THE ANTIHYPERTENSIVE EFFECT OF AQUEOUS EXTRACT OF *O AFRIicana* LEAVES

BY

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THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN DEPARTMENT OF MEDICAL BIOSCIENCE AT THE UNIVERSITY OF THE WESTERN CAPE, SOUTH AFRICA.

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Date submitted:       Nov 2007
KEYWORDS:

Hypertension

Blood pressure

O africana leaves

Aqueous extract

Captopril

Nifedipine

Angiotensin converting enzyme

Plasma angiotensin II
ABSTRACT

The incidence of cardiovascular diseases, including hypertension, is on the increase worldwide. Medicinal plants played an important role in the treatment of hypertension for centuries. Very few scientific studies have, however, been done to validate the use of these phytotherapies. *O africana* is one of the many phytotherapies that has been used indigenously for years to treat hypertension.

The objectives of this study were:

- To determine the most effective dose of *O africana* aqueous extract which will reduce blood pressure
- To determine whether chronic administration of *O africana* can be used
  - i) to prevent hypertension
  - ii) to treat hypertension
- To determine whether *O africana* exert its effects by modulation of the renin-angiotensin system.

To realize these objectives, 42 normotensive Dahl Salt-Sensitive (DSS) rats (n=6 in each group) and 12 hypertensive DSS rats (n=6 in each group) were used for single injection experiments to determine the acute blood pressure lowering effect of the aqueous extract of *O africana* leaves. Another 48 DSS rats were divided into 6 groups (n=8 in each group) to compare the chronic antihypertensive effect of aqueous extract of *O africana* leaves with that of the classic angiotensin converting enzyme inhibitor (captopril) and
calcium channel blocker (nifedipine). At the end of the experiments, blood samples were collected and assayed by ELISA to determine the plasma angiotensin II levels.

The blood pressure in hypertensive rats, induced by the administration of 2% NaCl in the drinking water for 2 weeks, was 204.83 ± 4.13/ 145.00 ± 2.58 mmHg. The results show that graded doses of aqueous extract of *O africana* leaves produced a significant acute lowering effect on blood pressure in normotensive rats, except at the lowest dose of 10 mg/kg (*p*<0.05 to *p*<0.001). The most effective dose was 1000 mg/kg. In hypertensive rats the dose of 1000 mg/kg was more effective than in normotensive rats (*p* <0.001).

Both aqueous extract of *O africana* leaves (1000 mg/kg) and captopril (50 mg/kg) prevented DSS rats from becoming hypertensive when administered 2% NaCl for 2 weeks. There was no significant difference between these groups. Nifedipine (10 mg/kg) gave a similar effect. An ELISA showed that plasma AII levels were significantly lower (*p*<0.001) in the extract group (13.57 ± 0.62 pg/ml) and captopril group (32.06 ± 2.97 pg/ml) in comparison with the high salt group (270.83 ± 9.76 pg/ml). Nifedipine, on the other hand, did not lower plasma AII levels (*p*>0.05).

After inducing hypertension by 2% NaCl in the drinking water for 1 week, *O africana* (1000 mg/kg) lowered blood pressure to normal levels within 1 week.

Thus, the results showed that graded doses of aqueous extract of *O africana* leaves have significant blood pressure lowering effects in both normotensive and hypertensive rats.
The most effective concentration was found to be 1000 mg/kg. Aqueous extract of *O africana* leaves produced better antihypertensive effects in hypertensive rats than in normotensive rats and prevented salt-sensitive rats from becoming hypertensive. The mechanism by which *O africana* exert its antihypertensive effects is by preventing AII formation, probably by acting as an ACE inhibitor.
DECLARATION

I, Xu Wang, do hereby declare that the thesis entitled:

“THE ANTIHYPERTENSIVE EFFECT OF AQUEOUS EXTRACT OF O AFRICANA LEAVES”

is the result of my own investigation and research, that it has not been submitted in part or in full for any other degree or to any other university. Where use was made of the work of others, it is duly acknowledged in the text.

Name: Xu Wang
Signature: ..............................
Date: ..............................
ACKNOWLEDGEMENTS

This work would not have been done without the generous support and help received from many different persons. I would like to acknowledge the following for the various roles they played during the study and write-up:

Prof. Daneel Dietrich. I am grateful for her guidance support and sedulous approach she upheld during my study.

Prof. Quinton Johnson for his supervision during the process of plant extraction, microbiology test and critical reviews.

Dr. Jeremy for his assistance during the process of extracting natural products.

Dr. Joanita Adams for her assistance during the process of microbiology test.

Dr. Samantha for numerous pieces of advice as well as critical reviews and for emendations with patience.

Mr. Andre Braaf for all the technical support.

All my friends for their support.
DEDICATION

I dedicate this master’s thesis to my mother ShengMei Dong and my father XueKai Wang for their love and support.
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ACC  Associated Clinical Conditions
ACE  Angiotensin Converting Enzyme
AHA  American Heart Association
AII  Angiotensin II
ANOVA Analysis Of Variance
CCB  Calcium Channel Blocker
CVD  Cardiovascular Diseases
DBP  Diastolic Blood Pressure
DSR  Dahl Salt-Resistant
DSS  Dahl Salt-Sensitive
ELISA Enzyme-Linked Immunosorbent Assay
HDL  High-Density Lipoprotein
HR  Heart Rate
HS  High Salt
IG  Intragastrically
ISH  International Society of Hypertension
LC\textsubscript{50} Lethal Concentration 50
LD\textsubscript{50} Lethal Dose 50
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL</td>
<td>Low-Density Lipoprotein</td>
</tr>
<tr>
<td>LVH</td>
<td>Left Ventricle Hypertrophy</td>
</tr>
<tr>
<td>MRC</td>
<td>Medical Research Council</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NS</td>
<td>Normal Salt</td>
</tr>
<tr>
<td>RAS</td>
<td>Renin-Angiotensin System</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized Controlled Trial</td>
</tr>
<tr>
<td>SADHS</td>
<td>South African Demographic and Health Survey</td>
</tr>
<tr>
<td>SBP</td>
<td>Systolic Blood Pressure</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>TCM</td>
<td>Traditional Chinese Medicine</td>
</tr>
<tr>
<td>TOD</td>
<td>Target Organ Damage</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
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CHAPTER 1

INTRODUCTION

Hypertension remains a major health problem in most countries because of its impact on the population mortality and morbidity. Worldwide, according to World Health Organization (WHO) report, hypertension is estimated to cause 7.1 million premature deaths and 4.5% of the disease burden annually (WHO, 2002-a).

The treatment of hypertension mainly relies on synthetic medicines. Several drug classes have been used in the treatment of hypertension in the past forty years. These include diuretics, beta blockers (β-blockers), calcium channel blockers (CCB’s) and more recently, angiotensin converting enzyme (ACE) inhibitors and angiotensin II receptor blockers. The data from more than 20 randomized controlled trials demonstrate reductions in both mortality and morbidity with these drug classes (Neal B, et al, 2000; WHO, 2003).

The use of these synthetic medicines however, has some negative effects. Most drugs used to treat hypertension have been evaluated for a number of specific patient populations; these include ACE inhibitors, β-blockers, CCB’s and diuretics in patients with concomitant diabetes, nephropathy, coronary and cerebrovascular disease, heart failure, and left ventricular hypertrophy (WHO, 2003). Side effects of these synthetic medicines have also been reported. For example, dry cough is a common side-effect of
ACE inhibitors and is a major limiting factor of their use (Ahmad M, et al, 2005). Secondly, despite the availability of useful non-drug therapy and potent medications, treatment is too often ineffective, mainly as a consequence of the patient’s lack of compliance with therapeutic regimens (WHO, 2003). Moreover, because of limited resources, synthetic drug treatment may not be affordable to the majority of hypertensive patients.

There are many herbal medicines traditionally used to treat hypertension in many countries. These herbal medicines are much easier and cheaper to obtain than the synthetic medicines, and fewer side effects are reported.

*O europaea* is one of these medicinal plants, and has shown significant antihypertensive effect (Khayyyal MT, et al, 2002). *O africana* is a subspecies of *O europaea*, which is indigenous to Africa. In previous scientific studies, the extract of roots and stems of *O africana* has been shown to possess antihypertensive activity (Osim E, et al, 1999), also the ethanol leaves extract was shown to be hypotensive (Somova LI, et al, 2003).

Very few studies have been done to determine the antihypertensive effects of the aqueous extract of *O africana* leaves. Considering that *O africana* leaves are used most often by drinking as a tea, more attention should be given to the aqueous extract of *O africana* leaves. Furthermore, no scientific articles are published on comparison of *O africana* extract with other classic antihypertensive drugs. The mechanism by which *O africana*
extract performs its hypotensive effect is also not clear (Rauwald HW, et al, 1994; Khayyyal MT, et al, 2002).

The objectives of this study were:

- To determine the most effective dose of *O africana* aqueous extract which will reduce blood pressure
- To determine whether chronic administration of *O africana* can be used
  
  i) to prevent hypertension
  
  ii) to treat hypertension
- To determine whether *O africana* exert its effects by modulation of the renin-angiotensin system.
CHAPTER 2

LITERATURE REVIEW

2.1 Hypertension

2.1.1 A global perspective

Hypertension is becoming one of the most prevalent diseases all over the world. Elevation of both systolic and diastolic blood pressures is associated with increased risk of cardiovascular diseases, like left ventricle hypertrophy (LVH), myocardial infarction, heart failure, strokes, and so on. Blood pressure depends on the amount of blood that the heart pumps out with each contraction, as well as the ease with which this blood flows through even the smallest blood vessels. The narrower the blood vessel, the more difficult it is for the blood to flow through and the higher the blood pressure gets. A definition of hypertension should therefore include both diastolic and systolic blood pressure criteria. The World Health Organization (WHO) defines hypertension as the situation when arterial pressure is greater than 140/90 mmHg for an extended period (Al-Nozha, et al, 1997).

The treatment of hypertension has been shown to prevent cardiovascular diseases and to extend and improve life, yet hypertension remains inadequately managed. Worldwide, the prevalence of hypertension is more than 600 million according to WHO (2002-b). In America, it affects around 50 million Americans (one in four American adults). Of those with hypertension, about 68% are aware of their condition, but only 27% have it under

### 2.1.2 Types of Hypertension

Primary hypertension and secondary hypertension are the two major types of systemic hypertension. Primary hypertension, which has no underlying cause, accounts for 95% of all cases of hypertension. This type of hypertension cannot be cured but can be kept under control by regular, ongoing treatment. Secondary hypertension, which is much less common, is caused by some other underlying condition (Fox SI, 2002). This kind of hypertension is often cured once the original medical problem is cured.

Based on the severity of the disease hypertensive individuals can be classified as indicated in **Table 2-1** as below:
Table 2-1. Classification of Hypertensive Individuals

<table>
<thead>
<tr>
<th>Stage</th>
<th>SBP (mmHg)</th>
<th>DBP (mmHg)</th>
<th>Presence of TOD or other risk factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimal</td>
<td>&lt; 120</td>
<td>&lt; 80</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Normal</td>
<td>120-134</td>
<td>80-85</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Stage 1u</td>
<td>135-149</td>
<td>86-95</td>
<td>No</td>
</tr>
<tr>
<td>Stage 1c</td>
<td>135-149</td>
<td>86-95</td>
<td>Yes</td>
</tr>
<tr>
<td>Stage 2u</td>
<td>150-180</td>
<td>96-110</td>
<td>No</td>
</tr>
<tr>
<td>Stage 2c</td>
<td>150-180</td>
<td>96-110</td>
<td>Yes</td>
</tr>
<tr>
<td>Stage 3u</td>
<td>&gt; 180</td>
<td>&gt; 110</td>
<td>No</td>
</tr>
<tr>
<td>Stage 3c</td>
<td>&gt; 180</td>
<td>&gt; 110</td>
<td>Yes</td>
</tr>
</tbody>
</table>

**TOD:** Target-organ damage; **SBP:** Systolic Blood Pressure; **DBP:** Diastolic Blood Pressure.

Subscript u indicates uncomplicated (free of TOD or other cardiovascular risk factors); subscript c, complicated (TOD or other cardiovascular risk factors are present)

Reproduced from: Black HR and Yi JY, 1996.

Apart from systemic hypertension described above the following also occurs:

**Hypertension during pregnancy:** Gestational hypertension is the early stages of high blood pressure during pregnancy. Preeclampsia is the severe high blood pressure during pregnancy. Eclampsia is very severe pregnancy hypertension leading to seizures (Baha M and Sibai MD, 2003).

**Pulmonary hypertension** is hypertension occurring in the pulmonary arteries.
2.1.3 Appraisal of risks

Hypertension is a significant risk factor for the development of other types of cardiovascular diseases (CVD’s), including congestive heart failure and cerebrovascular accidents. Of the 16.6 million deaths from cardiovascular disease every year, 7.2 million are due to ischaemic heart disease, 5.5 million to cerebrovascular disease, and an additional 3.9 million to hypertensive and other heart conditions (WHO/ISH Guidelines, 2003).

