Beneficial effect of *Tribulus terrestris* L. against ischemia in H9c2 cells and isoproterenol induced cardiac dysfunctions in rats

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By

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CERTIFICATE

This is to certify that the work embodied in the thesis entitled "**Beneficial effect of *Tribulus terrestris* L. against ischemia in H9c2 cells and isoproterenol induced cardiac dysfunctions in rats**" has been carried out by **Ms. Reshma P.L.**, under my supervision and guidance at Agroprocessing and Natural Products Division of Council of Scientific and Industrial Research-National Institute for Interdisciplinary Science and Technology (CSIR-NIIST), Thiruvananthapuram in partial fulfilment of the requirements for the award of degree of Doctor of Philosophy in Biotechnology under Faculty of Science, Cochin University of Science and Technology, Kochi, Kerala, India and the same has not been submitted elsewhere for any other degree. All the relevant corrections, modifications and recommendations suggested by the audience and the doctoral committee members during the pre-synopsis seminar of **Ms. Reshma P.L.** has been incorporated in the thesis.

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DECLARATION

I hereby declare that the thesis entitled "**Beneficial effect of *Tribulus terrestris* L. against ischemia in H9c2 cells and isoproterenol induced cardiac dysfunctions in rats** " embodies the results of investigations carried out by me at Agroprocessing and Natural Products Division of Council for Scientific and Industrial Research - National Institute for Interdisciplinary Science and Technology (CSIR-NIIST), Thiruvananthapuram as a full time research scholar under the supervision of Dr. K.G. Raghu and the same has not been submitted elsewhere for any other degree. In keeping with the general practice of reporting scientific observations, due acknowledgements has been made wherever the work described is based on findings of other investigators.

Reshma P. L.

Thiruvananthapuram,
May 2016.

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Reshma P L

…….TO MY FAMILY

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Chapter 2: Materials and Methods

PREFACE

Myocardial ischemia is caused by the inadequate supply of blood to the myocardium usually as a result of coronary artery disease. Cardiovascular disease is the leading cause of death worldwide and coronary artery disease is the greatest contributor, with 7.5 million deaths annually. Currently available drugs are able to alleviate the symptoms but have undesirable effects and secondary complications. *Tribulus terrestris* L. root and fruit are used in Ayurveda to treat heart ailments. But a scientific validation of the protective property of the herb against myocardial ischemia and the mechanism by which *Tribulus terrestris* L. mediates the protection has not been carried out. The objective of the present thesis is to elucidate the molecular mechanism of the beneficial property of *Tribulus terrestris* L. fruit and root against myocardial ischemia and cardiac dysfunctions by employing *in vitro* and *in vivo* models.

The entire thesis is divided into seven chapters. The introductory chapter deals with prevalence and history of heart diseases, the pathophysiology of coronary artery disease and myocardial ischemia, cell death and signaling pathways in myocardial ischemia, current drugs used to treat myocardial ischemia and traditional medicine from natural sources to treat heart diseases. The second chapter deals with the materials and methods. The third chapter deals with the anti-ischemic property of *Tribulus terrestris* L. root methanol extract (TTM) mediated by its antioxidant potential and regulation of apoptotic and necrotic cell death. Cell death markers like Bax, Bad and Bcl-2 and the MAPK signaling pathways (JNK, p38α and Akt) involved in the prevention of cell death in an *in vitro* model of myocardial ischemia using H9c2 cell line was also studied. Further, presence of chlorogenic acid, caffeic acid and 4-hydroxybenzoic acid enhanced its anti-ischemic potential. The fourth chapter deals with the beneficial property of *Tribulus terrestris* L. root methanol extract (TTM) in an isoproterenol (85 mg/kg, s.c.) induced myocardial infarction in rat model. Evaluation of cardiac biomarkers, histopathological alterations and alterations in physiological parameters such as ECG were studied. Effect of TTM in the modulation of pro-inflammatory cytokines IL-6, TNF-α, MCP-1, and IL-1β and anti-inflammatory cytokine IL-10 was also studied. The fifth chapter deals with the mitochondrial alterations induced during ischemia in H9c2 cardiomyoblasts and the possible amelioration with *Tribulus terrestris* L. fruit methanol

extract (TFM). Investigations were conducted on various mitochondrial parameters like activity of electron transport chain (ETC) complexes, oxygen consumption, ATP production, mitochondrial integrity, and mitochondrial membrane potential and mitochondrial dynamics. Further, presence of bioactives like ferulic acid, phloridzin and diosgenin in *Tribulus terrestris* L. fruit methanol extract contributes to its therapeutic potential. The sixth chapter deals with the protective property of TFM in an *in vivo* model of myocardial infarction using isoproterenol. The redox regulatory pathway, nitric oxide synthase, mitochondrial biogenesis and inhibition of a novel cardiac specific MAPK, TNNI3Kwas studied.

The seventh chapter deals with summary and conclusion. From this study we conclude that both fruits and roots of *Tribulus terrestris* L. are protective against ischemia and β - adrenergic overstimulation induced cardiac dysfunctions but the mechanism of protection may be different. Thus we conclude that both fruits and roots can be consumed for the prevention and management of heart diseases.

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LIST OF ABBREVIATIONS

ΔΨm	: mitochondrial transmembrane potential
Apaf1	: Apoptotic protease activating factor 1
Ask-1	: Apoptosis signal-regulating kinase 1
ATP	: Adenosine triphosphate
Bax	: Bcl-2 associated X protein
Bad	: Bcl-2 associated death promoter
Bcl-2	: B cell lymphoma 2
Bcl-xL	: B cell lymphoma - extra large
BSA	: Bovine serum albumin
CAT	: Catalase
CAD	: Coronary artery disease
CK-MB	: Creatine phosphokinase myocardial specific isoenzyme
CVD	: Cardiovascular disease
DCFH-DA	: Dichloro-dihydro-fluorescein diacetate
DMEM	: Dulbecco's Modified Eagle's medium
Drp1	: Dynamin related protein 1
DMSO	: Dimethyl sulfoxide
EDTA	: Ethylene diamene tetraacetic acid
EGTA	: Ethylene glycol tetraacetic acid
ELISA	: Enzyme linked immunosorbent assay
FBS	: Foetal bovine serum
FITC	: Fluorescein isothiocyanate
GPx	: Glutathione peroxidase
GRD	: Glutathione reductase

GSH : Reduced glutathione

HIF-1α : Hypoxia inducuble factor -1α

IκBα : Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor

IL-1β : Interleukin -1 beta

IL- 6 : Interleukin – 6

I/R : Ischemia / Reperfusion

JNK : c-Jun N-terminal kinase

LDH : Lactate dehydrogenase

MCP-1 : Monocyte chemo-attractant protein - 1

Mfn1 : Mitofusin -1

Mfn2 : Mitofusin-2

MAPK : Mitogen activated protein kinases

MI : Myocardial Infarction

mPTP : Mitochondrial permeability transition pore

MTT : 3-(4, 5-dimethylthiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide)

NAD : Nicotinamide adenine dinucleotide

NADPH : Nicotinamide adenine dinucleotide phosphate

NBT : Nitroblue tetrazolium

NFκB : Nuclear factor - κB

Nrf1 : Nuclear respiratory factor-1

Nrf2 : Nuclear respiratory factor-2

NOS : Nitric oxide synthase

OPA1 : Optic atrophy 1

PBS : Phosphate buffered saline

PI : Propidium iodide

PKC : Protein kinase C

PVDF : Polyvinylidene difluoride

qRT-PCR : quantitative real time polymerase chain reaction

RIPA buffer : Radio immunoprecipitation buffer

ROS : Reactive oxygen species

SDS : Sodium dodecyl sulphate

SGOT : Serum glutamic oxaloacetic transaminase

SGPT : Serum glutamic pyruvic transaminase

SOD : Superoxide dismutase

TBA : Thiobarbituric acid

TBST : Tris buffered saline containing tween 20

TCA : Trichloroacetic acid

TFC : Total flavonoid content

TGF-β1 : Transforming growth factor-β1

TNF-α : Tumour necrosis factor-α

Tfam : Transcriptional factor A, mitochondrial

TNNI3K : Cardiac troponin I-interacting kinase

TFC : Total flavonoid content

TPC : Total phenolic content

WHO : World health organization

XO : Xanthine oxidase

Introduction

1. Cardiovascular Disease

Cardiovascular disease (CVD) is the leading cause of death globally. Every year there are 17.3 million deaths due to CVD worldwide, and it is expected to increase to 23.6 million by 2030 (Mozaffarian et al., 2015b) . According to WHO reports, more than 75% of CVD deaths occur in low income and middle income countries. Coronary artery disease (CAD) is the greatest contributor among various diseases in cardiovascular disease.

1.1 Coronary artery disease

1.1.1 Epidemiology of coronary artery disease

CVD causes approximately 31% of the total deaths, of which 7.5 million are due to CAD (Mozaffarian et al., 2015a). CAD is the major epidemic of the 20th century and has increased by 41.7% in the past 25 years (Dalen et al., 2014). Among the cardiovascular diseases, CAD or ischemic heart diseases is the major contributor causing 46% of the total deaths (Figure 1.1). According to Framingham study, 1 in 2 men and 1 in 3 women are at a lifetime risk of CAD (Lloyd-Jones et al., 2002).

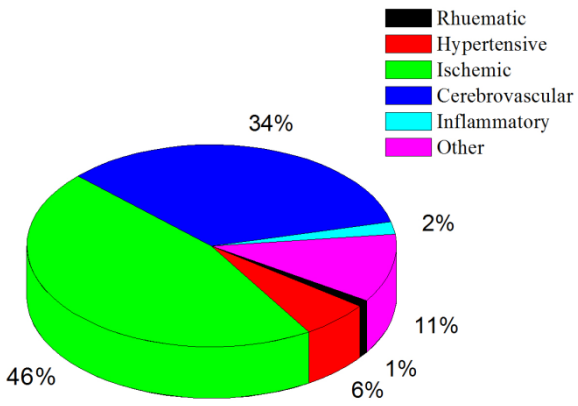

Figure 1.1 The proportions of cardiovascular deaths caused by various cardiovascular diseases. Ischemic heart disease causes 46% of the total death. (Wong, 2014).

India is going through an epidemiologic transition where the burden of communicable disease has declined and non-communicable diseases has risen rapidly (Krishnan, 2012). CVD is the top killer of Indians accounting for 23 per cent of all deaths in 2010-13 as

compared to 20 per cent in 2004-06. There has been a 4-fold rise of CAD prevalence in India during the past 40 years and currently there are 30 million cases of CAD in India. Studies show that there is a high prevalence of heart disease especially CAD in India with approximately 11 % in the urban population and 7 % in the rural population (Krishnan, 2012). The state of Kerala has the highest prevalence of CAD among the urban population (Figure 1.2). It is expected that CVD will be the largest cause of death and disability in India by 2020 which accounts to 2.6 million deaths annually (Nag and Ghosh, 2015). The increased burden of CAD in India can be explained by the alarming rise in the prevalence of coronary risk factors like diabetes, hypertension, atherogenic dyslipidemia, smoking, obesity and physical inactivity (Agyemang et al., 2009) (Figure 1.3).

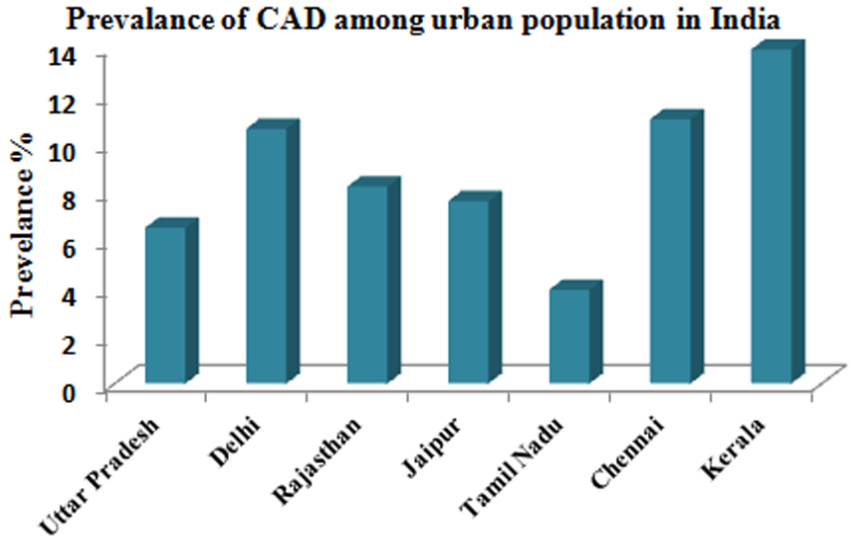

Figure 1.2 Prevalence of coronary artery disease among the urban population in India. The state of Kerala has the highest prevalence of coronary artery disease among the urban population. (Gupta, 2005)

The prevalence of coronary artery disease in Kerala has increased over the past two decades due to the rise in coronary risk factors (Krishnan et al., 2016). The prevalence is 15.1% in urban population and 16.2% in the rural population (Krishnan, 2012). Surprisingly, there is no difference between the urban and rural population, a scenario unique to Kerala (Zachariah et al., 2013). According to another study, CAD death was 31% in men and 17.6%

in women, 3-6% higher than Japan, rural China and the United States of America (Soman et al., 2011).

1.1.2 Coronary artery disease

It is caused by the blockage of the coronary artery that supplies blood to the heart. Heart is a vital organ that maintains life by supplying blood containing oxygen and nutrients to various organs of the body. The heart also needs a constant supply of oxygen and glucose for its proper function. This task is performed by the coronary artery that descends from the aorta and branches to the right and left coronary artery supplying blood to the right and left ventricles respectively. Blockage of the coronary artery is caused by the deposition of an atheromatous plaque at one or all of the coronary arteries, blocking blood supply and oxygen and nutrients to the heart.

1.1.3 Pathophysiology of coronary artery disease

The pathophysiological mechanism of CAD begins with the process of atherosclerosis. Atherosclerosis is the gradual thickening of the inner layers of the coronary arteries with plaque, which is accelerated by risk factors such as high blood pressure, high cholesterol, smoking, diabetes, and genetics (Bonomini et al., 2015) (Figure 1.3). This is continued for several years and narrows the lumen of the artery to various degrees until there is complete obstruction when symptoms are shown. Coronary artery disease is classified based on the type of atherosclerotic plaque and the stage of blockage (Cassar et al., 2009) (Figure 1.4).

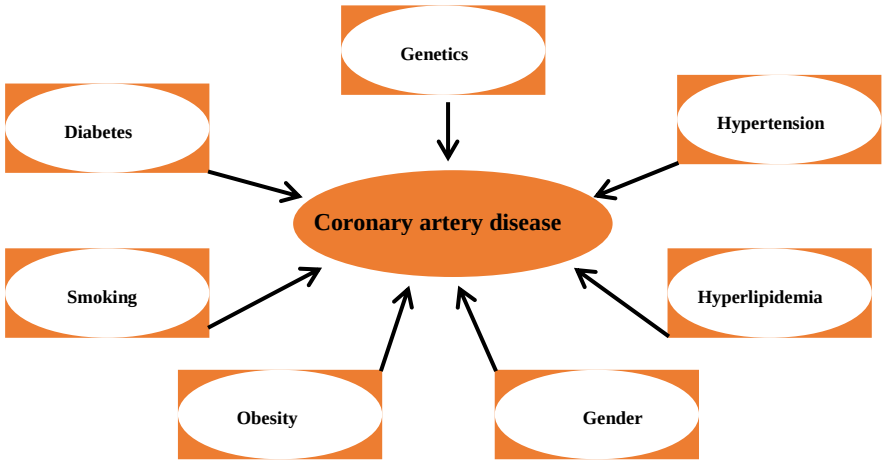

Figure 1.3 Risk factors for coronary artery disease. Hyperlipidemia and diabetes are the predominant risk factors associated with cardiovascular disease (Jousilahti et al., 1999)

An atherosclerotic plaque is composed of cellular debris, inflammatory cells, smooth muscle cells (SMCs) and cholesterol covered by a fibrous cap made of collagen, SMCs and elastin (Ambrose and Singh, 2015). Upto 70 % blockage of the coronary artery is asymptomatic; 70-75% blockage causes ischemia and shows symptoms of stable angina (chest pain) (Figure 1.4). The term angina is derived from the Greek word 'ankho" means" to choke". All other types of CAD come under acute coronary syndrome (ACS). The continuous inflammatory action on the fibrous cap of the atherosclerotic plaque causes thinning of the fibrous cap and ultimately ruptures the fibrous cap exposing the inner thrombolytic core (Ambrose and Singh, 2015). The plaque disruption causes further blockage of the coronary artery causing acute coronary syndrome (Falk et al., 1995). Rupture, fissure and unstable plaque causes unstable angina and 90% blockage to total blockage caused by plaque rupture causes acute myocardial infarction (AMI) and sudden cardiac death (SCD) (Finn et al., 2010).

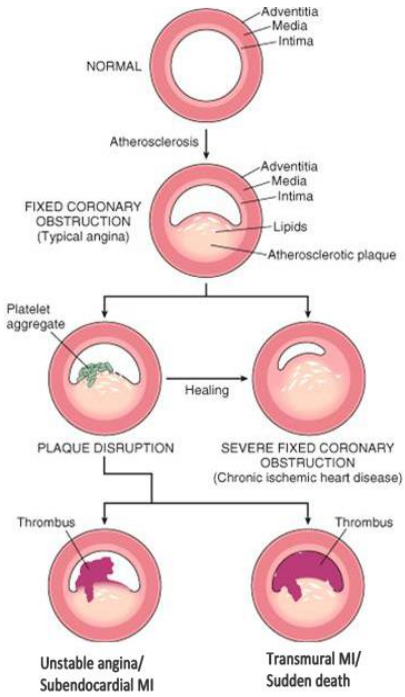

Figure 1.4 Coronary artery disease – pathogenesis. Schematic representation of sequential progression of coronary artery lesion, beginning with stable chronic plaque responsible for typical angina and leading to the various acute coronary syndromes. (Roberts, 1990)

1.2 History of heart diseases

Heart diseases were prevalent among the ancient Egyptians some 3500 years ago, specifically atherosclerosis and it is mentioned in the Ebers Papyrus (1500BC) (Petrovska and Cekovska, 2010). Ancient Indian physician Charaka (900BC) mentioned *Hrdroga* and Sushrutha (6BC) mentioned the concept of *Hritshoola* (angina), and hypertension. William Harvey was the first to describe circulation in 1628 and this paved the way for future research on the cardiovascular system (Silverman, 1985). The 20th century saw great achievements in the field of cardiology with the discovery of electrocardiography by William Einthoven (Schwartze, 1985), cardiac catheterization by Claude Bernard in 1929 (Nossaman et al., 2010), coronary angiography by Masons Sones in 1958 (Proudfit et al., 1966) and Helmuth Hertz and Inge Edler's discovery of echocardiography in 1952 (Edler, 1991). The current understanding of heart diseases is possible only because of these discoveries. The 1954 Framingham Heart Study by Paul Dudley White paved the way for preventive cardiology (Mahmood et al., 2014). In 1967 Christian Bernard performed the first human heart transplant (Bourassa, 2005). Discovery in the field of cardiovascular drugs was initiated with William Withering described the medical use of digitalis in heart in 1785 (Silverman, 1989). Discovery of β – blockers by James Black in 1960s (Quirke, 2006), Angiotensin converting enzyme inhibitor captopril by Cushman and Ondetti in 1970s (Cushman et al., 1978; Cushman and Ondetti, 1991) and the first HMG-CoA reductase inhibitor (statin) was isolated by Akira Endo in 1976 (Endo et al., 1976)

1.3 Myocardial ischemia and myocardial infarction

1.3.1 Pathophysiology of myocardial ischemia and myocardial infarction

Myocardial ischemia is caused by decrease in blood supply to the heart, which leads to a depletion of oxygen and nutrients in that region of the heart or increased myocardial metabolic demand (Hausenloy and Yellon, 2013). Onset of myocardial ischemia is the initial step in the development of myocardial infarction. Myocardial infarction is the endpoint of prolonged ischemia leading to death of the heart tissue (Kalogeris et al., 2012a). Thus, myocardial infarction and ischemia are both conditions defining the failing condition of the heart muscle (Buja, 2005).

A partial blockage of the coronary artery; leading to restriction of blood supply to an area of the heart, causing ischemia of the heart muscle in that area. The heart muscle or cardiomyocyte is greatly dependent on oxygen and consumes 30 % of the body's total oxygen in a resting state (Puente et al., 2014). Ischemia is caused by the imbalance in the demand and supply of oxygen (Shimokawa and Yasuda, 2008). It causes a cessation of aerobic respiration and a shift to anaerobic respiration, resulting in the depletion of glycogen stores and a resulting accumulation of hydrogen ions and tissue acidosis (Chiong et al., 2011; Sanada et al., 2011; Turer and Hill, 2010). This results in accumulation of lactate, a by-product of anaerobic respiration. The cessation of aerobic respiration and oxidative phosphorylation causes depletion of adenosine triphosphate (ATP) and the heart loses its contractility (Muravchick and Levy, 2006). ATP depletion has numerous detrimental effects on myocyte biochemistry and metabolism, including relaxation of myofilaments, glycogen depletion, disruption of ionic equilibrium and cell swelling (Perricone and Vander Heide, 2014). Prolonged ischemia causes irreversible damage leading to necrosis and apoptosis of the cardiomyocytes nourished by the blocked coronary artery. In addition, necroptosis or programmed necrosis, a form of cell death with characteristics of both necrosis and apoptosis has been suggested to contribute to myocyte death during ischemia (Lim et al., 2007; Smith and Yellon, 2011). Nevertheless, these effects can be reversed if the duration of ischemia is brief (less than 20 minutes) and by restoration of blood flow. But restoration of blood flow also leads to reperfusion injury (Eltzschig and Collard, 2004).

1.3.2 Neuro-humoral mechanism in myocardial infarction

The left ventricular damage caused by myocardial ischemia triggers a compensatory mechanism to restore cardiac output (Mann and Bristow, 2005). Activation of the sympathetic nervous system provides inotropic support to the failing heart (Triposkiadis et al., 2009). In early ischemia pain, anxiety and fall in cardiac output activate efferent sympathetic nerves (Schomig, 1990). However, released noradrenalin is rapidly removed. Ischemia for longer time causes accumulation of the catecholamine, noradrenalin. Myocardial ischemia of 15 min duration results in a 100-fold increase in catecholamine concentrations within the extracellular space of the ischemic zone, a two-fold increase in functionally coupled α-adrenoceptors, and a 30 % increase in β-adrenoceptors (Schomig et al., 1991). Increased β-adrenergic receptor

causes enhanced sensitivity of the heart to catecholamine. β_1 adrenergic receptor activates G_s proteins, while β_2 adrenergic receptors activate G_i and G_s proteins. G_s signaling stimulates adenyl cyclase, resulting in dissociation of adenosine triphosphate (ATP) into the second messenger adenosine 3, 5-cyclic monophosphate (cAMP), which in turn binds to cAMP-dependent protein kinase A. Targets of protein kinase A-induced phosphorylation's are L-type calcium channels and ryanodine receptors leading to increased entry of calcium into the cell and phospholamban a subunit of Na^{2+}/K^+ ATPase causes stimulation of sodium pump. The activation of β_1 and β_2 adrenergic receptors increases contractility (positive inotropic effect), frequency (positive chronotropic effect) and rate of relaxation (lusitropic effect) (Zakrzeska et al., 2005). The increased calcium produced by activation of β-adrenergic receptors leads to cell death by different mechanisms (Steinberg, 1999). ATP depletion occurs due to increased contractility and impaired ATP generation due to calcium overloading within the mitochondria (Griffiths and Rutter, 2009). ATP depletion disables the cardiomyocyte from performing energy dependent cell functions (Kuznetsov et al., 2011). Calcium overload results in the release of phospholipase and protease resulting in necrosis of the cardiomyocyte. Thus, the increased cell death causes further damage to the heart. The heart also possesses α adrenergic receptors and the binding of epinephrine and norepinephrine regulates blood flow and decreases contractility. Activation of α receptors activates phospholipase Cb. Phospholipase Cb hydrolyzes phosphatidylinositol (4, 5) bisphosphate to generate the second messengers inositol [1, 4, 5]-trisphosphate and 2-diacylglycerol. Inositol [1, 4, 5]-trisphosphate and 2-diacylglycerol contributes to the regulation of intracellular calcium responses.

1.3.3 Myocardial ischemia at the cellular level

Ischemia causes a cessation of aerobic respiration and a shift to anaerobic respiration causing depletion of glycogen stores and accumulation of hydrogen ions and lactic acidosis (Vander Heide and Steenbergen, 2013). The protons generated during ischemia are extruded from the cell by Na^+/H^+ exchange resulting in increase in intracellular sodium (Karmazyn et al., 1999). The accumulated sodium ions are eliminated by stimulation of Na^+/Ca^+ exchanger (NCX) which leads to the accumulation of intracellular calcium (Sanada et al., 2011). The ionic disturbances thus formed cause the depletion of high energy phosphates (ATP)

preventing the normal activity of Na^+/K^+ ATPase (Murphy and Steenbergen, 2008a). This cause further intracellular sodium and calcium accumulation (Figure 1.5). The high cytosolic calcium concentration causes the uptake of calcium by the mitochondria through the mitochondrial calcium uniporter (Demaurex et al., 2009). Mitochondrial calcium triggers the opening of mitochondrial permeability transition pore (mPTP). Intracellular calcium stimulates apoptosis (Halestrap et al., 2004) and activation of intracellular degradative enzymes such as proteases, phospholipase and endonuclease leading to damage of cellular membranes and subsequent disruption of osmotic balance and release of lysosomal enzymes causing necrosis (Halestrap, 2009).

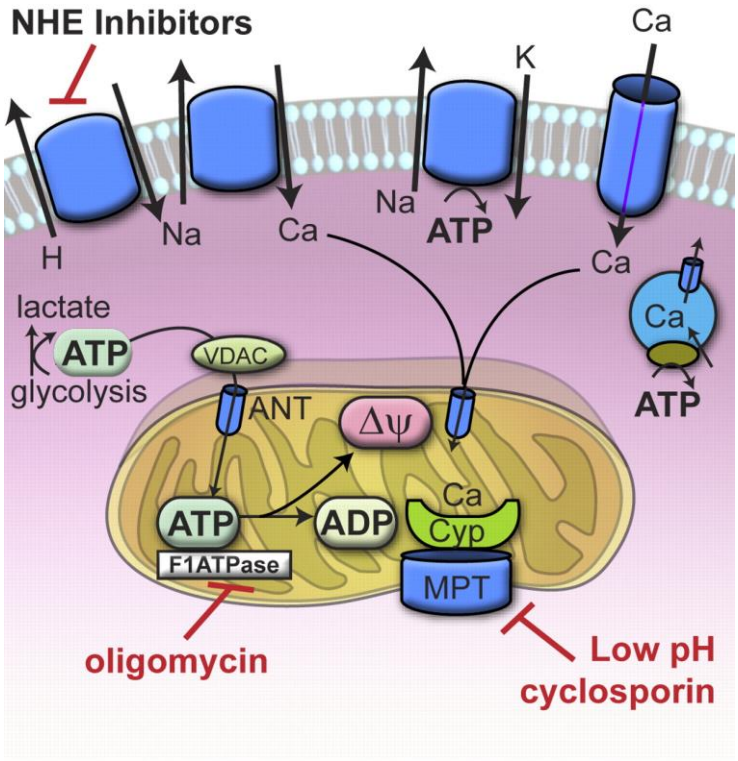

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Figure 1.5 Myocardial ischemia at the cellular level. During ischemia, ATP declines, causing decrease in pH due to anaerobic glycolysis. The increase in proton stimulates Na^+/H^+ exchange and Na^+/Ca^+ exchange, resulting in an increase in cytosolic calcium, resulting from more calcium entering the mitochondria, activating mitochondrial permeability transition pore. (Murphy and Steenbergen, 2008b)

Ischemia can be prevented by restoration of blood flow to the heart. But this causes further damage by reperfusion injury. Reperfusion causes rapid washout of accumulated protons greatly favoring influx of sodium ions resulting in greater elevation of intracellular calcium (Bers, 2008). This causes further increased mitochondrial calcium, mPTP opening,

outer membrane rupture and release of more calcium into the cytosol causing mPTP opening in other mitochondria in the myocyte triggering necrotic cell death (Di Lisa et al., 2001) (Griffiths and Halestrap, 1995)

Oxidative stress is another feature of ischemia reperfusion injury (Kalogeris et al., 2012a). While reactive oxygen species (ROS) are generated during normal metabolism and ischemia, there is a burst of ROS during reperfusion which overwhelms the capacity of the cell to scavenge these radicals as the activity of antioxidant enzymes are attenuated during ischemia (Kalogeris et al., 2014). This sudden burst of ROS along with mitochondrial calcium overload triggers the opening of mPTP (Kristian and Siesjo, 1998). The release of free oxygen radicals thus sensitizes other mitochondria to release ROS causing ROS-induced ROS release. ROS are capable of per oxidizing membrane lipids, cross linking proteins and creating DNA breaks.

Although protection can be initiated at reperfusion, injury also occurs during ischemia, and the relative proportion of injury occurring during ischemia versus reperfusion likely depends on the duration of ischemia. So, the best strategy for improving the morbidity and mortality during myocardial infarction is to minimize ischemic cell death of myocardial tissue. During ischemia, caspases are activated and there is occurrence of ion dysfunction. If cardioprotective strategies can be initiated before or during ischemia, it is likely that they will enhance protection, especially with longer durations of ischemia. In addition, events during ischemia can enhance the opening of the mPTP and thus the initiation of death at reperfusion. Thus, it is important to administer cardio protective agents as soon as possible in the ischemia stage itself.

1.3.4 ROS in myocardial ischemia

ROS are produced during ischemia. The main source of ROS during ischemia is the mitochondria and xanthine oxidase. ROS can activate ROS-sensitive mitogen activated protein (MAP) kinase kinases (MAPKKs) and apoptosis signal-regulating kinase 1 (ASK1), which activates the downstream MAP kinases, p38, and c-Jun N-terminal kinase (JNK). ROS activate nuclear factor kB (NF-kB) mediated through ASK1. The activation of NF-kB can produce TNF-α, leading to activation of extrinsic apoptotic pathway mediated by death receptors. It is of interest that TNF-α can also generate ROS which can further activate NFkB.

ROS also stimulate other transcription factors such as Ets and activator protein-1 (AP-1) mediated through Akt and protein kinase C pathways. Oxidative stress-induced activation of transcription factors leads to synthesis of antioxidant enzymes such as manganese-SOD and endothelial nitric-oxide synthase. Thus, ROS are key players for cell protection as well as cell injury in response to oxidative stress.

ROS generally produced as intermediates of oxidation-reduction (redox) reactions and are very reactive chemical species that comprise of various categories: free radicals (e.g. superoxide [O_2-], hydroxyl [OH−], nitric oxide [NO−]) and non-radical derivatives of O_2 which are capable of generating free radicals (e.g. H_2O_2, ONOO−) (Fridovich, 1997). The free radicals are highly reactive and unstable species with one or more unpaired electrons having the capacity of independent existence. Among the various free radicals, superoxide radicals are generated during the electron leakage from mitochondrial electron transport chain. It is an oxygen molecule having a free electron and is responsible for the production of other ROS like hydroxyl radicals and H_2O_2 (Maulik and Kumar, 2012).

Enzymatic detoxification of superoxide radicals leads to the formation of non-radical ROS and H_2O_2. H_2O_2 can readily cross the cellular and nuclear membrane and the various effects of H_2O_2 in the cardiomyocytes include the activation of NFκβ and the induction of intracellular calcium overload (Maulik and Kumar, 2012). In pathological conditions, the single-electron reduction of H_2O_2 may lead to the formation of highly reactive hydroxyl radicals (Seddon et al., 2007) . In addition, Fenton reaction of H_2O_2 in the presence of iron also leads to the formation of hydroxyl radicals (Tsutsui et al., 2011). Hydroxyl radicals are highly reactive and are capable of inducing severe damage to biomolecules and responsible for the initiation of lipid peroxidation (Lakshmi et al., 2009). Nitric oxide plays an important role in vascular homeostasis and in modulating cardiac function (Takimoto and Kass, 2007). Superoxide radical can reacts with nitric oxide to produce more toxic peroxynitrite radicals that can induce cell death. Reaction of superoxide radicals with nitric oxide leads to the inactivation of nitric oxide and subsequent loss of its biological activity (Seddon et al., 2007). Decomposition of peroxynitrite radicals yields hydroxyl and nitrogen dioxide radicals which are reported to activate lipid peroxidation reactions (Huie and Padmaja, 1993) .

1.3.5 Antioxidant defense system in the heart

In the biological system, two main classes of antioxidant defense system exist to scavenge and degrade ROS to non-toxic molecules. They are enzymatic antioxidants and non-enzymatic antioxidants. The major enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GRD) and glutathione peroxidase (GPx) and the non-enzymatic antioxidants, such as reduced glutathione (GSH), vitamins E, C, β-carotene etc (Giordano, 2005)(Gongora et al., 2006).

SOD acts as the first line of defense in the cell against oxidative stress and the major function in the biological system is the dismutation of superoxide radicals into H_2O_2 and O_2. Different isoforms of SOD in the mammalian tissue include manganese-containing SOD (Mn-SOD), copper containing SOD (Cu-SOD), and zinc containing SOD (Zn-SOD). Mn-SOD is the major isoform present in cardiac mitochondria and is responsible for the scavenging of superoxide anions in cardiac myocytes (Assem et al., 1997). CAT is another important antioxidant enzyme mainly located in cellular peroxisomes and in cytosol and catalyze the conversion of H_2O_2 to H_2O and molecular oxygen. It can also inhibit the initiation phase of free radical reaction (Maulik and Kumar, 2012)

GPx is a seleno enzyme present both in cytosol and mitochondria and the reduced activity of this enzyme is considered as prooxidant/antioxidant imbalance in the tissues (Alam et al., 2013). GPx catalyzes the decomposition of H_2O_2 to H_2O in the expense of oxidation of reduced glutathione to oxidized glutathione. Oxidized glutathione is reduced back to GSH by the action of another antioxidant enzyme, GRD (Peng and Li, 2002). One of the major non-enzymatic antioxidants in the cellular system is GSH. It is a tri-peptide critical for protective activities like detoxification of ROS and control of the inflammatory cytokine cascade in the cell (Abhilash et al., 2012). Depletion of GSH leads to oxidative injury due to the impairment in scavenging ROS (Kent et al., 2003). GSH can directly scavenge free radicals and acts as a co-substrate for glutathione peroxidase activity as well as cofactor for many enzymes including glutathione-s-transferase (GST) (Maritim et al., 2003). GST is found to exert its protective activity against free radical mediated cellular injury by catalyzing the decomposition of lipid peroxides (Peng and Li, 2002).

1.3.6 Cell death in myocardial ischemia and myocardial infarction

Cardiac myocyte death during myocardial ischemia and myocardial infarction occurs by apoptosis (Gottlieb et al., 1994) and necrosis (Baines et al., 2005; Kajstura et al., 1996) in association with autophagy. It has been reported that cardiac myocyte apoptosis becomes maximal 4.5 hours following myocardial infarction, whereas necrosis peaks at 24 hours (Kajstura et al., 1996). Reperfusion appears to accelerate the timing of apoptosis (Fliss and Gattinger, 1996). The balance between cardiomyocyte death and renewal plays an important role in heart failure (Mughal and Kirshenbaum, 2011). During myocardial ischemia and infarction three types of cell death occur. Apoptotic cell death during ischemia is mediated by two pathways; the intrinsic death receptor pathway mediated by cell surface death receptors FAS ligand and tumour necrosis factor alpha (TNF-α) and the intrinsic pathway that utilizes the mitochondria and the endoplasmic reticulum (Elmore, 2007). The activation of the mitochondrial apoptotic pathway leading to executioner caspase activation is an essential part of myocardial ischemia-induced heart injury (Crow et al., 2004). Cardiomyocytes express anti-apoptotic (Bax, Bad) and pro-apototic (Bcl-2) markers, which are transcriptionally regulated in heart disease (Dorn, 2009). The extrinsic pathway is activated by binding of death ligand to its receptor, which activates caspase-8 which cleaves downstream capases (Parrish et al., 2013). Caspase-8 also cleaves BH-3 only protein Bid, which translocates to the mitochondria to trigger apoptotic mitochondrial events (Kantari and Walczak, 2011).

The intrinsic pathway is activated by diverse biological, chemical, and physical stimuli (Whelan et al., 2010). These signals are transduced to the mitochondria by Bax, Bad and Bak. The key event in apoptosis is the permeabilization of the outer membrane which is triggered by Bax and Bcl-2 homologous antagonist/ killer (Bak). This triggers the release of apoptogens from the mitochondria into the cytosol (Gupta et al., 2009). Cytosolic cytochrome c, one such apoptogen binds to apoptotic protease activating factor-1 (Apaf-1) and triggers the formation of a second multiprotein complex, the apoptosome, in which procaspase-9 undergoes activation (Konstantinidis et al., 2012). Caspase-9 then cleaves and activates downstream procaspases. Apoptogen release is prevented by Bcl-2 and Bcl-xL (B cell leukemia/lymphoma-x, long isoform) (Crow et al., 2004) (Figure 1.6).

Necrosis is an unregulated cell death mechanism although regulated mechanism exists. A subset of cell death initiated by death receptor activation along with simultaneous caspase

inhibition, has been termed necroptosis (Whelan et al., 2010). The unique features of necrosis are loss of plasma membrane integrity and depletion of cellular ATP (Kung et al., 2011). Two distinct pathways are involved in necrotic cell death, the death receptor pathway (RIP) and the mPTP pathway. The death ligand binds to TNFR-1 (tumor necrosis factor-α receptor 1). Depending on context, activation of TNFR1 can promote cell survival or either apoptotic or necrotic cell death (Holler et al., 2000). TNF-α binding to the TNFR-1 stimulates the formation of complex-I. Polyubiquitination of RIP1 and components of complex I triggers activation of transcription factor NFκB and activation of survival genes (Ea et al., 2006). Death effectors of TNF-α signaling are mediated by complex II that endocytose complex I causing dissociation of TNFR1, de-ubiquitination of RIP1 and activation of procaspase-8 (Wang et al., 2008)(Hitomi et al., 2008). Procaspase-8 cleaves RIP1 unable to activate survival and necrotic pathways causing apoptosis. Caspase-8 also recruits downstream caspases causing apoptosis. If procaspase-3 is inhibited RIP cleavage does not occur recruiting RIP3 which leads to necrosis.

mPTP opening is triggered by increased calcium, oxidative stress, elevated phosphate concentration and adenine nucleotide depletion (Halestrap, 2009). mPTP pore opening causes the loss of electrical potential gradient due to proton gradient that normally exists across the inner mitochondrial membrane causing further ATP depletion. Pore opening also cause mitochondrial swelling due to the influx of water down its osmotic gradient into the mitochondrial matrix (Figure 1.6). Other factors that trigger cell death are the proteases, calpains and cathepsin. Calcium activates calpains that cleave and activate the pro-apoptotic Bid and Bax. Calpain can also inhibit apoptosis by cleaving upstream and downstream caspase. Cathepsins are lysosomal proteases that may be liberated during cellular stress (Boya and Kroemer, 2008).

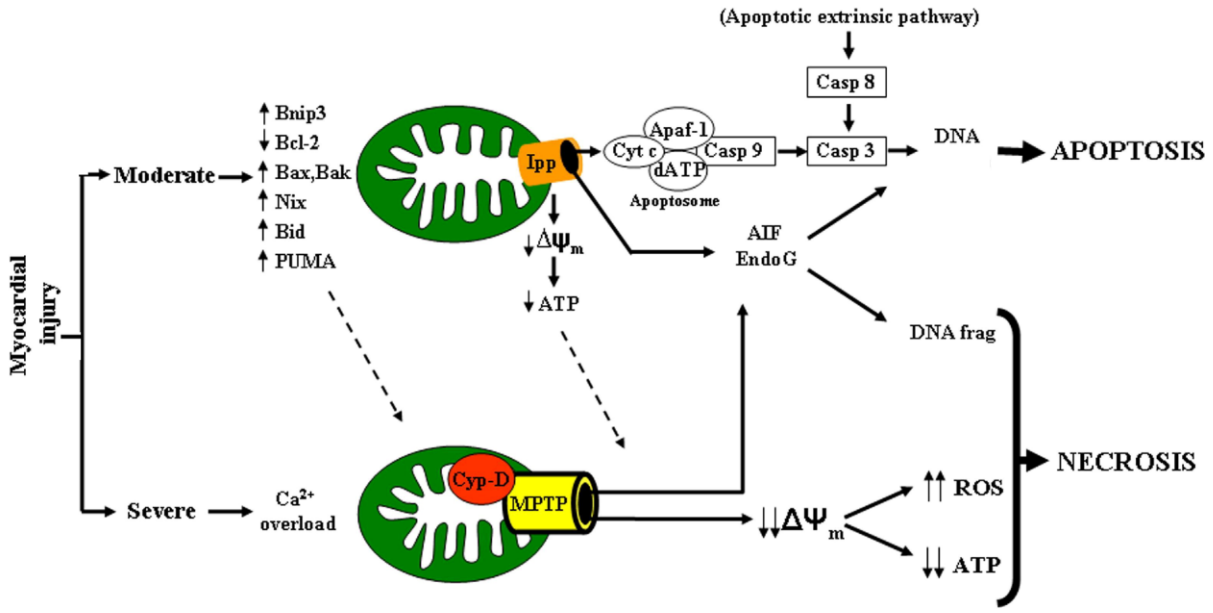

Figure 1.6 Cell death by apoptosis and necrosis in myocardial ischemia. Mechanism and potential relationship among apoptotic and necrotic pathways in myocardial ischemia. **(**González et al., 2011)

1.4 Signaling pathways in myocardial ischemia

1.4.1 Mitogen activated protein kinase (MAPK) signaling in myocardial ischemia.

Myocardial infarction and ischemia fall under the category of acute cardiac stress and the signaling pathway of various MAPKs are as follows.

Akt are MAPKs which induce anti-apoptotic proteins and which in turn cause cardioprotection from ischemic injury (Rose et al., 2010). Akt phosphorylates and degrades pro apoptotic Bax and promotes expression of anti-apoptotic Bcl-2 thus increasing Bcl-2/Bax ratio and preventing apoptosis (Das et al., 2008) Activation of Akt increased the expression of iNOS and eNOS, increasing NO production, opening of mitochondrial ATP sensitive potassium channel and thus inducing cardioprotection (Baines et al., 2002). Akt phosphorylation can also promote mitophagy and autophagy, thus inducing pro-death pathways. Akt activation and translocation to the nucleus induce pro-death pathways(Dagda et al., 2009b). Thus, the quality of Akt depends on the sub cellular compartmentalization and its interaction with other signaling pathways (Javadov et al., 2014) (Figure 1.7)

p38 consists of two isoforms α and β. p38- α is the dominant active isoform, activated by autophosphorylation, and contributes to myocardial infarction (Kumphune et al., 2010). Selective inhibition of p38α reduces ischemic injury. p38α activation may induce both detrimental and beneficial effects depending on variability of oxidative stress (Javadov et al., 2014). Oxidative stress induced by myocardial infarction and ischemia causes p38α activation. Short periods of activation such as in ischemic preconditioning may be beneficial, but prolonged periods of activation such as in myocardial infarction and ischemia are detrimental (Bell et al., 2008). p38α activation is pro-apoptotic and cause Bax translocation from the cytoplasm to the mitochondria. Also, activation of p38α reduced phosphorylation of Bad and stimulated translocation of Bad to the mitochondria (Capano and Crompton, 2006). Thus Bax and Bad translocate to the mitochondria and cause the release of cytochrome c inducing apoptosis. Also, p38α activation downregulated the expression of anti-apoptotic proteins Bcl-2 and Bcl-xl, thus promoting apoptosis (Kaiser et al., 2004) (Figure 1.7)

JNK is similar to p38α and poses dual role in ischemia and has both protective and detrimental effect. JNK1 inhibition prevented cardiomyocyte apoptosis but JNK2 inhibition had no effect (Dhanasekaran and Reddy, 2008; Hreniuk et al., 2001). Thus JNK signaling can induce both pro - apoptotic and anti - apoptotic effects in the heart. JNK phosphorylates anti-apoptotic proteins Bcl-2 and Bcl-xl and thus leads to their degradation, thus promoting apoptosis (Dhanasekaran and Reddy, 2008). JNK activation leads to increased ROS production in the mitochondria (Sucher et al., 2009). This increased ROS production along with calcium overload and ATP depletion leads to opening of the mPTP, increased ROS production, mitochondrial swelling and death by necrosis (Javadov et al., 2009) (Figure 1.7). However, inhibition of JNK increased apoptosis by further activation of p38 (Dougherty et al., 2002)

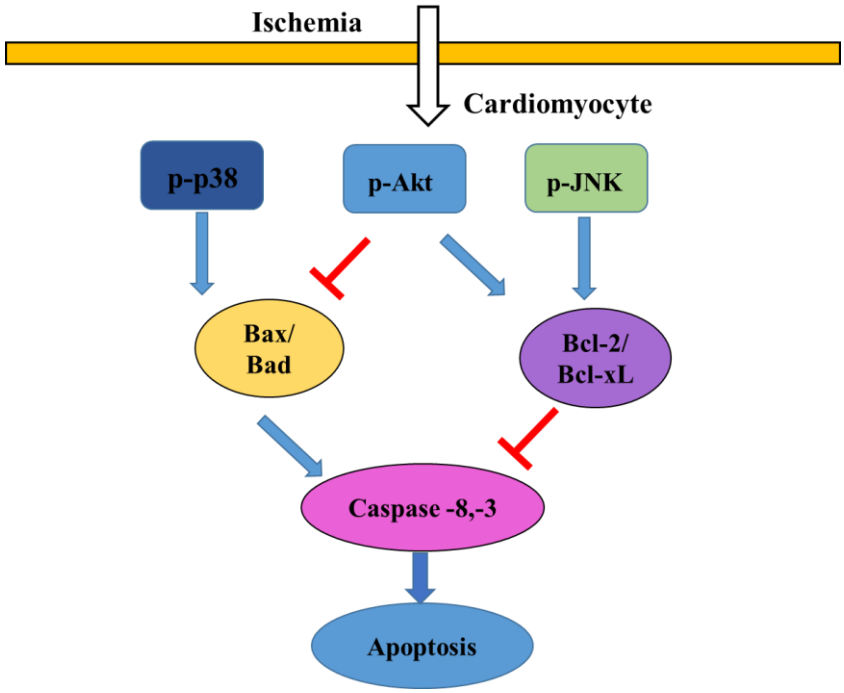

Figure 1.7. Schematic view of MAPK signaling in myocardial ischemia. Mitochondria mediate pro-survival and pro-death pathways by modulating MAPK activity (Javadov et al., 2014).

