SOME CARDIOVASCULAR ACTIVITIES OF THE AQUEOUS LEAF EXTRACT OF *CROTON ZAMBESICUS* LINN (EUPHORBIACEAE) IN LABORATORY ANIMALS

By

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Declaration

I declare that the work in the dissertation entitled "Some cardiovascular activities of the aqueous leaf extract of *Croton zambesicus* linn (Euphorbiaceae) in laboratory animals" has been carried out by me in the Department of Pharmacology and Therapeutics. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other Institution.

Rashidat Oluwafunke AYANNIYI ______________ __________

Signature Date

Certification

This dissertation titled "SOME CARDIOVASCULAR ACTIVITIES OF THE AQUEOUS LEAF EXTRACT OF *CROTON ZAMBESICUS* LINN (EUPHORBIACEAE) IN LABORATORY ANIMALS" by Rashidat Oluwafunke AYANNIYI meets the regulations governing the award of the degree of DOCTOR OF PHILOSOPHY IN PHARMACOLOGY of the Ahmadu Bello University, and is approved for its contribution to knowledge and literacy presentation.

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Abstract

Croton zambesicus Linn (Euphorbiaceae) is a highly valued medicinal plant in Nigeria and West African sub-region. It is used by traditional medicine practitioners in the treatment of various ailments including hypertension. This study aims to determine some cardiovascular activities of the aqueous leaf extract and subfractions of *Croton zambesicus* in laboratory animals. The leaves of *Croton zambesicus* were extracted with water and subsequently subjected to column chromatography to obtain named sub-fractions A preliminary phytochemical screening of the aqueous extract and pooled column subfractions was carried out. The median lethal dose (LD_{50}) of the aqueous extract was determined in mice and rats and the hypotensive activity was assessed in anaesthetized normotensive cats. The diuretic activity was determined in rats and the effect of the extract on uric acid concentration in fructose fed rats was also evaluated. The effect of the extract on isolated right atrium of the guinea pig and isolated perfused heart of the rabbit were determined. The smooth muscle relaxant activity was determined in isolated rabbit ileum while inhibition of angiotensin II, acetylcholine, calcium chloride and potassium chloride-induced contraction of the rat ileum were used to determine it's mechanism. The extract was screened for angiotensin converting enzyme inhibitory activity using bradykinin-induced contraction of rat ileum and spectrophotometry. The subfractions were screened for inhibitory effect on acetylcholine and angiotensin II-induced contraction of rat ileum and subfractions with inhibitory effect were further screened for hypotensive activity in anaesthetized normotensive cats. Preliminary phytochemical analysis of the aqueous leaf extract and subfractions revealed the presence of flavonoids, tannins, saponins, triterpenes and alkaloids. The oral median lethal dose (LD_{50}) was greater than 5,000 mg/kg in both rats and mice. The extract (20 and 40 mg/kg *i.v.*) produced a significant (P<0.001) reduction in the blood pressure of anaesthetized normotensive cat. At 10 mg/kg the extract had significant (P<0.01) diuretic activity in rats

comparable to that of hydrochlorothiazide (10 mg/kg). The extract (2.5 mg/ml) produced a significant ($P<0.05$) reduction in the tone and rate of contraction of isolated right atrium of the guinea pig. In addition, the extract (6.4 mg/ml) inhibited isoprenaline (10 nM)-induced contraction of the isolated rabbit heart. The aqueous extract (3.20 and 6.40 mg/ml) produced a significant ($P<0.001$) reduction in spontaneous contraction of the rabbit ileum. Furthermore, the extract (0.32-6.40 mg/ml) produced a significant ($P<0.001$) concentration-dependent inhibition of angiotensin II-induced contraction of the rat ileum with an IC_{50} of 0.50±0.03 mg/ml. Conversely the aqueous extract (0.32 and 0.64 mg/ml) had no significant inhibitory effect on acetylcholine and calcium chloride-induced contractions of the rat ileum. A ten times higher concentration of the extract (3.20 and 6.40 mg/ml) significantly ($P<0.001$) inhibited acetylcholine and calcium chloride-induced contraction with an IC_{50} of 2.3±0.04 and 2.7±0.10 mg/ml respectively. The extract (0.32-0.64 mg/ml) also significantly ($P<0.001$) inhibited potassium chloride-induced contraction with an IC_{50} of 0.3±0.01 mg/ml. The aqueous extract however, had no significant angiotensin converting enzyme inhibitory activity. Subfractions F11-13, F29, and F30-36 significantly ($P<0.05$) inhibited acetylcholine-induced contraction of the rat ileum with no significant inhibitory effect on angiotensin II-induced contraction. Furthermore, subfraction F11-13 caused a reduction in blood pressure (BP) at 0.8 mg/ml while subfractions F29 and F30-36 gave no BP reduction at the concentrations tested. Both the inhibitory effect on agonist-induced contraction and blood pressure reduction by the subfractions were lower compared with the aqueous leaf extract of *Croton zambesicus*.

The data obtained from this study revealed that the aqueous leaf extract of *Croton zambesicus* has hypotensive, diuretic, negative ionotropic and chronotropic effects and smooth muscle relaxant activities mediated possibly via inhibition of both angiotensin 1 receptor and extracellular calcium influx through receptor operated and voltage-dependent calcium channels. The phytochemicals present in the extract are possibly responsible for these pharmacological activities which may be beneficial in the management of cardiovascular disorders such as hypertension.

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Abbreviations

ABPM	Ambulatory blood pressure monitoring
ACE	Angiotensin converting enzyme
ACEI	Angiotensin converting enzyme inhibitor
ACh	Acetylcholine
AIDS	Aquired Immunodeficiency Syndrome
Ang II	Angiotensin II
ANOVA	Analysis of variance
ARB	Angiotensin receptor blocker
AT_1	Angiotensin 1 receptor
BMI	Body mass index
BP	Blood pressure
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
CCB	Calcium channel blocker
CKD	Chronic kidney disease
CVD	Cardiovascular disease
CZ	*Croton zambesicus*
DAG	Diacylglycerol
DALYs	Disability-adjusted life years
DBP	Diastolic blood pressure
ECG	Electrocardiograph
FAP	N-(3-[2-furyl]acryloyl]Phe
FAPGG	N-(3-[2-furyl]acryloyl]Phe-Gly-Gly)

$FeCl_3$	Ferric chloride
H_20	Water
HCT	Hydrochlothiazide
HIV	Human Immunodeficiency Virus
JNC	Joint National Committee
K^+	Potassium ion
KCl	Potassium chloride
LD_{50}	Median lethal dose
MAP	Mean arterial pressure
$MgCl_2$	Magnesium chloride
Na^+	Sodium ion
Na_2HCO_3	Sodium bicarbonate
Na_2HPO_4	Sodium hydrogen phosphate
NaCl	Sodium chloride
NBF	n-butanol fraction
NO	Nitric oxide
PCF	Pooled Column fraction
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
RAAS	Renin-angiotensin-aldosterone system
R_f	Retention factor
UV	Ultra violet
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Preamble

