# INVESTIGATING THE EFFICACY AND UNDERLYING MECHANISM OF CARDIOPROTECTION AFFORDED BY ROOIBOS (*ASPALATHUS LINEARIS*) IN ANGIOTENSIN-II INDUCED CARDIAC HYPERTROPHY & APOPTOSIS

By

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Thesis presented in fulfilment of the requirements for the Master of Science in the Faculty of Medicine and Health science at Stellenbosch University

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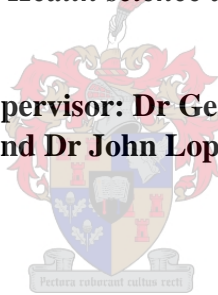
March 2021

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Abstract

Purpose: Rooibos (RB) has been reported to confer cardioprotection, but the underlying mechanisms are not fully elucidated. Furthermore, RB has never been tested against cardiac hypertrophy and apoptosis. Therefore, this study investigated the efficacy and underlying mechanisms of RB-induced cardioprotection in a model of angiotensin-II (Ang-II) induced cardiomyocyte hypertrophy & apoptosis.

Methods: Six groups of H9c2 cardiomyoblasts were either exposed to 2% FBS supplemented DMEM (control), Ang-II (20µM), RB (100µg/ml), Losartan (10µM) and co-treatment with RB + Ang-II or Ang-II + Losartan for 48 hrs. Cell viability, ATP levels, and hypertrophy and apoptosis signalling were measured with Western blotting. Cell size and mitochondrial membrane potential were measured using JC-1. Mitochondrial oxidative stress was measured with aconitase assay.

Results: Ang-II induced hypertrophy as well as apoptosis in H9c2 cardiomyoblasts by increasing cell size and upregulating growth signalling pathways, while decreasing cell viability, up-regulating of pro-apoptotic markers (Bax and cleaved caspase-3) and downregulating anti-apoptotic Bcl-2. In addition, Ang-II decreased mitochondrial membrane potential and ATP levels. RB co-treatment effectively antagonized Ang-II-induced hypertrophy and apoptosis of H9c2 cells. This was mediated by reducing cell size and dephosphorylating growth signalling pathways such as ERK 1/2, PKB, mTOR, Calcineurin and GSK-3ß. RB also increased cell viability by increasing Bcl-2, decreasing Bax and cleaved caspase-3 and thereby, inhibiting apoptosis. The administration of RB prevented depolarization of mitochondrial membrane potential and increased ATP activity. RB also enhanced the expression of mitochondrial respiratory chain complex IV and V, probably through the enhancement of mitochondrial biogenesis and the electron transfer system.

Conclusion: To our knowledge, the current study is the first to show that RB attenuates Ang-II induced cardiomyoblast hypertrophy and apoptosis by improving mitochondrial parameters and restoring GSK-3 beta and Bcl-2 signalling.

Opsomming

Doel: Daar word berig dat Rooibos (RB) kardiobeskerming verleen, maar die onderliggende meganismes is nog nie volledig toegelig nie. Verder is RB nog nooit getoets in kardiale hipertrofie en apoptose nie. Daarom het hierdie studie die doeltreffendheid en onderliggende meganismes van RB-geïnduseerde kardiobeskerming in 'n model van angiotensien-II (Ang-II) geïnduseerde kardiomiosiet-hipertrofie en -apoptose ondersoek.

Metodes: Ses groepe H9c2-kardiomioblaste is blootgestel aan 2% FBS aangevulde DMEM (kontrole), Ang-II (20 µM), RB (100 µg / ml), Losartan (10 µM) en gesamentlike behandeling met RB + Ang-II of Ang- II + Losartan vir 48 uur. Sellewensvatbaarheid, ATP-vlakke en hipertrofie en apoptose-sein is gemeet met Western blotting. Selgrootte en mitochondriale membraanpotensiaal is met behulp van JC-1 gemeet. Mitochondriale oksidatiewe stres is gemeet met akonitase-assai.

Resultate: Ang-II ïnduseerd hipertrofie sowel as apoptose in H9c2-kardiomyoblaste deur die selgrootte te verhoog en die groei van seinpaaie op te reguleer, terwyl dit die lewensvatbaarheid van die selle verminder, die pro-apoptotiese merkers (Bax en gekliefde caspase-3) opreguleer en die anti-apoptotiese Bcl-2 afreguleer. Verder het Ang-II die mitochondriale membraanpotensiaal en ATP-vlakke verlaag. Medebehandeling met RB het Ang-II-geïnduseerde hipertrofie en apoptose van H9c2-selle effektief geantagoniseer. Dit is bemiddel deur die vermindering van die selgrootte en die defosforilering van seinpaaie vir groei soos ERK 1/2, PKB, mTOR, Calcineurin en GSK-3ß. RB het ook die lewensvatbaarheid van die selle verhoog deur Bcl-2 te verhoog, Bax en gekliefde caspase-3 te verminder en dus apoptose te inhibeer. Die toediening van RB het depolarisasie van mitochondriale membraanpotensiaal voorkom en ATP-aktiwiteit verhoog. RB het ook die uitdrukking van mitochondriale respiratoriese kettingkompleks IV en V verbeter, waarskynlik deur die verbetering van mitochondriale biogenese en die elektronoordragstelsel.

Gevolgtrekking: Na ons mening is die huidige studie die eerste om aan te toon dat RB Ang-II geïnduseerde kardiomioblast hipertrofie en apoptose attenueer/onderdruk, deur mitochondriale parameters te verbeter en GSK-3 beta en Bcl-2 seine te herstel.

ACKNOWLEDGEMENTS

Writing this thesis has been an educational and joyful experience for me. In this section I would like to acknowledge the people who have contributed and made it possible for me to complete this thesis. It is my pleasure to express my gratitude to all those who have supported me.

This thesis would not have been possible without the excellent and constant support, guidance and encouragement of Dr John Lopez. I would like to take the opportunity and thank him very much for his unique ways of making me understand science and mentorship, which I am very indebted to. His scientific intuition has made him an important source of ideas, which have inspired me since I started my work in the laboratory.

Very special thanks go to Dr. G Maarman. This study would not have been successful without his excellent knowledge, gracious support and assistance. Further, I gratefully acknowledge Dr E Marais for her support, enthusiasm, advice and always willing to hear me out kept me going and making me want to achieve more.

I also would like to thank the National Research Foundation for supporting this research project. I would also like to give a great thanks to South African Rooibos council for their patience with me and their support for this project. I would like to further acknowledge the whole department of Medical Physiology for great support.

I very much would like to thank my friends for support, always willing to listen and advice. Last but not least I would like to thank my parents Yoliswa and Rodgers whose constant encouragement and support have helped me in every step of my studies at the University.

I would also like to thank the Almighty God for everyone He brought in my life in times of need. He has been good to me and will always be.

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List of abbreviations

2K1C	Two kidney, one clip
AcCoA	Acetyl-coenzyme A
ACE	Angiotensin converting enzyme
Acetyl-CoA	Acetyl-coenzyme A
ADH	Antidiuretic hormone
AgtrIA	Type 1A angiotensin II receptor gene
AIF	Apoptosis-inducing factor
ANF	Atrial natriuretic factor
Ang II	Angiotensin II
ANT	Adenine nucleotide translocator
ARF	Acute renal failure
AT1R	Angiotensin AT1 receptor
AT2R	Angiotensin II receptor type 2
ATP	Adenosine Tri-phosphate
Bcl-2	B-cell lymphoma **2**
βMHC	β-myosin heavy chain
BNP	Brain natriuretic peptide
CHD	Coronary heart disease
CHF.	Coronary heart failure
CI	Complex I
CII.	Complex II
CIII	Complex III
CIV	Complex IV
CO_2.	Carbon dioxide
CoQ	Coenzyme Q
CPT-1	Carnitine palmitoyl transferase I
CVD	Cardiovascular diseases
dH2O.	Distilled water
DNA	Deoxyribonucleic acid
ECF	Extracellular space
ERK	Extracellular signal-regulated kinase
ET-1	Endothelin-1
ETC	Electron transport chain
FADD	FAS-associated death domain protein
g.	Grams
GPCR	G-coupled protein receptor
GSK-3ß	Glycogen synthase kinase-3β
HF	Heart failure
HHD	Hypertensive heart disease
HTN.	Hypertension
IGF1R	Insulin-like growth factor 1 receptor
IHD	Ischemic heart disease
kDa	Kilo Dalton

LVH	Left ventricle hypertrophy
MAPK	Mitogen-activated protein kinase
McoA	Malonyl-coenzyme A
MEK	Mitogen-activated protein kinase kinas
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
Na-H	Sodium hydrogen
NADP.	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
nDNA.	Nuclear DNA
NFAT	Nuclear factor of activated T-cells
NO	Nitric oxide
NO-GC1 KO.	NO-Sensitive Guanylyl Cyclase 1 Knockout Mice
OO_2	Oxygen
OXPHOS	Oxidative phosphorylation
PDGF	Platelet-derived growth factor
PDH.	Pyruvate dehydrogenase
PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
PPAR-α	Peroxisome proliferator-activated receptor- alpha
RAAS	Renin-angiotensin-aldosterone system
RB	Rooibos
RHD	Rheumatic heart disease
ROS	Reactive oxygen species
Rpm	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEM	Standard Error of the Mean
SKA	Skeletal alpha-actin
ß-AR.	B-adrenergic receptor
T2D	Type 2 Diabetes mellitus
TBS.	Tris-buffered saline
TNF	Tumour Necrosis factor
TRADD	Tumour necrosis factor receptor type 1-associated DEATH domain protein
WHO	World health organization
μl	Microliter
GRE	Green rooibos extract

Chapter 1: Literature review

1.1 Introduction
1.1.1 Cardiovascular diseases

Cardiovascular diseases (CVDs) have been a leading cause of morbidity and mortality in modern nations for more than half a century (Quindry and Franklin, 2018; Maarman et al., 2020). In 2015, CVDs were responsible for an estimated 17.7 million deaths, 31% was number of deaths globally (WHO, 2013). Moreover, 7.4 million of these deaths were attributed to coronary heart disease, while 9.2% occurred in the African region (Kengne et al., 2005). Out of the 17 million global deaths in 2017, an alarming 82% of deaths were in low and middle-income countries (WHO, 2017). It is proposed that by 2030, the number of deaths attributable to CVD, will rise from 17 to 25 million (Kengne et al., 2005; WHO, 2012). It has further been reported that CVD are accountable for most of South African deaths compared to all cancers put together (Byrne et al., 2016). Thus, CVDs is a considerable health threat across the world, and increasingly so in countries like South Africa.

CVDs include a number of conditions that cause a disruption in either the structure or function of the cardiovascular system, and some of these conditions can either be due to genetic or lifestyle factors (e.g. diet and physical activity) (Zafar, 2015). CVDs can be classified into different categories including rheumatic heart disease (RHD), congenital heart defects, hypertensive heart disease (HHD), inflammatory heart disease, and ischemic heart disease (IHD) (Figure 1). A large proportion of CVDs have been attributed to atherosclerosis, and includes conditions like coronary artery disease and acute myocardial ischemia (World Heart Federation, 2016)
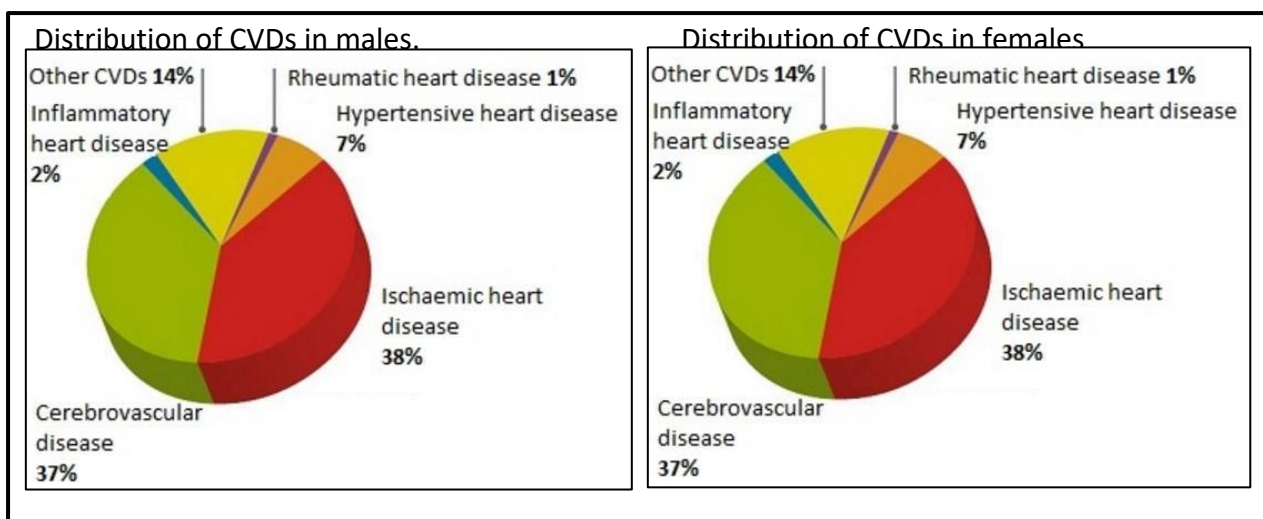
Figure 1*: **The statistical prevalence of CVD distribution among man and women.** (copied from The Heart and Stroke Foundation South Africa, 2016).

1.1.2 Categories of heart disease

1.1.2.1 Rheumatic heart disease

RHD is known as cardiac damage that occurs over a long period of time due to either one severe incident or several recurring incidents of acute rheumatic fever (ARF) (Bertrand *et al.*, 1988). It remains a critical cause of morbidity or mortality globally, notably in resource poorsettings (Muhamed et al., 2020). ARF is caused by the response of the body's autoimmunity to the infection of the throat due to Streptococcus pyogenes, also known as the group-A Streptococcus bacteria (Kumar and Tandon, 2013). ARF manifests as myocarditis, chorea, polyarthritis and erythema marginatum or a rash (Guilherme, Köhler and Kalil, 2011). The global burden of ARF and RHD is high, and is mainly observed in populations living in lowresource settings (Carapetis, 2007). In 2005, approximately 471,000 global AFR cases were reported and those were mostly children and teenagers between the ages of 5-15 years (Carapetis *et al.*, 2005). Furthermore, the prevalence of RHD cases worldwide ranged between 15.6 and 19.6 million (Carapetis *et al.*, 2005). An estimated 350,000 deaths occur each year due to ARF or RHD (Carapetis *et al.*, 2005). Approximately, 34.2 million cases concomitant with 10.1 million disability-adjusted life years lost in 2010 were due to RHD and this contributed to the high global burden (De Dassel et al., 2015). There are several risk factors responsible for ARF and RHD which includes gender, age and multiple environmental factors (Carapetis *et al.*, 2016). Although ARF statistics do not differ much between male and female, RHD is known to

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commonly affect females (Lawrence *et al.*, 2013). However, it is not fully elucidated whether the difference in RHD prevalence is caused by a higher susceptibility to autoimmune responses after *S. pyogenes* infection, or whether social factors (such as how the child was raised) increases susceptibility of *S. pyogenes* infection (Carapetis *et al.*, 2016). Rural areas, remote areas as well as urban slums are associated with household crowding because of low socioeconomic status or limited access to medical resources (Carapetis *et al.*, 2016). Therefore, one of the environmental factors that play a role is crowded households, which increases the likelihood of ARF and promotes the spread of *S. pyogenes* infections.

1.1.2.2 Ischemic heart disease

IHD also called coronary heart disease (CHD) comprises insufficient blood or oxygen circulation to the myocardium, and this is caused by thickening of coronary arteries causing them to narrow, these arteries usually supply blood to the myocardium (Gaze, 2013). Even though the narrowing of these coronary arteries can be due to vasoconstriction, most times it is caused by a build-up of plaque during called atherosclerosis (Soler and Ruiz, 2010). In the early stages of CHD, most people do not experience any symptoms (Soler and Ruiz, 2010). However, during the progression of atherosclerosis, especially if left untreated for a long period, symptoms are most likely to occur (Yelle, 2000). CHD is one of the key deaths causes in both men and women. From 2003 to 2006, approximately 17.6 million Americans aged 20 above had CHD, with an overall prevalence of 7.9 percent, and 9.1 percent of that men while 7 percent are women (Sani et al.,2006; National Centre for Health Statistics, 2016). CHD is accountable for more than half of all CV episodes in men and women under age 75 and above (Lloyd-Jones *et al.*, 2010). Age and gender are known risk factors of CHD. In term of age, CHD largely affects people between the ages 40-75. While when it comes to gender, CHD commonly affects men. In addition, the lifetime risk of developing CHD after age 40 is 49 percent for men and 32 percent for women (Lloyd-Jones *et al.*, 2009). According to an estimate by Global Burden of Diseases Project the death rate of men due to IHD is higher compared to women with CHD and this had created a financial burden worldwide and account for about $177.1 billion of direct and indirect cost in 2010 (Lloyd-Jones *et al.*, 2010). The World Health Organization (WHO) reported in 2005 that IHD was responsible for approximately 361 000 deaths in the African region and it has also been estimated that these projections will continue to increase almost as double in 2030 (WHO, 2009).

1.1.2.3 Hypertensive heart disease

HHD includes several complications associated with blood pressure. In HHD, the workload of the heart increases, consequently leading to enlargement of the myocardium. There are several factors documented that are believed to be responsible for the development of HHD and these factors continue to increase substantially (Diamond and Phillips, 2005). HHD is underpinned by increased myocardial fibrosis, apoptosis, endothelial dysfunction and inflammatory changes (Frohlich, 2009; Raman, 2010).

HHD is characterised by left ventricular hypertrophy in response to increased high blood pressure (Drazner, 2011). HHD includes complicated abnormalities such as left ventricular hypertrophy, impaired diastolic and systolic pressure. Clinically, HHD as arrhythmias and symptomatic heart failure (Drazner, 2011). Moreover, HHD is a result of the indirect and direct consequences of chronic high blood pressure (Tackling and Borhade, 2019), or the interplay between genetic and hemodynamic factors (Diamond and Phillips, 2005). Most of the factors responsible for HHD seem to involve the renin angiotensin aldosterone system (RAAS). However, recently discovered pathways also play a role, such as such as the human type-A natriuretic peptide receptor gene and G-coupled protein β 3-subunit gene (Diamond and Phillips, 2005). Most of the genetic factors responsible for HHD seem to target the renin angiotensin-aldosterone system (RAAS), these genes include the ACE and angiotensinogen genes. In a study conducted by (Brilla, Funck and Rupp, 2000), ACE inhibitors were used to treat myocardial fibrosis (which is one of the end-organ effects of HHD) and treatment minimised myocardium fibrosis by blocking the RAAS and thus, also reduce hypertension.

1.1.3 Risk factors of CVDs: Key role of hypertension

Most CVDs are preventable and therefore the increased awareness programs on behavioural risk factors such as the use of tobacco, obesity related to unhealthy diet, physical inactivity, and excessive use of alcohol are crucial (Maarman et al., 2020). These risk factors contribute to the development of diabetes, obesity, and hypertension (WHO, 2017). Hypertension is a condition that comprises elevated blood pressure in the absence of medical treatment (WHO, 2015). It can result in serious complications such as heart disease, stroke, and ultimately death (George et al., 2018). Hypertension remains the deadliest risk factor for CVD in low-income

countries (Ntuli et al., 2015), and the WHO reported that about 1.13 billion people worldwide have hypertension, mostly those living in low/middle-income countries (World Health Organization, 2011; Basu and Millett, 2013). Additionally, in Sub-Saharan Africa, the prevalence of hypertension ranges from 5%-50%, whereas in developed countries it ranges between 19%-30% (Woodiwiss et al., 2011). Hypertension in developed countries is a continuously growing health concern (Woodiwiss et al., 2011), and when comparing low, middle-income countries and high-income countries, hypertension is higher in low/middle-income than in high-income countries, with 28.5% (which is 349 million people) cases of hypertension (Mills, Stefanescu and He, 2020).

Data from the National Health and Nutrition Examination Survey (NHANES), spanning 20112014, demonstrated that in age groups 20 and above, 86 million adults had hypertension, with a prevalence of 34% (Benjamin et al., 2017). Data from the Centres for Disease Control and Prevention's (CDC) National Centre for Health Statistics (NCHS), spanning 2015-2016, the hypertension prevalence was 29% among people aged 18 and older (National Centre for Health Statistics, 2017). In African Americans, the prevalence of hypertension is one of the highest compared to any ethnic group worldwide, at 45% for males and 46.3% for females (National Centre for Health Statistics, 2017). The prevalence rate for Caucasian men is said to be 34.5% with 32.3% for women, while Hispanic men account for 28.9% and 30.7% for women (Benjamin et al., 2017). Moreover, black Americans are reported to have high prevalence of hypertension, and are therefore, at higher risk of developing heart failure (Tackling and Borhade, 2019). Worldwide, roughly 26% of the world's population, which is 972 million people, have hypertension (Mills, Stefanescu and He, 2020). This prevalence of hypertension is expected to increase to 29% by 2025, an increase that is attributed to the economic development of nations across the world (Mills, Stefanescu and He, 2020). The continuous increase in the prevalence of hypertension is a public health concern and financial burden (Forouzanfar et al., 2015).

1.1.4 Contribution of RAAS to the pathogenesis of hypertension

The RAAS is a major endocrine or paracrine system involved in the regulation of a myriad cardiovascular processes (Iravanian and Dudley., 2008). It is not only known for circulating hormones but plays a role in modulating numerous neurologic patterns in the brain (Labandeira-García *et al.*, 2014). This complex system has important physiological functions including control of water and electrolytes, systemic vascular resistance, blood pressure and cardiovascular homeostasis. The RAAS also regulates hemodynamic stability by controlling blood pressure through the regulation of vasoconstriction, sodium reabsorption, body fluid homeostasis (Atlas & Steven, 2007). Therefore, any disturbance in the RAAS has been implicated in the pathogenesis of hypertension. For example, differences in genes encryption for renin (Kurtz *et al.*, 1990), angiotensin-converting enzyme (Jacob *et al.*, 1991; Hilbert *et al.*, 1991), angiotensinogen (Hata *et al.*, 1994; Jeunemaitre *et al.*, 1992) and angiotensin receptor (ATR) (Bonnardeaux *et al.*, 1994) have been linked with hypertension in animal models and human populations of sodium-sensitive hypertension. Chronic stimulation of the RAAS can also lead to widespread oxidative stress, inflammation and endothelial dysfunction, which may consequently result in severe hypertension, kidney disorders and heart failure (Jackson *et al.*, 2018). The role of RAAS in the pathogenesis of hypertension (Cowan and Young, 2009), cardiac hypertrophy (Cowan and Young, 2009), and atherosclerosis has been confirmed previously. During the beginning stages of heart failure, the RAAS is stimulated as a compensatory mechanism to minimise to progression of cardiac pathology. However, as the disease progresses it assumes a harmful role which is responsible for an increase in cardiac preload and afterload, a hallmark of clinical heart failure syndrome (Iravanian and Dudley Jr., 2008).

The mechanism begins with activation of RAAS system in response to low BP, drop in intratubular sodium (Ren et al., 2019; Cowan and Young, 2009). The specialized cells called juxtaglomerular (JG) cells within the afferent arterioles of the kidney become activated, the activation of JG cells cause a release an inactivated form of renin enzyme. Once renin has been released in the circulation, it acts on its target, angiotensinogen, and catalyses the cleavage of angiotensinogen into Ang-I. Ang-I is normally inactive but acts as an Ang-II precursor. Thereafter, Ang-I is converted to Ang-II by an enzyme called angiotensin converting enzyme (ACE). ACE is situated primarily on the intimal side of the vascular endothelium in lungs and

kidneys. After conversion of Ang-I to Ang-II, Ang-II influences its target organs such as kidney, adrenal cortex, arterioles, and brain by attaching itself to Ang-II type-I (ATI) and type II (ATII) receptors.

Ang-II is a hormone of the RAAS known to play a crucial role in the progression of hypertension and contributes to the pathogenesis of CVD (Pushpakumar *et al.*, 2013). The physiological action of Ang-II is brought about by binding to specific extracellular receptors (Ito *et al.*, 1995). Ang-II receptors belong to rhodopsin-like G-protein family associated receptors and have been divided into two pharmacologically distinct types designated type-1 (AT1) and type-2 (AT2) (Chai *et al.*, 2012). Ang-II is responsible for stimulating the secretion and synthesis of aldosterone through the AT1-R in the adrenal cortex. Aldosterone is, in turn, responsible for sodium reabsorption, water retention, and loss of either potassium or magnesium. Furthermore, aldosterone modulates volume of extracellular space and blood pressure through a specific mechanism on the nephron of the kidney (Crowley *et al.*, 2006; Unger, 2002).

Ang-II in the brain has three effects to exert control over systemic blood pressure. Firstly, Ang-II attaches itself to the hypothalamus, activating thirst and thereby increasing water intake (Jackson *et al.*, 2018). Secondly, it causes release of antidiuretic hormone (ADH) through the posterior pituitary. ADH stimulate an increased water reabsorption in the kidney by putting aquaporin channels at the collecting duct (Young and Davisson, 2015). Lastly, Ang-II causes a decrease in baroreceptor reflex sensitivity. This reduces the response of baroreceptor to an increase in blood pressure, which would be counterproductive to the goal of the RAAS. Ang-II also acts on the proximal convoluted tubule of the kidney, it causes an increase in the Na-H exchange thereby increasing sodium reabsorption. Once the sodium is increased in the body it causes an increased osmolarity of blood, subsequently causing fluid to move into the blood volume and extracellular space. This in turn causes an increase in the arterial blood pressure (Wright and Harding, 2013; Jackson *et al.*, 2018).

Finally, Ang-II effects vasoconstriction in systemic arterioles. This begin by Ang-II binding to the G-protein-coupled receptors, causing a cascade of secondary messengers that lead to potent arteriolar vasoconstriction. This in turn causes an increase of total peripheral resistance, causing an increased blood pressure. On the other hand, though RAAS is responsible for such crucial

physiological processes, it can be inappropriately activated in many conditions that most likely promotes the development of hypertension. For example, in renal stenosis, which is the constriction of the arteries that carry blood to one or both kidneys, the juxtaglomerular cells become activated and detect a decrease in blood volume, and this activates the RAAS system. In turn, this can cause an inappropriate increase of circulating blood stream volume and arteriolar tone caused by poor renal perfusion (Ren et al., 2019; Santos *et al.*, 2019).

Collectively, recent findings have demonstrated that the primary action of Ang-II in the development of hypertension is mediated through AT1R (Mascolo *et al.*, 2017; Jackson *et al.*, 2018). In addition, the primary actions of AT1R are pre-hypertensive. Pre-hypertension is when blood pressure levels are above normal or rather optimal level. WHO categorizes prehypertension as a blood pressure measurement between 120/80 and 139/89 (Chamarthi *et al.*, 2010). The important role of AT1A receptors in pre-hypertensive was verified by Ito and colleagues (Ito *et al.*, 1995) in type-1A Ang-II receptor gene AgtrIA ($^{-/-}$) mutant animals. Compared with AgtrIA ($^{+/-}$) herezygotes systolic blood pressure was significantly reduced, and it was further reduced in AgtrIA$^{-/-}$. They further suggested that AgtrIA receptor function is needed for the response of blood pressure and hemodynamic to Ang-II. Their study has proven significance of the AgtrIA gene in controlling blood pressure in mice (Ito et al., 1995). The above data are in accordance with previous studies indicating that AT1A receptors play an essential role in blood pressure control (Červenka *et al.*, 2002). They found that targeted disturbance of the AT1A receptor gene eliminates the chances of development of 2K1C Gold blatt (two kidneys, one clip) hypertension model in mice, moreover, proposed that for the development of 2K1C hypertension, the presence of AT1A receptors is critical (Červenka *et al.*, 2002).

The study conducted by (Broekmans *et al.*, 2015) looking at Ang-II induced hypertension in nitric oxide (NO)-sensitive guanylyl cyclase-1 knockout mice (NO-GC1 KO), agrees with the study conducted by Muthalif and colleagues who also observed increased blood pressure in rat arteries due to Ang-II treatment (Muthalif *et al.*, 2000). Broekmans and his team treated male NO-sensitive guanylyl cyclase-1 NO-GC1 KO mice lacking the a-1 subunit of the heterodimeric NO-GC1 (a1b1) and wild type for two-weeks, at the end of the experiment they found that the treatment resulted in an increased systolic blood pressure, which was similar in wild type and NO-GC1 KO mice. Another study showed similar results with the two above studies with an exaggerated increase in blood pressure in rats that received a chronic infusion

of Ang-II (Nahey and Collister, 2007). These results indicate that Ang-II induced increased blood pressure independent of NO-GC as reduced sympathetic activity in the NO-GC1 KO mouse model prevents hypertension. Therefore, it was speculated that there could be a possibility of sympatho-excitatory action of NO-GC1 counteracting NO-GC1's dilatory effect in the vasculature (Broekmans *et al.*, 2015).

1.2 Impact of hypertension on the myocardium

Hypertension induces coronary arteriolar constriction and inflammatory changes in the myocardium (Raman, 2010). An increase in blood pressure involves alteration in the myocardium, which over time affects the cardiac structure (Bernardo *et al.*, 2010). This is manifested by cardiac growth and cardiac remodelling that may also occur as an outcome of pressure overload (such as systemic blood pressure) leading to pathological cardiac hypertrophy (Bernardo *et al.*, 2010). This remodelling may occur with the onset of hypertension or as the progressive evolution of the structure of the infarcted scar. During early stages, the myocardium continues to function normally as it compensates for the elevated workload/pressure by becoming hypertrophied. The consequential structural and functional changes in the myocardium leads to left ventricular hypertrophy (Diamond and Phillips, 2005). The process of cardiac hypertrophy is complex and comprises several role players. In response to diverse stimuli of cardiac stress, cardiac fibroblasts become stimulated, at the end, to undergo physical conversion to myofibroblasts (Czubryt, 2019), followed by enlargement of the interstitium, a major increase in accumulation of collagen in between myocytes and fascicles (Eckhouse and Spinale, 2012) and a decrease in intramyocardial capillary density which gives rise to impairments in diastolic filling (Drozdz and Kawecka-Jaszcz, 2014). Extracellular stress mediated by pressure overload and stretch-activated ion channels are accountable for transmission-intracellular signals into the nucleus, to increase hypertrophic gene expression (Drozdz and Kawecka-jaszcz, 2014).

During this myocardial remodelling, fibroblasts become the dominant cell type in the infarcted myocardium and undergo dramatic phenotypic changes to myofibroblasts (Pushpakumar *et al.*, 2013). Impairments associated with high blood pressure on the myocardium start small and build up over time if not detected early. It begins with blood vessels and major arteries that are responsible to carry blood throughout the body to the vital organs and tissues (Lifton RP, Gharavi AG, 2001). Thus, when the pressure exerted on the arterial wall in excessively increase,

as with hypertension, it causes disruption of the arterial wall (Nickerson et al., 1992). Over time, cholesterol deposits collect at the site of arterial damage, a plaque builds up and causes narrowing of the arterial lumen, reducing the blood flow that can lead to an acute myocardial infarction (Sathnur et al., 2013) (see Figure 2)
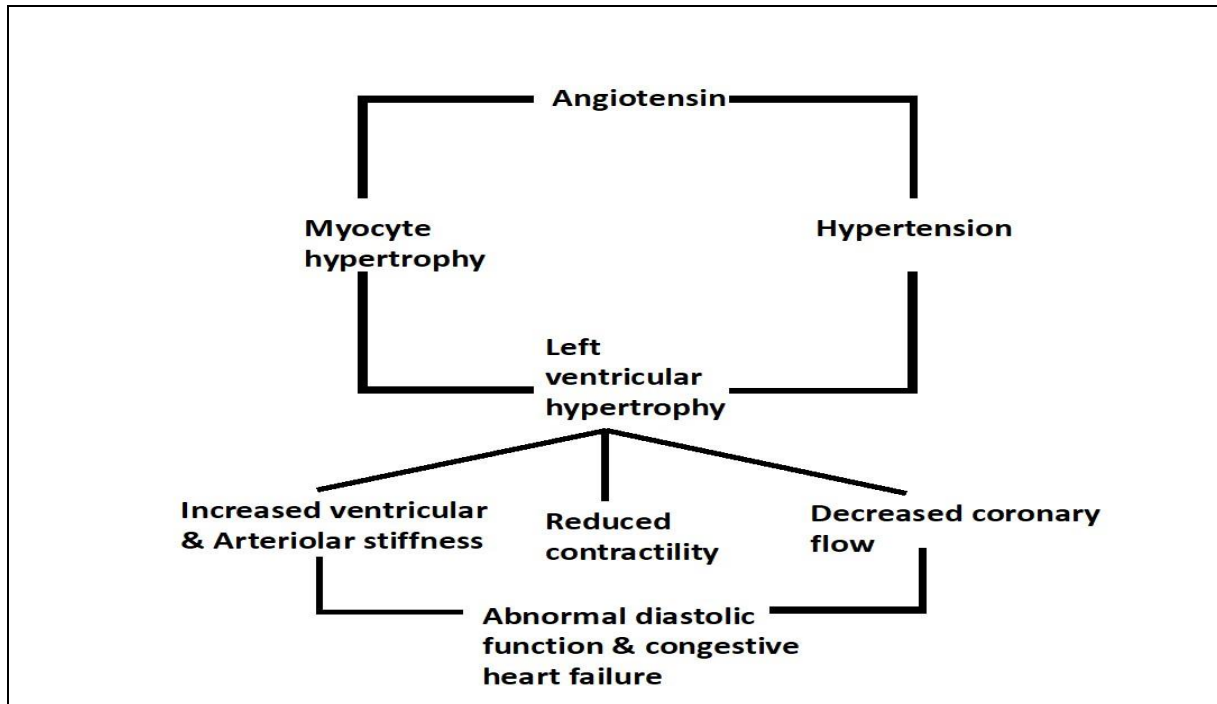
Figure 2: Representation of a balance between blood pressure and hypertrophy. *Increase in blood pressure lead to constriction of blood vessels, arterioles, decreased blood flow to the rest to the body and reduced contractility and subsequently formation of abnormal diastolic function and heart failure. At the same time the heart tries to compensate from increased blood pressure by enlarging the left ventricle to minimize wall stress in a process called cardiac hypertrophy.*

The chronic pressure overload that hypertension exerts on the myocardium predisposes to abnormal cardiac contraction and dilation of the ventricular wall (Petriz and Franco, 2014). Eventually, because the elevated blood pressure persists, the left ventricle compliance and diastolic filling pressure declines, leading to heart failure. Approximately 60% of left ventricular mass variance is most likely to be due to genetic factors independent of blood pressure (Diamond and Phillips, 2005). Hypertension activates mechano-sensing mechanisms in cardiomyoblasts via myocardial stretch that releases ion channels, growth factor receptors, and G-protein-coupled receptors, linking stress and pressure overload stimulus to gene

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regulation and protein synthesis (Bernardo *et al.*, 2010; Rosca et al., 2013) to induce intracellular transfer of signals to the nucleus, in order to increase hypertrophic markers (Drozdz and Kawecka-jaszcz, 2014).

The renin-angiotensin-aldosterone system (RAAS) is one of the key hormonal mechanisms that controls hemodynamic stability through regulation of blood pressure, fluid volume, and sodium-potassium balance. Thus, any destruction in one of the molecules that compose RAAS promote developing of hypertension (Te Riet *et al.*, 2015). One of the RAAS molecules is AngII which is the main vasoactive molecule known for inducing development of hypertension. Ang-II is known for its modulating function in contractile-related molecular expression (skeletal $\alpha\alpha$-actin, $\beta\beta$-myosin heavy chain, atrial natriuretic polypeptide, and fibronectin) and promote cardiac phenotype remodelling (Kim *et al.*, 1995) and hypertrophy. Initiation of biochemical events such as the translocation of NFAT, causes the modification of cardiac gene transcription in the nucleus (Crowley *et al.*, 2006). Apoptosis is an additional consequence of hypertension (González *et al.*, 2003). Hypertension is therefore responsible for many impairments in the heart, especially in relation to cardiac hypertrophy (Petriz and Franco, 2014). Left ventricular hypertrophy is a well-recognized characteristic of compensatory mechanism to try and adapt to increased pressure and an essential criterion of hypertensive heart disease (Frohlich, 1999).

1.2.1 Signalling pathways of hypertrophy

The regulation of cardiac hypertrophy can be categorised into two major pathways either as physiological or pathological (John *et al.*, 1999). Physiological hypertrophy is associated with athletes and is linked to corresponding incremental increase in length and width in cardiomyoblasts (John *et al.,* 1999), while pathological hypertrophy is linked to an increase in fetal gene expression, fibrosis, and cardiac dysfunction (McMullen and Jennings, 2007). Both of these types of hypertrophy are initiated in response to different stimuli, a difference in structure, molecular phenotype and each function different from one another (McMullen and Jennings, 2007). In pathological hypertrophy, the increased size in cardiac myocytes and how new sarcomeres are formed primarily serves to minimize wall stress and allow cardiovascular system to function normal at rest (i.e. Compensated growth). On the other hand, the way in which hypertrophied heart function may eventually decompensate, causing a left ventricle dilation and heart failure (Hort, 1985). While, physiological hypertrophy does not

decompensate into dilated cardiomyopathy or HF (Pluim *et al.*, 2000). Physiological and pathologic hypertrophy can further be subdivided into either concentric or eccentric, with regards to transformation of cardiomyoblasts in shape depending on the initiating stimulus (Pluim *et al.*, 2000; McMullen and Jennings, 2007). For example, a pathological stimulus such as hypertension results in chronic pressure overload that leads to concentric hypertrophy (Pluim *et al.*, 2000; McMullen and Jennings, 2007). On the other hand, the stimulus responsible for volume overload results in an increased diastolic wall dimension (Grossman et al., 1975). Also, pathologic cardiac hypertrophy development can also result from oxidative stress resulted from either led by either pressure or volume overload (Diwan and Dorn, 2007).
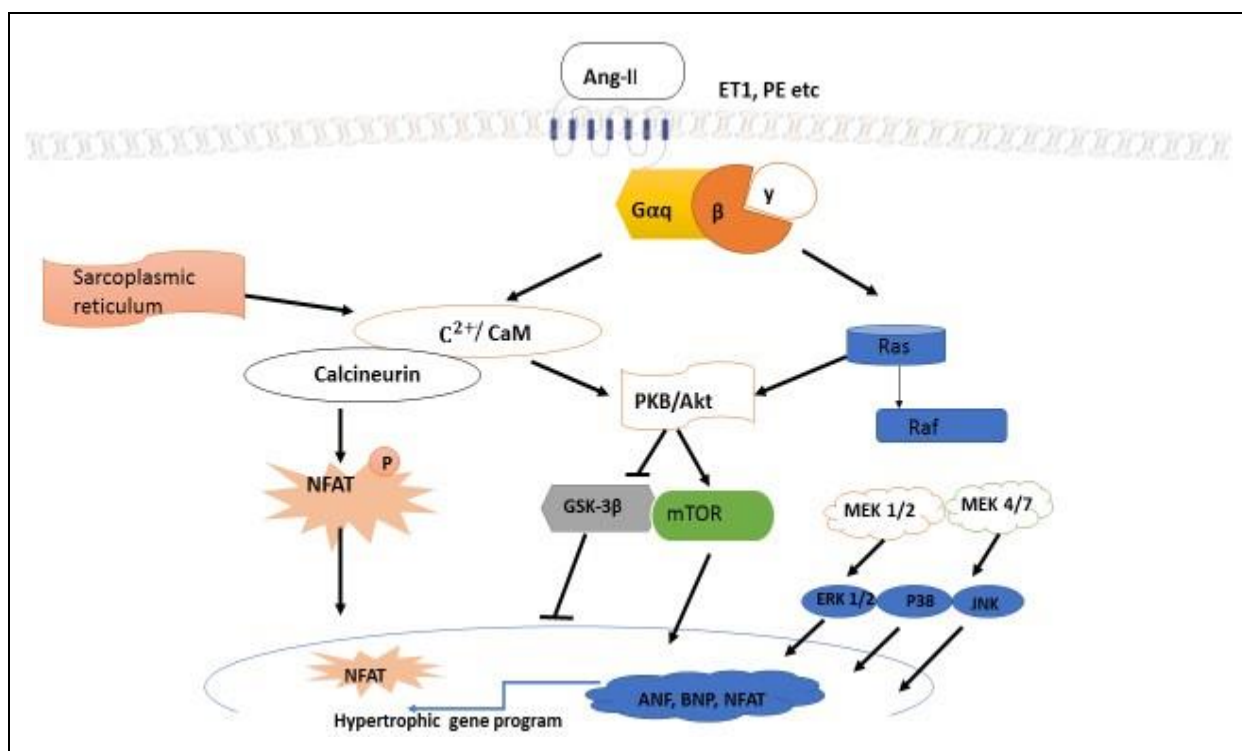
Figure 3: Schematic diagram of Ang-II on cardiomyocyte hypertrophy. *Ang-II stimulation caused an increase in intracellular calcium which activates calcium-calmodulin-activated protein phosphatase Calcineurin. Increase in calcium in turn activates PKC which increases activation of GSK-3β, thereby, inhibit hypertrophy. Calcineurin/NFAT pathway is also responsible for hypertrophy but is blocked by GSK-3β (including the decrease in hypertrophy markers such as mRNA level of BNP, ANF, and cell surface area). This diagram was adapted from* (McMullen and Jennings, 2007; Zhou *et al.*, 2013).

Cardiac hypertrophy is regulated by various stimuli, and these stimuli (Hunter and Chien, 1999) activates membrane receptors and intracellular signalling pathways to mediate the transcription of hypertrophy-related genes (Van Berlo *et al.*, 2013). These genes include c-fos, myc, as well as foetal genes which include atrial natriuretic factor (ANF), β-myosin heavy chain (βMHC), and skeletal alpha-actin (SKA) (Figure 3) (Van Berlo *et al.*, 2013). When the cardiac hypertrophic status is prolonged subsequent pathological cardiomyocyte growth occur, identified by an alteration in the extracellular matrix as well as abnormal adrenergic response, and alteration in the expression of proteins (Diwan and Dorn, 2007; Bernardo *et al.*, 2010).

Cardiomyoblasts have little or no ability to proliferate, and therefore, their only method of growth is hypertrophy (Diwan and Dorn, 2007). Generally, left ventricular hypertrophy is characterized by increased cardiomyocyte size, underpinned by elevated protein production and transformation in structures of the sarcomere (Carreño *et al.*, 2006). Many factors can induce left ventricular hypertrophy and these include endothelin-1 (ET-1), catecholamine, growth factors and Ang-II (Zhang *et al.*, 2004). Among these, Ang-II has commonly been used to induce left ventricular hypertrophy in experimental models, with concomitant pressure overload which leads to concentric hypertrophy (Marketou *et al.*, 2016). Other pathways involved in this effect of Ang-II includes activation of G-coupled protein receptors, the calcineurin-nuclear factor of NFAT signalling pathway, the mitogen-activated protein kinase (MAPK) signalling pathway and PI3K/Akt/GSK-3β signalling pathway (Figure 3) (Heineke & Molkentin, 2006).

Membrane bound ATR1, Ang-II binds to their ligands resulting in receptor colocalization that induces a significant increase in accumulation of inositol-3 phosphate which signals the sarcoplasmic/endoplasmic reticulum to release calcium into the cytosol (Unger, 2002) (Figure 3). The increase in cytosolic calcium activates calcium-calmodulin-activated protein phosphatase Calcineurin. Once activated, calcineurin directly dephosphorylates members of NFAT transcription family in the cytoplasm, promoting their translocation to the nucleus. Once the NFAT family members are in the nucleus, they contribute to the transcriptional induction of different immune response genes in T-cells, and genes with diverse functions in other cell types (Hogan et al., 2003; Gomes *et al.*, 2012). The NFAT in the nucleus can be phosphorylated by GSK-3ß thereby promoting its nuclear export (McKinsey and Olson, 2005). Calcineurin activity is negatively regulated by overexpression of GSK-3ß, which directly affects the N-

terminal regulatory domain of NFAT by phosphorylating it, thus counteracting the action of Calcineurin and blocking nuclear shuttling of NFAT (Carreño *et al.*, 2006). Once GSK-3ß is overexpressed in cultured cardiomyoblasts it inhibits agonist-induced hypertrophy, by preventing NFAT from translocating to the nucleus (Antos *et al.*, 2002). (Carreño *et al.*, 2006) (Figure 3).

Ca^{2+}/Calcineurin–NFAT signalling is one of the major pathways responsible for the development of cardiac hypertrophy (Guan et al., 2017). The Calcineurin–NFAT signalling pathway may also work with other pathways inducing hypertrophy, these pathways include MAPK/ERK, PI3K/AKT, P38 and JNK (Molkentin, 2004). MAPK/ERK and PKB/Akt pathways can be sensitive when it comes to reactive oxygen species (ROS) and they play a major part in the development of hypertrophy using a variety of stimuli such as volume/pressure overload, mechanical stretch and GPCR (Heineke & Molkentin, 2006). Accumulating evidence supports a physiological role for ROS as a "second messenger" in intracellular signalling cascades that control cell growth and proliferation (Hoidal, 2001). PKB/Akt pathway also induce hypertrophy via increase in expression mTOR (Shioi et al., 2002) and the inactivation of GSK-3β (Haq *et al.*, 2000). However, as compared to other kinases, GSK-3β is highly active in unstimulated cells and becomes inactivated in response to mitogenic stimulation (Starksen *et al.*, 1986). The activity of GSK-3β is regulated by phosphorylating the serine-9 site. The increase in phosphorylation of this site inhibits the activation of GSK-3β by creating an inhibitory pseudo-substrate for the enzyme (Dajani *et al.*, 2001). In contrast, once the serine-9 site is dephosphorylated it subsequently blocks phosphorylation of GSK-3β, leading to the activation of the kinase cascade. Several protein kinases, including Akt protein kinase B (PKB), have been shown to phosphorylate serine-9 of GSK-3β in response to mitogenic signalling, consequently inactivating the enzyme (Cross *et al.*, 1995). The ability of GSK-3β to inactivate NFAT proteins by preventing their accumulation in the nucleus suggests that GSK-3β might possess anti-hypertrophic activity (Antos *et al.*, 2002).

