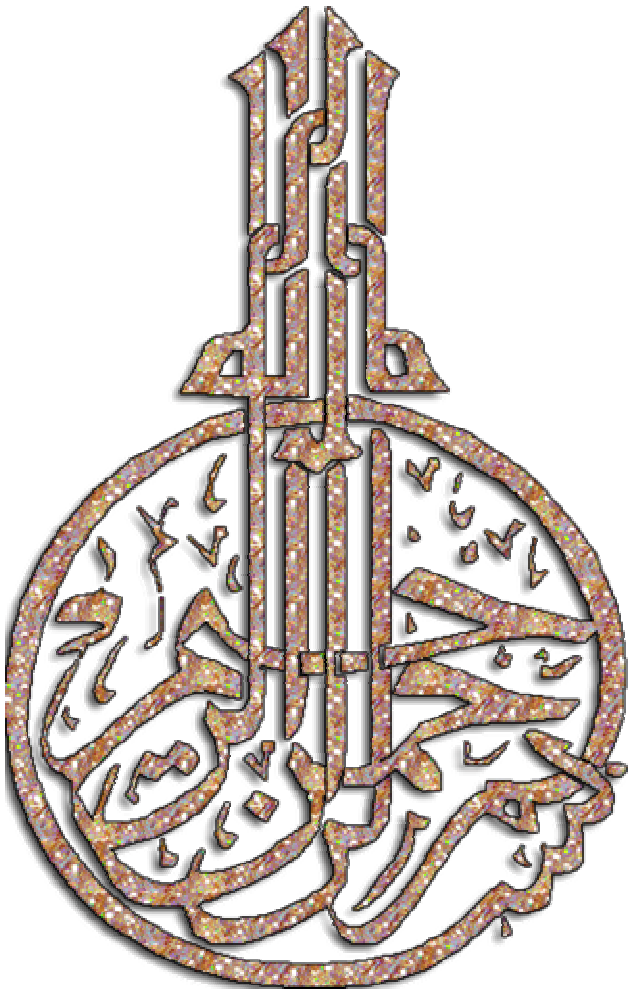
Effect of Pomegranate Juice on Lipid Profile and Antioxidant Enzymes in Hypercholesterolemic Rats

By:

Manal Moalla Dugilib AL-Moraie

A thesis Submitted for the Requirements of the Degree of Master of Science [Food and Nutrition]

FACULTY OF HOME ECONOMICS
KING ABDULAZIZ UNIVERSITY, JEDDAH
Rajab 1434 H- May 2013 G



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This thesis has been approved and accepted in partial fulfillment of the requirements for the degree of Master of Science [Food and Nutrition]

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1434H-2013G

Dedication to

This thesis is dedicated to:

My Parents

My Husband

My Daughter Hala & Sons Abdulrahman & Mohmmed

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First and foremost thanks are to **Allah**, the most beneficent and merciful, who without his aid this work couldn't be done.

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Abstract

Objective: The present study was carried out to investigate the effects of oral administration of Pomegranate juice at three dosage levels (1, 3 and 5 ml/kg b. wt.) to hypercholesterolemic rats for 28 days on body weight gain %, feed efficiency ratio, relative weights of some internal organs, serum levels of total cholesterol (TC), triglycerides (TG), lipoprotein fractions and liver enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were performed. Antioxidant enzymes, catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx,) were determined in homogenate liver. Histopathological examination of liver and heart were also carried out.

Methods: Thirty five male Wistar rats were distributed into five equal groups as follows: negative (normal rats), positive (hypercholesterolemic rats) control groups and positive groups orally given Pomegranate juice in doses of 1, 3 and 5 ml/kg b. wt., respectively. **Results:** The results showed that oral administration of Pomegranate juice to hypercholesterolemic rats for 28 days significantly decreased serum levels of TC, TG, low density lipoproteins cholesterol (LDL-c), very low density lipoproteins cholesterol (VLDL-c) and liver enzymes when compared to the control positive group. Levels of high density lipoprotein cholesterol (HDL-c) and antioxidant enzymes were significantly increased as compared to the control positive group. Histopathological examination of liver and heart of Pomegranate juice-treated groups showed amelioration of histological changes caused by high level of cholesterol in the positive control group. **Conclusion:** Results indicated that Pomegranate juice produces potent antiatherogenic and antioxidant effects in hypercholesterolemic rats. This study recommends that drinking Pomegranate juice may be beneficial for patients who suffer from hypercholesterolemia and/or arteriosclerosis.

تأثير عصير الرمان على صور ليبيدات الدم والإنزيمات المضادة للأكسدة لدى الفئران المصابة بارتفاع الكولسترول

منال معلا المورعي

المستخلص

الهدف: تم إجراء هذه الدراسة لمعرفة تأثير تناول عصير الرمان بثلاث جرعات عن طريق الفم للفئران المصابة بارتفاع مستوى الكولسترول بالدم لمدة ٢٨ يوم على معدل الزيادة في وزن الجسم ونسبة الكفاءة الغذائية والوزن النسبي لبعض الأعضاء الداخلية، وبعض التحليلات البيوكيميائية كمستوى الكولسترول الكلي، الجليسريدات الثلاثية، الليبوبروتينات وأنزيمات الكبد (أسبرتات أمينو ترانسفيراز و ألانين أمينو ترانسفيراز) في سيرم الدم ومستوى الإنزيمات المضادة للأكسدة (الكتاليز، سوبرأكسيد ديسميوتيز وجلوتاثيون بيروكسيديز) في أنسجة الكبد المتجانسة وكذلك الفحص الهستوباثولوجي للكبد والقلب.

الطريقة: تم توزيع خمسة وثلاثون فأر على خمس مجموعات بالتساوي كالتالي:مجموعة ضابطة سالبة،مجموعة ضابطة موجبة (مصابة بارتفاع مستوى الكولسترول بالدم)، ثلاث مجموعات أخرى مصابة بارتفاع مستوى الكولسترول بالدم وتم إعطاؤهم عصير الرمان عن طريق الفم بثلاث جرعات ١، ٣ و ٥ مل /كجم من وزن الجسم على التوالي.

النتائج: أظهرت النتائج أن تناول عصير الرمان عن طريق الفم للفئران المصابة بارتفاع مستوى الكولسترول بالدم لمدة ٢٨ يوم أدى إلى نقص معنوي في مستوى الكولسترول الكلي و الجليسريدات الثلاثية و الليبوبروتين المنخفض الكثافة و الليبوبروتين المنخفض الكثافة جدا وكذلك إنزيمات الكبد مقارنة بالمجموعة الضابطة الموجبة، بينما كانت هناك زيادة معنوية في كل من الليبوبروتين العالي الكثافة و الإنزيمات المضادة للأكسدة مقارنة بالمجموعة الضابطة الموجبة. وأظهر الفحص الهستوباثولوجي وجود تحسن ملحوظ في التغيرات المرضية التي أحدثها الكولسترول المرتفع بالدم مقارنة بالمجموعة الضابطة الموجبة.

الخلاصة: أوضحت النتائج أن عصير الرمان له تأثير فعال كمضاد للأكسدة وكذلك للكولسترول في الفئران المصابة بارتفاع الكولسترول بالدم. لذلك توصي الدراسة بتناول عصير الرمان للمرضى الذين يعانون من ارتفاع الكولسترول وكذلك تصلب الشرايين.

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LIST OF ABBREVIATION

AHA	American Heart Association
ALP	Alkaline phosphatase
ALT	Alanine transaminase
ANOVA	Analysis of Variance
AST	Aspartate transaminase
b. wt.	Body weight
BDL	Bile Duct Ligation
BWG %	Body Weight Gain percent
CAT	Catalase
CCl_4	Carbon tetrachloride
CEH	Cholesterol Ester Hydrolase
CES	Cholesterol Ester Synthetase
CHD	Coronary Heart Disease
cm	Centimeter
Con +ve	Control Positive
Con –ve	Control Negative
Cu Zn-SOD	Copper Zinc Superoxide Dismutase
CV	Central Vein
CVD	Cardiovascular Disease

DGAT1	Diacylglycerol acyltransferase 1
DNA	Deoxyribonucleic Acid
DPPH	1,1-Diphenyl-2-Picryl Hydrazyl
EA	Ellagic Acid
ET	Ellagitannin
Fe-NTA	Ferric Nitrilotriacetate
FER	Feed Efficiency Ratio
FH	Familial Hypercholesterolemia
FI	Feed Intake
g	Grams
g/l	Gram per Liter
GAEs	Gallic Acid Equivalents
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione Disulfide
GST	Glutathione Transferases
GT	Gallotannin
H & E	Hematoxylin and Eosin
H^+	Hydrogen Ions
H_2O_2	Hydrogen Peroxide
HCL	Hydrogen Acid
HDL-c	High Density Lipoprotein Cholesterol

HFD	High Fat Diet
IHD	Ischemic Heart Disease
IMT	Intima-Media Thickness
Kg	Kilogram
LDL-c	Low Density Lipoprotein Cholesterol
LSD	Least Significant Difference
MDA	Malondialdehyde
MEPP	Methanol Extract of Pomegranate Peel
mg	Milligrams
mg/dL	Milligrams per deciliter
Mg/kg	Milligrams per Kilogram
Mg/kg^{-1}	Milligrams per 1Kilogram of air
min	Minute
ml	Milliliters
Mm^{-1}	Millimolar (concentration), i.e. 10-3 moles per liter
MM-LDL	Minimally Modified Low-Density Lipoprotein
mmol/L	Millimoles per liter
Mn-SOD	Manganese Superoxide Dismutase
n-3	Omega 3 fatty acids
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
nm	Nanometer
nmol	Nanomole
NO	Nitric Oxide

NTB	Nitro Tetrazolium Blue
O_2	Oxygen
ox-LDL	Oxidized Low-Density Lipoprotein
P	Probability Values
P-CAD	Premature coronary artery disease
PFE	Pomegranate Fruit Extract
PH	Hydrogen Ion Concentration
PON1	paraoxonase 1
PPE	Pomegranate Peel Extract
ppm	Parts per million
PUFA	Polyunsaturated Fatty Acid
ROS	Reactive Oxygen Species
rpm	Revolution per minutes
SD	Standard Deviation
SFA	Saturated Fatty Acid
SOD	Superoxide Dismutase
SPSS	Statistical Package for Social Sciences
SR-A	Scavenger receptor A
TAS	Total Antioxidant Status
TBARS	Thiobarbituric acid reactive substances
TC	Total Cholesterol
TG	Triglycerides
U	Unit
VLDL-c	Very Low Density Lipoprotein Cholesterol

WHO	World Health Organization
μM	Micromolar (concentration), i.e. 10-6 moles per liter
μmol	Micromoles

CHAPTER I

INTRODUCTION

Chapter I

Introduction

Hypercholesterolemia has been well known as a proven risk factor for cardiovascular disease (CVD) (Wang *et al*., 2011). The Saudi Arabia Ministry of Health reported that in 2010, CVD are the second causes of death with 5,239 deaths (17.3% of the total number of deaths 30,289) (Health Statistical, 2010). It is estimated that by the year 2030 the death rates from CVD will increase and will remain as leading cause of death in the world (WHO, 2008). Hypercholesterolemia is generally, associated with an increase in plasma concentrations of low density lipoprotein (LDL-c) (bad cholesterol) and very low density lipoprotein (VLDL-c) and / or a decrease in high density lipoprotein cholesterol (HDL-c) (good cholesterol). Modification of oxidation of LDL-c is thought to play a key role during early atherogenesis i.e. formation of atheroma inside the walls of blood vessels that finally lead to arteriosclerosis (Kumar *et al.,* 2008a). Because of an increased resistance of LDL-c to oxidation after treatment with various synthetic pharmaceutical drugs (Breugnot *et al.,* 1992; Pentikainen *et al*., 1995), there is a great need to identify natural food products that can offer antioxidant protection against LDL-c oxidation.

Oxidative stress is a state in which oxidation exceeds the antioxidant systems in the body. Oxidative stress arises from an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defenses against them, which intensifies cellular damage. The antioxidant defenses enable the body system to remove ROS, restore the prevailing reducing environment and repair the tissue damage (Halliwell and Gutteridge, 1999). Oxidative stress plays an important role in the etiology and pathogenesis of many chronic diseases such as atherosclerosis, hypertension diabetes mellitus, and cancers (Reuter *et al*., 2010; Krajcovicova-Kudlackova, *et al*., 2012).

Dietary intake of antioxidants can inhibit or delay the oxidation of susceptible cellular substrates so prevent oxidative stress. Therefore, it is important to enrich our diet with antioxidants to protect against many chronic diseases. Antioxidants also play an important role in food quality preservation due to their ability to prevent oxidative deterioration of lipids (Erukainure *et al*., 2012). Antioxidants are important for health maintenance based on their modulation of the oxidation processes in the body (Lee *et al*., 2002). Therefore, the search for cheap and abundant sources of natural antioxidants is attracting worldwide interest.

Consumption of fresh fruits and vegetables to improve human health has been attributed mainly to their high contents of beneficial phytochemicals and other micronutrients (Opara and Al-Ani, 2010). These phytochemicals mainly phenolic compounds (such as flavonoids, phenolic acids, diterpenes, saponins and tannins) have received much attention for their high antioxidative activity by scavenging free radicals

which cause oxidative stress that can lead to cellular damage and many degenerative disorders (Lampe, 1999; Boyer and Liu, 2004).

The edible part of Pomegranate fruit represents 52% of total fruit weight, comprising 78% juice and 22% seeds. Fresh Pomegranate juice is rich in vitamin C (El-Nemr *et al.*, ١٩٩٠), and polyphenolic compounds such as anthocyanins, punicalagin, ellagic and gallic acid (Seeram *et al.*, 2005a; Lansky, 2006). Pomegranate fruit is widely considered as a healthy fruit due to its biological actions, most of these effects were attributed to its high phenolic content (Lansky and Newman, 2007). Previous studies on polyphenols of Pomegranate have been shown that these compounds are linked to the prevention of cardiovascular diseases, cancers and neurological damage in humans (Aviram *et al.*, 2002; Kuskoski *et al.*, 2004; Lansky and Newman, 2007). Flavonols and anthocyanins showed anti-carcinogenic, antimicrobial (Opara *et al.*, 2009), anti-inflammatory and antioxidant activities (Lansky and Newman, 2007). On the other hand, the phenyl propanoids as chlorogenic, caffeic and coumaric acids might be responsible of the inhibition of tumor initiation and development in rats as reported by Huang *et al.* (2005).

Several studies on Pomegranate extracts and its active constituents revealed that they have an antioxidant activity by scavenging free radicals, decreasing macrophage oxidative stress and preventing lipid peroxidation in animals as well as increasing plasma antioxidant capacity in elderly humans (Guo *et al.*, 2008). Studies in rats and mice confirm the antioxidant property of a Pomegranate by-product extract made from the whole fruit minus the juice, showed a 19 % reduction in oxidative stress in mouse

peritoneal macrophages, 42 % decrease in cellular lipid peroxides content, and 53% increase in reduced glutathione levels (Rosenblat *et al*., 2006).

The present study was designed to investigate the effect of oral administration of Pomegranate juice on hypercholesterolemic rats.

1.2 Aim of the Study:

The present study was performed to investigate the effect of oral administration of Pomegranate juice at three dosage levels on hypercholesterolemic rats after 4 weeks of treatment. The following parameters were tested:

- Chemical estimation of Gallic acid equivalents (polyphenolic compound) concentration in Pomegranate juice.
- Feed intake, body weight gain percent and feed efficiency ratio.
- Relative organs weight of liver and heart.
- Serum levels of total cholesterol (TC), triglycerides (TG), LDL-c, VLDL-c, HDL-c and liver enzymes (AST and ALT).
- Activities of antioxidant enzymes [catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx)].
- Histopathological examination of liver and heart of hypercholesterolemic rats orally given Pomegranate juice.

1.3 Objectives of the Study:

1. Assay of chemical composition of total polyphenols (Gallic acid equivalents) in Pomegranate juice.
2. Assay of the serum levels of total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL-c), high density lipoprotein (HDL-c), and activities of liver enzymes (AST and ALT) at the end of the intervention period.
3. Assay of activities of antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) at the end of the intervention period.
4. Histopathological examination of liver and heart at the end of the intervention period.

CHAPTER II

REVIEW OF LITERATURE

Chapter II

Review of Literature

2.1 Cholesterol:

Cholesterol is a sterol, a sort of fats, and its chemical structural formula is shown in Figure (2.1). It is one of the three major classes of lipids which all animal cells utilize to construct their membranes. It is also a precursor of steroid hormones, bile acids and vitamin D, which can be manufactured by all animal cells (Durrington, 2003; Gaziano and Gaziano, 2012).

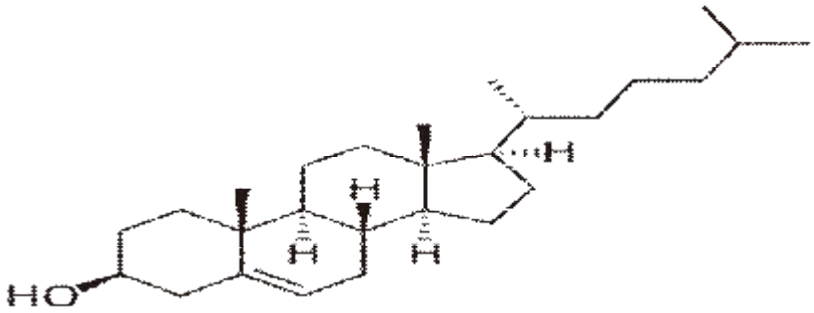


Figure 2.1 Chemical structural formula of Cholesterol (Durrington, 2003).

Cholesterol is essentially insoluble in water; it is transported in blood and in protein particles (Berg *et al*., 2007). The American Heart Association (AHA) describes the existence of two types of cholesterol :- (1) high density lipoproteins (HDL-c) which is considered good cholesterol, because it has the ability to carry excess cholesterol back to the liver for recycling, and (2) low density lipoproteins (LDL-c) which is the major carrier of cholesterol in humans. Low density lipoprotein (LDL-c), bad cholesterol, is harmful when its concentration is elevated in the blood. The ratio between these two types of cholesterol LDL/ HDL is defined an atherogenic index which is an important predictor of heart disease and atherosclerosis. The normal level cholesterol that should be found in the blood varies between 140 and 200 mg per deciliter (mg/dL). Elevated blood total cholesterol (hypercholesterolemia) occurs when its concentration become higher than 240 (mg/dL) (Schaefer, 2010; AHA, 2011).

2.1.1 Hypercholesterolemia:

Hypercholesterolemia is the presence of high level of total cholesterol in the blood. It is the form of "hyperlipidemia" (elevated levels of lipids in the blood) and "hyperlipoproteinemia"(elevated levels of lipoproteins in the blood). Hypercholesterolemia may lead to accumulation, oxidation and modification of lipids in the vascular endothelium leading to endothelial dysfunction, chronic inflammation and cardiovascular diseases (CVD) as mentioned by Insull (2009).

Epidemiological studies have shown that higher concentrations of LDL-c can be correlated to an increase risk of CVD and development of atherosclerosis (Catapano,

2009). The pathogenesis of atherosclerosis is initiated by elevated levels of LDL-c that accumulate in the arterial intima and are subject to oxidation by free radicals i.e. Reactive oxygen species (ROS) (Saini *et al*., 2004; Gropper *et al*., 2009). When the amount of ROS rises above normal levels, which has been associated with many chronic disease states including hypercholesterolemia and atherosclerosis, the antioxidant system of the body is overwhelmed resulting in the oxidation of particles such as proteins and lipids (Manach *et al*., 2005; Vincent and Taylor, 2006).

The oxidation of the lipid particle LDL-c is known to increase its atherogenicity since oxidized-low-density lipoprotein (ox-LDL) is recognized by macrophages and taken up via scavenger receptors. The macrophages are then thought to become foam cells which form plaque (atheroma) on the wall of arteries, and thus a case of arteriosclerosis developed (Stocker and Keaney, 2004) (Figure 2.2). Therefore, the oxidative modification of LDL-c associated with lipids is directly involved in the initiation process of atherosclerosis (Albertini *et al*., 2002).

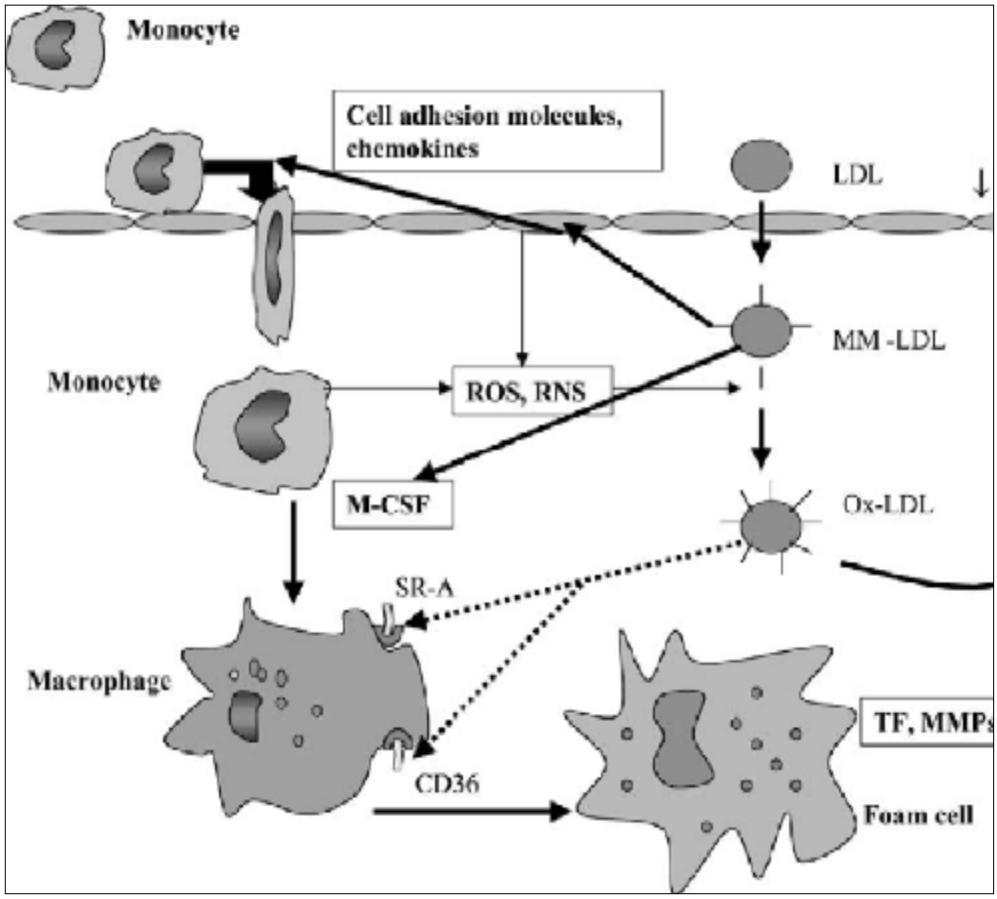


Figure 2.2 The development of atherosclerosis (Singh *et al.*, 2005).

LDL, Low-density lipoprotein; MM-LDL, minimally modified low-density lipoprotein; ox-LDL, oxidized-low-density lipoprotein; ROS, reactive oxygen species; SR-A, scavenger receptor A.

2.1.1.1 Signs and Symptoms of Hypercholesterolemia:

Hypercholesterolemia is asymptomatic, long standing elevation of serum total cholesterol can lead to atherosclerosis (Bhatnagar *et al*., 2008). Over a period of decades, chronically elevated serum total cholesterol contributes to formation of atheromatous plaques in the arteries. These processes cause progressive stenosis (narrowing) or even complete occlusion (blockage) of the involved arteries. Blood supply to the tissues and organs served by these stenotic or occluded arteries gradually diminishes until organ function becomes impaired. It is at this point tissue ischemia (restriction in blood supply) may manifest as specific symptoms. For example, temporary ischemia of the brain (commonly referred to as a transient ischemic attack) may manifest as temporary loss of vision, dizziness and impairment of balance, aphasia (difficulty speaking), paresis (weakness) and paresthesia (numbness or tingling), usually on one side of the body. Insufficient blood supply to the heart may manifest as chest pain, and ischemia of the eye may manifest as transient visual loss in one eye. Insufficient blood supply to the legs may manifest as limb pain when walking, while in the intestines it may present as abdominal pain after eating a meal (Grundy *et al*., 1998; Durrington, 2003).

Some types of hypercholesterolemia lead to specific physical findings. For example, familial hypercholesterolemia (FH) (Type IIa hyperlipoproteinemia) may be associated with xanthelasma palpebrarum (yellowish patches underneath the skin around the eyelids)(Shields and Shields, 2008), arcus senilis (white or gray discoloration of the peripheral cornea)(Zech and Hoeg, 2008).

Hypercholesterolemia is typically asymptomatic; certain physical changes may occur, including the appearance of cholesterol-rich skin deposits, called xanthomas. These can occur on the eyelids (xanthelasmas), or can develop on the elbows, knees, buttocks, tendons, and around the cornea of the eye (Zuliani and Renato, 2003; James and Berger, 2011).

2.1.1.2 Causes of Hypercholesterolemia:

Hypercholesterolemia is typically due to a combination of environmental and genetic factors (Bhatnagar *et al*., 2008). Environmental factors include: Elements such as diet, exercise, and smoking all affect levels of cholesterol as well as age, gender, and co-occurring diabetes and obesity (Gaziano *et al*., 2007). Genetic contributions are usually due to the additive effects of multiple genes, however occasionally may be due to a single gene defect such as in the case of familial hypercholesterolemia (Bhatnagar *et al*., 2008).

A strong association was found between hypercholesterolemia and number of secondary causes exist including: diabetes mellitus type 2, obesity, alcohol drinking, monoclonal gammopathy, dialysis, nephrotic syndrome, obstructive jaundice, hypothyroidism, Cushing's syndrome, anorexia nervosa and some medications (thiazide diuretics, cephalosporin, glucocorticoids, beta blockers, retinoic acid) (Zuliani and Renato, 2003; Bhatnagar *et al*., 2008).

