Role of Esculetin on Renin Angiotensin System and Epigenetics in the development of Cardiovascular and Renal Complications associated with Type 2 Diabetes

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Mr. Almesh Kadakol

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Dr. Gaikwad Anil Bhanudas

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CERTIFICATE

This is to certify that the thesis entitled **"Role of Esculetin on Renin Angiotensin System and Epigenetics in the development of Cardiovascular and Renal Complications associated with Type 2 Diabetes"** and submitted by **Mr. Almesh Kadakol**, ID No. **2012PHXF0405P** for award of Ph.D. degree of the Institute embodies original work done by him/her under my supervision.

Signature of the Supervisor:

Name:	Dr. GAIKWAD ANIL BHANUDAS
Designation:	Assistant Professor / Head, Department of Pharmacy, Birla Institute of Technology and Science, Pilani, Pilani campus, Rajasthan, India

Date:

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Date: Almesh Kadakol

Background:

Present westernized lifestyle contributing in the development of chronic metabolic syndrome. Among these, type 2 diabetes (T2D) has 85-90% burden of mortality. Grave worsening of T2D condition is associated with the progression of various cardiovascular complications. Despite, achieving normal glucose level, the complications such as progressive cardiovascular and renal complications are commonly observed which overwhelmed in complex chromosomal alteration mechanisms known as epigenetics. This motivated us to look into the natural treasure trove to find an appropriate treatment for these complicated, multifaceted diseases. Esculetin (6,7- dihydroxy coumarin derivative), a naturally available pleotropic coumarin derivative present in various medicinal plants and fruits such as *Artemisia capillaries, Artemisia scoparia, Citrus limonia, Cortex fraxini, Ceratostigma willmottianum, Viola yedoensis, Esculus hippocastanum* and *Cichorium intybus* which displays direct free radical quenching, lipooxygenase and cyclooxygenase inhibition, and anti-fibrotic activity. Owing to the aforementioned properties, it shows a potential therapeutic role in cancer, obesity, and diabetes. Based on such literature review, present study involves to depict the role of esculetin in epigenetic modifications in the expression of AT1, AT2, pro-inflammatory genes and fibrotic genes under exaggerated oxidative stress in the development of cardiovascular and renal complications under insulin resistance (IR) and T2D.

Methods:

Insulin resistance (IR) and T2D were induced by feeding alone high fat diet (HFD) and low dose Streptozotocin (STZ) (35 mg/kg, *i.p*) administration along with HFD feeding respectively. Esculetin was treated with doses of 50 and 100 mg/kg/day, *p.o* for 2 weeks. Primarily, IR, T2D and renal failure were assessed by analyzing blood plasma for glucose, triglyceride, cholesterol, creatinine, albumin and insulin levels using commercially available kits. Manipulated diet's possible additive, synergistic and antagonistic effects on esculetin were determined by *in vitro* antioxidant tests. Estimation of glutathione and lipid peroxidation were established for confirmation of oxidative stress in kidneys. Angiotensin II (Ang II) mediated vascular reactivity were performed to evidence irregularities in vascular toning. Histopathology and immunohistochemistry were performed to evaluate hypertrophy and protein quantifications. Western blotting was performed for post-translational histone

modifications (PTHMs) and real time polymerase chain reactions (RT-PCR) were performed for analyzing various gene expressions. Finally, chromatin immuno precipitation assays (ChIP) followed by RT-PCR were performed to determine occupancies of histone modifications at promoter regions of various genes.

Results:

Esculetin treatment significantly attenuates metabolic perturbations, alleviates insulin levels in hyperinsulinemic condition. Thoracic aorta of hyperinsulinemic and hyperglycemic rats showed hyper-responsiveness to Ang II mediated contraction and impaired acetylcholine mediated relaxation, and esculetin attenuate alterations in vascular reactivity to Ang II and acetylcholine challenges. In addition, immunohistochemical evaluations revealed that esculetin prevents increase in angiotensin II receptor type 1 (AT1), type 2 (AT2), Keap1, TGF-β, and decrease in ACE2 expression in aorta of hyperinsulinemic and hyperglycemic rats. Next, Esculetin treatment substantially improved vascular reactivity, increased *eNos* and decreased *Vcam1* mRNA levels, and reduced collagen deposition in rat thoracic aorta. Further, the fold changes in *At1* and *At2* receptor mRNA in IR and T2D were reversed by esculetin treatment. Modifications in histone H2B lysine 120 monoubiquitination (H2BK120Ub) were also reversed in esculetin treatment group aorta. Further, esculetin also modify the occupancy of H2AK120Ub at *At1, At2, Tgfβ1* and *Mcp1* promoter gene was evidenced in aorta. Besides this, for the first time we have demonstrated increased histone H2AK119Ub and H2BK120Ub levels in diabetic cardio myopathy (DCM). Esculetin treatment restored normal level of permissive PTHMs and H2A/H2B ubiquitination in IR and diabetic heart. In addition, esculetin attenuated alteration in the renin angiotensin system, oxidative stress (Keap1) and cell proliferation (Ki67); thus preventing DCM. Remarkably, esculetin bestows reno-protection owing to its renin angiotensin system modulating properties and also abrogating the hyper acetylation of histone H3 lysine (K) 14 and 18 and elevated H2AK119 monoubiquitination (Ub). This is the first report demonstrating that esculetin could be attributed to elevation of the occupancy of H2AK119Ub at the promoter regions of *Mcp1* and *Tgfb1*.

Conclusions:

In conclusion, our study provides comprehensive evidences for pleotropic effects of esculetin in the amelioration of IR, T2D and associated cardiovascular and renal complications by intervening renin angiotensin system (RAS) and post translational

histone modifications. Initially, the modified diet (HFD – High Fat Diet) induced IR and followed by low dose streptozotocin (35 mg/kg) administration induced T2D and its associated complications were significantly characterized by modifications in biochemical, morphological and hemodynamic parameters. Esculetin treatment showed improved metabolic and hemodynamic alterations in IR and T2D conditions. Further, esculetin ameliorated the impaired vascular toning along with improved angiotensin type 1 (AT1), angiotensin type 2 (AT2) and endothelial nitric oxide synthase (eNOS) expressions. Consequentially, esculetin also reduced vascular cell adhesion protein (VCAM) expression thereby reduced collage depositions in aorta. Furthermore a bore in the chromatin modifications, esculetin showed intervening property in H2B ubiquitination system including E3 ligases and deubiquitinases. Next, our data represented esculetin could have functional role in reverting of vascular perturbation by regulating *At1, At2, Tgfβ1 and Mcp1* genes under IR and T2D conditions in thoracic aorta.

It was observed, under oxidative stress and up-regulated RAS in IR and T2D conditions, the heart tissue possessed increased Kelch-like ECH-associated protein 1 (Keap 1), Ki67, AT1 and AT2, and reduced angiotensin converting enzyme 2 (ACE2) expressions. These findings could conclude a typical theory of augmentation of angiotensin converting enzyme (ACE)/angiotensin II (Ang II)/AT1 axis which may contribute in the development of cardiomyopathy. Such deleterious modifications could further involve in the activation of permissive (H3K27Ac, H3K56Ac, H3S10phospho, H3S28phosphor, H3T3phosphor, H3K4me2, H3K36me2, H3K79me2) and inhibition of repressive (H3K9me2 and H3K27me2) post translational histone modifications (PTHMs). Esculetin treatment reversed these modifications and restored the normal physiology of heart in IR and T2D rats. Further in type 2 diabetic nephropathy (T2DN) conditions, esculetin's ability to reduce disturbances in RAS may be responsible for its anti-inflammatory and anti-fibrotic potential. The reversal of PTHMs, especially H2AK119Ub may be an important underlying mechanism for the treatment of IR and T2DN. Thus, this study may help us improve our understanding of the basic mechanisms of action of esculetin behind its reno-protective action. It also emphasizes that epigenetic modifications play a significant role in regulation of renal fibrosis and macrophage infiltration and thus, a therapy for complete treatment of IR and T2DN must possess the ability to revert these gene specific PTHMs.

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AGEs	Advanced glycation end products
ACE	Angiotensin converting enzyme
ACE2	Angiotensin converting enzyme 2
Ang 1-7	Angiotensin 1-7
Ang 1-9	Angiotensin 1-9
Ang II	Angiotensin II
AT1	Angiotensin II type 1 receptor
AT2	Angiotensin II type 2 receptor
CCL	CC-chemokine ligand
Col1 a1	Alpha-1 type I collagen
DKK1	Dickkopf related protein 1
DN	Diabetic nephropathy
DUBs	De-ubiquitinases
ECL	Enhanced Chemiluminescence
ECM	Extra cellular matrix
ER	Endoplasmic reticulum
GMCs	Glomerular mesangial cells
H2AK119-Ub	H2A lysine 119 mono-ubiquitination
H2BK120-Ub	H2B lysine 120 mono-ubiquitination
H3K27Ac	H3 lysine 27 acetylation
H3K36me2	H3 lysine 36 di-methylation
H3K4me2	H3 lysine 4 di-methylation
H3K56Ac	H3 lysine 56 acetylation
H3K79me2	H3 lysine 72 di-methylation
H3K9Ac	H3 lysine 9 acetylation
H3K9me2	H3 lysine 9 di-methylation
H3S10Phospho	H3 serine 10 phosphorylation
H3S27Phospho	H3 serine 27 phosphorylation
HDACs	Histone de-acetylases
HFD	High fat diet
HMTs	Histone methyl transferases
IR	Insulin resistance
MCP-1	Monocyte chemo-attractant protein 1
NFκβ	Nuclear factor kappa-β
Nox	NADPH oxidase
NPD	Normal pellet diet
PTHMs	Post translational histone modification
RAGEs	Receptors for AGEs
RAS	Renin angiotensin system
Rnf	E3 ubiquitin-protein ligase ring finger protein
STZ	Streptozotocin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TGF-β	Transforming growth factor beta

TNF-α	Tumor necrosis factor alpha
UPS	Ubiquitin proteasome system
USP	Ubiquitin specific protease

Chapter I

Introduction and Review of Literature

A. Introduction:

Diabetes is a deteriorative and chronic disease that occurs either inability to produce enough insulin from pancreatic β cells (type 1 diabetes) or inability to effective utilization of produced insulin by different organs (type 2 diabetes) (WHO, 2016). Type 1 diabetes (T1D) (previously known as insulin-dependent, juvenile or childhood-onset diabetes) requires daily administration of insulin to regulate the level of glucose in their blood. If they do not have access to insulin, they cannot survive. The symptoms of T1D excessive urination and thirst, constant hunger, weight loss, vision changes and fatigue. The exact causes of T1D are unknown. It is generally agreed that T1D is the result of a complex interaction between genes and environmental factors, though no specific environmental risk factors have been shown to cause a significant number of cases. The effected population by type 1 diabetes are children and adolescents. Whereas, the type 2 diabetes (T2D), which results from the ineffective utilization of insulin by the body, accounts for the vast majority of people with diabetes around the world than type 1 diabetes (WHO, 2016). Symptoms may be similar to those of type 1 diabetes, but are often less marked or absent. As a result, the disease may go undiagnosed for several years, until complications have already arisen. For many years T2D was seen only in adults but it has begun to occur in children. The risk of T2D is an interplay of genetic and metabolic factors such as ethnicity, family history of diabetes and previous gestational diabetes combine with older age, overweight and obesity, unhealthy diet, physical inactivity and smoking. Overweight and obesity, together with physical inactivity are estimated to cause a large proportion of the global diabetes burden. Several dietary practices are linked to unhealthy body weight and/or T2D risk, including high intake of saturated fatty acids, high total fat intake and inadequate consumption of dietary fiber (DeSA, 2013; Mattei *et al.*, 2015; WHO, 2014). High intake of sugar-sweetened beverages, which contain considerable amounts of free sugars, increases the likelihood of being overweight or obese, particularly among children (Jeon *et al.*, 2008; Singh *et al.*, 2013). Recent evidence further suggests an association between high consumption of sugar-sweetened beverages and increased risk of T2D.

Over the time, raised blood glucose level leads to a serious consequential damage to the heart, blood vessels, eyes, kidneys and nerves. Acute metabolic complications associated with mortality include diabetic ketoacidosis from exceptionally high blood glucose concentrations (hyperglycemia) and coma as the result of low blood glucose (hypoglycemia) have generally seen in T2D patients. In associated with diabetes, long-term vascular complications arises due to chronic elevation of blood glucose levels, which

leads to damage of blood vessels (commonly observed in T2D patients). The resulting vascular complications are categorized as "microvascular complications" by damaging small blood vessels and "macrovascular complications" by damaging arteries. Microvascular complications include eye disease or retinopathy, kidney disease termed nephropathy and neural damage or neuropathy. The major macrovascular complications include accelerated cardiovascular disease resulting in myocardial infarction and cerebrovascular disease manifesting as strokes (Beckman *et al.*, 2016). Morrish et al in 2001 reported that, 44 % of mortality in T1D and 52 % in T2D is because of the cardiovascular disease. Further, on exploration of the role of renal diseases in diabetic patients, they found that it accounts for 21% of mortality in Type I diabetes and 11% in Type II diabetes (Morrish *et al.*, 2001). These complications further evidenced sooner in youth with T2D exhibit than the youth with T1D (Dart *et al.*, 2014). In T2D, the complications begin at early stage under insulin resistance by intervening vascular physiology. Simultaneously, insulin resistance and diabetes association changes in myocardial and renal structures and functions. These cardiovascular and kidney functional deteriorations are closely interlinked and considerable culprits are enhanced oxidative stress, up-regulated renin angiotensin system (RAS) and activation of inflammatory and fibrotic pathways (Boudina *et al.*, 2009; Bugger *et al.*, 2009; Reidy *et al.*, 2014). Even after normoglycemic, vascular complications are seen to be in the persistive development. The reasons were thought to be sustained inflammatory and fibrotic pathways. The emergence of such complications require molecular machineries which contribute in drastic cellular physiological modifications. From the recent decades, it is well understood that genetic predisposition alone is insufficient to explain the complexity of the disease entirely, hence this urge a necessity to emphasize on epigenetic mechanisms involved in the development of diabetic complications (Keating *et al.*, 2013). However until now, alteration in complete histone modifications in diabetic cardiovascular and renal complications is unexplored.

The currently available therapeutic interventions have various side-effects and have not yet been able to curb the root cause of IR and T2DN. This motivates us to look into the natural treasure trove to find an appropriate, side-effect free treatment for these complicated, multifaceted diseases. Esculetin (6,7- dihydroxy coumarin derivative), a naturally available pleotropic coumarin derivative present in various medicinal plants and fruits such as *Artemisia capillaries, Artemisia scoparia, Citrus limonia, Cortex fraxini, Ceratostigma willmottianum, Viola yedoensis, Aesculus hippocastanum,* and *Cichorium*

intybus, displays direct free radical quenching, lipooxygenase and cyclooxygenase inhibition, and anti-fibrotic activity (Galano *et al.*, 2016). Owing to the aforementioned properties, it shows a potential therapeutic role in cancer (Cho *et al.*, 2015), obesity (Kim *et al.*, 2015b; Sim *et al.*, 2015), and diabetes (Surse *et al.*, 2011b). Since, development of T2D complications involves elevated Ang II activity, oxidative stress and altered histone modifications, esculetin may prove instrumental in treatment of progressive renal fibrosis in IR and T2D. The present study may improve our understanding of post-translational histone modifications (PTHMs) occurring in T2D complications and the role of esculetin in reverting these alterations to elicit a reno-protective effect.

B. Clinical and Economical Impact of Type 2 Diabetes:

B.1. Clinical impact of diabetes and its complications:

Healthy people can produce healthy families in turn a healthy society and all together represent a healthy country. A depletion in public health could cost socioeconomic, political, and cultural devastations within entire world. Recognizing such future consequences world leaders initiated political commitments over global burden of public health. On effort of world health organization in collaborations with several other organization collectively reported global status reports on public health in different formats. Among these, two publication were published in 2010 and 2014, global status report on non-communicable diseases (NCD) highlighted as the leading cause of death globally (WHO, 2011; WHO, 2015). In 2012, 38 million deaths out of 56 million i.e. 68% were reasoned by NCDs. More than 40% of them (16 million) were premature deaths under age 70 years. Majorly, these NCDs are of 4 types which account for 82% of all related NCDs deaths. Distinguishably, cardiovascular diseases (CVDs) hold response for most NCDs deaths i.e. 17.5 million people annually, secondly cancers (8.2 million), followed by respiratory diseases (4 million) and diabetes (1.5 million). Almost three quarters of all NCD deaths i.e. 28 million and 82% of premature deaths occur in low- and middle-income countries (WHO, 2015). In such countries, it has been observed a rapid and ongoing transitions so that a huge population is being modified their lifestyle. Such modifications, including sedentary life, hypernutrition (Increased high fat consumption), reduced muscle mass and environmental pollutions which are being the etiological factors responsible for various diseases such as, obesity, hypertension, insulin resistance (IR), cardiovascular diseases, diabetes and renal failure (Nanditha *et al.*, 2016). Epidemically, the prevalence of cardiovascular and renal complications are largely diagnosed in people with diabetes.

According to world health organization (WHO) Diabetes is a major cause of blindness, kidney failure, heart attacks, stroke and lower limb amputation (Levitan *et al.*, 2004). Globally, an estimated 422 million adults were living with diabetes in 2014, compared to 108 million in 1980. The global prevalence (age-standardized) of diabetes has nearly doubled since 1980, rising from 4.7% to 8.5% in the adult population. This reflects an increase in associated risk factors such as being overweight or obese. Over the past decade, diabetes prevalence has risen faster in low- and middle-income countries than in high-income countries. Diabetes caused 1.5 million deaths in 2012. Higher-than-optimal blood glucose caused an additional 2.2 million deaths, by increasing the risks of cardiovascular and other diseases. Forty-three percent of these 3.7 million deaths occur before the age of 70 years. The percentage of deaths attributable to high blood glucose or diabetes that occurs prior to age 70 is higher in low- and middle-income countries than in high-income countries (WHO, 2015). On the other hand 2.2 million deaths from cardiovascular diseases, chronic kidney disease, and tuberculosis related to higher-than-optimal blood glucose. Such magnitude of diabetes highlights high blood glucose causes large burden of mortality beyond those deaths directly caused by diabetes itself.

B.2. Economic burden of diabetes and its complications:

In 2015, worldwide diabetic financial cost was reported to be USD 1.31 trillion in other sense 1 to 8 % global gross domestic product (GDP) (Bommer *et al.*, 2017). The global economic burden of diabetes, USD 857 billion costs direct medical bills and one third were indirect costs notably 34.7% of total burden such as lost productivity were observed (Bommer *et al.*, 2017). Such evidences exhibit a serious reminder that diabetes is not only a global health problem because of its effect on mortality and morbidity and quality of life, but also a major problem for national economies. In USA, it was found that the cost of the diabetes creates bigger economic burden for health care system, especially in northern states (Bommer *et al.*, 2017; Dieleman *et al.*, 2016). Direct health spending on diabetes worldwide has increased USD 427 billion from 2007 (Federation, 2009) to 2015 (Federation, 2015). This increased prevalence of expenditure was contributed to newly diabetes diagnosed population. In USA, the drugs and medical supplies for diabetes and its complications is increased by 321% from 1987 to 2011 (Zhuo *et al.*, 2015) and in 2016, minimum of 171 new drug therapies were reported to be developed (Report, 2016). Further such advance technologies for development of therapeutic parameters for diabetes probably increase the per capital medical expenditure per year of diabetic population. The other side of the burden i.e. indirect cost which is generated loss of productivity from

diabetic diseased population. The study is not yet conclusive and yet to studied detailed (Bommer *et al.*, 2017).

Among the developing economies, India holds title of world's diabetic capital. According to the International Diabetes Federation (IDF) diabetes atlas report 2015 India holds a second largest adult (69.2 million) population with diabetes. We found several studies in India which were carried out to evaluate either only direct cost or both direct and indirect cost burden. In 1999 Bangalore, among 611 diabetic patients were reported INR 15460 as direct and INR 3572 as indirect cost per patient annually (Rayappa, 1999). The total indirect cost for non-wage and wage earning respondents was estimated to be INR 9748 and INR 16 831 respectively (Kapur, 2007). According to 2005 estimations, the cost of treatment of diabetes in northern regions of India among 50 individuals was around INR 10000 and an additional INR 4000 as loss on account of disability due to the disorder (Grover *et al.*, 2005). In recent study at total of 368 hospitalized patients with only diabetes spent INR 4493 and associated complications burdened of INR 15280 (Kumpatla *et al.*, 2013). In government, private and rural clinics in south India a randomized diabetic 606 patients were reported direct highest cost of INR 19552 in private clinic and lowest in government clinic with INR 1204 (Sharma *et al.*, 2016).

The literature review over clinical and economic burden of diabetes and its complications have provided us plethoric evidences proving that immense population of world and India will be privileged with diabetic and its complications, producing larger socioeconomic and clinical burden. Current need of the day is multidimensional approach to reduce such burdens and improve social health. In this prospective, the attempt to approach with natural available antioxidants which were proven to be reduce the metabolic deteriorations and cost of the therapy. This purpose made us a thorough review current therapeutic strategies and role of naturally available antioxidants in therapeutic spectrum.

C. Persistent consequences in the development of type 2 diabetes

Type 2 diabetes (T2D) is categorically a condition developed by metabolic homeostasis insults which is usually follow obesity-insulin resistance (IR)-type 2 diabetes. The development of metabolic disturbances into T2D i.e. from insensitivity towards insulin to pancreatic β-cell dysfunction, is a consequence of inflammatory and hormonal factors, endoplasmic reticulum (ER) stress, and accumulation of by-products of nutritional overload in insulin-sensing tissues (Muoio *et al.*, 2008a).

C.1. An overview of insulin signaling pathway

Insulin on binding with insulin receptor (IR), a tyrosine kinase receptor, activates autophosphorylation leading to further phosphorylation of insulin receptor substrates (IRS - 1 to 4). Intern, IRS binds with various protein scaffolds such as Src-homology-2-containing protein (SHC), and the c-Cbl (CBL) proto-oncogene (Cohen, 2006; Taniguchi *et al.*, 2006). Phosphorylation of IRS1 and IRS2 results to their association with the p85 regulatory subunit of phosphatidylinositol 3 kinase (PI3K). This interaction recruits the p110 catalytic subunit of PI3K to the plasma membrane, resulting in conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3). Further PIP3 facilitates binding to PDK1 (phopshoinositide dependent protein kinase 1), PDK2 and Akt (Protein kinase B). Colocalization of PDKs and AKT facilitates activation of Akt by phosphorylation at Thr308 (PDK1) and Ser473 (PDK2), leading to phosphorylation of downstream targets such as glycogen synthase kinase-3 (GSK3) and the AS160 Rab GTPase-activating protein, which in turn interacts with the small GTPase RAB10 to facilitate translocation of glucose transporter-4 (GLUT4)-containing vesicles to the cell surface (Sano *et al.*, 2007) increases glucose uptake and stored as anabolic product. The insulin triggered pathway could be down regulated by various signaling dependent regulators. Initial autophosphorylation of IR can be reversed by protein Tyrosine phosphatase-1B (PTB1B or PTPN1), and Tyrosine kinase activity of the IR is inhibited by suppressor of cytokine signalling-1 (SOCS1) and SOCS2 (Muoio *et al.*, 2008a). Tyrosine phosphorylation and activation of IRS proteins is opposed by Serine phosphorylation of these proteins in response to over nutrition and activation of stress pathways (Draznin, 2006). Activation of lipid phosphatase PTEN (phosphatase and tensin homologue on chromosome-10) leads into conversion of PIP3 into PIP2 and results into down regulation of PIP3 and signaling through PDK1–PDK2–Akt (Kato *et al.*, 2009; Lazar *et al.*, 2006).

C.2. Molecular mechanism of insulin resistance

Insulin resistance is attributed by not only the tissue-dependent ectopic metabolic mechanisms but also inter-organ communication networks that are mediated by peptide hormones and inflammatory mediators and activation of intracellular stress responses. Inflammation is a physiological process characterized by elevated number of white blood cells or increased levels of pro-inflammatory cytokines in the circulation or tissue. Over reaction of inflammatory response usually leads to multiple side effects such as tissue injury and organ dysfunction. Obesity associated inflammation starts in adipose tissue and liver with elevated macrophage infiltration and expression of proinflammatory cytokines. The pro-inflammatory cytokines enter the blood stream to cause systemic inflammation.

The first observation that blood glucose was reduced by the anti-inflammation drug such as aspirin in type 2 diabetic patients (Shoelson *et al.*, 2006). Further observations, elevated fibrinogen circulation, pro-inflammatory cytokine and TNF-α in mice adipose tissue indicate involvement of inflammatory machineries in the development of IR. Then, C-reactive protein (CRP), interleukin 6 (IL-6), plasminogen activator inhibitor-1 (PAI-1) and many other inflammation mediators were found in the plasma of obese patients or animals in the last decade (Halberg *et al.*, 2008; Kershaw *et al.*, 2004).

In addition, activities of signaling molecules in the inflammatory pathways such as IκBα kinase β (IKKβ) and c-Jun N-terminal kinase 1 (JNK1) were found to be activated in adipose tissue and liver (Hirosumi *et al.*, 2002; Yuan *et al.*, 2001a). These intracellular signaling pathways are activated in obesity by TNF-α, free fatty acids, diaglyceride (DAG), ceramide, ROS and hypoxia (Ye, 2009). TNF-α acts through its p55 receptor to inhibit IRS-1 in the insulin signaling pathway (Peraldi *et al.*, 1996). Activation of IKKβ and JNK1 involves in IRS-1 inhibition by TNF-α (Aguirre *et al.*, 2000; Gao *et al.*, 2002). In addition, TNF-α also inhibits PPARγ function in the induction of insulin resistance in adipose tissue (Gao *et al.*, 2006; Ye, 2008). The IKKβ/NF-κB pathway is a dominant inflammation signaling pathway that is extensively studied in cancer and immunology studies. The serine kinase IKK has three major isoforms including IKKα (IKK1), IKKβ (IKK2) and IKKγ, which form an enzyme complex in the cytosol (Karin *et al.*, 2000). Of the three IKK isoforms, IKKβ catalyzes IκBα phosphorylation, which makes the IκBα protein ubiquitination and then degradation in proteasome. As a result, NF-κB translocates from cytoplasm into nucleus to induce gene expression. IKKβ is able to inhibit insulin signaling by phosphorylation of IRS-1 at multiple serine residues including Ser307 and Ser270 in adipocytes (Zhang *et al.*, 2008). In the JNK-AP1 pathway, JNK (c-JUN N-terminal kinase), a ubiquitous serine kinase, is extensively studied in cancer for carcinogenesis and apoptosis. The activity of JNK was first found in the pathogenesis of insulin resistance for phosphorylation of IRS-1 S307 (S312 in human), which inhibits the insulin signaling pathway in response to TNF-α signal (Rui *et al.*, 2001). JNK has several isoforms including JNK1, JNK2, and JNK3. JNK regulates gene expression through activation of transcription factor AP-1. In the JAK/STAT pathway, STAT (signal transducer and activator of transcription) is a latent transcription factor in the cytoplasm and the activation requires tyrosine phosphorylation by JAK kinases, which induces dimerization and translocation of STAT to the nucleus. STAT3 is closely related to obesity as it is activated by IL-6, leptin, and IL-10. The pathway regulates metabolism through

central and peripheral mechanisms. Inflammation inhibits the insulin signaling activity in adipocytes and hepatocytes through several mechanisms. The first is inhibition of IRS-1 (insulin receptor substrate 1) and insulin receptor in the insulin signaling pathway (White, 2002; Ye *et al.*, 2011). IRS- 1 receives signals from insulin receptor in the insulin signaling pathway. The second is inhibition of PPARγ function (Ye *et al.*, 2011). PPARγ is a nuclear receptor that drives lipid synthesis and fat storage in cells. Its activity is dependent on ligands, which include long chain fatty acids and thiazolidinedione (TZD). It induces expression of enzymes or proteins in lipogenesis or storage through transcriptional activation. Reduction of PPARγ activity contributes to insulin resistance. The third is to increase plasma free fatty acid (FFA) through stimulation of lipolysis and blocking TG synthesis. The three pathways mediate these effects of inflammation. These effects are primarily observed in adipose tissue and liver. Muscle insulin action is not sensitive to inflammation.

In obesity, several alterations contribute to the initiation of chronic inflammation, such as ER stress, adiponectin reduction, leptin elevation, adipocytes death, macrophage infiltration and lipolysis (Ye *et al.*, 2013). Adipose tissue hypoxia has been proposed as a common root for all of these changes (Ye, 2009). Hypoxia may directly or indirectly induce expression of pro-inflammatory cytokines in fat. The representative cytokines include TNF-α, IL-1, IL-6, MCP-1 (Monocyte Chemoattractant Protein-1) and PAI-1 (Plasminogen activator inhibitor-1). Macrophage infiltration is enhanced in the adipose tissue in obesity (Di Gregorio *et al.*, 2005; Fain, 2006; Odegaard *et al.*, 2007; Weisberg *et al.*, 2003; Xu *et al.*, 2003). Macrophage is derived from monocyte and controls local inflammatory responses in tissue. Adipose tissue macrophage is much more active than adipocytes in the production of TNF-α and other pro-inflammatory cytokines. Inhibition of macrophage by TZDs was found to enhance insulin sensitivity (Hevener *et al.*, 2007; Odegaard *et al.*, 2007).

Liver inflammation is associated with hepatic steatosis (fatty liver), a result of lipid accumulation in hepatocytes. Pro-inflammatory cytokines and Kupffer cell (liver macrophages) accumulation are elevated in the inflammatory response. Although these cytokines are known to block the insulin signaling pathway in hepatocytes, the biological significance of the inflammation remains to be investigated. Inflammation may also have beneficial effects in liver in terms of regulation of metabolism. Remodeling of extracellular matrix is required in the maintenance of hepatocyte function during liver expansion. IL-6 promotes hepatocyte proliferation that is required for hepatocyte regeneration to replace

the injured hepatocytes. Liver inflammation may be a feedback response in an effort to attenuate lipid accumulation in hepatocytes. One possibility is to enhance conversion of fatty acid into glucose in hepatic gluconeogenesis, in which fatty acid-derived acetyl CoA is used as building materials in the production of glucose. The gluconeogenesis is normally inhibited by insulin and is enhanced under insulin resistance. Inflammation induces hepatic insulin resistance to promote gluconeogenesis. IKKβ overexpression in liver led to hepatic inflammation and hepatic insulin resistance (Frantz, 2005). IKKβ knockout in liver reduced inflammation and protected mice from insulin resistance (Ye, 2013). In this way, inflammation protects liver from steatosis by stimulation of lipid utilization in hepatocytes.

C.3. Mechanisms of sudden β-cell failure in type 2 diabetes

Although obesity often leads to insulin resistance, only a subset of obese, insulin-resistant individuals progress to T2D. In both animal models and humans, the triggering factor is β-cell failure, which involves a decrease in β-cell mass and deterioration of key β-cell functions such as glucose-stimulated insulin secretion (GSIS). Obesity-related β-cell failure has both similar and distinct mechanistic elements compared with the development of insulin resistance in liver and muscle. β-cell mass is increased in obese non-diabetic humans compared with lean controls, and is decreased in obese patients with impaired fasting glucose or T2D (Butler *et al.*, 2003). Similarly, β-cell apoptosis is increased in obese humans with glucose intolerance or diabetes. Current evidences suggest that β-cell failure occurs as a combined consequence of metabolic overload, oxidative stress, increased rates of apoptosis, and loss of expression of fundamental components of the insulin granule secretory machinery, but specific genetic mutations that predispose to these events in patients with non-MODY (maturity onset diabetes of the young) T2D remain to be identified (Association, 2014; Vaxillaire *et al.*, 2017).

In metabolic overload, chronic exposure of pancreatic islets to elevated levels of nutrients induces β-cell dysfunction and ultimately triggers β-cell death. Exposure of isolated rodent islets to hyperglycaemia for several days raises basal insulin secretion but abrogates insulin secretion in response to stimulatory glucose (Khaldi *et al.*, 2004; Yan *et al.*, 2016). Similarly, exposure of islets to elevated levels of fatty acids does not impair GSIS unless the islets are cultured at or above a threshold concentration of glucose (Poitout *et al.*, 2002; Prentki *et al.*, 2002). These and other findings have led to the concept of β-cell functional impairment as a consequence of ‘glucolipotoxicity’ rather than as a consequence of exposure to either nutrient alone.

Further, changes in mitochondrial metabolism may synchronize with other effects of chronic lipid exposure in β-cells. Exposure of islets or insulinoma cell lines to elevated fatty acid levels increases uncoupling protein-2 (UCP2) expression. UCPs is one of the mitochondrial anion carrier proteins which separates oxidative phosphorylation from ATP synthesis with energy dissipated as heat, also referred to as the mitochondrial proton leak resulting in impaired ATP production during glucose stimulation(Chan *et al.*, 2004; Joseph *et al.*, 2004; Joseph *et al.*, 2002; Medvedev *et al.*, 2002). Subsequently this increases the oxidative stress in β-cells. Compelling evidence indicates that the accumulation of reactive oxygen species (ROS), such as hydroxyl radical and hydrogen peroxide (H2O2) generated by increased glucose and/or lipid metabolism, causes cell inactivation and death and such elevated levels of oxidative stress markers are significantly estimated in human T2D islets. Notably, islet β cells have unusually low antioxidant enzyme levels (e.g., glutathione and catalase), thus susceptible exposing their proteins, lipids, and/or DNA to oxidative modifications (Colombo *et al.*, 2002; Ebihara *et al.*, 2001). Importantly, antioxidant treatment can prevent the onset of diabetes in animal models of T1D (Oral *et al.*, 2002) as well as improve β cell function in T2D animal models (Abel *et al.*, 2001) and human T2D islets.

Furthermore, several important clues have been revealed interesting role of ER stress β-cells dysfunction. The protein kinase RnA (PKR)-like ER-associated kinase (PERK) is an important regulator of protein translation in mammalian cells because it phosphorylates and inhibits eukaryotic translation initiation factor-2a (eIF2a). Regulation of PERK–eIF2a is important for linking ER stress to the control of protein translation. Humans and mice that lack PERK have profound β-cell dysfunction and are severely diabetic, whereas mice with a mutation in the PERK phosphorylation site in eIF2a have fewer β-cells and are diabetic as a result of insulin deficiency. Moreover, feeding of heterozygous Eif2a-mutant mice on a high-fat diet causes distension of the ER lumen in β-cells, a reduction in islet insulin content and diabetes (Harding *et al.*, 2001; Scheuner *et al.*, 2001).

