Does *Olea africana* protect the heart against ischemia-reperfusion injury?

by

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Declaration

I, Asanda Maliza declare that the thesis entitled: “Does *Olea africana* protect the heart against ischemia-reperfusion injury?” is the result of my own investigation and research, that it has not been submitted for any other degree or to any university. All the resources I have used or quoted have been indicated and acknowledged by complete references.

Name: Asanda Maliza

Signed:

Date:

Dedication

I thank God (the Most High) for giving me the strength and making this effort a success. I also thank my parents, sisters and friends for their wonderful support throughout my study.

I can do all things through Christ who strengthens me. Philippians 4:13.

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Abstract

Cardiovascular disease is a major health problem and remains the number one cause of death worldwide. For centuries, medicinal plants have been used in different cultures as medicines for the treatment and control of various diseases. *Olea africana,* also known as the wild olive, is amongst the herbal plants used by people to treat many ailments. Recently, scientific studies on the hypotensive, vasodilatory and antidysarrhythmic effects of *O. africana* have been reported. Triterpenoids isolated from the *O. africana* leaves, for example, have antioxidant properties. The aqueous extract from the leaves of *O. africana* also have angiotensin-converting enzyme (ACE) inhibitory effects. ACE inhibitors and antioxidants protect the heart against ischemic-reperfusion injury. The serine / threonine protein kinase B (PKB) also known as Akt is activated downstream of phosphoinositide 3- (PI-3) kinase (PI-3-Kinase) and is involved in cardioprotection against ischemia-reperfusion injury. Angiotensin II (AII) decreases the intrinsic PI-3-kinase activity. In this study, we hypothesized that ACE inhibitors increase PI-3-kinase activity and thus activates PKB. The aims of this study were: 1) to determine whether treatment with the crude aqueous extract of leaves of *O. africana* protect the heart against ischemic-reperfusion injury and 2) if so, to determine whether the protection is mediated via the PKB signaling mechanism.

Hearts isolated from male Wistar rats were perfused with different concentrations of the plant extract. In one set of experiments, male Wistar rats were treated with the plant extract (1000 mg/kg/day) for 5 weeks for the evaluation of cardiac function before and after ischemia. At the end of the experiments, hearts were freeze-clamped and kept for

PKB / Akt determination. In another set of experiments, we determined the effect of *O. africana* extract (1000 mg/kg/day) or captopril (50 mg/kg/day) on infarct size. Rats fed jelly served as controls for captopril. In a subset of experiments, hearts were frozen immediately after treatment with *O. africana* extract (1000 mg/kg/day) or captopril (50 mg/kg/day) and PKB were determined.

Perfusion with the plant extract significantly decreased coronary flow ($p<0.05$). The heart function was decreased as evidenced by observed decreases in the force of contraction and heart rate, although these were not measured. Chronic treatment with the crude aqueous plant extract had no effect on cardiac function before ischemia, functional recovery (% left ventricular developed pressure and % rate pressure product) and PKB / Akt phosphorylation ($p>0.05$). Both the aqueous extract of *O. africana* leaves and captopril had no effect on infarct size compared to the control group ($p>0.05$). Captopril, however, improved the recovery of the left ventricular developed pressure. Non-perfused hearts isolated from rats treated with *O. africana* extract and captopril did not show any response to both captopril and the *O. africana* extract treatment as measured by PKB / Akt phosphorylation. The results of the present study suggest that the crude aqueous extract of *O. africana* is not cardioprotective against ischemia-reperfusion injury in this system of the isolated perfused rat heart.

Keywords

Angiotensin converting enzyme inhibitors

Antioxidants

Captopril

Cardiovascular

Ischemia-reperfusion

Olea africana

Protein Kinase B

Traditional Medicine

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List of Abbreviations

AII.....Angiotensin II

ACE.....Angiotensin converting enzyme

ADP.....Adenosine diphosphate

AIF.....Apoptosis inducing factor

ATP.....Adenosine triphosphate

BAD.....Pro-apoptotic protein BAD

Ca^{2+}.....Calcium

Ca^{2+} ATPase.....Calcium ion pump

CAT.....Catalase

CP.....Creatine phosphate

CPK.....Creatine phosphokinase

CVD.....Cardiovascular disease

GPx .....Glutathione peroxidase

H_2O_2.....Hydrogen peroxide

HDL.....High density lipoproteins

LC_{50}.....Lethal concentration that result in 50% death

LVDevP.....Left ventricular developed pressure

LDH.....Lactate dehydrogenase

LDL-C.....Low density lipoprotein cholesterol

MAP.....Mean arterial pressure

Na^+-K^+ ATPase.....Sodium-potassium ion pump

NO.....Nitric oxide

ND.....Not detectable

$ONOO^-$.....Peroxynitrite

PARP.....Poly ADP ribose polymerase

PDK.....Phosphoinositide dependent kinase

PI-3-kinase.....Phosphoinositide 3-kinase

PKB.....Protein kinase B

PRA.....Plasma renin activity

RAS.....Renin angiotensin system

RISK.....Reperfusion injury salvage kinase

ROS.....Reactive oxygen species

RPP.....Rate pressure product

SOD.....Superoxide dismutase

SR.....Sarcoplasmic reticulum

TBARS.....Thiobarbituric acid reactive substances

WHO.....World Health Organization

UWC.....University of the Western Cape

UTHSCSA.....University of Texas Health Science Center at San Antonio

% LVDevP.....Percentage left ventricular developed pressure

% RPP.....Percentage rate pressure product

CHAPTER 1

INTRODUCTION

Cardiovascular disease is a major health problem worldwide and remains the number one cause of death (http://www.who.org, June 2009). According to the World Health Organization (WHO), approximately 17.5 million people died from cardiovascular disease in 2005, representing 30% of all global deaths. Of these deaths, 7.6 million were due to heart attacks and 5.7 million due to stroke. It is estimated that around 80% of these deaths occurred in low and middle income countries (http://www.who.org, June 2009). The increased prevalence of cardiovascular disease has serious socioeconomic consequences. About 84% of the world's populations are in the middle and lower income classes (www.worldbank.org, 23 June 2009) and because clinical care of cardiovascular disease is costly and prolonged, there is a need to look at other medicines that will be easily accessible, less costly and have fewer side effects.

Medicinal plants play a key role in the global health issues. For centuries, medicinal plants have been used in different cultures as medicine for the treatment of various diseases. It is estimated that 65-80% of the world's population who lives in developing countries use plants for primary health care and this dependency is mainly due to poverty and lack of access to modern medicine (Akerele, 1993; Wood-Sheldon *et al*., 1997). In many remote areas in African countries, people consult the traditional healer of the village to deal with cases of illness. Hospitals and medicines are often beyond their reach and western medicine is too expensive (Wood-Sheldon *et al*., 1997). Scientific interest in

medicinal plants has grown rapidly due to increased effectiveness of new plant-derived drugs and rising concerns about the side effects of conventional medicine (Lee, 2004).

Olea africana, also known as the wild olive, is one of the herbal plants used by people for various illnesses. In southern Africa, the wild olive has been chosen amongst 120 plant species to be the most important plant in use in traditional medicine (Dold and Cocks, 1999). The beneficial effects of the wild olive on the cardiovascular system have been previously documented (Khayyal *et al*., 2002; Osim *et al*., 1999; Somova *et al*., 2003; Zarzuzelo *et al*., 1991). Hypotensive effects of *O. africana* (Khayyal *et al*., 2002; Osim *et al*., 1999; Somova *et al*., 2003) as well as vasodilatory effects (Zarzuzelo *et al*., 1991) have been reported. Likewise, the isolates (oleanolic acid, ursolic acid and uvaol) from the same plant have been shown to have anti-arrhythmic effects (Somova *et al*., 2004). Triterpenoids, namely oleanolic acid and ursolic acid, isolated from the *O. africana* leaves are known to have antioxidant properties (Somova *et al*, 2003). The aqueous extract from the leaves of *O. africana* have angiotensin converting enzyme (ACE) inhibitory effects (Adersen et al., 1997; Hansen *et al*., 1996). ACE inhibitors (Ozer *et al*., 2002; Eichhorn, 1998; Maulik *et al*., 2001; Takeda *et al*., 1997) and antioxidants (Venardos *et al*., 2004; Dhalla *et al*., 2000; Marczin *et al*., 2003) protect the heart against ischemic-reperfusion injury.

The serine / threonine kinase, PKB, also known as Akt, is a key mediator of many signal transduction process. PKB / Akt is involved in cardioprotection against ischemia-reperfusion injury and is activated downstream of phosphoinositide 3- (PI-3) kinase

(Engelbrecht *et al*., 2006). PI-3-kinase consists of an 85-kDa regulatory subunit (p85) and a 110-kDa catalytic subunit (p110). Angiotensin II (AII) increases phosphorylation of p85 and decreases the intrinsic PI-3-kinase activity associated with the p85/p110 complex (Folli *et al*., 1997). In this study, we hypothesized that ACE inhibitors, by decreasing AII levels, increase PI-3-kinase activity and thus activates PKB. We therefore assumed that *O. africana* by virtue of its ACE inhibitory properties will activate PKB.

Several studies have concentrated on the cardiovascular effects of *O. africana*, but none on the potential cardioprotection by *Olea africana* following ischemia-reperfusion injury. The present study was undertaken to evaluate the cardiovascular effects of a crude aqueous extract of *O. africana* leaves against ischemia-reperfusion injury on the isolated perfused rat heart.

The objectives of the study were to:

1. Determine whether treatment with the crude aqueous extract from leaves of *O. africana* protects the isolated rat perfused heart against ischemia-reperfusion injury.
2. Evaluate whether the protection referred to above is mediated via PKB signaling.

CHAPTER 2

LITERATURE RIVIEW

2.1 Use of plants in traditional medicine

Medicinal plants play a key role in world health. A medicinal plant is any plant which provides health-promoting characteristics or curative properties. Plant parts used include seeds, berries, roots, leaves, bark and flowers. For centuries, medicinal plants have been used in different cultures as medicine for the treatment of various diseases. Garlic (*Allium sativum*) has been used traditionally to treat cramps, worm infestation, hypertension, high cholesterol levels and warts (Banerjee *et al.,* 2002). Aloe vera has been applied externally for the healing of burns and wounds (Maenthaisong *et al*., 2007). Rooibos (*Aspalathus linearis*) has been reported to cure skin ailments, allergies, asthma and colic in infants (Joubert *et al*., 2008). Calendula (C*alendula officinalis*) has been used to treat abdominal cramps and constipation (Bashir *et al*., 2006). Indian Snakeroot (*Rauvolfia serpentina*) has been used in India to treat insomnia, anxiety and high blood pressure (Sukh, 1999).

It is estimated that around 80% of the world's population (Wood-Sheldon *et al*., 1997), 80-90% of the population in African countries (Hostettman *et al*., 2000) and 27 million people in South Africa (Meyer *et al*., 1996; Mander, 1998) depend on traditional medicine for primary health care. The use of various herbal remedies and preparations are described throughout human history representing the origins of modern medicine. Many conventional drugs originate from plant sources, such as aspirin derived from bark of willow, digoxin derived from foxglove, quinine derived from the bark of cinchona and

morphine derived from the opium poppy (Lee, 2004). Scientific interest in medicinal plants has grown and expanded rapidly due to an increased effectiveness of new plant-derived drugs and rising concerns about the side effects of conventional medicine (Lee, 2004). Plant constituents or ingredients vary considerable depending on several factors that affect the quality of a traditional medicine. These include environmental factors such as type of the soil, altitude and climate. Other factors that affect the quality of a traditional medicine are the use of fresh plants, water availability, period (season), time of collection, method of collecting, drying, storage, age and part of the plant collected.

2.2 Traditional plants with cardiovascular effects

2.2.1 Ginseng

Ginseng (*Panax ginseng*) is a root of the perennial herb which contains a series of tetracyclic triterpenoid saponins (ginsenosides) as active ingredients (Nocerino *et al*., 2000). The English word "ginseng" is derived from the Chinese term, renshen, meaning "man root". Red ginseng has been used as an antihypertensive agent in Korea (Braun *et al*., 2007) and, in animal studies, *Panax ginseng* was shown to decrease blood pressure (Morgan *et al*., 2000). The hypotensive effects of ginseng is claimed to be due to an angiotensin–converting enzyme (ACE) inhibitory effect (Persson *et al.*, 2006) and also due to nitric oxide (NO) release stimulated by ginsenosides (Morgan *et al.*, 2000). Nitric oxide in turn causes smooth muscle relaxation and vascular dilation (Morgan *et al.*, 2000). Red ginseng powder reduces plasma total cholesterol and triglycerides while elevating high density lipoproteins (HDL) (Yamamoto *et al.*, 1983).

2.2.2 Garlic

Garlic (*Allium sativum* L) is a perennial plant of the *Alliaceae* family, which includes onion, chive, shallot and leek. Allicin, the major component present in freshly crushed garlic, is one of the most biologically active compounds of garlic (Zhang *et al.*, 2001). Garlic extracts have been widely recognized as agents for the prevention and treatment of atherosclerosis, hyperlipidemia, thrombosis, hypertension and diabetes (Banerjee *et al.*, 2002).

Garlic's ability to significantly lower total blood cholesterol has been shown in many studies, suggesting that it may provide some protection against coronary artery disease and stroke (Auer *et al*., 1990; Jain *et al*., 1993; Warshafsky *et al*., 1993). A more recent meta-analysis of placebo controlled trials using standardized dried garlic powder showed a significant reduction in total cholesterol levels, low density lipoprotein and triglycerides levels at 8-12 weeks (Ackermann *et al*., 2001). Clinical studies have also shown that garlic has antihypertensive effects, decreasing both the systolic and diastolic pressures (Banerjee *et al.*, 2002). Garlic has also been found to decrease platelet aggregation (Rahman, 2007; Steiner *et al*., 1998). Chronic oral administration of garlic extract prevents oxidative stress and ultrastructural changes induced by myocardial ischemia-reperfusion injury (Banerjee *et al*, 2002).

2.2.3 Hawthorn

Hawthorn (C*rataegus species*) is an aromatic, sweet and sour herb that belongs to a member of the *Rosaceae* family. It contains oligomeric procyanidins, flavanoids,

chlorogenic, caffeic acid and triterpenes (Leung *et al.*, 2008). Hawthorn extracts from the leaves, berries and flowers are one of the safer remedies. In the 19th century, hawthorn berries have been used in Western Europe as a cardiotonic and are a recognized treatment for heart failure (Fugh-Berman, 2000). In clinical studies conducted on people with heart failure, hawthorn significantly improved heart function (Degenring *et al*., 2003; Zapfe, 2001). Animal and laboratory studies demonstrate that hawthorn has antioxidant properties that protects against the formation of atherosclerotic plagues (Bahorun *et al.*, 1994). The hawthorn extract administered intravenously in rats produced hypotensive effects by reducing mean arterial pressure (Leung *et al*., 2008).

2.2.4 Ginkgo

Ginkgo (*Ginkgo biloba*), also known as the Maidenhair tree, is a unique species with no living relatives. The leaf extract has been used for more than 3000 years by the Chinese and is the best selling remedy in the United States (Khan *et al*., 2006). It is believed to be rich in flavanoids and triterpenoids which exert their effects through free radical scavenging, antiplatelet activity, vasodilation, decreasing blood viscosity and anti-inflammatory activity (Khan *et al*., 2006). Ginkgo decreases platelet aggregation and causes vaso-relaxation by blocking nitric oxide metabolism (Gold and Farnsworth, 2002). Results from clinical trials demonstrate that a standardized leaf extract of Ginkgo is useful in preventing and treating cardiovascular disease (CVD), particularly ischemic cardiac syndromes (Mahad, 2002).

2.2.5 Soy

Soybean (*Glycine max*) is a legume native to East Asia. It has been used in China for 5000 years as a food and a component of drugs (Derbyshire *et al*., 1976). It contains significant amounts of amino acids essential for humans, and it is a good source of protein. In clinical trials, soy products consumption reduced total cholesterol and low density lipoprotein cholesterol (LDL-C) levels (Hasler, 2002).

2.3 Plants that protect against ischemia-reperfusion injury

Chronic oral administration of garlic extracts (25, 250 and 500 mg/kg) prevent oxidative stress by decreasing thiobarbituric acid reactive substances (TBARS), depletion of endogenous antioxidants (catalase, superoxide dismutase and glutathione) and ultrastructural changes induced by myocardial ischemia-reperfusion injury (Banerjee *et al*., 2002). Acute treatment with bagflower (*Clerondendron colebrookianum*) aqueous extracts (0.01% and 0.05%) administered at the time of reperfusion protects against oxidative stress and cellular injury associated with ischemia-reperfusion injury (Devi *et al*., 2005). The aqueous extracts from guava (*Psidium guajava*) and sea lavender (*Limonium wrightii*) have cardioprotective effects against myocardial ischemia-reperfusion injury in isolated rat hearts, primarily through their free radical-scavenging actions. The extracts attenuate ischemic contracture during ischemia and improved myocardial dysfunction after reperfusion (Yamashiro *et al*., 2003).

2.4 *Olea africana*

Olea europaea, subspecies *africana*, also known as the wild olive is a species belonging to the family *Oleaceae* (Green *et al*., 1979; Van Wyk *et al*., 1997). The wild olive is mainly located in Africa. In history, the wild olive is one of the most quoted in literature (Verdoorn, 1963). The olive fruit is of major agricultural importance in the Mediterranean region as a source of olive oil. In Southern Africa, the wild olive is one of the most popular plants used by Sotho, Xhosa and Zulu tribes (Watt and Breyer-Brandwijk, 1962; Hutchings, 1989 and Van Wyk and Gerike, 2000). Of 120 species, umNquma (wild olive) was designated the most important plant in traditional medicine (Dold and Cocks, 1999).

2.4.1 Vernacular names of the plant

The wild olive is named umNquma (Xhosa), Isadlulambazo (Zulu), Motholoari (Sotho) (Watt and Breyer-Brandwijk, 1962; Hutchings, 1989; Van Wyk and Gerike, 2000), Mutlhwari (Venda), Motlhware (Tswana) and Swartolienhout (Afrikaans) Green *et al*., 1979.

2.4.2 General description

The wild olive is a neatly shaped evergreen tree with a dense spreading crown (9 x 12 m) of glossy grey-green to dark-green foliage. Leaves are grey-green to dark-green above and greyish below. The tiny, lightly scented white to greenish flowers spray from October to February, followed from March to July with small, spherical, fleshy fruits,

either sweet or sour, which ripen purple-black (Green *et al*., 1979). The olive tree grows in the wide variety of soils with marked preference for calcareous (containing calcium carbonate, calcium, limestone and chalky) soil and coastal climate conditions. The tree is very tolerant to drought and high pH (Green *et al*., 1979).