Excessive increases in pressure stimulates neurohumoral mediators that trigger the activation of membrane bound receptors such as tyrosine kinases, cytokine receptors and GPCRs (Scott, 2004). Stimulation of GPCR-like adrenergic receptors can trigger activation of G-proteins, which in turn can lead to production of cAMP. The latter can lead to stimulation of Ca^{2+}

regulatory proteins and myofilament proteins. Moreover, this causes a transformation in sarcomeric protein production and hypertrophy (Perez et al., 2003; Small et al., 2002).

1.2.1.1 The role of mitochondria in cardiac hypertrophy

Mitochondria were the first subcellular organelles to be dissected. Mitochondria are made up of two bilayer membranes that create two recognizable compartments: an intermembrane space and a matrix space within the inner membrane. The outer mitochondrial membrane consists of proteins known as porins. Porins enable in and out movements of the ions from the mitochondrion. while the inner mitochondrial membrane contains different types of enzymes (El-Hattab and Scaglia, 2016). The inner part of the mitochondria consists of the energy generating multiple polypeptide enzyme complexes called the electron transfer system (ETS). (El-Hattab and Scaglia, 2016). Mitochondria are present in human cells containing a nucleus, and each cell type has several mitochondria based on their energy needs. Mitochondria are important sources of energy and they achieve this energy production through oxidative phosphorylation (Lu *et al.*, 2013). The heart has a high energy demand because it must contract from birth to death, and therefore the heart contains millions of mitochondria. There are several proteins accountable for keeping mitochondrial structure and function intact so that they can produce ATP optimally. However, few are coded from mitochondrial DNA (mtDNA), while about 99% or more of other proteins are coded from nuclear DNA (nDNA). This suggests that mitochondria are is regulated by two types of proteins, mitochondrial and nuclear (El-Hattab and Scaglia, 2016).

Ang-II, endothelin-1, platelet-derived growth factor (PDGF), and tumour necrosis factor (TNF)-α trigger production of mitochondrial ROS in the left ventricle via the NADPH oxidase, which results in cardiomyocyte apoptosis and hypertrophy (Mattera *et al.*, 2017). Ang-II induces cardiac hypertrophy and apoptosis by increasing a ROS dependent molecular signalling pathway (Figure 4). In addition, mitochondria act as a major regulator of cell death and hypertrophy. Therefore, Ang-II stimulates mitochondrial dysfunction, subsequently produce an excessive amount of ROS (Dao-Fu Dai et al., 2013). This ROS increases the outer mitochondrial membrane permeability, leads to a release of pro-apoptotic protein cytochromec into the cytosol. Furthermore, activating caspase proteases, this ultimately led to apoptosis (figure 5) (Susin & Zamzami, 1996). Also, the release of mitochondrial ROS can lead to the

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activation kinase cascades responsible for hypertrophy development such as PKB/Akt, GSK3ß, MAPK (Rosca et al., 2013; Kindo et al., 2012; Abel and Doenst, 2011).
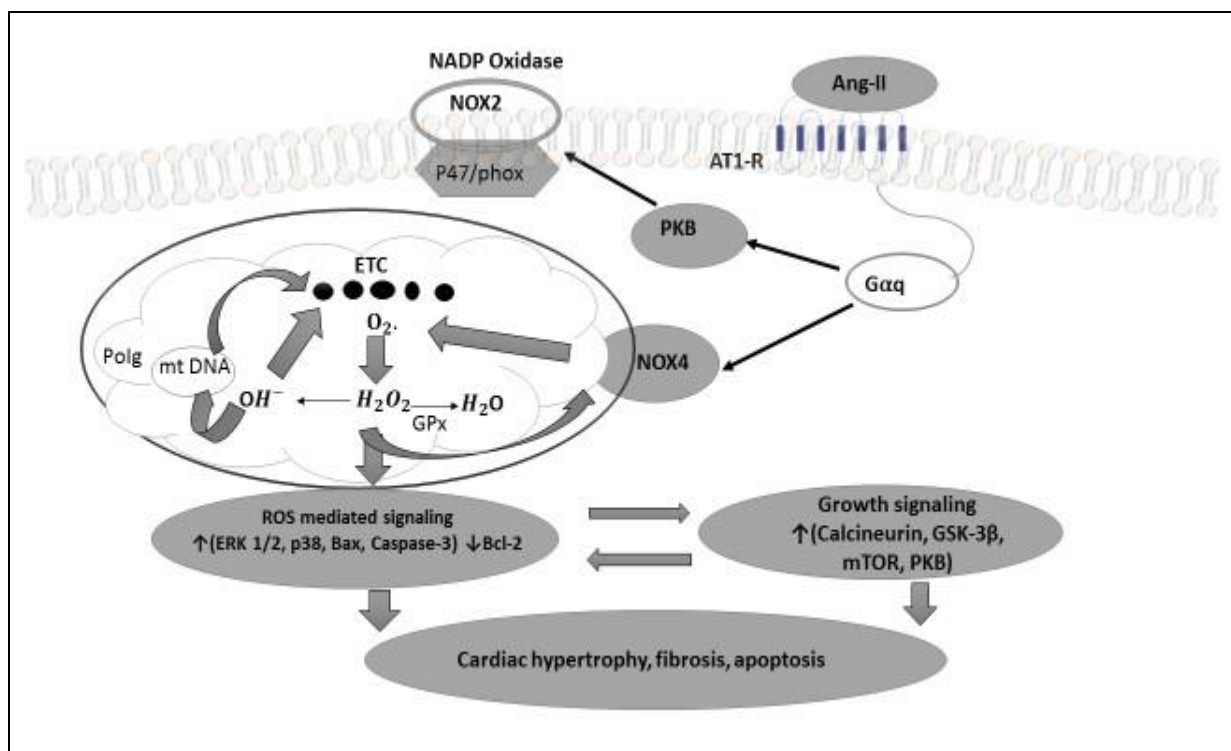
Figure 4: Ang-II induced hypertrophy and apoptosis converge into the mitochondria. *Ang-II induce both hypertrophy and apoptosis. This bioactive octapeptide in the RAAS, mediates its effect via AT1R. Ang-II-ATIR stimulates both growth and apoptosis by activating reactive oxygen (ROS) dependent molecular signalling pathways that involve the mitochondria and NADPH oxidase (NOX). There is a crosstalk between hypertrophy and apoptosis and it is mediated by Mitochondrial ROS-dependent pathways. This figure was adapted from (Dai et al., 2011).*

ROS acts as intracellular signalling molecules that control cardiac hypertrophy and are well recognized for their destructive effect, which vary with the concentration or macromolecular damage. Thus, ROS acts as one of the crucial contributors to the activation of apoptotic pathway of the cell (Azizi *et al.*, 2011). Cardiomyoblasts are known for undergoing apoptosis as a secondary mechanism to oxidative stress which in turn, promote a development CVD (Liu *et al.*, 2011). In addition, the overproduction of mitochondrial ROS is known to cause an increase in oxidation of membrane lipid and an increase in intracellular calcium. Accumulation of calcium in the heart can lead to increase mitochondrial membrane permeability, which in turn, accelerate apoptotic cascades (Liu *et al.*, 2011).

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Mitochondria are central to the regulation of cardiac apoptosis and hypertrophy, as they become abnormally stimulated to produce excessive concentrations of ROS (Liu *et al.*, 2004). Furthermore, the balance between cardiac apoptotic and hypertrophic signalling is essential for the determination of the cell fate, and mitochondria are the biological sites where the cell's fate will be determined (Pang *et al.*, 2004; Fortuño *et al.*, 2001). On the other hand, Ang-II also activates a redox-sensitive pathway during the development of cardiac hypertrophy and cardiomyocyte apoptosis (Siasos *et al.*, 2018). The mechanism through which NADPH oxidase (NOX) triggers these two pathways involves ROS-mediated activation of multiple kinase cascades that are responsible for both hypertrophy and apoptosis (figure 4) (Siasos *et al.*, 2018). Thus, an increase in NOX activity is known to be parallel to MAPK activation such as ERK 1/2 and P38. Once NOX activity is increased, MAPK is also activated simultaneously. Ang-II also binds to G-protein coupled receptors and activates cytoplasmic signalling such as increased release of intracellular calcium, which in turn either activates calcium-calmodulin calcineurin or alters mitochondrial membrane gradient increasing membrane permeability (Figure 4). Subsequently cardiac hypertrophy is caused by cellular apoptosis followed by changes in the organization of myofilaments, depolarization of mitochondrial membrane potential, alteration of Bcl-2 genes, DNA mutation, active caspase cascade, release of cytochrome-c and nuclear chromatin condensation (Adams *et al.*, 2000). Furthermore, evidence suggest that mitochondria are the major activators of caspase cascades in mammalian cells (Figure 4) (Green *et al.,* 1998). Depolarization of mitochondrial membrane concomitant with cytochrome-c release and other apoptotic proteins such as Smad into the cytoplasm, which in turn lead to activation of caspases and ultimately lead to apoptosis (Adams *et al.*, 2000).

In addition, under stressful conditions, mitochondria become the main source of ROS production, generated mainly in the ETS via electron leakage (Ide *et al.*, 2001). Superoxide is accountable for enlargement and death in cardiomyoblasts (Liu *et al.*, 2005; Tsutsui & Kinugawa, 2009). ROS production can also occur via superoxide (O^{2-}) producing enzymes and xanthine oxidase. Even though NADPH oxidase is the major site for synthesis of O^{2-} the role they play to overall elevation in ROS is not well elucidated (Ago *et al.*, 2010). Thus, mitochondria are assumed to be implicated in the pathology of multiple cardiovascular diseases, concerning the basic functions of the organelle, which therefore means they are central to both hypertrophy and apoptosis pathways (Dominic *et al.*, 2014). Mitochondria are therefore

regarded as central to all metabolic processes including oxidative phosphorylation, beta oxidation, steroid and heme biosynthesis, intermediary metabolism, programmed cell death and innate immunity (Suárez-rivero *et al.*, 2017). Various types of CVD are associated with mitochondrial damage such as inflammation, lysis of the mitochondria among those is cardiomyopathy (Dominic *et al.*, 2014; Trentadue *et al.*, 2012) and hypertrophy (Siasos *et al.*, 2018; (Adams *et al.*, 2000).

In a study conducted in an in vitro model it shown that mitochondria were central to cardiac apoptosis induced by Ang-II, endothelin-1, or pressure overload (Newmeyer & Farschon, 1994) and that adenine nucleotide translocator (ANT) is also likely to play a part in the process (Halestrap & Brenner, 2003). ANT, the mitochondrial proteins, exchanges cytosolic ADP for mitochondrial ATP and communicates with outer mitochondrial membrane proteins and proteins of the matrix to trigger the mitochondrial permeability transition pore (mPTP) (Ruck *et al.*, 1998; Halestrap & Brenner, 2003). Once the mPTP is open, it leads to depolarization of mitochondrial membrane potential, causing swelling of the matrix and outer membrane. This triggers a release of apoptotic proteins such as cytochrome-c, apoptosis-inducing factor, and pro-caspase (Susin *et al.*, 1999). In addition, ANT is also known as a regulator of apoptosis (Hang *et al.*, 2006).

Decreased articulation of cardiolipin, an important phospholipid responsible for making sure mitochondria function is intact, instigates cytochrome-c release into the cytosol leading to apoptosis (Paradies *et al.*, 2014; Iverson and Orrenius, 2004). A decrease in cardiolipin is associated with a loss in mitochondrial carrier activity, cytochrome levels including cytochrome-c. Therefore, it is important for cardiolipin to remain constant because of its important function in keeping the mitochondrial function maintained. This suggests that changes in cardiolipin directly influence cytochrome-c release from the inner mitochondrial membrane into the cytosol. In another study conducted in a hypertensive rodent's model, it was uncovered that Ang-II diminished the elements of complex-III, ATP production, creatine kinase and triggers an increased cytochrome-c release and activation of caspase-3 from the non-functional organelles (Atlas and Steven, 2007; Liu *et al.*, 2009). Overall, these studies illustrate that cardiac apoptosis and hypertrophy converge on the mitochondrion. This shows that mitochondria are central to the development of apoptosis and hypertrophy (Figure 4).

The ATP synthesized in the mitochondria is dependent on the ETS that comprises four complexes (I-IV) plus many molecules of coenzyme-Q. Stepwise electron transfer results in the active pumping of hydrogen ions through complexes I, II out of the mitochondrial matrix into the intramembranous space (Stanley et al., 2005). Complex III is where oxygen consumption actively takes place while complex IV and V partake in the final step of the ETS, which is oxidative phosphorylation. In addition, the expression and function of major proteins linked to the OXPHOS, such as ETC complexes I-V and other molecules are important in controlling what is going on with mitochondrial energy synthesis via posttranslational modification (Stoll et al., 2016). Moreover, alteration of these proteins adjusts the performance of the complexes and thus transforms the efficiency of mitochondria to come into physiological heart function. Mitochondria are involved in several processes and pathways such as metabolic and cellular signalling pathways cell survival and apoptosis. (Scheffler, 2002).

1.2.1.2 Signalling in the mitochondrion

Mitochondrial functions are achieved through the activation of intricate signalling pathways (Chandel, 2014). Several studies have reported mitochondria as signalling organelles and the mechanisms through which they perform this function is either through the release of proteins such as in regulation of cell death via the release of pro-apoptotic molecules such as cytochrome-c when the cell is damaged (Liu et al., 1996; Tait and Green, 2010), mitochondria evolved as the main site of production of ROS, major signalling molecules in the cell (Chandel et al., 1998; Schieber and Chandel, 2014).

Mitochondrial metabolites, which are normally regarded as intermediates of cardiac energy metabolism, can regulate post-translational modifications o proteins, or via affecting chromatin structure and function (Harada et al., 1999; Sciacovelli and Frezza, 2016), or by serving as a scaffold to configure signalling complexes (Biswas et al., 1999). Furthermore, mitochondria are key players in cardiac calcium signalling (Rizzuto et al., 2012). Mitochondria communicate with the rest of the cell via two signal transduction systems, namely retrograde and anterograde signalling (Rizzuto et al., 1993). Retrograde signalling is a signal transduction from mitochondria to the cytosol while anterograde signalling is a signal transduction from cytosol to the mitochondria. Anterograde signalling was mostly recognized through cytosolic calcium entering into mitochondria to regulate bioenergetics (Rizzuto et al., 1993). Mitochondria can

use their ROS to communicate with the rest of the cell under physiological conditions (Chandel, 2014). Under physiological conditions, retrograde signalling is observed in hypoxic conditions that stimulate mitochondrial ROS release, subsequently leading to the stabilization of hypoxia inducible factor (HIFs) and genes that are responsible for metabolic adaption to low oxygen are also induced (Chandel *et al.*, 1998). Based on an emerging model of mitochondrial specific ROS (mROS) and signalling, low levels of mROS are required to maintain homeostatic biological processes including oxygen sensing, epigenetics, autophagy, innate and adaptive immune responses, stem cell proliferation and differentiation, and hormone signalling (West et al., 2011; Hamanaka and Chandel, 2009), while once the mROS slightly increase it initiate pathways for adaptations to stress. Furthermore, low levels of ROS can also increase antioxidant production to counteract oxidative stress, while much higher increases in levels of mROS stimulate cell death or senescence (West et al., 2011; Hamanaka and Chandel, 2009). It is important to keep in mind that targets of mROS that relay signalling could be located whether in the mitochondrial matrix, the intermembrane space, or the cytosol (Chandel, 2014).

Mitochondria also use their outer membrane as a platform for signalling complexes. For example, A-kinase-anchoring proteins (AKAPs), located on the outer membrane, enabling phosphorylation of cAMP-dependent kinases (PKA). These AKAPs bind to the camp dependent serine/threonine kinase, which gather PKA with various signalling proteins in a scaffold to produce a signalling hub, which can be used as a target for multiple subcellular localization to enable specific target of PKA-dependent signalling (Taylor *et al.*, 2013). When the mitochondria are impaired, they can use several mechanisms to communicate with the rest of the cell, these include the release of metabolites, activation of AMPK, and changes in mitochondrial dynamics (Chandel, 2014). Firstly, the availability of metabolites has become an important mechanism to regulate signal transduction, in particular the acetylation and methylation and histone regulating epigenetic processes (Kaelin and McKnight, 2013). Moreover, mitochondria are responsible for generating acetyl-CoA and S-adenosyl-methionine (SAM) required for protein acetylation and methylation, respectively. Therefore, whatever changes or impairment in mitochondrial biology can have dangerous effects on the epigenetic state (Wellen *et al.*, 2009; Shyh-Chang *et al.*, 2013).

During the cardiac ischemic state mitochondria are unable to produce ATP and this can regulate signalling by increasing of AMP levels and adenosine as the breakdown product (Colgan and

Eltzschig, 2012). Increase in AMP levels are associated with a decline in ATP content leads to activation of kinase AMPK. In turn, AMPK abolishes various anabolic processes in the cell, promoting catabolic processes such as autophagy to ensure enough metabolic supply (Hardie et al., 2012). Adenosine, once is released into the cell, it participates in the stimulation of Gprotein-coupled receptors. As a result, metabolites and adenine nucleotides mostly thought to be implicated in biosynthetic and bioenergetic processes are also actively implicated in signalling (Colgan and Eltzschig, 2012).

The ability of mitochondria to properly move around in the cell is necessary to ensure a proper dissemination of their signals to the correct targets (Campello and Scorrano, 2010). For example, during hypoxia mitochondria become clamped into the perinuclear region, enabling ROS release into the nucleus for hypoxic gene expression (Al-Mehdi et al., 2012). Normally, mitochondria in healthy cells are elongated and packed together into a 'spaghetti-like" filamentous network. The rate of fusion and fission of mitochondria is properly controlled (Chan, 2012). However, when fission is out of control and becomes excessive it causes a destruction in the filaments network, thus, a punctate pattern and ultimately mitochondrial dysfunction. Furthermore, when fusion is also disrupted in the embryonic heart and stem cells in a mouse can lead to the disruption of development of the heart and also hinder differentiation of embryonic stem cells into cardiomyoblasts caused by an increase in Ca^{2+}-dependent calcineurin activity (Kasahara et al., 2013). Homeostasis and signalling of calcium is crucial for a cell to function normally. It plays an important role in the beginning of life after oocyte fertilization, cell differentiation during development, intracellular and intercellular signalling, and ultimately for initiation of cell death (Berridge et al., 1998). Mitochondria are known to contribute a critical role in signalling and homeostasis of calcium.

Mitochondria are divided into two groups depending no their primary function such as those found in excitable cells and those in non-excitable cells (Carafoli, 2012). In excitable cells, mitochondria can impact intracellular calcium in multiple ways. These cells include brain dendritic cells, cardiomyoblasts, and smooth muscle cells. Once the concentration of calcium increases, they store it in the mitochondrial matrix in the form of the main building block of bone called hydroxyapatite and tri-calcium phosphate. Therefore, mitochondria can propagate calcium-driven signals in two ways: one functioning as a calcium sink to block feedback inhibition, on the other hand, they function as a calcium source releasing a lot of calcium into the cytosol to intensify signals (Whelan and Zuckerbraun, 2013). Extended periods of

intracellular calcium increase are linked to an initiation of either necrotic cell death or activation of the apoptotic machinery (Bayir and Kagan, 2008).

1.2.2 Apoptosis

1.2.2.1 Signalling pathways of Apoptosis

Apoptosis is another consequence of hypertension and is defined as programmed cell death (D'Arcy, 2019). It is characterised by shrinking of the cell, followed by DNA fragmentation, chromatin condensation, blabbing of the membrane and finally formation of apoptotic bodies (Lemasters, 2018). It can be initiated by either extracellular or intracellular stimuli resulting in the activation of caspase cascade and consequently cell death. The regulation of apoptosis can be initiated through one of three pathways (1) the death receptor pathway, (2) the mitochondrial pathway and (3) the granzyme A or B pathway (Bennett, 2002; Gustafsson and Gottlieb, 2003). Apoptosis can be further regulated by two simple pathways, the intrinsic/mitochondrial pathway and extrinsic/ death receptor pathway (Bennett, 2002; Gustafsson and Gottlieb, 2003). In the intrinsic or mitochondrial pathway, the cell basically commits suicide because it senses stress, while in the extrinsic pathway, which is via death receptors the cell, the cell is given instruction to commit suicide via signal transduction activators from other cells (Bennett, 2002; Gustafsson and Gottlieb, 2003). The last pathway which includes perforin/granzyme is mediated by cytotoxic T cells. In this pathway, the induction of apoptosis occurs through granzyme B or granzyme A (Bennett, 2002; Gustafsson and Gottlieb, 2003). All three pathways, except granzyme A have one thing in common, they all induce apoptosis through the execution pathway that ends with the activation of caspase-3 (Bennett, 2002; Gustafsson and Gottlieb, 2003).

1.2.2.2 The death receptor pathway

Firstly, membrane bound death receptors which also form part of tumour necrosis receptor such as Fas or death receptors DR 3-6, bind to their trimerized ligand resulting in the receptors coming together and forming a homotrimeric complex, to recruit adapter proteins such as FasFADD, TNF-RI-TRADD via protein-protein communications (Wajant, 2003; Gustafsson and Gottlieb, 2003). In turn, these adapter proteins lead to the recruitment of caspase cascade such as caspase-8 (Boldin *et al.*, 1996; (Portt *et al.*, 2011) and caspase-2 to the homotrimeric complex (Penninger and Kroemer, 2003). Subsequent activation of caspase-8 and 2 lead to the

activation of downstream effector caspases, which include caspase-3, 6 and 7, responsible for the cleavage of intracellular substrates. These caspases are needed for culminating in cell death (Bennett et al., 1995), and for cellular survival, and metabolic function (Bennett, 2002) (figure 5).
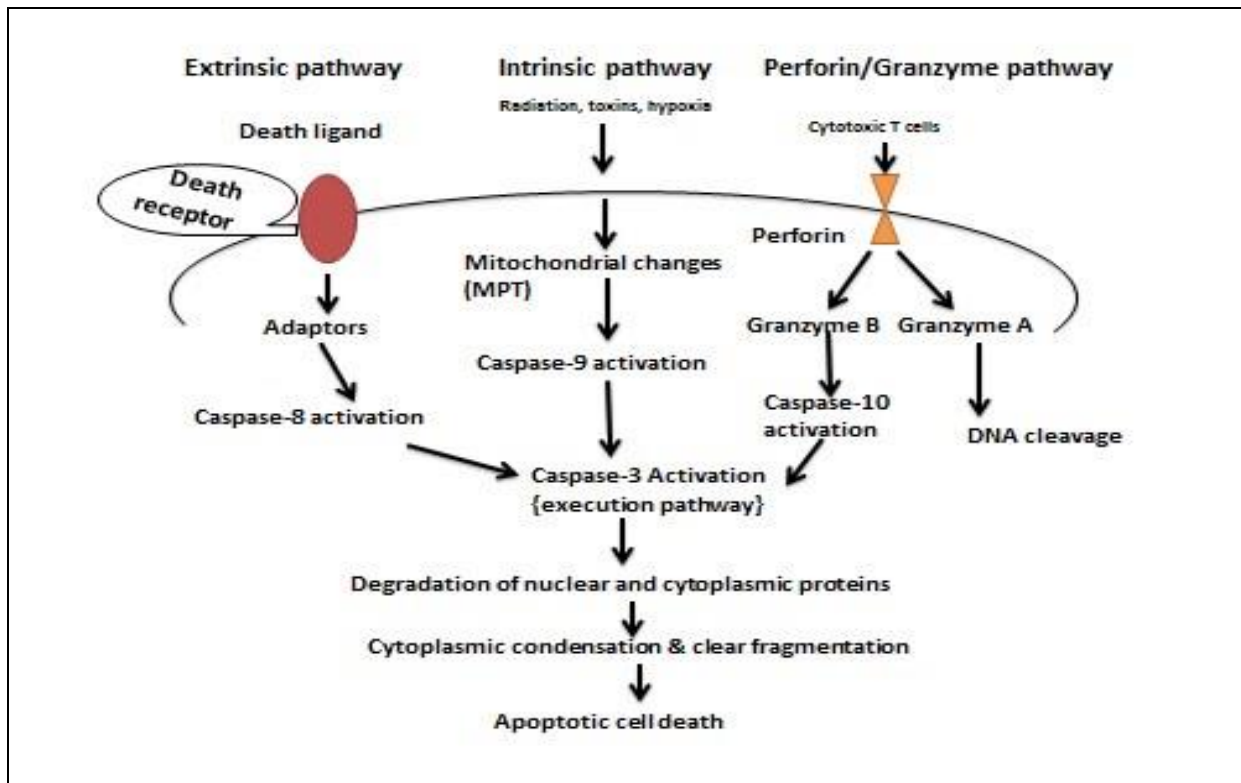
Figure 5: Signalling pathways of apoptosis. *Each pathway has a specific triggering signal to initiate its energy-dependent cascade of molecular events. Each pathway activates different initiator caspases such as 8, 9, and 10 and subsequently will activate the executioner caspase3. However, granzyme A works in a caspase-independent fashion. The activation of caspase-3 (also called execution pathway) leads to several cytomorphological changes including cell shrinkage, chromatin condensation, formation of cytoplasmic blebs and apoptotic bodies and finally phagocytosis of the apoptotic bodies by adjacent parenchymal cells, neoplastic cells or macrophages. This figure was adapted from (Elmore, 2007).*

1.2.2.3 The mitochondrial pathway

This pathway is also called the intrinsic pathway, during which mitochondria integrate different cellular stress signals such as growth factors, calcium or hypoxia (Gustafsson and Gottlieb, 2003). In response to these signals, directly after activation of caspase, the activation of caspase 8 leads to the cleavage of Bcl-2 and Bid. A family of Bcl-2 members can either be pro-apoptotic including Bax, Bid, Bik or anti-apoptotic such as Bcl-2 and Bcl-x (Saelens *et al.*, 2004). Once the pro-apoptotic Bcl-2 family is activated, they move to the mitochondria where they communicate with anti-apoptotic members that are mitochondrial membrane components (Salakou et al., 2007). The interconnection between pro-apoptotic and anti-apoptotic members cause the depolarization of a voltage dependent mitochondrial channel, an increase in the mitochondrial permeability and thus the release of mitochondrial pro-apoptotic factors, cytochrome-c and apoptosis-inducing factor into the cytosol (Penninger and Kroemer, 2003). After cytochrome c has been released into the cytosol, it is associated with adapter molecule apaf-1 and caspase-9 to form a macromolecular complex called the apoptosome. Activation of this complex triggers the activation of caspase 3 and apoptosis (Zou *et al.*, 1999; Jiang and Wang, 2000).

1.2.2.4 Physiological roles of apoptosis

Apoptosis also plays a physiological role in cell turnover, organ development, immunity and the nervous system (Empel *et al.*, 2004) It is responsible for the maintenance of homeostasis in living organisms by depleting cells as a response to variety of stimuli. After the cells have been depleted by apoptosis, the tissue undergoes remodelling through cell proliferation (Porrello et al., 2008). The degree to which the remodelling is achieved, depends on the natural balance between proliferation and apoptosis (Porrello et al., 2008). Apoptosis is, therefore, a necessary mechanism controlling the overall yield of cell proliferation, ensuring that homeostasis takes place in all tissues in a well-regulated manner to avoid adverse cellular consequences (Sinkovics, 1998). It regulates cell homeostasis by maintaining an equilibrium between cell proliferation and cell death, and performs other functions like global cardiac structure, removal of several vestigial structures, the timely renewal of cells (Gustafsson and Gottlieb, 2003; (Elmore, 2007)). This equilibrium is aimed to maintain the balance between new cells being formed as old cells die. Programmed cell death allows this balance through the phagocytosis stage which allows the cell contents to be reused whilst providing a competent mechanism to

extract loads of cells, essential for the equality of cell life and death (Ulukaya *et al.,* 2011; Mcwilliam, 2014).

The amount of cell death by apoptosis that takes place during the development years of a human being, and adulthood is astounding. A practical example of apoptosis is when fingers and toes in a developing human embryo separate, the cells between the digits go through apoptosis. Apoptosis does not only occur during development and aging stages but also functions as a defence mechanism when cells are attacked by a disease or by dangerous agents (Norbury, 2001). In addition, in adults, apoptosis is mainly significant in counteracting unrestricted proliferation and cyclic involution of several endocrine-dependent tissues. Apoptosis can also occur during the development of the nervous system, normally almost half or more of the nerve cells immediately die after their formation (Van Empel and De Windt, 2004). Moreover, apoptosis is considered a major role player in the development of the cardiovascular system, and it takes place at crucial time points during major developmental processes (Elmore, 2007). Apoptosis in the development of the nervous system occurs to minimize the number of nerve cells so they can match the targeted cell number that need innervation (Van Empel and De Windt, 2004).

Apoptosis also plays an important role in the regulation of the immune system. It does this through T-lymphocytes, which are responsible for getting rid of infected or damaged cells in the body (Sinkovics, 1998). The T-lymphocyte cells grow and mature in the thymus. However, before they can go through circulation, they must be inspected to make sure they are effective against foreign antigens and also to ensure that they will not react against healthy cells. Therefore, any self-reactive or ineffective T-lymphocytes are removed through apoptosis (Adams *et al.*, 2000). Lastly, apoptosis is involved in pregnancy, especially in the trophoblast cells which are responsible for the formation of the outer layer of a blastocyst. They invade the uterine environment to ensure maternal blood vessels are remodelled and also help to maintain a successful pregnancy (Sinkovics, 1998). Thus, apoptosis is needed to strictly control proliferation of cells. However, deregulation of apoptosis is commonly known and linked to diseases ranging from cancer, neurodegeneration, and CVD (Elmore, 2007).

1.2.2.5 Pathophysiological roles of apoptosis

Apoptosis plays a major role in the injury of the heart in IHD and HHD (De Meyer and Martinet, 2009). IDH, reperfusion of the myocardium inhibits apoptosis induced by ischemia, during coronary artery ligation and acute myocardial infarction in an in vivo model (De Meyer and Martinet, 2009). However, even though reperfusion can reduce apoptosis induced by ischemia, on its own, it can also elevate apoptosis in the re-perfused parts (Bennett, 2002). For this reason, inhibiting apoptosis in the heart, has become a therapeutic target during myocardial ischemia/reperfusion injury (Bennett, 2002).

Results from different in vitro, animal and human studies demonstrate that apoptosis of cardiomyoblasts plays an important part in the remodelling of cardiac hypertrophy (Wencker *et al.*, 2003). Apoptosis plays a significant part in the cardiac remodelling process (Baetz et al.,2005). and cardiac hypertrophy and apoptosis signalling entails several lateral and assembling pathways (Teiger *et al.*, 1996). Cardiac apoptosis plays an important role in controlling the shape and size of the heart during hypertrophy (Teiger *et al.*, 1996). Moreover, counteracts hypertrophy calcineurin-mediated NFAT activation, which is an important effector of hypertrophy, is critical in preventing cardiomyocyte apoptosis (Pu et al., 2003). The role of apoptosis in Ang-II-induced cardiac hypertrophy has lately been investigated and it was shown that there is an association between Ang-II, and cardiomyocyte hypertrophy and apoptosis. The effects of Ang-II are regulated through two G-protein-coupled receptors, AT1R and AT2R (Dasgupta & Zhang 2011, Da rosa Arauja *et al.*, 2010). The AT1R is responsible for major physiological mechanisms of Ang-II, such as stimulation of hypertrophy and aldosterone secretion from the adrenal gland, vasoconstriction, cardiac contractility and growth stimulation. AT1R induces cardiac hypertrophy, while AT2R is believed to induce opposite effects such as antigrowth by apoptosis and also a possible inhibition of AT1R which subsequently will inhibit hypertrophy (Dasgupta and Zhang, 2011).

1.3 Hypertrophy and apoptosis as therapeutic targets: Cardioprotection

It is possible to modify the signalling pathways of apoptosis and hypertrophy to confer cardioprotection (Guo *et al.*, 2010). Cardioprotection can be accomplished with the recruitment of endogenous mechanisms stimulated by physical or pharmacological interventions (Heusch, 2013), or by modifying hemodynamic and neurohumoral factors of injured cardiomyoblasts. Cardioprotection is therefore, the artificial manipulation of cellular processes with various therapeutic interventions to protect the heart before, during or after disease (Maarman, 2019). These interventions include pre- and post-conditioning, remote ischaemic conditioning, and intake of antioxidants (Maarman, 2019). Over the past years, several cardioprotective strategies have been developed, some of which provide a powerful protection against CVD (Crowley *et al.*, 2006). Other therapeutic strategies that have been examined include changes in the diet and medications which target the mechanisms of oxidative stress, inflammation, cardiac hypertrophy, fibrosis and apoptosis with, however, controversial findings (Bayeva et al., 2013; Papageorgiou *et al.*, 2011). Other routine cardioprotective drugs include ACE inhibitors, betaadrenergic receptor blockers, (McMurray & Pfeffer, 2005), Ang-II receptor antagonists, warfarin, aspirin, statins, hydralazine and nitrates (Quindry and Franklin, 2018). ACE inhibitors and adrenergic receptor inhibitors form part of the first line of the therapeutic intervention in patients with heart failure. Moreover, these two therapeutic interventions also lower blood pressure and block left ventricular remodelling by inhibiting signalling via the Ang-II receptors (Hunt *et al.*, 2009). Beta-blockers are used in conjunction with ACE inhibitors or adrenergic receptor antagonists to treat patients suffering with CVDs (Hunt *et al.*, 2009).

1.4 Natural cardioprotective therapies

The effectiveness of cardioprotective therapies depend on the type of CVD or at the stage of CVDs, and their efficacy can also yield different results in different patients. Regardless of the success of cardioprotective pharmacologic agents in attenuating CVD, death rates among patients continues to rise, with about one-third of patients who die due to heart failure (McMurray & Pfeffer, 2005). Existing pharmaceutical drugs that are routinely prescribed to confer cardioprotection against CVDs have several side effects after long-term administration. Side effects include persistent cough, fluid retention, joint pain, hypotension, fatigue, depression, renal insufficiency, anaemia and headaches (Bianca C Bernardo *et al.*, 2010). This

has led to a quest for alternative cardioprotective therapies, especially ones that are more natural as opposed to pharmaceutical (Ji *et al.,* 2009).

The search for better cardioprotective treatments has therefore placed the focus on medicinal plants (Maarman et al., 2020). These plants have a high polyphenol content, and are loaded with antioxidants (Akhlaghi and Bandy, 2009), which is what allow medicinal plants to prevent or alter disease processes (Mckay and Blumberg, 2007; Dludla *et al.*, 2017). This has been demonstrated in experimental diabetes, inflammation, obesity, cancer/tumour growth, allergies, and skin conditions (Mattera *et al.*, 2017). In recent years, great emphasis has been placed on medicinal plants and their ability to protect against CVDs.

There has been a steep rise in the utilization of herbal remedies in the treatment of clinical CVDs (Vasanthi *et al*., 2012; Atawodi, 2005). Several medicinal plants have been declared helpful against CVD (Akhlaghi and Bandy, 2009) and current evidence suggests that approximately 80% of people in low-income countries still depend on medicinal plants for the treatment of CVDs (Azwanida, 2015; Ji *et al.*, 2009). Medicinal plants have been well-known for their beneficial effects on health, long before their biochemical characterization (D'Andrea, 2015). Some of the plants that have been shown to be protective against CVDs include

Cyclopia spp. and Sutherlandia frutescens (Mckay and Blumberg, 2007; Drahansky *et al.*, 2016). Mainly because fewer side effects have been observed anecdotally, and due to their affordability even if administered long term (Azwanida, 2015; Ji *et al.*, 2009).

1.4.1 Mechanism of action in Apoptosis

Another herb, *Curcuma longa* (Cl) commonly known as turmeric has shown to be protective against myocardial apoptosis (Mohanty *et al.*, 2006). It was evaluated in an ischemia and reperfusion model of myocardial injury. Chronic treatment with *Curcuma longa* significantly inhibited apoptosis. The mechanism of action to attenuate apoptosis was via Bax downregulation and increased expression of the anti-apoptotic protein Bcl-2 as compared to control IR group (Mohanty *et al.*, 2006). The herbal extract can adjust cell signalling cascades (Williams *et al.*, 2004) and gene expression (Kuo, 2002; (Dong *et al.*, 2010), in the presence of CVD related processes (Dludla *et al*., 2017). The cardioprotective effects are associated with an increase in cardiomyocyte survival and contractile function (Budryn *et al.*, 2017). The

cardioprotection is also mediated via attenuated apoptotic signalling by reducing inflammation, lipid excessive increase and preventing impaired oxidation (Budryn *et al.*, 2017). In one study conducted by Malkapuram *et al.*, a hydroalcoholic extract of green coffee attenuated doxorubicin induced apoptosis in an H9C2 model. The data showed that green coffee is cardioprotective against doxorubicin-induced cardiac toxicity and this effect was mediated via the inhibition of apoptosis (Malkapuram *et al.*, 2016). The extract also had cytoprotective effects against oxidative stress in βTC3 cells (Budryn *et al.*, 2017). This extract of green and roasted coffee also applied its antioxidant properties in pro-oxidant-induced oxidative stress and lipid increase and utilized their bioactivity in rats fed a high-fat diet (Budryn *et al.*, 2017).

1.4.2 Mechanism of action on cardiac hypertrophy

In one study conducted by Prathapan *et al.*, 2014, *Boerrhaavia diffusa L.* extract also known as punarnava, conferred cardioprotection against impaired mitochondrial function caused by Ang-II induced hypertrophy in H9c2 cardiomyoblasts (Prathapan *et al.*, 2013). The group demonstrated that a phenolic compound of *Boerrhaavia diffusa L.* extract attenuated Ang-II induced hypertrophy, and this was mediated via reduced ROS, improved mitochondrial membrane potential, and improved activities of the mitochondrial ETS complexes, (Prathapan *et al.*, 2013). In another study, *Boerrhaavia diffusa L.* extract attenuated Ang-II induced hypertrophy, by down-regulating NF-κB and TGF-β1 signalling (Prathapan *et al.*, 2013). The extract also reduced oxidative stress via the decrease of ROS production and improved ETS function that improved cardiac function. Quercetin, a flavanol that occurs naturally in medicinal plants, has been shown to attenuate Ang-II-induced cardiac hypertrophy in H9c2 cardiomyoblasts as well as left ventricular hypertrophy in spontaneously hypertensive rats (Yan *et al.*, 2013). Quercetin inhibited hypertrophy by suppressing protein synthesis induced by Ang-II. It also inhibited changes in size by reducing the expression of hypertrophy markers (cfos, c-jun, ANP and BNP) (Yan *et al.*, 2013).

Vitexin a flavone glycoside isolated from the leaf of *Crataeguspinnatifida Bunge* attenuated isoproterenol induced cardiac hypertrophy in cultured neonatal rat ventricular myocytes in vitro and pressure overload-induced cardiac hypertrophy in mice (Lu *et al.*, 2013). The pathway through which the cardioprotective effects of Vitexin were mediated was the calcineurin-NFAT pathway. Vitexin inhibited the expression of Calcineurin and NFATc3 and increased the expression of calmodulin kinase-II in both models (Lu *et al.*, 2013). Rosmarinic acid (RA), one

of the natural polyphenol plants and ester of caffeic acid is found in mint and basil (Ito *et al.*, 1998) had inhibitory effects on adriamycin induced apoptosis in H9c2 cardiomyoblasts. Additionally, RA improved cell viability via the inhibition of intracellular ROS production and the restoration of cardiac mitochondrial membrane potential (Kim *et al.*, 2005). These data suggest that medicinal plants are effective at counteracting CVD, and they achieve this by altering cardiac hypertrophy and apoptosis.

1.4.3 The role of Rooibos (Aspalathus Linearis)

Rooibos (RB) (*Aspalathus linearis*) is a member of the plant family Fabaceae that is known to grow in South Africa's fynbos, it is broom-like structural. It is located to the north-western to western region of the Cape Floristic Region of South Africa in the Cederberg area and it is one of the most widely consumed herbal teas or tisanes (Mckay and Blumberg, 2007). Dating back to 1904, Benjamin Gunzberg recognized RB had a marketing potential and since then its popularity has steadily risen worldwide (Wilson, 2005). Its caffeine-free status contributed to the popularity of RB by modern consumers (Joubert *et al.*, 2009). However, it was not always seen as advantage to be caffeine-free, in 1917 it was reported by the Imperial Institute of London that "It does not look like this material would be acceptable in the United Kingdom as a substitute for ordinary tea, as it contains no caffeine or other alkaloids and would subsequently not have the stimulating effect of tea''. Later, the United Kingdom become one of the top importers of RB. There is another well-known export market for RB worldwide which include Germany, Japan, the Netherlands and the United States of America (South African Rooibos Council, 2017). Moreover, these countries represent more than 80% of the export market. RB is used as herbal infusion, health beverage, an ingredient in skin care products and cosmetics as well as a flavourant and colouring agent in a number of food applications (Windvogel, 2020).

The term 'RB' is used to reference the plant or the herbal beverage (tea) made from the plant (Hawkins et al., 2011). Other than RB being used as a beverage, it is also been recognized as used in numerous other products such as soaps, cosmetics and skin lotions (Chuarienthong et al., 2010). There are several other health beneficial effects of RB. The first documented use of RB was during the late 1700s by local Khoi-Khoi people who were observed using the plant medicinally (Von Gadow et al., 1997). More than 300 years ago, the indigenous Khoi-Khoi

tribe of the Cedarberg and the Elephants river valley region of the Western Cape first discovered that the leaves of the RB plant can be used as a tea, with an exceptional taste and aroma (Rooibos council, 2017). Following that research began to focus more on RB and the health benefits of RB were confirmed. RB has gained recognition as a functional health beverage both local and globally, because of its unique polyphenol profile (Beltrán-Debón *et al.*, 2011). It can be used in two forms, fermented and unfermented: fermented is oxidized and unfermented is unoxidized. The fermentation process gives fermented RB its unique brick red colour, while unfermented RB tea keeps its green colour (Gadow *et al.*, 1997; Gwashu, 2016).
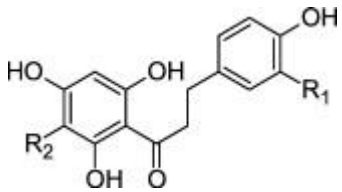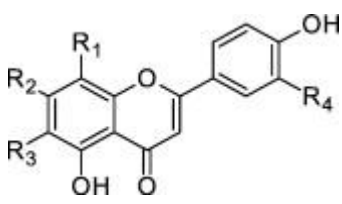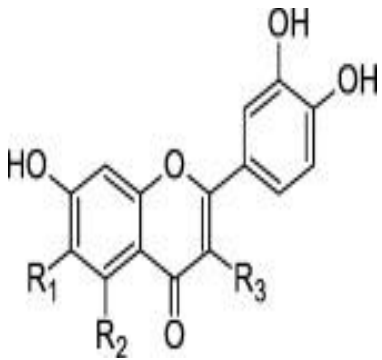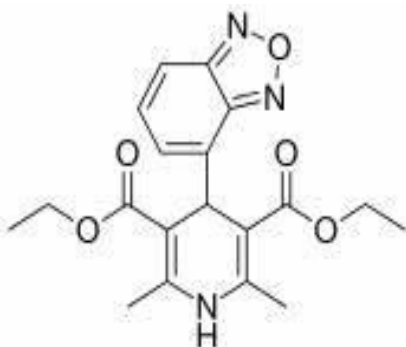
Prior to commercialization of the "unfermented" variant, also known as green RB or "unfermented" RB, the name "RB" referred to the fermented product. Recent protection of the name "RB" in South Africa (Anonymous, 2013) and the subsequent recognition of its status as a geographical indication (GI) in the European Union (Anon., 2014), not only offers ownership of this particular name to South Africa, but it will ensure that the term will be applicable only to RB products. Other common names used by international markets are red bush (translation of RB), red RB, and red tea. The latter name can lead to confusion with other herbal teas such as one prepared from the leaves of the hibiscus flower. Although a misnomer, the terms "unfermented" and "fermented" are widely used in the context of RB (Muller *et al.*, 2018). It has been shown to have anti-diabetic and hypoglycaemic effects (Jin *et al.*, 2013; Kamakura *et al.*, 2015) as well as antioxidant effects (Canda et al., 2014). In addition, RB also has demonstrated anti- inflammatory effects (Baba *et al.*, 2009), it has been shown to reduce colitis and modulate immune function *in vitro* (Hendricks and Pool, 2010) as well as *in vivo* where it has been shown to promote antigen-specific antibody production through augmentation of interleukin-2 production (Kunishiro et al., 2001). The chemoprotective effects of RB have been demonstrated in rat liver using the cancer initiator diethyl nitrosamine (Marnewick *et al.*, 2009).

1.4.3 Phytochemical and nutrient content of RB

Phytochemicals are non-nutritive compounds that are produced by plants and they possess different types of protective/ health properties (Gwashu, 2016). These phytochemicals are in essence, secondary plant metabolites and are mostly bound to sugar moieties that increase their solubility in water, thus allowing for storage in an inactive form (Perold, 2009). The most common phytochemical compounds found in RB are flavonoid and phenyl pyruvic acid glucosides listed in Table 1 (Mckay and Blumberg, 2007). RB has anti-inflammatory, anticancer, antidiabetic, and antioxidant properties (Atawodi, 2005). These properties of RB have been ascribed to its high phytochemical content.