These complexities what we reviewed may combined and combat the root causes of this disease by targeting over nutrition, energy imbalance and cellular stress responses that are induced by metabolic overload. On contrary the detailed studies are required to perceive a better insight on genetic modification and further cellular stress to identify the optimistic target for therapy. Not only progressive IR and β-cell dysfunction, but also the same root causes are contributing in parallel and consequential development of deteriorating vascular complications, majorly cardiovascular and renal complications.

D. Molecular pathology of diabetic cardiovascular and renal complications.

D.1. Diabetic cardiovascular complications:

An individual with diabetes is higher susceptible to the risk of cardiovascular disease (CVD) than non-diabetic person (Bugger *et al.*, 2014; Troncoso *et al.*, 2011). The risk is almost equivalent to that of person having previous history of a myocardial infarction. Such CVDs account for more than half of the mortality seen in the diabetic population. In type 1 diabetes, it is not common to see progression to CVD without an impairment in kidney function. In type 2 diabetes, kidney disease remains a major risk factor for premature CVD, in addition to dyslipidemia, poor glycemic control, and persistent elevations in blood pressure. Cardiovascular disorders in diabetes include premature atherosclerosis, manifest as myocardial infarction and stroke as well as impaired cardiac function, predominantly diastolic dysfunction. The CVD is a multifactorial consequence resulted from hyperglycemia and IR. The mediators by which cardiovascular insult leads into deteriorative physiology are advanced glycation end products (AGEs), fibrosis, impared Ca+2 handling, increased free fatty acid utilization, lipotoxicity, mitochondrial dysfunction altered insulin signaling, oxidative stress, inflammation, apoptosis / necrosis, autophagy, and ER stress ((Bugger *et al.*, 2014)).

Advanced glycation end products (AGEs) are predominantly long-lived proteins that become glycated after exposure to sugars, which alters their functional properties (Goldin *et al.*, 2006). The increased formation of AGEs secondary to hyperglycaemia may alter structural proteins and lead to increased myocardial stiffness. AGEs can cause crosslinks in collagen molecules, thereby impairing the ability of collagen to be degraded, leading to increased fibrosis with subsequent increased myocardial stiffness and impaired cardiac relaxation (Norton *et al.*, 1996). AGEs also act via AGE receptors (RAGE), the expression of which is induced in diabetic hearts by oxidative stress (Aragno *et al.*, 2006). Increased AGE and RAGE activation leads to activation of nuclear factor κB (NF-κB) signaling, which may contribute to the switch towards increased expression of the β-myosin heavy chain (MHC) isoform in diabetic hearts (Aragno *et al.*, 2006). Treatment with dehydroepiandrosterone counteracts oxidative stress-induced RAGE activation both in hearts of streptozotocin (STZ)-induced diabetic rats and Zucker diabetic fatty (ZDF) rats and normalizes NF-κβ signaling and the MHC isoform switch, which are early events in diabetic cardiomyopathy. In addition, hearts of STZ diabetic rats exhibit crosslinked AGEs on the sarcoplasmic / endoplasmic reticulum calcium ATPase (SERCA)-2a pump, which may impair sarcoplasmic reticulum (SR) Ca^{2+} reuptake in cardiac myocytes (Bidasee *et*

al., 2004; Kranstuber *et al.*, 2012). Long-term treatment with an AGE crosslink breaker partially normalizes SR-Ca^{2+} handling. Hemodynamic impairments were prevented by RAGE gene knockdown (Ma *et al.*, 2009).

Further, Diabetes is on the other hand known as a pro-inflammatory state and many groups have reported an increase in tissue concentrations of cytokines in various mouse models of diabetes suggesting an important contribution of inflammation to the development of diabetic cardiomyopathy. Studies demonstrated intra-myocardial inflammation in diabetic cardiomyopathy including increased expression of intra cellular adhesion molecules (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), increased infiltration of macrophages and leucocytes and increased expression of inflammatory cytokines (IL-1β, IL-6, IL-18, TNF-α and TGF-β1) (PACHER *et al.*, 2013; Tschöpe *et al.*, 2005; Westermann *et al.*, 2007). Other proposed mechanisms for increased inflammation in diabetic cardiomyopathy include oxidative stress via a Ras-related C3 botulinum toxin substrate 1 (RAC1)-mediated activation of NADPH oxidase and endoplasmic reticulum (ER) stress (Li *et al.*, 2010). Although most of these mechanisms have been studied in animal models of insulin-deficient (type 1) diabetes, a smaller number of studies in animal models of type 2 diabetes, such as the Zucker diabetic rat (Jadhav *et al.*, 2013), the low dose STZ and high-fat diet rat model (Ti *et al.*, 2011) or mice with diet induced obesity and diabetes (Monji *et al.*, 2013), also revealed increased myocardial inflammation that was driven in part by increased activation of M1 macrophages. Data on myocardial inflammation in humans with diabetes are lacking to date.

Next, Oxidative stress is widely accepted to play an important role in the development and progression of diabetes and its complications. Generated ROS can directly damage proteins or phospholipids by oxidation, or secondarily, by oxidising lipids to reactive lipid peroxides, or by generating reactive nitrogen species from nitric oxide. DNA is another major site of ROS induced damage and mitochondrial DNA has been proposed to be particularly susceptible to oxidative damage. Evidence of increased oxidative stress in human diabetic hearts has been provided by Anderson et al who demonstrated increased mitochondrial H2O2 emission and increased levels of 4-hydroxynonenal-modified proteins and 3-nitrotyrosinemodified proteins (Anderson *et al.*, 2009). In animal models of diabetes, several independent groups reported increased mitochondrial protein tyrosine nitration, increased levels of lipid peroxidation products, decreased levels of reduced glutathione and induction of the antioxidant defense system (Boudina *et al.*, 2007; Lashin *et al.*, 2006; Turko *et al.*, 2003; Ye *et al.*, 2004). More causal evidence for a role of ROS

in the pathogenesis of diabetic cardiomyopathy comes from studies by Epstein and colleagues, who demonstrated that transgenic overexpression of catalase or manganese superoxide dismutase in diabetic mice at least partially restored impaired mitochondrial function and cardiomyocyte contractility (Shen *et al.*, 2006). Interestingly, although short-term incubation of cardiomyocytes from non-diabetic control animals under high glucose conditions did not increase ROS production, similar treatment of cardiomyocytes isolated from the OVE26 mouse model of type 1 diabetes induced ROS overproduction. A mitochondrial source for these ROS was confirmed by overexpression of catalase or by inhibition of OXPHOS complex I or II. Thus diabetes-induced changes in mitochondria increase their propensity to overproduce superoxide, which is exacerbated in a hyperglycaemic environment. Further evidence for a mitochondrial source of ROS in diabetic hearts comes from Abel's group who directly demonstrated increased production of mitochondrial superoxide in diabetic db/db mouse hearts. Interestingly, some models of type 1 diabetes do not exhibit increased mitochondrial superoxide generation in the heart, including the Akita mouse model and STZ-induced diabetes, suggesting that mitochondria might not be the major or sole source of ROS across multiple diabetes models, or that certain mechanisms are distinct between animal models of type 1 and type 2 diabetes (Herlein *et al.*, 2008). Others have also shown increased myocardial NADPH oxidase-derived ROS production in STZ-induced diabetes, ob/ob mice and obese Zucker fa/fa rats; this is consistent with the model that oxidative stress in diabetic hearts results both from mitochondrial and extra mitochondrial sources (Serpillon *et al.*, 2009; Wold *et al.*, 2006). In beginning, the vulnerable metabolic risk factors including increased triglycerides and cholesterol levels, insulin resistance, and blood pressure simultaneously up regulates renin angiotensin system (RAS) and angiotensin converting enzyme receptor 1 (AT 1) expression resulting in cardiovascular deterioration (Napoli *et al.*, 2011). In vitro and in vivo experiments have shown growth promoting actions of Ang II, causing cardiac and vascular hypertrophy, cell differentiation, and apoptosis (Karnik *et al.*, 2015b). In general, angiotensin receptor type 1 blocker ARBs effectively prevent cardiac, vascular, and renal hypertrophy. Involvement of ERK1/2, PI3K, and CDK2 inhibition pathways, leading to G1-phase arrest, causes myocyte hypertrophy. Increase in protein synthesis involves activation of translation elongation factor-2 in cardiac myocytes via dephosphorylation by PP2A by a process that involves both PI3K and MAPK (Everett *et al.*, 2001). Increased protein synthesis through AT 1 receptors in human cardiac fibroblasts did not induce hypertrophy of cardiac fibroblast (Hou *et al.*, 2000). In the vasculature, DNA synthesis is

enhanced upon Ang II infusion through the activation of cyclin D1 and cdk4 and reduction in the expression of cell cycle kinase inhibitors p21 and p27 (Diep *et al.*, 2001). Thus, in the myocardium, regulation of growth effects by AT 1 receptor in myocytes and fibroblasts differs. Cardiac hypertrophy includes cardiac myocyte enlargement and proliferation of cardiac fibroblasts. Now it is generally believed that both hypertrophic response of myocytes and proliferative response of fibroblast may depend on other modifying factors such as production of ROS and secretion of various types of factors.

For instance, in pressure overload due to hypertension and myocardial infarction, cardiac remodeling process includes cardiomyocyte hypertrophy, extracellular matrix synthesis, fibrosis, and loss of compliance, leading to fatal outcomes. TGF β1 expression is increased in myocytes and fibroblasts of heart, which trans differentiate into a myofibroblast phenotype, resulting in myocardial remodeling (Baum *et al.*, 2011). The AT 1 receptor directly increases TGF β1 expression, translocation of Smad proteins Smad 2 and 4 into the nucleus, resulting in expression of fibrotic marker proteins, collagen, fibronectin, and connective tissue growth factor (CTGF) (Lim *et al.*, 2006; Zhang *et al.*, 2009). CTGF is a profibrotic factor that stimulates both Ang II and TGF β1 mediated fibrosis and apoptosis (Cabello-Verrugio *et al.*, 2011a). CTGF is involved in myocardial remodeling mediated via AT 1 receptors during transition to heart failure (Cabello-Verrugio *et al.*, 2011b). Gene expression analysis detected high CTGF mRNA expression in coronary artery biopsies from ischemic injury and coronary artery disease. Proteins involved in extracellular matrix remodeling, such as thrombospondin 4, collagen type 1 and 2, and fibronectin, and the inflammatory cytokines, such as IL-8, IL-6, vascular cell adhesion molecule-1, and monocyte chemoattractant protein-1 (MCP-1), are believed to couple cardiac remodeling with chronic angiotensin receptor stimulation (Gabrielsen *et al.*, 2007). IL-6 secretion by cardiac myocytes is regulated by Ang II. The effects of IL-6 on cardiomyocyte hypertrophy and fibroblast proliferation is inhibited by the AT 1 receptor antagonist losartan, suggesting that IL-6 contribution to cardiomyocyte hypertrophy is mediated by the AT 1 receptor (Fredj *et al.*, 2005). Ang II induced activation of the JAK/STAT pathway is involved in tissue remodeling after vascular injury and myocardial ischemia in rats (Omura *et al.*, 2001; Seki *et al.*, 2000). The AT 1 receptor activates STAT1 and GATA4 transcription factors in the development of myocyte hypertrophy (Wang *et al.*, 2005). Chronic activation of the AT 1 receptor in myocytes induces transcription of the Stat3 gene by pSTAT3 and overproduction of STAT3 protein, leading to nuclear accumulation of

STAT3 without tyrosine phosphorylation, which alters the transcriptional program of cardiac hypertrophy (Yue *et al*., 2010).

VSMC hypertrophy induced by Ang II involves PKC delta activation through Src-dependent Tyr phosphorylation, leading to Akt activation and signifying a novel molecular mechanism for enhancement of cardiovascular diseases induced by Ang II (Nakashima *et al.*, 2008). Anti-apoptotic effects of Ang II in cardiomyocytes and VSMCs are regulated by a mechanism involving PI3-kinase/Akt activation, subsequent upregulation of survivin, and suppression of caspase-3 activity (Ohashi *et al.*, 2004). Inhibitors of Akt and a dominant-negative mutant of Akt selectively block Ang II-induced proliferation of CHO-AT 1a cells (Dugourd *et al.*, 2003). Ang II activated reactive oxygen species acting through Src/caveolin-EGFR signaling pathway induces epithelialto-mesenchymal transition in renal epithelial cells. This may be a novel molecular mechanism involved in progressive renal injury caused by chronic exposure to Ang II (Chen *et al.*, 2012). Adenoviral-directed expression of the AT 1 receptor has defined the EGFR transactivation pathway for cardiac hypertrophy via PI3K/Akt signaling (Thomas *et al.*, 2002).

D.2. Diabetic nephropathy:

The deregulated metabolic lifestyle (including hyperglycemia, hyperlipidemia, and insulin resistance) initiates diabetic nephropathy (DN). Diabetes Complications and Treatment trial established that tight glucose control (HbA_{1c} level of 7% vs. 9%) reduces the development of DN risks by more than 50% in T1D patients (DCCT, 1993; Nathan *et al.*, 2014). Surprisingly, recent large clinical trials Action to Control Cardiovascular Risk in Diabetes (ACCORD), veterans affairs diabetes trial (VADT) and The Action in Diabetes and Vascular Disease: Preterax and Diamicron Controlled Evaluation (ADVANCE) failed to show a statistically significant benefit for reducing HbA_{1c} to less than 7% in T2D patients (Dluhy *et al.*, 2008; Group, 2008a; Group, 2008b). These results are unexpected and indicate that while hyperglycemia plays a critical role in DN initiation. One problem in interpretation may be that patients with T2D might have experienced years of metabolic alterations prior to being diagnosed with diabetes, which may contribute to DN even before they receive the diagnosis of T2D (Ko *et al.*, 2013).

Hyperglycemic initiated with environment provokers such as cellular alterations, including abnormalities in substrate delivery and altered ratios of cell-specific fuel sources (glucose intermediates, fatty acids, and amino acids, changes in respiratory chain protein function, and uncoupling of the respiratory chain). Several theories explain that cells that are unable to down-regulate their glucose transporters in the setting of extracellular hyperglycemia

experience an increase in their intracellular glucose concentration (Brasacchio *et al.*, 2009). Glucose can be oxidized in the cytoplasm via glycolysis; however, for efficient energy production, mitochondrial oxidative phosphorylation is preferred. In diabetes, glucose is shunted to the fructose 6-phosphate and hexosamine pathway (Kolm-Litty *et al.*, 1998). In this pathway, fructose 6-phosphate is diverted from glycolysis to provide substrate for the rate-limiting enzyme glutamine:fructose 6-phosphate aminotransferase. In diabetes, there is increased glucose oxidation by the polyol pathway as well. A family of aldo-keto reductase enzymes can use a wide variety of carbonyl compounds as their substrates and reduce these to their respective sugar alcohols (polyols) using NADPH. Animal studies indicate an early increase in metabolic flow via all these pathways and suggest their potential damaging roles (Mihalik *et al.*, 2012). Hyperglycemia also increases the non-enzymatic reaction of glucose and other glycating compounds derived both from glucose and from increased fatty acid oxidation, which generates advanced glycation end products (AGEs) in complication-prone cell types, including kidney cells (Wendt *et al.*, 2003). Further, increased reactive oxygen species (ROS) and superoxide generation by dysfunctional mitochondria in diabetes has been postulated as the primary initiating event in the development of diabetic complications (Brownlee, 2001; Brownlee, 2005). There are two major sites within the mitochondria where electron leakage can occur to produce superoxide, namely NADH dehydrogenase (complex I) and complex III (Forbes *et al.*, 2013). In addition to hyperglycemia, other metabolic factors, such as increased free fatty acid levels, changes in adiponectin, as well as insulin levels and resistance, contribute to metabolic imbalance and disease initiation (Rutkowski *et al.*, 2013; Sharma *et al.*, 2008; Susztak *et al.*, 2005). Fatty acid, insulin, and adiponectin levels are different in T1D versus T2D subjects; therefore, some of these metabolic differences may underlie different pathogenic pathways in type 1 diabetic nephropathy (T1DN) versus type 2 diabetic nephropathy (T2DN) development. For example, recent reports indicate that obesity (associated with T2D) induced modulation of adipokine levels might be an important factor in DN (Rutkowski *et al.*, 2013; Sharma *et al.*, 2008; Susztak *et al.*, 2005). Differences in insulin levels and resistance may also be central factors in DN. Animals with complete deletion of the insulin receptor from podocytes develop severe albuminuria and glomerulosclerosis, changes that are similar to human DN but occur in the absence of hyperglycemia (Welsh *et al.*, 2010). Overall, our understanding of the differences between T1DN and T2DN is poor.

Next, DN is usually classed as a microvascular complication of diabetes. Beginning metabolic injury-induced loss of microvascular endothelial cells is compensated by abnormal angiogenesis, inducing multiple fragile small blood vessels, which is likely mediated by VEGF, angiopoetins, endothelins, and nitrix oxide (Jeansson *et al.*, 2011). A tight balance of angiogenic factors is necessary for maintaining the glomerular filtration barrier. For example, both increased and decreased VEGF expression causes albuminuria and glomerular changes (Bertuccio *et al.*, 2011; Eremina *et al.*, 2008). Endothelial dysfunction and loss of glomerular and tubulointerstitial capillaries are key contributors to epithelial injury during DKD progression. While mesangial expansion and glomerular basement membrane thickening are the most commonly observed DN lesions. Cell analysis using samples from T1D and T2D patients indicated that podocyte number is highly correlated with proteinuria and appears to be one of the best disease predictors (White *et al.*, 2002). Podocyte loss may follow from hyperglycemia induced ROS generation causing podocyte apoptosis or detachment (Susztak *et al.*, 2006). ROS release occurs both from mitochondrial and plasma membrane NADPH oxidase (NOX) sources (Susztak *et al.*, 2006). Multiple lines of evidences indicate that podocyte dropout is a critical factor for DN development. Glomerular podocyte density is the best predictor of albuminuria (Meyer *et al.*, 1999; Pagtalunan *et al.*, 1997). Greater than 20% podocyte loss in animal models causes irreversible glomerular damage, manifesting as albuminuria; glomerulosclerosis then progresses to tubulointerstitial fibrosis and end-stage kidney failure. Because podocytes are terminally differentiated cells that are unable to replicate or significantly regenerate in adults, their injury appears to be the common link and the primary insult leading to glomerulosclerosis and DN.

In deoxycorticosterone acetate (DOCA)-salt hypertensive rats or SHRSP, which prominently exhibit progressive glomerulosclerosis, renal TGF-β1 and ECM protein RNA levels are higher than those in normotensive control rats (Kim *et al.*, 1994). Treatment of DOCA-salt hypertensive rats with candesartan cilexetil (1 mg/kg/day) (an angiotensin II receptor antagonist) or enalapril (10 mg/kg/day), although not decreasing blood pressure, significantly reduced urinary protein and albumin excretion and induced histological improvement in renal lesions, in association with decreases in renal cortical mRNA levels for TGF-β1, fibronectin, laminin, and collagen types I, III, and IV (Kim *et al.*, 2000). Also in SHRSP, candesartan cilexetil (0.1 mg/kg/day), without significantly decreasing blood pressure, significantly reduced renal TGF-β1, fibronectin, laminin, and collagen types I, III, and IV mRNA levels (Kim *et al.*, 2000). These findings provide in vivo evidence

implicating Angiotensin II (Ang II), via AT1 receptor, in renal injury in these hypertensive models, due to enhanced renal TGF-β1 and ECM expressions.

The given study examples represent important role of RAS in the development of DN. RAS plays an integral role in the homeostatic control of arterial pressure, tissue perfusion, and extracellular volume. In addition to its systemic and local renal hemodynamic effects. RAS also influences renal tissue cell infiltration and inflammation (Fujihara *et al*., 2000; Benigni *et al*., 2004; Graciano *et al*., 2004; Franco *et al*., 2007). Thus, the dysregulation of RAS may lead to hypertension and renal tissue injury. Ang II is the most powerful biologically active product of the RAS. In addition, Ang II directly induces podocyte injury via the activation of AT1 receptors, independent of hemodynamic changes (Durvasula *et al*., 2004; Liang *et al*., 2006; Liebau *et al*., 2006). Moreover, Ang II interacts with various local autocrine and paracrine factors, such as NO, eicosanoids, adenosine, and superoxide, to influence the glomerular filtration rate. It is of interest to note that glucose increases the expression of the angiotensinogen gene in proximal tubule cells and Ang II production in mesangial cells, suggesting that high glucose itself activates the renin-angiotensin system (Singh *et al*., 1999; Leehey *et al*., 2000; Vidotti *et al*., 2004). Numerous studies suggest that Ang II has been implicated in the progression of DN through multiple pathways. Ang II increases the level of mesangial TGF- β mRNA and increases the production of both latent and active TGF-β, which in turn results in extra cellular matrix accumulation by increasing the synthesis of matrix proteins, such as fibronectin, collagens, and laminin, and by inhibiting matrix degradation (Kagami *et al*., 1994; Egido *et al*., 1996). It has also been noted that TGF-β further increases the expression of PAI-1 (Kutz *et al*., 2001). It is still of interest to note that Ang II itself directly increases PAI-1 expression through an AT1 receptor dependent mechanism independent of TGF-β (Kagami *et al*., 1994; Kagami *et al*., 1997; Nakamura *et al*., 2000). Apart from fibrotic elements, such as TGF-β, PAI-1, Ang II has also been shown to cause renal fibrosis by upregulating the expression of Rho-A and activating the Rho/Rho kinase pathway (Ruiz-Ortega *et al*., 2006). Further, inflammatory cytokines such as interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) have been noted to be involved in the development and progression of DN (Wada *et al*., 2000; Mora-Fernandez *et al*., 2008). Interestingly, Ang II regulates the expression of inflammatory cytokines, such as IL-6 and MCP-1, in the kidney. The detrimental role of Ang II was confirmed when it was shown that the inhibition of Ang II improves DN through the suppression of renal MCP-1 (Amann *et al*., 2003). Moreover, VEGF has been implicated in the pathogenesis of DN, as VEGF is upregulated early in

diabetes mellitus, especially in podocytes (Cooper *et al*., 1999; Cha *et al*., 2000; Cha *et al*., 2004). Ang II stimulates the synthesis of VEGF in podocytes through the activation of the p38 mitogen-activated protein kinase (p38 MAPK) pathway, suggesting another mechanism through which Ang II may play a crucial role in the pathogenesis of DN (Kang *et al*., 2006). Numerous studies have also revealed that Ang II increases the renal production of reactive oxygen species (ROS) by activating NADPH oxidase (Giacchetti *et al*., 2005). Ang II also mediates ROS generation via the activation of NADPH, which in turn further stimulates VEGF synthesis (Feliers *et al*., 2006). Taken together, it may be concluded that Ang II is a master molecule of renal injury during diabetes.

E. Epigenetics and role in diabetes and its progressive complications

E.1. Epigenetics and histone code

Embryogenesis is a continuous cell division and cellular differentiation process from fertilized egg and associated with rapid transcriptions, repairing and replication of genetic material for introducing unique phenotypic identity for each cell type with the help of genetic regulation machineries. The regulation of genetic expression during continuous development of complex organisms without any change in the DNA sequence is known as epigenetics. Uninterruptable epigenetic regulations carry forward throughout the life and generation to generation to keep their identity or required modification according to the environment and life style. Further these chromatin modifications depend upon specific epigenetic modifications such as DNA methylation, post-translational modifications of histones, ATP dependent chromatin remodeling, histone variants and non-coding RNA regulations. These chromatin modification process, mark activation and silencing of various genes those could be pathogenic or non-pathogenic genes.

Chromatin is an assembly of eukaryotic genome with the help of histones that provides dynamic platform for controlling all DAN mediated process within the nucleus. The smallest basic unit of chromatin is nucleosome. In the nucleosome 147 bp of DNA wrapped approximately twice around the histone octamer. Histone octamer consists of two each of H2A, H2B, H3 and H4. Further each nucleosome is separated by 10-60 bp of linker DNA. This structural organization of chromatin hinders the accessibility to DNA for transcriptional factors, RNA polymerase and DNA polymerase enzymes. Each octamer of histone exhibit interactions within the histones and nucleosomal DNA that enables genuine wrapping around the octamer. Along with, each histone consists of residual segments, which extend form surface of the nucleosome known as histone tails. These are rich with basic amino acids, which contribute in the folding nucleosomal array to further

condensation of chromosome rather than providing stability to individual nucleosome. Histones are subject of post-translational modification with the help of several recruiting proteins those catalyze the acetylation, deacetylation and methylationof lysine (K) and arginine (R), phosphorylation of serin (S) and threonine (T), SUMOylation of K, ubiquitination of K and biotinylation of K to characterize transcriptional regulation, DNA repair and replication. Theses histone post-translational modifications are significant at amino and carboxy terminals of histone tail domain as compare to modifications within the histone central domain. Such histone modifications control chromatin fiber for unique DNA mediated processes. Certain pattern of post-translational modification of histone participate in global chromatin modifications such as acetylation of H3K9, H4K5 and H4K12 is associated with S phasic histone deposition and phosphorylation H2A at S1 and T119 and H3 at T3, S10 and S28 associated with mitotic chromatin condensation. In addition, histone acetylation correlates with transcriptional activity. Enriched methylation of H3 at K4, K36 and K79 fond in transcriptional active region and at K9 and K27 in silent region, specifically heterochromatin. This unique fashion of histone modifications provide framework to understand downstream consequences. Such pattern of modifications could referred as “mark”/ histone mark rather than code. For instance, H3K9 methylation after recruiting histone methyl transferase (HMT) enzyme Ash1 results into transcriptional activation (Fillingham *et al.*, 2008; Lee *et al.*, 2010; Turner, 2002).

E.2. Post-translational histone modifications in diabetes

Several studies have been devoted to the evaluation of genetic factors related to type 2 diabetes and associated complications. Although the avoidance of risk factors undoubtedly constitutes the most rewarding approach to the prevention of atherosclerosis, it has thus far been frustrated by inadequate patient compliance and the influence of genetic factors in determining an individual’s predisposition to atherosclerosis. The genetic factors which are utterly depend on the chromatin, which is not only scaffolding structure for genomic DNA but also a dynamic entity that can regulate gene expression and cellular functions. Nucleosomes of chromatin are composed of genetic DNA wrapped around an octamer of core histone proteins consisting of two copies of histones H2A, H2B, H3 and H4. This kind of packaging of genes into chromatin restricts access of transcription factors and the basal transcription machinery to target promoters (Allan *et al.*, 1982; Bradbury, 1978; Gasser *et al.*, 1998; Woroniecki *et al.*, 2011). The posttranslational modifications of histone proteins represent chemical variations to the chromatin template, which control sequence structure and gene function. The diversity of modifications as well as the pattern

and distribution can reflect different structural and functional roles mediated by epigenetic change. Combinatorial actions of these modifications form a ‘histone code’ that dictates the ‘repressed’ or ‘active’ states of chromatin (Reddy *et al.*, 2011b).

Histone H3 lysine acetylation (H3KAc) such as H3K9Ac, H3K14Ac, and H3K27Ac is generally associated with active gene promoters. Histone acetyltransferases (HATs) mediate H3KAc and histone deacetylases (HDACs) remove it. Histone H3 arginine methylation (H3Rme) is mediated by protein arginine methyltransferases (PRMT) such as co-activator-associated arginine methyltransferase 1 (CARM1) and generally activates gene expression (Kouzarides, 2007; Lee *et al.*, 2005). Histone lysine methyltransferases (HMTs) mediate histone lysine methylation (HKme), which can be associated with either active or repressive gene expression depending on the lysine modified. Furthermore, HMTs can mediate mono- (me1), di- (me2), or trimethylation (me3) of specific lysine residues to add an extra layer of regulation (Kouzarides, 2007; Shilatifard, 2006). H3K9me2, -me3 and H3K27me2, -me3 are generally repressive marks, whereas H3K4me is generally an active mark. H3Kme is relatively stable and evidence shows that it can be epigenetically transferred (Margueron *et al.*, 2010). The recent discovery of histone lysine demethylases (HDMs) that remove methylation marks from specific lysine residues demonstrated the dynamic nature of HKme. Emerging evidence shows an important regulatory role of HMTs and HDMs in diverse physiological processes and disease conditions (Portela *et al.*, 2010; Shi, 2007). Interestingly, some of these enzymes can also modify non-histone proteins including p53 and NF-κβ further re-enforcing their growing importance in cellular processes.

H3K4 demethylase lysine-specific demethylase 1 (LSD1), and an H3K9me2 methyltransferase SET domain bifurcated 1 (SETDB1) were implicated in adipogenesis (Musri *et al.*, 2010). Furthermore, on contrast with vascular complications under diabetic condition, it was demonstrate that H3 lysine-9 tri-methylation (H3K9me3) and H3K9me3 methyltransferase Suv39h1, a key repressive and relatively stable epigenetic chromatin mark involvement in metabolic memory in vascular smooth muscle cells (VSMC) derived from type 2 diabetic db/db mice (Villeneuve *et al.*, 2008b). On the other hand, protein levels of lysine-specific demethylase1 (LSD1), which negatively regulates H3K4 methylation and its occupancy at these gene promoters, were significantly reduced in db/db VSMCs (Reddy *et al.*, 2008a). A number of studies have now explored epigenetic mechanisms in inflammatory gene expression in vascular cells which is the key regulator for induction and progression of atherosclerosis. Increased inflammatory gene expression

required collaboration of transcription factors such as NF-kB and cAMP response element-binding protein with HATs including p300/CBP, steroid receptor co-activator-1, and pCAF (Reddy *et al.*, 2009). Also the epigenetic changes like decreased superoxide dismutase 2 expression in pulmonary hypertension (PH), as well as trimethylation of histones H3K4 and H3K9 in congestive heart failure has been found (Kim *et al.*, 2013). These multifactor epigenetic influences on VSMC, exhibit phenotype changes for progression of atherosclerosis.

F. Esculetin: a coumarin derivative antioxidant in multi therapeutic approach

F.1. Esculetin: an insight

F.1.1. Chemistry, Pharmacokinetics and Toxicity

Around 4,000 different phytochemicals are known to possess potential activity against various diseases like cancer and metabolic or degenerative diseases. Amongst all, coumarins are naturally occurring phytochemicals that are related to benzopyrones family (1, 2-benzopyrones or 2H-1-benzopyran-2-ones) and possess a broad range of therapeutic activities like anti-tumor (Huang *et al.*, 2011), anti-inflammatory (Fylaktakidou *et al.*, 2004), anti-oxidant (Kostova *et al.*, 2011), anti-diabetic (Patel *et al.*, 2012) and antidepressant effects (Sashidhara *et al.*, 2011). Structurally, coumarins are composed of a benzene ring with fused α-pyrone ring. In addition, it procures a conjugated system with ample electron and charge-transport properties (Matos *et al.*, 2015). So, many coumarin compounds have been actively studied for their robust quality with strong pharmacological activity, low toxic or side-effects, lesser drug resistance, high bioavailability, broad spectrum and better curative effects (Archier *et al.*, 2012; Budzynska *et al.*, 2015; Ciaravino *et al.*, 2001; Sim *et al.*, 2014).

Despite these compounds sorted out into various classifications, chemically they are categorized as: simple coumarins, furocoumarins, dihydrofurocoumarins, pyranocoumarins, phenylcoumarins and biscoumarins. Among these class of coumarins; esculetin is categorized under simple coumarins which also known as cichorigenin. Esculetin, a 6,7-dihydroxy coumarin found in many plants (Anand *et al.*, 2012; Bora *et al.*, 2010; Xin wei *et al.*, 2014) such as *Artemisia capillaries* (Xin wei *et al.*, 2014), *Artemisia scoparia (Yue et al., 1997),* the leaves of *Citrus limonia* (Chang, 1990), *Cortex fraxini* (Wu *et al.*, 2007; Zhang *et al.*, 2000), *Ceratostigma willmottianum* (Yue *et al.*, 1997), *Viola yedoensis* (Oshima *et al.*, 2012), *Aesculus hippocastanum* (Wilkinson *et al.*, 1999) and *Cichorium intybus* (Ahmed, 2009).

The pharmacokinetic studies of esculetin have also been done to evaluate its absorption, distribution, metabolism and excretion. The distribution of esculetin was evaluated with the help of high performance liquid chromatography (HPLC) and time of flight mass spectroscopy (TOF/MS) in the plasma and tissues of rats. The maximum plasma level of esculetin was found to be highest at 5 min post-oral administration and half-life in the plasma was reported to be of 45.0 min (Kim *et al.*, 2014). So, its pharmacokinetic profile suggests that its absorption and elimination from plasma is very fast and is also quickly distributed and eliminated from tissues.

Some of the coumarin derivatives were reported to be toxic but in a recent study Esculetin (6, 7-dihydroxycoumarin) was found to be non-mutagenic and non genotoxic or clastogenic/aneugenic in cultured human lymphocytes (Maistro *et al.* , 2015). In addition, several studies where esculetin have been used, found safe and effective in control animals and diseased animals, respectively, at higher dose(s).

F.1.2. Esculetin as anti-oxidant

Esculetin possesses structural coumarin moiety along with two hydroxyl substitutions which has potent anti-oxidant activity and showed evidence of direct radical quenching as shown by the DPPH radical and hydroxyl radical. ROS production is a natural phenomenon inside an organism with an aerobic metabolism which results in high influx of oxygen in the mitochondria. Oxidative stress is defined as an imbalance in the redox state, which can be because of increased reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) and reduced antioxidants' defense mechanisms. Altered redox balance forms one of the main axes for development of various pathophysiological conditions, including obesity, insulin resistance, diabetes, atherosclerosis, heart failure, renal failure, cancer and several neurological disorders (behavioral physiology and neurodegenerative disorders) (Bocci *et al.*, 2015). The elevation of oxidative stress may modify metabolic regulation, cell signaling, and other cellular functions accompanied by increased free radical attack to DNA. It is well known that the overproduction of ROS leads to the impairment of foremost cellular forms like lipid peroxidation (mainly in plasmatic membranes), damage in proteins (signaling proteins, enzymes and other proteins) and genomic damage (oxidation of nucleic acids). Moreover, it has been suggested that oxidative stress leads to the activation of p53, breakage in double strand of DNA, and apoptosis (Tornovsky Babeay *et al.*, 2014). Furthermore, oxidation is inextricably linked with inflammation which is a consequential progressive factor in all NCDs. This redox imbalance becomes even more harmful when genetic variation impairs

the normal degradation of cellular and nuclear proteins (Zhang, 2008). So, therapeutic approaches should be focused on the trimming of free radical formation and scavenging of free radicals.