Decisions about the management of patients with hypertension should not be based on the level of blood pressure alone, but also on the presence of other risk factors, concomitant diseases such as diabetes, target-organ damage and cardiovascular or renal disease, as well as other aspects of the patient's personal, medical and social situation. According to the 1999 WHO/ISH Guidelines (WHO/ISH Guidelines, 1999), four categories of absolute cardiovascular disease risk are defined (low, medium, high and very high risk). Each category represents a range of absolute disease risks. Among individuals in the category, the risk of a major cardiovascular event in the next 10 years is typically: less than 15% (low risk); 15-20% (medium risk); 20-30% (high risk); and greater than 30% (very high risk) (See Table 2-2 and Table 2-3).
Table 2-2 Factors influencing prognosis of patients with hypertension

<table>
<thead>
<tr>
<th>Risk factors for cardiovascular disease</th>
<th>Target-organ damage (TOD)</th>
<th>Associated clinical conditions (ACC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levels of systolic and diastolic blood pressure (grades 1-3)</td>
<td>Left ventricular hypertrophy (electrocardiogram or echocardiogram)</td>
<td>Diabetes</td>
</tr>
<tr>
<td>Males &gt; 55 years</td>
<td>Microalbuminuria (20-30 mg/day)</td>
<td>Cerebrovascular disease</td>
</tr>
<tr>
<td>Females &gt; 65 years</td>
<td>Radiological or ultrasound evidence of extensive atherosclerotic plaque (aorta, carotid, coronary, iliac and femoral arteries)</td>
<td>Ischemic stroke</td>
</tr>
<tr>
<td>Smoking</td>
<td>Hypertensive retinopathy grade III or IV</td>
<td>Cerebral hemorrhage</td>
</tr>
<tr>
<td>Total cholesterol &gt;6.1 mmol/l (240 mg/dl) or LDL-cholesterol &gt;4.0 mmol/l (160 mg/dl)</td>
<td></td>
<td>Transient ischemic attack</td>
</tr>
<tr>
<td>HDL-cholesterol M &lt;1.0, F &lt;1.2 mmol/l (&lt;40, 45 mg/dl)</td>
<td></td>
<td>Heart disease</td>
</tr>
<tr>
<td>History of cardiovascular disease in first-degree relatives before age 50</td>
<td></td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>Obesity, physical inactivity</td>
<td></td>
<td>Angina</td>
</tr>
</tbody>
</table>

Lower levels of total and low-density lipoprotein (LDL)-cholesterol are known to delineate increased risk but these were not used in the stratification table. HDL, high-density lipoprotein. Reproduced from: WHO/ISH Guidelines, 2003.

Table 2-3 Stratification of risk to quantify prognosis

<table>
<thead>
<tr>
<th>Blood Pressure (mmHg)</th>
<th>Grade 1 (mild hypertension)</th>
<th>Grade 2 (moderate hypertension)</th>
<th>Grade 3 (severe hypertension)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Other Risk Factors &amp; Disease History</td>
<td>SBP 140-159 or DBP 90-99</td>
<td>SBP 160-179 or DBP 100-109</td>
<td>SBP ≥ 180 or DBP ≥ 110</td>
</tr>
<tr>
<td>I no other risk factors</td>
<td>LOW RISK</td>
<td>MED RISK</td>
<td>HIGH RISK</td>
</tr>
<tr>
<td>II 1-2 risk factors</td>
<td>MED RISK</td>
<td>MED RISK</td>
<td>V HIGH RISK</td>
</tr>
<tr>
<td>III 3 or more risk factors or TOD or diabetes</td>
<td>HIGH RISK</td>
<td>HIGH RISK</td>
<td>V HIGH RISK</td>
</tr>
<tr>
<td>IV ACC</td>
<td>V HIGH RISK</td>
<td>V HIGH RISK</td>
<td>V HIGH RISK</td>
</tr>
</tbody>
</table>

2.1.4 Hypertension in South Africa.

The South African Demographic and Health Survey (SADHS) was first conducted in 1998 in a random sample of 13,802 subjects aged 15 years or older, of whom 76% were black people. The incidence of hypertension for the black South African population was 21%. For those over 65 years of age, 50% to 60% were hypertensive (SADHS, 1998). More than 6 million South Africans suffer from high blood pressure, and this figure is still on the increase (Steyn K, et al, 2000), yet fewer than one out of every five people with high blood pressure receive treatment of any kind in South Africa. In many cases where people have been diagnosed with high blood pressure and they are receiving treatment, the patient’s high blood pressure is not controlled nearly well enough. If the situation continues and not enough actions are taken to prevent and effectively treat hypertension in South Africa, more people will die from heart-related conditions than from Aids in the near future (http://www.health24.com). Contrary to popular opinion, high blood pressure often occurs in younger people.

2.1.5 The extent of the problem

Several community studies done by the Medical Research Council (MRC) showed that one out of every four people between the ages of 15 and 64 suffer from high blood pressure. Unhealthy lifestyle habits and poor eating habits play a great role in the development of high blood pressure, according to Dr Krisela Steyn, MRC researcher and project leader of several studies concerning blood pressure (Steyn K, et al, 2000).


2.2 Risk factors for hypertension

Risk factors for hypertension are factors that do not seem to be a direct cause of the disease, but seem to be associated in some way. Having a risk factor for hypertension makes the chances of getting the condition higher but does not always lead to hypertension. Also, the absence of any risk factors or having a protective factor does not necessarily guard people against getting hypertension.

The list of risk factors for hypertension includes:

**Sodium intake:** Excessive sodium intake is considered as an absolute and obvious risk factor of hypertension. Dahl first published scientific evidence for a positive association between salt consumption and blood pressure in the 1960’s (Dahl LK, 1961). He described a remarkable linear relationship between average sodium intake and prevalence of hypertension across five population groups. Since then, an abundance of evidence from observational studies among and within various populations have shown a positive relationship between sodium intake and arterial pressure in all age groups (MacGregor GA, 1983; Law MR and Frost CD, 1991; Campese, VM, 1994; Stamler J, 1997; Cutler JA, et al, 1997; Whelton PK, et al, 1998; Sacks FM, et al 2001).

In addition, there has been increasing circumstantial evidence from population and basic science studies that dietary sodium may cause cardiovascular target organ injury through blood pressure-independent effects. Et-taouil *et al* (2001) reported that a high-sodium diet decreases aortic hyaluronan content and large artery compliance through blood pressure-independent mechanisms (Et-taouil, *et al*, 2001). Now high sodium intake is one
of the fastest and most common methods to induce hypertension in animal models (Garrett MR, 2002).

**Obesity:** Obesity is a term applied to excess body weight with an abnormally high proportion of body fat (Grundy SM, 2002). Being overweight is a serious condition. There’s no doubt obesity is a significant risk factor to the development of hypertension. A large body of epidemiological data has supported a link between obesity and hypertension (Kannel WB, 1967; Bethesda MD, 1998; Eckel RH, *et al.*, 1998).

The pathophysiology of hypertension in obesity is complex and multifactorial and includes factors such as insulin, an overactive sympathetic nervous system, and so on. Though the mechanisms are complex, obesity is regarded to be characterized by maladaptive hemodynamics and abnormal responses to stress, and both defects could potentially contribute to hypertension (Nasser M, *et al.*, 1999).

**Lifetime Risk of Hypertension:** Lifetime risk statistics describes the long-term risk for developing hypertension in an individual, which is the probability of developing hypertension during the remaining years of life (either adjusted or unadjusted for competing causes of death). In both 55 and 65-year-old participants, the cumulative lifetime risk for the development of hypertension (at or above 140/90 mm Hg regardless of treatment) was 90% (*Figure 2-1*). Other studies showed that the age-related rise in SBP is primarily responsible for an increase in both incidence and prevalence of hypertension with increasing age (Franklin SS, *et al.*, 1997).
Alcohol intake: People believe that regular alcohol consumption can produce positive psychosocial effects and some beneficial effects on health, especially reduced atherothrombotic events and death. Excessive alcohol consumption produces the opposite effects and has been proven to be associated with cardiovascular disorders, including hypertension, coronary artery disease, and stroke (Maiorano G, et al, 1995; Fuchs FD, et al, 2001). MacMahon studied more than 30 cross-sectional epidemiologic studies and stated that an overwhelming majority of the studies had reported significant elevations in blood pressure in individuals who consumed excessive alcohol (MacMahon S, 1987).

Caffeine intake: Caffeine may be the world’s most commonly used pharmacologic substance. Caffeine has been proven to cause mental stimulation and increases blood
pressure (Rainnie DG, 1994). Caffeine intake corresponding to 1 to 4 cups of coffee can increase systolic and diastolic blood pressure by 14 mm Hg and 13 mm Hg respectively in caffeine-withdrawn subjects (Robertson D, 1978) at rest or during mental or exercise stress (Sung BH, 1990). Its pressor effect is greater in subjects with hypertension (Hartley TR, 2000). In men, caffeine increases BP by increasing vascular resistance (Pincomb GA, 1985), with no effect on cardiac output (Pincomb GA, 1991). In women who are regular caffeine consumers, the BP response is also sustained, but by greater cardiac output (Terry R, et al, 2004).

Smoking: The American Heart Association (AHA) estimates that about one in five deaths from cardiovascular diseases are attributable to smoking, about 37,000 to 40,000 nonsmokers die from CVD each year as a result of exposure to passive cigarette smoke (2002 Heart and Stroke Statistical Update). Individuals who smoke are two to six times more likely to develop coronary artery disease than nonsmokers. Smoking causes hardening of the arteries, which may increase blood pressure.

2.3 Treatment for hypertension

The goal of treatment for most patients is to lower the systolic blood pressure below 140 mm Hg and the diastolic blood pressure below 90 mm Hg. Treatment for high blood pressure involves lifestyle modifications and drug therapy.
2.3.1 Lifestyle modifications

A variety of lifestyle modifications have been shown, in clinical trials, to lower blood pressure (Ebrahim S and Smith GD, 1998) and to reduce the incidence of hypertension (Stevens VJ, *et al*, 2001). In many patients, particularly those whose blood pressure is moderately elevated, lifestyle modifications alone may achieve treatment goals. Patients who require drug therapy may also reduce the frequency and doses of medications through lifestyle modification. The following modifications in diet and physical activity should be carried out.

- **Weight loss in the overweight.** It has been widely proved that overweight patients can reduce blood pressure by losing weight (Leiter LA, *et al*, 1999). Gradual weight loss through modified calorie intake and increased physical activity is a good approach.

- **Physical activity.** Regular, moderate aerobic exercise can modestly decrease blood pressure and has many other beneficial effects (Hagberg JM, *et al*, 2000).

- **Salt (sodium chloride) restriction.** Since excessive salt intake can contribute to increase blood pressure, it is strongly suggested to limit salt consumption. Generally, a reduction to no more than approximately 2.4 grams of salt per day will have a much greater effect and should become the long-term target for population salt intake worldwide (Barry D and Stephen H, 2007).

- **Limited alcohol intake.** Moderate alcohol intake does not appear to cause hypertension. However, chronic heavy alcohol use elevates blood pressure (Xin X, *et al*, 2001). This may be the most common reversible cause of high blood

- **A diet with increased fresh fruit and vegetables and reduced saturated fat content.** Fresh fruit and vegetables contain rich and various vitamin and vegetable fibre, which can contribute to the elimination of deposit on the walls of blood vessel (Sacks FM, *et al*, 2001). They are much accessible for most communities and suggested to be a good life-long habit.

Other lifestyle changes have not been found in multiple clinical trials to have a significant or lasting antihypertensive effect. These include calcium (Griffith LE, *et al*, 1999) and magnesium supplements (Kawano Y, *et al*, 1998), reduction in caffeine intake (Jee SH, *et al*, 1999), quit smoking, and a variety of techniques designed to reduce stress (Leiter LA, *et al*, 1999), which are mainly performed by massage or relaxation therapy.

From the above it is clear that regardless of the blood pressure reading, all individuals should adopt appropriate lifestyle modifications. It is regarded as the foundation stone to prevent the prevalence of hypertension and cardiovascular diseases. The protective effects of modifying lifestyle include a reduction in the incidence of hypertension, heart attack and stroke, is likely to reduce cardiovascular morbidity and mortality. Furthermore, non-pharmacological therapy has much more advantages than drug therapy, such as no known harmful effects and it can improve the sense of well-being of the patient. Drug therapy on the other hand may cause adverse effects and reduce the quality of life in some patients.
2.3.2 Drug therapy

Generally there are several typical drug classes used against hypertension in the 20th century. Since 1967 more than 20 randomized, controlled trials (RCTs) have compared diuretics, β-blockers, and calcium channel blockers (CCBs) against placebo in hypertensive patients (Collins R, et al 1990; Neal B, et al, 2000; Psaty BM, et al, 1997). Later a newer drug class, angiotensin-converting enzyme (ACE) inhibitors, which plays an important role in the drug therapy of hypertension and attracts more and more attention were developed. In 2000 ACE inhibitors were compared with CCBs in almost 75,000 hypertensive patients and the related meta-analysis of data from the RCTs was published (Neal B, et al, 2000). For the endpoints of total cardiovascular mortality, the meta-analysis shows no significant convincing differences between drug classes or between the groups of old and new drugs.

Many clinical trials show that some patients need to reduce their blood pressure to lower levels than previously recognized and will often require more than one drug (Hansson L, et al, 1998; Dahlof B, et al, 2002). To achieve the best objectives, ACE inhibitors are also used together with other drug classes, such as diuretic and CCBs (Gilderman L, et al, 2005).