Diet has an important effect on blood cholesterol but the size of this effect varies substantially between individuals (Howell *et al.*,1997).Approximately 50% of dietary cholesterol is absorbed in the intestine but inter-individual variations in the efficiency of uptake, and the effect of other dietary components such as plant sterols and fiber content affect absorption (Lichtenstein,1990). Moreover, when dietary cholesterol intake goes down, production (principally by the liver) (Berg *et al*., 2007) typically increases, though not always with complete compensation, so that reductions in blood cholesterol can be modest. Reductions in fat intake, particularly saturated fats, also reduce blood cholesterol (Sacks and Katan, 2002). If a proper regimen of a healthier diet, increase in exercise and addition of medication is implemented cholesterol levels can be reduced especially for heterozygous hypercholesterolemic individuals (Gaziano *et al*., 2007; Ito *et al*., 2011).

Gender difference currently plays an important role in the etiology of hyperlipidemic induced disorders including CVD. For instance, men are more susceptible to coronary heart disease than age-matched women. However, postmenopausal women have an equal chance of CVD with men (Schwab *et al*., 2011).

Genetic variations are one factor that responsible for hypercholesterolemia, such as in FH where there are one or more genetic mutations happens for example, the LDL-c receptor (Sibley and Stone, 2006). Familial hypercholesterolemia (FH) is one of the most common genetic disorders and it provides the best evidence on the etiologic role of LDL cholesterol for arteriosclerosis development (Araujo *et al*., 2011). Familial hypercholesterolemia (FH) is caused by an autosomal dominant mutation of the LDL-c

receptor gene, resulting in high levels of LDL-c and premature coronary artery disease (P-CAD) (Yudi *et al*., 2012).

2.1.2 Antioxidant Enzymes Related to Hypercholesterolemia:

The preventative antioxidants include the metal chelating proteins and the endogenous antioxidant enzymes catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD). The concentrations and locations of these antioxidants are highly regulated because their main function is to suppress the formation of free radicals to prevent cellular damage or promote cell survival (Willcox *et al*., 2004).

Catalase (CAT) is an antioxidant enzyme which acts to detoxify hydrogen peroxide (H_2O_2) by converting it into water and oxygen (Oh *et al*., 2007) (Figure 2.3). It has one of the highest turnover rates of all enzymes and can convert millions of H_2O_2 molecules per second (Sendur *et al*., 2009). Depressed CAT is associated with an increased risk of chronic diseases associated with oxidative stress such as atherosclerosis, diabetes, neurodegenerative diseases and postmenopausal osteoporosis (Oh *et al*., 2007).

Glutathione peroxidase (GPx) is an antioxidant enzyme associated with selenium, which is required for its optimal function (Schwedhelm *et al*., 2003). It is found in the plasma and the cytosolic or membrane compartment of tissues (Willcox *et al*., 2004). It catalyzes the conversion of H_2O_2 into water and oxygen, using reduced

glutathione (GSH) as a substrate which is oxidized to glutathione disulfide (GSSG) in the process (Figure 2.3).

Superoxide dismutase (SOD) catalyzes the dismutation of $O_2^{\bullet-}$ to oxygen and H_2O_2, the latter of which is subsequently neutralized by CAT or GPx into water and oxygen (Figure 2.3). Superoxide dismutase (SOD) exists in three isoforms: manganese (Mn-SOD), copper zinc (Cu Zn-SOD) and extracellular. Superoxide dismutase (SOD) is localized in the intracellular space of tissues and is filtered through the kidney before being reabsorbed and catabolized within the proximal tubules (Schwedhelm *et al*., 2003). Free radicals which are not effectively suppressed by these preventative antioxidants initiate peroxidative chain reactions which must then be terminated by the chain-breaking, radical-scavenging antioxidants (Willcox *et al*., 2004).

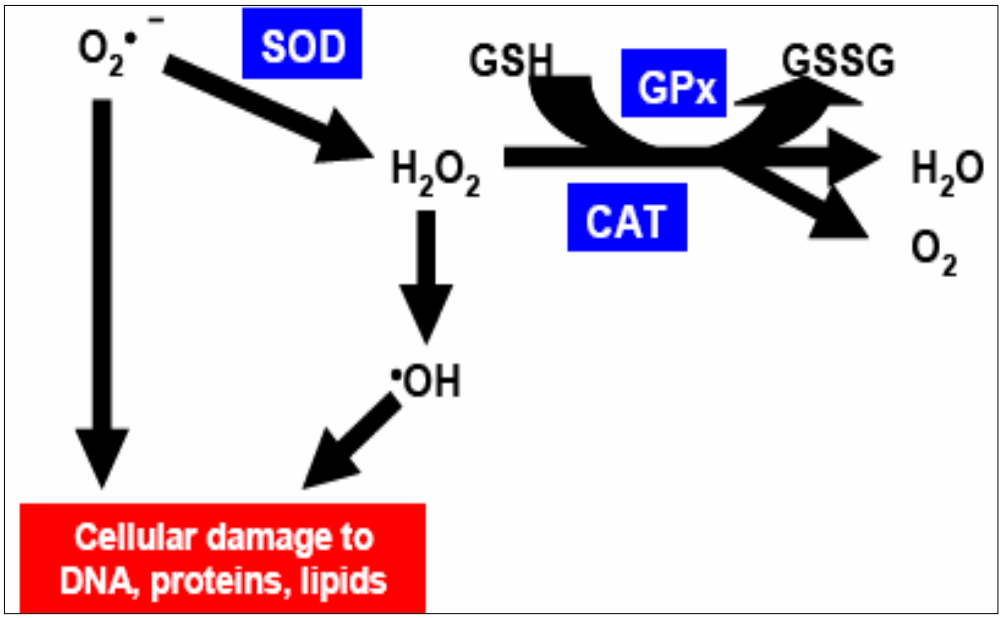


Figure 2.3 Schematic of formation of reactive oxygen species formation, including points of antioxidant enzyme action (Oh *et al*., 2007).

Several studies have shown that intake of high amounts of saturated fatty acids (SFAs) may increase oxidative stress by increasing lipid peroxidation and decreasing antioxidant enzymes (Diniz *et al*., 2004). This may be due to the hypercholesterolemic effect of SFAs, whereby elevated plasma LDL-c levels can lead to increased retention and oxidation of LDL-c in the arterial wall (Stocker and Keaney, 2004). Moreover, hypercholesterolemia is associated with increased superoxide production and nitric-oxide (NO) inactivation (Landmesser *et al*., 2000). Due to their high degree of saturation, polyunsaturated fatty acids (PUFAs) are highly susceptible to damaging, free radical-initiated lipid peroxidation. Consequently, PUFAs also tend to be positively associated with oxidative stress (Kris-Etherton *et al*., 2004).

Diniz *et al*. (2004) found that a high-SFA diet (28.8% total fat, 82% SFAs, 8% PUFAs; P: S ratio ~0.10) increased hydroperoxide concentrations and decreased glutathione peroxidase activity in cardiac tissue of male Wistar rats when compared to the control rats (3.8% total fat, 49% SFAs, 39% PUFAs; P: S ratio ~0.79).

2.2 Pomegranate:

Nowadays, it is widely accepted that the beneficial health effects of fruits and vegetables in the prevention of disease are due to the bioactive compounds they contain (Galaverna *et al*., 2008). The presence of significant amounts of bioactive compounds such as phenolic acids, flavonoids, and tannins in Pomegranate fruits assures them considerable nutritional value (Aviram *et al*., 2000).

The Pomegranate fruit has an ancient history and is mentioned in many Holy Scriptures such as the Torah, the Bible, and the Holy Quran (Langley, 2000; Longtin, 2003). The tree is native to the region of Persia and the Himalayan ranges of India and has been cultivated in Iran, Afghanistan, Pakistan, North India, Armenia, Azerbaijan, Georgia, and the Mediterranean region for several millennia (Jurenka, 2008). Saudi Arabia also is a famous on Pomegranate (El-Rashedy *et al.*, 2011). Pomegranate has been used in various regions and folk or traditional medical systems as a food supplement or a medicine because of its enormous compounds with lots of activities and without toxicity (Lansky and Newman, 2007). Sculptured representations of the fruit are found on the ancient monuments of Egypt and the Assyrian ruins (Jurenka, 2008).

The Pomegranate fruit could be considered a functional food because it has valuable compounds in different parts of the fruit that display functional and medicinal effects. These compounds can act as antioxidant (Cam *et al.*, 2009), as antitumor (Hamad and Al-Momene, 2009) as antihepatotoxic agents (Celik *et al.*, 2009), and improve cardiovascular health (Davidson *et al.*, 2009). They have been reported to have antimicrobial (Duman *et al.*, 2009), anti-inflammatory (Lee *et al.*, 2010), antiviral (Haidari *et al.*, 2009) and antidiabetic (Xu *et al.*, 2009) properties. They can improve oral (Di Silvestro *et al.*, 2009) and skin (Aslam *et al.*, 2006) health. They help to prevent Alzheimer's disease (Singh *et al.*, 2008), improve sperm quality (Turk *et al.*, 2008) and erectile dysfunction in male patients (Forest *et al.*, 2007).

2.2.1 Nutritional Value of Pomegranate:

The Pomegranate fruit has valuable compounds in different parts of the fruit. These parts can be divided into several anatomical origins: peel, seeds, and arils. An important product obtained from Pomegranate fruit is the juice that can be obtained from arils or from whole fruit. The chemical composition of Pomegranate fruits differs according to the cultivar, growing region, climate, maturity, cultivation practice, and storage conditions (Poyrazoglu *et al*., 2002; Barzegar *et al*., 2004; Fadavi *et al*., 2005). Significant variations in organic acids, phenolic compounds, sugars, water-soluble vitamins, and minerals of Pomegranates have been reported over the years by various researchers (Aviram *et al*., 2000; Mirdehghan and Rahemi, 2007; Cam *et al*., 2009; Davidson *et al*., 2009; Tezcan *et al.*, 2009). About 50% of the total fruit weight corresponds to the peel, which is an important source of bioactive compounds such as phenolic, flavonoids, ellagitannins (ETs), and proanthocyanidin compounds (Li *et al*., 2006), minerals, mainly potassium, nitrogen, calcium, phosphorus, magnesium, and sodium (Mirdehghan and Rahemi, 2007), and complex polysaccharides (Jahfar *et al*., 2003).

The edible part of Pomegranate fruit (50%) consists of 40% arils and 10% seeds. Arils contain 85% water, 10% total sugars, mainly fructose and glucose, and 1.5% pectin, organic acid such as ascorbic acid, citric acid and malic acid as well as bioactive compounds such as phenolic compounds and flavonoids, principally anthocyanins (Aviram *et al*., 2000; Tezcan *et al*., 2009).

The seeds are a rich source of total lipids; Pomegranate seed oil comprises 12% to 20% of total seed weight. The oil is characterized by a high content of polyunsaturated (n-3) fatty acids such as linolenic, linoleic, and other lipids such as punicic acid, oleic acid, stearic acid, and palmitic acid (Ozgul-Yucel, 2005; Fadavi *et al*., 2006). The seeds also contain protein, crude fibers, vitamins, minerals, pectin, sugars, polyphenols, isoflavones (mainly genistein), the phytoestrogen coumestrol, and the sex steroid estrone (El-Nemr *et al.*, 1990; Syed *et al*., 2007).

Pomegranate juice was reported to be composed of 85.4% water, 10.6% total sugars (fructose and glucose are present in similar quantities), 1.4% pectin, content in Pomegranate juice varies within the limits of 0.2–1.0%, depending on variety, and includes mainly anthocyanins (such as cyanidin-3-glucoside, cyanidin-3,5-diglucoside, and delphindin-3-glucoside), catechins . Several studies reported that minor compounds include calcium is 50% of its ash content, and the principal amino acids are glutamic and aspartic acids and organic acids, indoleamines, sterols, triterpenoids and α tocopherol (Poyrazoglu *et al.*, 2002; Ignarro *et al.*, 2006; Lansky and Newman, 2007; Heber *et al.*, 2007; Mousavinejad *et al.*, 2009; Jaiswal *et al.*, 2010; Krueger, 2012). Minerals in the Pomegranate juice and seed include iron , relatively prevalent, but not in so high concentrations as in watermelon, and calcium, chlorine, copper, potassium, magnesium, manganese, sodium, selenium and zinc (Waheed *et al*., 2004). In addition, Pomegranate juice contains 21 mg/ 100g vitamin C (Esmaillzadeh *et al.*, 2004).

Pomegranate juice contains approximately 5 mmol/L of total polyphenols in comparison to other fruit juices which contain approximately 1.3 to 4.0 mmol/L of total polyphenols (Seeram *et al*., 2006). Ellagitannins (ETs) and anthocyanins are the principal antioxidant polyphenols in Pomegranate juice (Gil *et al*., 2000). Anthocyanins, which are the water-soluble pigments that give Pomegranate juice its bright red color, are found in the arils of the Pomegranate (Seeram *et al*., 2006). The ETs are present in the peel, the membranes and piths of the fruit and account for approximately 92 % of the total antioxidant activity of Pomegranate juice (Seeram *et al*., 2006; Gil *et al*., 2000).

The major ETs present in the whole fruit are punicalagins, which can be hydrolyzed to ellagic acid (EA) and other smaller polyphenols *in vivo* (Seeram *et al*., 2004b). Commercial Pomegranate juice obtained by pressing whole Pomegranate fruit and its peels contains significant amounts of water soluble compounds, including punicalagins. However punicalagin levels vary widely in Pomegranate juice and can range from as low as 0.017 to 1.5 g/L of juice depending on the fruit cultivar as well as the processing and storage conditions (Gil *et al.,* 2000; Seeram *et al.,* 2004a).

2.2.1.1 Phenolic Compounds:

One of the main compounds responsible for most of the functional properties of many foods, among them Pomegranate fruit, are phenolic compounds in any of their forms (Viuda-Martos *et al*., 2011). Natural polyphenols can range from simple molecules (phenolic acids, phenylpropanoids and flavonoids) to highly polymerized compounds (lignins, melanins and tannins), with flavonoids representing the most

common and widely distributed subgroup (Soobrattee *et al*., 2005).Chemically, phenolic acids can be defined as substances that possess an aromatic ring bound to one or more hydrogenated substituents, including their functional derivatives (Marin *et al*., 2002).

Flavonoids are low-molecular weight compounds consisting of 15 carbon atoms, arranged in a C6-C3-C6 configuration. Essentially, the structure consists of 2 aromatic rings joined by a 3-carbon bridge, usually in the form of a heterocyclic ring (Balasundram *et al*., 2006).

Anthocyanins are the largest and most important group of flavonoids present in Pomegranate arils, which are used to obtain the juice. These pigments give the fruit and juice its red color (Afaq *et al*., 2005). There is a great variety of anthocyanins present in Pomegranate juice (Figure 2.4), principally cyanidin-3-O-glucoside, cyanidin-3, 5-di-O-glucoside, delphinidin-3-O-glucoside, delphinidin-3, 5-di-Oglucoside, pelargonidin-3-O-glucoside, and pelargonidin-3,5-di- O-glucoside (Lansky and Newman, 2007; Jaiswal *et al*., 2010). The main differences between them are the number of hydroxylated groups, the nature and the number of bonded sugars to their structure, the aliphatic or aromatic carboxylates bonded to the sugar in the molecule, and the position of these bonds (Kong *et al*., 2003).

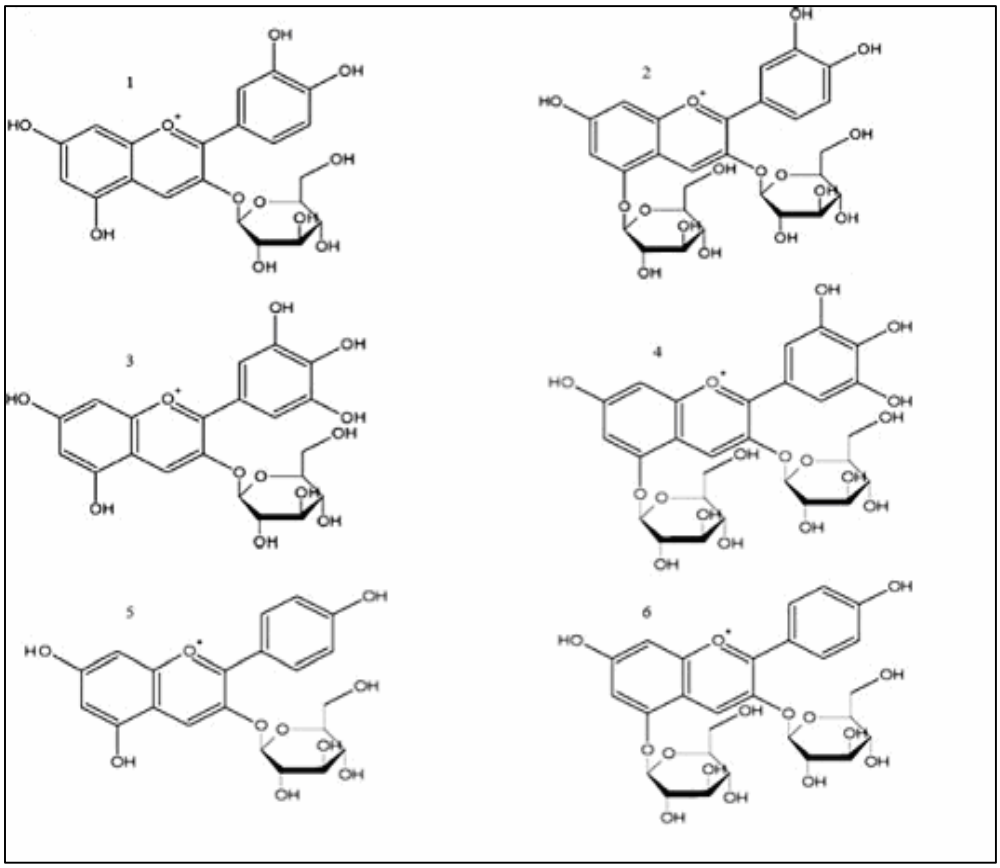


Figure 2.4 Principal anthocyanins present in Pomegranate juice (Viuda-Martos *et al.*, 201١).

1: cyanidin-3-O-glucoside; 2: cyanidin-3, 5-di-O-glucoside; 3: delphinidin-3-Oglucoside; 4: delphinidin-3,5-di-O-glucoside; 5: pelargonidin-3-O-glucoside; 6: pelargonidin-3,5-di-O-glucoside.

The phenolic acids present in Pomegranate juice (Figure 2.٥) can be divided into 2 groups: (1) hydroxybenzoic acids, mainly Gallic acid and EA (Amakura *et al*., 2000); and (2) hydroxycinnamic acids, principally caffeic acid, chlorogenic acid, and p-coumaric acid (Poyrazoglu *et al*., 2002).

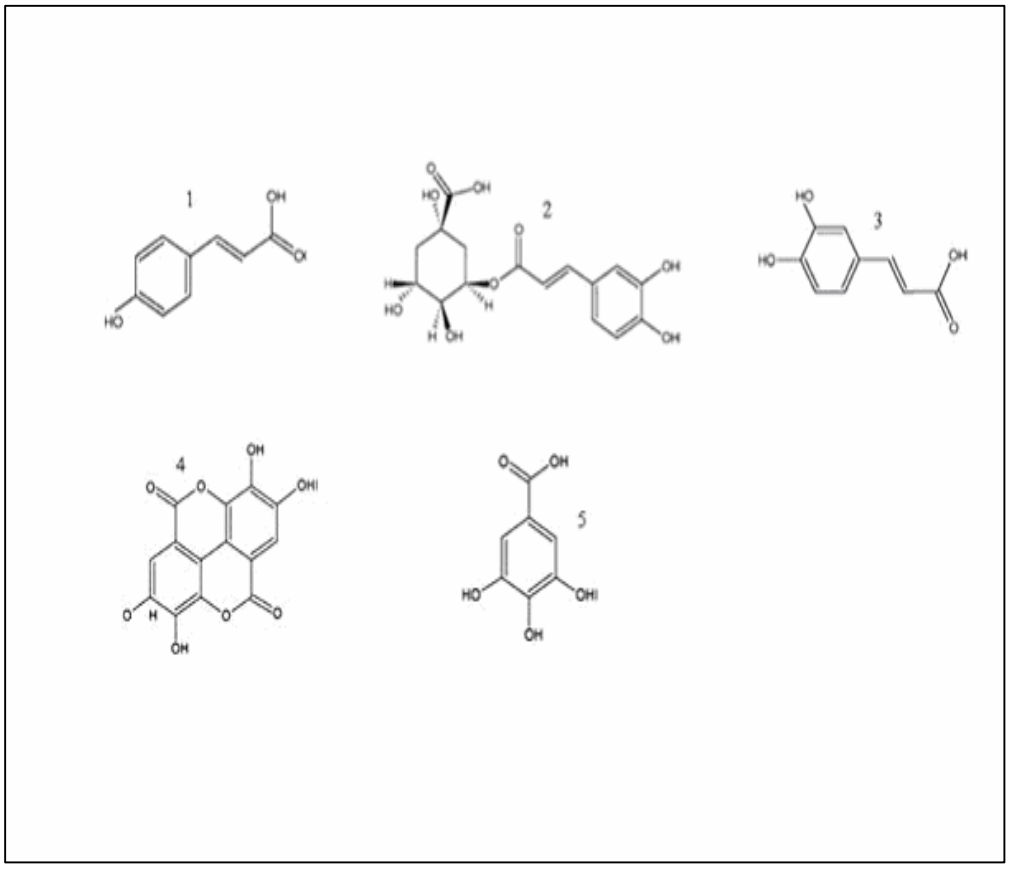


Figure 2.5 Principal phenolic acids present in Pomegranate juice (Viuda-Martos *et al*., 201١).

1: p-coumaric acid; 2: chlorogenic acid; 3: caffeic acid; 4: EA; 5: gallic acid.

2.2.1.2 Tannins:

Tannins are high-molecular-weight plant polyphenols divided into 3 chemically and biologically distinct groups: condensed tannins or proanthocyanidins (as found in tea, grapes, cranberries, and so on) and hydrolyzable tannins or ETs (as in raspberries, strawberries, and so on) as well as gallotannins (GTs) (Seeram *et al.*, 2005b). Pomegranate peel is rich in hydrolyzable tannins (Figure 2.6), mainly punicalin, pedunculagin, and punicalagin (Seeram *et al.*, 2005a). Tannins differ from proanthocyanidins in their chemical structures. ETs are esters of hexahydroxydiphenic acid and a polyol, usually glucose or quinic acid (Clifford and Scalbert, 2000). In addition to ETs, Pomegranate peel contains hydroxybenzoic acids such as gallagic, EA, and ellagic acid glycosides (Amakura *et al.*, 2000); anthocyanidins are principally cyanidin, pelargonidin, and delphinidin (Noda *et al.*, 2002) and flavonoids such as kaempferol, luteolin, and quercetin (Van Elswijk *et al.*, 2004).

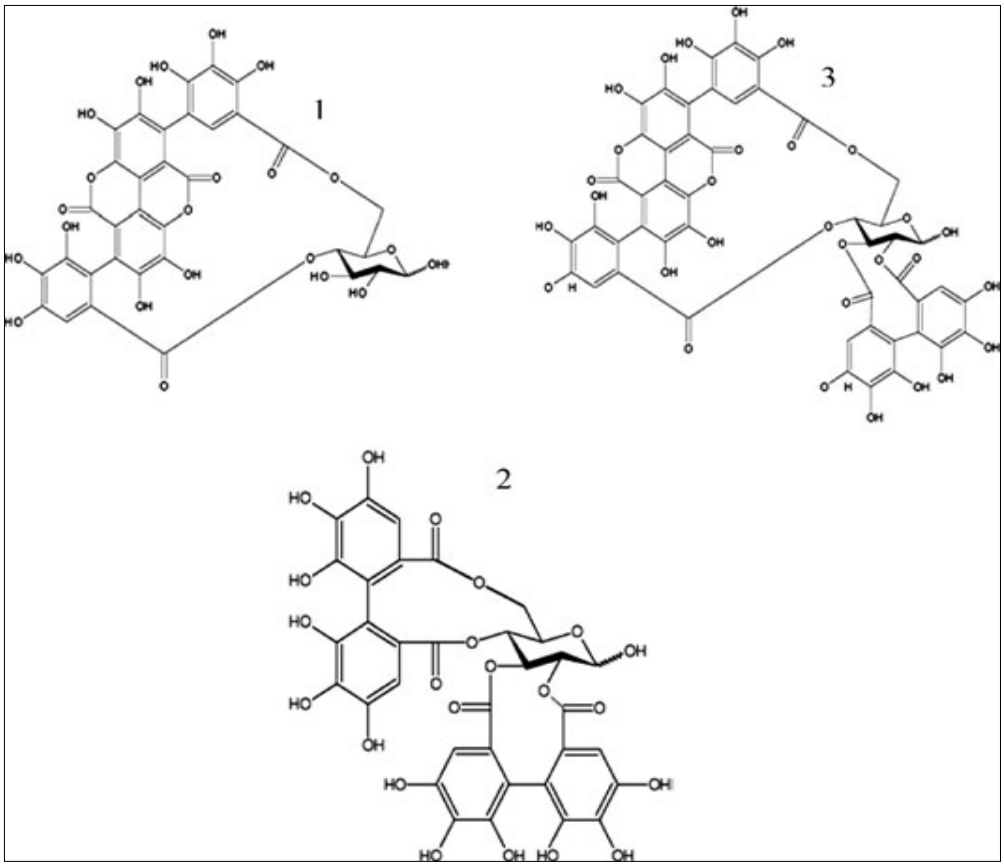


Figure 2.6 Principal ellagitannins (ETs) present in Pomegranate peel (Viuda-Martos *et al*., 2011).

1: punicalin; 2: pedunculagin; 3: punicalagin.

2.3 Effect of Pomegranate on Blood Lipid:

One of the major risk factors for the development of coronary heart disease (CHD) is dyslipidemia, which is mainly characterized by elevated levels of LDL-c and/or reduced HDL-c (Esmaillzadeh and Azadbakht, 2008). Oxidation LDL-c is thought to contribute to atherosclerosis and CVD (Heinecke, 2006). *In vitro*, animal and human trials have examined the effects of various Pomegranate constituents on the prevention and attenuation of atherosclerosis and LDL-c oxidation (Aviram *et al*., 2000; Fuhrman *et al*., 2005; Ignarro *et al*., 2006; Sezer *et al*., 2007; Basu and Penugonda, 2009; Davidson *et al*., 2009; Fuhrman *et al*., 2010).

