Therapeutic potential of *Coriandrum sativum L.* seed extract against experimentally induced insulin resistance, myocardial infarction and atherosclerosis

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CERTIFICATE

This is to certify that the thesis entitled "**Therapeutic potential of *Coriandrum sativum* L. seed extract against experimentally induced insulin resistance, myocardial infarction and atherosclerosis**" incorporates results of investigation carried out by the candidate himself under my supervision and guidance in the Department of Zoology, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara. The contents of the thesis, in full or parts have not beensubmitted to any other Institute or University for the award of any other degree or diploma.

Mr.Dipak K. Patel
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(Research guide)

List of publications:

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2. **Patel DK**, Desai SN., Gandhi HP., Devkar RV., Ramachandran AV., 2012. Cardio protective effect of *Coriandrumsativum* L. on isoproterenol induced myocardial necrosis in rats. Food and chemical toxicology 50(9), 3120–3125.

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Vadodara ***Dipak K. Patel***

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INTRODUCTION

Metabolic syndrome:

Metabolic syndrome (MS) is a concurrence of overweight and abdominal fat distribution, dyslipidemia, disturbed glucose and insulin metabolism and hypertension, is most important because of its association with subsequent development of type 2 diabetes mellitus and cardiovascular diseases (CVDs) (Reaven, 1988; Liese *et al*., 1998). In 1988, Gerald Reaven introduced the concept of syndrome X for the clustering of cardiovascular risk factors like hypertension, glucose intolerance, high triglycerides and low HDL cholesterol levels (Reaven, 1988). Etiology of this syndrome has also been referred by other researchers as 'the MS', 'the insulin resistance syndrome' and 'the deadly quartet' (Hanefeld and Leonhardt, 1981; DeFronzo and Ferrannini, 1991; Descovich *et al*., 1993; Bouchard and Perusse 1993; Kaplan, 1989). The name "insulin resistance syndrome" has been widely used and refers to insulin resistance as a common denominator of the syndrome (Modan *et al*., 1985; Haffner *et al*., 1992; Balkau and Charles, 1999). In 1998, World Health Organization (WHO) proposed a unifying definition for the syndrome and chose to call it the MS rather than the insulin resistance syndrome (Alberti and Zimmet, 1998).

WHO and the National Cholesterol Education Program's (NCEP's) Adult Treatment Panel (ATP) III guidelines (NCEP, 2001) have established the criteria for the diagnosis of MS (Table 1) (WHO, 1999; NCEP, 2001). MS-associated factors and its prevalence was defined by ATP III criteria wherein 3305 US black population, 3477 Mexican American, 5581 white men and non-pregnant or lactating women (aged 20 years

and older) were examined under the Third National Health and Nutrition Examination Survey. The survey revealed prevalence of MS in 22.8% and 22.6% of US men and women respectively. The MS was present in 4.6%, 22.4%, and 59.6% of normal-weight, overweight and obese men respectively with a similar pattern of distribution also observed in women. (Park *et al*., 2003).

Although, very few studies have been done to assess the prevalence of MS in India, the most significant of them have been the ones using the ATP-III guidelines. A study conducted to assess MS in southern Indian population (individuals > 20 yrs) revealed 25.8% prevalence as per the International Diabetes Federation (IDF) and 18.3% prevalence as per ATP-III guidelines (Deepa *et al*., 2006). Similarly, a study conducted at Bangalore revealed 34.9% (as per IDF) and 40.3% (as per ATP-III) prevalence of MS (Kanjilal *et al*., 2008). A survey of Bhatia community in Rajasthan concluded that the females are at a higher risk than males (36.2% in males and 47.8% in females as per ATP-III) (Gupta *et al*., 2004). A multi-centric study (20-69 yrs of age) of industrial employees (using ATP-III) revealed a higher prevalence of MS in Bangalore (38.8%) followed by Trivandrum (37.9%), Hyderabad (33.0%) and Lucknow (29.0%) but a lower prevalence was recorded in Nagpur and Dibrugarh (Reddy *et al*., 2004).

Genetic basis and a sedentary life style equally contribute towards the development of MS. Genetic factors include family history of type 2 diabetes mellitus, hypertension and a tendency to develop heart disease at an early age increases the risk of MS in an individual. Life style related factors such as smoking, high carbohydrate intake,

physical inactivity, sedentary life style, older age, postmenopausal status etc. account for larger prevalence of MS (Park *et al*., 2003).

Table 1: Criteria for development of MS as per ATPIII, WHO and IDF guidelines.

Clinical features	**NCEP ATPIII criteria ≥3 of the criteria below**	**WHO criteria Impaired glucose regulation/insulin resistance and ≥2 other criteria**	**International Diabetes Federation (IDF) definition**
Impaired glucose regulation/insulin resistance	Fasting plasma glucose ≥110 mg/dl	Type 2 diabetes mellitus or impaired fasting glycemia [≥6.1 mmol/L (110 mg/dl)] or impaired glucose tolerance or glucose uptake below lowest quartile under hyperinsulinemic, euglycemic conditions	(FPG) ≥ 100 mg/dL (5.6 mmol/L), or previously diagnosed type 2 diabetes If above 5.6 mmol/L or 100 mg/dL, OGTT is strongly recommended but is not necessary to define presence of the syndrome.
Abdominal obesity	Waist circumference >102 cm (40 in.) in men, >88 cm (35 in.) in women	Waist/hip ratio >0.90 in men, >0.85 in women or body mass index >30 kg/m2	If BMI is >30kg/m², central obesity can be assumed and waist circumference does not need to be measured.
Hypertriglyceridemia	≥150 mg/dl	≥1.7 mmol/L (150 mg/dl)	≥ 150 mg/dL (1.7 mmol/L) or specifi c treatment for this lipid abnormality
Low levels of HDL cholesterol	<40 mg/dl in men, <50 mg/dl in women	<0.9 mmol/L (35 mg/dl) in men, <1.0 mmol/L (39 mg/dl) in women	< 40 mg/dL (1.03 mmol/L) in males < 50 mg/dL (1.29 mmol/L) in females or specifi c treatment for this lipid abnormality
High blood pressure	≥130/85 mm Hg	≥140/90 mm Hg	systolic BP ≥ 130 or diastolic BP ≥ 85 mm Hg or treatment of previously diagnosed hypertension
Microalbuminuria	Not included	≥20 μg/min or albumin: creatinine ratio ≥30 mg/g	-

Diabetes mellitus is now a global health concern that has grown beyond manageable medical limits in last two decades. WHO estimates to have documented 177 million patients suffering from diabetes and the same are assumed to double in 2030 (WHO, 2000). Such a high prevalence of diabetes puts a burden on the society and the

public health sector that may seldom be managed by developing and economically poor countries (Leroith and Smith, 2005). Diabetes mellitus (DM) accounts for more than 90% of cases worldwide and is caused by a combination of insulin resistance and impaired insulin secretion (Warren, 2004). Patients suffering from DM are assumed to reach 350 million by 2030; with Africa (18.3 million) and Asia (119.6 million) expected to be at the epicenter (WHO, 2003; WHO, 2005). In developing countries, most of the people suffering from DM are between the age group of 45 to 65 years. However, in developed countries, people aged over 65 years are at a higher risk. A recent study shows that the global prevalence of DM will increase from 2.8% in the year 2000 to 4.4% in 2030 (Wild *et al*., 2004; Leahy, 2005).

It is a state of reduced responsiveness to the normal circulating levels of insulin (David *et al*., 2007). In this condition, the β cells secrete more insulin for glycemic homeostasis but, over a period of time, this condition amounts to insulin deficiency due to the destruction of β cells (Boden *et al*, 2001). Increasingly sedentary life-styles combined with ready access to energy-rich food sources creates an imbalance of energy, leading to storage of excess energy in adipose tissue. This scenario is further compounded in genetically susceptible individuals. Persistently over-nourished individuals witness sudden weight gain that ultimately leads to obesity. It also appears to induce lipid accumulation in "ectopic sites," such as the liver and skeletal muscle (Shulman, 2000; Unger, 2003; Danforth, 2000). This notion is supported by the almost universal finding of ectopic lipid accumulation in mice and humans with generalized lipodystrophy, an extreme example of limited adipose tissue storage capacity in the face of excess calorie

ingestion (Gavrilova *et al*, 2000; Kim *et al*., 2000).

Symptoms (Ikarashi *et al*., 2011)

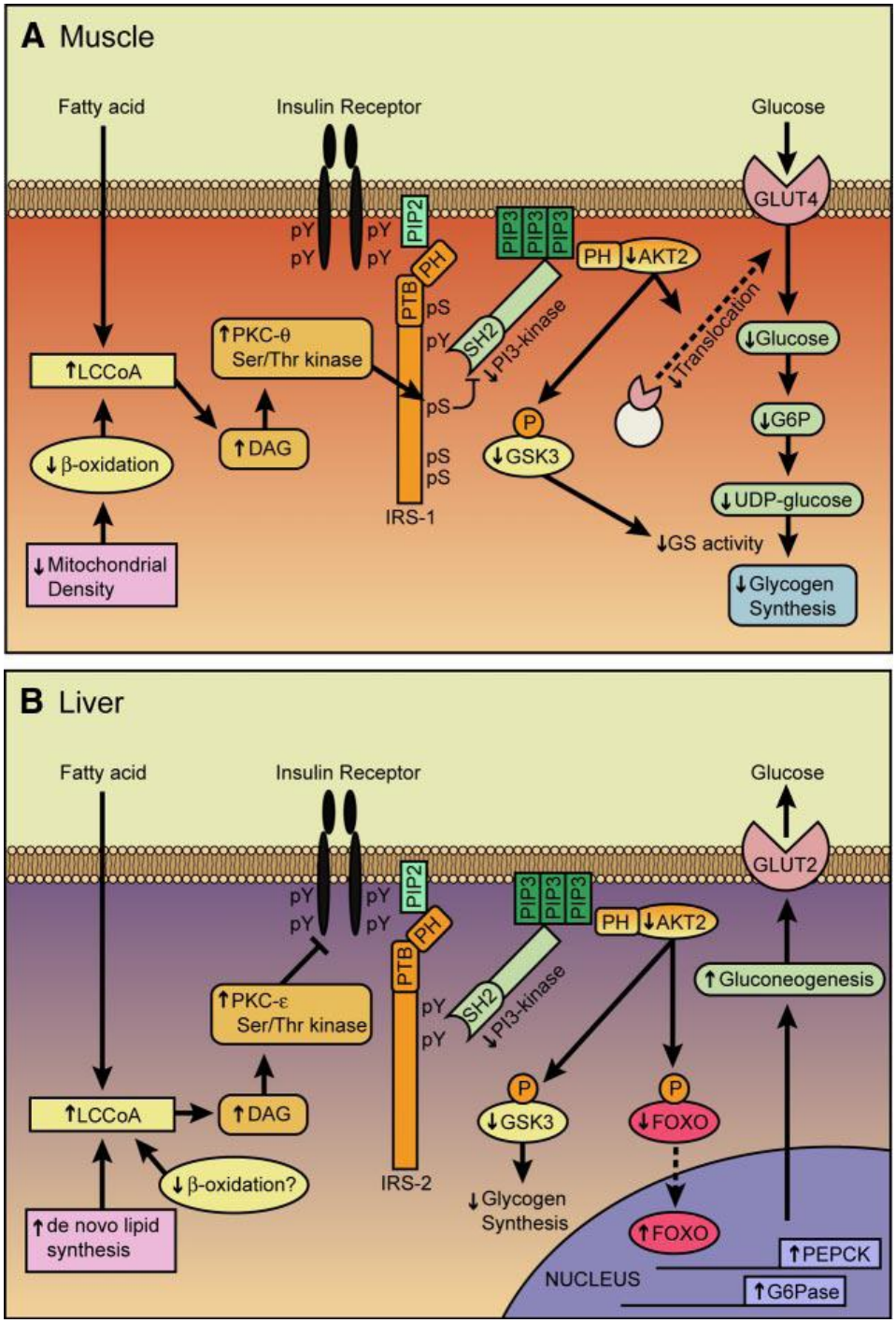
- Weight gain, abdominal adiposity in both males and females.
- Fatigue
- Increase blood sugar
- High blood triglyceride levels.
- Hypertension
- Increased pro-inflammatory cytokines associated with cardiovascular disease

Diagnosis

Fasting insulin levels: A fasting serum insulin level of greater than the upper limit of normal for the assay used (approximately 60 pmol/L) is considered evidence of insulin resistance.

Glucose tolerance testing (GTT): During a glucose tolerance test, a fasting patient takes a 75 gram oral dose of glucose. Blood glucose levels are then measured over the following 2 hours. Interpretation is based on WHO guidelines. After 2 hours a Glycemia less than 7.8 mmol/L (140 mg/dl) is considered normal, a glycemia of between 7.8 to 11.0 mmol/L (140 to 197 mg/dl) is considered as Impaired Glucose Tolerance (IGT) and a glycemia of greater than or equal to 11.1 mmol/L (200 mg/dl) is considered Diabetes Mellitus. (http://en.wikipedia.org/wiki/Insulin_resistance)

Molecular mechanism of insulin resistance

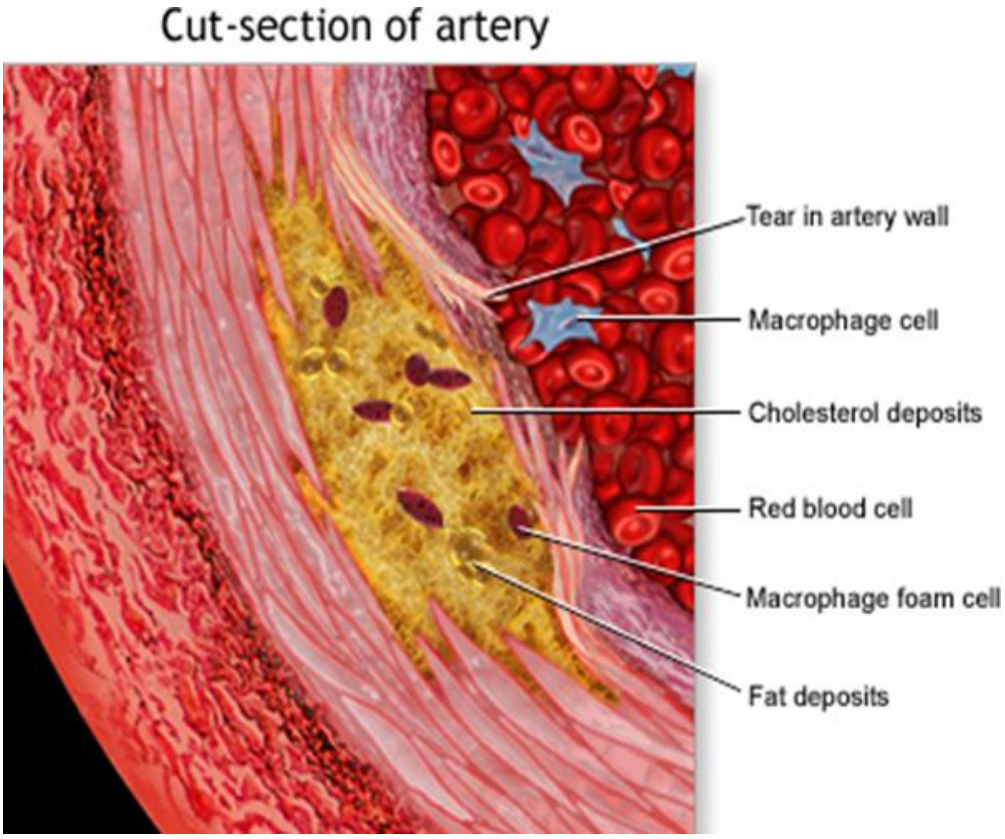


Mechanism of fatty acid-induced insulin resistance in skeletal muscle (*A*) and liver (*B*). *A*: muscle. Increases in intramyocellular LCCoA and DAG, due to increased fatty acid delivery and/or decreased mitochondrial fatty acid oxidation, trigger a serine/threonine kinase (Ser/Thr) cascade initiated by nPKCs and possibly involving IKK-β and/or JNK-1. This ultimately induces serine/threonine phosphorylation of critical IRS-1 sites in muscle, thereby inhibiting IRS-1 tyrosine phosphorylation and activation of PI 3-kinase, resulting in reduced insulin-stimulated muscle glucose transport and diminished muscle glycogen synthesis. *B*: liver. Increases in intracellular DAG, due to increased lipogenesis and/or decreased mitochondrial fatty acid oxidation activate PKC-ε, which binds to and inactivates the insulin receptor kinase resulting in reduced insulin-stimulated IRS-1 and IRS-2 tyrosine phosphorylation. This in turn results in reduced insulin activation of PI 3-kinase and AKT2. Reduced AKT2 activation results in lower GSK3 phosphorylation and lower FOXO phosphorylation, which in turn results in lower insulin-stimulated liver glycogen synthesis and decreased suppression of hepatic gluconeogenesis, respectively. DAG, diacylglycerol; FOXO, forkhead box protein O; GLUT, glucose transporter; G6P, glucose 6-phosphate; GSK3, glycogen synthase kinase- 3; IRS, insulin receptor substrate; IKK- β, IkB kinase- β; JNK-1, Jun kinase-1; LCCoA, long-chain acylcoenzyme A; nPKCs, novel protein kinase Cs; PEPCK, phospho*enol*pyruvate carboxykinase; PI 3-kinase, phosphoinositol 3-kinase; PTB, phosphotyrosine binding domain; PH, pleckstrin homology domain; SH2, src homology domain (Savage *et al*., 2007).

Treatment

Several anti-hyperglycemic pharmacological agents have been used to treat insulin resistance, *i.e*., α-glucosidase inhibitors, biguanides, thiazolidinediones, sulfonylureas, and insulin (Stumvoll and Häring, 2001; Brettenthaler *et al*., 2004). Lipid lowering drugs are also used to improve insulin sensitivity in diabetic patients. Fibrates have been shown to lower plasma triglycerides, reduce adiposity and improve hepatic and muscle steatosis, thereby improving insulin sensitivity. Fibrate drugs are widely used to treat hypertriglyceridemia in patients because it not only improves insulin sensitivity, but also reduces subclinical inflammation that otherwise, are a root cause for initiation of DM (Pahan, 2006).

Cardiac diseases



http://kardiol.com/?p=163

Atherosclerosis

It is a progressive disease in the wall of large and medium sized arteries that develop lesion called atherosclerotic plaques. Also, the arteries loose their elasticity and develop hardening. Another forms, such as arteriolosclerosis is characterized by general hardening (and loss of elasticity) of arterioles (small arteries) but atherosclerosis is a hardening of an artery specifically due to an atheromatous plaques. Atherosclerotic lesions are asymmetric focal thickening of the intima of artery, consisting of blood borne inflammatory and immune cells, vascular endothelial cells, smooth muscle cells, connective tissue elements

and lipids. Following are the risk factors for development of atherosclerosis (Glass and Witztum, 2001).

Genetic factors

- Elevated levels of LDL and VLDL
- Low levels of HDL
- Elevated lipoprotein (a)
- Hypertension
- Insulin resistance
- Diabetes Mellitus
- Male gender
- Elevated levels of homocysteine
- Elevated levels of hemostatic factors, e.g., Fibrinogen
- Metabolic syndrome

Environmental Factors

- Smoking
- Lack of exercise
- High fat diet
- Infectious agents

Elevated titre of serum cholesterol is probably unique in being sufficient to drive the development of atherosclerosis and hence, qualifies as the third important factor besides environmental and genetic factors. Inflammation, a defensive response of body to tissue damage, plays a key role in the development of atherosclerotic plaques (Goldstein and Brown, 1977). The formation of a plaque begins when excess LDLs from blood accumulate in the inner layer of an arterial wall, leading to the oxidation LDL present in

lipids or lipoproteins (Navab *et al*.,1996; Steinberg and Witztum, 1999). In response, Endothelial cells display adhesion molecules (VCAM-1, ICAM, P-Selectin and E-Selectin) on their luminal (inner) surface that attract and entangle inflammatory cells (monocytes) (Cybulsky and Gimbrone, 1991; Co lins *et al*., 2000). Smooth muscle cells of the intima secrete chemokines; monocyte chemoattractant proteins (MCP-1), which attract monocytes and convert them into macrophages (Navab *et al*.,1996). The macrophages then ingest oxidised LDL and form foam cells. T cells (lymphocytes) follow monocytes into the inner lining of an artery where they release inflammatory cytokines that intensify the inflammatory response (Yamada *et al*., 1998). Together, the foam cells, macrophages and T cells form a fatty streak that marks the beginning of an atherosclerotic plaque formation (Schwenke and Carew, 1989). Repeated cycles of inflammation lead to the accumulation of macrophages, some of which die in this location, producing the so-called necrotic core, and induce smooth muscle cell proliferation and migration in the lesion to form the thick fibrous cap of advanced, complicated, stable atherosclerotic lesions (Ross, 1999; Steinberg and Witztum, 1999; Paulsson *et al*., 2000). Expansion of a plaque in coronary artery obstructs the blood flow but most heart attack results due to plaque rupture. In addition, T cells induce foam cells to produce tissue factors (TF) that is considered to be the primary cofactor of cellular origin involved in activation of the coagulation pathway. The active form of TF has been shown to be present in specimens of human coronary artery in association both with acellular lipid areas and with macrophages and smooth muscle cells, which suggests that TF plays a major role in determining plaque thrombogenicity (Trimoli *et al*., 1999).

Diagnosis

Atherosclerosis does not trigger any signs and symptoms until it severely narrows or totally blocks an artery and hence, a medical emergency such as a heart attack or stroke is often a consequence. Elevated plasma levels of cholesterol and glucose are the main indicators for the detection of high risk of initiation and progression atherosclerosis (http://www.cardiacsolutions.net/pdf/education/Atherosclerosis.pdf).

Prevention of atherosclerosis

Lifestyle changes such as regular exercise, loss of excessive body weight, control of high blood pressure and diabetes mellitus may result in health benefits in terms of minimizing propensity of atherosclerosis. Many lipid lowering drugs such as statins, fibrates and bile sequestrants are available in the market that decreases the onset and progression of atheromatous plaque formation when detected in its early phase.

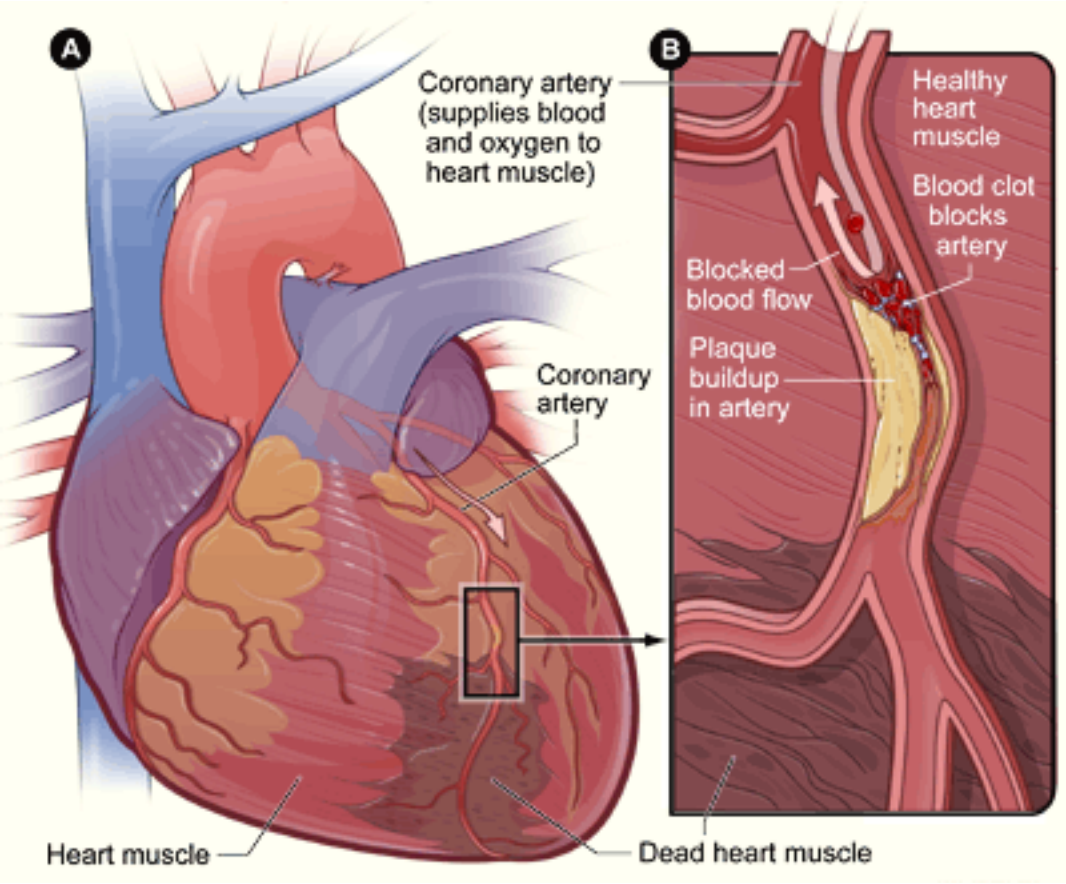
Statins: Statins are HMG-CoA reductase inhibitors that limit cholesterol synthesis hence, are most popularly used for treating atherosclerotic condition in patients. Statins (Lovastatin, Pravastatin, Simvastatin, Fluvastatin, Atorvastatin, and Rosuvastatin) block HMG-CoA reductase by competitive inhibition due to a higher affinity for the enzyme than its substrate (HMG-CoA). Inhibition of cholesterol synthesis, particularly in hepatocytes, decreases intracellular pools that further trigger proliferation of LDL receptors and their activity. This leads to an increased clearance of LDL particles. As a result, plasma concentrations of LDL-C and the number of LDL particles decrease in the blood stream with fewer LDL-C particles interacting with the intimal layer of blood vessels. Statins also lower plasma lipids, (including LDL-C) and triglycerides by

inhibition of hepatic VLDL synthesis. Statins are popular because they are effective group of drugs that manifest fewer side effects (tiredness, diarrhoea, sleeplessness and headache) (Wecker *et al*., 2010).

Fibrates: Fibrate is a popularly used term for Fibric acid or Phenoxyisobutyric acid (parent compound) that is used in several drugs that lower plasma cholesterol and triglyceride concentrations. Gemfibrozil, fenofibrate, and clofibrate are collectively known as fibrates or fibric acid derivatives. In contrast to statins, this group of drugs does not inhibit cholesterol biosynthesis. However, these drugs stimulate β-oxidation of fatty acids mainly in peroxisomes and partly in mitochondria thus lowering plasma fatty acid and triacylglycerol. However, nausea, diarrhoea and indigestion along with occasional skin rashes, impotency and weight gain may be the resultant side effects associated with these drugs (Staels *et al*., 1998).

Bile Acid Sequestrants (Resins): Bile acids are by-products of cholesterol metabolism in liver that are excreted via bile. Approximately 90% of it is reabsorbed from the intestine and reused for cholesterol biosynthesis in the liver. Bile Acid Sequestrants (Resins) (Cholestyramine, colestipol, and colesevelam) interfere with this intestinal re-absorption, by binding to the bile acids in the gut and thus promoting their elimination from the body along with faeces. These agents have no systemic effects and may lower the LDL cholesterol by 10-30% depending on their dose. Side effects include nausea, bloating, cramping and abnormal liver function (Guyton and Goldberg, 2009).

Myocardial Infarction



http://cidvascular.com/patients/coronaryartery/

Coronary artery diseases (CAD) and their end result, myocardial infarction (MI) contribute majorly to the overall statistics of mortality and morbidity in the western world. In UK alone, there are more than one million people who have suffered a heart attack. As per the updated statistics released by WHO, MI will be a major cause of death in the world by the year 2020 (Lopez and Murray, 1998). Myocardial infarction has witnessed a significant rise in India with a high frequency of occurrence amongst male patients (Krishnaswami, 1998; Ganesan and Anandan, 2009). Over the past 50 years, it has

become clear that the cascade of thrombotic events following atherosclerotic plaque rupture causes occlusion of the coronary artery, interrupting blood supply and oxygen to myocardium thus resulting in MI. Acute MI can be described based upon different perspectives such as clinical, electrocardiographic (ECG), biochemical and pathological characteristics (Thygesen *et al*., 2007). In MI, the first few hours witness ventricular tachyarrhythmia that results in considerable mortality because of their occurrence before a patient reaches the hospital. Ironically, these are easily preventable in an intensive care set. Ischemic myocardial pain is retrosternal, diffusive, radiating to left shoulder, left upper limb, right upper limb and back and is often described as crushing, constricting, squeezing or burning. Associated symptoms include dyspnea, fatigue, sweating, nausea and/or vomiting.

As per Thygesen *et al*., (2007) MI can be classified as:

Type 1: Spontaneous myocardial infarction

Type 2: MI secondary to an ischemic imbalance

Type 3: MI resulting in death when biomarker values are unavailable

Type 4: MI related to percutaneous coronary intervention

Type 5: MI related to coronary artery bypass grafting

Type 1: Spontaneous myocardial infarction related to atherosclerotic plaque rupture, ulceration, fissuring, erosion, or dissection with resulting intraluminal thrombus in one or more of the coronary arteries leading to decreased myocardial blood flow or distal platelet

emboli with ensuing myocyte necrosis. The patient may have underlying severe coronary artery disease but on occasion non-obstructive or no coronary artery disease.

Type 2: In instances of myocardial injury with necrosis where a condition other than coronary artery disease contributes to an imbalance between myocardial oxygen supply and/or demand, e.g. coronary endothelial dysfunction, coronary artery spasm, coronary embolism, tachyarrhythmias or bradyarrhythmias, anemia, respiratory failure, hypotension, and hypertension with or without left ventricular hypertrophy.

Type 3: Cardiac death with symptoms suggestive of myocardial ischemia and presumed new ischemic electrocardiographic changes or new left bundle branch block, but death occurring before blood samples could be obtained, before cardiac biomarkers could rise, or in rare cases cardiac biomarkers were not collected.

Type 4: Myocardial infarction associated with percutaneous coronary intervention is arbitrarily defined by elevation of cardiac troponin values >5×99 th percentile URL in patients with normal baseline values (≤99 th percentile URL) or a rise of cardiac troponin values >20% if the baseline values are elevated and are stable or falling. In addition, either (i) symptoms suggestive of myocardial ischemia, or (ii) new ischemic electrocardiographic changes or new left bundle branch block, or (iii) angiographic loss of patency of a major coronary artery or a side branch or persistent slow-flow or no-flow or embolization, or (iv) imaging demonstration of new loss of viable myocardium or new regional wall motion abnormality are required. MI associated with stent thrombosis is detected by coronary angiography or autopsy in the setting of myocardial ischemia and

with a rise and/or fall of cardiac biomarker values with at least one value above the 99th percentile URL.

Type 5: Myocardial infarction associated with coronary artery bypass grafting is arbitrarily defined by elevation of cardiac biomarker values >10×99 th percentile URL in patients with normal baseline cardiac troponin values (≤99 th percentile URL). In addition, either (i) new pathological Q waves or new left bundle branch block, or (ii) angiographic documented new graft or new native coronary artery occlusion, or (iii) imaging evidence of new loss of viable cells.

Electrocardiogram is the most important and basic diagnostic tool for the assessment of cardiac function. In acute myocardial infarction abnormality in ECG patterns include shortening of R-R intervals and QRS complex, ST segment elevation or depression, pathologic Q wave and loss of R wave. An X-Ray of the chest will show the heart size and state of pulmonary vasculature. Biochemical markers such as cardiac enzymes (Troponin-I, Troponin- T, Creatine kinase (Ck), Ck-MB and LDH) and lipid profile are elevated in plasma during cardiac damage (Jaffe *et al*., 2006). The measurement of Ck and Ck-MB levels has long been used for the diagnosis of MI. Ck, an enzyme present in many tissues, including the myocardium and skeletal muscle, has 3 isoenzymes: MM, MB, and BB. Ck-MB is present in a relatively high concentration in the myocardium (roughly 20% of the total myocardial Ck), whereas the concentration of Ck-MM is highest in skeletal muscle (98% of total muscle Ck) with only a small amount of Ck-MB (usually about 2%). However, healthy skeletal muscle can have up to 5% Ck-MB, and higher levels (up to 20%) of Ck- MB can be found in patients with renal failure and

chronic myopathic skeletal muscle injury. Following myocardial injury, the initial Ck-MB rise occurs 4 to 9 hours after the onset of chest pain, peaks at 24 hours, and returns to

baseline at 48 to 72 hours (Thygesen *et al*., 2007). Another important marker of MI is assessment of plasma titres of Troponins. They are made up of a regulatory complex of 3 protein subunits located on the thin filament of the myocardial contractile apparatus. The 3 subunits are designated troponin C (the calcium-binding component), TnT (tropomyosin-binding component), and TnI (the inhibitory component). The amino acid sequences of skeletal and cardiac isoforms of both TnT and TnI are sufficiently unique to permit development of monoclonal antibody–based immunoassays specific for cTnT and cTnI. Both cTnT and cTnI are stored in a 2compartment distribution in the myocyte, including a small cytosolic pool (4%-6%), with the majority of the remaining troponin found in the sarcomere. Thus, TnT and TnI have similar release kinetics from damaged myocardium. Both troponins increase in serum within 4 to 9 hours after AMI, peak at 12 to 24 hours, and remain elevated for up to 14 days (MacRae *et al*.,2006; Wu *et al*., 1999; Bertinchant *et al*., 1996).

Prevention

The risk of a recurrent myocardial infarction decreases with strict blood pressure management and lifestyle changes, chiefly smoking cessation, regular exercise, a sensible diet for those with heart disease, and limitation of alcohol intake. People are usually commenced on several long-term medications post-MI, with the aim of preventing secondary cardiovascular events such as further myocardial infarctions, congestive heart

failure or cerebrovascular accident (CVA). Unless contraindicated, such medications may include (Rossi, 2006; Smith *et al*., 2003).

- Antiplatelet drug therapy such as aspirin and/or clopidogrel should be continued to reduce the risk of plaque rupture and recurrent myocardial infarction. Aspirin is first-line, owing to its low cost and comparable efficacy, with clopidogrel reserved for patients intolerant of aspirin. The combination of clopidogrel and aspirin may further reduce risk of cardiovascular events, however the risk of hemorrhage is increased (Peters *et al*., 2003).
- Beta blocker therapy such as metoprolol or carvedilol should be commenced (Yusuf *et al*., 1985). These have been particularly beneficial in high-risk patients such as those with left ventricular dysfunction and/or continuing cardiac ischaemia (Dargie, 2001). β-Blockers decrease mortality and morbidity. They also improve symptoms of cardiac ischemia in NSTEMI.
- ACE inhibitor therapy should be commenced 24–48 hours post-MI in hemodynamically stable patients, particularly in patients with a history of MI, diabetes mellitus, hypertension, anteriorlocation of infarct (as assessed by ECG), and/or evidence of left ventricular dysfunction. ACE inhibitors reduce mortality, the development of heart failure, and decrease ventricular remodelling post-MI (Pfeffer *et al*., 1992).
- Statin therapy has been shown to reduce mortality and morbidity post-MI (Sacks *et al*., 1996; Sacks *et al*., 1998). The effects of statins may be more than their LDL lowering effects. The general consensus is that statins haveplaque stabilization and

multiple other ("pleiotropic") effects that may prevent myocardial infarction in addition to their effects on blood lipids (Ray and Cannon, 2005).

- The aldosterone antagonist agent eplerenone has been shown to further reduce risk of cardiovascular death post-MI in patients with heart failure and left ventricular dysfunction, when used in conjunction with standard therapies above (Keating and Plosker, 2004). Spironolactone is another option that is sometimes preferable to eplerenone due to cost.
- Evidence supports the consumption of polyunsaturated fats instead of saturated fats as a measure of decreasing coronary heart disease (Mozaffarian *et al*., 2010). Omega-3 fatty acids, commonly found in fish, have been shown to reduce mortality post-MI. While the mechanism by which these fatty acids decrease mortality is unknown, it has been postulated that the survival benefit is due to electrical stabilization and the prevention of ventricular fibrillation (Leaf *et al*., 2005). However, further studies in a high-risk subset have not shown a clear-cut decrease in potentially fatal arrhythmias due to omega-3 fatty acids (Brouwer *et al*., 2006; Raitt *et al*., 2005).

However, because most cases of sudden cardiac death occur in the population with coronary artery disease, it is logical that in recent years most attention has been given to secondary preventive therapy in patients with proven coronary artery disease and especially to survivors of a myocardial infarction.

In recent years, the prevention of cardiovascular diseases has been associated with the ingestion of fresh fruits, vegetables, spice or plants rich in natural antioxidants (Argolo *et*

al., 2004). The protective effects of plants can be due to the presence of flavonoids, anthocyanins and phenolic compounds (Sanchez-Moreno *et al*., 1998; Zhang and Wang, 2002). WHO recommends 500g of fresh fruits and vegetables per day (NCR, 1989). Antioxidant micronuctrients have attracted special attention, particularly vitamin E, Vitamin C, B carotene and other Carotenoids, such as lutin, zeazanthin and lycopene, which have the greatest singlet oxygen quenching properties (Halliwel and Gutteridge, 1989). More recently, there has been increased intrest in putative dietary antioxidants like bioflavonoids, anthocyanins, phenolic compounds and flavonals like quercetin or special phenol derivatives in red wine and oxygen sensitive B complexes, which are involved in the metabolism of homocysteine and L-arginine (Hirvonen *et al*., 2001).

Herbal medicines as an alternative therapy

Today we are witnessing a great deal of public interest in the use of medicines of herbal origin and therapies based on topical or ingestive uses of herbal extracts. Herbal medicines are based on the premise that plants contain natural substances that can promote health and alleviate illness. Plants have played a significant role in maintaining human health and improving the quality of human life for thousands of years and have served humans with valuable components of seasonings, beverages, cosmetics, dyes, and medicines. Estimates furnished by the WHO states that ~80% of the earth's inhabitants rely on traditional medicine for their primary health care needs and most of these therapies involve use of plant extracts or their active components. Furthermore, many Western drugs also share their lineage with herbal origin. Reserpine is widely used for the treatment of high blood pressure has its origin in *Rauwolfia serpentina*, whereas digitalis,

used as a heart stimulant was derived from *Digitalis purpurea*. Paclitaxel (TAXOL; Bristol-Myers Squibb, Princeton, NJ), a new chemotherapeutic agent is obtained from the bark of the Pacific yew, *Taxus brevifolia* and other yew species. Patients with metastatic breast cancer, advanced lung cancer, cancers of the head and neck, melanoma, ovarian

cancer, and lymphomas have responded positively to Taxol (Agrawal *et al*., 2010).

The modern therapeutic regimens are considered rigid, multi pharmaceutical and often associated with intolerable side effects. But in developing countries, these therapeutic options are expensive and not readily accessible to the poor people (Adeneye and Agbaje, 2008). These conditions demand an effective management/treatment of CVDs and related diseases. In view of these shortcomings, herbal pharmacotherapy is often explored by these patients as a cheaper and readily available alternative. Many traditional folklore medicines and herbal extracts have been used for the treatment of diabetes mellitus and CVD.

Plants/herbs/phytocompounds in the management of insulin resistance

Plant name	**Parameters protected**	**Reference**
Grape seed	Prevents body weight gain, hyperglycemia, hyperinsulinemia, attenuates impairments of insulin stimulated glucose disposal and possess insulinomimetic properties	Wannaporn *et al*., 2010
Tinispora cordifolia	Prevents hypertriglyceridemia hyperglycemia and partially prevents hyperinsulinemia	Reddy *et al*., 2009
Eucommia ulmoides	Improves plasma levels of insulin and index for insulin resistance HOMA-IR (Homeostasis Model Assessment ratio)	Xin Jin *et al*., 2010
Cinnamomum burmannii	Insulin like activity, increases insulin receptor beta autophosphorylation, potentiated insulin regulated glucose utilisation, and increase GLUT 1 expression	Cao *et al*., 2010
Hunteria umbellata	Hypoglycemic and anti hyperlipidemic, Improves insulin resistance and increases perioheral glucose uptake	Adeneye and Aenemi., 2009
Ananas comosus L.	Anti-diabetic,improves insulin sensitivity, hypoglycemic and Hypolipidemic	Xie *et al*., 2006
Biden spilosa	Anti hyperglycemic,protects pancreatic islets, augment serum insulin levels and reduced blood glucose levels	Hsu *et al*., 2009
Abutilum indicum	Reduces glucose absorption, hypoglycemic, antidiabetic and stimulates insulin secretion	Krisanapun *et al*., 2009
Eugenia jambolana	Stimulates insulin release,hypolipidemic, antihyper- -glycemic	Sharma *et al*., 2008
Artemisia princepspampanini	Anti-diabetic	Jung *et al*., 2007

Plants/herbs/phytocompounds in management of LDL oxidation

Plant/Herb	Parameters	References
Tropaeolum tuberosum	Peroxyl radical scavenger, chelating agent, protects against oxidation of LDL	Chirinos *et al*., 2008
Sida rhomboidea. Roxb	Reduces LDL oxidation, attenuates peroxyl radical formation	Thounaujam *et al*., 2011
Clerodendron glandulosum.Coleb	Prevents HMDM induced LDL oxidation and Cu^{+2}mediated LDL oxidation and foam cell formation	Jadeja *et al*., 2011
Terminalia bellerica	Prevents formation of superoxide, nitric oxide and hydroxyl radicals, potential inhibitor of LDL oxidation	Nampoothiri *et al*., 2011
Zanthoxy lumailanthoides	Prevents lipid accumulation in THP--1 cell line, deceases scavenger receptor expression and CD 36, prevents $CuSO_4$ mediated LDL oxidation	Chu *et al*., 2009
black soybean seed	Potent DPPH radical scavenger andattenuates LDL oxidation	Astadi *et al*., 2009
Hibiscus sabdariffa L.	Inhibition of ApoB fragmentation, Potent DPPH radical scavenger, inhibits TBARS formation, inhibits Ox-LDL induced apoptosis	Chang *et al*., 2006
Pinus morri sonicola Hay	Potent free radical scavenger, inhibits copper induced oxidation, decreases lipid accumulation and foam cell formation, inhibits nitric oxide production and decreases relative electrophoretic mobility	Yen *et al*., 2008
wheat bran	Reduces lipid peroxidation in LDL, potent DPPH and ABTS free radical scavenger	Yu *et al*., 2005

Plants/herbs/phytocompounds in management of atherosclerosis

Plants/Herbs/ Phytocompounds	**Parameters protected**	**Reference**
Olive leaf	It improves increment in serum levels of atherosclerosis related markers, TG, TC, VLDL, LDLtriglyceride (TG), total cholesterol, VLDL, LDL, HDL and Malondialdehyde (MDA). It prevented increment in lesions and thickness of intimasAnd also decrease mRNA expressions of inflammation factors, monocyte chemoattractant protein (MCP)-1, vascular cell adhesion molecule (VCAM)-1, nuclear factor-kappa B (NF-KB) and tumor necrosis factor a (TNF-a)	Wang *et al*., 2008
Curcumin	Curcumin, a polyphenolic natural compound prevents deposition of cholesterol and decrease plaque area.	Olszanecki *et al*., 2005
Moringa oleifera	The phenolic compound reduces plasma lipid and lipoprotein profile, conjugate diene formation and lipid peroxidation, and plaque formation.	Chumark *et al*., 2008
Garlic and Turmeric	They prevent increment in lipid and lipoprotein profile, ECG profile (Systolic Pressure , Diastolic Pressure, Mean Arterial Pressure, Heart Rate).	Ashraf *et al*., 2005
*Clerodendron glandulosum.*Coleb	It significantly prevents increment in serum cholesterol, triglycerides, and lipoproteins, markers of LDL-C oxidation, auto-antibody titer and aortic lipids. Also, LDL-C isolated from ATH+CG rats recorded mimimal aggregation and susceptibility to undergo ex vivo LDL-C oxidation. Microscopic evaluation of thoracic aorta reveled that prevention of	Jadeja *et al*., 2011

	atheromatous plaque formation, accumulation of lipid laden macrophages, calcium deposition, distortion/defragmentation of elastin, accumulation of macrophages and, down regulation of cell adhesion molecules (VCAM-1 and P-selectin) expression.	
*Sida rhomboidea***.**Roxb	It significantly reduces serum levels of TC, TG, LDL, VLDL, autoantibody against oxidized LDL (Ox-LDL), markers of LDL oxidation and aortic TC and TG. It also improves serum HDL levels. Histopathology of aorta and immunolocalization studies recorded decrement in atheromatous plaque formation, vascular calcification, significant elastin derangements and higher expression of macrophage surface marker (F4/80), vascular cell adhesion molecule-1 (VCAM-1) and p-selectin in ATH rats.	Thounaojam *et al*., 2012

Plants/herbs/phytocompounds in the management of IP induced myocardial infarction

Plants/Herbs/ Phytocompounds	**Parameters protected**	**Reference**
Oxalis corniculata	Improves cardiac damage enzyme marker (CPK and LDH), lipid profile (TC and TG), Reduce TBARS and prevented decrement in GSH and Ascorbic acid levels, and histopathological damages	Abhilash *et al*., 2011
Naringin	Prevents alteration in heart weight, ATPases, blood glucose, uric acid and histopathological damages	Rajadurai and Stanely Mainzen

		Psrince, 2007
Sida rhomboidea. Roxb	Improves heart weight, plasma lipid and lipoprotein profile, plasma cardiac damage marker enzymes, lipid peroxidation, endogenous enzymatic and non-enzymatic antioxidants, membrane bound ATPases and histopathology	Thounaojam *et al*., 2011
Cladosiphon okamuranus	Prevents increment in cardiac damage markers (CK-MB, LDH, AST, ALT), Improved serum HDL levels and decreased TC and TG levels and, also improved histopathological damages	Thomes *et al*., 2010
Punica granatum L.	Prevents increment in heart weight, infarct size, plasma marker enzymes of cardiac damage, lipid peroxidation, endogenous enzymatic and non-enzymatic antioxidants, cardiac ATPases and histopathological damages	Jadeja *et al*., 2010
Tribulus terrestris Linn.	Improves alteration in enzymatic and non- enzymatic antioxidants and cardiac damage markers, ECG pattern and histopathological damages	Ojha *et al*., 2008
Epigallocatechin-gallate (EGCG)	Prevents increment in TBARS and lipid Hydroperoxide, improved enzymatic and non-enzymatic antioxidants and also histopathological damages	Devika and Stanely Mainzen Prince, 2008
Rutin	Reduces mitochondrial damage and prevented cardiac mitochondrial dysfunction. Lipid peroxides, lipids and calcium, improving multienzyme activities, glutathione levels, adenosine triphosphate levels	Punithavathi *et al*., 2010
Gallic acid	Prevents increment in damage marker enzymes (ceatine kinase, creatine kinase-MB, aspartate transaminase, alanine transaminase and lactate dehydrogenase in serum and the levels of troponin-T) in plasma. It also prevented increment in the levels of lipid peroxidation improved enzymic antioxidants (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase) andnon-enzymic antioxidants (glutathione, vitamin C and E)	Priscilla and Stanely Mainzen Prince, 2009

Coriandrum sativum L.

Kingdom: Plantae
Subkingdom: Trachaeobionta (vascular plants)
Superdivision: Spermatophyta (seed plants)
Division: Magnoliophyta (flowering plants)
Class: magnoliopsida (dicotyledons)
Subclass: Rosidae
Order: Apiales
Family: Apiaceae (carrot family)
Genus: Coriandrum L. (coriander)
Species: Sativum

http://essentialoils.net/2012/09/coriander-oil-some-uses/

Coriandrum sativum L. Apiaceae (Umbelliferae) (coriander, also known as cilantro, cilantrillo, Arab parsley, Chinese parsley, Mexican parsley, Dhania and Yuen sai), is an annual herb commonly used in Middle eastern, Mediterranean, Indian, Latin American, African and Southeast Asian cuisine. All parts of the plant are edible, but the fresh leaves and the dried seeds are the most common parts used in cooking. It is a soft, growing to 50 centimetres (20 in) tall. The leaves are variable in shape, broadly lobed at the base of the plant, and slender and feathery higher on the flowering stems. The flowers are borne in small umbels, white or very pale pink, asymmetrical, with the petals pointing away from the centre of the umbel longer (5–6 mm) than those pointing towards it (only 1–3 mm long). The fruit is a globular, dry schizocarp 3–5 mm diameter. In the Indian traditional medicine, coriander is used in the disorders of digestive, respiratory and urinary systems, as it has diaphoretic, diuretic, carminative and stimulant activity (Grieve, 1971; PDR-HM, 2004). Its use is recommended in urethritis, cystitis, urinary tract infection, urticaria, rash, burns, sore throat, vomiting, indigestion, nosebleed, cough, allergies, hay fever, dizziness

and amoebic dysentery (Grieve, 1971; PDRHM, 2004). The use of coriander as a diuretic or in the treatment of renal disease has been described in several publications (Grieve, 1971; Nadkarni, 1976; Duke, 1992; Usmanghani *et al*., 1997; Eddouks *et al*., 2002; Azhar *et al*., 2003). Locally known as "Maadnouss," in Morocco, coriander has been documented as a traditional treatment of diabetes, indigestion, flatulence, insomnia, renal disorders and loss of appetite, and as a diuretic (Hmammouchi, 1999; Hassar, 1999; El-Hilaly *et al*., 2003). A large number of compounds have been identified in coriander, including flavonoids (quercetin and isoquercetin), polyphenols (rutin, caffeic acid derivatives, ferrulic acid, gallic acid and chlorogenic acid) and β-caroteinoids (Melo *et al*., 2003). The volatile oil derived from coriander seed (up to 1.7%), containing α-pinene, (−)-borneol, camphor, citronellol, coriandrol, *p*-cymene, geraniol, geranyl acetate, limonene, d-(+)-linalool, myrcene, α- and β- phellandrene, α- and β-pinene, α- and β-terpinene, trans-tridec-2-enale, and a number of fatty acids (Sergeeva, 1975; Ishikawa *et al*., 2003; PDR-HM, 2004), has been shown to stimulate gastric juices and is used as a carminative and spasmolytic agent. Other components identified in coriander include coriandrin, z-2-decenal, decanal, dodecanal, β -ionone, eugenol, hydroxycoumarins (umbelliferone and scopoletin), and a large number of water-soluble compounds, including, monoterpenoids, monoterpenoid glycosides, monoterpenoid glucose sulfate and other glycosides (Sergeeva, 1975; Ishikawa *et al*.,2003; Kobayashi *et al*., 2003; Esiyok *et al*., 2004; Kubo *et al*., 2004; PDR-HM, 2004; Bajpai *et al*., 2005; Eyres *et al*.,2005).

The predominant constituent of essential oil of coriander is linalool which forms approximately two thirds of the oil (Salzer, 1977; Lawrence, 1980a,b; Budavari *et al*., 1999; Gil *et al*., 2002; Grosso *et al*., 2008). Typical compositional analysis of coriander

oil is as follows: alcohols: linalool (60–80%), geraniol (1.2–4.6%), terpinen-4-ol (trace-3%), a-terpineol (<0.5%); hydrocarbons: c-erpinene(1–8%), r-cymene (trace-3.5%), limonene (0.5–4%), a-pinene (0.2–8.5%), camphene (trace-1.4%), myrcene (0.2–2%); Ketones (7–9%): camphor (0.9–4.9%); esters: geranyl acetate (0.1–4.7%), linalyl acetate (0–2.7%); coumarins/furanocoumarins: umbelliferone,bergapten. Coriander oil was reported to contain approximately 30% terpene hydrocarbons and 70% oxygenated compounds (Karlsen *et al*., 1971).

Studies have proved that coriander extracts and essential oil exhibit antioxidant activities (Melo *et al*., 2003). Wangensteen *et al*., (2004) demonstrated that coriander leaves showed stronger antioxidant activity, tested by DPPH, the inhibition of 15-lipoxygenase and inhibition of Fe2+-induced porcine brain phospholipid peroxidation, than the seeds. The aqueous and ethanol extracts of fresh coriander leaf strongly inhibit linoleic acid oxidation in an emulsion, while the essential oil obtained from fresh coriander leaf inhibits lipid oxidation in both model emulsion and bulk sunflower oil systems (Stashenko *et al*., 2002).

Pharmacological activities

Plant parts	Pharmacological activities
	Seeds
Powder	Hypolipidemic (Chithra and Leelamma, 1997; Chithra and Leelamma, 1999; Lal *et al*., 2004), hypocholesterolemic (Dhanapakiam *et al*., 2008).
Oil	Antifungal (Garg and Siddiqui, 1992) Antimicrobial (Baratta *et al*., 1998)
Extract	Insulin releasing and insulin like activity (Gray and Flatt, 1999) antihypertensive (Medhin *et al*., 1986), antioxidant (Melo *et al*., 2003; Ramadan *et al*., 2003; Bajpai *et al*., 2005, Hashim *et al*., 2005), Diuretic (Aissaoui *et al*., 2008), antidiabetic activity (Eidi *et al*., 2009)
	Leaves
Oil	Antimicrobial activity (Begnami *et al*., 2009; Matasyoh *et al*., 2009)
Extract	Antioxidant (Wong and Kitts, 2006; Melo *et al*., 2005) antibacterial activity (Wong and Kitts, 2006) Antidiabetic activity (Sreelatha and Inbavalli, 2012)
	Stem
Extract	Antioxidant, hypolipidemic and antidiabetic activity (Sreelatha and Inbavalli, 2012)

Polyphenols

Polyphenols are ubiquitous in plant kingdom and practically all plant foods and beverages contain at least some amounts of these compounds (3941). The richest sources are fruits, berries, vegetables, cereals, legumes, nuts, and beverages such as wine, tea, coffee and cocoa. However, the types and amounts of compounds may vary greatly between different foods.

