

**POTENTIAL CARDIOPROTECTIVE EFFECT OF CHRONIC, MODERATE
CONSUMPTION OF REGULAR AND REDUCED-ALCOHOL WINE IN A RAT
MODEL OF PULMONARY ARTERIAL HYPERTENSION**



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DIABA-NUHOHO PATRICK

DEDICATION

Dedicated to all who never give up in the pursuit of their dreams.

To God be the glory, awesome thing he has done. “Blessed be the God and Father of our Lord Jesus Christ, who hath blessed us with all spiritual blessings in heavenly places in Christ: According as he hath chosen us in him before the foundation of the world, that we should be holy and without blame before him in love: having predestined us unto the adoption of children by Jesus Christ to himself, according to the good pleasure of his will, to the praise of the glory of his grace, wherein he hath made us accepted in the beloved” *Ephesians 1: 3-6 KJV*.

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ABSTRACT

Background: Pulmonary arterial hypertension (PAH) is a severe disease which leads to right ventricular (RV) dysfunction and possibly death. The pathophysiological process of PAH remains unclear but oxidative stress is thought to contribute to arterial and ventricular dysfunction. Red wine has powerful antioxidant properties and is cardioprotective. However, side effects of alcohol may limit the use of wine as a therapeutic agent. The aim of this study was to test whether reduced-alcohol red wine (RARW) or regular red wine (RW) consumption would limit monocrotaline (MCT)-induced PAH in rats.

Methods: Long Evans male rats (150-175g) were randomly assigned into 6 groups: (1) Control: no subcutaneous injection of MCT; (2) MCT; (3) RARW; (4) MCT-Treated with RARW: 5.5% alcohol red wine (5) RW and (6) MCT-Treated with RW: 15% alcohol red wine. The wines were diluted with water (1:7), to supply an equivalent of 2-3 glasses daily consumed in humans ad libitum for 7 days before MCT treatment and for 28 days after MCT treatment with 80mg/kg. The stability of the wine was determined for 4 weeks by analysing ORAC values, total phenolic concentration, anthocyanin and catechin concentrations. Prior to randomisation, and at day 28, echocardiography (VEVO 2100, Visualsonics Inc.) was performed to evaluate pulmonary artery acceleration time (PAAT), an accurate estimate of pulmonary artery pressure, PAAT/Ejection fraction and RV thickness as a marker of RV hypertrophy. Oxidative stress was evaluated by measuring lipid peroxidation markers (conjugated dienes (CD), thiobarbituric acid reactive substances (TBARS)) and antioxidant measures of oxygen radical absorbance capacity (ORAC), superoxide dismutase (SOD) and catalase (CAT) activities in blood plasma were analysed.

Results: Baseline echocardiography showed similar cardiac function amongst all groups. MCT injection induced right ventricular hypertrophy compared to controls (1.22 ± 0.01 mm and 0.52 ± 0.04 mm; $p < 0.0001$). A decrease in PAAT was observed in the MCT group compared to controls (13.95 ± 0.95 vs 23.43 ± 1.64 ms, $p < 0.001$). However, MCT treatment with RARW ameliorated the trend in the MCT group (18.93 ± 1.80 vs 13.95 ± 0.95 ms, $p = 0.02$). Similarly, PAAT/Ejection fraction in the MCT group was reduced compared to the control group (0.18 ± 0.02 ms and 0.32 ± 0.18 ms; $p < 0.001$). Chronic moderate treatment with RARW in PAH animals improved hypertrophy and PAAT/Ejection fraction (0.85 ± 0.07 mm; $p < 0.001$ and 0.25 ± 0.30 ms, respectively; $p = 0.02$ versus the MCT group). Oxidative stress markers showed an increase in CD amongst animals with MCT compared to controls (671.60 ± 42.53 nmol/L and

453.10 $\pm$ 34.76 nmol/L; p=0.004). Chronic moderate treatment with RARW reduced lipid peroxidation (CD: 471.60 $\pm$ 27.45 nmol/L; p=0.004 versus the MCT group). Plasma TBARS, ORAC, SOD and CAT were not significantly affected by the condition or the treatment. The RARW had a consistently higher antioxidant status than the RW for the duration of the study. The mean concentration of RARW to the RW after 4 weeks in the total phenol was (291.90 $\pm$ 10.42 vs 235.80 $\pm$ 9.22 mg/L, p=0.006), that of the anthocyanins was (190.00 $\pm$ 3.53 vs 172.20 $\pm$ 5.13 mg/L M-3-G, p=0.0008), that of catechin was (12.06 $\pm$ 0.31 vs 10.26 $\pm$ 0.19 mg/L, p=0.157), and that of ORAC was (32.55 $\pm$ 2.75 vs 26.55 $\pm$ 2.37 nmol/L trolox equivalents, p=0.983).

Conclusion: This study suggests that a moderate and chronic treatment with RARW but not RW attenuates MCT-induced PAH in rats, an effect which may be mediated, at least in part, by reduction of lipid peroxidation. The use of RARW could be tested in a randomised controlled trial and may be more beneficial than RW. This simple, inexpensive treatment might represent a new therapeutic option for PAH

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ABBREVIATIONS

AAPH	2, 2'-azobis (-2-methyl-propionamidine) dihydrochloride
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AUC	Area under the curve
CD	Conjugated diene(s)
CHD	Coronary heart disease
CuSO₄	Copper II sulphate
CVD	Cardiovascular disease
DAOS	N-ethyl-N-3, 5 dimethoxy aniline sodium salt
dH₂O	De-ionised water
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine-tetra-acetic acid
eNOS	Endothelial nitric oxide synthase
GK	Glycerol kinase
GPO	Glycerol-3-phosphate kinase
HDL	High density lipoprotein
HDL-C	High density lipoprotein cholesterol
HIV	Human immunodeficiency virus
HIV/AIDs	Human immunodeficiency virus/Acquired immunodeficiency syndrome
LDL	Low density lipoprotein
LDLox	Oxidised low density lipoprotein
LV	Left ventricle
MCT	Monocrotaline
MDA	Malondialdehyde
mmHg	millimetres of mercury

MRI	Magnetic resonance imaging
MW	Molecular weight
M-3-G	Malvidin-3-glucoside
NADPH	Nicotinamide adenine dinucleotide phosphate
NCD	Non-communicable disease
NO	Nitric oxide
ORAC	Oxygen radical absorbance capacity
PAAT	Pulmonary artery acceleration time
PAH	Pulmonary arterial hypertension
PA	Pulmonary artery
PAP	Pulmonary arterial pressure
PBS	Phosphate buffered saline
PH	Pulmonary hypertension
PL	Phospholipid
POD	Peroxidase
RARW	Reduced-alcohol red wine
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RS	Reactive species
RV	Right ventricle
RVH	Right ventricular hypertrophy
RVDP	Right ventricle developed pressure
RW	Regular red wine
SCC	Spinning cone column
SOD	Superoxide dismutase
TAPSE	Tricuspid annular plane systolic excursion
TBA	Thiobarbituric acid

TBARS	Thiobarbituric acid reactive substance(s)
TC	Total cholesterol
TG	Triglyceride
UV	Ultraviolet
VLDL	Very low density lipoproteins
XOD	Xanthine oxidase
WHO	World health organisation

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

Pulmonary arterial hypertension (PAH) is characterised by a rise in pressure in the pulmonary artery due to increased resistance to which the right ventricle (RV) responds with pulmonary vascular resistance (Rose-Jones & V Mclaughlin, 2015). It is associated with a complex range of conditions and diseases such as chronic lung disease (chronic obstructive pulmonary disease, collagen vascular disease), infections (Human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS), schistosomiasis) and heart disease. All PAH-related diseases share common pathological features characterised by vasoconstriction, inflammation, pulmonary vascular remodelling, thrombosis *in situ* and hypertrophy (Xie et al., 2015). There is no cure for PAH and medical treatments are limited due, in part, to the lack of knowledge on the pathophysiology of the disease (Xie et al., 2015). In animal studies, for example in rats and mice, models have been established to mimic the disease such as the chronic hypoxia exposure model and the monocrotaline (MCT) lung injury model which have been extensively studied (Stenmark, Meyrick, Galie, Mooi, & McMurtry, 2009).

The MCT model is advantageous as it is reproducible, inexpensive and remains frequently used as an established model for PAH (Gomez-Arroyo et al., 2012). Monocrotaline is obtained from the seeds of the *Crotalaria spectabilis* plant and induces severe pulmonary injury as a single injection after hepatic generation of the MCT pyrrole (Maioli et al., 2011). MCT induces PAH because injury to small arteries leads to constriction and increased resistance in response to which higher pulmonary arterial pressure (PAP) is required for perfusion. This is achieved by right ventricular hypertrophy (RVH). Significant changes induced by this model include vascular remodelling, proliferation of pulmonary arterial smooth muscle cells, oxidative stress, endothelial dysfunction, inflammation and leukocyte infiltration (Csiszar et al., 2009). These changes may involve reactive oxygen species (ROS) and may subsequently cause damage to the heart muscles.

Moderate, chronic consumption of red wine is considered beneficial to health, with empirical evidence of this in both animal and human studies (Chiva-Blanch et al., 2013; Mihailovic-Stanojevic et al., 2016; Roig, Cascón, Arola, Bladé, & Salvadó, 1999; Schwarz et al., 2017). Of essential importance is its cardioprotective ability that seems to be related to the wine polyphenolic properties rather than the alcohol content (Lamont, Blackhurst, Albertyn, Marais, & Lecour, 2012). Previous research has focused on the individual antioxidant properties of red

wine as a therapeutic approach to many diseases (Labinskyy et al., 2008; Ludke et al., 2010; Morales-Cano et al., 2014). In this study wine as a ‘whole beverage’ is used, mimicking chronic and moderate human consumption: humans consume wine, not individual components of wine. Whether chronic, moderate consumption of wine protects against PAH has never been studied.

1.2 PROBLEM STATEMENT

Pulmonary arterial hypertension, though rare (the prevalence is mostly uncertain; but idiopathic PAH is estimated at 0.012% (Yang, Mardekian, Sanders, Mychaskiw, & Thomas, 2013)) is a global problem affecting patients with (and without) diseases such as schistosomiasis, sickle cell disease, chronic obstructive pulmonary disease, chronic left heart failure and HIV/AIDS. As a disease with rapid evolution, many patients eventually die from cardiac dysfunction. Due to unavailability of an effective treatment against PAH, animal models of PAH are essential in providing new therapeutic strategies, management, treatment and insight. No study has been carried out in relation to PAH with regular and reduced-alcohol red wine.

1.3 HYPOTHESIS

The hypothesis to be tested is that moderate, chronic consumption of regular or reduced-alcohol red wine consumption may offer protection against PAH.

1.4 JUSTIFICATION

It is not known whether moderate, chronic consumption of reduced-alcohol wine protects against PAH in animal studies, although a study using a specific component of wine, melatonin, as a treatment strategy for PAH has postulated the possible effect (Maarman et al., 2015). Reduced-alcohol wine is thought to confer similar cardioprotective benefits to regular wine (Lamont et al., 2012). Resveratrol exerts anti-inflammatory and anti-oxidative effects by reducing and preventing vascular remodelling and pulmonary hypertension (Labinskyy et al., 2008). In addition, resveratrol and melatonin are thought to induce cardioprotection (Lamont, Somers, Lacerda, Opie, & Lecour, 2011; Maarman et al., 2015). Grape juice was found to significantly increase endothelial nitric oxide synthase, decrease right ventricular end diastolic pressure and reduce lipid peroxidation in MCT-induced Cor pulmonale (Ludke et al., 2010). The flavonoid quercetin, a component of red wine, has also been shown to significantly reduce mortality, lower pulmonary arterial pressure (PAP), RVH, reduce vascular remodelling in rats injected with MCT and was found to be useful in the treatment of PAH (Morales-Cano et al.,

2014). Reduced-alcohol red wine may thus be a better alternative beverage, considering some of the adverse effects of alcohol, while still maintaining cardio-protective properties.

1.5 AIM

The aim of the present study is to determine whether regular and moderate consumption of regular and reduced-alcohol wine confers cardioprotection in PAH rats.

1.6 OBJECTIVES OF STUDY

To investigate this aim:

- PAH will be induced in Long Evans male rats by injecting a single dose of monocrotaline (MCT) subcutaneously before a 28-day study period.
- Analysis of oxidative stress markers (thiobarbituric acid reactive substances (TBARS) and conjugated dienes (CD)), endogenous antioxidants catalase and superoxide dismutase), plasma oxygen radical absorbance capacity (ORAC) and lipids (total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), triglycerides (TG), phospholipids (PL)) will be carried out.
- The stability of both regular- and reduced-alcohol red wine will be determined by analysing as the concentrations of total phenolic compounds, catechins and anthocyanins at regular intervals over a one-month period as well as determining the oxygen radical absorbance capacity (ORAC) over the same experimental period.

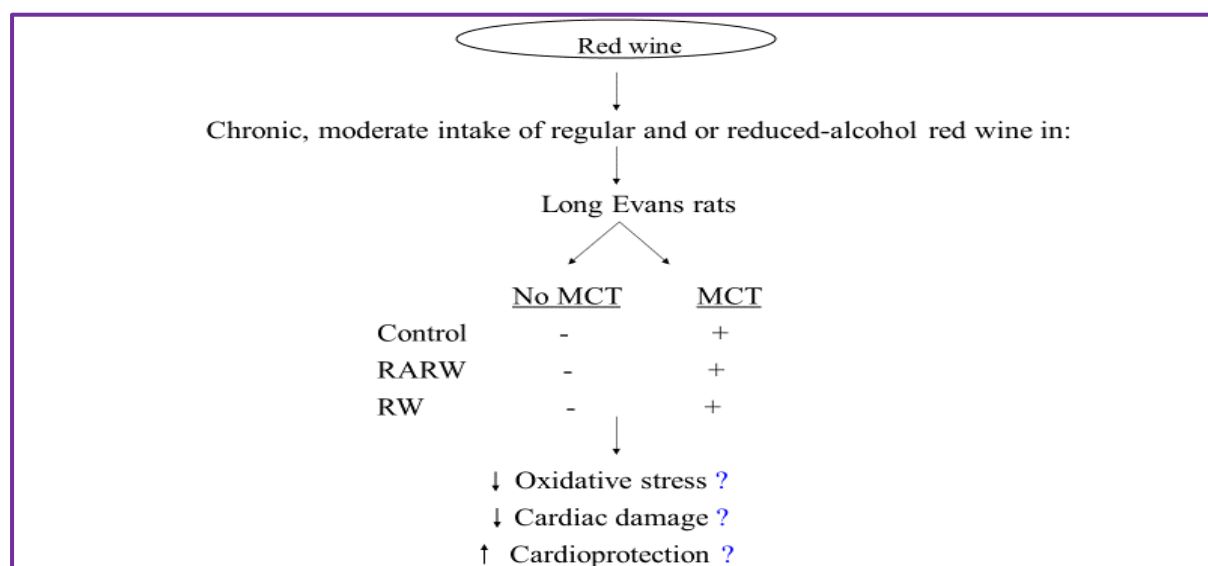


Figure 1: Hypothetical diagram for the experimental study.

Reduced-alcohol red wine (RARW); Regular red wine (RW); Monocrotaline (MCT)

CHAPTER 2: LITERATURE REVIEW

2.1 PREVALENCE OF NON-COMMUNICABLE DISEASE AND CARDIOVASCULAR DISEASE

The term non-communicable disease (NCD) refers to a non-infectious and non-transmissible disease, which develops over time and eventually leads to a chronic state (Kim & Oh, 2013). Diseases of such nature include but are not limited to cardiovascular disease (CVD), hypertension, diabetes, stroke, cancer, kidney disease and Alzheimer's disease. Globally, NCDs cause 68% of deaths and of these deaths, 40% are estimated to have occurred before the age of 70 years (Figure 2a) (WHO, 2014a). Approximately three-quarters of the global NCD deaths (82%) which occurred pre-maturely before the age of 70, happened in low and middle-income countries (WHO, 2014a). In 2012, CVD was ranked the lead cause of NCD deaths followed by cancer, respiratory disease (which includes asthma and chronic obstructive pulmonary disease) and diabetes. Collectively these four major NCDs were responsible for 82% of the deaths in Africa (WHO, 2014a).

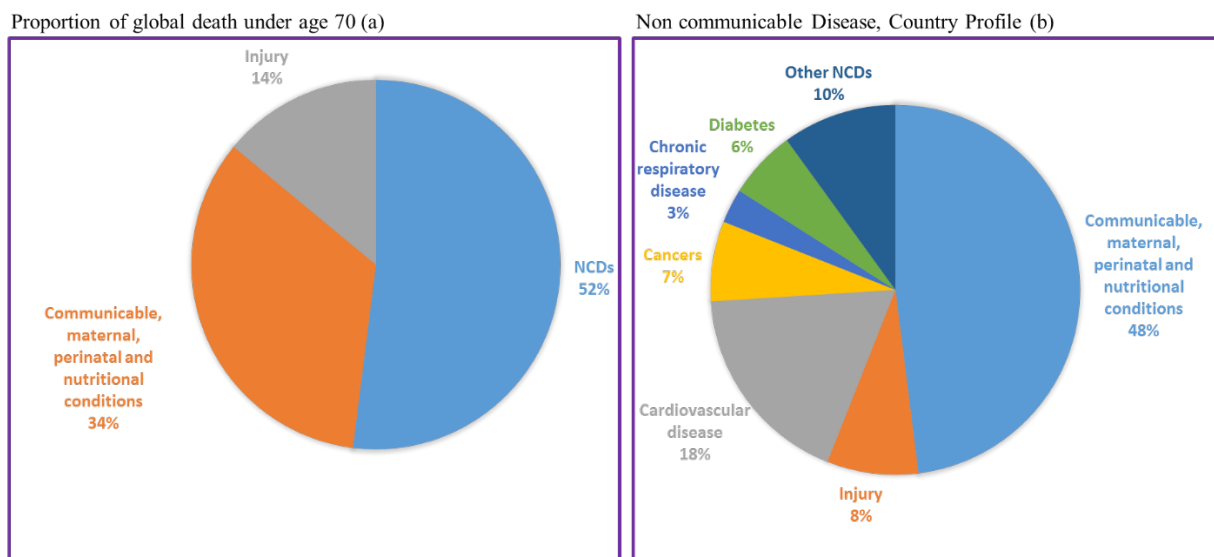


Figure 2: (a) Global cause of death: 2000-2012 (WHO, 2014a); (b) World Health Organization - Noncommunicable Disease (NCD), a chart of different NCDs in South Africa, 2014.

Within the African continent, adverse communicable, maternal, perinatal and nutritional conditions are the main causes of death and they are projected to rise approximately 65% by the year 2030 if certain pragmatic measures are not taken (Murray & Lopez, 1997). In South Africa, NCDs remain a major challenge and as one of the leading causes of deaths accounting for 39% of total death in the country with over 36% of these deaths occurring before age 60

(Nojilana, Bradshaw, Pillay-van Wyk, Msemburi, Laubscher, et al., 2016; Nojilana, Bradshaw, Pillay-van Wyk, Msemburi, Somdyala, et al., 2016). Cardiovascular disease in the sub region is fuelled by the persistent rise in HIV/AIDs, tuberculosis, maternal and perinatal conditions (Figure 2b) as well as behavioural and biologic risk factors (Hoy, Rao, Nhung, Marks, & Hoa, 2013). A double burden caused by alcohol abuse, smoking, unhealthy diet, physical inactivity leading to obesity, hypertension, diabetes and lipid disorders further complicate the trend (UNGA, 2012). Consequently, CVD becomes a major public health concern in a continent marked by limited resources and infrastructure (Sliwa, Acquah, Gersh, & Mocumbi, 2016). With infection and economic issues dominating the African continent, its future may be threatened, with hypertension probably posing one of the largest threats for the future. This research focuses on pulmonary arterial hypertension, a subclass of hypertension in general.

2.2 HYPERTENSION AND PULMONARY ARTERIAL HYPERTENSION

Hypertension is a condition in which there is raised blood pressure referring to the systemic circulation above a systolic pressure of 140mmHg and a diastolic pressure of 90mmHg (WHO, 2014b). The recent 2017 hypertension clinical guidelines by the American Heart Association (AHA) now defines hypertension as having a systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 80 mmHg (Whelton et al., 2017). It is estimated to cause approximately 9.4 million deaths globally, with a prevalence rate of 22% in adults aged 18 years and over (WHO, 2014b). As a major cardiovascular risk factor, causing stroke, myocardial infarction, cardiac failure and other complications, hypertension is associated with increased morbidity and mortality in sub-Saharan Africa (Figure 3A) (Sliwa et al., 2016) and is projected to increase by 60% by 2025 (Kearney et al., 2005).

During the 5th World Symposium on pulmonary hypertension (PH) held in France in 2013, PH was classified into five categories (Figure 3B) (Simonneau et al., 2013). Pulmonary arterial hypertension falls under group 1 as a category of conditions with injury to the pulmonary vessels that increases the pressure in the pulmonary artery resulting in right ventricular hypertrophy and heart failure (Chin, Kim, & Rubin, 2005). In the developed world, PAH occurs from 5 to 15 million adults (Rich and Rich, 2014) and additionally, PAH occurs in approximately 0.5% of HIV patients with poor prognosis. In SA where the burden of HIV is high, many cases of PAH may go undetected and its prevalence may not be different to the advanced countries (Essop, 2010).

The use of drugs and other chemicals such as monocrotaline (MCT) which can induce and mimic PAH in rats, causing right ventricular damage and hypertrophy, has provided valuable insight in understanding this pathologic condition.

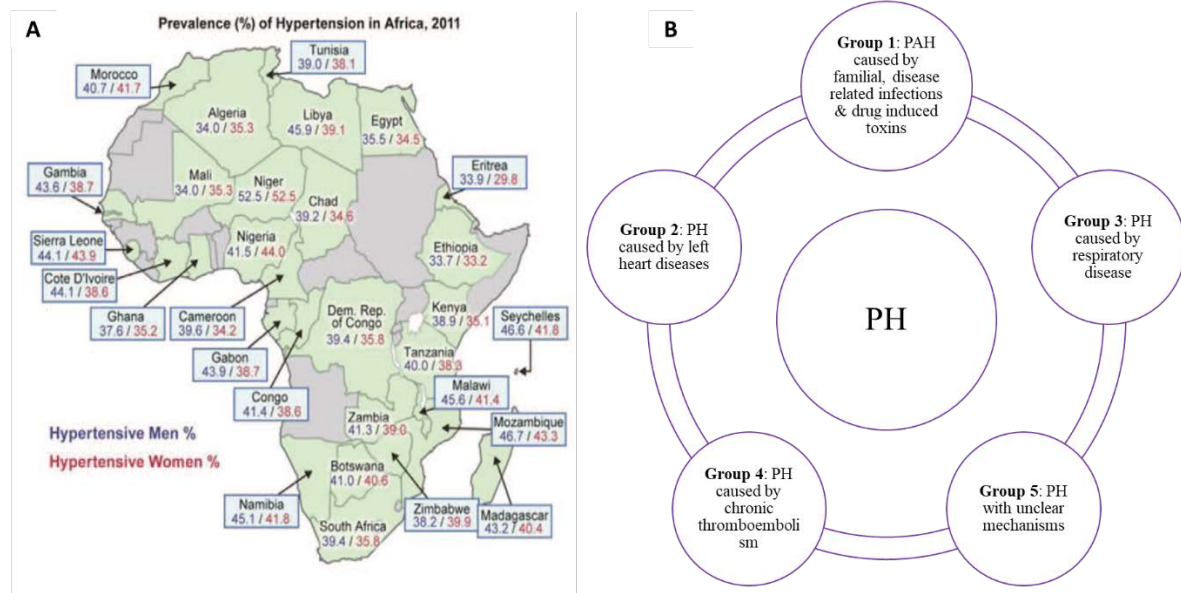


Figure 3: A) Mean prevalence rate of hypertension in adults aged ≥ 15 years in Africa as reported in WHO, Global status report in 2011. Adapted from (Sliwa et al., 2016). B) Classification of Pulmonary hypertension (Simonneau et al., 2013).