Aviram *et al*. (2000) reported that Pomegranate juice inhibited atherogenic modifications of LDL-c, including its retention, oxidation, and aggregation. The antiatherogenicity capability of Pomegranate juice is related to three components of atherosclerosis: plasma lipoproteins, arterial macrophages, and blood platelets. The potent antioxidative capacity of Pomegranate juice against lipid peroxidation may be the central link for the anti-atherogenic effects of Pomegranate juice on lipoproteins, macrophages, and platelets.

Sumner *et al.* (2005) examined whether daily consumption of Pomegranate juice for three months would affect myocardial perfusion (blood flow in the heart) in CHD and stress-induced ischemia (patients had undergone treadmill exercise or pharmacologic stress). The results showed that patients who consumed Pomegranate juice daily (240ml/day) for three months had a decrease in stress-induced ischemia

compared to the controls that had an increase in stress-induced ischemia. Also there was an average improvement of 17% in myocardial perfusion in the Pomegranate juice group and an average worsening of 18% in myocardial perfusion in the control group.

Esmaillzadeh *et al*. (2006) investigated the effect of concentrated Pomegranate juice consumption (40 g) on lipid profiles of type II diabetic patients with hyperlipidemia (total cholesterol or triglycerides ≥ 200 mg/dL). At the end of assay eight weeks, the results showed that there were no significant changes in serum triacylglycerol and HDL-c concentrations. However, reductions were obtained in total cholesterol (TC) (5.43%), LDL-c (9.24%), TC / HDL -c ratio (7.27%), and LDL / HDL ratio (11.76%).

Fuhrman *et al*. (2005) reported that Pomegranate juice exerts a direct effect on macrophage cholesterol metabolism by reducing cellular uptake of ox-LDL and inhibiting cellular cholesterol biosynthesis. Both of these processes eventually lead to a reduction in macrophage cholesterol accumulation and foam cell formation and attenuation of atherosclerosis development.

Basu and Penugonda (2009) suggested that the principal mechanisms of action of Pomegranate juice is anti-atherogenic and may include the following: decreased plasma lipids, lipid peroxidation, oxidized- LDL-c uptake by macrophages, intima media thickness, atherosclerotic lesion areas, inflammation, angiotensin converting enzyme activity and decreased systolic blood pressure; on the other hand Pomegranate juice increased serum antioxidant capacity enhanced biological actions of NO, which event

led to favorable effect on the progression of atherosclerosis and the subsequent potential development of CHD.

Anoosh *et al.* (2010) studied the effect of Pomegranate juice on plasma LDL-c in hypercholestolemic patients. In this investigation, patients were divided into three groups with twenty patients in each group. The treatments were including: Group one; using tabrizy variety of Pomegranate juice, Group two; black varieties of Pomegranate juice and Group three; a drug lovastatin (one of statins), for four weeks and then the authors compared the consumption of group one and two with group three. The results showed that there was no difference between group one and two with group three. As with the drug, the two groups (one and two) were effective in decreasing the level of LDL-c.

Rosenblat and Aviram (2011) demonstrated that consumption of fifty μM of Pomegranate juice is able to affects the triglyceride biosynthesis which could be attributed to a direct effect of Pomegranate juice on diacylglycerol acyltransferase 1(DGAT1) activity.

Hossin (2009) reported that supplementation of pomegranate peels powder and its extract to obese hypercholesteolemic male rats had a significant decreases on lipid metabolism , food consumption, body weight gain ratio and all tested lipid parameters except HDL-c compared to control positive group(hypercholesteolemic rats).

Chalfoun-Mounayar *et al.* (20١٢) demonstrated that pomegranate molasses or juice when added to the drinking water of mice (4 ml/L) during 11 weeks led to a significant decrease in weight curve compared to control animals; also triglycerides and peroxidation were decreased in the heart, lungs, and the liver, while SOD activity increased. In conclusion, Pomegranate molasses possesses a powerful antioxidant activity and a weight loss effect in mice.

2.٤ Effect of Pomegranate on Liver Function:

Epidemiological studies suggested the existence of a close association between liver disease and the prevalence of metabolic disorders such as dyslipidemia, insulin resistance, and hyperglycemia (Must *et al*., 1999). Although several lines of evidence suggested that hyperlipidemia may not play a causal role in liver injury, they may affect the severity of tissue damage (Jou *et al*., 2008). Indeed, liver damage is more severe when there are multiple coincidental insults. For example, excess lipid accumulation in the form of triglycerides in the cytosol of hepatocytes, which per se does not appear to impair liver function, significantly increases the vulnerability of the liver to the deleterious effects of cytokines, oxidative agents, and viral infections, predisposing this organ to inflammation and advanced fibrosis (Farrell and Larter, 2006).

Although hypercholesterolemia is a prominent metabolic disorder and a major risk factor for coronary heart disease and atherosclerosis, its precise contribution to the progression of hepatic steatosis, inflammation, and fibrosis has been practically overlooked. In this regard, apart from epidemiological studies suggesting

hypercholesterolemia as an independent risk factor for liver disease, a study suggesting that hypercholesterolemia increases susceptibility to virus-induced immunopathological liver disease experimental studies in rodents and rabbits demonstrating an association between the intake of high cholesterol and high-fat diets (HFD) with liver steatosis and inflammation (Ludewing *et al*., 2001; Pendino *et al*., 2005; Tous *et al*., 2005).

Lu *et al*. (2007); Prasad (2010) and Saki *et al*. (2011) studied the effect of hypercholesterolemia on serum levels of as alanine aminotransaminase (ALT), aspartate aminotransaminase (AST) and alkaline phosphatase (ALP). The results showed that a high cholesterol diet moderately elevated serum levels of ALT, AST and ALP in rats.

Toklu *et al*. (2007) assessed the effect of oral administration of Pomegranate peel extract (PPE) at 50 mg kg^{-1} or saline to rats for twenty-eight days on liver fibrosis induced by bile duct ligation (BDL). The results showed that the elevation of AST and ALT were significantly decreased after treatment. This effect may be due to antioxidant and antifibrotic properties of PPE and also from potential therapeutic value in protecting the liver from fibrosis and oxidative injury.

Osman *et al*. (2012) investigated the antioxidant effect of Pomegranate peel and juice on diabetes mellitus induced by alloxan in female rats. Thirty two rats were allocated into four groups as follows: Group one; control without any treatment; Group two: diabetic animals injected with alloxan; Group three: diabetic peel group animals injected with alloxan and then fed on peel Pomegranate; Group four: diabetic juice group animals injected with alloxan and then gavage with Pomegranate juice, for four

weeks treatment. The results showed that AST and ALT were significantly increased in the diabetic group. After treatment with peel and juice, AST and ALT levels decreased and become near to the control levels, especially ALT value. Furthermore, levels of TC, TG, LDL-c and total lipids increased, while HDL decreased in diabetic group. Administration of peel and juice of Pomegranate prevented these changes.

Fyiad *et al*. (2012) studied the effect of Pomegranate juice on nucleic acids alterations and oxidative stress in rats with experimentally hepatitis. Results revealed that the pretreatment with Pomegranate juice (twenty ml/ kg^{-1} b. wt., day^{-1} for fourteen days) effectively hindered the adverse effect of D-Galactosamine / lipopolysaccharide and protect against hepatic damage via suppression of oxidative stress. Histopathological studies of the liver of different groups also support the protective effects exhibited by Pomegranate juice through restoring the normal hepatic architecture. These were also significant decreases in the serum level of diagnostic marker enzymes (AST, ALT and ALP) as compared to the alleviation of D-Galactosamine / lipopolysaccharide –induced hepatitis control group.

2.٥ Effect of Pomegranate on Antioxidant Enzymes:

The antioxidant and sensory qualities of Pomegranates depend on several factors, such as cultivar, climatic conditions during fruit maturation and ripening and the part of the fruit used (Borochov-Neori *et al*., 2009). Thus, Singh *et al*. (2002) reported that a methanol extract of Pomegranate peel had much higher antioxidant capacity than that of

seeds, as demonstrated by using both β-carotene-linoleate and 1, 1-diphenyl-2-picryl hydrazyl(DPPH) model assays.

Tzulker *et al*. (2007) reported that the homogenate prepared from the whole Pomegranate fruit exhibited an approximately twenty-fold higher antioxidant activity than the level of that prepared from the aril juice.

The antioxidant activity of Pomegranate components has been the subject of many studies (Naveena *et al.*, 2008; Cam *et al*., 2009; Mousavinejad *et al*., 2009; Tezcan *et al*., 2009), most conducted *in vitro* and *in vivo*. All these activities may be related to the diverse phenolic compounds present in Pomegranate, including punicalagin isomers, EA derivatives, and anthocyanins (delphinidin, cyaniding, pelargonidin 3- glucosides, and 3, 5-diglucosides). These compounds are known for their properties to scavenge free radicals and to inhibit lipid oxidation *in vitro* (Gil *et al*., 2000; Noda *et al*., 2002).

However, Tzulker *et al*. (2007) suggested that punicalagin originating from the peels is one of the major phytochemicals contributing to the total antioxidant capacity of Pomegranate juice, while anthocyanins play only a minor role in this activity. The action and mechanism of action set in motion by the antioxidant activity of these compounds are still not clearly understood, although it is a known fact that antioxidant mechanisms involved in biological matrixes are quite complex and several different factors may play a role (Cam *et al*., 2009).

Aviram *et al.* (2004) conducted a study with nineteen patients suffering from carotid artery stenosis, patients were divided into two groups: Nine patients that did not consume Pomegranate juice served as a control group; Ten patients were included in the Pomegranate juice in a dose of 50 ml/day for one year as treated group. The results showed that there was an increased in common carotid intima-media thickness (IMT) by 9% during one year for the control group; Whereas, Pomegranate juice consumption resulted in a significant IMT reduction, by up to 30%, after one year. Furthermore, serum levels of antibodies against ox-LDL were decreased by 19%, and in parallel serum total antioxidant status (TAS) was increased by 130% after one year of Pomegranate juice consumption.

Madrigal-Carballo *et al.* (2009) suggested that Pomegranate polyphenolic molecules undergo redox reactions because phenolic hydroxyl groups readily donate hydrogen for reducing agents. Negi and Jayaprakasha (2003) have also reported a significant increase in the antioxidant reducing power of Pomegranate peel extracts with increases in concentration from 50 to 400 ppm. Reducing properties are generally associated with the presence of reducing agents (Pin-Der, 1998).

Gordon (1990) reported that the antioxidative action of reducing agents is based on the breaking of the free radical chain by the donation of a hydrogen atom. Reducing agents also react with certain precursors of peroxides, thus preventing peroxide formation (Naveena *et al*., 2008). However, Amarowicz *et al.* (2004) suggested that the antioxidant activity of phenolic compounds is due to their ability to scavenge free radicals or chelate metal cations.

Gil *et al*. (2000) reported that Pomegranate juice possessed a three-folds higher antioxidant activity than that of red wine or green tea, and two-, six-, and eight-folds higher levels than those detected in grape/cranberry, grape fruit, and orange juices, respectively (Azadzoi *et al*., 2005; Rosenblat and Aviram, 2006).

Guo *et al.* (2008) found that consumption of 250 mL Pomegranate pulp juice daily for four weeks by healthy elderly subjects resulted in increased plasma antioxidant capacity, while subjects consuming apple juice experienced no significant increase. In addition, subjects consuming the Pomegranate pulp juice exhibited significantly decreased plasma carbonyl content, a biomarker for oxidant/antioxidant barrier impairment in various inflammatory diseases.

Seeram *et al*. (2008) demonstrated that Pomegranate juice had the greatest antioxidant potency composite index among such beverages as apple juice, aćaí juice, black cherry juice, blueberry juice, cranberry juice, grape juice, orange juice, red wines, and iced tea; and the antioxidant activity was at least 20% greater than any of the other beverages tested. Indeed, Aviram and Dornfeld (2001) reported that consumption of Pomegranate juice, which is rich in tannins; possess anti-atherosclerotic properties that could be related to its potent antioxidative characteristics.

Flavonoids also make a great contribution to the antioxidant activity of Pomegranate due to their effect on free radicals elimination (Wang *et al.*, 2006; Suo *et al.*, 2009). It seems that flavonoids from Pomegranate possess had a significant antiperoxidative activity. It was evidenced by that the concentrations of

malondialdehyde, hydroperoxides and conjugated dienes in the liver, heart and kidney were significantly reduced and the activities of the enzymes such as CAT, SOD, GPx, glutathione reductase (GR) and the concentration of glutathione in the tissue were significantly enhanced after the orally administered with total flavonoids from Pomegranate (Sudheesh and Vijayalakshmi, 2005).

A separate study on rats with carbon tetrachloride (CCl_4) induced liver damage demonstrated that pretreatment with a Pomegranate peel extract enhanced or maintained the free radicals scavenging activity of the hepatic enzymes CAT, SOD, and peroxidase, and resulted in 54% reduction of lipid peroxidation values compared to control groups (Chidambara *et al.*, 2002).

Ajaikumar *et al.* (2005) studied the effect of consumption of 70% methanolic extract of *Punica granatum* fruit at 250 mg/kg and 500 mg/kg on rats that suffer from gastric ulceration. The results showed that SOD, CAT, GSH and GPx levels were increased and found more or less equal to the normal values. The tissue lipid peroxidation level was found to be decreased in treated groups of animals as compared to the control group. The authors concluded that gastroprotective activity of the extract may be due to its antioxidant mechanism.

Faria *et al.* (2007) investigated the antioxidant activity, which has been related to beneficial health properties of Pomegranate juice in male mice. For this purpose, mice ingested Pomegranate juice (or water in control group) during four weeks, after which damage to lipids; proteins and DNA were evaluated as oxidative cell biomarkers. Levels

of hepatic glutathione and the activities and expression of enzymes involved in its metabolism were determined. Catalase (CAT) and SOD activities were quantified as these enzymes have a crucial role in antioxidant defense. Protection against protein and DNA oxidation was found in Pomegranate juice group. There was also a significant decrease in all studied enzymatic activities (GPx, GST, GR, SOD and CAT) by Pomegranate juice treatment.

The effect of Pomegranate juice consumption on antioxidant activity of male healthy rats was studied by Turk *et al.* (2008). Twenty-eight healthy adult male Wistar rats were divided into four groups; each group containing seven rats. One mL distilled water, 0.25 mL Pomegranate juice plus 0.75 mL distilled water, 0.50 mL Pomegranate juice plus 0.50 mL distilled water and 1 mL Pomegranate juice were given daily for seven weeks by gavage to rats in the first, second, third and fourth groups, respectively. Body organ weights, antioxidant enzyme activities were investigated. The results showed that there was a decrease in malondialdehyde (MDA) level and marked increases in GSH, GPx and CAT activities, were observed in rats treated with different concentrations of Pomegranate juice.

Moreover, Mohan *et al.* (2010) studied the effect of Pomegranate juice extract which was orally given in a dose of 100 mg/kg and 300 mg/kg; orally for four weeks on blood pressure in diabetic Wistar rats. The results showed that there was a reduction in oxidative stress induced by diabetes and Angiotensin II. Pomegranate juice treatment also caused a significant decrease in levels of thiobarbituric acid reactive substances

(TBARS) in kidney tissues and pancreas, while the activity of enzymes SOD, CAT and GSH showed significant elevation.

Abdel Moneim *et al*. (2011) investigated the antioxidant properties of Pomegranate in hepatic and renal tissues of rats. Eighteen adult male albino rats were randomly divided into three groups, six rats of each. The first group served as control and received saline (0.2 ml saline/ rat) by oral administration via stomach (gastric) tube. The second group received oral administration of three ml/kg Pomegranate juice for twenty-one days and served as Pomegranate juice group. The third group received oral administration of 200 mg/kg methanol extract of Pomegranate peel for twenty-one days and served as methanol extract of Pomegranate peel (MEPP) group. The authors concluded that administration of Pomegranate juice and MEPP reduces lipid peroxidation and NO in homogenate of both liver and kidney tissues. A significant increase in SOD and CAT activities of rats received Pomegranate was observed. These findings demonstrated that Pomegranate has a potent antioxidative effect.

2.6 Safety of Pomegranate:

Pomegranate and its constituents have been safely consumed for centuries without adverse effects. Studies of Pomegranate constituents in animals at concentrations and levels commonly used in folk and traditional medicine note no toxic effects (Vidal *et al*., 2003). Toxicity of the polyphenol antioxidant punicalagin, abundant in Pomegranate juice, was evaluated in rats. The results showed neither toxic effects nor significant differences in the treatment group compared to controls were reported. This

finding was confirmed by the normal histopathological picture of rat organs (Cerda *et al*., 2003).

Aviram *et al.* (2004) studied the effect of Pomegranate juice consumption (50 ml/day which contain 1.5 mmoles of total polyphenols) for up to three years on ten patients with carotid artery stenosis. The results showed that there were no toxic effects on blood chemistry and kidney, liver, and heart functions.

Heber *et al*. (2007) investigated the safety of Pomegranate fruit extract (PFE) tablets in amounts up to 1.420 mg/day (870 mg gallic acid equivalents (GAEs)) for twenty-eight days on eighty-six overweight human volunteers. The results showed that there were no adverse events reported or adverse changes in blood and urine laboratory values were observed.

CHAPTER III

MATERIAL AND METHODS

Chapter III

Material and Methods

In the present study, the biological and histopathological effects of Pomegranate juice at three concentration levels were investigated on hypercholesterolemic rats.

3.1 Material:

3.1.1 Pomegranate:

Ripe Pomegranate fruits used in this study were purchased from a local market, Jeddah, Kingdom of Saudi Arabia. The Pomegranate plant was grown in Taif city, a west province of the Kingdom of Saudi Arabia.

3.1.2 Cholesterol:

Cholesterol was purchased from Sigma-Aldrich Company, St. Louis, Missouri, USA, in the form of white crystalline powder in plastic bottles each containg 100 gram.

3.1.3 Rats:

A total number of thirty five adult male albino rats of Wistar strain of 6-8 weeks old and weighed 150 ± 30 grams were used in this study. The rats were obtained from Faculty of Pharmacy, King Abdul-Aziz University, Jeddah, Saudi Arabia.

3.1.4 Kits for Biochemical Analysis:

Commercial diagnostic kits for estimating serum lipid profile (total cholesterol, triglycerides and lipoprotein fractions) were obtained from Randox Laboratories, U.K. The kits for estimating liver function enzymes (AST and ALT) were obtained from Diamond Company, Hannover, Germany. Antioxidant enzymes commercial kits were purchased from Roche Diagnostic laboratories, Germany.

3.2 Methods:

3.2.1 Preparation of the Basal Diet:

The basal diet for rats was prepared using AIN-93 according to Reeves *et al.* (1993). The basal diet consists of the following: Protein (Casein) 20%; Sucrose 10%; Corn Oil 4%; Choline Chloride 0.2%; Vitamin mixture 1%; Salt mixture 3.5%; Fibers (Cellulose) 5% and the remainder is Corn Starch up to 100%.

3.2.2 Induction of Hypercholesterolemia:

Induction of hypercholesterolemia was done by feeding rats on cholesterol containing diet (experimental diet) which was prepared by formulated basal diet with

2% Cholesterol and 0.5% Cholic acid for 4 weeks according to the method described by Shinnick *et al*. (1990).

3.2.3 Preparation of Pomegranate Juice:

The fruits of fresh Pomegranate were washed and manually peeled, without separating the seeds. Pomegranate juice was obtained using a commercial blender (Moulinex, France), filtrated with a Buchner funnel (Faria *et al*., 2007).

3.2.4 Experimental Design and Grouping of Rats:

The experiment was performed on thirty five male mature Wistar rats. Animals were distributed randomly into 5 equal groups. Rats were housed in standard plastic cages at a room temperature maintained at 24 ± 2 °C, with fixed 12 hour lighting system.

All rats were allowed to free access to basal diet and water for one week before starting the experiment for acclimatization. After acclimatization period, the rats were allocated in to the following groups:-

Group (1): Rats fed on the basal diet only, kept as a negative control group (Con -ve) and received oral gavage of distilled water.

The other four groups were fed on experimental diet for four weeks. After this period, blood samples were taken for measuring total cholesterol level. Rats with blood cholesterol level ≥ 5.2 mmol /L were considered to be hypercholesterolemic (Iqbal *et al*., 2011). These rats were distributed in to the following groups:-.

Group (2): Rats fed on experimental diet only, kept as a positive control group (Con +ve) and received oral gavage of distilled water.

Group (3): Rats fed on experimental diet and orally given Pomegranate juice in a dose of 1 ml/kg body weight (b. wt.).

Group (4): Rats fed on experimental diet and orally given Pomegranate juice in a dose of 3 ml/kg b. wt.

Group (5): Rats fed on experimental diet and orally given Pomegranate juice in a dose of 5 ml/kg b. wt.

3.2.5 Determination of Feed Intake, Body Weight Gain percent (BWG %) and Feed Efficiency Ratio (FER):

Daily feed intake (FI) per group was calculated throughout the experimental period (28 days).The biological values of different diets were assessed by the determination of body weight gain percent (BWG %) which was calculated at the end of the experimental period as well as feed efficiency ratio (FER) was calculated twice a week, according to the method of Chapman *et al*. (1959).Using the following equations:

$$\textbf{Body weight gain percent (BWG \%)} = \frac{\text{Final body weight} - \text{Initial body weight}}{\text{Initial body weight}} \times 100.$$

Feed efficiency ratio was calculated as follows:

$$\textbf{Feed efficiency ratio (FER)} = \frac{\text{Gain in body weight (g)}}{\text{Feed consumed (g)}}$$

At the end of the experimental period, all rats were fasted overnight then sacrificed. Blood samples were immediately collected from the retro orbital plexus with

capillary tubes under mild ether anesthesia, into clean dried centrifuge tubes. The tubes were then centrifuged at 3000 rpm for 15 minutes. Clear serum samples were carefully separated using Pasteur pipettes, and frozen at - 20° C until biochemical analysis (Margoni *et al.,* 2011). The liver and heart were removed by careful dissection and blotted free of adhering blood immediately after sacrificing the rats. The organs were washed with cold saline solution and dried between two filter papers then weighed and they saved for the histopathological examination. Calculation of the relative organs weight was done according to the following equation:

$$\textbf{Organ relative weight} = \frac{\text{Organ weight}}{\text{Animal final bodyweight}} \times 100.$$

Livers and hearts were kept in 10% neutral buffered formalin pending for the histopathological examination.

3.2.6 Biochemical Analyses:

The biochemical analyses were measured using different methods as explained below:

3.2.6.1 Estimation of Total Polyphenol Concentration in Pomegranate Juice:

Total polyphenol concentrations in Pomegranate juice were determined spectrophotometrically according to the method of Singleton and Rossi (1965) and modified by Narr Ben *et al.* (1996) for small volumes. Gallic acid stock solution was prepared in ethanol at a concentration of 1mM and used as standard solution. The results were recorded as gallic acid equivalents (GAEs).

3.2.6.2 Serum Analysis:

3.2.6.2.1 Determination of Serum Total Cholesterol (TC):

Serum cholesterol was determined according to the method described by Allain *et al*. (1974), using a Spectrophotometer (Thermo Fisher Scientific Genesys, USA) adjusted at 500 nm wave length. The concentration of the sample was calculated from the following equation:-

$$\textbf{TC concentration (mmol/L)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 5.17.$$

3.2.6.2.2 Determination of Serum Triglycerides (TG):

Concentrations of serum triglycerides were determined according to the method described by Trinder (1969), using a Spectrophotometer (Thermo Fisher Scientific Genesys, USA) at 500 nm wave length. The concentration of the sample was calculated from the following equation:

$$\textbf{TG concentration (mmol/L)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 2.25.$$

3.2.6.2.3 Determination of High Density Lipoprotein Cholesterol (HDL-c):

Serum high density lipoprotein cholesterol was calorimetrically determined according to the method described by Lopes-Virella *et al*. (1977), using a Spectrophotometer (Thermo Fisher Scientific Genesys, USA) at 500 nm wave length. The concentration of the sample was calculated from the following equation:-

$$\textbf{HDL-c concentration (mmol/L)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 4.65.$$

3.2.6.2.4 Determination of Low Density Lipoprotein Cholesterol (LDL-c):

Serum low density lipoproteins cholesterol was calorimetrically determined according to the method described by Fridewald *et al.* (1972). The concentration of the sample was calculated from the following equation:-

$$\textbf{LDL-c concentration (mmol/L)} = \text{Total cholesterol} - \left(\frac{\text{TG}}{2.2} + \text{HDL-c}\right).$$

3.2.6.2.5 Determination of Very Low Density Lipoprotein Cholesterol (VLDL-c):

Serum very low density lipoproteins cholesterol was calorimetrically determined according to the method described by Fridewald *et al.* (1972). The concentration of the sample was calculated from the following equation:

$$\textbf{VLDL-c concentration (mmol/L)} = \frac{\text{Triglycerides}}{2.2}.$$

3.2.6.2.6 Determination of Liver Enzyme Activity:

3.2.6.2.6.1 Determination of AST and ALT enzyme activity:

Serum aspartate aminotransferase (AST) activity and alanine aminotransferase (ALT) activity were estimated enzymatically based on color reaction formation. The developed color was measured according to the method described by Bergmeyer *et al.* (1978) using a Spectrophotometer (Thermo Fisher Scientific Genesys, USA) adjusted at

505 nm wave length. The concentration was calculated by matching the reading of optical density of concentration of the sample with that of the standard solution.

3.2.6.3 Determination of Antioxidant Enzyme Activity:

The frozen liver samples were thoroughly homogenized on ice with Tri- HCL buffer solution (PH 7.4) to obtain 10% tissue homogenate. The prepared liver homogenates were used for measurement of activities of antioxidant enzymes.

