An Evaluation of the Chemical Composition, and the *in vitro* and *in vivo* Antihypertensive Activity of Extracts of *Tulbaghia acutiloba* Harv. in an L-NAME induced Hypertensive Model

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Doctor of Philosophy in Medical Sciences (Human Physiology)

Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences,

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Supervisor: Professor Irene Mackraj

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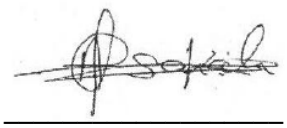
Preface

The experimental work described in this thesis was conducted at the University of KwaZulu-Natal (Durban, South Africa), under the supervision of Professor Irene Mackraj.

This work has not been submitted in any form for any degree to any tertiary institution, where use has been made of the work of others, it is duly acknowledged in the text.



Isaiah Kofi Arhin **28th October, 2019**

Date

As the candidate's supervisor, I agree to the submission of this thesis.

Professor Irene Mackraj **28th October, 2019**

Date

Declaration

I Isaiah Kofi Arhin declare that:

(i) The research reported in this thesis, except where otherwise indicated, is my original work.

(ii) This thesis has not been submitted for any degree or examination at any other university.

(iii) This thesis does not contain other persons' data, pictures, graphs or other information unless specifically acknowledged as being sourced from other persons.

(iv) That my contribution to the project was as follows:

Identification of research topics, experimental design, execution, data analysis and interpretation, manuscript and thesis write-up.

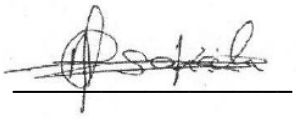
(v) That the contributions of others to the project were as follows:

Professor Irene Mackraj (supervisor) has reviewed the entire project and the write-up of the manuscript and thesis. This project was funded by the College of Health Sciences, UKZN (Grant no.: 636801).

Dr Kogi Moodley, Dr Depika Dwarka and Mr. Ajay Bissesseur have reviewed the laboratory design of the project and manuscripts.

Professor Himansu Baijnath assisted with identification and authentication of the plant material

Simoene Eche and Delon Naicker assisted with the laboratory experimentations.



Isaiah Kofi Arhin **28th October, 2019**

Date

Publications and Presentations

Peer-reviewed Publications contributing to this thesis

Arhin Isaiah, Dwarka Depika, Bissesseur Ajay, Naicker Delon, Mackraj Irene. Biochemical, Phytochemical Profile and Angiotensin-1 Converting Enzyme Inhibitory activity of the Hydro-methanolic extracts of *Tulbaghia acutiloba* Harv. Accepted: Journal of Natural remedies, Volume 19, Issue 3, 2019 **(Chapter 3 of this thesis)**

Arhin Isaiah, Dwarka Depika, Moodley Kogi and Mackraj Irene. Ethnopharmacological Review of South African Indigenous Plants for Angiotensin Converting Enzyme (ACE) Inhibitory Activity. *Under Review:* Indian Journal of traditional knowledge (Manuscript number: 9687) **(Chapter 2 of this thesis)**

Conference Presentations

Cardioprotective effects of the hydro-methanolic leaf extract of *Tulbaghia acutiloba* and Ramipril in an L-NAME induced hypertensive rat model. Poster presentation at the Gordon Research Conference on Natural Products and Bioactive Compounds (GRS) Gordon Research Seminar, July 27, 2019 - July 28, 2019, Proctor Academy in Andover, NH United States (abstract accepted).

Manuscripts in preparation and submitted:

Arhin Isaiah, Dwarka Depika, Eche Simeone, Moodley Kogi and Mackraj Irene. Cardioprotective effects of the hydro-methanolic leaf extract of *Tulbaghia acutiloba* and Ramipril in an L-NAME induced hypertensive rat model. Submitted to *Records of Natural products* (**Chapter 4 of this thesis**)

Arhin Isaiah, Moodley Kogi and Mackraj Irene. Haematological and Renal function markers as indicators of antihypertensive efficacy of the leaf hydro-methanolic extract of *Tulbaghia acutiloba* Harv in an L-NAME induced hypertensive rats (**Chapter 5 of this thesis**).

Dedication

I dedicate this thesis to my parents and siblings.

Acknowledgements

With great ebullience and utmost reference to the Almighty God, this phenomenal accomplishment would not have been possible without divine assistance.

To my supervisor, Professor Irene Mackraj, I deeply appreciate your unremitting support, patience, excellent mentorship and guidance throughout the project.

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THESIS OUTLINE

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The principal findings of this PhD research study have been compiled into an article format and presented as a thesis by manuscript.

Chapter One: Provides background information with a brief review of selected topics relevant to the study. Study aims and objectives, hypotheses and potential benefits of this research are also highlighted.

Chapter Two: Forms part of the literature review and describes the ethnopharmacological benefits and screening of South African plants with antihypertensive properties through the inhibition of ACE. This review has been submitted to the Indian Journal of traditional knowledge (an ISI accredited journal) and is currently under review (Manuscript number: 9687)

Chapter Three: Reports on the phytochemical components and *in vitro* preliminary screening of *Tulbaghia acutiloba* for potential therapeutic effects. This forms the first part of the PhD experimental research and has been accepted for publication by the Journal of Natural remedies (an ISI accredited journal), Volume 19, Issue 3, 2019.

Chapter Four: Forms part of experimental research that evaluated the antihypertensive and cardioprotective effect of *Tulbaghia acutiloba* extracts in an L-NAME induced hypertensive rats.

Chapter Five: Reports on the effect of *Tulbaghia acutiloba* on renal function and haematological parameters in an L-NAME induced hypertensive rats.

Chapter Six: Provides general conclusions and synthesis of the research findings. It also highlights limitations and makes recommendations for future studies.

ABSTRACT

The increasing prevalence of hypertension over the years has been identified as a major contributor to high morbidity and mortality, globally and locally, in Africa, posing a serious global health threat. The demand for novel therapeutic strategies has become increasingly important, given that conventional options may be inaccessible and costly, and are often associated with side effects, hampering patient compliance. The scientific validation of alternative strategies, such as phytotherapy has, therefore, become a major focus in the treatment and management of hypertension, as it is perceived as being cheap, accessible, and possessing minimal side effects. Hence the investigation of medicinal plants, within the field of novel drug discovery, is of interest as plants possess various phytochemicals displaying biological activities, which may be beneficial in hypertension, and it's associated complications. Since there is no existing scientific data available to validate its medicinal usage, this study, therefore, evaluated the *in vitro* and *in vivo* antihypertensive effects of *Tulbaghia acutiloba.* The hydro-methanolic extracts of the plant parts (i.e. leaves, flowers, rhizomes and roots) were initially evaluated *in vitro* for their phytochemistry, antioxidant potential, angiotensin-converting enzyme (ACEI) inhibition activities, and heavy metal content. The phytochemical investigation of the various parts of the plants showed the presence of phenols, amino acids and alkaloids in all parts, with the leaves exhibiting a higher total phenolic content, in comparison to the other parts. Further analysis, using gas chromatography–mass spectrometry (GC-MS), revealed the presence of bioactive compounds, such as *α-linolenic acid,* which was found only in the leaves. Other compounds such as *oleic acid* and *palmitic acid* were found in all the parts of the plants. All parts of the plant showed antioxidant activity *in vitro.* Heavy metal toxicity analysis revealed the safety profile for all parts of the plants. All parts also showed a potential ACE inhibitory effect of greater than 50%, with the leaves showing the most significant effects, comparable to the conventional drug, Ramipril. We further investigated the effect of the hydro-methanolic leaf extract on oxidative stress, endothelial function, cardiovascular, renal and haematological parameters, associated with hypertension, in an L-NAME induced hypertensive rat model. The administration of the hydro-methanolic leaf extract of *Tulbaghia acutiloba* at different concentrations of 40, 60 and 80mg/kg b.w., reduced systolic, diastolic and mean arterial pressure in the model, with a pronounced effect at the dosage of 80mg/kg b.w. Additionally, the leaves of *T. acutiloba* significantly enhanced bradykinin receptor levels (B1 and B2), nitric oxide (NO) availability, promoted antioxidant activities and significantly reduced ACE activity in serum and cardiac tissues in hypertensive rats. Cardioprotection was significantly enhanced at 80mg/kg b.w. of *T.acutiloba,* as depicted by the cardiac function and morphology, and cardiac gene expression in experimental rats. There was no evidence of toxicity as depicted in the liver enzymatic activity after the administration of *T.acutiloba* in the hypertensive rats. Administration of *T. acutiloba* improved renal function as evidenced by the increased creatinine clearance (Ccr), improved

fractional excretion of sodium and decreased urine protein-creatinine ratio (UPr/UCr). Additionally, decreased levels of leucocyte infiltration, decrease in both, neutrophil to lymphocyte ratio (NLR) and lymphocyte to monocyte ratio (LMR), was found after administration of *T.acutiloba*, with a maximal effect occurring at a dose of 80mg/kg b.w. Together, these findings provide scientific validation for *T.acutiloba* as a medicinal plant that has cardioprotective and antihypertensive properties, and is able to improve renal function and haematological parameters in an L-NAME induced hypertensive rat model. Overall, the data also provides substantive evidence for the possible usage of *T.acutiloba* as an alternate antihypertensive agent, in resource limited areas where conventional drugs are inaccessible.

Key Words: *Tulbaghia acutiloba*, phytotherapy, cardioprotection, L-NAME, renal function, haematology

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Background

Over the last decade, the prevalence of hypertension has risen at a faster rate than recorded previously in both developing and developed countries, with dire outcomes. There is consistent evidence of the growing health and economic repercussions of hypertension and its associated complications, specifically in Africa (1). Distinctive efforts intended to focus on preventing and managing hypertension remains less than optimum (2). Hypertension (HTN) is a major risk factor for many cardio-renal diseases and remains one of the major causes of mortality worldwide (3). Several conventional treatment options are available to manage HTN, but remain inaccessible and unaffordable to many who need them, specifically in low- and middle-income countries, where the prevalence of HTN is increasing at a faster rate compared to the high-income countries (3). Current conventional regimens have a number of setbacks, as they present with severe side effects that may pose mild to severe discomfort to hypertensive patients when administered. There is therefore a focus to scale up access to affordable anti-hypertensive therapy that have minimal side effects in the global fight against the burden of hypertensive conditions and other non-communicable diseases (4).

According to the World Health Organization (WHO), there is a growing global interest and high demand for phytopharmaceutical products, with many pharmaceutical companies now focusing on producing phytotherapeutic products and herbs in the fight to reduce hypertensive cases (5). Reports from the WHO further suggests that the global non-communicable disease (NCD) goal for hypertension is a 25% relative reduction in prevalence by 2025 (5). Phytotherapy, which is scientifically proven to be of good quality, efficacy and safety, facilitates access to healthcare and has the added benefit of minimal perceived side effects (5). In Africa, it is estimated that the ratio of traditional medicine healers to the population is approximately 1:500, while clinicians have a ratio of 1:40 000 (6). As many medicinal plants have numerous bioactive compounds that work synergistically to produce antihypertensive and antioxidant effects, investigating regional medicinal plants will enhance access to affordable treatment (6).

In South Africa, it is reported that over 27 million people (more than 50%) rely on indigenous medicinal plants for healing of various ailments, including HTN, (7) with the scientific validation of existing and novel medicinal plants being essential for managing and treating hypertension.

1.2 Definition and Classification of Hypertension

Hypertension (HTN), or high blood pressure, has been defined as repeated elevated blood pressure of more than a systolic pressure of 140mm Hg and a diastolic pressure of 90mm Hg (>140/90mm Hg) (8). According to a report of the American College of Cardiology (ACC) and the American Heart Association (AHA) Task Force on Clinical Practice Guidelines on Prevention, Detection, Evaluation, and Management of High Blood Pressure in Adults, 2017, HTN is now defined as persistent rise in blood pressure of more than 130/90 mm Hg on at least three different occasions in a relaxed physiological state (9). This condition has been regarded as major risk for cardiovascular and renal diseases through its effect on target organs, such as the heart and kidneys (10). Based on repeated measurements of the systolic (SBP) and diastolic blood pressure (DPB), the ACC and AHA Task Force on Clinical Practice now classifies HTN as normal, elevated, stage 1 and stage 2 hypertension (Table 1.1)

Table 1: Categories of hypertension as defined by the American College of Cardiology and the American Heart Association Task Force (10)

BP Category	SBP		DPB
Normal	<120 mm Hg	and	<80mm Hg
Elevated	120-129mm Hg	and	<80mm Hg
Hypertension			
Stage 1	130-139mm Hg	or	80-89mm Hg
Stage 2	≥140mm Hg	or	≥90mm Hg
Hypertensive crisis	>180mm Hg	and/or	>120mm Hg

*SBP = systolic blood pressure**,** DBP = diastolic blood pressure

Source: the American College of Cardiology and the American Heart Association Task Force on Clinical Practice Guidelines on Prevention, Detection, Evaluation, and Management of High Blood Pressure in Adults, 2017 (10).

Known as a ‘silent killer’, HTN may exist for an extended period as an asymptomatic condition, which may gradually affect the quality of life of a person before symptoms begin to manifest (11). These usually occurs after a mild or serious complication has been caused by the influence of the persistent asymptomatic elevated blood pressure (12). These manifestations can include persistent headaches, fatigue, chest pains, irregular heartbeats and poor vision (12). Untreated HTN in the midst of these symptoms may lead to serious complications, such as stroke, heart attack, renal failure and blindness (13). Hypertension is broadly grouped under two main types based on the cause and disease progression namely: primary or essential hypertension and secondary hypertension (14).

1.2.1 Primary or essential Hypertension

This type of HTN accounts for approximately 95% of all hypertensive cases worldwide. Sometimes known as 'idiopathic hypertension', this form has previously been described as having an unknown underlying cause, although it is mainly attributed to hereditary and of a genetic origin (15). While many theories have tried to establish the specific cause of essential HTN, they have not been able to clearly define the pathway of the disease. Many researchers have therefore adopted a multifactorial approach to determining the cause of the disease (16). The elusive etiology of essential HTN makes it a critical factor for therapeutic intervention, as most treatments are targeted at the symptoms rather than the main cause (16). Studies have supported a natural progression in the disease process, implying that the initial rise in cardiac output and blood volume may set off successive increases in systemic vascular resistance, thereby resulting in increased blood pressure (15). Moreover, certain predisposing factors are known to initiate multiple complex genetic and molecular mechanisms that could lead to the rise in arterial blood pressure. Such predisposing factors include obesity, insulin resistance, high alcohol and salt intake, ageing, sedentary lifestyle, stress, low potassium levels, and a lack of fruit and vegetables as sources of antioxidants (17). It has therefore become necessary to incorporate a multidisciplinary research approach to explain the mechanism, progression and management of this multifaceted disorder, and hence the focus of our present study.

1.2.2 Secondary hypertension

Secondary hypertension (HTN) is due to a known cause, enabling an easier and effective intervention (18), the condition accounting for approximately 5% or less of all cases. Some of the conditions that could cause secondary HTN include, but are not limited to, pregnancy, certain medications and supplements, tumours, thyroid and hormonal disorders, such as Cushing's Syndrome (19).

1.3 Epidemiology of Hypertension

Despite concerted efforts by local and international agencies to reduce the prevalence of HTN globally, the rate remains unacceptably high at 22% (14), being estimated to cause 7.5 million deaths annually, representing 12.8% of the total deaths globally. It is estimated that one in every four men, and one in five women, globally has raised blood pressure, representing 22% of the adult population (20). This trends indicates that there was a rise in the number of adults with HTN from 594 million in 1975, to 1.13 billion in 2015, implying a growing rate of HTN with the increasing ageing population (1). The prevalence of hypertensive cases are known to be highest in Africa, with 27% of the adult population having high blood pressure (20). The high-income countries, such as the United States of America, have declining HTN prevalence from 25% to 18% of the adult population over the past two decades. These estimations are

predicted to decline with an increase in the availability of healthcare facilities, and more easily accessible and affordable therapeutic interventions (21).

Hypertension (HTN) is currently under focus in Africa, and is regarded as the most common cause of cardiovascular and renal disease, being a key contributor to high mortality and morbidity in adults (22). A prevalence rate of 30-32% is reported in most rural and middle-income urban areas in Africa. The studies show a 20.8% prevalence rate in Eastern and Southern Africa, and it is postulated that NCDs, such as HTN and its complications, may soon surpass communicable diseases as a major cause of death if left untreated (22). The greater risk of hypertensive cases among the Black African population should lead to a more intense investigation of HTN in Africa (23). In South Africa, it has been reported that one in three adults live with elevated blood pressure; accounting for one in every two strokes and two in every five heart attacks (24). In 2017, it was estimated that 42% to 54% of the adult population were living with HTN in South Africa, this figure being expected to rise if critical interventions are not implemented (25), necessitating the search for novel therapeutic interventions.

1.4 Aetiology and Pathogenesis of Essential Hypertension

The human body has several mechanisms to ensure that the blood pressure is maintained within a homeostatic range. Blood pressure is known to be a product of cardiac output (CO) and peripheral vascular resistance, and its control is therefore exerted by regulating one or two of these components (26). A rise in blood pressure develops as a result of disturbances of the components and mechanisms involved in its regulation, with an acute or chronic rise possibly involving the dysregulation of the cardiorenal, nervous and circulatory systems (27). Several contributing factors have been associated with interruptions in the functioning of the blood pressure regulation, leading to the pathogenesis of essential HTN. These factors include: the overproduction of reactive oxygen species (ROS); endothelial-derived constricting factors leading to endothelial dysfunction; genetic alterations, reduction in the bioavailability of nitric oxide (NO), dysregulation of the renin-angiotensin system (RAS) or renin- angiotensin aldosterone system (RAAS), increased activation of the sympathetic nervous system, renal sodium and water retention, variations in the kallikrein-kinin system expression, chloride and potassium homeostatic imbalance; haematological disorders and environmental factors, such as chronic stress, alcohol intake and physical inactivity (28)

1.5 The role of the Renin-Angiotensin System in the pathogenesis of Hypertension

1.5.1 The Classical RAS

The renin-angiotensin system (RAS) functions as a key modulator to regulate blood pressure in both the physiological and pathological states (29). The production of renin from the juxtaglomerular apparatus (JGA) cells of the kidney is the initial step for regulating blood pressure. Renin, produced as a circulatory enzyme, is involved in maintaining the extracellular volume and arterial integrity (30) in the hydrolytic catabolism of angiotensinogen, which is produced and secreted by the liver cells. The hydrolyses of angiotensinogen by renin leads to the formation of a decapeptide angiotensin I (Ang1), which is further cleaved by the angiotensin converting enzyme (ACE) and primarily situated in the pulmonary circulation that is bound to the endothelium (31). The cleavage of Ang I by ACE leads to the hydrolyses of Ang I to form the most vasoactive peptide, angiotensin II (Ang II), which activates the G-protein coupled angiotensin II receptor type 1 (AT1) and increases the peripheral vascular resistance, leading to vasoconstriction and a subsequent rise in blood pressure (32). The effects of angiotensin II are also mediated by another receptor, AT2, which has been demonstrated to be abundant in foetal tissues, although adult cardiac and renal tissues have recently been reported to be actively involved in its activities (33).

Ang II also acts on the adrenal gland, activates the stimulation of aldosterone, which causes the epithelial lining of the kidneys to increase the re-absorption of sodium and water, thereby increasing blood volume (34). It is also known to increase vasopressin release through the activation of the pituitary gland (34). The local tissue production of Ang II in the heart, brain, kidneys, adrenals and blood vessels is regulated by ACE and other key enzymes. The activity of RAS at the local level plays a crucial role in cardiovascular and renal remodeling in HTN (35).

1.5.2 The Novel RAS

The classical view of the RAS presents a direct signalling pathway that begins with the conversion of angiotensinogen to Ang I by renin, as described earlier. For the past few decades, the classical RAS has been considered to be active in circulation, with the vasoactive Ang I exerting various mechanisms in the pathophysiology of HTN (35). However, the RAS has become much more complex, with the discovery of novel peptides that demonstrates several biological effects (36). As RAS is also locally produced in a number of tissues, such as the kidneys and heart, there are novel pathways of Ang II expression that are independent of angiotensin converting enzyme (ACE) (36). In 2000, ACE2 was discovered, with characteristics of an enzyme similar to ACE, the description of a homologue for ACE called ACE2, building on the emerging premise of complexity (37). While the classical ACE converted Ang I to Ang II, ACE2 results in Ang (1-9) formation and Ang (1-9) can be converted to Ang (1-7), which acts as a

vasopressor agent. The ACE2 local expression correlates with Ang (1-7) concentration and leads to the antagonism of Ang II. Therefore, ACE inhibition may increase Ang (1-7) levels and reduce Ang II (38), with ACE2 being mainly expressed in the heart and kidney, and to some degree in the vascular endothelium (38).

Almost all pathophysiologic and physiologic functions that are Ang II induced, such as vasoconstriction, tubular transport stimulation, aldosterone release, growth stimulatory actions and proinflammatory effects, are mediated by AT1, although information regarding the exact role of AT2 is not clear (39). It has been postulated that the AT1 blockade may result in Ang II formation and increased concentration, with free Ang II binding to AT2, AT3 and AT4. While there is little information regarding AT3 and AT4 stimulation, they have been suggested to promote thrombosis (40). Unlike ACE, with ACE 2, there is no conversion of Ang I to Ang II, and the ACE inhibitors do not inhibit its enzymatic activity directly. Thus, ACE2 is an inhibitor in Ang II formation by stimulating alternate pathways for the degradation of Ang I, which may lead to a possible vasodilatory effect (40, 41).

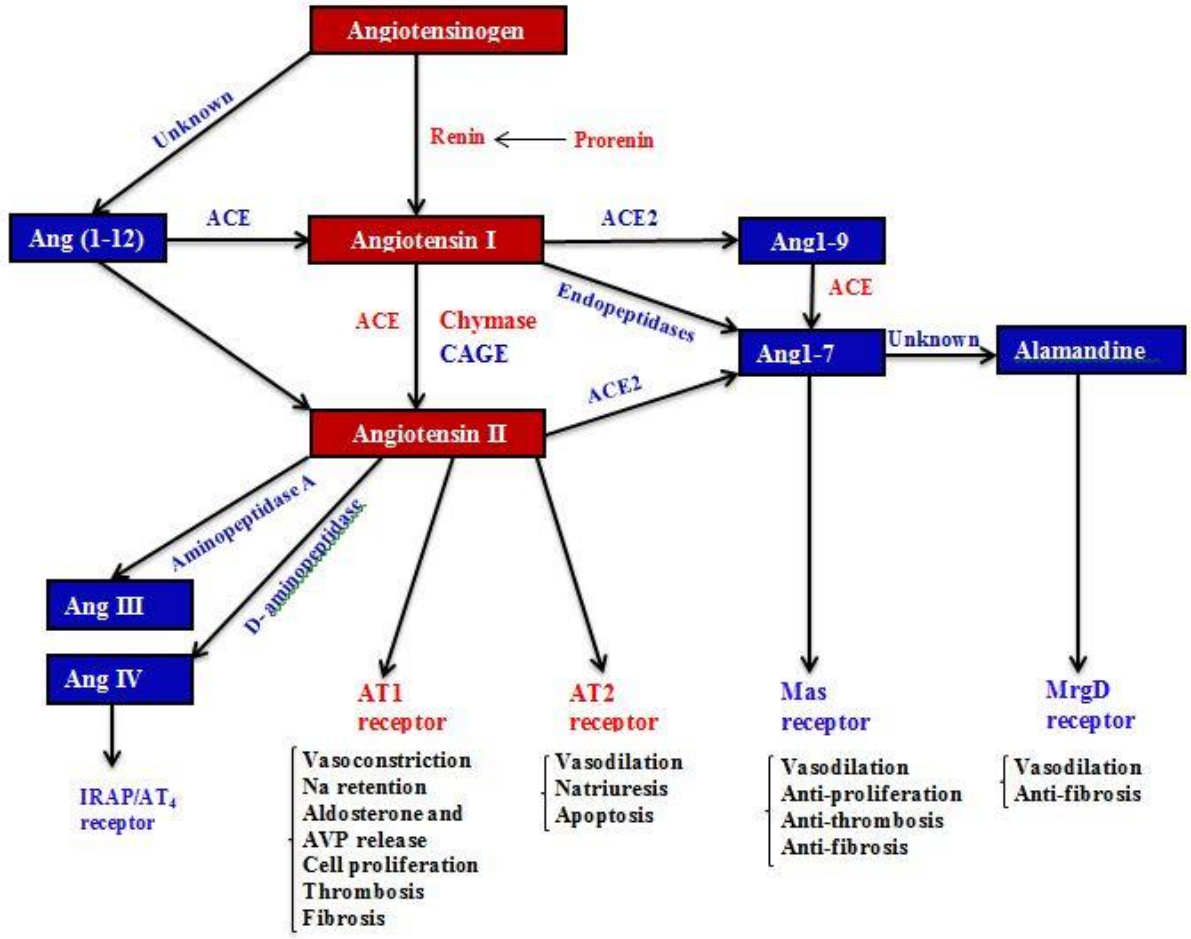

Figure 1: Representation of the classical (RED) and alternative pathways (BLUE) of the renin-angiotensin system (RAS) and their interactions. ATI, angiotensin II type 1; AT2, angiotensin II type 2, NO, nitric oxide, CAGE, chymostatin sensitive AngII-generating enzyme. (Adapted from (42-44)

1.6 The Role of the kallikrein-kinin system (KKS) in the pathogenesis of Hypertension

An imbalance in the endogenous vasoconstrictor and vasodilator systems may lead to the development and maintenance of high blood pressure (41). Kinins (bradykinin, lys-bradykinin and bradykinin receptors) are classified as endogenous vasodilators, and are known for antagonising angiotensin II-induced vasoconstriction and sodium retention (45). Bradykinin and lys-bradykinin are known to act mainly through the bradykinin type1 (B1) and type 2 (B2) receptors. Studies have suggested that the bradykinin type 2 receptor (B2) is the main inducer of changes in the cardiovascular and renal function (46). The beneficial effect of kinins in renal and cardiovascular diseases is mainly mediated through the bioavailability of nitric oxide and prostaglandins, extending their role to cardioprotection and nephroprotection (47). Importantly, the angiotensin-converting enzyme (ACE), which catalyses the conversion of angiotensin I to angiotensin II, is also known as kininase II, and is responsible for the

degradation of bradykinin into inactive metabolites (48). This implies that high serum ACE activity will result in the inactivation of bradykinin and a reduction in its receptor activity, hence leading to the vasoconstrictive and end organ damaging activities of uncontrolled Ang II (46). In the human body, therefore, ACE inhibitors are known to lower blood pressure and enhance the activity of the kallikrein-kinin system (KKS), leading to the accumulation of kinin in circulation and target tissue organs (47). Therefore, development of novel therapeutic interventions to augment the kinin activity in the vasculature and specific compartments in the heart and kidney may be a highly effective approach to manage and treat HTN and its complications.

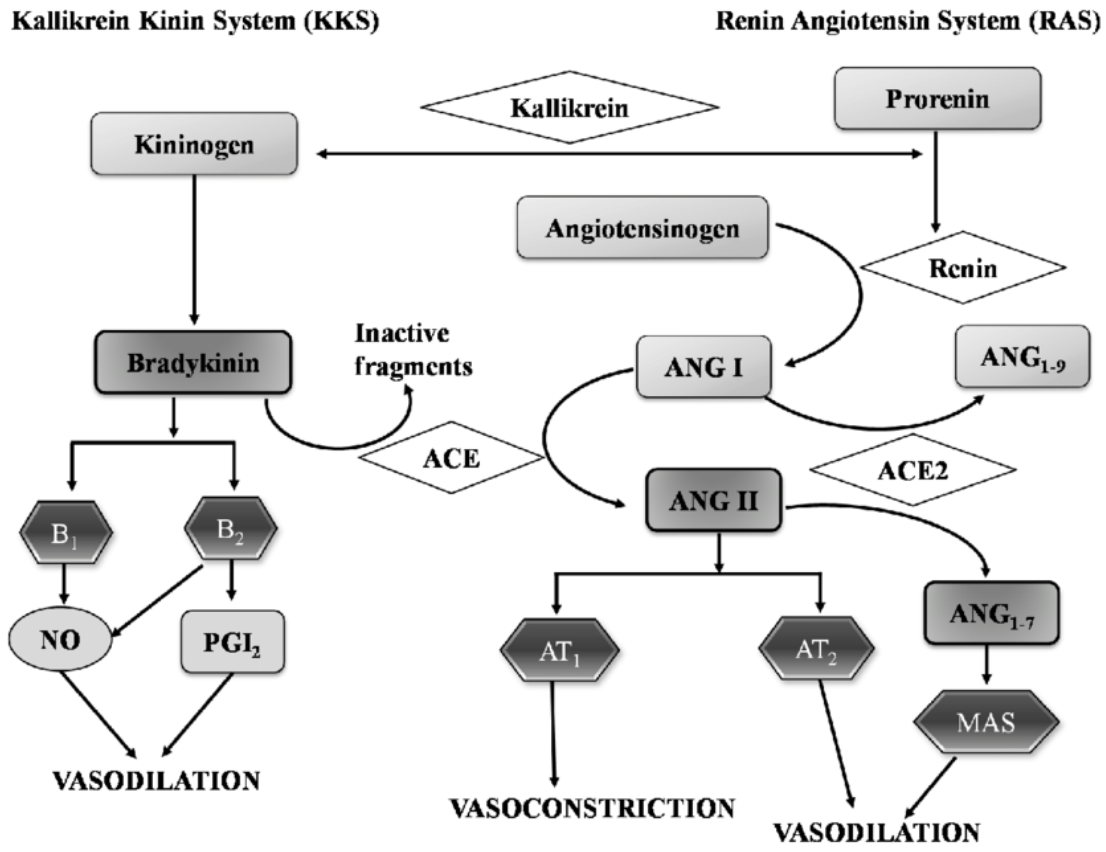

Figure 2: Renin-angiotensin system and kallikrein-kinin system in the regulation of blood pressure. Angiotensin I (Ang I), Angiotensin II (Ang II), Angiotensin converting enzyme (ACE), Angiotensin converting enzyme 2 (ACE 2), Angiotensin receptor 1 (AT1), Angiotensin receptor 2 (AT2), Bradykinin receptor 1 (B1), Bradykinin receptor 2 (B2), Nitric oxide (NO), Prostaglandins 2 (PgI2). (Adapted from Majumder and Wu, 2015 (49).

1.7 Endothelial dysfunction in Hypertension

Endothelial dysfunction, defined as the biological and physical alteration of the vascular endothelial lining, is characterized by the release of pro-constrictive, pro-inflammatory and pro-thrombotic agents (50). Essential HTN is associated with an imbalance between vasodilators and vasoconstrictors, which upsets the integrity of the endothelial cells, leading to a vicious cycle that may result in the maintenance of high blood pressure (51). Reports indicate that endothelial activation and injury could lead to the alteration in vascular reactivity and tone, fibrinolytic pathways, as well as coagulation. These alterations are reliable indicators in the prognosis of HTN and end-organ damage (51).

Evidence from research over the past few decades has identified nitric oxide (NO) as a central modulator of vascular endothelial function, and while it is highly versatile, it is a structurally simple molecule that plays a key role in various activities in the vasculature (52). It is known to regulate vasodilation, the permeability of the vascular bed and platelet activity. Endothelial cell-derived NO, at an optimum level, is known to cause vasodilation and inhibition of platelet activity in the blood vessels through the activation of soluble guanylyl cyclase, which, coupled with a decrease in cytosolic Ca^{2+} concentration, facilitates the biosynthesis of 3,5-cyclic guanosine monophosphate (cGMP) (53). The decrease in cytosolic Ca^{2+} concentration is caused by the ability of NO to inhibit the voltage-gated Ca^{2+} channels and the activation of protein kinases that phosphorylates proteins in the sarcoplasmic reticulum. The release of cGMP, coupled with a decrease in cytosolic Ca^{2+} concentration, facilitates the inhibition of calcium-calmodulin myosin light chain kinase in vascular smooth muscle cells, which further promotes vasorelaxation and platelet inhibition (54). The biosynthesis of endothelial NO increases in response to external and internal stimuli, such as serotonin, bradykinin, acetylcholine, adenosine disphosphate (ADP) and shear stress. Reports indicate that acute or chronic inhibition of NO synthesis in the endothelial lining could lead to hypertension, myocardial ischaemia or stroke (54). Recent studies have also shown that the excessive elevation of NO may be associated with the general cytotoxicity, and its maintenance in its accepted cellular range is therefore crucial for therapeutic targets and endothelial function (55).

Adequate NO production may not be able to meet the target of protecting the vascular integrity due to the presence of reactive oxygen species. Several reports from studies suggest that oxidative stress caused by reactive oxygen species (ROS) alters the biological functions of the endothelium through their inactivation of endothelial-derived NO (56). An increase in oxidative stress that is caused by superoxide (O_2^-), hydroxyl radical (OH^-) and other reactive oxygen species (ROS) increases the NO destruction, hence reducing its physiological activities in the endothelium. There are further indications that Ang II promotes the formation of the oxidant, superoxide (O_2^-) at concentrations that affect blood pressure

minimally (57). Previous reports show that despite the presence of oxidative stress, the activities of NO could be enhanced by administering a potent antioxidant, such as superoxide dismutase (SOD) and reduced glutathione (GSH), which are known to effectively scavenge oxidants, i.e. superoxide and hydroxyl radical, to restore the endothelial function (58).

There is, however, a controversial viewpoint on whether endothelial dysfunction remains a cause or an effect of HTN. Some studies have shown a bi-directional relationship, with the evidence indicating that endothelial dysfunction leads to HTN, while others suggest that this could be a risk factor for HTN (59). Despite this, there is a clear relationship between HTN and endothelial dysfunction, although the exact mechanism to establish cause and effect needs to be ascertained through further investigations (59). Based on the possible therapeutic benefits of an enhanced endothelial function in hypertensive patients due to the bioavailability of NO, a reduction of blood pressure, antioxidant activity and other measures to improve the vasculature; the current studies focus on assessing the effective mechanism of novel antihypertensives, which promotes NO production, coupled with antioxidant activity (60). There is a growing interest in investigating the role of ACE inhibitors, which are known to lower blood pressure, increase NO production, and also have therapeutic and antioxidant effects on the endothelial function (61).

