"PHYTOPHARMACOLOGICAL EVALUATION OF THE BARK OF *AILANTHUS EXCELSA* ROXB. WITH SPECIAL REFERENCE TO ITS ANTI HYPERLIPIDEMIC ACTIVITY"

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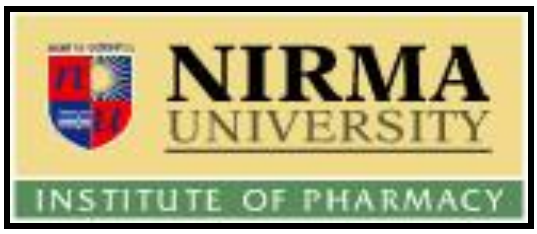
PHYTOPHARMACEUTICALS & NATURAL PRODUCTS

BY

DIVYANG PATEL. (10MPH502), B. Pharm.

UNDER THE GUIDANCE OF

PROF. (DR.) VIMAL KUMAR,
M. Pharm., Ph.D.



DEPARTMENT OF PHYTOPHARMACEUTICALS & NATURAL PRODUCTS
INSTITUTE OF PHARMACY
NIRMA UNIVERSITY
AHMEDABAD 382481
GUJARAT, INDIA

MAY 2012

CERTIFICATE

This is to certify that MR. DIVYANG PATEL has prepared his thesis entitled " Phytopharmacological Evaluation of the bark of *Ailanthus excelsa* Roxb. with Special Reference to its Anti Hyperlipidemic Activity ", in partial fulfillment for the award of M. Pharm. degree of the Nirma University, under my guidance. He has carried out the work at the Department of Phytopharmaceuticals and Natural Products, Institute of Pharmacy, Nirma University, Ahmedabad.

Guide:

Prof.(Dr.) Vimal Kumar
M. Pharm., Ph.D.
Professor & Head,
Department of
Phytopharmaceuticals
& Natural Products,
Institute of Pharmacy,
Nirma University, Ahmedabad

Forwarded Through:

Dr. Manjunath Ghate
M. Pharm., Ph.D.
Director
Institute of Pharmacy,
Nirma University, Ahmedabad

Date: 19th May, 2012

DECLARATION

I declare that the thesis " Phytopharmacological Evaluation of the bark of *Ailanthus excelsa* Roxb. with Special Reference to its Anti Hyperlipidemic Activity ", has been prepared by me under the guidance of Prof. (Dr.) Vimal Kumar, Professor and Head, Department of Phytopharmaceuticals and Natural Products, Institute of Pharmacy, Nirma University. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

DVP.

MR. DIVYANG PATEL (10MPH502)
Department of Phytopharmaceuticals
And Natural Products
Institute of Pharmacy
Nirma University
Sarkhej - Gandhinagar Highway
Ahmedabad-382481
Gujarat, India

Date: 19th May, 2012

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Date: ***Divyang Patel***

Place*:* ***Institute of Pharmacy,***

Nirma University,

Ahmedabad.

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List of Abbreviations :

Short form	Full form
AIDS	Acquired Immune Deficiency Syndrome
TLC	Thin Layer Chromatography
R_f	Retention Factor
AQ	Aqueous extract of *A.excelsa*
µ	Micro
g.	Gram
Ml	Millilitre
WHO	World Health Organization
%	Percentile
Kg	Kilogram
p.o.	Per os (mouth, orally)
i.p.	Intra peritoneal
i.v.	Intra venous
H	Hour
Min	Minute(s)
STZ	Streptozotocin
H_2SO_4	Sulphuric acid
NaCl	Sodium chloride
$FeCl_3$	Ferric chloride
KOH	Potassium hydroxide
$NaCo_3$	Sodium carbonate
$AlCl_3$	Aluminium chloride
CPCSEA	Committee for the purpose of control and supervision of experiments on animals
ANOVA	The analysis of variance
%w/w	Percentage weight by weight
TC	Total Cholesterol
TG	Triglycerides
LDL	Low Density Lipoprotein
HDL	High Density Lipoprotein
TP	Total Proteins
HFD	High Fat Diet
CCD	Charged Couple Device
AQ200	Aqueous Extracts 200 mg/kg
AQ400	Aqueous Extracts 400 mg/kg
ME200	Methanolic Extracts 200mg/kg
ME400	Methanolic Extracts 200 mg/kg
Quassi100	Quassinoid Rich Fraction 100 mg/kg
Quassi 200	Quassinoid Rich Fraction 200 mg/kg
LCAT	Lecithin-Cholesterol Acyltransferase
CETP	Cholesteryl Ester Transfer Protein
VLDL	Very Low Density Lipoproteins

IDL,	Intermediate-Density Lipoproteins
LDLR	Low-Density Lipoprotein Receptor
SR-B1	Scavenger Receptor Class B1

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Chapter 1

Abstract

Abstract

The importance of serum lipoprotein disturbances and abnormal lipid metabolism characterized by hyperlipidemia or hyperlipoproteinemia as etiological factors in the development of coronary heart diseases and potentiating of arteriosclerosis is now supported by a considerable body of evidence amassed from epidemiological and population studies (Anonymous, 1971 and 1984; Turpanen, 1979). Infect, it is almost accepted that arteriosclerosis is a disorder of lipid transport and metabolism. Cholesterol by-product would form thick, tough deposit called plague on the inner wall of the arteries, stiffening them and then starving the heart of blood, creating choke point where a clot could stop the flow entirely (Duff and Macmillian, 1951; Goldstein *et al*., 1979).

Ailanthus is a deciduous tree belonging to the family Simaroubaceae and is widely distributed in Asia and north Australia. Its native origin is China and it is known as 'tree of heaven', 'tree of sun' or 'Persian sumach' (Adamik and Brauns, 1957). In traditional medicine *A. excelsa* is used to cure wounds and skin eruptions and is used in the indigenous system of medicine as a antipyretic, and for bronchitis, asthma and in conditions of diarrhoea and dysentery (British Pharmacopoeia, 1988).

Macroscopical and microscopical characters were studied first for authentication of the bark of *A.excelsa*. Various physicochemical parameters like ash values, extractive values, moisture content and swelling index were also evaluated for the bark powder. Preliminary phytochemical screening showed the presence of phytoconstituents like flavonoids, triterpenoids, phenolics, tannins, carbohydrates etc. Estimations of flavonoids, triterpenoids and phenolics showed higher amount of presence of all above three classes of constituents in aqueous extracts as compared to methanolic extracts.

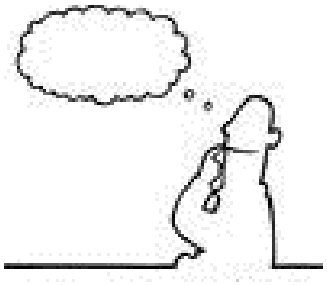
High fat diet (containing 20% ground nut oil, 1% cholesterol, and 0.5 % cholic acid) model was used for *in-vivo* evaluation of anti hyperlipidemic activity. Both aqueous and methanolic extracts showed significant reduction in total cholesterol, triglyceride and LDL levels and increase in HDL and total protein levels in serum when compared to HFD group. Both the extracts showed anti hyperlipidemic activity

comparable with standard drug atorvastatin. Antioxidant parameters for liver were also measured at the end of the study. Significant lower levels of MDA and Glutathion were found in all treated groups when compared to disease induced group. Histopathology studies also showed the less amounts of fatty infiltration in treated groups when compared to disease induced group. Both aqueous (200mg and 400mg) and methanolic (200mg and 400mg) extracts produced no significant effects in all lipid parameters. Significant reduction in body weight and food intake was also observed in all treated groups as compared to high fat diet groups.

In intravenous lipid tolerance test in rats, all the treated and control group animals showed rise in the triglyceride levels at 20 minute interval, and then in all the groups significant reduction in triglyceride levels in next 40 and 60 minute time intervals. Methanolic extracts (200 mg/kg) and quassinoid rich fraction (200 mg/kg) showed significant reduction in triglyceride levels at 60 minutes when compared to 20 min time interval. The aqueous extracts also produced decrease in triglyceride levels at 60 min when compared to 20 min triglyceride levels.

In-vitro lipase assay was also performed and IC 50 value of the aqueous extracts was found to be 400 µg/ml. DPPH free radical scavenging activity was also carried out for anti oxidant activity. Quassinoid rich fraction showed highest anti oxidant activity (with IC 50 value of 85.77± 0.13 µg/ml) as compared to aqueous and methanolic extracts.

All three above observed effects may be due to presence of the predominant phytoconstituents like triteprenoids and flavonoids. In conclusion, aqueous and methanolic extracts of *A.excelsa* possesses anti hyperlipidemic and anti obesity potentials. Hence, it can be suggested that the bark of *A. excelsa* could be useful for the treatment of lipid disorders.



Chapter 2

Introduction

Introduction

Diseases of the cardiovascular system are the most common cause of death. Lifestyle changes have a significant impact on the health of the people. The modernization of societies appears to result in a dietary pattern that is high in saturated fats and refined sugars; and is low in fibres content (Barai et al., 2007). It is now established that hyperlipidemia represents a major risk factor for the premature development of atherosclerosis and its cardiovascular complications.

Ischemic heart disease (IHD) which includes angina pectoris, myocardial infarction and chronic post ischemic cardiac failure is a most common and leading causes of morbidity and mortality in developed and developing countries (Carnethon et al., 2006). Hyperlipidemia, hypertension, obesity, raised coagulation factor and homocysteine are modified risk factors for atherosclerosis, in that hyperlipidemia is a most common risk factor causes IHD in elderly population (Rang et al., 2005).

Atherosclerosis of arteries is a generalized disease of the arterial network known as progressive and silent killer disease characterised by the formation of lesions called atherosclerosis plaques in the walls of large and medium sized coronary arteries which reduces blood flow to the myocardium – called coronary artery disease (Brown et al, 1990). The incidence of IHD has increased dramatically in the last 60 years and such diseases are now recognized as one of the leading causes of death in technically advanced societies (Anderson et al., 1987, Schaefer et al., 1994).

The hyperlipidemia comprises a heterogeneous group of disorders whose characteristic expression is an elevation in the plasma concentration of cholesterol and triglyceride (Goldstein et al, 1973). Hyperlipidemia is a major cause of atherosclerosis and atherosclerosis-associated conditions, such as coronary heart disease (CHD), ischemic cerebro vascular disease, and peripheral vascular disease. These conditions account for most morbidity and mortality among middle-aged and older adults. Hyperlipidemia (elevated levels of triglycerides or cholesterol) and reduced HDL-C levels occur as a consequence of several interrelated factors that affect the concentrations of the various plasma lipoproteins. These factors may be lifestyle or behavioral (*e.g.*,diet or exercise), genetic (*e.g.*,mutations in a gene regulating

lipoprotein levels), or metabolic (*e.g.*, diabetes mellitus or other conditions that influence plasma lipoprotein metabolism)(Brunton et al, 2008).

Recent studies have shown that lipid associated disorders are not only attributed to the total serum cholesterol, but also to its distribution among different lipoproteins. The low density lipoproteins (LDL) are the major carriers of cholesterol towards tissues having atherogenic potential, while the high density lipoproteins (HDL) carrycholesterol from peripheral tissues to the liver. HDL thus gives protection against many cardiac problems and obesity. Although genetic factors recline behind these lipid disorders (Sattivel et al., 2000).

Epidemiological studies have identified numerous risk factors for atheromatous disease. Some of these cannot be altered (e.g. a family history of ischaemic heart disease), but others are modifiable and are potential targets for therapeutic drugs (Rang et al, 2005). The list of modifiable risk factors includes the raised low density lipoprotein, reduced high density lipoprotein, hypertension, diabetes mellitus, cigarette smoking, obesity, physical inactivity, raised C-reactive protein, raised homocystein. Recent studies have demonstrated that increased formation of free radicals / reactive oxygen species (ROS) contribute to cardiovascular disease (CVD) progression (Kaliora et al., 2006). Reactive oxygen species induce cardiac dysfunction and cardiac apoptosis and/or necrosis in heart failure (Griendling et al., 1998). Reactive oxygen species are formed intracellularly and are controlled by antioxidant defense. The generation of large amounts of reactive oxygen species can overwhelm the intracellular antioxidant defense, causing activation of lipid peroxidation, protein modification, and DNA breaks (Hiroi et al ., 1999). Reactive oxygen species induced depletion of antioxidants is a key factor for the initiation of atherosclerosis and the development of CVD (Kaliora et al., 2006).

Clinical trials showed conclusively that lowering serum cholesterol reduces morbidity and mortality from coronary artery diseases in the patients with established coronary artery disease and also reduces new coronary artery disease events and mortality in the patients without established coronary artery disease (Amundenson et al,. 2002). Hyperlipidemia following oxidative stress may cause oxidative modifications in low

density lipoproteins, which play an important role in the initiation and progression of atherosclerosis and related cardiovascular diseases (Parthasarthy et al, 1992).

Currently available allopathic treatments for hyperlipidemic conditions are statins, nicotinic acid, fibric acid derivatives and bile acid binding resins. Although statins have been found to be effective in lowering the serum low-density lipid levels by as much as 21% to 43% and they have been found to cause many adverse side effects. Statins are basically enzyme inhibitors, so it is likely that they may be inhibiting other critical enzymes in the body that have not been investigated so far, causing serious adverse side effects. Statins are ingested on a long-term basis to produce and maintain the desirable effect; therefore, there may be a risk of chronic toxic effects, including carcinogenic, teratogenic, and mutagenic, over a lifetime of use (Carlson 2006, Clark 2003). Major side effect seen with fibric acid derivatives is rhabdomyolysis. Resins are not absorbed properly therefore, systemic toxicity is low but gastrointestinal symptoms-especially diarrhoea is most common side effect. High doses of Nicotinic acid can disturb liver function, impair glucose tolerance, and precipitate gout by increasing circulating urate concentration (Rang et al, 2005).

There is a continuous search for alternative drugs. Therefore it is prudent to look for options in herbal medicine for CVD treatment as well. India is in the cutting edge of well-recorded and traditionally well practiced knowledge of herbal medicine. More than 80% of Indian population still use traditional folk medicine therapies for treating their ailments (Vaidhya et al 2007). In the traditional system of Indian medicine, plant formulation and combined extracts of plants are used as drug of choice rather than individual. The herbal ingredients in these formulations are selected based on their healing property with respect to the disease condition. There are several such medicinal plant formulations and recommendations for their preparation which are mentioned in antique literature for treating various ailments with no side effects to the individual. Since, the information is too old, there is the need to revive these recommendations carefully by making use of modern scientific tools in order to advocate their scientific merit and supremacy over the existing drugs. WHO also recommended for further investigation into traditional method of treatment (WHO 1991).

Some of the major limitations in the effective pharmacological treatment of hyperlipidemia are the constraints imposed on health care resources, particularly in the low and middle income countries (Bergman et al., 2005). There is a need to tackle this physiological problem as it is attaining grave proportions globally. In this scenario , the problem may be tackled by the use of natural agents due to their cost effectiveness and minimal side effects (Oluwatosin et al., 2008).

In recent times, much research intrest has been focused on various herbs that possess hypolipidemic property that may be useful in reducing the risk factor of CVD (Craig et al.,1999). Currently the use of complimentary and alternative medicines and especially the consumption of phytochemicals have been rapidly increasing worldwide. As herbal medicines are less damaging than synthetic drugs they have better compatibility thus improving patient tolerance even on long term use. (Kaliora et al., 2006). Condiments, medicinal plants, fruits, used in day to day preparation of food in Indian kitchen have been identified as hypolipidemic in Ayurveda (Bishayee et al., 1994) .

Medicinal plants have a significant role in maintaining human health and improving the quality of human life for thousands of years and served humans as valuable components of medicine. Further more many western drugs had their origin in plant extracts. There are many herbs which are predominantly used to treat cardiovascular, liver, central nervous system, digestive and metabolic disorders and they can be used as drug or supplement in management of various disease. Medicinal plant are also important source of other beneficial compounds such as antioxidants, oligosaccharides, essential fatty acids, vitamins, minerals, lignins, fibers (Arulmozhi et al., 2007).

In many developing countries most of hyperlipidemic individuals use medicinal plants to treat hyperlipidemia and atherosclerosis. Therefore, there is a strong interest to search for natural hyperlipidemic substances derived from medicinal plants. A vast number of medicinal plants has received attention in this regard and they have been shown to lower plasma lipid levels (Harnafi et al., 2007). The wide use of these herbal drugs had now lead to carry out research in institutions and universities on the potential benefits of herbal drugs.