2.2.1 Pathophysiology of PAH

Though the pathophysiology of PAH is complex, subclasses of the disease, share a common pattern such as vasoconstriction, inflammation, endothelial dysfunction, RV hypertrophy, and right ventricle heart failure. Despite several studies in this field, understanding and treatment strategies are still inadequate (Hoeper et al., 2013). Experimental evidence suggests ROS play a pathogenic role in the development of PAH while antioxidants could play a therapeutic role in improving PAH (Wong, Bansal, Pavlickova, Marcocci, & Suzuki, 2013). Animal models of PAH, particularly drug induced models in rats, are of interest in broadening the understanding of this pathologic condition. Oxidative stress is an imbalance occurring as a result of an excess of reactive species (RS) reacting with biological systems to cause damage, requiring repair and detoxification and associated with an increase in oxidants such as hydrogen peroxide and superoxide relative to the concentration of antioxidants (Bowers et al., 2004). Oxidative stress is associated with most diseases and PAH may be no exception. Oxidative stress in PAH is associated with increased production of reactive oxygen species (ROS), reactive nitrogen

species (RNS) and mitochondrial dysfunction. These oxidants cause damage to DNA and biological tissues by oxidation through lipid peroxidation. Production of RS has been demonstrated in animal PH models and PAH patients (Chrobak, Haeger, Maracle, & Fredenburgh, 2014).

The cardioprotection of wine is largely attributed to the polyphenolic compounds in the wine with their antioxidant capacity that may increase plasma and or antioxidant capacity after ingestion, scavenging the free radicals induced by oxidative stress (Sánchez-Moreno, Larrauri, & Saura-Calixto, 1999). Treatments rich in flavonoids used during MCT-induced PAH are known to decrease lipid peroxidation, decrease hydrogen peroxide, increase protein expression of eNOS (endothelial nitric oxide synthase) and decrease RV remodelling by reducing the activity of cleaved Caspase-3 response in MCT-induced PAH (Ludke et al., 2010; Mosele et al., 2012). Antioxidant activity could also be important in the tissue experiencing oxidative stress. This phenomenon may confer protection against PAH, protecting the heart.

2.2.2 Diagnosis and current treatment of PAH

The diagnosis of PAH requires several investigations to determine the cause, severity and progression of the disease. These investigations include electrocardiogram, chest radiograph, echocardiogram, exercise testing, haemodynamic and pulmonary function tests, measures of arterial blood gases, ventilation and perfusion lung scans, pulmonary angiography and high resolution computed tomography as well as blood tests for assessment of underlying disease and right ventricle heart catheterisation for pressure studies (Montani et al., 2013).

The first line of treatment against ROS induced by PAH includes superoxide dismutase (SOD) and catalase known to ameliorate MCT-induced PAH (Kamezaki et al., 2008). Overexpression of SOD in the lung tissue ameliorated PAH induced by MCT (Kamezaki et al., 2008). The activity of catalase is however known to be variable for different PH aetiologies. For instance, its activity was decreased at one stage of the disease progression and increased in lung tissue of MCT-treated rats (Aggarwal, Gross, Sharma, Fineman, & Black, 2013; Jin et al., 2008; Ludke et al., 2010). The treatment strategies consist of general management and use of non-specific and specific drug therapies. General management includes physical activities, avoiding high altitude and hypoxia, care during pregnancy and contraception use, avoiding unnecessary anaesthesia and surgery and avoiding drugs like vasoconstrictors and beta blockers. The non-specific drug therapies include diuretics, oral anticoagulants, calcium blockers and digitalis.

Current treatment of PAH includes the use of more specific drugs such as prostanoids (epoprostenol, treprostinol, inhaled illoprost), endothelin receptor antagonists (bosentan, ambrisentan) and phosphodiesterase type-5 inhibitors (sildenafil, tadalafil, vardenafil). Combined therapies and emerging treatment such as the use of tyrosine kinase inhibitors, macitentan, soluble guanylate cyclase activator (riociguat), prostacyclin receptor agonists (selexipag) and non-medical treatments such as balloon atrial septostomy and lung transplantation are current possible treatment options in humans (Montani et al., 2013).

The use of rat models (with MCT, MCT pneumonectomy, chronic hypoxia, chronic hypoxia with SUGEN-516, endothelin B receptor deficiency) have provided histological, molecular mechanisms and haemodynamic measurements into the pathobiology of PAH (Ryan, Bloch, & Archer, 2011). Drug trials in animal studies have produced significant improvement of PAH although there seem to be suggestions that differences still exist between rat pulmonary vascular disease and human PAH. Key concern is the rate of disease progression in rats compared with humans (weeks versus years) respectively, absence of plexiform lesions in rats, and genetic mutations (Stenmark et al., 2009).

2.3 MCT-INDUCED PAH

Pulmonary arterial hypertension is characterised by gradual vascular and endothelial changes which lead to vasoconstriction and remodelling of the pulmonary vessels. Animal models such as rats, mice, dogs, monkeys and pigs have been used (Stenmark et al., 2009). The most commonly used is the MCT in a rat model as it is inexpensive and reproducible. In this model, a single subcutaneous injection of monocrotaline (MCT), a toxic pyrrolidine alkaloid (Figure 4), induces endothelial cell damage. The liver enzyme cytochrome P450 3A activates the alkaloid and the animal model resembles the human primary pulmonary hypertension model when examined (Reid, Lane, Morin, Wilson, & Segall, 1998). One of the mechanisms which involves the pathogenesis of PAH is oxidative stress. The superoxide radical, a potent NADPH (nicotinamide adenine dinucleotide phosphate) oxidase activator, seems to be the most important ROS induced in PAH as a result of its inflammatory process (Belló-Klein, Araujo, Schenkel, & de Lima Seolin, 2016). There are suggestions that MCT induction causes the catalytic gp 91 phox of NADPH oxidase to be upregulated and increases the expression of complex II subunit B of mitochondrial respiratory chain which is very significant in the ROS generation process (Belló-Klein et al., 2016). Other sources of ROS generation for superoxide radical such as uncoupled NOS, xanthine oxidase and hydrogen peroxide have been discussed

(Aggarwal et al., 2013; Colombo et al., 2013; Mosele et al., 2012). Several doses of MCT have been described in which a PAH response has been elicited: ranging from 2.4-300mg/kg (Gomez-Arroyo et al., 2012). However, the dose-symptom response may be uncertain as this may largely depend on the research objectives, experimental protocol and the animal model being used. Signs of inflammation, hypertrophy, right ventricular damage, oxidative stress, vascular remodelling and thrombosis have occurred (Csiszar et al., 2009). Though PAH is a devastating disease causing damage to vital systemic organs, especially the heart, kidney and lungs, wine with its antioxidant properties can lessen the burden imposed by inducing protection.

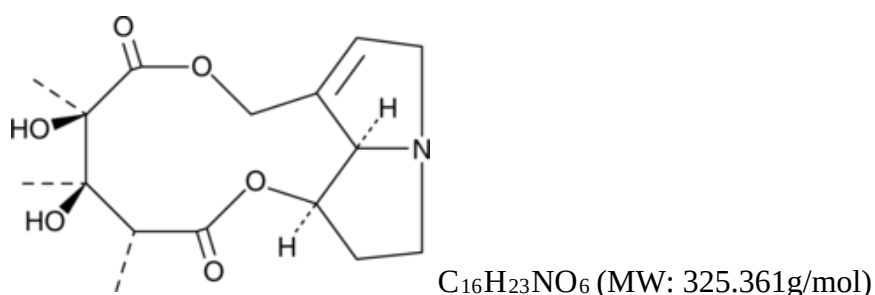


Figure 4: Molecular structure and formula of monocrotaline.

2.4 WINE AND CARDIOVASCULAR DISEASE

Chronic and moderate consumption of red wine is usually considered more beneficial and healthier than liquor, white wine or beer owing to its high concentration of phenolic compounds. Polyphenols as a major component in red wine are known to have cardiovascular benefits (Khodja, Chataigneau, Auger, & Schini-Kerth, 2012). These phytochemicals derived from red wine have varying biochemical and molecular targets and are able to induce cardioprotection through the action of endothelial production of nitric oxide to reduce oxidative stress, the production of antioxidants and anti-inflammatory mechanisms to reducing inflammation in blood vessels and prevention of platelet aggregation (Iriti & Faoro, 2009; Li & Förstermann, 2012). Countless researchers focused on the components of red wine as treatment strategies for different diseases (Chicoine, Stewart, & Lucchesi, 2009; Lamont et al., 2011; Maarman et al., 2015; Pasinetti, Wang, Ho, Zhao, & Dubner, 2015; Ray et al., 1999). Significant evidence shows that red wine is indeed a drink for the heart (Saleem & Basha, 2010). Melatonin, a component of red wine which has been extensively studied, helps regulate the pattern of sleep and appetite in humans (Fabrizio et al., 2013); reduces blood pressure (Grossman, Laudon, & Zisapel, 2011) and improves the heart overall function of the heart by

improving nitric oxide metabolism and endothelial function (Cicero & Borghi, 2013). However, it is worth noting that the alcoholic beverage is an important area worth investigating. Beneficial and positive attributes from red wine components may still be unfounded by variation in humans possibly due to the counter-effect of ethanol in wine from excessive consumption which counteracts or nullifies the protective effects of the polyphenols (Barbero-Becerra, López-Velázquez, Sánchez-Valle, Uribe, & Méndez-Sánchez, 2012).

2.4.1 Red wine: a potent antioxidant and cardioprotective agent

Red wine is a beverage with its alcohol content obtained from fermented red grapes. The yeast naturally present in the skin of the grapes converts the sugar to carbon dioxide and alcohol (releasing heat as a by-product). Different categories of red wine can be made depending on, for example, the processing and the grape type. The grape fruit contains compounds called polyphenols of which flavonoids are a major component. Polyphenolic antioxidants consist of non-flavonoid and flavonoid compounds with antioxidant activity (Mitsubishi, Ikeuchi, & Nojima, 2001). These polyphenols are also present in foods, beverages, tea or vegetables and their consumption is associated with lower CVD incidence (Gémes et al., 2016; Hertog, Hollman, & van de Putte, 1993; Nardini et al., 1995). The concentrations of polyphenols in wine are dependent on factors such as the seed, grape skin, the vine stem, products of yeast metabolism, the grape variety, geology, environment and wine processing techniques (Frankel, Waterhouse, & Teissedre, 1995). The major flavonoids in wine include conjugates of the flavonols (quercetin and myricetin), the flavanols (catechin and epicatechin) and anthocyanins. The non-flavonoids include the hydroxybenzoates (hydroxybenzoic acid and gallic acid) the hydroxycinnamates (caffeic, caftaric, and coumaric acids (Gálvez, Barroso, & Pérez-Bustamante, 1994)) and resveratrol (Woraratphoka, Intarapichet, & Indrapichate, 2007). These have been shown to have protective effects (Blackhurst & Marais, 2009; López et al., 2007) against heart diseases and oxidative damage. The phenolic compounds such as monomeric flavonoids, anthocyanins, phenolic acids and polymeric tannins have antioxidant properties, play an inhibitory role at different stages of tumour development (Hertog et al., 1993), inhibit oxidation of LDL *in vitro* (Frankel, Waterhouse, & Kinsella, 1993) and are effective in scavenging oxygen free radicals and protecting against lipid peroxidation (Gambelli & Santaroni, 2004; Nardini et al., 1995).

These compounds, though available in other beverages (Figure 5) but are of greater availability in red wine, can slow down disease progression and inhibit damaging oxidative processes

(Ames, Gold, & Willett, 1995) either by scavenging oxygen-derived radicals or by donating hydrogen, binding metal ion or stabilizing phenoxyl radicals (Hugel, Jackson, May, Zhang, & Xue, 2016). They also can act as signaling molecules to induce physiological responses or gene expression (Williams, Spencer, & Rice-Evans, 2004).

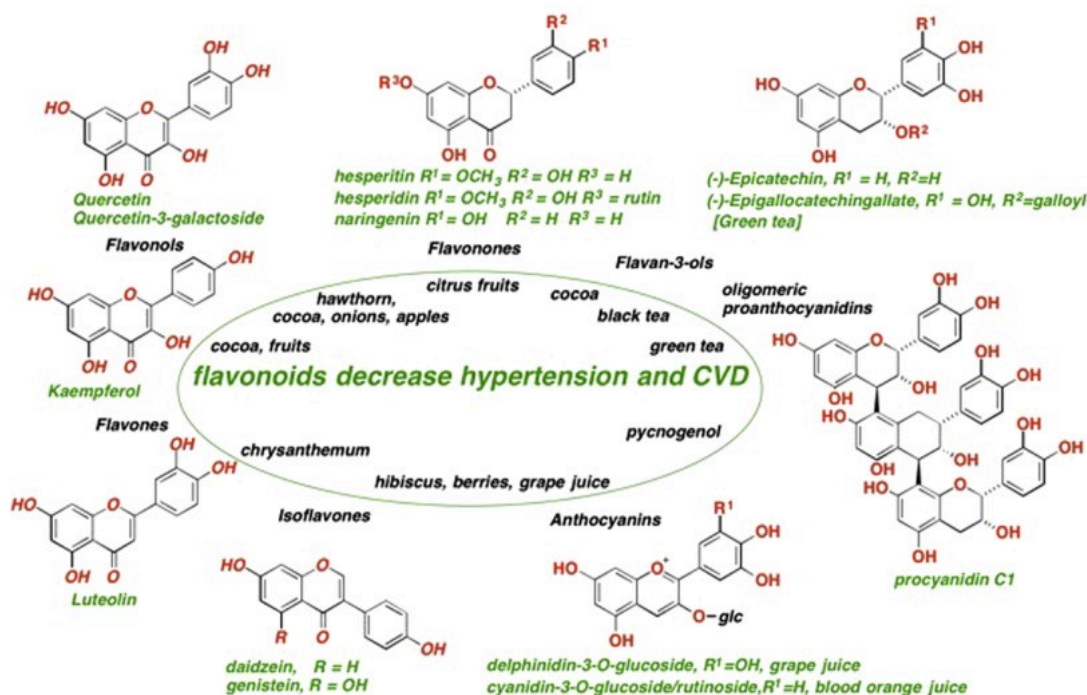


Figure 5: Protective dietary flavonoids influencing hypertension and cardiovascular disease (Hugel et al., 2016).

2.4.2 Evidence of red wine induced cardioprotection

Evidence of cardioprotection by red wine is demonstrated in Figures 5 and 6. Both experimental and epidemiological studies have been reported (Croft, 2016). The effects of red wine on hypertension and blood pressure can be used as a guide in assessing cardioprotection. A study found inhibition of oxidative stress, endothelial dysfunction and arterial hypertension in pathologic arteries of rats as a result of red wine polyphenols (López-Sepúlveda et al., 2008). Though alcohol consumption may be associated with some risk to health, moderate regular red wine intake is considered beneficial (Iriti & Varoni, 2014). In subjects with cardiovascular risk, moderate intake of red wine had no effect on blood pressure compared to those with dealcoholised red wine which reduced the blood pressure (Chiva-Blanch et al., 2012). Chiva-Blanch et al. (2013) largely attributed the difference in cardiovascular risk protection to the different phenolic contents of the alcoholic drinks.

2.5 ALCOHOL

There is little doubt that excessive consumption of alcohol is harmful but moderate and frequent consumption of a low alcoholic beverage like red wine is considered beneficial (Blackhurst & Marais, 2009; Koppes, Dekker, Hendriks, Bouter, & Heine, 2005). Ethanol is the most abundant alcohol in wine with concentrations between 10-13% v/v and some alcohol content going up to 16% v/v dependent on the strain of yeast, sugar, density, temperature present, fermentation process and country policy. The ethanol gives the wine stability against aging and impacts positively on its sensory properties (Soleas, Diamandis, & Goldberg, 1997).

2.5.1 Epidemiological evidence of alcohol-induced cardioprotection

Globally, alcohol contributes to 4% of the disease burden (Rehm et al., 2010) and consumption patterns may range from irregular to moderate to binge intakes depending on individual lifestyle. Bingeing and infrequent alcohol intake are considered more harmful and a prevalent practice in Africa, while moderate and frequent patterns seem to be the norm in other continents, conferring protection evident in the Mediterranean diet and the French Paradox phenomenon (Rehm et al., 2010; Renaud & Lorgèril, 1993). These lifestyle patterns may have consequential effect on the heart or other organs in the body causing stroke, cardiomyopathies, liver damage and cancers (Leong et al., 2014). Data on alcohol and its pattern of use in Africa is limited while alcohol abuse and intake is ever-increasing due to the influence, rebranding and promotional activities of the alcohol industries booming on the African market (Connor & Hall, 2015). For instance, in South Africa, widespread alcohol abuse is reported among all sectors including students, leading to high numbers of patients reporting at the trauma units, increased accidents on the road and high mortality (Parry et al., 2002). A recent cohort based population study has shown that moderate alcohol drinking is usually associated with a lower risk of presenting with several CVDs while heavy drinking is associated with a greater risk (Bell et al., 2017).

2.5.2 Experimental evidence of alcohol-induced cardioprotection

Despite red wine widely being known to be beneficial, the reason for benefit in the wine is unclear. It is generally accepted that moderate to light drinking of red wine gives protection to the heart whereas abstention and excessive chronic intake do not. Both alcohol and red wine are known to provide cardiovascular benefits (Figure 6). Red wine in moderation has long been thought of as heart-healthy, indicating ethanol may offer some level of cardioprotection.

Lamont et al. (2012) reported that reducing the alcohol concentration to 6% v/v in red wine did not reduce its antioxidant capacity nor its cardioprotective properties when the drinking water of rats was supplemented with wine at concentrations equivalent to 2-3 glasses per day consumed in humans. This is possibly one of a few studies that used whole red wine, suggesting that moderate consumption of alcohol from any beverage may be considered beneficial, but wine confers more benefit (Thornton, Symes, & Heaton, 1983). In one study, moderate ethanol intake of 18 mL or less per day seems to keep the risk of hypertension at low levels (Okubo, Suwazono, Kobayashi, & Nogawa, 2001) as opposed to regular intake of 50 g or 100 g per day which had a linear effect on hypertension (Taylor et al., 2009).

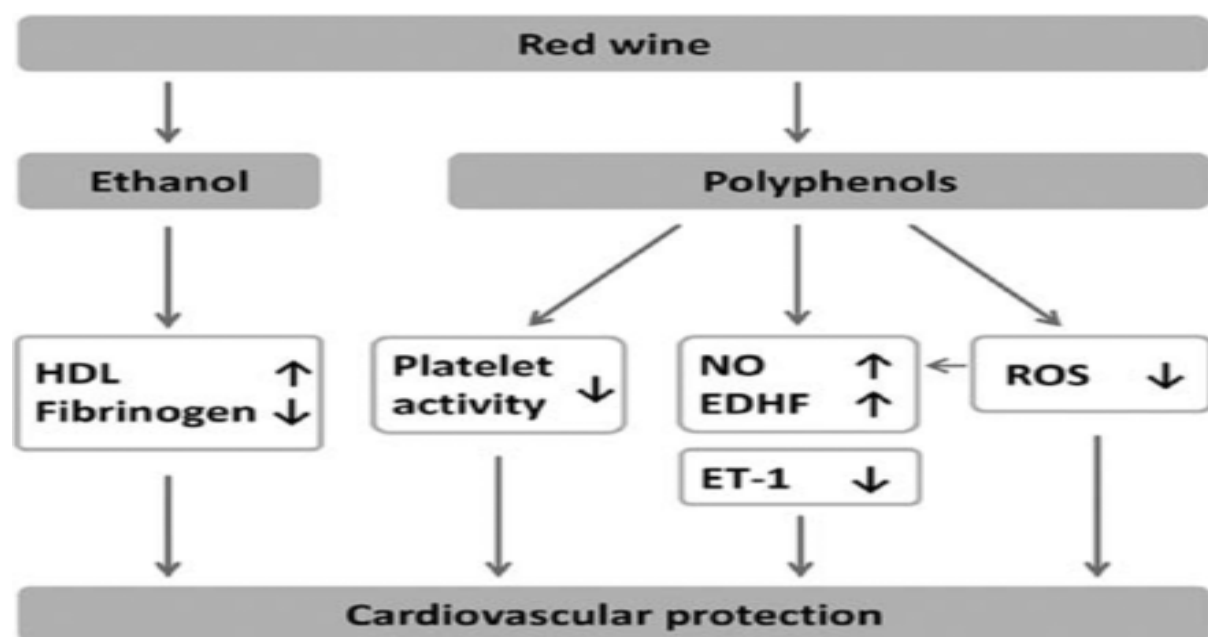


Figure 6: Factors potentially contributing to the protective effects of red wine in the blood vessels. Adapted from (Li & Förstermann, 2012).

High density lipoprotein (HDL); Nitric oxide (NO); Endothelium-derived hyperpolarizing factor (EDHF); Reactive oxygen species (ROS); Endothelin 1 (ET-1)

2.5.3 Reduced-alcohol wine and cardioprotection

Low alcohol wine consists of three categories; no-alcohol (<0.5% v/v), low-alcohol (0.5-1.2% v/v) and reduced-alcohol (1.2% to 5.5-6.5% v/v) (Pickering, 2000) although definitions may vary. Reducing the alcohol content of wine does not seem to affect the antioxidant properties of reduced-alcohol wine (Lamont et al., 2012) as it still contains antioxidant-active components such as resveratrol, melatonin, quercetin, anthocyanin and catechins. A study carried out on

rats showed that dealcoholised white and red wine regulated oxidative stress and inflammatory response *in vivo*, with the red wine showing more cardioprotection by decreasing oxidative stress associated with inflammation (López et al., 2007) and this was attributed to the higher content of polyphenolic compounds in the red wine. Also, chronic administration of alcohol-free lyophilised red wine induced antihypertensive and vasodilatory effects in normal and hypertensive rats (de Moura et al., 2004). A key phenomenon is that the components making up the wine can interact synergistically and reinforce the antioxidant properties, a model proven by (Frémont, Belguendouz, & Delpal, 1999) and confirmed in a recent postgraduate thesis ((Dlamini, 2015) unpublished data). Reduced-alcohol wine may afford a similar protective effect that most researchers have indicated about red wine.

CHAPTER 3: MATERIALS AND METHODS

3.1 STUDY SITE FOR ANIMAL STUDY

The experiments were conducted at the Hatter Institute in male Long Evans rats weighing 150-175g, housed in the Faculty of Health Science animal unit of the University of Cape Town, in a 12-hour dark/light cycle. The room temperature in the animal house was maintained between 20 °C - 23 °C and the humidity at 40%.

3.1.1 Ethical Issues

Approval for this study was given by the Faculty of Health Sciences Animal Ethics Committee, University of Cape Town (ethics number 016/001). All protocols were carried out in compliance with the guide, care and use of laboratory animals published by the United States National Institute for Health in 1996.

