UNIVERSITA' DEGLI STUDI DI VERONA

GRADUATE SCHOOL OF LIFE AND HEALTH SCIENCES

DEPARTMENT OF SURGERY, DENTISTRY, PAEDIATRICS
&
GYNAECOLOGY

DOCTORAL PROGRAM IN CARDIOVASCULAR SCIENCES

Cycle XXIX January 2014 – December 2017

"Cardioprotective Effects of Cocoa Extract Polyphenols against Ischemia/Reperfusion Injury in Rats"

SAJEELA AHMED

Ph.D. Coordinator – **Prof. Giovanni Battista Luciani**
Ph.D. Supervisor – **Prof. Giuseppe Faggian**
Ph.D. Co-Supervisor – **Alessio Rungatscher**

6th April, 2018

CARDIOPROTECTIVE EFFECTS OF COCOA EXTRACT POLYPHENOLS AGAINST ISCHEMIA/REPERFUSION INJURY IN RATS – SAJEELA AHMED

PhD Thesis

VERONA, 6th April 2018

ISBN ……

UNIVERSITA' DEGLI STUDI DI VERONA

GRADUATE SCHOOL OF LIFE AND HEALTH SCIENCES

DEPARTMENT OF SURGERY, DENTISTRY, PAEDIATRICS, AND GYNAECOLOGY

DOCTORAL PROGRAM IN CARDIOVASCULAR SCIENCES

Cycle XXIX January 2014 – December 2017

Cardioprotective Effects of Cocoa Extract Polyphenols against Ischemia/Reperfusion Injury in Rats

A Ph.D. thesis submitted to the University of Verona in partial fulfillment of the requirements for the Cardiovascular Sciences Ph.D. degree of the Graduate School of Life and Health Sciences, Department of Surgery, Dentistry, Pediatrics, and Gynecology, University of Verona, Verona, Italy

Ph.D. Coordinator: Prof. Giovanni Battista Luciani

Signature: _____

Ph.D. Tutor: Prof. Giuseppe Faggian

Signature: _____

Ph.D. Co-Tutor: Prof. Alessio Rungatscher

Signature: _____

Doctoral candidate: Sajeela Ahmed

Signature: _____

UNIVERSITA' DEGLI STUDI DI VERONA

GRADUATE SCHOOL OF LIFE AND HEALTH SCIENCES

DEPARTMENT OF SURGERY, DENTISTRY, PAEDIATRICS, AND GYNAECOLOGY

DOCTORAL PROGRAM IN CARDIOVASCULAR SCIENCES

External examiners

Prof.

Signature: _____

Prof.

Signature: _____

Prof.

Signature: _____

Doctoral candidate: Sajeela Ahmed

Signature: _____

SOMMARIO

I polifenoli dietetici possiedono proprietà antiossidanti e antinfiammatorie e sono noti per la prevenzione delle malattie degenerative del cancro e delle malattie cardiovascolari, ma i loro benefici sulla salute dipendono dalla quantità consumata e dalla loro biodisponibilità. Lo scopo di questo studio era di valutare l'effetto del trattamento esogeno (orale) del cacao sul danno miocardico acuto dopo ischemia coronarica / riperfusione nei ratti a livello istopatologico e biochimico.

Estratti metanolici di composti fenolici totali dal quarto di età disponibile in commercio di Recioto e polvere di estratto di cacao sono stati analizzati utilizzando sia l'HPLC-DAD che l'HPI ESI-MS. 41 diversi composti fenolici sono stati rilevati e identificati nel Recioto mentre nel cacao 19 sono stati trovati diversi composti fenolici, principalmente derivati dalla catechina e dall'epicatechina. Nell'analisi quantitativa (HPLC-DAD) del cacao, sono stati trovati 250 ± 1 mg di flavanoli in 1 grammo di estratto di cacao (capsula). Sebbene 90 ± mg di flavanoli rilevati in 1 litro di vino rosso Recioto. La quantità di flavonoidi presenti nell'estratto di cacao commercialmente è tre volte superiore a quella trovata nel vino rosso Recioto.

L'estratto di cacao è stato somministrato per via orale in cinque diversi gruppi I, II, III, IV e V di ratti (n = 1) in modo dose-dipendente 5 mg / kg (peso corporeo), 10 mg / kg, 15 mg / kg e 25 mg / kg per 15 giorni. Il gruppo VI era servito come controllo. Dopo 1 ora di somministrazione orale di cacao, è stato raccolto 1 ml di sangue per l'analisi dei polifenoli di cacao nel siero del sangue mediante spettrometria di massa HPLC. Questo ha prodotto un corrispondente aumento dei polifenoli del siero del sangue ed è diventato costante dopo una dose di 15 mg / kg.

Un altro esperimento è stato progettato dopo aver ottenuto una dose ottimale di polifenoli di cacao nel siero del sangue. In questo esperimento, trenta ratti sono stati divisi in gruppi trattati e di controllo. Il gruppo trattato (n = 15) ha ricevuto la dose ottimale selezionata (cioè 15 mg / kg) di estratto di cacao ogni giorno per 15 giorni oralmente e il gruppo di controllo (n = 15) non è stato trattato. Entrambi i gruppi sono stati sottoposti ad occlusione chirurgica dell'arteria coronaria discendente anteriore sinistra per 30 minuti e 24 ore di riperfusione. Infine, i cuori sono stati asportati e trattati per l'analisi biomolecolare.

Lo stato antiossidante nei tessuti del miocardio è stato stimato determinando l'attività di perossidasi (POD), catalasi (CAT), superossido dismutasi (SOD) e il livello di perossidazione lipidica (TBARS), glutatione perossidasi (GSH) è stato misurato mediante spettrofotometro. Sono stati osservati effetti marcati migliorati con il trattamento con estratto di cacao (15 mg / kg) ei valori di questi biomarcatori sono stati invertiti verso il gruppo di controllo.

L'entità dell'infiammazione di CCL5 / RANATES e IL-6, NF-κB2, marcatori di nitro-tirosina e fosforilazione a valle della via signale (sopravvivenza) di Erk ½ e Akt è stata osservata mediante immunoistochimica e l'apoptosi dei nuclei miocardici è stata analizzata mediante saggio TUNEL. Trattamento con estratto di cacao, marcatori infiammatori diminuiti (CCL5 / RANTES, IL-6 e NF-KB) e apoptosi miocardica (TUNEL, $p < 0,01$) rispetto al gruppo di controllo.

L'attivazione di entrambi p-Akt e p-ERK1 / 2 è stata osservata anche nel gruppo trattato con cacao. Il diminuito marcatore infiammatorio e l'apoptosi nel cuore di ratto trattato con estratto di cacao potrebbero essere modulati dall'attività p-Akt e p-ERK1 / 2. I nostri risultati hanno dimostrato che l'estratto di cacao una volta al giorno per 15 giorni nei ratti attenua la lesione I / R miocardica e limita lo stress ossidativo e nitrosativo e l'infiammazione con riduzione dell'apoptosi miocardica.

La dose ottimale di estratto di cacao che si trova nel modello di ratto può avere anche un valore traslazionale nell'uomo. Ad esempio, se il peso corporeo medio di 70 kg di esseri umani richiede 3.000 mg di estratto di cacao per 15 giorni può avere effetti simili come riduzione dello stress ossidativo, apoptosi e marcatori infiammatori che possono essere modulati dalle attività di Akt e ERK1/2.

I risultati di questo studio sono riassunti nella figura 1 seguente:

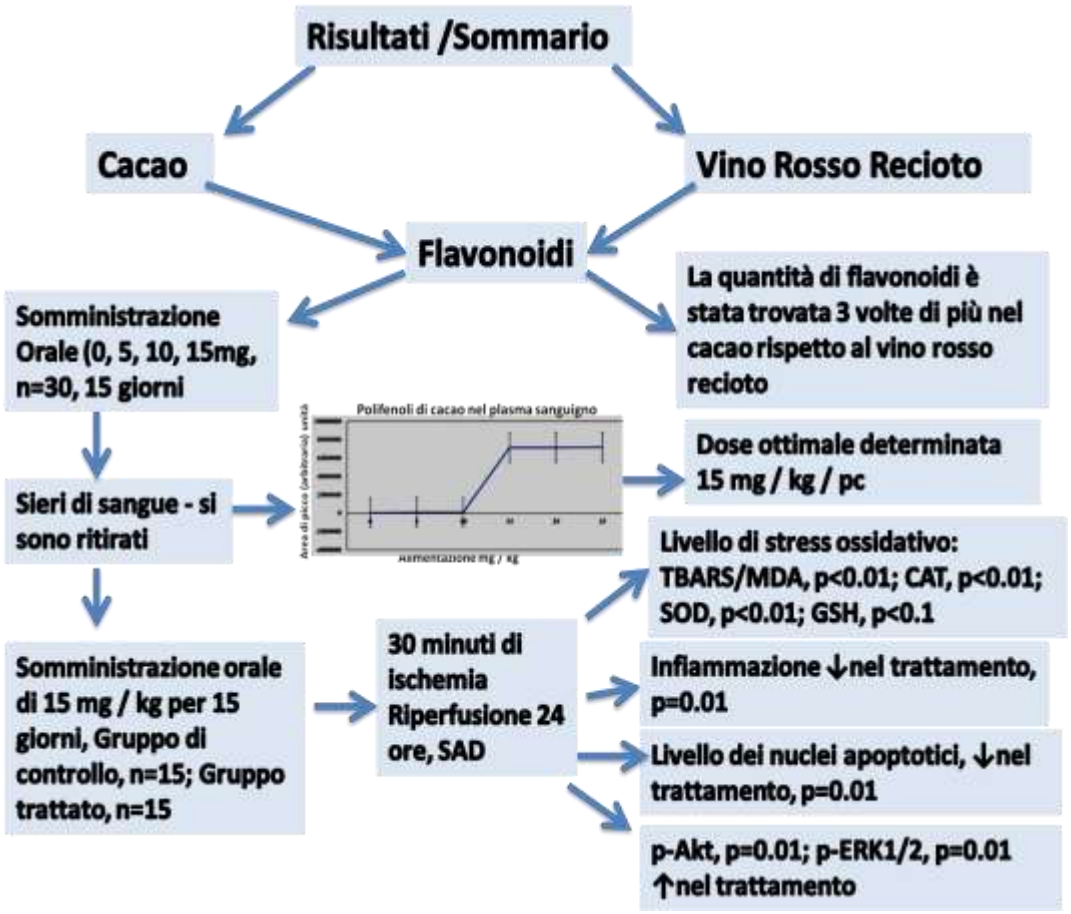

Figura: 1. Riepilogo / risultati dello studio: il grafico illustra le quantità di flavonoidi nell'estratto di cacao e nel vino rosso recioto; effetti dell'estratto di cacao su stress ossidativo, infiammazione, apoptosi e chinasi pro-sopravvivenza.

SUMMARY

Dietary Polyphenols possess antioxidant and anti-inflammatory properties and known for the prevention of degenerative diseases cancer and cardiovascular diseases, but their health benefits depend upon the amount consumed and their bioavailability. The aim of this study was to evaluate the effect of exogenous (oral) cocoa extract treatment on acute myocardial injury following acute coronary ischemia/reperfusion in rats at histopathological and biochemical level.

Methanolic extracts of total phenolic compounds from the commercially available four years old Recioto red wine and the cocoa extract powder were analyzed by using both HPLC-DAD and HPLC ESI-MS. 41 different phenolic compounds were detected and identified in Recioto while in cocoa 19 different phenolic compounds, mainly derived from catechin and epicatechin, were found. In quantitative analysis (HPLC-DAD) of cocoa, 250 ± 1 mg of flavanols were found in 1 gram of cocoa extract (capsule). Although 90± mg of flavanols detected in1litre of Recioto red wine, the amount of flavonoids found in commercially cocoa extract are three times more than those found in Recioto red wine.

The cocoa extract was orally administrated into five different groups I, II, III, IV and V of rats (n=1) in dose-dependent manner 5mg/kg (body weight), 10mg/kg, 15mg/kg and 25mg/kg for 15 days. Group VI was served as control. After 1 hour of oral administration of cocoa, 1ml of blood was collected for the analysis of cocoa polyphenols in blood serum through HPLC-mass spectrometry. This produced a corresponding increase in blood serum polyphenols and became constant after 15 mg/kg dose.

Another experiment was designed after obtaining an optimal dose of cocoa

polyphenols in blood serum. In this experiment, thirty rats were divided into treated and control groups. Treated group (n=15) received the optimal selected dose (i.e. 15 mg/kg) of cocoa extract daily for 15 days orally and the control group (n=15) was remained untreated. Both groups underwent surgical occlusion of the left anterior descending coronary artery for 30 minutes and 24 hours of reperfusion. Finally, hearts were excised and processed for biomolecular analysis.

Antioxidant status in myocardial tissues was estimated by determining the activities of peroxidase (POD), catalase (CAT), superoxide dismutase (SOD), and the level of lipid peroxidation (TBARS), glutathione peroxidase (GSH) was measured by a spectrophotometer. Marked ameliorated effects were observed with cocoa extract (15mg/kg) treatment and the values of these biomarkers were reversed towards the control group.

The extent of inflammation of CCL5/RANATES and IL-6, NF-κB2, Nitro-tyrosine markers and downstream phosphorylation of signally pathway (survival pathway) of Erk ½ and Akt was observed by immunohistochemistry and myocardial nuclei apoptosis was analyzed by TUNEL assay. Treatment with cocoa extract diminished inflammatory markers (CCL5/RANTES, IL-6 and NF-KB2) and myocardial apoptosis (TUNEL, $p < 0.01$) as compared to control group. Activation of both p-Akt and p-ERK1/2 was also observed in the cocoa treated group. The diminished inflammatory marker and apoptosis in the cocoa extract treated rat heart could be modulated by the activity p-Akt and p-ERK1/2. Our results demonstrated that cocoa extract once a day for 15days in rats attenuates myocardial I/R injury and limits oxidative and nitrosative stress and inflammation with reduction of myocardial apoptosis.

The optimal dose of the cocoa extract that is found in rat model may have also a translational value in human. For example, if 70kg average body weight of human takes 3,000mg of cocoa extract for 15days may have similar effects such as reduction of oxidative stress, apoptosis, and inflammatory markers that may

be modulated by activities of Akt and ERK1/2.

The findings of this study are summarized in the following figure 2:

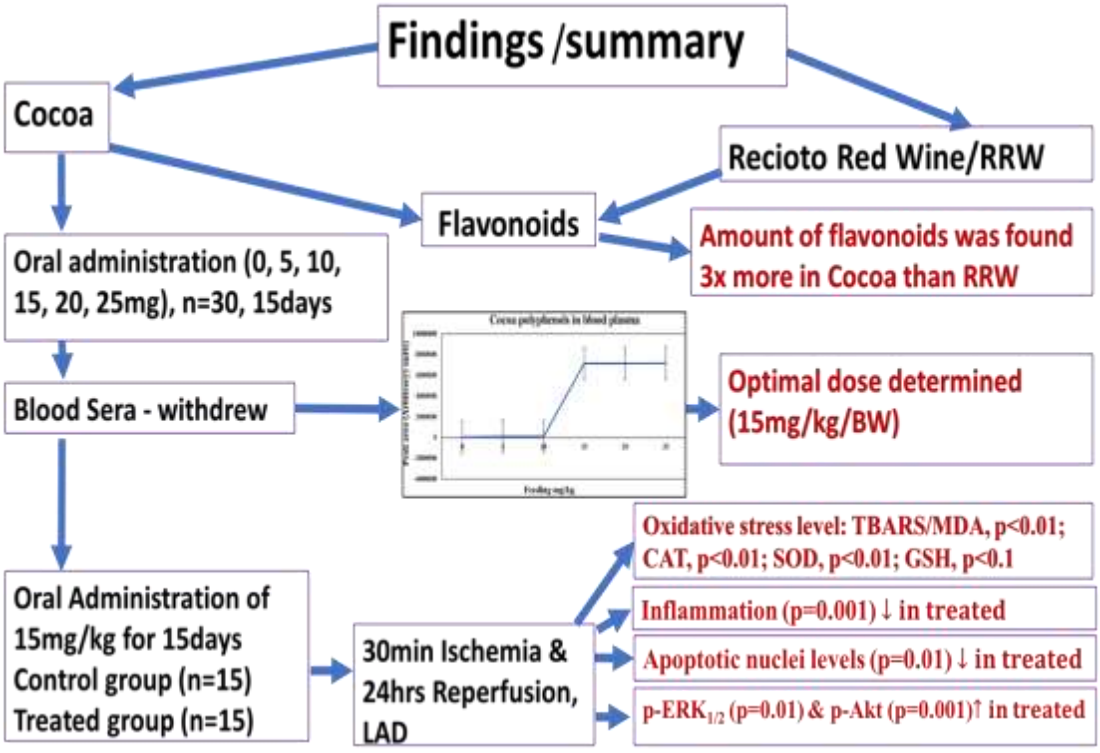

Figure: 2. Summary/findings of the study: the chart illustrates amounts of flavonoids in the cocoa extract and recioto red wine; effects of cocoa extract on oxidative stress, inflammation, apoptosis, and pro-survival kinases.

ABSTRACT

Background: Consumption of flavonoid-rich nutraceuticals has been associated with a reduction in coronary events. The present study analyzed the effects of cocoa extract flavonols on myocardial injury following acute coronary ischemia/reperfusion.

Materials and methods: A commercially available cocoa extract was identified by chromatographic mass spectra. Nineteen different phenolic compounds were identified and 250 mg of flavan-3-ols (procyanidin) were isolated in 1 gram of extract. Oral administration of cocoa extract in incremental doses from 5mg/kg up to 25 mg/kg were given to five group of Sprague-Dawley rats (5 each group) daily for 15 days and additional 5 rats were used as a control group. This produced a corresponding increase in blood serum polyphenols and became constant after 15 mg/kg dose. Thirty rats were divided into treated and control groups. Treated group (n=15) received the optimal selected dose (i.e. 15 mg/kg) of cocoa extract daily for 15 days orally and the control group (n=15) was remained untreated. Both groups underwent surgical occlusion of the left anterior descending coronary artery for 30 minutes and 24 hours of reperfusion. Finally, hearts were excised and processed for biomolecular analysis.

Results: Cocoa extract reversed ($p < 0.001$) membrane peroxidation, nitrosative stress and superoxide dismutase and catalase activity caused by myocardial I/R injury. Treatment with cocoa extract diminished inflammatory markers (CCL5/RANTES, IL-6 and NFkB2) and myocardial apoptosis (TUNEL, $p < 0.01$). Cocoa treatment also showed activation of both p-Akt and p-ERK1/2. The diminished inflammatory marker and apoptosis in cocoa extract treated rat heart could be modulated by the activity p-Akt and p-ERK1/2.

Conclusion: The augmentation of cocoa extract once a day for 15days in rats attenuates myocardial I/R injury and limits oxidative and nitrosative stress and inflammation with reduction of myocardial apoptosis.

Keywords: Flavonoids, Cocoa extract, Recioto red wine, Ischemia-reperfusion injury, oxidative stress, apoptosis, inflammatory markers, downstream signaling.

ACKNOWLEDGEMENT

I would like to thank Allah Almighty, who gave me the strength, courage and energy to accomplish my research as without His help this project would have not been possible to complete.

I feel highly privileged and obliged to express my sincere thanks to my supervisor Prof. Giuseppe Faggian. His efforts tower the inculcation of spirit of hard work and maintenance of professional integrity besides other valuable suggestions will always serve as beacon of light through the course of my life.

I would like to gratefully thank my co-supervisor Dr. Alessio Rungatscher for his expertise and involvement in the study and his follow-up in the experiment and evaluating manuscript and reports which help me to improve the paper. I would also like to thank Prof. Luciani Giovanni Battista for giving me valuable comments on each presentation of seminars.

I am grateful to Dr. Flavia Merigo for their technical assistance and collaboration in laboratory of Department of Neuroscience, Biomedicine, and Movement and Dr. Flavia Guzzo Department of Biotechnology, University of Verona Italy (laboratory of Plant Biology and Metabolomics) and her nice group Dr. Mauro commisso, Stefno negari and Stefania for supporting and providing material for pursing the research work in her laboratory.

I would also like to thank Department of Pathology and Diagnostics Research Team: Dr. Giulio Innamorati for assisting in reagents order and Dr. Laura Marcazzan and Dr. Stefania Baschirotto for their up to date information of seminars and administrative issues.

I would like to thank also to my colleagues Dr. Dmitry Kosenkov, Dr. Mahmoud Ismail, Dr. Laura Saracino, Dr. Naseer Ahmed, Dr. Alice Giacomazzi, and Dr. Mebratu Alebachew Gebrie for their cooperation during the experimental work in the laboratory.

Everlasting and heartful thanks to my dear beloved husband Mr. Muhammad Omer Tahir for leaving a positive impact on my life by his endearment support, cooperation, consolatory behavior and prayers not only in academics but in every aspect of my life. No amount of words could ever describe the love I have for my cute and adorable son Ibraheem Omer.

I seem it a great honor and privilege to record profound sense of gratitude and extend my compliments to my affectionate parents Mr. Ahmed Din and Ms. Ghazala Ahmed and brothers Dr. Shehzad Qasmi, Sajjad and Nadeem for their encouragement, inspiration for higher ideas, love, devotion, moral and financial support, amicable attitude and their countless prays for my glorious success about my pursuits throughout my life. I can do no more than to reaffirm my external devotion to all the members of my life.

Finally, I would also like to thank the University of Verona for giving me an opportunity and financial support to follow my doctoral study.

MAY ALLAH BLESS YOU ALL!

DECLARATION

I, the under sign, state that this Ph.D. thesis was carried out in accordance with the regulations of the University of Verona. It hasn`t been submitted for any other academic award or is being published elsewhere. This work is original and every contribution and effort of others is acknowledged. Any views expressed in the thesis are those of the author, except where indicated by reference in the text.

SAJEELA AHMED

Signature: _____

ABBREVIATIONS

ABTS, 2, 2‘-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)

BSA - Bovine Serum Albumin

CAT - Catalase

CCLS 5 - C-C Chemokine Ligand 5

DAPI - 4`, 6-Diamidino-2-Phenylindole

DPPH, 2, 2-diphenyl-1-picrylhydrazyl

GSH - Glutathione

IHC - Immunohistochemistry

IL-1 - Interleukin 1

IRI - Ischemia-Reperfusion Injury

ITIS - Integrated Taxonomic Information System

MDA - Malondialdehyde

NF-kB2 - Nuclear Factor Kappa B2

PBS - Phosphate Buffered Saline

P-Akt - Phosphorylated Serine-Threonine Protein Kinase.

P-ERK1/2 - Phosphorylated Extracellular Signal Regulated Kinases ½

POD - Peroxidase

RANTES - Regulated on Activation Normal T Cell Expression and Secreted

ROS - Reactive Oxygen Species

SOD - Superoxide Dismutase

SPSS - Statistical Package for the Social Science

TBARS - Thiobarbituric Acid Reactive Substances

TUNEL - Terminal Deoxynucleotidyl Transferase, 2`-Deoxyuridine 5`-Triphosphate Nick-End Labeling

Tris - Trihydroxymethylaminomethane

CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

1.1. INTRODUCTION

1.2. Cocoa

Cocoa is dry and non-fat powdered manufacture from seeds of *Theobroma cacao* L. Tree and originally, they were found and widely produced in tropical regions of Central and South America (Alamu, 2013). Four West African countries, Ivory Coast, Ghana, Nigeria, and Cameron are the main cocoa producing countries. Ivory Coast is one of the world's number one Cocoa exporters (Agarwal, 2013). Chocolates, Cocoa Liquor, cocoa powder, confectionary, and ganache are made from Cocoa bean (Grivetti et al., 2009). Flowers produce directly on the trunk and older branches of a cocoa tree in the form of clusters called 'cauliflory'. Fruits 'cacao pod' are formed after pollination and 20 to 60 seeds called 'cocoa beans' embedded in a white pulp are present in each pod (Agarwal, 2013).

Cocoa beans were considered as food of heaven by the Mayans, as they were presumably revealed by god Quetzalcoatl (Agarwal, 2013). They believed that cocoa beans are more energetic and make them sturdy. Spaniards noticed that they can walk long distances without any fatigue and allowed the Aztecs greater stamina due to the cocoa consumption. Some Mexican, Mayan and Olmec tribes also have well-known the medical importance of these beans (Agarwal, 2013). Products made from cocoa have been enjoyed by humans for centuries (Samman et al., 2001).

Cocoa contains a naturally occurring compound that is flavonoids, theobromine, and magnesium. Cocoa extract is bioactive compounds found in cocoa products. Cocoa products are consumed all over the world for its pleasant taste and have very stimulating and pleasurable effects (Samman et al., 2001). It has been described in many European historical documents that chocolate was not

drunk only for favorable drink or dessert but it was also used to treat various types of diseases such as cardiac pain and angina (Keen, 2001). It has been found that Kuna Indians, live on Panamas San Blas Islands consume more than four cups of cocoa in a week does not show any age-related blood pressure and heart related diseases (Nsor-Atindana et al., 2012) Polyphenols are bioactive compounds naturally found in plant sources and have gained a point of interest in recent years due to their unique nutritional and functional values including antioxidant, antimutagenic, antimicrobial and antitumor effects (Atindana et al., 2012; Tomas-Barberan et., 2007).

In a recent study reported by Akinmoladun et al., (2016), antioxidant containing extracts of cocoa and kola nut tree has shown the protective effect against myocardial ischemia-reperfusion injury/IRI using Langerdorff-perfused rat hearts. In another study, flavonoids (5-hydroxy derivatives: 5-hydroxy flavone, apigenin, chrysin and naringenin) have been stated in improving post-ischemic functional recovery and lowering of myocardial tissue injury (Testai et al., 2013). On the other hand, in earlier in vitro study of the peripheral blood mononuclear human cell, inhibition of IL-1 mRNA expression and T-cells secretion of IL-2 has been reported from polyphenol containing Cocoa liquor (Sanbongi et al., 1997). However, the previous studies lack biochemical analysis of inflammatory markers and signaling proteins activities, and evaluation of myocardial nuclei apoptotic levels in rat hearts after IRI.

Additionally, comparison of Cocoa and Recioto red wine/RRW flavonoid contents has not been done. Therefore, in this study after comparing flavonoid contents in cocoa and RRW, we employed some inflammatory, apoptotic, downstream signaling proteins (p-Akt & p-ERK1/2) biomolecular analysis by designing immunohistochemistry, TUNEL and additional oxidative stress markers approach to investigate the effects of cocoa against IRI in rats which underwent LAD induction.

1.3. Scientific Classification of Cocoa

The Botanical name of Cocoa is *Theobroma Cacao* and belongs to family Malvaceae (alternatively Sterculiaceae) and is differentiated into three main cultivar groups: Criollo, Forastero, and Trinitario, produced in the subtropical regions worldwide. Cocoa is one of the world's most popular crops, cultivated about 8.2 million hectares, having a leading role in the social and economic lives of many peoples in poor rural areas (Pohlan and Perez, 2011). The cocoa plant is scientifically classified as shown in the table below:

Table: 1. Scientific Classification of Cocoa plant

1	**Kingdom**	→	**Plantae**
2	Subkingdom	→	Viridaeplantae
3	Infrakingdom	→	Streptophyta
4	Division	→	Tracheophyta
5	Subdivision	→	Subspermatophytina
6	Infradivision	→	Angiospermae
7	Class	→	Magnoliopsida
8	Superorder	→	Rosanae
9	Order	→	Malvales
10	Family	→	Malvaceae
11	Genus	→	Theobroma L.
12	Species	→	Cocoa

Adopted from Integrated Taxonomic Information System (ITIS, 2011).

Figure: 3. Cocoa seeds in the fruit or Cocoa pod and Cocoa tree with pods: Fig.1.A shows the cocoa seeds attached on its stem of Theobroma cocoa L. Tree; (source Wikipedia) Fig.1.B illustrates the cross-section of cocoa with its pods (Adopted from Keith Weller 2004, United States Department of Agriculture).