Herbal drugs are probably the most common source of samples for evaluation in high throughout screening of natural products. They have yielded many useful compounds and plant-derived ingredients, which are an important component of modern pharmaceuticals (Miller and Gereau, 2000). A large number of medicinal plants useful in CVD have been described in traditional and folklore medicines including *Ailanthus excelsa*. Therefore, the *Ailanthus excelsa* Roxb. bark was selected for the anti-hyperlipidemic activity. In the near future we can expect the introduction of a range of novel chemical entities in the various therapies for hyperlipidemia from this medicinal plant.

Chapter 3

Literature Review

3. Literature Review

3.1. Literature Review of Hyperlipidemia

3.1.1. Epidemiology of Hyperlipidemia

Hyperlipidemia and its associated consequences are undisputed risk factors in the development of atherosclerosis. Moreover, a World Health Organization (WHO) survey reveals that India is predicted to have a large number of mortalities due to coronary artery disease by the year 2015 (Singh et al.,2002). Hyperlipidemia; the disorder of lipid metabolism have been ranked as one of the greatest risk factors contributing to the prevalence and severity of atherosclerosis, stroke and coronary heart diseases (Saravanan et al., 2003 & Grundy et al., 1986).

Hyperlipidemia characterized by hypercholesterolemia is the most prevalent indicator for susceptibility to cardiovascular diseases. World Health Organization (WHO) report suggested that high blood cholesterol contributes to approximately 56 % cases of cardiovascular diseases worldwide and causes about 4.4 million deaths each year. (3^{rd} report of the National Cholesterol Education Program, 2002). The term "dyslipidaemia" now a days is increasingly being used to describe abnormal changes in lipid profile, replacing the old term hyperlipidaemia.

Hyperlipidemia is a metabolic disorder, specifically characterized by alterations occurring in serum lipid and lipoprotein profile due to increased concentrations of Total Cholesterol (TC) , Low Density Lipoprotein Cholesterol (LDL-C), Very Low Density Lipoprotein Cholesterol (VDLD-C) , and Triglycerides (TAG) with a concominant decrease in the concentration of High Density Lipoprotein Cholesterol (HDL-C) in the blood circulation (Dhuley et al., 1999).

A relationship between total cholesterol and the development of coronary heart disease is recognized, as is its contribution to the 1 in 4 deaths from coronary heart disease that occurs each year in the UK. A reduction in the mean level of total cholesterol in the population will reduce the development of coronary atherosclerosis and the prevalence of coronary heart disease (Walker et al, 2007).

3.1.2. Etiology of Hyperlipidemia

It can be caused by primary causes or secondary causes.

1. Primary causes:

- Familial or Genetic due to single gene defect,
- Multifactorial or polygenic which have multiple genetic defect, dietary and physical activity related causes.

2. Secondary causes :

- Anabolic steroid use
- Anorexia nervosa
- Cigarette smoking
- Diabetes
- Glycogen storage diseases
- Hypothyroidism
- Liver disease
- Medications: corticosteroids, anticonvulsants and certain oral contraceptives,
- Overweight or obesity
- Renal disease
- Therapeutic diet: ketogenic; high carbohydrate
- Transplant (bone marrow, heart, kidney and liver)

Risk factors

- Family history of premature death in first-degree relative (male relative <55 years, female relative <65 years)
- Hypertension(blood pressure ≥140/90 mm Hg or on antihypertensive medication)
- Diabetes mellitus
- Cigarette smoking
- Low HDL cholesterol (<35 mg/dl)
- Overweight or obesity
- Physical inactivity

NCEP redefined low HDL for adults as <40 mg/dl and considers high levels (>60 mg/dl) as protective. Similar adjustments of values for adolescents have not been

set, however. Recent research is elucidating the roles of other variables or "emerging risk factors" that are considered to confer greater risk of CHD:

- Increased fibrinogen PA1-1 levels
- Elevated levels of homocysteine
- Increased levels of c-reactive protein
- Increased Lp(a) level

3.1.3. Pathophysiology

Lipoprotein Metabolism

Lipoprotein Classification and Composition:

Lipoproteins are large, mostly spherical complexes that transport lipids (primarily triglycerides, cholesteryl esters, and fat-soluble vitamins) through body fluids (plasma, interstitial fluid, and lymph) to and from tissues. Lipoproteins play an essential role in the absorption of dietary cholesterol, long-chain fatty acids, and fat-soluble vitamins; the transport of triglycerides, cholesterol, and fat-soluble vitamins from the liver to peripheral tissues; and the transport of cholesterol from peripheral tissues to the liver (Daniel et al,2000, Rang et al,2005),

Lipoproteins contain a core of hydrophobic lipids (triglycerides and cholesteryl esters) surrounded by hydrophilic lipids (phospholipids, unesterified cholesterol) and proteins that interact with body fluids.

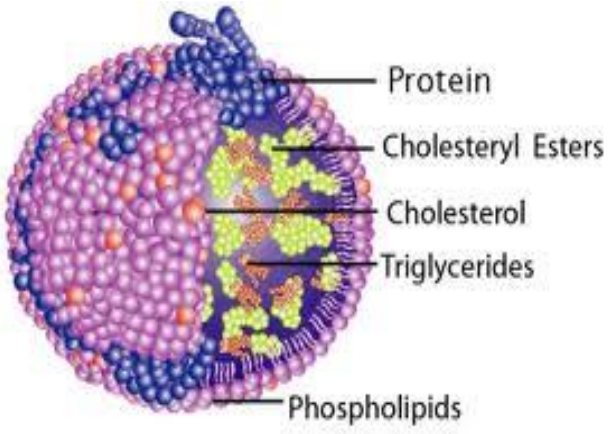


Fig 1 : Structure of lipoprotein

Each lipoprotein class comprises a family of particles that vary slightly in density, size, migration during electrophoresis, and protein composition. The density of a lipoprotein is determined by the amount of lipid and protein per particle.

The plasma lipoproteins are divided into six major classes based on their relative densities (Segrest et al 2000).

- **Chylomicrons :** Produced by the gut in response to dietary fat.
- **VLDL (Very Low Density Lipoprotein):** Produced by the liver.
- **IDL(Intermediate Density Lipoprotein):** An intermediate in VLDL catabolism.
- **LDL(Low Density Lipoprotein):** End product of VLDL catabolism ("Bad" cholesterol).
- **HDL(High Density Lipoprotein):** Produced by the liver and gut ("Good" cholesterol).
- **Lp(a):** Consists of LDL plus a protein called apo(a) ("Heart attack" cholesterol).

A. Chylomicrons

They are formed in the intestine and carry triglycerides of dietary origin, unesterified cholesterol, and cholesteryl esters. They transit the thoracic duct to the blood stream. Triglycerides are removed in extrahepatic tissues through a pathway shared with VLDL that involves hydrolysis by the lipoprotein lipase system. Decrease in particle diameter occurs as triglycerides are depleted. Surface lipids and small lipoprotein are transferred to HDL. The resultant chylomicron are taken up by receptor mediated endocytosis into hepatocytes.

B. Very Low Density Lipoprotein

They are secreted by liver and export triglycerides to peripheral tissues. VLDL triglycerides are hydrolyzed by LPL-yielding free fatty acids for storage in adipose tissue and for oxidation in tissues such as cardiac and skeletal muscles. Depletion of triglycerides produces remnants (IDL), some of which undergo endocytosis directly by liver. The remainder is converted to LDL by further removal of triglycerides mediated by hepatic lipase. This process explains the “beta shift “ phenomenon, the increase of LDL (beta lipoprotein) in serum as hypertriglyceridemia. Increased level

of LDL can also result from increased secretion of VLDL and from decreased LDL catabolism.

C. Intermediate Density Lipoprotein

Intermediate-density lipoproteins are formed from the degradation of very low-density lipoproteins. IDL enables fats and cholesterol to move within the water-based solution of the bloodstream. Each native IDL particle consists of protein that encircles various fatty acids, enabling, as a water-soluble particle, these fatty acids to travel in the aqueous blood environment as part of the fat transport system within the body. They are cleared from the plasma into the liver by receptor-mediated endocytosis, or further degraded to form LDL particles. In general, IDL, somewhat similar to low-density lipoprotein (LDL), transports a variety of triglyceride fats and cholesterol like LDL and can also promote the growth of atheroma. VLDL is a large, triglyceride-rich lipoprotein secreted by the liver that transports triglyceride to adipose tissue and muscle. The triglycerides in VLDL are removed in capillaries by the enzyme lipoprotein lipase and the VLDL returns to the circulation as a smaller particle with a new name, intermediate-density lipoprotein (IDL). The IDL particles have lost most of their triglyceride, but they retain cholesteryl esters. Some of the IDL particles are rapidly taken up by the liver; others remain in circulation, where they undergo further triglyceride hydrolysis and are converted to LDL.

D. Low Density Lipoproteins

LDL is catabolised chiefly in hepatocytes and other cells by receptor-mediated endocytosis. Cholesteryl esters from LDL are hydrolyzed yielding free cholesterol for the synthesis of cell membranes. Cells also obtain cholesterol by synthesis via a pathway involving the formation of mevalonic acid by HMG-CoA reductase. Production of this enzyme and of LDL receptors is transcriptionally regulated by the content of cholesterol in the cell. Normally, about 70% of LDL is removed from plasma by hepatocytes. Even more cholesterol is delivered to the liver via IDL and chylomicrons. Unlike other cells, hepatocytes can eliminate cholesterol by secretion in bile and by conversion to bile acids.

E . High Density Lipoproteins

The apoproteins of HDL are secreted by the liver and intestine. Much of the lipid comes from the surface monolayers of chylomicrons and VLDL during lipolysis. HDL also acquires cholesterol from peripheral tissues, protecting the cholesterol homeostasis of cells. Free cholesterol is transported from the cell membrane by a transporter, ABCA1, acquired by a small particle termed prebeta-1 HDL, and then esterified by lecithin: cholesterol acyltransferase (LCAT), leading to the formation of larger HDL species. Cholesterol is also exported from macrophages by the ABCG1 transporter to large HDL particles. The cholesteryl esters are transferred to VLDL, IDL, LDL, and chylomicron remnants with the aid of cholesteryl ester transfer protein (CETP). Much of the cholesteryl ester transferred is ultimately delivered to the liver by endocytosis of the acceptor lipoproteins. HDL can also deliver cholesteryl esters directly to the liver via a docking receptor (Scavenger receptor, SR-BI) that does not cause endocytosis of the lipoproteins.

F . Lp (a)

Lp(a) is a major independent genetic risk factor for cardiovascular disease. Lp(a) particles are similar to LDL consisting of a cholesterol-rich core, with an apoB-100 protein attached. However, Lp(a) uniquely differs to LDL in that it also has an apo(a) protein attached via a disulfide bond. The apo(a) is comprised of a series of kringle structures. Apo(a) is synthesised in liver and binds to newly synthesised apoB-100. The size of the apo(a) protein is genetically determined and levels of Lp(a) can vary up to 1000-fold between individuals. Plasma levels rise shortly after birth up to a consistent level within several months, typical plasma levels of Lp(a) are similar in men and women: one in five (20%) have levels above 50 mg/dL. How an increased Lp(a) contributes to heart disease is not clear. The lesions in artery walls contain substances that may interact with Lp(a), leading to the build up of lipids in atherosclerotic plaques.

Table 1: Characteristics of Plasma Lipoproteins : (Brunton et al,2008)

Lipo.P Class	Density of flotation g/ml	Major lipid constituent	TG: Chol Ratio	Significant apoprotein	Site of synthesis	Mechanism of Catabolism
Chylo microns	<<1.006	Dietary triglycerides & cholesterol	10:1	B-48,E.A-I, A-IV,C-I, C-II, C-III	Intestine	TG hydrolysis by LPL, Apo–E mediate remnant uptake by liver
VLDL	<1.006	Hepatic triglycerides	5:1	B-100, E, C-II, C-III	Liver	TG hydrolysis by LPL
IDL	1.006 to 1.009	Cholesterol esters	1:1	B-100, E, C-II, C-III	Product of VLDL catabolism	50% Converted to LDL mediated by HL, 50 % Apo – E mediated uptake by liver
LDL	1.019 to 1.062	Cholesteryl esters	NS	B-100	Product of VLDL catabolism	Apo-B 100 mediated uptake by LDL receptor
HDL	1.063 to 1.21	Phospholipid, Cholesteryl esters	NS	A-I ,A-II, E, C-I, C-II, C-III	Intestine Liver Plasma	Uptake of HDL cholesterol by hepatocytes
Lp (a)	1.05 to 1.09	Cholesteryl esters	NS	B-100, Apo (a)	Liver	Unknown

HDL - High Density Lipoprotein

LDL - Low Density Lipoprotein

IDL - Intermediate Density Lipoprotein

VLDL - Very Low Density Lipoprotein

Lp(a) - Apoliprotein

3.1.4 Lipoprotein Families and its Clinical Significance :

Classification of plasma lipoproteins on the basis of apolipoprotein (apo) composition recognizes two lipoprotein (Lp) classes, one of which is characterized by apo A-I and the other by apo B as major protein constituents. The former lipoprotein class consists of three major subclasses referred to (according to their apolipoprotein constituents) as Lp-A-I, Lp-A-I:A-II, and Lp-A-II, and the latter one of five subclasses called Lp-B, Lp-B:E, Lp-B:C, Lp-B:C:E, and Lp-AII: B:C:D:E.

As polydisperse systems of particles, the apoA-I-containing lipoproteins overlap in high-density segments and apo B- containing lipoproteins in low-density segments of the density gradient. Each subclass is characterized by a specific chemical composition and metabolic property. Normolipidemia and dyslipoproteinemias are characterized by quantitative rather than qualitative differences in the levels of apoA- and apoB-containing subclasses. Furthermore, apoA-containing subclasses seem to differ with respect to their relative antiatherogenic capacities, and apoB-containing subclasses regarding their relative atherogenic potentials. Whereas Lp-A-I may have a greater antiatherogenic capacity than other apoA containing subclasses, the cholesterol-enriched Lp-B:C appears to be the most atherogenic subclass among apoB-containing lipoprotein families. The use of pharmacologic and/or dietary interventions to treat dyslipoproteinemias has already shown that these therapeutic modalities may affect selectively individual apolipoprotein-defined lipoproteins, and thus allow the selection of individualized treatments targeted at decreasing harmful and/or increasing beneficial lipoprotein subclasses (Alaupovic et al, 2003).

Table 2: Types of Apolipoproteins : (Daniel et al, 1998).

Apolipoprotein	**Primary source**	**Lipoprotein association**	**Function**
ApoA-I	Liver	HDL, chylomicrons	Structural protein for HDL, activates LCAT
ApoA-II	Intestine	HDL, chylomicrons	Structural protein for HDL
ApoA-IV	Liver	HDL, chylomicrons	Unknown
ApoA- V	Intestine	VLDL	Unknown
ApoB-48	Liver	Chylomicrons	Structural protein for chylomicrons
ApoB-100	Liver	VDLD,IDL,LDL,LP(a)	Structural protein for VDLD,LDL,LP(a), ligand for binding to LDL receptors
ApoC-I	Liver	Chylomicrons VLDL,HDL	Unknown
ApoC-II	Liver	Chylomicrons VLDL,HDL	Cofactor for LPL
ApoC-III	Liver	Chylomicrons VLDL,HDL	Inhibits lipoprotein binding to receptors
ApoD	Spleen, brain, testes, adrenals	HDL	Unknown
ApoE	Liver	Chylomicron remnants, IDL,HDL	Ligand for binding to LDL receptor
ApoH	Liver	Chylomicrons VLDL,LDL,HDL	B2 glycoprotein I
ApoJ	Liver	HDL	Unknown
ApoL	Unknown	HDL	Unknown
Apo(a)	Liver	Lp(a)	Unknown

Each class of lipoprotein has a specific role in lipid transport, and there are different pathways for exogenous and for endogenous lipids, as well as a pathway for reverse cholesterol transport. The pathways are distinguished by the main apoproteins (apoB-48, apoB-100 and apoA1, respectively) that are ligands for the key receptors.

