EVALUATION OF HYPOLIPIDEMIC ACTIVITY OF *TERMINALIA ARJUNA* LEAF EXTRACT IN RAT MODELS OF HYPERLIPIDEMIA

By

D. BALA SUNDER REDDY

B.V.Sc. & A.H.,

THESIS SUBMITTED TO THE
ACHARYA N.G. RANGA AGRICULTURAL UNIVERSITY
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF
MASTER OF VETERINARY SCIENCE
(VETERINARY PHARMACOLOGY AND TOXICOLOGY)
IN THE FACULTY OF VETERINARY SCIENCE

DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY
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CERTIFICATE

Mr. D. BALA SUNDER REDDY has satisfactorily prosecuted the course of research and that the thesis entitled **"EVALUATION OF HYPOLIPIDEMIC ACTIVITY OF *TERMINALIA ARJUNA* LEAF EXTRACT IN RAT MODELS OF HYPERLIPIDEMIA"** submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or the part thereof has not been previously submitted by him for a degree of any university.

Date : 8-12.2005

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CERTIFICATE

This is to certify that the thesis entitled **"EVALUATION OF HYPOLIPIDEMIC ACTIVITY OF *TERMINALIA ARJUNA* LEAF EXTRACT IN RAT MODELS OF HYPERLIPIDEMIA"** submitted in partial fulfillment of the requirements for the degree of **"MASTER OF VETERINARY SCIENCE"** of the Acharya N.G. Ranga Agricultural University, Hyderabad, is a record of the bonafide research work carried and by **MR. D. BALA SUNDER REDDY** under my guidance and supervision. The subject of the thesis has been approved by the student's advisory committee.

No part of the thesis has been submitted for any other degree or diploma. The published part has been fully acknowledge. The author of the thesis has duly acknowledged all assistance and help received during the course of investigation.

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LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

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D. Bala Sunder Reddy

(D. BALA SUNDER REDDY)

ABBREVIATIONS

5' ND	-	5' Nucleotidase
@	-	At the rate of
<	-	Lesser than
>	-	Greater than
%	-	Percent
µg	-	Microgram
□C	-	□Centigrade
ACP	-	Acid Phosphatase
ALP	-	Alkaline Phosphatase
ALT	-	Alanine Aminotransferase
ANOVA	-	Analysis of Variance
apo	-	Apolipoprotein
AST	-	Aspertate Aminotransferase
b.wt	-	Body Weight
CHO	-	Cholesterol
dl	-	Decilitre
g	-	Gram(s)
H & E	-	Hematoxyline and Eosin
HDL	-	High Density Lipoproteins
HMG-CoA	-	3-Hydroxy-3-Methyl Glutaryl Coenzyme A
IDL	-	Intermediate Density Lipoprotein
i. p.	-	Intraperitoneal

Kg	-	Kilogram(s)
L	-	Litre(s)
LCAT	-	Lecithin Cholesterol Acyl Transferase
LDH	-	Lactate Dehydrogenase
LDL	-	Low Density Lipoproteins
Lp (a)	-	Lipoprotein (a)
MIC	-	Minimum Inhibitory Concentration
mμ	-	Millimicron (s)
mg	-	Milligram (s)
ml	-	Milliliter
N	-	Normality
nm	-	Nanometer(s)
P	-	Probability
PHO	-	Phospholipids
p. o.	-	Per os
rpm	-	Revolutions per minute
SE	-	Standard Error
TGL	-	Triglycerides
UV-VIS	-	Ultra Violet- Visual
VLDL	-	Very Low Density Lipoproteins
V/V	-	Volume/ Volume
W/V	-	Weight/ Volume
W/W	-	Weight/ Weight

DECLARATION

I, **D. BALA SUNDER REDDY**, hereby declare that the thesis entitled **"EVALUATION OF HYPOLIPIDEMIC ACTIVITY OF *TERMINALIA ARJUNA* LEAF EXTRACT IN RAT MODELS OF HYPERLIPIDEMIA"** submitted to Acharya N.G. Ranga Agricultural University, Hyderabad, for the degree of **"MASTER OF VETERINARY SCIENCE"** is the result of original research work done by me. I also declare that the materials contained in this thesis have not been published earlier.

Date: 8-12-2005

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ABSTRACT

Recognition of hypercholesterolemia as a risk factor for atherosclerosis forced the development of drugs that reduce the cholesterol levels in the serum. Plant kingdom is a rich natural source for many therapeutic molecules. *Terminalia arjuna* bark is reported to posses hypolipidemic activity. However no such reports are available on the leaves and hence the leaves were screened for their hypolipidemic potential in two rat models viz. triton induced and diet induced hyperlipidemic rats. Methanolic leaf extract was used @ 125, 250 and 500 mg/kg b.wt and for comparison bark extract was used @ 125 mg/kg b.wt.

In triton induced model hypolipidemic effect was evaluated by monitoring the serum levels of total cholesterol and triglycerides. In diet induced model hypolipidemic effect was evaluated by monitoring the serum levels of total cholesterol, triglycerides, HDL cholesterol, VLDL cholesterol, LDL cholesterol and phospholipids on day 15 and day 30. The same parameters were also estimated in liver tissues at necropsy on day 30. Cholic acid and desoxycholic acid in feces were estimated at the end of the study on day 30. Results indicated that leaf extract exhibited hypolipidemic activity on day 30 at dose levels 250 and 500 mg/kg b.wt. However bark extract exhibited the hypolipidemic effect on day 15 and day 30 at the tested dose of 125 mg/kg b.wt.

Thus it was evident that though leaves posses hypolipidemic activity, this activity is less potent and less efficacious compared to the activity present in the bark.

CHAPTER 1

Introduction

CHAPTER I

INTRODUCTION

The World Health Organization (WHO) estimates that every year 12 million people worldwide die from cardiovascular diseases, with most of them being from the developing world (Kmietowicz, 2002). Coronary heart disease is an important cause of death, and the atherosclerosis accounts for the majority of these deaths. Atherosclerosis also results in significant cardiac morbidity such as anginal syndromes, myocardial infarctions and in non-cardiac morbidity such as cerebrovascular accidents and peripheral vascular disease.

The hallmarks of atherosclerosis are the deposition of lipids in the arterial intima, recruitment of inflammatory cells into the intima, smooth muscle cell accumulation and the elaboration of collagen matrix proteins and then sticking of platelets to it. Since the hypercholesterolemia, characterized by an increase in serum cholesterol, has been generally recognized to contribute significantly to the progression of atherosclerosis, this fact points to the importance of reducing plasma cholesterol levels (Erkkila Arja *et al.,* 1999). Animal studies and many randomized double blind studies in the human beings prove beyond doubt, the cause and effect relationship between hypercholesterolemia and morbidity and mortality from coronary artery disease (Brown *et al.,* 1997, Superko and Krauss, 1994).

Recognition of hypercholesterolemia as a risk factor forced the development of drugs that reduce the cholesterol levels. Present pharmacotherapy of hypercholesterolemia / atherosclerosis includes mostly the statins that inhibit hepatic cholesterol biosynthesis, fibrates with complex actions, the bile acid sequestrant resins that sequester bile acids in the intestines, the cholesterol absorption inhibitors and LDL oxidation inhibitors.

Investigation of traditional medicine by sound scientific methods is desirable and highly relevant in any field of drug development. Different hypocholesterolemic agents of plant origin, such as alfa-alfa (Malinow *et al.*, 1980), soya beans (Kritchevsky, 1979), garlic (Kamanna and Chandrashekhara, 1982) and fenugreek seeds (Yves Sauvaire *et al.*, 1984) have been used in regression studies of atherosclerosis.

The fruit and bark of ***Terminalia arjuna*** (Roxb) Whight and Arnot (Family: Combretaceae, Hindi name: Arjuna; Telugu name: Erramaddi, Tellamaddi) have been used since the Vedic period for the treatment of heart diseases. It forms an essential ingredient of many patent ayurvedic proprietary preparations sold as cardiotonics (Nesamony, 1988; Chopra *et al.*, 1986). More than twenty compounds including glycosides with a modified steroid ring have been isolated from *T. arjuna* (Kumar and Prabhakar, 1987). It has been reported to possess multiple actions with regard to usage in hypercholesterolemia and dyslipidemia. (Tiwari *et al.*, 1989; Khanna *et al.*, 1996; Ram *et al.*, 1997; Shaila *et al.*, 1997, Shaila *et al.*, 2000).

However it is evident from careful perusal of available literature that all these studies have been conducted using the bark of *T. arjuna.* Studies on the effectiveness of *T. arjuna* leaves in the above conditions are lacking. Availability of bark is limited and collection is laborious compared to the leaves. Keeping this in view it is planned to screen the leaves of *T. arjuna* for their potential in the treatment of hyperlipidemias.

Hence, the present study is proposed with following objectives.

- To study the hypolipidemic effect of *T. arjuna* leaves in rats,
- To compare the hypolipidemic effect of *T. arjuna* leaves with the bark,
- To compare the hypolipidemic effect of *T. arjuna* leaves with standard drugs.

CHAPTER II

Review of Literature

CHAPTER II

REVIEW OF LITERATURE

2.1 Hyperlipidemia/ Hyperlipoproteinemia/ Dyslipidemia

The term hyperlipidemia or more precisely hyperlipoproteinemia refer to a condition in which the concentrations of cholesterol and/or triglyceride rich lipoproteins are elevated above the normal levels. There is strong evidence from several studies that the extent of reduction of the incidence of coronary heart disease is directly related to the reduction of serum cholesterol (Lipids Research Clinics Programme, 1984; Martin *et al.*, 1986). Since high lipid content of plasma is believed to be the incriminating agent in atherothrombotic diseases, pharmacological agents have been developed which reduce the concentration of plasma lipids. To understand how these hypolipidemic drugs work, it is imperative to know, how the lipids are handled in the body, i.e., lipid metabolism and the patho-physiology of various hyperlipidemic states.

2.1.1 Cholesterol

Cholesterol is present in tissues and in plasma lipoproteins either as free cholesterol or combined with a long chain fatty acids as cholesteryl esters. Cholesterol is a precursor of all other steroids in the body such as corticosteroids, sex hormones, bile acids and vitamin- D. It is typically a product of animal metabolism and therefore occurs in foods of animal origin such as egg yolk, meat,

liver and brain. Cholesterol is an amphipathic lipid and as such is essential structural component of membranes and of the outer layer of plasma lipoproteins (Mayes, 2000).

Lipoproteins transport free cholesterol in the circulation, where it readily equilibrates with cholesterol in other lipoproteins and in membranes. Cholesteryl ester is a storage form of cholesterol found in many tissues. LDL is the mediator of cholesterol and cholesteryl ester uptake into in many tissues. Free cholesterol is removed from tissues by HDL and transported to the liver for conversion to bile acids in the process known as reverse cholesterol transport. Cholesterol is a major constituent of gallstones. However its chief role in pathologic process is as a factor in the genesis of arterioscleroses of vital arteries, causing cerebrovascular, coronary and peripheral vascular diseases. Coronary arteriosclerosis correlates with a high plasma LDL: HDL cholesterol ratio (Mayes, 2000).

2.1.2 Lipoproteins

Lipoproteins are macromolecules that contain lipids and proteins known as apolipoproteins/apoproteins. The lipid constituents include free and esterified cholesterol, triglycerides, and phospholipids. In all spherical lipoproteins, the most water insoluble lipids (cholesteryl esters and triglycerides) are core components and the more polar water soluble components (apoproteins, phospholipids and unesterified cholesterol) are located on the surface (Robert and Bersot *et al.*, 2001). The major classes of lipoproteins with their characteristics are given in the Table 1

Table: 1 Major Classes of Plasma Lipoproteins with their Characteristics (Robert and Bersot *et al.*, 2001)

LIPOPROTEIN CLASS	DENSITY OF FLOTATION (g/ml)	MAJOR LIPID CONSTITUENT	TG/CHO RATIO	SIGNIFICANT APOPROTEINS	SITE OF SYNTHESIS	MECHANISM (S) OF CATABOLISM
Chylomicrons and remnants	<< 1.006	Dietary tri-glycerides and cholesterol	10:1	B-48, E, A-I, A-IV, C-I, C-II, C-III	Intestine	Triglyceride hydrolysis by lipoprotein lipase, apo E-mediated remnant uptake by liver
VLDL(very low density lipoproteins)	< 1.006	Endogenous or hepatic triglycerides	5:1	B-100, E, C-I, C-II, C-III	Liver	Triglyceride hydrolysis by lipoprotein lipase
IDL (intermediate density lipoproteins)	1.006–1.019	Cholesteryl esters and endogenous triglycerides	1:1	B-100, E, C-II C-III	Catabolic product of VLDL	50% converted to LDL mediated by hepatic lipase, 50% apoE-mediated uptake by liver
LDL (low density lipoproteins)	1.019–1.063	Cholesteryl esters	NS	B-100	Catabolic product of VLDL	apo B-100 mediated uptake by LDL receptor (~75% in liver)
HDL (high density lipoproteins)	1.063 – 1.21	Phospholipids cholesteryl esters	NS	A-I, A-II, E, C-I, C-II, C-III	Intestine, liver and plasma	Complex transfer of cholesteryl ester to VLDL and LDL uptake to HDL cholesterol by hepatocytes
Lp (a) (lipoprotein(a))	1.05 – 1.09	Cholesteryl esters	NS	B-100, apo (a)	Liver	Unknown

NS: not significant (triglyceride is <5% of LDL and HDL).

2.1.2.1 Apolipoproteins /Apoproteins

Apolipoproteins /apoproteins are the surface proteins of various lipoproteins and in addition to providing structural stability to the lipoproteins, they play critical roles in determining the metabolic fate of particles on which they reside.

The apoproteins are named in an arbitrary alphabetical order and the apoproteins that have well defined roles in plasma lipoprotein metabolism include apo A-1, apo A-II, apo A-IV, apo B-100, apo B-48, apo C-I, apo C-II, apo C-III and apo-E.

apo A-1 is the major protein present on HDL constituting about 70-80% of the protein mass. It is an activator of LCAT, which esterifies free cholesterol in plasma. apo A-II is the second most abundant apoprotein in HDL. Its function is still unknown. apo A-IV is a minor component of HDL and chylomicrons. It may play a role in activation of LCAT.

apo B-48 is the major apoprotein in VLDL, IDL and LDL. It is the apoprotein, which is essential for assembly and secretion of chylomicrons. apo B-100 is the largest of apoproteins and is absolutely necessary for the assembly and secretion of VLDL from the liver and serves as the binding protein for the LDL receptors on cells through out the body.

apo C-I is a minor component of VLDL, IDL and HDL. Its exact function is unknown. apo C-II is a constituent of VLDL and is also present on IDL, HDL and chylomicrons. It is an essential activator of enzyme LPL that hydrolyses

triglycerides present in chylomicrons and VLDL. Subjects lacking apo C-II have severe hypertriglyceridemia. apo C-III is a major component of VLDL in which it accounts for about 40% of the protein. It is also present on IDL, HDL and chylomicrons. Some studies suggest that apo C-III is an inhibitor of LPL action.

apo E is found on all the lipoproteins and it appears to regulate the removal of remnant lipoproteins from the plasma by liver. Subjects lacking apo E have severe hypercholesterolemia and early atherosclerosis (Ghatak and Asthana, 1995).

2.1.3 Metabolism of Lipids

A little more than half the cholesterol of the body arises by synthesis (about 700 mg/dl), and the remainder is provided by the average diet. The liver accounts for approximately 10% of total synthesis in humans, the intestines for about another 10%. Virtually all tissues containing nucleated cells are capable of synthesizing cholesterol (Mayes, 2000).

There are different pathways for metabolism of exogenous and endogenous lipids as depicted in Plate 1.

2.1.3.1 Exogenous Pathway

In the exogenous pathway, cholesterol and triglycerides absorbed from the gastrointestinal tract are transported in the lymph and then in the plasma as chylomicrons (diameter 100-1000 nm) to capillaries in muscle and adipose tissue.