Pathways which participate in oxidative stress conditions like epidermal growth factor receptor/phosphatidylinositol/Akt (EGFR/PI3K/Akt) pathway, B-cell lymphoma 2/Bcl2-like protein (Bcl-2/Bax) pathway, mitogen activated protein kinase/ extracellular signal-regulated kinase (MAPK/ERK) pathway and peroxisome proliferator-activated receptor gamma/ transforming growth factor beta (PPAR-γ/TGF-β) pathway has been reported to be suppressed by esculetin treatment in various NCDs (Chang *et al.*, 2016; Kang *et al.*, 2014b; Surse *et al.*, 2011a; Won *et al.*, 2012). Moreover, in renin angiotensin system (RAS), AT1R and AT2R expressions have also been suppressed by esculetin (Figure 1). Oxidative stress related pathways leads to the activation of several downstream molecules like caspase 3, 9; Cyclooxygenase (COX); Tumor necrosis factor-α (TNF-α); Hemeoxygenase-1 (HO-1); Monocyte Chemoattractant Protein (MCP-1); PPAR-γ and fibronectin which has to be effectively suppressed by Esc. Lipid peroxidation is one of the major mechanisms of cellular injury caused by H_2O_2. However, esculetin was found to decrease H_2O_2 induced lipid peroxidation, indicating the cytoprotective properties of esculetin against H_2O_2 induced lipid damage (Zhang, 2008). Thus, above mentioned evidences show that esculetin possesses a powerful anti-oxidant activity which helps in curbing several NCDs.

F.2. Esculetin in multi therapeutic approach

F.2.1. Cancer

Cancer is characterized by unrestrained growth and spread of cells. It can occur in any part of the body. Frequently, the cancer growths conquer nearby tissues and further metastasize to different sites (Mendis, 2014). Despite its treatment through surgery, radiotherapy or chemotherapy, it still remains as one of the most dangerous and leading causes of morbidity and mortality.

Esculetin has shown a promising chemopreventive and chemotherapeutic activity in different cancers (Chang *et al.*, 1996). It has been reported that in HL-60 leukaemia cells, esculetin impedes growth and cell cycle progression by inducing arrest of the G1 phase, upshot from the inhibition of retinoblastoma protein phosphorylation (Lacy *et al.*, 2004). Esculetin induces apoptosis in HT-29 colon cancer cells via endoplasmic reticulum (ER) stress which was confirmed by mitochondrial calcium glut and expression of ER stress-related proteins such as glucose regulated protein 78, phosphorylated ribonucleic acid-

dependent protein kinase-like ER kinase, phosphorylated inositol requiring enzyme 1, phosphorylated eukaryotic initiation factor-2α, spliced X-box binding protein 1 and cleaved activating transcription factor 6.

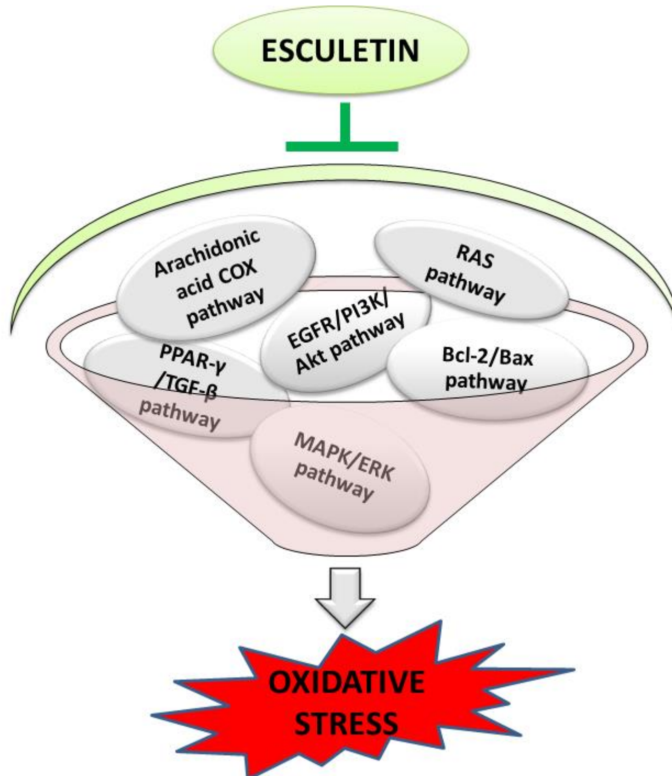


Figure 1: Several oxidative pathways result in oxidative stress which is targeted by esculetin to exert its anti-oxidant property.

Esculetin also persuaded the expression of the CCAAT/enhancer-binding protein-homologous protein (CHOP) and pro-apoptotic factors caspase-12 thereby induced apoptosis in same cancer cells (Kim *et al.*, 2015a). In ZR-75-1 human breast cancer cells, esculetin tends to rises in Ca^{2+} level by liberating Ca^{2+} from the ER and resulting Ca^{2+} influx through 2-APB-sensitive store-operated Ca^{2+} entry. Moreover, it has also been found to be involved in G2/M cell cycle arrest by activating Ca^{2+}-associated mitochondrial apoptotic pathways (Chang *et al.*, 2016). In oral squamous cell carcinoma (OSCC), esculetin has shown to significantly inhibit EGFR/PI3K/Akt pathway and it has been demonstrated that nucleophosmin which tends to be higher in cancer cells as compared to normal, gets decreased with esculetin treatment (Won *et al.*, 2012). Human malignant melanoma is the most deadly type of skin cancer which results in more than three-fourth deaths of skin cancer (Su *et al.*, 2009). In G361 cell lines of human malignant melanoma, Sp1 (Specificity Protein) transcription factor and its targeted proteins like p27, p21, and cyclinD1 which are overexpressed in cancer condition get reduced by esculetin treatment.

Moreover, it has also shown a remarkable effect in human hepatoma HepG2 cells by increasing the nuclear accumulation of the nuclear factor-like 2 (Nrf2) and further elevating the expression of NAD(P)H: quinone oxidoreductase (NQO1) at both protein as well as mRNA levels (Subramaniam *et al.*, 2011).

F.2.2. Cardiovascular diseases

Cardiovascular diseases (CVDs), the disorders of heart and blood vessels involve coronary heart disease, cerebrovascular disease, rheumatic heart disease, myocardial infarction (MI) and other conditions. A number of studies have shown the capability of controlling individual cardiovascular risk factors in preventing or slowing CVDs. Numerous natural products and their derivatives are used in order to treat these diseases (López Alarcón *et al.*, 2013). Oral pre-treatment with esculetin possesses an anti-lipoperoxidative and antioxidant effect in isoproterenol-induced MI (Karthika *et al.*, 2012). This might be due to its free radical scavenging and antioxidant properties; therefore exerts defensive role against isoproterenol-induced MI in rats (Karthika *et al.*, 2012). Esculetin effect has also been investigated in balloon angioplasty rat model where it inhibit three signaling pathways including (a) the stimulation of p42/44 MAPK and the downstream effectors of c-fos and c-jun immediate early genes, (b) the activation of nuclear factor-kappa B (NF-kappa B) and activator protein-1 (AP-1), and (c) PI3K activation and cell cycle progression (Pan *et al.*, 2003). It also suppresses MMP-9 expression through the down regulation of NF-κB and AP-1 binding activity and dynamically stops the migration and invasion of TNF-α treated vascular smooth muscle cells without any toxicity (Lee *et al.*, 2011). These results prove a promising therapeutic approach of esculetin in the treatment of cardiovascular diseases and atherosclerosis.

F.2.3. Kidney dysfunction

Kidney dysfunction is characterised by microalbuminuria, increased creatinine level and hyperuricemia. Oxonate-induced (O-I) animal model has served as a good model to assess drugs or possible therapeutic agents that reduce the overall plasma uric acid level due to associated hyperuricemia and renal function impairment (Hu *et al.*, 2013). Hyperuricemia is the main biochemical cause of gout and has been known to be associated with renal injury (Dehghan *et al.*, 2008). When esculetin and its glycosylation product esculin were used, they have shown upregulation of renal organic anion transporter 1 (mOAT1), organic cations (mOCT1 & mOCT2) and carnitine transporters (mOCTN1 & mOCTN2). It confirmed the potential role of esculetin in ameliorating hyperuricemia and kidney

function impairment (Hu *et al.*, 2013). Till now, less work has been done to evaluate the role of esculetin in kidney dysfunction, so there is plenty of scope for work in this field.

F.2.4. Neurological disorders

Neurological disorders is a broad term including the diseases of central nervous system such as Parkinson's disease (PD), stroke, dementia, Alzheimer's disease (AD), Huntington's chorea (WHO, 2006). The existing literature shows that complete cure for these ailments are still not available. Apart from synthetic molecules, several natural products have been used to treat these disorders and among them, esculetin is one of the natural products which have been used in PD, stroke and AD. Various monogenic forms of PD have been discovered with leucine-rich repeat kinase-2 (LRRK2) mutations as the major cause of sporadic and familial PD. LRRK2 is a complex protein possess the catalytic domain mutations engaged in the pathogenesis of PD (Chan *et al.*, 2013). With this study, it has been proved that natural antioxidants (like esculetin) with antioxidant as well as kinase inhibitor properties might be applicable for LRRK2-linked PD (Angeles *et al.*, 2016). Ischemic stroke is defined as a neurological disorder which actually is a ruinous form of stroke with particularly high disability that affects more than 6,90,000 adults each year (Shih *et al.*, 2005). Neuronal apoptosis induced by cerebral I/R (Ischemic/Reperfusion) injury has been reported to be protected by Esc; targeting main apoptosis-related proteins Bcl-2 and Bax (Wang *et al.*, 2012). As it is used clinically and has a safety profile, esculetin may hold a therapeutic prospective for the treatment of stroke in the clinical trials. Esculetin has shown a potential therapeutic approach towards neurodegenerative diseases which are the outcomes of brain cell death and takes place via axonal/dendritic degeneration (Ali *et al.*, 2016; Jameel *et al.*, 2016). It has remarkable ability to inhibit β-site amyloid precursor protein cleaving enzyme 1 (BACE1), butyrylcholinesterase (BChE), and acetylcholinesterase (AChE) which are responsible for the progression of AD. And hence, esculetin serve as a therapeutic agents for the treatment and prevention of AD (Ali *et al.*, 2016). Acute restraint stress (ARS) is the condition which affects both mature adult and aged mice, resulting in inability of contextual memory in the step-through passive avoidance and it has been partially treated by esculetin only in the 11 months old mice by restoring anti-oxidant enzyme activity (Martín Aragón *et al.*, 2016).

F.2.5. Diabetes and its complications

Diabetes is a chronic disease that occurs either when the pancreas does not produce enough insulin (type 1) or when the body cannot effectively use the insulin (type 2) it produced.

T2D comprises a huge percentage of people with diabetes around the world (Magliano *et al.*, 2015) and is largely the result of obesity and reduced physical activity. It is triggered by a set of multifactorial metabolic and physiological abnormalities characterized by at least four of the major components of its pathophysiology, such as central obesity, hypertension, dyslipidemia and hyperglycemia. These components lead to higher susceptibility for the progression of debilitating micro and macro-vascular complications (Miller *et al.*, 2011; Savica *et al.*, 2010). Esculetin has been proved for its capacity to increase insulin sensitivity which could partially reduce the pressure of insulin production on pancreatic β-cells and prevent further cellular damages. Under pre-diabetic condition, up-regulation of adverse intracellular cascade (PI3K/Akt), renin angiotensin system (RAS) and oxidative stress results in progression of cardiovascular diseases. Initial effects of upregulated RAS trigger the vascular perturbation characterized by reduced vascular relaxation followed by cardiac fibrosis. In this condition, esculetin protected the cardiovascular system by reducing the angiotensin mediated vascular reactivity and fibronectin/TGF-β in cardiac myocytes . When esculetin role was evaluated in obese conditions, it has shown a remarkable effect, like in 3T3-L1 adipocytes it increases HO-1 and suppresses nitric oxide, TNF-α, MCP-1, PPARγ expressions (Yang *et al.*, 2006). Moreover, under diabetic conditions, hyperglycemia is associated with the over production of advanced glycogen end products (AGE). The diabetes-related in vitro studies showed that esculetin inhibits formation of AGE-products and activities of α-glucosidase and protein tyrosine phosphatase 1B (Islam *et al.*, 2013). Recently, it was reported that esculetin showed protective effect in HFD fed low dose streptozotocin (STZ) treated rats by appreciably increasing the insulin sensitivity followed by decreasing the angiotensin II type 1 receptor (AT1R), angiotensin II type 2 receptor (AT2R), Keap1 and TGF-β expression . Esculetin also been explored for its effect on carbohydrate metabolic enzymes in STZ treated rats, where it markedly increased the activities of glucokinase, glucose-6-phosphate dehydrogenase and reduced glucose-6-phosphatase and fructose-1, 6-bisphosphatase activities. Additionally, it decreased plasma glucose, glycated haemoglobin levels and augmented the level of insulin (Prabakaran *et al.*, 2012). Esculetin treatment was also reported to revert the lipid peroxidation markers such as lipid hydroperoxides, thiobarbituric acid reactive substances and conjugated dienes; enzymatic antioxidants like catalase, superoxide dismutase, glutathione peroxidase, glutathione-S-transferase; and non-enzymatic antioxidants like Vitamin C, E to near normal levels (Prabakaran *et al.*, 2013). Esculetin not only showed a defensive role in type 2 diabetes

but also has potential role in type 1 diabetes (T1D). In STZ induced T1D mice model, esculetin reduces renal damage by decreasing the hepatic glucose 6-phosphatase and Caspase-3 levels and increasing glucose uptake, phosphorylation of insulin receptors, Akt and GSK-3β expressions (Kang *et al.*, 2014b). Another study suggested that the renoprotective effect ushered by esculetin could be related to its ability to decrease and increase the mRNA levels of *mmp13* and *bmp6* respectively. It also inhibits PPARγ/TGF-β1 pathway by suppressing the PPAR-γ and TGF-β1 expressions leading to reduction in renal fibrosis (Surse *et al.*, 2011a). Evidences suggest that complete cure for diabetes calls for an attention towards its epigenetic basis.

Based on the above literature survey, it may be concluded that esculetin (6,7-dihydroxy-coumarin) has pleotropic effects in the treatment or prevention of NCDs. The main phenomenon behind the development of NCDs is oxidative stress and its devastating downstream pathways. Esculetin being a potent anti-oxidant and intracellular ROS scavenging agent may be involved in activation of enzymes which are hostile to ROS. Esculetin has shown a protective effect in various NCDs including cancer, metabolic diseases, neurological disorders, CVDs, and renal dysfunction by altering the underlying pathological pathways. The implementation of esculetin as a therapeutic intervention, either as mono-therapy or in combination with other drugs for these complications may lead us to unprecedented results. Despite all the enduring research, there is a large amount of pending work to be done for development of this molecule.

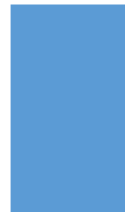
This review may provide an insight into mechanisms elicited in the progression of IR, T2D and associated cardiovascular and renal complications, and esculetin to treat to prevent NCDs and thus it may pave a path for the present research regarding further therapeutic development in T2D associated complications.

Table 1. Different pathways/targets influenced by esculetin in different non-communicable disorders (NCDs).

NCDs	**Study models**	**Mechanism of action**	**References**
Cancer	HL-60 Leukemia cells	Inhibit retinoblastoma protein phosphorylation & arrest cell cycle	(Lacy *et al.*, 2004)
	HT-29 colon cancer cells	↑ BAX, caspase 9 & 3 and ROS ↑ ER stress related proteins, CHOP & caspase 12	(Kim *et al.*, 2015a)

	ZR-75-1 human breast cancer cells	↑ Intracellular Ca2+, mitochondrial apoptotic pathway & G2/M phase arrest	(Chang *et al.*, 2016)
	HN22 & HSC2 oral squamous cell lines	Supresses EGFR/PI3K/Akt pathway ↓ Nucleophosmin levels	(Won *et al.*, 2012)
	G361 Human malignant melanoma	↓ Sp1, cyclin D ↑ Caspase 3, PARP, BAX	(Su *et al.*, 2009)
CVDs	ISO-induced MI in rats	Free radical scavenging & antioxidant properties	(Karthika *et al.*, 2012)
	Balloon vascular injury in rats	↓ p42/44 MAPK activity and the downstream effectors of c-fos & c-jun ↓ NF-kappaB, AP-1 & PI3K expressions	(Pan *et al.*, 2003)
Kidney dysfunctions	Oxonate-induced mice model	Upregulate renal organic anion transporter 1 (mOAT1), organic cations (mOCT1 & mOCT2) and carnitine transporters (mOCTN1 & mOCTN2).	(Hu *et al.*, 2013)
Neurological diseases	Cerebral Ischemic/ Reperfusion injury model of stroke	↑ Bcl-2, ↓ Bax and caspase-3	(Wang *et al.*, 2012)
	In vitro ChE and BACE1 enzyme assays for AD (Alzheimer's disease)	Inhibit β-site amyloid precursor protein cleaving enzyme 1 (BACE1), BChE and AChE	(Ali *et al.*, 2016)
	Acute restraint stress mice model	↑ GSH/GSSG ratio & COX activity	(Martín Aragón *et al.*, 2016)

Obesity	3T3-L1 adipocytes	↓ Nitric oxide, TNF-α, MCP-1, PPARγ and ↑ HO-1 expression	(Yang *et al.*, 2006)
	HFD- induced obesity in mice	↑ AMPK, ↓ PPARγ in adipocytes	(Ejaz *et al.*, 2009)
Diabetes	STZ induced type 1 diabetic mice	↑ Glucose uptake, and phosphorylation of insulin receptors, Akt, GSK-3β	(Kang *et al.*, 2014b)
	STZ induced type 1 diabetic rats	Inhibits PPARγ/TGF-β1 pathway,↑Mmp13 & ↓ Bmp6	(Surse *et al.*, 2011a)



Chapter I

Gaps in Existing Research

- ***Vascular complications;*** under pre-diabetic and diabetic conditions begins with endothelial and vascular smooth muscle cell dysfunction. Further substantial reinforcement in the vascular deterioration observed by activation of renin angiotensin system (RAS) (Karnik *et al.*, 2015; Karpe *et al.*, 2012). Under chronic metabolic aberrations stimulate locally produced tissue generated angiotensin II (Ang II) and provides critical paracrine or autocrine control in the progressive pathophysiological conditions such as insulin resistance (IR), hypertension, inflammation, thrombosis, atherosclerosis, diabetes, end-stage renal disease, coronary artery disease, cardiovascular hypertrophy, and heart failure (Karnik *et al.*, 2015). Studies suggested that vascular smooth muscle cells and fibroblasts are harbor the Ang II production pathway (Karnik *et al.*, 2015; Kaschina *et al.*, 2003; Paneni *et al.*, 2013a). The effect of produced Ang II on the target tissues depends on the presence of type and abundance of receptors mainly angiotensin II type 1 receptor (AT1) and angiotensin II type 2 receptor (AT2) (Karnik *et al.*, 2015). Among these, chronic activation of the AT1 receptor, leads to hypertension, cardiac arrhythmia, stroke, diabetic nephropathy, and metabolic disorders through activation of pleotropic cellular signaling cascades which include enzymes, adapter proteins, transcription factors, and small GTP binding proteins and downstream kinases. On contrary, activation of AT2 receptor in vasculature is suggested to be protective (Habashi *et al.*, 2011; Kukida *et al.*, 2016), which was demonstrated by deletion of AT2 receptor in mice, observed inhibition of pressure natriuresis, vascular hypertrophy, and exacerbation of heart failure (Adachi *et al.*, 2003; Brede *et al.*, 2001; Gross *et al.*, 2000). Similarly, gene for AT2 receptor is regulated by various growth factors, inflammatory mediators, and the growth phase of the cells. (Karnik *et al.*, 2015). Extended serum depletion combined with insulin or insulin like growth factor 1 (IGF-1) or interleukin-1b (IL-1b) stimulate the expression of AT 2 receptor in VSMCs, whereas growth factors, like platelet derived growth factor (PDGF) and phorbol ester, inhibit expression of AT 2 receptor (Karnik *et al.*, 2015). The plasma insulin concentrations regulate AT 2 receptor expression in aorta. Tissue-specific expression of the AT 2 receptor has been traced to enhancer elements in the AT 2 receptor gene promoter consisting of the activator protein 1 AP-1 (inhibitor) (Wu *et al.*, 2005), CCAAT-enhancer-binding protein (C/EBP), nuclear factor for interleukin 6 (NF/IL-6) and interferon regulatory factor IRF-2 (activator) (Karnik *et al.*, 2015). Both

activation and inhibition of AT1 and AT2 receptors have been reported for covalent histone modifications and contribute in active participation in pro-inflammatory and pro-fibrotic gene expression in the development of diabetic nephropathy in rats (Pandey *et al.*, 2016). Among the post translational histone modifications, H2A and H2B monoubiquitination (Ub) system have been reported for its active participation in diabetic nephropathy (Goru *et al.*, 2016a) and oxidative stress (Cotto-Rios *et al.*, 2012).

- ***Diabetic Cardiomyopathy;*** Type 2 diabetes is a complex disease with a number of pathways involved in its development and progression. Based on recent findings, now it is well understood that genetic predisposition alone is insufficient to explain the complexity of the disease entirely, hence this urge a necessity to emphasize on epigenetic mechanisms involved in the development of diabetic complications (Keating *et al.*, 2013). Epigenetics can be defined as the heritable changes in gene expression patterns without any alteration in the underlying DNA sequences; this includes DNA methylation, post translational histone modifications (PTHMs) and, micro-RNA regulation of mRNA translation (Asrih *et al.*, 2013; Keating *et al.*, 2013; Mahmoud *et al.*, 2013). Among the above mentioned epigenetic changes, the current study focuses on post translational histone modifications (PTHMs) in diabetic cardiomyopathy (DCM). PTHMs includes methylation, acetylation, ubiquitination, and phosphorylation on amino terminal tail of core histone such as H3, H4, H2A, and H2B thereby forming a 'histone code' which regulates the transcriptional outcome of gene(s) (Asrih *et al.*, 2013; Cooper *et al.*, 2010). Generally, increase in histone H3 acetylation (H3K9Ac, H3K14Ac and H3K27Ac) and phosphorylation (H3S10phospo and H3S28phospo) results in the transcriptional activation of the gene, while methylation can lead to activation or repression depending on the position of lysine modified; for instance, histone H3K4me, H3K36me, and H3K79me leads to activation, while H3K9me and H3K27me causes repression of gene(s) (Backs *et al.*, 2006; Ernst *et al.*, 2011; Papait *et al.*, 2013). Alteration in PTHMs leads to changes in the expression of numerous genes related to inflammation (Reddy *et al.*, 2008), endothelial dysfunction (Okabe *et al.*, 2012; Paneni *et al.*, 2013b), and extracellular matrix (ECM) accumulation (Sun *et al.*, 2010) which play a crucial role in the development of chronic diabetic complications. It has been reported that diabetic nephropathy (db/db mice) is associated with increased permissive (H3K9/14Ac, H3K36me3, and H3K4me 1, 2, and 3) and decreased

repressive (H3K9me2, H3K9me3, and H3K27me3) PTHMs, which can enhance chromatin unfolding and thereby increase gene(s) expression (Reddy *et al.*, 2011). Furthermore, hyperglycemia alters the histone H2A/H2B ubiquitination in rat glomerular mesangial cells (GMCs), which can activate transforming growth factor-β (TGF-β) signalling pathway involved in the pathogenesis of diabetic nephropathy (Gao *et al.*, 2013). However until now, alteration in histone H2A/H2B ubiquitination in diabetic cardiomyopathy is mysterious (Figure 2).

- ***Diabetic nephropathy;*** Insulin resistance (IR) and type 2 diabetes (T2D) show an array of common underlying pathways including chronic inflammation, extracellular matrix (ECM) deposition and fibrosis. Type 2 diabetic nephropathy (T2DN), a long term major complication of hyperglycaemia is one of the most important causes of chronic kidney disease and end stage renal failure globally (Mima, 2013; Rask-Madsen *et al.*, 2010). Oxidative stress is one of the major factors involved in progression of these diseases which in turn activates various downstream pathways, including Renin angiotensin system (RAS). The balance between the two axes- deteriorative, Angiotensin II /Angiotensin converting enzyme/ Angiotensin II Type 1 Receptor (Ang II /ACE/AT1R) and protective, ACE2/ Ang (1-7) and (1-9)/ Mas receptor arm is responsible for limiting inflammation and fibrosis under physiological conditions (Batlle *et al.*, 2012; Kalupahana *et al.*, 2012). It was seen that in streptozotocin (STZ) induced diabetic rats, tubular (Tikellis *et al.*, 2003) and glomerular (Ye *et al.*, 2006) expression of ACE2 was decreased. Also, chronic treatment with ACE2 inhibitor, MLN-4760, in *db/db* mice or in STZ induced diabetic mice results in albuminuria (Santos *et al.*, 2008; Ye *et al.*, 2006). These aberrations in gene expression leading to pathological conditions can't be explained completely by genetic dogma itself, epigenetics plays a significant role in regulation of gene expression by remodelling the chromatin structure.

  In DN, increased permissible (H3K9/14Ac, H3K36me3, and H3K4me1/2/3) and reduced repressive (H3K9me2, H3K9me3, and H3K27me3) posttranslational histone modifications (PTHMs) have been reported (Goru *et al.*, 2016b). For instance, H3K9me2 demethylase, *Jhdm2a* knockout increased expression of inflammatory markers leading to obesity and hyperlipidaemia (Reddy *et al.*, 2011) and TGF-β mediated H3K4me increases expression of fibrotic genes (Sasaki *et al.*, 2016).

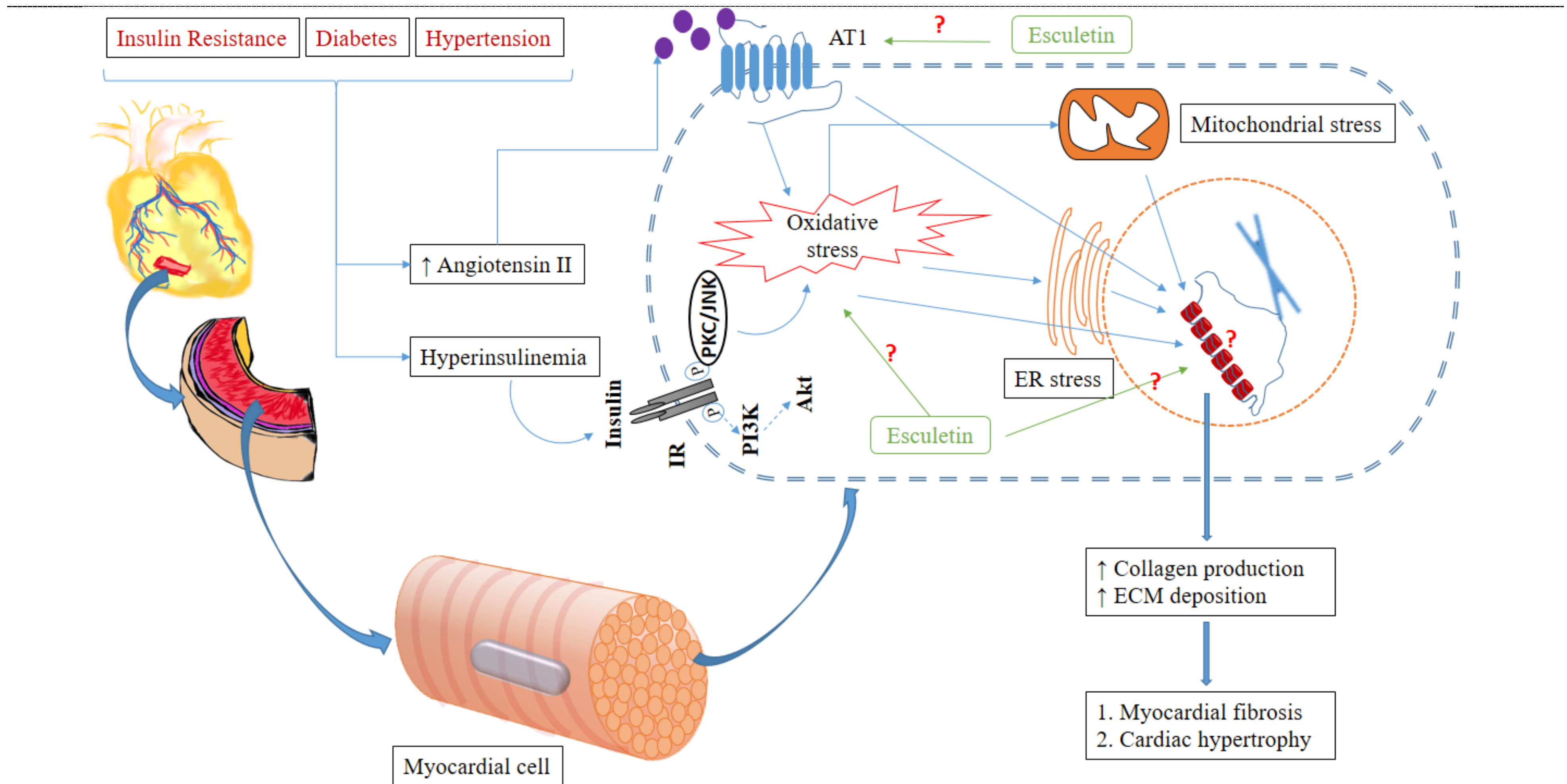


Figure 2: Existing gaps and study hypothesis in diabetic cardiomyopathy.

Further, increase in renal H3Ac at lysine 9, 14, 18, 23 and 27, H3K4me2 and H3 serine 10 phosphorylation leads to transcriptional activation in advanced stages of DN (Kato *et al.*, 2013). Recently, it was found that Losartan mediated amelioration of T2D in *db/db* mice could be attributed to its ability to attenuate the increased H3K9/14Ac at *Rage*, *Pai-1*, and *Mcp1* promoters in cultured mesangial cells (Reddy *et al.*, 2014). Histone ubiquitination (Ub) also plays an important role in cellular functioning guiding the substrates proteins to undergo degradation or to trigger biological cycles. Recent discovery of ubiquitin ligases and deubiquitinases has unveiled the role of H2A lysine ubiquitination (H2AKUb) in X inactivation and Polycomb group complex dependent gene silencing (de Napoles *et al.*, 2004). However, the correlation of H2AUb and gene expression has not yet been established completely, especially in pathogenesis of IR and T2DN (Figure 3). These studies indicate that the therapeutic agents which could reverse the epigenetic modifications associated with diseases' pathogenesis may emerge as promising treatments.

- ***Esculetin;*** currently available therapeutic interventions have various side-effects and have not yet been able to curb the root cause of IR and T2DN. This motivates us to look into the natural treasure trove to find an appropriate, side-effect free treatment for these complicated, multifaceted diseases. Esculetin (6,7- dihydroxy coumarin derivative), a naturally available pleotropic coumarin derivative present in various medicinal plants and fruits such as *Artemisia capillaries, Artemisia scoparia, Citrus limonia, Cortex fraxini, Ceratostigma willmottianum, Viola yedoensis, Aesculus hippocastanum,* and *Cichorium intybus,* displays direct free radical quenching, lipooxygenase and cyclooxygenase inhibition, and anti-fibrotic activity (Galano *et al.*, 2016). Owing to the aforementioned properties, it shows a potential therapeutic role in cancer (Cho *et al.*, 2015), obesity (Kim *et al.*, 2015; Sim *et al.*, 2015), and diabetes (Surse *et al.*, 2011). In literature review, we have reviewed that esculetin attenuates insulin sensitivity, vascular perturbation and cardiomyopathy in IR and T2D. Based upon such reviews on esculetin, we used esculetin as therapeutic intervention in diabetic cardiovascular and renal complications and drawn objectives to achieve the same.

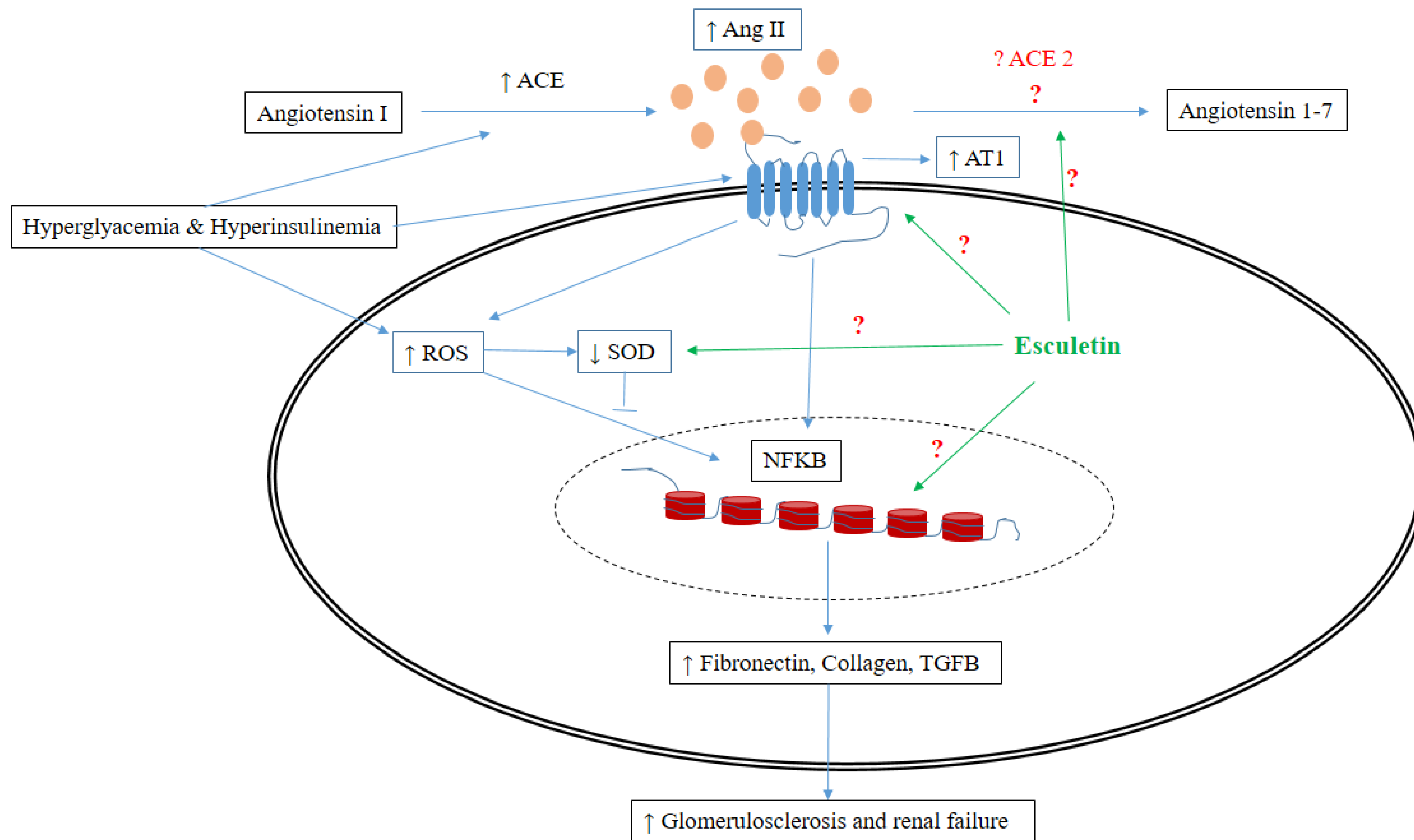


Figure 3: Existing gaps and hypothesis in diabetic nephropathy.