Polyphenols can have indirect antioxidant effects altering antioxidant gene expression and endogenous antioxidants enzymatic defenses (Stevenson and Hurst, 2007; Forman *et al.*, 2014). In a study conducted by Beltrán-Debón and colleagues, administration of RB evoked similar protective effects to resveratrol, the effects include improved metabolic rate and increased mitochondrial biogenesis (Beltrán-Debón *et al.*, 2011). Dihydrochalcone, aspalathin and nothafagin are the distinct polyphenolic compounds found in RB (Dludla *et al.*, 2016). They possess antithrombotic activity and also help to reduce inflammation induced by high glucose. Fruit and vegetables have been recognized to for their beneficial effect on human health (Joshipura *et al.*, 2001), but the underlying mechanism of dietary polyphenols are not fully elucidated.

Table 1: Flavonoid and phenyl pyruvic acid glucoside content of green and fermented RB
(Mckay and Blumberg, 2007; Walters, *et al.*, 2017)

STRUCTURE	TYPE	COMPOUND
	Dihydrochalcone	Aspalathin
		Nothafagin
	C-Glucosyl flavones/Flavones	Orientin
		Isoorientin
		Vitexin
		Isovitexin
		Luteolin
		Chrysoerio
	O-Glycosyl flavonols	Quercetin-3-O-rutinoside Quercetin-3-O-robinobioside
		Quercetin-3-O-galactoside
		Quercetin-3-O-glucoside
	Phenylpropenoic acid glucoside	PPAG

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1.4.4 The link between rooibos and cardioprotection

The major source of dietary phenolic consumption has been reported to reduce CVD risk (Mattera *et al.*, 2017). The flavonols, Rutin and quercetin, have been proven to enhance cardiac function in streptozotocin-induced diabetic rats (Krishna *et al.*, 2005). Baba *et al.*, 2009 and Pantsi *et al.*, 2011b demonstrated that fermented and unfermented RB improved aortic output and that this was associated with high flavonol content.

RB has been reported to be anti-apoptotic due to the presence of PPAG, one of the RB components which has been shown to be protective against apoptosis against STZ induced apoptosis (Himpe *et al.*, 2016). Beneficial effects of dietary RB extract significantly decreased PARP cleavage which strongly suggests that RB offers this protection by inhibiting apoptosis (Pantsi *et al.*, 2011). Accumulating data have shown that flavonols inhibit apoptosis by downregulating the pro-apoptotic proteins, caspase-3 and Bax, in return inhibits apoptosis (Johnson *et al.*, 2016; Dludla *et al.*, 2016; Himpe *et al.*, 2016) One study, also found that cardiomyoblasts pre-treated with FRE for six hours caused a reduction intracellular ROS production and blocked apoptosis caused by exogenous H_2O_2 and ischemia (Dludla *et al.*, 2014). Moreover, cardioprotective effects of RB and its flavonoids also appear to involve a reduction in lipid peroxidation and up-regulation of the antioxidant enzymes through Nrf2 activation, and a reduction in apoptosis which may also be associated with increases in the Bcl2: Bax ratio (Johnson *et al.*, 2017; Pantsi *et al.*, 2011b; Dludla, *et al.*, 2017) (Table 2).

Another study has reported that protective effect is dose-dependent (Kawano *et al.*, 2009).

Polyphenols are suggested to have pro-oxidant effect in dose response (Bennetau-pelissero, 2005). However, the protective effect of the fermented RB extract depends on dose response and efficacy. Increasing the dose from 1 to 10 g/ml reduced its protective effect against exogenous HH_2OO_2(Dludla *et al.*, 2014). The effect of RB being dose dependent has been found to be interesting because one would expect that higher dose should increase its activity. Also, in the current study during dose-response test, a high concentration 500 µg/ml seemed to reduce the protective effect of RB, these finding were unexpected. In addition, it has been reported that polyphenols are more likely to have pro-oxidant effects at higher doses (Mennen *et al.*, 2005). In addition, an aqueous extract of fermented rooibos (Aspalathus linearis) also demonstrated anti-diabetic effects in cardiomyoblasts. The anti-diabetic effect was induced by

improving decreased of GSH and ATP concentration which are tightly related to generation of oxidative stress and metabolic dysfunction (Dludla *et al.*, 2014). Several other studies have also reported on the anti-diabetic properties of RB. For example (Ulicna *et al.*, 2006) showed that an aqueous extract of RB improved oxidative stress in streptozotocin induced Wistar rats by significantly lowering malondialdehyde levels in plasma and tissues of rats. In rodent models of streptozotocin induced diabetes, RB exerted antioxidant effects through increases in the activity of superoxide dismutase, catalase, glutathione peroxidase and decreasing lipid peroxidation (Dludla *et al.*, 2017; Ayeleso et al., 2014) (Table 2).

Table 2: Cardioprotective effect of RB compounds in different experimental models. This table was designed and constructed and adapted from (Dludla, Joubert, *et al.*, 2017)

Compound	Experimental outcome	Reference
Aspalathin and Nothafagin	RB offers cardiac protection against ischaemia/reperfusion in the isolated perfused rat heart	(Pantsi et al., 2011)
	Prevented cell apoptosis by reducing phosphorylation of AMPK; decreasing inflammation and lipid accumulation; and attenuated oxidative damage via increasing Nrf2 expression	(Johnson et al.,2016;)
	Protective properties of RB flavonoids on the prevention of skin cancer	(Gwashu, 2016)
Orientin and isoorientin	Prevented myocardial infarction	(Ku et al, 2014)
	Attenuated ventricular remodelling associated with myocardial infarction	(Ku, Kwak and Bae, 2014)
	Protected vascular barrier integrity by inhibiting hyper permeability	(Lee et al., 2014)
Vitexin and isovitexin	Prevented ischemia-reperfusion injury by reducing calcium overload and modulating ERK1/2 signalling and MAPK pathway.	(Che et al., 2016)
	Prevented cardiac hypertrophy by inhibiting calcineurin and CaMKII signalling pathways	(Lu et al., 2013)
Luteolin and chrysoeriol	Prevented ischemia-reperfusion injury and increased of coronary flow.	(Liao et al., 2011)
	Reduced arterial blood pressure and protected against doxorubicininduced cardiotoxicity	(Liu et al., 2009)
	Prevented hypertensive vascular remodelling	(Su et al., 2015)
Quercetin and rutin	Alleviated vascular complications associated with insulin resistance through the PPARγ pathway	(El-Bassossy et al., 2014)
	Quercetin inhibits left ventricular hypertrophy in spontaneously hypertensive rats and inhibits Ang-II-induced H9C2 cells hypertrophy by enhancing PPAR-c expression and suppressing AP-1 activity	(Yan et al., 2013)
Hyperoside and rutin	Quercetin prevents in vivo and in vitro myocardial hypertrophy through the proteasome-GSK-3 pathway	(Chen et al., 2018)
	Hydrogen peroxide induced cell damage and ischemia reperfusion injury	(Li et al.,2012 ; (Piao et al., 2008)
	Protected against advanced glycation end products, oxidative stress, and myocardial infarction	(Wen et al.,2013 ; (Hou et al., 2016)

RB was also demonstrated to improve oxidative stress and lipid profiles after chronic consumption of six cups of RB tea for six weeks, these two are both associated with CVDs in healthy adults at risk for developing CVD (Marnewick *et al.*, 2011). RB was also demonstrated to exert anti-hyperglycaemic effects. Aspalathin from green RB tea was found to prevent postprandial hyperglycaemia by suppressing glucose absorption and inhibiting carbohydrate hydrolysing enzymes (Mikami *et al.*, 2015). Recently, Dludla and co-workers (Dludla *et al.*, 2020) demonstrated that RB also ameliorated glycemia-induced oxidative damage in a model of H9c2 cardiomyoblasts exposed to high glucose concentrations, by improving FFA utilization and enhanced levels of intracellular antioxidants such as glutathione and superoxide dismutase.

RB was also demonstrated to reduce hepatic insulin resistance by modulating the PI3K/Akt and AMPK pathways (Mazibuko-Mbeje *et al.*, 2019). The PI3K/Akt pathway is a complex insulin regulated pathway implicated in glucose and lipid metabolism. This pathway is also involved in other cellular processes, which include protein synthesis, cell signalling, cell growth and apoptosis (Huang *et al.*, 2018; Mackenzie and Elliott, 2014).

Cardiovascular protective effects of RB have also been established in several other studies. RB inhibited ACE in 17 healthy volunteers receiving a single oral dose of RB. This suggests that RB achieves cardioprotection through inhibition of ACE activity (Persson *et al.*, 2010). It has been suggested that the effect of RB on ACE could be due to the presence of the double bond between C2 and C3, the catechol group at the B-ring and the acetone group on C4 on the Cring of flavonoids (Guerrero *et al.*, 2012). The inhibition induced by RB may be due to quercetin, one of the RB flavonols, which has been shown to exhibit an inhibitory effect on ACE activity in vitro (Persson *et al.*, 2008). ACE contains two active sites of $Zn2^+$and therefore, the ability of quercetin to bind the $Zn2^+$ ion results in the inhibition of ACE activity (Berecek and Zhang, 1995). In an in vitro study conducted by (Persson *et al.*, 2006) RB inhibits ACE and increases nitric oxide production in human umbilical vein endothelial cells.

1.5 Mechanisms of RB-induced cardioprotection

The use of RB, particularly in its extract/tea form has become an attractive, simple and affordable approach to potentially treating disease (Hu, 2003; Vasanthi *et al*., 2012). RB extract has been reported to confer cardioprotection against a model of ischaemia/reperfusion in the isolated perfused rat heart (Pantsi *et al.*, 2011). Another study used RB extract to show its cardioprotective effect (Dludla *et al.*, 2020) in a hyperglycemia-induced myocardial oxidative damaged model. Several other studies also showed cardioprotective effects of RB (Mazibuko *et al.*, 2013; Smit, 2004; Mikami *et al.*, 2015). In addition, separate RB compounds have been documented and proven that they may also confer cardioprotection on their own such as Cglucosyl flavonoids, and Z-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid (Muller *et al.*, 2018), rutin (Jeong *et al.*, 2009; Krishna *et al.*, 2005), and quercetin (Chen *et al.*, 2018; Panicker *et al.*, 2010). However, when comparing effects of separated RB compounds on their own versus the whole RB extract different result effects have been shown. For example, one group investigated effect of quercetin, while another used RB extract against ischemia-reperfusion and the cardioprotective mechanisms were different. Rooibos extract prevented ischemia reperfusion injury by inhibiting apoptosis (Pantsi *et al.*, 2011), while the other group using quercetin prevented ischemia reperfusion injury by reducing the activity of Src kinase and intracellular ROS production (Chen *et al.*, 2013). Therefore, interest is accumulating to elucidate the underlying mechanisms of RB-induced cardioprotection. To date, these mechanisms have not been fully elucidated. Furthermore, despite the association between CVDs, hypertrophy and apoptosis, the effect of RB extract has not been investigated in a combined model of cardiac hypertrophy and apoptosis. Therefore, the purpose of the present study is to fully describe and elucidate the mechanisms of an aqueous extract of fermented RB in such a complex model.

1.5.1 Hypothesis

We hypothesize that RB confers cardioprotection in an Ang-II induced cardiomyocyte apoptosis and hypertrophy model.

1.5.2 Aim

To establish the cardioprotective effects and underlying mechanisms of RB.

1.5.3 Objectives

- Establish a model of Ang-II-induced hypertrophy and apoptosis using H9c2 cardiomyoblasts

- Investigate the cardioprotective effects of RB extract by measuring cardiomyocyte apoptosis, viability and cell growth/size using live cell imaging.

- Investigate the involvement of in RB-induced mitochondrial protection by measuring ATP production and expression of mitochondrial ETS complexes.

- Delineate the effect of RB on cardiomyocyte hypertrophic and apoptotic signalling pathways with Western Blotting.

Chapter 2: Materials and Methods

2.1 Plant material:

A cold water-soluble RB extract 02 (fermented), batch number: E1CCJ / 23/PP/A was kindly supplied by Rooibos Limited® (Rooibos Avenue, Clanwilliam 8135). The procedure for preparation was as follows: A powdered cold-water RB extract was weighed off for each experiment (10mg/ml) and dissolved in a cold water to make up the stock. The dissolved extract was diluted freshly for each experiment in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2% fetal bovine serum (FBS), at the acquired concentration before treatment for each experiment.

2.2 Cell culturing and maintenance

H9c2 rat embryonic heart-derived ventricular cells were originally obtained from the European Collection of Cell Cultures. Cells were maintained in a monolayer at 37°C and in a 95% O_2, 5% CO_2, humidified air atmosphere and were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen Gibco), supplemented with 10% fetal bovine serum (FBS, Invitrogen, Gibco) and 1% penicillin/streptomycin solution (Invitrogen, Gibco). Cells were allowed to proliferate in T75 flasks (75 cm^2 flasks, Nest Scientific, USA) and the growth medium was refreshed every 72 hours. When cultures reached 70-80% confluence they were split to the next generation by washing the cell monolayer with pre-warmed (37°C) phosphate-buffered saline (PBS) followed by trypsinization using 0.25% Trypsin-ethylene-diaminetetra-acetic acid (trypsin-EDTA) (Invitrogen Gibco). Cells were gently agitated to ensure complete detachment from the flask and were transferred to a sterile tube for centrifugation at 700 rpm for 4 minutes. Cell pellets were re-suspended in fresh warm growth medium and a cell counter was used to count the cells. Cells were then seeded at the desired density in fresh warm growth medium for experiments or further passaging. Cells between passages 15 and 20 were used in all experiments.

2.3 Cell culture treatments

Procedure: H9c2 cells were seeded in a 100mm petri dish at 500,000 cells per plate and allowed to attach and recover overnight in the incubator in a DMEM. The next day, DMEM was removed and replaced with a fresh DMEM (0% FBS) for an hour and this is known as double starvation. After 1 h of double starvation with plain DMEM, the first group was treated with Ang-II added to a final concentration of 20µM, next group treated with RB (100 µM), Losartan (Los) only (10µM), co-treatment with RB + Ang-II and co-treatment with Ang-II + Los. Vehicle control cells received DMEM with 2% FBS only. The cells in all the treatment groups were incubated for 48 h (see Figure 6). At the end of the treatment, media was removed and 10 ml of fresh Dulbecco's phosphate-buffered saline (DPBS) containing 10 µl of JC-1 dye as mitochondrial membrane potential indicator was added and incubated for 30min to measure surface area. Cells were washed with DPBS and imaged in DMEM without phenol red (Thermo Fisher Scientific, Paisley, UK). Live cells were imaged using a Zeiss inverted confocal microscope (Nikon, UK) taking a total of 6 images per treatment in 2X 100 mm petri dish 20X magnification. Individual images were analysed using an Image J Analyzer, ± 100 cells were analysed per treatment. The mean surface area per cell were measured and compared using two-way ANOVA followed by Bonferroni's multiple comparisons test.
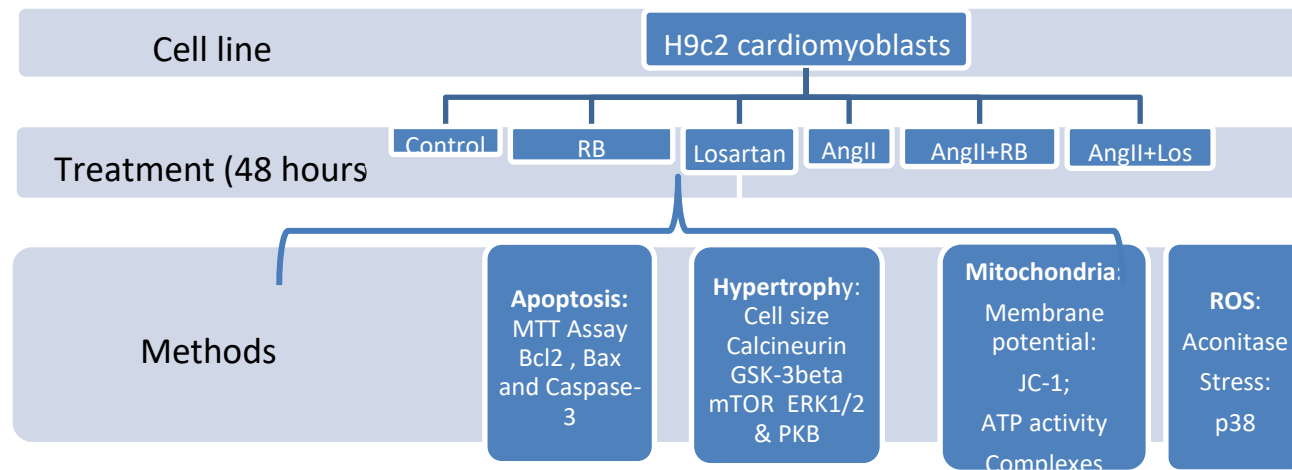
2.4 Study design.

Figure 6: Schematic representation of the study design. *H9c2 cells were treated as indicated above, followed by the assessment of various mitochondrial and cellular parameters*

2.5 Preparation of the drugs

6.5.1 Angiotensin II Acetate human (SLBX8991) from Sigma Aldrich (MW: 1046.2) at 1mg/ml was dissolved in 1 mL cell culture-tested water (MilliQ) to give a final concentration of 0.956 mM stock solution which was stored at –80°C. Working concentration of 20 and 5 µM were made up from the initial stock using DMEM. For experimental purposes, the final concentration used was 20µM, as significantly increased cell size was obtained at this concentration compared to 5µM. Even though 5µM also increased cell size it was less effective than 20µM

6.5.2 Losartan from Sigma (MW: 461 g/mole) at 5mg was dissolved in 10mL cell culture tested water (MilliQ water) to give a final concentration of 1mM stock solution which was stored at –80°C. Serial dilution of working concentration of 10µM was made from to initial stock using DMEM to make up for final concentration of 0.1µM and 1µM from 10µM. However, 10µM worked better as compared to other concentrations, as it significantly reduced increased cell sized caused by 20µM Ang-II treatment.

2.5.3 JC-1 from Abcam (ab113850) (MW=652,23g/mol) at 5mg was dissolved into 2.5ml DMSO to give final concentration of 3mM stock solution which was stored at –80°C. The working concentrations for the dose-response tests were 1.65µM and 3.25µM from the initial stock to give dilution of final concentration 5µM and 10µM. However, we chose 10µM as it was more effective for the time of 30 min and analysis was much easier, while 5µM could not reach equilibrium in 30 min making it hard for the stain to penetrate the mitochondria. The incubation period 30 min found in the literature was not effective. This was due to the fact that it was not enough time for such a small amount of stain (5µM) to penetrate into the mitochondria and reach equilibrium to show the fluorescence. Thus, poor images, which were difficult to analyse. For that reason, we chose 10µM for 30 min. The working concentration of 10µM was used and diluted with DPBS.

2.5.4 The cold water-soluble RB extract 02 (fermented), batch number: E1CCJ / 23/PP/A at 10mg/ml was in dissolved 1 ml cell culture-tested water (MilliQ) stock solution stored at-80°C. The working concentrations were 50µg/ml and 100µg/ml. However, we chose to work

with 100µg/ml as it was more effective when compared with 50µg/ml. The viability tests showed more live cells at 100µg/ml compared to 50µg/ml.

2.6 Measurement of hypertrophic growth in H9c2 Cardiomyoblasts

Hypertrophy was assayed by the measurement of cell size of H9c2 cells as previously described (Hwang *et al*., 2006; Alvarez *et al*., 2007). Fifty percent confluent cells were seeded at a density of $5x10^5$ cells /mL in 100mm petri dish. After treatment, cells in the 100mm petri dish plates were washed with PBS. 10µM JC-1 prepared in DPBS was added to each plate for 20 minutes at 37˚C incubation. The JC-1 solution was aspirated, and cells were washed twice with PBS. DPBS was then added for imaging using a Zeiss fluorescence microscope, random photographs were taken from each sample. Three images per sample were taken, 100 cells were selected for analysis to avoid biasness and images were analysed using image J for cell size.

2.7 MTT Assay

Cell viability assay were performed in intact H9c2 cells using the MTT cell viability assay. The MTT assay is a colorimetric reaction. It is also a quantitative and sensitive detection of cell proliferation by measuring the growth rate of cells by virtue of a linear relationship between cell activity and absorbance (Mahajan et al., 2012). The reduction of 3- [4, 5-dimethylthiazole2-yl]-2, 5-diphenyltetrazolium bromide (MTT) enzyme to MTT-formazan is catalysed by mitochondrial succinate dehydrogenase (Chacon et al., 1997). Therefore, MTT cellproliferation assay measures the reduction of a tetrazolium component (MTT) into an insoluble formazan product by the mitochondria of viable cells.

H9c2 were seeded in 24-well cell culture microplate at an optimized density of $1.5x10^5$cells/well and were incubated overnight at 37°C. Cells were then treated as previously described. At termination of treatment, medium was removed and cells were refreshed with 200µl of Assay buffer (containing 1ml DPBS and 2mg/ml MTT assay powder) purchased from Sigma-Aldrich, and placed into a CO_2 - free incubator at 37°C for 2 hrs, followed by addition of Sorensen's buffer (01 M Glycine, 0.1M NaCl, 50 ml sterile water; pH 10.5) and DMSO to lyse the cells and solubilize the coloured crystals. The colorimetric detection is done at a wavelength of 570 nm in a spectrophotometer plate reader. Therefore, the amount of colour generated is directly proportional to the number of viable cells.

2.8 Immunofluorescence staining

Differences in mitochondrial membrane potential were detected by using 5, 5', 6, 6', tetrachloro-1, 1', 3, 3', -tetraethylbenzimidazolylcarbocyanine (JC-1). JC-1 is a ratiometric (red/green), cationic, lipophilic fluorescence probe. Due to its cationic nature, JC-1 easily enters the mitochondria and forms aggregates (J-aggregates) that emit red fluorescence in the phycoerthrin (PE - red) channel. However, in cells with decreased mitochondrial membrane potential or depolarized mitochondria, JC-1 remains in the cytoplasm in its monomeric form, which emits green fluorescence in the fluorescein (FITC - green) channel. Since the monomers and aggregates of JC-1 have different emission spectra, changes in mitochondrial membrane potential can be assessed by comparing the ratio of PE to FITC (red/green). Therefore, the ratio of red/green decreases as the mitochondrial membrane potential decreases (Keil *et al.*, 2011)

2.9 Measurement of aconitase

Aconitase is an iron–sulfur-containing protein that isomerizes citrate and is activated during mitochondrial oxidative stress (Prathapan et al., 2014; Abcam, 2014). Aconitase consists of two isoenzymes, mitochondrial aconitase and cytosolic aconitase. The key role mitochondrial aconitase is to regulate ATP production through intermediate flux in the Krebs cycle. Moreover, the cytosolic aconitase when in its reduced state, functions as an enzyme, while in the oxidized state it is implicated in regulation of iron homeostasis as iron regulatory protein 1 (IRP1). ROS is believed to play a critical role in the regulation of aconitase functions. Catalytic aconitase action is regulated by [4Fe-4S] 2+ cluster reversible oxidation and cysteine residues, so redox-dependent posttranslational modifications (PTMs) have gained increasing consideration as regards possible regulatory effects. In the presence of a sustained ROS flux, redox-dependent PTMs are most likely to cause enzyme damage and cell stress through altered energy and iron metabolism.

At the termination of treatment activity of aconitase was assayed in control and treated cells using an Aconitase assay kit (ab109712, from Abcam® USA) as per manufacturer's instructions. After 48hrs of the treatment, cells were washed and gently scraped to ensure fully detachment and transferred into a sterile 15ml tube for centrifugation at 700 rpms, 4 min at 4˚C.

Cell pellets were re-suspended in fresh ice cold Aconitase preservation solution supplemented with 1/10 detergent and centrifuged at 20000X g for 10 minutes. Protein concentration was determined using the Bradford assay to determine whether the proteins extraction efficiency is highly variable between samples being compared. After protein determination aconitase activity was determined by adding 50µl of the diluted sample and 200µl of assay buffer into the wells of 96-well plate consist with isocitrate and manganese. Measurements were taken using plate reader with kinetic program for 30 minutes.

2.10 ATP Activity

Cell were grown and treated as previously described. ATP activity was measured using the luminescence-based Cell-Titre-Glo luminescence assay *kit* (Lonza Inc., Rockland, USA). Cells were cultured in 96 well plates overnight and treated for 48 hrs. Samples were prepared according to the manufacturer´s instructions, at the termination of the treatment 100 µl ATP content was added in each well for ATP levels. Measurements were performed in an iMark (Biorad) microplate reader, using white plate, clear bottom-bottom with lid 96 well plates, recording the absorbance at 450 nm after 30 min incubation at 37˚C. ATP standards were prepared in the range of 0.01–1 µl. Each sample and standard were prepared in triplicate in the assay, which was performed for each independent experiment.

2.11 Fractionation of cytosol/Nucleus

H9c2 cells were grown and treated as previously described in 100mm petri dishes. At the termination of treatment, growth medium was removed, and cells were washed with cold PBS (4°C). Cells were then gently scraped from the bottom of culture flasks using a sterile cell scraper whilst maintained on ice, and then centrifuged in a 15 ml tube at 700 rpm for 4 min at 4˚C. The supernatant was discarded. Proteins were extracted from cell pellets by adding 200µl of cold Radioimmuno precipitation assay (RIPA) buffer, pH 7.4, containing: Tris-HCL (2.5mM), EDTA (1mM), Sodium orthovanadate (Na$_3$VO, 1mM), Protease inhibitors (Cocktail of (10mg/ml)), phenyl-methyl-sulphonyl fluoride (PMSF, 0.1mM), Nonidet-P40 (NP-40, 1%), NaCl (0.1M). Samples were then put on ice for 20 min to weaken the cell membranes. After they have been thawed, 0.5 Zirconium beads were mixed with cells and bullet blended 3 times for 1 min and rested for 5 min after each spin. After bullet blending, samples were incubated

on ice for another 20 min and then centrifuged at 2500 rpm for 10 min (4˚C). The supernatant (cytosolic fraction) was transferred in a pre-cooled 2 ml Eppendorf tube. The pellets known as the nuclear fraction was centrifuged again to extract nuclear protein. To extract nuclear proteins, 200μl of cold Radioimmunoprecipitation assay (RIPA) buffer plus 1% Triton and 0.1 % SDS were added to the cell pellets. The pellets were bullet blended as previously described to get nuclear extracts. After bullet blending, samples were left to rest for 20 min on ice. Samples were then centrifuged at 13,000 rpm for 30 minutes at 4ºC. After 30 min the nuclear proteins in the supernatant were collected and the concentration of the proteins was determined by the Bradford method.

2.12 Western blot

Proteins were loaded and separated using gradient polyacrylamide gels with a 4% stacking gel (4 – 20% Mini-PROTEAN® TGX Stain-Free™ Protein Gels, Bio-Rad). A BLU eye prestained protein ladder (GeneDirex, PM007-0500) was loaded in the first lane on each gel as a reference of molecular weights of specific bands. The proteins samples were boiled for 5 min and briefly centrifuged before loading each sample (20μg/well) into the remaining gel lanes. Proteins were separated by SDS-PAGE (Criterion-PROTEAN® Tetra cell, Bio-Rad) at a constant voltage of 100 V and current of 200 mA for 10 minutes and thereafter at a constant voltage of 140 V (200mA) for 50 minutes (Power Pac 1000, Bio-Rad). Following SDS-PAGE, the stain-free gels were imaged using the ChemiDoc™ XRS + System (Bio-Rad) to determine equal loading and activation of the gel. The proteins will then be transferred to a polyvinyl dine fluoride (PVDF) membrane (Trans-Blot® midi transfer pack, Bio-Rad) at a constant voltage of 200 V and 200 mA for 1 hour using the Trans-Blot Turbo System (Bio-Rad). The proteins on the PVDF membranes were imaged using the ChemiDoc™ XRS + System (Bio-Rad).

Nonspecific binding sites were blocked with 5% fat free milk in Tris-buffered saline solution with 0.1% Tween (pH 7.4) for 1-2 hours at 24°C. Membranes were incubated with Phosphospecific antibodies (1:1000) overnight at 4°C. Phospho-specific antibodies were as follows: Phospho-mTOR (Ser2448), Phospho-GSK-3 beta (Ser 9), phospho-PKB/ Akt (Ser-473), phospho-ERK1/2 or p44/p42 (Thr-202/Tyr-204) Phospho-p38 (Cell Signalling, Danvers, MA, USA). The corresponding antibodies for total protein expression (1:2000) were also used: mTOR, GSK-3 beta, PKB/Akt, ERK1/2 or p44/p42, as well as Calcineurin A/B, 3, Caspase-3,

Bax, Bcl-2, and p38 MAPK. Histone was also used as nuclear marker. After incubation with horseradish peroxidase-linked secondary antibody (1:2000 to 1:5000), signals were enhanced with a chemiluminescent agent (ECL) and visualized by ChemiDoc using Image Lab. Results were normalized to the total protein loaded via the house-keeping protein beta tubulin, to correct for unequal loading and expressed as a ratio of phosphorylated over total proteins. For each gel, all values were expressed as a fold-change to the untreated control samples. For each gel there were 6 groups, and all were loaded in duplicate. We also did three biological repeats.

2.13 Quantification of individual flavonoids

High performance liquid chromatography (HPLC) analysis with diode-array detection as previously described on an Agilent 1200 system (Agilent, Santa Clara, CA, USA) was used to determine the compounds present in the fermented rooibos extract. All the compounds were determined using the method described in Walters et al. (2017). However, Aspalathin and ferulic acid were determined using the method described in (Muller *et al.*, 2020).

The peak height or area for different peaks cannot be compared directly as different compounds have different spectra with different wavelengths for maximum absorbance. We use 288 nm for compounds with maximum absorbance near 288 nm (e.g. PPAG, Aspalathin), while 350 nm is used for compounds with their maximum absorbance near 350 nm (e.g. orientin and isoorientin). Furthermore, compounds with their maximum absorbance at a similar wavelength could still have different extinction coefficients, i.e. different concentrations of the compound are needed to give the same peak area. Therefore, we used authentic reference standards to generate calibration curves against which each compound is quantified.

2.14 Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc.). Comparisons between treatment groups were performed using a one-way ANOVA analysis followed by a Bonferroni multiple comparison as a post hoc test or an unpaired Student's t-test where appropriate. A P-value of ≤0.05 was deemed as statistically significant. Results were expressed as the mean ± SEM of 3 independent biological experiments

Chapter 3: Results

3.1 Ang-II induces cardiomyocyte hypertrophy/apoptosis in a dose-dependent manner

Cardiomyoblasts were stimulated with either 5 µM Ang-II or 20 µM for 48 h before analysis of changes in cell morphology. To study the dose–response effect of Ang-II on cardiomyocyte hypertrophy/apoptosis, H9c2 cardiomyoblasts were exposed to various concentrations of AngII, and hypertrophy/apoptosis was estimated 48 h after culture by JC-1 Staining (Figure 7). The results showed that the pro-hypertrophy and pro-apoptotic effect of Ang-II on cardiomyocyte viability was gradually enhanced between 0.1 and 20 µM, and Ang-II at 20µM appeared to have the strongest action. The concentration of 20mM was therefore chosen for Ang-II for the subsequent experiments (Figure 8).
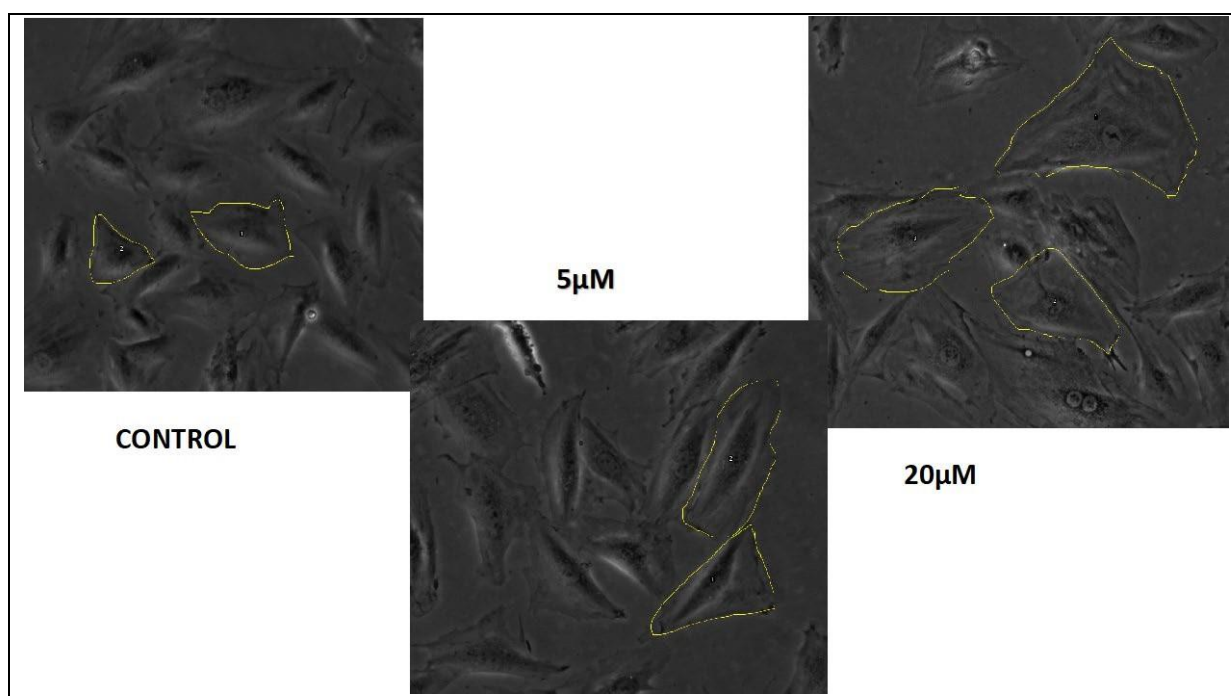

Figure 7: Ang-II induced hypertrophic growth of H9C2 cells. *Dose–response effect of ANGII on cardiomyocyte hypertrophy, H9c2 cardiomyoblasts were exposed to two concentrations of ANGII (5µM & 20µM), and hypertrophy was estimated 48 h after culture by JC-1 Staining. The yellow borders delineate the size of the cell. A bright field microscope was used to take the pictures with 20X magnification.*
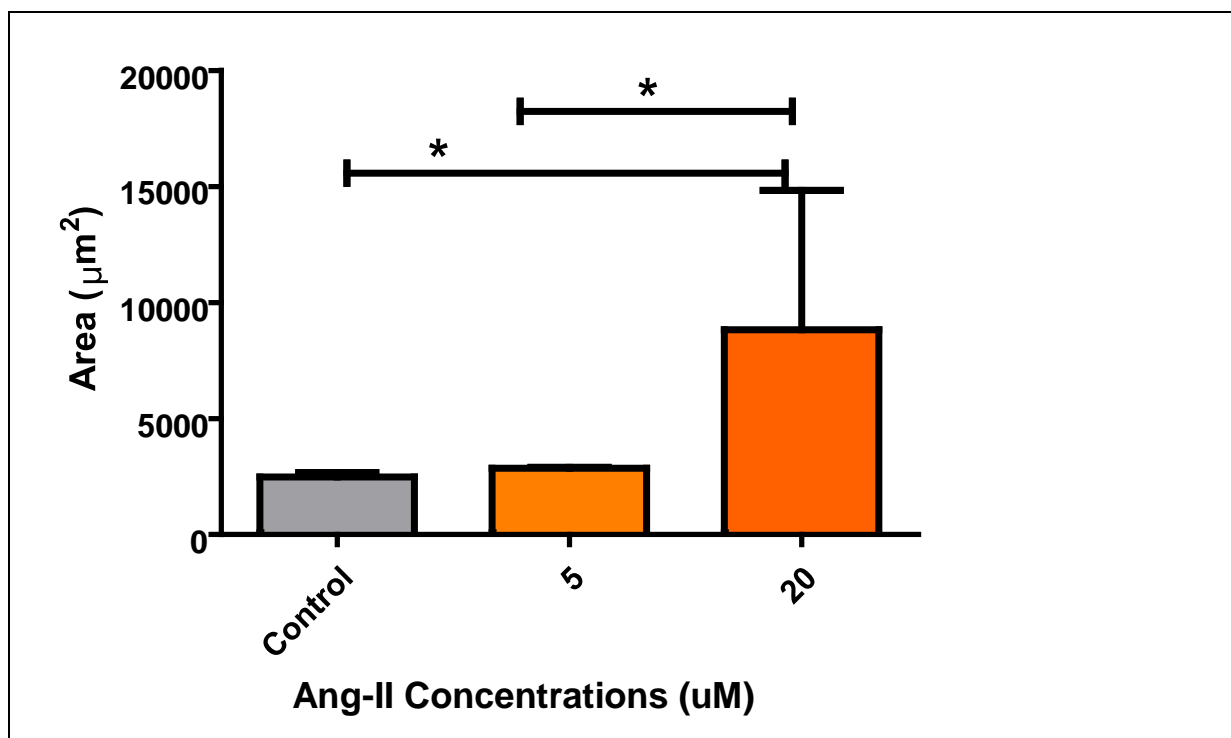
Figure 8: Ang-II induce hypertrophy in a dose-dependent manner. *After treated with Ang-II (5 and 20µM) alone for 48 h. Ang-II induced hypertrophy in a dose-dependent manner and 20 µM proved to be more effective as compared to 5µM.The experiments had three biological repeats (n=3), each of the three repeats had two technical repeats. The values denote the average area ± SEM; *P < 0.05.*

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3.2 Rooibos extract HPLC analysis

Our HPLC analysis of the extract showed high levels of the flavonoids iso-orientin (0.396 g/100 g), orientin (0.515 g/100 g), Bioquercetin (0.455 g/100 g) and as well as the flavanone Z-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid (PPAG), (0.421 g/100 g), (R)-8-β-Dglucopyranosyl-eriodictyol (0.221g/100g), (S)-8-β-D-glucopyranosyl-eriodictyol (0.225g/100g), (R)-6-β-D-glucopyranosyl-eriodictyol (0.269g/100g), (S)-6-β-Dglucopyranosyl-eriodictyol (0.284g/100g) (Table 3). However, other flavonoids i.e. Aspalathin, nothofagin, vitexin, hyperoside, rutin, isovitexin, isoquercitrin and luteolin-7-Oglucoside, and ferulic acid were present at less than 0.2 g/100 g plant material.

Table 3: soluble solids content (g/100g plant material) of fermented rooibos plant material. *The data represent means.*

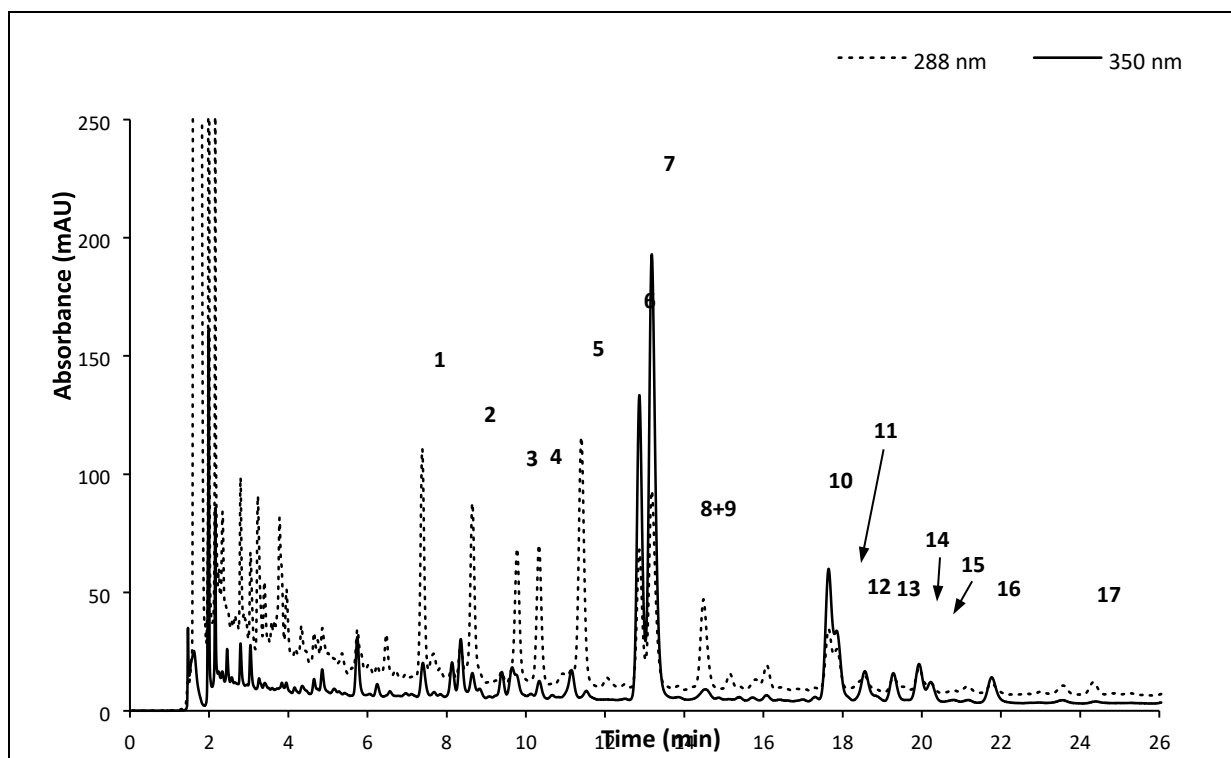| Compounds	Numbers	Concentration [compound] (g compound/100 g SS)
(S)-6-β-D-glucopyranosyleriodictyol	1	0,284
(R)-6-β-D-glucopyranosyleriodictyol	2	0,269
(S)-8-β-D-glucopyranosyleriodictyol	3	0,225
(R)-8-β-D-glucopyranosyleriodictyol	4	0,221
Z-2-(β-Dglucopyranosyloxy)-3phenylpropenoic acid (PPAG)	5	0,421
Isoorientin	6	0,396
Orientin	7	0,515
Aspalathin	8	0,161
Ferulic acid	9	0,030
Bioquercetin	10	0,445
Vitexin	11	0,164
Hyperoside	12	0,120
Rutin	13	0,101
Isovitexin	14	0,074
Isoquercitrin	15	0,071
Luteoloside	16	0,115
Nothofagin	17	0,020

Figure 9: HPLC for fermented cold-water rooibos extract. *High performance liquid chromatography diode-array detection chromatograms for fermented cold-water rooibos extract obtained using the developed method described by* (Villiers, *et al.*, 2017) *and (Muller et al., 2020) at 288 nm and 350 nm wavelengths. Peak numbers correspond to numbers in Table 3.*

3.2 Toxicity dose-response of Rooibos

The potential cytotoxicity of Rooibos was analysed using a cell viability assay (MTT Assay). H9c2 cells were incubated with various concentrations of Rooibos (50, 100 or 500μg/ml) for 48 h. Cell viability in Rooibos-treated cells exhibited no significant differences when compared with the control cells, indicating that Rooibos at these concentrations did not possess any cytotoxicity in H9c2 cells.
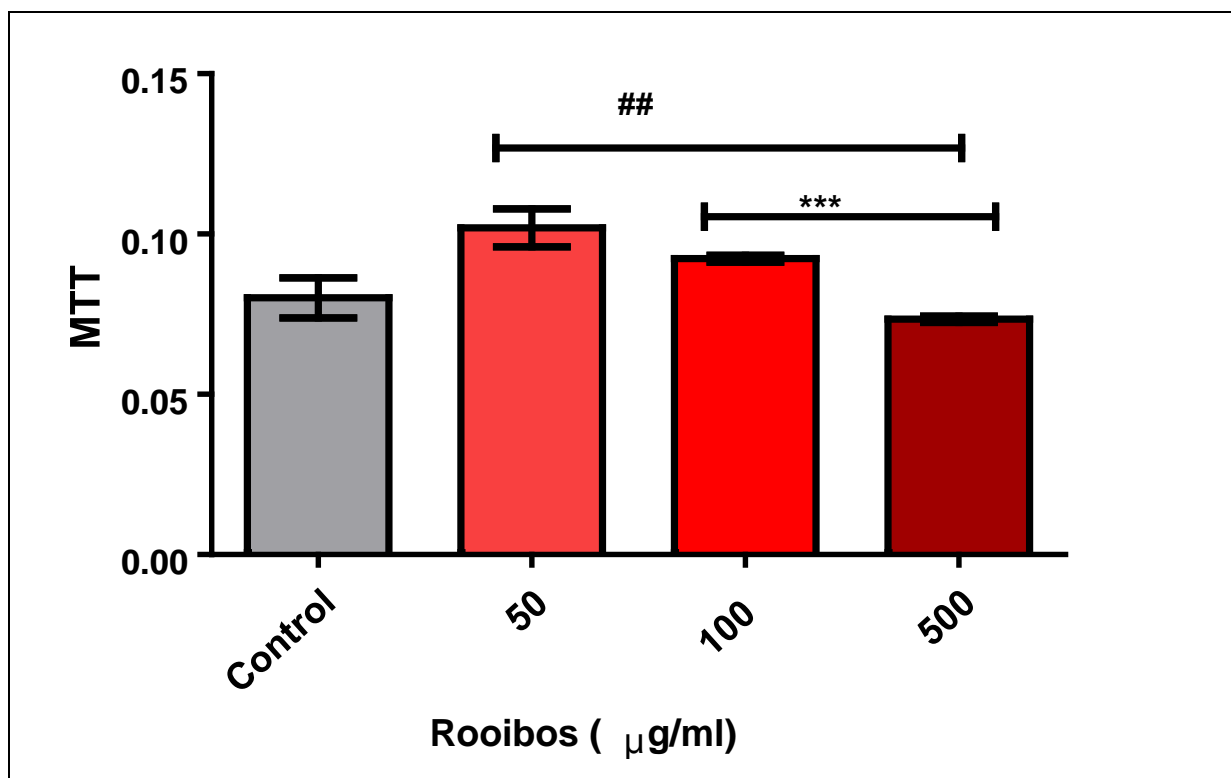
56

Figure 10: The effects of Rooibos (RB) on H9c2 cardiomyoblasts. *RB toxicity was measured in H9c2 cells 48 hrs. RB toxicity was measured using MTT Assay. The data was represented using ANOVA, n=4.The experiments were repeated three times (n=3), with each repeat done in duplicate. The values denote the average area ± SEM; ## P < 0.01; *** P < 0.001*

3.3 RB decreased Cell Surface Area in Hypertrophic Cardiomyoblasts

The inhibitory effect of RB in hypertrophic cardiomyoblasts was investigated by measuring cell size after cells were exposed to RB + Ang-II co-treatment. The duration of the treatment was 48 hrs with Ang-II (20μM) in the presence or absence of losartan (Los) or RB. (A) Representative average area of four individual experiments.