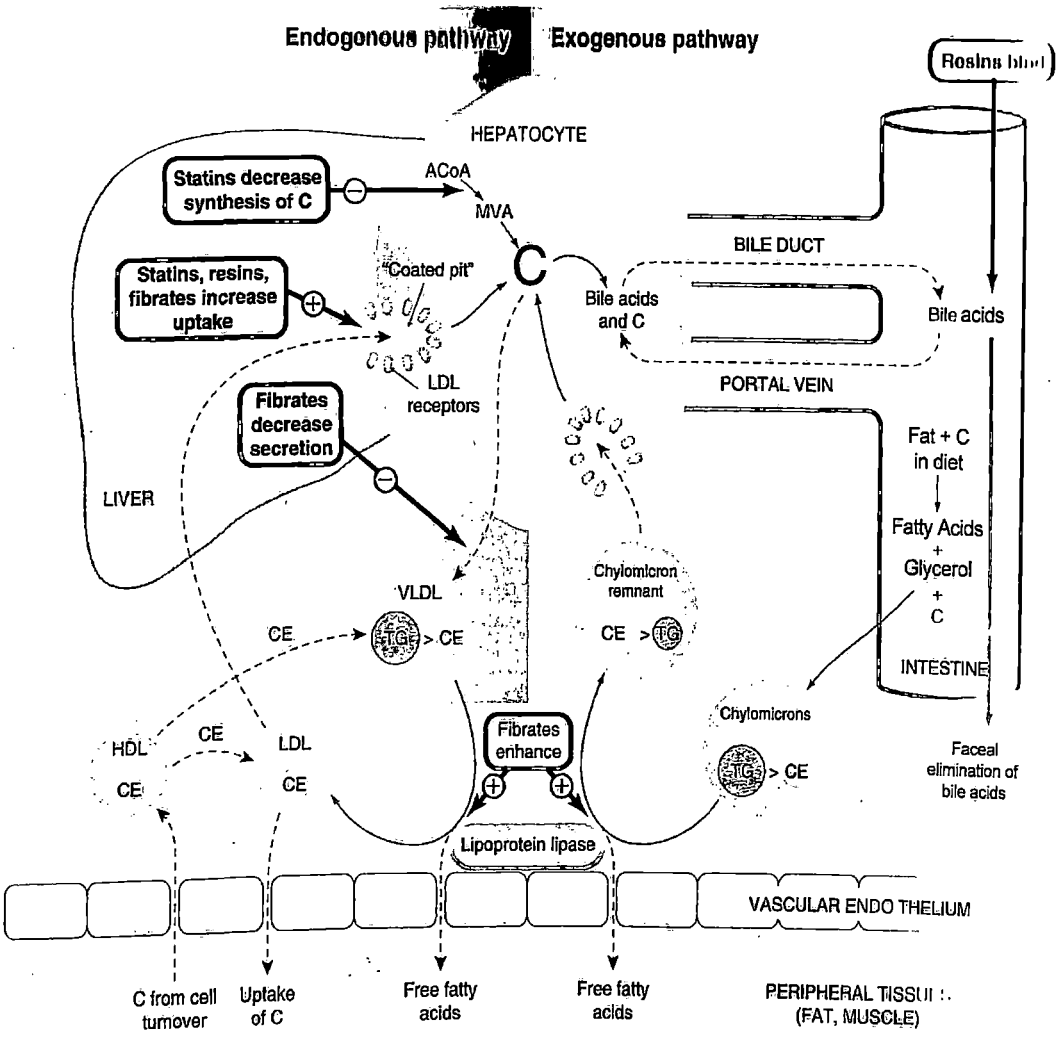


Plate : 1 Schematic diagram of cholesterol transport in the tissues, with sites of action of the main drugs affecting lipoprotein metabolism.

Here the core triglycerides are hydrolysed by lipoprotein lipase, and the tissues take up the resulting free fatty acids. The chylomicron remnants (diameter 30-50 nm), still containing their full complement of cholesteryl esters, pass to the liver, bind to receptors on hepatocytes and undergo endocytosis. Cholesterol is liberated with in the liver cell and may be stored or oxidized to bile acids or secreted in the bile unaltered. Alternately, it may enter the endogenous pathway of lipid transport via VLDL (Rang *et al.*, 2003).

2.1.3.2 Endogenous Pathway

In endogenous pathway, cholesterol and newly synthesized triglycerides are transported from the liver as VLDL (diameter 30-80 nm) to muscle and adipose tissue, where the triglycerides are hydrolysed and the resulting fatty acids enter into the tissue. During this process, the lipoprotein particles become smaller (diameter 20-30 nm), but still have a full complement of cholesteryl esters and ultimately become LDL, which provides the source of cholesterol for incorporation into cell membranes and for synthesis of steroids and bile acids. Cells take up LDL by endocytosis via LDL receptors that recognize LDL apolipoproteins. Cholesterol can return to plasma from the tissues in HDL particles (diameter 7-20 nm). Cholesterol is esterified with long-chain fatty acids via HDL particles, and the resulting cholesteryl esters are subsequently transferred to VLDL or LDL particles by a transfer protein present in the plasma (Rang *et al.*, 2003).

2.1.4 Classification of Hyperlipidemias

The phenotypic classification of the hyperlipidemias based upon serum electrophoresis as per WHO is as follows (Seth and Sandeep, 2000)

Type I Hyperlipidemia: Characterized by severe elevation of chylomicrons and triglycerides due to congenital deficiency of lipoprotein lipase or apo C-II. Deposition of fat in the skin represents the clinical manifestations of the disorder.

Type II A Hyperlipidemia: Characterized by the elevation of LDL cholesterol. Genetic conditions responsible are familial hypercholesterolemia, polygenic hypercholesterolemia, familial combined hyperlipidemia and familial defective apolipoprotein B-100. These individuals are at high risk for developing premature coronary artery disease.

Type II B Hyperlipidemia: Characterized by the elevation of both LDL cholesterol and triglyceride levels. Familial combined hyperlipidemia is the most common genetic cause of this disorder where both VLDL and LDL are elevated.

Type III Hyperlipidemia: Develops due to a defect in VLDL remnant clearance; also known as familial dysbetalipoproteinemia. These individuals have difficulty in removing triglyceride rich VLDL remnant particles and consequently have elevations of cholesterol and triglycerides.

Type IV Hyperlipidemia: Characterized by hypertriglyceridemia (levels generally between 250 and 500 mg/dl). Causes are multiple such as genetic and diseases contributing are diabetes, nephritis along with administered medications.

Type V Hyperlipidemia: Characterized by elevated levels of chylomicrons and VLDL levels. Defective lipolysis and an over production of VLDL are responsible. Causes can be genetic or secondary to diabetes mellitus, obesity or alcohol consumption.

2.1.5 Antihyperlipidemic Drugs

Based on mechanism of action the antihypolipidemic drugs can be classified as follows

2.1.5.1 HMG–Co A Reductase Inhibitors (Statins)

HMG-Co A reductase inhibitors are the most effective and well tolerated agents for treating hyperlipidemias. These exert their major effect i.e. reduction of LDL levels through a mevalonic acid like moiety that competitively inhibits HMG-Co A reductase by product inhibition (Alberts *et al.*, 1980). Drugs under this group include atorvastatin, cerivastatin, lovastatin, pravastatin, simvastatin *etc.*

The major adverse effect of clinical significance associated with statin use is myopathy and all statins have been associated with myopathy and rhabdomyolysis (Pogson *et al.*, 1999).

2.1.5.2 Bile Acid Sequestrants

The bile acid sequestrants are highly positive charged and bind to negative charged bile acids. Because of their large size, resins are not absorbed, and the bound bile acids are excreted in the stool. Since over 95% bile acids are normally

reabsorbed, interruption of this process depletes the liver's pool of bile acids. As a result, hepatic cholesterol content declines, stimulating the production of LDL receptors, an effect similar to that of statins (Bilheimer *et al.*, 1983). Drugs under this group include cholestyramine, colistipol and colesevalem.

2.1.5.3 Fibric Acid Derivatives

The mechanism of action of the fibrates is not currently well understood. Many researchers consider stimulation of lipoprotein lipase to be the mechanism of action, but VLDL and triglyceride production may also be retarded or its catabolism increased. It also promotes the transfer of cholesterol to HDL. Others have maintained that the lipid-lowering activity is tied to the peroxisomal proliferation activity of fibrates. Clinically these agents have the primary effect of lowering triglycerides. Another major beneficial effect is they elevate HDL levels, while LDL can be reduced or elevated. Fibrates, especially clofibrate, have a potential to increase biliary lithogenicity (Ghatak and Asthana, 1995). Drugs under this group include clofibrate, benzafibrate, ciprofibrate, fenofibrate, gemfibrozil *etc.*

2.1.5.4 Niacin (Nicotinic acid)

Nicotinic acid is one of the oldest drugs used to treat dyslipidemia and is the most versatile in that it favourably affects virtually all lipid parameters. The adverse effects associated with niacin therapy are flushing and dyspepsia which limit patient compliance (Altschul *et al.*, 1955). Niacin is the only lipid-lowering drug that reduces Lp (a) levels significantly, by about 40% (Carlson *et al.*, 1989).

2.1.5.5 LDL Oxidation Inhibitors

These drugs act by preventing oxidation of LDL cholesterol thus slow the development of atherosclerosis (Illigworth, 1991). Probucol is the best studied agent of this group. Adverse effects include mild gastro-intestinal disturbances that disappear with continued treatment and a more serious problem is its tendency to prolong the Q-T interval (Dujovne, 1991).

2.2 Animal Models of Hyperlipidemia

Experimentally hyperlipidemia can be induced in laboratory animals by administering some agents orally in the diet or systemically.

2.2.1 Diet Induced Models

These models, involve incorporating a hyperlipidemia inducing substance or chemical in the normal feed. Such models simulate well the clinical models of hyperlipidemia and are often associated with an increase in activity of the exogenous cholesterol pathway.

Cholesterol feeding has been often used to elevate serum or tissue cholesterol levels in studies designed to assess hypercholesterolemia related metabolic disturbances in different animal models (Holmgren and Brown 1993; Hozumi *et al.*, 1995). Watts *et al.* (1994) reported that a high level of saturated fat in addition to cholesterol is required in the rat model. Induction of hyperlipidemia in cholesterol and fat fed rats occur as a result of alteration in several aspects of

lipoprotein metabolism. Kris-Etherton and Cooper (1980) observed that in cholesterol and fat administered rats, the disappearance rate of chylomicron remnants was markedly prolonged. They also noted that the delay in removal was due to an increase in the circulatory remnant concentration without the removal defect of hepatocytes. In addition the abnormal cholesterol content of the lipoprotein particles of d < 1.006 g/ml was particularly accounted for the hepatic secretion of VLDL poor in triglycerides and rich in cholesterol. Some of the established diet induced hyperlipidemic rat models are,

- Daily administration by gavage of 1ml/100 gm body weight for 7 days of a cocktail containing cholesterol @ 100 g, propyl thiouracil @ 30 g and cholic acid @ 100 g suspended in 1 litre peanut oil (Fillios *et al.*, 1956).

- Daily administration by oral gavage a hyperlipidemic diet of arachis oil (10 ml/kg) containing 5% cholesterol and 0.5% cholic acid for 21 days (Arichi *et al*; 1982).

- Feeding cholesterol @ 4.5%, sodium cholate @ 1.8%, butter @ 37% and propylthiouracil @ 0.3% added to normal rat feed (Michele Coutard and Mary, 1982).

- Some authors have induced hyperlipidemia in male albino rats, by feeding a hyperlipidemic diet containing starch, casein, sugar, groundnut oil and cholesterol (Seetharamaiah and Chandrasekhara, 1989), and by feeding a hyperlipidemic diet containing of casein, sucrose, hydrogenated oil, salt, cellulose and cholic acid (Sharma, 1980).

- Rats were fed a lipogenic diet consisting of 2% cholesterol, 20% sunflower oil and 0.5% cholic acid added to normal chow and were given 3% ethanol for 42 days for induction of hyperlipidemia (Bolkent *et al.*, 2005).

2.2.2 Chemical Induced Models

In chemical induced models, a particular chemical or substance is systemically administered to induce hyperlipidemia. These correlate well to the increased activity of endogenous pathway of cholesterol, often accompanied by increase in cholesterol biosynthesis in the liver. Drugs, which reduce lipid levels by decreasing synthesis of cholesterol and triglycerides, can be evaluated well using these models.

2.2.2.1 Triton Induced Hyperlipidemia

Systemic administration of the surfactant triton WR-1339 (Iso octylpolyoxy ethylene phenol) to fasted or non-fasted mice and rats results in elevation of plasma cholesterol and triglyceride levels (Vogel and Vogel, 1997).

This hypercholesterolemia is biphasic. Initially there will be a sharp increase in serum cholesterol levels reaching a peak, 2 to 3 times the control value, by 24 hours after administration of triton (phase I). The hypercholesterolemia falls off nearly to control values with in the next 24 hours (phase II). The mechanism of the triton hypercholesterolemia in phase I is thought to be due to increased hepatic synthesis of cholesterol through the ability of triton to interfere with the hepatic uptake of plasma lipids by the tissues. Drugs interfering with endogenous

cholesterol biosynthesis were shown to be active in the phase I of the test, while drugs interfering with excretion and metabolism of cholesterol were active in phase II (Vogel and Vogel, 1997).

2.3 Medicinal Plants with Hypolipidemic Activity

All currently available hypolipidemic/ anti hyperlipidemic drugs for use are neither fully effective, nor totally free from side effects. Hence there is a need to probe for new hypolipidemic agents. Plant kingdom is a rich natural source for many therapeutic molecules. Certain plants have been reported to have hypolipidemic activity in various animal models and human clinical trials. Of these some plants like, *Allium sativum, Cicer arietinum, Commiphora mukul, Curcuma longa, Emblica officinalis, Inula racemosa, Terminalia arjuna, Trigonella foenumgraecum, Zingiber officinale etc.*, seem to be promising in lowering cholesterol levels (Sharma and Dwivedi, 1997).

Oral administration of petroleum ether extract of *Allium sativum, Allium cepa* and ethyl acetate fraction of *Commiphora mukul* to albino rats significantly prevented the rise in levels of serum cholesterol and triglycerides, caused by atherogenic diet. All the three agents were also found to confer significant protection against atherogenic diet induced atherosclerosis (Lata *et al.*, 1991).

A water soluble protein fraction of garlic was investigated for its effect on hyperlipidemia induced by alcohol (3.76 g/kg/day). Garlic protein (500 mg/kg/day) showed significant hypolipidemic action comparable with a standard

dose of gugulipid (50 mg/kg/day). It was opined that the hypolipidemic action is mainly due to an increase in cholesterol degradation to bile acids and neutral sterols and mobilization of triacyl glycerols in treated rats (Rajasree *et al.*, 1999).

Gum guggul, a resin obtained from an ethyl acetate fraction of *Commiphora mukul*, possess marked hypolipidemic activity (Nityanand and Kapoor, 1973). A standardized fraction from this resin containing a mixture of lipid sterols (Guggulsterones) is named as guggulipid, and is marketed as a hypolipidemic/lipid-lowering agent (Nityanand and Kapoor, 1984). The lipid lowering action of guggulsterone, the active constituent of gugulipid, was studied in triton injected and cholesterol fed hyperlipidemic rats. Serum lipids were found to be lowered by guggulsterone @ 50 mg/kg in triton WR-1339 induced hyperlipidemia. Chronic feeding of this drug @ 5 mg/kg to animals simultaneously fed with cholesterol @ 25 mg/kg for 30 days, lowered serum lipid and apoprotein levels (Chander *et al.*, 1996).

The hypolipidemic effect of ginger was studied in rats that were fed atherogenic diet. A significant decrease in the levels of cholesterol, phospholipids and free fatty acids in the tissues (liver, intestine, kidney, and aorta) and serum were observed in ginger treated rats. Serum triglyceride levels, LDL and VLDL levels were also significantly lowered while HDL levels were elevated in the ginger treated groups (Murugaiah *et al.*, 1999).