3.2.6.3.1 Determination of Catalase (CAT):

Catalase activity was measured by monitoring the decomposition of H_2O_2 at 240 nm wave length (extinction coefficient 0.00394 ± 0.0002 mM^{-1} mm^{-1}) according to the method described by Sinha (1972). CAT enzyme activity was expressed as U of catalase/mg protein (1 unit of catalase is defined as the amount of enzyme required to hydrolyze 1 μmol of H_2O_2 per min).

3.2.6.3.2 Determination of Superoxide Dismutase (SOD):

Superoxide dismutase (SOD) activity was assessed using a Xanthine oxidase system to generate superoxide radicals (O_2^-) as described by Kakkar *et al*. (1984). The rate of suppression of the reduction of Nitro tetrazolium blue (NTB) by O_2^- was monitored at 550 nm wave length. SOD enzyme activity was expressed as U of SOD/mg of protein (1 unit of SOD is defined as the amount of enzyme required to inhibit the rate of reduction of NTB by 50%.

3.2.6.3.3 Determination of Glutathione Peroxidase (GPx):

Glutathione peroxidase (GPx) activity was assayed by NADPH oxidation at 340 nm wave length when GSSG is reduced back by glutathione reductase as described by Paglia and Valentaine (1967), using cumene hydroperoxide (relatively stable organic peroxide, acts as oxidizing agent) as a substrate. Glutathione peroxidase activity was calculated using an extinction coefficient of 6.22 mM^{-1} cm^{-1} and expressed as U of GPx /mg of protein (1 unit of GPx is defined as the amount of enzyme required to convert 1 nmol NADPH to $NADP^{+}$ per min).

3.2.7 Histopathological Examination:

Specimens from the halves of liver and heart were taken immediately after weighed the organs of the rats and immersed in 10% neutral buffered formalin. The fixed specimens were then trimmed, washed, and dehydrated in ascending grades of alcohol, then cleared in xylene, and stained with Hematoxylin and Eosin (H&E) and examined microscopically according to Bancroft and Gamble (2008).

3.3 Statistical Analysis:

Statistical analyses were carried out using Statistical Package for the Social Sciences (SPSS) for Windows, version 18 (SPSS Inc., Chicago, IL, USA). The obtained data were presented as means ± standard deviation (SD). Statistical analysis of variance between mean values of different groups was performed using one way ANOVA test followed by the least significant difference (LSD) test to determine the variance between all treatments. Differences were considered significant at $P<0.05$.

CHAPTER IV

RESULTS

Chapter IV

RESULTS

In the current study, the results of estimation of total polyphenols content of Pomegranate juice, effect of Pomegranate juice on initial body weight, final body weight, daily feed intake, body weight gain percent (BWG %), feed efficiency ratio (FER), relative organs weight to body weight as well as some biochemical constituents in serum of hypercholesterolemic rats and histopathological examination of liver and heart are depicted in Tables 4.1 to 4.8 and illustrated in Figures 4.1 to 4.28. These effects were explored when Pomegranate juice was orally given to hypercholesterolemic rats at three dosage levels for 28 days.

4.1 Estimation of total polyphenols content of Pomegranate juice:

The total polyphenols content of Pomegranate juice as gallic acid equivalents was 3.9 ± 0.1 mg /ml as recorded in Table 4.1.

Table 4.1 Total polyphenols as gallic acid equivalents (GAEs), of Pomegranate juice

Beverage	Gallic acid equivalents GAEs (mg/ml)
Pomegranate juice	3.9 ± 0.1

Mean ± SD of triplicate measurements.

4.2 Effect of oral administration of Pomegranate juice on initial body weight, final body weight and body weight gain percent (BWG %) in hypercholesterolemic rats:

The initial body weight, final body weight and body weight gain percent (BWG %) of hypercholesterolemic rats treated with Pomegranate juice at three dosage levels (1, 3 and 5 ml/kg b. wt.) are presented in Table 4.2 and Figures 4.1, 4.2 and 4.3.

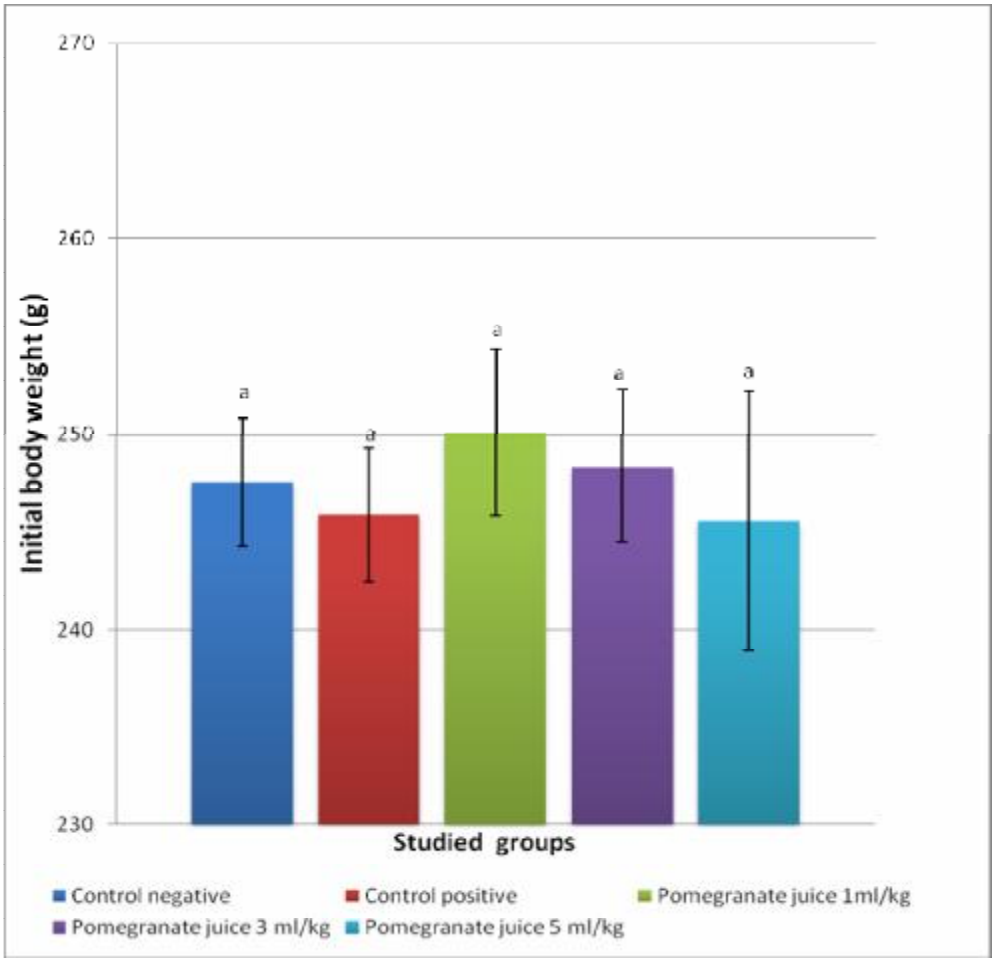
Data recorded in Table 4.2 and Figure 4.1 showed that there was no significant differences in initial body weight between all experimental groups. A significant ($P< 0.05$) increase was observed in the final body weight of hypercholesterolemic rats (positive control group), compared to the normal rats (negative control group) as shown in Table 4.2 and Figure 4.2. Oral administration of Pomegranate juice in doses of 3 and 5 ml/kg b. wt., significantly ($P< 0.05$) decreased the final body weight when compared to the hypercholesterolemic rats (positive control group) by 7.8 and 9.03 % respectively. No significant difference was observed between the group given the low dose (1 ml/kg b. wt.) of Pomegranate juice and the positive control group.

Concerning body weight gain percent, the results showed that there was a significant increase in the hypercholesterolemic rats (positive control group) when compared to normal rats (negative control group) by 14.87 %. Oral administration of Pomegranate juice in doses of 1, 3 and 5 ml/kg b. wt., significantly ($P < 0.05$) decreased the BWG % by 5.4, 11.15 and 11.4 % respectively, when compared to hypercholesterolemic rats (positive control group) as shown in Table 4.2 and Figure 4.3.

Table 4.2 Effect of oral administration of Pomegranate juice on initial body weight, final body weight and body weight gain %(BWG %) in hypercholesterolemic rats

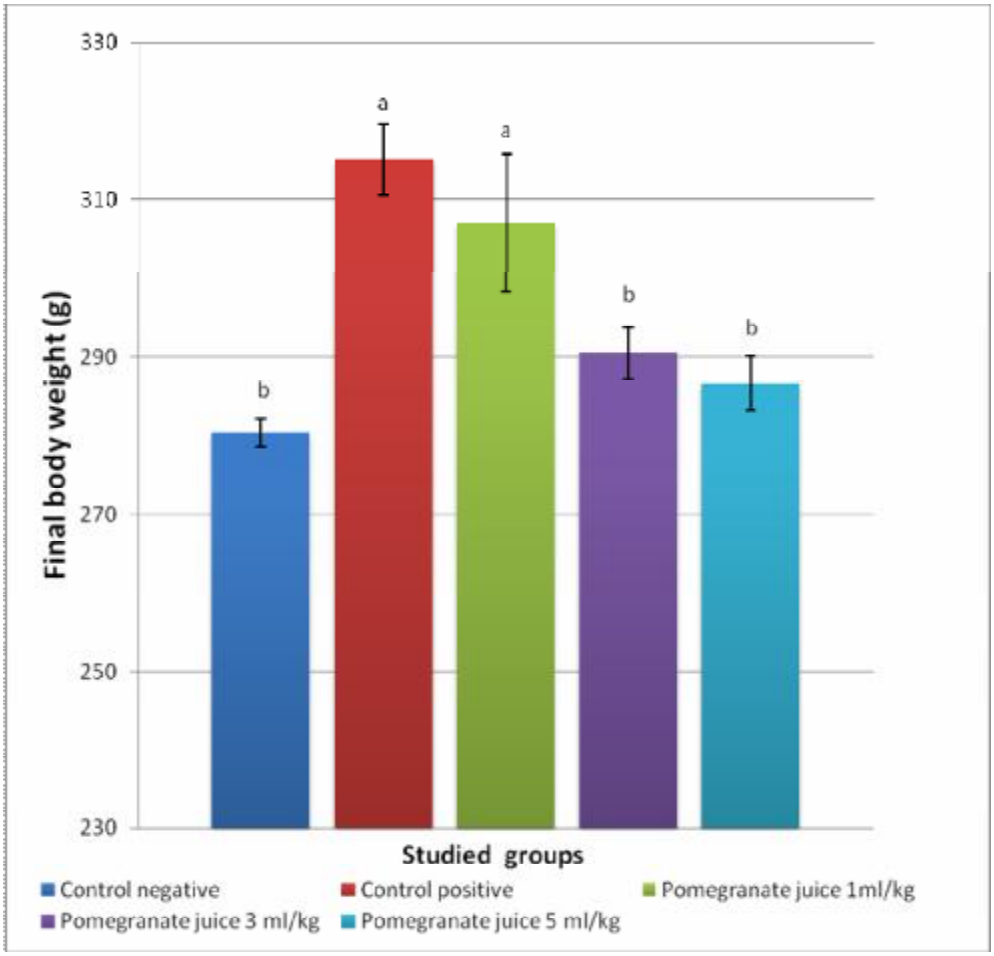
Parameter / Groups	Initial weight (g)	Final weight (g)	Body weight gain (%)
Negative control	247.54 ± 3.29[a]	280.40 ± 1.87[b]	13.29 ± 1.15[c]
Positive control	245.88 ± 3.40[a]	315.10 ± 4.45[a]	28.16 ± 1.30[a]
Pomegranate juice 1 ml/kg	250.08 ± 4.24[a]	307.02 ± 8.78[a]	22.76 ± 2.18[b]
Pomegranate juice 3 ml/kg	248.34 ± 3.89[a]	290.50 ± 3.28[b]	17.01 ± 3.05[c]
Pomegranate juice 5 ml/kg	245.56 ± 6.60[a]	286.62 ± 3.43[b]	16.76 ± 1.99[c]

Data are presented as means ± standard deviation, (n = 7 for each group).
Values with different superscripts within each column are significantly different at $P < 0.05$.
Values with similar or partially similar superscripts are non significant.



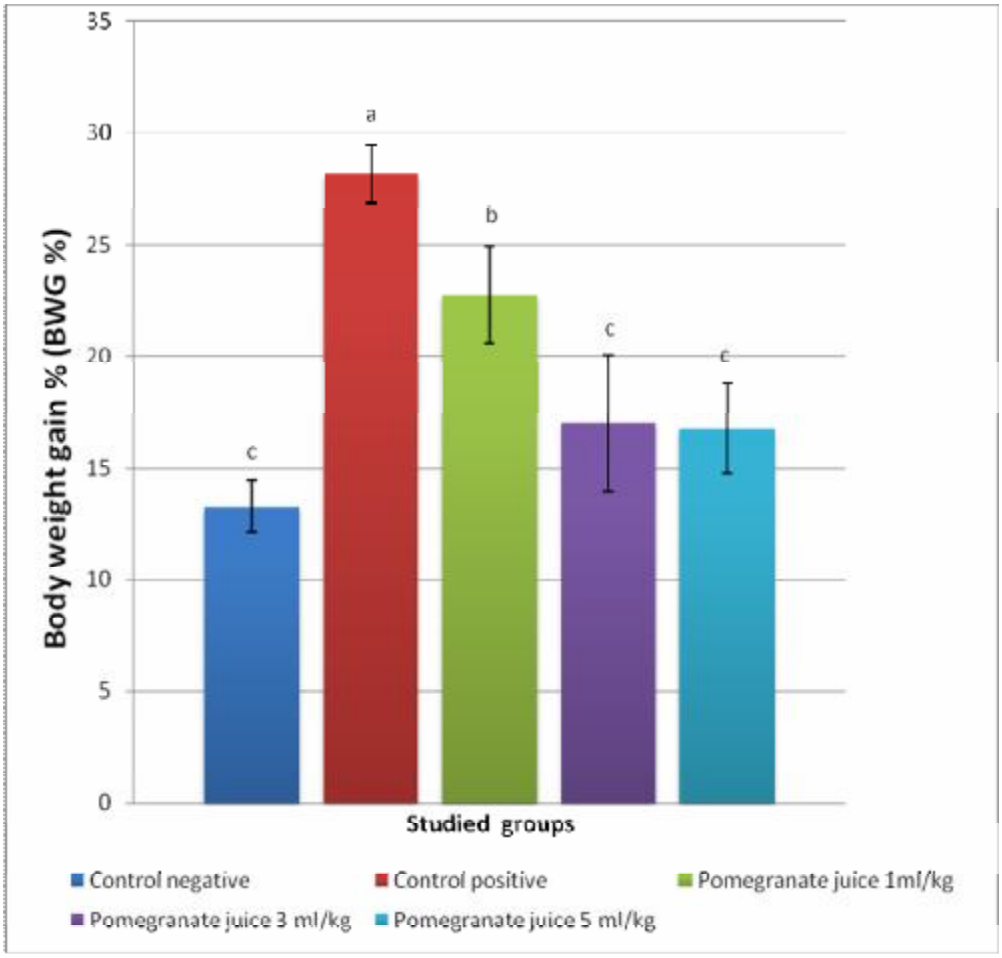
Values with different superscripts within the column are significantly different at P< 0.05.

Figure 4.1 Effect of oral administration of Pomegranate juice on the initial body weight in hypercholesterolemic rats



Values with different superscripts within the column are significantly different at P< 0.05.

Figure 4.2 Effect of oral administration of Pomegranate juice on the final body weight in hypercholesterolemic rats



Values with different superscripts within the column are significantly different at P< 0.05.

Figure 4.3 Effect of oral administration of Pomegranate juice on the body weight gain percent (BWG %) in hypercholesterolemic rats

4.3 Effect of oral administration of Pomegranate juice on feed intake and feed efficiency ratio (FER) in hypercholesterolemic rats:

Feed intake and feed efficiency ratio (FER) of hypercholesterolemic rats orally given Pomegranate juice at three dosage levels (1, 3 and 5 ml/kg b. wt.) are shown in Table 4.3 and Figures 4.4 and 4.5.

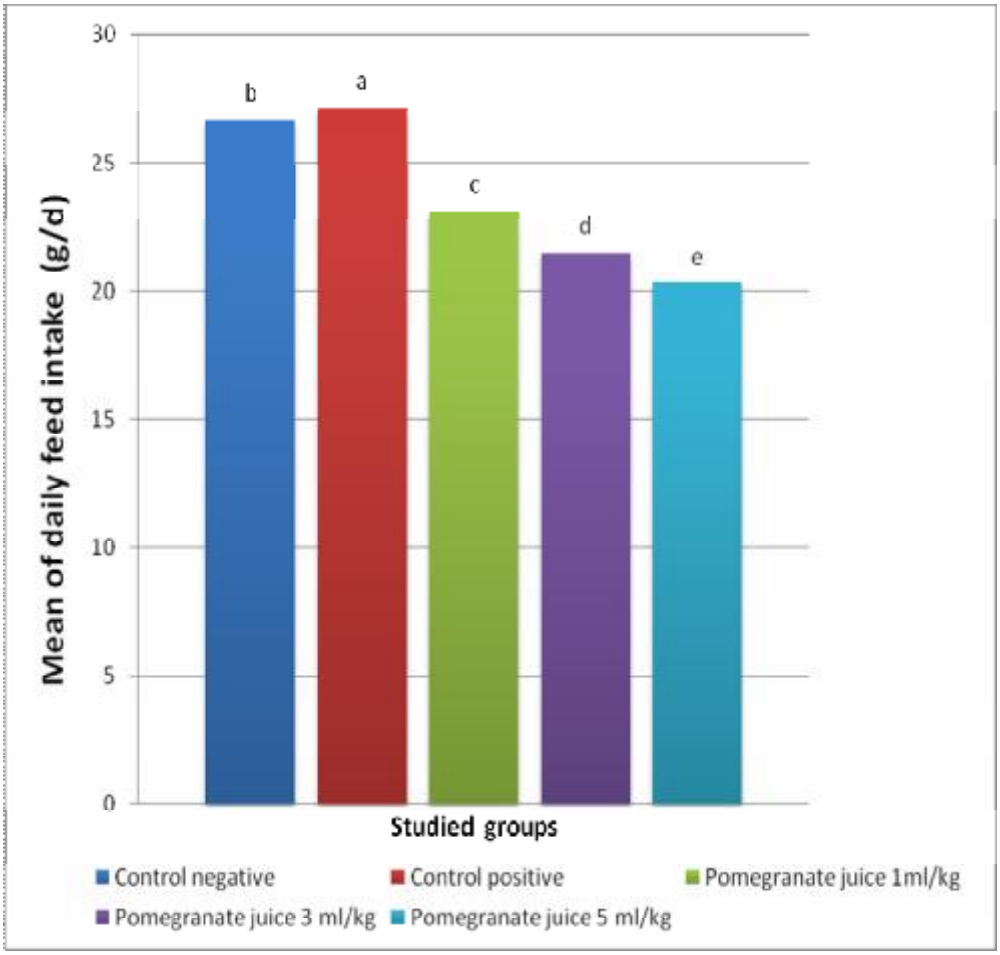
Feed intake was significantly ($P< 0.05$) increased in the hypercholesterolemic rats (positive control group), compared to normal rats (negative control group) by 1.61 %.Oral administration of Pomegranate juice at three dosage levels 1, 3 and 5 ml/kg b. wt., decreased feed intake as compared to hypercholesterolemic rats (positive control group) by 14.76, 20.66 and 25.10 % respectively.

It is clear from Table 4.3 and Figure 4.5 that FER in hypercholesterolemic rats (positive control group) significantly ($P< 0.05$) increased when compared to normal rats (negative control group) by 93.6 %. Significant ($P< 0.05$) decreases were observed in rats orally given Pomegranate juice in doses of 3 and 5 ml/kg b. wt., as compared to the positive control group by 23.07 and 20.87 % respectively. The dose 1 ml/kg b. wt., didn’t cause a significant change when compared to the positive control group.

Table 4.3 Effect of oral administration of Pomegranate juice on feed intake and feed efficiency ratio (FER) in hypercholesterolemic rats

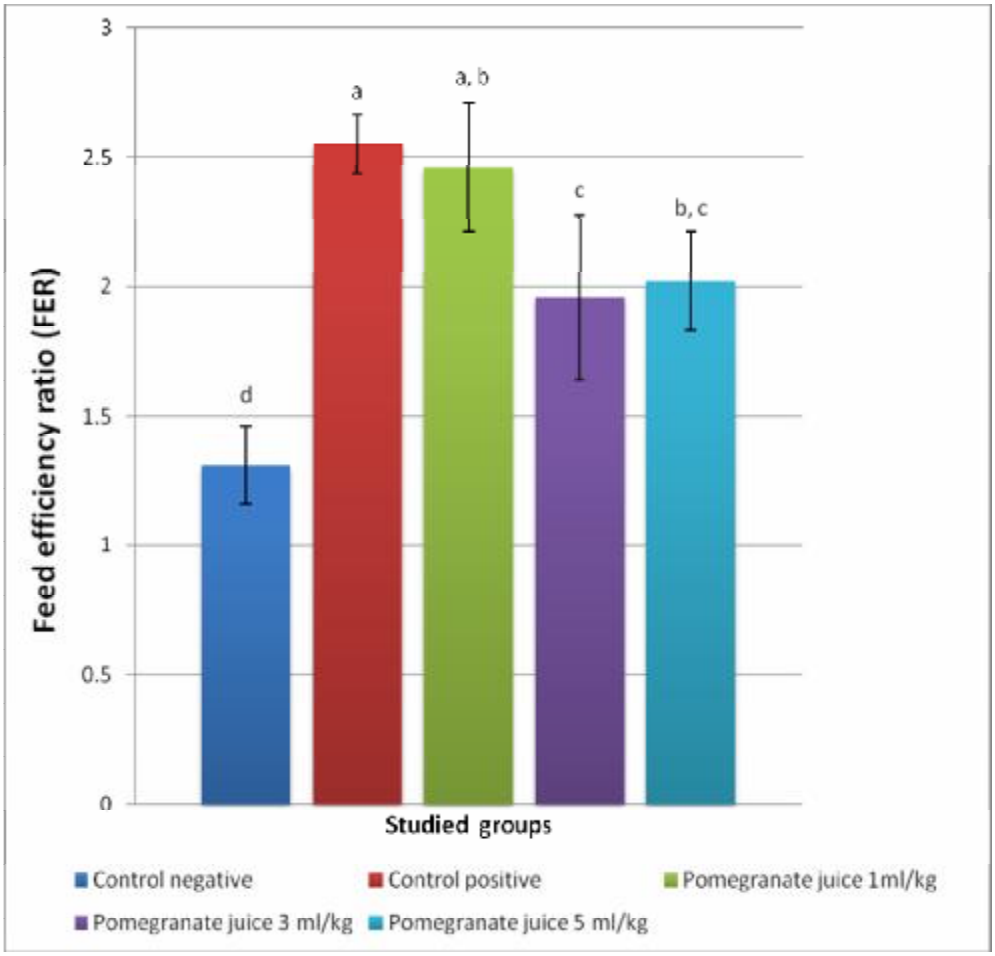
Parameter / Groups	Mean of daily feed intake (g/d)	Feed efficiency ratio (FER)
Negative control	26.67 [b]	0.047 ± 0.005 [d]
Positive control	27.10 [a]	0.091 ± 0.002 [a]
Pomegranate juice 1 ml/kg	23.10 [c]	0.088 ± 0.009 [a, b]
Pomegranate juice 3 ml/kg	21.50 [d]	0.070± 0.01 [c]
Pomegranate juice 5 ml/kg	20.30 [e]	0.072 ± 0.007 [b, c]

Data are presented as means ± standard deviation, (n = 7 for each group).
Values with different superscripts within the column are significantly different at P< 0.05.
Values with similar or partially similar superscripts are non significant.



Values with different superscripts within the column are significantly different at P< 0.05.

Figure 4.4 Effect of oral administration of Pomegranate juice on feed intake in hypercholesterolemic rats



Values with different superscripts within the column are significantly different at P< 0.05.

Figure 4.5 Effect of oral administration of Pomegranate juice on feed efficiency ratio (FER) in hypercholesterolemic rats

4.4 Effect of oral administration of Pomegranate juice on liver and heart relative weight to the body weight in hypercholesterolemic rats:

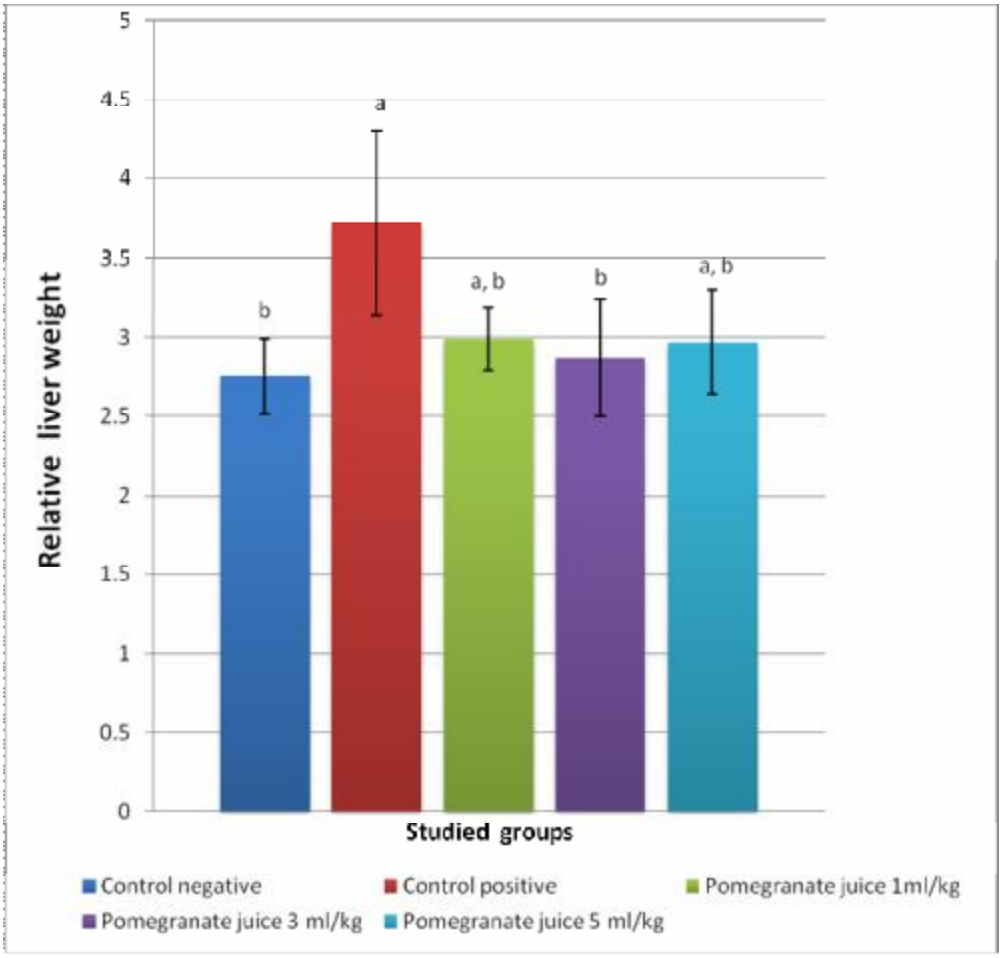
Concerning the relative organs weight to the body weight of rats, the results showed that hypercholesterolemic rats had a significant ($P< 0.05$) increase in the relative weights of liver and heart as compared to normal rats (negative control group) by 0.97 and 0.11 % respectively as depicted in Table 4.4 and Figures 4.6 and 4.7.