1.4. Types of Wines in Verona

There are different types of grapes in Verona, such as Garganega, Corvina, Molinara and Rondinella grapes, Barbera, Sangiovese, Marzemino, Merlot and Cabernet Sauvignon etc. These grapes are involved in making many alcoholic beverages and juices (Patricia Guy, 2003; Amarone Tours)**.** To know the amount and type of flavonoids that are present in them are unknown. Not surprisingly, Verona with the important role it had in Roman age has a very old tradition in vine and winemaking. The Valpolicella DOC region is located between Bardolino and Soave, just north of Verona. Valpolicella is a red wine made primarily from Corvina, Rondinella, and Molinara. Corvina is considered the superior grape and usually, makes up a majority of the blend **(**Patricia Guy; Amarone tours).

Figure: 4. Map of Verona regions producing wines (Source Amarone Tours)

Soave is a wine produced from Garganega grapes as a single varietal or blend that is one of the most famous of the Italian whites. The Soave DOC is located about 10 miles to the east of Verona. Soave is a dry white wine produced from 70% Garganega grapes with Pinot Bianco, Trebbiano di Soave (Verdicchio), and Chardonnay making up most of the remaining 30%. The Bardolino region is about 30 miles West of Verona, close to Lake Garda. Bardolino is a light red wine made primarily from a blend of Corvina, Rondinella, and Molinara grape varieties (the same grapes as Valpolicella) (source Amarone Tours). Barbera, Sangiovese, Marzemino, Merlot and Cabernet Sauvignon are allowed in small amounts as well.

The three wine regions in the north-eastern corner of Italy are often referred to as the Tre Venezie, the Three Venices because they were once part of the Venetian Empire. Each of these regions produces red and white wines that are among the most popular Italian wines outside of Italy (source Amarone Tours).

1.5. Recioto Red wine

Recioto has been classified as one of the high-quality red wine of the Italian Valpolicella region (Verona, Veneto, Italy) and has been obtained Denomination of Controlled and Guaranteed Origin (DOCG) (Dall'Asta et al., 2011). Recioto is an intensely flavored, sweet red wine made from dried *(passito)* grapes and most ideal wine (Ferrarini, 1982; Ferrarini & Zironi, 1987; Barbanti et al., 2008). Epidemiological studies have indicated that regular and moderate consumption of red wine can reduce the risk of cardiovascular-related diseases (Lippi et al., 2010**;** Szmitko and Verma, 2005).

Similarly, resveratrol has been considered as a major component present in red wine shows anticarcinogenic activity (Kundu et al., 2004; Shankar et al., 2007; He et al., 2008) but exists at a low level in red wine as compared to other flavonoids such as catechin and epicatechin (Balga et al., 2014). Red wine polyphenols show protective effects against angiotensin II-induced hypertension and endothelial dysfunction by reducing the NADPH oxidase-dependent formation of superoxide anions (Sarr et al., 2006).

1.6. Comparison of flavonoids in Recioto Red wine and Cocoa extract

Presence of flavonoids in different foodstuff and drinks are indicators protective effects of the materials on peripheral and central tissues. Therefore, in this study, it is important to determine the number of flavonoids in recioto red wine and cocoa extract to study further the protective effect against ischemia and reperfusion injury. The study evaluating the total antioxidant activity in cocoa, red wine, green and black teas has shown highest antioxidant activity in cocoa than the other three foodstuffs using the ABTS and DPPH radical scavenging assays (Lee et al., 2003).

1.7. Polyphenols

Phenolic compounds belong to a group of natural substances with variable phenolic structures and are found in fruit, vegetables, grains, bark, roots, stems, flowers, tea, and wine (Middleton, 1998; Vinson et al. 2001). These natural products were known for their beneficial effects on health from centuries before flavonoids were isolated as the effective compounds (Stefani et al., 1999). We are consuming these abundant micronutrients in our diet with an average of about 1 gram a day (Scalbert et al., 2005). These phenolic components of fruits and vegetables vary not only between different types but also among the cultivars of the same type and can even contingent on growing condition and harvesting time. Fruits and vegetables are the main sources of phenolic components and many of them are well known for their antioxidant and anti-inflammatory activities (Ghosh, 2005).

Polyphenols consist of a group of chemical compounds, distinguish by the presence of more than one phenolic group; however, the phenolic acids are phenols, have only one ring. The polyphenols are included in one of the important classes of plant secondary metabolites that contain flavonoids, lignans, stilbenes, coumarins and tannins (Harborne, 1993).

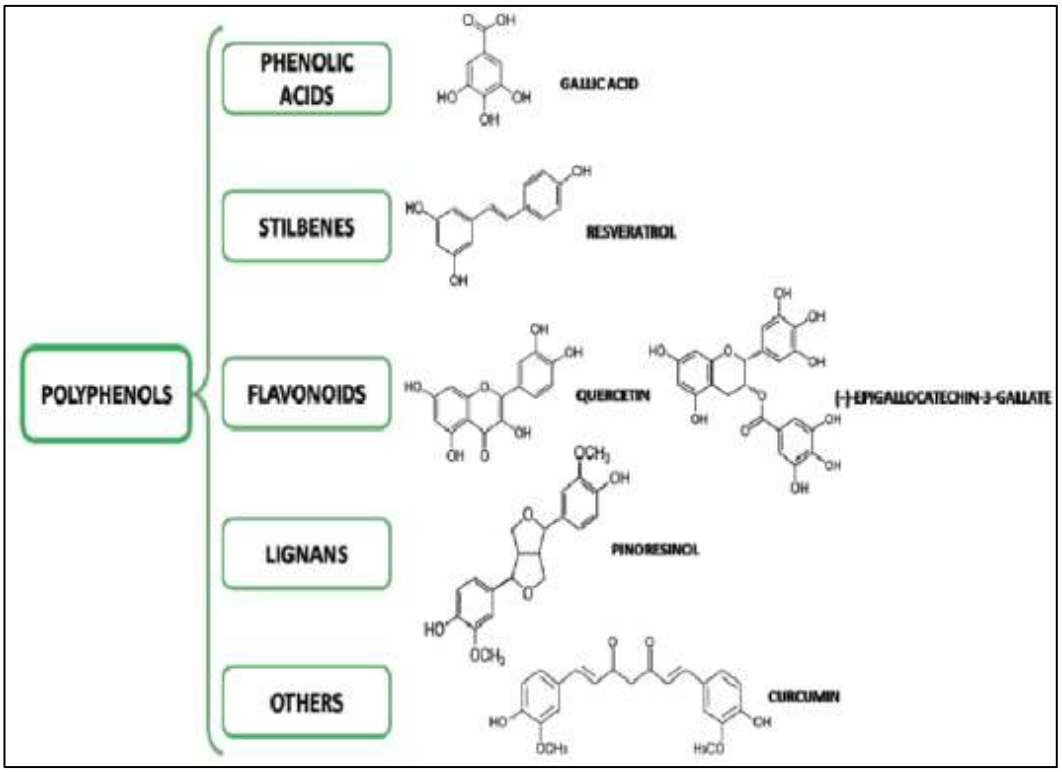

Figure: 5. Represents Classification and Structure of Polyphenols: (Spagnuolo et al., 2016).

The polyphenols belong to wide a range of the heterogeneous group of biologically active secondary metabolites in plants, where they help in cell wall protection, colourful attractants for birds and insects, defence against various environmental stress conditions (intense light, infection, wounding etc) (Hakkinen, 2000). The polyphenols are divided into four groups according to their phenolic rings and on the structural elements that link these rings: include phenolic acids, lignans (known as phytoestrogens; flaxseeds are their oil major source), flavonoids (the richest source of human diet), and stilbenes (resveratrol is still under observation due to its anti-carcinogenic properties) (Tomas-Barberan, 2012).

The flavonoids have a basic structure consist of three rings and they are subdivided into six families according to their substitution pattern: anthocyanins, flavonols, flavanols, flavanones, flavones and isoflavones which are present in the form of catechins in tea, red wine and chocolate, citrus fruits and soya (Shahidi

and Naczk, 1995). Although the term ‘polyphenols’ represents more than 8000 different structures, only a few of them have been investigated pharmacologically. For example, moderate and continuous consumption of red wine has shown beneficial effects against onset of cardiovascular diseases due to the presence of natural phytoalexin resveratrol, quercetin, delphinidin and (+)-catechin in red wine (Kirimlioglu et al., 2008; Kiviniemi et al., 2007; Nasissides et al., 2006).

Three main types of polyphenols are present in the unfermented cocoa bean are flavan-3ols or catechins, epicatechins anthocyanins and proanthocyanidins and (Misnawi, et al., 2004a; Jalil and Ismail, 2008). The phenolic contents in cocoa beans depend upon its genetic makeup, crop season and its processing time (Saltini et al., 2013). During the fermentation process, the polyphenols combined with cell liquid for oxidation, polymerization, and reaction with different proteins (Misnawi et al., 2004b; Wollgast and Anklam, 2000).

Anthocyanins are converted into anthocyanidins and sugar components, leucocyanidins are dimerized after hydrolyzation, and (-)- epicatechin are reduced to 10-20% due to its soluble in nature (Misnawi et al., 2004a; Wollgast and Anklam, 2000). It has been reported that ripe and unripe cacao pods contain exclusively amount of (-)- epicatechin and (+)-catechin but after the fermentation, levels these compounds were decreased, but (-)-catechin were found due to heat-induced epimerization. Additional loss of polyphenols occurs due to some nonenzymatic browning reaction, after drying of cacao beans (Hurst et al., 2011). A significant loss of polyphenols due to thermolabile flavanols and oxidation of catechin and epicatechin with amino acids and proteins to quinones, polymerize with other polyphenols in the roasting process (Rusconi and Conti, 2010; Li et al., 2012).

Antioxidant properties of polyphenols may differ accordingly to the arrangement of functional groups around the nuclear structure and their aglycones are strong antioxidant than their glycosides (Heim et al., 2002). The polyphenols are proton-donor-scavenging radicals (Rice Evans et al., 2002) and act as

inhibitors to reduce the oxidative stress, bind carbohydrates, chelate metals and proteins (Heim et al., 2002). Because of these properties, they possess anti-inflammatory, anticarcinogenic, antibacterial, antihepatotoxic, antiviral, and antiallergenic compounds (Zaveri, 2006; Vita, 2005; Arts and Hollman, 2005; Rice-Evans et al., 1997). It has been shown that during the process of chocolate making, there is a significant reduction in antioxidant activity and loss of polyphenols due to alkalization, high temperatures and presence of oxygen (Wollgast and Anklam, 2000).

The polyphenols give a bitter taste to cocoa; however, new approaches have been developed for the preservation of cocoa polyphenols contents during its processing whilst keeping satisfactory aroma (Schinella et al., 2010). Various health benefits inherent in cocoa polyphenols distinguish it and making cocoa as a potential functional food.

1.8. Bioavailability of Polyphenols

Bioavailability and absorption of polyphenols depend on their chemical structure, food matrix and many other factors like food processing, individual variability (genetic makeup of individuals, age, gender and physiological disorders), biodiversity of their own intestinal microbiota taxa and enzymatic activity in the colon (D'Archivio et al., 2010; Tomas-Barberan, 2012), are the key elements concerning the ability of a person to take almost total amount of bioactive derivatives from ingested polyphenols (Marin et al., 2015).

Natural products like vegetables and fruits, green and black tea, red wine, cocoa, coffee, spices and some herbs, as well as nuts and algae, are the most important food sources of polyphenols (Quinones et al., 2013; Sankno et al., 2005). Some polyphenols are present in all plant products and some are specific to food, so that, generally, food consists of complex mixtures of polyphenols (D'Archivio et al., 2010). Isoflavones and phenolic acids show the highest bioavailability, followed by catechins, flavanones and quercetin glucosides, while

anthocyanidins, proanthocyanidins, and galloylated tea catechins are not absorbed in all or poorly absorbed (Han et al., 2007).

The polyphenols are conjugated in the form of sulphate, glucuronide and methyl groups in the gut mucosa and inner tissues once they are ingested and absorbed, where epicatechin and epigallocatechin are transformed into as glucuronide and sulphate conjugates. Once flavonoids are ingested sugar moieties have been removed from their phenolic backbones and absorbed in the small intestine. Only 55% and 22% of catechin and epicatechin are absorbed in small intestine. Proanthocyanidins are in the form of polymers, have high molecular weight, and therefore its trimer and dimer are unlikely absorbed in the small intestine in their native form, transported into the liver where, they undergo methylation, glucuronidation, and sulfation which possess antioxidant capacity (Han et al., 2007).

Some unabsorbed polyphenols, they are esterified to sugars, lipids and organic acids in the small intestine. There are no enzymes to break these ester links in human tissues, then the main site for their metabolism is colon, where they meet colonic microbiota then cleaves the conjugating mioties, but most of their absorption occurs in small intestine (Marín et al., 2015) When final product as aglycone from small intestine and colon enter blood stream by the portal vein, transported into liver, where they may have subjected to conjugated, from where they again enter the blood stream until they are excreted in urine (Monagas et al., 2010; Selma et al.,2009; Stalmach et al., 2010; Nielsen et al.,1998; lee et al., 2002; Tilgmann et al., 1996; Donovan et al., 2001; Vaidyanathan et al., 2002; Crespy et al; 2001; Ouzzine et al., 2003; Marín et al., 2015). Some of the liver conjugates back into small intestine as bile product, before being reabsorbed again, gut microbial enzymes regenerated these deconjugated compound (Rechner et al., 2004; Aura et al., 2008; Cardona et a., 2013). The metabolites that are not absorbed are eliminated as feces.

All these conjugation reactions are extremely efficient and free aglycones are either present in low concentration or absent at all in blood plasma after nutritional doses (D'Archivio et al., 2010; Marín et al., 2015).

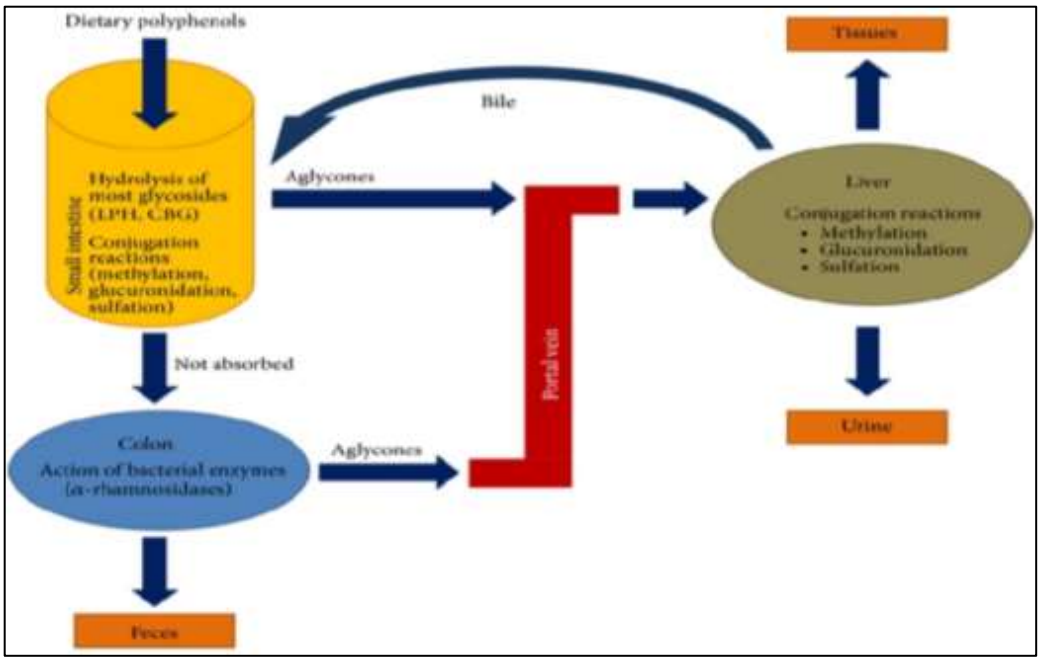

Figure: 6. The absorption and metabolism of polyphenols: This figure shows bioavailability of flavonoids indicating from dietary flavonoids intake, their absorptions, and conjugation reactions into the liver and end products through tissues, urine, and feces (Marín et al., 2015)

Epicatechin showed a higher absorption in human and its concentration in blood plasma detected after 30 mins of the oral dose, reach peak after 2-3 hours and back to baseline after 6-8 hours. Generally, polyphenols with low molecular weight showed a higher concentration of blood and it higher the chance to reach quickly to the target organ (Cooper et al., 2008). The chemical structure might also effect the bioavailability of polyphenols, (-) - a form of catechin is almost 10 times less absorbed than its (+) - catechin form (Cooper et al., 2008).

The bioavailability of polyphenols generally increases with the presence of sugars and oils, whereas it decreases in the presence of proteins and lipids (Tomas-Barberan, 2012). Milk proteins decrease the bioavailability of epicatechin

in chocolate products (Neilson et al., 2010). Serafini et al. (2003) reported inhibition of the antioxidant activity of chocolate in vivo due to the presence of milk in it. However, this effect has not found in chocolate drinks (Neilson et al., 2010).

Proteomic techniques demonstrate the study of interactions of cocoa polyphenols with milk proteins that have shown the presence of covalent binding of free SH-group of the free cysteine residue of protein in protein-polyphenol complex formation (Neilson et al., 2010). The study supported by fact that alkylation form of peptide is not reacted with flavanols, while lactosylation has shown polyphenol binding. Since bioavailability of polyphenols is not significantly affected, the only little portion of proteins interacts with polyphenols (Gallo et al., 2013).

This study was reported by Roura et al. (2007) and Keogh et al. (2007) showed that milk does not have an influence on the absorption and metabolism of cocoa powder polyphenols in healthy adults. Sucrose increased the bioavailability of polyphenols, but manufacturing process can also affect the extent of sucrose impact. It has been observed that bioavailability of polyphenols increases when they are consumed after a carbohydrate-rich meal (Schramm et al., 2003). Peters et al. (2010) revealed that addition of sucrose in green tea might slow down the catechin absorption, partly due to higher viscosity, but it also enhanced catechin uptake by the intestine.

1.9. Significance of Cocoa Polyphenols in Cardiovascular Health

Cocoa contains polyphenolic flavonoids components and traditionally used by native Indian as a raw unsweetened drinks and dried cocoa powder (Grivetti and Howard-Yana, 2009). After reaching to Europe sugar was added and other processing steps became popular to minimize bitterness and to give European taste and flavor. These alterations resulted in lowering of flavanol

content which is a possible polyphenolic with potential antioxidant activity (Hristova et al., 2012).

American researchers noted the medicinal importance of cocoa beans. When residents of the Island Kuna in South America are observed, are consumed a large amount of home-prepared cocoa, full of flavonoids contents and stayed hypertension free lives. However, people on Kuna mainland consume commercially available cocoa lacking flavonoids. It is found that they develop cardiovascular-related diseases and hypertension (Fisher and Hollenberg, 2005). On examining the renal hemodynamics, the Kuna Island people have shown a high level of NO level, consistent with the positive antioxidant influence of cocoa polyphenols on the endothelium (Fisher et al., 2003). Cacao beans contain flavanols, the main flavonoids that are present are catechins and epicatechins (Steinberg et al., 2003).

The antioxidant effects of cocoa and chocolate have shown a positive correlation with their procyanidin and catechin contents (Wan et al., 2001). These are effective antioxidant and enhance cardiovascular system (Desch et al., 2010; Corti et al., 2009; Ding et al., 2006). Many studies have grown a point of interest for many medical and basic scientists to investigate the effects of cocoa in cardiovascular diseases (Corti et al., 2009; Ella et al., 2012).

Cocoa has been considered as a potential medicine from the seventh century. It has been described in the European historical documents that chocolate is not drunk only for favorable drink or dessert but it is used to treat various types of diseases such as cardiac pain and angina (Tomas-Barberan et al., 2007). Furthermore, in vivo studies indicate that consumption of chocolate can increase the total antioxidant capacity of human blood plasma (Serafini et al., 2003). There is a reduction in cardiovascular disease by 37%, diabetes by 31% and stroke by 29% by the high level of chocolate consumption, shown in recent meta-analysis study (Adriana et al., 2011).

Various studies have indicated that cocoa has many beneficial effects on the risk of stroke, hypertension, insulin resistance, coronary artery disease (CAD), cognitive function, dementia and metabolic syndrome (Skelhon et al., 2012; Larsson et al., 2012; Hooper et al., 2012; Tokede et al., 2011; Desch et al., 2010; Reid et al., 2010; Armitage et al., 2009; Taubert et al., 2007; Henderson et al., 2007).

Consumption of cocoa on daily basis exerts many useful effects on cardiovascular health (Corti et al., 2009). Many researchers have investigated that polyphenols rich food or polyphenols have a key role in health protection due to their antioxidant activities (Awe et al., 2013; Cooper et al., 2008; Han et al., 2007). Epidemiologically, it has been found that cocoa intake reduces the cardiovascular diseases and all-cause mortality but an inverse relationship with the blood pressure (Buijsse et al., 2006). This study has been confirmed in a large population (Buijsse et al., 2010).

1.10. Influence of Polyphenols on Health

Polyphenols have shown to scavenge free oxygen radicals and reduce oxidative stress by generation and increasing activity of pro-oxidant and antioxidant enzymes in the cells (Wang et al., 2006). The well-known property of almost every group of flavonoids is their capacity to act as antioxidants. The antioxidant ability of flavonoids has been broadly studied in both biological tissues and cell-free medium (Woodman et al.,2005; Magnani et al., 2000; Rice-Evans et al., 1995; Salah et al., 1995).

Many flavonoids, including quercetin, have shown a reduction in ischemia-reperfusion injury by inhibiting with inducible nitric-oxide synthase activity (Middleton, 1998; Shoskes, 1998). Silibinin is a flavonoid and act as an antioxidant and has been testified to inhibit nitric oxide dose-dependently (Sanhueza et al., 1992). It has been observed that quercetin and myricetin present in red wine can also enhanced the glutathione/glutathione disulphide

(GSH/GSSH) level in rat's kidney tissues, showing an antioxidant activity in the cell after giving redwine to adult rats (Rodrigo et al., 2002).

Improvement of endothelial dysfunction has been reported in isolated rat aorta after treatment of quercetin and isorhamnetin and can reduce oxidative stress in the cell (Romero et al., 2009; Sanchez et al., 2007). Because of their best-described antioxidant ability, several studies have been taken to determine the benefical effects of flavonoids for the treatment of many pathological diseases such as atherosclerosis, dementia diabetes, cancer and others, these diseases are caused due to high level of oxidative stress in the cells (Nijveldt et al., 2001).

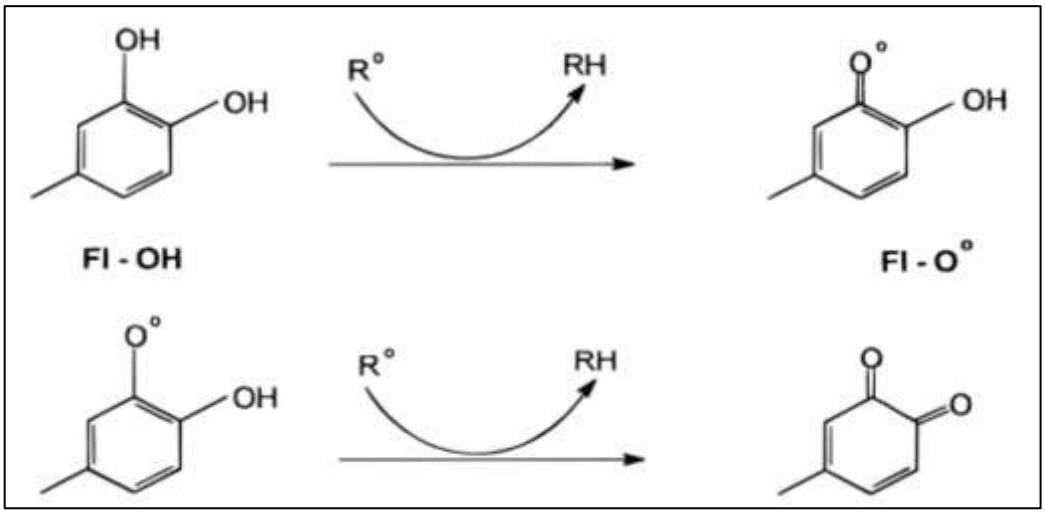

Figure: 7. Flavonoids (Fl-OH) scavenging the reactive oxygen species (R°): A stable quinone structure will form after Fl-OH gives an H atom to the radical (Procházková et al., 2011; Pietta, 2000).

Polyphenols are also active vasodilators. Many studies reported that flavonoids play an important role in the vasorelaxation and it is mostly facilitated through endothelium-independent systems. (Ajay et al., 2003; Chan et al., 2000; Herrera et al., 1996). The activity of phosphodiesterases is supressed by the flavanone naringenin and provoked vasorelaxation in rat endothelium-denuded aorta and caused hyperpolarisation (Qin et al., 2008; Orallo et al., 2005).

Various studies have reported that the flavonoids also possess anti-aggregatory and anti-inflammatory properties (Tomas-Barberan et al., 2007; Samman et al., 2001; Serafini et al., 2003). Flavonoids are capable to minimise the action of inflammatory signalling pathways, finally preventing the unnecessary release of nitric oxide (NO), leukocyte activation and the COX-2 expression (Zhang et al., 2011; Jakubowski et al 2009; Sio et al., 2006; Xia et al., 2016) Flavonoids play a key role in the prevention of NF-kB pathway activation by reducing the iNOS protein synthesis (Tsai et al.,1999; Yamamoto & Gaynor, 2001; Testai 2015)

Flavonoids especially flavones, block key enzymes associated pathways including lipoxygenase, cyclooxygenase and phospholipase A2 therefore help in decreasing the formation of inflammatory markers like leukotrienes and prostaglandins (Harris et al., 2006; Kimata et al., 2000; Lindahl & Tagesson, 1993; Baumann et al., 1980). Various studies have indicated that flavonoids can also inhibit the formation of pro-inflammatory cytokines such as IL-6 , TNF-α and IL-1β (Cho et al., 2003b; Gerritsen et al., 1995;).

Resveratrol has shown anti-thrombotic effects and anti-atherosclerotic effects in a NO-independent pathway by reducing platelet aggregation, activation, and adhesion, thus inhibiting thrombogenesis. Besides, resveratrol also acts as a potential inhibitor of both nuclear factor kappa B (NF-k B) dependent gene expression and NF-k B activation by preventing inhibitor kappa B (IkB) kinase (Holmes-McNary & Baldwin, 2000).