Chapter I

Objectives

Based on the literature reviews and vacuity in the current studies, we carried out the studies to achieve following objectives:

- To study the effect of esculetin on the development of cardiovascular and renal complications in insulin resistance and type 2 diabetes.
- To study the effect of esculetin on major artery toning in insulin resistant and type 2 diabetic rats associated with nephropathy.
- To study the effect of esculetin on expression of angiotensin type I (AT1) and II (AT2) receptors in the development of cardiovascular and renal complications under insulin resistance and type 2 diabetic conditions.
- To delineate the role of esculetin on post-translational histones modifications in the development of cardiovascular and renal complications under insulin resistance and type 2 diabetic conditions.
- To study the effect of esculetin on expression of the pathogenic genes that are differentially expressed in accelerated cardiovascular and renal complications under insulin resistance and type 2 diabetes.

To achieve above mentioned objectives we performed following experimental procedures:

1. Determination of morphological, biochemical and hemodynamic variations
2. Evaluation of angiotensin II (Ang-II) and acetylcholine (ACh) mediated vascular reactivity
3. Histopathology and immunohistochemistry to delineate pathological conditions and protein quantification
4. Western blotting to determine post translational histone modifications
5. Quantitative real time polymerase chain reaction (RT-PCR) to quantify AT1, AT2, inflammatory and fibrotic gene expressions
6. Chromatin immune precipitation assay (Chip assay) to determine the occupancies of histone modifications on various gene promoter regions.

Chapter II

Materials and Methods

Materials:

1. Drugs and reagents:

Esculetin and Streptozotocin (STZ) were procured from Sigma-Aldrich (St. Louis, MO, USA). Glucose, triglyceride, total cholesterol, blood urea nitrogen (BUN), Creatinine and Albumin kits were purchased from Accurex (Mumbai, Maharashtra, India). Ultra-sensitive rat insulin ELISA kit was obtained from Crystal Chem (Downer's Grove, IL, USA). The antibody used against Fibronectin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and remaining antibodies were procured from Cell Signaling Technology (Danvers, MA, USA). For the Western blotting, enhanced chemiluminescence (ECL) reagent was used from Thermo Fisher Scientific (Waltham, MA, USA). All the rest of the chemicals were procured from Sigma-Aldrich, Thermo Fisher Scientific, Merck, HiMedia, CDH, S D Fine-Chem and unless otherwise mentioned..

2. Instruments:

Major instruments used to conduct present experiments methods are enlisted bellow (Table 2).

Table 2: List of instruments used in the study

Name of instruments	Make	Country
Non-invasive blood pressure (NIBP) system	ADInstruments	Australia
Two Muscle Chamber water-jacketed organ bath Isometric force transducer (77005F) Data Capsule Evo 4 Channel Digital Recorder	UGO Basile	Italy
Microtome	Leika	Germany
Microscope	Olympus	USA
Vertical gel electrophoresis unit	Bio-Rad	USA
Semi-Dry transfer apparatus	Bio-Rad	USA
Amersham Hypercassette	GE Healthcare	USA
Horizontal gel electrophoresis unit	Tarsons	India
Thermocycler	BR Biochem	India
Light cycler-96	Roche	Germany
DynaMag-2	Thermofisher Scientific	USA

Methods:

1. Evaluation of In vitro antioxidant capacity of esculetin:

1.1. Sample preparation and extraction:

Preparation of sample and extraction was performed as described by (Cilla et al., 2012; Wang et al., 2011a). Two different and manipulated diets, normal pellet diet (NPD) and high fat diet (HFD) are divided into four parts of 6 g each (i) alone NPD, (ii) NPD mixed with 10 mg of esculetin (NPD+E), (iii) alone HFD and (iv) HFD mixed with 10 mg of esculetin (HFD+E). All four dietary parts were then made to pass through an in vitro gastrointestinal digestion procedure mimicking the physiological situation in the upper digestive tract (simulate gastric and intestinal fluid) which were prepared according to USP29-NF24 (USP29-NF24, 2008). First, incubation of dietary mixture into simulated gastric fluid pH 1.2 for 2 h and followed by incubation into simulated intestinal fluid pH 6.8 for 6 h in orbital shaker incubator with 100 rpm. After incubation in simulated gastro-intestinal fluid, the suspension was then centrifuged at 4000 rpm for 15 min at 4 °C and the pellet was collected. Further, esculetin was extracted from the pellets by incubating with 100 ml of methanol on an orbital shaker for 12 h at room temperature. Next, the suspension was centrifuged at 4000 rpm for 30 min at 4 °C, the supernatant was filtered through a 0.2 μm syringe filter (Axiva, Delhi, India) into amber glass flask and then used in all assays.

1.2.Total phenolic content (TPC) assay:

The total phenolic content (TPC) was determined as described by (Wang *et al.*, 2011) briefly, 25 µl of the extract was mixed with 125 µl of the Folin - Ciocalteu reagent (FCR) and 100 µl of 7.5% sodium carbonate. The mixture was gently shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 765 nm. A standard curve of pure esculetin was prepared with a concentration range from 50 to 250 µg/ml. All tests were performed in triplicates and the TPC in the extract of all four dietary mixtures was expressed in micrograms of pure esculetin per gram sample.

1.3. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging capacity assay:

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was performed as described by (Wang *et al.*, 2011). All methanolic extract samples were added (10 µl each) to 250 µl of DPPH (2.5 µM) in a 96-well plate and shaken vigorously incubated for 30 min at room temperature. The remaining DPPH was quantified by measuring the absorbance at 517 nm. The percentage inhibition of sample radical scavenging capacity

was calculated. Blank solution was used with corresponding extract without DPPH. The mean values were obtained from triplicate determinations.

1.4. Hydrogen peroxide (H_2O_2) scavenging assay:

The ability of dietary extracts to scavenge hydrogen peroxide (H_2O_2) was estimated according to the method of (Ruch *et al.*, 1989). Hydrogen peroxide (40 mM) is prepared in phosphate buffer saline (50 mM pH 7.4). Esculetin 100 µg/ml and extract were added to H_2O_2 and absorbance at 230 nm is determined after 10 min of incubation at 37 °C. A corresponding blank solution containing phosphate buffer and esculetin or extract without H_2O_2 were used. The percentage scavenging capacity was calculated. The experiments were performed in triplicates.

2. Experimental animals:

Male Wistar rats (160 -180 g) were procured from Central Animal Facility, Birla Institute of Technology and Science (BITS), Pilani campus, Pilani. All the procured animals were maintained under standard laboratory environmental conditions with food and water *ad libitum*. The animals were acclimatized for one week by feeding normal pellet diet (NPD). All the experiments performed in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experimentations on Animals, (CPCSEA) India. Before conducting experiments all procedures were approved by Institutional Animal Ethics Committee (IAEC, BITS Pilani, Protocol approval No. IAEC/RES/17/03/Rev-2/19/35 and IAEC/RES/17/05Rev-1/18/25).

3. Development non-genetic model for insulin resistance, type 2 diabetes and associated cardiovascular and renal complications in rats:

Insulin resistance (IR) and type 2 diabetes (T2D) were induced by feeding alone high fat diet (HFD) and low dose Streptozotocin (STZ) (35 mg/kg, *i.p*) administration along with HFD feeding respectively. High fat diet is a manipulated diet regimen with nutrition facts of 58% fat, 25% protein and 17% carbohydrate, as a percentage of total kcal. Each rat consumes approximately 65 Kcal/day of normal pellet diet (NPD) whereas rats fed with HFD consumed approximately 105 kcal/day.

After initial acclimatization, for developing IR, group of animals (n=72) were fed with alone HFD *ad libitum* for 4 (n=18) and 24 weeks (n=18) (Figure 1). To this group we abbreviated as HFD group. Further, For the development of T2D first, animal diet was manipulated with HFD for initial 2 weeks then low dose of STZ (n=36) and for all control animals citrate buffer (0.1M, pH-4.4) were injected. Then the animals were fed with HFD for further 4 (n=18) and 24 weeks (n=18). This group of animals were abbreviated as

HFD+STZ group (Figure 1). Finally, a group of rats were fed with NPD throughout the study and abbreviated as NC (Figure 1) (Gaikwad *et al.*, 2010a; Pandey *et al.*, 2015; Pandey *et al.*, 2016; Pandey *et al.*, 2017).

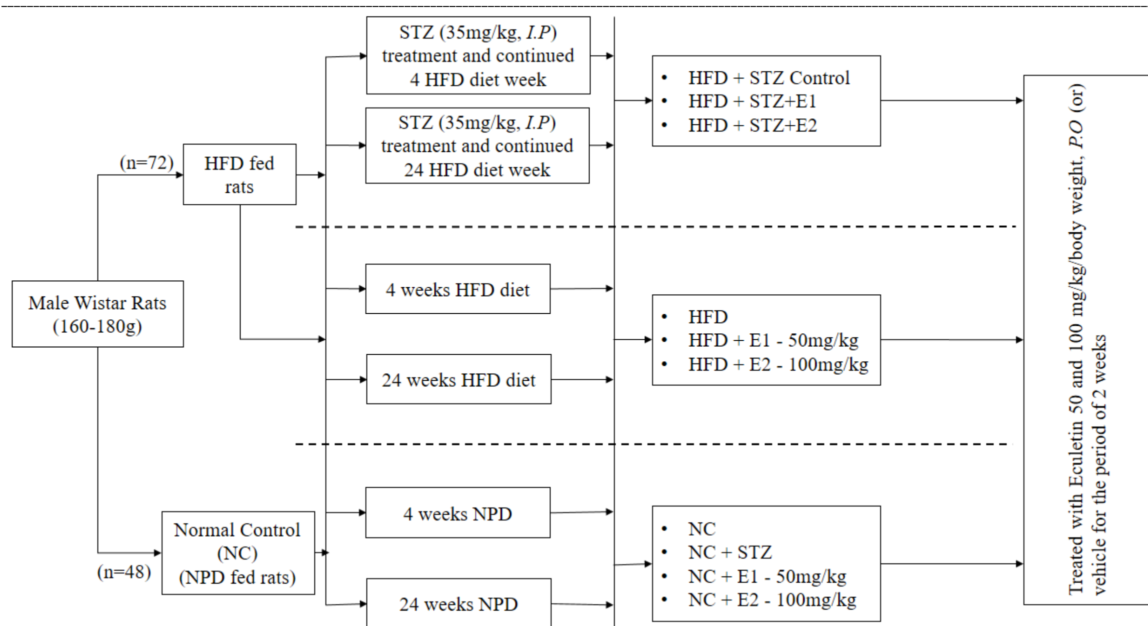


Figure 1: Schematic representation of animal study design

Representation of animal study protocol to describe the experimental groups and their respective treatments. Experimental groups: NC – Normal pellet diet fed Control, HFD – high fat diet fed control, NC+STZ– Streptozotocin (35 mg/kg, *i.p*) administered to normal control animals, HFD+STZ – Streptozotocin (35 mg/kg, *i.p*) administered to high fat diet animals, NC+E1, NC+E2, HFD + E1, HFD + E2, HFD+STZ+E1 and HFD+STZ+E2 – Esculetin (50 mg/kg/day, P.O) and Esculetin (100 mg/kg/day, P.O) treatment to NC, HFD and HFD+STZ animals respectively.

4. Treatment with Esculetin

Esculetin at doses of 50 and 100 mg/kg/day *p.o* was reported to prevent glomerulosclerosis in STZ induced type 1 diabetic rats (Surse *et al.*, 2011). Therefore, in the present study, esculetin was administered with same doses. After 4 and 24 weeks, rats fed with NPD, HFD and HFD+STZ were treated with esculetin 50 and 100 mg/kg/day, *p.o* and respective control animals treated with vehicle (0.5% sodium carboxy methyl cellulose) for 2 weeks and the rats were allowed to feed on their respective diet till end of the study (Figure 1). Body weight, biochemical estimations and blood pressure measurements were performed at the end of the studies (i.e. after two weeks treatment – 6 weeks and 26 weeks).

5. Determination of morphological, biochemical and hemodynamic variations in insulin resistance, type 2 diabetes and associated cardiovascular and renal complications:

After 4 and 24 weeks of the study, body weight was measured and plasma was collected. Animals were sacrificed by decapitation method and required organs were collected. Kidney and heart were cleaned, weighed and kept in -08°C freezer. All plasma samples were analyzed for glucose (pGL), triglycerides (pTG), total cholesterol (pTC), blood urea nitrogen (BUN), creatinine (pCr), albumin (pAL) using commercially available kits [Accurex, Mumbai, Maharashtra, India]. Plasma insulin level was determined by ultra-sensitive insulin ELISA kit [Crystal Chem, Downers Grove, IL, USA]. Systolic blood pressure (SBP) was recorded on the last day of the treatment in all groups using a tail cuff blood pressure recorder [ADInstruments, NSW, Australia] (Gaikwad et al., 2010b; Pandey et al., 2017).

6. Vascular reactivity experiments:

6.1. Isolation and tissue preparation:

After the sacrifice of animals, thoracic aorta (from the arch of aorta to the diaphragm) was quickly excised and placed in ice-cold oxygenated (95% O_2 + 5% CO_2) Krebs-Henseleit buffer (KHB) (NaCl – 119 mM, KCl – 4.75 mM, NaH_2PO_4 – 1.19 mM, $MgSO_4$ – 1.19 mM, $CaCl_2$ – 2.54 mM, $NaHCO_3$ – 25 mM, and glucose – 11 mM) of pH 7.4. The aorta was cut into 5 mm segments by sharp micro scissors (Vannas scissor or scalpel blade) and was cleaned by removing adhering fat and adventitial tissues. Care was taken not to stretch the vessel. Aortic ring was suspended by a pair of stainless steel hooks in water-jacketed organ bath [Ugo Basile, Varese, Italy], filled with 10ml of oxygenated Krebs-Henseleit buffer (KHB). The tissue in KHB was continuously provided with carbogen (95% O_2 + 5% CO_2) and maintained temperature of 37°C. Tissue was subjected to equilibration for 120 min under the tension of 2 g. The bath fluid was changed for every 15min. Then, contraction was measured isometrically by using force transducer [77005F, UGO Basile, Varese, Italy] (Karpe *et al.*, 2012; Karpe *et al.*, 2014).

6.2. Evaluation of Angiotensin II (Ang-II) and Acetylcholine (ACh) mediated vascular reactivity:

After 120 min exposure to 2 g tension, the aorta was exposed to 80mM KCl, a depolarizing solution. After two such challenges, cumulative contractile responses to increasing concentrations of Ang II (1nM to 30μM) were recorded. Next, aortic rings were pre-

contracted with sub-maximal concentration of phenylephrine (100 nM) to evaluate acetylcholine (1nM to 30µM) induced vasodilatation. At the end of every experiment tissue was subjected to drying. Then, dried weight of tissue was taken for the calculation of contraction in terms of tension. The responses were normalized to cross sectional area of the tissue and the tension developed was calculated (Karpe *et al.*, 2012; Karpe *et al.*, 2014).

7. Measurement of oxidative stress markers:

Oxidative stress markers such as reduced glutathione (GSH) and lipid peroxidation were estimated in homogenized renal tissue samples of 24 week study. GSH was estimated as mentioned by Ellman GL et.al. (Ellman, 1959). Further, Lipid peroxidation was determined by measuring the renal malondialdehyde (MDA) equivalents levels through the thiobarbituric acid (TBA) reactive substances estimation method, used by Ohkawa H et. al (Ohkawa *et al.*, 1979). Data were expressed as concentration of GSH and TBA per microgram of total protein.

8. Histopathology and immunohistochemistry:

Histopathology and immunohistochemistry was performed as per the protocol described by Gaikwad et al., (Gaikwad *et al.*, 2010a; Gaikwad *et al.*, 2010b). Briefly, from each rat, portion of heart, kidney and aorta tissues were fixed in 10% (v/v) formalin in phosphate buffer solution (PBS) and embedded in paraffin after completing the routine processing. For, histopathology, sections of 5µm were stained with haematoxylin/eosin (H&E) and Picro-Sirius Red (PSR). At least 25 sections were observed under microscope (Olympus BX41, NY, USA) and images were captured. For immunohistochemistry, sections of 5µm were taken from paraffin blocks and deparaffinised with xylene, followed by antigen retrieval by heating in citrate buffer (10 mmol/L). The following primary antibodies were used: anti - AT1, anti - AT2, anti - ACE2, anti - Keap1, anti - fibronectin (rabbit, 1:50 dilution; Santa Cruz Biotechnology, USA), anti - Ki-67, anti - TGF-β, (rabbit, 1:50 dilution; Cell Signaling Technology, Danvers, MA, USA) (Table 2) and HRP linked anti-rabbit secondary antibody was used, followed by detection with diaminobenzidine (DAB) as a chromogen. Slides were counterstained (haematoxylin), dehydrated (alcohols and xylene), and mounted in DPX (Sigma). All histopathological images were analyzed using Image J software for calculating nuclei positive area, collagen positive area, and DAB positive area.

Table 3. List of antibodies used in immunohistochemistry

Primary antibody (used against)	Dilutions	Company	Country
AT1 AT2 ACE2 Keap1 Fibronectin	1:50	Santa Cruz Biotechnology	USA
Ki67 TGF β	1:50	Cell Signalling Technology	USA

9. Histone isolation and western blotting:

Histone isolation and western blotting were performed as described earlier (a; Gaikwad *et al.*, 2010b; Pandey *et al.*, 2015). Briefly, tissues was dissected manually, homogenized in Buffer-A [12% w/v sucrose, 10mM EDTA, 5mM Nacl, 10mM Tris, 1% PMSF (0.1M), 0.1% NaBr (1M), and pH-7.2]. After filtration this homogenate was layered on Buffer-B [15%w/v sucrose, 10mM EDTA, 5mM Nacl, 10mM Tris, 1% PMSF (0.1M), 0.1% NaB (1M), and pH-7.4] and centrifuged at 4000 rpm. Subsequent layering was done by adding 1% Triton-X solution, after centrifugation nuclear palette was re-suspended in modified LSB. Further treatment with concentrated HCl, this solution was sonicated and centrifuged at max speed, then supernatants was collected and 25% Trichloro acetic acid (20% w/v) was added for precipitation of protein. After final centrifugation at max speed pellet which contain histone protein was dissolved in water.

Isolated histones were subjected to 14% SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Amersham, GE healthcare Bio-Science, Pittsburgh, PA, USA). Immunoblot analysis was performed by using rabbit monoclonal antibodies against: histone ubiquitination- H2AK119ub and H2BK120ub; histone H3 phosphorylation- H3S10phospho, H3S28phospho, and H3T3phospho; histone H3 dimethylation- H3K4me2, H3K9me2, H3K27me2, H3K36me2, and H3K79me2; histone H3 acetylation- H3K9Ac, H3K14Ac, H3K18Ac, H3K28Ac, and H3K56Ac; all antibodies were used in 1:1000 (v/v) dilution. Anti-rabbit IgG, HRP-linked antibody was used as secondary in 1:20,000 (v/v) dilution (Cell Signaling Technology, Danvers, MA, USA) (Table 3). Proteins were detected by the ECL system and ECL Hyperfilm. Immunoblots were quantified by densitometric analysis using ImageJ software and the exposures were in linear dynamic range, each modification was normalized by respective total H3, H2A and H2B blot, then data analysis was performed by using Prism software (version 5.0; GraphPad, San Diego, CA, USA) and results were expressed as fold over NC.

Table 4. List of antibodies used in western blotting

Primary antibody (used against)	Dilutions	Company	Country
H2AK119ub H2BK120ub H3S10phospho H3S28phospho H3T3phospho H3K4me2 H3K9me2 H3K27me2 H3K36me2 H3K79me2 H3K9Ac H3K14Ac H3K18Ac H3K28Ac H3K56Ac H3 Total H2A Total H2B Total	1:1000	Cell Signalling Technology	USA

10. Real time polymerase chain reaction (RT-PCR) experiments:

Isolation of RNA from frozen tissue samples and Real time polymerase chain reaction was performed as described previously (S. K. Goru et al., 2016; A. Pandey et al., 2016). In brief, RNA was isolated (Ambion™ PureLink™ RNA Mini Kit, Life Technologies, Carlsbad, CA, USA) and incubated with recombinant DNase1 [Ambion™ Recombinant DNase I (RNase-free), Life Technologies, Carlsbad, CA, USA] to remove the single or/and double stranded DNA, chromatin and RNA:DNA hybrids present in the sample.

On continuing, cDNA was synthesized by using cDNA kit (GeneSure™ First Strand cDNA Synthesis Kit, Puregene, Genetix Biotech Asia Pvt. Ltd., New Delhi, India). Quantitative real-time polymerase chain reaction was performed on LightCycler® 96 Real-Time PCR System (Roche Diagnostics GmbH, Mannheim, Germany) using the FastStart Essential DNA Green Master (Roche Diagnostics GmbH, Mannheim, Germany) and gene specific primers (Eurofins, Genomics India Pvt. Ltd, Bangalore, Karnataka, India). Primers for *Sod1, At1, At2, Vcam, eNos, Ace, Ace2, Usp21, Usp16, Rnf2, Rnf168, Tgfb1, Mcp1* and *Col1a1* were designed, the forward and reverse primers were enlisted in the following table (Table 4). After amplification, a melting curve analysis was performed to verify the specificity of the reaction. Levels of mRNA in the samples were normalized

to their respective 18s contents. Experiments were carried out in triplicate (n=3) for each sample and results were expressed as fold change over respective control. Finally, results were analysed by LightCycler® Software (Roche Diagnostics GmbH, Mannheim, Germany).

11. Chromatin Immuno-precipitation (ChIP) assays:

Chromatin Immunoprecipitation (ChIP) assay was performed as described previously (Goru et al., 2016a) using Magnify Immunoprecipitation kit (Invitrogen, CA, USA). Briefly, frozen tissues were quickly minced and suspended in Dulbecco's phosphate buffer saline (DPBS) and incubated for 10 min. in 1% formaldehyde for crosslinking the chromatin. Glycerine (0.1M) was added and incubated for 5 min. to stop the crosslinking reaction. Further, sonication was used (6 cycles, each cycle with minimal amplitude for 10s keeping on ice) for shearing the chromatin. Part of sheared chromatin was incubated in anti-H2AK119Ub antibody for 2 h and remaining was kept in -80°C for input sample. Histone-DNA complex was eluted after incubation and reverse crosslinking was performed using reverse crosslinking buffer. DNA was recovered and purified by magnetic bead supplied in the kit. Purified DNA was analysed by qRT-PCR using FastStart Essential DNA Green Master (Roche Diagnostics GmbH, Mannheim, Germany) on LightCycler® 96 Real-Time PCR System (Roche Diagnostics GmbH, Mannheim, Germany). Promoter specific primers were used for At1, At2, Tgfβ and Mcp1 (Eurofins, Genomics India Pvt. Ltd, Bangalore, Karnataka, India) (Table 4). Anti-IgG antibody and input DNA were used as negative and positive control for the experiment respectively. Results were expressed as fold change over non-treated groups.

Table 5: List of designed primers for qRT-PCR and ChIP-RT-PCR

Gene Name	Primer sequence for qRT-PCR	Accession ID No.
Sod	Forward 5'-CACTCTAAGAAACATGGCG-3' Reverse 5'-CTGAGAGTGAGATCACACG-3'	NM_017050.1
At1	Forward 5'-CTCTGCCACATTCCCTGAGTT-3' Reverse 5'-CTTGGGGCAGTCATCTTGGA-3'	NM_030985.4
At2	Forward 5'-AACCGGCAGATAAGCATTTG-3' Reverse 5'- CAGCCACAGCCAGATTGAAG-3'	NM_012494.3
Vcam-1	Forward 5'-AAGTCTACACCTCCCCCAAGA-3' Forward 5'-CATGTCATCGTCACAGCAGC-3'	NM_012889.1

eNos	Forward 5'-GCCCCCAGAACTCTTCACTC-3' Reverse 5'-CCGGGTGTCTAGATCCATGC-3'	NM_021838.2
Ace	Forward 5'-CGCAGCTCTTCGCTGAC-3' Reverse 5'-TCTCCTCCGTGATGTTGGTG-3'	NM_012544.1
Ace2	Forward 5'-ATGAAGCGGGAGATCGTTGG-3' Reverse 5'-TGGAACAGAGATGCAGGGTC-3'	NM_001012006.1
Usp 21	Forward 5'-TGGAGCGAGAAGACAGCAAG-3' Reverse 5'-CGGTCACATACTGGGGCATT-3'	NM_001127638.1
Usp 16	Forward 5'-GCCGTCTCACCGGATTGTA-3' Reverse 5'-CCCCTTTGTTCGTTTCTTTCCC-3'	NM_001100501.1
Rnf 2	Forward 5'-ACAGCGCACAGACCAGATACA-3' Reverse 5'-AGACCCCACCACACCACTTG-3'	NM_001025667.1
Rnf 168	Forward 5'-CCACACGCTCTGTAACCCAT-3' Reverse 5'-CTGGCTGGTACTCATCAACGAT-3'	NM_001127597.2
Tgf-β	Forward 5'-CTGCTGACCCCCACTGATAC-3' Reverse 5'-AGCCCTGTATTCCGTCTCCT-3'	NM_021578.2
Mcp-1	Forward 5'-GTCTCAGCCAGATGCAGTTA-3' Reverse 5'-CCTTATTGGGGTCAGCACAG-3'	NM_031530.1
Collagen1 a1	Forward 5'-TGGCAACCTCAAGAAGTCCC-3' Reverse 5'-ACAAGCGTGCTGTAGGTGAA-3'	NM_053304.1
18s	Forward 5'-GCAATTATTCCCCATGAACG-3' Reverse 5'-AGGGCCTCACTAAACCATCC-3'	NR_003278.3

Gene Name	Primer sequence for ChIP-qRT-PCR	Accession ID No.
Tgf-β	Forward 5'-TTCGCGCTCTCCGAAGTT-3' Reverse 5'-CGGGCGTCAGCACTAGAA-3'	NM_021578.2
Mcp-1	Forward 5'-TGTCACAAGCTCTTCGGTTT-3' Reverse 5'-GTCCACTGAGTCCTTGGTTATC-3'	NM_031530.1
At1	Forward 5'-CTCTGCCACATTCCCTGAGTT-3' Reverse 5'-CTTGGGGCAGTCATCTTGGA-3'	NM_030985.4
At2	Forward 5'-CCTTCCATGTTCTGACCTTCTT-3' Reverse 5'-GCCAGGTCAATGACTGCTATAA-3'	NM_012494.3

12. Statistical analysis

Experimental values were expressed as means ± S.E.M. Statistical comparison between different groups was performed using one way analysis of variance to detect the difference in observations between all groups. If F value was significant then multiple comparisons were done by Tukey test using Prism software (version 5.0; GraphPad, San Diego, CA) for Windows. Data was considered to be statistically significant if $p<0.05$.Cumulative concentration response curves were analyzed for pD2 value (the negative log concentration required to produce 50% of the maximal response) and maximal contraction (Emax), and statistical differences between the means were determined by one-way ANOVA followed by Tukey test. Immunohistochemical scores for aorta (4 weeks study) were analyzed using Kruskal e Wallis ANOVA on ranks, followed by the Tukey test. Data was considered statistically significant if $p < 0.05$.

Chapter III

Experimental Results

1. Effect of dietary matrix manipulations on antioxidant property of esculetin.

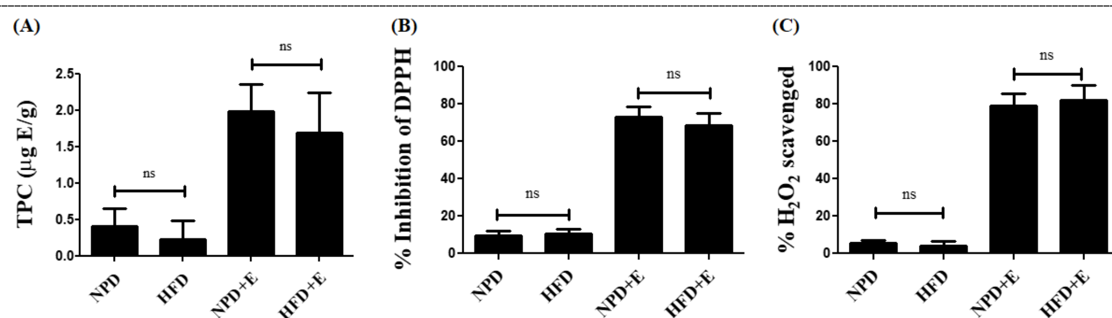


Figure 5: In vitro evaluation of possible additive, synergistic and antagonistic effects of food matrix on antioxidant capacity of esculetin.

(A) Total phenolic content (TPC), (B) DPPH radical scavenging capacity assay and (C) Hydrogen peroxide (H2O2) scavenging assay. Note: Results were represented as (A) microgram equivalent of pure esculetin per gram sample, (B) and (C) percentage DPPH and H2O2 scavenging capacity. [All the experiments were performed differently in triplicate and represented as means ± SEM, P<0.05]; NPD – Normal pellet diet; HFD – High fat diet; NPD+E – NPD mixed with 10 mg of Esculetin; HFD+E – HFD mixed with 10 mg of Esculetin.

To determine the possible additive, synergistic and antagonistic effects on total antioxidant capacity of esculetin by manipulated dietary matrix, we performed *In Vitro* simulated gastro-intestinal digestion followed by extraction and evaluation of antioxidant capacity extracts. In the alone normal pellet diet (NPD) and high fat diet (HFD) extractions were evidenced in no changes in the TPC, DPPH and H_2O_2 scavenging assays. Further, the extractions of blended NPD and HFD with esculetin (NPD+E and HFD+E) have showed a significantly higher antioxidant capacity than alone NPD and HFD dietary extracts and on other hand, the results of TPC, DPPH and H_2O_2 scavenging assays demonstrated no significant difference between antioxidant capacity of NPD+E and HFD+E dietary blends ($p<0.05$) (Figure 5A, B and D).

2. Esculetin attenuates alterations in biochemical, morphometric and hemodynamic perturbations in cardiovascular and renal complications under insulin resistance and type 2 diabetes.

The development of IR, T2D and initial cardiovascular insults were characterized and confirmed by assessing the plasma metabolic parameters such as plasma glucose, triglyceride, total cholesterol and insulin levels. In both 4 and 24 weeks animals, it was found that glucose, triglycerides and total cholesterol were significantly increased in both

HFD fed and HFD+STZ groups as compared to the NC rats (Table 6 and 7). Unlikely, Insulin levels were significantly raised by HFD feeding and reduced by HFD+STZ groups. In addition to initial pathological condition in the development of cardiovascular complications, measurement of hemodynamic changes holds an importance where we found significant inclined systolic blood pressure (SBP) in HFD and HFD+STZ rats which was measured by noninvasive blood pressure measurement technique using tail cuff blood pressure recorder [ADInstruments, NSW, Australia] (Table 8 and 9).

Further, estimation of plasma biochemical parameters such as plasma creatinine (pCr), blood urea nitrogen (BUN) and plasma albumin (pA) levels revealed the association of T2D in the development of renal complications. We found, pCr and BUN levels were increased while pA was reduced significantly in HFD+STZ group animals. The HFD fed group did not show any significant alterations in renal functioning parameters which shows that IR is not yet been associated with renal dysfunction. Such aberrations in plasma parameters and hemodynamic changes were significantly reversed by the treatment with both the doses of esculetin in 4 and 24 week animals (Table 10 and 11).

The change in body weight (BW), heart weight (HW) and kidney weight (KW) are yet another morphological markers for pathogenesis of cardio-renal syndrome under IR and T2DN. At the end of the both studies, animal BW, HW and KW were measured. In HFD fed rats, both BW, HW and KW were considerably higher whereas in the HFD+STZ treated rats we observed significantly reduced BW and HW whereas KW was observed to be increased when compared with NC (Table 8 and 9). Esculetin treatment effectively normalized both BW, HW as well as KW. The relative heart [(HW/BW) * 1000] and kidney weight [(Kidney Weight / Body Weight) x 1000)] were found to be increased in HFD and HFD+STZ treated animals and significantly reduced by esculetin treatment at both doses in 4 week and 24 week animals (Table 8 and 9).

All together the data represent the ability of esculetin to improve insulin sensitivity, hyperglycaemia and followed by cardiovascular and renal dysfunction. In these initial parameters, we did not find any significant changes in plasma biochemical, morphological and hemodynamic measurements in STZ and esculetin treated NPD fed rats as compared to NC, therefore these 3 groups were omitted in further evaluations (Table 7).

Table 6: Amelioration in metabolic perturbation on treatment with Esculetin in insulin resistance and type 2 diabetic rats (4 weeks).