Oral administration of Pomegranate juice in a dose of 3 ml/ kg b. wt., significantly decreased liver and heart weight compared to hypercholesterolemic rats (positive control group) by 0.85 and 0.09 % respectively. No significant changes were observed in rats orally given Pomegranate juice in doses of 1 and 5 ml/ kg b. wt., for both organs when compared to hypercholesterolemic rats (positive control group).

Table 4.4 Effect of oral administration of Pomegranate juice on liver and heart relative weight in hypercholesterolemic rats

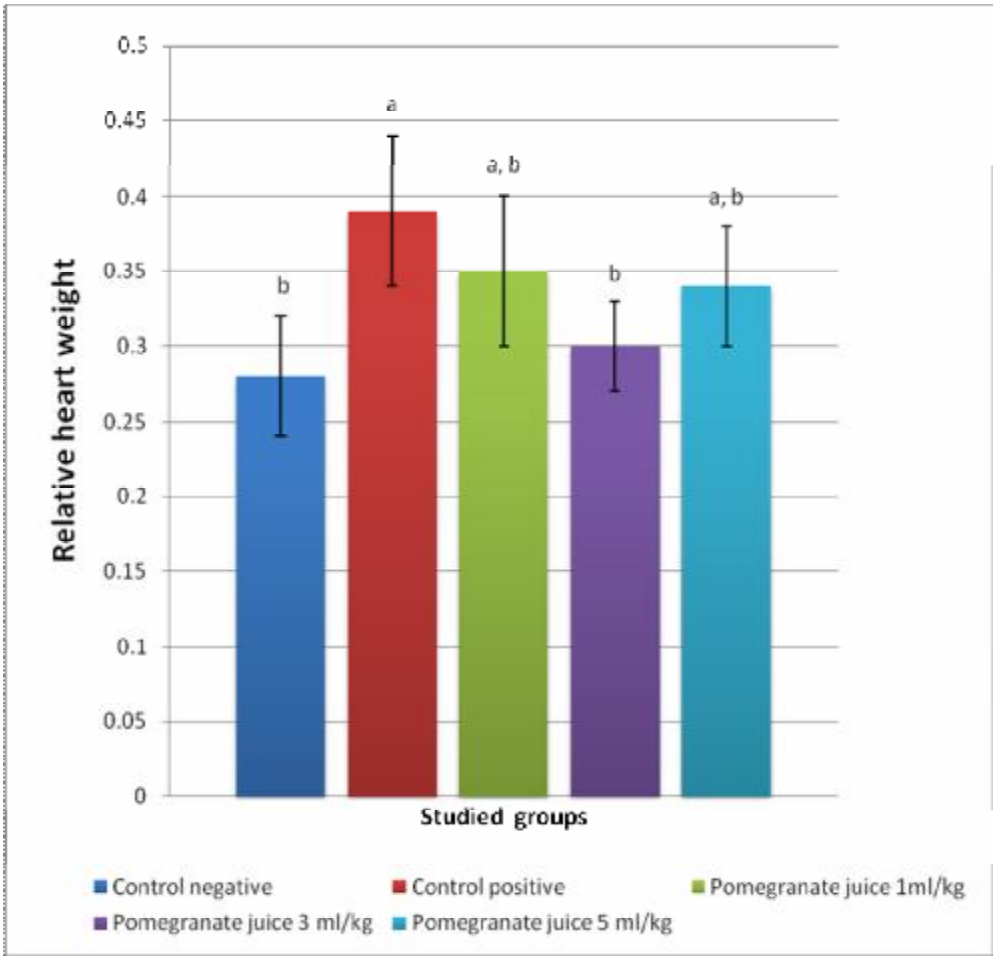
Parameter / Groups	Relative liver weight	Relative heart weight
Negative control	2.75 ± 0.24^{b}	0.28 ± 0.04^{b}
Positive control	3.72 ± 0.58^{a}	0.39 ± 0.05^{a}
Pomegranate juice 1 ml/kg	$2.99 \pm 0.20^{a, b}$	$0.35 \pm 0.05^{a, b}$
Pomegranate juice 3 ml/kg	2.87 ± 0.37^{b}	0.30 ± 0.03^{b}
Pomegranate juice 5 ml/kg	$2.97 \pm 0.33^{a, b}$	$0.34 \pm 0.04^{a, b}$

Data are presented as means ± standard deviation, (n = 7 for each group).
Values with different superscripts within the column are significantly different at $P < 0.05$.
Values with similar or partially similar superscripts are non significant.



Values with different superscripts within the column are significantly different at P< 0.05.

Figure 4.6 Effect of Pomegranate juice on relative weight of liver of hypercholesterolemic rats



Values with different superscripts within the column are significantly different at P< 0.05.

Figure 4.7 Effect of Pomegranate juice on relative weight of heart of hypercholesterolemic rats

4.5 Effect of oral administration of Pomegranate juice on the serum level of total cholesterol (TC) and triglycerides (TG) in hypercholesterolemic rats:

Results of biochemical analyses revealed that hypercholesterolemic rats (positive control group) had a significant ($P<0.05$) increase in total cholesterol (TC) by 177.4 % and triglycerides (TG) by 77.77 % compared to normal rats (negative control group) as recorded in Table 4.5 and shown in Figures 4.8 and 4.9.

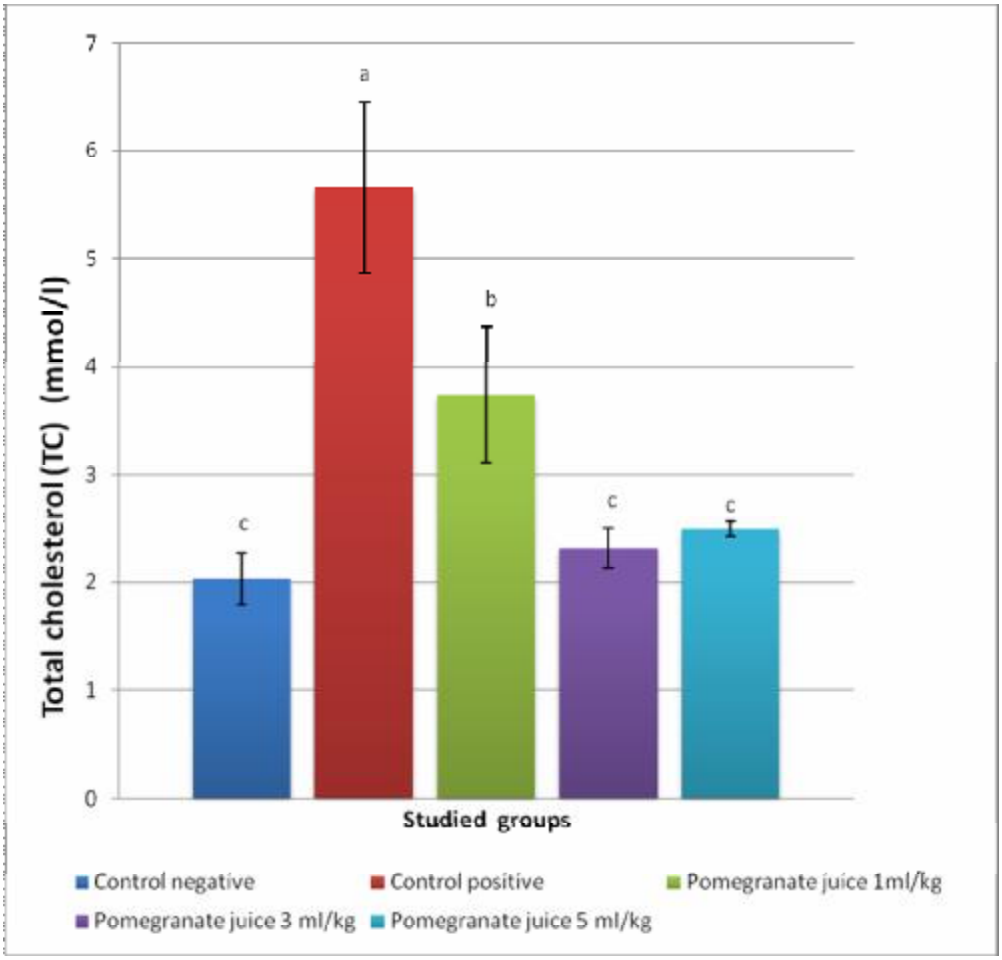
Oral administration of Pomegranate juice at the tested doses 1, 3and 5 ml/kg b. wt., significantly ($P<0.05$) decreased serum TC by 33.92, 59.01 and 55.83 % respectively compared to hypercholesterolemic rats (positive control group) as shown in Table 4.5 and Figure 4.8.

Oral administration of 1 and 5 ml/kg b. wt., of Pomegranate juice to hypercholesterolemic rats significantly ($P<0.05$) reduced serum TG levels by 40 and 40.62 % respectively when compared to hypercholesterolemic rats (positive control group). The dose of 3 ml/kg b. wt., caused no significant change when compared to the positive control group as shown in Table 4.5 and Figure 4.9.

Table 4.5 Effect of oral administration of Pomegranate juice on the serum levels of total cholesterol (TC) and triglycerides (TG) in hypercholesterolemic rats

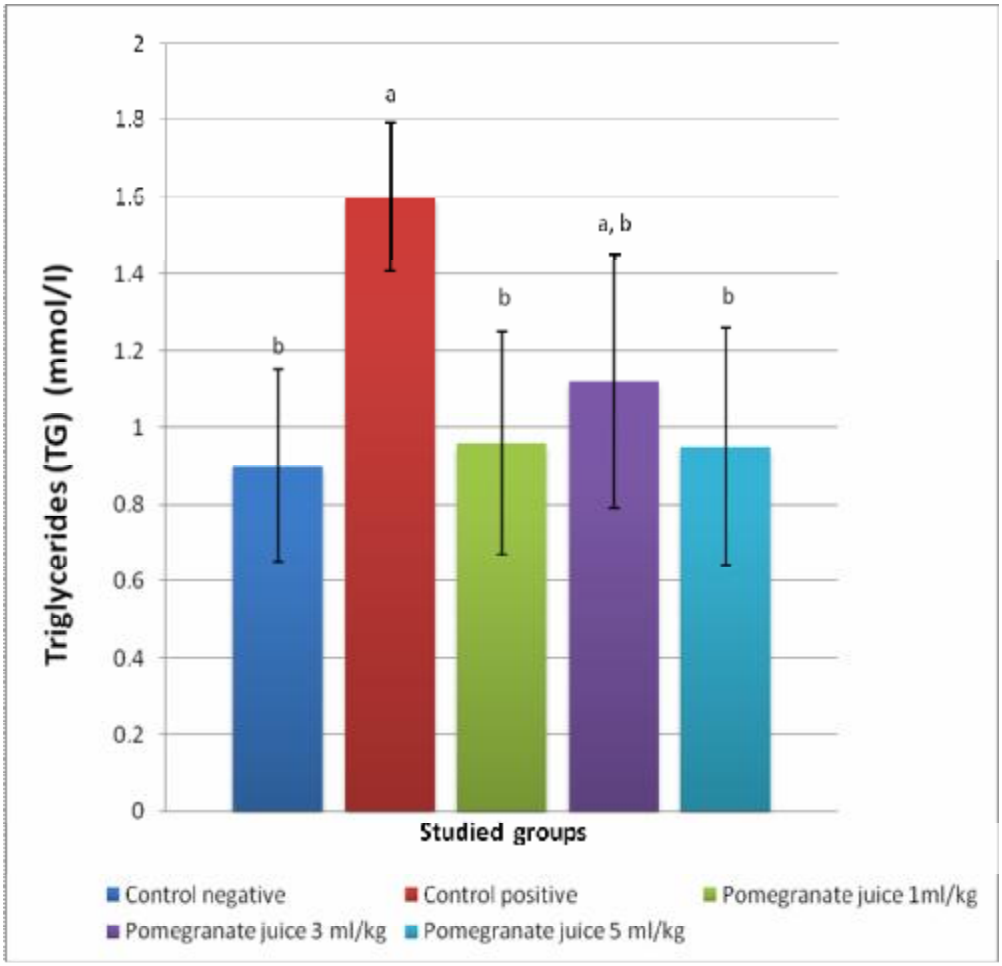
Parameter / Groups	TC (mmol/L)	TG (mmol/L)
Negative control	2.04 ± 0.24^{c}	0.90 ± 0.25^{b}
Positive control	5.66 ± 0.79^{a}	1.60 ± 0.19^{a}
Pomegranate juice 1 ml/kg	3.74 ± 0.62^{b}	0.96 ± 0.29^{b}
Pomegranate juice 3 ml/kg	2.32 ± 0.19^{c}	$1.12 \pm 0.33^{a, b}$
Pomegranate juice 5 ml/kg	2.50 ± 0.07^{c}	0.95 ± 0.31^{b}

Data are presented as means ± standard deviation, (n = 7 for each group).
Values with different superscripts within the column are significantly different at $P< 0.05$.
Values with similar or partially similar superscripts are non significant.



Values with different superscripts within the column are significantly different at P< 0.05.

Figure 4.8 Effect of oral administration of Pomegranate juice on serum total cholesterol (TC) in hypercholesterolemic rats



Values with different superscripts within the column are significantly different at P< 0.05.

Figure 4.9 Effect of oral administration of Pomegranate juice on serum triglycerides (TG) in hypercholesterolemic rats

4.6 Effect of oral administration of Pomegranate juice on the serum levels of lipoprotein fractions in hypercholesterolemic rats:

Results in Table 4.6 and Figures 4.10, 4.11 and 4.12 illustrate the effect of different doses of Pomegranate juice on serum levels of lipoprotein fractions in hypercholesterolemic rats.

Results of high density lipoprotein cholesterol (HDL-c) are recorded in Table 4.6 and shown in Figure 4.10. The obtained data indicated that hypercholesterolemic rats (positive control group) had a significant ($P< 0.05$) decrease in serum of hypercholesterolemic rats when compared with the normal rats (negative control group) by 22 %. Oral administration of 1, 3 and 5 ml/kg b. wt., of Pomegranate juice to hypercholesterolemic rats significantly ($P<0.05$) increases serum HDL-c level by 54.69, 50 and 71.88 % respectively when compared to hypercholesterolemic rats (positive control group), as shown in Table 4.6 and Figure 4.10.

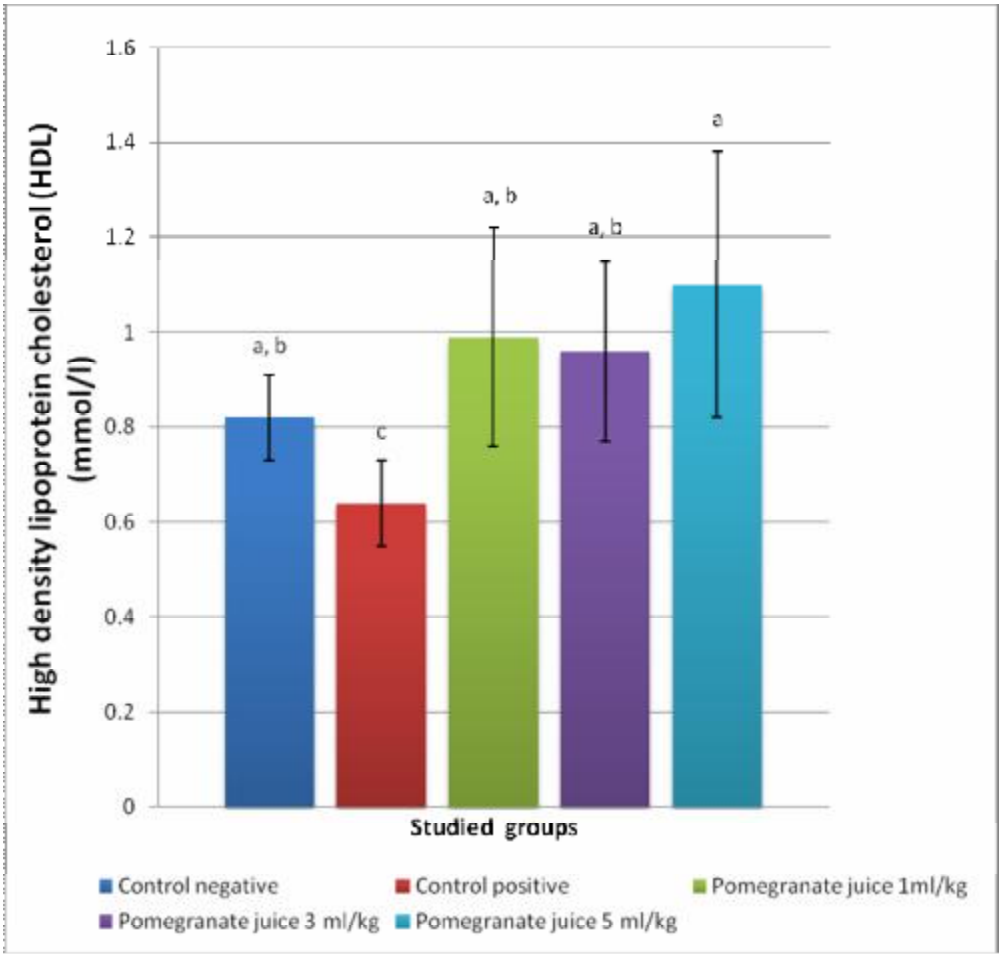
It is clear from Table 4.6 and Figure 4.11 that hypercholesterolemic rats (positive control group) had a significant ($P<0.05$) increase in serum level of low density lipoprotein cholesterol (LDL-c) when compared to the normal rats (negative control group) by 382.9 %. Oral administration of Pomegranate juice at three dosage levels significantly ($P<0.05$) decreased serum levels of LDL-c when compared to hypercholesterolemic rats (positive control group). The decreases in serum levels of LDL-c in rats given Pomegranate juice at doses 1, 3 and 5 ml/kg b. wt., groups were 41.4, 75.75 and 75.25 % respectively.

Concerning serum levels of very low density lipoprotein cholesterol (VLDL-c), the results revealed that hypercholesterolemic rats (positive control group) had a significant ($P<0.05$) increase in serum level of VLDL-c when compared to normal rats (negative control group) by 75.6 %. Oral administration of Pomegranate juice at 1, 3 and 5 ml/kg b. wt., for 28 days produced significant ($P<0.05$) decreases in serum levels of VLDL-c by 40.27, 43.05 and 40.27% respectively, when compared to the hypercholesterolemic rats(positive control group) as shown in Table 4.6 and Figure 4.12.

Table 4.6 Effect of oral administration of Pomegranate juice on the serum levels of lipoprotein fractions in hypercholesterolemic rats

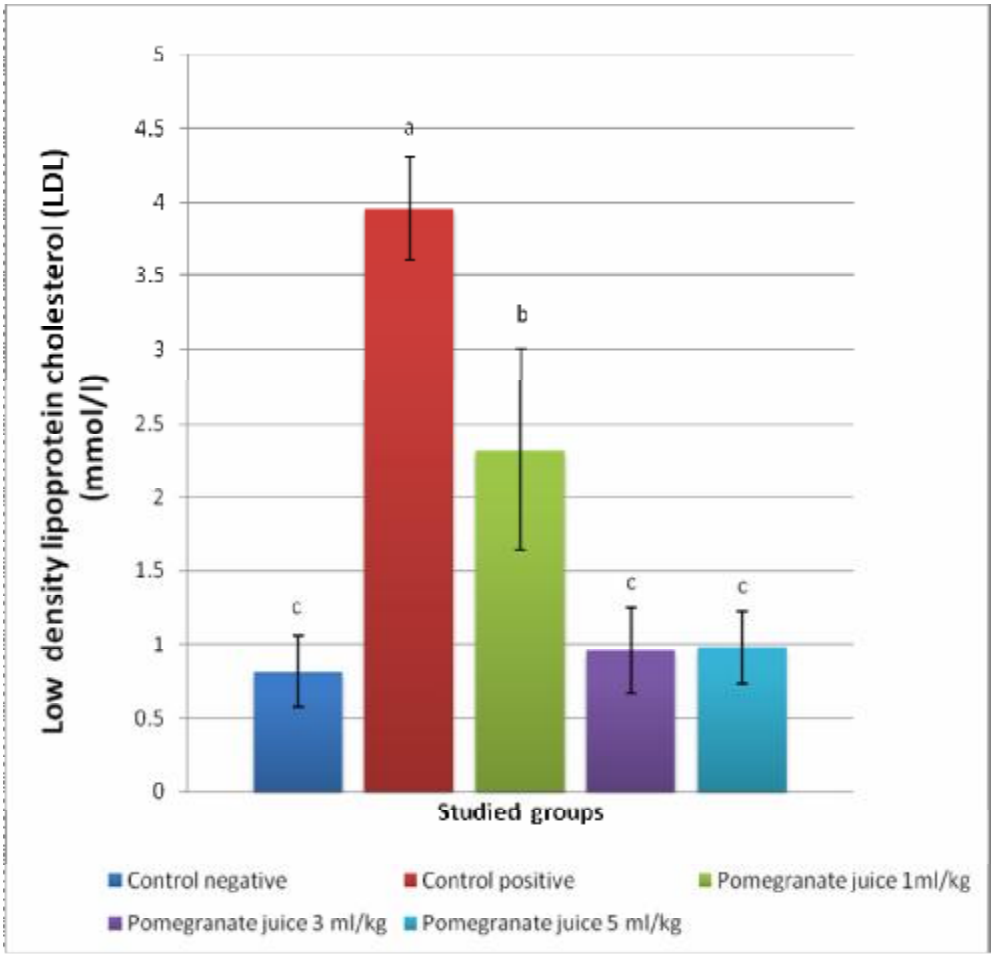
Parameter / Groups	HDL-c (mmol/L)	LDL-c (mmol/L)	VLDL-c (mmol/L)
Negative control	$0.82 \pm 0.09^{a, b}$	0.82 ± 0.24^{c}	0.41 ± 0.11^{b}
Positive control	0.64 ± 0.09^{c}	3.96 ± 0.35^{a}	0.72 ± 0.19^{a}
Pomegranate juice 1 ml/kg	$0.99 \pm 0.23^{a, b}$	2.32 ± 0.68^{b}	0.43 ± 0.13^{b}
Pomegranate juice 3 ml/kg	$0.96 \pm 0.19^{a, b}$	0.96 ± 0.29^{c}	0.41 ± 0.16^{b}
Pomegranate juice 5 ml/kg	1.10 ± 0.28^{a}	0.98 ± 0.24^{c}	0.43 ± 0.15^{b}

Data are presented as means ± standard deviation, (n = 7 for each group).
Values with different superscripts within the column are significantly different at $P < 0.05$.
Values with similar or partially similar superscripts are non significant.



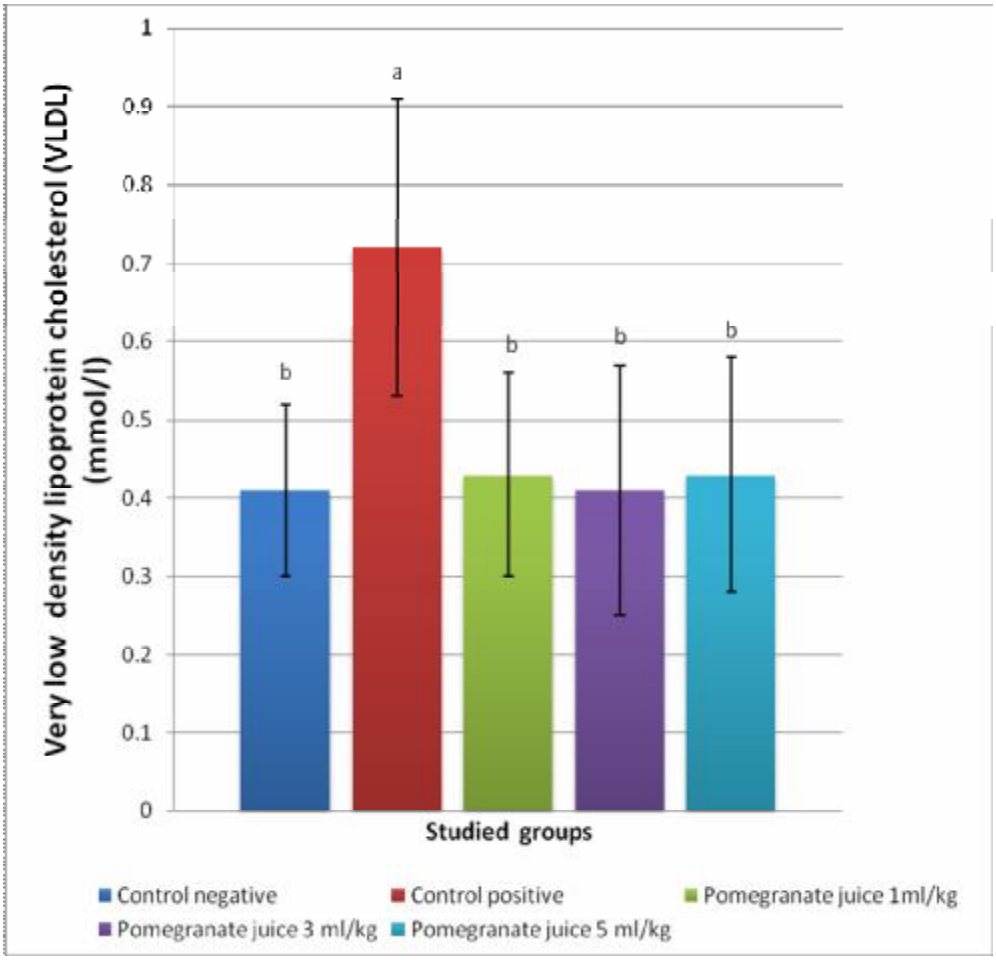
Values with different superscripts within the column are significantly different at P< 0.05.