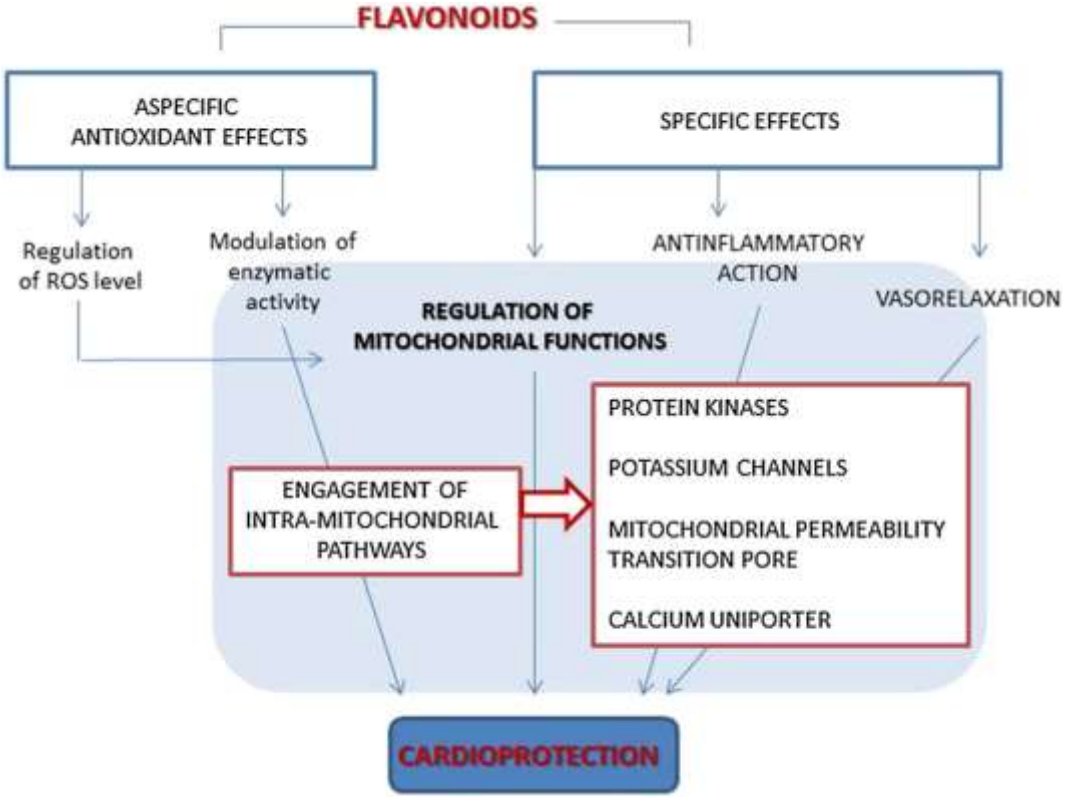

Figure: 8. Effects of flavonoids on the mechanism of cardioprotection: The chart represents flavonoids` antioxidant, anti-inflammatory and vasorelaxation effects on regulatory mitochondrial functions that benefits cardioprotection (Testai, 2015).

The antitumor activity of flavonoids is still under discussion and point of interest. Antioxidant systems are often inadequate, reactive oxygen species may cause carcinogenesis after any damage or injury (Loft& Poulsen 1996; Pryor, 1997). Reactive oxygen species can cause mutation by damaging of DNA and unrepaired or misrepaired division of cells. If these changes occur in critical genes, such as in tumor suppressor genes or oncogenes then initiation or progression of cells may result (Stefani et al., 1999; Fotsis et al., 1997).

Grapes contain a diversity of antioxidants, including catechin, epicatechin, resveratrol, quercetin and proanthocyanidins, resveratrol found mainly in the skin of grapes and proanthocyanidins in seeds (Vinson et al., 2001). Some flavonoids such as apigenin, fisetin, and luteolin have stated as potential inhibitors of cell proliferation (Stefani et al., 1999).

1.10.1. Mechanisms of Free Radical Induced Injury Prevention by Flavonoids

Injury that is caused by free radicals can be prevented through many mechanisms such as direct scavenging of reactive oxygen species (ROS) by hydrogen atom donation, interaction with other antioxidant enzymes, metal chelating activity, reduction of α-tocopheryl radicals, inhibition of oxidases, mitigation of oxidative stress caused by nitric oxide, by increasing, plasma urate levels and antioxidant properties of other low molecular antioxidants (Procházková et al., 2011).

1.10.2. Flavonoids as Pro-Oxidant

Pro-oxidant activities of a flavonoid rely on the number of hydroxyl substitutions in its backbone structure. That means the more hydroxyl substitutions, the stronger the pro-oxidant activities of the flavonoids (Cao et al., 1997).

1.10.3. Antioxidant Property

Many researchers have shown that polyphenols possess many biological properties and have an important role in health preservation (Awe et al., 2013; Cooper et al., 2008; Han et al., 2007). The important influence of flavonoids is the scavenging of oxygen-derived free radicals. The antioxidant property of cocoa and chocolate is associated with their catechin and procyanidin contents (Wan et al., 2001).

The catechins and flavones seem to be the most potent flavonoids for defending the body against reactive oxygen species. In acute situations of oxidative stress, it has been reported that quercetin and epigallocatechin gallate were the strong antioxidants amongst other flavonoids (Qiu et al., 2012). They have shown a correlation with cell-death survival signaling pathway which may

vary with a dose that inhibits or promote apoptosis, cytoprotective effects or exhibiting chemo-preventive respectively (Ramos 2007; Mandel et al., 2004).

1.10.4. Vasodilation and Anti-Inflammatory Properties

A consistent and striking peripheral vasodilation have been observed in healthy people due to the short-and long-term intake of cocoa and dark chocolate (Bayard et al., 2007; Fisher et al., 2006). Polyphenols, especially flavanols in cocoa-related products, have been shown a high level of endothelial nitric oxide, which contributes in vasodilation and thus may lower blood pressure (Fisher and Hollenberg, 2006; Fisher et al., 2003; Karim et al., 2000).

Cocoa and chocolate products contributes high level of flavonoids among commonly consumed foods and also was historically used as a medicine to cure of inflammation, pain and numerous other diseases (Tomas-Barberan et al., 2001)Various evidence indicate that they occur in the form of monomeric flava-3-ol or procyanidins in cocoa, regular intake of cocoa drinks rich in flavanols can strengthen the photoprotective properties of skin, lowers the risk of cardiovascular diseases, potent antioxidant, anti-inflammatory and anti-allergic properties (Schneider et al., 2001; Reddy et al., 2006; Samman et al., 2001).

Cocoa increase NO-dependent vasodilatation in patients with cardiovascular risk factors, diabetes and as well as in coronary arteries in healthy adults (Balzer et al., 2008; Flammer et al., 2007; Grassi et al., 2005a; Heiss et al., 2003; Fisher et al., 2003). A study of 18-65 years of women has shown high resistance against UV-induced erythema and as well as significant increase in blood flow in cutaneous and subcutaneous tissue after 12week continually intake of 329 mg cocoa drink (Neukam el al., 2007).

This study is associated with other human trails which show high flow-mediated dilation (FMD) of conduit arteries and enhanced microcirculation after consumption of flavanol-rich cocoa (Schroeter et al., 2006; Heiss et al., 2003).

Another study conducted on smokers also demonstrated enhanced flow-mediated vasodilation after ingestion of cocoa-rich diet (Heiss et al., 2005).

Cocoa flavanols show promising effects in increasing of cerebral blood flow to grey matter up to 60% after a single dose of 450mg of cocoa by 2-3 h post-consumption (Francis et al., 2006). Researchers from the University Hospital of Cologne collected data from five studies related to the influence of cocoa on blood pressure consist of 173 participants, that shows the consumption of cocoa rich food has a significant decrease in blood pressure (Taubert et al., 2007).

1.10.5. Effects of Cocoa on Nervous System

The nervous system is one of the body systems, comprises of central and peripheral nerves. Many studies have shown neuroprotective effects of polyphenols containing foods like cocoa and tea against neuronal cell death (Jain, 2011; Ramiro-Puig E et al., 2011). In vitro oxidative stress study has also shown a neuroprotective effect of cocoa extract and (-)-epicatechin by reducing ROS production and modulating MAPK activation (Ramiro-Puig E et al., 2011). Human Alzheimer`s disease in vitro study demonstrated neuroprotection effect of cocoa polyphenolic extract through brain-derived neurotrophic factor (BDNF) survival pathway that is related with canonical nerve growth factor (Cimini et al., 2013).

Clinical and epidemiological data have also flavonoids and polyphenols against neurodegenerative disease in animal models (Cimini et al., 2013). The cocoa extract flavonoids interaction with protein and lipid kinases signaling cascades inhibit neuronal death via neurotoxicants such as oxygen radicals and promote neuronal survival and synaptic plasticity. Thus, it has not only neuroprotective effects but also impacts on cognitive performance (Nehlig et al., 2013).

1.10.6. Effects of Cocoa on Cancer

Cancer is one of the main causes of death and disability all over the world and it is commonly divided into three stages: initiation, promotion, and progression within the cells (Pritchard & Grady 2011). The cell cycle is controlled by many regulatory proteins. Any alteration occurs in these specific cell cycle regulatory protein or any mutation can result due to unrepaired or misrepaired in DNA or during cell division because of many external or internal environmental factor may lead to cancer many other carcinogenic diseases (Pan et al.,2011) It has been indicated by various epidemiological and experimental studies that dietary polyphenols such as, diet that is rich with fruits and vegetables play as important role as chemopreventive agents, in the reduction of many cancer-related diseases in humans (Mahmoud et al.,2000; Ramos, 2008; Surh, 2003). It has been observed that some flavonoids like fisetin, apigenin, and luteolin can help in the inhibition of cell proliferation (flav.5) Dietary polyphenols showed a chemopreventive effects in colorectal cancer ((Pan et al.,2011; Ramos, 2008).

1.10.7. Effect of Cocoa on Cholesterol Levels

Various evidence indicated that oxidation of Low-density lipoprotein (LDL) is a very serious factor in the development of atherosclerosis (Steinberg et al., 1989). Fatty streaks will appear by the Uptake of oxidized LDL by macrophages and smooth muscle cells and it's an early stage of atherosclerosis. Due to these vascular changes accumulate an excess amount of lipids like cholesterol ester can cause a release of many growth factors by smooth muscle cells and fibroblasts (Kume et al., 1994; Kume et al., 1992). Chocolate is a major source of catechins and epicatechins revealed by a Dutch study, especially in the young population (Arts et al., 1999). In previous studies showed that intake of cocoa extract from its powder can increase the resistance against the oxidation of LDL and helps in the inhibition of atherosclerosis in hypercholesterolemic rabbits (Kurosawa et al., 2005). Intake of dairy cocoa powder can enhance the resistance of LDL to oxidation, a study carried out on healthy human adults (Osakabe et al.,

2001 & Baba et al., 2004). Daily consumption of cocoa powder can decrease oxidation of LDL and increase the HDL-cholesterol levels in plasma in humans (Baba et al., 2007).

1.10.8. Anti-Diabetic Effect of Cocoa

Recent studies are indicating the anti-diabetic effect of the cocoa extract. Flavonoids that is found in cocoa has an anti-diabetic effect through different mechanisms either by enhancing insulin secretion, improving insulin sensitivity in peripheral tissues such as myocardium, exerting a lipid-lowering effect and preventing the oxidative and inflammatory damages that are shown in our study that can be associated to diabetes (Ramos et al., 2017).

1.10.9. Effects of Cocoa on Blood Pressure

Cocoa plays a role in the vascular system and on cerebral blood flow (Nehlig et al., 2013) that regulate blood pressure. In a study of 856 healthy subjects, a significant reduction of blood pressure in 2-18 weeks due to flavonol-rich cocoa products are reported (Ried et al., 2012). According to University Hospital Düsseldorf, 2015 report, the blood pressure lowering potential of flavonols that increase flow-mediated vasodilation may be able to reduce the age-related risk of developing cardiovascular diseases (Sansone et al., 2015). Contradicting data are also reported by different studies showing flavonoids pro-oxidant toxic properties (Procházková et al., 2011).

1.11. Cardiovascular Diseases

Cardiovascular disease (CVD) is one of the important source of death both in developing and under developing countries. In 2012, The World Health Organisation (WHO) predicted that, seventeen and half million people died from CVDs, which accounting roughly about thirty one percent of all worldwide deaths. Among these 7.4 million deaths cases were results of ischemic heart

disease (IHD), which cause 46% of deaths in men and 38% in females (Wong, 2014) and it is estimated by WHO its percentage become double between 1985 and 2015 (Reddy KS & Yusuf, 1998).

Coronary heart disease is due to development of atherosclerosis, which is considered by the increase of cholesterols, white blood cells and fatty acids which leads to obstruction of vessels due to limited supply of blood to the heart (Bolli et al., 1998). Major problems of coronary heart disease contain ischemia heart disease (IHD) and heart failure. There are many risk factors for the occurrence of cardiovascular diseases. A recent study that is done in the United States has shown in a data of body mass index and waist circumference, the flavonoid intake is related to reducing cardiovascular disease risk in the adult population (Sebastian et al., 2017).

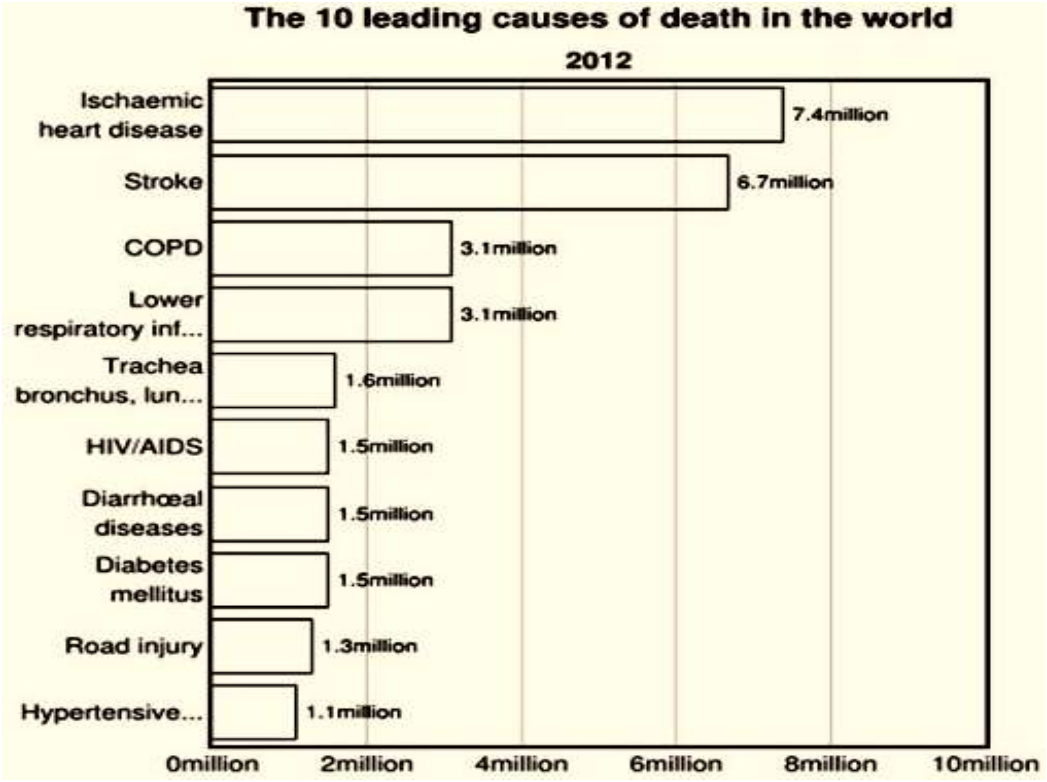

Figure: 9. Leading Causes of death Worldwide: Ischemic heart disease represents number one cause of deaths, 2000-2012. Data adopted from World Health Organization, 2014.

1.12. Oxidative Stress in Heart

Endothelial dysfunction is associated with premature atherothrombotic disease and it is a pathophysiological condition, the most important type of endothelial dysfunction is increased in oxidative stress and decreased in NO bioavailability in the cells (Oemar et al., 1998; Munzel et al., 2010). Congestive heart failure (CHF) is the most common medical condition, representing the last stage of many cardiovascular disorders and is related to high mortality and morbidity (Swedberg et al., 2005). Much of evidence has assessed that patients with CHF typically display endothelial dysfunction and increased oxidative stress (Katz et al., 2005).

Furthermore, patients with reduced Flow Mediated Dilatation (FMD) are at high risk for cardiovascular disorders and death (Katz et al., 2005); increased in platelet activation results in many atherosclerosis-related problems. Most of the complications of Heart Failure (HF) are thrombus-related and CHF showed improve platelet activation (Gibbs et al., 2001). Congestive heart failure is the last stage of many cardiac disorders and at the time it is impossible to reverse many cardiovascular alterations. For example, a decrease of low-density lipoproteins by 3- hydroxy1-3-methyl-glutaryl (HMG) coenzyme reductase inhibitors, no more minimize the incident rates in ischemic cardiomyopathy patients (Tavazzi et al., 2008; Kjekshus et al., 2007). Therefore, alternative measurements should be taken to improve the condition of these high-risk patients.

1.13. Ischemic Heart Disease

Ischemic heart disease is considered as a common cause of death and disability worldwide (World Health Organization, 2008). IHD, which occurs as result of limited blood supply to cardiac myocytes due to partial or total blockage of coronary arteries, it may occur in any part of the body such as in any organ, muscle and tissue (VDF, 2012) from atherosclerosis due to many risk factors such as high blood pressure, smoking, high cholesterol, diabetes, advanced age, a

family history of cardiovascular disease, obesity and sedentary lifestyle (VDF, 2012).

In 1960, Jennings and his co-workers were the first researchers those proposed the Myocardial reperfusion injury, they detected unreasonable changes after ischemia/reperfusion (I/R) in the canine heart Morphologically, these changes were cell swelling calcification in the mitochondria and contracture of myofibrils in the canine heart (Jennings et al., 1960).

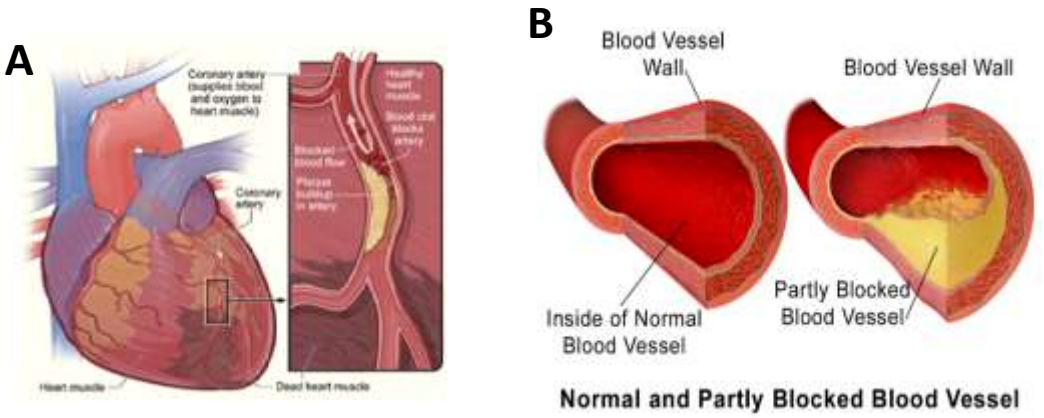

Figure: 10A. Representing an overview of heart and artery that shows coronary artery disease (ischemia heart area) **Fig: 10 B** shows the cross-section of normal coronary artery with a normal blood flow and partially blocked with plaque (Blausen, 2014).

The ischemic region suffers very fast and anoxic cell death in minutes of ischemia formation. Irreversible procedures cause rapid energy depletion, mitochondrial malfunction, ion pump collapse that results from more than calcium accumulation, extracellular potassium, cell swelling which are symptoms of necrotic cell death.

During ischemia, hypoxanthine forms by cellular ATP degradation. Normally, oxidation of hypoxanthine occurs to xanthine through xanthine dehydrogenase. Therefore, during ischemia, xanthine dehydrogenase transforms into xanthine oxidase. Despite, xanthine dehydrogenase uses nicotinamide

adenine dinucleotide as xanthine dehydrogenase but in ischemia, xanthine oxidase uses oxygen as its substrate, and hence, during ischemia, is not possible to catalyze the conversion of hypoxanthine to xanthine, resulting in a production of the excess amount of hypoxanthine in tissues. When oxygen is restored during reperfusion, the formation of toxic ROS occurs due to a conversion of hypoxanthine by xanthine oxidase.

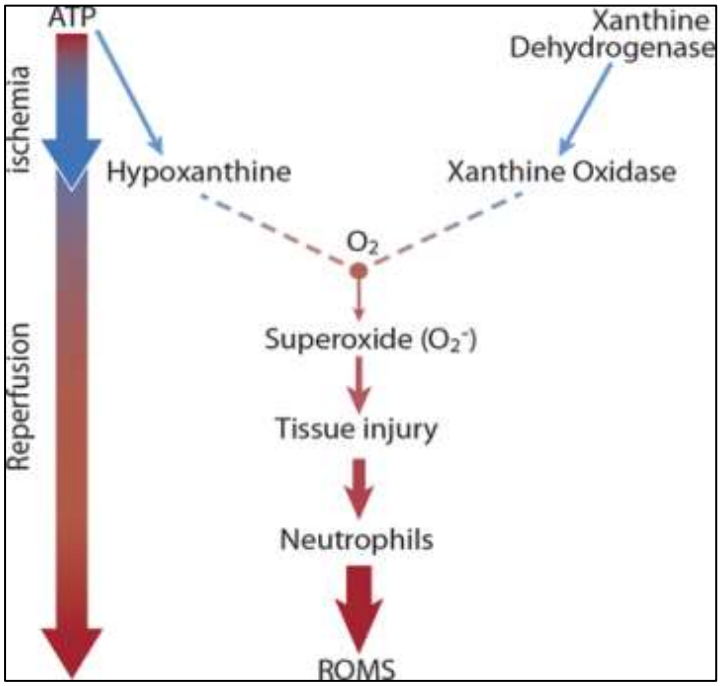

Figure: 11. The figure represents the mechanism of ischemic-reperfusion injury and production of reactive oxygen metabolites (ROMS): Adopted from (Gonzalez et al., 2015.

It promotes various serious pathophysiological conditions like stroke, myocardial infarction, hypovolemic shock, and peripheral vascular insufficiency (Collard and Gelman, 2001). In response to ischemia-reperfusion-induced stress, cellular processes become rapidly activated. The ischemic region appears immediately in the tissue near to a blocked artery which undergoes quick, anoxic cell death within minutes of ischemia (Collard and Gelman, 2001).

Ischemic heart disease (IHD) is the major precursor of myocardial infarction (MI). Now common therapies have been developed as primary coronary

angioplasty and thrombolysis, to reduce infarct size of heart, restore blood supply to heart and decrease mortality (Verma et al., 2002&Chan et al., 2011) Various studies indicate that, MI as the most lethal symptom of cardiovascular diseases and point of critical investigation of many clinicians and basic medical scientist (Timmers et al., 2012). Reperfusion of the ischemic myocardium is the possible source for prevention of tissue damage in patients with IHD (Karunakaran et al., 2006). Therefore, reperfusion, itself responsible for several mechanisms including leukocyte infiltration, endothelial dysfunction and accumulation of reactive oxygen species (ROS) in cardiomyocytes that leads to apoptosis (Park & Lucchesi, 1905; Frangogiannis et al; 2002; Collard &Gelman, 2001)

The ischemic region can be diagnosed by high values of biochemical markers of myocardial necrosis, electrocardiographic (ECG) findings, and inflammation by images and can be analyzed by pathology. MI may be the primary symptom of coronary artery disease (CAD) or it may appear, frequently, in patients with the determined cardiac disease. It has been investigated that reperfusion injury causes 50% of irreversible myocardial infarct size (Yellon & Hausenloy, 2007) indicating that possible pharmacological treatments should be required at the time of reperfusion to prevent or reduce myocardial ischemia.

1.14. Inflammatory Response during I/R injury

Even though acute inflammation is a pathophysiological healing response triggered during myocardial I/R, accumulation of various evidence shows that the inflammatory response that is stimulated while ischemia, and critically increased during reperfusion, could itself cause apoptosis of tissues leading to myocardial disorders (Hansen, 1995).

Various Inflammatory reactions become activated after I/R injury due to the release of cytokines and leukocyte infiltration into the endangered myocardial region (Entman et al., 1994). Neutrophils, monocytes, and lymphocytes are the main immune cells that are mainly involved in this process; therefore,

inflammatory cells produce proteolytic enzymes that cause the development of injury (D'Amico et al., 2000; Frangogiannis et al., 2007; Hansen, 1997). Chemokines are small chemotactic cytokines that elevate the level of leukocyte recruitment to inflammatory sites (Charo & Ransohoff, 2006) However, many clinical and pre-clinical studies have shown the contribution of various inflammatory markers such as CCL5 and IL-6 in acute cardiac injury (Reichel et al., 2006; Empana et al., 2010; Fuchs et al., 2003; Manten et al., 1998).

Endothelium dysfunction may also result due the interface of neutrophils with the endothelium and this process occurs by the soluble adhesion molecules like E-selectin, P-selectin, intracellular adhesion molecules-1, vascular cell adhesion molecule-1 and many more (Entman & Smith, 1994). Clinical studies also indicated that production of high level of white blood cells are directly related with the increase the number of mortality rate in patients with acute mycardical infarction, demonstrating a close link between a poor prognosis post-myocardial infarction and systemic inflammation(Grzybowski et al., 2004).

1.15. Signalling Cascades Associated with I/R Injury

Various signalling cascades could activate during myocardial I/R injury due to increase of oxidative stress and Ca2+ overload that's leads to cell apoptosis or survival. Many strategies have been developing to cure myocardial I/R injury to regulate the activation or inhibition of pro-survival and pro-injurious signalling transduction cascades in the heart tissue to minimise damaging of cardiomyocyte during ischemia reperfusion.

1.15.1. PI3K/Akt Pathway

Phosphoinositide-3-kinase–protein kinase B/Akt (PI3K-PKB/Akt) pathway became a point of investigation and identified in the early 1980s during characterizing the insulin growth receptor signaling pathway (Alessio et al., 2001; Hemmings and Restuccia et al., 2012). Many signaling cascades are participating

in cardioprotection. PI3K/Akt signaling transduction cascade is well known for one of the major signaling pathway that is directly associated with cell growth or cell survival. Phosphorylation of Akt can inhibit cardiomyocytes apoptosis at the time of I/R injury (Xia et al., 2016) Previous studies also observed the cardioprotective effects of this pathway during I/R injury (Karthikeyan et al., 2007; Ahmed et al., 2017).

Akt is a serine/threonine kinase signaling protein and belongs to a group of protein kinases (protein kinase A/protein kinase G/protein kinase C-like) those contain Threonine308 and Serine473 phosphorylation sites in the regulatory and kinase domain (Vivanco and Sawyers, 2002). Once Akt becomes activated, transfer from plasma membrane to cytoplasm and enter the nucleus, where it has many substrates to attach. Phosphorylation by Akt works according to the target protein, it can be stimulatory or inhibitory, either enhancing or suppressing the effect of proteins (Guertin et al. 2006; Hemmings and Restuccia et al., 2012). When PK13/Akt is fully activated it mediates various cellular mechanisms such as cell growth, proliferation, cell survival, translation, transcription and cell death (Vivanco and Sawyers, 2002). Over stimulation or activation of P13K/Akt signaling pathway leads to abnormal cell growth or proliferation cause cancer or other infectious diseases (Calvo et al., 2009). Akt 1 regulation enhanced physiological cardiac hypertrophy (growth of cardiac cells), while another study in mice, it showed an opposite signaling pathological hypertrophy (DeBosch et al., 2006; Walsh, 2006).

1.15.2. ERK1/2 Signaling Cascade

In cellular biology, ERK signalling pathway is widely studied among other signaling pathways. There are two isomers of Erk: Erk 1 and Erk 2, and they are almost 83% similar with each other, showed many common effects during signalling pathways, normally it is known as Erk 1/2 (Rose *et al.*, 2010). This is also known as p42/p44 MAPK pathway and it contains a chain of proteins that transfer a signal from receptor to DNA and form proteins that undergo changes in

cells. Upon Stimulation, Ras protein becomes activated then it activates c-Raf/MAPKKK, which then phosphorylates the Erk1/2 (Rose *et al.*, 2010). When it becomes activated it phosphorylates many downstream substrates including MAPK-activated protein kinase-1, 90 kDa ribosomal S6 kinases (Frodin & Gammeltoft, 1999) and also transcription factor Elk-1 (Davis, 1993). They will mediate several regulatory molecules that cause cell differentiation, proliferation, and cell survival (Frodin & Gammeltoft, 1999) and various human diseases may occur due to abnormal regulation of MAPK cascades (Roberts and Der, 2007).