Another kinase which promotes oxidative stress induced I / R injury is the cardiac troponin I-interacting protein kinase (TNNI3K). TNNI3K was identified as interacting with cardiac troponin (cTn1) and hence the name. There is over-expression of TNNI3K during myocardial infarction and ischemia (Lal et al., 2014). TNNI3K regulates mitochondrial ROS production, mitochondrial membrane potential and mitochondrial calcium influx (Lal et al., 2014). p38α is the downstream effector of TNNI3K and the signaling pathway by which TNNI3K exerts its detrimental effects (Tang et al., 2013). So, pharmacological inhibition of TNNI3K a cardiac specific kinase could be a therapeutic strategy to prevent myocardial ischemia-induced alterations. TNNI3K is heart specific (Vagnozzi et al., 2013) and hence inhibiting TNNI3K will not induce any side effects.

1.5 Mitochondrial dysfunction in ischemia

Due to high energy demand, the cardiomyocytes are rich in mitochondria (Gustafsson and Gottlieb, 2008) and they make up 30 % of the volume of the cardiomyocytes (Hausenloy and Ruiz-Meana, 2010). They are the source of ATP that fuels the excitation–contraction

coupling (Yaniv et al., 2010). They sense endoplasmic reticulum calcium release and prevent calcium induced cytotoxicity (Eisner et al., 2013). They are the major source of ROS (Eisner et al., 2013). Mitochondria are the 'gatekeepers' of apoptosis mediated by Bcl2 family protein and necrosis mediated by opening of the permeability transition pore (Kinnally et al., 2011). They play a crucial role in energy production in cells, but they are involved in other phenomena such as ion homeostasis, free radical production, and ultimately cell death (O'Rourke et al., 2005).

Mitochondria are dynamic organelles able to change their morphology in response to different signals (Piquereau et al., 2013). Mitochondria undergo continuous fission, fusion and mitophagy (Iglewski et al., 2010). Mitofusins 1 and 2 (Mfn1 and Mfn2) regulate fusion of the outer mitochondrial membrane, and optic atrophy protein 1 (Opa1) regulates fusion of the inner mitochondrial membrane of juxtaposed mitochondria. Mitochondrial fusion is promoted by increased oxidative phosphorylation, increased respiration and limits mitophagy and apoptosis (Pellegrino and Haynes, 2015). Dynamin-related protein 1 (Drp1) interacts with fission protein 1(Fis1) in the outer mitochondrial membrane to promote mitochondrial fission (Kubli and Gustafsson, 2012) (Figure 1.8). Mitochondrial fission depolarizes mitochondria. Failure to restore membrane potential, targets mitochondria for degradation by autophagy or apoptosis. Mitochondrial biogenesis is required for cell growth and is promoted during nutrient deprivation and oxidative stress and requires the expression of genes by transcription factors Nrf1/2, PPARγ and transcriptional cofactor PGC-1α. Mitophagy is the fusion of mitochondria with autophagosomes to undergo lysis. Mitochondrial fragmentation is required for mitophagy and fusion protects mitochondria from fragmentation (Gomes and Scorrano, 2013). Modulation of mitochondrial dynamics appears as a novel pharmacological strategy for cardioprotection, in particular to protect after a heart attack, and in ischemia-reperfusion (Ong and Gustafsson, 2012)

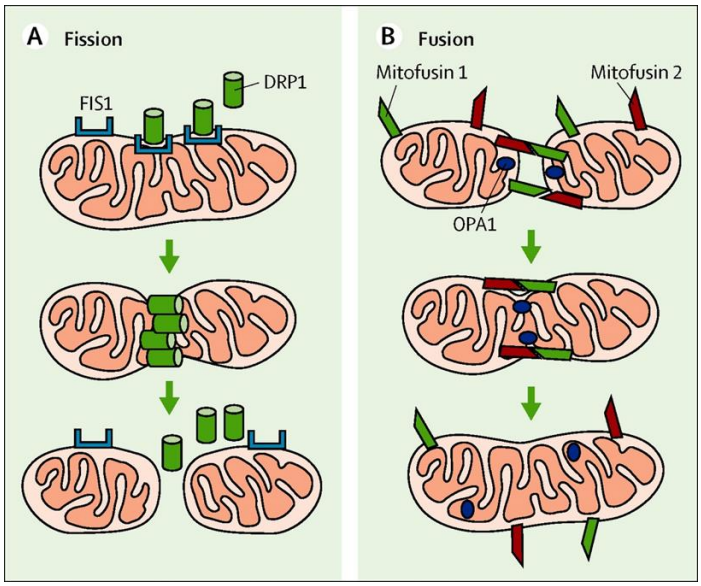

Figure 1.8 Mitochondrial dynamics. Mitochondrial fission is regulated by mitochondrial fission protein 1 (Fis1) and dynamin related protein (Drp1). Mitochondrial fusion is regulated by fusion proteins Mitofusin 1 and 2 (Mfn1 and Mfn2) and optic atrophy 1 (OPA1) (Hagberg et al., 2014)

1.6 Inflammatory response in myocardial infarction

The myocardium has less endogenous regenerative capacity and therefore loss of a significant amount of cardiac muscle ultimately leads to formation of a scar. Cardiac repair is dependent on inflammatory response that serves to clear the wound from dead cells and matrix debris, but also provides key molecular signals for activation of reparative cells. But timely containment of inflammatory signals is needed to ensure optimal formation of a scar in the infarcted area and to prevent development of adverse remodeling. The predominant mechanism of cardiomyocytes death in the infarcted area is by necrosis. Necrotic cells release their intercellular contents and initiate an inflammatory response by activating innate immune pathways leading to NFκB activation that leads to the production of inflammatory cytokines and chemokines. The genes regulated by the NFκB family of transcription factors are diverse and include those involved in the inflammatory response, cell adhesion and growth control (Irwin et al., 1999). NFκB activation by oxidative stress and hypoxia in the setting of ischemia rapidly induce cytokines such as tumor necrosis factor (TNF-α), interleukin-6 (IL-6) and IL-1β (Kapadia et al., 1997) (Figure 1.9). Increased cytokine upregulation leads to a chronic remodeling phase where there is increased matrix metalloproteinase activity (MMP)

and increased natriuretic peptide ANP and BNP in the infracted myocardium (Deten et al., 2002). During the chronic phase post infarction, the activation of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) contribute to the laying down of collagen and wound repair. Interleukin IL1β signaling mediates chemokine synthesis in the infarcted myocardium and stimulates infiltration of the infarct with leukocytes. Neutrophils are recruited very early after cardiac injury, followed by pro-inflammatory monocytes and lymphocytes. Monocytes promote angiogenesis and collateral growth in a paracrine manner, by secreting vascular endothelial growth factor (VEGF). They are also a major source of MMP-9, which is involved in the emergence and branching of the newly formed vascular network (Johnson et al., 2004). Once the monocytes have infiltrated the inflamed tissues, they can differentiate into macrophages. One macrophage population (M1) expresses the inducible NOS-2 and pro-inflammatory cytokines, such as IL-1 and IL-12, whereas another population (M2) produces large amounts of arginase 1, the anti-inflammatory cytokine IL-10, and VEGF. IL-10 inhibits the production of IL-1α, IL-1β, TNF-a, IL-6 and IL-8, thus suppressing the inflammatory response. Macrophages, mast cells and lymphocytes create an environment rich in inflammatory cells, capable of regulating neo vessel formation, fibroblast proliferation and extracellular matrix metabolism, through the production of a variety of cytokines and growth factors. Myocardial infarction is also associated with an early release of angiogenic factors, VEGF and IL-8 and they have a role in enhancing infarct neo vascularization (Lee et al., 2000; Li et al., 1996).

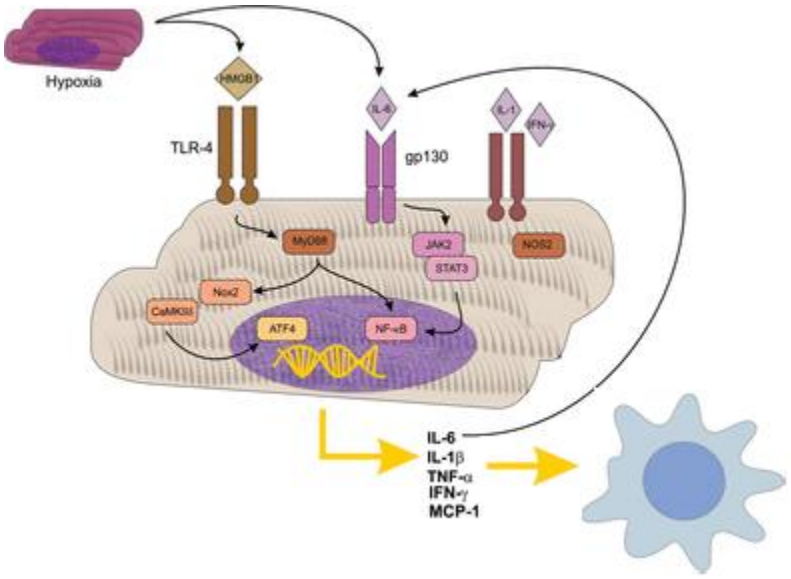

Figure 1.9 Inflammatory response in myocardial infarction. NFκB activation by oxidative stress, and hypoxia in the setting of ischemia, rapidly induce cytokines such as tumor necrosis factor-α, interleukin-6, interleukin-1β which leads to chronic remodeling of the myocardium (Ghigo et al., 2014)

1.7 *In vitro* and *in vivo* models of myocardial ischemia

Anti-ischemic and cardio protective effect of a plant can be studied in a laboratory using *in vivo* animal models and *in vitro* cell based models. A variety of *in vitro* models are available to test the efficacy of a plant material against ischemia. These models are based on any one of the property of the human disease. Some models are used to screen for anti-ischemic property in selected medicinal plants. Other models can be used to study the mechanism of protection of traditionally used plants against various aspects of ischemia. The advantages of using *in vitro* assays are cost effectiveness, requirement of less test material and rapid output (Doke and Dhawale, 2015). *In vitro* assays can reduce the use of animal testing and has reduced variability. The parameters used to screen the anti – ischemic property of a plant *in vitro* are:

- Antioxidant activity of the test material
- Maintenance of ion homeostasis
- Protection of cell organelles important for cardiomyocyte survival
- Inhibition of cell death caused by ischemia

Small animal models of myocardial ischemia have been used extensively to study the protective effect of plants and their active principles. These animal model systems offer quick

and straightforward method to verify the effectiveness of a therapy and can also be used for extensive studies to optimize the therapy based on dose–effect relationship and the route and times of application. Due to heterogeneity of ischemic condition, no single model of heart failure perfectly recreates the human disease (Zaragoza et al., 2011). Thus, many different animal models have been used each having different set of features of human myocardial ischemia. Myocardial damage in rat hearts is induced by two procedures: surgical and pharmacological

1.7.1 Surgical model

The surgical method of left coronary artery ligation was first developed by Pfeffer (Pfeffer et al., 1979). The left coronary artery is ligated and the heart returned to the thorax immediately and closed or the coronary artery undergoes temporary occlusion and then reperfusion allowing flow recovery through the previously occluded coronary artery. Thus this method can be used to evaluate diverse parameters resulting from either permanent ischemia or ischemia/ reperfusion injury.

1.7.2 Pharmacological model

Pharmacological induction of heart damage was first implemented by Bagdon in 1963 and is achieved by treatment with the β-1 adrenergic receptor agonist, isoproterenol (Zbinden and Bagdon, 1963). Isoproterenol administration before ischemia exerts a cardioprotective action in rats, but at the right dose it induces cardiac myocyte necrosis and extensive left ventricular dilation. Isoproterenol treatment and left coronary artery ligation in rats are efficient and reproducible methods that provide valuable information about the underlying mechanisms implicated in human heart disease.

1.8 Medications and therapies used in coronary artery disease

The treatment methodology depends on the extent of damage. If the damage is only 70% medications are used. Medications used to treat coronary artery disease are drugs that can control high blood pressure and promote blood flow in the artery. Coronary vasodilators such as nitroglycerin dilate blood vessels and make blood flow more easily (Duncker and Bache, 2008). Drugs that suppress platelet activity such as aspirin prevent blood clot and thereby prevent the likelihood of heart attack (Gregg and Goldschmidt-Clermont, 2003).

Drugs that reduce cholesterol levels such as bile acid sequestrants, statins, fibrates and niacin are used (Safeer and Lacivita, 2000). Beta blockers that slow down heart rate and decrease blood pressure such as propranolol (Frishman, 2003) and calcium channel blockers such as amlodipine (Hagar et al., 1992) are also used. Angiotensin converting enzyme inhibitors, such as captopril and angiotensin receptor blockers such as losartan prevent increase in blood pressure and prevent remodeling of the heart.

Procedures used to treat coronary artery disease are percutaneous coronary intervention (PCI) and coronary artery by-pass graft (CABG). PCI commonly called angioplasty is a procedure that opens blocked or narrowed coronary arteries. CABG is a surgery in which arteries from other parts of the body are used to bypass the narrowed coronary artery, thus improving blood flow.

1.8.1 Medicinal plants used in the treatment of heart diseases

The use of plants to treat diseases dates back to prehistoric age. The earliest records for usage of plant as drugs are used in the Atharva Veda (2000 BC). In India, Ayurveda and indigenous medicine is used by 65% of the population for their primary health care needs. The ancient Ayurvedic texts used the term "*hrdroga*" for heart diseases and "*hritshoola*" for heart pain. It also links heart diseases to obesity "*medoroga*". Several plants are used in Ayurveda and indigenous systems of medicine for the treatment of heart diseases. The first plant used to prepare drug against heart disease is digitalis. The active constituents are the cardiac glycosides such as deslanoside, digitoxin and digoxin which are used to treat congestive heart failure and atrial fibrillation. Reserpine and ajmalicine derived from *Rauwolfia serpentina* are used as antihypertensive drugs. The stem bark of *Terminalia arjuna* is used as a cardioprotective agent against ischemia and hypertrophy. The main constituents are arjunoside, arjunin and arjunic acid. Inula racemosa is very effective against angina, controls cholesterol levels and prevents heart attack. The chief constituents are sesquiterpene lactone and alantolactone. *Commiphora mukul* resin guggul strengthens the heart and lowers cholesterol levels.

1.9 *Tribulus terrestris* L.: An overview

Tribulus terrestris L. is an annual herb of the Zygophyllaceae family (Fig. 1.10). It is a prostrate herb growing in dry conditions throughout the world. It grows throughout India as a weed along roadsides upto an elevation of 5,400m. The herb has many slender spreading branches and silky villous young parts, leaves abruptly simple, pinnate, opposite, leaflets almost sessile, rounded or oblique at the base, mucronate at the apex; flowers bright yellow, solitary, leaf opposed; fruits, a 5-angled or wing spinous tuberculate woody schizocarp, separating into five cocci, each coccus having two long, stiff, sharp divaricate spines towards the distal half and two shorter ones near the base; seed one or more in each cocci (Varier, 1996). The fruit and root are sweet, cooling, diuretic, aphrodisiac, emollient, appetizer, digestive, anti-helminthic, expectorant, anodyne, anti-inflammatory, anti-diabetic, alterant, laxative, cardio-tonic, styptic, lithonotriptic and tonic (Varier, 1996). Various parts of the plant are used to treat several diseases and in Ayurvedic texts several formulations contain *Tribulus terrestris* L. as the main ingredient. *Tribulus terrestris* L. roots are a constituent of *Dashamoola* and it is mentioned in Ayurvedic text *Sahasrayogam* (a nonSamhitā, Kerala *Materia Medica)* to be *Hrdya* (cardiotonic). It is an important constituent of *Dashamoola Hareetaki* (*Sahasrayogam*), which is used against heart diseases. *Tribulus terrestris* L. fruits are effective against *Hrdroga* (heart diseases) and are mentioned in several Ayurvedic texts (*Bhavaprakash nighantu*, *Dhanwantri nighantu*). So, both the root and fruit were analyzed and the mechanism of cardio protection of root as well as fruit was studied in an *in vitro* and *in vivo* model of myocardial ischemia.

Figure 1.10 Plant material used in this study. *Tribulus terrestris* L.

1.9.1 Vernacular names of *Tribulus terrestris* L.

Tribulus terrestris is known by various names. Few of them are listed below in Table 1.1

Table 1.1 Vernacular names of *Tribulus terrestris* L.

Language	Name
Sanskrit	Gokhsura, Svadamstra
Malayalam	Nerinnil
Tamil	Nerinci
English	Land-caltrops, Puncture-vine
Hindi	Gokharu

1.9.2 Scientific classification of *Tribulus terrestris* L. is given in Table 1.2. *Tribulus terrestris* belongs to the Zygopyllaceae family and this family is commonly known as caltrop family.

Table 1.2: Scientific classification of *Tribulus terrestris* L.

Categories	Taxa
Kingdom	Plantae
Division	Tracheophyta
Class	Magnoliopsida
Order	Zygophyllales
Family	Zygophyllaceae
Genus	*Tribulus*
Species	*terrestris*

1.9.3 Chemical constituents reported in *Tribulus terrestris* L. fruits and roots

Phytochemical constituents of *Tribulus terrestris* L. root and stem bark are diosgenin, neotigogenin, tigogenin, hecogenin gitogenin, campesterol, stigmasterol, β-Sitosterol, four glycosides of kaempferol, three glycoside of quercitin and 8 glycoside of isorhamnetin (Chhatre et al., 2014). Phytochemical constituents of the *Tribulus terrestris* L. fruit are

kaempferol, kaempferol-3-glucoside, kaempferol-3-rutinoside, tribuloside , quercetin, quercetin 3-*O*- glycoside, quercetin 3-*O*-rutinoside, and kaempferol 3-*O*-glycoside, tribulasamide A, tribulasamide B, feruloytyramine, terestriamide, coumaroyltyramine,7-methyldroindanone , rutin, terrestrosins A-E, terrestrosins F-K, neotigogenin, desgalctotigonin, F-gitonin, ruscogenin, desglucolanatigonin, gitonin, diosgenin, hecogenin, chlorogenin, tribulosaponin A-E, isoterrestrosin-B, 25-D spirosta-3,5 diene, protodioscin and prototribestin (Kumar et al., 2012; Ponnusamy et al., 2011; Singh et al., 2013)

1.10 Objectives of the present study

Coronary artery disease and the associated myocardial ischemia and myocardial infarction are the leading cause of death worldwide. Currently available medications and treatment procedures can alleviate some aspects of ischemic injury. But they have secondary complications and are unable to cure ischemic heart disease completely. Traditional medicine has been used since ancient times to prevent and treat heart diseases. But, the biochemical and molecular mechanism of protection from ischemia is unknown. So, the objective of the present study is the elucidation of molecular and biochemical mechanism of the anti-ischemic and cardio protective property of *T. terrestris* L. fruit and root.

1.11 Work flow

The thesis is divided into 7 chapters. Chapter 1 is introduction; chapter 2 contains the materials and methods. Chapter 3 and chapter 4 deals with anti-ischemic property of *T. terrestris* root respectively. Chapter 5 and chapter 6 deal with the anti-ischemic property of *T.terrestris* fruit respectively. Chapter 7 deals with the summary and conclusion. The flow chart of the entire work of the thesis is given below (Figure 1.11)

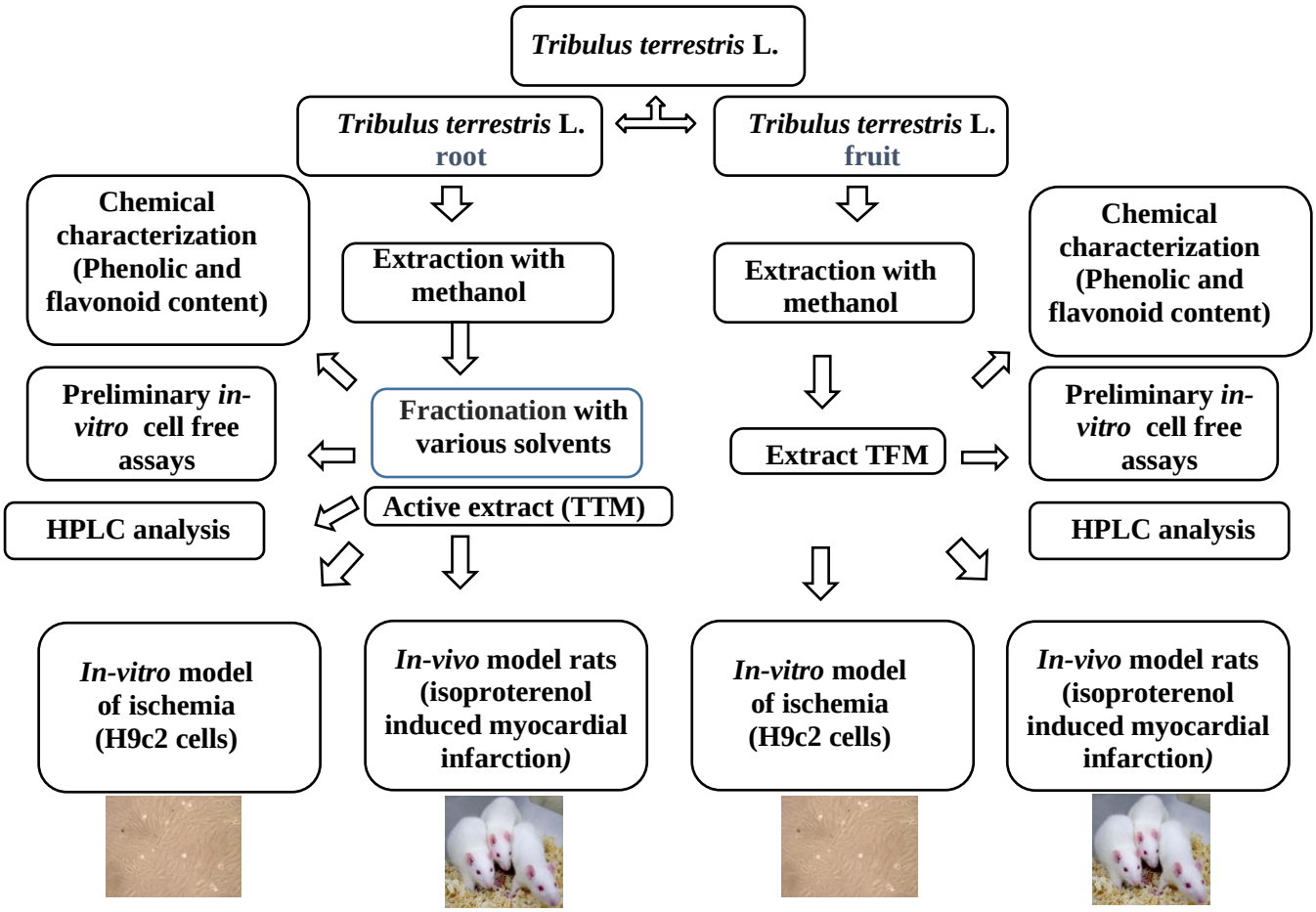

Figure 1.11 Work flow of the thesis.

References

Abhilash PA, Harikrishnan R and Indira M (2012) Ascorbic acid supplementation causes faster restoration of reduced glutathione content in the regression of alcohol-induced hepatotoxicity in male guinea pigs. *Redox report* 17: 72-79.

Agyemang C, Addo J, Bhopal R, Aikins Ade G and Stronks K (2009) Cardiovascular disease, diabetes and established risk factors among populations of sub-Saharan African descent in Europe: a literature review. *Globalization and Health* 5: 7-9

Alam MN, Bristi NJ and Rafiquzzaman M (2013) Review on *in vivo* and *in vitro* methods evaluation of antioxidant activity. *Saudi Pharmaceutical Journal* 21: 143-152.

Ambrose JA and Singh M (2015) Pathophysiology of coronary artery disease leading to acute coronary syndromes. *F1000 prime Reports* 7: 1-5.

Assem M, Teyssier JR, Benderitter M, Terrand J, Laubriet A, Javouhey A, David M and Rochette L (1997) Pattern of superoxide dismutase enzymatic activity and RNA changes in rat heart ventricles after myocardial infarction. *The American Journal of Pathology* 151: 549-555.

Baines CP, Kaiser RA, Purcell NH, Blair NS, Osinska H, Hambleton MA, Brunskill EW, Sayen MR, Gottlieb RA, Dorn GW, Robbins J and Molkentin JD (2005) Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature* 434: 658-662.

Baines CP, Zhang J, Wang GW, Zheng YT, Xiu JX, Cardwell EM, Bolli R and Ping P (2002) Mitochondrial PKCepsilon and MAPK form signaling modules in the murine heart: enhanced mitochondrial PKCepsilon-MAPK interactions and differential MAPK activation in PKCepsilon-induced cardioprotection. *Circulation Research* 90: 390-397.

Bell JR, Eaton P and Shattock MJ (2008) Role of p38-mitogen-activated protein kinase in ischaemic preconditioning in rat heart. *Clinical and Experimental Pharmacology & Physiology* 35: 126-134.

Bers DM (2008) Calcium cycling and signaling in cardiac myocytes. *Annual Review of Physiology* 70: 23-49.

Bonomini F, Rodella LF and Rezzani R (2015) Metabolic syndrome, aging and involvement of oxidative stress. *Aging and Disease* 6: 109-120.

Bourassa MG (2005) The history of cardiac catheterization. *The Canadian Journal of Cardiology* 21: 1011-1014.

Boya P and Kroemer G (2008) Lysosomal membrane permeabilization in cell death. *Oncogene* 27: 6434-6451.

Buja LM (2005) Myocardial ischemia and reperfusion injury. *Cardiovascular Pathology* 14: 170-175.

Capano M and Crompton M (2006) Bax translocates to mitochondria of heart cells during simulated ischaemia: involvement of AMP-activated and p38 mitogen-activated protein kinases. *The Biochemical Journal* 395: 57-64.

Cassar A, Holmes DR, Jr., Rihal CS and Gersh BJ (2009) Chronic coronary artery disease: diagnosis and management. *Mayo Clinic Proceedings* 84: 1130-1146.

Chhatre S, Nesari T, Somani G, Kanchan D and Sathaye S (2014) Phytopharmacological overview of *Tribulus terrestris*. *Pharmacognosy Reviews* 8: 45-51.

Chiong M, Wang ZV, Pedrozo Z, Cao DJ, Troncoso R, Ibacache M, Criollo A, Nemchenko A, Hill JA and Lavandero S (2011) Cardiomyocyte death: mechanisms and translational implications. *Cell Death & Disease* 2 :1-11.

Crow MT, Mani K, Nam YJ and Kitsis RN (2004) The mitochondrial death pathway and cardiac myocyte apoptosis. *Circulation Research* 95 :957-970.

Cushman DW, Cheung HS, Sabo EF and Ondetti MA (1978) Design of new antihypertensive drugs: potent and specific inhibitors of angiotensin-converting enzyme. *Progress in Cardiovascular Diseases* 21: 176-182.

Cushman DW and Ondetti MA (1991) History of the design of captopril and related inhibitors of angiotensin converting enzyme. *Hypertension* 17: 589-592.

Dagda RK, Zhu J and Chu CT (2009) Mitochondrial kinases in Parkinson's disease: converging insights from neurotoxin and genetic models. *Mitochondrion* 9: 289-298.

Dalen JE, Alpert JS, Goldberg RJ and Weinstein RS (2014) The epidemic of the 20(th) century: coronary heart disease. *The American Journal of Medicine* 127: 807-812.

Das A, Xi L and Kukreja RC (2008) Protein kinase G-dependent cardioprotective mechanism of phosphodiesterase-5 inhibition involves phosphorylation of ERK and GSK3beta. *The Journal of Biological Chemistry* 283: 29572-29585.

Demaurex N, Poburko D and Frieden M (2009) Regulation of plasma membrane calcium fluxes by mitochondria. *Biochimica et Biophysica Acta* 1787: 1383-1394.

Deten A, Volz HC, Briest W and Zimmer HG (2002) Cardiac cytokine expression is upregulated in the acute phase after myocardial infarction. Experimental studies in rats. *Cardiovascular Research* 55: 329-340.

Dhanasekaran DN and Reddy EP (2008) JNK signaling in apoptosis. *Oncogene* 27: 6245-6251.

Di Lisa F, Menabo R, Canton M, Barile M and Bernardi P (2001) Opening of the mitochondrial permeability transition pore causes depletion of mitochondrial and cytosolic NAD+ and is a causative event in the death of myocytes in postischemic reperfusion of the heart. *The Journal of Biological Chemistry* 276: 2571-2575.

Doke SK and Dhawale SC (2015) Alternatives to animal testing: a review. *Saudi Pharmaceutical Journal* 23: 223-229.

Dorn GW, 2nd (2009) Having a change of heart: reversing the suicidal proclivities of cardiac myocytes. *Transactions of the American Clinical and Climatological Association* 120: 189-198.

Dougherty CJ, Kubasiak LA, Prentice H, Andreka P, Bishopric NH and Webster KA (2002) Activation of c-Jun N-terminal kinase promotes survival of cardiac myocytes after oxidative stress. *The Biochemical Journal* 362: 561-571.

Duncker DJ and Bache RJ (2008) Regulation of coronary blood flow during exercise. *Physiological Reviews* 88: 1009-1086.

Ea CK, Deng L, Xia ZP, Pineda G and Chen ZJ (2006) Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. *Molecular Cell* 22: 245-257.

Edler I (1991) Early echocardiography. *Ultrasound in Medicine & Biology* 17: 425-431.

Eisner V, Csordas G and Hajnoczky G (2013) Interactions between sarco-endoplasmic reticulum and mitochondria in cardiac and skeletal muscle - pivotal roles in Ca^{2+} and reactive oxygen species signaling. *Journal of Cell Science* 126: 2965-2978.

Elmore S (2007) Apoptosis: a review of programmed cell death. *Toxicologic Pathology* 35: 495-516.

Eltzschig HK and Collard CD (2004) Vascular ischaemia and reperfusion injury. *British Medical Bulletin* 70: 71-86.

Endo A, Kuroda M and Tsujita Y (1976) ML-236A, ML-236B, and ML-236C, new inhibitors of cholesterogenesis produced by *Penicillium citrinium*. *The Journal of Antibiotics* 29: 1346-1348.

Falk E, Shah PK and Fuster V (1995) Coronary plaque disruption. *Circulation* 92: 657-671.

Finn AV, Nakano M, Narula J, Kolodgie FD and Virmani R (2010) Concept of vulnerable/ unstable plaque. *Arteriosclerosis, Thrombosis, and Vascular Biology* 30: 1282-1292.

Fliss H and Gattinger D (1996) Apoptosis in ischemic and reperfused rat myocardium. *Circulation Research* 79: 949-956.

Fridovich I (1997) Superoxide anion radical (O_2^-), superoxide dismutases, and related matters. *The Journal of Biological Chemistry* 272: 18515-18517.

Frishman WH (2003) Cardiology patient page. Beta-adrenergic blockers. *Circulation* 107: 117-119.

Giordano FJ (2005) Oxygen, oxidative stress, hypoxia, and heart failure. *The Journal of Clinical Investigation* 115: 500-508.

Gomes LC and Scorrano L (2013) Mitochondrial morphology in mitophagy and macroautophagy. *Biochimica et Biophysica Acta* 1833: 205-212.

González A, Ravassa S, Beaumont J, López B, Díez J (2011) New Targets to Treat the Structural Remodeling of the Myocardium. *Journal of the American College of Cardiology*. 58: 1833-1843.

Gongora MC, Qin Z, Laude K, Kim HW, McCann L, Folz JR, Dikalov S, Fukai T and Harrison DG (2006) Role of extracellular superoxide dismutase in hypertension. *Hypertension* 48: 473-481.

Gottlieb RA, Burleson KO, Kloner RA, Babior BM and Engler RL (1994) Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *The Journal of Clinical Investigation* 94: 1621-1628.

Gregg D and Goldschmidt-Clermont PJ (2003) Cardiology patient page. Platelets and cardiovascular disease. *Circulation* 108: 88-90.

Griffiths EJ and Halestrap AP (1995) Mitochondrial non-specific pores remain closed during cardiac ischaemia, but open upon reperfusion. *The Biochemical Journal* 307: 93-98.

Griffiths EJ and Rutter GA (2009) Mitochondrial calcium as a key regulator of mitochondrial ATP production in mammalian cells. *Biochimica et Biophysica Acta* 1787: 1324-1333.

Gupta R (2005) Burden of coronary heart disease in India. *Indian Heart Journal* 57: 632-638

Gupta S, Kass GE, Szegezdi E and Joseph B (2009) The mitochondrial death pathway: a promising therapeutic target in diseases. *Journal of Cellular and Molecular Medicine* 13: 1004-1033.

Gustafsson AB and Gottlieb RA (2008) Heart mitochondria: gates of life and death. *Cardiovascular Research* 77: 334-343.

Hagar JM, Newman LG and Kloner RA (1992) Effects of amlodipine on myocardial infarction, infarct expansion, and ventricular geometry in the rat. *American Heart Journal* 124: 571-580.

Halestrap AP (2009) What is the mitochondrial permeability transition pore? *Journal of Molecular and Cellular Cardiology* 46: 821-831.

Halestrap AP, Clarke SJ and Javadov SA (2004) Mitochondrial permeability transition pore opening during myocardial reperfusion--a target for cardioprotection. *Cardiovascular Research* 61: 372-385.

Hagberg H, Mallard C, Rousset CI, Thornton C (2014). Mitochondria: hub of injury responses in the developing brain. *Lancet Neurology* 13: 217-232

Hausenloy DJ and Ruiz-Meana M (2010) Not just the powerhouse of the cell: emerging roles for mitochondria in the heart. *Cardiovascular Research* 88: 5-6.

Hausenloy DJ and Yellon DM (2013) Myocardial ischemia-reperfusion injury: a neglected therapeutic target. *The Journal of Clinical Investigation* 123: 92-100.

Hitomi J, Christofferson DE, Ng A, Yao J, Degterev A, Xavier RJ and Yuan J (2008) Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. *Cell* 135: 1311-1323.

Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, Bodmer JL, Schneider P, Seed B and Tschopp J (2000) Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. *Nature Immunology* 1: 489-495.

Hreniuk D, Garay M, Gaarde W, Monia BP, McKay RA and Cioffi CL (2001) Inhibition of c-Jun N-terminal kinase 1, but not c-Jun N-terminal kinase 2, suppresses apoptosis

induced by ischemia/reoxygenation in rat cardiac myocytes. *Molecular Pharmacology* 59: 867-874.

Huie RE and Padmaja S (1993) The reaction of NO with superoxide. *Free Radical Research Communications* 18: 195-199.

Iglewski M, Hill JA, Lavandero S and Rothermel BA (2010) Mitochondrial fission and autophagy in the normal and diseased heart. *Current Hypertension Reports* 12: 418-425.

Irwin MW, Mak S, Mann DL, Qu R, Penninger JM, Yan A, Dawood F, Wen WH, Shou Z and Liu P (1999) Tissue expression and immunolocalization of tumor necrosis factor-alpha in postinfarction dysfunctional myocardium. *Circulation* 99: 1492-1498.

Javadov S, Jang S and Agostini B (2014) Crosstalk between mitogen-activated protein kinases and mitochondria in cardiac diseases: therapeutic perspectives. *Pharmacology & Therapeutics* 144: 202-225.

Javadov S, Karmazyn M and Escobales N (2009) Mitochondrial permeability transition pore opening as a promising therapeutic target in cardiac diseases. *The Journal of Pharmacology and Experimental Therapeutics* 330: 670-678.

Johnson C, Sung HJ, Lessner SM, Fini ME and Galis ZS (2004) Matrix metalloproteinase-9 is required for adequate angiogenic revascularization of ischemic tissues: potential role in capillary branching. *Circulation Research* 94: 262-268.

Jousilahti P, Vartiainen E, Tuomilehto J, Puska P (1999) Sex, Age, Cardiovascular risk factors, and coronary heart disease: A prospective follow-up study of 14786 middle-aged men and women in Finland *Circulation* 99: 1165-1172

Kaiser RA, Bueno OF, Lips DJ, Doevendans PA, Jones F, Kimball TF and Molkentin JD (2004) Targeted inhibition of p38 mitogen-activated protein kinase antagonizes cardiac injury and cell death following ischemia-reperfusion in vivo. *The Journal of Biological Chemistry* 279: 15524-15530.

Kajstura J, Cheng W, Reiss K, Clark WA, Sonnenblick EH, Krajewski S, Reed JC, Olivetti G and Anversa P (1996) Apoptotic and necrotic myocyte cell deaths are independent contributing variables of infarct size in rats. *Laboratory Investigation* 74: 86-107.

Kalogeris T, Baines CP, Krenz M and Korthuis RJ (2012) Cell biology of ischemia/reperfusion injury. *International Review of Cell and Molecular Biology* 298: 229-317.

Kalogeris T, Bao Y and Korthuis RJ (2014) Mitochondrial reactive oxygen species: a double edged sword in ischemia/reperfusion vs preconditioning. *Redox Biology* 2: 702-714.

Kantari C and Walczak H (2011) Caspase-8 and Bid: caught in the act between death receptors and mitochondria. *Biochimica et Biophysica Acta* 1813: 558-563.

Kapadia SR, Oral H, Lee J, Nakano M, Taffet GE and Mann DL (1997) Hemodynamic regulation of tumor necrosis factor-alpha gene and protein expression in adult feline myocardium. *Circulation Research* 81: 187-195.

Karmazyn M, Gan XT, Humphreys RA, Yoshida H and Kusumoto K (1999) The myocardial Na(+)-H(+) exchange: structure, regulation, and its role in heart disease. *Circulation Research* 85: 777-786.

Kent KD, Harper WJ and Bomser JA (2003) Effect of whey protein isolate on intracellular glutathione and oxidant-induced cell death in human prostate epithelial cells. *Toxicology in vitro* 17: 27-33.

Kinnally KW, Peixoto PM, Ryu SY and Dejean LM (2011) Is mPTP the gatekeeper for necrosis, apoptosis, or both? *Biochimica et Biophysica Acta* 1813: 616-622.

Konstantinidis K, Whelan RS and Kitsis RN (2012) Mechanisms of cell death in heart disease. *Arteriosclerosis, Thrombosis, and Vascular Biology* 32: 1552-1562.

Krishnan MN (2012) Coronary heart disease and risk factors in India - on the brink of an epidemic? *Indian Heart Journal* 64: 364-367.

Krishnan MN, Zachariah G, Venugopal K, Mohanan PP, Harikrishnan S, Sanjay G, Jeyaseelan L and Thankappan KR (2016) Prevalence of coronary artery disease and its risk factors in Kerala, South India: a community-based cross-sectional study. *BMC Cardiovascular Disorders* 16: 2-12.

Kristian T and Siesjo BK (1998) Calcium in ischemic cell death. *Stroke* 29:705-718.

Kubli DA and Gustafsson AB (2012) Mitochondria and mitophagy: the yin and yang of cell death control. *Circulation Research* 111: 1208-1221.

Kumar D, Arya V, Kaur R, Bhat ZA, Gupta VK and Kumar V (2012) A review of immunomodulators in the Indian traditional health care system. *Journal of Microbiology, Immunology, and Infection* 45: 165-184.

Kumphune S, Bassi R, Jacquet S, Sicard P, Clark JE, Verma S, Avkiran M, O'Keefe SJ and Marber MS (2010) A chemical genetic approach reveals that p38alpha MAPK activation by diphosphorylation aggravates myocardial infarction and is prevented by the direct binding of SB203580. *The Journal of Biological Chemistry* 285: 2968-2975.

Kung G, Konstantinidis K and Kitsis RN (2011) Programmed necrosis, not apoptosis, in the heart. *Circulation Research* 108: 1017-1036.

Kuznetsov AV, Margreiter R, Amberger A, Saks V and Grimm M (2011) Changes in mitochondrial redox state, membrane potential and calcium precede mitochondrial dysfunction in doxorubicin-induced cell death. *Biochimica et Biophysica Acta* 1813: 1144-1152.

Lakshmi SV, Padmaja G, Kuppusamy P and Kutala VK (2009) Oxidative stress in cardiovascular disease. *Indian Journal of Biochemistry & Biophysics* 46: 421-440.

Lal H, Ahmad F, Parikh S and Force T (2014) Troponin I-interacting protein kinase: a novel cardiac-specific kinase, emerging as a molecular target for the treatment of cardiac disease. *Circulation Journal* 78: 1514-1519.

Lee SH, Wolf PL, Escudero R, Deutsch R, Jamieson SW and Thistlethwaite PA (2000) Early expression of angiogenesis factors in acute myocardial ischemia and infarction. *The New England Journal of Medicine* 342: 626-633.

Li J, Brown LF, Hibberd MG, Grossman JD, Morgan JP and Simons M (1996) VEGF, Flk-1, and Flt-1 expression in a rat myocardial infarction model of angiogenesis. *The American Journal of Physiology* 270: 1803-1811.

Lim SY, Davidson SM, Mocanu MM, Yellon DM and Smith CC (2007) The cardioprotective effect of necrostatin requires the cyclophilin-D component of the mitochondrial permeability transition pore. *Cardiovascular Drugs and Therapy* 21: 467-469.

Lloyd-Jones DM, Larson MG, Leip EP, Beiser A, D'Agostino RB, Kannel WB, Murabito JM, Vasan RS, Benjamin EJ, Levy D and Framingham Heart S (2002) Lifetime risk for developing congestive heart failure: the Framingham Heart Study. *Circulation* 106: 3068-3072.

Mahmood SS, Levy D, Vasan RS and Wang TJ (2014) The Framingham Heart Study and the epidemiology of cardiovascular disease: a historical perspective. *Lancet* 383: 999-1008.

Mann DL and Bristow MR (2005) Mechanisms and models in heart failure: the biomechanical model and beyond. *Circulation* 111: 2837-2849.

Maritim AC, Sanders RA and Watkins JB, 3rd (2003) Diabetes, oxidative stress, and antioxidants: a review. *Journal of Biochemical and Molecular Toxicology* 17: 24-38.

Maulik SK and Kumar S (2012) Oxidative stress and cardiac hypertrophy: a review. *Toxicology Mechanisms and Methods* 22: 359-366.

Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, Das SR, de Ferranti S, Despres JP, Fullerton HJ, Howard VJ, Huffman MD, Isasi CR, Jimenez MC, Judd SE, Kissela BM, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Magid DJ, McGuire DK, Mohler ER, 3rd, Moy CS, Muntner P, Mussolino ME, Nasir K, Neumar RW, Nichol G, Palaniappan L, Pandey DK, Reeves MJ, Rodriguez CJ, Rosamond W, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Woo D, Yeh RW and Turner MB (2015a) Heart Disease and Stroke Statistics-2016 Update: A Report From the American Heart Association. *Circulation*. 133: 38-360

Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, de Ferranti S, Despres JP, Fullerton HJ, Howard VJ, Huffman MD, Judd SE, Kissela BM, Lackland DT, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Matchar DB, McGuire DK, Mohler ER, 3rd, Moy CS, Muntner P, Mussolino ME, Nasir K, Neumar RW, Nichol G, Palaniappan L, Pandey DK, Reeves MJ, Rodriguez CJ, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Willey JZ, Woo D, Yeh RW, Turner MB, American Heart Association Statistics C and Stroke Statistics S (2015b) Heart disease and stroke statistics--2015 update: a report from the American Heart Association. *Circulation* 131: 29-322.

Mughal W and Kirshenbaum LA (2011) Cell death signalling mechanisms in heart failure. *Experimental and Clinical Cardiology* 16: 102-108.

Muravchick S and Levy RJ (2006) Clinical implications of mitochondrial dysfunction. *Anesthesiology* 105: 819-837.

Murphy E and Steenbergen C (2008) Ion transport and energetics during cell death and protection. *Physiology* 23: 115-123.

Nag T and Ghosh A (2015) Cardiovascular disease risk factor clustering among rural adult population in West Bengal, India. *Obesity Research & Clinical Practice* 10:124-32

Nossaman BD, Scruggs BA, Nossaman VE, Murthy SN and Kadowitz PJ (2010) History of right heart catheterization: 100 years of experimentation and methodology development. *Cardiology in Review* 18: 94-101.