2.3.3 Drugs in different populations

Most drugs used to treat hypertension have also been evaluated for a number of specific indications. In addition, different classes may have different effect levels when treating the same disease. For regression of LVH, ACE inhibitors and CCBs was found to be
more effective than β-blockers and diuretics (Dahlof B, et al, 2002; Schmieder RE, et al, 1998; Devereux RB, et al, 2001). Further comparative studies showed a greater reduction in proteinuria has been found with initial therapy with ACE inhibitors than with other classes (Brenner BM, et al, 2001; Agodoa LY, et al, 2001). Multiple placebo-controlled trials have shown significant reductions in proteinuria and a slowing of progression of renal damage in both non-diabetic and type-I diabetic nephropathies with ACE inhibitors (Jafar TH, et al, 2001).

Moreover, certain drugs may logically be chosen for other reasons. For example, a diuretic or CCBs may lower blood pressure more in Africa-American and older patients than an ACE inhibitors or a β-blocker (Cushman WC, et al, 2000; Radevski IV, et al, 2000).

2.4 Mechanisms of ACE inhibition therapy

2.4.1 Renin-Angiotensin-Aldosterone System (RAS)
Figure 2-2 The pathway of ACE inhibition in Renin-Angiotensin-Aldosterone system.

Sites of pharmacologic blockade in the rennin-angiotensin-aldosterone system: 1) renin inhibitors, 2) ACE inhibitors, 3) angiotensin II type I receptor antagonists, 4) angiotensin II type II receptor antagonists. Reproduced from Garry PR and John HB, 2001.
The RAS plays an important role in regulating blood volume, arterial pressure, and cardiac and vascular function (Figure 2-2). While the pathways for the RAS have been found in a number of tissues, such as brain (Johnston CI, et al, 1992), blood vessel wall (Müller DN and Luft FC, 1998), etc, the most important site for renin release is the kidney. Sympathetic stimulation, renal artery hypotension, and decreased sodium delivery to the distal tubules stimulate the release of renin by the kidney. Renin is an enzyme that acts upon a circulating peptide substrate, angiotensinogen, which undergoes proteolytic cleavage to form the decapeptide angiotensin I (AI). Vascular endothelium, particularly in the lungs, has an enzyme, angiotensin converting enzyme (ACE), which cleaves off two amino acids to form the octapeptide, angiotensin II (AII).

AII has several very important functions:

- Constricts resistance vessels (via AII receptors) thereby increasing systemic vascular resistance and arterial pressure
- Acts upon the adrenal cortex to release aldosterone, which in turn acts upon the kidneys to increase sodium and fluid retention
- Stimulates the release of vasopressin (antidiuretic hormone) from the posterior pituitary which acts upon the kidneys to increase fluid retention
- Stimulates thirst centers within the brain
- Facilitates norepinephrine release from sympathetic nerve endings and inhibits norepinephrine re-uptake by nerve endings, thereby enhancing sympathetic adrenergic function
- Stimulates cardiac hypertrophy and vascular hypertrophy
Therapeutic manipulation of this pathway is very important in treating hypertension and heart failure. ACE inhibitors and AII receptor blockers, for example, are used to decrease arterial pressure, ventricular afterload, blood volume and hence ventricular preload, as well as inhibit and reverse cardiac and vascular hypertrophy.

2.4.2 ACE inhibitors

ACE inhibitors are valuable agents for the treatment of hypertension, heart failure, and other cardiovascular and renal diseases. The cardioprotective effects of ACE inhibitors are mediated by blockade of both conversion of AI to AII and kinin hydrolysis, as displayed in Figure 2-2. A recent study shows that in AII-induced hypertension, the cardiac antifibrotic effect of ACE inhibitors is a result of the inhibition of N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) hydrolysis, resulting in a decrease in cardiac cell proliferation (probably fibroblasts), inflammatory cell infiltration, TGF-beta expression, Smad2 activation, and collagen deposition (Peng H, et al, 2005).

Elevated plasma renin is not required for the actions of ACE inhibitors, although ACE inhibitors are more efficacious when circulating levels of renin are elevated. As the RAS is found in many tissues mentioned above, ACE inhibitors may act at these sites in addition to blocking the conversion of angiotensin in the circulating plasma.

2.4.3 Therapeutic Uses

Hypertension. ACE inhibitors are effective in the treatment of primary hypertension and hypertension caused by renal artery stenosis, which causes renin-dependent hypertension owing to the increased release of renin by the kidneys. Reducing
angiotensin II formation leads to arterial and venous dilation, which reduces arterial and venous pressures. By reducing the effects of angiotensin II on the kidney, ACE inhibitors cause natriuresis and diuresis, which decreases blood volume and cardiac output, thereby lowering arterial pressure.

Some of the older literature indicated that ACE inhibitors (and angiotensin receptor blockers, ARBs) were less efficacious in African American hypertensive patients, which unfortunately led to lower utilization of these important, beneficial drugs in African Americans. While it is true that African Americans do not respond as well as other races to monotherapy with ACE inhibitors or ARBs (Matthew RW, et al, 1995), the differences are eliminated with adequate diuretic dosing. Therefore, current recommendations from the 7th report of the Joint National Committee are that ACE inhibitors and ARBs are appropriate for use in African Americans, with the recommendation of adequate diuretic dosing to achieve the target blood pressure (Aram VC, et al, 2003).

**Heart Failure.** ACE inhibitors have proven to be very effective in the treatment of heart failure caused by systolic dysfunction, like dilated cardiomyopathy (Eichhorn EJ, 1998). Beneficial effects of ACE inhibition in heart failure include:

- Reduced afterload, which enhances ventricular stroke volume and improves ejection fraction.
- Reduced preload, which decreases pulmonary and systemic congestion and edema.
Reduced sympathetic activation, which has been shown to be deleterious in heart failure.

Improving the oxygen supply/demand ratio primarily by decreasing demand through the reductions in afterload and preload.

Prevents AII from triggering deleterious cardiac remodeling.

Finally, ACE inhibitors have been shown to be effective in patients following myocardial infarction because they help to reduce deleterious remodeling that occurs post-infarction.

2.4.4 Specific Drugs

The first ACE inhibitor marketed, captopril, is still widely in use today. Although newer ACE inhibitors differ from captopril in terms of pharmacokinetics and metabolism, all the ACE inhibitors have similar overall effects on blocking the formation of AII. ACE inhibitors include the following specific drugs:

- Benazepril
- Captopril
- Enalapril
- Fosinopril
- Lisinopril
- Perindopril
- Ramipril
- Trandolapril
2.4.5 Unwanted effects

Although ACE inhibitors affect capacitance and resistance vessels and reduce cardiac load as well as arterial pressure, one should be aware of the existence of unwanted effects. For example, captopril was initially used in doses that, in retrospect, were excessive. In these large doses, it caused dry coughs, rashes, taste disturbance and heavy proteinuria (Ahmad M, et al, 2005). Currently dry coughs are still a common side effect of ACE inhibitors and remain the major limiting factor of their use. Secondly, despite the availability of useful non-drug therapy and potent medications, treatment is too often ineffective, mainly as a consequence of the patient’s lack of compliance with therapeutic regimens (WHO, 2003). Moreover, because of limited resources, synthetic drug treatment may be not affordable to the majority of hypertensive patients.

2.5 Medical Plants with antihypertensive effects

2.5.1 Traditional medical plants

A medicinal plant is any plant which provides health-promoting characteristics, temporary relief or symptomatic problems or has curative properties. (http://davesgarden.com)

Medicinal plants play a very important role since the existence of human beings. As early as 2800 B.C., the first independent system about Traditional Chinese Medicine (TCM) appeared on the earth, which is called “The Medical Classic of the Yellow Emperor” (Zhu M, 2001). This book is written in the form of a dialogue in which the Yellow Emperor (the legendary first ancestor of the Chinese nation) discusses medicine with his
ministers and some well-known doctors. No researchers of TCM in China could escape knowing about this text. Till now over 40% of medicines prescribed in the United States contain chemicals derived from plants (http://www.nps.gov). Furthermore, most developing countries are endowed with vast resources of medicinal and aromatic plants, much of which are still under-explored, especially in the rural areas.

Africa has a long and impressive list of medicinal plants that are used based on local knowledge. There are 70-80% of Africa’s population relies on medicinal plants (Cunningham AB, 1993). Considering of being precluded from the luxury of access to modern therapy, mainly for economic reasons, this number won’t change much now. All over, including Africa, it is claimed that the use of traditional plant medicines offer many advantages.

The scientific literature relating medicinal plants to blood pressure is extensive and can be dated back to more than 100 years ago. With the development of technology, people do not settle for taking infusions or decoctions orally any more. They start to locate the effective components and look for the right doses. More and more traditionally used medicinal plants have been studied to investigate the principles or mechanisms of their blood pressure lowering effect.

Medicinal plants, including Olive, Camellia, Claviceps purpurea, Coffea arabica (Bruneton J, 1995), Hawthorn, Linden blossom, Yarrow, Cramp bark and Valerian (http://www.healthy.net), are a broad range of remedies that have the observed effect of
lowering elevated blood pressure. They appear to work in a variety of ways, such as cardiac tonics, diuretics, nervines and anti-spasmodics (David LH, 2005).

In South Africa, traditional medicine is an integral part of cultural life. It is estimated that between 12 and 15 million South Africans still depend on traditional herbal medicine from as many as 700 indigenous plant species (Meyer JJM, et al, 1996).

The traditional use of wild olive in South Africa has lasted for hundreds of years (Walter HL, et al, 2003). The dried leaves are most often used, followed by the roots or the stem bark. The scientific study seems to start from 1960’s, which determined the main use of this plant as a hypotensive to lower blood pressure and to enhance renal function (Watt JM, et al, 1962). Researchers screened 20 Zulu medicinal plants and indicated that 6 of them have high level of ACE inhibition activities, including *Adenopodia spicata*, *Dietes iridioides*, *Mesembryanthemum*, etc (Andrew C, et al, 1999).

### 2.5.2 Advantages and disadvantages of medicinal plant medicine

Traditional medicinal plants are potential sources of new drugs, sources of cheap starting materials for synthesis of known drugs. It is claimed that the human body better accepts drugs derived from natural sources than synthetic substances invented in the laboratory (Muhizi, 2002). Furthermore, traditional plant medicines have fewer side effects when compared with synthetic products.
Traditional plant medicines also have some disadvantages. Plants from different geographic areas may have biologic diversity (Fabricant and Farnsworth, 2001). Secondly, for most traditional medicines, the specific constituents that cause a therapeutic effect is often not known. There exist many different constituent in the plant, and it is likely that they work together to produce the desired therapeutic effect. The exact combination of active ingredients is often the crucial problem for the traditional plant medicine development.

Olives have traditionally been used for hundreds of years and more and more researches proved its lowering blood pressure effect, it should get our more attention on the mechanism and comparative effects with classic synthetic medicines.

2.6 Current studies using olives

Olives have many different species in different geographic areas. Among these olives species, *O europaea*, the origin of the cultivated olives, is very widespread in Mediterranean countries, Africa, the Arabian Peninsula, the Indian subcontinent and Asia.

Previously, most studies were performed on *O europaea*. Studies on the active principles of the European olive leaf, the two secoiridoids *oleuropein* and *oleacein*, have been conducted since 1960. It was reported that the bitter glycoside *oleuropein* had a hypotensive, coronary dilating and antiarrhythmic action (Petkov V and Manolov P, 1972). Recently, a bioassay-directed fractionation showed that another component of
European olive leaf, beta-(3, 4 dihydroxyphenyl) ethanol was a potent calcium-antagonist (Rauwald HW, et al., 1994). The isolate by fractionation from the olive leaf, secoiridoid oleacein, was reported to have distinct ACE inhibitory effect (Hansen K, et al., 1995) and anti-oxidant activity (Bruneton J, 1995).

The antiarrhythmic activity of 95% ethanol, glycerine and ethanol:glycerine (50:50) extracts of European leaf and shoot has been demonstrated in the rat administrated intragastrically (IG) at doses of 25mg/kg, following aconite-induced arrhythmia (Cicosta, et al, 1990). In the same study, antihypertensive activity was demonstrated by glycerine:ethanol (50:50) extracts given IG to the rat at dosages of 125-250mg/kg, following desoxycorticosterone acetate-induced hypertension. Positive inotropic effects of 95% ethanol, glycerine and ethanol:glycerine (50:50) extracts were demonstrated in the rabbit at dosages of 5.0 mg/ml (heart).

Spasmolytic activity of similar extracts was demonstrated in the guinea pig at doses of 50mg/kg against vasopressin-induced coronary spasm and hypotensive activity in the rat at doses of 100mg/kg, given IG. Maximum hypotensive activity effect was seen 60-120 minutes after administration of each extract. Positive chronotropic effects of glycerine:ethanol (50:50) extracts were noted, when given IG to the desoxycorticosterone acetate - induced hypertensive rabbit at a dose of 125mg/ml (Cicosta, et al, 1990).
Leaf decoctions or lyophilised extracts administered to the rat showed spasmolytic activity against phenylephrine-induced contractions, both in the presence of and without endothelium (Zarzuelo, et al, 1991).

Antihypercholesterolaemic activity has been shown in rats given a daily dose (IG) of 500mg/kg of a glycerine:ethanol leaf extract for 15 days. Activity was noted both in diet-induced and triton-induced hypercholesterolaemic animals (Pasquale D, et al, 1991). Some of the cardio-vascular effects noted for *O europaea* have been attributed to the secoiridoids *oleuropein* and *oleacein* (ACE inhibitory activity).