It has been observed in many studies that activation of Erk ½ signaling pathway showed very strong cardioprotective effects during I/R injury. It showed a great importance in pharmacological studies, many pharmacological drugs have been given during I/R injury to activate the Erk ½ pathway to exerts their cardioprotective effects, there was a reduction of the myocardial infract size in rat hearts due to the phosphorylation of Erk ½ (Ikeda *et al.*, 2006). Erk ½ signaling pathway showed a positive effect in reducing myocardial infarct size and improving post-ischemic cardiac contractility in rat isolated hearts during to I/R (Yao *et al.*, 2010). Various studies shows that activation of Erk1/2 causes phosphorylation and inhibition of cell pro-apoptotic (BAD (B-cell lymphoma-2-associated death promoter), caspase-3 andcaspase-9) and cell survival proteins such as Bax (B-cell lymphoma-2-associated X protein) in the cells (Hausenloy & Yellon, 2007).

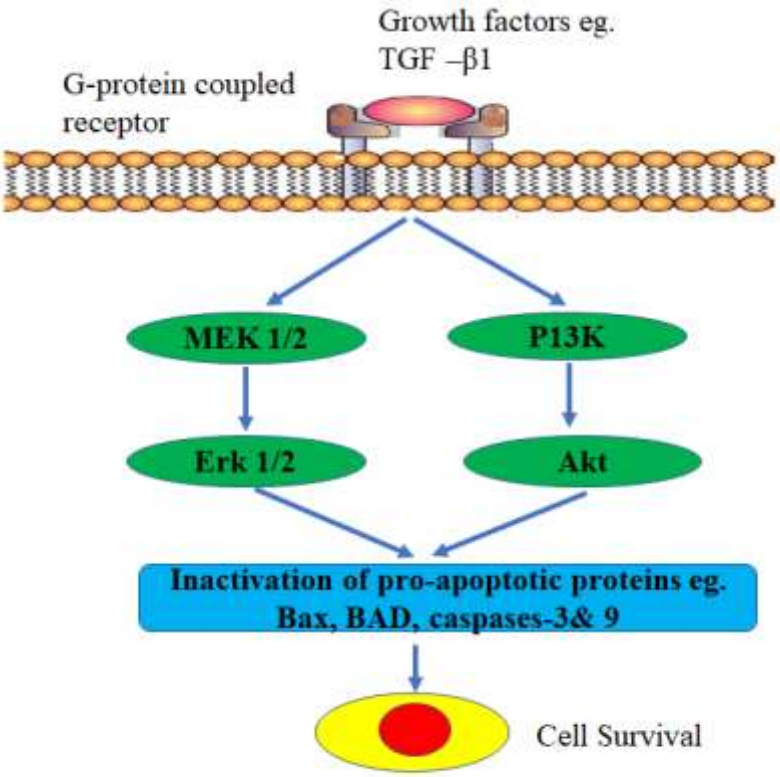

Figure: 12. Represents Schematic diagram of Cell survival or Reperfusion Injury RISK Pathway: During ischemia/reperfusion, due to stimulation of Growth factors e.g TGFβ 1 and G-protein coupled receptor, two cardioprotective signalling pathways Erk 1/2 phosphatidylinositol 3′-kinase (PI3K)/protein kinase B (Akt) pathways become activated. They are extracellular signal-regulated kinases and congregate at the mitochondria, where phosphorylation and inactivation of pro-apoptotic proteins Bax (Bcl-2-associated X protein, BAD (Bcl-2-associated death promoter), Caspases-3 & 9 occurs and that result in cell survival (Hausenloy & Yellon, 2004). MEK= mitogen-activated protein kinase kinase; TGF= transforming growth factor**.**

1.16. Aims of the Study

1.16.1. General Objective

The aim of this study was to investigate the effects of cocoa extract in ischemia-reperfusion-induced myocardial tissues in Sprague-Dawley rats.

1.16.2. Specific Objectives

- To compare the flavonoid amount in both Cocoa extract and Recioto Red Wine
- To determine optimal cocoa extract dose in Sprague-Dawley rats
- To translate cocoa extract optimal dose from rats to human
- To measure oxidative stress in myocardial tissue of Cocoa treated rats following ischemia and reperfusion
- To observe histopathological changes in myocardial tissue sections using hematoxylin and eosin staining
- To analyze the sensitivity of inflammatory markers on myocardial tissue in Cocoa extract treated rats
- To demonstrate the apoptotic levels of myocardial nuclei in Cocoa treated rats
- To analyze phosphorylation of ERK1/2 and Akt signaling proteins in myocardial tissue of Cocoa treated rats

CHAPTER TWO

2.1. MATERIALS AND METHODS

2.2. Design of the study

This study was experimentally designed to investigate cardioprotective effects of cocoa on the heart of rats. In this study, it was first designed to compare amounts of flavonoids in cocoa and Recioto red wine and then, oral administration of cocoa for 15days to determine the optimal dose at which saturation dose begins from the sera of the blood. Finally, it was planned to administer optimal dose orally for 15 days to study biomolecular analysis after 30min of ischemia and 24 hrs of reperfusion (Fig. 1.) after observation of heart and blood samples.

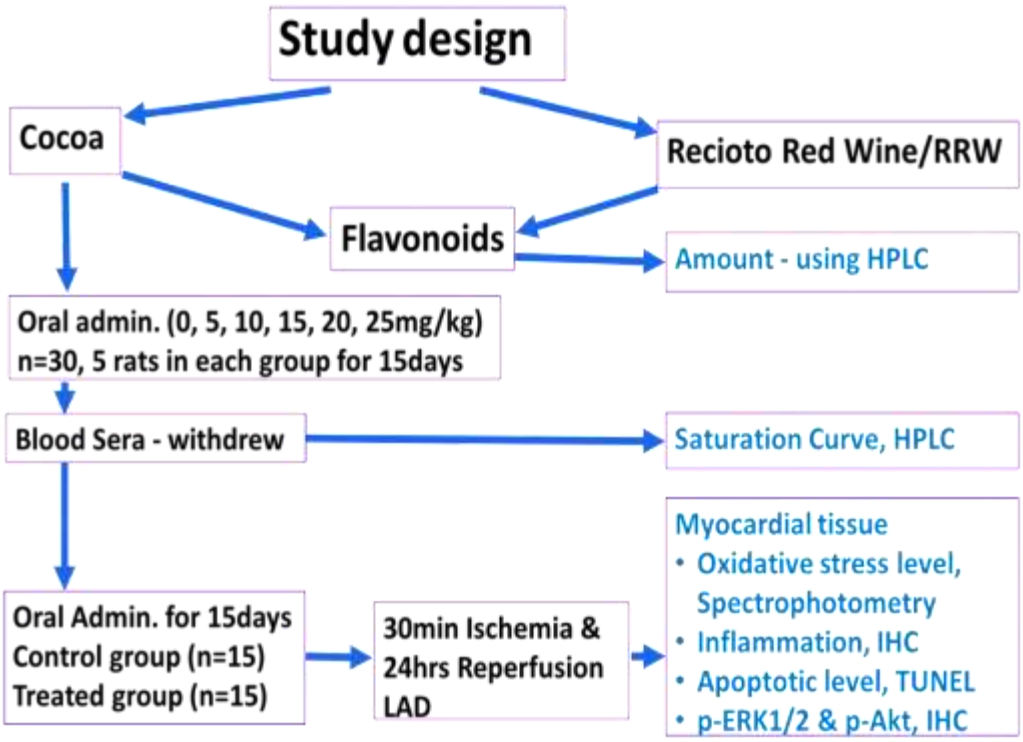

Figure: 13. Experimental study design: The sketch showed the experimental design of the study using a total of 60 Sprague-Dawley rats divided into 30 rats for flavonoids amount identification and 30 rats for effects of cocoa extracts on the myocardial tissue after induction of 30min ischemia and 24hrs reperfusion.

2.3. Chemicals & Materials Used

Cocoavia® and Recioto red wine (four-year-old) were purchased from Trademarks Mars Inc, USA and by Masi Agricola, Verona Italy. Reduced glutathione (GSH), glutathione reductase, gamma-glutamyl p-nitroanilide, glycylglycine, bovine serum albumin (BSA), 1,2-dithio-bis nitro benzoic acid (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), reduced nicotinamide adenine dinucleotide phosphate (NADPH), CCl4, flavine adenine dinucleotide (FAD), glucose-6-phosphate, 2,6-dichlorophenolindophenol, thiobarbituric acid (TBA), picric acid, sodium tungstate, sodium hydroxide, trichloroacetic acid (TCA) were purchased from Sigma. Primary antibodies CCL5/RANTES, ICAM1, Nitro-tyrosine, IL-6, NFkB2, P-Erk, and P-Akt antibody were obtained from BIOSS antibodies, Novusbio and cell signaling technology. In Situ Cell Death Detection Kit, AP was from Roche.

Figure: 14. The figure represents Cocoavia® extract and Recioto red wine used for the study.

2.4. Animals

Eighteen healthy Sprague-Dawley (SD) male rats weighting 300-350g were employed and kept in a setup cage system in a 21±2^{o}C room. They fed with food (ad libitum) and water. They were adapted to the laboratory conditions for a week prior to the experiment. First rats were used for dose-response experiment and thirty SD male rats used for the biochemical and immunohistochemistry analysis after animal treatment and ischemia-reperfusion. All animal experiments

were done per ethical guidelines reviewed and approved by University of Verona Ethical Committee and the Italian Ministry of Health (341/2016-PR) at C.I.R.S.A.L. (Interdepartmental Research Centre for Laboratory Animals) of the Biological Institutes, University of Verona, and Verona, Italy.

2.5. Extraction of Recioto Red wine and Cocoavia®

Extraction of total Phenolic compounds from the four-year-old age Recioto red wine (provided by MASI AGRICOLA, Verona Italy) was carried out by diluted with 100% LC-MS methanol, prior to that filtered the wine through Minisart RC4 0.2µm pore filter. In case of cocoa pills-powder (provided by Trademarks Mars, Inc) was weighted and diluted into three times of ice-cold methanol and vortex. Sonification was carried out for 15 min at 40 kHz in ultrasonic bath (Falc Instruments, Bergamo, Italy) at room temperature, centrifuged for 10 min at 16,000 g at 4°C and filtered through 0.2 µm pore filters following protocol (Dal Santo et al., 2016; Calderón et al., 2009). For the LC-MS chromatograph analysis, made serial dilutions with methanol both for wine sample (1:2, 1:3, 1:10, 1:20, and 1:25) and cocoa sample (1:2,1:10, 1:20,1:50,1:100 and 1:200). Before the injection the samples were diluted 1:2 with LC-MS water (Fluka) and injected volume was 20µl.

The chromatographic analyses were carried out by using a Beckman CoulterGold 127 HPLC system (Beckman Coulter, Fulletron, CA) equipped with a C18 guard column (7.5 x 2.1 mm) and an analytical Alltima HP C18 column (150 x 2.1 mm, particle size 3 µm) (Alltech Associates Inc, Derfield, IL). Two eluents were used: 0.5% (v/v) formic acid, 5% (v/v) acetonitrile in water (buffer A), and 100% acetonitrile (buffer B) using gradient program from (-); flow rate 200 µl min^{-1}

Each sample was analyzed in two technical and three biological replicates, with 20 µl injection volumes and 31 min equilibration between samples. The HPLC system was coupled on-line with a Bruker ion trap mass spectrometer

Esquire 6000, equipped with either electrospray ionization (ESI). MS data were collected using the Bruker Daltonics Esquire 5.2- Esquire Control 5.2 software, and processing using the Bruker Daltonics Esquire 5.2-Data Analysis 3.2 software (Bruker Daltonics GmbH, Bremen, and Germany). Negative and positive ion mass spectra and MS/MS and MS3 spectra were recorded at target mass of 1000 m/z; range of 50-3000 m/z with the fragmentation amplitude set at 1 V. In addition, the parameters were nebulizing gas was N2:50 psi at 350°C and dry gas for both of ion sources10 l min^{-1} for ESI; ESI: capillary source were: 4000 V; end plate offset-500 V; skimmer: -40 V; cap exit -121 V; Oct 1 DC: -12 V; Oct 2 DC: -1.70 V: lens 1: 5 V; lens 2: 60 V.

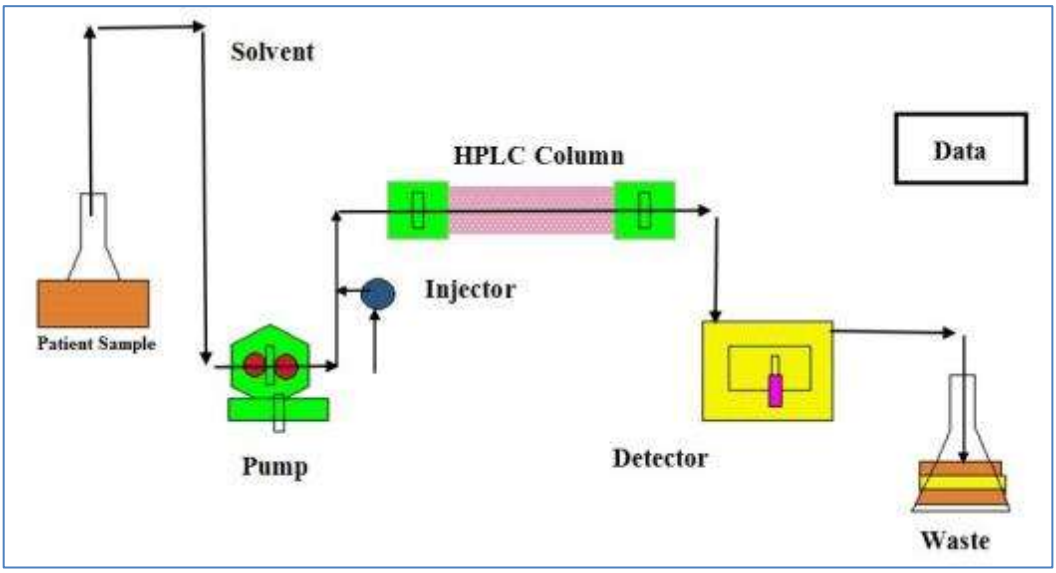

Figure: 15. Represents High-Performance Liquid Chromatography (HPLC) Basic Instrumentation: It contains, Mobile phase (solvent), pump, injector, HPLC column (stationary phase), Detector and data analysis. (Source: Thyrocare.com).

2.6. Identification of Polyphenols

Metabolites were identified by comparing their retention times, m/z values and MS fragmentation patterns with those of commercial standards in our in-house library. UV/vis spectra recorded by HPLC-DAD were also used to support the LC-MS identification as described in (Calderón et al., 2009). Fragmentation

patterns collected in online databases such as MassBank (www.massbank.jp) or reported in the literature were also considered, especially when no authentic standard compounds were available. Neutral losses of 132, 146 and 162 Da were considered diagnostic of the loss of pentose, deoxyhexose and hexose sugar, respectively.

2.7. Quantification of Polyphenols

The quantification of the Cocoavia® and RDW metabolites in the methanolic extracts was carried through HPLC-DAD (Beckman Coulter Gold 126 Solvent Module coupled to a Gold 168 Diode Array Detector), relying on calibration curves of authentic standard compounds.

2.8. Animal Treatment in Dose-dependent Manner

Eighteen SD male rats were divided into six groups of three rats each. Among them, group 1 served as control fed with normal rat diet, while the animals of other groups were administrated orally with 5,10,15,20 and 25mg/kg body weight of Cocoavia® extract dissolved in water, five times per week for 15 days respectively. All animals were kept at a temperature of 22–24°C and fed with a regular pellet diet ad libitum.

2.8.1. Blood Collection

One ml blood was collected, 1 hour after oral administration of Cocoa extract from each rat, when showed a higher concentration of flavonoids in plasma between 30-60 min as described in (Baba et al., 2000) through lateral tail vein following (Lee et al.,2005) on the 1^{st} day, 7^{th} day and 15^{th} day of administration. Blood was deposited in clean heparinized glass tubes, centrifuged (3500 rpm for 15 min) stored at -80°C for the HPLC-ESI-MS analysis.

2.8.2. Preparation and HPLC-ESI-MS Analysis of Sera Samples

The following protocol of rat sera preparation for HPLC-ESI-MS analysis was optimized from the deproteination method proposed by (Polson et al., 2003) by introducing an initial delipidation step.

For each sample, 200 µL of sera were added to an isovolume of chloroform and, after vigorous mixing, the samples were centrifuged (16000 rcf, 15, 4°C). Chloroform phases including the lipid fraction were discarded, whereas the upper phases were recovered and added to three volumes of cold ethanol to precipitate the protein fraction. After vigorous mixing, the samples were let for 10 min at room temperature and then centrifuged as above.

Supernatants were passed through Minisart 0.2-µm filters and 20 µL was injected in a column for the HPLC-ESI-MS analysis. The latter was performed as described in the previous paragraph using the same chromatographic method and keeping the MS parameters unvaried. The absence of ion suppression due to possible matrix effect in HPLC-ESI-MS analysis was evaluated by spiking 0.01 µg of (+)-catechin in one sera sample. The presence of characteristic metabolites detected in Cocoavia® extracts and of those potentially derived from their catabolism in mice was investigated in chromatograms recorded in negative ionization.

2.9. Animal Groups and Ischemia-reperfusion Surgery

Ten SD male rats were divided into two groups control and treated five in each group. The selected dose 15mg/kg of cocoa from our previous results was given to a treated group (n=5) once in a day for 15 days. Control group remained untreated. At the end of the experiment, after 12 hours, 30 min of ischemia was given to left anterior descending artery (LAD) of heart and 24hrs of perfusion was given to both groups.

2.10. Surgical Procedure for Ischemia and Reperfusion

Thirty rats were divided into two groups (control and treated). The selected dose 15mg/kg of cocoa extract from the dose-response study was given to a treated group (n=15) once a day for 15 days. Control group (n=15) remained untreated. Subsequently, both groups underwent 30 minutes of myocardial ischemia by ligation of the left anterior descending (LAD) coronary artery followed by 24 hours of reperfusion. The surgical procedure was produced as previously described (Mohanty etl., 2004). Briefly, the rats were anesthetized with an intramuscular injection of ketamine (70 mg/kg) and xylazine (5 mg/kg), and after a left thoracotomy, the heart was exteriorized by lateral compression of the thorax and connected to the descending artery approximately 2 mm from the origin with wired polyvinyl (5-0 Ethicon) between the border of the left atrium and pulmonary artery. The heart was replaced in the thorax, the lungs were expanded with positive pressure, and the pneumothorax was aspirated. All surgical procedure was done with the help of surgeons at department of Surgery, University of Verona using the protocol indicated by Kolk et al., (2009) and Samsamshariat et al., (2005)

Figure: 16. Surgical procedures of ischemia and reperfusion using LAD: A, The figure represents the surgical procedure of ischemia-reperfusion injury performing ligation of the left anterior descending coronary artery (LAD) indicating by the yellow arrow.

2.10.1. Tissue Collection

After 24hrs, hearts were excised, separated the ischemic part (left ventricle) and divided into two portions:

1. One portion of LV (left ventricle) tissue stored in 4% Formalin for immunohistochemistry and tunnel analysis.

2. The second portion of the tissue LV was treated with liquid Nitrogen and stored at -80 ºC for measurement of oxidative stress.

2.11. Tissue Processing, Embedding, and Sectioning

Heart tissues (left ventricle) were quickly washed three times for 1 or 2 min each with phosphate buffer saline (PBS) (pH 7.35, 0.1M) to remove all the blood contents from the tissues after collection, then quickly and sufficiently fixed with 4% paraformaldehyde and stored at 4 C for long time or at room temperature for 24 hours.

For performing immunohistochemistry and TUNEL assays, washed samples three times for 10 min each with phosphate buffer saline (PBS) (pH 7.35, 0.1M) and then dehydrated with three grades of ethanol concentrations (70%, 90%, 100%) for one hour, one and half hour and one hour each, respectively. Delocalization of samples was done by two changes of xylene (100%) for one hour. To remove xylene, samples were infiltrated with two changes of molten paraffin wax at 56^{o}c for one hour.

After the infiltration process, embedding of myocardial tissues was done for better sectioning. In the first step, put a small amount of paraffin into cassettes, and then transferred the samples with warm forceps into the mold and set in the middle of the cassettes according to your desired orientations, and then filled the cassettes with melted molten-paraffin wax; handle with care to prevent over or

under filling. At last, embedded tissues were placed on ice, and at 4^{o}c for overnight to make strong paraffin blocks. These paraffin-embedded tissues were used to perform different histological and biochemical analysis.

All the embedded samples were cut into 3 µm thickness sections by Leica RM 2255 CSA® US digital microtome. To obtained unwrinkled sections, it is better to place blocks on ice before sectioning. Put the sections on cold water bath and picked up very carefully with back of brushes and put evenly on the FLEX IHC coated microscope slides with white-painted label area (Code: K8020, K802021-2, Wo503900201, 75mm, W x 25mm, D x 1mm, H), put them on the warm water bath to remove wrinkles of sections.

2.12. Immunohistochemistry

Immunohistochemistry is considered as the best method for the analysis of inflammation and localization of target molecules in tissues or cells and also playing an important role in the research and diagnostics of many diseases (Duraiyan et al., 2012). To investigate the inflammation and status of pro-survival signaling kinases in cardiomyocytes the following antibodies obtained from Bioss antibodies, Novusbio, Abcam and Cell signaling were used:

- CCL5/RANTES (Cat.No.BS1324R),
- Nitro-tyrosine (Cat. No. BS 8551),
- IL-6 (Cat. No. 10E5),
- NFkB2 (Cat, No, ab16502),
- P-Erk1/2 (Monoclonal Anti-MAP Kinase Activated / monophosphorylated / Phosphothreonine, #M7802 Ab produced in mouse)
- P-Akt (Ser473, 193H12, #4058) rabbit-mAb.

2.12.1. Immunohistochemistry Staining

To access the inflammation in ischemic part (LV) of heart tissues (n=10) were decalcified then embedded in paraffin, section 3(μm), deparaffinized and rehydrated with xylene (20 min) and graded ethanol concentration (5 min) and, then two changes of distilled water (2mins). For antigen retrieval, a microwave was used and then the sections were boiled in 10Mm sodium citrate buffer (Ph 6.0) for 30 mins, sections were then cooled and rinsed three times in distilled water and PBS (5min). To suppress Endogenous peroxidase activity, immersed all the tissue sections with 3.0% hydrogen peroxide in methanol for 15 mins. To permeabilize the tissues, they were rinsed three times in PBS containing 0.1 Triton X (PBS-TX) and incubated in 5% of horse serum in PBS-TX for 30 mins. All sections were incubated with primary antibodies CCL5/RANTES, ICAM1, Nitrotyrosine and IL-6 with dilutions 1:300, 1:100, 1:300, 1:100 respectively in antibody diluent for 1hour at room temperature and then left over at 4 C for overnight.

The tissue sections were rinsed with PBS-TX after an incubation period of primary antibodies and then incubated in Biotinylated anti-rabbit secondary antibody (Dako Corp.) with 1:400 dilution for 1hour at room temperature. After series of washes with PBS-TX, sections were incubated with Avidin-biotin complex (The VECTASTAIN® Elite ABC Kit (Standard), Cat. No. PK-6100 Vector Laboratories, Inc. Burlingame, CA 94010 USA) on each section of tissue for 1hr, followed instruction of manufactures guidelines for complex preparation. All the sections were rinsed and placed in diaminobenzidine (DAB) in PBS containing 0.75% H_2O_2 and 3% nickel for 5-10 mins to monitor the intensity of desired staining. Wash the sections two times, mounted after dehydration in graded ethanol series and cleared in xylene. The negative control was used to confirm and check the absence of the signal or specificity of staining. Image acquisition of all histological sections was done under Olympus System BX51 Universal research microscopy. The images were analyzed by using ImageJ

software NIH, Bethesda, MD) to quantify the strength of immune-peroxidase staining in heart tissue.

2.13. Hematoxylin and Eosin Staining

For hematoxylin and eosin staining procedure, ischemic part of myocardial tissues was collected and processed in 4% paraformaldehyde and ethanol and then embedded in paraffin-wax. The tissue containing blocks were sectioned at 3microns. After removing wax from tissues, they were stained by hematoxylin-eosin staining and silver nitrate. Eosin and Hematoxylin staining solutions were prepared by following the procedure shown in appendix 1.

The hematoxylin was dissolved in ethanol. Thoroughly prepare ammonium alum solution in distilled water and boiled. After the addition of hematoxylin solution to ammonium alum solution, carefully added the glycerol and sodium iodide and then added acetic acid one by one in given amount, mix thoroughly.

2.14. TUNEL Assay

TUNEL (Terminal deoxynucleotidyl transferase, 2`-deoxyuridine 5`-Triphosphate Nick End Labeling) assay is the key tool for the detection of apoptotic nuclei of a cell within tissue samples. Apoptosis is a process of programmed cell death and as a part of normal development and highly controlled and regulated processes during organism life cell cycle. The apoptotic study is very important because any changes occur in these processes can lead to cancer, autoimmune and degenerative diseases and many others. Fragmentation of DNA is the whole mark of late apoptosis. For the detection of DNA fragmentation, a very specific DNA polymerase (terminal deoxynucleotidyl transferase/TdT) attach with non-labeled and fluorescently labeled nucleotide 2`-deoxyuridine 5`-triphosphate (adds dUTP) at the fragmented end of DNA. When there is a breakdown of double-stranded DNA, a great amount of recruitment of the independent template (TdT) nucleotide occurs.

Cell undergoes apoptosis if there is a breakdown of DNA and this apoptosis can be measured by the amount of TdT active within cell through the fluorescein-dUTP from double-stranded breaks.

2.15. Measurement of Oxidative Stress

The TBARS (specifically, malondialdehyde – MDA) levels were measured spectrophotometrically at 532 nm in the cell lysates using the methodology described by (Ben Mansour et al., 2011). GSH content was estimated according to the method of (Beutler et al., 1963). The reaction is based on the thiol group of GSH reacts with the –SH reagent (DTNB) to form the thionitrobenzoic acid. The reaction mixture contained 0.1 M of phosphate buffer (pH 7.4), 10 mM of DTNB and 15 µL of the supernatant. The yellow color developed was immediately read at 412 nm on a spectrophotometer. The values were expressed as ng GSH/µg protein. The catalytic activity of CAT was determined as described by (Aebi et al., 1984). The decomposition of the substrate H2O2 was monitored spectrophotometrically at 240 nm. The CAT activity was calculated in terms of µmol of H2O2 consumed/min/mg protein. The SOD activity assay was based on the SOD ability to inhibit the auto-oxidation of epinephrine at alkaline medium (Goya et al., 2016). The assay mixture contained 50 mM glycine buffer (pH 10.0), cell extract, and 60 mM epinephrine (pH 2.0). The SOD activity was indirectly measured by the variation in absorbance of the oxidized product of epinephrine, i.e., adrenochrome, at 480 nm.

2.16. Microscopy

All sections were studied and acquired images for immunohistochemistry, TUNEL and H&E stained sections under Olympus System BX51 Universal research microscopy at 10x, 20x, and 40x magnifications. The photographs were taken for immunoreactivity and pathological examination. The images were analysed by ImageJ software (NIH, Bethesda, MD), the average mean fluorescent

intensities (MFIs) were measured by dividing the intensities by area of each image.

2.17. Statistical Analysis

All the results were expressed as mean ± SE. Comparison between control and treated groups was statistically evaluated by Students t-test and a P value < 0.05 was considered as statistically significant.

CHAPTER THREE

3.1. RESULTS

3.2. Identification of Phenolic Compounds in Cocoavia® and Recioto red wine

The unknown metabolites of Recioto red wine and Cocoavia® samples were analyzed by using HPLC-ESI-MS followed ESI-base peaks, MS/MS, and MS3 fragmentation at their respective retention time and mass to charge ratio (m/z).

3.2.1. Identification of Phenolic Compounds in Recioto red wine

The metabolites of Recioto red wine samples were analyzed by using HPLC-ESI-MS followed ESI-base peaks, MS/MS, and MS3 fragmentation at their respective retention time and mass to charge ratio (m/z). As it is seen from the Figure 15 A (+) positive and fig 15 B. (–) negative ion mass LC-ESI/MS chromatogram spectra of Recioto red wine and table no.2.Twenty-three at a positive and fourteen at a negative ion mass spectrum, different phenolic compounds were detected and identified from commercially available Recioto red wine.