O'Rourke B, Cortassa S and Aon MA (2005) Mitochondrial ion channels: gatekeepers of life and death. *Physiology* 20: 303-315.

Ong SB and Gustafsson AB (2012) New roles for mitochondria in cell death in the reperfused myocardium. *Cardiovascular Research* 94: 190-196.

Parrish AB, Freel CD and Kornbluth S (2013) Cellular mechanisms controlling caspase activation and function. *Cold Spring Harbor Perspectives in Biology* 5: 1-24

Pellegrino MW and Haynes CM (2015) Mitophagy and the mitochondrial unfolded protein response in neurodegeneration and bacterial infection. *BMC Biology* 13: 1-9.

Peng X and Li Y (2002) Induction of cellular glutathione-linked enzymes and catalase by the unique chemoprotective agent, 3H-1,2-dithiole-3-thione in rat cardiomyocytes affords protection against oxidative cell injury. *Pharmacological Research* 45: 491-497.

Perricone AJ and Vander Heide RS (2014) Novel therapeutic strategies for ischemic heart disease. *Pharmacological Research* 89: 36-45.

Petrovska BB and Cekovska S (2010) Extracts from the history and medical properties of garlic. *Pharmacognosy Reviews* 4: 106-110.

Pfeffer MA, Pfeffer JM, Fishbein MC, Fletcher PJ, Spadaro J, Kloner RA and Braunwald E (1979) Myocardial infarct size and ventricular function in rats. *Circulation Research* 44: 503-512.

Piquereau J, Caffin F, Novotova M, Lemaire C, Veksler V, Garnier A, Ventura-Clapier R and Joubert F (2013) Mitochondrial dynamics in the adult cardiomyocytes: which roles for a highly specialized cell? *Frontiers in Physiology* 4: 102-104.

Ponnusamy S, Ravindran R, Zinjarde S, Bhargava S and Ravi Kumar A (2011) Evaluation of traditional Indian antidiabetic medicinal plants for human pancreatic amylase

inhibitory effect in vitro. *Evidence-Based Complementary and Alternative Medicine :* 2011: 1-10

Proudfit WL, Shirey EK and Sones FM, Jr. (1966) Selective cine coronary arteriography. Correlation with clinical findings in 1,000 patients. *Circulation* 33: 901-910.

Puente BN, Kimura W, Muralidhar SA, Moon J, Amatruda JF, Phelps KL, Grinsfelder D, Rothermel BA, Chen R, Garcia JA, Santos CX, Thet S, Mori E, Kinter MT, Rindler PM, Zacchigna S, Mukherjee S, Chen DJ, Mahmoud AI, Giacca M, Rabinovitch PS, Aroumougame A, Shah AM, Szweda LI and Sadek HA (2014) The oxygen-rich postnatal environment induces cardiomyocyte cell-cycle arrest through DNA damage response. *Cell* 157: 565-579.

Quirke V (2006) Putting theory into practice: James Black, receptor theory and the development of the beta-blockers at ICI, 1958-1978. *Medical History* 50: 69-92.

Rose BA, Force T and Wang Y (2010) Mitogen-activated protein kinase signaling in the heart: angels versus demons in a heart-breaking tale. *Physiological Reviews* 90: 1507-1546.

Roberts WC (1990) Interventional and surgical cardiovascular pathology: clinical correlations and basic principles. *Annals of Surgery* 212: 645

Safeer RS and Lacivita CL (2000) Choosing drug therapy for patients with hyperlipidemia. *American Family Physician* 61: 3371-3382.

Sanada S, Komuro I and Kitakaze M (2011) Pathophysiology of myocardial reperfusion injury: preconditioning, postconditioning, and translational aspects of protective measures. *American Journal of Physiology Heart and Circulatory Physiology* 301: 1723-1741.

Schomig A (1990) Catecholamines in myocardial ischemia. Systemic and cardiac release. *Circulation* 82: 13-22.

Schomig A, Haass M and Richardt G (1991) Catecholamine release and arrhythmias in acute myocardial ischaemia. *European Heart Journal* 12: 38-47.

Schwartze D (1985) Willem Einthoven. Thoughts on the occasion of his 125th anniversary. *Zeitschrift fur die gesamte innere Medizin und ihre Grenzgebiete* 40: 356-360.

Seddon M, Looi YH and Shah AM (2007) Oxidative stress and redox signalling in cardiac hypertrophy and heart failure. *Heart* 93: 903-907.

Shimokawa H and Yasuda S (2008) Myocardial ischemia: current concepts and future perspectives. *Journal of Cardiology* 52: 67-78.

Silverman ME (1985) William Harvey and the discovery of the circulation of blood. *Clinical Cardiology* 8: 244-246.

Silverman ME (1989) William Withering and an account of the foxglove. *Clinical Cardiology* 12: 415-418.

Singh R, Hussain S, Verma R and Sharma P (2013) Anti-mycobacterial screening of five Indian medicinal plants and partial purification of active extracts of Cassia sophera and Urtica dioica. *Asian Pacific Journal of Tropical Medicine* 6: 366-371.

Smith CC and Yellon DM (2011) Necroptosis, necrostatins and tissue injury. *Journal of Cellular and Molecular Medicine* 15: 1797-1806.

Soman CR, Kutty VR, Safraj S, Vijayakumar K, Rajamohanan K, Ajayan K and Group PS (2011) All-cause mortality and cardiovascular mortality in Kerala state of India: results from a 5-year follow-up of 161,942 rural community dwelling adults. *Asia-Pacific Journal of Public Health* 23: 896-903.

Steinberg SF (1999) The molecular basis for distinct beta-adrenergic receptor subtype actions in cardiomyocytes. *Circulation Research* 85: 1101-1111.

Sucher R, Gehwolf P, Kaier T, Hermann M, Maglione M, Oberhuber R, Ratschiller T, Kuznetsov AV, Bosch F, Kozlov AV, Ashraf MI, Schneeberger S, Brandacher G, Ollinger R, Margreiter R and Troppmair J (2009) Intracellular signaling pathways control mitochondrial events associated with the development of ischemia/ reperfusion-associated damage. *Transplant International* 22: 922-930.

Takimoto E and Kass DA (2007) Role of oxidative stress in cardiac hypertrophy and remodeling. *Hypertension* 49: 241-248.

Tang H, Xiao K, Mao L, Rockman HA and Marchuk DA (2013) Overexpression of TNNI3K, a cardiac-specific MAPKKK, promotes cardiac dysfunction. *Journal of Molecular and Cellular Cardiology* 54: 101-111.

Triposkiadis F, Karayannis G, Giamouzis G, Skoularigis J, Louridas G and Butler J (2009) The sympathetic nervous system in heart failure physiology, pathophysiology, and clinical implications. *Journal of the American College of Cardiology* 54: 1747-1762.

Tsutsui H, Kinugawa S and Matsushima S (2011) Oxidative stress and heart failure. *American Journal of Physiology Heart and Circulatory Physiology* 301: 2181-2190.

Turer AT and Hill JA (2010) Pathogenesis of myocardial ischemia-reperfusion injury and rationale for therapy. *The American Journal of Cardiology* 106: 360-368.

Vagnozzi RJ, Gatto GJ, Jr., Kallander LS, Hoffman NE, Mallilankaraman K, Ballard VL, Lawhorn BG, Stoy P, Philp J, Graves AP, Naito Y, Lepore JJ, Gao E, Madesh M and Force T (2013) Inhibition of the cardiomyocyte-specific kinase TNNI3K limits oxidative stress, injury, and adverse remodeling in the ischemic heart. *Science Translational Medicine* 5: 207-215.

Vander Heide RS and Steenbergen C (2013) Cardioprotection and myocardial reperfusion: pitfalls to clinical application. *Circulation Research* 113: 464-477.

Varier PK, Nambiar, V. P. K., Ramankutty, C. (1996) Indian Medicinal Plants: A compendum of 500 species, pp 311-315, Orient Longman Limited, 160, Anna Salai, Chennai 600031, Chennai.

Wang L, Du F and Wang X (2008) TNF-alpha induces two distinct caspase-8 activation pathways. *Cell* 133: 693-703.

Whelan RS, Kaplinskiy V and Kitsis RN (2010) Cell death in the pathogenesis of heart disease: mechanisms and significance. *Annual Review of Physiology* 72: 19-44.

Wong ND (2014) Epidemiological studies of CHD and the evolution of preventive cardiology. *Nature Reviews Cardiology* 11: 276-289.

Yaniv Y, Juhaszova M, Nuss HB, Wang S, Zorov DB, Lakatta EG and Sollott SJ (2010) Matching ATP supply and demand in mammalian heart: *in vivo, in vitro,* and *in silico* perspectives. *Annals of the New York Academy of Sciences* 1188: 133-142.

Zachariah G, Harikrishnan S, Krishnan MN, Mohanan PP, Sanjay G, Venugopal K, Thankappan KR, Cardiological Society of India Kerala Chapter Coronary Artery D and Its Risk Factors Prevalence Study I (2013) Prevalence of coronary artery disease and coronary risk factors in Kerala, South India: a population survey design and methods. *Indian Heart Journal* 65: 243-249.

Zakrzeska A, Schlicker E, Kwolek G, Kozlowska H and Malinowska B (2005) Positive inotropic and lusitropic effects mediated via the low-affinity state of beta1-adrenoceptors in pithed rats. *British Journal of Pharmacology* 146: 760-768.

Zaragoza C, Gomez-Guerrero C, Martin-Ventura JL, Blanco-Colio L, Lavin B, Mallavia B, Tarin C, Mas S, Ortiz A and Egido J (2011) Animal models of cardiovascular diseases. *Journal of Biomedicine & Biotechnology* 20: 497841- 497851.

Zbinden G and Bagdon RE (1963) Isoproterenol-Induced heart necrosis, an experimental model for the study of angina pectoris and myocardial infarct. *Revue canadienne de biologie / editee par l'Universite de Montreal* 22: 257-263.

Materials and methods

2.1Chemicals and reagents

2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 4-hydroxybenzoic acid, ethylene diaminetetraacetic acid (EDTA), phenazine methosulphate (PMS), nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide reduced (NADH), 2-deoxyribose, (dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), gallic acid, catechin, trolox, α-tocopherol, caffeic acid, chlorogenic acid, ferulic acid, phloridzin, diosgenin, 2,7-dihydro dichlorofluorescein diacetate (H_2DCFDA), dihydroethidium(DHE), mitochondria staining kit-JC1, bongkrekic acid, cobalt chloride, Dulbecco's Modified Eagle's Medium (DMEM), ethidium bromide (EtBr), 3,3'-diaminobenzidine (DAB), haematoxylin, eosin, dimethyl sulphoxide (DMSO) and all other chemicals unless otherwise mentioned were from Sigma Aldrich (St. Louis, MO, USA). Kits for biochemical assays (lactate dehydrogenase-LDH), creatinine kinase- CK, creatinine kinase myocardial b fraction CK-MB, serum glutamic oxaloacetic transaminase -SGOT, serum glutamic pyruvic transaminase -SGPT and calcium) were from Agappe Diagnostics, Kerala, India. Kits for real time PCR, were obtained from Invitrogen, Life Technologies, Carlsbad, USA. Ascorbic acid, butylated hydroxyl toluene (BHT), ferric chloride, thiobarbituric acid (TBA), trichloroacetic acid (TCA) and folin-ciocalteu reagent were from Sisco Research Laboratories (Mumbai, India) and fetal bovine sera (FBS), antibiotic/antimycotic solution was from Gibco (Langley, OK, USA).The cell culture flasks and plates were from BD Biosciences (USA). All solvent of both analytical and HPLC grade were from Merck (USA).

2.2 Plant material and preparation

Tribulus terrestris L. whole plant was collected during the month of April from Virudhunagar district (Latitude 9° 35' 1.0032"Longitude:E 77° 57' 30.0384") of Tamil Nadu, India, as per advice from Ayurvedic physician, identified and authenticated by Dr. Biju Haridas, from the Jawaharlal Nehru Tropical Botanical Garden and Research Institute, Palode, Trivandrum. For future reference, voucher specimen (AC/4-2011) has been deposited at the herbarium of our Institute.

The *Tribulus terrestris* L. fruit and root were separated, washed and dried in an oven at 37 °C, and pulverized. One kilogram of the ground root was macerated with methanol at room temperature (27 ± 1˚C) under continuous stirring for 8 hrs, filtered and concentrated under vacuum at reduced pressure to yield TTM extract. TTM was further fractionated with solvents according to their polarity to yield T. *terrestris* root methanol extract DCM fraction (TTMD), *T. terrestris* root methanol extract ethyl acetate fraction (TTME) and *T. terrestris* root methanol extract methanol fraction (TTMM). Fruits were processed separately. One kilogram of fruit was macerated with methanol at room temperature (27 ± 1˚C) under continuous stirring for 8 hrs, filtered and concentrated under vacuum at reduced pressure using a rotary evaporator (BUCHI R-215, Switzerland) to yield TFM extract. TFM extract was not further fractionated. The yield of the extracts of TTM and TFM and fractions TTMD, TTME and TTMM were also calculated and checked for its antioxidant potential.

2.3 Chemical characterization

2.3.1 Quantification of total phenolic content

The total phenolic content (TPC) of TTM and TFM was determined using the Folin-Ciocalteu reagent (Singleton, 1965). Different concentrations of TTM and TFM and its different fractions were taken and 500 µL of Folin-Ciocalteu reagent was added. After 5 minutes of incubation, 1 mL of sodium carbonate (20%) was added and incubated at ambient temperature (25-27 °C) for 90 minutes. The color developed was measured at 765 nm using a UV-VIS spectrophotometer (UV-2450 PC; Shimadzu, Kyoto, Japan). Results were expressed as milligram gallic acid equivalents per g of extract (mg GAE/ g of extract).

2.3.2 Quantification of total flavonoid content

The total flavonoid content (TFC) was determined by a colorimetric method (Maksimovic et al., 2005) with minor modifications. Different concentration of TTM, its fractions and TFM were added to 1ml of 5 % (w/v) sodium nitrite and placed for 6 min, followed by reaction with 1 mL of 10 % (w/v) aluminium chloride to form a flavonoid-aluminium complex. After 6 minutes, 10 mL of 4.3 % (w/v) NaOH was added and the total volume was made up to 25 ml. with distilled water. After 15 minutes at room temperature, the absorbance was measured against a blank at 510 nm with a UV-VIS spectrophotometer (UV-

2450 PC, Shimadzu, Japan) and the TFC was expressed as milligram catechin equivalents per g of extract.

2.3.3 Quantification of total saponin content

The total saponin content was determined as described previously (Xi et al., 2008). Different concentrations of TFM were mixed with 0.1 ml vanillin (8 %) and 1 ml sulphuric acid (72 %). The mixture was incubated at 60 °C for 10 minutes and cooled on an ice bath for 15 minutes. The absorbance was read at 560 nm. Diosgenin was used as reference standard and the total saponin content was expressed as mg diosgenin equivalents per g dry weight of extract.

2.4. Determination of antioxidant potential

Free radical scavenging activity of the extract was determined by the following assays.

2.4.1 DPPH radical scavenging activity

The ability of the extract to scavenge DPPH radical was determined (Shimada et al., 1992). Briefly, 3 mL of the reaction mixture containing 2.9 mL of 0.01 M DPPH in methanol (OD should be between 0.9 - 1.1) and 0.1 ml of extract at various concentrations were mixed and read at 517 nm after 30 min using a UV-VIS spectrometer. When DPPH reacts with an antioxidant compound that can donate hydrogen, it gets reduced, and the resulting decrease in absorbance at 517 nm after 30 minutes was recorded using a UV-VIS spectrophotometer (UV-2450 PC, Shimadzu, Japan). Gallic acid was used as standard compound.

2.4.2 ABTS radical scavenging activity

The ability of the extracts and standard compound to scavenge ABTS radical was determined (Re et al., 1999).OD was measured at 734 nm (BioTek Instruments, Winooski, USA), 7 min after the initial mixing of different concentrations of extract with 7mM ABTS solution (Stock solution – 38.4 mg ABTS and 6.62 mg potassium persulphate in 10 ml distilled water and diluted in ethanol to obtain OD between 0.65 and 0.75). Trolox, the water soluble analogue of vitamin E was used as a reference standard. Percentage radical scavenging activity was plotted against the corresponding concentration of extract to obtain the IC_{50}.

2.4.3 Superoxide radical scavenging activity assay

Superoxide anions were generated in a non-enzymatic phenazine methosulphate (PMS)-NADH system through the reaction of PMS, NADH and oxygen (Liu et al., 1997) and it was assayed by the reduction of NBT. In this assay, the superoxide anions were generated in Tris-HCl buffer (100 mM, pH 7.4) containing 0.75 ml of NBT (300 mM) solution, 0.75 ml of NADH (936 mM) solution and 0.3 ml of different concentrations of the extract and its fractions. The reaction was initiated by adding 0.75 ml of PMS (120 mM) solution to the mixture. After 5 minutes of incubation at room temperature, the absorbance at 560 nm was measured in a spectrophotometer (UV-2450 PC; Shimadzu, Kyoto, Japan).

2.4.4 Hydroxyl radical scavenging activity assay

The hydroxyl radical scavenging activity was measured by the deoxyribose method (Halliwell et al., 1987) and compared with that of catechin. The reaction mixture containing TTM and TFM at various concentrations (12.5-100 mg), deoxyribose (3.75 mM), H_2O_2 (1 mM), potassium phosphate buffer (20 mM, pH 7.4), $FeCl_3$ (0.1 mM), EDTA (0.1 mM) and ascorbic acid (0.1 mM) was incubated in a water bath at 37 °C for 1 h. The extent of deoxyribose degradation was measured by adding TBA (1 %) and TCA (2 %) and heated in a water bath at 100 °C for 20 minutes. The absorbance of the resulting solution was measured spectrophotometrically at 532 nm (UV-2450 PC; Shimadzu, Kyoto, Japan)

2.4.5 Determination of LDL oxidation

Oxidation of LDL and production of malondialdehyde (MDA) was measured by reaction with thiobarbituric acid (TBA) (Scoccia et al., 2001). LDL (50 µg/ml) was incubated with different concentrations of extract and the oxidation of LDL was initiated by the addition of 50 µl copper sulphate (2 mM) and incubation at 37 °C for two hrs. Final volume of the reaction mixture was made up to 1.5 ml with phosphate buffer (pH 7.4). After incubation, 500 µl of reaction mixture was mixed with 250 µl of TBA (1 % in 50 mM of NaOH) and TCA (0.28 %). Samples were again incubated at 95 °C for 45 min. After cooling and centrifugation at 2000 rpm (10 min) fluorescence was taken at 515 nm excitation and 553 nm emission. Solutions without test samples were used as controls.

2.4.6 Determination of antiperoxidative activity in linoleic acid emulsion system

Antiperoxidative activity was determined by thiocyanate method in linoleic acid emulsion system (Duh et al., 1997). Linoleic acid emulsion was prepared by mixing 0.28 g linoleic acid, 0.28 g Tween 20 and 50 ml of phosphate buffer (0.2 M, pH 7.0). Different concentrations of the test sample were mixed with linoleic acid emulsion in potassium phosphate buffer (0.02 M, pH 7.0) and the reaction mixture was incubated at 37 °C for 24 hrs. After incubation, the degree of oxidation was measured by sequentially adding ethanol (5 ml, 75 %), ammonium thiocyanate (0.1 ml, 30 %) and ferrous chloride (0.1 ml, 0.02 M in 3.5 % HCl) to the linoleic acid emulsion containing test sample (0.1 ml), and the absorbance was read at 500 nm (UV 2450 PC; Shimadzu, Kyoto, Japan). Solutions without test samples were used as control samples.

2.5 High performance liquid chromatography for identification of compounds

2.5.1 Chemicals and standard solution preparation

All the solvents and chemicals used in the experiments were of HPLC grade. The stock solutions of caffeic acid, ferulic acid, chlorogenic acid, 4-hydroxybenzoic acid, phloridzin and diosgenin were prepared separately in methanol. Working solution was prepared from stock solution at appropriate concentration range of (10-100 ppm) for each sample.

2.5.2 Instrumentation and chromatographic conditions

Chromatographic separation and analysis was performed with Agilent HPLC equipped with C-18 column (250 * 4.6 mm, 5 µm) (Agilent Technologies, USA) for phenolics and flavonoids. The mobile phase comprising of 1 % aqueous acetic acid: acetonitrile in the ratio (60:40) v/v maintaining a flow rate of 1.0 ml/min and peaks were detected at 254 nm with UV detector. For separation of the saponin, diosgenin a C8 column (250 * 3 mm, 5 µm) was used. Mobile phase was 1 % aqueous acetic acid (A) and methanol (B) in the ratio 15:85. Flow rate was 1ml/ min and chromatogram was acquired at 254 nm. The sample working solutions (10 µl) was injected and the area was measured for all compounds. From the calibration curve (peak area *vs* concentration), the amount of caffeic

acid, ferulic acid, chlorogenic acid, 4-hydroxybenzoic acid, phloridzin and diosgenin were calculated.

2.6 *In vitro* cell based assays

2.6.1 Cell culture

H9c2 cell line, embryonic rat heart myoblasts were purchased from the National Centre for Cell Science, Pune, India. The cells were maintained in a 37^{o} C incubator (Sanyo, Japan) in an atmosphere of 5% CO_2 in DMEM with 10% FBS, antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) and amphotericin B (0.25 µg/ml) and grown to sub confluence prior to the experiments. Cells were pretreated with TTM and TFM for 24 hours prior to induction of ischemia. 0.1% DMSO was used as the vehicle and 0.1 µM cyclosporin A was used as the positive control.

2.6.2 Induction of ischemia in H9c2 cardiomyobalsts

2.6.2.1 Experimental details

To induce ischemia, H9c2 cells were maintained at 37 °C, 0.1 % O_2, 5 % CO_2,, 95 % N_2 in a hypoxia incubator (Galaxy 48 R, New Brunswick Scientific, USA), in an ischemic buffer (composition in mM :137 NaCl, 12 KCl, 0.5 $MgCl_2$, 0.9 $CaCl_2$, 20 HEPES, 20 2-deoxy-D-glucose, pH-6.2) according to a previously reported method (Loos et al., 2011) for 1 hour. Morphological changes during ischemia were visualized (Nikon, NY, USA). After induction of ischemia for 1 hour, cells were removed from the hypoxia chamber and was either used for performing experiments or harvested immediately to isolate the RNA and protein for further experiments.

2.6.3 MTT assay for determination of cell viability

The cell viability with various concentrations of TTM and TFM (10-175 µg/ml) were tested by MTT assay to rule out toxicity (Mosmann, 1983). For this, cells were seeded in 24 well plates at a concentration of 5x 10^4 cells per well. Cells grown to 70-85 % confluence were exposed to various concentrations of TTM and TFM (10-175 µg/ml). TTM and TFM was dissolved in dimethyl sulphoxide (DMSO) and the final concentration of DMSO used was less than 0·1 % (v/v) for each treatment. The same concentration of DMSO was used in

control cells as vehicle. The control and treated cells were incubated for 24 h in 37 °C, 5 % CO_2 incubator. After incubation, media was removed and replaced with MTT and incubated for 3-4 hours. After incubation, DMSO was added to dissolve insoluble crystals and the absorbance was read at 570 nm.

2.6.4 MTT assay for determination of protective property of compounds against ischemia

Protective effect of pretreatment with phenolic compounds; caffeic acid, chlorogenic acid, 4-hydroxybenzoic acid, ferulic acid, phloridzin and saponin; diosgenin (1, 2.5, 5, 10, 25 µg/ml) against ischemia, were also analyzed by MTT assay. Cells were seeded in 96 well plates at a density of $5x10^3$ cells pr well. They were pretreated with different compounds (1-25 µg/ml) for 24 hours, followed by ischemia for 1 hour. MTT assay was performed and absorbance measured at 570 nm.

2.6.5 LDH release assay

Release of LDH into the medium by the cells from all experimental groups (ischemia group and pretreated with different concentrations TTM and TFM (10-25 µg/ml) was measured using an LDH assay kit (Cayman Chemical, Ann Arbor, Michigan, USA) following manufacturer's instructions. Briefly, 100 µl of supernatant was collected from cultured cells and is added with 100 µl of LDH reaction solution containing NAD+, lactic acid, iodonitrotetrazolium (INT) and diaphorase (Decker and Lohmann-Matthes, 1988). The mixture was incubated with gentle shaking for 30 minutes at room temperature and the absorbance was taken at 490 nm.

2.6.6 Detection of apoptosis using Annexin V-FITC/PI double staining assay

Analysis of cell death by apoptosis was done by Annexin V-FITC/ PI technique (Fadok et al., 1992). For this, the cells were seeded in a 96 well plate at a density of $5x10^3$ cells/well. Intensity of fluorescence was measured after 24 hours of pretreatment with TTM and 1 hour ischemia. Dead cells were stained by propidium iodide and detected at excitation, emission of 540 and 570 nm respectively. Apoptotic cells stained by Annexin V-FITC were detected at 485/ 535 nm. The apoptotic cells exhibited green fluorescence, while necrotic

cells showed red and green fluorescence on staining with Annexin V- FITC/ PI. On the other hand, live cells presented little or no fluorescence.

2.6.7 Preparation of cell lysate for antioxidant enzyme activities

The harvested cells were homogenized with 20 mM Tris/ HCl buffer (pH 7.5) containing 0.2 % Triton X-100 and 0.5 mM PMSF and sonicated for 30 seconds on ice. Total cell lysates were centrifuged at 3000 rpm at 4° C for 15 minutes and aliquots of the supernatant were utilized for subsequent enzymatic assays.

2.6.7.1 Activity of superoxide dismutase

Superoxide dismutase activity was measured according to the spectrophotometric method (Kakkar et al., 1984). Cell lysate (250 µl) was added to 0.5 ml water, 1.25 ml ethanol, 0.75 ml chloroform and centrifuged at 3000 rpm for 5 minutes. To the supernatant, sodium pyrophosphate (0.025 M, pH 8.3), phenazine methosulphate (PMS) (186 µM), nitroblue tetrazolium (NBT) (300 µM) and NADH (780 µM) was added and incubated at 30 °C for 90 seconds. After incubation, glacial acetic acid and butanol was added to the reaction mixture and the absorbance was read at 560 nm. One unit of the enzyme activity is defined as the enzyme reaction which gave 50 % inhibition of NBT reduction in one minute under the assay conditions and expressed as specific activity in units/ mg protein.

2.6.7.2 Activity of catalase

Activity of catalase was assayed by monitoring the disappearance of H_2O_2 at 240 nm (Aebi, 1984). Briefly, 0.1 ml of cell lysate was added to a cuvette containing 1.9 ml of 50 mM phosphate buffer (pH 7.0). The reaction was started by addition of 1 ml of freshly prepared 30 mM H_2O_2 and the catalase activity was expressed as amount of enzyme that catalyzed the conversion of one µM H_2O_2 in a minute/mg protein.

2.6.7.3 Activity of glutathione peroxidase

Glutathione peroxidase activity was assayed according to the method of Flohe and Gunzler (Flohe and Gunzler, 1984). The assay mixture contained 500 µl of potassium phosphate (0.1 mM), 1 mM of Na_2EDTA (pH 7.0), 100 µl of 1 mM NaN_3, 100 µl of cell lysate, 100 µl of glutathione reductase (0.24 U), and 100 µl of 10 mM reduced glutathione.

After 10 minutes of incubation at 37° C, 100 µl of 1.5 mM NADPH in 0.1% $NaHCO_3$ was added. The reaction was started by adding 100 µl of H_2O_2 (1.5 mM) and the decrease in absorbance at 340 nm was monitored for about 5 minutes. The enzyme activity was calculated and one unit of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 µM NADPH per minute.

2.6.7.4 Estimation of reduced glutathione (GSH)

The cellular GSH content in control and treated cells was measured according to the method of Hissin and Hilf (Hissin and Hilf, 1976). In brief, cells after respective treatment were pelleted by centrifugation (750 × g) for 5 minutes and were washed with PBS. To the pellet, 50 µl of 25 % HPO_3 and 188 µl of 0.1 M sodium phosphate buffer containing 5 mM EDTA (pH 8.0) were added, and then the cells were homogenized on ice. After homogenization, the samples were centrifuged at 13000 × *g* for 5 minutes at 4 °C. The supernatant was diluted with the above phosphate buffer; 0.1 ml of diluted sample was incubated with 0.1 ml of o-phthalaldehyde solution (0.1 % in methanol) and 1.8 ml of phosphate buffer for 15 minutes at room temperature. Fluorescence was then read with an excitation wavelength of 350 nm and emission wavelength of 420 nm. Cellular GSH content was expressed as nanomoles of GSH per milligram of cellular protein.

2.6.7.5 Total antioxidant capacity

The total antioxidant capacity was determined in cell lysate using total antioxidant assay kit. The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS (2, 2'-azino-di-[3-ethylbenzthiazoline sulphonate]) to reduced $ABTS^{**++}$ by metmyoglobin (Miller et al., 1993). The capacity of the antioxidants in the sample to prevent ABTS oxidation is compared with that of trolox, a water-soluble tocopherol analogue, and is quantified as molar trolox equivalents. The cell after treatment were harvested and centrifuged at 2000 rpm for 10 minutes. The pellet thus formed is homogenized in ice using lysis buffer, centrifuged and the supernatant was used for the study. Metmyoglobin (10 µl), chromogen (150 µl), samples or standard (trolox) are added to appropriate wells of the plate. The reaction is initiated by adding 40 µl of hydrogen peroxide and the absorbance was measured at 750 nm using a plate reader. The antioxidant concentration of the sample was

calculated using the equation obtained from the linear regression of the standard curve by substituting the average absorbance for each sample and the antioxidant concentration are expressed in mM.

2.7 Detection of intracellular ROS

Analysis of ROS production in ischemia was determined employing DCFH-DA (Choi et al., 2007). DCFH-DA is cleaved intracellularly by non-specific esterase and it becomes highly fluorescent upon oxidation by ROS. The cells were seeded in 96 well black plates, at a density of $5x10^3$ cells per well. After 24 hours of pretreatment with TTM and TFM, the cells were exposed to ischemia for 1 hour. DCFH-DA (20 µM) dissolved in PBS was added to the medium and incubated at 37 °C, in dark, in a CO_2 incubator for 20 minutes. The cells were then washed with PBS (pH-7.4) and subjected to fluorometric analysis at excitation and emission wavelength of 490 and 525 nm, in a multimode reader (BioTek, USA). Images of the cells were taken by fluorescent microscope (BD Pathway 855 Bio-imaging system, BD Biosciences, USA) equipped with filters in the FITC range (i.e. excitation, 490 nm; and emission, 525 nm) and analyzed using AttoVision 1.5.3 software.

For flow cytometry analysis, cells were incubated with DCFH-DA (20 µM) for 20 minutes at 37 °C in a humidified atmosphere of 5 % CO_2 incubator. After incubation, cells were trypsinized and suspended in Krebs-Ringer-phosphate buffer (pH 7.4). For each measurement, data from 10000 single cell events were collected using a flow cytometer (FACS Aria II, BD Bioscience, San Jose, USA) and analyzed using BD FACS Diva ™ software (BD Bioscience, San Jose, USA)

2.8 Studies on mitochondria

2.8.1 Detection of mitochondrial superoxide radical production

Mitochondrial superoxide productions in the H9c2 cells were detected with a fluorescent dye, MitoSOX (Dagda et al., 2009a). Briefly after respective treatments, cells were washed with PBS (pH 7.4) and loaded with MitoSOX (5 µM) in the medium and incubated for 20 minutes at 37 °C in CO_2 incubator. Live cell bioimaging was done with (BD Pathway 855 Bioimaging System, BD Biosciences) and the excitation/emission range of the dye was 514/ 580 nm.

2.8.2 Detection of mitochondrial transmembrane potential

Change in mitochondrial transmembrane potential (ΔΨm) was detected using a mitochondria staining kit (Sigma- Aldrich, St. Louis, MO, USA) that uses JC-1, a cationic fluorescent dye. The cells from various treated groups were incubated with JC-1 stain for 20 minutes. In normal cells, because of the electrochemical potential gradient, the JC-1 dye concentrates in the mitochondrial matrix, where it forms red fluorescent aggregates. Dissipation of ΔΨm prevents the accumulation of JC-1 in the mitochondria and thus it is dispersed throughout the entire cell, leading to a shift from red (J-aggregates) to green fluorescence (JC-1 monomers). For imaging of JC-1 monomers, the live cell bioimager (BD Pathway 855 Bio-imaging System, BD Biosciences, USA) was set at 490 nm excitation and 530 nm emission wavelengths, and for J-aggregates, the wavelengths were set at 525 nm excitation and 590 nm emission (Javadov et al., 2006b). Valinomycin was used as the positive control.

2.8.3 Integrity of mitochondrial permeability transition pore

To examine the mitochondrial permeability transition pore (mPTP) opening, the cells were loaded with calcein-AM (0.25 μM) in the presence of 8 mM cobalt chloride for 30 minutes to quench cytosolic and nuclear calcein fluorescence (Javadov et al., 2006a). The calcein fluorescence was then compartmentalized within mitochondria until PTP opening permits the distribution of cobalt inside mitochondria, which results in the quenching of calcein fluorescence in the mitochondrial matrix. Images of the cells were taken at 488 nm excitation and 525 nm emission (BD Pathway 855 Bioimaging System, BD Biosciences, USA).

2.8.4 Determination of the activity of mitochondrial respiratory complexes (OXPHOS)

After respective treatments, mitochondria were isolated using a mitochondrial isolation kit (Sigma-Aldrich, St. Louis, MO, USA) and suspended in 50 mM phosphate buffer (pH 7.0). Then it was frozen and thawed 3-5 times to release the enzymes (except complex IV, which was extracted with 0.5 % Tween 80 in phosphate buffer, v/v). The effect of TFM on complex I mediated electron transfer (NADH dehydrogenase) was studied using NADH as the substrate and menadione as electron acceptor. The reaction mixture containing 200 μM

menadione and 150 µM NADH was prepared in phosphate buffer (0.1 M, pH 8.0). To this, mitochondria (100 µg) was added, mixed immediately and observed quickly for change in the absorbance at 340 nm for 8 minutes (UV-2450 PC; Shimadzu, Kyoto, Japan) (Paul et al., 2008).

Complex II (succinate dehydrogenase) mediated activity was measured spectrophotometrically at 600 nm using DCPIP as an artificial electron acceptor and succinate as substrate. The extent of decrease of absorbance was considered as the measure of the electron transfer activity of complex II (Paul et al., 2008). The reaction mixture was prepared in 0.1 M phosphate buffer (pH 7.4) containing 10 mM EDTA, 50 µM DCPIP, 20 mM succinate and mitochondria (50 µg). The change in absorbance was observed immediately for 8 minutes at 30 °C.

Complex III (ubiquinol-cytochrome C reductase) activity was determined as per the method described previously (Sudheesh et al., 2009). In brief, mitochondrial protein (50 µg) was mixed with 100 µM EDTA, 2 mg BSA, 3 mM sodium azide, 60 µM ferricytochrome C, decylubiquinol (1.3 mM) and phosphate buffer (50 mM, pH 8.0) in a final volume of 1 ml. The reaction was started by the addition of decylubiquinol and monitored for 2 minutes at 550 nm and again after the addition of 1 µmol/ l of antimycin A. The activity was calculated from the linear part of absorption-time curve, which was not less than 30 seconds. Activity of complex III was expressed as µmoles of ferricytochrome C reduced/minute/mg protein.

Activity of complex IV (cytochrome C oxidase) was determined as per previous method (Sudheesh et al., 2009). Briefly, 1 ml of ferrocytochrome C solution was mixed with approximately 50 µg of mitochondrial protein and phosphate buffer (30 mM, pH 7.4) in a net volume of 1.3 ml. The reaction was started by the addition of enzyme source and was monitored at 550 nm with an interval of 15 seconds for 4 minutes. The difference in absorbance was calculated from the linear part of the absorption-time curve. KCN (5 µM) was used as inhibitor of complex IV. Complex IV activity was expressed as micromoles of ferrocytochrome C oxidized/min/mg protein using the extinction coefficient 21 mM^{-1} cm^{-}

.2.8.5 Oxygen consumption assay

Oxygen consumption rate in control and treated cells were assayed using Cayman's cell based oxygen consumption rate assay kit using antimycin A as standard inhibitor

(Cayman Chemicals, Ann Arbor, USA). The kit utilizes a phosphorescent/fluorescent oxygen probe, MitoXpress to measure oxygen consumption rate in living cells (Vander Heiden et al., 2009).

MitoXpress probe is chemically stable and is excitable between 360-390 nm and emits at 630-680 nm. In order to measure oxygen consumption rate in living cells, cells were seeded in black clear bottom 96 well cell culture plates at a density of $5x10^3$ cells/well in 200 µl culture medium. Cells were treated with TFM and ischemia was induced as per the protocol. After treatment period, wells were loaded with fresh culture medium. Well containing culture medium alone served as blank wells. Then MitoXpress solution was added to all the wells except blank wells. Then, mineral oil was dispensed to overlay each well and the fluorescence was measured for 150 minutes. The phosphorescence of Mito Xpress, a phosphorescent oxygen probe is quenched by oxygen and the phosphorescent signal is inversely proportional to the amount of oxygen present. The oxygen consumption rate was calculated by the change in MitoXpress probe signal overtime.

2.8.6 Determination of ATP content in the cells

ATP content in the cells was determined by using a kit (Molecular Probes, Inc., Eugene, USA). The kit provides a bioluminescence assay for the quantitative determination of ATP with recombinant firefly luciferase and its substrate D-luciferin. The assay is based on luciferase's requirement for ATP in producing light (emission maximum ~560 nm) (Walenta et al., 1990). Briefly, after respective treatments, cells were lysed with buffer (pH 7.8) containing 100 mM potassium phosphate, 2 mM EDTA, 1 mM dithiothreitol (DTT) and 1 % triton X-100. Then, 10 µl of the sample was added to standard reaction mixture which contains deionized water, 20X reaction buffer, 0.1 M DTT, 10 mM D-luciferin and luciferase (5 mg/ml) and the luminescence was recorded using microplate reader (Tecan Infinite 200 PRO, Mannedorf, Switzerland).

2.9 Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using Pure link RNA Mini Kit (Invitrogen, Carlsbad, USA). 2 µg of total RNA was reverse transcribed using Superscript III First strand cDNA synthesis kits (Invitrogen, Carlsbad, USA) using random hexamer primers. The relative mRNA

expression levels of gene of interest compared to housekeeping gene β- actin was analyzed by quantitative real time PCR (qRT-PCR) in a CFX96 Real Time PCR system (Bio-rad, USA) using a C1000 Thermo cycler and using CFX Manager Software (Bio-rad, USA) . The conditions used for PCR was initial denaturation for 10 min at 95 °C, followed by 39 cycles of 15 s denaturation at 95 °C, 30 s annealing at the optimal primer temperature and 10s extension at 72 °C. Each sample was assayed in triplicate in a 20 µl reaction volume containing 1 µl cDNA, 10 µl SYBR green master mix (Power SYBR Green PCR Master Mix, Invitrogen, Carlsbad, USA), 5.81 µl DEPC water and 1.6 µl of each primer. No template controls were also run to ensure the absence of contamination. Analysis was performed according to the ΔΔCt method using β-actin as the housekeeping gene. Specific primers for each gene were designed to amplify a single product, as confirmed by dissociation curve analysis after the real-time PCR run.

$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

Where ΔCt = Ct (gene of interest) – Ct (β-actin)

$\Delta\Delta Ct = \Delta Ct$ treatment – ΔCt control, where Ct- threshold cycle.

Table 2.1Nucleotide sequence of qRT-PCR primers

mRNA	Amplicon	Forward primer	Reverse primer
OPA1	183	TGAAGGCAAGCTCTTCCCAATG	TGTTCATTAGCCCACAGGCATC
Mfn1	124	AACCCAGCAGCACCAGATAATG	TTTTCCAAATCACGCCCCCAAC
Mfn2	199	TTCCACAAAGTGAGTGAGCGTC	TGGCAGACACAAAGAAGATCC
Drp1	186	ACAGCACACAGGAACTGTTACG	ACGGCAAGGCTTAGATTTCTCGG
Fis1	143	AATTTCAGTCTGAGCAGGCAGC	TTTGCTACCTTTGGGCAACAGC
ERK2	123	TGACCTCAAGCCTTCCAAC	GCTACATACTCTGTCAAGAACC
JNK2	147	AGCACCCGTACATCAACGTCTG	TCGCTCCTCCAAATCCATGACC
p38α	187	GGCAGACCTGAACAACATCGTG	AGCCAGCCCAAAATCCAGAATC
TNNI3K	139	TCAGCGAGACAGCTTTTCACAG	TGGTAGCAAGCAGAGTGCAATC
HIF-1α	150	ATCATATCACTGGACTTCGGC	AGTTTCAGAGGCAGGTAATGG

2.10 Western blotting

Total protein was extracted from H9c2 cells after treatment and heart tissue (Details of animal experiments are given in section 2.11) by lysis in RIPA buffer containing protease inhibitor cocktail (Sigma Aldrich, St Louis, MO, USA). After incubation at 4 °C for 1 hour, with constant agitation to ensure complete lysis, the cell suspension was centrifuged at 12,000 rpm from 15 min at 4 °C and the supernatant was collected. Protein content in the supernatant was quantified using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) in accordance with the manufacturer's instructions. Equal amount of proteins (50 µg) was separated by 10 % Sodium dodecyl sulphate – polyacrylamide electrophoresis (SDS – PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes using turbo trans blot apparatus (BD Bioscience, USA). The membrane was blocked with 5 % BSA in TBST for 1 hour at room temperature. The membrane was washed 3 times in TBST for 10 minutes each.

The membrane was incubated at 4 °C overnight in 5 % BSA in TBST containing primary antibodies (Santa Cruz Biotechnology, USA) to one of the following: anti HIF1α, , anti p38α, anti p-p38α, anti JNK, anti p-JNK, anti ERK1/2, anti p-ERK1/2, anti Bax, anti Bad, anti Bcl-2, anti IL-6, anti TNF-α, anti NFκB, anti HO-1, anti Nrf2, anti NOS-2, anti VEGF, anti β-actin. After washing with TBST, the membrane was incubated with peroxidase conjugated secondary antibodies for 1 hour at room temperature. After washing the membrane was developed using Clarity ™ Western ECL Substrate (BIO-RAD, USA). The immunoblot images were analyzed in ChemiDoc XRS system (BIO-RAD, USA) using Image Lab software.

2.11 *In vivo* experiments

2.11.1 Induction of myocardial infarction in rats by isoproterenol

Male albino rats of wistar strain (10 weeks old, 200 ± 20 g), bred at animal facility of Sree Chitra Tirunal Institute Medical Science and Technology (SCTIMST), Trivandrum were selected for this study. Rats were housed in polypropylene cages (5 rats per cage) under an ambient temperature of 23 ± 20 °C; 50-60 % relative humidity; light 300 lux at floor level with regular 12 h light/ dark cycle. Animals were maintained on a standard pellet diet and

water *ad libitum*. According to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) formed by the Government of India in 1964, proper sanction had been obtained for animal experiments from Mahatma Gandhi University institutional animal ethics committee (Ethics Committee Approval Reference No. - School of Biosciences, M.G University, IAEC Clearance No. B21032014-21). Animals were sacrificed by cervical dislocation under light ether anesthesia as per ethics committee guidelines. All the animal experiments were carried out by me at School of Biosciences, Mahatma Gandhi University, Kottayam.

Rats were divided into six groups

Group 1: Normal control animals (NC) treated with saline alone

Group 2: Normal animals pre-treated with TTM/TFM 500 mg/kg body weight (treatment control) (T CON for toxicity evaluation of extract).

Group 3: Isoproterenol control animals (ISO) treated with isoproterenol (85 mg/kg).

Group 4: Animals pre-treated with TTM/TFM 250 mg/kg body weight (TTM/TFM 250).

Group 5: Animals pre-treated with TTM/TFM 500 mg/kg body weight (TTM/TFM 500).

Group 6: Animals pre-treated with propranolol 15 mg/kg body weight (PC).

Animals of experimental groups were orally administered with TTM/TFM (250 or 500 mg/kg body weight) or propranolol (15 mg/kg body weight) dissolved in saline for 21 days. Propranolol a widely used β - blocker was used as the positive control. After 21 days myocardial dysfunction was induced in rats by subcutaneous injection of isoproterenol (85 mg/kg in saline) for two consecutive days (22^{nd} and 23^{rd} day). Normal animals received subcutaneous injection of saline. After 24 hours of treatment rats were subjected to ECG analysis and then sacrificed. Blood and heart tissue were stored in -20 °C for future studies.

2.12 Cardiac biomarkers

2.12.1 Serum lactate dehydrogenase (LDH)

Activity of LDH in the serum of experimental rats was measured using a kit as per manufacturer's instruction (Agappe Diagnostics, Mumbai, India). Briefly, 10 µl of serum was mixed with working reagent containing Tris buffer (80 mM; pH 7.4), pyruvate (1.6 mM), NaCl (200 mM) and NADH (240 mM), mixed well and incubated at 37 °C for 1 minute and measured the change in absorbance per minute for 3 minutes duration at 340 nm.