Transport of Dietary Lipids (Exogenous Pathway)

The exogenous pathway of lipoprotein metabolism permits efficient transport of dietary lipids. Dietary triglycerides are hydrolyzed by pancreatic lipaseswithin the intestinal lumen and are emulsified with bile acids to form micelles. Dietary cholesterol and retinol are esterified (by the addition of a fatty acid) in the enterocyte to form cholesteryl esters and retinyl esters, respectively. Longer-chain fatty acids (12 carbons) are incorporated into triglycerides and packaged with apoB-48, cholesteryl esters, retinyl esters, phospholipids, and cholesterol to form chylomicrons. Nascent chylomicrons are secreted into the intestinal lymph and delivered directly to the systemic circlation, where they are extensively processed by peripheral tissues before reaching the liver. The particles encounter lipoprotein lipase (LPL), which is anchored to proteoglycans that decorate the capillary endothelial surfaces of adipose tissue, heart, and skeletal muscle . The triglycerides of chylomicrons are hydrolyzed by LPL, and free fatty acids are released; apoC-II, which is transferred to circulating chylomicrons, acts as a cofactor for LPL in this reaction. The released free fatty acids are taken up by adjacent myocytes or adipocytes and either oxidized or reesterified and stored as triglyceride. Some free fatty acids bind albumin and transported to other tissues, especially the liver. The chylomicron particle progressively shrinks in size as the hydrophobic core is hydrolyzed and the hydrophilic lipids (cholesterol and phospholipids) on the particle surface are transferred to HDL. The resultant smaller, more cholesterol ester – rich particles are referred to as *chylomicron remnants.* The remnant particles are rapidly removed from the circulation by the liver in a process that requires apoE. Consequently, few, if any, chylomicrons are present in the blood after a 12-h fast, except in individuals with disorders of chylomicron metabolism.

Transport of Hepatic Lipids (Endogenous Pathway)

The endogenous pathway of lipoprotein metabolism refers to the hepatic secretion and metabolism of VLDL to IDL and LDL (Fig.2).VLDL particles resemble chylomicrons in protein composition but contain apoB-100 rather than apoB-48 and have a higher ratio of cholesterol to triglyceride (1 mg of cholesterol for every 5 mg of triglyceride). The triglycerides of VLDL are derived predominantly from the esterification of long chain fatty acids. The packaging of hepatic triglycerides with the other major components of the nascent VLDL particle (apoB-100, cholesteryl esters, phospholipids, and vitamin E) requires the action of the enzyme microsomal transfer protein (MTP). After secretion into the plasma, VLDL acquires multiple copies of apoE and apolipoproteins of the C series. The triglycerides of VLDL are hydrolyzed by LPL, especially in muscle and adipose tissue. As VLDL remnants undergo further hydrolysis, they continue to shrink in size and become IDL, which contain similar amounts of cholesterol and triglyceride. The liver removes 40 to 60% of VLDL remnants and IDL by LDL receptor–mediated endocytosis via binding to apoE. The remainder of IDL is remodelled by hepatic lipase (HL) to form LDL; during this process, most of the triglyceride in the particle is hydrolyzed and all apolipoproteins except apoB-100 are transferred to other lipoproteins. The cholesterol in LDL accounts for 70% of the plasma cholesterol in most individuals. Approximately 70% of circulating LDLs are cleared by LDL receptor–mediated endocytosis in the liver. Lipoprotein(a) [Lp(a)] is a lipoprotein similar to LDL in lipid and protein composition, but it contains an additional protein called apolipoprotein (a) [apo(a)]. Apo(a) is synthesized in the liver and is attached to apoB-100 by a disulfide linkage. The mechanism by which Lp(a) is removed from the circulation is still unknown.

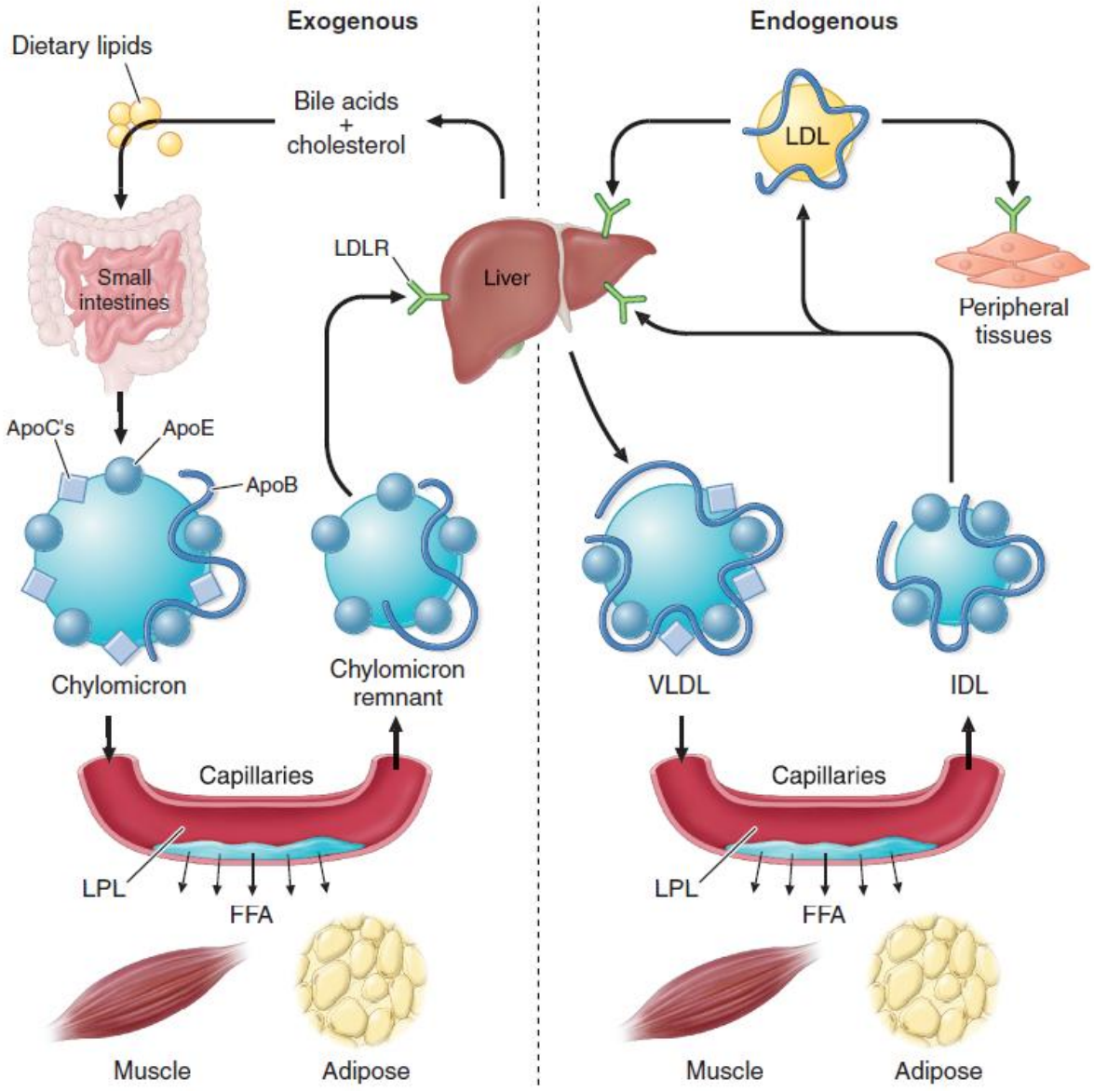


Fig 2 : The exogenous and endogenous lipoprotein metabolic pathways.

The exogenous pathway transports dietary lipids to the periphery and the liver. The exogenous pathway transports hepatic lipids to the periphery. **LPL**, lipoprotein lipase; **FFA,** free fatty acids; **VLDL**, very low density lipoproteins; **IDL**, intermediate-density lipoproteins; **LDL**, low density lipoproteins; **LDLR,** low-density lipoprotein receptor

HDL Metabolism and Reverse Cholesterol Transport

All nucleated cells synthesize cholesterol but only hepatocytes can efficiently metabolize and excrete cholesterol from the body. The predominant route of cholesterol elimination is by excretion into the bile, either directly or after conversion to bile acids. Cholesterol in peripheral cells is transported from the plasma membranes of peripheral cells to the liver by HDL-mediated process termed reverse cholesterol transport . Nascent HDL particles are synthesized by the intestine and the liver. The newly formed discoidal HDL particles contain apoA-I and phospholipids (mainly lecithin) but rapidly acquire unesterified cholesterol and additional phospholipids from peripheral tissues via transport by the membrane protein ATP-binding cassette protein A1 (ABCA1). Once incorporated in the HDL particle, cholesterol is esterified by lecithincholesterol acyltransferase (LCAT), a plasma enzyme associated with HDL. As HDL acquires more cholesterylester it becomes spherical, and additional apolipoproteins and lipids are transferred to the particles from the surfaces of chylomicrons and VLDL during lipolysis. HDL cholesterol is transported to hepatoctyes by both an indirect and a direct pathway. HDL cholesterylesters are transferred to apoB-containing lipoproteins in exchange for triglyceride by the cholesteryl ester transfer protein (CETP). The cholesteryl esters are then removed from the circulation by LDL receptor– mediated endocytosis. HDL cholesterol can also be taken up directly by hepatocytes via the scavenger receptor class BI (SR-BI), a cell-surface receptor that mediates the selective transfer of lipids to cells. HDL particles undergo extensive remodeling within the plasma compartment as they transfer lipids and proteins to lipoproteins and cells. For example, after CETP-mediated lipid exchange, the triglyceride enriched HDL becomes a substrate for HL, which hydrolyzes the triglycerides and phospholipids to generate smaller HDL particles.

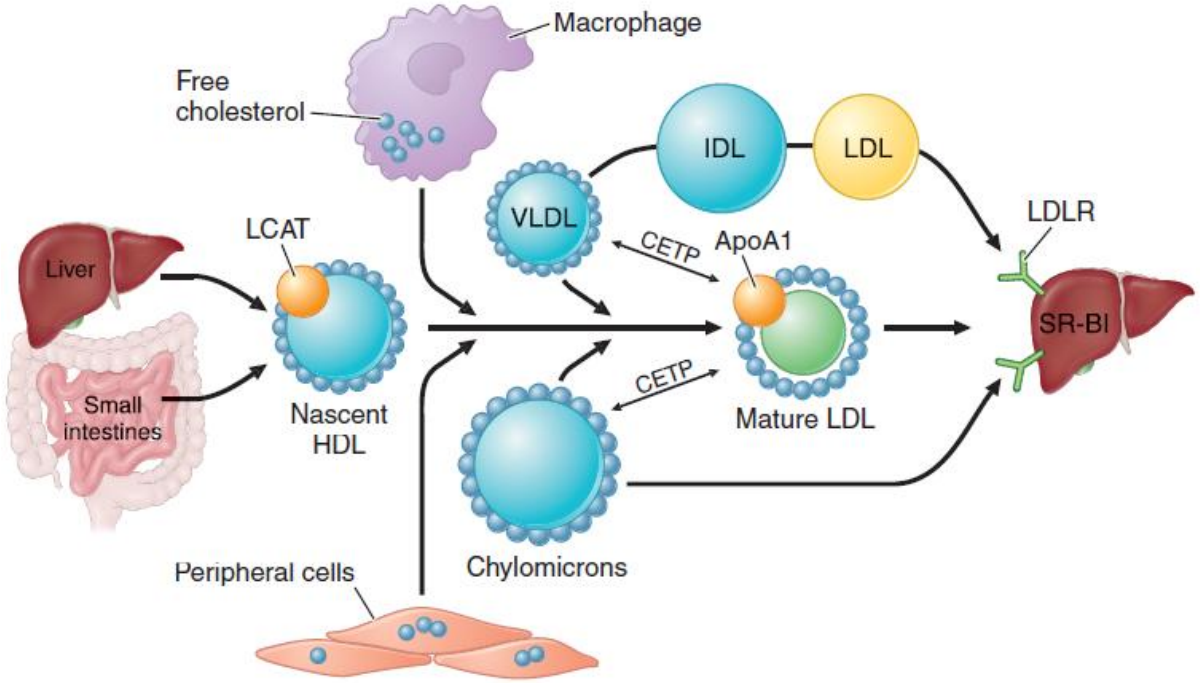


Fig 3 : HDL metabolism and reverse cholesterol transport.

LCAT, lecithin-cholesterol acyltransferase**; CETP,** cholesteryl ester transfer protein; **VLDL**, very low density lipoproteins; **IDL**, intermediate-density lipoproteins; **LDL**, low-density lipoproteins; **HDL**, high-density lipoproteins; **LDLR**, low-density lipoprotein receptor; **TG**, triglycerides; **SR-B1**, scavenger receptor class B1

HDL metabolism pathway transports excess cholesterol from the periphery back to the liver for excretion in the bile. The liver and the intestine produce nascent HDL. Free cholesterol is acquired from macrophages and other peripheral cells and esterfied by LCAT and forming mature HDL. HDL cholesterol can be selectively taken up by the liver via SR-BI. Alternatively, HDL cholesteryl ester can be transferred by CETP from HDL to VLDL and chylomicrons, which can then be taken up by the liver.

3.1.5. Classification of Hyperlipidemia

Hyperlipidemias are classified according to the **Fredrickson classification** which is based on the pattern of lipoproteins on electrophoresis or ultracentrifugation. It was later adopted by the World Health Organization (WHO). It does not directly account for HDL, and it does not distinguish among the different genes that may be partially responsible for some of these conditions. It remains a popular system of classification, but is considered out dated by many experts now.

Following five types of hyperlipidemia described by Fredrickson. (Frederickson et al , 1965).

Hyperlipoproteinemia type I :

This very rare form (also known as Buerger-Gruetz syndrome, primary hyperlipoproteinaemia, or familial hyperchylomicronemia) is due to a deficiency of lipoprotein lipase (LPL) or altered apolipoprotein C2, resulting in elevated chylomicrons, the particles that transfer fatty acids from the digestive tract to the liver. Its prevalence is 0.1% of the population.

Hyperlipoproteinemia type II :

Hyperlipoproteinemia type II, the most common form, is further classified into type IIa and type IIb, depending mainly on whether there is elevation in the triglyceride level in addition to LDL cholesterol.

Type IIa :

This may be sporadic (due to dietary factors), polygenic, or truly familial as a result of a mutation either in the LDL receptor gene on chromosome 19 (0.2% of the population) or the ApoB gene (0.2%). The familial form is characterized by tendon xanthoma, xanthelasma and premature cardiovascular disease.

Type IIb :

The high VLDL levels are due to over production of substrates, including triglycerides, acetyl CoA, and an increase in B-100 synthesis. They may also be caused by the decreased clearance of LDL. Prevalence in the population is 10%

Hyperlipoproteinemia type III :

This form is due to high chylomicrons and IDL (intermediate density lipoprotein). It is also known as broad beta disease or dysbetalipoproteinemia, the most common cause for this form is the presence of ApoE E2/E2 genotype. It is due to cholesterol-rich VLDL (β-VLDL). Its Prevalence in the population is 0.02% .

Hyperlipoproteinemia type IV :

This form is due to high triglycerides. It is also known as hypertriglyceridemia (or pure hypertriglyceridemia). According to the NCEP-ATPIII definition of high triglycerides (>200 mg/dl). Its prevalence in adult population about 16% .

Hyperlipoproteinemia type V :

This form is a rare condition characterized by increased synthesis of very low density lipoproteins (VLDL) and reduced levels of lipoprotein lipase. It is also associated with glucose intolerance and hyperuricemia.

3.1.6. Treatment of Hyperlipidemia

Table 3. National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATPIII) guidelines for desirable serum lipid levels in adults : (NCEP guidelines, 2002)

LDL CHOLESTEROL	
<100 mg/dl	Optimal
100-129 mg/dl	Near optimal /Above optimal
130-159 mg/dl	Borderline high
160-189 mg/dl	High
≥190 mg/dl	Very high
TOTAL CHOLESTEROL	
<200 mg/dl	Desirable
200-239 mg/dl	Borderline high
≥240 mg/dl	High
HDL CHOLESTEROL	
<40 mg/dl	Low
≥60 mg/dl	High
TRIGLYCERIDE LEVELS	
<150 mg/dl	Normal
150-199 mg/dl	Borderline high
200-499 mg/dl	High

Non- Pharmacological Treatment

Dietary management in hyperlipoproteinemia :

1. Restriction of fat calories to less than 20 % of total calories.
2. Saturated, Monounsaturated and polyunsaturated fats should form 1/3rd each of the total dietary fat.
3. Increased consumption of vegetables, fresh fruit, cereals, nuts especially walnuts and almonds (in moderation) and whole grain products.
4. Increased consumption of fish, where possible/permitted.
5. Drastic reduction in alcohol consumption (Satoskar et al, 2009).