3.2 EXPERIMENTAL INDUCTION OF PULMONARY ARTERIAL HYPERTENSION

The total study duration was 35 days, including 7 days of pre-treatment with reduced-alcohol wine, regular wine, or water, and 28 days for PAH development after MCT injection. Echocardiography was performed on all the rats at the end of day -7 and again on day 28 before sacrificing the animals (Figure 7). MCT was administered as a single subcutaneous injection (80mg/kg) after pre-treatment. Wine with alcohol concentrations of 5.5% and 15% was obtained from Conetech South Africa using the spinning column technique to lower the alcohol content and provided as drinking liquid described in Sampling Population and Technique (Section 3.2.1). The stability of the wine was tested during the experimental period twice a week for 4-weeks. After the 28 days, echocardiography was performed, the animals were sacrificed, blood samples were collected by cardiac puncture, biometric measurements were carried out and the hearts were separated into RV and LV plus septum for histology.

3.2.1 Sample population and techniques

Adults rats on average drink between 10mL and 12mL of water per 100g body mass per day (Animal care; John Hopkins University (web.jhu.edu/animalcare/procedures/rat.html)). The drinking water of the rats in this study was supplemented with one part of either 5.5% reduced-

alcohol or 15% regular red wine to seven parts of water, at an amount equivalent to 2-3 glasses of wine as per day in humans. Always water and wine were replenished daily. The groups were pre-treated with wine or water for 7 days before the start of the single MCT injection. A maximum of 3-4 rats was housed per cage and markings were made on their tails with permanent marker (and repeated when faded) to allow for individual follow up of every rat. 80mg/kg of MCT was administered by subcutaneous injection using a 25G needle at day 0 (Figure 7).

3.2.2 Sample size and groups

To assess the effect of MCT on PAH in 35 Long Evans rats, the following randomised groups of rats ($n \geq 4$) were used:

1. Control rats receiving only water ($n=5$)
2. MCT control rats receiving only water ($n=7$)
3. Rats receiving reduced-alcohol only wine (5.5%) ($n=5$)
4. MCT treated rats receiving only reduced-alcohol wine (5.5%) ($n=7$)
5. Rats receiving only regular wine (15%) ($n=4$)
6. MCT treated rats receiving only regular wine (15%) ($n=7$)

3.2.3 Blood sample collection and euthanasia

Once deep unconsciousness was induced using 0.4mL/kg of pentobarbitone mixed with phosphate buffered saline (1x PBS), reflex reactions from pedal and tail pinch were checked to ensure adequate depth of anaesthesia prior to blood sampling by cardiac puncture after dissection. A blood sample of about 6mL was drawn from the heart after thoracotomy (McClure, 1999). The blood was drawn slowly to prevent collapsing the heart using a 25G needle of length 12-19mm into a serum gel separator tube and an EDTA anticoagulant tube. The EDTA tube was gently tilted to mix the blood with the anticoagulant and immediately put on ice. The tubes were transferred to a precooled (4 °C) centrifuge and centrifuged for 15 minutes at 2500G force. The serum and plasma were transferred into 2mL micro reaction vials in aliquots of 1mL in duplicates. The tubes were labelled and stored at -80 °C until the analyses were performed. Once the blood was collected, the heart was excised from the chest cavity for the biometric data.

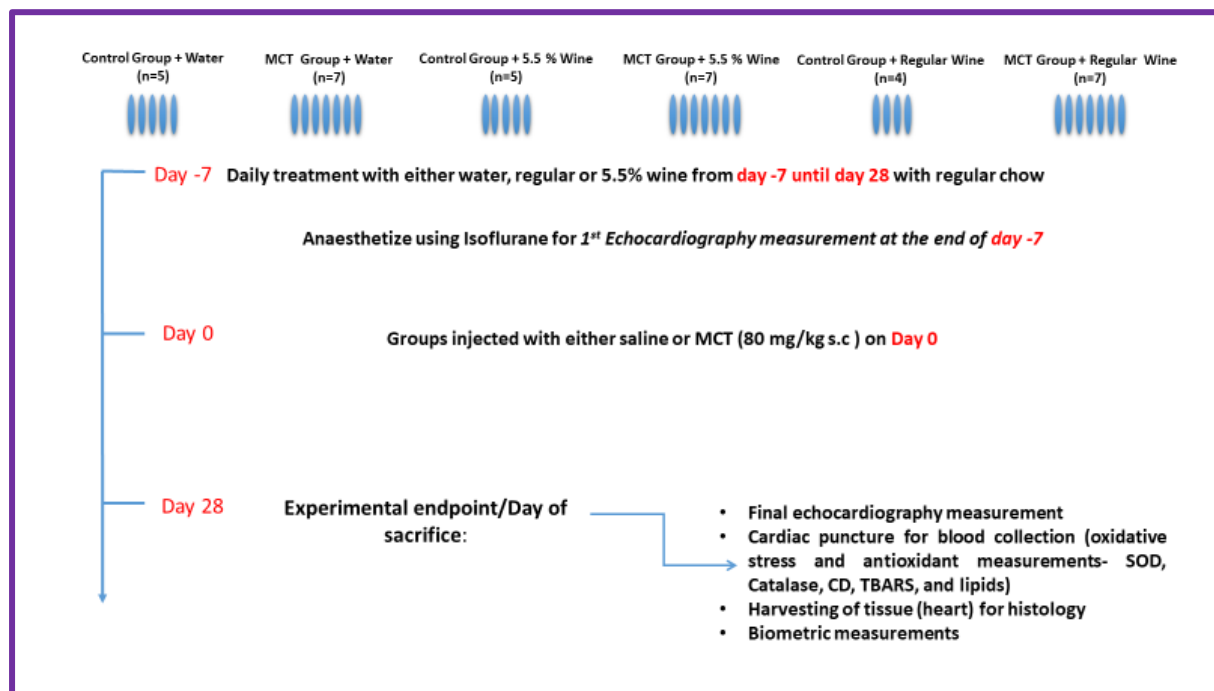


Figure 7: Summarised protocol for this study.

3.3 DATA COLLECTION AND BIOMETRIC MEASUREMENTS

The body weights of all the rats were measured and recorded every day throughout the experimental study. In addition, the volumes of liquid consumed were recorded daily in a monitoring sheet (Appendix V).

3.3.1 Biometric measurements

The heart was weighed and the mass recorded. The RV was separated from the LV plus septum, and their weights recorded. The length of the right tibia of every rat was measured and recorded. The ratios of the mass of the RV to the LV plus septum, to tibia length and to the whole heart were calculated and used for different indices of RV hypertrophy (Kosanovic et al., 2011). The liver was removed, rinsed with 1% PBS, lightly dried with a paper tissue and weighed. Loss of liver mass was determined by dividing the weight of the liver by the body weight (Handoko et al., 2010).

3.3.2 Echocardiography: Cardiac function

Echocardiography was performed on all groups of rats at the end of day -7 (Figure 7) for a baseline assessment prior to the MCT injections, and again on the day of sacrificing (day 28). The rats were anaesthetised with 4% isoflurane gas, to induce deep unconsciousness and this was maintained with 2% isoflurane via nose cone delivery, along with oxygen at a flow rate of 1.5L/minute. Rats were reclined in a dorsal position and secured to a warm pad to keep their body temperatures at 36.5 °C to 37.5 °C. Their tails were covered to reduce heat loss. The rats' chests were shaved using a hair remover gel applied for less than a minute, cleaned and a warm ultrasound gel was applied to the chests to obtain good images over the chest. Parameters such as diastolic thickness of RV from a long and short axis view, pulmonary artery acceleration time (PAAT) and peak flow from the pulmonary artery, A and E waves, PAAT/ejection fraction as well as tricuspid annular plane systolic excursion (TAPSE) from a 4-chamber apical view using (VEVO 2100 Visualsonics, Inc) were measured.

3.3.3 Histology of heart for determination of the extent of cardiac fibrosis

Separated RV and LV plus septa were rinsed in three changes of 1% PBS and dried with soft tissue paper before weighing. Afterwards, the heart tissue was fixed in 4% buffered formalin (Appendix I) in small labelled histology containers until ready for processing. The processing stages are described in Table 1.

Table 1: Processing of tissues before embedding

Steps	Process	Comments	Time
1	70% Alcohol	Dehydration	60 minutes
2	90% Alcohol	Dehydration	60 minutes
3	100% Alcohol	Dehydration	60 minutes
4	100% Alcohol	Dehydration	60 minutes
5	100% Alcohol	Dehydration	60 minutes
6	3X changes of Iso-Octane	Clearing	60 minutes each
7	2X changes of Paraffin wax at 60°C	Paraffinisation	60 minutes each
8	1X change of Paraffin wax at 60°C	Paraffinisation	120 minutes

The RV and LV plus septum were grouped and marked with colours as indicated in Figure 8 before sectioning. The heart sections were embedded in paraffin and sectioned longitudinally

at a thickness of 5µm for histological analysis. Sections were stained with 0.1% sirius red in picric acid (Sigma-Aldrich, SA) for collagen deposits. Sections were examined microscopically with a Nikon Eclipse 90i microscope with a 20X objective. Images were captured and analysed with image J software (<https://imagej.nih.gov/ij/index.html>).

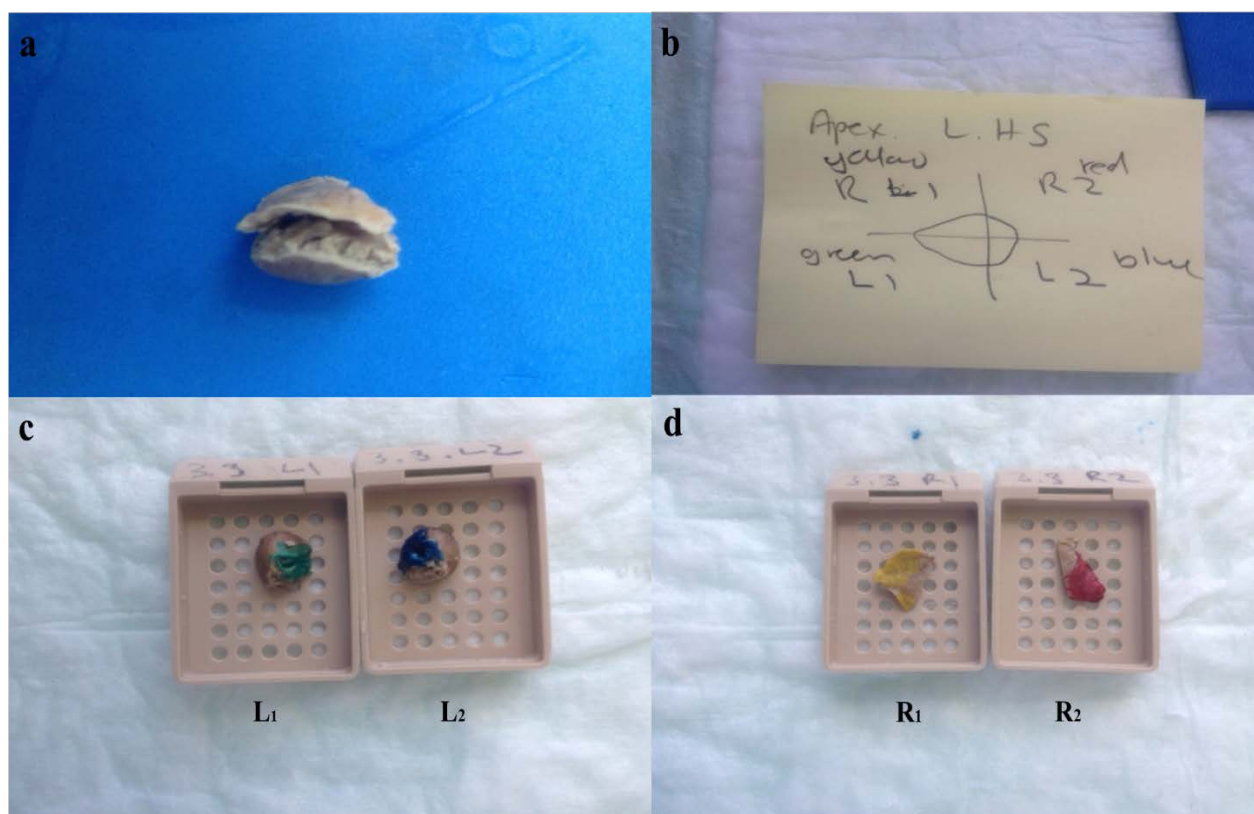


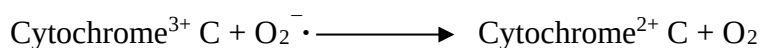
Figure 8: Illustration of the position of the heart before sectioning; (a) RV (R) and LV plus septum (L), (b) longitudinal sections with colours, (c) LV plus septum in both L1 (green) and L2 (blue), (d) RV in both R1 (yellow) and R2 (red).

3.4 ANTIOXIDANT AND OXIDANT ANALYSIS

3.4.1 Superoxide dismutase (SOD) activity determination

The SOD assay used was a modified method of McCord and Fridovich (1969). The superoxide radical is produced enzymatically by the reaction catalysed by xanthine oxidase (XOD). Oxidised cytochrome C is reduced by the superoxide radical and the rate of reduction is measured spectrophotometrically over time at 550nm. Superoxide dismutase inhibits the reduction of cytochrome C by competing for the superoxide radical as indicated below:





The reagents required for the SOD assay: potassium phosphate buffer (216mM), ethylenediaminetetraacetic acid (EDTA), cytochrome C (1.1mM) and xanthine (0.108mM), were prepared and made into one solution called the Cocktail Reagent (Appendix III). All reagents were purchased from Sigma Aldrich, South Africa. Secondly, the SOD standard containing 10mg/10.5mL (5000IU/mL) was prepared and aliquots of 150µL were made and stored at -20 °C. For an aliquot of 150µL a further 1: 10 dilution was prepared by adding 1350µL de-ionised water as the Working SOD standard, (500IU/mL) from which a further set of standards were prepared as below, while keeping on ice (Appendix IV).

Thirdly, the xanthine oxidase (5IU/mL) was prepared by first adding 6.76µL of cold de-ionised water to 5µL of xanthine oxidase, followed by a final addition of 209µL of cold de-ionised water. The solution was mixed and immediately stored on ice. To ensure the reaction slope of the diluted xanthine oxidase was within the range of 0.025 ± 0.005 , 280µL of the prepared Cocktail Reagent was added to wells in a Greiner 96 well plate and de-ionised water was added to wells as shown in Table 2. The absorbance at 550nm was checked until stable and immediately, 10µL of the diluted xanthine oxidase was added as shown in Table 3. The timer was immediately started. The absorbance was read at 550nm with a spectrophotometer at 1 minute intervals for 5 minutes from time point zero. The reaction slope of the xanthine oxidase was calculated by the difference in time between the zero and final readings after subtracting the blank. Once the slope was within the 0.025 ± 0.005 range, the rest of the assay was performed.

Table 2: Checking for the stability of the xanthine oxidase.

	Blank/H ₂ O	XOD
Cocktail Reagent	280µL	280µL
dH ₂ O	20µL	10µL

Finally, the Cocktail Reagent (280µL), de-ionised water (10µL), sample volume of (10µL) and standards (10µL) were added to well plates and xanthine oxidase (10µL) was added and readings taken using a spectrophotometer for 5 minutes (Table 3). The blank reaction well was made up of 280µL of Cocktail Reagent and 20µL of water alone. The final reaction slope was determined, and the SOD activity calculated and expressed as IU/mL protein.

Table 3: SOD assay protocol.

	Blank	Test (sample)	Standard (SOD)
Cocktail Reagent	280µL	280µL	280µL
dH ₂ O	20µL	-	-
Text/Sample	-	10µL	-
Standard/SOD	-	-	10µL
XOD	-	10µL	10µL

3.4.2 Catalase activity determination



Catalase activity was evaluated following the reduction of H₂O₂ at a 240nm wavelength using a modified method of Aebi (1984). Catalase catalyses the reduction of hydrogen peroxide to oxygen gas and water. Plasma samples are diluted 1:10 in 50mM phosphate buffer, pH 7.0. The diluted samples (10µL) were added to a Costar® (Corning) 96-well, UV-transparent plate (Sigma-Aldrich, SA), plus 10 µL of 50mM phosphate buffer, pH 7.0 and 220µL of freshly prepared 40mM (30%) H₂O₂ (made by adding 410µL of neat H₂O₂ to 100mL of 50mM phosphate buffer, pH 7.0). The rate of decomposition of H₂O₂ was measured spectrophotometrically from changes in absorbance at 240 nm over 5 minutes, relative to a blank (20µl phosphate buffer plus 220µL of the 40mM H₂O₂). Activity of catalase was expressed as units/mg protein using a molar extinction coefficient of 30% H₂O₂ at 240nm = 43.6 M⁻¹.cm⁻¹. Activity was calculated as ΔA/min x 240/10 X 1/0.031 (molar absorption of H₂O₂, but reading only 240µL)

3.4.3 Oxygen radical absorbance capacity (ORAC) assay of blood plasma

The ORAC measures the oxidative breakdown of the fluorescent molecule fluorescein and demonstrates an association between ROS and antioxidants in various pathologies for example PAH (Ames, Shigenaga, & Hagen, 1993). The actions of ROS are counteracted by the antioxidants found both endogenously and by the consumption of antioxidants in the red wine. Antioxidants are considered to protect the fluorescein against damage slowing down the fluorescence decay. The free radical generator used in this assay is AAPH, which produces peroxy radicals by heating, damaging the fluorescent molecule and leading to a gradual loss of fluorescence over time. The higher the concentrations of antioxidants, the more the free

radicals are scavenged, thus maintaining the intensity of the fluorescent signal observed. This function is quantified using the antioxidant trolox, a water-soluble vitamin E analogue as a standard (Huang, Ou, & Prior, 2005). Samples were loaded into a 96-well, white flat-bottomed, Polysorp®-Nunc microtitre plate (Sigma-Aldrich, SA) and readings recorded at an excitation wavelength of 485nm and emission wavelength of 520nm, using a fluorometer with a micro plate data acquisition program (Advanced Reads®). Decay curves of fluorescence intensity against time were recorded at various intervals (described below) over approximately 2-3hrs, the time taken in this study for all fluorescence values to reach very close to zero. (Time intervals: 0min, 5, 20, 40, 60, 80, 100, 120min). The AUC (area under the curve) was generated by computing data into GraphPad prism software.

At the start of the ORAC assay, 7850µL of phosphate buffer (0.075M, pH 7.4) (Appendix II) were pre-warmed in a 37°C incubator for approximately 30 minutes; 0.696g of AAPH was weighed; fluorescein stock 1 and 2 were prepared and kept in dark bottles protected against light (Appendix II). A standard curve using trolox (6-OH-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) (Sigma-Aldrich, SA) was prepared (Appendix IV).

The plasma samples were deproteinized by adding 100µL of ice cold methanol (Merck Chemicals, SA) to 50µL of plasma. This was vortexed and microcentrifuged at 12000G force, for 5 minutes. To 40µL of supernatant, 250µL of phosphate buffer (0.075M, pH 7.4) was added. Then, the supernatant was diluted further by taking 80µL and adding it to 420µL of phosphate buffer and vortexed. A final dilution was made by adding 50µL of supernatant to 350µL of phosphate buffer, becoming the final sample for the assay. Using a 96-well, white flat bottomed, micro titre plate Polysorp®-Nunc (Sigma-Aldrich, SA), 300µL of phosphate buffer was added to wells A₁ and A₂ as the blanks, with 200µL of phosphate buffer in A₃ and A₄. To wells A₅ and A₆, 100µL of phosphate buffer were added. In wells A₇ to B₈, 50µL of phosphate buffer as well as 50µL of their respective standards as prepared above were added. All the samples were then added into the remaining wells in duplicate. The working solution of fluorescein was made by adding 320µL of stock 2 solution to a final volume of 20mL with buffer and 100µL of the working fluorescein was added to all wells except wells A₁ and A₂. 50µL of AAPH was quickly added to wells A₅ to H₁₂, the timer was started, and readings were recorded at the excitation and emission wavelengths as described above.

3.4.4 Blood plasma concentration of lipid peroxidation products (TBARS and CDs)

Lipid peroxidation was determined by measuring MDA, a lipid peroxide product which is measured by the thiobarbituric acid reactive substances (TBARS) assay using a modified method of Asakawa and Matsushita (1979). MDA is formed during the process of lipid peroxidation and reacts by linking two molecules of thiobarbituric acid (TBA), generating a red dye which is measured at a wavelength of 532nm and the absorbance measured is proportional to the concentration of MDA. The samples were loaded into a 96-well, flat bottomed, microtitre Greiner plate (Sigma-Aldrich, SA) and absorbance readings were recorded at 532nm. (Appendix IV).

Conjugated dienes (CDs) as an initial marker of lipid peroxidation were measured in cyclohexane at a wavelength of 234nm in 96-wells, UV-transparent, flat-bottomed, microtitre plates (Costar® Corning (Sigma-Aldrich, SA)) using a molar extinction value of $2.95 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ after first undergoing Folch extraction (Folch, Lees, & Sloane-Stanley, 1957). 50µL of plasma sample were added to 203µL (a mixture of 10mL chloroform to 5mL methanol). This was vortexed for 1-minute and centrifuged at 8000G force for 15 minutes at 4°C. The top aqueous layer was removed leaving a precipitate of protein on top of the organic solvent. Using a fine pipette to plunge through the protein layer, the organic layer was collected into separate labelled Eppendorf tubes. The organic layer was dried with Nitrogen gas for approximately 2 minutes and 500µL of cyclohexane was added and vortexed for 1 minute. The absorbance was read after aliquots of 300µL were dispensed into the Costar® plate (Sigma-Aldrich, South Africa) and the CD were calculated using $A_{234}/0.0295 \times 10 = \text{nmol CDs/mL plasma}$.

3.5 LIPID ANALYSIS

3.5.1 Triglyceride assay

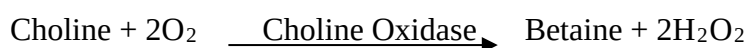
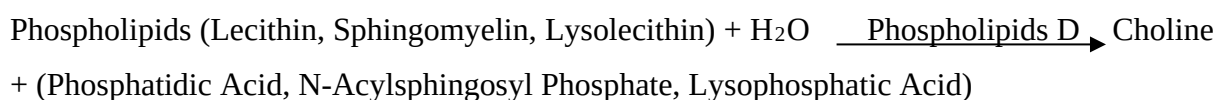
Triglycerides (TG) are a major component of very low-density lipoproteins (VLDL) and chylomicrons, and play an important role in metabolism (as an energy source) and transport of dietary fat. An enzymatic GPO-DAOS method from Wako LabAssay™ (Wako Chemicals GmbH, Germany) was used for this assay. TG are hydrolysed in serum samples to glycerol and free fatty acids in the presence of lipoprotein lipase (LPL). Glycerol is converted to glycerol 3-phosphate by glycerol kinase (GK) in the presence of ATP. Glycerol-3-phosphate is oxidized by glycerol-3-phosphate oxidase (GPO) producing hydrogen peroxide. The hydrogen peroxide produced causes N-ethyl-N-3,5 dimethoxy aniline sodium salt (DAOS) and 4-Amino-

antipyrine to undergo a quantitative oxidative condensation catalysed by peroxidase (POD) producing a blue pigment. The concentration of TG produced in the sample is determined by measuring the absorbance of the blue colour at a wavelength of 600nm (Appendix IV).