Bhandari *et al.*, (1998) recorded the protective action of ethanolic ginger extract against hypercholesterolemia in cholesterol fed rabbits in comparison with standard drug gemfibrozil. Both the agents reduced the serum and tissue cholesterol, serum triglycerides, LDL, VLDL, phospholipids and increased serum HDL cholesterol levels. Aortic atherosclerosis was seen at a lower degree in both ethanolic ginger extract and gemfibrozil treated groups.

Oral administration of bergenin, isolated from the leaves of *Flueggea microcarpa,* to hyperlipidemic rats for 14 days significantly decreased serum total lipid without much change in serum cholesterol and triglycerides. However, after 21 days of administration, the serum cholesterol, triglycerides, LDL cholesterol and VLDL cholesterol levels were significantly reduced while the serum HDL cholesterol level was elevated. Bergenin treated animals also showed a significant decrease in atherogenic index (Jahromi *et al.*, 1992).

The methanolic extract of leaves of *Aleurites moluccana* had significant hypocholesterolemic activity and provided considerable protection against insurgence of high fat diet (cholesterol 1%, sodium cholate 2%, vitamin mixture 2%, oligoelements 0.2%, salt mixture 5.8%, corn oil 20%, cellulose 4%, sucrose 44%, casein 5%, protein 15% for 30 days) induced hyperlipaemia and triton induced hyperlipidemia (Pedrosa *et al.*, 2002). It was opined that these effects could be due to several mechanisms involving inhibition of intestinal absorption of lipids or cholesterol synthesis, enhancement of cholesterol degradation or interference with lipoprotein distribution.

Ajit *et al.* (2000) evaluated seven herbal preparations designated as SSB, B/Sj/K, S/Pg/K, Ttmn, Tmfg, FCBN and TBLH for their lipid lowering ability in triton induced hyperlipidemic rats along with their antioxidant potential against generation of O_2 free radicals *in vitro.* The herbal samples were prepared from garlic, onion, *Carum copitum* (*Tachyspermum ammi*), *Terminalia bellarica, T. chebula, Achyranthes aspera, Tridax procumbens, Andrographis paniculata, Gymnema sylvestre, Curcuma zedoaria, Mikania cordata*, ginger, fenugreek and *Picorhiza kurroa,*. Administration of these drugs at 200 mg/kg *p.o. l*owered the levels of serum total cholesterol, phospholipids and triglycerides to varying extents in triton WR-1339 induced hyperlipidemia. Hypolipidemic action of SSB, B/Sj/K, FCBN and TBLH were more significant and comparable to that of gugulipid @100 mg/kg *p.o.,* a standard drug from *Commiphora mukul.* All these preparations at 0.1- 1.0 mg/ml inhibited the *in vitro* generation of hydroxyl radicals in a concentration dependent manner. However SSB, B/Sj/K, FCBN and TBLH were more potent antioxidants against the formation of O_2 free radicals.

2.4 TERMINALIA ARJUNA

Botanical Name

Terminalia arjuna (Roxb.)Wt.&Arn.

Classical Names

Arjuna, Dhavala, Kakubha, Veeravriksha, Nadisarja, Partha (Sharma *et al.*, 2003)

Plate: 2 *Terminalia arjuna* (Roxb.) Wt. & Arn. (Combretaceae)

LEAVES & FRUITS

BARK

Vernacular Names

Telugu – Erramaddi, Tellamaddi and Yermuddi. **English** – Arjun **Hindi** – Arjun, Kahu, Kahua, Arjan, Khawa, Anjani and Jamla. **Kannada** – Maddi, Vaidairya, Billi matti and Holematti. **Tamil** –Attumarutu, Irmarutu, Vellaimarutu and Vellamatti (Sharma *et al.*, 2003)

Botanical Description

It is a large, evergreen tree. Leaves are sub opposite, oblong or elliptic, coriaceous, cordate, shortly acute or obtuse at the apex. Flowers in panicled spikes. Fruits are ovoid or ovoid-oblong, 2.5-5.0 cm long, nearly glabrous, with 5-7 hard winged angles (Sharma *et al.*, 2003)

Distribution

The plant is common throughout the greater part of the Indian peninsula along rivers, streams, ravines and dry water courses, sub himalayan tract, chota nagpur, orissa, west bengal, punjab, deccan and konkan tracts. Plant is also found in Sri Lanka and Myanmar (Sharma *et al.*, 2003)

2.4.1 Phytochemistry

Various chemical constituents that have been isolated from *T. arjuna* and their structures have been established which belong to different classes such as hydrolysable tannins, triterpenoid glycosides, cardenolides, triterpenoid carboxylic acids, flavanoids, phenolics, phytosterol, mineral salt and sugar (Rahman *et al.*, 2004). Phytochemical constituents of *T.arjuna* and the parts in which they are present are given in the Table 2.

Table: 2 Phytochemical Constituents of T. arjuna **and Parts in which they are Present**

TYPE OF CHEMICAL	PART	ACTIVE PRINCIPLE	REFERENCE
Tannins	Bark	Punicalin, punicalgin, terchebulin, terflavin C, castalgin, casuariin, casuarinin	Linn *et al.*, 1996
		Pentagalloyl glucose, tetragalloyl glucose	Kandi F E and Nassar M I, 1998
		(+)-catechol, (+)-gallocatechol, epicatechol and epigallocatechol	Madhusudhanamma *et al.*, 1980
	Leaves	Arjunin I	Kandi F E and Nassar M I, 1998
Triterpenoid glycosides	Bark	Arjunoglucoside I, arjunoglucoside II	Murae *et al.*, 1976
		Arjunoglucoside III	Truyuki *et al.*, 1979
		Arjunoside I, arjunoside II	Anjaneyulu and Prasad, 1982
		Arjunoside III, arjunoside IV	Anjaneyulu and Prasad, 1982
		Arjunetoside	Upadhyay *et al.*, 2001
	Leaves	Arjunetin	Chauhan *et al.*, 1997
Triterpenoids	Roots	Friedelin	Nagar *et al.*, 1979
	Leaves	β-amyrin	Chauhan *et al.*, 1997

Flavanone	Fruit Bark Leaves	Arjunone Quercetin, kaempferol, pelargonidin and luteolin Apigenin-7-0-neohesperidoside	Nagar *et al.*, 1979, Nagar *et al.*, 1979 Dwivedi and Jouhari, 1997 Chauhan *et al.*, 1998
Phenolics	Bark, root leaves, fruit	Gallic acid Ellagic acid	Anjaneyulu and Prasad, 1982 Row *et al.*, 1970
Phytosterol	Bark, fruit leaves	β-sitosterol	Singh and Pandey, 1995

2.4.2 Hypolipidemic Activity of *T. arjuna* Tree Bark

It was evident from the perusal of the available literature that all hypolipidemic studies on *T. arjuna* were carried out employing the bark and no such reports are available on the leaves of the plant. Hence the works done on the *T. arjuna* tree bark are reviewed here under.

The hypolipidemic effects of *T. arjuna* tree bark and cholestyramine were studied in hypercholesterolemic rabbits. Both reduced the total and LDL cholesterol significantly in comparison to control. HDL levels were reduced significantly with cholestyramine treated group, but no alteration was noticed in *T. arjuna* treated group (Tiwari *et al.*, 1989).

The hypolipidemic activity of *T. arjuna* bark was studied by Khanna *et al.*, (1996) in triton induced and cholesterol fed hyperlipidemic rats. In *T. arjuna* bark (100 mg/kg *p.o.*) treated group there was a significant reduction in total cholesterol, phospholipids and triglycerides compared to triton group. When *T. arjuna* bark powder (100 mg/kg *p.o.*) was administered for 30 days to rats on cholesterol diet, significantly lowered serum lipids and protein levels of β-lipoproteins and a smaller increase in HDL cholesterol was noticed compared to only cholesterol fed group.

Serum cholesterol, triglycerides and phospholipids levels were reduced by the administration of *T. arjuna* bark to hypercholesterolemic rabbits. Although pretreatment with *T. arjuna* did not have any effect on serum lipid levels, there was a significant reduction in atherosclerotic plaque formation. Tissue lipids also showed a marked decrease (Shaila *et al.*, 1997).

Diet induced hyperlipidemic rabbits were given a normal diet including a 50% ethanolic extract of *T. arjuna* bark in doses of 100 mg/kg and 500 mg/kg and compared with control group. There was a significant reduction in total and LDL cholesterol levels in hypercholesterolemic rabbits. At 500 mg/kg it also reduced total: HDL and LDL: HDL ratios (Ram *et al.*, 1997).

In a randomized placebo-controlled clinical trial the hypocholesterolemic effects of *T. arjuna* tree bark powder was compared with vitamin-E. There was a significant decrease in total cholesterol (12.7%) and LDL cholesterol (25.6%) in *T. arjuna* treated group. There was also a significant decrease in lipid peroxide levels in both groups. However the decrease was more in vitamin-E group (Gupta *et al.*, 2001).

2.4.3 Other Properties of *T. arjuna* Tree Bark

The effects of aqueous extracts containing tannin related compounds from the stem bark of *T. arjuna* on the blood pressure and heart rate of rats were examined. The aqueous extract produced a transient decrease in blood pressure accompanied by a slight decrease in heart rate. The hypotensive effect was observed with a fraction containing tannin related compounds (F2) separated from the aqueous extract. The hypotensive effect of F2 was not influenced by pretreatment of rats with propronolol, but it was attenuated by pretreatment with atropine. It was suggested that the hypotensive effect could be mediated by cholinergic mechanisms (Takahashi *et al.*, 1997).

The antibacterial activity of crude drug from the tree bark of *T. arjuna* was recorded against bacteria using the hole plate diffusion method with concentrations of 5-25 mg/ml (Samy and Ignacimuthu, 2001). The antibacterial activity was also confirmed by the dilution method (1.25-20 mg/ml) in MIC. The above results were supported by photochemical analysis. Specific activity against pathogenic bacteria *Bacillus subtilis* and *Staphylococcus aureus* confirm the traditional usage of bark of *T. arjuna.*

The anti cancer potency of the ethanolic extract of *T. arjuna* tree bark was screened on N- nitroso diethyl amine (DEN) induced hepatocellular carcinoma in wistar albino rats (Sivalokanathan *et al.*, 2004). The cancer bearing animals were treated with ethanolic extract of *T. arjuna* @ 400 mg/kg for 28 days. The DNA and RNA content in the liver and kidney of cancer bearing animals were higher than those of control animals. Enzymes like ALT, AST, ACP, ALP, LDH and 5' nucleotidase (5'ND) were significantly increased in the serum of cancer bearing animals. On the other hand, ALT and AST were found decreased, while ACP, ALP, LDH and 5' ND were increased in the liver and kidney of the cancer bearing animals. These changes were reversed to normal levels in animals treated with the extract of *T. arjuna*, indicating its anticancer activity.

CHAPTER III

Materials and Methods

CHAPTER III

MATERIALS AND METHODS

3.1 Experimental Animals

Healthy, male, Wister strain albino rats weighing 150-200 g for cholesterol induced model and 250-300 g for triton induced model of hyperlipidemia were procured from the Department of Laboratory Animal Medicine, TNVASU, Madhavaram milk colony, Madhavaram, Chennai. The animals were housed in solid bottom polypropylene cages (four animals in each) at an ambient temperature of 25±2□C and 45-55% relative humidity with 12-12 h light and dark cycle. The rats were kept on *ad libitum* feed and water. Permission was obtained from the Institutional Animal Ethics and Biosafety Committee before the start of the experiment. The experiment was conducted in the Department of Pharmacology and Toxicology, College of Veterinary Science, Tirupati.

3.2 Feed Composition

As per NRC, 1995, requirement of lab animals specifications

Table: 3 Feed Composition

S.No.	Ingredient	Parts
1.	Wheat	40
2.	Maize	30
3.	Soybean meal	23
4.	Skim milk powder	5
5.	Mineral mix	1
6.	Salt	1

3.3 Plant Extracts

The bark and leaves of *T. arjuna* were collected locally in the month of April and were shade dried. The dried and pulverized bark powder (1 kg) was extracted with methanol (90%) by hot continuous percolation over 72 hours by using Soxhlet apparatus, where as dried and pulverized leaf powder (600 gm) was extracted with methanol (90%) by the same process over 24 hours. The methanolic extract was filtered and concentrated to a dry mass by using vacuum distillation and evaporation. A dark brownish red shiny crystal like residue of 13.89% (W/W) yield was obtained from the bark and pale greenish dry mass of 11.34% (W/W) yield was obtained from the leaves. The extracts were stored in a vacuum desiccator until use.

3.4 Experimental Design

Two models of hyperlipidemia were employed in the study. Gemfibrozil and gugulipid were used as standard hypolipidemic drugs for triton and diet induced models of hyperlipidemia respectively.

3.4.1 Triton Induced Rat Model of Hyperlipidemia

Thirty-six adult male rats weighing 250-300 g were randomly assigned to six groups each having six animals (n=6) and were treated as follows. Triton WR-1339 (10% W/V) solution was prepared in normal saline and given as single administration intra peritoneally to 18h fasted rats @ 200 mg/kg (Pedrosa *et al.*,

2002). The plant extracts and gemfibrozil were prepared in 2% carboxy methylcellulose and administered orally 1h prior to administration of triton WR – 1339.

Table: 4 Triton Induced Rat Model of Hyperlipidemia

S. No.	Group	Treatments
1.	I	Normal saline *i.p.*
2.	II	2% C.M.C. + Triton @ 200 mg/kg in n.s. *i.p.*
3.	III	*T. arjuna* leaf extract @ 125 mg/kg *p.o.* + Triton @ 200 mg/kg in n.s. *i.p.*
4.	IV	*T. arjuna* leaf extract @ 250 mg/kg *p.o.* + Triton @ 200 mg/kg in n.s. *i.p.*
5.	V	*T. arjuna* leaf extract @ 500 mg/kg *p.o.* + Triton @ 200 mg/kg in n.s. *i.p.*
6.	VI	Gemfibrozil (Lopid capsules)- Pfizer, Bangalore @ 250 mg/kg *p.o.* + Triton @ 200 mg/kg in n.s. *i.p.*

Blood was collected at 0, 18, 24 and 40 hours after triton administration by retro-orbital puncture under ether anesthesia to monitor serum total cholesterol and triglycerides levels.

3.4.2 Diet Induced Rat Model of Hyperlipidemia

Fifty-six adult male rats weighing150-200 g were randomly assigned to seven groups having eight animals in each (n=8) and were treated as follows.

For the induction of hyperlipidemia in groups II through VII, cholesterol (Hi Media, Mumbai) @ 500 mg/kg + cholic acid (Hi Media, Mumbai) @ 50 mg/kg suspended in groundnut oil was administered daily @ 10 ml/kg orally for 30 days (Arichi *et al,* 1982). The plant extracts and gugulipid were prepared in 2% carboxy methylcellulose and administered orally one hour prior to administration of ground nut oil (Jahromi *et al.,* 1992).

Table : 5 Diet Induced Rat Model of Hyper Lipidemia

S. No.	Group	Treatments
1.	I	Normal diet
2.	II	2% C.M.C. + Hyperlipidemic diet
3.	III	*T. arjuna* leaf extract @ 125 mg/kg *p.o.* + Hyperlipidemic diet
4.	IV	*T. arjuna* leaf extract @ 250 mg/kg *p.o.* + Hyperlipidemic diet
5.	V	*T. arjuna* leaf extract @ 500 mg/kg *p.o.* + Hyperlipidemic diet
6.	VI	*T. arjuna* bark extract @ 125 mg/kg *p.o.* + Hyperlipidemic diet
	VII	Guggulosterones (Gugulipid) Natural Remedies Pvt. Ltd, Bangalore @ 10 mg/kg *p.o.* + Hyperlipidemic diet

Blood was collected on days 0, 15 and 30 of the experiment by retro-orbital puncture under ether anesthesia. All the animals were sacrificed in each group, on day 30 for collection of livers and aorta. The serum (Days 0, 15 and 30) and liver

(Day 30) samples were analyzed for total cholesterol, triglycerides, HDL cholesterol, VLDL cholesterol, LDL cholesterol and phospholipids. Fecal material collected over the entire experimental period was assayed for cholic acid and desoxycholic acid.