Another study also indicates the antihypertensive effects of olive leaf extracts in vivo in the rat following IG administration of 50mg/kg of an 80% ethanol extract (Khayyyal, et al, 2002).

Effects, including renal effects via diuretic activities (Capretti G. and Bonaconza E. 1949; Ribeiro RA, et al, 1988), antimicrobial activity (Grange et al, 1990; Anesini C and Perez C, 1993), effects on the endocrine system (Eskander et al, 1995), effects on the inflammatory response (Fehri B et al, 1996), as well as hepatic activity (Han Y.M et al, 2001), have been published.

Inside *O europaea*, several subspecies are recognized, one of which is the small-fruited subspecies *africana* (formaly *O africana*), which is mainly located in African regions.
2.6.1 Geographical distribution

*O africana* are widespread in a variety of habitats, from forest and riverside bush to open grassveld, stony flats, mountain kloofs and rocky ledges throughout Southern Africa and northwards through east Tropical Africa into Eritrea (Figure 2-4).

**Figure 2-4 Distribution map of *O africana***

From: Breitenbach VF, 1986
2.6.2 Major chemical constituents

Figure 2-5 Major chemical constituents


2.6.3 Medicinal uses

Traditionally *O africana* was mainly used to lower blood pressure and to treat related cardiovascular diseases by using the dried leaves, sometimes the roots or the stem bark. Several other traditional uses have been recorded (Watt JM, 1962; Hutchings A, 1996; Iwu MM, 1993). Leaf infusions are used elsewhere as a lotion to treat eye infections or as a gargle to relieve sore throat; it is also taken internally as a remedy for colic or urinary tract infections. The powdered leaf is also used as a styptic.

Much research have been done on *O europaea* (Trovato A, et al, 1993; Rauwald HW, et al, 1994; Al-Qarawi SA, et al, 2002; Khayyyal MT, et al, 2002). However, studies on *O africana* are seldom published. Recently, Somova LI and co-workers published their findings on *O africana* (Somova LI, et al, 2003 and 2004). They indicate that *O africana* leaves can prevent the development of severe hypertension and atherosclerosis of the
experimental animals in a dose 60 mg/kg b.w. for 6 weeks treatment. It provides an effective and cheap treatment of this particular, most common type of salt-sensitive hypertension in the African population. Osim EE, et al. (1999) investigated the effects of crude extracts of root and stem of *O africana* and concluded that the extracts lowered the blood pressure and heart rate in both normotensive and hypertensive rats (Osim EE, *et al*, 1999).

2.7 Research problems

From section 2.6 it is clear to conclude from the above screening of previous studies that, although *O africana* has been observed to produce blood pressure lowering effects, the aqueous extract of *O africana* leaves has not been systematically tested. Considering that *O africana* is traditionally used with hot water, we consider the aqueous extract as very important. Moreover, does the aqueous extract of *O africana* produce better antihypertensive effect than synthetic medicine or not? It seems no such studies have been done yet. The most effective dose of aqueous extract has also not been determined.

2.8 The Dahl Salt-Sensitive (DSS) and Salt-resistant (DSR) rats in hypertension research.

The arterial pressure of some human hypertensives is very sensitive to the changes in sodium intake, they therefore have been classified as “salt-sensitive”. Since the number of salt-sensitive hypertensives is quite big (Myron HW, *et al*, 2001), it is necessary to choose salt-sensitive animal model to perform this study.
2.8.1 Dahl Salt-Sensitive (DSS) Rats

The Dahl Salt-Sensitive rat may be the animal model of choice. The Dahl rat is one of the most widely studied models of hypertension with over 1600 references in the literature over the past 35 years. There is already evidence for the Dahl rats’ value in probing the genetics of human hypertension (Dahl LK, et al. 1961, 1962, 1963). Selective breeding of rats for susceptibility or resistance to the hypertensive effect of high salt intake was originally done in 1960s. Dahl’s study showed that chronic excess salt ingestion leads to sustained hypertension (Dahl LK, et al. 1961). Dahl and his partners then found that not all rats responded to salt with similar changes in blood pressure, then they selectively breed rats for susceptibility (S rats) or resistance (R rats) to the hypertensive effects of high salt (8% NaCl) diet. After only three generations of selective breeding, the S and R lines were clearly separated. The blood pressures of R rats were essentially similar on control or high salt diets, while S rats responded to salt with a pronounced increase in blood pressure (Dahl LK, et al. 1962). Thus, the two strains yielded an interesting model for the interaction of an environmental factor (salt) with genotype.

2.8.2 Characteristics of Dahl rat

The DSS rat can also be used to determine other related cardiovascular diseases like cardiac hypertrophy, heart failure, as well as insulin resistance, hyperinsulinemia, hypertriglyceridemia, hypercholesterolemia and nephropathy (Somova LI and Channa ML, 1999).
Secondly, the Dahl rat model has many characteristics in common with salt-sensitive humans, such as decreased NO production and a suppressed renin-angiotensin system (Chen PY and Sanders PW, 1993).

Many studies that have used the DSS rat as their animal models, especially in those articles which studied the antihypertensive effects of olives, obtained great results and regarded the DSS rat as one of the standard hypertensive animal models (Yasuki K, et al, 1997; Somova LI. et al, 2003). To use the same rat model will be helpful to determine the comparison of different extracts.

### 2.8.3 Induction of hypertension

Two methods are used to induce hypertension in this model:

Firstly, via adding salt to the diet or water:

*Diet: 8% NaCl diet (high salt).* Treating rats with a diet containing 8% NaCl is the main method to induce hypertension in the DSS model. It has been used for many years and is accepted by most of researchers (Inoko M, *et al*, 1994; Mozaffari M.S, *et al*, 2000). Previous studies show that LVH was observed 5 weeks after putting the DSS rats on a diet containing 8% NaCl (DSS rats were fed a diet with 8% NaCl diet after the age of 6 weeks), and left ventricle dilatation was marked at the 15-20 weeks. During the latter stage, the DSS rats showed labored respiration with left ventricle global hypokinesis. All the DSS rats died within 1 week by massive pulmonary congestion (Inoko M, *et al*, 1994).
Water: 2%-4% NaCl in drinking water (high salt). The high salt diet is normally specially made, is not always easily available and is expensive. In recent years researchers indicated that treating animals with 2%-4% NaCl in drinking water also can induce hypertensive rat model (Li P, et al, 1998; Ojewole JAO, et al, 2006). Ojewole JAO and his team successfully induced hypertension in the DSS rat by giving 4% saline to drink. Rats with an arterial blood pressure of 170/120 mmHg and above were considered as hypertensive and used in that study.

Secondly, via injection (intraperitoneally or intravenously): This method is seldomly used (Li XN, et al, 1998).

2.9 Toxicity test

The brine shrimp assay is a very useful tool to determine the toxicity for the isolation of bioactive compounds from plant extracts (Sam TW, 1993). Since formally published in 1982, this method has been one of the most widely used tests (Meyer BN, et al, 1982; Alluri AK, et al, 2005).

Lethal Concentration 50 (LC$_{50}$) and Lethal Dose 50 (LD$_{50}$) values are the common measurements of the acute toxicity. LC$_{50}$ is the concentration needed to kill 50% of a sample population or hosts exposed (Stephen R and Joseph S, 2004). This measure is generally used when exposure to a chemical is through the animal breathing it in, while the LD$_{50}$ is the measure generally used when exposure is by swallowing, through skin contact, or by injection.
2.10 Blood pressure measurements in rats

Laboratory techniques used to measure blood pressure in rats involves both invasive and non-invasive techniques. The former includes radiotelemetry or methods in which the animal is anesthetized and several blood vessels are cannulated in order to allow for placement of a BP transducer and injection of drugs and saline. Non-invasive techniques include photoplethysmography, piezoplethysmography and volume pressure recordings. The invasive techniques are very accurate and are the gold standard used to compare the accuracy of non-invasive techniques.

We used the CODA II non-invasive system, (Kent Scientific, Connecticut, USA). This system uses volume pressure technology. The volume pressure recording sensor uses a specially designed differential pressure transducer to non-invasively measure the blood volume in the tail. With these measurements there are no artifacts related to eg. ambient temperature and movement artifacts are minimal and greatly reduced.
CHAPTER 3

MATERIALS AND METHODS

3.1 Plant material

Leaves of *O africana* were collected from trees growing on the campus of University of the Western Cape (UWC) in South Africa, between September and October 2004. They were identified and authenticated by Mr. Benny Mouers of the Department of Botany, UWC. Specimens for *O africana* bearing voucher numbers 4849 and 4850 were used in this project.

3.1.1 Preparation of aqueous extract

Fresh leaves, 4,630 g, were washed twice, air dried for 2 weeks and ground into a powder. Of the powder 3,310 g was used to prepare an aqueous extract.

The aqueous extract was obtained by shaking the powder in distilled water and allowing it to stand for 24 hours. It was then filtered, using Whatman filter paper and evaporated to 10% of its original volume at a temperature of 40°C, using a rotary evaporator. The reduced volume of the filtrate was first frozen overnight and then freeze dried for 24 hours to obtain a fine powder. The powder was weighed and dissolved in a known volume of distilled water and then serially diluted. The aqueous extracts were stored at 4 °C until use.
3.2 Animals

Dahl salt-sensitive (DSS) rats and control Dahl salt-resistant (DSR) rats were obtained from the University of Kwazulu-Natal, South Africa. Male rats (5 weeks old), weighing 200–220g, were used. Rats were maintained in the Medical Bioscience Department animal house at UWC and housed in polyethylene cages with water and standard diet provided ad libitum for 2 weeks to allow the rats to acclimatize to the new environment. Animal room temperature was maintained at 26 ± 2°C, with constant humidity and a 12-h light/dark cycle. During the 2 weeks the rats were trained in the experimental procedure to prevent stress induced hypertension during the experiments.

3.3 Drugs

Captopril and nifedipine were obtained from a local pharmacy. Jelly and gelatin were purchased from a local supermarket.

3.4 Blood pressure determination

An automated computerized tail-cuff blood pressure monitor, the CODA II Non-Invasive Blood Pressure System (from Kent Scientific Corporation, Connecticut, USA) was used to record the blood pressure and heart rates of rats.

Rats were placed in restraining holders with a nose cone to calm the animals. The restrainers were placed on a heating pad (32 ± 2°C) to warm the rat’s tail and maintain
blood flow to the tail. Animals were placed in the restrainers for at least 5 minutes before monitoring the blood pressure.

**Figure 3-1 Picture shows a rat in restraining holder ready for blood pressure determination.**

3.5 Toxicity Test

The brine shrimp (Artemia salina) toxicity test was used to investigate the toxicity of the aqueous extract of *O africana*. The materials included brine shrimp eggs, sea water, Petri dishes, liquid pipettes, test compounds (*O africana* aqueous extract) and filter paper.

3.5.1 Procedure

Hatching of the brine shrimp:

Brine shrimp eggs were obtained from a local pet shop. The standard procedure described by Meyer *et al.* (1982), was followed. The eggs were hatched in a 1L glass bottle filled with seawater. The sea water was filtered twice to get rid of any contaminants. The brine shrimp were kept under constant aeration at 25 ºC for 48 hours. After hatching, active
nauplii completely free from egg shells were collected by pipette. The nauplii were transferred into a shallow dish and a light was put on the side. The most active nauplii were chosen and used for the assay.

Preparation of the plant material:
To prepare a stock of *O africana* extract 20 mg of freeze-dried aqueous extract of *O africana* was dissolved in 2 ml of distilled water. From the stock solution, 5μl was sampled and directly pipetted on a circular filter paper disc (2 cm in diameter) in each of three Petri dishes. The Petri dishes with discs were briefly placed in the oven to allow evaporation of the distilled water and 5 ml sea water was added to each Petri dish to make up a concentration of 10μg/ml. From the stock solution 50μl was added to each of 3 Petri dishes containing circular discs and the same procedure, as described above, was performed to attain a new concentration of 100μg/ml in each Petri dish. To obtain a concentration of 1000μg/ml, the above procedure was followed using 500μl of the stock solution in each Petri dish.

Brine shrimp bioassay:
Toxicity was determined by using three concentrations of *O africana*: 10, 100 and 1000μg/ml. The test was performed in triplicate at each of the concentrations. To account for accidental deaths a control, without extract, was included. Twelve Petri dishes each containing a filter paper circular disc (2cm in diameter) were thus prepared as indicated below:
Table 3-1 Dish preparation for toxicity test

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<th>Control</th>
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<td><img src="image5.png" alt="Image" /></td>
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<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
</tbody>
</table>

“○”: dish.

Ten active shrimp larvae were carefully transferred into each Petri dish by pipette. The Petri dishes were maintained at room temperature and constant lighting for 24 hours. The surviving larvae were counted after 24 hours and the percentage deaths at each dose were determined. Only those larvae that were immotile after being touched were considered dead.

LC$_{50}$ determination:
Concentration was plotted against the percentage mortality. In cases where deaths occurred in the control solution, the data were corrected using Abbott’s formula (Abbott, W.S, et al, 1925):

\[
% \text{ deaths} = \left( \frac{\text{test} - \text{control}}{\text{control}} \right) \times 100
\]

LC\text{50} value was obtained from the best-fit line where the concentration of the extract caused 50% mortality in brine shrimp test.

### 3.6 Induction of Experimental Hypertension

The method for inducing experimental hypertension was similar to the way used by Ojewole JAO, et al (2005). All animals were fed normal laboratory chow. To induce hypertension animals had free access to water containing 2% NaCl (high salt group). Animals in the control group received normal tap water.