3.5.2 Phospholipid assay

The concentration of phospholipids in serum was determined by an enzymatic choline oxidase-DAOS method from Wako LabAssayTM (Wako Chemicals GmbH, Germany). Phospholipids are a major component of biological membranes, along with glycolipids, cholesterol and proteins. Phospholipids in samples are hydrolysed to choline in a reaction catalysed by phospholipase D. The formed choline is oxidized by choline oxidase producing hydrogen peroxide. The hydrogen peroxide produced causes DAOS and 4-amino-antipyrine to undergo quantitative oxidative condensation in the presence of peroxidase (POD) producing a blue colouration. The concentration of phospholipids contained in sample is determined by measuring the absorbance of the blue colour at a 600nm wavelength (Appendix IV).



3.5.3 Cholesterol assay

Total cholesterol was measured by an enzymatic cholesterol oxidase-DAOS method from Wako LabAssayTM (Wako Chemicals GmbH, Germany). Cholesterol esters in plasma or serum samples are decomposed into free cholesterol and fatty acids by cholesterol esterase whereafter the sample reacts with the chromogen reagent. The cholesterol is oxidised with existing free

cholesterol by cholesterol oxidase, producing hydrogen peroxide. In the presence of hydrogen peroxide DAOS and 4-Aminoantipyrin are oxidised and undergo quantitative condensation by peroxidase producing a blue pigment. Quantitation of total cholesterol in the samples is measured by the absorbance at 600nm wavelength using a spectrophotometer.

Cholesterol esters $\xrightarrow{\text{Cholesterol esterase}}$ Free Cholesterol + Fatty Acid

Cholesterol $\xrightarrow{\text{Cholesterol oxidase}}$ Cholestenone + H₂O₂

2H₂O₂ + 4-Aminoantipyrine + DAOS $\xrightarrow{\text{POD}}$ OH-(Blue Pigment) + 3H₂O

The assay in a transparent 96-well Greiner microplate was performed as indicated in Appendix IV.

3.5.4 High density lipoprotein cholesterol (HDL-C) concentration assay

Total high-density lipoprotein (HDL) and subclasses (HDL₂ and HDL₃) are separated by a simple precipitation method modified from Gidez, Miller, Burstein, Slagle, and Eder (1982). Heparin-manganese chloride (heparin (sodium): 135mg/mL (2000 U/mL) and MnCl₂·4H₂O: 1.12M in dH₂O) causes precipitation of Apo B containing lipoproteins in serum samples and HDL₂ is selectively precipitated by addition of dextran sulphate (0.0143g in 1mL dH₂O). HDL₂ cholesterol is calculated as the difference between total HDL cholesterol (heparin-manganese chloride supernatant) and HDL₃ cholesterol (dextran sulphate supernatant). These are transferred into a 96 well transparent plate in duplicate along with the cholesterol standard as described in section 3.5.3, and the cholesterol assay is used to determine cholesterol concentration in each HDL fraction. A chromogen reagent is added to the well plates, gently shaken as before and incubated at room temperature for 30 minutes. The absorbance is read on a spectrophotometer at a wavelength of 600nm (Appendix IV). The concentrations of HDL₃ and total HDL are calculated from the set of standards and the concentration of HDL₂ is obtained by the difference between total HDL and HDL₃.

3.6 WINE STABILITY ANALYSIS (REGULAR AND REDUCED-ALCOHOL RED WINE)

3.6.1 Techniques in producing reduced-alcohol and alcohol-free wines

Low alcohol wines can be produced by different techniques such as distillation under vacuum or atmospheric pressure, evaporation, freeze concentration, membrane processes (dialysis, reverse osmosis and membrane contactors (Diban, Athes, Bes, & Souchon, 2008), adsorption (on resins or on silica gels) and extraction using organic solvents or supercritical carbon dioxide (Pickering, 2000). The most common systems utilised in the industry are the spinning cone column technique (Makarytchev, Langrish, & Fletcher, 2004) and reverse osmosis (Bui, Dick, Moulin, & Galzy, 1986). The spinning cone column is operated by two stages: aroma recovery and ethanol removal (Sykes, Casimir, & Prince, 1992). It consists of a gas-liquid contacting device with a vertical counter current flow system that uses the action of gravity and centrifugal force in its operation through a column which alternates and rotates when the upper surface is wetted with wine, allowing vapour to flow up the column as shown in Figure 9 (Pickering, 2000). This spinning cone process was used by Conetech to produce the reduced-alcohol wine in this study.

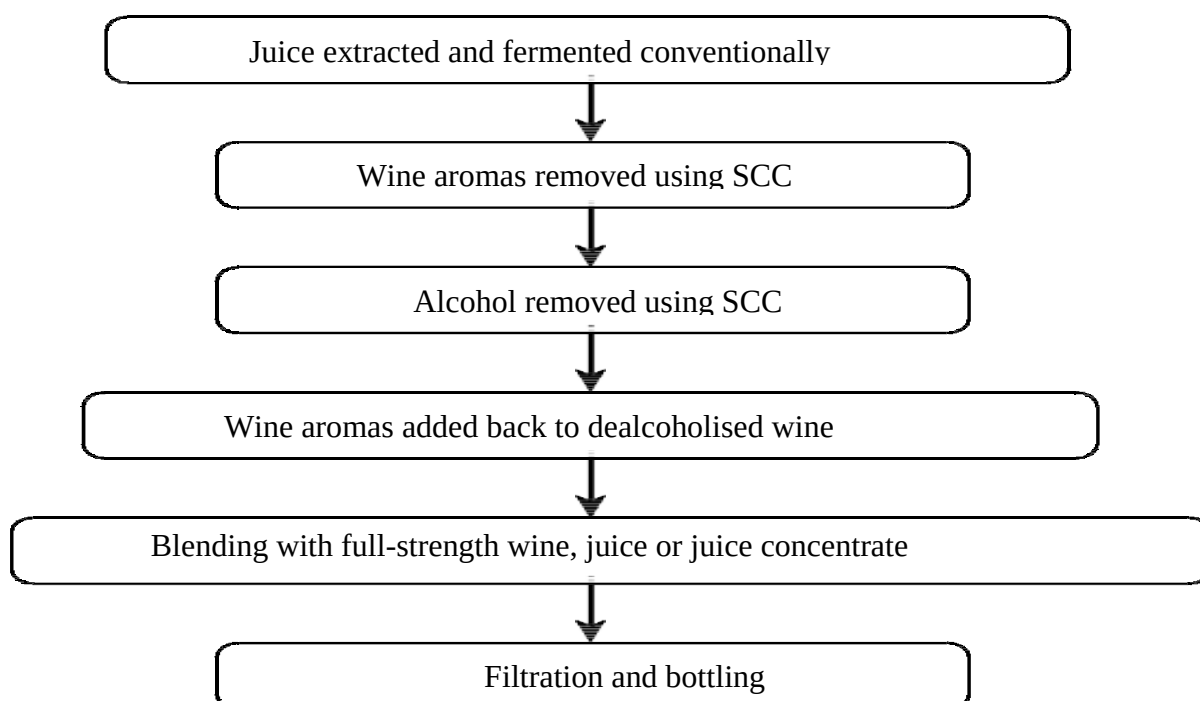


Figure 9: Process involving the spinning cone column (SCC). Adapted from (Pickering, 2000).

3.6.2 Total phenolic compound concentration in wine

The concentration of total phenolic compounds in red wine was determined spectrophotometrically by the method of Waterhouse (2002). This method is based on the chemical reduction of the Folin-Ciocalteu reagent, a mixture of tungsten and molybdenum oxides, and produces a blue colour that exhibits a broad absorption at a maximum wavelength of 765nm when measured with a spectrophotometer. The intensity of the light absorbed at this wavelength is proportional to the concentration of total phenolic compounds in the sample.

Freshly opened red wine (5.5%: Shiraz-Darling and 15%: Shiraz-Darling, Conetech, SA) was diluted 1: 10 in de-ionised water. A standard curve stock (gallic acid (0.125g in 2.5 mL ethanol) was prepared and used. Using 20 μ L of each of the prepared standards, and 1.58mL dH₂O, steps b to e were followed (Appendix IV).

3.6.3 Concentration of anthocyanins in wine

The method of Heredia, Adams, Fields, Held, and Harbertson (2006) was adapted for this assay. Anthocyanins are the main source of red pigment in red wines, forming polymeric pigments upon reacting with certain compounds. Polymeric pigments are the stable form of the colour compound and resist pH changes. Freshly opened red wine (5.5%: Shiraz-Darling and 15%: Shiraz Darling, Conetech, SA) was diluted 1: 10 in distilled water. 500 μ l of the diluted wine samples were aliquoted into labelled Eppendorf tubes with buffer A (Appendix I) and immediately mixed. The reaction mixture was transferred (300 μ L) in duplicate into a Greiner 96 well transparent plate (Sigma-Aldrich, SA) and incubated at room temperature for 10 minutes. The absorbance at 520nm was measured for polymeric pigments (measurement A) with buffer A as blank. For measurement B, 50 μ L of diluted wine plus 50 μ L buffer B (Appendix I) plus 200 μ L of buffer D (Appendix I) were added subsequently into separate wells and incubated for 10 minutes at room temperature. Readings for measurement B were obtained after measuring absorbance at 520nm. Concentrations of anthocyanins present in wine were calculated using the formula: (measurement A - measurement B)/factor. That is, ((measurement B*10) - (measurement A*5))/0.0102 in mg/L M-3-G, (malvidin-3-glucoside). (The expected range in red wine: 41-270mg/L M-3-G).

3.6.4 Concentration of catechins in wine

The concentration of catechins in red wine was analysed by the method of Heredia et al. (2006). Freshly opened red wine (5.5%: Shiraz-Darling and 15%: Shiraz-Darling, Conetech, SA) samples were diluted 1: 10 in de-ionised water. A catechin standard (0.3µg/µL) was added to wells in duplicate in volumes of 0 to 20µL as indicated in (Appendix IV). Volumes were made up to 30µL with buffer C_i (Appendix I). 30µL of diluted wine were aliquoted into separate wells in duplicate and subsequently 230µL of buffer C_{ii} (Appendix I) was added to all volumes in the wells followed by a final 40µL of iron (III) chloride. The reaction mixture was incubated at room temperature on a plate shaker for 10 minutes. The absorbance was read at 510nm on a spectrophotometer with buffer C_i as blank and using GraphPad prism software. The standard curve of the catechins was expressed as mg/L catechin equivalents. The expected range in red wine: 870- 2600mg/L catechin equivalents.

3.6.5 Oxygen radical absorbance capacity (ORAC) assay of wine

A similar method to that used for plasma (section 3.4.3) was used, with a few differences. The red wine (5.5%: Shiraz-Darling and 15%: Shiraz-Darling, Conetech, SA) was first diluted 1: 80 by adding 10µL of red wine to 790µL of phosphate buffer. Then a further dilution (1: 800) from the first was done by adding 100µL of the first diluted wine to 900µL of phosphate buffer. A final dilution (1:1600) was made by adding 200µL of the second dilution to 200µL of phosphate buffer and vortexed. Using a 96-well, white flat bottomed, microtitre plate Polysorp®-Nunc (Sigma-Aldrich, SA), 300µL of phosphate buffer was added to wells A₁ and A₂ as the blank, with 200µL of phosphate buffer in A₃ and A₄. To wells A₅ and A₆, 100µL of phosphate buffer was added. In wells A₇ to B₈, 50µL of phosphate buffer as well as 50µL of their respective standards (Appendix IV) were added. All the wine samples were then added into the remaining wells B₉ and H₁₂ (with the number of wells used dependent on the number of wine samples) in duplicates. The working solution of fluorescein was made by adding 320µL of stock 2 solution to 20mL of buffer and 100µL of the working fluorescein was added to all wells except wells A₁ and A₂. 50µL of AAPH was quickly added from wells A₅ to H₁₂. The timer was started and readings were recorded at an excitation wavelength of 485nm and emission wavelength of 520nm, using a microplate data acquisition program (Advanced Reads®) for approximately 2-hours at specified time intervals (details in Appendix IV). Data

was computed on GraphPad Prism version 6.00 for windows, GraphPad software, La Jolla, CA USA)

3.7 STATISTICAL ANALYSIS

The data obtained was entered and analysed using GraphPad Prism version 6.00 for windows, GraphPad software, La Jolla, CA USA). ANOVA was used for comparing several groups. Where significant differences were observed using the two-way ANOVA, post-hoc Bonferroni's test was used to evaluate differences within groups. The data was reported as mean $\pm$ standard error (SE) of the mean. Statistical significance was set at $p < 0.05$.

CHAPTER 4: RESULTS

All conditions regarding the experiments were optimal for the study: temperature, light/dark cycle, feeding and drinking volumes. The body masses and drinking volume of all rats were recorded daily in a monitoring sheet (Appendix V). Though 2 deaths occurred in the MCT control rats, the number of total rats that survived and used for the experiment was 35. The cause of these 2 deaths was assessed by a veterinarian and reported to the animal unit faculty and subsequently a report was drafted and submitted to the Animal Ethics Committee.

4.1 Biometric measurements

Biometric measurements taken at the end of the experiment showed MCT rats developed significant RV hypertrophy compared to controls (0.39 ± 0.03 vs 0.23 ± 0.03 g, $p=0.018$). RV hypertrophy was also seen in the MCT+RW rats compared to controls (0.44 ± 0.02 vs 0.23 ± 0.03 g, $p=0.001$) while MCT+RARW did not result in significant RV hypertrophy compared to the controls (0.29 ± 0.04 vs 0.23 ± 0.03 g, $p>0.05$). However, MCT+RARW significantly decreased RV hypertrophy compared to the MCT+RW (0.29 ± 0.04 vs 0.44 ± 0.03 g, $p=0.023$). MCT and MCT+RW rats showed a significant decrease in LV+ septal weight to heart ratio compared to controls (0.52 ± 0.01 vs 0.59 ± 0.02 , $p=0.048$; 0.50 ± 0.02 vs 0.59 ± 0.02 , $p=0.008$) respectively and increased the RV to LV+ septal weight ratio (0.45 ± 0.02 vs 0.24 ± 0.03 , $p=0.0004$; 0.52 ± 0.04 vs 0.24 ± 0.03 , $p<0.001$) respectively when compared to the controls. Interestingly MCT+RARW increased the LV+ septal weight to heart ratio compared with MCT rats (0.61 ± 0.02 vs 0.52 ± 0.01 , $p=0.005$) and compared with MCT+RW rats (0.61 ± 0.02 vs 0.50 ± 0.02 , $p=0.0005$) respectively. Again, MCT+RARW decreased the RV to LV+ septal weight ratio in both MCT rats (0.32 ± 0.03 vs 0.45 ± 0.02 , $p=0.038$) and MCT+RW rats (0.32 ± 0.03 vs 0.52 ± 0.04 , $p=0.0002$) respectively (Table 4).

Table 4: Biometric measurements

	C	MCT	RARW	MCT+RARW	RW	MCT+RW
Body Mass (g)	311.9 ± 21.6	292.6 ± 4.2	291.0 ± 16.3	283.9 ± 12.6	314.6 ± 14.3	281.5 ± 10.7
Whole Heart (g)	1.59 ± 0.11	1.65 ± 0.08	1.44 ± 0.07	1.47 ± 0.10	1.63 ± 0.05	1.70 ± 0.06
RV (g)	0.23 ± 0.03	0.39 ± 0.03*	0.23 ± 0.04	0.29 ± 0.04‡	0.28 ± 0.01	0.44 ± 0.02*
Liver (g)	18.18 ± 1.30	17.29 ± 0.51	17.49 ± 0.62	17.13 ± 0.89	20.01 ± 1.17	16.03 ± 0.87
Tibia length (cm)	4.80 ± 0.20	4.81 ± 0.07	5.02 ± 0.02	4.64 ± 0.12	5.13 ± 0.12	4.64 ± 0.05
LV + Septal Weight (g)	0.93 ± 0.09	0.86 ± 0.03	0.91 ± 0.03	0.89 ± 0.06	1.01 ± 0.09	0.84 ± 0.04
(LV + Septal Weight)/Heart (g/g)	0.59 ± 0.02	0.52 ± 0.01*	0.64 ± 0.02	0.61 ± 0.02†‡	0.62 ± 0.05	0.50 ± 0.02*
Heart Weight/Tibia Length (g/cm)	0.33 ± 0.03	0.34 ± 0.02	0.29 ± 0.01	0.32 ± 0.02	0.32 ± 0.02	0.37 ± 0.01
RV/ (LV + Septal Weight) (g/g)	0.24 ± 0.03	0.45 ± 0.02*	0.25 ± 0.03	0.32 ± 0.03†‡	0.28 ± 0.02	0.52 ± 0.04*
Heart Weight/Body Weight (g/g)	0.0052 ± 0.0008	0.0057 ± 0.0003	0.0050 ± 0.0002	0.0052 ± 0.0004	0.0052 ± 0.0002	0.0061 ± 0.0002
Liver Weight/Body Weight (g/g)	0.061 ± 0.009	0.059 ± 0.002	0.060 ± 0.002	0.060 ± 0.001	0.064 ± 0.003	0.057 ± 0.002

C: Control, MCT: Monocrotaline, RARW: Reduced-alcohol red wine, RW: Regular wine. *p< 0.05 (vs C); †p<0.05 (vs MCT); ‡p<0.05 (vs MCT+RW), n ≥ 4 per group. Values are mean ± standard error of the mean.

4.1.1 Drinking volume of water, reduced alcohol and regular wine

Rats were divided into 3 groups: those receiving water, those receiving RARW and those receiving RW. Their drinking patterns were recorded daily for 35 days. In week 1 only, rats consumed more RW than water (170 ± 13 vs 124 ± 5 mL, $p=0.003$) and RARW (170 ± 13 vs 121 ± 5 mL, $p=0.0011$). On average the rats weekly consumed, 120 ± 3 mL of water, 109 ± 4 mL of RARW and 143 ± 7 mL of RW ($p>0.05$) as indicated in Figure 10.

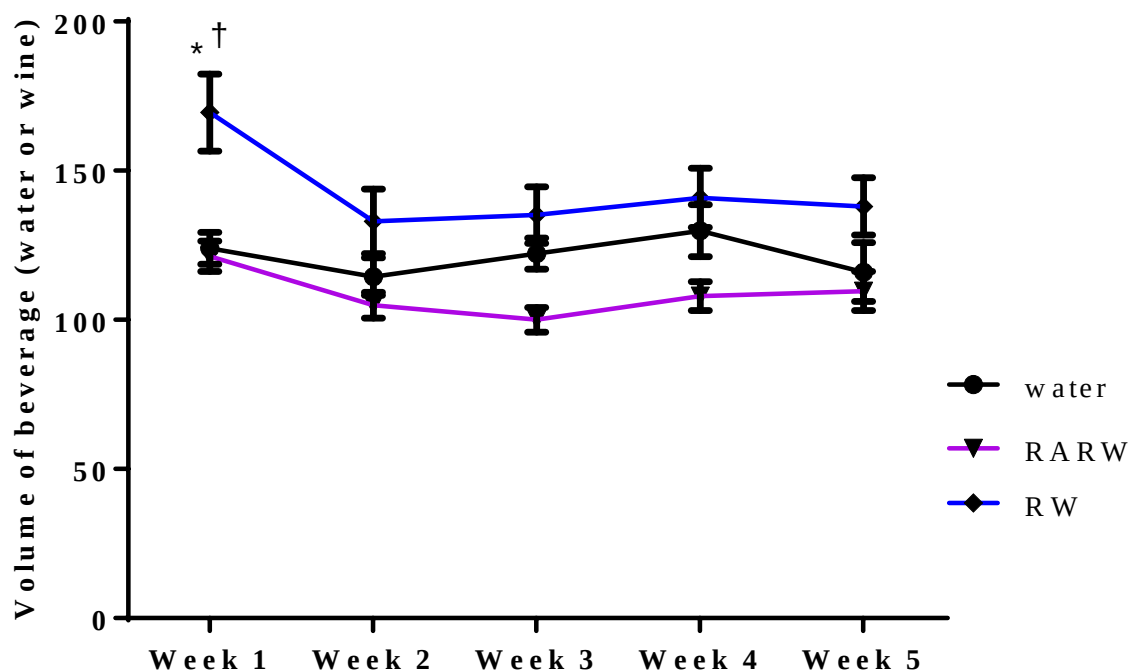


Figure 10: Drinking patterns of the rats over the experimental period

RARW: Reduced-alcohol red wine, RW: Regular wine.

* $p < 0.05$ (vs Water); † $p < 0.05$ (vs RARW). $n \geq 4$ per group. The results represent pooled data for the three groups. Values are mean $\pm$ standard error of the mean.

4.2 Echocardiography measurements

Echocardiography measurements taken at the end of the study period showed RARW treated controls and RW treated controls had similar levels compared to untreated controls (Figures 22 and 23) and (Table 5). MCT rats had significant decrease in PAAT compared to controls (13.95 ± 0.95 vs 23.43 ± 1.64 ms, $p=0.0001$). The MCT+RW rats compared to controls (14.58 ± 1.20 vs 23.43 ± 1.64 ms, $p=0.0003$) and MCT+RARW compared to the controls (18.93 ± 1.80 vs 23.43 ± 1.64 ms, $p=0.0391$) showed similar trends. However, RARW treatment increased PAAT in both MCT (18.93 ± 1.80 vs 13.95 ± 0.95 ms, $p=0.0193$) and MCT+RW groups (18.93 ± 1.80 vs 14.58 ± 1.20 ms, $p=0.0382$). MCT rats had a significant decrease in PAAT/ejection fraction compared to controls (0.18 ± 0.02 vs 0.32 ± 0.18 ms, $p=0.0004$). A similar trend was seen in the MCT+RW rats compared to controls (0.19 ± 0.01 vs 0.32 ± 0.18 ms, $p=0.0012$) and MCT+RARW compared to the controls (0.25 ± 0.03 vs 0.32 ± 0.18 ms, $p=0.0391$). MCT treatment with RARW ameliorated the harm compared to both MCT (0.25 ± 0.03 vs 0.18 ± 0.02 ms, $p=0.0193$) and MCT+RW groups (0.25 ± 0.03 vs 0.19 ± 0.01 ms, $p=0.038$). Levels of maximum velocity were significantly decreased in both MCT (978 ± 62 vs 1235 ± 71 m/s, $p=0.0076$) and MCT+RW rats (975 ± 72 vs 1235 ± 71 m/s, $p=0.0069$) compared to controls. Also, the VTI was significantly decreased in both MCT (36 ± 5 vs 56 ± 2 cm, $p=0.0029$) and MCT+RW rats (33 ± 3 vs 56 ± 2 cm, $p=0.0006$) compared to controls.