3.5 Serum Lipid Profile

3.5.1 Estimation of Serum Total Cholesterol

Bhat Biotech diagnostic kit was used for estimation of total cholesterol, which followed CHOP/POD method (Allain *et al.*, 1974).

3.5.2 Estimation of Triglycerides

Triglycerides were estimated employing Bhat Biotech diagnostic kit, which followed GPO and Peroxide method (Bucolo G and David H, 1973; Wermern *et al.*, 1981).

3.5.3 Estimation of HDL Cholesterol

HDL cholesterol was estimated employing Bhat Biotech diagnostic kit, which followed phosphotungstic precipitation method (Friedewald, *et al.*, 1972).

3.5.4 Estimation of VLDL and LDL Cholesterol

VLDL and LDL levels were estimated as per the Friedewald formula.

VLDL = Triglyceride/5

LDL = Total cholesterol – (HDL + VLDL)

3.5.5 Estimation of Phospholipids

Serum phospholipids were estimated as per the method of Youngberg and Youngberg (1930).

Principle

The lipids are extracted into an ethanol-ether mixture and digested with sulphuric acid and hydrogen peroxide. The phosphorus, now present as phosphate, is then determined colorimetrically by ammonium molybdate method. In acidic solution, phosphorus reacts with molybdate to form phosphomolybdate complex. The change in absorbance due to complex formation is measured at 680 mμ, which is directly proportional to the concentration of phosphorus.

Extraction of Lipid Phosphorus

Reagents

1. Ethanol-ether mixture: 3 volumes of ethanol and 1 volume of ether.
2. Sulphuric acid, 10 N solution
3. Hydrogen peroxide, 100 volumes (30%)

Technique

1. About 16 ml of ethanol-ether mixture was taken in a 6×1 inch tube and 1 ml of serum was added drop by drop, with constant shaking.
2. Heated carefully to boiling in a boiling water bath.

3. Cooled and volume made up to 20 ml with further ethanol-ether mixture.

4. Mixed and filtered taking care to avoid loss by evaporation.

5. Pipetted 8 ml of the filtrate (equivalent to 0.4 ml of serum) into another 6×1 inch tube and evaporated to dryness.

6. Added 1 ml of 10 N sulphuric acid and heated gently.

7. When the mixture turned brown, flame was removed and a drop of hydrogen peroxide was added.

8. Heated further and repeated the addition of hydrogen peroxide at intervals until the digestion was complete and the liquid turned colorless.

9. Added 1 or 2 ml of water and boiled for a few seconds.

10. The liquid in the tube was transferred to a 10 ml stoppered cylinder, made upto the mark with water, taken 5 ml of the resulting solution (=0.2 ml of serum), added 1 ml of ammonium molybdate solution (7.5 grams in 400 ml of aqueous solution). Allowed to stand for thirty minutes.

11. A standard was prepared using 0.5 ml of a solution containing 5 mg phosphorus per 100 ml (0.2197 gram of potassium dihydrogen phosphate per liter) plus 4.5 ml of 10% trichloroacetic acid, and a 5 ml of 10% trichloroacetic acid was used as a standard blank and treated each in the same way as the test.

12. Read the test and standard against the standard blank at 680 mμ using UV – VIS spectrophotometer.

Calculation

Since 5ml of the diluted digest is equivalent to 0.2 ml of the serum and the standard contains 0.025 mg of phosphorus:

$$\text{Phospholipid phosphorus (mg/dl)} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times \frac{100}{0.2} \times 0.025$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 12.5$$

3.6 Liver Lipid Profile

3.6.1 Extraction of Total Lipids from Liver Tissue

Total lipids were extracted from the liver tissue employing the method of Folch *et al.* (1957).

Reagents

1. Chloroform-methanol mixture- 2:1 (V/V)
2. Chloroform-methanol mixture- 1:1 (V/V)
3. Hexane-isopropyl alcohol mixture- 3:2 (V/V)

Procedure

- Weighed 1 gm of liver tissue and transferred it to a boiling tube. To this added 20 ml of 2:1 chloroform: methanol.
- Homogenized for 2 minutes. Agitated the tubes for 10 minutes using orbital shaker.
- Centrifuged at 3000 rpm for 5 minutes. Supernatant was transferred to another tube.
- To this added 4 ml of distilled water. Shaken well for few seconds and again centrifuged at 3000 rpm for 5 minutes.
- Then the 3 phases were separated. Removed the upper phase and rinse the inter phase carefully once or twice with 1:1 chloroform: methanol, with out disturbing the lower phase.
- Then transferred the lower chloroform phase containing lipids into round bottom flask. Before transferring, weight of round bottom flask was recorded. Chloroform is evaporated under vacuum using rotatary evaporator. Allowed it to dry and again weight of the round bottom flask was recorded. The difference in weight gives the total lipid content in 1 gm of liver tissue.
- Added 30 ml of hexane- isopropyl alcohol to each round bottom flask. Kept it over night by sealing the round bottom flask with aluminum foil.
- Next day removed the aluminum foil and heated the round bottom flask gently using a water bath (50-60□C). To dissolve the white precipitate, rinsed the flask with 2-3 ml of 2:1 chloroform: methanol. Transferred the contents of round bottom flask to a measuring cylinder and make up the volume to 30 ml with 2:1 chloroform: methanol in all estimations to keep the final volume constant.

3.6.2 Estimation of Liver Lipid Profile

From the final extracted portion, total cholesterol, triglycerides, HDL, LDL, VLDL and phospholipids were estimated using the methods described for the serum estimations.

3.7 Determination of Desoxycholic Acid and Cholic Acid in Feces

- Rat feces were collected over 30 days. Bile acids in feces were estimated employing the method of Mosbach *et al.* (1954).
- Weighed 1gm of fecal sample and placed in a 50 ml Erlenmeyer flask.
- 5 ml of absolute ethanol was added to each flask, triturated using glass rod and the solution was heated to boiling on a steam bath, and filtered into a 25 ml glass stoppered centrifuge tube.
- Two additional 5 ml portions of hot ethanol were used to rinse the Erlenmeyer flask and filter paper.
- The ethanol was then evaporated on a steam bath under a current of air. To each tube add 5 ml of 5% sodium hydroxide solution and samples in each tube were hydrolyzed by boiling for 30 minutes.
- The samples were then cooled to room temperature and acidified (phenol red) by the drop wise addition of concentrated hydrochloric acid.
- The acidified solution was extracted four times with 10 ml of diethyl ether using a separating funnel, and the combined extracts were washed with 2 ml of water.
- The ether solution containing bile acids was then dried over sodium sulphate for over night.
- Sodium sulphate was then filtered off and washed once with 5 ml of ether. The ether was then evaporated on a steam bath.

- The residue was dissolved in 10 ml of acetone and from this pipette out an aliquot of 1ml solution into a centrifuge tube.
- The acetone was evaporated on the steam bath under a current of air. Five milliliters of 65% sulphuric acid was added to each tube and the samples were heated in a water bath at 60□C for 15 minutes.
- The samples were then cooled to room temperature and allowed to stand at this temperature for 15 minutes.
- At the end of this period absorption measurements were made at 320 nm (cholic acid) and 385 nm (desoxycholic acid) using UV-VIS Spectro Photometer.
- At all the determinations cholic acid and desoxycholic acid standards (0.10 mg in 5 ml of 65% sulphuric acid) were run.
- Standard curves of cholic acid and desoxycholic acid were drawn using 0.02, 0.04, 0.06, 0.08 and 0.10 mg of cholic acid and desoxy cholic acid in 5 ml of 65% sulphuric acid.
- The bile acid concentrations were calculated from the standard curve by noting the concentration that is corresponding to absorbance of each test sample from the standard curve.

3.8 Gross and Histopathology

A detailed post mortem examination of the rats sacrificed from each group was conducted. The gross pathological changes if any, were noted. Tissue pieces of liver and aorta were collected in 10% formalin, processed and stained with H & E stain as described by Singh and Sulochana (1997).

3.9 Statistical Analysis

The results were analyzed statistically using one-way ANOVA, followed by Dunnett's test, at $P < 0.05$ (SPSS 12.0,2004).

CHAPTER IV

Results

CHAPTER IV

RESULTS

4.1 Triton Induced Rat Model of Hyperlipidemia

4.1.1.1 Serum Total Cholesterol

Serum total cholesterol (mg/dl) levels in triton induced hyperlipidemic rat model are presented in table 6 and figure 1. It was evident that at "O" hour the cholesterol levels did not differ significantly ($P<0.05$) among various groups and the values ranged from 71.8±4.5 to 81.2±2.3.

At 18th hour the cholesterol levels in group I was 73.9±4.9, which was found to be significantly ($P<0.05$) elevated to 613.0±88.1, 474.5±62.7, 571.8±74.9, 474.3±67.2 and 208.7±13.1 in groups II through VI. However in treatment group VI the levels were significantly ($P<0.05$) lowered than that noticed in group II.

At 24th hour the cholesterol levels were found to be significantly ($P<0.05$) elevated in groups II through VI when compared to group I. However in treatment group VI the levels were significantly ($P<0.05$) decreased than that noticed in group II.

At 40th hour it was observed that the cholesterol levels in groups II through V were significantly ($P<0.05$) elevated compared to group I. In treatment groups IV, V and VI the levels were significantly ($P<0.05$) lowered than that noticed in group II. However the level was not decreased in group III when compared to group II.

4.1.1.2 Serum Triglycerides

Serum triglycerides (mg/dl) levels in triton induced hyperlipidemic rat model are presented in table 7 and figure 2. It was evident that at "O" hour the levels of triglycerides did not differ significantly ($P<0.05$) among various groups and the values ranged from 93.2±8.4 to 110.5±4.5.

At 18th hour the levels of triglycerides in group I was 86.7±4.9, which was found to be significantly ($P<0.05$) elevated to 1005.4±45.8, 1025.4±25.4, 904.4±57.2, 867.8±45.7 and 643.7±50.4 in groups II through VI. However in treatment group VI the levels were significantly ($P<0.05$) lowered than that noticed in group II.

By 24th hour the levels of triglycerides in group I was 92.8±2.9, which was found to be significantly ($P<0.05$) elevated in groups II through VI. In treatment group VI the levels were significantly ($P<0.05$) decreased than that noticed in group II.

At 40th hour it was observed that in groups II through V the triglycerides were significantly ($P<0.05$) elevated compared to group I. But in treatment groups IV, V and VI the levels were significantly ($P<0.05$) lowered than that noticed in group II. However the level was not decreased in group III when compared to group II.

Table: 6 Effect of *T. arjuna* leaf extract on serum total cholesterol (mg/dl; mean±_SE) levels in triton induced hyperlipidemic rat model

		Hours after triton administration			
S. No	Group	O	18	24	40
1.	I	71.8±4.5	73.9±4.9*	76.4±3.3*	73.6±3.4*
2.	II	75.4±5.1	613.0±88.1#	822.9±48.7#	219.1±21.3#
3.	III	77.6±3.4	474.5±62.7#	792.4±55.7#	144.2±15.2#
4.	IV	74.5±2.3	571.8±74.9#	665.1±79.8#	112.3±7.6#*
5.	V	81.2±2.3	474.3±67.2#	560.4±70.7#	98.8±4.9#*
6.	VI	72.8±2.3	208.7±13.1#*	395.8±27.5#*	107.2±8.8*

# $p < 0.05$ significant vs. group I

* $p < 0.05$ significant vs. group II

Table: 7 Effect of *T. arjuna* leaf extract on levels of serum triglycerides (mg/dl; mean± SE) in triton induced hyperlipidemic rat model

		Hours after triton administration			
S. No	Group	O	18	24	40
1.	I	93.2±8.4	86.7±4.9*	92.8±2.9*	89.0±5.2*
2.	II	96.6±5.7	1005.4±45.8#	1218.8±22.1#	485.2±43.9#
3.	III	103.9±3.5	1025.4±25.4#	1131.6±24.1#	339.2±22.8#
4.	IV	103.2±2.5	904.4±57.2#	961.8±68.3#	267.8±36.1#*
5.	V	110.5±4.5	867.8±45.7#	917.8±72.3#	204.1±6.7#*
6.	VI	101.4±6.8	643.7±50.4#*	726.4±43.6#*	148.6±20.0*

# $p < 0.05$ significant vs. group I

* $p < 0.05$ significant vs. group II

Figure : 1 Effect of *T. arjuna* leaf extract on serum cholesterol (mg/dl; mean± SE) levels in triton induced hyperlipidemic rat model

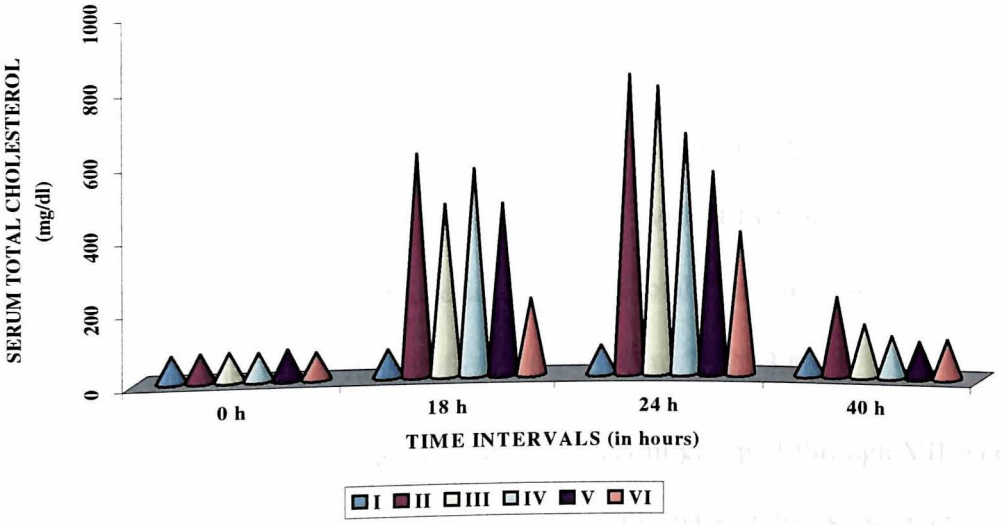
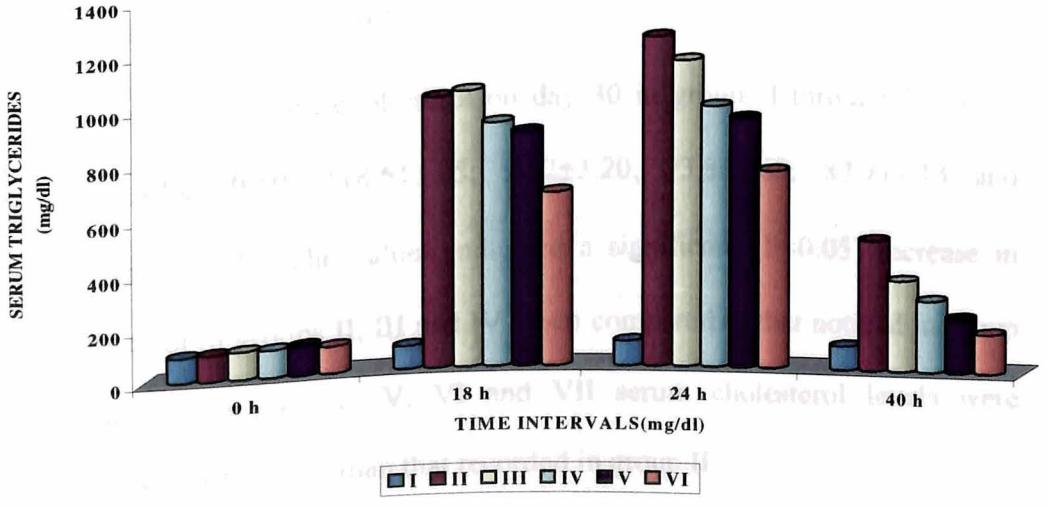


Figure : 2 Effect of *T. arjuna* leaf extract on levels of serum triglycerides (mg/dl; mean± SE) in triton induced hyperlipidemic rat model



4.2 Diet Induced Rat Model of Hyperlipidemia

4.2.1 Serum Lipid Profile

4.2.1.1 Serum Total Cholesterol

Serum total cholesterol (mg/dl) levels in diet induced hyperlipidemic rat model are presented in table 8 and figure 3. It was evident that on day "O" there was no significant ($P<0.05$) difference in the serum total cholesterol levels among various groups and the values ranged from 70.2±3.45 to 76.7± 3.66.

