard, Naveed Sattar. Effects of Prior Moderate Exercise on Postprandial Metabolism and Vascular Function in Lean and Centrally Obese Men. *J Am Coll Cardiol*; 2004, 44, 2375-2382.

Jenner P. Oxidative stress in Parkinson's disease. *Annals Neurol*; 2003, 53, 26-36.

Jialal I, Stein D, Balis D. Effect of hydroxymethyl glutaryl coenzyme a reductase inhibitor. *Circulation*; 2001,103, 1933-1935.

Jignasa S P, Seema K S, Chakraborty M, Jagadish V K. Evaluation of cardioprotective activity of *Medoharvati* by isoproterenol induced myocardial damage in rats. *Int Res J Pharm*; 2012, 3, 210-217.

Johnson D, Lardy H. Isolation of liver or kidney mitochondria. *Methods in Enzymology*; 1967, 10, 94-96.

Johnson W J, Mahlberg F H, Rothblat G H, Phillips M C. Cholesterol transport between cells and high density lipoproteins. *Biochim Biophys Acta;* 1991, 1085, 273-298.

Joshi A, Bhobe M, Saatarkar A. Phytochemical investigation of the roots of *Grewia microcos* Linn. *J Chem Pharm Res*; 2013, 5, 80-87.

Julie H, Campbell M, Johnny L, Efendy J, Gordon R. Molecular basis by which garlic suppresses atherosclerosis. *J Nutr*; 2001, 131, 1006-1009.

Junichiro Y, Path F C, Kaori Y, Aki N, Tsutomu Y, Ryo A. Testing various herbs for antithrombotic effect. *Nutrition*; 2005, 21, 580-587.

Kalemba D, Kunicka A. Antibacterial and antifungal properties of essential oils. *Curr Med Chem*; 2003, 10, 813-829.

Kamagate A, Dong H H. FoxO1 integrates insulin signaling to VLDL production. *Cell Cycle*; 2008, 7, 3162-3170.

Kamanna V S, Kashyap M L. Mechanism of action of niacin. *Am J Cardiol*; 2008, 101, 20-26.

Karlsson J. Introduction to Naturology and Radical Formation. In: Antioxidants and Exercise. Illinois: Human Kinetics Press, 1997.

Karwowski W, Naumnik B, Szczepanski M, Mysliwiec M. The mechanism of vascular calcification- a systematic review. *Med SciMonit*; 2012, 18, 1-11.

Kawakami A, Osaka M, Tani M. Apolipoprotein CIII links hyperlipidemia with vascular endothelial function. *Circulation*; 2008, 118, 731-742.

Kawshik K C, Achinto S, Sitesh C B, Joydeb K K. Analgesic and anti-inflammatory activities of *Desmodium triflorum* DC. *J Biol Sci*; 2005, 5, 581-583.

Kersten S, Seydoux J, Peters J M, Gonzalez F J, Desvergne B, Wahli W. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature*; 1990, 347, 645-654.
Kersten S, Seydoux J, Peters J M, Gonzalez F J, Desvergne B, Wahli W. Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *J Clin Invest*. 1999,103, 1489-1498.

Kersten S. Integrated physiology and systems biology of PPARα. *Molecular Metabolism*; 2014, 3, 354-371.

Kevin J W, Ira T. The response-to-retention hypothesis of early atherogenesis. *Arterioscler Thrombo Vasc Biol*; 1995, 15, 551-561.

Kim K, Kim H, Kwon J, Lee S, Kong H, Im S A, Lee Y H, Lee Y R, Oh S T, Jo T H, Park Y I, Lee C K, Kim K. Hypoglycemic and hypolipidemic effects of processed Aloe vera gel in a mouse model of noninsulin- dependent diabetes mellitus. *Phytomedicine*; 2009, 16, 856-863.

Kimura Y, Okuda H, Arichi S. Effects of stilbenes on arachidonate metabolism in leukocytes. *Biochem Biophys Acta*; 1985, 834, 275-278.

Kinra P, Tewari V, Raghu Raman T. Role of bleeding time and clotting time in preoperative hemostasis evaluation. *Ind J Aerospace Med*; 2009, 53, 56-61.

Kmietowicz Z. WHO warns of heart disease threat to developing world. *BMJ*; 2002, 12, 325-853.

Koes R E, Quattrocchio F, Mol J M. The flavonoid biosynthetic pathway in plants: function and evolution. *BioEssays*; 1994, 16, 123-132.

Kohno M, Yokokawa K, Yasunari K, Minami M, Kano H, Hanehira T, Yoshikawa J. Induction by lysophosphatidylcholine, a major phospholipid component of atherogenic lipoproteins, of human coronary artery smooth muscle cell migration. *Circulation*; 1998, 98, 353-359.

Kohno T, Takahashi M, Manda R, Yokota J. Inactivation of the PTEN/MMAC1/TEP1 gene in human lung cancers. *Genes Chromosomes Cancer*; 1998, 22, 152-156.

Korn E D. Methods in enzymology (S. Fleischer ed.). Academic press, New York, USA, 1974.

Kovacic P, Jacintho J D. Mechanisms of carcinogenesis: focus on oxidative stress and electron transfer. *Curr Med Chem*; 2001, 8, 773-796.

Krol W, Czuba Z P, Threadgill M D, Bernadetted M, Cunningham, Pietsz G. Inhibition of nitric oxide production in murine macrophage by flavones. *Biochem pharmacol*; 1995, 50, 1031-1035.

Krone W, Naegele H, Behnke B, Greten H, Opposite effects of insulin and catecholamines on LDL-receptor activity in human mononuclear leukocytes. *Diabetes*; 1988, 37, 1386-1391.

Kukreja R C, Hess M L. The oxygen free-radical system-From equations through membrane–protein interactions to cardiovascular injury and protection. *Cardiovasc Res*; 1992, 26, 641-655.

Kulkarni S K, Handbook of Experimental Pharmacology (2nd ed.), Vallabh Prakashan Publication, New Delhi, India, 1993.

Kumar S N, Swamy V M, Kumar K G, Behera M G, Review on *Careya arborea* Roxb. *Int J of Res in Ayurveda Pharm*; 2010, 1, 306-315.

Kurian G A, Philip S, Varghese T. Effect of aqueous extract of the *Desmodium gangeticum* DC root in the severity of myocardial infarction. *J Ethnopharmacol*; 2005, 97, 457-461.

Kurian G S, Padikkala J. Administration of aqueous extract of *Desmodium gangeticum* (L) root protects rat hearts against ischemic reperfusion injury induced oxidative stress. *Ind J Exp Biol*; 2009, 47, 129-135.

Lai S C, Peng W H, Huang S C, Ho Y L, Huang T H, Lai Z R, Chang Y S. Analgesic and anti-inflammatory activities of methanol extract from *Desmodium triflorum* DC in mice. *Am J Chin Med*; 2009, 37, 573-588.

Lai S C, Ho Y L, Huang S C, Huang T H, Lai Z R, Wu C R, Lian K Y, Chang Y S. Antioxidant and antiproliferative activities of *Desmodium triflorum* DC. *Am J Chin Med*; 2010, 38, 329-342.

Lamb D J, Reeves G L, Taylor A, Ferns G A. Dietary copper supplementation reduces atherosclerosis in the cholesterol-fed rabbit. *Atherosclerosis*; 1999, 146, 33-43.

Lan Z, Alvin H S. Platelet aggregation testing in platelet rich plasma. *Am J Clin Path*; 2005, 123, 172-183.

Lancaster J E, Shaw M L, Joyce M D. A novel alliinase from onion roots. Biochemical characterization and cDNA cloning. *Plant Physiol*; 2000, 122, 1269-1279.

Lassegue B, Clempus R E. Vascular NAD(P)H oxidases: specific features, expression, and regulation. *Am J Physiol Regul Integr Comp Physiol*; 2003, 285, 277-297.

Laura R, Thomas S H, Rebecca J R, Jane R, Karen N, Laura A M, David A G, Edward B, Gerard D, Clement E F, Gail P J. The correlation of paraoxonase (PON1) activity with lipid and lipoprotein levels differs with vascular disease status. *J Lip Res*; 2005, 46, 1888-1895.

Lehr D. Healing of myocardial necrosis caused by sympathomimetic amines. *Rec Adv Studies Card Structmetab*; 1972, 1, 526-550.

Levine G N, Keaney J F, Vita J A. Cholesterol reduction in cardiovascular diseases: clinical benefits and possible mechanism. *N Engl J Med*; 1995, 332, 512-515.

Lewis W, Bruce B, John C, Robert D, Valentin F. Coronary artery calcification: pathophysiology, epidemiology, imaging methods, and clinical implications. *Circulation*; 1996, 94, 1175-1192

Li HG, Forstermann U. Nitric oxide in the pathogenesis of vascular disease. *J Pathol*; 2000, 190, 244-254.