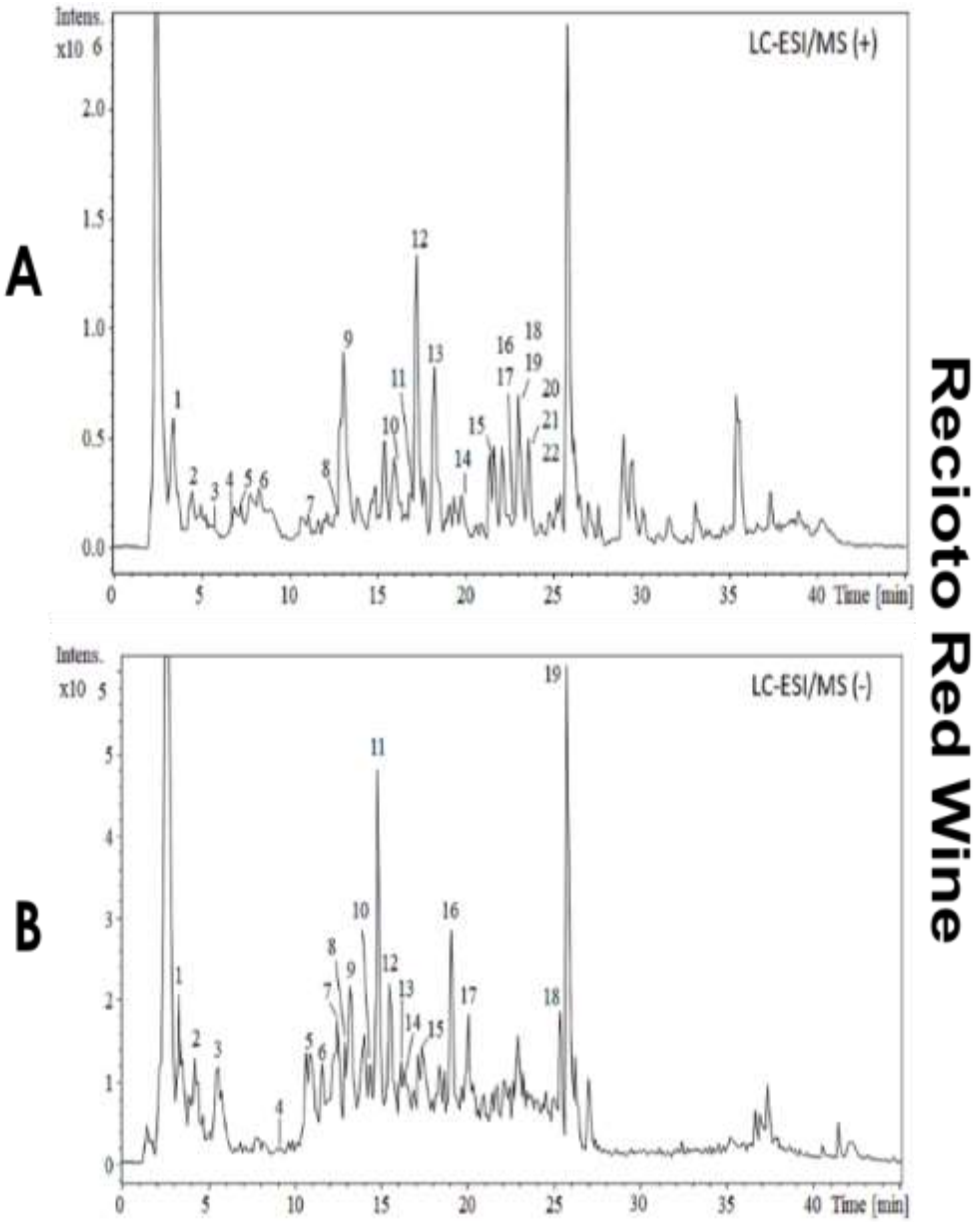

Figure: 17. Identification of phenolic compounds in Recioto red wine: Fig: 17A. Shows Twenty-three different phenolic compounds were identified at (+) ion mass LC-ESI/MS chromatogram and **Fig: 17 B.** shows fourteen different phenolic compounds at (–) ion mass LC-ESI/MS chromatogram were identified.

Table: 2. Identification of Phenolic Compounds and Molecular Ions in Recioto Red Wine/RRW:

Identified Phenolic components and Molecular ions were detected by MS analysis of RRW samples at positive and negative ion mass spectra are under below.

No.	m/z (+)	m/z (-)
1	Proline	Epicatechin
2	Leucine hexose dehydrated	Quercetin
3	Phenylalanine derivative	Hydroxytyrosol glucoside
4	Tryptophane hexose	Gallic acid derivative
5	Adenylosuccinic acid pentose	(Epi)catechin ethyl derivative
6	2-S-glutathionyl-trans-caffeic acid	resveratrol tetramer
7	Malvidin hexose isotope	Benzoyl alcohol hexose pentose
8	Peonidin hexose pyruvate	Caffeic acid derivative
9	Malvidin hexose	Dihydroquercetin deoxyhexose
10	Delphinidin hexose pyruvate	Kaempferol hexose hydrated
11	Malvidin hexose pyruvate	Dihydromyricetin desoxyhexose
12	Procyanidin B1	Malvidin derivative
13	Delphinidin hexose vinylphenol	Caffeoyl tartaric acid, 2M-1
14	Petunidin hexose vinylphenol	Catechin derivative
15	Malvidin acetylhexose pyruvate	Dihydromyricetin deoxyhexose
16	Peonidin hexose vinylguaiacol	(Epi)catechin ethyl derivative
17	2-S-glutathionyl-trans-caftaric acid	Dihydroquercetin deoxyhexose
18	Malvidin hexose vinylguaiacol	Quercetin

19	Malvidin dihexose	Resveratrol tetramer
20	Malvidin coumaroylhexose pyruvate	
21	Malvidin hexose vinyl (epi)catechin	
22	Malvidin coumaroyl hexosyl hexose hydrated	
23	Malvidin acetylhexose vinyl(epi)catechin	

3.2.2. Identification of Phenolic Compounds in Cocoa Extract

HPLC-ESI-MS followed ESI-base peaks, MS/MS, and MS3 fragmentation at their respective retention time and mass to charge ratio (m/z) analysis was performed to identify phenolic compounds in Cocoavia®. As it is seen from the Figure (-) negative ion mass LC-ESI/MS chromatogram spectra of Cocoavia®, twenty different phenolic compounds from cocoa extract were identified as shown in figure (16, c) and in Table 3.

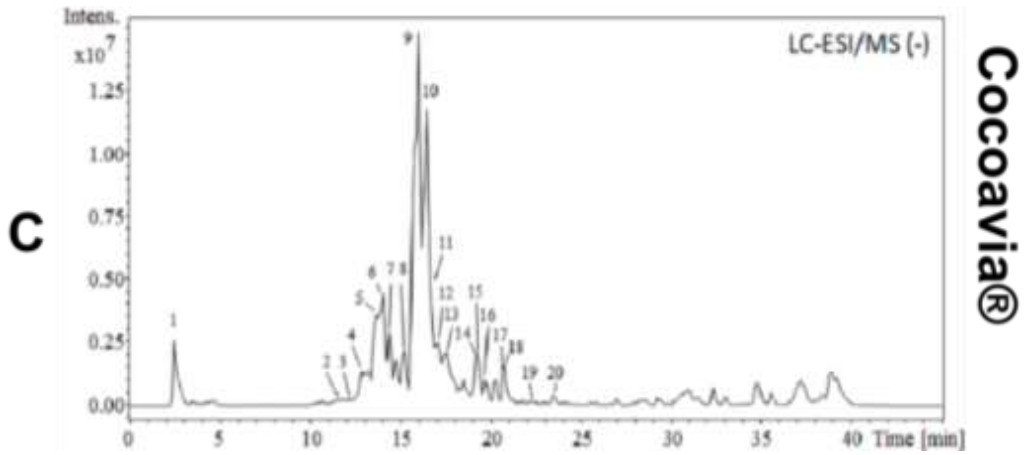

Figure: 18. Identification of phenolic compounds: Samples of cocoa extract (Cocoavia) were analyzed by using HPLC-ESI-MS followed ESI-base peaks, MS/MS, and MS3 fragmentation at their respective retention time and mass to charge ratio (m/z). Nineteen different phenolic compounds were identified fig 18 C.

Table: 3. Identification of phenolic compounds in Cocoa extract: Identified Phenolic molecules from the Cocoavia® samples were analyzed by chromatographic mass spectra in negative ion mode.

No.	(-) m/z	No.	(-) m/z
1	Di-hexose, Di-hexose [2M-H]	11	Procyanidin pentamer
2	N-[3′,4′-dihydroxy-(Z)-cinnamoyl]-L-aspartic acid	12	Procyanidin monomer
3	Catechin hexose	13	Procyanidin aptamer
4	Catechin	14	Procyanidin hexamer
5	Procyanidin trimer	15	Procyanidin
6	Procyanidin tetramer	16	Quercetin hexose
7	Procyanidin B2	17	Procyanidin derivative
8	Epicatechin	18	Ellagic acid pentose
9	Procyanidin trimer, Catechin derivative	19 &20	(Epi) catechin ethyl trimers
10	Procyanidin tetramer		

3.3. Determination of Phenolic Compounds

Figure 17 a & b shows the quantitative analysis of RDW and Cocoa extract, through HPLC-DAD array, with the same protocol as used for HPLC-ESI/MS analysis. Statistical analysis was carried out to calculate the amounts of phenolic molecules by recording the area of their respective peaks, wavelengths,

obtaining the coefficient equation and value of R^2 for both standards and samples. (+)-Catechin (y=5E+06x - 20037, R^2=0.997) and procyanidin A2 (y=3E+06x+8794, R^2=0.9998) were used to quantify flavan-3ols and their oligomers (absorbance at 280 nm). Caffeic acid (y=3E+07x+231629, R^2=0.9999), quercetin (y=7E+06x+79102, R^2=0.9993) and Resveratrol (y=3E+07x – 54374, R^2=0.996) were used to quantify hydroxycinnamic acid derivatives (320 nm), flavonols (350 nm) and stilbenes respectively as shown in table 4.

Table: 4. The table represents the coefficient equation and value of R^2 for standards at the respective wave lengths.

No.	Standard used	Equation	R^2
1	Catechin	y=5E+06x - 20037	0.997
2	Caffeic acid	y=3E+07x - 36306	0.997
3	Resveratrol	y=3E+07x - 54374	0.996
4	Quercetin	y = 1E+07x + 12001	0.997
5	Gallic acid	y=1E+07x +19805	0.999

.

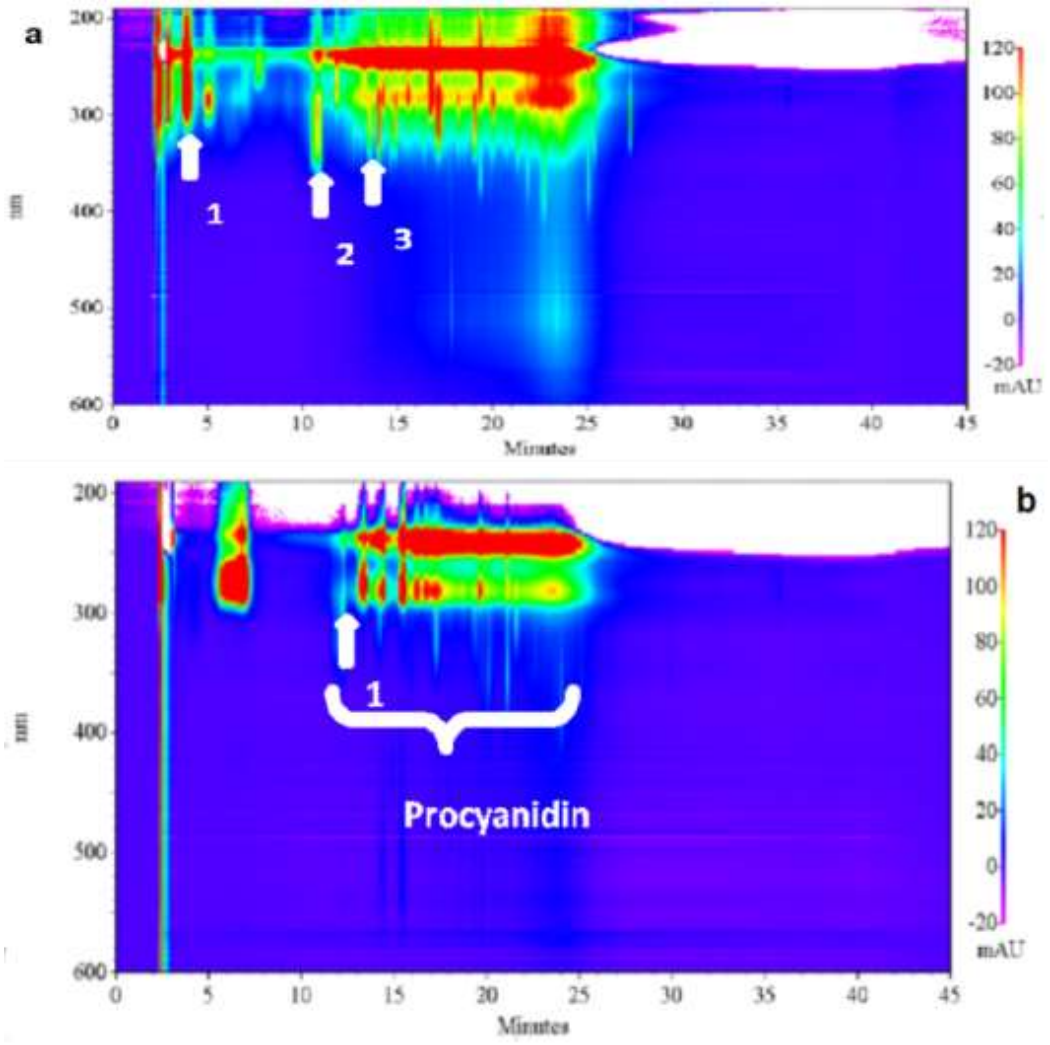

Figure: 19. HPLC- DAD analysis of RDW and Cocoavia®: a=Recioto red wine & b=Cocoavia®: (a) representing 1: Gallic acid equivalents, 2: caffeoyl tartaric acid and 3: catechin derivatives (b) procyanidin 1: catechin.

In Cocoavia® 250 ± 1 mg of flavan-3ols (procyanidin) were found in 1300mg of cocoa extract capsule, while in case of RDW, the amounts of identified molecules were 15±2.69 mg/100ml Gallic acid equivalents, 3.36±1.39 mg/100ml Caffeoyl tartaric acid, and 9.04±2.69mg/100ml of Catechin derivatives. So, in one liter of a bottle of Recioto wine contains 150mg, 33.6mg, and 90.4mg of Gallic acid equivalents, Caffeoyl tartaric acid, and Catechin derivatives. Online data bases and in-house library was followed to check the amount of respectively identified molecules in Recioto red wine.

3.4. Determination of Flavonoids in Rat Serum

Figure 18 showed the total phenolic contents in rat serum calculated by HPLC-MS mass spectrometry, between Cocoa extract treated groups (5mg/kg, 10mg/kg, 15mg/kg, 20mg/kg, and 25mg/kg) and untreated group. Results were indicated as (average mean among different time points I st,7th,15th day of blood collection of treated and untreated groups) cocoa polyphenols increased in blood serum as the dose of Cocoa extract increases and became almost remain constant after the 15mg/kg of treatment and this dose was considered as optimal dose for this study. This optimal does (15mg/kg/bw) could be translated to average human body weight (70kg/kg/bw) as 3,000mg of cocoa extract for 15days.

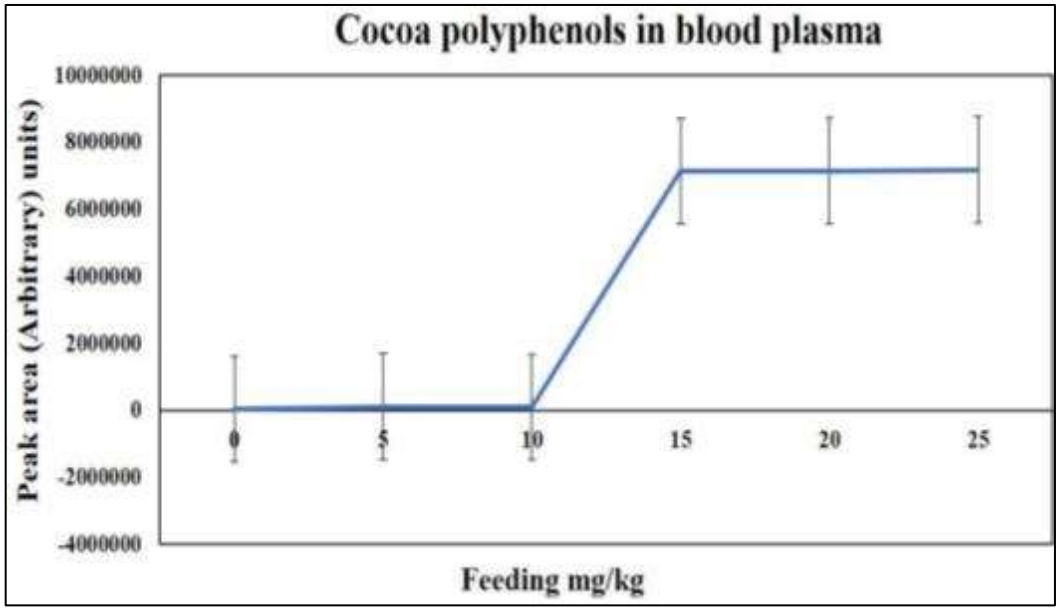

Figure: 20. Determination of Cocoa Extract Dose-response in the Blood Sera: Quantification of total polyphenols of cocoa extract in blood serum by HPLC-Mass Spectrometry: Data was expressed as average mean of different time points (1st, 7th, 15th day of blood collection of treated and untreated group) and cocoa extract (mg/kg) in incremental dose demonstrating 15 mg/kg to be the optimal dose of cocoa extract (six groups, n=30) and each group (n=5).

3.5. Histopathological Evaluation of Cocoa Treated Rat Myocardial Tissue after Ischemia and Reperfusion Induction

Hematoxylin and Eosin stained myocardial tissue of cocoa treated and control rats were evaluated under bright field light microscope. Cocoa extract treated rat myocardial tissue observed with fewer lymphocytes compared to control after induction of ischemia and reperfusion for both groups Fig. 19.

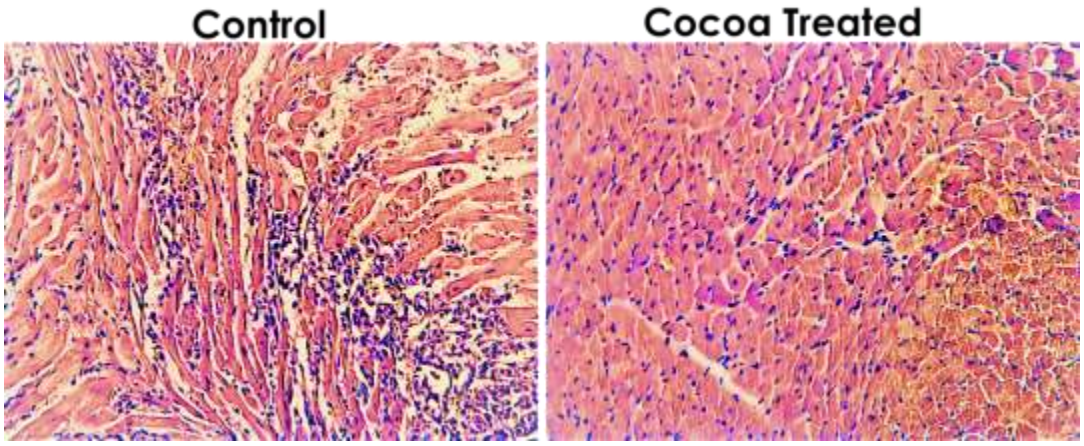

Figure: 21. Hematoxylin and Eosin Stained Myocardial Tissue in Cocoa Treated and Control Rats: interstitial infiltration of lymphocytes which are indicators of inflammation was observed in less in cocoa treated myocardial tissue section compared to untreated rat heart tissue (x20).

3.6. Expression of Inflammatory Markers (CCL5/RANATES and IL-6) in Cocoa Treated Rat Cardiac Tissues

The extent of inflammation of CCL5/RANATES and IL-6 are expressed as brown particles in myocardial tissues after 30min of ischemia and 24hr of reperfusion in left ventricle through paraffin-embedded samples by immunohistochemistry. Myocardial CCL5/RANATES and IL-6 expression were significantly elevated in Cocoavia® treated group as compared to control group as in Fig. 20 (A, B, C, and D). The reduction of myocardial CCL5/RANATES immunoreactivity was found lower in cocoa extract treated rats related to control,

p=0.000135. The same result was also found in IL-6 that showed lower IL-6 immunoreactivity related to control and p=0.001682.

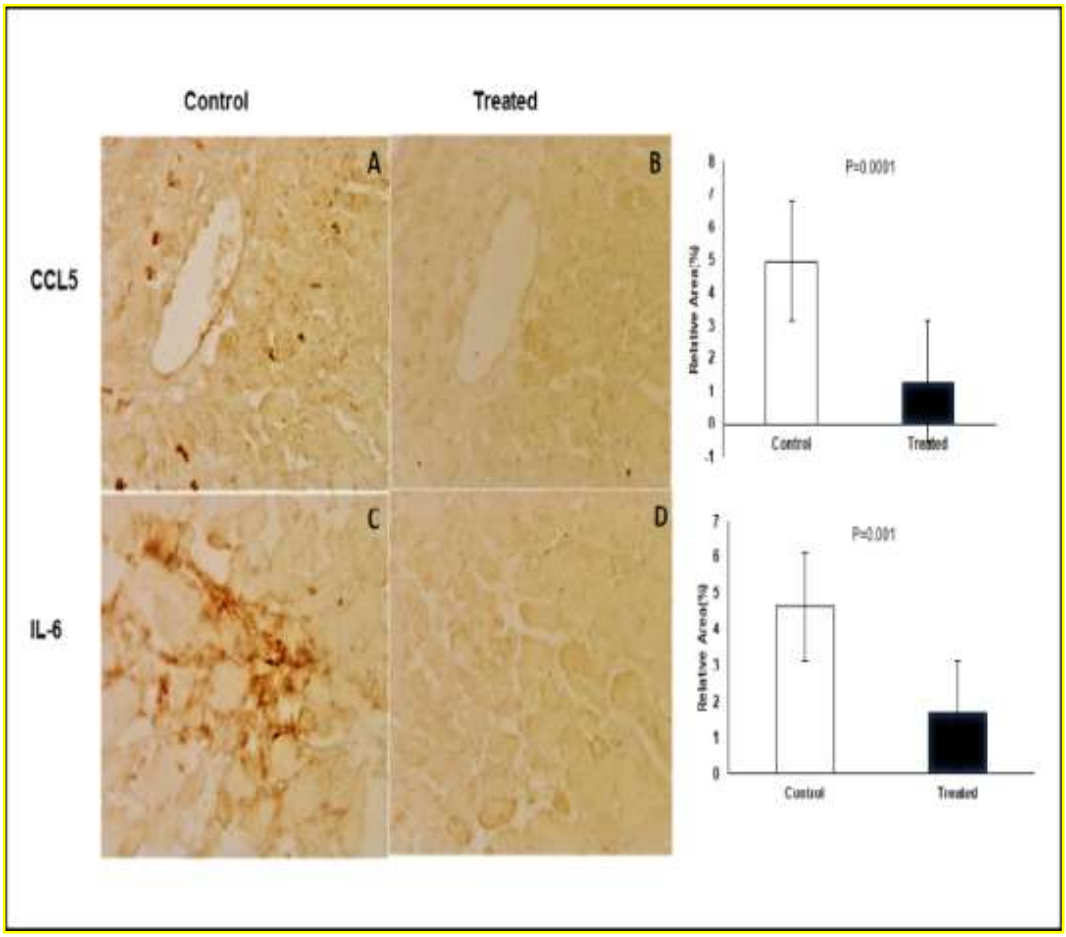

Figure: 22. Immunohistochemical Analysis of Inflammatory Markers (CCL5 /RANTES) on Cocoa Treated Rat Myocardial Tissue Compared to Control: The myocardial tissues were stained for CCL5/RANTES (A and B) and IL-6 (C and D). The graph illustrates increased immunoreactive CCL5/RANTES (A and B) and IL-6 (C and D) inflammatory cells in cocoa treated rat (n=15) myocardial tissue compared to control (n=15). Representative images (20x). Data illustrated in the graphs were presented as mean ± SE. P value less than 0.05 considered as statistically significant. Note: CCLS/RANTES, C-C Chemokine Ligand 5/Regulated on Activation Normal T Cell Expression and Secreted; IL-1, Interleukin1.

3.7. Expression of Nuclear Factor Kappa B Subunit 2 (NF-kB2) in Myocardial Tissue of Cocoa Treated Rats Compared to Control

In our immunoperoxidase analysis, immunoreactivity of Nuclear Factor Kappa B Subunit 2 (Nf-kB2) which is expressed in different cell type and functions as a central activator of genes involved in inflammation and immune function was determined. The immunoreactive Nf-kB2 was significantly decreased in a group of 15 cocoa treated rat heart tissue compared to control and p=0.01. The reduced in Nf-kB2 in cocoa extract treated rat myocardial tissue implies the activation of inflammation and immune function. This result was related to the less observed inflammatory lymphocytes in hematoxylin and eosin stained myocardial tissue section in cocoa treated rat that is shown in fig 21.

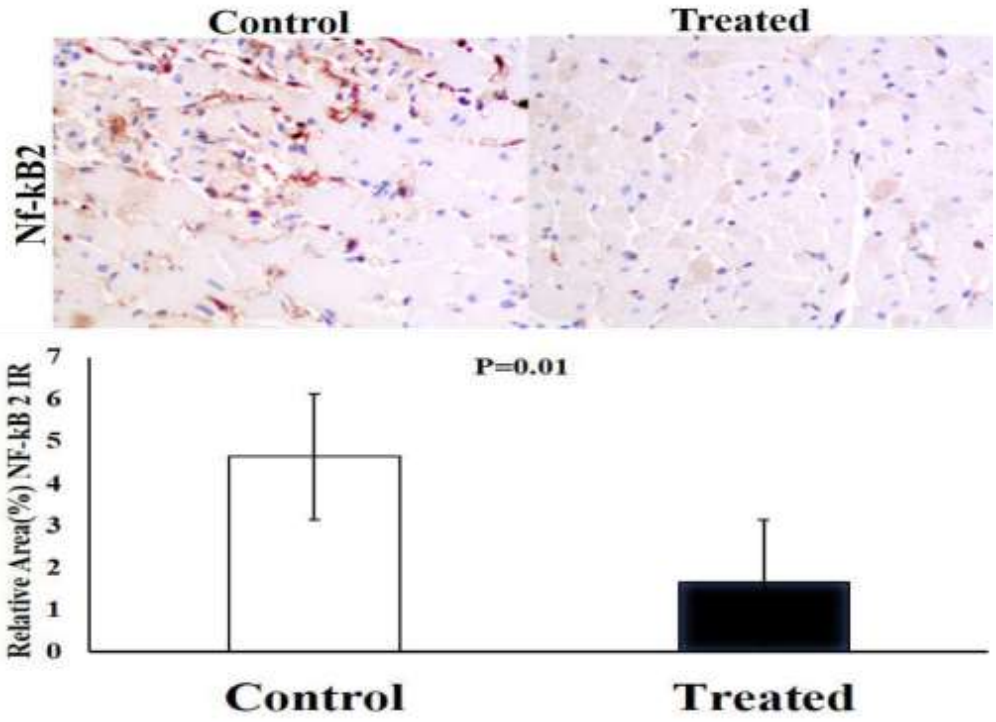

Figure: 23. Immunohistochemical Analysis showing expression of NF-kB2 in Myocardial Tissue of Cocoa treated Rats Compared to Control: The myocardial tissues were stained with NF-kB2. The graph exemplifies increased NF-kB2 immunoreactive cells in cocoa treated rat (n=15) myocardial tissue compared to control (n=15). Representative images (x20). Data illustrated in the graphs were presented as mean ± SE. P value less than 0.05 considered as

statistically significant. Note: NFkB2, Nuclear Factor Kappa Subunit B2; IR, Immunoreactivity.