The systolic and diastolic blood pressure and heart rate were recorded by a tail cuff using the CODA II™ Non-Invasive Blood Pressure System, as described above.

### 3.7 Protocols

#### 3.7.1 Effect of salt supplementation on the blood pressure of DSS and DSR rats

Blood pressure and heart rate were measured twice weekly in two groups (n=8 each) of DSS rats and two groups (n=8 each) of DSR rats. One group of DSS rats and one group of DSR rats received normal tap water (NS, normal salt/control groups) while another
group of DSS rats and DSR rats received 2% NaCl in the drinking water (HS, high salt groups).

3.7.2 Effect of acute treatment with *O africana*

3.7.2.1 Effect of single injection of *O africana* on normotensive rats

Seven groups (n=6 each) of normotensive DSS rats were used for these experiments. Rats received a single injection of one of six different doses (10, 40, 75, 200, 1000 and 1200mg/kg i.p.) of *O Africana*. The seventh group received normal saline by injection (i.p) and served as the control group. Systolic and diastolic blood pressures, as well as heart rate were monitored for 80 minutes after the injection.

3.7.2.2 Effect of single injection of *O africana* on hypertensive rats

Two groups (n=6 each) of DSS rats received 2% NaCl drinking water (high salt) for 2 weeks to induce hypertension. At the end of the second week, one group received 1000mg/kg of *O africana* extract by injection (i.p.), while the other group (control group) received normal saline by injection (i.p). Systolic and diastolic blood pressures, as well as heart rates were monitored for 150 minutes after the injection treatment.

3.7.3 Effect of chronic treatment with *O africana*

48 DSS rats were divided into 6 groups (n=8 in each group). One group received normal tap water (control group) and another received 2% NaCl in the drinking water (HS group). A third group received 2% NaCl + extract (1000 mg/kg/day) in the drinking water. To ensure that the rats received the correct dosage of the extract two animals were housed per cage. The average volume of water consumed by the rats per day was
measured twice weekly in order to adjust the captopril content in the water if necessary. Animals were also weighed twice weekly. Group four received 2% NaCl in the drinking water and were given 50 mg/kg captopril per day (HS + captopril group). Group five received water containing 2% NaCl and were given 10 mg/kg nifedipine per rat per day (HS + nifedipine group). Captopril and nifedipine were administered in blocks containing gelatine and jelly. Group six received 2% NaCl in the drinking water in the first week and were given 2% NaCl + extract (1000 mg/kg/day) in the drinking water in the second week (HS + extract in 2nd week).

Blood pressure and heart rates were monitored prior to treatment and thereafter twice per week for two weeks.

3.7.4 Angiotensin converting enzyme inhibition activity of *O africana*

In order to determine whether the *O africana* extract has ACE inhibitor properties two types of biochemical determinations were performed. Firstly, ACE inhibitors can reduce blood pressure by reducing the level of ACE. The level of ACE in blood is described in an addendum to the thesis. Secondly, here we determined the angiotensin II level in the plasma to illustrate ACE inhibiting properties of the extract.

After two weeks treatment as described in section 3.7.3 rats were fasted overnight. The next day the animals were anesthetized with sodium pentobarbital (50 mg /kg i.p.). The chests were quickly opened and blood was collected from the trunk. After 10 minutes centrifugation at 5 000g, plasma was collected and stored at -20 °C for determination of AII levels.
To extract AII plasma samples were defrosted and brought to room temperature. Phenyl cartridges were pre-washed with 1 ml of methanol, followed by 1 ml of water. Two ml of plasma was passed through the cartridge. The cartridge was again washed with 1 ml of water. Angiotensin II peptides were eluted from the cartridge with 0.5 ml of methanol. The methanol was then evaporated to dryness in a fume hood. 0.5 ml of EIA buffer was added and each sample centrifuged at 3000 g for 10 minutes at 4°C. AII was determined by an EIA assay (Société de Pharmacologie et d'Immunologie-BIO, France) according to the manufacturer’s instructions. The plate was read at 405 nm.

### 3.8 Statistical analysis

Results are shown as the mean±SEM. Two groups were compared using a Student’s t-test. Multiple groups were compared by ANOVA by the Bonferroni post hoc tests. P<0.05 is considered significant. All tests were performed by using the SPSS V13.0 statistical package.
CHAPTER 4

RESULTS

4.1 Percentage yield of the extract

For the aqueous extract, 3,310 g of the dried ground plant material was used and the extract powder obtained after freeze drying was 86.2 g thus, producing a 2.6% yield.

4.2 Brine shrimp toxicity test

The brine shrimp toxicity test showed that the aqueous extract of *O africana* has low toxicity with LC$_{50}$ >5000 µg/ml (See Table 4-1 and Figure 4-1).

Table 4-1 Brine shrimp death during toxicity testing

<table>
<thead>
<tr>
<th>% Deaths</th>
<th>LC$_{50}$ µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µg/ml</td>
<td>100 µg/ml</td>
</tr>
</tbody>
</table>
No of dead brine shrimp is indicated as a percentage of the total no of brine shrimp present at each concentration.

**Figure 4-1 Toxicity of aqueous extract of *O africana*.**

### 4.3 Water intake

Prior to salt loading the water intake for the rats was 35 ± 2 ml/day per rat. At the end of the 2 week experimental period the water intake of rats receiving normal tap water was 36 ± 2 ml/day per rat, while the rats receiving 2% NaCl in drinking water drank 40 ± 2 ml/day per rat, which was significant more than control (*p*<0.001).

### 4.4 Effect of salt loading on SBP, DBP and HR of DSS and DSR rats
The systolic and diastolic blood pressure of rats receiving normal tap water (NS) or 2% NaCl (HS) in the drinking water were monitored for up to 7 weeks. For the first two weeks all animals received normal tap water and were acclimatized to the experimental procedure. From week 3 onwards rats received either normal tap water (NS) or 2% NaCl (HS) in the drinking water. At the end of the training period SBP, DBP and HR were within the normal range for all groups (Table 4-2-1, 4-2-2 and 4-2-3). The following are considered normal values for rats: BP = 130/90 (http://www.williams.edu/) and HR = 350 - 450 beats per minute (Piotr J and Jolanta Z, 2001).

**Figure 4-2 Picture shows a representative recording obtained when blood pressure was measured with the CODA II™ system.**

High salt treatment produced a quick and significant increase in SBP (130.83 ± 2.66 to 163 ± 2.79, \( p < 0.001 \)) and DBP (89.50 ± 2.81 to 119.33 ± 3.47, \( p < 0.001 \)) in the salt-sensitive rats after 1 week, and these values continued to increase in the second week of treatment. Heart rate was also significantly increased (Table 4-2-3: 423.33 ±
3.00 VS 413.38 ± 8.58 after 1 week; 429.67 ± 10.35 VS 406.88 ± 4.16 after 2 weeks, p<0.05), but the value remained within the normal range. By the end of the second week the animals were weak and we decided to terminate the experiment.

In the DSR rats salt loading produced a significant increase in SBP after one week and the value continued to increase in the second week (Table 4-2-1, p< 0.05 to 0.001). Diastolic pressure and HR remained unchanged.

In DSR rats receiving normal tap water DBP and HR remained constant throughout the experimental period. Systolic pressures record increased slightly in week 5 and 6 (133.38 ± 1.74 and 136.13 ± 2.11 Table 4-2-1), but the value was again normal in week 7.

DSS rats receiving normal tap water showed a slow but significant increase in SBP and DBP over the experimental period (SBP 180.63 ± 4.81 VS 130.83 ± 2.66; 133.28 ± 1.25 VS 89.50 ± 2.81, p< 0.001); the values, however, remained lower than in the corresponding HS group.

Table 4-2-1. Average Weekly Systolic Blood Pressure
Values shown as Mean ± SEM. Numbers in brackets indicates the number of animals (n) in each group.
Blood pressure is given in mmHg.
NS = normal salt; HS = HS; - Salt loading with 2% NaCl;
*: p<0.05; **: p<0.01; ***: p<0.001 (Significance is relative to week 2 data)

Table 4-2-2 Average Weekly Diastolic Blood Pressure

<table>
<thead>
<tr>
<th>Strain / Week</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
<th>Week 6</th>
<th>Week 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSS NS (8)</td>
<td>128.25 ± 0.98</td>
<td>127.38 ± 1.38</td>
<td>131.75 ± 1.97</td>
<td>149.00 ± 2.05***</td>
<td>161.50 ± 2.90***</td>
<td>169.88 ± 3.98***</td>
<td>180.63 ± 4.81***</td>
</tr>
<tr>
<td>DSR NS (8)</td>
<td>120.88 ± 1.13</td>
<td>128.13 ± 1.66</td>
<td>129.38 ± 1.54</td>
<td>129.63 ± 1.69</td>
<td>133.38 ± 1.74*</td>
<td>136.13 ± 2.11**</td>
<td>131.25 ± 2.30</td>
</tr>
<tr>
<td>DSS HS (6)</td>
<td>126.83 ± 2.68</td>
<td>130.83 ± 2.66</td>
<td>163.00 ± 2.79***</td>
<td>204.83 ± 4.13***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSR HS (8)</td>
<td>120.86 ± 1.32</td>
<td>127.43 ± 2.31</td>
<td>136.29 ± 1.80*</td>
<td>142.14 ± 2.14***</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values shown as Mean ± SEM. Numbers in brackets indicates the number of animals (n) in each group.
Blood pressure is given in mmHg
NS = normal salt; HS = HS; - Salt loading with 2% NaCl;
*: p<0.05; **: p<0.01; ***: p<0.001 (Significance is relative to week 2 data)

Table 4-2-3 Average Weekly Heart Rate

<table>
<thead>
<tr>
<th>Strain / Week</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
<th>Week 6</th>
<th>Week 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSS NS (8)</td>
<td>91.13 ± 2.00</td>
<td>91.63 ± 1.63</td>
<td>90.75 ± 1.71</td>
<td>109.13 ± 2.43***</td>
<td>116.13 ± 4.23***</td>
<td>125.63 ± 1.93***</td>
<td>133.38 ± 1.25***</td>
</tr>
<tr>
<td>DSR NS (8)</td>
<td>86.38 ± 2.87</td>
<td>89.38 ± 2.42</td>
<td>91.50 ± 1.48</td>
<td>87.63 ± 2.52</td>
<td>90.13 ± 1.30</td>
<td>90.38 ± 1.34</td>
<td>90.38 ± 1.89</td>
</tr>
<tr>
<td>DSS HS (6)</td>
<td>86.17 ± 2.09</td>
<td>89.50 ± 2.81</td>
<td>119.33 ± 3.47***</td>
<td>145.00 ± 2.58***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSR HS (8)</td>
<td>88.57 ± 1.21</td>
<td>86.14 ± 3.38</td>
<td>91.86 ± 2.11</td>
<td>93.00 ± 2.46</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values shown as Mean ± SEM. Numbers in brackets indicates the number of animals (n) in each group.
Blood pressure is given in mmHg
NS = normal salt; HS = HS; - Salt loading with 2% NaCl;
*: p<0.05; **: p<0.01; ***: p<0.001 (significance is relative to week 2 data)
<table>
<thead>
<tr>
<th>Strain / Week</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
<th>Week 6</th>
<th>Week 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSS NS (8)</td>
<td>403.75 ± 3.99</td>
<td>402.63 ± 17.31</td>
<td>413.38 ± 8.58</td>
<td>406.88 ± 4.16</td>
<td>417.00 ± 14.42</td>
<td>424.75 ± 13.00</td>
<td>418.63 ± 3.43</td>
</tr>
<tr>
<td>DSR NS (8)</td>
<td>400.25 ± 8.29</td>
<td>413.25 ± 9.21</td>
<td>397.75 ± 10.49</td>
<td>403.63 ± 6.51</td>
<td>394.25 ± 11.56</td>
<td>418.25 ± 7.67</td>
<td>411.50 ± 2.77</td>
</tr>
<tr>
<td>DSS HS (6)</td>
<td>407.50 ± 6.91</td>
<td>414.67 ± 6.75</td>
<td>423.33 ± 3.00*</td>
<td>429.67 ± 10.35*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSR HS (8)</td>
<td>407.14 ± 4.88</td>
<td>403.29 ± 9.38</td>
<td>403.14 ± 9.97</td>
<td>416.43 ± 8.41</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values shown as Mean ± SEM. Numbers in brackets indicates the number of animals (n) in each group.
Heart rate is given in beats per minute
NS = normal salt; HS = HS;
- Salt loading with 2% NaCl;
*: p<0.05; **: p<0.01; ***: p<0.001 (significance is relative to week 2 data)

4.5 Effect of acute treatment with *O africana*

4.5.1 Normotensive experiments: Single injection of *O africana* aqueous extract on normotensive rats

The SBP, DBP and HR of normotensive DSS rats before injection of the extract were: 130.83 ± 2.66 mmHg, 89.50 ± 2.81 mmHg and 422.17 ± 7.43 respectively. Injection of saline did not significantly influence SBP or DBP. Although fluctuations in HR was observed, it was not significant.