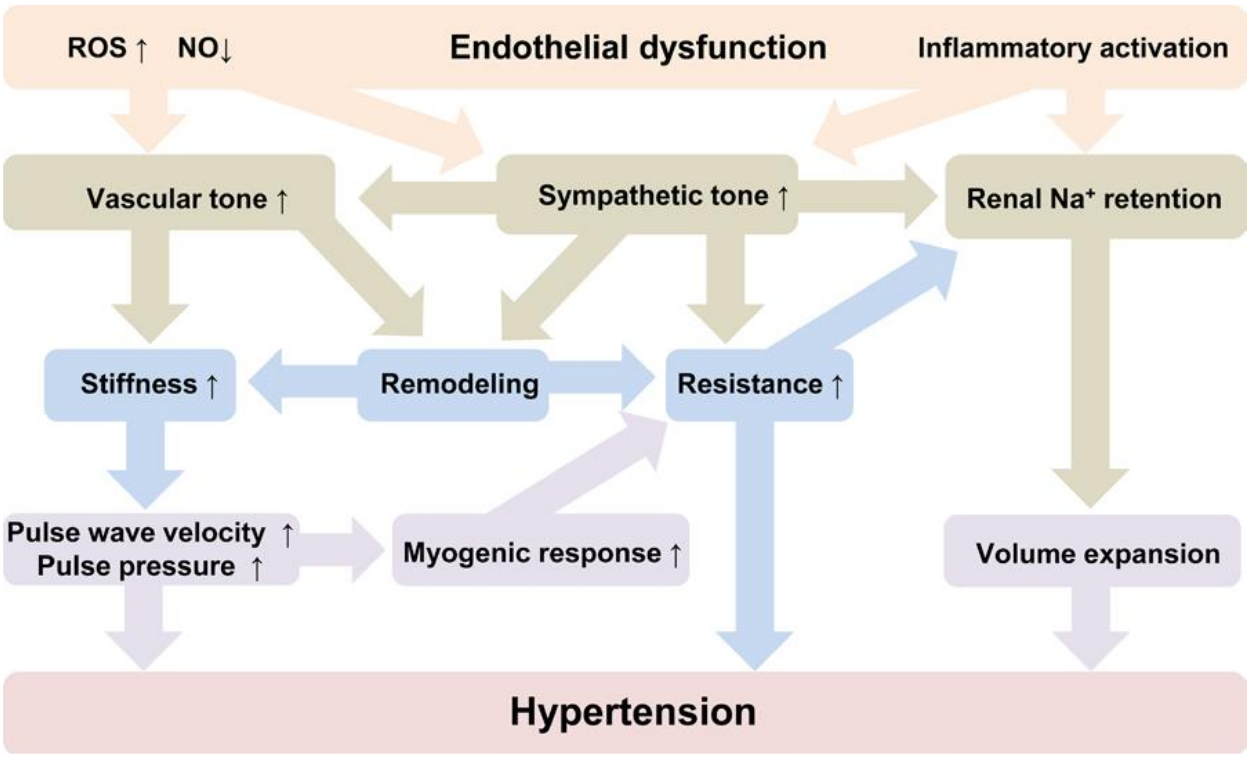

Figure 3: Pathways from endothelial dysfunction (ED) to hypertension. The link between ED and hypertension is clearly illustrated with oxidative stress and inflammation as the key role players. ROS indicates reactive oxygen species. (Adapted from Brandes, 2014(62**).**

1.8 Haematological Indices in the Pathogenesis of Hypertension

In recent years, there has been a growing interest in the role of haematological indices in the pathogenesis of HTN, with the exact mechanism and relationship between them being under investigation and the clear directives yet to be ascertained (63). It has been suggested that oxidative stress and decreased endogenous NO concentrations, associated with a dysfunctional endothelium, could lead to a number of functional and structural abnormalities of organs, that are involved in the hematopoietic process during HTN (64). The kidney is one of the major organs involved in the hematopoietic process, as the active hormone, erythropoietin, associated with the production of red cells, is produced in the kidneys. Reports indicate that renovascular HTN is accompanied by abnormalities of red blood cells that may adversely impact the microcirculation and exacerbate end-organ damage (65). Haematocrit levels have been identified as being generally higher in hypertensive patients, and the occurrence of HTN and stroke, are known to double with every 10% increase in the haematocrit level (65). The mechanism behind blood pressure and haematocrit is most likely related to the blood viscosity that is associated with peripheral resistance during HTN. Higher blood viscosity is known to affect the peripheral resistance to blood flow, and this increased resistance could lead to raised blood pressure (66). Although the specific pathways for this relationship are unclear, it is hypothesized that the reduction in red cell deformability, and an increase in

the aggregability, size and numbers of red blood cells, may cause a circulatory defect that could lead to target organ damage (67). It is also suggested that increased haemoglobin levels may lead to left ventricular hypertrophy, while low haemoglobin levels could cause anemia and heart failure, therefore compounding the effect of HTN (65).

As a chronic inflammatory condition, HTN is also known to be associated with elevated white blood cells (leucocytes) in vessels. Several studies have reported on the activation and infiltration of leucocytes in the vascular bed, as an indication of tissue ischaemia and vascular injury, in the pathogenesis of HTN (68). Leucocytes are also reported to play a major role in the initiation and progression of atherosclerosis and its deleterious effect on endothelial function. The infiltration of leucocytes in the blood vessels is known to be enhanced by the activation of inflammatory genes, such as NF-κB, Heme Oxygenase-1 (HO1), and increased activation of AT1and AT2 receptors (69). Current research has indicated that the ratio of specific white blood cells differentials may be a better marker in the progression of HTN than individual differentials. The ratios of differential white blood cell markers, such as neutrophils, basophils, lymphocytes and monocytes, have recently been identified as a novel yet inexpensive method of identifying the prognosis and pathogenesis of HTN. Such ratios include the neutrophil-lymphocyte ratio (NLR), lymphocyte-monocyte ratio (LMR) and the platelet-lymphocyte ratio (PLR) (70).

Endothelial dysfunction during HTN may also lead to platelet activation due to vascular complications. During vascular injury, activation of platelet at the injured site leads to the over production of platelets, which could result in coronary thrombosis and myocardial infarction (71). Haematological findings during HTN are generally considered a strong indicator for the prognosis and the pathogenesis of HTN. The number of researchers, who aim to identify therapeutic agents that possess the potential of improving relative haematological functions during HTN, have risen recently. Their findings will lead to an improved understanding the role of haematological parameters to treat and manage essential HTN (72).

1.9 TREATING AND MANAGING HYPERTENSION

A number of pharmacologically active compounds are conventionally used to treat and manage HTN, these drugs are generally being classified into three main groups, namely: (1) Direct vasodilators (2) Adrenergic inhibitors and (3) Diuretics (73).

1.9.1 Direct Vasodilators

This group of antihypertensive drugs acts directly by inhibiting the ACE, calcium channel blockade or angiotensin receptor blockade.

1.9.2 Angiotensin Converting Enzyme Inhibitors

Studies have established the crucial role that the ACE plays in the formation of Ang II, a potent vasoconstrictor (74), and their key role having been extensively investigated. ACE inhibitors are known to be the first line of therapy in most hypertensive cases (75). The mechanism underlying the lowering of blood pressure by ACE inhibitors, is by reducing the circulating levels of Ang II (76). Simultaneously, the activity of ACE in the vessel walls and various tissues, including the kidney and heart, are known to be inhibited to varying degrees by diverse ACE inhibitors. They also inhibit the breakdown of bradykinin, and together with the kinin, stimulate NO production, leading to a direct vasodilatory effect and an improved endothelial function (77). In addition, ACE inhibitors lessen the anticipated rise in the sympathetic activity observed after vasodilation, and results in a decreasing heart rate and cardiac output (77).

Additionally, studies show that the multiple effects of ACE inhibitors could lead to dampening the arterial wave reflection and increasing the aortic distensibility. The dynamics of blood flow in managing HTN with ACE inhibitors contributes to the reversal of hypertrophy in the heart (78). This action of ACE inhibitors surpasses that of other antihypertensive agents, therefore making it a drug of choice in preventing end-organ damage associated with HTN (78). A large clinical trial supports the use of ACE inhibitors for all patients who are at a higher risk of developing various heart diseases and renal abnormalities associated with hypertension. Apart from their haemodynamic effects, they are also able to prevent interstitial collagen deposition (79).

Side effects associated with ACE inhibitors

Despite its beneficial role in preventing end-organ damage, conventional ACE inhibitor, such as Captopril, Ramipril, Lisinopril and Enapril have a number of disadvantages, as they have various side-effects (80). A dry hacking and sometimes obstinate cough is the most common side-effect reported with ACE inhibitor therapy, which is suggested to be caused by the increase in kinin levels. Other reported

side effects include increased blood-potassium level (hyperkalemia), fatigue, dizziness, persistent headaches and a loss of taste. Studies have also linked conventional ACE inhibitors to loss of libido as a side-effect (81).While these side-effects, such as coughing, could be managed with inhaled sodium cromoglycate and aspirin, it would be desirable to replace ACE inhibitors with potent therapeutic agents that have minimal side effects.

1.9.3 Calcium channel blockers

The antihypertensive mechanism of calcium channel blockers is by binding to the L-type high voltage calcium channels, leading to an inhibited influx of calcium ions in vessels, causing vasodilation (82). Previous studies also indicate that calcium channel blockers may increase NO bioavailability and hence improve endothelial function (83). Drugs in this antihypertensive category include Nifedipine, which is known to exert an effect on blood vessels and a lesser action on cardiac output (84). Due to the important role of calcium in several cellular functions, studies have raised concerns over the potential adverse effects of blocking these channels (84).

Side effects associated with calcium channel blockers

Common side effects associated with calcium channel blockers include excessive hypotension, headache, tachycardia and oedema. Studies have also reported excessive bradycardia and a reduction in the electrical conduction (85).

1.9.4 Angiotensin receptor blockade

Angiotensin receptor blockers (ARBs) are known to displace ang II from its target receptor, angiotensin II receptors (AT1), in blood vessels, thereby antagonizing its effect (86). Reports indicate that ARBs cause a decrease in aldosterone production, leading to increased sodium excretion (87). Although both act on the renin-angiotensin system (RAS), the major disparity between ARBs and ACE inhibitors is that, unlike the latter, the former do not increase kinin levels (88), their increase possibly, a key factor accounting for some of the beneficial effects of ACE inhibitors over ARBs (88).

Side effects associated with angiotension receptor blockade

Current treatment guidelines for HTN recommends a combined use of ACEIs and ARBs, especially in cases where patients are intolerant to the persistent dry cough caused by ACEI (89, 90). This combination has been shown to offer additional benefits to managing HTN. However, angioedema and ageusia have also been reported as a side-effect of ARBs (89).

1.9.5 Adrenergic inhibitors

This group of antihypertensive drugs acts by several different mechanisms (91), including by inhibiting the sympathetic nervous system, thereby leading to a decreased force and rate of cardiac contractility (92), and a reduction in peripheral vascular resistance that decreases blood pressure (93). Beta-adrenergic blockers are known to act on the renin angiotensin system (RAS) by decreasing the renin production in the juxtaglomerular cells of the kidney (91).

Side effects associated with adrenergic inhibitors

Common side-effect reported in the usage of adrenergic inhibitors include orthostatic hypotension, ejaculatory disorders, and a severe hepatotoxicity that could be life threatening (94). In recent times, adrenergic inhibitors are rarely used to treat HTN due to its life threatening side-effects, which could increase mortality in hypertensive cases (94). It is therefore recommended that patients on adrenergic inhibitors be monitored closely for any lethal effect (95).

1.9.6 Diuretics

Diuretics form a common initial therapeutic strategy for most hypertensive cases, despite a decline in their use in the early 1990s. The short-term use of diuretics has been reported to induce natriuresis, with a simultaneous reduction in blood volume, hence reducing cardiac output (96). Moreover, the evidence suggests that chronic use of diuretics may also lead to a reduction of peripheral vascular resistance and therefore decreasing blood pressure (97). Most published works recommend thiazide-type diuretics as the first-line therapy, in cases where specific antihypertensive agents are not indicated, which act on the distal tubules of the kidney where they increase urinary excretion of sodium and potassium (98).

Side effects associated with diuretics

Common side effects reportedly associated with thiazide-duiretics include hyperlipidemia, hyperglycaemia, hypokalaemia, glycosuria and hyperuricaemia (99). The need for diuretics has been shown to be lessened with the use of ACE inhibitors and ARBs, which alters the renin-angiotensin-aldosterone mechanism with a natriuretic effect (100).

1.10 ANIMAL MODELS USED IN EXPERIMENTAL HYPERTENSION

A number of experimental animal models have been developed that mimic the pathophysiology and mechanism of HTN in humans, most of which share many features with HTN in humans (101). These models have suitable features to better understand the aetiology and progression of HTN, as well as to pharmacologically screen potential antihypertensive drugs for better treatment options. The aetiological factors that contribute to human HTN (102) include excessive salt intake, genetic predisposition, hyperactivation of the renin-angiotensin system (RAS) and nitric oxide bioavailability (102).

Studies have shown that a single animal model may not have all the answers to the pathogenesis and treatment of HTN, as its aetiopathology is multifactorial (103, 106). However, each model explains a particular pathway for the progression and treatment of HTN, and the studied model is therefore associated with the aetiology and treatment of interest (103). The currently preferred animal model for HTN is the rat (104, 105). As essential HTN is the most common presentation of HTN in humans, and is generally associated with a genetic and pharmacological interest, most experimental models are developed for essential HTN (106). Examples of such models include the spontaneous hypertensive strain (SHR), Dahl-salt sensitive strain, and Milan, Lyon and Sabra strain. Among these rat models, the SHR has been generally used, although it explains only a specific type of HTN involving mainly the RAS (101).

1.11 Chronic nitric oxide inhibition-induced Hypertension: The L-NAME model

Studies have demonstrated several roles for NO in the development and treatment of HTN. As a vasodilator, NO has been demonstrated to be involved in several pathways involved in the pathophysiology of essential HTN (107). A deficiency of nitric oxide synthase (NOS) leads to a reduction of NO, causing reduced endothelium-dependent vasorelaxation, and eventually leading to HTN. As NO is involved in several pathways and mechanisms leading to essential HTN, it is a major focus area in hypertensive research (108). The chronic inhibition of NO synthesis with *N^{ω}-nitro-L-arginine methyl ester* (L-NAME), has great potential for elucidating a number of mechanisms involved in the development and treatment of essential HTN (108).

The L-NAME induced hypertensive rat model has been shown to involve more than the removal of the tonic vasodilatory action of NO, as there is also evidence for contributions from the RAS, prostaglandins, sympathetic nervous system (SNS), superoxide anions, oxidative stress and the kallikrein-kinin system in the pathology of hypertension (109). It is therefore currently one of the main models for cardiovascular and renal studies associated with essential HTN (110), as NO has been implicated in a number of

pathological conditions that are associated with cardio-renal HTN, especially cardiac hypertrophy, end-organ damage and inflammatory processes (111). Indeed, NO has been demonstrated to also inhibit monocyte adhesion, platelet function and vascular smooth muscle proliferation (112). Reports also show the involvement of NO in the haematological alterations during HTN (113). Given that drugs are more likely be highly efficient in the prevention and reversal of early, rather than late, vascular alterations in HTN; this model could be most appropriate for testing vascular protective effects and renal effects of antihypertensive drugs, in the context of NO deficiency (114).

1.12 Medicinal and Nutritive Plants in Hypertension

Medicinal plants generally refers to the whole or parts of plants or herbal materials that contain one or more active compounds, that are essential for therapeutic purposes, or that could serve as an essential ingredient in drug development (115).They possess various phytochemicals, and have unique nutritional value, that may show potential biological and therapeutic effects. Current research into the treatment of chronic diseases, such as HTN, has focused largely on the efficacy of medicinal plants, especially in Africa, due to the high cost and inaccessibility of the conventional pharmaceutical drugs. Additionally, conventional pharmaceutical drugs have a number of side effects and setbacks that make them difficult to adhere to (115, 116). The WHO, in its strategic goal to combat chronic diseases, such as HTN, supports the discovery of a novel therapeutic agent, that could effectively reduce systemic blood pressure with prolonged duration of action, to allow for once-daily dosing, as well as to reduce adverse reactions or untoward metabolic effects, to reduce end-organ damage (117, 118).

Unlike medicinal plants, conventional drugs may contain a single active compound that works to produce a pharmacological effect, with its several side effects and contraindications with adverse reactions (119). Medicinal plants, on the other hand, are known to contain a number of bioactive compounds that work synergistically to produce a net therapeutic effect, with minimal side effects in most cases (120). Studies have shown that most plants produce a better therapeutic effect, when consumed in their crude form by indigenous populations, due to the presence of multiple bioactive compounds (119, 121). Moreover, these bioactive compounds could also be precursors to novel drug development, as 40% of conventional drugs are plants-derived (119).

According to Van Wyk and Gericke (2000), local medicinal plants represent almost one eighth of the African plant population, many of which can provide important leads for new drug discoveries that have not been investigated (122). Due to the renewed interest in medicinal plants, they are universally being recognized as beneficial to the economic, social and health needs of societies (123). Both local and

international initiatives are currently exploring South African botanical resources, with a focus on screening indigenous plants to identify the pharmacologically bioactive compounds (124). In the KwaZulu-Natal (KZN) Province, the Traditional Health Services are supported with more than $30 million USD annually, due to medicinal plants. An estimated 700 000 tons of plant material is consumed annually in South Africa, amounting to almost $150 million USD (125). The recognition and evaluation of medicinal plants in South Africa, and Africa, aims to improve the availability, efficacy, safety and affordability (126). In Africa, it is estimated that, approximately 90% of the population still rely exclusively on medicinal plants due to their efficacy, accessibility and affordability. South Africa has a strong history relating to traditional healing and hosts a variety of about 24 000 temperate and tropical flora, which also accounts for approximately 10% of the higher plant species, globally (127).

1.12.1 Tulbaghia acutiloba Harv.

Tulbaghia acutiloba Harv. (Alliaceae) is a small, cluster forming, bulbous perennial herb that is found mainly in the Southern African countries, such as Botswana, Lesotho, Swaziland, South Africa and Zimbabwe (128). The pointed petals of this plant species confer the name '*acutiloba',* which in latin means 'sharply pointed lobes'. It has sweet fragrant flowers with garlic scented edible leaves, and grows best in loamy soil, all year round (129). In South Africa, it is mainly located in the Eastern Cape, KwaZulu-Natal, Free State and Gauteng Provinces, where it is used by the indigenous populations for several purposes (130). It is also known as 'wild garlic in English, 'isihihi' in Xhosa, 'wildeknoffel' in Afrikaans, 'ishaladi lezinyoka' in isiZulu, and 'motsuntsunyane, sefothafotha' in Sesotho (131). *Tulbaghia acutiloba* is one of the very few members of the family Alliaceae that has not been investigated biologically especially in an *in viv*o model, despite its various therapeutic uses.

1.12.2 Traditional and Nutritive uses

Tulbaghia acutiloba is mostly used therapeutically, as food, or as a protective ornament. In traditional medicine, it is used to treat diseases and conditions such as HTN, stroke, chest pains, diabetes, infectious diseases, flu, halitosis as well as an aphrodisiac (132). The leaves and the roots are used in the form of a decoction or extraction using alcohol, and in the case of some infectious diseases, may be used as a poultice (132). As a food, the leaves and flowers of *Tulbaghia acutiloba* are found in the diet of most indigenous people as a culinary herb. It is believed that by eating the leaves as food, it prevents the development of chronic and infectious diseases (133). *Tulbaghia acutiloba* is also known to repel snakes and other poisonous reptiles, and they are therefore planted around houses as repellants. This practice is based on the belief that snakes and other poisonous reptiles, dislike the intense aromatic smell produced in the plant's leaves and flowers (134).

1.12.3 Phytochemistry and biological activity

In spite of a number of reports on the biological, pharmacological and phytochemical profiles of various plants of the *Alliaceae* family, there is a dearth of information on the scientific validation of the phytochemical profile, antihypertensive, cardioprotective, hematological and renal effects of *Tulbaghia acutiloba*. Generally, the presence of sulfur, flavonoids, phenols and saponins in the plants of the *Alliaceae* family, have been previously reported, although each plant may possess distinctive phytochemical components and biological activities that may augment its therapeutic effects (135, 136). Moreover, there is a dearth of information regarding the antioxidant and biological activities of *Tulbaghia acutiloba,* and hence the need to confirm its usage both *in vitro* and *in vivo*.

1.13 Motivation and rationale of the study

Natural product research is an exciting platform for novel drug discovery initiatives in the pharmaceutical industry. Hypertension, a growing public health threat, could have severe repercussions on the economy and the disease burden of South Africa and the region. Medicinal plants still constitute a considerable reservoir of new compounds and therapeutic options. As a treatment and preventive option, phytotherapy is readily accessible, localized and presents with minimal side effects. Amongst the 24 000 variety of species found in South Africa, less than 10%, have been subjected to thorough scientific and pharmacological investigations (123). Despite the lack of scientific evidence on the efficacy of medicinal plants, the general public uses herbal preparations to meet their primary health care needs. Reliance on herbal preparations without any scientific validation has potential health risks, as they may contain harmful substances. Biological testing for cytotoxity, chemical screening and functional studies are important to validate the use of the compounds. It is within this framework that this study was conducted, as there is little or no scientific validation of *Tulbaghia acutiloba*, a plant that has great potential, given that it is categorised within the well-known Alliaceae family

1.14 Aim of the study

This study aimed to investigate the chemical composition, and the *in vitro and in vivo* antihypertensive activities of the extracts of *Tulbaghia acutiloba* Harv. in a hypertensive experimental model, to understand the biological activity and therapeutic benefits of the plant extracts.

1.15 Study objectives

The objectives of the study (Chapters three, four and five) were:

1. To identify the phytochemical constituents, *in vitro* antioxidant potential and analyse the heavy metal toxicity of the crude extracts of *Tulbaghia acutiloba*

2. To evaluate the *in vitro* angiotensin-converting enzyme (ACE) inhibition potential of the crude extracts of *Tulbaghia acutiloba*
3. To evaluate the antihypertensive and cardioprotective effect of *Tulbaghia acutiloba* extracts in an L-NAME model of hypertension
4. To examine the effects of *Tulbaghia acutiloba* extract on renal function in an L-NAME model of hypertension
5. To determine the effects of *Tulbaghia acutiloba* extract on heamatological indices in an L-NAME model of hypertension

1.16 Brief overview of methodology and study design

To accomplish the objectives, standardized laboratory methods and treatment protocols were strictly adhered to, as illustrated in Chapters 3, 4 and 5. Animal experimental work was conducted as per the approval of the Animal Ethics Committee of UKZN (Ref: AREC/062/016PD) according to the guidelines of National Institutes of Health for the Care and Use of Laboratory Animals, South Africa.

1.17 Potential benefit of this research

This study is among the first to showcase the scientific validation of *Tulbaghia acutiloba,* and unravelling the pharmacological significance of this plant, offers new avenues in the search for improved therapeutic approaches for essential hypertension. Therefore, the outcomes from this study are novel and also contribute to the global understanding of the pathophysiology and treatment of essential hypertension and its associated complications, which will serve as a precursor for the possible development of drugs within WHO approved recommendations and guidelines.

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Chapter 2: Manuscript One

Ethnopharmacological Review of South African Indigenous Plants for Angiotensin Converting Enzyme (ACE) Inhibitory Activity

(Submitted for peer review: Indian Journal of Traditional Knowledge, Manuscript Number: 9687)

This chapter reviews South African Indigenous plants with ACE inhibitory activities within the context of their phytochemical profile and biological activities. This formed the background to evaluate *Tulbaghia acutiloba* for its therapeutic functions.

Formats used in this chapter are according to the Journal specifications

Ethnopharmacological Review of South African Indigenous Plants for Angiotensin Converting Enzyme (ACE) Inhibitory Activity

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Abstract

South Africa has one of the highest prevalence of hypertension, with over 6 million people diagnosed as hypertensive. This being a major factor for cardiovascular diseases, needs to be critically managed to diminish the disease burden. Angiotensin converting enzyme (ACE) plays an imperative functional protagonist in regulating blood pressure by converting angiotensin I to angiotensin II, a robust vasoconstrictor. A review of traditional plants involved in ACE inhibition was conducted with evidence from published scientific journals, books and conference proceedings. Despite the availability of pharmaceutical drugs, underprivileged, predominantly rural sectors in South Africa facing socioeconomic challenges have opted for cost effective herbal concoctions with fewer perceived side effects. Whilst a number of plants and their formulations are used traditionally to treat hypertension, more scientific studies are needed to authenticate the efficacy and toxicity of these herbal remedies.

Keywords: Hypertension, Cardiovascular disease, Indigenous medicine, Renin Angiotensin System.

Introduction

Cardiovascular disease (CVD) is a rapidly growing multifactorial disease and major public health threat, despite the advancements in research made over the past few decades[1]. Hypertension is still the major leading risk factor of cardiovascular diseases, with serious and sometime fatal outcomes, accounting for 16.5% of annual global deaths[2]. Previously hypertension was defined as elevated blood pressure (BP) of more than 140/90 mm Hg. However according to the American Heart Association 2017[3], hypertension is now defined as elevated BP of more than 130/80 mm Hg. An estimated 23.5 million people will be die as a result of the condition by 2030 if the current interventions are not successful or new ones not established[1].

The renin angiotensin system (RAS) is considered to play an important role in the pathogenesis of cardiovascular diseases, including hypertension. Recent reports strongly support the use of RAS inhibiting agents, such as angiotensin receptor blockers, (ARB) as well as angiotensin converting enzyme (ACE) to clinically manage cardiovascular diseases. Angiotensin converting enzyme, is an enzyme responsible for the conversion of Angiotensin I to Angiotensin II. Angiotensin II is known to trigger a cascade of pathways that leads to vasoconstriction, which ultimately progresses to hypertension[4].

Conventional drugs are effective in treating hypertension, specifically those that acts as an ACE inhibitor (ACEI), including Captopril, Ramipril, Benazepril, Enalapril, Lisinopril and Perindopril[5,6]. However, reports of uncontrolled BP in patients who adhere to the routine of these antihypertensive drugs, together with the adverse side effects, has been established[5,6]. Furthermore, these chemical drugs are not easily available in developing countries, thereby contributing to non-adherence of therapeutic regimens[6]. These factors heightens the urgency to find cheaper, easily accessible and effective therapy[7]. According to the World Health Organisation (WHO), poverty and lack of modern medicine forces 65-80% of the world's population to depend exclusively on plants for principle health care. Moreover, the isolation of natural compounds from medicinal plants, as rich sources of novel drugs with diverse potential therapeutic benefits, has gained considerable scientific attention[7].

Etiopathogenisis of cardiovascular diseases and hypertension

Blood pressure is defined as the pressure of the blood flow that is exerted against the blood vessel wall as it is pumped by the heart. Cardiac output and peripheral vascular resistance are regulated by the interface of several influences, such as genetic, environmental and demographic factors[4]. Arterial BP is the product of cardiac output (CO) and total peripheral resistance (TPR)[8], with BP maintenance being dependent on the balance of both TPR and CO, as illustrated in Figure 1.

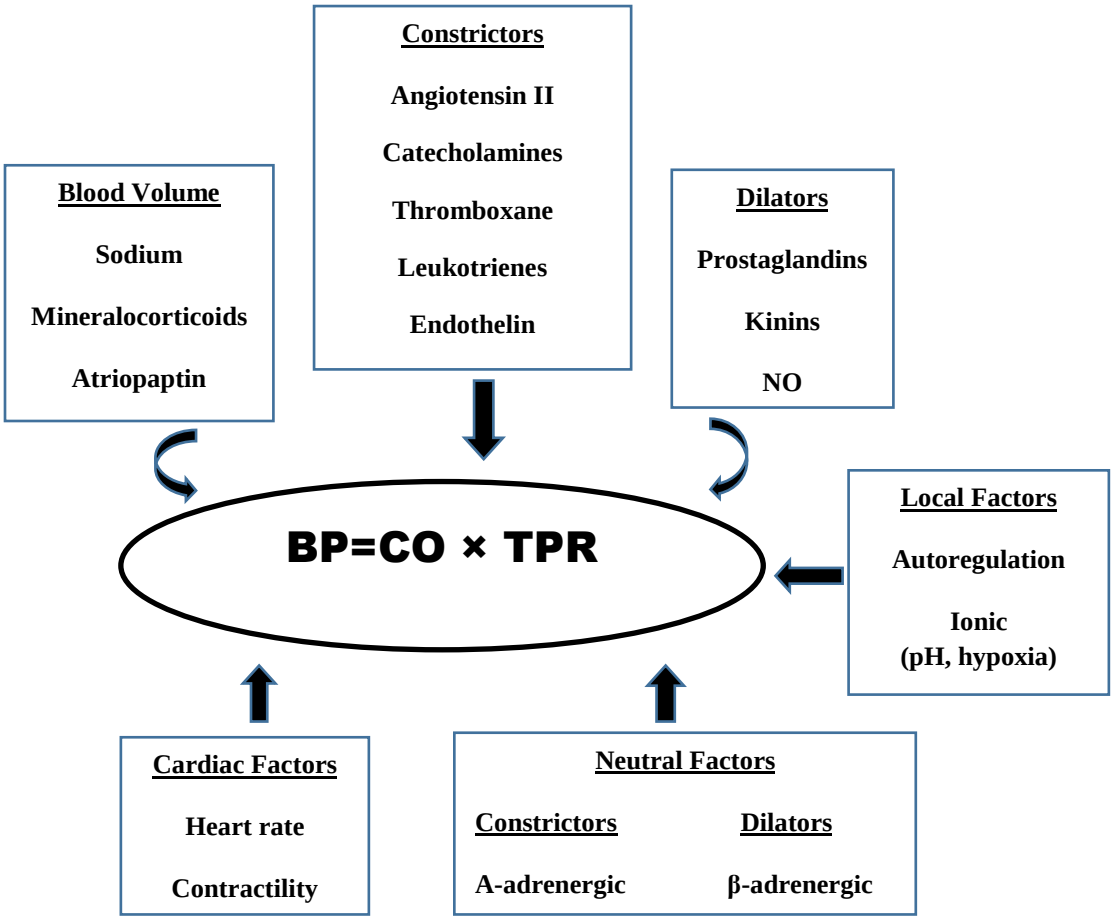

Figure 1: Role cardiac output and total peripheral resistance in blood pressure regulation (Adapted from Kumar *et al*., 2005 [9]).

Primary or idiopathic hypertension is well-defined as elevated BP, wherein secondary contributors, for example renovascular disease, renal failure, pheochromocytoma, aldosteronism, or other causes are not present[10]. Essential hypertension accounts for 90 – 95% of all hypertensive[11]. Key pathophysiological alterations associated with essential hypertension include: endothelial dysfunction; increased generation of reactive oxygen species (ROS); decreased bioavailability of the key vasodilator, nitric oxide (NO); increased sodium intake; altered expression of the kallikrein-kinin system that influences vascular tone and renal salt handling, as well as excessive activation of the renin angiotensin aldosterone system (RAS)[12].

The Classical Renin Angiotensin Aldosterone System (RAAS)

The central role of the RAS in regulating BP is well recognized[13], with Angiotensin II, the main effector octapeptide, being essential for its control. Angiotensin II (Ang II) directly activates the angiotensin II type 1 (AT1) receptor that leads to the stimulation of various tyrosine kinases, which in turn causes the phosphorylation of the tyrosine residues in a number of proteins, leading to vasoconstriction[14]. Abnormal RAS activity leads to an array of cardiovascular diseases, such as hypertension, atherosclerosis, myocardial infarction, congestive heart failure and renal disease[14].

The Novel Renin Angiotensin System (RAS)

According to the long-held classical theory, the RAS is viewed as an endocrine system, with circulating Ang II as the active metabolite[15]. Recent attention has focused on the tissue-specific paracrine and/or autocrine functions of Ang II[16]. Evidence strongly suggests that the essential components of the RAS are present in all organs and tissues of the body, and that the tissue/local RAS is regulated independently of the circulatory RAS, although they can interact[15,17] which has important implications for therapeutic interventions.

Local Ang II can be generated directly from angiotensinogen by the actions of the enzymes cathepsin G and chymostatin-sensitive-Ang II-generating (CAGE) enzyme, or from Ang I through the action of the enzyme chymase. Reports suggest that chymase is the major catalyst of tissue Ang II formation in the human heart (>80%) and the arteries (>60%)[17,18]. The alternative RAS pathway also identifies the enzyme ACE II, which is located in the heart, kidney, testes and the gastrointestinal tract. ACE II, a homologue of ACE, catalyses the conversion of Ang I to inactive angiotensin 1-9 [Ang (1-9)], and Ang II to angiotensin 1-7 [Ang (1-7)][19]. Ang I can also be converted to Ang (1-7) by the action of endopeptidases [18]. The 'classic' ACE catalyses the conversion of Ang (1-9) to the vasodilator and anti-proliferative Ang (1-7) [19]. The Ang (1-7) G protein-coupled protein Mas receptor is a key component of the Ang 1-7/ACE II/Mas axis, which is believed to counter-balance the pressor effects of the ACE/Ang II /AT1 receptor pathway[19].

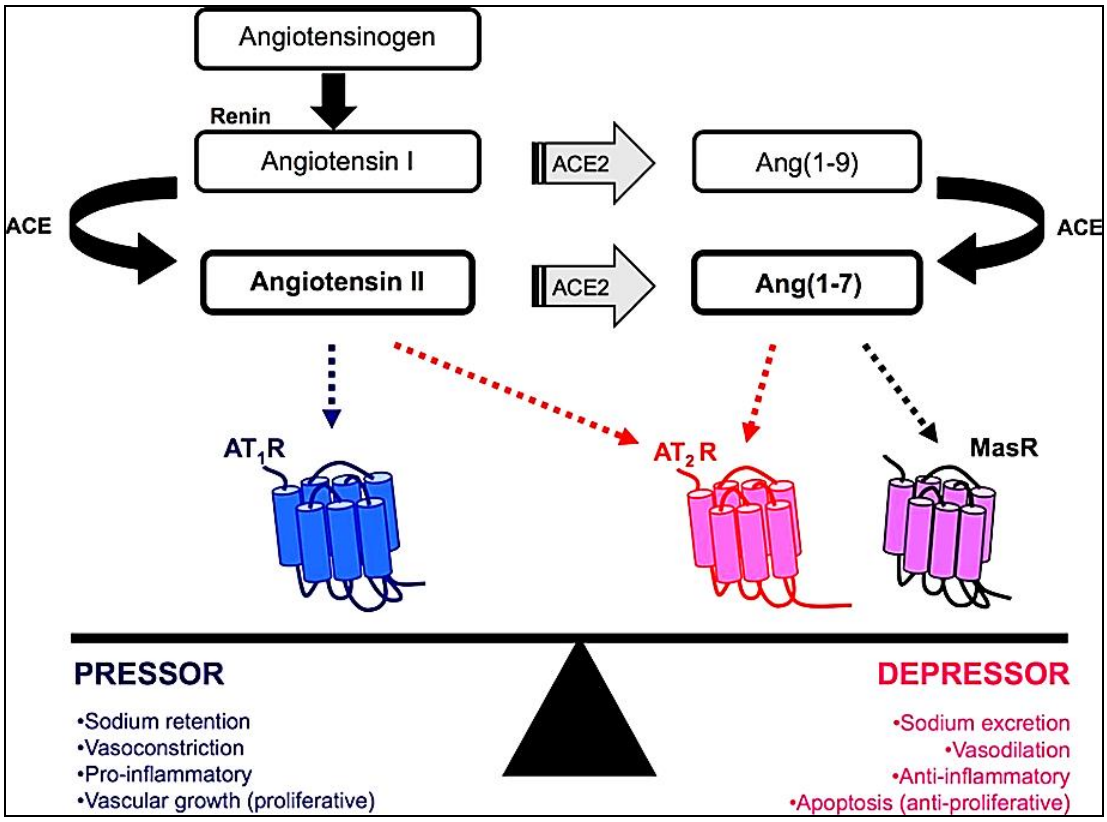

Figure 2: Components of the classical and alternative pathways of the renin-angiotensin system (RAS) and their interactions [20]

ACE inhibition and its Implication on RAS and Hypertension

ACE is a dipeptidyl-liberating expeptidase that catalyses the cleavage of histidyl-leucine from angiotensin I to produce the potent vasopressor octapeptide angiotensin II [21]. Ang II is produced in various tissues, such as the blood vessels, heart, adrenals and brain, which are regulated by ACE as well as other enzymes, including rennin [13]. Although the conversion of Ang I to Ang II by ACE is a normal regulatory process, unrestrained elevated ACE activity leads to increased levels of the latter, which leads to the development of hypertension [14]. This occurrence is essential for the development of inhibitors that are involved in converting Ang I to Ang II to treat hypertension. Additionally, ACE is identical to kininase II, the enzyme that facilitates the degradation of bradykinin, a critical component of the kallikrein-kinin system (KKS). The increased levels of the potent vasodilator, bradykinin, due to kininase II inhibition,

stimulates the production of NO and prostaglandins, including prostacyclin and prostaglandin E2, resulting in increased local blood flow and the relaxation of smooth muscles[18, 22].