Polyphenols in food

Sub class	Individual compounds
Hydroxybenzoic acids	Gallic, vanillic, syringic, *p*hydroxybenzoicacid
Hydroxycinnamic acids	Caffeic, ferulic, *p*coumaric, sinapic acid
Anthocyanidins	Cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin
Flavonols	Quercetin, kaempferol, myricetin, isorhamnetin
Flavones	Luteolin, apigenin
Flavanones	Hesperetin, naringenin, eriodictyol
Flavan3ol Monomers	Catechin, epicatechin, gallocatechin, epigallocatechin, epicatechin3gallate, epigallocatechin3gallate, theaflavin, theaflavin gallate, theaflavin digallate, thearubigins

Epidemiological studies have explored the relation between polyphenol intake and the subsequent decrement in the risk of CVDs in human subjects. Animal and clinical studies have further detailed the underlying protective mechanism(s) of polyphenols induced reduction in risk of CVDs. A study done on the Greek population by Lagiou *et al*., (2004) reported that the intake of flavan-3-ols was associated with a decreased risk of CHD and later peripheral arterial occlusive disease (Lagiou *et al*., 2006). Marniemi and his co-workers found that the intake of two flavonoids, luteolin and kaempferol, inverted the risk of acute myocardial infarction (AMI) in a Finnish population (Marniemi *et al*., 2005). Recently, high intake of anthocyanidins was found to be related with the decreased risk of acute MI (Tavani *et al*., 2006).

The most popular hypothesis for the protective mechanism of polyphenols against CVD is their ability to act as antioxidants. Polyphenols have been suggested to decrease the oxidative stress in human body particularly by inhibiting oxidation of LDL (Fuhrman and Aviram, 2001). Flavonoids may inhibit oxidative stress by: 1) scavenging free radicals, acting as reducing agents, hydrogen atom donating molecules or singlet oxygen quenchers; 2) chelating metal ions; 3) sparing other antioxidants (e.g. βcarotene, vitamin C and E); and 4) preserving HDL associated serum paraoxonase activity (Fuhrman and Aviram, 2001). Antioxidant properties of polyphenols are related to their chemical structure and are dependent on the number and arrangement of phenolic hydroxyl groups (Heim *et al*., 2002; RiceEvans, 2001).

In vitro antioxidant properties of various polyphenols have been well documented and most of the compounds have been found to be powerful antioxidants. The evidence is especially extensive for monomeric (catechins) and polymeric (proanthocyanidins) flavan-3-ols. Antioxidant properties and beneficial effects of food items rich in flavan-3-ols, such as black tea (Ishikawa *et al*., 1997; McAnlis *et al*., 1998), green tea (Ishikawa *et al*., 1997; van het Hof *et al*., 1997, Serafini *et al*., 1996; Princen *et al*., 1998), red wine (van het Hof *et al*., 1997, Fuhrman *et al*., 2001), cocoa (Sanbongi *et al*. 1998; Waterhouse *et al*., 1996), and chocolate (Vinson *et al*., 1998 , Waterhouse *et al*., 1996) are elaborately reported against oxidative stress or atherosclerosis. Also flavonols, especially quercetin, have been found to be effective antioxidants *in vitro* (Das and Ray, 1988; Williamson *et al*., 2005).

CS and polyphenols

Hashim and his research group had isolated polyphenol from CS using 80% methanol and estimated the levels of polyphenolic compounds in each fraction using standard quercetin (Hashim *et al*., 2005). It was found that the higher polyphenolic content was present in the said hydromethanolic CS extract. Various fractions were prepared from polyphenol rich hydromethanolic extract of CS and subjected to the hydrogen peroxide-induced oxidative damage in human lymphocytes. Pretreatment resulted in protection of human lymphocytes against H2O2 -induced oxidative damage. H2O2 treatment significantly decreased the activities of antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase and caused decreased glutathione content and increased thiobarbituric acid-reacting substances (TBARS). Treatment with polyphenolic fractions (50µg/ml) increased the activities of antioxidant enzymes and glutathione content and reduced the levels of TBARS significantly. Observed reduction in the level of lipid peroxides showed a decreased tendency of peroxidative damage. This study suggested that polyphenolic compounds present in hydromethanolic extract of CS effectively suppress hydrogen peroxide-induced oxidative stress.

Aim of the study

Lipid lowering and antioxidant properties of the CS extract are well documented. Although, majority of the studies are centered on essential oils extracted from CS seed extract, a report on preliminary phytochemical analysis of hydromethanolic extract of CS has been reported to be containing high levels of polyphenols. Due to the existing lacunae in information and going by the merits of hydromethanolic extract of CS the same was chosen for our study. This thesis is a compile of preventive role of hydromethanolic extract of CS against experimentally induced insulin resistance, atherosclerosis and myocardial infarction using relevant *in vivo* and *in vitro* experimental models. The results obtained herein have been discussed in detail to document merits/demerits of hydromethanolic extract of CS as a therapeutic agent and to decipher the underlying mechanism of its therapeutic potential.

CHAPTER 1

Acute and sub-chronic toxicological evaluation of hydro-methanolic extract of *Coriandrum sativum* L. seeds

INTRODUCTION

The traditional systems of medicine such as the Ayurveda, Unani and Sidda have been a treasure trove for development of majority of modern medicines. Also the medicinal research relies on ethnobotany and ethnopharmacognocy for discovery of new molecules for that conventionally result in drugs developments (Gurib-Fakim, 2006). World Health Organization (WHO) estimates that approximately 80 % of the developing world's population is using traditional medicine for primary healthcare (Bannerman, 1982). However, there is a prevalent misunderstanding that herbal medicines are devoid of toxic effects (WHO, 2004). Adverse effects of herbs have been reported including allergic reactions, hepatotoxicity (Saad *et al*., 2006), nephrotoxicity (Colson and De Broe, 2005; Kwan *et al*., 2006; Zhu, 2002; Vanherweghem, 1998), cardiac toxicity (Horowitz *et al*., 1996; Moritz *et al*., 2005; Gaibazzi *et al*., 2002), neurotoxicity (Ernst, 2003; Benjamin *et al*., 2001) and even death (Jensen and Allen, 1981) have been reported. There are several potential causes of toxicity due these medications, they include:

• Use of inherent toxic herbs

• Variability in active or toxic ingredients due to growing conditions, processing, or preparation

• Misidentification of herbs

• Contamination or adulteration

Reported Cases of hepatic, renal and cardiac toxicity and, cancer related to herb Consumption

Common Name	Scientific Name	Suggested Active Compounds	Uses	Side-Effects
Celandine	*Chelidonium majus*	Isoquinoline Alkaloids	Externally for skin conditions (warts, eczema); internally for liver & gallstones.	Ten cases of hepatitis.
Comfrey	*Symphytum officinale* *Symphytum asperum*	Pyrrolizidine alkaloids*	Internally for blunt injuries (bruises, sprains, and broken bones), digestive tract problems (ulcers, diarrhea, inflammation), rheumatism and pleuritis. Externally as a gargle for gum disease, pharyngitis, and strep throat.	Vino-occlusive disease, liver toxicity and failure, & liver cancer.
Germander	*Teucrium chamaedrys*	Diterpenes	Weight loss, gout, digestive aid, fever.	Liver toxicity, fatal hepatitis. France banned it in 1992 after 26 hepatitis cases.
Groundsel	*Senecio vulgaris*	Pyrrolizidine alkaloids	Colic, epilepsy, worms.	Not recommended for internal use due to its toxic and carcinogenic pyrrolizidine alkaloids.
Chocolate Vine (Mu Tong)	*Caulis aristolochiae*	Aristolochic acid	Urinary tract infections, ascites, laryngitis, & kidney stones.	Acute renal failure, permanent renal failure, renal-function impairment, Fanconi syndrome.
Foxglove	*Digitalis lanata*	Cardiac glycosides	Congestive heart failure.	Tachycardia; ventricullar fibrillation, & death.

Henbane	*Hyoscyamus niger*	Tropane alkaloids – Hyoscyamine	Internally for stomach complaints, toothaches, ulcers, & tumors.	Impaired vision, constipation, flushed skin, irregular heartbeat; 19 Bedouin children hospitalized after ingestion – restlessness and hallucinations (3 went into a coma).
Licorice	*Glycyrrhiza glabra*	Triterpenesaponins, Hydroxycoumarins	Approved by the German Commission E for gastritis, cough, & bronchitis. Also used for ulcers, inflammation & epilepsy.	Hypertension, hypokalemia, hypernatremia, edema, heart failure, death.
Squill	*Urginea maritime*	Cardiac glycosides	Approved by the German Commission E for cardiac insufficiency, arrhythmia, nervous heart complaints, & venous conditions. Other uses include bronchitis, asthma, whooping cough, and wounds.	Nausea, vomitting, hyperkalemia, arrhythmias, and atroventricular block, 1 case of death.
Coltsfoot	Coltsfoot tussilagofarfara	Pyrrolizidine alkaloids – Senkirkine	Approved by German Commission E for cough, bronchitis, inflammation of mouth & pharynx. Other uses include smoking cessation treatment.	Cancer in rats. Flowers not recommended due to possible hepatoxic and carcinogenic effects. Germany limits dosages. Austria prohibits leaves.

Therefore, a pre- clinical toxicity study is indispensible to validate their safe medicinal use. Preclinical toxicological evaluations of synthetic or herbal medicines or herbs should be done either of guidelines such as (a) Organization for the Economic Co-operation and Development (OECD) Guidelines, (b) WHO GCP Guidelines (c) UCSF IACUC POLICY– Laboratory Housing and Study Areas for Research Animals and (d) Schedule "Y" in Drugs and Cosmetics (Eighth Amendment) Rules 1988, India.

We had recently reported anti-insulin resistance (Patel *et al*., 2011) and cardioprotective (Patel *et al*., 2012) potentials of CS seed extract. Since toxicological evaluation of CS seed extract is not studied, the present study evaluates possible toxicity of CS seed extract using OECD guidelines.

MATERIALS AND METHODS

Plant material and preparation of extract

Seeds of CS were collected (in the month of February and March) and identified by Dr. P.S. Nagar, Department of Botany, The M.S. University of Baroda. A herbarium of plant was deposited in the Department of Botany. One hundred grams of powdered dry seeds were soaked in methanol:water (80:20 v/v) at room temperature and allowed to stand for seven days. The resultant extract was filtered through a muslin cloth and then concentrated in a rotary evaporator under reduced pressure to obtain a thick semisolid brown paste (Hashim *et al*., 2005). The final yield was 8.3 g (w/w).

Experimental animals

Adult female Swiss albino mice (20-25 g) were obtained from Zydus Cadila Research Centre, Ahmedabad, Gujarat, India. They were housed under standard animal house conditions (temperature: 23 ± 2 °C; photoperiod: 12 h light and 12 h dark; humidity: 45-50 %). They were fed with standard laboratory pellets (M/S Pranav agro, Ltd., Baroda, India) and water *ad libitum*. The animals were maintained as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) India and the experimental protocol approved by the animal ethical committee of the Department of Zoology, The M. S. University of Baroda, Vadodara (Approval No.827/ac/04/ CPCSEA).

Acute oral toxicity

Acute oral toxicity study was conducted according to the guidelines of OECD, 401. Twenty four animals were randomly allocated into four groups of six animals each. Group I (Control): animals were administered orally with vehicle (0.05 % Carboxy methyl cellulose; CMC). Remaining groups (II, III and IV) were administered with

1000, 3000 and 5000 mg/ kg body weight of CS extract respectively via gastric intubation. Doses were prepared using 0.05 % CMC and dose volume was not more than 1 ml/kg body weight. Cage side observations (tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma) were recorded during first four hours and mortality was recorded after 24 h (Jadeja *et al*., 2011).

Sub-chronic oral toxicity

The sub-chronic oral toxicity study was conducted according to the guidelines of the OECD, 407. Twenty four animals were randomly divided into four groups of six animals each. Group I (CS_0) served as a control and received 0.5 % CMC (vehicle) for 28 days whereas; the remaining groups (Group II- CS_1, Group III- CS_2 and Group IV- CS_3) were orally administered 1000, 2000 and 3000 mg/kg body weight respectively of CS extract daily for 28 consecutive days. Food and water intake were recorded daily, whereas; body weight was recorded once in a week throughout study period *(*Thounaojam *et al*., 2010b; Thounaojam *et al*., 2011*).*

Plasma isolation and hematology

At the end of 28 days, blood samples were collected from overnight fasted animals through retro-orbital sinus puncture in ethylene diamine tetra acetic acid (EDTA) coated vials and plasma was separated by cold centrifugation (Plasto Crafts Superspin-R centrifuge) at 3000 rpm for 10 min. Blood was also collected for the analysis of haematological parameters such as white blood cell (WBC) count, red blood cell (RBC) count, haemoglobin (Hb) levels, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and red cell distribution width (RCDW) using BC 2300 Hematology Analyzer (Shezhen Mindray Biomedical Electronics Co., Ltd., China).

Plasma biochemical parameters

Creatinine kinase-MB (CK-MB; cardiac damage), aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, total protein (liver damage) and urea and creatinine (kidney damage) were analyzed using commercially available kits (Recon diagnostic Ltd., Vadodara, India). Also, plasma glucose and lipid profile [total cholesterol (TC), triglyceride (TG) and high density lipoprotein (HDL-C)] were assessed and low density lipoprotein (LDL-C) and very low density lipoprotein (VLDL-C) were calculated by Friedewald's formula (Friedewald *et al*., 1972).

Relative organ weights and histopathology

Animals were later sacrificed by cervical dislocation under mild ether anesthesia for autopsy and liver, kidney, heart, lung and spleen were excised, rinsed in 0.9 % saline and weighed. After sacrifice, organ weights (lungs, heart, liver, kidney and spleen) were recorded and relative organ weights (ROW) were calculated as follows.

$$\text{ROW} = \frac{\text{Absolute organ weight (g) X 100}}{\text{Body weight on the day of sacrifice (g)}}$$

Tissue pieces of vital organs (heart, liver and kidneys) were fixed in 10 % paraformaldehyde for paraffin histology and processed in paraffin embedding as per the standard protocol. 7 µm thick sections of each tissue were stained with hematoxylin and eosin, and observed for possible histopathological damages.

RESULTS

Acute oral toxicity

Cage side observations did not record any behavioral changes such as tremor, convulsion, salivation, diarrhea, lethargy or sleep during the first four hours of CS extract (1000, 2000 or 3000 mg/kg body weight) administration. After 24 h there was no mortality recorded in plant extract administered groups. However, urine output was found to be increased in CS treated animals (1000, 2000 or 3000 mg/kg body weight) as compared to the control (data not shown).

Sub-chronic oral toxicity

Body weight gain, food and water intake (Table 1.1; Figure 1.1a-1.1b)

CS_1 and CS_2 groups did not record any significant alterations in body weight gain. However, CS_3 group (3000 mg/kg body weight) recorded significant ($p<0.001$) decrement in body weight gain. Further, there was significant ($p<0.05$ and $p<0.001$ respectively) reduction in food intake of CS_2 and CS_3 groups as compared to CS_0. Water intake was significantly ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) increased in all the CS extract administered groups as compared to CS_0 group.

Haematology (Table 1.2; Figure 1.2a-1.2d)

The haematological parameters (RBC, WBC, Hb, MCV, MCH, MCHC, RCDW, monocytes, lymphocytes, eosinophil) did not record any significant alterations in any of CS administered groups.

Biochemical parameters (Table 1.3 &1.4; Figure 1.3a-1.3c and 1.4a &1.4b)

Plasma glucose recorded moderate non-significant decrement in CS_2 and CS_3 groups. Also, plasma TC, TG, LDL-C and VLDL-C levels recorded moderate to significant reductions in all the CS treated groups but, plasma HDL-C levels were unaltered. Plasma marker of creatinine kinase-MB, AST, ALT, bilirubin, total protein, urea and

creatinine did not record significant alterations in any of the CS treated groups as compared to the CS_0 group.

Relative organ weights and histopathology (Table 1.5; Figure 1.5a &1.5c, 1.6, 1.7 and 1.8)

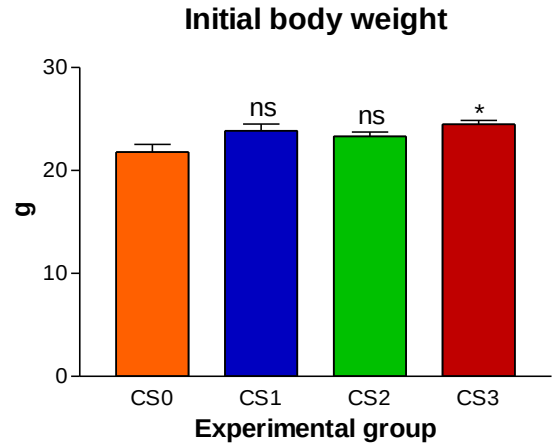
There were no significant changes in ROW of CS treated groups as compared to CS_0 group. A detailed scrutiny of histoarchitecture of the heart, liver and kidney did not reveal any observable cellular damage. The cellular morphology, nuclear characteristics and tissue integrity of organs of CS treated groups were comparable to the CS_0 group.

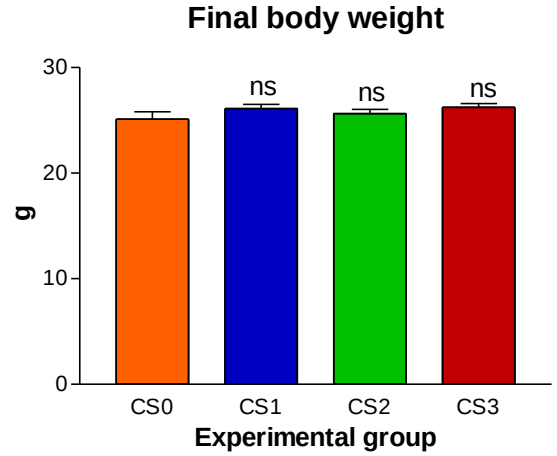
Table 1.1: Effect of CS on food intake, water intake and body weight

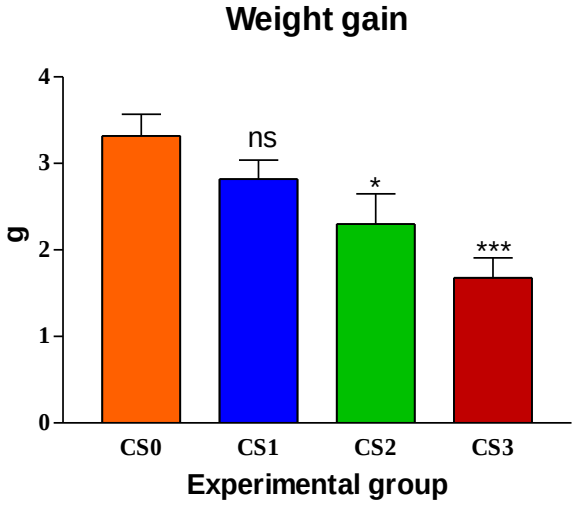
Groups	Body weight (g)		Weight gain g	Food intake g/day	Water intake ml/day
	Initial	Final			
CS_0	21.78±0.76	25.10±0.70	3.32±0.25	5.22±0.21	7.47±0.83
CS_1	23.87±0.65ns	26.12±0.38ns	2.82±0.22^{ns}	4.43±0.33^{ns}	10.80±0.63*
CS_2	23.32±0.41ns	25.64±0.40ns	2.30±0.35*	3.78±0.39*	11.27±0.39**
CS_3	24.48±0.39*	26.24±0.34ns	1.68±0.23***	3.24±0.24***	11.46±0.56**

Where, n=6. Data were expressed as mean ± S.E.M. * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and ns (not significant) when CS_0 v/s CS_1, CS_2 and CS_3

Figure 1.1a: Effect of CS extract on food intake, water intake and body weight

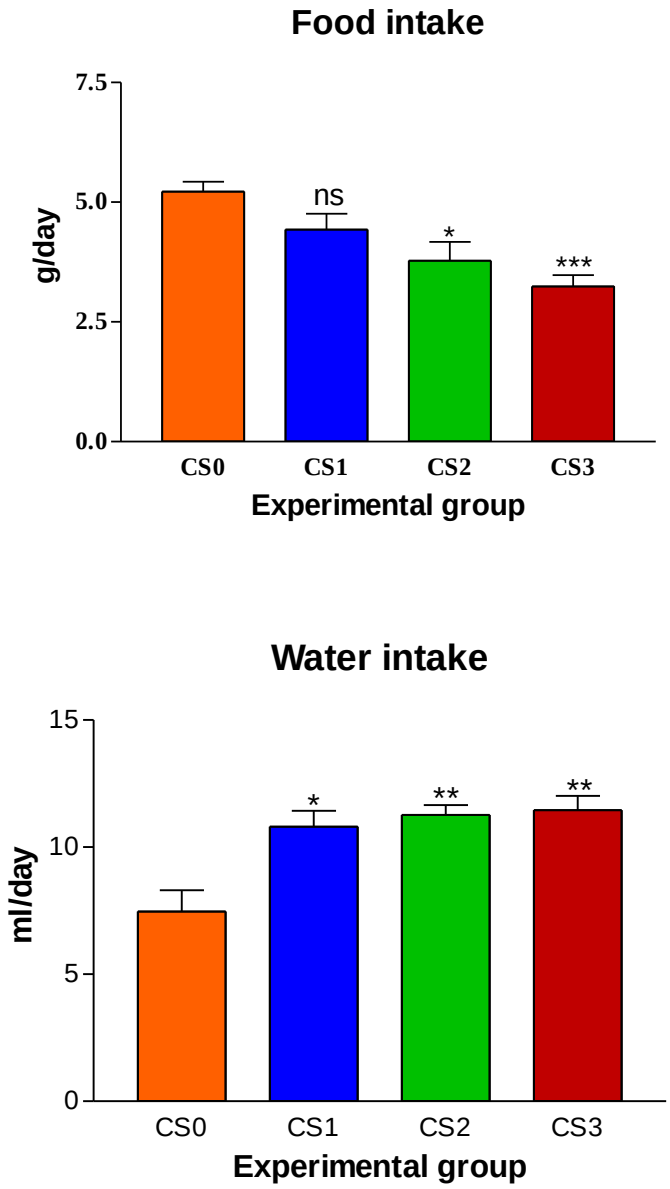






Where, n=6. Data were expressed as mean ± S.E.M. * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and ns (not significant) when CS_0 v/s CS_1, CS_2 and CS_3

Figure 1.1b: Effect of CS extract on food intake, water intake and body weight



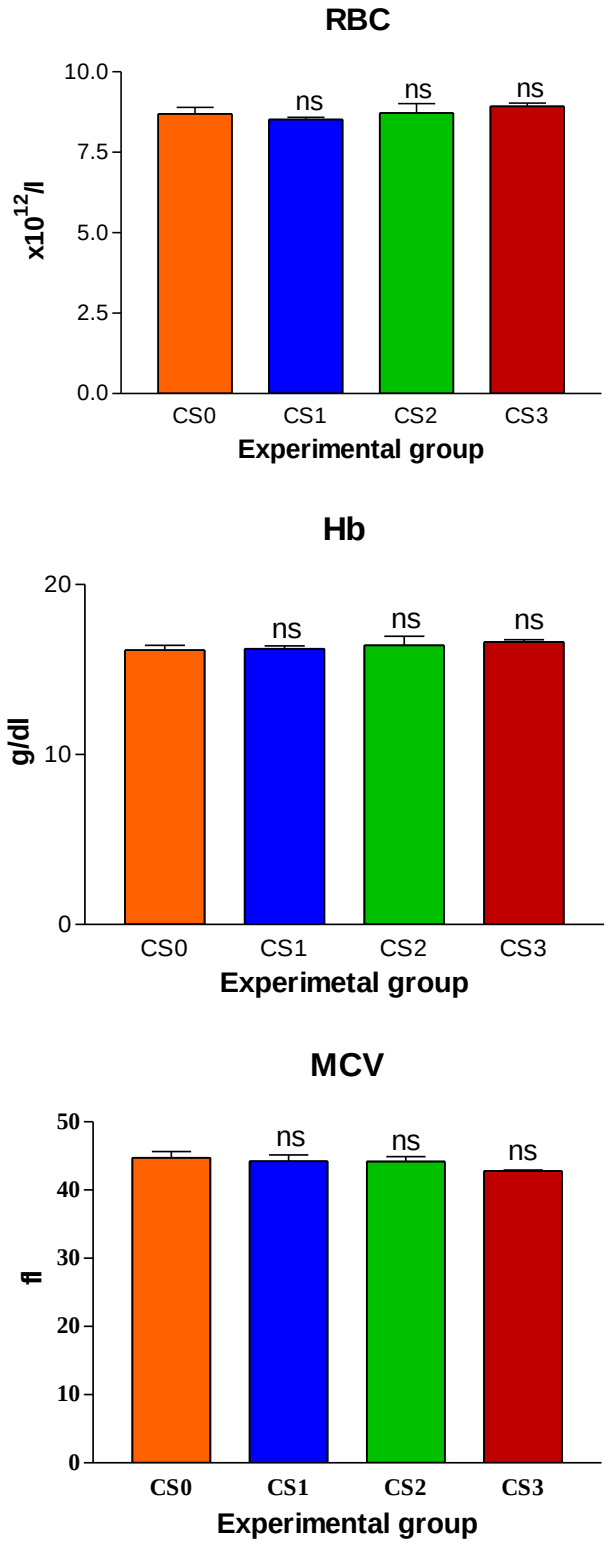
Where, n=6. Data were expressed as mean ± S.E.M. * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and ns (not significant) when CS_0 v/s CS_1, CS_2 and CS_3

Table 1.2: Effect of CS on haematological parameters

Parameter	Groups			
	CS_0	CS_1	CS_2	CS_3
RBC (x 10^{12}/l)	8.70±0.20	8.52±0.07^{ns}	8.72±0.29^{ns}	8.93±0.10^{ns}
Hb (g/dl)	16.14±0.29	16.22±0.17^{ns}	16.42±0.53^{ns}	16.62±0.13^{ns}
MCV (fl)	44.73±0.92	44.23±0.93^{ns}	44.17±0.73^{ns}	42.80±0.15^{ns}
MCH (pg)	18.72±0.19	18.94±0.18^{ns}	19.02±0.34^{ns}	18.64±0.26^{ns}
MCHC (g/dL)	44.56±0.55	44.62±0.59^{ns}	43.78±0.98^{ns}	43.00±0.57^{ns}
RCDW (%)	18.00±0.19	17.60±0.30^{ns}	17.67±0.26^{ns}	17.45±0.25^{ns}
WBC (x 10^3/µl)	16.33±0.48	16.27±0.23^{ns}	16.43±0.83^{ns}	16.53±0.21^{ns}
Monocytes (%)	2.02±0.15	2.16±0.20^{ns}	2.12±0.15^{ns}	2.05±0.16^{ns}
Lymphocytes (%)	7.34±0.15	7.30±0.25^{ns}	7.18±0.24^{ns}	7.06±0.18^{ns}
Eosinophils(%)	2.34±0.23	2.10±0.43^{ns}	1.79±0.21^{ns}	1.88±0.27^{ns}
Platelet (x 10^3/µl)	678.60±29.43	669.40±24.14^{ns}	667.40±20.00^{ns}	661.20±15.83^{ns}
MPV (fl)	10.06±0.09	9.98±0.06^{ns}	10.02±0.14^{ns}	10.00±0.09^{ns}

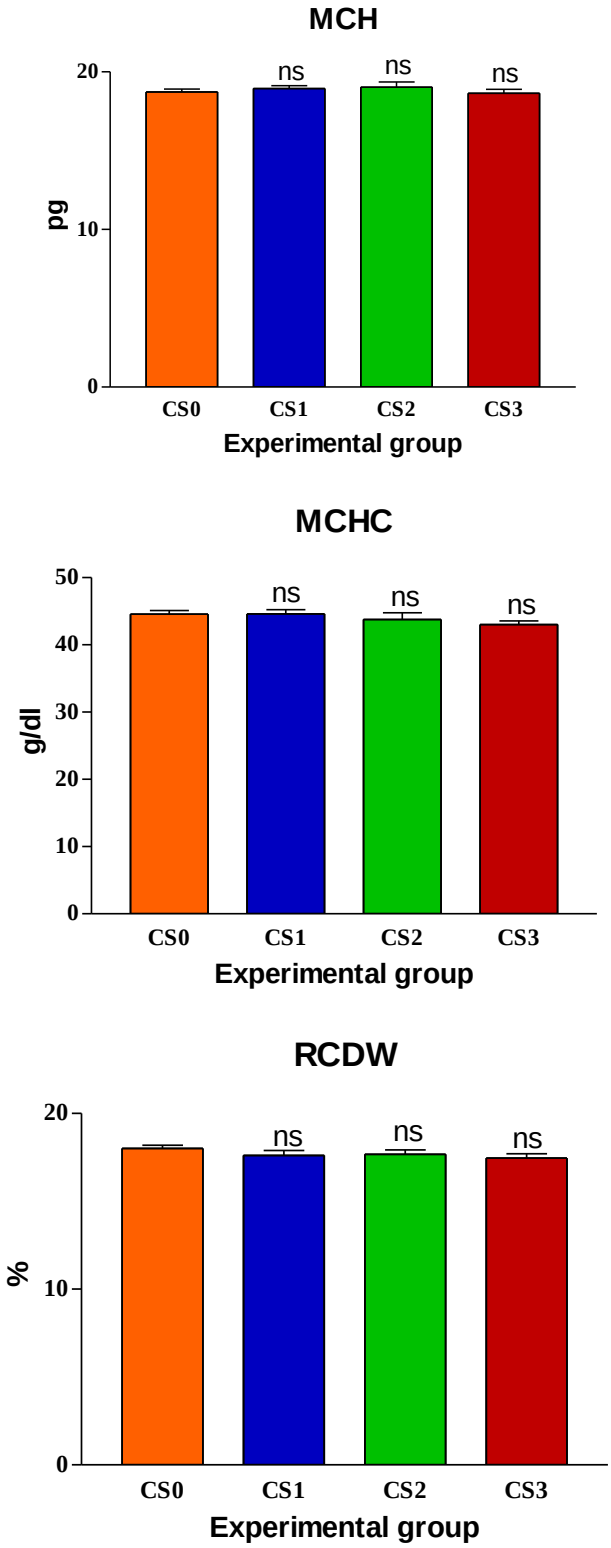
Where, n=6. RBC: Red blood corpuscle, Hb: Haemoglobin; MCV: Mean corpuscular volume, MCH: Mean corpuscular haemoglobin, MCHC: Mean corpuscular haemoglobin concentration, RCDW: Red cell distribution width, WBC: white blood corpuscle, MPV: Mean platelet volume, where n=6. Data were expressed as mean ± S.E.M. ns (not significant) when CS_0 v/s CS_1, CS_2 and CS_3

Figure 1.2a: Effect of CS extract on haematological parameters



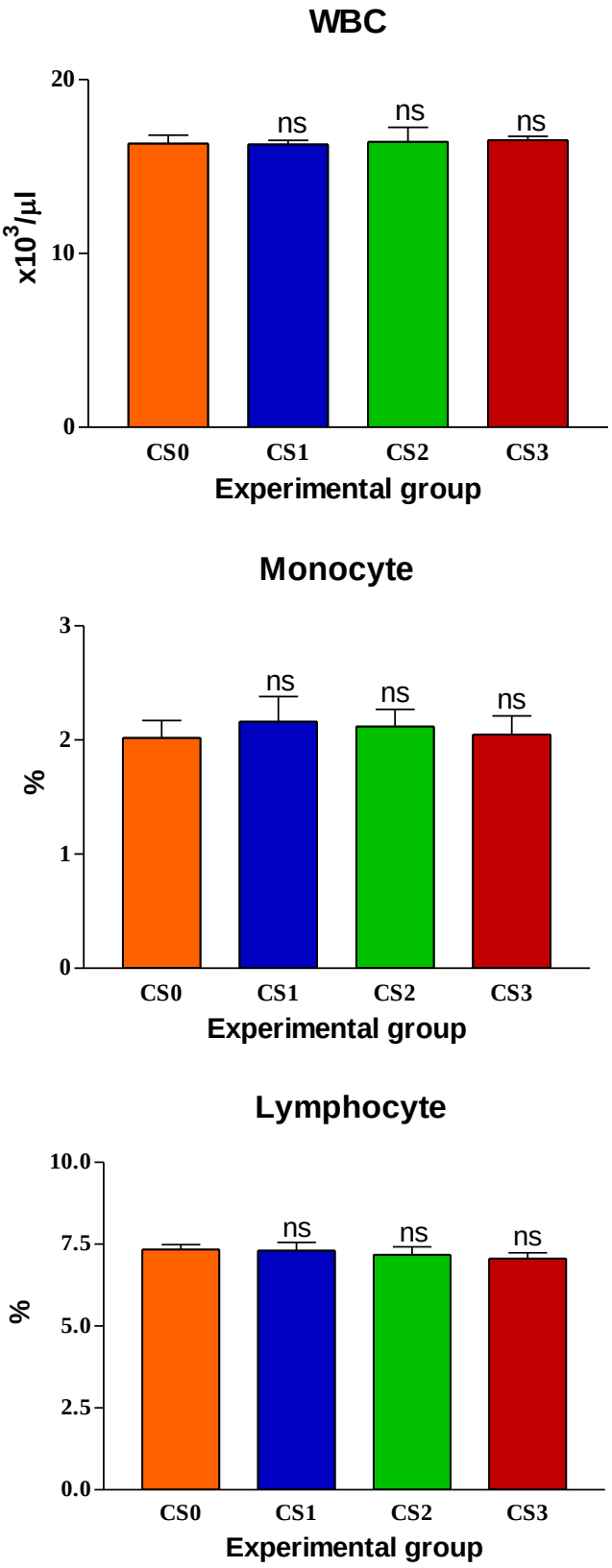
Where, n=6. Data were expressed as mean ± S.E.M. * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and ns (not significant) when CS_0 v/s CS_1, CS_2 and CS_3

Figure 1.2b: Effect of CS extract on haematological parameters



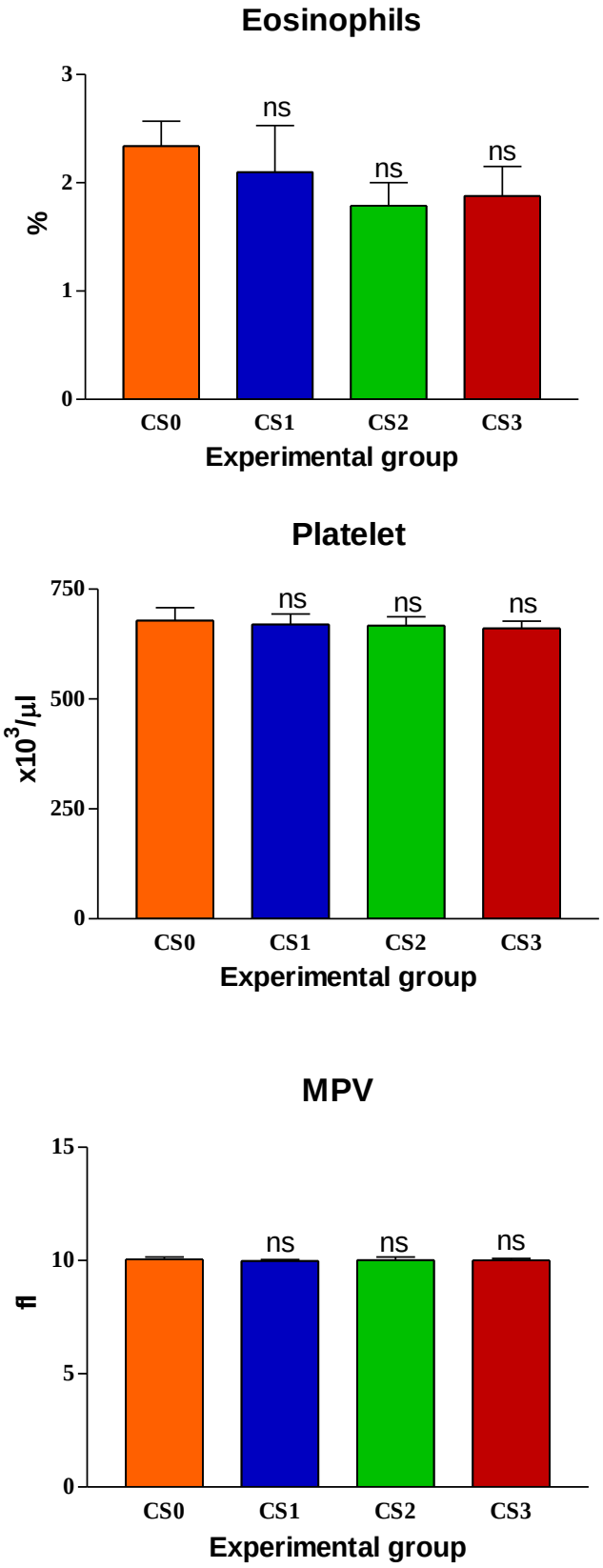
Where, n=6. Data were expressed as mean ± S.E.M. * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and ns (not significant) when CS_0 v/s CS_1, CS_2 and CS_3

Figure 1.2c: Effect of CS extract on haematological parameters



Where, n=6. Data were expressed as mean ± S.E.M. * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and ns (not significant) when CS_0 v/s CS_1, CS_2 and CS_3

Figure 1.2d: Effect of CS extract on haematological parameters



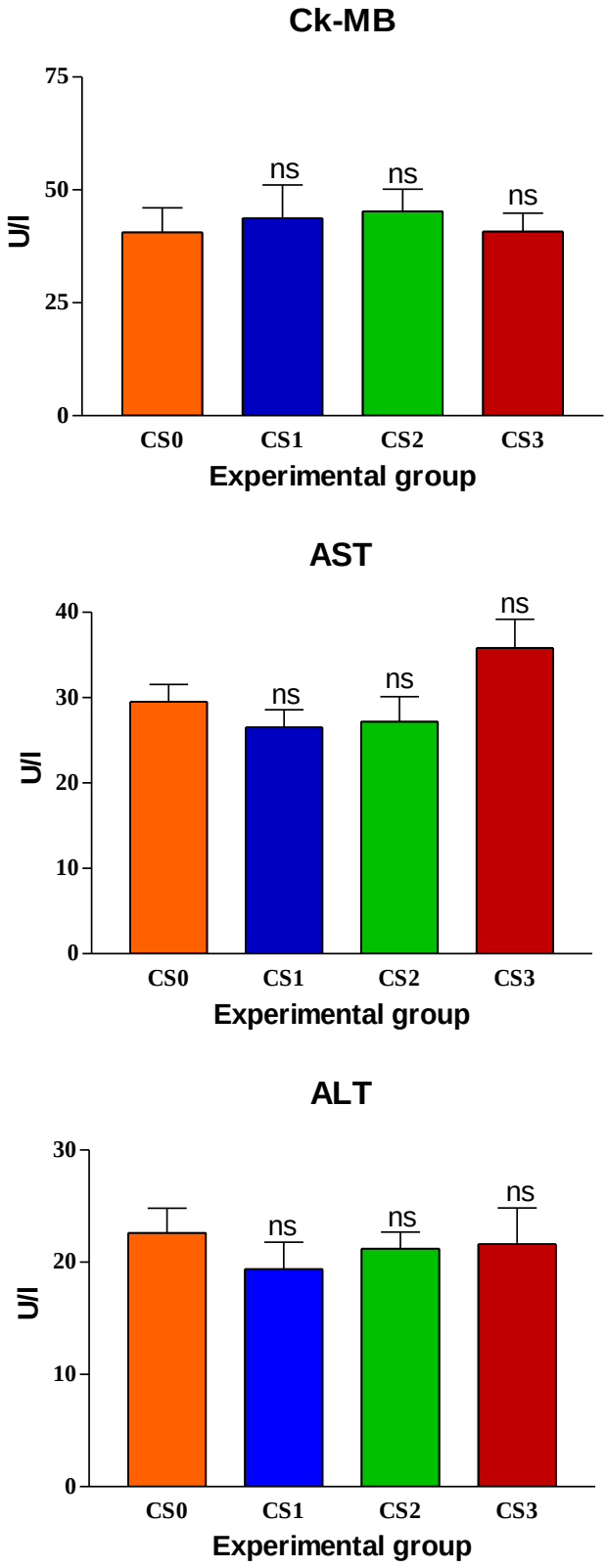
Where, n=6. Data were expressed as mean ± S.E.M. * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and ns (not significant) when CS_0 v/s CS_1, CS_2 and CS_3

Table 1.3: Effect of CS extract on plasma markers of heart, liver and kidney damage

			Groups			
			CS_0	CS_1	CS_2	CS_3
1.	Cardiac damage marker	Creatinine kinase-MB (U/L)	40.60±5.42	43.72±7.40 ^{ns}	45.27±4.88^{ns}	40.79±4.06^{ns}
2.	Hepatic damage markers	Aspartate trans-aminase (U/L)	29.50±2.06	26.50±2.07^{ns}	27.17±2.94^{ns}	30.83±3.34^{ns}
		Alanine trans-aminase (U/L)	22.60±2.20	19.40±2.40^{ns}	21.20±1.49^{ns}	21.60±3.23^{ns}
		Bilirubin (mg/dl)	1.86±0.22	1.79±0.14^{ns}	1.83±0.15^{ns}	1.97±0.10^{ns}
		Total protein (g/dl)	4.66±0.12	4.59±0.03^{ns}	4.85±0.25^{ns}	4.86±0.19^{ns}
3.	Kidney damage markers	Urea (mg/dl)	58.69±6.39	58.92±4.44^{ns}	65.45±6.92^{ns}	62.30±6.96^{ns}
		Creatinine (mg/dl)	0.32±0.03	0.32±0.05^{ns}	0.36±0.04^{ns}	0.38±0.05^{ns}

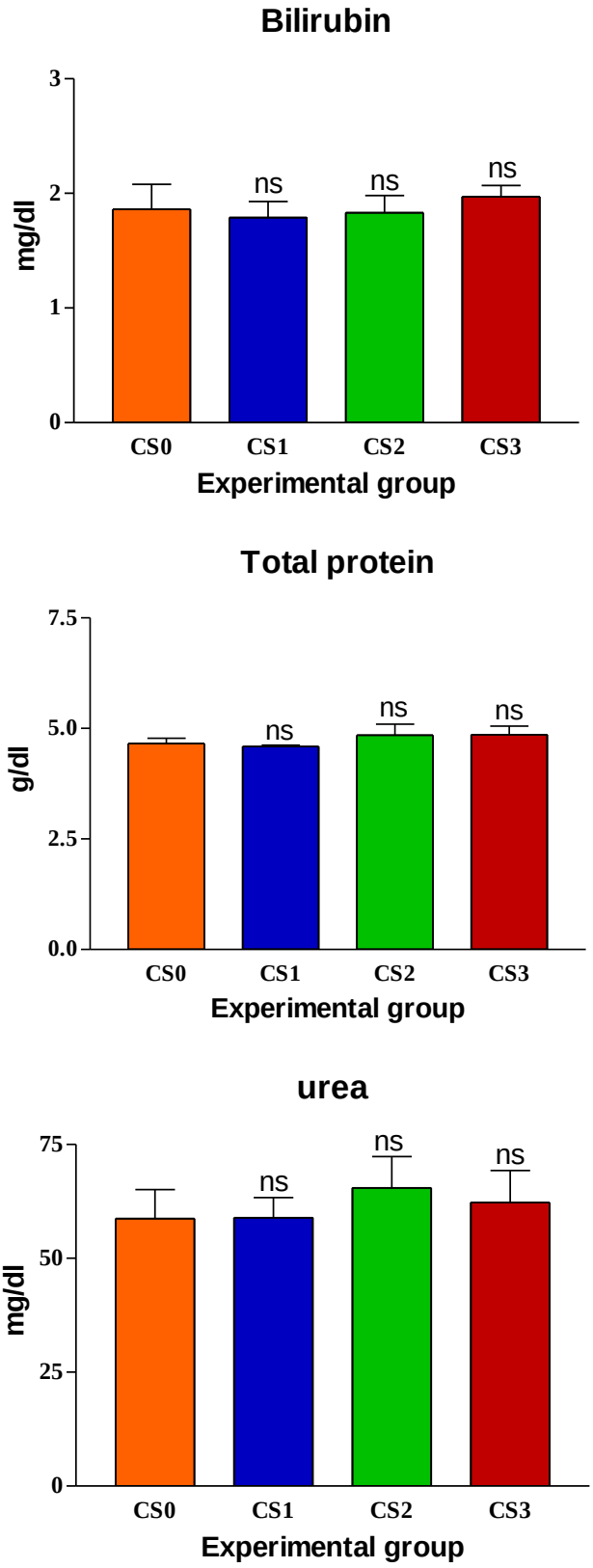
Where, n=6. Data were expressed as mean ± S.E.M. ns (not significant) when CS_0 v/s CS_1, CS_2 and CS_3

Figure 1.3a: Effect of CS extract on plasma markers of heart, liver and kidney damage



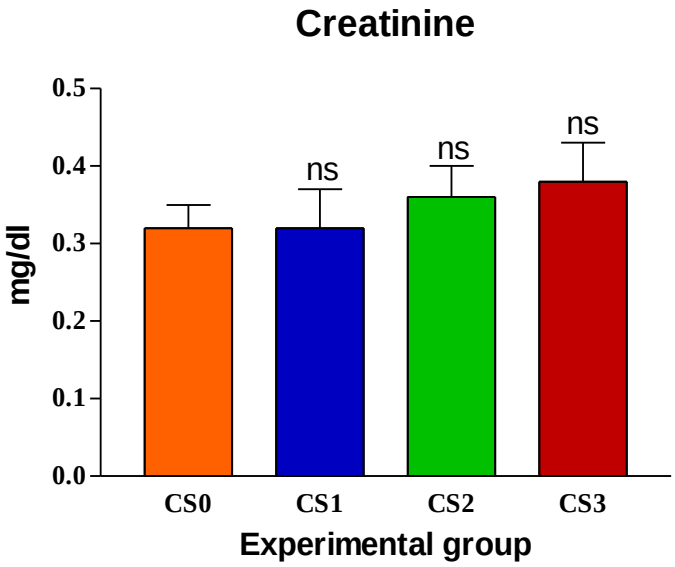
Where, n=6. Data were expressed as mean ± S.E.M. ns (not significant) when CS_0 v/s CS_1, CS_2 and CS_3

Figure 1.3b: Effect of CS extract on plasma markers of heart, liver and kidney damage



Where, n=6. Data were expressed as mean ± S.E.M. ns (not significant) when CS_0 v/s CS_1, CS_2 and CS_3

Figure 1.3c: Effect of CS extract on plasma markers of heart, liver and kidney damage



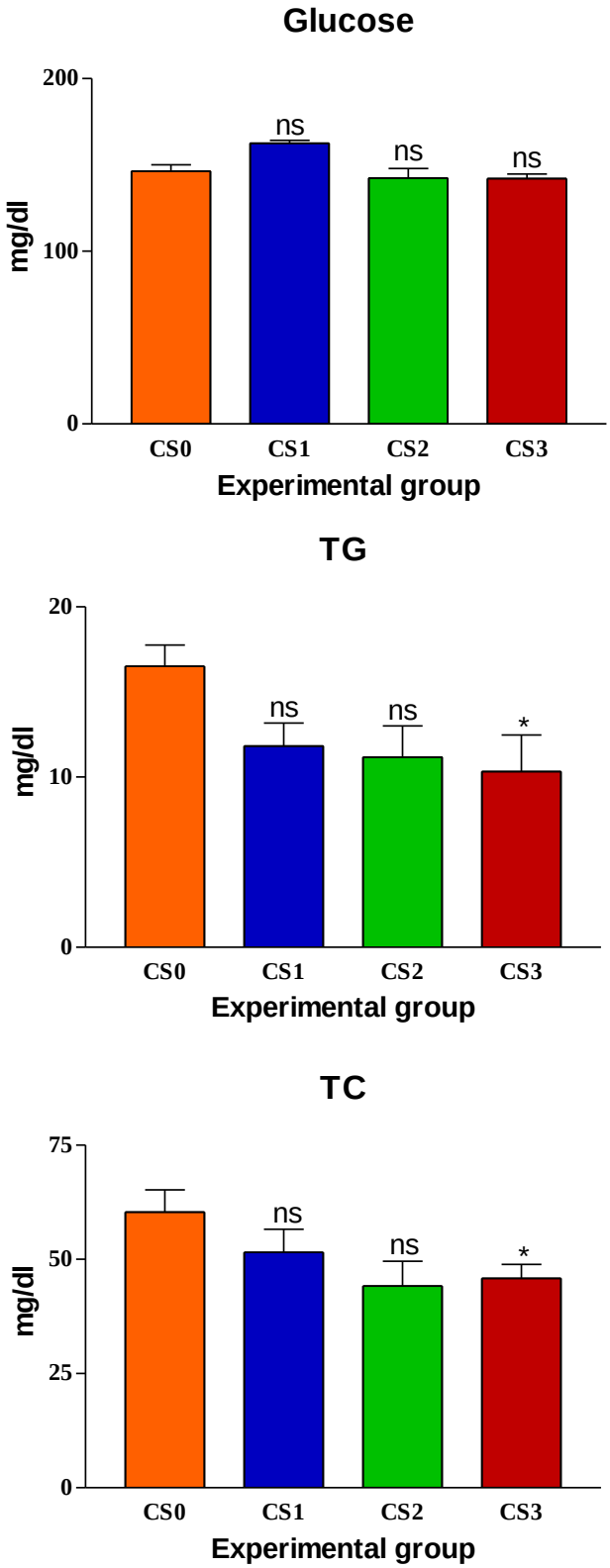
Where, n=6. Data were expressed as mean ± S.E.M. ns (not significant) when CS_0 v/s CS_1, CS_2 and CS_3

Table 1.4: Effect of CS on plasma glucose, lipid profile and lipoprotein profile

	Groups			
	CS_0	CS_1	CS_2	CS_3
Blood glucose (mg/dl)	146.20±4.97	155.7±7.06^{ns}	139.2±5.52^{ns}	134.2±4.71^{ns}
TC (mg/dl)	60.33±3.99	51.50±5.11^{ns}	44.17±5.40^{ns}	45.83±3.09*
TG (mg/dl)	16.50±1.26	11.83±1.38*	11.17±1.32*	10.33±2.15*
VLDL-C (mg/dl)	3.30±0.25	2.36±0.31*	2.23±0.23*	2.06±0.30*
LDL-C (mg/dl)	33.21±4.57^{ns}	25.61±5.62^{ns}	20.52±4.24^{ns}	20.74±3.34^{ns}
HDL-C (mg/dl)	23.55±1.14^{ns}	24.03±1.03^{ns}	23.30±0.66^{ns}	23.03±0.78^{ns}

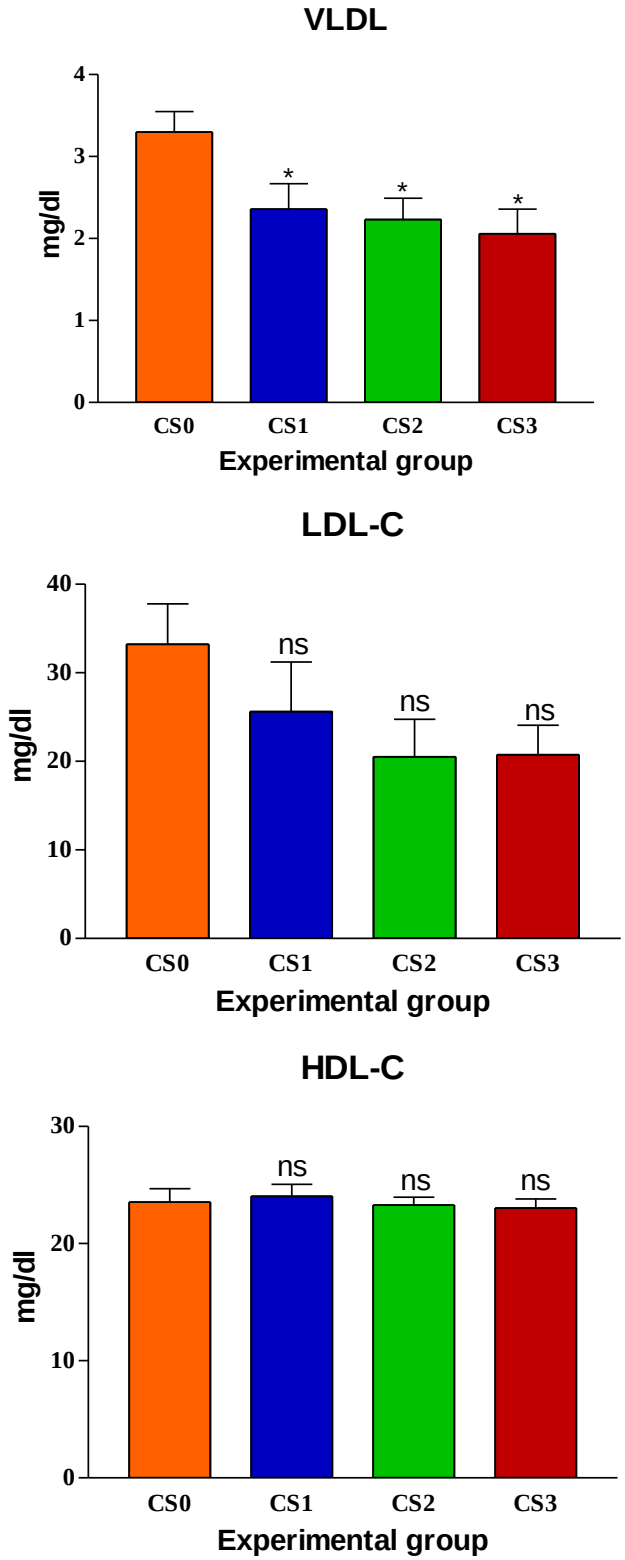
Where, n=6. Data were expressed as mean ± S.E.M. *$p<0.05$, ns (not significant) when CS_0 v/s CS_1, CS_2 and CS_3