Cardiovascular diseases (CVDs) are a group of disorders of the heart and blood vessels including; coronary heart disease, cerebrovascular disease and peripheral artery disease (WHO, 2013a). CVDs are currently a global medical and public health issue and leading cause of death with estimated 17.3 million deaths in 2008 representing 30 % of all global deaths (Addo *et al*., 2007; Alwan, 2011). They are projected to remain the leading cause of death with more than 23 million people dying annually by 2030 (Mathers and Loncar, 2006). Underlying determinants include: globalization, urbanization, population ageing, poverty, stress and hereditary factors (WHO, 2013a). Behavioural risk factors responsible for about 80% of coronary heart disease and cerebrovascular disease include; unhealthy diet, physical inactivity, tobacco use and harmful use of alcohol (Alwan, 2011). According to findings from the INTERHEART Africa study, hypertension, diabetes and abnormal lipids are clinical risk factors for CVDs (Steyn *et al*., 2005).

In Africa, increasing urbanization and changing lifestyles have raised the incidence of cardiovascular diseases to near epidemic proportion (Kadiri, 2005). Hypertension is one of the most prevalent noncommunicable conditions worldwide, which is responsible for an estimated 45% of deaths due to heart disease and 51% of deaths due to stroke globally (WHO, 2013b).

1.1.1 Hypertension

Hypertension affects about 1 billion people worldwide and is a major risk factor for stroke, myocardial infarction, vascular disease, and chronic kidney disease (Madhur, 2013). The prevalence rate in sub-Saharan African particularly in urban societies seems to be as high as those seen in developed countries (Addo *et al*., 2007). Prevalence of hypertension among Nigerians is high (Ekwunife and Aguwa, 2011) and has been put at 38.6% and 41.2% respectively for adult males and females aged ≥25 years (WHO, 2012).

Awareness, treatment and control of hypertension is extremely low in developing countries where majority of health care resources are directed to HIV/AIDS, tuberculosis and malaria control and treatment (Tesfaye *et al*., 2009, Ulasi *et al*., 2011). Managing this chronic condition and its resultant complications constitutes a great financial burden on individual patient and the health system of many countries (Ganiyu and Suleiman, 2014). For this reason majority of people living in developing countries relies on traditional remedies (mainly herbs) for their health care needs (WHO, 2013b).

1.1.2 Traditional medicine

Traditional and complementary medicine is an important and often underestimated part of health-care. It is found in almost every country in the world and the demand for its services is increasing (WHO, 2013b). Traditional medicine of proven quality, safety and efficacy, contributes to the goal of ensuring that all people have access to health care (WHO, 2013b).

At an International Conference on Traditional Medicine for South-East Asian

Countries in February 2013, the WHO Director-General, Dr Margaret Chan, made the following statement on traditional medicine:

> Traditional medicines, of proven quality, safety, and efficacy, contribute to the goal of ensuring that all people have access to health care. For many millions of people, herbal medicines, traditional treatments, and traditional practitioners are the main source of health care, and sometimes the only source of care. This is care that is close to homes, accessible and affordable. It is also culturally acceptable and trusted by large numbers of people. The affordability of most traditional medicines makes them all the more attractive at a time of soaring health-care costs and nearly universal austerity. Traditional medicine also stands out as a way of coping with the relentless rise of chronic non-communicable diseases
> (WHO, 2013b).

Many countries in Africa are conducting research on traditional medicines for malaria, HIV/AIDS, sickle-cell anaemia, diabetes and hypertension using WHO guidelines (WHO, 2011).

Croton zambesicus is a medicinal plant that is widely used amongst traditional medicine practitioners in Nigeria and Benin Republic. It is used in the management of chronic ailments including hypertension (Adjanohaun *et al*., 1989) but with limited scientific information on its ethnomedicinal use in the management of cardiovascular disorders.

1.2 Statement of research problem

High blood pressure causes 9.4 million premature deaths annually (Lim *et al*., 2012) and 92 million disability-adjusted life years (DALYs) worldwide (Lopez *et al*., 2006). Hypertension also contributes to the burden of heart disease, stroke, kidney failure, premature death and disability *(WHO, 2013b)*. African region, out of the six WHO's regions has the highest prevalence of hypertension estimated at 46% of adults aged 25 and above, according to WHO's *Global status report on noncommunicable diseases 2010 (WHO 2013 b).*

The general economic, security and political crisis experienced by developing nations like Nigeria leads to a gradual decline in the provision of health-care services. The people have less access to effective and affordable health-care services (Ganiyu and Suleiman, 2014). *Over 80 % of cardiovascular deaths have been reported to take place in low- and middle-income countries (WHO, 2013a). There is thus a need for more research in medicinal plants that will be safe and effective in the management of cardiovascular diseases.*

1.3 Justification for study

Non optimal blood pressure is the leading cause of death globally (WHO, 2013a). Most of these deaths and disability caused by blood pressure-related diseases are suffered by people in resource-limited countries. The resulting burden threatens already fragile health systems as well as social and economic development as very few people receive blood pressure-lowering therapy in these countries (Perkovic *et al*., 2007).

Pharmacological therapy is costly and associated with multiple side effects resulting in patient non-compliance. There is thus a need to explore alternative therapies particularly from herbal sources as these are easily assessable and affordable (Siddiqi *et al*., 2012).

About 75 to 80% of the world population use herbal medicines for primary health care because of their acceptability by the people and minimal side effects (Tabassum and

Ahmad, 2011). A considerable number of bioactive compounds including flavonoids and terpenoids derived from plants, in addition to possessing cardio protective effect, have been shown to reduce the risk of cardiovascular disease (Vasanthi *et al*., 2012).

A pragmatic way to maintain optimal arterial pressure would be to administer foods and herbs that reduce cardiac output and or systemic vascular resistance. This is important because dietary factors play a key role in the development of cardiovascular disease (Odugbemi, 2006).

1.4 Aim and objectives of the Study

1.4.1 Aim of the study

To investigate some cardiovascular activities of the aqueous leaf extract and fractions of *Croton zambesicus* in laboratory animals.