Graded doses of aqueous extracts of *O africana* (40 to 75 mg/kg) produced a mild fall in systolic and diastolic blood pressure (p < 0.05 in comparison with control). In contrast, 10 mg/kg of the extract did not produce any significant lowering of systolic or diastolic blood pressure in comparison with the pretreatment value (Table 4-3-1 and 4-3-2).
At higher doses, the *O africana* extract produced a greater and quicker significant lowering of systolic and diastolic blood pressure compared to the lower doses (*p*<0.05). At a dose of 1000mg/kg, the aqueous extract of *O africana* reduced the systolic and diastolic blood pressure to 87.67 ± 2.59 and 57.17 ± 1.93 mmHg respectively. At a dose of 1200 mg/kg, blood pressure was reduced faster than in the dose of 1000mg/kg (13.50 ± 0.67 VS 15.00 ± 0.45 min in SBP and 13.33 ± 0.76 VS 16.17 ± 1.11 min in DBP, *p*<0.001), but also came back to previous level quicker (SBP in 35.33 ± 2.23 VS 56.67 ± 1.63 mins and DBP in 35.17 ± 1.80 VS 55.17 ± 3.15 mins, *p*<0.001, **Figure 4-3-1 and 4-3-2**). We thus chose to administer *O africana* at a dose of 1000 mg/kg/day in experiments in which *O africana* was administered over a prolonged period of time.

Injection of the *O Africana* extract affected heart rate (*p*<0.05) only when injected at the higher doses (200, 1000 and 1200 mg/kg). The effect was not as lasting as the blood pressure lowering effect (**Figure 4-3-3**).

**Table 4-3-1 Effect of different doses of *O africana*, injected in a single dose, on systolic pressure of normotensive rats.**

<table>
<thead>
<tr>
<th>Extract conc. (mg/kg)</th>
<th>Max reduction (mmHg)</th>
<th>% of reduction</th>
<th>Reaction start time (min)</th>
<th>Lasting time (mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>6.50 ± 1.34</td>
<td>4.97 ± 0.99</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>40</td>
<td>13.67 ± 3.72</td>
<td>10.53 ± 2.90</td>
<td>31.33 ± 0.56</td>
<td>16.67 ± 1.05</td>
</tr>
<tr>
<td>75</td>
<td>22.17 ± 3.68**</td>
<td>16.96 ± 2.72**</td>
<td>27.33 ± 0.92***</td>
<td>21.00 ± 1.39</td>
</tr>
<tr>
<td>Dose (mg/kg)</td>
<td>Systolic Blood Pressure (mmHg)</td>
<td>SEM</td>
<td>p-Value</td>
<td>Systolic Blood Pressure (mmHg)</td>
</tr>
<tr>
<td>-------------</td>
<td>--------------------------------</td>
<td>------</td>
<td>---------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>200</td>
<td>28.83 ± 3.94***</td>
<td>22.08 ± 2.81***</td>
<td>24.50 ± 0.72***</td>
<td>35.00 ± 1.48***</td>
</tr>
<tr>
<td>1000</td>
<td>42.33 ± 2.76***</td>
<td>32.54 ± 2.03***</td>
<td>15.00 ± 0.45***</td>
<td>56.67 ± 1.63***</td>
</tr>
<tr>
<td>1200</td>
<td>26.50 ± 2.92***</td>
<td>20.32 ± 2.11***</td>
<td>13.50 ± 0.67***</td>
<td>35.33 ± 2.23***</td>
</tr>
</tbody>
</table>

Values are shown as Mean ± SEM; n=6 in each group
n/a: not available; *: p<0.05; **: p<0.01; ***: p<0.001

Figure 4-3-1: Effect of different doses of *O africana* extract, administered as a single injection, on systolic blood pressure of normotensive rats.

*: p<0.05; **: p<0.01; ***: p<0.001 (relative to saline injection); n=6 in each group
Table 4-3-2. Effect of different doses of *O africana* extract, injected as a single dose, on diastolic blood pressure of normotensive rats.

<table>
<thead>
<tr>
<th>Extract conc. (mg/kg)</th>
<th>Max reduction (mmHg)</th>
<th>% of reduction</th>
<th>Reaction start time (mins)</th>
<th>Lasting time (mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>10.83 ± 2.39</td>
<td>11.06 ± 2.44</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>40</td>
<td>13.67 ± 7.54</td>
<td>13.94 ± 7.69</td>
<td>29.17 ± 1.25</td>
<td>16.33 ± 0.99</td>
</tr>
<tr>
<td>75</td>
<td>21.33 ± 7.76</td>
<td>21.77 ± 7.62</td>
<td>25.50 ± 1.18</td>
<td>23.83 ± 0.79</td>
</tr>
<tr>
<td>200</td>
<td>32.00 ± 5.22**</td>
<td>32.65 ± 5.32**</td>
<td>26.50 ± 1.78</td>
<td>38.17 ± 1.72***</td>
</tr>
<tr>
<td>1000</td>
<td>36.50±3.25**</td>
<td>37.25 ± 3.32**</td>
<td>16.17 ± 1.11***</td>
<td>55.17 ± 3.15***</td>
</tr>
<tr>
<td>1200</td>
<td>34.67 ± 2.81**</td>
<td>40.79 ± 3.31**</td>
<td>13.33 ± 0.76***</td>
<td>35.17 ± 1.80***</td>
</tr>
</tbody>
</table>

Values are shown as Mean ± SEM; n=6 in each group
n/a: not available; *: p<0.05; **: p<0.01; ***: p<0.001

Figure 4-3-2: Effect of different doses of *O africana* extract, administered as a single injection, on diastolic blood pressure of normotensive rats.

Figure 4-3-3: Effect of different doses of *O africana* extract, administered as a single injection on the heart rate of normotensive rats.
4.5.2 Hypertensive Experiments: Single injection of *O africana* aqueous extract on hypertensive rats

The SBP and DBP of hypertensive DSS rats before injection of the extract or saline were: 204.83 ± 4.13 mmHg and 145.00 ± 2.58 mmHg respectively.

Graded volumes of normal saline, equivalent to the volumes of extract injected, did not significantly lower SBP or DBP (Figure 4-4-1 and Figure 4-4-2). At a dose of 1000mg/kg, the *O africana* extract reduced SBP by 76.25 ± 2.38 mmHg (*p* < 0.001) compared to saline injection (control). Diastolic pressure was reduced from 137.00 ± 4.43 to 83.38 ± 2.65 mmHg (*p*<0.001). The reduction in SBP and DBP was significantly (*p*=0.001) better in the hypertensive rats compared to the normotensive rats (SBP in
76.25 ± 4.40 VS 42.33 ± 2.76; DBP in 53.63 ± 5.53 VS 36.50 ± 3.25; Figure 4-4-3 and Figure 4-4-4).

Figure 4-4-1 Effect of injection of 1000mg/kg O africana aqueous extract on systolic blood pressure in hypertensive DSS rats.
*: p<0.05; **: p<0.01; ***: p<0.001; n=6 in each group

Figure 4-4-2 Effect of injection of 1000mg/kg *O africana* extract on diastolic blood pressure in hypertensive DSS rats.

Figure 4-4-3 Reduction in SBP in the hypertensive rats and normotensive rats.

**: p<0.01; ***: p<0.001; n=6 in each group
Figure 4-4-4 Reduction in DBP in the hypertensive rats and normotensive rats.

**: p<0.01; ***: p<0.001; n=6 in each group

Injection of the extract reduced HR significantly (p<0.001) after 25 minutes, an effect that lasted 15 minutes (Figure 4-4-5).

Figure 4-4-5 Effect of injection of 1000mg/kg *O africana* aqueous extract on heart rate on hypertensive DSS rats.
4.6 Effect of chronic treatment with *O africana* extract

Figure 4-5-1 shows that the SBP in the HS DSS group was 163.00 ± 2.78 mmHg after 1 week and 204.83 ± 4.14 mmHg after 2 weeks of salt loading. Diastolic pressure over the same period increased to 119.33 ± 3.47 and 145.00 ± 6.33 mmHg, respectively.

*O africana* extract (1000 mg/kg/day) prevented the increase in SBP or DBP (Figure 4-5-1 and 4-5-2) At the end of the 2nd week, the *O africana* extract decreased the systolic blood pressure to 128.88 ± 2.36 mmHg, which is not only significant lower than the high salt group (*p*<0.001), but also not significant from control group (128.88 ± 2.36 VS 127.38 ± 1.69 mmHg, *p*>0.05).
The antihypertensives captopril (50 mg/kg/day) and nifedipine (10 mg/kg/day) also prevented an increase in SBP and DBP during salt loading of DSS rats (Figure 4-5-1). At the doses used, both the *O africana* extract and captopril had better blood pressure lowering effects on SBP than nifedipine (extract 128.88 ± 2.36, captopril 134.38 ± 2.30 VS nifedipine 141.25 ± 2.34 mmHg), no significant difference was observed between the *O africana* extract and captopril.

To determine whether the *O africana* can be used to effectively treat hypertension rats were given 2% NaCl in the drinking water for 1 week before they were treated with the extract. Systolic blood pressure was increased significantly (*p*<0.001) after 1 week salt loading (164.75 ± 2.30 VS 131.75 ± 1.31 mmHg). The SBP did not differ with the high salt group (164.75 ± 2.30 VS 163.00 ± 1.97 mmHg, *p*>0.05). After 1 week’s treatment with extract, the SBP was reduced back to the control level (133.25 ± 1.94 VS 127.38 ± 1.69 mmHg, *p*>0.05), which was significantly lower than high salt group (*p*<0.001) (Figure 4-5-1).

![Figure 4-5-1](image)

Figure 4-5-1 Effect of chronic treatment with *O africana*, captopril and nifedipine on systolic pressure of salt loaded DSS rats.
Salt loading for 2 weeks increase DBP from 89.50 ± 4.60 to 145.00 ± 6.86 mmHg. *O africana* extract (1000 mg/kg/day) prevented the increase in DBP. In fact, the DBP was significantly reduced (85.63 ± 2.49 VS 145.00 ± 6.86 mmHg, *p*<0.001; Figure 4-5-2). Both captopril and nifedipine also prevented the increase in DBP during salt loading (Figure 4-5-2).

Diastolic blood pressure following salt loading for 1 week was 120.38 ± 4.72 mmHg. After a week of treatment with *O africana* extract, DBP was significantly reduced to 93.75 ± 5.18 mmHg (*p*<0.001). This value was not significant from the initial value (91.63 ± 1.63 mmHg; *p*>0.05).
Figure 4-5-2 Effect of chronic treatment with *O africana*, captopril and nifedipine on diastolic pressure of salt loaded DSS rats.

![Graph showing diastolic blood pressure over weeks with different treatments](image)

***: $p<0.001$ compared to high salt group; ·: $p<0.05$ compared to corresponding to week 0 group; …: $p<0.001$ compared to corresponding to week 0 group; NS-C: not significant in comparison with control; NS-S: not significant in comparison with high salt group. n=8 in each group.

The HR was $429.67 \pm 11.75$ beats per minute after 2 weeks of salt loading. *O africana* decreased the HR to $379.50 \pm 8.57$ beats per minute ($p<0.001$). This level was also significant lower than in the control group. Similar results were obtained in the captopril treated group ($387.25 \pm 3.68$ beats per minute; $p<0.001$) and the nifedipine treated group ($394.75 \pm 5.01$ beats per minute; $p<0.001$). The HR in all treatment groups were not significant from the values at the start of the experiment ($p>0.05$) (Figure 4-5-3).
Similar to SBP and DBP, HR in salt loaded animals was reduced after 1 week treatment with *O africana* extract (396.50 ± 15.16 beats per minute, *p*<0.001). Although changes in HR were noted, the HR remained within in normal range.

**Figure 4-5-3** Effect of chronic treatment with *O africana* extract, captopril and nifedipine on heart rate of salt loaded DSS rats.

![Heart rate graph](image)

***: *p*<0.001 compared to high salt group; *: *p*<0.05 compared to corresponding to week 0 group; NS-C: not significant in comparison with control; NS-S: not significant in comparison with high salt group. n=8 in each group.

### 4.7 Plasma Angiotensin II levels

The plasma AII level of DSS rats in the control group (no salt loading) was 62.31 ± 1.18 pg/ml, as shown in **Figure 4-6**. Following 2 weeks salt loading the plasma AII levels reached to 270.83 ± 9.76 pg/ml. Administration of *O africana* extract (1000mg/kg/day) prevented the increase in plasma AII level (13.57 ± 0.62 pg/ml, *p*<0.001, **Figure 4-6**).
The ACE inhibitor, captopril, also prevented the increase in plasma AII level (32.06 ± 2.97 pg/ml, \( p < 0.001 \), Figure 4-6). AII levels in the nifedipine treated group did not differ from that of the salt loaded group (265.80 ± 4.90 VS 270.83 ± 9.76 pg/ml; \( p > 0.05 \)).

Both the \textit{O africana} extract and captopril decreased the plasma AII levels to values below control (\( p < 0.001 \), Figure 4-6). The plasma AII level following administration of the \textit{O africana} extract was also significantly lower than obtained with captopril administration (\( p < 0.01 \), Figure 4-6).

**Figure 4-6 Effect of \textit{O africana}, captopril and nifedipine on plasma AII levels in salt loaded DSS rats**

***: \( p < 0.001 \) compared to high salt group; ...: \( p < 0.001 \) compared to control. \( n=7 \) in each group. NS-S: not significant in comparison with high salt group.
CHAPTER 5

DISCUSSION

*Olea africana,* drunk as a tea, has been used to treat hypertension in Africa for many years (Breitenbach VF, 1986), yet not many studies have been done to determine the scientific basis of its use. Furthermore, previous studies on the antihypertensive effects of *O africana* involved the use of either ethanol extracts (Somova LI, *et al.*, 2003) or an aqueous extract from the roots and stems (Osim EE, *et al.*, 1999) of the plant. This may be the first scientific report on the antihypertensive effect of an aqueous extract from the leaves of *O africana*. The leaves are the part of the plant most often used by traditional users (Breitenbach VF, 1986).