Table 5: Echocardiography measurements

	C	MCT	RARW	MCT+RARW	RW	MCT+RW
Heart rate	414 ± 26	362 ± 22	415 ± 39	397 ± 18	366 ± 11	392 ± 20
Left ventricle						
IVS, d	1.75 ± 0.08	1.56 ± 0.23	1.61 ± 0.06	1.59 ± 0.09	1.72 ± 0.06	1.70 ± 0.05
IVS, s	3.08 ± 0.14	2.85 ± 0.30	2.82 ± 0.07	2.75 ± 0.27	2.86 ± 0.12	2.76 ± 0.18
LVID, d	8.28 ± 0.32	7.90 ± 0.32	8.19 ± 0.29	8.12 ± 0.34	8.72 ± 0.17	7.45 ± 0.32
LVID, s	4.98 ± 0.41	4.86 ± 0.46	5.02 ± 0.27	4.80 ± 0.37	5.20 ± 0.28	4.45 ± 0.34
LVPW, d	1.79 ± 0.16	1.79 ± 0.16	1.81 ± 0.12	1.73 ± 0.09	1.81 ± 0.10	2.00 ± 0.10
LVPW, s	2.76 ± 0.29	2.78 ± 0.20	2.84 ± 0.21	2.66 ± 0.15	2.98 ± 0.11	3.05 ± 0.15
Shortening fraction	40 ± 3	35 ± 19	39 ± 2	44 ± 3	41 ± 2	41 ± 3
Ejection fraction	67 ± 4	68 ± 5	67 ± 2	72 ± 3	69 ± 3	69 ± 4
Mitral flow						
Peak E	1143 ± 178	979 ± 89	1127 ± 97	1080 ± 57	1101 ± 114	1035 ± 91
Peak A	1040 ± 193	871 ± 53	855 ± 79	987 ± 71	754 ± 50	1003 ± 108
E/A ratio	1.1 ± 0.1	1.1 ± 0.2	1.3 ± 0.1	1.1 ± 0.1	1.5 ± 0.2	1.2 ± 0.2
Pulmonary artery flow						
PAAT	23.43 ± 1.64	13.95 ± 0.95*	22.14 ± 1.40	18.93 ± 1.80*†‡	20.33 ± 3.07	14.58 ± 1.20*
PAAT/Ejection time	0.32 ± 0.18	0.18 ± 0.02*	0.34 ± 0.02	0.25 ± 0.03*†‡	0.33 ± 0.01	0.19 ± 0.01*
Max velocity	1235 ± 71	978 ± 62*	1218 ± 54	1111 ± 32	1301 ± 80	975 ± 72*
VTI	56 ± 2	36 ± 5*	53 ± 2	46 ± 2	53 ± 3	33 ± 3*

Heart rate (bpm); IVS, d: inter-ventricular septum, diastole (mm); IVS, s: inter-ventricular septum, systole (mm); LVID, d: left ventricle internal diameter, diastole (mm); LVID, s: left ventricle internal diameter, diastole (mm); LVPW, d: left ventricle posterior wall, diastole (mm); LVPW, s: left ventricle posterior wall, systole (mm); Shortening fraction (%); Ejection fraction (%); Peak E: maximum velocity of mitral E wave (m/s); Peak A: maximum velocity of mitral A wave (m/s); PAAT: pulmonary artery acceleration time (ms); VTI: velocity time integral (cm); C: Control, MCT: Monocrotaline, RARW: Reduced-alcohol red wine, RW: Regular wine. *p< 0.05 (vs C); †p<0.05 (vs MCT); ‡p<0.05 (vs MCT+RW), n ≥ 4 per group. Values are mean ± standard error of the mean.

4.2.1 Echocardiography changes in RV Thickness

Right ventricular thickness was significantly increased in all MCT treated groups compared to controls (Figure 11). MCT vs controls (1.22 ± 0.01 vs 0.45 ± 0.02 mm, $p < 0.0001$), MCT+RARW vs controls (0.85 ± 0.07 vs 0.45 ± 0.02 mm, $p = 0.0009$) and MCT+RW vs controls (1.26 ± 0.01 vs 0.45 ± 0.02 mm, $p < 0.0001$). MCT treatment with RARW significantly decreased the levels of RV thickness compared to MCT (0.85 ± 0.07 vs 1.22 ± 0.10 mm, $p = 0.0009$) and compared to MCT+RW rats (0.85 ± 0.07 vs 1.26 ± 0.01 mm, $p = 0.0005$).

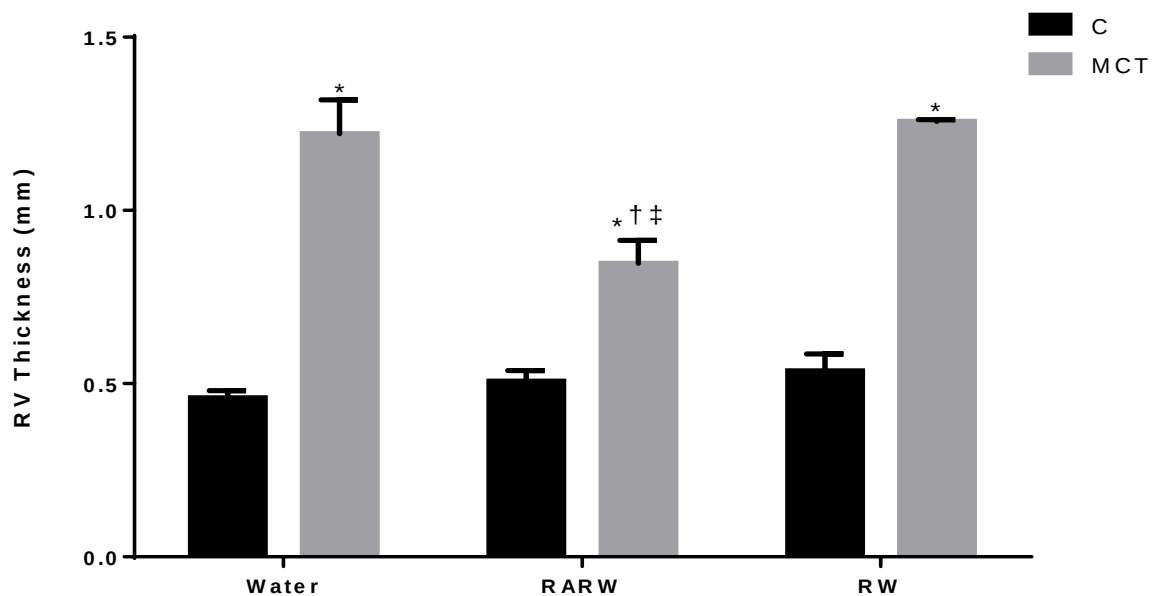


Figure 11: Effect of moderate, chronic treatment of RARW and RW on the right ventricular free wall in MCT-induced PAH.

C: Control, MCT: Monocrotaline, RARW: Reduced-alcohol red wine, RW: Regular wine, RV: Right ventricle. * $p < 0.05$ (vs C); † $p < 0.05$ (vs MCT); ‡ $p < 0.05$ (vs MCT+RW), $n \geq 4$ per group. Values are mean $\pm$ standard error of the mean.

4.2.2 Echocardiography changes in RV diameter/LV diameter

Echocardiography measurements showed RV diameter/LV diameter ratio was higher in MCT vs controls (1.13 ± 0.14 vs 0.31 ± 0.02 , $p < 0.0001$), MCT+RARW vs controls (0.78 ± 0.09 vs 0.31 ± 0.02 , $p < 0.0001$) and MCT+RW vs controls (1.17 ± 0.14 vs 0.31 ± 0.02 , $p = 0.0268$). MCT treatment with RARW showed significant decrease in the levels of RV diameter/LV diameter when compared to MCT (0.78 ± 0.09 vs 1.13 ± 0.14 , $p = 0.0411$) and MCT+RW rats (0.78 ± 0.09 vs 1.17 ± 0.14 , $p = 0.0169$) in Figure 12.

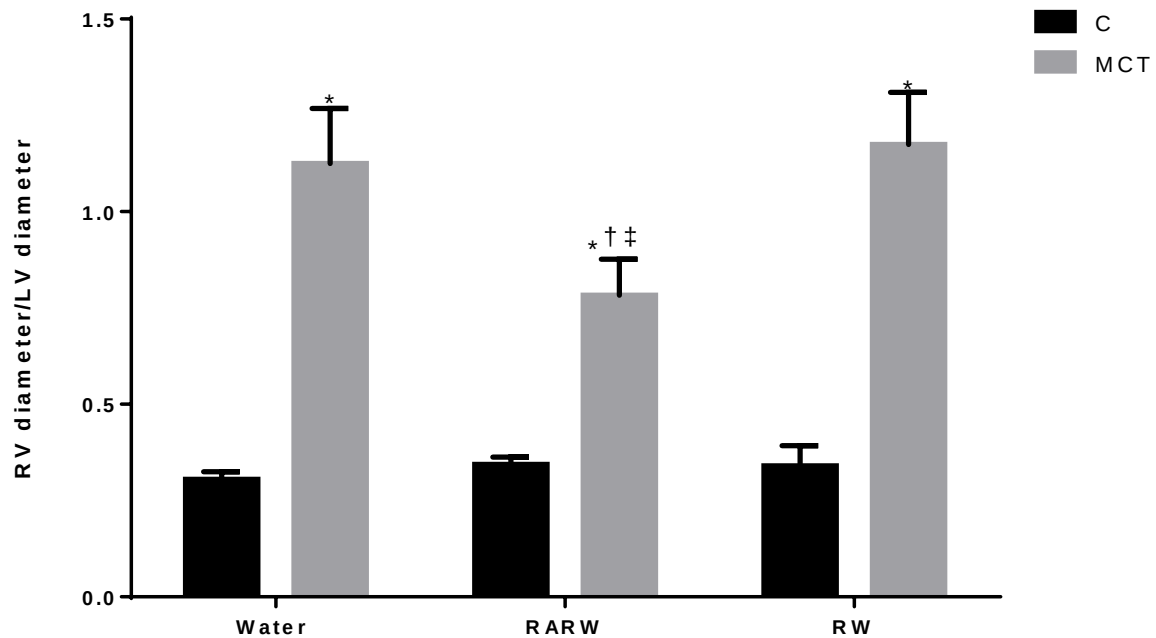


Figure 12: Effect of moderate, chronic treatment of RARW and RW on the ratio of the RV diameter to LV diameter in MCT-induced PAH.

C: Control, MCT: Monocrotaline, RARW: Reduced-alcohol red wine, RW: Regular wine, RV: Right ventricle, LV: Left ventricle. * $p < 0.05$ (vs C); † $p < 0.05$ (vs MCT); ‡ $p < 0.05$ (vs MCT+RW), $n \geq 4$ per group. Values are mean $\pm$ standard error of the mean.

4.2.3 Echocardiography changes in Tricuspid Annular Plane Systolic Excursion (TAPSE)

MCT rats had a significant decrease in TAPSE compared to controls (2.68 ± 0.21 vs 3.88 ± 0.19 mm, $p=0.0118$). A significant decrease was also observed between MCT+RW and controls groups (2.14 ± 0.30 vs 3.88 ± 0.19 mm, $p=0.0001$). MCT treatment with RARW significantly increased the levels of TAPSE when compared to MCT (3.69 ± 0.20 vs 2.68 ± 0.21 mm, $p=0.0280$) and MCT+RW rats (3.69 ± 0.20 vs 2.14 ± 0.30 mm, $p=0.0002$) in Figure 13.

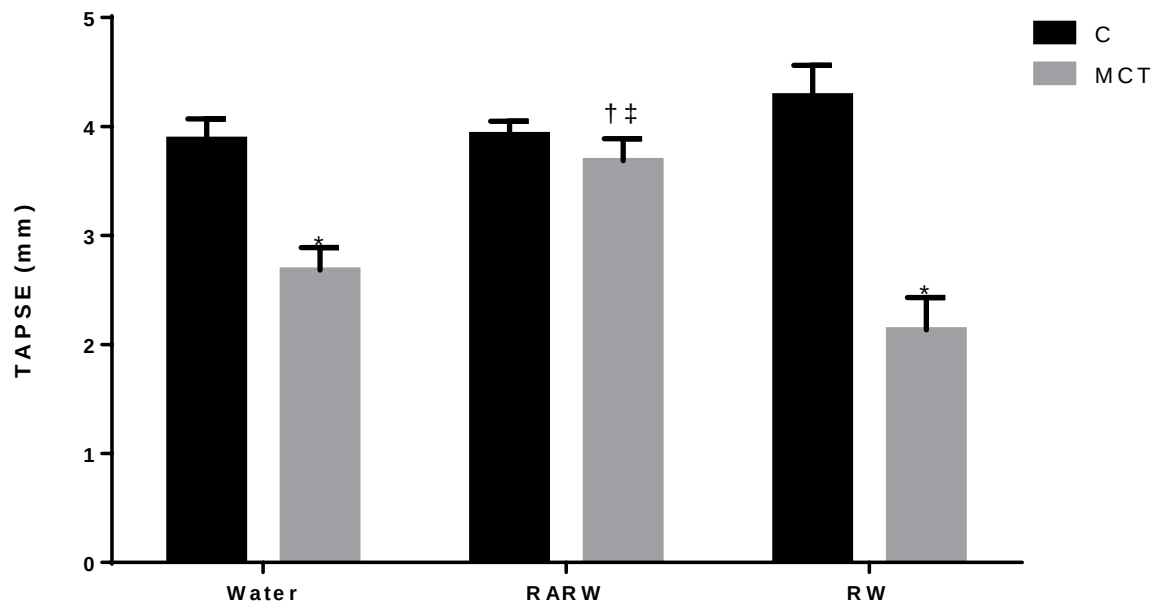


Figure 13: Effect of moderate, chronic treatment of RARW and RW on the Tricuspid Annular Plane Systolic excursion in MCT-induced PAH.

C: Control, MCT: Monocrotaline, RARW: Reduced-alcohol red wine, RW: Regular wine, TAPSE: Tricuspid annular plane systolic excursion. * $p < 0.05$ (vs C); † $p < 0.05$ (vs MCT); ‡ $p < 0.05$ (vs MCT+RW), $n \geq 4$ per group. Values are mean $\pm$ standard error of the mean.

4.3 Plasma lipid peroxidation markers and antioxidant activity

4.3.1 Conjugated dienes

Blood plasma was analysed for conjugated diene (CD) concentrations in all groups (Figure 14). Concentrations were significantly increased in MCT vs controls (671.60 ± 42.53 vs 453.10 ± 34.76 nmol/mL, $p=0.0039$) while MCT treatment with RARW significantly decreased CD compared to MCT rats (471.60 ± 27.45 vs 671.60 ± 42.53 nmol/mL, $p=0.0041$).

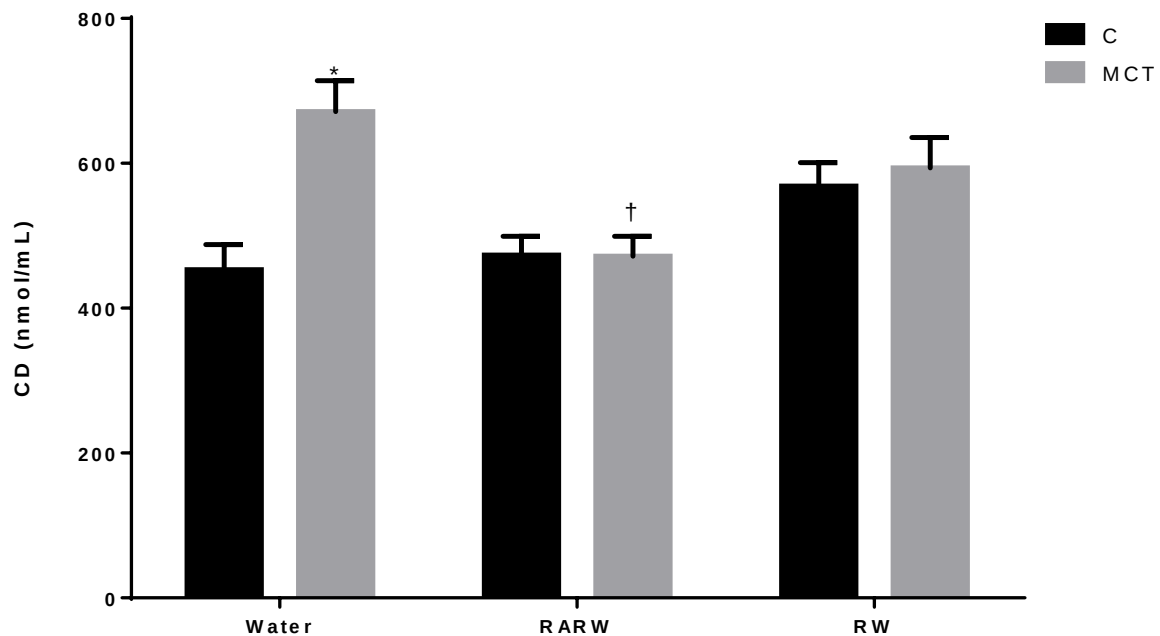


Figure 14: Effect of moderate, chronic treatment of RARW and RW on plasma conjugated diene in MCT-induced PAH.

C: Control, MCT: Monocrotaline, RARW: Reduced-alcohol red wine, RW: Regular wine, CD: Conjugated diene.

* $p < 0.05$ (vs C); † $p < 0.05$ (vs MCT); $n \geq 4$ per group. Values are mean $\pm$ standard error of the mean.

4.3.2 Thiobarbituric acid reactive substances (TBARS)

Thiobarbituric acid reactive substance concentrations in blood plasma were significantly increased in MCT vs control (10.63 ± 2.92 vs 4.19 ± 0.25 $\mu\text{mol/L}$, $p=0.0454$). The consumption of RARW and RW by control rats had no significant effect on TBARS (4.27 ± 0.38 vs 4.19 ± 0.25 $\mu\text{mol/L}$, $p>0.05$; 4.27 ± 0.36 vs 4.19 ± 0.25 $\mu\text{mol/L}$, $p>0.05$) respectively in Figure 15.

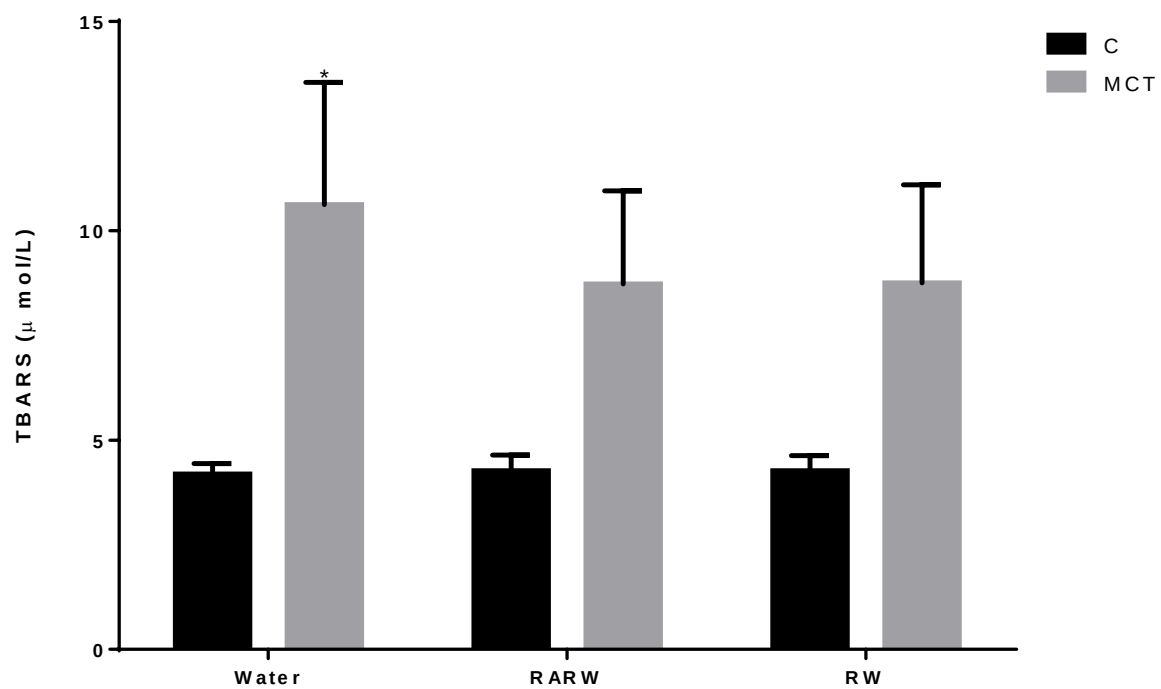


Figure 15: Effect of moderate, chronic treatment of RARW and RW on plasma TBARS in MCT-induced PAH.

C: Control, MCT: Monocrotaline, RARW: Reduced-alcohol red wine, RW: Regular wine, TBARS: Thiobarbituric acid reactive substances. * $p < 0.05$ (vs C); $n \geq 4$ per group. Values are mean $\pm$ standard error of the mean.

4.3.3 Antioxidant activity of superoxide dismutase (SOD)

No significant difference in SOD activity was found between the MCT rats and controls (66.84 ± 12.54 vs 83.67 ± 27.06 IU/mg protein, $p > 0.05$). In RARW treated controls with and without MCT, the SOD activity seemed to decrease but this was not significant. RW had no significant effect on SOD activity. Figure 16.

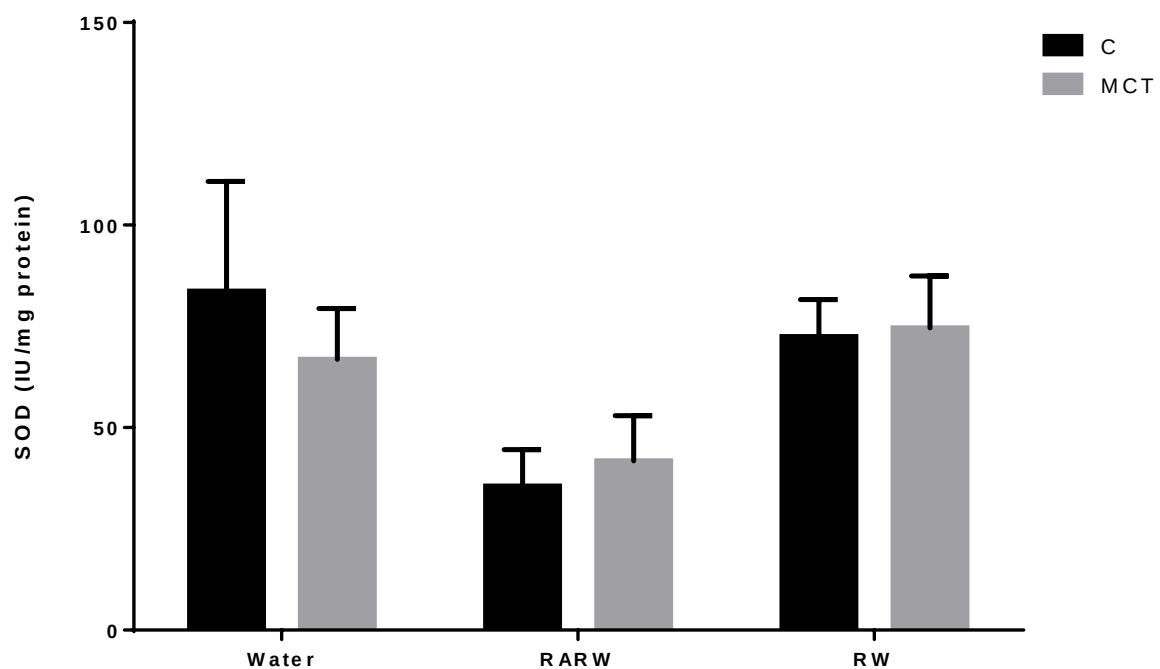


Figure 16: Effect of moderate, chronic treatment of RARW and RW on plasma SOD in MCT-induced PAH.

C: Control, MCT: Monocrotaline, RARW: Reduced-alcohol red wine, RW: Regular wine, SOD: Superoxide dismutase. $p > 0.05$; $n \geq 4$ per group. Values are mean $\pm$ standard error of the mean.