1.4.2 Objectives of the study are to determine the:

i. Effect of aqueous extract and column fractions of *Croton zambesicus* on the blood pressure of anaesthetized normotensive cats
ii. Diuretic activity of aqueous extract of *Croton zambesicus* in rats
iii. Effect of aqueous extract of *Croton zambesicus* on isolated right atrium of the guinea pig perfused heart of the rabbit
iv. Mechanism of action of aqueous extract of *Croton zambesicus* using isolated rat ileum
v. Angiotensin converting enzyme inhibitory activity of the aqueous extract of *Croton zambesicus* in rat
vi. The effect of column fractions of the aqueous leaf extract of *Croton zambesicus* on isolated rat ileum

1.5 Research Hypothesis

The aqueous leaf extract of *Croton zambesicus* and fractions has no pharmacologically active constituents which are beneficial in the management of cardiovascular disorders.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Hypertension

Hypertension (HTN) or high blood pressure (BP) is a chronic medical condition in which the BP in the arteries is elevated. Hypertension is defined as a systolic blood pressure (SBP) of 140 mm Hg or more, or a diastolic blood pressure (DBP) of 90 mmHg (Roger *et al*., 2012). Hypertension is not a disease but an important risk factor for cardiovascular complications. These complications include; stroke, congestive heart failure, myocardial infarction, pulmonary embolism, cerebral aneurysm, kidney failure (Pierdomenico *et al*., 2009). The most common of these are stroke and myocardial infarction. An increase of 5 mmHg in diastolic blood pressure is associated with a 35-40 % increase risk of stroke (Walker and Whittlesea, 2008).

2.1.1 Classification of hypertension

Based on recommendations of the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC7), the classification of BP for adults aged 18 years or older is as follows (Chobanian *et al*., 2003):

- Normal: Systolic lower than 120 mm Hg, diastolic lower than 80 mm Hg
- Prehypertension: Systolic 120-139 mm Hg, diastolic 80-89 mm Hg
- Stage 1: Systolic 140-159 mm Hg, diastolic 90-99 mm Hg
- Stage 2: Systolic 160 mm Hg or greater, diastolic 100 mm Hg or greater

2.1.2 Etiology of hypertension

Abnormally high blood pressure is generally divided into two main categories: essential hypertension and secondary hypertension. Primary or essential hypertension accounts for 90-95 % of adult cases, while secondary hypertension accounts for 2-10 % of cases (Carretero *et al*., 2000; Rule *et al*., 2009).

Secondary hypertension results from an identifiable cause which include atherosclerosis, diabetes, kidney disease, adrenal gland tumors, Cushing's syndrome, hyperthyroidism, hypothyroidism, obesity, metabolic disorders, preeclampsia during pregnancy, sleep apnea, congenital defects of the aorta and heart, alcoholism, and toxicity from prescribed and illicit drugs, especially cocaine and methamphetamines (Mayo, 2011).

The etiology of essential hypertension is unknown and its pathophysiology is assumed to be multifactorial. Many causal factors have been linked to essential hypertension including a sedentary lifestyle, tobacco smoking, excessive stress, visceral obesity, hypokalemia, high sodium intake and other poor dietary habits, sodium sensitivity, alcohol consumption and vitamin D deficiency (Wofford *et al*., 2004; Kyrou *et al*., 2006; Wu *et al*., 2006).

2.2 Pathophysiology of Hypertension

A number of interrelated factors contribute to the raised blood pressure in hypertensive patients, and their relative roles may differ between individuals. Among the factors that have been intensively studied are salt intake, obesity and insulin resistance, the renin-angiotensin system, and the sympathetic nervous system. Other factors that have been evaluated, include; genetics, endothelial dysfunction (as changes in endothelin and nitric oxide), low birth weight, intrauterine nutrition, neurovascular anomalies and hyperuricemia (Batkai and Thum, 2012; Johnson *et al.*, 2013).

Physiological mechanisms involved in development of essential hypertension include: Increased cardiac output, increased peripheral resistance, overactivity of renin-angiotensin-aldosterone system and autonomic nervous system (Armstrong *et al*., 2012).

2.2.1 Cardiac output and peripheral resistance

Maintenance of a normal blood pressure is dependent on the balance between the cardiac output and peripheral vascular resistance. Most patients with essential hypertension have a normal cardiac output but a raised peripheral resistance (Harvey, 2012). Peripheral resistance is determined by small arterioles, the walls of which contain smooth muscle cells. Contraction of smooth muscle cells is thought to be related to a rise in intracellular calcium concentration, which may explain the vasodilatory effect of drugs that block the calcium channels. Prolonged smooth muscle constriction induces structural changes with thickening of the arteriolar vessel walls possibly mediated by angiotensin, leading to an irreversible rise in peripheral resistance (Chong and Michel, 2012).

In early hypertension the peripheral resistance is not raised and the elevation of the blood pressure is caused by a raised cardiac output, which is related to sympathetic over activity (Waller *et al*., 2010). The subsequent rise in peripheral arteriolar resistance might therefore develop in a compensatory manner to prevent the raised pressure being transmitted to the capillary bed where it would substantially affect cellular homeostasis (Waller *et al*., 2010).

2.2.2 Renin-angiotensin system

The renin-angiotensin system is one of the most important of the endocrine systems that affect the control of blood pressure. Renin is secreted from the juxtaglomerular apparatus of the kidney in response to glomerular under perfusion or hyponatremia. It is also released in response to stimulation from the sympathetic nervous system (Beevers *et al*., 2001). Renin is responsible for converting renin substrate (angiotensinogen) to angiotensin I, a physiologically inactive substance which is rapidly converted to angiotensin II in the lungs by angiotensin converting enzyme (ACE). Angiotensin II is a potent vasoconstrictor and thus causes a rise in blood pressure (Guibert *et al*., 2008). In addition it stimulates the release of aldosterone from the zona glomerulosa of the adrenal gland, which results in a further rise in blood pressure related to sodium and water retention (Golan *et al*., 2012).

There is, however, increasing evidence that there are important non-circulating "local" renin-angiotensin epicrine or paracrine systems, which also control blood pressure. Local renin systems have been reported in the kidney, the heart, and the arterial tree. They may have important roles in regulating regional blood flow (Golan *et al*., 2012).

2.2.3 Autonomic nervous system

Sympathetic nervous system stimulation can cause both arteriolar constriction and arteriolar dilatation. Thus the autonomic nervous system has an important role in maintaining a normal blood pressure. It is also important in the mediation of short term changes in blood pressure in response to stress and physical exercise (Guzik., *et al*., 2007; Madhur *et al*., 2010).

There is, however, little evidence to suggest that epinephrine and norepinephrine have any clear role in the aetiology of hypertension. Nevertheless, their effects are important because drugs that block the sympathetic nervous system do lower blood pressure and have a well established therapeutic role. Hypertension can be linked to an interaction between the autonomic nervous system and the renin-angiotensin system, together with other factors such as sodium, circulating volume, and some hormones (Batkai and Thum, 2012).

2.2.4 Endothelial dysfunction

Vascular endothelial cells play a key role in cardiovascular regulation by producing a number of potent local vasoactive agents, including the vasodilator molecule nitric oxide and the vasoconstrictor peptide endothelin. Dysfunction of the endothelium has been implicated in human essential hypertension (Waller *et al*., 2010). Modulation of endothelial function is an attractive therapeutic option in attempting to minimize some of the important complications of hypertension (Waller *et al*., 2010).