The cell surface area significantly increased after 48 h stimulation with 20μM Ang-II (Figure 11*). The cell area of the control group was increased from 1931 ± 78.04μm^2 to 2389 ± 104.9μm^2 after treatment with Ang-II (n=4, *p<0.05;* Figure 12*). Measurement of the area of the cells after treatment with RB (100μg/ml) revealed that RB decreased the Ang-II induced increased growth from 2389 ± 104.9μm^2 to 1886 ± 30.37μm^2. (n=4, *p<0.01*) Moreover, a similar effect was found with the classic Ang-II inhibitor, Losartan (10μM) which decreased

the cell area from $2389 \pm 104.9 \mu m^2$ to 1931 ± 40.38, (n=4, *p<0.05*). Compared with the control group, we found that the area of cells treated with RB and Los only were not increased, there was no significant difference.
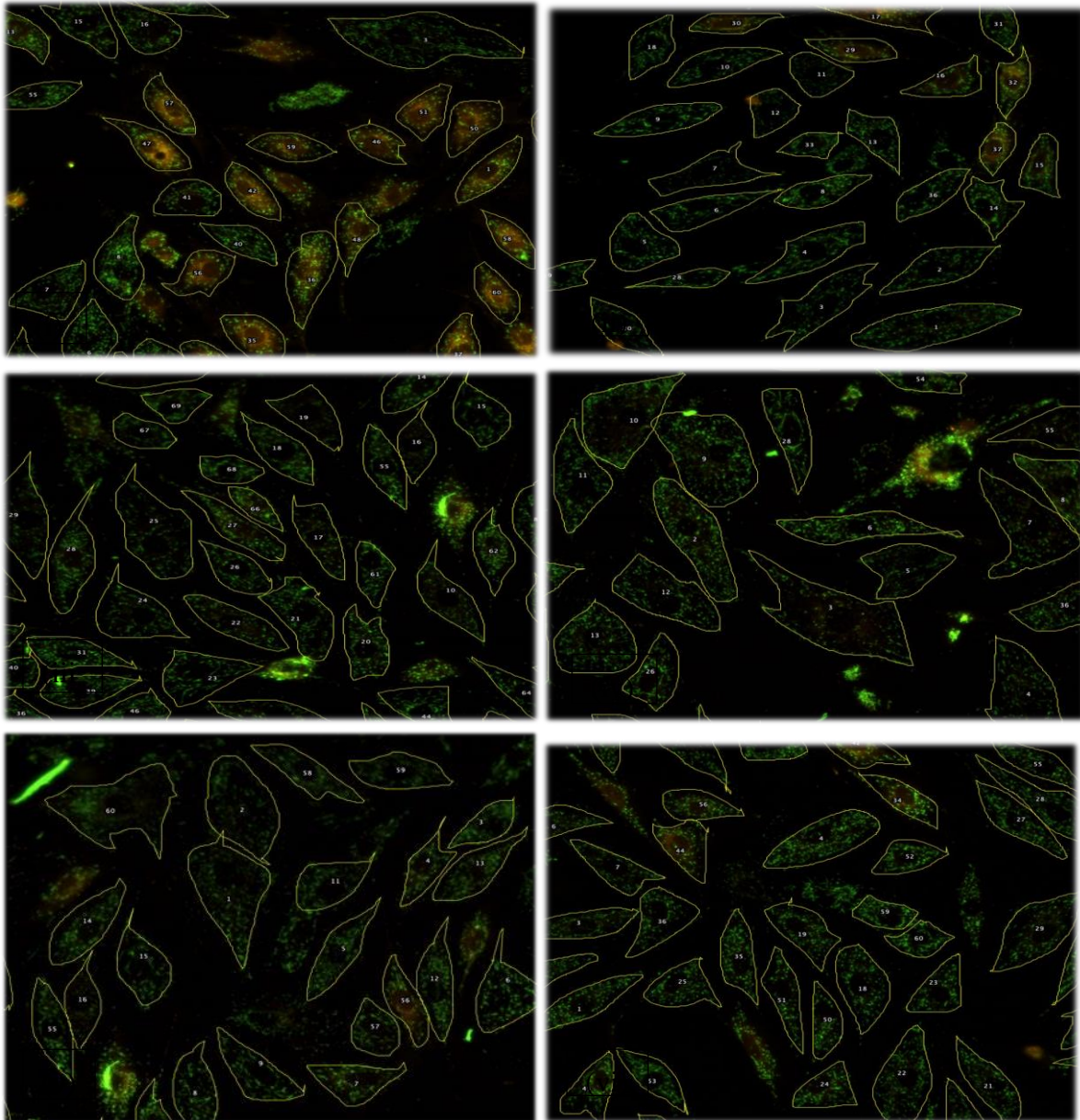

Figure 11: Ang-II induced hypertrophic growth of H9C2 cells was inhibited by Rooibos (RB). *Representative images of individual cells conditions. (i) control cells, (ii) Rooibos, (iii) Losartan, (iv) Ang-II, (v) RB+ Ang-II, Los+ Ang-II, (vi) RB + RB. The yellow borders delineate the size of the cell. Microscopic observation of hypertrophy formation following increase in cell size (original magnification 20X). After treated with RB (100 µg/ml) and Ang-II (20µM) alone or in combination for 48 h. H9C2 cells were stained with JC-1, hypertrophy was analysed by measuring cell surface area using Image J software 7.0.*
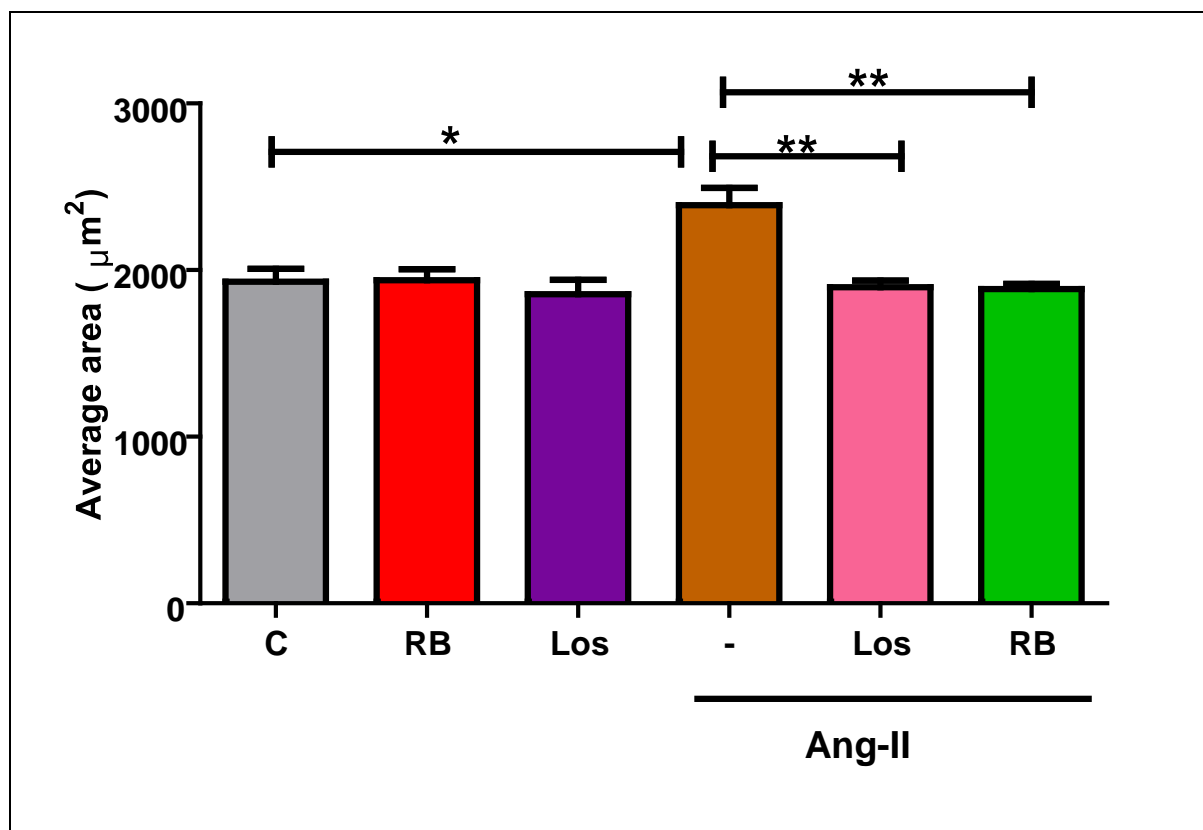
58

Figure 12: Rooibos inhibited Ang-II induced hypertrophic growth of H9c2 cells.
Quantification of cell size change. Ang II (20µM) caused an increase in H9c2 cell size and was attenuated by RB (100µg/mL). The values denote the average area ± SEM (n = 4) with duplicate determinations in each experiment. (* P < 0.05; ** P < 0.01)

3.4 RB prevents Cell death in Hypertrophic/Apoptotic Cardiomyoblasts

Cells treated with Ang-II only and those treated with RB + Ang-II was evaluated for viability. Our results have shown that Ang-II decreased cell viability in apoptotic cells. Our results revealed that treatment with RB extract protected the cells from Ang-II-induced cell death. The viability significantly increased after treatment with RB. RB had similar effect as the Ang-II receptor inhibitor, Los.
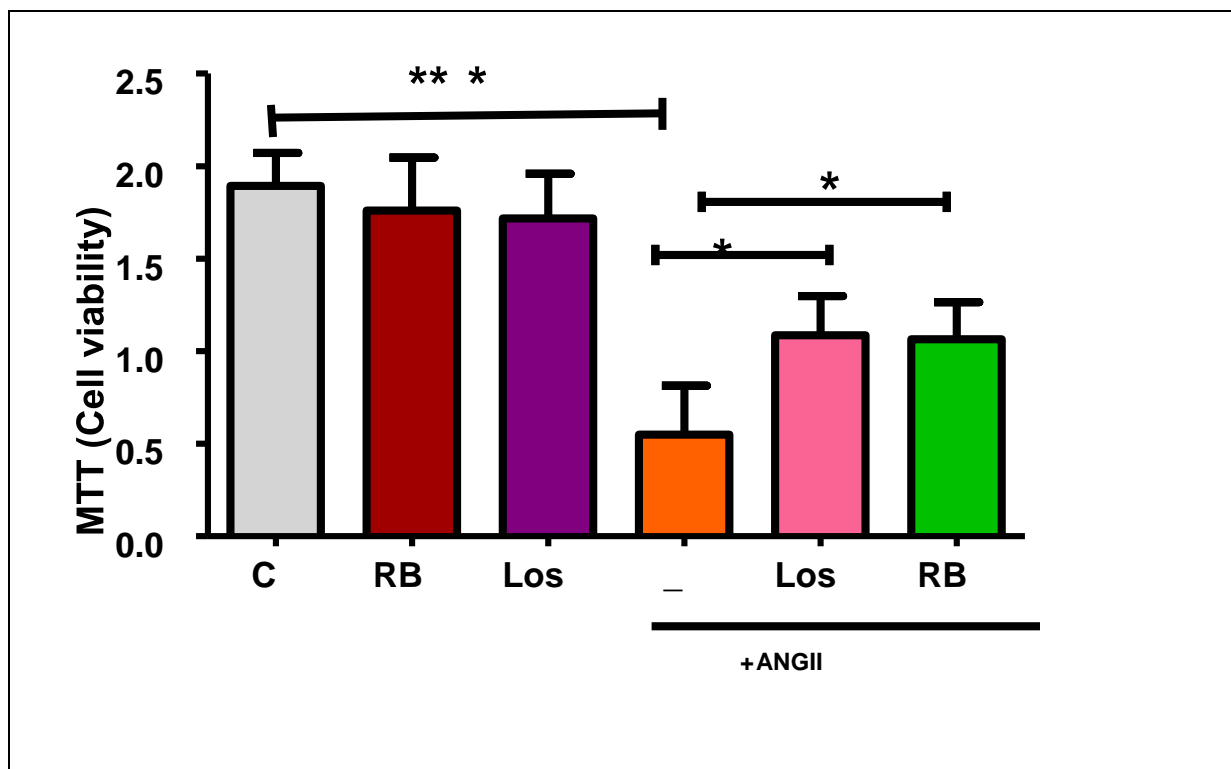

Figure 13: The cardioprotective effect of Rooibos (RB) investigated on cell viability. *Cell viability was measured in H9c2 cells 48 hrs after treatment with Ang-II (20µM). Cell viability test showed that RB increased cells' viability when compared to Ang-II treated group. Cell viability was measured using MTT Assay. The data was represented using ANOVA, n=4. The values denote the average area ± SEM. *P<0.05, ***P<0.001*

3.5 Western blot

3.5.1 Effect of RB in hypertrophic signalling pathways

Western Blotting was performed on H9c2 cells treated with the RB, Ang-II, Losartan classical inhibitor of Ang-II, to measure the expression and activation of important hypertrophy signalling intermediates such as PKB, GSK-3β, ERK 1/2 and mTOR. The expression of Calcineurin was also determined in H9c2 cells. For each gel, all values were normalized to Btubulin, expressed as a fold-change to the untreated control samples and presented as mean ± SEM All the blots were repeated three times. Each blot used for display represents one of the three biological repeats. Each biological repeat is blotted in duplicate.

3.5.1.1 RB increases the Activity of GSK-3ß in Hypertrophic/Apoptotic H9c2 cells

GSK-3ß is also known as one of the important inducers of cardiac hypertrophy (Antos et al., 2002; Hardt & Sadoshima, 2002). To determine whether the phosphorylation of GSK-3ß was involved in development of Ang-II-induced cardiac hypertrophy we used western blot measured by phosphorylated specific antibody (Figure 14). Compared to the control, the level of phosphorylated GSK-3ß was increased in the Ang-II-treated group, but this increase was blocked by RB treatment. Additionally, there was no significant difference in total GSK-3ß between control and the Ang-II group (Figure 15). Also, no significant difference was observed after RB treatment and Ang-II group in total GSK-3ß. The Ratio of phosphorylated vs. total GSK-3β from the Ang-II treated cells significantly decreased in RB treated cells (Figure 16).
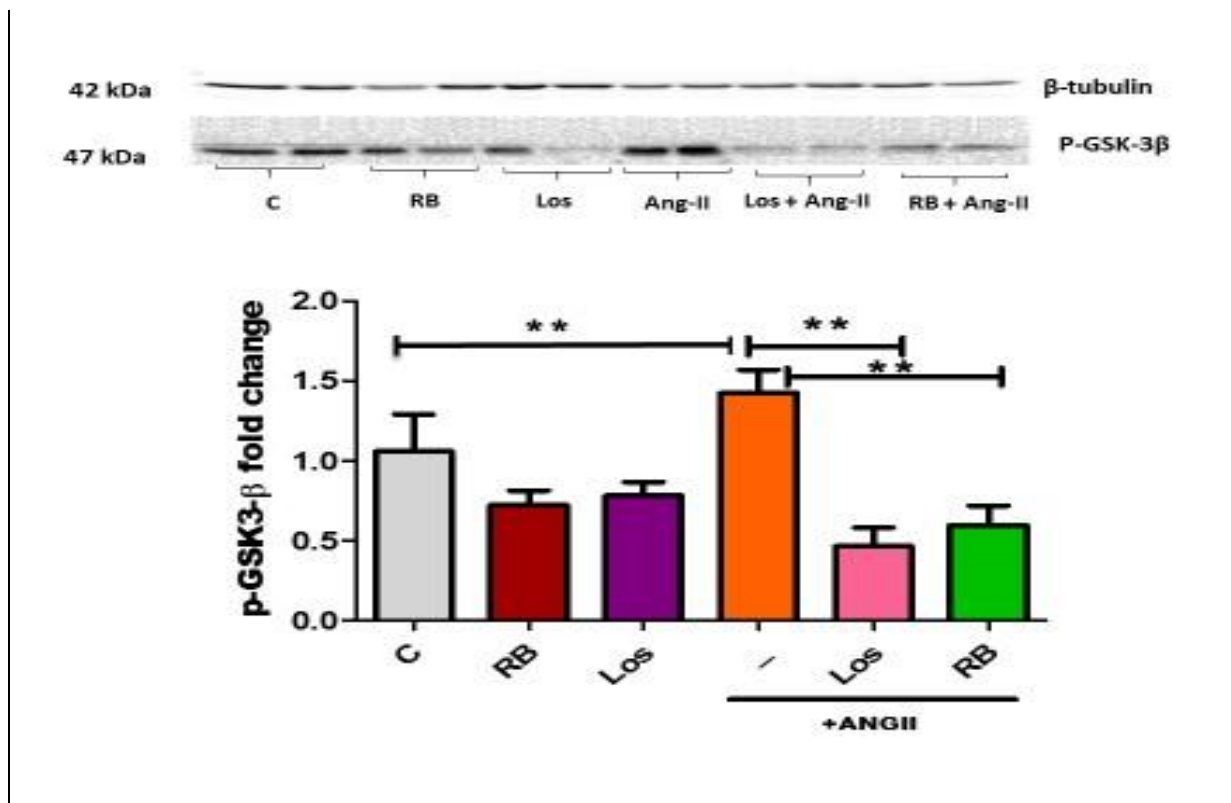
Figure 14: Effect of RB on phosphorylated-GSK-3β levels in Ang-II induced hypertrophy.
H9c2 cells were treated for 48 hrs with Ang-II (20µM) in the presence or absence of Rooibos (100µg/ml) or Losartan (10µM). Each experiment was repeated three times in H9c2 cardiomyoblasts. Representative Western blot of phosphorylated GSK-3β and beta-tubulin. This blot represents one of the three biological repeats, each is in duplicate. All values were normalized to beta-tubulin, expressed as a fold-change to the untreated control samples and presented as mean ± SEM (n=3). **P<0.001.
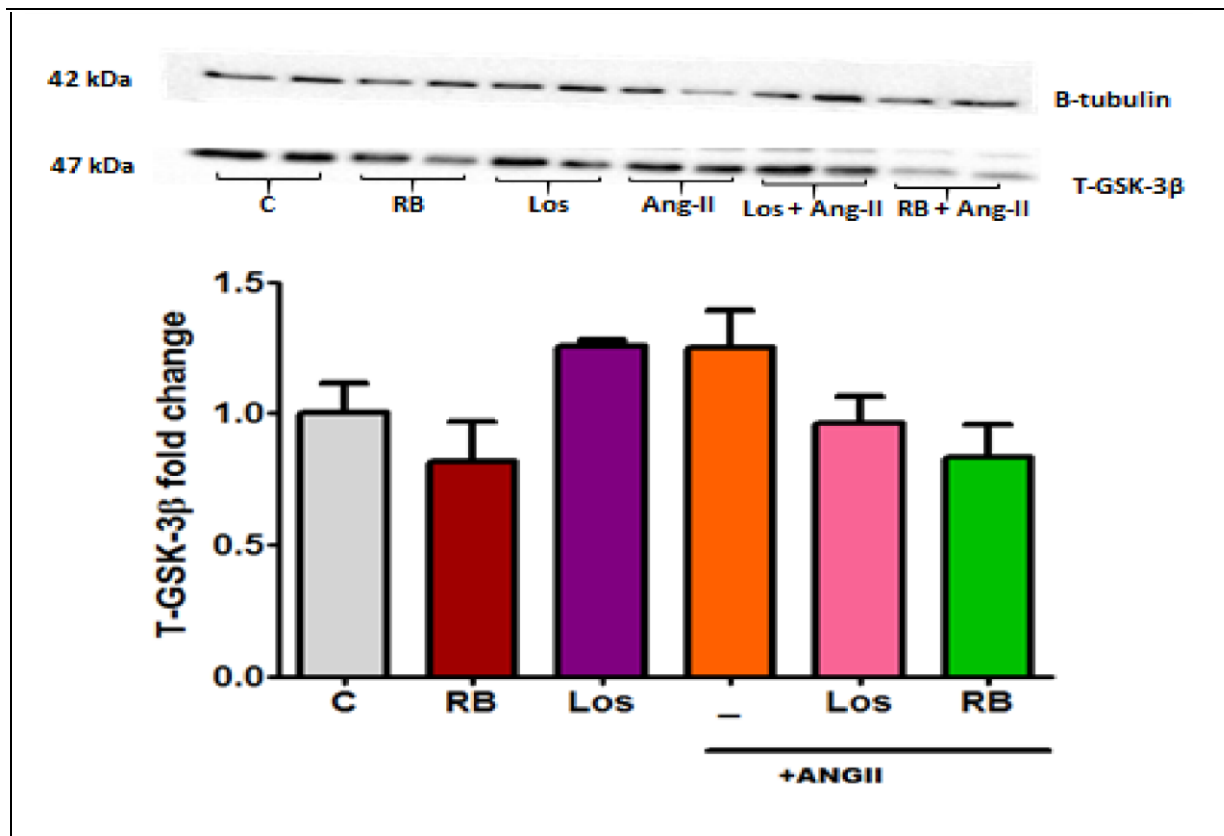
Figure 15: Effect of RB on total-GSK-3β protein expression in hypertrophy/Apoptotic cells. *H9c2 cells treated for 48 hrs with Ang-II (20μM) in the presence or absence of Rooibos (100μg /ml) or Losartan (10μM). Each experiment was repeated three times in H9c2 cardiomyoblasts. Representative Western blot of Total GSK-3β and beta-tubulin. This blot represents one of the biological repeats (n=3) in the appendix, each biological repeat is in duplicate. All values were normalized to beta-tubulin, expressed as a fold-change to the untreated control samples and presented as mean ± SEM.*
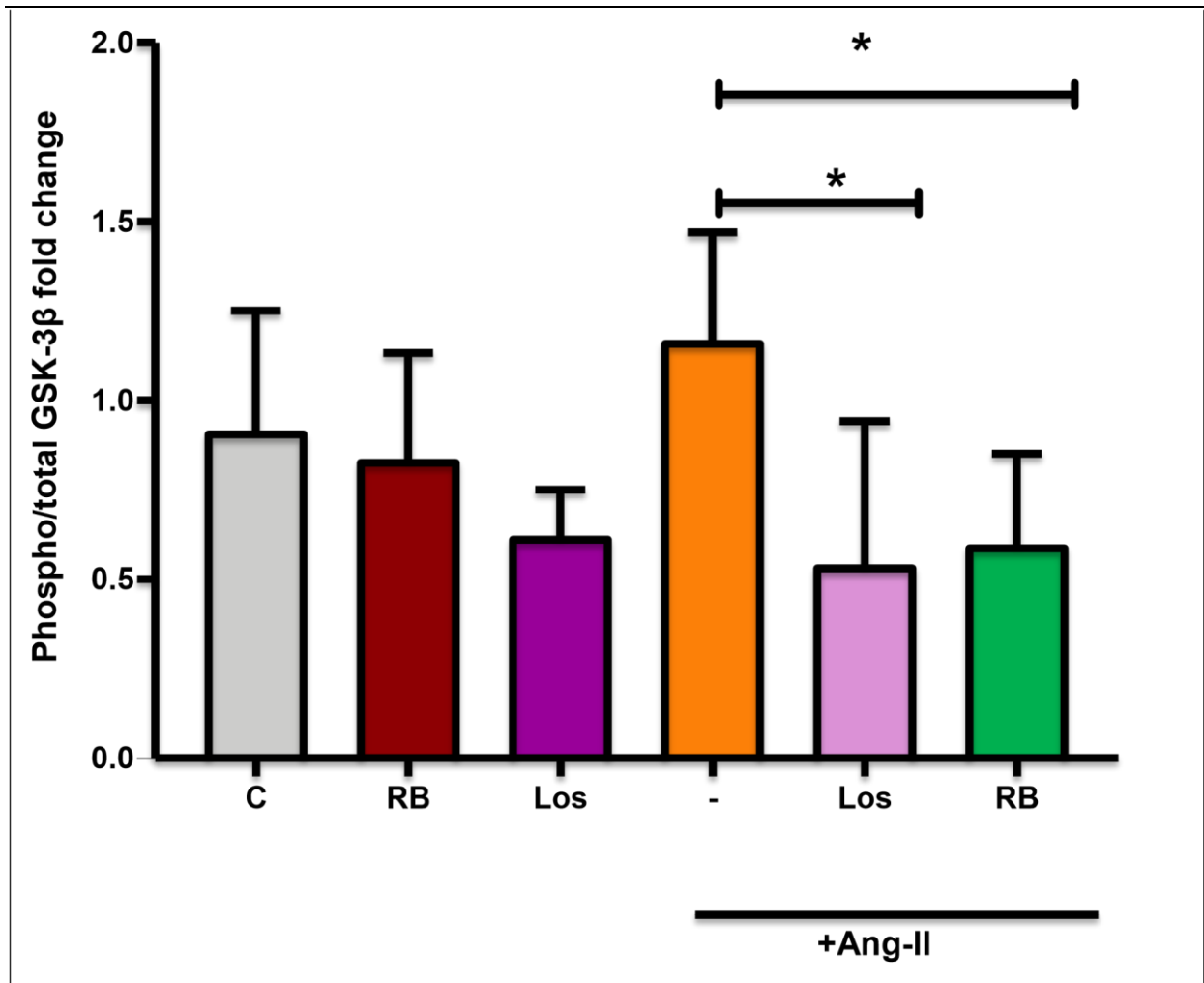
Figure 16: Effect of RB on Phosphorylated/Total-GSK-3β ratio expression in hypertrophy/Apoptotic cells. *H9c2 cells treated for 48 hrs with Ang-II (20 µM) in the presence or absence of Rooibos (100µg/ml) or Losartan (10µM). Each experiment was repeated three times in H9c2. The data is presented as mean ±SEM. * P < 0.05*

3.5.1.2 RB inhibit phosphorylation of PKB/Akt

Several intracellular signalling pathways have been reported to play a role in the promotion of cardiac hypertrophy, these include PKB/Akt pathway. To confirm that PKB was activated in response to a model of Ang-II induced hypertrophy, we assessed the phosphorylation of PKB/Akt pathway by western blotting with phosphorylated specific antibody (Figure 17). We found that Ang-II increased phosphorylation of PKB/Akt. However, the increased phosphorylation was downregulated by treatment with RB + Ang-II co-treatment. We also found that when RB alone is compared with the control group there was no significant difference (Figure 18). Same results were observed with Losartan an Ang-II classical inhibitor. The Ratio of phosphorylated vs. total PKB in cardiomyoblasts from the Ang-II treated cells significantly increased when treated with RB (Figure 19).

Figure 17: Effect of RB on Phospho-PKB protein levels in Ang-II-induced hypertrophy.
*H9c2 cells treated for 48 hrs with Ang-II (20μM) in the presence or absence of Rooibos (100μg/ml) or Losartan (10μM). Each experiment was repeated three times in H9c2 cardiomyoblasts. Representative Western blots of Phosphorylated and beta-tubulin. Each blot represented above represents one of the three biological repeats, each biological repeats the treatments are in duplicates. All values were normalized to beta-tubulin, expressed as a fold-change to the untreated control samples and presented as mean ± SEM (n=3). * P<0.05, **P<0.01*
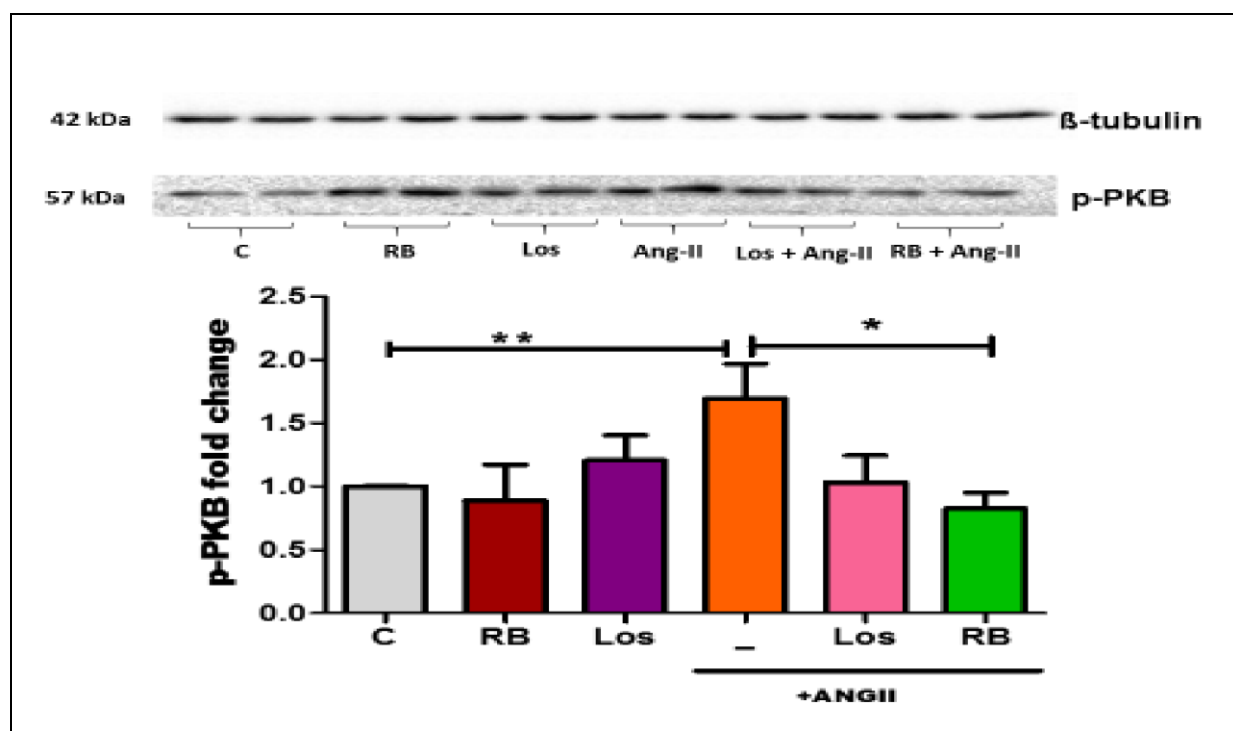
Figure 18: Effect of RB on total PKB protein expression in Ang-II-induced hypertrophy. *H9c2 cells treated for 48 hrs with Ang-II (20μM) in the presence or absence of Rooibos (100μg /ml) or Losartan (10μM). Each experiment was repeated three times in H9c2 cardiomyoblasts. Representative Western blot of Total PKB/Akt and beta-tubulin. Each blot represented above represent one of the three biological repeats, each biological repeat are in duplicates. All values were normalized to beta-tubulin, expressed as a fold-change to the untreated control samples and presented as mean ± SEM (n=3).*

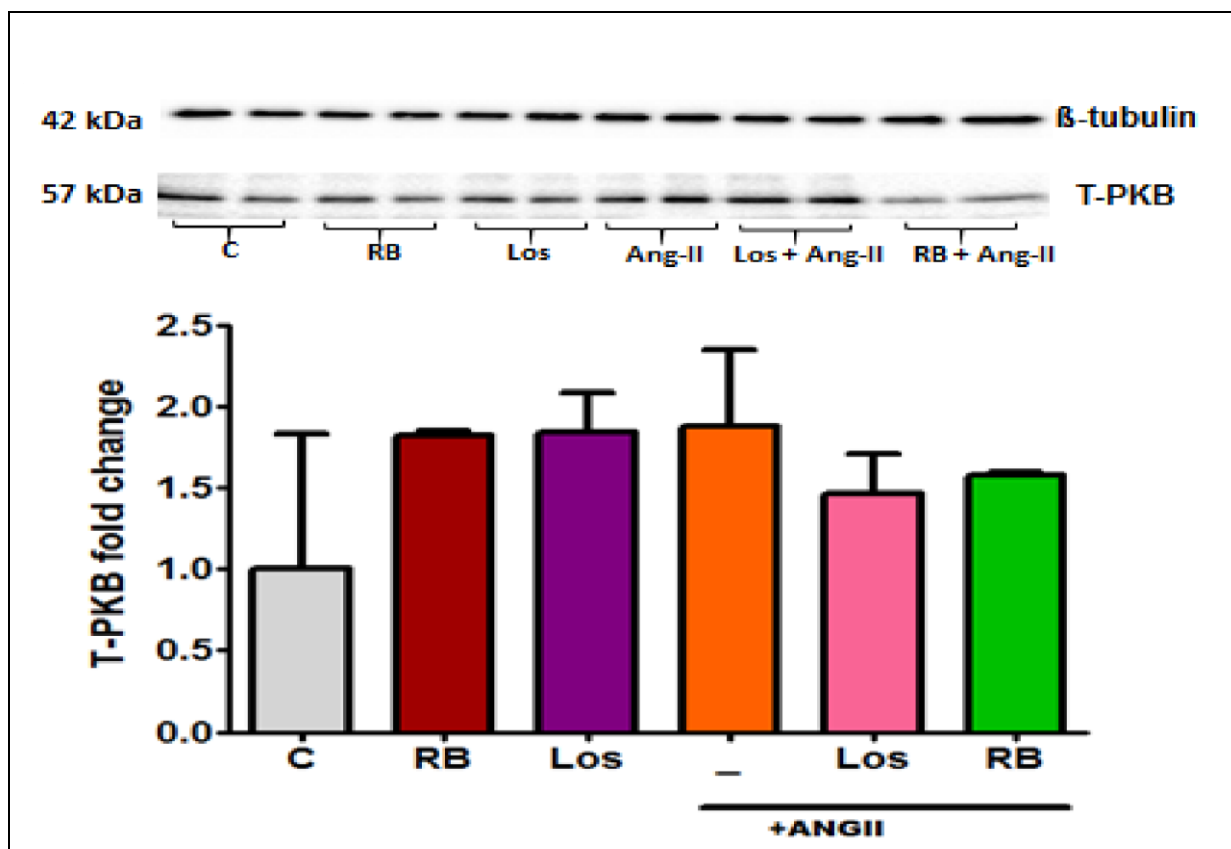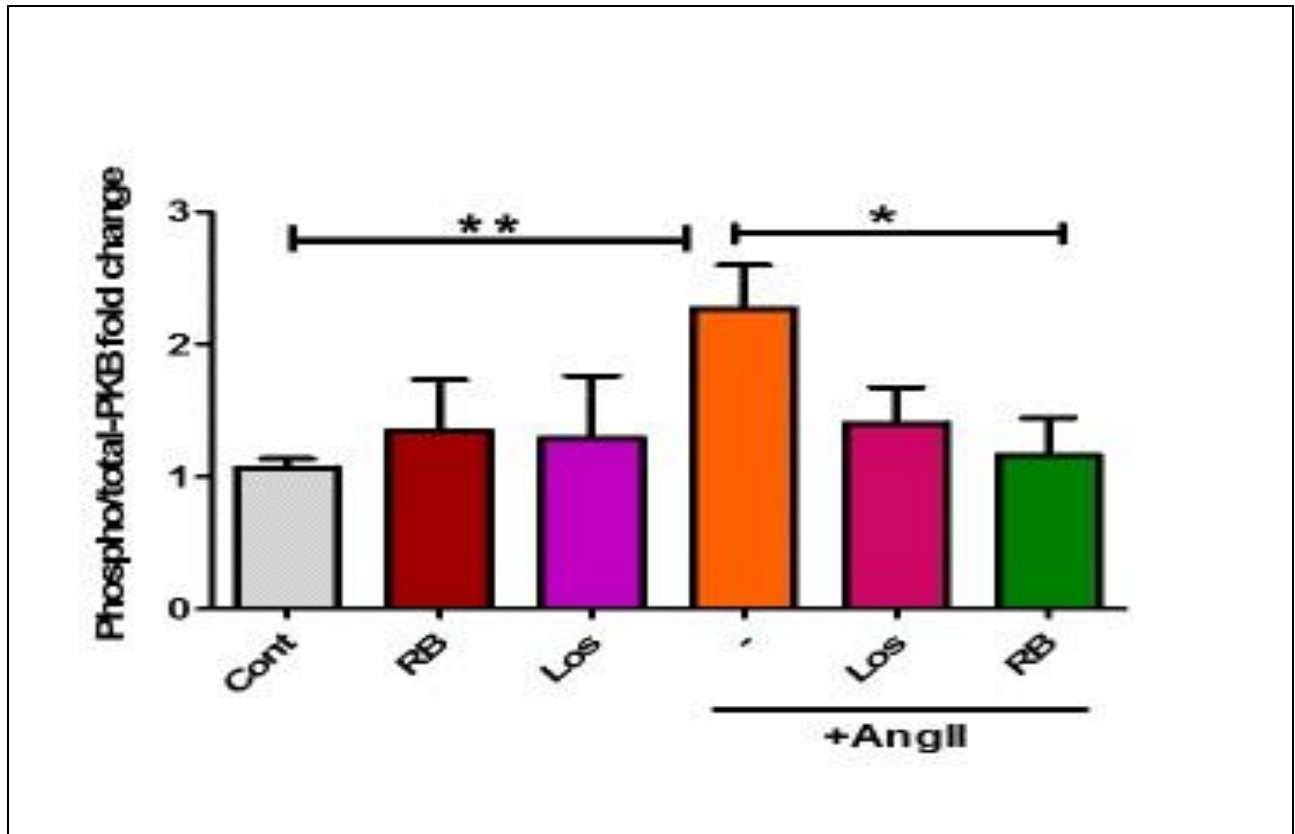**Figure 19: Effect of RB on Phosphorylated/total-PKB ratio expression in hypertrophy/apoptotic cells.** *H9c2 cells treated for 48 hrs with Ang-II (20µM) in the presence or absence of Rooibos (100µg /ml) or Losartan (10µM). Each experiment was repeated three times (n=3) in H9c2 cells.* *****P<0.05, **P<0.01

3.5.1.3 Effect RB on Ang-II stimulated phosphorylation of ERK1/2

The stimulation of cells with Ang-II provoked an increase in the expression of ERK1/2. This effect was attenuated by RB from 1.879 ± 0.114 to 1.419 ± 0.123 (Figure 20). We also found that Losartan significantly inhibited increased phosphorylation by Ang-II. There was no significant difference between Ang-II and RB + Ang-II co-treatment in total expression (Figure 21). The Ratio of phosphorylated vs. total ERK 1/2 showed no significant differences in the cells from the Ang-II treated and RB co-treated cells (Figure 22).