4.3.4 Antioxidant activity of catalase

RARW treated controls and RW treated controls had similar levels of catalase activity compared to untreated controls (0.89 ± 0.31 vs 1.09 ± 0.38 IU/mg protein, $p>0.05$; 0.75 ± 0.49 vs 1.09 ± 0.38 IU/mg protein, $p>0.05$) respectively. Catalase activity seems to be higher in MCT compared to controls (2.02 ± 0.75 vs 1.09 ± 0.38 IU/mg protein, $p>0.05$) but was not statistically significantly different. MCT treated with RW had similar catalase activity compared to MCT rats (2.13 ± 1.15 vs 2.02 ± 0.75 IU/mg protein, $p>0.05$) shown in Figure 17.

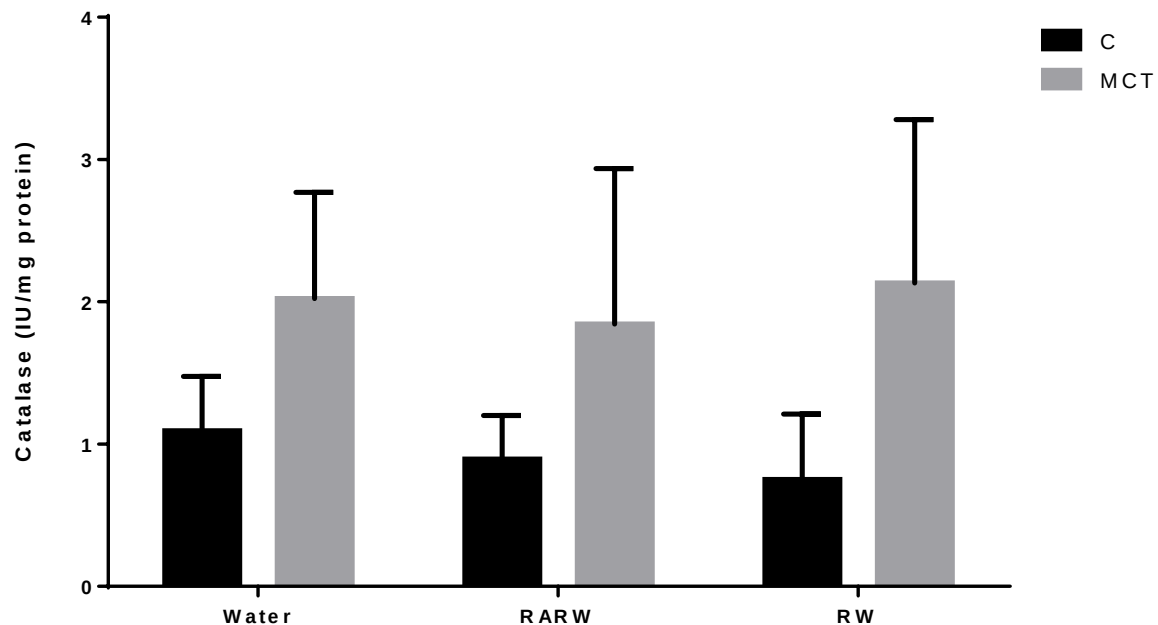


Figure 17: Effect of moderate, chronic treatment of RARW and RW on plasma antioxidant catalase in MCT-induced PAH.

C: Control, MCT: Monocrotaline, RARW: Reduced-alcohol red wine, RW: Regular wine. $p>0.05$; $n \geq 4$ per group. Values are mean $\pm$ standard error of the mean.

4.3.5 Oxygen radical absorbance capacity (ORAC) activity

MCT rats had decreased ORAC activity compared to controls (2.10 ± 0.72 vs 3.13 ± 0.46 nmol/L trolox equivalents, $p>0.05$). MCT rats treated with RARW had similar ORAC values compared to controls (2.53 ± 0.58 vs 3.13 ± 0.46 nmol/L trolox equivalents, $p>0.05$). MCT rats treated with RW had similar ORAC activity compared to MCT rats (1.85 ± 0.58 vs 2.10 ± 0.72 nmol/L trolox equivalents, $p>0.05$) in Figure 18.

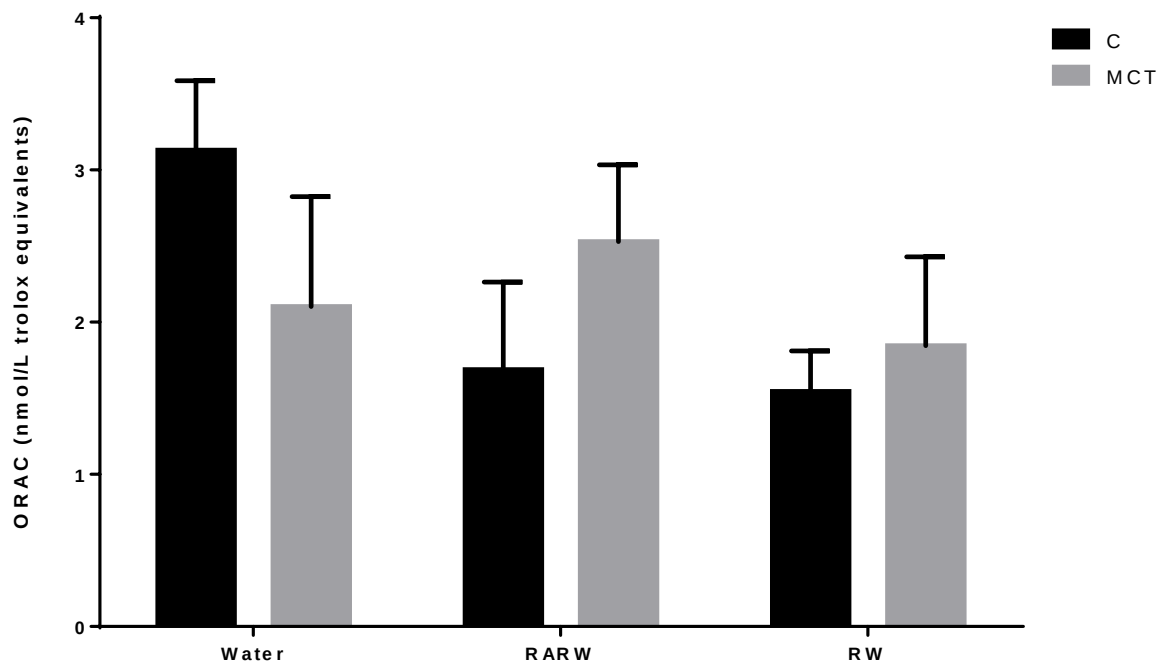


Figure 18: Effect of moderate, chronic treatment of RARW and RW on plasma oxygen radical absorbance capacity in MCT-induced PAH.

C: Control, MCT: Monocrotaline, RARW: Reduced-alcohol red wine, RW: Regular wine, ORAC: oxygen radical absorbance capacity. $p>0.05$; $n \geq 4$ per group. Values are mean $\pm$ standard error of the mean.

4.4 Lipid concentrations

Lipid concentrations in rats were measured in serum for triglyceride (TG), phospholipid (PL), total cholesterol (TC), total high-density lipoprotein cholesterol (HDL-C), high-density lipoprotein-3 cholesterol (HDL₃) and high-density lipoprotein-2 cholesterol (HDL₂). There was no statistical significant difference between TG, PL, TC, HDL-C, HDL₃-C, HDL₂-C groups treated with MCT, MCT+RARW, MCT+RW and the control group for the lipids. Within the total cholesterol group, MCT appears to be increased compared to the control group (18.17 ± 0.96 vs 14.42 ± 0.78 $\mu\text{g}/\mu\text{L}$, $p>0.05$). MCT treated with RARW showed no significant difference compared to the MCT group (14.38 ± 1.03 vs 18.17 ± 0.96 $\mu\text{g}/\mu\text{L}$, $p>0.05$) Figure 19.

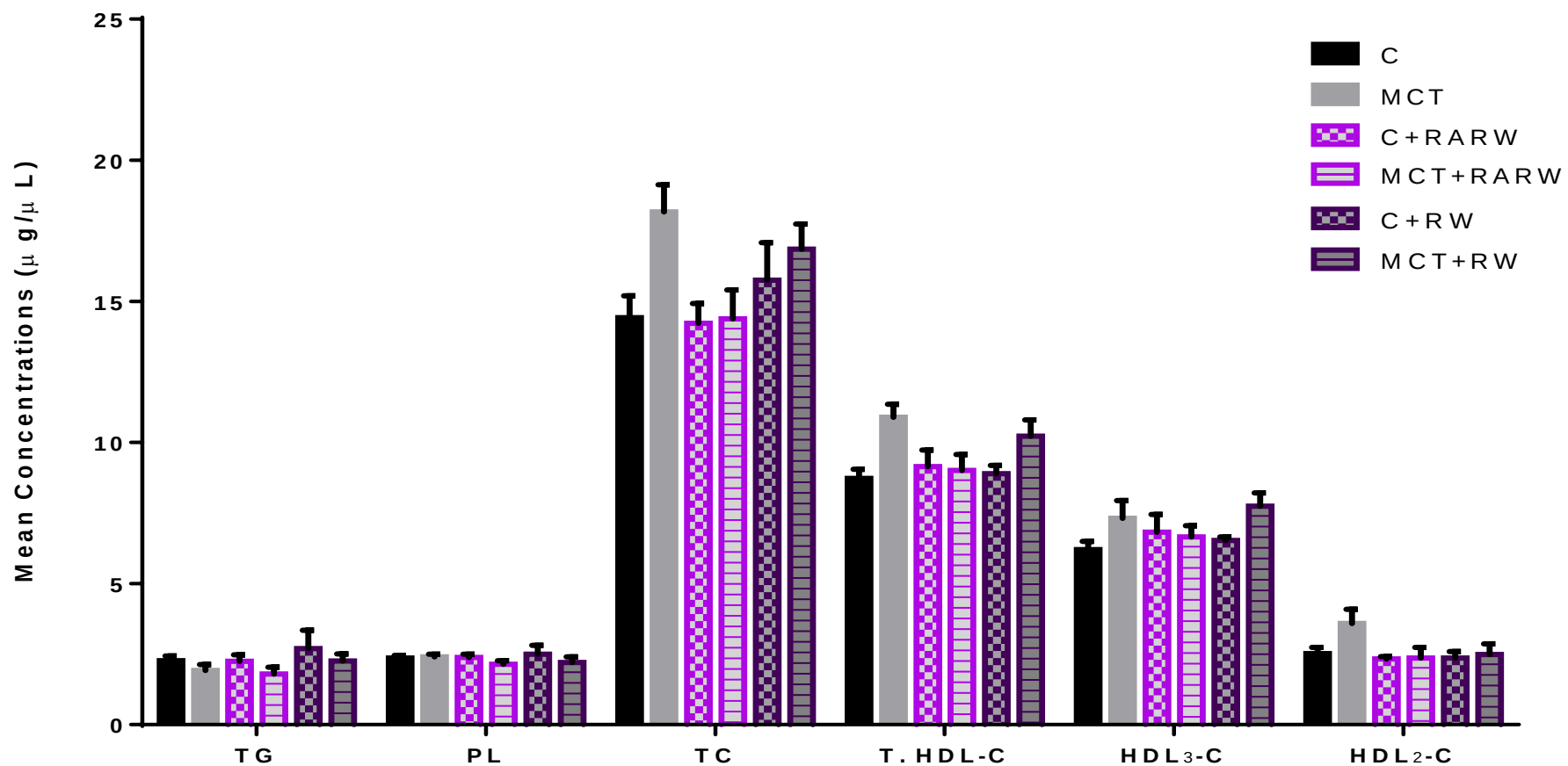


Figure 19: Effect of moderate, chronic treatment of RARW and RW on serum lipids in MCT-induced PAH.

C: Control, MCT: Monocrotaline, RARW: Reduced-alcohol red wine, RW: Regular wine, TG: Triglyceride, PL: Phospholipid, TC: Total cholesterol, T. HDL-C: Total high-density lipoprotein cholesterol, HDL₃-C: High density lipoprotein-3 cholesterol, HDL₂-C: High density lipoprotein-2 cholesterol. $p > 0.05$; $n \geq 4$ per group. Values are mean $\pm$ standard error of the mean.

4.5 Determination of cardiac fibrosis

There was no statistically significant difference between the percentage of collagen deposits in MCT rats compared to the control rats (0.92 ± 0.13 vs 0.73 ± 0.42 %, $p > 0.05$). MCT treatment with RARW was similar to untreated control (0.56 ± 0.14 vs 0.73 ± 0.42 %, $p > 0.05$). Also, MCT had a greater percentage of collagen deposits in the RV compared to MCT+RW rats (0.92 ± 0.13 vs 0.64 ± 0.15 %, $p > 0.05$) but this was not significantly different. Comparison of the percentage of collagen deposits in the RV to the LV showed no significant difference as indicated in Figures 20 and 21.

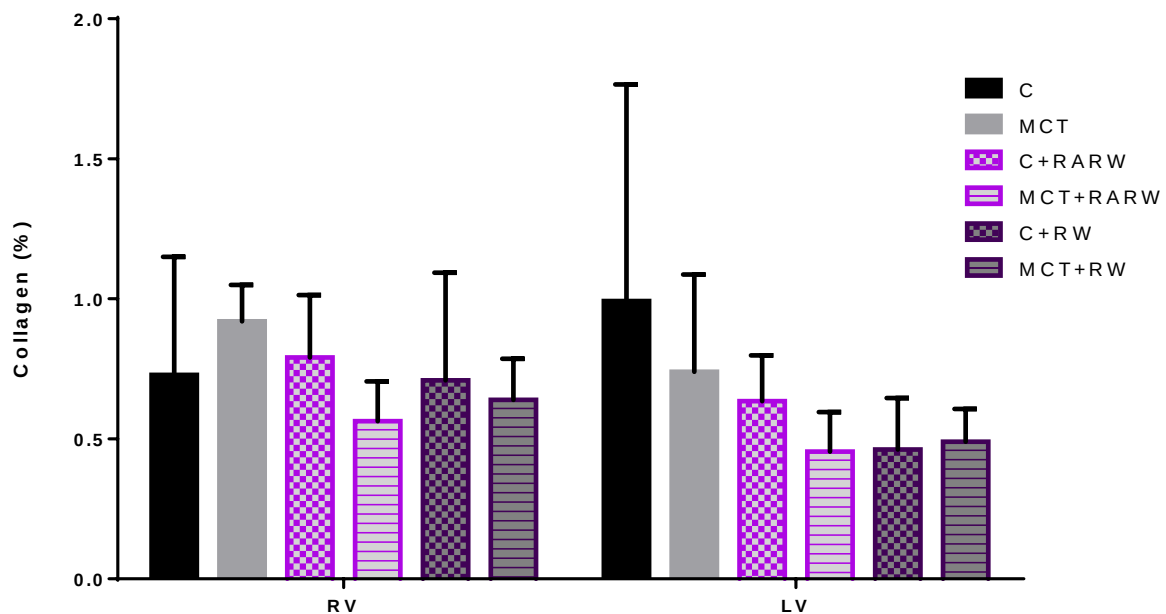


Figure 20: Effect of moderate, chronic treatment of RARW and RW on cardiac fibrosis in MCT-induced PAH.

C: Control, MCT: Monocrotaline, RARW: Reduced-alcohol red wine, RW: Regular wine, RV: Right ventricle, LV: Left ventricle. $p > 0.05$; $n \geq 4$ per group. Values are mean $\pm$ standard error of the mean.

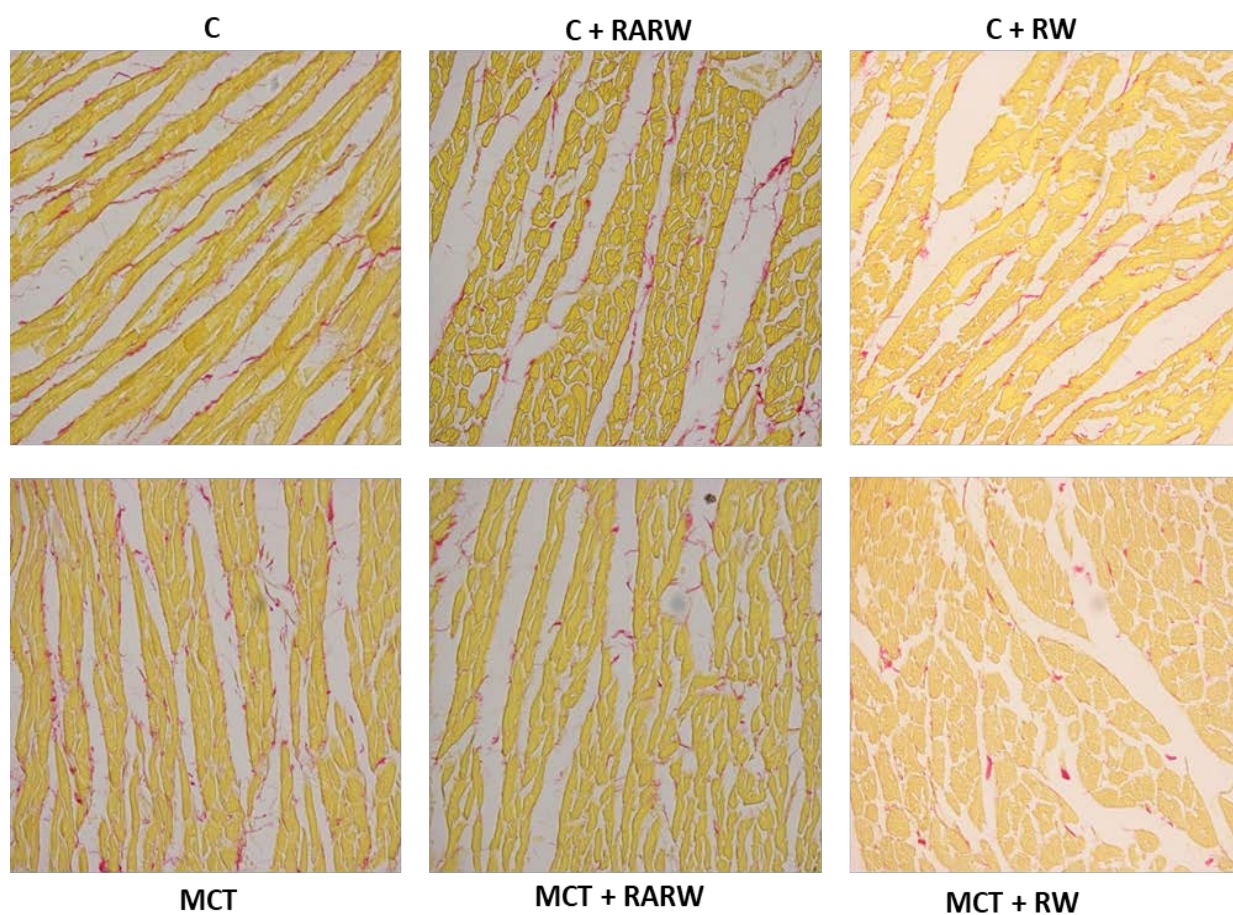


Figure 21: Histology section of hearts (RV) stained with 0.1% sirius red in picric acid to access cardiac fibrosis at 20X magnification.

C: Control, MCT: Monocrotaline, RARW: Reduced-alcohol red wine, RW: Regular wine, RV: Right ventricle.
 $p > 0.05$; $n \geq 4$ per group.

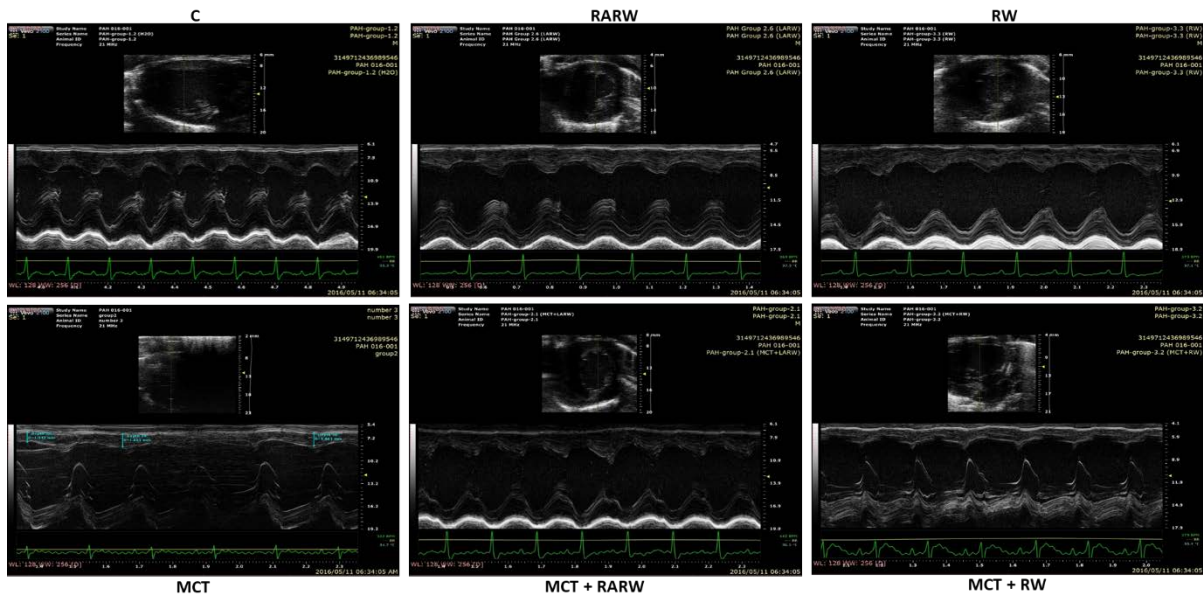


Figure 22: M-mode echocardiography analysis for RV and LV wall thickness.

Representative images of echocardiography for measuring RV and LV free wall thickness in Control, MCT, RARW, RARW+MCT, RW and RW+MCT rats in a PAH model. Data are from Long Evans control rats treated with monocrotaline, RARW and/or RW. RV: right ventricle; LV: Left ventricle.

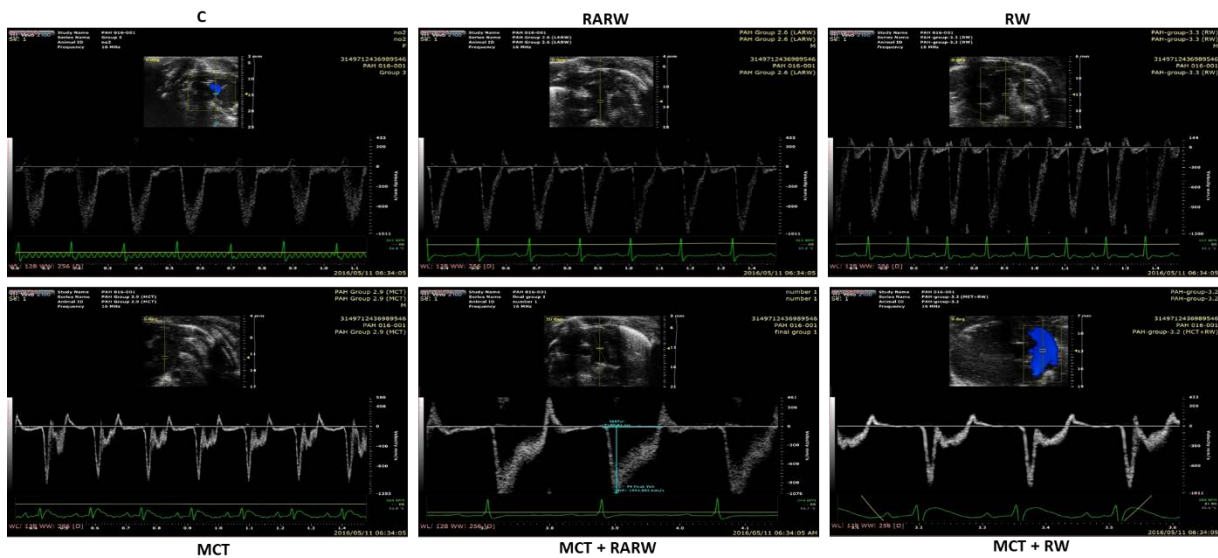


Figure 23: Pulse wave (PW) Doppler recordings.