Clinically effective antihypertensive therapy appears to restore impaired production of nitric oxide, but does not seem to restore the impaired endothelium dependent vascular relaxation or vascular response to endothelial agonists. This indicates that such endothelial dysfunction is primary and becomes irreversible once the hypertensive process has become established (Faxon *et al.*, 2004).

2.2.5 Vasoactive substance

Vasoactive systems and mechanisms affecting sodium transport and vascular tone are involved in the maintenance of a normal blood pressure. It is not clear, however, what part these play in the development of essential hypertension (Chong and Michel, 2012).

Bradykinin: is a potent vasodilator that is inactivated by angiotensin converting enzyme. Consequently, the ACE inhibitors may exert some of their effect by blocking bradykinin inactivation (Vogel, 2002).

Endothelin: is a vascular endothelial vasoconstrictor which may produce a salt sensitive rise in blood pressure. It also activates local renin-angiotensin systems (Faxon *et al*., 2004).

Atrial natriuretic peptide: is a hormone secreted from the atria of the heart in response to increased blood volume. It increases sodium and water excretion from the kidney as a sort of natural diuretic. A defect in this system may cause fluid retention and hypertension (Beevers *et al*., 2001).

2.2.6 Hypercoagulability

Hypertension is often associated with abnormalities of vessel wall (endothelial dysfunction or damage), the blood constituents (abnormal levels of haemostatic factors, platelet activation, and fibrinolysis), and blood flow, suggesting that hypertension confers a prothrombotic or hypercoagulable state. These components appear to be related to target organ damage and long term prognosis, and some may be altered by antihypertensive treatment (Golan *et al*., 2012).

2.2.7 Insulin sensitivity

Epidemiological studies have shown that there is a clustering of several risk factors, including obesity, hypertension, glucose intolerance, diabetes mellitus, and hyperlipidaemia. This has led to the suggestion that these represent a single syndrome (metabolic syndrome X or Reaven's syndrome), with a final common pathway to cause raised blood pressure and vascular damage (Armstrong *et al*., 2012).

2.2.8 Genetic factors

Although separate genes and genetic factors have been linked to the development of essential hypertension, multiple genes most likely contribute to the development of the disorder in a particular individual. Hypertension is about twice as common in subjects who have one or two hypertensive parents (Dickson *et al.*, 2006). Epidemiological studies suggest that genetic factors account for approximately 30 % of the variation in blood pressure in various populations. This figure was derived from comparisons of parents with their monozygotic and dizygotic twin children, as well as their other children, and with adopted children. Some familial concordance is however, due to shared lifestyle (dietary) factors (Rahmouni *et al.*, 2005).

Experimental models of genetic hypertension have shown that the inherited tendency to hypertension resides primarily in the kidney (Beevers *et al*., 2001). Animal and human studies show that a transplanted kidney from a hypertensive donor raises the blood pressure and increases the need for antihypertensive drugs in recipients coming from “normotensive” families. Conversely a kidney from a normotensive donor does not raise the blood pressure in the recipient. Increased plasma levels of angiotensinogen have also been reported in hypertensive subjects and in children of hypertensive parents (Beevers *et al*., 2001).

2.2.9 Hyperuricemia

Uric acid has been proposed to have a causal role in hypertension and may be an important risks factor for hypertension (Johnson *et al*., 2013). Studies in animal models suggest that hyperuricemia may be particularly important in early hypertension (Watanaba *et al.,* 2002). Experimentally, the mechanism by which uric acid causes hypertension is via oxidative stress, endothelial dysfunction and activation of the renin angiotensin system (Johnson *et al*., 2013).

2.3 Management of Hypertension

2.3.1 Non drug measures

Lifestyle modification is a non drug measure employed in the management of hypertension. This involves increased physical activity, weight loss in overweight patients, diet low in sodium and high in potassium, consumption of fruits, vegetables, and low-fat dairy product (Chobanian *et al*., 2003). The Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC7) recommend lifestyle modification as the first step in managing hypertension (Chobanian *et al*., 2003).

2.3.2 Drugs used in the management of hypertension

When lifestyle modifications are insufficient to achieve the goal BP, several drugs are available for treating and managing hypertension (Waller *et al*., 2010). Drugs used in the management of hypertension include; thiazide diuretics, angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs), calcium channel blocker (CCBs), beta-blockers, vasodilators and centrally acting antihypertensives (Chobanian *et al*., 2003). These drugs decrease cardiac output and or peripheral vascular resistance (Harvey, 2012).

2.3.2.1 Thiazide diuretics

Diuretics such as bendrofluazide and hydrochlorothiazides are recommended as first-line therapy for hypertension because of their effectiveness and ability to dilate arteries (Wright *et al*., 2009). Diuretics increase the rate of urine formation along with net loss of sodium and water resulting in decreased cardiac output and reduced vascular resistance (Armstrong *et al*., 2012). They are beneficial in elderly patients, low-renin hypertension, obese patients with volume overload and isolated systolic hypertension (Jain, 2008). Thiazides are cheap and effective but often associated with increased risk of hypokalemic metabolic alkalosis, hyperglycemia, hyperuricemia, amongst others (Zillich *et al*., 2006).

2.3.2.2 Beta adrenoceptor antagonist

Beta adrenoceptor antagonist such as; atenolol, propanolol, labetalol and celiprolol act by reducing cardiac output via negative inotropic and chronotropic actions. They specifically block the action of catecholamines on beta-adrenergic receptors (Cruickshank, 2010). In addition, they block sympathetic nervous system stimulation of renin release by the juxtaglomerular cells. Beta-blockers when used in combination with diuretics lower the risk of stroke by approximately 40 percent (Psaty *et al*., 1997). The most commonly reported adverse effects are lethargy, erectile dysfunction and nightmares which occur with lipid-soluble beta-blockers (Cruickshank, 2010).

2.3.2.3 Calcium channel blockers

Calcium channel blockers (CCB) decrease the influx of calcium through voltage-gated L-type in the plasma membrane. The resulting decrease in intracellular calcium concentration leads to reduced contraction of both cardiac and vascular smooth muscle cells. There are three subclasses; the dihydropyridines (nifedipine-like), the phenylalkylamine derivatives (verapamil), and the benzothiazepines (diltiazem). Calcium channel blockers are more effective than ACE inhibitors or beta-blockers alone in blacks and the elderly hypertensive patient. Side effects include ankle edema, headache, and flushing (Nelson, 2010).

2.3.2.4 Angiotensin Converting Enzyme (ACE) inhibitors

These drugs inhibit the activity of ACE responsible for the conversion of angiotensin-I into angiotensin-II, a potent vasoconstrictor. As a consequence, ACE inhibitors lower arteriolar resistance and increase venous capacity, cardiac output, and stroke volume, (Waller *et al*., 2010). Examples include; captopril, lisinopril, perindopril and ramipril (Walker and Whittlesea, 2008). They are more effective when combined with a thiazide diuretic and beneficial in treating hypertension in people with coronary artery disease, heart failure or kidney failure (Walker and Whittlesea, 2008).