Figure 4.10 Effect of oral administration of Pomegranate juice on serum levels of high density lipoprotein cholesterol (HDL-c) in hypercholesterolemic rats



Values with different superscripts within the column are significantly different at P< 0.05.

Figure 4.11 Effect of oral administration of Pomegranate juice on serum levels of low density lipoprotein cholesterol (LDL-c) in hypercholesterolemic rats



Values with different superscripts within the column are significantly different at P< 0.05.

Figure 4.12 Effect of oral administration of Pomegranate juice on serum levels of very low density lipoprotein cholesterol (VLDL-c) in hypercholesterolemic rats

4.7 Effect of oral administration of Pomegranate juice on serum levels of liver function enzymes in hypercholesterolemic rats

Results of liver function tests of hypercholesterolemic rats orally given Pomegranate juice at three dosages levels 1, 3 and 5 ml/kg b. wt., are shown in Table 4.7 and Figures 4.13 and 4.14.

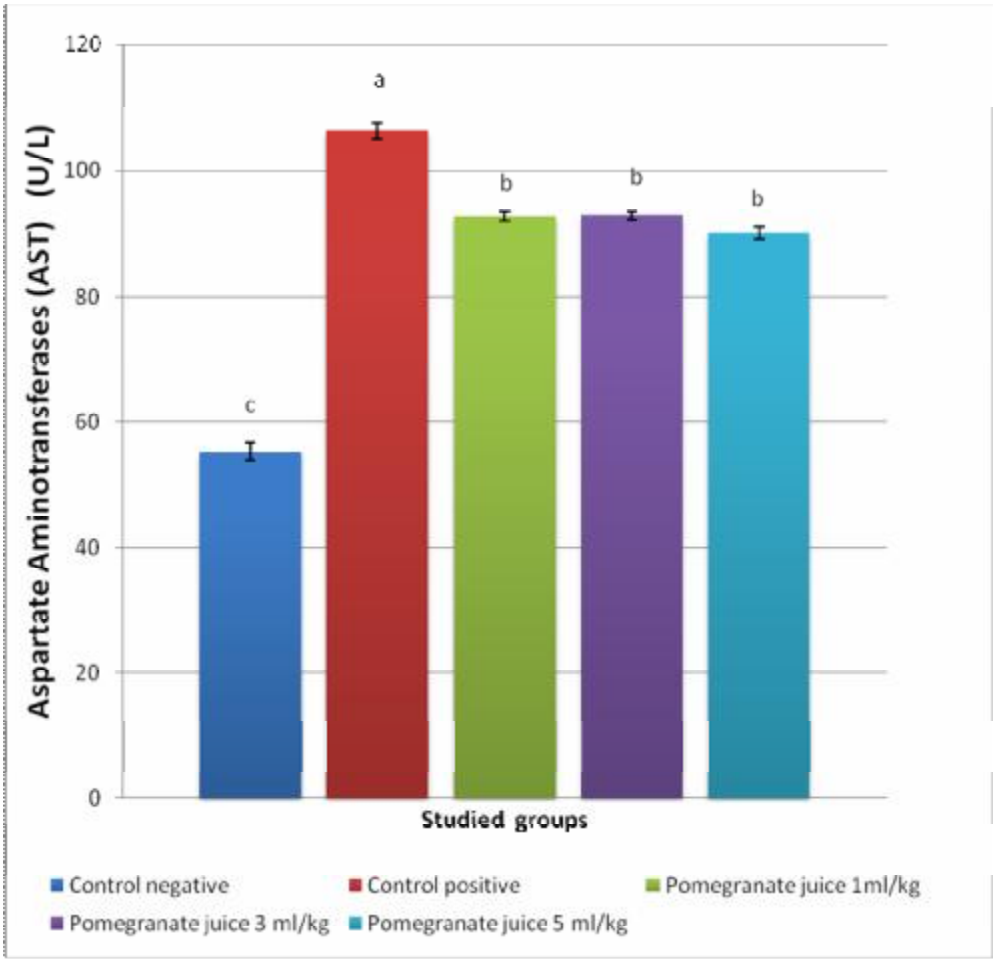
It is clear from Table 4.7 and Figures 4.13 and 4.14 that hypercholesterolemic rats (positive control group) had significant ($P< 0.05$) increases in serum levels of AST and ALT enzymes in serum of hypercholesterolemic rats when compared to the normal rats (negative control group) by 92.57 and 86.9 % respectively.

Oral administration of 1, 3 and 5 ml/kg b. wt., of Pomegranate juice to hypercholesterolemic rats significantly ($P<0.05$) reduced serum AST enzyme level by 12.84, 12.77 and 15.38 % respectively as compared to hypercholesterolemic rats (positive control group), as shown in Table 4.7 and Figure 4.13. The corresponding percentages of decreased ALT were 13.40, 16.40 and 23% respectively as compared to the hypercholesterolemic rats (positive control group) as shown in Table 4.7 and Figure 4.14.

Table 4.7 Effect of oral administration of Pomegranate juice on serum levels of liver function enzymes in hypercholesterolemic rats

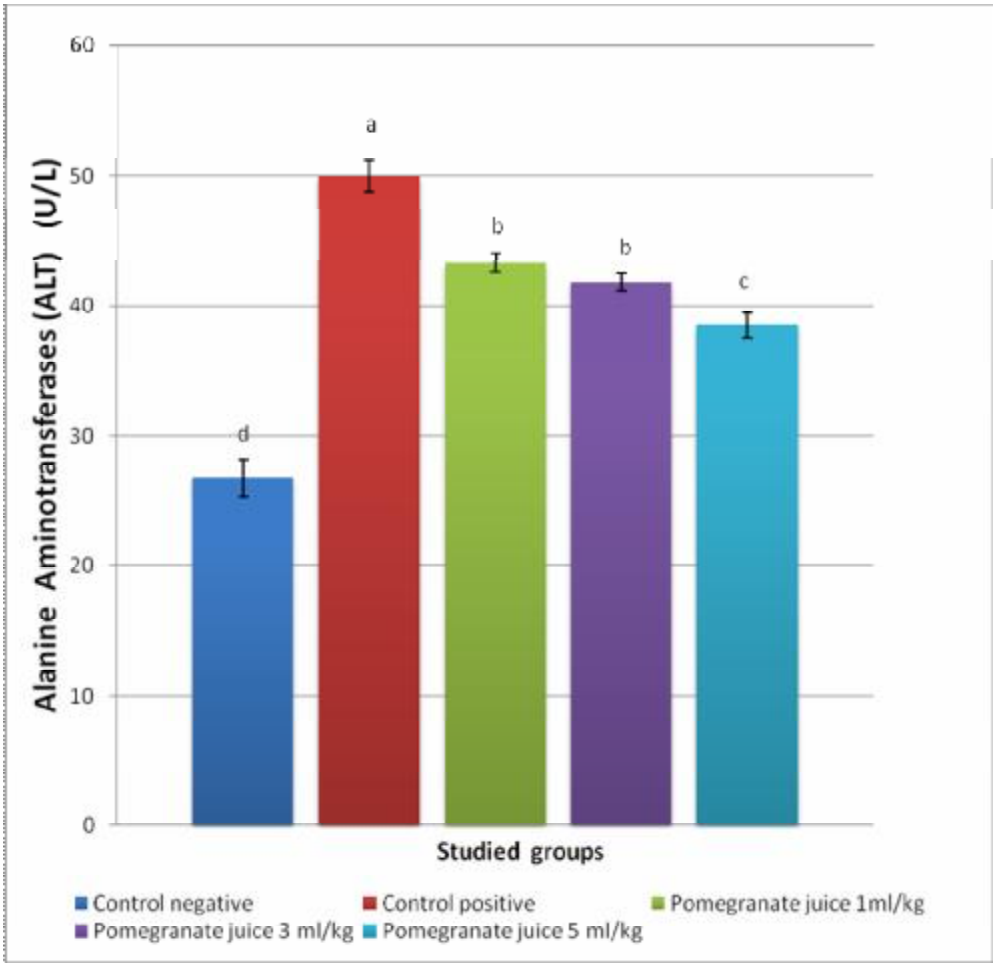
Parameter / Groups	AST (U/L)	ALT (U/L)
Negative control	55.24 ± 2.33 [c]	26.74 ± 0.88 [d]
Positive control	106.38 ± 4.33 [a]	50.00 ± 1.01 [a]
Pomegranate juice 1 ml/kg	92.72 ± 1.59 [b]	43.30 ± 0.47 [b]
Pomegranate juice 3 ml/kg	92.80 ± 1.72 [b]	41.80 ± 0.62 [b]
Pomegranate juice 5 ml/kg	90.02 ± 0.22 [b]	38.50 ± 1.10 [c]

Data are presented as means ± standard deviation, (n = 7 for each group).
Values with different superscripts within the column are significantly different at $P < 0.05$.
Values with similar or partially similar superscripts are non significant.



Values with different superscripts within the column are significantly different at P< 0.05.

Figure 4.13 Effect of oral administration of Pomegranate juice on serum levels of Aspartate aminotransferases (AST) in hypercholesterolemic rats



Values with different superscripts within the column are significantly different at P< 0.05.

Figure 4.14 Effect of oral administration of Pomegranate juice on serum levels of Alanine aminotransferases (ALT) in hypercholesterolemic rats

4.8 Effect of oral administration of Pomegranate juice on liver homogenates levels of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) in hypercholesterolemic rats:

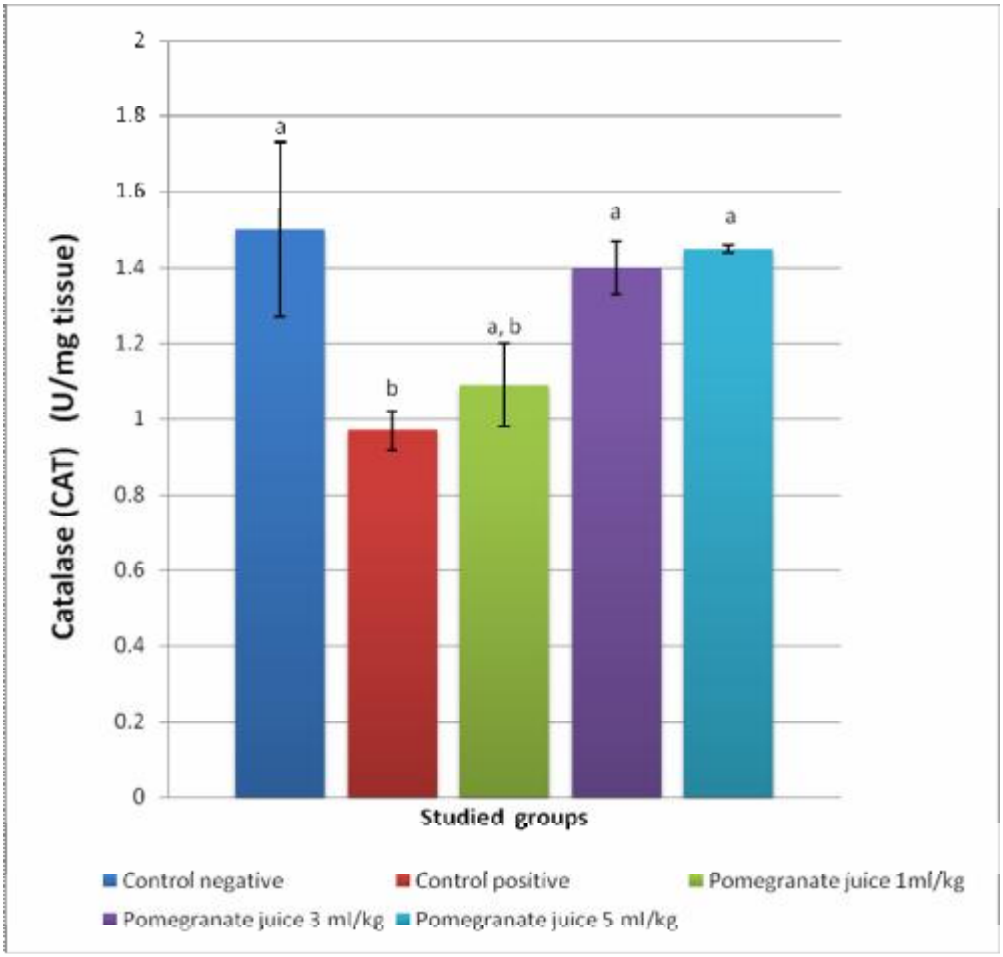
From data recorded in Table 4.8 and Figures 4.15, 4.16 and 4.17 it could be noticed that hypercholesterolemic rats (control positive group) had significant ($P<0.05$) decreases in liver homogenates levels of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzymes when compared to normal rats (negative control group) by 35.33, 32.81 and 23.65 % respectively. Oral administration of Pomegranate juice at the small dose 1 ml/kg b. wt., showed non significant changes in liver homogenates levels of CAT, SOD and GPx serum when compared to hypercholesterolemic rats (positive control group).

Administration of Pomegranate juice at doses 3 and 5 ml/kg b. wt., showed significant ($P< 0.05$) increase in CAT enzyme liver homogenates level by 44.32 and 49.48 % respectively when compared to hypercholesterolemic rats (positive control group). The corresponding percentages of increased of SOD were 41.86 and 58.14 % respectively when compared to hypercholesterolemic rats (positive control group). Regarding GPx enzyme level, a significant ($P<0.05$) increase was recorded in rats orally given Pomegranate juice in doses of 3 and 5 ml/kg b. wt., by 30.15 and 30.32 % respectively as compared to hypercholesterolemic rats (positive control group).

Table 4.8 Effect of oral administration of Pomegranate juice on liver homogenates levels of catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) enzymes in hypercholesterolemic rats

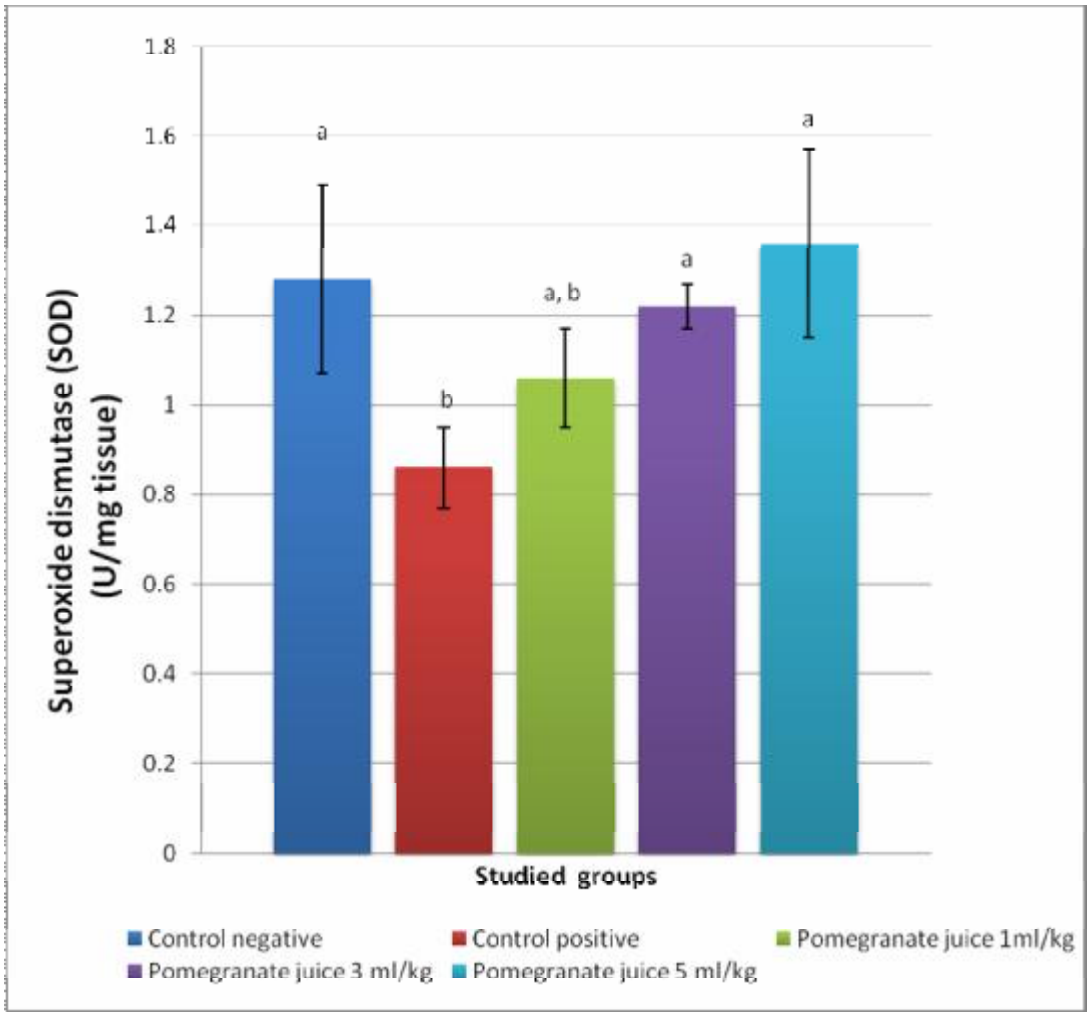
Parameter / Groups	CAT (U/mg tissue)	SOD (U/mg tissue)	GPx (U/mg tissue)
Control negative	1.50 ± 0.45^{a}	1.28 ± 0.21^{a}	62.90 ± 1.41^{a}
Control positive	0.97 ± 0.05^{b}	0.86 ± 0.09^{b}	48.02 ± 1.19^{b}
Pomegranate juice 1 ml/kg	$1.09 \pm 0.11^{a, b}$	$1.06 \pm 0.11^{a, b}$	49.42 ± 0.73^{b}
Pomegranate juice 3 ml/kg	1.40 ± 0.07^{a}	1.22 ± 0.05^{a}	62.50 ± 0.67^{a}
Pomegranate juice 5 ml/kg	1.45 ± 0.01^{a}	1.36 ± 0.21^{a}	62.58 ± 0.97^{a}

Data are presented as means ± standard deviation, (n = 7 for each group).
Values with different superscripts within the column are significantly different at $P < 0.05$.
Values with similar or partially similar superscripts are non significant.



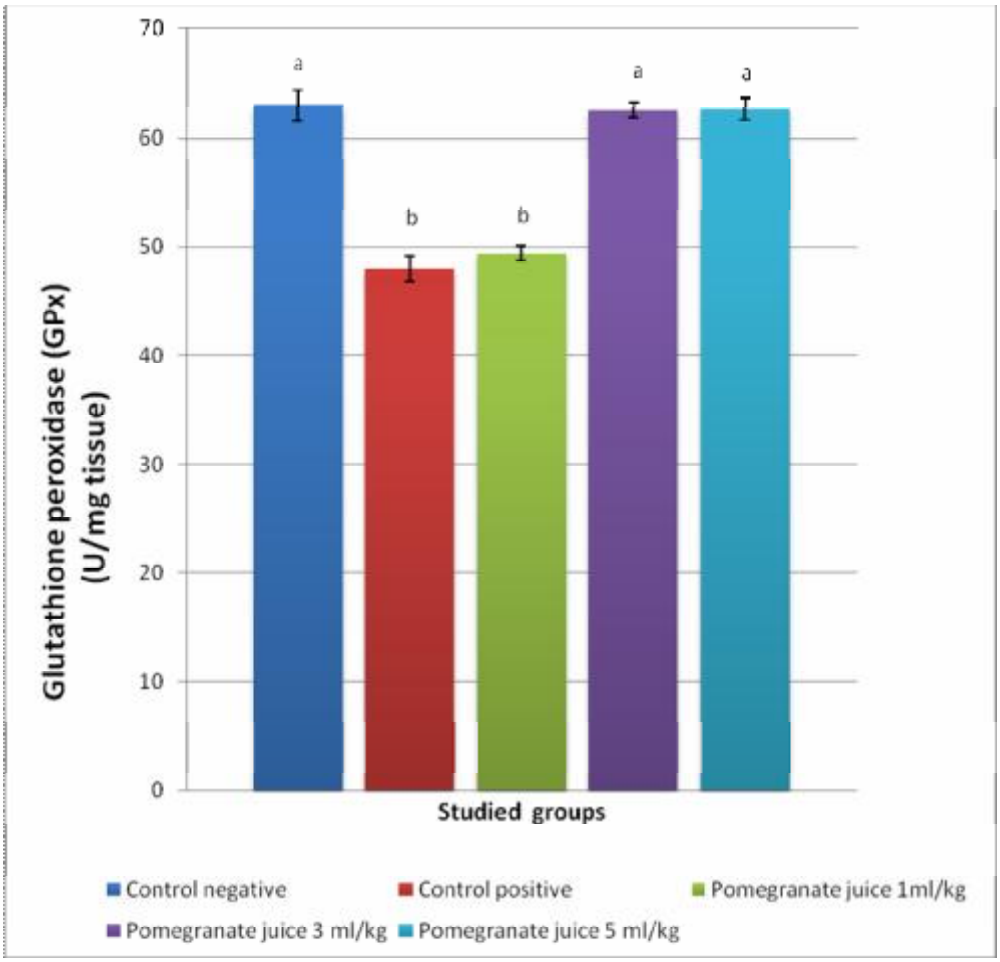
Values with different superscripts within the column are significantly different at P< 0.05.

Figure 4.15 Effect of oral administration of Pomegranate juice on liver homogenates levels of catalase (CAT) in hypercholesterolemic rats



Values with different superscripts within the column are significantly different at P< 0.05.

Figure 4.16 Effect of oral administration of Pomegranate juice on liver homogenates levels of superoxide dismutase (SOD) in hypercholesterolemic rats



Values with different superscripts within the column are significantly different at P< 0.05.

Figure 4.17 Effect of oral administration of Pomegranate juice on liver homogenates levels of glutathione peroxidase (GPx) in hypercholesterolemic rats

4.9 Histopathological Examination:

4.9.1 Liver:

Histological examination of liver of the negative control group demonstrated normal histological pattern where hepatic lobules appeared as hexagonal masses of liver cells (hepatocytes) radiating form a central vein. Blood sinusoids appeared between cords of hepatocytes. The hepatocytes had a hexagonal outline with central rounded nucleus. The cytoplasm showed some vacuoles Figure 4.18.

Compared to negative control Figure 4.18, examination of liver sections of the positive control (hypercholesterolemic rats) group revealed marked impairment of the normal structural organization of hepatic lobules in many areas and deposition of large lipid droplet in cells. Hepatocytes showed vacuolar degeneration, swollen and vacuolated cells, and some nuclei revealed clear signs of dark small pyknosis as illustrated in Figures 4.19 and 4.20.

Examination of liver of hypercholesterolemic rat treated by Pomegranate juice in a dose of 1 ml/kg b. wt., revealed a marked improvement with normal hepatocytes, congested central vein when compared to the positive control group Figure 4. 21. The hypercholesterolemic rat orally given Pomegranate juice in a dose of 3 mg/kg b. wt., showed marked improvement from changes caused by cholesterol except of few residual cells with fine lipid droplets, or scattered dark apoptotic cells and some sections showed bile duct proliferation Figure 4.22.

Sections from liver of hypercholesterolemic rat after treated by Pomegranate juice in a dose of 5 ml/kg b. wt., showed more improvement in histological structure comparing with section of rats that orally given Pomegranate juice in doses of 1 and 3 ml/kg b. wt., The examination section showed almost normal structure with regular arrangement of hepatocyte cell cords and exhibited a reduction in fat accumulation. The hepatocytes around the central vein (CV) showed rounded nuclei and vesicular indicating active cells. Blood sinusoids between the cells had also normal appearance Figure 4.23.

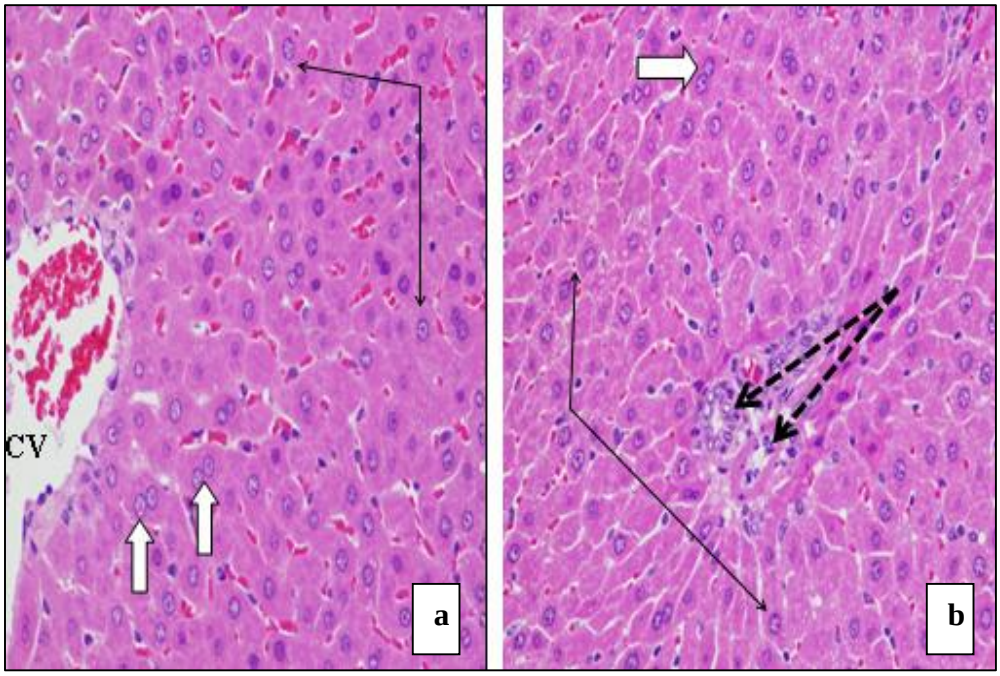


Figure 4.18 (a-b) Section in the liver of a normal rat (negative control) showing liver cells surrounded by central vein (CV) and peripheral portal structures (dotted black arrows), normal hepatocytes with large central vesicular (black arrows), some cells are binucleated (white arrows) (H & E x 400).