2.12.2 Serum creatinine kinase (CK)

Serum creatinine kinase was estimated using a kit (Agappe Diagnostics, Mumbai, India) as per manufacturer's instructions. 40 µl serum was mixed with 1 ml of working reagent containing a mixture of reagent A and B in the ratio 4:1. Reagent A contains Imidazole, D - Glucose, N - acetyl L cysteine, magnesium sulphate, NADP, EDTA, hexokinase. Reagent B contains creatine phosphate, ADP, AMP, diadenosine pentaphosphate and glucose 6 phosphate dehydrogenase. Mixed and incubated at 37 °C for 100 seconds and the change in absorbance per minute was recorded at 340 nm for 3 minutes.

2.12.3 Serum creatinine kinase myocardial B fraction (CK-MB)

Serum CK-MB activity was estimated using a kit (Agappe Diagnostics, Mumbai, India) as per manufacturer's instructions. 40 µl of serum was mixed with 1 ml of working reagent. The constituents of working reagent include imidazole (125 mM), D-glucose (25 mM), N-acetyl-L-cysteine (25 mM), magnesium acetate (12.5 mM), NADP (2.52 mM), EDTA (2.02 mM), hexokinase (6800 U/L), antibody to CKM monomer, creatine phosphate (250 mM), ADP (15.2 mM), AMP (25 mM), diadenosine pentaphosphate (103 mM), glucose-6-phosphate dehydrogenase (8800 U/L). Contents were mixed well and incubated at 37 °C for 100 seconds and the change in absorbance per minute was recorded at 340 nm for 5 minutes.

2.12.4 Serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic-pyruvic transaminase (SGPT)

Activity of SGOT and SGPT were determined using the kits from Agappe Diagnostics, Mumbai, India. For SGOT, working reagent consisted of Tris buffer (pH 7.8, 88

mM), L-aspartate (260 mM), LDH (1500 U/L), MDH (900 U/L), α-ketoglutarate (12 mM) and NADH (0.24 mM). Working reagent for SGPT consisted of Tris buffer (pH 7.5, 110 mM), L-alanine (600 mM), LDH 1500 U/L, alpha-ketoglutarate (16 mM) and NADH (0.24 mM). 100 µl of serum was mixed with 1000 µl of working reagent and incubated for 1 minute at 37 °C. Change in absorbance per 60 seconds during 3 minutes at 340 nm was recorded

2.12.5 Serum calcium

Calcium ion concentration was measured using a kit from Agappe Diagnostics, Mumbai, India. The procedure is based on the reaction of calcium ions with O-cresolphthalein complex in an alkaline solution to form an intense violet coloured complex which shows maximum absorbance at 578nm. The serum sample or the standard (10 µl) was mixed with working reagent (1000 µl) and incubated for 5 min at room temperature. The absorbance was measured at 578 nm and the calcium concentration was determined as the ratio of the absorbance of the sample to the absorbance of the standard.

2.13 Estimation of inflammatory cytokines

Inflammatory cytokines (IL-6, IL-1β, TNF-α, IL-10 and MCP-1) were estimated using ELISA method (BD Biosciences, USA). For performing these assays, 100 µl captured antibody were added to the wells and incubated overnight at 4 °C. The supernatant was then aspirated and the wells were washed with wash buffer three times. 200 µl blocking buffer was added to each well and incubated in room temperature for 1 hour. After washing, 100 µl of media from respective treatment groups were added to each well and incubated at room temperature for 2 hours. After incubation washing steps were repeated. 100 µl working detector was added to all the wells and incubated for 1 hour at room temperature. After washing, 100 µl substrate solutions was added and incubated for 30 minutes in dark. The reaction was then stopped by addition of stop solution and the absorbance was read at 450 nm.

2.14 NF-κB translocation assay

NF-κB translocation was determined using a kit from Cayman Chemicals (ANN Arbor, USA). The method is based on an ELISA technique and a specific double stranded DNA sequence containing NF-κB response element immobilized on the bottom of micro

plate wells. After respective treatment, the nuclear and cytoplasmic fractions were added to these coated wells and NF-κB present in the sample specifically binds to NF-κB response element. NF-κB was detected by the addition of a specific primary antibody. Secondary antibody conjugated with HRP was added to provide a sensitive colorimetric reading at 405 nm.

2.15 Histopathological evaluation

Immediately after dissection, heart was taken out, cleaned and fixed in 10 % neutral buffered formalin solution for the preparation of histopathological slides. After fixation, tissues were dehydrated in graded ethanol series, cleared in xylene and embedded in paraffin wax. The solid sections were prepared at 5 µm thickness using a microtome, stained with Hematoxylin-eosin (H&E) / masson's trichrome. The sections were examined under light microscope and photomicrographs were taken.

2.16 Measurement of ECG and heart rate

Electrocardiography is a transthoracic interpretation of the electrical activity of the heart over a period of time, as detected by electrodes attached to the surface of the skin and recorded by a device external to the body. Briefly, rats were anesthetized with ketamine (65 mg/kg body weight) and xylazine (5 mg/kg body weight) at the end of experimental period (after 24 hours of second ISO injection) and leads were connected to the dermal layer. ECG recording was performed using Power lab data acquisition system and analysed with ECG Analysis Module (AD Instruments, Australia).

2.17 Statistical analysis

Statistical analysis were done using SPSS statistical program (SPSS/PC$^+$, Version 11.0, Chicago, IL, USA). The results were presented as the mean value ± standard deviation (SD) for the control and experimental groups. Data were subjected to one-way analysis of variance (ANOVA) and the differences among the means for the groups were assessed using Duncan's multiple range tests to determine which mean values were significantly different at $p \leq 0.05$.

References

Aebi H (1984) Catalase in vitro. *Methods Enzymology* 105: 121-126.

Choi EH, Chang HJ, Cho JY and Chun HS (2007) Cytoprotective effect of anthocyanins against doxorubicin-induced toxicity in H9c2 cardiomyocytes in relation to their antioxidant activities. *Food and Chemical Toxicology* 45: 1873-1881.

Dagda RK, Cherra SJ, 3rd, Kulich SM, Tandon A, Park D and Chu CT (2009) Loss of PINK1 function promotes mitophagy through effects on oxidative stress and mitochondrial fission. *The Journal of Biological Chemistry* 284: 13843-13855.

Decker T and Lohmann-Matthes ML (1988) A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *Journal of Immunological Methods* 115: 61-69.

Duh PD, Yen WJ, Du PC and Yen GC (1997) Antioxidant activity of mung bean hulls. *Journal of the American Oil Chemists Society* 74: 1059-1063.

Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL and Henson PM (1992) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *Journal of Immunology* 148: 2207-2216.

Flohe L and Gunzler WA (1984) Assays of glutathione peroxidase. *Methods in Enzymology* 105: 114-121.

Halliwell B, Gutteridge JM and Aruoma OI (1987) The deoxyribose method: a simple "test-tube" assay for determination of rate constants for reactions of hydroxyl radicals. *Analytical Biochemistry* 165: 215-219.

Hissin PJ and Hilf R (1976) A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Analytical Biochemistry* 74: 214-226.

Javadov S, Baetz D, Rajapurohitam V, Zeidan A, Kirshenbaum LA and Karmazyn M (2006a) Antihypertrophic effect of Na+/H+ exchanger isoform 1 inhibition is mediated by reduced mitogen-activated protein kinase activation secondary to improved mitochondrial integrity and decreased generation of mitochondrial-derived reactive oxygen species. *The Journal of Pharmacology and Experimental Therapeutics* 317: 1036-1043.

Javadov S, Purdham DM, Zeidan A and Karmazyn M (2006b) NHE-1 inhibition improves cardiac mitochondrial function through regulation of mitochondrial biogenesis during postinfarction remodeling. *American Journal of Physiology Heart and Circulatory Physiology* 291: 1722-1730.

Kakkar P, Das B and Viswanathan PN (1984) A modified spectrophotometric assay of superoxide dismutase. *Indian Journal of Biochemistry & Biophysics* 21: 130-132.

Liu F, Ooi VE and Chang ST (1997) Free radical scavenging activities of mushroom polysaccharide extracts. *Life Sciences* 60: 763-771.

Loos B, Genade S, Ellis B, Lochner A and Engelbrecht AM (2011) At the core of survival: autophagy delays the onset of both apoptotic and necrotic cell death in a model of ischemic cell injury. *Experimental Cell Research* 317: 1437-1453.

Maksimovic Z, Malencic D and Kovacevic N (2005) Polyphenol contents and antioxidant activity of *Maydis stigma* extracts. *Bioresource Technology* 96: 873-877.

Miller NJ, Rice-Evans C and Davies MJ (1993) A new method for measuring antioxidant activity. *Biochemical Society Transactions* 21: 95-100.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* 65: 55-63.

Paul MK, Kumar R and Mukhopadhyay AK (2008) Dithiothreitol abrogates the effect of arsenic trioxide on normal rat liver mitochondria and human hepatocellular carcinoma cells. *Toxicology and Applied Pharmacology* 226: 140-152.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M and Rice-Evans C (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine* 26: 1231-1237.

Scoccia AE, Molinuevo MS, McCarthy AD and Cortizo AM (2001) A simple method to assess the oxidative susceptibility of low density lipoproteins. *BMC Clinical Pathology* 1: 1-8.

Shimada K, Fujikawa K, Yahara K and Nakamura T (1992) Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *Journal of Agricultural and Food Chemistry* 40: 945-948.

Singleton VL, Rossi Jr. Joseph, A. (1965) Colorimetry of total phenolics with phosphomolybdic- phosphotungstic acid reagents. *American Journal of Enology and Viticulture* 16: 144-158.

Sudheesh NP, Ajith TA and Janardhanan KK (2009) Ganoderma lucidum (Fr.) P. Karst enhances activities of heart mitochondrial enzymes and respiratory chain complexes in the aged rat. *Biogerontology* 10: 627-636.

Vander Heiden MG, Cantley LC and Thompson CB (2009) Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 324: 1029-1033.

Walenta S, Dotsch J and Mueller-Klieser W (1990) ATP concentrations in multicellular tumor spheroids assessed by single photon imaging and quantitative bioluminescence. *European Journal of Cell Biology* 52: 389-393.

Xi M, Hai C, Tang H, Chen M, Fang K and Liang X (2008) Antioxidant and antiglycation properties of total saponins extracted from traditional Chinese medicine used to treat diabetes mellitus. *Phytotherapy Research* 22: 228-237.

Beneficial effect of *Tribulus terrestris* L. root methanol extract (TTM) against ischemia induced apoptosis in H9c2 cardiomyoblasts

3.1 Introduction

Cardiovascular disease is the leading cause of death worldwide and myocardial ischemia caused by coronary artery disease is a significant contributor (Ambrose and Singh, 2015). Prolonged myocardial ischemia i.e. reduction in the availability of oxygen and substrates can damage heart muscle reducing the ability to effectively pump blood from the heart, leading to heart failure (Kalogeris et al., 2012a). Myocardial ischemia causes disruption of ionic homeostasis, especially sodium and calcium, mitochondrial permeability transition, energetic and oxidative stress and induction of cell death by apoptosis, and necrosis (Kalogeris et al., 2012b). To understand the many aspects of cardiac ischemic injury several models are used. H9c2 cells are rat embryonic cardiomyoblasts and are similar to primary cardiomyocytes with regard to their energy metabolism features and electrophysiological properties. They are also sensitive to hypoxia. It is appropriate to use H9c2 cells for the study of ischemia and myocardial infarction and to evaluate the efficiency of various therapeutic interventions. So, an *in vitro* model of ischemia was used, by maintaining H9c2 cells in an ischemic buffer and limiting oxygen conditions.

Cell death during myocardial ischemia occurs by the mechanism of apoptosis and necrosis. During apoptosis there is cell shrinkage, blebbing, nuclear condensation and fragmentation. The apoptotic bodies thus formed are removed by phagocytes (Kerr et al., 1972). A cell can undergo apoptosis by two pathways, extrinsic death receptor pathway and intrinsic mitochondrial pathway. Extrinsic death pathway is caused by the binding of death domain (TNFα or Fas-L) to its death receptor in the plasma membrane, leading to activation of caspase-8 and subsequently caspase-7 and caspase-3 causing proteolytic cleavage of critical proteins in the cell (Gustafsson and Gottlieb, 2007). The intrinsic pathway is activated by intracellular stress signals such as hypoxia, oxidative stress and DNA damage. The anti-apoptotic proteins Bcl-2 and Bcl-xL maintain mitochondrial integrity while the pro-apoptotic proteins Bid, Bim, Bad, BNIP3, PUMA, NOXA and the effector proteins Bax and Bak cause apoptosis (Cheng et al., 2001). The mitochondria integrate the pro- and anti-apoptotic signals. Bax and Bak oligomerises to form pore in the mitochondrial outer membrane causing the

release of cytochrome c, second mitochondria derived activator of caspase (SMAC), endonuclease G and apoptosis inducing factor (AIF) form the intermembrane space to the cytosol. This leads to activation of caspase 9 and other downstream caspases 3 and 7 leading to apoptosis (Jiang and Wang, 2000).

A number of mechanisms have been proposed to explain the initiation and execution of necrosis, including death receptors, reactive oxygen species (ROS), calcium, and mitochondrial permeability transition pore (mPTP) opening. Opening of the mPTP leads to necrotic cell death. It causes dissipation of the proton gradient responsible for oxidative phosphorylation and ATP synthesis. It also leads to mitochondrial swelling and rupture due to sudden influx of water and solutes.

A characteristic feature of heart failure following myocardial ischemia and myocardial infarction is the progressive reduction in the number of cardiomyocytes and cardiac dysfunction. Apoptosis levels are lower in heart failure, but an apoptotic rate of 0.1% causes a reduction in cell number by 37% in one year (Mani, 2008). Studies have shown that, over expression of Bcl-2, inhibition of Bax and deletion of PUMA reduced infarct size (Hochhauser et al., 2007; Toth et al., 2006). So, apoptosis has a role to play in ischemic cell death in the myocardium. Necrosis is more prominent in failing heart and contributes more to disease pathogenesis than apoptosis (Guerra et al., 1999). Increased calcium stress along with persistent activation of adrenergic receptors triggers necrotic cell death (Chiong et al., 2011). Cyclosporin, a potent inhibitor of mPTP administered during reperfusion after percutaneous coronary intervention reduced infarct size and reduced creatinine kinase and tropoinin I release (Piot et al., 2008). In our study, cyclosporin was used as the positive control. From this it is clear that preventing cell death by a pharmacological agent will have protective effect on the heart against ischemia.

Current medications available for myocardial ischemia and myocardial infarction are cholesterol lowering agents, nitrates and aspirin that prevent blockage and improve blood flow, beta blockers and calcium channel blockers that reduce the heart rate (Riccioni, 2013) and angiotensin converting enzyme inhibitors or angiotensin receptor antagonists that relax blood vessels (Lincoff, 2014). These medications are used alone or in combination to reduce the symptoms of myocardial ischemia. But, these medications have adverse effects of long term use. So, there is a quest for safer therapeutic agents of natural sources. Alternative

medicines are used by 60% of the world population to cure diseases. In India, 65% of the rural population use Ayurveda to meet their healthcare needs (Gogtay et al., 2002). A detailed investigation of the mode of action of traditional medicine is necessary for wider acceptability. Hence in our study, attempts were made to elucidate the mechanism of action of *T. terrestris* roots against myocardial ischemia-induced alterations and cell death in an *in vitro* model using H9c2 cells. Moreover, an attempt was also made to decipher some of the constituents present in *T. terrestris* L. roots.

3.2 Experimental details

3.2.1 Preparation of *T. terrestris* root extracts and its fractions

The fresh whole roots were air-dried and extracted with methanol at ambient temperature (37 ± 1 °C) under continuous stirring for 6 hours, and the extraction process was repeated until the solvent became colourless. The supernatant was filtered through Whatman no. 1 filter paper and concentrated *in vacuo* under reduced pressure in a rotavapor (Heidolph, Germany) followed by lyophilisation. The lyophilized *T. terrestris* L. root methanol extract (TTM) was stored at 4 °C until use. The yield of the extract was found to be 14.26 g /1kg of plant material.

For fractionation, TTM was dissolved in distilled water and was partitioned with hexane and the organic layer was collected and evaporated. The remaining aqueous solution was evaporated under reduced pressure and again dissolved in water and further fractionated successively with dichloromethane, ethyl acetate and methanol. The resulting extracts were evaporated to dryness in vacuum to yield the fractions of dichloromethane (TTMD), ethyl acetate (TTME) and methanol (TTMM). The detailed procedures for the estimation of total phenolic content (TPC), total flavonoid content (TFC) and *in vitro* antioxidant activities were given in Chapter-2. *T. terrestris* L. root methanol extract (TTM) was used for further studies.

3.2.2 Cell culture treatment

H9c2 cells were pretreated with different concentrations of TTM for 24 hours prior to the induction of ischemia for 1 hour. Schematic representation of treatment parameters are given in Figure 3.1. Ischemia was induced by maintaining cells in an ischemic buffer pH 6.4, containing (in mM): 137 NaCl, 12 KCl, 0.5 $MgCl_2$, 0.9 $CaCl_2$, 20 HEPES, 20 2-deoxy-D-

glucose at 37 °C, 0.1% O_2 , 95% N_2 and 5% CO_2. TTM was dissolved in DMSO and the final concentration of DMSO used was less than 0.1% (v/v) for each treatment. The same concentration of DMSO was used in control cells as vehicle. Cyclosporin A at a concentration of 0.1µM was used as positive control (PC) and was administered 24 hours before the induction of ischemia. After simulated ischemia, control and treated cells were subjected to viability assays using MTT and LDH release assay. After the optimization of effective dose of TTM based on MTT and LDH assay, the experimental groups consist of (a) control cells, (b) cells treated with ischemic conditions , (c)TTM (10µg/ml) pre-treatment before ischemia, (d) TTM (25µg/ml) pre-treatment before ischemia, (e) TTM (50µg/ml) pre-treatment before ischemia, (f) positive control (0.1µM) pre-treatment before ischemia (Figure 3.1). After respective treatments, cells were analyzed for various parameters relevant to ischemia. Details of all the analytical procedures are given in chapter - 2.

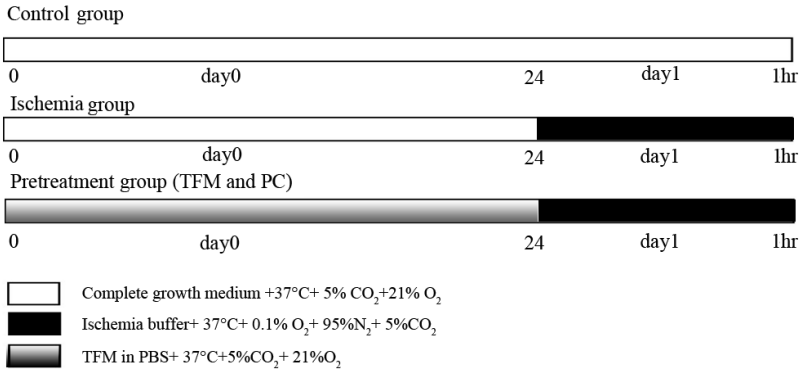

Figure 3.1: Schematic representation of treatment conditions. Control group, ischemia group and pre-treatment group (*T. terrestris* root methanol extract pre-treatment 10, 25, 50 µg/ml and Cyclosporin A [PC]) 24 hours prior to ischemia**.**

3.3Results

3.3.1 Total phenolic content (TPC), total flavonoid content (TFC) and yield of TTM

The yield, total phenolic content and flavonoid content of TTM and its fractions are shown in Table 3.1 and the results show that TTM is rich in phenolic compounds (45.43 ± 0.6 mg gallic acid equivalents g^{-1} dry weight) and flavonoid compounds (67.33 ± 3.3mg

catechin equivalents g^{-1} dry weight) when compared to its different fractions. The methanol extract of *T. terrestris* root (TTM) has a yield of 14.36 g from 1kg of the material.

Table3.1: Yield, total phenolic and flavonoid content in methanol water extract of *Tribulus terrestris* L. roots (TTM) and its fractions.

Extract	Yield	Total phenolic compounds	Total flavonoid compounds
TTM	14.36g	45.43 ± 0.6	67.33 ± 3.3
TTMD	17.83g	34.4 ± 1.3	4.99 ± 0.34
TTME	13.08g	38.36 ± 0.8	54.37 ± 3.7
TTMM	71.81g	34.3 ± 3.3	39.33 ± 1.3

Total phenolic compounds were determined as mg gallic acid equivalents g^{-1} dry weight

Total flavonoid compounds were determined as mg catechin equivalents g^{-1} dry weight

Yield, total phenolic and total flavonoid content of *T. terrestris* root methanol extract and its fractions. TTM-*Tribulus terrestris* methanol *extract*, TTMD- *Tribulus terrestris* methanol extract DCM fraction, TTME- *Tribulus terrestris* methanol extract ethyl acetate fraction, TTMM- *Tribulus terrestris* methanol extract methanol fraction. Results for total phenolic were expressed as gallic acid equivalents/g extract (mg GAE/g) and for flavonoids as quercetin equivalents/g extract (mg QE/g).

3.3.2 Free radical scavenging potential of TTM

Table 3.2 shows the free radical scavenging potential of TTM and its fractions in terms of its ability to scavenge DPPH, ABTS, hydroxyl and superoxide radicals and is compared with standard compounds like gallic acid, trolox, ascorbic acid and catechin. The free radical scavenging ability is expressed as IC_{50}. IC_{50} values stand for the effective concentration of antioxidant required to scavenge 50 % of radicals in the reaction mixture and the lower IC_{50} values indicate higher the activity. TTM exhibited significant scavenging potential against different free radicals (IC_{50} 74.86 ± 0.06 for DPPH, 118 ± 0.33 for ABTS, 358.34 ± 0.06 for superoxide and 186.58 ± 0.04 for hydroxyl radicals) and so TTM was used for further studies.

Table3.2Free radical scavenging potential of TTM and its fractions

	IC_{50} (µg/ml)			
Sample	**DPPH RSA***	**ABTS RSA***	**Superoxide RSA***	**Hydroxyl RSA***
TTM	74.86 ± 0.06	118 ± 0.33	358.34 ± 0.06	186.58 ± 0.04
TTMD	133.33 ± 0.08	Above 300	534.33 ± 0.33	550.17 ± 0.06
TTME	78.33 ± 0.33	173 ± 0.06	343.43 ± 0.13	355.83 ± 0.34
TTMM	163.33 ± 0.08	69.3 ± 0.08	436.85 ± 0.14	333.41 ± 0.38
Gallic acid	15.67 ± 0.31			
Trolox		5.3 ± 0.31		
Ascorbic acid			89.81 ± 0.51	
Catechin				64.67 ± 0.3

Estimated IC_{50} values of DPPH, ABTS superoxide and hydroxyl radical scavenging activities of TTM its fractions and standards. TTM-methanolic extract of *T.terrestis*, TTMD- *Tribulus terrestris L.* methanol extract DCM fraction, TTME- *Tribulus terrestris L.* methanol extract ethyl acetate fraction, TTMM- *Tribulus terrestris L.* methanol extract methanol fraction,* RSA- radical scavenging activity. Values expressed as mean ± SD (n=6).

3.3.3 Cell viability study using MTT assay

Relative cell viability after treatment with different concentrations of TTM was shown in Figure. 3.2A. Data showed that TTM, concentration up to 50 µg did not affect cell viability significantly ($p \leq 0.05$) (TTM at 50 µg/ml had 85 ± 9.3 % viable cells).

3.3.4 LDH release during ischemia and amelioration with TTM

LDH release to the medium is a significant marker of cardiac cell necrosis. Analysis showed that ischemia caused significant release of LDH to the medium (99.9 µU/ml) compared to the same in control (20.34 µU/ml), indicating necrotic type of cell death. Pretreatment with extract significantly ($p \leq 0.05$) reduced LDH release. TTM at 10 µg reduced LDH release to 80.9 µU/ml and high concentrations (25 µg) further reduced LDH release (76.5 µU/ml). This indicates that pretreatment with TTM was protective against ischemia-induced plasma membrane alterations (Figure 3.2B)

3.3.5 Morphological changes in H9c2 cells during ischemia and amelioration by TTM

Ischemia for 1 hour caused a change in morphology of the H9c2 cells. The cells showed shrinkage, change in nuclear morphology and blebbing characteristic of apoptotic cell death. TTM pretreatment at concentration of 25 and 50 μg/ml was able to prevent to some extent the changes in morphology produced by ischemia (Figure 3.2 C)

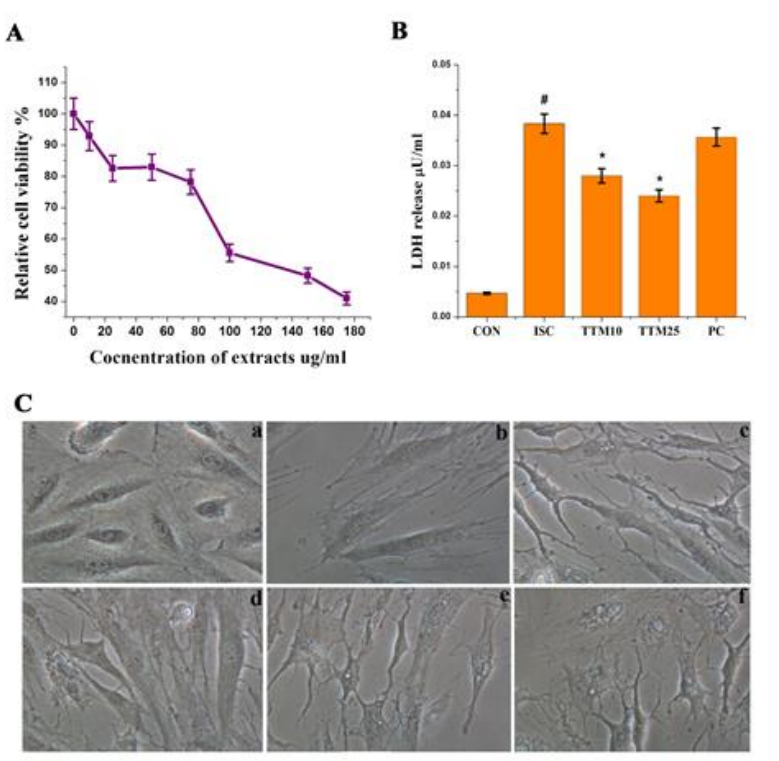

Figure 3.2 Analysis of toxicity, LDH release and morphology in H9c2 cells during ischemia. A. Analysis of toxicity of *T. terrestris* root methanol extract (0-180 μg/ml) in H9c2 cells by MTT assay, B. Lactate dehydrogenase release in H9c2 cells after simulated ischemia and amelioration with *T. terrestris* root methanol extract. C. Morphology of H9c2 cells after simulated ischemia and amelioration with *T. terrestris* root methanol extract pretreatment a. control, b. ischemia, c. *T. terrestris* root methanol extract 10 μg/ml + ischemia, d. *T. terrestris* root methanol extract 25 μg/ml + ischemia, e. *T. terrestris* root methanol extract 50 μg/ml + ischemia, f. positive control cyclosporin A (0.1 μM) + ischemia.

3.3.6 Characterization of TTM by HPLC

HPLC fingerprint of TTM clearly reveals the presence of chlorogenic acid (0.53 mg/g extract), caffeic acid (12.4mg/g extract) and 4-hydroxybenzoic acid (0.60 mg/g extract). (Figure 3.3)

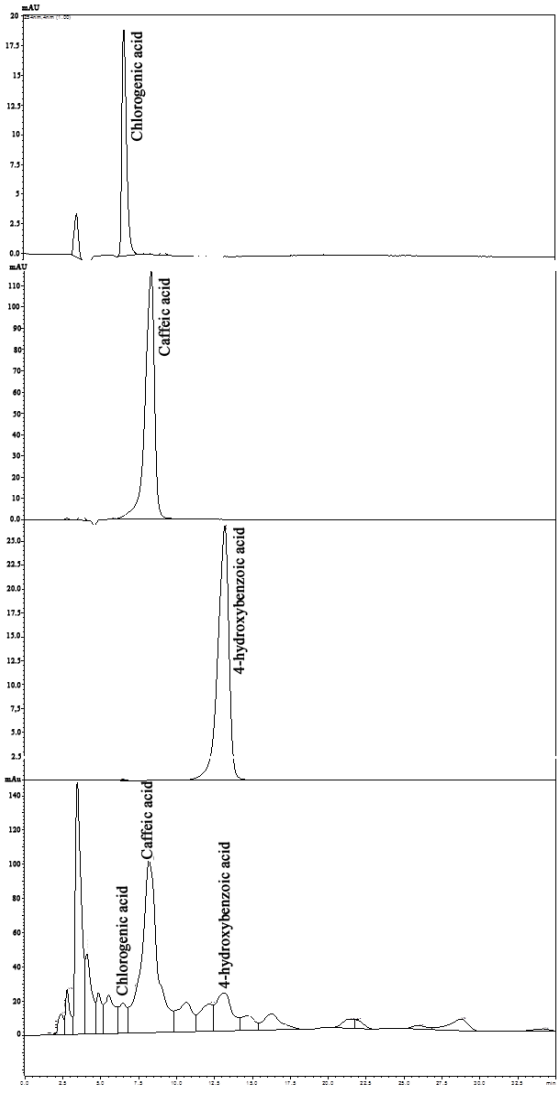

Figure 3.3 HPLC Chromatogram of *Tribulus terrestris* L. root methanol extract and standard compounds A. chlorogenic acid (retention time -6.561 min.), B. caffeic acid (retention time -7.853 min.) C. 4-hydroxy benzoic acid (retention time18.160 min.) D.HPLC fingerprint of *Tribulus terrestris* L. root methanol extract, Peaks were detected at 254nm

3.3.7 Cytoprotective effect of caffeic acid, chlorogenic acid and 4-hydroxy benzoic acid against ischemia in H9c2 cells

Cytoprotective effect of compounds identified from TTM, against ischemia in H9c2 cells was evaluated by MTT assay. All the phenolic acids significantly ($p \leq 0.05$) protected the cells from ischemia-induced cell death. Of which, chlorogenic acid was the most effective (99 ± 9.3% viable cells when pretreated with 1 µg/ml chlorogenic acid).

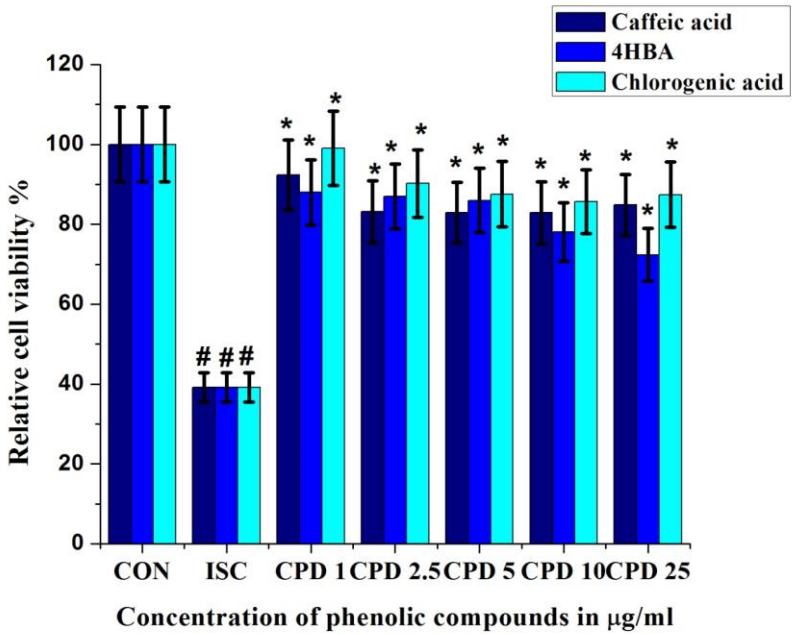

Figure 3.4 Protective property of phenolic compounds against ischemia. Relative cell viability of control cells, after ischemia and after treatment with phenolic compounds: caffeic acid, 4 hydroxybenzoic acid and chlorogenic acid at concentrations 1, 2.5, 5, 10 and 25 µg/ml 24 hours before induction of ischemia for 1 hour, in H9c2 cells. Values are expressed as mean ± SD. (n = 6). # indicates significant difference from control, * indicates significant difference from ischemia. Significance was accepted at $p \leq 0.05$.

3.3.8 Effect of TTM on intracellular ROS generation

Imaging and fluorometry experiments revealed significant generation of oxidative stress in ischemia cells compared to control (Figure. 3.5 A, B). Fluorometry analysis showed 56 % increase of fluorescence in ischemic cells compared to control. TTM at 10, 25 and 50 µg reduced ROS by 11 %, 10 % and 7 %, respectively. Flow cytometry data further confirmed this result (Figure. 3.5C).

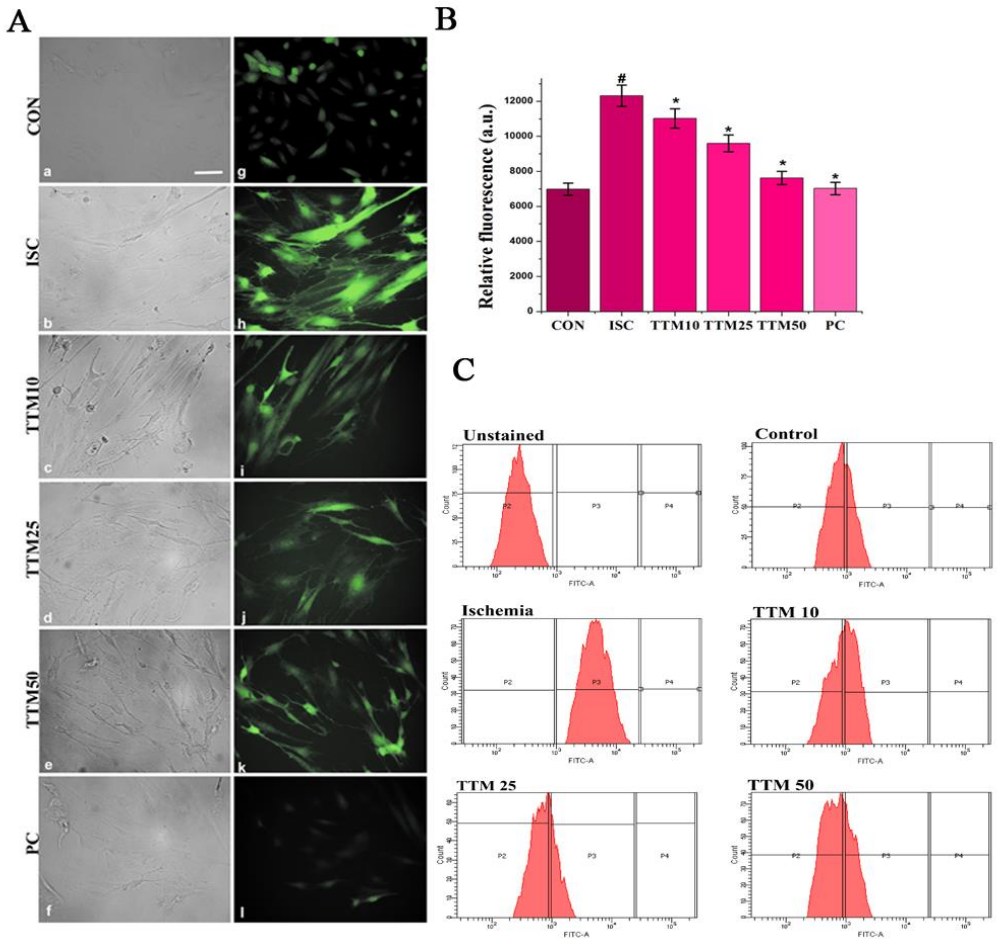

Figure 3.5 Reactive oxygen species generation during ischemia. A. Reactive oxygen species generation during ischemia and its scavenging by *T. terrestris* root methanol extract (10, 25, 50 µg/ml) and positive control (PC), Cyclosporin A (0.1 µM). B. Representative histograms of relative fluorescence. C. Reactive oxygen species (ROS) accumulation during ischemia measured by FACS: Unstained control, control, ischemia, cells pretreated with *T. terrestris* root methanol extract (10, 25, and 50 µg/ml) prior to ischemia. Values are expressed as mean ± SD. (n=6). # indicates significant difference from the normal, * indicates significant difference from ischemia. Significance was accepted at $p \leq 0.05$.

3.3.9 Effect of TTM on intracellular superoxide generation

Superoxide anion generation was assessed employing dihydroethidium (DHE) (Sankar et al., 2013) (Figure 3.6 A, B). There was an increase in superoxide generation (18 %) in ischemia group compared to control group. Extract pretreatment reduced superoxide generation compared to ischemia group, but there was no clear cut concentration dependent effect.

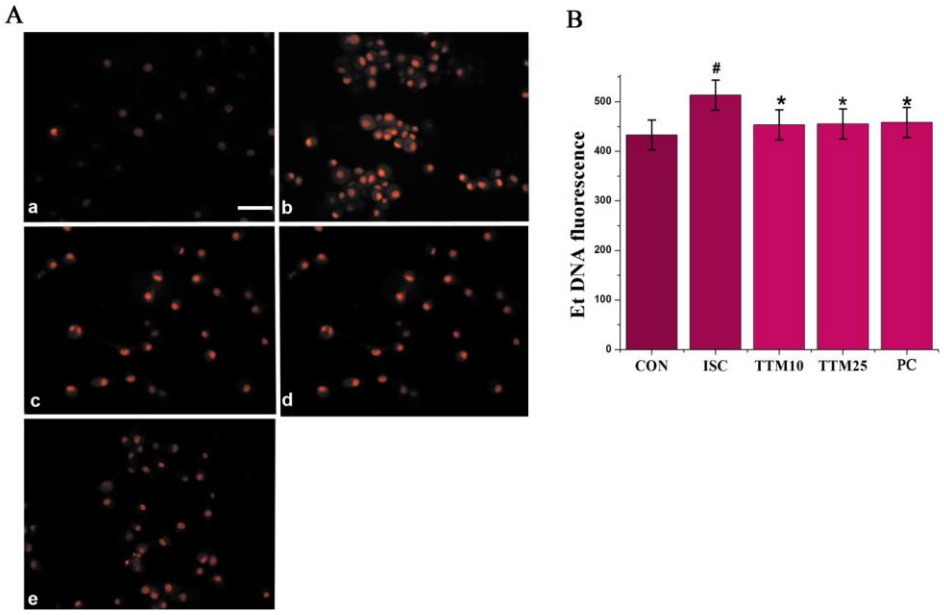

Fig 3.6 Superoxide generation during ischemia A-Representative fluorescent images of superoxide generation during ischemia and amelioration by *T. terrestris* root methanol extract (TTM). a - control cells, b - ischemia, c - cells pre-treated with *T. terrestris* root methanol extract 10 µg/ml prior to ischemia, d - cells pretreated with *T. terrestris* root methanol extract 25 µg/ml prior to ischemia, e - cells pre-treated with positive control Cyclosporin A (0.1 µM) prior to ischemia B. Representative histogram of ethidium fluorescence of control, ischemia, cells pretreated with *T. terrestris* root methanol extract (10, 25 µg/ml) and positive control Cyclosporin A (0.1 µM) prior to ischemia. Values are expressed as mean ± SD. (n = 6). # indicates significant difference from control, * indicates significant difference from ischemia. Significance was accepted at $p \leq 0.05$.

3.3.10 Analysis of cell death during ischemia by Annexin V-FITC/PI double staining

In order to examine the cell death during simulated ischemia in H9c2 cells, Annexin V-FITC/ propidium iodide staining and fluorescent microscopy was performed. Annexin V-FITC stained apoptotic cells and propidium iodide is a cell impermeable dye that can enter only dead cells. There was a significant increase (54 %) in apoptotic cells during ischemia and the number of apoptotic cells was significantly reduced (28 %) on treatment with TTM 10 µg/ml. Necrotic or dead cell stained by propidium iodide was also increased during ischemia and was reduced on treatment with TTM. (Figure 3.7)

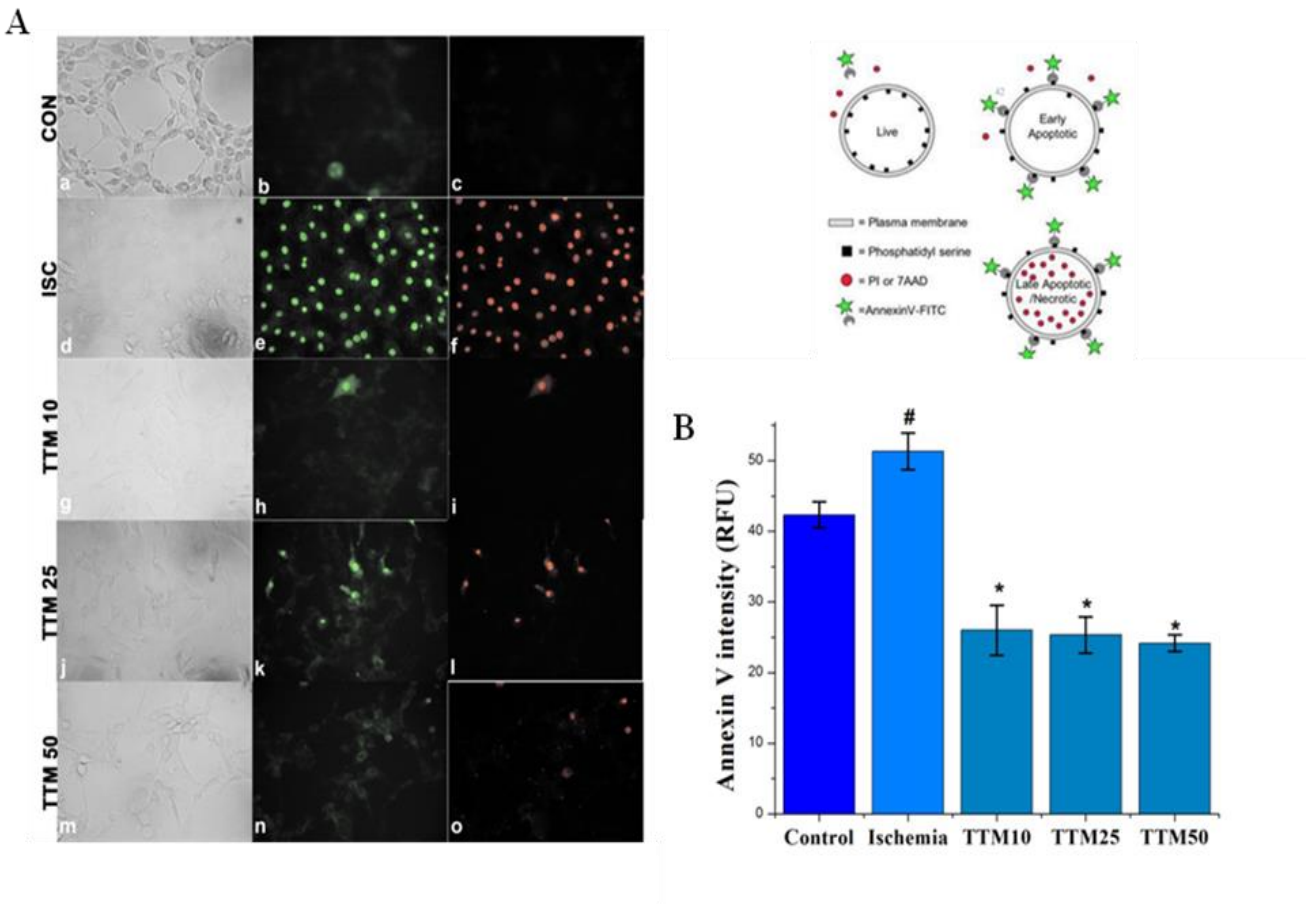

Figure 3.7 Cell death during ischemia A. Representative fluorescent images of apoptotic (FITC stained) and necrotic (propidium iodide stained) cells during ischemia and reduction in cell death with *T. terrestris* root methanol extract a-c control cells, d-f ischemia, g-i cells pre-treated with *T. terrestris* root methanol extract 10 μg/ml prior to ischemia, j-l cells pretreated with *T. terrestris* root methanol extract 25 μg/ml prior to ischemia, m-o cells pretreated with positive control Cyclosporin A (0.1μM) prior to ischemia, B. Representative histogram of Annexin V FITC fluorescence of control, ischemia, cells pretreated with *T. terrestris* root methanol extract (10, 25, 50 μg/ml) prior to ischemia. Values are expressed as mean ± SD. (n = 6). # indicates significant difference from control, * indicates significant difference from ischemia. Significance was accepted at $p \leq 0.05$.

3.3.11 Activity of caspase-3

Ischemia caused a significant increase in the caspase-3 activity (800 %) compared to control, whereas pretreatment of the cells with TTM prevented the increased activity of capase-3 induced by ischemia in a dose dependent manner (TTM 10 μg/ml - 55.55 %, TTM 25 μg/ml - 77.77 %, TTM50 μg/ml - 88.88 %) (Figure 3.8)

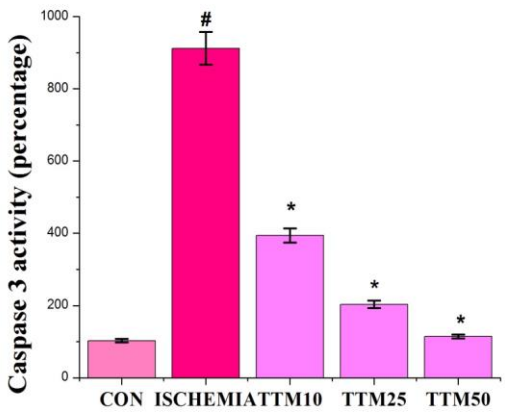

Figure 3.8 Caspase 3 activity measured during ischemia. Representative histogram of the percentage of caspase -3 activity in control, ischemia, and cells pretreated with *T. terrestris* root methanol extract (10, 25 and 50 µg/ml) prior to ischemia. Values are expressed as mean ± SD. (n = 6). # indicates significant difference from control, * indicates significant difference from ischemia. Significance was accepted at $p \leq 0.05$.