On day 15, the serum total cholesterol levels in groups I through VII were 75.1±5.49; 132.9 ±11.17; 113.0±5.88; 104.0±4.40; 94.5±3.29; 82.9±2.47 and 80.8±3.55 respectively. The values indicated a significant ($P<0.05$) increase in serum cholesterol in groups II, III and IV when compared with that of group I. In treatment groups VI and VII serum cholesterol levels were significantly ($P<0.05$) lower than that noticed in group II.

The levels of serum cholesterol on day 30 in groups I through VII were 73.4±3.89; 142.6±10.10; 118.6±3.25; 99.2±3.20; 93.8±1.69; 82.7±2.13 and 80.8±2.71 respectively. The values indicated a significant ($P<0.05$) increase in serum cholesterol in groups II, III and IV when compared to that noticed in group I. In treatment groups IV, V, VI and VII serum cholesterol levels were significantly ($P<0.05$) lower than that recorded in group II.

4.2.1.2 Serum Triglycerides

The levels of serum triglycerides (mg/dl) in diet induced hyperlipidemic rat model are presented in table 9 and figure 4. It was evident that on day "O" there was no significant ($P<0.05$) difference in levels of serum triglycerides among various groups and the values ranged from 95.2±3.29 to 102.1±5.55.

On day 15, the serum triglycerides in groups I through VII were 98.3±2.94; 192.0±10.21; 181.2±11.56; 145.9±4.83; 138.3±4.53; 142.0±7.18 and 132.1±7.67 respectively. The values indicated a significant ($P<0.05$) increase in serum triglycerides in groups II, III, IV and V when compared to that noticed in group I. In treatment groups VI and VII serum triglycerides were significantly ($P<0.05$) decreased when compared to group II.

The values of serum triglycerides on day 30 in groups I through VII were 102.1±6.99; 194.6±9.99; 152.6±10.15; 142.9±5.10; 137.2±8.03; 128.5±6.97 and 132.4±6.60 respectively. The values indicated a significant ($P<0.05$) increase in their levels in groups II and III when compared to that noticed in group I. In treatment group IV, V, VI and VII there was a significant ($P<0.05$) reduction in serum triglycerides compared to that recorded in group II.

Table: 8 Effect of *T. arjuna* leaf extract on serum total cholesterol (mg/dl; mean±SE) levels in diet induced hyperlipidemic rat model

S. No.	Group	Day 0	Day 15	Day 30
1.	I	76.7±3.66	75.1±5.49*	73.4±3.89*
2.	II	76.0±3.08	132.9±11.17#	142.6±10.10#
3.	III	74.0±3.68	113.0±5.88#	118.6±3.25#
4.	IV	70.2±3.45	104.0±4.40#	99.2±3.20# *
5.	V	72.5±3.52	94.5±3.29	93.8±1.69*
6.	VI	74.6±3.61	82.9±2.47*	82.7±2.13*
7.	VII	70.6±2.07	80.8±3.55*	80.8±2.71*

# $p < 0.05$ significant vs. group I

*$p < 0.05$ significant vs. group II

Table: 9 Effect of *T. arjuna* leaf extract on levels of serum triglycerides (mg/dl; mean±SE) in diet induced hyperlipidemic rat model

S.No.	Group	Day 0	Day 15	Day 30
1.	I	98.5±5.09	98.3 ±2.94*	102.1 ±6.99*
2.	II	102.1±5.55	192.0±10.21#	194.6±9.99#
3.	III	97.1±2.27	181.2±11.56#	152.6±10.15#
4.	IV	95.2±3.29	145.9±4.83#	142.9±5.10*
5.	V	96.5±3.68	138.3±4.53#	137.2±8.03*
6.	VI	99.2±6.47	142.0±7.18*	128.5±6.97*
7.	VII	99.2±5.19	132.1±7.67*	132.4±6.60*

# $p < 0.05$ significant vs. group I

* $p < 0.05$ significant vs. group II

Figure : 3 Effect of *T. arjuna* leaf extract on serum cholesterol (mg/dl; mean± SE) levels in diet induced hyperlipidemic rat model

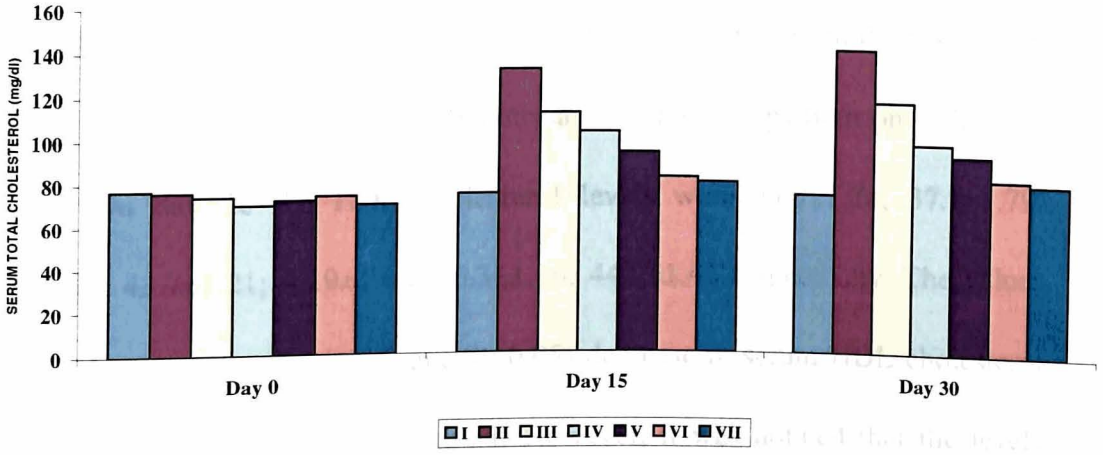
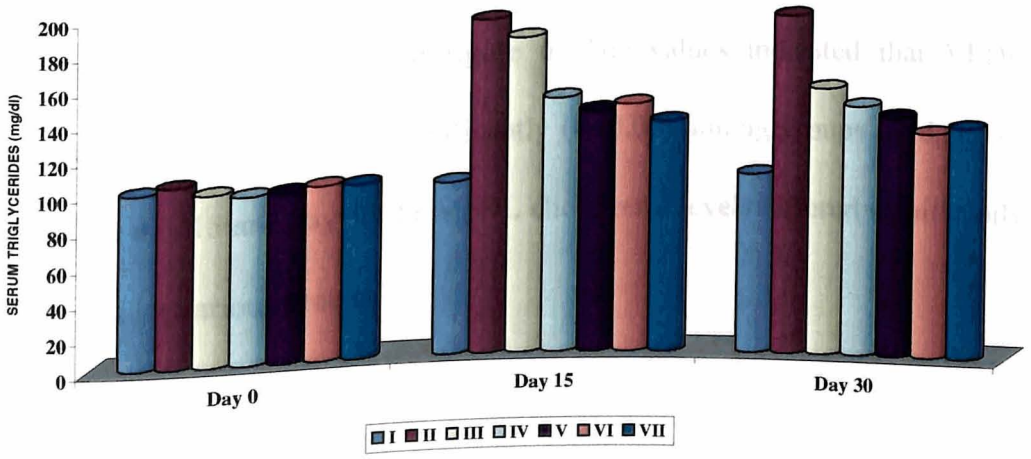


Figure: 4 Effect of *T. arjuna* leaf extract on levels of serum triglycerides (mg/dl; mean± SE) in diet induced hyperlipidemic rat model



4.2.1.3 Serum HDL Cholesterol

Serum HDL cholesterol (mg/dl) levels in diet induced hyperlipidemic rat model are presented in table 10 and figure 5. It was observed from the results that the HDL levels did not differ significantly among the groups both on 0 day and day 15. On day 30 the HDL cholesterol levels were 44.9±1.24; 37.9±1.79; 42.8±1.56; 43.7±1.21; 42.9±1.86; 46.3±1.86; 44.9±1.42 respectively. The values indicate that there was a significant ($P<0.05$) decrease in serum HDL cholesterol in group II when compared to group I. However, it was noticed that the levels were significantly ($P<0.05$) increased in group VI when compared to group II.

4.2.1.4 Serum VLDL Cholesterol

Serum VLDL cholesterol (mg/dl) levels in diet induced hyperlipidemic rat model are given in table 11 and figure 6. The values indicated that VLDL cholesterol levels did not differ significantly ($P<0.05$) among groups on day "0". However, on day 15 and day 30 the VLDL cholesterol levels differed significantly ($P<0.05$) among various groups.

On day 15, the serum VLDL cholesterol level in group I was 19.6±0.58, which was significantly ($P<0.05$) elevated to 38.4 ± .04; 36.2 ± 2.31; 29.2±1.36and 27.0±0.90 in groups II, III, IV and V respectively. However there was a significant ($P<0.05$) reduction in levels in groups VI and VII when compared to that of group II.

On day 30, the serum VLDL cholesterol level in group I was 20.4±1.39, which was significantly elevated to 38.9±1.99 and 30.5±2.03 in groups II and III respectively. However there was a significant ($P<0.05$) reduction in levels in treatment groups IV, V, VI and VII when compared to that of group II.

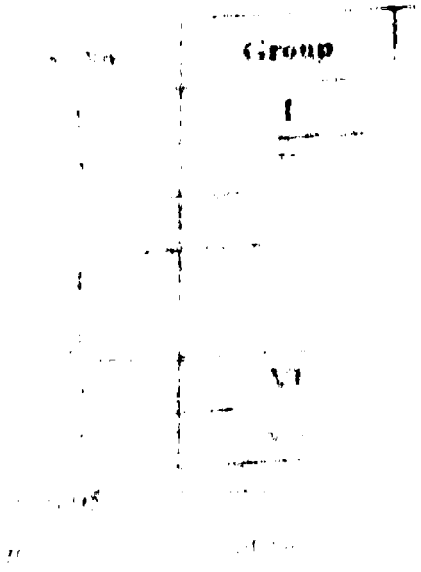


Table: 10 Effect of *T. arjuna* leaf extract on serum HDL cholesterol (mg/dl; mean±SE) levels in diet induced hyperlipidemic rat model

S. No.	Group	Day 0	Day 15	Day 30
1.	I	43.7±1.64	42.71±1.00	44.9±1.24*
2.	II	42.7±2.16	39.4±1.44	37.9±1.79#
3.	III	41.9±1.00	43.2±0.85	42.8±1.56
4.	IV	39.4±1.97	42.5±1.58	43.7±1.21
5.	V	40.2±2.68	41.8±1.82	42.9±1.86
6.	VI	43.9±1.79	45.1±2.87*	46.3±1.86*
7.	VII	40.4±1.91	43.7±1.94	44.9±1.92

# $p < 0.05$ significant vs. group I

* $p < 0.05$ significant vs. group II

Table: 11 Effect of *T. arjuna* leaf extract on serum VLDL cholesterol (mg/dl; mean±SE) levels in diet induced hyperlipidemic rat model

S. No.	Group	Day 0	Day 15	Day 30
1.	I	19.7±1.01	19.6 ±0.58*	20.4±1.39 *
2.	II	20.4±1.11	38.4±2.04#	38.9±1.99#
3.	III	19.4±0.45	36.2±2.31#	30.5±2.03#
4.	IV	19.0±0.65	29.2±1.36#	28.6±1.02*
5.	V	19.3±0.73	27.0±0.90#	27.4±1.60*
6.	VI	19.8±1.29	28.4±1.43*	25.7±2.19*
7.	VII	19.8±1.03	26.4±1.53*	26.4±1.32*

# $p < 0.05$ significant vs. group I

* $p < 0.05$ significant vs. group II

Figure: 5 Effect of *T. arjuna* leaf extract on serum HDL cholesterol (mg/dl; mean± SE) levels in diet induced hyperlipidemic rat model

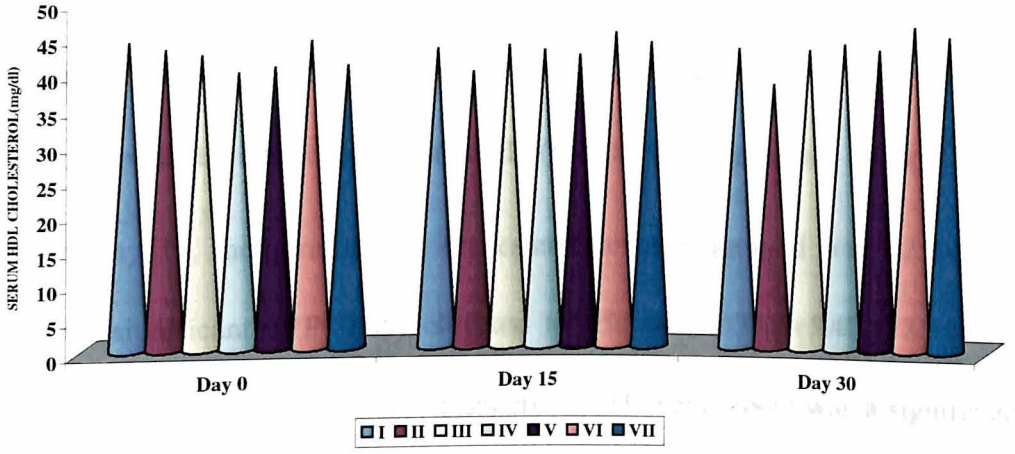
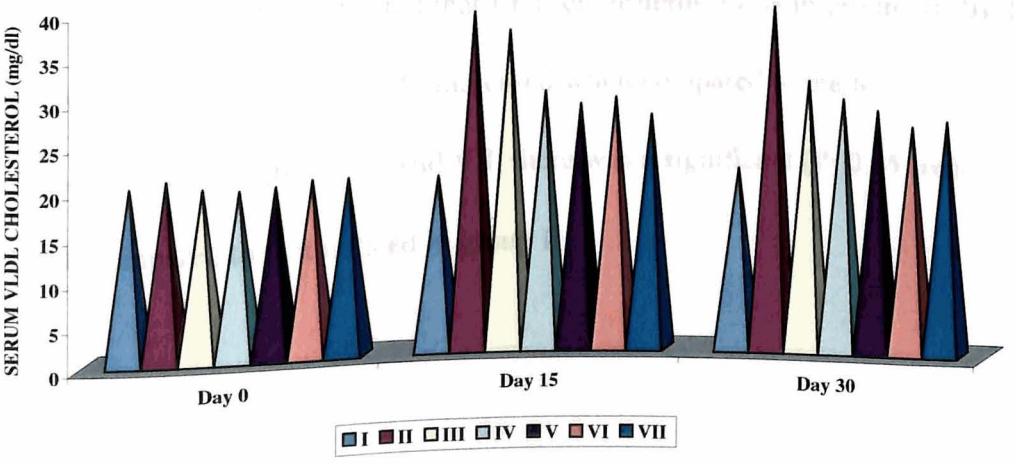


Figure: 6 Effect of T. arjuna leaf extract on serum VLDL cholesterol (mg/dl; mean± SE) levels in diet induced hyperlipidemic rat model



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4.2.1.5 Serum LDL Cholesterol

Serum LDL cholesterol (mg/dl) levels in diet induced hyperlipidemic rat model are tabulated in table 12 and figure 7. The values indicated that LDL cholesterol levels did not differ significantly ($P<0.05$) among groups on day 0.

On day 15, the serum LDL cholesterol level in group I was 12.7±5.20, which was significantly ($P<0.05$) elevated to 55.1±4.17, 33.5±5.40, 32.3±5.64, 25.0±3.25 in group II, III, IV and V respectively. However there was a significant ($P<0.05$) reduction in levels in groups VI and VII when compared to that of group II.