2.3.2.5 Angiotensin receptor blockers (ARBs)

ARBs, such as losartan, valsartan and irbesartan lower blood pressure in hypertensive patients and slow the progression of cardiovascular disease (Coffman, 2011; Crowley *et al*., 2007). They block the activation of angiotensin 1 (AT1) receptors, which directly causes vasodilation and reduces the secretion of aldosterone, among other actions. The combined effect reduces blood pressure (Golan *et al*., 2012).

Like ACE inhibitors, ARBs are recommended for people with coronary artery disease, heart failure, and kidney failure. ARBs are chosen for treatment of hypertension when a patient is intolerant of ACE inhibitor therapy (Golan *et al*., 2012). ARBs do not inhibit the breakdown of bradykinin or other kinins, and are thus only rarely associated with the persistent dry cough and angioedema that limit ACE inhibitor therapy. ARBs such as irbesartan and losartan have proven to be of benefit to hypertensive patients with type-2 diabetes mellitus and may delay the progression of diabetic nephropathy (Kassler-Taub *et al*., 1998).

2.3.2.6. Alpha-adrenoceptor antagonists

Alpha$_1$-antagonists (such as doxazosin and prazosin) reduce total peripheral resistance by blocking the sympathetic activation of α_1-receptors on resistance vessels (Waller *et al*., 2010). These drugs are used for some patients in the management of hypertension. They have a good side-effect profile (palpitations and occasional postural hypotension). In addition, they are reported to have beneficial effects on lipid profile and insulin resistance, and lack the negative effects on sexual potency of other antihypertensives, which is an advantage for diabetics ((Remedica, 2004).

2.3.2.7 Centrally acting sympathomimetics

Clonidine and α-methyldopa, centrally acting α_2-adrenoceptor agonists, were once popular choices, but are now less widely prescribed outside specialist scenarios such as pre-eclampsia. They carry a risk of rebound hypertension on withdrawal (Remedica, 2004).

2.3.2.8 Direct acting vasodilators

Vasodilators, such as hydralazine and the very potent minoxidil, are mostly used in resistant hypertension when standard agents fail. Use of the latter is generally restricted by unpleasant side effects such as hypertrichosis (Remedica, 2004)

2.3.2.9 Imidazoline type 1 receptor agonists

Selective imidazoline type-1 receptor agonists like Moxonidine are well tolerated, with dry mouth being the only frequently reported unwanted effect (Golan *et al*., 2012). This is an advantage over centrally acting α_2-adrenoceptor agonist in the management of hypertension (Golan *et al.*, 2012).

2.4 Current Trend and Treatment Guideline for Management of Hypertension

Hypertension is multifactorial in nature and as a result most patients require treatment with 2-3 or more antihypertensive agents to achieve target pressure (Cushman *et al*., 2002; Milani, 2005; Jamerson *et al*., 2008).

Based on recommendations of the report of Joint National Committee 8 (JNC 8), the initial therapy of hypertension is CCBs, ACE inhibitors, ARBs, or diuretics. In nonblack hypertensive patients, initial therapy is a thiazide-type diuretic, CCB, ACE inhibitor, or ARB. In hypertensive black patients, initiate therapy with a thiazide-type diuretic or CCB. Regardless of race or diabetes status, in patients 18 years or older with chronic kidney disease, initial or add-on therapy should consist of an ACE inhibitor or ARB (Wood, 2013; James *et al*., 2014).

2.4.1 The JNC 8 recommendations

The Eight Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC 8) recommendations (Wood, 2013 and James *et al*., 2014). In patients aged 60 years or older, therapy should be initiated in those with systolic BP levels at 150 mm Hg or greater or whose diastolic BP levels are 90 mm Hg or greater; and treated to below these thresholds. In patients younger than 60 years as well as those older than 18 years with either chronic kidney disease (CKD) or diabetes, the BP treatment initiation and goals should be 140/90 mm Hg. In nonblack hypertensive patients, treatment should be initiated with a thiazide-type diuretic, CCB, ACE inhibitor, or ARB. In hypertensive black patients, therapy is initiated with a thiazide-type diuretic or CCB. Regardless of race or diabetes status, in patients 18 years or older with CKD, initial or add-on therapy should consist of an ACE inhibitor or ARB. An ACE inhibitor should not be used in conjunction with an ARB in the same patient.

If a patient's goal BP is not achieved within 1 month of treatment, the dose of the initial agent should be increased or an agent from another of the recommended drug classes should be added; if 2-drug therapy is unsuccessful for reaching the target BP, a third agent from the recommended drug classes should be added. In patients whose goal BP cannot be reached with 3 agents from the recommended drug classes, agents from other drug classes are used and or the patients are referred to a hypertension specialist.

Antihypertensive regimens that include two agents with complementary mechanisms of action may result in greater reductions in BP than the single-agent components. JNC 8 in its recent report recommends the use of 2-3 drugs if BP reduction is not achieved with a single agent (James *et al*., 2014). Therapy with a calcium channel blocker, an angiotensin receptor blocker, and a thiazide diuretic has been advocated to be a logical combination in the management of hypertension (Calhoun *et al*., 2009). Calcium channel blocker inhibits the transmembrane influx of calcium ions into vascular smooth muscle and cardiac muscle, angiotensin receptor blocker inhibits angiotensin II-mediated vasoconstriction and renal sodium retention, while thiazide diuretic reduces intravascular volume and total body sodium (Calhoun *et al*., 2009). The benefits of combination therapy in hypertension include increased efficacy and decreased severity of adverse effects (Neutel, 2006; Sica, 2002). Dual therapy with valsartan and hydrochlorothiazide (HCT) provides a greater antihypertensive effect compared with either agent alone. In addition valsartan attenuates the HCT-induced hypokalemia (Pool *et al*., 2007).

A study by Calhoun *et al*., (2009), demonstrated the efficacy and safety of triple therapy amlodipine/valsartan/hydrochlorothiazide (10/320/25 mg) combination in a single pill formulation compared with dual therapy valsartan/HCT, amlodipine/valsartan and amlodipine/HCT in patients with moderate to severe hypertension. Triple therapy was well tolerated and BP control was significantly better than dual therapies (Calhoun *et al*., 2009). The study confirmed the benefit of using drugs with complementary mechanisms of action to treat hypertension.

The use of single-pill combinations is also of additional benefit since it has the potential to improve patient adherence to therapy compared with administration of multiple-pill combinations (Padwal *et al*., 2010).

2.4.2 Collaborative AHA/ACC/CDC advisory recommendations

A science advisory on the treatment of hypertension was issued in November 2013 via a collaborative effort by the American Heart Association (AHA), the American College of Cardiology (ACC), and the Centers for Disease Control and Prevention (CDC). It described criteria for successful hypertension management algorithms and advocates the creation of algorithms that can be incorporated into a system-level approach to high BP, as well as modified to accommodate different practice settings and patient populations (Go, *et al*., 2013).