Figure 2.1: *O. africana* plant
(From: Green *et al*., 1979)

2.4.3 Traditional medicinal uses

Infusions prepared from the leaves of *O. africana* are used to treat eye infections or as a gargle to relieve sore throat (Green *et al*., 1979). The infusion is also taken internally as a remedy for colic or urinary tract infection and to improve kidney function. The powdered leaf is used as a styptic. The fruit is used to treat diarrhoea (Green *et al*., 1979). Traditional remedies prepared from the leaves, roots or stem bark are used to lower blood pressure and to treat related cardiovascular diseases (Watt, 1962; Hutchings, 1996).

2.4.4 Bioactive compounds in the olive leaf

The active ingredients found in the plant are chemical compounds that are responsible for the specific activity of the plant by acting directly or indirectly to prevent or treat disease and maintain health.

2.4.4.1 Phenolic compounds

Oleuropein, a member of secoiridoids, is the major principal phenolic compound present in the leaves of the plant. Bourquelot and Vintilesco (1908) isolated the compound which is a heterosidic ester of oleanolic acid and hydroxytyrosol. The oleuropein content in the olive leaves was found to be high, around 60 - 90 mg per gram of the dry material (Le Tutour *et al*., 1992).

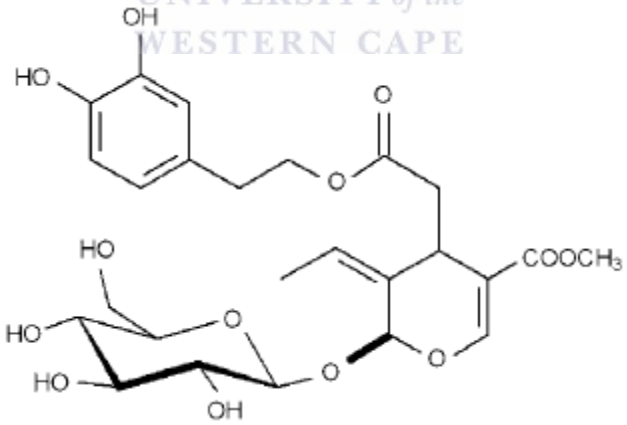


Figure 2.2: Chemical structure of Oleuropein
(From: Andreadou *et al*., 2006)

Other phenolic compounds have been isolated and identified in the olive leaves through the process of hydrolysis but are present in lesser quantity. These compounds include demethyloleuropein, ligstroside (Soler-Rivas *et al*., 2000), verbascoside (Amiot *et al*.,

1986). Flavanoids, another group of phenolic compounds found in the olive leaves are luteolin-7-0-glucoside, luteolin-7-0-rutinoside, apigenin-7-0-glucoside, rutuin, luteolin and apigenin (De Laurentis, 1997).

2.4.4.2 Terpenic compounds

Another group of bioactive compounds found in the olive leaf is terpenic compounds (Khan *et al*., 2007). Mussini *et al*. (1975) isolated alpha-amyrine and confirmed the presence of maslinic acid. Oleanolic acid (3-beta-hydroxyoleon), found extensively throughout the plant kingdom, is another terpenic compound found in the olive leaf. Oleanolic acid in the olive leaf occurs in free acid form accounts for 3% of the dry leaf weight (Khan *et al*., 2007).

2.5 Toxicity of the plant

The triterpenoids, oleanolic acid (OA) and ursolic acid (UA) isolated from African wild olive leaves have low toxicity on brine shrimps, LC_{50} 0.10 mg/ml (OA) and 0.95 mg/ml (UA) (Somova *et al*., 2003). The crude aqueous extract of the same plant have low toxicity-LC_{50} > 5000 µg/ml (Wang, 2008).

2.6 Cardiovascular effects of *Olea africana*

In an isolated perfused rabbit heart using the Langendorff system, the ethanol extract from the leaves of *Olea europaea* decreased systolic pressure, heart rate and caused an increase in relative coronary flow (ratio between coronary flow and rate pressure product) (Scheffler *et al*., 2008). Using the same extract in cultured neonatal rat cardiomyocytes,

the extract caused a significant decrease in maximum L-type calcium channel ($I_{ca.L}$) peak currents and this was reversible upon washout (Scheffler *et al*., 2008).

2.6.1 Antihypertensive / hypotensive effects

The triterpenoids namely oleanolic acid and ursolic acid, isolated from *Olea africana* leaves were reported to have antihypertensive and anti atherosclerotic effects. The effects were studied on the Dahl salt-sensitive (DSS) and insulin-resistant genetic rat model of hypertension (Somova *et al*., 2003). Ribeiro *et al*. (1986) showed that a crude aqueous ethanol (50:50 volume) extract of the olive leaves when given orally at a dose of 40 ml/kg produced antihypertensive effects in spontaneous hypertensive rats. Osim *et al*. (1999) reported the hypotensive effects of the crude aqueous and ethanol extracts in normo and hypertensive rats. The aqueous extract was more potent than the ethanol extract. Oral administration of the extract (100 mg/kg) in hypertensive rats was shown to have antihypertensive effects (Khayyal *et al*., 2002).

Zarzuelo *et al*. (1991) reported vasodilatory effects of a decoction made from *Olea europaea* leaf on isolated rat aorta preparations. Oleuropeoside was shown to be the active compound responsible for the vasodilatory action. The aqueous leaf extract of *Olea africana* was found to posses ACE inhibitory effects, inhibiting ACE activity by 82% (Adersen *et al*., 1997). Using whole blood from normotensive and hypertensive rats, the crude aqueous extract of the same plant caused a decrease in ACE levels and prevented hypertension from Dahl salt-sensitive rats by decreasing plasma angiotensin II

levels (Wang, 2008). In *O. europaea* aqueous leaf extract, Oleacein was shown to be an active ingredient responsible for the ACE inhibitory activities (Hansen *et al*., 1996).

2.6.2 Antidysrhythmic effects

Somova *et al*. (2004) examined the cardiotonic and antidysrhythmic activity of the triterpenoids, namely, oleanolic acid, ursolic acid and uvaol isolated from leaves of the Africana wild olive and methyl maslinate isolated from leaves of *Olea europaea*. Arrhythmias were chemically induced with calcium chloride and adrenaline, and mechanically induced through ischemia-reperfusion. The isolates showed antidysrhythmic activity on both the chemical and mechanical types of arrhythmia and displayed a significant dose-response vasodepressor effect and sinus bradycardia.

2.7 Ischaemic-reperfusion injury

Ischemia-reperfusion represents a clinically relevant problem associated with thrombolysis, angioplasty and coronary bypass surgery (Dhalla *et al*., 2000). Ischemia is described as an inadequate flow of blood to the myocardium due to constriction of the blood vessels supplying the heart muscle. Myocardial ischemia can result in tissue damage leading to necrosis and apoptosis. Restoration of blood flow (reperfusion) is the only way to save the myocardium from eventual tissue death, however, reperfusion has been shown to exacerbate myocardial damage (Takeda *et al*., 1997; Ozer *et al*., 2002; Narang *et al*., 2004). Myocardium ischemia-reperfusion injury includes contractile dysfunction, arrhythmias and irreversible myocyte damage (Dhalla *et al*., 2000). The changes in the myocardium due to ischemic-reperfusion injury are considered to be due

to increase formation of reactive oxygen species (ROS), calcium (Ca^{2+}) overload and the activation of the renin-angiotesin system (Liang, *et al*., 1981; Wang *et al*., 2001; Murphy and Steenbergen, 2008).

Oxidative stress has been largely implicated in the etiopathogenesis of ischemic-reperfusion injury as evidenced by increase in thiobarbituric acid reactive substances (TBARS) and depletion of endogenous antioxidants (Narang *et al*., 2004; Devi *et al*., 2005; Vanden Hoek *et al*., 1997; Marczin *et al*., 2003, Dhalla *et al*., 2000; Benerjee *et al*., 2002, Jan-Kan *et al*., 2005; Venardos *et al*., 2004). During reperfusion, oxygen undergoes sequential reduction to form reactive oxygen species (ROS) such as superoxide radical, hydrogen peroxide (H_2O_2) and hydroxyl radical (Banerjee *et al*., 2002; Dhalla *et al*., 2000; Narang *et al*., 2004). Due to increased ROS production, the antioxidant defense mechanism is overwhelmed resulting in an imbalance between oxidants and endogenous antioxidant defense mechanisms, causing oxidative stress (Molyneux *et al*., 2002; Marczin *et al*., 2003; Vanden Hoek *et al*., 1997). Oxidative stress modifies phospholipids and proteins leading to lipid peroxidation and oxidation of thiol groups which alter membrane permeability. The alteration in membrane permeability may result in cellular defects, including a depression in the sarcolemmal Ca^{2+} ATPase and Na^{+}-K^{+} ATPase pumps, which leads to decreased Ca^{2+} efflux and increased Ca^{2+} influx in cardiac cells. The depression in Ca^{2+} regulatory mechanism by ROS ultimately results in intracellular Ca^{2+} overload (Dhalla *et al*., 2000). A rise in Ca^{2+} leads to activation of calpains, which may be involved in the cleavage of proteins resulting in plasma membrane rupture and activation of pro-apoptotic BID, thus resulting in apoptotic cell death. Also, an increase

in Ca^{2+} leads to activation of inner mitochondrial large-conductance channel, MPT, which leads to loss of ATP and mitochondrial function, resulting in mitochondrial swelling and the release of cytochrome c, thus activating apoptosis (Murphy and Steenbergen, 2008).

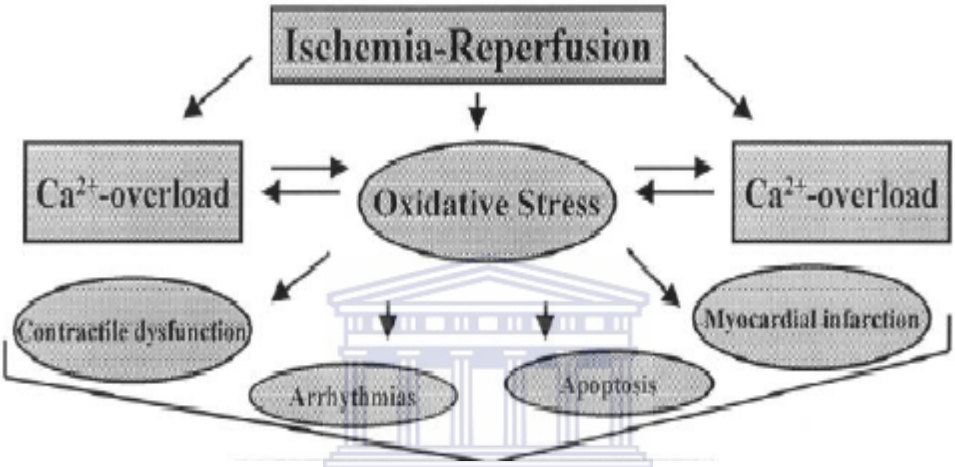


Figure 2.3: Pathophysiologic implications of oxidative stress in ischemia-reperfusion injury in the heart
(From: Dhalla *et al.*, 2000)

In addition to ROS, oxidative stress may also be caused by nitrogen oxygen species. Peroxynitrite ($ONOO^-$), a nitrogen oxide species, has also been shown to cause deleterious effects in the heart following ischemia and reperfusion (Dhalla *et al.*, 2000). It is formed by fast biradical reaction of nitric oxide and superoxide anion, mainly in the vascular endothelium, myocytes and neutrophils. Peroxynitrite hydroxylates, nitrates aromatic and compounds induce cellular damage by lipid peroxidation, DNA fragmentation, damage of proteins and plasma lipids, depleting antioxidants such as glutathione and cysteine and nitrating proteins leading to cellular and organ dysfunction,

thus resulting in cell death (Ronson *et al*., 1999). Peroxynitrite induces apoptotic cell death in a variety of cell types in culture such as pheochromocytoma-derived PC12 cells, cortical neurons, HL-60 cells and rat thymocytes (Wang *et al*., 2007).

Specific proteases that belong to the caspase family are crucial effectors of apoptosis. In response to an apoptotic stimulus (i.e. ROS), activation of the pro-apoptotic Bcl-2 family members such as BAD, Bak and Bax trigger a sequence of events leading to the release of mitochondrial cytochrome c into the cystol. Cytochrome c forms a complex with, dATP, pro-caspases and Apaf-1, resulting in activation of caspase 9, and then caspase 3. Once activated, caspase 3 cleaves poly ADP ribose polymerase (PARP) and induces cytoplasmic and nuclear apoptosis, including DNA fragmentation (Hausenloy *et al*., 2004; Castanenda *et al*., 2003; Kumar and Jugdutt, 2003). Mitochondrial dysfunction also causes release of an alternative protein (apoptosis inducing factor, AIF), which activates caspase 3 for the initiation of apoptotic pathway.

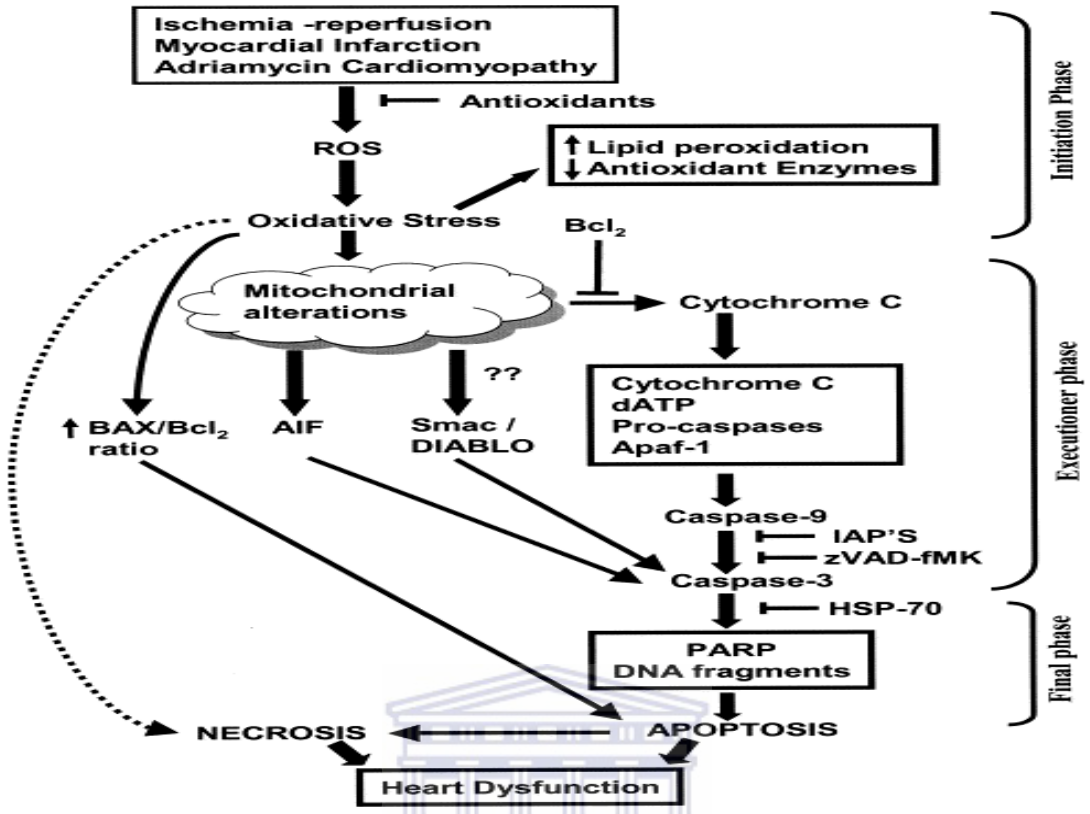


Figure 2.4: Oxidative stress-induced apoptotic pathways involved in the heart disease

(From: Kumar and Jugdutt, 2003)

The rennin-angiotensin system (RAS) is known to be involved in the pathogenesis of ischemia-reperfusion induced myocardial injury (Liang, *et al*., 1981; Wang *et al*., 2001; Ferreira *et al*., 2007). Activation of RAS leads to increased formation of Angiotensin II, which, in turn, causes vasoconstriction, reperfusion arrhythmias and generation of oxygen free-radicals resulting in increased ischemic-reperfusion injury (Maulik *et al*., 2001). Angiotensin II increases intracellular calcium levels of myocytes and smooth muscle cells through activation of ryanodine–sensitive Ca^{2+} release channels in the sarcoplasmic reticulum (SR), leading to positive inotropism, impairment of diastolic

function and coronary vasoconstriction. At pathophysiological levels, angiotensin II is cardiotoxic and induces myocyte death (Moens *et al*., 2005).

2.8 Mechanisms that protect the heart against ischemia-reperfusion injury

Angiotensin converting enzyme (ACE) inhibitors protect against ischemia-reperfusion injury (Maulik *et al*., 2001; Ozer *et al*., 2002; Takeda *et al*., 1997; Linz *et al*., 1986; De Graeff *et al*., 1984). Antioxidants through their free radical scavenging effects inhibit the generation of reactive oxygen species and reduce injury associated with ischemia-reperfusion (Moens *et al*., 2005; Banerjee *et al*., 2205; Dhalla *et al*., 2000; Andreadou *et al*., 2006; Jan-Kan *et al*., 2005; Marczin *et al*., 2003; Venardos *et al*., 2004; Banerjee *et al*., 2002). Endogenous antioxidants such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are the major antioxidant defense mechanisms in the heart and have a protective function in maintaining thiol groups of enzymes and other proteins in their reduced state and in preventing peroxidation of membrane lipids (Molyneux *et al*., 2002).

Activation of the prosurvival kinases such as Akt and Erk 1/2 which are termed reperfusion injury salvage kinase (RISK) pathway has been demonstrated to confer powerful cardioprotection against myocardial ischemia-reperfusion injury (Haunseloy *et al*., 2004; Haunseloy *et al*., 2005; Engelbrecht *et al*., 2006; du Toit *et al*., 2008). The RISK pathway is activated down stream of phosphatidylinositol-3-OH kinase (PI3-K). The pathway has been implicated in cellular survival through recruitment of anti-apoptotic pathway of protection (Haunseloy *et al*., 2004). Activation of Akt or Erk 1/2

cascades phosphorylate the pro-apoptotic protein BAD. Phosphorylation of BAD results in its binding to 14-3-3 proteins, which confiscate it from its mitochondrial targets, thereby preventing apoptosis. Also, phosphorylation of Akt or Erk 1/2 inhibits conformational changes in BAX required for its translocation to the mitochondria, thereby preventing apoptosis. ACE inhibitors (Frolkis *et al*., 2001), antioxidants (Onimaru *et al*., 2006) and the RISK pathway (Akt and Erk 1/2) (Hausenloy *et al*., 2005) prevent cell death by inhibiting factors that mediate apoptosis and necrosis (ROS and AII).