Figure 20: Effect of RB on phospho-ERK1/2 protein levels in hypertrophy/Apoptotic cells. *H9c2 cells treated for 48 hrs with Ang-II (20μM) in the presence or absence of Rooibos (100μg /ml) or Losartan (10μM). Each experiment was repeated three times in H9c2 cardiomyoblasts. Representative Western blot of Phosphorylated ERK 1/2 and beta-tubulin. This blot above is one of the three biological repeats (n=3) with two technical repeats for each biological repeat. All values were normalized to beta-tubulin, expressed as a fold-change to the untreated control samples and presented as mean ± SEM.* P<0.05, **P<0.01.*

Figure 21: Effect of RB on total-ERK1/2 protein expression in hypertrophy/Apoptotic cells. *H9c2 cells treated for 48 hrs with Ang-II (20µM) in the presence or absence of Rooibos (100µg /ml) or Losartan (10µM). Each experiment was repeated three times in H9c2 cardiomyoblasts. Representative Western blot of Total ERK 1/2 and beta-tubulin. This blot above is one of the three biological repeats (n=3) each technical repeat is in duplicates. All values were normalized to beta-tubulin, expressed as a fold-change to the untreated control samples and presented as mean ± SEM (n=3).*
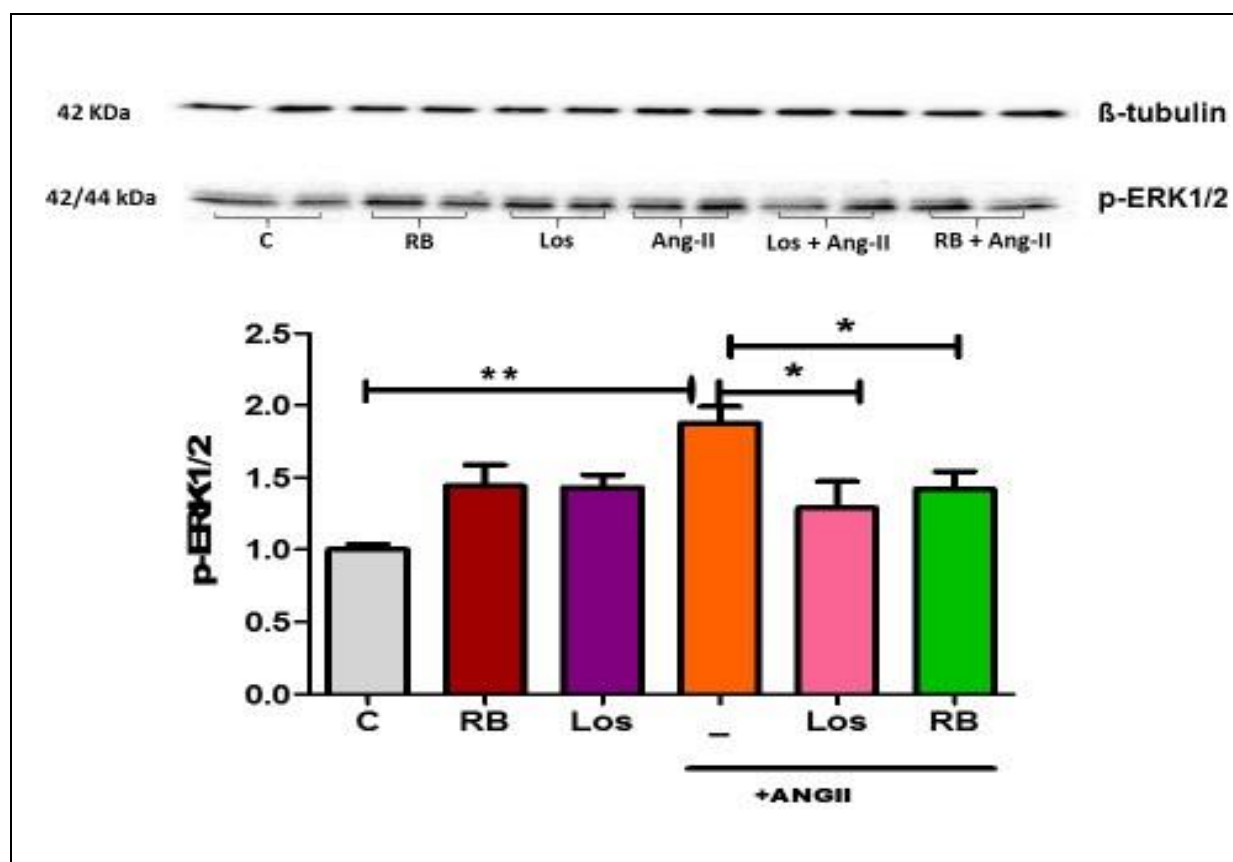
Figure 22: Effect of RB on Phosphorylated/Total-ERK1/2 ratio levels in hypertrophy/Apoptotic cells. *H9c2 cells treated for 48 hrs with Ang-II (20μM) in the presence or absence of Rooibos (100μg /ml) or Losartan (10μM). Each experiment was repeated three times in H9c2 cardiomyoblasts. The data was presented as mean ± SEM (n=3).*

3.5.1.4 Effect of RB on mTOR expression

The expression of mTOR was evaluated and the results showed that the phosphorylation of mTOR protein was significantly unregulated in the hypertrophy induced by Ang-II. However, after adding RB, we found that RB co-treatment could significantly downregulate the increased phosphorylation levels of mTOR protein induced by Ang-II (Figure 23). In addition, we also found that Losartan Ang-II inhibitor could also significantly downregulate the expression of mTOR. We also found same trend with Losartan, it significantly attenuated increased phosphorylation by Ang-II. We also observed a significant decrease in mTOR levels when compared to the control group (Figure 23). We found no significant difference in total mTOR expression (Figure 24). The ratio of phosphorylated vs. total mTOR in the Ang-II treated cells significantly increased when exposed to RB co-treatment (Figure 25).
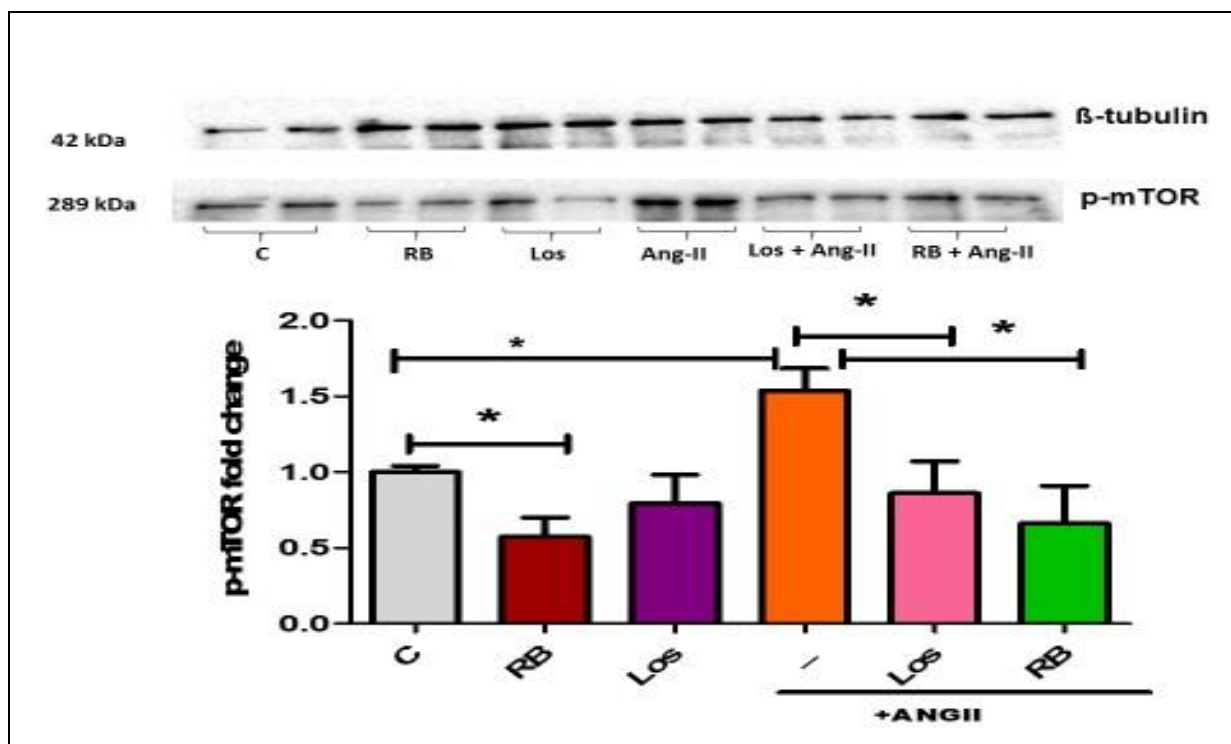
Figure 23: Effect of RB on Phospho-mTOR protein levels in Ang-II induced hypertrophy. *H9c2 cells treated for 48 hrs with Ang-II (20μM) in the presence or absence of Rooibos (100μg/ml) or Losartan (10μM). Each experiment was repeated three times in H9c2 cardiomyoblasts. Representative Western blot of Phosphorylated mTOR and beta-tubulin. This blot above is one of the three biological repeats (n=3) each is in duplicate. All values were normalized to beta tubulin, expressed as a fold-change to the untreated control samples and presented as mean ± SEM (n=3). * P<0.05.*
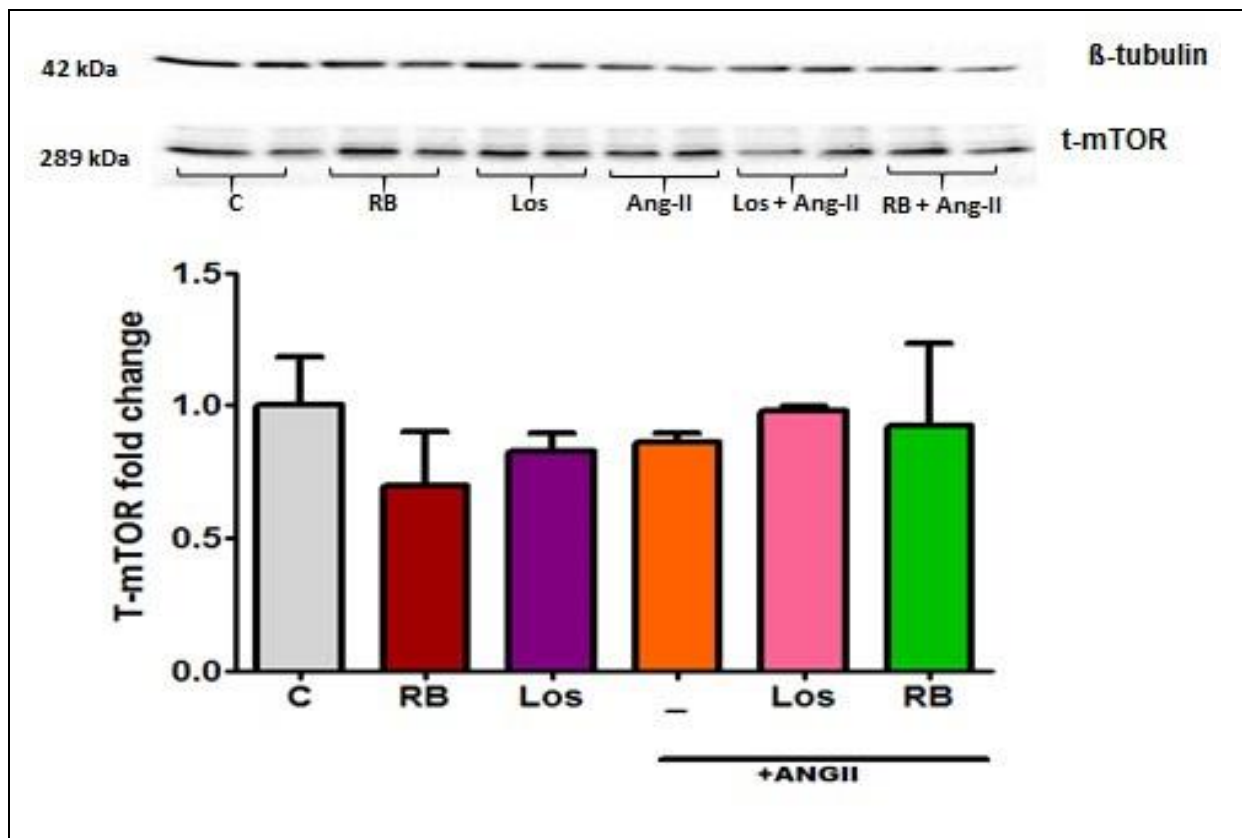
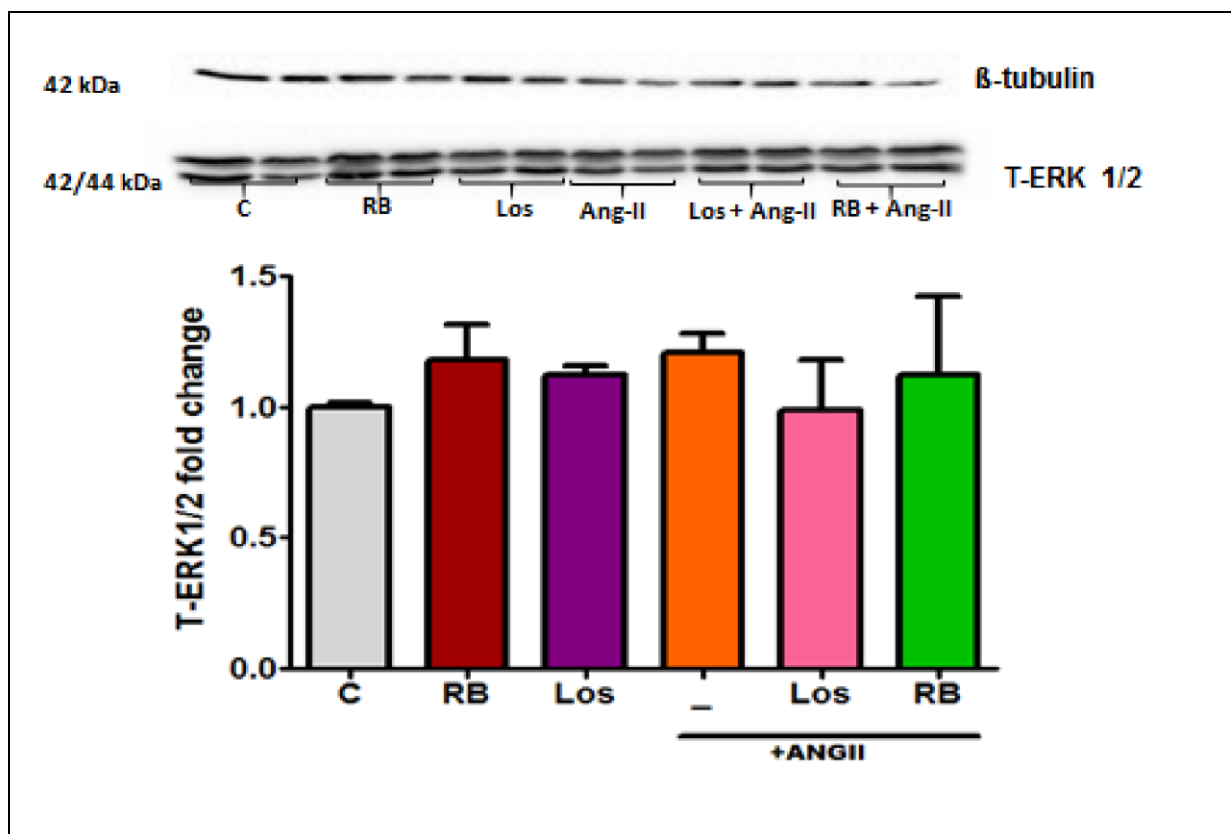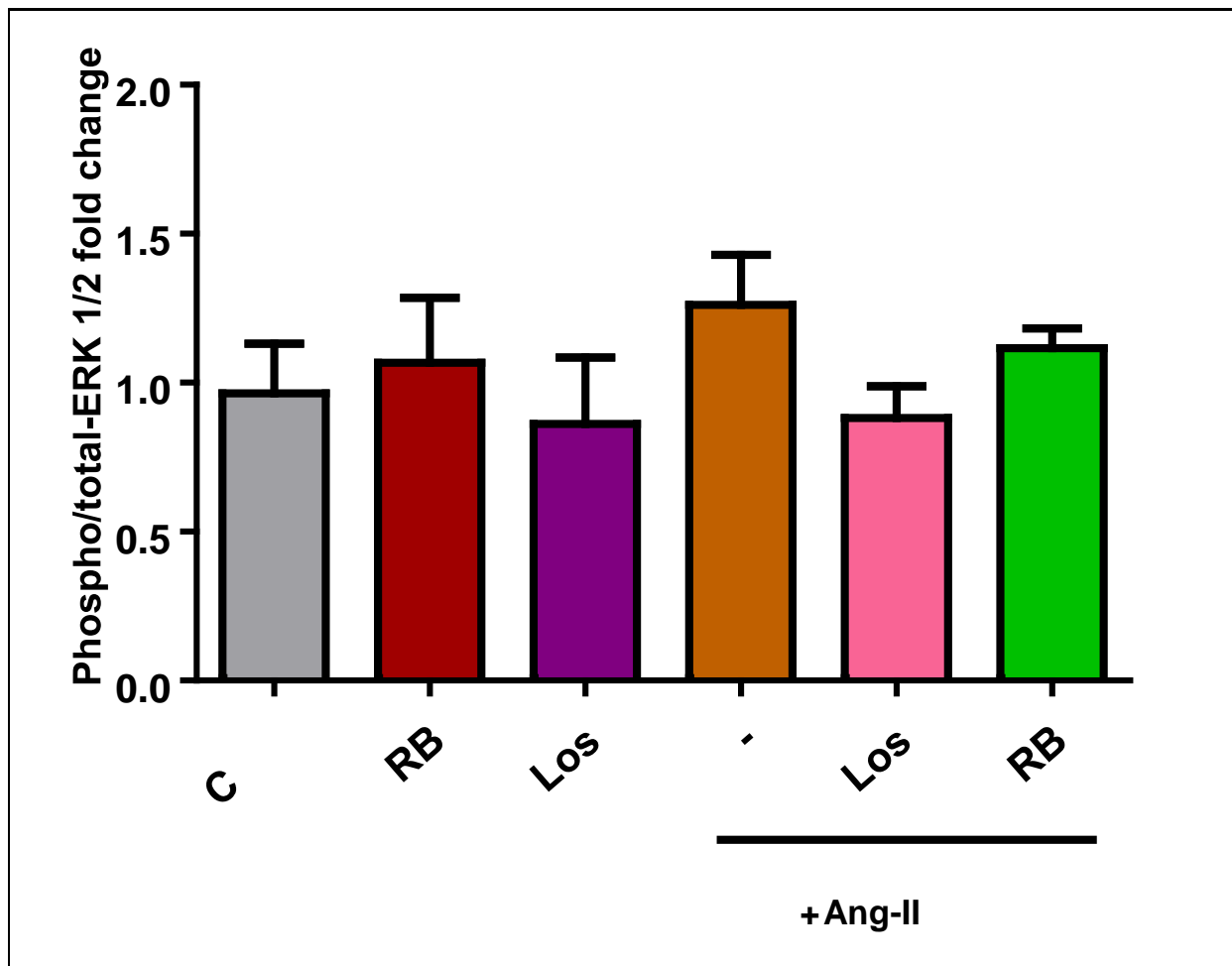**Figure 24: Effect of RB on total-mTOR protein expression in Ang-II-induced hypertrophy**. *H9c2 cells treated for 48 hrs with Ang-II (20µM) in the presence or absence of Rooibos (100µg /ml) or Losartan (10µM). Each experiment was repeated three times in H9c2 cardiomyoblasts. Representative Western blots of Total mTOR and beta-tubulin. The blot above represents one of the three biological repeats (n=3) with two technical repeats. All values were normalized to beta-tubulin, expressed as a fold-change to the untreated control samples and presented as mean ± SEM.*
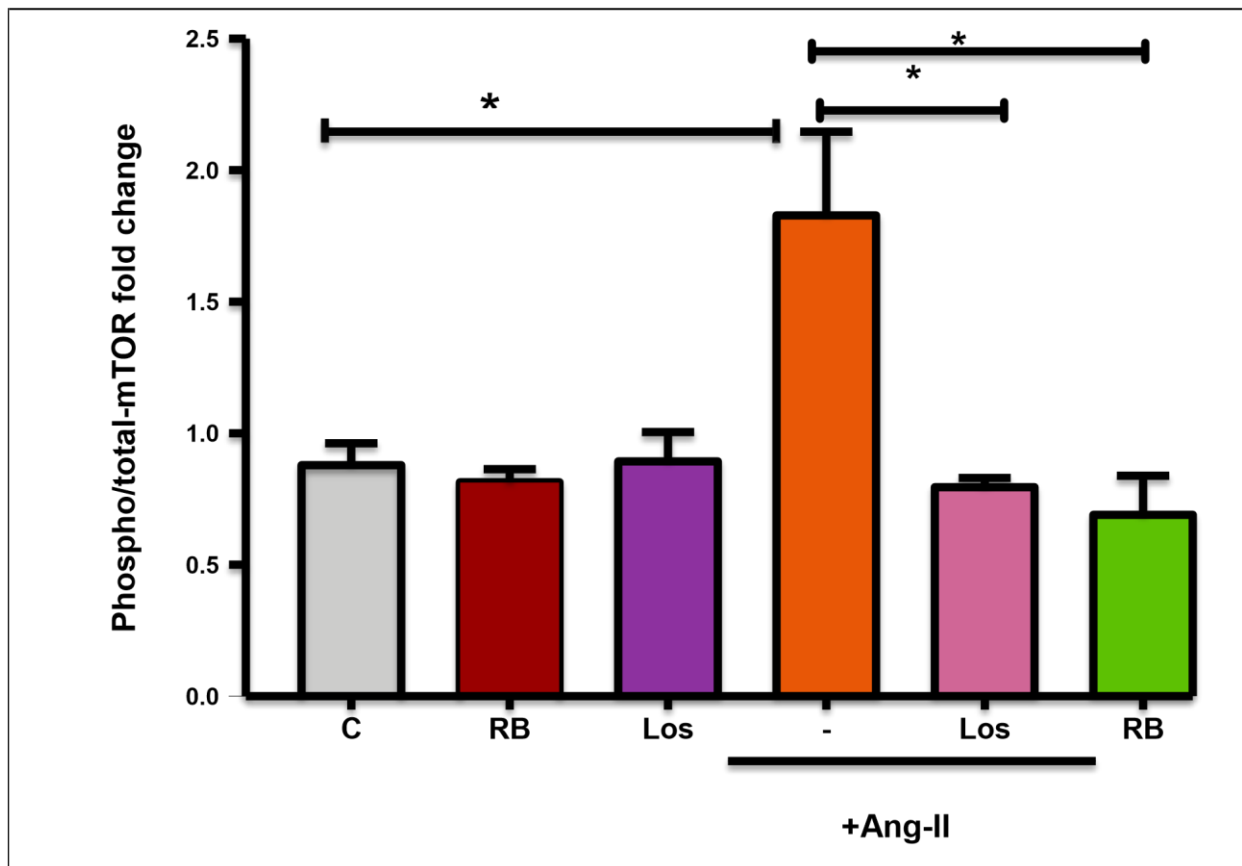
Figure 25: Effect of RB on Phosphorylated/total-mTOR ratio expression in hypertrophy. *H9c2 cells treated for 48 hrs with Ang-II (20μM) in the presence or absence of Rooibos (100μg/ml) or Losartan (10μM). Each experiment was repeated three times in H9c2 cardiomyoblasts. The data was presented as mean ± SEM (n=3). *P<0.05.*

3.5.1.5 RB down-regulated phosphorylation of Calcineurin

We investigated the possible involvement of Calcineurin–NFAT pathway in the cardioprotective activity of RB against ANGII-induced cardiac hypertrophy using specific total calcineurin antibody. We observed an increase in expression in Ang-II-treated cells compared to the control. However, we found that after treatment with RB co-treatment the increased expression of calcineurin was downregulated. We observed that Losartan also down-regulated the increase in calcineurin caused by Ang-II (Figure 26).
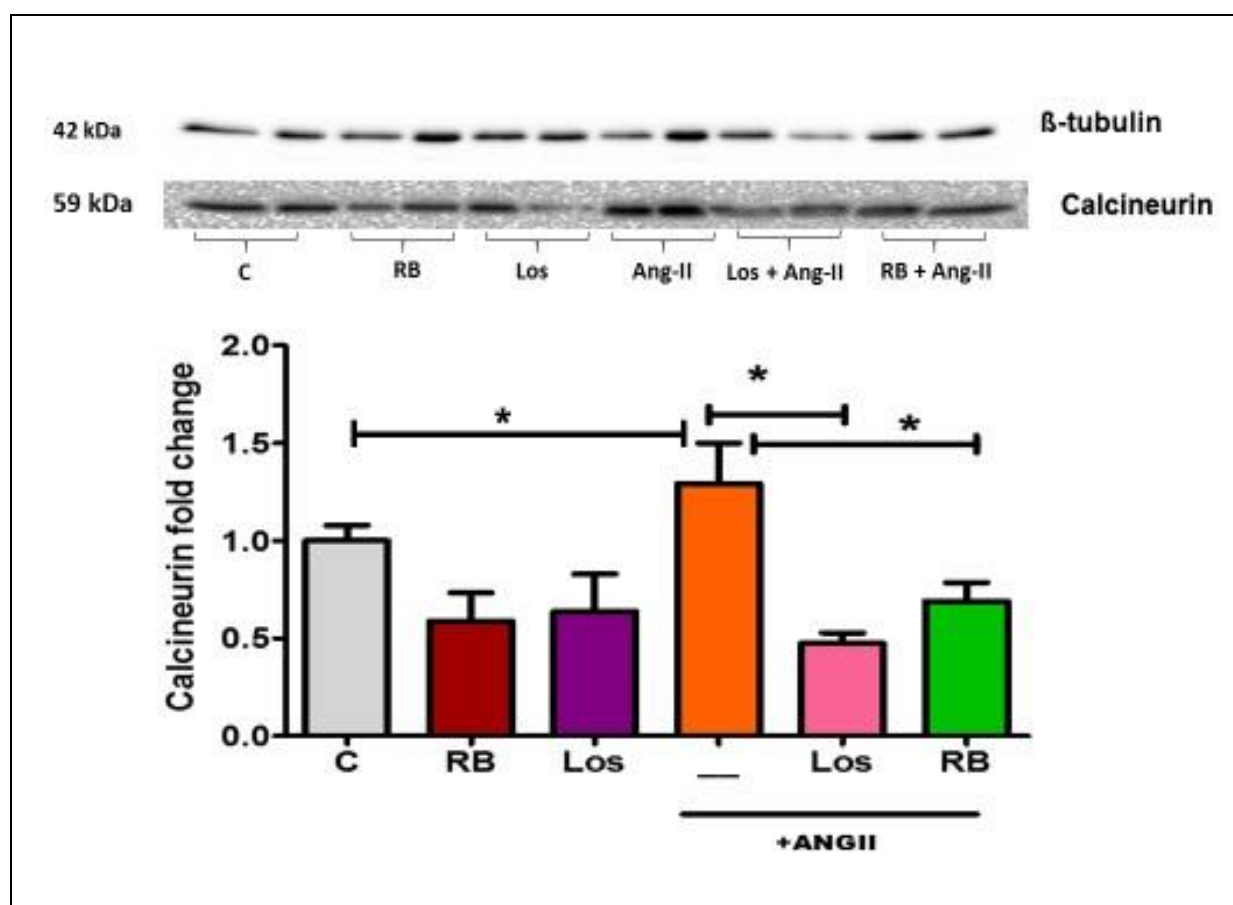
Figure 26: Effect of RB on Calcineurin protein expression in Ang-II-induced hypertrophy. *H9c2 cells treated for 48 hrs with Ang-II (20µM) in the presence or absence of Rooibos (100µg/ml) or Losartan (10µM). Each experiment was repeated three times in H9c2 cardiomyoblasts. Representative Western blot of Calcineurin expression and beta-tubulin. The blot above represents one of the three biological repeats (n=3) each is in duplicate. All values were normalized to beta-tubulin, expressed as a fold-change to the untreated control samples and presented as mean ± SEM (n=3). * P<0.05.*

3.5.2 Effect of RB in apoptotic pathways

To confirm which of the pro-apoptotic and anti-apoptotic proteins were activated in Ang-II induced apoptosis, we used western blot to measure the expression and activation of the following apoptotic signalling intermediates such as Bax, Caspase-3, P38 and Bcl-2, Bax/Bcl2. The results showed that compared with the control group apoptosis was increased in the Ang-II-treated group. However, RB inhibited the apoptosis induced by Ang-II. All values were normalized to beta-tubulin. The experiment was repeated three times. Each of the blots below represents one of the three biological repeats (n=3) in the Appendix, with each repeat consisting of two technical repeats.

3.5.2.1 Effect of RB on Bax expression

The H9c2 cells from the Ang-II treated group showed significantly up-regulated protein expression of the Apoptotic executor Bax when compared to the cardiomyoblasts from control group from the 1.000 ± 0.278 to 1.850 ± 0.394 to 1.617 ± 0.183. Furthermore, the cells after treatment with RB presented with significantly decreased expression of Bax from 1.617 ± 0.183 to 0.674 ± 0.250 (Figure 27).

Figure 27: Effect of RB on Bax in H9c2 cells. *H9c2 cells treated for 48 hrs with Ang-II (20μM) in the presence or absence of Rooibos (100μg/ml) or Losartan (10μM). Each experiment was repeated three times in H9c2 cardiomyoblasts. Representative Western blots of Bax expression and beta-tubulin. The blot above represents one of the biological repeats (n=3) in the Appendix, with each biological repeat with two technical repeats. All values were normalized to beta tubulin, expressed as a fold-change to the untreated control samples and presented as mean ± SEM.* P<0.05, ***P<0.001.*

3.5.2.2 Effect of RB co-treatment on Bcl-2 expression

The expression of Bcl-2 was significantly downregulated in the Ang-II-treated group. On the other hand, RB co-treatment significantly prevented the down-regulation of Bcl-2 compared to Ang-II stimulated cells (Figure 28).

Figure 28: Effect of RB on Bcl-2 in H9c2 cardiomyoblasts. *H9c2 cells treated for 48 hrs with Ang-II (20μM) in the presence or absence of Rooibos (100μg /ml) or Losartan (10μM). Each experiment was repeated three times in H9c2 cardiomyoblasts. Representative Western blot of Bcl-2 and beta-tubulin. The blot above represents one of the biological repeats (n=3) in the Appendix, with each biological repeat with two technical repeats. All values were normalized to beta-tubulin, expressed as a fold-change to the untreated control samples and presented as mean ± SEM (n=3). ***P<0.001.*

3.5.2.3 Effect of RB co-treatment in the Bax/Bcl-2 ratio

The results demonstrated that the expression of Bax/Bcl-2 ratio was significantly upregulated in the Ang-II treated cells. However, after addition of RB, we found that RB could significantly downregulate the expression of Bax/Bcl-2 ratio (Figure 29).
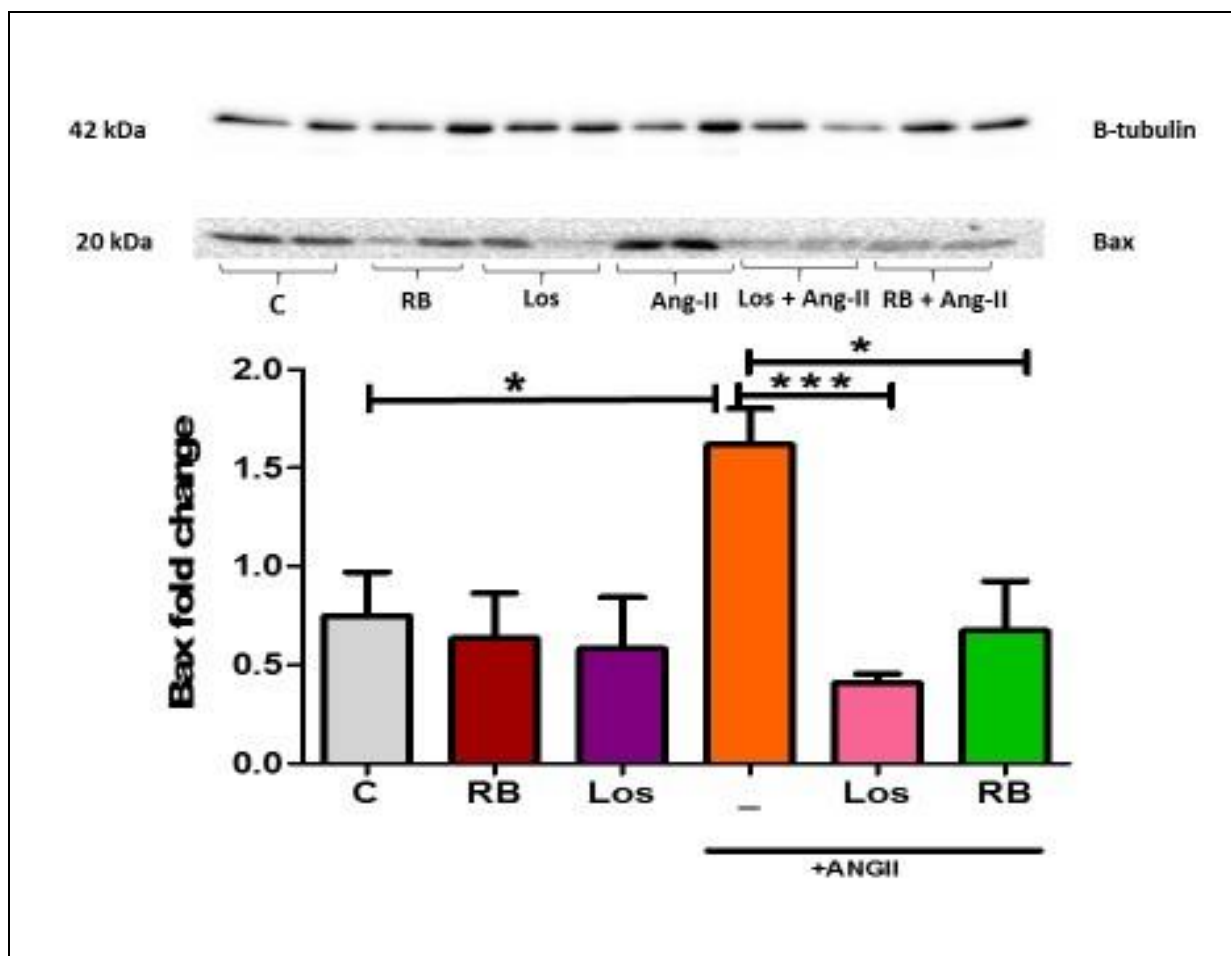

Figure 29: Effect of RB on Bax/Bcl-2 ratio in H9c2 cells. *H9c2 cells treated for 48 hrs with Ang-II (20µM) in the presence or absence of Rooibos (100µg /ml) or Losartan (10µM). Each experiment was repeated three times in H9c2 cardiomyoblasts. Representative Western blot of Bax/Bcl-2 expression and beta-tubulin. The blot above represents one of the biological repeats (n=3) in the Appendix, with each biological repeat with two technical repeats. All values were normalized to beta-tubulin, expressed as a fold-change to the untreated control samples and presented as mean ± SEM (n=3). ***P<0.001.*

3.5.2.4 Effect of RB on Caspase-3 expression

The H9c2 cardiomyoblasts from the Ang-II treated group showed a significant increase in the basal expression of Apoptotic executor cleaved caspac-3 when compared to the cells from control group from 1.000 ± 0.278 to 1.85 ± 0.394. However, co-treatment with RB mitigated the increased cleavage of caspase-3 from 1.85 ± 0.394 to 1.146 ± 0.03.
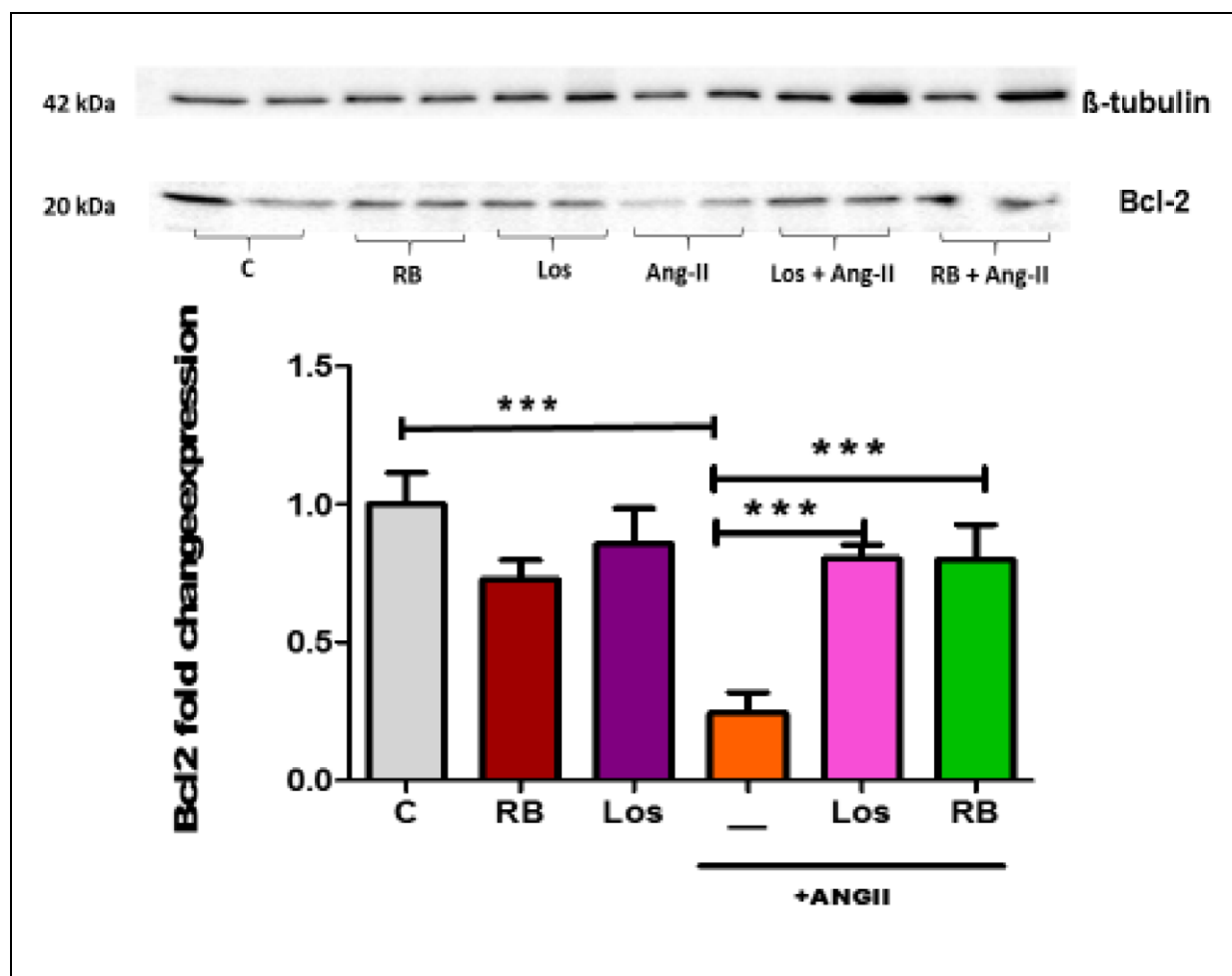

Figure 30: Effect of RB on Cleaved caspase-3 in H9c2 cells. *H9c2 cells treated for 48 hrs with Ang-II (20μM) in the presence or absence of Rooibos (100μg /ml) or Losartan (10μM). Each experiment was repeated three times in H9c2 cardiomyoblasts. Representative Western blots of Cleaved caspase expression and beta-tubulin. This blot represents one of the three biological repeats (n=3), each biological repeat has two technical repeats. All values were normalized to beta-tubulin, expressed as a fold-change to the untreated control samples and presented as mean ± SEM.* P<0.05.*

3.5.2.5 Inhibitory effect of RB on up regulated p38 levels

In order to prove whether the anti-apoptotic effect of RB is mediated via p38 MAPK, expression level of p38 MAPK and phosphorylated p38 were also analysed in treated cells and control. Ang-II induced a rapid increase in the phosphorylation of p38 when compared to the control. However, Ang-II-induced phosphorylation was inhibited by RB from 1.492 ± 0.1387 to 0.5038 ± 0.07465 (Figure 31). We did not observe any notable difference in the expression of total p38 MAPK in both control and treated cells (Figure 32). The ratio of phosphorylated vs. total P38 showed no significant differences in the cardiomyoblasts from the Ang-II treated and RB treated cells (Figure 33).

*Figure 31: Effect of RB on Phosphorylated p38, in H9c2 cardiomyoblasts. H9c2 cells treated for 48 hrs with Ang-II (20µM) in the presence or absence of Rooibos (100µg /ml) or Losartan (10µM). Each experiment was repeated three times in H9c2 cardiomyoblasts. Representative Western blot of Phosphorylated p38 and beta-tubulin. This blot represents one of the three biological repeats (n=3), each biological repeat has two technical repeats. All values were normalized to beta-tubulin, expressed as a fold-change to the untreated control samples and presented as mean ± SEM.** P<0.01, *** P<0.001.*
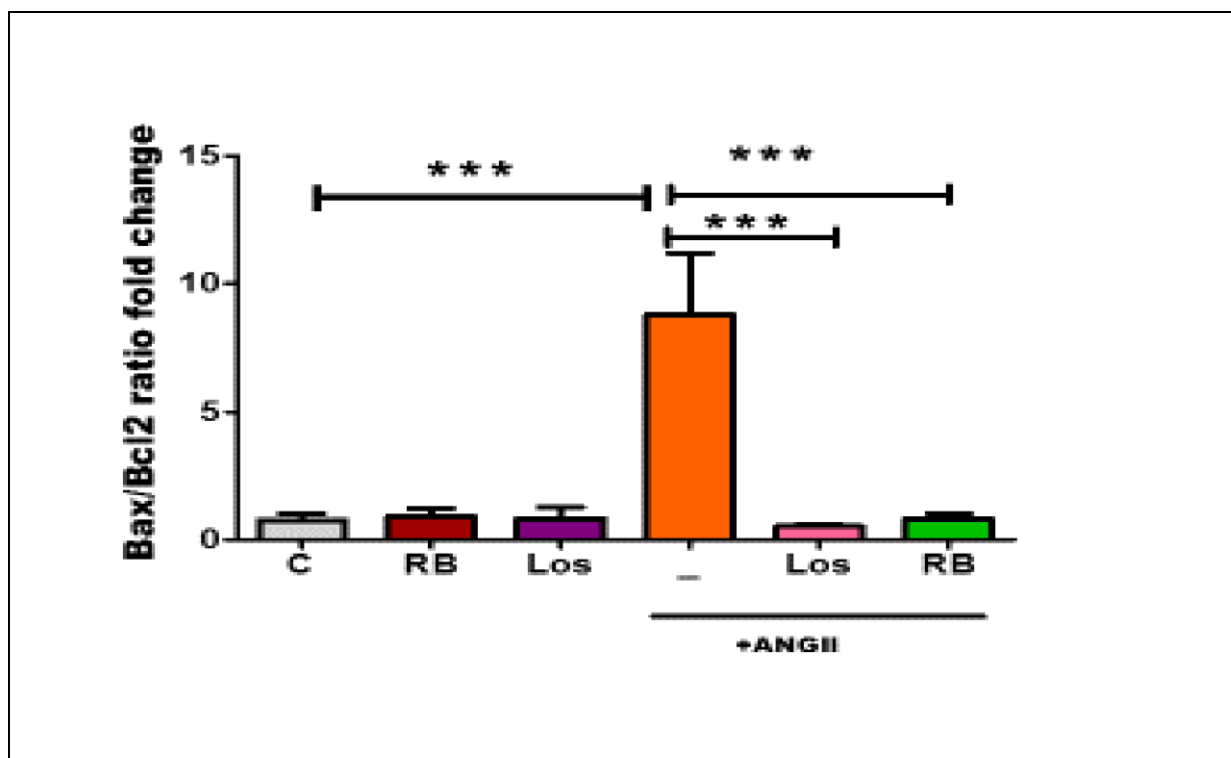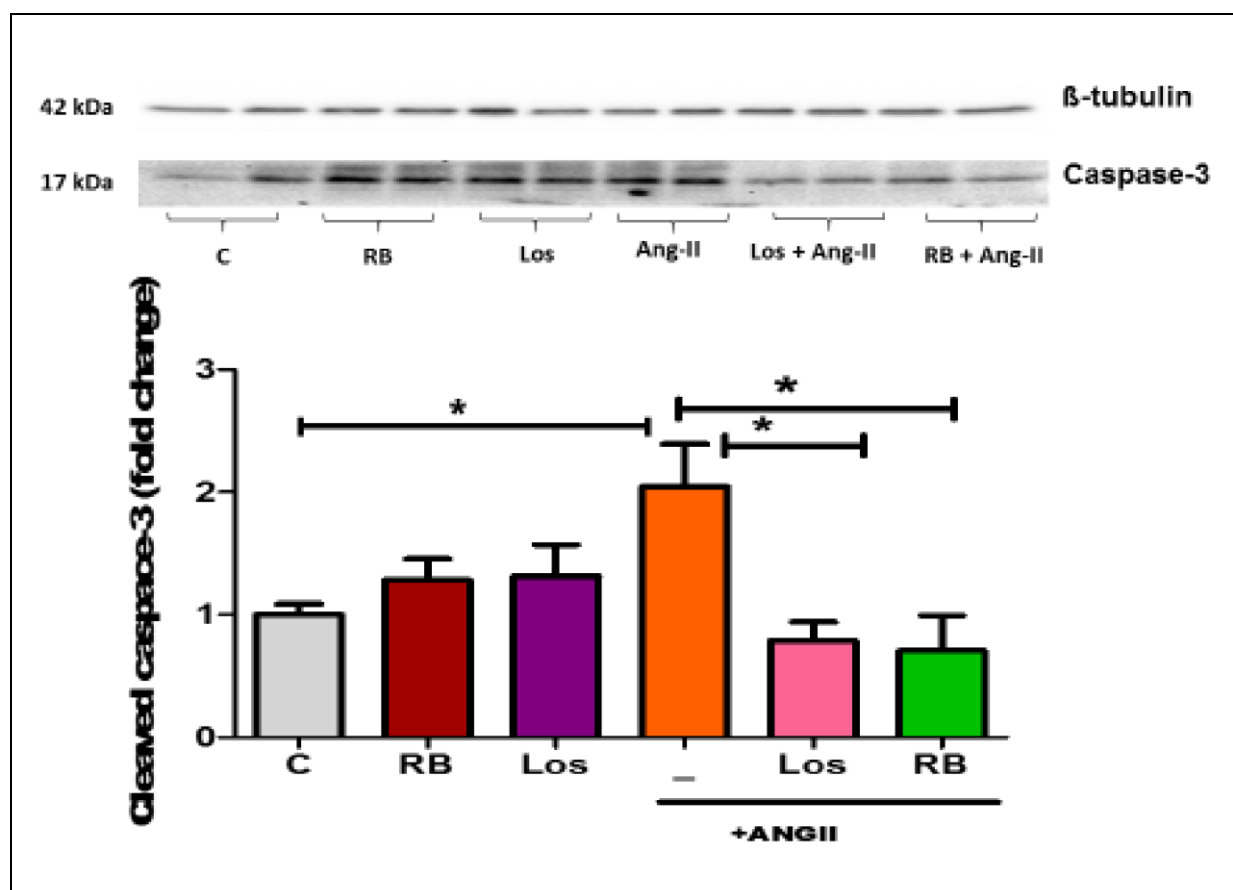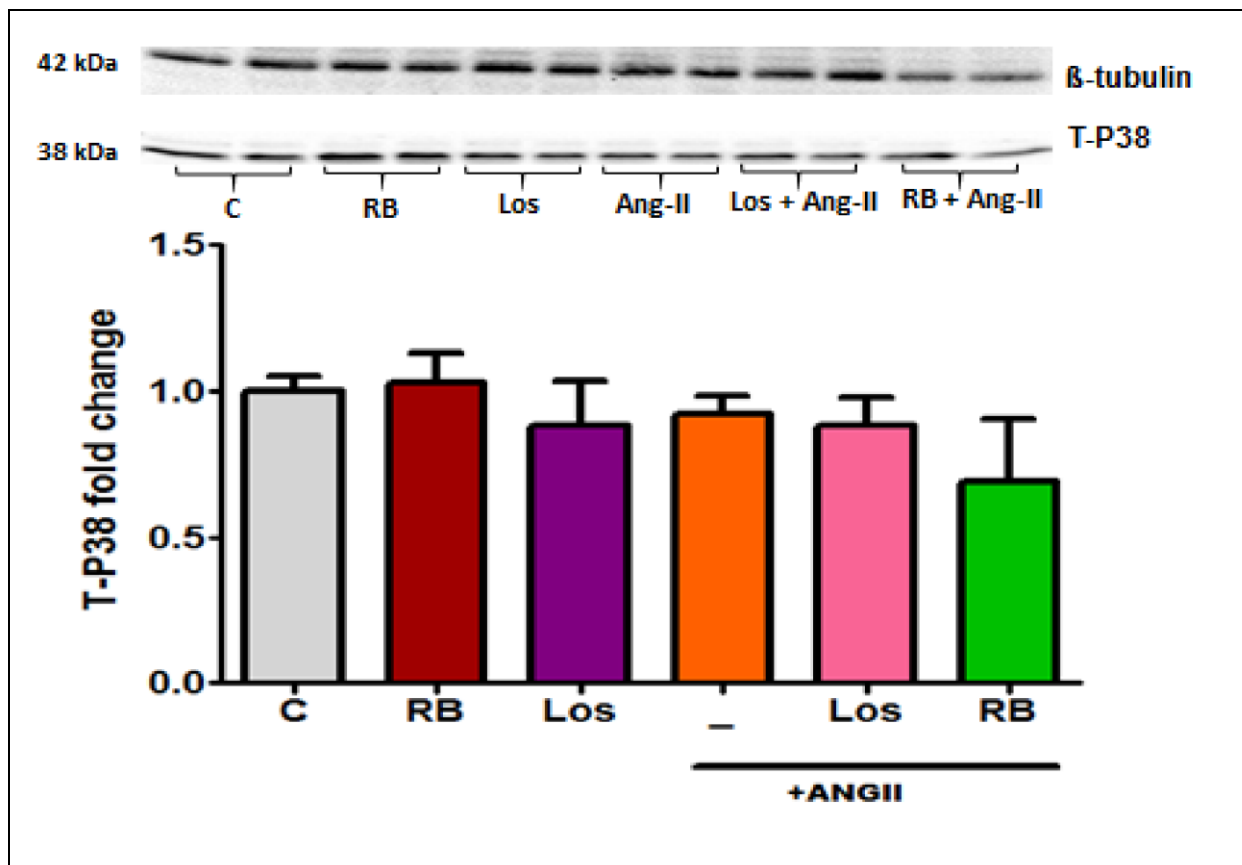
Figure 32: Effect of RB on t-p38 in H9c2 cells. *H9c2 cells treated for 48 hrs with Ang-II (20μM) in the presence or absence of Rooibos (100μg /ml) or Losartan (10μM). Each experiment was repeated three times in H9c2 cardiomyoblasts. Representative Western blots of Total p38 and beta-tubulin. The blot above represents one of the three biological repeats (n=3). All values were normalized to beta-tubulin, expressed as a fold-change to the untreated control samples and presented as mean ± SEM.*
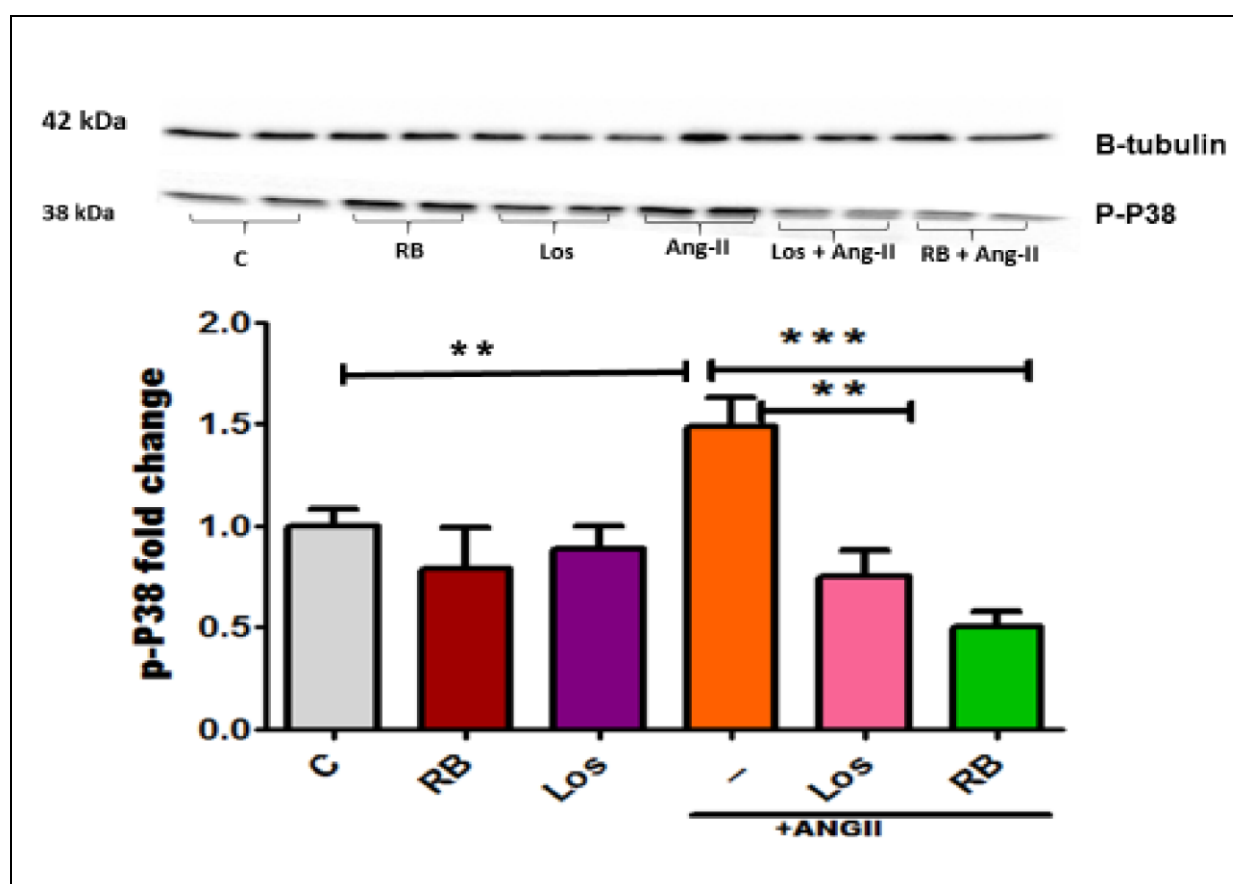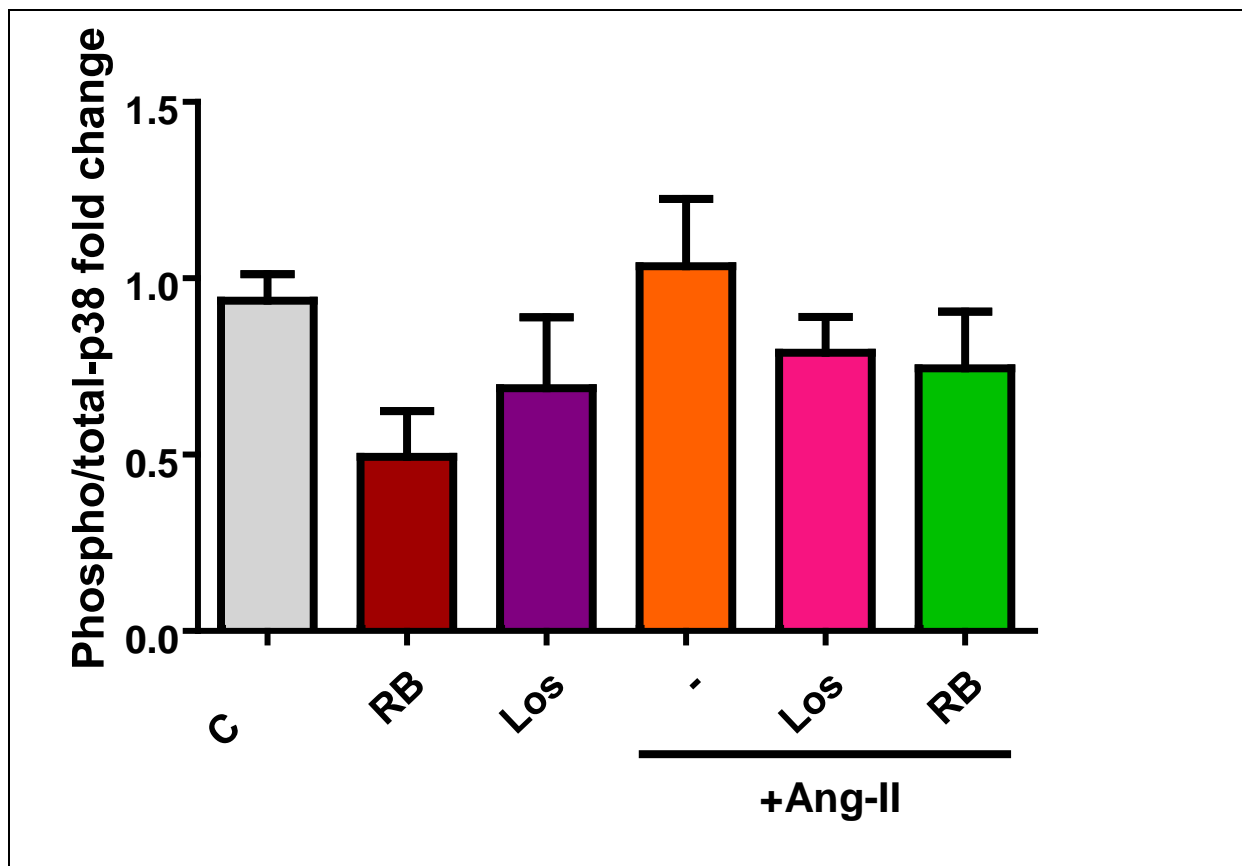
Figure 33: Effect of RB on Phosphorylated/total-p38 ratio in H9c2 cells. *H9c2 cells treated for 48 hrs with Ang-II (20 µM) in the presence or absence of Rooibos (100µg /ml) or Losartan (10µM). Each experiment was repeated three times in H9c2 cardiomyoblasts. The data was presented as mean ± SEM (n=3).*