Figure 1.4a: Effect of CS on plasma glucose, lipid and lipoprotein profiles



Where, n=6. Data were expressed as mean ± S.E.M. * $p<0.05$, ns (not significant) when CS_0 v/s CS_1, CS_2 and CS_3

Figure 1.4b: Effect of CS on plasma glucose, lipid and lipoprotein profiles



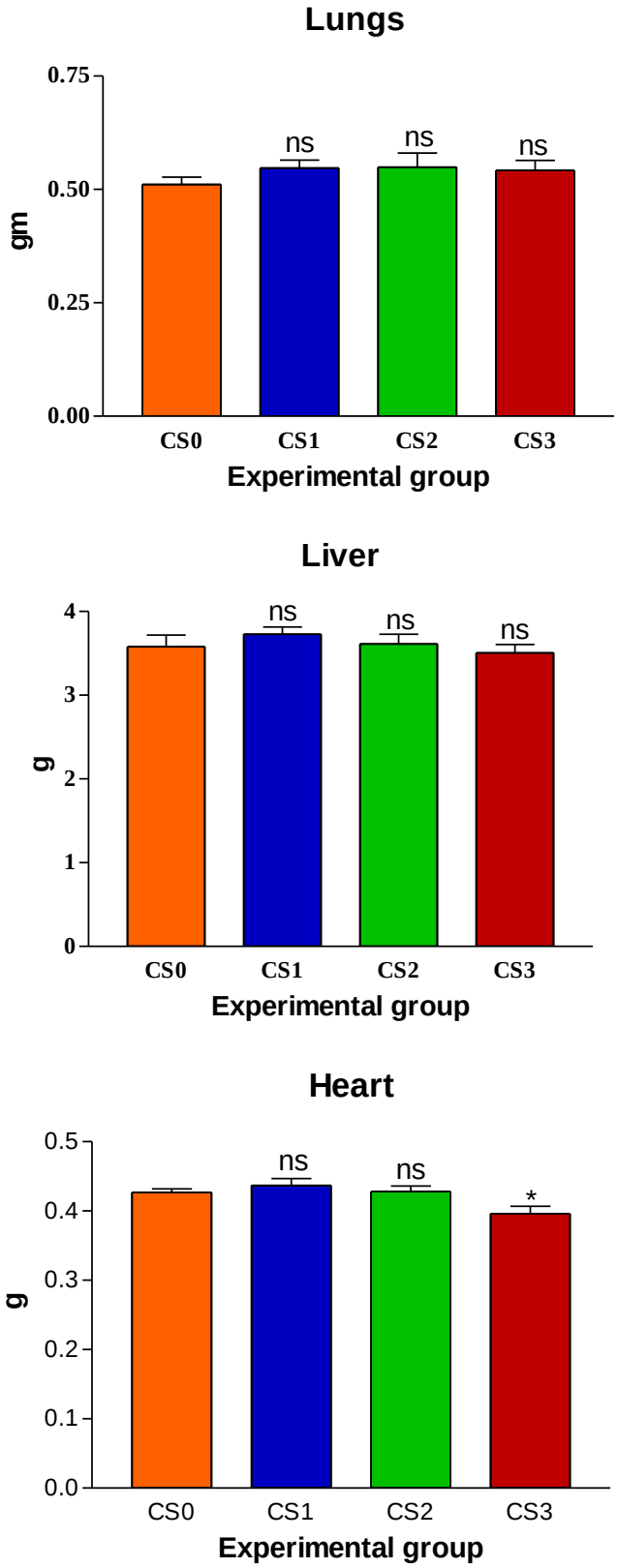
Where, n=6. Data were expressed as mean ± S.E.M. *p<0.05, ns (not significant) when CS_0 v/s CS_1, CS_2 and CS_3

Table 1.5: Effect of CS on relative organ weight

Organs (g)	Groups			
	CS_0	CS_1	CS_2	CS_3
Lungs	0.51±0.01	$0.54±0.01^{ns}$	$0.54±0.03^{ns}$	$0.54±0.02^{ns}$
Heart	0.42±0.01	$0.43±0.01^{ns}$	$0.42±0.01^{ns}$	0.40±0.01*
Liver	3.58±0.13	$3.72±0.08^{ns}$	$3.61±0.11^{ns}$	$3.50±0.10^{ns}$
Kidney	1.10±0.05	$1.16±0.05^{ns}$	$1.12±0.02^{ns}$	$1.00±0.03^{ns}$
Spleen	0.30±0.01	$0.27±0.01^{ns}$	$0.29±0.02^{ns}$	$0.30±0.17^{ns}$

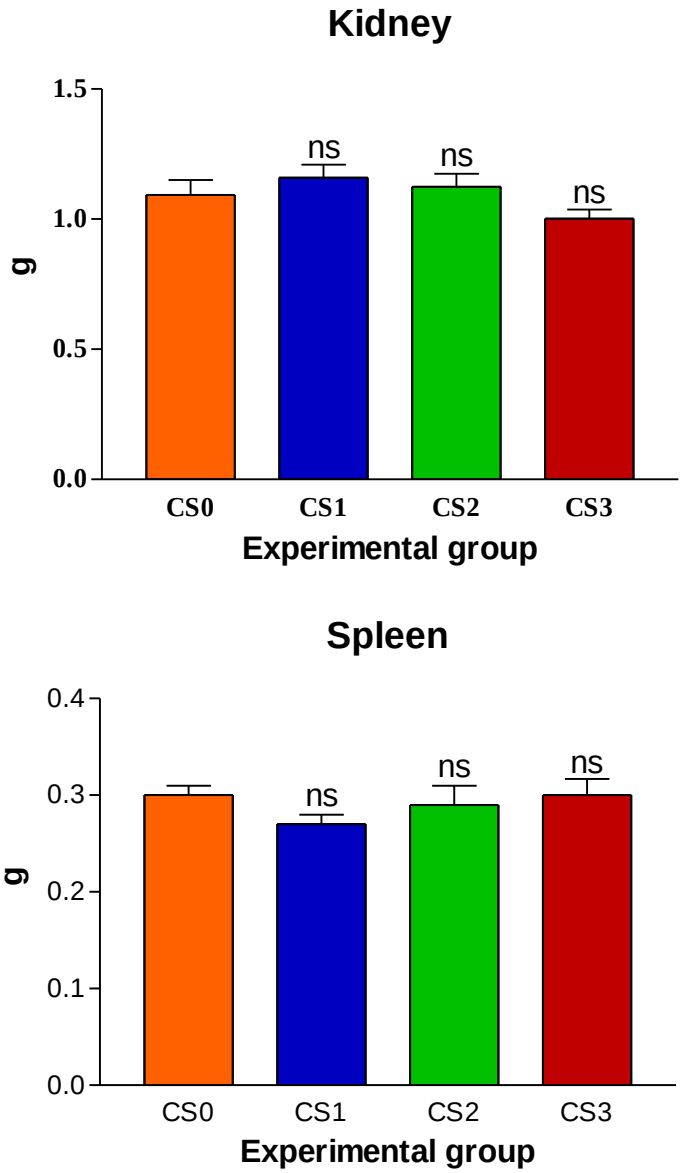
Where, n=6. Data were expressed as mean ± S.E.M. ns (not significant) when CS_0 v/s CS_1, CS_2 and CS_3

Figure 1.5a: Effect of CS on relative organ weight



Where, n=6. Data were expressed as mean ± S.E.M. ns (not significant) when CS_0 v/s CS_1, CS_2 and CS_3

Figure 1.5b: Effect of CS on relative organ weight



Where, n=6. Data were expressed as mean ± S.E.M. ns (not significant) when CS_0 v/s CS_1, CS_2 and CS_3

Figure 1.6: Photomicrographs of the sections of the heart of control (CS_0) and CS administered (CS_1, CS_2 and CS_3) mice for 28 days showing no histoarchitecture change in CS treated groups as compared to control

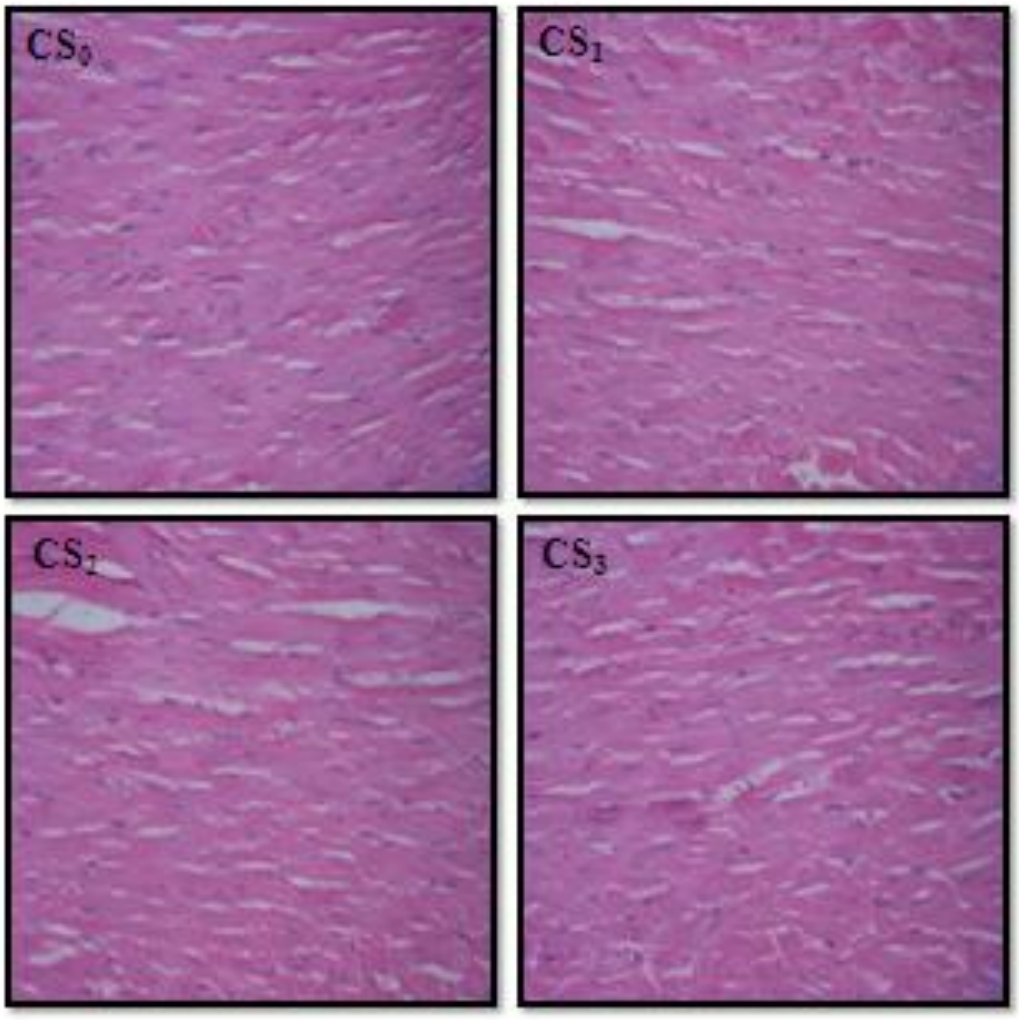


Figure 1.7: Photomicrographs of the sections of the liver of control (CS_0) and CS administered (CS_1, CS_2 and CS_3) mice for 28 days showing no histoarchitecture change in CS treated groups as compared to control

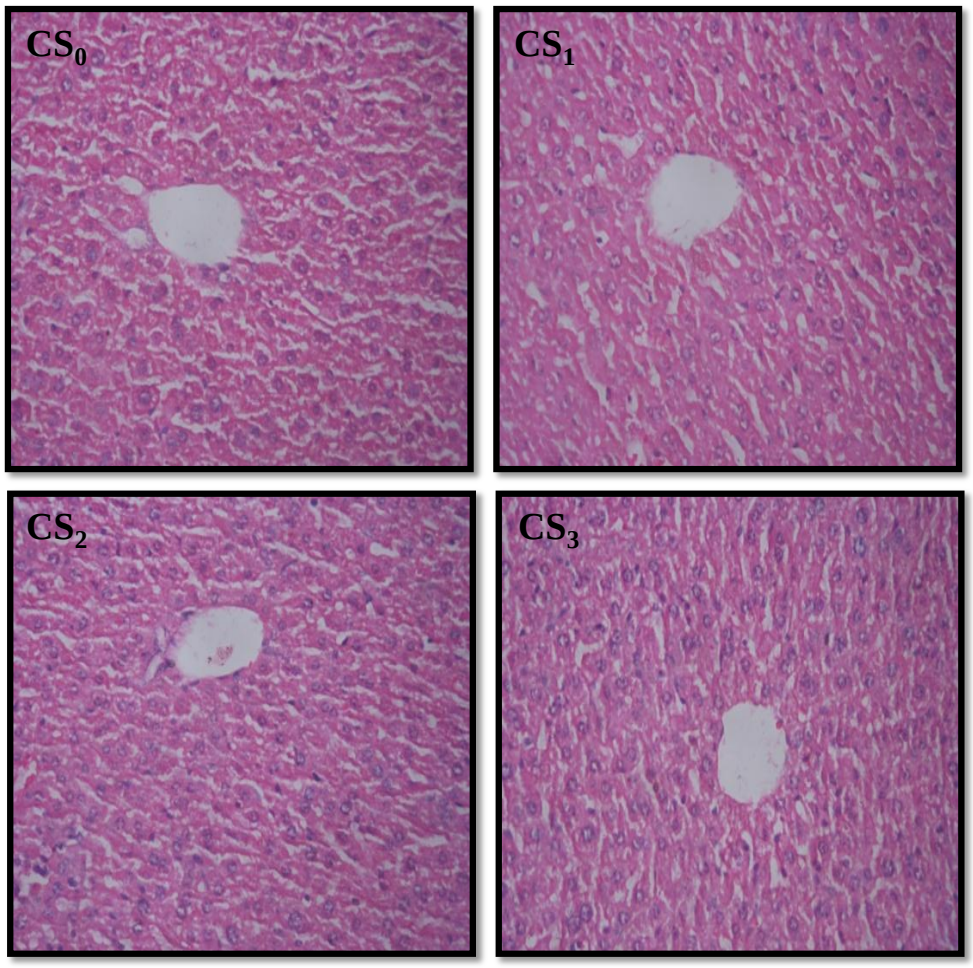
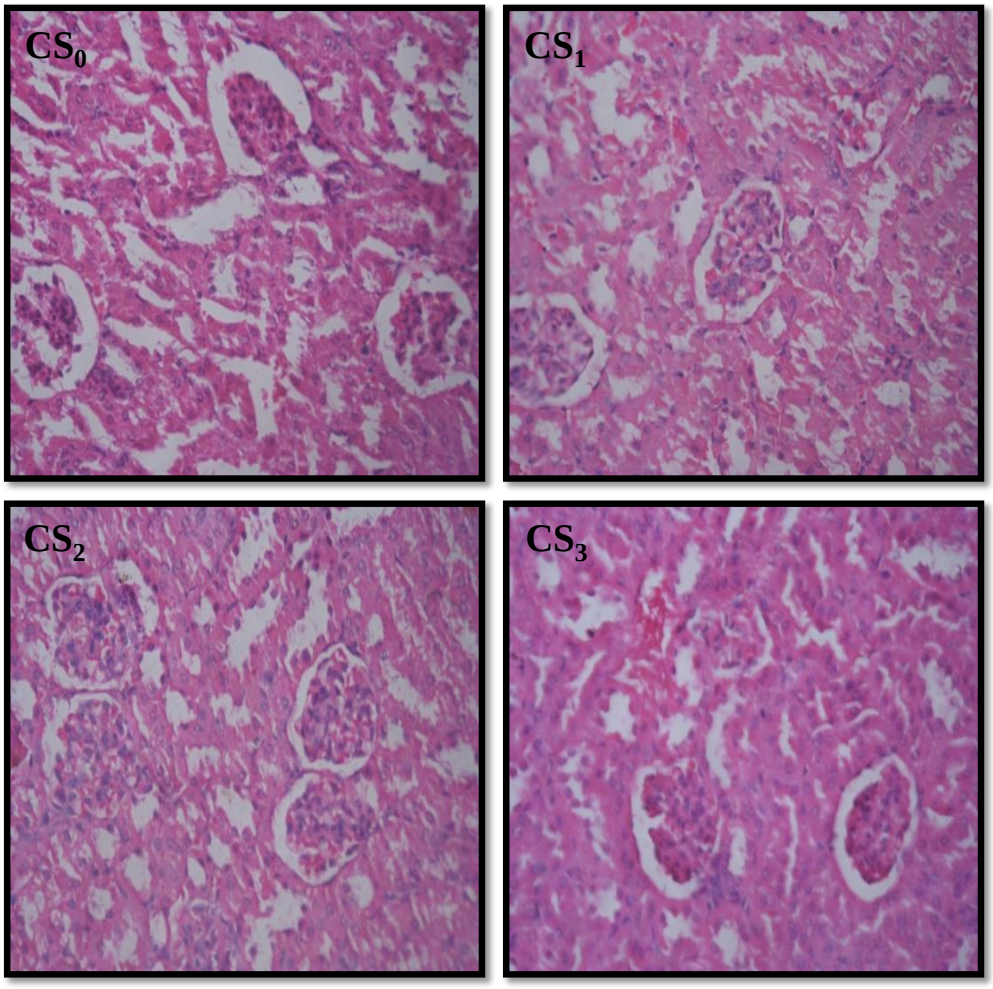


Figure 1.8: Photomicrographs of the sections of the kidney of control (CS_0) and CS administered (CS_1, CS_2 and CS_3) mice for 28 days showing no histoarchitecture change in CS treated groups as compared to control



DISCUSSION

Acute toxicity study recorded zero mortality at the end of 24 h period, following CS extracts administration. No behavioral alterations were recorded during the first four hours after administration of CS extract. Hence, the LD50 of CS extract is thought to be greater than 5000 mg and therefore CS extract can be considered as non-toxic up to the said dose (OECD 401).

Sub-chronic oral toxicity studies have provided information on drugs that can possibly pose health risks (Ministério de Saúde/Brasil, 2004). Twenty eight days of oral administration of CS (CS2 and CS3) extract showed significant decrement in food intake and body weight gain as compared to CS0 mice. Significant reduction in food intake is suggested as responsible for the observed decrement in body weight gain. Loss of appetite is often synonymous with weight loss due to disturbances in carbohydrate, protein or fat metabolisms (Klaassen, 2001) and the same might be a possible reason for the weight loss in our study. CS (CS2 and CS3) treated mice also showed significant decrement in plasma TC, TG, LDL-C and VLDL-C whereas; glucose and HDL levels were unchanged. These results indicate that higher doses of CS (CS2 and CS3) results in a reduction of food intake and subsequent decrement in lipid profile whereas; a lower dose (CS1) does not lead to any such negative impact on metabolism.

CK-MB is an enzyme present in the myocardium that leaks out only under conditions of massive myocardial damage resulting from disintegration of contractile apparatus and increased sarcoplasmic permeability (Mair *et al*., 1994; Jadeja *et al*., 2010). Observed normal levels of CK-MB under all doses CS administration is reflective of its normal

functional status and negligible damage. The same is further validated through the histology of heart of CS treated groups that reveals presence of intact myocardium. However, marginal increment in ROW of heart in CS3 group is inexplicable and warrants further scrutiny.

High levels of AST and ALT are reported in liver diseases or hepatotoxicity (Brautbar and Williams, 2002; Desai *et al*., 2012). Plasma AST, ALT and bilirubin of CS0 and CS treated groups were comparable thus indicative of normal functional status of liver. The ROW and histopathological observations of liver showed no significant changes following CS treatment.

Renal dysfunction can be assessed by concurrent measurements of urea and creatinine and their normal levels reflect at reduced likelihood of renal problems (Davis and Bredt, 1994; Thounaojam *et al*., 2010a). In the present study, changes in plasma urea and createnine levels in CS treated groups showed non-significant differences on a dose dependent manner indicating a normal renal function. Healthy status of the kidneys of CS treated groups was further confirmed by their histoarchitecture and ROW. However, higher urine output observed in CS2 and CS3 treated groups can be attributed to its diuretic property as reported earlier by other research groups (Aissaoui *et al*., 2008; Jabeen *et al*., 2009).

The haematopoietic system is one of the most sensitive targets for toxic compounds and hence it is mandatory to record any possible alterations resulting from a test substance (Olson *et al*., 2000). Change in haematological parameters has a higher predictive value, when the data of drug toxicity on animal studies are translated for clinical usage

(Adeneye and Adokiye, 2008). A normal haematological profile of CS treated groups also further justified the non-toxic nature of CS extract.

In light of these findings, we may conclude that CS extract is not toxic in all the doses studied herein. This study is the first report that evaluates toxicity of CS extract and defines it as non-toxic up to a dose of 3000 mg/kg body weight.

SUMMARY

Coriandrum sativum L. (CS) seeds are known to possess therapeutic potentials against a variety of physiological disorders. This study assesses acute and sub-chronic toxicity profile of hydro-methanolic extract of CS seeds using OECD guidelines. In acute toxicity study, mice were once orally administered 1000, 3000 and 5000 mg/kg body weight of CS extract. There were no any behavioral alterations or mortality recorded in CS treated groups. The LD50 value was more than 5000 mg/kg body weight. In the sub-chronic oral toxicity study, the animals were orally administered with CS extract (1000, 2000 and 3000mg/kg body weight) daily for 28 days whereas; vehicle control group received 0.5 % carboxy methyl cellulose. There was significant reduction in food intake, body weight gain and plasma lipid profiles of CS2 and CS3 (2000 and 3000 mg/kg body weight respectively) groups as compared to the control group. However, there were no alterations in haematological profile, relative organ weights, histology and plasma markers of damage of vital organs (heart, liver and kidney). The overall finding of this study indicates that CS extract is non-toxic up to 3000 mg/kg body weight and can be considered as safe for consumption.

CHAPTER 2

Coriandrum sativum L. aqueous extract mitigates high fat diet induced insulin resistance by controlling visceral adiposity in C57BL/6J mice

INTRODUCTION

Changes in lifestyle and intake of caloric rich diets have predisposed the populace to obesity. Type 2 diabetes (T2D) is also closely associated with obesity and is characterized by an insulin resistance (IR) severely affecting glucose disposal (Frőde and Medeiros, 2008). Treatment of IR involves popular lipid lowering and insulin sensitizing drugs that have been associated with many side effects. Multiple drug usage is the necessary option for maintaining glycemic levels and other associated manifestation in T2D patients (Gerich, 2001). These compelling reasons provide impetus for investigating the medicinal properties of herbs for use as alternatives in the treatment of T2D on a global scale. In this context, World Health Organization (WHO) has estimated that, about 25% of modern medicines are derived from plants and that, the global market for herbal medicine currently stands at over 60 million US$ annually (WHO, 2009).

As *Coriandrum sativum*L.(CS) has been used as traditional antidiabetic herbal agent and it's hypoglycemic and insulin secretor effects have been evaluated in streptozotocin induced diabetic rats (Eidi *et al*., 2008; Swanston-Flatt *et al*., 1990), the present study was designed to assess the efficacy of aqueous extract of CS in alleviating IR in high fat diet fed model of T2D.

MATERIALS AND METHODS

Plant Extract

CS seeds were procured from local ayurvedic medicinal shop of Vadodara, India. Hundred grams of powdered seeds were boiled at 100°C for 3 h in distilled water. Resulting filtrate was concentrated in a hot air oven until it formed a semisolid paste, which was then freeze dried. The yield was 12% w/w. Two doses (1% and 3%) of aqueous extract of CS were mixed with high fat diet.

Experimental Animals

Male C57BL/6J mice (6-8 weeks of age) were purchased from the National Centre for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition (NIN), Hyderabad, INDIA. They were housed and maintained in clean polypropylene cages and fed with standard laboratory diet (SLD) and water *ad libitum*. The experiments were carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and approved by the animal ethical committee of the Department of Zoology, The Maharaja Sayajirao University of Baroda, Vadodara (Approval No.827/ac/04/CPCSEA).

Experimental Design

30 animals were randomly allocated into 5 groups of 6 animals each. Mice were fed with standard laboratory diet (SLD) or high fat diet (HFD) for 12 weeks (Jadeja *et al*., 2010; Thounaojam *et al*., 2010). CS or Rosiglitazone (ROS) were given to the experimental animals by mixing with HFD.

Group I (SLD): Mice were fed with SLD

Group II (HFD): Mice were fed with HFD

Group III (HFD+CS1%): Mice fed with HFD containing CS extract (1% w/w)

Group IV (HFD+CS3%): Mice fed with HFD containing CS extract (3% w/w)

Group V (HFD+ROS): Mice fed with high fat diet containing Rosiglitazone (0.05% w/w).

At the end of the experimental period, blood was collected from retro orbital sinus in EDTA coated vial under mild ether anesthesia. Plasma was obtained by cold centrifugation (4°C) of the vials for 10 min at 3000 rpm. Later, animals were sacrificed by cervical dislocation and epididymal fat pad were excised, weighted and fixed in 4% buffered paraformaldehyde.

Body weight, food intake and feed efficiency

Known quantity of food (SLD or HFD) was given to the respective experimental groups and food intake was measured daily. Feed efficiency ratio (FER) was expressed as the total weight gain of an experimental animal during 12 weeks ÷ the total food intake.

Plasma and hepatic lipids

Plasma free fatty acid (FFA) content was estimated by the method of Itaya and Ui, (1965) while, triglyceride (TG) and total cholesterol (TC) contents were estimated by using enzymatic kits (Reckon Diagnostics. Ltd, Vadodara, India) in a semi autoanalyser (Micro lab 300 L, Merck). Total lipids were extracted from liver of control and experimental animals with chloroform: methanol (2:1) (Folch *et al*., 1957) and hepatic Free fatty acids were assayed in the same (Itaya and Ui, 1965). Known quantity of lipid extract was than dissolved in 1% Triton X-100 (Thounaojam *et al*., 2010) and TC and TG were assayed using above kits.

Blood glucose, plasma insulin and fasting insulinresistance index (FIRI)

Animals were fasted overnight (for 12 h) and later blood glucose was measured in whole blood sample obtained from tail vein (by one touch glucometer, Sugar Scan, HMD BIOMEDICAL INC., India). Plasma insulin was assayed using Mouse ELISA kit (Mercodia Developing Diagnostics Ltd, Sweden). Fasting insulin resistance index (FIRI) was expressedas:

Fasting insulin (pmol/l) x Fasting blood glucose (mg/dl) ÷ 25

Intraperitoneal glucose tolerance test (IPGTT)

Fasting (12 h) blood glucose was measured in whole blood (by one touch glucometer, Sugar Scan, HMDBIOMEDICAL INC., India) obtained from tail vein (0min). Later, glucose solution was injected intraperitoneally (2 g/kg) and blood glucose was assayed at 30, 60, 90 and 120 min and the tolerance curves plotted. Area under the curve ($AUC_{glucose}$) was calculated based on the trapezoid rule (Graph PadPrism version 3.0).

Intraperitoneal insulin response test (IPIRT)

Overnight fasted mice received insulin (Aventis Pharma Deutschland GmbH, Mumbai, India) 0.2 U/kg body weight by intraperitoneally. Blood samples werecollected from tail vein at 0 min (before administration) and subsequently at 10, 20, 30 and 60 min after administration of insulin. Blood glucose was measured in whole blood (by one touch glucometer, Sugar Scan, HMD BIOMEDICAL INC., India). KITT was determined with the formula:

KITT= 0.693×100 ÷T½.

Where T½ is half-life of plasma glucose decay was obtained with the formula: T½= ln2 ÷ ω. Where, ω constant of plasma glucose disintegration was obtained

with the formula: $\omega = \ln C1 - \ln C2 \div T2 - T1$ with glucose concentration C1 at time T1 (10 min) and C2 at T2 (60 min) (Thounaojam *et al*., 2010).

Histology of epididymal fat pad

Epididymal fat pad was fixed in 4% buffered paraformaldehyde, dehydrated in graded alcohol seriesand embedded in paraffin wax. Five μm sections were cut (by Leica RM 2115 Microtome) and stained with hematoxyline and eosin (H&E) and examined underLeica microscope. Photographs of adipocytes weretaken with Canon power shot S7 digital Camera (400X). To quantify adipocyte number and diameter, the H&E stained sections were analyzed using an image analysis system (Image Pro-Plus, Silver Spring, MD).

Statistical analysis

Statistical analysis of the data was done by one way ANOVA followed by Bonferroni's multiple comparison test and results were expressed as mean ±S.E.M (Using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego California, USA).

RESULTS

Body weight gain and feed efficiency ratio (Table: 2.1; Figure: 2.1)

HFD group recorded significant increase in body weight gain but not in food intake and feed efficiency ratio as compared to SLD ($p<0.01$). HFD+CS (CS 1% & CS 3%) recorded significantly dose dependent decrement in body weight gain, food intake and feedefficiency ratio as compared to HFD fed mice. HFD+ROS group also showed decrement of the said parameters which were comparable to HFD+CS1% but, HFD+CS3% was the most efficient in inducing.

Plasma and hepatic lipid profile (Table 2.2 and 2.3; Figure 2.2a and 2.3)

Plasma and hepatic TC and TG levels and plasma FFA level were significantly elevated in HFD group as compared to SLD group ($p<0.01$). CS supplemented HFD fed groups (CS 1% & CS 3%) and HFD+ROS were significantly ($p<0.01$) able to attenuate the effect of HFD as was evident in form of decrement in levels of hepatic and plasma TC and TG and plasma FFA.

Fasting blood glucose (FBG) and serum Insulin levels (Table 2.2; Figure 2.2b)

FBG, plasma insulin level and FIRI were significantly higher in HFD group ($p<0.01$) as compared to SLD group. However, CS supplemented HFD groups recorded significantly lowered levels of these parameters in dose dependent manner as compared to HFD group ($p<0.01$).

Intraperitoneal glucose tolerance test andIntraperitoneal insulin response test (Figure 2.4)

IPGTT of HFD fed mice recorded significant elevation in glucose level at 30 min that failed to return to its normal level at 120 min. $AUC_{glucose}$ of HFD fed mice was significantly higher compared to SLD mice ($p<0.01$). However, IPGTT of CS supplemented HFD mice showed dose dependent decrement in $AUC_{glucose}$ compared to that of HFD fed mice ($p<0.01$). HFD+ROS group also recorded a decrement in $AUC_{glucose}$ values compared to HFD group. IPIRT plots of glucose levels of HFD and SLD fed mice were comparable, however, CS supplemented HFD fed mice showed significant improvement in the IPIRT curves. The same was evident in the form of higher KITT values in these groups compared to the HFD group ($p<0.01$). HFD+ROS group also recorded higher KITT values of IPIRT as compared to HFD group.

Adipocyte diameter, number and surface area (Table 2.4; Figure 2.5 & 2.6)

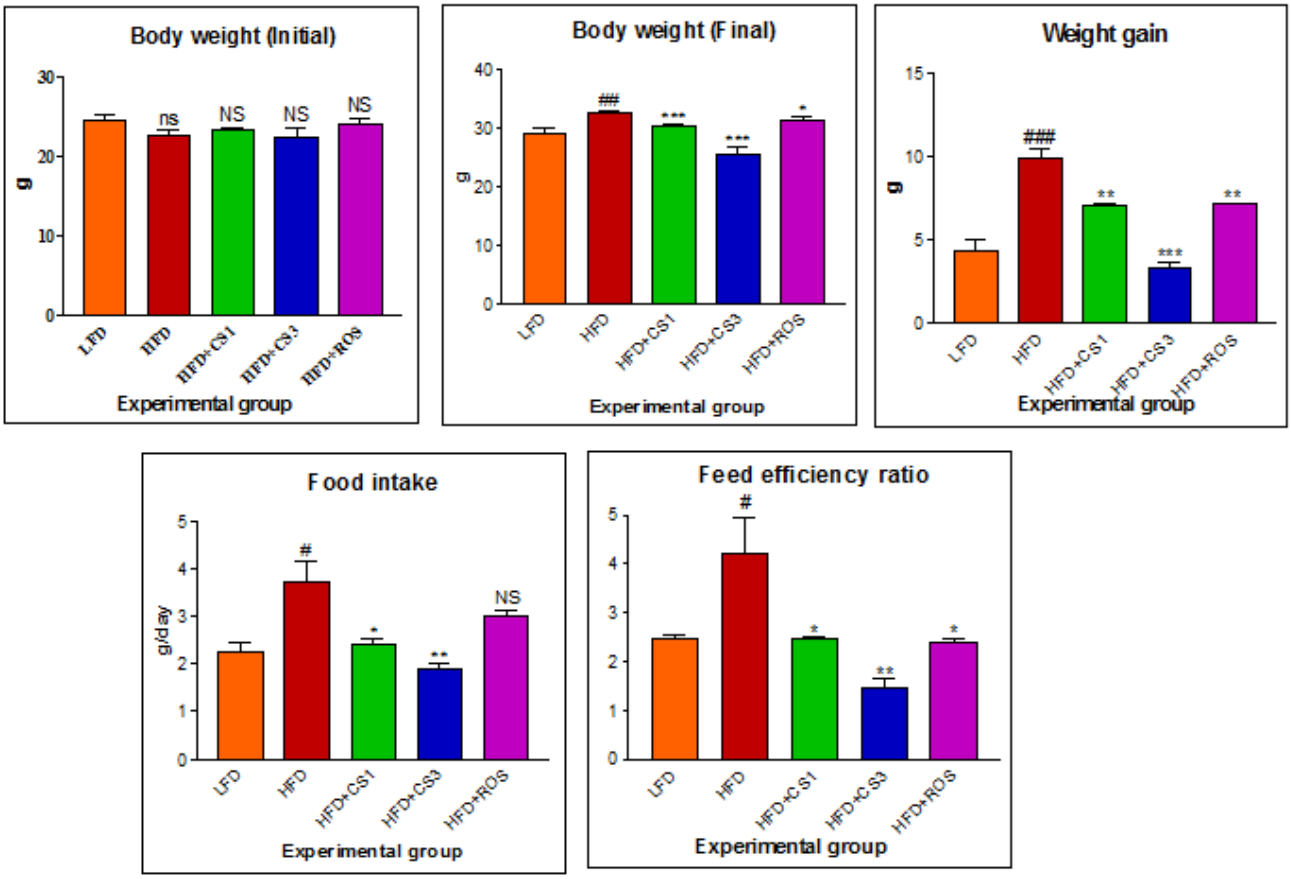
Microscopic examination of epididymal fat pad of HFD group recorded a significant increase in diameter and surface area of adipocytes compared to the adipocytes of SLD group ($p<0.01$). CS supplemented HFD groups showed adipocytes with mixed dimensions. However, the overall score of measurements of diameter and surface area of adipocytes recorded in HFD+CS (1% & 3%) were significantly lower than that of HFD group. The total number of adipocytes counted in a unit area in HFD+CS (1% & 3%) groups were significantly higher ($p<0.01$) than in the HFD group. These numbers were comparable to SLD or HFD+ROS groups. HFD+ROS group also showed moderate decrement in diameter of adipocyte compared to HFD group.

Table 2.1: Effect of CS extract and Rosiglitazone on Body weight, Food intake and Feed efficiency ratio

	SLD	**HFD**	**HFD+CS1%**	**HFD+CS3%**	**HFD+ROS**
Initial body weight(g)	24.40±0.86	22.53±0.79^{ns}	23.32±0.24^{NS}	22.22±1.34^{NS}	24.00±0.64^{NS}
Final body weight(g)	29.00±0.70	32.42±0.28$^{\#\#}$	30.32±0.30***	25.50±1.12***	31.16±0.59*
Weight gain (g)	4.320±0.69	9.867±0.59$^{\#\#\#}$	7.033±0.16**	3.280±0.36***	7.160±0.074**
Food intake (g/day)	2.263±0.19	3.725±0.41$^{\#}$	2.392±0.12*	1.880±0.11**	3.020±0.08^{NS}
Feed Efficiency ratio	2.480±0.05	4.228±0.73$^{\#}$	2.458±0.03*	1.464±0.17**	2.372±0.09*

Where, n=6. # $p<0.05$, ## $p<0.01$, ### $p<0.001$ and ns: non significance when, LFD vs HFD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and NS: non significance when, HFD vs HFD+CS1, HFD+CS3 and HFD+ROS

Figure 2.1: Effect of CS extract and Rosiglitazone on Body weight, Food intake and Feed efficiency ratio



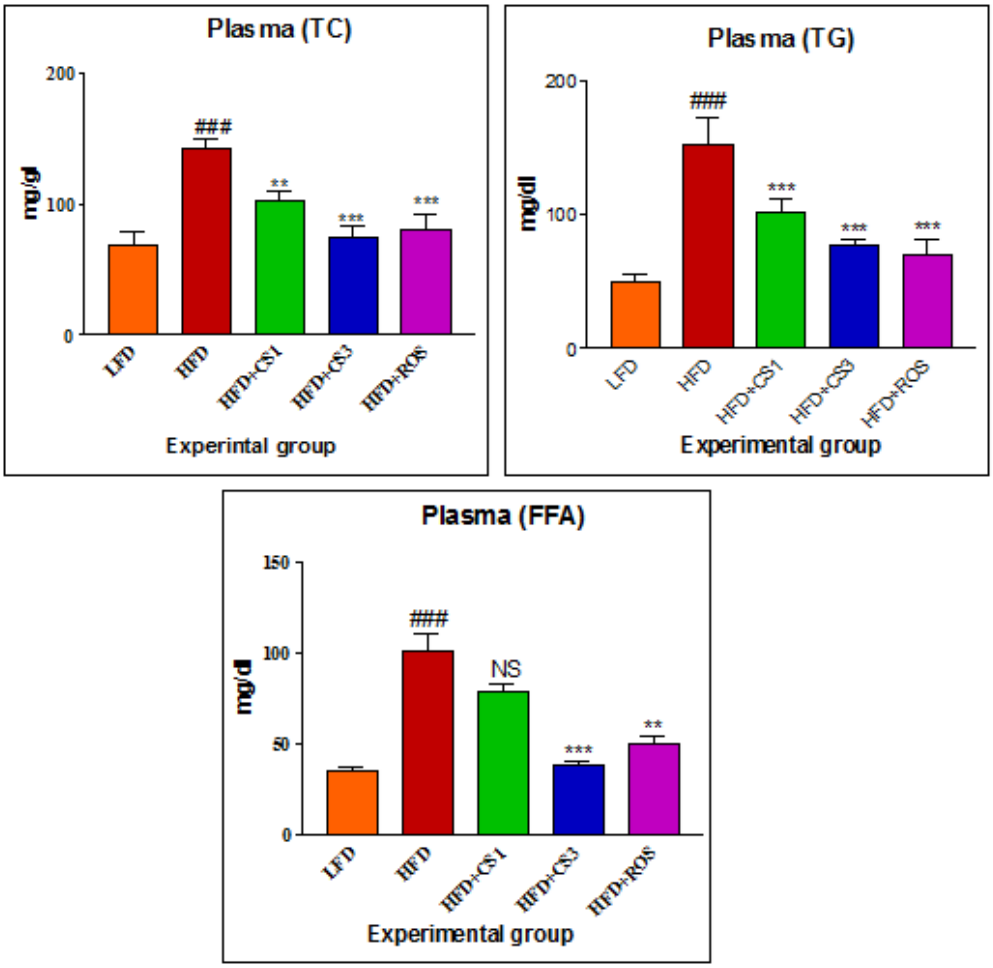
Where, n=6. # p<0.05, ## p<0.01, ### p<0.001 and ns: non significance when, LFD vs HFD. * p<0.05, ** p<0.01, *** p<0.001 and NS: non significance when, HFD vs HFD+CS1, HFD+CS3 and HFD+ROS

Table 2.2: Effect of CS extract and Rosiglitazone on Plasma lipid profile and glucose and insulin levels

	SLD	HFD	HFD+CS1%	HFD+CS3%	HFD+ROS
TC (mg/dl)	68.50±10.56	142.7±7.63$^{###}$	103.0±7.0**	74.8±8.23***	81.25±11.09***
TG(mg/dl)	50.0±5.22	152.3±20.67$^{###}$	102.3±9.24**	76.5±4.85***	70.25±11.6***
FFA(mg/dl)	35.32±2.32	101.00±10.15$^{###}$	79.23±3.84^{NS}	37.97±2.08***	50.28±3.70**
Glucose(mg/dl)	110.0±5.21	162.8±3.47$^{###}$	131.3±10.71*	117.0±8.31***	128.0±4.35***
Insulin (pmol/l)	38.89±3.81	79.0±4.34$^{###}$	56.43±2.96**	44.97±4.44***	46.7±3.41***
FIRI	168.7±8.42	513.7±26.10$^{###}$	309.2±21.93***	201.1±25.35***	243.7±21.48***

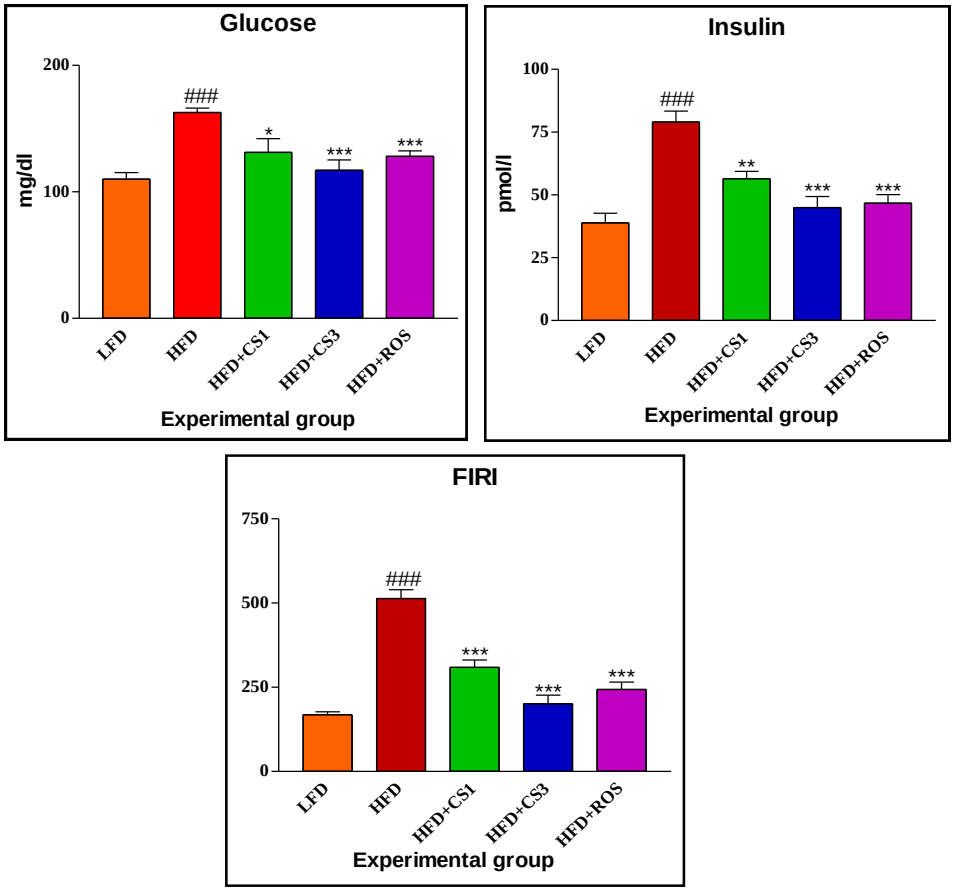
Where, n=6. # $p<0.05$, ## $p<0.01$, ### $p<0.001$ and ns: non significance when, LFD vs HFD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and NS: non significance when, HFD vs HFD+CS1, HFD+CS3 and HFD+ROS

Figure 2.2a: Effect of CS extract and Rosiglitazone on Plasma lipid profile



Where, n=6. # $p<0.05$, ## $p<0.01$, ### $p<0.001$ and ns: non significance when, LFD vs HFD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and NS: non significance when, HFD vs HFD+CS1, HFD+CS3 and HFD+ROS

Figure 2.2b: Effect of CS extract and Rosiglitazone onplasma glucose and insulin levels



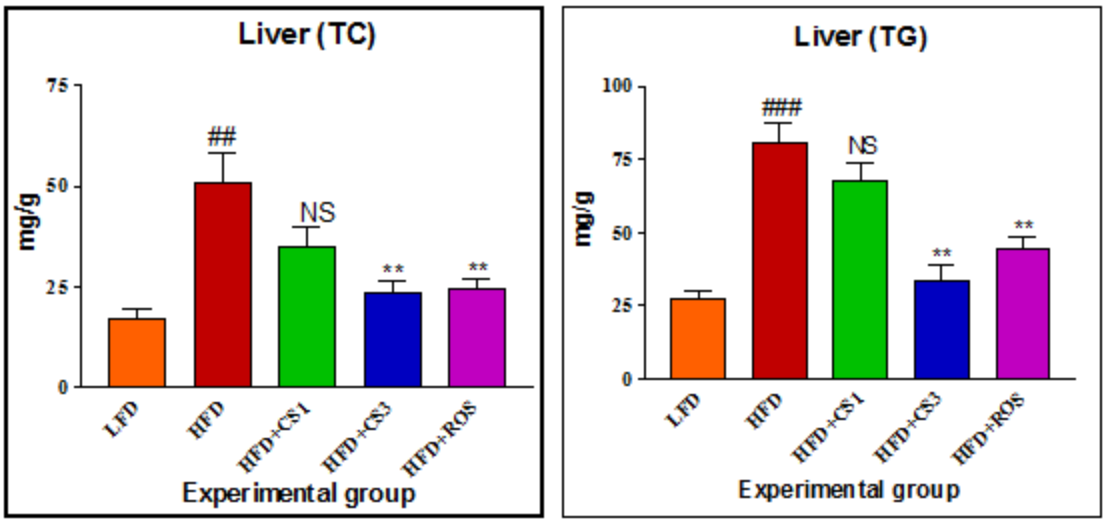
Where, n=6. # $p<0.05$, ## $p<0.01$, ### $p<0.001$ and ns: non significance when, LFD vs HFD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and NS: non significance when, HFD vs HFD+CS1, HFD+CS3 and HFD+ROS

Table 2.3: Effect of CS extract and Rosiglitazone on hepatic lipid profile

	SLD	HFD	HFD+CS1%	HFD+CS3%	HFD+ROS
TC (mg/g)	17.23±2.34	50.75±7.33$^{\#\#}$	34.75±5.07^{NS}	23.50±2.90**	24.44±2.66**
TG (mg/g)	27.00±3.06	80.25±7.46$^{\#\#\#}$	67.5±6.41^{NS}	33.65±5.49**	44.25±4.34**

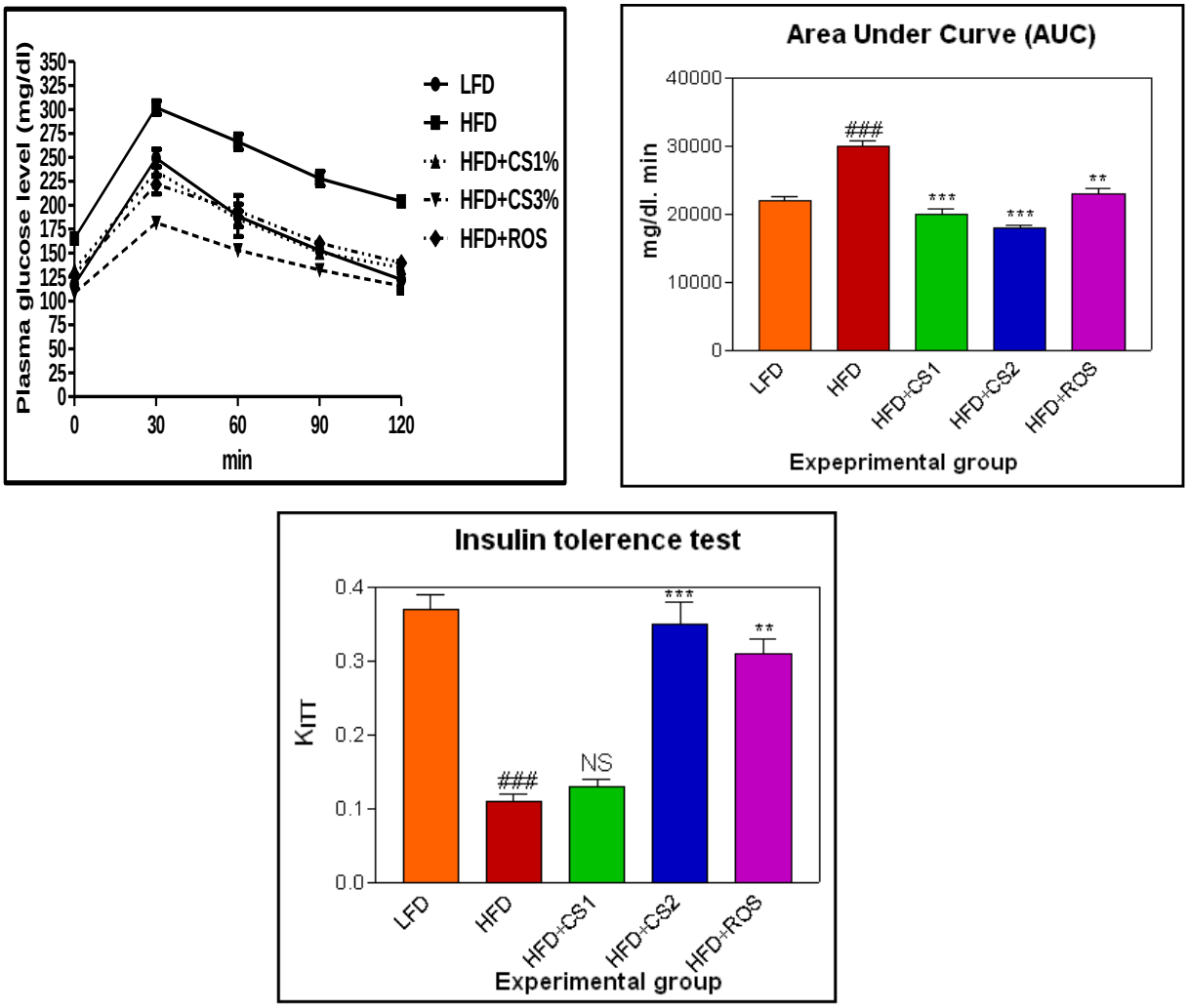
Where, n=6. # $p<0.05$, ## $p<0.01$, ### $p<0.001$ and ns: non significance when, LFD vs HFD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and NS: non significance when, HFD vs HFD+CS1, HFD+CS3 and HFD+ROS

Figure 2.3: Effect of CS extract and Rosiglitazone on hepatic lipid profile



Where, n=6. # $p<0.05$, ## $p<0.01$, ### $p<0.001$ and ns: non significance when, LFD vs HFD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and NS: non significance when, HFD vs HFD+CS1, HFD+CS3 and HFD+ROS

Figure 2.4: Effect of CS extract and Rosiglitazone on IPGTT and IPIRT



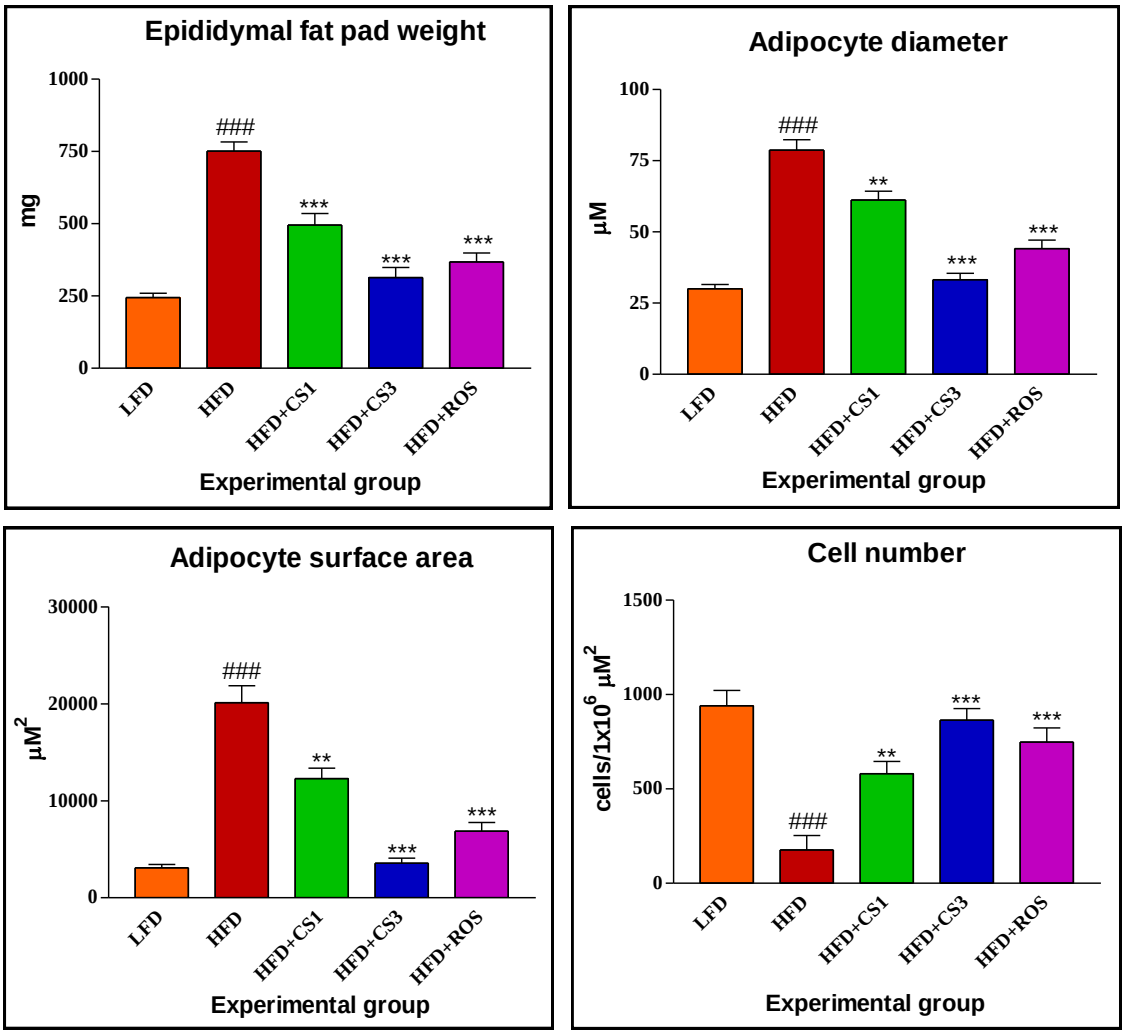
Where, n=6. # $p<0.05$, ## $p<0.01$, ### $p<0.001$ and ns: non significance when, LFD vs HFD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and NS: non significance when, HFD vs HFD+CS1, HFD+CS3 and HFD+ROS

Table 2.4: Effect of CS extract and Rosiglitazone on Epididymal fat pad weight, Adipocyte Diameter, Adipocyte Surface area, Adipocyte number

	SLD	HFD	HFD+CS1%	HFD+CS3%	HFD+ROS
Epididymal fat pad@	244.5±15.39	751.5±31.7$^{###}$	495.8±39.95***	313.8±35.12***	368.3±30.68***
Adipocyte Diameter$	30.00±1.57	78.7±3.71$^{###}$	61.20±3.13**	33.20±2.2***	44.1±3.05***
Adipocyte Surface area¥	3099.0±326.5	20117.0±1751$^{###}$	12287.0±1084**	3550.0±526***	6865.0±889***
Adipocyte Number€	941±81	177±76$^{###}$	580±66**	864±62***	748±75***

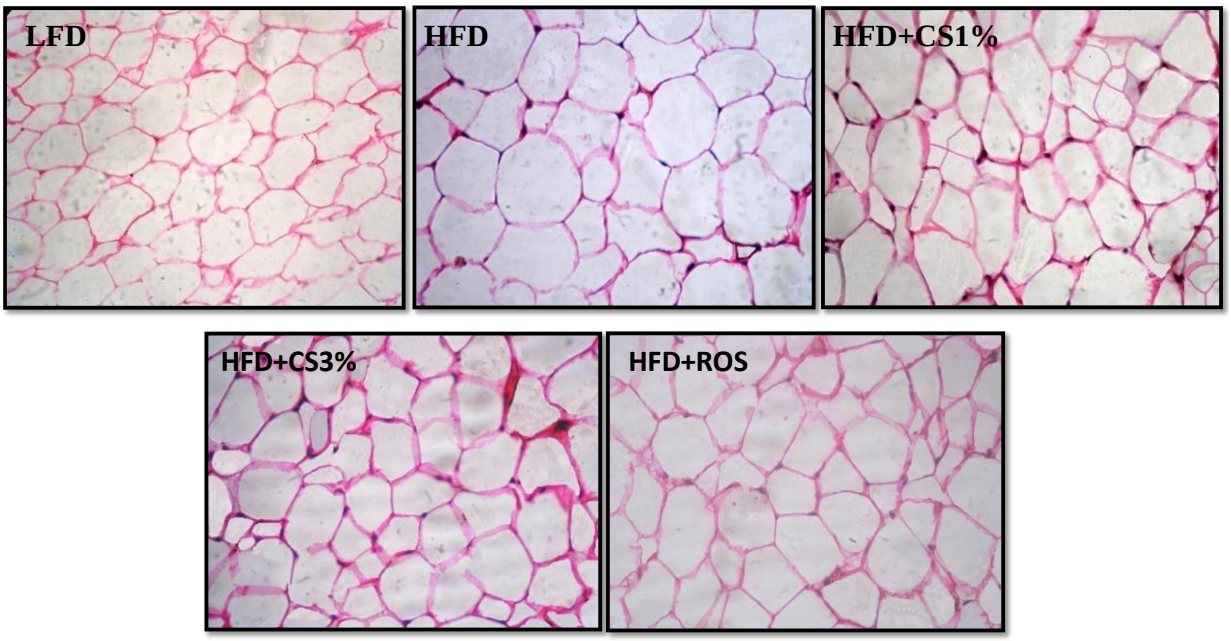
Where, n=6. @=mg, $=μm,¥=μm^2, €= cells/1x10^{6}μm^2. #p<0.05, ##p<0.01, ###p<0.001 and ns: non significance when, LFD vs HFD. * p<0.05, ** p<0.01, *** p<0.001 and NS: non significance when, HFD vs HFD+CS1, HFD+CS3 and HFD+ROS

Figure 2.5: Effect of CS extract on epididymal fat pad weight, adipocyte diameter, adipocyte surface area and cell number



Where, n=6. # $p<0.05$, ## $p<0.01$, ### $p<0.001$ and ns: non significance when, LFD vs HFD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and NS: non significance when, HFD vs HFD+CS1, HFD+CS3 and HFD+ROS

Figure 2.6: Photomicrograph of epididymal fat pad showing the effect of CS extract and Rosiglitazone on adipocyte morphology. Magnification X 400.