2.9 ACE inhibitors and ischemia-reperfusion

Clinical and experimental studies have established the therapeutic benefits of ACE inhibitors not only in treating hypertension and congestive heart failure but also in reducing reinfarction (Eichhorn, 1998), limiting infarct size (Liu *et al*., 1996; Ozer *et al*., 2002) and reducing reperfusion arrhythmias (Ozer *et al*., 2002, Olmez *et al*., 1995; Westlin *et al*., 1998; van Gilst *et al*., 1986). The beneficial effects of ACE inhibitors are mediated through vasodilation by preventing or reduction in both local AII generation and bradykinin degradation (Ferreira *et al*., 2007; Ozer *et al*., 2002). ACE inhibitors facilitate salt and water excretion by complex effects on the kidney and these include attenuation of secondary hyperaldosteronism with a reduction in mineralocorticoid-stimulated sodium reabsorption. ACE inhibitors also inhibit angiotensin-mediated thirst by an action on the hypothalamus. The attenuation of aldosterone effects reduces hypokalemia, and this may contribute to the antiarrythmic effect of ACE inhibitors (Fletcher, 1996).

In an *in vivo* model of myocardial ischemia-reperfusion injury, acute treatment with captopril (4 mg/kg), an ACE inhibitor, when administered before ischemia rather than before reperfusion prevents loss of haemodynamic function (LVDEP and MAP), rise in TBARS and depletion of endogenous antioxidants (glutathione, superoxide dismutase and catalase) (Maulik *et al*., 2001). Similarly, using the isolated perfused heart, acute treatment with captopril (8 µg/ml or 80 µg/ml) for few minutes before ischemia and during the first few minutes of reperfusion improved post-ischemic cardiac function (rate pressure product and coronary flow) and levels of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and creatine phosphate (CrP) (Takeda *et al*., 1997). Using an *in vivo* model of myocardial ischemia-reperfusion injury, acute treatment with captopril (3 mg/kg) before ischemia reduced the incidences of ventricular fibrillation and myocardial infarct size (Ozer *et al*., 2002).

2.10 PKB / Akt pathway

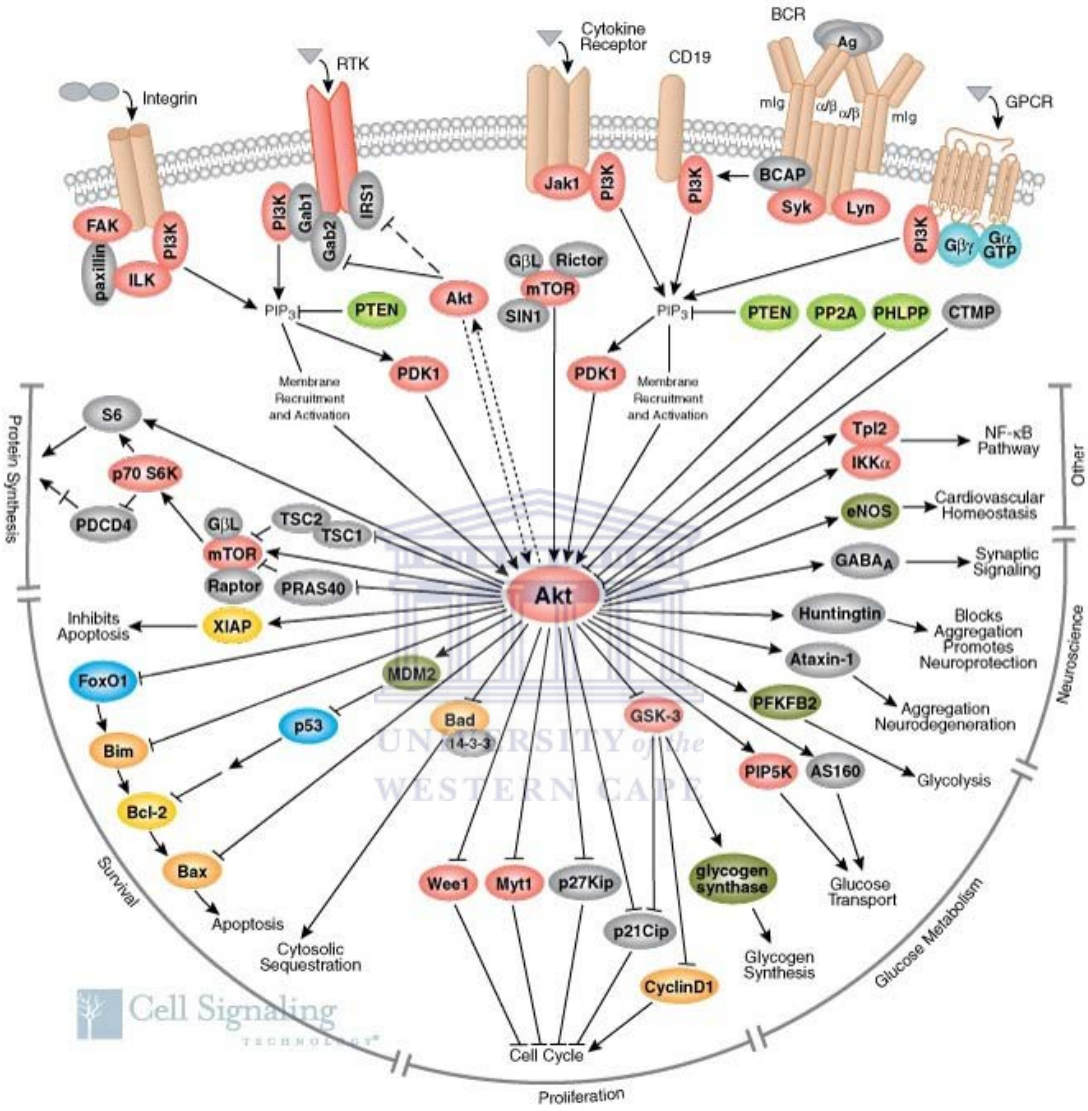


Figure 2.5: Schematic representation of the PI-3 kinase-PKB / Akt signaling pathway
(From: www.cellsignal.com, Feb 2010)

The serine / threonine protein kinase B also known as Akt is a key mediator of signal transduction (cell proliferation, survival and apoptosis) processes (Lawlor *et al*., 2001). The protein has three isoforms, namely, Akt1 / PKB-alpha, Akt2 / PKB-Beta and Akt3 /

PKB-gamma. Akt has three domains, the N-terminal pleckstrin homology (PH) domain, a kinase domain and a C-terminal regulatory domain. The binding of ligands such as growth factors, cytokines, mitogens, insulin or insulin like growth factors and hormones to the receptor tyrosine kinase (RTK) in the cell membrane results in autophosphorylation of tyrosine residues on the intracellular domain of the receptor. Phosphoinositol 3-kinase (PI-3-kinase) which consists of an 85-kDa regulatory subunit (p85) and a 110-kDa catalytic subunit (p110) is then recruited to the phosphotyrosine residues via Src Homology 2 (SH2) domains in the regulatory subunit. The binding of the p85 subunit to the phosphorylated RTK leads to conformational change in the catalytic subunit and consequent kinase activation (Brar *et al*., 2002). PI-3-kinase phosphorylates membrane bound phosphatidylinositol-4,5-diphosphate (PIP2) to produce phosphatidylinositol-3,4,5-triphosphate (PIP3) and this can be reversed by phosphatase and tension homology (PTEN). The binding of PIP3 to the pleckstrin homology (PH) domain of Akt anchors Akt to the plasma membrane and allows its phosphorylation (at T308 and S473 sites) and activation by phosphoinositide–dependent kinases (PDK-1 and PDK-2) (Anderson *et al*., 1998). Akt mediate many of the downstream events (protein synthesis, survival, proliferation and glucose metabolism) regulated by PI-3-kinase. PKB / Akt protein kinase promotes cell survival by inhibiting proteins that mediate apoptosis, such as the Bcl2 family member BAD and the Forkhead family of transcription factors. BAD promotes apoptosis by interacting with Bcl-X_L on the mitochondrial membrane. Phosphorylation of BAD by PKB enables it to interact with 14-3-3 proteins which prevent it from binding to Bcl-$X_{L,}$ thereby preventing apoptosis (Lawlor *et al*., 2001). Akt regulates cell growth through its effects on the mTOR and p70 S6 kinase pathways, the cell cycle and cell

proliferation through its direct action on the CDK inhibitors p21 and p27, and its indirect effect on the levels of cyclin D1 and p53 (Manning and Cantley, 2007). Akt has been shown to regulate proteins involved in neuronal function including GABA receptor, ataxin-1 and huntingtin proteins (Manning and Cantley, 2007).

2.10.2 PKB / Akt in ischemia-reperfusion

In response to ischemia-reperfusion-induced injury, several signal transduction pathways in the heart are triggered (Engelbrecht *et al*., 2006). The PI-3-kinase-PKB / Akt pathway is activated in response to ischemia-reperfusion injury and initiate myocardial protection through its anti-apoptotic action (Haunseloy *et al*, 2004). Activation of PKB via PI-3-K inase has been shown to protect cells against hypoxia / reoxygenation induced cell death (Brar *et al*., 2002). PKB / Akt promotes survival of cardiomyocytes *in vitro* and protects against ischemia-reperfusion injury in the mouse heart (Fujio *et al*., 2000). Haunseloy *et al*. (2005) has shown that activation of prosurvival kinase PKB / Akt in both pre-conditioning and post-conditioning to be cardioprotective against myocardial ischemia-reperfusion injury.

2.11 Research problem

The aqueous extract of the leaves of *O. africana* has angiotensin converting enzyme (ACE) inhibitory properties (Adersen *et al*., 1997; Wang, 2008). The triterpenoids isolated from the *O. africana* leaves have also been shown to have antioxidant properties (Somova *et al*, 2003). ACE inhibitory and antioxidants properties are noteworthy since ACE inhibitors (Ozer *et al*., 2002; Eichhorn, 1998; Maulik *et al*., 2001; Takeda *et al*.,

1997) and antioxidants (Venardos *et al*., 2004; Dhalla *et al*., 2000; Marczin *et al*., 2003) protect the heart against ischemia-reperfusion injury.

To our knowledge, there is currently no study in which cardioprotection by *Olea* has been demonstrated in ischemia-reperfusion injury. Therefore, the present study aims to investigate the cardioprotective effects of *O. africana* in ischemia-reperfusion injury. The PKB / Akt pathway which is involved in cardioprotection against ischemia-reperfusion injury (Haunseloy *et al*., 2004; Haunseloy *et al*., 2005, Fujio *et al*., 2000), is activated downstream of PI-3-kinase (Engelbrecht *et al*., 2006). Angiotensin II decreases the intrinsic PI-3-kinase activity (Folli *et al*., (1997)). Furthermore, Wang (2008) showed that the crude aqueous extract of the *O. africana* leaves decrease plasma AII levels in hypertensive rats. We therefore hypothesized that ACE inhibitors increase PI-3-kinase activity and thus activate PKB. Based on our hypothesis on ACE inhibitors, we will also determine whether the protection is mediated via the PKB / Akt signaling mechanism, since the aqueous extract of *O. africana* has ACE inhibitory effects (Adersen *et al*., 1997; Wang, 2008). *O. africana* is traditionally used with hot water as a tea, hence it is important for the aqueous extract to be considered.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Preparation of the extract

Fresh leaves of *Olea europaea* subsp. *africana* were collected between April and May 2006 at the University of the Western Cape (UWC) and ground powder from the leaves collected in June 2006 were bought from Parceval pharmaceutical company in Cape Town, South Africa. The leaves that were harvested at UWC were identified by Mr Frans Weitz of the Department of Botany. The leaves were washed and air-dried for three weeks. The dried leaves were milled to a fine powder. The aqueous extract was obtained by shaking the powder (100 g) in distilled water (900 ml) and allowing the mixture to stand for 24 hours before filtering. The filtrate was frozen overnight at -80 ℃ and freeze dried until a fine powder was obtained. The freeze dried powder was stored in a sealed container and kept in the fridge.

3.1.1 Toxicity test

Toxicity of the aqueous extract of *Olea africana* was investigated using the brine shrimp (*Artemia salina*) toxicity test, according to the method of Meyer *et al*. (1982). Artemia eggs (30 – 40 g) obtained from a local pet shop were hatched in a 1-liter bottle filled with fresh filtered sea water. The eggs were kept under constant aeration at 30 ℃ until the eggs were fully hatched. After hatching, 100 µl containing between 10 - 20 active nauplii, was transferred to petri dishes containing sea water and different concentrations of the extract and made up to a final volume of 5000 µl with sea water. The toxicity of the

plant extract was determined in duplicates using nine concentrations: 2.5, 10, 25, 50, 250, 750, 1250, 1750 and 2250 mg/ml. To account for accidental deaths, controls without extract were included. The petri dishes were maintained at room temperature for 24 hours under a light. For each petri dish, the number of larvae were recorded. After 24 hours, the number of surviving larvae were counted and the percentage deaths at each concentration were determined.

LC_{50} determination

The percentage mortality was plotted against the *O. africana* concentration. The LC_{50} value (value which causes 50% mortality) was obtained by regression analysis.

3.2 Chemicals and drugs

Sodium chloride, sodium bicarbonate, potassium chloride, potassium di-hydrogen phosphate, magnesium sulphate, sodium sulphate, calcium chloride, glucose, Evans Blue, 2, 3, 5 – triphenyltetrazolium chloride, sodium di-hydrogen phosphate, di-sodium hydrogen phosphate, 37% formaldehyde solution, potassium phosphate, hydrochloric acid and ethyl acetate were obtained from Kimix Chemical and Laboratory supplies (South Africa).

Tris, EDTA, ß- glycerolphosphate, tetra sodium pirophosphate, sodium orthovanadate, leupeptin, aprotinin, phenylmethyl sulphonyl fluoride, triton, Commassie Brilliant Blue, phosphoric acid, ethanol, sodium dodecyl sulphate, acrylamide, ammonium persulfate solution, Tetramethylethylenediamine, butanol, glycine, methanol, Ponseau stain and

antibodies were purchased from Cell Signaling Technology (Boston, United States of America).

3.3 Animals

All animals received humane care in accordance with the Principles of Laboratory Animal Care of the National Society of Medical Research and the Guide for Care and use of laboratory Animals of the National Academy of Sciences (National Institute of Health Publications no. 80 - 23, revised 1978). Male Wistar rats weighing between 220 – 250 g were used in preliminary experiments and rats weighing from 160 – 200 g were used for the chronic treatment with the plant extract. The animals were obtained from the University of Cape Town and the University of KwaZulu-Natal, South Africa. The animals were maintained in the Medical Biosciences Department animal house at University of the Western Cape and given free access to normal tap water and standard rat chow. The temperature of the animal room was maintained at 26 °C, with constant humidity and a 12-h light/dark cycle. The animals were kept in these conditions for a week to acclimatize to the new environment.

3.4 Isolation of the rat heart

The animals were anaesthetized by intraperitoneal injection (sodium pentabarbitone 40 mg/kg). The diaphragm was accessed by a trans-abdominal incision to expose the thoracic cavity. The thorax was opened by bilateral incision along the lower margin of the last to the first rib to expose the heart. The heart was rapidly excised and immediately immersed in ice cold Krebs-Henseleit bicarbonate buffer solution containing (mM):

sodium chloride (119.00), sodium bicarbonate (25.00), potassium chloride (4.75), potassium di-hydrogen phosphate (1.2), magnesium sulphate (0.6), sodium sulphate (0.6), calcium chloride (1.25) and glucose 10, at pH 7.4.

3.5 Preliminary experiments (Perfusion with *O. africana* extract)

To determine whether treatment with *O. africana* aqueous extract is cardioprotective against ischemia-reperfusion injury, the working heart model according to Neely *et al*. (1967) was used. In all the preliminary experiments, the concentration of the extract used for the isolated heart was calculated based on the assumption that a 250 g rat has a blood volume of 16 ml (Diehl e*t al*., 2001).

3.5.1 Perfusion with different concentrations of *O. africana* extract

Hearts were isolated as described in section 3.4 and perfused with Krebs-Henseleit buffer via the aortic cannula in a retrograde manner at 100 cm H_2O. After 10 min, the perfusion was switched to the working mode for 20 min. Hearts were then perfused for 10 min with one of the following concentrations of the extract: 10, 12, 20, 40 or 200 mg/kg rat. Parameters determined were coronary flow and aortic output using a measuring cylinder.

3.5.2 Perfusion with *O. africana* extract at constant flow

Hearts were perfused with *O. africana* extract (200 mg/kg) as described above except that the coronary flow was maintained at a constant rate (10.5 ml/min) using a peristaltic

pump. Parameters measured were aortic systolic and diastolic pressures using a pressure transducer connected to the data-acquisition system.

3.5.3 Infusion with *O. africana*

Hearts were also perfused with the infusion (200 mg/kg) prepared from *O. africana* powder from the leaves that were obtained in Parceval pharmaceutical company. The powder (50 mg) was dissolved in 16 ml of boiling water. The infusion was left overnight to allow it to steep and filtered before use. The perfusion protocol as described in section 3.5.1 was followed. The parameter measured was coronary flow using a measuring cylinder.

3.5.4 Light sensitivity of *O. africana* extract

To determine whether the extract is light sensitive, hearts were perfused with the extract (1000 mg/kg) as described in section 3.5.1. The reservoir was covered with foil to protect the extract from the light. Parameters determined were coronary flow using a measuring cylinder and temperature using a thermal probe inserted into the coronary sinus.

3.6 Chronic treatment of rats with *O. africana* extract

To determine whether chronic treatment with the crude aqueous extract of *Olea africana* will protect the heart against ischemia-reperfusion injury, rats were subjected to one of the following protocols.

3.6.1 Effect of chronic treatment with *O. africana* extract on cardiac function

For the evaluation of cardiac function, 16 rats were used. They were divided into the control group (n=8) and the *O. africana* group (n=8). The control group received normal tap water. The *O. africana* group received the extract of *O. africana* in the drinking water (1000 mg/kg). The average volume of water consumed by the animals per cage and the body weights were recorded twice a week to determine the correct quantity of the extract to be used. Rats were treated for 5 weeks.

The Langendorff isolated perfused heart as described by Langendorff (1895) was used as our experimental model. After removal, the hearts were perfused via the aortic cannula in a retrograde manner at 100 cm H_2O with Krebs-Henseilet buffer oxygenated with 95% O_2 / 5% CO_2 at 37 °C. During the initial perfusion, excess tissue from the heart was removed. A latex balloon filled with normal saline was inserted into the left ventricle via the left atrium. The balloon was connected to a pressure transducer which was connected to a data-acquisition system to record cardiac function. Left ventricular end-diastolic pressure was set between 4 - 10 mmHg. The temperature of the heart was monitored by a thermal probe inserted in the coronary sinus and was maintained at 37 °C. After 30 min perfusion, global ischemia was induced for 20 min by occluding the perfusion inflow lines to the aorta. During ischemia the temperature of the heart was maintained at 36.6 °C. After 20 min ischemia, reperfusion was initiated by opening the inflow lines to the aorta. At the end of 30 min reperfusion, hearts were freeze-clamped with Wollenberger clamps pre-cooled in liquid nitrogen. The hearts were then kept at –80 °C for PKB / Akt determination. PKB / Akt were determined as described in section 3.6.3.1.