3.6 Effect of RB on metabolic activity

3.6.1 Mitochondrial membrane potential

In this present study we determined whether RB exerts its protection on mitochondria. Ang-II at 20 µM caused a remarkable and statistically significant decrease in JC-1 ratio in Ang-IItreated group indicating a loss of mitochondrial membrane potential. JC-1 in the untreated group was 1.53 ± 0.38 and that was decreased to 0.44 ± 0.05 upon addition of Ang-II. Evidence in the present study indicate that RB co-treatment significantly restored mitochondrial membrane potential and JC-1 ratio to 0.92 ± 0.186, this indicated positive activity of RB against Ang-II induced mitochondrial toxicity (Figure 34). This was statistically significant compared to Ang-II-treated group.

JC-1 Aggregates	JC-1 Monomers	Merged
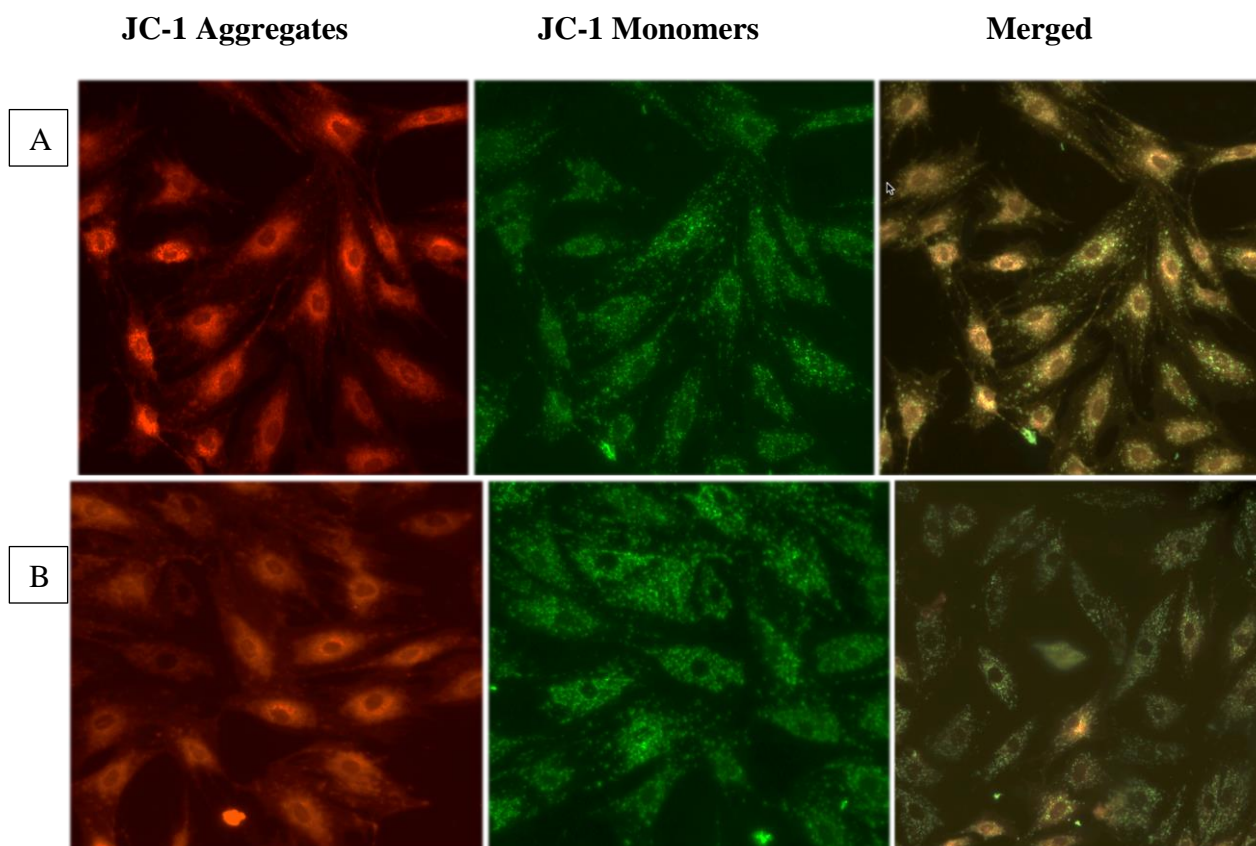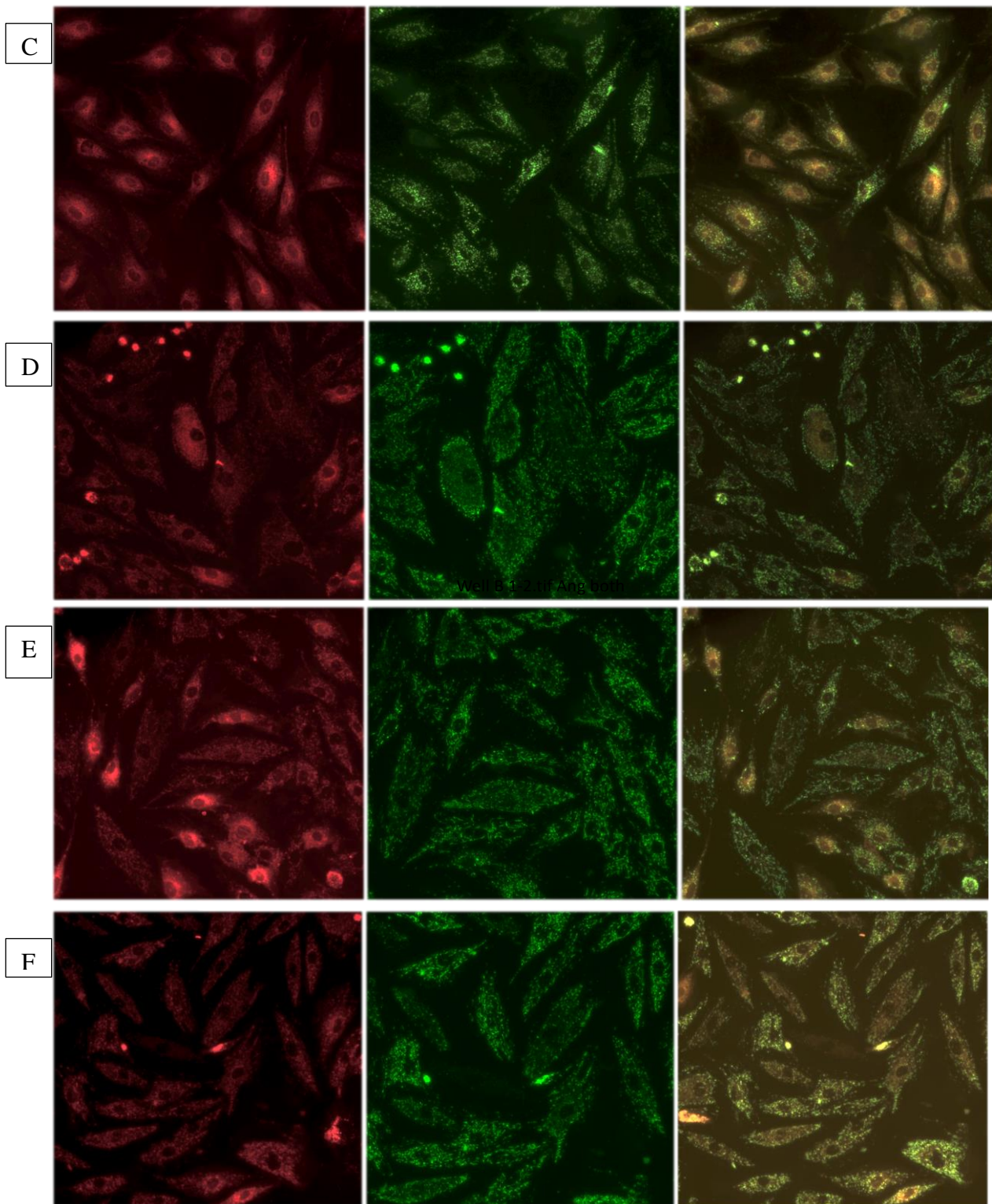
Figure 34: Effect of RB on mitochondrial membrane potential in hypertrophy/apoptotic cells.

The representative images show JC-1 aggregates, JC-1 monomers and merged images of both. (a) Control cells, (b) Rooibos, (c) Losartan, (d) Ang-II, (e) Los + Ang-II, (f) RB + Ang-II. The cell is stained with JC-1, then analysed with Image J software to determine mitochondrial membrane potential.20X magnification was used to take pictures.
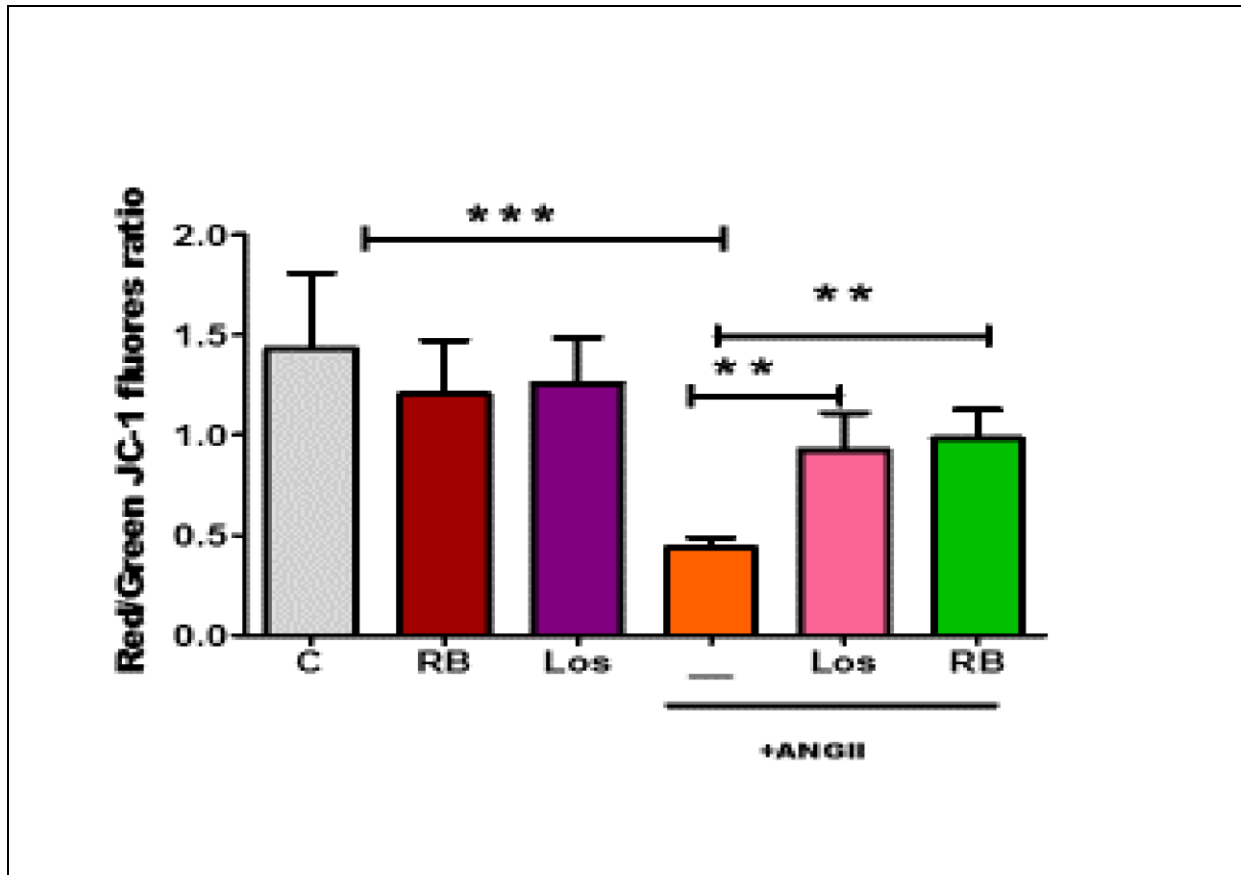
Figure 35: Change in mitochondrial membrane potential in different groups. *(Ratio of JC-1 aggregates (Red) to JC-1 monomers (Green). The graph represents the ratio of JC-1 aggregates to JC-1 monomers (ratio of 590:530 nm emission intensity) demonstrate alteration of membrane potential Ang-II while maintenance by RB. The values are fold-changes to untreated controls (n=3). Data presented as mean ± SEM.** P<0.01, *** P<0.001.*

3.6.2 ATP Activity

The results showed that ATP levels in the Ang-II-treated cells were decreased by 37% compared with the control group. Comparison between the RB co-treated cells and Ang-II treated cells the results revealed that the ATP level was increased by 21% (Figure 36).
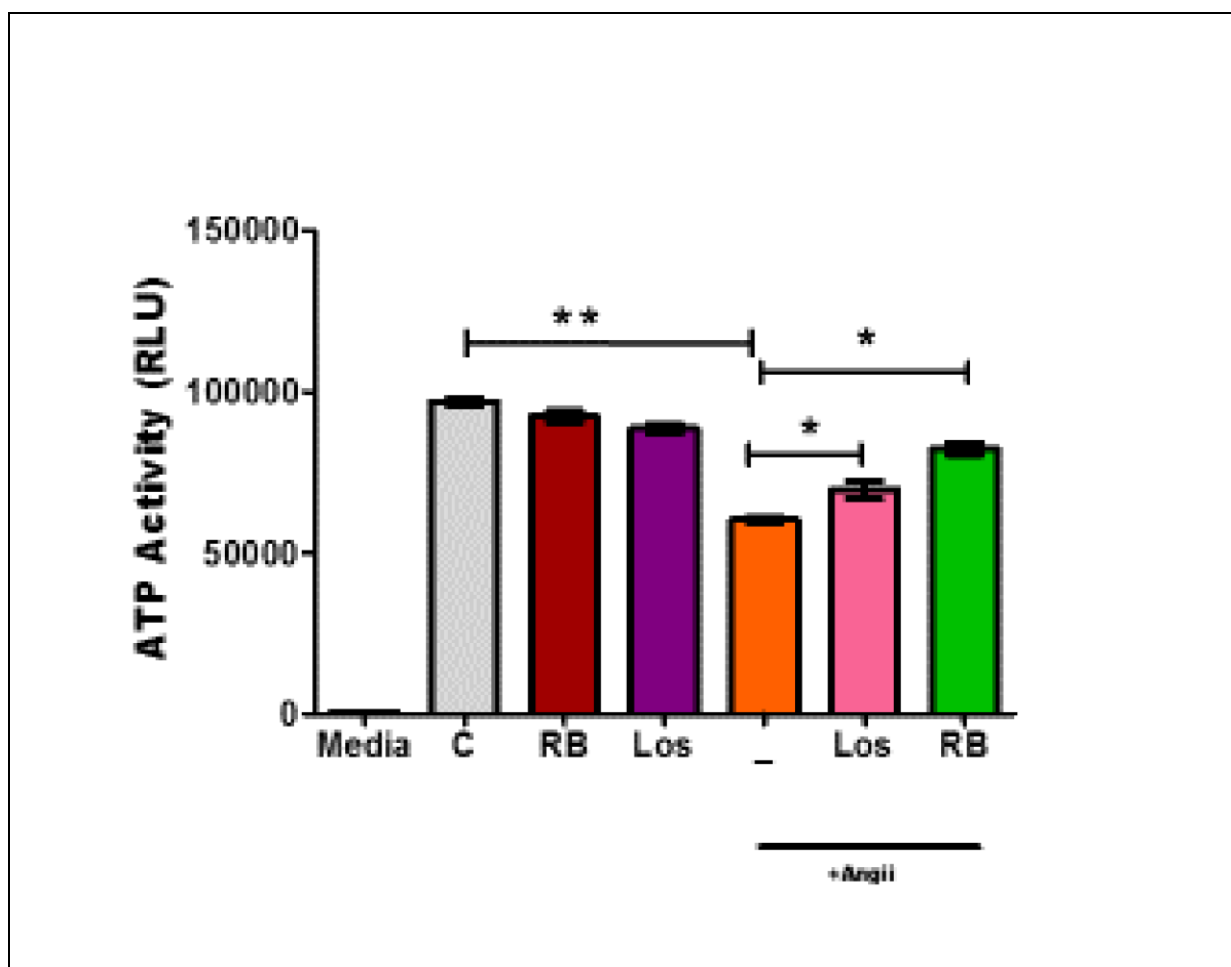
Figure 36: Effect of RB on the ATP activity in Ang-II-induced hypertrophy/apoptosis. *Cellular viability was measured using Cell-Titre-Glo luminescence assay kit. Relative light unit (RLU). The experiments were repeated three times. Data presented as mean ± SEM.* P<0.05, ** P<0.01.*

3.6.3 Mitochondrial respiratory complexes

The expression of respiratory chain complexes such as complexes IV and V were significantly decreased in Ang-II treated cells compared to control cells. There were reductions in expression of complex IV and V in Ang-II-treated cells while the co-treatment with RB improved the

reduction in the expression of the respiratory chain complexes compared to the Ang-II-treated cells (Figure 40 & 41). In addition, we found no significant difference in complex II and III between co-treated RB and Ang-II treated cells (Figure 38, 39). We further found that complex II & IV were significantly difference in RB only compared to the control (Figure 36, 38). We also observed that complex III, IIV & V were significantly different in Losartan alone compared to the control (Figure 37, 38 & 39). All these values are fold-changes to untreated controls.
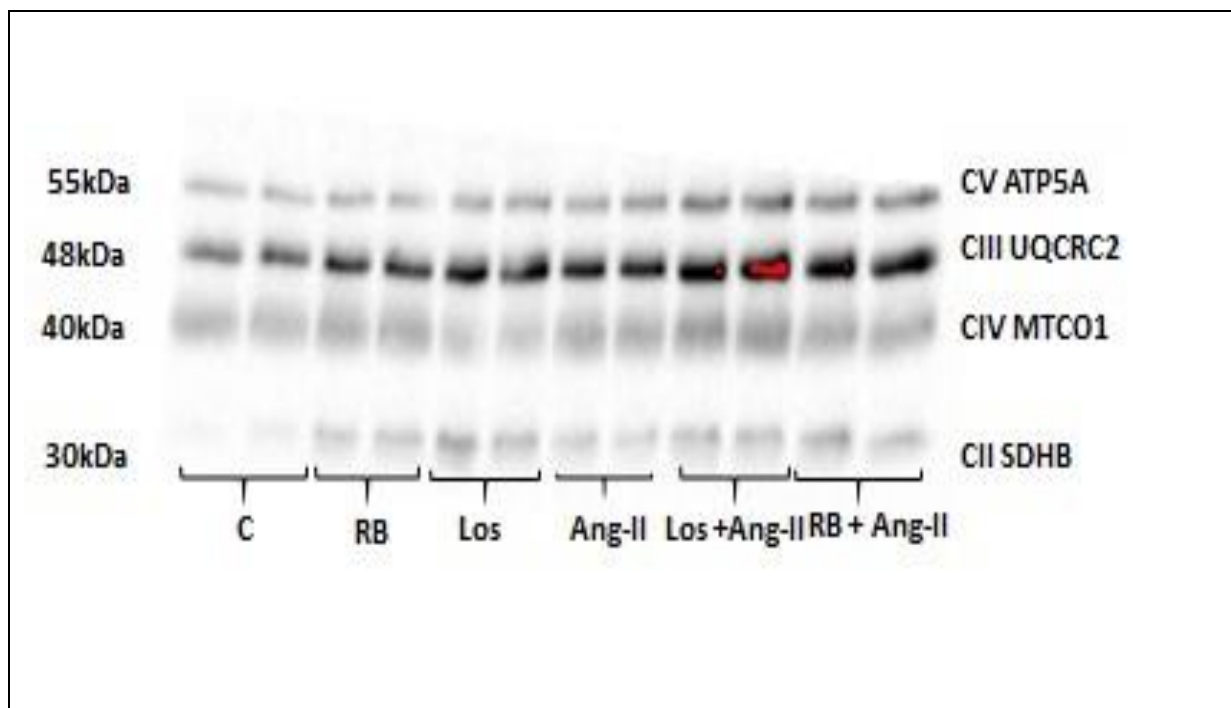
Figure 37: Effect of RB on mitochondrial complex chain. *H9c2 cells treated for 48 hrs with Ang-II (20μM) in the presence or absence of Rooibos (100μg /ml) or Losartan (10μM). Each experiment was repeated three times in H9c2 cardiomyoblasts. The blot represents one of the three biological repeats, each in duplicate. The data was presented as mean ± SEM (n=3).*
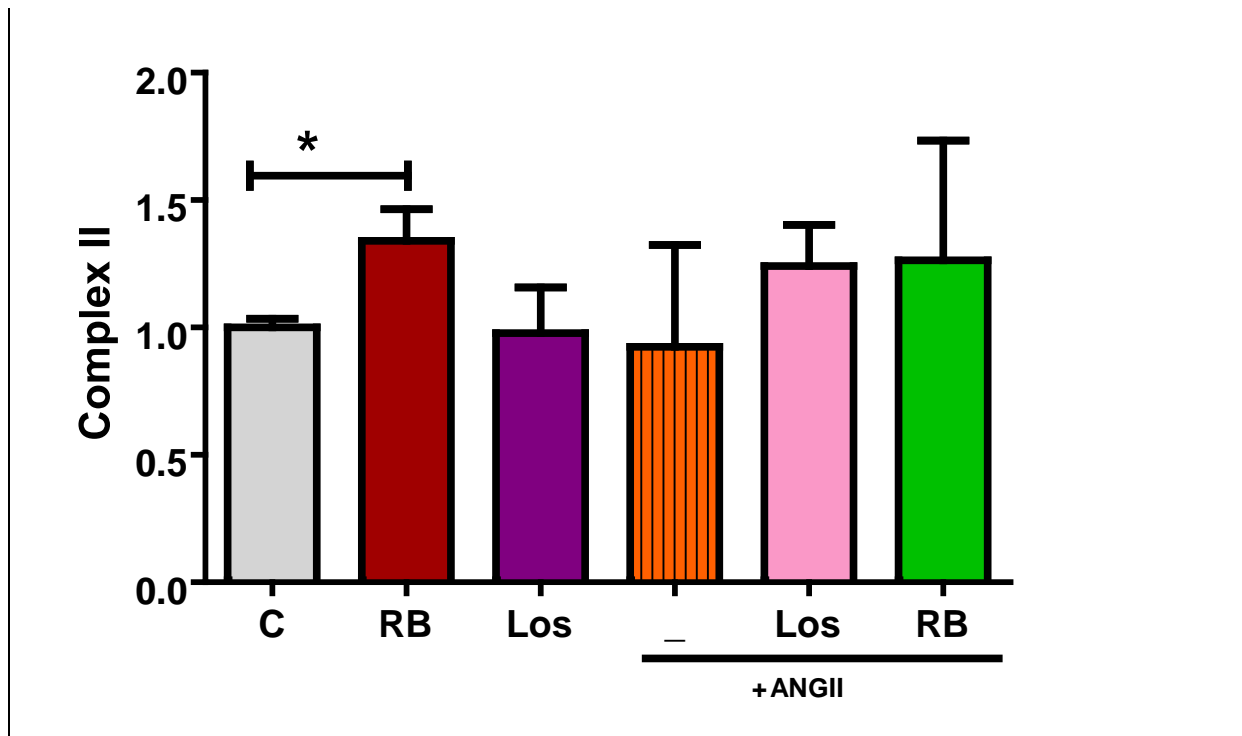
Figure 38: Effect of RB on complex II. *Graphical representation of complex II. H9c2 cells treated for 48 hrs with Ang-II (20µM) in the presence or absence of Rooibos (100µg /ml) or Losartan (10µM). Each experiment was repeated three times in H9c2 cardiomyoblasts. The data was presented as mean ± SEM (n=3). *P<0.05*
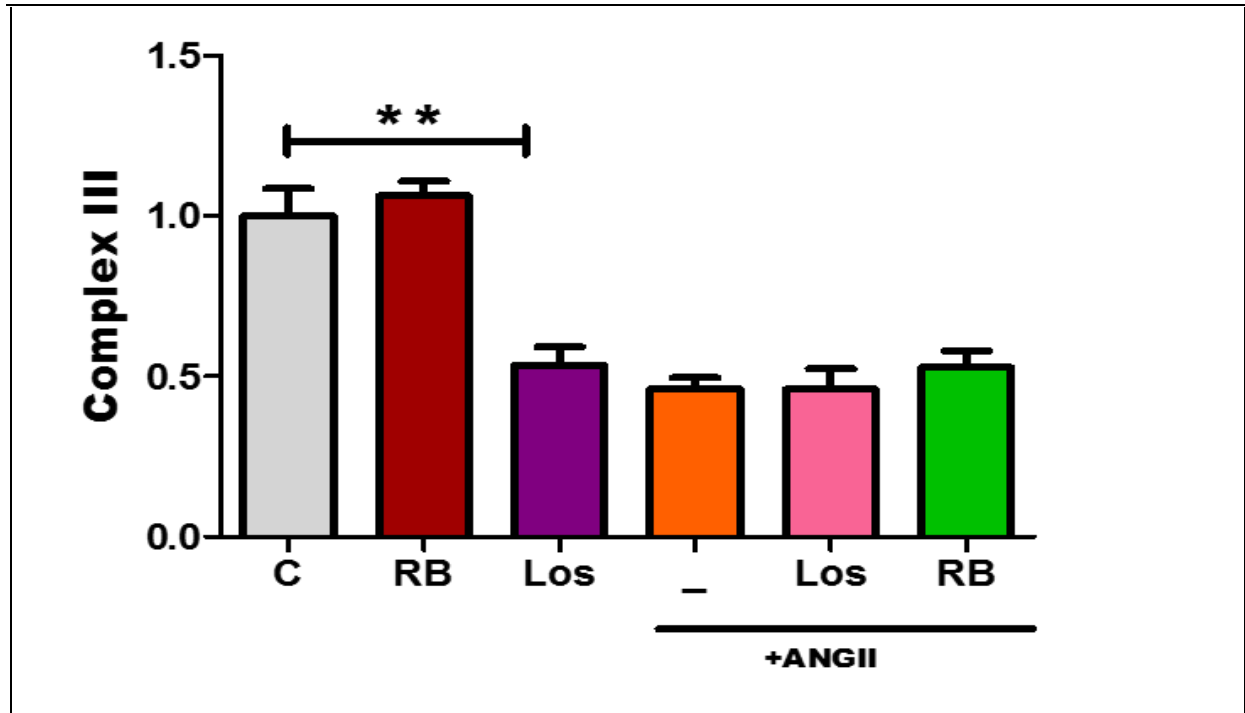
Figure 39: Effect of RB on complex III expression. Graphical representation of complex III. *H9c2 cells treated for 48 hrs with Ang-II (20µM) in the presence or absence of Rooibos (100µg/ml) or Losartan (10µM). Each experiment was repeated three times in H9c2 cardiomyoblasts. The data was presented as mean ± SEM (n=3). **P<0.01*
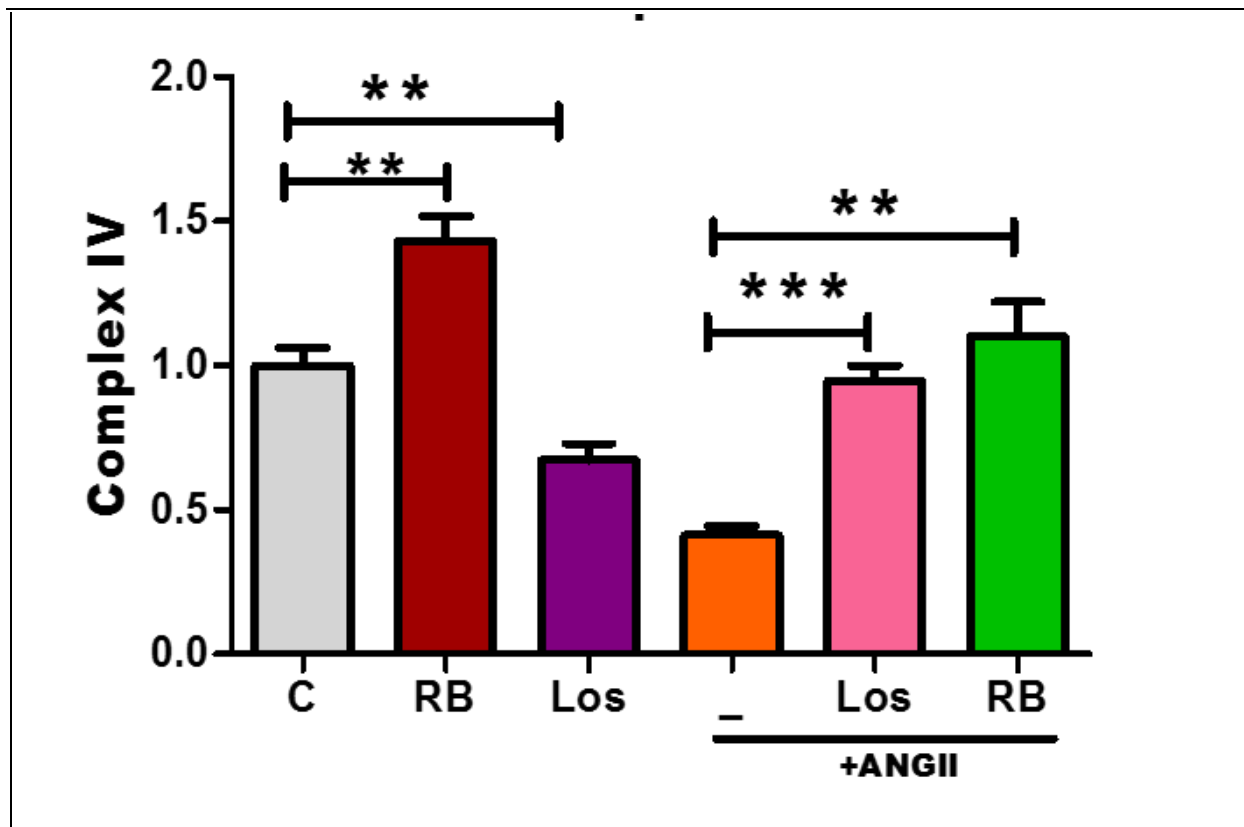
Figure 40: Effect of RB on complex IV expression. *Graphical representation of complex IV. H9c2 cells treated for 48 hrs with Ang-II (20µM) in the presence or absence of Rooibos (100µg/ml) or Losartan (10µM). Each experiment was repeated three times in H9c2 cardiomyoblasts. The data was presented as mean ± SEM (n=3). **P<0.01, ***P<0.001*
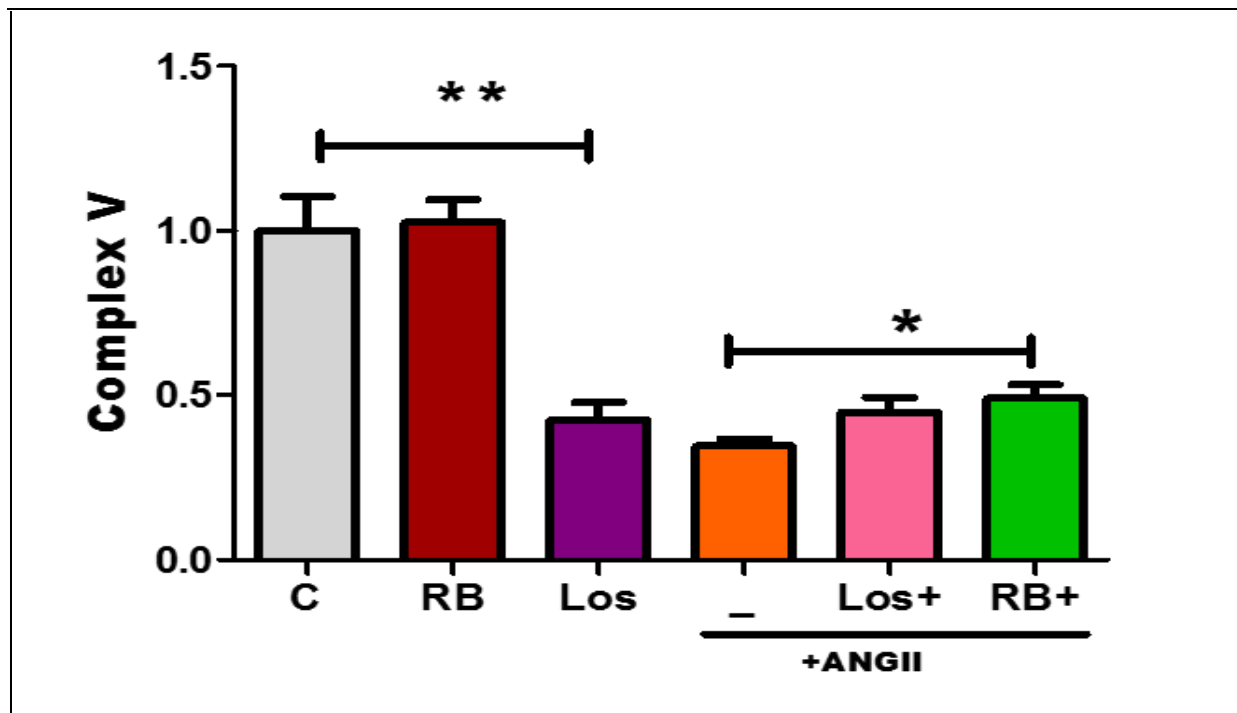
Figure 41: Effect of RB on complex V expression. Graphical representation of complex VI. *H9c2 cells treated for 48 hrs with Ang-II (20µM) in the presence or absence of Rooibos (100µg/ml) or Losartan (10µM). Each experiment was repeated three times in H9c2 cardiomyoblasts. The data was presented as mean ± SEM (n=3). *P<0.05, **P<0.01*

7.7 Effect of RB on Aconitase activity

Aconitase activity was measured using the Aconitase assay kit at an absorbance of 240 nm. To determine catalytic activity of Aconitase the conversion of the substrate isocitrate to cisaconitate was measured as an increase in absorbance over a period of 30 minutes (Figure 42). The results showed no significant difference in Aconitase activity in Ang-II treated cells, while co-treatment with RB showed a slight increase but this was not significant.
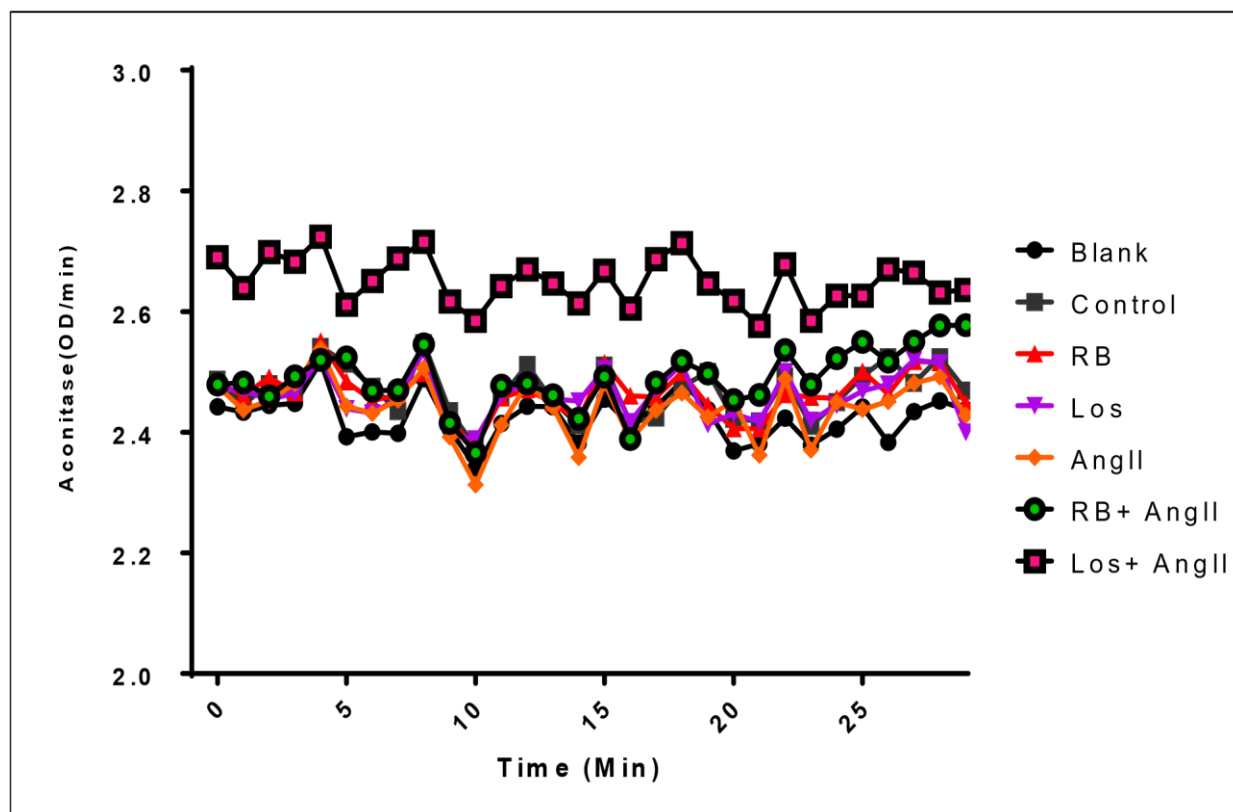
Figure 42: Effect of RB on aconitase activity. *Representation of Aconitase activity over a 30-minute time period. The experiment was repeated three times with two technical repeats for each treatment. The data was analysed using ANOVA. This data represents the mean.*

Chapter 4: Discussion

In the current study we investigated the cardioprotective effects of RB in an H9c2 model of Ang-II-induced hypertrophy and apoptosis. We have provided evidence that Ang-II in fact did cause hypertrophy (Prathapan et al, 2014; Li et al., 2017) and apoptosis (Liu et al., 2009; Pang et al., 2004) as reported previously. Firstly, apoptosis induced by Ang-II was observed as decreased cell viability, up-regulation of pro-apoptotic markers Bax, caspase-3 and downregulation of anti-apoptotic Bcl-2. Secondly, Ang-II stimulated hypertrophy was validated by observing increased cell size and upregulating growth signalling pathways. Thirdly, Ang-II reduced the expression of mitochondrial complexes IV and V, decreased mitochondrial membrane potential and reduced cellular ATP content. RB co-treatment effectively antagonized Ang-II-induced apoptosis and hypertrophy of H9c2 cells. This was mediated by increasing Bcl-2, decreasing Bax and caspase-3, reducing cell size and increasing cell viability. RB dephosphorylated growth signalling pathways such as ERK 1/2, PKB, mTOR, Calcineurin and GSK-3ß. Lastly, RB improved mitochondrial membrane potential, increased ATP content and restored expression of mitochondrial complexes IV and V.