Groups	Plasma Glucose (mmol/l)	Plasma Insulin (pM)	Plasma Triglycerides (mg/dl)	Total Cholesterol (mmol/l)
Normal Control **(NC)**	5.75 ± 0.22	2.35 ± 0.07	42 ± 2.2	1.53 ± 0.02
NC/STZ(35mg/kg) **(NC+STZ)**	5.91 ± 0.15	2.47 ± 0.19	44 ± 2.2	1.54 ± 0.11
NC/Esculetin(50mg/kg) **(NC+E1)**	5.65 ± 0.29	2.24 ± 0.15	42 ± 4.3	1.51 ± 0.07
NC/Esculetin(100mg/kg) **(NC+E2)**	5.98 ± 0.24	2.22 ± 0.08	42 ± 2.5	1.67 ± 0.11
High Fat Diet Control **(HFD)**	6.78 ± 0.08	6.24 ± 0.44*	61 ± 2.1*	4.01 ± 0.10*
HFD/Esculetin(50mg/kg) **(HFD+E1)**	5.91 ± 0.20	6.55 ± 0.50	51 ± 2.4	$2.98 \pm 0.12^{\#}$
HFD/Esculetin(100mg/kg) **(HFD+E2)**	5.69 ± 0.30	$2.61 \pm 0.12^{\#}$	$45 \pm 1.8^{\#}$	$2.67 \pm 0.06^{\#}$
HFD/Streptozotocin(35mg/kg) **(HFD+STZ)**	23.68 ± 0.16*	2.08 ± 0.10*	156 ± 9.8*	11.11 ± 0.38*
HFD/STZ/Esculetin(50mg/kg) **(HFD+STZ+E1)**	$17.89 \pm 0.61^{\$}$	$3.02 \pm 0.15^{\$}$	$110 \pm 3.1^{\$}$	$6.68 \pm 0.41^{\$}$
HFD/STZ/Esculetin(100mg/kg) **(HFD+STZ+E2)**	$8.26 \pm 0.62^{\$}$	$3.94 \pm 0.19^{\$}$	$54 \pm 3.4^{\$}$	$3.00 \pm 0.31^{\$}$

Note: Data are represented as means ± SEM, n = 6 rats/group [(*) vs NC; (#) vs HFD; ($) vs HFD+ STZ].

Table 7: Amelioration in metabolic perturbation on treatment with Esculetin in insulin resistance and type 2 diabetic rats (24 weeks).

Groups	Plasma Glucose (mmol/l)	Plasma Insulin (pM)	Plasma Triglycerides (mg/dl)	Total Cholesterol (mmol/l)
Normal Control **(NC)**	6.11 ± 0.18	3.13 ± 0.06	45 ± 3.2	1.53 ± 0.02
NC/STZ(35mg/kg) **(NC+STZ)**	6.30 ± 0.21	3.58 ± 0.18	43 ± 2.5	1.87 ± 0.14
NC/Esculetin(50mg/kg) **(NC+E1)**	6.19 ± 0.29	3.11 ± 0.13	44 ± 2.3	1.82 ± 0.01
NC/Esculetin(100mg/kg) **(NC+E2)**	6.09 ± 0.18	3.02 ± 0.10	45 ± 1.5	1.78 ± 0.02
High Fat Diet Control **(HFD)**	7.24 ± 0.20*	9.26 ± 0.03*	96 ± 3.5*	6.71 ± 0.13*
HFD/Esculetin(50mg/kg) **(HFD+E1)**	6.63 ± 0.11#	5.63 ± 0.13#	74 ± 6.2#	3.54 ± 0.21#
HFD/Esculetin(100mg/kg) **(HFD+E2)**	6.11 ± 0.37#	3.44 ± 0.14#	45 ± 3.4#	1.80 ± 0.18#
HFD/Streptozotocin(35mg/kg) **(HFD+STZ)**	30.03 ± 1.73*	2.06 ± 0.04*	185 ± 4.1*	18.42 ± 0.32*
HFD/STZ/Esculetin(50mg/kg) **(HFD+STZ+E1)**	21.68 ± 0.42$	3.75 ± 0.06$	125 ± 6.5$	10.29 ± 0.04$
HFD/STZ/Esculetin(100mg/kg) **(HFD+STZ+E2)**	12.26 ± 0.39$	4.02 ± 0.16$	77 ± 1.5$	3.18 ± 0.15$

Note: Data are represented as means ± SEM, n = 6 rats/group [(*) vs NC; (#) vs HFD; ($) vs HFD+ STZ].

Table 8: Effect of esculetin treatment on morphometric changes in insulin resistance and type 2 diabetic rats (4 weeks).

Groups	**Body Weight (BW)(g)**	**Heart Weight (HW) (g)**	**Kidney Weight (KW) (g)**	**(HW/BW)*10^3**	**(KW/BW)*10^3**	**Systolic Blood Pressure (mmHg)**
Normal Control **(NC)**	165 ± 1.85	0.58 ± 0.043	3.45 ± 0.13	1.06 ± 0.02	3.11 ± 0.09	87.67 ± 0.49
NC/STZ(35mg/kg) **(NC+STZ)**	166 ± 5.27	0.59 ± 0.036	3.51 ± 0.14	1.18 ± 0.04	3.20 ± 0.10	87.50 ± 0.34
NC/Esculetin(50mg/kg) **(NC+E1)**	165 ± 2.51	0.58 ± 0.016	3.49 ± 0.10	1.16 ± 0.14	3.23 ± 0.14	86.67 ± 0.21
NC/Esculetin(100mg/kg) **(NC+E2)**	167 ± 2.75	0.57 ± 0.033	3.48 ± 0.06	1.14 ± 0.07	3.33 ± 0.11	87.17 ± 0.40
High Fat Diet Control **(HFD)**	194 ± 3.77*	0.73 ± 0.031	4.13 ± 0.18	1.26 ± 0.02	3.46 ± 0.08	97.50 ± 0.50*
HFD/Esculetin(50mg/kg) **(HFD+E1)**	193 ± 2.55	0.63 ± 0.054	3.76 ± 0.21	1.25 ± 0.04	3.28 ± 0.14	93.50 ± 0.84
HFD/Esculetin(100mg/kg) **(HFD+E2)**	180 ± 3.39#	0.57 ± 0.036	3.53 ± 0.20	1.25 ± 0.04	3.46 ± 0.08	88.00 ± 0.51#
HFD/Streptozotocin(35mg/kg) **(HFD+STZ)**	117 ± 4.92*	0.79 ± 0.033*	5.56 ± 0.13	1.96 ± 0.10*	6.60 ± 0.27*	116.00 ± 0.63*
HFD/STZ/Esculetin(50mg/kg) **(HFD+STZ+E1)**	120 ± 3.11	0.72 ± 0.056	4.40 ± 0.14	1.34 ± 0.06$	4.80 ± 0.25$	101.70 ± 1.11$
HFD/STZ/Esculetin(100mg/kg) **(HFD+STZ+E2)**	157 ± 5.36$	0.58 ± 0.028$	3.64 ± 0.14	1.29 ± 0.06$	3.46 ± 0.16$	88.33 ± 0.91$

Note: Data are represented as means ± SEM, n = 6 rats/group [(*) vs NC; (#) vs HFD; ($) vs HFD+ STZ].

Table 9: Effect of esculetin treatment on morphometric changes in insulin resistance and type 2 diabetic rats (24 weeks).

Groups	**Body Weight (BW) (g)**	**Heart Weight (HW) (g)**	**Kidney Weight (KW) (g)**	**(HW/BW)*10^3**	**(KW/BW)*10^3**	**Systolic Blood Pressure (mmHg)**
Normal Control **(NC)**	263 ± 2.75	0.79 ± 0.03	1.51 ± 0.02	2.93 ± 0.06	5.77 ± 0.09	87.88 ± 0.03
NC/STZ(35mg/kg) **(NC+STZ)**	260 ± 3.24	0.75 ± 0.04	1.50 ± 0.02	2.87 ± 0.15	5.76 ± 0.13	87.53 ± 0.04
NC/Esculetin(50mg/kg) **(NC+E1)**	261 ± 2.49	0.75 ± 0.02	1.51 ± 0.03	2.89 ± 0.07	5.77 ± 0.12	87.93 ± 0.16
NC/Esculetin(100mg/kg) **(NC+E2)**	263 ± 2.75	0.75 ± 0.03	1.51 ± 0.02	2.92 ± 0.07	5.75 ± 0.15	87.64 ± 0.22
High Fat Diet Control **(HFD)**	338 ± 2.53*	1.11 ± 0.04*	1.93 ± 0.02*	3.34 ± 0.07*	5.72 ± 0.08*	105.15 ± 1.50*
HFD/Esculetin(50mg/kg) **(HFD+E1)**	305 ± 3.28#	1.03 ± 0.03	1.72 ± 0.03#	3.31 ± 0.08	5.63 ± 0.10#	98.42 ± 0.43
HFD/Esculetin(100mg/kg) **(HFD+E2)**	262 ± 2.77#	0.89 ± 0.02#	1.51 ± 0.04#	3.05 ± 0.02$	5.77 ± 0.17#	87.60 ± 0.14#
HFD/Streptozotocin(35mg/kg) **(HFD+STZ)**	183 ± 1.35*	0.64 ± 0.02*	2.20 ± 0.05*	3.67 ± 0.05*	12.0 ± 0.31*	124.03 ± 1.13*
HFD/STZ/Esculetin(50mg/kg) **(HFD+STZ+E1)**	230 ± 2.30$	0.73 ± 0.04	1.88 ± 0.02$	3.63 ± 0.13	8.21 ± 0.18$	111.68 ± 1.03
HFD/STZ/Esculetin(100mg/kg) **(HFD+STZ+E2)**	263.0 ± 1.84$	0.79 ± 0.02	1.51 ± 0.02$	3.40 ± 0.06$	5.76 ± 0.09$	89.59 ± 1.20$

Note: Data are represented as means ± SEM, n = 6 rats/group [(*) vs NC; (#) vs HFD; (#) vs HFD+ STZ].

Table 10: Amelioration in renal function on treatment with Esculetin in insulin resistance and type 2 diabetic rats (4 weeks).

Groups	**Plasma Albumin (g/l)**	**Plasma Creatinine (mg/dl)**	**Blood Urea Nitrogen (g/dl)**
Normal Control **(NC)**	39.95 ± 1.67	1.29 ± 0.11	5.17 ± 0.32
NC/STZ(35mg/kg) **(NC+STZ)**	39.27 ± 2.40	1.29 ± 0.12	5.11 ± 0.25
NC/Esculetin(50mg/kg) **(NC+E1)**	39.03 ± 1.58	1.32 ± 0.10	5.35 ± 0.34
NC/Esculetin(100mg/kg) **(NC+E2)**	39.13 ± 2.97	1.18 ± 0.11	5.17 ± 0.46
High Fat Diet Control **(HFD)**	39.47 ± 1.99	1.34 ± 0.14	6.86 ± 0.54
HFD/Esculetin(50mg/kg) **(HFD+E1)**	39.05 ± 1.90	1.35 ± 0.25	5.49 ± 0.43
HFD/Esculetin(100mg/kg) **(HFD+E2)**	39.57 ± 2.57	1.27 ± 0.12	5.35 ± 0.44
HFD/Streptozotocin(35mg/kg) **(HFD+STZ)**	21.15 ± 2.43*	4.60 ± 0.14*	9.73 ± 0.36*
HFD/STZ/Esculetin(50mg/kg) **(HFD+STZ+E1)**	30.17 ± 2.31$	4.28 ± 0.35$	9.05 ± 0.79
HFD/STZ/Esculetin(100mg/kg) **(HFD+STZ+E2)**	39.42 ± 2.36$	1.70 ± 0.12$	5.47 ± 0.57$

Note: Data are represented as means ± SEM, n = 6 rats/group [(*) vs NC; (#) vs HFD; ($) vs HFD+ STZ].

Table 11: Amelioration in renal function on treatment with Esculetin in insulin resistance and type 2 diabetic rats (24 weeks).

Groups	Plasma Albumin (g/l)	Plasma Creatinine (mg/dl)	Blood Urea Nitrogen (g/dl)
Normal Control **(NC)**	38.73 ± 1.5	1.54 ± 0.12	5.28 ± 0.33
NC/STZ(35mg/kg) **(NC+STZ)**	37.97 ± 1.3	1.59 ± 0.18	5.15 ± 0.26
NC/Esculetin(50mg/kg) **(NC+E1)**	38.58 ± 1.6	1.48 ± 0.07	5.24 ± 0.35
NC/Esculetin(100mg/kg) **(NC+E2)**	38.81 ± 1.5	1.53 ± 0.01	5.38 ± 0.44
High Fat Diet Control **(HFD)**	37.95 ± 2.9	1.50 ± 0.05	7.46 ± 0.72
HFD/Esculetin(50mg/kg) **(HFD+E1)**	39.10 ± 2.9	1.55 ± 0.14	6.17 ± 0.48
HFD/Esculetin(100mg/kg) **(HFD+E2)**	38.67 ± 1.4	1.59 ± 0.12	5.27 ± 0.25
HFD/Streptozotocin(35mg/kg) **(HFD+STZ)**	18.48 ± 1.4*	11.37 ± 0.05*	18. 96 ± 0.37*
HFD/STZ/Esculetin(50mg/kg) **(HFD+STZ+E1)**	27.41 ± 2.6$	6.49 ± 0.01$	10.74 ± 0.21$
HFD/STZ/Esculetin(100mg/kg) **(HFD+STZ+E2)**	37.68 ± 1.6$	2.42 ± 0.04$	6.52 ± 0.50$

Note: Data are represented as means ± SEM, n = 6 rats/group [(*) vs NC; (#) vs HFD; ($) vs HFD+ STZ].

3. Esculetin attenuates vascular dysfunction in contraction and relaxation under insulin resistance and type 2 diabetes.

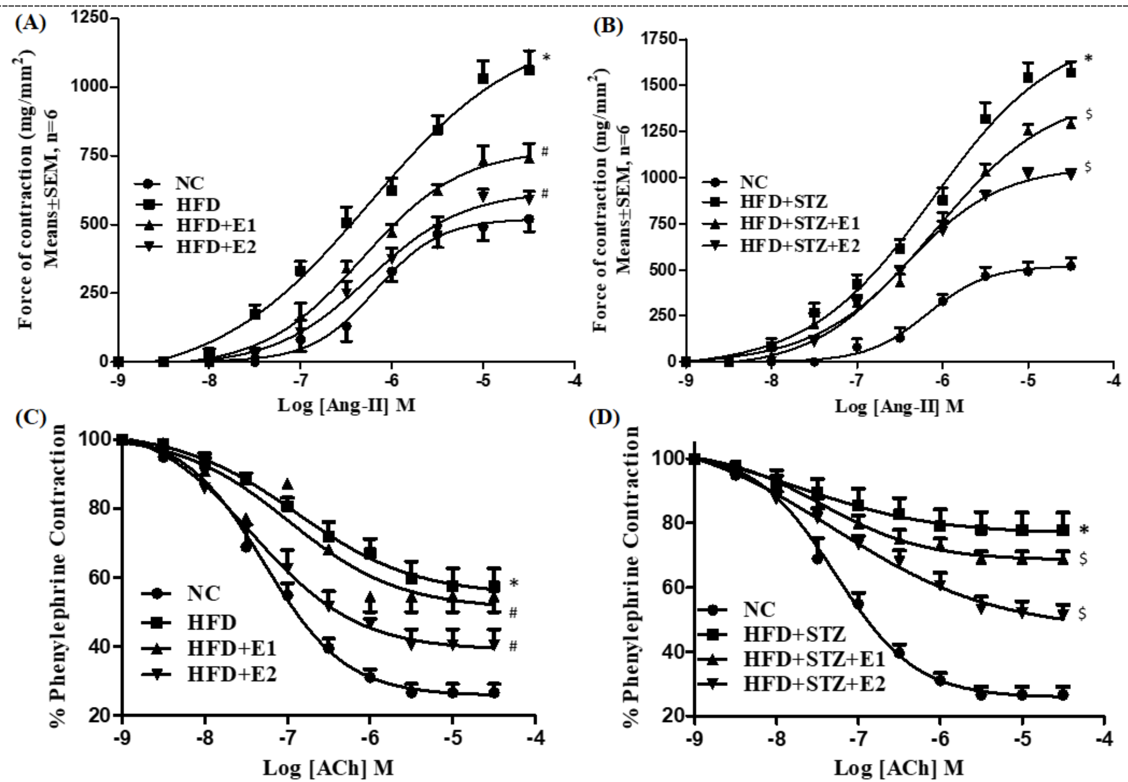


Figure 6: Effect of esculetin on Angiotensin II and Acetylcholine mediated vascular reactivity under insulin resistance and type 2 diabetes (4 weeks).

Cumulative response curves (CRCs) to Ang II mediate contractions on (A) NC, HFD, HFD+E1 and HFD+E2, and (B) NC, HFD+STZ, HFD+STZ+E1 and HFD+STZ+E2; Acetylcholine mediated relaxations on (C) NC, HFD, HFD+E1 and HFD+E2, and (D) NC, HFD+STZ, HFD+STZ+E1 and HFD+STZ+E2 rat thoracic aorta (n ¼ 6 rats/group). Note: (*) vs NC; (#) vs HFD; ($) vs HFD+STZ.

Under IR and T2D conditions the primary complications begin with vascular perturbations and immediately results in the deteriorated vascular toning. To establish vascular dysfunctions and effect of esculetin on the same, we performed cumulative concentration response curves (CRCs) to Ang II mediated vasoconstriction and ACh mediated vasorelaxation on aortic ring preparation. In both 4 and 24 weeks studies, CRCs of HFD and HFD+STZ group rats showed an upward shift indicating an increase in maximal contractile (E_{max}) response to Ang II when comparison to NC rats (Figure 6A, 4B, 5A and 5B) (Table 12). Further, esculetin significantly attenuated the exaggerated vascular responsiveness to Ang II in both IR and T2D conditions (Figure 6A and B). There was no

significant difference among all the groups of 2 different studies in terms of the pD2 value (Table 12), indicating that the sensitivity of aortic rings from different groups to Ang II remain unchanged. On the other hand, both HFD and HFD+STZ group showed reduced acetylcholine mediated relaxation which was significantly alleviated by esculetin treatment (Figure 6C, 4D, 5C and 5D).

Table 12: pD2 value and E_{max} of all group of animals to Ang II mediated aortic contraction

Groups	4 Weeks		24 Weeks	
	pD2 Value	E_{max} (mg/mm^2)	pD2 Value	E_{max} (mg/mm^2)
NC	6.21 ± 0.08	520 ± 45.52	7.331±0.11	649±14
HFD	6.28 ± 0.18	1037 ± 79.95*	7.15±0.21	1414±14*
HFD+E1	6.35 ± 0.16	755 ± 50.26$^{\#}$	7.027±0.18	1105±12$^{\#}$
HFD+E2	6.27 ± 0.13	600 ± 30.73$^{\#}$	7.369±0.19	623±96$^{\#}$
HFD+STZ	6.08 ± 0.07	1544 ± 51.27*	7.67±0.189	2304±12*
HFD+STZ+E1	6.04 ± 0.09	1304 ± 30.41$^{\$}$	7.657±0.12	1779±19$^{\$}$
HFD+STZ+E2	6.46 ± 0.12	1019 ± 37.27$^{\$}$	7.184±0.10	1136±16$

Note: Data are represented as means ± SEM, n = 6 rats/group [(*) vs NC; (#) vs HFD; ($) vs HFD+ STZ].

On the other hand, deflected vascular toning also been reported to be associated with dysregulation of endothelial derived nitric oxide synthase (eNOS), a profound molecule and highly restricted to endothelial cells which is required for vascular toning. However, in 24 week study, prolonged HFD fed and HFD + STZ rats showed significant reduction in aortic mRNA expression of *eNos*. On contrary, treatment with both the doses of esculetin inclined the expression *eNos* of mRNA levels in aorta.

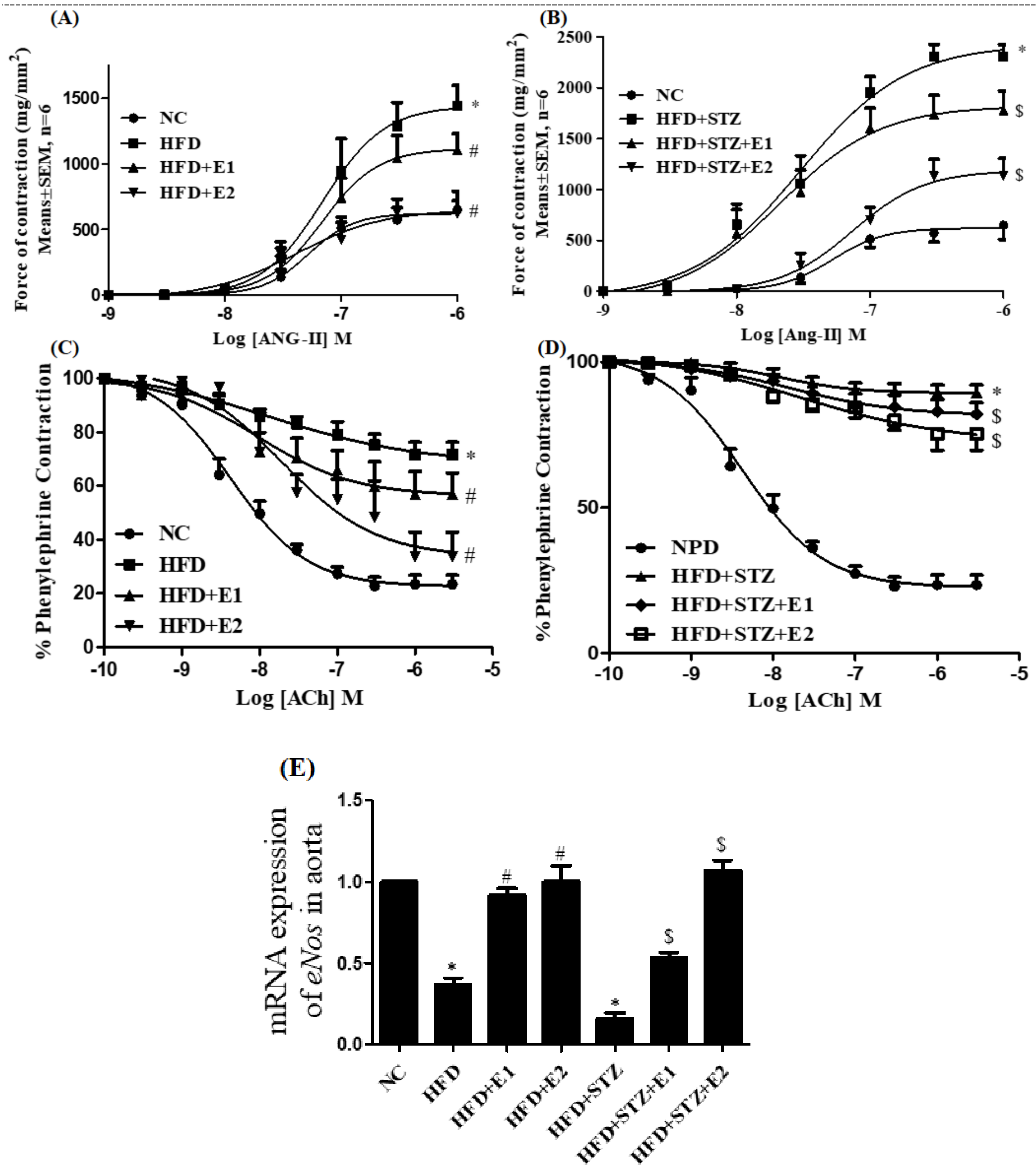


Figure 7: Effect of esculetin on Angiotensin II and Acetylcholine mediated vascular reactivity under insulin resistance and type 2 diabetes (24 weeks).

Cumulative response curves (CRCs) to Ang II mediate contractions on (A) NC, HFD, HFD+E1 and HFD+E2, and (B) NC, HFD+STZ, HFD+STZ+E1 and HFD+STZ+E2; Acetylcholine mediated relaxations on (C) NC, HFD, HFD+E1 and HFD+E2, and (D) NC, HFD+STZ, HFD+STZ+E1 and HFD+STZ+E2 rat thoracic aorta (n ¼ 6 rats/group). Endothelial nitric oxide synthase (eNos) mRNA levels in rat thoracic aorta; Results were analyzed by 2-ΔΔCt method and represented as relative fold changes Vs 18s. Each data

point is triplicated and represented as means ± S.E.M, [(*P < 0.05 compared with control), (*) vs NC; (#) vs HFD; ($) vs HFD+STZ].

4. Esculetin alleviates oxidative stress in cardiovascular and renal complications under insulin resistance and type 2 diabetes.

Hyperglycemia and IR have been found to be intricately linked with abruption of anti-oxidative defense mechanisms by raise in free radicals and weakening of antioxidants (Nishikawa, 2015; Santos et al., 2008). Similarly, renal GSH level measurement was significantly reduced in HFD and HFD+STZ group (Figure 6A). In addition, lipid peroxidation determination, significantly evidenced by increased renal TBA reactive substance in HFD and HFD+STZ group (Figure 6B). Nonetheless, esculetin treatment was able to strongly reduce this deleterious effects (Figure 6A and B).

Under basal conditions, nuclear factor-like 2 (NRF2), a transcriptional activator which binds antioxidant response elements (ARE) of target gene promoter, has been found co-localize with inhibitor INrf2 (Keap-1) and retain in the cytoplasm. Under oxidative stress, Keap-1 releases NRF2, thereby allowing the activator to translocate to the nucleus and

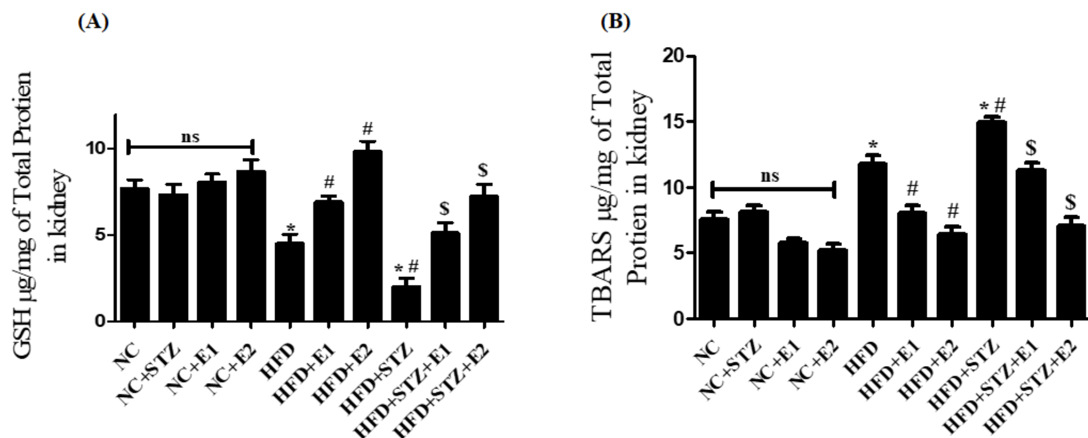


Figure 8: Effect of esculetin treatment on oxidative stress parameters such as reduced glutathione (GSH) and thiobarbituric acid reactive substrate (TBARS).

Esculetin decreased the oxidative stress, indicated by (A) increased GSH levels, (B) reduced TBA reactive substance levels Each data point is triplicated and represented as means ± S.E.M, [(*P < 0.05 compared with control), (*) vs NC; (#) vs HFD; ($) vs HFD+STZ].

bind to ARE containing genes. Here, estimation of Keap-1 is one of the markers to evidence oxidative stress and we examined the levels of Keap1 in 24 and 4 weeks animal heart and aorta respectively. Augmentation of Keap-1 protein was observed in HFD and

HFD+STZ group of both 24 (heart) and 4 (aorta) weeks animals (Figure 7A and B). Esculetin treatment significantly reduced the Keap-1 protein expressions in heart and aorta tissues (Figure 7A and B). Furthermore, superoxide dismutase 1 (SOD1) (CuZnSOD), a cytoplasmic isoform is a major antioxidant enzyme for removal of free radicals. In order to check the effect of esculetin on expression of Sod1, we measured the mRNA expression of Sod1 in 24 week rat kidney. Compared with NC, the Sod1 mRNA expression was reduced by 0.5 and >0.5 fold in HFD and HFD+STZ groups respectively. Esculetin treatment, at both the doses, showed significant improvement in Sod1 mRNA expression (Figure 6E). On the bases of above observations, esculetin has a potential to ameliorate the oxidative stress and radical scavenging effect which is considered essential to slow down the progression of cardiovascular and renal complications associated with IR and T2D.

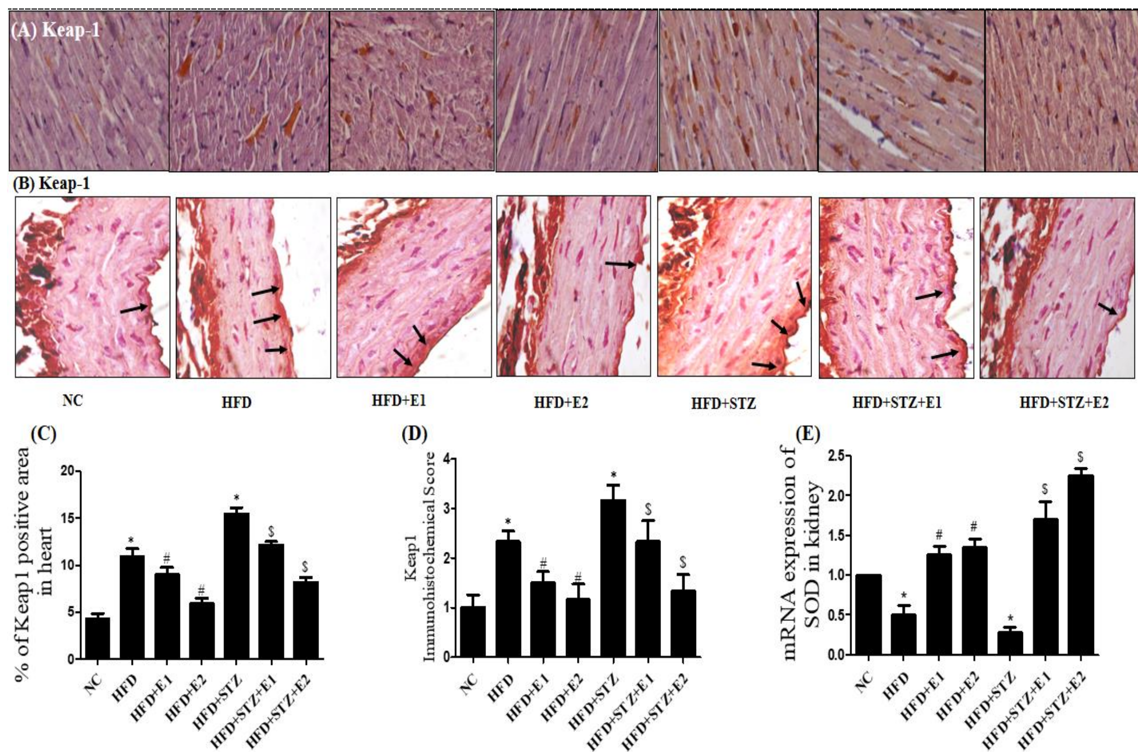


Figure 9: Esculetin suppresses Keap-1 protein expression and increases Sod 1 mRNA levels under insulin resistance and type 2 diabetes conditions.

Light microscopic images illustrating immune staining for Keap-1 protein expression (40X magnification) in (A) 24 weeks study animal heart; (B) 4 weeks study animal aorta; and quantification of (C) % Keap-1 positive area; and (D) Keap-1 immunohistochemistry scores. All values are represented as means ± SEM from at least 25 sections per group. Each data point is represented as means ± SEM, n = 6 rats/group. Esculetin reduces mRNA

expression of (E) *Sod 1* in both HFD fed and HFD+STZ treated rats. Results are represented as relative fold changes Vs 18s. Each data point is triplicated and represented as means ± S.E.M, [(*P < 0.05 compared with control), (*) vs NC; (#) vs HFD; ($) vs HFD+STZ].

5. Effect of esculetin on up-regulated renin angiotensin system (RAS) in cardiovascular and renal complications under insulin resistance and type 2 diabetes.

Renin angiotensin system (RAS) play a central role in the pathophysiology of cardiovascular and renal diseases in human. In IR and T2D, up-regulation of RAS and its consequences reinforce in the development of devastating cardio-renal syndrome. To evidence such conditions and effect of esculetin treatment on the same, we observed protein and mRNA expression of angiotensin II receptor type 1 (AT1), angiotensin II receptor type 2 (AT2), angiotensin converting enzyme (ACE) and angiotensin converting enzyme 2 (ACE2).

In HFD and HFD+STZ animals' heart, protein expressions of AT1 and AT2 were increased, whereas on contrary, ACE2 protein was reduced (Figure 10A, B and C). Similarly, mRNA levels of AT1 and ACE were observed to be increased in kidneys of HFD and HFD+STZ animal, and ACE2 mRNA level was declined (Figure 10G, H and I). In the aorta, both AT1 protein and mRNA expressions were increased in HFD and HFD+STZ groups (Figure 11A and G). The conditions of AT2 in the aorta are different, where we observed increased protein and mRNA expression in HFD group, but on the other hand, in HFD+STZ, even though increased AT2 protein expression, we observed reduced mRNA levels (Figure 11B and H). Next, the ACE2 protein was reduced in aorta of HFD and HFD+STZ animals. The treatment with esculetin, especially with higher dose, significantly reduced protein and mRNA expressions of AT1 in aorta, heart and kidney. Further, in heart, the AT2 was reduced and ACE2 protein expression was increased in both heart and kidney. In aorta, esculetin treated group showed increased mRNA expression of AT2 receptors.