DISCUSSION

C57BL/6J mouse is a popular experimental model used in pre-clinical investigations of herbal and synthetic therapeutic agents against diabetes and obesity as there mice undergo a series of physiological changes when fed with high fat diet for 12-15 weeks. These changes are similar to the onset and progression of T2D and IR (Ahrén *et al*., 1997). In these mice, HFD induced IR is preceded by hyperlipidemia and visceral adiposity.

Our study has shown significant increment inbody weight gain, food intake and feed efficiency ratio in HFD mice, well reflected in the form of significant increment in the weight of epididymal fat pad. However, CS (1% & 3%) supplementation of HFD mice significantly prevented the characteristic body weight gain and increase inepididymal fat pad mass possibly due to decreased food intake. Previous studies have reported that insulin sensitivity in T2D patients improved with weight loss (DeFronzo and Ferrannini, 1991). CS supplemented HFD fed mice minimized the increase in hepatic and plasma TC and TG levels characteristic of HFD. These observations are in accordance with a previous report of Chithra and Leelamma (1997) on CS induced decrement in lipid profile of *Sprauge dawly* rats maintained on a hyperlipidemic diet. It has been reported that, increase in circulating level of plasma FFA contributes to an increase in IR and inhibition of glucose uptake by skeletal muscles and other peripheral tissues (Boden *et al*., 1997). Also, in ob/ob mice, hyperinsulinemia develops due to decreased sensitivity towards insulin in liver, muscle and adipose tissues (Genuth *et al*., 1971). CS supplemented HFD mice (1% & 3%) were able to improve IR bydecreasing levels of plasma FFA and insulin titer. Also, an improvement in fasting plasma glucose

levels and FIRI values provide ample testimony to CS induced improvement of IR in HFD fed mice. IPGTT and IPIRT tests were carried out in control and experimental mice to assess CS induced possible improvement in insulin sensitivity and the results obtained were compared with HFD group. Lower $AUC_{glucose}$ values and higher KITT indices recorded in HFD+CS (1% & 3%) groups are attributable to improved insulin sensitivity in CS supplemented HFD fed mice. These mitigating changes are comparable to ROS induced changes in visceral adiposity and IR. The histological manifested changes in adipocyte mass and size of HFD are attributable to diet induced lipogenesis. Co-presence of CS in HFD is able to resist the adiposity changes caused due to fat rich diet. Adipocyte hypertrophy is a strong evidence of visceral obesity and IR (Flier, 2004; Wellen and Hotamisligil, 2005) and in fact, the larger adipocytes are associated with IR and smaller ones with better insulin sensitivity (Okuno *et al*., 1998; Kubota *et al*., 1999; Kodowaki, 2000). The sizes of adipocytes in HFD and HFD+CS groups of mice in this context could easily reflect the higher IR in the former and greater insulin sensitivity in the later. The observations on adipocyte number and size in HFD+CS (1%) and HFD+CS (3%) also tend to suggest the dose dependent favorable influence of the extract inpreventing diet induced visceral adiposity and IR. The observed significant anti-hyperglycemic effect of CS extracts might also suggest increased peripheral glucose uptake as well as decreased transport of glucose across the intestinal epithelium (Gallagher *et al*., 2003). *In vivo* studies on this line and the possible role of CS extract to regulate the expression of PPARγ and other related genes in HFD mice are in progress.

This inventory is however first report that hasinvestigated role of CS extract in improvement of HFD induced IR. These results are attributable to multiple physiological

processes such as CS extract induced decrement in food intake, lowering of plasma and tissue lipids and decrement in size of adipocytes. Also, CS extract induced lowering of insulin, eventually leading to improvement of IR, improved insulin sensitivity and efficient clearance of glucose load, further corroborate these observations. Since, protective role of CS extract against STZ induced type I diabetes has already been established, this study is an addition to its already established pharmacotherapeutic uses.

SUMMARY

This study investigates the effect of dietary supplementation with *Coriandrum sativum* L. seed aqueous extract (CS) to a high fat diet (HFD), for induced insulin resistance (IR) C57BL/6J mice. Changes in body weight, food intake, feed efficiency ratio, fasting blood glucose (FBG), plasma insulin, fasting insulin resistance index (FIRI), plasma and hepatic triglyceride (TG), total cholesterol (TC) and, plasma free fatty acid (FFA) levels were evaluated in control and treated groups. Also, the diameter, surface area and number of adipocytes and, intraperitoneal glucose tolerance test (IPGTT) and intraperitoneal insulin response test (IPRTT) were performed. CS supplementation (1% and 3% w/w) to HFD fed mice (for 12 weeks) significantly prevented HFD induced increment in body weight gain, food intake, feed efficiency, FBG, plasma insulin, FIRI, plasma and hepatic TG and TC and, plasma FFA, adipocyte diameter and surface area along with decrement in adipocyte number. Also, improved responses were recorded in the IPGTT and IPRTT in CS supplemented HFD fed mice. These set of changes were comparable to the rosiglitazone (0.05%) supplemented HFD fed mice. Our findings suggest that CS improves insulin sensitivity primarily by mitigating plasma and tissue lipids and, adipocyte hypertrophy.

CHAPTER 3

Cardio protective effect of *Coriandrum sativum* L. on isoproterenol induced myocardial necrosis in rats

INTRODUCTION

Epidemiological studies predict an ominous prevalence of cardio vascular diseases globally as well as in India during next decade (Lopez and Murray, 1998; Gilski and Borkenhagen, 2005). Myocardial infarction, a highly prevalent ischemic condition characterized by tissue necrosis develops essentially due to an imbalance between oxygen need and actual supply (De Bono and Boon, 1992) and results in irreversible histopathological damages and subsequent cardiovascular complications (Gross and Auchampach, 2007). Isoproterenol (IP), a synthetic catecholamine and β-adrenergic agonist increases heart rate and exhaust energy reservoir of cardiac myocytes leading to cell death. It induces myocardial necrosis via multiple modes of action in experimental animals. They are i. Functional hypoxia and ischemia, ii. Coronary insufficiency, iii. Alteration in metabolism, iv. Decreased level of high energy phosphate store, v. intracellular Ca^{+2} overload, vi. Changes in electrolyte contents and vii. Oxidative stress.

Oxidative stress is more probably one the main mechanisms through which catecholamines exert their toxic effects. Spontaneous oxidation of catecholamines results in the formation of catecholamine-o-quinones, which generate aminochromes through cyclization. Adrenochrome (which results from the cyclization of epinephrine-o-quinones) can be oxidized to several other compounds such as adrenolutin 5,6, dihydroxy 1-methylindole or adrenochrome adrenolutin dimmer. All these redox reactions generate

free radicals. Consequently, catecholamine-o-quinones and aminochromes and the radical species resulting from the oxidation of catecholamines are thought to be involved in catecholamine related toxicity (Dhalla *et al*., 1992).

The oxidation products have ability to interact with sulphhydril groups of various proteins and also lead to the production of superoxide ions and subsequently hydrogen peroxide. This results in changes in microsomal permeability, mitochondrial Ca^{+2} uptake, decrease in ATP production and formation of the highly reactive hydroxyl radicals which caused protein, lipid and DNA damage (Takeo *et al*.). IP also produce number of biochemical and electrophysiological alterations which preceed histological alteration in histology of heart. The primary disturbances of IP induced myocardial infarction has been reported to enhance adrenal cyclase activity, resulting in increased cAMP production, which in turn would lead to the higher lipid accumulation the myocardium (Subash *et al*., 1998). Several early events such as ultrastructural changes; histological, biochemical, electrolyte and membrane changes have been shown to occur within 48hr after the injection of IP. Histological changes induced by excessive amounts of IP include degeneration and necrosis of myocardial fibers, accumulation of imflammatory cells, interstitial edema, lipid droplets and endocardial hemorrhage (Lehr, 1972).

Biochemical alterations in IP induced cardiomyopathy represents a complex pattern of changes in cardiac marker enzymes, lipid profile, lipid metabolizing enzymes, enzymatic and non-enzymatic antioxidant levels, glycoprotein levels, decrease in ATP store, and changes in electrolyte levels in the blood as well as in the myocardial tissue (Fleckenstein *et al*., 1974; Lehr, 1972). Changes including those in sarcolemma, sarcoplasmic reticulum and mitochondria are mainly mediated by oxidative stress, which

is known to result in alterations of enzyme activity and transport systems and cause disturbances in cellular homeostasis (Takeo *et al*., 1980). Lipolysis is also one of the important determinants of IP induced myocardial injury. Study also provides evidences that chronic β-AR stimulation markedly shows iNOS up-regulation, CRP release an nitrate stress and that iNOS mediated nitrative stress function as a main interface linking chronic β-AR activation and myocardial cell apoptosis (Hu *et al*., 2006)

IP-induced myocardial necrosis serves as an excellent experimental model to study catecholamines induced cardiac dysfunction and also to evaluate the possible cardioprotective efficacy of various natural and synthetic agents. Several pre-clinical and clinical studies involving pretreatment with vitamins and antioxidants have demonstrated their potential to prevent myocardial damage (Singh *et al*., 1994; Senthil *et al*., 2004). Previously Hashim *et al*., (2005) have investigated that hydro-methanolic extract of *Coriandrum sativum* L. (CS) seed had strong antioxidant property and it had prevented oxidative damage induced by H_2O_2 to lymphocytes. The present study was designed to assess cardioprotective potential of hydro-methanolic extract of the customarily used spice CS seeds in IP induced multifocal myocardial necrosis in rats.

MATERIALS AND METHODS

Plant material and preparation of extract

CS plants were collected in the seedling months (February and March) and Dr. P.S. Nagar, Department of Botany, The M.S. University of Baroda identified the plant and a sample specimen was deposited in the herbarium of the Department of Botany. Hundred grams of powdered dry seeds soaked in methanol:water (80:20 v/v) at room temperature was allowed to stand for seven days. Resultant extract filtered through a muslin cloth was concentrated in a rotary evaporator under reduced pressure to obtain a thick semisolid brown paste (Hashim *et al*., 2005). The final yield obtained was 8.3 g (w/w).

Experimental animals

Adult male *Wistar* rats (150-200 g; obtained from Zydus Cadila Research Centre, Ahmedabad, Gujarat, India) were housed under standard animal house conditions (23±2°C; LD 12:12 and 45- 50% humidity) and provided with pelleted diet (M/S Pranav agro, Ltd., Baroda, India) and water *ad libitum*. The animals were maintained as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) India and the experimental protocol approved by the animal ethical committee of the Department of Zoology, The M. S. University of Baroda, Vadodara (Approval No.827/ac/04/CPCSEA).

Experiment design

Thirty animals were randomly divided into five groups of six animals each. Group I (NC) served as control and received 0.5% Carboxy methyl cellulose (CMC; *p.o.*) for 28 days and normal saline (*s.c.*) on days 29 and 30. Group II (IP) served as positive control rats

and received 0.5 CMC (*p.o.*) for 28 days and isoproterenol (85 mg/kg body weight, *s.c.*) on days 29 and 30 while, the remaining groups [Group III (IP+CS100), Group IV (IP+CS200) and group V (IP+CS300)] received respectively 100, 200 and 300 mg/kg body weight of CS extract daily for 28 days (*p.o.*) and IP (85 mg/kg, *s.c.*) on days 29 and 30. The protocol for IP treatment schedule was as per the previous works from this laboratory (Jadeja *et al*., 2010; Thounaojam *et al*., 2011). At the end of the experimental period (i.e. 31st day), animals were fasted overnight (12h) and blood samples were collected from retro-orbital sinus under mild ether anesthesia. Plasma was obtained by cold centrifugation of samples at 3000 rpm for 10 min. Later, animals were sacrificed by cervical dislocation under mild anaesthesia and heart was excised and stored at -80^{o}C for further evaluations. A piece of cardiac tissue was fixed in 10% paraformaldehyde for paraffin wax histology.

Plasma markers of cardiac damage

Plasma levels of creatine phospokinase- MB (CK-MB), lactate dehydrogenase (LDH), aspartate transaminase (AST), alanine transaminase (ALT) and uric acid were ascertained by using commercially available kits (Reckon Diagnostic Ltd., Vadodara, India).

Plasma lipid profile

Triglyceride (TG), total cholesterol (TC) and high density lipoprotein (HDL) content were assayed by using commercially available kits (Recon Diagnostic, Ltd., Vadodara, India). Lowdensity lipoprotein (LDL) and Very low-density lipoprotein (VLDL) were calculated by Friedewald's formula (Friedewald *et al*., 1972).

Cardiac antioxidants and Lipid peroxidation (LPO)

Cardiac tissue from control and treated groups was weighed and homogenized (10%w/v) in chilled Tris buffer (10mM; pH 7.4) and centrifuged at 10,000 g for 20 min at 0^{o}C. Clear supernatant was used to assay superoxide dismutase (SOD; Marklund and Marklund, 1974), catalase (CAT; Aebi *et al*., 1983), glutathione peroxidase (GPx; Rotruck *et al*., 1973), glutathione s-transferase (GST; Habig, 1974), reduced glutathione (GSH; Beutler, 1963), vitamin E (Vit. E; Baker and Frank, 1968), total protein content (Lowry *et al*., 1951) and lipid peroxidation levels (LPO; Buege and Aust, 1978). Total ascorbic acid content (AA) was measured as per Roe and Küether (1943) by preparing homogenates of fresh cardiac tissue in 6% Trichloro acetic acid.

Cardiac ATPases

Pellets obtained from tissue homogenate after centrifugation was re-suspended in ice-cold Tris buffer (10 mM, pH 7.4) to get a final concentration of 10% and was used for the estimation of Na^{+} K^{+} ATPase (Bonting, 1970), Ca^{2+} ATPase (Hjerken and Pan, 1983) and Mg^{2+}ATPase (Ohinishi *et al*., 1982). Protein was estimated according to the method of Lowry *et al*., (1951).

Macroscopic and microscopic evaluation of cardiac tissue:

Heart tissue slices (approx. 2-3 mm thick) transversely cut across the ventricle were kept in a covered glass dish containing 1% TTC (2, 3, 5- triphenyltetrazolium chloride; Sigma, St. Louis, MO) solution and incubated at 37°C for 20 min for differentiation of viable tissue from necrotic areas (Li *et al*., 2011). Heart samples from control and treated rats

were fixed in 4% buffered paraformaldehyde, dehydrated in graded alcohol series and embedded in paraffin wax. Five µm thick sections cut (by Leica RM2155 Microtome) and stained with haematoxylin-eosin, were photographed with Canon power shot S72 digital Camera (200X) attached to a Leica microscope.

Statistical analysis:

Statistical analysis of data was done by one way ANOVA followed by Bonferroni‟s multiple comparison test and results were expressed as mean ± S.E.M (Using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego California USA).

RESULTS

Plasma markers of cardiac damage (Table 3.1; Figure 3.1a & 3.1b)

IP treated rats showed significant ($p<0.005$) increment in the plasma levels of CK-MB, LDH, AST, ALT and uric acid compared to NC rats. Pretreatment of IP rats with CS prevented the IP induced increase in the serum levels of these parameters in a dose dependent manner.

Plasma lipid profile (Table 3.2; Figure 3.2a & 3.2b)

IP treatment recorded significant ($p<0.005$) increase in plasma TG, TC, LDL, and VLDL and decrement in HDL levels compared to the NC group. CS treatment showed dose dependent decrement in TC, TG, LDL, VLDL and significant increment in HDL compared to IP treated rats.

Cardiac anti-oxidants and LPO (Table 3.3; Figure 3.3a-3.3d)

IP treated group recorded significant ($p<0.001$) increment in LPO level, as well as significant ($p<0.001$) decrement in the activities of enzymatic antioxidants (SOD, CAT, GPx and GST) and content of non-enzymatic antioxidants (GSH, AA and Vit. E) compared to NC rats. Administration of CS (100, 200 and 300 mg/kg body weight, respectively) markedly prevented all the alterations with respect to antioxidants and LPO in IP treated rats and maintained them to the near normal levels.

Cardiac ATPases (Table 3.4; Figure 3.4a)

The cardiac tissue of IP treated rats depicted significant ($p<0.005$) decrement in the activities of $Na^{+}/K^{+,}$ Mg^{2+} and Ca^{2+}ATPases compared to that of NC rats while, IP+CS

treated cardiac tissue recorded significant resistance.

TTC and HE staining of cardiac tissue (Figure 3.5 and 3.6)

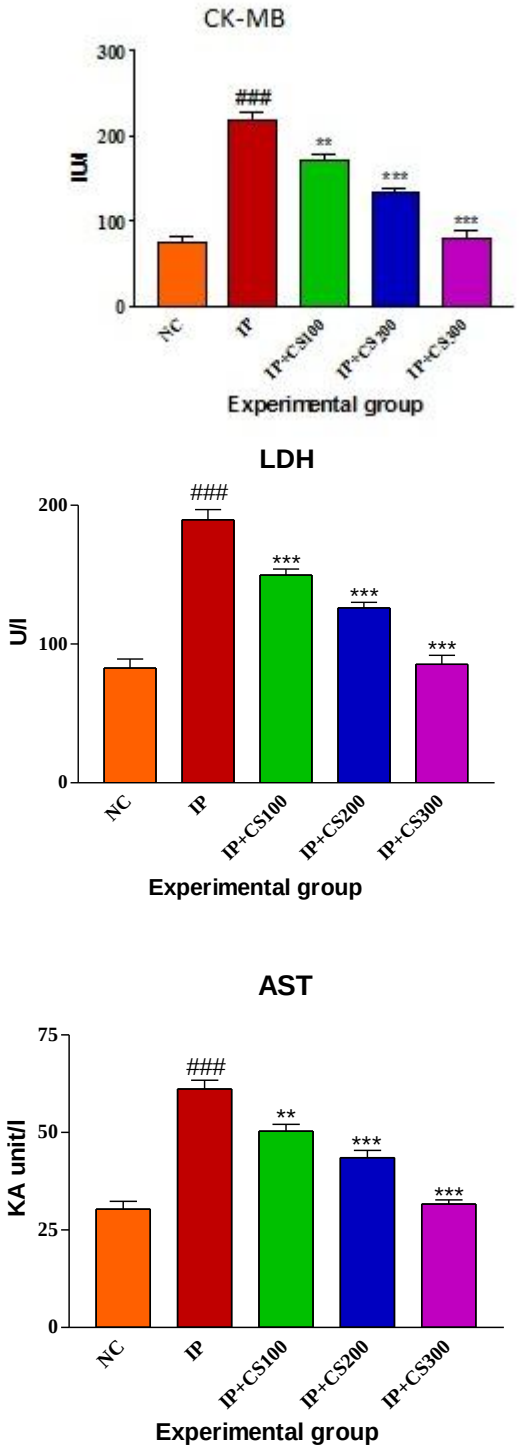
TTC staining of heart of control rats showed brick red coloration indicative of more number of viable cells whereas, IP treated rats showed large area of pale yellow coloration was suggestive of necrosis. However, IP rats pretreated with CS showed a protective effect with a minimal or no pale yellow coloration in a dose dependent manner. HE staining of cardiac tissue from NC rats showed histoarchitecture of myofibers that were characteristically multinucleated and intact. IP treatment resulted in focal myocardial necrosis (encircled area) and disrupted myofibers. However, IP+CS treated groups showed relatively less disruption of myofibers with IP+CS 300 showing maximum fiber integrity.

Table 3.1**:** Effect of CS seed extract on plasma markers of cardiac damage

Parameters	**NC**	**IP**	**IP+CS100**	**IP+CS200**	**IP+CS300**
CkMB (IU/l)	75.66±6.91	218.20±29.16$^{###}$	171.20±9.19**	133.10±6.16***	80.10±9.03***
LDH (U/l)	82.71±6.50	189.60±7.36$^{###}$	149.70±4.32***	126.00±4.15***	85.60±6.35***
AST (KA units/l)	30.33±1.99	61.17±2.24$^{###}$	50.33±1.76**	43.50±1.91***	31.67±1.02***
ALT (KA units/l)	19.33±1.11	44.83±2.18$^{###}$	36.67±1.82*	31.00±1.73***	22.17±1.99***
Uric acid (mg/dl)	1.91±0.21	7.01±0.47$^{###}$	5.24±0.41*	3.72±0.19***	2.14±0.21***

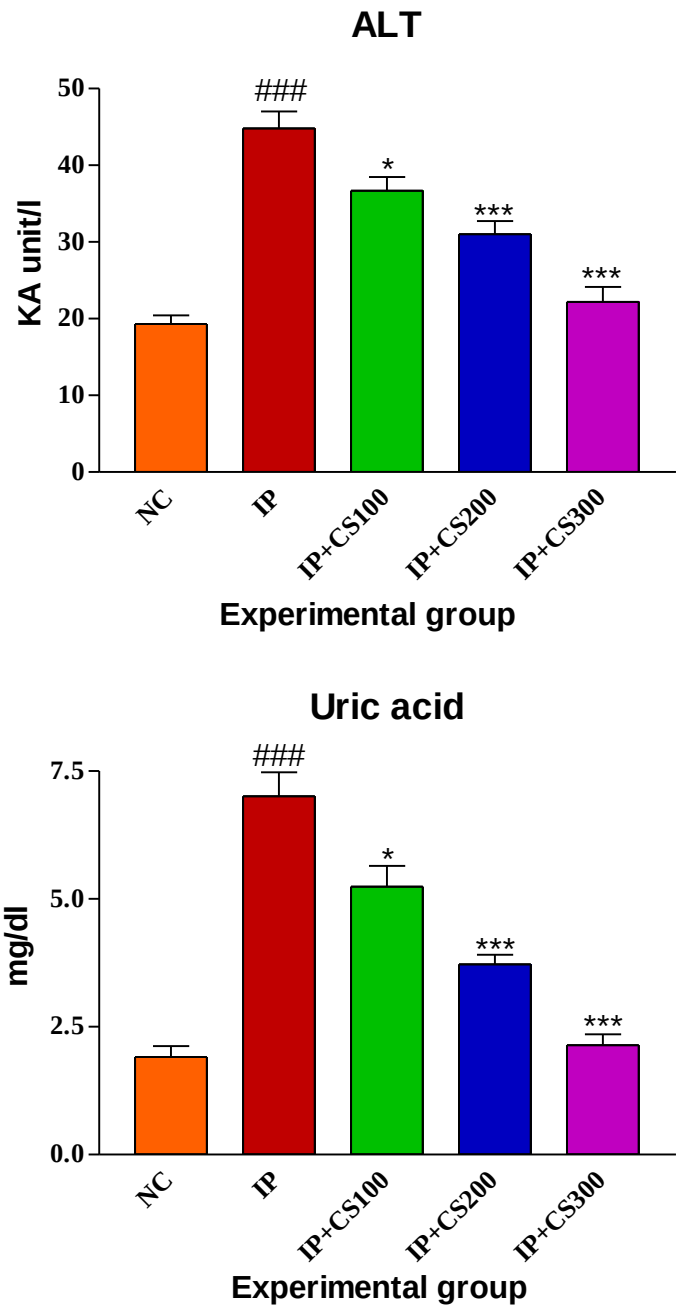
Where n=6. Data were expressed as mean ± S.E.M. ### ($p<0.001$) when NC vs. IP and * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) when IP vs. IP+CS

Figure 3.1a**:** Effect of CS seed extract on plasma markers of cardiac damage



Where n=6. Data were expressed as mean ± S.E.M. ### ($p<0.001$) when NC vs. IP and * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) when IP vs. IP+CS

Figure 3.1b: Effect of CS seed extract on plasma markers of cardiac damage



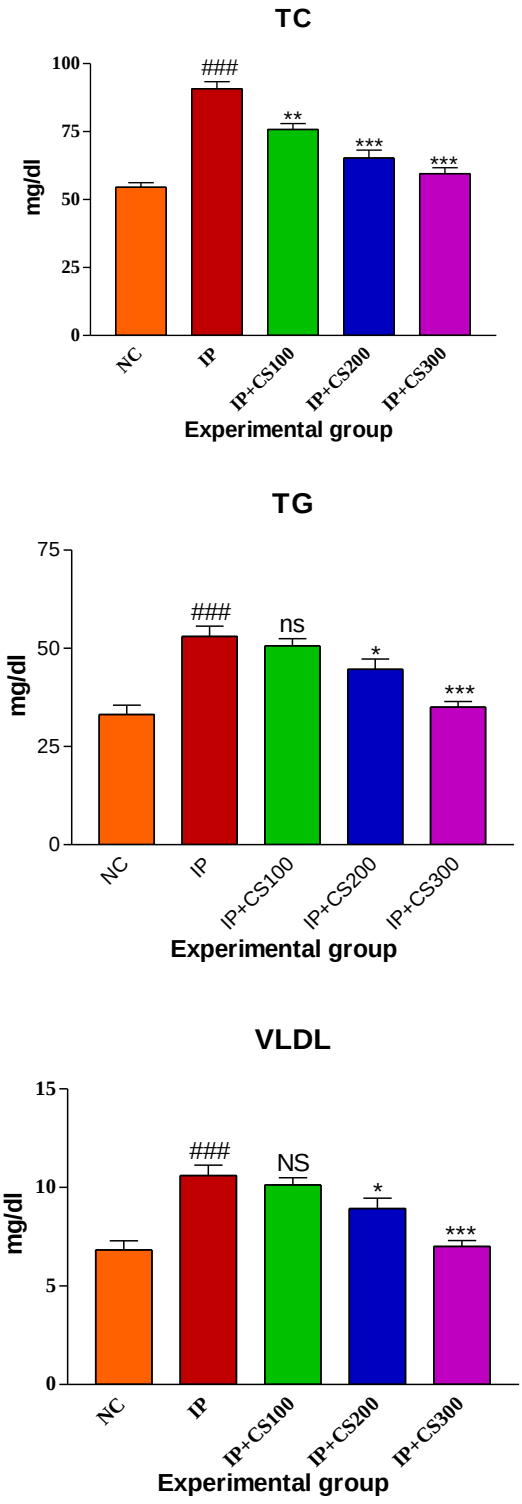
Where n=6. Data were expressed as mean ± S.E.M. ### ($p<0.001$) when NC vs. IP and * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) when IP vs. IP+CS

Table 3.2: Effect of CS seed extract on plasma lipid profile

Parameters	NC	IP	IP+CS100	IP+CS200	IP+CS300
TC$^{@}$	54.50±1.72	90.83±2.58$^{###}$	75.83±2.16**	65.33±2.88***	59.50±2.23***
TG$^{@}$	33.17±2.37	53.00±2.67$^{###}$	50.67±1.82^{NS}	44.67±2.60*	35.00±1.48***
VLDL$^{@}$	6.63±0.47	10.60±0.53$^{###}$	10.13±0.36^{NS}	8.93±0.52*	7.00±0.29***
LDL$^{@}$	34.30±0.52	90.27±2.83$^{###}$	70.97±2.62***	58.10±3.92***	43.67±2.75***
HDL$^{@}$	26.83±0.98	11.17±1.04$^{###}$	15.00±0.73^{NS}	16.17±1.35*	22.83±0.79***

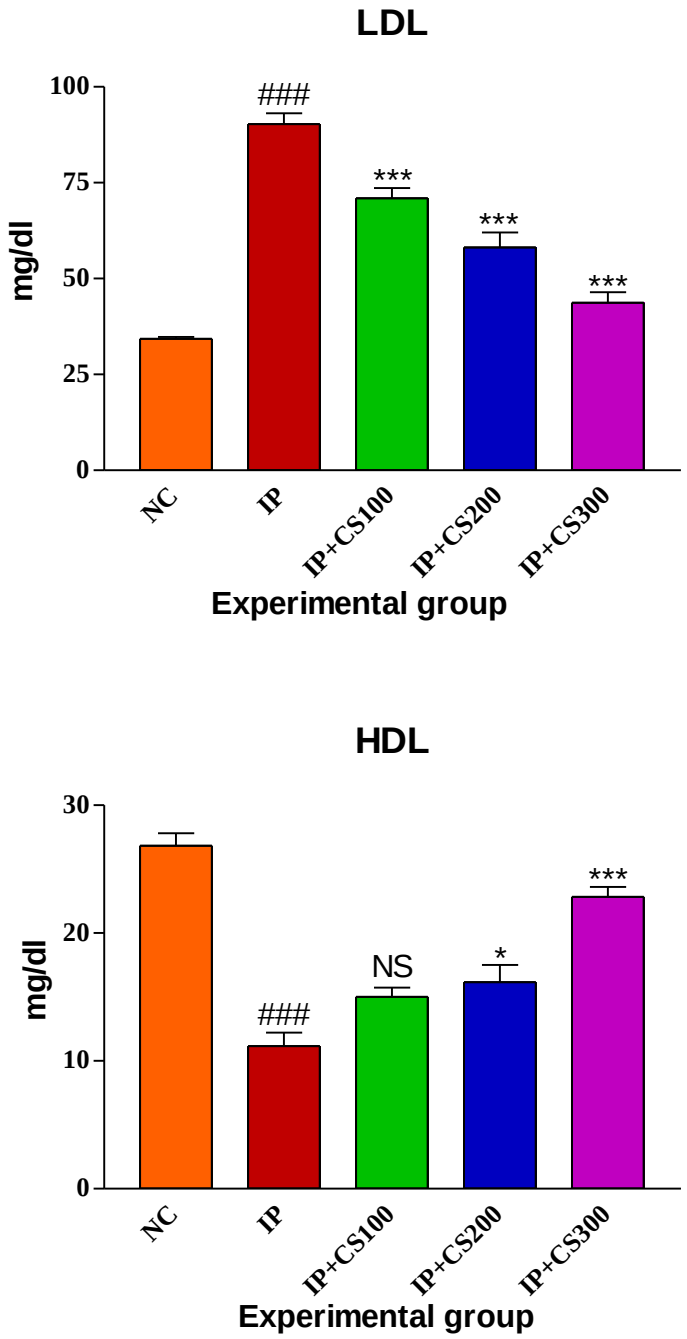
Where, @=mg/dl. n=6. Data were expressed as mean ± S.E.M. ### ($p<0.001$) when NC vs. IP and * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) when IP vs. IP+CS

Figure: 3.2a: Effect of CS seed extract on plasma lipid profile



Where, n=6. Data were expressed as mean ± S.E.M. ### ($p<0.001$) when NC vs. IP and * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) when IP vs. IP+CS

Figure: 3.2b: Effect of CS seed extract on plasma lipid profile



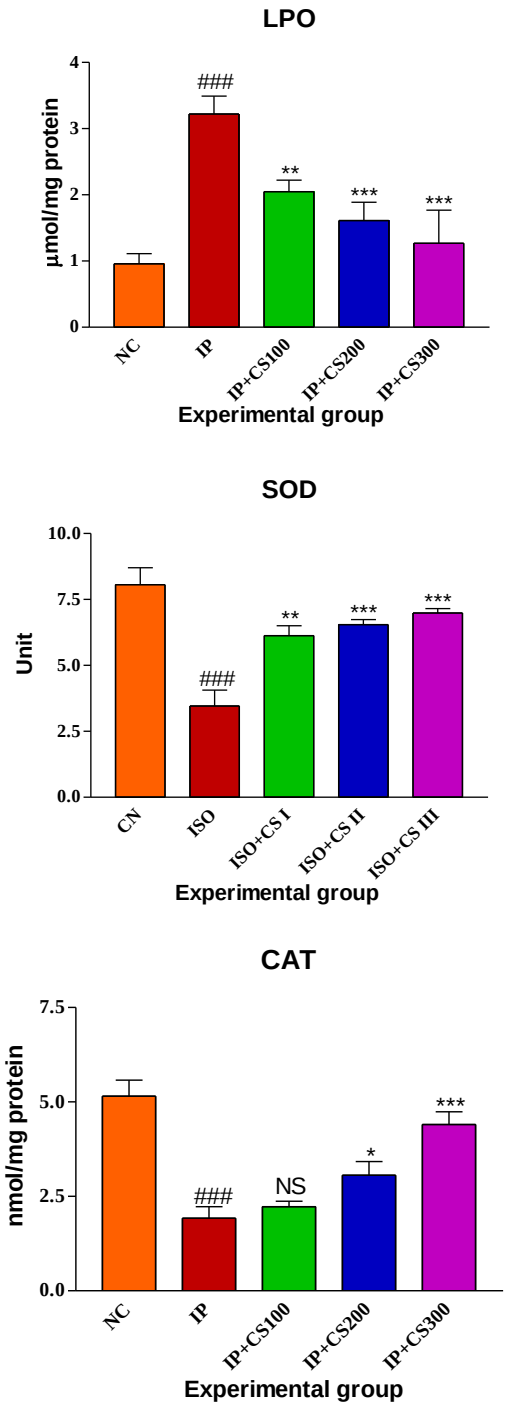
Where, n=6. Data were expressed as mean ± S.E.M. ### ($p<0.001$) when NC vs. IP and * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) when IP vs. IP+CS

Table 3.3: Effect of CS seed extract on Cardiac LPO levels and enzymatic and non-enzymatic anti-oxidants

Parameters	NC	IP	IP+CS100	IP+CS200	IP+CS300
LPO*	0.96±0.53	3.22±0.27###	2.05±0.73**	1.61±0.28***	1.27±0.57***
SOD	8.06±0.63	3.46±0.60###	6.12±0.37**	6.54±0.19***	6.98±0.17***
CAT@	5.15±0.43	1.92±0.31###	2.23±0.14NS	3.06±0.29*	4.40±0.34***
GPx$	3.08±0.05	1.03±0.06###	1.22±0.06*	1.81±0.07***	2.80±0.04***
GST#	787.4±14.41	423.6±12.63###	541.4±10.19***	625.7±11.34***	760.7±16.06***
GSH@	9.27±0.17	3.81±0.22###	4.79±0.26*	5.87±0.31***	7.81±0.18***
AA€	250.0±5.0	114.0±7.0###	140.0±8.0*	195.0±12.0***	229.0±9.0***
Vit E€	5.29±0.23	1.33±0.31###	2.65±0.36NS	3.32±0.21***	4.91±0.29***

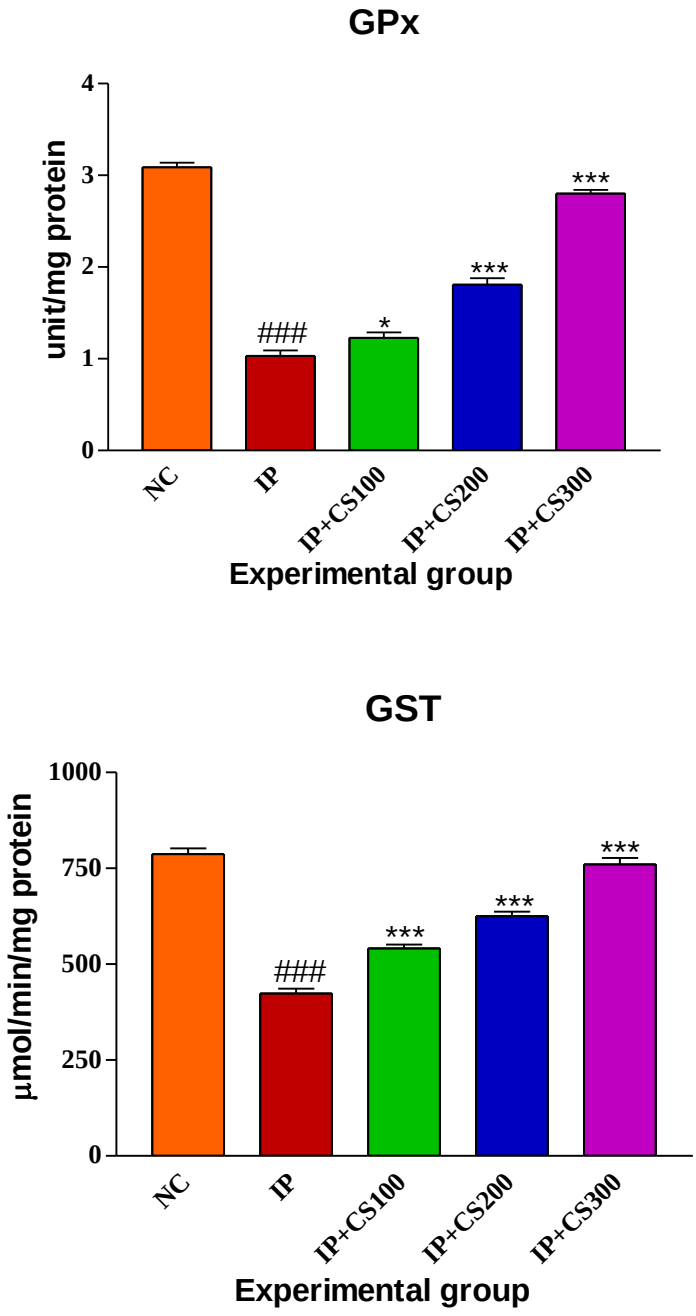
*=µmol/mg protein, @=nmol/mg protein, $=unit/mg protein, #=µmol/min/mg protein, €= mg/100 g tissue Where, n=6. Data were expressed as mean ± S.E.M. ### ($p<0.001$) when NC vs. IP and * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) when IP vs. IP+CS

Figure 3.3a: Effect of CS seed extract on Cardiac LPO levels and enzymatic and non-enzymatic anti-oxidants



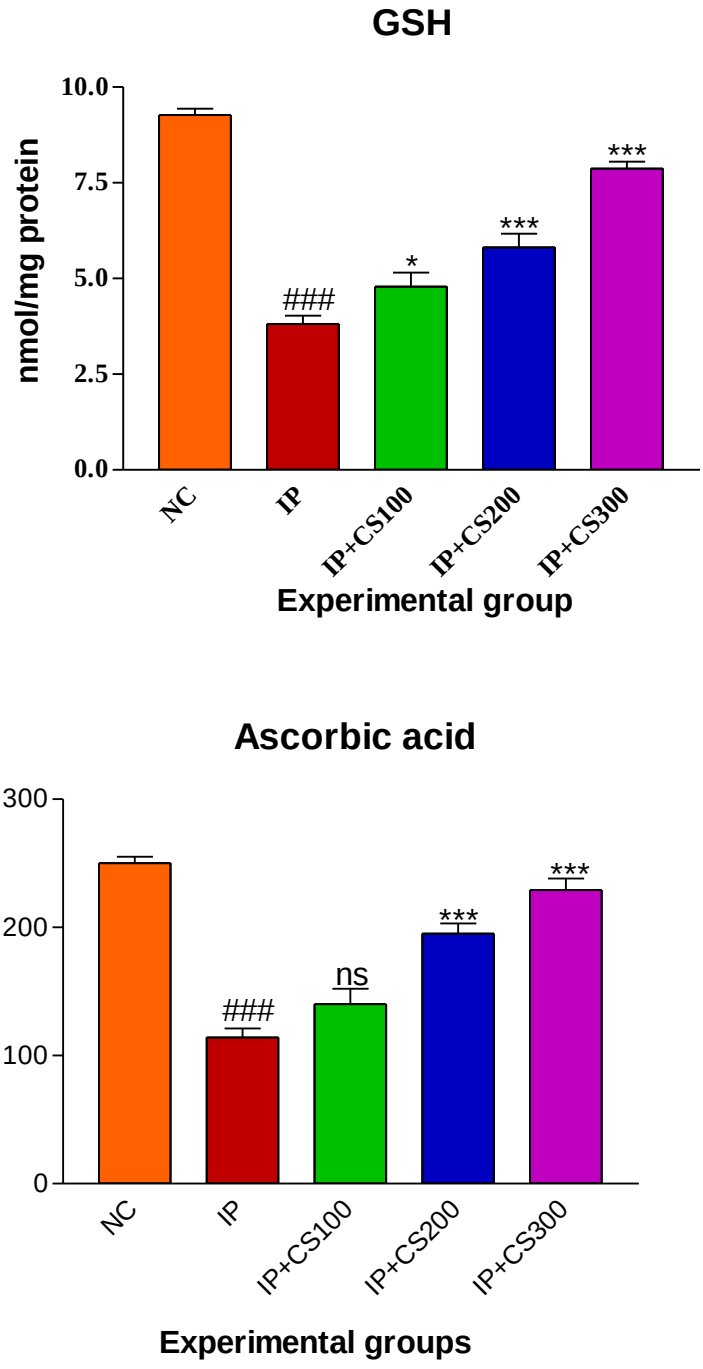
Where, n=6. Data were expressed as mean ± S.E.M. ### ($p<0.001$) when NC vs. IP and * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) when IP vs. IP+CS

Figure 3.3b: Effect of CS seed extract on Cardiac LPO levels and enzymatic and non-enzymatic anti-oxidants



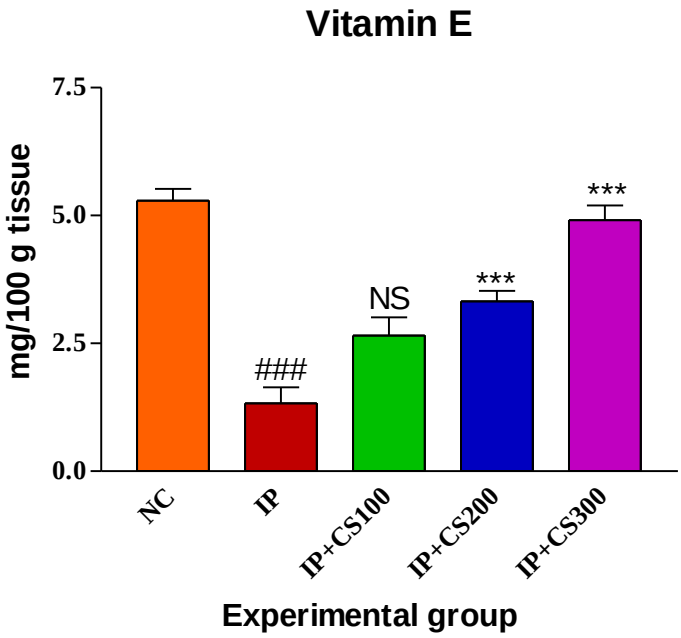
Where, n=6. Data were expressed as mean ± S.E.M. ### ($p<0.001$) when NC vs. IP and * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) when IP vs. IP+CS

Figure 3.3c: Effect of CS seed extract on Cardiac LPO levels and enzymatic and non-enzymatic anti-oxidants



Where, n=6. Data were expressed as mean ± S.E.M. ### ($p<0.001$) when NC vs. IP and * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) when IP vs. IP+CS

Figure 3.3d: Effect of CS seed extract on Cardiac LPO levels and enzymatic and non-enzymatic anti-oxidants



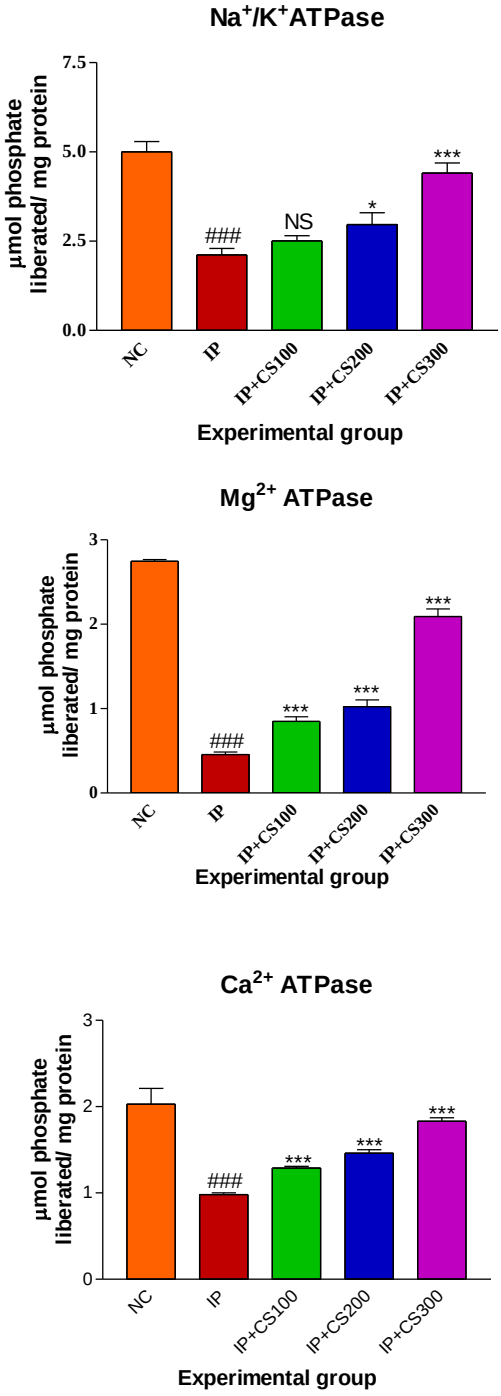
Where, n=6. Data were expressed as mean ± S.E.M. ### ($p<0.001$) when NC vs. IP and * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) when IP vs. IP+CS

Table 3.4: Effect of CS seed extract on Cardiac ATPases

Parameters	NC	IP	IP+CS100	IP+CS200	IP+CS300
Na^+/K^+ATPase@	5.00±0.50	2.11±0.17###	2.51±0.14NS	2.96±0.33*	4.40±0.28***
Mg^{2+} ATPase@	2.74±0.02	0.45±0.03###	0.84±0.05***	1.02±0.08***	2.09±0.09***
Ca^{2+} ATPase@	2.03±0.18	0.98±0.02###	1.29±0.02***	1.46±0.04***	1.83±0.04***

Where, @=µmol phosphate liberated/ mg protein. Where, n=6. Data were expressed as mean ± S.E.M. ### ($p<0.001$) when NC vs. IP and * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) when IP vs. IP+CS

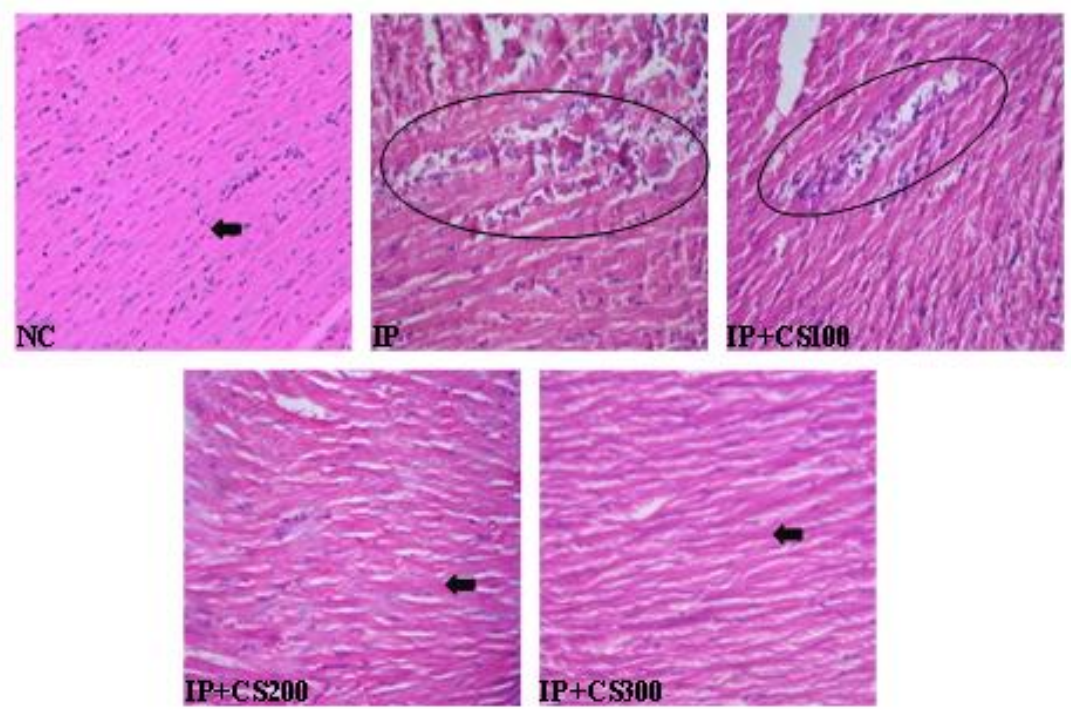
Figure 3.4a: Effect of CS seed extract on Cardiac ATPases



Where, n=6. Data were expressed as mean ± S.E.M. ### ($p<0.001$) when NC vs. IP and * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) when IP vs. IP+CS

Figure 3.5: Effect of CS seed extract on triphenyltetrazolium chloride (TTC) stained cardiac tissue slices. Arrows indicate necrotic tissue.