A cold-water RB extract was used in this study, and our HPLC analysis showed that the major flavonoid in our extract was Aspalathin (0.161g/100g). Aspalathin and its 3-deoxy derivative nothofagin (0.020g/100g), as well as their flavone derivatives, isoorientin (0.396g/100g), orientin (0.515g/100g), vitexin (0.164) and isovitexin (0.074g/100g) comprised 1.33g/100g of the extract. The major quercetin glycoside was bioquercetin (quercetin-3-O-robino-bioside; 0.445g/100g) as well as the phenolic precursor Z-2-(β-D- glucopyranosyloxy) −3phenylpropenoic acid which was (0.421g/100g). We also quantified the flavanones compound 1-4. Other compounds being quantified included luteoloside (a flavone) and quercetin glycosides (rutin, hyperoside and isoquercitrin), all present at levels less or equal to one, in the extract. HPLC analysis of fermented RB extract showed that our extract contained high levels of Z-2-(β-D- glucopyranosyloxy) −3-phenylpropenoic acid (PPAG) and the flavonoids isoorientin, orientin and bio-quercetin. Compared with previous data concerning Aspalathin in fermented RB, our extract contained less Aspalathin (0.161g/100g) which was comparable the one found in a study (0.17g/100g) (Walters et al., 2017). In another study (Joubert et al., 2009) authors also found low levels of Aspalathin in fermented RB extract beverages on the market. They speculated that either their extract had a very poor quality or

low quantities of Aspalathin were used by the manufacturers. Moreover, when compared to unfermented RBs extract the Aspalathin is much higher with 1.2g/100g, while our extract contains about 10 times less Aspalathin *0,161g/100g*.

Previous studies have also shown that RB contains number of pharmacologically active compounds such as mentioned above which we were also able to quantify. In contrast, the content values in the present study are relatively low in comparison to previously analysed hot water extracts of RB (Joubert and Beer, 2012), as well as those in (Muller et al., 2016). It seems that boiling increased the flavonoid content of RB tea, irrespective of the type (Muller et al., 2020). Most of the identified compounds in our extract have been shown to attenuate metabolic disturbances related to CVDs. PPAG has been shown to be very effective in protecting depolarization of the mitochondria and cell apoptosis and against substrate (Dludla *et al.*, 2016). Aspalathin and nothofagin have been reported to prevent inflammation and thrombosis by suppressing TNF-α, IL-6 and NF-κB in high glucose-induced vascular in HUVECs and mice model (Ku et al., 2014). In addition, orientin and iso-orientin are known for their potential to maintain vascular barrier integrity through hyperpermeability inhibition (Lee et al., 2014). Vitexin has also been shown to exhibit protective properties against cardiac hypertrophy by interrupting calcineurin and CaMKII signalling pathways (Lu *et al.*, 2013). Moreover, quercetin exhibits antihypertensive properties and alleviates cardiac hypertrophy by increasing antioxidant capacity (Chen et al., 2018; Yan et al., 2013).

Our initial Ang-II concentration was based on a previous study that exposed H9c2 cells to Ang-II at a 100nM concentration that increased hypertrophy and apoptosis (Prathapan *et al.*, 2013). However, this concentration and others did not work in our laboratory (Wu *et al.*, 2014; Zhou *et al.*, 2014; Li *et al.*, 2017), as it had no impact on cell size. However, we managed to find an effect at 20μM Ang-II. We used cell surface area with JC-1 staining as proxy for hypertrophy. However, an increase in cell size does not necessarily equate to hypertrophy. Therefore, we also assessed hypertrophy signalling pathways. Accumulating data suggest that oxidative stress is implicated as one of the key contributing factors in the development of hypertrophy (Fan *et al.*, 2019). Ang-II induced ROS activates a wide variety of hypertrophy signalling kinases and transcription factors (Maulik and Kumar, 2012). Several studies have demonstrated that Ang-II elevates ROS and activates Calcineurin/NFAT and PKB/Akt cascades (Fan et al., 2019;

Molkentin, 2004; Rohini et al., 2010) these pathways are considered to be important promoters of cardiac hypertrophy. In the present study, we demonstrated that Ang-II induced hypertrophy. Hypertrophy caused by an up-regulation of the PKB/Akt signalling pathway contains two molecular target proteins: GSK-3β and mTOR. Disruption of both PKB/Akt & mTOR could explain impaired survival signalling and promote pathological cardiac hypertrophy as previously reported by (Aoyagi *et al.*, 2012). Our study demonstrated that Ang-II treatment induced phosphorylation of PKB/Akt subsequently increased GSK-3β phosphorylation, which in-turn activated an increased cell protein synthetic machinery. Accumulating evidence suggest that GSK-3β is well-known as a negative regulator of cardiac hypertrophy that blocks increased levels of protein synthesis and expression of hypertrophic genes (Hardt and Sadoshima, 2002). Once the active GSK-3β expression is increased in the heart it blocks hypertrophy mediated by pressure overload, activated calcineurin and β-adrenergic receptors (Antos et al., 2002). Activation of PKB/Akt also resulted in an up-regulation of mTOR, leading to cell enlargement. Our findings corroborated previous findings from Wu et al. Their experimental results showed that Ang-II induced hypertrophy was mediated by PKB/Akt signalling pathways using H9c2 cardiomyoblasts (Wu *et al.*, 2014). A study conducted by Chen et al., 2018 also demonstrated that Ang-II induced cardiac hypertrophy which was related to activation of GSK-3α/β in Neonatal SD rats (1–2 days old).

To our knowledge we show for the first time that RB attenuates hypertrophy in a complex model of Ang-II induced hypertrophy in H9c2 cells. From our JC-1 staining data the size of H9c2 cells was significantly reduced after treatment with RB compared with the Ang-II treated group. Furthermore, we found that RB inhibited Ang-II induced hypertrophy by decreasing the PKB/Akt intracellular signalling pathways. Subsequently inhibiting increased expression of downstream proteins of PKB/Akt such as GSK-3β and mTOR, which are probable mediator for protective effect against Ang-II induced cardiac hypertrophy in H9c2 cells.

Both unfermented and fermented RB have been reported to confer cardioprotection by decreasing phosphorylation of PKB/Akt (Pantsi *et al.*, 2011). The decrease in phosphorylation of PKB/Akt on serine 473 and threonine 308 by RB extract could be due to the fact that the RB extract contains a lot of flavonoids and high polyphenol content level (Schroete et al., 2002). Flavonoids in RB, particularly aspalathin, a C-glucosyl dihydrochalcone can modulate

PI3K/Akt to maintain substrate metabolism in the adipose tissue, skeletal & cardiomyoblasts (Mazibuko. *et al.*, 2015, Son *et al.*, 2013 & Johnson *et al.*, 2016). Similarly, other plant flavonoids such as Vitex and Quercetin can also inhibit Ang-II cardiac hypertrophy and apoptosis, with Vitex preventing hypertrophy by inhibiting Calcineurin (Lu *et al.*, 2013). On the other hand, quercetin prevent hypertrophy by activating survival pathways which include GSK-3ß and Akt (Chen *et al.*, 2019). Recent evidence also shows that Fisetin, a plant polyphenol found in apples and cucumber is able to attenuate hypertrophy by activating survival signalling pathways such as IGF-IR-PI3K-Akt signaling in H9c2 cells (Chen *et al.*, 2019). The fact that RB mediates its cardioprotection through PKB/Akt signalling was supported by our previous findings, in a model of lipid-induced hepatic insulin resistance using palmitate-exposed C3A liver cells, it has also been reported that RB has beneficial health effects by modulating survival signalling such as PI3K/Akt and AMPK Pathways (MazibukoMbeje *et al.*, 2019). They indicated that treatment with green rooibos extract (GRE) significantly increased PI3K/Akt and AMPK phosphorylation while concurrently enhancing glucose transporter 2 expression. Another study also reported that green RB extract inhibited the proliferation of castrated prostate cancer patients by suppressing the PKB/Akt signalling pathway (Huang *et al.*, 2019). These observations suggest that THE /Akt signalling pathway is one of the main targets for RB treatments, an effect also observed in our study.

Mitogen-activated protein kinases (MAPKs) have been reported to be involved in primary cellular function with regard to both cellular growth and apoptosis. A member of the MAP kinase family, ERK1/2, is well-known as a key player in cell proliferation (Su *et al.*, 2015) and promoting cardiac hypertrophy (Chen *et al.*, 2018). To confirm the involvement of ERK1/2 as one of the growth pathways, we used immunoblotting to assess the phosphorylation of ERK1/2. Ang-II increased the phosphorylation of ERK1/2 as compared to the control group, suggesting that MAPK/ERK pathway activation had a role in driving the development of cardiac hypertrophy in H9c2 cardiomyoblasts. ERK1/2 activation is widely associated with antiapoptotic functions but it has also been reported that sustained ERK1/2 activation mediates apoptosis (Sun *et al.*, 2012; Wei *et al.*, 2012). Given that activation of the ERK 1/2 signalling pathway has been reported can be both pro- and/or anti-apoptotic (Sun *et al.*, 2012). The way in which ERK signalling functions can be contradictory as it is believed to be host of both cell death and cell survival. Furthermore, this can only be explained and understood through the basic effects of the MAPK signalling system. The extremely sensitive biochemical properties

of MAPKs and the presence of threshold levels for activation explain why robust or sustained stimulation of MAPKs is pro-apoptotic (Yue and López, 2020). It has been reported that robust ERK activation suppresses the cell cycle via the expression of cell-cycle inhibitor proteins, including p21Cip/Waf and p27KIP (Marshall, 1999). On the other hand, weak stimulation is anti-apoptotic. Further studies investigating the temporal ordering of phosphorylation, early for anti-apoptotic targets and late for pro-apoptotic targets, are required to better understand the behaviour of MAPKs (Yue and López, 2020). Our study is in support of (Sun *et al.*, 2012) who observed apoptosis mediated by the mitochondria-dependent intrinsic apoptotic pathway, ERK signalling pathway and pro-apoptotic Bax. This could also suggest that the anti-apoptotic effect linked with RB is responsible for the suppression of Ang-II induced ERK1/2 activation. This suggests that ERK activation could also be responsible for intracellular ROS generation, which is readily suppressed by RB.

Cardiac hypertrophy is believed to be one of main predictors of continuous heart diseases which frequently leads to heart failure associated with alteration in the intracellular calcium handling (Wilkins and Molkentin, 2004). Accumulating data suggests that continued pressure overload leads to an increased sustained intracellular Ca^{2+} in cardiomyoblasts (Wang et al., 2012). Furthermore, Ca^{2+} is both a regulatory factor and second messenger and it has been reported to play a crucial role in the progression of cardiac hypertrophy (Vicencio et al., 2011). Several studies have confirmed that Ca^{2+} activated kinase, Calcineurin, acts as an important factor in promoting the development of hypertrophy (Lu et al., 2013; Heineke & Molkentin, 2006). One of the significant signalling pathways responsible for cardiac hypertrophy, calcineurin responds to altered calcium is calcineurin–NFAT. Calcineurin is a strong executor of cardiac growth in this pathway and it has been reported to be sufficient to promote development of cardiac hypertrophy (Molkentin, 2004; Fischer et al., 2013). In the present study, there was a significant increase in calcineurin in hypertrophied cells upon the stimulation of H9c2 with Ang-II. The increase in calcineurin is associated with an increase in intracellular Ca^{2+}. RB treatment reduced expression of calcineurin. This suggest that the cardioprotection of RB was also mediated by inhibition of the Ca^{2+}-calcineurin pathway. The negative regulation of calcineurin expression by RB, also inhibited its downstream substrates such as GSK-3ß, ERK 1/2 and mTOR and thereby blocking Ang-II induced hypertrophy (Molkentin, 2004; Hogan et al., 2003).

In addition, high concentrations of intracellular calcium may lead to mitochondrial swelling subsequently to a sudden permeability increase to inner mitochondrial membrane, this in turn stops the chemiotic gradient across the inner mitochondrial membrane. This leads to uncoupling of oxidative phosphorylation and subsequently decreased ATP production and increases ROS production (Hunter and Haworth, 1979). Moreover, when the outer mitochondrial membrane ruptures it leads to a release of cytochrome-c, a main contributor in the initiation of apoptosis (Kroemer et al., 2007). In the present study Ang II-induced apoptosis in H9c2 cardiomyoblasts as seen with increased expression of Bax and cleaved caspase-3 and subsequent the deceased cell viability. Transcription factors of anti-apoptotic genes Bcl-2 and Bc-xL prevent the release of cytochrome-c and protect cardiac cells from apoptosis (Portt et al., 2011). However, Bid and Bax are transcription factors that promote the expression of cell death proteins affecting the mitochondrial membrane permeability in a negative way (Raisova *et al.*, 2001). Dimmeler et al., 1996 showed that AngII increased apoptosis of human umbilical venous endothelial cells. Another study conducted by Condoreli et al., 1999 showed that pressure stimulated an increase of apoptosis in cardiomyoblasts and contributed to the transition from left ventricular hypertrophy to heart failure, which may be associated with upregulation of Bax and reduction of Bcl-2. The present study, confirmed results reported by (Condoreli et al., 1999) in their study. We observed an up-regulation of pro-apoptotic mediators including Bax and caspase-3, while the anti-apoptotic protein was reduced. The increased expression of Bax and caspase-3 was downregulated by RB.

Both apoptosis and hypertrophy can be stimulated by similar factors and there is a cross talk between the regulatory mechanisms of the two signalling pathways. Hypertrophy is believed to be mediated via increased apoptosis which in turn leads to cardiac fibrosis and cardiac muscle damage (Wang *et al.*, 2016). PI3K/Akt is one of the mediators of both apoptosis and hypertrophy and its mechanism of action depends on its phosphorylation (Nicholson and Anderson, 2002). Our results also showed similar results to those of Chen et al., 2019 who observed AngII induced apoptosis by activation of IGF/IR/PI3K/Akt signalling pathways in H9C2 cardiomyoblasts and spontaneous hypertensive rats. The PI3K/Akt pathway is important for both cell survival when stimulated by Ang-II that influences many other factors that are involved in apoptosis, either through direct phosphorylation or transcription regulation (Nicholson and Anderson, 2002). It has been documented that Akt, once activated, moves from the plasma membrane to the cytosol and nucleus (vanhaesebroeck & Alessi, 2000). Once in the

nucleus, Akt blocks transcription factors that promote the expression of pro-apoptotic genes such as caspases, Bax and Bid, and increases the transcription of anti-apoptotic genes such as Bcl-2. This suggests that the ability of RB to decrease apoptosis in our study, is mediated by direct phosphorylation or by enhancing expression of survival genes. Herein we suggest that Bcl2 is the main regulatory network used by which RB protect the cardiomyocyte against AngII-induced apoptosis. This further suggests that the Bcl-2 family proteins were directly regulated by RB and may have an effect on mitochondrial membrane pore formation, leading to the blockage of cytochrome c release, causing caspase-3 inhibition of the intrinsic apoptotic pathway (Sun *et al.*, 2012). Activation of the caspase-3 was inhibited by RB in our study.

Comparable results were observed in H9c2 cells exposed to chronic hyperglycaemia. Treatment with aspalathin, a major phenolic compound in RB extract, was seen to protect cardiomyoblasts against Fas ligand-induced apoptosis by activating the Igf-PI3K-Akt prosurvival pathway by increasing expression of Bcl-2 (Johnson *et al.*, 2016). An alteration in the Bcl-2/Bax ratio is most likely to lead to increased mitochondrial depolarization, subsequently leading to a release of cytochrome-c, which is an important molecule to promote development of apoptosis. We observed that Ang-II resulted in a decrease in Bcl-2/Bax ratio, thus, an increase in apoptosis. However, treatment with RB reversed induced apoptosis by increasing the Bcl-2/Bax ratio. These observations are consistent with TUNEL staining and western blotting results for Bax and Bcl-2 found in isolated heart cells of diabetic mice using fermented rooibos extract (Dludla et al., 2017). These findings therefore strongly suggest that RB protects against oxidative stress-induced myocardial apoptosis.

Previous studies have reported that protection provided by RB extract is most likely to be attributed to an alteration of apoptotic promotors (Pantsi *et al.*, 2011). Studies also showed that Quercetin, a flavonol, alters apoptotic signalling pathways by maintaining mitochondrial membrane potential, down-regulating increased expression of Bax, activating Bcl-2 and further altering increased expression of caspase-3 (Choel et al., 2009; Hsu et al., 2006). Several studies also showed that treatment with Rutin, another flavonoid, offered anti-apoptotic effect by altering the increased expression of caspase-3 and upregulating Bcl-2 levels (Jeong *et al.*, 2009; Gong *et al.*, 2010). Similarly, a study by (Pantsi *et al.*, 2011b; Dludla *et al.,* 2016), showed a significant decrease in caspase-3 in the RB treated group. Therefore, our results agree with the literature on the apoptosis suppressing effect of RB.

Our study further suggests that the apoptosis that develop during Ang-II treatment associated with p38 MAPK. Besides the fact that p38 is well-known for its role in the cellular stress response and inflammation, p38 MAPK has also been implicated in promoting regulated cell death (Cuenda and Rousseau, 2007). P38, the stress-response protein, a member of MAPK implicated in execution of apoptosis was also up-regulated after cells were treated with Ang-II (Zhou *et al.*, 2014). Ang-II induced increase in p38 MAPK phosphorylation has been identified to be crucial in cellular responses such as inflammatory, apoptosis (Bagchi *et al.*, 2017) also responsible for mediating the development of other CVDs such hypertrophy (Chan *et al.*, 2005). As reported by (Prathapan *et al.*, 2013), our experiments have also showed that Ang II induced apoptosis in H9c2 and the mechanism of action might be mediated by the activation of p38 MAPK and other apoptotic executors. Normally, stimulation of p38 MAPK is executed during its phosphorylated state. For us to confirm whether the anti-apoptotic effects of RB had anything to do with p38 MAPK, we used specific antibody to look at phosphorylated and total p38 MAPK in Ang II- exposed H9c2 cells. We observed a significant decrease in phosphorylation of P38 after co-treatment with RB. For this reason, the anti-apoptotic action of RB may also be dependent on its ability to inhibit increased phosphorylation of p38 MAPK. The mechanism of action of RB's anti-apoptotic effect is assumed to be by blocking ROSdependent p38 signalling pathways (Zhou *et al*., 2014). Subsequent increase of p38 MAPK phosphorylation led to an increase in expression of pro-apoptotic protein; Bax, and caspase-3. However, RB inhibited all the pro-apoptotic proteins and down-regulated Bcl-2 an antiapoptotic protein.

Moreover, increased expression of Bcl-2 or Bcl-xL has been shown to ameliorate stressmediated accumulation of p38 at the mitochondria and thereby prevent apoptosis (Perfettini *et al.*, 2005). On the other hand, ROS is suggested to be another possible link between p38 MAPK signalling and the intrinsic pathway. Mitochondrial ROS may cause DNA damage and protein oxidation, subsequently activating the redox-sensitive kinase p38 MAPK to promote host cell death by apoptosis (Jia *et al.*, 2007).

To gain insight into RB effects and understand some mechanism of action on oxidative stress amelioration in Ang-II-induced hypertrophy and apoptosis in H9c2 cells, we investigated mitochondrial membrane potential comparing Ang-II-treated group and group with or without

RB using JC-1 staining. JC-1 dye in normal cells becomes more concentrated in the mitochondrial matrix by emitting a red fluorescence aggregates due to existence of electrochemical potential gradient. Any alteration in the mitochondrial membrane potential prevents JC-1 from accumulation in the mitochondria, thus, it gets dispersed all over the cell resulting in monomers emitting green fluorescence. Our data shows more JC-1 aggregates (red fluorescence) in healthy control cells revealing intact mitochondria. Ang-II treated cells displayed a depolarized membrane potential which was evident from significantly increased, formation of JC-1 monomers (green fluorescence) in hypertrophied cells. RB maintained the membrane potential, which was associated with increased amount of red fluorescence. The significant increase in mitochondrial membrane potential observed with RB in our Ang-II treated H9c2 cells, was supported by observing increased cell viability. Therefore, we demonstrated the mitochondrial protective effect of RB extract on Ang-II-induced hypertrophy and apoptosis. This suggests that RB treatment can effectively prevent Ang-II-induce mitochondrial damage such as depolarization of mitochondrial membrane potential. Thus, the treatment with RB can prevent loss of mitochondrial structure and functional integrity in the cell culture model.

We also found that the ATP content was significantly increased with RB and AngII cotreatment of H9c2 cardiomyoblasts, this was supported by the data obtained for mitochondrial membrane potential studies. These findings suggest that mitochondrial dysfunction was restored by RB, illustrated by maintenance in mitochondrial membrane potential and greater capacity to improve ATP activity. RB-treated cells are thought to maintain their mitochondrial membrane potential through less demand for ATP consumption in their mitochondria. Similarly, an increase in mitochondrial membrane potential was also observed in 3T3-Li preadipocytes after 48 hrs of treatment with green RB extract and fermented RB extract also illustrated by an increase in percentage cellular ATP content. (Hattingh *et al.,* 2019; Pantsi *et al.*, 2011b).

Furthermore, our study demonstrated that cardiac hypertrophy was associated with decreased mitochondrial respiratory chain complex IV and V. The decrease in the expression of complex IV has been reported to have a direct influence on oxygen consumption rate and electron transport chain in vivo (Sohal, 1993; Schwarze et al., 1998). This decrease in complex IV is also most likely to increases the upstream carriers in a decreased state, which in turn increase the speed at which univalent decrease of oxygen and consequently the production of O^{2-}/H_2O_2

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(Ferguson et al., 2005). There are several mechanisms proposed to explain hypertrophy/ apoptosis related complex IV activity. Firstly, adding to the fact that complex IV causes an increase in generation of ROS, the observed decrease in expression of complex IV could also be directly subsequent to increased ROS production (Ferguson et al., 2005). Mitochondria are both sources and targets of ROS, and production of H_2O_2 by the mitochondria might be increased as a result of cardiac hypertrophy-associated mitochondrial dysfunction.

A second possible explanation for the decreased complex IV expression could be damage in the subunits encoded by mitochondrial DNA. Mitochondrial DNA is known to be the first target of oxidative damage because it is very close to the ETS and could also result from the absence of histone-like protein protective layer (Caron et al., 1979; Clayton, 1982). In addition, there is the possibility of a loss of cytochrome-c because cytochrome-c interact with both complex III and IV. Therefore, loss of cytochrome-c indicate that apoptosis may have played a part (Fosslien, 2003). However, our results showed no significant difference in mitochondrial complex II and III after incubation with RB as compared to Ang-II-treated group. In addition, Pereira *et al.*, 2019, also observed similar results by failing to show significant difference in mitochondrial complex III after incubation with different concentrations of mitogen-activated protein kinase MEK inhibitors. However, in another study using a rabbit model of neonatal pressure overload induced hypertrophy, they observed a decline in mitochondrial complex I and III activities during compensated hypertrophy. In addition, this was accompanied by a decrease in the mitochondria ROS and oxidative damage that only manifested itself after progression to heart failure (Griffiths *et al.*, 2010). RB improved mitochondrial function by restoring complex IV expression after it was negatively affected by Ang-II. Previous studies suggest that ROS production at complex II is believed to be low at physiological concentrations of succinate. Therefore, this supports the notion that complex II is not the main source of ROS involved in hypertrophy and apoptosis (Stoll et al., 2018; Zorov et al., 2014). We also observed no significant difference between Ang-II-treated and RB treated groups.

The decrease in the complex V expression may lead to a decrease in the ability of the heart to meet energy demands. The demand of energy being weighed down by energy supply is then followed by a moderate but continuous decrease in the activity of mitochondrial respiration, subsequently diminishes the rate at which the ATP is generated (Jonckheere et al., 2012; MedlinePlus, 2017). Our results agree with this as we observed a decrease in the ATP activity

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in Ang-II treated group. However, to compensate for the loss of ATP during this abnormal enlargement of the cardiomyoblasts, the mitochondria have to increase the production of ATP and RB seemed to significantly increases ATP content as needed (Stoll et al., 2016). The increase in complex V after treatment with RB in hypertrophic cardiomyoblasts, could mean that RB increased the capacity of the mitochondrial oxidative phosphorylation of the cells during hypertrophy. However, we did not measure mitochondrial respiratory capacity or oxidative phosphorylation in this study.

Chapter 5: Conclusions

Our cold water fermented RB extract conferred cardioprotection against the toxic effects of Ang-II treated H9c2 cells. RB achieved this effect by reducing hypertrophy and apoptosis, improving mitochondrial membrane potential, restoring expression of complex IV and V, and enhancing ATP activity. These effects of RB were further supported by inhibiting the phosphorylation of hypertrophy signalling pathways including GSK-3ß, ERK1/2, PKB/Akt, Calcineurin and mTOR, while reducing expression of Bax, cleaved caspase-3, and p38 and increasing Bcl-2. Our study is the first to demonstrate RB-induced cardioprotection in a model of both hypertrophy and apoptosis induced by Ang-II with clear underlying mechanisms.
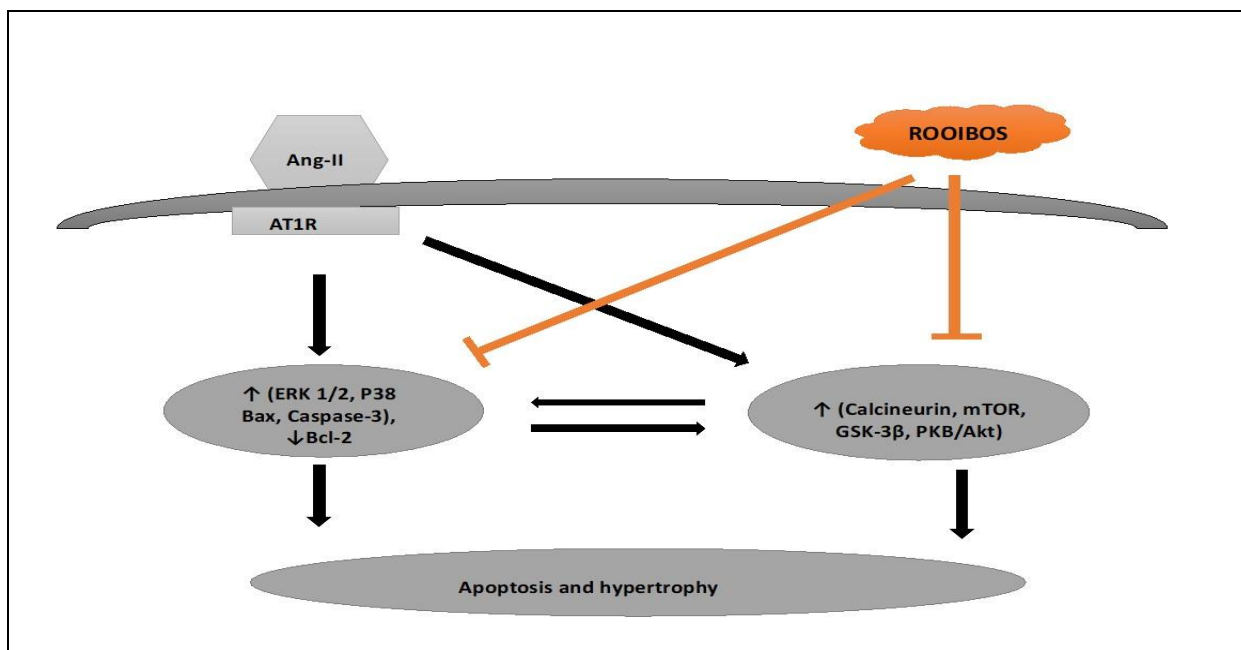

Figure 43: Summary of Anti-hypertrophic and anti-apoptotic effect of RB. *This figure shows a summary of signalling pathways RB used to inhibit Ang-II induced-cardiac hypertrophy and apoptosis.*

6.0 Novelty and significance

We established a new model of both hypertrophy and apoptosis induced by Ang-II in H9c2 cardiomyoblasts.

RB is widely used against heart diseases in the traditional system of medicine. However, its cardioprotective properties related to hypertrophy have not been evaluated. Therefore, our study is the first to give evidence of the cardioprotective effect of RB in a model of Ang-II induced hypertrophy.

7.0 Limitations

Our experiments had some limitations. In this study we could not detect complex I, because the band was too faint to analyse. Moreover, during fractionation we could not get enough proteins from the nucleus which made it impossible for us to observe translocation of NFAT from the cytosol to the nucleus during hypertrophy. In our attempt to yield more protein in the nuclear fraction we added different concentrations of SDS and NP-40. However, probing with actin and histone for cytosol and nucleus, respectively, we were unable to obtain a pure nuclear sample.

For our aconitase measurement the assay provided inconsistent readings i.e. extreme differences among the biological repeats, which made it difficult to determine aconitase activity. Therefore, the data of the aconitase assay could not be used as an indication of mitochondrial stress. This method was a kinetic assay that was time dependent and more optimisation is needed. We also measured whole cell lysates and therefore, the mitochondria concentration might be very low. Thus, in the future we plan to use a mitochondrial isolation kit to directly determine mitochondrial aconitase activity.

8.0 Future directions

We will be looking at mitochondrial function with high-resolution respirometer (Oroboros®), as this will allow us to comprehensively assess mitochondrial respiratory pathways and the effects of RB on them, to confer cardioprotection. This is important, as mitochondrial respiratory pathways are central to the development of cardiac hypertrophy and apoptosis. We would also like to confirm hypertrophy with other hypertrophic markers such as NFAT translocation (from cytosol to nucleus) or GATA4 expression and to further look at mRNA levels of hypertrophic genes like BNP, ANP and ßMHC. We are also planning measure SOD and CAT, the two scavenging enzymes and MitoSox staining a novel fluorogenic dye that is specifically used to target the mitochondrial superoxide in live cells. We will also measure NOX activity to see generation and involvement of mitochondrial ROS. We will measure NOX using total ROS detection kit (Enzo, US) following the manufacture's protocol. The quantitative analysis of intracellular ROS production will be done using cytometry (Sun *et al.*, 2012).

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10.0 Appendix

Herewith the western blot results of each antibody used in 3 biological repeats. On the left side is the chemiluminescence blot and corresponding the membrane on the right (to account for the protein loading).