On day 30 the LDL cholesterol levels in groups I through VII were 12.1±1.85; 65.7±9.63; 45.2±3.42; 26.9±2.85; 23.4±1.96; 10.7±1.32 and 9.4±1.74 respectively. The values indicated that LDL cholesterol levels in groups II, III, IV and V were significantly ($P<0.05$) increased when compared to the levels in group I. While in treatment groups VI and VII, there was a significant ($P<0.05$) reduction in levels compared to that noticed in group II.

4.2.1.6 Serum Phospholipids

Serum phospholipids (mg/dl) levels in diet induced hyperlipidemic rat model are given in table 13 and figure 8. The values indicated that the levels of phospholipids did not differ significantly ($P<0.05$) among groups on day 0.

On day 15, the levels of phospholipids in group I was 92.1±2.72 while it was significantly elevated to 207.1±6.86; 193.5±8.58, 185.4±2.69; 175.7±8.27; 181.8± 4.37 and 123.4±4.26 in groups II through VII respectively. In treatment group VII there was a significant ($P<0.05$) reduction in levels when compared to group II. On day 30, the levels of phospholipides in group I was 91.4±2.13 while it was significantly elevated to 216.9±9.52; 185.1±9.96; 157.6±6.61; 125.9±5.89; 145.9±8.23 and 121.1±4.55 in groups II through VII respectively. In treatment groups IV, V, VI and VII there was a significant ($P<0.05$) reduction in levels when compared to that noticed in group II.

Table: 12 Effect of *T. arjuna* leaf extract on serum LDL cholesterol in (mg/dl; mean±SE) levels in diet induced hyperlipidemic rat model

S. No.	Group	Day 0	Day 15	Day 30
1.	I	13.3±1.82	12.7±5.20	12.1±1.85*
2.	II	12.9±1.74	55.1± 4.17#	65.7±9.63#
3.	III	12.6±2.86	33.5±5.40#	45.2±3.42#
4.	IV	11.7±2.27	32.3±5.64#	26.9±2.85#
5.	V	13.0±2.04	25.0±3.25#	23.4±1.96#
6.	VI	10.8±3.84	9.4±3.75*	10.7±1.32*
7.	VII	10.3±1.31	10.6±2.65*	9.4±1.74*

# $p < 0.05$ significant vs. group I

* $p < 0.05$ significant vs. group II

Table: 13 Effect of *T. arjuna* leaf extract on serum phospholipids (mg/dl; mean±SE) levels in diet induced hyperlipidemic rat model

S. No.	Group	Day 0	Day 15	Day 30
1.	I	91.5±1.97	92.1 ± 2.72*	91.4 ± 2.13*
2.	II	92.8±2.55	207.1± 6.86#	216.9± 9.52#
3.	III	85.5±2.15	193.5± 8.58#	185.1± 9.96#
4.	IV	86.4±2.45	185.4± 2.69#	157.6± 6.61#*
5.	V	87.4±2.47	175.7± 8.27#	125.9± 5.89#*
6.	VI	85.6±1.88	181.8± 4.37#	145.9± 8.23#*
7.	VII	91.2±2.04	123.4± 4.26#*	121.1± 4.55#*

# $p< 0.05$ significant vs. group I

* $p < 0.05$ significant vs. group II

Figure : 7 Effect of *T. arjuna* leaf extract on serum LDL cholesterol in (mg/dl; mean± SE) levels in diet induced hyperlipidemic rat model

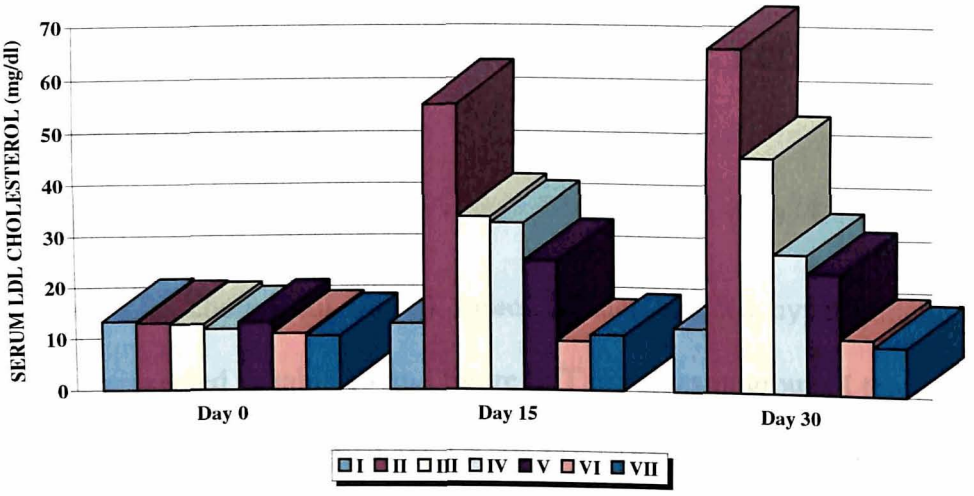
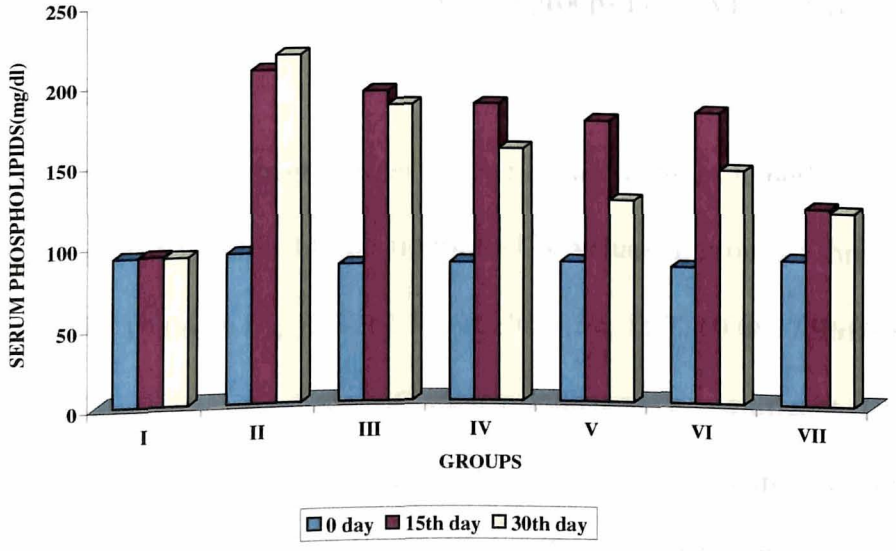


Figure: 8 Effect of *T. arjuna* leaf extract on serum phospholipids (mg/dl; mean±SE) levels in diet induced hyperlipidemic rat model



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4.2.2 Liver Lipid Profile

Lipid profile of liver tissue in diet induced hyperlipidemic rat model was estimated by assessing total cholesterol, triglycerides, HDL, VLDL, LDL, phospholipids (mg/g) content.

Liver Total Cholesterol

Liver total cholesterol (mg/g) values in diet induced hyperlipidemic rat model were presented in table 14 and figure 9. The values in groups I through VII are 5.52±0.38; 9.85±0.83; 8.38±0.44; 7.27±0.31; 7.16±0.45; 6.78±0.38; 6.59±0.39 respectively. Analysis of the values revealed that the liver total cholesterol level was significantly ($P<0.05$) increased only in group II when compared to group I. But when the values were compared against group II, it was observed that there was a significant ($P<0.05$) reduction in levels in groups IV, V, VI and VII.

Liver Triglycerides

The values of liver triglycerides (mg/g) in diet induced hyperlipidemic rat model are presented in table 14 and figure 9. The values in groups I through VII were 7.31±0.79; 17.06±1.16; 12.32±1.14; 10.74±0.59; 11.77±0.68; 7.96±0.34 and 7.50±0.73 respectively. Analysis of values indicated a significant ($P<0.05$) increase in liver triglycerides in group II when compared to group I. In treatment groups IV, V, VI and VII there was significant ($P<0.05$) decrease in levels when compared to group II.

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Liver HDL Cholesterol

Liver HDL cholesterol (mg/g) values in diet induced hyperlipidemic rat model are presented in table 14 and figure 9. The values in groups I through VII were 2.98±0.20; 3.07±0.64; 3.67±0.31; 3.08±0.21; 3.31±0.29; 3.98±0.18 and 4.05±0.3 respectively. The values revealed that HDL cholesterol did not significantly ($P<0.05$) altered in group II when compared to group I. But when the values were compared against group II, it was observed that, the increase in groups VI and VII was significant ($P<0.05$).

Liver VLDL Cholesterol

Liver VLDL cholesterol (mg/g) values in diet induced hyperlipidemic rat model are presented in table 14 and figure 9. Liver VLDL cholesterol values in groups I through VII were 1.46±0.15; 3.41±0.23; 2.46±0.22; 2.14±0.11; 2.35±0.13; 1.59±0.06 and 1.50±0.14 respectively. The values indicated a significant ($P<0.05$) increase in group II when compared to group I. However in treatment groups IV, V, VI and VII there was significant ($P<0.05$) decrease in liver VLDL cholesterol when compared to group II.

Liver LDL Cholesterol

Liver LDL cholesterol (mg/g) values in diet induced hyperlipidemic rat model are presented in table 14 and figure 9. The values in groups I through VII were 1.08±0.51; 3.37±0.54; 2.25±0.66; 2.05±1.01; 1.50±0.59; 1.21±0.17and 1.04±0.09 respectively. The values revealed that there was a significant ($P<0.05$)

raise in LDL cholesterol value in group II when compared to group I. However, in treatment groups VI and VII there was significant ($P<0.05$) decrease in liver LDL cholesterol when compared to group II.

Liver Phospholipids

Liver phospholipids (mg/g) values in diet induced hyperlipidemic rat model are presented in table 14 and figure 9.The values in groups I through VII were 6.70±0.33; 9.26±0.43; 7.27±0.25; 7.19±0.30; 7.03±0.29; 7.12±0.36 and 6.76±0.27 respectively .The values indicated a significant ($P<0.05$) increase in group II when compared to group I. However in treatment groups IV, V, VI and VII there is significant ($P<0.05$) decrease in liver phospholipids when compared to group II.

4.2.3 Faecal Bile Acids

Faecal cholic acid (µg/g) and desoxycholic acid (µg/g) concentration in diet induced hyperlipidemic rat model are presented in table 15 and figure 10.

4.2.3.1 Faecal Cholic Acid

Faecal cholic acid (µg/g) values in diet induced hyperlipidemic rat model are presented in table 15 and figure 10. The faecal cholic acid values in groups I through VII were 79.1±3.76; 56.3±2.85; 59.5±1.83; 65.7±2.73; 68.8±3.59; 79.3±1.58 and 81.2±1.60 respectively. The values indicated a significant ($P<0.05$) decrease in groups II and III when compared to group I. However in treatment groups VI and VII there was a significant ($P<0.05$) increase in fecal cholic acid levels when compared to group II.

Table: 14 Effect of *T. arjuna* leaf extract on liver lipid profile (mg/g; mean ± SE) in diet induced hyperlipidemic rat model

S. No	Group	CHO	TGL	HDL	VLDL	LDL	PHO
1	I	5.52±0.38*	7.31±0.79*	2.98±0.20*	1.46±0.15*	1.08±0.51*	6.70±0.33*
2	II	9.85±0.83#	17.06±1.16#	3.07±0.64	3.41±0.23#	3.37±0.54#	9.26±0.43#
3	III	8.38±0.44	12.32±1.14	3.67±0.31	2.46±0.22	2.25±0.66	7.27±0.25`
4	IV	7.27±0.31*	10.74±0.59*	3.08±0.21	2.14±0.11*	2.05±1.01	7.19±0.30*
5	V	7.16±0.45*	11.77±0.68*	3.31±0.29	2.35±0.13*	1.50±0.59	7.03±0.29*
6	VI	6.78±0.38*	7.96±0.34*	3.98±0.18*	1.59±0.06*	1.21±0.17*	7.12±0.36*
7	VII	6.59±0.39*	7.50±0.73*	4.05±0.3*	1.50±0.14*	1.04±0.09*	6.76±0.27*

# $p < 0.05$ significant vs. group I
* $p < 0.05$ significant vs. group II

Abbreviations: CHO: Total cholesterol; TGL: Triglycerides; HDL: HDL cholesterol; VLDL: VLDL cholesterol; LDL: LDL cholesterol; PHO: Phospholipids.

4.2.3.2 Faecal Desoxycholic Acid

Faecal desoxycholic acid (µg/g) values in diet induced hyperlipidemic rat model were presented in table 15 and figure 10. The values in groups I through VII were 67.8±2.30; 49.6±2.95; 48.1±2.56; 51.0±2.53; 56.1±2.67; 64.1±1.48 and 63.5±1.98 respectively. The values indicated a significant ($P<0.05$) decrease in groups II, III and IV compared to group I. However in treatment groups VI and VII there was a significant ($P<0.05$) increase in fecal desoxycholic acid when compared to group II.

Table: 15 Effect of *T. arjuna* leaf extract on faecal cholic acid (µg/g) and desoxy cholic acid (µg/g) in diet induced hyperlipidemic rat model

S.No	Group	Cholic acid (µg/g) (mean± SE)	Desoxy cholic acid (µg/g) (mean± SE)
1	I	79.1±3.76	67.8±2.30*
2	II	56.3±2.85#	49.6±2.95#
3	III	59.5±1.83#	48.1±2.56#
4	IV	65.7±2.73	51.0±2.53#
5	V	68.8±3.59	56.1±2.67
6	VI	79.3±1.58*	64.1±1.48*
7	VII	81.2±1.60*	63.5±1.98*

# $p< 0.05$ significant vs. group I

* $p < 0.05$ significant vs. group II

Figure : 9 Effect of *T. arjuna* leaf extract on liver lipid profile (mg/g; mean±SE) in diet induced hyperlipidemic rat model

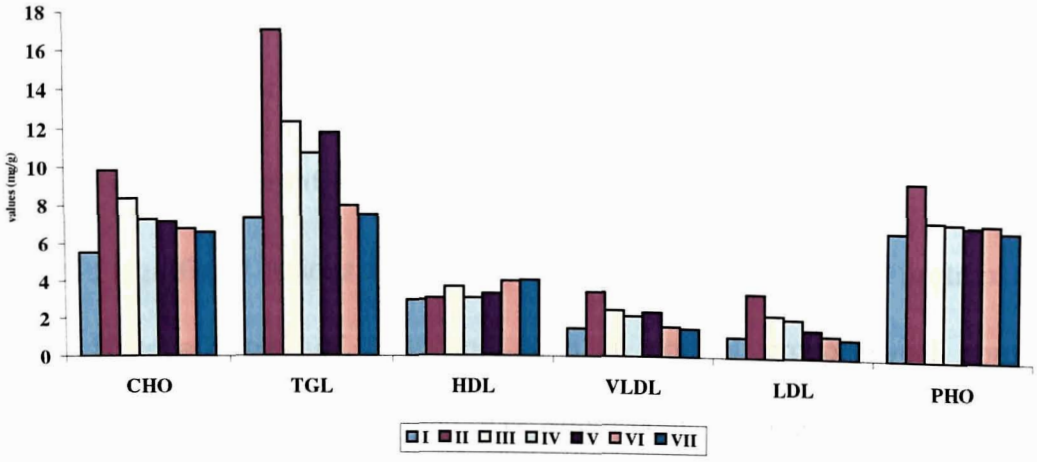
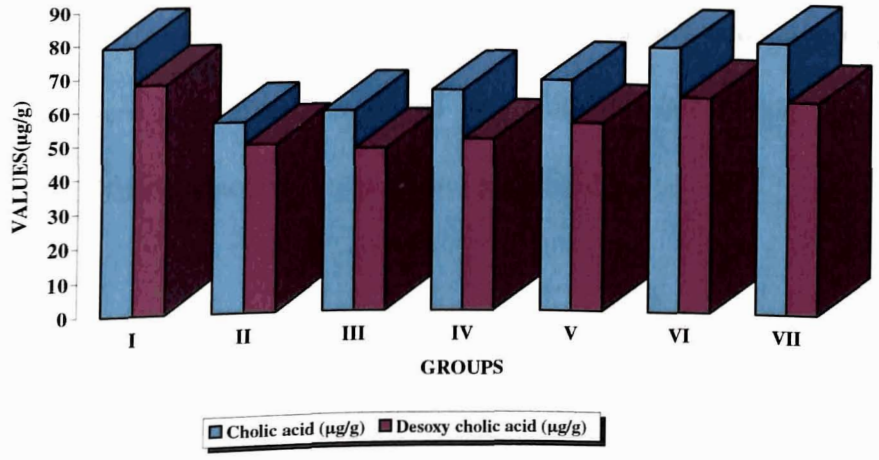


Figure: 10 Effect of *T. arjuna* leaf extract on faecal cholic acid (µg/g) and desoxy cholic acid (µg/g) in diet induced hyperlipidemic rat model



4.2.4 Clinical Signs

All the animals were in good health throughout the experimental period and average body weight of the animals did not change significantly during the study.