Exercise :

Moderate amounts of aerobic exercise (brisk walking, jogging, swimming, cycling) on a regular basis have a desirable effect on the lipid profile of an individual. These beneficial effects have been demonstrated within 2 months in middle aged men exercising for 30 minutes, three times a week. Current advice for adults who are not routinely active is to undertake 30 minutes of moderate intensity activity on at least 5 days of the week. For active individuals, additional aerobic exercise of vigorous intensity is recommended for 20-30 minutes three times a week. Exercise per se probably has little effect on total cholesterol levels in the absence of a reduction in body weight, body fat or dietary fat. Perhaps the most important effect of regular exercise is to raise levels of HDL-C in a dose dependent manner according to energy expenditure (Durstine et al, 2002).

Stanol Esters & Plant Sterols :

The availability of margarines and other foods enriched with plant sterols or stenol esters increases the likelihood that LDL-C can be reduced by dietary change. Both Stenol esters and Plant sterols at a maximum effective dose of 2g/day inhibit cholesterol absorption from the gastrointestinal tract and reduced LDL-C by an average of 10 %. They compete with cholesterol for incorporation into mixed micelles, thereby impairing its absorption from the intestine. However, as with other dietary changes the reduction seen varies between individuals and is probably dependent on the initial cholesterol level. Single meal studies show that phyto-sterols

are bioactive at doses as low as 150 mg and the small amounts that naturally occur in foods are also probably important in the management of total cholesterol.

Anti - Oxidants :

Antioxidants occur naturally in fruits and vegetables and are important components of a healthy diet. Their consumption is thought to be beneficial in reducing the formation of atherogenic, oxidized LDL-C. Primary and secondary prevention trials with anti-oxidant vitamin supplements, however, have not been encouraging. Neither vitamin E nor β carotene supplements would appear to reduce the risk of coronary heart disease but likewise have not been shown to be harmful (Walker et al,2007).

Pharmacological Treatment:

The recent guidelines for detection and treatment of hypercholesterolemia together with specific therapeutic goals have stimulated interest by using lipid lowering agents. The last decade has seen an explosive growth in the drug discovery area which is now translating into clinical trials with many new lipid lowering agents. In addition, clinical trials assessing clinical outcomes and cost effectiveness are resulting in a changing approach to how current lipid lowering drugs are used, especially in terms of dosing and combination therapies. At present only four classes of lipid altering agents remain in wide use; bile acid binding resins, niacin, fibrates and HMG CoA reductase inhibitors.

Although only a decade has passed since the first HMG CoA reductase inhibitor, lovastatin, entered clinical trials this group of compounds are not only the most widely used lipid lowering agents, but more than six such agents have been currently developed. In addition to pharmacological therapies, there have been significant advances in the non-pharmacological treatment of hypercholesterolemia, the most important being LDL-receptor gene replacement and selective LDL-apheresis (Stein et al, 1994).

3.1.7.Current Drugs Therapy Used to Treat Hyperlipidemia

HMG-CoA Reductase Inhibitors : Statins

Statins, inhibitors of 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase, have revolutionized the treatment of hypercholesterolemia. They are the most efficient agents for reducing plasma cholesterol, being also appreciated for their good tolerance. Angiographic studies have demonstrated that these compounds reduce the progression and may induce the regression of atherosclerosis. These effects were translated in significant cardiovascular morbidity and mortality reductions in many clinical trials (Vaughan et al, 2000).

Recently completed primary and secondary intervention trials have shown that the significant reductions in low-density lipoprotein (LDL) cholesterol achieved with statins result in significant reductions in morbidity and mortality associated with coronary artery disease as well as reductions in the incidence of stroke and total mortality. Experimental studies have also shown statin-induced improvements in endothelial function, decreased platelet thrombus formation, improvements in fibrinolytic activity, and reductions in the frequency of transient myocardial ischemia **(**Farnier et al,1998).

Adverse Effects of Statin Therapy:

Statins are generally well tolerated. The most important adverse effect is liver and muscle toxicity. Myopathy can happen if inhibitors of cytochrom P450 or other inhibitors of statins metabolism are administered together with statins, determining the increase of their blood concentration (Maron et al,2000).

Fibric Acid Derivatives :

Fibrates are generally effective in lowering elevated plasma triglycerides and cholesterol. The magnitude of lipid changes depends on the patient's pre treatment lipoprotein status (Tikkanen M., 1992) as well as the relative potency of the fibrate use (Zimetbaum et al,1991). The most pronounced effects of fibrates are a decrease in plasma triglyceride-rich lipoproteins (TRLs). Levels of LDL cholesterol (LDL-C) generally decrease in individuals with elevated baseline plasma concentrations, and HDL cholesterol (HDL-C) levels are usually increased when baseline plasma concentrations are low (Tikkanen , 1992).

Tolerability and Safety :

In general, fibrates are considered to be well tolerated, with an excellent safety profile. A low incidence of fibrate-associated toxicity has been reported in almost every organ system (Sgro et al, 1991). Members of the two most popular classes of lipid-lowering drugs, HMG CoA reductase inhibitors and fibrates, cause cancer in rodents. (Newman et al, 1996). Although the mechanism may be related to peroxisome proliferation, a definite link has not yet been established. In humans, long-term administration of various fibrates does not cause peroxisome proliferation or any other morphological changes in the liver (Gariot et al,1983, Angelin et al,1984). Extrapolation of this evidence of carcinogenesis from rodents to humans is uncertain. Clinically relevant interactions of fibrates with other antihyperlipidemic drugs include rhabdomyolysis (reported in combination with HMG CoA reductase inhibitors) and decreased bioavailability when combined with some bile acid sequestrants. Finally, the potentiation of the anticoagulant effect of coumarin derivatives may cause bleeding (Blum et al, 1992).

Bile Acid Binding Resins:

The bile acid binding resins are effective for primary and secondary prevention of coronary artery disease (Insull, 2006). Clinical experience indicates that bile acid binding resins can be beneficial for various patients, including different ages and both sexes.

The bile acid binding resins form non-absorbable complexes with bile acids in the gastrointestinal tract and increase their fecal excretion, thereby preventing their reabsorption and removing them from the enterohepatic circulation. Thus, these agents have a dual effect. The bile acid binding resins diminish the ability of bile acids to solubilise dietary lipids and stimulate the liver to convert endogenous cholesterol into bile acids in an attempt to maintain bile acid pool size (Staels et al, 2007).

Safety and Tolerability

The major adverse effects associated with colestyramine and colestipol include constipation and flatulence, leading to high patient discontinuation rates, between 40% and 60% (Andrade SE, et al,1995, Avorn et al, 1998) .

Till date, there is no serious adverse effects of colestimide have been found. However, colestimide is known to cause constipation (Kajiyama et al, 1996, Suzuki, 2007).

Some patients treated with colestimide need laxatives, such as magnesium oxide, to prevent constipation. Although these may lead to better patient compliance, bile acid binding resins are contraindicated in patients with bowel obstruction. Twenty-five years of controlled clinical trials have shown that treating dyslipidemia with bile acid binding resins, alone and in combination with other lipid-lowering drugs such as statins, is safe and effective (Insull , 2006) .

Niacin : (Nicotinic acid)

Nicotinic acid is a particularly effective in lowering the blood concentrations of low and very low density lipoprotein cholesterol and in increasing the concentration of high density lipoprotein cholesterol. Intake at quantities of one gram or more not only provide pharmacological benefits but also carries significant risk of adverse effect , thus requiring medical supervision and monitoring.

Serious side effects of nicotinic acid have occasionally occurred when gram quantities were taken to lower serum lipids. More severe reactions may be jaundice, fatigue, fulminant liver failure (Rader et al ,1992) (Clementz et al,1989) .

Table 4. Medications for Hyperlipidemia

Drug Class	Agents	Effects (% change)	Side Effects
HMG CoA reductase inhibitors	Lovastatin Pravastatin Atorvastatin	↓LDL (18-55),↑ HDL (5-15) ↓ Triglycerides (7-30)	Myopathy, increased liver enzymes
Cholesterol absorption inhibitor	Ezetimibe	↓ LDL(14-18), ↑ HDL (1-3) ↓Triglyceride (2)	Headache, GI distress
Nicotinic Acid	Niacin	↓LDL (15-30), ↑ HDL (15-35) ↓ Triglyceride (20-50)	Flushing, Hyperglycemia, Hyperuricemia, GI distress, hepatotoxicity
Fibric Acids	Gemfibrozil Fenofibrate	↓LDL (5-20), ↑HDL (10-20) ↓Triglyceride (20-50)	Dyspepsia, gallstones, myopathy
Bile Acid sequestrants	Cholestyramine	↓ LDL ↑ HDL No change in triglycerides	GI distress, constipation, decreased absorption of other drugs

Combination of Drugs Treatment in Hyperlipidemia

The combination of two lipid lowering drugs is very useful in the treatment of severe hyperlipoproteinemia, particularly with heterozygous familial hypercholesterolemia (type II a) in whom single drug therapy often fails to achieve satisfactory plasma levels of LDL-C.

The commonly used drug combinations are as follows :

1.Bile Acid Binding Resins plus Fibrates :

Recommended for familial combined hyperlipidemia.(type II b).

2.Bile Acid Binding Resins plus Niacin :

Effective in familial hypercholesterolemia (type II a) and also in familial combined Hyperlipidemia (type II b).

3.Bile Acid Binding Resins plus Statins :

Highly effective in reducing LDL-C in patients of familial hypercholesterolemia (type II a)

4.Bile Acid Binding Resins plus Niacin plus Statins :

Specially useful in patients with severe disorders due to elevated LDL (type II a & II b)

5.Niacin plus Statin :

An efficacious combination for familial combined hyperlipidemia.(type II b) and familial hypercholesterolemia (type II a).

6.Statins plus Ezetimibe :

Synergistic combination for treating primary hypercholesterolemia and can also be used for the treatment of individuals with homozygous familial hypercholesterolemia (type II a) (Sharma et al, 2007).

3.2 Literature Review of the Plant

The bark of *Ailanthus excelsa* was selected for its phytopharmacological evaluation with special respect to its anti-hyperlipidemic activity. The plant was short-listed based on literature review as follows.

3.2.1 Introduction to Genus

The genus *Ailanthus* consists of approximately ten species, which have a wide distribution ranging from Asia to north Oceania. Five species and two varieties have been found in South western, south eastern, central, and northern China.

3.2.2. Introduction to Family

Simaroubaceae, the quassia family of flowering plants, in the order Sapindales, comprising 25 genera of pantropical trees, including *Ailanthus*, or the tree of heaven . Members of the family have leaves that alternate along the stem and are composed of a number of leaflets arranged along an axis. Most species have small flowers, bitter bark, and fleshy fruits that are sometimes winged. The tree of heaven is often planted as an ornamental along city streets because it is smoke- and insect-resistant. Female plants are preferred because the male flowers release a disagreeable odour. Several varieties have colourful, twisted fruits and coloured leafstalks. Bark of species of the genera Quassia and Picrasma yields quassia, a bitter substance used in medicines.

3.2.3. Taxonomical Profile

Kingdom : Plantae (Plants)

Subkingdom : Tracheobionta (Vascular Plant)

Superdivision : Spermatophyta (Seed Plant)

Division : Magnoliophyta (Flowering Plants)

Class : Magnoliopsida (Dicotylendons)

Subclass : Rosidae

Order : Sapindales

Family : Simaroubaceae (Quassia family)

Genus : Ailanthus

Species : Ailanthus excelsa . Roxb (tree of heaven)

Other species with their distribution

- *Ailanthus altissima* (Tree of Heaven) – northern and central mainland China, Taiwan, arguably the best known species.
- *Ailanthus vilmoriniana*, which according to an unpublished checklist is a synonym of *Ailanthus altissima*
- *Ailanthus excelsa* – India and Sri Lanka
- *Ailanthus integrifolia* – New Guinea and Queensland, Australia
- *Ailanthus triphysa* (White Siris) – Northern and Eastern Australia. Ailanthus malabarica is a synonym of Ailanthus triphysa

Table 5. Vernacular names of *Ailanthus excelsa*

Region/Language/ System of medicine	**Name(s)**
Sanskrit	Madala, Katvariga, Dirghavrnta
Assam	Aralu
Hindi	Maharukha, Limbado, Maharuk, Mahanimb,Ghodakaranj
Bengali	Mahanim
Gujarati	Moto ardusa, Adusa, Arduri, Arlabo, Moto adusa
Kannada	Doddamaru, Hemaraheera mara, Dodumani
Malayalam	Perumaram, Mattipongilyam
Marathi	Mahanimb, Maharukh
Punjabi	Arua
Tamil	Peruppi, Perumaruttu, Peru, Pee
Telugu	Pedu, Pey, Pedda, Peddamamanu, Putta
Oriya	Mahanim, Mahala, Gorni-Kawat, Palamow, Ghokaram
Kashmiri	Merumaram, Mattipongilyam
Rajasthan	Arua

3.2.4 .Therapeutic uses

The bark is bitter, astringent, anthelmentic, febrifuge, appetizer, bitter tonic, taste bud stimulant. It is useful in diarrhoea, amoebic dysentery, chronic giardiasis, dyspepsia, abdominal spasm anorectal disease, haemorrhoids, fistula, fissures, ulcerative colitis and worm infection. It is also used as blood purifier in skin diseases, typhoid fevers, blood coagulation disorders, gouty arthritis, boils, carbuncle, scabies and allied skin disease, chronic bronchitis, bronchial asthma, pulmonary kochs, bronchiectasis, polyurea, **diabetes mellitus, obesity,** uterine disorders like dysmenorrhoea and leucorrhoea. The bark and leaves have great repute as postnatal tonic. Leaf juice is administered along with milk for post labour pains **(**The Ayurvedic Pharmacopoeia of India, 2001; Database, 2002).

Ayurvedic properties

Rasa -Tikta, kashaya

Guna - Ruksha

Veerya -Sheeta

Veepaka -Katu

Doshaghnata -Kaphapittashamaka

Ragaghnata - Kaphapittajaroga,Charmaroga, Dushtavrana, Apasmara, Manasavikara, Balagraha, Pravahika, Atisara, Grahani, Arsha, Bhagandara, Krimi, Raktavikara, Raktavata, Jeernakasa, Urahkshata, Kshaya, Pradara, Yonivyapada, Jeernajwara, Vishamajwara, Medoroga, Madhumeha.

Karma -Twagdoshahara, Vranshodhana, Deepana, Pachana, Grahi, krimighna, Raktashodhaka, Raktambhana, Kasahara, Sandhaneeya, yonidoshahara, Jwaranashaka, Lekhana, Madhuryanashaka, Vishaghna.

Doses

Leaf juice -10-20 ml; Bark powder-1-3 gm; Extract- (Ghanasatva)-1 gm.

3.2.5. Formulation and preparations

Ailanthus is an important ingredient in most of the formulations and preparations. Ailanthus is an important ingredient in most of theayurvedic preparations like, Pusyanuga churna, a herbo-mineral Ayurvedic preparation of which *Ailanthus excelsa*

is one of the constituent, was found effective in vataja and kaphaja pradara. Brahat Gangadhara churna and Aralu putpaka, used in the management of atisara, krimi, arsa, sannipatajwara, brama, tvakroga, chardi, kustha, pravahika, grahani, prameha, gulma, swasa, musaka and visaja roga. Dashmularista, a highly prized ayurvedic formulation for fatigue, is actually a mixture of ten different herbs out of which one is Shyonak/Sonapatha. It aids in cellular regeneration to hasten removal of dead or weak cells and replace them with fresh, vital ones.