Li J M, Shah A M. Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology. *Am J Physiol Regul Integr Comp Physiol*; 2004, 287, 1014-1030.

Liang H Q, Rye K A, Barter P J. Dissociation of lipid-free apolipoprotein A-I from high density lipoproteins. *J Lipid Res*; 1994, 35, 1187-1191.

Liao Hui, Banbury Linda K, Leach David N. Antioxidant activity of 45 Chinese herbs and the relationship with their TCM characteristics. *Evid Based Complemen Alter Med*; 2008, 5, 429-434.

Libby P, Ridker P M, Maseri A. Inflammation and Atherosclerosis. *Circulation research*; 2002, 105, 1135-1143.

Libby P. Inflammation and cardiovascular disease mechanisms. *Am J Clin Nutr*; 2006, 83, 456-460.

Libby P. Inflammation in atherosclerosis, *Nature*; 2002, 420, 868-874.

Lindholm L H, Ibsen H, Dahlof B. Cardiovascular morbidity and mortality in patients with diabetes in the losartan intervention for endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol. *Lancet*; 2002, 359, 1004-1010.

Lindner V, Lappi D A, Baird A, Majack R A, Reidy M A. Role of basic fibroblast growth factor in vascular lesion formation. *Circ Res*; 1991, 68, 106-113.

Lipnick R L, Cotruvo J A, Hill R N. Comparison of the up-and-down, conventional LD_{50} and fixed dose acute toxicity procedures. *Fd Chem Toxicol*; 1995, 33, 223-231.

Liu S, Manson J E, Lee I M, Cole S R, Hennekens C H, Willett W C, Buring J E. Fruit and vegetable intake and risk of cardiovascular disease: the women's health study. *Am J Clin Nutr*; 2000, 72, 922-928.

Liu X, Chen Z, Chua C C, Ma Y S, Youngberg G A, Hamdy R, Chua B L.Melatonin as an effective protector against doxorubicin-induced cardiotoxicity. *Am J of Physiol*; 2002, 283, 254-263.

Liuzzo G, Biasucci L M, Gallimore J R. The prognostic value of C-reactive protein and serum amyloid a protein in severe unstable angina. *N Engl J Med*; 1994, 331, 417-424.

Li X, Higashida K, Kawamura T, Higuchi M. alternate day high fat diet induces an increase in mitochondrial enzyme activities and protein content in rat skeletal muscle. *Nutrients*; 2016, 8(4), 203-214.

Lusis A J, Atherosclerosis. *Nature*; 2000, 40, 233-241.

Ma X Zheng, Hu C, Rahman K, Qin L. The genus Desmodium (Fabaceae)- traditional uses in Chinese medicine, Phytochemistry and pharmacology. *J Ethnopharmacol*; 2011, 138, 314-32.

Ma Y, Henderson H E, Liu M S, Zhang H, Forsythe I J, Clarke-Lewis I, Hayden M R, Brunzell J D. Mutagenesis in four candidate heparin binding regions (residues 279-282, 291-304, 390-393, and 439-448) and identification of residues affecting heparin binding of human lipoprotein lipase. *J Lipid Res*; 1994, 35, 2049-2059.

Mace Gorbach S L. Anaerobic bacteriology for clinical laboratories. *Pharmacognosy*; 1963, 23, 89-91.

Mach F, Schonbeck U, Sukhova G K, Atkinson E, Libby P. Reduction of atherosclerosis in mice by inhibition of CD40 signalling. *Nature*; 1998, 394, 200-203.

Mach F. Statins as immunomodulatory agents. *Circulation*; 2004, 109, 15-18.

Mackness M I, Bouiller A, Hennuyer M, Mackness B, Hall M, Tailleux A, Duriez P, Delfly B, Durrington P N, Fruchart J C. Paraoxonase activity is reduced by a pro-atherosclerotic diet in rabbits. *Biochem Biophys Res Commun*; 2000, 269, 232-236.

Mackness M I, Mackness B, Durrington P N, Connelly P W, Hegele R A. Paraoxonase: Biochemistry, genetics and relationship to plasma lipoproteins. *Curr Opin Lipidol*; 1996, 7, 69-76.

MacMahon S, Peto R, Cutler J, Collins R, Sorlie P, Neaton J, Abbott R, Godwin J, Dyer A, Stamler J. Blood pressure, stroke, and coronary heart disease. Part 1. Prolonged differences in blood pressure: prospective observational studies corrected for the regression dilution bias. *Lancet*; 1990, 335, 765-774.

Madamanchi N R, Vendrov A, Runge M S. Oxidative stress and vascular disease. *Arterioscler Thromb Vas Biol*; 2005, 25, 29-38.

Madhesh M, MuthuR, Selvaraj N, Ramalingam R, Vaiyapuri M. Protective role of fenugreek on isoproterenol induced myocardial infarction in rats. *Int Res J Pharm*; 2012, 3, 211-216.

Maeda H, Akaike T. Nitric oxide and oxygen radicals in infection, inflammation, and cancer. *Biochemistry* (USSR); 1998, 63, 854-865.

Maehly A C, Chance B. The assay of catalases and peroxidases. *Methods Biochem Anal*; 1954, 1, 357-424.

Malmendier C, Delcroix C. Effects of fenofibrate on high and low densitylipoprotein metabolism in heterozygous familial hypercholesterolemia. *Atherosclerosis*; 1985, 55, 161-169.

Mancini G B, Henry G C, Macaya C. Angiotensin converting enzyme inhibition with quinapril improves endothelial vasomotor dysfunction in patients with coronary artery disease. *Circulation*; 1996, 94, 258-265.

Manninen V, Tenkanen L, Koskinen P. Joint effects of serum triglyceride and LDL cholesterol and HDL cholesterol concentrations on coronary heart disease risk in the Helsinki Heart Study: implications for treatment. *Circulation*; 1992, 85, 37-45.

Manson J E, Rimm E B, Stampfer M J, Colditz G A, Willett W C, Krolewski A S. Physical activity and incidence of non-insulin-dependent diabetes mellitus in women. *Lancet*; 1991, 338, 774-778.

Marathe G K, Zimmerman G A, McIntyre T M. Platelet-activating factor acetylhydrolase, and not paraoxonase-1, is the oxidised phospholipid hydrolase of high density lipoprotein particles. *J Biol Chem*; 2003, 278, 3937-3947.

Marberger H, Huber W, Bartsch G, Schulte T, Swoboda P. Orgotein: a new antiinflammatory metalloprotein drug evaluation of clinical efficacy and safety in inflammatory conditions of the urinary tract. *Int Urol Nephrol*; 1974, 6, 61-74.

Marchegiani F, Marra M, Olivieri F, Cardelli M, James R W. Paraoxonase 1: genetics and activities during aging. *Rejuvenation Res*; 2008, 11, 113-127.

Maria J H, Michel R M, Flavia A C, Stephen E F, Alvaro F B, Valdir G G, Tatiana M D, Luiz G N. Effectiveness of Two Methods for Preparation of Autologous Platelet-Rich Plasma: An Experimental Study in Rabbits. *Eur J Dentistry*; 2010, 8, 107-117.

Martin G, Duez H, Blanquart C, Berezowski V, Poulain P. Statin-induced inhibition of the Rho-signaling pathway activates PPARα and induces HDL apoA-I. *J Clin Investig*; 2001, 107, 1423-1432.

Martindale J L, Holbrook N J. Cellular response to oxidative stress: signaling for suicide and survival. *J Cell Physiol*; 2002, 192, 1-15.

Marx N, Imhof A, Froehlich J. Effect of rosiglitazonetreatment on soluble CD40L in patients with type 2 diabetesand coronary artery disease. *Circulation*; 2003,107, 1954-1957.

Maryam Z, Farrukh A, Iqbal A. The *in vitro* antioxidant activity and total phenolic content of Fourindian medicinal plants. *Int J Pharm Pharmaceut Sci*; 2009, 1, 88-95.

Mattiello T, Trifiro E, Jotti G S, Pulcinelli F M. Effects of pomegranate juice and extract polyphenols on platelet function. *J Med Food*; 2009, 12, 334-339.

Mattsson H L M, Lindmark H, Diczfalusy U, Bjorkhem I, Ottosson M, Liu Y, Bondjers G, Wiklund O. Oxysterols present in atherosclerotic tissue decrease the expression of lipoprotein lipase messenger RNA in human monocyte-derived macrophages. *J Clin Invest*; 1996, 97, 461-468.

Maulik N, Das D K. Redox signaling in vascular angiogenesis. *Free Radic Biol Med*; 2002, 33, 1047-1060.

McCord J M, Fridovich I. Superoxide Dismutase, an Enzymic Function for Erythrocuprein (Hemocuprein). *J Biol Chem*; 1969, 244, 6049-6055.