3.6.1.1 Parameters measured

A pressure transducer connected to the balloon was connected to a data-acquisition system to record the left ventricular systolic and diastolic pressures and heart rate. A measuring cylinder was used to collect and measure coronary flow. The readings were taken at the end of stabilization and reperfusion periods. Cardiac function was determined by left ventricular developed pressure (LVDevP) and rate pressure product (RPP). Left ventricular developed pressure is equal to systolic pressure minus diastolic pressure. Rate pressure product is equal to heart rate multiplied by left ventricular developed pressure. Functional recovery was expressed as percentage of the pre-ischemic value using the following formulae:

% RPP recovery = (RPP post-ischemic/RPP pre-ischemic) ×100

% LVDevP recovery = (LVDevP post- ischemic/LVDevP pre-ischemic) × 100

3.6.2 Effect of chronic treatment with *O. africana* extract on cardiac infarct size

For the determination of infarct size, 24 rats were used. They were divided into the control group (n=5), *O. africana* group (n=6), captopril group (n=5) and the jelly (vehicle) group (n=6). The first (control) group received normal tap water. The second (*O. africana*) group received the extract of *O. africana* in the drinking water (1000 mg/kg/day). The average volume of water consumed by the animals per cage and the body weights were recorded twice a week to determine the correct quantity of the extract. The third (captopril) group received captopril (50 mg/kg/day) in jelly blocks. The fourth

(jelly) group received jelly blocks daily as a vehicle control for captopril. All rats received normal laboratory rat chow. Rats were treated for 5 weeks.

At the end of treatment period, hearts were isolated and perfused according to the method described in section 3.6.1. After 30 min perfusion, regional ischemia was induced for 45 min by left coronary artery ligation using a silk suture. During regional ischemia, the temperature of the heart was maintained at 36.6 °C. Reperfusion was initiated for 35 min by loosening the suture. At the end of the experiment, the suture around the left coronary artery was securely tied and ± 500 µl of 0.2% Evans Blue suspension was slowly injected via the aortic cannula. The hearts were removed and frozen at -20 °C for 24 - 48 hours before analysis. After freezing, the heart was cut into 2-mm thick slices and stained in the dark with 1% w/v triphenyltetrazolium chloride in phosphate buffer (pH 7.4) at 37 °C for 15 - 20 min. Slices were then fixed in 10% v/v formaldehyde solution to enhance the contrast between stained viable tissue and unstained necrotic tissue. The area at risk and the area of infarcted tissue in the risk zone were determined using The University of Texas Health Science Center at San Antonio (UTHSCSA) Image tool software version 3. The infarct size was expressed as a percentage of the risk zone.

3.6.3 Effect of chronic treatment with *O. africana* extract, captopril and jelly on PKB / Akt

For PKB / Akt analysis 20 rats were used. They were divided and treated according to the groups described in section 3.6.2. Each group comprised of 5 animals.

3.6.3.1 Protein determination

At the end of treatment period, animals were sacrificed and prepared according to the method described in 3.4. The hearts were then kept at -80 °C. The tissue from the left ventricle was homogenized for 8 sec in a lysis buffer containing: Tris 20 mM, EDTA 1mM, sodium chloride 150 mM, ß-glycerolphosphate 1mM, tetra sodium pirophosphate 2.5 mM, sodium orthovanadate 1mM, leupeptin 10 µg/ml, aprotinin 10 µg/ml, phenylmethyl sulphonyl fluoride 50 µg/ml and triton 1% and then centrifuged at 14500 rpm for 10 min. The supernatant was drawn and the lysate protein content was determined using the Bradford assay (Bradford, 1976). After protein determination, lysates were diluted in Laemmli sample buffer, boiled for 5 min and stored at –20 °C for western blot analysis. Lysates were boiled for 5 min and the protein (25 ug/ul) was separated on a 12% Sodium dodecyl sulphate gel. The separated proteins were transferred to the PVDF membrane (Immobilon ^{TM} P, Millipore) and the membrane was stained with Ponceau red for visualization of the protein. The membrane was then washed 3 times with 10 X TBS Tween (TBST) and non-specific binding sites were blocked by 5% fat free milk in TBST. The membrane was then washed 6 times with TBST and incubated with the primary antibody that recognizes phosphospecific PKB (Ser ^{473} and Thr ^{308}). The membrane was then washed 6 times with TBST for 5 min before incubated with the secondary antibody. After washing with TBST, the membrane was covered with ECL ^{TM} detecting reagents for 1 min and quickly exposed to an autoradiography film (Hyperfilm ECL, RPN 2103) to detect light emission (ECL ^{TM} Western Blotting). Films were densitometrically analyzed (UN-SCAN-IT, silkscience).

3.7 Statistical analysis

Results are expressed as the mean ± SEM (standard error of the mean). Two groups were compared using either the Mann-Whitney or t test, when appropriate. Multiple groups were compared using either Kruskal-Wallis or One-way ANOVA test, when appropriate. A p-value < 0.05 was considered statistically significant. Tests were performed using SPPS V16.0 statistical package (http://www.spss.com) and graphs were plotted using Graph Pad Prism version 5.02 (http://www.graphpad.com).

CHAPTER 4

RESULTS

4.0 Percentage yield of the extract

From the leaves that were harvested at the University of the Western Cape, 1039.14 g of the ground powder was used and 79.98 g of the lyophilysate was obtained, thus producing a 8% yield. Of the ground powder obtained from Parceval pharmaceutical company, 2400 g was used and the powder obtained after freeze drying was 326.45 g, thus producing a 13.6% yield.

4.1 The brine shrimp toxicity test

The toxicity of the plant extract was determined using nine concentrations of *O. africana* crude aqueous extract (Table 4.1). The LC_{50} was 1269 mg/ml and the associated 95% confidence interval was 1016 to 1586 mg/ml (Appendix I). At a concentration of 2250 mg/ml all shrimps were killed.

Table 4.1 The percentage lethality of the different concentration of *O. africana* extract on brine shrimps

	Concentration (mg/ml)								
	2.5	10	25	50	250	750	1250	1750	2250
Control	0	0	0	0	0	0	0	0	0
O. africana	0	0	0	8	18	25	40	68	100

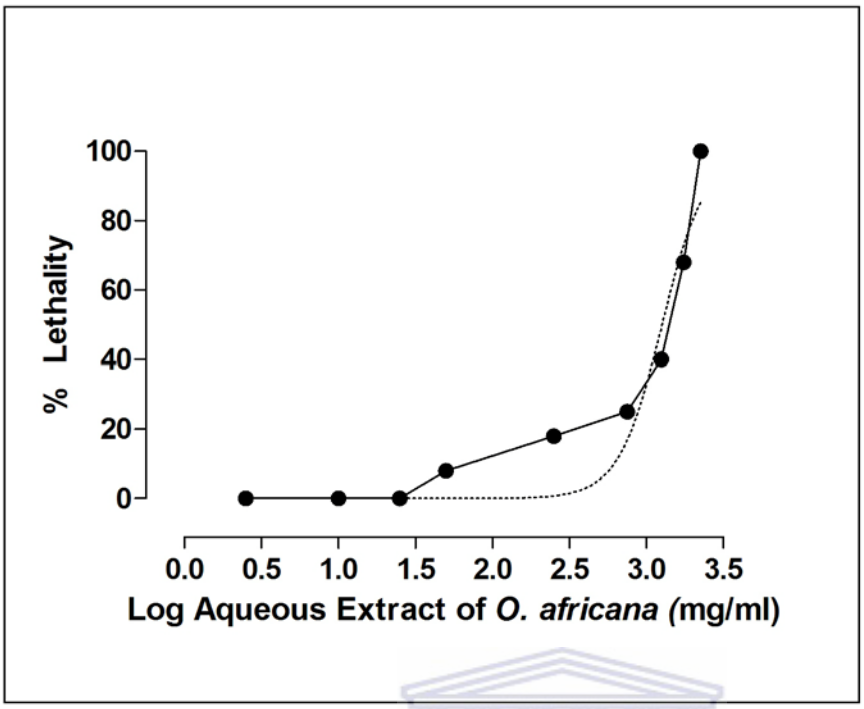


Figure 4.1 Toxicity of the aqueous extract of *O. africana*. The stippled line show the non-linear regression

4.2 Preliminary experiments

In all the experiments in which rat hearts were perfused with the plant extract, no statistics are presented due to the small sample size used. The reason for the small sample size is because the coronary flow decreased dramatically, we therefore, switched to a model where the animals were treated with the plant extract.

4.2.1 Perfusion with different concentrations of *O. africana* extract

To determine a suitable concentration of the extract to be used, rat hearts were perfused with Krebs-Henseleit buffer for 10 min in the Langendorff mode, followed by 20 min perfusion in the working mode. Hearts were then perfused with different concentrations of *O. africana* extract in the Langendorff mode for 10 min. The coronary flow and aortic output of hearts perfused with the Krebs-Henseleit buffer was not different between

groups, averaging from 16.6±1.8 ml/min to 17.5±0.8 ml/min and between 35.0±1.1 ml/min and 40.2±4.3 ml/min respectively (Table 4.2). When hearts were perfused with the extract, coronary flow averaged from 0.3±0.0 ml/min to 0.6±0.1 ml/min and aortic output could not be measured. When hearts were perfused with the extract, coronary flow (Table 4.2), decreased significantly at all concentrations.

Table 4.2 Effect of perfusion with different concentrations of *O. africana* extract on cardiac function

Number of hearts	Working heart Perfusion		Langendorff Perfusion		
	CF (ml/min)	AO (ml/min)	Extract conc (mg/kg)	CF (ml/min)	AO (ml/min)
2	17.5±0.8	40.2±4.3	10	0.6±0.1	ND
2	17.3±1.3	36.4±2.0	12	0.3±0.4	ND
2	17.3 ±1.1	38.1±1.7	20	0.4±0.0	ND
3	15.6±0.6	35.0±1.1	40	0.3±0.1	ND
4	16.6±1.8	35.0 ±3.0	200	0.3±0.0	ND

Results are expressed as the mean±SEM, ND= not detectable (Langendorff mode)

4.2.2 Perfusion with *O. africana* extract at constant flow

To determine the effect of the plant extract on cardiac function when perfused at constant flow, hearts were perfused with Krebs-Henseleit buffer for 10 min in the Langendorff mode, followed by 20 min perfusion in the working mode. Hearts were then perfused with *O. africana* (200 mg/kg) extract in the Langendorff mode for 10 min with coronary

flow set at 10.5 ml/min using a peristaltic pump. During perfusion with the Krebs-Henseleit buffer, systolic aortic pressure was 90.1±0.3 mmHg and diastolic aortic pressure was 40.0±1.1 mmHg. When hearts were perfused with the extract, the contraction of the heart became weaker until, after 4 - 7 min, the heart stopped beating. Coronary flow decreased from 10.5 ml/min to 0.3±0.1 ml/min, despite the peristaltic pump being used.

Table 4.3 Effect of perfusion with *O. africana* extract at constant flow

	Systolic aortic pressure (mmHg)	Diastolic aortic pressure (mmHg)
Perfusion with Krebs-Henseleit Bicarbonate buffer	90.1±0.3	40.0±1.1
Perfusion with *O. africana*	ND	ND

Results are expressed as the mean±SEM, n=3; ND= not detectable

4.2.3 Effect of *O. africana* infusion

To determine the effect of *O. africana* infusion on cardiac function, hearts were perfused with Krebs-Henseleit buffer for 10 min in the Langendorff mode, followed by 20 min perfusion in the working mode. Hearts were then perfused with the infusion of *O. africana* (200 mg/kg) in the Langendorff mode for 10 min. When hearts were perfused with the infusion of *O. africana*, coronary flow decreased from 16.1±0.4 ml/min to 0.5±0.3 ml/min (Table 4.4).

Table 4.4 Effect of *O. africana* infusion on coronary flow

	Coronary Flow (ml/min)
Perfusion with Krebs-Henseleit Bicarbonate buffer	16.1±0.4
Perfusion with *O. africana*	0.5±0.3

Results are expressed as the mean±SEM, n=2

4.2.4 Light sensitivity of *O. africana* extract

To determine whether the effect of *O. africana* extract described above was due to light sensitivity, the reservoir was covered with foil to protect the extract from light. Hearts were perfused with Krebs-Henseleit buffer for 10 min followed by 10 min perfusion with *O. africana* extract. When hearts were perfused with Krebs-Henseleit bicarbonate buffer, coronary flow was 9.6±0.4 ml/min and decreased to 0.4±0.0 ml/min during perfusion with *O. africana* extract. Temperature also decreased from 35.6±0.0 °C during perfusion with the Krebs-Henseilet buffer to 31.2±0.7 °C when hearts were perfused with the plant extract (Table 4.5).

Table 4.5 Effect of perfusion with *O. africana* extract on coronary flow and temperature

	Coronary Flow (ml/min)	Temp (°C)
Perfusion with Krebs-Henseleit Bicarbonate buffer	9.6±0.4	35.6±0.0
Perfusion with *O. africana*	0.4±0.0	31.2±0.7

Results are expressed as the mean±SEM, n=4

4.3 Effects of chronic treatment with *O. africana* extract on cardiac function

After the rats were treated with *O. africana* extract for 5 weeks, hearts were isolated and perfused with Krebs–Henseilet bicarbonate buffer for 30 min in the Langendorff mode, followed by 20 min global ischemia. Hearts were then reperfused for 30 min in the Langendorff mode.

4.3.1 Coronary flow

Coronary flow was similar before and after ischemia in the control (11.4±0.9 versus 10.1±1.0 ml/min, p>0.05) and *O. africana* (10.6±0.8 versus 10.1±0.6 ml/min, p>0.05) groups. There was no significance difference between the control and *O. africana* groups before ischemia (11.4±0.9 ml/min versus 10.6±0.8 ml/min) and after ischemia (10.1±1.0 ml/min versus 10.1±0.6 ml/min, p>0.05) (Figure 4.2).

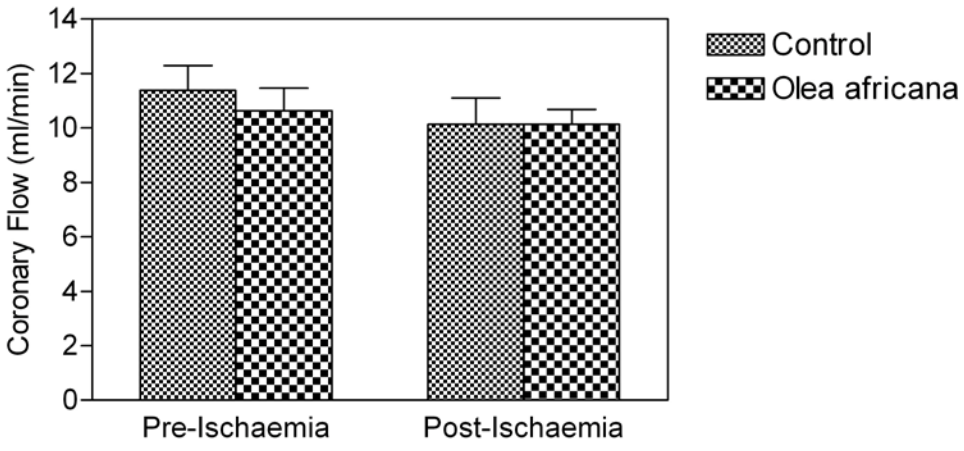


Figure 4.2 Effect of chronic treatment with *O. africana* extract on coronary flow. Values are expressed as the mean±SEM; n=8 in each group

4.3.2 Systolic ventricular pressure

In the control group, systolic ventricular pressure before ischemia was 94.9±3.4 mmHg and 105.1±5.1 mmHg after ischemia, p>0.05. In the *O. africana* group, systolic ventricular pressure before ischemia was 90.5±1.9 mmHg and 89.3±4.1 mmHg after ischemia, p>0.05. Systolic ventricular pressure was similar in *O. africana* and the control groups before ischemia (90.5±1.9 versus 94.9±3.4 mmHg), p>0.05. After ischemia, systolic ventricular pressure differed significantly between the groups (89.3±4.1 versus 105.1±5.1 mmHg), p<0.05, (Figure 4.3).

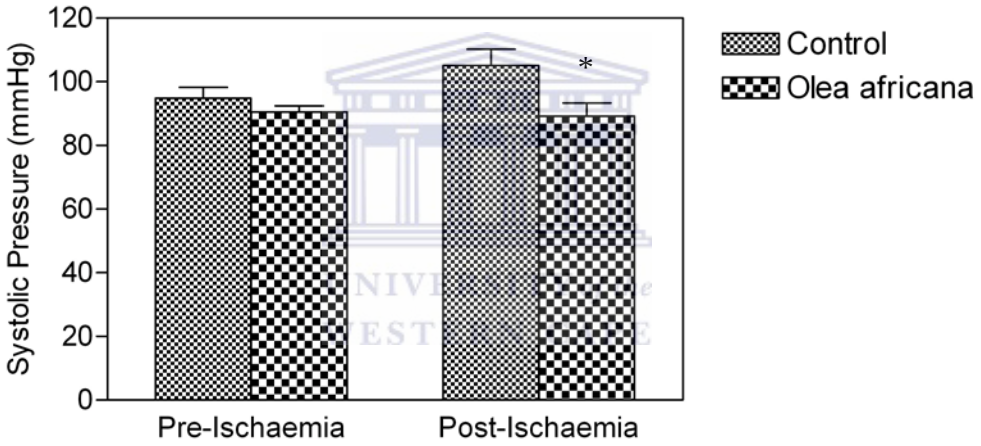


Figure 4.3 Effect of chronic treatment with *O. africana* extract on systolic ventricular pressure. Values are expressed as the mean±SEM; n=8 in each group, *p<0.05 compared to the Post-ischemic control group

4.3.3 Diastolic Ventricular Pressure

The diastolic pressure in the control group before ischemia was 10.3±0.3 mmHg and increased to 32.5±5.3 mmHg after ischemia, p<0.05. Diastolic ventricular pressure in the *O. africana* group before ischemia was 10.0±0.4 mmHg and increased to 21.4±2.5 mmHg after ischemia, p<0.05. There was no significance difference between the control

and *O. africana* groups before (10.3±0.3 versus 10.0±0.4 mmHg) and after ischemia (32.5±5.3 versus 21.4±2.5 mmHg), p>0.05 (Figure 4.4).