3.3.12 Expression of pro-apoptotic markers Bax and Bad during ischemia

Ischemia caused a significant increase in pro-apoptotic marker proteins Bax by 30 %, but Bad were increased by only 5 %. While, TTM pre-treatment significantly prevented the increase in expression of Bax by 23 % and 20 % for TTM 10 and 25 µg/ml respectively (Figure 3.9A, B). Pro-apoptotic protein Bad was increased by only 5 % in ischemia group compared to control. While, TTM pretreatment prevented the upregulation of Bad by 4.5 % and 9 % for TTM 10 and 25 µg/ml respectively (Figure 3.9 C, D).

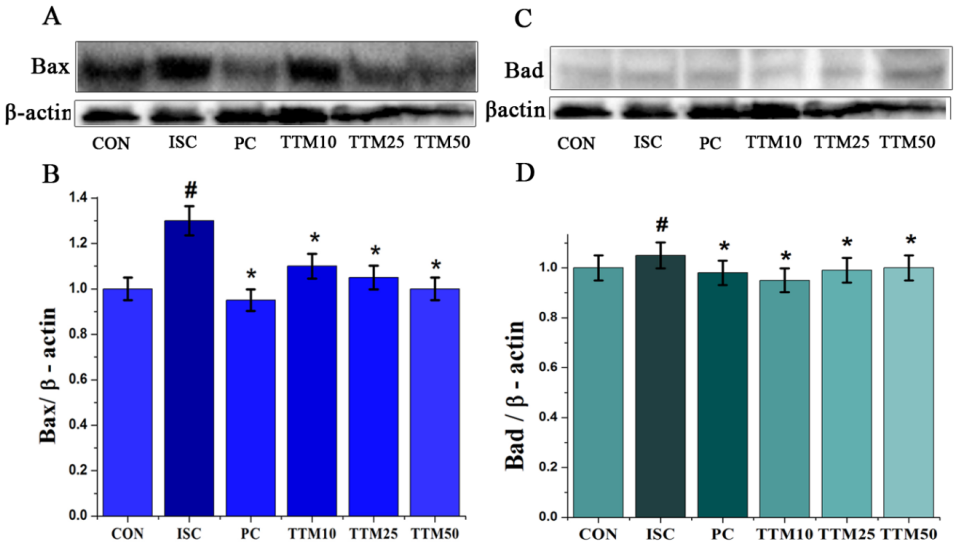

Figure 3.9 Bax and Bad expression during ischemia A. Western blot showing increased expression of Bax during ischemia and amelioration with *T. terrestris* root methanol extract. B. Densitometric quantification ratio of Bax to β - actin. C. Western blot image showing increased expression of pro-apoptotic protein Bad during ischemia and amelioration with *T. terrestris* root methanol extract., D. Densitometric quantification of protein levels of Bad relative to β - actin. Values expressed as mean ± SD (n = 6). # indicates values are significantly different from control cells. * indicates values are significantly different from ischemia and the significance accepted at $p \leq 0{\cdot}05$.

3.3.13 Expression of anti-apoptotic markers Bcl-2 during ischemia

Ischemia caused a significant decrease in anti-apoptotic marker Bcl-2 by 30 %. While, TTM pretreatment significantly prevented the down regulation of Bcl-2 caused by ischemia by 42 %, 28 % and 22 % for TTM 10, 25 and 50 µg/ml respectively (Figure 3.10 A, B)

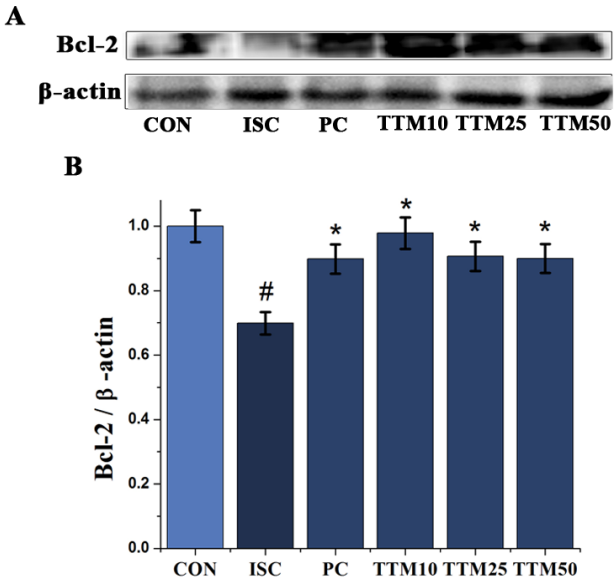

Figure 3.10 Bcl-2 expression during ischemia A. Western blot image showing decreased expression of Bcl-2 during ischemia and amelioration with *T. terrestris* root methanol extract. B. Densitometric quantification of protein levels of Bcl-2 relative to β-actin. Values expressed as mean ± SD (n=6). # indicates values are significantly different from control cells. * indicates values are significantly different from ischemia and the significance accepted at $p \leq 0{\cdot}05$.

3.3.14 Effect of TTM on ischemia-induced activation of p38α MAPK

In order to identify the signaling pathway by which TTM exhibits its anti-apoptotic potential, the mRNA as well as protein level expression of total p38 α and phosphorylated p38 α was determined in control and treated cells. Blotting showed ischemia activated p38 α expression via phosphorylation but not at the level of mRNA. There was an increase in phosphorylation by 100% during ischemia and TTM treatment was able to significantly downregulate the increased phosphorylation of p38α (TTM 10 µg/ml – 35 %, TTM 25 µg/ml – 34 %, TTM 50 µg/ml – 30 %) which was not dose dependent.

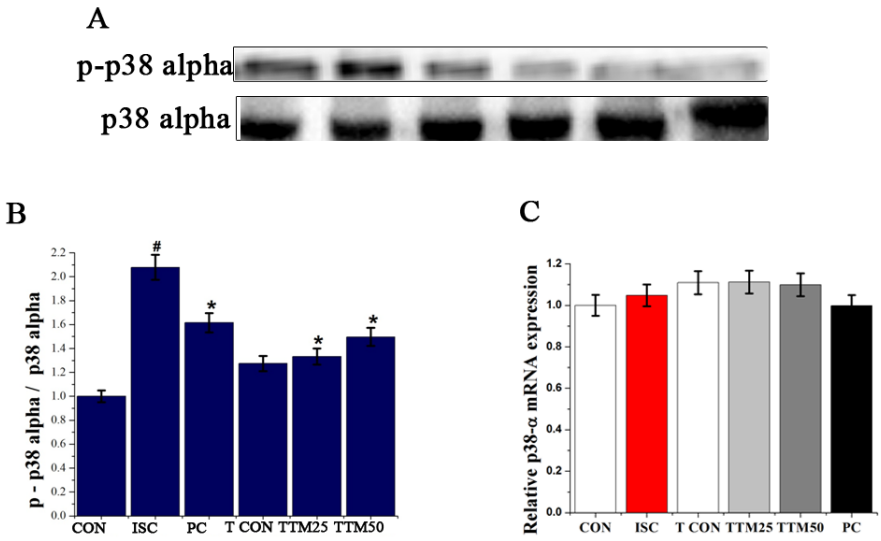

Figure 3.11 p38 activation during ischemia A. Western blot image showing phosphorylation and activation of p38 α during ischemia and amelioration with *T. terrestris* root methanol extract. B. Densitometric quantification of protein levels of phosphorylated p38 α relative to total p38 α. C. mRNA expression of p38 α. Values expressed as mean ± SD (n=6). # indicates values are significantly different from control cells. * indicates values are significantly different from ischemia and the significance accepted at $p \leq 0{\cdot}05$.

3.3.15 Effect of TTM on ischemia-induced activation of JNK1/2 MAPK

Ischemia also caused phosphorylation and activation of JNK1/2 MAPK compared to control. Expression studies show that JNK was also activated by phosphorylation during ischemia (90% increase) and not at the mRNA level. TTM also significantly downregulated the increase in phosphorylation caused by ischemia (TTM 10 µg/ml - 36.8 %, TTM 25 µg/ml - 31.57 %, TTM 50 µg/ml - 26.31 %) which was not dose dependent. (Figure 3.12)

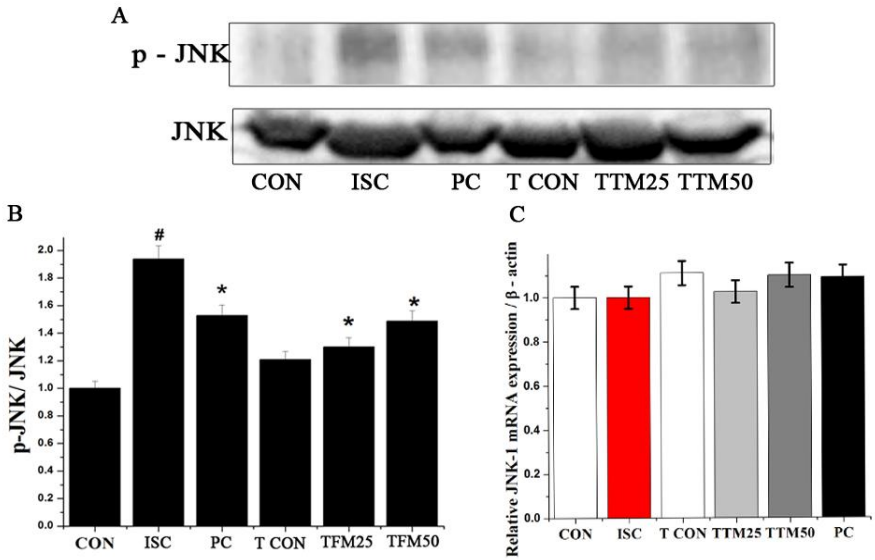

Figure 3.12 JNK activation during ischemia A. Western blot image of phosphorylation and activation of JNK during ischemia and amelioration with *T. terrestris* root methanol extract. B. Densitometric quantification of protein levels of phosphorylated JNK relative to total JNK. C. mRNA expression of JNK-1. Values expressed as mean ± SD (n = 6). # indicates values are significantly different from control cells. * indicates values are significantly different from ischemia and the significance accepted at $p \leq 0{\cdot}05$.

3.3.16 Effect of TTM on ischemia-induced activation of Akt

Ischemia caused deactivation of Akt (there was a decrease in phosphorylation by 25 %) compared to control and no change at the mRNA level. TTM pretreatment also significantly prevented the decrease in phosphorylation of Akt caused by ischemia. (TTM 10 µg/ml - 6.6 %, TTM 25 µg/ml - 12.3 %, TTM 50 µg/ml - 26.6 %) (Figure 3.13)

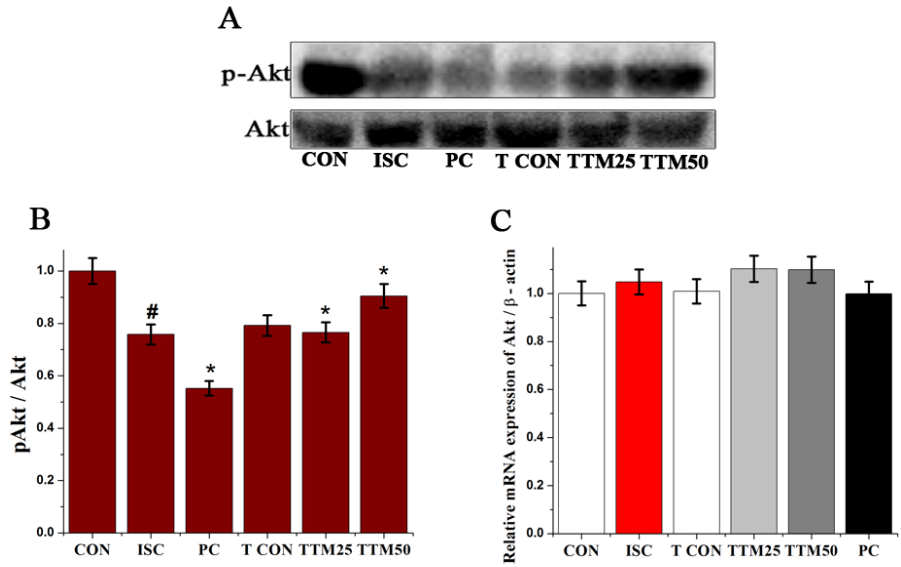

Figure 3.13 Akt phosphorylation during ischemia A.Western blot image of phosphorylation of Akt during ischemia and amelioration with *T. terrestris* root methanol extract. B. Densitometric quantification of protein levels of phosphorylated Akt relative to total Akt C. mRNA expression of Akt. Values expressed as mean ± SD (n = 6). # indicates values are significantly different from control cells. * indicates values are significantly different from ischemia and the significance accepted at $p \leq 0{\cdot}05$.

3.4 Discussion

It has been well accepted that increased ROS, subsequent oxidative damage and eventually cell death by necrosis and apoptosis are some of the major causes of ischemic injury in heart (Assaly et al., 2012). There is evidence that increased intake of antioxidants as food and medicine may protect against cardiovascular diseases (Morton et al., 2000). *Tribulus terrestris* L. root is a well-known traditional medicine reported in Ayurveda, widely used for heart related problems (Ukani et al., 1997). But, the molecular mechanism underlying the therapeutic ability against heart ailments is not known in detail, except a few reports utilizing the saponins of *Tribulus terrestris* L. (Zhang et al., 2010; 2011). The scientific validation of traditional medicine for its mechanism of action is very much essential, for universal acceptance and wider use by public. A detailed investigation has been conducted on the efficacy of *Tribulus terrestris* L. roots against cardiac ischemia, emphasizing the role of this plant in reducing oxidative stress and cell death.

The phenolic, flavonoid content and radical scavenging activity with respect to various radicals have been performed. It is a well-established fact that a positive correlation exist between the phenolic content and antioxidant activity of the material (Croft, 1998).

There is an increasing interest in the efficacy of antioxidants to prevent deleterious effect of oxidants in the human body. DPPH method assessed the primary radical scavenging activity of the antioxidant while ABTS assay measures ability of an antioxidant to inhibit radical formation. In the body, superoxide and hydroxyl radicals are generated as a byproduct of cellular respiration (Kirkinezos and Moraes, 2001). TTM exhibited more phenolic content and better radical scavenging activities than the other fractions (Table 3.1 and 3.2).

The efficacy of the polyphenol rich TTM to protect against ischemia was checked in an *in vitro* model of cardiac ischemia using H9c2 cells. MTT assay showed that TTM was most effective in protecting the cell from ischemia. Thus, TTM was used for further studies. LDH release to the medium, during ischemia, revealed alterations in the integrity of the plasma membrane. In addition, DCFDA incorporation in ischemia-induced H9c2 cells, showed significant increase in intracellular ROS generation. This is in line with previous reports (Madamanchi and Runge, 2013). Pretreatment with TTM significantly suppressed LDH release and ROS generation in the cells subjected to ischemia. Likewise, TTM attenuated superoxide production in ischemic cells (Figure 3.6 A, B).

Prolonged ischemia, reperfusion and myocardial infarction cause cell death. Cell death is mostly by apoptosis, necrosis, necro-apoptosis and autophagy. The heart has a limited capacity to regenerate and in order to maintain normal contraction and myocardial function the cardiac cell death should be controlled and cardiac cell number should be maintained. Manipulation of the mechanism by which cell death occurs can protect the heart. Cardiac-specific overexpression of antiapoptotic Bcl-2 reduced infarct size, cardiac apoptosis, and cardiac dysfunction caused by ischemia / reperfusion injury (Chen et al., 2001). Deletion of pro-apoptotic Bax reduced infarct size and lessens dysfunction occurring during ischemia/ reperfusion injury (Hochhauser et al., 2003). Rats treated with upstream caspase inhibitors reduced infarct size by 31-53 % (Yaoita et al., 1998).

Multiple death and survival pathways are activated during ischemia and subsequent reperfusion (Wei et al., 2011). Mitogen activated protein kinase (MAPK) pathways plays both pro and anti-apoptotic role in cell death. Adenoviral transduction of constitutively active Akt reduced myocyte apoptosis, infarct size and cardiac dysfunction caused by I/R injury (Matsui et al., 2001). The possible mechanism is by phosphorylation and activation of pro-apoptotic Bax, Bad and Foxo (Fujio et al., 2000). JNK-1 can activate both survival and death

signals that may complement or antagonize Akt and other survival kinases. It depends on the duration of ischemia (Yu et al., 2004). Brief periods of ischemia phosphorylate Akt, reduced the infarct size and activated the survival pathway (Tournier et al., 2000). Prolonged ischemia followed by reperfusion phosphorylated IRS-1 inhibits Akt activation and causes cell death by regulating mPTP (Sunayama et al., 2005). Adenovirus induced transduction of p38α reduced infarct size, reduced apoptosis and induced angiogenesis (Tenhunen et al., 2006).

T. terrestris L. root methanol extract mediated protection against ischemia. Ischemia for 1 hour in H9c2 cardiomyoblasts phosphorylated and activated the MAPKs: p38α, JNK and Akt. In the previous studies, p38α inhibition upregulated Bcl-2 whereas activation of p38α downregulated Bcl2 protein levels (Kaiser et al., 2004). In our study, TTM pretreatment reduced the phosphorylation of p38α thereby causing upregulation of Bcl-2 and triggering the anti-apoptotic pathway and thus prevents cell death by apoptosis. JNK1/2 phosphorylation was increased during ischemia, in our study. But, there was a reduction of Akt phosphorylation following ischemia. This phenomenon may be due to IRS phosphorylation and inhibition of Akt phosphorylation thereby inducing cell death by necrosis. However, TTM pretreatment decreased JNK phosphorylation and subsequently activated Akt and thus prevented cell death.

Previous studies have shown that *T. terrestris* contains a number of pharmacologically active compounds. In this study, identification and quantification of phenolic compounds like caffeic acid, chlorogenic acid and 4-hydroxybenzoic acid in *T. terrestris* roots has been done by HPLC (Figure.3.3). There are reports that, chlorogenic acid attenuated chronic ventricular remodeling after myocardial infarction and protects against ischemia reperfusion injury in the rat liver, through its antioxidant properties (Yun et al., 2012). Caffeic acid ameliorates brain injury in focal cerebral ischemia (Zhou et al., 2006). 4-hydroxybenzoic acid is a scavenger of hydroxyl radical and protects against cerebral ischemia and reperfusion (Liu et al., 2002). The presence of these phenolic acids in *Tribulus terrestris* L. extract (TTM) is expected to contribute to the protective property as they were individually beneficial in preventing cell death during ischemia.

In the present study, the *Tribulus terrestris* L. root extract was treated before the induction of ischemia, thereby slowing down the rate of development of ischemia by protecting the mitochondria from injury. Thus the plant can be used to develop a lead

molecule that can be used as a preventive against ischemia-induced cardiovascular ailments. On the basis of these observations, it is indisputable that polyphenol mediated antioxidant and anti-apoptotic potential of this plant plays a significant role in its medicinal properties against heart problems. Importantly, this is the first report to elucidate partially, the biochemical basis of the therapeutic use of TTM.

The overall study reveals the significant antioxidant potential of polyphenol rich *Tribulus terrestris* L. extract. Pre-treatment with the extract significantly prevented apoptosis caused by ischemia in H9c2 cells via its antioxidant capacity.

References

Ambrose JA and Singh M (2015) Pathophysiology of coronary artery disease leading to acute coronary syndromes. *F1000prime Reports* 7: 8-14.

Assaly R, de Tassigny A, Paradis S, Jacquin S, Berdeaux A and Morin D (2012) Oxidative stress, mitochondrial permeability transition pore opening and cell death during hypoxia-reoxygenation in adult cardiomyocytes. *European Journal of Pharmacology* 675: 6-14.

Chen Z, Chua CC, Ho YS, Hamdy RC and Chua BH (2001) Overexpression of Bcl-2 attenuates apoptosis and protects against myocardial I/R injury in transgenic mice. *American Journal of Physiology Heart and Circulatory Physiology* 280: 2313-2320.

Cheng EH, Wei MC, Weiler S, Flavell RA, Mak TW, Lindsten T and Korsmeyer SJ (2001) BCl-2, BCl-(xL) sequester BH3 domain-only molecules preventing Bax and Bak mediated mitochondrial apoptosis. *Molecular Cell* 8: 705-711.

Chiong M, Wang ZV, Pedrozo Z, Cao DJ, Troncoso R, Ibacache M, Criollo A, Nemchenko A, Hill JA and Lavandero S (2011) Cardiomyocyte death: mechanisms and translational implications. *Cell Death & Disease* 2: 244-250.

Croft KD (1998) The chemistry and biological effects of flavonoids and phenolic acids. *Annals of the New York Academy of Sciences* 854: 435-442.

Fujio Y, Nguyen T, Wencker D, Kitsis RN and Walsh K (2000) Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart. *Circulation* 101: 660-667.

Gogtay NJ, Bhatt HA, Dalvi SS and Kshirsagar NA (2002) The use and safety of non-allopathic Indian medicines. *Drug Safety* 25: 1005-1019.

Guerra S, Leri A, Wang X, Finato N, Di Loreto C, Beltrami CA, Kajstura J and Anversa P (1999) Myocyte death in the failing human heart is gender dependent. *Circulation Research* 85: 856-866.

Gustafsson AB and Gottlieb RA (2007) Bcl-2 family members and apoptosis, taken to heart. *American Journal of Physiology Cell physiology* 292: 45-51.

Hochhauser E, Cheporko Y, Yasovich N, Pinchas L, Offen D, Barhum Y, Pannet H, Tobar A, Vidne BA and Birk E (2007) Bax deficiency reduces infarct size and improves long-

term function after myocardial infarction. *Cell Biochemistry and Biophysics* 47: 11-20.

Hochhauser E, Kivity S, Offen D, Maulik N, Otani H, Barhum Y, Pannet H, Shneyvays V, Shainberg A, Goldshtaub V, Tobar A and Vidne BA (2003) Bax ablation protects against myocardial ischemia-reperfusion injury in transgenic mice. *American Journal of Physiology Heart and Circulatory Physiology* 284: 2351-2359.

Jiang X and Wang X (2000) Cytochrome c promotes caspase-9 activation by inducing nucleotide binding to Apaf-1. *The Journal of Biological Chemistry* 275: 31199-31203.

Kaiser RA, Bueno OF, Lips DJ, Doevendans PA, Jones F, Kimball TF and Molkentin JD (2004) Targeted inhibition of p38 mitogen-activated protein kinase antagonizes cardiac injury and cell death following ischemia-reperfusion in vivo. *The Journal of Biological Chemistry* 279: 15524-15530.

Kalogeris T, Baines CP, Krenz M and Korthuis RJ (2012) Cell biology of ischemia/ reperfusion injury. *International Review of Cell and Molecular Biology* 298: 229-317.

Kerr JF, Wyllie AH and Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer* 26: 239-257.

Kirkinezos IG and Moraes CT (2001) Reactive oxygen species and mitochondrial diseases. *Seminars in Cell & Developmental Biology* 12: 449-457.

Lim SY, Davidson SM, Mocanu MM, Yellon DM and Smith CC (2007) The cardioprotective effect of necrostatin requires the cyclophilin-D component of the mitochondrial permeability transition pore. *Cardiovascular Drugs and Therapy* 21: 467-469.

Lincoff AM (2014) Managing acute coronary syndromes: decades of progress. *Cleveland Clinic Journal of Medicine* 81: 233-242.

Liu M, Liu S, Peterson SL, Miyake M and Liu KJ (2002) On the application of 4-hydroxybenzoic acid as a trapping agent to study hydroxyl radical generation during cerebral ischemia and reperfusion. *Molecular and Cellular Biochemistry* 234-235: 379-385.

Madamanchi NR and Runge MS (2013) Redox signaling in cardiovascular health and disease. *Free Radical Biology & Medicine* 61: 473-501.

Mani K (2008) Programmed cell death in cardiac myocytes: strategies to maximize post-ischemic salvage. *Heart Failure Reviews* 13: 193-209.

Matsui T, Tao J, del Monte F, Lee KH, Li L, Picard M, Force TL, Franke TF, Hajjar RJ and Rosenzweig A (2001) Akt activation preserves cardiac function and prevents injury after transient cardiac ischemia *in vivo*. *Circulation* 104: 330-335.

Morton LW, Abu-Amsha Caccetta R, Puddey IB and Croft KD (2000) Chemistry and biological effects of dietary phenolic compounds: relevance to cardiovascular disease. *Clinical and Experimental Pharmacology & Physiology* 27: 152-159.

Piot C, Croisille P, Staat P, Thibault H, Rioufol G, Mewton N, Elbelghiti R, Cung TT, Bonnefoy E, Angoulvant D, Macia C, Raczka F, Sportouch C, Gahide G, Finet G, Andre-Fouet X, Revel D, Kirkorian G, Monassier JP, Derumeaux G and Ovize M (2008) Effect of cyclosporine on reperfusion injury in acute myocardial infarction. *The New England Journal of Medicine* 359: 473-481.

Riccioni G (2013) Ivabradine: the hope for a good treatment of ischemic heart disease. *Current Medicinal Chemistry* 20: 1817-1823.

Sankar V, Pangayarselvi B, Prathapan A and Raghu KG (2013) Desmodium gangeticum (Linn.) DC. exhibits antihypertrophic effect in isoproterenol-induced cardiomyoblasts via amelioration of oxidative stress and mitochondrial alterations. *Journal of Cardiovascular Pharmacology* 61: 23-34.

Sunayama J, Tsuruta F, Masuyama N and Gotoh Y (2005) JNK antagonizes Akt-mediated survival signals by phosphorylating 14-3-3. *The Journal of Cell Biology* 170: 295-304.

Tenhunen O, Soini Y, Ilves M, Rysa J, Tuukkanen J, Serpi R, Pennanen H, Ruskoaho H and Leskinen H (2006) p38 Kinase rescues failing myocardium after myocardial infarction: evidence for angiogenic and anti-apoptotic mechanisms. *FASEB Journal* 20: 1907-1909.

Toth A, Jeffers JR, Nickson P, Min JY, Morgan JP, Zambetti GP and Erhardt P (2006) Targeted deletion of Puma attenuates cardiomyocyte death and improves cardiac function during ischemia-reperfusion. *American Journal of Physiology Heart and Circulatory Physiology* 291: 52-60.

Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, Bar-Sagi D, Jones SN, Flavell RA and Davis RJ (2000) Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. *Science* 288: 870-874.

Ukani MD, Nanavati DD and Mehta NK (1997) A review on the ayurvedic herb tribulus terrestris L. *Ancient Science of Life* 17: 144-150.

Wei J, Wang W, Chopra I, Li HF, Dougherty CJ, Adi J, Adi N, Wang H and Webster KA (2011) c-Jun N-terminal kinase (JNK-1) confers protection against brief but not extended ischemia during acute myocardial infarction. *The Journal of Biological Chemistry* 286: 13995-14006.

Yaoita H, Ogawa K, Maehara K and Maruyama Y (1998) Attenuation of ischemia /reperfusion injury in rats by a caspase inhibitor. *Circulation* 97: 276-281.

Yu C, Minemoto Y, Zhang J, Liu J, Tang F, Bui TN, Xiang J and Lin A (2004) JNK suppresses apoptosis via phosphorylation of the proapoptotic Bcl-2 family protein BAD. *Molecular Cell* 13: 329-340.

Yun N, Kang JW and Lee SM (2012) Protective effects of chlorogenic acid against ischemia/reperfusion injury in rat liver: molecular evidence of its antioxidant and anti-inflammatory properties. *The Journal of Nutritional Biochemistry* 23: 1249-1255.

Zhang S, Li H and Yang SJ (2010) Tribulosin protects rat hearts from ischemia/reperfusion injury. *Acta Pharmacologica Sinica* 31: 671-678.

Zhang S, Li H and Yang SJ (2011) Tribulosin suppresses apoptosis via PKC epsilon and ERK1/2 signaling pathway during hypoxia/reoxygenation in neonatal rat ventricular cardiac myocytes. *Journal of Asian Natural Products Research* 13: 1135-1145.

Zhou Y, Fang SH, Ye YL, Chu LS, Zhang WP, Wang ML and Wei EQ (2006) Caffeic acid ameliorates early and delayed brain injuries after focal cerebral ischemia in rats. *Acta Pharmacologica Sinica* 27: 1103-1110

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Effect of *Tribulus terrestris* L. root methanol extract (TTM) on isoproterenol induced myocardial dysfunction in rats

4.1 Overview of chapter 3

In the previous chapter, *T.terrestris* root methanol extract (TTM) was found effective against ischemic complications in a cell line model of myocardial ischemia. The partial characterization of TTM revealed the presence of bioactives like caffeic acid, ferulic acid and 4-hydroxybenzoic acid. This prompted for further exploration of the protective property of TTM in isoproterenol model of myocardial infarction in rat. Also, an *in vivo* study is necessary to check the translation of *in vitro* results to *in vivo* and understand the mechanism underlying its mode of action. Isoproterenol induced myocardial infarction is one of the standard models used to determine the protective effect of a drug against infarction.

4.2 Introduction

Coronary heart disease is the leading cause of death worldwide. For better prognosis, it is necessary to understand the etio-pathogenic mechanism of myocardial infarction. To study the protective effect of a test material against myocardial infarction, a widely used experimental model is the catecholamine model i.e. induction of infarction by administration of isoproterenol in rats, as it causes a myocardial damage similar to the one observed in MI in humans (Mladenka et al., 2009). During myocardial infarction there is complete blockage of the coronary artery leading to death of heart muscle in the left ventricle. This results in the decrease in pumping ability of the heart. Lack of sufficient pumping leads into various pathological alterations in heart function and shift to compensatory responses to maintain adequate cardiac output (Young, 2010). The cell switches to myocardial hyper function by stimulating the neuro-humoral compensatory mechanism i.e. activation of the sympathetic nerves, increase both in chronotropism and inotropism as well as hypotension in the coronary bed (Anthonio et al., 1998). Secondly, there is an elevation of calcium inside the cell, activation of the adenylate cyclase enzyme and the depletion of ATP levels (Bhagat et al., 1976). Eventually, there is an oxidative stress resulting in the complex functional, structural, biochemical, and molecular changes leading to progression of heart failure. Beta blockers are usually administered to reduce the increase in

heart rate caused by the activation of β-adrenergic receptors (Frishman, 2003). But they have adverse effects and secondary complications. Beta-blockers may trigger severe asthma attack and effect cholesterol and triglyceride levels (Kaiser et al., 2014). So, therapeutic agents from natural sources that can act as effective β-blockers without the adverse effects and thus prevent the progression of heart failure are the need of the day. The mechanisms of benefit include anti-arrhythmic effects, slowing or reversing the detrimental ventricular remodeling caused by sympathetic stimulation and decreased myocyte death and inflammation.

We found that TTM is an effective antioxidant. It protects from ischemia-induced generation of ROS, protecting cells from apoptotic and necrotic death by up-regulating transcription factors and MAPK signaling. So, studies have been conducted to check whether TTM is effective against myocardial infarction induced secondary complications. There is high demand for medication in the form of phytoceuticals to treat secondary complications such as cardiac remodeling, tachycardia, inflammation and heart failure associated with myocardial infarction. In this background, methanol extract of *Tribulus terrestris* L. roots (TTM) were evaluated against isoproterenol induced pathophysiological alterations in the heart of Wistar rats with more emphasis on oxidative stress, electrical conduction, and inflammation related parameters including histopathological changes in the heart.

4.3 Methods

4.3.1 Experimental Design

Male wistar rats weighing (190-220 g) was used for the experiment. Rats were divided into six groups of six rats each.

Group I - Vehicle control (saline)

Group II - Treatment control (TTM 500 mg/kg body weight), to check the toxicity of the extract)

Group III - Isoproterenol induced myocardial infarction (85 mg/kg s. c.)

Group IV - TTM (250 mg/kg body weight) (pre-treatment) + isoproterenol

Group V - TTM (500 mg/kg body weight) (pre-treatment) + isoproterenol

Group VI - Propranolol (15 mg/kg body weight) (pre-treatment) + isoproterenol

In this experiment, TTM and positive control propranolol was given as pretreatment for 21 days before the induction of myocardial infarction by isoproterenol administration.

TTM (250 mg/kg body weight and 500 mg/kg body weight) and propranolol (15 mg/kg body weight) was administered orally to the rats for 21 days (pre-treatment). On the 22nd and 23rd day, animals in this group were administered isoproterenol subcutaneously (85 mg/kg). We followed this regime of experiment to check the efficiency of test material as a preventive agent against myocardial infarction. At the end of the experiment, rats were anaesthetized (by subcutaneous administration of ketamine (65 mg/kg) and xylazine (5 mg/kg)) and ECG was performed. Then, rats were sacrificed by cervical dislocation under deep ether anaesthesia and blood samples and hearts were collected in pre-cooled containers for estimation of various biochemical parameters and histopathological analysis. Detailed analytical procedures are given in chapter 2.

Various parameters studied include

- Lactate dehydrogenase (LDH), creatinine kinase (CK), creatinine kinase -isoenzyme of myocardial specificity (CK-MB), serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) and calcium.
- Activity of antioxidant enzymes in the heart
- Histopathology of the heart
- ECG variations
- Activation and translocation of NFκB and activation of pro-inflammatory cytokines, TNF-α, IL-6 and IL-1β, chemokines MCP-1 and anti-inflammatory cytokines, IL-10.

4.4 Results

4.4.1 Cardiac biomarkers LDH, CK, CK-MB, SGOT, SGPT and calcium in the serum of control and treated rats

Isoproterenol administration resulted in increase of LDH (133.33 ± 1.2 %), CK-MB (150 ± 2.2%), SGOT (57.14 ± 0.5 %), and SGPT (22.22 ± 0.2 %) in the blood when compared to normal rats (Figure 4.1). However, TTM administration prevented the abnormal release of these cardiac biomarkers. There was no significant change in calcium levels. Also, rats treated with TTM alone did not show any abnormal release of the cardiac marker enzymes into the blood. These results show that TTM at this concentration (500 mg/kg body weight) does not have any toxic effect on the animal

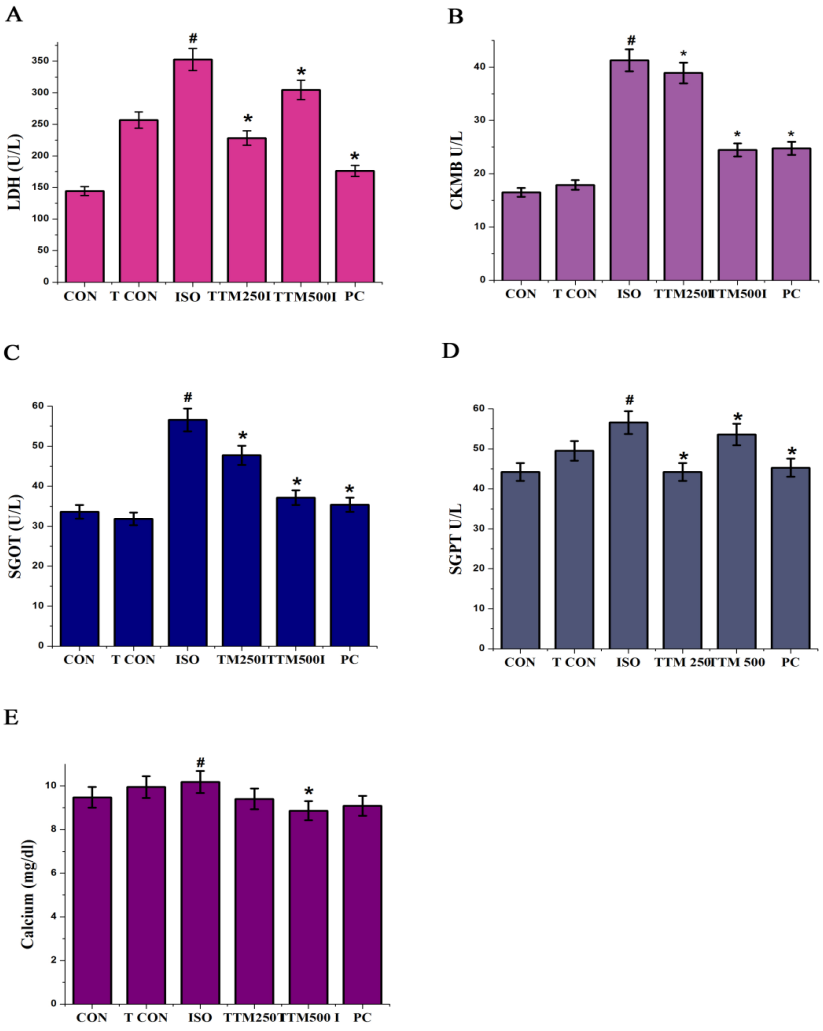


Figure 4.1 Cardiac biomarker quantification from serum of rats from different groups. A. lactate dehydrogenase, B. creatine kinase - isoenzyme of myocardial specificity, C. serum glutamic oxaloacetic transaminase, D. serum glutamic pyruvic transaminase and E. calcium in control group, treatment control group, isoproterenol group, *T. terrestris* L. root methanol extract treatment (250 and 500 mg/kg body weight) + isoproterenol and positive control (propranolol treatment 15 mg/kg body weight) + isoproterenol groups respectively. Results are expressed as mean ± SD, n = 6, # $p \leq 0.05$ vs control,* $p \leq 0.05$ vs ischemia.

4.4.2 Activities of antioxidant enzymes in the heart

Table 4.1 shows the activities of endogenous antioxidant enzymes in the heart of control and isoproterenol treated rats. Isoproterenol treatment decreased significantly ($p \leq 0.05$) the activities of superoxide dismutase by 44. 6%, catalase by 45.2 % and glutathione peroxidase by 49 % in the heart. Whereas, treatment with TTM (250 and 500 mg/kg body

weight) and propranolol (15 mg/kg body weight) significantly ($p \leq 0.05$) prevented the decrease in activity of antioxidant enzymes. Decrease in SOD activity was prevented by 51 %, 70 % and 48 %, decrease in CAT activity was prevented by 62 %, 72 % and 55 % and decrease in GPx activity was prevented by 68 %, 79 % and 66 % with TTM 250 mg/kg, TTM 500 mg/kg and propranolol 15 mg/kg respectively.

Table 4.1 Activities of antioxidant enzymes in the heart.

Groups	SOD (units/mg protein)	CAT (µmoles of H_2O_2 decomposed/ min/mg protein)	GPx (units/mg protein)
I	6.23 ± 0.55	0.53 ± 0.04	5.76 ± 0.54
II	5.92 ± 0.76	0.58 ± 0.06	5.54 ± 0.59
III	3.45 ± 0.43#	0.29 ± 0.03#	2.93 ± 0.33#
1V	5.23 ± 0.45*	0.47 ± 0.05*	4.93 ± 0.56*
V	5.89 ± 0.59*	0.50 ± 0.05*	5.25 ± 0.48*
VI	5.13 ± 0.49*	0.45 ± 0.04*	

Group I - control group, Group II - treatment control group, Group III - isoproterenol group, Group IV - *T. terrestris* L. root methanol extract treatment (250 mg/kg body weight) + isoproterenol, Group V - *T. terrestris* L. root methanol extract treatment (500 mg/kg body weight) + isoproterenol, Group VI - propranolol treatment (15 mg/kg body weight) + isoproterenol. Results are expressed as mean ± SD (n = 6). # indicates significant difference from control group. * indicates significant difference from isoproterenol treatment group. Significance accepted at $p \leq 0.05$

4.4.3 Histopathology of infarction

Myocardial infarction results in characteristic metabolic and structural alterations of the myocytes involving mitochondria, nuclei, myofilaments and sarcolemma. Hematoxylin - eosin staining of the isoproterenol treated rat heart showed profound inflammatory infiltration, interstitial edema, engorged capillaries and contraction band necrosis (Figure 4.2b). However, TTM treatment (250 mg/kg and 500 mg/kg body weight) and positive control (propranolol 15 mg/kg body weight) showed normal myocardial fibers and reduced levels of inflammatory infiltration edema and necrosis (Figure 4.2 c, d)

Masson's trichrome staining (for collagen visualization) of the isoproterenol treated rat heart revealed severe fibrosis when compared to the control rats (Figure 4.3 b). The collagen present in the fibrotic tissue appears blue on Masson's trichrome staining. However, TTM pre-treatment (250 mg/kg and 500 mg/kg body weight) and positive control (propranolol 15 mg/kg body weight) was effective in attenuating cardiac fibrosis in isoproterenol treated myocardial infarction (Figure 4.3 c, d)

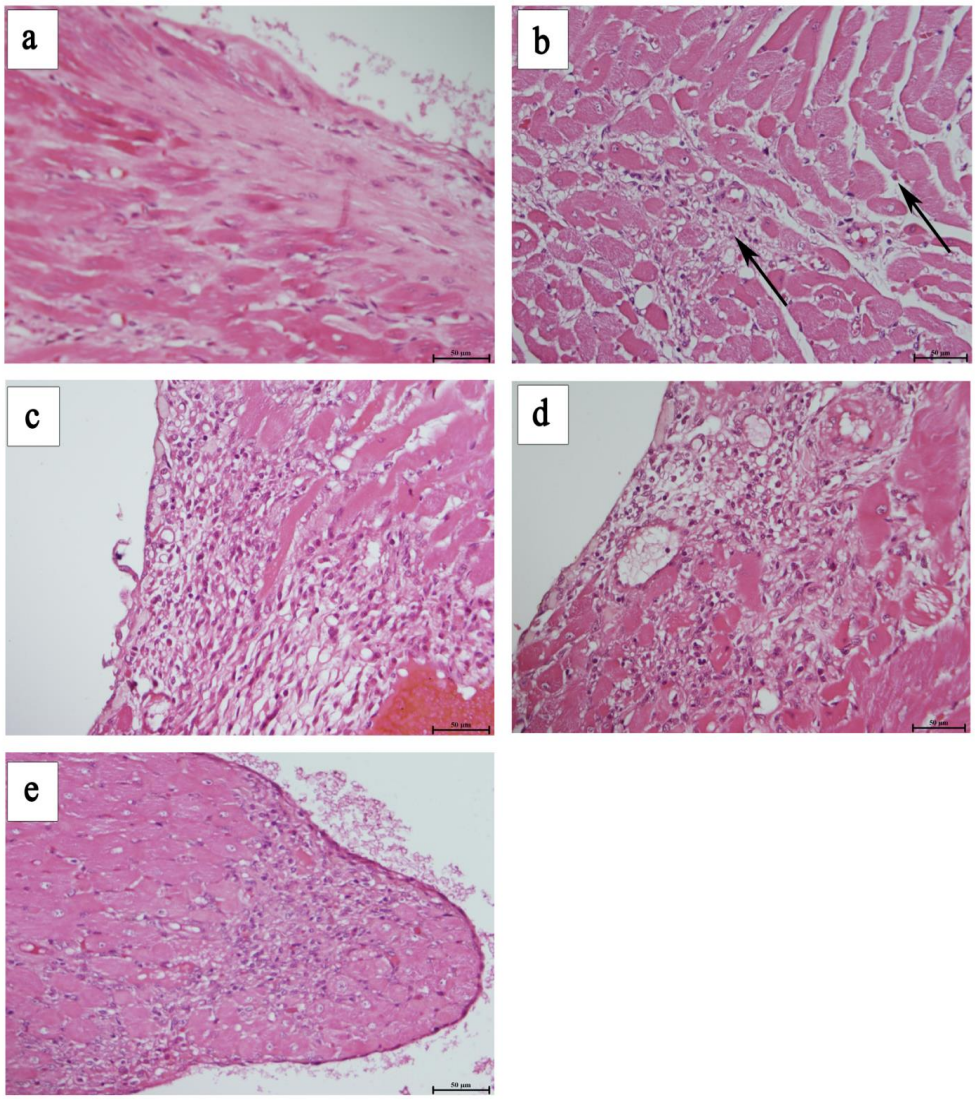


Figure 4.2: Hematoxylin-eosin staining of heart tissue (40X) a. control, b. isoproterenol, c. *T. terrestris* L. root methanol extract treatment (250 mg/kg body weight) + isoproterenol, d. *T. terrestris* L. root methanol extract treatment (500 mg/kg body weight) + isoproterenol, e. Propranolol treatment (15 mg/kg body weight) + isoproterenol.