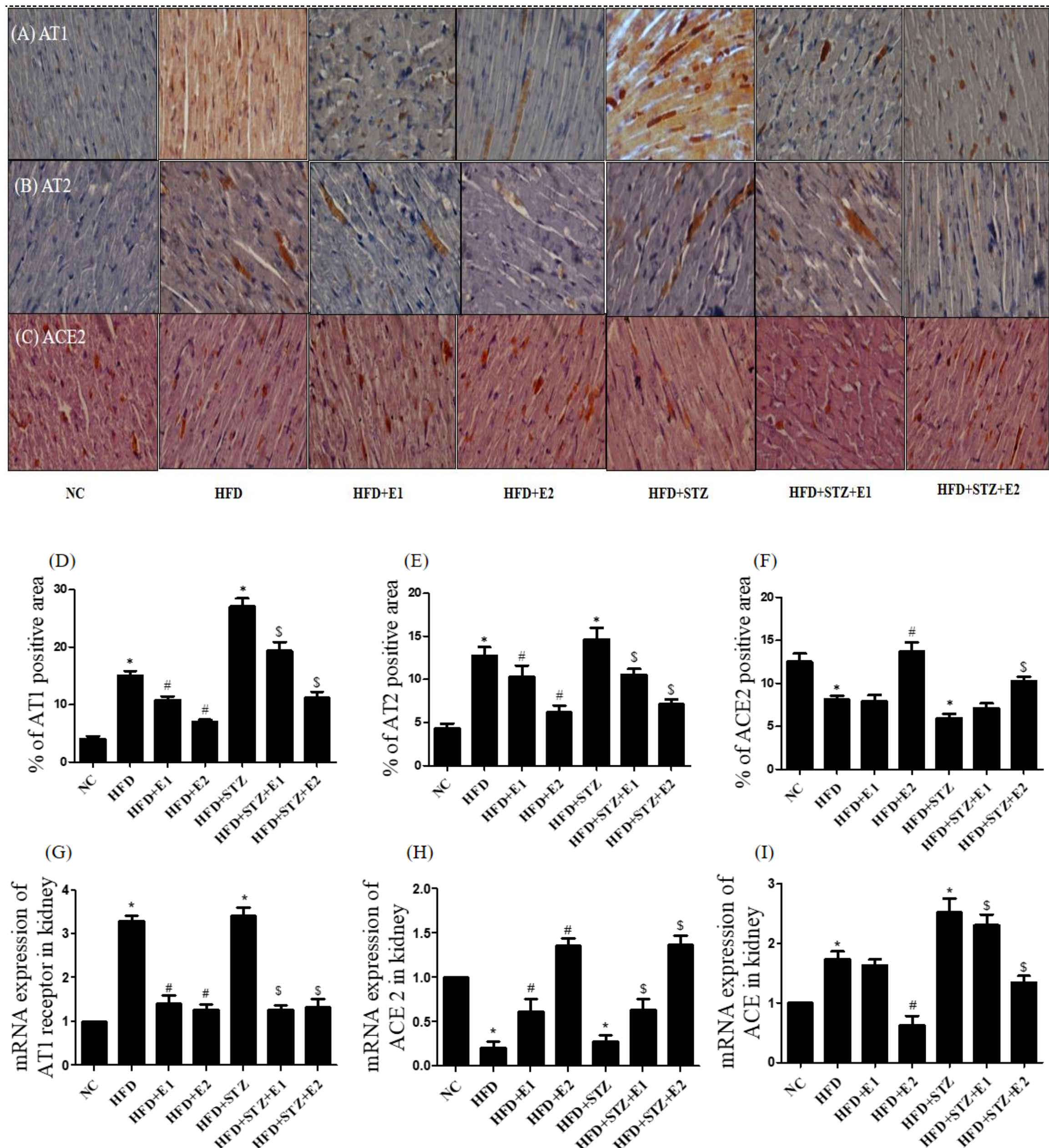


Figure 10: Effect of esculetin treatment on up-regulated renin angiotensin system (RAS) in heart and kidney tissues under insulin resistant and type 2 diabetes

Immunohistochemical analysis of (A) AT1, (B) AT2 and (C) ACE2 were performed (original magnification, ×100). Expression level of (D) AT1, (E) AT2 and (F) ACE2 were represented in % positive area. Furthermore, esculetin reduced the mRNA expressions of (G) angiotensin II receptor type 1 *(At1)*, (I) angiotensin II converting enzyme *(Ace)* and elevated the mRNA expression of (H) Angiotensin I converting enzyme 2 *(Ace2)* in HFD fed and HFD+STZ treated rats. Results were represented as relative fold changes Vs 18s. Each data point is triplicate. [Note: Each data point is represented as means ± S.E.M, *P < 0.05 compared with control, (*) vs NC; (#) vs HFD; ($) vs HFD+STZ].

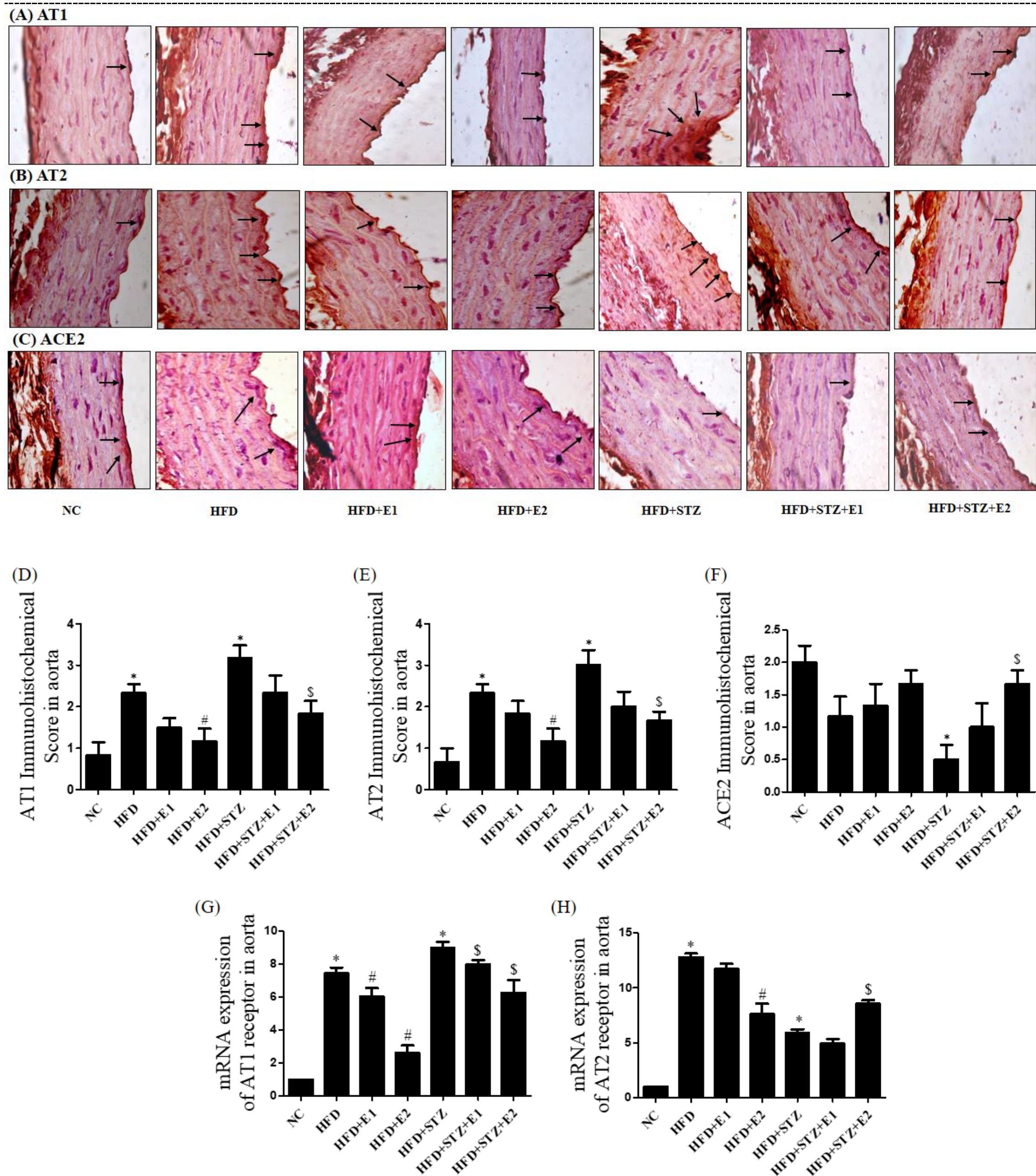


Figure 11: Effect of esculetin treatment on up-regulated renin angiotensin system (RAS) in the aorta under insulin resistant and type 2 diabetes

Immunohistochemical analysis of (A) AT1, (B) AT2 and (C) ACE2 were performed (original magnification, ×100). Expression level of (D) AT1, (E) AT2 and (F) ACE2 were represented in % positive area. Furthermore, esculetin modulated the mRNA expressions of (G) angiotensin II receptor type 1 *(At1)* and (H) angiotensin II receptor type 2 *(At2)* in HFD fed and HFD+STZ treated rats. Results were represented as relative fold changes Vs 18s. Each data point is triplicate. [Note: Each data point is represented as means ± S.E.M, *P < 0.05 compared with control, (*) vs NC; (#) vs HFD; ($) vs HFD+STZ].

6. Intervention of esculetin in the progressive inflammatory, proliferative and fibrotic pathways in cardiovascular and renal complications under insulin resistance and type 2 diabetes.

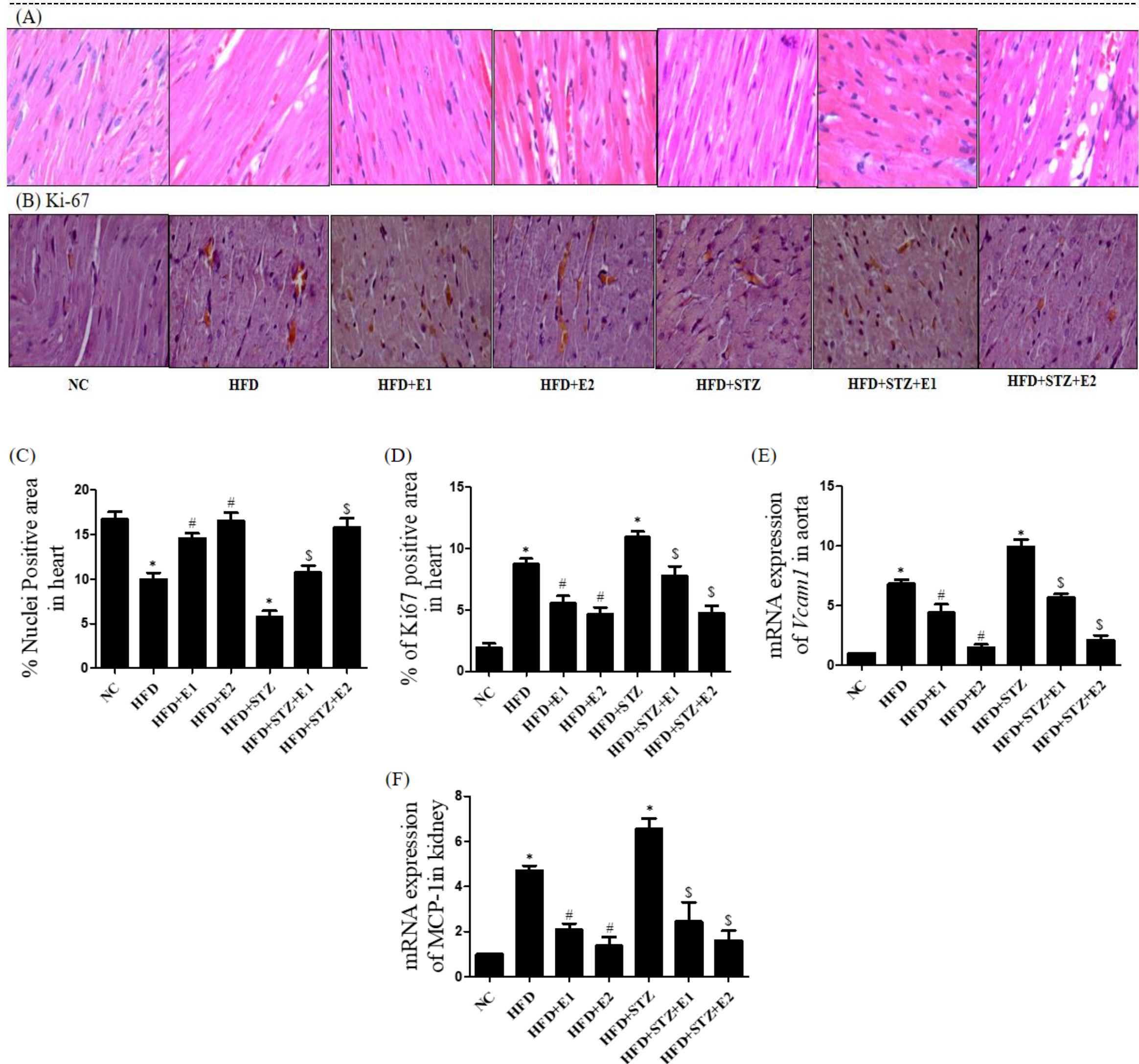


Figure 12: Effect of esculetin treatment on hypertrophy, proliferation and inflammatory markers in heart, aorta and kidney under insulin resistant and type 2 diabetes

Heart sections stained with (A) hematoxylin/eosin (H&E) stain; and immuno staining with (B) anti - Ki-67 (original magnification, ×100) were analyzed semi-quantitatively as % of Nuclei positive area for (C) H&D and (D) Ki-67. Next, mRNA expressions of (E) Vascular cell adhesion molecule 1 (Vcam1) in aorta and (F) Monocyte Chemoattractant Protein-1 (Mcp1) in kidney. Note: Results were represented as relative fold changes Vs 18s. Each data point is triplicate. Note: [Each data point is represented as means ± S.E.M, *P < 0.05 compared with control, (*) vs NC; (#) vs HFD; ($) vs HFD+STZ].

The up-regulated RAS has enough multifunctional machineries which influence many cellular and molecular processes, including cell growth, proliferation, inflammation and fibrosis. In general, we the characterized myocardial hypertrophy by haematoxylin and eosin (H&E) staining in heart sections where marked reduction in nuclei positive area and considerable increase in extracellular matrix was evidenced in HFD and HFD+STZ rats (Figure 12A). On contrary, esculetin treatment marked increased the nuclei positive area and reduced extracellular matrix in HFD and HFD+STZ treated animals (Figure 12A).

Further, followed by cell growth, proliferation is another characteristic feature of cardiovascular diseases. On this account, we measured protein expression of Ki-67 (a marker for determining growth of a given cell population). The myocardial expression of Ki-67 protein was significantly higher in HFD and HFD+STZ group animals, which was significantly reduced by esculetin treatment (Figure 12B).

In parallel, the inflammatory markers such as VCAM1 and MCP-1 were symbolized the deteriorating progression in diabetic complication. A marked increased in mRNA expressions of *Vcam1* and *Mcp1*in aortic and kidney tissues respectively were observed in HFD and HFD+STZ group rats (Figure 12E and F). Esculetin treatment resulted into a significant drop in *Vcam1* (Figure 12E) and *Mcp1* (Figure 12F) mRNA expressions.

As a consequence of these molecular changes, activated fibrotic cascade was determined under IR and T2D conditions. We measured aortic protein deposition of TGF-β and Collagen, heart collagen and renal TGF-β, collagen and fibronectin. Similarly, we measured mRNA expression of *Tgfβ1* and *Collagen1a1* in kidney. The results showed increased TGF-β protein expressions in aorta and kidney, and increased mRNA expression in kidney in HFD and HFD+STZ rats (Figure 13A, B and D). Further, the collagen protein deposition was observed to be increased in aorta, heart and kidney along with mRNA levels in kidney of only HFD+STZ rats (Figure 14A, B, C and I).

Surprisingly, the mRNA expression of *Collagen1a1* was not modified kidney samples of HFD fed group rats (Figure 14I). Furthermore, the protein expression of fibronectin was also increased in kidneys of HFD and HFD+STZ group animal (Figure 14D). The esculetin treatment abrogated all measured markers of fibrosis in IR and T2D developed rats. Finally, in compared with HFD and STZ control group animals, esculetin treated groups represented anti - hypertrophic, proliferative inflammatory and fibrotic activity of esculetin in IR and T2D conditions.

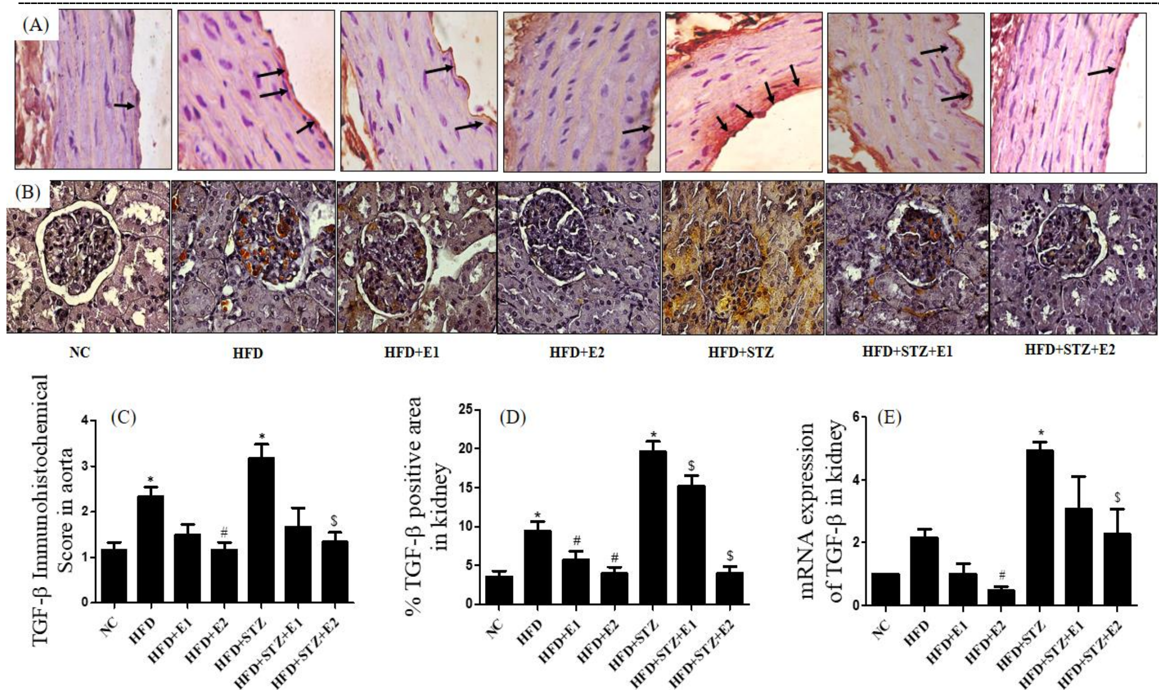


Figure 13: Effect of esculetin treatment on tissue TGF-β under insulin resistant and type 2 diabetes

Immunohistochemistry of Transforming growth factor beta (TGF-β) in (A) aortic (original magnification, X100) and (B) renal sections (original magnification, X40) which were analysed in (D) aorta as TGF-β immuno staining scores and in (E) kidney semi-quantitatively as % of TGF-β positive area. Further estimated mRNA levels of *Tgfβ* in kidneys and results were represented as relative fold changes Vs 18s. [Note: each data point is triplicated and represented as means ± S.E.M, *P < 0.05 compared with control, (*) vs NC; (#) vs HFD; ($) vs HFD+STZ].

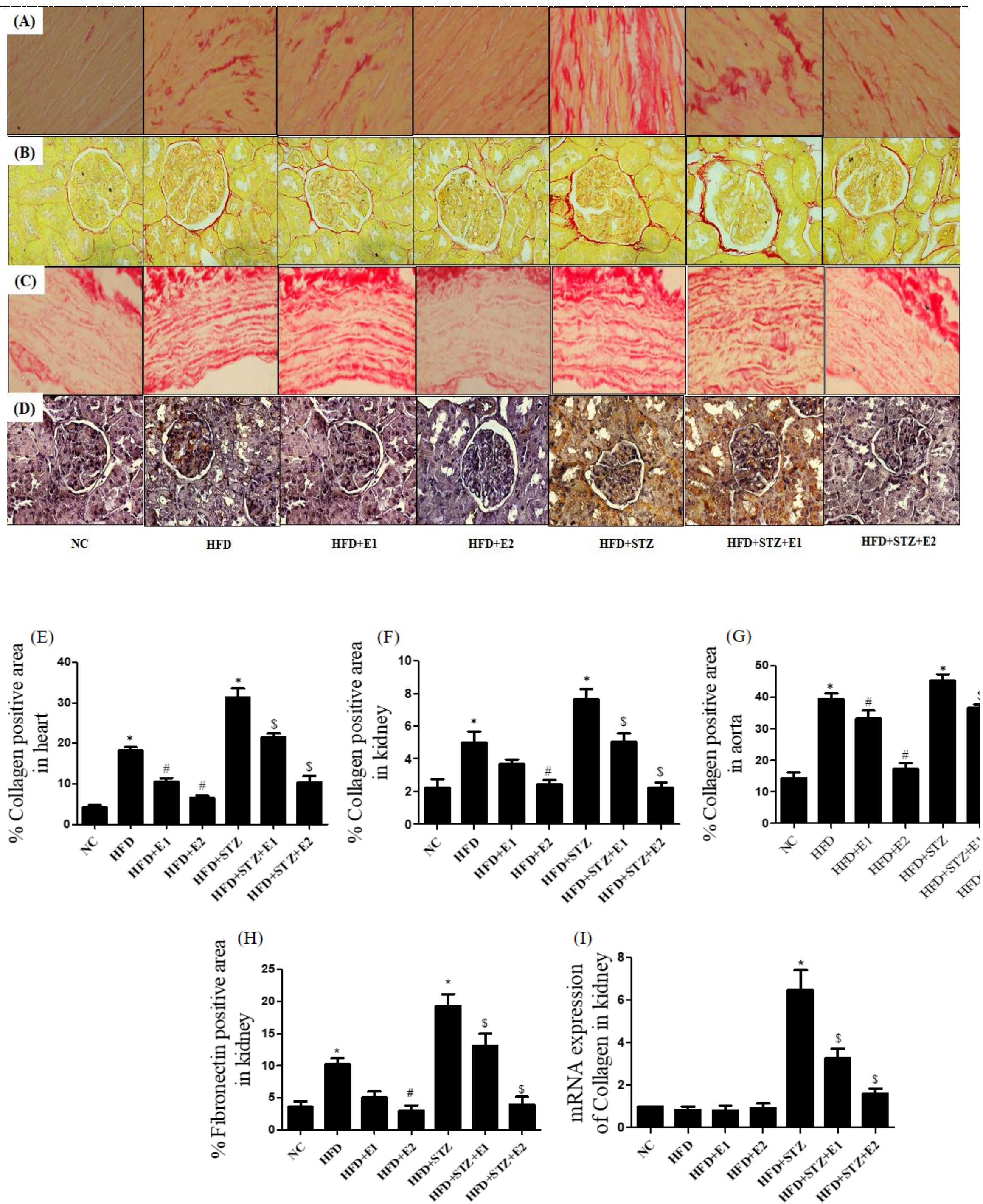


Figure 14: Effect of esculetin treatment on collagen and fibronectin under insulin resistant and type 2 diabetes

The microscopic illustrations representing collagen depositions in histopathological sections of (A) heart, (B) kidney and (C) aorta which were stained with picro-sirius red (PSR) (original magnification, X100); (D) Fibronectin immunostaining was estimated (40X magnification) and depositions were estimated by using Image J software as (E, F

and G) % collagen positive area and (H) % fibronectin positive area. Further, estimation of (I) collagen (*Col1a1*) mRNA was represented as relative fold changes Vs 18s. [Note: Each data point is triplicated and represented as means ± S.E.M, *P < 0.05 compared with control, (*) vs NC; (#) vs HFD; ($) vs HFD+STZ].

7. Esculetin modulates post-translational histone modulations in heart, kidney and aorta in cardiovascular and renal syndrome under insulin resistance and type 2 diabetes.

Post translational histone modifications (PTHMs) alter the chromatin states thereby modifying the accessibility of transcriptional machineries which play a crucial role in gene(s) and protein expression. It has been reported that change in histone H3 methylation could account for TGF-β induced over expression of ECM genes and plays a pivotal role in the development of diabetic nephropathy. In addition, in type 2 diabetic (db/db) mice renal failure can increase H3K9/K14Ac, H3S10phospho, and H3K4me in the heart, which can be a contributing factor for cardiac hypertrophy and fibrosis. Therefore, to examine chromatin unfolding in IR and T2D conditions, we have checked various permissive PTHMs by western blotting.

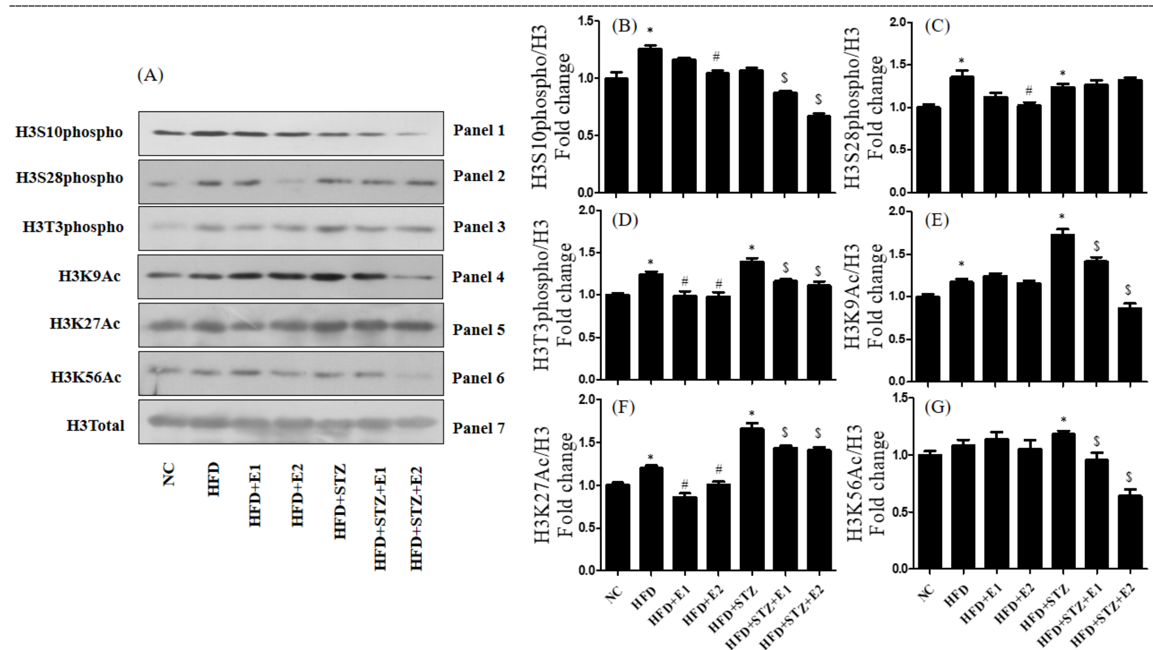


Figure 15: Effect of esculetin treatment H3 phosphorylation and acetylation immunoblotting in whole heart of Insulin resistance and type 2 diabetes rats.
(A) PTHMs – H3S10phospho (panel 1), H3S28phospho (panel 2), H3T3phospho (panel 3), H3K9Ac (panel 4), H3K27Ac (panel 5), H3K56Ac (panel 6) and H3Total (panel 7) were performed and represented; Protein levels were quantified by scanning densitometry using Image J software **(B, C, D, E, F and G)**. Each data point is represented as mean ± SEM, n = 3 blots/protein [(*) vs NC; (#) vs HFD; ($) vs HFD+ STZ].

In cryopreserved heart samples, significant increase in H3S10phospho (Figure 15 - panel 1), H3S28phospho (Figure 15 - panel 2), H3K9Ac (Figure 15 - panel 4), H3K4me2 (Figure 16 - panel 1) and H3K9me2 (Figure 16 - panel 2) was observed in HFD and HFD+STZ treated rats compared to NC. In addition, for the first time we found augmented levels of other permissive PTHMs– H3T3phospho (Figure 15 - panel 3), H3K27Ac (Figure 15 - panel 5), H3K56Ac (Figure 15 - panel 6), H3K36me2 (Figure 16 - panel 3), and H3K79me2 (Figure 16 - panel 4) in HFD and HFD+STZ treated rats compared to NC rats. This result suggests the state of the transcriptional activation under IR and type 2 diabetes. Esculetin was found to be more effective in attenuating permissive PTHMs at 100 mg/kg/day dose (Figure 15 & 14).This novel finding suggests that esculetin's ability to reverse permissive PTHMs might be contributed to its cardio protective potential in IR and type 2 diabetes.

It has been established that augmented H2B monoubiquitination may lead to increased more stable PHTMs such as histone H3K4 and H3K79 methylation. To the best of our knowledge, there is no in vivo report available regarding alteration in histone ubiquitination in type 2 diabetic condition. Hence, we examined the expression of H2AK119 and H2BK120 ubiquitination in the heart of IR and T2D rats by immunoblotting (Figure 16). H2AK119Ub (Figure 16 - panel 6) and H2BK120Ub (Figure 16 - panel 7) levels were significantly increased in HFD and HFD+STZ treated rats as compared to NC (Figure 16). This finding clearly matches the observed changes in H3 dimethylation in the same animals. In addition, we found that esculetin effectively reduces H2AK119Ub and H2BK120Ub levels in the heart of IR and T2D rats (Figure 16). This novel finding further helps us to understand the basic mechanism of the cardio protective role of esculetin in T2D condition.

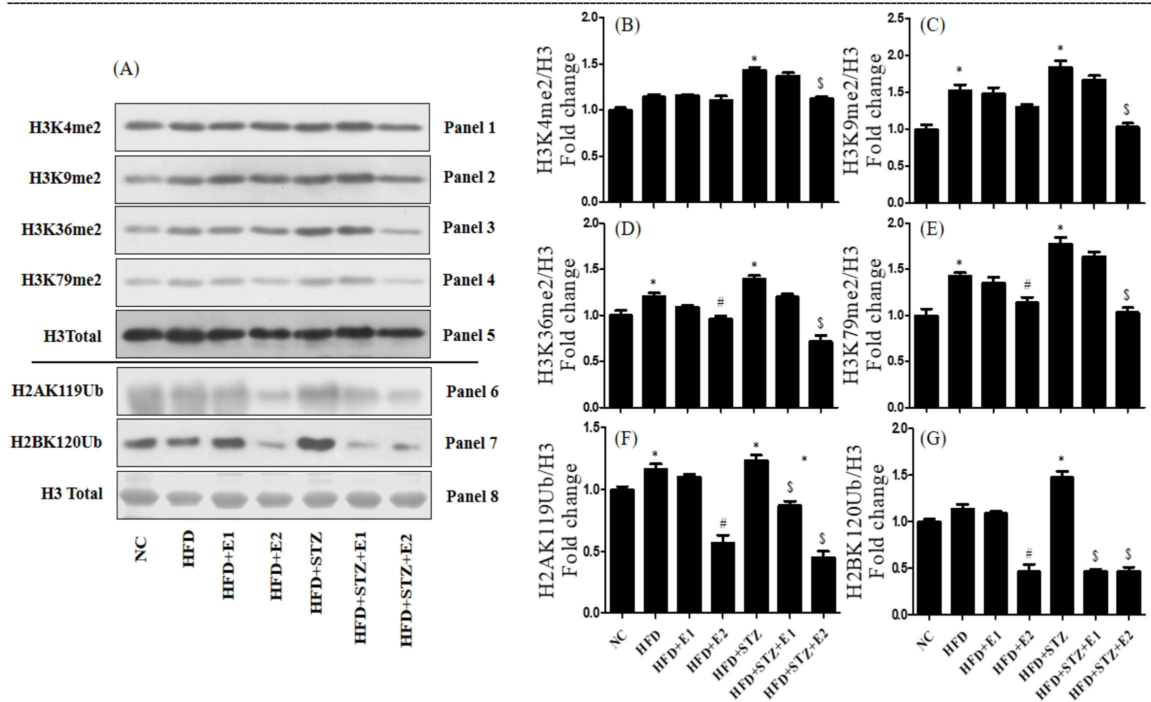


Figure 16: Esculetin treatment modulates H3 dimethylation and H2A/H2B ubiquitination in whole heart of Insulin resistance and type 2 diabetic rats.

Immunoblotting represented (A) PTHMs – H3K4me2 (panel 1), H3K9me2 (panel 2), H3K36me2 (panel 3), H3K79me2 (panel 4), H3Total (panel 5), H2AK119Ub (panel 6), and H2BK120Ub (panel 7) and H3Total (panel 8); Protein levels were quantified by scanning densitometry using Image J software and represented in **B, C, D, E, F** and **G**. Each data point is represented as mean ± SEM, n = 3 blots/protein [(*) vs NC; (#) vs HFD; ($) vs HFD+STZ].

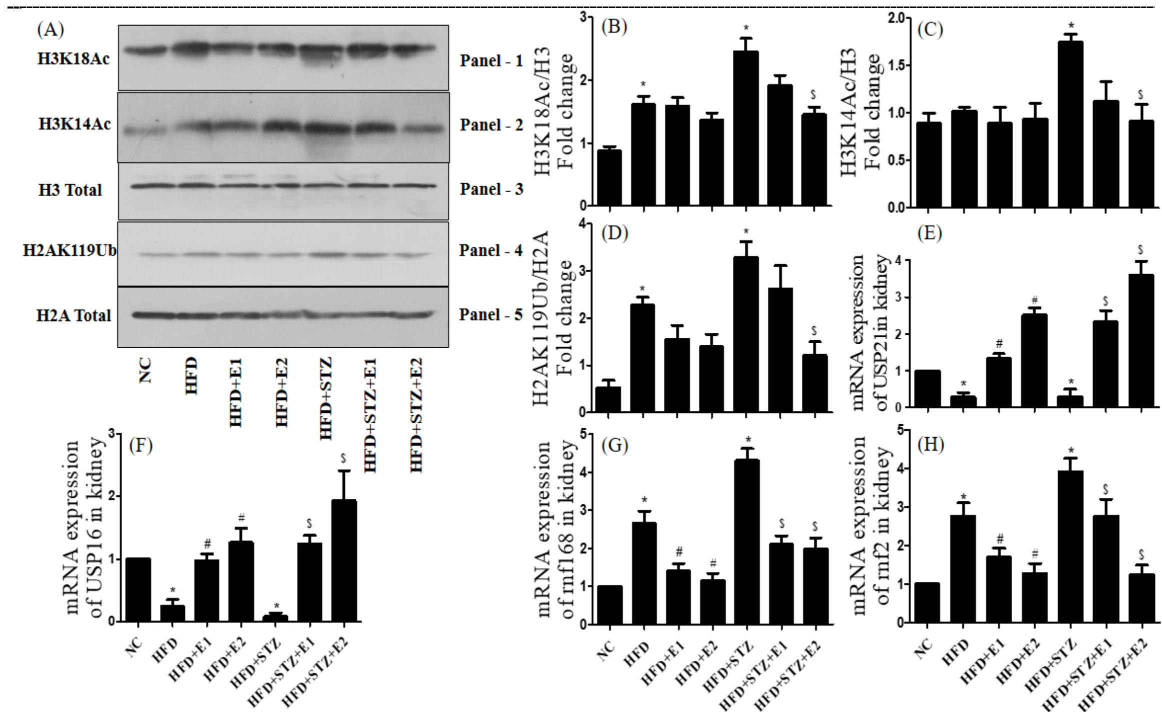


Figure 17: Effect of esculetin treatment on PTHMs - H3 acetylation, H2AK119 monoubiquitination and H2A specific mRNA expression of E3 ubiquitin ligases and deubiquitinases in insulin resistance and hyperglycemic rat kidneys.