McGinness J E, Proctor P H, Demopoulos H B, Hokanson J A, Kirkpatrick D S. Amelioration of cis-platinum nephrotoxicity by orgotein (superoxide dismutase). *Physiol Chem Phys*; 1978, 10, 267-277.

McMurray H F, Parthasarathy S, Steinberg D. Oxidatively modified low density lipoprotein is a chemoattractant for human T lymphocytes. *J Clin Invest*; 1993, 92, 1004-1008.

Mead J R, Irvine S A, Ramji D P. Lipoprotein lipase: structure, function, regulation, and role in disease. *J Mol Med*; 2002, 80, 753-769.

Medh J D, Bowen S L, Fry G L, Ruben S, Andracki M, Inoue I, Lalouel J M, Strickland D K, Chappell D A. Lipoprotein lipase binds to low density lipoprotein receptors and induces receptor-mediated catabolism of very low density lipoproteins in vitro. *J Biol Chem*; 1996, 271, 17073-17080.

Mellies M, Stein E, Khoury P, Lamkin G, Glueck C. Effects of fenofibrateon lipids, lipoproteins and apolipoproteins in 33 subjects withprimary hypercholesterolaemia. *Atherosclerosis*; 1987, 63, 57-64.

Meng C Q. Atherosclerosis is an inflammatory disorder after all. *Curr Top Med Chem*; 2006, 6, 93-102.

Mertens A, Hobvoet P. Oxidised LDL and HDL: antagonists in atherothrombosis. *Faseb J*; 2001, 15, 2073-2084.

Messmore H L, Wehrmacher W H. Therapeutic use of low molecular weight heparins. *Seminar in Thrombosis Hemostasis*; 1993, 1, 97-100.

Methe H, Weis M. Atherogenesis and inflammation-was Virchow right? *Nephrol Dial Transplant*; 2007, 22, 1823-1827.

Meyers C D, Carr M C, Park S, Brunzell J D. Varying cost and free nicotinic acid content in over-the-counter niacin preparations for dyslipidemia. *Ann Intern Med*; 2003, 139, 996-1002.

Miller A M, Xu D, Asquith D L, Denby L, Li Y. IL-33 reduces the development of atherosclerosis. *J Exp Med*; 2008, 205, 339-346.

Miller D M, Buettner G R, Aust S D. Transition metals's catalysts of "autoxidation" reactions. *Free Radic Biol Med*; 1990, 8, 95-108.

Misiewicz I, Skupinska K, Kasprzycka-Guttman T. Sulforaphane and 2-oxohexyl isothiocyanate induce cell growth arrest and apoptosis in L-1210 leukenia and ME-18 melanoma cells. *Oncol Rep*; 2003, 10, 2045-2050.

Mitchell R S, Kumar V, Abbas A K, Fausto N. Robbins Basic Pathology: with student consult Online Access (8th ed.), Saunders, Philadelphia, 2005.

Molavi B, Mehta J L. Oxidative stress in cardiovascular disease: Molecular basis of its deleterious effects, its detection, and therapeutic considerations. *Curr Opin Cardiol*; 2004, 19, 488-493.

Monetecucco F, Quercioli A, Dallergi F, Viviani G L, Mach F. New evidence for nicotinic acid treatments to reduce atherosclerosis. *Expert Rev Cardiovasc Ther*; 2010, 8, 1457-1467.

Monika S, Meenakshi B, Razdan B K. Toxicity studies of a developed hepatoprotective polyherbal formulation in experimental rats. *Asian J Pharm Clin Res*; 2013, 6, 47-50.

Moore R E, Navab M, Millar J S, Zimetti F, Hama S, Rothblat G H, Rader D J. Increased atherosclerosis in mice lacking apolipoprotein A-I attributable to both impaired reverse cholesterol transport and increased inflammation. *Circ Res*; 2005, 97, 763-771.

Moreno P R, Fuster V. The year in atherothrombosis. *J Am Coll Cardiol*; 2004, 44, 2099-2110.

Morrow D A, Ridker P M. C-reactive protein, inflammation, and coronary risk. *Med Clin North Am*; 2000, 84, 149-161.

Mozaffarian D. Does alpha-linolenic acid intake reduce the risk of coronary heart disease? a review of the evidence. *Altern Ther Health Med*; 2005, 11, 24-30.

Muhlestein J B, Anderson J L. Chronic infection and coronary artery disease. *Cardiol Clin*; 2003, 21, 333-362.

Muller F L, Lustgarten M S, Jang Y, Richardson A, Van Remmen H. Trends in oxidative aging theories. *Free Radic Biol Med*; 2007, 43, 477-503.

Munzel T, Sinning C, Post F, Warnholtz A, Schulz E, Warnholtz, Schulz. Athophysiology, diagnosis and prognostic implications of endothelial dysfunction. *Annals Med*; 2008, 40, 180-196.

Myers C E, McGuire W P, Liss R H, Ifrim I, Grotzinger K, Young RC. Adriamycin: the role of lipid peroxidation in cardiac toxicity and tumor response. *Science*; 1977, 197, 165-167.

Nabavi S M, Ebrahimzadeh M A, Nabavi S F, Hamidinia A, Bekhradnia A R. Determination of antioxidant activity, phenol and flavonoid content of *parrotiapersicamey. Pharmacologyonline*; 2008, 2, 560-567.

Naidu M, Kumar K V, Mohan, Sundaram C, Singh S. Protective effect of *Gingko biloba* extract against I.K doxorubicin-induced cardiotoxicity in mice. *Indian J Exp Biol*; 2002, 40, 894-900.

Nakajima K, Nakano T, Tanaka A. The oxidative modification hypothesis of atherosclerosis: the comparison of atherogenic effects on oxidized LDL and remnant lipoproteins in plasma. *Clinica Chimica Acta*; 2006, 367,. 36-47.

Napoleone E, Di Santo A, Camera M. Angiotensin converting enzyme inhibitors down regulate tissue factor synthesis in monocytes. *Circ Res*; 2000, 86, 139-143.

Navab M, Hama S Y, Anantharamaiah G M, Hassan K, Hough G P, Watson A D, Reddy S T, Sevanian A, Fonarow G C, Fogelman A M. Normal high density lipoprotein inhibits three steps in the formation of mildly oxidised low density lipoprotein: steps 2 and 3. J. *Lipid Res*; 2000, 41, 1495-1508.

Newnham H H, Barter P J. Changes in particle size of high density lipoproteins during incubation with very low density lipoproteins, cholesteryl ester transfer protein and lipoprotein lipase. *Biochim Biophys Acta*; 1992, 1125, 297-304.

Nishimura H, Higuchi O, Tateshita K. Antioxidative activity of sulfur-containing compounds in Allium species for human LDL oxidation in vitro. *Biofactors*; 2004, 21, 277-280.

Nobuhiro Y. Control of triglycerides. *Asian Med J*; 2001, 44, 42-47.

Nofer J R, van der Giet M, Tolle M. HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3. *J Clin Invest*; 2004,113, 569-581.

Nofer J R, Walter M, Kehrel B. HDL3-mediated inhibition of thrombin-induced platelet aggregation and fibrinogen binding occurs via decreased production of phosphoinositide-derived second messengers 1,2-diacylglycerol and inositol 1,4,5-tris-phosphate. *Arterioscler Thromb Vasc Biol*; 1998, 18, 861-869.

Nordestgaard B G, Varbo A: Triglycerides and cardiovascular disease. *Lancet*; 2014, 384, 626-635.

Nykjaer A, Nielsen M, Lookene A, Meyer N, Roigaard H, Etzerodt M, Beisiegel U, Olivecrona G, Gliemann J. A carboxyl-terminal fragment of lipoprotein lipase binds to the low density lipoprotein receptor-related protein and inhibits lipase-mediated uptake of lipoprotein in cells. *J Biol Chem*; 1994, 269, 31747-31755.

OECD guideline, 2002; Organisation for Economic Cooperation and Development. Guidelines for the Testing of Chemicals/Section 4: Health Effects Test No. 423: Acute oral toxicity - acute toxic class method. 2002.

OECD guidelines, 2008;Organization for Economic Co-operation and Development for testing of chemicals, Toxicity guideline no. 407, repeated dose 28 day oral toxicity study in rodents. Adopted: 2008

Ohashi R, Mu H, Wang X, Yao Q, Chen C. Reverse cholesterol transport and cholesterol efflux in atherosclerosis. *Quarterly J Med*; 2005, 98, 845-856.

Okawa H. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem*; 1979, 95, 351-358.

Olson R D, Mushlin P S. Doxorubicin cardiotoxicity: analysis of prevailing hypotheses. *FASEB J*; 1990, 4, 3076-3086.

Ooi E M, Barrett P H, Chan D C. Apolipoprotein C-III: understanding an emerging cardiovascular risk factor. *Clin Sci*; 2008, 114, 611-624.

Oram J F, Yokoyama S. Apolipoprotein-mediated removal of cellular cholesterol and phospholipids. *J Lipid Res*; 1996, 37, 2473-2491.