3.8. Evaluation of Nitro-tyrosine Immunoreactivity on Myocardial Tissue in Cocoa treated Rat after I/R

In our immunohistochemistry analysis, immunoreactivity of Nitro-tyrosine which is an indicator of cell damage was evaluated. The immunoreactivity of nitro-tyrosine was significantly reduced in a group of fifteen cocoa treated rat heart tissue compared to control and p=0.01. This showed lower cell damage/oxidative stress that could be due to the treatment of rats with cocoa extracts for 15 days.

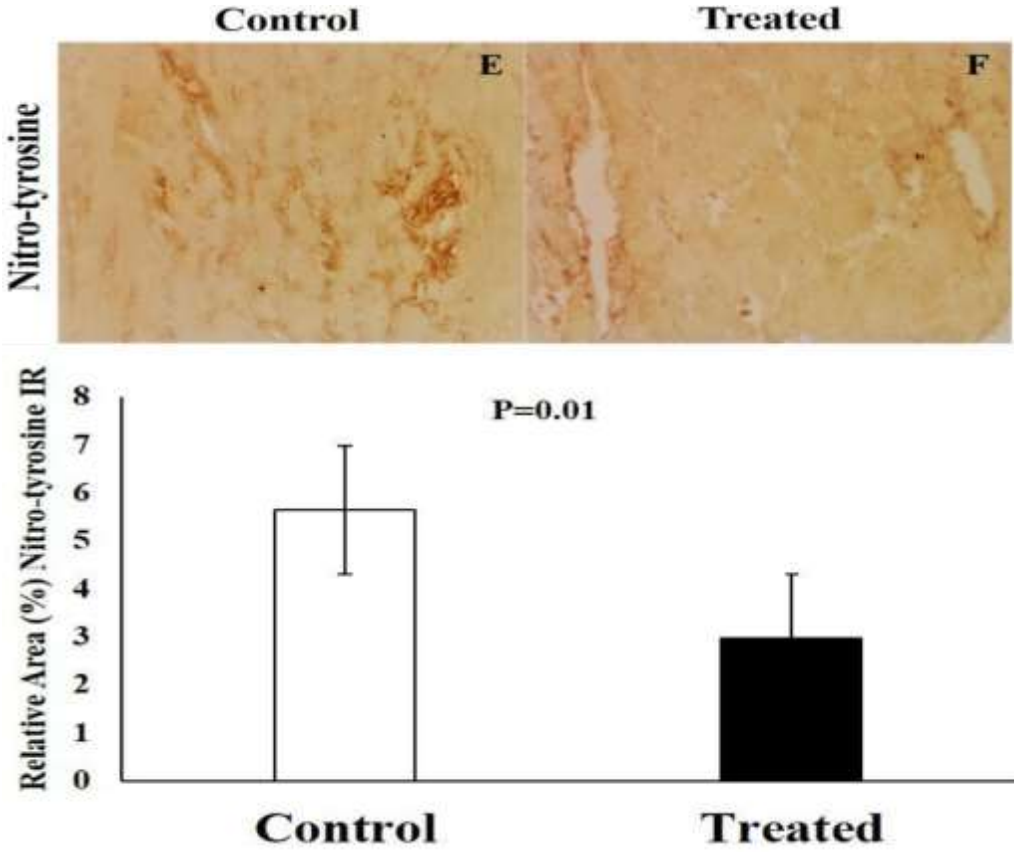

Figure: 24. Evaluation of Nitrosative Stress on Myocardial Tissue in Cocoa treated Rat: Relative expression of nitrotyrosine in myocardial tissue of cocoa treated (n=15) rats compared to control (n=15). Representative images (x20). Data illustrated in the graph were presented as mean ± SE. P value less than 0.05 considered as statistically significant. IR, Immunoreactivity.

3.9. Effects of Cocoa Extract on Oxidative Stress on Myocardial Tissue of Rats after I/R injury

Oxidative stress was analyzed by detecting levels of TBARS, GSH, and the enzymatic activity of both SOD and CAT. In the cocoa extract treated groups, a decrease in the TBARS/ MDA levels was observed. The reduction indicated lower cardiac membrane lipid peroxidation compared to the control (Figure 23, a). Redox status was significantly elevated after cocoa extract administration as indicated by GSH levels (Figure 23, b). The activity of SOD and CAT was significantly increased ($p < 0.001$) in the cocoa treated group as compared to control group after induction of I/R (Figure 23, c-d).

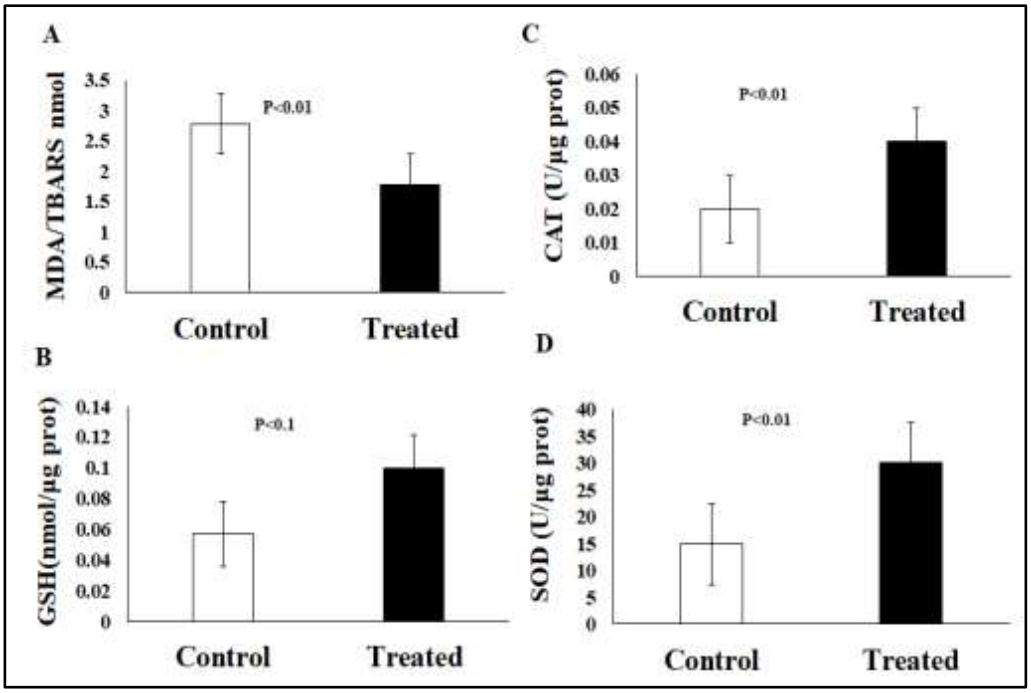

Figure: 25. Effects of Cocoa Extract on Oxidative Stress on Myocardial Tissue Rats: Lower oxidative stress was recorded in cocoa treated rat (n=15) myocardial tissue compared to control (n=15). Lipid peroxidation index (TBARS) expressed as malondialdehyde (MDA) concentration (A) measured in nmol/µg. Reduced glutathione (GSH) content (B), Catalase (CAT) activity (C), and Superoxide dismutase (SOD) activity (D). Data illustrated in the graphs were presented as mean ± SE. P value less than 0.05 considered as statistically significant.

3.10. Effect of Cocoa Extract Phenols on Myocardial Nuclei Apoptosis in Rats

The level of cardiomyocytes nuclei apoptotic was determined by TUNEL assay. The TUNEL assay showed a significant decline of apoptosis nuclei in Cocoa extract treated group (figure 24B) compared to control after 30min of ischemia and 24hr of reperfusion episode, p=0.015481.

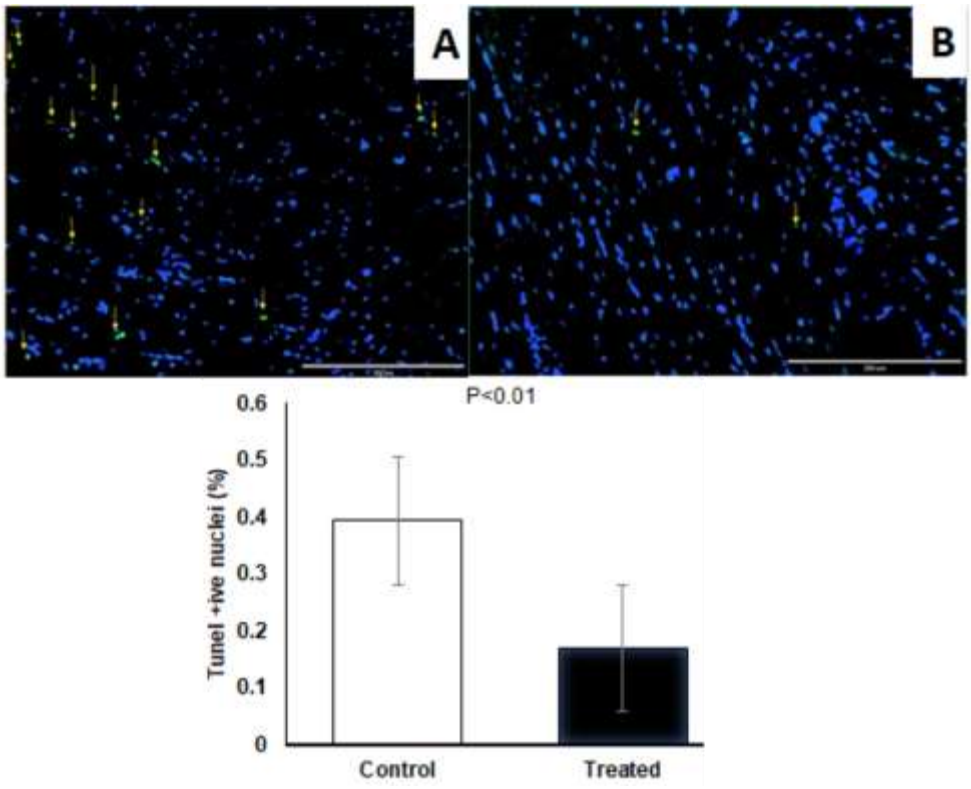

Figure: 26. Analysis of Apoptosis by TUNEL Staining on Cocoa Treated Rats of Myocardial Tissue Compared to Control: TUNEL fluorescent staining showing TUNEL positive nuclei in cocoa extract treated and control (x20). Counter staining was performed using DAPI nuclear staining. Cocoa treated (Fig. 7 A, n=15) myocardial tissue showed higher number of TUNEL-positive nuclei compared to control group (Fig. B, n=15). Yellow arrows indicate apoptotic nuclei. Data illustrated in the graph were presented as mean ± SE. P value less than 0.05 considered as statistically significant. Scale bar = 100µm. TUNEL, Terminal deoxynucleotidyl transferase, 2`-deoxyuridine 5`- Triphosphate Nick-End Labelling.

3.11. Effects of Cocoa on Activities of Phosphorylated Extracellular Signal Regulated Kinases ½ and Akt Kinases

The phosphorylated extracellular signal-regulated kinases ½ and Akt are regulatory protein kinases that involve in proliferation and cell-survival. In our immunoperoxidase analysis, the phosphorylation of Akt was significantly elevated in cocoa treated rats of myocardial tissue compared to untreated groups, p= 0.001. Similarly, the activity p-ERK1/2 was higher compared to control, p= 0.01. These might show the modulating activities of these two protein kinases in cocoa treated rat heart tissue after ischemia and reperfusion.

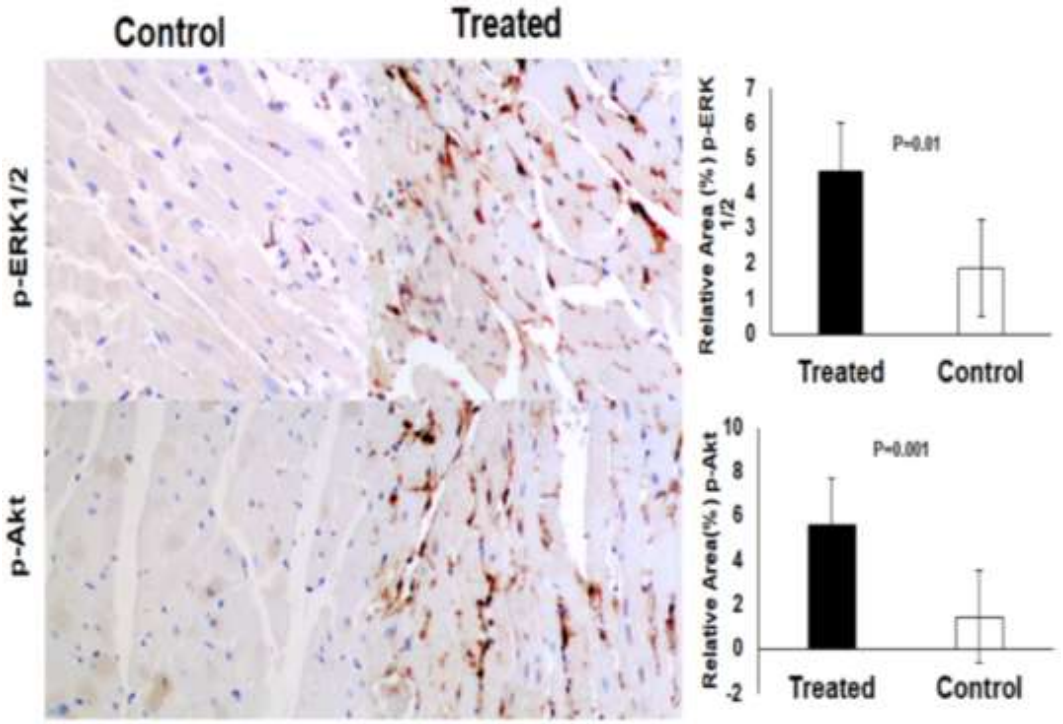

Figure: 27. Immunohistochemical labeling of p-Akt and p-ERK1/2 in Cocoa Treated Rat Myocardial Tissue Compared to Control: The graphs showed a significant elevation on activation of p-Akt and p-ERK1/2 in myocardial tissue of cocoa treated rats (n=15) compared to control (n=15). Data illustrated in the bar graph were presented as mean ± SE. P value less than 0.05 considered as statistically significant. Note: P-ERK1/2, Phosphorylated Extracellular Signal-regulated Kinases ½; P-Akt, Phosphorylated Serine-Threonine Protein K, x20.

CHAPTER FOUR

4.1. DISCUSSION AND CONCLUSION

4.2. Discussion

Cocoa has the highest flavonol contents of all foodstuffs and its extract contains a considerable concentration of proanthocyanidins (Goya et al., 2016). They are polymeric flavan-3-ols whose antioxidant property hinges on their ability to serve as electron donors to terminate the radical chain reaction (Lesschaeve et al., 2005). Flavonoids represent a major subset of phenolics, and they are highly effective scavengers of most oxidizing molecules and free radicals implicated in several diseases (Luo et al., 2017) including cardiovascular diseases.

In our study, comparison of flavonoids` amount has been done in recioto red wine and cocoa extract. Commercially available the recioto red wine and the cocoa extract powder have been determined to give rise to forty-one and nineteen phenolic compounds, respectively. Even though higher number of flavonoids is detected in recioto red wine, the amounts of flavonoids are three times higher than recioto red wine. This finding goes in line with the study evaluating the total antioxidant activity in cocoa, red wine, green and black teas using ABTS and DPPH that has shown the highest antioxidant activity in cocoa than the other three foodstuffs (Lee et al., 2003).

In the present study, to the best of our knowledge, various concentrations of a commercially available epicatechin and catechin containing cocoa extract are determined in a dose-response manner in Sprague-Dawley rats for the first time and the 15mg/kg/BW dose has been found stable in the bloodstream for 15days. This concentration of cocoa extract dose-response assessment is used to establish the 15mg/kg optimal dose in a daily administration regimen in order to have maximal plasma concentration of polyphenols for cardioprotective characterization of cocoa against acute myocardial I/R.

In our observational study of rats treated with cocoa extract at higher doses (30 and 35mg/kg/bw) has resulted in higher rate of mortality and coagulation of blood. The coagulation and higher rate of mortality effects of cocoa extract at higher doses might be due to high blood pressure, higher number of platelets and/or dehydration. This result shows that rats when treated with cocoa extract at 30 and 35mg/kg/bw may have adverse effects as compared to 15mg/kg/bw treatment for 15 days which has been indicated in our study with protective effects against ischemia and reperfusion injury. The implication of this is, lower doses (15mg/kg/bw) of cocoa extract have better cardioprotective effect than higher doses (30-35mg/kg/bw) in rats treated for 15days.

In our study, a total of 250 mg of flavan-3-ols (procyanidin) have been determined in 1gram of cocoa extract. Among them, nineteen different phenolic compounds are identified (Figure 16, c and Table 3). We have been hypothesized that these phenolic compounds which are found in cocoa extract might have a role in the reduction of inflammatory markers, oxidative stress, myocardial apoptosis, and in the greater activation of pro-survival downstream proteins in cocoa extract treated rat heart. Many studies have shown the protective effect of myocardial ischemia-reperfusion injury (Akinmoladun et al., 2016) and in improving post-ischemic functional recovery (Testai et al., 2013).

Even though flavonoids have many health-related benefits, their bioavailability is a major concern and can be affected by absorption and chemical structure (Scalbert et al., 2002). The optimal dose could give clue in the evaluation of the bioavailability of flavonoids of cocoa in the bloodstream in rats.

Inflammation is a protective functional response of the body tissues to harmful stimuli (Goya et al., 2016). Findings of this study have presented a reduction in the expression of CCL5/RANATES which recruit leukocytes into inflammatory sites in the cocoa treated rat heart (Figure 20). Moreover, the significant reduction of inflammatory markers (IL-6 and NFkB) in cocoa extract

rat heart (Figure 20 and Figure 21) which are involved in pro-inflammatory signaling pathway have been identified in this study. The lower lymphocytes observed in histopathological evaluation of cocoa treated rat heart tissue has also supported these lowered inflammatory markers in the presence of cocoa extract.

As indicated by different studies (Goya et al., 2016), body tissue responds to unfavorable/harmful stimuli by increasing the number of inflammatory markers such as IL-6 and NF-κB2. This implies that the detected reduction of inflammatory markers in cocoa treated rat myocardium has an advantage in protecting the heart against ischemia and reperfusion injury. On the other hand, in earlier in vitro study of the peripheral blood mononuclear human cell, inhibition of IL-1 mRNA expression and T-cells secretion of IL-2 has been reported from polyphenol containing cocoa liquor (Sanbongi et al., 1997). The reduced inflammatory markers in cocoa treated rat heart suggest flavonoids found in cocoa as a therapeutic target for anti-inflammatory drugs for the treatment of cardiovascular diseases.

This study on levels of TBARS, GSH, and the enzymatic activity of both SOD and CAT has also shown a decrease in the TBARS/MDA levels in cocoa extract treated rat myocardium that implies lower cardiac membrane lipid peroxidation compared to the control (Figure 23, a). The redox status was also significantly attenuated after cocoa extract administration as indicated by GSH levels (Figure 23, b). Similarly, in another study, the decline in TBARS/MDA levels is also used as an indicator of lower cardiac membrane lipid peroxidation that hampers the cardioprotective effects (Ahmed et al., 2017).

The present study has also shown the elevated activities of SOD and CAT in the cocoa treated rat myocardium after induction of I/R (Figure 23, c-d). Recently, a study in extracts of cocoa and kola nut tree in the protective effect against myocardial ischemia-reperfusion injury/IRI is reported by Akinmoladun et al., (2016). This study goes in line with our finding that shows the reduction of oxidative stress in cocoa treated rat heart. Another supporting data has stated that

improvement of post-ischemic functional recovery and lowering of myocardial tissue injury due to the presence of higher amount of flavonoids (Testai et al., 2013).

In our study on measurement of nitrotyrosine levels, the nitrotyrosine levels which indicates nitrosative stress in myocardial tissue has declined in cocoa treated rat heart as shown in the figure. 22. The decrease in nitrotyrosine levels which is highly associated with lower glucose (Bo et al., 2005) and correlated with oxidative stress after ischemia and reperfusion. The reduction of oxidative stress and cardioprotective potential of cocoa which are observed in our study could be due to the potent antioxidant and free scavenging activities of flavonoids (Procházková et al., 2011) or proton-donor-scavenging radicals' property of polyphenols are also indicated by Rice Evans et al., (2002). These can occur in nitrite donors or boosters in the setting of I/R in the potent vasodilator and anti-inflammatory agent (Darra et al., 2010; Rungatscher et al., 2015) and act as inhibitors to reduce the oxidative stress, bind carbohydrates, chelate metals and proteins (Heim et al., 2002).

Although intake of cocoa elevates the blood pressure, many studies are strengthened the antioxidant effects of polyphenols containing food with respect to health benefits (Awe et al., 2013; Cooper et al., 2008; Han et al., 2007). Moreover, two observational studies have shown a reduction of the cardiovascular diseases and mortality due to the intake of cocoa (Buijsse et al., 2006; Buijsse et al., 2010).

In our TUNEL assay that analyzes myocardial apoptosis in cocoa treated rat myocardium; myocardial apoptosis has been reduced in rats treated with cocoa extract after induction of ischemia and reperfusion (Figure 24). The higher apoptosis augments myocardial apoptosis and heart failure in the contrary the lower apoptosis protects the heart (Jose et al., 2016). The lower myocardial apoptosis observed in cocoa treated rats in this study demonstrates the limiting potential of flavonoids containing cocoa in myocardial apoptosis which could be

due to lipotoxicity that changes fuel oxidation and mitochondrial dysfunction which lead to low energy for myocyte contraction and higher levels of oxidative stress and ROS that directly damage and destruct cellular structures that induce myocyte apoptosis (Munzel et al., 2015; Sawyer et al., 2002). The toxicological study also indicates the contribution of epicatechin and catechin in cocoa in the reduction of apoptosis via inhibition of amyloid-β protein (Heo and Lee, 2005). The reduced myocardial apoptosis could be due to the antioxidant content of cocoa that might contribute to the protection against ischemia-reperfusion injury.

In our study, immuno-peroxidase analysis of p-Akt and p-ERK1/2 in myocardial tissue from rats treated with cocoa extract has shown elevation in the activity of p-Akt and p-ERK1/2 (Figure 25). Pro-survival downstream proteins (Akt and ERK1/2) are known for their contribution to cell survival after ischemia and reperfusion injury. They are also known for their mediating action for preconditioning in cardioprotection (Zhang et al., 2014). According to Zhang and colleagues (Zhang et al., 2014), activation of contributing to the reduction of myocardial infarct size and heart dysfunction after reperfusion in rats.

Myocardial ischemia produces a neurohormonal response, phagocyte recruitment, mitochondrial dysfunction, and reactive oxygen species formation (Giordano et al., 2005) and from infarct area, over time remote sites are affected by oxidative stress, energy metabolism abnormalities, extracellular matrix alterations and myocytes apoptosis. Therefore, this study suggests that treating rats with flavonoids containing cocoa extract diminish CCL5/RANTES, IL-6, NFkB, oxidative stress, and apoptosis in the myocardium and the process can be modulated by the activities of p-Akt and p-ERK1/2. It is also known sometimes flavonoids act as pro-oxidant. Although pro-oxidant properties could be considered toxic, these properties could be associated with activities of Akt and ERK1/2 protein kinases by which flavonoids work for cardiac cell physiology (Procházková et al., 2011).

Cocoa contains procyanidins mainly catechin and epicatechin. It is still a point of discussion and/or interest that polyphenols don't have their own direct protective effects to tissues, it might be due to action of microbiota in colon, where undergoes methylation, glucuronidation, and sulfation of polyphenols, and that are transformed into their aglycones which possess antioxidant capacity (Han et al.,2007). These stable forms of aglycones later enter into bloodstream and reach to the target tissues and act as antioxidant, anti-allergic, anti-inflammatory effects within the tissues. Cardio protective effects of cocoa might be due to the indirect by-product of cocoa called aglycones when they are transformed into their methylated, glucuronidated, and sulphated forms in myocardial tissue after ischemia and reperfusion.

4.3. Conclusion

In this study, cocoa extract oral administration in rats at the optimal 15mg/kg/BW is found to reduce oxidative stress, myocytes apoptotic nuclei levels, and inflammatory markers. It is also found to elevate activities of the pro-survival protein kinases (Akt and ERK1/2) after induction of ischemia and reperfusion.

Based on the findings of this study, it can be concluded that the augmentation of cocoa extract once a day for 15days in rats attenuates myocardial I/R injury and limits oxidative and nitrosative stress and inflammation with reduction of myocardial apoptosis. The optimal dose of cocoa extract that is found in rat model may have also a translational value in human. For example, if 70kg average body weight of human takes 3,000mg of cocoa extract for 15days may have similar effects such as reduction of oxidative stress, apoptosis, and inflammatory markers that may be modulated by activities of Akt and ERK1/2; however, further study is required.

4.4. Recommendations

The above findings are obtained using optimal dose (15mg/kg/bw) at moderated chronic duration (15days) after I/R. However, this study recommends studies to be done using short-term treatment of rats with cocoa extract to evaluate the effect in oxidative stress, apoptosis, inflammatory markers and different signaling proteins levels in I/R-induced myocardial tissue of rats.

CHAPTER FIVE

5.1. REFERENCES

Adriana B, Jean S, Laura J, Samantha W, Angela W, Di Angelantonio E and Oscar H F. Chocolate consumption and cardiometabolic disorders. Systematic review and meta-analysis. British Medical Journal.2011; 343: 44884495.

Aebi H. Catalase in vitro. Methods Enzymology. 1984;121–126.

Agarwal S K. Dark chocolate: a therapeutic food. Medical Science. 2013: 1(1): 13-15.

Ahmed N, Linardi D, Decimo I, Mehboob R, Gebrie MA, Innamorati G, Luciani GB, Faggian G, Rungatscher A. Characterization and Expression of Sphingosine 1-Phosphate Receptors in Human and Rat Heart. Frontiers in Pharmacology. 2017; 312.

Ajay M, Gilani AU, Mustafa MR. Effects of flavonoids on vascular smooth muscle of the isolated rat thoracic aorta. Life Sciences.2003; 74**:** 603-612.

Akinmoladun AC, Olowe JA, Komolafe K, Ogundele J, Olaleye MT. Antioxidant activity and protective effects of cocoa and kola nut mistletoe (Globimetula cupulata) against ischemia/reperfusion injury in Langendorff-perfused rat hearts. Journal of Food and Drug Analysis. 2016; 24(2): 417-426.

Alamu S A. Analysis of seedling subsidy policy and cocoa production in south-west Nigeria. Nigerian Institute of Social and Economic Research. 2013;3(4): 59-68.

Alessi DR. Discovery of PDK1, one of the missing links in insulin signal transduction. Colworth Medal Lecture. Biochemical Society Transactions. 2001; 29: 1–14.

Amarone Tours. http://www.amaronetours.it/verona_wine_regions.

Armitage ME, Wingler K, Schmidt HH and La M. Translating the oxidative stress hypothesis into the clinic: NOX versus NOS. Journal of Molecular Medicine.2009; 87(11): 1071-1076.

Arts ICW and Hollman PCH. Polyphenols and disease risk in epidemiologic studies. The American Journal of Clinical Nutrition.2005; 81(1): 317-325.