(A) Representative Western blot for histone H3 acetylation on lysine (K) 18 (panel 1) and lysine 14 (panel 2); total histone H3 (panel 3); histone H2A ubiquitination on K119 (panel 4) total H2A (panel 5). For quantification, each band was normalized to the respective total histone H3 for H3 acetylation and total H2A for H2A ubiquitination. Protein levels were quantified by scanning densitometry using ImageJ software (B), (C) and (D). Each data point is represented as means ± SEM, n = 3 blots/protein [(*) vs NC; (#) vs HFD; ($) vs H + S]. Further, esculetin decreased mRNA levels of H2A specific deubiquitinases (E) Usp21 and (F) Usp16, and E3 ligases (G) Rnf168 and (H) Rnf2. Results are represented as relative fold changes Vs 18 s. Each data point is triplicated and represented as means ± S.E.M, [(*P < 0.05 compared with control), (*) vs NC; (#) vs HFD; ($) vs HFD + STZ].

Next we examined the impact of IR and T2D PTHMs in rat kidney samples. Histone H3 acetylation (H3Ac) is a permissive PTHM associated with expression of various chemokines and cytokines. Therefore, to understand the effect of esculetin on H3Ac under IR and T2D conditions, we examined H3K14Ac and H3K18Ac modifications. In HFD and HFD + STZ groups, prominent increase in H3K14Ac and H3K18Ac was observed, which is concordant with the previous reports. A significant reduction in H3Ac was evidenced

on treatment with esculetin (Figure 17 - Panel 1 and 2). Further, results demonstrated that esculetin treatment shows histone deacetylating potential and reverses permissive PTHMs in the kidneys of IR and T2D rats. Furthermore, our studies reveal that H2AK119Ub is increased significantly in both HFD fed and HFD+STZ treated as compared to NC which could be controlled markedly by esculetin treatment (Figure 17 Panel 4). The elevation in H2AK119Ub could be further correlated with the alterations in mRNA expression of H2A specific ubiquitination machineries. We found that in HFD and HFD + STZ groups, mRNA levels of E3 ligases- Rnf168 and Rnf2 (Figure 17G and H) were higher and deubiquitinases - Usp21 and Usp16 [Figure 17E and F] were significantly lower than NC and these aberrations were significantly reversed by the esculetin treatment.

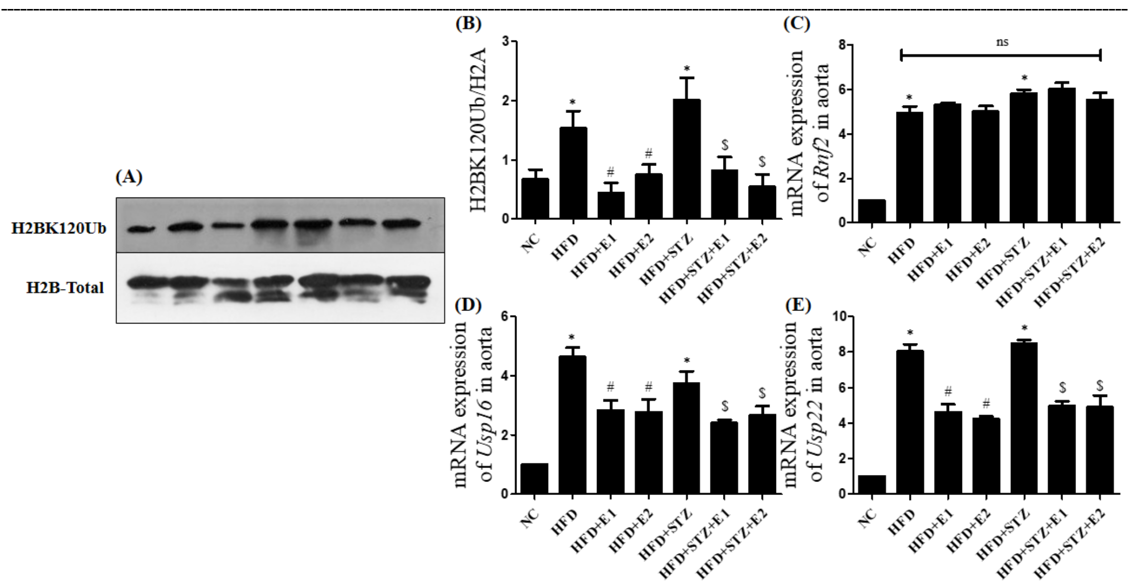


Figure 18: Effect of esculetin on histone H2B lysine 120 monoubiquitination (H2BK120Ub) in thoracic aorta under insulin resistance and type 2 diabetic conditions. **(A)** Representative Western blot for histone H2BK120 and total H2B. **(B)** Quantification was performed by scanning densitometry using Image J software and normalizing each band to the respective total histone. Each data point is represented as means ± SEM, n = 3 blots/protein. Further, mRNA expression of H2A specific E3 ligase **(C)** Rnf2, and H2A/H2B specific deubiquitinases **(D)** Usp16 and (E) Usp22 were analyzed by 2-ΔΔCt method and represented relative fold changes Vs 18s. [Note: (*) vs NC; (#) vs HFD and ($) HFD+STZ].

Since we extensively studied and reported histone H2A and H2B monoubiquitination in the development of diabetic cardiac and renal complication. In extension of such work, we observed increased H2BK120 in HFD and HFD+STZ rats (Figure 18A) thoracic aorta. On the other hand, esculetin reversed the modification in H2BK120Ub. Further, elevated mRNA levels of Rnf2, (Figure 18C) a H2AK119 specific E3 ubiquitin-protein ligase required for H2A monoubiquitination, were observed in both HFD and HFD+STZ rat aorta (Figure 18D). However, esculetin treatment showed no such significant impact on Rnf2 mRNA levels in rat thoracic aorta. With prospective of H2A/H2B specific deubiquitinases such as USP16 and USP 22, mRNA levels of both Usp16 (Figure 18D) and Usp22 (Figure 18E) were elevated in aorta of HFD and HFD+STZ rats (Figure 18E and F). In esculetin treated groups, the mRNA levels of Usp16 and Usp22 were significantly declined compared with HFD and HFD+STZ group animals.

8. Esculetin alters H2B lysine 120 monoubiquitination (H2BK120Ub) enrichment at promoters of AT1 and AT2 receptors, inflammatory and pro-fibrotic genes in the rat aorta under insulin resistance and type 2 diabetes.

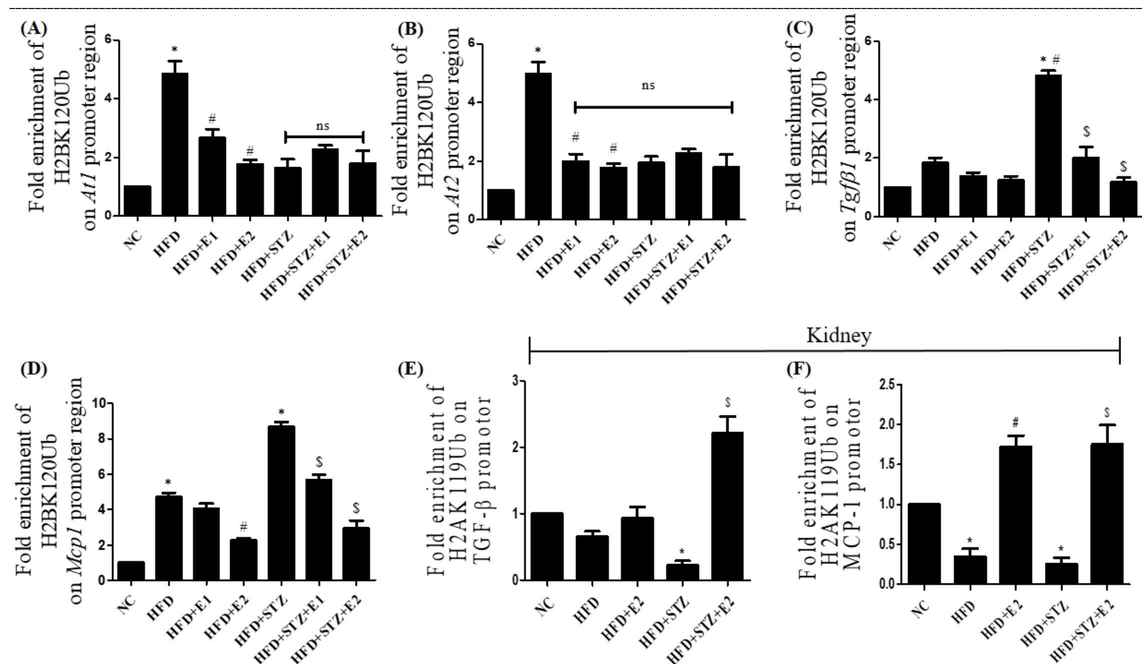


Figure 19: Effect of esculetin on the occupancy of H2A/H2B at promoter regions of fibrotic and inflammatory genes under insulin resistance and type 2 diabetic conditions.

Occupancies of H2BK120Ub on the promoters of **(A)** *At1*, **(B)** *At2*, **(C)** *Tgfb1* and **(D)** *Mcp1* were demonstrated by ChIP assay in thoracic aorta samples. Further in kidney samples, the effect of esculetin treatment on the occupancy of H2AK119Ub at promoter regions of **(E)** *Tgfb1* and **(F)** *Mcp1* was ddetermined. Fold enrichment was calculated after normalisation with input for ChIP-qPCR. Finally results are expressed as fold change over normal control group. Each data point is triplicated and represented as means ± S.E.M, [(*P < 0.05 compared with control), (*) vs NC; (#) vs HFD and ($) vs HFD+STZ].

In continuation with novel approach, the occupancy of H2BK120Ub at At1, At2, Tgfβ1 and Mcp1 gene were determined and evidenced the role of H2BK120Ub in AT1 and AT2 receptor expression, and TGF β and MCP-1 protein expression. In HFD group, the occupancy of H2BK120Ub at *At1, At2, Tgfβ1* and *Mcp1* promoter regions were high (Figure 19A, B, C and D), whereas in HFD+STZ group, occupancy of H2BK120Ub is reduced at At1 and At2, and increased at *Tgfβ1* and *Mcp1* promoter regions. Interestingly, intervened effect of esculetin significantly reduced in the occupancy of H2BK120Ub at *At1, At2, Tgfβ1* and *Mcp1* promoter regions in HFD group animals. Further we observed no significant difference in H2BK120Ub occupancy at *At1* and *At2* promoter regions of esculetin treated HFD+STZ rats. But surprising occupancy at *Tgfβ1* and *Mcp1* promoter regions in esculetin treated HFD+STZ group animals were marked reduced (Figure 19A, B, C and D).

9. Esculetin modifies H2A lysine 119 mono-ubiquitination (H2AK119Ub) enrichment at inflammatory and pro-fibrotic promoter regions in kidney under insulin resistance and type 2 diabetes conditions.

To determine the role of H2AK119Ub in renal fibrosis and macrophage infiltration in IR and T2D kidneys, we conducted chromatin immunoprecipitation (ChIP) assays which is explained in methods section. Our results demonstrate that promoter region of fibrotic (*Tgfb1*) and inflammatory (*Mcp1*) genes were less occupied with immune-precipitated H2AK119Ub in HFD and HFD + STZ rats. The occupancy of H2AK119Ub on promoter regions of both *Tgfb1* and *Mcp1* was found to be significantly improved in esculetin treated groups as compared with HFD and HFD + STZ rats (Fig. 17E and F).

Chapter IV

Discussion

The present study shows that esculetin treatment in HFD fed as well as HFD+STZ treated rats ameliorated metabolic and morphometric alterations associated with hyperglycaemia, IR and renal failure which could be ascribed to its ability to regularise the oxidative stress and disturbances in RAS activity.

1. Food matrix effect and possible additive, synergistic and antagonistic effects on antioxidant properties of esculetin.

Esculetin (6,7-dihydroxy-2H-1-benzopyran-2-one), a natural coumarin derivative present in various plants- *Citrus limonia* (Chinese lemon), *Artemisia capillaris* (Red stem and capillary wormwood), *Ceratostigma willmottianum* (Chinese Plumbago), *Matricariaria chamomilla* L. (Chamomile) possesses antioxidant, anti-inflammatory, anti-proliferative, anti-cancer, anti-adipogenic and anti-hyperglycaemic potential (Kim *et al.*, 2015c). The availability of esculetin could be vary due to phytochemical variations which further depends on cultivars, growing region, harvest season, maturity stage and storage conditions of naturally available food products (Wang *et al.*, 2011b). It is necessary to incorporate or to attain such immense additive value of esculetin in functional foods. The "functional food", at present defined in various manner with different health oriented professional organizations. The amalgamated definitions from International Food Information Council (IFIC), International Life Sciences Institute of North America (ILSI), Health Canada and The Nutritional Business of Journal (NBJ), we may define functional food as a food or dietary component which provides benefits beyond basic nutrition such as to reduce the risk of chronic diseases. Such product may range from isolated nutrients, dietary supplements and diets to genetically engineered foods, herbal products and processed food such as cereals, soups, milk and milk products, egg, oils, meat and products, sea foods, nuts and oilseeds, functional fruits and vegetables, herbs and spices, beverages (tea and wine) and fermented foods. The Nutritional Business of Journal considers functional foods and nutraceuticals are substitute words for each other. But, remaining IFIC, ILSI and Health Canada draw a segregated line between functional foods and nutraceuticals (Bigliardi *et al.*, 2013; Ghosh *et al.*, 2014; Wildman, 2016).

It has been well studied that, in the betterment of dietary significance as functional food one should know about the consequential modifications possessed by active nutraceuticals towards pharmacological activity for example, different fruits and vegetables consumed together, the antioxidant capacity may be modified by additive, synergistic and antagonistic interactions among the food components, which may further alter

physiological property (Wang *et al.*, 2011b). Since we are using dietary manipulations to develop IR and T2D, it is necessary to explore possible additive, synergistic and antagonistic effects of food matrix on the basic antioxidant property of esculetin. We studied *in vitro* antioxidant capacity of esculetin after an in vitro gastrointestinal digestion procedure mimicking the upper digestive tract (stomach and small intestine) with two different manipulated diets such as normal pellet diet (NPD) and high fat diet (HFD). The tests conducted after (TPC, DPPH and H_2O_2 scavenging assay) were represented no significant difference in antioxidant capacity of esculetin either in presence of NPD or HFD as compared to pure esculetin. In support with our findings, plasma analysis by LC/MS/MS after oral administration of esculetin to mice, there was a rapid and short-lasting increase in plasma esculetin levels (Kang *et al.*, 2014a). Further, *in vivo* studies on esculetin has been reported to improve oxidative stress in diabetic Sprague Dawley rats by countering reactive oxygen species (ROS). Several studies reported over association of deteriorative ROS production under IR and hyperglycaemia.

2. Phenotype alleviation by esculetin in non-genetic model of insulin resistance, type 2 diabetes and diabetic nephropathy.

Development of IR by HFD feeding is a feasible and ideal model. This method was adapted (Gaikwad *et al.*, 2010b; Sayyed *et al.*, 2010) and performed with temporal dimensions of 4 and 24 weeks. This kind of dietary manipulation is commonly employed to simulate human situation of obesity, metabolic syndrome and T2D (Brito-Casillas *et al.*, 2016).

A typical rodent diets are formulated attending to their daily caloric needs (approximately 50 – 65 kcal/day) and are provided ad libitum. Rat maintenance normal diet (NPD) we provide contains 16% crude protein, 4-5% crude fat, 5% crude fiber, 10% moisture and 5% total ash (RATATOUILLE SOLUTIONS, Alwar, Rajasthan, India) with approximate calories of 2.7 – 3.4 kcal/gm. For manipulated high fat diet (HFD), we prepared and provided diet with nutritional facts of 58% fat, 25% protein and 17% carbohydrate, for an approximate calorie intake of 105kcal/day. Lard (pig fat) is the major ingredient in HFD as major source for fat. The generated metabolic products from lard, elevate the plasma triglycerides and free fatty acids. The free fatty acids are the major contributors for the development of IR.

The mechanism begins with two different intermediate metabolites diacylglycerol (DAG) and ceramides. On HFD feeding, DAGs activate protein kinase C (PKC) family leading to

the activation of serine/threonine kinases followed impairment of Insulin receptor-substrate (IRS) tyrosine phosphorylation. Ceramides mediate activation of protein phosphatase A2 (PPA2) which can phosphorylate AKT2. Ceramides can also activate inflammatory pathways including JNK and NFkb. Free fatty acids like palmitate and linoleic acids activate inflammatory IKK/NFkb pathway through interaction with Toll-like receptor 4 (TLR4) and thus induce IR (Sah *et al.*, 2016). Thus, the feeding of HFD for a period of initial 2 weeks produced rats with insulin resistance syndrome as it was phenotypically characterized by the increased body weight (obesity), mild hyperglycaemia, hypertriglyceridemia, hypercholesterolemia and compensatory hyperinsulinemia, a condition similar to pre-diabetic, insulin-resistant state in humans. Similarly, T2D was induced by along with HFD feeing, administration of low dose streptozotocin (STZ) (35 mg/kg) (HFD+STZ) to group of rats. In this HFD+STZ group we observed significant increase in plasma glucose, triglyceride, cholesterol levels and alongside reduced plasma insulin level. Eventually this HFD+STZ groups show type 2 diabetic conditions. This resembles the conversion of pre-diabetes to frank hyperglycaemia in patients with T2D is associated with decline in secretory capacity of pancreatic beta cells to compensate for the existing insulin resistance. In brief the mechanism follows, increased endoplasmic reticulum (ER) workload on increased desire for insulin leads into up-regulated ER stress and increased protein misfolding. Such deleterious effects of ER stress lead to cell death, mediated by Inositol-requiring enzyme 1 (IRE1). Next, hyperinsulinemia is accompanied by accumulation of amyloid fibrils at the surface of β-cells to induce dysfunction and apoptotic death (Kahn *et al.*, 2006; Muoio *et al.*, 2008b; Özcan *et al.*, 2004; Srinivasan *et al.*, 2005).

On prolonged feeding of HFD after low dose STZ administration (24 weeks in present study), we observed substantial changes in renal phenotype which was evidenced by estimation of plasma biochemical parameters such as plasma creatinine (pCr), blood urea nitrogen (BUN) and plasma albumin (pA) levels revealed the association of T2D in the development of renal complications. We found, pCr and BUN levels were increased while pA was reduced significantly in HFD+STZ group animals. The HFD fed group did not show any significant alterations in renal functioning parameters which shows that IR is not yet been associated with renal dysfunction. Initial abrogated hemodynamic pressure and hyperglycemia are the credit contributors in up-regulated RAS system, production of reactive oxygen species (ROS), pro-inflammatory and inflammatory cytokines. They work together to contribute to tissue fibrosis, promoting cell proliferation and ECM synthesis

resulting into renal failure. Diabetic nephropathy is one of most common diabetic related complications in human. Albuminuria is the first indicator for renal damage in the early onset of diabetic nephropathy and is constantly monitored in the clinic. The clearance rate of creatinine calculated by the ratio of serum over urine creatinine which correlates with glomerular filtration rate and is also used as indicator for kidney function (Zhang *et al.*, 2015). However, esculetin reverses the metabolic changes observed in HFD and HFD+STZ animals. The possible mechanism could be down regulating oxidative stress and inflammatory pathway. Indeed, it has been recognized for the past century that high doses of salicylates (aspirin) reverse insulin resistance and diabetes (Kim *et al.*, 2001; Yuan *et al.*, 2001b).

3. Esculetin attenuates alterations in angiotensin II (Ang II) and acetylcholine (ACh) mediated vascular reactivity associated with hyperinsulinemia and hyperglycemia

One of the objectives of the study is to investigate the protective effect of esculetin on altered vascular reactivity associated with hyperinsulinemia and hyperglycemia. Till this discussion, following findings provided convincing evidence of the beneficial effect of esculetin in prevention of metabolic and morphological perturbations. First target tissue in IR and T2D conditions is blood vessel. All the harmful factors breach the vascular defense machineries of endothelium. Hence, such utter compromise in the integrity of vascular structure initiates targeting vascular endothelial (EC) and smooth muscle cells (VSMC) (Pasquier *et al.*, 2015). Further, consequential imbalance in renin angiotensin system (RAS) and increased angiotensin II (Ang II) production trigger reactive oxygen species generation which further initiates and reinforce in generation reactive nitrogen species, expression of adhesive, pro-inflammatory molecules such as nuclear factor – κB (NF-κB) in EC and VSCM, resulting in endothelial dysfunction and cell migration (Karnik *et al.*, 2015a; Kaschina *et al.*, 2003). Considering these facts, the present study unveils the protective effect of esculetin on vascular dysfunction associated with IR and type 2 diabetes. Initially, hyperinsulinemia and hyperglycemia were associated with significant rise in systemic blood pressure (SBP) (hypertension) (Gaikwad *et al.*, 2007), which were attributed, at least in part, to vascular dysfunction. We observe increased SBP in HFD and HFD+STZ group rats. Esculetin treatment abrogated the elevations in SBP in both the conditions. Accumulated body of evidence suggests that, hyperinsulinemia and hyperglycemia can modulate the physiological responses to Ang II, which may contribute to the pathogenesis of vascular dysfunction associated with diabetes (Amiri *et al.*, 1999;

Gaikwad *et al.*, 2007). The thoracic aorta isolated from HFD and HFD+STZ treated rats showed exaggerated vasoconstriction responses to Ang II but the sensitivity (pD2 value) remain unchanged, which are consistent with previous reports (Gaikwad *et al.*, 2007; Karpe *et al.*, 2012b). Ang II- an extremely potent hormone of RAS modulates various deleterious actions through AT1R. Over activation of RAS and increased Ang II level result into increased oxidative stress and uncoupling of the endothelial nitric oxide synthase (eNOS) which largely account for vascular dysfunctions (Hink *et al.*, 2001; Viswanad *et al.*, 2006).

Ang II induced exaggerated contraction of aorta was significantly attenuated by esculetin treatment in both the conditions. In the vascular tissues, acetylcholine stimulates production and release of endothelial derived relaxing factors (e.g. nitric oxide and prostacyclin), and produce endothelium dependent and nitric oxide mediated relaxation (Baluchnejadmojarad *et al.*, 2013; Roghani *et al.*, 2009). In addition, aortic ring of HFD and HFD + STZ treated rats showed impaired endothelium dependent relaxation to acetylcholine challenge as compared to normal control rats. Hyperinsulinemia and hyperglycemia causes tissue damage by several mechanisms including oxidative stress, increased advanced glycation end products (AGEs) formation, and apoptosis which might be the contributing factors for impaired endothelium dependent relaxation and vascular dysfunctions (Gray *et al.*, 2014; Hartge *et al.*, 2007). It has been reported that, an AMPK activator metformin attenuated impaired acetylcholine induced endothelial dependent relaxation in aorta of HFD fed mice, and recently AMPK modulating effect of esculetin has been reported (Kim *et al.*, 2015c; San Cheang *et al.*, 2014). Our results showed that, esculetin treatment improves endothelial dependent relaxation of aortic rings in both the conditions, which might be, in a part, contributed to its protective effect on vascular dysfunctions. To investigate molecular mechanisms behind protective effect of esculetin on vascular dysfunction in hyperinsulinemia and hyperglycemia, we checked the expression of AT1R, AT2R and ACE2. It has been reported that, in diabetes AT1R and AT2R were increased and ACE2 expression was decreased in aorta (Karpe *et al.*, 2012b; Karpe *et al.*, 2014b; Romero *et al.*, 2014; Tikoo *et al.*, 2015). In the present study, we also observed similar changes in the aorta of hyperinsulinemic and hyperglycemic rats. Besides RAS, TGF-b also plays an important role in the extracellular matrix accumulation and vascular remodeling by up-regulating connective tissue growth factor (CTGF) and fibroblast growth factor (Lan *et al.*, 2013; Subramanian *et al.*, 2012). Vidagliptin (dipeptidyl peptidase-IV inhibitor) found to be protective against vascular injury in

diabetes by reducing TGF-b expression and attenuating the deleterious effects of AGEs (Matsui *et al.*, 2011). In addition, HFD fed rats treated with Oltipraz (a nuclear respiratory factor 2 (NRF2) activator) restored impairment of the endogenous redox system (decrease in NRF2 and increase in Keap1) which is important in the evelopment of obesity and insulin resistance (Yu *et al.*, 2011). Our results also show increased expression of TGF-b and Keap1 in the aorta of diabetic rats, which was significantly reduced by esculetin treatment, hence improving the insulin resistance and vascular dysfunction in diabetes.

4. Esculetin ameliorates vascular perturbation by intervening in the occupancy of H2BK120Ub at At1, At2, Tgfβ1 and Mcp1 promoter gene in thoracic aorta of IR and T2D rats

The progressive micro or macro vascular complications in insulin resistance (IR) and type 2 diabetes (T2D) have been extensively studied at complex chromatin modifications in vivo and in vitro (Paneni *et al.*, 2013b). Further, epigenetic pathways have also been recently implicated in other facets of vascular gene expression, such as the diabetic complications, pathophysiology of atherosclerosis, cytokine-inducible gene expression in vascular endothelium (Gimbrone *et al.*, 2016; S Xu *et al.*, 2014) and developmental regulation of vascular remodeling (Tarantini *et al.*, 2016). In a recent study, Expression of endothelial nitric oxide synthase (eNOS) mRNA was observed in association with nucleosomes that encompassed the eNOS core promoter and proximal downstream coding regions were highly enriched in particularly acetylated histone H3 lysine 9, histone H4 lysine 12, and di- and tri-methylated lysine 4 of histone H3 (Fish *et al.*, 2005). In addition, the normal physiology of smooth muscle cells plasticity is preserved by active regions of chromatin which exhibit increased histone methylation and acetylation in particularly by increased H3K4me2, H3K79me2, H3K9Ac and H4Ac in the regulation of smooth muscle alpha actin (ACTA2), smoothelin, h-caldesmon, calponin, transgelin (TALGN), and smooth muscle myosin heavy chain (MYH11) protein expressions (Liu *et al.*, 2015).

Histone H2A and H2B monoubiquitination was reported the functional role in the regulation of H3K4Me2 and H3K9Me2 through modulating the expression of SET7/9 and SUV39H1 in the development of diabetic renal fibrosis (Goru *et al.*, 2016a). Furthermore, on AT1 receptor blockade and AT2 receptor activation enhanced the occupancy of H2AK119Ub at promoter regions of monocyte chemoattractant protein-1 (*Mcp1*) and transforming growth factor beta 1 (*Tgfβ1*) genes in type 2 diabetic rat kidney (Pandey *et al.*, 2016). Similarly, under the influence of hyperlipidemia, hyperglycemia and change in

hemodynamic turbulence, the epigenetic histone modifications in the regulation of AT1, AT2, pro-inflammatory and pro-fibrotic chromatin promoter region is still unknown. However, for the first time, the current study demonstrate the chromatin based modification with respect to H2BK120Ub in the expression of *AT1, AT2, TGFβ* and *MCP1* genes in rat thoracic aorta under pre-diabetic and type 2 diabetic conditions. Esculetin, a proven functional food , has studied its pharmacological activity in diabetes and its complications. Previously we reported the intervention of esculetin in amelioration of metabolic and morphometric alterations in with hyperglycemia, IR and renal failure through abrogating the H2AK119Ub modifications in expression of inflammatory and pro-fibrotic genes . Based on such previous evidences, we further continued our study to evaluate the role of esculetin in the amelioration of vascular perturbation under IR and T2D.

Considering thoracic aorta is one of the major arteries, further cellular and epigenetic modifications were evaluated in thoracic aorta. Based on vascular reactivity data, for substantially raised question about the density of AT1 receptors, we determined the mRNA levels of AT1 receptors in thoracic aorta. Here we observed the increased levels of *At1* mRNA levels in early IR conditions. Whereas in HFD+STZ groups, *At1* mRNA levels were observed no significantly different with HFD group indicating activation of intracellular deteriorating downstream signaling pathways (Higuchi *et al.*, 2007). However, esculetin treatment in insulin resistant and type 2 diabetic rats, attenuated the Ang II mediated aggravated vasoconstriction. Further, on evaluation of *At1* mRNA levels, esculetin significantly reduced AT1 expression. Under IR and diabetic nephropathy conditions, esculetin was reported to beneficial by reducing the *At1* mRNA in kidneys . On the other hand, the evidently reduced vascular relaxation was observed when thoracic aorta was challenged to ACh mediated relaxation in both HFD and HFD+STZ significantly compared with NC. These results suggest that, there could be a possibility of reduced vascular relaxing factor such as endothelial derived nitric oxide (eNOS) and AT2 receptor density which is reported for vascular relaxation property through activation of protein kinase A (PKA) and cGMP followed by activation of eNOS. This was further confirmed by reduced mRNA expression of eNOS in HFD and HFD+STZ rats. On contrary the elevated mRNA levels of AT2 in HFD group rats indicates the defensive mechanism for altered physiological condition in aorta and further progression of diseased condition can be indicated through reduced mRNA levels of AT2 receptors in HFD+STZ rats.

Interesting, esculetin reduced *At2* mRNA levels in HFD, but conflicting to the same, esculetin increased *At2* mRNA levels in HFD+STZ group animals. Followed by, esculetin significantly elevated the eNOS mRNA levels in IR and T2D condition and further substantiating by escalating ACh mediated aortic relaxation. Further, altered vascular toning results into initial activation of endothelial adhesive molecules such as VCAM and ICAM (Paneni *et al.*, 2013a). In which we measured mRNA expression levels of *Vcam1*. As expected, the *Vcam1* mRNA expression was elevated in HFD and HFD +STZ rats. Furthermore, precipitation of vascular dysfunction, we observed significantly higher collagen deposition in HFD and HFD+STZ rat thoracic aorta. By esculetin treatment, it was assured the vascular protective property since we contemplated *Vcam1* mRNA expression which intern resulted in reduced collagen deposition in aortic strip.

Next part of the study explores the association of histone H2B monoubiquitination in the progression of vascular dysfunction under IR and T2D. We observed and increased H2BK120Ub in HFD and HFD+STZ groups. Further, we observed increase mRNA expression of histone H2A specific E3 ligase, Rnf2 in HFD and HFD+STZ groups. Rnf2 belongs to the polycomb group (PcG) protein family and is involved in the transcriptional repression through histone H2A ubiquitination (Wang *et al.*, 2004). Recently, deletion of Bmi1, another member of PcG family, was found to improve insulin sensitivity and T2D (Cannon *et al.*, 2014). Esculetin treatment in HFD and HFD+STZ group animals showed no significant change in elevated Rnf2 mRNA expression indicating sustained H2A ubiquitination on histones which further associated with cell proliferation (Li *et al.*, 2016). Along with E3 ligase we observed increased mRNA levels of H2A/H2B specific deubiquitinases (DUBs) USP 16 and USP 22. On the other hand these DUBs have been explored the functional role in normal physiology and diseased conditions. For example, bursts of reactive oxygen species (ROS) reversibly inactivate DUBs through the oxidation of the catalytic cysteine residue (Cotto-Rios *et al.*, 2012). In diabetic nephropathy, the increased expression of USP22 reduced the Sirt1 ubiquitination and degradation, and decreased fibronectin and TGF-β1 expression in glomerular mesangial cells under both basal and AGE-treated conditions (Huang *et al.*, 2014).

Furthermore, interlink between H2BK120Ub and AT1, AT2, pro-inflammatory (MCP-1) and Pro-fibrotic (TGFβ-1) was explored by chromatin immuno precipitation assay (ChIP assay). We found the increased occupancy of H2BK120Ub at promoter regions of *Tgfβ1* and *Mcp1* genes in both HFD and HFD+STZ group. In different manner, the occupancy of H2BK120Ub at promoter regions of *At1* and *At2* genes was observed as high in HFD

group, whereas in HFD+STZ group the occupancy was significantly reduced. The promoter region of *At1* and *At2* genes were found to be hyper monoubiqitinated on H2B histones in HFD fed animals. This increased histone monoubiquitination could be functionally relevant in the unfolding of reconstituted chromatin templates under insulin resistance. Concurrently, we observed increased mRNA expressions in HFD fed rat aorta. On the other hand, in HFD+STZ group animals, enrichment of ubiquitination at H2B lysine 120, on promoter regions of *At1* and *At2* genes was observed to be reduced. But, *At1* mRNA expression was increased even though reduced declined occupancy of H2BK120Ub on promoter region of *At1* gene. Further elaborated chromatin modifications studies are required to understand the extent of involvement of H2BK120Ub in the *At1* and *At2* under IR and T2D conditions. Specifically, the present study provides evidence for impoverished H2BK120Ub by esculetin treatment and reduced the occupancy of H2BK120Ub in HFD and HFD+STZ rats on *Tgfβ1* and *Mcp1* which could be the interventional mechanisms in abrogating vascular condition under IR and T2D conditions. The occupancy of H2BK120Ub at *At1* and *At2* promoter gene regions was reduce in HFD by esculetin whereas in HFD+STZ we observed nothing significant changes with esculetin treatment. The reduction in the occupancy of H2BK120Ub in esculetin treated rats could be the result of reduction in inflammatory and oxidative stress pathways. Since, prolonged circulating triglycerides and hyperglycemia leads into activation of cellular inflammatory and oxidative stress pathways were reported in the post translational histone modifications (Chervona *et al.*, 2012). It has been suggested that, such inflammatory cytokines, reactive oxygen species (ROS) and reactive nitrogen species (RNS) induce the endothelial and vascular smooth muscle cell damage of atherosclerotic lesion formation (Karnewar *et al.*, 2016). Esculetin possesses structurally polyphenolic moiety and evidenced for its direct radical quenching as shown by the DPPH radical and hydroxyl radical. Further a recent study which explored that esculetin can interact with DNA to the minor grooves of the Ct-DNA by electrostatic interactions (Sarwar *et al.*, 2015). Such studies indicate the possibility of direct involvement of esculetin in the chromatin modifications for the expression or suppression of gene expression. With respect to esculetin and DNA interactions, the study need to expand to determine the direct involvement in post translational histone modifications such as H2BK120Ub. Taken all together, the protective role of esculetin under initial IR and T2D conditions, could be attribute through reversing the modified histone ubiquitination and intervening in the occupancy of H2BK120Ub at promoter regions of *At1, At2, Tgfβ1 and Mcp1* genes.