Representative pulse wave (PW) echocardiography in control rats, MCT, RARW, RARW+MCT, RW and RW+MCT rats with PAH. Data are from control rats and post MCT rats at day 28.

4.6 Wine stability (for regular and reduced-alcohol red wine)

At the end of the 4 weeks the average mean concentration of total phenolic compounds in RARW compared with RW was 291.90 ± 10.42 vs 235.80 ± 9.22 mg/L, $p=0.006$; the concentration of the anthocyanins was 190.00 ± 3.53 vs 172.20 ± 5.13 mg/L M-3-G, $p=0.0008$, that of catechin was 12.06 ± 0.31 vs 10.26 ± 0.19 mg/L, $p=0.157$, and the ORAC values were 32.55 ± 2.75 vs 26.55 ± 2.37 nmol/L trolox equivalents, $p=0.983$ respectively.

4.6.1 Determination of concentration of total phenolic compounds in regular and reduced-alcohol red wine

Reduced alcohol red wine had higher total phenol concentration values compared to regular wine, and this was consistent over 4 weeks. In week 1, concentrations of total phenolic compounds in RARW was higher compared to RW (293.99 ± 7.49 vs 238.64 ± 3.99 mg/L, $p<0.0001$). In week 2, concentrations of total phenolic compounds in RARW was greater compared to RW (305.52 ± 2.06 vs 259.51 ± 2.18 mg/L, $p<0.0001$). A similar trend was observed in the 3rd week with increased concentrations of total phenolic compounds in RARW compared to RW (306.19 ± 8.85 vs 229.52 ± 3.79 mg/L, $p<0.0001$). In week 4, RARW showed a significant difference in concentration compared to the RW (261.73 ± 3.71 vs 215.52 ± 2.16 mg/L, $p<0.0001$) as shown in Figure 24. A coefficient of variation (CV) of 7.14% and 7.82% was determined for RARW and RW respectively.

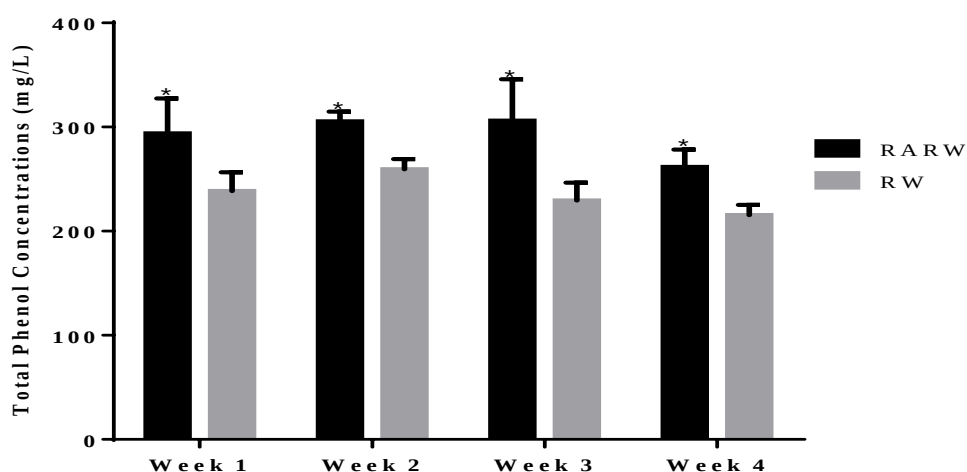


Figure 24: Total phenol concentration in RARW and RW over 4 weeks.

RARW: Reduced-alcohol red wine, RW: Regular red wine. * $p<0.05$ (vs RW). Values are mean $\pm$ standard error of the mean.

4.6.2 Determination of concentration of anthocyanins in regular and reduced-alcohol red wine

Reduced alcohol red wine had higher anthocyanin concentration values compared to regular wine over 4 weeks. In week 1, concentrations of anthocyanins in RARW was higher compared to RW (198.95 ± 0.79 vs 183.57 ± 1.37 mg/L M-3-G, $p < 0.0001$). In week 2, concentrations of anthocyanins in RARW was greater compared to RW (189.57 ± 1.06 vs 178.10 ± 1.28 mg/L M-3-G, $p = 0.0003$). Similar trend was observed in the 3rd week with increased concentrations of anthocyanins in RARW compared to RW (181.69 ± 1.17 vs 163.00 ± 2.78 mg/L M-3-G, $p < 0.0001$). In week 4, RARW showed significantly difference in concentration compared to the RW (189.73 ± 1.13 vs 163.98 ± 3.08 mg/L M-3-G, $p < 0.0001$) in Figure 25. A coefficient of variation (CV) of 3.71% and 5.96% was determined for RARW and RW respectively.

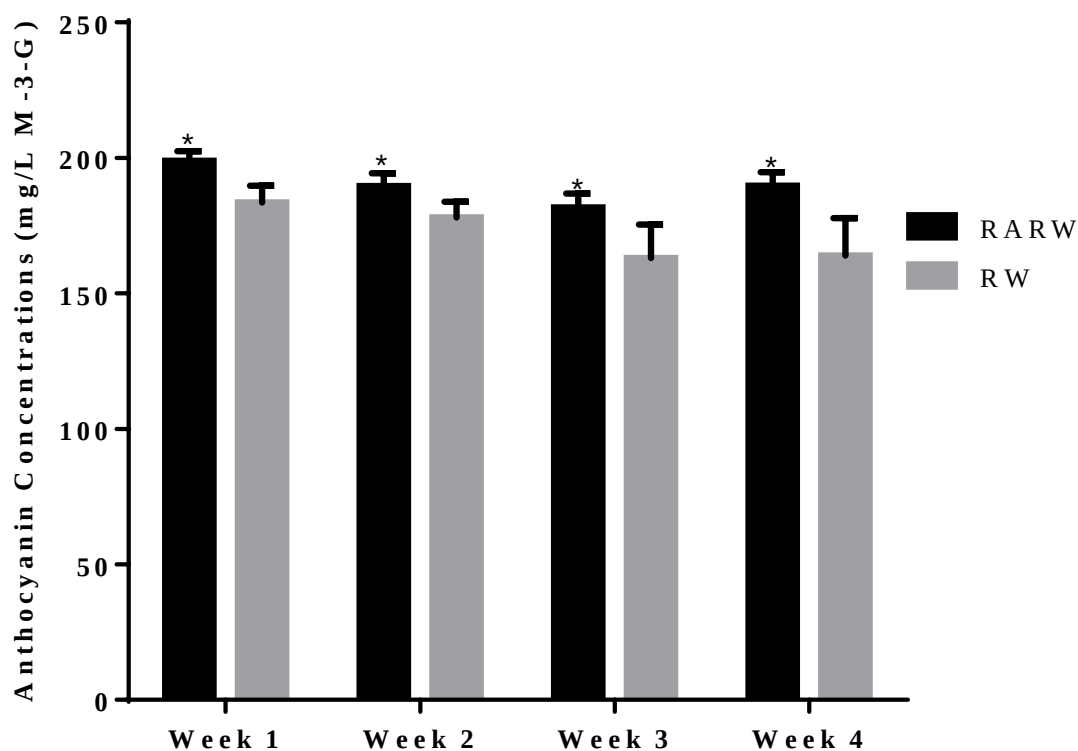


Figure 25: Anthocyanins concentration in RARW and RW over 4 weeks.

RARW: Reduced-alcohol red wine, RW: Regular red wine. * $p < 0.05$ (vs RW). Values are mean $\pm$ standard error of the mean.

4.6.3 Determination of concentration of catechins in regular and reduced-alcohol red wine

Reduced alcohol red wine had higher concentration values of catechins compared to regular wine over 4 weeks. In week 1, concentrations of catechins in RARW was higher compared to RW (12.08 ± 0.11 vs 10.19 ± 0.09 mg/L, $p < 0.0001$). In week 2, concentrations of catechins in RARW was greater compared to RW (11.19 ± 0.11 vs 9.75 ± 0.10 mg/L, $p < 0.0001$). Similar trend was observed in the 3rd week with increased concentrations of catechins in RARW compared to RW (12.36 ± 0.17 vs 10.52 ± 0.10 mg/L, $p < 0.0001$). In week 4, RARW showed significantly difference in concentration compared to the RW (12.64 ± 0.21 vs 10.59 ± 0.16 mg/L, $p < 0.0001$) in Figure 26. A coefficient of variation (CV) of 5.21% and 3.77% was determined for RARW and RW respectively.

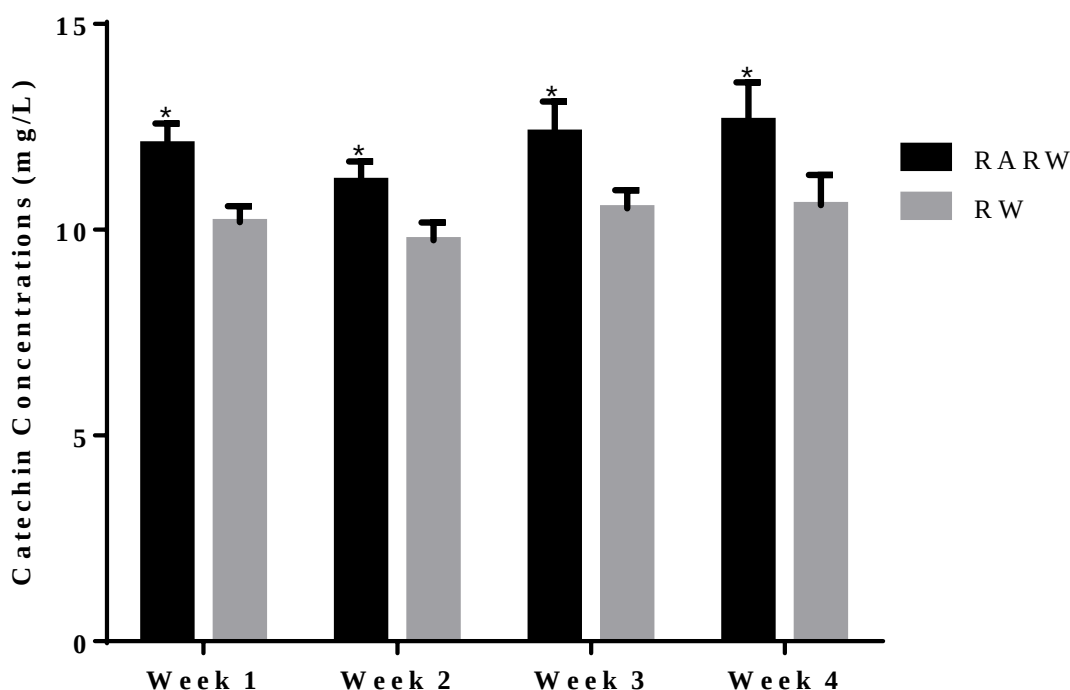


Figure 26: Catechins concentration in RARW and RW over 4 weeks.

RARW: Reduced-alcohol red wine, RW: Regular red wine. * $p < 0.05$ (vs RW). Values are mean $\pm$ standard error of the mean.

4.6.4 Oxygen radical absorbance capacity in regular and reduced-alcohol red wine

Reduced alcohol red wine had higher ORAC values compared to regular wine over 4 weeks. However, values of ORAC in both types of wine showed no significant difference over the weeks. ($p>0.05$) in Figure 27. A coefficient of variation (CV) of 16.90% and 17.82% was determined for RARW and RW respectively.

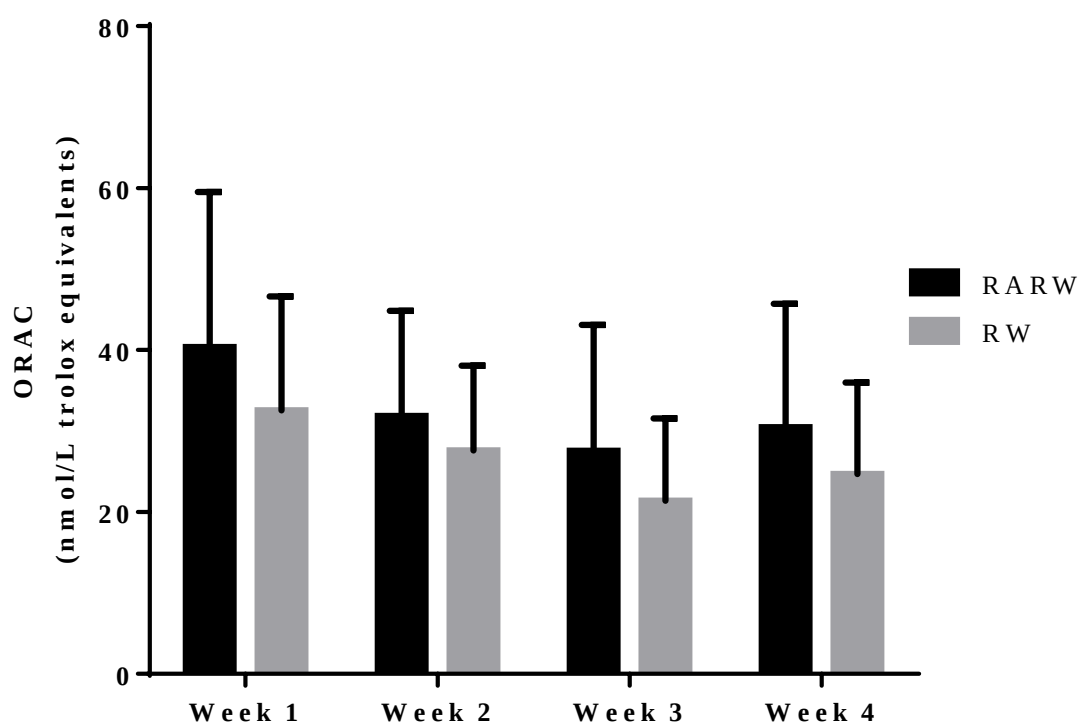


Figure 27: ORAC determination in RARW and RW over 4 weeks.

RARW: Reduced-alcohol red wine, RW: Regular red wine. * $p>0.05$ (vs RW). Values are mean $\pm$ standard error of the mean.

CHAPTER 5: DISCUSSION

5.1 Summary

In this study, it has been demonstrated that chronic consumption of RARW but not RW over 28 days by rats can potentially ameliorate adverse effects of MCT on the RV and lead to cardioprotection. This study did not support the concept of alcohol in red wine contributing to cardioprotection. Furthermore, the stability of reduced-alcohol and regular red wine over 4 weeks (determined by analysing ORAC values, total phenol concentration, anthocyanin concentration and catechin concentration) demonstrated that the wines were stable with reduced-alcohol red wine (RARW) having significant concentrations of total phenol, anthocyanin and catechin over the regular red wine (RW). Monocrotaline injected PAH rats showed signs of oxidative stress, significant RV hypertrophy and decrease of pulmonary artery flow confirmed by echocardiography. MCT increased oxidative stress by the end of the experiment but this was attenuated by consuming RARW.

5.2 Stability of regular and reduced-alcohol red wine

Wine consists of different components: polyphenolic and non-polyphenolic agents (Hugel et al., 2016). Many of these components have been extensively reviewed (Burin, Arcari, Bordignon-Luiz, & Costa, 2011; Chicoine et al., 2009; Croft, 2016) and have been shown to have antioxidant properties. The concentrations of total phenolic compounds, anthocyanins, and catechins as well as their ORAC values over the 4 weeks was done to test if their contents were stable once the bottles were opened. This study used a 1 in 7 dilution of all the wine samples to mimic what is considered moderate consumption as a daily drinking volume to the rats. RARW had significant and higher concentrations of total phenols, catechins and anthocyanins over the RW, while ORAC concentration values showed no differences. Thus, stability of the wines was not compromised by diluting out the alcohol in the wines. While one study compared the ORAC values from 12% to 6% in red wine and found no changes (Lamont et al., 2012), another also looked at stability of red wine over time by measuring ORAC and total phenols and found no changes in concentration ((Dlamini, 2015) thesis). This study however is peculiar in itself as it compares the antioxidant contents in both RARW and RW from the same winery and batch. The explanation for the differences in antioxidant measurement in both RARW and RW may be explained by several factors. Firstly, a study by Gambelli and Santaroni (2004) have shown that the differences in antioxidant concentrations

may be due to the age of the wine. Also Goldberg (1995) suggest that wine with alcohol content of 10% or more gives stability in wine as complex polymeric compounds may be broken to monomeric forms through fermentation further reducing their concentration levels. This may explain the decrease in antioxidant levels as observed in the RW. It is possible that changes in the bioavailability of polyphenols in both reduced-alcohol and regular red wine may suggest the differences in the levels of oxidative and antioxidant measurements observed (Benito, Buxaderas, & Mitjavila, 2004; Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004; Scalbert & Williamson, 2000). In fact, it was surprising to find changes in antioxidant concentrations as they were from the same winery. However, a longer study on the stability is warranted to firmly draw conclusions. The findings in this study relating to the stability of wine may explain that RARW have more antioxidants than RW and may have better cardioprotection.

5.3 Effect of chronic, moderate regular- and reduced-alcohol red wine on antioxidants and oxidative stress in PAH rats

Oxidative stress is an imbalance caused by excess reactive species that may cause damage to biological systems, requiring repair and detoxification (Bowers et al., 2004). Though the role of reactive species cannot be downplayed due to its importance in the physiology of health and disease (Wong et al., 2013) many studies have demonstrated their effects in disease progression (Zhang et al., 2015). However, not much information is available on the use of reduced-alcohol and regular red wine as therapeutic treatments and especially in PAH. In this study, MCT increased oxidative damage (TBARS and CD) in the rats. It is possible that the alcohol in the RW also increased oxidative damage but due to the lower alcohol concentration in the RARW, it did not significantly increase CD concentration. In a study conducted in male Sprague-Dawley rats, red wine with less than 3g/L (0.3%) ethanol modulated the oxidative stress and inflammatory changes and this they tested using TBARS (López et al., 2007), supporting the notion that red wine with reduced-alcohol content can be cardioprotective. Also, a recent study that used polyphenol rich powdered red wine as a treatment strategy in male hypertensive rats ameliorated oxidative damage by reducing malondialdehydes (Del Pino-García, Rivero-Perez, González-SanJosé, Croft, & Muniz, 2017). Though the study by Del Pino-García et al. (2017) did not involve alcohol, a diluted (1 in 7) RARW of 5.5% alcohol content in this study indicates a very low alcohol concentration of less than 1%. Nevertheless, this may limit the use of RARW as therapeutic treatment due to its alcohol content. Possibly, a powdered RARW and RW formulation would provide alcohol-free antioxidants.

Another significant finding was the changes in the right ventricle and pulmonary artery flow parameters observed in this study. Possibly suggesting a dysfunction in the endothelium because oxidative stress is known to induce inflammatory changes leading to endothelial damage (Ishibashi, 2013; Touyz, 2005). The link between oxidative stress and endothelial dysfunction caused by vascular inflammation has been implicated in the pathophysiology of several disease including hypertension and cardiovascular disease (Griendling & FitzGerald, 2003; Mazor et al., 2010; Touyz, 2005) leading to decreased bioavailability of nitric oxide (NO) causing vasoconstriction in the endothelium. In one study, chronic administration of red wine polyphenols reduced hypertension and vascular dysfunction by reducing vascular oxidative stress (Rush, Quadrilatero, Levy, & Ford, 2007). Indeed, the use of RARW in this study reversed the effect of oxidative stress as measured by CDs similar to the findings from Maarman et al. (2015). However, the source of CDs is unknown and could be systemic and ameliorated mostly by RARW. In effect, red wine through the synergistic action of the complex polyphenols may mediate pulmonary artery relaxation by acting directly on smooth muscles (Leblais et al., 2008).

This study did not find any significant difference in the plasma concentrations of SOD, ORAC and catalase. These findings were contrary to what was expected. As SOD, ORAC and catalase belong to the antioxidant groups (Aebi, 1984; Huang et al., 2005; McCord & Fridovich, 1969), it was expected that consumption of antioxidant rich drinks like RARW and RW should raise the plasma antioxidant contents, but this was not so. Unfortunately, tissue penetration and antioxidant activity in rat tissue was not evaluated. Similar findings were published by Bertelli, Giovannini, Stradi, Bertelli, and Tillement (1996) who found lower levels of resveratrol (a major antioxidant component of red wine) in different tissues of the body after an oral dose of red wine was given to rats for 15 days. It is thus possible that the amount of antioxidants absorbed into the rat circulations was not sufficient to raise the plasma concentrations or even if they were enough, metabolic and cellular changes as well as the route of administration might have affected the synergistic or pleiotropic effects of the antioxidants.

5.4 Effect of chronic, moderate regular- and reduced-alcohol red wine on lipid concentrations

Alcohol had no significant effect on blood lipids. This study used TG, PL, TC and HDL-C as the commonly-measured plasma lipids, and found no significant changes. Blood lipid

concentration measurements were essential for this study as it is known that alcohol may trigger lipid changes, physiological findings may also have a double effect (Huang et al., 2017; Park & Kim, 2012; Pikaar et al., 1987; Veenstra, Ockhuizen, van de Pol, Wedel, & Schaafsma, 1990).

5.5 Effect of chronic, moderate regular- and reduced-alcohol red wine on biometric and cardiac parameters

Histological changes of MCT-induced PAH are known to present several patterns in both heart and lungs. A key feature of MCT-induced PAH is the presence of intima and media proliferation in the pulmonary vessels known to induce pulmonary obstruction and RV hypertrophy (Montani et al., 2013). In this study, MCT effect on cardiac fibrosis was not significant. Both the MCT+RARW and MCT+RW decreased fibrosis but not significantly differently (Figure 20). This, however was not significant to warrant any difference as observed with RV hypertrophy and LV+ septal atrophy. Previous studies found significant collagen deposits in MCT injected rats with the reason that cardiac changes caused by cardiomyocytes microtubules proliferation, atrophic remodelling in the LV, fibrosis, reactive oxygen species and dysfunction in the pulmonary arteries can trigger these changes (Hardziyenka et al., 2012; Maarman et al., 2015; Stones, Benoist, Peckham, & White, 2013). Also, several factors such as platelet aggregation, vasoconstriction and remodelling of the blood vessels either by molecular and cellular mechanisms are known to play a key role in the disease pathophysiology (Humbert, Sitbon, & Simonneau, 2004; O'callaghan et al., 2011). A known non-invasive method for the assessment of cardiac parameters is echocardiography, which gives a better correlation with magnetic resonance imaging (MRI) assessment of pulmonary artery pressure and catheterisation- the gold standard method (Urboniene, Haber, Fang, Thenappan, & Archer, 2010). In this study, which high-lights the obstruction of the pulmonary artery with the use of echocardiography is evident in the estimates of PAAT, PAAT/Ejection time, maximum velocity flow, VTI, TAPSE measurements and images in Figures 22 and 23. This finding is similar to Thibault et al. (2010) who found that PAAT and PAAT/Ejection time correlates well with increases in right ventricular systolic pressure.