4.2.5 Gross and Histopathology

No significant lesions were observed grossly in the livers and aorta of all groups. Histopathology of liver and aorta in control group reveal normal histological picture (Plates 3 and 4). Microscopic lesions in the liver of hyperlipidemic diet alone fed group included mild congestion, degenerative changes in hepatic cells, moderate proliferation of bile ducts and focal infiltration of inflammatory cells (Plate 5). The aorta of this group revealed the presence of mild tunica intimal degeneration and plaque formation (Plate 6). Following treatment with *T. arjuna* leaf extract, *T. arjuna* bark extract and gugulipid the above changes were substantially reduced and could be due to hypolipidemic effect of leaf and bark extract of *T. arjuna* and gugulipid.

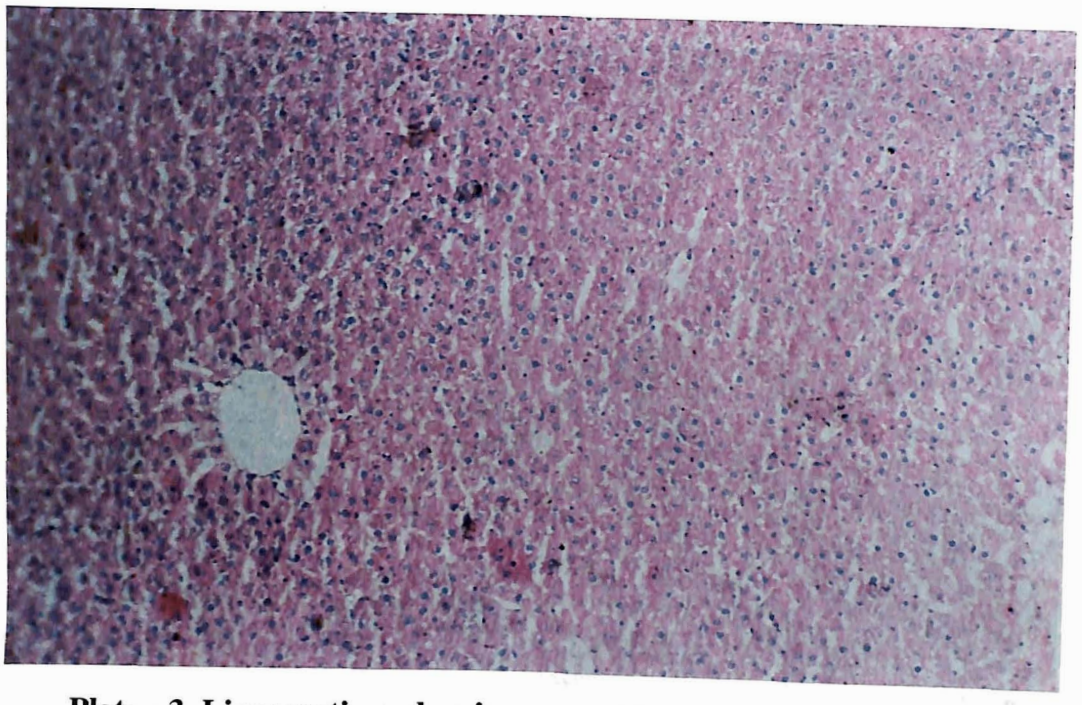


Plate : 3 Liver section- showing normal histological picture (10×7)

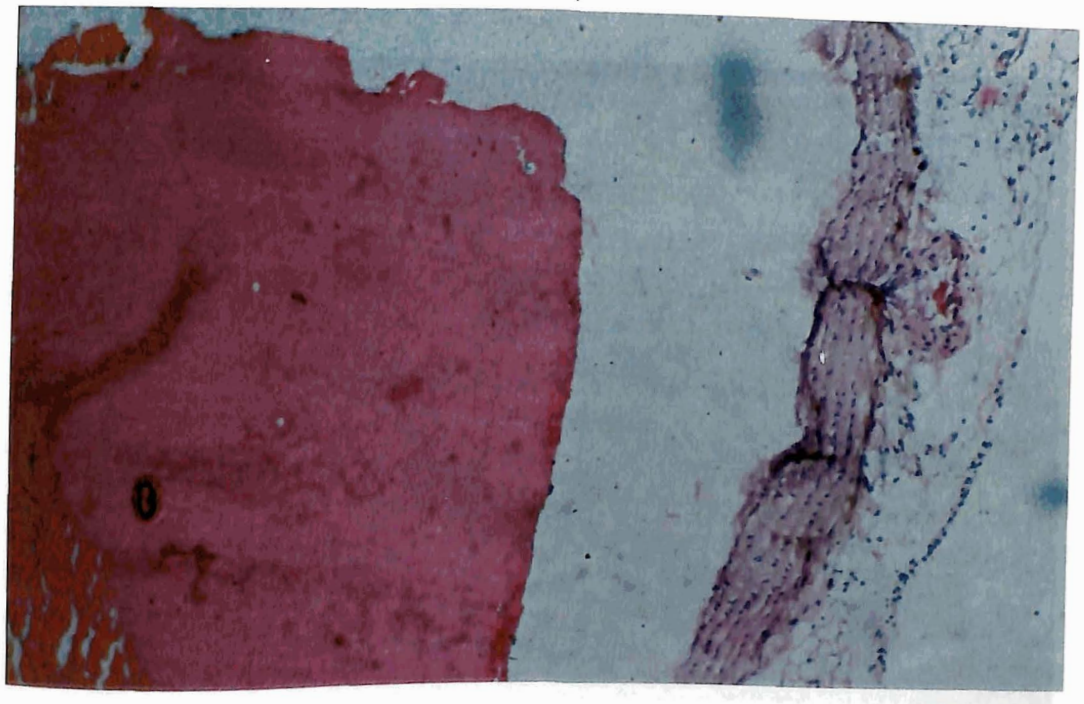


Plate : 4 Aorta section – showing normal histological picture (10×7)

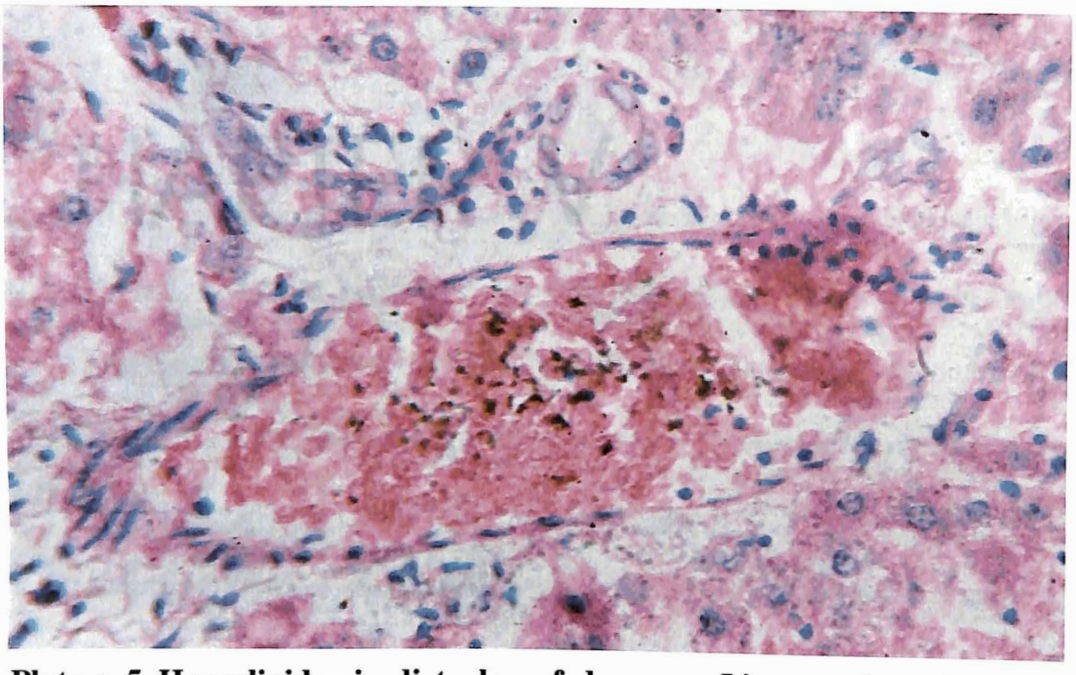


Plate : 5 Hyperlipidemic diet alone fed group- Liver section- showing moderate proliferation of bile ducts, congestion, degenerative changes in hepatic cells (40×7).

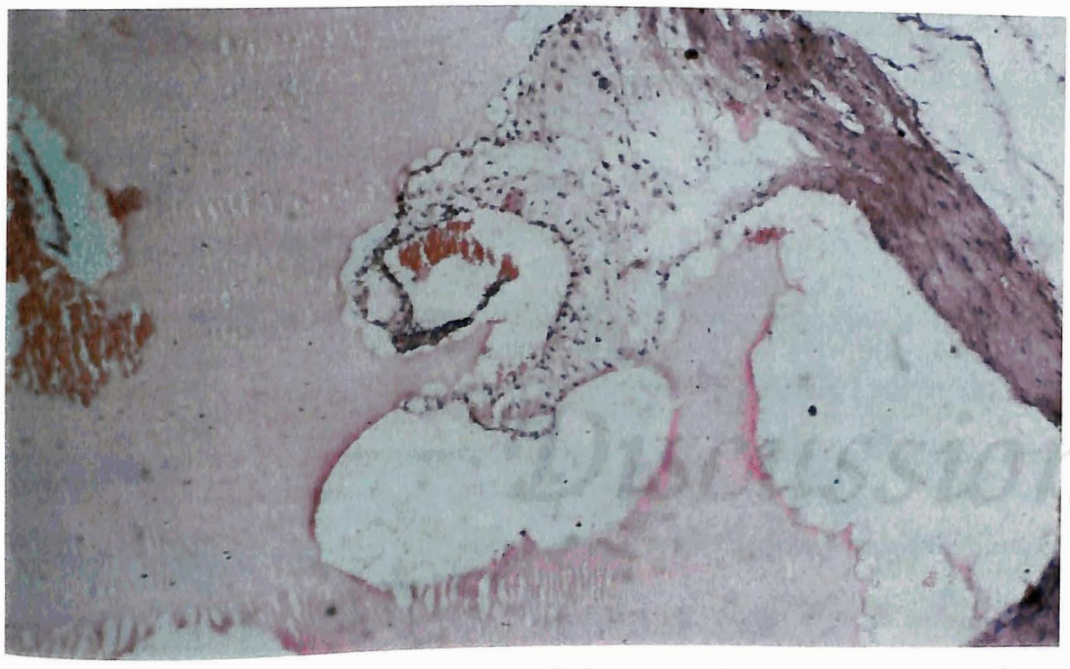


Plate : 6 Hyperlipidemic diet alone fed group- Aorta section- showing Tunica intimal degeneration and plaque formation (10×7).

CHAPTER V

Discussion

CHAPTER V

DISCUSSION

Many of the currently available hypolipidemic /anti-hyperlipidemic drugs are neither fully effective, nor totally free from side effects. Hence there is a need to probe for new hypolipidemic agents. Plant kingdom is a rich natural source for many therapeutic molecules. Though many reports are available on the efficiency of *T. arjuna* bark extract in hyperlipidemic states, no such reports are available on the efficiency of leaves of the same plant. It was reported that the leaves of *T. chebula*, another species of Terminalia genus possess hypolipidemic activity (Khanna *et al.*, 1993). Compared to bark, collection of leaves is easy and are available in abundance. More over drying of leaves is easy and complete, which facilitates their storage. It is known that any moisture content in the stored material will favor mould growth hampering the quality.

Keeping this in view, the present study was designed to screen the hypolipidemic potential of *T. arjuna* leaves. The study was conducted in two hyperlipidemic rat models viz. triton induced and diet induced rat models of hyperlipidemia.

The rat model was selected because in this species conjugation of bile acids occurs preferentially with taurine and to a lesser extent with glycine as in human beings, where as in rabbits conjugation of bile acids occurs exclusively with

glycine (Huxtable, 1986). The hypolipidemic/ anti-hyperlipidemic potential of the methanolic extract of *T. arjuna* leaves was evaluated against the standard drugs gemfibrozil in triton induced model and gugulipid in diet induced model.

5.1 Triton Induced Rat Model of Hyperlipidemia

Triton administration resulted in significantly elevated levels of serum total cholesterol and triglycerides at 18, 24 and 40 hours post administration. Significant elevation in levels of serum total cholesterol and triglycerides in triton treated rats was also reported earlier by Gandhi and Mulky (1993), Khanna *et al.* (2002) Pedrosa *et al.* (2002), and Seok *et al.* (2004). Paoletti (1962) suggested the use of triton WR-1339 Isooctyl polyoxyethylene phenol) induced hyperlipidemia as an approach to screen the action of hypolipidemic drugs.

It was suggested that the hypercholesterolemia occurring after injection of triton was due to the latter's ability to retain excess triglycerides and phospholipids which then in turn mobilizes and sequestrates cholesterol from the extra hepatic and hepatic sources (Friedman and Byers, 1957). Catanozi *et al,* (2001) reported that triton WR - 1339 blocked the removal of triglycerides from plasma.

Gemfibrozil @ 250 mg/kg was used as a standard hypolipidemic drug for comparison. Chander *et al.* (1996) and Khanna *et al.* (2002) also used gemfibrozil as a standard drug in a similar type of study.

In the present study there was a significant ($P<0.05$) reduction in the levels of cholesterol and triglycerides at 18, 24 and 40 hours after triton administration in

the group treated with triton and gemfibrozil, when compared with triton alone treated group, indicating that gemfibrozil effectively antagonized the triton induced hyperlipidemia.This effect of gemfibrozil on serum levels of cholesterol and triglycerides was in accordance with the earlier reports (Chander *et al.* 1996; Khanna *et al.* 2002).

Treatment of triton injected rats with *T. arjuna* leaf extract @ 250 and 500 mg/kg had no significant effect at 18^{th} and 24^{th} hour. However at 40^{th} hour, a significant ($P<0.05$) reduction in serum cholesterol and triglycerides were recorded. Thus it was evident that gemfibrozil is effective in countering triton induced hyperlipidemic changes in serum from 18^{th} hour it self while the *T. arjuna* leaf extract could produce the same only at 40^{th} hour.

Since there are no published reports on the hypolipidemic activity of *T. arjuna* leaf extract in triton induced hyperlipidemic rat model, it was not possible to compare the results of present study with previous works.

5.2 Diet Induced Rat Model of Hyperlipidemia

A reasonable assessment of a new hypolipidemic agent cannot be made merely by measurement of cholesterol and triglycerides levels and it is of great importance to secure information about the effect of the drug on individual lipoproteins like high density lipo protein (HDL), low density lipoprotein (LDL) and very low density lipoprotein (VLDL) cholesterol levels (Miller *et al.* 1977; Rossner *et al.*, 1978).