ACEIs have also been reported to prevent the progression of albuminuria to proteinuria, reduce proteinuria in patients with glomerular disease, and/or delay the progression of renal insufficiency to end-stage renal disease [23]. Moreover, ACEIs are reported to reduce total peripheral vascular resistance without significantly altering the heart rate[23].

Conventional prescription drug generally used as ACE inhibitor

The most commonly used western drug for ACE inhibition is captopril, with studies having found that PCR analysis of rat renal tissue, following its use demonstrated down regulation of the AT1 gene, indicating that ACE inhibitors play an important role in AT1 receptor regulation [24, 25]. Captopril bioavailability in adults is approximately 75%, with the majority of the drug being eliminated via the kidney together with inactive metabolites (50:50 ratio)[26]. Captopril is usually well tolerated when administered orally, although occasional side effects have been reported. These adverse effects include metallic taste, rash, neutropenia, liver toxicity oliguria, acute renal failure, hypotension, hyperkalemia, anaemia and neurological complications [23, 27]. Furthermore, hypotension, hyperkalemia and pregnancy related abnormalities have also been associated with ACEI therapy. The most common side effects caused by an impaired metabolism of bradykinin due to ACE inhibitors is hypertensive patients manifesting a dry hacking cough and angioedema[28].

Novel drug therapy that mimics the hypotensive effects of captopril, but without the adverse side effects, is therefore essential to treat hypertension[29]. Additionally, in the plant world, active substances coexist with the other compounds that mitigate their negative side effects, with a recent return to phytotherapy having been observed. There has therefore been a renewed call by the WHO to screen plant material for the presence of biologically active compounds. It is firmly believed that a great, yet still not fully revealed, therapeutic potential exists in plants, because, thus far only a few percent out of 250,000 plant species have been investigated with regard to their usefulness in medicine[30].

The Role of Herbal Medicines In Managing Cardiovascular Disease And Hypertension

The use of medicinal plants to treat hypertension and other cardiovascular diseases has gained much attention by the scientific world over the last decade[7]. This is mainly due to medicinal plants being a cheaper alternative to manufactured formulations, with fewer, or no undesired side effects[31]. However, their use in primary health care is not a reflection of the economic status of a person, with 70% of the population in developed countries relying on complementary and alternative medicine (CAM) for treatment purposes, of which herbal medicines plays a significant role[32].

The chemical constituents of the plant cells that exert biological activities on human and animal cells fall into two distinct groups, depending on their relative concentration in the plant body, as well as their major function: (a) primary metabolites, the accumulation of which satisfies nutrition and structural needs, and (b) secondary metabolites, which act as hormones, pharmaceuticals and toxins[33].

Medicinal plants which are rich in a variety of secondary metabolites, such as flavonoids, alkaloids, polyphenols, tannins and terpenoids, have been explored for their cardio protective and antihypertensive properties[34-37]. The probability of plants secondary metabolites as a source of novel ACE inhibitors continues to be under investigated, whereby only a restricted amount of species have been examined[33]. Table 1 below shows common plant extracts and molecules isolated from them that have previously shown inhibitory effects on ACE.

Table 1: Medicinal plants from around the world displaying ACE inhibition

Plant name (family)	Active Ingredients	Medicinal uses	Bioactivity tested	Reference
Crataegus spp (hawthorn) 	Glycosides, proanthocyanidins, flavonoids	Stomach ailments, Chronic heart failures, Hypertension	Antihypertensive, antiparasitic	34
Cassytha filiformis 	Alkaloids, phenols	Male sexual dysfunction, Jaundice, Cardiovascular diseases,	Aphrodisiac, antihypertensive	35
Jatropha curcas 	Saponins, phytate, curcins	Contraceptive, Laxative	Antioxidant, anti constipative	36
Moringa oleifera 	Amino acids, flavonoids, polyphenols	Stomach ailments, Microbial infections	anti-inflammatory, anti-parasitic,	37

Passiflora edulis 	Cardiac glycosides, terpenoids, alkaloids, steroids	Jaundice, Sedative, Tranqulizer	anti-depressant, antihypertensive	[39]
Psathura Borbonica 	Flavonoids, phenols	Diuretic, Laxative,	Anti-constipation, anti-hypertensive	[40]
Apium graveolens 	Polynnes, apiin, apigenin, lunularin	Arthritis, inflammation, Antimicrobial	Anti-inflammatory, cardio protective, antibacterial	[41]
Leea guineensis 	Phenylpropanoid, terpenoids, aldehydes	Rheumatism, Oedema	Anti-arthritic, anti-inflammatory	[42]

Poupartia borbonica				
	Cyclohexenone, phenols, flavonoids	Malaria infection, Microbial infections	Antiplasmodial, anti-bacteria, anti pesticidal	[43]

South African Indigenous Plants with ACE inhibition

In South Africa, many people still use plants as medicines as an alternative or supplement to visiting a western health care practitioner [44]. This is not surprising due to South Africa's cultural diversity as well as its large floral biodiversity. South Africa is home to over 30,000 species of higher plants and 3000 of these species have been found to be used in traditional medicine across the country [44]. There are over 27 million users of indigenous medicine [45] and an estimated 200,000 indigenous traditional healers, which up to 60% of the population consult with[44].

Within the wealth of plants in South Africa, scientists have reported on several with ACE inhibitory activities (≥50%), which may serve as a foundation for drug discovery to treat hypertension[46,47]. Moreover, they represent an untapped reservoir of potentially useful chemical compounds, unique templates that could serve as starting points to achieve a better understanding of biological processes involved in hypertension [47]. The local plants of particular importance for ACE inhibition are stated below, these being found in various provinces of South Africa.

Tulbaghia violacea Harv.

A very popular garden plant, *T. violacea* belongs to the Alliacaea family, and is one of the fastest growing bulbous plants, which could grow to a height of 0.5m. It has long narrow leaves with a strong garlic smell and pinkish mauve tubular flowers that are mainly seen from January to April[48]. It is a well distributed plant, stretching from the Eastern Cape, to KwaZulu-Natal and Limpopo, and extends as far north as Zimbabwe [48] (Figure 4).

Figure 4: *T. violacea* [48]

Ethnomedicinal uses and studies

T. violacea is an edible plant that is usually prepared in salads and other dishes, with decoctions of the bulbs used to treat coughs and colds, as well as pulmonary tuberculosis. Findings on its antimicrobial and antifungal properties are well documented [49-51]. It is also used traditionally to treat numerous other ailments, including fits, paralysis, headaches, high blood pressure, fevers, rheumatism, heart problems, chest complaints, stomach cramps and constipation[52]. Several studies have confirmed the *in vitro* ACE inhibition activities of *T. violacea*, with both aqueous and methanolic extracts showing ACE inhibition activities[4, 53, 54] .

Phytochemicals in this genus

Phytochemical analysis revealed the presence of flavonoids, glycosides, tannins, terpenoids saponins and steroids, these compounds being biologically active that may contribute to the antioxidant activities of the *Tulbaghia* species[52]. *T. violacea* has been found to be rich in sulphur-containing compounds, which in most cases account for the characteristic odours and medicinal properties of both the *Tulbaghia* and *Alluim* species[55].

***Protorhus longifolia* Bernh.**

P. longifolia is a medium to large, mostly dioecious class of tree belonging to the Anacardiaceae (Mango) family. It is commonly called red beech and is often distributed from the Eastern Cape to Limpopo. This species is an evergreen indigenous tall tree and mainly occurs in coastal, scarp and mist belt forests, rock ridges, cliffs, riparian frontiers, or in woodland[56]. *P. longifolia* is commonly known as uNhlangothi (isiZulu). It is one of the top 10 most important and frequently traded medicinal plants in the Eastern Cape Province[57], and has patently yellow or red leaves, with flowers that are greenish-white (male) or pink to red (female) (Figure 5).

Figure 5: *P. Longifolia* [58]

Ethnomedicinal uses and studies

The bark has traditionally been used to treat numerous ailments, such as the bacterial infections of *Ehrlichia ruminantium* and diarrhea in cows [57], hemiplegic paralysis, heartburn and bleeding from the stomach. Some parts of the plants have also been used to fortify the heart[59], while Suleiman et al. (2009)[60] claims that the leaf extracts possess antimicrobial activity. Mosa et al. (2011)[61] showed that the stem bark has an anti-platelet aggregation. Mosa et al. (2016)[62] examined the *in vivo* cardio protective effect of methyl-3b-hydroxylanosta-9, 24-dien-21-oate (RA-3) that was isolated from *P. longifolia* in ISO-induced myocardial injury in rats, while Duncan et al. (1999)[54] reported a 77% *in vitro* ACE inhibition activity of *P. longifolia* extracts.

Phytochemicals in this genus

Triterpenes isolated from the bark *P. longifolia,* have been linked to its cardio protective activity.

Adenopodia spicata E. Mey.

A. spicata belongs to the *Fabaceae* family, being frequently known as 'spiny splinter bean' (English), lugagane (siSwati), umlungumabele (isiXhosa) and umlungumabele (isiZulu). It is a typical climbing shrub/tree found mainly in the coastal forests of KwaZulu-Natal as well as the Eastern Cape to Limpopo[57]. It is an evergreen shrub that grows up to 10m height comprising of small slender branches, with the leaf axis being mostly round or flattened, and often prickly. It contains bisexual flowers and gives rise to a glabrous pod fruit that breaks into segments[57], (Figure 6).

Figure 6: *A. spicata* [58]

Ethnomedicinal uses and studies

The roots of *A. spicata* is traditionally used to treat chest pains and syphilis [63]. In Zululand, a root infusion is used by traditional healers as an emetic to fatten goats, while Zulu sangomas use the crushed roots to increase their spiritual powers[63]. Duncan et al. (1999)[54], found *in vitro* ACE inhibitory activity in both the aqueous (97%) and methanolic (72%) extracts of the leaves of *A. spicata*. According to theses authors, *A. spicata* generated the highest *in vitro* ACE inhibition activity in South Africa to date. A detailed *in vivo*

study is necessary to establish the bioavailability of the active compounds in animal gut and hence an implication for drug discovery in man

Phytochemicals in this genus

The active compounds from *A. spicata* have not been isolated and identified.

Catha edulis Vahl.

C. edulis belongs to the Celastraceae family, and is commonly called Bushman's tea (Eng.), Boesmanstee (Afr.), umhlwazi (Zulu), iqgwaka (Xhosa) and khat (Arabic). This is a small deciduous tree with bright green leaves that grows up to 10 m tall, has an upright crown, and somewhat drooping branches that resemble a eucalypt from a distance. It is dispersed in KwaZulu-Natal and Eastern Cape, mostly extending inland from the coastal belt, although it is also found in the Western Cape, Mpumalanga, Swaziland, Mozambique and through to tropical Africa and the Arab countries[64] (Figure 7).

Figure 7: *C. Edulis* [65]

Ethnomedicinal uses and studies

C. edulis young buds and tender leaves are chewed to attain a state of euphoria and stimulation. Traditional use of this plant includes constipation, urine retention, acute cardiovascular and nervous

system effects, increased alertness, patience, dependence problems, as well as psychiatric symptoms [66]. Duncan et al. (1999)[54] established that the ethanolic extracts of the leaves of *C. edulis* produced an 82% ACE inhibition *in vitro*.

Phytochemicals in this genus

Many different compounds are found in *C. edulis*, including alkaloids, terpenoids, flavonoids, sterols, glycosides, tannins, amino acids, vitamins and minerals[66]. The phenylalkylamines and cathedulins are the major alkaloids, which are structurally related to amphetamines (chemical, nervous system stimuli), while cathinone has vasoconstrictor activity in isolated perfused hearts from guinea pig[66].

Justicia flava Vahl.

J. flava belongs to the Acanthaceae family, and usually grows in disturbed habitats, on a wide range of soil types, in full sun or semi shady areas. It is prevalent in tropical and Southern Africa, being a perpetual herb that grows up to 450 mm high. Is commonly called “ipela” in Zulu. The leaves are lanceolate or broadly ovate, with flowers that are subtended by large, leaf-like bracts, while the tubular flowers are pale to greenish with a bright yellow lower lip (Figure 8).

Figure 8*:* *J. flava* [58]

Ethnomedicinal uses and studies

J. flava is used traditionally to treat coughs, paralysis, fever, epilepsy, convulsions, spasm, as well as skin disorders. A concoction of the roots are used for diarrhea and dysentery[68]. Agyare et al. (2013)[69] reported that methanolic leaf extracts significantly increased the tensile strength and the rate of contraction of wounds, with intense granulation tissue formation. Ramesar et al. (2008)[70] reported that aqueous extracts of the leaves showed 53% ACE inhibition activity.

Phytochemicals in this genus

Steroids, including campesterol, stigmasterol, sitosterol, and sitosterol-D-glucoside, were isolated from its leaves and roots [71].

Dietes iridioides L.

D. iridioides is a part of the Iridaceae family, and is commonly called indawo-yehlathi, isiqiki-sikatokoloshe or isishuphe somfula (isiZulu). It is an evergreen herb that usually grows up to 600 mm in height, has green leaves that are arranged in a loose fan, with small flowers, each bloom lasting a single morning. This plant is very commonly used in South African landscapes and gardens[59], being distributed throughout the country, specifically in the less dry provinces of KwaZulu-Natal, the Eastern Cape and Western Cape (Figure 9).

Figure 9: *D. Iridioides*[72]

Ethnomedicinal uses and studies

D. iridioides infusions made from of the rhizome are traditionally used as an enema, to treat dysentery and hypertension. The rhizomes also being used to facilitate childbirth[73]. Ground rhizomes are ingredients in tonics for goats[74], while the roots are used to treat menstruation disorders. It is widely used in traditional medicine to treat flu, cold, toothache, malaria, and bruise[75]. Duncan et al. (1999)[54], found that the aqueous leave extracts produced an *in vitro* ACE inhibition activity of 80%.

Phytochemicals in this genus

The main secondary metabolites isolated from extracts of Iridaceae include isoflavonoids, flavonoids, triterpenoids, naphthoquinones, anthraquinones, naphthalene derivatives, xanthones and phenolics[76]. Active compounds found in this family were reported by Lucena et al. (2007)[77], to have an extensive variety of biological activities, including antibacterial, antiprotozoal, antiviral, antioxidant, antinociceptive, anti-inflammatory cytotoxic, and immunomodulatory benefits.

Dombeya rotundifolia Hochst.

D. rotundifolia belongs to the *Sterculiaceae* family, often referred to as the chestnut family[78], being frequently known as wild pear (English) or inhliziyonkhulu (isiZulu). The wild pear is a deciduous tree that grows approximately 1 - 1.5 m per year and can reach heights of up to 10 m. The bark is dark brown, bumpy and corky on fully developed trees. The leaves are almost round and are shielded with tiny spiky hairs, which is a distinguishing characteristics[79]. *D. rotundifolia* are distributed throughout KwaZulu-Natal and Mpumalanga, where they grow in woodlands, grasslands or bushel as well as on rock-strewn slopes (Figure 10).

Figure 10: *D. rotundifolia* [58]

Ethnomedicinal uses and studies

D. rotundifolia is traditionally used to treatment heart problems, vomiting in pregnant women, intestinal ulcers, headaches, stomach ailments, haemorrhoids, diarrhoea and dyspepsia, as well as to accelerate the onset of labour[80]. Duncan et al. (1999)[54], established that the ethanolic leave extracts exhibited an 83% ACE inhibition activity.

Phytochemicals in this genus

Reid et al. (2001) [80], isolated two main types of tannins i.e. condensed and hydrolysable tannins. Condensed tannins have traditionally been used to assist in wound healing and scorches, and produce a waterproof layer when applied to the skin[80]. These tannins also have particular shielding significance against toxins when ingested, with hydrolysable tannins being thought to have antiviral and anti-tumour properties[81].

Searsia chirindensis Baker f.

S. chirindensis (previously called *Rhus chirindensis)* belongs to the Anacardiaceae family, and is generally called red currant (English), umhlakothi (Xhosa), inhlokoshiyane enkulu (Xhosa) or muvhadela-phanga (Venda). It is widely distributed in Limpopo, Mpumalanga, KwaZulu-Natal, Eastern

Cape, and Western Cape. *S. chirindensis* is a semi-deciduous shrub to small tree that grows up to 6-10 m in height (Figure 11).

Figure 11: *S. chirindensis* [82]

Ethnomedicinal uses and studies

Traditional healers usually use the sap of this plant to treat heart conditions, strengthen the body and encourage blood circulation, while the bark is used to treat rheumatism as well as mental disorders[83]. Ojewole (2008)[84] showed that the aqueous extract of the stem-bark portentously postponed the commencement of seizures brought by pentylenetetrazole (PTZ), or antagonized picrotoxin (PCT)-induced seizures, suggesting that *S. chirindensis* acts as an analgesic and anti-inflammatory. Other studies have reported that the stem bark is used ethno-medically as an agent to control type 2 diabetes. Duncan et al. (1999)[54], established that the ethanolic leaf extracts of *S. chirindensis* could be used as an anti-hypertensive drug, as it possessed an 85% ACE inhibitory activity.

Phytochemicals in this genus

The stem bark contains flavonoids, small quantities of saponins, tannins, triterpenoids, traces of alkaloids and cardiac glycosides, which are thought to act as an anticonvulsant.

Can South African Indigenous Plants be Harnessed Therapeutically for ACE inhibition?

Despite centuries of traditional use of medicinal plants, several scientific based studies still remain poorly understood, debateable, or unrecognized. The human body may be regarded as multifaceted, with the ability to mend itself. However, in the modern world, the variables are almost infinite. It is well recognized that the existing global population face greater health concerns, particularly life-threatening diseases such as hypertension. In light of this, scientists are currently reviewing the historical use of plant extracts to cure ailments in order to produce medicines[85].

During the last few eras, it has become evident that there exists a superfluity of plants with medicinal potential and it is increasingly being accepted that the African traditional medicinal plants offer probable prototype molecules in the search for novel antihypertensive drug agents. Many of the plants presented in this review show boundless potential for ACE inhibition thus justifying supplementary clinical investigations. Nevertheless, very limited scientific and clinical evidence is available, thereby necessitating comprehensive screening and scientific investigations before exposure to the global market[86].

Conclusion

Inhibition of ACE is a contemporary beneficial objective in the management of hypertension. Synthetic ACE inhibitors are understood to have numerous side effects. Consequently, the investigation for non-toxic, innocuous, novel and cost-effective ACE inhibitors as substitutes to conventional treatment is needed. Medicinal plants are likely to play an increasingly important role in providing health care in South Africa. In this review, investigational as well as the traditional knowledge of South African plants used for ACE inhibitory activity were identified, each with their benefits and limitations. Ongoing studies are required to investigate their therapeutic potential in the management and treatment of hypertension.

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Conflict of Interest

The authors agree to the contents of this review and have declared that there is no conflicting interest.

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Chapter 3: Manuscript Two

Biochemical, Phytochemical Profile and Angiotensin-1 Converting Enzyme Inhibitory activity of the Hydro-methanolic extracts of *Tulbaghia acutiloba* Harv.

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Literature reviewed at the beginning of the study indicated the importance of assessing novel medicinal plants through preliminary screening and identification of phytochemical contents, and investigating antihypertensive effects and biochemical parameters as part of a holistic approach to finding accessible alternate remedies in the treatment of hypertension. Thus Chapter 3 presents a detailed *in vitro* study conducted to assess the phytochemical constituents, antioxidant activity, heavy metal toxicity and angiotensin-converting enzyme inhibition properties of the indigenous herb, *Tulbaghia acutiloba*.

Formats used in this chapter are according to the Journal specifications.

Biochemical, Phytochemical Profile and Angiotensin-1 Converting Enzyme Inhibitory activity of the Hydro-methanolic extracts of *Tulbaghia acutiloba* Harv.

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ABSTRACT

Phytotherapeutic treatments have been on the ascendancy over the years as the quest for alternate and easily accessible health care is on the rise. *Tulbaghia acutiloba* has been used by both traditional healers and the South African population for the management of chronic conditions, but lacks scientific authentication for usage. Hydro-methanolic extracts (roots, rhizomes, leaves and flowers) of the plant were evaluated for their antioxidant activities, biochemical and phytochemical profile. Additionally, the heavy metal content and antihypertensive effects including angiotensin 1-converting enzyme inhibitory activities (ACEI) were investigated. The antioxidant ability of the hydromethanolic extracts were determined by *2, 2-diphenyl-1-picryl hydrazyl (DPPH),* hydrogen peroxide and nitric oxide scavenging activities. Phytochemical profile was assessed using qualitative and quantitative methods whereas the heavy metal toxicity was determined by using inductively-coupled plasma optical emission spectrometry (ICP-OES). *In vitro* ACEI activity was determined by the hydrolysis of the tripeptide, *hippuryl –L-Histidyl-L-leucine (Hip-His-Leu)*. All the extracts showed potent antioxidant activities which was concentration dependent. Phytochemical analysis showed the presence of phenols, amino acids and alkaloids in all the extracts. The leaves showed a relatively higher total phenolic content of 43.26±1.15 mgGAE/g. Gas chromatography–mass spectrometry (GC-MS) analysis showed the presence of major compounds such as *α-linolenic acid* in the leaves as well as *oleic acid* and *palmitic acid* in other parts of the plants. Toxicity of heavy metal was undetected in all extracts of the plant. All the extracts of the plants showed a >50% ACE inhibition at different concentrations with the leaves showing a relatively higher inhibitory activity (76.66 ± 1.65, *IC50;*154.23*μg/ml)* compared with the other parts of the plants. It is therefore concluded in this study that the biological activities and phytochemical component of the hydro-methanolic extracts of *T. acutiloba* is indicative of its possible use for the treatment as well as prevention of hypertension and oxidative stress-related diseases.

Key words: *Tulbaghia acutiloba,* Angiotensin 1-Converting Enzyme, Phytochemical, Heavy Metal Toxicity, Antioxidant.

1. Introduction

Chronic lifestyle-related metabolic diseases such as hypertension (HTN) cause double the number of deaths compared to infectious diseases[1]. Hypertension, as the major risk factor of cardiovascular diseases, continues to be on the ascendancy with increased numbers of patients with high blood pressure seeking herbal and naturopathic treatments, especially in developing countries such as South Africa[2]. Phytotherapy is an integral part of the traditional healing system in developing countries, a trend that is rapidly rising in developed countries owing to the belief that herbal remedies and indigenous medicine are generally considered safer, affordable, easily accessible and with no or minimal side effect as compared to the drugs used in conventional medicine[3]. Moreover, several conventional drugs are derived from herbal medicines, but the major variation between the two is that herbal drugs mostly contain a number of bioactive compounds, whereas the conventional drugs mostly contain an isolated bioactive substance; therefore, the constituents of both herbal and conventional medicines may act on one another to augment or enhance an effect. In South Africa, it is estimated that between 12 and 15 million people still depend on traditional herbal medicine, from as many as 700 indigenous plant species[4,5]. New remedies discovered by traditional healers are increasingly being used (especially for cardiovascular diseases), increasing the need for scientific validation of mechanism of action and drug dosage formulations[6].

Bioactive compounds such as flavonoids, phenols, saponins, steroids and tannins obtained from medicinal plants, show high antioxidant activities that are key to the therapeutic management of chronic conditions such as hypertension, cancer and diabetes[7]. It is now widely reported that central to the pathology of these diseases is oxidative stress, caused by free radicals. Free radicals are either generated in the human body, *in situ* or from the external environment through air pollutants[8], however an increase in free radicals in the body could lead to oxidation of biomolecules [9], and other pathological conditions[10]. Prolonged oxidative stress leads to heart diseases, and in particular the risk factor, hypertension[11]. Moreover, the reducing capacity or scavenging activity of an extract or compound against free radicals may serve as a significant indicator of its potential antioxidant activity[12].

It is important to note that a drawback of phototherapy may include heavy metal accumulation, absorbed from the environment by the plant. The continuous ingestion of toxic heavy metals

such as lead, chromium, barium, arsenic and cadmium, into the human body may have genetic and epigenetic effects leading to numerous disorders[13]. Therefore, there is the need to evaluate medicinal plants for toxic heavy metal content.

The increasing use of plants therapeutically, with little scientific data to verify their safety profile and dosage could lead to side effects. One such plant is *Tulbaghia acutiloba. T. acutiloba* is commonly known as 'wild garlic", "*ishaladi lezinyoka"* in Zululand, "*sefothafotha"* in South Sotho and lisela in Swaziland. It is indigenous to the Eastern Cape, KwaZulu-Natal, Gauteng, Free State and Mpumalanga regions of South Africa[14]. Generally, It is used as a herbal treatment for a number of health conditions such as fungal infections, bruised skin, sores and also forms part of many delicacies in the Eastern Cape region[15]. Traditionally, the Basotho's, cook the leaves, bulb and roots of *T. acutiloba* to make a lotion. This lotion is used to wash incisions to avoid bacterial contamination and aid in wound healing. A concoction of this plant is made and drunk for the treatment of high blood pressure as well as diabetes[16,17]. They are also grown domestically by the Zulu's and Xhosa's around their homes as a protective charm to repel snakes and other harmful reptiles [17].

We have previously investigated *Tulbaghia violacea* or 'wild garlic' which is closely related to the *Tulbaghi acutiloba*. We were able to show antidiabetic, antihypertensive and renoprotective effects which could be attributed to its organosulfur compounds[18].

In spite of the therapeutic herbal properties and the wide use of *T. acutiloba*, there is a lack of scientific information concerning the pharmacological and biological activities of its extracts. This study investigated the antihypertensive properties, antioxidant activities, toxic heavy metal content and phytochemical profile of *T. acutiloba*.

2. Material and Methods

2.1 Preparation of Plant Material and Extract

Fresh rhizomes, roots, leaves and flowers of *T. acutiloba* were collected from Durban, KwaZulu-Natal and authenticated by Prof Himansu Baijnath (Botanist) from the University of KwaZulu-Natal (UKZN). The parts collected were washed thoroughly under tap water and allowed to air dry at room temperature over a period of 72 hours. Each part of the plant was crushed in a Waring blender. The powdered form (50g) of each part was immersed in separate 500ml hydro-methanolic solution (80% methanol; 20% water) and shaken at 80rpm for 48 hours. The crude extract for each part was filtered and concentrated in a rotary evaporator, thereafter air dried to form a sludge. The remaining extracts were weighed and the percentage yield determined for each part of the plant.

2.2 Antioxidant activity

2.2.1 2, 2-Diphenyl-1-Picryl Hydrazyl (DPPH) Radical Scavenging Activity

The scavenging activity of each part of the plant on the stable radical DPPH was determined according to the method by Murthy et al., (2002) with some modifications[19]. A volume of 150 µl of the hydro-methanolic solution of each extract at different concentrations (1000, 500, 250, 125, 62.5, 31.3 and 15.6 µg/ml) were mixed with 2.85 ml of a methanolic solution of DPPH (0.1 mM). Equal amount of MeOH and DPPH with no extract was used as control. The reaction was kept at room temperature for 30 minutes in the dark and thereafter the absorbance measured at 517 nm against methanol as a blank using a UV spectrophotometer (SPECTROstar^{nano} BMG LABTECH, Germany). The positive control used in the study was Ascorbic acid. A triplicate run of all tests was performed. The free radical scavenging activity was expressed as percentage in accordance with the equation as follows:

% scavenging activity (DPPH) = Ac-As/Ac × 100.

Where As = absorbance of sample and Ac = absorbance of control

2.2.2 Hydrogen Peroxide Scavenging Activity

The potential of the *T. acutiloba* extracts in the scavenging of hydrogen peroxide was evaluated using a method according to Ruch et al., (1989) with some modifications[20]. Hydrogen peroxide solution of 40 mM was made in a phosphate buffer of pH 7.4. Briefly, 0.2 ml of the various concentrations of the extracts (1000, 500, 250, 125, 62.5, 31.3 and 15.6 µg/ml) were added to a hydrogen peroxide solution (0.6 ml, 40mM) for 10 minutes. The absorbance of hydrogen

peroxide was determined at a wavelength of 560 nm after 10 minutes. A solution of phosphate buffer without hydrogen peroxide was used as a blank before each reading. The positive control used in the study was Ascorbic acid. The hydrogen peroxide scavenging activity of the extracts was expressed as percentage as follows:

% scavenging activity (H_2O_2) = Ac-As/Ac × 100.

Where As = absorbance of sample and Ac = absorbance of control

2.2.3 Nitric Oxide Scavenging Activity

The scavenging activity of *T. acutiloba* against Nitric oxide was measured in accordance with the method developed by Govindarajan et al., (2003) with some revisions[21]. A mixture of 0.5ml of a 10 mM sodium nitroprusside solution in a phosphate buffered saline was added to 1ml of different concentrations of the extracts (1000, 500, 250, 125, 62.5, 31.3,15.6 μg/ml) and incubated at 25°C for 180 min. Control samples without the extracts but with an equal volume of buffer were prepared in a similar manner to test samples. After 180 min, the incubated mixture (1.5ml) was taken out. A solution of 1.5 ml Griess reagent made up of 1% sulphanilamide, 0.1% N-1-naphthylethylenediamine dihydrochloride and 2% phosphoric acid was used to dilute the incubated solution. The absorbance of the resulting chromophore formed after the addition of the Griess reagent to the incubated solution was measured at 546 nm. The positive control used in the study was Ascorbic acid. The radical scavenging activity of the extracts was expressed as a percentage using the formula as follows:

% scavenging activity (NO_2) = Ac-As/Ac × 100.

Where As = absorbance of sample and Ac = absorbance of control

2.3 Determination of *In-vitro* Angiotensin-1 Converting Enzyme (ACE) Inhibition Activity

Stock Substrate Solution: A 100ml stock substrate solution of 25mM hippuryl –L-Histidyl-L-leucine (Hip-His-Leu) was freshly made by the dissolution of 1.12g of Hip-His-Leu (sigma Aldrich, schnelldorf, Germany) in 25mM sodium hydroxide (NaOH).

Sodium Borate Buffer: Sodium borate buffer was freshly made up by the dissolution of 30.5g of boric acid (H_3BO_4) and 65.7g of sodium chloride (NaCl) in distilled water. The final volume prepared was 1L and this preparation gave a 1.25 –fold concentration (0.5M). The pH of the solution was adjusted to 8.3 using 13N NaOH.

ortho-phthaldialdehyde (*o*- phthaldialdehyde): ortho-phthaldialdehyde (OPA) (sigma Aldrich) solution was prepared by dissolving 200mg of OPA in 10ml of methanol.

Assay Buffer: An assay buffer used in the study was made by mixing one volume of stock substrate solution to four volumes of 0.5M sodium borate buffer

The activity of ACE was determined on the principle that the tripeptide hydrolysis is blocked at the amino terminal. The tripeptide (Hip-His-Leu) has a sequential terminus of Angiotensin I (Ang I) and its subsequent chemical breakdown due to the reaction with water, could be identified by the resultant hippuric acid formation. The ACE inhibition activity of each plant extract was performed in accordance with the method developed by Santos et al (1985) with amendments[22]. Briefly, 10µl of ACE from rabbit lung (Sigma Aldrich) was incubated in 480µl of assay buffer and 10µl of different concentrations of plant extracts (1000µg/ml, 500µg/ml, 250µg/ml and 125µg/ml) in a test tube for 15 mins at 37ºC. The reaction was terminated by adjusting the pH with 1.2ml of 3.4N NaOH. Ortho-phthaldialdehyde (100µl) was then added to the reaction mixture and left for 10mins. In order to acidify the solution thereafter, 200µl of 3N hydrochloric acid was mixed to the solution. The measurement of the resulting chemical Histidine-Leucine (His- Leu) was made fluorometrically at 365nm excitation and 495nm emission wavelengths using a fluorescence spectrophotometer (F-2500/F-4500 FLsolutions). A blank solution prepared by the addition of the ACE from rabbit lung after 3.4N NaOH without the test compounds was used. Ramipril, a conventional ACE inhibitor drug was used as control.

Percentage Inhibition was expressed as % Inhibition: = Ab-As/Ab x 100;

Where As = absorbance of sample and Ab = absorbance of blank

2.4 Phytochemical anlaysis

2.4.1 Alkaloids

The presence of alkaloids was determined by mixing 0.5g of the crude extract in 5 ml of 1% hydrochloric acid solution. The solution was stirred and thereafter, positioned in a water bath for 10 minutes. The solution was then filtered and three drops of the Mayer's reagent (1.36g of mercuric chloride; 5.0g potassium iodide in 100ml of water) were added to the filtrate. A yellowish brown colour indicated the presence of alkaloids[23].

2.4.2 Tannins

The ferric chloride test[24] was performed to determine the presence of tannins. Ten milliliter (10ml) of water was used to dissolve the plant extracts (0.25 g) and thereafter, three drops of 1% ferric chloride solution was added in a test tube. The reaction was observed for colour changes. A greenish black or a black color formation showed that tannins were present.

2.4.3 Terpenoids

The Salkowski test[25] was performed to determine the presence of terpenoids. Five milliliter (5ml) of the extract was mixed with 2 ml of chloroform in a test tube. Thereafter, 3ml of concentrated sulphuric acid was carefully added. The development of a colour change was observed. The formation of a reddish brown colour confirmed the presence of terpenoids.

2.4.4 Steroids

Test for steroids was determined by dissolving 1mg of each plant extract in 10 ml of chloroform in a test tube. Afterwards, 10ml of concentrated sulphuric acid was added and the colour change was noted. Red ring formation indicated the presence of steroids[26].

2.4.5 Flavonoids

The extracts (1g) were dissolved in 5ml of distilled water in a test tube. Three drops of a diluted lead acetate solution were added and the development of colour was noted and recorded. The formation of a yellow coloration confirmed the presence of flavonoids[27].

2.4.6 Saponins

Determination of saponins was evaluated using the foam test[28]. Each extract (0.5 g) was dissolved in 2 ml of distilled water and shaken for 15 minutes. The formation of a foam layer point to the presence of saponins.

2.4.7 Phenols

The ferric chloride test was used to determine the presence of phenolic compounds[24]. One (1) gram of each extract was placed to dissolve in 2 ml of distilled water. Thereafter, three drops of a 10% ferric chloride solution was added. A black colour formation specified the presence of phenols.

2.4.8 Glycosides

The Borntrager test was used to determine the presence of glycosides[29]. Fifty (50) mg of each extract was put in a 5 ml of hydrochloric acid to dissolve. The solution was immersed in a warm water bath for 2 hours. After 2 hours the samples were filtered, 3 ml of chloroform was added to the filtrate and shaken. The chloroform layer was then separated and 5 drops of 10% ammonia solution was added to this layer to observe the resulting colour change. A pinkish red colour indicated the presence of glycosides.

2.4.9 Cardiac Glycosides

The legal's test was used to determine the presence of cardiac glycosides[30]. One (1) ml of each extract was placed in 1 ml of water to dissolve. Thereafter, three drops of 10% sodium hydroxide, 1 ml of 0.3% nitroprusside sodium and 1 ml of pyridine reagent. A blood red colour disclosed the presence of cardiac glycosides.

2.4.10 Amino Acid

The ninhydrin test for the determination of amino acid was used in this study[31]. Briefly, 1g of each extract was added to 0.25% w/v of the ninhydrin reagent and boiled for 5 minutes. The development of a dark blue coloration indicated the presence of amino acid.

2.4.11 Reducing Sugar

Reducing sugars were determined using the Benedict's test[32]. Three to four drops of Benedicts reagent was added to 0.5g of each extract and heated gently for 5 minutes to observe for any possible colour change. The formation of an orange red precipitate verified the presence of reducing sugars.