In Ayurvedic literature there happens to be a controversy between the common name used for both *Ailanthus excelsa* and *Oroxylum indicum* mentioned as Shyonak. In the Bhavprakashnighantu also *Ailanthus excelsa*, is described under the name of Araluand Sonapatha/Shyonak is mentioned as its synonym. In Amarkosh aralu, shyonak and tintuk are the names given to the same plant. In the title some controversial drugs in Indian medicine the Nighantu writers have confounded it with *Oroxylum indicum* Bignoniaceae). As per the Adarsha Rajniguntakar Nighantu, the description under Shyonakyugal mentioned that in case of two Shyonakas, one should be aralu and other is tintuk, whereas European practitioners consider both *Ailanthus excelsa* and *Oroxylum indicum* as totally different plants; however *Ailanthus excelsa* is said to be a substitute for Shyonak. Pilex, the most popularly used ointments for piles contains bark of *Ailanthus excelsa* and is indicated in hemorrhoids, anal fissures, fistulae, proctitis, venous stasis, varicose veins, thrombophlebitis, varicocele and varicosity. Lukol tablets used in leucorrhoea contains Loh Bhasma, along with extracts of *Withania somnifera*, *Saraca indica*, *Woodfordia floribundi*, *Symplocos racemosa*, *Ailanthus excelsa*, *Leptadenia reticulata* and *Asparagus racemosus* which acts synergistically as uterine tonics, nervine sedatives and have a stimulating action on the endometrium and ovarian tissues. Sports massage oil prepared from the bark of *A. excelsa* is used to keep muscles relaxed. Rain tree's Simarouba extract the preparation of *Simarouba amara*, contain quassinoids like ailanthinone and glaucarubinone as the main active constituents, which are also present in *Ailanthus excelsa* and are considered to be the main therapeutic constituents for dysentery (amoebic and bacterial) and diarrhoea; intestinal worms and internal parasites; malaria; as an astringent to stop internal bleeding (stomach ulcers, hemorrhages) and externally for wounds and in viral infections. Katabheyadi taila, asthadashanga kashaya, Hreeberadi kavatha. *Ailanthus excelsa* bark infusion (1 in 20), dose: 1 to 2 ounces. Ailantic acid,

dose: 1 to 3 grains; in large doses it causes nausea, vomiting and purging (Anonymous, 1985; Database, 2000; Lavhale and Mishra, 2007).

3.2.6. Botanical discription

Ailanthus excelsa is a large deciduous tree, 18-25 m tall; trunk straight, 60-80 cm in diameter; bark light grey and smooth, becoming grey-brown and rough on large trees, aromatic, slightly bitter.

Leaves are alternate, pinnately compound, large, 30-60 cm or more in length; leaflets 8-14 or more pairs, long stalked, ovate or broadly lance shaped from very unequal base, 6-10 cm long, 3-5 cm wide, often curved, long pointed, hairy gland; edges coarsely toothed and often lobed.

Flower clusters droop at leaf bases, shorter than leaves, much branched; flowers many, mostly male and female on different trees, short stalked, greenish-yellow; calyx 5 lobed; 5 narrow petals spreading 6 mm across; stamens 10; on other flowers, 2-5 separate pistils, each with elliptical ovary, 1 ovule, and slender style.

Fruit a 1-seeded samara, lance shaped, flat, pointed at ends, 5 cm long, 1 cm wide, copper red, strongly veined, twisted at the base The generic name ‘Ailanthus’ comes from ‘ailanthos’ (tree of heaven), the Indonesian name for *Ailanthus moluccana*.

Fig 4. Tree and Bark of *Ailanthus excelsa*

3.2.7. Ecology and distribution

History of cultivation:

The tree is native to Central, Western and Southern India, but is now being spread to other semi-arid and subtropical areas. It is fairly common in Central Sudan and is found planted on riverine and sandy soils.

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Natural habitat:

Ailanthus excelsa grows well in semi-arid and semi-moist regions and has been found suitable for planting in dry areas with annual rainfall of about 400 mm. It is commonly found in mixed deciduous forests and some sal forests, but is rare in moist areas with high salt-tolerant species.

Geographic distribution

This plant is indigenous to Central and South India, Rajasthan, Bihar, Bengal, Orissa, Bundelkhand, throughout Madhya Pradesh, Bharuch and Panchmahal district of Gujarat and dry deciduous forests of Maharashtra, and Karnataka and forests of Tamilnadu.

Biophysical limits:

Altitude: 0-900 m, mean annual temperature: 0 - 45^0C, mean annual rainfall: 500-2500 mm. Soil type: Grows in a wide variety of soils, but thrives best in porous sandy loams. It avoids clayey soils with poor drainage and waterlogged areas. Its growth is poor on shallow dry soils.

Reproductive biology:

The flowers appear in large open clusters among the leaves towards the end of the cold season. Male, female and bisexual flowers are intermingled on the same tree. The fruits ripen just before the onset of the monsoon. The seeds are very light and are dispersed far and wide by the wind propagation and management (Vogt, 1995).

3.2.7. Phytochemistry

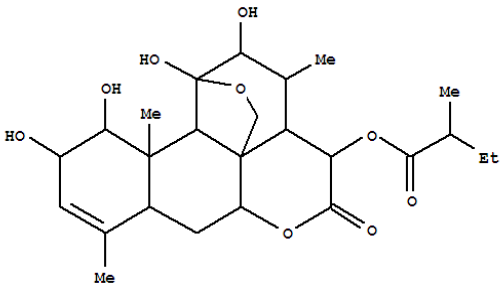
Quassinoids

The plants of the Simaroubaceae family contain the bitter principles known as quassinoids which are degraded triterpenes and are highly oxygenated. Some of these plants are used in folk medicine for anthelmintic and anti amoebic properties (Polonsky, 1973). In recent years attention has been focused on quassinoids as several of them have shown promising antitumor, antiviral, anti malarial, anti leukemic and anti feedant properties (Polonsky, 1985). Chemical examination of *Ailanthus excelsa*, a Simaroubaceous plant, has been carried out by several workers resulting in the

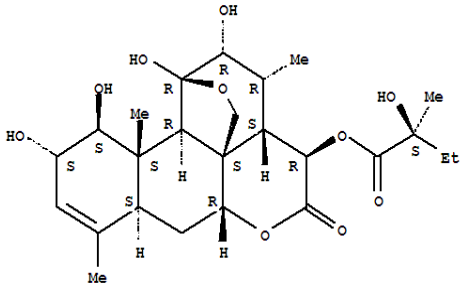
isolation of quassinoids (Ogura *et al*., 1977; Khan and Shamsuddin, 1978, 1980; Khan and Zuberi, 1980; Sahai and Bhatia1985). Three quassinoids, 1, 2 and3, 4-dihydro excelsin were isolated from the stem bark of *Ailanthus excelsa*, along with five known quassinoids excelsin, glaucarubine, ailanthinone, glaucarubinone and glaucarubolone. The glaucarubolone has been isolated for the first time from this plant in 2003. The structural elucidation is based on the analysis of spectroscopic data. Five known compounds were identified as excelsin 5(Khan and Shamsuddin, 1980); glaucarubine (Ogura *et al*., 1977) and glaucarubolone (Gaudemer and Polonsky, 1965) by comparing their physical and spectral data with those reported in the literature. Glaucarubolone has been isolated for the first time from this plant.

Stem bark of *A. excelsa* contains quassinoids like excelsin, 1,4-dihydroexcelsin, 2,4-dihydroexcelsin, 3,4-dihydroexcelsin, 13,18 dehydroexcelsin, glaucarubin, glaucarubol, ailanthinone, 1,12-deoxy-13-formyl ailanthiol, ailanex A, ailanex B, polyandrol and glaucarubolone while the root bark is reported to contain ailanthinone, glaucarubinone and mixture of glaucarubin -15- isovalerate, 13, 18-dehydroglaucarubol15-isovalerate .

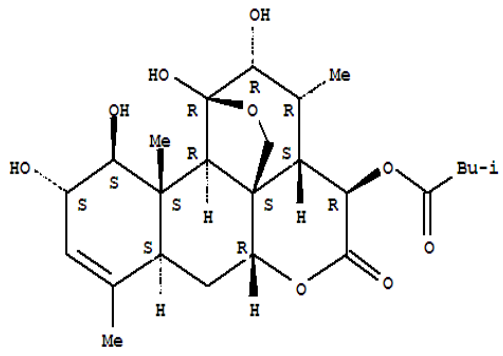
2, 6-dimethoxy benzoquinone and malanthin: yellowishgreen viscous oil was obtained by percolation of air dried powder of trunk bark from an old tree of *A. excelsa*. This oil after refrigeration in minimum amount of benzene and light petroleum gives colorless crystalline malanthin. Saponification of the mother liquor left after malanthin crystallization gives 10% saponifiable matter and 90%unsaponifiable material. The unsaponifiable material upon column chromatography on alumina gives 2, 6 dimethoxybenzoquinone and β-sitosterol (Lavhale and Mishra 2007).



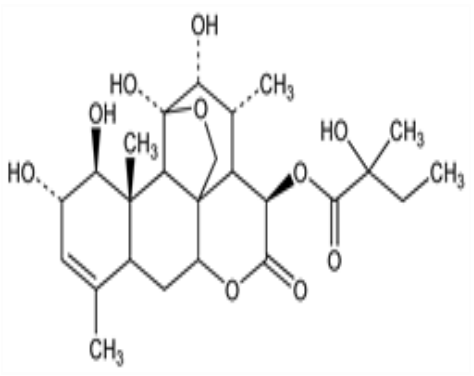
Excelsin



Glaucarubine



Ailanthinone



Glaucarubinone

Steroidal compounds

The petroleum ether extract of stem bark on column chromatography over silica gel gives β sitosterol and Stigmasta-4, 22-diene-3-one with hexane-ethyl acetate (9:1) (Lavhale and Mishra, 2007).

Triterpine

Root bark showed the presence of a new triterpene alcohol, 3S, 24S and 25-trihydroxytirucall-7-ene. Triacontane and Hexatriacontane: Stem Bark showed the presence of triacontane and hexatriacontane (Lavhale and Mishra, 2007).

Alkaloids

Methanol extract from root bark after solvent extraction with chloroform gave four alkaloids viz., canthin- 6-one, 1-methoxy canthin -6-one, 5-methoxy canthin -6-one and 8-hydroxy canthin-6-one. these alkaloids have shown significant cytotoxicity against 12-O-tetradecanoylphorbol-13- acetate induced Epstein-Barr virus early antigen (EBV-EA). Canthin-6-one and 4-methoxy canthin-6-one showed potent antiulcerogenic activity in gastric lesions induced animals, as well as significant anti nociceptive activity in mice.

Flavonoids

The leaves were reported to contain different flavonoids like kaempferol (5, 4', 5, 7-Tetrahydroxy flavone), luteolin (3',4',5,7-tetrahydroxy flavone), apigenin (4'.5, 7-trihydroxy flavone) while fruits contains quercetin. These flavonoids were reported to possess many biological activities such as antibacterial, antiinflammatory,

antiallergic, antimutagenic, antiviral, antineoplastic, anti-thrombotic and vasodilatory properties. The flavon-C glycosides like vitexin show antioxidant, analgesic and antithyroid activities, where as quercetin inhibits the growth of leukemic cells, ehrlich ascites tumor cells and other ascites tumor cells. Bark contains wax like, reddish brown, water soluble bitter principle, known as ailantic acid. It is given as a tonic and protein synthesis in mammalian cells as well as in malaria alterative in dyspepsia and constipation.

Volatile constituents

The volatile constituents from fresh aerial parts of *Ailanthus excelsa* were analysed by GC and GC-MS. Fatty acids and their esters represented the main fraction (40.9%) of the obtained oil, with 9, 12, 15-octadecatrienoic acid methyl ester (linolenic acid) being the main component (13.7%). The dominant component of the oil was phytol (26.7%) A novel triterpenoid isolated from the root bark of *Ailanthus excelsa* Roxb AECHL-1 as a potential Anti-Cancer agent (Lavhale *et al*., 2009).

Proteins

Leaves contain considerable amount of proteins where, cytoplasmic protein fraction can be used for human consumption; while the unfractionated and chloroplastic fractions could be utilized as a nutritious feed for ruminants and nonruminants. Proximate analysis of various fractions of fresh leaves showed 62.71% crude protein in cytoplasmic protein fraction, while whole leaf showed 20.86% protein. The unfractionated and fractions from chloroplastic protein contained more crude fat than the whole leaf and pressed cake. Compared to whole leaf and pressed cake, protein fractions were low in crude fiber content. The amino acid compositions of protein sample, showed an excellent balance of essential amino acids. The leaf protein fractions were nutritionally superior to the whole leaf, pressed cake as well as soyabean protein (Lavhale and Mishra, 2007). Fractionation and some chemical studies on *Ailanthus excelsa* seed protein has been carried out (Kundu and Laskar, 2008).

3.2.8. Pharmacological Review

Antifungal activity

Chloroform fraction of the methanol extract of stem bark of *A.excelsa* showed significant fungistatic and fungicidal activity against *Aspergillus fumigatus*, *Penicillium requentence*, *Aspergillus niger*, *Penicillium notatum* and *Botrytis cinerea* (Joshi et al., 2003).

Antibacterial activity

Ethyl acetate fraction of dried stem bark of *A.excelsa* inhibited the growth of *Staphylococcus aureus, Escherichia coli* and *Bacillus subtilis*. Three active principles, excelsin, 13, 18 dihydroexcelsin and 1, 12- deoxy-13-formylailanthinol, isolated from bark are responsible for this activity. The antibacterial activity of all three compounds was more pronounced than the antifungal activity (Lavhale and Mishra, 2007).

Anti-amoebic activity

The aqueous, petroleum ether and defatted ethanolic extracts (Quassinoid fraction) of stem bark of *Ailanthus excelsa* were tested against the laboratory cultured *Entamoeba histolytica* for its anti-amoebic action using metronidazole as standard drug. The EC value for aqueous, petroleum ether and defatted 50 ethanolic extracts (Quassinoid fraction) were 195, 185 and 150 µg/ mL against *E. histolytica* respectively (Yoganandam *et al*., 2009b).

Antiasthmatic activity

Methanolic extract of stem bark of *A.excelsa* at a dose of 100mg/kg body wt. showed antiasthmatic activity in isolated guinea pig ileum models. Methanolic extracts also significantly inhibited clonidine induced catalepsy in pre treated animals and the duration of catalepsy was found to be 48.6±4.74 at 150 min and 35.5±4.59 at 120 min after the administration clonidine (Kumar *et al*., 2010).

Anti allergic activity

Methanolic extract of leaves of *A. excelsa* at dose of (100, 200 and 400 mg/kg) showed significant inhibition of milk-induced Leucocytosis and eosinophilia in pre-treated animals (Kumar et al., 2011).

Anti inflammatory activity

Methanolic extracts of *A. excelsa* leaves at a dose of 250 mg /kg & 500 mg/kg showed remarkable anti inflammatory activity in carrageenan induced rat paw edema model (Said et., al. 2010).

Antifertility activity

The alcoholic extract of the leaf and stem bark at a dose of 250 mg/kg body wt. exhibited a remarkable anti implantation and early abortificient activity in female albino rats (Lavhale and Mishra, 2007).

Hydro-alcoholic extracts of *A.excelsa* produced dose dependent anti implantation response in rats. With an increase in the dose of the hydro-alcoholic extracts, the percentage of implantation failure increased and was significant at doses of 200 and 400 mg/kg (Ravichandran et al., 2007).

Antipyretic activity

Ethanolic extract of *A. excelsa*, showed moderate to significant degree of antipyretic activity against yeast suspension induced hyperthermia in an experimental rat model (Lavhale and Mishra, 2007).

Analgesic activity

Methanolic extracts of *A. excelsa* bark at a dose of 100 mg / kg showed significant increase in mean basal reaction time in Hot plate method & increase in latency to flick tail compared with control group of animals (Chauhan et al., 2011).

Hepatoprotective activity

Ethanolic extract of leaves showed protective effects against CCL_4 induced liver injury as evidenced by a significant reduction in the CCL_4 induced elevated enzyme levels of serum glutamate oxaloacetate transaminase, serum glutamate pyruvate transaminase and serum alkaline phosphatase. The presence of phenolics might be the

responsible factor for the above activity (Lavhale and Mishra, 2007). Ethanol extract of stem bark of *Ailanthus excelsa* also showed protective effects against CCL_4 induced hepatotoxicity in rats (Yoganandam *et al*., 2009a).

Anti Plasmodial activity

The methanolic extract of *A.excelsa* inhibited *in vitro* growth of chloroquine-sensitive (D10) and resistant strains (W2) of *Plasmodium falciparum* (IC50 4.6 and 2.8 μg/ml, respectively). Chloroform fraction inhibited recombinant PLM II, PLM IV, and the formation of β-haematin. Chloroform fraction reduced PLM II activity in a concentration dependent manner (IC50 of 13.4±1.7 μg/ml, mean±SD), and it was inactive against PLM IV. The Choroform fraction inhibited β-hematin formation dose dependently. At the highest dose (5 mg/ml) the inhibition was 37.7% (Mario et al., 2007).