Oshima R, Ikeda T, Watanabe K, Itakura H, Sugiyama N. Probucol treatment attenuates the aortic atherosclerosis in Watanabe heritable hyperlipidemic rabbits. *Atherosclerosis*; 1998, 137, 13-22.

Ostad S N, Soodi M, Shariffzadch M, Khorshidi N, Marzban H. The effect of fennel essential oil on uterine contraction as a model for dysmenorrheal, pharmacology and toxicology study. *J Ethnopharmacol*; 2001, 76, 299-304.

Pacher P, Beckman J S, Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev*; 2007, 87, 315-424.

Paffenbarger R S Jr., Hyde R T, Wing A L, Hsieh C C. Physical activity, all cause mortality and longevity of college alumni. *N Engl J Med*; 1986, 314, 605-613.

Pai J K, Pischon T, Ma J. Inflammatory markers and the risk of coronaryheart disease in men and women. *N Engl J Med*; 2004, 351, 2599-2610.

Palmer A M, Murphy N, Graham A. Triglyceride-rich lipoproteins inhibit cholesterol efflux to apolipoprotein (apo) A1 from human macrophage foam cells. *Atherosclerosis*; 2004, 173, 27-38.

Panthong A, Kanjanapothi D, Taesotikul T, Phankummoon A, Panthong K, Reutrakul V. Anti-inflammatory activity of methanolic extracts from *Ventilago harmandiana* Pierre. *J Ethnopharmacol*; 2004, 91, 237-242.

Parhami F, Fang Z T, Fogelman A M, Andalibi A, Territo M C, Berliner J A. *J Clin Invest*; 1993, 92, 471-478.

Parthasarathy S, Wieland E, Steinberg D. A role for endothelial cell lipoxygenase in the oxidative modification of LDL. *Proc Natl Acad Sci USA*; 1989, 86, 1046-1050

Pastore A, Piemonte F, Locatelli M, Lo R, Anna L, Laura M, Tozzi, G, Federici G. Determination of blood total, reduced, and oxidized glutathione in pediatric subjects. *Clin Chem*; 2003, 47, 1467-1469.

Patel S, Puranik R, Nakhla S. Acute hypertriglyceridaemia in humans increases the triglyceride content and decreases the anti-inflammatory capacity of high density lipoproteins. *Atherosclerosis*; 2009, 204, 424-428.

Patil R H, Prakash K, Maheshwari V L. Hypolipidemic effect of *Terminalia arjuna* (L.) in experimentally induced hypercholesteremic rats. *Acta Biologica Szegediensis*; 2011, 55, 289-293.

Patsch J R, Gotto A M Jr., Olivecrona T, Eisenberg S. Formation of high density lipoprotein like particles during lipolysis of very low density lipoproteins in vitro. *J of Clin Invest*; 1978, 29, 161-179.

Paulo J O, James A B, Maria S S, Richard L L, Froberg M K, Antonio J M, Kendall B W. Carvedilol-mediated antioxidant protection against doxorubicin-induced cardiac mitochondrial toxicity. *Tox Applied Pharmacol*; 2004, 200, 159-168.

Pejic R N, Lee D T. Hypertriglyceridemia. *J Am Board Fam Med*; 2006, 19, 310-316.

Perzborn E, Strassburger J, Wilmen A, Pohlmann J, Roehrig S, Schlemmer K H, Straub A. *In-vitro and in-vivo* studies of the novel antitrombotic agent BAY 59-793- an oral, direct Factor Xa inhibitor. *J Thromb Haemostasis*; 2005, 3, 514-521.

Petit L, Lesnik P, Dachet C, Moreau M, Chapman M J. Tissue factor pathway inhibitor is expressed by human monocyte-derived macrophages: relationship to tissue factor induction by cholesterol and oxidised LDL. *Arterioscler Thromb Vasc Biol*; 1999, 9, 309-315.

Pham-Huy L A, He H, Pham-Huyc C. Free radicals, antioxidants in disease and health. *Int J Biomed Sci*; 2008, 4, 89-96.

Pierpoint W S. Why do plants make medicines. *Biochemist*; 2000, 22, 37-40.

Pinn G. Herbs and cardiovascular disease-from past to present. *Aust Fam Physician*; 2000, 29, 1149-1153.

Pittman R C, Knecht T P, Rosenbaum M S, Taylor C A Jr. A nonendocytotic mechanism for the selective uptake of high density lipoprotein-associated cholesterol esters. *J Biol Chem*; 1987, 262, 2443-2450.

Podrez E A, Abu-Soud H M, Hazen S L. Myeloperoxidase-generated oxidants and atherosclerosis. *Free Radic Biol Med*; 2000, 28, 1717-1725.

Pohanka M. Alzheimer´s disease and oxidative stress: a review. *Current Medicinal Chemistry*; 2013, 21, 356-364.

Pompella A, Visvikis A, Paolicchi A, Tata V, Casini A F. The changing faces of glutathione, a cellular protagonist. *Biochem Pharma*; 2003, 66, 1499-1503.

Purushothman K K, Kishore V M, Narayanaswamy V. The structure and sterochemistry of Gangetin, a new pterocarpan from *Desmodium gangeticum* (Leguminosae). *J Chem Soc*; 1971, 81, 2420-2422.

Quinn M T, Parthasarathy S, Fong L G, Steinberg D. Oxidatively modified low density lipoproteins: a potential role in recruitment and retention of monocyte/macrophages during atherogenesis. *Proc Natl Acad Sci*; 1987, 84, 2995-2998.

Raghavan G, Subha R, Madhavan V, Annie S, Ajay S R, Shanta M. Studies on the antioxidant activities of *Desmodium gangeticum. Biol Pharm Bull*; 2003, 26, 1424-1427.

Rajadurai M, Stanley P P. Preventive effect of naringinon lipid peroxidase and antioxidants in isoproterenol-induced cardiotoxicity in Wister rats: biochemical and histopathological evidences. *Toxicology*; 2006, 228, 259-268.

Rajavashisth T B, Andalibi A, Territo M C, Berliner J A, Navab M, Fogelman A M, Lusis A J. Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins. *Nature*; 1990, 344, 254-257.

Rallidis L S. The effect of diet enriched with alpha linolenic acid in dyslipidemic patients. *Atherosclerosis*; 2004, 174, 127-132.

Ramond A, Godin-Ribuot D, Ribuot C, Totoson P, Koritchneva I, Cachot S, Levy P, Joyeux-Faure M. Oxidative stress mediates cardiac infarction aggravation induced by intermittent hypoxia. *Fundam Clin Pharmacol*; 2011, 27, 252-261.

Rashidlamir A, Ghanbari Niaki A, Saadatnia A. The Effect of eight weeks of wrestling and wrestling technique based circuit training on lymphocyte ABCA1 gene expression and plasma apolipoprotein AI. *World J Sport Sci*; 2011, 2, 144-150.

Rastogi R P, Mehrotra B N. Compendium of Indian Medicinal Plants. Lucknow and New Delhi: Central Drug Research Institute, Publications and Information Directorate, 1993.

Rathore N, John S, Kale M. Lipid peroxidation and antioxidant enzymes in isoproterenol induced oxidative stress in rat tissues. *Pharmacol Res*; 1998, 38, 297-303.

Raza H. Modulation of xenobiotic metabolism and oxidative stress in chronic streptozotocin-induced diabetic rats fed with *Momordica charantia* fruit extract. *J Biochem Mol Toxicol*; 2000, 14, 131-139.

Reis S E, Holubkov R, Smith A C, Kelsey S F, Sharaf B L, Reichek N, Rogers W J, Merz N B, Sopko G, Pepine C J. Coronary microvascular dysfunction is highly prevalent in women with chest pain in the absence of coronary artery disease: Results from the NHLBI WISE Study. *Am Heart J*; 2001, 141, 735-741.

Relling D P, Esberg L B, Fang C X, Johnson W T, Murphy E J, Carlson E C. High-fat diet–induced juvenile obesity leads to cardiomyocyte dysfunction and up-regulation of Foxo3a transcription factor independent of lipotoxicity and apoptosis. *J Hypertens*; 2006, 24, 549-561.

Remaley A T, Warnick G R. HDL-C: The Changing Testing Paradigm-Part 1. *Am Association for Clinical Chemistry*; 2007, 33, 11-19.

Renan O S, Francisca B S, Samara R D, Nathalia S C, Valdelania G S. Phytol, a diterpene alcohol, inhibits the inflammatory response by reducing cytokine production and oxidative stress. *Fund Clin Pharmacol*; 2014, 28, 455-464.

Renard C B, Kramer F, Johansson F, Lamharzi N, Tannock L R. Diabetes and diabetes associated lipid abnormalities have distinct effects on initiation and progression of atherosclerotic lesions. *J Clin Investig*; 2004, 114, 659-668.