2.4.12 Total Phenolic Content

The method developed and standardized by Singleton and Rossi (1965)[33] with minor modifications was used in the determination of the total phenolic content of each extract of the plant. The Folin and Ciocalteu reagent was used in the study. Each plant extract (0.2 ml) was dissolved in a 0.6 ml of distilled water and 0.2 ml of Folin-Ciocalteu's phenol reagent. The reaction was timed for 5minutes and thereafter, 1 ml of sodium carbonate solution saturated (8% w/v in water) was placed into the mixture and the resulting mixture volume was topped up to 3 ml with distilled water and kept in the dark for 40 min for the development of a colour. After incubation the absorbance of the resulting colour was determined at 750 nm wavelength. The phenolic content was expressed as Gallic acid equivalents (GAE/g) of dry plant material with reference to a Gallic acid standard curve with concentrations 25 - 200µg/l. All experimental procedures were conducted in triplicates. Readings for the various extracts and standards were carried out using a spectrophotometer (Cary 50 Bio UV-Vis Spectrophotometer, Varian) at 725 nm with a reagent blank.

2.5 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis of Plant Extracts

The gas chromatography-mass spectrometry analysis of each part of *T. acutiloba* extract was performed using the Perkin-Elmer Gas Chromatography (Clarus 580) which has an MSD spectrometer (Clarus SQ8S) instrument with in-built auto sampler. Samples were analyzed on Elite 5MS columns (30m × 0.25mm id × 0.25µm). The temperature of the oven was programmed from 37ºC to 320ºC at an 18-25ºC/min rate and held for 0.5; 1.85min and 18 at 320ºC, respectively. The temperature of the injector was 250ºC with an MS Ion source temperature of 280ºC with a full scan and a solvent delay of 0 – 2.3min. The scan range of the MS scan was m/z 35-500 in 0.10s. Each sample (1µl) was introduced into a helium carrier gas with a split flow of 20ml/min.

2.6 Heavy Metal Toxicity Testing

Heavy metal toxicity analysis was performed using the inductively-coupled plasma optical emission spectrometry (ICP-OES). The test is based on the principle that when energy from a plasma source is transferred to a sample, there is an excitation of the component elements (atoms) and they move to a higher energy state. Once these stimulated atoms come back to a low

energy state, there are rays of emission that are released. There is therefore the measurement of the released emitted rays that characterises the wavelength of the photons. The constituent elements are identified by their characteristics emission lines and quantified by the intensity of the same lines[34].The following heavy metals were analysed; Arsenic (As), Cadmium (Cd), Chromium (Cr), Mercury (Hg), Lead (Pb), and Nickel (Ni). Each extract (0.5g) was weighed out. The digestion was carried out on a hot plate (60-70 ° C) using 10 ml of Aqua Regia. The mixture was then filtered into a 10ml volumetric flask. The solution was made up to the mark with double distilled water. The solution was then filtered into ICP vials with a 0.45 micro-filter and analysis was performed using ICP-OES. Analysis was performed in triplicate.

2.7 Data Analysis

All experimental observations were performed in triplicate and were expressed as an average of three analyses ± standard error of the mean (SEM). One sample t-test was used in the statistical analyses for comparing two observations and one way ANOVA for multiple comparisons. The p-value<0.05 were regarded as significant.

3. RESULTS

3.1 Plant Extraction and Physicochemical properties

The percentage yield and the physicochemical properties of the parts of *T. acutiloba* are shown in Table 1. The plant is well known for its sweet-scented nature that is inherent in the flowers and leaves.

Table 1. Physicochemical properties and Percentage yield of extracts of *Tulbaghia acutiloba*

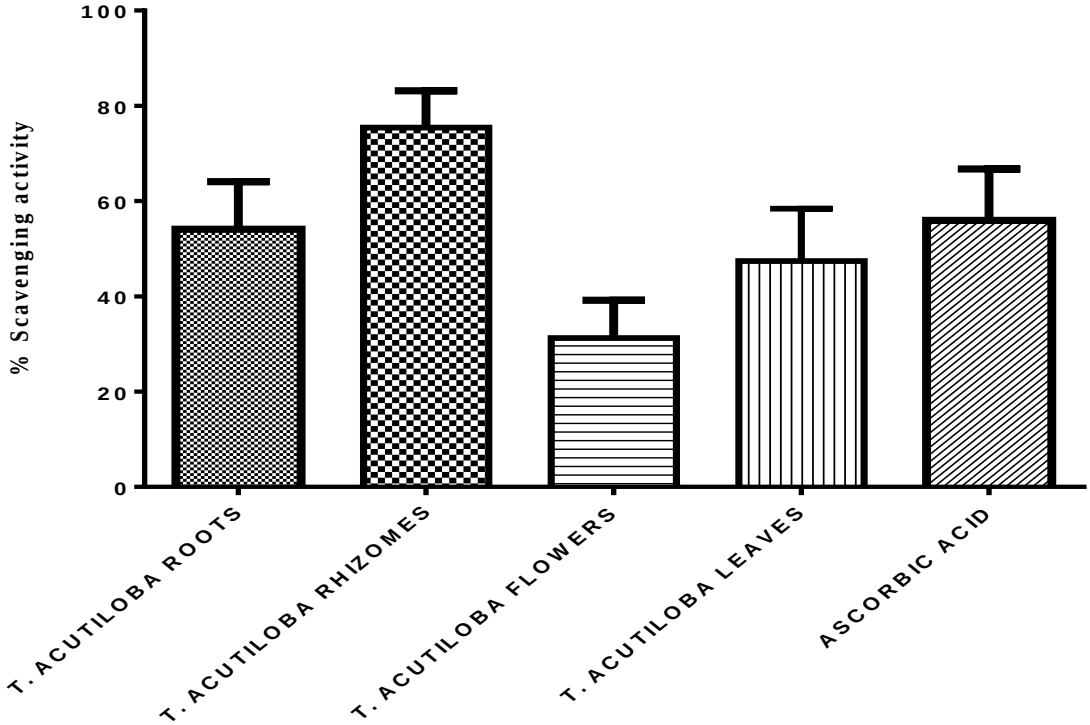
Plant Part	Percentage yield (%)	Physical appearance	odour
Roots	2.62	 Pale brown	Garlic-like odour *(alliaceous odour)*
Rhizomes	8.00	 Brown	Garlic-like odour *(alliaceous odour)*
Flowers	3.62	 Green with fleshy orange to reddish brown ring	Sweet scented
Leaves	12.8	 Dark green	Sweet scented

3.2 Antioxidant Activities

3.2.1 2, 2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity

It is observed from the results that there is a concentration dependent activity for the DPPH radical scavenging activity for the various parts (roots, rhizomes, leaves, flowers) of *T. acultiloba*. Interestingly, the rhizomes showed a relatively higher antioxidant activity at the various concentrations than all the other parts of the plants with an IC50 value of 201.99µg/l and a peak scavenging activity of 95.18± 0.20. However, there was no significant difference in the activities of the various parts of the plants as compared to the positive control, ascorbic acid ($p>0.05$) as shown in figure 1.

Figure 1: DPPH scavenging activity

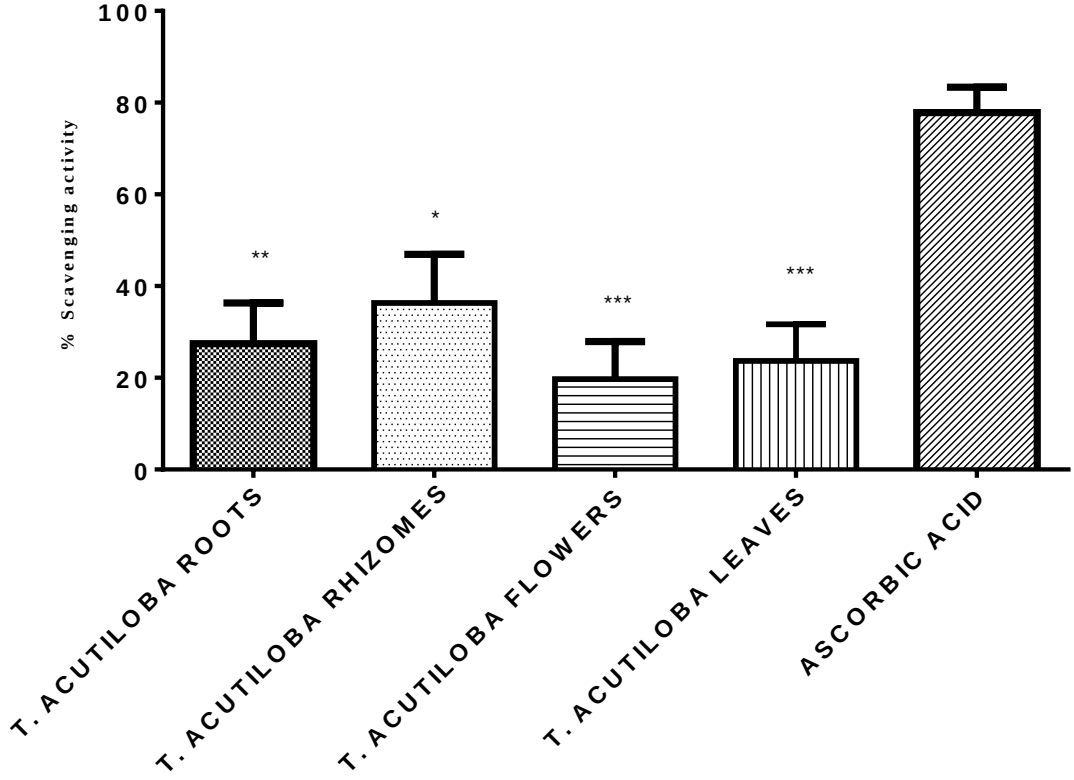


3.2.2 Hydrogen peroxide scavenging activity

Figure 2 shows the hydrogen peroxide scavenging activities of each part of the plant and the positive control. The result indicated a dose dependent inhibition of hydrogen peroxide with the rhizomes once again showing a relatively higher scavenging activity than the other parts of the

plants with a peak value of 72.42±0.35 occurring at the concentration of 1000µg/l and an IC50 value of 518.16µg/l. Interestingly, there was no scavenging activity at 15.6µg/l for all the parts Moreover, all the parts of the plants showed a significantly lower scavenging activities (Leaves ($p<0.001$), flowers ($p<0.001$), roots ($p<0.01$), rhizomes ($p<0.05$) when compared with the scavenging activities of Ascorbic acid.

Figure 2: H_2O_2 Scavenging activity

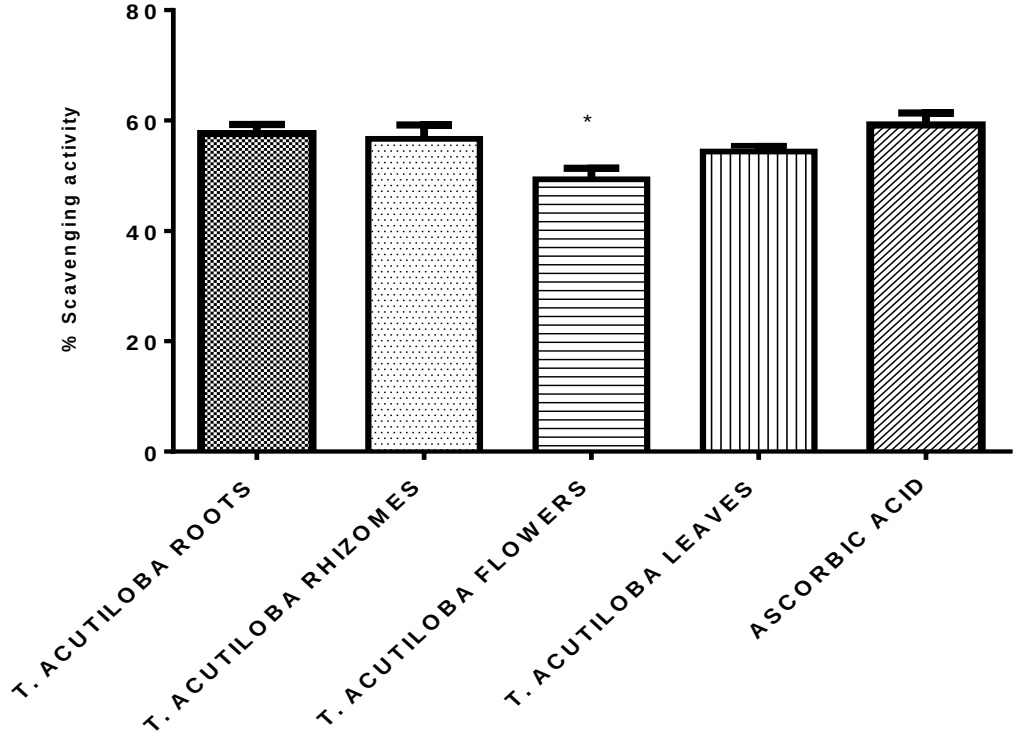


*significantly different ($p<0.05$) from the value of standard compound; ** significantly different($p<0.01$) from the value of standard compound; *** significantly different ($p<0.001$) from the value of standard compound

3.2.3 Nitric oxide scavenging activity of T. acutiloba

The outcome of the free radical scavenging potential of each extract tested is presented in figure 3. There was a generally good scavenging activity for all the extracts though the results did not show a strong dose-dependent activity. Interestingly, the lowest concentration of 15.6µg/l, produced an effective antioxidant activity. The leaves, rhizomes, and roots did not show any significant difference ($p>0.05$) from the activity of Ascorbic acid with the exception of the flowers that showed a significantly lower activity ($p<0.01$).

Figure 3: Nitric Oxide Scavenging Activity

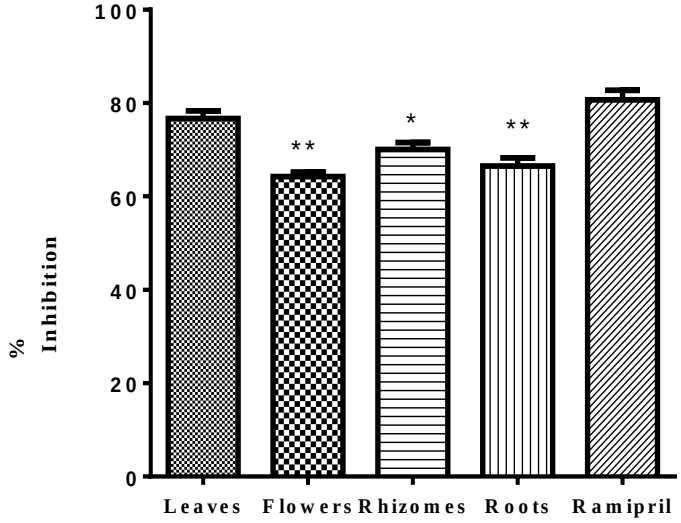


*significantly different ($p<0.05$) from the value of standard compound

3.3 *In vitro* Angiotensin-1 Converting Enzyme (ACE) Inhibitor activity

Figure 4 shows the percentage ACE inhibition of the hydro-methanolic extract of the various parts of the plants. All extracts showed a percentage inhibition of >50% within a concentration range of 125 - 1000µg/ml. The conventional ACEI drug used as a positive control in the study, showed a mean percentage inhibition of 80.67% with an IC50 value of 112.91µg/ml. There were no significant difference from the activity of the leaves (76.6% ± 1.65; IC50:154.23) as compared to the value of the conventional drug, Ramipril. Moreover, the roots and flowers showed a significantly lower activity when compared to both the conventional drug and the leaves. Conversely, the rhizomes showed a significantly lower activity as compared to Ramipril but no significant difference when compared to the activity of the leaves. This observation from the study indicates a comparable activity of the leaves to the activity of the conventional drug, Ramipril.

Figure 4: ACE Inhibition activity of *T. acutiloba*



*Significantly different (P<0.05) from the value of Ramipril; ** Significantly different (p <0.05) from the value of both Ramipril and Leaves.

3.4 Phytochemical analysis and total phenolic content of *T. acutiloba*

The phytochemical analysis of the extracts of the plants is shown in table 2. Our findings indicate the presence of tannins, phenols, alkaloids and amino acids in all parts of the extract of the plant used in the study. Moreover, saponins were found only in the roots of the plants. The rhizomes and leaves showed the presence of terpenoids. However, there was an absence of glycosides, cardiac glycosides, flavonoids and reducing sugars in all parts of the plant. The presence of steroids was identified in the rhizomes only.

Table 2: Phytochemical analysis of *T. acutiloba*

Phytochemical	Rhizomes	Roots	Flowers	Leaves
Tannins	+	+	+	+
Saponins	-	+	-	-
Terpenoids	+	-	-	+
Phenols	+	+	+	+
Glycosides	-	-	-	-
Cardiac Glycosides	-	-	-	-
Steroids	+	-	-	-
Alkaloids	+	+	+	+
Flavonoids	-	-	-	-
Amino Acids	+	+	+	+
Reducing sugars	-	-	-	-

'+' indicates the presence of the compound; '-' Indicates the absence of the compound

3.5 Total Phenolic Content of *T. acutiloba*

The total phenolic content was expressed as mgGAE/g extract as $y = 0.0072x + 0.0819$ ($R^2 = 0.9981$). The total phenolic content of the various extract is represented in table 3. The leaves showed a relatively higher phenolic content of 43.26 ± 1.15 mg/g as shown in table 3.

Table 3: Total phenolic content of parts of *T. acutiloba*

Sample	Total Phenolic content (mg/g)
Rhizomes	28.04 ± 0.36
Roots	31.54 ± 8.28
Flowers	20.80 ± 1.22
Leaves	43.26 ± 1.15

3.6 GC/MS analysis of hydro-methanolic extracts of *T. acutiloba*

Gas chromatography–mass spectrometry (*GC-MS*) was performed to determine the individual constituents in each extract. The various chemical components were detected by corresponding their retention time and mass spectra with that of an inherent citations library. The outcome of the analysis is shown in Table 4. A total of 14 compounds were isolated in the extracts. Major compounds identified included acetic acid, *α-linolenic acid,* palmitic acid, oleic acid, *9,12-octadecadienoic acid* and decanoic acid among others.

Table 4: Compounds Identified in the Gas Chromatographic analysis of *T.acutiloba*

Sample / compound name	**Chemical formula**	**Retention time (mins)**	**Relative abundance height (%)**
4a. Leaves			
Acetic Acid	CH3COOH	9.89	10.07
α-linolenic acid	$C_{18}H_{30}O_2$	21.87	4.24
Palmitic acid	$C_{16}H_{32}O_2$	22.61	5.92
Oleic Acid	$C_{18}H_{34}O_2$	24.22	54.56
4b. Roots			
Acetic Acid	CH3COOH	9.73	5.75
Decanoic acid	$C_{10}H_{20}O_2$	20.16	3.12
1-Tetradecyne	$C_{14}H_{26}$	21.80	3.22
9,12-octadecadienoic acid	$C_{18}H_{32}O_2$	21.81	3.22
Hexanamide	$C_6H_{13}NO$	22.61	8.37
Oleic Acid	$C_{18}H_{34}O_2$	24.22	61.54
4c. Rhizomes			
1-methylcyclopropanemethanol	$C_5H_{10}O$	10.14	3.10
Dodecanamide	$C_{14}H_{29}NO_2$	22.60	7.86
Lauramide	$C_{12}H_{25}NO$	22.61	8.78
9-octadecanamide	$C_{18}H_{37}NO$	24.21	69.44
Oleamide	$C_{18}H_{35}NO$	24.22	71.50
4d Flowers			
Acetic acid	CH3COOH	9.76	8.32
myristimide	$C_{14}H_{29}NO$	22.60	8.15
Palmitic acid	$C_{16}H_{32}O_2$	22.61	6.41
Oleic acid	$C_{18}H_{34}O_2$	24.21	65.79

3.7 Heavy metal toxicity analysis

Table 5 shows the analysis of heavy metals that are of great concern to public health. From the results, it is evident that there were no toxic heavy metals detected in *T. acutiloba*. Moreover, the levels of barium, chromium and nickel were not significant.

Table 5: Toxic heavy metal analysis of *T. acutiloba*

Metal	Root	Leaves	Rhizomes	Flowers	*Minimum toxicity levels*
Arsenic	Not Detected	Not Detected	Not Detected	Not detected	*5-10ng/ml*
Barium	**0.25ng/ml***	**0.29ng/ml***	**0.131ng/ml***	**0.20ng/ml***	*8-14ng/ml*
Cadmium	Not Detected	Not Detected	Not Detected	Not detected	*3-5 ng/ml*
Chromium	**0.12ng/ml***	Not Detected	Not Detected	Not detected	*3.26- 7ng/ml*
Lead	Not Detected	Not Detected	Not Detected	Not detected	*10-15 ng/ml*
Mercury	Not Detected	Not Detected	Not Detected	Not detected	*20-32 ng/ml*
Nickel	**2.06ng/ml***	**0.22ng/ml***	**0.424ng/ml***	**0.15ng/ml***	*4-8.6ng/ml*

*values are far below the minimum toxicity levels

3.8 Correlation between the Antioxidant activites, Total phenolic content and ACE inhibition activities of *T. acutiloba*.

Table 6 indicates the correlation between the antioxidant activities, Total phenolic content and ACE inhibition activities of *T. acutiloba* using the Pearson correlation coefficient analysis. From the results obtained, there was generally a weak positive correlation between the antioxidant activities and ACE inhibition activities. However, a moderate positive correlation was observed between the Total Phenolic content and ACE inhibition activities (r=0.464).

Table 6. **Pearson correlation coefficient between Antioxidant activities, Total Phenolic content and ACE Inhibition activities of *Tulbaghia acutiloba***

	ACE Inhibition Activity	**Total Phenolic Content**
DPPH scavenging activity	0.092	0.168
Nitric Oxide scavenging activity	0.035	0.033
Hydrogen peroxide scavenging activity	0.077	0.226
ACE Inhibition activity		0.464

4. Discussion

The prevention and treatment of hypertension involves several mechanisms in the body that contributes significantly to the regulation of blood pressure[35]. These mechanisms include the reduction of oxidative stress in the body, inhibition of Angiotension-I converting enzyme (ACE), the presence of bioactive compounds as well as the regulation of nitric oxide in the human body[36].

Since most diseases are related to increased free radicals or reactive oxygen species, the potential scavenging activity will reduce excess free radicals which may otherwise lead to disease[37]. Our current findings indicate an active scavenging activity of the various parts of *T. acutiloba*, as seen in the DPPH assay. Our findings in the scavenging activities of the DPPH is in corroboration with other studies done on similar plant species of the same family of *Tulbaghia* where effective scavenging activity was observed[38,39].

Additionally, though our findings indicate a significantly lower scavenging activity as compared to Ascorbic acid, there was generally a good scavenging activity of hydrogen peroxide especially at the highest concentration of 1000µg/L of each part of the plant. This finding is in line with other studies performed on similar species of plants within the family of *Tulbaghia*[38,40]. Hydrogen peroxide is known to be a weak oxidizing agent that incapacitates some enzymes in the body directly, usually through the oxidation of essential thiol (-SH) groups. It is permeable to cells and can cross cell membranes rapidly and once inside the cell, it could form a bond with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radicals and this could account for the basis of many of its toxic effects[41,42]. Therefore, the reducing capacity of an extract or compound may serve as a significant indicator of its potential antioxidant activity[12].

With respect to Nitric Oxide scavenging activities, it was generally observed that there were no significant differences between the activities of the parts of the plants and Ascorbic acid with the exception of the flowers. This findings seems to be in agreement with other studies conducted on *Tulbaghia violacea* which was observed to have a good scavenging activity against Nitric oxide[43,44]. There is a substantial evidence that Nitric oxide (NO) is a potent pleiotropic mediator of physiological processes such as neuronal signaling, smooth muscle relaxation, regulation of cell mediated toxicity and inhibition of platelet aggregation[45]. It is known to have a biphasic

action at the cellular level; optimal concentrations of NO are known to safeguard the tissues against oxidative killing whereas higher doses promotes cell death, and these dual effects occur through distinct mechanisms[46-48]. At higher doses, it is a permeable free radical which has been proven to have a crucial role in the pathogenesis of inflammation and pain. Nitric oxide inhibitors that could reduce excessively high dose of nitric oxide to optimal levels, have been shown to have anti inflammatory effects on inflammatory tissues and tissue changes seen in models of inflammatory bowel disease[49,50]. This may be one of several reasons for its usage by traditional healers in the hypertensive and wound healing process; as anti-inflammatory agents are very crucial in wound healing and the management of high blood pressure.

The phytochemical analysis showed the presence of phenols in all parts of the plant. Phenolic compounds are well known for their antimicrobial and antioxidant protection against cancer and heart diseases. Phenolic compounds are known to exert their antioxidants effect by chelating ions and scavenging free radicals particularly, superoxide ($O_2^{\bullet-}$), peroxyl and hydroxyl radicals ($OH^{\bullet}$). This process inhibits both DNA damage and lipid peroxidation, which can cause membrane damage hence maintaining cellular integrity and body function[53]. The high concentrations of phenolic compounds may account for the medicinal value of *T. acutiloba* in the management and treatment of oxidative stress induced disorders. Moreover, tannins are water soluble polyphenols that bind and precipitate proteins and other organic compounds[51,52]. There are controversial views of the role of tannins in the human body. In the past years, tannins were known to be anti-nutritional because it was suggested that they bind to proteins and amino acids, hence reducing their absorption in the body[53,54]. However recent studies have supported their potential benefit in inhibiting the ACE and hence reducing blood pressure in the vessels[55-57]. Studies have also suggested that low doses of tannins in the human body may be very beneficial[58].

T. acutlioba also has alkaloids. The presence of alkaloids in plants is known for their numerous health benefits and therapeutic functions. Various studies has confirmed the effective role of alkaloids as antiinfectious, antipyretic, aphrodisiac, vasodilator, antihypertensive and anticarcinogenic[59,60]. The vasodilatory mechanism of alkaloids is not well established, however, it is known to lower intracellular calcium concentration which contributes to smooth muscle cell relaxation leading to vasodilation and a subsequent decrease in blood pressure[60-61]. Other studies

conducted on similar species of the plants indicates an active role of such plants in the management of hypertension and infectious diseases[61,62].

Heavy Metal analysis of *T. acutiloba* also revealed that all metals analysed were either undetected or were within the safe limit, therefore, we can deduce that different parts of the plant are safe for human consumption provided it is sampled from a non-contaminated geographical location. Heavy metals are naturally occurring elements with a high atomic weight and density[63] and are considered toxic based on their dose and route of exposure in the human body[8]. Persistent exposure to heavy metals could lead to heart attacks, lung damage, chronic kidney diseases and tremors[8, 45]. Due to the irreversible effects that heavy metals could pose on the health of human beings, this study confirms the validity of the safe usage of *T. acutiloba*[45].

In this study we also found that all parts of the plants at different concentrations produced more than 50% ACE inhibition. Additionally, the activities of the leaves were comparable to the conventional ACEI. Similar studies conducted on the parts of plants in the same family of *Tulbaghia* and related species, also exhibited more than 50% inhibition of ACE[64-66]. Importantly, unlike other studies conducted on other species of *Tulbaghia* and related families, *T. acutiloba* showed very promising ACE inhibition that is comparable to the conventional ACEI, Ramipril. The significance of this is multifold; including the possible modification of these plants derived novel compounds to avoid the debilitating side effects of conventional ACEIs.

Furthermore, it was observed that the correlation between the antioxidants and ACE inhibitory effect was positively associated, although a weak correlation was recorded. However, since a moderately strong positive correlation was observed between the total phenolic content of the plant and ACE inhibitory effect, it suggests that the ability of the plant to inhibit ACE could be due to the synergistic effect of the antioxidant potential, total phenolic content and the presence of other bioactive compounds indentified in the GCMS analysis. Key among these bioactive compounds is the presence of α-linolenic acid (ALA), which was found mainly in the leave extract and absent in the other parts of the plant extracts. Palmitic acid and oleic acid were also identified as bioactive compounds which have supporting literature confirming their therapeutic effect especially in regards to cardiovascular diseases[67,68]. Interestingly, the higher concentration of total phenolic content, antioxidant potential and the presence of ALA and other bioactive compounds in the leaves may account for the comparable ACE inhibition activity of the leaves to

the conventional drug, Ramipril. This is worth noting since none of the parts of the plants exhibited a comparable activity to Ramipril. Several studies conducted on ALA (also known as omega-3-fatty acids) has proven to be an effective therapeutic agent especially in relation to cardiovascular diseases such as hypertension [69-71]. Studies conducted on other species of plants of Tulbaghia and similar plants revealed compounds such as palmitic acids, oleic acids, octadecanoic acids among others which were also identified in our studies[72-74].

Though there are several studies to suggest that isolated compounds may produce therapeutic effects, current studies has also confirmed that bioactive compounds in certain plants may produce superior therapeutic effects when they act synergistically with other bioactive compounds[75-78].

5. Conclusion

The current study has so far shown that *T. acutiloba* contains bioactive compounds that are essential for therapeutic effects. It further shows that *T. acutiloba* may have an antihypertensive effect since it showed a comparable ACE inhibition activity to Ramipril. This finding therefore, support the ethnopharmacological use of *T. acutiloba* Harv.(Alliaceae) in the management and regulation of essential hypertension particularly in areas of the world where access to conventional ACEI drugs are limited for several reasons. *T. acutiloba* is a nutraceutical that can easily be harvested and obtained for its therapeutic effects. However, further studies are needed to explicate its effect on body organs, biochemical profile and relevant gene expressions *in vivo* in order to ascertain its comprehensive effect on hypertension.

6. Study Limitations

The study evaluated the *in vitro* activities of *Tulbaghia acutiloba* and therefore its effect *in vitro* may not be an indication of its activities *in vivo*. Therefore further studies are needed to assess its effect in an *in vivo* experimental model of hypertension.

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Chapter 4: Manuscript Three

Cardioprotective effects of *Tulbaghia acutiloba* hydro-methanolic leaf extract and Ramipril in an L-NAME induced hypertensive rat model

The phytochemical content, antihypertensive potential, antioxidant effect and heavy metal toxicological safety exhibited by *Tulbaghia acutiloba* in the *in vitro* study identified in chapter 3, raises an important concern for its effect in a long term *in vivo* administration of the plant on antioxidant activities, systolic and diastolic blood pressure as well as its effect on cadioprotection. The leave extract showed a better therapeutic potential as evident in the ACE inhibition effect which was higher than all the other parts of the plants and comparable to Ramipril. In addition, the leaves showed the presence of essential bioactive compounds such as α-linolenic acid as well as high phenolic content and antioxidant activity as observed in the study conducted in chapter 3 and hence the need to investigate further in an *in vivo* model of hypertension. Since hypertension is closely associated with oxidative stress, cardiovascular diseases, cardiovascular end-organ damages and NO bioavailability, there was the need to evaluate the antihypertensive and cardioprotective effect of *Tulbaghia acutiloba* hydro-methanolic leaf extract in an L-NAME induced hypertensive rats. Thus chapter 4 presents a detailed *in vivo* study and analysis of *Tulbaghia acutiloba* hydro-methanolic leaf extract on hypertension and its associated cardiovascular function and protection.

Cardioprotective effects of *Tulbaghia acutiloba* hydro-methanolic leaf extract and Ramipril in an L-NAME induced hypertensive rat model

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ABSTRACT

The indigenous herb, *Tulbaghia acutiloba*, has been used traditionally in the management of hypertension, but to date there is no scientific validation of its cardiovascular effects. We evaluated the effect of *T. acutiloba* (TA) on *N^{ω}-Nitro-L-arginine methyl ester hydrochloride* (L-NAME)-induced hypertension and its effect on cardioprotection. Experimental hypertension was induced by orally administering L-NAME (50mg/kg b.w.) in rats for 5 weeks. Rats were treated with varying doses of hydro-methanolic leaf extracts of TA (40, 60 and 80mg/kg b.w.) and Ramipril (10mg/kg b.w.) in separate groups for 5 weeks. Systolic (SBP) and diastolic blood pressure (DBP) were measured weekly using the tail-cuff method. The rats were sacrificed on Day 36 by halothane overdose. Blood and heart tissues were collected for biochemical and molecular assays, as well as histological examinations. Treatment of TA in the hypertensive rats significantly reduced SBP and DBP. Rats with hypertension showed cardiac hypertrophy and this condition was attenuated with the administration of TA and Ramipril. TA and Ramipril administration further reduced cardiac troponin I (cTnI) levels, creatine phosphokinase (CK), CK-MB(creatine phosphokinase- muscle/brain), and angiotensin-converting enzyme (ACE) activity in the L-NAME induced rats. Concentrations of hepatic enzymes including alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) were also reduced significantly with the treatment of TA and Ramipril. There was a significant increase in plasma and tissue nitric oxide (NO) concentrations as well as increased superoxide dismutase(SOD), catalase(CAT) and glutathione (GSH) concentrations with the treatment of TA. Lipid peroxidation, measured in TBARS, was significantly reduced in the TA treated groups. TA significantly decreased cardiac tissue mRNA expressions of ACE, AT1 and AT2 while increasing tissue expressions of B1 and B2 significantly. Additionally, histopathological examination confirmed the biochemical findings of the protective effect of TA on cardiac tissues. In all investigations, the TA dose of 80mg/kg showed a maximum effect as compared to the other dosages. These results suggest that TA dose of 80mg/kg, may have beneficial role in the treatment of hypertension and act as a protective agent against a long term cardiovascular abnormalities associated with hypertension.

Key words: *Tulbaghia acutiloba*, Cardioprotection, Hypertension, L-NAME, Antioxidant enzymes.

Introduction

Cardiovascular disease and its associated complications remain a leading cause of morbidity and mortality in both developed and developing countries (1). Hypertension is a leading risk factor for cardiovascular disease, accounting for 7.6 million deaths globally. Recent studies indicate that 54% of stroke and 47% of coronary heart disease are attributed to hypertension (1, 2).

The renin–angiotensin aldosterone system (RAAS) plays a key physiological role in the maintenance and regulation of blood pressure and body fluid homeostasis (3). The octapeptide angiotensin II (Ang II) is the main effector in RAAS. Classically, by binding to type I angiotensin II receptor (AT1), Ang II activates a cascade of actions and reactions that results in vasoconstriction and increased sodium (Na) reabsorption leading to high blood pressure. Ang II is produced when angiotensin-converting enzyme (ACE), a zinc containing metalloprotease hydrolyses peptides by removing a dipeptide from the C-terminus from inactive decapeptide angiotensin I (4). RAAS, therefore, provides crucial targets for the treatment of hypertension and its related end-organ diseases. Angiotensin-converting enzyme is well recognized therapeutic target, whose levels are known to increase in myocardial infarction and atherosclerosis (3). Reports indicate that chronic administration of nitric oxide synthase inhibitor, *N*ω-nitro-L-arginine methyl ester hydrochloride (L-NAME) activates the RAAS (5-7). Furthermore, blockade of the RAAS with a potent ACEI has been shown to avert increased blood pressure in L-NAME-treated animals (8).

The L-NAME model is widely used to show the therapeutic efficacy of putative antihypertensive agents. This model is based on the chronic inhibition of nitric oxide synthase (NOS) by, an L-Arginine analogue, L-NAME leading to a cascade of reactions that elevates blood pressure and its pathophysiological process has been closely linked to essential hypertension (9). Nitric oxide (NO) is a well-known biological mediator involved in several physiologic and pathological processes, notably in the regulation of vascular function, blood pressure and body fluid homeostasis (10). In view of this, the reduction in the biosynthesis of NO basal levels due to its chronic inhibition with an orally active nitric oxide synthase (NOS) inhibitor, L-NAME, makes it a suitable hypertensive model for experimental studies (11). ACE inhibitors, such as Ramipril and Captopril, have been extensively used in the treatment and management of hypertension. In addition to ACE inhibition, Ramipril and Captopril are also known to decrease blood pressure by preventing the breakdown of bradykinin, a potent endothelium-dependent vasodilator (12). Importantly, several studies have suggested that an increase in bradykinin levels with ACE inhibitors, is accompanied by a potential augmentation of NO levels leading to a vasodilatory effect (13).,

Although Ramipril and Captopril are conventional ACE inhibitors in the treatment of hypertension, there are still setbacks, such as high cost and side effects including persistent dry cough, nephropathies and targeted organ dysfunctions (14). Hence there has been a focus on 'herbal alternatives' with minimal perceived side effects (15) such as *Tulbaghia acutiloba.*

Tulbaghia acutiloba, belonging to the family, Amaryllidaceae, is traditionally used as a culinary herb, infusion for chest and gastric ailments, bacteria and fungi infections as well as management of hypertension (16). Despite its classification in the well-studied *Alliaceae* family (17, 18) this plant's biological and therapeutic activities have not been investigated. Previous studies in our laboratory showed a very potent *in vitro* antioxidant activity of the plant. We showed that all parts of the plants (leaves, flowers, rhizomes, roots) showed a >50% *in vitro* ACE inhibition activity, with the leaves showing a significantly higher inhibitory activity, comparable to Ramipril (19). Therefore, this study was undertaken to examine the *in vivo* antihypertensive and cardioprotective effect of *T. acutiloba* (TA) hydro-methanolic leaf extracts in an L-NAME hypertensive model.