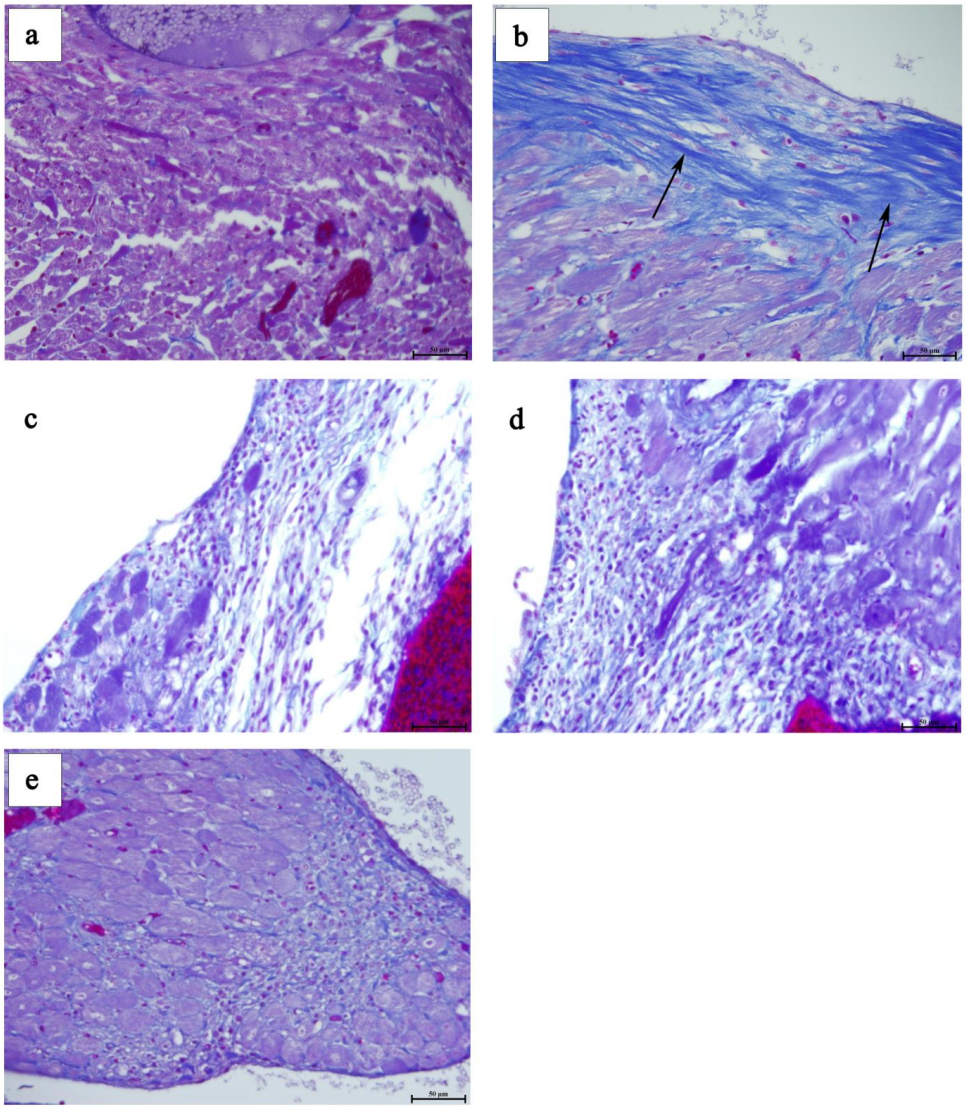


Figure 4.3 Masson's trichrome staining of heart tissue (40X) a. control, b. isoproterenol, and c. *T. terrestris* L. root methanol extract treatment (250 mg/kg body weight) + isoproterenol, d. *T. terrestris* L. root methanol extract treatment (500 mg/kg body weight) + isoproterenol, e. Propranolol treatment (15 mg/kg body weight) + isoproterenol

4.4.4 ECG recording

Isoproterenol treated animals showed significant ($p \leq 0.05$) ST segment depression (4.4 fold) when compared to control group (Figure 4.4). ST segment depression is a clinical marker of myocardial infarction. Pretreatment with TTM, showed significant ($p \leq 0.05$) prevention of ST segment depression (1.26 fold for TTM 250mg/kg body weight and 1.14

fold for TTM 500 mg/kg body weight) in ECG when compared to isoproterenol group (Figure 4.4)

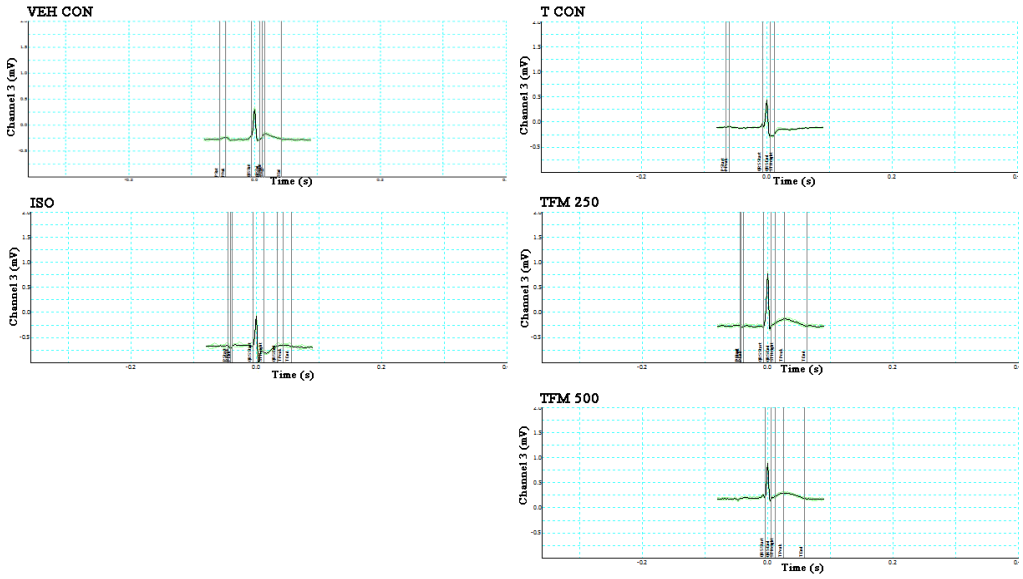


Figure 4.4 Electrocardiogram. Electrocardiogram of control, treatment control, isoproterenol, *T. terrestris* L. root methanol extract treatment (250 mg/kg body weight) + isoproterenol , *T. terrestris* L. root methanol extract (500 mg/kg body weight) + isoproterenol treatment groups.

4.4.5 ECG parameters

Heart rate was increased significantly ($p \leq 0.05$) by 50 % in isoproterenol group and the increase in heart rate was partially ameliorated on TTM and positive control treatment (9 % and 13 % for TTM 250 and TTM 500 mg/kg body weight respectively and 26 % for positive control). There was significant ($p \leq 0.05$) QT prolongation, 39 % on isoproterenol treatment and TTM (250 and 500 mg/kg body weight) administration prevented QT prolongation by 5 % where as positive control prevented QT prolongation by 12 % (Table 4.2)

Table 4.2 Electrocardiogram parameters

Parameters	CON	T CON	ISO	TTM250	TTM500	PC
Heart rate (bpm)	229.65	271.12	342.2	311.25	297.52	250.1517
PR interval (s)	0.049867	0.47994	0.044001	0.041896	0.04759	0.042994
QRS interval (s)	0.017904	0.013254	0.022103	0.02065	0.018552	0.019975
QTc interval (s)	0.09629	0.112817	0.1335	0.1259	0.1267	0.117686
P amplitude (mV)	0.08052	0.054523	0.077127	0.116433	0.110558	0.089842
Q amplitude (mV)	0.019525	0.012363	0.032057	0.024775	0.019125	0.00669
R amplitude (mV)	0.618817	0.48996	0.82955	0.870522	0.617	0.852617
S amplitude (mV)	-0.08725	-0.0436	-0.13855	-0.08547	-0.08093	-0.08319
ST height (mV)	0.018614	0.009866	-0.06351	0.016928	0.009488	-0.00681
T amplitude (mV)	0.8052	0.116433	0.054523	0.110558	0.089842	0.077127

Electrocardiogram parameters of control, treatment control, isoproterenol, *T. terrestris* L. root methanol extract (250 mg/kg body weight) + isoproterenol, *T. terrestris* L. root methanol extract (500 mg/kg body weight) + isoproterenol, and positive control propranolol (15 mg/kg body weight) + isoproterenol

4.4.6 Effect of *Tribulus terrestris* L. root on isoproterenol induced secretion of MCP-1, IL-10, and IL-1β.

To assess how isoproterenol affects the inflammatory markers, the presence of pro-inflammatory chemokines, monocyte chemo attractant protein -1(MCP-1), and cytokines, IL-10, IL-1β, IL-6 and TNF-α was measured in the serum of control and treated rats. Isoproterenol caused significant ($p \leq 0.05$) increase in the level of inflammatory cytokine, IL -1β and chemokine, MCP-1 and a significant ($p \leq 0.05$) decrease in cytokine, IL-10 compared to control (Figure 4.5). MCP-1 was increased significantly by 83.3 % on isoproterenol treatment while TTM administration prevented the increase in MCP-1 levels (27.27 % for TTM 250 mg/kg and 31.81 % for TTM 500 mg/kg). IL-1β was increased by 166.67 % on isoproterenol treatment, while TTM pre-treatment prevented the decrease of IL-1β levels by 37. 5% and 18. 75% for TTM 250 mg/kg and TTM 500 mg/kg respectively, which was not dose dependent. IL-10 was decreased by 33.33 % on isoproterenol treatment while TTM

prevented the decrease of IL-10 levels by 20 % and 30 % for TTM 250 mg/kg and TTM 500 mg/kg respectively (Figure 4.5).

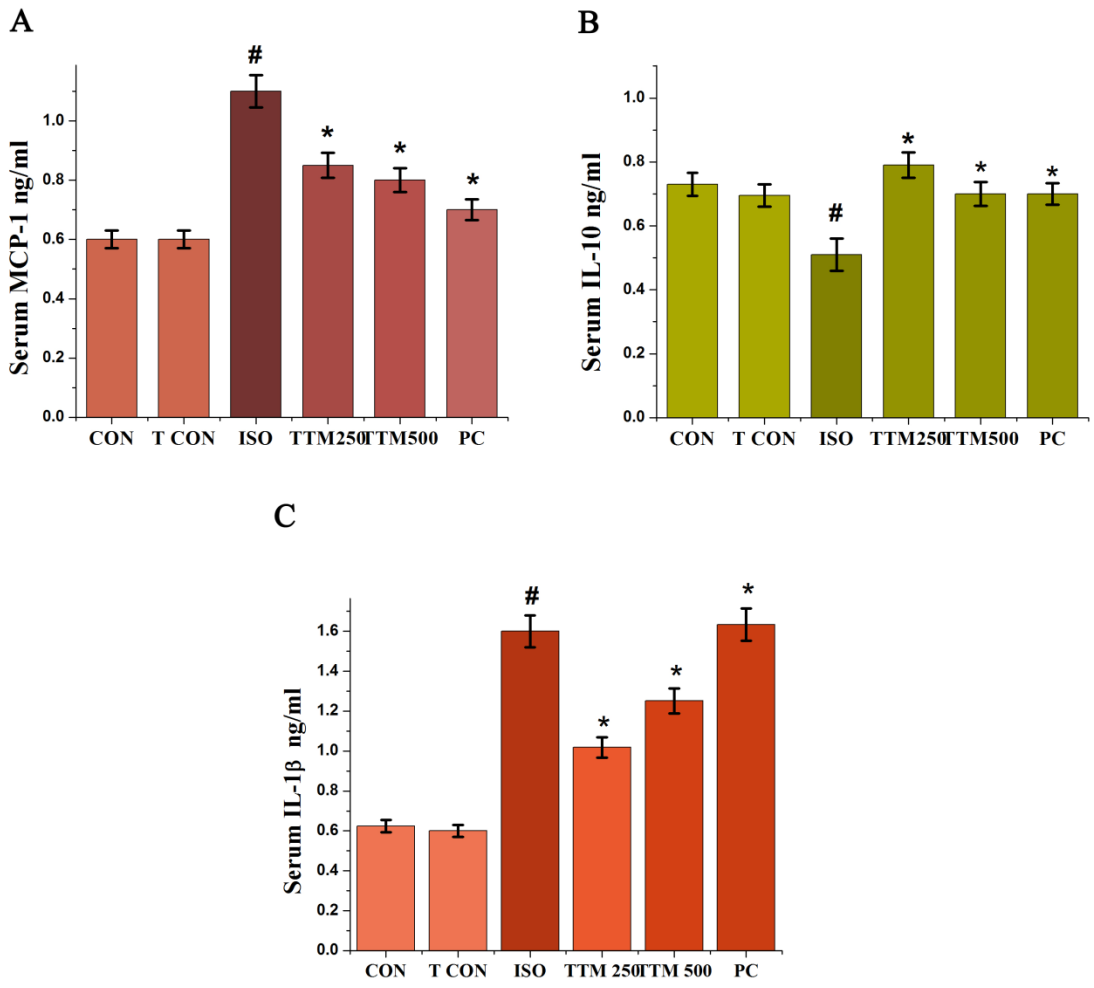


Figure 4.5: Expression of inflammatory cytokines and chemokines. Effect of *Tribulus terrestris* L. root methanol extract on isoproterenol induced secretion of A. monocyte chemo attractant protein -1, B. Interleukin-10 and C. Interleukin-1β levels in control, treatment control, isoproterenol, *T. terrestris* L. root methanol extract 250 mg/kg body weight and isoproterenol treatment, *T. terrestris* L. root methanol extract 500 mg/kg body weight and isoproterenol treatment and propranolol 15 mg/kg body weight and isoproterenol treatment groups. Results are expressed as mean ± SD (n = 6). # indicates significant difference from control group. * indicates significant difference from isoproterenol treatment. Significance accepted at $p \leq 0.05$

4.4.7 Effect of *Tribulus terrestris* L. on cardiac tissue expression of IL-6 and TNF-α

Isoproterenol treatment caused a significant ($p \leq 0.05$) increase in tissue level expression of inflammatory markers IL - 6 and TNF - α. There was a 100 % increase in IL - 6

expression with isoproterenol treatment and TTM pre-treatment prevented significantly ($p \leq 0.05$) the upregulation of tissue inflammatory markers IL- 6 by 30 % and 75 % for TTM 250 and 500 mg/kg respectively. TNF - α expression was increased by 60 % and TTM 250 and TTM 500 mg/kg treatment prevented the up-regulation of TNF-α by 12.5 % and 12 % respectively. There was no dose dependent change in TNF-α expression on TTM treatment (Figure 4.6)

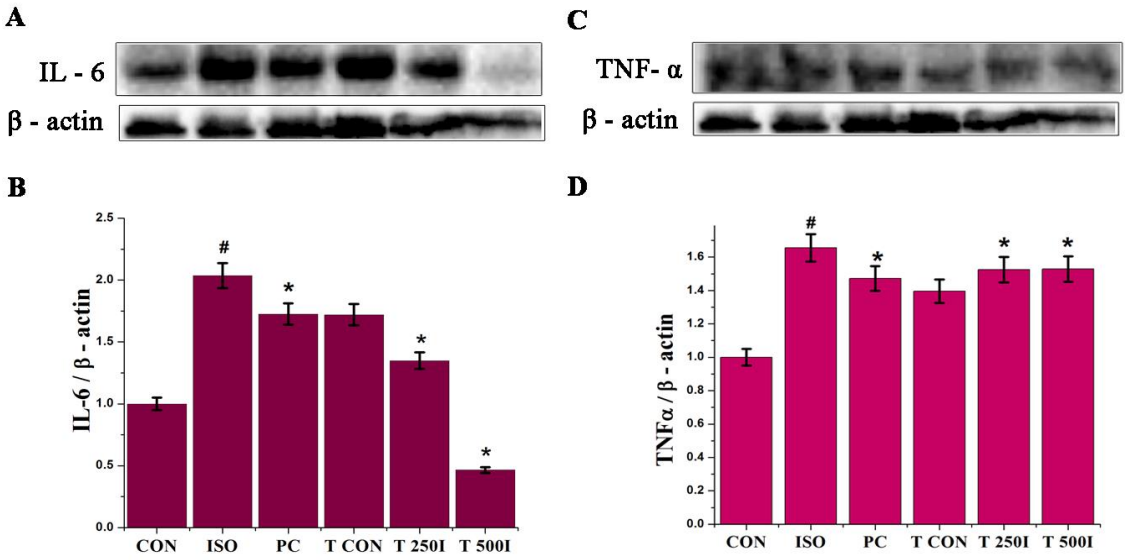


Figure 4.6 Expression of IL-6 and TNF-α. A. Immuno-blot analysis of interleukin - 6 in control, isoproterenol, propranolol (15 mg/kg body weight), treatment control, *T. terrestris* root methanol extract 250 mg/kg body weight and isoproterenol treatment, *T. terrestris* root methanol extract 500 mg/kg body weight and isoproterenol treatment. B. Densitometric quantification of protein level expression of IL- 6 relative to β - actin. C. Immuno-blot analysis of tumor necrosis factor - α in control, isoproterenol, positive control propranolol 15 mg/kg body weight, treatment control, *T. terrestris* methanol extract 250 mg/kg body weight and isoproterenol treatment, *T. terrestris* methanol extract 500 mg/kg body weight and isoproterenol treatment. D. Densitometric quantification of protein level expression of tumor necrosis factor - α relative to β - actin. Values are expressed as mean ± standard deviation, n = 6, # mean value significantly different from control $p \leq 0.05$, * mean value significantly different from isoproterenol treated $p \leq 0.05$

4.4.8 NFκB expression and translocation

Isoproterenol caused a significant ($p \leq 0.05$) up-regulation of NFκB expression in the nuclear fraction. There was a 30 % increase of NFκB expression in the nuclear fraction with isoproterenol treatment (Figure 4.7 A, B). TTM pre-treatment was able to prevent significantly ($p \leq 0.05$) the increase in expression of NFκB in the nuclear fraction by 61.53 %

and 38.46 % for TTM 250 and TTM 500 mg/kg body weight respectively (Figure 4.7 A, B). The NFκB translocation assay showed that isoproterenol treatment caused the phosphorylation of IκB and increased translocation of transcription factor NFκB from the cytoplasm to the nucleus and subsequent binding of NFκB to the promoter or enhancer region of target genes such as cytokines thereby increasing their expression. The NFκB expression in the nuclear fraction was significantly, ($p \leq 0.05$) increased (116 %) during isoproterenol treatment and TTM pre-treatment (250 and 500 mg/kg body weight) prevented significantly ($p \leq 0.05$) the translocation of NFκB to the nucleus by 30 % and 46 % respectively (Figure 4.7 C)

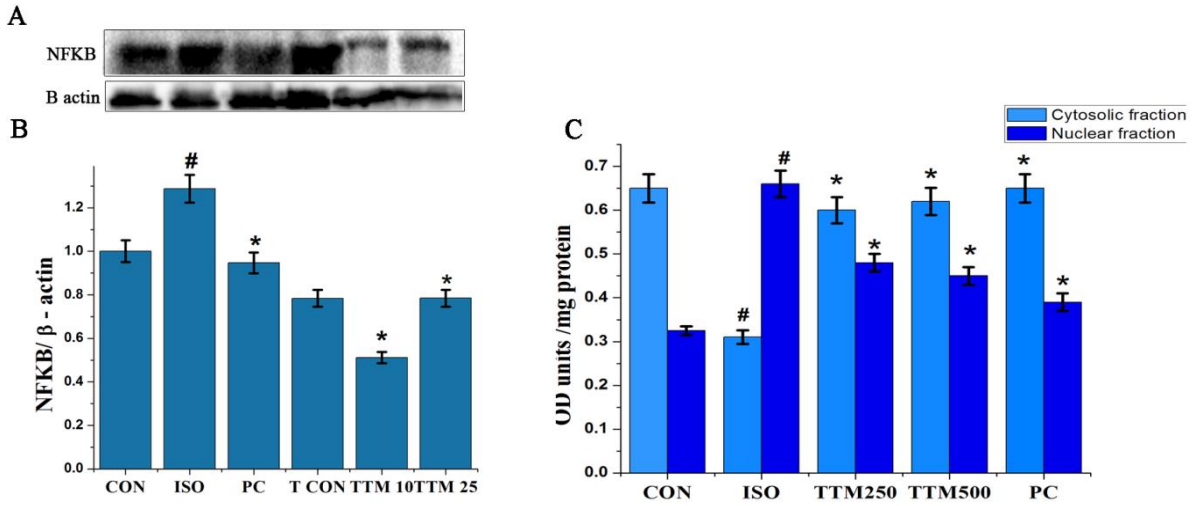


Figure 4.7 Nuclear factor - kappa B expression in the nucleus and translocation. A. Immunoblot analysis of nuclear factor - kappa B expression in control, isoproterenol, positive control (propranolol) 15 mg/kg and isoproterenol, *T. terrestris* root methanol extract 250 mg/kg and isoproterenol treatment, *T. terrestris* root methanol extract 500 mg/kg and isoproterenol treatment. B. Densitometric quantification of protein levels of NF-κB relative to β - actin. C. NF-κB translocation assay in control, isoproterenol, positive control propranolol 15 mg/kg and isoproterenol, *T. terrestris* root methanol extract 250 mg/kg and isoproterenol treatment, *T. terrestris* root methanol extract 500 mg/kg and isoproterenol treatment. Values are mean ±standard deviation, n = 6, # mean value significantly different from control $p \leq 0.05$, * mean value significantly different from isoproterenol treated $p \leq 0.05$

4.5 Discussion

Myocardial infarction is associated with an inflammatory response which is a prerequisite for healing and scar formation but is implicated in the pathogenesis of post infarction remodeling and heart failure (Frangogiannis, 2014). Excessive early inflammation

causes matrix degradation leading to cardiac rupture (Tao et al., 2004). Prolongation of the inflammatory reaction may impair collagen deposition leading to formation of a scar with reduced tensile strength, thus increasing chamber dilation (Frangogiannis, 2012). Enhanced expression of pro-inflammatory mediators may activate pro-apoptotic pathways inducing further loss of cardiomyocytes. Finally defective containment of the inflammatory reaction may lead to extension of the inflammatory infiltrate into the non-infarcted myocardium enhancing fibrosis and worsening diastolic function (Bujak and Frangogiannis, 2007). Thus, proper containment of the inflammatory response is necessary for preventing further damage to the myocytes.

The innate or natural immune system is the body's first line of defense against threats such as physical or chemical injury. A series of reactions are induced that prevent ongoing tissue damage and activate repair process to restore homeostasis (Pickup, 2004). Activation of NFκB pathway is one of the major nuclear factor which leads to the activation of pro-inflammatory cytokines. NFκB is the regulator of more than 200 pro inflammatory genes and widely recognized as the master switch of inflammation (Schwartz and Reaven, 2006). Elevated level of pro inflammatory cytokines is the hall mark sign of inflammation. Inflammatory response following AMI serves to clear the wound and facilitate wound healing and scar formation, but excessive inflammatory response causes adverse left ventricular remodeling and heart failure.

The initiation of the inflammatory process begins with complement activation (Pinckard et al., 1975). Complement activation further recruits neutrophils and monocytes to the injured myocardium (Rossen et al., 1994). ROS have the ability to directly injure cardiac myocytes and also trigger the inflammatory cascade through induction of cytokines (Dhalla et al., 2000; Lefer and Granger, 2000). The antioxidant enzymes Mn SOD and catalase are involved in the regulation of ROS. Studies demonstrated that mouse over-expressing SOD caused a significant decrease in infarct size which is mediated by reducing inflammatory response (Uraizee et al., 1987). In our study, there was a reduction in antioxidant enzymes SOD, catalase and glutathione peroxidase following isoproterenol treatment and TTM administration caused an increase in antioxidant enzyme activity thus protecting from ROS induced injury. Damaged cells and ROS also cause the release of danger associated

molecular patterns (DAMP) recognized by Toll like receptors (TLRs). TLR recruitment leads to phosphorylation and degradation of IκB, release and translocation of NFκB to the nucleus leading to transcription of pro inflammatory genes such as cytokines and adhesion molecules (Oeckinghaus and Ghosh, 2009). ROS also leads to inflammasome activation which leads to inflammatory cytokine production (Harijith et al., 2014). Thus, signals in the infarcted myocardium activate toll-like receptor signaling, while complement activation and generation of reactive oxygen species induce cytokine up regulation. Pro-inflammatory cytokines activate chemokines and adhesion molecules and activate the innate immune response.

Cytokines such as tumor necrosis factor-alpha (TNF-α) or interleukin-6 (IL-6) are recruited soon after myocardial injury and can acutely regulate myocyte survival or apoptosis and trigger additional cellular inflammatory response (Nian et al., 2004). The production of IL-6 from macrophages is induced by IL-1 and TNF-α. Endothelial cells are capable to produce IL-6 on stimulation with a variety of inflammatory mediators. In acute MI IL-6 levels were found to be elevated before the appearance of detectable signs of necrosis. As a conclusion of these findings a primary role of IL-6 in the pathogenesis of acute MI has been suggested. IL-1β, as a gatekeeper of inflammation, is an early and prominent mediator for inflammatory response in MI (Dinarello, 2011). Increased plasma IL-1β levels were strongly associated with impaired myocardial function and left ventricular hypertrophy following reperfused myocardial infarction (Orn et al., 2012).

The recruitment of inflammatory cells is a crucial step of inflammatory response after MI, which is mediated by the interactions between chemokines and cell adhesion molecules expressed on activated endothelial cells and their receptors on inflammatory cells. CC chemokines monocyte chemoattractant protein-1 (MCP-1) induces the infiltration of mononuclear phagocytes while CXC chemokines such as IL-8 and C5a mediate the infiltration of the infarct with neutrophils. Monocyte Chemoattractant Protein (MCP-1), a chemokine is also markedly up regulated in the infarcted myocardium inducing recruitment of mononuclear cells in the injured areas. IL-10 an inhibitory cytokine has a role in suppressing acute inflammatory response and in regulating extracellular matrix metabolism(Frangogiannis et al., 2000). IL-10 inhibits the production of IL-1α, IL-1β, TNF-

α, IL-6 and IL-8 by LPS-activated monocytes, suppressing the inflammatory response. Thus IL-10 has a protective role.

In this study there was increased expression of pro-inflammatory cytokines IL-6, TNF-α and IL-1β on isoproterenol treatment, which was down regulated by *T. terrestris*. There was also increased expression of chemokines MCP-1 which was down regulated by *T. terrestris* treatment. IL-10 an inhibitory cytokine, which has a protective effect against inflammation, was down regulated in isoproterenol group. The downregulation of IL-10 was prevented with *T. terrestris* treatment thus protecting the myocardium from further inflammation.

ECG showed a depression of ST segment (negative T wave) in isoproterenol treatment group indicating myocardial ischemia, similar to previous report (Krenek et al., 2009). ST segment depression is a feature of sub-endocardial ischemia. It is mostly caused by demand ischemia where energy supply to cardiomyocytes is insufficient for the work force. *Tribulus terrestris* L. root was also able to prevent the ST depression caused by isoproterenol treatment. Heart rate was also increased on isoproterenol treatment and *Tribulus terrestris* L. root (TTM) was able to prevent the increase in heart rate. There was also increased tissue necrosis, inflammatory infiltration, edema and tissue fibrosis on isoproterenol treatment which was evident from the histopathological examination of the heart tissue. TTM pre-treatment was able to prevent to some extent the damage caused to heart tissue by isoproterenol administration. Thus, *T. terrestris* root is also protective in an *in vivo* model of myocardial infarction. This protective property may be due to the compounds present in *T. terrestris* roots.

References

Anthonio RL, Van Veldhuisen DJ and Van Gilst WH (1998) Left ventricular dilatation after myocardial infarction: ACE inhibitors, beta-blockers, or both? *Journal of Cardiovascular Pharmacology* 32: 1-8.

Bhagat B, Sullivan JM, Fischer VW, Nadel EM and Dhalla NS (1976) cAMP activity and isoproterenol-induced myocardial injury in rats. *Recent Advances in Studies on Cardiac Structure and Metabolism* 12: 465-470.

Bujak M and Frangogiannis NG (2007) The role of TGF-beta signaling in myocardial infarction and cardiac remodeling. *Cardiovascular Research* 74: 184-195.

Dhalla NS, Temsah RM and Netticadan T (2000) Role of oxidative stress in cardiovascular diseases. *Journal of Hypertension* 18: 655-673.

Dinarello CA (2011) A clinical perspective of IL-1beta as the gatekeeper of inflammation. *European Journal of Immunology* 41: 1203-1217.

Frangogiannis NG (2012) Regulation of the inflammatory response in cardiac repair. *Circulation Research* 110: 159-173.

Frangogiannis NG (2014) The inflammatory response in myocardial injury, repair, and remodelling. *Nature Reviews Cardiology* 11: 255-265.

Frangogiannis NG, Mendoza LH, Lindsey ML, Ballantyne CM, Michael LH, Smith CW and Entman ML (2000) IL-10 is induced in the reperfused myocardium and may modulate the reaction to injury. *Journal of Immunology* 165: 2798-2808.

Frishman WH (2003) Cardiology patient page. Beta-adrenergic blockers. *Circulation* 107: 117-119.

Harijith A, Ebenezer DL and Natarajan V (2014) Reactive oxygen species at the crossroads of inflammasome and inflammation. *Frontiers in Physiology* 5: 352-358.

Kaiser EA, Lotze U and Schafer HH (2014) Increasing complexity: which drug class to choose for treatment of hypertension in the elderly? *Clinical Interventions in Aging* 9: 459-475.

Krenek P, Kmecova J, Kucerova D, Bajuszova Z, Musil P, Gazova A, Ochodnicky P, Klimas J and Kyselovic J (2009) Isoproterenol-induced heart failure in the rat is associated

with nitric oxide-dependent functional alterations of cardiac function. *European Journal of Heart Failure* 11: 140-146.

Lefer DJ and Granger DN (2000) Oxidative stress and cardiac disease. *American Journal of Medicine* 109: 315-323.

Mladenka P, Hrdina R, Bobrovova Z, Semecky V, Vavrova J, Holeckova M, Palicka V, Mazurova Y and Nachtigal P (2009) Cardiac biomarkers in a model of acute catecholamine cardiotoxicity. *Human and Experimental Toxicology* 28: 631-640.

Nian M, Lee P, Khaper N and Liu P (2004) Inflammatory cytokines and postmyocardial infarction remodeling. *Circulation Research* 94: 1543-1553.

Oeckinghaus A and Ghosh S (2009) The NF-kappaB family of transcription factors and its regulation. *Cold Spring Harbor Perspectives in Biology* 1:1-6.

Orn S, Ueland T, Manhenke C, Sandanger O, Godang K, Yndestad A, Mollnes TE, Dickstein K and Aukrust P (2012) Increased interleukin-1beta levels are associated with left ventricular hypertrophy and remodelling following acute ST segment elevation myocardial infarction treated by primary percutaneous coronary intervention. *Journal of Internal Medicine* 272: 267-276.

Pickup JC (2004) Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes. *Diabetes Care* 27: 813-823.

Pinckard RN, Olson MS, Giclas PC, Terry R, Boyer JT and O'Rourke RA (1975) Consumption of classical complement components by heart subcellular membranes in vitro and in patients after acute myocardial infarction. *Journal of Clincal Investigation* 56: 740-750.

Rossen RD, Michael LH, Hawkins HK, Youker K, Dreyer WJ, Baughn RE and Entman ML (1994) Cardiolipin-protein complexes and initiation of complement activation after coronary artery occlusion. *Circulation Research* 75: 546-555.

Schwartz EA and Reaven PD (2006) Molecular and signaling mechanisms of atherosclerosis in insulin resistance. *Endocrinology and Metabolism Clinics of North America* 35: 525-549

Tao ZY, Cavasin MA, Yang F, Liu YH and Yang XP (2004) Temporal changes in matrix metalloproteinase expression and inflammatory response associated with cardiac rupture after myocardial infarction in mice. *Life Sciences* 74: 1561-1572.

Uraizee A, Reimer KA, Murry CE and Jennings RB (1987) Failure of superoxide dismutase to limit size of myocardial infarction after 40 minutes of ischemia and 4 days of reperfusion in dogs. *Circulation* 75: 1237-1248.

Mitochondrial dysfunction in H9c2 cells during ischemia and amelioration with *Tribulus terrestris* L. fruit methanol extract (TFM)

5.1 Introduction

Mitochondria are key organelles and occupy about 35-40 % of the cardiomyocytes volume and produce 95 % of the ATP most essential for the proper functioning of the heart (Pham et al., 2014). Mitochondria are multifunctional organelles and serve as site for electron transport chain (ETC), oxidative phosphorylation, citric acid cycle, β - oxidation, oxidant generation and programmed cell death (Ballinger, 2005). They rely on ETC and oxidative phosphorylation to produce ATP (Lodish, 2000). During ischemia, deprivation of oxygen causes a decrease in the activity of electron transport chain (ETC) complexes and increased free radical production (Zhao et al., 2013). Protecting ETC complexes will be a protective measure in decreasing the oxidative damage. The innate antioxidant system of the myocardium also prevents damage caused by oxidative stress (Rahal et al., 2014). Myocardial antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase act in concert to protect the myocardium from ischemia-induced oxidative insult (Dhalla et al., 2000). Although, the myocardium expresses endogenous free radical scavenging enzymes, these defense systems are insufficient during pathological conditions like ischemia due to depletion of their content (Roth et al., 2004). Thus, focusing on the prevention of surplus ROS mediated mitochondrial dysfunction will be a promising therapeutic target. Oxidative damage to mitochondrial membranes, enzymes, and ETC components culminate in impaired mitochondrial ATP production, which facilitates mPTP opening (Waldmeier et al., 2003) that leads to cellular apoptosis and necrosis. Thus, protecting the mitochondria by a therapeutic agent is an effective strategy to prevent the deleterious effects of myocardial ischemia.

During physiological conditions mitochondria exhibit a high inner mitochondrial transmembrane potential ($\Delta\psi m$) by electron transfer from the electron transport chain and simultaneous proton pumping from the matrix (Kadenbach, 2003). During ischemia, the change in transmembrane potential causes increased calcium uptake impaired ATP synthesis that lead to a loss of ion homeostasis, stimulation of ROS generation, mPTP opening, matrix swelling, and outer mitochondrial membrane rupture and finally death of the myocyte (Jones

et al., 2003; Teshima et al., 2003). Therefore, maintaining mitochondrial transmembrane potential and preventing mPTP opening is protective against ischemia.

Maintaining integrity of the mitochondria is very essential in protecting the mitochondria from ischemic injury. Mitochondrial dynamics and mitophagy have an important role in maintaining the quality of the mitochondria (Ni et al., 2015). During ischemia there is an increase in mitochondrial fission, making the mitochondria more susceptible to mitophagy and a decrease in mitochondrial fusion (Ikeda et al., 2014). The alteration of integrity of mitochondria affects its function. So maintaining the integrity of the mitochondria is essential for prevention of damage caused by ischemia. Thus, a detailed study on the effect of *Tribulus terrestris* L. fruit methanol extract on mitochondrial function during cardiac ischemic injury was studied with special emphasis on mitochondrial bioenergetics and integrity.

5.2 Methods

5.2.1 Experimental details

The fresh whole fruits were air-dried and extracted with methanol at ambient temperature (37 ± 1 °C) under continuous stirring for 6 hours, and the extraction process was repeated until the solvent became colorless. The supernatant was filtered through Whatman no. 1 filter paper and concentrated *in vacuo* under reduced pressure in a rotavapor (Heidolph, Germany) followed by lyophilisation. The lyophilized *T. terrestris* L. fruit methanol extract (TFM) was stored at 4 °C until use. The yield of the extract was found to be 17.34 g /1kg of plant material. The antioxidant property of the extract (TFM) was evaluated. The detailed procedures for the estimation of total phenolic content (TPC), total flavonoid content (TFC) and *in vitro* antioxidant activities were given in chapter-2. Cell culture and treatment is similar to chapter-3. Cyclosporin A at a concentration of 0.1µM was used as positive control (PC) and was administered 24 hours before the induction of ischemia. After simulated ischemia, control and treated cells were subjected to viability assays (MTT and LDH release assay). After the optimization of effective dose of TFM based on MTT and LDH assay, the experimental groups consist of (a) control cells, (b) ischemia treated cells, (c) cells pretreated with TFM alone without ischemia (treatment control cells), (d) TFM

(10 µg/ml) pre-treatment before ischemia, (e) TFM (25 µg/ml) pre-treatment before ischemia, (f) TFM (50 µg/ml) pre-treatment before ischemia, (g) positive control (cyclosporin A 0.1 µM) pre-treatment before ischemia (Figure 5.1). After respective treatments, cells were analyzed for various parameters relevant to ischemia which affected the mitochondria. Details of all the analytical procedures are given in chapter - 2.

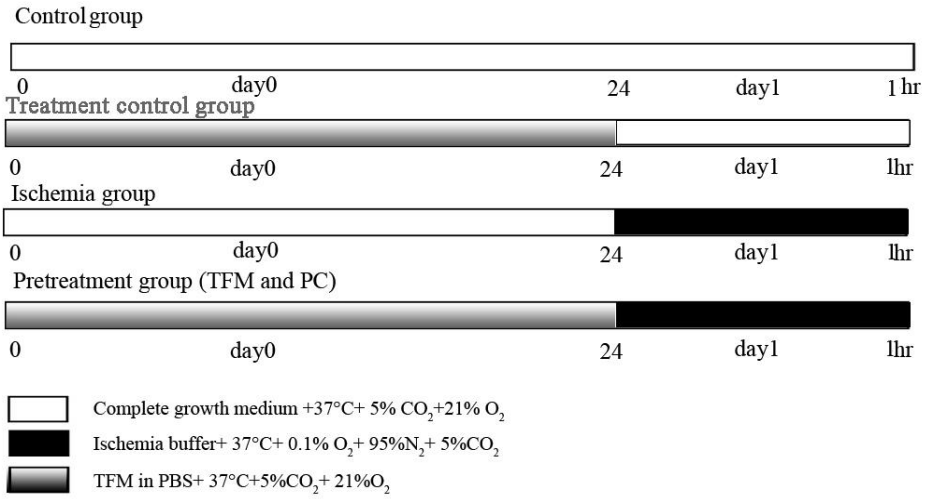


Figure 5.1 Schematic representation of treatment parameters. Control group, treatment control group, ischemia group and pre-treatment group (*Tribulus terrestris* L. fruit methanol extract pre-treatment 10, 25 µg/ml and Cyclosporin A [PC]) 24 hours prior to ischemia**.**

The various parameters specific to mitochondria studied further are.

- Detection of intracellular ROS
- Alteration in mitochondrial transmembrane potential (ΔΨm)
- Integrity of mitochondrial permeability transition pore (mPTP)
- Activities of mitochondrial respiratory complexes (Complex I-IV)
- Oxygen consumption rate, ATP content and expression of HIF-1α
- Mitochondrial dynamics, gene and protein expression of mitochondrial fission (Drp-1 and Fis1) and fusion proteins (Mfn1, Mfn2, OPA-1)

5.3 Results

5.3.1 HPLC analysis of TFM

HPLC (Figure 5.1) analysis of TFM showed the presence of phenolic acid, ferulic acid (Rt-12.703min) (5.420 ± 0.29 mg $g^{-1)}$; flavonoid, phloridzin (Rt-22.063 min) (3.6702 ± 0.18 mg $g^{-1)}$ and saponin, diosgenin (Rt-12.892 min) (17.346 ± 0.87 mg g^{-1})

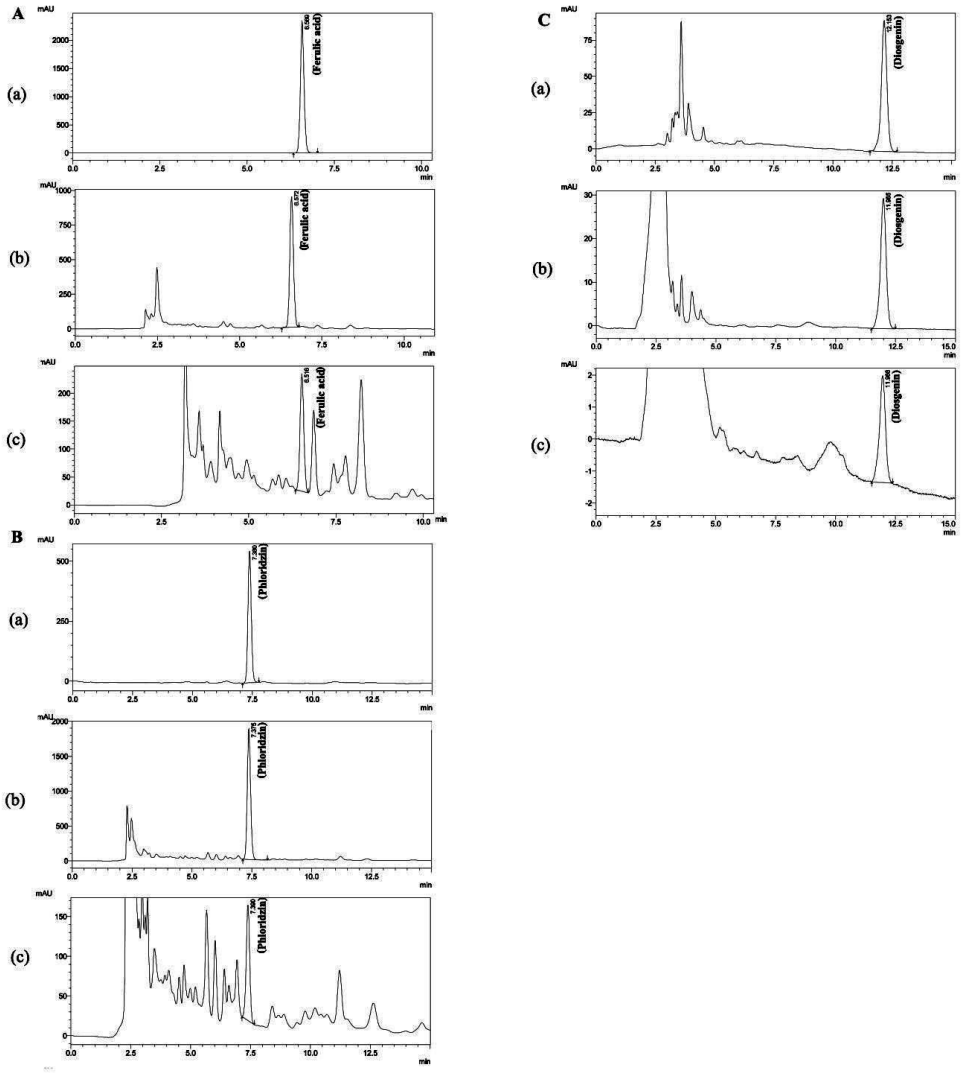


Figure 5.2 HPLC analysis of *T. terrestris* fruit methanol extract and standard compounds (A) HPLC chromatograph of (a) ferulic acid standard, (b) ferulic acid spiked *T. terrestris* fruit methanol extract, (c) *T. terrestris* fruit methanol extract. (B) HPLC chromatograph of (a) phloridzin standard, (b) phloridzin spiked *T. terrestris* fruit methanol extract, (c) *T. terrestris* fruit methanol extract. (C) HPLC chromatograph of (a) diosgenin standard, (b) diosgenin spiked *T. terrestris* fruit methanol extract, (c) *T. terrestris* fruit methanol extract.

5.3.2 Total phenolic content (TPC) and total flavonoid content (TFC)

The TPC in the extract was calculated as 27.75 mg gallic acid equivalents/g dry weight of plant extract and TFC was calculated as 23.05 mg catechin equivalents/g dry weight of extract.

5.3.3. Total antioxidant capacity

Total antioxidant capacity of TFM was calculated as 32.48 mg ascorbic acid equivalents/g dry weight of extract.

5.3.3.1 DPPH, ABTS, superoxide, hydroxyl radical scavenging assays

The IC_{50} values of DPPH, ABTS, and hydroxyl and superoxide radical scavenging activities of extract and standard compounds are given in Table 5.1. TFM showed significant DPPH scavenging activity with an IC_{50} of 198.07 µg/ml compared to 147.15 µg/ml for gallic acid. ABTS scavenging showed IC_{50} of 63.27 µg/ml compared to standard trolox 29.25 µg/ml. Hydroxyl and superoxide radical scavenging activities showed IC_{50} of 731.25 and 331.5 µg/ml compared to standards ascorbic acid and catechin showing IC_{50} of 380.25 and 185.25 µg/ml respectively.

5.3.3.2 Determination of LDL oxidation

The percentage inhibition of LDL oxidation showed significant potential of extract with an IC_{50} value of 429 µg/µl for the sample extract whereas the IC_{50} value for the standard catechin was found to be 415.66 µg/µl. (Table 5.1)

5.3.3.3 Determination of anti-peroxidative capacity in lecithin lipid micelles system

The estimated IC_{50} value of the extract was calculated as 468 µg/ml and that of α-tocopherol as 351 µg/ml. (Table 5.1)

Table 5.1 IC_{50} values of DPPH, ABTS, superoxide and hydroxyl radical scavenging activities

	IC_{50} µg/ml					
Sample	**DPPH RSA**	**ABTSRSA**	**Superoxide RSA**	**Hydroxyl RSA**	**LDL oxidation**	**Antiperoxidative capacity**
TFM	198.0 ± 18	63.37 ± 6	331.5 ± 31	731 ± 70	429 ± 41	468 ± 44
Gallic acid	147.1 ± 13					
Trolox		29.25 ± 2				
Ascorbic acid			185.25 ±17			
Catechin				380 ± 36	415± 35	
α-tocopherol						351 ± 33

IC_{50} values of DPPH, ABTS, superoxide and hydroxyl radical scavenging activities exhibited by *Tribulus terrestris* L. fruit methanol extract and standard compounds gallic acid, trolox, ascorbic acid, catechin and **α** - tocopherol in µg/ml. Results are expressed as mean ± SD, n = 6.

5.3.4 Cell line studies

5.3.4.1 MTT assay

Cell viability after treatment with different concentrations of TFM (10, 25, 50, 75, 100, and 150 µg/ml) is shown in Figure 5.3A of which, concentration up to 50 µg/ml did not affect cell viability significantly ($p \leq 0.05$). Thus, concentrations of 10, 25, 50 µg/ml were used for further studies.