Hypoglycemic activity

A single administration of leaves or stem bark extracts of *A.excelsa* lowered the blood glucose of normal rats in a glucose tolerance test. Administration of each extract for 60 days produced a significant hypoglycemic effect on STZ-induced diabetic rats with improved renal parameters which suggest its potential use in the treatment of diabetes (Lavhale and Mishra, 2007).

Insect feedent-deterrent

Bioassay directed fraction of the methanol extract of the stem bark led to the isolation and identification of antifeedent constituent excelsin. A leaf disc method of bioassay showed the potency of excelsin to prevent feeding was 75.94% at a concentration of 1000 ppm against *Spilosoma oblique*. This insect is a destructive lepidopterous pest in the Northern parts of India, attacking a wide range of crops. The ED of excelsin was found to be 0.563% (Tripathi and Jain, 1993). Structure activity correlation indicates that cytotoxicity might be involved in the mode of action of these compounds. Ailanthone acts as a feeding deterrent to herbivores because of its extremely bitter taste (Lavhale and Mishra, 2007).

Anti hypertensive activity

Methanolic extract of *A. excelsa* leaves were reported to have inhibitory effect on ACE with 53.78% inhibition at the screened concentration of 330 g/mL. The Flavanoids were isolated from ethyl acetate fraction and all showed inhibition of ACE activity by 60% – 90% at a concentration of 330 µg/mL (Lacaille-Dubois *et al*., 2001; Kameda *et al.*, 1987).

Antitumor and cytotoxicity

Aqueous extracts of roots of *A.excelsa* when screened by the brine shrimp lethality assay, it showed significant toxicity to the brine shrimp (<60 Rg mLG). The quassinoids like Ailanthione glaucarubinone and a mixture of glaucarubol 15 - isovalerate have shown substantial antitumor and cytotoxic activities against the P 388 lymphocytic leukemia and KB test system respectively. The observed antitumor activity is by inhibiting the protein synthesis of ribosomal peptidyl transferase leading to termination of chain elongation (Lavhale and Mishra,2007).

Gastroprotective and anti secretory effects

The pretreatment of animals with methanolic, petroleum ether, and chloroform extracts (100 mg/kg,(p.o.) from *A. excelsa* significantly reduced gastric lesion induced by ulcerogenic agent (56, 47, and 70%, respectively) when compared with animals pretreated with vehicle. The diethyl ether pretreatment led to the least gastric lesion damage (83%), similar to the standard antiulcer drug, cimetidine, at the same dose (100 mg/kg, p.o.). The lower effective dose of diethyl ether extract, as well as cimetidine, given by intraduodenal route significantly increased the pH values and reduced the acid output of gastric juice (Melanchauksi et al.,2010).

Toxicity studies

Higher dosage of drug are said to lead queasiness, dizziness, headache, tingling in limbs and diarrhea, myocarditis associated with fever, chills, epigastric pain, substernal chest pressure and shortness of breath which may likely due to exposure to quassinoids present in tree sap (Bisognano *et al*., 2005).

3.3. Medicinal Plants in the Treatment of Hyperlipidemia

Natural remedies have been used for centuries for a wide variety of ailments by various cultures. Herbal medicines are still used by about 75 – 80 % of world population, mainly in the developing countries, for primary health care because of better cultural acceptabilities, better compatibility with the human body and lesser side effects (Khamboj et al.,2000). However, the last few years have seen a major increase in their use in the developed world. The World Health Organization (WHO) has recently defined traditional medicine (including herbal drugs) as comprising therapeutic practices that have been in existence from hundreds of years , before the development and spread of modern medicine and are still in use today (WHO 1991).

The chemical constituents present in the herbal drugs are a part of the physiological functions of living flora and hence they are believed to have better compatibility with human body. These drugs are made from renewable resources of raw materials by eco – friendly processes and will bring economic prosperity to the masses growing these raw materials.

Table 6. Medicinal plants experimentally investigated for Anti Hyperlipidemic activity

Biological Name	**Plant Part used**	**Extract/ Active Constituent**	**Animal Model**	**Reference**
Achyranthus aspera	Leaf	Alcoholic extracts	Triton model	Khanna et al.,1992
Acorus calamus	Rhizome	Ethanolic, Aqueous, Saponins	HFD model	Parab et al., 2002
Adenocalymma alliaceum	Flower	Raw dried flowers	HFD model	Srinivasan et al.,1995
Aleurites moluccama	Leaf	Methanolic extracts	Triton model	Pedrosa et al., 2002
Allium cepa	Root	Pet. Ether extracts	HFD model	Lata et al., 1991

Allium sativum	Rhizome	Pet. Ether extracts	HFD model	Lata et al.,1991
Alpinia officinarum	Rhizome	5- hydroxyl-7-(4'hydroxy- 3' methoxypheny l)-1- phenyl- 3-heptanone	Triton model	Shine et al., 2003
	Rhizome	3- methyl-ether galangin		
Amphypterygim adstingens	Stem bark	Hexan extract	HFD model	Mata et al., 1991
Ananas comosus	Leaf	Ethanolic extract	Alloxan induced dyslipidemic rats	Xie et al., 2005
Apium graveolens	Celery	Aqueous extract	HDF model	Tsi et al., 1995
Asparagus racemosus	Root	Raw dried root powder	STZ induced diabetic rats	Visavadiya et al., 2005
Boerhavia diffusa	Leaf	Aqueous extracts	Alloxan induced diabetic rats	Pari et al., 2004
Caesalpinia bonducella	Seed	Ethanolic extracts	STZ induced diabetic rats	Sharma et al.,1997
Capparis deciduas	Fruit	Ethanolic extract	STZ induced diabetic rats	Purohit et al.,2005
Capparis spinosa	Fruit	Aqueous extracts	STZ induced diabetic rats	Eddouks et al.,2005
Carlina thistle	Root	Aqueous extracts	HFD model	Ludina et al.,2005
Casearia esculenta	Root	Aqueous extracts	STZ induced diabetic rats	Prakasam et al.,2003
Cassia tora	Seed	Ethanol extracts	HFD model	Patil et al.,2004

Clerodendron colebrookianum	Leaf	Methanolic extracts	HFD model	Devi et al., 2004
Coccinia indica	Leaf	Ethanolic extracts	STZ induced diabetic rats	Pari et al.,2003
Commiphora mukul	Resin	Ethanolic extracts	HFD rats	Dixit et al., 1980
Convolvulus microphyllus	Whole plant	Ethanolic extracts	Cholesterol fed rabbits	Guassin et al.,1995
Coriander stivum	Seed	Raw seeds	HFD rats	Lal et al., 2004
Crataegus oxyacantha	Berry	Alcohol extracts	HFD rats	Shanti et al., 1994
Cuminum cyminum	Seed	Raw	Alloxan induced diabetic rats	Dhandapani et al., 2002
Cupressus sempervirens	Cone	Hydro alcohol extracts	HFD rats	Karkabounas et al ., 1999
Curcuma comosa	Rhizome	Ethyle acetate extracts	HFD rats	Piyachaturavat et al ., 1999
Curcuma longa	Rhizome	Ethanol aqueous extract	HFD rats	Ramirez et al ., 1999
Desmodium gangeticum	Root	Aqueous extract	Dexamethasone induced hyperlipidemia	Kurian et al., 2005
Dolichos biflorus		Methanol extract	HFD model	Muthu et al., 2005
Emblica officinalis	Fruit	Fruit juice	HFD model	Mathur et al., 1996
Eugenia jabolana	Seed Seed Kernel	Ethanol extract Ethanol extract	Alloxan induced diabetic rabbits STZ induced diabetic rats	Sharma et al., 2003 Ravi et al., 2005

Ficus bengalensis	Bark	Flavonoids: leucopelargonin derivatives, quercetin	HFD model	Daniel et al., 2003
Ficus carica	Leaf	Leaf decoction	Triton induced hyperlipidemia	Perez et al., 1998
Garcinia cambogia	Fruit	Methanol extract	Dexamethasone administered rats	Mahendran et al., 2001
Garcinia kola	Seed	Kolaviron	HFD model	Adaramoye et al., 2005
Gardenia jasminoides	Fruits	Water extract, crocetin, crocin	HFD model	Lee et al., 2005
Glycine tomoentella	Root	Aqueous extract	HFD model in hamsters	Ko et al., 2004
Helicteres isora	Root	Ethanol extract	STZ induced diabetic rats	Chakrabati et al., 2002
Hibiscus rosa	Flower	Ethanol extract	STZ induced diabetic rats	Sachdeva et al., 2003
Hypericum perforatum	Whole plant	Flavonoid	HFD model	Zou et al., 2005
Iris germanica	Rhizome	Ethanol extract	HFD model	Chaudhary et al., 2005
Kalopanax pictus	Stem Bark	Kalopanax saponin A hedearagenin	STZ induced diabetic rats	Park et al., 1998
Lupinus albus	Seed	Isoflavones	STZ induced diabetic rats	Sirtori et al., 2004
Lupinus angustifolius	Seed	Raw in powder form	STZ induced diabetic rats	Martin et al., 2005

Lyengaria stllata	Leaves	Ethanol extract	Triton induced and diet induced hyperlipidemic rats	Ara et al., 2002
Mangifera indica	Leaf	Mangiferin	STZ induced diabetic rats	Muruganandan et al., 2005
Momordica charantia	Fruit	Aqueous extracts	STZ induced diabetes rats	Ahmed et al., 2001
Moringa oleifera	Leaf	Crude extract	HFD rats	Ghasi et al., 2000
Myristica fragrans	Fruit Seed	Ethanol extract Methanol extract	HFD rabbits	Ram et al., 1996 Sharma et al., 1995
Nardostachys jatamansi	Whole plant	50% ethanol extract	Triton induced hyperlipidemia	Dixit et al., 1998
Nigella sativa	Seed	Petroleum ether extract	STZ induced diabetic rats	Le et al., 2004
Nostoc commune	Whole	Raw	HFD model	Hori et al., 1994
Olea europea	Leaves	Triterpenoids	HFD model	Somova et al., 2003
Opuntia ficus	Lyophilized	Water decoction	Genetic diabetic model	Galati et al., 2003
Paeonia lactiflora	Leaves	Methanol	HFD model	Yang et al., 2004
Plumbago zeylanica	Root	Plumbagin 2-methyl-5-1:4 hydroxy naphthoquinone	Hypolipidemic and antiathero -sclerotic rabbits	Sharma et al., 1991
Prunus davidiana	Stem	Methanol extract	HFD model	Choi et al., 1991

Pterocarpus marsupium	Stem	EtOAc extract, flavonoids: marsupsin, pterosupin, liquiritigenin	HFD rats	Jahromi et al., 1993
Retama raetam	Fruit	Aqueous extract	HFD rats	Maghrani et al., 2004
Salvadora persica	Stem	Lyophilized decoction	Triton induced hypercholesterolemia rats	Galati et al., 1999
Semecarpus anacardium	Nut shell	Methanol extract	Hypocholesterolemic rabbit	Sharma et al., 1995
Solanum melongena	Fruit	Flavonoids	HFD rats	Sudheesh et al., 1997
Solieria robusta	Leaves	Ethanol extract	Triton induced hypercholesterolemia rats	Ara et al., 2002
Spergularia purpurea	Whole plant	Aqueous plant	STZ induced diabetic rats	Jouad et al., 2003
Suaeda fructicosa	Whole plant	Aqueous extract	STZ induced diabetic rats	Bennani et al., 1999
Terminalia arjuna	Bark	Ethanol , ethyl acetate fractions	Poloxamer 407 induced hyperlipidemia	Subramanium et al.,2011
Teucrium polium	Aerial parts	Aqueous extract	HFD model	Rasekh et al., 2001
Tinospora cordifolia	Root	Aqueous extract	Alloxan induced diabetic rats	Prince et al., 1998
Tribulus terrestris	Fruits	Saponins	HFD model	Chu et al., 2003

Trigonella foenum-graecum	Seed	Saponins, alkaloids-trigonelline	Triton induced and HFD model	Habori et al., 1998
Vaccinium myrtillus	Leaf	Alcoholic extract	STZ induced diabetic rats	Cignarella et al., 1996
Zingiber officinale	Rhizome	Ethanol extract	HFD rabbit	Bhandari et al., 1998

HFD – High Fat Diet , **STZ -** Streptozotocin

3.4. Polyherbal Formulations for Treatment of Hyperlipidemia

Major formulations used in Ayurveda are based on herbs and used as decoctions, infusion, tinctures and powders. Drug formulation in Ayurveda (As mention in Ayurvedic treatise like Charaka Samhita, Sushruta Samhita) is based on two principles: (a) Use as single drug, and (b) Use of more than two drugs. When two or more herbs are used in formulation they are known as polyherbal formulation. Sometimes herbs are combined with mineral preparation. The concept of polyherbalism is peculiar to Ayurveda although it is difficult to explain in term of modern parameter.

The herbs are selected according to disease other herbs are used to prevent side effect arising from chief herb. It is evident that there are many herbal formulations of varying potency since these preparation act by different mechanism, (Shrivastva et al., 2012) it is theoretically possible that different combination of these extract will do better job in producing the effect. In the traditional system of plant medicine it is usual to use plant formulation and combined extract of plants are used as a drug of choice rather than individual ones (Kumar, 2010), to get the benefit of synergism and to find suitable anti hyperlipidemic and antioxidant combination therapy. Different polyherbal formulations those are scientifically investigated for their potential are listed below. All are appeared to be most effective, relatively nontoxic and have substantial documentation of efficacy.

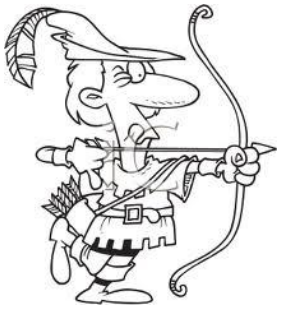
Table 7. Polyherbal Formulations Investigated for their Anti Hyperlipidemic Activity

Name of Preparation	Herbal Components	Model studies/ Mechanism of Action	Reference
Diarun Plus	*Nelumbo nucifera* *Terminalia chebula* *Zingiber officinale* *Glycyrrhiza glabra* *Hibiscus rosasinensis* *Eclipta Alba* *Rosa damascene* *Quercus infectoria* *Hemidesmus indicus*	HFD Model	Ashokan et al., 2010
Antichol	*Commiphora mukul* *Curcuma longa* *Emblica officinalis* *Terminalia arjuna* *Terminalia belerica* *Terminalia chebula* *Garcinia cambojia* *Ptreocarpus marsupium*	ISO induced hyperlipidemia model	Venu P. et al.,2010
Diacure	*Syzigium cumini* *Azardiricta indica* *Ocimum tenuiflorum* *Abitulon indicum* *Cassia auriculata* *Ficus bengalensis* *Tinospora cardifolia* *Phyllanthus emblica* *Trigonella foenum graceum* *Curcuma longa* *Phyllanthus niruri*	STZ induced Diabetic rats	Arun N. et al., 2011
Dihar	*Syzygium cumini* *Momordica charantia* *Embelica officinalis* *Gymnema sylvestre*	STZ induced diabetic rats	Patel et al., 2009

	Enicostemma littorale *Azadirachta indica* *Tinospora cordifolia* *Curcuma longa*		
Glyoherb	*Gymnema sylvestre* *Picrorrhiza kurroa* *Swertia chirata* *Momordica charantia* *Holarrhena antidysenterica* *Phyllanthus embilica* *Tribulus terrestris* *Terminalia chebula* Euginea jambolana *Trigonella foenumgraecum* *Azadirachta indica* *Cedrus deodara* *Terminalia belletica* Bang bhasma	STZ induced Diabetic rats	Patel et al.,2009
Triglize	*Terminalia arjuna* *Cissus qandrangularis* *Boerhaavia diffusa* *Commiphora mukul* *Phyllanthus embilica* *Terminalia belletica* *Terminalia chebula* *Tribulus terrestris* *Allium Satirum* *Trigonella foenumgraecum*	HFD model in rats	Parshuraman et al., 2010

Sugnil	*Aristolochia bracteata* *Shorea roxburghii* *Cassia auriculata* *Casearia esculanta* *Coscinium fenestratum* *Curcuma longa* *Eugenia jambolana* *Gymnema sylvestre* *Triphala*	STZ induced diabetic rats	Paranthaman et al ., 2011

STZ : Streptozotocin ; **HFD :** High Fat Diet



Chapter 4

Aim and Objective

4. Aim and Objectives

By the literature survey, it is very obvious that the bark of *Ailanthus excelsa* is having multiple uses, and traditionally it is reported for its use in treatment of diabetes mellitus and obesity (Database 2005). Therefore it was decided to correlate these uses with its anti hyperlipidemic potential. There are no scientific reports available regarding anti hyperlipidemic activity of the bark of *A.excelsa*. Hence it was selected to explore its phytopharmacological activity with special reference to its anti hyperlipidemic activity. The aim and objectives of the present work are :

- To perform phytopharmacological investigation of *Ailanthus excelsa* bark with special reference to its anti- hyperlipidemic activity.
- Pharmacognostic evaluation of the crude drug with the aim of establishing the diagnostic standards.
- Physico-chemical evaluation and phytochemical analysis of various extracts using various chemical tests and quantitative estimations.
- Evaluation of anti hyperlipidemic activity of the aqueous and methanolic extract of *Ailanthus excelsa* bark at different doses using high fat diet induced hyperlipidemia model and intravenous lipid tolerance test in rats.
- To explore the possible mechanisms responsible for the anti hyperlipidemic activity.