Richard J F. Inflammatory atherosclerosis characteristics of the injurious agent; Heart Research Foundation of Sacramento, CA, USA. 2002, 819-825.

Ridker P M, Cannon C P, Morrow D, Rifai N, Rose L M. C-reactive protein levels and outcomes after statin therapy. *N Engl J Med*; 2005, 352, 20-28.

Ridker P M, Rifai N, Clearfield M. Measurement of C-reactive protein for the targeting of statin therapy in the primary prevention of acute coronary events. *N Engl J Med*; 2001, 344, 1959-1965.

Ridker P M. High sensitivity C-reactive protein, inflammation and cardiovascular risk: from concept to clinical practice to clinical benefit. *Am Heart J*: 2004, 148 19-26.

Ridnour L A, Isenberg J S, Espey M G, Thomas D D, Roberts D D, Wink D A. Nitric oxide regulates angiogenesis through a functional switch involving thrombospondin-1. *Proc Natl Acad Sci*; 2005, 102, 13147-13152.

Rinninger F, Kaiser T, Mann W A, Meyer N, Greten H, Beisiegel U. Lipoprotein lipase mediates an increase in the selective uptake of high density lipoprotein-associated cholesteryl esters by hepatic cells in culture. *J Lipid Res*; 1998, 39, 1335-1348.

Ritu M, Sharma A, Dixit V P, Mira S. Hypolipidemic effect of fruit juice of *Embilica officinalis* in cholesterol-fed rabbits. *J Ethnopharmacol*; 1996, 50, 61-68.

Robertson A K, Hansson G K. T cells in atherogenesis: for better or for worse? *Arterioscler Thromb Vasc Biol*; 2006, 26, 2421-2432.

Robins S J, Collins D, Wittes J T. Relation of gemfibrozil treatment and lipid levels with major coronary events: VA-HIT: a randomized controlled trial. *JAMA*; 2001, 285, 1585-1591.

Romero J C, Reckelhoff J F. Role of angiotensin and oxidative stress in essential hypertension. *Hypertension*; 1999, 34, 943-949.

Rosenblat M, Volkova N, Ward J, Aviram M. Paraoxonase 1 (PON1) inhibits monocyte-to-macrophage differentiation. *Atherosclerosis*; 2011, 219, 49-56.

Rosenson R S. Management of high-density lipoprotein abnormalities. *Atherosclerosis*; 2009, 207, 328-335.

Ross R. Atherosclerosis - an inflammatory disease. *N Engl J Med*; 1999, 340, 115-126.

Rotllan N, Calpe Berdiel L, Guillaumet Adkins A, Suren Castillo S, Blanco Vaca F. CETP activity variation in mice does not affect two major HDL anti-atherogenic properties: Macrophage-specific reverse cholesterol transport and LDL antioxidant protection. *Atherosclerosis*; 2008, 196, 505-513.

Rubin T A, Murdoch M, Nelson D B. Acute GI bleeding in the setting of supratherapeutic international normalized ratio in patients taking warfarin: endoscopic

diagnosis, clinical management, and outcomes. *Gastrointestinal Endoscopy*; 2008, 58, 369-373.

Rutan G H, McDonald R H, Kuller L H. Historical perspective of elevated systolic vs diastolic blood pressure from an epidemiological and clinical trial viewpoint. *J Clin Epidemiol*; *1*989, 42, 663-673.

Sacks F M, Alaupovic P, Moye L A. Effect of pravastatin on apolipoproteins B and C-III in very low-density lipoproteins and low-density lipoproteins. *Am J Cardiol*; 2002, 90, 5-7.

Saidu Y, Nwachukwu F C, Bilbis L S, Faruk U Z, Abbas A Y. Toxicity Studies of the Crude Aqueous Root Extract of *Albizzia chevalieri* Harms in Albino Rats. *Nigerian Journal of Basic and Applied Science*; 2010, 18, 308-314.

Sanjib Bhattacharya, K K Mueen Ahmed, Subhankar Chakraborty. Free Radicals and Cardiovascular Diseases: An Update. *Free Radicals and Antioxidants*; 2011, 1, 17-22.

Sarwar N, Danesh J, Eiriksdottir G. Triglycerides and the risk of coronary heart disease: 10,158 incident cases among 262,525 participants in 29 Western prospective studies. *Circulation*; 2007, 115, 450-458.

Sayre L M, Smith M A, Perry G. Chemistry and Biochemistry of oxidative stress in neurodegenerative disease. *Curr Med Chem*; 2001, 8, 721-738.

Schneider C A, Voth E, Moka D. Improvement of myocardial blood flow to ischemic regions by angiotensin converting enzyme inhibition with quinaprilat IV: a study using [^{15}O] water dobutamine stress positron emission tomography. *J Am Coll Cardiol*; 1999, 34, 1005-1011.

Scholz R W, Graham K S, Gumpricht E, Reddy C C. Mechanism of interaction of vitamin E and glutathione in the protection against membrane lipid peroxidation. *Ann NY Acad Sci*; 1989, 570, 514-517.

Schonbeck U, Libby P. Inflammation, immunity, and HMG CoA reductase inhibitors: statins as anti-inflammatory agents? *Circulation*; 2004, 109, 18-26.

Schoonjans K, Peinado Onsurbe J, Lefebvre A M, Heyman R A, Briggs M, Deeb S, Staels B, Auwerx J. PPARa and PPARg activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. *EMBO J*; 1996, 15, 5336-5348.

Schroeder P E, Hasinoff B B. The doxorubicin-cardioprotective drug dexrazoxane undergoes metabolism in the rat to its metal ion-chelating form ADR-925. *Cancer Chemother Pharmacol*; 2002, 50, 509-513.

Schulte H, Cullen P, Assmann G. Obesity, mortality and cardiovascular disease in the Munster Heart Study (PROCAM). *Atherosclerosis*; 1999, 144, 199-209.

Scott R, O'Brien R, Fulcher G. Effects of fenofibrate treatment on cardiovascular disease risk in 9,795 individuals with type 2 diabetes and various components of the metabolic

syndrome: the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study. *Diabetes Care*; 2009, 32, 493-498.

Segui J, Gironella M, Sans M, Granell S, Gil F, Gimeno M, Coronel P, Pique JM, Panes J. Superoxide dismutase ameliorates TNBS-induced colitis by reducing oxidative stress, adhesion molecule expression, and leukocyte recruitment into the inflamed intestine. *J Leukoc Biol*; 2004, 76, 537-544.

Sendobry S M, Cornicelli J A, Welch K, Bocan T, Tait B, Trivedi B K, Colbry N, Dyer R D, Feinmark S J, Daugherty A. Attenuation of diet-induced atherosclerosis in rabbits with a highly selective 15-lipoxygenase inhibitor lacking significant antioxidant properties. *Br J Pharmacol*; 1997, 120, 1199-1206.

Seethanathan P, Vijayagopalan P, Augusti K T, Kurup P A. Changes in hepatic free NAD and NADP levels in rats fed a hypercholeterolemic diet. *Atherosclerosis*; 1970, 11(2), 339-342.

Seyyed Majid Bagheri, Leila Keyhani, Mehrangiz Heydari, Mohammad Hossein Dashti. Antinociceptive activity of *Astragalus gummifer* gum (gum tragacanth) through the adrenergic system, a *in vivo* study in mice. *JAIM*; 2015, 6, 19-23.

Shahriyary L, Yazdanparast R. Inhibition of blood platelet adhesion, aggregation and secretion by *Artemisia dracunculus* leaves extracts. *J Ethnopharmacol*; 2007, 114, 194-198.

Shanthi M, Pekka P, Norrving B. Global atlas on cardiovascular disease prevention and control (1st ed.), Geneva: World Health Organization in collaboration with the World Heart Federation and the World Stroke Organization, 2011.

Sharma H M. Phytochemical synergism: beyond the active ingredient model. *Altern Ther Clin Pract*; 1997, 4, 91-96.

Sher T, Yi H F, McBride O W, Gonzalez F J. cDNA cloning, chromosomal mapping, and functional characterization of the human peroxisome proliferator activated receptor. *Biochemistry*; 1993, 32, 5598-5604.

Shih D M, Gu L, Xia Y R, Navab M, Li W F, Hama S, Castellani L W, Furlong C E, Costa L G, Fogelman A M. Mice lacking serum paraoxonase is susceptible to organophosphate toxicity and atherosclerosis. *Nature*; 1998, 394, 284-287.

Shinyashiki M, Pan C J, Lopez B E, Fukuto J M. Inhibition of the yeast metal reductase heme protein fre1 by nitric oxide (NO): a model for inhibition of NADPH oxidase by NO. *Free Radic Biol Med*; 2004, 37, 713-723.

Shirwaikar A, Shirwaikar A, Rajendran K, Punitha I S R. *In vitro* antioxidant studies on the benzyl tetra tsoquinoline alkaloid berberine. *Bio Pharm Bull*; 2006, 29, 1906-1910.