5.6 Limitations of the study and recommendations

MCT-induced PAH model is relatively simple, inexpensive and easy to use; however, a major challenge is the toxic effect of the MCT drug on the metabolic system of the rats causes dehydration, decreased motility, decreased appetite, isolation from the group and early death.

Also, due to financial constraints and time, the measurement of oxidative stress and antioxidant activities was assessed only in the blood and not in the tissues, for example lung, heart and liver. Since MCT affects these organs the most, it would perhaps have given more insight on the processes and mechanisms if tissues had been analysed. Tissue analyses might also have been able to confirm the results from the blood. Also, rats could not be housed individually in a cage because it was too costly.

Furthermore, molecular measures (such as the role of red wine in anti-inflammatory, antithrombotic and antihypertrophic effect as well as protecting against endothelial dysfunction by inhibiting angiotensin II and upregulating nitric oxide synthase) can be determined and draw conclusions on the mechanisms of the PAH in the rat body were not determined. These could perhaps have provided explanations for the mechanistic pathways of the polyphenolic effect of red wine on the body.

Future studies could include investigation of the morphology of pulmonary artery and pulmonary arterioles, studies in an aortic banding model and in larger animal models as RARW and RW given in drinking water gavage would better mimic human setting. Studies similar in design to this study could include a comparison of consumption of undiluted wine (to maximise the antioxidant concentration) by the rats. Red wine could also be dried and pulverised, and the resulting powder be given as treatment capsules to the rats. Tissue and blood from the treatment-fed rats could then be analysed for oxidative stress analyses in future studies. Thus, compounds in wine could further be investigated as the reduced-alcohol may still potentially be harmful or inadequate for some patients.

5.7 Conclusions

A single subcutaneous injection of MCT (80mg/kg) induced pulmonary arterial hypertension as observed from an increase in right ventricular thickness, a decrease in pulmonary acceleration time (PAAT), pulmonary artery maximum velocity (max velocity), velocity time

integral (VTI) and tricuspid annular plane systolic excursion (TAPSE). Chronic, moderate consumption of reduced alcohol red wine lessens right ventricular mass, improved the levels of PAAT, max velocity, VTI and TAPSE and reduced oxidative stress (CD) levels.

In conclusion, the results of this study suggest that moderate and chronic consumption of reduced-alcohol red wine may ameliorate the course and outcome of PAH attributable to MCT in rats (Figure 28). If this is an antioxidant basis and if most human diseases involve oxidative damage, the use of RARW could be tested in a randomised controlled trial and may be more beneficial as compared with RW.

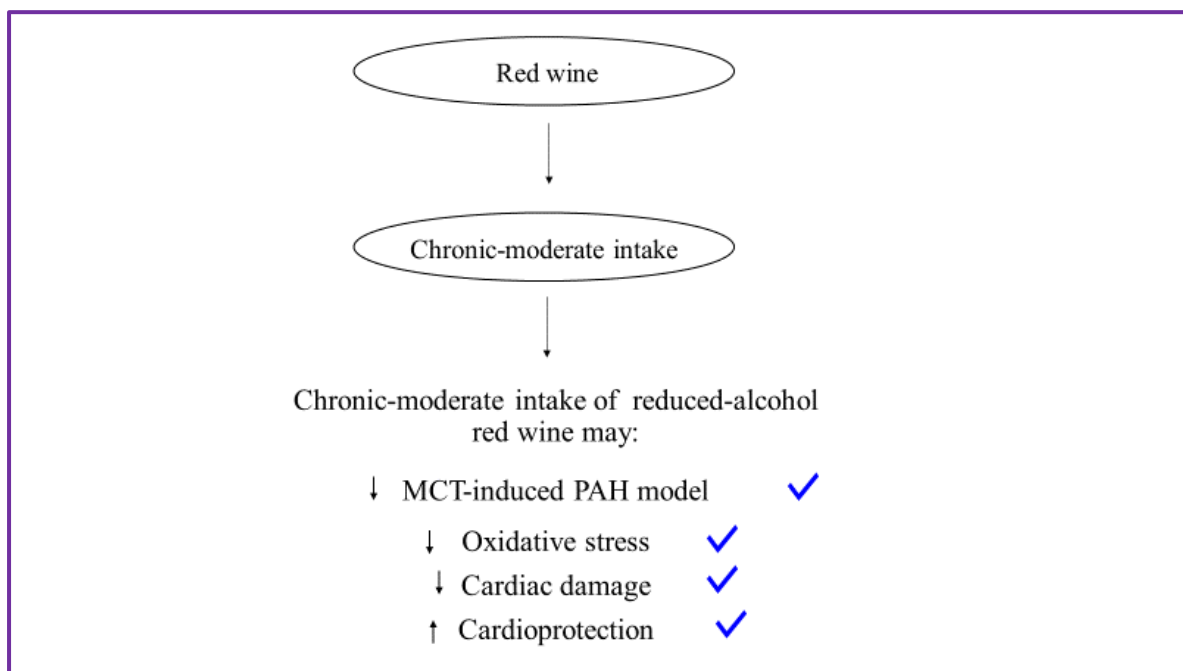


Figure 28: Schematic diagram of the experimental study outcome.

Monocrotaline (MCT); Pulmonary arterial hypertension (PAH)

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APPENDIX I: REAGENT PREPARATION (ANTHOCYANIN, CATECHIN, TBARS, CATALASE, TOTAL PHENOLIC COMPOUND ASSAYS)

Reagent preparation for Anthocyanin assay

Buffer A (stored at RT)	Buffer B (stored at RT)	Buffer D (stored at RT)
6mL glacial acetic acid	2.5g potassium bitartrate	Weigh 11.61g maleic acid
Add 4.97g NaCl	Add 60mL ethanol	Add 4.97g NaCl
pH to 9.4 with 10% NaOH	pH to 3.3 with 2.0 M HCl	pH to 1.8 with 10% NaOH
Make up to 500mL dH ₂ O	Make up to 500mL dH ₂ O	Make up to 500mL dH ₂ O

Reagent preparation for Catechin assay

Prepare catechin stock (10µg/µL): 50mg (0.05g) in 500µL ethanol, mix, then make up to 5mL with dH₂O (aliquot and freeze at -20°C). Standard Concentration range: 0-300mg/L = 300µg/mL = 0.3 µg/µL

Buffer C _i (store at RT)	Buffer C _{ii}	FeCl ₃ .6H ₂ O (store at RT)
5% w/v 25g SDS	20mL buffer C _i	Weigh 0.27g of FeCl ₃ .6H ₂ O
5% v/v 25mL triethanolamine	Add 3mL ethanol	Add 80µL of 12.1M HCl
pH to 9.4 with HCl.	Comment: Make up shortly before use	Make up to 100mL with dH ₂ O
Make up to 500mL with dH ₂ O		

Reagent preparation for Thiobarbituric Acid Reactive Substance (TBARS) assay

Ferric Chloride (store at RT)	0.2M Glycine buffer (store at RT)	2-Thiobarbituric acid (TBA) solution
Weigh 0.27g FeCl ₃ .6H ₂ O	Weigh 15.01g glycine	Weigh 0.5g TBA and 0.3g SDS
Make up to 100mL with dH ₂ O	pH with HCl to 3.6	Make up to 100mL dH ₂ O
	Make to 1L with dH ₂ O	
Ethanolic butylated hydroxytoluene (BHT)		Glacial acetic acid
0.22g BHT		
Dissolve in 100mL ethanol		

Reagent preparation for Catalase assay

50mM KH₂PO₄	50mM HPO₄.2H₂O	50mM Phosphate buffer
Weigh 6.81g KH ₂ PO ₄	Weigh 8.90g HPO ₄ .2H ₂ O	Make buffer in a ratio of 1:1.15 (KH ₂ PO ₄ : HPO ₄ .2H ₂ O)
Make up with dH ₂ O to 1L	Make up with dH ₂ O to 1L	pH to 7.0
30% H₂O₂ (8.82M)		
Make up 40mM (410μL + 100mL phosphate buffer)		

Reagent preparation for Total phenolic compound assay

Gallic Acid	Folin-Ciocalteu reagent (neat)	Na₂CO₃ (20% v/v)
Weigh 0.125g gallic acid in 2.5mL C ₂ H ₅ OH (ethanol)	Add 100μL	20g of anhydrous Na ₂ CO ₃ in 80mL dH ₂ O. Bring to boil after cooling and add few crystals of Na ₂ CO ₃
Prepare freshly for use		Leave for 24 hours then filter (Whatman#1) and add to volume of 100mL with dH ₂ O
		Store indefinitely at room temperature.

Reagent preparation for 4% buffered formalin in PBS (for use in tissue fixation)

To make 4% formaldehyde, add 800mL of 1XPBS	Mix and stir in a ventilated hood. Heat and stir to 60 °C
Add 40g of paraformaldehyde powder to the heated PBS solution	pH with 1M NaOH to dissolve the powder
Top up with 1XPBS to 1L	Recheck pH with HCl to 6.9
Freeze and store at 2-8 °C for a month	

APPENDIX II: REAGENT PREPARATION (ORAC)

Reagent preparation for Oxygen Radical Absorbance Capacity (ORAC) assay

0.075M Phosphate Buffer		Fluorescein
Make up 0.75M $K_2HPO_4 \cdot 3H_2O$ (weigh 85.6g to 500mL dH_2O)		Stock 1: Weigh 0.0225g in 50mL phosphate buffer
Make up 0.75M $NaH_2PO_4 \cdot H_2O$ (weigh 51.8g to 500mL dH_2O)		Stock 2: Weigh 50 μ L stock 1 in 10mL phosphate buffer
80mL K_2HPO_4 (0.75M) + 20mL NaH_2PO_4 (0.75M)		Working solution: 320 μ L stock 2 in 20mL phosphate buffer
Make to 900mL, pH to 7.4, make up to 1L with dH_2O		
AAPH	Trolox (100mM)	
Weigh either (0.087g + 980 μ L warm buffer OR	Weigh 0.0050g in 200 μ L ethanol	
0.348g + 3920 μ L warm buffer OR	100 μ L of 100mM + 9.9mL buffer = 1000 μ M	
0.696g + 7840 μ L warm buffer	1mL of 1000 μ M + 9mL buffer = 100 μ M (5nmol)	
Dependant on sample numbers	For the 5nmol trolox stock, aliquot into small volumes for storage at 4°C	

APPENDIX III: REAGENT PREPARATION (SOD)

Reagent preparation for superoxide dismutase (SOD) assay

216mM Potassium phosphate buffer	10.7mM EDTA solution	1.1mM Cytochrome C (Sigma C7752)	0.108mM Xanthine (Sigma X0626)
4.93g of potassium phosphate buffer in 100mL dH ₂ O	0.04g in 10mL	0.0146g in 1mL dH ₂ O	0.0016g in 90mL dH ₂ O
pH to 7.8 at 25°C			Stir and add small drops of 1M KOH until xanthine dissolves and Make up to 100mL with dH ₂ O in a volumetric flask
pH with 1M KOH or 1M HCl			
Cocktail Reagent	Reagents		Volumes
	dH ₂ O		4.6mL
	Potassium Phosphate buffer		5.0mL
	10.7mM EDTA		0.2mL
	1.1mM Cytochrome C		0.2mL
	0.108mM Xanthine		10.0mL
Cocktail Reagent: (stable for 3 days)	Total:		20mL for 48samples

APPENDIX IV ASSAY PROCEDURE

Protocol for staining with PicroSirius red

Steps	Process	Comments	Time
1	Xylene	Deparaffination	10 minutes
2	Xylene	Deparaffination	10 minutes
3	Xylene	Deparaffination	10 minutes
4	100% Alcohol	Rehydration	5 minutes
5	100% Alcohol	Rehydration	5 minutes
6	90% Alcohol	Rehydration	5 minutes
7	70% Alcohol	Rehydration	5 minutes
8	Distilled water	Washing	3 minutes
9	0.1% PicroSirius red	Staining	60 minutes
10	1% Glacial acetic acid	Washing	5 minutes
11	1% Glacial acetic acid	Washing	5 minutes
12	Distilled water	Washing	1 minute
13	70% Alcohol	Dehydration	5 minutes
14	90% Alcohol	Dehydration	5 minutes
15	100% Alcohol	Dehydration	5 minutes
16	100% Alcohol	Dehydration	5 minutes
17	Xylene	Clearing	3 minutes
18	Xylene	Clearing	3 minutes
19	Xylene	Clearing	3 minutes
20	Mount with Xylene based medium	—	—
21	Dry slides at room temperature	—	Overnight

Superoxide dismutase (SOD) Standard (Sigma S2515) curve determination

- 2µL of Working SOD standard + 98µL dH₂O (10IU/mL)
- 3µL of Working SOD standard + 97µL dH₂O (15IU/mL)
- 5µL of Working SOD standard + 95µL dH₂O (25IU/mL)
- 10µL of Working SOD standard + 90µL dH₂O (50IU/mL)
- 15µL of Working SOD standard + 85µL dH₂O (75IU/mL)
- 20µL of Working SOD standard + 80µL dH₂O (100IU/mL)
- 30µL of Working SOD standard + 70µL dH₂O (150IU/mL)

Oxygen radical absorbance capacity (ORAC) assay standard determination

- 0.0050g of Trolox in 200 μ L absolute ethanol = 100mM
- 100 μ L of 100mM + 9.9mL phosphate buffer = 1000 μ M
- 1mL of 1000 μ M + 9mL phosphate buffer = 100 μ M (5nmol)
- 300 μ L of 100 μ M + 300 μ L phosphate buffer = 50 μ M (2.5nmol)
- 300 μ L of 50 μ M + 300 μ L phosphate buffer = 25 μ M (1.25nmol)
- 300 μ L of 25 μ M + 300 μ L phosphate buffer = 12.5 μ M (0.625nmol)
- 300 μ L of 12.5 μ M + 300 μ L phosphate buffer = 6.25 μ M (0.313nmol)
- 300 μ L of 6.25 μ M + 300 μ L phosphate buffer = 3.13 μ M (0.156nmol)
- 300 μ L of 3.13 μ M + 300 μ L phosphate buffer = 1.37 μ M (0.078nmol)

ORAC assay showing the arrangement on a 96-well, white flat bottomed, microtitre plate Polysorp®-Nunc (Sigma-Aldrich, SA)

Wells	Buffer: μ L	Fluorescein: μ L	AAPH: μ L	50 μ L Trolox or 50 μ L Plasma or 50 μ L Red Wine
A1	300			
A2	"			
A3	200	100		
A4	"	"		
A5	100	"	100	0
A6	"	"	"	
A7	50	"	"	0.078 nmol Trolox
A8	"	"	"	"
A9	"	"	"	0.156 nmol Trolox
A10	"	"	"	"
A11	"	"	"	0.313 nmol Trolox
A12	"	"	"	"
B1	"	"	"	0.625 nmol trolox
B2	"	"	"	"
B3	"	"	"	1.25 nmol Trolox
B4	"	"	"	"
B5	"	"	"	2.5 nmol Trolox
B6	"	"	"	"
B7	"	"	"	5 nmol Trolox
B8	"	"	"	"
B9 - H12	"	"	"	

Blood plasma concentration of malondialdehyde (TBARS) assay

- Using 5mL macrotubes (MTCBio, Lasec SA), 50uL of plasma sample was added. Then 50uL $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution (the reagents used are described in appendix I) and 50uL of ethanolic butylated hydroxytoluene (BHT) solution were added. The reaction mixture was vortexed for 1 minute. A blank of 50uL of water was used.
- 0.75mL of glycine buffer was added and vortexed. Then 0.75mL of TBA solution was added and vortexed.
- The reaction mixture in the macro tubes was heated at 90°C for 15minutes on a heating block and cooled for 2 minutes on ice.
- 0.5mL of glacial acetic acid and 1mL chloroform were added, and the mixture was vortexed and then centrifuged at 3000G force for 15 minutes.
- 300uL of supernatants in duplicate were added to a Greiner 96-well transparent plate (Sigma-Aldrich, South Africa) and absorbance of the pink colour formed was measured at 532 nm wavelength, with water as blank. Using 300uL per well of 1.56 molar extinct coefficient and 50uL sample, TBARS in the samples were calculated as $A_{532} - \text{blank} \times 50.7 = \text{nmol/mL } (\mu\text{mol/L})$.

Triglyceride assay protocol with a step-by-step procedure

- Transparent 96-well Greiner microplates (Sigma-Aldrich, South Africa) were used for the assay.
- Aliquots of 0, 1, 2, 5, 7, 10, 15 and 20 μ L respectively of glycerol standard (corresponding to 3 μ g/ μ L triolein) were pipetted in duplicate into well plates and made up with physiological saline to 50 μ L.
- 5 μ L of sample plus 45 μ L of 0.9% physiological saline were added to separate empty well plates in duplicate.
- Thereafter, 250 μ L of freshly reconstituted TG reagent was added to each well using a multi-pipette dispenser.
- The microplate was placed onto a shaker to be gently shaken for 30minutes, and the absorbance was then read on a spectrophotometer at a wavelength of 600nm
- Using the standard curve, the concentration of TG in the sample was determined from regression analysis using GraphPad prism software.

Phospholipid assay protocol with a step-by-step procedure

- Using transparent 96-well Greiner microplate, aliquots of 0, 1, 2, 3, 5, 8, 10, 15, 20 and 25 μ L standard (choline chloride corresponding to 3 μ g/ μ L) were pipetted in duplicates into wells and made up to a final volume of 30 μ L with physiological saline.
- Into separate wells, aliquots of 10 μ L of sample plus 20 μ L physiological saline were added in duplicates.
- Then 220 μ L of freshly reconstituted phospholipid reagent was added to all wells using a multi-pipette dispenser
- The microplates with 300 μ L total volumes in each well was transferred onto a shaker to be gently shaken for 30 minutes at room temperature
- The absorbance at 600nm using a spectrophotometer was read and the concentration of samples were extrapolated from linear analysis of the standard curve using GraphPad Prism software.

Cholesterol assay protocol with a step-by-step procedure

	Sample	Standards									
Standard content (μ g)		0	2	4	6	10	14	20	26	32	
Sample volume (μ L)	20	0	1	2	3	5	7	10	13	16	
Volume of H ₂ O added (μ L)	30	50	49	48	47	45	43	40	37	34	
Chromogen reagent (μ L)	250	250									

- The microtitre plate was placed onto a shaker to be gently shaken for 30 minutes, where after the absorbance was read on a spectrophotometer at a wavelength of 600nm.
- Concentrations of sample were calculated from linear regression analysis of the standard curve using GraphPad prism software.

High density lipoprotein (HDL) assay protocol with a step-by-step procedure

- Eppendorf tubes (1) were labelled and 30µL of mixture (1 volume heparin + 2 volumes MnCl₂) were added to 300µL of sample
- The reaction mixture was mixed gently to avoid foaming and left at room temperature for 20 minutes.
- Subsequently, the mixture was centrifuged for 60 minutes at 12,000G force, 4°C.
- With a fine pipette, the clear supernatant from Eppendorf (1) was transferred into another labelled tube (Eppendorf 2), labelled as total HDL.
- 100µL supernatant (from Eppendorf 2) and 10µL dextran sulphate were gently mixed to avoid foaming and left at room temperature for 20 minutes.
- The mixture was centrifuged for 30 minutes at 12,000G force, 4°C
- Using a fine pipette, the supernatant was transferred into Eppendorf 3 and labelled as HDL₃.
- Using a clear transparent 96 well Greiner microplate, 20µL of total HDL and 20µL HDL₃ were pipetted into wells. A cholesterol standard (2µg/µL) was also used (section 3.8.3). Volumes were made up to 50µL with dH₂O.
- After adding 250µL of cholesterol reagent, the microplate was incubated at room temperature for 30 minutes on a plate shaker.
- The absorbance at 600nm wavelength was read using a spectrophotometer and concentrations of cholesterol were extrapolated from the standard curve, using the same assay described in Cholesterol assay protocol above.

Total phenolic compound assay protocol with a step-by-step procedure

Preparation of the standard curve for the assay

- 100µL of prepared stock gallic acid + 9.9mL dH₂O = 500mg/L

- 400µL of the 500mg/L +100µL dH₂O = 400mg/L
- 300µL of the 500mg/L +200µL dH₂O = 300mg/L
- 200µL of the 500mg/L +300µL dH₂O = 200mg/L
- 100µL of the 500mg/L +400µL dH₂O = 100mg/L
- 50µL of the 500mg/L +450µL dH₂O = 50mg/L
- 0µL +500µL dH₂O = 0mg/L

Using 20 µL of each of the prepared standards above, and 1.58mL dH₂O, steps b to e were followed as below:

- 20µL of diluted wine + 1.58mL dH₂O was added to labelled plastic tubes.
- Then 100µL Folin-Ciocalteu reagent (neat) was added to each labelled tube.
- The reaction mixture was vortexed and incubated for 8 minutes at room temperature.
- 300µL of sodium carbonate (Na₂CO₃ (20% v/v)) was added to all labelled tubes, vortexed and incubated at 40°C for 30 minutes
- The absorbance at 765nm was measured using the spectrophotometer after adding 300µL of each sample into a 96-well Greiner plate (Sigma-Aldrich, SA).

Catechin assay

Simplified catechin assay in tabular form showing the volumes and amounts pipetted into well plates.

Concentrations in well (µg)	Volume (µL)	Buffer C _i	Buffer C _{ii}	FeCl ₃ (µL)
0	0	30	230	40
10	1	29	230	40
20	2	28	230	40
30	3	27	230	40
40	4	26	230	40
50	5	25	230	40
100	10	20	230	40
150	15	15	230	40
200	20	10	230	40

Wine Sample volume	30	0	230	40
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APPENDIX V: ANIMAL WELFARE MONITORING SHEET

FHS AEC number		Short title	Low alcohol wine and pulmonary arterial hypertension
Name of monitor(s)		Monitoring frequency	Once daily; Additional as below*

Date:														
Cage Number:														
Animal Number:														
Time of day:	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM
Baseline Weight:														
Weight:														
Volume of Drink:														
Discomfort or Stress (Yes/No)														
Discomfort or Stress Score 0 (None), 1 (mild), 2 (moderate) and 3 (Severe) <i>If moderate (2), monitor twice daily</i>														

Scoring Definitions:

- 0. (None)** No discomfort or stress:
Normally active; inquisitive; hair-coat clean; normal appetite.
- 1. (Mild)** Discomfort or stress: Hair-coat ruffled (hair erect); decreased or increased activity; avoidance behaviour; isolation from the group; depressed; decreased appetite.
- 2. (Moderate) Physical deterioration: piloerection; cyanosis; lethargic;** weight loss $\geq 10\%$; dehydration (tenting skin); weakness; decreased motility.
- 3. (Severe)** Distress: Very weak; unresponsive to touch; unconscious; convulsing; difficulty breathing.

* Additional action to take

If more than **mild Discomfort or Stress**, score as 2 on the sheet and monitor twice daily.

If the animal show signs of becoming **Distressed**, and not likely to recover and no action can be taken to alleviate

Physical deterioration or Distress															its distress, the animal must immediately be euthanized.
Distress (euthanize immediately)															
Euthanized (E) or Found dead (FD)															
Time of death															
Signature of monitor															