Hence in diet induced hyperlipidemic rat model, detailed lipid profiles of serum and liver tissue were monitored besides the estimation of cholic acid and desoxycholic acid in feces. Lipid profile was assessed by estimating total cholesterol, triglycerides, HDL, VLDL, LDL cholesterol and phospholipids in both serum and liver tissue. In serum the above estimations were carried out on days 0, 15 and 30, while they were estimated only on day 30 in liver tissues that were collected at necropsy. The results obtained in all the drug treated groups were compared with the hyperlipidemic diet alone fed group.

5.2.1 Serum Lipid Profile

In the present study it was observed that the serum levels of total cholesterol, triglycerides, VLDL, LDL cholesterol and phospholipids were significantly ($P<0.05$) elevated in rats that received cholesterol and cholic acid suspended in groundnut oil (Arichi *et al.* 1982).

Cholesterol feeding has been often used to elevate serum or tissue cholesterol levels in studies designed to assess hypercholesterolemia related metabolic disturbances in different animal models (Holmgren and Brown; 1993; Hozumi *et al.* 1995). Watts *et al.* (1994) reported that a high level of saturated fat in addition to cholesterol is required in the rat model.

Induction of hyperlipidemia in cholesterol and fat fed rats occur as a result of alteration in several aspects of lipoprotein metabolism. Kris-Etherton and Cooper (1980) observed that in cholesterol and fat administered rats the disappearance rate

of chylomicron remnants was markedly prolonged. They also noted that the delay in removal was due to an increase in the circulatory remnant concentration without a removal defect of hepatocytes. In addition the abnormal cholesterol content of the lipoprotein particles of d < 1.006 g/ml was particularly accounted for the hepatic secretion of a triglyceride poor, cholesterol rich VLDL.

Rats that received the leaf extract @ 125, 250 and 500 mg/kg dose level along with hyperlipidemic diet also exhibited a significant ($P<0.05$) increase in serum cholesterol levels on day 15. However on day 30, the cholesterol level were significantly ($P<0.05$) lowered in rats that received leaf extract @ 250 and 500 mg/kg dose level when compared to rats that received only hyperlipidemic diet. Where as in rats that received the *T. arjuna* bark @ 125 mg/kg and gugulipid @ 10 mg/kg, it was observed that the cholesterol levels were significantly ($P<0.05$) lowered both on day 15 and day 30.

Serum triglycerides estimation also revealed a similar trend. The three doses of *T. arjuna* leaf extract could not significantly ($P<0.05$) lower the elevated triglycerides on day 15, while the two higher doses could significantly ($P<0.05$) lower the same on day 30. As seen with cholesterol, triglycerides levels were also significantly ($P<0.05$) lowered by bark and gugulipid on day 15 and day 30.

Serum HDL cholesterol estimation revealed that the HDL levels did not differ significantly ($P<0.05$) among the groups on day 15. However, on day 30 the HDL levels were significantly ($P<0.05$) lowered in rats that were maintained only

on hyperlipidemic diet. But no such lowered HDL levels were observed in leaf extract treated rats and the levels were on par with that observed in rats that received normal diet. In rats that received *T. arjuna* bark extract @ 125 mg/kg dose level, a significant ($P<0.05$) increase in HDL levels were noticed on day 15 and day 30 when compared to rats that received only hyperlipidemic diet.

Rats that received the leaf extract at all three dose levels along with hyperlipidemic diet exhibited significantly ($P<0.05$) elevated serum VLDL cholesterol on day 15. However on day 30 the cholesterol levels were significantly ($P<0.05$) lowered in rats that received leaf extract @ 250 and 500 mg/kg dose level when compared to rats on hyperlipidemic diet alone. However the bark and gugulipid significantly ($P<0.05$) lowered the VLDL levels both on day 15 and day 30 when compared to hyperlipidemic diet alone received rats.

In rats that received the leaf extract, it was observed that LDL cholesterol levels were significantly ($P<0.05$) increased on day 15 and day 30. However LDL levels were significantly ($P<0.05$) lowered by bark and gugulipid on day 15 and day 30 when compared to hyperlipidemic diet alone received rats.

Rats that received the leaf extract at all three dose levels along with hyperlipidemic diet exhibited significantly ($P<0.05$) elevated levels of serum phospholipids on day 15. However on day 30 the levels were significantly ($P<0.05$) lowered in rats that received leaf extract @ 250 and 500 mg/kg dose level when compared to rats that received only hyperlipidemic diet. In rats that

received the *T. arjuna* bark a significant ($P<0.05$) reduction was noticed on day 30, when compared to hyperlipidemic diet alone received rats. However, the phospholipids levels were significantly ($P<0.05$) lowered by gugulipid on day 15 and 30 when compared to hyperlipidemic diet alone received rats.

It was evident from the above that in rats that received the methanolic extract of *T. arjuna* leaves @ 125, 250 and 500 mg/kg dose level along with hyperlipidemic diet had significantly ($P<0.05$) elevated levels of serum total cholesterol, triglycerides, LDL, VLDL cholesterol and phospholipids on day 15. However on day 30 the total cholesterol, triglycerides, VLDL cholesterol and phospholipids levels were significantly ($P<0.05$) lowered in rats that received leaf extract @ 250 and 500 mg/kg, while their levels were still elevated in rats treated with 125 mg/kg dose level. It was also observed that leaf extract could not lower the LDL cholesterol at any dose level during the entire study period. While administration of hyperlipidemic diet also resulted in significantly ($P<0.05$) lowered HDL levels, leaf extract co-administration prevented such lowering. co-administration of bark extract significantly increased the HDL levels.

Thus it can be stated that leaf extract could neutralize the effect of hyperlipidemic diet administration at 250 and 500 mg/kg dose level. However this was noticed only on day 30, while no such effect was seen on day 15. But such neutralization effect was seen with bark extract at 125 mg/kg dose level by day 15 itself and this is comparable with the protective effect seen with gugulipid in the study.

The hypolipidemic effect of *T. arjuna* bark in hyperlipidemic rat models was also reported earlier by Khanna *et al.* (1996), Shaila *et al.* (2000). Similar effects were also reported in rabbit models by Tiwari *et al.* (1989), Ram *et al.* (1997) and Shaila *et al.* (1997).

5.2.2 Liver Lipid Profile

The levels of liver total cholesterol, triglycerides, VLDL, LDL cholesterol and phospholipids were significantly ($P<0.05$) elevated in rats that received hyperlipidemic diet alone. It was evident that liver total cholesterol, triglycerides, VLDL cholesterol and phospholipids levels were significantly ($P<0.05$) lowered in rats that received leaf extract @ 250 and 500 mg/kg, while their levels were still elevated in rats treated with 125 mg/kg dose level. It was also observed that leaf extract could not lower the liver LDL cholesterol at any dose level.

Administration of hyperlipidemic diet resulted in no change in HDL levels, while leaf extract co-administration caused moderate increase in HDL levels. Thus it can be stated that leaf extract could neutralize the effect of hyperlipidemic diet administration at 250 and 500 mg/kg dose level.

In rats that received *T. arjuna* bark @ 125 mg/kg and gugulipid @ 10 mg/kg, a significant ($P<0.05$) decrease in liver levels of total cholesterol, triglycerides, VLDL, LDL cholesterol and phospholipids levels were noticed. A significant ($P<0.05$) increase in HDL cholesterol level was also noticed in bark and gugulipid received rats. The hypolipidemic effect of *T. arjuna* bark extract was comparable with the protective effect seen with gugulipid in the study.

5.2.3 Fecal Bile Acids

Fecal cholic acid and desoxycholic acid values in diet induced hyperlipidemic rat model indicated a significant ($P<0.05$) decrease in their levels in rats receiving hyperlipidemic diet alone when compared to control rats. Rats that received *T. arjuna* bark @ 125 mg/kg and gugulipid @ 10 mg/kg, indicated a significant ($P<0.05$) increase in fecal cholic acid and desoxycholic acid levels when compared to their levels in rats receiving hyperlipidemic diet alone. In rats that received *T. arjuna* leaf extract also an increased fecal cholic acid and desoxycholic acid were observed, which were statistically not significant.

Increase in fecal bile acid excretion in rats that received *T. arjuna* bark @ 125 mg/kg and gugulipid @ 10 mg/kg was in agreement with previous studies reported by Khanna *et al.* (1996) and Chander *et al.* (1996) respectively. They opined that *T. arjuna* bark extract and guggulsterones interfered with the enterohepatic circulation of bile acids and reduce the absorption of dietary cholesterol from small intestine.

5.2.4 Gross and Histopathology

In the present study, no significant gross lesions were observed in liver and aorta of all the groups. Microscopic lesions in the liver of hyperlipidemic diet alone fed group included mild congestion, degenerative changes in hepatic cells, moderate proliferation of bile ducts and focal infiltration of inflammatory cells. The aorta of this group revealed the presence of mild tunica intimal degeneration

and plaque formation. Following treatment with *T. arjuna* leaf extract, *T. arjuna* bark extract and gugulipid the above changes were substantially reduced and could be due to hypolipidemic effect of leaf and bark extract of *T. arjuna* and gugulipid.

5.3 Hypolipidemic Potential of *Terminalia arjuna* Leaves

The hypolipidemic activity produced by the *Terminalia arjuna* leaves and bark might have resulted from the multiple actions of various active principles present in the plant.

Lecithin Cholesterol Acyl Transferase (LCAT) is synthesized in the liver and it catalyzes the esterification of free cholesterol with in the circulation. The esters thus formed move into the core of the HDL, enabling the HDL particle to acquire more free cholesterol from other lipoproteins and cell membranes facilitating reverse cholesterol transport (Shepherd, 1994).

Hepatic triglyceride lipase is located on the hepatic endothelial cells. It plays a role in removing triglycerides from the partially catabolized VLDL or IDL and there fore plays a role in the conversion of VLDL to LDL (Kostner, 1991).

Higher activity of HMG Co-A reductase, the rate-limiting enzyme in cholesterol biosynthesis was noticed in the livers of rats fed with hypercholesterolemic diet (Bradley-Hillgartner *et al.*, 1995). This increased activity could be associated with down regulation of LDL receptors by cholesterol and saturated fatty acids in the diet, which also contribute to elevated serum LDL cholesterol (Stucchi *et al.*, 1995; Mustad *et al.*, 1997).

Khanna *et al.*, (1996) opined that stimulation of plasma LCAT, hepatic lipases, receptor mediated catabolism of LDL and possible inhibition of HMG Co-A reductase might be responsible for the hypolipidemic effect of *T. arjuna*. They further reported that administration of *T. arjuna* increased the fecal cholic acid and desoxycholic acid, thus limiting the enterohepatic cycling of bile acids. In the present study also an increased fecal cholic acid and desoxycholic acid were observed, which were statistically not significant.

Ntanios and Jones (1998) reported that hypolipidemic effect of certain plant extracts was due to the phytosterols, which might interfere with various biochemical pathways of lipids. Singh and Pandey (1995) reported the presence of β- sitosterol, a phytosterol in the leaves of *T. arjuna*. Lees and Lees (1976) have also reported that β- sitosterol is useful in the treatment of type-II hyperlipidemia.

It could be concluded from the present study that the methanolic extract of *T. arjuna* leaves posses hypolipidemic activity. However when compared to bark, leaves appeared to be less potent and less efficacious in hyperlipidemic conditions.

CHAPTER VI

Summary

CHAPTER VI

SUMMARY

Terminalia arjuna (Roxb.)Wt.&Arn. is a large, evergreen tree and the plant is common throughout the greater part of the Indian peninsula. Bark of *T. arjuna* is a popular ingredient of many ayurvedic formulations used in the treatment of heart ailments. Many reports are available on the efficiency of *T. arjuna* bark extract in hyperlipidemic states. However no such reports are available on the efficiency of leaves of the same plant. Hence the present study was designed to evaluate the hypolipidemic activity of methanolic extract of *T. arjuna* leaves in two hyperlipidemic rat models viz. triton induced hyperlipidemia and diet induced hyperlipidemia.

In triton induced model, thirty six male albino wistar rats were randomly assigned to six groups, each having six animals. Group I served as a control. Rats in group II through VI were given triton WR-1339 @ 200 mg/kg intraperitonially. In addition rats in group III, IV, V and VI received *T. arjuna* leaf extract @ 125, 250 and 500 mg/kg, and gemfibrozil @ 250 mg/kg, respectively through oral route. Blood was collected at 0, 18, 24 and 40 hours after triton administration by retro-orbital puncture under ether anesthesia to monitor serum total cholesterol and triglycerides levels.

In diet induced model, fifty six male albino wistar rats were randomly assigned to seven groups each having eight animals. Group I served as a control. Rats in group II through VII were given cholesterol (500 mg/kg) and cholic acid (50 mg/kg) suspended in groundnut oil daily @ 10 ml/kg orally for 30 days. In addition rats in group III, IV and V received *T. arjuna* leaf extract @ 125, 250 and 500 mg/kg respectively. Rats in group VI received *T. arjuna* bark extract @ 125 mg/kg orally while those in group VII were fed gugulipid @ 10 mg/kg daily for 30 days.

Blood was collected for biochemical estimations on days 0, 15 and 30 and liver tissues were collected at necropsy on day 30 to estimate their lipid profile. Fecal material collected over the entire experimental period was assayed for cholic acid and desoxycholic acid.

Triton administration resulted in significantly elevated levels of serum total cholesterol and triglycerids at 18, 24 and 40 hours post administration. Treatment of triton injected rats with *T. arjuna* leaf extract @ 250 and 500 mg/kg had no significant effect at 18^{th} and 24^{th} hour. However at 40^{th} hour, a significant ($P<0.05$) reduction in serum cholesterol and triglycerides was recorded, when compared with triton alone treated group. There was a significant ($P<0.05$) reduction in the levels of total cholesterol and triglycerides at 18, 24 and 40 hours after triton administration in the group that received gemfibrozil, when compared with triton alone treated group.

In diet induced model, serum levels of total cholesterol, triglycerides, LDL, LDL cholesterol and phospholipids were significantly ($P<0.05$) elevated in rats that received hyperlipidemic diet. Rats that received the methanolic extract of *T. arjuna* leaves @ 125, 250 and 500 mg/kg dose level along with hyperlipidemic diet exhibited significantly ($P<0.05$) elevated levels of serum total cholesterol, triglycerides, LDL, VLDL cholesterol and phospholipids on day 15. However on day 30 the cholesterol, triglycerides, VLDL cholesterol and phospholipids levels were significantly ($P<0.05$) lowered in rats that received leaf extract @ 250 and 500 mg/kg, while their levels were still elevated in rats treated with 125 mg/kg dose level. It was also observed that leaf extract could not lower the LDL cholesterol at any dose level during the entire study period. While administration of hyperlipidemic diet also resulted in significantly ($P<0.05$) lowered HDL levels, leaf extract co-administration prevented such lowering. Thus it was evident that leaf extract could neutralize the effect of hyperlipidemic diet administration at 250 and 500 mg/kg dose level. However this was noticed only on day 30, while no such effect was seen on day 15. But such neutralization effect was seen with bark extract at 125 mg/kg dose level by day 15 it self and this was comparable with the protective effect seen with gugulipid in the study.

It was apparent that liver total cholesterol, triglycerides, VLDL cholesterol and phospholipids levels were significantly ($P<0.05$) lowered in rats that received leaf extract @ 250 and 500 mg/kg, while their levels were still elevated in rats

treated with 125 mg/kg dose level. It was also observed that leaf extract could not lower the liver LDL cholesterol at any dose level. Administration of hyperlipidemic diet resulted in no change in HDL levels, while leaf extract co-administration caused moderate increase in HDL levels.

In rats that received *T. arjuna* leaf extract, an increased fecal cholic acid and desoxycholic acid was observed, which were however statistically not significant, when compared to their levels in rats receiving hyperlipidemic diet alone.

Thus it could be concluded from the present study that, the methanolic extract of *T. arjuna* leaves posses hypolipidemic activity. However when compared to bark, leaves appeared to be less potent and less efficacious in hyperlipidemic conditions.

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LITERATURE CITED

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