Shivani G, Manish K G, Vinod K J, Raj K G. Anti-inflammatory activity of two classical formulations of Laghupanchamula in rats. *JAIM*; 2013, 4, 23-27.

Siddiqua J, Mohammad I, Shoaib A. Paraoxonase activity in patients with atherosclerosis. *Pak J Biochem Mol Biol*; 2010, 43, 189-199.

Simunek T, Klimtova I, Kaplanova J, Sterba M, Mazurova Y, Adamcova M, Hrdina R, Gersl V T, Ponka P. Study of daunorubicin cardiotoxicity prevention with pyridoxal isonicotinoyl hydrazone in rabbits. *Pharmacol Res*; 2005, 51, 223-231.

Singer J M and Plotz C M. The latex fixation test. I. Application to the serologic diagnosis of rheumatoid arthritis. *Am J Med*; 1956, 21(6), 888- 892.

Singh R P, Sharad S, Kapur S. Free radicals and oxidative stress in neurodegenerative diseases: Relevance of Dietary Antioxidants. *JIACM*; 2004, 5, 218-225.

Singleton V L, Rossi J A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Viticult*; 1965, 16(3), 144-158.

Sirat H M, Susanti D, Ahmad F, Takayama H, Kitajima M. Amides, triterpenes and flavonoids from the leaves of *Melastoma malabathricum* L. *J Nat Med*; 2010, 64, 492-495.

Smith C, Mitchinson M J, Aruoma O I, Halliwell B. Stimulation of lipid peroxidation and hydroxyl-radical generation by the contents of human atherosclerotic lesions. *Biochem J*; 1992, 286, 901-905.

Smythe M A, Stephens J L. Koerber J M, Mattson J C. A Comparison of Lepirudin and Argatroban Outcomes. *Clin* and *Appl Thromb- Hemostasis*; 2005, 11, 371-374.

Soejima H, Ogawa H, Yasue H-. Angiotensin converting enzyme inhibition reduces monocyte chemoattractant protein 1 and tissue factor levels in patients with myocardial infarction. *J Am Coll Cardiol*; 1999, 34, 983-988.

Soleas G J, Diamandis E P, Goldberg D M. Resveratrol: a molecule whose time has come and gone? *Clin Chem*; 1997, 30, 91-113.

Somchit M N, Nur S H. Anti-inflammatory property of ethanolic and aqueous extracts of *Zingiber zerumbet*. *Indian J Pharmacol*; 2003, 35, 181-183.

Soran H, Younis N N, Charlton-Menys V, Durrington P, Variation in paraoxonase-1 activity and atherosclerosis. *Curr Opin Lipidol*; 2009, 20, 265-274.

Sotiriou S N, Orlova V V, Al Fakhri N, Ihanus E, Economopoulou M, Isermann B, Bdeir K, Eisaf M, Tselepis A D. Effect of hypolipidemic drugs on lipoprotein-associated platelet activating factor acetylhydrolase: implication for atherosclerosis. *Biochem Pharmacol*; 2003, 66, 2069-2073.

Soufi M, Sattler A M, Herzum M, Maisch B, Schaefer J R. Molecular basis of obesity and the risk for cardiovascular disease. *Herz*; 2006, 31, 200-206.

Souza C F, Georetti S R, Salvador M J, Fonseca M V, Oliveira W P. Antioxidant activity and physical- chemical properties of spray and spouted bed dried extracts of *Bauhinia forficate*. *Brazilian Journal of Pharmaceutical Sciences*; 2009, 45, 209-218.

Spady D K. Reverse cholesterol transport and atherosclerosis. *Circulation;* 1999,100, 576-578.

Speer M Y, McKee M D, Guldberg R E, Liaw L, Yang H Y, Tung E, Karsenty G, Giachelli C M. Inactivation of the osteopontin gene enhances vascular calcification of matrix Gla protein-deficient mice: evidence for osteopontin as an inducible inhibitor of vascular calcification *in vivo*. *J Exp Med*; 2002, 196, 1047-1055.

Stadler N, Lindner R A, Davies M J. Direct detection and quantification of transition metal ions in human atherosclerotic plaques: evidence for the presence of elevated levels of iron and copper. *Arterioscler Thromb Vasc Biol*; 2004, 24, 949-954.

Staels B, Vu Dac N, Kosykh V, Saladin R, Fruchart J C, Dallongeville J, Auwerx J. Fibrates down-regulate apolipoprotein C-III expression independentof induction of peroxisomal acyl co-enzyme A oxidase. *J Clin Invest*; 1995, 95, 705-712.

Stary H C, Chandler A B, Dinsmore R E, Fuster V, Glagov S, Insull W J, Rosenfeld M E, Schwartz C J, Wagner W D, and Wissler R W. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arterioscler Thromb Vasc Biol*; 1995, 15, 1512-1531.

Stein E A, Lane M, Laskarzewski P. Comparison of statins in hypertriglyceridemia. *Am J Cardiol*; 1998, 81, 66-69.

Sterba M, Popelova O, Simunek T, Mazurova Y, Potacova A, Adamcova M, Kaiserova H, Ponka P, Gersl V. Cardioprotective effects of a novel iron chelator, pyridoxal 2-chlorobenzoyl hydrazone, in the rabbit model of daunorubicin-induced cardiotoxicity. *J Pharmacol Exp Ther*; 2006, 319, 1336-1347.

Stiko Rahm A, Hultgardh Nilsson A, Regnstrom J, Hamsten A, Nilsson J. Native and oxidised LDL enhances production of PDGF AA and the surface expression of PDGF receptors in cultured human smooth muscle cells. *Arterioscler Thromb*; 1992, 12, 1099-1109.

Stocker R, Keaney J F Jr. New insights on oxidative stress in the artery wall. *J Thromb and Haemost*; 2005, 3, 1825-1834.

Stocker R, Keaney J F. Role of oxidative modifications in atherosclerosis. *Physiological Reviews*; 2004, 84, 1381-1478.

Suarna C, Dean R T, May J, Stocker R. Human atherosclerotic plaque contains both oxidized lipids and relatively large amounts of α-tocopherol and ascorbate. *Arterioscler Thromb Vasc Biol*; 1995, 15, 1616-1624.

Sudheesh N P, Ajith T, Janardhanan K K. *Ganoderma lucidum* (Fr.) enhances activities of heart mitochondrial enzymes and respiratory chain complexes in the aged rat. *Biogerontology*; 2009, 10, 627-636.

Suessenbacher A, Potocnik M, Dorler J, Fluckinger G, Wanitschek M, Pachinger O, Frick M, Alber H F. Comparison of peripheral endothelial function in shift versus non-shift workers. *Am J Cardiol*; 2011, 7, 945-948.

Superko H R, Krauss R M. Coronary artery disease regression convincing evidence for the benefit of aggressive lipoprotein management. *Circulation*; 1994, 90, 1056-1069.

Surh Y J. NF-kappa B and Nrf2 as potential chemoprotecive targets of some anti-inflammatory and antioxidative phytonutrients. *Asia Pac J Clin Nutr*; 2008, 17, 269-272.

Suresh C, Joshi, Pratibha K. Jain, Priyanka Sharma. Antiatherosclerotic and lipid lowering effects of *cinnamomum verum* in cholesterol-fed rabbits. *Int J Curr Pharm Res*, 2017, 9 (1), 75-80.

Surjeet K, Lincy J, Mathew G, Anmol S. *Int J Pharmaceu Sci Rev Res*; 2011, 18, 8-15.

Swirski F K, Libby P, Aikawa E, Alcaide P, Luscinskas F W. Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. *J Clin Investig*; 2007, 117, 195-205.

Szmitko P E, Wang C, Weisel R D, De Almeida J R, Anderson T J, Verma S. *Circulation*; 2003, 108, 1917-1923.

Thippeswamy B S, Thakker S P, Tubachi S, Kalyani G A, Netra M K, Patil U S, Gavimath C C, Veerapur V P. Cardioprotective effect of *Cucumistrigonus* Roxbon isoproterenol-induced myocardial infarction in rat. *Am J of Pharmacol Toxicol*; 2009, 4, 29-37.

Thompson G R. Management of dyslipidaemia. *Heart*; 2004, 90, 949-55.

Tintut Y, Patel J, Territo M, Saini T, Parhami F, Demer L L. Monocyte macrophage regulation of vascular calcification in vitro. *Circulation*; 2002, 105, 650-655.

Tomas M, Senti M, Garcia F, Vila J, Torrents A, Covas M, Marrugat J. Effect of Simvastatin Therapy on Paraoxonase Activity and Related Lipoproteins in Familial Hypercholesterolemic Patients. Arteriosclerosis, *Thromb Vasc Biol*; 2000, 20, 2113-2119.