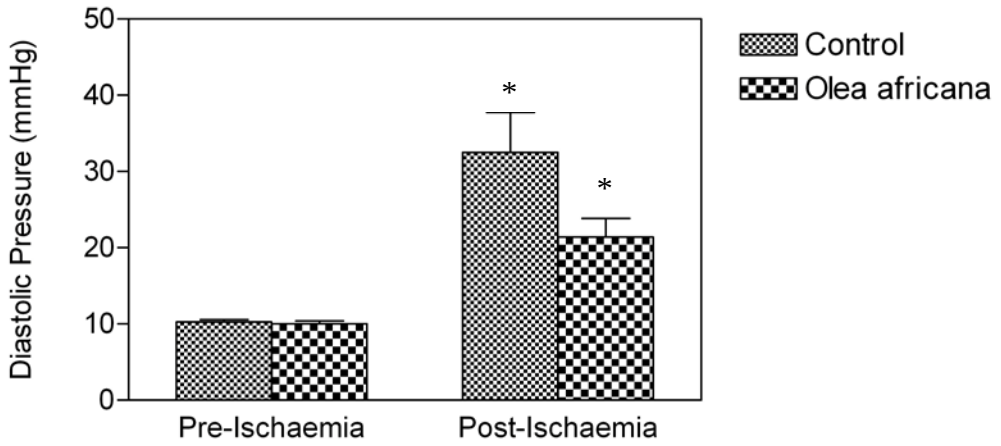


Figure 4.4 Effect of chronic treatment with *O. africana* extract on diastolic ventricular pressure. Values are expressed as the mean±SEM; n=8 in each group, *p <0.05 compared to before ischemia in the same group

4.3.4 Heart rate

The heart rate in the control group was similar before and after ischemia (289.3±11.7 versus 267.3±12.8 bpm), p>0.05. Heart rate in the *O. africana* group before ischemia was 312.5±12.1 bpm and decreased to 292.0±6.8 bpm after ischemia, p<0.05. When comparing the groups, heart rate was similar before ischemia (289.3±11.7 versus 312.5±12.1 bpm, control vs *O. africana*) and after ischemia (267.3±12.8 versus 292.0±6.8 bpm, control vs *O. africana*), p>0.05 (Figure 4.5).

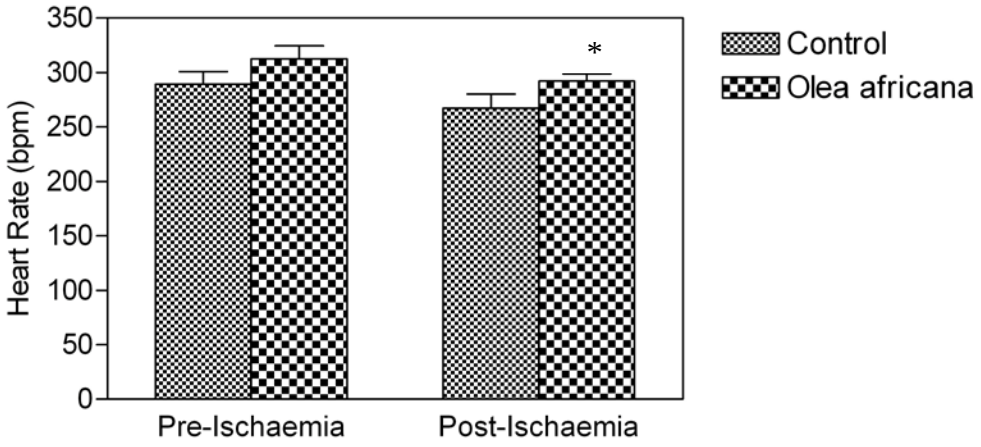


Figure 4.5 Effect of chronic treatment with *O. africana* extract on heart rate. Values are expressed as the mean±SEM; n=8 in each group, *p<0.05 compared before ischemia in the same group

4.3.5 Left ventricular developed pressure (LVDevP)

The LVDevP in the control group before ischemia was 84.6±3.4 mmHg and decreased to 72.6±5.2 mmHg after ischemia, p<0.05. In the *O. africana* group, LVDevP before ischemia was 80.5±2.0 mmHg and decreased to 67.9±4.3 mmHg after ischemia, p<0.05. There was no significance difference in LVDevP between *O. africana* and control groups before (80.5±2.0 versus 84.6±3.4 mmHg respectively) and after ischemia (67.9±4.3 versus 72.6±5.2 mmHg respectively), p>0.05 (Figure 4.6).

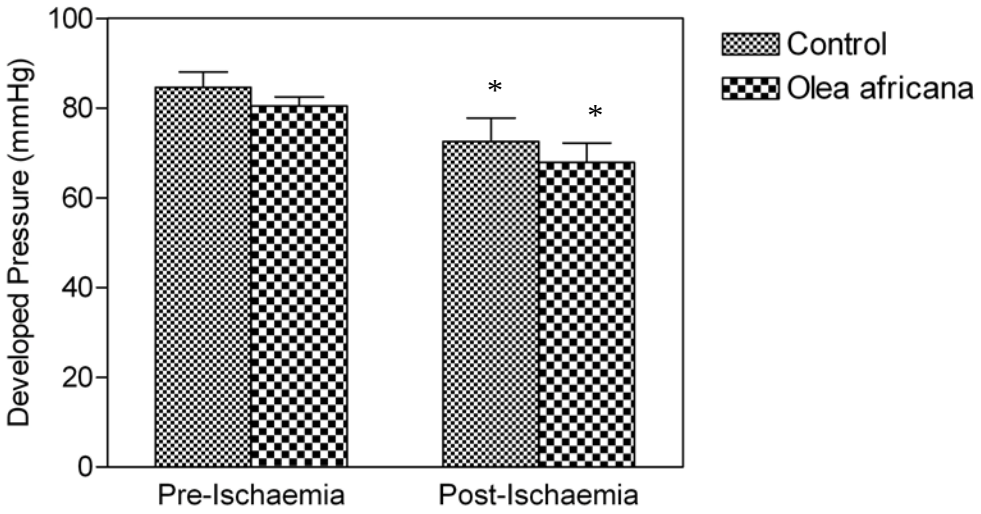


Figure 4.6 Effect of chronic treatment with *O. africana* extract on left ventricular developed pressure. Values are expressed as the mean±SEM; n=8 in each group, *p <0.05 compared to before ischemia in the same group

4.3.6 Rate pressure product (RPP)

The RPP in the control group before ischemia was 24346.8±938.1 mmHg/min and decreased to 19367.5±1581.1 mmHg/min after ischemia, p<0.05. In the *O. africana* group, the rate pressure product before ischemia was 25129.5±1077.7 mmHg/min and decreased to 19893.3±1504.6 mmHg/min after ischemia, p<0.05. Rate pressure product was similar before ischemia in the control group (24346.8±938.1 mmHg/min) and *O. africana* group (25129.5±1077.7 mmHg/min, p>0.05). There was no significance difference after ischemia between the control group (19367.5±1581.1 mmHg/min) and the *O. africana* group (19893.3±1504.6 mmHg/min, p>0.05) (Figure 4.7).

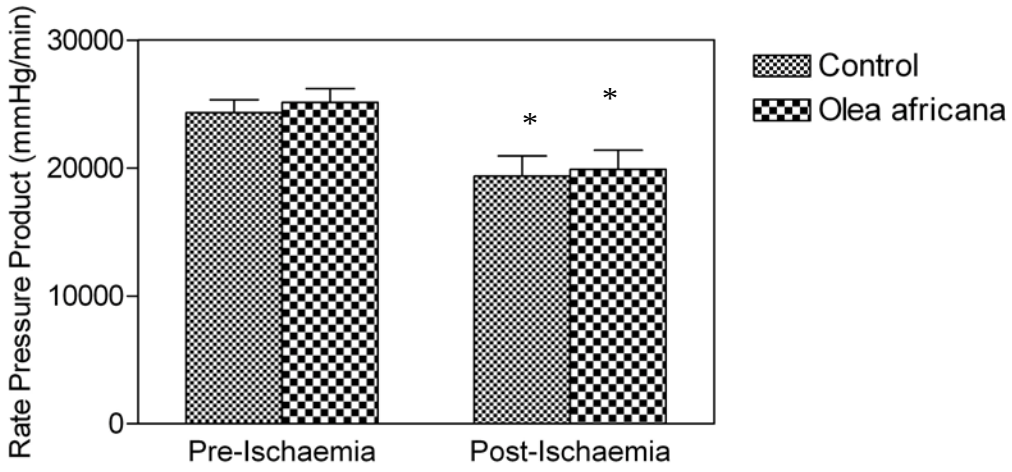


Figure 4.7 Effect of chronic treatment with *O. africana* extract on rate pressure product. Values are expressed as the mean±SEM; n=8 in each group, *p <0.05 compared to before ischemia in the corresponding group

4.3.7 Recovery of the left ventricular developed pressure

The percentage recovery of the LVDevP was similar in the control group (85.6±4.6 %) and the *O. africana* group (84.4±5.1 %) p>0.05 (Figure 4.8).

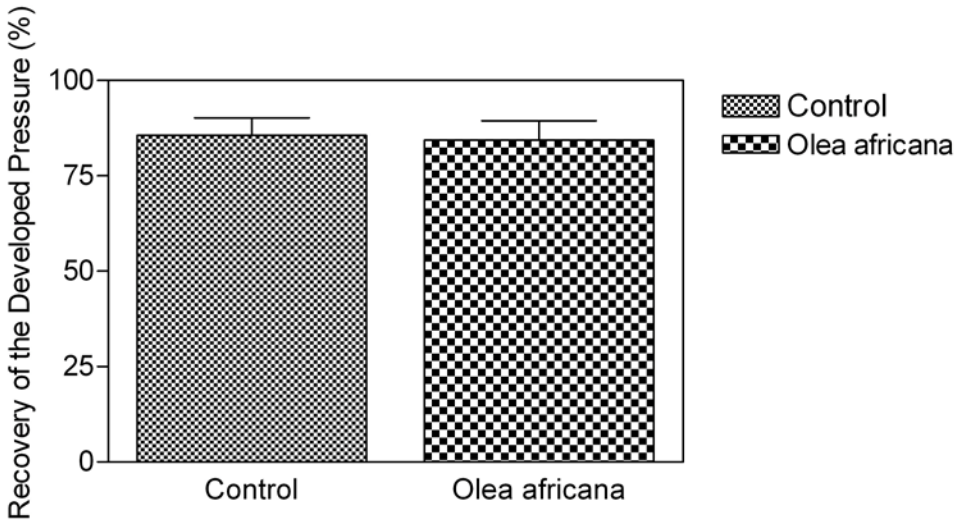


Figure 4.8 Effect of chronic treatment with *O. africana* extract on percentage recovery of the left ventricular developed pressure. Values are expressed as the mean±SEM; n=8 in each group

4.3.8 Recovery of rate pressure product

There was no significance difference in the rate pressure product recovery between the control group (79.4±5.1 %) and the *O. africana* group (79.3±5.7 %), p>0.05 (Figure 4.9).

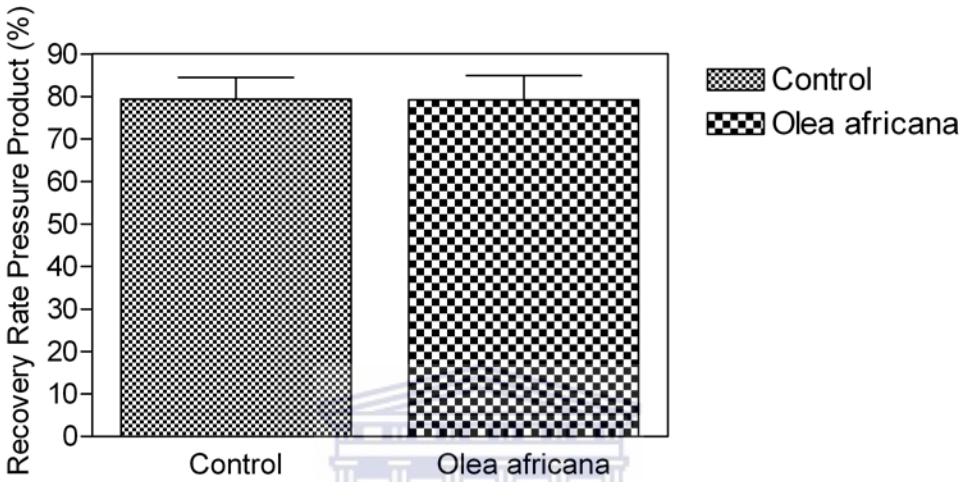


Figure 4.9 Effect of chronic treatment with *O. africana* extract on percentage recovery of rate pressure product. Values are expressed as the mean±SEM; n=8 in each group

4.4 The effect of ischemia-reperfusion injury on total PKB / Akt

Figure 4.10A shows the Western blot of total PKB / Akt of the control and *O. africana* groups. The total PKB / Akt was similar in the *O. africana* (1.0±0.1) and the control group (1.0±0.0), p>0.05 (Figure 4.10B). The values are expressed relative to the control (control =1).

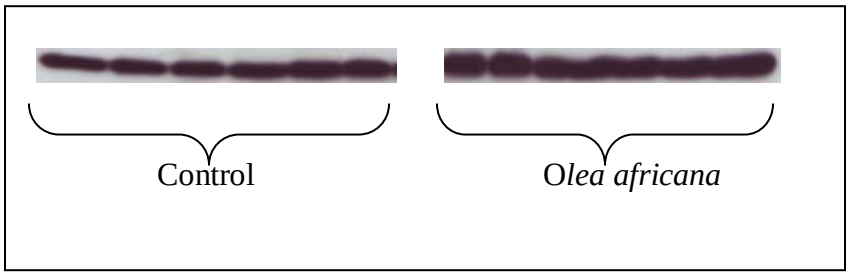


Figure 4.10A Western blot of total PKB / Akt in hearts subjected to ischemia-reperfusion injury.

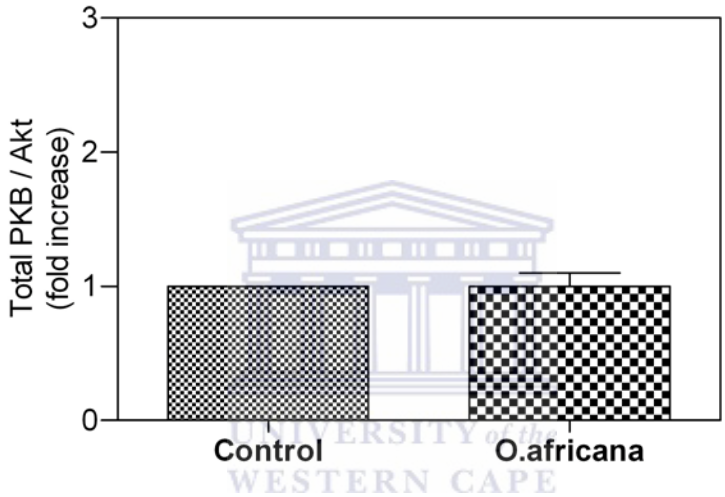


Figure 4.10.B The effect of ischemia-reperfusion on total PKB / Akt. Values are expressed as the mean±SEM; control n=6, *O. africana* n=7

4.4.1 The effect of ischemia-reperfusion injury on PKB / Akt phosphorylation

Figure 4.11A shows the Western blot of phosphorylated PKB / Akt of the control and *O. africana* groups. The PKB / Akt phosphorylation was similar in the *O. africana* (1.1±0.2) and the control groups (1.0±0.0), $p>0.05$, (Figure 4.11B). Values are expressed relative to the control (control=1).

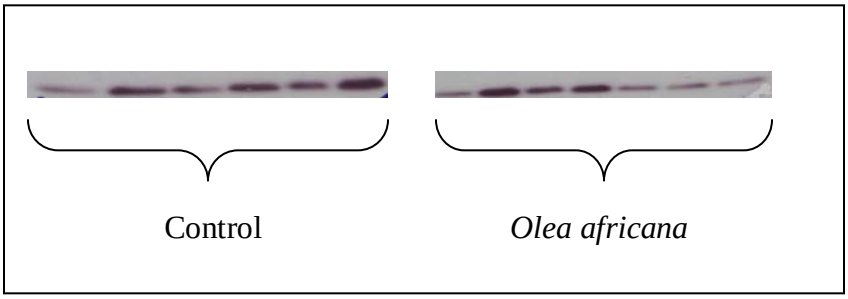


Figure 4.11A Western blot of PKB / Akt phosphorylation in hearts subjected to ischemia-reperfusion.

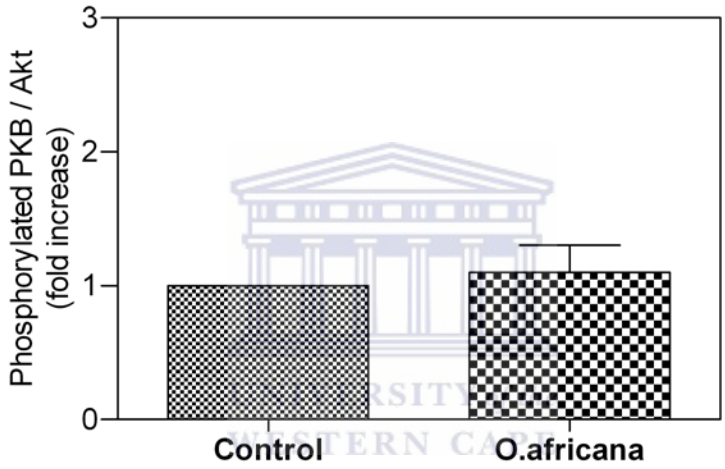


Figure 4.11B The effect of ischemia-reperfusion on PKB / Akt phosphorylation. Values are expressed as the mean±SEM; control n=6, *O. africana* n=7

4.5 Chronic treatment with *O. africana* extract, captopril and jelly on cardiac function and infarct size

4.5.1 Coronary flow

The average coronary flow before ischemia was 11.7±0.9 ml/min in the control group, 11.6±0.7 ml/min in the *Olea africana* group, 11.4±0.8 ml/min in the captopril group and 11.8±0.5 ml/min in the jelly group, $p>0.05$. During ischemia the average coronary flow decreased in the control (6.3±0.6 ml/min), *O. africana* (6.1±0.5 ml/min), captopril

(5.9±0.5 ml/min) and jelly groups (6.4±0.3 ml/min), $p<0.05$. In all the groups, the average coronary flow during ischemia decrease to 53%.

4.5.2 Left ventricular developed pressure (LVDevP)

The LVDevP was similar before ischemia in the control group (106.8±4.8 mmHg), *O. africana* group (109.7±3.7 mmHg), captopril group (96.6±2.6 mmHg) and the jelly group (105.0±2.8 mmHg), $p>0.05$. The LVDevP in the control group before ischemia was 106±4.8 mmHg and decreased during ischemia to 70.4±2.4 mmHg, and after ischemia to 59.8±2.6 mmHg, $p<0.05$. The LVDevP in the *O. africana* group during and after ischemia (64.4±3.1 mmHg and 64.0±1.9 mmHg respectively) differed significantly ($p<0.05$) from the pre-ischemic value (109.7±3.7 mmHg). There was a significant difference in LVDevP in the captopril group before, during and after ischemia (96.6±2.6 mmHg, 66.4±3.1 mmHg and 67.0±3.6 mmHg respectively), $p<0.05$. The LVDevP differed significantly in the jelly group before ischemia (105±2.8 mmHg), during ischemia (79.5±2.4 mmHg) and after ischemia (68.5±2.3 mmHg), $p<0.05$. During ischemia, LVDevP in the control group was 70.4±2.4 mmHg, in the *O. africana* group 64.0±2.5 mmHg, in the captopril group 66.4±3.1 mmHg and in the jelly group 79.5±4.2 mmHg, $p>0.05$. There was no significant difference after ischemia in the control group (59.8±2.6 mmHg), the *O. africana* group (64.0± 1.9 mmHg), the captopril group (67.0±3.6 mmHg) and the jelly group (68.5±2.3 mmHg), $p>0.05$, (Figure 4.12).