Figure 3.6: Effect of CS seed extract on cardiac histopathology of cardiac tissue. Tissue sections (7μM) are stained with hematoxylin-eosin (400X). Encircled area indicates focal myocardial necrosis whereas, arrows indicate healthy myofibers.



DISCUSSION

Administration of higher doses of IP to rats induces increment in heart rate, systolic and diastolic irregularities and abnormal ECG pattern (Rona, 1985; `Karthick and Prince, 2006). These events epitomized by hypoxia, calcium over load and increased production of reactive oxygen species (ROS) lead to degenerative changes in cardiac tissue that culminate in necrosis. Accordingly, IP treated rats herein recorded significant increment in plasma levels of CK-MB, LDH, AST, ALT and uric acid, which is in keeping with the known IP induced deficiency of oxygen supply and increased sarcolemmal permeability and consequent leaching of CK-MB and LDH into the blood stream along with increased plasma levels of AST, ALT and uric acid (Mathew *et al*., 1985; Weir *et al*., 2003). The recorded ability of CS to effectively prevent these alterations clearly points towards its cardio-protective competence and maintenance of sarcolemmal integrity. Also the activity levels of 3-hydroxy-3-methyl-glutaryl-CoA (HMG CoA) reductase and Lecithin-cholesterol acyltransferase (LCAT) have been reported to undergo significant alterations following IP treatment which resulted in altered lipid and lipoprotein profiles (Rajadurai and Stanely Mainzen Prince, 2006). Hence, the observed decrement in lipid profile in IP+CS treated groups indicates at possible modulatory influence of CS on activity levels of HMG CoA and LCAT that requires further investigations.

SOD and CAT are enzymatic antioxidants that act as the first line of cellular defense and help in scavenging free radicals. Therefore, a decrement in their activity levels results in free radical induced cellular damage. Other enzymatic antioxidants GPx

and GST and non-enzymatic GSH also help maintain healthy cell functions by scavenging free radicals like peroxy radicals, superoxide ions and singlet oxygen formed by toxicants (Rathore *et al*., 1998). AA is a water soluble vitamin that acts as an antioxidant and scavenger of superoxide and other free radicals, getting transformed in the process to dehydroascorbate (Frei *et al*., 1986; Packer *et al*., 1979). Vitamin E is a lipid soluble antioxidant that protects membrane polyunsaturated fatty acids and other components from oxidation by free radicals (Tappel, 1972). Presently, we have observed increased LPO and decreased endogenous antioxidants (both enzymatic and non-enzymatic) in IP treated rats. Apparently, IP causes heightened oxidative damage of cellular macromolecules marked by elevated level of LPO by way of increased generation of free radicals as has also been inferred by Gokkusu and Mostafazadeh (2003). However, pretreatment of IP animals with CS prevented the decrease in antioxidant levels and increase in LPO significantly in a dose dependent manner. Plant based extracts that are rich in polyphenols and flavonoids are supposedly strong antioxidants and CS seed extract has been reported to be rich in flavonoids, terpenoids (Wangensteen *et al*., 2004) and polyphenols (Hashim *et al*., 2005). The latter workers have opined that alcoholic extract of CS has maximal content of the said antioxidants compared to other types of extract. The currently observed effects of CS may be attribuTable to the presence of these secondary metabolites.

ATPases, by maintaining differential levels of ions play important roles in the regulation of contraction - relaxation cycles of cardiac muscles and consequently, peroxidation of sarcolemmal lipids can result in their inactivation as suggested by Kako *et*

al. (1988). Reduced activity of Na+/K+ ATPase with compromised Na+ efflux can result in altered membrane permeability (Finotti *et al*., 1986). A decrement in Ca2+ATPase expectantly would decrease sarcoplasmic Ca2+ concentration and weaken the contractility of heart. Hence, loss of ATPase activity in the ischemic state could contribute to myofibrillar necrosis and functional damage. Even Chernysheva *et al*. (1980) have reported IP induced decrement in the activity levels of Na+/K+, Mg2+ and Ca2+ ATPase in rats. However, IP+CS treated rats show a dose dependent significant up keep of these ATPases, essentially attribuTable to the membrane stabilizing aproperty of CS extract that protects the sarcolemma and intracellular membranes from the deleterious effect of IP and consequent myocardial damage (Hashim *et al*., 2005).

TTC is a redox indicator that is commonly used to differentiate between metabolically active and inactive cells and tissues (Altman, 1976). Staining of cardiac tissue slices with TTC provides insight regarding the infarct size and is a well accepted method to assess necrosis of myocardial tissue (Prabhu *et al*., 2006). TTC is enzymatically reduced to brick red precipitates of formazan dye or TPF (1,3,5-triphenylformazan). Active mitochondrial respiration generating reduced coenzymes is responsible for the reduction of TTC to TFP in all tissues including the cardiac tissue (Ramkissoon, 1966). Hence, appearance of patches of pale white color in cardiac tissue slices of IP treated rats indicates areas of focal necrosis due to non-reduction of TTC as observed in the present study in IP treated rats. The IP+CS rats (especially CS300) depicted minimal pale yellow patches suggestive of normal myocardial structure.

Histological observations further confirm the IP induced necrotic changes

affecting myofiber disruption and fraying of fibers. These deleterious changes seem ably resisted by pretreatment with CS with the highest dose affording maximal protection. These observations provide compelling macroscopic and microscopic evidences regarding the cardioprotective potential of CS seed extract. Parameters investigated here in indicate that hydro-methanolic extract of CS is potent in mitigating IP induced myocardial necrosis. The same is evidenced inform of CS induced favourable alterations in biochemical and histo-morphological parameters. Although the observed results have been attributed to high content of polyphenols in hydro-methanolic extract of CS. Our further studies are aimed that isolating the active component of CS and to reassess its cardioprotective potential in more appropriate experimental model (coronary ligation) and using gold standard marker enzyme such as cardiac Troponin I that underlying mechanism of CS induced cardioprotection.

It can be concluded from the present study that hydro-methanolic extract of CS seeds has cardioprotective potential. The same is attributable to high polyphenol content in CS seeds.

Summary

The preventive effect of *Coriandrum sativum* L. (CS) on cardiac damage was evaluated by Isoproterenol (IP) induced cardiotoxicity model in male *Wistar* rats. Rats were pretreated with methanolic extract of CS seeds at a dose of 100, 200 or 300 mg/kg orally for 30 days and they were subsequently administered (*s.c.*) with IP (85 mg/kg body weight) for the last two days. IP treated rats showed increased LPO, decreased levels of endogenous antioxidants and ATPases in the cardiac tissue together with increased plasma lipids and markers of cardiac damage. TTC staining showed increased infarct areas while HXE staining showed myofibrillar hypertrophy and disruption. CS (200 and 300 mg/kg body weight) pretreatment significantly prevented or resisted all these changes. Our results show that methanolic extract of CS is able to prevent myocardial infarction by inhibiting myofibrillar damage. It is also concluded that, the rich polyphenolic content of CS extract is responsible for preventing oxidative damage by effectively scavenging the IP generated ROS.

CHAPTER 4

Protective effect of *Corianandrum sativum* L. seed extract against H_2O_2 induced oxidative stress in rat cardiac H9C2 cells

INTRODUCTION

Reactive oxygen species (ROS) are natural byproducts of cellular aerobic metabolism and biological functions (Šimunek *et al*., 2005; Das and Maulik, 2003; Oldenburg *et al*., 2002; Tang *et al*., 2002). Overproduction of ROS creates an imbalance in the cellular redox potential that overwhelms anti-oxidant capacity and leads to oxidative stress (Halliwell and Gutteridge, 1999) culminating in lipid peroxidation, protein cross-linking and DNA cleavage (Wang *et al*., 1999). Oxidative stress is associated with pathophysiology of a number of diseases such as Parkinson's disease, Alzheimer's disease, atherosclerosis, myocardial ischemia, cardiac arrhythmias, congestive heart failure and cardiomyopathy (Bolli *et al*., 1995; Ferrari *et al*., 1998; Tsutsui, 2001; Minotti *et al*., 2004). Cardiomyocytes have higher levels of metabolic activity and an ischemic condition following reperfusion, greatly aggravates the generation of ROS leading to mitochondrial dysfunction and cell death (Zweier and Talukder, 2006). Heart is particularly more vulnerable to oxidative damage because the cardiomyocytes have been reported to possess a weak endogenous antioxidant defense system (Kehrer, 2000). Under such circumstances, an external supplementation of natural antioxidants plays an important role in imparting cardioprotection.

Previously, we had reported the *in vivo* cardioprotective potential of polyphenol rich fraction of CS in isoproterenol induced myocardial necrotic rats (Chapter 3; Patel *et al*., 2012). CS was able to prevent the myocardial injury induced by isoproterenol and the same was tentatively attributed to its antioxidant property. In continuation of our previous study, this inventory showcases the potential of CS in protecting oxidatively stressed rat cardiomyocytes in culture. The protocol investigates CS mediated mitigation of oxidative stress and prevention of death of rat cardiomyocytes (H9C2) cells.

MATERIALS AND METHODS

Chemicals

Dulbeco's Modified Eagle Medium (DMEM), Trypsin Phosphate Verses Glucose (TPVG) solution, antimycotic-antibiotic solution and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were purchased from HiMedia Laboratories Pvt. Ltd. (Bombay, India). Fetal bovine serum (FBS) was purchased from Biosera (Ringmer, East Sussex UK) and dimethyl sulfoxide (DMSO) was purchased from the Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Hydrogen peroxide (H_2O_2) was purchased from Merck and, 4',6-diamidino-2-phenylindole (DAPI), Propidium iodide (PI), Rhodamine 123 and 2',7' –dichlorofluoresceindiacetate (DCFDA) were purchased from Sigma (Delhi, India).

Plant material and preparation of extract

CS plants were collected in the seedling months (February and March) and Dr. P.S. Nagar, Department of Botany, The M.S. University of Baroda identified the plant and a sample specimen was deposited in the herbarium of the Department of Botany. Hundred grams of powdered dry seeds were soaked in methanol:water (80:20 v/v) at room temperature and was allowed to stand for seven days. Extract was filtered through a muslin cloth and was concentrated in a rotary evaporator under reduced pressure to obtain a thick semisolid brown paste (Hashim *et al*., 2005). The final yield obtained was 8.3 g (w/w).

Cell culture and treatment protocol

Rat cardiomyocytes were obtained from National Centre for Cell Sciences, Pune, India, seeded (1.0 $x10^5$ cells/ ml in T-25Flask) and cultured in DMEM containing 10% fetal bovine serum and 1% antimycotic-antibiotic solution at 37^oC with 5% CO_2 (Thermo Scientific, forma II water jacketed CO_2 incubator). Cells were sub-cultured every third day by trypsinization with 0.25% TPVG solution. All the agents were filtered through 0.22 mm filter (Laxbro Bio-Medical Aids Pvt. Ltd.) prior to use for the experiment. Confluent H9C2 cells were treated with 100 mM H_2O_2 in presence or absence of CS (10–125µg ml^{-1}) for 24 h and used for further analysis.

Cell viability assay by MTT

H9C2 cells (7.0 x 10^3cells/well) were maintained in 96 well cell culture plates (Tarson India Pvt. Ltd) for a period of 24 h as mentioned earlier. At the end of incubation period, 10 ml of MTT (5 mg ml^{-1}) was added to the wells and the plates were incubated for 4 h at 37 $^{\circ}$C. At the end of 4 h, the culture media was discarded and all the wells were washed with phosphate buffer saline. This was followed by addition of 100 µl of DMSO and incubated for 30 min. Absorbance was read at 540 nm in ELX800 Universal Microplate Reader (Bio-Tek instruments, Inc, Winooski, VT) and cell viability was calculated (Devkar *et al*., 2012).

Cellular integrity by lactate dehydrogenase(LDH) release assay

H9C2 cells (7.0 $x10^3$ cells per well) were maintained in 96 well cell culture plates for 24 h as mentioned above. Later, the supernatant from each well was collected and activity levels of LDH were assayed using commercially available kit (Reckon Diagnostics Pvt.

Ltd., Baroda, India). Readings were recorded on Merck micro lab L300 semi autoanalyzer.

Lipid peroxidation (LPO) assay

H9C2 cells (1.0 x 10^5 cells per well) were maintained in 6 well cell culture plates as described earlier for 24 h. Subsequently, cells were collected using TPVG solution from the plate (Tarson India Pvt. Ltd.) in 2 ml centrifuge tubes and levels of malonaldehydewere assayed in the cell suspension using thiobarbituric acid–trichloro acetic acid–hydrochloric acid reagent (Buege and Aust, 1978).

Intracellular reactive oxygen species (ROS) generation

H9C2 cells (1.0 x 10^5 cells per well) were grown on cover slips using 12 well cell culture plates for 24 h as mentioned earlier. At the end of incubation, cells in cover slips were incubated with 7.5 mM CM-H_2DCFDA (5-(and-6)-chloromethyl-20,70-dichlorodihydrofluoresceindiacetate, acetyl ester) at 37 ˚C for 30 min in the dark. Later, the cells were observed using a Leica DMRB florescence microscope (Jadeja *et al*., 2011).

Mitochondrial membrane potential (MMP)

Mitochondrial membrane potential was measured using the fluorescent cationic dye Rhodamine 123 (rho123) as previously described. H9C2 cells (1.0 x 10^5cells/well) were maintained in 6 well culture plates as described above for 24 h. The cells were then incubated with 1 mM rho123 for 10 min at 37 ˚C. The fluorescence was determined at excitation and emission wavelengths of 485 and 530 nm, respectively using spectrofluorometer (Jasco FP-6350) (Thounaojam *et al*., 2011).

Acridine orange/ethidium bromide staining for apoptosis

H9C2 cells (1.0 x 10^5 cells per well) were maintained in 6 well cell culture plates as described earlier for 24 h. At the end of the experimental period, cells were collected using TPVG solution. One microlitre of acridine orange (AO) and ethidium bromide (EB)dye mixture (1 mg $ml^{-}1$ AO and 1 mg $ml^{-}1$ EB in phosphate buffer saline) was mixed with 9 ml of cell suspension (5.0 x 10^5 $cellsml^{-}1$) on a clean microscope slide and immediately examined using Leica DMRB florescence microscope and photographed (Devkar *et al*., 2012).

PropidiumIodide (PI) staining

Propidium iodide (PI), a DNA intercalating fluorescent probe, was used to assess nuclear morphology for distinguishing patterns of cell death. H9C2 cells (1.0 x10^5 cells per well) were grown on glass cover slips and maintained in 6 well cell culture plates for 24 h as mentioned above. The media was removed and the cells were washed with ice cold PBS. Later, the cells were incubated with PI staining solution (5 µg/ml in 10 mmol/L PBS) for 3-5 minutes at room temperature in dark. Cover slips were removed placed on a clean glass slide and examined using Leica DMRB florescence microscope and photographed (Canon cybershot 72 digital camera) (Zamai L, *et al*., 1996).

Statistical analysis

Statistical evaluation of the data was done by one way ANOVA followed by Bonferroni's multiple comparison test. The results were expressed as mean±S.E.M using Graph Pad Prism version3.0 for Windows, Graph Pad Software, SanDiego California USA.

RESULTS

Cytotoxicity assay (Table 4.1 a and 4.b; Figure 4.1 a and 4.b)

Cytotoxicity assay of H9C2 cells with CS (10, 25, 50, 100, and 125 μg/mL) revealed a non-significant dose dependent decrement as compared to the control group. However, H_2O_2 treatment significantly reduced the cell viability to 30.23% as compared to the control group. However, presence of CS accounted for significant reduction in the impact of H_2O_2 treatment as the results showed a dose dependent improvement in cell viability with 125 μg/mL dose being more effective.

LDH release assay (Table 4.2; Figure 4.2a & 4.2b)

H9C2 cells recorded significant increment ($p<0.001$) in activity levels of LDH in the supernatant following H_2O_2 treatment. However, the impact of H_2O_2 was significantly reduced in presence of CS in dose dependent manner. The highest CS dose of 125µg/ml showed most significant decrement ($p<0.001$) LDH release as compared to cells treated with H_2O_2 alone.

Lipid peroxidation assay (Table 4.2; Figure 4.3)

H9C2 cells treated with H_2O_2 alone showed significant increment ($p<0.001$) in LPO levels as compared to untreated cells. However, H_2O_2+CS treated cells recorded dose dependent decrement in LPO levels and it was highest in cells treated with 125µg/ml of CS as compared to H_2O_2 alone treated cells ($p<0.001$).

Mitochondrial membrane potential (Table 4.2; Figure 4.4)

A loss in mitochondrial membrane potential ($p<0.001$) was recorded following treatment of H9C2 cells with H_2O_2. However, this decrement was significantly prevented in

presence of CS with the highest dose (125µg/ml) accounting for significant increment ($p<0.001$) compared to H_2O_2 treated cells. The same is also comparable to the control cells.

Intracellular reactive oxygen species (ROS) generation (Figure 4.6)

The fluorescence microscopy data showed that H_2O_2 alone treated cells enhanced the fluorescence intensity of DCF-DA, an indicator of increased $O_2^{\cdot -}$ and NO radicals, compared to the controls. The enhanced fluorescence intensity of DCF-DA was prevented by CS treatment, compared to the H_2O_2 alone treated cells.

Acridine orange/ethidium bromide (Figure 4.7)

Acridine orange/ethidium bromide (AO/EB) staining of H_2O_2 treated H9C2 cells showed higher number of EB positive (red) cells as compare to the control wherein, more number of AO positive (green) cells was observed. But, co-supplementation of CS recorded lesser number of EB positive and more AO positive cells.

PI staining for nuclear morphology (Figure 4.8)

H_2O_2 treated H9C2 cells showed nuclear condensation and fragmented nuclei, whereas H_2O_2+CS treatment resulted in less number of cells with condensed or fragmented nuclei as compared to H_2O_2 treated cells.

Table 4.1: Effect of CS on cell viability of H9C2. (a) The cell viability of H9C2 cells treated with various concentration of CS (10, 25, 50, 75, 100 and 125 μg/mL) for 24 h. (b) The cell viability of H9C2 cells treated with various concentration of CS (10, 25, 50, 75, 100 and 125 μg/mL) and 100 μM of H_2O_2 for 24 h.

4a

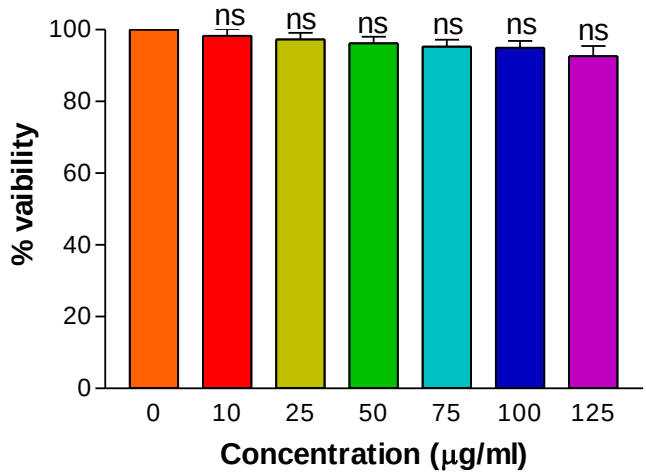
CS (Concentration in µg/ml)	**% cell viability**
0	100.0
10	98.25 ± 1.99^{NS}
25	97.25 ± 1.90^{NS}
50	96.23 ± 1.78^{NS}
75	95.33 ± 1.87^{NS}
100	94.94 ± 1.94^{NS}
125	92.65 ± 2.79^{NS}

4b

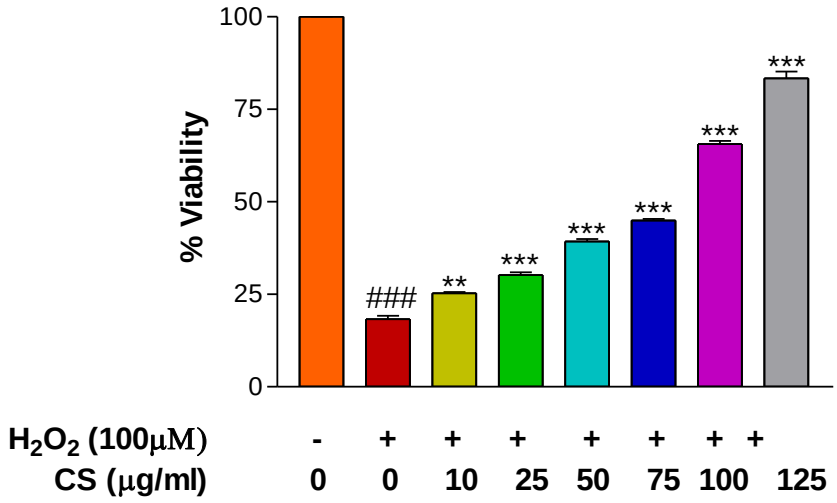
CS (Concentration in µg/ml) + H_20_2 (100µM)	**% cell viability**
0+0	100.0
0+100µM	$18.25\pm 0.93^{###}$
10+100µM	$25.25\pm 0.35^{**}$
25+100µM	$30.23\pm0.70^{***}$
50+100µM	$39.33\pm 0.62^{***}$
75+100µM	$44.94\pm 0.46^{***}$
100+100µM	$65.65\pm0.80^{***}$
125+100µM	$83.41\pm1.78^{***}$

Results are expressed as mean±S.E.M for n=3. Where, ns = non-significant, ### $p< 0.001$ compared to cells deprived of H_2O_2 and CS. **$p < 0.01$ and ***$p < 0.001$ compared to cells treated with H_2O_2 alone.

Figure 4.1**:** Effect of CS on cell viability of H9C2. (a) The cell viability of H9C2 cells treated with various concentration of CS (10, 25, 50, 75, 100 and 125 μg/mL) for 24 h. (b) The cell viability of H9C2 cells treated with various concentration of CS (10, 25, 50, 75, 100 and 125 μg/mL) and 100 μM of H_2O_2 for 24 h



4a



4b

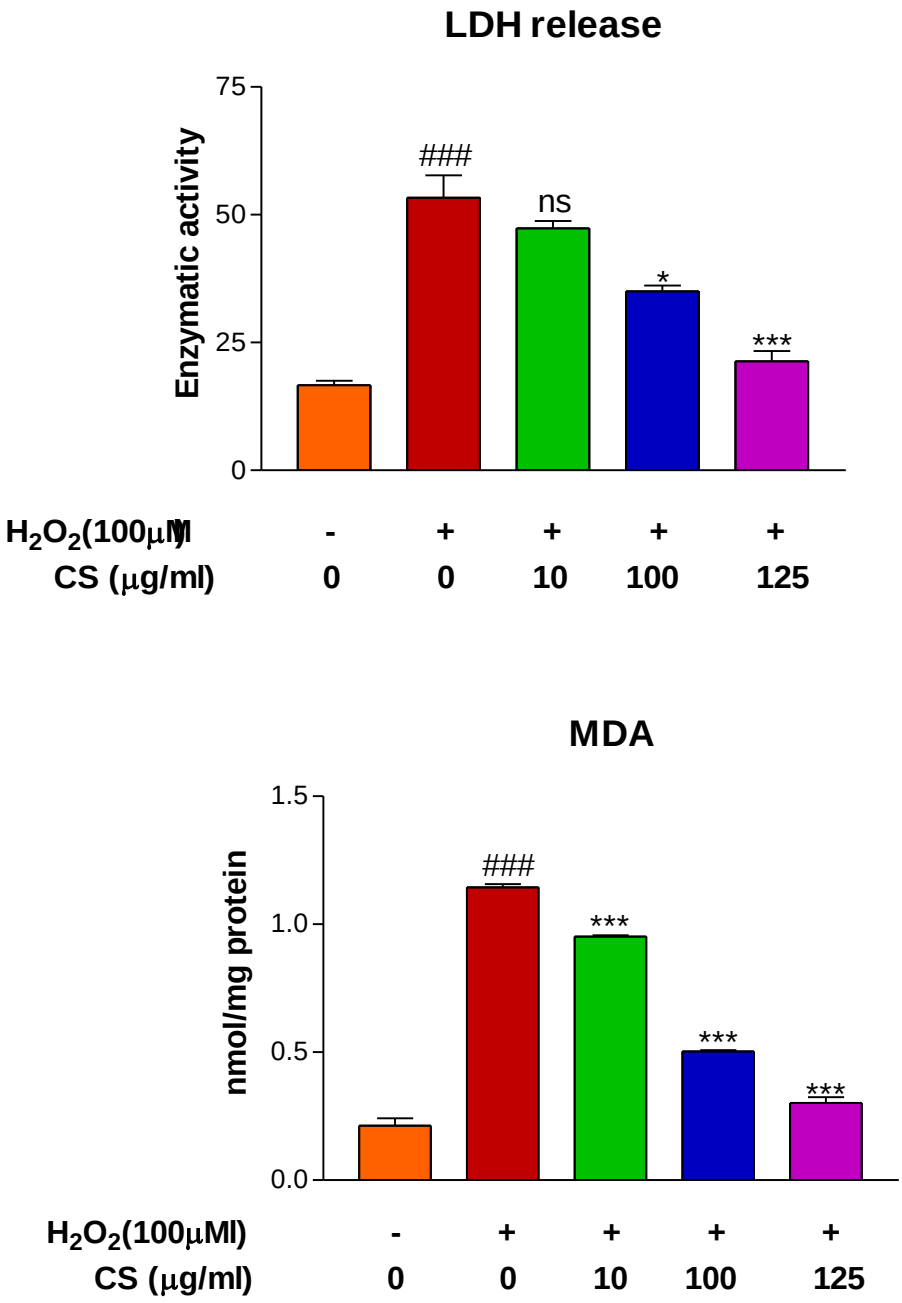
Results are expressed as mean±S.E.M for n=3. Where, ns = non-significant, ### p< 0.001 compared to cells deprived of H_2O_2 and CS. **p < 0.01 and ***p < 0.001 compared to cells treated with H_2O_2 alone.

Table 4.2: Effect of CS on LDH leakage, LPO and MMP of H9C2 cells treated with or without H_2O_2.

CS (µg/ml)+H_2O_2 (µM)	**LDH release (Enzyme activity)**	**LPO (nmols/mg protein)**	**MMP (Fluorescence intensity unit)**
0+0	16.67± 0.88	0.21±0.02	160.0± 2.08
0+ 100	53.33± 4.41$^{###}$	1.14±0.01$^{###}$	38.67± 2.60$^{###}$
10+ 100	47.33±1.45^{NS}	0.95±0.01***	65.33± 2.72**
100+ 100	35.0±1.15*	0.50±0.01***	95.33± 2.60***
125+ 100	21.33± 2.02***	0.30±0.02***	149.7± 3.18***

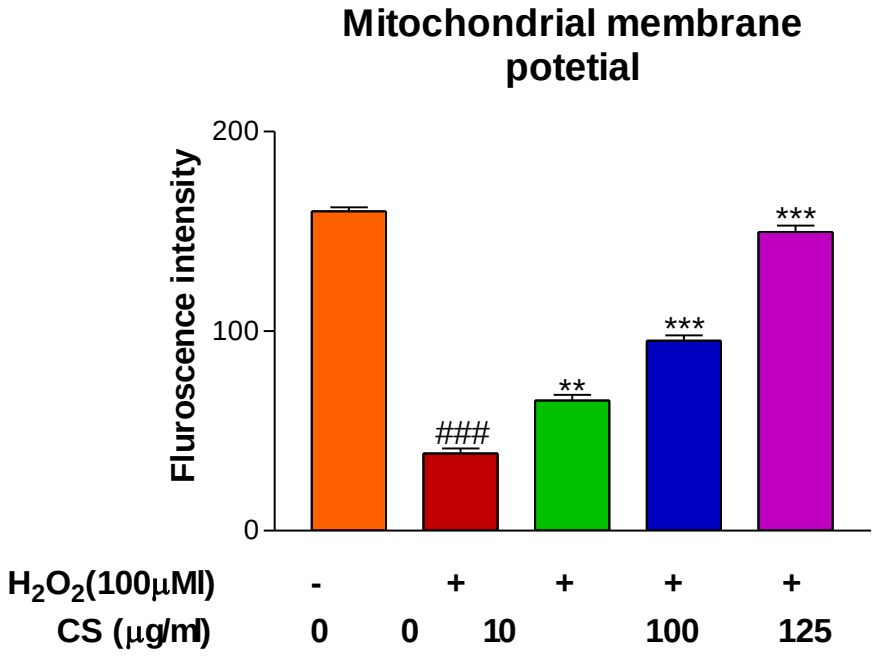
Results are expressed as mean±S.E.M for n=3. Where, ns = non-significant, ### $p< 0.001$ compared to cells deprived of H_2O_2 and CS. **$p < 0.01$ and ***$p < 0.001$ compared to cells treated with H_2O_2 alone.

Figure 4.2a: Effect of CS on LDH leakage, LPO and MMP of H9C2 cells treated with or without H_2O_2



Results are expressed as mean±S.E.M for n=3. Where, ns = non-significant, ### p< 0.001 compared to cells deprived of H_2O_2 and CS. **p < 0.01 and ***p < 0.001 compared to cells treated with H_2O_2 alone.

Figure 4.2b: Effect of CS on MMP of H9C2 cells treated with or without H_2O_2



Results are expressed as mean±S.E.M for n=3. Where, ns = non-significant, ### $p < 0.001$ compared to cells deprived of H_2O_2 and CS. **$p < 0.01$ and ***$p < 0.001$ compared to cells treated with H_2O_2 alone.

Figure 4.3: Phase contrast photomicrograph of H9C2 cells; untreated (NC), treated with 100 µM H_2O_2 (H_2O_2), treated with 100 µM H_2O_2 in presence of 125 µg/ml of CS (H_2O_2+CS)

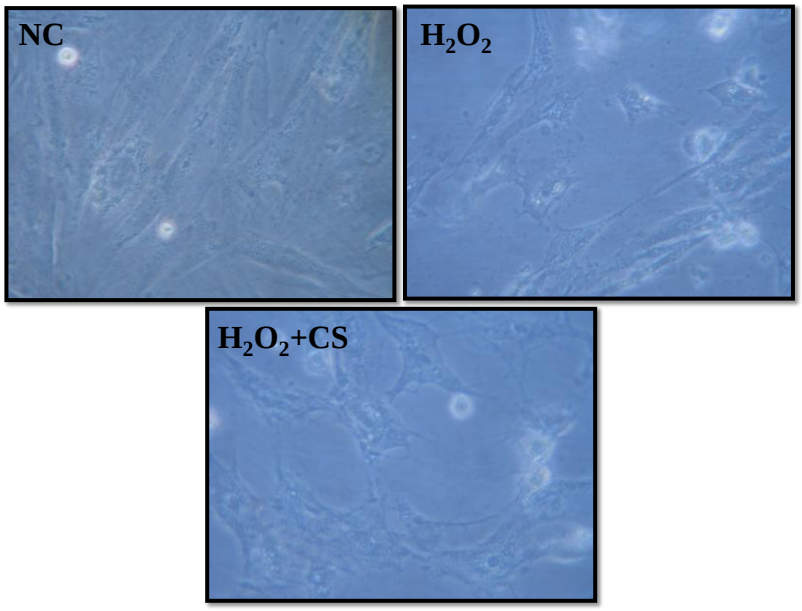


Figure 4.6: Photomicrographs of DCF-DA stained H9C2 cells; untreated (NC), treated with 100 µM H_2O_2 (H_2O_2), treated with 100 µM H_2O_2 in presence of 125 µg/ml of CS (H_2O_2+CS)

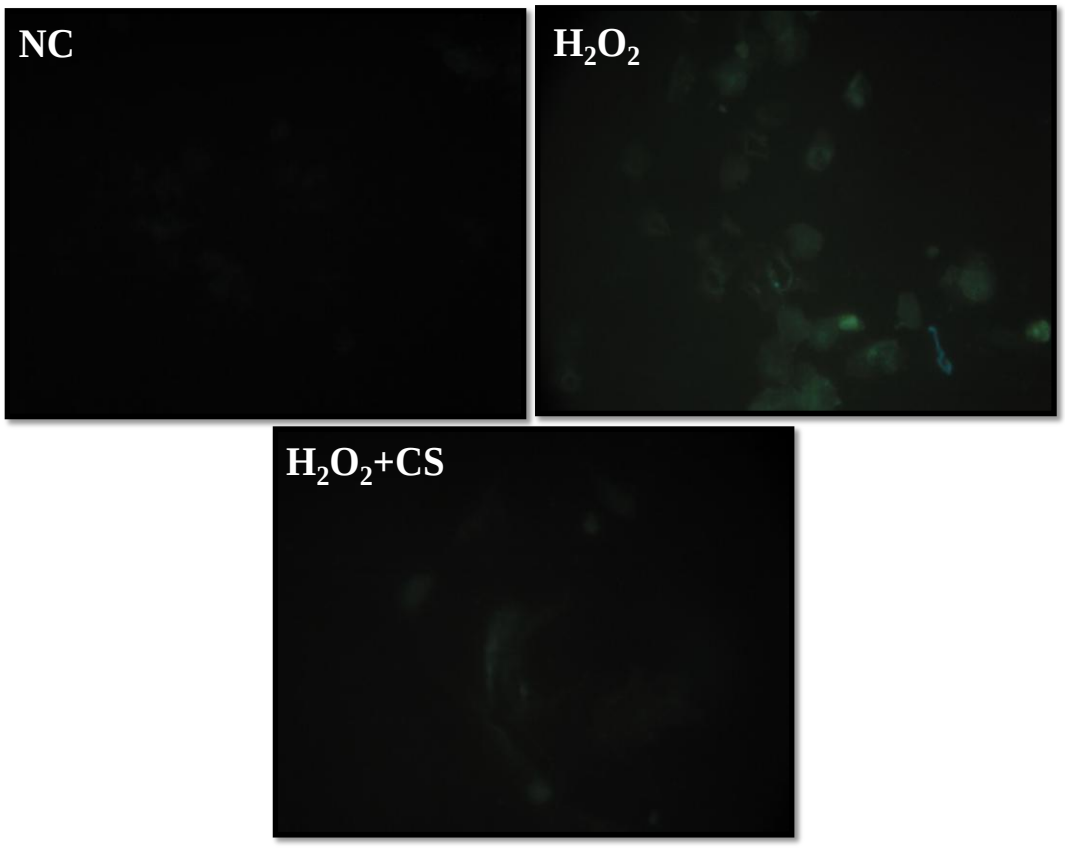


Figure 4.7: Photomicrographs of AO-EB stained H9C2 cells; untreated (NC), treated with 100 µM H_2O_2 (H_2O_2), treated with 100 µM H_2O_2 in presence of 125 µg/ml of CS (H_2O_2+CS)

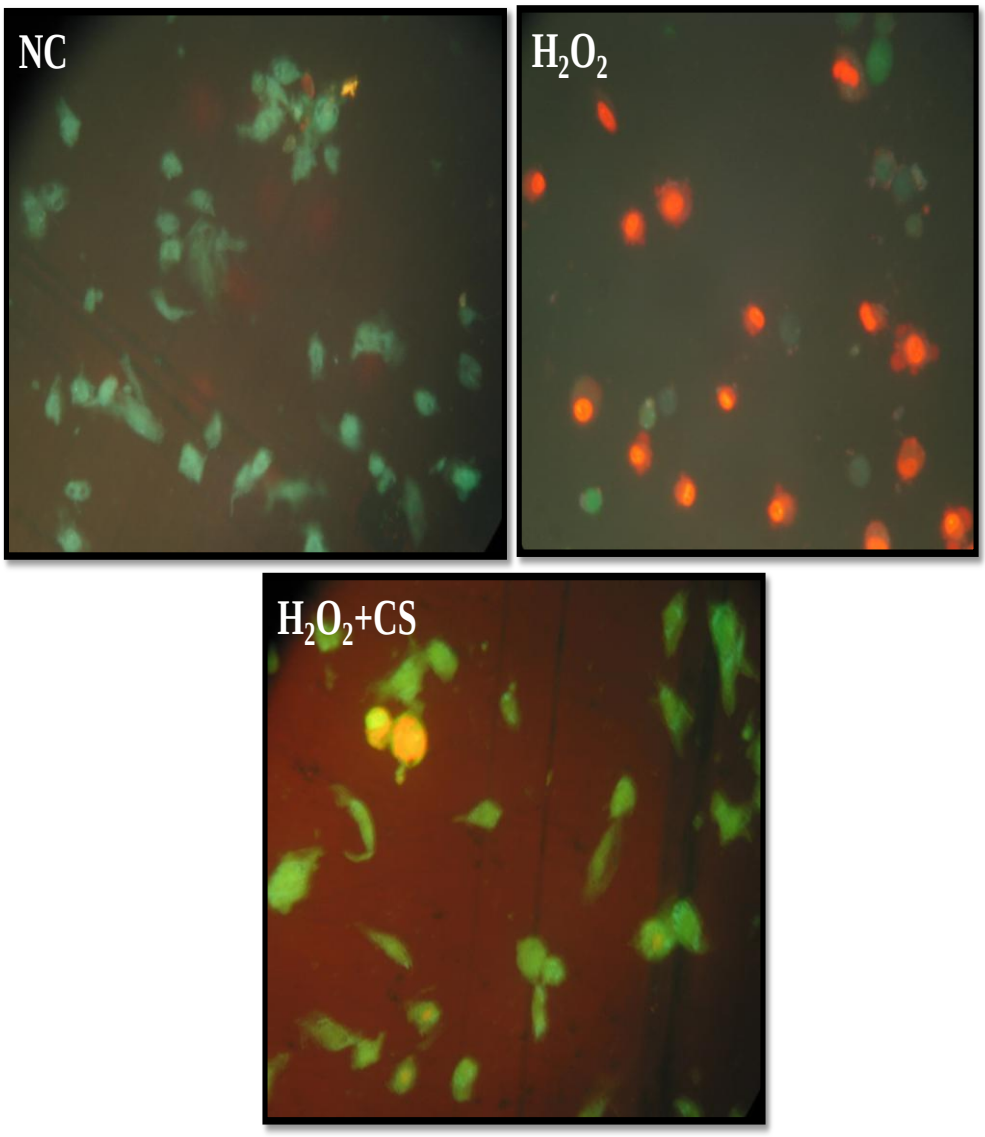
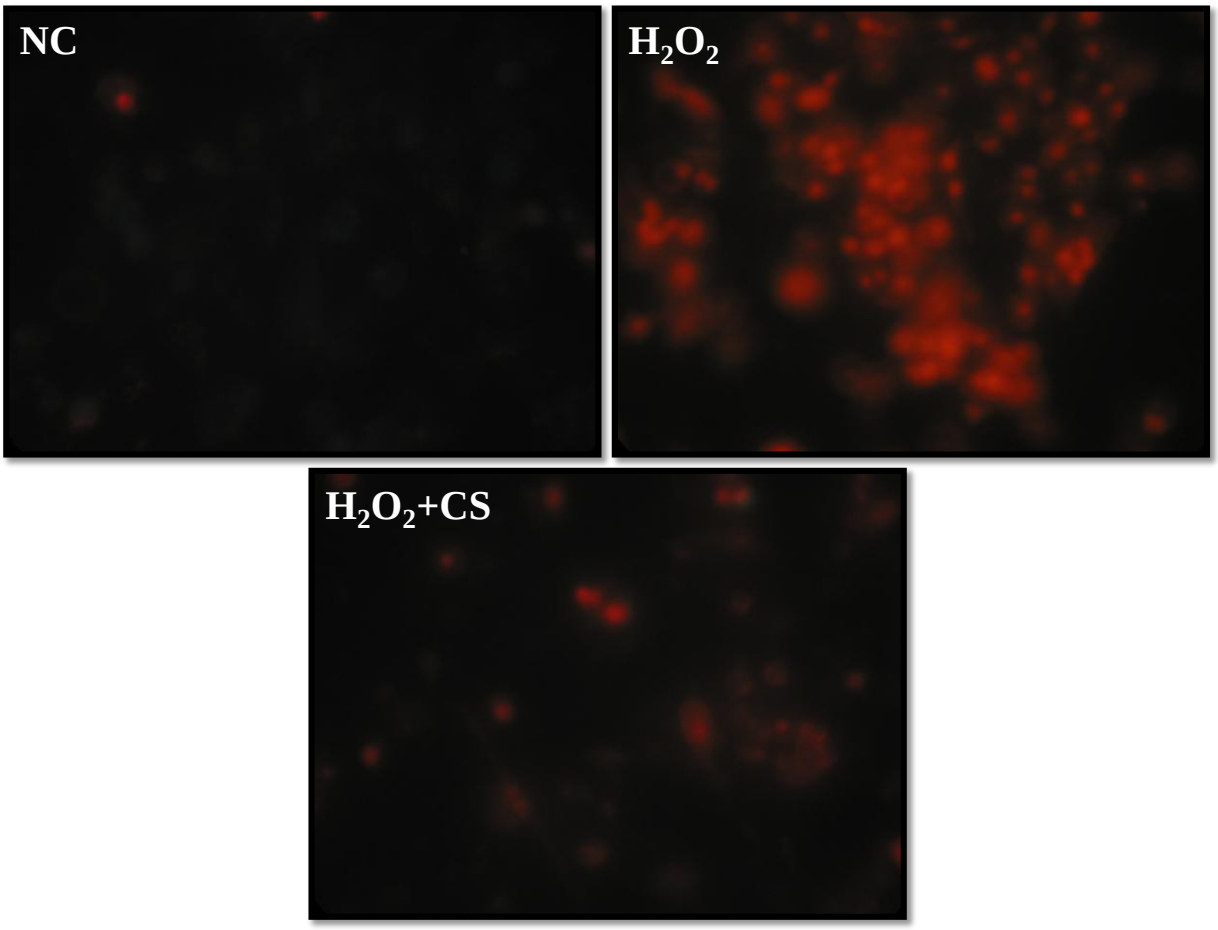


Figure 4.8: Photomicrographs of PI stained H9C2 cells; untreated (NC), treated with 100 μM H_2O_2 (H_2O_2), treated with 100 μM H_2O_2 in presence of 125 μg/ml of CS (H_2O_2+CS)



DISCUSSION

Acute myocardial ischemia is characterized by arrhythmias, transient mechanical dysfunction and death of cardiomyocytes and the same is attributed to oxygen (superoxide- $O_2^{-\bullet}$ and hydroxyl OH^{-}) radicals (Bolli, 1991). Copious amount of oxygen free radicals are generated during reperfusion or re-oxygenation of ischemic or hypoxic myocardium. In order to mimic this scenario of oxidative damage, H_2O_2 is used as a source of oxygen free radicals (Wu *et al*., 1996). Also, H_2O_2 induces remarkable increment in generation of intracellular ROS, coupled with compromised antioxidants, loss of mitochondrial membrane potential and apoptosis of cardiomyocytes (Kumar and Gupta, 2011). Myocardium comprises of heterogenous cell types such as cardiomyocytes, fibroblasts and endothelial cells that are supported by an extracellular matrix (ECM). Cardiomyocytes have a dominant presence and hence, are an important cell type that directly accounts for the overall performance of myocardium (Kumar and Gupta, 2011). Hence, H_2O_2 induced oxidative stress to rat cardiomyocytes (H9C2 cells) in culture is a popularly used experimental model for the assessment of cardioprotective potential of a test compound or herbal extract (Devkar *et al*., 2012).

Cardiomyocytes treated with H_2O_2 causes generation of intracellular ROS, especially $O_2^{-\bullet}$ that mimics the ischemia/reperfusion-induced *in vivo* injury in an infracted myocardium. Hydrogen peroxide produced due to the action of Superoxide dismutase (SOD) on $O_2^{-\bullet}$ further generates hydroxyl (OH^{-}) radicals in the presence of Fe^{2+} ions. In such cases, OH^{-} has been reported to have most damaging effects such as cytotoxicity and cardiac stunning, during ischemic reperfusion (Liochev, 1999; Reif, 1992; Chevion *et al*., 1993). These ROS ($O_2^{-\bullet}$ and OH^{-}) are able to oxidize biological macromolecules such

as DNA, proteins and lipids causing extensive intracellular damages (Zweier and Talukder, 2006).

The elevation of MDA is considered as important indicators of lipid peroxidation induced by H_2O_2 in cultured cells (Palmer *et al.*, 1988; Yang *et al.*, 2007). There was significant increment in LPO levels following H_2O_2 treatment indicating increased oxidative stress. However, H_2O_2+CS treated cells recorded lower indices of LPO that was comparable to that of control group. These observations suggest that presence of CS is able to prevent H_2O_2 induced oxidative damage. Further confirmation of intracellular oxidative stress was gauged with 2',7' –dichlorofluoresceindiacetate (DCFDA), a fluorogenic dye that measures intracellular hydroxyl, peroxyl and other reactive oxygen species (ROS). After diffusion in to a cell, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2', 7' – dichlorofluorescin (DCF) and gives green fluorescence (Royall and Ischiropoulo, 1993). In the present study, H_2O_2 treated cells showed significant increase in intracellular oxidative stress as evidenced by higher number of fluorescent cells as compared to the control. Whereas; H_2O_2+CS treatment significantly prevented generation of intracellular ROS as evidenced by less number/weak intensity of fluorescent positive cells.

Intracellular lactic acid concentration is indicative of loss of membrane integrity due to lipid peroxidation in a condition of heightened oxidative stress (Devkar *et al*., 2012). In the present study, LDH levels were significantly increased following H_2O_2 treatment whereas; CS co-supplementation significantly reduced LDH release which indicates less damage to the cell membrane. These observations also are in agreement

with results obtained in cytotoxicity assay wherein, H_2O_2+CS group had recorded improvement in cell viability following MTT assay.

A distinctive feature of the early stages of programmed cell death is the disruption of active mitochondria thus altering its membrane potential. These changes are presumed to be due to the opening of the mitochondrial permeability transition pore (MPTP), allowing passage of ions and small molecules. The resulting equilibration of ions, in turn leads to the decoupling of the respiratory chain and the release of cytochrome c into the cytoplasm thus causing activation of apoptosome and triggering apoptosis (Wigdal *et al*., 2002; Kroemer *et al*., 2007). These set of changes can be surmised by using a dye called as RHO 123 that accumulates in mitochondria and produces fluorescence. Its fluorescent intensity is directly proportional to the mitochondrial membrane potential of a cell and hence, RHO 123 is popularly used to assess the energy state of functional mitochondria (Royall *et al*., 1994). In the present study, mitochondrial membrane potential was significantly reduced in H_2O_2 treated cells whereas; CS co-supplementation was able to prevent loss of mitochondrial membrane potential. These results are indicative of restoration of mitochondrial function in presence of CS.

Dual staining method of acridine Orange (live cells-green) and ethidium Bromide (dead cells-red-to-orange) enables rapid and easy recognition/differentiation of live-dead cells under a fluorescence microscope (Spector *et al*., 1998) In the present study, higher number red to oranges cells were observed following H_2O_2 treatment whereas, H_2O_2+CS treatment accounted for more number of green cells. These observations clearly suggest that CS accounts for an improved cell viability.

It has been reported that cells undergoing apoptosis exhibit cytoplasmic blebbing, nuclear shrinkage, chromatin condensation, irregularity in shape and fragmentation of nuclei (Kerr *et al*., 1972; Choi *et al*., 2002). Since, PI is not taken up by viable cells and hence, is commonly used for identifying dead cells (Moore *et al*., 1998). In the present study, PI staining of control cells remained unstained while H_2O_2 alone treated cell showing nuclear condensation and fragmentation. CS treated group showed less number of nuclear condensation and fragmented nuclei as compared to H_2O_2 alone treated cells.

SUMMARY

Oxidative stress is a common denominator in many aspects of cardiovascular pathogenesis. The present study assesses the cardioprotective potential of polyphenol rich methanolic extract of *Coriandrum sativum* L. (CS) against hydrogen peroxide (H_2O_2) induced oxidative stress in H9C2 cells. CS co-supplementation has reduced H_2O_2 (100 μmol/l) mediated LDH release, lipid peroxidation levels and also prevented in decrement of mitochondrial membrane potential. It has prevented intracellular ROS generation, chromatin condensation and cell death. These results are indicative of cardioprotective efficacy of CS due the presence of polyphenols. Polyphenols are strong antioxidants and they might have quench free radicals generated by H_2O_2 and reduce oxidative stress mediated cell death.

CHAPTER 5

Coriandrum sativum L. seed extract mitigates lipotoxicity in RAW 264.7 cells and prevents atherogenic changes in rats

INTRODUCTION

Hyperlipidemia is a major cause of cardiovascular disorders such as atherosclerosis and coronary heart disease (Chobanian, 1991). High levels of circulating cholesterol have been identified as a potential risk factor for atherosclerosis and coronary heart disease (Kannele *et al*., 1979). World Health Organization has predicted that, almost 23.6 million people will die due to cardiovascular diseases (CVD) by 2030 (Halliwell, 1995). Hence, hypercholesterolemia characterized by elevated plasma low-density lipoprotein cholesterol (LDL) levels is an important risk factor for the development and progression of atherosclerosis (Kannel *et al*., 1975; Keys, 1970) wherein, oxidative modification of LDL plays a pivotal role (Heinecke, 1997). Formation of Ox-LDL is triggered by transition metals, plasma enzymes, vascular endothelium or smooth muscle cells (Sparrow and Olszewski, 1993). It has been reported that the oxidatively modified LDL is cytotoxic, chemotactic and chemostatic (Napoli *et al*., 1997; Steinberg, 1997). LDL particles (<70nm) are more prone to enter the tunica intima of thoracic aorta and undergo cell mediated oxidation (Simionescu and Simionescu, 1993). This process is coupled with expression of cell adhesion molecules on vascular endothelium to trigger recruitment of monocyte that soon differentiate into macrophages (Glass and Witztum, 2001). At this state, the macrophages exhibit scavenger receptor mediated Ox-LDL uptake and subsequently get transformed into lipid laden foam cells. These cells soon undergo

apoptosis to form apoptotic bodies that initiate formation of fatty streak. As a result, more macrophages are recruited and undergo similar fate and contribute towards formation of an atherosclerotic lesion (Bjorkerud and Bjorkerud, 1996; Harada-Shiba *et al*., 1998). Since, the sequence of events is irreversible and hence, reduction in the level of triglyceride, cholesterol and LDL by naturally occurring compounds is often considered as an effective alternative in alleviating development of an atherosclerostic lesion (Novo *et al*., 2011).

Hashim *et al* (2005) reported that polyphenol rich fractions of CS possess strong anti-oxidant potential and an ability to alleviate H_2O_2 mediated oxidative damage in human lymphocytes. Also, CS administration resulted in preventing hyperlipidemia in high fat diet fed rats (Chithra and Leelamma, 1997). Owing to these credentials of CS seed extract, the present study was designed to assess its efficacy in preventing *in vitro* LDL oxidation mediated macrophage modification. Also, the *in vivo* study is aimed at further confirmation of the efficacy of CS seed extract in alleviating pathophysiological alterations in high fat diet fed atherogenic rats.