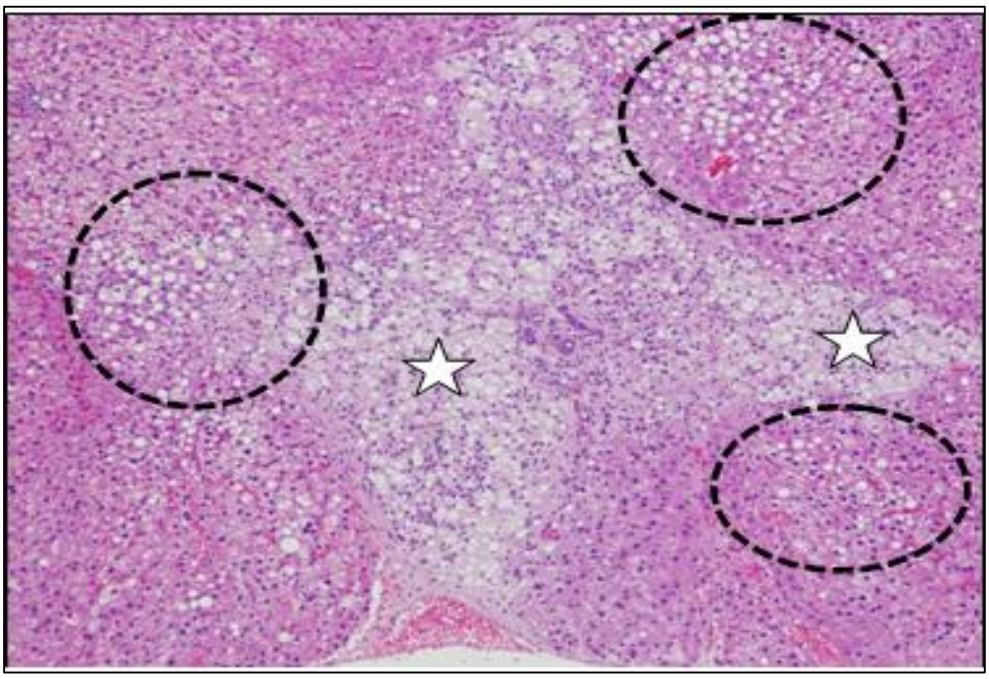


Figure 4.19 Section in the liver of hypercholesterolemic rat (positive control) showing foci of lipid droplets deposition within hepatocytes (dotted circles) and the neighboring cells showed vacuolar degeneration (stars) (H & E x 100).

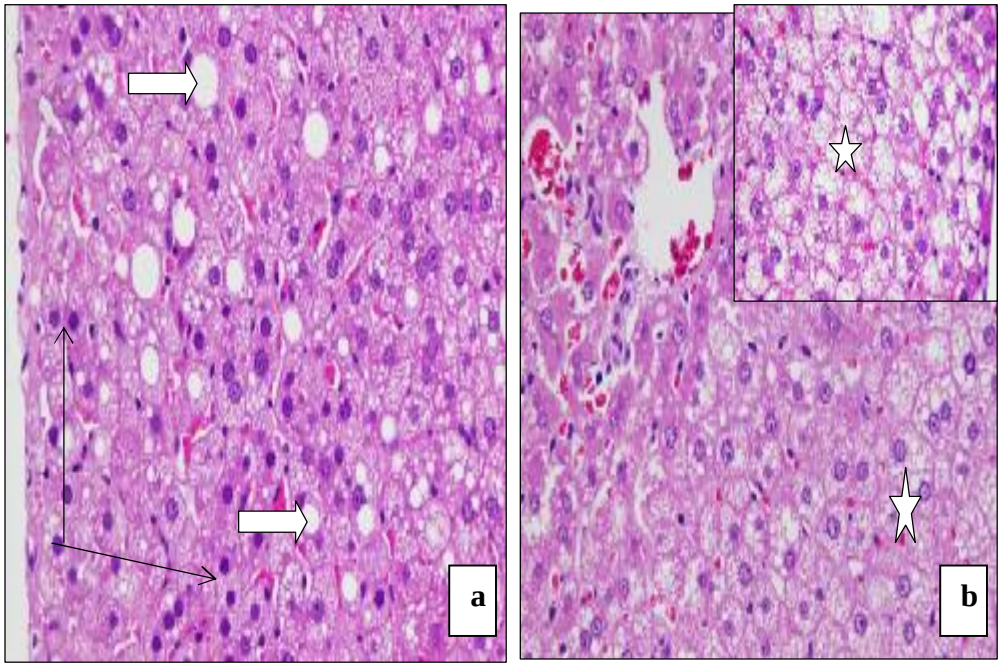


Figure 4.20 (a-b) Section in the liver of hypercholesterolemic rat (positive control) showing deposition of large lipid droplet in some cells (white arrows). In other cells marked vacoulations were observed (stars), some cells showed dark small degenerated nuclei (black arrows) (H & E x 400).

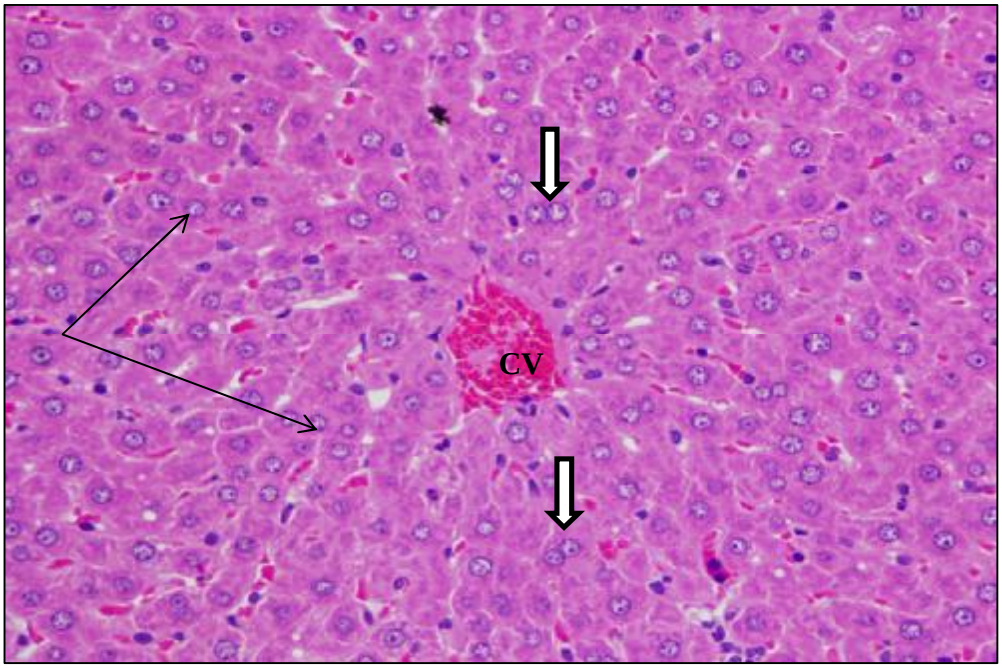


Figure 4.21 Section in the liver of hypercholesterolemic rats after treatment with 1 ml/ kg b. wt., Pomegranate juice showing mild improvement with congested central vein (CV), normal hepatocytes (black arrows) and numerous binucleated cells (white arrows) (H & E x 400).

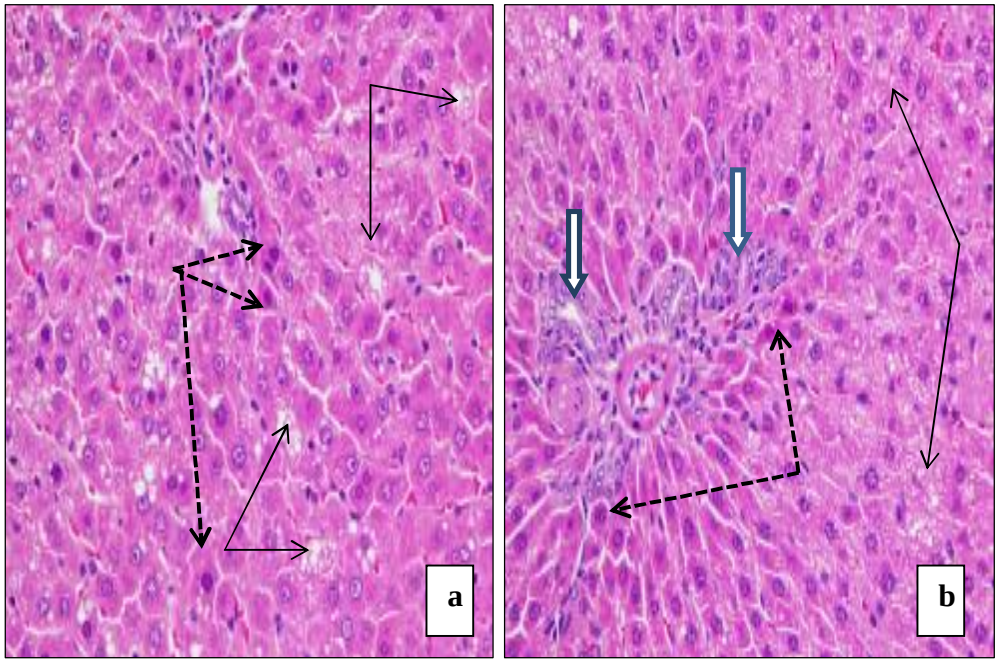


Figure 4.22 (a-b) Section in the liver of hypercholesterolemic rat after treatment with 3 ml/kg b. wt., Pomegranate juice showing moderate improvement from degenerative changes except presence of few residual cells with lipid droplets (thin black arrows) or scattered dark apoptotic cells(dotted arrows) and some showed bile duct proliferation (white arrows) (H & E x 400).

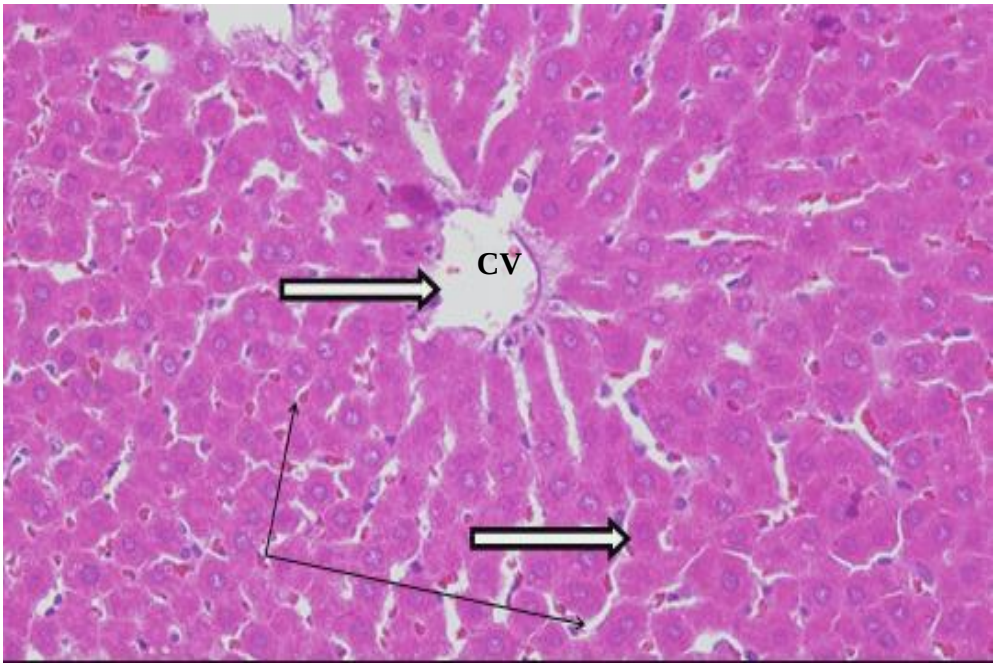


Figure 4.23 Section in the liver of hypercholesterolemic rat after treatment with 5 ml/ kg b. wt., Pomegranate juice showing almost normal structure with regular arrangement of hepatic cell cords (black thin arrows) around the central vein (CV), hepatocytes showed rounded and vesicular nuclei indicating active cells. Hepatic sinusoids between the cells showed normal appearance (white arrows) (H & E x 400).

4.9.2 Heart:

The histological examination of the heart tissue of normal healthy rats showed normal histological architecture manifested by normal cardiac vessels wall thickness, normal size and appearance of cardiac muscles and blood capillaries as illustrated in Figure 4.24. In rats fed on high - cholesterol diet, the examination of the heart revealed some degenerative changes with inflammatory cell infiltration and marked congestion of blood capillaries as demonstrated in Figure 4.25.

The heart sections of the hypercholesterolemic rat orally given Pomegranate juice in a dose of 1ml/kg b. wt., showed a slight improvement of pathological lesions with presence of thickened walls and cholesterol deposition in cardiac vessels. Degeneratd dark muscles and congested vessels Figure 4.26. Treatment with Pomegranate juice in a dose of 3 ml/kg b. wt., showed a moderate improvement except cardiac vessels still had focal thickening and some cardiac muscles looked dark Figure 4.27.

Oral administration of Pomegranate juice in a dose of 5 ml/kg b. wt., revealed a marked improvement in histological architecture of the heart tissue except presence of few apoptotic dark cells in the cardiac muscle as shown in Figure 4.28.

From the above mentioned histological findings in liver and heart it seems that the effects of Pomegranate juice on histological pictures of liver and heart were in a dose – dependent manner.

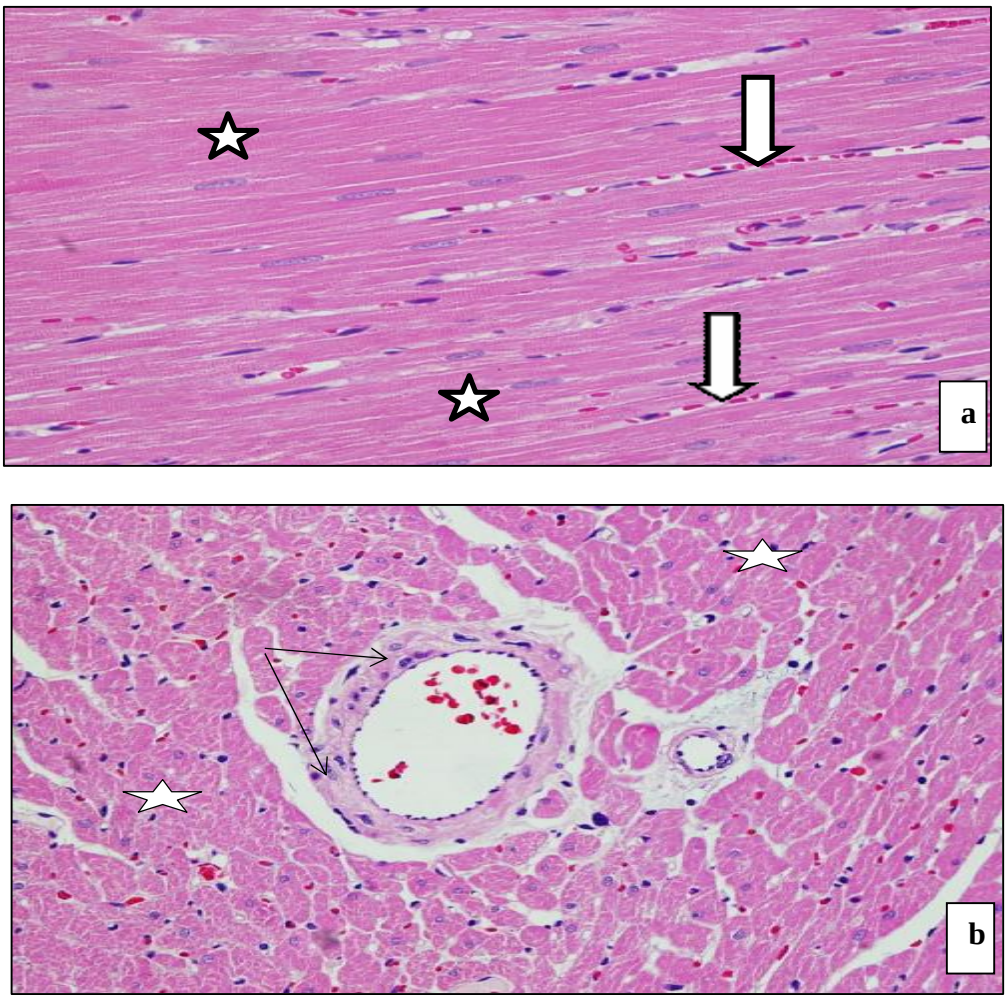


Figure 4.24 (a-b) Section of heart of normal rats (negative control) showing cardiac vessels with normal wall thickness (black arrows). Cardiac muscles (stars) with normal size and appearance (no degeneration), Blood capillaries were also normal (white arrows) (H &E x 400).

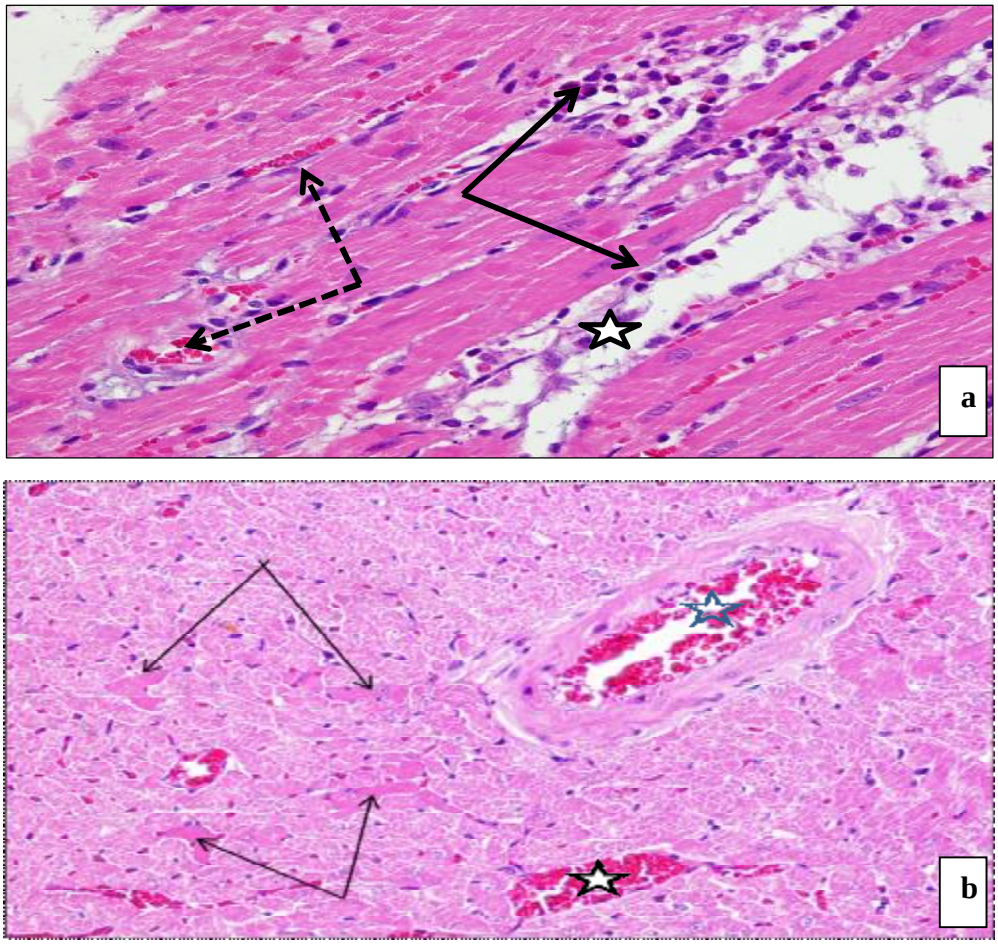


Figure 4.25 (a-b) Section from rat heart of hypercholesterolemic rat (positive control) showing degenerative changes of some cardiac muscles (stars) with inflammatory cells infiltration (black arrows). Marked congestion of blood capillaries (dotted arrows) was seen (H & E x 400).

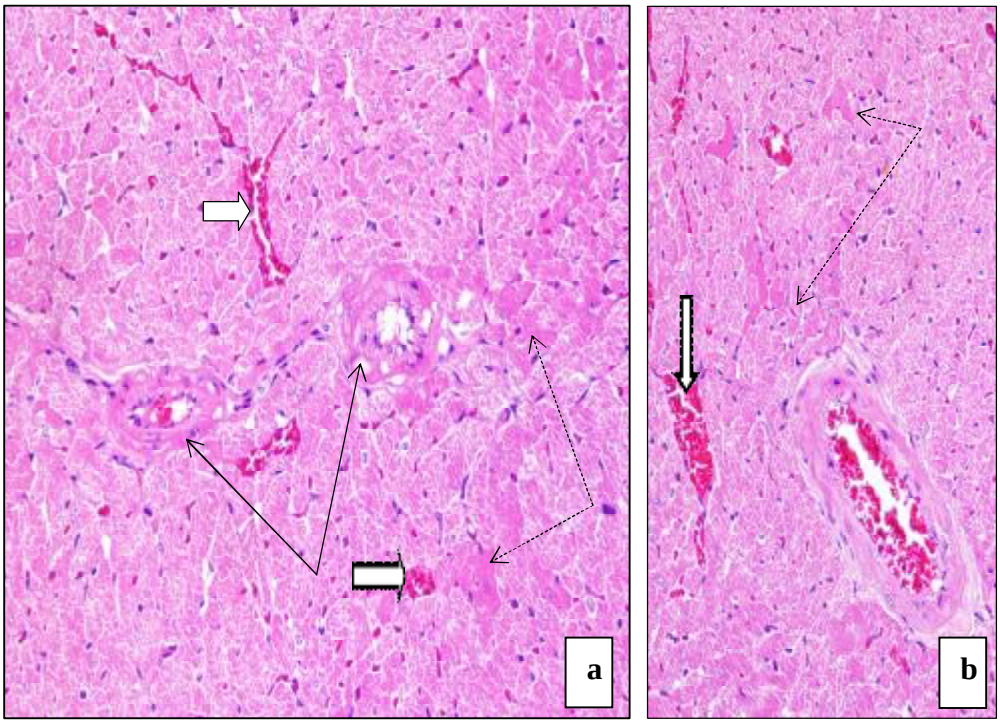


Figure 4.26 (a-b) Section from rat heart of hypercholesterolemic rat after treated with 1ml/kg b. wt., Pomegranate juice showing a slight improvement except presence of thickened walls and cholesterol deposition in cardiac vessels (black arrows). Degenerated cardiac muscle (dotted arrows) and congested vessels (white arrows) were also seen (H & E x 400).

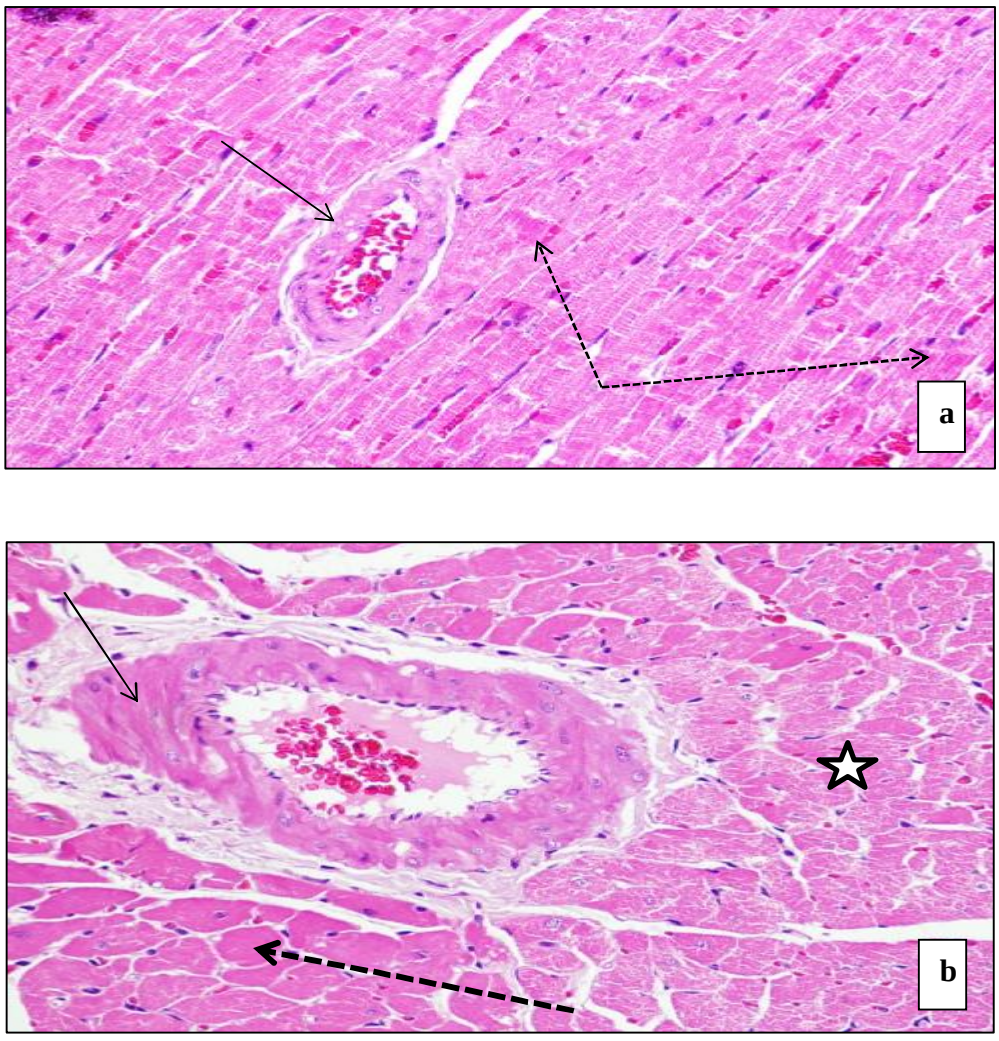


Figure 4.27 (a-b) Section in cardiac muscles of hypercholesterolemic rat after treatment with 3 ml/kg b. wt., Pomegranate juices showing a moderate improvement (white star). Cardiac blood vessels still showed focal thickening (black arrows). Cardiac muscle showed degeneration (dotted arrows) (H&E x 400).