A joint AHA/ACC/CDC algorithm in the report included the following recommendations (Go, *et al*., 2013) BP: Recommended goal of 139/89 mm Hg or less.

Stage 1 hypertension (systolic BP 140-159 mm Hg or diastolic BP 90-99 mm Hg): Can be treated with lifestyle modifications and, if needed, a thiazide diuretic. Stage 2 hypertension (systolic BP >160 mm Hg or diastolic BP >100 mm Hg): Can be treated with a combination of a thiazide diuretic and an ACE inhibitor, an angiotensin receptor blocker, or a calcium channel blocker. Patients who fail to achieve BP goals: Medication doses can be increased and/or a drug from a different class can be added to treatment

2.4.3 Joint ESH and ESC guidelines

In June 2013, the European Society of Hypertension (ESH) and the European Society of Cardiology (ESC) released new guidelines for the management of hypertension, recommending that all patients, except special populations such as patients with diabetes and the elderly, be treated to below 140 mm Hg systolic BP (Mancia *et al*., 2013). The guidelines advise that physicians should make decisions on treatment strategies based on the patient's overall level of cardiovascular risk.

Recommendations of the new ESH and ESC guidelines include (O'Riordan, 2013 and Mancia *et al*., 2013):

In patients younger than 80 years, the systolic BP target should be 140 to 150 mm Hg, but BP can be lower than 140 mm Hg if the patient is fit and healthy. Patients with diabetes should be treated to below 85 mm Hg diastolic BP. Salt intake should be limited to approximately 5 to 6 g per day. Body-mass index (BMI) should be reduced to 25 kg/m^2 and waist circumferences should be reduced to less than 102 cm in men and less than 88 cm in women. Ambulatory BP monitoring (ABPM) should be incorporated into the assessment of risk.

Effective combination therapies include thiazide diuretics with ARBs, calcium-channel antagonists, or ACE inhibitors; or, calcium-channel antagonists with ARBs or ACE inhibitors. Dual renin-angiotensin system blockade (ARBs, ACE inhibitors, and direct renin inhibitors) is not recommended because of the risks of hyperkalemia, low BP, and kidney failure. Although additional data is needed, renal denervation is a promising therapy in the treatment of resistant hypertension.

2.4.4 American Diabetic Association (ADA) 2011 guideline

The ADA 2011 standard of medical care states that in individuals with diabetes and mild hypertension, it may be reasonable to begin treatment with a trial of nonpharmacologic therapy (diet, exercise, and other lifestyle modifications.) Mild hypertension as defined by the ADA guideline (systolic BP 130-139 mm Hg or diastolic BP 80-89 mm Hg) may be classified as prehypertension by other organizations (ADA, 2011).

The ADA 2011 standards of medical care in diabetes also indicate that a majority of patients with diabetes mellitus have hypertension. In patients with type 1 diabetes, nephropathy is often the cause of hypertension, whereas in type 2 diabetes, hypertension is one of a group of related cardiometabolic factors (ADA, 2011). Hypertension remains one of the most common causes of congestive heart failure (CHF). Antihypertensive therapy has been demonstrated to significantly reduce the risk of death from stroke and coronary artery disease.

2.5 Traditional Medicine

Traditional medicine has been in existence for hundreds of years and the World Health Organisation estimates that about 80 % of the population in developing countries relies on traditional plant medicines for primary health care needs. (Sampson *et al*., 2000; Elujoba *et al.*, 2005). The regular use of traditional therapies demands scientific evidence for the principles behind therapies so as to improve efficacy and safety (Patwardhan *et al.,* 2005).

2.5.1 Medicinal plants with beneficial cardiovascular activity

Some medicinal plants and herbs reported to have hypotensive and or antihypertensive effects include;

2.5.1.1 Allium sativum (Family: Liliaceae; Common name: Garlic)

Allium sativum has long been used for a variety of cardiovascular conditions, especially hyperlipidemia. It has also been reported to have hypotensive action. It is thought to increase nitric oxide production, resulting in smooth muscle relaxation and vasodilatation (Reinhart *et al*., 2008; Dhawan and Jain 2005).

2.5.1.2 Carum copticum (Family: Umbelliferae; Common name: Ajwain)

The crude extract of *C. copticum* produced a fall in BP and heart rate (HR) of anaesthetized normotensive rats. Hypotension produced is very brief and returns to normal within a minute (Gilani *et al*., 2005).

2.5.1.3 Cassia occidentalis

(Family: Caesalpiniaceae; Common name: Coffee weed)

The leaf of the plant is used in traditional medicine as an antihypertensive agent. *In vitro* studies of the leaf extract showed a relaxant effect on aortic rings. The study revealed that cassia extract may be relaxing smooth muscle and reducing BP by inhibiting Ca^{2+} influx through receptor-operated and voltage-sensitive channels (Ajagbonna *et al*., 2001).

2.5.1.4 Hibiscus sabdariffa (Family: Malvaceae; Common name: Roselle)

This is one of the most extensively studied plants for antihypertensive properties. A study reported the water extract of dry *Hibicus sabdariffa* calyx produced a fall in the BP of experimentally-induced hypertensive rats (Mojiminiyi *et al*., 2007). The antihypertensive effect of the crude extract has been attributed to direct vasorelaxant effects, mediated through acetylcholine and histamine dependent mechanisms (Adegunloye *et al*., 1996 and Mojiminiyi *et al*., 2007).

2.5.1.5Moringa oleifera (Family: Moringaceae; Common name: Murungai)

The crude extract of the leaves of *M. oleifera* caused dose-dependent fall in systolic, diastolic, and mean arterial BP which was brief, returning to normal within two minutes (Faizi *et al*., 1998).

2.5.1.6 Ocimum basilicum (Family: Lamiaceae; Common names: Basil)

The crude extract of *Ocimum basilicum* has been reported to cause a fall in systolic, diastolic and mean arterial BP. This cardiovascular effect of the extract was attributed to eugenol, which exerts its effect by blocking the calcium channels (Azhar *et al.,* 1995).

The cardiovascular activities of *Viola odorata* Linn (Violaceae), (Siddiqi *et al*., 2012) and *Hymenocardia acida* (Manga *et al*., 2013) have also been reported.

These plants may be beneficial to health when consumed or added to food, while isolation of bioactive compounds in the plants may enhance their development into safe, effective and affordable therapeutic agents for the management of hypertension (Tabassum and Ahmed, 2011).

2.5.2 Description of the plant-*Croton zambesicus*

Croton zambesicus Muell Arg. (Euphorbiaceace) (syn C. amabilis muell. Arg., C. gratissimus Burch.) is an ornamental tree grown in villages and towns in Nigeria. It is a slender dioecious tree up to 30 feet high, sometimes forming a bole, with scaly bark. The leaves have a pointed apex and are silvery rusty-scaly below. It is commonly called bushveld, and referred to koriba or ichen maser in Hausa, Ajekobale in Yoruba, mfam in Ekoi and Moramora in Kilba (Hutchinson and Daziel, 1972).