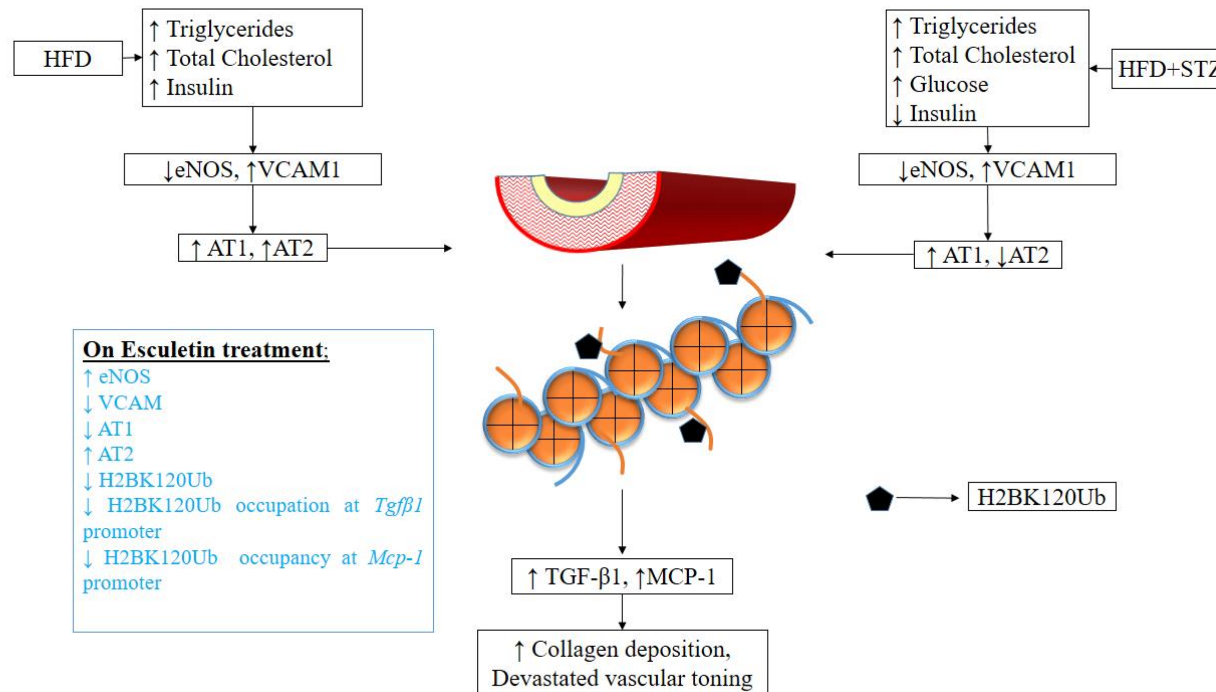


Figure 20: Association of H2BK120Ub in vascular damage and possible mechanisms of esculetin under insulin resistance and type 2 diabetes
Insulin resistance (IR) and hyperglycemia commonly share in the elevation of blood triglycerides and total cholesterol. Increased insulin i.e. hyperinsulinemia is signature consequence of IR, whereas hyperglycemia is of diabetes. Such etiological contributions trigger in the reduction of endothelial nitric oxide synthase (eNOS) and stimulation in the production of vascular cell adhesion molecule 1 (VCAM1); these contribute in elevation of angiotensin II receptor type 1 (AT1) and type 2 (AT2) receptor gene expression in IR. Since AT2 receptor is known for vascular relaxation activity and elevated gene expression could be conclude as defensive machinery towards vascular insult in IR. In hyperglycemic condition, failure of such mechanisms leads into increased AT1 and down regulate AT2 gene expressions; in chromatin level such stimulations activates post-translational histone modifications such as H2BK120Ub which intern activates transcriptional gene expression for TGF β1 and MCP-1 proteins by increased occupancy at promoter regions. This results into increased vascular collagen depositions flowed by deteriorating vascular toning; Esculetin treatment reduces vascular deterioration by increasing eNOS and AT2 and reducing VCAM1 and AT1. Further, esculetin also reduces the occupancy of H2BK120Ub at promoter regions of TGF β1 and MCP-1 thereby reduces collagen deposition and improves vascular toning.

5. Esculetin reverses histone H2A/H2B ubiquitination, H3 dimethylation, acetylation and phosphorylation in preventing type 2 diabetic cardiomyopathy.

The accumulated body of evidence suggests that, epigenetic PTHMs occurs in diabetes and play important role in modulating pathological gene expression associated with diabetic renal (Reddy *et al.*, 2013; Sun *et al.*, 2010; Yuan *et al.*, 2013) and vascular complications (Okabe *et al.*, 2012a; Paneni *et al.*, 2013d; Reddy *et al.*, 2011b). There is wide range of PTHMs and they act in concert with each other, so combinatorial effect of PTHMs reflect on the chromatin status which regulates translation of gene(s) (Kouzarides, 2007). Generally, increased permissive (active) and decreased repressive (inactive) PTHMs leads to chromatin decondensation, which ease the access of transcription machinery to the promoter and transcribed regions of genes and ultimately leads to transcriptional amplification (Kouzarides, 2007). It has been reported that, TGF-β treatment to rat mesangial cells (RMCs) can augment H3K4me1, H3K4me2, H3K4me3 and reduces H3K9me2 & H3K9me3 levels at the promoter of fibrotic genes [e.g. connective tissue growth factor (CTGF), PAI-1, and Collagen-α1 (I) chain (Col1α1)] and was well correlated with increased fibrotic genes and protein expression in TGF-β treated RMCs, which considered to be pathologically important in the development of diabetic nephropathy (Sun *et al.*, 2010). It has been demonstrated that in neonatal rat cardiomyocytes activation of Calcium/calmodulin-dependent protein kinase II (CaMKII) signaling by hypertrophic agonists (e.g. Phenylephrine, and Xylometazolin) increases H3 phosphorylation (H3S10phospho) which results in to primary cardiac cell hypertrophy (Awad *et al.*, 2013).

In genetically diabetic animals (db/db mice) renal failure can increase cardiac histone H3 acetylation (H3K9Ac and H3K23Ac), dimethylation (H3K4me2), and phosphorylation (H3S10phospho) which might be a contributing factor for increased expression of genes [myosin light chain 3 (Myl3), myosin heavy chain 3, 6, and 7 (Myh3, Myh6, and Myh7), matrix metallopeptidase 1b (Mmp1b), and TGF-β] related to cardiac hypertrophy and fibrosis (Gaikwad *et al.*, 2010a). Furthermore, in non-genetic type 2 diabetic (HFD+STZ treated) rats, there was increased H3K9/14Ac, H3S10phospo, and H3K4me2 in heart tissue, which might account for elevated fibrillin 1 (Fbn1) and collagen type III *α*1 (Col3A1) gene expression in diabetic cardiomyopathy (Gaikwad *et al.*, 2010a). Our result matches with these findings- increased level of H3K9Ac, H3K14Ac, H3S10phospo, and H3K4me2 in the heart of HFD and H+S treated rats (mimics insulin resistance and type 2 diabetic condition, respectively) compared to NC rats. In addition, we have shown increase

in other permissive PTHMs (e.g. H3K36me2, H3K79me2, H3S28phospho, H3T3phospho, and H3K18Ac) in the heart of IR and diabetic rats. Further, it has been reported that, TNF-α treatment reduced levels of repressive (H3K9me3) chromatin marks at key inflammatory gene promoter [e.g. interleukin-6 (IL-6), macrophage colony stimulating factor-1 (MCSF), and monocyte chemoattractant protein-1 (MCP-1)], which was inversely correlated with the increased expression of these genes in VSMCs derived from diabetic *db/db* mice (Villeneuve *et al.*, 2008b), and similar results were obtained in human VSMCs and endothelial cells cultured under high glucose conditions (Brasacchio *et al.*, 2009). We also studied alterations in heterochromatin marks in IR and type 2 diabetic cardiac complications. Notably level of repressive histone H3 modification (H3K9me2 and H3K27me2) diminished in IR and type 2 diabetic hearts, which can further contribute to increased protein expression involved in diabetic cardiomyopathy.

Till date, a connecting link between alterations in histone H2A/H2B ubiquitination and their relation with diabetic cardiovascular complications is not completely established. Recently, Gao C et al., demonstrated increase in histone H2AK119ub and decrease in histone H2BK120ub in rat glomerular mesangial cells (HBZY-1) under high glucose condition (Gao *et al.*, 2013a). Histone H2B ubiquitination (H2BK123ub) is a dynamic modification which can promote more stable PTHMs e.g. histone H3K4me2, H3K4me3, H3K79me2, and H3K79me3 (Shahbazian *et al.*, 2005; Vlaming *et al.*, 2014). Our results provide the first evidence of augmented levels of histone H2AK119 and H2BK120 ubiquitination, which clearly match with increased H3K4me2 and H3K79me2 in the heart of IR and type 2 diabetic rats. Taken together, we have identified several permissive and repressive PTHMs in the heart of IR and type 2 diabetic rats, which play their part in the pathogenesis of diabetic cardiac complications. These findings suggest that, a therapeutic intervention which can reverse epigenetic alteration associated with diabetes by might be helpful to reduce progression of diabetic cardiomyopathy; in the present study we utilized this novel approach by esculetin treatment to IR and diabetic rats.

Existing reports suggest that, due to the pleiotropic nature of esculetin has a protective effect on diabetes associated hepatic and renal dysfunction (Prabakaran *et al.*, 2013), and potential to reveres epigenetic changes prevent glomerulosclerosis in STZ induced type 1 diabetic nephropathy (Surse *et al.*, 2011b). However, the cardio protective effect of esculetin in IR type 2 diabetes was not studied till now. The present study results showed that esculetin treatment effectively reduces protein expression of AT_1R and AT_2R, increase ACE2 expression in heart of IR and type 2 diabetic animals. So, augmentation of protective

the ACE2 (ACE2/Ang 1–7)/Mas axis) (Karpe *et al.*, 2014b; Santos *et al.*, 2013) and inhibition of deleterious AT_1R (ACE/Ang II/AT_1R axis) (Hayden *et al.*, 2011) may contribute to cardio protective potential of esculetin in IR and type 2 diabetes. Esculetin also inhibits the expression of Keap1 (oxidative stress) and Ki-67 (cell proliferation) in IR and type 2 diabetic heart. Considering as whole, the esculetin has cardio protective effect by virtue of its anti-hypertensive, anti-proliferative, radical scavenging, anti-fibrotic and anti-hypertrophic potential on IR and type 2 diabetic heart.

The accumulated body of evidence suggests that, potential to reverse epigenetic PTHMs in the pathological condition might be the basic mechanism of the protective effect of a drug. For example, Losartan can reverse increased H3K9/14Ac and H3K36me3 level by inhibiting histone acetyl transferases (HATs) and H3K36-methyl transferase, respectively, at RAGE and PAI-1 genes in kidney of type 2 diabetic db/db mice (Reddy *et al.*, 2013), and atorvastatin can increase histone H3K9/14Ac in the promoter region of ACE2 in the heart of atherosclerotic NZW rabbit (Tikoo *et al.*, 2014). In addition, Curcumin analog C66 prevents diabetic nephropathy via p300/CBP-mediated reduction of histone H3K9/14Ac in kidney of STZ induced type 1 diabetic mice (Wang *et al.*, 2014). Esculetin potentially reverses PTHMs- H3K4me and H3K9/14Ac in STZ induced diabetic nephropathy and attenuated development of glomerulosclerosis (Surse *et al.*, 2011b). Based upon these reports, we have demonstrated the effect of esculetin on PTHMs in heart of non-genetic model of IR and type 2 diabetic. To the best of our knowledge, this is the first report which shows that esculetin treatment inhibited permissive PTHMs (e.g. H3K4me2, H3K36me2, H3K79me2, H3S10phospho, H3S28phospho, H3T3phospho, H3K9Ac, H3K14Ac and H3K18Ac) and augmented repressive PTHMs (e.g. H3K9me2 and H3K27me2) in heart of IR and type 2 diabetic rats. In addition, new reported epigenetic changes- increased H2AK119 and H2BK120 was attenuated by esculetin in heart of IR and type 2 diabetic rats. Overall, the current study result suggests that due to the pleiotropic nature of esculetin, it could reverse epigenetic changes in the heart associated with type 2 diabetes and which might be the basic mechanism contributed to its' cardio protective effect. In conclusion, our studies showed that in insulin resistance (pre-diabetic) and type 2 diabetic conditions there was chromatin decondensation (opening) which may have detrimental consequences in transcription of pathological gene(s) which account for diabetic cardiomyopathy. Further, the esculetin has a cardio protective role in type 2 diabetes by reducing oxidative stress, hypertension, cell proliferation, cardiac hypertrophy, and ECM accumulation in IR and type 2 diabetic heart.

Esculetins' potential to reverse epigenetic alteration which might be the basic mechanism of its cardio protective effect in type 2 diabetes.

6. Esculetin ameliorates insulin resistance and type 2 diabetic nephropathy through reversal of histone H3 acetylation and H2A lysine 119 monoubiquitination.

This is the first study showing that increased expression of key inflammatory (Mcp1) and pro-fibrotic (Tgfb1) genes is associated with alterations in H2AK119Ub at these genes, as well as with altered expression of related E3 ligases (Rnf2 and Rnf168) and deubiquitinases (Usp16 and Usp21) in the kidney of non-genetic models for IR and T2D. It also shows that esculetin mediated reversal of T2D and IR may be partially attributed to its ability to elevate H2AK119Ub at Mcp1 and Tgfb1 promoter sites

Under IR and diabetes, breakdown of anti-oxidant defence mechanisms which includes superoxide dismutase system (SOD); copper and zinc SOD (SOD1 and 3) and manganese SOD (SOD2) and glutathione system cause lipid peroxidation and further trigger renal fibrosis, inflammation and cell death (Jha *et al.*, 2016). Superoxide dismutase 1 SOD1 (CuZnSOD) is an enzyme for superoxide removal, which converts superoxide into hydrogen peroxide and molecular oxygen. The hydrogen peroxide is further detoxified to water (H_2O) by catalase or glutathione peroxidase (Fujita *et al.*, 2009). Existing reports state that SOD activity in peripheral blood cells is reduced in the diabetic patients with DN as compared with non-diabetic patients (Hodgkinson *et al.*, 2003). Estimations of SOD1 reduces glutathione (GSH) and thiobarbituric acid (TBA) reactive substances for lipid peroxidation are widely recognised marker for determination of oxidative stress in laboratory animals. Interestingly, in the current study, we found that esculetin attenuated oxidative stress, as evidenced by improvement in tissue GSH and TBA reactive substances, and mRNA expression of *Sod1* in IR and T2D rats' kidney.

The reversal in the oxidative stress may be responsible for abrogating the *At1* and *Ace* expression, thus reducing the hypertrophic, pro-fibrotic and pro-inflammatory activities of Ang II. Our results showed that esculetin improves mRNA expression of *Ace2*, a monocarboxypeptidase cleaving an amino acid from the C terminus of Ang I and Ang II, to produce Ang 1–9 and Ang 1–7, respectively. ACE2 expression in renal tubular cells after 24 weeks of STZ-induced diabetes in *Sprague Dawley* rats has been found to be reduced significantly (Tikellis *et al.*, 2003). Another study showed that genetic *Ace2* ablation in male mice leads to an age-dependent development of glomerular mesangial expansion with increased deposition of fibrillary collagens I/III and ECM protein fibronectin (Oudit *et al.*, 2006). Male ACE2 mutant mice or in db/db mice with long term

blockade of ACE2 with MLN-4760 or streptozotocin induced diabetic mice have been seen to be associated with increased deposition of collagen type I and III and fibronectin in the glomeruli and increased urinary albumin excretion (Santos *et al.*, 2008). Such actions of ACE2 provide a rationale for embarking on strategies that might increase ACE2 in diabetes. By the action of ACE2, produced Ang 1-7 appears to have anti-proliferative effects, and also has diuretic and natriuretic effects. Ang 1-7 has recently been shown to inhibit Ang II – stimulated phosphorylation of NF-B and MAP kinases in the proximal renal tubular cells, which is protective peptide (Simões e Silva *et al.*, 2016). Ang 1-7 also increases nitric oxide and prostacycline release and potentiates the effects of bradykinin. (Simões e Silva *et al.*, 2016). Taken together, the ACE2/Ang (1–7)/Mas receptor axis emerges as a novel therapeutic target in the development of renal fibrosis under IR and diabetes.

The activation of deteriorative axis of RAS triggers downstream inflammatory and fibrotic pathways. Our results showed that renal fibrosis (indicated by *Tgfb1*, *col1a1* and fibronectin) and inflammation (*Mcp1*) markers were significantly elevated in both HFD fed and HFD+STZ treated and esculetin could markedly control these factors. Accumulating evidences show that these aberrations in gene expression are linked with changes in PTHMs.

Histone acetylation is known to cause transcriptional activation by promoting recruitment of protein complexes/transcription factors essential for gene expression (Reddy *et al.*, 2015). In *db/db* mice, renal failure was seen to increase cardiac H3K9Ac, H3K23Ac, H3K4me2, and H3S10phospho levels which in turn altered the expression of genes involved in fibrosis and hypertrophy (*Fbn1* and *Col3A1*) (Gaikwad *et al.*, 2010b). In our previous study, we had demonstrated that treatment with esculetin reversed the PTHMs including H3K4me2, H3K36me2, H3K79me2, H3S10phospho, H3S28phospho, H3T3phospho, H3K27Ac, H3K56Ac, H2AK119 and H2BK120 ubiquitination in the hearts of IR and T2D rats . It is well known that histone deacetylating agents have proved beneficial in chronic kidney diseases including diabetic nephropathy, lupus nephritis, aristolochic acid nephropathy, and transplant nephropathy. Our results show that esculetin attenuates the hyper-acetylation of histone H3 at K14 and 18, associated with both IR as well as T2D rats' kidneys, and this may be one of the underlying mechanisms for the reno-protective activity.

Another histone modification, H2AUb, orchestrates cellular events, like transcription initiation and elongation, silencing, and DNA repair and its alteration results in human

diseases, such as cancer. Histone Ub, a reversible process is guided by ubiquitin ligases and deubiquitinases which are related with vital cellular responses. For instance, H2A specific deubiquitinase, *Usp21* is required for transcription initiation and subsequent H3K4me2/me3 and E3 ubiquitin ligase, *Rnf8* regulates DNA-damage response. A recent study states that hyperglycaemia increases H2AK119Ub and reduces H2BK120Ub in glomerular mesangial cells and could be associated with activation of TGF-β mediated signalling pathway. Our results indicate that disturbances in H2AK119Ub and associated machinery could be alleviated by esculetin treatment. Moreover, the reduced occupancy of H2AUbK119 at the promoter sites of *Tgfb1* and *Mcp1* in IR and T2D kidney could also be reverted by esculetin treatment. Esculetin's ability to ameliorate IR, T2D and associated renal dysfunctions could partially be attributed to its ability to increase occupancy of H2AK119Ub at *Tgfb1* and *Mcp1* promoter regions.

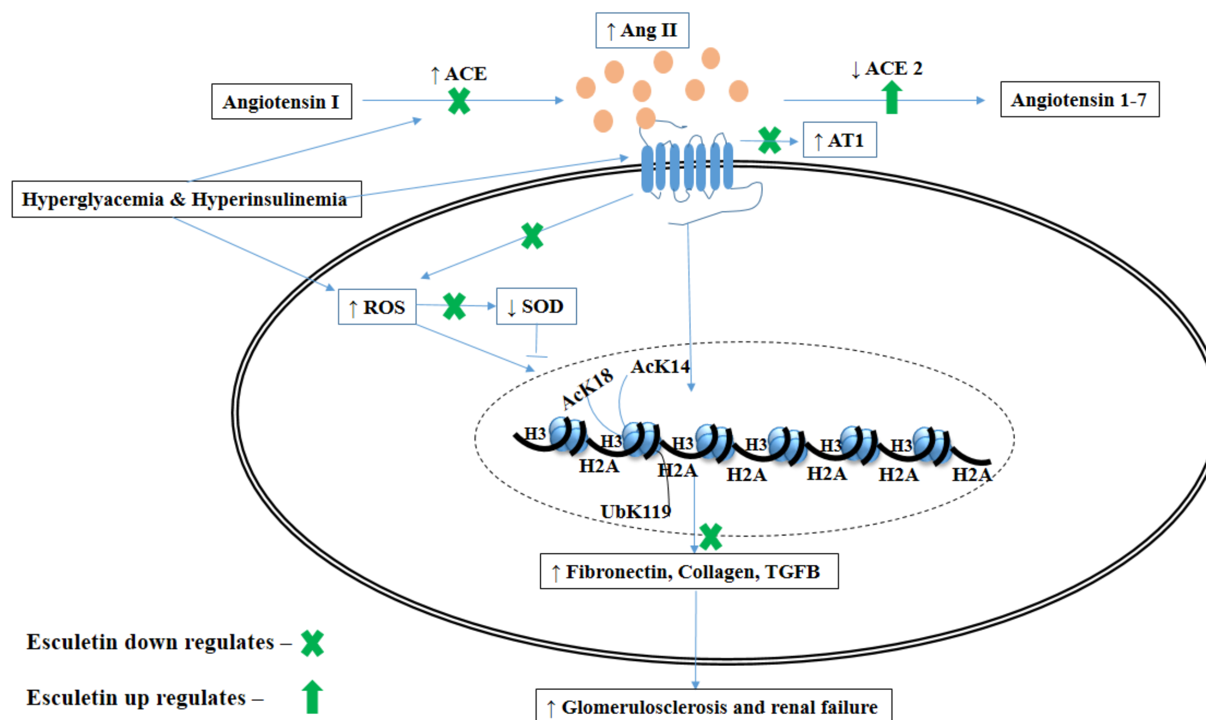


Figure 21: Possible mechanisms of esculetin in amelioration of nephropathy under hyperinsulinemic and hyperglycemic conditions.
The illustration depicts the pathways which causes progression in type 2 diabetic nephropathy. In hyperinsulinemic and hyperglycemic conditions increase the expression of angiotensin converting enzyme (ACE), angiotensin II (Ang II) and reduce angiotensin converting enzyme 2 (ACE2). All together produce oxidative stress and reduce the super oxide dismutase (SOD). Such consequential assembly of stimuli activated histone modifications such as H3K14Ac, H3K18Ac and H2AK119Ub. Among these histone modifications, H2A119KUb is established as a part of relaxation in promoter regions of transforming growth factor beta 1(TGF-β1) and monocyte chemoattractant protein-1 (MCP1) genes which leads to glomerulsclerosis and renal failure. Esculetin inhibits the stimuli along with up regulation of angiotensin 1-7 expressions which is recognized for its cellular protective properties.

Conclusions

In conclusion, our study provides comprehensive evidences for pleotropic effects of esculetin in the amelioration of insulin resistance (IR), type 2 diabetes (T2D) and associated cardiovascular and renal complications by intervening renin angiotensin system (RAS) and post translational histone modifications.

- Initially, the modified diet (HFD – High Fat Diet) induced IR and followed by low dose streptozotocin (35 mg/kg) administration induced T2D and its associated complications were significantly characterized by modifications in biochemical, morphological and hemodynamic parameters. Esculetin treatment showed improved metabolic and hemodynamic alterations in IR and T2D conditions.
- Further, esculetin ameliorated the impaired vascular toning along with improved angiotensin type 1 (AT1), angiotensin type 2 (AT2) and endothelial nitric oxide synthase (eNOS) expressions. Consequentially, esculetin also reduced vascular cell adhesion protein (VCAM) expression thereby reduced collage depositions in aorta. Furthermore a bore in the chromatin modifications, esculetin showed intervening property in H2B ubiquitination system including E3 ligases and deubiquitinases. Next, our data represented esculetin could have functional role in reverting of vascular perturbation by regulating *At1, At2, Tgfβ1 and Mcp1* genes under IR and T2D conditions in thoracic aorta.
- It was observed, under oxidative stress and up-regulated RAS in IR and T2D conditions, the heart tissue possessed increased Kelch-like ECH-associated protein 1 (Keap 1), Ki67, AT1 and AT2, and reduced angiotensin converting enzyme 2 (ACE2) expressions. These findings could conclude a typical theory of augmentation of angiotensin converting enzyme (ACE)/angiotensin II (Ang II)/AT1 axis which may contribute in the development of cardiomyopathy. Such deleterious modifications could further involve in the activation of permissive (H3K27Ac, H3K56Ac, H3S10phospho, H3S28phosphor, H3T3phosphor, H3K4me2, H3K36me2, H3K79me2) and inhibition of repressive (H3K9me2 and H3K27me2) post translational histone modifications (PTHMs). Esculetin treatment reversed these modifications and restored the normal physiology of heart in IR and T2D rats.
- Further in type 2 diabetic nephropathy (T2DN) conditions, esculetin's ability to reduce disturbances in RAS may be responsible for its anti-inflammatory and anti-fibrotic potential. The reversal of PTHMs, especially H2AK119Ub may be an important underlying mechanism for the treatment of IR and T2DN. Thus, this study may help

us improve our understanding of the basic mechanisms of action of esculetin behind its reno-protective action. It also emphasizes that epigenetic modifications play a significant role in regulation of renal fibrosis and macrophage infiltration and thus, a therapy for complete treatment of IR and T2DN must possess the ability to revert these gene specific PTHMs.

Future Scope of Work

Based on our research findings and limitations, this work may be extended for further research in the following dimensions / environments:

I. Dissection of molecular mechanisms in the development of cardiovascular and renal complications under type 2 diabetes.

Present study has provided evidences on RAS in whole tissue such as aorta, heart and kidney. In RAS, angiotensin II (Ang-II) a biological active peptide acts on different receptors subtypes AT1 and AT2 majorly. The molecular and cellular perspectives of Ang-II on AT1 & AT2 are differ whit individual organ and different cell types. Further, Ang II expression has been reported to be subjective for each different cells present in blood cells, cardiac myocytes and renal cells. Present study can be extend to evaluate with respect to ACE/Ang- II/AT1 axis and ACE2/Ang 1-7/Mass receptor axis in each isolated cells.

II. Dissecting epigenetic modifications in the development of cardiovascular and renal complications under type 2 diabetes.

Present study has spotted light on modifications in histone H2A lysine 119 and H2B lysine 120 monoubiquitination in aorta, heart and kidney under IR and T2D conditions. Molecular mechanism underlying the epigenetic regulation of vascular AT1 and AT2 expression in insulin resistance and diabetes remains a kind of global observations. Still it needs to substantial evidences with respect to protein recruiting systems in histone monoubiquitination.

III. A systemic approach to clinical and applied research on esculetin as functional foods.

Functional foods is a new concept to provide food products covering with various enriched essential substance so as to provide people with an additional physiological benefit presumed to prevent disease or promote health. Although the role of oxidative stress in aging, neurodegenerative and vascular diseases, cancer, diabetes, and other related diseases is largely accepted, the value of antioxidant strategies is still debatable. Since, we have shown the health benefits of esculetin as an antioxidant and therapeutic substance in insulin resistance, diabetes and its complications. This becomes more important when, apart from foods or reasonable lifestyle changes, antioxidant supplements such as esculetin are considered. A long term trials are still needed to assess the efficacy of esculetin intervention. Any persistent reduction in oxidative stress should be monitored using appropriate, reliable, and sensitive biomarkers, and the real health benefits of lowering the oxidative stress should be assessed critically.

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Appendix

List of publications from thesis:

1. **Kadakol A,** Goru SK, Malek V and Gaikwad AB. (2017) “Esculetin ameliorates vascular perturbation by intervening in the occupancy of H2BK120Ub at At1, At2, Tgfβ1 and Mcp1 promoter gene in thoracic aorta of IR and T2D rats”. ***Biomedicine & Pharmacotherapy***. 95: 1461-1468. [Impact Factor: 2.759].
2. **Kadakol A**, Malek V, Goru SK, Pandey A, Sharma N, Gaikwad AB (2017) "Esculetin ameliorates insulin resistance and type 2 diabetic nephropathy through reversal of histone H3 acetylation and H2A lysine 119 monoubiquitination". ***Journal of Functional Foods***. 35, 256-266. [Impact Factor: 3.973].
3. **Kadakol A**, Sharma N, Kulkarni YA, Gaikwad AB (2016) "Esculetin: A phytochemical endeavor fortifying effect against non-communicable diseases". ***Biomedicine & Pharmacotherapy***. 84: 1442-1448 [Impact Factor: 2.326].
4. **Kadakol A**, Malek V, Goru SK, Pandey A, and Gaikwad AB (2015) "Esculetin Reverses Histone H2A/H2B Ubiquitination, H3 Dimethylation, Acetylation and Phosphorylation in preventing Type 2 Diabetic Cardiomyopathy". ***Journal of Functional Foods***. 17:127–136. [Impact Factor: 3.973].
5. **Kadakol A**, Malek V, Goru SK, Pandey A, Bagal MB and Gaikwad AB (2015) "Esculetin Attenuates Alterations in Ang II and Acetylcholine Mediated Vascular Reactivity Associated with Hyperinsulinemia and Hyperglycemia". ***Biochemical and Biophysical Research Communication***. 461(2): 342–347. [Impact Factor: 2.371].
6. **Kadakol A**, Goru SK, Pandey A and Gaikwad AB (2014) "Reno Protective Activity of Esculetin in Insulin Resistant and Type 2 Diabetic Rats". ***Journal of the American Society of Nephrology Supplement***. 25, 962A-PUB 291.

List of publications apart from thesis:

1. **Kadakol A**, Malek V, Goru SK, Pandey A and Gaikwad AB (2017) “Telmisartan and esculetin combination ameliorates type 2 diabetic cardiomyopathy by reversal of H3, H2A, and H2B histone modifications”. ***Indian Journal of Pharmacology***. (Accepted) [Manuscript ID: IJP_170_16].
2. Goru SK, **Kadakol A**, Malek V, Pandey A, Sharma N, Gaikwad AB (2017) "Diminazene aceturate prevents type 1 diabetic nephropathy through increasing glomerular ACE2 and

AT2 receptor expression". ***British Journal of Pharmacology***. 174: 3118-3130. [Impact Factor: 5.491].

3. Goru SK, **Kadakol A**, Gaikwad AB (2017) "Hidden targets of ubiquitin proteasome system: To prevent diabetic nephropathy". ***Pharmacological Research.*** 120: 170-179. [Impact Factor: 4.816].
4. Pandey A, Raj P, Goru SK, **Kadakol A**, Malek V, Sharma N, Gaikwad AB (2017) "Esculetin ameliorates hepatic fibrosis in high fat diet induced non-alcoholic fatty liver disease by regulation of FoxO1 mediated pathway". ***Pharmacological Reports.*** 69: 666–672. [Impact Factor: 2.251].
5. Pandey A, Goru SK, **Kadakol A**, Malek V, Sharma N, Gaikwad AB (2016) "H2AK119 monoubiquitination regulates angiotensin II receptor mediated macrophage infiltration and renal fibrosis in type 2 diabetic rats". ***Biochimie.*** 131: 68-76. [Impact Factor: 3.017].
6. Pandey A, Kumar GS, **Kadakol A**, Malek V, Gaikwad AB (2016) "FoxO1 inhibitors: The future medicine for metabolic disorders?". ***Current Diabetes Reviews.*** 12:1-8.
7. Goru SK, **Kadakol A**, Pandey A, Malek V, Sharma N, Gaikwad AB (2016) "Histone H2AK119 and H2BK120 Monoubiquitination Modulate SET7/9 and SUV39H1 in Type 1 Diabetes Induced Renal Fibrosis". ***Biochemical Journal***. 473(21): 3937-3949. [Impact Factor: 3.562].
8. **Kadakol A**, Pandey A, Goru SK, Malek V and Gaikwad AB (2015) "Insulin Sensitizing and Cardioprotective Effects of Esculetin and Telmisartan combination by Attenuating Ang II Mediated Vascular Reactivity and Cardiac Fibrosis". ***European Journal of Pharmacology***. 765:591-7. [Impact Factor: 2.730].
9. Pandey A, Goru SK, **Kadakol A**, Malek V, and Gaikwad AB (2015) “Differential Regulation of ACE2 and NF-κB by Ang II Receptor Subtypes in Type 2 Diabetic Kidney”. ***Biochimie***. 118: 71-81. [Impact Factor: 3.017].
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Brief Biography of the Supervisor:

Dr. Gaikwad Anil Bhanudas is currently working as Head/Assistant Professor in Department of Pharmacy, at BITS Pilani, Pilani campus, Rajasthan. He has first awarded by NIPER for Doctoral Fellowship and during his doctoral studies, Doctoral Sandwich Fellowship award from DAAD (German Academic Exchange Services). He visited reputed overseas institutes as visiting scientist in Department of Medicine/Nephrology, Albert Einstein College of Medicine, Bronx, NY, USA and Nephrological Center, MedizinischePoliklinik, Ludwig-Maximilians-University, Munich, Germany, in the year of 2008 to 2009 and in 2010 respectively. His enthusiasm in research was recognized by various government agencies and accepted his research proposals. The title of the proposals and their respective sponsored Govt. agencies are; (1) "Esculetin a Therapeutic Intervention Targeted at Cardio-Renal Syndrome in Type 2 Diabetes" - SERB, DST Govt of India, Empowerment and Equity Opportunities for Excellence in Science; (2) "Understanding the Epigenetic Regulation in modulating Inflammatory Response mediated by NF-kB through AT1 or AT2 receptor in Type II diabetic nephropathy" - SERB, DST Govt of India, Fast Track for Young Scientist; (3) "Effect of Esculetin on Angiotensin II in rat thoracic aorta under hyperglycemia and hyperinsulinemic conditions" - UGC, Govt of India, Major Research Proposal; (4) "Role of Histone Ubiquitination& Angiotensin Converting Enzyme 2 in the Development of Renal Fibrosis under Type I diabetic Condition" - CSIR, Govt of India, EMR. Till the date he has provided essential and novel evidences on histone post-translational modifications in type 2 diabetic heart, regulation of nuclear factor kappa B by angiotensin receptor type 1 and 2 in diabetic nephropathy, therapeutic intervention of esculetin in development of cardio-renal syndrome under type 2 diabetes and so on. Dr. Gaikwad Anil Bhanudas is not only contributed in research but also in scientific writing by being a one of the authors in book chapter, which are published by Elsevier. His review publications are the valuable contribution towards scientific community and in addition he has more than 30 peer-reviewed research publications. Along with research achievement, he has proven himself for his administrative skills. At present he is guiding five PhD students and guided more than four M. Pharm students in the fulfillment of their dissertation.

Brief Biography of the Candidate:

Mr. Almesh Kadakol currently working as research scholar and pursuing PhD in Department of Pharmacy, BITS Pilani, Pilani campus, Rajasthan. He completed his graduation (Pharmacy) and post-graduation (Pharmacology) from Rajiv Gandhi University of Health and Sciences, Bangalore, Karnataka. Till date he has published 11 research and 4 review articles in peer-reviewed journals.