It is argued that *O africana* causes blood pressure lowering effect by modifying the RAS, probably by acting as an ACE inhibitor. This study investigated the changes in the RAS during treatment via determining the angiotensin II levels in the blood and comparing it with the values obtained with the ACE inhibitor captopril, and nifedipine which is not an ACE inhibitor.

5.1 Toxicity of the *O africana* extract

Our results indicate that the plant extract has a very low toxicity (LC$_{50}$ > 5000 µg/ml). We are thus satisfied that the dose of the extract used in our chronic treatment experiments is not toxic to the rats.
5.2 Induction of hypertension in the rat model by addition of salt to the drinking water

Most studies in which the DSS rat is used to study hypertension, the animals are fed a high salt diet (8% NaCl in the diet) (Inoko M, et al, 1994; Mozaffari M.S, et al, 2000). It is undoubtedly a well established model that is well characterized. It does however require the use of synthetic diets which has to be specially formulated and is expensive. The time required before hypertension is achieved is at least 5 weeks (Inoko M, et al, 1994).

In this study hypertension was induced by adding 2% NaCl to the drinking water. This technique was first published by Ojewole JAO, et al (2003). In our experiments all DSS rats given 2% NaCl in the drinking water were hypertensive after only one week (Table 4-2-1 and Table 4-2-3), The systolic pressure increased quickly and significantly from 130.83 ± 2.66 mmHg to 163.00 ± 2.79 mmHg (p<0.05) and the diastolic pressure from 89.50 ± 2.81 mmHg to 119.33 ± 3.47 mmHg (p<0.05). After 2 weeks of high salt treatment, these levels increased to reach 204.83 ± 4.13 mmHg and 145.00 ± 2.58 mmHg respectively, which is about 56.9% higher than normal blood pressure level and 25.2% (p<0.01) higher than after one week’s treatment.

It was observed that at the end of 2 weeks of salt loading, all DSS rats were trembling and seemed very weak. Much more urine was discharged, compared to the normotensive animals, so that the bedding was often wet and had to be changed more frequently.
Normotensive rats in other groups were still active and didn’t show such weakness. 2 of 8 DSS rats receiving salt in the drinking water died after 2 weeks, which is the reason that the data was recorded only from 6 rats in the DSS HS group. The rats in the DSS HS group probably developed heart failure or other serious cardiovascular diseases by the end of 2 weeks of salt loading. This is in comparison with the 8% NaCl dietary model where the rats develop LVH after about 5 weeks and heart failure after about 9-12 weeks (Inoko M, et al, 1994).

An interesting finding is that the DSS group receiving normal tap water (i.e. normal salt) also became hypertensive after 4 to 5 weeks with systolic pressure increasing to over 160 mmHg after 5 weeks. This may be because of some genetic drift in the colony since it is quite an old colony. On the contrary, the blood pressure of the DSR rats did not increase significantly.

As indicated earlier, the DSS rats receiving 2% NaCl in the drinking water became weak after only 2 weeks. It can thus be argued that the salt content of the water should be reduced. In previous experiments of this nature in which DSS rats were used, the water salt content was as high as 4% (Ojewole JAO, et al, 2006). The rapid deterioration of the rats in our experiments can perhaps be due to the fact that also the DSS rats receiving normal tap water developed hypertension after a few weeks. It is thus necessary to monitor the rate at which hypertension develops closely in future experiments, especially if one also wants to study the progressive deterioration from hypertension to LVH and
subsequent heart failure. It might then be necessary to reduce the salt concentration in the water to 1%.

**5.3 Effect of acute treatment with *O africana* in normotensive rats**

Intraperitoneally administration of the aqueous extracts of *O africana* leaves caused an immediate and dose dependant fall in both systolic and diastolic blood pressure, as well as heart rate in normotensive rats. The administration of equivalent volumes of normal saline, used as control for the extract, had no significant blood pressure or heart rate lowering effect. The decrease in blood pressure and heart rate can thus not be attributed to blood volume changes upon injection.

All doses of aqueous extract of *O africana* leaves produced significant blood pressure and heart rate lowering effect (*p*<0.05 to 0.01) in normotensive rats, except the lowest dose (10mg/kg). The percentage reduction in systolic blood pressure differs from 13.07 (40 mg/kg) to 34.02 (1000 mg/kg) from the lower doses to higher doses. The lasting time of the effect was from 15 to 55 minutes respectively, which indicates that aqueous extract of *O africana* leaves caused a dose dependant lowering effect of systolic blood pressure and heart rate.

Although at doses of 40, 75 and 200 mg/kg, mean systolic and diastolic blood pressure was reduced significantly (*p*<0.05), it was observed that the maximum effect occurred at dose of 1000 mg/kg (87.67 ± 2.59 and 57.17 ± 1.93 mmHg), while the quickest lowering effect occurred at dose of 1200 mg/kg (13 mins). *Figure 4-3-1 – Figure 4-3-3* show that
though the dose of 1000 mg/kg did not decrease the systolic pressure as rapidly as the 1200 mg/kg (15 VS 13 mins), it reduced the blood pressure to the lowest level, as well as heart rate. Furthermore, the decrease in systolic pressure and heart rate was maintained for a longer period of time compared to the dose of 1200 mg/kg (Table 4-4-1). We thus concluded that the dose of 1000 mg/kg caused a more stable lowering effect on systolic blood pressure.

A previous study (Osim EE, et al, 1999) reported that the aqueous extract from the roots and stems of *O africana* have a significant lowering effect on mean blood pressure at doses of 200 mg/kg and 1000 mg/kg (oral treatment). Our study suggests that the dose of 1000 mg/kg should be the maximum effective concentration of aqueous extract of *O africana*.

### 5.4 Effect of acute treatment with *O africana* in hypertensive rats

In most cases, drugs are used to treat hypertensive patients, not normotensive people. It is thus important to test the hypotensive effects in hypertensive rats. In the normotensive rats 1000 mg/kg was considered the best concentration tested. This concentration was thus also tested in our hypertensive rats. A dose of 1000 mg/kg reduced the systolic blood pressure significantly from 204.83 ± 4.13 mmHg to 124 ± 6.3 mmHg (*p* <0.001) and the diastolic pressure from 145.00 ± 2.58 mmHg to 90.00 ± 3.62 mmHg (*p* <0.001). This dose thus effectively decreased the blood pressure back to normotensive level. The blood pressure was reduced significantly from 13 to 50 mins, which means that the dose of 1000 mg/kg can produce quick and lasting antihypertensive effect in hypertensive rats.
In comparison to the effect on normotensive rats, the dose of 1000 mg/kg has a more pronounced effect on hypertensive rats. Systolic blood pressure of hypertensive rats was reduced 76.25 \pm 4.40 \text{ mmHg}, which is 80\% more (p<0.001) than in normotensive rats (42.33 \pm 2.77 \text{ mmHg}). It has previously been shown that ACE inhibitors can cause a more pronounced blood pressure lowering effect in hypertensive patients than in normotensive people (Urata H, et al, 1990; Giulio SD, et al, 1996; Weber MA, 1997). This view is supported in our rat model.

5.5 Effect of chronic treatment

In our acute treatment experiments 1000 mg/kg was found to be an effective dose to treat hypertension in our animals. As shown in Figure 4-4-1 and 4-4-2 this dose effectively decreased the blood pressure of the hypertensive rats back to normal values. We thus also wanted to determine whether this dose if administered on a daily basis can be used to (i) treat our hypertensive animals (ii) prevent our DSS rats from becoming hypertensive. We housed two animals per cage (this was done for practical reasons since we had a large number of animals at the time). Twice weekly the body weights and the average daily water intake for the animals in the cage was determined. The values were used to determine the amount of extract that had to be added to the drinking water to administer the extract at 1000mg/kg. It can be argued that the approach followed is not accurate or proper however, as will be seen in the sections to follow, the approach was effective.
5.5.1 Treatment of hypertensive animals

After one week on the high salt regime our rats became hypertensive with the systolic pressure reaching $164.75 \pm 2.31$ mmHg and diastolic pressure $120.38 \pm 1.67$ mmHg. After one week *O africana* (1000 mg/kg/day) decreased the systolic pressure to $133.25 \pm 1.94$ mmHg and the diastolic pressure to $93.75 \pm 1.83$ mmHg. The level is not significant from the normotensive pressure (before any treatment: $133.25 \pm 1.94$ systolic and $93.73 \pm 1.83$ mmHg diastolic). This indicates that in *O africana* at 1000 mg/kg/day was effective in reducing blood pressure of hypertensive rats back to normal values.

5.5.2 Preventative effects of *O africana*

As knowledge increases and the public becomes more health conscious more people recognizes the value of preventative treatment either by a modification of lifestyle or by the use of supplements. Many people, especially those that are not very disciplined, find the latter option more attractive.

After 2 weeks of salt loading the systolic pressure of the DSS rats reached $204.83 \pm 4.14$ mmHg and diastolic pressure reached $145.00 \pm 2.58$ mmHg in the high salt group ($p<0.001$ compared to value prior to salt treatment). The corresponding values in the extract treated group were $128.88 \pm 2.36$ mmHg and $85.63 \pm 2.49$ mmHg respectively ($p>0.05$ compared to values prior to salt treatment). Unlike the rats in the high salt group rats in the extract treated group were active and healthy in appearance.
In previous experiments the ACE inhibitor, captopril at a dose of 50 mg/kg (Stephen JL, et al, 2006) and the calcium channel blocker, nifedipine at 10 mg/kg (Hisakazu I, et al, 1980) were found to be effective in preventing hypertension in rats. We thus compared the antihypertensive effects of our aqueous $O$ africana extract with the referred doses of captopril and nifedipine. In our experiments 1000 mg/kg/day $O$ africana extract was at least equally effective as captopril (50 mg/kg) and nifedipine (10 mg/kg). In our experiments captopril and nifedipine was administered using gelatine and jelly as vehicle. Results have shown that the vehicle did not affect the blood pressure readings in the animals (Unpublished article).

5.6 Mechanism of the antihypertensive effects of $O$ africana

It was previously suggested that ethanolic extracts of $O$ africana may exert its antihypertensive effects via modulation of the sympathetic nervous system or by acting as an ACE inhibitor (Somova LI, et al, 2003). Chemical analysis of $Olea$ extracts prepared from roots and stems suggests that also the aqueous extract of $Olea$ species may have ACE inhibitor properties (Osim EE, et al, 1999). One of our objectives was thus to determine whether our $O$ africana aqueous extract exert its antihypertensive effects by modulating the activity of the RAS.

To achieve this objective we compared the plasma AII levels in our extract treated rats with that of captopril (ACE inhibitor) treated rats and nifedipine (calcium channel blocker) treated rats.
DSS rats receiving tap water (and normal salt) had plasma AII levels of 62.31 ± 1.18 pg/ml (Figure 4-6). After oral 2% salt loading for 2 weeks plasma AII levels increased to 270.83 ± 9.76 pg/ml. Administration of the ACE inhibitor, captopril or the *O africana* extract not only prevented the increase in plasma AII levels, but in both cases significantly reduced the levels to 32.06 ± 2.97 pg/ml and 13.57 ± 0.62 pg/ml respectively (Figure 4-6). The non – ACE-inhibitor antihypertensive, nifedipine, did not prevent the decrease in plasma AII levels (Figure 4-6). We thus conclude that the aqueous *O africana* extract exerts its hypotensive effects at least in part by modulating the RAS system. A study (see appendix) by the author of this thesis suggests that the extract has ACE inhibitor properties. Our study does not allow us to make such a firm conclusion. We were unable to source an angiotensin I assay. Angiotensin I levels, together with the angiotensin II levels would allow us to make a firm statement regarding the ACE inhibitor activity of the extract.

The *O africana* extract decreased the heart rate of our rats (Figure 4-5-3) significantly compared to control. Previous experiments indicate that captopril may also decrease HR (Konstam MA, *et al*, 2000). These results are confirmed in this study. We can thus not exclude the possibility that the extract exerts its hypotensive effects also in part by modulating the sympathetic nervous system. One can however argue that also captopril appears to have an apparent heart rate lowering effect. However, although there were fluctuations in HR, all values were within in normal range.
5.7 Conclusions and recommendations

This study indicates that:

- An aqueous extract prepared from leaves of *O africana* has hypotensive effects in rats
- A dose of 1000 mg/kg/day can be used to effectively treat or prevent hypertension induced by high salt loading in rats. It is possible that lower doses may be equally effective.
- If the extract is administered by injection the dose which decreased the blood pressure most effectively was found to be 1000 mg/kg
- The extract exerts its hypotensive effects at least in part by modulating the RAS, probably by acting as an ACE inhibitor.

Traditionally *O africana* is not only used to treat hypertension. It is also used to treat ailments such as colic and urinary tract infections (Watt JM, 1962; Hutchings A, 1996; Iwu MM, 1993). In this study, using bolus injection experiments, we show that the *O africana* extract may decrease the blood pressure of normotensive rats. Normotensive persons using *O africana* injections for ailments such as colic must be made aware of its potential hypotensive effects. We recognize such effects will be dose dependent.