Arts ICW, Hollman PCH, Kromhout D. Chocolate as a source of tea flavonoids. Lancet. 1999; 354:488.

Aura A M. Microbial metabolism of dietary phenolic compounds in the colon. Phytochemistry Reviews.2008;7(3):407–429, 2008.

Awe F B, Fagbemi T N, Ajibola, C F and Badejo A A.The antioxidant capacity of beverage blends made from cocoa, zobo and ginger. World Academy of Science, Engineering, and Technology. 2012; 68: 2082-2086.

Awe F B, Fagmebi TN, Olawunmi B, Ifesan T and Badejo AA. Antioxidant properties of cold and hot water extract of cocoa, Hibiscus flower extract, ginger beverage blend. Food Research International.2013; 52(2): 490-495.

Baba S, Natsume M, Yasuda A Nakamura Y, Tamura T, Osakabe N, Kanagae M and Kondo K. Plasma LDL and HDL cholesterol and oxidized LDL concentrations are altered in normo- and hypercholesterolemic humans after intake of different levels of cocoa powder. The Journal of Nutrition.2007;137(6): 1436-1441.

Baba S, Osakabe N, Natsume M, et al. Long-term intake of cocoa powder reduce the oxidative susceptibility of low-density lipoprotein in healthy humans. Japan Journal Medicine of Pharmacy Sci.2004; 52:947–63.

Baba S, Osakabe N, Natsume M, Yasuda A, Takizawa T, Nakamura T, Terao J. Cocoa powder enhances the level of antioxidative activity in rat plasma. British Journal of Nutrition. 2000;673-680.

Baba S, Osakabe N, Kato Y, Natsume M, Yasuda A, Kido T, Fukuda

K, Muto Y, and Kondo K. Continuous intake of polyphenolic compounds containing cocoa powder reduces LDL oxidative susceptibility and has beneficial effects on plasma HDL-cholesterol concentrations in humans. American Journal of Clinical Nutrition.2007;85(3)709-717.

Balga I, Leskó A, Ladányi M, Kállay M. Influence of aging on changes in polyphenolic compounds in red wines. Czech Journal of Food Sciences. 2014; 32: 563–569.

Balzer J, Rassaf T, Heiss C, Kleinbongard P, Lauer T, Merx M, Heussen N, Gross H B, Keen C L, Schroeter H and Kelm M.Sustained benefits in vascular function through flavanol-containing cocoa in medicated diabetic patients a double-masked, randomized, controlled trial. Journal of the American College of Cardiology.2008; 1: 2141-2149.

Barbanti D, Mora B, Ferrarini R,Tornielli G B, Cipriani M. Effect of various thermo-hygrometric conditions on the withering kinetics of grapes used for the production of ‘‘Amarone” and ‘‘Recioto” wines. Journal of Food Engineering. 2008; 85; 350 -358.

Baumann J, von Bruchhausen F, Wurm G.Flavonoids and related compounds as inhibition of arachidonic acid peroxidation. Prostaglandins.1980; 20: 627-639.

Bayard V, Chamorro F, Motta J and Hollenberg N K. Does flavanol intake influence mortality from nitric oxide-dependent processes? Ischemic heart disease, stroke, diabetes mellitus, and cancer in Panama. International Journal of Medical Science.2007; 4: 53-58.

Ben Mansour R, Gargouri B, Bouaziz M, Elloumi N, Belhadj Jilani I, Ghrabi Z, Lassoued S. Antioxidant activity of ethanolic extract of inflorescence of Ormenis Africana in vitro and in cell cultures. Lipids Health Diseases. 2011;78.

Beutler E, Duron O, Kelly B M. Improved method for the determination of

blood glutathione. Laboratory of Clinical. Medicine.1963;882–888.

Blausen.com staff. Medical gallery of Blausen Medical. Wiki Journal of Medicine. 2014.

Bo S, Gambino R, Guidi S, Silli B, Gentile L, Cassader M, Pagano GF. Plasma nitrotyrosine levels, antioxidant vitamins, and hyperglycemia. Diabet Med. 2005; 22(9):1185-9.

Bolli R. Basic and clinical aspects of myocardial stunning. Progress in Cardiovascular Diseases.1998; 40: 477-516.

Branen AL. Toxicology and biochemistry of butylated hydroxyanisole and butylated hydroxytoluene. J Am Oil Chem Soc. 1975; 59-63.

Buijsse B, Feskens E J, Kok FJ and Kromhout D.Cocoa intake, blood pressure, and cardiovascular mortality: The Zutphen Elderly Study. The Archives of International Medicine.2006; 166: 411-417.

Buijsse B, Weikert C, Drogan D, Bergmann M and Boeing H. Chocolate consumption in relation to blood pressure and risk of cardiovascular disease in German adults. The European Heart Journal.2010; 31: 1616-1623.

Calderón AI, Wright BJ, Hurst WJ and van Breemen R B, Screening Antioxidants Using LC-MS: A Case Study with Cocoa, Journal of Agricultural Food Chemistry.2009; 57(13)5693–5699.

Caliceti C, Rizzo P, Cicero AF. Potential benefits of berberine in the management of perimenopausal syndrome. Oxid Med Cell Longev. 2015; 723093.

Cao G, Sofic E, Prior RL. Antioxidant and Prooxidant Behavior of Flavonoids: Structure-activity Relationships. Free Radical Biology & Medicine, 1997; 22 (5): 749–760.

Cardona F, Andr´es-Lacueva C, Tulipani S, Tinahones FJ and Queipo-

Ortu˜no M I. Benefits of polyphenols on gut microbiota and implications in human health. Journal of Nutritional Biochemistry.2013; 24 (8):1415–1422.

Carl L Keen. Chocolate: Food as Medicine/Medicine as Food. Journal of the American College of Nutrition.2001; 20(5); 436–439.

Chan E, Liu X, Guo D, Kwan Y, Leung G P, Lee SM and Chan S. Extract of Scutellaria baicalensis Georgi root exerts protection against myocardial ischemia-reperfusion injury in rats. The American Journal of Chinese Medicine.2011; 39(4): 693-704.

Chan EC, Pannangpetch P, Woodman OL.Relaxation to flavones and flavonols in rat isolated thoracic aorta: mechanism of action and structure-activity relationships. Journal of Cardiovascular Pharmacology.2000; 35: 326-333.

Charo I F and Ransohoff R M.The many roles of chemokines and chemokine receptors in inflammation. New England Journal of Medicine.2006;354(6):610-21.

Cho SY, Park SJ, Kwon MJ, Jeong TS, Bok SH, Choi WY. Quercetin suppresses proinflammatory cytokines production through MAP kinases and NF-kappaB pathway in lipopolysaccharide-stimulated macrophage. Molecular Cellular Biochemistry.2003b; 243: 153-160.

Cimini A, Gentile R, D'Angelo B, et al. Cocoa Powder Triggers Neuroprotective and Preventive Effects in a Human Alzheimer's Disease Model by Modulating BDNF Signaling Pathway. Journal of cellular biochemistry. 2013;114(10):2209-2220.

Collard CD, Gelman S. Pathophysiology, Clinical Manifestations, and Prevention of Ischemia–Reperfusion Injury. Anesthesiology, Society of Anesthesiologists, 2001; 94:1133–8, Inc. Lippincott Williams & Wilkins, Inc.

Cooper KA, Donovan JL, Waterhouse A I and Williamson G. Cocoa and

health: A decade of research. British Journal of Nutrition. 2008; 99(1): 1-11.

Corti R, Flammer AJ, Hollenberg N K and Luscher T F.Cocoa and cardiovascular health. Circulation.2009; 119: 1433-1441.

Cos P, Ying L, Calomme M, Hu JP, Cimanga K, Van Poel B, et al. Structure-activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. Journal of Natural Products.1998; 61: 71-76.

Crespy V, Morand C, Besson C, Manach C, D´emign´e and R´em´esy C. Comparison of the intestinal absorption of quercetin, phloretin and their glucosides in rats. Journal of Nutrition.2001;31(8): 2109–2114.

D'Archivio M, Filesi C, Var`I R, Scazzocchio B, and Masella R. Bioavailability of the polyphenols: Status and controversies. International Journal of Molecular Sciences. 2010; 11(4):1321–1342.

Dal Santo S, Fasoli M, Negri S, D'Incà E, Vicenzi N, Guzzo F, Tornielli G B, Pezzotti M, and Zenoni S, Plasticity of the Berry Ripening Program in a White Grape Variety, Frontier of Plant Science. 2016;7, 970.

Dall'Asta M, Cirlini E, Morini G. Galaverna. Brand-dependent volatile fingerprinting of Italian wines from Valpolicella C. Journal of Chromatography. 2011; A, 1218; 7557–7565.

D'Amico M, Di Filippo C, La M, Solito E, McLean PG, Flower RJ, Oliani SM, and Perretti M, "Lipocortin 1reduces myocardial ischemia-reperfusion injury by affecting local leukocyte Recruitment", FASEB Journal, vol.14, no. 13, pp. 1867-9, 2000.

D'Archivio M, Filesi C, Vari R, Scazzocchio B and Masella R.Bioavailability of the polyphenols: status and controversies. International Journal of Molecular Sciences.2010; 11(4): 1321-1342.

Darra E, Rungatscher A, Carcereri de Prati A, Podesser BK, Faggian G, Scarabelli T, Mazzucco A, Hallström S, Suzuki H. Dual modulation of nitric oxide production in the heart during ischemia/reperfusion injury and inflammation. Thromb Haemost. 2010; 200-6.

Desch S, Schmidt J, Kobler D, Sonnabend M, Eitel I, Sareban M, Rahimi K, Schuler G and Thiele H.Effect of cocoa products on blood pressure: systematic review and meta-analysis. American Journal of Hypertension.2010; 23(1): 97-103.

Ding EL, Hutfless SM, Ding X and Girotra S.Chocolate and prevention of cardiovascular disease: a systematic review. Nutrition and Metabolism.2006; 3: 2-13.

Donovan J L, Crespy V, C. Manach C.et al., Catechin is metabolized by both the small intestine and liver of rats. Journal of Nutrition.2001; 131:(6) 1753–1757.

Disciplinare D.O.C., D.M. Vini della Valpolicella, Gazzetta Ufficiale della Repubblica Italiana n◦ 84 del 12/04/2010),2011.

Duraiyan J, Govindarajan R, Kaliyappan K and Palanisamy M. Applications of immunohistochemistry. Journal of Pharmacy Bioallied Sciences. 2012; 4:307–309.doi: 10.4103/0975-7406.100281

Ella Z, Alice 0, Dianna J M, Danny L and Reid C M. The effectiveness and cost effectiveness of dark chocolate consumption as prevention therapy in people at high risk of cardiovascular disease. British Medical Journal.2012; 344: 3657-3665

Empana JP, Jouven X, Canoui-Poitrine F, Luc G, Tafflet M, Haas B, Arveiler D, Ferrieres J, Ruidavets JB, Montaye M, Yarnell J, Morange P, Kee F, Evans A, Amouyel P, and Ducimetiere P.C-reactive protein, interleukin 6, fibrinogen and risk of sudden death in European middle-aged men: the PRIME study. Arteriosclerosis, Thrombosis and Vascular

Biology.2010;30(10):2047-52.

Entman ML, Smith CW. Postreperfusion inflammation: a model for reaction to injury in cardiovascular disease. Cardiovascular Research.1994; 28: 1301-1311.

Falany C N. Enzymology of human cytosolic sulfotransferases. The FASEB Journal.1997;11 (4)206–216.

Ferrarini R, & Zironi R. La surmaturazione delle uve destinate alla vinificazione. Vignevini.1987; 4, 31–45.

Ferrarini R. Nuova tecnologia per la surmaturazione delle uve. Vignevini.1982;9: 34–40.

Fisher ND and Hollenberg NK. Aging and vascular responses to flavanol-rich cocoa. Journal of Hypertension. 2006;24:1575-1580.

Fisher ND and Hollenberg N K.Flavanols for cardiovascular health: the science behind the sweetness. Journal of Hypertension. 2005; 23(8): 1453-1459.

Fisher ND, Hughes M, Gerhard-Herman M. and Hollenberg N K.Flavanol-rich cocoa induces nitric-oxide-dependent vasodilation in healthy humans. Journal of Hypertension. 2003; 21: 2281-2286.

Flammer AJ, Hermann F, Sudano I, Spieker L, Hermann M, Cooper KA, Serafini M, Luscher T F, Ruschitzka F, Noll G, and Corti R. Dark chocolate improves coronary vasomotion and reduces platelet reactivity. Circulation.2007;116: 2376-2382.

Forte M, Conti V, Damato A, Ambrosio M, Puca AA, Sciarretta S, Frati G, Vecchione C, Carrizzo A. Targeting Nitric Oxide with Natural Derived Compounds as a Therapeutic Strategy in Vascular Diseases. Oxid Med Cell Longev. 2016; 7364138.

Fotsis T, Pepper MS, Aktas E, et al. Flavonoids, dietary-derived inhibitors of cell proliferation and in vitro angiogenesis. Cancer Research.1997; 57: 2916–21.

Francis ST, Head K, Morris PG and Macdonald I A. The effect of flavanol-rich cocoa on the fMRI response to a cognitive task in healthy young people. Journal of Cardiovascular Pharmacology.2006; 47: 215-220.

Frangogiannis NG, Dewald O, Xia Y, G. Ren G, Haudek S, Leucker T. Critical role of monocyte chemoattractant protein-1/CC chemokine ligand 2 in the pathogenesis of ischemic cardiomyopathy. Circulation.2007;115(5)584-92.

Frangogiannis NG, Smith CW and Entman ML.The inflammatory response in myocardial infarction. Cardiovascular Research.2002;53(1): 31-47.

Fuchs M, Hilfiker A, Kaminski K, Hilfiker-Kleiner D, Guener Z, Klein G, Podewski E, Schieffer B, Rose-John S and Drexler H.Role of interleukin-6 in left ventricular remodeling and survival after experimental myocardial infarction. Faseb Journal.2003;17 (14) 2118-20.

Gallo M, Vinci G, Graziani G, de Simone C and Ferranti P. The interaction of cocoa polyphenols with milk proteins studied by proteomic techniques. Food Research International. 2013; 54: 406-415.

Gerritsen ME, Carley WW, Ranges GE, Shen CP, Phan SA, Ligon GF et al. Flavonoids inhibit cytokine-induced endothelial cell adhesion protein gene expression. American Journal of Pathology.1995; 147: 278-292.

Ghosh D. Anthocyanins and anthocyanin-rich extracts in biology and medicine: Biochemical, cellular, and medicinal properties, Current in Topics Nutraceutical Research. 2005; 3: 113 —124.

Gibbs C R, Blann AD, Watson RD and Lip GY. Abnormalities of hemorheological, endothelial, and platelet function in patients with chronic heart failure in sinus rhythm: effects of angiotensin-converting enzyme

inhibitor and beta-blocker therapy. Circulation.2001; 103: 1746-1751.

Giordano FJ. Oxygen, oxidative stress, hypoxia, and heart failure. J Clin Invest. 2005; 500- 8.

Gonzalez LM, Moeser AJ, Blikslager AT. Animal models of ischemia-reperfusion-induced intestinal injury: progress and promise for translational research. 2015.

Goya L, Martín M Á, Sarriá B, Ramos S, Mateos R, Bravo L. Effect of Cocoa and Its Flavonoids on Biomarkers of Inflammation: Studies of Cell Culture, Animals and Humans. Nutrients. 2016; 8(4): 212.

Goya L, Martín M Á, Sarriá B, Ramos S, Mateos R, Bravo L. Effect of Cocoa and Its Flavonoids on Biomarkers of Inflammation: Studies of Cell Culture, Animals and Humans. Nutrients. 2016; 8(4): 212.

Grassi D, Necozione S, Lippi C, Croce G, Valeri L, Pasqualetti P, Desideri G, Blumberg J B and Ferri C. Cocoa reduces blood pressure and insulin resistance and improves endothelium-dependent vasodilation in hypertensives. Hypertension.2005a; 46: 398-405.

Grivetti LE and Howard-Yana S. Chocolate: History, Culture, and Heritage. 1st edn, John Wiley and Sons, Chicester United Kingdom. 2009;1-975.

Grzybowski M, Welch RD, Parsons L, Ndumele CE, Chen E, Zalenski R, et al. The association between white blood cell count and acute myocardial infarction in-hospital mortality: findings from the National Registry of Myocardial Infarction. Academic Emergency Medicine.2004; 11: 1049-1060.

Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, Brown M, Fitzgerald KJ, Sabatini DM. Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCα, but not S6K1. Developmental Cell. 2006; 11: 859–871.

Gülçin I, Oktay M, Küfrevioğlu OI and Aslan I. Determination of antioxidant activity of lichen. Cetraria islandica (L) Ach. Journal of Ethnopharmacology.2002;79(3)325-329.

Hakkinen S. Flavonols and phenolic acids in berries and berry products. Ph.D. dissertation, Faculty of Medicine, Kuopio, Finland, 2000.

Han X, Shen T and Lou H. Dietary polyphenols and their biological significance. International Journal of Molecular Sciences.2007; 8(9): 950-958.

Hansen PR. Role of neutrophils in myocardial ischemia and reperfusion. Circulation. 1995;91: 1872-1885.

Harborne J B. The Flavonoids: Advances in research since 1986. Chapman and Hall, London. 1993.

Harris GK, Qian Y, Leonard SS, Sbarra DC, Shi X. Luteolin and chrysin differentially inhibit cyclooxygenase-2 expression and scavenge reactive oxygen species but similarly inhibit prostaglandin-E2 formation in RAW 264.7 cells. Journal of Nutrition.2006; 136: 1517-1521.

Hausenloy DJ and Yellon DM. Reperfusion injury salvage kinase signaling: taking a RISK for cardioprotection. Heart Failure Reviews.2007; 12: 217-234.

He S, Sun C and Pan Y. Red Wine Polyphenols for Cancer Prevention. International Journal of Molecular Science.2008; 9(5): 842–853.

Heim K E Tagliaferro A R and Bobilya D J. Flavonoid antioxidants: chemistry, metabolism, and structure-activity relationships. Journal of Nutritional Biochemistry.2002; 13(10): 572-584.

Heiss C, Dejam A, Kleinbongard P, Schewe T, Sies H and Kelm M. Vascular effects of cocoa rich in flavan-3-ols. The Journal of the American Medical Association. 2003; 290: 1030-1031.

Heiss C, Kleinbongard P, Dejam A and Pen S. Acute consumption of flavanol-rich cocoa and the reversal of endothelial dysfunction in smokers. Journal of the American College of Cardiology.2005; 46: 1276-1283.

Hemmings BA and Restuccia DF. PI3K-PKB/Akt Pathway. Cold Spring Harbor Perspectives in Biology. 2012; 4(9):doi: 10.1101/cshperspect.a011189.

Henderson JS, Joyce R A, Hall GR, Hurst W J and McGovern P E. Chemical and archaeological evidence for the earliest cacao beverages. Proceedings of the National Academy of Sciences.2007; 104: 18937-18940.

Heo HJ, Lee CY. Epicatechin and Catechin in Cocoa Inhibit Amyloid Protein Induced Apoptosis. J. Agric. Food Chem. 2005; 53 (1445):1448-1445.

Herrera MD, Zarzuelo A, Jimenez J, Marhuenda E, Duarte J. Effects of flavonoids on rat aortic smooth muscle contractility: structure-activity relationships. General Pharmacology.1996; 27: 273-277.

Holmes-McNary M, Baldwin AS Jr. Chemopreventive properties of trans-resveratrol are associated with inhibition of activation of the IkappaB kinase. Cancer Research. 2000;60(13):3477-83.

Hooper L, Kay C, Abdelhamid A, Kroon PA, Cohn J S, Rimm E B and Cassidy A. Effects of chocolate, cocoa, and flavan-3-ols on cardiovascular health: a systematic review and meta-analysis of randomized trials. American Journal of Clinical Nutrition. 2012;95(3): 740-751.

Hristova K, Nakaoka T, Otsuka K, Fedacko J, Singh R, Singh RB, Wilczynska F A and Wilson D W.Perspectives on chocolate consumption and risk of cardiovascular diseases and cognitive function. The Open Nutraceuticals Journal.2012;5: 207-212.

Hurst W J, Krake S H, Bergmeier SC, Payne M J, Miller KB and Stuart D A. Impact of fermentation, drying, roasting and Dutch processing on flavan-

3-ol stereochemistry in cacao beans and cocoa ingredients. Chemistry Central Journal.2011; 5(1): 53-62.

Integrated Taxonomic Information System (ITIS). Malvales of North America Update Database. Flora of North America Expertise Network, in connection with an Update for USDA PLANTS (2007-2010); (version 2011).

Jain DKK. The Handbook of Neuroprotection. Jain Pharma Biotech, Springer Science+ Business Media, Humana press. 2011;779-048-5.

Jakubowski A, N. Maksimovich , Olszanecki R. S-nitroso human serum albumin given after LPS challenge reduces acute lung injury and prolongs survival in a rat model of endotoxemia. Naunyn-Schmiedeberg's Archives of Pharmacology. 2009; 379: 281.

Jalil A B M M and Ismail A. Polyphenols in cocoa and cocoa products: is there a link between antioxidant properties and health? Molecules.2008; 13(9): 2190-2219.

Jennings RB, Sommers HM, Smyth GA, Flack HA, Linn H. Myocardial necrosis induced by temporary occlusion of a coronary artery in the dog. Archives of Pathology.1960;70: 68-78.

Jose CJ, Vatner DE, Vatner SF. Myocardial apoptosis in heart disease: does the emperor have clothes? Basic Res Cardiol. 2016; 111(3):31.

Karim M, McCormick K and Kappagoda C T. Effects of cocoa extracts on endothelium-dependent relaxation. Journal of Nutrition.2000; 130(8): 2105-2108.

Karthikeyan K, Sarala bai B R and Niranjali Devaraj S. Grape seed proanthocyanidins ameliorate isoproterenol-induced myocardial injury in rats by stabilizing mitochondrial and lysosomal enzymes: an in vivo study, 2007.Life Sciences, vol.81, pp.1615-21.

Karunakaran KG, Muhammad TS, Peter, Vinoth VP, Karthikeyan KK, Niranjali ND and Jayaprakash SS. Cardioprotective effect of the Hibiscus rosa sinensis flowers in an oxidative stress model of myocardial ischemic reperfusion injury in rat. BMC Complementary and Alternative Medicine.2006;6-32.

Katz SD, Hryniewicz K, Hriljac I, Balidemaj K, Dimayuga C, Hudaihe, A and Yasskiy A. Vascular endothelial dysfunction and mortality risk in patients with chronic heart failure. Circulation. 2005; 111: 310-314.

Keogh J B, McInerney J, and Clifton P M. The effect of milk protein on the bioavailability of cocoa polyphenols. Journal of Food Science. 2007; 72(3): 230233.

Kimata M, Shichijo M, Miura T, Serizawa I, Inagaki N, Nagai H. Effects of luteolin, quercetin and baicalein on immunoglobulin E-mediated mediator release from human cultured mast cells. Clinical Experimental of Allergy.2000; 30: 501-508.

Kirimlioglu V, Sozen H, Turkoglu S and Haberal M. Protective effect of resveratrol, a red wine constituent polyphenols, in rats subjected to portal vein thrombosis, Transplantation Proceeding 2008; 40: 290-292.

Kiviniemi T 0, Saraste A, Toikka J 0 and Saraste M. A moderate dose of red wine, but not de-alcoholized red wine increases coronary flow reserve. Atherosclerosis.2007; 195: 176-181.

Kjekshus J, Apetrei E, Barrios V, Bohm M, Cleland J G, Cornel J H, Dunselman P, Fonseca C, Goudev A, Grande P, Gullestad L, Hjalmarson A, Hradec J, Janosi A, Kamensky G, Komajda M, Korewicki J, Kuusi T, Mach F, Mareev V, McMurray J J, Ranjith N, Schaufelberger M, Vanhaecke J, van Veldhuisen D J, Waagstein F, Wedel H and Wikstrand J. Rosuvastatin in older patients with systolic heart failure. The New England Journal of Medicine.2007; 357: 2248-2261.

Kolk MVV, Meyberg D, Deuse T, Tang-Quan KR, Robbins RC, Reichenspurner H, Schrepfer S. LAD-Ligation: A Murine Model of Myocardial Infarction. Journal of Visualized Experiments. 2009; 32:1-3. doi: 10.3791/1438.

Kume N, Cybulsky MI, Gimbrone MA. Lysophosphatidylcholine, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured human and rabbit arterial endothelial cells. Journal of Clinical investigation.1992;90:1138-44.

Kume N, Gimbrone MA Jr. Lysophosphatidylcholine transcriptionally induces growth factor gene expression in cultured human endothelial cells. Journal of Clinical investigation.1994;93:907–11.

Kundu JK, Surh Y-J. Molecular basis of chemoprevention by resveratrol: NF-κB and AP-1 as potential targets. Mutation Research. 2004; 555:65–80.

Kurosawa T, Itoh F, Nozaki A, et al. Suppressive effects of cacao liquor polyphenols (CLP) on LDL oxidation and the development of atherosclerosis in Kurosawa and Kusanagi-hypercholesterolemic rabbits. Atherosclerosis.2005; 179:237–46.

Lara Testai. Flavonoids and mitochondrial pharmacology: A new paradigm for cardioprotection Life Sciences. 2015; 135: 68–76.

Larsson SC, Virtamo J and Wolk A. Chocolate consumption and risk of stroke: A prospective cohort of men and meta-analysis. Neurology.2012; 79: 12231229.

Lee G and Goosens K A.Sampling Blood from the Lateral Tail Vein of the Rat.Journal of Visualized Experiments. 2015: 99, e52766, doi:10.3791/52766, 2015.

Lee KW, Kim YJ, Lee HJ, Lee CY. Cocoa Has More Phenolic Phytochemicals and a Higher Antioxidant Capacity than Teas and Red Wine. American Chemical Society. J. Agric. Food Chem., **2003**; 51 (25): 7292–7295.

Lee M J, Maliakal P, Chen L. et al., Pharmacokinetics of tea catechins after ingestion of green tea and (-)-epigallocatechin- 3-gallate by humans: Formation of different metabolites and individual variability. Cancer Epidemiology Biomarkers and Prevention. 2002;11(10):1025–1032.

Lesschaeve I, Noble AC. Polyphenols: factors influencing their sensory properties and their effects on food and beverage preferences. American Journal of Clinical and Nutrition. 2005;330S-335S.

Li Y, Feng Y, Zhu S, Luo C, Ma J and Zhong F. The effect of alkalization on the bioactive and flavor-related components in commercial cocoa powder. Journal of Food Composition and Analysis.2012; 25(1): 17-23.

Liara M. Gonzalez,1 Adam J. Moeser,2 and Anthony T. Blikslager. Animal models of ischemia-reperfusion-induced intestinal injury: progress and promise for translational research.

Lindahl M, Tagesson C. Selective inhibition of group II phospholipase A2 by quercetin. Inflammation.1993; 17: 573-582.

Lippi G, Franchini M, Favaloro E J and Targher G. Moderate Red Wine Consumption, and Cardiovascular Disease Risk: Beyond the "French Paradox", Seminar Thrombosis Hemostasis. 2010;36(1): 059-070.

Loft S, Poulsen HE. Cancer risk and oxidative DNA damage in man. Journal of Molecular Medicine. 1996; 74:297–312.

Luo Y, Shang P, Li D. Luteolin: A Flavonoid that Has Multiple Cardio-Protective Effects and Its Molecular Mechanisms. Frontiers of Pharmacology. 2017;692.

Magnani L, Gaydou EM, Hubaud JC.Spectrophotometric measurement of antioxidant properties of flavones and flavonols against superoxide anion. Analytica Chimica Acta. 2000; 411: 209-216.