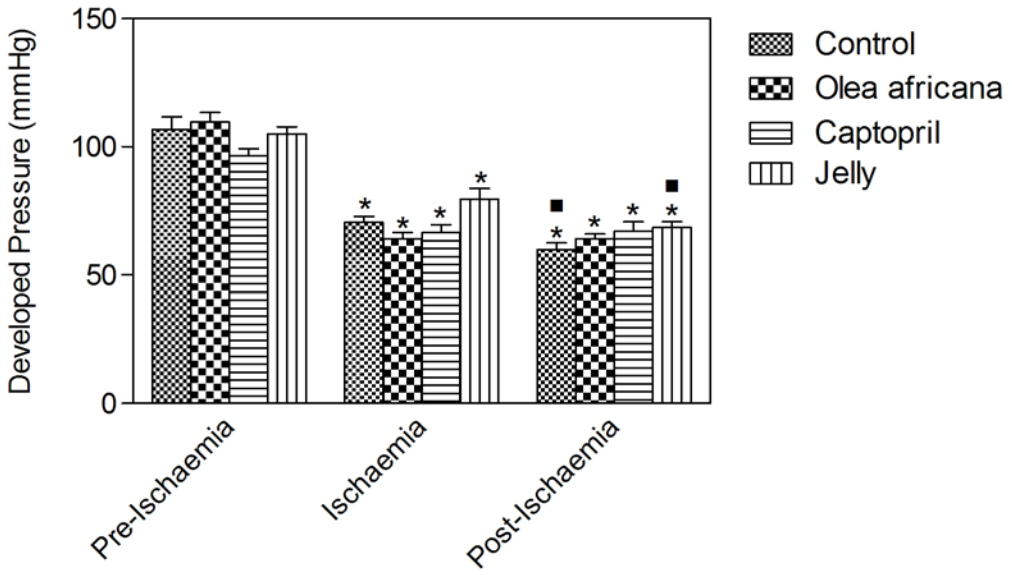


Figure 4.12 Effect of chronic treatment with *O. africana* extract, captopril and jelly on left ventricular developed pressure. Values are expressed as the mean±SEM; control group n=5, *O. africana* n=6, captopril n=5, jelly (vehicle, n=6), *p<0.05 compared to pre-ischemia, ▪p<0.05 (post-ischemia compared to ischemia).

4.5.3 Rate pressure product (RPP)

The RPP was similar before ischemia in the control group (40912.4±1801.1 mmHg/min), *O. africana* group (35787.2±1397.0 mmHg/min), captopril group (35934.2±1380.4 mmHg/min) and jelly group (34942.2±1316.2 mmHg/min) p>0.05. The RPP in the control group before ischemia was 40912.4±1801.1 mmHg and decreased during ischemia to 30626±2126.8 mmHg, and after ischemia to 24142±1187.7 mmHg, p<0.05. The RPP in the *O. africana* group during and after ischemia (20214.7±1302.4 mmHg and 20083.7±676.1 mmHg, respectively) differed significantly (p<0.05) from the pre-ischemic value (35787.2±137 mmHg). There was a significance difference in RPP in the captopril group before, during and after ischemia (35934.2±1380.4 mmHg, 23749±1221 mmHg and 23172.8±547.8 mmHg, respectively, p<0.05). The RPP differed significantly

in the jelly group before ischemia (34942.2±1316.2 mmHg), during ischemia (26546.2.5±957.7 mmHg) and after ischemia (21691.3±1283.2 mmHg, $p<0.05$). After ischemia, the RPP was similar in the control group (24142.0±1187.7 mmHg/min), *O. africana* group (20083.7±676.1 mmHg/min), captopril group (23172.8±547.8 mmHg/min) and the jelly group (21691.3±1283.2 mmHg/min) $p>0.05$. RPP during ischemia was lower in the *O. africana* group (20214.2±1302.4 mmHg/min) and captopril group (23749.0±1221.2 mmHg) compared to the control group (30626.2±2126.8 mmHg/min), $p<0.05$ (Figure 4.13).

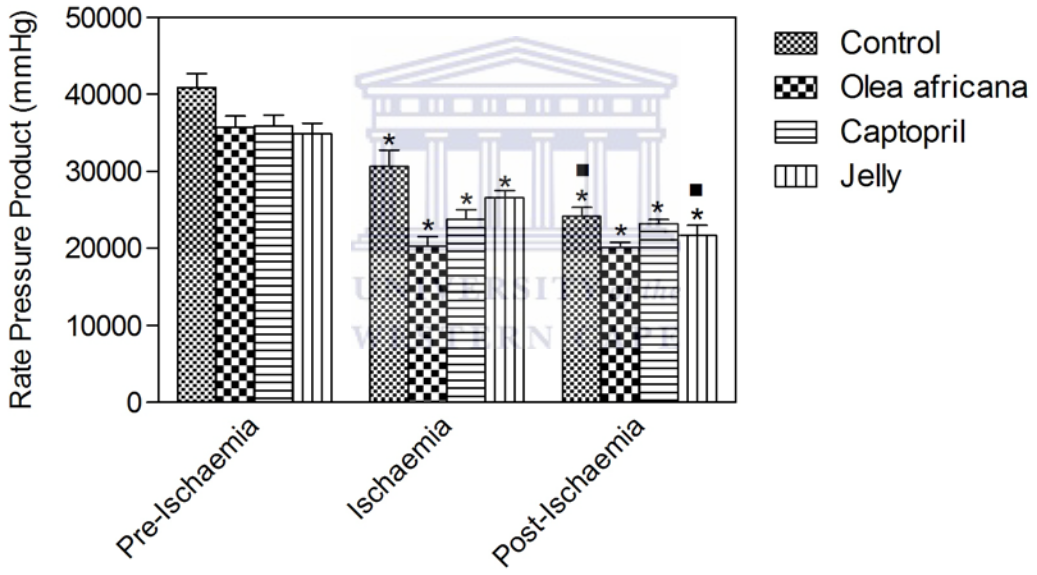


Figure 4.13 Effect of chronic treatment with *O. africana* extract, captopril and jelly on rate pressure product. Values are expressed as the mean±SEM; Control group n=5, *O. africana n*=6, captopril n=5, jelly (vehicle, n=6), *$p<0.05$ compared to pre-ischemia, ▪$p<0.05$ (post-ischemia compared to ischemia)

4.5.4 Recovery of the left ventricular developed pressure

There was no significance difference in percentage recovery of the left ventricular developed pressure between the *O. africana* (58.8±3.2 %), jelly (65.3±0.8 %) and the

control groups (56.2±3.0 %,), p>0.05. Percentage recovery of the left ventricular developed pressure differed significantly in the captopril group (69.4±3.0 %) compared to the control group (56.2±3.0 %, p<0.05) (Figure 4.14).

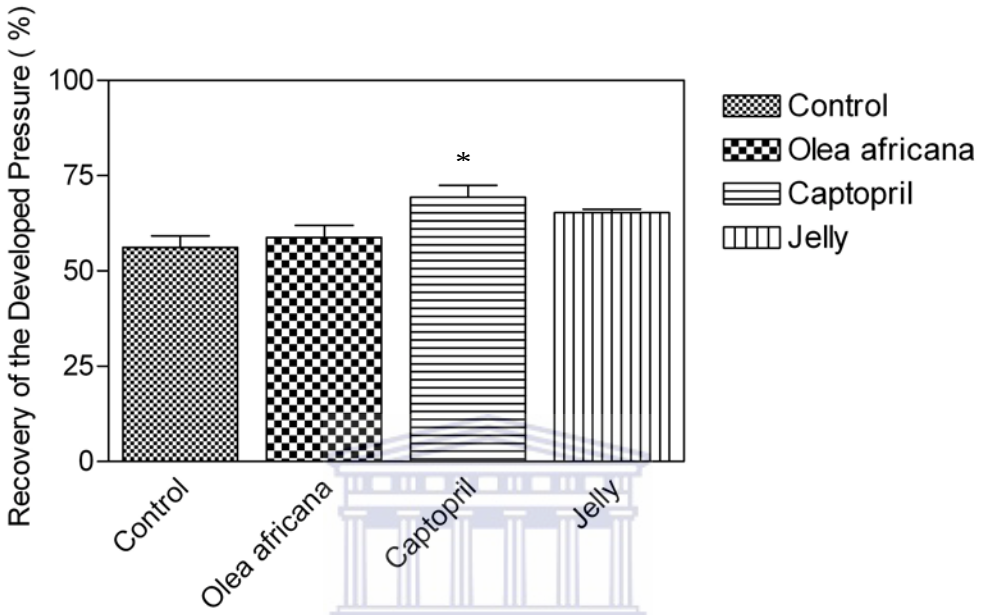


Figure 4.14 Effect of chronic treatment with *O. africana* extract, captopril and jelly on percentage recovery of left ventricular developed pressure. Values are expressed as the mean±SEM; control group n=5, *O. africana n*=6, captopril n=5, jelly (vehicle, n=6), *p<0.05 compared to control group

4.5.5 Recovery of rate pressure product (RPP)

The percentage recovery of the rate pressure product was the same in the *O. africana* (56.8±3.7 %), captopril (64.6±1.2 %), jelly (62.2±1.7 %) and the control groups (59.2±2.3 %), p>0.05 (Figure 4.15).

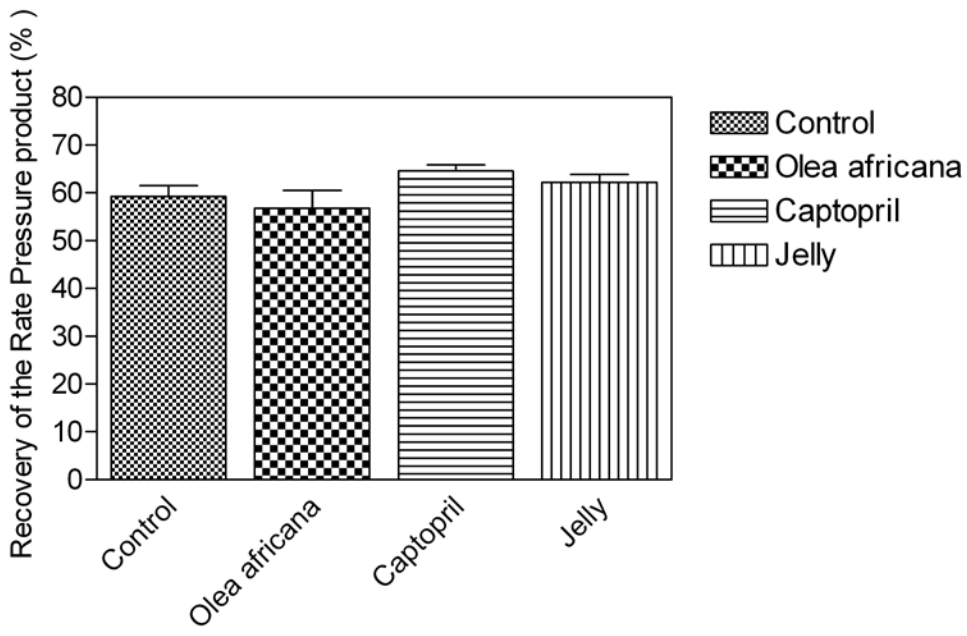


Figure 4.15 Effect of chronic treatment with *O. africana* extract, captopril and jelly on percentage recovery of rate pressure product. Values are expressed as mean±SEM; control group n=5, *O. africana n*=6, captopril n=5, jelly (vehicle, n=6)

4.5.6 Infarct size

The infarct size (expressed as a % of the area at risk) was the same in *O. africana* (41.3±3.5 %), captopril (41.0±3.8 %), jelly (42.5±4.1 %) and the control groups (40.6±3.2 %), p>0.005, (Figure 4.15). There was no significance difference in the percentage of the area at risk in *O. africana* (54.2±5.4 %), captopril (51.8±4.0 %), jelly (47.2±2.2 %) and control groups (48.4±3.5 %), p>0.005, (Figure 4.16).

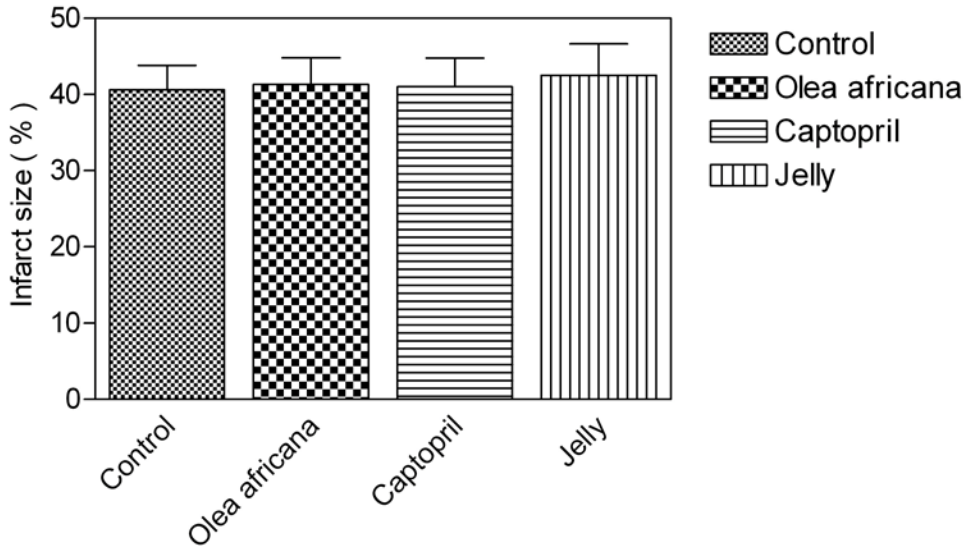


Figure 4.16 Effect of chronic treatment with *O. africana* extract, captopril and jelly on infarct size of perfused hearts. Values are expressed as the mean±SEM; control group n=5, *O. africana* n=6, captopril n=5, jelly (vehicle, n=6)

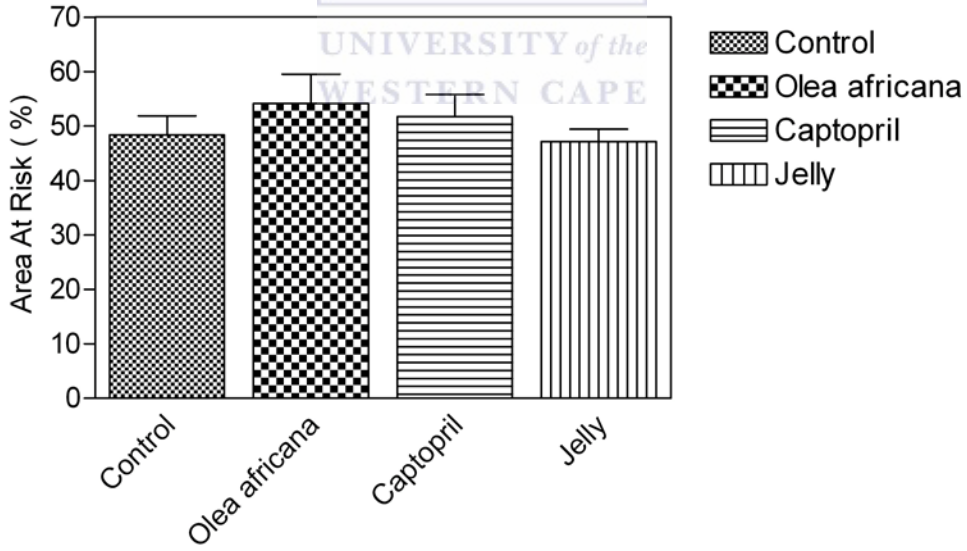


Figure 4.17 Effect of chronic treatment with *O. africana* extract, captopril and jelly on area at risk. Values are expressed as the mean±SEM; control group n=5, *O. africana* n=6, captopril n=5, jelly (vehicle, n=6)

4.6 Chronic administration of *O. africana* extract, captopril and jelly on total PKB/Akt

Figure 4.18A shows the Western blot of total PKB / Akt of the control, *O. africana*, captopril and jelly groups. The total PKB / Akt was similar in *O. africana* (0.7±0.1), captopril (0.7±0.1), jelly (0.7±0.1) compared to the control group (1.0±0.0), $p>0.05$, (Figure 4.18B). The values are expressed relative to the control (control=1).

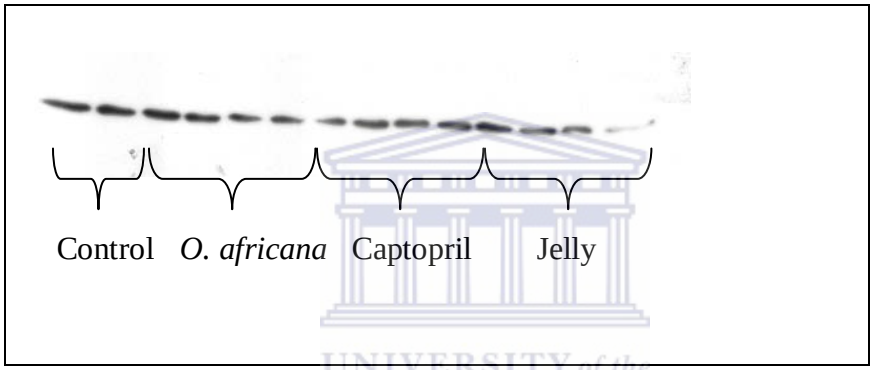


Figure 4.18A Western blot of total PKB / Akt expression in non perfused hearts

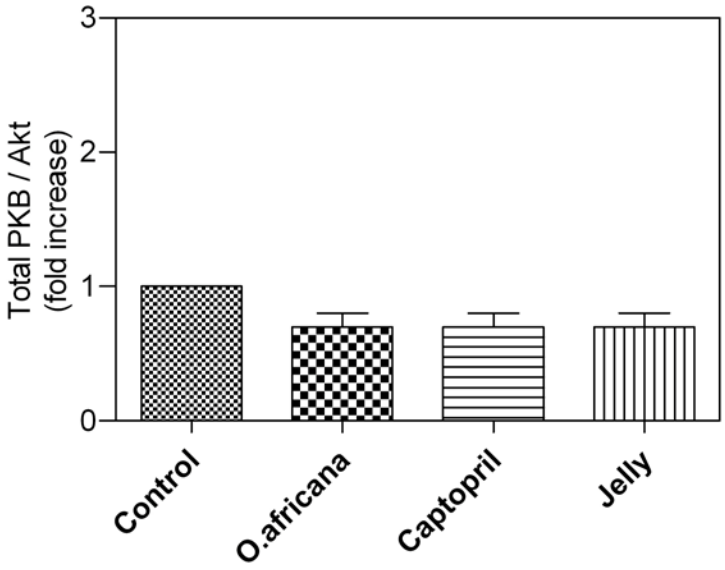


Figure 4.18B The effect of chronic administration of *O. africana* extract, captopril and jelly on total PKB / Akt. Values are expressed as the mean±SEM; control group n=2, *O. africana n*=4, captopril n=4, jelly (vehicle, n= 4)

4.6.1 Effect of chronic administration of *O. africana*, captopril and jelly on PKB/Akt phosphorylation

Figure 4.19A shows the Western blot of phosphorylated PKB / Akt of the control, *O. africana*, captopril and jelly groups. Phosphorylated PKB / Akt was similar in the *O. africana* group (3.1±0.4), captopril (3.1±0.5), jelly *(*4.2±0.8) and the control groups (1.3±0.0), p>0.05, (Figure 4.19B).