We did not use a tea or infusion prepared from the *O africana* leaves. We do, however, believe this study currently most closely represents the form in which the plant is used traditionally.
5.8 Future studies

Future studies should investigate the effects of the extract on rats with complications of hypertension, such as LVH and heart failure. One also has to determine whether the extract can protect the myocardium.
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Appendix

The antihypertensive effects of *Olea africana* phytotherapy

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ABSTRACT

Hypertension is becoming an increasingly common global health problem, despite the use of many synthetic drugs for this condition. *Olea africana* is one of the many phytotherapies that has been used indigenously to modulate hypertension for years. In the current study, the inhibitory activity of ethanol extracts of *Olea africana* (OAE), pure oleanolic acid (OA), aqueous extracts of *Olea africana* (OAW) and the synthetic drug Captopril (Cap) on angiotension converting enzyme (ACE) levels in whole blood in normotensive and hypertensive rats were compared *in vitro*. The results indicate that OA produced mild inhibitory activity of ACE levels, while Captopril, OAE and OAW produced more significant inhibition effect. The $C_{\text{max}}$ of Captopril was 0.04mg/ml, which reduced ACE levels to $976.87\pm25.38$pg/ml (control level in normotensive rats was $1172.24\pm28.62$ pg/ml) and $1397.52\pm87.95$ pg/ml (control level in hypertensive rats was $1810.36\pm32.11$ pg/ml). The peak inhibition effect in normotensive and hypertensive rats was observed in OAW at dose of 1.00mg/ml, which reduced ACE levels $28.94\pm5.27\%$ and $34.01\pm9.89\%$. In conclusion, aqueous and ethanol extracts of *Olea africana* produced greater lowering of ACE levels compared to Captopril and OA. OAW showed most significant ACE inhibitory effect among all the four tested reagents. Hence, further research on the anti-hypertensive effects of *Olea africana* extracts is recommended.
INTRODUCTION

Hypertension is becoming an increasingly common health problem worldwide because of increasing longevity and prevalence of contributing factors such as obesity, physical inactivity and an unhealthy diet. Worldwide, hypertension is estimated to cause 7.1 million premature deaths and 4.5% of the disease burden annually.

The main treatment of hypertension still relies on synthetic medicines. Three main drug classes have been used in the treatment of hypertension in the past forty years. They are diuretics, Beta blockers (β-blockers) and calcium channel blockers (CCBs). The data from more than 20 randomized controlled trials conclusively demonstrate reductions in both mortality and morbidity with these three drug classes. Now, a newer class, angiotensin converting enzyme inhibitors (ACEI’s), is being widely used against the older classes.

The use of these synthetic medicines however, has some negative effects. Most drugs used to treat hypertension have been evaluated for a number of specific populations; these include ACEI’s, B-blockers, CCB’s and diuretics in patients with concomitant diabetes, nephropathy, coronary and cerebrovascular disease, heart failure, and left ventricular hypertrophy. Other side effects of these synthetic medicines have also been reported; for example, dry cough is a common side-effect of ACEI’s and is a major limiting factor of their use. Secondly, despite the availability of useful non-drug therapy and potent medications, treatment is too often ineffective, mainly as a consequence of the patient’s lack of compliance with therapeutic regimens. Moreover, because of limited resources, synthetic drug treatment may be not affordable to the majority of hypertensive patients.

On the other hand, there are many herbal medicines traditionally used to treat
hypertension in countries such as China, Japan, South Africa, Morocco and Cameroon. For example, Chinese *Peristrophe roxburghiana*, *Hibiscus sabdariffa*, Japanese *Toki-shakuyaku-san* and Cameroon’s *Mitragyna ciliate* have been traditionally used to treat hypertension in local regions for years. These herbal medicines are much easier and cheaper to obtain than the synthetic medicines, and fewer side effects are reported.

Oleanolic acid (OA) is a triterpenoid compound, found in more than 120 plant species. OA has been shown to have many pharmacological properties, including antihypertensive activity. *Olea europaea* is a medicinal plant that has shown significant antihypertensive effects by it’s ACE inhibitory activity. *Olea africana* is a subspecies of *Olea europaea* and is indigenous to Africa. In traditional medicine, this plant is used as a diuretic, hypotensive, emollient, febrifuge and tonic, for urinary and bladder infections and for headaches. In scientific studies, the extract of the leaves, root and stem of *Olea africana* has been shown to possess antihypertensive activity.

**MATERIALS AND METHODS**

**Plant material**

Leaves of *Olea africana* were collected on the campus of the University of the Western Cape (UWC) in South Africa, between September and October 2004. Fresh leaves were washed, air dried and ground into powder.

**Ethanol extract** was prepared by shaking the ground powder in twice its weight of ethanol and allowing it to stand for 12 hours, after which it was vacuum filtered. The filtrate was then evaporated to dryness at 40°C, using a rotary evaporator. Before use, the extract was weighed and dissolved in a known volume of distilled water and then serially diluted to 0.02mg/ml, 0.04mg/ml and 0.08mg/ml. The ethanol extracts were stored at 4°C until use.
**Aqueous extract** was obtained by shaking the powder in distilled water and allowing it to stand for 24 hours before filtering, using a filter paper. The filtrate was then evaporated to 10% of its original volume using a rotary evaporator at a temperature of 40°C. The reduced volume of the filtrate was first frozen overnight and then freeze dried for 24 hours to obtain a fine powder. The powder was weighed and dissolved in a known volume of distilled water and then serially diluted to 0.50 mg/ml, 1.00 mg/ml and 2.00 mg/ml. The aqueous extracts were stored at 4°C until use.

**Oleanolic acid**

Pure oleanolic acid (Sigma Aldrich) was stored at 4°C. Before use, OA was brought to room temperature and then serially diluted to 0.50 mg/ml, 1.00 mg/ml and 2.00 mg/ml.

**Animals**

Dahl salt-sensitive (DSS) genetically hypertensive rats and control normotensive Dahl salt-resistant (DSR) rats were ordered from the University of Kwazulu-Natal, South Africa. Male rats (6 weeks old), weighing 200–250g at the beginning of the experiment, were used. Rats were bred in the Medical Bioscience Department animal house, UWC and housed individually in polyethylene cages with water and standard food provided ad libitum. Animal room temperature was maintained at 26±2°C, with constant humidity and a 12-h light/dark cycle.

The control normotensive DSR rats received normal saline for 3 weeks. The DSS rats received high-salt diet (8% NaCl) for 3 weeks to induce hypertension.22

Before use, rats were anesthetized with sodium pentobarbital (40 mg /kg i.p.) and the chests were quickly opened. Injectors were used to collect blood (2ml) from the most strongly beating position of hearts.

**Whole Blood Assay**
Collected blood was immediately transferred into lithium heparin glass tubes (BD Company) and lightly inverted several times. Blood was diluted 1:5 with RPMI 1640 medium. Cell culture was carried out in sterile 96-well (NUNC) plates under aseptic conditions. 180µl of diluted blood was transferred to wells of the 96 well plate. The negative control consisted of 20µl of medium added to 180µl of blood. The positive control consisted of 20 µl of Captopril (Adcock Ingram Ltd.) added to the blood culture, as Captopril is an ACEI and has been proven to inhibit ACE levels.23 The experiment consisted of 20µl of either *Olea africana* ethanol extract (OAE) or pure oleanolic acid (OA) added to the diluted blood. The cultured blood was then incubated for 1-1.5 hours in a humidified incubator at 37°C and 5% CO2. The blood was stimulated in the presence and absence of Captopril or OAE or OA. After incubation, the supernatants were collected and stored at -20°C for determination of blood ACE levels.

**ACE Assay**

The human ACE Quautikine immunoassay kit (R&D Systems, Catalog No.DACE00) was used for the analysis of ACE levels. Before use, the reagents, samples and ACE standards were brought to room temperature. First 100µl Assay Diluent was added to each well. Then 50µl ACE standard, supernatants of the negative control, positive control or plant extract was added to different wells. The plate was then incubated for 2 hours on a shaker at room temperature. The wells were then aspirated and washed 4 times before 200µl ACE conjugate was added to each well. The plate was incubated for 2 hours again on the shaker at room temperature. The wells were then aspirated and washed 4 times again. 200µl substrate solution was then added to each well and the plate was incubated for 30 minutes at room temperature on the benchtop, protected from light. Then 50µl stop solution was added to each well. The data was read at 450nm on a microplate reader (Labsystems Multiskan® MCC/340).

**Statistical analysis**
All data are expressed as mean± standard error of the mean (SEM) and analysed by analysis of variance (ANOVA) test. The SPSS V12.0 was used, including one-way student’s $t$-test. A $p$ value of 0.05 or less was considered statistically significant.

**RESULTS**

**Comparison of Captopril, OAE, OA and OAW against Control**

**Normotensive Rat Experiments**

The control mean ACE level in whole blood in normotensive rats was 1172.24±57.37 pg/ml.

Graded doses of Captopril (0.02mg/ml to 0.08mg/ml), OAE, OA and OAW (0.50mg/ml to 2.00mg/ml) produced significant drops of ACE levels ($p < 0.05$, Figure 1&2). At dose of 0.04mg/ml, Captopril displayed best ACE levels reduction, which decreased ACE levels down to 976.87±25.38pg/ml. OAW, OA and OAW showed best ACE inhibitory effect at dose of 1.00mg/ml, and the lowest ACE level was observed in OAW, which was 831.14±45.55pg/ml.

![Figure 1. ACE levels reduction effect of Captopril in normotensive rats](image1.png)

![Figure 2. ACE levels reduction effect of OAE, OA and OAW in normotensive rats](image2.png)
Hypertensive Rat experiments
The control mean ACE level in whole blood in hypertensive rats was 1810.36±70.11 pg/ml.
ACE levels drops were observed and expressed in Figure 3&4. All these 4 tested reagents at different doses showed significant drops ($p < 0.01$). The lowest ACE level was also observed in OAW at dose 1.00mg/ml, which was 1193.26±69.76pg/ml.

Comparison of OAE, OAW and Captopril
When compared to Captopril, at all the doses administrated (0.50, 1.00 and 2.00mg/ml), OAE and OAW produced greater decrease in ACE levels than corresponding doses of Captopril (0.02, 0.04 and 0.08mg/ml) in both normotensive and hypertensive rats (Figure 5-8, $p < 0.05$ to 0.01). For normotensive rat experiments, the decrease percentage of ACE levels in OAE and OAW (1.00mg/ml) were observed as 22.85±3.84% and 28.94±5.27% against 16.72±3.23% in Captopril (0.04mg/ml). For hypertensive rat experiments, the numbers come up to 28.32±7.25% and 34.01±9.89% (OAE and OW at 1.00mg/ml) against 22.79±4.14% (Captopril at 0.04mg/ml).
Comparison of OAE, OAW and OA

Although OA also produced significant ACE inhibitory effect, data showed that OA is not as powerful as OAE or OAW. At all doses, OAE and OAW displayed greater lowering of ACE levels, especially at dose of 1.00mg/ml in hypertensive rat experiments, where OA produced 11.20±4.44% of reduction, while OAE and OAW produced 28.32±7.25% and 34.01±9.89% (Figure 6&8, \( p < 0.001 \)).
Comparison of OAE and OAW

Even OAE and OAW come from same plant and both produced greater ACE inhibitory effects than Captopril and OA, however, their effect are not exactly same. In fact, at all the doses administered, OAW showed greater reduction of ACE levels than corresponding doses of OAE (Figure 6&8, $p < 0.05$).

DISCUSSION

The results from previous studies have confirmed that two inhibitors of ACE (Captopril and OA) produce an antihypertensive response in rats\textsuperscript{1,6,7}, however, ACE levels were seldom tested in these studies. This may be the first time to determine the ACE levels reduction in whole blood by comparing Captopril, \textit{Olea africana} extracts, and oleanolic acid.

As presented above, all these four reagents administrated showed significant inhibition of ACE levels in whole blood. $C_{\text{max}}$ of Captopril was 0.04mg/ml. $C_{\text{max}}$ of OAE, OA and OAW was 1.00mg/ml. However, both OAE and OAW produced greater reduction of ACE levels when compared to Captopril and OA. In addition, OAW seems to be more effective than OAE according to the results.

Oleanolic acid is one of the compounds of \textit{Olea africana} extract, but the results showed that OA is not as effective as OAE or OAW. It seems that OA alone might not produce significant anti-hypertensive effect as OAE or OAW. The interactions of OA and other compounds in \textit{Olea africana} extract appear to be complex and additional work need to be done to more fully understand this biology.

As classic ACE inhibitor for the treatment of hypertension, Captopril produced significant ACE inhibition in whole blood. However, OAE and OAW produced greater effect than Captopril. Considering the side effect of Captopril which can’t be ignored,
Olea africana extract seems to be a better choice to treat hypertension for its less negative effect. Furthermore, OAE and OAW are also easier to be prepared.

Olea africana is widespread in Africa. It is easier to obtain and costs less than synthetic medicines.\textsuperscript{18} The cost of antihypertensive treatment, especially in developing countries with rich herbal resources and declining economies, might be reduced by resorting to scientifically proven herbal treatments such as the Olea africana extracts studied here. This study may validate the use of the aqueous and ethanol extracts of Olea africana in the treatment of some forms of hypertension.

ACKNOWLEDGMENTS

The authors thank Mr. James Mukinda for assistance with blood collection from rats.

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