Phosphorylated GSK-3ß blots
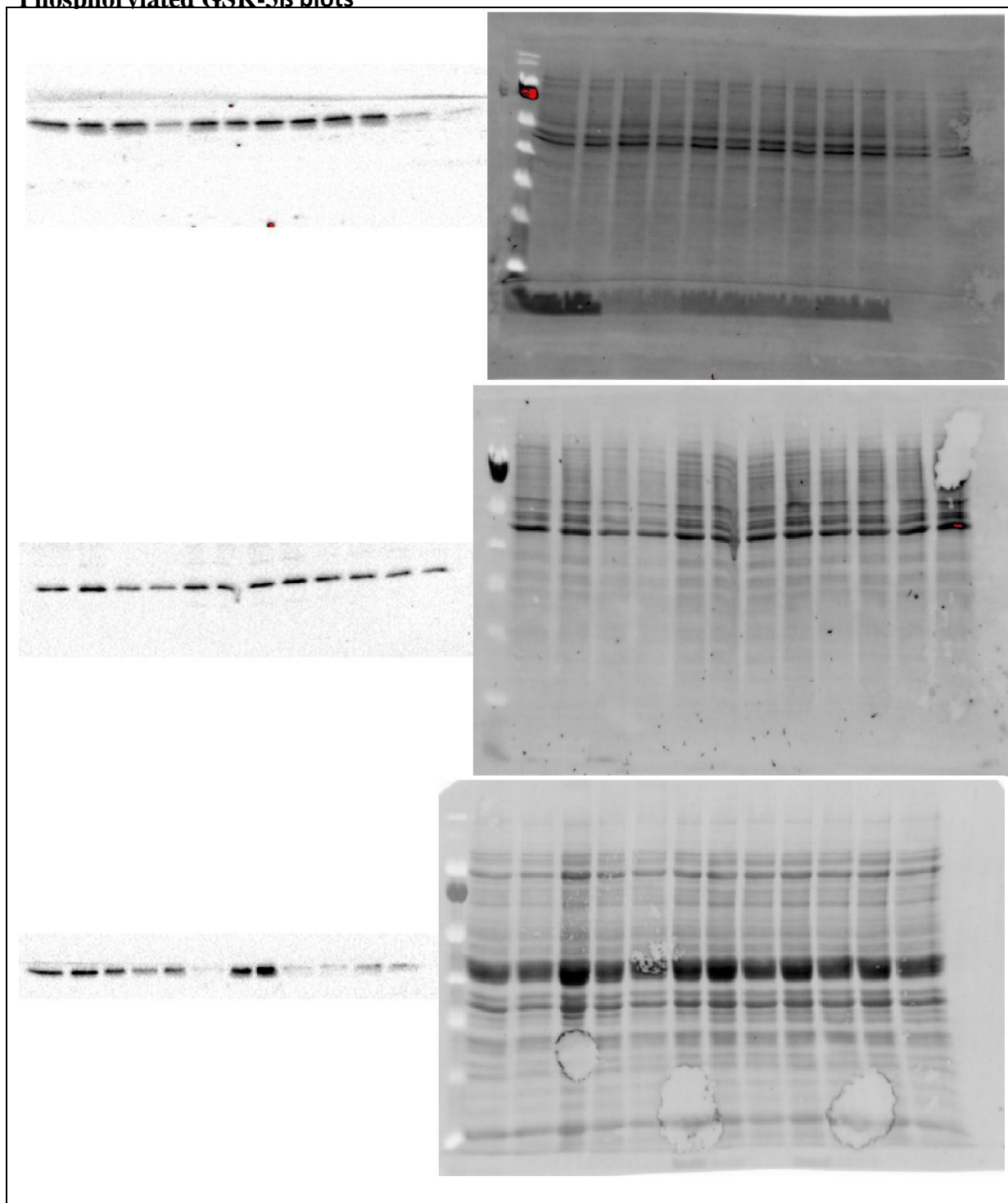
Total GSK Blots
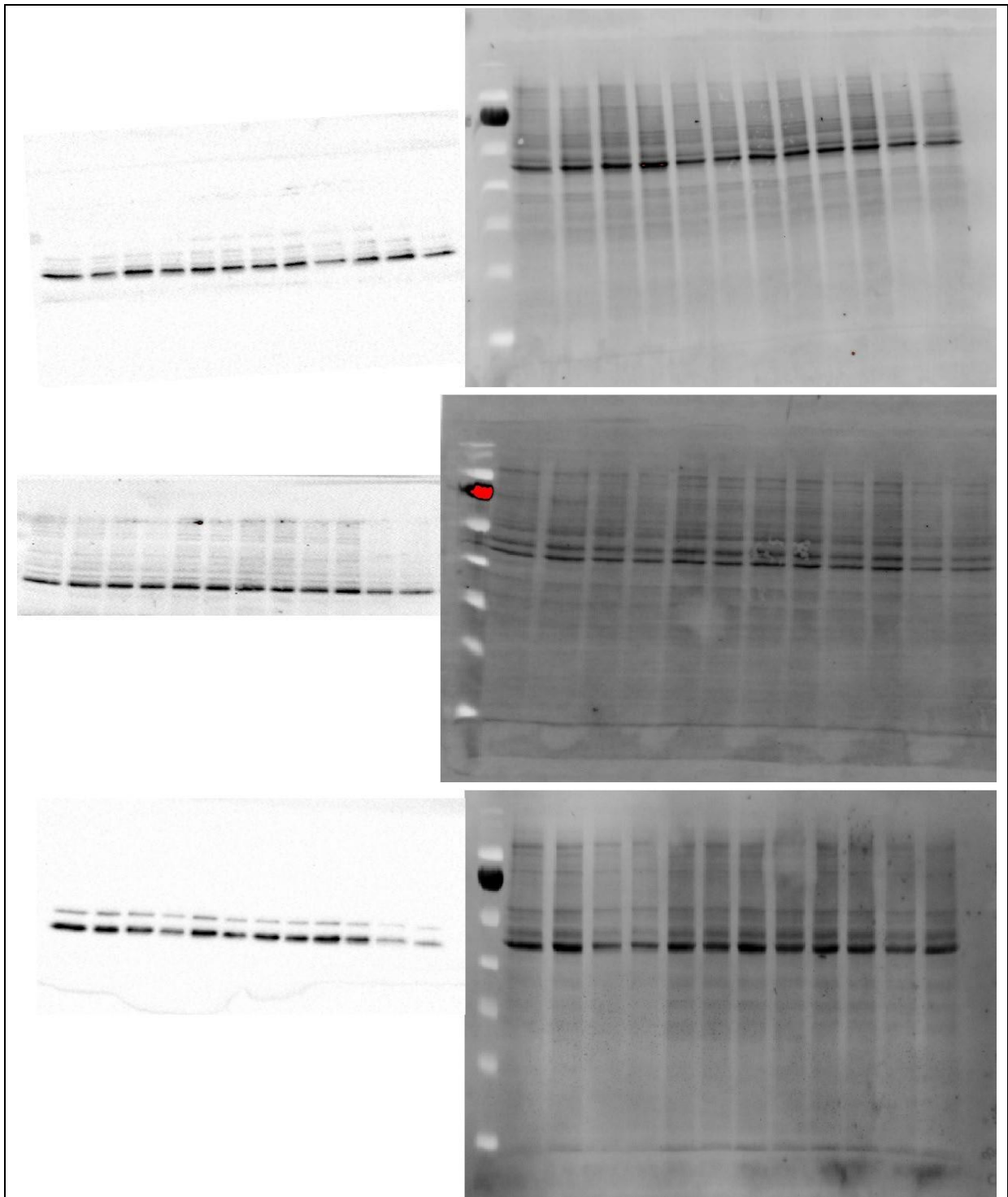
Phosphorylated PKB blots
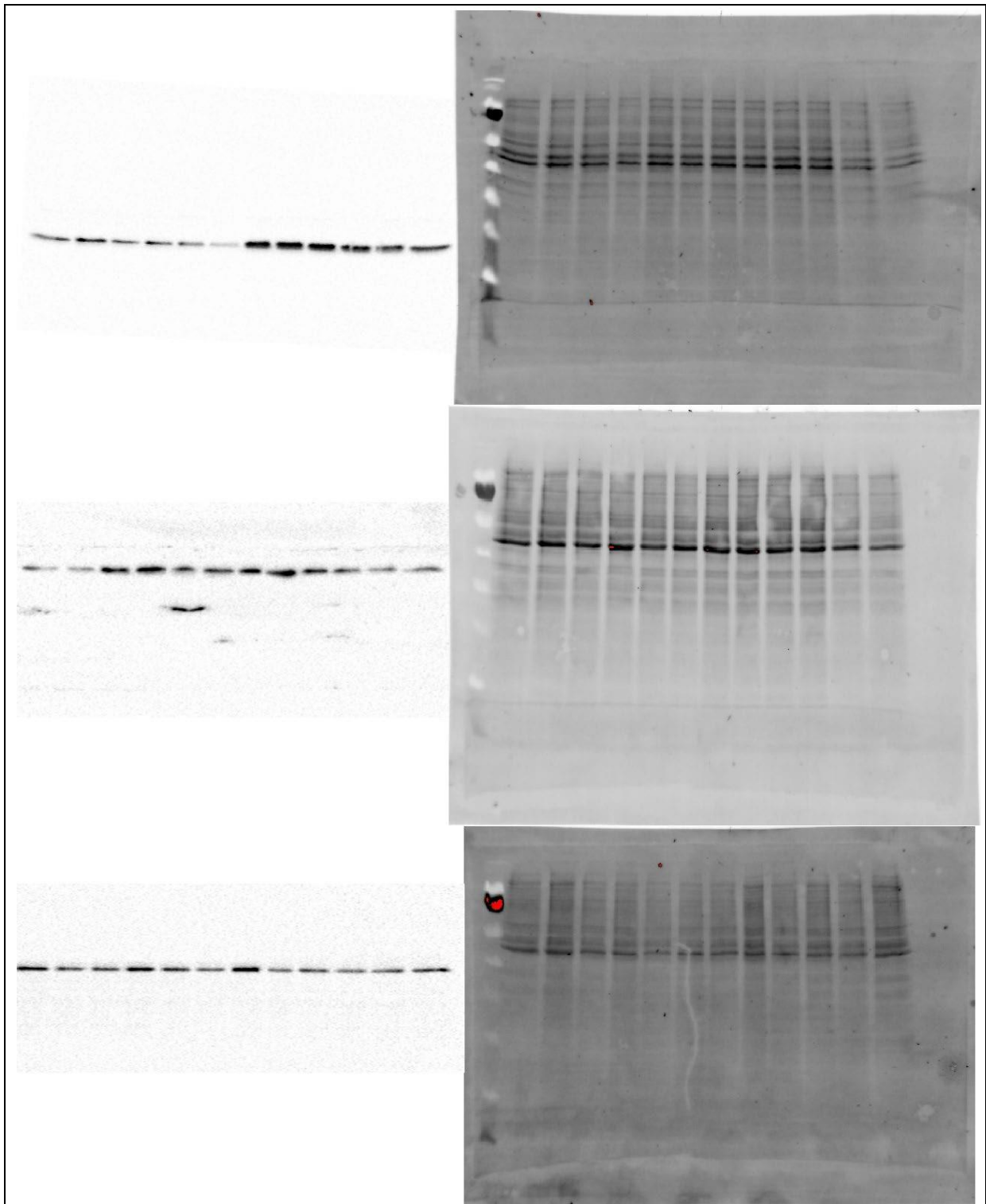
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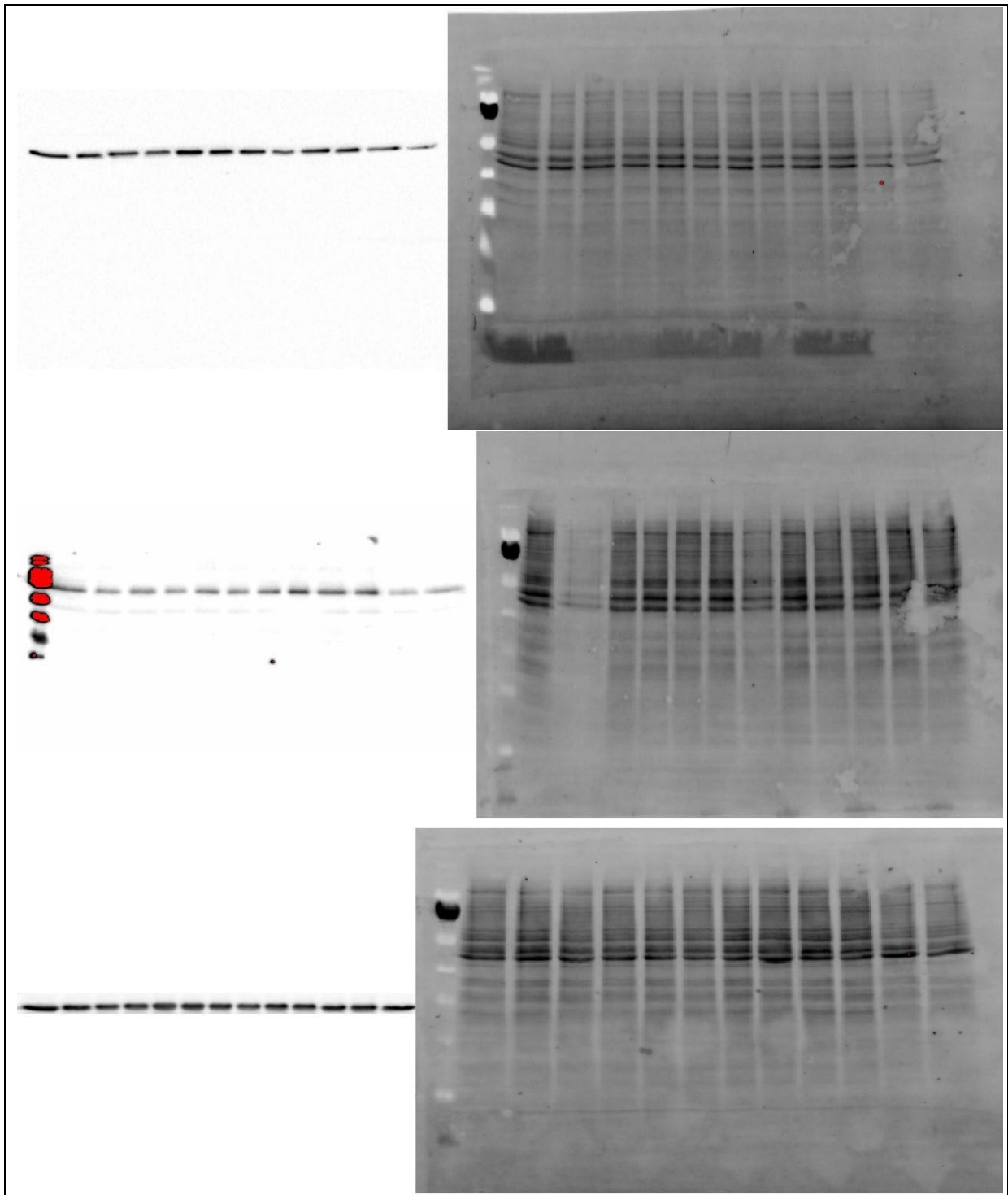
Phosphorylated ERK 1/2
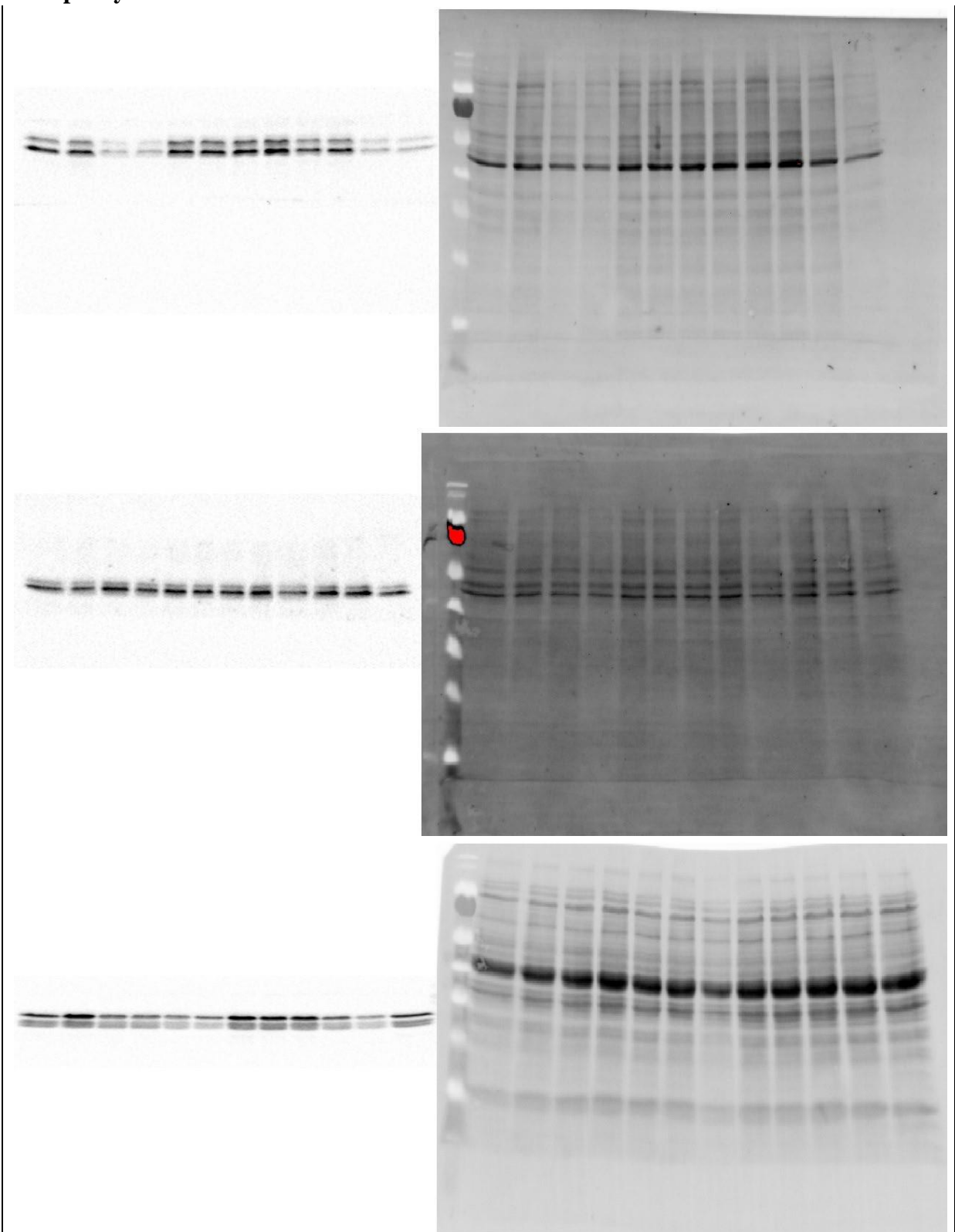
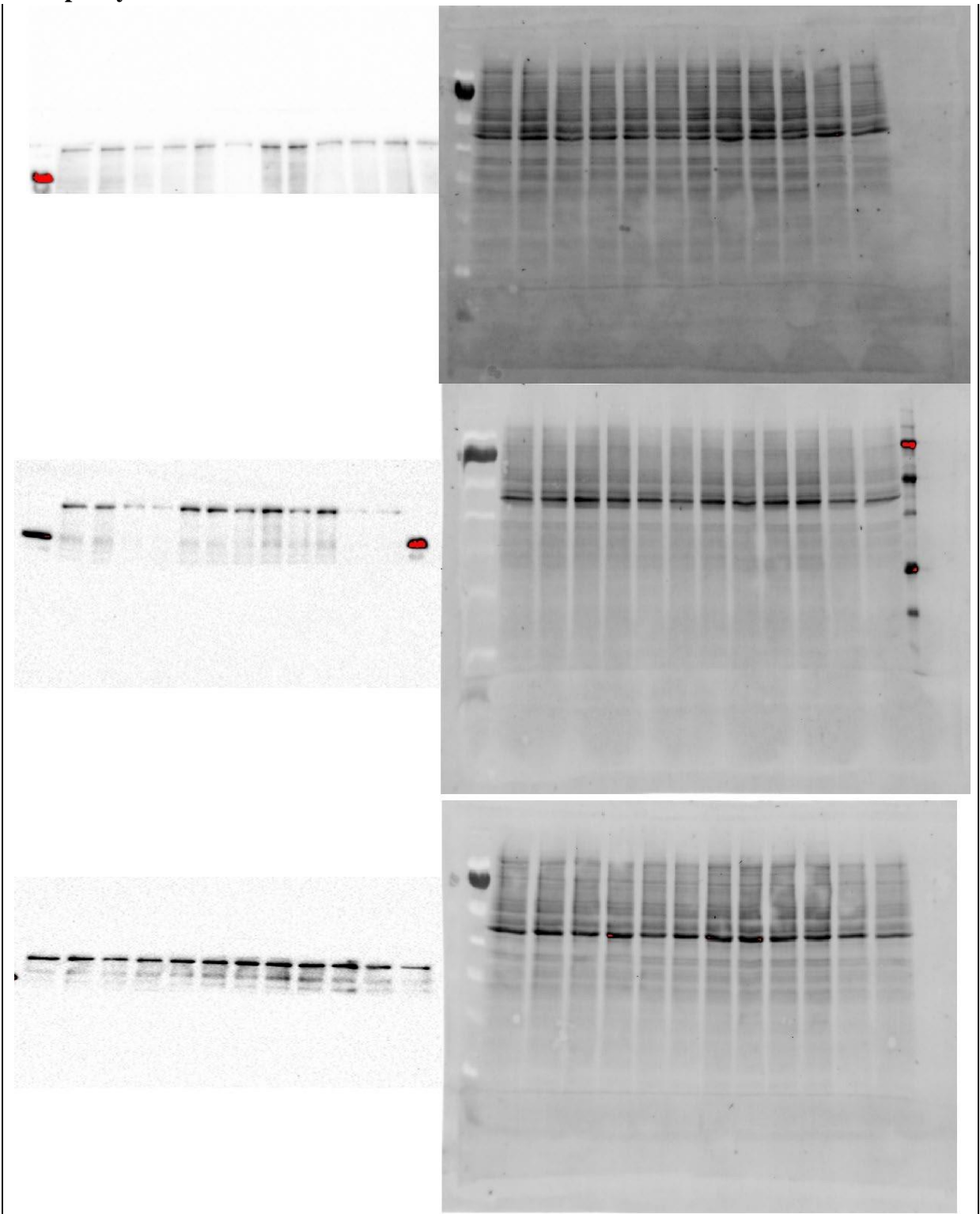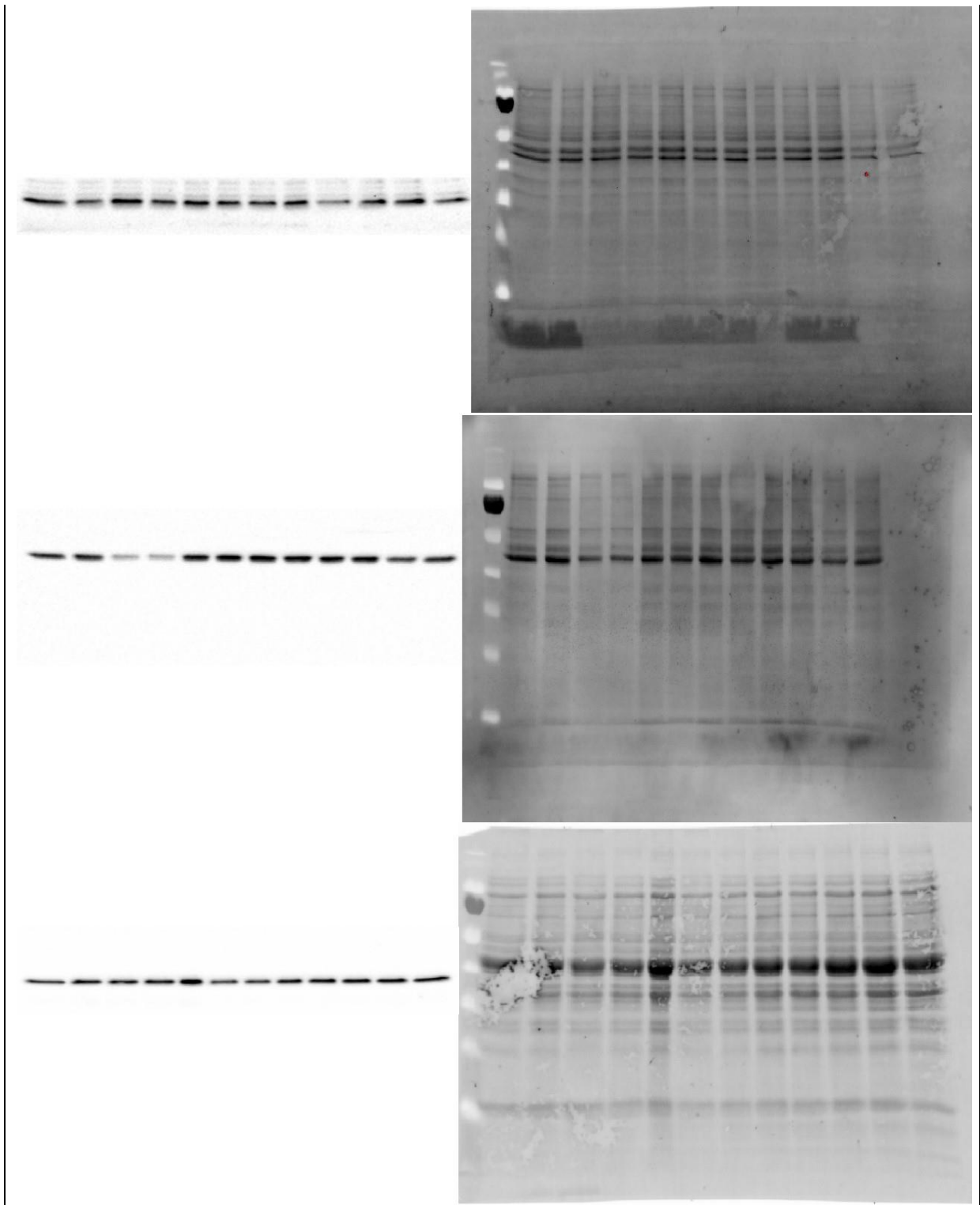**Total ERK 1/2**
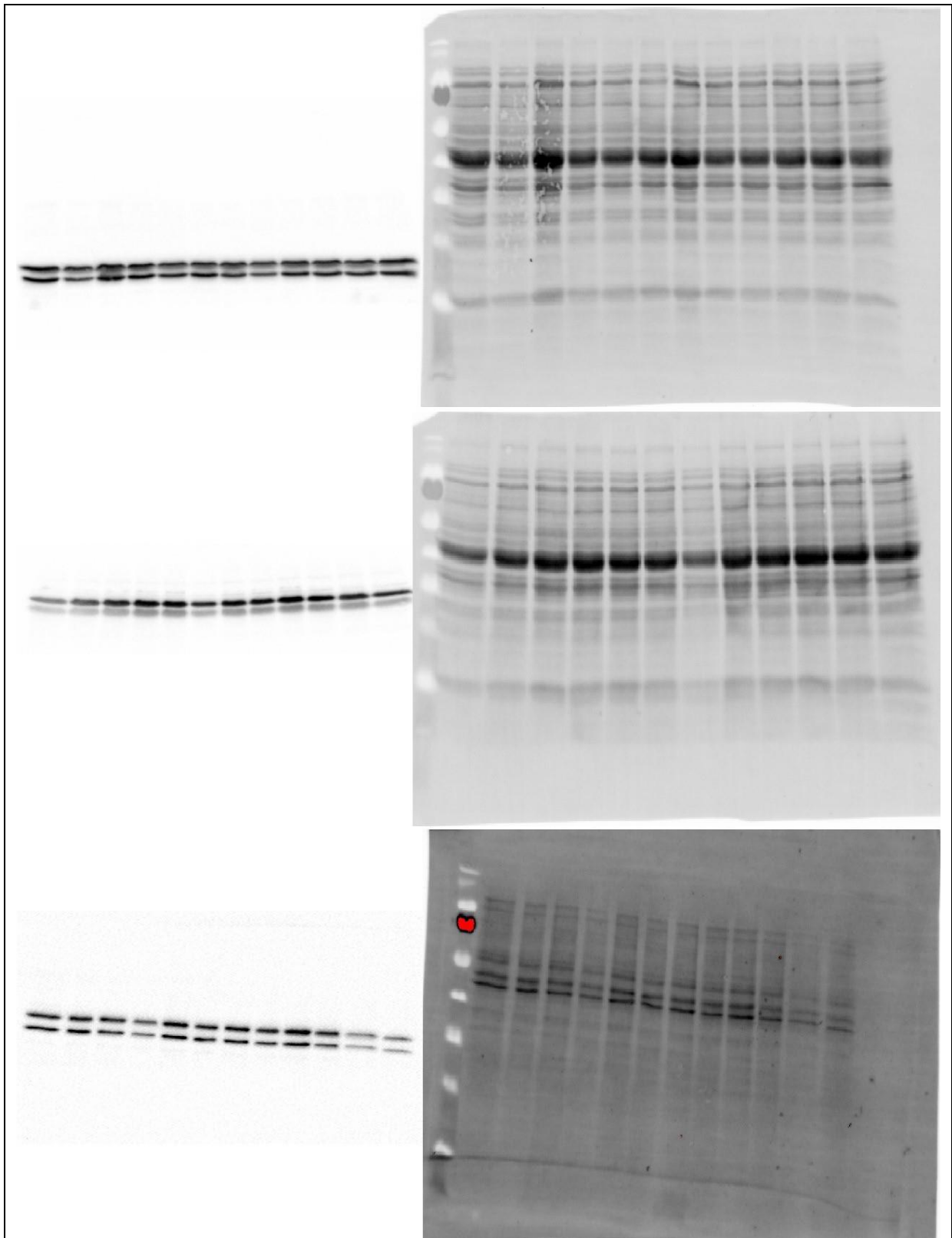
Calcineurin
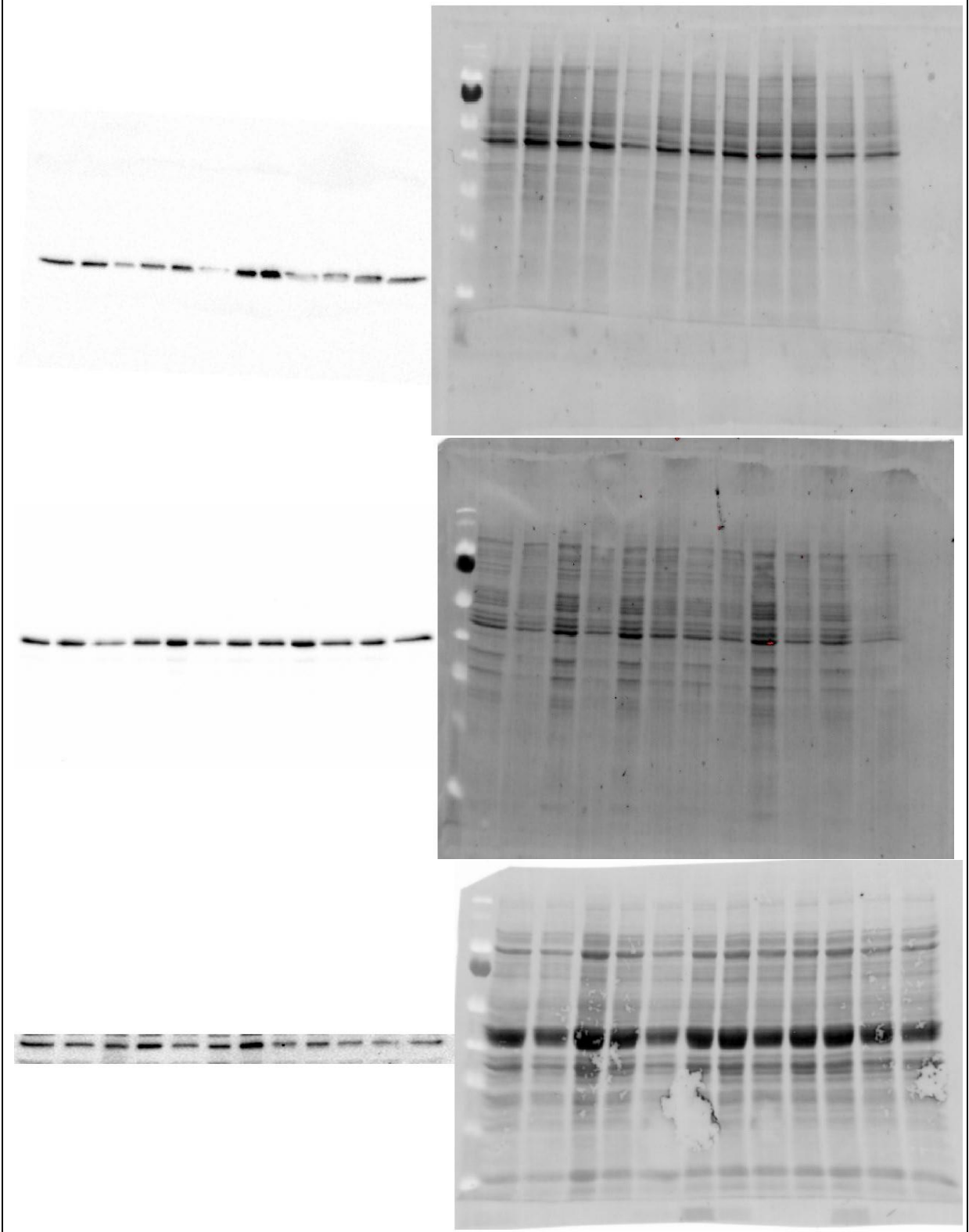
Phosphorylated mTOR

Total mTOR

Apoptosis blots

Bax blots
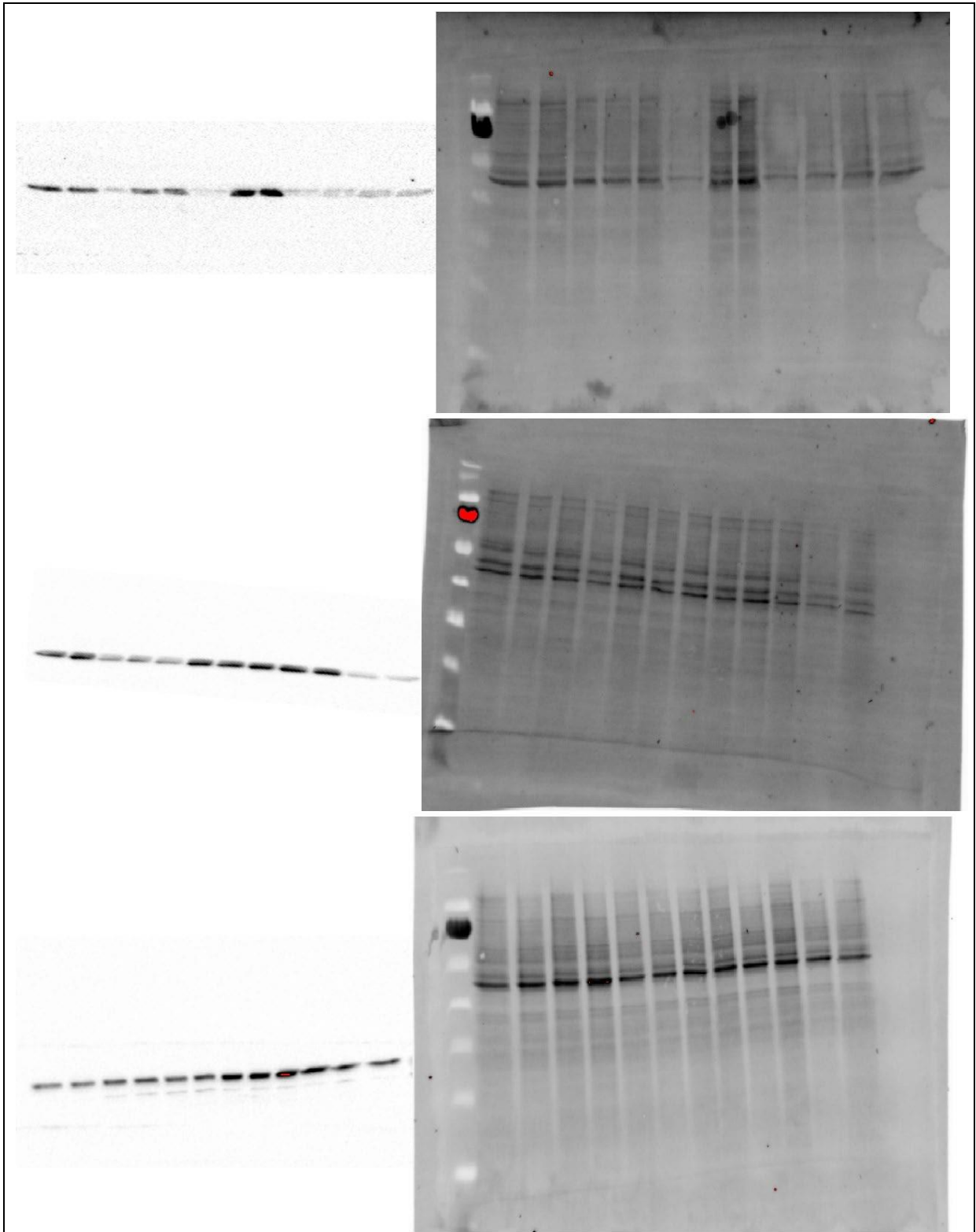
Bcl-2 Blots
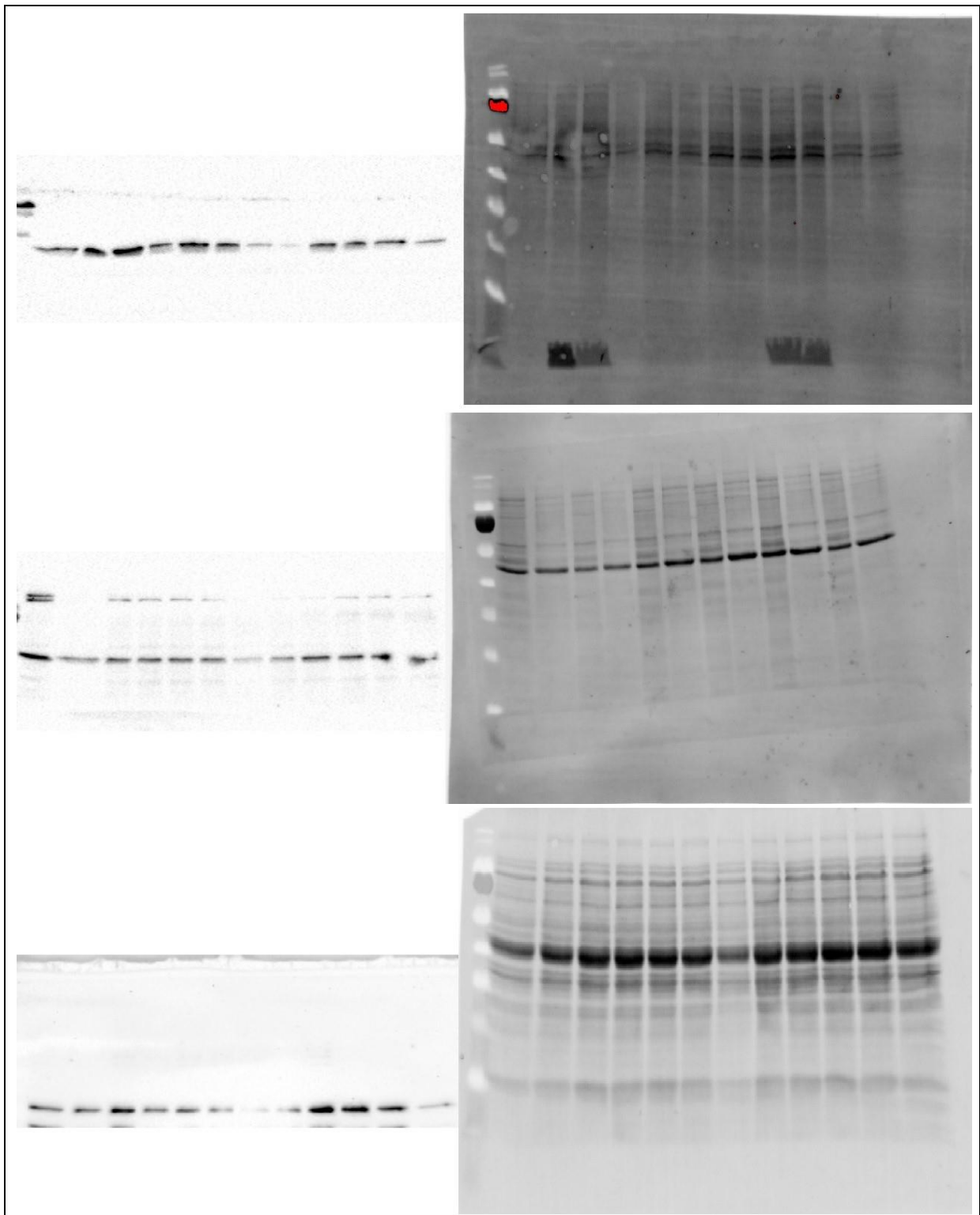
Cleaved caspase 3
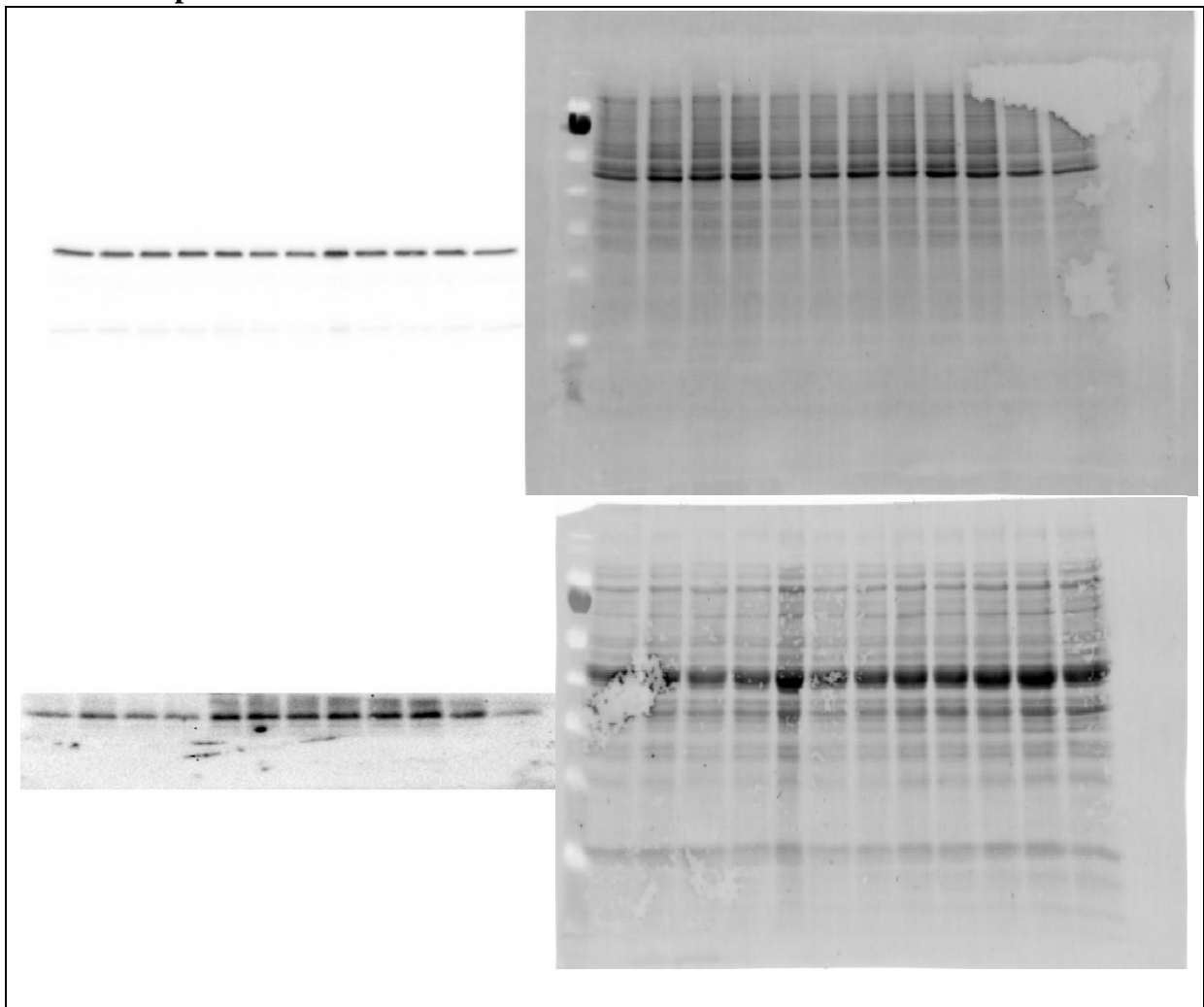
Phosphorylated p38
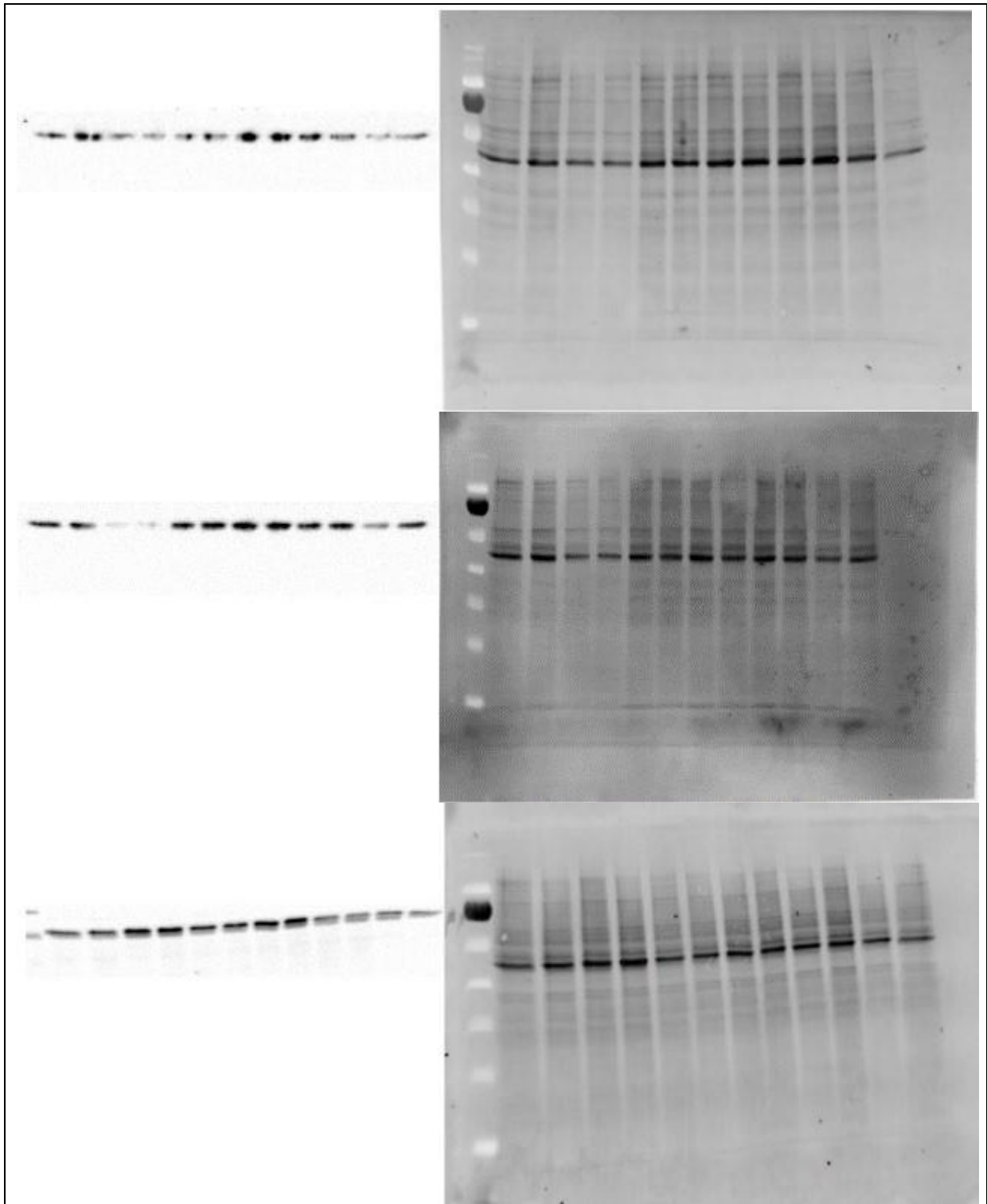
Total p38
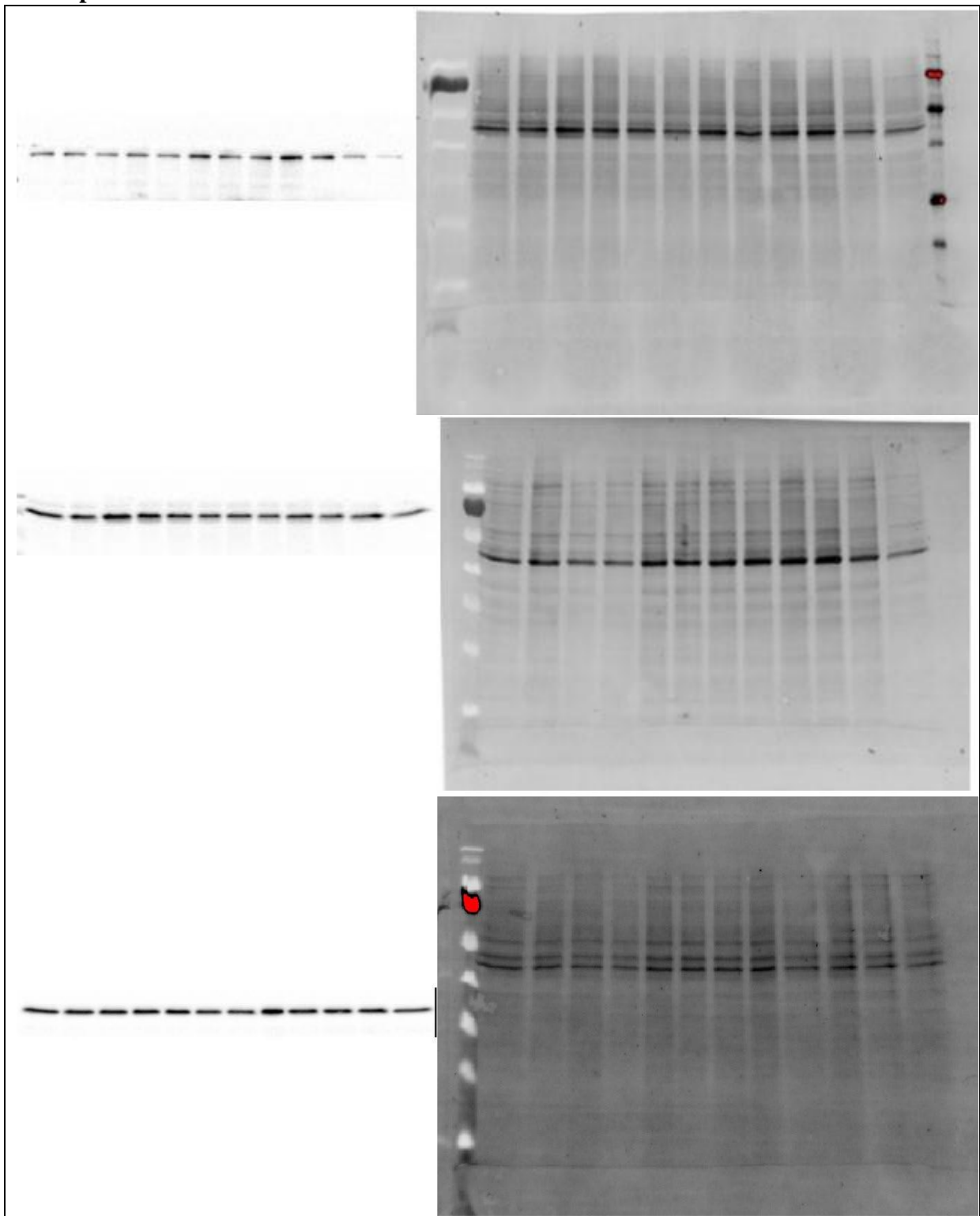
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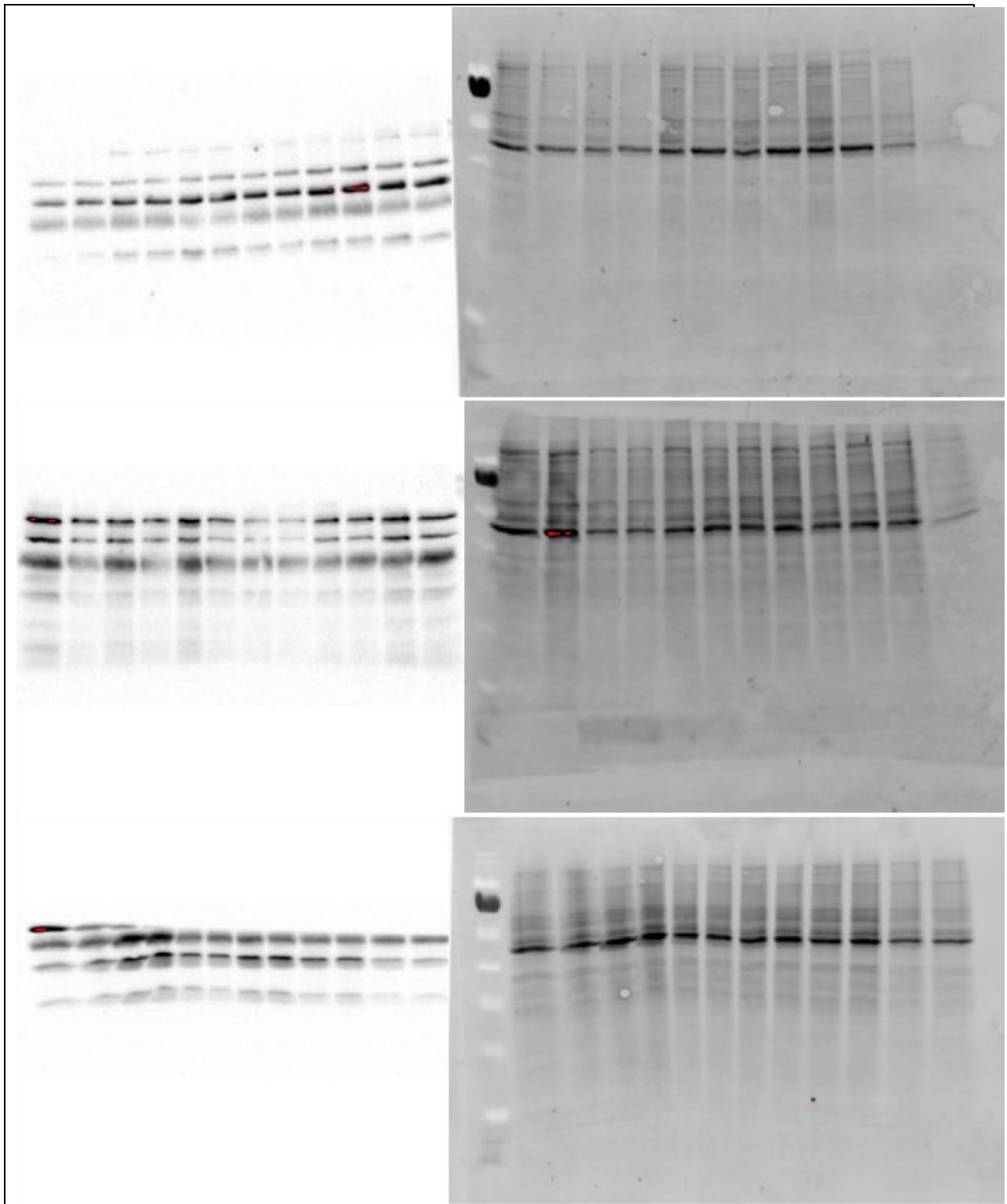
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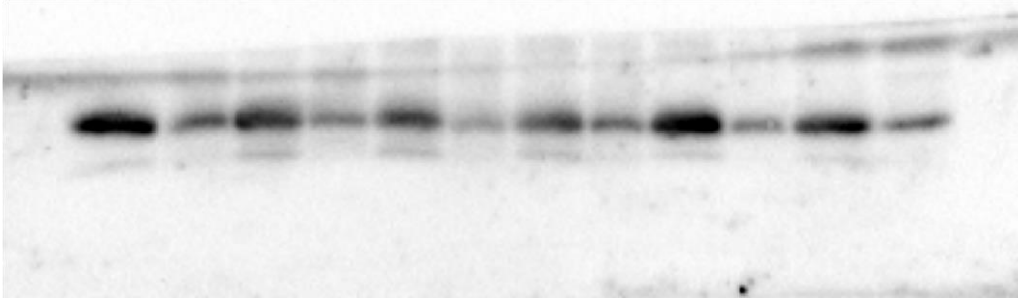
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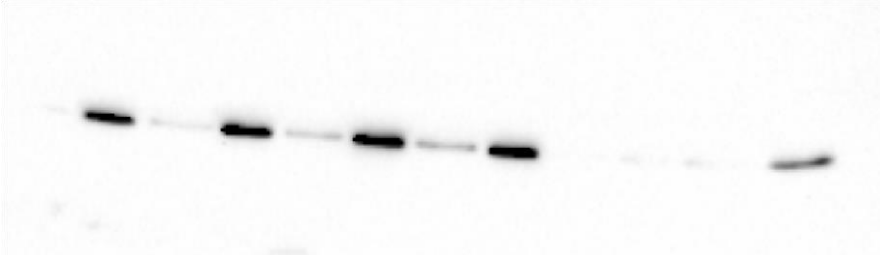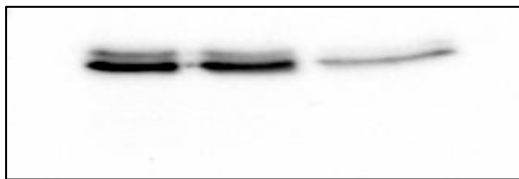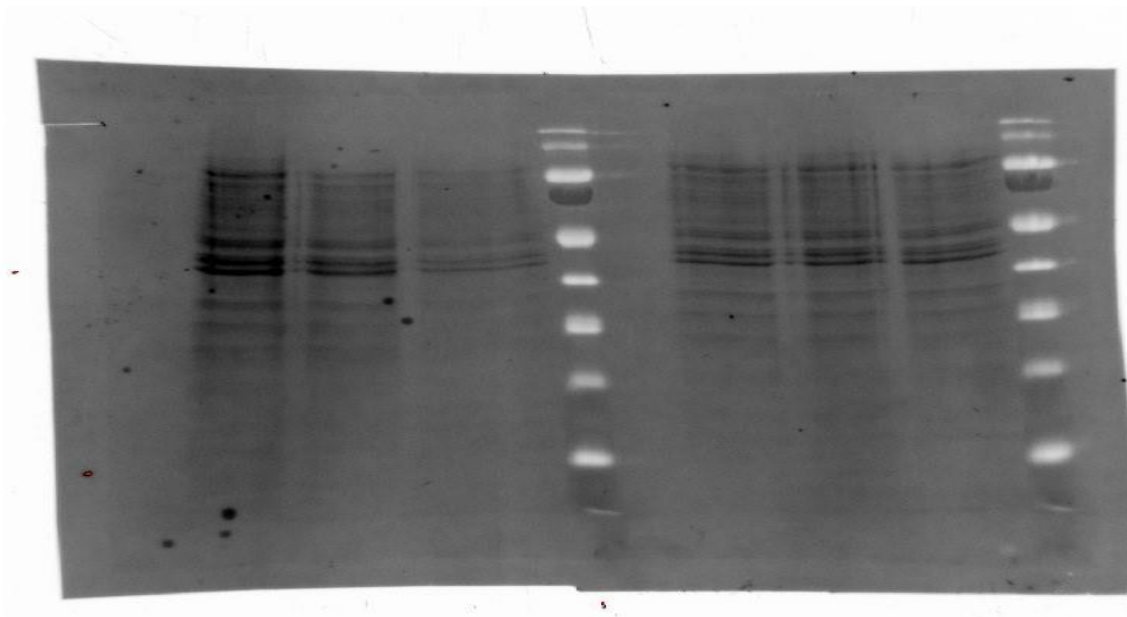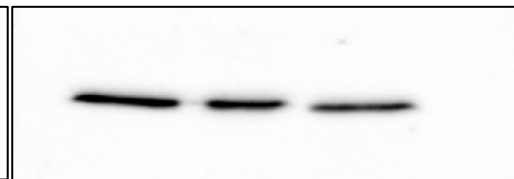

MODEL OPTIMIZATION of ERK, BAX and P38:

Phospho-ERK   Bax

Phospho-P38