MATERIALS AND METHODS

Plant material and preparation

The leaves of *T. acutiloba* were freshly harvested and collected after being authenticated by a botanist, Prof. Himansu Baijnath, at the School of Life Sciences, University of KwaZulu-Natal (UKZN), Durban, South Africa. They were thoroughly washed under running water and dried at room temperature for 72 hours. Subsequently, the leaves were crushed in a Waring blender. The powder (300g) obtained after crushing was immersed in 3L hydro-methanolic solution (80% methanol and 20% water). The mixture was agitated at 80rpm for 48 hours and thereafter filtered. The filtrate was concentrated in a rotary evaporator and freeze-dried at -40ºC to yield an extract for experimental use. The hydro-methanolic extract of the leaves showed a yield of 12.8%.

Animal Study

Animals

Forty-two, healthy, male albino Wistar rats (155-210g) were acquired from the Biomedical Resource Unit (BRU) of the University of KwaZulu-Natal (UKZN), South Africa. The animals were housed in a propylene cage at a temperature range of 19-23ºC with a 12h light/12h dark cycle. Water and standard rat chow were provided *ad libitum* to all animals. Ethical approval was obtained from the UKZN Animal Ethics Committee (Reference number: AREC/062/016PD).

Chemicals

N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) and Ramipril were procured from Sigma-Aldrich (St. Louis, Missouri, USA).

L-NAME –induced arterial hypertensive model and Experimental protocol.

The experimental rats were given L-NAME orally at a dosage of 50mg/kg body weight (b.w) daily for 5 weeks (20, 21). Ramipril and *T. acutiloba* were dissolved in distilled water and all doses were dispensed, orally, daily (at the same time every morning) via an intragastric tube, for 5 weeks. The animals were randomly grouped into 6 groups of 7 animals each, as outlined below;

Group 1: Control (distilled water only)
Group 2: L-NAME control (50mg/kg b.w.)
Group 3: L-NAME + Ramipril (10mg/kg b.w.)
Group 4: L-NAME + *T. acutiloba* leaves (40mg/kg b.w.)
Group 5: L-NAME + *T. acutiloba* leaves (60mg/kg b.w.)

Group 6: L-NAME + *T. acutiloba* leaves (80mg/kg b.w.)

Blood pressure, heart rate and body weights measurements

Systolic and diastolic blood pressure measurements as well as heart rates were evaluated using the non-invasive tail-cuff method (IITC, Life Sciences Inc. Model 31, Woodland Hills, CA, USA). Prior to the initiation of the procedure, the animals were pre-conditioned for 7 days in order to acclimatize the animals to the conditions of the experiment. They were placed in restrainers in heating chambers and exposed to a temperature of 31-34 ºC for 15-20 minutes. The blood pressure measurements and heart rate were recorded every week during the experimental period. Values reported are the mean value of three consecutive readings. Body weights were determined weekly for the duration of study using a pre-calibrated electronic scale.

Animal Sacrifice

The duration of the animal study was 35 days (5 weeks). On day 36, the rats were sacrificed by the administration of halothane overdose and blood was collected via cardiac puncture. Plasma and serum samples were obtained through centrifugation at 224 x g for 10minutes prior to biochemical analysis. The heart, liver and kidneys of each rat was harvested, washed to remove blood and weighed. Thereafter they were snap-frozen in liquid nitrogen. All samples were stored temporarily at -70ºC before use. The heart was further divided into two; the left heart was used for molecular assays while the right heart was fixed in a neutral buffered formalin (10%) for 72 hours and used for histological assessment

NOx estimation

Plasma and heart tissue nitric oxide (NO) estimation was determined based on the principle of the Griess reaction (22, 23). The reaction of nitrate with a mixture of sulfanilamide and naphthlethylenediamine, leads to the formation of a chromophore which is measured at an absorbance of 550nm. There is a reduction of nitrate to nitrite after incubating the mixture for 30 minutes in the presence of NADPH with nitrate reductase. A standard curve was used to estimate the amount of nitrite/nitrate in the plasma and heart tissue homogenates. Units were expressed as μmol/L.

Antioxidant Assay in heart tissues

Enzymatic antioxidant activities of catalase (CAT) and superoxide dismutase (SOD) in heart tissues were determined using a method adapted from those developed by Sinha (1972) and Kakkar *et al*., (1984) respectively. Non-enzymatic antioxidant determination of reduced glutathione (GSH) in heart tissues was

estimated by the method of Ellman (1959) with modifications. Thiobarbituric acid-reactive substances (TBARS) concentration was estimated using the method developed by Niehaus & Samuelsson (1968).

Serum ACE activity and cardiac function

Serum angiotensin-converting enzyme (ACE) activity was measured by an enzymatic rate method using available commercial kit purchased from Trinity Biotech, USA and experimental procedures performed according to manufacturer's instructions. Cardiac markers including creatine phospokinase (CK), creatinine phosphokinase-muscle-brain (CK-MB- the bound combination of the isoenzymes CKM and CKB of the enzyme phosphocreatine kinase) and Troponin I (cTnI) were measured in the serum using an automated chemistry analyser (Beckman Coulter Synchron, DXC 600, Access Clinical System, USA).

Estimation of Serum Lipids and hepatic function markers

Serum Triglyceride (TG), HDL-Cholesterol and Total Cholesterol concentration were measured using an automated chemistry analyser (Beckman Coulter Synchron, DXC 600, Access Clinical System, USA). Low-density Lipoprotein (LDL-C) was calculated according to the formula developed by de Cordova and de Cordova, 2013 (24) as follows: LDL-C = ¾(TC-HDL-C). Very Low-density Lipoproteins (VLDL) were estimated using the formula developed by Friedwald *et al*, 1972 (25) as follows VLDL= TG/2.2 (in mmol/l). Hepatic function markers including alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) were measured in the serum using an automated chemistry analyser (Beckman Coulter Synchron, DXC 600, Access Clinical System, USA).

Analysis of mRNA Expression

Total mRNA was extracted from left cardiac tissues using the Zymo Research Quick-RNA Miniprep kit (ZymoResearch, Irvine, USA) according to the protocol of the manufacturer. The purity and concentration of RNA were determined by a nanodrop spectrophotometer (Thermoscientific Nanodrop 1000 Spectrophotometer). Thereafter a total RNA of 1µg was reverse transcribed to cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratory (Pty) Ltd. USA) according to the manufacturer's protocol.

Real time-Polymerase chain reaction (RT-PCR) was carried out using the Lightcycler 96® (Roche Diagnostics, Germany). Conditions used for the RT-PCR analysis were as follows: 95ºC initial denaturation cycle for 10minutes thereafter a PCR run consisting of 40 cycles at 95ºC for 15s, 60ºC for 30s and 72ºC for 30s with a single fluorescent measurement. The housekeeping gene used in the analysis was Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the fluorescent dye utilized in the analysis was SYBR Green 1 (SSO Advanced Univers SYBR GR. SUPRMIX, Bio-Rad Laboratory (Pty)

Ltd. USA). The oligonucleotide primer sequences for the various genes investigated in the RT-PCR analysis were as follows:

Gene	Forward Primer	Reverse Primer
ACE	5'-TCGGCCATGTTGAGCTACTTC-3'	5'-TCCCCATGCAGCTCGTTC-3'
AT1a	5'-TATCACAGTGTGCGCGTTTCA-3'	5'-TGGTAAGGCCCAGCCCTAT-3'
AT2	5'-CCTTCCTGTATTGTTTCGTTGGA-3	5'-CGGCAAGACATAGTCTCTCTCTTG-3'
B1	5' - AGG GTT CGT CAT CAC TAT -3'	5'- AGG TAG ATT TCC GCT ATG -3'
B2	5'-TGA GGAACAACGAGATGAAGAAG -3'	5'- GGAAACCAACACAGCACAAAG AC-3'
GAPDH	5'-TGACAACTCCCTCAAGATTGTCA-3'	5'-GGCATGGACTGTGGTCATGA-3'

Histological Examination of cardiac tissues

Sections of 5µm thickness was prepared from the heart tissues of each animal from processed paraffin-embedded samples. The tissue sections of the heart were stained with Haematoxylin and Eosin (H&E) and examined under a light microscope for indications of tissue changes. Micrographs of the cross-sectional area of tissue samples were taken at 40x magnification and analyzed using the Leica SCN 400 scanner (Leica SCN 400, Germany).

Statistical Analysis

All values are presented as means ± SEM for seven rats in each group. Data analysis was computed using analysis of variance (ANOVA), followed by post-hoc tests for multiple comparisons using either the Bonferroni and Dunnett's test (as appropriate), on GraphPad Prism Version 6. A p-value of <0.05 was considered significant.

RESULTS

Effect of TA on body weight, heart weight and heart rate

The effect of *T. acutiloba* (TA) at three different concentrations (40, 60 and 80mg/kg) on the body weight, heart weight and heart rate is shown in Table 1. There were no significant differences in body weights (g) in the 6 experimental groups at baseline and at week 5 (P>0.05). The L-NAME untreated rats (group 2) showed a significantly increased heart weight and heart weight-to-body weight ratio (P>0.05), while treatment with TA or Ramipril showed a significant decrease (P <0.05). Moreover, there were no significant differences in the heart weight and heart weight-to-body weight ratio between the control group, Ramipril group and the three different concentrations of TA. The baseline heart rate (bpm) of the 6 experimental groups were not significantly different (P>0.05), however, the L-NAME untreated group showed a significant increased heart rate (bpm) at week 5 as compared to the other experimental groups. This increase was attenuated by the treatment of Ramipril and TA at the three different doses (40,60, 80mg/kg).

Table 1: Body weight(b.w.), Heart weight(HW) and Heart Rate(HR) of the various experimental groups (1-6)

Parameter / Samples	Groups 1	2	3	4	5	6
B.W.(g) (baseline)	170.14 ± 9.15	170.57 ±7.20	176.43 ± 5.20	173.57 ± 1.80	173.86 ± 6.01	172.83 ± 12.62
B.W.(g) (Week 5)	316.86 ± 9.85	295.43 ±8.08	305.57 ± 12.39	292.14 ± 7.38	315.14 ± 8.62	308.00 ± 2.97
HW(g)	1.02 ± 0.05	1.56 ± 0.08 [a]	1.02 ± 0.04 [b]	1.00 ± 0.04 [b]	1.11 ± 0.03 [b]	1.01 ± 0.09 [b]
HW/b.w. (%)	0.34 ± 0.02	0.56 ± 0.02 [a]	0.35 ± 0.02 [b]	0.37 ± 0.02 [b]	0.38 ± 0.02 [b]	0.36 ± 0.03 [b]
HR(bpm)(baseline)	368.71 ± 9.21	373.43 ±7.07	373.71 ± 11.05	381.29 ± 7.46	382.57 ± 8.54	381.83 ± 4.81
HR(bpm) (Week 5)	396.86 ± 0.91	456.71 ±4.84[a]	386.00 ± 2.32[b]	400.00 ±2.45[b]	392.00 ±1.18[b]	392.83 ± 1.56[b]

Group 1- Control; Group 2-L-NAME(50mg.kg); Group 3- L-NAME + Ramipril(10mg.kg); Group 4- L-NAME + TA(40mg/kg); Group 5- L-NAME + TA(60mg/kg); Group 6- LNAME + TA(80mg/kg). Values are expressed as mean ± SEM for seven rats per group. [a] P <0.05 vs. control; [b] P <0.05 vs. L-NAME.

Effect of TA on blood pressure

The effect of TA at concentrations of 40, 60 and 80mg/kg on systolic and diastolic blood pressure in L-NAME induced hypertensive rats are shown in Figures 1 and 2. It was observed that there was a significant (P<0.05) increase in systolic and diastolic blood pressure in the L-NAME induced rats from week 2 to week 5 and this increase was attenuated by the treatment of TA and Ramipril. The effect of TA

on systolic and diastolic blood pressure was more pronounced at doses of 60 and 80mg/kg. There was no significant difference in systolic and diastolic blood pressure between the TA treated rats at 60 and 80mg/kg, Ramipril and the control group throughout the study.

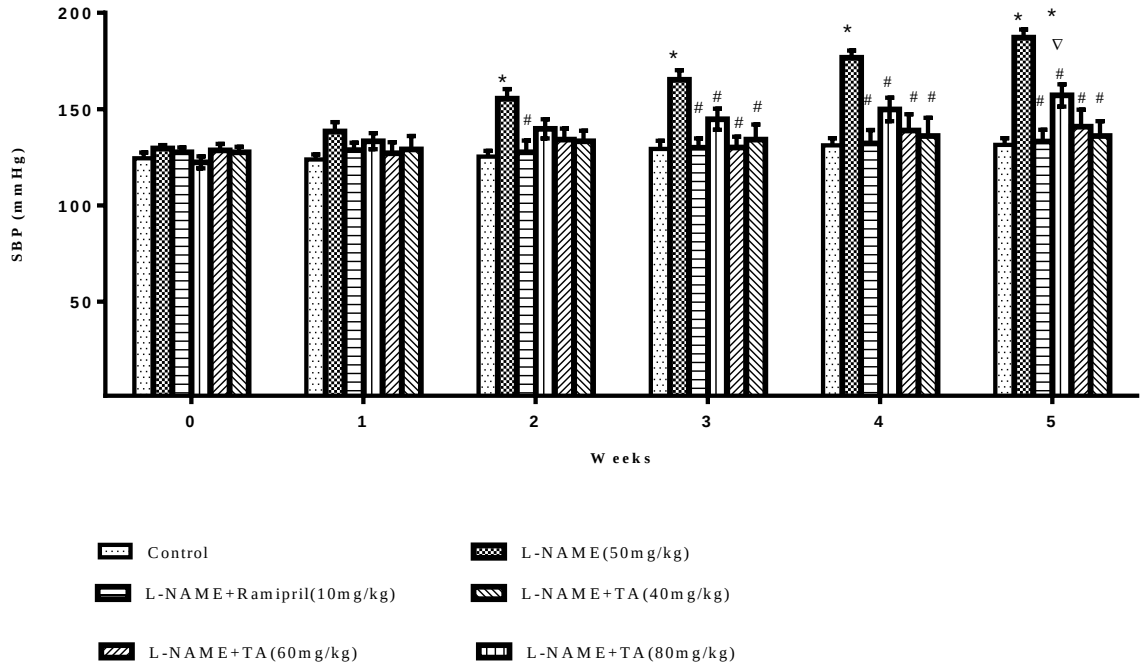

Figure 1: Effect of TA on systolic blood pressure (SBP) in L-NAME induced hypertensive rats, Values are expressed as mean ± SEM for seven rats per group. * *P*< 0.05 vs. Control; # *P* <0.05 vs. L-NAME; ▽ *P*<0.05 vs. Ramipril

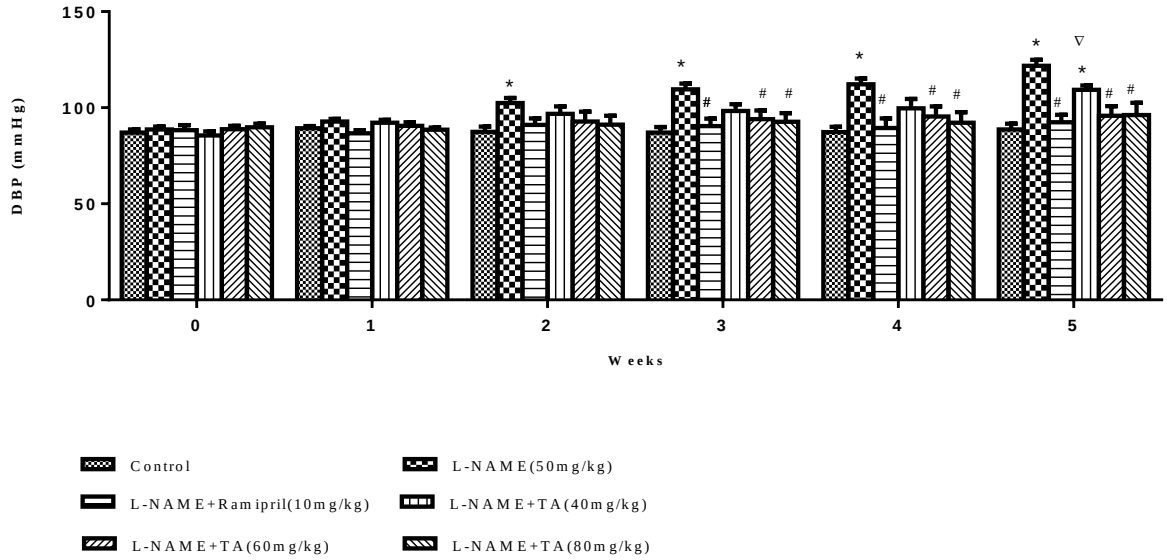

Figure 2: Effect of TA on diastolic blood pressure (DBP) in L-NAME induced hypertensive rats, Values are expressed as mean ± SEM for seven rats per group. * *P*< 0.05 vs. Control; # *P* <0.05 vs. L-NAME; ▽ *P*<0.05 vs. Ramipril

Effect of TA on plasma NOx concentration

The effect of TA on plasma nitric oxide metabolites (NOx) concentration in L-NAME induced hypertensive rats are shown in figure 3. It was observed that the L-NAME administration significantly (P<0.05) decreased the plasma NOx concentration when compared to the control, whereas the treatment with TA and Ramipril significantly restored the plasma NOx concentration. There was no significant difference in the plasma NOx concentration of the TA treated rats, Ramipril and the control groups.

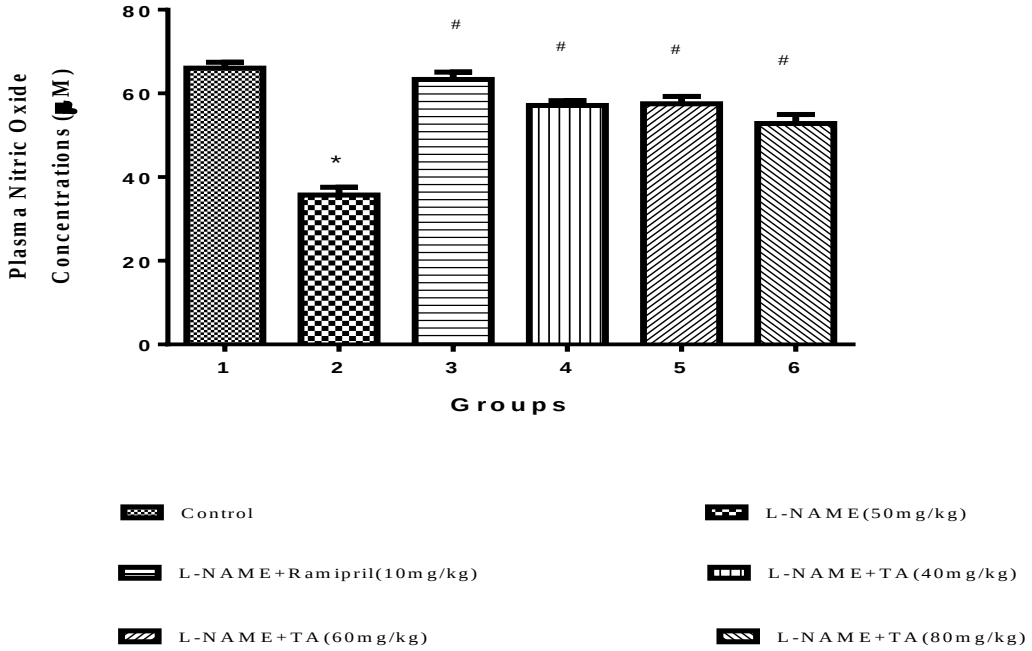

Figure 3: The effect of TA on plasma NOx concentration in L-NAME induced hypertensive rats. Values are expressed as mean ± SEM for seven rats per group. * *P*< 0.05 vs. Control; # *P* <0.05 vs. L-NAME.

Effect of TA on enzymatic and non-enzymatic antioxidant activity in heart tissue

The effect of TA on enzymatic and non-enzymatic antioxidants in heart tissue samples is shown in Table 2. The L-NAME untreated rats showed a significant (P<0.05) reduction in the activities of SOD and CAT when compared to the control whiles the administration of TA and Ramipril significantly increased the activities of these enzymatic antioxidants. It was also observed that GSH levels were significantly (P<0.05) reduced in the L-NAME untreated group and the treatment with TA and Ramipril significantly increased the levels with maximum effect at 80mg/kg of TA. Moreover, the levels of TBARS were significantly increased in the L-NAME untreated group and the administration of TA and Ramipril significantly (P<0.05) reduced the levels of TBARS with a more pronounced effect at 80mg/kg of TA. However, the levels of TBARS at 40mg/kg of TA was significantly increased when compared to the Ramipril treated group and the control group. Similar to plasma NO levels, NOx concentration at the heart tissue level of the L-NAME untreated group significantly decreased (P<0.05) when compared to the control. There was also a reduction in all the treated groups (Ramipril, 80, 40 and 60mg/kg). However, none were significantly different compared to the control.

Table 2: Effect of TA on superoxide dismutase (SOD), catalase (CAT), reduced Glutathione(GSH), Thiobarbutiric acid reactive substances(TBARS) and NOx activities in heart tissue

Parameter Samples	Groups					
	1	2	3	4	5	6
SOD(U[a]/mg protein)	13.17 ± 1.10	7.75 ± 0.99[a]	12.64 ± 0.72[b]	10.77 ± 0.74	12.11 ± 0.56[b]	16.68 ± 0.79[b,c,d,e]
CAT(U[b]/mg protein)	63.54 ± 2.07	38.10 ± 0.28[a]	61.63 ± 1.11[b]	60.75 ± 1.64[b]	49.93 ± 0.31[b]	44.97 ± 1.12[b]
GSH (µg/mg protein)	0.023 ± 0.00	0.016 ± 0.00[a]	0.023 ± 0.00	0.03 ± 0.01[b]	0.02 ± 0.00	0.04 ± 0.01[a,b]
TBARS (mmol/100g wet tissue)	0.49 ± 0.04	1.28 ± 0.12[a]	0.63 ± 0.02[b]	0.95 ± 0.03[a,b,c]	0.79 ± 0.02[a,b]	0.61 ± 0.03[b]
NOx(µmol/l)	92.73 ± 1.02	74.59 ± 4.94[a]	79.07 ± 0.93[a]	82.09 ± 1.10	76.47 ± 5.08[a]	91.47 ± 1.10[b]

Group 1- Control; Group 2-L-NAME(50mg.kg); Group 3- L-NAME + Ramipril(10mg.kg); Group 4- L-NAME + TA(40mg/kg); Group 5- L-NAME + TA(60mg/kg); Group 6- LNAME + TA(80mg/kg). Values are expressed as mean ± SEM for seven rats per group. [a] *P* <0.05 vs. control; [b] *P* <0.05 vs. L-NAME; [c] P<0.05 vs. Ramipril; [d] P<0.05 vs. TA(40mg/kg); [e] P<0.05 vs. TA(60mg/kg)

Effect of TA on serum ACE activity and cardiac function markers

The effect of TA on serum ACE activity and cardiac function markers is shown in Table 3. Serum ACE activity increased significantly (P<0.05) in the L-NAME untreated group when compared to the control group and this increase was attenuated by the administration of TA and Ramipril with maximum effect at 80mg/kg of TA. There was no significant difference between the control, Ramipril and TA treated groups. Serum cardiac markers CKMB and Troponin I (cTnI) were significantly increased (P<0.05) in the L-NAME group whiles the treatment with TA and Ramipril led to a significant decrease with maximal effect at 60 and 80mg/kg of TA. However, the 40mg/kg dose of TA showed a significant increase in serum CKMB levels when compared to the control, Ramipril group and TA treated group at dose 80mg/kg. There was no significant difference in the serum CKMB and cTnI in the control, Ramipril and TA dose of 80mg/kg. There was no significant difference in the serum creatine phosphokinase (CK) in all the experimental groups, although there was a tendency for decreased levels in the Ramipril and TA treated groups of 60 and 80mg/kg dose.

Table 3: Effect of TA on serum ACE activity and cardiac function markers.

Parameter / Samples	Groups 1	2	3	4	5	6
ACE (U/L)	158.00 ± 15.18	261.50 ±23.64[a]	222.7 ± 22.59	216.10 ±9.34	244.60 ±23.43	206.50 ± 15.57
CKMB (IU/L)	58.81 ± 3.38	99.60 ± 4.09[a]	70.69 ± 3.77[b]	98.84 ±3.84[a,c]	60.10 ± 4.98[b,d]	58.33 ± 4.47[b,d]
CK (IU/L)	906.4 ± 72.04	1064 ± 149.9	949.1 ± 90.76	1094 ± 131.2	869.5 ± 103.9	705.8 ± 66.70
cTnI (ng/ml)	0.37 ± 0.03	0.88 ± 0.13[a]	0.51 ± 0.05[b]	0.77 ± 0.04[a]	0.53 ± 0.02[b]	0.55 ± 0.05[b]

Group 1- Control; Group 2-L-NAME(50mg.kg); Group 3- L-NAME + Ramipril(10mg.kg); Group 4- L-NAME + TA(40mg/kg); Group 5- L-NAME + TA(60mg/kg); Group 6- LNAME + TA(80mg/kg). Values are expressed as mean ± SEM for seven rats per group. [a] P <0.05 vs. control; [b] P <0.05 vs. L-NAME; [c] P<0.05 vs. Ramipril; [d] P<0.05 vs. TA(40mg/kg).

Effect of TA on lipid profile and hepatic function

The effect of TA on the lipid profile and hepatic function is presented in Table 4. There was no significant difference in the lipid profile in all the experimental groups in the study, although total cholesterol, LDL-C and VLDL-C were slightly elevated in the L-NAME group as compared to the control and the TA treated groups. The Ramipril treated group showed a slightly increased triglyceride and HDL-C levels when compared to the control, L-NAME group and the TA treated groups. Hepatic function markers were represented by a significant (P<0.05) increase in ALT levels in the L-NAME untreated group but this increase was attenuated by the administration of Ramipril and TA with a more pronounced effect at the 80mg/kg dose. However, the TA treatment at 40mg/kg was significantly (P<0.05) increased, when compared to the control and Ramipril group. There was no significant difference in ALT levels between the Ramipril and the TA dosages of 60 and 80mg/kg, respectively, although Ramipril showed a greater effect (P<0.05). There were no significant differences in AST and ALP levels in all experimental groups, although the L-NAME untreated group showed a slightly increased concentration.

Table 4: The effect of TA on lipid profile and hepatic function markers

Parameter / Samples	Groups 1	2	3	4	5	6
Total Cholesterol (mmol/l)	0.86 ± 0.05	1.00 ± 0.04	0.90 ± 0.06	0.94 ± 0.05	0.95 ± 0.05	0.85 ± 0.06
Triglycerides (mmol/l)	1.331 ± 0.09	1.37 ± 0.16	1.87 ± 0.23	1.43 ± 0.31	1.69 ± 0.13	1.45 ± 0.33
HDL-C (mmol/l)	0.69 ± 0.03	0.74 ± 0.04	0.75 ± 0.04	0.67 ± 0.03	0.70 ± 0.02	0.69 ± 0.02
LDL-C (mmol/l)	0.15 ± 0.03	0.19 ± 0.02	0.11 ± 0.02	0.21 ± 0.02	0.18 ± 0.02	0.14 ± 0.04
VLDL-C(mmol/l)	0.61 ± 0.04	0.66 ± 0.06	0.85 ± 0.11	0.65 ± 0.14	0.66 ± 0.13	0.57 ± 0.16
AST (IU/L)	128.9 ± 4.93	130.6 ± 6.90	120.0 ± 7.16	147.4 ± 13.72	120.5 ± 2.60	116.0 ± 2.98
ALT (IU/L)	59.14 ± 4.17	98.40 ± 8.51a	49.43 ± 3.44b	84.29±5.45a,c	78.33 ± 4.49c	70.00 ± 3.49b
ALP (IU/L)	152.4 ± 7.37	186.8 ± 13.92	176.4 ± 10.98	175.3 ± 7.35	163.2 ± 7.24	178.7 ± 8.01

Group 1- Control; Group 2-L-NAME(50mg.kg); Group 3- L-NAME + Ramipril(10mg.kg); Group 4- L-NAME + TA(40mg/kg); Group 5- L-NAME + TA(60mg/kg); Group 6- LNAME + TA(80mg/kg). Values are expressed as mean ± SEM for seven rats per group. [a] P <0.05 vs. control; [b] P <0.05 vs. L-NAME; [c] P<0.05 vs. Ramipril.

Expression of mRNAs in heart tissue

ACE mRNA expression

Figure 4 presents the levels of ACE mRNA expression in the heart tissues of the six experimental groups. The L-NAME rats showed a significant (P<0.05) elevated mRNA ACE expression (10 folds) when compared to the control. However, treatment with Ramipril and TA suppressed the elevated expression with the maximum effect at 80mg/kg of TA (5.8 folds). There was no significant difference in mRNA ACE expression between the Ramipril and TA treated rats (P>0.05).

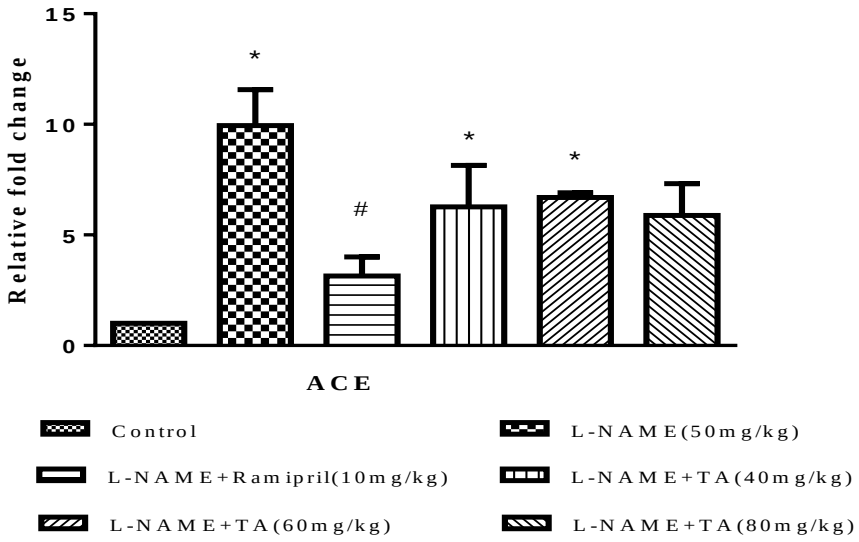

Figure 4: The mRNA levels of ACE in L-NAME induced hypertensive rats. Values are expressed as mean ± SEM for seven rats per group. * *P*< 0.05 vs. Control; # *P* <0.05 vs. L-NAME.

Expression of Bradykinin receptors B1 and B2 mRNA

The effect of TA on the mRNA expression of bradykinin receptors B1 and B2 in heart tissues are represented in Figure 5. The L-NAME group showed a decreased bradykinin receptor 1 (B1) mRNA expression (downregulation of 0.4 fold) when compared to the control. Ramipril and TA treatment significantly increased the mRNA expression of B1 with a pronounced effect at 80mg/kg of TA (4.4 fold increase). The TA dose of 80mg/kg had a greater effect as compared to the Ramipril treated group. Interestingly, all experimental groups showed a significant increase of bradykinin receptor 2 (B2) expression when compared to the control, with greater effect in the TA 80mg/kg dose group (6.8 fold increase). There was no significant difference between the Ramipril group and the TA treated groups. However, the TA dose of 80mg/kg was significantly increased when compared to the L-NAME group.

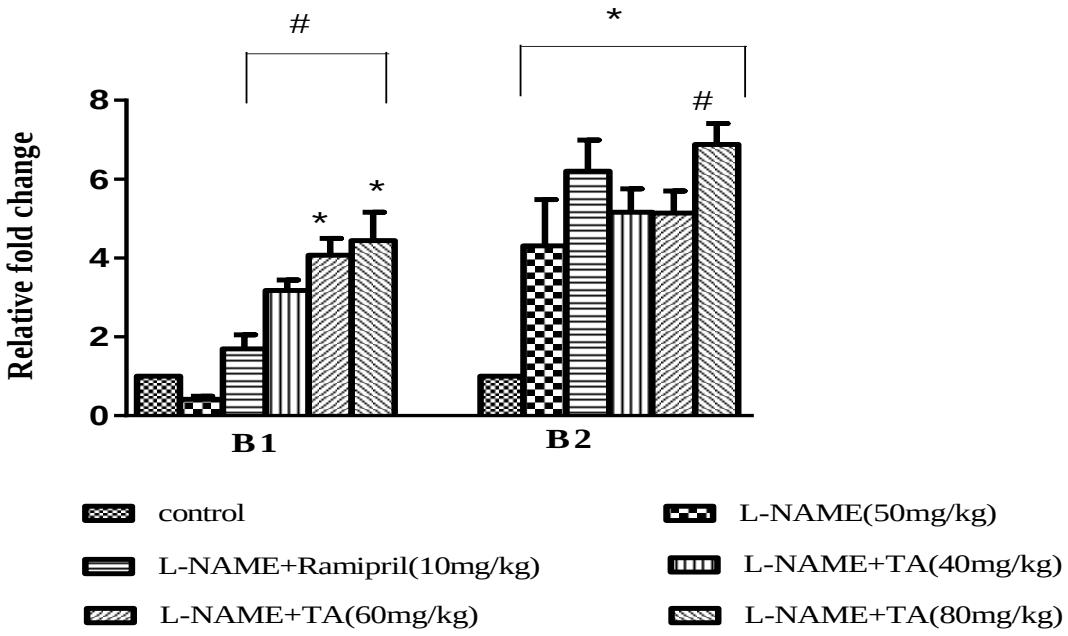

Figure 5: The mRNA levels of bradykinin receptors B1 and B2 in L-NAME induced hypertensive rats. Values are expressed as mean ± SEM for seven rats per group. * *P*< 0.05 vs. Control; # *P* <0.05 vs. L-NAME.

Expressions of AT1a and AT2 mRNA

The expressions of AT1a and AT2 receptors in the heart tissue of the experimental and control groups are presented in Figure 6. The L-NAME untreated group showed a significant (P<0.05) 3.8 fold increase in AT1a receptor expression when compared to the control. Interestingly, treatment with Ramipril and TA showed a significant down regulation of AT1a receptor expression, with more pronounced effect at 80mg/kg of TA (0.3 fold decrease). There was no significant difference in AT1a receptor expression between the Ramipril and TA treated groups.

Similarly, the L-NAME group showed a significant 3 fold increase in AT2 receptor expression when compared to the control. Treatment with Ramipril and TA showed a significant down regulation of AT2 receptor expression. There were no significant difference in the AT2 receptor expression between the Ramipril and the TA treated groups (P>0.05).