MATERIALS AND METHODS

Plant material and preparation of extract

Whole plant of CS was collected during its seedling months (February and March) and was identified by Dr. P.S. Nagar, Department of Botany, The M.S. University of Baroda. A sample specimen was deposited in the herbarium of the Department of Botany. Hundred grams of powdered dried seeds were soaked in methanol:water (80:20 v/v) at room temperature and allowed to stand for seven days. The resultant extract was filtered through a muslin cloth and concentrated in a rotary evaporator under reduced pressure to obtain a thick semisolid brown coloured paste (Hashim *et al*., 2005). The final yield obtained was 8.3 g (w/w).

Isolation of LDL

Venous blood was collected by a pathologist from fasting healthy volunteers with normal levels of cholesterol as per the standard guidelines. These samples were kept at room temperature for 45 min and serum was obtained by centrifuging at 3000 rpm for 10 min at 4°C. LDL was isolated using heparin–citrate buffer (64 mM trisodium citrate at pH 5.05 containing 50,000 IU/l heparin) precipitation method (Ahotupa *et al*., 1998). A mixture of 0.1 ml of serum and 1 ml of the heparin–citrate buffer was vortexed and allowed to stand for 10 min at room temperature and later centrifuged (3,000 rpm for 10 min at 20°C) to remove insoluble lipoproteins. The resultant pellet was suspended in 0.1 ml of phosphate-buffered saline (PBS; 0.1 M, pH 7.4, containing 0.9% NaCl). Protein concentration of the

LDL was estimated by the method of Lowry *et al*. (1951) using bovine serum albumin as standard

LDL Oxidation Kinetics

0.1 ml of LDL (100 µg protein) was diluted to 0.9 ml with PBS and was incubated in presence or absence of 0.1 ml of CS extract (10–100 µg/ml) at 37°C for 30 min. Later, freshly prepared 0.167 mM $CuSO_4$ solution was added to initiate LDL oxidation. The LDL oxidation kinetics was determined by continuously monitoring (every 10 min) the absorbance for 180 min (at 37°C) at 234 nm in a UV/VIS Perkin Elmer spectrophotometer. Lag time (min) was determined from the intercepts of lines drawn through the linear portions of the lag phase and propagation phase. The rate of oxidation was determined from the slope of the propagation phase. The concentration of CD in the samples was calculated by using a molar extinction coefficient of 2.95 9 104 $M^{-1}cm^{-1}$. Maximum concentration of CD formed was calculated from the difference in the concentration of CD at zero time and at diene peak (absorption maxima) (Esterbauer *et al*., 1989).

LDL oxidation products

Three sets of tubes were prepared to assess the rate of LDL oxidation in presence or absence of CS extract. Copper-mediated LDL oxidation was carried out in presence or absence of CS extract (10–100µg/ml) for 24 h as mentioned above. Later, 0.01 ml of 10 mM EDTA was added in each tube to stop oxidation reaction and each samples were processed for measurement of malondialdehyde (MDA), lipid hydroperoxide (LHP) and protein carbonyl (PC) as follows:

Malondialdehyde: 0.1 ml of aliquot was mixed with 1 ml TBA reagent (0.37% TBA, 15% TCA in 0.25N HCl) and placed in water bath at 100°C for 30 min, cooled to room temperature and centrifuged at 3000 rpm for10 min. The absorbance of the supernatant was measured at 532 nm with UV–VIS Perkin Elmer spectrophotometer and MDA was calculated using a molar extinction coefficient of 1.56 x 10^5 $M^{-1}cm^{-1}$ (Buege and Aust, 1978).

Lipid hydroperoxide: 0.1 ml of aliquot was mixed with 0.9 ml of Fox reagent (0.25 mM ammonium sulphate, 0.1 mM xylenol orange, 25 mM H_2SO_4, and 4 mM BHT in 90% (v/v) HPLC-grade methanol) and incubated at 37°C for 30 min. The absorbance was read at 560 nm and LHP content was determined using the molar extinction coefficient of 4.3 x 10^4 M^{-1} cm^{-1} (Nourooz-Zadeh *et al*., 1996).

Protein carbonyls: 0.1 ml of aliquot was mixed with 0.2 ml of DNPH (in 2 M HCl). After incubation at room temperature for 60 min, 0.6 ml of denaturing buffer (150 mM sodium phosphate buffer containing 3% SDS) was added and mixed thoroughly. Later, ethanol and heptane (1.8 ml of each) to precipitate protein and the contents were centrifuged. The protein was washed three times with 1.5 ml of ethyl acetate/ethanol (1:1, v/v), dissolved in 1 ml of denaturing buffer, and read at 360 nm in a spectrophotometer. The carbonyl content was calculated using molar extinction absorption coefficient of 22.000 $M^{-1}cm^{-1}$ (Reznick and Packer, 1994).

Relative Electrophoretic Mobility (REM)

LDL was subjected to Cu^{2+}-mediated oxidation in presence or absence of CS extract (10–100µg/ml) for 24 h as mentioned above. Later, 0.01 ml of 10 mM EDTA was added in each tube to stop oxidation and the contents were centrifuged to obtain a pellet. The

electrophoretic mobility of native or oxidized LDL (with or without CS extract) was detected using agarose gel electrophoresis (Reid and Mitchinson, 1993). Samples were loaded on 0.6% agarose gel and electrophoresed (100 V) in 50 mM barbituric acid (pH 8.6) for 40 min. After electrophoresis, the gels were fixed in a solution containing 60% methanol, 30% water and 10% glacial acetic acid for 30 min, dried at 50°C for 40 min and stained with 0.6% Sudan black B for 60 min. Gels were photographed and the results were expressed in terms of distance (meter) travelled by LDL from origin.

Apolipoprotein B100 (ApoB) Fragmentation

Copper-mediated LDL oxidation was carried out in presence or absence of CS extract (10–100µg/ml) for 24 h as mentioned above. Later, 0.01 ml of 10 mM EDTA was added in each tube to stop oxidation. Samples were centrifuged and LDL obtained was denatured with 3% SDS, 10% glycerol, and 5% bromophenol at 95°C for 5 min and cooled to room temperature. Later, LDL samples were loaded on 8% SDS-polyacrylamide gels, and electrophoresis was performed at 100 V for 60 min. The gels were stained with 2% coomassie brilliant blue solution for 6 h at 4°C and de-stained (20% glacial acetic acid and 10% methanol in water) for 30 min. Later, gels were cleared in 15 and 10% acetic acid for 10 min each, fixed in 10% glycerol and photographed using canon power shot S70 digital camera (Lee *et al*., 2002).

Preparation of oxidized LDL and culture of RAW 247.6 cells

0.1 ml of LDL (100 µg protein) was diluted to 0.9 ml with PBS and incubated for 24 h at 37°C. LDL was oxidized with 0.01 ml freshly prepared CuSO4 (0.167 mM). Analysis of MDA and CD were carried out in the LDL samples. Samples with MDA 50 ± 5 nmol/mg

LDL protein and CD 80 ± 8 nmol/mg LDL protein were used for further studies. RAW 247.6 cells (Macrophages) were purchased from National Centre of Cell Sciences, Pune, India. Cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotic–antimycotic solution in a humidified incubator at 37°C with 5% CO_2.

Cell mediated LDL Oxidation

RAW 247.6 cells (1 x 10^5/ml) were incubated in 1 ml of Ham's F-12 medium (without phenol red) containing LDL (100 μg/ml) at 37°C for 24 h. Cell-free control well was used for all conditions. At the end of incubation, oxidation was arrested by chilling the medium and adding 0.2 mM EDTA and 0.04 mM BHT. Later, 0.1 ml of each supernatant was used for the assay of MDA described earlier (Thounaojam *et al*., 2011).

Ox-LDL induced foam cell formation

RAW 247.6 cells treated with CS extract (100 μg/ ml) were incubated in presence of 100 μg/ ml of Ox-LDL for 24 h. Later, medium was decanted and cells fixed in 4% paraformaldehyde for 15 min. The cells were then washed twice with PBS, and stained in 1% Oil red O solution for 30 min. At the end of staining, excess Oil red O was removed and 1 ml of glycerin added. Photographs were taken on Leica DMIL inverted microscope using canon power shot S 70 digital camera (Jadeja *et al*., 2011).

Intracellular oxidative stress

Macrophage cells (1 x 10^5/ml) treated with CS (100 μg/ ml) were incubated in presence of 100 μg/ ml of Ox-LDL for 24 h. Later, the cells were further incubated for 60 min at 37°C and incubated with 0.0075 mM 2',7'-dichlorfluorescein-diacetate (DCF-DA) for 30 min in

dark (Silva *et al*., 2010). Photographs were taken using canon power shot S70 digital camera in Leica DMRB florescence microscope.

Mitochondrial Membrane Potential assay

Mitochondrial membrane potential in control and CS treated cells was measured using a fluorescent cationic dye Rhodamine123 (RHO123) (Pereira and Oliveira (Pereira and Oliveira, 2000). RAW 247.6 cells (1 x 10^5/ml) pretreated with CS extract (10–100 μg/ml) for 30 min were incubated in presence of 100 μg/ml of Ox-LDL for 24 h. The cells were then incubated with 0.001 mM RHO123 for 10 min at 37°C and the fluorescence was determined (485 and 530 nm excitation and emission respectively) using spectroflurometer (Jasco FP-6350).

Cytotoxicity assay

RAW 247.6 cells (1 x 10^4) pre-treated with CS extract (10–100 μg/ml) for 30 min were incubated in presence of 100 μg/ml of Ox-LDL for 24 h. Further incubation of the cells was carried out in a culture medium containing 0.5 mg/ml3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) for 160 min. Later, 0.15 ml of dimethyl sulphoxide was added to all the wells and was incubated for 30 min at room temperature with constant shaking. Absorbance was read at 540 nm using ELX800 Universal Microplate Reader (Bio-Tek instruments, Inc, Winooski, VT) and % cell viability was calculated.

Nuclear condensation study

Control or pre-treated (CS extract 100 μg/ml for 30 min) RAW 247.6 cells (1 x 10^5/ml) were incubated with Ox-LDL (100 μg/ml) of for 24 h. A Single-cell suspension of treated cells was washed in PBS, fixed in 70% ethanol. Cells were washed again with PBS and

incubated with DAPI stain (0.6 μg/ml in PBS) for 5 min. Chromatin fluorescence was observed under a Leica DMRB 2000 florescence microscope. Apoptotic cells were morphologically defined by cytoplasmic and nuclear shrinkage and chromatin condensation (Hsieh *et al*., 2007).

Cell Cycle analysis

RAW 247.6 cells (1 x 10^6/ml) were cultured in presence of CS extract (100 μg/ml) and 100 μg/ml of Ox-LDL for 24 h. At the end of incubation, cells were collected, washed twice with PBS, fixed overnight in cold 70% ethanol at 4°C and re-suspended in PBS. Cells were incubated with RNase A for 45 min and stained with propidium iodide (1 mg/ml) in the dark at 37°C for 30 min (Pozarowski and Darzynkiewicz, 2004). The suspension was analyzed with on Flow Cytometer (BD FACSAria III, USA). The apoptosis was determined based on the ‘‘sub-G1’’ peak.

***In vivo* studies**

Experimental animals

Age matched (9–10 weeks old) male *Sprague dawley* rats weighing 300 ± 20gms (Zydus research centre, Ahmedabad) were maintained in clean polypropylene cages and fed with laboratory chow (M/S Pranav agro, Ltd. Baroda, India) and water *ad libitum*. The experimental protocol was executed according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and approved by the animal ethical committee of the Department of Zoology, The M.S. University of Baroda, Vadodara (Approval No.827/ ac/04/CPCSEA).

Induction of atherosclerosis

In the present study, a total of 24 rats were divided into three groups of eight animals each. Group I: CON; rats were given single dose of 0.9% saline (0.1 ml) intraperitoneally and maintained on standard laboratory chow and simultaneously administered with 0.5% CMC (0.1 ml) orally for 8 weeks. Group II: ATH; rats were given single dose of Vitamin D3 (600,000 unit/kg) intraperitoneally and later, fed with an atherosclerotic diet (3% cholesterol, 0.5% cholic acid, 0.2% 6-propyl 2-thiouracil, 5% sucrose, 10% lard, and 81.3% powdered laboratory chow) (23–25) and simultaneously administered with 0.5% CMC (0.1 ml) orally for 8 weeks. Group III: ATH+CS; rats were given single dose of Vitamin D3 (600,000 unit/kg) intraperitoneally and later fed with an atherosclerotic diet and simultaneously administered with 200 mg/kg of CS extract by oral feeding for 8 weeks (Cai *et al.,* 2005; Huang *et al*., 2004). At the end of the experimental period, blood was collected from overnight fasted rats (12 h) via retro-orbital sinus puncture under mild ether anaesthesia in 2 ml centrifuge tubes and serum was separated by cold centrifugation (4°C) at 1500 rpm for 10 min. Later, animals were sacrificed by cervical dislocation under mild ether anaesthesia and thoracic aorta of control and experimental animals were collected. Two small pieces of thoracic aorta from aortic arch were collected and processed for paraffin wax histology. The remaining piece of thoracic aorta was stored at –80°C (Cryo Scientific Ltd., India) for further use.

Serum lipids

Serum triglyceride (TG), total cholesterol (TC) and high density lipoprotein (HDL) contents were estimated withcommercially available enzymatic kits (Reckon Diagnostics

Ltd., Baroda, India) using a semi auto-analyser (Micro lab 300 L, Merck) and levels of LDL and very LDL (VLDL) were calculated(Friedewald *et al*., 1972).

Isolation of LDL from rats and MDA assay

LDL was isolated from serum samples of control and experimental rats by heparin-citrate buffer precipitation method as described earlier (Ahotupa *et al*., 1998). The Protein concentration of LDL was estimated by the method of Lowry *et al*. (1951) using BSA as standard. Oxidation state was evaluated by assaying malonaldehyde (MDA) levels in the LDL samples of control and experimental groups as mentioned above and the absorbance was measured at 532 nm with UV/VIS Perkin Elmer spectrophotometerand, MDA was calculated using a molar absorption coefficient of 1.56×10^5 $M^{-1}cm^{-1}$ (Buege and Aust, 1978).

Microscopic evaluation of thoracic aorta

Thoracic aorta of control and experimental rats were fixed in 4% buffered paraformaldehyde, dehydrated in graded alcohol series and embedded in paraffin wax using automated tissue processor. Five to seven μm sections were cut (on a Leica RM 2155 Microtome), stained with haematoxylin and eosin (H&E). Another set of sections of aorta from control and experimental rats was incubated in Von kossa stain solution (1% silver nitrate) under ultraviolet light for 20 min. The sections were then rinsed repeatedly in distilled water, placed in 5% sodium thiosulphate for 5 min and rinsed in distilled water again (to remove un-reacted silver). The sections were then counterstained with 1% eosin for 5 min (Hsieh *et al*., 2007). All sections were examined under a Leica DMRB microscope (100 X) and photographed with a canon power shot S70 digital camera at 100 X magnification.

Immunohistochemistry of thoracic aorta

Paraffin embedded sections of thoracic aorta of control and experimental rats were deparaffinised in xylene and hydrated using graded series of alcohol and water. Sections were then washed in phosphate buffer saline (PBS) and antigen retrieval step was carried out by immersing slides in sodium citrate buffer at 80°C for 10 min. Later, endogenous peroxidases were removed by incubation of sections in 3% H_2O_2 for 20 min in dark. Non-specific binding sites were blocked by incubation of slides with 1% fetal bovine serum (FBS) for 30 min. Localization of vascular cell adhesion molecule-1 (VCAM-1), and P-selectin was carried out by incubating sections with rabbit anti-rat IgG at a dilution of 1:100 (SantaCruz Biotechnology, Inc.) and goat anti-rat P-selectin IgG at a dilution of 1:100 (Santa Cruz Biotechnology,Inc.), respectively for overnight at 4°C in a humidified chamber. At the end of incubation, slides were washed with PBS and then, the sections were incubated with respective horseradish peroxidise (HRP) conjugated secondary antibodies for 4 h at room temperature. Goat anti-rabbit IgG-HRP 1:100 (Bangalore Genei Pvt Ltd.) for VCAM-1, and rabbit anti-goat IgG-HRP 1:100 (Bangalore Genei Pvt Ltd.) for P-selectin were used. At the end of incubation, sections were thoroughly washed with PBS and final detection step was carried out using diaminobenzydine (DAB) detection system (Bangalore Genei Pvt Ltd.) and counterstained with haematoxylin. Sections were examined under Leica DMRB microscope and photographed using a canon Power shot S70 digital camera (Thounaojam *et al*., 2012).

Statistical Analysis

Statistical evaluation of the data was done by one-way ANOVA followed by Bonferroni's multiple comparison tests. The results were expressed as mean ± S.E.M using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego California USA.

RESULTS

LDL oxidation kinetics (Figure 5.1)

LDL subjected to treatment with 10μM $CuSO_4$ showed significantly ($p<0.001$) increased CD formation and reduced lag time. In contrast, CS (25, 50, 75, and 100 μg/ml) was able to reduce CD formation and delay lag time in a dose-dependent manner.

LDL oxidation products (MDA, LHP, and PC) (Figure 5.2)

There was significant ($p<0.001$) elevation in formation of MDA, LHP and PC after treatment of LDL with 10 μM $CuSO_4$ as compared to the control group. However, presence of CS (25, 50, 75, and 100 μg/ml) accounted for a dose dependent reduction in formation of LDL oxidation products.

Relative Electrophoretic Mobility (REM) and ApoB fragmentation (Figure 5.3a & 5.3b)

The degree of $CuSO_4$ mediated LDL oxidation was assessed by the extent of altered REM and ApoB fragmentation. There was significant increment in electrophoretic mobility in Ox-LDL whereas; the same showed a dose dependent reduction following CS treatment. Also, the OX-LDL showed fragmentation of ApoB and the same was evidenced by complete absence of the band in electrophoretic pattern observed herein. However, CS (10, 25, 50, 75, and100 μM) treatment accounted for reappearance of the ApoB band in a dose dependent manner.

Cell mediated LDL Oxidation (Figure 5.4)

Oxidation of LDL mediated by RAW 247.6 cells recorded significantly ($p<0.001$) increased MDA levels as compared to the control group whereas, presence of CS (10, 25,

50, 75, and100 μM) accounted for a decrement in MDA levels with highest doses being most significant.

Foam cells (Figure 5.5)

Incubation of RAW 247.6 cells with Ox-LDL (100 μg/ml) for 24 h resulted in significant uptake of Ox-LDL, leading to higher intracellular cholesterol accumulation compared to Ox-LDL deprived cells. Addition of CS extract to Ox-LDL treated macrophages significantly reduced intracellular cholesterol accumulation.

Intracellular oxidative stress (Figure 5.6)

As shown in Figure 5.6 Ox-LDL (100 μg/mL) treated RAW 247.6 cells showed a visible increment in the intensity of green color. The same is indicative of high content of ROS generated and elevated levels of intracellular oxidative stress. However, presence of CS (100 μg/mL) was instrumental in recording observable decrement in the intensity of green color within the cytoplasm of cells.

Mitochondrial membrane potential and cytotoxicity assay (Figure 5.7 & 5.8)

RAW 247.6 cells treated with OX-LDL (100 μg/mL) showed a significant decrement ($p<0.001$) in cell viability and mitochondrial membrane potential whereas, the same showed a dose dependent improvement following CS treatment.

Nuclear Condensation and cell cycle analysis (Figure 5.9 & 5.10)

Cells incubated with Ox-LDL were stained with DAPI and PI showed typical characteristics of chromatin condensation and accumulation of cells in sub-G_0 phase. However, CS treatment recorded visibly less number of cells with condensed nuclei. Also,

the cell cycle analysis of Ox-LDL+CS treated cells accounted for significantly less number of cells in G_0 phase suggesting improved cell survival.

***In vivo* studies**

Serum lipid profile and MDA assay (Figure 5.11 & 5.12)

There was significant ($p<0.001$) elevation in serum TC, TG, VLDL and LDL levels and, a decrement in HDL level recorded in ATH rats as compared to the control rats. However, ATH+ CS rats showed significant ($p<0.001$) decrement in TC, TG, VLDL and LDL levels and increment in HDL level as compared to ATH group. There was significant ($p<0.001$) increment in MDA level the LDL isolated from ATH rats as compared to the control rats. However, ATH+CS treated rats recorded significant ($p<0.001$) decrement in MDA level.

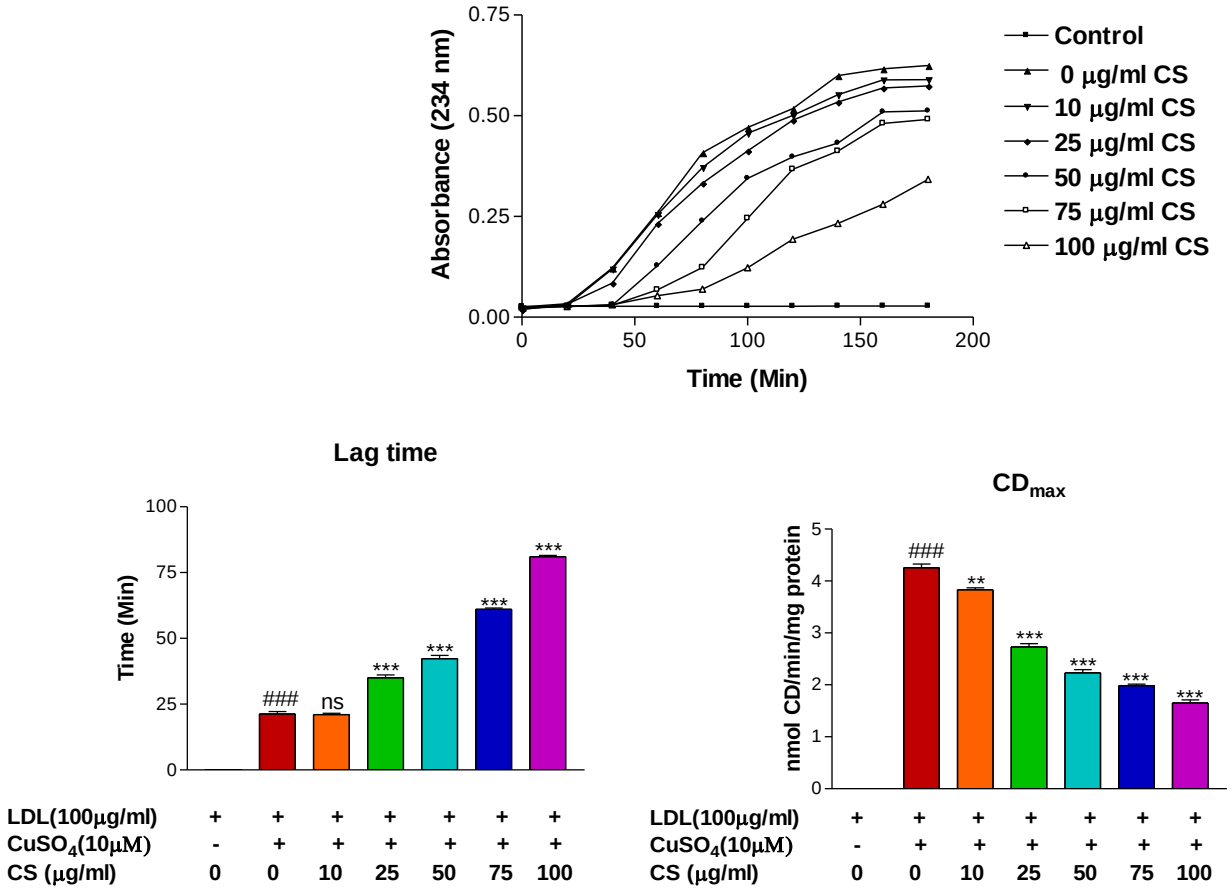
Microscopic evaluation of thoracic aorta (Figure 5.13a & 5.13b)

The photomicrographs of thoracic aorta of control rats stained with hematoxylin and eosin showed normal histo-architecture with intact intima and linear pattern of smooth muscles in all observed tissue sections. However, there was a significant distortion of intima, smooth muscle derangement, degenerative changes in media and atheromatous plaque formation observed in ATH rats. The ATH+CS rats showed relatively less distortion of intima and smooth muscles with small patches of atheromatous plaques as compared to ATH rats. The Von kossa stained sections of aorta showed a high level of calcification in ATH rats as compared to control rats whereas, the same showed minimal calcification in aorta of ATH+CS treated rats.

Immunohistochemical (IHC) localization of adhesion molecules in aorta (Figure 5.14a & 5.14b)

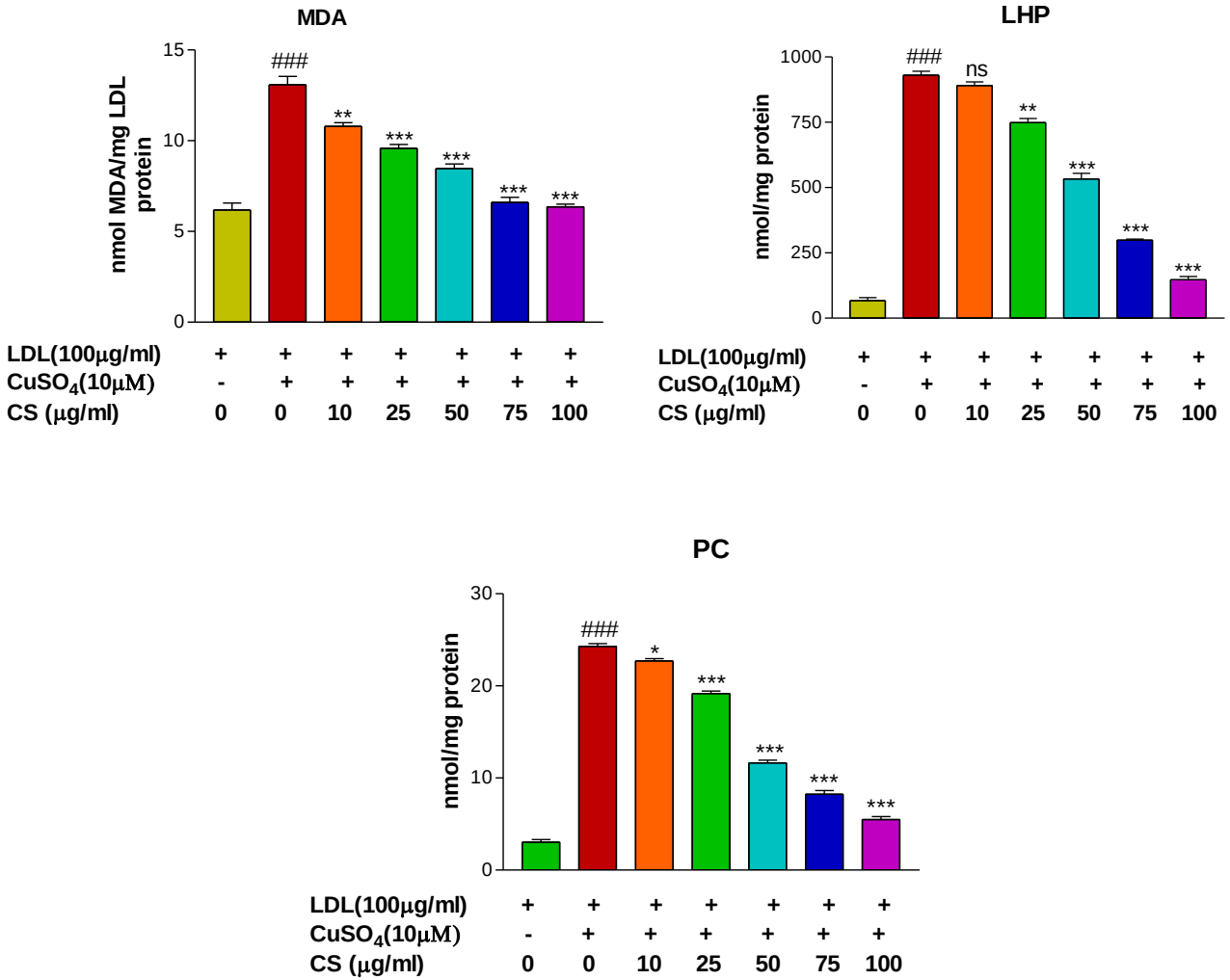
Cell adhesion molecules (CAMs; VCAM-1 and p-selectin) were immunolocalized in section of thoracic aorta of control and treated rats. The endothelial lining of aorta of control rats showed no evidence of immunostaining of both CAMs but the same were visibly intense in ATH rats. However, ATH+CS rats recorded lower grade of expression and the same was evident in their stained sections.

Figure 5.1: Effect of CS on copper-mediated LDL oxidation kinetics



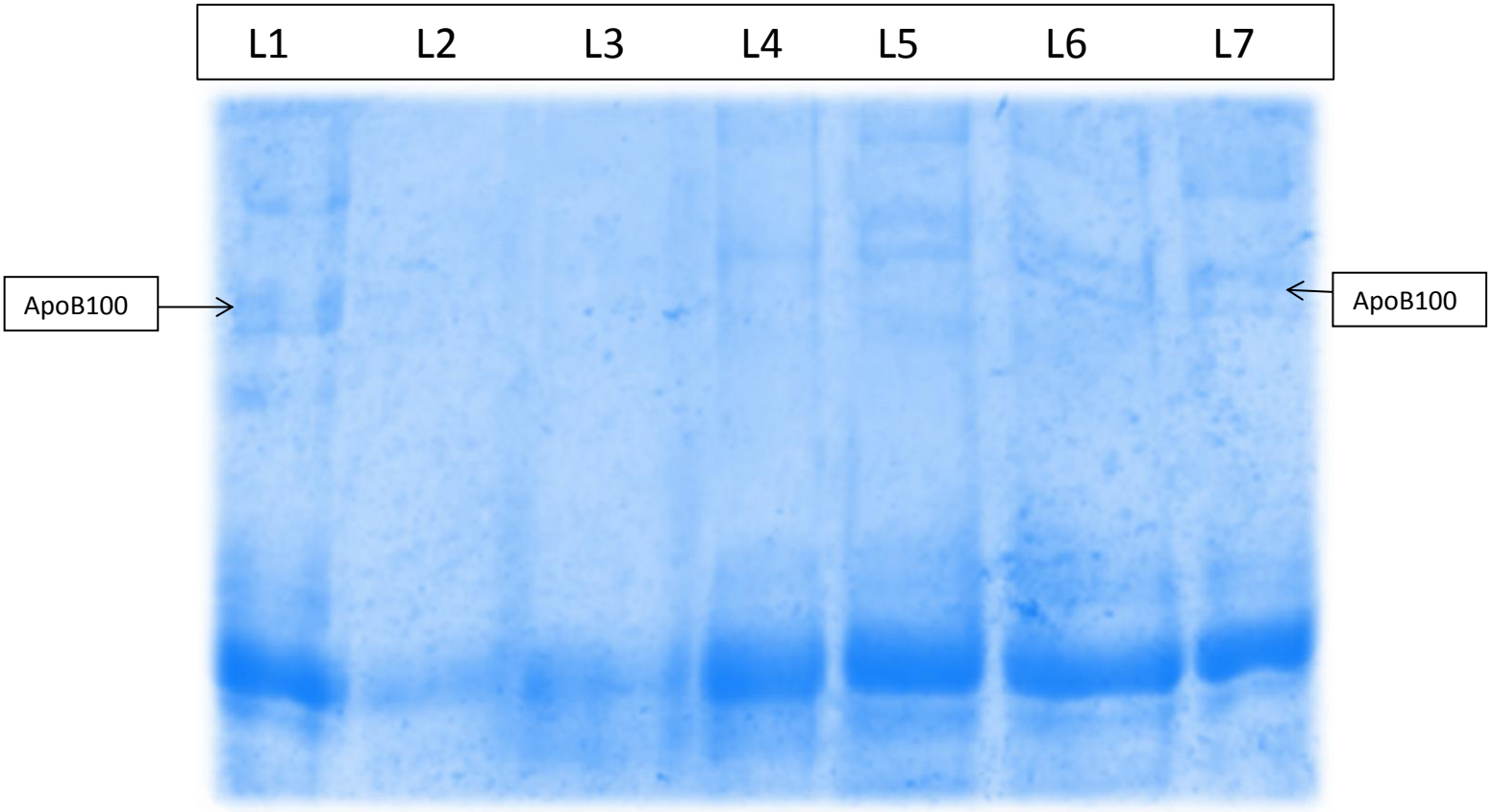
Data expressed as mean±S.E.M. for n=3. ### p<0.001 compared to LDL alone and * p<0.01, ** p<0.05, *** p<0.001 and ns = non significant compared to LDL+ $CuSO_4$.

Figure 5.2: Effect of CS on formation of LDL oxidation products (MDA, LHP and PC)



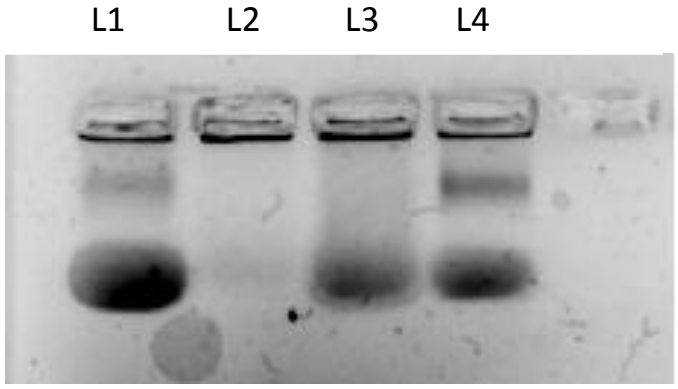
Data expressed as mean±S.E.M. for n=3. ### p<0.001 compared to LDL alone and * p<0.01, ** p<0.05, *** p<0.001 and ns = non significant compared to LDL+ CuSO4.

Figure 5.3a: Effect of CS on copper-mediated Apo B fragmentation



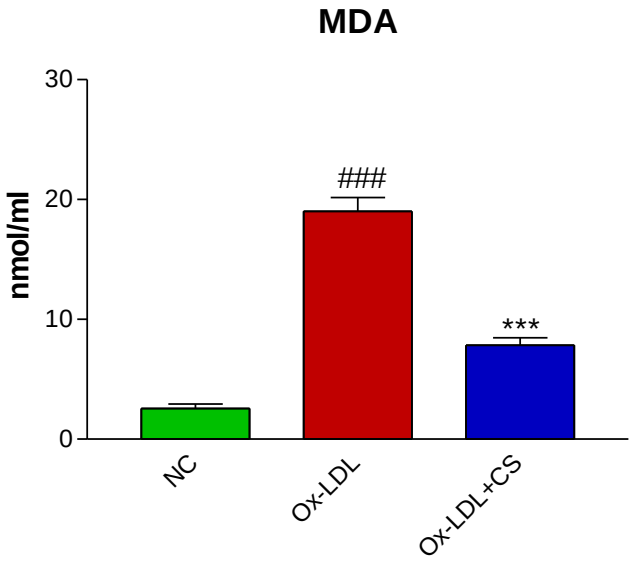
L1=nLDL, L2=Cu+LDL, L3=Cu+LDL+CS10µg/ml, L4=Cu+LDL+CS25µg/ml, L5=Cu+LDL+CS50µg/ml, L6=Cu+LDL+CS75µg/ml, L7=Cu+LDL+CS100µg/ml

Figure 5.3b: Relative electrophoretic mobility



L1=nLDL, L2=Cu+LDL, L3=Cu+LDL+CS10µg/ml, L4=Cu+LDL+CS125µg/ml

Figure 5.4: Effect of CS on cell mediated LDL oxidation



Data expressed as mean±S.E.M. for n=3. ### $p<0.001$ compared to control and $p<0.001$ compared to ox-LDL

Figure 5.5: Effect of CS extract on intracellular cholesterol accumulation (Foam cell formation assay; Oil red O staining) in Ox-LDL-treated RAW 264.7 cells. NC; RAW 264.7 cells, Ox-LDL; RAW 264.7 cells exposed to Ox-LDL and Ox-LDL + CS; RAW 264.7 cells exposed to Ox-LDL in presence of 100 μg/ml CS extract.

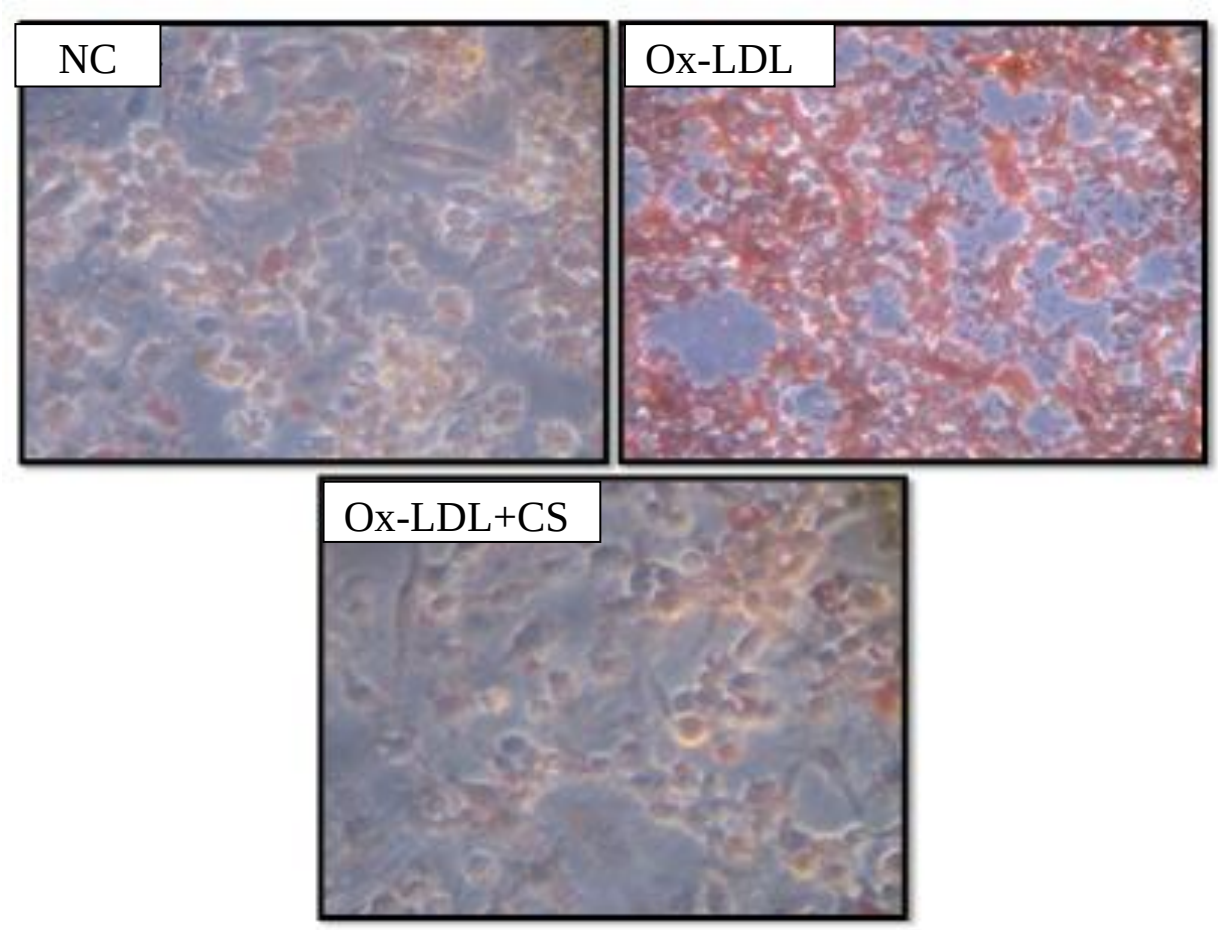


Figure 5.6: Effect of CS extract on peroxyl radical generation (DCF-DA staining) in Ox-LDL-treated RAW 264.7 cells. NC; RAW 264.7 cells, Ox-LDL; RAW 264.7 cells exposed to Ox-LDL and Ox-LDL + CS; RAW 264.7 cells exposed to Ox-LDL in presence of 100 μg/ml CS extract

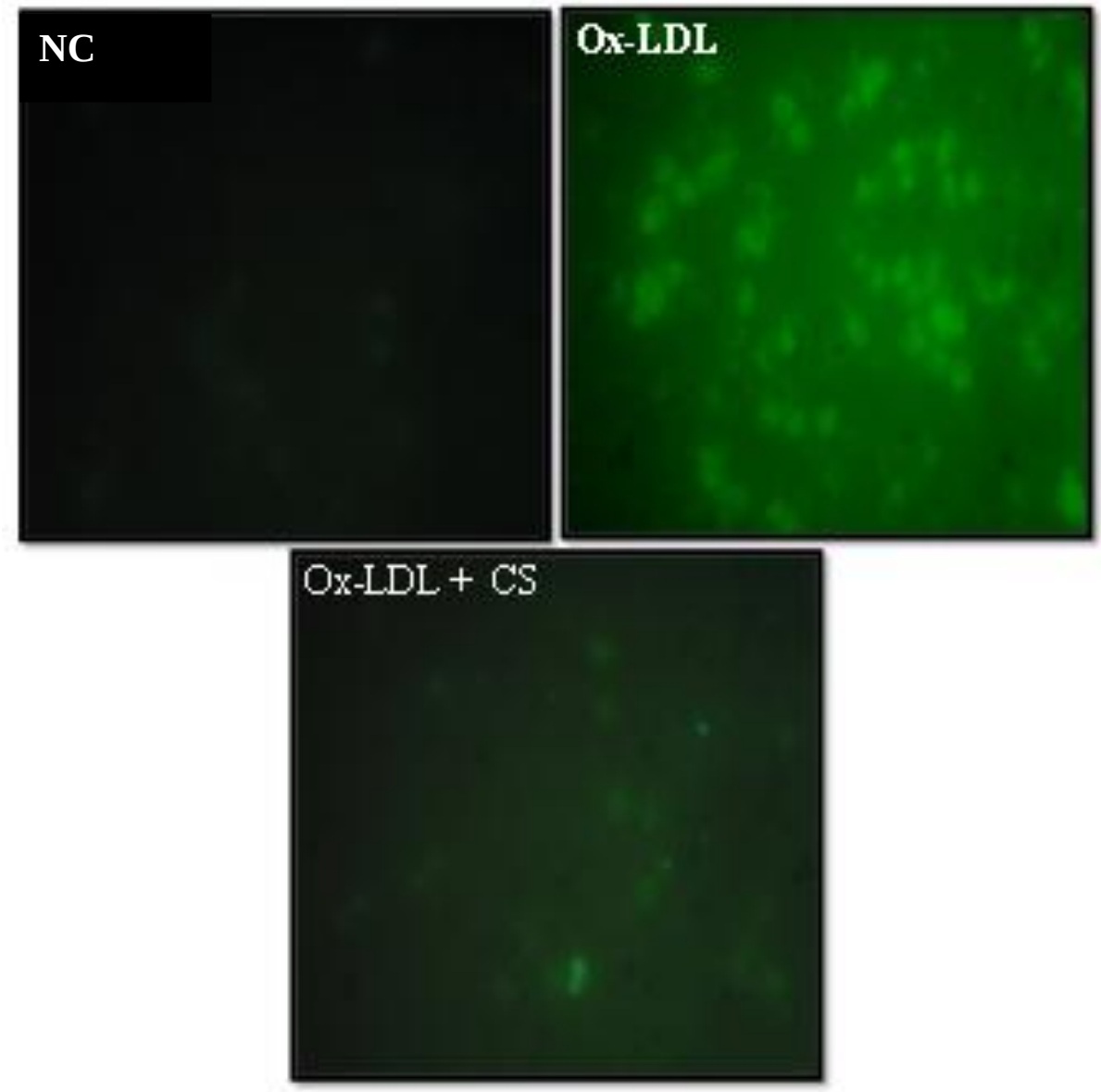


Figure 5.7: Effect of CS extract on mitochondrial membrane potential (Rhodamine 123 staining) in Ox-LDL-treated RAW 264.7 cells. Data expressed as mean ± S.E.M for n = 3. ###P <0.001 compared with n-LDL and *P<0.05, **P<0.01, ***P<0.001 and ns non-significant compared Ox-LDL

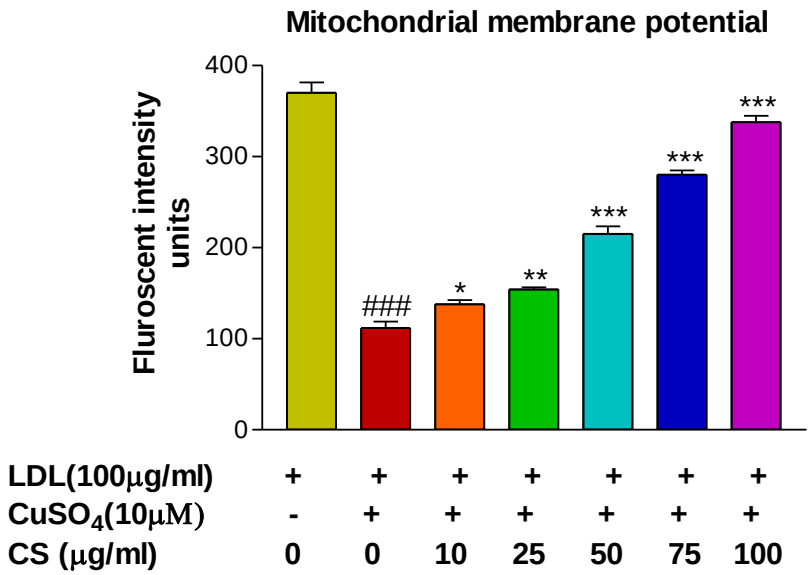
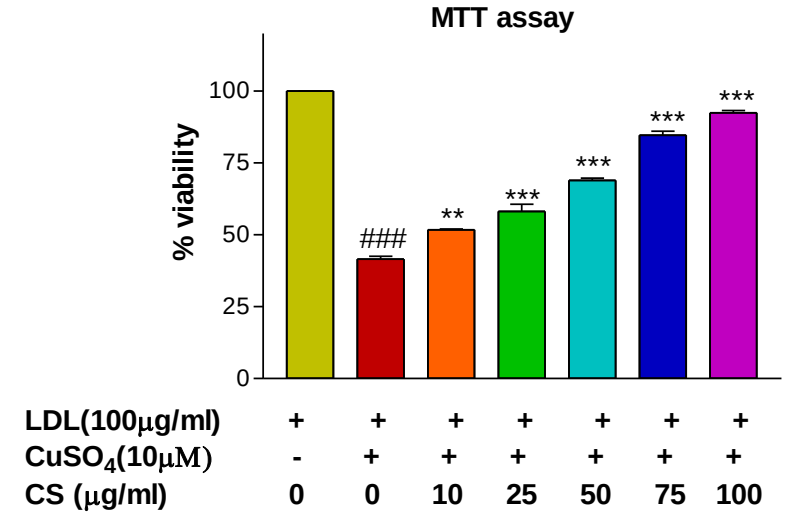


Figure 5.8: Effect of CS extract on cell viability (MTT assay).
Data expressed as mean ± S.E.M for n = 3. ###P <0.001 compared with Ox-LDL and CS untreated and *P<0.05, **P<0.01, ***P<0.001 and ns non-significant compared Ox-LDL



Data expressed as mean±S.E.M. for n=3. ### p<0.001 compared to control and p<0.001 compared to ox-LDL

Figure 5.9: Effect of CS extract on nuclear condensation (DAPI staining) in Ox-LDL-treated RAW 264.7 cells. CN; RAW 264.7 cells, Ox-LDL; RAW 264.7 cells exposed to Ox-LDL and Ox-LDL + CS; RAW 264.7 cells exposed to Ox-LDL in presence of 100 μg/ml CS extract.