Chapter 5

Materials and Methods

5. Material and Methods

5.1. Collection and authentication of the plant material

The dried bark material of *Ailanthus excelsa* was procured from Lallubhai Vrajlal Gandhi Ahmedabad, Gujarat, India, in the month of October 2011. The bark was identified by comparing their morphological description as described in various standard texts (Nadkarni, 1996; The Wealth of India, 1972). Further, the voucher specimen (IPS/PCOG/MPH11-12/502) was authenticated by Dr. B.L. Punjani, Ethanobotanist, P.G. Centre in Botany, Smt. S.M. Panchal Science College, Talod, Gujarat, India. The bark was then shade dried and coarsely powdered, and stored in airtight plastic container at room temperature until needed.

5.1. Macroscopic evaluation

The bark of *Ailanthus excelsa* was subjected to macroscopic observations, which comprised of the organoleptic characters of the crude drugs viz. size, shape, odour, appearance, surface, taste and fracture etc.

5.2. Microscopic evaluation

Free hand transverse and surface sections of the dried bark of *Ailanthus excels*a were cut. The section was cleared with chloral hydrate solution and then stained with phloroglucinol hydrochloric acid (1:1) and mounted with glycerin. A separate section was prepared and stained with iodine solution for the identification of starch grains.

5.3. Powder study

Small quantity of powder of bark of *Ailanthus excelsa* was taken and decolorize by using chloral hydrate. Then these decolourised powders stained with different reagents like HCl, phloroglucinol and iodine. All observations of the microscopic evaluation were made and recorded with the help of special CCD (charged couple device, Lawrence and Mayo) camera attached with microscope.

5.4. Physico-Chemical Evaluation (The Ayurvedic Pharmacopoeia of India, 2001).

5.4.1. Determination of total ash

2gm of accurately weighed bark powder was incinerated in a tarred platinum or silica dish at a temperature not exceeding 450^{o}C until free from carbon, cooled and weighed. If a carbon free ash could not be obtained in this way, the charred mass was exhausted with hot water, the residue was collected on an ash less filter paper, incinerated, along with filter paper, evaporated to dryness and ignited at a temperature not exceeding 450^{o}C. The ash thus obtained was then cooled, weighed and percentage of ash was calculated with reference to the air-dried drug.

5.4.2. Determination of water soluble ash

The ash obtained from above procedure was boiled for 5 min. with 25ml of distilled water and the insoluble matter was collected in a Gooch crucible, or on an ash less filter paper. The insoluble matter thus obtained was washed with hot water and filter paper was ignited to a constant weight along with filter paper. The weight of insoluble matter was substracted from total ash giving water soluble ash. The percentage of water soluble ash was calculated with reference to the air-dried drug.

5.4.3. Determination of acid insoluble ash

The ash obtained from above procedure was boiled for 5 min. with 25ml of dilute hydrochloric acid and the insoluble matter was collected in a Gooch crucible, or on an ash less filter paper. The insoluble matter thus obtained was washed with hot water and filter paper was ignited to a constant weight along with filter paper. The percentage of acid-insoluble ash was calculated with reference to the air-dried drug.

5.4.4. Determination of alcohol soluble extractive

5gm of the air-dried bark powder was macerated with 100ml of alcohol of the specified strength in a closed flask for 24 hours, shaking at an interval of six hours. It was then allowed to stand for 18 hours. The macerate was filtered rapidly taking precaution against any loss of solvent. 25 ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish and dried at 105^{o}C to a constant weight and finally weighed. The percentage of alcohol soluble extractive was calculated with reference to the air-dried drug.

5.4.5. Determination of water soluble extractive

5gm of the air-dried bark powder was macerated with 100ml of chloroform water of the specified strength in a closed flask for 24 hours, shaking at an interval of six hours. It was then allowed to stand for 18 hours. The macerate was filtered rapidly to prevent any loss of water. 25ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish and dried at 105^{o}C to a constant weight and finally weighed. The percentage of water-soluble extractive was calculated to the air-dried drug.

5.4.6. Determination of moisture content (Loss on drying)

About 10 gm of bark powder was placed in a tared evaporation dish. It was then dried at 105^{o}C for 5 hours and weighed. Drying was continued and the bark was weighed at 1 hr interval until the difference between two successive weighing corresponded to not more than 0.25 percent. Constant weight was reached when two consecutive weighing after drying for 30min. and cooling for 30min. in a desiccators, did not show more than 0.01gm difference.

5.4.7. Determination of Swelling index (WHO 2002)

The specified quantity of bark material (powdered) was introduced into a 25 ml glass stoppered measuring cylinder. 25 ml of water was added and the mixture was shaken thoroughly every 10 minutes for 1 hour. It was then allowed to stand for 3 hours at room temperature. The volume in ml occupied by the plant material, including any sticky mucilage was measured. The mean value of the individual determinations, related to 1 g of plant material was calculated.

5.5. Preliminary Phytochemical Screening

The extract prepared from *Ailanthus excelsa* bark was analyzed qualitatively for the detection of the major groups of chemical constituents and individual components using standard and specific detecting reagents as described by Evans (2002) and Harbone (1998).

5.5.1.Tests for Alkaloids

About 500 mg of each of the dried extract was stirred with about 5 ml of dilute hydrochloric acid and filtered. The filtrate was tested with the following reagents:

(i) Mayer's reagent: Few drops of Mayer's reagent (Potassium mercuric iodide solution) were added separately to each filtrate and observed for the formation of white or cream colored precipitates.

(ii) Dragendroff's reagent: Few drops of Dragendroff's reagent (Solution of potassium bismuth iodide) were added separately to each filtrate and observed for the formation of orange yellow precipitates.

(iii) Hager's reagent: Few drops of Hager's reagent (Saturated aqueous solution of picric acid) were added separately to each filtrate and observed for the formation of yellow precipitates.

(iv) Wagner's reagent: Few drops of Wagner's reagent (Solution of iodine in potassium iodide) were added separately to each filtrate and observed for the formation of reddish brown precipitates.

5.5.2. Tests for Carbohydrates

(i) Molisch's test: A small amount of each extract was dissolved in ethanol and two drops of a 20% w/v solution of α-napthol in ethanol were added to it. Now, about 1 ml of concentrated H_2SO_4 was slowly added along the sides of the test tube. Appearance of red-violet ring at the junction of the two layers indicated the presence of carbohydrates.

(ii) Fehling's test: A small amount of the each extract was dissolved in about 2 ml of distilled water and filtered. An equal amount of Fehling's solution was added to the

filtrate and the contents were boiled. Appearance of brick red precipitates confirmed the presence of reducing sugars.

(iii) Benedict test: A small amount of the each extract was dissolved in about 2 ml of distilled water and filtered. About 1 ml of Benedict solution was added to the filtrate. The contents were boiled and observed for the appearance of brick-red precipitates which confirmed the presence of reducing sugars.

5.5.3.Tests for Glycosides

(i) Borntrager's test: A small amount of each extract was hydrolysed with dilute HCl for a few min on water bath. To the hydrolysate, about 1 ml of benzene and 0.5 ml of dilute ammonia solution was added. Appearance of reddish-brown color at the junction of the two layers confirmed the presence of glycosides.

5.5.4.Tests for Sterols

(i) Liebermann-Burchard's test: A small amount of each extract was dissolved separately in chloroform and few drops of acetic anhydride were added. Now, concentrated sulphuric acid was added drop-wisely along the sides of the test tube and observed for the appearance of blue to blood red color as the indication of sterols.

(ii) **Salkowski test:** A small amount of each extract was dissolved in chloroform. Concentrated Sulphuric acid was added drop wise along the sides of test tube and observed for presence of red or yellow colour at lower layer.

5.5.5 Tests for Saponins

(i) Foam Test: About 1 ml of each extract (in the respective solvents) was separately diluted to 20 ml with distilled water and further shaken in a graduated cylinder for 15 minutes. Formation of about 1 cm thick layer of foam confirmed the presence of saponins.

5.5.6Tests for Phenolic compounds and Tannins

(i) Ferric chloride test: Small amount of each extracts were separately shaken with water and warmed. Now about 2 ml of 5% ferric chloride solution was added and observed for the formation of green or blue color.

(ii) Lead acetate test: A few milligrams of each extract were separately stirred with about 2 ml distilled water and filtered. To the filtrate, few drops of 10% w/v lead acetate solution was added and observed for the formation of white precipitates.

5.5.7. Tests for Amino acids and Proteins

(i) Millon's test: A small amount of each extract was separately dissolved in about 5 ml of distilled water and filtered. To 2 ml of the filtrate, 5-6 drops of Million's reagent (solution of mercury nitrate and nitrous acid) were added and observed for formation of red precipitates as an indication of the presence of proteins.

5.5.8 Tests for flavonoids

(i) Shinoda test: A few milligrams of each extract were separately shaken with ethanol in different test tubes. Now, small pieces of metallic magnesium or zinc were added followed by addition of 2 drops of concentrated HCl and observed for the formation of pink color.

(ii) Aqueous NaOH test: To test solution add 10 % NaOH, yellow color is obtained.

(iii) Mineral acid test: To test solution add conc. H_2SO_4, yellow-orange color is obtained.

(iv) Lead acetate solution test: To the test solution add 10 % of lead acetate solution, yellowish precipitates are obtained.

5.9. TLC Profile of *Ailanthus excelsa*

1 gm of powdered bark of *Ailanthus excelsa was* extracted with 10 ml methanol and 10 ml of water using reflux for 15 min., filtered and concentrated up to 2-3 ml. This methanolic extract and aqueous extracts were spotted for different classes of Phytochemicals like, flavonoids and triterpenoids, etc. (Harborne, 1998; Wagner *et al.*, 2004, API, 2001) using different mobile systems.

A. Flavonoids:

Mobile phase: Ethyl acetate:Formic acid:Glacial acetic acid:Water (100:11:11:26)

Detection: UV 364 (Blue- green spot), Vanillin- Phosphoric acid (Brown spot)

B. Trierpenoids:

Mobile phase: Chloroform: Methanol(18:1)

Detection: UV 364 (yellow green florescence), Sulphuric acid reagent (Brown color spot)

5.10. Estimations

A. Estimation of Phenolic Substances (Singleton and Rosi, 1965):

1g of air-dried powdered bark of *A.excelsa* was extracted with 100ml methanol and 100 ml of water by maceration process for 24 hours, separately and filtered. The final volume of the filtrate was adjusted up to100 ml using water and methanol with repected extracts. 5ml of each extract was diluted with an equal volume of water and methanol with respected extracts and was used for the estimation of phenols. 10ml of the each extract were taken and 10ml of distilled water and 1.5ml of diluted (1:2) Folin Ciocaulteu reagent were added in both extract and the mixture was kept for 5min. After adding 4ml of 20% Na_2CO_3 solution, the final volume was adjusted to 25ml using distilled water. The absorbance was measured at 765nm at an interval of 30min. up to 2 hours using distilled water as a blank. The data were compared with similarly prepared set of standard substance- Gallic acid, in concentration range of 5 µg to 30 µg per 25ml.

B. Estimation of Flavonoids (Baharam *et al.,* 1996):

1g of air-dried powdered bark of *A.excelsa* was extracted with 100ml methanol and 100 ml of water separately by maceration process for 24 hours and filtered. The final volume of the filtrate was adjusted to 100ml using water and methanol with respected extracts. Each of One ml of these extracts was diluted up to 10ml with methanol and was used for the estimation of flavonoids. To 3ml of the each extract, 3ml of methanolic $AlCl_3$ was added. After 10min, absorbance was measured at 430nm.

C. Estimation of Terpenoids (Dai et al.,1999).

1g of air-dried powdered bark of *A.excelsa* were extracted with 100ml methanol and water by maceration for 24 hours and filtered. The final volume of the filtrate was adjusted to 100ml using methanol. Each of One ml of these extracts was diluted up to 10ml with methanol and was used for the estimation of terpenoids. To 1ml of the each extract, Add 0.2 ml of the vanillin solution to each tube and swirl. Stand for 2 minutes at room temperature. Add 0.3 mL of concentrated H_2SO_4 to each tube in three portions (0.1 ml each) and mix carefully. Add 0.7 ml of methanol. Stand for 5 minutes at room temperature. The absorbance was measured at 577 nm using a glass (1 ml) microcuvette.

5.11.Pharmacological Activity: (*In- Vivo* Models)

5.11.1. Evaluation of Anti Hyperlipidemic Activity of Aqueous and Methanolic Extracts of Bark of *Ailanthus excelsa* in High Fat Diet induced Hyperlipidemic Rats

1. Plant material:

- Powdered bark of *A.excelsa*

2. Chemicals:

- Cholesterol and Cholic acid were acquired from Finar Chemicals Ltd, Ahmedabad (India) and Chemdyes Corporation, Ahmedabad (India) respectively.
- Atrovastatin was acquired from was obtained as a gift sample from Torrent Research Center, Gandhinagar, Gujarat, (India)..
- Atorvastatin at a dose of 10 mg/kg was given after dissolving in distilled water orally.
- Aqueous extract of powdered bark was dissolved in distilled water and given orally at dose of 200 and 400 mg/kg.
- Methanolic extracts of powdered bark was suspended in 0.5% CMC and given orally at dose of 200 and 400 mg/kg.

3. Preparation of Extracts:

The powdered bark of *A.excelsa was* sieved through 40 # mesh sieve. The measured amount of the powder was then subjected to continuous and sequential; hot solvent extraction using two solvents distilled water and methanol in a soxhlet apparatus seperately. Filtrates were evaporated and dried at 40^0C in hot air oven and stored in air tight container.

4. Preparation of High Fat Diet (HFD) Food

Normal pellets were powdered in pulverizer and from that powder high fat diet food containing 20% ground nut oil, 0.5% cholesterol, 1% cholic acid was prepared**.**

5. Animals

Healthy adult wistar rats weighing between 150 - 250 gm were selected for the study. Animals were maintained at 25 ± 2^0C and kept in well ventilated animal house under natural photoperiodic condition in polypropylene cages with free access to food and water. They were kept for 1 week in laboratories before the experiments for acclimatization to the laboratory conditions. Six animals were used for each group of study. All experiments and protocols described in present study were approved by the Institutional Animal Ethics Committee (IAEC) of Institute Of Pharmacy, Nirma University, Ahmedabad and with permission from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Protocol number for the present study was given IPS/PCOG/MPH11- 12/2019.

6. Experimental Procedure:

The experiment was conducted for 28 days, in which rats (n=6) were randomly divided into 7 groups. Treatment schedule given below was followed. Body weight was measured every week (Sai et al., 2010).

Table 8. Treatment schedule for anti Hyperlipidemic activity

Sr No	Groups	Treatment
1	Normal control	Distilled water
2	Disease control	HFD (diet containing 20% ground nut oil, 0.5% cholesterol, 1% cholic acid)
3	Standard	HFD + Atorvastatin (10 mg/kg, p.o)
4	AQ 200	HFD + Aqueous extracts of *A.excelsa* bark (200mg/kg, p.o.)
5	AQ 400	HFD + Aqueous extracts of *A.excelsa* bark (400mg/kg, p.o.)
6	ME 200	HFD + Methanolic extracts of *A.excelsa* bark (200 mg/kg, p.o.)
7	ME 400	HFD + Methanolic extracts of *A.excelsa* bark (400 mg/kg, p.o.)