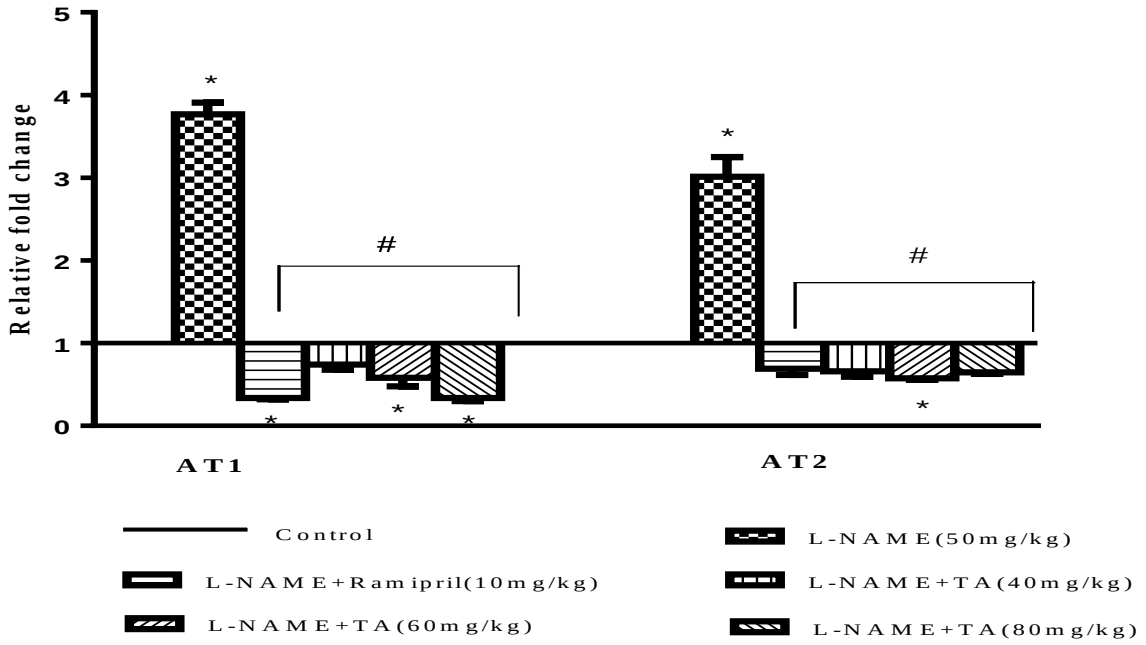

Figure 6: The mRNA levels of AT1a and AT2 receptors in L-NAME induced hypertensive rats. Values are expressed as mean ± SEM for seven rats per group. * *P*< 0.05 vs. Control; # *P* <0.05 vs. L-NAME.

Histopathological examination of cardiac tissues

The histology of myocardial tissues of all groups is represented in Figure 7(A-F). There were clearly defined, well-organised normal, cardiac fibers with nucleated cells of similar sizes observed in the normal untreated group (figure 7A). The L-NAME group showed a gross disorganization of cardiac fibers and inflammatory cells (7B). Interestingly, there was a marked change in the histological appearance in the Ramipril and TA treated groups (7C-E). The Ramipril and TA doses of 60mg/kg and 80mg/kg produced profound vascular changes as compared to the L-NAME untreated group. Cardiac fibers in the Ramipril and TA doses of 60mg/kg and 80mg/kg looked normal with mild indications of vascular degeneration (7 D, E). These effects were much pronounced in the TA dose of 80mg/kg (7E) which was similar to the control untreated group. However, there was a level of cardiac fiber disorganization with remarkable inflammatory cells in the TA dose of 40mg/kg, indicating a possible poor regeneration of cells following inflammation and vascular degeneration.

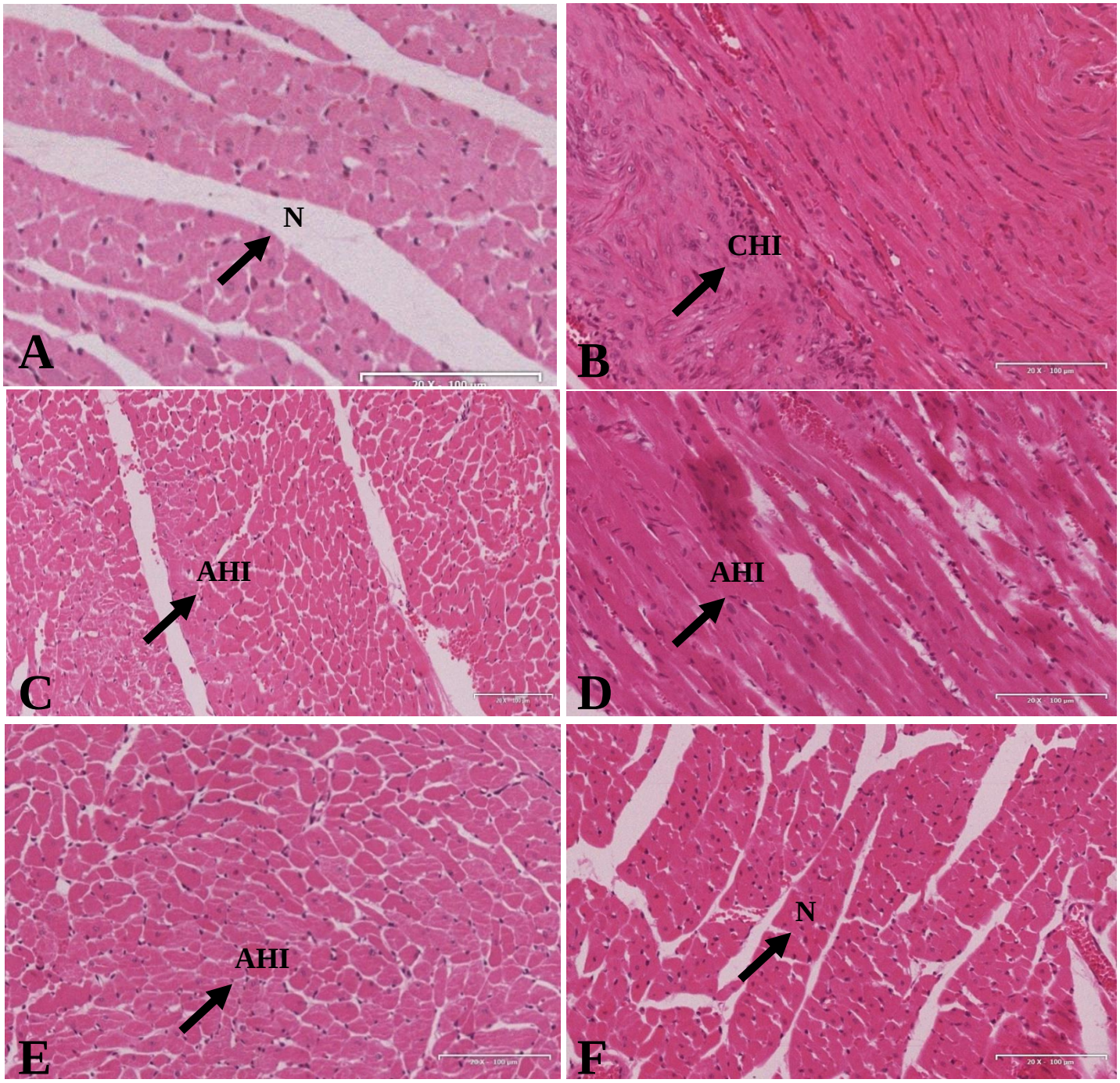

Figure 7: Photomicrographs representing histological changes in myocardial tissue sections (H & E, x40) (A)Control showing normal cardiac fibers; (B) L-NAME treated group showing gross myocardial fiber degeneration and thickened cardiac fibers with inflammatory cells; (C) Ramipril + L-NAME group similar to control showing attenuated degeneration of cardiac cells with normal cardiac fibers. (D) TA (40mg/kg) + L-NAME showing remarkable degeneration of cardiac cells with mild attenuated degeneration. (E) TA (60mg/kg) + L-NAME similar to control showing marked attenuated degeneration; (F) TA(80mg/kg) + L-NAME similar to control showing attenuated degeneration with normal cardiac fibers(N- Normal; CHI- Cardiac hypertrophy/inflammation;AHI-AttenuatedHypertrophy/inflammation).

DISCUSSION

Chronic inhibition of NO synthase (eNOS) by L-NAME is known to produce systemic vasoconstriction and increase blood pressure. Additionally, reports indicate that NO deficiency in rats causes cardiovascular remodeling, endothelial dysfunction, increased production of ROS and decreased antioxidant enzyme systems (28).

The successful induction of hypertension in L-NAME model was confirmed by the observed significant progressive rise in systolic and diastolic blood pressure as well as heart rate. This was further confirmed by the reduced plasma and tissue NO levels and accompanied by significant increase in oxidative stress, cardiac biomarkers and progressive long term cardiac hypertrophy. The treatments by Ramipril and the plant extracts of TA attenuated the rise of blood pressure and alleviated key pathological manifestations

Cardiac hypertrophy is an adaptive response to both physiological and pathological stimuli. The pathological hypertrophy, caused by nitric oxide deficiency, may be attributed to the increase in cardiac muscle mass as a result of the increase in myocyte size, in order to adapt to the overload caused by the NO deficient hypertension (26). Our findings show that this condition was attenuated by the treatment of TA and Ramipril, hence the absence of cardiac hypertrophy in the treated groups (27). We also demonstrated that the five week L-NAME treatment caused significantly decreased plasma NOx level, in keeping with others (28) while treatment with TA and Ramipril restored NOx levels to levels comparable to the control group.

Furthermore, the development of oxidative stress caused by the excessive production of reactive oxygen species is a major contributor to the progression of cardiovascular disorders coupled with hypertension (29). In this study, we observed a significant reduction in the SOD, CAT and GSH levels in the L-NAME untreated groups, which is in keeping with Berenyiova *et al,* (2018). This depletion is attributable to increased ROS such as superoxide anion (O_2^-) and hydroxyl radical (OH^-), due to L-NAME administration (30). Moreover, TA and Ramipril significantly increased antioxidants levels, with maximal effects at 60 and 80mg/kg. These antioxidants, in L NAME group, may have a short duration in scavenging ROS, hence prolonged oxidation exposure in the absence of antioxidant activity, could lead to deterioration in tissue antioxidant levels (30). Our study suggests that treatment with Ramipril and TA (60 and 80mg/kg) may have an active role in promoting the bioavailability of these antioxidants, hence maintaining steady state physiologic conditions. Additionally, lipid peroxidation, is known to alter the cellular integrity of tissues and organs, especially in the heart (31). The end product of lipid peroxidation, measured as Thiobarbutiric Acid Reactive Substances (TBARS) (32), was seen to increase significantly in

the heart in the L-NAME untreated rats, a clear sign of oxidative stress in these tissues. This was attenuated by the administration of Ramipril and TA treatment, with maximum effect at 80mg/kg. From these observations, it is evident that the protective role and antihypertensive effect of TA, would be partially due to its antioxidant potential.

We also found increased levels of cardiac biomarkers such as CKMB, CK and cTnI in the L-NAME group, which is consistent with previous findings by others (33, 34). High concentrations of cTnI and cardiac markers indicate the presence of myocardial cell injury, which could lead to infarction (34). The early rise in cTnI and cardiac enzymes CKMB and CK after myocardial injury is known to occur about the same time (35). Cardiac troponin I is currently known as one of the most sensitive cardiac markers, and together with a rise in CK and CKMB levels, provides evidence of myocardial injury in the L-NAME group (36). However, this was alleviated by the administration of TA and Ramipril.

The process involved in the L NAME hypertensive induction involves multiple pathways. The processes involved in the induction of hypertension by L-NAME, entails more than the reduction or elimination of a tonic vasodilator action of NO through NO synthase inhibition (37). We observed increased cardiac and serum ACE activity, as well as ACE mRNA gene expression in heart tissues in the L-NAME untreated group. In contrast, treatment with TA and Ramipril decreased these parameters. Our findings are similar to other studies, that have demonstrated that increased NO bioavailability are effective in inhibiting the activity of ACE in a dose-dependent manner (38, 39). Though the exact mechanism of NO-stimulated-ACE inhibition remains unclear, it is well known that there is an inverse relationship between ACE activity and the NO system, suggesting that the RAS is activated by inhibition of NO (40). Notably, our studies also indicated a significant upregulation in cardiac AT1 and AT2 receptor gene expression in the L-NAME untreated group. This was followed by a significant down regulation when treated with Ramipril and TA. This finding concurs with similar studies in L-NAME hypertensive rats (41, 42). Evidence from several reports also show that NO has an antagonistic effect on the biological function of Ang II (42, 43). Therefore the upregulation of AT1 and AT2 in the L-NAME model, with its subsequent downregulation in the treated groups, may be due to the multiple feedback mechanisms involved in the NO release and the ACE/Ang II/AT1 association (44).

We further examined the effect of TA and Ramipril on the kinin-kallikrein system (KKS), through the expression of the bradykinin receptors B1 and B2. It was observed that bradykinin receptor B1 gene was significantly decreased in the L-NAME hypertensive rats, and significantly increased with the treatment of TA and Ramipril, with a profound effect in the 80mg/kg dose of TA. These findings are in line with other similar studies, that seems to suggest that ACE inhibitors promote the expression of bradykinin

receptors B1 and B2, through the bioavailability of NO production (45). Studies have shown that ACE inhibitors directly or indirectly affect bradykinin B1 and B2 receptor signaling, which may be the basis of some of their therapeutic effectiveness (45). There is also a strong evidence that B1 and B2 enhancement through ACE inhibition may activate endothelial NO synthase, and hence promoting the bioavailability of NO in the vascular system (46). Indeed, our studies confirmed the above-mentioned phenomenon, with an upregulation of B1 and B2 receptors in TA and Ramipril treated groups, coupled with an increase NO levels, and a significant reduction of B1 coupled with low levels of NO. Hence, TA (80mg/kg) and Ramipril may mediate their therapeutic role via the KKS.

We further evaluated liver function to ascertain the toxicological effect of therapeutic agents (47). Our study showed that the liver enzymes ALT, AST and ALP in the Ramipril and TA (60mg/kg and 80mg/kg) treated groups were not significantly different from the control group, an indication of the absence of toxicity, although there was a tendency towards an increase in the TA dose of 40mg/kg and the L-NAME untreated rats. We examined the lipid parameters and found no significant difference in lipid profiles amongst the experimental groups, although triglycerides level were increased slightly in the Ramipril group. Our findings corroborates with other findings, where NO deficient induced hypertension may have led to progression of cardiovascular diseases independent of lipid profile (48)(49).

Histopathological examination of cardiac tissue showed vascular degeneration and marked inflammation the L-NAME group. This is in keeping with other studies, which suggests that activation of RAS coupled with oxidative stress and decreased NO availability may cause inflammation and cardiovascular injury (50). On the contrary, TA (80 mg/kg b.w.) and Ramipril treated hypertensive rats revealed a near normal morphology of cardiac fibres. The TA and Ramipril treated group showed no evidence of degeneration or cell death. Therefore, these observations demonstrate the protective role of TA at a dose of 80mg/kg and Ramipril on cardiovascular integrity.

Conclusion

To the best of our knowledge, this is the first study to examine the cardiovascular effects of TA. In conclusion, it is observed that the dose of 80mg/kg TA possesses a strong cardioprotective role, comparable to Ramipril, which may be attributed to its strong antioxidant and antihypertensive properties through the inhibition of ACE in an L-NAME induced hypertensive rats. TA shows promise as a possible therapeutic agent in the treatment and management of essential hypertension. However, further studies are needed to elucidate the precise underlying mechanisms of action of *Tulbaghia acutiloba*.

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Chapter 5: Manuscript Four

Haematological and Renal function markers as indicators of antihypertensive efficacy of the leaf hydro-methanolic extract of Tulbaghia acutiloba Harv. in an L-NAME induced hypertensive rats

Chapter 4 demonstrated the antihypertensive and cardioprotective effect of *Tulbaghia acutiloba* in an L-NAME induced hypertensive rats and the reduction of oxidative stress. Chapter 5 was therefore intended to assess the antihypertensive efficacy of *T. acutiloba* (TA) on renal function and haematological parameters. Since renal function and haemtological parameters are indicated as prognostic markers in pathophysiology of hypertension, it was therefore necessary to understand the effect of TA on these crucial parameters and functions to better appreciate the antihypertensive efficacy of TA in an L-NAME induced hypertensive rats.

Haematological and Renal function markers as indicators of antihypertensive efficacy of the leaf hydro-methanolic extract of *Tulbaghia acutiloba* Harv in an L-NAME induced hypertensive rats

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ABSTRACT

Haematological assessment and renal function are essential tools in assessing the antihypertensive efficacy of therapeutic agents including medicinal plants. The effect of the indigenous plant, *Tulbaghia acutiloba* (TA) on renal and haematological parameters has not been investigated. Therefore, we evaluated the effect of TA on these parameters as indicators of its antihypertensive efficacy in L-NAME induced hypertensive rats. Male albino Wistar rats were treated, orally, with L-NAME (50mg/kg b.w.), hydro-methanolic leaf extracts of TA at different doses (40, 60 and 80mg/kg b.w.), Ramipril (10mg/kg b.w.) and water (control) in separate groups for 5 weeks. Mean arterial blood pressure was measured weekly using the tail-cuff method. A 24-hour urine sample was collected for each rat weekly. On day 36, the rats were sacrificed, and blood samples collected for the determination of renal function and haematological analysis. Kidney mRNA gene expression was performed for NF-kB, HO-1 and eNOS. The treatment of the hypertensive rats with TA and Ramipril resulted in a significant reduction in the mean arterial blood pressure, with a pronounced effect observed in the 80mg/kg dose of TA. The TA treated group showed increased creatinine clearance (Ccr), urine volume and a reduction in serum creatinine, proteinuria and Urine protein-creatinine ratio (UPr/UCr). TA treatment also decreased lipid peroxidation in renal tissues and erythrocytes whiles increasing SOD, CAT, GSH and NO levels. Moreover, red cell distribution width (RDW), white blood cells (WBC), neutrophil to lymphocyte ratio (NLR), lymphocyte to monocyte ratio (LMR), platelet and Mean Platelet volume (MPV) were significantly reduced in the TA and Ramipril treated groups with maximum effect occurring at the dose of 80mg/kg of TA. No significant difference was observed in the haemoglobin levels in all experimental groups.

TA administration resulted in a significant decrease in renal NF-kB gene expression, while increasing HO-1 and NO gene expression in renal tissues. Generally, our results indicate improved renal function and haematological profile as markers of the antihypertensive efficacy of *Tulbaghia acutiloba.*

Key words: *Tulbaghia acutiloba,* renal function, haematological parameters, hypertension, antioxidant

Introduction

The aetiology and pathophysiology of systemic arterial hypertension, of which 95% is essential, is currently under investigation, as it poses a major health concern, globally (1). By 2030, it is estimated that the global annual death toll, due to hypertension, is predicted to reach 23.5 million (2, 3). Notably, hypertension is associated with oxidative stress and increased inflammatory responses, and is a major risk factor for renal and cardiovascular disorders (4). Moreover, endothelial dysfunction is a major role player in the pathogenesis of hypertension, together with these markers (5). Several animal models of hypertension, displaying these key pathological features have been developed. In this regards the chronic administration of $N^{(\omega)}$-nitro-L-arginine methyl ester (*L-NAME*), in a rat model, is known to block the endothelial nitric oxide synthase (NOS), leading to the reduction in the biosynthesis of NO, with downstream effects on endothelial function (6). In turn, impaired endothelial function increases oxidative stress, which may lead to chronic inflammation, and subsequently lead to severe, end-organ damage (7). The kidney, a major target organ of hypertension, is associated with changes in biochemical and haematological parameters, which are emerging as suitable prognostic indicators of the disease (8). In recent years, the primary focus of antihypertensive treatment has been to decrease the risk of organ damage associated with elevated blood pressure, in order to decrease morbidity and mortality (9). The study of inflammatory status, oxidative stress and biochemical changes associated with organ function, will increase functional understanding, and enable better prediction of the prognosis of hypertension (10). Recently, there has been an increased interest in the investigation of haematological parameters in chronic diseases, such as hypertension, as they may adversely affect blood cells in the body (11). Haematological indices such as neutrophil-lymphocyte ratio (NLR), lymphocyte-monocyte ratio (LMR), red cell distribution width (RDW), white blood cells (WBC), mean platelet volume (MPV) and mean cell volume (MCV) have emerged as a novel, yet inexpensive, means of determining the systemic inflammatory-immunological markers for the prognosis of hypertension and the determination of vascular and renal integrity (12). The prognostic value of these indices has been demonstrated in stroke, coronary heart diseases and nephropathies (13).

Haematological indices, used as indicators of the prognosis of hypertension, in conjunction with renal markers, are of great interest since most of the enzymes and biochemical elements necessary for haematological function are produced in the kidney (13). For instance, erythropoietin, a hormone required for the synthesis of red blood cells, is produced in the juxtaglomerular apparatus of the kidney (14). Moreover, the formation of other lineages of blood cells, including white blood cell differentials, as well as platelets, are known to be directly or indirectly linked to the normal functioning of the renal organ.

Hence, these factors, together with biochemical parameters and relevant inflammatory gene expression such as NF-KB, eNOS, and Haem Oxygenase 1 (HO-1) in the kidney, can be used to determine the prognosis of hypertension (15, 16).

Natural product research is key to finding novel therapeutic applications, to lower risk of mortality and morbidity in hypertension (17). Despite the progress made in the use of orthodox medicine, phytotherapy still remains as an important contributor to health care systems because of cost, access and reported minimal side effects (17).

Tulbaghia acutiloba is used traditionally in Southern Africa for the management of various ailments, including hypertension, as well as, infectious diseases (18). It is also used as a culinary herb in the Eastern Cape and KwaZulu-Natal of South Africa (18). Despite its widespread usage, there is a lack of reports on the effect of the plant on hypertension, in particular, pertaining to the renal system and haematological parameters. Earlier studies in our laboratory reported (unpublished data) on the cardiovascular effects of *Tulbaghia acutiloba*. Therefore, the present study was conducted to examine the efficacy of the hydro-methanolic leaf extract of *Tulbaghia acutiloba* on renal function, oxidative stress and haematological parameters in an L-NAME induced hypertensive rats.

MATERIAL AND METHODS

Plant sampling and preparation

Tulbaghia acutiloba (TA) leaves were identified, collected and authenticated by Professor Himansu Baijnath, a botanist at the University of KwaZulu-Natal (UKZN), Durban, South Africa. The leaves of TA were washed thoroughly under running water and allowed to air dry for 72 hours at room temperature, under sterile conditions. The air-dried leaf material was crushed into a powder using a Waring blender. Thereafter the air-dried powdered material was extracted using a hydro-methanolic solution (80% methanol, 20% water) on a shaker, at 80rpm for 48 hours. The supernatant solution was filtered and placed in a rotary evaporator. Thereafter the concentrate was freeze dried at -40ºC. The leaves were then ready for use in this study. There was a 12.8% yield of the leaf extract.

Animal Experimental protocol

Male albino rats (n=42) in good health were obtained from the Biomedical Resource Unit (BRU) of the University of KwaZulu-Natal (UKZN). Standard housing conditions of 23±2˚C room temperature, 50-60% humidity, and a 12h light/12h dark cycle were maintained throughout the study period. The animals had unrestricted access to standard rat food and water. Ethical consent was sought from the Animal Ethics Committee of UKZN (Ref: AREC/062/016PD), according to the guidelines of National Institutes of Health for the Care and Use of Laboratory Animals.

Chemicals

N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) and Ramipril were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

Animal Model of L-NAME induced hypertension and TA and Ramipril treatment

N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) was administered to the animals orally at a dosage of 50mg/kg body weight(b.w.) for 5 weeks. Each dose of Ramipril and TA leaves in powdered form were mixed and completely dissolved in distilled water, and the various doses were administered to the rats orally, on a daily basis, using an intragastric tube for the entire study period. There were 6 groups, into which, each of the animals were randomly assigned. There were 7 animals in each group designated as follows:

Group 1: Control (distilled water only)
Group 2: Rats were treated with L-NAME only (50mg/kg b.w.)
Group 3: Rats were co-administered with L-NAME (50mg/kg) and Ramipril (10mg/kg b.w.)
Group 4: Rats were co-administered with L-NAME (50mg/kg) and TA leaves (40mg/kg b.w.)
Group 5: Rats were co-administered with L-NAME (50mg/kg) and TA leaves (60mg/kg b.w.)
Group 6: Rats were co-administered with L-NAME (50mg/kg) and TA leaves (80mg/kg b.w.)

Measurement of Mean arterial pressure

Animals were subjected to the measurement of mean arterial blood pressure using the non-invasive tail-cuff method (IITC, life Sciences Inc. Model 31, Woodland Hills, CA, USA), as previously reported. Prior to the measurement, the rats were given a pre-conditioning treatment for a week to ensure that the rats are exposed and adapted to the experimental conditions to avoid interference. The mean arterial blood pressure was measured weekly during the experimental period. Results reported represent the mean value of three consecutive readings.

Animal Sacrifice and specimen collection

On the next the day following the 5 week period, the animals were sacrificed using halothane overdose, thereafter cardiac puncture was performed in order to remove blood from rats. The kidney tissues were quickly collected, washed, weighed, and frozen in liquid nitrogen. Whole blood samples were collected into an EDTA tubes for haematological analysis. Plasma and serum samples were obtained after blood collection through centrifugation at 224 x g for 10 minutes for biochemical analysis.

Lipid peroxidation and Antioxidant assay

Lipid peroxidation in kidney and erythrocytes was assessed through the estimation of Thiobarbutirc acid-reactive substances (TBARS) according to the method by Niehaus & Samuelsson (1968).
Kidney tissue and erythrocyte catalase and superoxide dismutase (SOD) activities were determined by the method developed by Sinha (1972) and Kakkar et al., (1984) respectively, with slight modifications. Reduced glutathione (GSH) in erythrocytes and kidney tissues estimation was estimated by the method of Ellman (1959) with modifications. The NOx concentration estimation in the kidney tissues and erythrocytes was based on the Griess reaction (Green *et al.,* 1982; Foresi *et al.,* 2016).

Haematological analysis

A full blood count was carried out on whole blood samples using an automated haematology analyser (Beckman Coulter Ac.T diff., Coulter Electronics, USA) as per manufacturer's instruction. The NLR was calculated by dividing the neutrophil count by the lymphocyte count (Neutrophil count ÷ lymphocyte count). Similar ratios used in the study such as the LMR, were calculated in a similar manner.

Renal function markers

The urine volume for each animal was determined weekly, by placing the animals in metabolic cages for a 24-hour urine volume collection and measurement. Urinary and serum/plasma electrolytes and protein concentration were analysed using an electrolyte and chemistry analyzer (Beckman Coulter Synchron, DXC 600, Access Clinical System, USA). Creatinine clearance was evaluated as a measure of Glomerular filtration rate based on the formula:

Creatinine clearance rate (ml/min) = (Urine volume x urine creatinine concentration) / plasma creatinine concentration / time.

Fractional excretions of sodium (FeNa) and potassium (FeK^+) were measured to evaluate the urine sodium and potassium excretions, and were expressed as percentages of sodium and potassium absorbed by the kidney using the standard equation according to the method of Steiner(1984) and Hare (1950), respectively, as follows:

FeNa (%) = (Urinary sodium x Plasma creatinine) / (Plasma sodium x Urinary creatinine) X 100

FeK^+ (%) = (Urine Potassium x Plasma creatinine) / (Plasma potassium x Urine creatinine) X 100

Relative mRNA expression in the kidney

Total mRNA was extracted from kidney tissues using the Zymo Research Quick-RNA Miniprep kit (Zymo Research, Irvine, USA) according to manufacturer's instruction. A nanodrop spectrophotometer (Thermoscientific Nanodrop 1000) was used to assess the purity and concentration of RNA extracted. Thereafter, a total RNA of 1µg was reverse transcribed into cDNA according to the manufacturer's protocol using the iScript cDNA synthesis kit (Bio-Rad Laboratory Pty, Ltd. USA).

Realtime polymerase chain reaction (RT-PCR) was carried out using the Lightcycler 96 (Roche Diagnostics, Germany). Conditions used for RT-PCR analysis were as follows: 95ºC initial denaturation cycle for 10minutes, thereafter a PCR run consisting of 40 cycles at 95ºC for 15s, 60ºC for 30s and 72ºC

for 30s with a single fluorescent measurement. The house keeping gene used in the analysis was Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the fluorescent dye utilized in the analysis was SYBR Green 1 [SSO Advanced Univers SYBR GR. SUPRMIX, Bio-Rad Laboratory (Pty) Ltd. USA]. The oligonucleotide primer sequences for the various genes investigated in the RT-PCR analysis were as follows:

Gene	Forward Primer (5' – 3')	Reverse Primer (5' – 3')
NF-κB	GCGGCCAAGCTTAAGATCTGCCGCCGAGTAAAC	CGCTGCTCTAGAGAACACAATGGCCACTTG
HO-1	ACTTTCAGAAGGGTCAGGTGTCC	TTGAGCAGGAAGGCGGTCTTAG
eNOS	CTCGTCCCTGTGGAAAGACAA	TGACTTTGGCTAGCTAGCTGGTAACTGT

Statistical Analysis

Values reported are means ± SEM for seven rats per group. Analysis of data was done by analysis of variance (ANOVA) followed by post hoc tests for multiple comparisons using Bonferroni and Dunnett's test were appropriate on GraphPad Prism Version 6. Data values were considered significant at $P<0.05$.

RESULTS

Effect of TA on Mean Arterial Pressure (MAP).

Figure 1 shows the effect of TA on mean arterial pressure at three different doses (40, 60 and 80mg/kg b.w.) in L-NAME induced hypertensive rats. A significant ($P<0.05$) increase in the mean arterial pressure was observed in the untreated L-NAME group from week 1 to week 5, while treatment with TA and Ramipril significantly reduced the mean arterial pressure, with a more pronounced effect at 80 mg/kg b.w. of TA (vs. untreated L-NAME). No significant difference was observed between the effect of TA (80mg/kg b.w.) and Ramipril for mean arterial pressure.

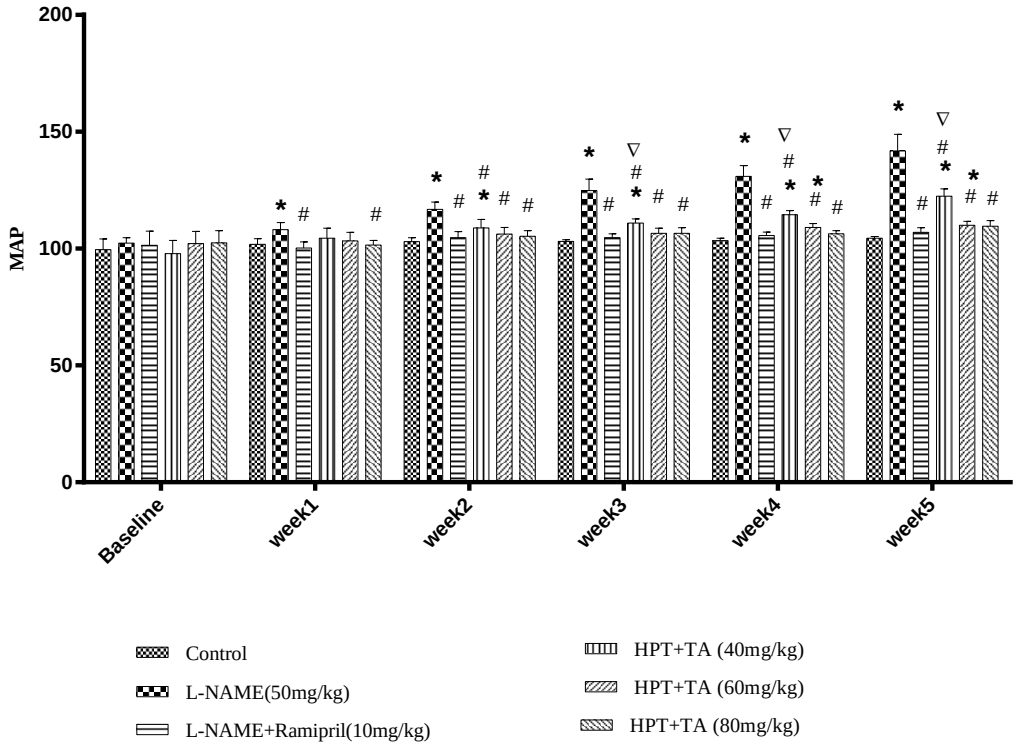

Figure 1: Effect of TA on Mean Arterial Pressure (MAP) in L-NAME induced hypertensive rats, Values are expressed as mean ± SEM for seven rats per group. * *P*< 0.05 vs. Control; # *P* <0.05 vs. L-NAME; ∇ *P*<0.05 vs. Ramipril

Effect of TA on antioxidant activities in Kidney tissues and Erythrocytes

The effect of TA on the antioxidant activities in the kidney tissues and erythrocytes are shown in table 1. The activities of superoxide dismutase (SOD) and catalase (CAT) were significantly ($P<0.05$) reduced in the L-NAME group of rats as compared to the control, while the treatment with TA and Ramipril significantly ($P<0.05$) increased these enzymatic antioxidants, with a maximum effect at 80mg/kg of TA. Furthermore, the levels of reduced glutathione (GSH) and nitric oxide (NOx) were decreased significantly in the L-NAME untreated rats, whiles it was increased significantly with the administration of TA and Ramipril, with a maximum effect occurring at the dose of 80mg/kg of TA. Lipid peroxidation, measured using Thiobarbutric acid reactive substances (TBARS), was also increased significantly ($P<0.05$) in the L-NAME untreated rats when compared with the control. This effect was significantly reduced with the treatment of TA and Ramipril, with a pronounced effect at 80mg/kg of TA.

Table 1: Effect of TA on superoxide dismutase (SOD), catalase (CAT), reduced Glutathione (GSH), Thiobarbutric acid reactive substances (TBARS) and NOx activities in kidney tissues and erythrocytes

Parameter	Samples	Groups					
		1	2	3	4	5	6
SOD	**Erythrocytes (U/mg Hb)**	17.95±1.18	7.53±0.69[a]	17.31±0.18[b]	15.60±0.20[b]	15.36±0.11[b]	16.22±0.08[b]
	Kidney (U/mg protein)	17.35±0.21	12.24±0.08[a]	16.12±0.10[b]	13.53±0.05[a,b,c]	14.60±0.13[b,c]	15.89±0.06[b]
CAT	**Erythrocytes(U/mgHb)**	176.9±2.90	115.3±6.38[a]	144.7±2.14[a,b]	120.5±6.83[a,c]	159.9±0.95[b,d]	166.1±6.38[b,c,d]
	Kidney (U[b]/mgprotein)	91.63±1.04	56.12±.0.59[a]	128.0±0.52[a,b]	109.0±1.51[a,b,c]	117.6±6.29[a,b]	179.1±1.60[a,b,c,d,e]
GSH	**Erythrocytes (µmol/g Hb)**	0.07±0.01	0.06±0.00[a]	0.07±0.00[b]	0.05±0.00[a,b,c]	0.06±0.00[a,d]	0.06±0.01[a,d]
	Kidney (µg/mg protein)	0.03±0.00	0.02±0.00[a]	0.03±0.00[b]	0.03±0.00[b]	0.03±0.00[b]	0.03±0.00[b]
TBARS	**Erythrocytes(nmol/g Hb)**	8.19±0.06	19.47±0.43[a]	9.12±0.16[b]	11.32±0.06[a,b,c]	8.65±0.11[b,d]	8.25±0.14[b,d]
	Kidney(mmol/100g wet tissue)	1.61±0.05	3.77±0.10[a]	2.17±0.06[a,b]	3.22±0.05[a,b,c]	2.29±0.05[a,b,d]	1.95±0.05[b,d]
NOx	**Erythrocytes(µmol/g Hb)**	92.72±1.03	74.59±4.94[a]	79.07±0.93[a]	82.09±1.10	76.47±5.08[a]	91.47±1.09[b,e]
	Kidney (µmol/l)	103.9±2.44	80.84±1.81[a]	92.09±1.09	100.7±2.10[b]	102.4±2.03[b]	137.1±5.78[a,b,c.d.e]

Group 1- Control; Group 2-L-NAME(50mg.kg); Group 3- L-NAME + Ramipril(10mg.kg); Group 4- L-NAME + TA(40mg/kg); Group 5- L-NAME + TA(60mg/kg); Group 6- LNAME + TA(80mg/kg). Values are expressed as mean ± SEM for seven rats per group. [a] *P* <0.05 vs. control; [b] *P* <0.05 vs. L-NAME; [c] P<0.05 vs. Ramipril; [d] P<0.05 vs. TA(40mg/kg); [e] P<0.05 vs. TA(60mg/kg)

Effect of TA on red blood cell and platelet parameters

Table 2 presents the effect of TA on red cell and platelet parameters. No significant differences were observed in the Haemoglobin concentration (Hb), red blood cell counts (RBC) and the haematocrit (HCT) values in all the experimental groups. However, the red cell distribution width (RDW) significantly increased in the L-NAME untreated group when compared to the control. This was significantly ($P<0.05$) decreased in the TA and Ramipril treated groups, with a pronounced effect at the 80mg/kg dose of TA. No significant difference was observed in the RDW between the TA treated groups, the control and Ramipril groups. Furthermore, a significant ($P<0.05$) increase in the platelet levels were observed in the L-NAME untreated group as compared to the control, whiles the administration of TA and Ramipril decreased the platelet levels. This decrease was significant ($P<0.05$) at concentrations of 40mg/kg and 80mg/kg doses of TA. Again, the mean platelet volume (MPV) increased significantly ($P<0.05$) in the L-NAME untreated group when compared to the control, and this was significantly alleviated with the treatment of TA and Ramipril, with maximum effect at 80mg/kg of TA. No significant difference was observed in MPV values between the TA treated groups, Ramipril treated and control groups (Table 2).