Plate I. ***Croton zambesicus*** **(Euphorbiaceae) in its natural habitat**

Plate IIa: Upper surface of *Croton zambesicus* leaf with fruits

Plate IIb: Lower surface of *Croton zambesicus* leaf

2.5.3 Ethnomedicinal uses of *Croton zambesicus*

The tree is widely distributed in tropical Africa and in times past it was planted as a fetish tree *(Hutchinson and Daziel, 1972).* It has a reputation of conferring protection, and is often planted near the entrance of houses to ward off evil influences both in Sierra Leone and Nigeria *(Hutchinson and Daziel, 1972).*

The belief that it is protective against witches, explains its Yoruba name, *àje kò bàlé,* means 'witches do not dare to perch on it', and the plant enters an incantation for the placation of witches. To the *Ejagham* of Southern Nigeria it is a symbolic tree, known, at least in its religious significance, as *mfam* (name of the Juju cult). It is powerful to restore health to an important person, or the leaves drawn gently over the face of the dying to cause the spirit to pass painlessly. A soup made from the leaves is used for dysentery cases in Southern Nigeria (Burkill, 1985).

A leaf-decoction is used as a wash in both Nigeria and Sierra Leone and is taken internally for dysentery, fever, convulsions (Adjanohaun *et al*., 1989*). In Benin Republic and Nigeria, the leaf is used as antihypertensive and antimicrobial for urinary tract infections* (Adjanohaun *et al*., 1989*). The Ibibios in Uruan area of Akwa Ibom State of Nigeria use the leaf traditionally as a remedy for malaria (Okokon et al., 2005). In* Nigeria it is used for headache and to expel intestinal worms *(Hutchinson and Daziel, 1972)*. The fruits, like the bark, are aromatic. They are used in the Adamawa region of Nigeria to spice food and to prepare a sort of scent *(Hutchinson and Daziel, 1972).*

2.5.4 Phytochemical and Pharmacological studies on Croton zambesicus

*The phytochemistry of Croton species is diverse. Terpenoids are reported to be the predominant secondary metabolite in the genus, and these are mainly diterpenoid. Volatile oils containing mono and sesquiterpenoids, alkaloids, and phenolic substances including flavonoids, lignoids and proanthocyanidins have also been reported to be present in Croton zambesicus (*Salatino *et al., 2007).*

The antidiabetic, (Okokon et al., 2006), anticonvulsant and neuropharmacological (Ayanniyi and Wannang, 2008a and 2008b), antiulcer, (Okokon and Nwafor, 2009), anti-inflammatory, analgesic and antipyretic (Okokon and Nwafor, 2010), and anticoagulant activities (Robert et al., 2010) have been report scientifically. Vascular smooth muscle relaxant activity of a natural diterpene isolated from Croton *zambesicus (*Baccelli *et al., 2007; Martisen et al*., 2010) and antioxidant activity (Aderogba *et al.*, 2011) *of Croton zambesicus have also been reported.*

The ent-trachyloban-3β-ol, a trachylobane diterpene isolated from dichloro-methane extract of the leaves has cytotoxic activity on Hela cells (Block et al., 2002). The alkaloidal fractions of the leaf have been reported to possess weak antimicrobial activity (Abo et al., 1999).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Animals

Male Albino rats (150-200 g) were obtained from the Animal House of the Department of Pharmacology and Therapeutics, Ahamadu Bello University Zaria. Guinea pigs of either sex (300-500 g), adult cats (1-1.5 kg) and rabbits (600-800 g) were obtained from Samaru market in Zaria and kept in the animal house to acclimatize. The experiments were carried out in accordance with the Guidelines for Laboratory Procedures laid down by the International Animal Care and Use Committee (IACUC) in Nigeria. All the animals were fasted for 16 hours, but allowed free access to water before the commencement of experiments.

3.1.2 Collection and identification of plant material

The leaves of *Croton zambesicus* were collected between April and June, 2011 from Iwo, Iwo local government area of Osun State, Nigeia. The plant was identified by Mr. I.A. Kareem, a taxonomist at the Federal College of Forestry, Jos, Nigeria and further authenticated at the Herbarium Section in the Department of Pharmacognosy, University of Jos, Nigeria. The plant was found to correspond with voucher specimen number UJ/PGPH/HSP/0801.

3.1.3 Drugs

Acetylcholine chloride, losartan, *N*-(3-[2-furyl]acryloyl]Phe-Gly-Gly) (FAPGG), nifedipine (Sigma Aldrich Chemical, USA). Angiotensin II, bradykinin and lisinopril

(Bachem AG, Switzerland), were prepared by dissolving the powder in deionized water prior to administration.

3.1.4 Equipment and apparatus

These include: Recording microdynanometer (Ugo Basile), 10 ml, 20 ml organ baths, blood pressure transducer, centrifuge, chromatographic column, data capsule digital recorder 17400 (Ugo basile, Italy), isolated perfused heart set up, spectrophotometer (Jenway), metabolic cages, micro pipettes (VWR, England), potter tube, separating funnel, stop watches, ultra violet (UV) lamp and vortex mixer

3.2 Methods

3.2.1 Preparation of the aqueous leaf extract of *Croton zambesicus* (AECZ)

The leaves were removed from the stem, shade dried for 2 weeks and reduced to fine powder using mortar and pestle. The powdered leaves (300 g) were extracted by maceration with distilled water for 24 hours and filtered with Whatman filter paper (No.1). The filtrate was evaporated to dryness on a water bath at 40 - 45 ^{o} C. The residue obtained was weighed and stored in a dessicator prior to use. A stock solution of the crude extract was prepared by weighing a quantity of the extract, triturated with a mortar and pestle and dissolved with deionized water.

3.2.2 Partitioning of the aqueous leaf extract of *Croton zambesicus*

Partitioning of the extract was carried out using n-butanol, ethyl acetate and hexane. Fourteen grams of the extract was weighed and dissolved in deionized water. This was transferred into a separating funnel with an equal volume of n-butanol. The separating funnel was shaken gently, left to stand for a few minutes and the aqueous and n-butanol fractions collected respectively. This procedure was repeated three times. The n-butanol fraction was concentrated over a water bath at 50-55 ^{o}C.

3.2.3 Purification of n-butanol fraction

The n-butanol fraction (3.4 g) was subjected to column chromatography using silica gel (60 mesh size). Distilled hexane, dichloromethane, ethyl acetate and methanol were used as eluents. The column was packed with dry silica gel, then n-butanol fraction was dissolved in methanol and impregnated on Silica gel before introducing onto the Column. Gradient elution was started with pure hexane, thereafter the eluent polarity was increased gradually with dichloromethane, ethylacetate and completed with 100 % methanol. A total of fifty-three (53) eluates were collected and screened using thin layer chromatography. Eluates with similar retention factor (R_f) values were combined and distilled off to obtain named residues. They were subsequently referred to as pooled column fractions (PCF) 10, 11-13, 14, 15-26, 27, 28, 29, 30-36, 40-45, 46-51 and 52-53. Some crystals were formed at the bottom of the flask containing PCF 28. These were separated by filtration and the filtrate named PCF 28(1). The crystals were dissolved successively with n-butanol and methanol and the filtrate obtained were named PCF 28(2) and PCF 28(3), respectively while the residue was named PCF 28 crystal. The PCF were stored in the refrigerator before use.