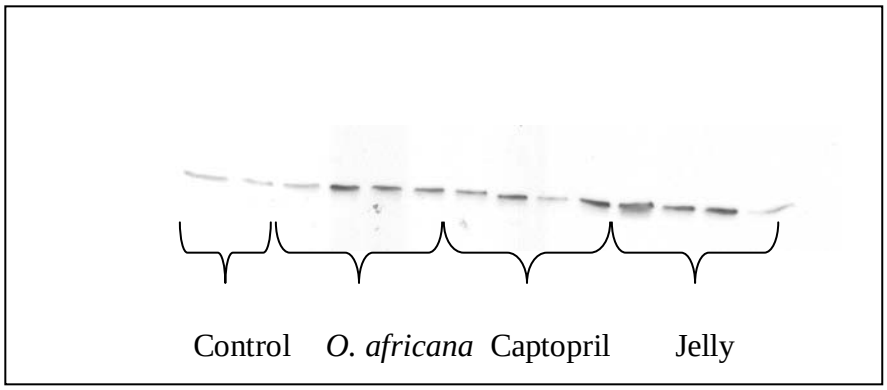


Figure 4.19A Western blot of PKB/Akt phosphorylation in non perfused hearts

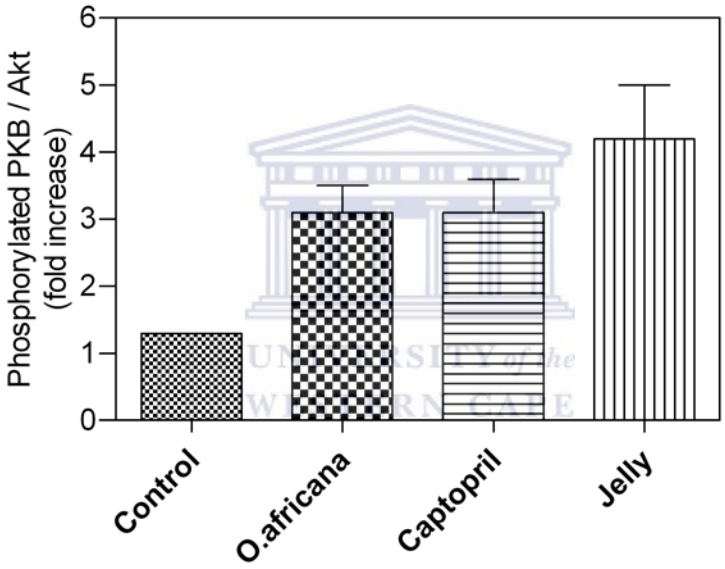


Figure 4.19B Effect of chronic administration of *O. africana*, captopril and jelly on PKB/Akt phosphorylation. Values are expressed as the mean±SEM; control group n=2, *O. africana n*=4, captopril n=4, jelly (vehicle, n= 4)

CHAPTER 5

DISCUSSION

5.1 Toxicity of *O. africana* extract

A concentration of 2250 mg/ml killed all (100%) the brine shrimps. The concentrations used for the preliminary experiments or acute treatment (2.5 mg/ml to 50 mg/ml) and chronic treatment experiments (1000 mg/kg or 250 mg/ml) are not toxic to the rats (LC_{50}=1269 mg/ml) (Table 4.1; Figure 4.1).

5.2 Acute treatment with *O. africana* extract

The initial objective was to determine the effects of *O. africana* extract on ischemia-reperfusion injury on the isolated perfused rat heart. Initially, hearts were perfused at constant hydrostatic pressure (100 mmHg) with different concentrations of the *O. africana* extract to determine the suitable concentration of the plant extract. In all the different concentrations of the plant extract used, cardiac function decreased as evidenced by a significant decrease in coronary flow (Table 4.2). The heart rate and force of contraction also decreased (not shown in the results). The findings suggest that the plant extract might contain toxic effects, however, our results from the brine shrimp toxicity test indicate that the concentrations that were used to perfuse the heart are not toxic (Table 4.1; Figure 4.1). The effects of the plant extract suggest that the extract has vasoconstritory properties on the coronary arteries.

Due to the observed effects above of the plant extract, hearts were then perfused with the plant extract (200 mg/kg) at constant flow rate (10.5 ml/min) to force the extract through the heart to determine whether the extract will have the same observed effects. Cardiac function again decreased until the heart stopped beating (Table 4.3). It was also observed that after 4 - 7 min of perfusion with the plant extract, coronary flow decreased completely despite the peristaltic pump being used. We then perfused the hearts with an infusion (200 mg/kg) of the plant extract to determine whether there is any difference due to the extraction methods used. The same effects were observed when the plant extract was used, i.e., coronary flow decreased significantly, $p< 0.05$ (Table 4.4). Hearts were then perfused with the plant extract (200 mg/kg), covering the reservoir containing the plant extract to determine whether the effects observed with the plant extract were due to the light sensitivity of the plant extract. Cardiac function again decreased significantly (Table 4.5). In the isolated perfused heart, perfusion with *Olea europaea* ethanol leaf extract decreased systolic left ventricular pressure and heart rate, but increased relative coronary flow (ratio between coronary flow and rate pressure product) (Scheffler *et al*., 2008). The findings of these authors disagree with our results and this might be due to the different extracts (aqueous versus ethanol) and the species (*Olea europaea* versus *Olea africana*) used.

5.3 Effect of chronic treatment with *O. africana* extract

Due to the fact that we could not perfuse the heart with the plant extract, we then treated the rats with the *O. africana* extract daily for 5 weeks.

5.3.1 Effect of chronic treatment with *O. africana* extract on cardiac function

Chronic treatment with *O. africana* aqueous extract (1000 mg/kg/day) had no effect on cardiac function of the isolated heart in terms of coronary flow, left ventricular systolic pressure, left ventricular diastolic pressure, heart rate, left ventricular developed pressure and rate pressure product compared to the control group. In this study, we showed that 20 min ischemia decreased cardiac function during reperfusion as evidenced by an increase in left ventricular diastolic pressure, a decreased in left ventricular developed pressure and rate pressure product. These findings are in agreement with studies that have shown that ischemia decrease cardiac function (Apstein *et al*., 1977; Takeda *et al*., 1997, Narang *et al*., 2004). The increase in end-diastolic pressure reflects diastolic dysfunction and is a representative of the left ventricle's inability to relax during diastole (Venardos *et al*., 2004). *Olea africana* treatment did not affect post-ischemic coronary flow (Figure 4.2), left ventricular diastolic pressure (Figure 4.4), left ventricular developed pressure (Figure 4.6), rate pressure product (Figure 4.7), percentage recovery of the left ventricular developed pressure (Figure 4.8) and percentage recovery of the rate pressure product (Figure 4.9) compared to the control group. Although it appears that the post-ischemia left ventricular systolic pressure (Figure 4.3) and heart rate (Figure 4.5) decreased in the *O. africana* treated group, it was not significant. These results demonstrate that chronic treatment with the crude aqueous extract of *O. africana* leaves did not offer cardioprotection against ischemia-reperfusion injury as evidenced by lack of improvement in functional recovery (% LVDP and % RPP).

5.3.2 Effect of ischemia-reperfusion injury on PKB / Akt

In this study, we hypothesized that ACE inhibitors, by decreasing AII levels, increase PI-3-kinase activity and thus activates PKB (for motivation refer to page 2-3). In response to ischemia, cells activate various signal transduction pathways which may either be harmful to the organism or allow it to adapt to the stressful environment (Engelbrecht *et al*., 2006). Ischemia followed by reperfusion has been shown to activate the pro-survival Akt, p42/p44 and Erk 1/2 kinases, which are termed reperfusion injury salvage kinase (RISK) pathways. The RISK pathways has been implicated in cellular survival through the recruitment of anti-apoptotic mechanism by phosphorylating the pro-apoptotic protein BAD. Phosphorylation of BAD by p42/p44, Erk 1/2 or PKB results in its binding to 14-3-3 proteins, which prevent it from binding to its mitochondrial target, thereby preventing apoptosis (Lawlor *et al*., 2001; Hausenloy *et al*., 2004). Activation of the RISK pathways also inhibits the conformational change in BAX (pro-apoptotic protein) required for its translocation to the mitochondria, thereby preventing apoptosis (Hausenloy *et al*., 2004). We therefore investigated the involvement of the PKB / Akt protein in the cellular response to treatment with *O. africana* crude aqueous extract. We have chosen to investigate PKB / Akt involvement because, to our knowledge, there is no study showing a link between ACE inhibitors and PKB activity in ischemia-reperfusion injury. The results demonstrated that there was no change in total PKB /Akt, which showed that PKB expression was the same in all groups. Chronic treatment with *O. africana* extract had no effect on PKB / Akt, phosphorylation compared to the control group, $p>0.05$ (Figure 4.11B). In another set of experiments (discussed later), PKB activity was increased 3 fold in hearts isolated from *O. africana* treated rats. In this set of experiments, however,

animals were treated with *O. africana* crude aqueous extract and hearts were then isolated and perfused with Krebs-Henseleit buffer before being subjected to an ischemic-reperfusion protocol. PKB / Akt activity was measured at the end of the reperfusion period. Perfusing the hearts with Krebs-Henseleit buffer prior to ischemia, and in the absence of *O. africana*, could have resulted in the loss of PKB activity

5.4. Effect of chronic treatment with *O. africana* extract, captopril and jelly on infarct size

ACE inhibitors have been shown to protect against ischemia-reperfusion injury (Ozer *et al.*, 2002; Eichhorn, 1998; Maulik *et al*., 2001; Takeda *et al*., 1997). The aqueous extract from the leaves of *O. africana* have angiotensin converting enzyme (ACE) inhibitory effects (Adersen *et al*., 1997; Hansen *et al*., 1996) and we therefore assumed that *O. africana* by virtue of its ACE inhibitory effects will protect against ischemia-reperfusion injury. In our system, chronic treatment with the *O. africana* extract did not protect against ischemia-reperfusion. We therefore treated rats with captopril (an angiotensin converting enzyme inhibitor) as a positive control to determine whether it protects against ischemia-reperfusion injury in our experiments. Our results showed that chronic treatment with *O. africana* crude aqueous extract and captopril did not limit infarct size (Figure 4.16). These findings are not in agreement with pervious results by Ozer *et al*. (2002) who showed that acute administration of captopril (3 mg/kg) in an *in vivo* model of myocardial ischemia-reperfusion injury decreases infarct size. Similarly, Maulik *et al*. (2001) using the same model showed that captopril (4 mg/kg) improves post-ischemia cardiac function. Treatment with captopril (8 µg/ml or 80 µg/ml) before ischemia provided protection in isolated perfused hearts (Takeda *et al*., 1997). The reason for the

different findings may be due to the different protocols followed. In our experiments, rats were treated with captopril and in the referenced studies, hearts were perfused with captopril. In our experiments, captopril improved recovery of the left ventricular developed pressure (Figure 4.14), but not the rate pressure product (Figure 4.15). Chronic treatment with *O. africana* extract had no effect on cardiac function and infarct size. The findings of this study, suggest that chronic treatment with *O. africana* extract in our experimental setting does not improve cardiac function and also does not have an effect on infarct size and this may be due to loss of PKB activation in our experimental setting (cf. page 63).

5.5 Effect of chronic treatment with *O. africana* extract, captopril and jelly extract on PKB / Akt

Our experiments were based on the hypothesis that ACE inhibitors, by decreasing AII levels, increase PI-3-kinase activity, required for PKB phosphorylation. The crude aqueous extract of the *O. africana* leaves decreases plasma AII levels in hypertensive rats (Wang, 2008). In rats treated with *O. africana* extract and subjected to ischemia-reperfusion, the extract had no effect on PKB / Akt phosphorylation. We, therefore, treated rats for 5 weeks with *O. africana* extract and isolated the hearts without perfusing them. This was to determine whether chronic treatment would activate the PKB / Akt protein in cardiac tissue *in vivo*. Captopril and the jelly (vehicle for captopril) groups were also added.

Our results showed that chronic treatment with *O. africana* extract and captopril had no effect on PKB / Akt, although there was a 3-fold increase in PKB / Akt phosphorylation.

The fact that the 3-fold increase in PKB / Akt was not statistical significant may be due to the small sample size used for the Western blot experiments (Figure 4.19B).

5.6 Conclusion

The results of this study showed that treatment with the crude aqueous extract of *O. africana* leaves did not protect against ischemia-reperfusion injury in the isolated perfused rat heart.

5.7 Recommendations

We have demonstrated that chronic treatment with the crude aqueous extract of the *O. africana* is not cardioprotective against ischemia-reperfusion injury on isolated perfused heart in our experimental setting. When the plant extract was present in the blood (non-perfused hearts), there was a 3-fold increase in PKB / Akt phosphorylation, although this was not statistical significant because of the small sample size used for the Western blot experiments. In future experiments, an *in vivo* model of ischemia-reperfusion should thus be used. One can then also use leaves harvested in different seasons to determine whether there is a seasonal effect.

REFERENCES

Ackermann RT, Mulrow CD, Ramirez G, Gardner CD, Morbidoni L, Lawrence VA (2001). Garlic shows promise for improving some cardiovascular risk factors. *Archives of International Medicine*. 161:813-824.

Adersen A, Adersen H (1997). Plants from Reunion Island with alleged antihypertensive and diuretic effects – an experimental and ethnobotanical evaluation. *Journal of Ethnopharmacology*. 58:189-206.

Akerele O (1993). Summary of WHO guidelines for the assessment of herbal medicines. *Herbal Gram*. 28:13-19.

Amiot MJ, Fleuriet A, Macheix JJ (1986). Importance and evolution of phenolic compounds in olive during growth and maturation. *Journal of Agricultural and Food Chemistry*. 34:823-826.

Anderson KE, Coadwell J, Stephens LR, Hawkins PT (1998). Translocation of PDK-1 to the plasma membrane is important in allowing PDK-1 to activate protein Kinase B. *Current Biology*. 8:684-691.

Andreadou I, Iliodromitis EK, Mikros E, Constantinou M, Agalias A, Magiatis P, Skaltsounis AL, Kamber E, Tsantili-Kakoulidou A, Kremastimos D (2006). The olive constituent Oleuropein exhibits anti-ischemic, anti-oxidative and hypolipedemic effects in anesthetized rabbits. *The journal of Nutrition*. 136:2213-2219.

Apstein CS, Delckelbaum L, Muller M, Hagopian L, Hood WB (1997). Graded global ischemia and reperfusion. Cardiac function and lactate metabolism. *Circulation*. 55(6):864-872.

Auer W, Eiber A, Hertkorn E, Hoehfeld E, Koehrle U, Lorenz A, Mader F, Merx W, Otto G, Schmid-Otto B, & *et al* (1990). Hypertension and hyperlipidaemia: garlic helps in mild cases. *British journal of clinical practice*. *Supplement*. 69: 3-6.

Bahorun T, Trotin F, Pommery J (1994). Antioxidant activities of *Crataegus monogyna* extracts. *Planta Medicine*. 60:323-328.

Banerjee SK, Dinda AK, Manchanda SC, Maulik SK (2002). Chronic garlic administration protects rat heart against oxidative stress induced by ischemic reperfusion injury. *BMC Pharmacology*. 2:16.

Banerjee SK, Maulik SK. (2002). Effect of garlic on cardiovascular disorders: a review. *Journal of Nutrition*. 1:4-14.

Bashir S, Janbaz KH, Jabeen Q & *et al* (2006). Studies on spasmogenic and spasmolytic activities of Calendula officinalis flowers. *Phytotherapy Research*. 20:906-910.

Bradford MM (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. 71:248-254.

Brar BK, Stephanou A, Knight R, Latchman DS (2002). Activation of protein kinase B / AKt by Urocortin is essential for its ability to protect cardiac cells against hypoxia / reoxygenation-induced cell death. *Journal of Molecular Biology*. 34:483-492.

Braun L, Cohen M (2007). Herbs and natural supplements: An evidence-based guide. 2nd edition. Medical. Pages 791.

Bourquelot E, Vintilesco JS (1908). On oleuropein, a newly discovered glucoside of the Olive tree. C.R. *Academy of Sciences*. 147:533-535.

Castaneda MP, Swiatecka-Urban A, Mitsnefes MM, Feuerstein D, Kaskel FJ, Tellis V, Devarajan P (2003). Activation of mitochondrial apoptotic pathways in human renal allografts after ischemiareperfusion injury. *Transplantation*. 76(1):50-54.

Degenring FH, Suter A, Weber M (2003). A randomised double blind placebo controlled clinical trial of a standardized extract of fresh *Crataegus* berries (*Crataegisan*) in the treatment of patients with congestive heart failure NYHA II. *Phytomedicine*. 10(5):363-369.

De Graeff PA, van Gilst WH, De Langen CDJ, Wesseling H (1986). Concentration dependent protection by captopril against reperfusion injury in the isolated rat heart. *Circulation.*70: II-89.

De la Ribeiro R, Fiuza de Melo, de Barros F, Gomes C, Trolin G (1986). Acute antihypertensive effect in conscious rats produced by some medicinal plants used in the state of Sao Paolo. *Journal of Ethnopharmacology*. 15(3):261-269.

De Launteris N, Crescenzo G, Lai OR, Milillo MA (1997). Investigation on the extraction and concentration of oleuropein and Flavanoids in *Olea europaea* L. based products. *Journal of Pharmacy and Pharmacology*. 7:27-30.

Derbyshire E, Wright DJ, Boulter D (1976). Legumin and vicilin, storage proteins of legume seeds. *Phytochemistry*.15:3-24.

Devi R, Banerjee SK, Sood S, Dinda AK, Maulik SK (2005). Extracts from Clerondendron colebrookianum Walp protects rat heart against oxidative stress induced by ischemic-reperfusion injury. *Life Sciences*. 77:2999-3009.