Tornvall P, Olivecrona G, Karpe F, Hamsten A, Olivecrona T. Lipoprotein lipase mass and activity in plasma and increase after heparin are separate parameters with different relations to plasma lipoproteins. *Artm'oscler. Thromb Vmc Bio*; 1995, 15, 1086-1093.

Toth L, Muszbek L, Komaromi I. Mechanism of the irreversible inhibition of human cyclooxygenase-1 by aspirin as predicted by QM/MM calculations. *J Molecular Graph Model*; 2013, 40, 99-109.

Toth P P, Gotto A M Jr. High-density lipoprotein cholesterol. Comprehensive Management of High Risk Cardiovascular Patients. New York, NY: *Informa Healthcare USA Inc*; 2007, 17, 295-340.

Toth P P. Subclinical atherosclerosis: what it is, what it means and what we can do about it. *Int J Clin Practice*; 2008, 62, 1246-1254.

Ueda M, Sethi A A, Freeman L A, Vaisman B L, Amar M, Shamburek R D, Remaley A T. High density lipoproteins: Overview and update on new findings from diagnostics to therapeutics. *Journal of Clinical Ligand Assay*; 2006, 28, 216-232.

Upadhye A S, Misar A V. Studies on antidiarrhoeal activity of *Jatropha curcas* roots extract in albino mice. *J Ethnopharmacol*; 2000, 70, 183-187.

Upaganlawar A, Gandhi H, Balaraman R. Isoproterenol induced myocardial infarction: protective role of natural products. *J pharmacol toxicol*; 2011, 6, 11-17.

Vaccari C S, Hammoud R A, Nagamia S H, Ramasamy K, Dollar A L, Khan B V. Revisiting niacin: reviewing the evidence. *J Clin Lipid*; 2007, 1, 248-255.

Valipasha K, Sadasivudu B. Intracellular content of thiol compounds, thiobarbituric acid reactive substances and gamma-glutamyl transpeptidase in rat brain during anoxia. *Neuroscience Letter*; 1984, 46, 209-214.

Valko M, Leibfritz D, Moncol J, Cronin M D, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol*; 2007, 39, 44-84.

Valko M, Morris H, Mazur M, Rapta P, Bilton R F. Oxygen free radical generating mechanisms in the colon: Do the semiquinones of Vitamin K play a role in the aetiology of colon cancer. *Biochim Biophys Acta*; 2001, 1527, 161-166.

Van Der Wall A C, Becker A E, Van der Loos C M, Das T K. Site of intimal rupture or erosion of thrombolised coronary atherosclerotic plaque is characterized by an inflammatory process irrespective of the dominant plaque morphology. *Circulation*; 1994, 89, 36-44.

Van Eck M, Zimmermann R, Groot P H. Role of macrophage-derived lipoprotein lipase in lipoprotein metabolism and atherosclerosis. *Arterioscler Thromb Vasc Biol*; 2000, 20, 53-62.

Van Gaal L F, Mertens I L, De Block C E. Mechanisms linking obesity with cardiovascular disease. *Nature*; 2006, 444, 875-880.

Van Vleet J F, Ferrans V J, Weirich W E. Cardiac disease induced by chronic adriamycin administration in dogs and evaluation of vitamin E and selenium as cardioprotectants. *Am J Pathol*; 1980, 99, 13-42.

Vega G L, Grundy S M. Effect of statins on metabolism of apo-B-containing lipoproteins in hypertriglyceridemic men. *Am J Cardiol*; 1998, 81, 36-42.

Velez Carrasco W, Lichtenstein A H, Barrett P H R. Human Apolipoprotein A-I kinetics within triglyceride-rich lipoproteins and high density lipoproteins. *J Lipid Res*; 1999, 40, 1695-1700.

Venturi S, Venturi M. Evolution of Dietary Antioxidant Defences. *European EPI-Marker*; 2007, 11, 3, 1-7.

Venugopala Rao A, Ramakrishnan S. Indirect assessment of HMG-CoA reductase enzyme in liver tissue. *Clin chem*; 1975, 21, 1523-1525.

Verma S, Wang C H, Li S H. A self-fulfilling prophecy: C-reactive proteinattenuates nitric oxide production and inhibits angiogenesis. *Circulation*; 2002, 106, 913-919.

Vikramadithyan R K, Hu Y, Noh H L, Liang C P, Hallam K. Human aldose reductase expression accelerates diabetic atherosclerosis in transgenic mice. *J Clin Investig*; 2005, 115, 2434-2443.

Vinegar R, Truax J F, Selph J H, Johnstone P R, Venable A L, McKenzie K K. Pathway to carrageenan induced inflammation of the hind-limb of rat. *Fed Proc*; 1987, 6, 118-126.

Vipin P S, Johannah N M, Seema Menon, Lincy Lawrence, Achuthan C. Raghavamenon, Jose Padikkala. Enhancement of functional HDL contributing to anti-atherogenic efficacy of *Desmodium gyrans* DC. *WJPPS*; 2014, 4, 1-16.

Von Hoff D D, Layard M W, Basa P, Davis H L, Von Hoff A L, Rozencweig M, Muggia F M. Risk factors for doxorubicin-induced congestive heart failure. *Annals of Internal Med*; 1979, 91, 710-717.

Vu Dac N, Schoonjans K, Kosykh V, Dallongeville J, Fruchart J C, Staels B, Auwerx J. Fibrates increase human apolipoprotein A-II expression through activation of the peroxisome proliferator-activated receptor. *J Clin Invest*; 1995, 96, 741-750.

Vu Dac N, Schoonjans K, Laine B, Fruchart J C, Auwerx J, Staels B. Negative regulation of the human apolipoprotein A-I promoter by fibrates can be attenuated by the interaction of the peroxisome proliferator-activated receptor with its response element. *J Biol Chem*; 1994, 269, 1012-1018.

Wahab M A, Akoul E M S, Abdelaziz A H. Modulatory effects of melatonin and vitamin-E on doxorubicin-induced cardiotoxicity in Ehrlich ascites carcinoma bearing mice. *Tumorigenesis*; 2000, 86, 157-162.

Waldi D. Spray reagents for thin layer chromatography: A laboratory book, Academic press, New York, USA, 1965.

Walzem R L, Wong J S, Blaner W S, Ding Z M, Melford K, Wongsiriroj N, Shu X, de Sauvage F, Ryan R O, Fong L G, Bensadoun A, Young SG. Glycosyl phosphatidyl inositol-anchored high-density lipoprotein-binding protein 1 plays a critical role in the lipolytic processing of chylomicrons. *Cell Metabolism*; 2007, 5, 279-291.

Wang C S, Hartsuck J, McConathy W J. Structure and functional properties of lipoprotein lipase. *Biochim Biophys Acta*; 1992, 1123, 1-17.

Wang L, Gill R, Pedersen T L. Triglyceride-rich lipoprotein lipolysis releases neutral and oxidized FFAs that induce endothelial cell inflammation. *J Lipid Res*; 2009, 50, 204-213.

Wannamethee S G, Shaper A G, Walker M. Changes in physical activity, mortality, and incidence of coronary heart disease in older men. *Lancet*; 1998, 351, 1603-1608.

Warrier P K. Indian Medicinal Plants: A Compendium of 500 Species (1st ed.), Orient Longman, Chennai, 1995.

Watson A D, Berliner J A, Hama S Y, La Du B N, Fault K F, Fogelman A M, Navab M. Protective effect of high density lipoprotein associated paraoxonase: inhibition of the biological activity of minimally oxidized low-density lipoprotein. *J Clin Invest*; 1995, 96, 2882-2891.

Weeler-Aceto A, Cowan. Neurogenic and tissue-mediated components of formalin-induced edema: Evidence for supra spinal regulation. *Agents and actions*; 1991, 34, 264-269.

Wexler B C. Myocardial infarction in young vs old male rats: Pathophysiological changes. *Am Heart J*; 1978, 96, 70-80.

Whitney E J, Krasuski R A, Personius B E. A randomized trial of a strategy for increasing high-density lipoprotein cholesterol levels: effects on progression of coronary heart disease and clinical events. *Ann Intern Med*; 2005,142, 95-104.

Wierzbicki A S, Have we forgotten the pivotal role of the high density lipoprotein cholesterol in atherosclerosis prevention. *Curr Med. Res. Opin*; 2005, 21, 299-306.

Wilcox C S. Effects of tempol and redox-cycling nitroxides in models of oxidative stress. *Pharmacol Ther*; 2010, 126, 119-145.

Willcox J K, Ash S L, Catignani G L. Antioxidants and prevention of chronic disease. *Review Crit Rev Food Sci Nutr*; 2004, 44, 275-295.

Willems F F, Aengevaeren W R, Boers G H, Blom H J, Verheugt F W. Coronary endothelial function in hyperhomocysteinemia: improvement after treatment with folic acid and cobalamin in patients with coronary artery disease. *J Am Coll Cardiol*; 2002, 40, 766-772.