HFD – High Fat Diet

Sample collection and estimation of serum lipid profile

Blood samples were collected on o day, 7th day, 15th day, 21st day and 28th day by retro orbital plexus puncture method using glass capillary under light ether anaesthesia after 8 hr fasting and allowed to clot for 30 min. Then blood samples were centrifuged at 3,000 rpm for 15 min. The serum was separated and stored at -20^0 C until biochemical estimation of lipid profile. Serum lipid profile was then estimated using diagnostic kits. (Labcare Diagnostics (India) Pvt.Ltd) (Israni et al.,2010).

Histopathology

One lobe of liver was stored in 10% formalin solution for histopathological studies.

Parameters Assessed:

Body weight

Lipid profile in serum:

- Total cholesterol
- Triglycerides
- HDL cholesterol
- LDL cholesterol
- Total protein

Oxidative stress parameters in liver homogenate:

- Pro- oxidant :- Malondialdehyde.(MDA)
- Anti-oxidant :- Reduced Glutathione (GSH)

5.11.2. Intravenous Lipid Tolerance Test in Rats (Carlson et al.,1972)

Purpose and retionale

Intravenous injection of a lipid emulsion results in an increase of triglycerides in serum. The lipolytic activity can be determined by measuring lipid elimination.

Procedure

Male Wistar rats weighing 200–240 g were treated daily with 200mg/kg and 400 mg/kg doses of aqueous and, methanolic extracts, and chloroform fraction of methanolic extracts (qaussinoid rich fraction) of *A. excelsa* and the vehicle over a period of 5 days. On the fifth day, two hours after the last administration of the test compound, the animals were anesthetized with 125 mg/kg sodium hexobarbital i.p. Then they were injected intravenously with 2 ml/kg of a 20% lipid emulsion (Sino – Swed Pharmaceuticals , Pune (India)). Prior to the injection and 20, 40, and 60 min time interval blood was withdrawn by retro-orbital puncture for determination of triglycerides.

5.11.3. Estimation of Lipid Profile in Serum :

Estimation of Total Cholesterol :

In vitro quantitative determination of the activity of cholesterol in serum was done using enzymatic kit (Lab Care Diagnostics, India Limited).

Principle

Cholesterol esters are hydrolyzed by Cholesterol esterase to produce cholesterol. Hydrogen Peroxide is then produced from oxidation of cholesterol by cholesterol oxidase. The indicator quinoneimine is formed from hydrogen peroxide and 4 - aminoantipyrine in the presence of phenol and peroxide. The absorption of the red quinoneimine dye is proportional to the concentration of cholesterol in the sample.

Procedure

Pipette into 3 test tubes labeled Blank (B), Standard (S) and sample as shown below.

	Blank	**Standard**	**Sample**
Sample	-	-	10µl
Standard	-	10µl	-
Reagent	1000µl	1000µl	1000µl

Mixed and incubated for 5 mins at 37°C (or 10 mins at 20 - 25°C). The absorbance of the sample (AT) and standard (AS) against reagent blank was measured at 505 nm. The colour was stable for 30 mins at 20 - 25°C.

Calculations:-

AT/AS x Conc. Standard = mg/dl Total Cholesterol

Estimation of Triglycerides:

In vitro quantitative measurement of triglyceride (neutral fat) concentration in serum was done by using kit (Lab Care Diagnostics, India Limited).

Principle

Triglycerides are determined after enzymatic hydrolysis with lipases. The quinonemine indicator is formed from hydrogen peroxide, 4- aminophenazone, and 4-chlorophenol under the catalytic influence of peroxidase.

Procedure

Pipette into 3 test tubes labeled Blank (B), Standard (S) and Sample as shown below:-

	Blank	**Standard**	**Sample**
Sample	-	-	10µl
Standard	-	10µl	-
Reagent	1000µl	1000µl	1000µl

Mixed and incubated for 5 mins at 37°C (or 10 mins at 20 - 25°C). The absorbance of the sample (AT) and standard (AS) against reagent blank was measured at 505 nm. The colour was stable for 30 mins at 20 - 25°C.

Calculations:- AT/AS x Conc. Std. = mg/dl Triglycerides

Estimation of LDL Cholesterol :

In vitro quantitative measurement of LDL-C concentration in serum was done by using kit (Lab Care Diagnostics, India Limited.)

Principle

Direct determination of serum LDL (low-density lipoprotein cholesterol) levels without the need for any pre-treatment or centrifugation steps.

The assay takes place in two steps.

1. Elimination of lipoprotein non-LDL.

$$\text{Cholesterol esters} \xrightarrow{\textbf{CHE}} \text{Cholesterol + Fatty acids}$$

$$\text{Cholesterol + } O_2 \xrightarrow{\textbf{CHOD}} \text{4-Cholestenone + } H_2O_2$$

$$2H_2O_2 \xrightarrow{\textbf{Catalase}} 2H_2O + O_2$$

2. Measurement of LDLc :-

$$\text{Cholesterol esters} \xrightarrow{\textbf{CHE}} \text{Cholesterol + Fatty acids}$$

$$\text{Cholesterol + } O_2 \xrightarrow{\textbf{CHOD}} \text{4-Cholestenone + } H_2O_2$$

$$2H_2O_2 + \text{TOOS + 4-AA} \xrightarrow{\textbf{POD}} 2H_2O + O_2$$

The Intensity of the color formed is proportional to the LDL concentration in the sample.

Procedure

Pipette into 3 test tubes labeled Blank (B), Standard (S) and Sample as shown below.

Blank Standard Sample

	Blank	**Standard**	**Sample**
R1(µl)	750	750	750
Standard(µl)		10	
Sample(µl)			10
Mix and incubate for for 5 min			
R2(µl)	250	250	250

Mixed and incubated for 5 mins at 37°C. The absorbance (A) was measured against the Blank at 546 nm.

Calculation:

$$\frac{\text{A Sample}}{\text{A Calibrator}} \text{ x Calibrator conc. = mg/dl of LDL C in the sample}$$

Concentration of Calibrator is 56 mg/dl.

Estimation of HDL Cholesterol :

In vitro quantitative measurement of HDL-C concentration in serum was done by using kit (Lab Care Diagnostics, India Limited)

Principle

Direct determination of serum HDL (high-density lipoprotein cholesterol) levels without the need for any pre-treatment or centrifugation of the sample. The method depends on the properties of a detergent which solubillizes only the HDL so that HDL-C is released to react with the cholesterol esterase, cholesterol oxidase and chromogens to give colour. The non HDL lipoprotein LDL, VLDL and chylomicrons are inhibited from reacting with the enzymes due to absorption of the detergents on their surfaces. The intensity of the color formed is proportional to the HDL concentration in the sample

Procedure

Pipette into 3 test tubes labeled Blank (B), Standard (S) and sample as shown below:-

	Blank	**Standard**	**Sample**
R1(µl)	750	750	750
Standard(µl)		10	
Sample(µl)			10
Mix and incubate for for 5 min			
R2(µl)	250	250	250

Read absorbance A1.

Mixed and incubated for 5 min at 37^{o}C. The absorbance A2 was measured against blank at wavelength 600 nm. The increase of the absorbance ΔA=A2-A1 was calculated.

Calculations:

HDL-C (mg/dl) = ΔA sample / ΔA Calibrator X Calibrator conc.

Estimation of Total Protein (Colorimetric test – Biuret test):

Principle:

Cupric ions, in an alkaline medium, interact with protein peptide bonds resulting in the formation of a color complex.

Assay procedure:

Pipette into test tubes

	Blank	Standard	Sample
Sample	-	-	20 µl
Standard	-	20 µl	-
Reagent	1000µl	1000 µl	1000 µl

Mix well, Incubate for 10 min at 20-25°C. Measure absorbance of the Serum sample (Ac) and standard (As) against reagent blank.

Calculation:

gm/dl Protein = Ac/As x Conc. Standard

Estimation of Oxidative Stress Parameters in Liver

Isolation of liver :

All the animals were euthanasiously sacrificed by the excess dose of ether. Liver was collected and was blotted free of blood and tissue fluids. Then it was weighed on balance and the weight was noted down. The liver was stored in a deep freezer at -20 ˚ C temperature for estimation of oxidative stress parameters.

Preparation of the tissue homogenate :

Liver, kept in cold conditions (pre-cooled in inverted petridish on ice) was removed. It was cross chopped with surgical scalpel into fine slices and was chilled in the cold 0.25 M sucrose, quickly blotted on a filter paper. The tissue was minced and homogenized in 10 mM Tris-HCl buffer, pH 7.4 (10% w/v) with 25 strokes of tight teflon pestle of glass homogenizer at a speed of 2500 rpm. The clear supernatant was used for enzymes assays.

Measurement of oxidative stress parameters

Lipid peroxidation:- (MDA) :

Malondialdehyde (MDA) was estimated by the method of **Slater et al., 1979.**

Principle:

The method estimates malondialdehyde (MDA), a product of lipid peroxidation process. One molecule of MDA reacts with two molecules of thiobarbituric acid (TBA) under mildly acidic conditions to form a pink coloured chromogen, whose intensity was measured colorimetrically at 535 nm.

Reagents:

1. **Sodium lauryl sulphate (SLS) (8%) :** 8 gm of SLS in 100 ml of distilled water.
2. **Acetic acid (20 %) :** Prepared in 0.27 M hydrochloric acid (2.29 ml HCL in 100 ml water)
3. **Thiobarbituric acid(TBA) (1% in Tris hydrochloride, pH 7): (Freshly prepared):-** 1 gm of thiobarbituric acid in 100 ml of Tris hydrochloride buffer pH 7.
4. **Trichloroacetic acid (TCA) (10%) :** 10 gm of trichloroacetic acid in 100 ml of distilled water.

Procedure:

Following steps were performed for the test.

Blank	**Test**
0.2 ml of D.W	0.2 ml of Homogenate
0.2 ml of SLS	0.2 ml of SLS
1.5 ml acetic acid in HCl	1.5 ml acetic acid in HCl
1.5 ml TBA	1.5 ml TBA
0.6 ml DW	0.6 ml DW
Heated for 45 min in water bath at 95^{0}C and cool	
2 ml mixture + 2 ml TCA	2 ml mixture + 2 ml TCA
Centrifuge on 1000 RPM for 5 min	

The absorbance of the developed pink colour was measured at 532 nm.

Calculation:

A = a × b × c

A = Absorbance of test sample

a = Molecular Extinction coefficient ($1.56 * 10^5$ cm^{-1})

b = Path length (1 cm)

c = Conc. of sample

Units:- nanomoles of MDA / mg of protein

Reduced Gluathione :

Reduced of glutathione (GSH) was estimated by the method of **Moran et al , 1979.**

Principle :

Glutathione present in RBC consist of sulfhydryl groups. 5,5 dithiobis 2- nitro benzoic acid (DTNB), A disulphide compound, gets readily attacked by these sulfhydryl groups and forms a yellow coloured anion which measured colorimetrically at 412 nm.

Reagents :

1. Trichloroacetic acid (TCA) (10%) : 10 gm of TCA in 100 ml of distilled water.

2. 0.3 M Na_2HPO_4 : 4.26 gm of Na_2HPO_4 in distilled water.

3. DTNB (Dithiobis nitro benzoic acid) (Fresh) : 40 mg in 100 ml of 1% Sodium citrate & cover with aluminium foil.

Procedure:

Blank	**Test**
0.2 ml of D.W	0.2 ml of Homogenate
1 ml of TCA (10%)	1 ml of TCA (10%)
Keep in ice bath for 30 min & Centrifuge for 10 min at 4 ˚C at 3000 RPM, take 0.5 ml of Supernatant	
0.5 ml of Supernatant	0.5 ml of Supernatant
2 ml di-sodium hydrogen phosphate	2 ml di-sodium hydrogen phosphate
0.25 ml DTNB(Cover with aluminium foil)	0.25 ml DTNB(Cover with aluminium foil)

Mixed well and then the absorbance was measured against blank at 412 nm using spectrophotometer.

Calculation: **Y = 0.015X - 0.0265**

Where, X = Conc. of reduced of glutathione**.**

Y = Absorbance of test sample,

Units: µg of GSH / mg of protein.

5.11.4. *In-Vitro* Model

Lipase Inhibitory Assay

The substrate emulsion was prepared by blending 15 mL tributyrin, 50 mL emulsification reagent (17.9 g NaCl, 0.41 g KH2PO4, 540 mL glycerol, 6.0 g gum Arabic in 1000 mL deionized water) and 235 mL deionized water. The assay system contained 9.0 mL substrate emulsion, 0.50 mL plant extract in 50% DMSO and 0.50 ml lipase preparation (1.0 U/mL) in a total volume of 10 mL. The final concentrations of the reactants were: tributyrin 50.0 ml/l; lipase 0.05 U/ml; plant extract 5, 50, 500 μg/ml. The reaction was linear during the first 10 min, and run for 10 min. The concentration of DMSO in the reaction (2.5%) did not affect the enzyme activity.

Lipase activity was calculated as

Specific lipase activity,

$$\mathbf{U/mL = (T/t_T - B/t_B) * 0.05 * 1/0.5}$$

Where the specific lipase activity was defined in lipase units (U) per mL i.e. μmoles released butyric acid per ml enzyme preparation (pH 7.0; 30 °C) per min, T and B represent the volume of a 50 mmol/l solution of NaOH in microliters, used to titrate the test and blank, respectively; t_T and t_B are the reaction times, in min, for the test and blank, respectively; and 1/0.5 converts the enzyme preparation volume to mL. The concentration of each test sample giving 50% inhibition of the enzyme activity (IC50) compared with the reaction of the control was calculated from the least squares regression line of the semi-logarithmic plot against the remaining activity. Sibutramine was assayed as a positive control. The assay was run in triplicate for each sample.

5.11.5. *In-Vitro* Antioxidant activity:

1. Plant material:

Powdered bark of *A.excelsa*

2. Preparation of Extract:

Same as mentioned above

3. DPPH (1, 1-diphenyl-2-picryl hydrazyl) radical scavenging activity:

The antioxidant activity of aqueous and methanolic extracts were assessed on the basis of the radical scavenging effect of the stable DPPH free radical (Bloi, 1958). About 25 - 200 µl of aqueous and methanolic extracts were added to 3 ml of DPPH in methanol (0.33%) in a test tube. After incubation at 37^{o}C for 30 minutes the absorbance of each solution was determined at 517 nm using spectrophotometer (Hwang *et al.*, 2001, Ghosh *et al.*, 2009). The corresponding blank reading were also taken and the remaining DPPH was calculated by using the following formula,

DPPH radical scavenging activity (%) = [Abs (control) – Abs (test)] × 100.

Where, Abs (control) : Absorbance of DPPH radical + methanol

Abs (test) : Absorbance of DPPH radical + test.

IC50 value is the concentration of the sample required to scavenge 50% DPPH free radical.

5.11.6 Statistical analysis

All the values are expressed as Mean ± S.E.M. Statistics was applied using Graph Pad Prism 5.0 version. Statistical significance between all the groups was carried out using one way ANOVA analysis followed by Tukey's multiple comparision test. Differences were considered to be statistically significant when $P < 0.05$.

Chapter 9

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Dr. Bhaskar L. Punjani

Ph.D.(Botany), FES, CES
Ethanobotanist, Associate Professor
Head, P.G. Centre in Botany,
Smt. S.M. Panchal Science College Talod- 383215
Gujarat , India
email id- blpunjani1958@gmail.com

CERTIFICATE

This is to certify that the specimens of the plants provided by following students of the Institute of Pharmacy, Nirma University, are authentified by me .

Sr No	Student Name	Plant Name
1	Devang Shelat	*Pterocarpus marsupium*
2	Divyang Patel	*Ailanthus excelsa*
3	Sangeeta Gupta	*Typha angustata*
4	Shaival Shah	*Symplocos racemosa*
5	Chaitanya Sarvaiya	*Achyranthus aspera*

(Dr. Bhaskar L. Punjani)
Associate Professor
Head, PG Centre in Botany
Smt. S. M. Panchal Science College
TALOD-383 215 (Dist.: Sabarkantha), Gujarat