Table 2: Effect of TA on Red cell and platelet parameters

Parameter / **Samples**	**Groups** 1	2	3	4	5	6
Hb (g/dl)	15.26±0.38	15.73±0.77	15.20±0.53	15.89±0.41	15.63±0.58	15.87±0.28
RBC (x 10^6/µL)	8.570 ±0.14	8.89±0.35	8.36±0.25	9.06±0.15	8.66±0.17	8.68±0.24
HCT (%)	45.73±0.69	47.79±2.15	45.21±0.72	47.63±0.31	46.24±1.09	47.35±0.83
RDW (SD)(fl)	10.14±0.31	13.64±0.56[a]	10.49±0.20[b]	10.39±0.25[b]	9.92±0.15[b]	9.88±0.14[b]
PLT (x10^3/Ml)	875.9±34.68	952.9±9.45	859.4±39.34	827.4±29.05[b]	849.9±12.70	823.0±19.24[b]
MPV(fl)	6.23±0.13	8.09±0.23[a]	6.30±0.04[b]	6.14±0.06[b]	6.06±0.06[b]	6.15±0.05[b]

Group 1- Control; Group 2-L-NAME(50mg/kg); Group 3- L-NAME + Ramipril(10mg/kg); Group 4- L-NAME + TA(40mg/kg); Group 5- L-NAME + TA(60mg/kg); Group 6- LNAME + TA(80mg/kg). Values are expressed as mean ± SEM for seven rats per group. [a] P <0.05 vs. control; [b] P <0.05 vs. L-NAME;

Effect of TA on white blood cell counts

Figure 2 shows the effect of TA on white cell counts. There was a significant ($P<0.05$) increase in the white cell count in the L-NAME untreated group when compared to the control group, while the administration of TA and Ramipril significantly ($P<0.05$) reduced the white cell count. No significant difference was observed in the TA treated groups and the Ramipril groups.

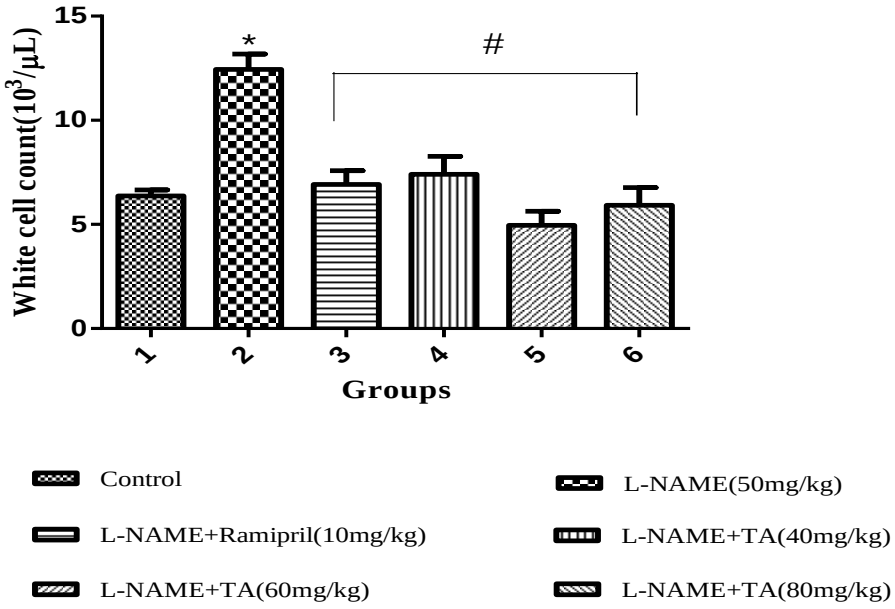

Figure 2: Effect of TA on white cell count in L-NAME induced hypertensive rats, Values are expressed as mean ± SEM for seven rats per group. * *P*< 0.05 vs. Control; # *P* <0.05 vs. L-NAME.

Effect of TA on Neutrophil-lymphocyte ratio (NLR) and Lymphocyte-monocyte ratio (LMR)

The effect of TA on neutrophil-lymphocyte ratio (NLR) and lymphocyte-monocyte ratio (LMR) is shown in figures 3 and 4 respectively. A significant (P<0.05) increase in the NLR and LMR was seen in the L-NAME group as compared to the control group, and this was significantly attenuated with the administration of TA and Ramipril. No significant difference was seen in the NLR and LMR between the TA treated groups, Ramipril and the control group.

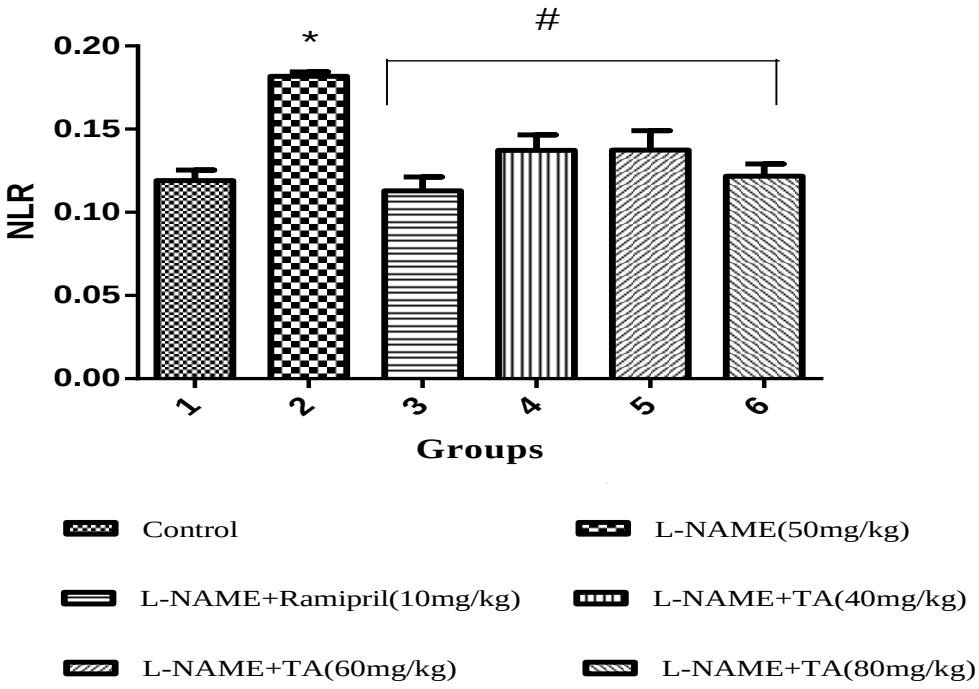

Figure 3: Effect of TA on neutrophil - lymphocyte ratio (NLR) in L-NAME induced hypertensive rats, Values are expressed as mean ± SEM for seven rats per group. * *P*< 0.05 vs. Control; # *P* <0.05 vs. L-NAME

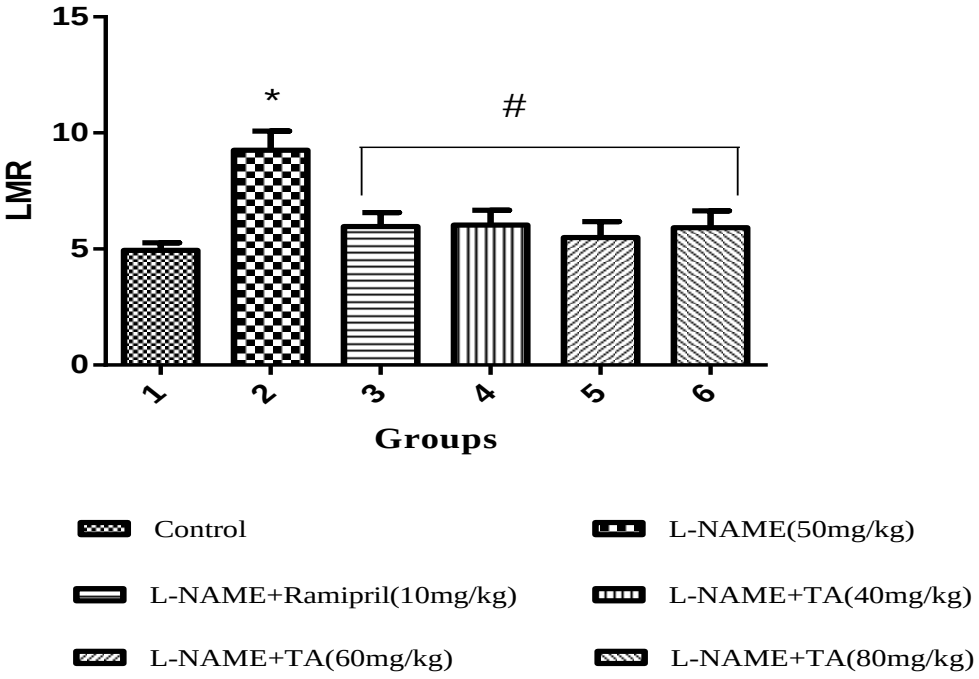

Figure 4: Effect of TA on lymphocyte-monocyte ratio (LMR) in L-NAME induced hypertensive rats, values are expressed as mean ± SEM for seven rats per group. * *P*< 0.05 vs. Control; # *P* <0.05 vs. L-NAME

Effect of TA on renal function

Tables 3, 4 and 5 shows the effect of TA on renal function markers

Effect of TA on Urine electrolytes

It was observed that urine sodium and potassium levels were decreased significantly ($P<0.05$) in the L-NAME untreated group when compared to the control group, whiles the administration of TA increased the levels of sodium and potassium levels, with maximum levels at 80mg/kg of TA, though this increase was not significant as compared to the L-NAME untreated group (Table 2). Notably, the Ramipril group showed decreased levels of sodium and potassium levels, as compared to the L-NAME untreated group, and the TA treated groups (Table 2). Moreover, a similar pattern was observed in chloride levels in all the groups, with maximum level occurring at the 40mg/kg dose of TA (Table 2).

Effect of TA on Urine creatinine, protein and urea levels

Creatinine and urea levels decreased in the L-NAME untreated group as compared to the control group, while the treatment with TA increased in the creatinine and urea levels, although it was not significant (Table 3). However, the Ramipril group showed a decrease in creatinine and urea levels, as compared to the control group and the TA treated groups (Table 3). Protein levels increased in the L-NAME untreated group as compared to the control, and this was decreased in the Ramipril and TA treated groups when compared with the L-NAME. Interestingly, the Ramipril group showed the lowest levels of protein as compared to the other treated groups (Table 3).

Table 3: Effect of TA on renal parameters measured in Urine

Parameter / Samples	Groups 1	2	3	4	5	6
Sodium Urine (mmol/l)	164.7±12.38	98.00±4.84[a]	90.0±13.91[a]	109.0±14.87[a]	107.4±7.74[a]	120.4±4.26
Potassium(mmol/l)	451.5±12.39	280.0±22.93[a]	207.3±26.94[a]	325.3±30.99[a,c]	308.9±18.24[a]	311.2±40.91[a]
Chloride (mmol/l)	329.2±12.11	234.4±17.32	155.3±27.39[a]	216.0±21.72[a]	209.4±12.60[a]	207.8±25.41[a]
Creatinine(mmol/l)	9.75±0.57	7.24±0.71	4.73±0.59[a]	7.21±0.52	7.31±0.42[c]	7.87±0.91[c]
Protein(g/l)	2.42±0.21	3.89±0.50	1.40±0.07[b]	2.57±0.46	2.35±0.11[b]	2.69±0.44
Urea(mmol/l)	955.3±89.19	702.6±52.65	529.6±73.57[a]	769.1±74.80	715.7±36.65	792.8±78.84
Volume(baseline) (ml/24h)	8.243±0.46	8.46±0.15	8.50±0.91	7.64±0.82	7.71±0.68	8.20±0.46
Volume(week 5) (ml/24hr)	14.29±0.38	21.21±1.93[a]	23.00±0.82[a]	19.29±1.66	24.24±1.08[a]	24.00±1.60[a]

Group 1- Control; Group 2-L-NAME(50mg.kg); Group 3- L-NAME + Ramipril(10mg.kg); Group 4- L-NAME + TA(40mg/kg); Group 5- L-NAME + TA(60mg/kg); Group 6- LNAME + TA(80mg/kg). Values are expressed as mean ± SEM for seven rats per group. [a] P <0.05 vs. control; [b] P <0.05 vs. L-NAME; [c] P<0.05 vs. Ramipril;

Effect of TA on urine volumes

Urine volume at baseline levels showed no significant difference amongst all the experimental groups. However, at week 5, there was a significant (P<0.05) increase in urine volume in the Ramipril and TA treated groups, as compared to the control, with a maximum level recorded at 80mg/kg of TA (Table 3). Furthermore, there was an increase in urine volume in the Ramipril and TA treated groups, as compared to the L-NAME untreated group, though this was not significant (Table 3).

Effect of TA on Plasma/Serum electrolytes

Plasma/serum sodium levels showed no significant difference (P>0.05) in all the experimental groups. Moreover, there were no significant difference in potassium levels between the TA and Ramipril treated groups and the control (Table 4). No significant difference was also observed potassium levels between the TA treated group and the L-NAME untreated group. Interestingly, there was a significant increase (P<0.05) in potassium levels in TA (40mg/kg) as compared to TA doses of 60 and 80 mg/kg (Table 4). Additionally, there was an increase in chloride levels in the TA and Ramipril treated groups, as compared to the L-NAME group, but this did not differ significantly; with the exception of TA dose of 80mg/kg which increased significantly as compared to the L-NAME treated group (Table 4).

Effect of TA on serum/plasma creatinine, protein and blood urea nitrogen (BUN) levels

Serum creatinine levels in the Ramipril and TA treated groups were generally decreased, as compared to the L-NAME untreated group, although this was not significant (Table 4). However, a significant reduction was seen in TA dose of 40mg/kg when compared to the control, L-NAME group and Ramipril treated groups (Table 4). No significant difference was also seen in protein levels in all the experimental groups, although there was a slight increase ($P<0.05$) in the L-NAME untreated group (Table 4). Blood urea nitrogen levels was increased in the Ramipril group, as compared to the other experimental groups, however, no significant difference was observed between the TA treated group vs. L-NAME and the control groups (Table 4).

Table 4: Effect of TA on Renal parameters measured in Plasma/serum

Parameter	Groups					
	1	2	3	4	5	6
Sodium (mmol/l)	142.4±0.65	142.6±1.16	142.7±0.72	140.7±0.64	142.7±0.62	143.7±1.06
Potassium(mmol/l)	5.33±0.14	5.48±0.20	5.26±0.14	5.99±0.32	5.02±0.07[d]	4.93±0.17[d]
Chloride (mmol/l)	97.86±0.77	95.60±0.68	97.43±0.81	97.75±0.59	98.33±0.76	99.67±1.12[b]
Creatinine(mmol/l)	35.29±2.47	37.80±2.29	31.71±1.36	23.00±1.31[a,b,c]	30.00±1.69	29.00±1.93[b]
Protein(g/l)	60.86±1.46	65.80±2.97	63.43±0.78	63.43±1.36	65.50±0.67	64.83±1.01
BUN (mmol/l)	6.77±0.25	7.22±0.61	8.51±0.21[a]	7.76±0.14	7.48±0.22	7.70±0.33

Group 1- Control; Group 2-L-NAME(50mg.kg); Group 3- L-NAME + Ramipril(10mg.kg); Group 4- L-NAME + TA(40mg/kg); Group 5- L-NAME + TA(60mg/kg); Group 6- L-NAME + TA(80mg/kg). Values expressed as mean ± SEM for seven rats per group. [a] P <0.05 vs. control; [b] P <0.05 vs. L-NAME; [c] P<0.05 vs. Ramipril; [d] P<0.05 vs. TA(40mg/kg).

Effect of TA on Creatinine Clearance, fractional excretion of sodium, fractional excretion of potassium, Urine protein-creatinine ratio and kidney weight

Creatinine clearance increased in the TA treated groups as compared to the L-NAME induced group, and the Ramipril group, with a maximum effect at 80mg/kg of TA (Table 5). There was no significant difference between the TA treated groups and the control group. Additionally, there was no significant difference in the fractional excretions of sodium and potassium between the TA treated groups and the L-NAME group, although there was a slight increase in the L-NAME group (Table 5). Furthermore, there was an increase in the Urine protein-creatinine ratio (UPr/UCr) in the L-NAME group as compared to the control. This was decreased with the treatment of TA and Ramipril although it was not significant (Table

5). No significant difference was observed in UPr/UCr between the TA treated groups, the Ramipril and control group (Table 5). Left Kidney weight per body weight (LKW/BW) did not differ significantly in all the experimental groups (Table 5).

Table 5: Effect of TA on Creatinine Clearance (Ccr), fractional excretion of sodium (FeNa), fractional excretion of potassium (FeK^+), Urine Protein-Creatinine ratio (UPr/UCr) and left kidney weight-body weight ratio (LKW/BW)

Parameter	Groups					
	1	2	3	4	5	6
Ccr (ml/min)	0.31±0.03	0.26±0.03	0.24±0.04	0.42±0.03[c]	0.42±0.04[c]	0.46±0.09[b,c]
FeNa (%)	4.00±0.19	3.49±0.30	4.14±0.47	2.44±0.27[a,c]	3.04±0.11	3.35±0.61
FeK^+ (%)	301.1±33.03	263.5±16.45	266.2±21.69	175.3±14.88[a]	249.9±13.78	237.7±29.45
UPr/UCr	0.26±0.03	0.54±0.11[a]	0.33±0.05	0.38±0.07	0.32±0.02	0.34±0.04
LKW/BW (%)	0.42±0.01	0.44±0.02	0.42±0.02	0.43±0.03	0.43±0.02	0.44±0.02

Group 1- Control; Group 2-L-NAME(50mg.kg); Group 3- L-NAME + Ramipril(10mg.kg); Group 4- L-NAME + TA(40mg/kg); Group 5- L-NAME + TA(60mg/kg); Group 6- LNAME + TA(80mg/kg). Values are expressed as mean ± SEM for seven rats per group. [a] P <0.05 vs. control; [b] P <0.05 vs. L-NAME; [c] P<0.05 vs. Ramipril;

Relative fold changes in the mRNA expressions in the Kidney

HO-1 mRNA expressions

Figure 5 shows the levels of HO-1 gene expressions in the kidney tissues of the experimental groups. The L-NAME group showed a 0.5 fold decrease in HO-1 gene expression, when compared to the control. Interestingly, treatment with Ramipril and TA showed a significant ($p<0.05$) upregulation of HO-1 gene expression, with more pronounced effect at 80mg/kg of TA (5.4 fold increase). However, the upregulated gene expression of HO-1 in the Ramipril and TA doses of 60mg/kg and 80mg/kg were significantly higher than that of the 40mg/kg. No significant difference was observed in the HO-1 gene expression in the TA treated groups (60mg/kg and 80mg/kg) and the Ramipril group (Figure 5).

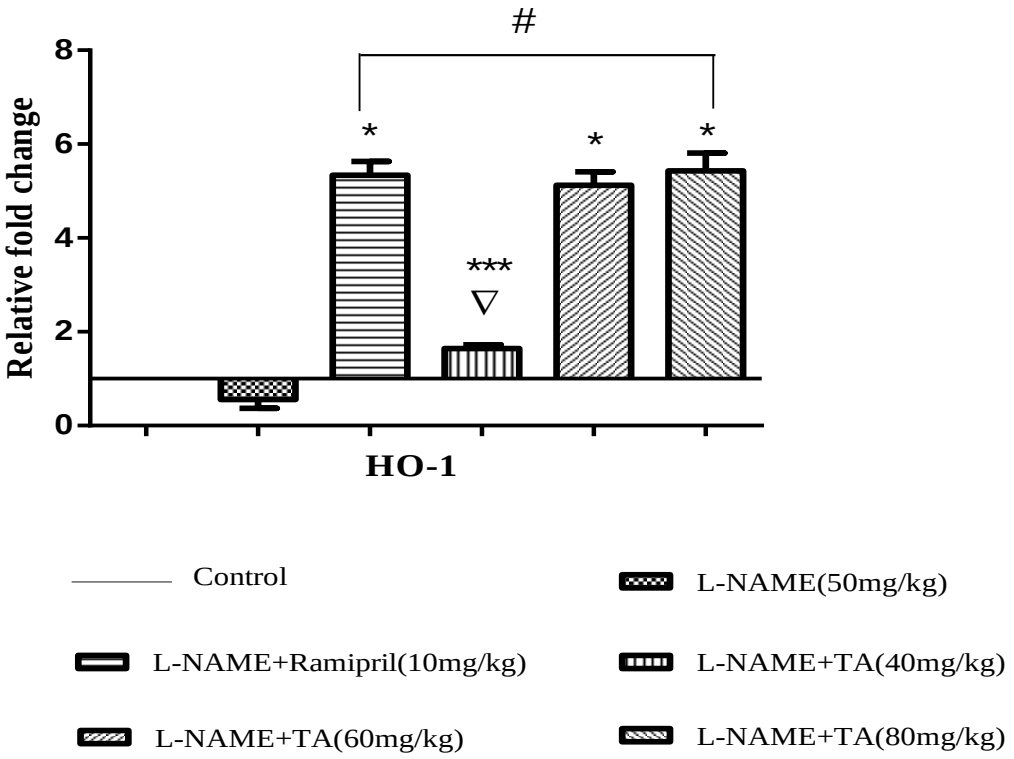

Figure 5: Graph showing the renal mRNA levels of HO-1 in L-NAME induced hypertensive rats. Values are expressed as mean ± SEM for seven rats per group. * *P*< 0.05 vs. Control; # *P* <0.05 vs. L-NAME, * *P <0.001* vs. TA (60mg/kg and 80mg/kg), ∇ P<0.05 vs. Ramipril**

NF-κB mRNA gene expression

The mRNA gene expression of NF-κB in kidney tissues is represented in figure 6. There was a significant (P<0.05) 3 fold increase in the gene expression of NF-κB in the L-NAME induced hypertensive group, when compared to the control. This increased expression was attenuated with the administration of TA and Ramipril, where there was a significant (P<0.05) downregulation with a maximum effect occurring at TA doses of 60 (0.4 fold decrease) and 80mg/kg (0.5 fold decrease). No significant difference was seen in the NF-κB gene expressions between the TA treated groups and the Ramipril treated group.

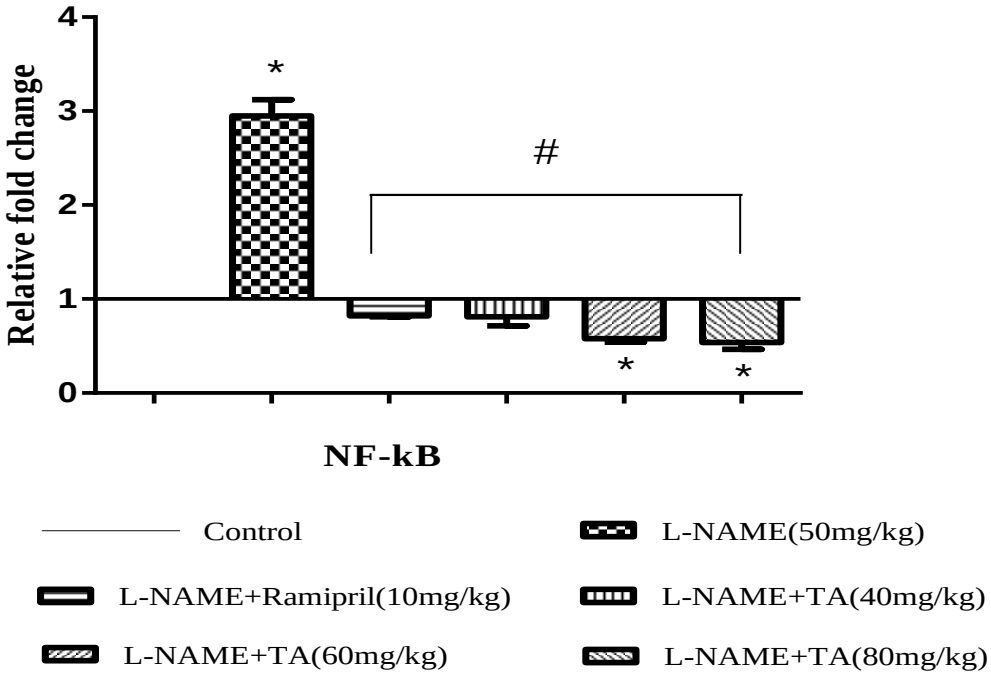

Figure 6: Graph showing the renal mRNA levels of NF-κB in L-NAME induced hypertensive rats. Values are expressed as mean ± SEM for seven rats per group. * *P*< 0.05 vs. Control; # *P* <0.05 vs. L-NAME.

eNOS mRNA gene expression

The eNOS mRNA gene expression in the kidney is represented in figure 7. There was a significant (P<0.05) 2-fold increase in the gene expression of eNOS in the L-NAME induced hypertensive group, when compared to the control. Interestingly, this increased expression was significantly (P<0.05) upregulated with the treatment of TA and Ramipril with the maximum effect occurring at the dose of 80mg/kg of TA (5.8 fold increase). No significant difference was seen in the eNOS gene expressions between the TA treated groups (60 and 80mg/kg) and the Ramipril treated group.

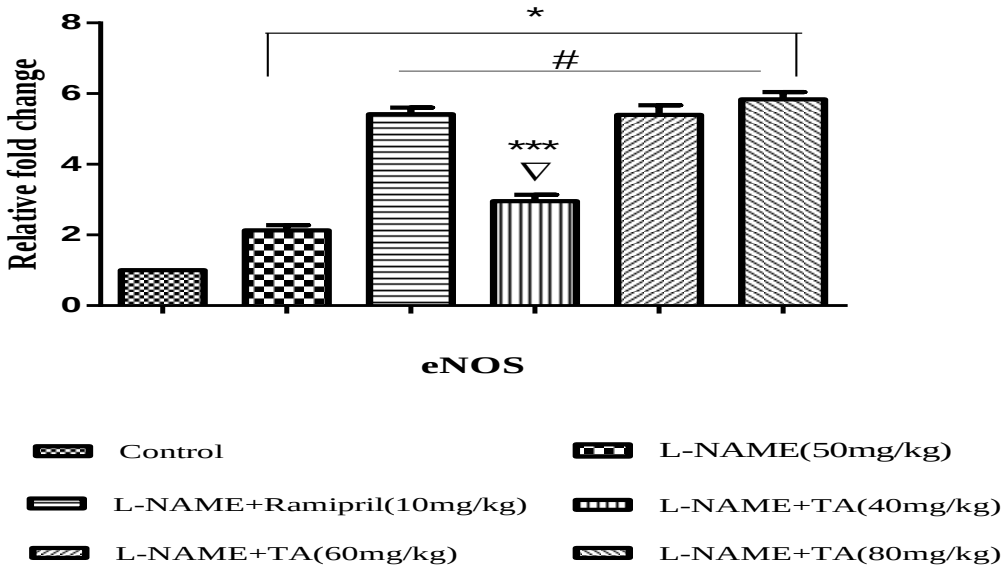

Figure 7: Graph showing the renal mRNA levels of eNOS in L-NAME induced hypertensive rats. Values are expressed as mean ± SEM for seven rats per group. * *P*< 0.05 vs. Control; # *P* <0.05 vs. L-NAME, *** P <0.001 vs. TA (60mg/kg and 80mg/kg), ▽ P<0.05 vs. Ramipril

Discussion

We have previously demonstrated a long term lowering of the systolic and diastolic blood pressure in an L-NAME hypertensive model and its subsequent effect on the maintenance of the cardiac function (unpublished data). In this study, we examined the long-term effects of TA and Ramipril on haematology and renal function, associated with hypertension, in an L-NAME induced hypertensive rat model. The L-NAME rat model showed a significant progressive increase in the mean arterial pressure (MAP) over the period of the study, as compared to the experimental groups, thereby affirming the expected L-NAME induced chronic inhibition of NO, resulting in arterial hypertension (19). The alleviation of this rise in MAP with the treatment of TA and Ramipril confirms their antihypertensive effect, with a pronounced effect at a TA dose of 80mg/kg.

Arterial hypertension is commonly associated with uncontrolled production of reactive oxygen species (ROS) leading to oxidative stress (20). This study, therefore, evaluated the free radical activities of superoxide dismutase (SOD), catalase (CAT), GSH and NO concentrations in kidney tissues and erythrocytes of hypertensive rats. The decreased activities of SOD, CAT and GSH in the L-NAME rats, in both erythrocytes and the kidney tissues, as observed in this study, may be due to their over utilization in ROS scavenging, as a result of increased blood pressure (21). However, their increased levels in the Ramipril and TA treated groups, demonstrates their role in promoting the bioavailability of these antioxidants, to aid the body to reduce oxidative stress (22).

Furthermore, lipid peroxidation, as a measure of oxidative damage in tissues, is measured using thiobarbituric acid reactive substances (TBARS) (23). Our study showed a significant increase in TBARS in the L-NAME group, indicating possible cellular damage in the kidneys and the erythrocytes. However, the decreased levels of TBARS in the Ramipril and TA treated groups, further supports their possible protective role in the maintenance of cellular integrity in the kidney, and the prevention of possible haemolysis in the erythrocytes due to oxidative stress (23).

We further examined the role of heamatological parameters in the development and progression of hypertension. It is worth noting that our study is amongst the few, recent studies that have focused on the investigations of haematological parameters, as indicators of the progression of hypertension, especially in the L-NAME hypertensive model. The tendency towards increased levels of haemoglobin (Hb), red blood cell (RBC) and haematocrit (HCT) in the L-NAME group, is in keeping with others (24, 25). This may be partially attributed to increased oxygen requirements, as a result of increased metabolic activity, during hypertension (25). Moreover, the significant increase in red cell distribution width (RDW)

observed in the L-NAME group in this study corroborates with other findings (26, 27). RDW is a measure of the variation of the size and volume of the circulating erythrocyte (27). The mechanism by which hypertension leads to an increased RDW is still under investigation (28). It may be attributed to increased arterial pressure which leads to the activation of RAS, causing angiotensin II induced erythropoietin activity, leading to the early proliferation of erythroid progenitors, therefore accounting for the variation in sizes of circulating erythrocytes (29). Therefore, the decreased RDW in the TA and Ramipril treated groups, may be probably due to the RAS blockade by TA and Ramipril in L-NAME induced rats. We also postulate that the compromised perfusion during hypertension, leading to hypoxia, may also cause erythropoietin secretion.

Furthermore, in our study, the platelet and mean platelet volume (MPV) increased significantly in the L-NAME group, as compared to the other experimental groups. This could be due to increased platelet activation, associated with endothelial damage, during high blood pressure (30). Other studies that are in keeping with our study, suggest that the consumption of platelets increases at the site of blood vessel injury, and this occurrence enhances the release of larger platelets which are known to be more physiologically active, and hence accounting for the increased MPV in hypertension (31). TA and Ramipril treatment are shown in our study to reduce blood cell and vessel injury, thereby preventing the over production of platelets and increased MPV, as reflected in the reduction of platelets and MPV values in these groups.

Additionally, white blood cell (WBC) indices are also known as prognostic markers of hypertension (32). The findings in our study showed a significantly increased WBC in the L-NAME hypertensive rats when compared to the control group. Treatment with TA and Ramipril significantly reduced the WBC, in agreement with recent findings of increased WBC among hypertensive patients (32, 33). This occurrence could be explained mostly due to the chronic inflammatory conditions associated with hypertension (34). WBC is a well-known inflammatory marker and tends to increase in hypertension (34). However, TA and Ramipril treatment, as shown in this study, attenuates the complications that arise from inflammation associated with hypertension, hence decreasing WBC.

To critically examine the role of white blood cell indices in hypertension, the neutrophil-lymphocyte ratio (NLR) and the lymphocyte-monocyte ratio (LMR), are recognized as emerging prognostic indicators for hypertension (35). Interestingly, our study shows a significant increase in NLR and LMR in L-NAME hypertensive rats, which was significantly attenuated with the administration of TA and Ramipril. This finding is in corroboration with other findings that indicate that increased NLR and LMR are predictive

markers of mortality in hypertension (36, 37). A combination of neutrophil and lymphocyte, as well as lymphocyte and monocyte, are known to be better indicators as compared to the individual parameters (36). Our study further supports the findings from other studies, that support the protective role of antihypertensives such as TA and Ramipril in decreasing NLR and LMR, hence, ultimately impacting on the complications and mortalities associated with hypertension (38).

It is established that hypertension causes biochemical and electrolyte changes in renal function, which also predicts the severity of the disease (39). Renal dysfunction is usually typified by a decreased glomerular filtration rate (GFR), increased serum creatinine, and progressive increase in proteinuria (40). In this study, we found improved renal function under TA treatment as evidenced by the decrease in serum creatinine and proteinuria. Additionally, we found increased creatinine clearance as well as a slight increase in the urine and serum electrolytes. Although most of these biomarkers were not significant, it indicates a tendency for TA to improve renal function, especially at a dose of 80mg/kg.

Surprisingly, TA and Ramipril treatment produced a non-significant decrease in fractional excretion of sodium (FeNa) and potassium (FeK^+) as compared to the L-NAME hypertensive rats, suggesting that chronic inhibition of NO may increase FeNa and FeK^+. Variable effects of NO inhibition, on sodium and potassium excretion, have been reported. Several authors have reported that L-NAME enhances sodium and potassium reabsorbtion, resulting in decreased FeNa and FeK^+ whereas, others postulate that chronic inhibition of NO with L-NAME, may override the anti-natriuretic effect initially stimulated, and lead to increased FeNa and FeK^+ (40, 41). In view of this discrepancy, we postulate that NO may play a crucial role in renal function, independent of changes in sodium and potassium excretion. Additionally, chronic inhibition of NO, has been demonstrated to decrease GFR in some studies, and not in others (42, 43). Previous studies also suggest that NO inhibition may decrease the blood flow in the renal system without affecting the GFR(44). This may explain in part the reason for the non significant decrease in GFR, as measured by the creatinine clearance, in the L-NAME rats, as compared to the other experimental groups in our study.