Wilson P W, D'Agostino R B, Levy D, Belanger A M, Silbershatz H, Kannel W B. Prediction of coronary heart disease using risk factor categories. *Circulation*; 1997, 81, 1837-1847.

Wink M. A short history of alkaloids. In: Alkaloids: Biochemistry, ecology and medicinal applications, Plenum, New York, 1998, 11-44.

Witting P K, Pettersson K, Letters J, Stocker R. Site-specific antiatherogenic effect of probucol in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol*; 2000, 20, 26-33.

Witztum J L, Steinberg D. The oxidative modification hypothesis of atherosclerosis: does it hold for humans. *Trends Cardiovasc Med*; 2001, 11, 93-102.

Wong H, Davis R C, Thuren T, Goers J W, Nikazy J, Waite M, Schotz M C. Lipoprotein lipase domain function. *J Biol Chem*; 1994, 269, 10319-10323.

Wong H, Schotz M C. The lipase gene family. *J Lipid Res*; 2002, 43, 993-999.

Woo K V, Scott Baldwin H. Role of Tie1 in Shear Stress and atherosclerosis trends. *Cardiovasc Med*; 2011, 21, 118-123.

Wu B J, Kathir K, Witting P K, Beck K, Choy K. Antioxidants protect from atherosclerosis by a heme oxygenase-1 pathway that is independent of free radical scavenging. *J Exp Med;* 2006, 203, 1117-1127.

Wu B J, Yan L, Charlton F Witting P, Barter P J, Rye K A. Evidence that niacin inhibits acute vascular inflammation and improves endothelial dysfunction independent of changes in plasma lipids. *Arterioscler Thromb Vasc Biol*; 2010, 30, 968-975.

Wu K K, Wu T J, Chin J, Mitnaul L J, Hernandez M. Increased hypercholesterolemia and atherosclerosis in mice lacking both ApoE and leptin receptor. *Atherosclerosis*; 2005, 181, 251-259.

Xia P, Vadas M A, Rye K A, Barter P J, Gamble J R. High density lipoproteins (HDL) interrupt the sphingosine kinase signaling pathway. A possible mechanism for protection against atherosclerosis by HDL. *J Biol Chem*; 1999, 274, 143-147.

Xu M F, Tang P L, Qian Z M, Ashraf M. Effects by doxorubicin on the myocardium is mediated by oxygen free radicals. *Life Science*; 2001, 68, 889-901.

Yamada K, Naemura A, Sawashita N, Noguchi Y, Yamamoto. An onion variety has natural antithrombotic effect as assessed by thrombosis models in rodents. *J Thromb Res*; 2004, 213-220.

Yangsoo J, Lincoff M, Plow E F, Topol E J. Cell adhesion molecules in coronary artery disease. *J Am Coll Cardiol*; 1994, 24, 1591-1601.

Yarnell J W, Patterson C C, Sweetnam P M. Do total and high density lipoprotein cholesterol and triglycerides act independently in the prediction of ischemic heart disease? Ten-year follow-up of Caerphilly and Speedwell Cohorts. *Arterioscler Thromb Vasc Biol*; 2001, 21, 1340-1345.

Yasojima K, Schwab C, McGeer E G, McGeer, P L. *Am J Pathol*; 2001, 158, 1039-1051.

Yasuhiko Homma, Koichiro Homma, Shinichi Iizuka, Kamon Iigaya. Effects of Fluvastatin on Plasma Levels of Low-Density Lipoprotein Subfractions, Oxidized Low-Density Lipoprotein, and Soluble Adhesion Molecules: A Twenty-Four–Week, Open-Label, Dose-Increasing Study *Curr Ther Res Clin Exp*; 2003, 64, 236-247.

Yip C Y, Chen J H, Zhao R, Simmons C A. Calcification by valve interstitial cells is regulated by the stiffness of the extracellular matrix. *Arterioscler Thromb Vasc Biol*; 2009, 29, 936-942.

Yi R C, Jin H, Rajashree K, Tony K A N, Tse H T. Phenylethyl isothiocyanate induces apoptotic signaling via suppressing phosphatase activity against c-Jun N-terminal kinase. *J Biol Chem*; 2002, 277, 334-342.

Yost T J, Jensen D R, Haugen B R, Eckel R H. Effect of dietary macronutrient composition on tissue-specific lipoprotein lipase activity and insulin action in normal-weight subjects. *Am J Clin Nutr*; 1998, 68, 296-302.

Young J L, Libby P, Atherosclerosis, Pathophysiology of heart diseases: A collaborative project of medical student and faculty. Baltimore, Lippinocolt Wiiliams & Wilkins, USA, 2007, 123, 132-138.

Yu R, Mandlekar S, Harvey K. Chemopreventive isothiocyanates induce apoptosis and caspase-3-like protease activity. *Canc Res*; 1998, 58, 402-408.

Yui Y, Aoyama T, Morishita H, Takahashi M, Takatsu Y, Kawai C. Serum prostacyclin stabilizing factor is identical to apolipoprotein A-I. A novel functions of Apo A-I. *J Clin Invest*; 1988, 82, 803-807.

Yusuf M, Begum J, Hoque M N, Chowdhury J U. Medicinal plants of Bangladesh, *Bangladesh Council Sci Ind Res*; 2009, 8, 462-463.

Yusuf S, Sleight P, Pogue J. Effects of an angiotensin converting enzyme inhibitor, ramipril, on cardiovascular events in high risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. *N Engl J Med*; 2000, 342,145-153.

Yves C, Jean P B, Claire T, Luc S, Kjell S, Bernard B. Antithrombotic effect with combined aspirin and a loading dose of clopidogrel on experimental arterial thrombogenesis in humans early potent. *Circulation*; 2000, 101, 2823-2828.

Zakaria M M, Islam M W, Radhakrishnan R, Chen H B, Kamil M, Al-Gifri A N. Antinociceptive and anti-inflammatory properties of *Caralluma arabica*. *J Ethnopharmacol*; 2001, 76, 155-158.

Zhang Y, Zanotti I, Reilly M P, Glick J M, Rothblat G H, Rader D J. Overexpression of Apolipoprotein A-I promotes reverse transport of cholesterol from macrophages to feces in vivo. *Circulation*; 2003, 108, 661-663.

Zheng C, Furtado J, Khoo C. Apolipoprotein C-III and the metabolic basis for hypertriglyceridemia and the dense LDL phenotype. *Circulation*; 2010, 121, 1722-1734.

Zipes D P, Libby P, Bonow R O, Braunwald E. Braunwald's heart disease: A textbook of cardiovascular medicine (7th ed.), Elsevier Saunders, Pennsylvania, USA, 2005.

Zirlik A, Maier C, Gerdes N, MacFarlane L, Soosairajah J. CD 40 ligand mediates inflammation independently of CD40 by interaction with Mac-1. *Circulation*; 2007, 115, 1571-1583.

LIST OF PUBLICATIONS

1. **VIPIN P. S**, Johannah N. M, Seema Menon, Lincy Lawrence, Indu M. S and Jose Padikkala. Study of antioxidant and anti-inflammatory activities of methanol extract of *Desmodium gyrans* DC. *Journal of Chemical and Pharmaceutical Research*, 2015, 7(9), 227-234.

2. **VIPIN P. S**, Johannah N. M, Seema Menon, Lincy Lawrence, Achuthan C. Raghavamenon and Jose Padikkala. Enhancement of functional HDL contributing to anti-atherogenic efficacy of *Desmodium gyrans* DC.

World journal of pharmacy and pharmaceutical sciences, 2015, 4(1), Research Article ISSN 2278 – 4357.

3. **VIPIN P. S**, Johannah N. M, Seema Menon, Lincy Lawrence, Achuthan C. Raghavamenon and Jose Padikkala. Antithrombotic and anticoagulant activities of *Desmodium gyrans* DC. *Journal of Chemical and Pharmaceutical Research*, 2015, 7(5), 973-980.

4. Seema Menon, Lincy Lawrence, **VIPIN P. S** and Jose Padikkala. Phytochemistry and evaluation of *in vivo* antioxidant and anti-inflammatory activities of *Oroxylum indicum* Vent. root bark. *JOCPR*, 2015, 7(10), 767-775.

5. Lincy L, Seema M, Sheka V, **VIPIN P S** and Jose P. Radical scavenging and gastroprotective ativity of methanolic extract of *Gmelina arborea* stem bark. *Journal of Ayurvedic and Integrative Medicine*, 2016, 7, 78-82.

6. Lincy L, Seema M, Divya M K, **VIPIN P S** and Jose P. Inhibition of dimethyl benz (a) anthracene (DMBA) - croton oil induced mouse skin tumorigenesis by *Gmelina arborea* with potential anti-inflammatory activity. *Journal of Environmental Pathology, Toxicology and Oncology*, 2016, 35(3), 263–272.