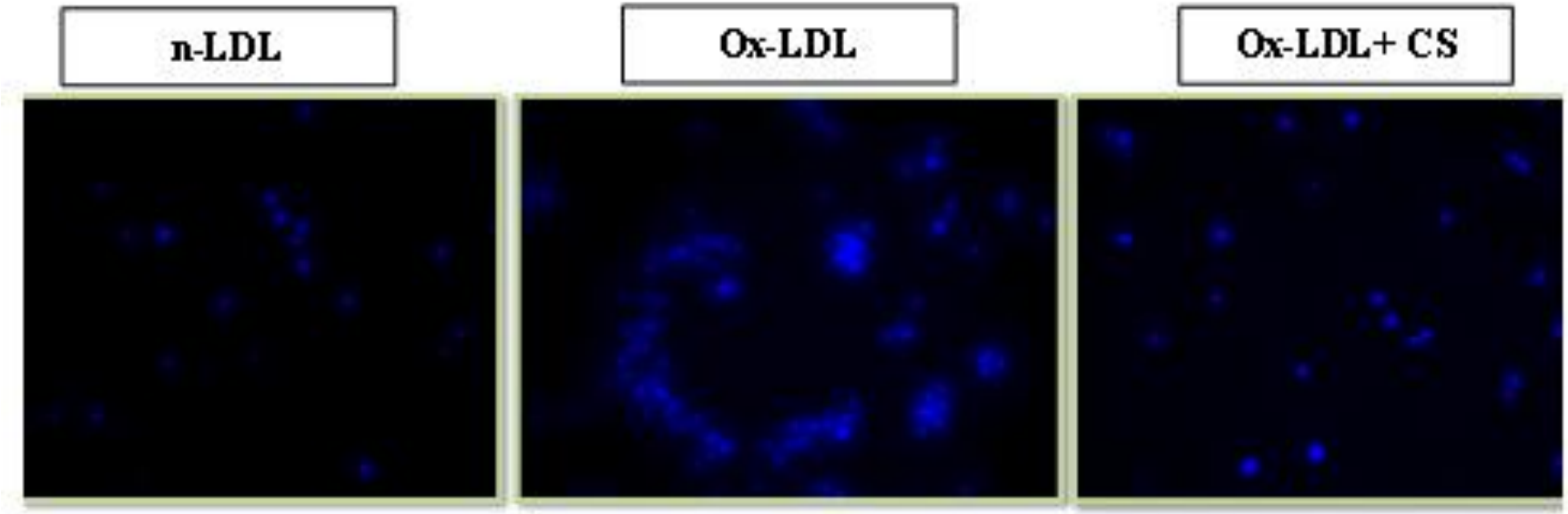


Figure 5.10: Effect of CS extract on cell cycle distribution in Ox-LDL-treated RAW 264.7 cells. NC; RAW 264.7 cells, Ox-LDL; RAW 264.7 cells exposed to Ox-LDL and Ox-LDL+CS; RAW 264.7 cells exposed to Ox-LDL in presence of 100 µg/ml CS extract

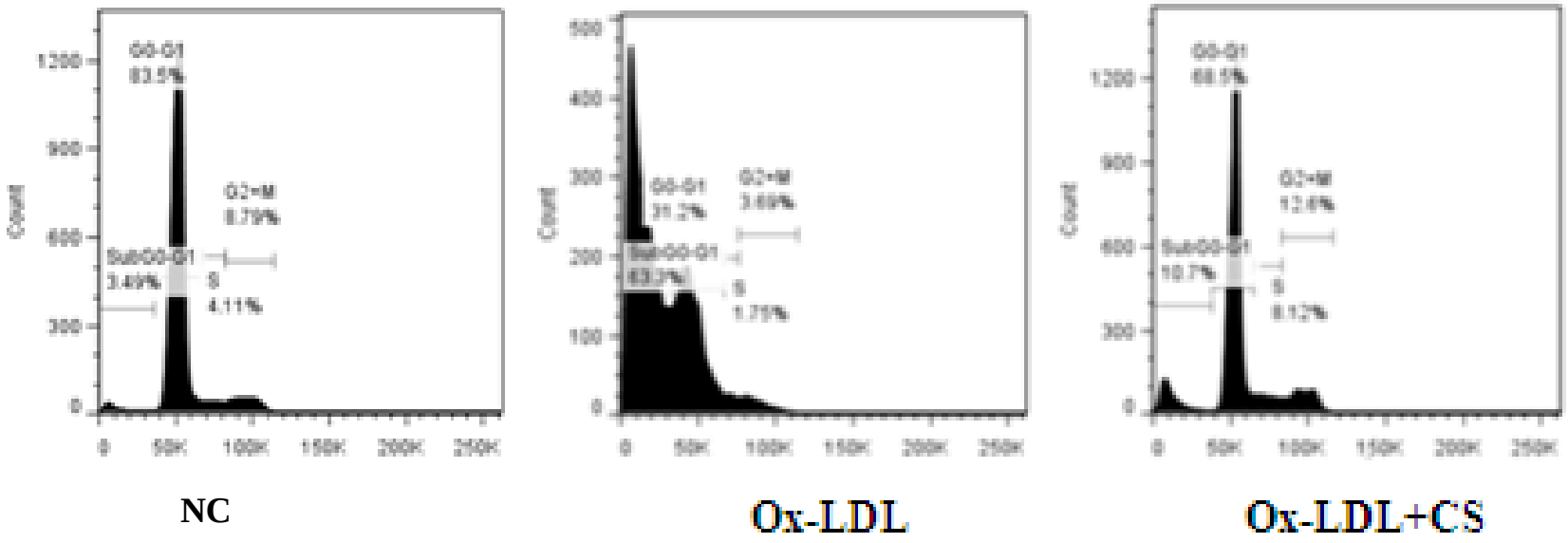
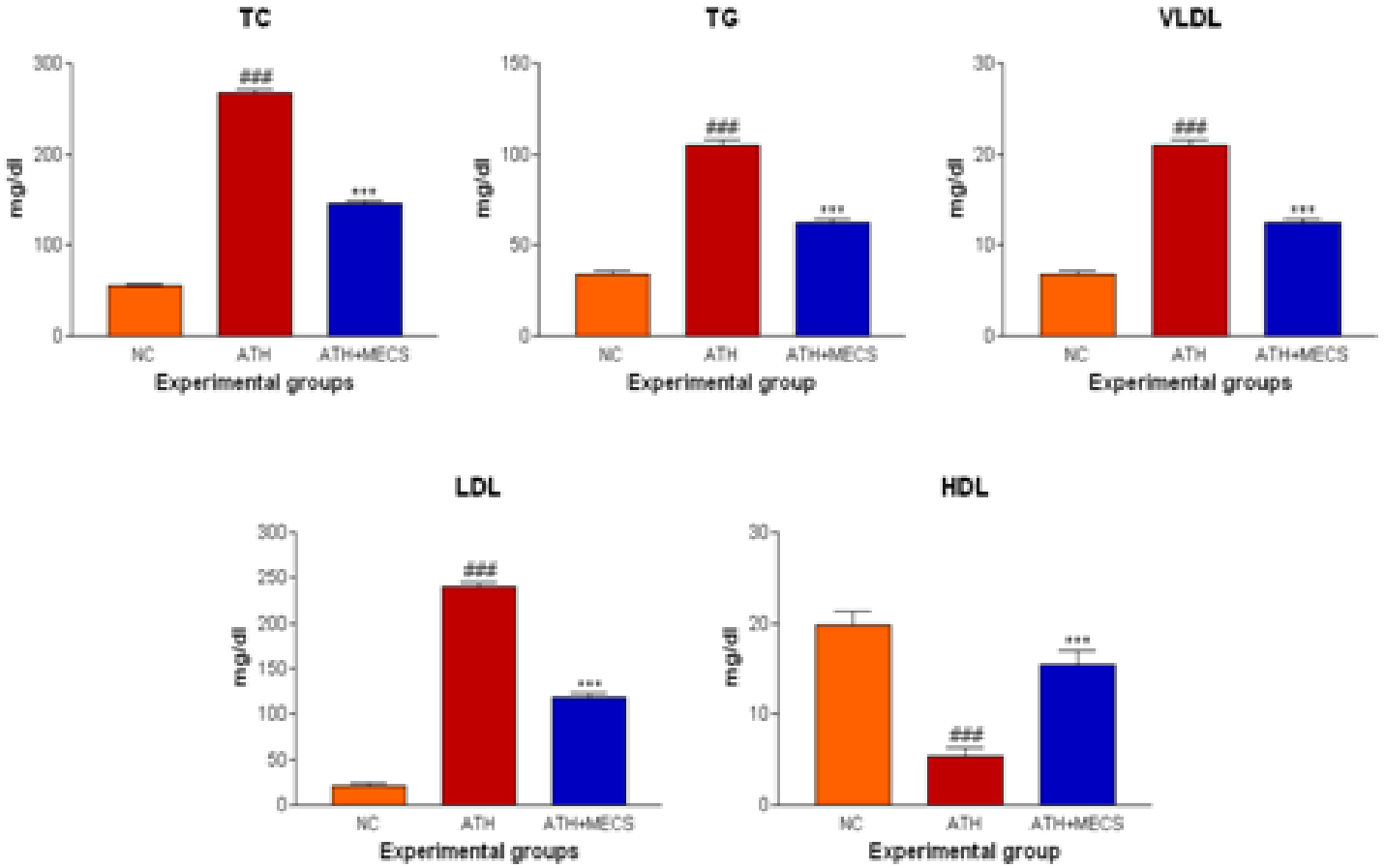
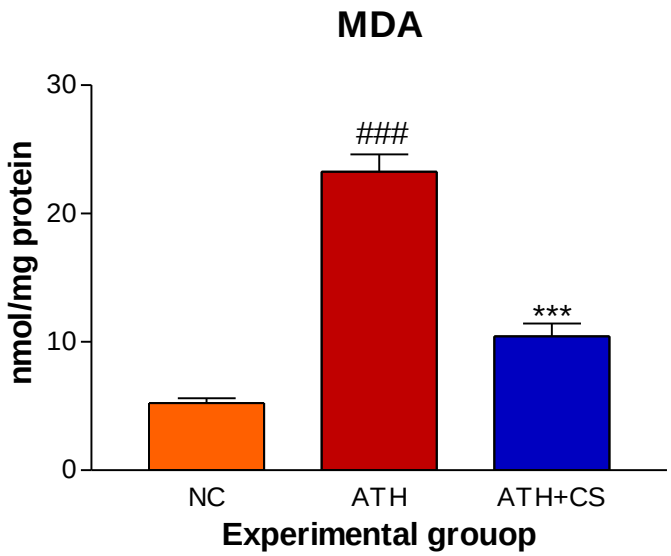


Figure 5.11: Effect of CS on lipid and lipoprotein profiles.



Data expressed as mean±S.E.M. for n=6. ### p<0.001 compared to ATH and * p<0.01, ** p<0.05, *** p<0.001 and ns = non-significant compared to ATH

Figure 5.12: Effect of CS on serum MDA level of control rats and rats fed with atherogenic diet in presence or absence of CS extract



Data expressed as mean±S.E.M. for n=6. ### p<0.001 compared to ATH and * p<0.01, ** p<0.05, *** p<0.001 and ns = non significant compared to ATH+C

Figure 5.13a: Effect of CS extract on histopathology of thoracic aorta of atherogenic diet fed rat

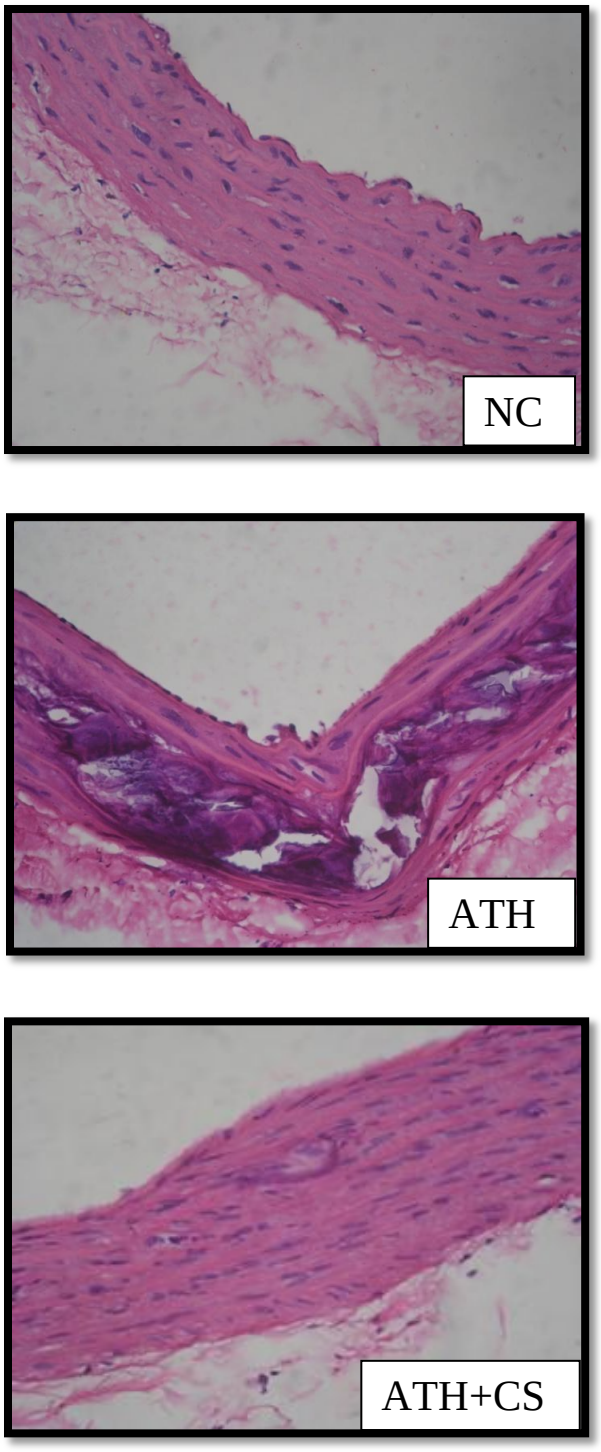


Figure 5.13b: Effect of CS extract on histopathology showing extent of calcification on atherogenic diet fed rats using vonkossa stain.

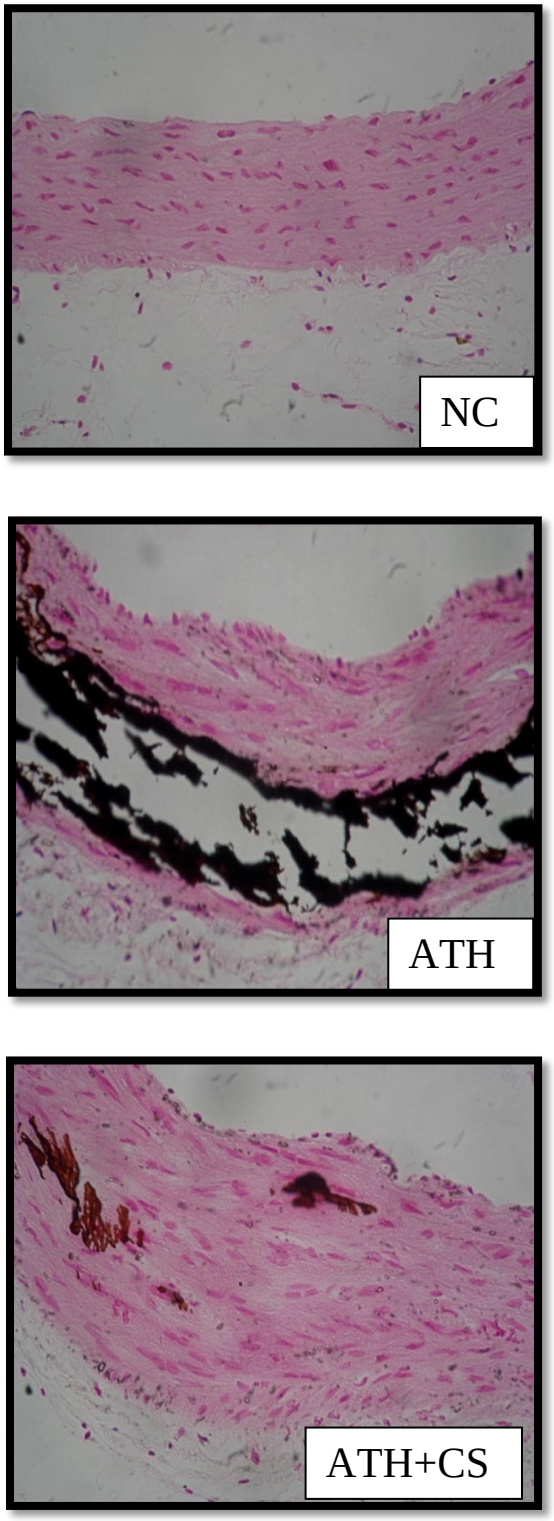


Figure 5.14a: Effect of CS extract on expression of VCAM1 in thoracic aorta of atherogenic diet fed rat using imunohistochemistry

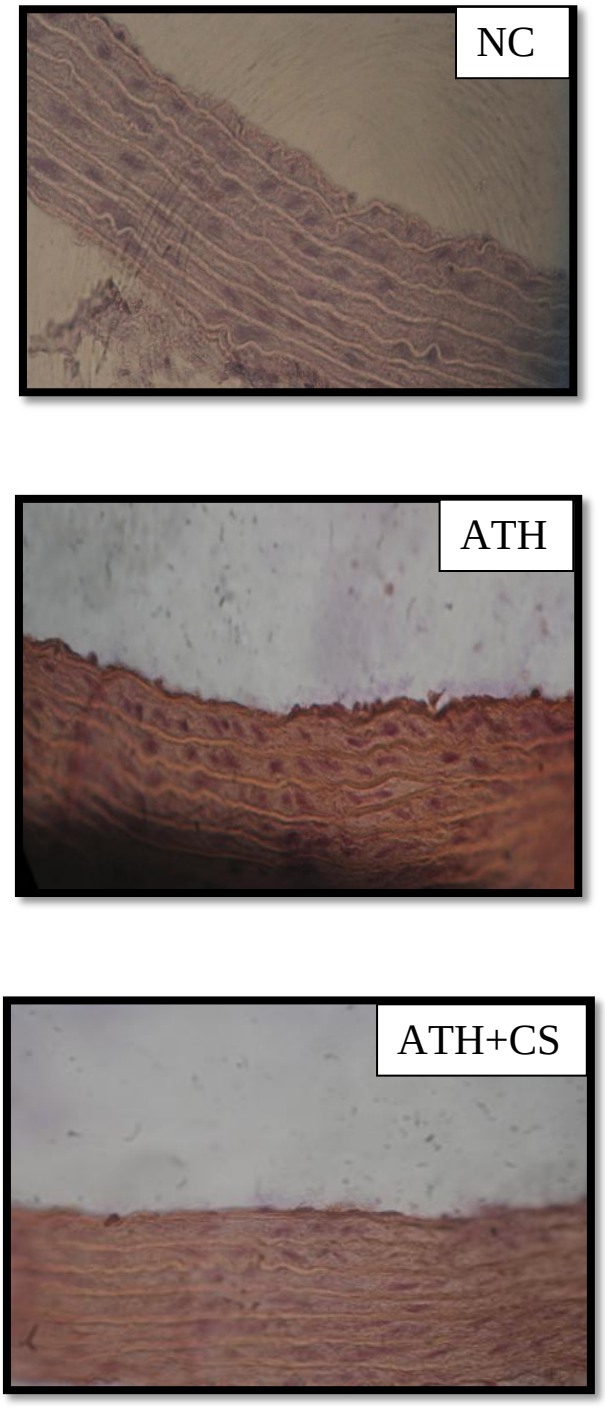
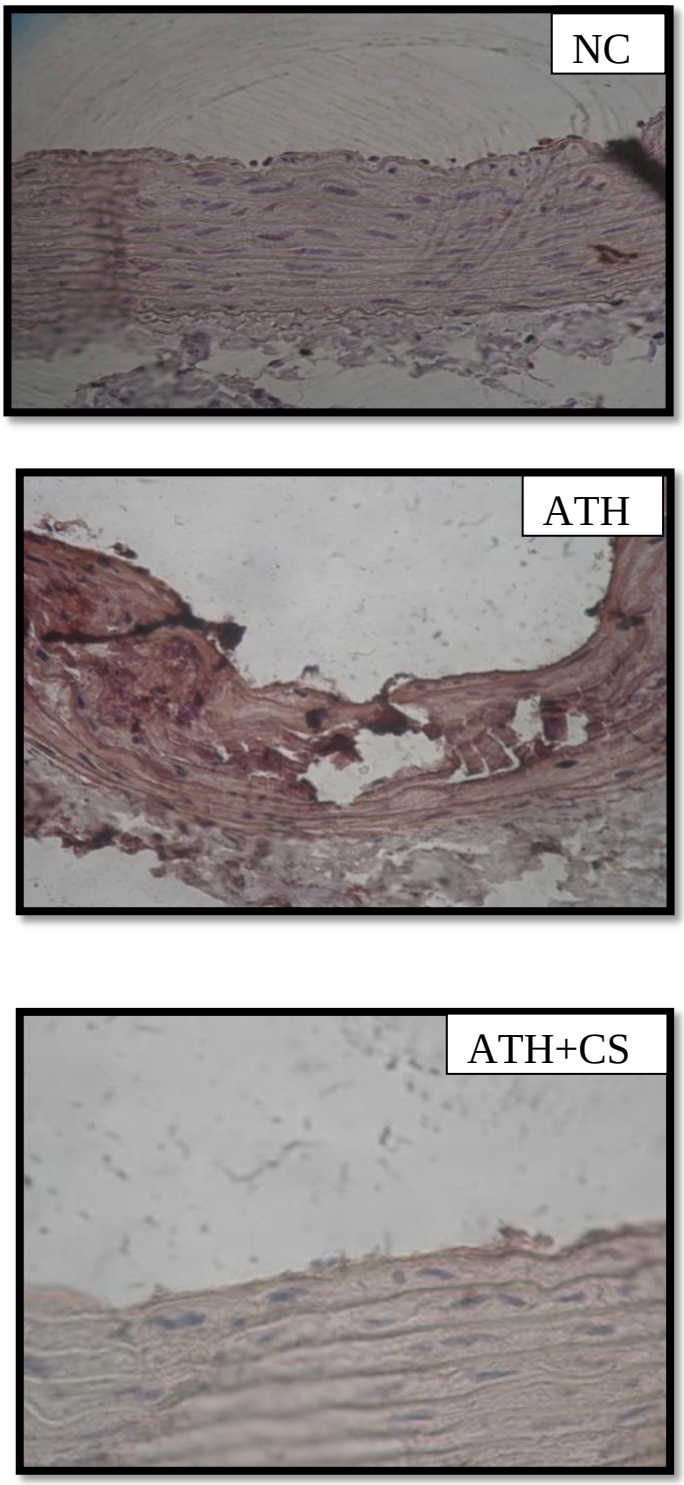


Figure 5.14b: Effect of CS extract on the expression of p-selectin in thoracic aorta of atherogenic diet fed rats



DISCUSSION

Atherosclerosis is multifactorial disease wherein hypercholesterolemia and oxidative modification of LDL are the key initiation factors in its development and progression (Zha *et al*., 2009). Oxidative modification in n-LDL in vivo results from an imbalance between the pro-oxidant challenges and antioxidant defences (Moriel *et al*., 2002). Various in vitro methods have been developed to evaluate the contributions exerted by intrinsic factors in triggering oxidative modifications of LDL particles (Lobato *et al*., 2009). In the present study, LDL was isolated from human serum by precipitation method and subsequently challenged with copper ion so as to obtain ox-LDL. The kinetics of LDL oxidation was subsequently evaluated by assessing its oxidation products such as CS whereas; the non-kinetic indices such as MDA, LHP and PC have also been assessed (Thounaoujam *et al*., 2011). These parameters have been extensively used by various research groups to establish the efficacy of a drug or herbal extract in preventing LDL oxidation (Thounaoujam *et al*., 2011, Yu *et al*., 2005, Visavadiya *et al*., 2009).In the present study Cu^{+2} treatment accounted for elevated indices of MDA, LHP and PC a progressive increment in CD levels. These results confirm Cu^{+2} mediated oxidation of LDL. These results are in accordance with studies performed by other research group as well as previous studies from our lab (Thounaoujam *et al*., 2012, Jadeja *et al*., 2012). Presence of CS extract accounted for significant decrement in LDL oxidation products. The same was evidence in form of increased lag time (100 min) as compared to Cu^{+2} mediated LDL oxidation. It is assumed that the high polyphenol content present in CS extract scavenges

the free radicals thus accounting for a prolonged lag time and significant decrement in formation of the LDL oxidation products.

Fragmentation of fatty acids during atherogenic progression is associated with generation of highly reactive intermediates such as aldehydes and ketones that are instrumental in causing imperative modifications of LDL. This is known to oxidatively delete peptide bonds and cause derivatization of the lysine residue causing fragmentation of Apo B (Stocker and Keaney, 2004). The same process also confirms covalent adducts and an increase the negative charge of LDL molecules. Since, these sequence of events are difficult to monitor in vivo, REM and Apo B fragmentation by electrophoresis is popularly adopted in vitro protocol for assessing anti-atherogenic potential of the test compound on Cu^{+2}mediated oxidatively modified LDL molecules. In our study, CS treatment recorded for restoration of REM in a dose dependent manner with the highest dose (100 µg/ml) showing results comparable to that of n-LDL (Control). Also, the ox-LDL witnessed complete disappearance of the Apo B fragment. The same made reappearance with the highest dose accounting for results comparable to n-LDL (Control). These observations strongly suggest that CS extract is efficient in preventing LDL oxidation due to rich polyphenol content that prevents oxidative damage. These credentials strengthen the claim that CS extract has the anti-atherogenic potentials.

Several studies have shown that major cell types such as the endothelial cells smooth muscle cells and monocyte derived macrophages have ability to oxidise LDL (Aviram *et al*., 1994; Rusinol *et al*., 2000; Yen *et al*., 2008). Hence, RAW 264.7 macrophages were fed with n-LDL and incubated for 24 h in presence or absence of CS extract to assess the efficacy of CS extract in preventing LDL oxidation. Decreased levels

of LDL oxidation products (MDA) recorded in the present study provide testimony to the efficacy of CS extract in significantly preventing cell mediated LDL oxidation. The antioxidant property of hydro-methanolic extract of CS has already been established in our lab (Patel *et al*., 2012) and observed effects in the present study are attributable to the efficacy of CS extract in preventing oxidative modification of LDL.

In the progression of atherosclerosis monocytes cross the endothelial barrier and differentiate into resident macrophages (Boyle, 2005).The endothelial and smooth muscle cells of thoracic aorta oxidised the native LDL. This is crucial step wherein the macrophages take up Ox-LDL via scavenger receptor (SRB1) to transform into lipid laden foam cells (Shashkin *et al*., 2005). In our study Ox-LDL fed RAW 264.7 cells showed clear evidences of intracellular accumulation. However, visibly less number of foam cells observed in CS treated macrophages possibly indicates at the ability of CS in preventing SRB1 expression. The same needs further experimental validation.

Pathogenesis of atherosclerosis is also marked by elevated levels of intracellular oxidative stress due to formation of peroxy radicals prior to the trigger apoptotic pathway (Asmis and Begley, 2003). In the present study, a peroxy radical specific stain (H_2-DCFDA) produces powerful green fluorescence in macrophages that have been fed with Ox-LDL (Thounaojam *et al*., 2011). Results obtained in our study are in accordance with other study wherein cells show high intensity of green fluorescence due to conversion of H_2-DCFDA (colourless) to DCFDA (green colour) in presence of peroxy radicals. Polyphenols are free radicals scavenger and polyphenol rich CS extract used in the present study possibly scavenges peroxy radicals as evidenced by a weak fluorescence observed in Ox-LDL +CS treated group.

Metabolic performance of the cell including macrophages rest upon the metabolic normalcy of its mitochondrial (Newsholme and Newsholme, 1989). Intracellular oxidative stress triggers depolarization of mitochondrial membrane and the same has been reported in Ox-LDL treated macrophages using RHO 123 stain (Huigsloot *et al*., 2002). In the present study, RAW 264.7 cells exposed to Ox-LDL recorded a significant decrement in mitochondrial membrane potential whereas; the same was restored to normalcy in a dose dependent manner following CS treatment. These results are in accordance with the cytotoxicity assay in which the CS treated cells showed a dose dependent improvement in cell viability, possibly due to the CS extract in alleviating Ox-LDL induced mitochondrial damage.

An association between Ox-LDL to apoptosis and necrosis of foam cells has been hypothesized by several workers (Tabas, 2005) and has also been demonstrated in vitro (Chang *et al*., 2006). In the pathophysiological progression of atherosclerosis elevated levels of intracellular cholesterol play critical role in regulation of Ox-LDL mediated apoptosis (Ryan *et al*., 2005). In the present study, Ox-LDL treated RAW 246.7 cells recorded nuclear condensation, cell cycle arrest and apoptosis. However, CS treatment showed dose dependent increment in cell viability with more number of cells in G_0 phase. CS extract has been reported to be possessing cholesterol lowering properties (Chitrhra and Leelamma, 1997). Such efficacies of herbal extracts have discreetly associated with their ability to induce reverse cholesterol transport and massive cholesterol efflux (Kaplan *et al*., 2001). Observations recorded herein such as reduced foam cells formation and improved cell viability are attributable to hypolipidemic property of CS extract that coupled with its antioxidant potential provides multi-pronged safety to the cells. These

results provide compelling evidence of anti-atherogenic potential of CS extract and hence, its efficacy was further validated in an in vivo atherogenic rodent model.

In the present study, Vit D3 + sodium Cholet + PTU and SD rat model was used to induce atherosclerosis (Cai *et al*., 2005). In this model, PTU induced hypothyroidism and disruption of LDL receptors coupled with sodium Cholet induced increased cholesterol absorption and lower Cholesterol 7 alpha-hydroxylase activity results in hypercholesterolemia. In such circumstances vit. D3 ha a detrimental effect on the structure and function of aorta, a condition that mimics in vivo atherosclerosis in humans (Cai *et al*., 2005). Significantly elevated lipid profile of serum and aorta of atherosclerotic rats in the present study further corroborate the said hypothesis. However, lowered lipid profile following CS treatment is in agreement with the lipid lowering property of CS reported by (Chithra and Leelamma, 1997). Serum LDL isolated from atherosclerotic rats showed elevated levels of MDA suggesting that serum LDL had undergone in vivo oxidation. However, co-supplementation of CS in atherosclerotic rats accounted for already established ability of CS in preventing LDL oxidation in an in vitro assay performed in this study.

Increased expression of cell adhesion molecules on an activated atherogenic endothelium governs and initiation and progression of atherosclerosis (Thounaojam *et al*., 2012). VCAM-1 and p-selectin expression in aorta facilitates adhesion of leucocyte that ends in a cascade of events (Carlos and Harlan, 1994; Gebuhrer *et al*., 1995). In the present study endothelium of thoracic aorta of CS treated atherosclerotic rats recorded significantly lowering immune localization of CAMs as capered to atherosclerotic rats.

These results are in agreement with our previous report (Thounaojam *et al*., 2012). These results further emphasized upon the anti-atherogenic potential of CS extract.

Pathological evaluation of thoracic aorta of atherosclerotic rats showed prominent atheromatous plaque formation and related damage to the vascular endothelial and smooth muscle cells. Also, calcium deposition is clearly evident. These results were greatly reduced in CS co-supplemented group and the results were comparable to that of control group. Endothelial damage, smooth muscle cells migration, plaque formation and calcium deposition are extensively reported pathological damages in ahterogenic rats. And the same are attributable to vitamin D3 injection. CS induced prevention of plaque formation; smooth muscle cell migration safe guarding of vascular endothelium and preventing calcium deposition suggest that CS extract has potential of mitigating in vivo induction of experimental atherosclerosis. These results are in synergy with previously reported therapeutic potential of CS extract and add further value to its established reputation as a medicinal dietary ingredient.

SUMMARY

This study was designed to assess the efficacy of *Coriandrum sativum* L. (CS) in preventing *in vitro* LDL oxidation mediated macrophage modification. Further, an *in vivo* study was also conducted to confirm upon the efficacy of CS seed extract in alleviating pathophysiological alterations of high fat diet induced atherosclerosis in rats. Copper mediated cell free oxidation of LDL (low density lipoproteins) accounted for elevated indices of MDA, LHP and PC and a progressive increment in CD levels whereas, reverse set of changes were recorded in presence of CS extract. Cell mediated LDL oxidation (using RAW 264.7 cells) accounted for lowered MDA production and oxidized LDL (ox-LDL) mediated cell death in presence of CS extract and the same was attributed to its potent antioxidant and free radical scavenging potentials. High fat diet fed atherogenic rats showed elevated lipid indices, evidences of LDL oxidation, plaque formation in thoracic aorta. The same was further validated with immunostaining of cell adhesion molecules and HXE staining. However, co-supplementation of CS to atherogenic rats recorded significant lowering of the above mentioned parameters further strengthening the claim that CS extract is instrumental in preventing onset and progression of atherosclerosis.

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Original article:

ACUTE AND SUB-CHRONIC TOXICOLOGICAL EVALUATION OF HYDRO-METHANOLIC EXTRACT OF *CORIANDRUM SATIVUM* L. SEEDS

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ABSTRACT

Coriandrum sativum L. (CS) seeds are known to possess therapeutic potentials against a variety of physiological disorders. This study assesses acute and sub-chronic toxicity profile of hydro-methanolic extract of CS seeds using OECD guidelines. In acute toxicity study, mice were once orally administered 1000, 3000 and 5000 mg/kg body weight of CS extract. There were no any behavioral alterations or mortality recorded in CS treated groups. The LD_{50} value was more than 5000 mg/kg body weight. In the sub-chronic oral toxicity study, the animals were orally administered with CS extract (1000, 2000 and 3000 mg/kg body weight) daily for 28 days whereas; vehicle control group received 0.5 % carboxy methyl cellulose. There was significant reduction in food intake, body weight gain and plasma lipid profiles of CS_2 and CS_3 (2000 and 3000 mg/kg body weight respectively) groups as compared to the control group. However, there were no alterations in haematological profile, relative organ weights, histology and plasma markers of damage of vital organs (heart, liver and kidney). The overall finding of this study indicates that CS extract is non-toxic up to 3000 mg/kg body weight and can be considered as safe for consumption.

Keywords: *Coriandrum sativum* L., hydro-methanolic seed extract, acute toxicity, sub-chronic toxicity

INTRODUCTION

The traditional systems of medicine such as the Ayurveda, Unani and Sidda have been a treasure trove for development of majority of modern medicines. Also the medicinal research relies on ethnobotany and ethnopharmacognocy for discovery of new molecules for that conventionally result in drugs developments (Gurib-Fakim, 2006). World Health Organization (WHO) estimates that approximately 80 % of the developing world's population is using traditional medicine for primary healthcare (Bannerman, 1982). However, there is a prevalent misunderstanding that herbal medicines are devoid of toxic effects (WHO, 2004). Adverse effects of herbs have been reported including allergic reactions, hepatotoxicity (Saad et al., 2006), nephrotoxicity (Colson and De Broe, 2005; Kwan et al., 2006; Zhu, 2002; Vanherweghem, 1998), cardiac toxicity (Horowitz et al., 1996; Moritz et al., 2005; Gaibazzi et al., 2002), neurotoxicity (Ernst, 2003; Benjamin et al., 2001) and even death (Jensen and Allen, 1981) have been reported. Therefore, a pre-clinical toxicity study is

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indispensible to validate their safe medicinal use.

Coriandrum sativum L. (Apiaceae) (CS) is an annual herb, that is widely distributed. Its fresh leaves and dried seeds are extensively used in Middle Eastern, Mediterranean, Indian, Latin American, African and Southeast Asian cuisines. Decoction and tincture of powdered seeds of CS alone or in combination with other herbal agents are recommended for dyspeptic complaints, loss of appetite, convulsion, insomnia and anxiety (Grieve, 1971). It is also used as medication against diabetes, indigestion, flatulence, renal disorders and a diuretic agent (Grieve, 1971; Emamghoreishi et al., 2005). Its therapeutic potential in the treatment of urethritis, cystitis, urinary tract infection, urticaria, rashes, burns, sore throat, vomiting, indigestion, nosebleed, cough, allergies, hay fever, dizziness and amoebic dysentery has also been reported (Grieve, 1971; PDR for Herbal Medicines, 1998).

Phytochemical constituents of CS seeds have been studied extensively and their analysis had revealed presence of polyphenols (rutin, caffeic acid derivatives, ferulic acid, galic acid, and chlorogenic acid), flavonoids (quercetin and isoquercetin) and β-carotenoids (Melo et al., 2003). The essential oil obtained from CS seeds contains α and β-pinene, camphor, citronellol, coriandrol, *p*-cymene, geraniol, geranyl acetate, limonene, linalool, myrcene, α and β phellandrene and α and β-terpinene along with many fatty acids. Presence of water soluble compounds such as monoterpenoid glycosides, monoterpenoid glucose sulfate and other glycosides have been reported (Sergeeva, 1975; Ishikawa et al., 2003). The pharmacological activities of various extracts and essential oils of CS seeds have been studied wherein; the essential oils have been found to possess antimicrobial (Baratta et al., 1998) and antifungal properties (Garg and Siddiqui, 1992). Its efficacy as a hypoglycemic (Gray and Flatt, 1999), hypolipidemic (Chithra and Leelamma, 1997, 1999; Lal et al., 2004), hypocholesterolemic (Dhanapakiam et al., 2008), antihypertensive (Medhin et al., 1986), antioxidant (Melo et al., 2003; Ramadan et al., 2003; Bajpai et al., 2005), antimutagenic (Cortes-Eslava et al., 2004), anxiolytic (Emamghoreishi et al., 2005), antimicrobial (Kubo et al., 2004; Cantore et al., 2004), larvicidal (Consoli et al., 1988) and post-coital antifertility agent (Al-Said et al., 1987) have also been reported.

We had recently reported anti-insulin resistance (Patel et al., 2011) and cardioprotective (Patel et al., 2012) potentials of CS seed extract. Since toxicological evaluation of CS seed extract is not studied, the present study evaluates possible toxicity of CS seed extract using Economic Co-operation and Development (OECD) guidelines.

MATERIAL AND METHODS

Plant material and preparation of extract

Seeds of CS were collected (in the month of February and March) and identified by Dr. P.S. Nagar, Department of Botany, The M.S. University of Baroda. A herbarium of plant was deposited in the Department of Botany. One hundred grams of powdered dry seeds were soaked in methanol:water (80:20 v/v) at room temperature and allowed to stand for seven days. The resultant extract was filtered through a muslin cloth and then concentrated in a rotary evaporator under reduced pressure to obtain a thick semisolid brown paste (Hashim et al., 2005). The final yield was 8.3 g (w/w).

Experimental animals

Adult female Swiss albino mice (20-25 g) were obtained from Zydus Cadila Research Centre, Ahmedabad, Gujarat, India. They were housed under standard animal house conditions (temperature: 23 ± 2 °C; photoperiod: 12 h light and 12 h dark; humidity: 45-50 %). They were fed with standard laboratory pellets (M/S Pranav agro, Ltd., Baroda, India) and water *ad libitum*. The animals were maintained as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) India and the experimental protocol approved by the

animal ethical committee of the Department of Zoology, The M. S. University of Baroda, Vadodara (Approval No.827/ac/04/ CPCSEA).

Acute oral toxicity

Acute oral toxicity study was conducted according to the guidelines of Organization for Economic Co-operation and Development (OECD, 401). Twenty four animals were randomly allocated into four groups of six animals each. Group I (Control): animals were administered orally with vehicle (0.05 % Carboxy methyl cellulose; CMC). Remaining groups (II, III and IV) were administered with 1000, 3000 and 5000 mg/ kg body weight of CS extract respectively via gastric intubation. Doses were prepared using 0.05 % CMC and dose volume was not more than 1 ml/kg body weight. Cage side observations (tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma) were recorded during first four hours and mortality was recorded after 24 h.

Sub-chronic oral toxicity

The sub-chronic oral toxicity study was conducted according to the guidelines of the Organization for Economic Co-operation and Development (OECD, 407). Twenty four animals were randomly divided into four groups of six animals each. Group I (CS_0) served as a control and received 0.5 % CMC (vehicle) for 28 days whereas the remaining groups (Group II- CS_1, Group III- CS_2 and Group IV- CS_3) were orally administered 1000, 2000 and 3000 mg/kg body weight respectively of CS extract daily for 28 consecutive days. Food and water intake were recorded daily, whereas, body weight was recorded once in a week throughout study period.

Plasma isolation and haematology

At the end of 28 days, blood samples were collected from overnight fasted animals through retro-orbital sinus puncture in ethylene diamine tetra acetic acid (EDTA) coated vials and plasma was separated by cold centrifugation (Plasto Crafts Superspin-R centrifuge) at 3000 rpm for 10 min. Blood was also collected for the analysis of haematological parameters such as white blood cell (WBC) count, red blood cell (RBC) count, haemoglobin (Hb) levels, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and red cell distribution width (RCDW) using BC 2300 Haematology Analyzer (Shezhen Mindray Biomedical Electronics Co., Ltd., China).

Plasma biochemical parameters

Creatinine kinase-MB (cardiac damage), aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, total protein (liver damage) and urea and creatinine (kidney damage) were analyzed using commercially available kits (Recon diagnostic Ltd., Vadodara, India). Also, plasma glucose and lipid profile [total cholesterol (TC), triglyceride (TG) and high density lipoprotein (HDL-C)] were assessed and low density lipoprotein (LDL-C) and very low density lipoprotein (VLDL-C) were calculated by Friedewald's formula (Friedewald et al., 1972).

Relative organ weights and histopathology

Animals were later sacrificed by cervical dislocation under mild ether anesthesia for autopsy and liver, kidney, heart, lung and spleen were excised, rinsed in 0.9 % saline and weighed. After sacrifice, organ weights (lungs, heart, liver, kidney and spleen) were recorded and relative organ weights (ROW) were calculated as follows.

$$\text{ROW} = \frac{\text{Absolute organ weight (g)} \times 100}{\text{Body weight on the day of sacrifice (g)}}$$

Tissue pieces of vital organs (heart, liver and kidneys) were fixed in 10 % paraformaldehyde for paraffin histology and processed in paraffin embedding as per the standard protocol. 7 µm thick sections of each tissue were stained with hematoxylin and eosin, and observed for possible histopathological damages.

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RESULTS

Acute oral toxicity

Cage side observations did not record any behavioral changes such as tremor, convulsion, salivation, diarrhea, lethargy or sleep during the first four hours of CS extract (1000, 2000 or 3000 mg/kg body weight) administration. After 24 h there was no mortality recorded in plant extract administered groups. However, urine output was found to be increased in CS treated animals (1000, 2000 or 3000 mg/kg body weight) as compared to the control (data not shown).

Sub-chronic oral toxicity

Body weight gain, food and water intake

CS_1 and CS_2 groups did not record any significant alterations in body weight gain. However, CS_3 group (3000 mg/kg body weight) recorded significant ($p<0.001$) decrement in body weight gain. Further, there was significant ($p<0.05$ and $p<0.001$ respectively) reduction in food intake of CS_2 and CS_3 groups as compared to CS_0. Water intake was significantly ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) increased in all the CS extract administered groups as compared to CS_0 group (Table 1).

Haematology

The haematological parameters (RBC, WBC, Hb, MCV, MCH, MCHC, RCDW, monocytes, lymphocytes, eosinophil) did not record any significant alterations in any of CS administered groups (Table 2).

Biochemical parameters

Plasma glucose recorded moderate non-significant decrement in CS_2 and CS_3 groups. Also, plasma TC, TG, LDL and VLDL levels recorded moderate to significant reductions in all the CS treated groups but, plasma HDL levels were unaltered (Table 4). Plasma marker of creatinine kinase-MB, AST, ALT, bilirubin, total protein, urea and creatinine did not record significant alterations in any of the CS treated groups as compared to the CS_0 group (Table 3).

Relative organ weights and histopathology

There were no significant changes in ROW of CS treated groups as compared to CS_0 group (Table 5). A detailed scrutiny of histoarchitecture of the heart, liver and kidney did not reveal any observable cellular damage. The cellular morphology, nuclear characteristics and tissue integrity of organs of CS treated groups were comparable to the CS_0 group (Figure 1).

Table 1: Effect of *Coriandrum sativum* L. seed extract sub-chronic oral administration on food intake, water intake and body weight

Groups	Body weight (gm)		Weight gain gm	Food intake gm/day	Water intake ml/day
	Initial	Final			
CS_0	21.78±0.76	25.10±0.70	3.32±0.25	5.22±0.21	7.47±0.83
CS_1	23.87±0.65	26.12±0.38	$2.82±0.22^{ns}$	$4.43±0.33^{ns}$	$10.80±0.63^{*}$
CS_2	23.32±0.41	25.64±0.40	$2.30±0.35^{*}$	$3.78±0.39^{*}$	$11.27±0.39^{**}$
CS_3	$24.48±0.39^{*}$	26.24±0.34	$1.68±0.23^{***}$	$3.24±0.24^{***}$	$11.46±0.56^{**}$

where n=6. Data were expressed as mean ± S.E.M. * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and ns (not significant) when CS_0 v/s CS_1, CS_2 and CS_3

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Table 2: Effect of *Coriandrum sativum* L. seed extract sub-chronic oral administration on haematological parameters

Parameter	Groups			
	CS_0	CS_1	CS_2	CS_3
RBC (x 10^{12}/l)	8.70±0.20	8.52±0.07^{ns}	8.72±0.29^{ns}	8.93±0.10^{ns}
Hb (g/dl)	16.14±0.29	16.22±0.17^{ns}	16.42±0.53^{ns}	16.62±0.13^{ns}
MCV (fl)	44.73±0.92	44.23±0.93^{ns}	44.17±0.73^{ns}	42.80±0.15^{ns}
MCH (pg)	18.72±0.19	18.94±0.18^{ns}	19.02±0.34^{ns}	18.64±0.26^{ns}
MCHC (g/dL)	44.56±0.55	44.62±0.59^{ns}	43.78±0.98^{ns}	43.00±0.57^{ns}
RCDW (%)	18.00±0.19	17.60±0.30^{ns}	17.67±0.26^{ns}	17.45±0.25^{ns}
WBC (x 10^3/µl)	16.33±0.48	16.27±0.23^{ns}	16.43±0.83^{ns}	16.53±0.21^{ns}
Monocytes (%)	2.02±0.15	2.16±0.20^{ns}	2.12±0.15^{ns}	2.05±0.16^{ns}
Lymphocytes (%)	7.34±0.15	7.30±0.25^{ns}	7.18±0.24^{ns}	7.06±0.18^{ns}
Eosinophils (%)	2.34±0.23	2.10±0.43^{ns}	1.79±0.21^{ns}	1.88±0.27^{ns}
Platelet (x 10^3/µl)	678.60±29.43	669.40±24.14^{ns}	667.40±20.00^{ns}	661.20±15.83^{ns}
MPV (fl)	10.06±0.09	9.98±0.06^{ns}	10.02±0.14^{ns}	10.00±0.09^{ns}

RBC: Red blood corpuscle, Hb: Haemoglobin; MCV: Mean corpuscular volume, MCH: Mean corpuscular haemoglobin, MCHC: Mean corpuscular haemoglobin concentration, RCDW: Red cell distribution width, WBC: white blood corpuscle, MPV: Mean platelet volume, where n=6. Data were expressed as mean ± S.E.M. ns (not significant) when CS_0 v/s CS_1, CS_2 and CS_3

Table 3: Effect of *Coriandrum sativum* L. seed extract sub-chronic oral administration on plasma markers of heart, liver and kidney damage

		Groups			
		CS_0	CS_1	CS_2	CS_3
Cardiac damage markers	Creatinine kinase-MB (U/L)	40.60±5.42	43.72±7.40^{ns}	45.27±4.88^{ns}	40.79±4.06^{ns}
Hepatic damage markers	Aspartate transaminase (U/L)	29.50±2.06	26.50±2.07^{ns}	27.17±2.94^{ns}	30.83±3.34^{ns}
	Alanine transaminase (U/L)	22.60±2.20	19.40±2.40^{ns}	21.20±1.49^{ns}	21.60±3.23^{ns}
	Bilirubin (mg/dl)	1.86±0.22	1.79±0.14^{ns}	1.83±0.15^{ns}	1.97±0.10^{ns}
	Total protein (g/dl)	4.66±0.12	4.59±0.03^{ns}	4.85±0.25^{ns}	4.86±0.19^{ns}
Kidney damage markers	Urea (mg/dl)	58.69±6.39	58.92±4.44^{ns}	65.45±6.92^{ns}	62.30±6.96^{ns}
	Creatinine (mg/dl)	0.32±0.03	0.32±0.05^{ns}	0.36±0.04^{ns}	0.38±0.05^{ns}

where n=6. Data were expressed as mean ± S.E.M. ns (not significant) when CS_0 v/s CS_1, CS_2 and CS_3

Table 4: Effect of *Coriandrum sativum* L. seed extract sub-chronic oral administration on plasma glucose, lipid profile and lipoprotein profile

	Groups			
	CS_0	CS_1	CS_2	CS_3
Blood glucose (mg/dl)	146.20±4.97	155.7±7.06^{ns}	139.2±5.52^{ns}	134.2±4.71^{ns}
TC (mg/dl)	60.33±3.99	51.50±5.11^{ns}	44.17±5.40^{ns}	45.83±3.09*
TG (mg/dl)	16.50±1.26	11.83±1.38*	11.17±1.32*	10.33±2.15*
VLDL-C (mg/dl)	3.30±0.25	2.36±0.31*	2.23±0.23*	2.06±0.30*
LDL-C (mg/dl)	33.21±4.57^{ns}	25.61±5.62^{ns}	20.52±4.24^{ns}	20.74±3.34^{ns}
HDL-C (mg/dl)	23.55±1.14^{ns}	24.03±1.03^{ns}	23.30±0.66^{ns}	23.03±0.78^{ns}

where n=6. Data were expressed as mean ± S.E.M. ns (not significant) when CS_0 v/s CS_1, CS_2 and CS_3

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Table 5: Effect of *Coriandrum sativum* L. seed extract sub-chronic oral administration on relative organ weight

Organs (gm)	Groups			
	CS_0	CS_1	CS_2	CS_3
Lungs	0.51±0.01	0.54±0.01^{ns}	0.54±0.03^{ns}	0.54±0.02^{ns}
Heart	0.42±0.01	0.43±0.01^{ns}	0.42±0.01^{ns}	0.40±0.01*
Liver	3.58±0.13	3.72±0.08^{ns}	3.61±0.11^{ns}	3.50±0.10^{ns}
Kidney	1.10±0.05	1.16±0.05^{ns}	1.12±0.02^{ns}	1.00±0.03^{ns}
Spleen	0.30±0.01	0.27±0.01^{ns}	0.29±0.02^{ns}	0.30±0.17^{ns}

where, n=6. Data were expressed as mean ± S.E.M. ns (not significant) when CS_0 v/s CS_1, CS_2 and CS_3

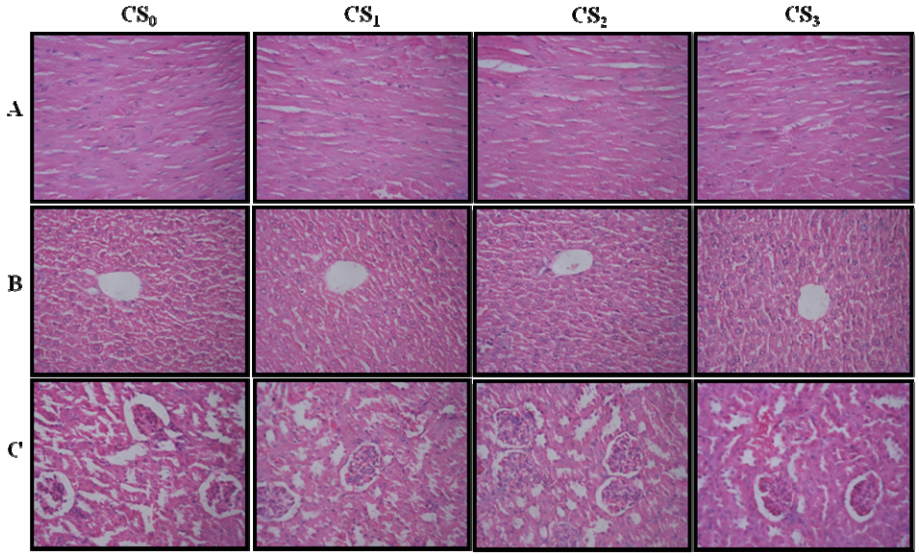

Figure 1: Photomicrographs of the sections of the heart (A), liver (B) and kidney (C) of control (CS_0) and CS administered (CS_1, CS_2 and CS_3) mice for 28 days showing no histoarchitecture change in CS treated groups as compared to control

DISCUSSION

Acute toxicity study recorded zero mortality at the end of 24 h period, following CS extracts administration. No behavioral alterations were recorded during the first four hours after administration of CS extract. Hence, the LD_{50} of CS extract is thought to be greater than 5000 mg and therefore CS extract can be considered as non-toxic up to the said dose (OECD 401).

Sub-chronic oral toxicity studies have provided information on drugs that can possibly pose health risks (Ministério de Saúde/Brasil, 2004). Twenty eight days of oral administration of CS (CS_2 and CS_3) extract showed significant decrement in food intake and body weight gain as compared to CS_0 mice. Significant reduction in food intake is suggested as responsible for the observed decrement in body weight gain. Loss of appetite is often synonymous with weight loss due to disturbances in carbohydrate, protein or fat metabolisms (Klaassen, 2001) and the same might be a possible reason for the weight loss in our study. CS (CS_2 and CS_3) treated mice also showed significant decrement in plasma TC, TG, LDL and VLDL whereas glucose and HDL levels were unchanged. These results indicate that higher doses of CS (CS_2 and CS_3) results in a reduction of food

intake and subsequent decrement in lipid profile whereas a lower dose (CS_1) does not lead to any such negative impact on metabolism.

CK-MB is an enzyme present in the myocardium that leaks out only under conditions of massive myocardial damage resulting from disintegration of contractile apparatus and increased sarcoplasmic permeability (Mair et al., 1994). Observed normal levels of CK-MB under all doses CS administration is reflective of its normal functional status and negligible damage. The same is further validated through the histology of heart of CS treated groups that reveals presence of intact myocardium. However, marginal increment in ROW of heart in CS_3 group is inexplicable and warrants further scrutiny.

High levels of AST and ALT are reported in liver diseases or hepatotoxicity (Brautbar and Williams, 2002). Plasma AST, ALT and bilirubin of CS_0 and CS treated groups were comparable thus indicative of normal functional status of liver. The ROW and histopathological observations of liver showed no significant changes following CS treatment.

Renal dysfunction can be assessed by concurrent measurements of urea and creatinine and their normal levels reflect at reduced likelihood of renal problems (Davis and Bredt, 1994). In the present study, changes in plasma urea and createnine levels in CS treated groups showed non-significant differences on a dose dependent manner indicating a normal renal function. Healthy status of the kidneys of CS treated groups was further confirmed by their histoarchitecture and ROW. However, higher urine output observed in CS_2 and CS_3 treated groups can be attributed to its diuretic property as reported earlier by other research groups (Aissaoui et al., 2008; Jabeen et al., 2009).

The haematopoietic system is one of the most sensitive targets for toxic compounds and hence it is mandatory to record any possible alterations resulting from a test substance (Olson et al., 2000). Change in haematological parameters has a higher predictive value, when the data of drug toxicity on animal studies are translated for clinical usage (Adeneye and Adokiye, 2008). A normal haematological profile of CS treated groups also further justified the non-toxic nature of CS extract.

CONCLUSION

In light of these findings, we may conclude that CS extract is not toxic in all the doses studied herein. This study is the first report that evaluates toxicity of CS extract and defines it as non-toxic up to a dose of 3000 mg/kg body weight.

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Coriandrum sativum L. aqueous extract mitigates high fat diet induced insulin resistance by controlling visceral adiposity in C57BL/6J Mice

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Coriandrum sativum L. aqueous extract mitigates high fat diet induced insulin resistance by controlling visceral adiposity in C57BL/6J Mice

[Coriandrum sativum L. extracto acuoso mitiga dieta rica en grasas de alta resistencia a la insulina inducida por el control de la adiposidad visceral en ratones C57BL/6J]

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Abstract

This study investigates the effect of dietary supplementation with *Coriandrum sativum* L. seed aqueous extract (CS) to a high fat diet (HFD), for induced insulin resistance (IR) C57BL/6J mice. Changes in body weight, food intake, feed efficiency ratio, fasting blood glucose (FBG), plasma insulin, fasting insulin resistance index (FIRI), plasma and hepatic triglyceride (TG), total cholesterol (TC) and, plasma free fatty acid (FFA) levels were evaluated in control and treated groups. Also, the diameter, surface area and number of adipocytes and, intraperitoneal glucose tolerance test (IPGTT) and intraperitoneal insulin response test (IPRTT) were performed. CS supplementation (1% and 3% w/w) to HFD fed mice (for 12 weeks) significantly prevented HFD induced increment in body weight gain, food intake, feed efficiency, FBG, plasma insulin, FIRI, plasma and hepatic TG and TC and, plasma FFA, adipocyte diameter and surface area along with decrement in adipocyte number. Also, improved responses were recorded in the IPGTT and IPRTT in CS supplemented HFD fed mice. These set of changes were comparable to the rosiglitazone (0.05%) supplemented HFD fed mice. Our findings suggest that CS improves insulin sensitivity primarily by mitigating plasma and tissue lipids and, adipocyte hypertrophy.