Dhalla NS, Elmoselhi AB, Hata T, Makino N (2000). Status of myocardial antioxidants in ischemia-reperfusion injury. *Cardiovascular Resea*rch. 47:446-456.

Diehl KH, Hull R, Morton D, Pfister R, Rabemampianina Y, Smith D, Vidal JM, van de Vorstenbosch C (2001). A good practice guide to the administration of substances and removal of blood, including routes and volumes. *Journal of Applied Toxicology*. 21:15–23.

Dold T, Cocks M (1999). Imithi yamasiko-useful plants in the Peddie District of the Eastern Cape with specific reference to *O.europaea subsp. Africana*. *Planta Life*. 21:24-25.

Don SE, Bennion GR, Randall DJ, Shelton G (1972). Factors affecting arterial and blood flow from the heart in intact, unrestrained lingcod, Ophiodonelongates. *Comparative Biochemistry and Physiology Part A*: physiology. 43(3):681-695.

du Toit EF, Genis A, Opie LH, Pollesello P, Lochner A (2008). A role for the RISK pathway and K_{ATP} channels in pre- and post-conditioning induced by levosimendan in isolated guinea pig heart. *British Journal of Pharmacology*. 154:41-50.

Eichhorn EJ (1998). Medical therapy of chronic heart failure. Role of ACE inhibitors and beta-blockers. *Clinical Cardiology*. 97:2099-2100.

Engelbrecht AM, Esterhyuse J, du Toit EF, Lochner A, van Rooyen J (2006). $_{P}$38-MAPK and PKB / AKt, possible role players in red palm oil-induced protection of isolated perfused rat heart. *Journal of Nutritional Biochemistry*. 17:265-271.

Ferreira AJ, Santos RAS, Almeida AP (2001). Angiotensin-(1-7): Cardioprotective effect in myocardial ischemia-reperfusion. *Hypertension*. 38:665-668.

Fletcher PJ (1996). ACE inhibitors in the treatment and prevention of heart failure. *Australian Prescriber*. 19:2-3.

Folli F, Kahn CR, Hansen H, Bouchie JL, Feener EP (1997). Angiotensin II inhibits insulin signaling in aortic smooth muscle cells at multiple levels: A potential role for serine phosphorylation in insulin / Angiotensin II crosstalk. *Journal of Clinical Investigation*. 100(9):2158-2169.

Frolkis I, Gurevitch J, Yuhas Y, Iaina A, Wollman Y, Chernichovski T, Paz Y, Matsa M, Pevni D, Kramer A, Shapira I, Rephael M (2001). Interactions between Paracrine tumor necrosis factor-alpha and Paracrine angiotensin II during ischemia. *Journal of American College of Cardiology*. 37(1):316-322.

Fugh-Berman A (2000). Herbs and dietary supplements in the prevention and treatment of cardiovascular disease. *Preventive Cardiology*. 3(1):24-32.

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

Gold ME, Farnsworth N (2002). Alternative medicines for cardiovascular disease. *Journal of Cardiovascular Nursing*. 16(4)v-viii.

Green PS, Kupicha FK (1979). Notes on the genus *Olea*. *Kew Bulletin*. 34:69-75.

Hansen k, Adersen A, Brogger CS, Rosendal JS, Nyman U, Wagner SU (1996). Isolation of angiotensin converting enzyme (ACE) inhibitor from *Olea europaea* and *Olea lancea*. *Pythomedicine*. 2(4):319-325.

Hasler CM (2002). The cardiovascular effects of Soy products. *Journal of Cardiovascular Nursing*. 16(4):50-63.

Hausenloy DJ, Tsang A, Yellon DM (2005). The reperfusion injury salvage kinase pathway: A common target for both ischemic preconditioning and postconditioning. *Cardiovascular Medicine*. 15(2):69-75.

Hausenloy DJ, Yellon DM (2004). New directions for protecting the heart against ischemia-reperfusion injury: targeting the reperfusion salvage kinase (RISK)-pathway. *Cardiovascular Research*. 61:448-460.

Heimier D, Cimato A, Sani G, Pieroni A, Galardi C, Romani A (2002). Flavanoids from olive leaves (*Olea europaea* L.) as affected by light. *Journal of Commodity Science*. 41(1): 31-39.

Hostettman K, Terreaux C (2000). Search for new lead compounds from higher plants. *Chimia*.54:652-657.

Hutchings A (1989). Observations on plant usage in Xhosa and Zulu medicine. *Botalia*. 19:225-235.

Hutchings A (1996). Zulu medicinal plants. *Natal University Press*, Pierermaritzberg. Pages 450.

Jain AK, Vargas R, Gotzkowsky S, McMahon FG (1993). Can garlic reduce levels of serum lipids? A controlled clinical study. *American Journal of Medicine*. 94(6):632-635.

Jan-Kan C, Shu-Er C (2005). Antioxidants and myocardial ischemia: reperfusion injuries. *Chang Gung Medical Journal*. 28:369-77.

Joubert E, Gelderblom WC, Louw A & *et al* (2008). South African herbal teas: Aspalathus linearis, Cyclopia spp., Athrixia phylicoides - a review. *Journal of Ethnopharmacology*. 119:376-412.

Khan GMI, Culver DC, William B (2006). Encyclopedia of heart diseases. Hilary Rowe. Ontario, Canada. Pages 653.

Khan Y, Panchal S, Vyas N, Butani A, Kumar V (2007). *Olea europaea*: A Phyto-Pharmacological Review. *Pharmacognosy Review.* 1(1):114-118.

Khayyal MT, el-Ghazaly MA, Abdallah DM, Nassar NN, Okpanyi SN, Kreuter MH (2002). Blood pressure lowering effect of an olive leaf extract (*Olea europaea*) in L-NAME induced hypertension in rats. *Arzneimittelforschung*. 52:797-802.

Kumar D, Jugdutt BI (2003). Apoptosis and oxidants in the heart. *Journal of Laboratory and Clinical Medicine*. 142(5):288-297.

Lawlor MA, Alessi DR (2001). PKB / AKt: a key mediator of cell proliferation, survival and insulin responses? *Journal of Cell Science*. 114:2903-29010.

Lee JS (2004). Medicinal plants: A powerful health aid? The science creative quarterly. Plant Science Bulletin. 50(4).

Le Tutour B, Guedon D (1992). Antioxidative activities of O*lea europaea* leaves and related phenolic compounds. *Phytochemistry*. 31:1173-1178.

Leung SWS, Man RYK (2008). Effect of hawthorn, a herbal medicine on arterial blood pressure in anaesthetized rats. *Journal of the Federation of American Societies for Experimental Biology*. 22:1129-17.

Liang C, Yi IM, Sherman LG, Black I, Garvas H, Hood WB (1981). Dobutamine infusion in conscious dogs with and without acute myocardial infarction. Effects on systemic hemodynamics, myocardial blood flow and infarct size. *Circulation Research*. 49:170-80.

Linz W, Scholkens BA, Han YF (1986). Beneficial effects of the converting enzyme inhibitor, ramipril, in ischemic rat hearts. *Journal of Cardiovascular Pharmacology*. 8:S91-S99.

Liu YH, Yang XP, Sharov VG, Sigmon DH, Sabbath HN, Carretero OA (1996). Paracrine system in the cardioprotective effect of angiotensin-converting enzyme inhibitors on myocardial ischemia-reperfusion injury in rats. *Hypertension*. 27:7-13.

Maenthaisong R, Chaiyakunapruk N, Niruntraporn S (2007). "The efficacy of aloe vera for burn wound healing: a systematic review." *Burns*. 33:713-718.

Mahad GB (2002). *Ginkgo Biloba* for prevention and treatment of cardiovascular disease: A review of literature. *Journal Cardiovascular of Nursing*. 16(4):21-32.

Mander M (1998). Marketing of indigenous medicinal plants in South Africa. A case study in KwaZulu-Natal. Food and Agricultural Organisation of the United Nations, Rome. 67-71.

Manning BD, Cantley LC (2007). Akt / PKB signaling: Navigating downstream. *Cell*. 129:1261-1274

Marczin N, El-Habashi N, Hoare GS, Bundy RE, Yacoub M (2003). Antioxidants in myocardial ischemia-reperfusion injury: therapeutic potential and basic mechanisms. *Archives of Biology and Biophysics*. 420:222-236.

Maulik SK, Kumari R, Maulik M, Manchanda SC, Gupta SK (2001). Captopril and its time on administration in myocardial ischaemic-reperfusion injury. *Pharmacological Research*. 44(2):123-128.

Meyer BN, Ferrigni NR , Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL (1982). Brine shrimp: a convenient general bioassay for active plant constituent. *Planta Medicine*. 45:31-34.

Meyer JJM, Afolayan AJ, Taylor MB, Engelbrecht L (1996). Inhibition of herpes simplex virus type 1 by aqueous extracts from shoots of *Helichrysum queronites*. *Journal of Ethnopharmacology*. 52:41-43.

Moens AL, Claeys JP, Timmermans JP, Vrints CJ (2005). Myocardial ischemia-reperfusion-injury, a clinical view on a complex pathophysiological process. *International Journal of Cardiology*. 100:179-190.

Molyneux CA, Glyn MC, Ward BJ (2002). Oxidative stress and cardiac microvascular structure in ischemia and reperfusion: the protective effect of antioxidant vitamins. *Microvascular Research*. 64:265-277.

Morgan A, Cupp MJ (2000). *Panax ginseng*. Toxicology and Clinical Pharmacology of Herbal Products. *Humana Press*. 141-150.

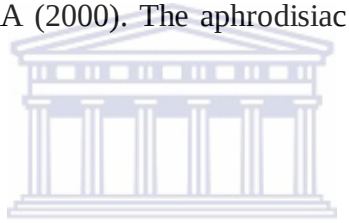
Murphy E, Steenbergen C (2008). Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. *Physiological Reviews*. 88:581-609.

Mussini P, Orsini F, Pelizzoni F (1975). Triterpens in leaves of *Olea europaea*. *Phytochemistry*. 14:1135-1139.

Narang D, Sood S, Thomas MK, Dinda AK, Maulik SK (2004). Effects of dietary palm olein oil on oxidadive stress associated with ischemic-reperfusion injury in isolated rat heart. *BMC Pharmacology*. 4:29-36.

Neely JR, Liebermeister H, Battersby EJ, Morgan HE (1967). Effect of pressure development on oxygen consumption by isolated rat heart. *American Journal of Physiology*. 212:804-814.

Nocerino E, Amato M, Izzo AA (2000). The aphrodisiac and adaptogenic properties of ginseng. *Fitoterapia*. 71:S1-S5.



Olmez E, Birincioglu M, Aksoy T, Acet A (1995). Effects of the captopril on ischemia-reperfusion-induced arrhythmias in an *in vivo* rat model. *Pharmacological Research*. 32:37-41.

Onimaru S, Nakamura K, Kariyazono H, Ikeda R, Ueno T, Fukumoto Y, Yabuki A, Sakata R, Yamada K (2006). Inhibitory effects of edaravone on the production of tumor necrosis factor-α in the isolated heart undergoing ischemia and reperfusion. *Heart Vessels*. 21:108–115.

Osim EE, Mbajiorgu EF, Mukarati G, Vaz RF, Makufa B, Munjeri O, Musabayane CT (1999). Hypotensive effects of crude extract *Olea.africana* (*Oleaceae*) in normo and hypertensive rats. *The Central African Journal of medicine*. 45(10):269-74.

Ozer MK, Sahna E, Birincioglu M, Acet A (2002). Effects of captopril and losartan on myocardial ischemia–reperfusion induced arrhythmias and necrosis in rats. *Pharmacological Research*. 45(4):257-263.

Persson IA, Dong L, Persson K (2006). Effect of *Panax ginseng* extract (G115) on angiotensin–converting enzyme (ACE) activity and nitric oxide (NO) production. *Journal of Ethnopharmacology*. 105(3):321-325.

Rahman K (2007). "Effects of garlic on platelet biochemistry and physiology". *Molecular Nutrition and Food Research*. 51(11):1335–44

Ronson RS, Nakumara M, Vinten-Johansen J (1999). The cardiovascular effects and implications of peroxynitrite. *Cardiovascular Research*. 44:47-59.

Scheffler A, Rauwald HW, Kampa B, Mann U, Mohr FW, Dhein S (2008). *Olea europaea* leaf extract exerts L-type Ca^{2+} channel antagonistic effects. *Journal of Ethnopharmacology*. 120:233-240.

Soler-Rivas C, Espin JC, Wichers HJ (2000). Oleuropein and related compounds. *Journal of the science of Food and Agriculture*. 80:1013-1023.

Somova LI, Shode FO, Ramnanan P, Nadar A (2003). Antihypertensive, antiatherosclerotic and antioxidant activity of triterpenoids isolated from *Olea europaea, subspecies africana* leaves. *Journal of Ethnopharmacology*. 84(2-3):299-305.

Somova LI, Shode, Mipando, M (2004). Cardiotonic and Antidysrhythmic effects of oleanolic and ursolic acids, methyl maslinate and uvaol. *Phytomedicine*. 11:121-129.

Steiner M, Lin RS (1998). "Changes in platelet function and susceptibility of lipoproteins to oxidation associated with administration of aged garlic extract". *Journal of Cardiovascular Pharmacology*. 31(6):904–8.

Sukh Dev (1999). Ancient-modern concordance in Ayurvedic plants: some examples. *Environmental Health Perspectives*. 107(10):783–789.

Sutherland FJ, Hearse DJ (1999). The isolated blood and perfusion fluid perfused heart. *Pharmacological Research*. 41(6):613-647.

Takeda H, Haneda T, Kikuchi K (1997). Protective effects of angiotensin-converting enzyme inhibitor Captopril on postischemic myocardial damage in perfused rat heart. *Japanese Journal of Corrections and Rehabilitation Research Center*. 61:687-694.

Vanden Hoek TL, Li C, Shao Z, Schumacker PT, Becker LB (1997). Significant levels of oxidants are generated by isolated cardiomyocytes during ischemia prior to reperfusion. *Journal of Molecular Biology*. 29:2571-2583.

van Gilst WH, de Graeff PA, Wesseling H, de Langen CD (1986). Reduction of reperfusion arrhythmias in the ischemic isolated rat heart by angiotensin converting enzyme inhibitors: a comparison of captopril, enalapril and HOE 498. *Journal of Cardiovascular Pharmacology*. 8:722-728.

Van Wyk BE, Gerike N (1997). Medicinal plants of South Africa. Briza Publication, Pretoria. 38-39.

Van Wyk BE, Gerike N (2000). Peoples plants. A guide to useful plants of Southern Africa. Briza Publication, Pretoria. 128.

Venardos K, Harrison G, Headrick J, Perkins A (2004). Effects of dietary selenium on glutathione peroxidase and thioredoxin reductase activity and recovery from cardiac ischemia-reperfusion. *Journal of Trace Elements in Medicine and Biology*.18:81-88.

Verdoorn IC (1963). The genus *Olea*. Flora of Southern Africa. 26:112-119.

Wang J (2008). The antihypertensive effects of aqueous extract of *O africana* leaves. MSc dissertation. University of the Western Cape, South Africa.

Wang LX, Ideishi M, Urata H, Arakawa K, Saku K (2001). Mechanism of the cardioprotective effect of inhibition of the rennin-angiotensin system on ischemia-reperfusion-induced myocardial injury. *Hypertension Research*. 24(2):179-87.

Wang X, Feng Liang F, Jiao X, Liu L, Bai X , LI M, Zhi J, Liu H (2007). Diverse effects of *L*-arginine on cardiac function of rats subjected to myocardial ischemia and reperfusion *in vivo*. *Acta Biochimica et Biophysica Sinica*. 39:201–207.

Warshafsky S, Kamer RS, & Sivak SL. (1993). Effect of garlic on total serum cholesterol. A meta-analysis. *Annals of Internal Medicine*. 119:599-605.

Watt JM, Breyer-Brandwijk MG (1962). The medicinal and poisonous plants of Southern and Eastern Africa. 2nd edition. Livingstone, London.

Westlin W, Mullane K (1988). Does captopril attenuate reperfused-induced myocardial dysfunction by scavenging free radical? *Circulation*. 77:130-139.

Wood-Sheldon J, Balick MJ, Laird SA (1997). Medicinal plants: Can utilization and conservation coexist? The New York Botanical Garden, Bronx, New York, USA.

Yamamoto M, Uemura T, Nakama S, Uemia M, Kumagai A (1983). Serum HDL-cholesterol increasing and fatty olive improving actions of *Panax ginseng* in high cholesterol diet-fed rats with clinical effect on hyperlipidaemia in man. *American Journal of Chinese Medicine*. 11:96-101.

Yamashiro S , Noguchi K, Matsuzaki T, Miyagi K, Nakasone J, Sakanashi M, Sakanashi M, Kukita I, Aniya Y, Sakanashi M (2003). Cardioprotective effects of extracts from *Psidium guajava* L. and *Limonium wrightii*, Okinawan medicinal plants, against ischemia-reperfusion injury in perfused rat hearts. *Pharmacology*. 67(3):128-135.

Zapfe JG (2001). Clinical efficacy of *crataegus* extract WS 1442 in congestive heart failure NYHA class II. *Phytomedicine*. 8(4):262-266.

Zarzuelo A, Duarte J, Jimenez j, Gonzalez M, Utrilla MP (1991). Vasodilator effect of olive leaf. *Planta Medica*. 57(5):417-419.

Zhang XH, Lowe D, Giles P, Fell S, Connock MJ, & Maslin DJ (2001). Gender may affect the action of garlic oil on plasma cholesterol and glucose levels of normal subjects. *Journal of Nutrition*. 131(5):1471-1478.

htpp://www.cellsignaling.com/reference/pathway/Akt_PKB.html.Feb 2010

http://www.graphpad.com

http://www.spss.com

http://www.who.int/cardiovascular_diseases/en.June 2009

http://www.worldbank.org/psd/complete.nsf .23 June 2009

APPENDIX

Appendix I. Regression analysis of dose-response relationship between log transformed doses of the aqueous extract of *O. africana* and % lethality

	Nonlin fit	A
		% Lethality
		Y
1	log(agonist) vs. normalized response -- Variable slope	
2	Best-fit values	
3	LogEC50	3.104
4	HillSlope	3.055
5	EC50	1269
6	Std. Error	
7	LogEC50	0.04094
8	HillSlope	0.8775
9	95% Confidence Intervals	
10	LogEC50	3.007 to 3.200
11	HillSlope	0.9801 to 5.131
12	EC50	1016 to 1586
13	Goodness of Fit	
14	Degrees of Freedom	7
15	R square	0.9231
16	Absolute Sum of Squares	752.4
17	Sy.x	10.37
18	Number of points	
19	Analyzed	9

The table was taken from Graph Pad Prism (version 5.02; http://www.graphpad.com), the program in which the regression analysis was performed. The LC_{50} of the aqueous extract of *O. africana* was 1269 mg/ml and the associated 95% confidence interval was 1016 to 1586 mg/ml.

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