5.3.4.2 LDH release assay

Lactate dehydrogenase secretion of all experimental groups (control, ischemia and cells pretreated with TFM prior to ischemia) was measured. The LDH secretion to the medium had been increased significantly in ischemia group (200 %) compared to control, while TFM treatment significantly ($p \leq 0.05$) reduced LDH secretion compared to ischemia group (TFM 10 µg/ml - 98%, TFM 25 µg/ml -156 % and TFM 50 µg/ml -174 %; Figure 5.3 B)

5.3.4.3 Morphology of H9c2 cells

Simulated ischemia for 1 hour caused a change in morphology of the H9c2 cells. The cells showed shrinkage, change in nuclear morphology and blebbing characteristic of apoptotic cell death. TFM pretreatment at concentration of 10, 25 and 50 µg/ml was able to prevent to some extent the changes in morphology produced by ischemia. (Figure 5.3C)

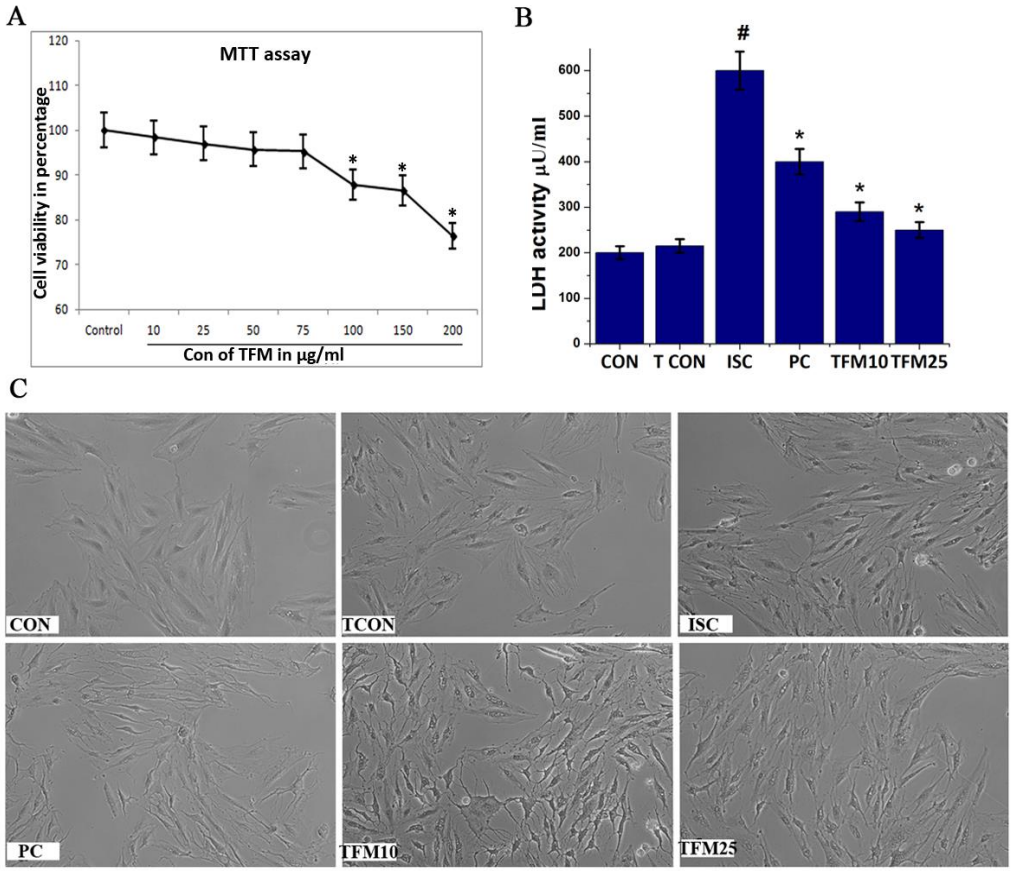


Figure 5.3 Analysis of toxicity, LDH release and morphology of H9c2 cells during ischemia *Tribulus terrestris* L. fruit methanol extract ameliorates ischemia-induced cell injury in H9c2 cells (A) Relative cell viability determined by MTT assay on pre-treatment with different concentrations of *Tribulus terrestris* L. fruit methanol extract (10, 25, 50, 75, 100, 150, 200 µg/ml). (B) LDH release in control, ischemia, positive control and cells treated with *Tribulus terrestris* L. fruit methanol extract (10, 25 µg/ml) for 24 hours prior to ischemia for 1 hour. All values are represented as mean ± SD, n = 6, # $p \leq 0.05$ Vs control, * $p \leq 0.05$ Vs ischemia (C) Morphological changes of H9c2 cells during ischemia: a. control, b. ischemia, c. treatment control, d. positive control and e,f. cells pre-treated with different concentrations of *Tribulus terrestris* L. fruit methanol extract (10, 25 µg/ml), 24 hours before induction of ischemia.

5.3.5 TFM reduced ROS

Imaging and fluorometry experiments revealed significant ($p \leq 0.05$) generation of oxidative stress in ischemic cells compared to control (Figure. 5.4 A, B, C). Fluorometry analysis exhibited considerable increase of fluorescence in ischemic cells compared to control (77 %). TFM at 10 and 25 µg/ml reduced ROS levels significantly ($p \leq 0.05$) compared to control (TFM 10 µg/ml- 55 %, TFM 25 µg/ml - 73 %, PC - 33 %). Flow cytometry data further confirmed this result (Figure. 5.4B).

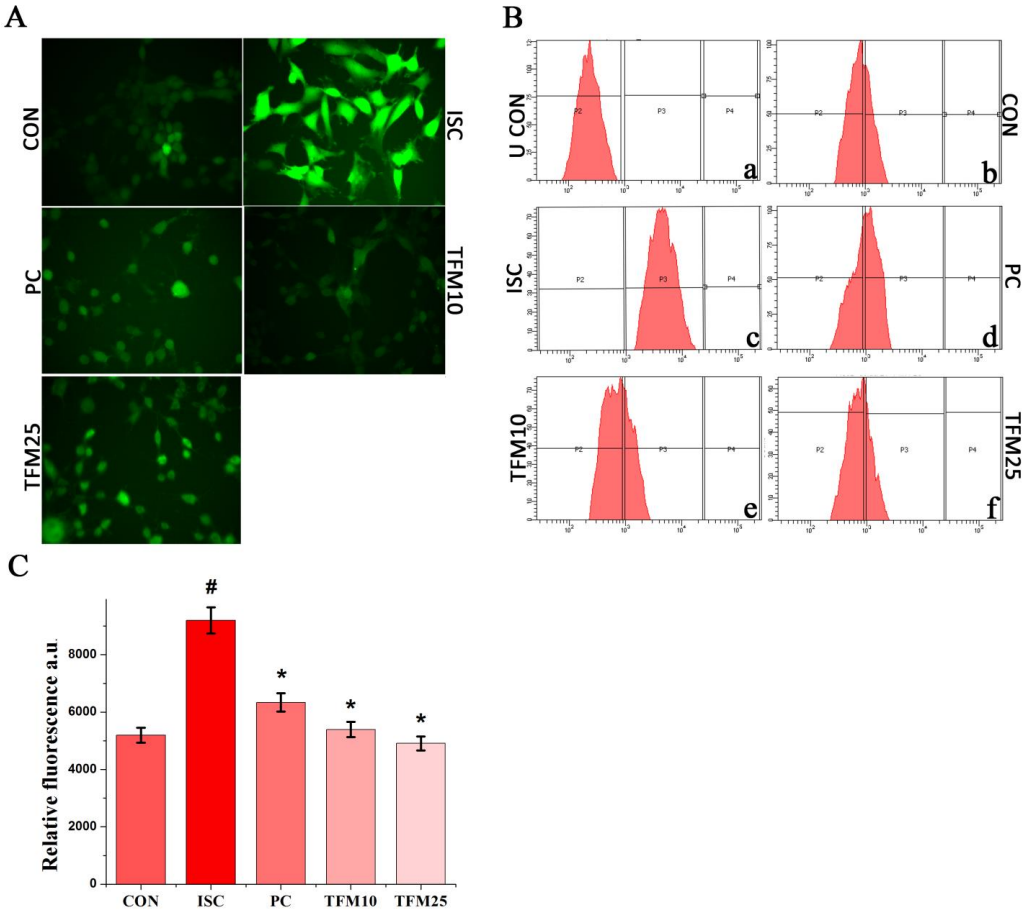


Figure 5.4 Reactive oxygen species production during ischemia A. Shown are fluorescent images of control, ischemia and cells treated with positive control and 10 and 25 µg/ml *Tribulus terrestris* L. fruit methanol extract respectively prior to ischemia. B. Flow cytometry analysis of intracellular reactive oxygen species generation measured using H_2DCFDA. Ischemia shows increased reactive oxygen species generation (population P2) which is prevented by pre-treatment with *Tribulus terrestris* L. fruit methanol extract (10, 25, µg/ml).C. Histograms showing relative fluorescence emitted by H9c2 cells in control, ischemia and cells pre-treated with positive control and 10 and 25 µg/ml *Tribulus terrestris* L. fruit methanol extract respectively prior to ischemia. All values are represented as mean ± SD, n = 6, # $p \leq 0.05$ Vs control, * $p \leq 0.05$ Vs ischemia

5.3.6 TFM restored activities of antioxidant enzymes

5.3.6.1 TFM restored activities of antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase

During severe oxidative stress, there is instability in the antioxidant defense system of the cell. Simulated ischemia for 1 hour caused a significant ($p \leq 0.05$) decrease in intracellular antioxidant enzymes. There was 88 % decrease in SOD activity with ischemia for 1 hr compared to control group and TFM pre-treatment significantly ($p \leq 0.05$) prevented depletion of SOD activity (TFM 10 µg/ml - 200%, TFM 25 µg/ml - 300 %, TFM 50 µg/ml - 400 % and PC - 425 %) (Figure 5.5 A). There was a significant ($p \leq 0.05$) decrease of 39 % in catalase activity during ischemia compared to control and pre - treatment with TFM prevented the decrease in catalase activity significantly ($p \leq 0.05$) (TFM 10 µg/ml – 12 %, TFM 25 µg/ml - 25 % , TFM 50 µg/ml - 40 % and PC - 48 %) (Figure 5.5 B). In the case of glutathione peroxidase enzyme also, there was a significant ($p \leq 0.05$) decrease in activity during ischemia (54 %) compared to control and TFM pre-treatment prevented the depletion caused by ischemia (TFM 10 µg/ml - 15 %, TFM 25 µg/ml - 40 %, TFM 50 µg/ml - 20 % and PC-50 %) (Figure 5.5 C).

5.3.6.2 TFM restored glutathione levels

Ischemia caused a significant ($p \leq 0.05$) decrease in intracellular glutathione content (56 %) compared to control. However, pre-treatment with TFM (10, 25, 50 µg/ml) prevented the depletion of GSH significantly ($p \leq 0.05$), during ischemia (TFM 10 µg/ml – 10 %, TFM 25 µg/ml – 50 %, TFM 50 µg/ml - 20 % and PC - 75 %) (Figure 5.5 D).

5.3.6.1 Total (Trolox equivalent) antioxidant capacity

Ischemia caused a significant ($p \leq 0.05$) decrease (90 %) in antioxidant capacity compared to control. Pre-treatment with TFM prevented the decrease in antioxidant capacity significantly ($p \leq 0.05$), compared to ischemia group (TFM 10 µg/ml – 200 %, TFM 25 µg/ml – 360 %, TFM 50 µg/ml - 600 % and PC – 500 %) (Figure 5.5 E).

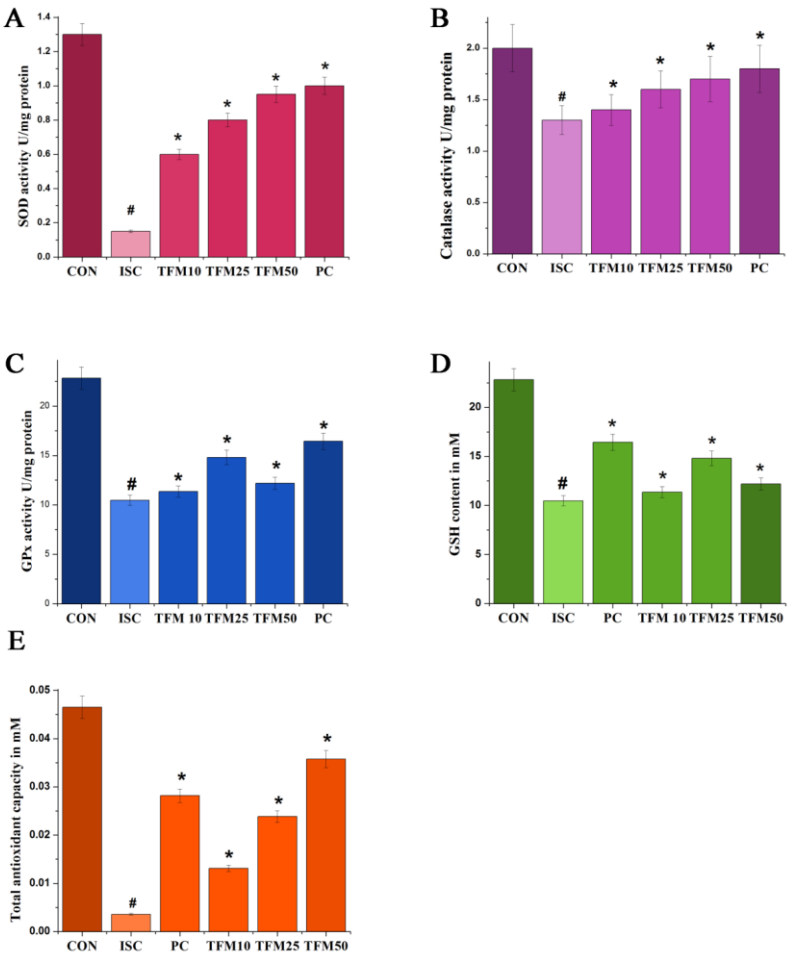


Figure 5.5 Reduction in anti-oxidant enzyme activity A. superoxide dismutase, B. catalase, C. glutathione peroxidase, D. reduced glutathione, E. total antioxidant capacity exhibited by H9c2 cells after ischemia and amelioration by treatment with *Tribulus terrestris* L. fruit methanol extract (10, 25, 50 µg/ml) and positive control (Cyclosporin A 0.1µM). Results are expressed as mean ± SD, n = 6, # $p \leq 0.05$ Vs control,* $p \leq 0.05$ Vs ischemia

5.3.7 TFM restored the activities of mitochondrial respiratory complexes

The activity of mitochondrial respiratory complexes, complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (coenzyme Q: cytochrome c - reductase) and complex 1V (cytochrome c oxidase) were significantly ($p \leq 0.05$) decreased in ischemia. The activities of complex I, II, III and IV were decreased by 44 %, 14 %, 91 % and 90 % respectively in ischemia compared to control. However, pre-treatment with TFM prevented the reduction in activities of these respiratory complexes significantly ($p \leq 0.05$) in H9c2 cells exposed to ischemia (Table 5.2).

Table 5.2 Effect of simulated ischemia on the activities of mitochondrial respiratory complexes

	Complex I (ΔOD 340nm)	Complex II (ΔOD 600nm)	Complex III (ΔOD 550nm)	Complex IV (ΔOD 550nm)
Control	0.320 ± 0.02	0.187037 ± 0.017	0.167 ± 0.01	7.102272 ± 0.66
T. control	0.315 ± 0.03	0.185026 ± 0.019	0.163 ± 0.01	7.242272 ± 0.62
Ischemia	0.180 ± 0.01#	0.15926 ± 0.007#	0.015 ± 0.001#	0.71022 ± 0.06#
Isc + PC	0.312 ± 0.29*	0.187037 ± 0.017*	0.029 ± 0.002*	0.71022 ± 0.06*
Isc + TFM 10	0.260 ± 0.24*	0.224074 ± 0.020*	0.031 ± 0.009*	1.065341±0.09*
Isc + TFM 25	0.321 ± 0.30*	0.19 ± 0.008*	0.041 ± 0.008*	1.4204 ± 0.13*

Effect of simulated ischemia on the activities of mitochondrial respiratory complexes and amelioration with *Tribulus terrestris* L. fruit methanol extract 10, 25 µg/ml and cyclosporin A (0.1 µM). Units: complex I- µmoles of DCIP reduced/ min/ mg protein; complex II - µmoles of DCIP reduced/ min/ mg protein; complex III - µmoles of ferricytochrome C reduced/ min/ mg protein; complex IV - µmoles of ferrocytochrome C oxidized/ min/ mg protein. Values are expressed as mean ± SD, (n = 6), # ($p \leq 0.05$) significant difference from the control group and,*($p \leq 0.05$) significant difference from ischemia.

5.3.8Assessment of mitochondrial membrane potential and integrity of permeability transition pore

There was a dissipation of trans-membrane potential during ischemia. Cells pre-treated with TFM, showed a significant protection against ischemia-induced mitochondrial transmembrane changes (Figure 5.6 A, B). The dissipation of transmembrane potential was visualized by JC-1 dye. JC-1 dye has the property of reversible formation of JC-1 aggregates upon membrane depolarization that causes shifts in emitted light from 530 nm (*i.e.*, emission of JC-1 monomeric form) to 590 nm (*i.e.*, emission of J-aggregate) when excited at 490 nm. The color of the dye changes reversibly from green to red as the mitochondrial membrane becomes depolarized. The image showed that JC1 monomers formed were significantly less and J aggregates were more in control cells, whereas in ischemia and valinomycin treated cells JC1 monomers were more and J aggregates were less (Figure 5.6). This indicates dissipation of transmembrane potential. Interestingly in cells pre-treated with TFM and positive control, the monomers formed were significantly less ($p \leq 0.05$) compared to ischemia cells, showing protection against ischemia-induced mitochondrial transmembrane changes (Figure 5.6 A, B).

Cells stained with calcein and $CoCl_2$ showed integrity of permeability transition pore. In normal cells calcein enter the mitochondria and appear as punctate. Ischemia caused a decompartmentalization of calcein fluorescence indicating mPTP opening. Pre-treatment with TFM (10 and 25 µg/ml) and PC showed no calcein decompartmentalization and the cells appeared with punctiform fluorescence similar to control. (Figure 5.6 C)

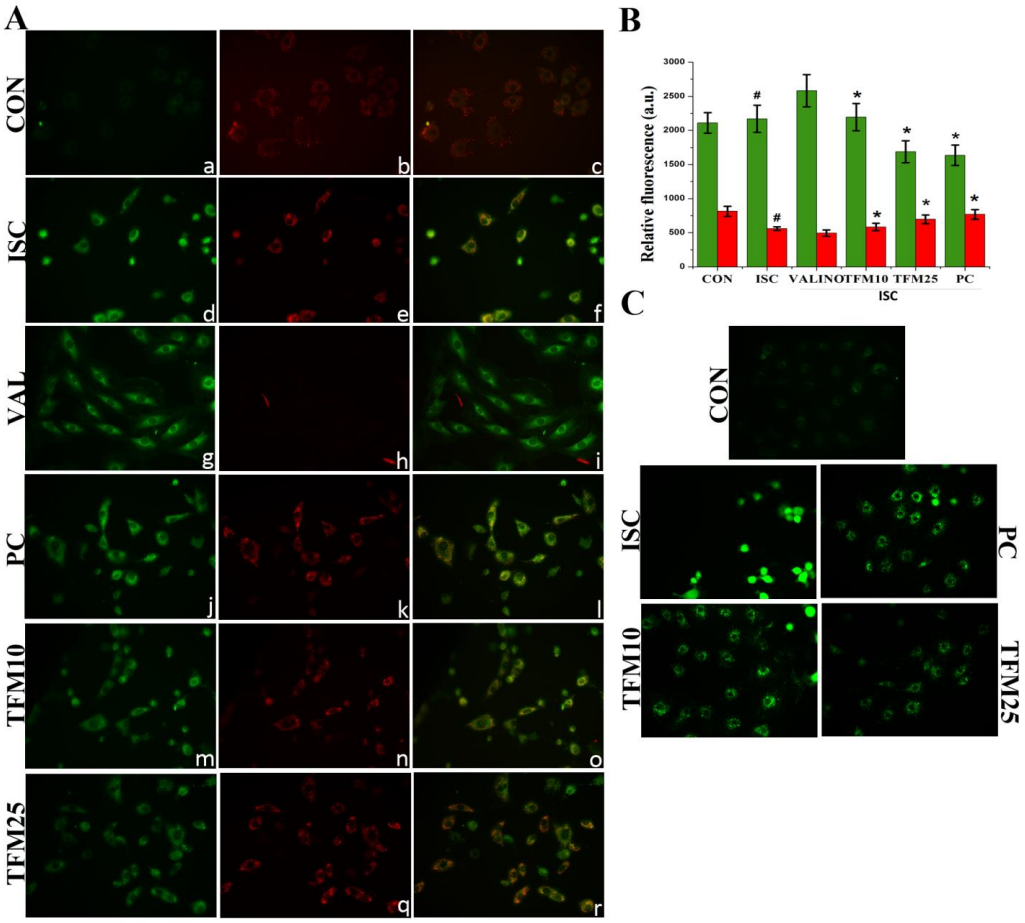


Figure 5.6 Change in mitochondrial transmembrane potential and alteration in integrity of permeability transition pore during ischemia. A. Images showing a, d, g, j, m, p JC1 monomers b, e, h, k, n, q J - aggregates and c, f, i, l, o, r overlay image of monomers and aggregates in control, ischemia, valinomycin treated and cells pre-treated with positive control and 10 and 25 µg/ml *Tribulus terrestris* L. fruit methanol extract prior to ischemia. B. Histogram showing JC1 monomers and J - aggregates of control, ischemia, valinomycin and cells pre-treated with positive control and *Tribulus terrestris* L. fruit methanol extract (10 and 25 µg/ml) 24 hours before the induction of ischemia, n = 6, Values are represented as mean ± SD, # $p \leq 0.05$ Vs control, * $p \leq 0.05$ Vs ischemia C. *Tribulus terrestris* L. fruit methanol extract attenuates permeability transition pore opening in H9c2 cells subjected to ischemia. mPTP opening was visualized using calcein - $CoCl_2$ staining. The following groups were present control, ischemia, cells pre - treated with PC and 10 and 25 µg/ml *Tribulus terrestris* L. fruit methanol extract prior to ischemia.

5.3.9 Oxygen consumption, ATP production and expression of HIF-1α

Ischemia caused a significant ($p \leq 0.05$; 86.6 %) reduction in oxygen consumption rate compared to control (Figure 5.7 A). TFM and positive control pre-treatment prevented the reduction in oxygen consumption significantly ($p \leq 0.05$; 250 % and 200 % with TFM 10 and 25 µg/ml respectively and 350 % with PC; Figure 5.7 A). ATP levels were reduced significantly ($p \leq 0.05$) in ischemia by 18 % and TFM and positive control pre-treatment prevented the reduction in ATP content (11 % and 15 % with TFM 10 and 25 µg/ml respectively and 8 % with PC) (Figure 5.7 B). HIF-1α expression was up-regulated significantly ($p \leq 0.05$), 15 % during ischemia and TFM pre-treatment and positive control prevented the increase in expression of HIF-1α by 5 % and 13 % with TFM 10 and 25 µg/ml respectively and 17 % with positive control.

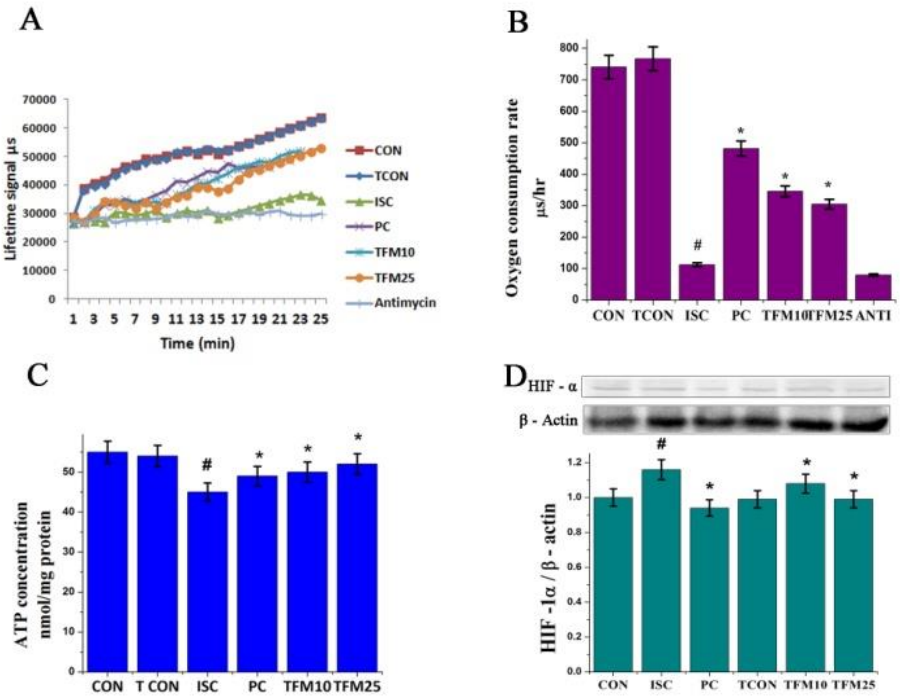


Figure 5.7 Measurement of oxygen consumption rate, ATP content and expression of HIF-1α in ischemia (A, B) Oxygen consumption rate was measured in control, treatment control, ischemia and after pre-treatment with positive control and *Tribulus terrestris* L. fruit methanol extract 10, 25 µg/ml and antimycin prior to ischemia. (C) ATP content was measured in control, treatment control, and ischemia and after pre-treatment with cyclosporin A and *Tribulus terrestris* L. fruit methanol extract 10, 25 µg/ml prior to ischemia. (D) Immunoblotting and densitometric quantification of HIF-1α expression relative to β – actin, n = 6, Values are represented as mean ± SD, # $p \leq 0.05$ Vs control, * $p \leq 0.05$ Vs ischemia.

5.3.10 Regulation of mitochondrial genes expression of fission and fusion proteins by TFM

Ischemia significantly ($p \leq 0.05$) upregulated expression of genes involved in mitochondrial fission and down regulated the expression of genes involved in mitochondrial fusion. Drp1 and Fis1 mRNA was upregulated by 0.94 fold and 0.6 fold in ischemia. TFM pre-treatment significantly ($p \leq 0.05$) prevented the upregulation of mitochondrial fission genes (Drp1 expression was decreased by 0.8 F, 0.9 F and 0.45 F and Fis1 expression was decreased by 0.7 F. 0.8 F and 0.4 F for TFM 10, 25 µg/ml and positive control respectively) (Figure 5.8 A). OPA1, Mfn1 and Mfn2 (mitochondrial fusion proteins) mRNA expression was down regulated by 0.75, 0.98 and 0.97 fold respectively in ischemia compared to control. TFM and positive control pre-treatment significantly ($p \leq 0.05$) prevented the downregulation of these genes (Mfn1 expression was decreased by 7 F, 3 F and 3F, Mfn2 expression was decreased by 1 F, 1 F and 13 F, OPA1 expression was decreased by 0.75 F, 3.25 F and 1F for TFM 10, 25µg/ml and positive control respectively) (Figure 5.8 B).

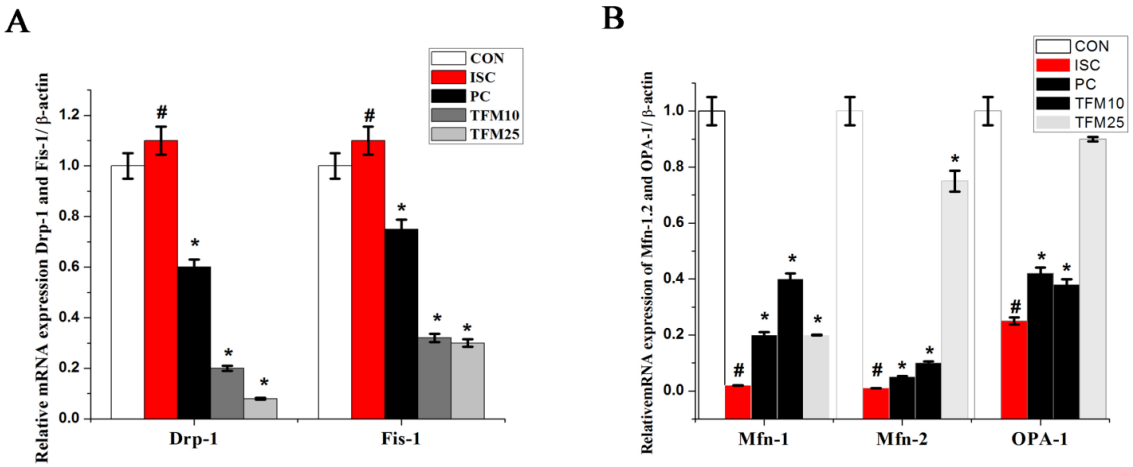


Figure 5.8 Relative mRNA expression of mitochondrial fission and fusion proteins by qRT PCR. Ischemia caused a change in relative mRNA expression of mitochondrial fission and fusion proteins. A. Shown are representative mRNA relative expression profile of mitochondrial fission proteins Drp1 and Fis1 in control, treatment control, ischemia and after pretreatment with positive control and *Tribulus terrestris* L. fruit methanol extract 10, 25 µg/ml prior to ischemia. B. Shown are representative mRNA relative expression profile of mitochondrial fusion proteins Mfn1, Mfn2 and OPA-1 in control, treatment control, ischemia and after pretreatment with positive control and *Tribulus terrestris* L. fruit methanol extract 10, 25 µg/ml prior to ischemia. Results shown are mean ± SD of six independent experiments. # $p \leq 0.05$ relative to control,* $p \leq 0.05$ relative to ischemia group.

5.3.11 Effect of TFM on the expression of mitochondrial proteins

Ischemia significantly ($p \leq 0.05$) up regulated the expression of genes involved in mitochondrial fission and down regulated the expression of genes involved in mitochondrial fusion. Ischemia caused an upregulation of fission proteins, Drp1 (0.2 fold) and Fis1 (0.92 fold) and TFM and positive control pre-treatment prevented significantly ($p \leq 0.05$) the upregulation. Drp1 expression decreased by 0.2, 0.4 and 0.25 fold, while Fis1 expression was decreased 0.3, 0.81 and 0.22 fold for TFM 10, 25 µg/ml and positive control respectively (Figure 5.9 A, C). Ischemia caused a downregulation of fusion proteins Mfn2 (0.18 fold) and OPA1 (0.2 fold) and TFM and positive control pre-treatment prevented significantly ($p \leq 0.05$) the decrease in expression caused by ischemia (Mfn2 expression decreased by 0.62 fold, 0.12 fold and 0.37 F and OPA1 expression decreased by 0.12 F, 0.18 F and 0.125 F for TFM 10, 25 µg/ml and positive control pre-treatment respectively) compared to ischemia.

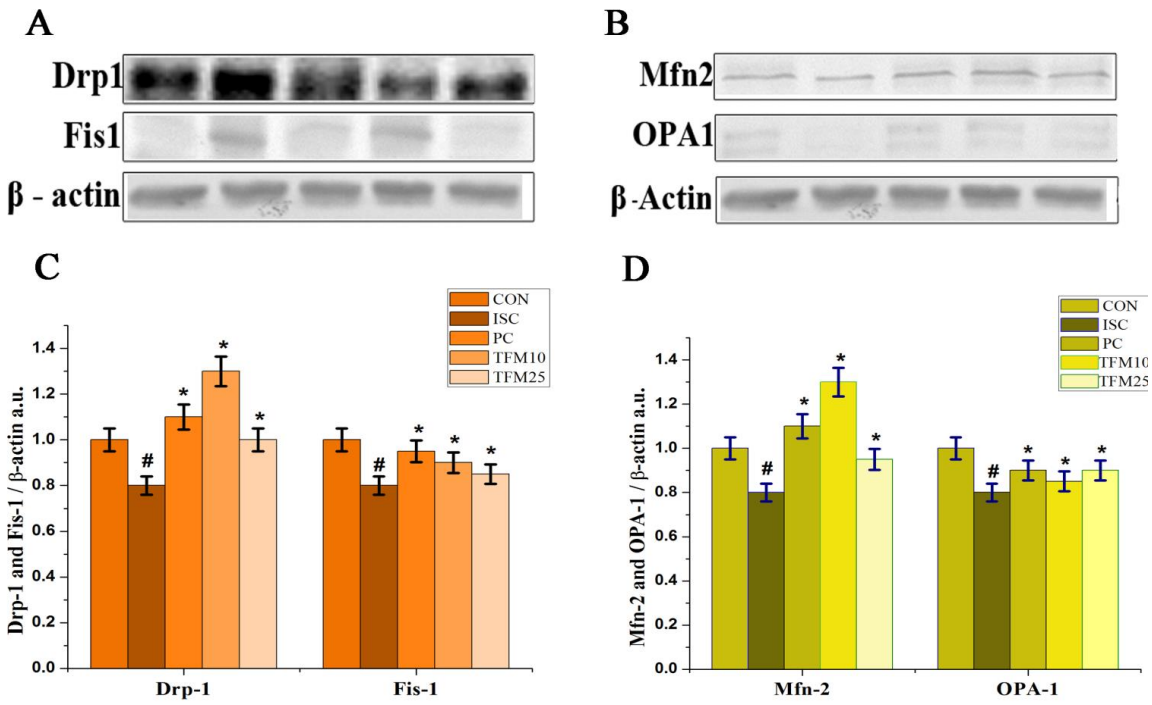


Figure 5.9 Expression of mitochondrial fission and fusion proteins. Ischemia caused a change in protein expression of mitochondrial fission and fusion proteins. A. Shown are representative immunoblot of mitochondrial fission proteins Drp1 and Fis1 of control, treatment control, ischemia and after pretreatment with positive control and *Tribulus terrestris* L. fruit methanol extract 10, 25 µg/ml prior to ischemia. B. Densitometric quantification of mitochondrial fission proteins Drp1 and Fis1 relative to β - actin. C. Shown are representative immunoblot of mitochondrial fusion proteins Mfn2 and OPA-1 of control, treatment control, ischemia and after pretreatment with positive control

and *Tribulus terrestris* L. fruit methanol extract 10, 25 µg/ml prior to ischemia. D. Densitometric quantification of mitochondrial fusion proteins Mfn2 and OPA-1 relative to β - actin. Results shown are mean ± SD of six independent experiments. # $p \leq 0.05$ relative to control,* $p \leq 0.05$ relative to ischemia group.

5.4. Discussion

The mitochondria are a vital component of cellular energy metabolism and intracellular signaling processes. Mitochondria have a role as a "friend and foe" (Camara et al., 2011). Under normal conditions, mitochondria exist in a metabolic and cellular ion homeostatic state that is crucial for normal myocardial function. Deviation from the physiological condition, that occurs in ischemia, can result in abnormally high mitochondrial calcium, increased oxidative stress, damage to mitochondrial membranes, enzymes, ETC components that causes impaired mitochondrial ATP production, which facilitates mPTP opening (Waldmeier et al., 2003) that leads to cellular apoptosis and necrosis. Thus, mitochondrial dysfunction and the resulting oxidative stress are central in the pathogenesis of several maladies including cardiac ischemic injury. This chapter focuses on understanding the role of mitochondria in the etiology and progress of cardiac ischemia and potential therapeutic benefits of targeting the organelle in attenuating cardiac ischemia. The *Tribulus terrestris* L. fruits have been reported to be protective against nephro-toxicity, diabetic neuropathy, spermatogenesis defects (Kumari and Singh, 2015; Raoofi et al., 2015). This chapter explores whether the administration of TFM can protect the mitochondria from ischemic injury and prevent the deleterious effects of ischemia.

In this study, ischemia has been induced in cell culture by exposing the cells to ischemic solution. It is reported that ischemia produce ketosis, acidosis and depletion of ATP in the tissue (Lu et al., 2005). H9c2 cells are rat embryonic myoblast cells and retain several elements of the electrical and hormonal signaling pathway of cardiac cells and have therefore become widely accepted *in vitro* model to study the ischemia of the heart (Kuznetsov et al., 2015). They are highly sensitive to hypoxia-reoxygenation injury in terms of loss of cell viability and mitochondrial respiration and thus the efficacy of TFM to protect against ischemia was checked in this cell line. LDH release to the medium, during ischemia, revealed alterations in the integrity of the plasma membrane. Pretreatment with TFM significantly suppressed LDH release in the cells subjected to ischemia (Figure 5.3). It is

reported that diosgenin pre-administration reduced LDH levels (Badalzadeh et al., 2014; Jastroch et al., 2010). The reduction in LDH levels on treatment with TFM may be due to the presence of diosgenin in TFM.

The heart needs oxygen avidly and, although it has powerful defense mechanisms, it is susceptible to oxidative stress. Reactive oxygen species are produced by oxidative phosphorylation and maintained in permissible levels by antioxidant enzymes. The cell has an innate antioxidant system including enzymes like manganese superoxide dismutase (Mn SOD), thioredoxin reductase, glutathione peroxidase and catalase which inhibit the formation of ROS or enhance endogenous antioxidant system (Stowe and Camara, 2009). Mn SOD catalyses the reduction of superoxide radicals to H_2O_2 (Ohta et al., 1994). Glutathione peroxidase acts as the H_2O_2 scavenger of the heart. It catalyses the per oxidation of H_2O_2 in the presence of reduced glutathione (GSH) to form H_2O and oxidized glutathione GSSG (Paglia and Valentine, 1967). The GSSG recycles back to give GSH by glutathione reductase, which requires NADPH (Carlberg and Mannervik, 1985). The redox homeostasis of the mitochondria is thus maintained by GSH. Catalase and glutathione peroxidase convert H_2O_2 to H_2O. Glutathione peroxidase is present in mitochondrial matrix and is very essential in protecting the myocardium from ischemia and enhancing the activity of glutathione peroxidase is effective in protecting the heart (Werns et al., 1992). In the present study, innate antioxidant system of heart myoblast was found significantly depleted (Table 5.1). However, TFM administration for 24 hours prior to the onset of ischemia was found to significantly prevent the depletion of antioxidant enzymes that occurred during ischemia (Table 5.1) similar to other reports in animal study (Sailaja et al., 2013).

Cardiomyocytes, depend entirely on oxidative phosphorylation (OXPHOS) for their function. Therefore, structural preservation of the ETC complexes is paramount in maintaining a high bioenergetic status to provide efficient cellular homeostasis with minimal ROS production. In ischemia oxygen delivery to the mitochondria is not sufficient to meet the need of substrate oxidation leaving the ETC in a reduced state. Increased electron leakage from ETC oxidizes O_2 to O_2- and subsequent production of H_2O_2 and OH^-. Complex I (NADH: ubiquinone oxidoreductase) is a major contributor of mitochondrial ROS metabolism in cells (Jastroch et al., 2010). Electrons are transferred from NADH and $FADH_2$

from complex I and II, respectively, to downstream redox centers. ROS from complex I are proposed to mediate the physiological effects related to cardiac signaling. A decrease in complex I activity observed during I/R is associated with decrease in state 3 respiration, protein nitration and oxidative damage to mitochondrial proteins and phospholipids (Paradies et al., 2004). Complex II (succinate ubiquinone reductase) is involved in the oxidation of succinate to fumarate and thereby reducing ubiquinone in the inner membrane space (Chen et al., 2007). To date, there is no evidence indicating that complex II–mediated ROS production is involved in cardiac signaling. Complex III (coenzyme Q : cytochrome c - oxidoreductase) is one of the major sources for mitochondrial $\cdot O_2$- production in post ischemic heart. Oxidative damage to complex I and II can weaken the interaction with complex III thereby increasing $\cdot O_2$- production (Lee et al., 2012). A significant decrease in complex IV activity has been monitored in the mitochondria of ischemic hearts (Lee et al., 2012; Prabu et al., 2006) mainly by the hyper phosphorylation of complex IV by protein kinase A (PKA). A previous study have inferred that a specific region in ETC, most likely between complex III and IV is liable to get damaged during ischemia. Therefore preserving the activity of complex III and IV may have protective effects against ischemia. In the present study also complexes have shown significant alterations in their activities during ischemia and TFM was effective in preventing the decrease in activity of these respiratory chain complexes (Table 5.2). Interestingly, it has been reported that ferulic acid ameliorates mitochondrial dysfunction in the rat brain by altering the activity of ETC complexes (Kumar et al., 2014) and the same is expected to have role in the beneficial action of TFM against altered ETC complexes during ischemic insult.

During ischemia the increase in mitochondrial calcium can impair ATP synthesis and lead to a loss of ion homeostasis, stimulation of ROS generation, mPTP opening, matrix swelling, and outer mitochondrial membrane (OMM) rupture (Perrelli et al., 2011). Cumulatively, these factors results in collapse of $\Delta\Psi m$, release of cytochrome c and finally cell death. Simulated ischemia, in this study, caused dissipation in mitochondrial transmembrane potential and TFM treatment was able to prevent the change caused in transmembrane potential. The mPTP is a highly dynamic, nonselective pore which spans the inner mitochondrial membrane (IMM) and outer mitochondrial membrane (Higgins and

Coughlan, 2014). Pathologic opening of the pore during ischemia is triggered by excess mitochondrial calcium, increased ROS production, increased Pi and Mg^{2+} (Adam-Vizi and Starkov, 2010). Pretreatment of a therapeutic agent before ischemia can prevent mPTP opening by preserving integrity of outer mitochondrial membrane (OMM), preventing cytochrome c release and attenuating the production of oxidative stress similar to ischemia preconditioning. In our study, TFM attenuated the mPTP opening occurring during ischemia thereby preserving the integrity of the mitochondria.

Mitochondria are never static; they continuously change their shape by the process of fission, fusion and movement (Dorn, 2013). Fission and fusion are quality control mechanisms by which mitochondria repair damage (Ni et al., 2015). Mitochondrial fission and fusion are regulated by dynamin Drp1, Fis1, Mfn1, 2 and OPA1. Mitochondrial fusion is promoted by assembly of proteins Mfn1 and Mfn2 at the outer mitochondrial membrane (OMM) and OPA1 at the inner mitochondrial membrane, thereby fusion of juxtaposed mitochondria (Suen et al., 2008). Fission is promoted by recruitment of Drp1 and its interaction with receptor Fis1 (Ni et al., 2015). These proteins (Drp1, Fis1, Mfn1, 2 and OPA1) are not only involved in maintaining mitochondrial dynamics, they are also involved in oxidative phosphorylation. In the present study also there was a disruption of fission, fusion balance i.e. there is increased fission and decreased fusion during ischemia and this disruption in balance was ameliorated by TFM pretreatment. (Figure 5 .8, 5.9)

Tribulus terrestris L. is reported beneficial against defective oxidative phosphorylation and ATP production during mitochondrial damage with high energy diet (Berkman et al., 2009). In addition, stabilization of mitochondrial transmembrane potential and amelioration of mitochondrial mediated apoptosis has been seen in rat (Liu et al., 2008). Moreover, ferulic acid is found to be beneficial against oxidative stress and mitochondrial dysfunction during isoproterenol induced cardiac dysfunction (Yogeeta et al., 2006) .Besides, phloridzin is found to be beneficial against mitochondrial mediated apoptosis. In addition, diosgenin is effective against cardiac disorder via mitochondrial K^+ ATP channel modulation (Badalzadeh et al., 2015). From this data, it is possible to postulate that these compounds and other bioactives present contribute for the beneficial effect of TFM. Finally we conclude that mitochondria is potential drug target for future research on cardiac ischemia and the potent

"mitochondriotropic" property of the constituents of *Tribulus terrestris* L. fruits is the main scientific basis of its use in traditional medicine for heart ailments.

References

Adam-Vizi V and Starkov AA (2010) Calcium and mitochondrial reactive oxygen species generation: how to read the facts. *Journal of Alzheimer's Disease* 2: 413-426.

Badalzadeh R, Yavari R and Chalabiani D (2015) Mitochondrial ATP-sensitive K+ channels mediate the antioxidative influence of diosgenin on myocardial reperfusion injury in rat hearts. *General Physiology and Biophysics* 34: 323-329.

Badalzadeh R, Yousefi B, Majidinia M and Ebrahimi H (2014) Anti-arrhythmic effect of diosgenin in reperfusion-induced myocardial injury in a rat model: activation of nitric oxide system and mitochondrial KATP channel. *The Journal of Physiological Sciences* 64: 393-400.

Ballinger SW (2005) Mitochondrial dysfunction in cardiovascular disease. *Free Radical Biology & Medicine* 38: 1278-1295.

Berkman Z, Tanriover G, Acar G, Sati L, Altug T and Demir R (2009) Changes in the brain cortex of rabbits on a cholesterol-rich diet following supplementation with a herbal extract of Tribulus terrestris. *Histology and Histopathology* 24: 683-692.

Camara AK, Bienengraeber M and Stowe DF (2011) Mitochondrial approaches to protect against cardiac ischemia and reperfusion injury. *Frontiers in Physiology* 2: 13-18.

Carlberg I and Mannervik B (1985) Glutathione reductase. *Methods in Enzymology* 113: 484-490.

Chen YR, Chen CL, Pfeiffer DR and Zweier JL (2007) Mitochondrial complex II in the post-ischemic heart: oxidative injury and the role of protein S-glutathionylation. *The Journal of Biological Chemistry* 282: 32640-32654.

Dhalla NS, Temsah RM and Netticadan T (2000) Role of oxidative stress in cardiovascular diseases. *Journal of Hypertension* 18: 655-673.

Dorn GW, 2nd (2013) Mitochondrial dynamics in heart disease. *Biochimica et Biophysica Acta* 1833: 233-241.