Keywords: *Coriandrum Sativum* L., high fat diet, Insulin resistance, adipocyte hypertrophy

Resumen

En este estudio se investigó el efecto de un extracto acuoso de semillas de *Coriandrum sativum* L. (CS), adicionado a una dieta con alto contenido graso en ratones C57BL/6J, con resistencia a la insulina inducida. Los cambios en el aumento de peso corporal, consumo de alimento, eficiencia alimenticia, glicemia, insulina plasmática, índice de resistencia a la insulina, triglicéridos hepáticos y plasmáticos, colesterol total y concentración plasmática de ácidos grasos libres, fueron evaluados en grupos control y tratados. Adicionalmente se controló, el diámetro, superficie y número de adipocitos, prueba de tolerancia a la glucosa intraperitoneal y la prueba de respuesta de la insulina por vía intraperitoneal. La adición de CS (1% y 3% w / w) a la dieta con alto contenido graso a ratones (12 semanas) previno de manera significativa el incremento de peso, la ingesta de alimentos, la eficiencia alimenticia, FBG, la insulina plasmática, FIRI, los triglicéridos hepáticos y plasmáticos, el colesterol total, ácidos grasos libres plasmáticos, el diámetro de los adipocitos y la superficie junto con el decremento en el número de los adipocitos. Además, mejoras de la respuesta se registraron en el IPGTT y IPRTT. Este conjunto de cambios fue comparable al obtenido con rosiglitazona (0,05%), adicionada a la dieta con alto contenido graso. Estos hallazgos sugieren que el CS mejora la sensibilidad a la insulina principalmente por la mitigación de los lípidos del plasma, del tejido y la hipertrofia del adipocito.

Palabras Clave: Dieta rica en grasas, resistencia, a la insulina, hipertrofia de los adipocitos

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INTRODUCTION

Changes in lifestyle and intake of caloric rich diets have predisposed the populace to obesity. Type 2 diabetes (T2D) is also closely associated with obesity and is characterized by an insulin resistance (IR) severely affecting glucose disposal (Frőde and Medeiros, 2008). Treatment of IR involves popular lipid lowering and insulin sensitizing drugs that have been associated with many side effects. Multiple drug usage is the necessary option for maintaining glycaemic levels and other associated manifestation in T2D patients (Gerich, 2001). These compelling reasons provide impetus for investigating the medicinal properties of herbs for use as alternatives in the treatment of T2D on a global scale. In this context, World Health Organization (WHO) has estimated that, about 25% of modern medicines are derived from plants and that, the global market for herbal medicine currently stands at over 60 million US$ annually (WHO, 2009).

Coriandrum sativum L. (Apiaceae) (CS) is an annual herb, the fresh leaves and dried seeds of which form part of Middle Eastern, Mediterranean, Indian, Latin American, African and Southeast Asian cuisines. Decoction and tincture of powdered seeds of CS alone or in combination with other herbal agents are recommended for dyspeptic complaints, loss of appetite, convulsion, insomnia and anxiety (Grieve, 1971). It is also used as medication against diabetes, indigestion, flatulence, renal disorders and as a diuretic agent in India and Morocco (Grieve, 1971; Emamghoreishi *et al*., 2005). It is also used in urethritis, cystitis, urinary tract infection, urticaria, rashes, burns, sore throat, vomiting, indigestion, nosebleed, cough, allergies, hay fever, dizziness and amoebic dysentery (Grieve, 1971; PDR-HM, 2004).

Phytochemical constituents of CS seeds have been studied extensively and its analysis has revealed the presence of polyphenols (rutin, caffeic acid derivatives, ferulic acid, galic acid, and chlorogenic acid), flavonoids (quercetin and isoquercetin) and β-carotenoids (Melo *et al*., 2003). The essential oil obtained from CS seeds contains α and β- pinene, camphor, citronellol, coriandrol, *p*-cymene, geraniol, geranyl acetate, limonene, linalool, myrcene, α and β phellandrene and α and β-terpinene. Also many fatty acids have been identified in the seeds oil. A large number of water soluble compounds have been identified including monoterpenoid glycosides, monoterpenoid glucose sulfate and other glycosides (Sergeeva, 1975; Ishikawa *et al*., 2003). The pharmacological activities of various extracts and of the essential oil of CS seeds have been also studied. The essential oil has been found to possess antimicrobial (Baratta *et al*., 1998) and antifungal properties (Garg and Siddiqui, 1992). Its efficacy as a hypoglycemic (Gray and Flatt, 1999), hypolipidemic (Chithra and Leelamma, 1997; Chithra and Leelamma, 1999; Lal *et al*., 2004), hypocholesterolemic (Dhanapakiam *et al*., 2008), antihypertensive (Medhin *et al*., 1986), antioxidant (Melo *et al*., 2003; Ramadan *et al*., 2003; Bajpai *et al*., 2005), antimutagenic (Cortes-Eslava *et al*., 2004), anxiolytic (Emamghoreishi *et al*., 2005), antimicrobial (Kubo *et al*., 2004; Cantore *et al*., 2004), larvicidal (Consoli *et al*., 1988) and post-coital antifertility (Al-Said *et al*., 1987) agent have also been reported.

As CS has been used as traditional anti-diabetic herbal agent and it's hypoglycemic and insulin secretor effects have been evaluated in streptozotocin induced diabetic rats (Eidi *et al*., 2009; Swanston-Flatt *et al*., 1990), the present study was designed to assess the efficacy of aqueous extract of CS in alleviating IR in high fat diet fed model of T2D.

MATERIALS AND METHODS

Plant Extract

CS seeds were procured from local ayurvadic medicinal shop of Vadodara, India. Hundred grams of powdered seeds were boiled at 100°C for 3 hrs in distilled water. Resulting filtrate was concentrated in a hot air oven until it formed a semisolid paste, which was then freeze dried. The yield was 12% w/w. Two doses (1% and 3%) of aqueous extract of CS were mixed with high fat diet.

Experimental Animals

Male C57BL/6J mice (6-8 weeks of age) were purchased from the National Centre for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition (NIN), Hyderabad, INDIA. They were housed and maintained in clean polypropylene cages and fed with standard laboratory diet (SLD) and water *ad libitum*. The experiments were carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and approved by the animal ethical committee of the Department of Zoology, The Maharaja Sayajirao University of Baroda, Vadodara (Approval No.827/ac/04/CPCSEA) .

Experimental Design

30 animals were randomly allocated into 5 groups of 6 animals each. Mice were fed with standard laboratory diet (SLD) or high fat diet (HFD) for 12 weeks (Jadeja *et al*., 2010; Thounaojam *et al*., 2010). CS or Rosiglitazone (ROS) were given to the experimental animals by mixing with HFD.

Group I (SLD): Mice were fed with SLD
Group II (HFD): Mice were fed with HFD
Group III (HFD+CS1%): Mice fed with HFD containing CS extract (1% w/w)
Group IV (HFD+CS3%): Mice fed with HFD containing CS extract (3% w/w)
Group V (HFD+ROS): Mice fed with high fat diet containing Rosiglitazone (0.05% w/w).

At the end of the experimental period, blood was collected from retro orbital sinus in EDTA coated vial under mild ether anesthesia. Plasma was obtained by cold centrifugation (4°C) of the vials for 10 min at 3000 rpm. Later, animals were sacrificed by cervical dislocation and epididymal fat pad were excised, weighted and fixed in 4% buffered paraformaldehyde.

Body weight, food intake and feed efficiency

Known quantity of food (SLD or HFD) was given to the respective experimental groups and food intake was measured daily. Feed efficiency ratio (FER) was expressed as the total weight gain of an experimental animal during 12 weeks ÷ the total food intake.

Plasma and hepatic lipids

Plasma free fatty acid (FFA) content was estimated by the method of Itaya and Ui, (1965) while, triglyceride (TG) and total cholesterol (TC) contents were estimated by using enzymatic kits (Reckon Diagnostics. Ltd, Vadodara, India) in a semi autoanalyser (Micro lab 300 L, Merck). Total lipids were extracted from liver of control and experimental animals with chloroform: methanol (2:1) (Folch *et al*., 1957) and hepatic Free fatty acids were assayed in the same (Itaya and Ui, 1965). Known quantity of lipid extract was than dissolved in 1% Triton X-100 (Thounaojam *et al*., 2010) and TC and TG were assayed using above kits.

Blood glucose, plasma insulin and fasting insulin resistance index (FIRI)

Animals were fasted overnight (for 12 hrs) and later blood glucose was measured in whole blood sample obtained from tail vein (by one touch glucometer, Sugar Scan, HMD BIOMEDICAL INC., India). Plasma insulin was assayed using Mouse ELISA kit (Mercodia Developing Diagnostics Ltd, Sweden). Fasting insulin resistance index (FIRI) was expressed as: - Fasting insulin (pmol/l) x Fasting blood glucose (mg/dl) ÷ 25.

Intraperitoneal glucose tolerance test (IPGTT)

Fasting (12 hrs) blood glucose was measured in whole blood (by one touch glucometer, Sugar Scan, HMD BIOMEDICAL INC., India) obtained from tail vein (0 min). Later, glucose solution was injected intraperitoneally (2 g/kg) and blood glucose was assayed at 30, 60, 90 and 120 min and the tolerance curves plotted. Area under the curve ($AUC_{glucose}$) was calculated based on the trapezoid rule (Graph Pad Prism version 3.0).

Intraperitoneal insulin response test (IPIRT)

Overnight fasted mice received insulin (Aventis Pharma Deutschland GmbH, Mumbai, India) 0.2 U/kg body weight by intraperitoneally. Blood samples were collected from tail vein at 0 min (before administration) and subsequently at 10, 20, 30 and 60 min after administration of insulin. Blood glucose was measured in whole blood (by one touch glucometer, Sugar Scan, HMD BIOMEDICAL INC., India). K_{ITT} was determined with the formula: $K_{ITT}= 0.693\times100 \div T_{½}$. Where $T_{½}$ is half-life of plasma glucose decay was obtained with the formula: $T_{½}= \ln2 \div \omega$. Where, ω constant of plasma glucose disintegration was obtained with the formula: $\omega = \ln C1 - \ln C2 \div T2 - T1$ with glucose concentration C1 at time T1 (10 min) and C2 at T2 (60 min) (Thounaojam *et al*., 2010).

Histology of epididymal fat pad

Epididymal fat pad was fixed in 4% buffered paraformaldehyde, dehydrated in graded alcohol series and embedded in paraffin wax. Five µm sections were cut (by Leica RM 2115 Microtome) and stained with hematoxyline and eosin (H&E) and examined under Leica microscope. Photographs of adipocytes were taken with Canon power shot S7 digital Camera (400 X). To quantify adipocyte number and diameter, the H&E stained sections were analyzed using an image analysis system (Image Pro-Plus, Silver Spring, MD).

Statistical analysis

Statistical analysis of the data was done by one way ANOVA followed by Bonferroni's multiple

comparison test and results were expressed as mean ± S.E.M (Using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego California USA).

RESULTS

Body weight gain and feed efficiency ratio

HFD group recorded significant increase in body weight gain but not in food intake and feed efficiency ratio as compared to SLD ($p<0.01$). HFD+CS (CS 1% & CS 3%) recorded significantly dose dependent decrement in body weight gain, food intake and feed efficiency ratio as compared to HFD fed mice (Table 1). HFD+ROS group also showed decrement of the said parameters which were comparable to HFD+CS1% but, HFD+CS3% was the most efficient in inducing.

Table 1. Effect of CS extract and Rosiglitazone on Body weight, Food intake and Feed efficiency ratio

	SLD	HFD	HFD+CS1%	HFD+CS3%	HFD+ROS
Initial body weight(g)	24.40±0.86	22.53±0.79	23.32±0.24	22.22±1.34	24.00±0.64
Final body weight(g)	29.00±0.70	32.42±0.28	30.32±0.30	25.50±1.12	31.16±0.59
Weight gain (g)	4.32±0.69	9.86 ± 0.59^{c}	7.03 ± 0.16^{B}	3.28 ± 0.36^{C}	7.16 ± 0.074^{B}
Food intake (g/day)	2.26±0.1979	3.72 ± 0.4195^{a}	2.39 ± 0.1234^{A}	1.88 ± 0.1137^{B}	3.02 ± 0.089^{NS}
Feed efficiency ratio	0.02±0.002	0.03 ± 0.001^{b}	0.03 ± 0.002^{NS}	0.02 ± 0.002^{A}	0.02 ± 0.002^{A}

Data are expressed as the mean ± S.E.M. Where, a) $p<0.05$, b) $p<0.01$, c) $p<0.001$ and ns: non significance when, SLD vs HFD. A) $p<0.05$, B) $p<0.01$, C) $p<0.001$ and NS: non significance when, HFD vs HFD+CS1%, HFD+CS3% and HFD+ROS

Plasma and hepatic lipid profile

Plasma and hepatic TC and TG levels and plasma FFA level were significantly elevated in HFD group as compared to SLD group ($p<0.01$). CS supplemented HFD fed groups (CS 1% & CS 3%) and HFD+ROS were significantly ($p<0.01$) able to attenuate the effect of HFD as was evident in form of decrement in levels of hepatic and plasma TC and TG and plasma FFA (Table 2).

Fasting blood glucose (FBG) and serum Insulin levels

FBG, plasma insulin level and FIRI were significantly higher in HFD group ($p<0.01$) as compared to SLD group. However, CS supplementation of HFD mice groups recorded significantly lowered levels of these parameters in dose dependent manner as compared to HFD group ($p<0.01$; Table 2).

Intraperitoneal glucose tolerance test and Intraperitoneal insulin response test

IPGTT of HFD fed mice recorded significant elevation in glucose level at 30 min that failed to return to its normal level at 120 min. $AUC_{glucose}$ of HFD fed mice was significantly higher compared to SLD mice ($p<0.01$). However, IPGTT of CS supplemented HFD mice showed dose dependent decrement in $AUC_{glucose}$ compared to that of HFD fed mice ($p<0.01$). HFD+ROS group also recorded a decrement in $AUC_{glucose}$ values compared to HFD group (Fig. 1).

IPIRT plots of glucose levels of HFD and SLD fed mice were comparable, however, CS supplemented HFD fed mice showed significant improvement in the IPIRT curves. The same was evident in the form of higher K_{ITT} values in these groups compared to the HFD group ($p<0.01$). HFD+ROS group also recorded higher K_{ITT} values of IPIRT as compared to HFD group (Fig. 2).

Table 2. Effect of CS extract and Rosiglitazone on plasma Glucose and serum Insulin

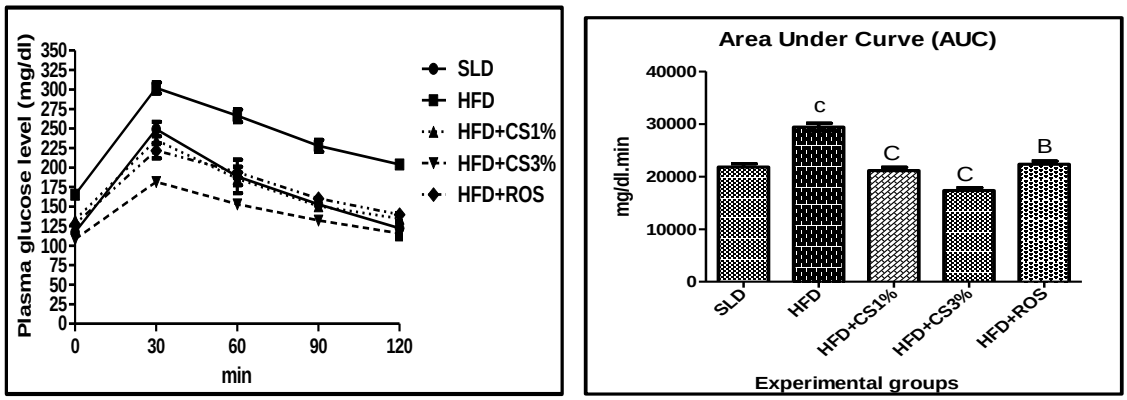
	SLD	HFD	HFD+CS1%	HFD+CS3%	HFD+ROS
Plasma					
TC(mg/dl)	68.50±10.56	142.70±7.63^{c}	103.00±7.0^{B}	74.80±8.23^{C}	81.25±11.09^{C}
TG(mg/dl)	50.00±5.22	152.30±20.67^{c}	102.30±9.24^{C}	76.50±4.85^{C}	70.25±11.69^{C}
FFA(mg/dl)	35.32±2.32	101.00±10.15^{c}	79.23±3.84^{NS}	37.97±2.08^{C}	50.28±3.70^{B}
Glucose(mg/dl)	110.00±5.21	162.80±3.47^{c}	131.30±10.71^{A}	117.00±8.31^{C}	128.00±4.35^{C}
Insulin(pmol/L)	38.89±3.81	79.00±4.34^{c}	56.43±2.96^{B}	44.97±4.44^{C}	46.70±3.41^{C}
FIRI	168.70±8.42	513.70±26.10^{c}	309.20±21.93^{C}	201.10±25.35^{C}	243.70±21.48^{C}
Liver					
TC(mg/dl)	17.23±2.34	50.75±7.33^{b}	34.75±5.07^{NS}	23.50±2.90^{B}	24.44±2.66^{B}
TG(mg/dl)	27.00±3.06	80.25±7.46^{c}	67.50±6.41^{NS}	33.65±5.49^{B}	44.25±4.34^{B}
Adipocyte					
Diameter(µm)	30.00±1.57	78.70±3.71^{c}	61.20±3.13^{B}	33.20±2.26^{C}	44.10±3.05^{C}
Surface area(µm^{2})	706.00±86	4862.00±125^{c}	2940.10±108^{B}	860.00±93^{C}	1520.60±122^{C}
Number(cells/1x10^{6} µm^{2})	941±81	177±76^{c}	580±66^{B}	864±62^{C}	748±75^{C}

Data are expressed as the mean ± S.E.M Where, p<0.05, b) p<0.01, c) p<0.001 and ns: non significance when, SLD vs HFD. A) p<0.05, B) p<0.01, C) p<0.001 and NS: non significance when, HFD vs HFD+CS1%, HFD+CS3% and HFD+ROS

Adipocyte diameter, number and surface area

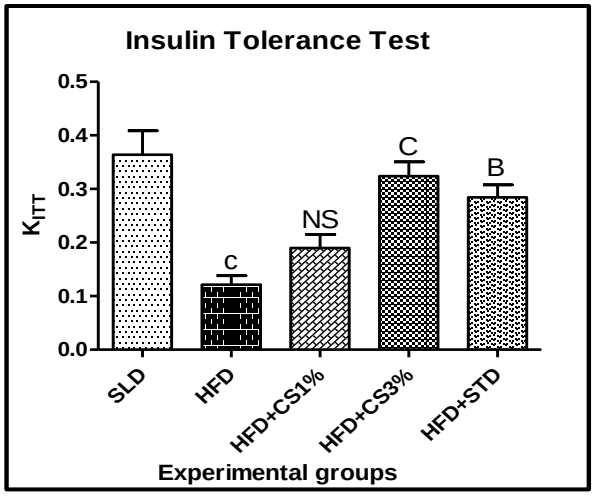
Microscopic examination of epididymal fat pad of HFD group recorded a significant increase in diameter and surface area of adipocytes compared to the adipocytes of SLD group (p<0.01). CS supplemented HFD groups showed adipocytes with mixed dimensions. However, the overall score of measurements of diameter and surface area of adipocytes recorded in HFD+CS (1% & 3%) were significantly lower than that of HFD group. The total number of adipocytes counted in a unit area in HFD+CS (1% & 3%) groups were significantly higher (p<0.01) than in the HFD group. These numbers were comparable to SLD or HFD+ROS groups (Table 2). HFD+ROS group also showed moderate decrement in diameter of adipocyte compared to HFD group.

Fig.1. Effect of CS extract and Rosiglitazone on IPGTT and AUC



Data are expressed as the mean ± S.E.M a) p<0.05, b) p<0.01, c) p<0.001 and ns: non significance when, SLD vs HFD. A) p<0.05, B) p<0.01, C) p<0.001 and NS: non significance when, HFD vs HFD+CS1%, HFD+CS3% and HFD+ROS

Fig.2. Effect of CS extract and Rosiglitazone on IPIRT and K_{ITT}



Data are expressed as the mean ± S.E.M a) p<0.05, b) p<0.01, c) p<0.001 and ns: non significance when, SLD vs HFD. A) p<0.05, B) p<0.01, C) p<0.001 and NS: non significance when, HFD vs HFD+CS1%, HFD+CS3% and HFD+ROS

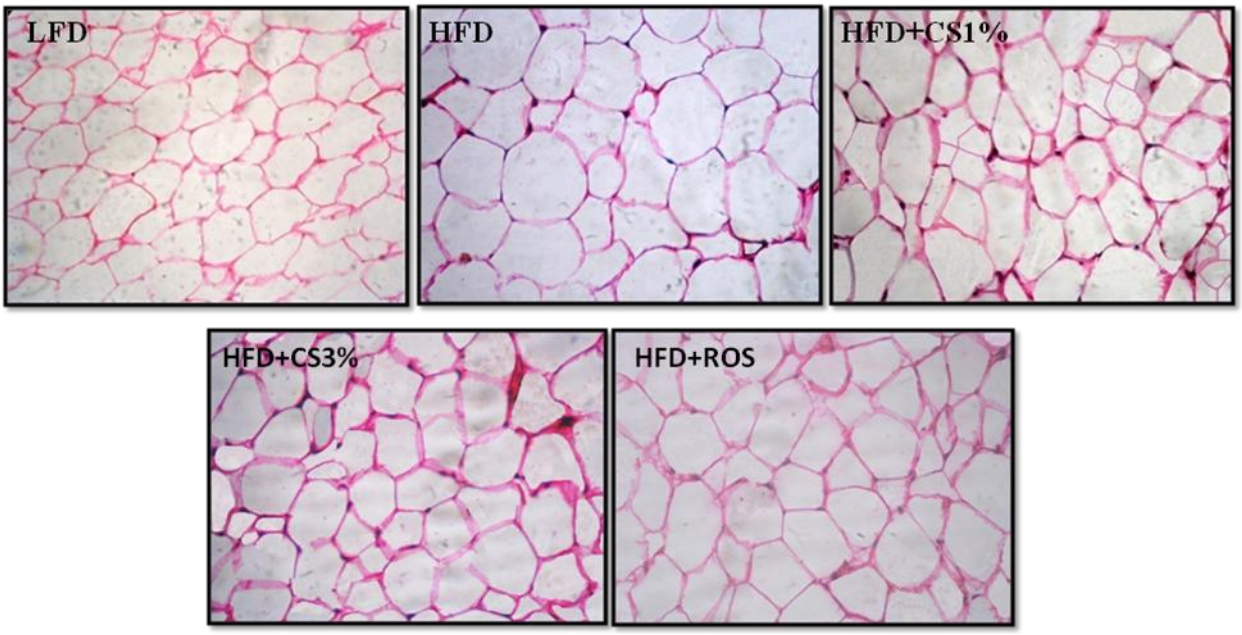
DISCUSSION

C57BL/6J mouse is a popular experimental model used in pre-clinical investigations of herbal and synthetic therapeutic agents against diabetes and obesity as there mice undergo a series of physiological changes when fed with high fat diet for 12-15 weeks. These changes are similar to the onset and progression of T2D and IR (Ahrén *et al*., 1997). In these mice, HFD induced IR is preceded by hyperlipidemia and visceral adiposity.

Our study has shown significant increment in body weight gain, food intake and feed efficiency ratio in HFD mice, well reflected in the form of significant increment in the weight of epididymal fat pad (personal communication). However, CS (1% & 3%) supplementation of HFD mice significantly prevented the characteristic body weight gain and increase in epididymal fat pad mass possibly due to decreased food intake. Previous studies have reported that insulin sensitivity in T2D patients improved with weight loss (DeFronzo and Ferrannini, 1991). CS supplemented HFD fed mice minimized the increase in hepatic and plasma TC and TG levels characteristic of HFD. These observations are in accordance with a previous report of Chithra and Leelamma (1997) on CS induced decrement in lipid profile of *Sprauge dawly* rats maintained on a hyperlipidemic diet. It has been reported that, increase in circulating level of plasma

FFA contributes to an increase in IR and inhibition of glucose uptake by skeletal muscles and other peripheral tissues (Boden *et al*., 1997). Also, in ob/ob mice, hyperinsulinemia develops due to decreased sensitivity towards insulin in liver, muscle and adipose tissues (Genuth *et al*., 1971). CS supplemented HFD mice (1% & 3%) were able to improve IR by decreasing levels of plasma FFA and insulin titer. Also, an improvement in fasting plasma glucose levels and FIRI values provide ample testimony to CS induced improvement of IR in HFD fed mice. IPGTT and IPIRT tests were carried out in control and experimental mice to assess CS induced possible improvement in insulin sensitivity and the results obtained were compared with HFD group. Lower $AUC_{glucose}$ values and higher K_{ITT} indices recorded in HFD+CS (1% & 3%) groups are attributable to improved insulin sensitivity in CS supplemented HFD fed mice. These mitigating changes are comparable to ROS induced changes in visceral adiposity and IR.

Figure 3. Photomicrograph of Epididymal fat pad showing the effect of CS extract and Rosiglitazone on adipocyte morphology



The histological manifested changes in adipocyte mass and size of HFD are attributable to diet induced lipogenesis. Co-presence of CS in HFD is able to resist the adiposity changes caused due to fat rich diet. Adipocyte hypertrophy is a strong evidence of visceral obesity and IR (Flier, 2004; Wellen and Hotamisligil, 2005) and in fact, the larger adipocytes are associated with IR and smaller ones with better insulin sensitivity (Okuno *et al*., 1998; Kubota *et al*., 1999; Kodowaki, 2000). The sizes of adipocytes in HFD and HFD+CS groups of mice in this context could easily reflect the higher IR in the former and greater insulin sensitivity in the later. The observations on adipocyte number and size in HFD+CS (1%) and HFD+CS (3%) also tend to suggest the dose dependent favorable influence of the extract in preventing diet induced visceral adiposity and IR.

The observed significant anti-hyperglycemic effect of CS extracts might also suggest increased peripheral glucose uptake as well as decreased transport of glucose across the intestinal epithelium (Gallagher *et al*., 2003). *In vivo* studies on this line and the possible role of CS extract to regulate the expression of PPARγ and other related genes in HFD mice are in progress.

CONCLUSION

This inventory is however first report that has investigated role of CS extract in improvement of HFD induced IR. These results are attributable to multiple physiological processes such as CS extract induced decrement in food intake, lowering of plasma and tissue lipids and decrement in size of adipocytes. Also, CS extract induced lowering of insulin, eventually leading to improvement of IR, improved insulin sensitivity and efficient clearance of glucose load, further corroborate these observations. Since, protective role of CS extract against STZ induced type

I diabetes has already been established, this study is an addition to its already established pharmacotherapeutic uses.

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Cardio protective effect of *Coriandrum sativum* L. on isoproterenol induced myocardial necrosis in rats

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ABSTRACT

The preventive effect of *Coriandrum sativum* L. (CS) on cardiac damage was evaluated by Isoproterenol (IP) induced cardiotoxicity model in male *Wistar* rats. Rats were pretreated with methanolic extract of CS seeds at a dose of 100, 200 or 300 mg/kg orally for 30 days and they were subsequently administered (*s.c.*) with IP (85 mg/kg body weight) for the last two days. IP treated rats showed increased LPO, decreased levels of endogenous antioxidants and ATPases in the cardiac tissue together with increased plasma lipids and markers of cardiac damage. TTC staining showed increased infarct areas while HXE staining showed myofibrillar hypertrophy and disruption. CS (200 and 300 mg/kg body weight) pretreatment significantly prevented or resisted all these changes. Our results show that methanolic extract of CS is able to prevent myocardial infarction by inhibiting myofibrillar damage. It is also concluded that, the rich polyphenolic content of CS extract is responsible for preventing oxidative damage by effectively scavenging the IP generated ROS.

1. Introduction

Epidemiological studies predict an ominous prevalence of cardio vascular diseases globally as well as in India during next decade (Lopez and Murray, 1998; Gilski and Borkenhagen, 2005). Myocardial infarction, a highly prevalent ischemic condition characterized by tissue necrosis develops essentially due to an imbalance between oxygen need and actual supply (De Bono and Boon, 1992) and results in irreversible histopathological damages and subsequent cardiovascular complications (Gross and Auchampach, 2007).

Isoproterenol (IP), a synthetic catecholamine and β-adrenergic agonist increases heart rate and exhaust energy reservoir of cardiac myocytes leading to cell death. It induces myocardial necrosis via multiple modes of action in experimental animals. It is essentially manifest by its stimulation of sarcolemmal adenylate cyclase and Na^+ and Ca^{2+} channels resulting in exaggerated influx of Ca^{2+} and energy consumption and consequent cell death (Milei et al., 1978). Free radicals produced by IP initiate the peroxidation of membrane bound polyunsaturated fatty acids (PUFAs) leading to both structural and functional myocardial injury (Thompson and Hess, 1986). IP-induced myocardial necrosis serves as an excellent experimental model to study catecholamines induced cardiac dysfunction and also to evaluate the possible cardioprotective efficacy of various natural and synthetic agents.

Coriandrum sativum L. (Apiaceae) (CS) is an ubiquitous annual herb, the leaves and seeds of which form a key ingredient of Middle Eastern, Mediterranean, Indian, Latin American, African and Southeast Asian cuisines. Apart from its usage as a condiment, decoction and tincture of powdered seeds of CS find usage either alone or in combination with other herbals in the treatment of cough, dysentery, sore throat, convulsion, insomnia and anxiety (Grieve, 1971). An extract of CS seeds is also reported to have therapeutic potential against diabetes, cardiovascular and urinary disorders (Eguale et al., 2007; Emamghoreishi et al., 2005). Phytochemical analysis of CS seeds has revealed the presence of polyphenols (rutin, ferulic acid, galic acid, chlorogenic acid and caffeic acid derivatives), flavonoids (quercetin and isoquercetin) and β-carotenoids (Melo et al., 2003). The oil of CS seeds is rich in α and β-pinene, camphor, citronellol, coriandrol, *p*-cymene, geraniol, geranyl acetate, limonene, linalool, myrcene, α and β phellandrene and terpinene besides many water soluble compounds such as monoterpenoid glycosides and their derivatives (Sergeeva, 1975; Ishikawa et al., 2003). The reported pharmacological actions of CS are many with its oil shown to possess antifungal (Garg and Siddiqui, 1992) and antimicrobial (Baratta et al., 1998) properties and seed extract shown to possess hypoglycemic (Gray and Flatt, 1999), hypolipidemic (Chithra and Leelamma, 1997; Chithra and Leelamma, 1999; Lal et al., 2004), hypocholesterolemic (Dhanapakiam et al., 2008),

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anti-insulin resistance activity (Patel et al., 2011), antihypertensive (Medhin et al., 1986) and antioxidant (Melo et al., 2003; Ramadan et al., 2003; Bajpai et al., 2005) competence.

Several pre-clinical and clinical studies involving pretreatment with vitamins and antioxidants have demonstrated their potential to prevent myocardial damage (Singh et al., 1994; Senthil et al., 2004). Previously Hashim et al. (2005) have investigated that hydro-methanolic extract of CS seed had strong antioxidant property and it had prevented oxidative damage induced by H_2O_2 to lymphocytes. The present study was designed to assess cardioprotective potential of hydro-methanolic extract of the customarily used spice CS seeds in IP induced multifocal myocardial necrosis in rats.

2. Materials and methods

2.1. Plant material and preparation of extract

CS plants were collected in the seedling months (February and March) and Dr. P.S. Nagar, Department of Botany, The M.S. University of Baroda identified the plant and a sample specimen was deposited in the herbarium of the Department of Botany. Hundred grams of powdered dry seeds soaked in methanol:water (80:20 v/v) at room temperature was allowed to stand for seven days. Resultant extract filtered through a muslin cloth was concentrated in a rotary evaporator under reduced pressure to obtain a thick semisolid brown paste (Qaiser et al., 2009). The final yield obtained was 8.3 g (w/w).

2.2. Experimental animals

Adult male *Wistar* rats (150–200 gm; obtained from Zydus Cadila Research Centre, Ahmedabad, Gujarat, India) were housed under standard animal house conditions (23 ± 2 °C; LD 12:12 and 45–50% humidity) and provided with pelleted diet (M/S Pranav agro, Ltd., Baroda, India) and water *ad libitum*. The animals were maintained as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) India and the experimental protocol approved by the animal ethical committee of the Department of Zoology, The M.S. University of Baroda, Vadodara (Approval No.827/ac/04/CPCSEA).

2.3. Experiment design

Thirty animals were randomly divided into five groups of six animals each. Group I (NC) served as control and received 0.5% Carboxy methyl cellulose (CMC; *p.o.*) for 28 days and normal saline (*s.c.*) on days 29 and 30. Group II (IP) served as positive control rats and received 0.5 CMC (*p.o.*) for 28 days and isoproterenol (85 mg/kg body weight, *s.c.*) on days 29 and 30 while, the remaining groups [Group III (IP + CS100), Group IV (IP + CS200) and group V (IP + CS300)] received respectively 100, 200 and 300 mg/kg body weight of CS extract daily for 28 days (*p.o.*) and IP (85 mg/kg, *s.c.*) on days 29 and 30. The protocol for IP treatment schedule was as per the previous works from this laboratory (Jadeja et al., 2010; Thounaojam et al., 2011). At the end of the experimental period (i.e. 31st day), animals were fasted overnight (12 h) and blood samples were collected from retro-orbital sinus under mild ether anesthesia. Plasma was obtained by cold centrifugation of samples at 3000 rpm for 10 min. Later, animals were sacrificed by cervical dislocation under mild anesthesia and heart was excised and stored at −80 °C for further evaluations. A piece of cardiac tissue was fixed in 10% paraformaldehyde for paraffin wax histology.

2.4. Plasma markers of cardiac damage

Plasma levels of creatine phospokinase- MB (CK-MB), lactate dehydrogenase (LDH), aspartate transaminase (AST), alanine transaminase (ALT) and uric acid were ascertained by using commercially available kits (Reckon Diagnostic Ltd., Vadodara, India).

2.5. Plasma lipid profile

Triglyceride (TG), total cholesterol (TC) and high density lipoprotein (HDL) content were assayed by using commercially available kits (Recon Diagnostic, Ltd., Vadodara, India). Lowdensity lipoprotein (LDL) and Very low-density lipoprotein (VLDL) were calculated by Friedewald"s formula (Friedewald et al., 1972).

2.6. Cardiac antioxidants and Lipid peroxidation (LPO)

Cardiac tissue from control and treated groups was weighed and homogenized (10%w/v) in chilled Tris buffer (10 mM; pH 7.4) and centrifuged at 10,000 g for 20 min at 0 °C. Clear supernatant was used to assay superoxide dismutase (SOD; Marklund and Marklund, 1974), catalase (CAT; Aebi, 1983), glutathione peroxidase (GPx; Rotruck et al., 1973), glutathione s-transferase (GST; Habig et al., 1974), reduced glutathione (GSH; Beutler, 1963), vitamin E (Vit. E; Baker and Frank, 1968), total protein content (Lowry et al., 1951) and lipid peroxidation levels (LPO; Buege and Aust, 1978). Total ascorbic acid content (AA) was measured as per Roe and Küether (1943) by preparing homogenates of fresh cardiac tissue in 6% Trichloro acetic acid.

2.7. Cardiac ATPases

Pellets obtained from tissue homogenate after centrifugation was re-suspended in ice-cold Tris buffer (10 mM, pH 7.4) to get a final concentration of 10% and was used for the estimation of Na^+ K^+ ATPase (Bonting et al., 1970), Ca^{2+} ATPase (Hjerken and Pan, 1983) and Mg^{+2}ATPase (Ohinishi et al., 1982). Protein was estimated according to the method of Lowry et al. (1951).

2.8. Macroscopic and microscopic evaluation of cardiac tissue

Heart tissue slices (approx. 2–3 mm thick) transversely cut across the ventricle were kept in a covered glass dish containing 1% TTC (2, 3, 5- triphenyltetrazolium chloride; Sigma, St. Louis, MO) solution and incubated at 37 °C for 20 min for differentiation of viable tissue from necrotic areas (Li et al., 2011).

Heart samples from control and treated rats were fixed in 4% buffered paraformaldehyde, dehydrated in graded alcohol series and embedded in paraffin wax. Five micrometer thick sections cut (by Leica RM2155 Microtome) and stained with haematoxylin-eosin, were photographed with Canon power shot S72 digital Camera (200×) attached to a Leica microscope.

2.9. Statistical analysis

Statistical analysis of data was done by one way ANOVA followed by Bonferroni's multiple comparison test and results were expressed as mean ± S.E.M (Using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego California USA).

3. Results

3.1. Plasma markers of cardiac damage

IP treated rats showed significant ($p < 0.005$) increment in the plasma levels of CK-MB, LDH, AST, ALT and uric acid compared to NC rats. Pretreatment of IP rats with CS prevented the IP induced increase in the serum levels of these parameters in a dose dependent manner (Table 1).

3.2. Plasma lipid profile

IP treatment recorded significant ($p < 0.005$) increase in plasma TG, TC, LDL, and VLDL and decrement in HDL levels compared to the NC group. CS treatment showed dose dependent decrement

Table 1
Effect of CS seed extract on plasma markers of cardiac damage.

Parameters	NC	IP	IP + CS100	IP + CS200	IP + CS300
CkMB$^{\$}$	75.66 ± 6.91	218.20 ± 29.16^{c}	171.20 ± 9.19^{B}	133.10 ± 6.16^{C}	80.10 ± 9.03^{C}
LDH$^{\#}$	82.71 ± 6.50	189.60 ± 7.36^{c}	149.70 ± 4.32^{C}	126.00 ± 4.15^{C}	85.60 ± 6.35^{C}
AST*	30.33 ± 1.99	61.17 ± 2.24^{c}	50.33 ± 1.76^{B}	43.50 ± 1.91^{C}	31.67 ± 1.02^{C}
ALT*	19.33 ± 1.11	44.83 ± 2.18^{c}	36.67 ± 1.82^{A}	31.00 ± 1.73^{C}	22.17 ± 1.99^{C}
Uric acid$^{@}$	1.91 ± 0.21	7.01 ± 0.47^{c}	5.24 ± 0.41^{A}	3.72 ± 0.19 ^{C}	2.14 ± 0.21^{C}

Where, $ = IU/l, # = U/l, * = KA Units/l, @ = mg/dl. $n = 6$. Data were expressed as mean ± S.E.M. a ($p < 0.05$), b ($p < 0.01$), c ($p < 0.001$) when NC vs. IP and A ($p < 0.05$), B ($p < 0.01$), C ($p < 0.001$) when IP vs. IP + CS.

Table 2
Effect of CS seed extract on plasma lipid profile.

Parameters	NC	IP	IP + CS100	IP + CS200	IP + CS300
TC[@]	54.50 ± 1.72	90.83 ± 2.58[c]	75.83 ± 2.16[B]	65.33 ± 2.88[C]	59.50 ± 2.23[C]
TG[@]	33.17 ± 2.37	53.00 ± 2.67[c]	50.67 ± 1.82[NS]	44.67 ± 2.60[C]	35.00 ± 1.48[C]
VLDL [@]	6.63 ± 0.47	10.60 ± 0.53[c]	10.13 ± 0.36[NS]	8.93 ± 0.52[A]	7.00 ± 0.29[C]
LDL[@]	34.30 ± 0.52	90.27 ± 2.83[c]	70.97 ± 2.62[C]	58.10 ± 3.92[C]	43.67 ± 2.75[C]
HDL[@]	26.83 ± 0.98	11.17 ± 1.04[c]	15.00 ± 0.73[NS]	16.17 ± 1.35 [A]	22.83 ± 0.79[C]

Where, @ = mg/dl. $n = 6$. Data were expressed as mean ± S.E.M. a ($p < 0.05$), b ($p < 0.01$), c ($p < 0.001$) when NC vs. IP and A ($p < 0.05$), B ($p < 0.01$), C ($p < 0.001$) when IP vs. IP + CS.

Table 3
Effect of CS seed extract on Cardiac LPO levels and enzymatic and non-enzymatic anti-oxidant.

Parameters	NC	IP	IP + CS100	IP + CS200	IP + CS300
LPO*	0.96 ± 0.53	3.22 ± 0.27[c]	2.05 ± 0.73[B]	1.61 ± 0.28[B]	1.27 ± 0.57[B]
SOD	8.06 ± 0.63	3.46 ± 0.60[c]	6.12 ± 0.37[B]	6.54 ± 0.19[C]	6.98 ± 0.17[C]
CAT[@]	5.15 ± 0.43	1.92 ± 0.31[c]	2.23 ± 0.14[NS]	3.06 ± 0.29[A]	4.40 ± 0.34[C]
GPx[$]	3.08 ± 0.05	1.03 ± 0.06[c]	1.22 ± 0.06[A]	1.81 ± 0.07[C]	2.80 ± 0.04[C]
GST[#]	787.4 ± 14.41	423.6 ± 12.63[c]	541.4 ± 10.19[C]	625.7 ± 11.34[C]	760.7 ± 16.06[C]
GSH[@]	9.27 ± 0.17	3.81 ± 0.22[c]	4.79 ± 0.26[A]	5.87 ± 0.31[C]	7.81 ± 0.18[C]
AA[€]	250.0 ± 5.0	114.0 ± 7.0[c]	140.0 ± 8.0[A]	195.0 ± 12.0[C]	229.0 ± 9.0[C]
Vit E[€]	5.29 ± 0.23	1.33 ± 0.31[c]	2.65 ± 0.36[NS]	3.32 ± 0.21[C]	4.91 ± 0.29[C]

* = µmol/mg protein, @ = nmol/mg protein, $ = unit/mg protein, # = µmol/min/mg protein, € = mg/100 g tissue Where, $n = 6$. Data were expressed as mean ± S.E.M. a ($p < 0.05$), b ($p < 0.01$), c ($p < 0.001$) when NC vs. IP and A ($p < 0.05$), B ($p < 0.01$), C ($p < 0.001$) when IP vs. IP + CS.

Table 4
Effect of CS seed extract on Cardiac ATPases.

Parameters	NC	IP	IP + CS100	IP + CS200	IP + CS300
Na^+/K^+ATPase[@]	5.00 ± 0.50	2.11 ± 0.17[c]	2.51 ± 0.14[NS]	2.96 ± 0.33[A]	4.40 ± 0.28[C]
Mg^{2+} ATPase[@]	2.74 ± 0.02	0.45 ± 0.03[c]	0.84 ± 0.05[C]	1.02 ± 0.08[C]	2.09 ± 0.09[C]
Ca^{2+} ATPase[@]	2.03 ± 0.18	0.98 ± 0.02[c]	1.29 ± 0.02[C]	1.46 ± 0.04[C]	1.83 ± 0.04[C]

Where, @ = µmol phosphate liberated/ mg protein. $n = 6$. Data were expressed as mean ± S.E.M. a ($p < 0.05$), b ($p < 0.01$), c ($p < 0.001$) when NC vs. IP and A ($p < 0.05$), B ($p < 0.01$), C ($p < 0.001$) when IP vs. IP + CS.

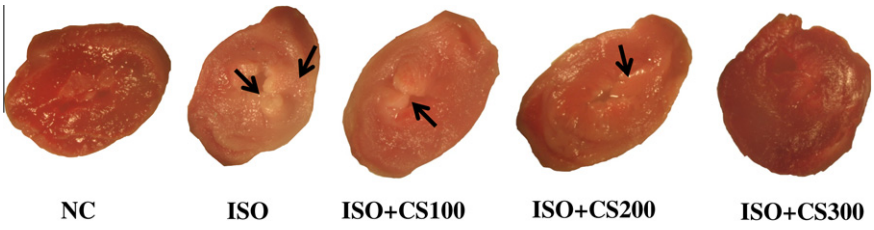

Fig. 1. Effect of CS seed extract on triphenyltetrazolium chloride (TTC) stained cardiac tissue slices. Arrows indicate necrotic tissue.

in TC, TG, LDL, VLDL and significant increment in HDL compared to IP treated rats (Table 2).

3.3. Cardiac anti-oxidants and LPO

IP treated group recorded significant ($p < 0.001$) increment in LPO level, as well as significant ($p < 0.001$) decrement in the activities of enzymatic antioxidants (SOD, CAT, GPx and GST) and content of non-enzymatic antioxidants (GSH, AA and Vit. E) compared to NC rats. Administration of CS (100, 200 and 300 mg/kg body weight, respectively) markedly prevented all the alterations with respect to antioxidants and LPO in IP treated rats and maintained them to the near normal levels (Table 3).

3.4. Cardiac ATPase

The cardiac tissue of IP treated rats depicted significant ($p < 0.005$) decrement in the activities of Na^+/K^+, Mg^2 and Ca^{2+}ATPases compared to that of NC rats while, IP + CS treated cardiac tissue recorded significant resistance (Table 4).

3.5. TTC and HE staining of cardiac tissue

TTC staining of heart of control rats showed brick red coloration indicative of more number of viable cells whereas, IP treated rats showed large area of pale yellow coloration was suggestive of necrosis. However, IP rats pretreated with CS showed a protective effect with a minimal or no pale yellow coloration in a dose dependent manner (Fig. 1).

HE staining of cardiac tissue from NC rats showed histoarchitecture of myofibers that were characteristically multinucleated and intact. IP treatment resulted in focal myocardial necrosis (encircled area) and disrupted myofibers. However, IP + CS treated groups showed relatively less disruption of myofibers with IP + CS 300 showing maximum fiber integrity. (Fig. 2).

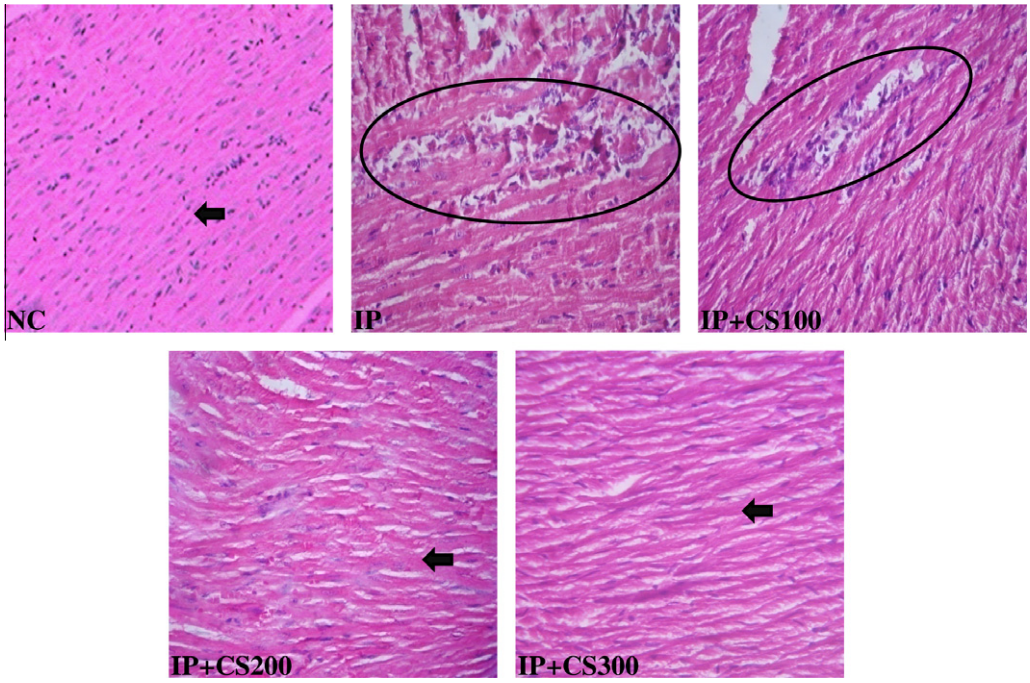

Fig. 2. Effect of CS seed extract on cardiac histopathology of cardiac tissue. Tissue sections (7 μM) are stained with hematoxylin-eosin (400×). Encircled area indicates focal myocardial necrosis whereas, arrows indicate healthy myofibers.

4. Discussion

Administration of higher doses of IP to rats induces increment in heart rate, systolic and diastolic irregularities and abnormal ECG pattern (Rona, 1985; Karthick and Prince, 2006). These events epitomized by hypoxia, calcium over load and increased production of reactive oxygen species (ROS) lead to degenerative changes in cardiac tissue that culminate in necrosis.

Accordingly, IP treated rats herein recorded significant increment in plasma levels of CK-MB, LDH, AST, ALT and uric acid, which is in keeping with the known IP induced deficiency of oxygen supply and increased sarcolemmal permeability and consequent leaching of CK-MB and LDH into the blood stream along with increased plasma levels of AST, ALT and uric acid (Mathew et al., 1985; Weir et al., 2003). The recorded ability of CS to effectively prevent these alterations clearly points towards its cardio-protective competence and maintenance of sarcolemmal integrity.

Also the activity levels of 3-hydroxy-3-methyl-glutaryl-CoA (HMG CoA) reductase and Lecithin-cholesterol acyltransferase (LCAT) have been reported to undergo significant alterations following IP treatment which resulted in altered lipid and lipoprotein profiles (Rajadurai et al., 2006). Hence, the observed decrement in lipid profile in IP + CS treated groups indicates at possible modulatory influence of CS on activity levels of HMG CoA and LCAT that requires further investigations.

SOD and CAT are enzymatic antioxidants that act as the first line of cellular defense and help in scavenging free radicals. Therefore, a decrement in their activity levels results in free radical induced cellular damage. Other enzymatic antioxidants GPx and GST and non-enzymatic GSH also help maintain healthy cell functions by scavenging free radicals like peroxy radicals, superoxide ions and singlet oxygen formed by toxicants (Rathore et al., 1998). AA is a water soluble vitamin that acts as an antioxidant and scavenger of superoxide and other free radicals, getting transformed in the process to dehydroascorbate (Frei et al., 1986; Packer et al., 1979). Vitamin E is a lipid soluble antioxidant that protects membrane polyunsaturated fatty acids and other components from oxidation by free radicals (Tappel, 1972). Presently, we have observed increased LPO and decreased endogenous antioxidants (both enzymatic and non-enzymatic) in IP treated rats. Apparently, IP causes heightened oxidative damage of cellular macromolecules marked by elevated level of LPO by way of increased generation of free radicals as has also been inferred by Gokkusu and Mostafazadeh (2003). However, pretreatment of IP animals with CS prevented the decrease in antioxidant levels and increase in LPO significantly in a dose dependent manner. Plant based extracts that are rich in polyphenols and flavonoids are supposedly strong antioxidants and CS seed extract has been reported to be rich in flavonoids, terpenoids (Wangensteen et al., 2004) and polyphenols (Hashim et al., 2005). The latter workers have opined that alcoholic extract of CS has maximal content of the said antioxidants compared to other types of extract. The currently observed effects of CS may be attributable to the presence of these secondary metabolites.

ATPases, by maintaining differential levels of ions play important roles in the regulation of contraction–relaxation cycles of cardiac muscles and consequently, peroxidation of sarcolemmal lipids can result in their inactivation as suggested by Kako et al. (1988). Reduced activity of Na^+/K^+ ATPase with compromised Na^+ efflux can result in altered membrane permeability (Finotti and Palatini, 1986). A decrement in Ca^{2+}ATPase expectantly would decrease sarcoplasmic Ca^{2+} concentration and weaken the contractility of heart. Hence, loss of ATPase activity in the ischemic state could contribute to myofibrillar necrosis and functional damage. Even Chernysheva et al. (1980) have reported IP induced decrement in the activity levels of Na^+/K^+, Mg^{2+} and Ca^{2+} ATPase in rats. However, IP + CS treated rats show a dose dependent significant up keep of these ATPases, essentially attributable to the membrane stabilizing aproperty of CS extract that protects the sarcolemma and intracellular membranes from the deleterious effect of IP and consequent myocardial damage (Hashim et al., 2005).

TTC is a redox indicator that is commonly used to differentiate between metabolically active and inactive cells and tissues (Altman, 1976). Staining of cardiac tissue slices with TTC provides

insight regarding the infarct size and is a well accepted method to assess necrosis of myocardial tissue (Prabhu et al., 2006). TTC is enzymatically reduced to brick red precipitates of formazan dye or TPF (1,3,5-triphenylformazan). Active mitochondrial respiration generating reduced coenzymes is responsible for the reduction of TTC to TFP in all tissues including the cardiac tissue (Ramkissoon, 1996). Hence, appearance of patches of pale white color in cardiac tissue slices of IP treated rats indicates areas of focal necrosis due to non-reduction of TTC as observed in the present study in IP treated rats. The IP + CS rats (especially CS300) depicted minimal pale yellow patches suggestive of normal myocardial structure. Histological observations further confirm the IP induced necrotic changes affecting myofiber disruption and fraying of fibers. These deleterious changes seem ably resisted by pretreatment with CS with the highest dose affording maximal protection. These observations provide compelling macroscopic and microscopic evidences regarding the cardioprotective potential of CS seed extract.

Parameters investigated here in indicate that hydro-methanolic extract of CS is potent in mitigating IP induced myocardial necrosis. The same is evidenced inform of CS induced favourable alterations in biochemical and histo-morphological parameters. Although the observed results have been attributed to high content of polyphenols in hydro-methanolic extract of CS. Our further studies are aimed that isolating the active component of CS and to reassess its cardioprotective potential in more appropriate experimental model (coronary ligation) and using gold standard marker enzyme such as cardiac Troponin I that underlying mechanism of CS induced cardioprotection.

5. Conclusion

It can be concluded from the present study that hydro-methanolic extract of CS seeds has cardioprotective potential. The same is attributable to high polyphenol content in CS seeds.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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