Furthermore, our assessment of mRNA expression of renal inflammatory markers such as renal HO-1, NF-κB, and eNOS revealed a significant upregulation of renal HO-1 and e-NOS in the TA treated group. We also found a significant downregulation of NF-κB in the TA and Ramipril treated groups, compared to the untreated L-NAME group. This observation is in support of improved renal function in an L-NAME induced hypertensive rat model. The significant up-regulation of HO-1 in TA and Ramipril treated groups, when compared to the control, is in keeping with other studies, that suggest that increased

expression of HO-1 in renal tissue confers a protective role on renal integrity (45). HO-1 is known to be the rate limiting enzyme responsible for the degradation of heme (46). It must be noted that free heme could be a highly toxic compound, and could lead to oxidative stress (47). Although free heme was not measured in the current study, its presence could lead to increased influx of leukocytes (WBC) into organs during inflammation (48), which was evident in our study. Finally, heme forms part of several pro-inflammatory enzymes such as cytochrome p450 mono-oxygenases, cyclooxygenase, and inducible nitric oxide synthase. Therefore, when HO-1 eliminates excessive free heme, it compromises the optimal activity of those enzymes, leading to an attenuation of inflammation (49). In view of this, we can postulate that TA and Ramipril may prevent inflammation associated with hypertension, through the expression of HO-1 and eNOS in kidney tissues.

Furthermore, NF-κB, as an inflammatory marker, plays a crucial role in the progression of hypertension (50). NF-κB is known to promote WBC infiltration in renal tissues during hypertension (51). Therefore, the increased white blood cells observed in our study, together with the increased expression of NF-κB in the L-NAME treated rats, confirms the inflammatory basis of hypertension. Additionally, a decreased expression of NF-κB, as well as decreased WBC in the TA and Ramipril treated groups, also confirms the anti-inflammatory effects.

Conclusion

In the current study, it has been demonstrated for the first time, that *Tulbaghia acutiloba* has a beneficial effect on renal function in an L-NAME induced hypertensive rat model, based on the haematological and biochemical parameters, which may be a good prognostic indicator of its antihypertensive efficacy. The dose of 80mg/kg of TA possessed a strong antihypertensive effect which is most likely related to the reduction in oxidative stress, increased bioavailability of NO, decreased heamatological parameters such as NLR, LMR, MPV, RDW, WBC, as well as, upregulation of HO-1 and downregulation of NF-κB. We can infer that TA (80mg/kg) may have a crucial role in maintaining renal function as well as maintaining the cellular integrity of circulating blood cells. Our study confirms the direct and indirect relationship between haematological and renal function in the disease progression and treatment of arterial hypertension. However, further studies are needed to ascertain the role of each bioactive compound responsible for the antihypertensive, renoprotection and haematological function of *Tulbaghia acutiloba*.

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CHAPTER 6

SYNTHESIS AND CONCLUSION

This study aimed to investigate the chemical composition, and *in vitro and in vivo* antihypertensive activities of the extracts of *Tulbaghia acutiloba* Harv. in an hypertensive experimental model to understand the biological activity and therapeutic benefits of the plant extracts.

The objectives to address the studies were:

1. To identify the phytochemical constituents, *in vitro* antioxidant potential and analyse heavy metal toxicity of the crude extracts of *Tulbaghia acutiloba*
2. To evaluate the *in vitro* Angiotensin-converting enzyme (ACE) Inhibition potential of the crude extracts of *Tulbaghia acutiloba*
3. To evaluate the antihypertensive and cardioprotective effect of *Tulbaghia acutiloba* extracts in an L-NAME hypertensive rats
4. To examine the effects of *Tulbaghia acutiloba* extract on renal function in an L-NAME hypertensive rats
5. To determine the effects of *Tulbaghia acutiloba* extract on heamatological indices in an L-NAME hypertensive rats

A comprehensive review and analysis of *Tulbaghia acutiloba* was conducted on its use to manage blood pressure. This formed the theoretical framework to evaluate *Tulbaghia acutiloba* phytochemicals using *in vitro* tests of antioxidant activity, and its inhibition of the angiotensin converting enzyme (ACE). Moreover, the heavy metal toxicological properties of the plant were evaluated to ascertain the acceptable limits for safe traditional use. Based on the promising *in vitro* findings obtained, the long term antihypertensive effects of *Tulbaghia acutiloba* was evaluated in an L-NAME induced hypertensive model.

A review of reports pertaining to alternative antihypertensive therapies, specifically phytotherapy, revealed a number of potential antihypertensive phytochemicals, many having been identified in South Africa, with its rich traditional knowledge systems and botanical diversity (Chapter 2). While many medicinal plants have been screened for *in vitro* biological activity, the studies lacked mechanistic insight into their *in vivo* effects. Furthermore, a holistic approach was applied to address the involvement of the

kallikrein-kinin system in combination with the renin-angiotensin system in the pathogenesis and treatment of hypertension.

Our findings showed that *Tulbaghia acutiloba* contains phytohemicals with strong antioxidant activity *in vitro* and *in vivo*, that it inhibits ACE activities under both conditions, and has antihypertensive and cardioprotective effects *in vivo.* The plant was also observed to improve renal function and haematological parameters associated with hypertension. This study provides evidence of the efficacy of *Tulbaghia acutiloba* and a basis for its further evaluation as a candidate for novel drug discovery. The key findings of the study and the relevant conclusions are summarized below.

***In vitro* preliminary investigations**

Objectives 1 and 2 entailed identifying the phytochemical constituents to establish the *in vitro* antioxidant potential and heavy metal toxicity analysis as well as the ACE inhibition activities of the crude extracts of *Tulbaghia acutiloba.* The preliminary screening of *Tulbaghia acutiloba* phytochemicals from the roots, rhizomes, flowers and leaves showed promising biological activity (Chapter 3). The *in vitro* antioxidant analysis was conducted to evaluate the scavenging activity of the plant on DPPH, hydrogen peroxide and nitric oxide. Moreover, as a culinary herb, there was a need for a heavy metal toxicity analysis to shed more light on its safety profile. Additionally, its antihypertensive potential was evaluated *in vitro* using the ACE inhibition activity assay, with all extracts being evaluated against the conventionally used ACEI drug, Ramipril.

The findings indicated the presence of α-linolenic acid (ALA), an omega-3- fatty acid, in the plant's leaves. This is notable, as ALA, an essential fatty acid, is not produced by the body, but is obtained from dietary sources, and has several beneficial effects, including lowering blood pressure (1), and is unique in the genus, *Tulbaghia*. While this was identified only in the leaves as a major compound, and not in other parts of the plants; *palmitic acid*, *oleic acid* and other bioactive compounds were observed in the other parts of the plant. Other compounds identified included phenols, saponins, alkaloids and amino acids in all parts of the plant, which also showed significant antioxidant activities and a strong ACE inhibitory activity, with the maximum effect being observed in the leaves that was comparable to the conventional ACEI, Ramipril, used in the study. Additionally, heavy metals were not detected in all parts of the plants, confirming their safety for consumption, when harvested from KwaZulu-Natal Province. It is of interest to note that the leaves, which contained ALA, coupled with the highest phenolic content, showed significant ACE inhibition, indicating that this effect may be related to the presence of these compounds. These findings led to further investigations of its potential therapeutic effect, *in vivo.*

Antihypertensive and cardioprotective effects of *Tulbaghia actuliloba* leaves

Objective 3 entailed evaluating the *in vivo* antihypertensive and cardioprotective efficacy of the crude leaf extracts of *Tulbaghia acutiloba*. It is well documented that untreated hypertension can lead to end-organ damage, including major organs in the cardiovascular system, especially the heart (2). This study therefore evaluated the antihypertensive efficacy and cardioprotective role of *Tulbaghia acutiloba* leaf extracts at different concentrations (40, 60 and 80mg/kg b.w.) in an L-NAME induced hypertensive rat model (Chapter 4). *Tulbaghia acutiloba* decreased the systolic and diastolic blood pressure levels, with maximal effect observed at the dose of 80mg/kg, which was comparable to the activity of Ramipril. Moreover, cardiac hypertrophy was not observed in the L-NAME hypertensive rats treated with *Tulbaghia acutiloba*. The plant's effect on the renin-angiotensin system and the kallikrein-kinin system in hypertension was observed, and a decrease in serum and cardiac tissue ACE activity identified in the hypertensive rat model. This was coupled with an increase in bradykinin receptor levels (B1 and B2) and a subsequent decrease in the AT1 and AT2 levels. The cardioprotective role of *Tulbaghia acutiloba* was also supported by a significant decrease in serum cardiac troponin I, CK and CKMB levels in the treated hypertensive model. This was further confirmed by histological analysis by attenuating cardiac hypertrophy and inflammation after administering *Tulbaghia acutiloba*. Generally, these findings indicate the beneficial therapeutic effects of the plant, as it decreased hypertensive vasoconstrictors, such ACE and ATI, whiles increasing vasodilatory effects via B1 and B2. These effects lower blood pressure with an impact on cardiac remodeling, and may account for the near normal histomorphology observed in the *Tulbaghia acutiloba* treated hypertensive model.

The role of oxidative stress in the progression of hypertension cannot be over emphasised, making its evaluation necessary. The findings indicated a significant increase in antioxidant activity in superoxide dismutase (SOD), catalase and GSH levels, as well as increased cardiac tissue NO bioavailability when treated with *Tulbaghia acutiloba*, and that lipid peroxidation was significantly reduced. Even at a high concentration of 80mg/kg b.w, the plant had no toxic effect on the liver, as indicated in the pattern of liver enzymes comparable to the control groups, with the lipid profiles indicating a non-significant difference in all treatment groups. Generally, the trends observed corroborate other studies conducted in potentially therapeutic medicinal plants (3-5). It is also important to note, based on biological activity, that the dose of 80mg/kg b.w. of *Tulbaghia acutiloba* leaf extract showed an optimal therapeutic effect, comparable with the activity of Ramipril.

Effects of *Tulbaghia acutiloba* (TA) on renal function

Objective 4 entailed evaluating the effect of *Tulbaghia acutiloba* on renal function. Untreated hypertension may also alter the physiologic functioning of the kidney, and the markers of kidney function were therefore evaluated (Chapter 5). The plant extract was found to improve renal function, at different concentrations, as evidenced in the increased urine volume and creatinine clearance, coupled with decreased Urine protein-creatinine ratio (UPr/UCr) and proteinuria. Other studies on garlic extracts have exhibited a similar pattern in hypertensive rat models (6, 7).

As the role of oxidative stress in the body during hypertension may be localised, the antioxidant effect of *Tulbaghia acutiloba* in the kidney was also examined, with a similar antioxidant trend being observed in both the kidney and heart tissues. There was a significant increase in the activities of SOD, catalase and GSH, coupled with the increased bioavailability of NO and reduced lipid peroxidation in the renal tissues after treatment, with pronounced effect at 80mg/kg b.w. These parameters work synergistically in an organism to improve the physiological function by impacting positively on various biochemical pathways, which could otherwise lead to pathology. Other reports also suggest that plants in the *Alliaceae* family inhibit Ang II-induced ROS generation, decrease lipid peroxidation in rat kidneys, improve antioxidative status, increase NO production and prevent vascular inflammation (8, 9).

Furthermore, a significant decrease in renal NF-kβ expression in the *Tulbaghia acutiloba* and Ramipril treated groups was found. A number of studies have suggested that activation of NF-kB is mediated through Ang II (9-11), with the decreased expression of this gene alluding to the effects related to RAS. Additionally, Heme oxygenase-1 (HO-1), an emerging gene of interest in the pathogenesis of hypertension, was investigated. HO-1 has become a recent gene of focus in hypertension due to its protective role in the kidney when highly expressed (12, 13). Our study found a significant increase of HO-1 expression in renal tissues in the *Tulbaghia acutiloba* treated L-NAME induced hypertensive group, which is a significant finding, as the role of HO-1 gene in hypertension is an emerging tool for assessing the therapeutic potentials of medicinal plants (14, 15).

Effects of *Tulbaghia acutiloba* (TA) on haematological function

Objective 5 entailed evaluating the effect of the plant on haematological function. Current accumulating evidence has underscored the importance of haematological parameters in the treatment and prognosis of hypertension (16, 17). The haemoglobin and haematocrit levels, red cell distribution width (RDW),

platelet, white blood cells and differential ratios, such as neutrophil-lymphocyte ratio (NLR) and lymphocyte-monocyte ratio (LMR), have become a major focus area, as a novel yet inexpensive marker to determine the progress and treatment efficiency in hypertension. Haematocrit levels are related to blood viscosity, and therefore affects haemodynamics (17). Additionally, white blood cells, and their relative differential ratios, mark the progress of the inflammatory processes associated with hypertension, with an elevation leading to possible end-organ damage (17). This study therefore evaluated the effect of *Tulbaghia acutiloba* on haematological parameters to better understand the mechanism of its antihypertensive efficacy (Chapter 5). Administering *Tulbaghia acutiloba* significantly reduced the RDW and haematocrit levels, while the white blood cells, NLR, LMR, platelet and mean platelet levels were significantly reduced in the plant and Ramipril-treated groups. Generally, the haematological findings in this study indicate that *Tulbaghia acutiloba* has a beneficial effect on circulation, as represented by reduced inflammatory markers, as well as platelet levels. These findings may be integrated with increased expression of HO-1 and NO levels, which are known to positively impact the vasculature and haematological parameters (Chapter 5). This study is among the few emerging reports that highlight the effect of medicinal plants on haematological function as a marker of its antihypertensive efficacy, and therefore serves to contribute immensely to investigations of this nature.

Conclusion

The study findings constitute multiple mechanisms in assessing the antihypertensive potential of *Tulbaghia acutiloba*. Our evaluation of its phytochemical constituents and biological activities has supported the therapeutic efficacy of this plant in lowering blood pressure, and therefore has the potential to be used to treat hypertension. Our results show that chronic administration of *T.acutiloba* leaf extracts produce cardioprotective, antihypertensive, improved renal and haematological function with no toxicological effect in an L-NAME induced hypertensive model. We may therefore conclude that the evidence produced in this study provides a strong basis and lays the groundwork for further scientific validation of the traditional use of *T.acutiloba* in primary health care to treat and manage hypertension and its associated complications.

Recommendations and future studies

The following recommendations are made to further understand the use of *T.acutiloba* to treat and manage hypertension.

1. To perform *in vivo* studies on the antihypertensive effect of *T.acutiloba* in other *in vitro* and *in vivo* hypertensive models to fully understand the integrative pathways in the pathogenesis and treatment of hypertension.

2. To isolate and test individual phytochemical bioactive compounds, *both in vitro* and *in vivo*, in order to identify novel therapeutic agents.
3. To determine the effect of *T.acutiloba* and Ramipril in the novel RAS pathway, such as the Ang 1-7/ACE2/Mas axis.
4. To evaluate the safety and efficacy of *T.acutiloba* on pregnancy-induced hypertension, as traditional healers may administer this herbal plant under these circumstances.
5. To evaluate the effect of *T.acutiloba* on gut microbiota, a relatively novel target, in the progression and treatment of hypertension.

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APPENDICES

Animal Ethics Approval

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

22 September 2017

Mr Isaiah Kofi Arhin (217073071)
School of Laboratory Medicine & Medical Sciences
Westville Campus

Dear Mr Arhin,

Protocol reference number: AREC/062/016PD
Project title: Beneficial effects of nutritive plants in South Africa with ACE inhibition activity

Full Approval – Renewal Application

With regards to your renewal application received on 14 August 2017. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

CONDITION:
New PI will not be allowed to start animal work without the PROOF of Basic Animal Handling Training. Please provide once obtained.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 22 September 2018.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

.....

Prof S Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Supervisor: Professor Irene Mackraj
Cc NSPCA: Ms Jessica Light
Cc Acting Academic Leader Research: Dr Michelle Gordon
Cc BRU – Dr Linda Bester
Cc Registrar: Mr Simon Mokoena

Animal Research Ethics Committee (AREC)
Ms Mariette Snyman (Administrator)
Westville Campus, Govan Mbeki Building
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Telephone: +27 (0) 31 260 8350 **Facsimile:** +27 (0) 31 260 4609 **Email:** animalethics@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

Animal Handling Certification

UNIVERSITY OF KWAZULU-NATAL

Biomedical Resource Unit

November 16, 2017

Dear Prof Islam
Chair: Animal Research Ethics Committee
c/o School of Life Sciences

RE: COMPETENCE TRAINING ON RATS ONLY

This letter confirms that Mr Isaiah Kofi Arhin,217073071, has undergone an evaluation for invasive procedures on the 16th of November 2017 and shows competence regarding the following:

a. IP Administration
b. Oral gavage
c. Subcutaneous procedures

Kind Regards

Dr SD Singh BVSc. (Mumbai) MS (Illinois) LAS (Utrecht) CVE (Pretoria)
HOD: Biomedical Resource Unit
Veterinarian

Research Ethics Certification

TRREE

Zertifikat
Certificat
Certificado
Certificate

Promouvoir les plus hauts standards éthiques dans la protection des participants à la recherche biomédicale
Promoting the highest ethical standards in the protection of biomedical research participants

Clinical Trials Centre
The University of Hong Kong

Certificat de formation - Training Certificate

Ce document atteste que - this document certifies that

Isaiah Arhin

a complété avec succès - has successfully completed

Introduction to Research Ethics

du programme de formation TRREE en évaluation éthique de la recherche
of the TRREE training programme in research ethics evaluation

July 21, 2017
CID : Ğ4etPzKrfB

Professeur Dominique Sprumont
Coordinateur TRREE Coordinator

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Programme de Formation continue (5 Crédits)

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[REV : 20170310]

Research Ethics Evaluation

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Research Ethics Evaluation

du programme de formation TRREE en évaluation éthique de la recherche
of the TRREE training programme in research ethics evaluation

July 21, 2017
CID : SNy2Cqeh7P

Professeur Dominique Sprumont
Coordinateur TRREE Coordinator

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Promoting the highest ethical standards in the protection of biomedical research participants

Clinical Trials Centre
The University of Hong Kong

Certificat de formation - Training Certificate

Ce document atteste que - this document certifies that

Isaiah Arhin

a complété avec succès - has successfully completed

Good Clinical Practice (GCP)

du programme de formation TRREE en évaluation éthique de la recherche
of the TRREE training programme in research ethics evaluation

July 21st, 2017
CID : YldNSI2YIG

Professeur Dominique Sprumont
Coordinateur TRREE Coordinator

FMH Continuing Education Program (5 Credits)
Programme de Formation continue (5 Crédits)

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Continuing Education Programms
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[REV : 20170310]

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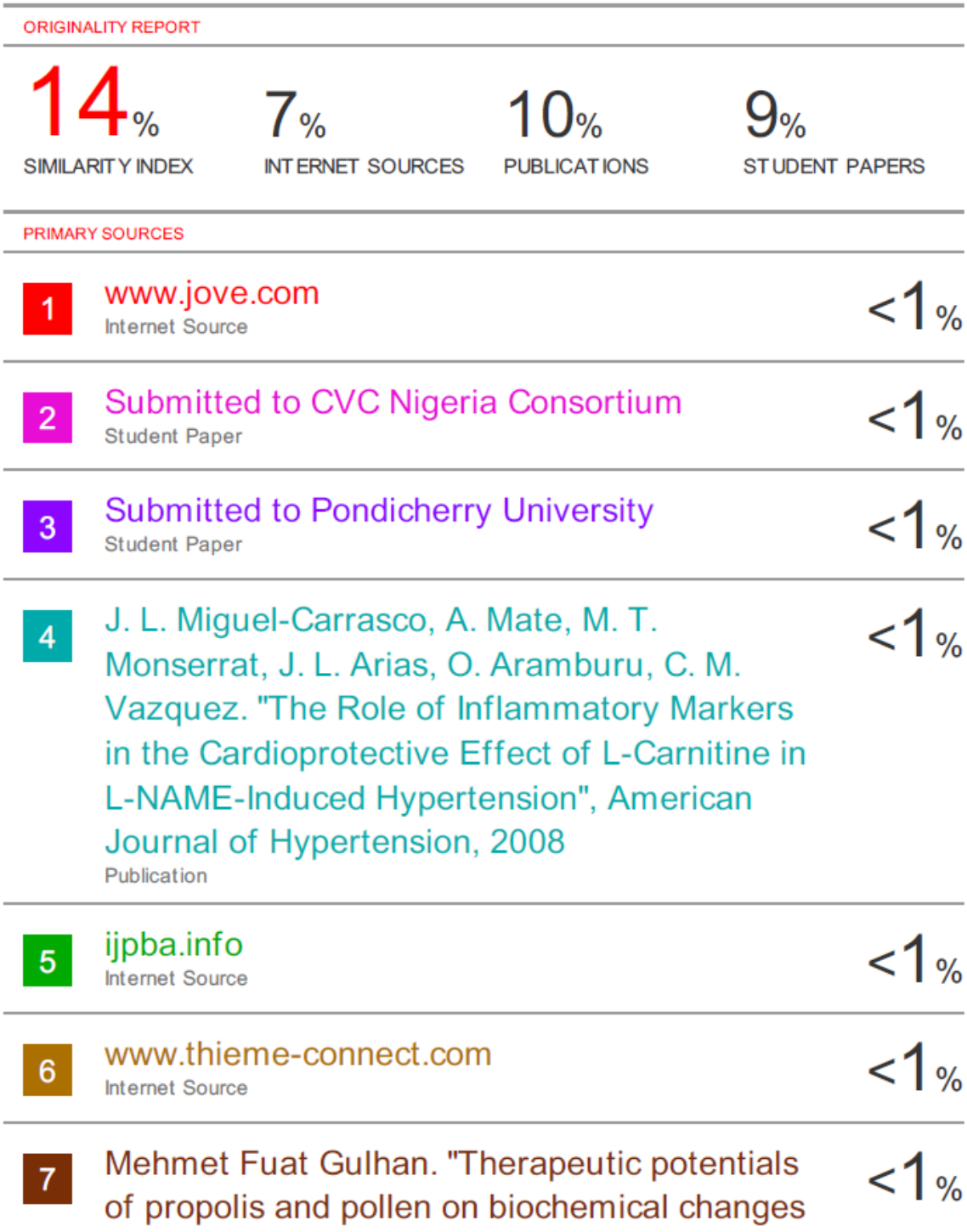

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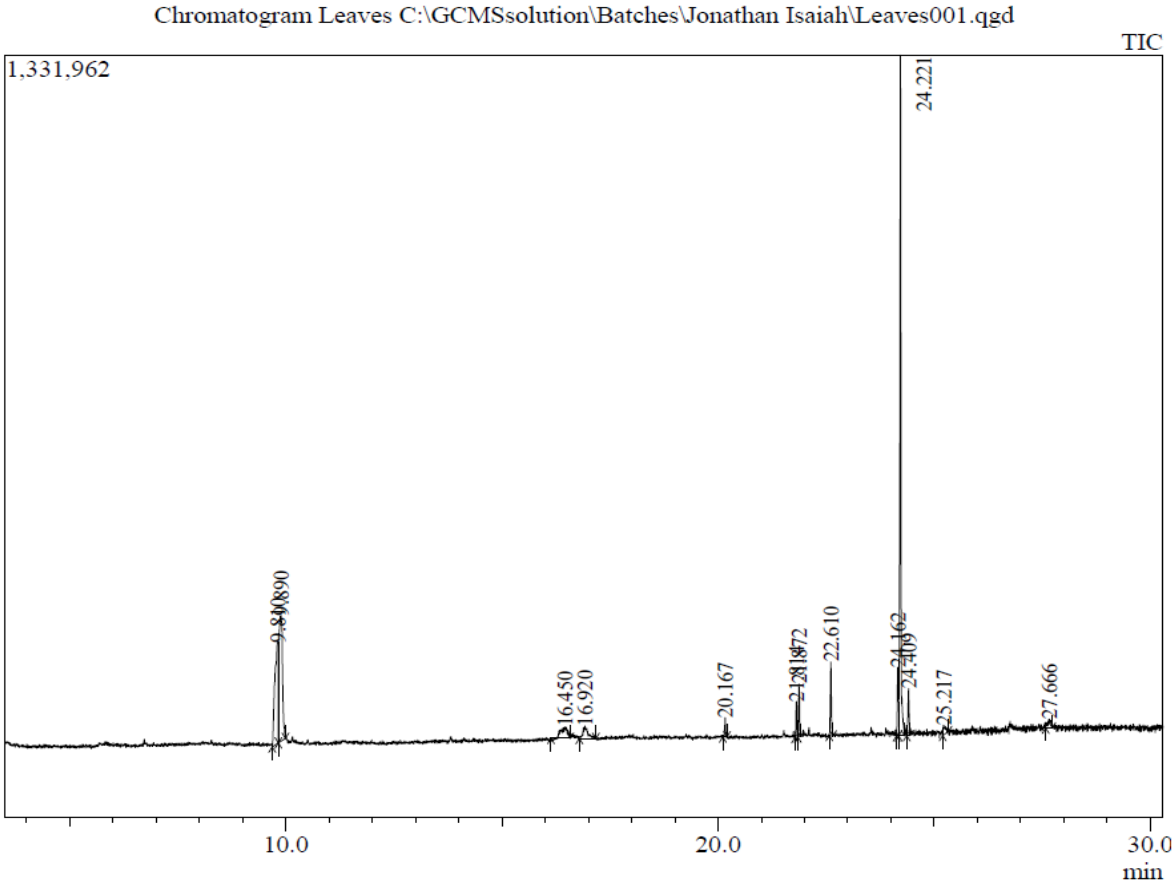
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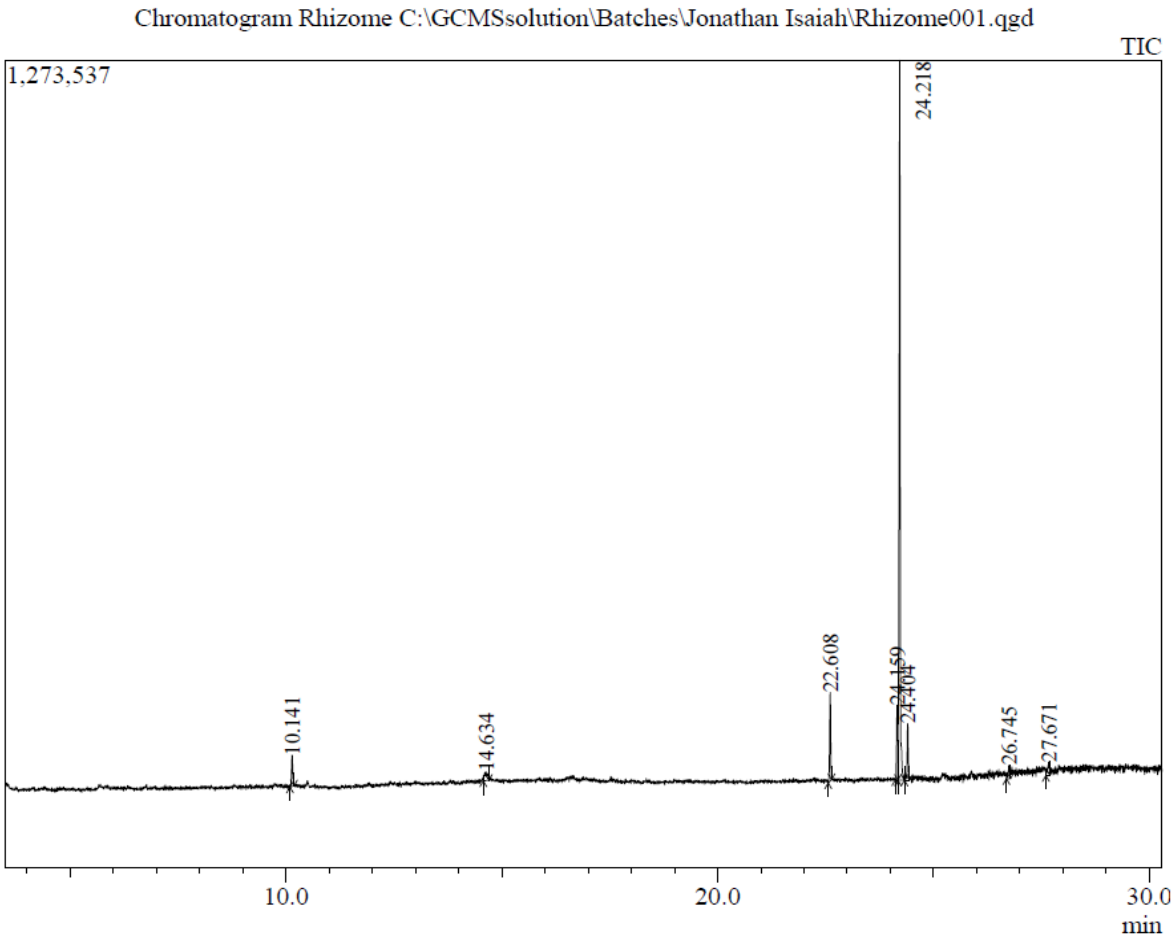
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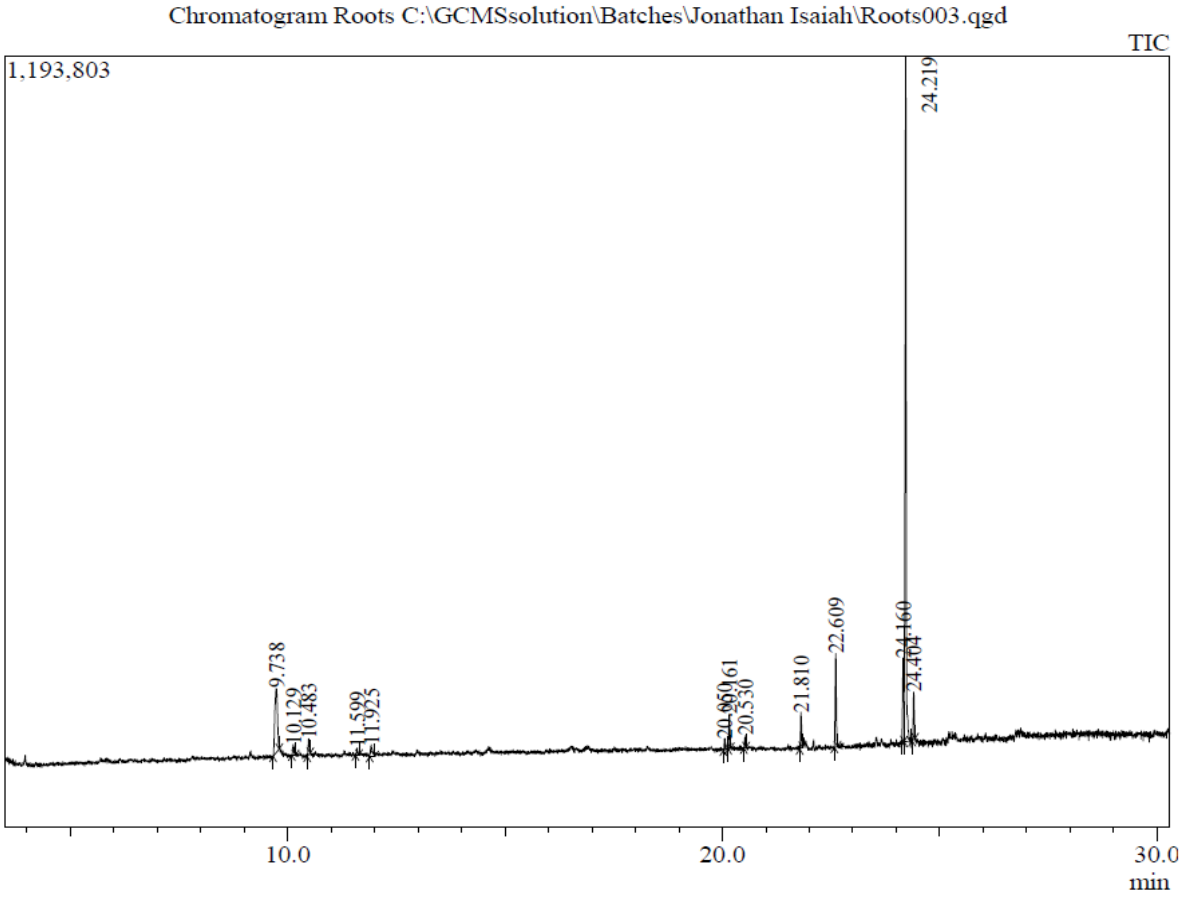
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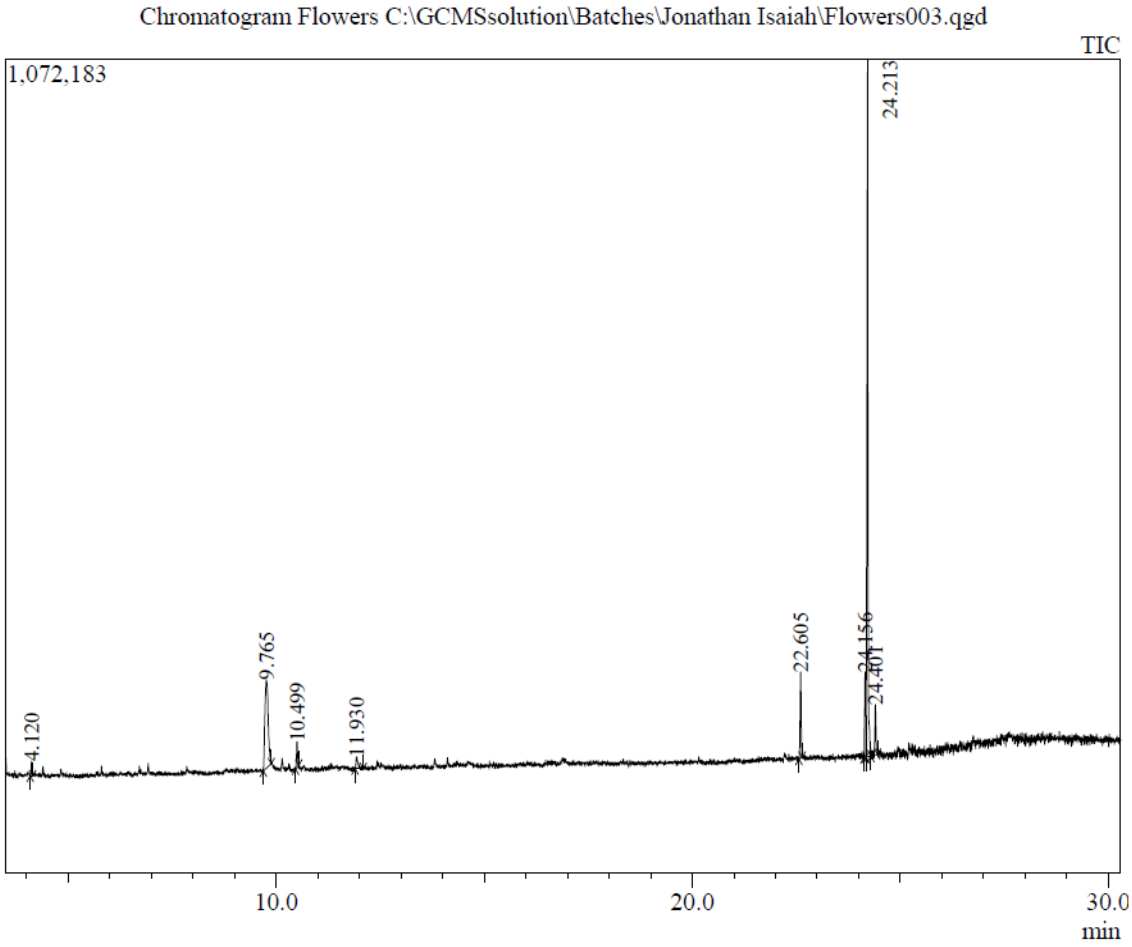
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