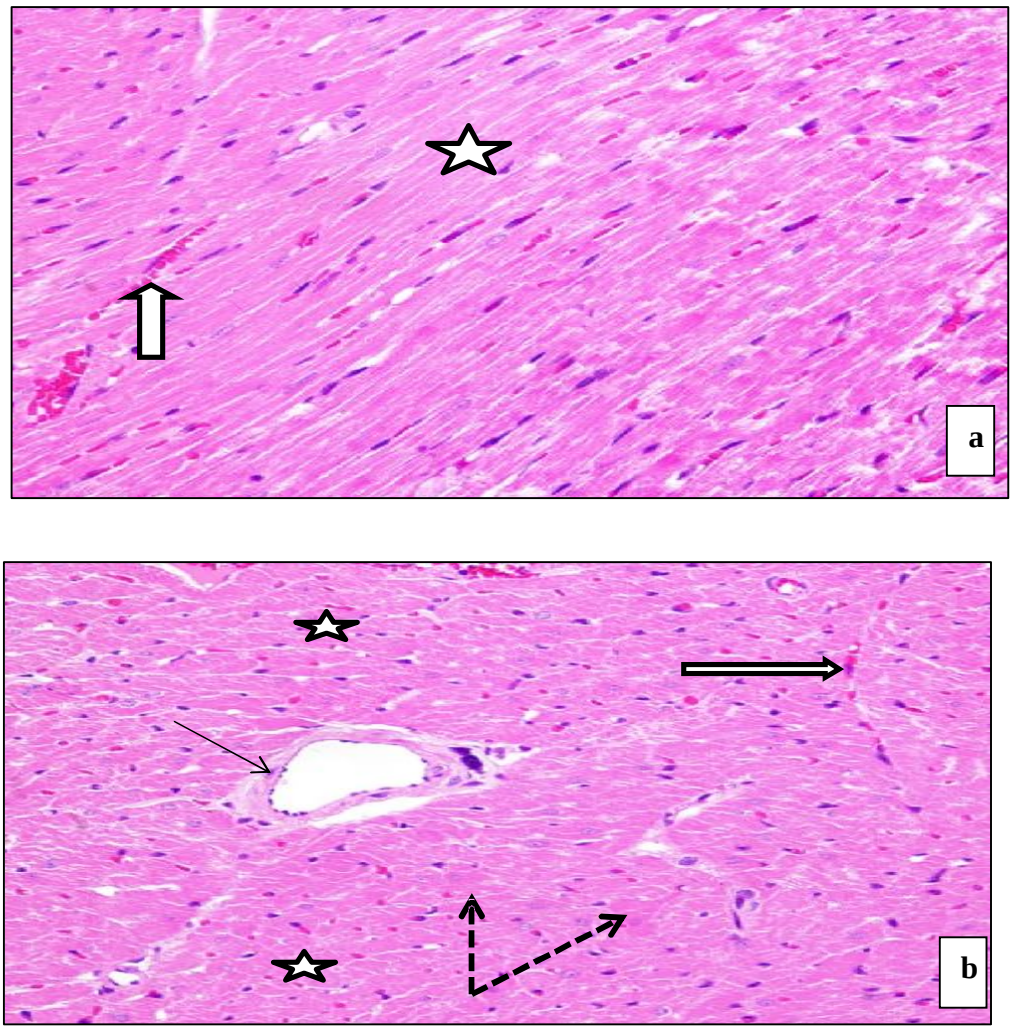


Figure 4.28 (a-b) Section in cardiac wall of hypercholesterolemic rat after treated with 5ml/kg b. wt., Pomegranate juice showing almost normal appearance of cardiac muscles (stars) except few apoptotic dark cells (dotted arrows). Cardiac blood vessels showed normal thickness (thin black arrow). Blood capillaries were normal and not congested (white arrow) (H & E x400).

CHAPTER V

DISCUSSION

Chapter V

Discussion

The present study was performed to elucidate the effect of oral administration of Pomegranate juice at three dosage levels to hypercholesterolemic rats during the experimental period (28 days) on feed intake, body weight gain percent (BWG %), feed efficiency ratio (FER), relative weights of some internal organs and serum levels of total cholesterol (TC), triglycerides (TG), lipoprotein fractions, liver enzymes (AST and ALT) and antioxidant enzymes (CAT, SOD and GPx) as well as the histopathological examination of liver and heart were also carried out.

Concerning feed intake and BWG %, there was a controversy about the effect of high cholesterol supplemented diet on feed intake and BWG % of rats. Ramachandran *et al.* (2003) and Harnafi *et al*. (2009) reported that there were no significant changes of weight gain or there was a linear increase in the weight gain between healthy and hypercholesterolemic rats. Results of the present study revealed incidence of significant increases in feed intake and BWG % of hypercholesterolemic rats when compared to the negative control rats. These findings were in agreement with those obtained by Matos *et al.* (2005); Hossin (2009); Otunola *et al*. (2010); Amin *et al*. (2011) and Nwozo *et al*.

(2011) who confirmed our results. The increase in body weight of hypercholesterolemic rats might be due to the increase of feed and caloric intake by rats. On contrary, the observed body weight loss could be attributed to the reduction in nutrient intake caused by high cholesterol content of the diet which might impair intestinal absorption of protein and other nutrients as suggested by Matos *et al*. (2005).

With regard to the effects of Pomegranate juice when orally given to hypercholesterolemic rats at three dosage levels for 28 days on feed intake and body weight gain, the results revealed that Pomegranate juice significantly reduced feed intake and BWG % when compared to the positive control group. These findings might be due to decreased appetite (anorexia) of rats and/or reduction of intestinal fat absorption or due to an inhibition of pancreatic lipase activity. It is well known that dietary fat is not directly absorbed from the intestine unless it has been subjected to the action of pancreatic lipase (Lei *et al*., 2007).

In relation to relative weights of liver and heart, there were significant increases in relative weights in liver and heart of hypercholesterolemic rats as compared to the normal group. These results might be due to the accumulation of fat in the liver and heart cells leading to an increase in their weight. These results were confirmed by histopathological examination of these internal organs which showed presence of fatty changes of hepatocytes and focal sarcoplasmic granularity in cardiac myocytes. Our findings were in accordance with those obtained by Matos *et al*. (2005) who reported that the increase in liver weight of hypercholesterolemic rats could be a consequence of the higher fat content (fat/liver). Moreover, Puskas *et al*. (2004) reported that in response

to high cholesterol diet there was an intracellular lipid accumulation in cardiomyocytes of rats, thus leading to an increase in its weight.

Concerning relative weights of liver and heart of Pomegranate juice-treated hypercholesterolemic rats; the results showed that weight of these organs decreased when compared to the hypercholesterolemic group. Our results were in agreement with those of Chidambara *et al*. (2002) who reported that Pomegranate peel when given to rats exhibited protective effects on liver and heart weights.

Results of the present study revealed that feeding of rats on high - cholesterol diet resulted in significant increases in serum levels of TC, TG, LDL-c and VLDL-c accompanied with a significant decrease in HDL-c level as compared to the negative control group. The increases in serum concentrations of the above mentioned parameters and the reduction in serum HDL-c as a result of feeding high - cholesterol diet have been pointed out as risk factors for the development of atherosclerosis and related cardiovascular diseases, which was represented by the decrease in HDL/TC ratio. These results were confirmed by histopathological examination of heart which showed degenerative changes of some cardiac muscles with inflammatory cell infiltration associated with a marked congestion of blood capillaries, compared to the negative control group. The present findings were in the same line as with those reported by Wang and Chen (2004); Gorinstein *et al.* (2006); Frantz *et al*.(2012) who demonstrated that lipid metabolism in rats fed high fat - diet (HFD) presented disorders and levels of serum TC and TG increased significantly, compared with the negative control group.

These results could be explained on the basis that feeding of rats on atherogenic diet leads to increase in cholesterol absorption and hence serum cholesterol increment.

Concerning serum TG level, the present findings agreed with the study of Yugarani *et al.* (1992) who demonstrated that plasma TG level increased significantly after feeding rats on HFD indicating that the increasing in triglycerides is of dietary origin.

Regarding to serum LDL-c and HDL-c levels in rats fed with high cholesterol diet, the current results were in agreement with those of Boden and Pearson (2000); Glass and Witztum (2001); Witztum and Steinberg (2001) and Kumar *et al*. (2010). The previous authors concluded that oxidation of LDL-c resulted in formation of a wide range of biologically active products, including peroxides and malondialdehyde. Moreover, Sezer *et al*. (2011) demonstrated that the oxidative modified lipids and their degradation products are believed to have adverse effects such as pro-inflammatory, immunogenic and cytotoxic activities which contribute to both the initiation and progression of atherosclerotic lesions. Furthermore, Tebib *et al*. (1994) found that activity of the lipoprotein lipase enzyme augmented in hypercholesterolemic rats. Lipase transforms VLDL-c into LDL-c that would lead to an increase in serum concentration of LDL-c. However, Shanmugasundaram *et al*. (1986) reported that the increment of plasma LDL-c level after HFD consumption could be explained via involvement of two enzymes namely cholesterol ester hydrolase (CEH) and cholesterol ester synthetase (CES). These enzymes balance the cholesterol levels in the blood. Hence, it is logical to assume that the elevation in plasma cholesterol is mediated through increased

cholesterol turnover and influenced by the relative balance between CEH and CES activity. With increased estrifying activity (when CEH: CES is lowered) cholesterol will be predominantly in its ester form (as in LDL-c) and can lead to the development and progression of atherosclerosis.

The results concerning serum HDL-c level in hypercholesterolemic group, the present result is well documented by the study of Yugarani *et al*. (1992). It has been reported that cholesterol transport to extra-hepatic tissues is primarily ensured by LDL-c (bad cholesterol); while HDL-c (good cholesterol) has an important role in reversing the cholesterol transport process (Gurr *et al*., 1989). Hypercholesterolemia is an important etiological factor in coronary heart disease (CHD). Studies have shown that the risk of developing CHD is linearly related to serum cholesterol concentration and LDL-c. On the other side, HDL-c exerts a protective effect (Mattson and Grundy, 1985).

Phenols and flavonoids are very important plant constituents because of their antioxidant activity (Annegowda *et al*., 2010 and Abdel Moneim, 2012). The antioxidant activity of phenolic compounds is mainly due to their redox properties which play an important role as free radical scavengers, reducing agents, quenchers of singlet oxygen and complexes of pro-oxidant metals (Mustafa *et al*., 2010). The plant phenolics are commonly present in fruits, vegetables, leaves, nuts, seeds, barks, roots and in other plant parts (Kaviarasan *et al*., 2007). Pomegranate is an important source of phenols and flavonoids such as anthocyanins, hydrolysable tannins punicalagin and punicalin (Afaq *et al*., 2005), ellagic and gallic acids (Lansky and Newman, 2007). Pomegranate also contains vitamin C (Turk *et al*., 2008). The antioxidant and free radicals scavenging

activities of phenolic compounds derived from Pomegranates (Rosenblat *et al*., 2006) and vitamin C (Sonmez *et al*., 2005) have been reported.

Furthermore, Rice-Evans *et al*. (1996); Schwenke and Behr (1998); Gil *et al*. (2000); Aviram *et al*. (2002) and Noda *et al*. (2002) concluded that Pomegranate juice is rich in polyphenols and demonstrate high capability in scavenging free radicals and inhibiting LDL-c oxidation *in vitro* and *in vivo*. The present study showed that oral administration of Pomegranate juice at three dosage levels significantly decreased serum level of TC, TG, LDL-c and VLDL-c, but increased HDL-c as compared to the positive control group. These findings correlated with those obtained by Aviram *et al*. (2000) who reported that Pomegranate juice consumption by atherosclerotic mice significantly reduced cholesterol accumulation and foam cell formation in heart tissues. Pomegranate juice treatment significantly and substantially inhibited the progression of atherosclerotic lesions by inhibition of atherogenic modifications of LDL-c, including its retention, oxidation, and aggregation.

Moreover, Tezcan *et al*. (2009) demonestrated that Pomegranate juice increased the level of serum HDL associated paraoxonase 1 (PON1) and decreased the LDL-c susceptibility to aggregation and oxidation. Esmaillzadeh *et al*. (2006) reported that diabetic patients with elevated blood lipids who were supplemented with Pomegranate juice for eight weeks experienced significant reductions in their TC and LDL-c. The results of Rosenblat and Aviram (2006) demonstrated that Pomegranate juice can inhibit LDL-c oxidation in 3 ways:

1. Pomegranate juice polyphenols inhibit copper ion-induced LDL-c oxidation, and thus reduce the oxidized LDL (ox-LDL) content.

2. Pomegranate juice polyphenols also increase the activity of serum HDL-c associated paraoxonase 1 (PON1).

3. Paraoxonase 1 (PON1) can in turn hydrolyze lipid peroxides in ox-LDL and convert them to a less atherogenic LDL-c thus causing further reduction in ox-LDL content.

Histopathological examination of heart of hypercholesterolemic rats treated with Pomegranate juice showed improvement in histological structure. Cardiac vessels showed normal thickness. Blood capillaries are normal and not congested as compared to the hypercholesterolemic group. The present results agreed with the study of Rosenblat and Aviram (2006) who reported that the antioxidant and free radicals scavenging property of Pomegranate juice seem to protect the myocardium against oxidative damage in heart tissue.

There has been conflicting reports on the effect of high cholesterol diet on serum biochemical parameters related to hepatic function (AST and ALT). Some reports on the effects of hypercholesterolemia on serum levels of AST and ALT enzymes. In this concern, studies of Lu *et al*. (2007); Prasad (2010) and Saki e*t al*. (2011) showed that a high cholesterol diet moderately elevated serum levels of ALT, AST and ALP in rats. On contrary, Molgaard *et al*. (1989) reported that there were no changes in the serum levels of AST and ALT. The discrepancy in the serum levels of these enzymes could be attributed to the levels and duration of hypercholesterolemia (Lu *et al*., 2007). Our

results revealed that feeding rats on cholesterol-enriched diet produced liver injury as indicated by marked elevation in serum levels of AST and ALT enzymes associated with markedly histopathological changes. These changes consisted of diffuse vacuolar degeneration; fat vacuoles and necrosis of hepatocytes and markedly focal fibrosis as well as a marked decrease in the antioxidant defense system. This decrease was manifested by the significant increase in lipid peroxidation and significant decreases in the activity of antioxidant enzymes namely superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). These results were in agreement with the previous studies of Miura *et al*. (2000); Arhan *et al*. (2009) and Saki *et al*.(2011) who reported that high cholesterol diet significantly increased serum hepatic enzyme levels (AST and ALT). This increase was attributed to increase the production of free radicals, which initiate lipid peroxidation leading to cellular damage as a result of induction of cytochrome P-450 in the liver producing highly reactive trichloromethyl free radical. This in turn, in the presence of oxygen generated by metabolic leakage from mitochondria causes lipid peroxidation of membrane lipid leading to loss of integrity of cell membranes and damage of hepatic tissue with subsequent increase in serum of these enzymes (AST and ALT).

Results of the present study showed that there were significant decreases in serum levels of AST and ALT enzymes in hypercholesterolemic rats orally given Pomegranate juice in a dose of 1, 3 and 5 ml/kg b. wt., compared to the positive control group. The present results partially agreed with the results obtained by Osman *et al.* (2012) who examined the antioxidant effect of Pomegranate peel and juice on diabetes mellitus induced by alloxan in Female Rats. The results showed that AST and ALT

were significantly increased in diabetic group, but after treatment with peel and juice, AST and ALT levels decreased and become near to the control level especially ALT value. This effect is due to antioxidant content of Pomegranate peel and juice.

Kaur *et al*. (2006) reported that Pretreatment with Pomegranate flower extract, at a dose regimen of 50-150 mg / kg b. wt., for a week, have a protective effect against ferric nitrilotriacetate (Fe-NTA)-induced oxidative stress, as well as hepatic injury. The results showed that there was an inhibition in serum of AST and ALT enzymes which may be due to potent antioxidant and hepatoprotective properties of Pomegranate juice.

The biochemical results of our study were confirmed by histopathological findings, which seen in liver sections. The histological findings of liver of the treated rats showed almost completely normal structure with regular arrangement of hepatocyte cell cords and exhibited reduction in fat accumulation. The nuclei of hepatocytes around the central vein (CV) were rounded and vesicular indicating active cells. Blood sinusoids between the cells had also normal appearance when compared to the positive control group. These histological findings agreed with the study of Fyiad *et al.* (2012) who investigated the effect of Pomegranate juice on nucleic acids alterations and oxidative stress in experimentally hepatitis rats. Results of the previous study revealed that pretreatment with Pomegranate juice (20 ml kg^{-1} b. wt., day^{-1} for 14 days) effectively hindered the adverse effect of D-Galactosamine / lipopolysaccharide and protected against hepatic damage via suppression of oxidative stress. Histopathological studies of the liver of different groups also supported the protective effects exhibited by Pomegranate juice through restoring the normal hepatic architecture. A significant

decrease in the serum level of diagnostic enzyme markers (AST, ALT and ALP) was also detected as compared to the positive control group.

As indicated in the present study, the untreated hypercholesterolemic rats had significant decrease in the level of antioxidant enzyme system (CAT, SOD and GPx). Consistent with our results, Kumar *et al*. (2008b) reported that hypercholesterolemia enhanced the free radical generation in various ways. Several studies suggested that disorders of lipid metabolism, hyperlipidemia and obesity are associated with overproduction of oxygen free radicals (Rehman *et al*., 2003). The enhanced accumulation of these free radicals and dysfunction of antioxidant defense system resulted in oxidative stress (Giao *et al*., 2008). These radicals can bind covalently to the macromolecules and induce peroxidative degradation of the membrane lipids rich in polyunsaturated fatty acids, leads to the formation of lipid peroxides followed by multiple pathological changes (Shyamala *et al*., 2003).

The antioxidant activity of Pomegranate components has been the subject of many studies (Naveena *et al*., 2008; Cam *et al*., 2009; Mousavinejad *et al*., 2009; Tezcan *et al*., 2009). The antioxidant capacity of Pomegranate juice was shown to be three times higher than that of red wine and green tea, based on the evaluation of the free radicals scavenging activity and iron reducing capacity of the juice (Gil *et al*., 2000). Pomegranate juice was also shown to have significantly higher levels of antioxidants in comparison to commonly consumed fruit juices, such as grape, cranberry, grapefruit, or orange juice (Azadzoi *et al*., 2005; Rosenblat and Aviram, 2006). The principal antioxidant polyphenols in Pomegranate juice include the ellagitannins and anthocyanins

which have been shown to be the antioxidant responsible for the free radicals scavenging ability of Pomegranate juice (Gil *et al*., 2000).

Chidambara *et al*. (2002) concluded that Pomegranate extract has also been shown to protect the antioxidant enzymes CAT, GPx and SOD from the effects of toxic chemicals. Turk *et al*. (2008) reported that there was a significant decrease in malondialdehyde (MDA) level and marked increases in reduced glutathione (GSH), GPx and CAT activities, and vitamin C level were observed in rats treated with different doses of Pomegranate juice.

In the present study, Oral administration of Pomegranate juice in doses of 3 and 5 mg/kg b.wt., caused significant increases in activities of CAT, SOD and GPx enzymes when compared to the positive control group. The improvement of CAT, SOD and GPx enzyme activities could be possibly explained by antioxidant properties of Pomegranate juice due to presence of bioactive polyphenolic compounds which play a role in scavenging free radicals and also prevent DNA damage (Fyiad *et al*., 2012).

Valadares *et al*. (2010) confirmed the ability of Pomegranate extract to protect DNA and preventing chromosomal damage in mice. In addition, Kaur *et al*. (2006) demonstrated that Pomegranate extract afforded up to 60 % protection against hepatic lipid peroxidation due to maintenance of the GSH and serum levels and activities of CAT, GPx and glutathione reductase (GR) enzymes.

CHAPTER VI

CONCLUSION AND RECOMMENDATIONS

Chapter VI

Conclusion and Recommendations

6.1 Conclusion:

The present study concluded that oral administration of Pomegranate juice at three dosage levels for 28 days to hypercholesterolemic rats reduces body weight gain and feed efficiency ratio; lowers the elevated serum levels of liver enzymes; improves lipid profile and serum level of antioxidant enzymes in hypercholesterolemic rats. These effects are associated with amelioration of degenerative histopathological changes in liver and heart tissues induced by high-cholesterol diet. Therefore, fortification of food products with Pomegranate seeds or drinking of Pomegranate juice may be beneficial for patients who suffer from elevated liver enzymes or hypercholesterolemia or arteriosclelorosis or oxidative stress.

6.2 Recommendations:

The study recommended the following:

1- Pomegranate treatment improved lipid profile, increased serum antioxidant levels, improved serum levels of liver enzymes and ameliorated the degenerative changes seen in liver and heart tissues of hypercholesterolemic rats.

2- Patients suffering from hypercholesterolemia and /or cardiovascular disease (CVD) should consume Pomegranate juice because of its health beneficial effect on serum total cholesterol (TC), triglycerides (TG) and LDL-c.

3- Patients suffering from acute hepatitis are advised to consume Pomegranate juice owing to its marked hepatoprotective, excellent antioxidant activities.

4- Encouragement of cultivation of Pomegranate trees in Kingdom of Saudi Arabia and other Arabic countries for increasing production of Pomegranate fruits.

5- Nutritional and health educational programs should be organized and directed to the public to be informed about health benefits of Pomegranate.

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ARBIC SUMMARY

تأثير عصير الرمان على صور ليبيدات الدم والإنزيمات المضادة للأكسدة لدى الفئران المصابة بارتفاع الكولسترول

منال معلا المورعي

الملـــــــــخص العربي

استهدفت الدراسة الحالية تأثير تناول عصير الرمان بثلاث جرعات عن طريق الفم لمدة 28 يوم للفئران المصابة بارتفاع مستوى الكولسترول بالدم. وشملت هذه التأثيرات الوزن النسبي للجسم،نسبة كفاءة الغذاء، والأوزان النسبية لبعض الأعضاء الداخلية(الكبد والقلب) و مستوى إنزيمات الكبد في سيرم الدم، صورة دهون الدم، ومستوى الإنزيمات المضادة للأكسدة في أنسجة الكبد المجانسة، وكذلك الفحص الهستوباثولوجي لأنسجة الكبد والقلب.

و تم تطبيق الدراسة على 35 فأر ذكر بالغ من فصيلة الألبينو ويستر، تم توزيعها إلى خمس مجموعات بالتساوي، وكانت المجموعة الأولى ضابطة سالبة، تناولت على الغذاء الأساسي. أما المجموعات الأربعة الأخرى تناولت غذاء مدعم بنسبة 2 % كولسترول لإحداث ارتفاع في كولسترول الدم لهذه الفئران. وتركت إحداها مجموعة ضابطة موجبة (مصابة بارتفاع كولسترول الدم)، وتم إعطاء المجموعات الثلاثة الأخرى عصير الرمان عن طريق الفم بثلاثة جرعات هي 1، 3 و5 مل/ كجم من وزن الجسم على التوالي وذلك لمدة 28 يوم. تم حساب

أوزان الفئران قبل بدء التجربة وفى نهايتها لمعرفة معدل الزيادة النسبية في وزن الجسم، وكذلك حساب كمية الغذاء المستهلك يوميا وحساب نسبة كفاءة الغذاء. وفى نهاية فترة التجربة تم أخذ عينات من الدم لإجراء التحليلات البيوكيميائية، وحساب الوزن النسبي لبعض الأعضاء الداخلية، وكذلك تم أخذ الكبد والقلب لإجراء الفحص الهستوباثولوجى.

وأظهرت النتائج أن تناول عصير الرمان عن طريق الفم للفئران المصابة بارتفاع مستوى الكولسترول بالدم قد أدى إلى نقص معنوي في الوزن النسبي للجسم، نسبة كفاءة الغذاء ومستويات إنزيمات الكبد، الكولسترول الكلي، الجليسريدات الثلاثية و الكولسترول المنخفض الكثافة والكولسترول المنخفض الكثافة جدا، بينما أدى إلى زيادة معنوية في نشاط الإنزيمات المضادة للأكسدة. وقد أظهر الفحص الهستوباثولوجى لأنسجة الكبد والقلب وجود تحسن واضح يعتمد على الجرعة المعطاة في التغيرات الهستوباثولوجية (المرضية) التي سببها الكولسترول المرتفع بهذه الأنسجة.

وتدل نتائج هذه الدراسة أن عصير الرمان له تأثيرات علاجية هامة وهى تأثير خافض لإنزيمات الكبد المرتفعة، وخافض للكولسترول والدهون الثلاثية ومضاد للأكسدة في الفئران المصابة بارتفاع كولسترول الدم. وتوصى هذه الدراسة بأن تناول عصير الرمان الطازج قد يكون مفيدا للمرضى الذين يعانون من ارتفاع إنزيمات الكبد وارتفاع الكولسترول والجليسريدات الثلاثية وكذلك في حالات الإجهاد التأكسدي.

تأثير عصير الرمان على صور ليبيدات الدم والإنزيمات المضادة للأكسدة لدى الفئران المصابة بارتفاع الكولسترول

منال معلا دغيليب المورعي

بحث مقدم لنيل درجة الماجستير في الإقتصاد المنزلي (الغذاء والتغذية)

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