Mahmoud NN, Carothers AM, Grunberger D, Bilinski RT, Churchill MR,

Martucci C, Newmark HL, Bertagnolli MM. Plant phenolics decrease intestinal tumors in an animal model of familial adenomatous polyposis. Carcinogenesis 2000; 21:921–927.

Mandel S, Weinreb 0, Amit T and Youdim M B H.Cell signaling pathways in the neuroprotective actions of the green tea polyphenol (-)-epigallocatechin-3-gallate: implications for neurodegenerative diseases. Journal of Neurochemistry.2004; 88(6): 1555-1569.

Manten A, de Winter R J, Minnema MC, ten Cate H, Lijmer J G, Adams R, Peters RJ and van Devante S J.Procoagulant and proinflammatory activity in acute coronary syndromes.Cardiovascular Research.1998;40(2):389-95.

Marín L, Miguélez E L, Villar C J and Lombó F. Bioavailability of Dietary Polyphenols and Gut Microbiota Metabolism: Antimicrobial Properties BioMed Research International.2015; 905215: 1-18.

Middleton EJ. Effect of plant flavonoids on immune and inflammatory cell function. Advances of Experimental Medical Biology. 1998; 439:175–82

Misnawi S J, Jamilah B and Nazamid S. Effect of polyphenol concentration on pyrazine formation during cocoa liquor roasting. Food Chemistry.2004a; 85(1): 73-80.

Misnawi S J, Jamilah B and Nazamid S. Sensory properties of cocoa liquor as affected by polyphenol concentration and duration of roasting. Food Quality and Preference.2004b; 15(5): 403-409.

Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. Journal of Biology and Chemistry. 1972; 3170–3175.

Mohanty I, Singh Arya D, Dinda A, Joshi S, Talwar KK, Gupta SK. Protective effects of Curcuma longa on ischemia-reperfusion induced myocardial injuries and their mechanisms. Life Sci. 2004;1701-11.

Monaga M, Urpi-Sarda M, S´anchez-Pat F. et al.,.Insights into themetabolism and microbial biotransformation of dietary flavan-3-ols and the bioactivity of their metabolites. Food and Function. 2010;1, (3):233–253.

Munzel T, Gori T, Bruno R M and Taddei S. Is oxidative stress a therapeutic target in cardiovascular disease? The European Heart Journal.2010; 31: 2741-2748.

Munzel T, Gori T, Keaney JF Jr, Maack C, Daiber A. Pathophysiological role of oxidative stress in systolic and diastolic heart failure and its therapeutic implications. Eur Heart J. 2015; 2555-64.

Naissides M, Pal S, Mamo JCL, James AP and Dhaliwal S. The effect of chronic consumption of red wine polyphenols on vascular function in postmenopausal women. The European Journal of Clinical Nutrition.2006;60: 740 -745.

Nehlig A. The neuroprotective effects of cocoa flavanol and its influence on cognitive performance. Br J Clin Pharmacol. 2013; 75(3): 716–727.

Neilson AP, Song BJ, Sapper TN, Bomser JA and Ferruzzi MG. Tea Catechin Auto-oxidation Dimers are Accumulated and Retained by Caco-2 Human Intestinal Cells. Nutrition Research.2010; 30(5): 327-340.

Neukam K, Stahl W, Tronnier H, Sies H and Heinrich U. Consumption of flavanol-rich cocoa acutely increases microcirculation in human skin. European Journal of Nutrition. 2007. 46: 53 —56.

Nielsen S E, Breinholt V, Justesen U, Cornet C T, and Dragsted L O.In vitro biotransformation of flavonoids by rat liver microsomes. Xenobiotica.1998: 28(4):389–401, 1998.

Nijveldt RJ, van Nood E, van Hoorn DE, Boelens PG, van Norren K, van Leeuwen PA. Flavonoids: a review of probable mechanisms of action and potential applications. American Journal of Clinical Nutrition.2001; 74:

418-425.

Nsor-Atindana J, Zhong F, Mothibe K J, Bangoura M L and Lagnika C. Quantification of Total Polyphenolic Content and Antimicrobial Activity of Cocoa (Theobroma cacao L.) Bean Shells. Pakistan Journal of Nutrition. 2012; 11(7): 574-579.

Oemar B S, Tschudi M R, Godoy N, Brovkovich V, Malinski T and Luscher TF. Reduced endothelial nitric oxide synthase expression and production in human atherosclerosis. Circulation.1998; 97: 2494-2498.

Orallo F, Camina M, Alvarez E, Basaran H, Lugnier C. Implication of cyclic nucleotide phosphodiesterase inhibition in the vasorelaxant activity of the citrus-fruits flavonoid (+/-)-naringenin. Planta Medicine.2005; 71: 99-107.

Osakabe N, Baba S, Yasuda A, et al. Daily cocoa intake reduces the susceptibility of low-density lipoprotein to oxidation as demonstrated in healthy human volunteers. Free Radical Research. 2001; 34:93–9.

Ouzzine M, Barr´e L, Netter P, Magdalou J, and Fournel- Gigleux S. The human UDP-glucuronosyltransferases: structural aspects and drug glucuronidation. Drug Metabolism Reviews. 2003;35(4):287–303.

Pan MH, Lai CS, Wu JC, Ho CT. Molecular mechanisms for chemoprevention of colorectal cancer by natural dietary compounds. Molecular Nutrition Food Research. 2011;55(1):32.

Park J L and Lucchesi B R. Mechanisms of myocardial reperfusion injury. The Annals of Thoracic Surgery.1095;8(5): A1-A26.

Patricia Guy. Wines of Italy, Windsor 2003. Tide-Mark Press

Peters CM, Green RJ, Janle EM and Ferruzzi MG.Formulation with ascorbic acid and sucrose modulates catechin bioavailability from green tea. Food Research International. 2010; 43(1): 95-102.

Pietta PG. Flavonoids as antioxidants. Journal of Natural Production.2000; 63: 1035-1042.

Pohlan H A J J and Perez V S. Growth and production of cocoa. Soils, Plant Growth and Crop Production.2011; 3: 1-11.

Polson C, Sarkar P, Incledon B, Raguvaran V and Grant R. Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography–tandem mass spectrometry. Journal of Chromatography.2003;785:(2):263-275.

Pritchard C C and Grad W M. Colorectal Cancer Molecular Biology Moves into Clinical Practice. Gut. 2011; 60(1): 116–129.

Procházková D, Boušová I, Wilhelmová N. Antioxidant and prooxidant properties of flavonoids. Fitoterapia. 2011; 82:513–523.

Pryor WA. Cigarette smoke radicals and the role of free radicals in chemical carcinogenicity. Environmental Health Perspectives 1997; 105: 875–82.

Qin CX, Chen X, Hughes RA, Williams SJ, Woodman OL. Understanding the cardioprotective effects of flavonols: discovery of relaxant flavonols without antioxidant activity. Journal Medicine of Chemistry.2008; 51: 1874-1884.

Qiu H, Liu J, Wei D, Li N, Yamoah EN, Hammock BD. and Chiamvimonvat N. Cardiac-generated prostanoids mediate cardiac myocyte apoptosis after myocardial ischaemia. Cardiovascular Research.2012; 1-10.

Quinones M, Miguel M and Aleixandre A. Beneficial effects of polyphenols on cardiovascular disease. Pharmacological Research.2013; 68: 125131.

Ramiro-Puig E, Casadesús G, Lee H, Zhu X, McShea A, Perry G, Pérez-Cano FJ, Smith MA, Castell M. Neuroprotective effect of cocoa flavonoids on in vitro oxidative stress. European Journal of Nutrition. 2009; 48 (1):54–

61.

Ramos S, Martin MA, Goya L. Effects of Cocoa Antioxidants in Type 2 Diabetes Mellitus. Antioxidants. 2017;6(4).

Ramos S. Cancer chemoprevention and chemotherapy: dietary polyphenols and signaling pathways. Molecular Nutrition. Food Research. 2008; 52: 507–526.

Ramos S. Effects of dietary flavonoids on apoptotic pathways related to cancer chemoprevention. Journal of Nutritional Biochemistry.2007;18(7): 427-442.

Rechner AR, Smith MA, Kuhnle G et al., Colonic metabolism of dietary polyphenols: influence of structure on microbial fermentation products. Free Radical Biology and Medicine.2004;36(2) 212–225.

Reddy KS, Yusuf S. Emerging epidemic of cardiovascular disease in developing countries. Circulation. 1998;97(6):596-601.

Reddy M B, Hurrel RF, and Cook J D. Meat consumption in a varied diet marginally influence non-heme iron absorption in normal individuals. Journal of Nutrition.2006;136, (3)576-581.

Reichel CA, Khandoga A, Anders HJ, Schlondorff D, Luckow B and Krombach F. Chemokine receptors Ccr1, Ccr2, and Ccr5 mediate neutrophil migration to postischemic tissue. Journal of Leukocyte Biology. 2006;79(1):114-22

Reid K, Sullivan T, Fakler P, Frank, O R and Stocks N P.Does chocolate reduce blood pressure? A meta-analysis. BioMed Central Medicine.2010; 8: 39.

Rice-Evans CA, Miller N J and Paganga G. Antioxidant properties of phenolic compounds. Trends in Plant Science.1997; 2(4): 152-159.

Rodrigo R, Rivera G, Orellana M, Araya J, Bosco C. Rat kidney antioxidant response to long-term exposure to flavonol-rich red wine. Life Sciences.2002; 71: 2881-2895.

Romero M, Jimenez R, Sanchez M, Lopez-Sepulveda R, Zarzuelo MJ, O'Valle F. Quercetin inhibits vascular superoxide production induced by endothelin-1: Role of NADPH oxidase, uncoupled eNOS and PKC. Atherosclerosis.2009; 202: 58-67.

Roura E, Andres-Lacueva C, Estruch R, Mata MLB, Izquierdo-Pulido M, Waterhause AL and Lamuela-Raventos RM. Milk does not affect the bioavailability of cocoa powder flavonoid in healthy human. Annals of Nutrition and Metabolism.2007; 51(6): 493-498.

Rungatscher A, Hallström S, Linardi D, Milani E, Gasser H, Podesser BK, Scarabelli TM, Luciani GB, Faggian G. S-nitroso human serum albumin attenuates pulmonary hypertension, improves right ventricular-arterial coupling, and reduces oxidative stress in a chronic right ventricle volume overload model. J Heart Lung Transplant. 2015; 479-88.

Rusconi M. and Conti, A. Theobroma cacao L., the Food of the Gods: a scientific approach beyond myths and claims. Pharmacological Research.2010; 61(1): 5-13.

Saha MR, Alam A, Akter R, and Jahangir R. In vitro free radical scavenging activity of Ixora coccinea L. Bangladesh Journal of Pharmacology.2008;3:90-6, 2008.

Sakano K, Mizutani M, Murata M, Oikawa S, Hiraku Y, Kawanishi S. Procyanidin B2 has anti- and pro-oxidant effects on metal-mediated DNA damage. Free Radic Biol Med. 2005; 39:1041–1049.

Salah N, Miller NJ, Paganga G, Tijburg L, Bolwell GP, Rice-Evans C.Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. Archives of Biochemistry and Biophysics.

1995; 322: 339-346.

Saltini R, Akkerman R and Frosch S. Optimizing chocolate production through traceability: a review of influence of farming practices on cocoa bean quality. Food Control.2013; 29: 167-187.

Samman S, Sandstrom B, Toft M B, Bukhave K, Jenzen , Sorenzen SS, Hazen M. Green tea or rosemary extract added to food reduces non-heme iron absorption,.American Society for Clinical Nutrition.2001;73:607-6012.

Samsamshariat SA, Samsamshariat ZA, Movahed M. A novel method for safe and accurate left anterior descending coronary artery ligation for research in rats. Cardiovascular Revascularization Medicine.2005;6:121–123.

Sanbongi C, Suzuki N, Sakane T. Polyphenols in Chocolate, Which Have Antioxidant Activity, Modulate Immune Functions in Humans in Vitro. Cellular Immunology. 1997; 177: (CI971109): 129-136.

Sanchez M, Lodi F, Vera R, Villar IC, Cogolludo A, Jimenez R, et al. Quercetin and isorhamnetin prevent endothelial dysfunction, superoxide production, and overexpression of p47phox induced by angiotensin II in rat aorta. Journal of Nutrition. 2007; 137: 910-915.

Sanhueza J, Valdes J, Campos R, Garrido A, Valenzuela A. Changes in the xanthine dehydrogenase/xanthine oxidase ratio in the rat kidney subjected to ischemia-reperfusion stress: preventive effect of some flavonoids. Res Commun Chem Pathol Pharmacol 1992; 78: 211–8.

Sansone R, Rodriguez-Mateos A, Heuel J, Falk D, Schuler D, Wagstaff R, Kuhnle GGC, Spencer JPE, Schroeter H, Merx MW Kelm M, Heiss C. Cocoa flavanol intake improves endothelial function and Framingham Risk Score in healthy men and women: a randomised, controlled, double-masked trial: The Flaviola Health Study. British Journal of Nutrition, 2015; 1DOI: 10.1017/S0007114515002822.

Sarr M, Chataigneau M, Martins S, Schott C, Bedoui J El, Oak M H, Muller B, Chataigneau T, Schini-Kerth V. B.Red wine polyphenols prevent angiotensin II-induced hypertension and endothelial dysfunction in rats: Role of NADPH oxidase. Cardiovascular Research. 2006; 71 (4); 794–802.

Sawyer DB, Siwik DA, Xiao L, Pimentel DR, Singh K, Colucci WS. Role of oxidative stress in myocardial hypertrophy and failure. J Mol Cell Cardiol. 2002; 379-88.

Scalbert A, Manach C, Morand C, Remesy C and Jimenez L. Dietary polyphenols and the prevention of diseases, Critical Reviews in Food Science and Nutrition.2005;45: 287-306.

Scalbert A, Morand C, Manach C, Remesy C. Absorption and metabolism of polyphenols in the gut and impact on health. Biomed. Pharmacother. 2002; 56:276–282.

Schinella G, Mosca S, Cienfuegos-Jovellanos E, Pasamar MA, Muguerza B, Ramon D and Rios J L. Antioxidant properties of polyphenol-rich cocoa products industrially processed. Food Research International.2010; 43(6): 1614-1623.

Schneider CL, Cowles R L, Stuefer-Powell CL, Carr T P.Dietary stearic acid reduces cholesterol absorption and increases endogenous cholesterol excretion in hamsters fed cereal-based diets. Journal of Nutrition.2002;130(5):1232-8.

Schramm DD, Karim M, Schrader HR, Holt RR, Kirkpatrick NJ, Polagruto JA, Ensunsa JL, Schmitz HH and Keen CL. Food effects on the absorption and pharmacokinetics of cocoa flavanols. Life Sciences.2003; 73: 857869.

Schroeter H, Heiss C, Balzer J. and Kleinbongard P. (-)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. Proceedings of the National Academy of Sciences, USA. 2006; 103: 10241029.

Sebastian RS, Enns CW, Goldman JD, Moshfegh AJ. Dietary flavonoid intake is inversely associated with cardiovascular disease risk as assessed by body mass index and waist circumference among adults in the united states. NDPI, Nutrients. 2017; 9:827.

Selma V, Esp´ın JC, and Tom´as-Barber´an F A. Interaction between phenolics and gut microbiota: role in human health. Journal of Agricultural and Food Chemistry. 2009; 57(15): 6485–6501.

Serafini M, Bugianesi R, Maiani G, Valtuena S, De Santis S, Crozier A. Plasma antioxidants from chocolate. Nature. 2003;1013-7.

Serafini M, Bugianesi R, Maiani G, Valtuena S, De Santis S, Crozier A.Plasma antioxidants from chocolate" Nature, vol. 424 , no.6952, pp.1013,2003.

Shahidi F and Naczk M, Shahidi F, Naczk M. Food Phenolics: Sources, Chemistry, Effects, Applications. Technomic Publishing Co. Inc., Lancaster Pennsylvania, USA.1995; 281-319.

Shankar S, Singh G, Srivastava RK. Chemoprevention by resveratrol: molecular mechanisms and therapeutic potential. Frontier Biosciences. 2007; 12:4839–4854.

Shoskes DA. Effect of bioflavonoids quercetin and curcumin on ischemic renal injury: a new class of renoprotective agents. Transplantation.1998; 66:147–52.

Sio S W, Ang S F, Lu J, Moochhala S, and Bhatia M. Substance P upregulates cyclooxygenase-2 and prostaglandin E metabolite by activating ERK1/2 and NF-kappaB in a mouse model of burn-induced remote acute lung injury. Journal of Immunology.2010;185: 6265.

Skelhon T, Grossiord N, Morgan A and Bon S. Quiescent water-in-oil Pickering emulsions as a route toward healthier fruit juice infused chocolate confectionary. Journal of Materials Chemistry.2012; 22: 19289-19295.

Spagnuolo C, Napolitano M, Tedesco I, Moccia S, Milito A, Russo G L. Neuroprotective Role of Natural Polyphenols Source: Current Topics in Medicinal Chemistry.2016; 16 (17)1943-1950.

Stalmach A, Steiling H, Williamson G, and Crozier A.Bioavailability of chlorogenic acids following acute ingestion of coffee by humans with an ileostomy. Archives of Biochemistry and Biophysics.2010;501(1): 98–105.

Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. New England Journal of Medicine. 1989; 320:915–24.

Steinberg F M, Bearden MM and Keen C L. Cocoa and chocolate flavonoids: implications for cardiovascular health. Journal of American Dietetic Association.2003; 103 (2): 215-23.

Surh Y J. Cancer chemoprevention with dietary phytochemicals. Nature Reviewers. Cancer.2003; 3: 768–780.

Swedberg K, Cleland J, Dargie H, Drexler H, Follath F, Komajda M, Tavazzi L, Smiseth O A, Gavazzi A, Haverich A, Hoes A, Jaarsma T, Korewicki J, Levy S, Linde C, Lopez-Sendon J L, Nieminen MS, Pierard L and Remme W J.Guidelines for the diagnosis and treatment of chronic heart failure: executive summary (update 2005): The Task Force for the Diagnosis and Treatment of Chronic Heart Failure of the European Society of Cardiology. The European Heart Journal.2005; 26: 11151140.

Szmitko PE, Verma S. Red wine, and your Heart. Circulation. 2005; 111:2.

Taubert D, Roesen R and Schomig E. Effect of cocoa and tea intake on blood pressure: a meta-analysis. Archives of International Medicine.2007; 167(7): 626-634.

Tavazzi L, Maggioni AP, Marchioli R, Barlera S, Franzosi MG, Latini R, Lucci D, Nicolosi G L, Porcu M and Tognoni G. Effect of rosuvastatin in patients with chronic heart failure (the GISSI-HF trial): a randomized,

double-blind, placebo-controlled trial. The Lancet.2008; 372: 12311239.

Testai L, Martelli A, Cristofaro M, Breschi MC, Calderone V. Cardioprotective effects of different flavonoids against myocardial ischemia/reperfusion injury in Langendorff-perfused rat hearts. Royal Pharmaceutical Society. Journal of Pharmacy and Pharmacology. 2013; 65:750–756.

Testai L, Martelli A, Cristofaro M, Breschi MC, Calderone V. Cardioprotective effects of different flavonoids against myocardial ischemia/reperfusion injury in Langendorff-perfused rat hearts. Royal Pharmaceutical Society. Journal of Pharmacy and Pharmacology 2013; 65:750–756.

Thyrocare.comhttp://www.thyrocare.com/High-Performance-Liquid Chromatography.html

Tilgmann C and Ulmanen I. Purification methods of mammalian catechol-O-methyltransferase. Journal of Chromatography B: Biomedical Applications. 1996; 684, (1-2) 147–161.

Timmers L, Pasterkamp G, de Hoong VC, Arsalan F, Appelman Y, de Kelijin DP.The innate immune response in reperfused myocardium. Cardiovascular Research. 2012; 276-283.

Tokede O A, Gaziano J M and Djousse L.Effects of cocoa products/dark chocolate on serum lipids: a meta-analysis. European Journal of Clinical Nutrition.2011; 65(8): 879-886.

Tomas-Barberan F A, Cienfuego- jovellano E, Marín A, Muguerza B, Gil-Izquierdo A, Cerda B, Zafrilla P, Morillas J, Mulero J, Ibarra A, Pasamar M A,Ramón D and Espín J C. A new process to develop a cocoa powder with higher flavonoid monomer content and enhanced bioavailability in healthy humans. Journal of Agricultural Food Chemistry. 2007. 16, (10): 3926-35.

Tomas-Barberan F A. Types, food sources, consumption, and

bioavailability of dietary polyphenols nutrinsight, in Proceedings of the Symposium 11th Nutrition Conference, Kraft Foods. 2012; 5-13.

Tsai SH, Lin-Shiau SY, Lin JK. Suppression of nitric oxide synthase and the down-regulation of the activation of NF-κB in macrophages by resveratrol. British Journal of Pharmacology. 1999; 126:673–680.

University Hospital Düsseldorf. "Cocoa flavanols lower blood pressure and increase blood vessel function in healthy people: Cocoa flavanols could help maintain cardiovascular health as we age." Science Daily, 10 September2015.<www.sciencedaily.com/releases/2015/09 /150910110832.htm>.

Vaidyanathan J B and Walle T. Glucuronidation and sulfation of the tea flavonoid (-)-Epicatechin by the human and rat enzymes. Drug Metabolism and Disposition. 2002;30(8): 897–903.

Vascular Disease Foundation/VDF. Focus on Ischemia. 2012, 8206 Leesburg Pike, Suite 301, Vienna, VA 22182. 26vdf2012.

Verma S, Fedak PWM, Weisel RD, Butany J, Rao V, Maitland A, Li R, Dhillon B, Yau TM. Fundamentals of reperfusion injury for the Clinical Cardiologist. Circulation. 2002; 105:2332-2336. doi: 10.1161/01.CIR.0000016602.96363.36.

Vinson J A, Proch J and Zubik L. Phenol antioxidant quantity and quality in foods: cocoa, dark chocolate, and milk chocolate. Journal of Agricultural and Food Chemistry. 1999; 47(12): 4821-4824.

Vinson JA, Teufel K, Wu N. Red wine, dealcoholized red wine, and especially grape juice, inhibit atherosclerosis in hamster model. Atherosclerosis. 2001; 156:67–72.

Vita J A. Polyphenols and cardiovascular disease: effects on endothelial and platelet function. The American Journal of Clinical Nutrition.2005; 81(1): 292-297.

Vivanco I, Sawyers CL. The phosphatidylinositol 3-kinase Akt pathway in human cancer. Nature Reviews Cancer. 2002; 2:489–501.

Wan Y, Vinson J A, Etherton T D, Proch J, Lazarus S A and Kris-Etherton P M. Effects of cocoa powder and dark chocolate on LDL oxidative susceptibility and prostaglandin concentrations in humans. American Journal of Clinical Nutrition.2001; 74(5): 596-602.

Wang J, Liu Y T, Xiao L, Zhu L, Wang Q, and Yan T.Anti-Inflammatory Effects of Apigenin in Lipopolysaccharide-Induced Inflammatory in Acute Lung Injury by Suppressing COX-2 and NF-kB Pathway. Inflammation. 2014;37(6).

Wang-Polagruto J F, Villablanca AC, Polagruto J A, Lee L, Holt R R, Schrader HR, Ensunsa J L, Steinberg FM, Schmitz HH and Keen C L.Chronic consumption of flavanol-rich cocoa improves endothelial function and decreases vascular cell adhesion molecule in hypercholesterolemic postmenopausal women. Journal of Cardiovascular Pharmacology.2006; 4(2): 177-186.

WHO. Global status report on noncommunicable diseases: “the nine global noncommunicable disease targets; a shared responsibility”. 2014; ISBN. 978-92-4-156485-4.

Wollgast J and Anklam E. Polyphenols in chocolate: is there a contribution to human health? Food Research International.2000;33(6): 449-459.

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

Woodman C R, Muller J M and Rush J W E. Flow regulation of eNOS and CuZn SOD mRNA expression in porcine coronary arteries. American Journal of Physiology.1999; 276: 1058-1063.

Xia P, Liu Y, Cheng Z. Signaling Pathways in Cardiac Myocyte Apoptosis. Biomedical Research Institute. 2016; 9583268.

Yamamoto Y and Gaynor R B. Therapeutic potential of inhibition of the NF-κB pathway in the treatment of inflammation and cancer. Journal of Clinical Investigation. 2001; 5:107(2): 135–142.

Yellon DM, Hausenloy DJ (2007). Myocardial reperfusion injury. The New England Journal of Medicine.2007; 357: 1121-1135.

Zaveri N T. Green tea and its polyphenolic catechins: medicinal uses in cancer and noncancer applications. Life Sciences.2006;78(18): 2073-2080.

Zhang H, Xue G, Zhang W, Wang L, Li H, Zhang L, Lu F, Bai S, Lin Y, Lou Y, Xu C, Zhao Y. Akt and Erk1/2 activate the ornithine decarboxylase/polyamine system in cardioprotective ischemic preconditioning in rats: the role of mitochondrial permeability transition pores. Mol Cell Biochem. 2014; 390:133–142.

Zhang LN, Zheng J J, L. Zhang L. Protective effects of asiaticoside on septic lung injury in mice. Experimental and Toxicological Pathology.2001 63: 519.

Zhang N, Pei F, Wei H, Zhang T, Yang C, Ma G, et al. Isorhamnetin protects rat ventricular myocytes from ischemia and reperfusion injury. Experimental Toxicology Pathology. 2011; 63: 33-38.

Zhao D, Yang J, Yang L. Insights for Oxidative Stress and mTOR Signaling in Myocardial Ischemia/Reperfusion Injury under Diabetes. Oxid Med Cell Longev. 2017; 6437467

CHAPTER SIX

6.1. APPENDICES

6.2. Appendix I

6.2.1. Hematoxylin and Eosin Staining Protocol

Following chemicals were used in hematoxylin preparation:

1.	Hematoxylin	2g
2.	Ethanol absolute	100ml
3.	Ammonium alum	3g
4.	Distilled water	100ml
5.	Glycerol	100ml
6.	Sodium Iodide	0.24g
7.	Acetic acid	10ml

6.2.2. Eosin

It was prepared by dissolving 1g of Eosin in 100 ml of 70% of ethanol.

6.2.3. Hydration

These slides were deparaffinized in xylene as follows:

1.	Xylene 1	2-5 minutes at room temperature
2.	Xylene 2	2-5 minutes at room temperature

Tissues were hydrated in graded solutions of alcohol in descending order.

3.	100% Alcohol	2-5 minutes at room temperature
4.	90% Alcohol	2-5 minutes at room temperature
5.	70% Alcohol	2-5 minutes at room temperature
6.	50% Alcohol	2-5 minutes at room temperature
7.	30% Alcohol	2-5 minutes at room temperature
8.	Hematoxylin	1-2 dips
9.	Wash in tap water	5-10 minutes until the tissues were blue

6.2.4. Dehydration

Now cross sections were dehydrated in ascending order in alcoholic grades for 3-5 minutes.

1.	30% Alcohol	2-5 minutes at room temperature.
2.	50% Alcohol	2-5 minutes at room temperature.
3.	70% Alcohol	2-5 minutes at room temperature.
4.	90% Alcohol	2-5 minutes at room temperature.
5.	Eosin	1-2 dips.
6.	90% Alcohol	1 dip.
7.	100% Alcohol	2-5 minutes at room temperature.
8.	Xylene	5-10 minutes.

6.3. Appendix II

6.3.1. Immunohistochemistry Staining

Two changes of xylene for **10 min each**

Two changes of ethanol (100%)/absolute for **5 min each**

Block peroxidase activity with blocking reagent (ready to use) for **10 min**

Wash with PBS and agitate three times for **5 min each**

Blocking solution for **1hr** (ready to use)

Incubate with 1^{o}Ab **overnight**

Incubate with 2^{o}Ab for **1hr**

Incubate with ABC kit for **1hr** (prepare 30min earlier)

Develop with DAB for **3 min**

Apply DepeX in middle of slide and cover with coverslip