Higgins GC and Coughlan MT (2014) Mitochondrial dysfunction and mitophagy: the beginning and end to diabetic nephropathy? *British Journal of Pharmacology* 171: 1917-1942.

Ikeda Y, Sciarretta S, Nagarajan N, Rubattu S, Volpe M, Frati G and Sadoshima J (2014) New insights into the role of mitochondrial dynamics and autophagy during oxidative stress and aging in the heart. *Oxidative Medicine and Cellular Longevity* 2014: 934-947.

Jastroch M, Divakaruni AS, Mookerjee S, Treberg JR and Brand MD (2010) Mitochondrial proton and electron leaks. *Essays in Biochemistry* 47: 53-67.

Jones SP, Hoffmeyer MR, Sharp BR, Ho YS and Lefer DJ (2003) Role of intracellular antioxidant enzymes after in vivo myocardial ischemia and reperfusion. *American Journal of Physiology Heart and Circulatory Physiology* 284: 277-282.

Kadenbach B (2003) Intrinsic and extrinsic uncoupling of oxidative phosphorylation. *Biochimica et Biophysica Acta* 1604: 77-94.

Kumari M and Singh P (2015) *Tribulus terrestris* ameliorates metronidazole-induced spermatogenic inhibition and testicular oxidative stress in the laboratory mouse. *Indian Journal of Pharmacology* 47: 304-310.

Kuznetsov AV, Javadov S, Sickinger S, Frotschnig S and Grimm M (2015) H9c2 and HL-1 cells demonstrate distinct features of energy metabolism, mitochondrial function and sensitivity to hypoxia-reoxygenation. *Biochimica et Biophysica Acta* 1853: 276-284.

Lee HL, Chen CL, Yeh ST, Zweier JL and Chen YR (2012) Biphasic modulation of the mitochondrial electron transport chain in myocardial ischemia and reperfusion. *American Journal of Physiology Heart and Circulatory Physiology* 302: 1410-1422.

Liu XM, Huang QF, Zhang YL, Lou JL, Liu HS and Zheng H (2008) Effects of *Tribulus terrestris* L. saponion on apoptosis of cortical neurons induced by hypoxia-reoxygenation in rats. *Journal of Chinese Integrative Medicine* 6: 45-50.

Lodish H, Berk A, Zipursky S L (2000) Molecular Cell Biology., in *Electron Transport and Oxidative Phosphorylation* (Freeman WH ed), New York.

Lu J, Zang WJ, Yu XJ, Chen LN, Zhang CH and Jia B (2005) Effects of ischaemia-mimetic factors on isolated rat ventricular myocytes. *Experimental Physiology* 90: 497-505.

Ni HM, Williams JA and Ding WX (2015) Mitochondrial dynamics and mitochondrial quality control. *Redox Biology* 4: 6-13.

Ohta H, Adachi T and Hirano K (1994) Internalization of human extracellular-superoxide dismutase by bovine aortic endothelial cells. *Free Radical Biology & Medicine* 16: 501-507.

Paglia DE and Valentine WN (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *The Journal of Laboratory and Clinical Medicine* 70: 158-169.

Paradies G, Petrosillo G, Pistolese M, Di Venosa N, Federici A and Ruggiero FM (2004) Decrease in mitochondrial complex I activity in ischemic/reperfused rat heart: involvement of reactive oxygen species and cardiolipin. *Circulation Research* 94: 53-59.

Perrelli MG, Pagliaro P and Penna C (2011) Ischemia/ reperfusion injury and cardioprotective mechanisms: Role of mitochondria and reactive oxygen species. *World Journal of Cardiology* 3: 186-200.

Pham T, Loiselle D, Power A and Hickey AJ (2014) Mitochondrial inefficiencies and anoxic ATP hydrolysis capacities in diabetic rat heart. *American Journal of Physiology Cell Physiology* 307: 499-507.

Prabu SK, Anandatheerthavarada HK, Raza H, Srinivasan S, Spear JF and Avadhani NG (2006) Protein kinase A-mediated phosphorylation modulates cytochrome c oxidase function and augments hypoxia and myocardial ischemia-related injury. *The Journal of Biological Chemistry* 281: 2061-2070.

Rahal A, Kumar A, Singh V, Yadav B, Tiwari R, Chakraborty S and Dhama K (2014) Oxidative stress, prooxidants, and antioxidants: the interplay. *BioMed Research International* 2014: 1-19.

Raoofi A, Khazaei M and Ghanbari A (2015) Protective effect of hydroalcoholic extract of *Tribulus terrestris* on Cisplatin induced renal tissue damage in male mice. *International Journal of Preventive Medicine* 6: 11-15.

Roth E, Hejjel L, Jaberansari M and Jancso G (2004) The role of free radicals in endogenous adaptation and intracellular signals. *Experimental and Clinical Cardiology* 9: 13-16.

Sailaja KV, Shivaranjani VL, Poornima H, Rahamathulla SB and Devi KL (2013) Protective effect of *Tribulus terrestris* L. fruit aqueous extracton lipid profile and oxidative stress

in isoproterenol induced myocardial necrosis in male albino wistar rats. *EXCLI Journal* 12: 373-383.

Stowe DF and Camara AK (2009) Mitochondrial reactive oxygen species production in excitable cells: modulators of mitochondrial and cell function. *Antioxidants & Redox Signaling* 11: 1373-1414.

Suen DF, Norris KL and Youle RJ (2008) Mitochondrial dynamics and apoptosis. *Genes & Development* 22: 1577-1590.

Teshima Y, Akao M, Li RA, Chong TH, Baumgartner WA, Johnston MV and Marban E (2003) Mitochondrial ATP-sensitive potassium channel activation protects cerebellar granule neurons from apoptosis induced by oxidative stress. *Stroke* 34: 1796-1802.

Waldmeier PC, Zimmermann K, Qian T, Tintelnot-Blomley M and Lemasters JJ (2003) Cyclophilin D as a drug target. *Current Medicinal Chemistry* 10: 1485-1506.

Werns SW, Fantone JC, Ventura A and Lucchesi BR (1992) Myocardial glutathione depletion impairs recovery of isolated blood-perfused hearts after global ischaemia. *Journal of Molecular and Cellular Cardiology* 24: 1215-1220.

Yogeeta SK, Raghavendran HR, Gnanapragasam A, Subhashini R and Devaki T (2006) Ferulic acid with ascorbic acid synergistically extenuates the mitochondrial dysfunction during beta-adrenergic catecholamine induced cardiotoxicity in rats. *Chemico-Biological Interactions* 163: 160-169.

Zhao Q, Wang S, Li Y, Wang P, Li S, Guo Y and Yao R (2013) The role of the mitochondrial calcium uniporter in cerebral ischemia/reperfusion injury in rats involves regulation of mitochondrial energy metabolism. *Molecular Medicine Reports* 7: 1073-1080.

Isoproterenol induced myocardial dysfunctions in rats and amelioration with *Tribulus terrestris* L. fruit methanol extract (TFM)

6.1 Overview of Chapter 5

In the previous chapter, *T. terrestris* fruit methanol extract (TFM) was found effective against antioxidant and mitochondrial alterations in ischemia. The partial characterization of TFM revealed the presence of bioactives like ferulic acid, phloridzin and diosgenin. This prompted for further exploration of the protective property of TFM in an *in vivo* model of myocardial infarction using isoproterenol with special emphasis on mitochondrial redox regulation, mitochondrial biogenesis, cardiac specific MAPK, TNNI3K and cardiac remodeling

6.2 Introduction

Myocardial infarction is the leading cause of death worldwide. During myocardial infarction the adrenergic receptors of the heart are stimulated by catecholamines. Catecholamines at low concentration are beneficial in regulating heart function by exerting a positive inotropic action on the myocardium, whereas high concentration of catecholamines or chronic exposure produces deleterious effects on the cardiovascular system. The mechanism by which isoproterenol exerts its deleterious effect is relative cardiac hypoxemia due to increased cardiac work and myocardial oxygen demand, coronary artery vasoconstriction causing ischemia, hypoxia, interference with mitochondrial oxidative phosphorylation by free fatty acids, intracellular calcium overload, potassium and magnesium ion depletion and ATP depletion.

Mitochondria are key organelle in the cardiovascular system and agents that maintain mitochondrial integrity have been used successfully to treat cardiovascular dysfunctions. Carvedilol has been demonstrated to have additional effects independent of blocking the beta-adrenergic receptor and has been shown to directly protect against mitochondrial permeability transition by reducing oxidative stress (Kametani et al., 2006; Oliveira et al., 2004). As highly metabolic cells, cardiomyocytes maintain a high cellular store of phosphocreatine and adenosine triphosphate (ATP), which is required for continuous cardiac function. The large-

scale process of generating ATP leads to the production of ROS and reactive nitrogen species ROS/ RNS by the mitochondrial electron transport chain. Under physiological conditions the ROS are removed by the innate antioxidant system. But, under stress conditions H_2O_2 in concert with endogenous production of carbon monoxide (CO) and nitric oxide (NO) serve as important redox signals for anti-oxidant protection and for the cellular repair mechanisms. The adaptive mechanism in response to oxidative stress is the activation of the antioxidant response element (ARE) transcriptional pathway (Itoh et al., 1997). In the nucleus, Nrf2 binds to ARE promoter regions of genes that encode antioxidant enzymes such as superoxide dismutase (SOD), cytoprotective enzymes such as heme oxygenase-1 (HO-1), and genes for signaling proteins required for mitochondrial biogenesis such as Nuclear Respiratory Factor-1 (Nrf1) and mitochondrial transcription factor A (Tfam) (Favreau and Pickett, 1995; Jaloszynski et al., 2007; Rushmore et al., 1991). The cytoprotective responses that have evolved to allow heart cells to respond, survive, and adapt to oxidative and nitrosative stress involve several novel, redox-based, signaling mechanisms. This adaptive mechanism along with mitophagy protect and preserves a well-functioning population of mitochondria and allows the cell to reduce the ongoing contribution of damaged mitochondria to overall cellular oxidative stress.

Cardiac specific MAPK, cardiac troponin I-interacting kinase (TNNI3K) is a novel MAPK that is upregulated during ischemia, myocardial infarction and cardiac hypertrophy. TNNI3K is cardiac specific and expressed only in the cardiomyocytes and not in fibroblasts and endothelial cells. So, inhibitors of this MAPK are effective in protecting cardiomyocytes from oxidative stress induced damage. Histological changes in isoproterenol induced cardiomyopathy include degeneration and necrosis, accumulation of inflammatory cells such as leucocytes, interstitial edema, lipid droplet accumulation and endocardial hemorrhage. Agents that protect the heart against these changes are effective against isoproterenol induced myocardial infarction.

6.3 Methods

Male wistar rats weighing (190-220g) was used for the experiment. Rats were divided into six groups of six rats each.

Group I - vehicle control (saline)

Group II - treatment control (TFM (500 mg/kg) to check the toxicity of the extract)

Group III - isoproterenol induced myocardial infarction (85 mg/kg s.c.)

Group IV - TFM (250 mg/kg body weight) (pre-treatment) + isoproterenol

Group V - TFM (500 mg/kg body weight) (pre-treatment) + isoproterenol

Group VI - Propranolol (15 mg/kg body weight) (pre-treatment) + isoproterenol

In this experiment TFM and propranolol were administered as pre-treatment for 21 days before the induction of myocardial infarction by isoproterenol. TFM (250 mg/kg body weight and 500 mg/kg body weight) and propranolol (15 mg/kg body weight) was administered orally to the rats for 21 days (pre-treatment). On the 22^{nd} and 23^{rd} day animals of these group were administered isoproterenol subcutaneously (85 mg/kg body weight). We followed this regime to check the efficacy of the test material as a preventive agent against myocardial infarction. At the end of the experiment, rats were anaesthetized by subcutaneous administration of ketamine (65 mg/kg) and xylazine (5 mg/kg) and ECG was performed. Then rats were sacrificed by cervical dislocation under deep ether anesthesia and blood samples and hearts were collected in pre-cooled containers for estimation of various biochemical parameters and histopathological analysis. Detailed analytical procedures are given in chapter 2.

Various parameters studied include

- Redox regulation pathway (Nrf2, HO-1)
- Nitric oxide synthase (NOS-2)
- Mitochondrial biogenesis (Nrf1 and Tfam)
- Cardiac TNNI3K expression

6.4 Results

6.4.1 Nrf-2 and HO-1 expression in myocardial infarction

There was a significant ($p \leq 0.05$), 10 % decrease in Nuclear factor - 2 (Nrf2) expression, in the nuclear fraction of the infarcted hearts, indicating reduced translocation of Nrf2 to the nucleus (Figure 6.1 A, B). TFM pre-treatment (250 mg/kg and 500 mg/kg) and positive control treatment increased the translocation of Nrf2 to the nucleus by 50 %, 45 % and 40 % respectively, when compared to isoproterenol group (Figure 6.1 A, B). Thus there is evidence for increased activation and translocation of Nrf2 to the nucleus after TFM treatment. Hemoxygenase -1 (HO - 1), a downstream effector of Nrf2 activation was also downregulated significantly ($p \leq 0.05$) (10 %) following isoproterenol treatment and TFM pre-treatment prevented the downregulation of HO -1 by 22.2 %, 21.9 % for TFM 250 and 500 mg/kg body weight respectively and 22 % for positive control (Figure 6.1 C, D).

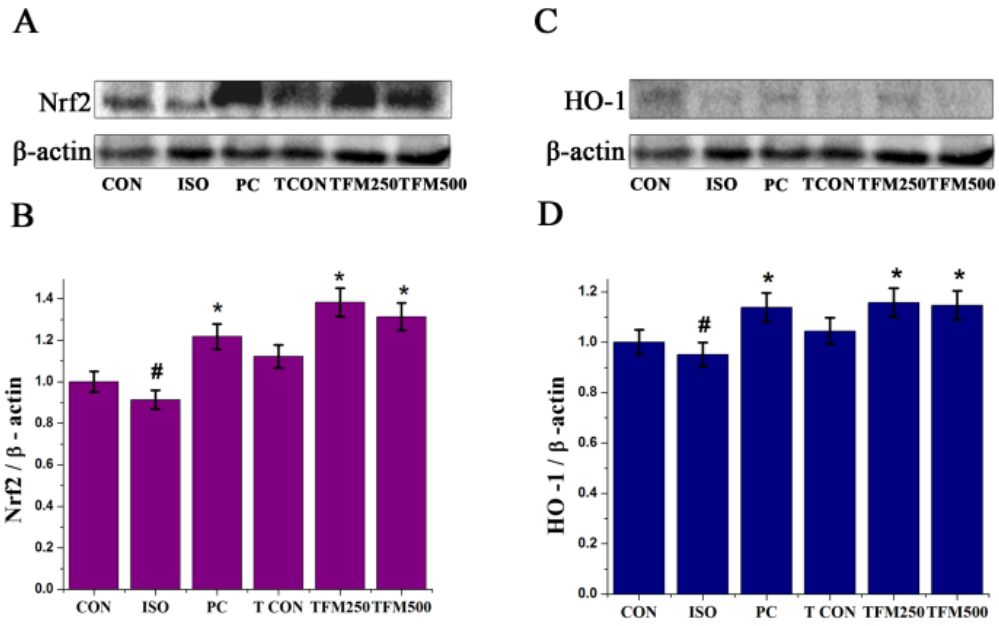


Figure 6.1 Nrf2 and HO-1 expression. A. Expression of Nrf2 in the nuclear fraction by western blotting of vehicle control, isoproterenol, treatment control, *Tribulus terrestris* L. fruit methanol extract 250 mg/kg and isoproterenol treatment and *Tribulus terrestris* L. fruit methanol extract 500 mg/kg and isoproterenol treatment groups. B. Densitometric analysis of protein expression of Nrf2 relative to β - actin C. Expression of HO -1 by western blotting of vehicle control, isoproterenol, treatment control, *Tribulus terrestris* L. fruit methanol extract 250 mg/kg and isoproterenol treatment and *Tribulus terrestris* L. fruit methanol extract 500 mg/kg and isoproterenol treatment groups. D.

Densitometric analysis of protein expression of HO-1 relative to β - actin. Results shown are mean ± SD of six independent experiments. # $p \leq 0.05$ relative to control,*$p \leq 0.05$ relative to ischemia group.

6.4.2. Mitochondrial biogenesis

The expression of mitochondrial biogenesis markers Nrf1 and Tfam was studied at the mRNA level. The expression of Nrf1 and Tfam was downregulated significantly ($p \leq 0.05$) following myocardial infarction by 0.3 F and 0.4 F respectively (Figure 6.2). TFM pre-treatment significantly ($p \leq 0.05$) prevented downregulation of Tfam and Nrf1 genes. Nrf1 gene dowregulation was prevented by 0.85 F, 1.1 F and 1.14 F for TFM 250, 500 mg/kg and positive control respectively compared to isoproterenol and Tfam gene downregulation was prevented by 1 F, 1.3 F and 1.33 F for TFM 250, 500 mg/kg and positive control respectively compared to isoproterenol group (Figure 6.2)

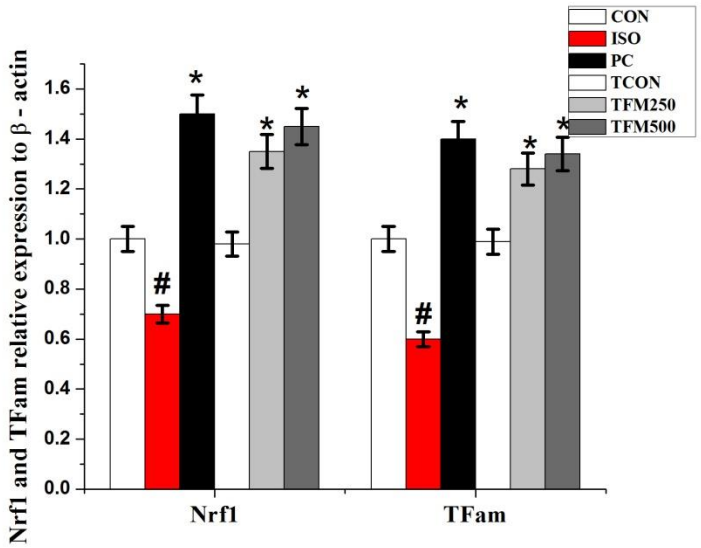


Figure 6.2 Relative mRNA expression of mitochondrial biogenesis markers Nrf1 and Tfam in vehicle control, isoproterenol, treatment control, positive control and isoproterenol treatment, *Tribulus terrestris* L. fruit methanol extract 250 mg/kg and isoproterenol treatment and *Tribulus terrestris* L. fruit methanol extract 500 mg/kg and isoproterenol groups. Results shown are mean ± SD of six independent experiments. # $p \leq 0.05$ relative to control, * $p \leq 0.05$ relative to ischemia group.

6.4.3 NOS-2 and myocardial infarction

There was a significant ($p \leq 0.05$) 50 % up regulation of inducible nitric oxide synthase expression (NOS-2/ iNOS) in isoproterenol induced myocardial infarction group (Figure 6.3). TFM (250 mg and 500 mg/kg) and positive control pre-treatment prevented up

regulation of NOS-2 significantly ($p \leq 0.05$) by 33 %, 46 % and 25 % respectively (Figure 6.3).

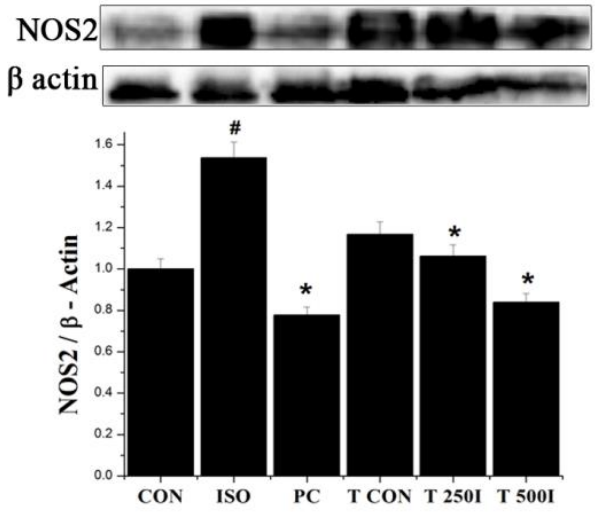
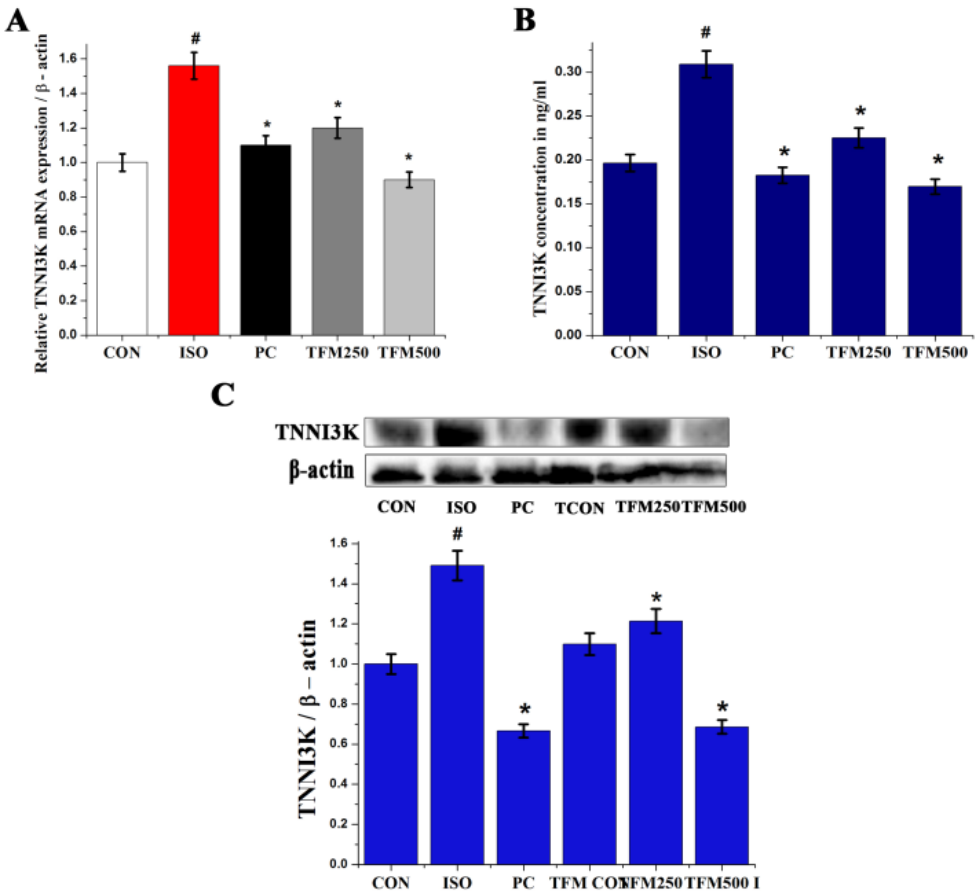


Figure 6.3 NOS-2 expression in myocardial infarction. A. NOS-2 expression in control , isoproterenol, positive control, treatment control, *Tribulus terrestris* L. fruit methanol extract 250 mg/kg and isoproterenol treatment and *Tribulus terrestris* L. fruit methanol extract 500 mg/kg and isoproterenol groups, B. Densitometric quantification of NOS-2 expression relative to β - actin. Results shown are mean ± SD of six independent experiments. # $p \leq 0.05$ relative to control,*$p \leq 0.05$ relative to ischemia group.

6.4.4 TNNI3K expression in myocardial infarction

Isoproterenol induced myocardial infarction caused an over expression of MAPK, TNNI3K at the mRNA level and protein level. There was a significant ($p \leq 0.05$) 50% increase in TNNI3K expression on isoproterenol treatment when measured by ELISA and western blotting when compared to vehicle control (Figure 6.4B, C). TFM pre-treatment at 250 mg/kg and 500 mg/kg and positive control significantly ($p \leq 0.05$) prevented upregulation of TNNI3K by 40.6 % , 46.6 % and 47 % respectively when compared to isoproterenol treatment; measured by western blot (Figure 6.4 C). ELISA measurement showed a significant ($p \leq 0.05$) inhibition of 26.6 % and 56.6 % for TFM 250 and 500 mg/kg pre-treatment and 40 % with positive control pre-treatment when compared to isoproterenol

group (Figure 6.4 B). Expression of TNNI3K at the mRNA level was also measured and was found to be significantly ($p \leq 0.05$) increased on isoproterenol treatment. mRNA level expression of TNNI3K increased by 0.55 F compared to control and TFM pre-treatment (250



and 500 mg/kg) and positive control prevented the up regulation of TNNI3K gene expression by 0.2 F, 0.6 F and 0.4 F respectively (Figure 6.4A).

Figure 6.4 TNNI3K expression in isoproterenol induced myocardial infarction TNNI3K expression in isoproterenol induced myocardial infarction and inhibition of TNNI3K expression by *Tribulus terrestris* L. fruit methanol extract. A. Relative mRNA expression profile of TNNI3K gene in vehicle control, isoproterenol, treatment control, positive control, *Tribulus terrestris* L. fruit methanol extract 250 mg/kg and isoproterenol treatment and *Tribulus terrestris* L. fruit methanol extract 500 mg/kg and isoproterenol groups. B. TNNI3K protein expression in ng/ml measured by ELISA technique in vehicle control, isoproterenol, treatment control, positive control, *Tribulus terrestris* L. fruit methanol extract 250 mg/kg and isoproterenol treatment and *Tribulus terrestris* L. fruit methanol extract 500 mg/kg and isoproterenol groups. C. TNNI3K expression by western blotting and densitometric quantification in vehicle control, isoproterenol, treatment control, positive control, *Tribulus terrestris* L. fruit methanol extract 250 mg/kg and isoproterenol treatment and *Tribulus terrestris* L. fruit methanol extract 500 mg/kg and isoproterenol groups. Results shown are mean ± SD of six independent experiments. # $p \leq 0.05$ relative to control,*$p \leq 0.05$ relative to ischemia group.

6.5 Discussion

In the previous chapter *T. terrestris* fruit (TFM) was found to protect cardiomyoblat from ischemic injury by protecting mitochondria. This chapter deals with effect of TFM in an *in vivo* model of myocardial infarction by isoproterenol with special emphasis on mitochondrial biogenesis, redox regulation and cardiac specific MAPK, TNNI3K.

Cardiomyocytes suffer oxidative and nitrosative stress following myocardial infarction. However, the major site of damage during such stress is on bioenergetics and associated pathways (Jennings and Ganote, 1976). Maintenance of mitochondrial homeostasis for required energy production through the balance of mitochondrial turnover, fission and fusion, and generation of new mitochondria via mitochondrial biogenesis is critical for tissue health. Pharmacological activation of mitochondrial biogenesis can enhance oxidative metabolism and tissue bioenergetics, and improve organ function in conditions characterized by mitochondrial dysfunction. Oxidative stress regulates redox regulated pathways present in mitochondria the Nrf2 /HO1 /CO pathway and mitochondrial biogenesis.

Nrf2 is a basic leucine zipper transcription factor that binds to promoter region of various genes (Nqo1, GST and Hmox1) (Itoh et al., 2010). It forms ternary docking complex with Keap1 (cytoplasmic inhibitor). In non-stressed cells, Nrf2 is rapidly degraded by the kelch-like ECH-associated protein 1 (Keap1) - mediated ubiquitin proteasome system (Itoh et al., 2010). Nrf2 Ser40 phosphorylation by protein kinase C (PKC) dissociates Nrf2 from Keap1 in response to oxidative stress. The Nrf2 translocates to the nucleus and binds to antioxidant response element of various genes (ARE) and induce transcription of various genes. Results show that there was decreased expression of Nrf2 in the isoproterenol treatment group. Nrf2 expression was increased on *T. terrestris* (TFM) treatment prior to isoproterenol. The binding of Nrf2 to H*mox*1gene causes expression of hemoxygenase (HO-1) protein. HO-1 catalyzes the conversion of heme into biliverdin, iron and carbon monoxide (CO) (Alam and Cook, 2007). HO-1 gene expression is up-regulated in response to the substrate heme as well as to various environmental stressors such as cadmium, lipopolysaccharide, nitric oxide (NO) and oxidative stress. CO binds to the reduced heme of cytochrome c oxidase (COX) and inhibits cellular respiration and generates hydrogen

peroxide (H_2O_2) (D'Amico et al., 2006; Piantadosi, 2002). HO-1 over expression, which leads to CO generation, activates nuclear respoiratory factor 1 (Nrf-1) transcription (Suliman et al., 2007). In this cascade, CO-induced H_2O_2 leads to the activation of the PI3K/AKT pathway, activating Nrf2, which directly binds to the AREs in the Nrf-1 gene promoter (Piantadosi et al., 2008). Therefore, Nrf2 is indispensable for HO-1/CO-mediated mitochondrial biogenesis. Thus, CO provokes the ROS-mediated activation of Nrf1, which induces mitochondrial biogenesis (Piantadosi and Suliman, 2006). Our results show that HO-1 expression was also increased on treatment with TFM. Investigations have shown that CO generated from HO-1 overexpression stimulates mitochondrial SOD-2 upregulation and increases H_2O_2 production, which activates Akt/ PKB, deactivates GSK3b and induces Nrf-1 leading to the stimulation of mitochondrial biogenesis. Markers of mitochondrial biogenesis Nrf-1 and Tfam were downregulated in infarction and TFM upregulated these genes increasing mitochondrial biogenesis and thus protecting the myocardium from injury.

Following ischemia and myocardial infarction the myocardium heals the ischemic zone and undergoes cardiac remodeling. Cardiac specific NOS2 overexpression caused positive inotropic response in isoproterenol induced β-adrenergic overstimulation adverse remodeling and increased mortality (Mungrue et al., 2002). Pharmacological inhibition of NOS-2 decreased β-adrenergic inotropic responsiveness (Gealekman et al., 2002). Our study showed that there is increased expression of NOS-2 following isoproterenol induced β-adrenergic stimulation and TFM treatment inhibited the upregulation of NOS-2.

Cardiac Troponin I-interacting Kinase or TNNI3K is a novel cardiac specific MAPK expressed only in the cardiac myocytes and not in the fibroblasts vascular endothelial cells and skeletal muscle. TNNI3K showed increased expression during cardiac dysfunctions and myocardial infarction (Vagnozzi et al., 2013). Mice overexpressing TNNI3K had a large infarct size when compared to mice that did not over express TNNI3K. TNNI3K was also associated with cardiac hypertrophy (Wang et al., 2013). The reason for the increased cell death was that TNNI3K interacts with the mitochondria and caused increased ROS production (Lal et al., 2014). The increased ROS production activates the p38 pathway causing apoptotic cell death (Lal et al., 2014). Mice given small molecule TNNI3K inhibitors showed reduced scarring, reduced remodeling and better ventricular function. Thus inhibiting

TNNI3K can reduce pathological effects of a heart attack (Vagnozzi et al., 2013). Pharmacological TNNI3K inhibition is cardiac selective and prevents potential adverse effects of systemic kinase inhibition. Our results show that isoproterenol induced myocardial infarction caused an increase in expression of TNNI3K and TFM inhibited the expression of TNNI3K. Thus, the constituents of *T. terrestris* fruit individually or synergistically have the ability to inhibit a cardiac specific MAPK, TNNI3K causing decreased ROS production, decreased expression of p38 and reduced cell death by apoptosis.

Thus, pretreatment with TFM maintains mitochondrial homeostasis, maintain mitochondrial turnover by increasing mitochondrial biogenesis, regulated NOS-2 expression and inhibit the expression of a cardiac specific MAPK, TNNI3K. Thus TFM has the ability to protect against the adverse effects of myocardial infarction in an *in vivo* model also.

References

Alam J and Cook JL (2007) How many transcription factors does it take to turn on the heme oxygenase-1 gene? *American Journal of Respiratory Cell and Molecular Biology* 36: 166-174.

D'Amico G, Lam F, Hagen T and Moncada S (2006) Inhibition of cellular respiration by endogenously produced carbon monoxide. *Journal of Cell Science* 119: 2291-2298.

Favreau LV and Pickett CB (1995) The rat quinone reductase antioxidant response element. Identification of the nucleotide sequence required for basal and inducible activity and detection of antioxidant response element-binding proteins in hepatoma and non-hepatoma cell lines. *Journal of Biological Chemistry* 270: 24468-24474.

Gealekman O, Abassi Z, Rubinstein I, Winaver J and Binah O (2002) Role of myocardial inducible nitric oxide synthase in contractile dysfunction and beta-adrenergic hyporesponsiveness in rats with experimental volume-overload heart failure. *Circulation* 105: 236-243.

Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M and Nabeshima Y (1997) An Nrf2/ small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochemical and Biophysical Research Communications* 236: 313-322.

Itoh K, Mimura J and Yamamoto M (2010) Discovery of the negative regulator of Nrf2, Keap1: a historical overview. *Antioxidants & Redox Signaling* 13: 1665-1678.

Jaloszynski P, Murata S, Shinkai Y, Takahashi S, Kumagai Y, Nishimura S and Yamamoto M (2007) Dysfunction of Nrf2 decreases $KBrO_3$-induced oxidative DNA damage in Ogg1-null mice. *Biochemical and Biophysical Research Communications* 364: 966-971.

Jennings RB and Ganote CE (1976) Mitochondrial structure and function in acute myocardial ischemic injury. *Circulation Research* 38: 80-91.

Kametani R, Miura T, Harada N, Shibuya M, Wang R, Tan H, Fukagawa Y, Kawamura S and Matsuzaki M (2006) Carvedilol inhibits mitochondrial oxygen consumption and

superoxide production during calcium overload in isolated heart mitochondria. *Circulation Journal* 70: 321-326.

Ko D, Wang Y, Berger AK, Radford MJ and Krumholz HM (2002) Nonsteroidal antiinflammatory drugs after acute myocardial infarction. *American Heart Journal* 143: 475-481.

Lal H, Ahmad F, Parikh S and Force T (2014) Troponin I-interacting protein kinase: a novel cardiac-specific kinase, emerging as a molecular target for the treatment of cardiac disease. *Circulation Journal* 78: 1514-1519.

Mehta JL and Li DY (1999) Inflammation in ischemic heart disease: response to tissue injury or a pathogenetic villain? *Cardiovascular Research* 43: 291-299.

Mullane KM, Read N, Salmon JA and Moncada S (1984) Role of leukocytes in acute myocardial infarction in anesthetized dogs: relationship to myocardial salvage by anti-inflammatory drugs. *The Journal of Pharmacology and Experimental Therapeutics* 228: 510-522.

Mungrue IN, Gros R, You X, Pirani A, Azad A, Csont T, Schulz R, Butany J, Stewart DJ and Husain M (2002) Cardiomyocyte overexpression of iNOS in mice results in peroxynitrite generation, heart block, and sudden death. *The Journal of Clinical Investigation* 109: 735-743.

Oliveira PJ, Bjork JA, Santos MS, Leino RL, Froberg MK, Moreno AJ and Wallace KB (2004) Carvedilol-mediated antioxidant protection against doxorubicin-induced cardiac mitochondrial toxicity. *Toxicology and Applied Pharmacology* 200: 159-168.

Piantadosi CA (2002) Biological chemistry of carbon monoxide. *Antioxidants & Redox Signaling* 4: 259-270.

Piantadosi CA, Carraway MS, Babiker A and Suliman HB (2008) Heme oxygenase-1 regulates cardiac mitochondrial biogenesis via Nrf2-mediated transcriptional control of nuclear respiratory factor-1. *Circulation Research* 103: 1232-1240.

Piantadosi CA and Suliman HB (2006) Mitochondrial transcription factor A induction by redox activation of nuclear respiratory factor 1. *The Journal of Biological Chemistry* 281: 324-333.

Romson JL, Hook BG, Kunkel SL, Abrams GD, Schork MA and Lucchesi BR (1983) Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. *Circulation* 67: 1016-1023.

Rushmore TH, Morton MR and Pickett CB (1991) The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *The Journal of Biological Chemistry* 266: 11632-11639.

Suliman HB, Carraway MS, Tatro LG and Piantadosi CA (2007) A new activating role for CO in cardiac mitochondrial biogenesis. *Journal of Cell Science* 120: 299-308.

Sun Y (2009) Myocardial repair/remodelling following infarction: roles of local factors. *Cardiovascular Research* 81: 482-490.

Vagnozzi RJ, Gatto GJ, Jr., Kallander LS, Hoffman NE, Mallilankaraman K, Ballard VL, Lawhorn BG, Stoy P, Philp J, Graves AP, Naito Y, Lepore JJ, Gao E, Madesh M and Force T (2013) Inhibition of the cardiomyocyte-specific kinase TNNI3K limits oxidative stress, injury, and adverse remodeling in the ischemic heart. *Science Translational Medicine* 5: 207-214.

Wang X, Wang J, Su M, Wang C, Chen J, Wang H, Song L, Zou Y, Zhang L, Zhang Y and Hui R (2013) TNNI3K, a cardiac-specific kinase, promotes physiological cardiac hypertrophy in transgenic mice. *PLoS one* 8: 585-592

Weisman HF, Bush DE, Mannisi JA, Weisfeldt ML and Healy B (1988) Cellular mechanisms of myocardial infarct expansion. *Circulation* 78: 186-201.

Weisman HF and Healy B (1987) Myocardial infarct expansion, infarct extension, and reinfarction: pathophysiologic concepts. *Progress in Cardiovascular Diseases* 30: 73-110.

Willems IE, Havenith MG, De Mey JG and Daemen MJ (1994) The alpha-smooth muscle actin-positive cells in healing human myocardial scars. *The American Journal of Pathology* 145: 868-875.

Summary and Conclusion

The prevalence of cardiovascular disease (CVD) is increasing worldwide. CVD has assumed epidemic proportions in India and cause 23% of the total death. Among various heart diseases, CAD is the greatest contributor causing 46% of the total death due to CVD. Coronary artery disease is caused by the blockage of the coronary artery that supplies blood to the heart, causing death of the heart muscle in that area of the heart, leading to myocardial ischemia, myocardial infarction and finally heart failure. Various treatment approaches such as early detection, lifestyle modifications, medications, coronary interventions reduce the risk of heart failure. The medications available, only alleviate the symptoms and there is a risk of secondary complications. Several of the drugs available to treat CVD are derived from medicinal plants. Medicinal plants are now used widely for the management of heart disease and as an adjunct to conventional therapy. *Tribulus terrestris* L. is widely used in traditional medicine, as a cardiotonic to treat heart diseases. Both the fruit and the root are mentioned in Ayurveda to be protective against heart diseases. So, both the fruit and root were analyzed and the mechanism of cardio protection of *T. terrestris* L. fruit and root was studied in an *in vitro* and *in vivo* model of myocardial ischemia.

Tribulus terrestris L. whole plant was collected, fruit and root separated, washed, dried and processed for extraction. *T. terrestris* root was macerated with methanol to yield *Tribulus terrestris* L. root methanol extract (TTM). Methanolic extract (TTM) and its different solvent fractions viz., hexane, dichloromethane, ethyl acetate and methanol water fractions were screened for their antioxidant potential using various in vitro chemical assays like DPPH, ABTS, superoxide and hydroxyl radical scavenging activities. Results showed that TTM showed better radical scavenging activity then its fractions. TTM was also partially characterized by HPLC. The protective property of partially characterized TTM against cell death in ischemia was evaluated in an *in vitro* model of myocardial ischemia using H9c2 cell lines. Markers of cell death, caspase-3 activity, pro-apoptotic markers Bax, Bad and anti-apoptotic markers Bcl-2 were evaluated. The results showed that pre-treatment with TTM reduced apoptotic cell death which was evident from the reduction in caspase-3 activity and

expression of Bax and Bad. To study the mechanism of protection, several MAPK pathways were studied and it was found that TTM mediates its protection by activating MAPKs, p38α and JNK and inactivating Akt. TTM treatment also reduced intracellular ROS, superoxide and decreased LDH release. Results of the study clearly establish the protective property of TTM against cell death in myocardial ischemia.

The protective property of TTM in and *in vitro* model of myocardial ischemia prompted for further exploration of the protective property of TTM in an *in vivo* model of myocardial infarction employing isoproterenol. Various parameters like release of cardiac biomarkers, endogenous antioxidant status, ECG and histopathological alterations of the heart tissue was evaluated. Activation and translocation of NFκB and activation of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β, chemokines MCP-1 and anti-inflammatory cytokine IL-10 were also studied. TFM pre-treatment was found to reduce the levels of serum LDH, CK-MB, SGOT and SGPT. In addition, TFM treatment prevented oxidative damage in the heart tissue, which was evident from enhanced activities of various antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase. ECG evaluation showed increase heart rate and ST segment depression, characteristic of myocardial infarction, which was reduced by TTM pre-treatment. Inflammatory infiltration, interstitial edema, necrosis and myocardial collagen content (marker of cardiac fibrosis) was significantly higher in isoproterenol induced rats, while TTM treatment reversed these changes which was evident by histopathological analysis. In our study there was translocation of NFκB to the nucleus and increased expression of pro-inflammatory cytokines IL-6, TNF-α and IL-1β, chemokines MCP-1 and decreased expression of anti-inflammatory cytokine IL-10 which was prevented with TTM treatment. Thus, TTM was found effective in an *in vitro* and in vivo model of ischemia.

Mitochondrial dysfunction and resulting oxidative stress are central in the pathogenesis of several maladies including cardiac ischemic injury. Thus, TFM was evaluated for its protective property against mitochondrial injury in cardiac ischemia. Various parameters relevant to mitochondria such as ROS production, alteration in mitochondrial transmembrane potential, integrity of mitochondrial permeability transition pore (mPTP), activities of mitochondrial respiratory complexes (Complex I-IV), oxygen consumption rate,

ATP content and expression of HIF-1α and mitochondrial dynamics was studied. The results revealed that pre-treatment with TFM significantly prevented the generation of intracellular ROS and maintained innate antioxidant status and protected the mitochondria by preventing the dissipation of ΔΨm, opening of mPTP, mitochondrial swelling and enhanced the activities of respiratory chain complexes and oxygen consumption rate, ATP production and expression of HIF-1α in H9c2 cell line. During ischemia there is increased fission and decreased fusion of mitochondria and this disruption in fission fusion balance was ameliorated with TFM pre-treatment. TFM was also partially characterized by HPLC. Thus, TFM protects the mitochondria and the protective property of TFM may be due to the bioactives present.

The mitochondria protective property of TFM prompted for further exploration of the protective property of TFM in an *in vivo* model of myocardial infarction using isoproterenol with special emphasis on mitochondrial redox regulation, mitochondrial biogenesis, cardiac specific MAPK TNNI3K and cardiac remodeling. Thus, pretreatment with TFM maintained mitochondrial homeostasis by increased Nrf2 translocation to the nucleus and increased expression of antioxidant response genes, maintain mitochondrial turnover by increased mitochondrial biogenesis, regulated NOS-2 expression and inhibited the expression of a cardiac specific MAPK, TNNI3K. Thus TFM was able to protect from the adverse effects of myocardial infarction in an *in vivo* model also.

Overall results reveal that both *Tribulus terrestris* L. fruit and root have cardio protective property. But, increased usage of the root of the plant, makes it more susceptible to overexploitation and loss of the plant species. So, usage of the fruit of *Tribulus terrestris* L. for medicine is recommended. Based on the results from the present study, we propose detailed investigation on *Tribulus terrestris* L. for the development of a phytoceutical for cardiac problems.

List of Publications

- **Reshma P L**, Neethu S Sainu, Anil K Mathew, Raghu K G. Mitochondrial dysfunction in H9c2 cells during ischemia and amelioration with *Tribulus terrestris* L. **Life Sci.** 2016; 152: 220-30
- **Reshma P L**, Lekshmi V S, Sankar V, Raghu K G. *Tribulus terrestris* (Linn.) Attenuates Cellular Alterations Induced by Ischemia in H9c2 Cells Via Antioxidant Potential. **Phytother Res**. 2015; 29: 933-43
- Soumya RS, VineethaVP, **Reshma PL**, Raghu KG. Preparation and characterization of selenium incorporated guar gum nanoparticle and its interaction with H9c2 cells. **PLoS One.** 2013; 8: e7441.

Presentations in scientific conferences

- Oral presentation "Evaluation of protective property of extracts of *Tribulus terrestris* L. against ischemia reperfuison injury in H9c2 cell line" 25th Annual Conference of the Indian Society for Atherosclerosis Research , Annamalai University, Chidambaram, September 1-3, 2012.
- Oral presentation on *"*Evaluation of protective effect of *Tribulus terrestris* L. against ischemia – an *in vitro* study" 26th Annual Conference of Indian Society for Atherosclerosis Research and National Seminar on Innovations in Atherosclerosis and Cardiac Diseases. Amala Cardiac Centre, Thrissur, November 22-23, 2013
- Poster presentation on *"Tribulus terrestris* L. attenuates alterations in cardio myoblast (H9c2) during ischemia by up-keeping innate antioxidant status and mitochondrial function by inhibiting kinase TNNI3K". **Best poster award** at the 7th International Conference on Recent Advances in Cardiovascular Sciences, Amity University, Noida March 10-11, 2015.
- Oral presentation on *"Tribulus terrestris* L. attenuates alterations in cardio myoblast (H9c2) during ischemia by up-keeping innate antioxidant status and mitochondrial function by inhibiting kinase TNNI3K" Indo - Canadian Symposium on Heart Failure: Progress & Prospects. Rajiv Gandhi Centre for Biotechnology, Trivandrum, Kerala, March 12-14, 2015