3.2.4 Preliminary phytochemical screening of the aqueous leaf extract of *Croton zambesicus* and column fractions

The aqueous extract and column fractions of *Croton zambesicus* were screened for phytochemical constituents according to the methods of Evans, (1989) and Sofowora, (1993).

3.2.4.1 Carbohydrate (Molisch test)

A few drops of Molisch reagent were added to an aqueous solution of the extract in a test tube. Concentrated sulphuric acid (1ml) was added down the side of the test tube. The presence of a reddish ring at the interface of the two liquids indicated the presence of carbohydrate.

3.2.4.2 Anthraquinones (Borntrager test)

The extract 0.5 g was shaken in 5 ml of chloroform in a test tube. This was filtered and the filtrate shaken with an equal volume of dilute ammonia solution. The appearance of a reddish pink colour in the lower ammoniacal layer denotes the presence of anthraquinones.

3.2.4.3 Alkaloids (Dragendoff test)

Aqueous solution of the extract (1 ml) was treated with strong ammonia and extracted with chloroform. The chloroform extract was concentrated and a few drops of Dragendorff reagent was added to it. The appearance of an orange-yellow precipitate indicated the presence of alkaloid.

3.2.4.4 Steroids and triterpenes (Liebermann Burchards test)

The extract (0.1 g) was extracted with 5 ml of methanol and filtered. The filtrate was evaporated to dryness on a water bath. The residue was shaken with chloroform and filtered into a clean test tube. Acetic anhydride (2 ml) was added to the filtrate and shaken. Concentrated sulphuric acid (1ml) was added carefully down the side of the tube to form a lower layer. A brownish-red or violet ring at the interface between the two liquids with the upper layer turning green indicated the presence of steroids and triterpenes.

3.2.4.5 Cardiac glycosides (Keller-Killiani test)

An aqueous solution of the extract was shaken with 0.5 ml lead acetate and filtered. The filtrate was shaken with 5 ml of chloroform and allowed to separate. The chloroform was pipetted off and evaporated to dryness. The residue was dissolved in 3 ml glacial acetic acid containing sulphuric acid. A reddish brown layer formed at the interphase of the two liquids with the upper layer turning to bluish-green on standing.

3.2.4.6 Saponins (Frothing test)

An aqueous solution of the extract was shaken and a froth that persisted on warming indicated the presence of saponins.

3.2.4.7 Tannins ($FeCl_3$ test)

An aqueous solution of the extract was filtered. Two drops of 5 % ferric chloride was added to the filtrate. The presence of a dark green precipitate indicated the presence of tannins.

3.2.4.8 Flavonoids (Sodium hydroxide test)

Acetone was added to 0.1 g of the extract and stirred. The mixture was evaporated, residue extracted with warm water and filtered. To an equal volume of the filtrate, 5 ml of 20 % sodium hydroxide was added. The appearance of a yellow colour indicated the presence of flavonoids.

3.2.5 Composition of physiological solutions

Ringer Locke Solution was prepared by weighing the following salts; NaCl (9 g), KCl (4.2 g), D-glucose (1 g), Na_2HCO_3 (5 g), 1M $CaCl_2$ (10.8 ml) and dissolved in 1 liter of deionized water.

Tyrode Solution was prepared by weighing the following salts; NaCl (9 g), KCl (2 g), D-glucose (1 g), Na_2HCO_3 (0.5 g), $CaCl_2$ (2 g), Na_2HPO_4 (0.5 g), $MgCl_2.6H_20$ (1ml) and dissolved in 1 liter of deionized water.

3.3 Acute Toxicity Study

The median lethal dose (LD_{50}) of the aqueous leaf extract of *Croton zambesicus* (AECZ) was determined using the method described by Lorke (1983) in rats and mice. This test was carried out in two phases. In the first phase, three (3) groups each containing three animals were treated with AECZ at doses of 10, 100, and 1000 mg/kg body weight *p.o.* and observed for signs of toxicity and death for 48 hours. In the second phase, four (4) groups each containing one mouse each were administered with four more specific doses of the extract, based on the result of the first phase. The LD_{50} value was determined by calculating the geometric mean of the lowest dose that caused death and the highest dose at which all the animal survived.

$$LD_{50} = \sqrt{MLD \times MTD}$$

Where LD_{50} = median lethal dose

MLD = minimum lethal dose

MTD = maximum tolerated dose

3.4 Effect of Aqueous Leaf Extract of *Croton zambesicus* on Blood Pressure in Normotensive Cats

An adult cat of weighing 1.0 to 1.5 kg was anaesthetized by intraperitoneal administration of thiopentone sodium (50 mg/kg). The right carotid artery and left femoral vein were cannulated for measurement of arterial blood pressure and systemic administration of drugs, respectively. In order to minimize blood coagulation, heparin (100 I.U.) was administered intravenously and flushed with 0.9 % w/v sodium chloride. The arterial cannular was connected to a pressure transducer and the blood pressure (BP) readings were recorded with a micro-dynamometer. After 10 minutes stabilization period, graded doses (5-40 mg/kg) of the AECZ were infused. Each dose was flushed with 0.1 ml normal saline and the BP was recorded. BP was allowed to return to baseline values before further doses were infused (Ojewole *et al.,* 2007). One centimeter (1 cm) on the recording sheet corresponds to 10 mmHg pressure change in glass sphygmomanometer (Bako *et al*., 2010).

3.5 Determination of Diuretic Activity of the Aqueous Leaf Extract of *Croton zambesicus* in Rats

The method of Freitas et al., (2011) with modifications was employed. Male albino rats weighing 100 to 150 g were maintained at room temperature and 12 hour dark-light cycle. Food and water were given *ad libitum*.

Ten rats were used in a crossover study and were divided into 5 groups of 2 animals each. An interval of 2 days was allowed between each treatment. The experiment was carried out between 8 am and 12 noon.

All the groups were given a salt loading of 2 ml of 5 % dextrose-saline solution subcutaneously (*s.c.*) at 0 and 1hour and were placed in metabolic cages. The urine voided after 30 minutes was discarded. The control (NaCl 0.9 %), extract and drugs were administered intraperitoneally (*i.p.*) as shown in Table 3.1.

Table 3.1: Study Design for Diuretic activity of the aqueous extract of *Croton zambesicus* in rats

Day	Group 1	Group 2	Group 3	Group 4	Group 5
1	NaCl (0.9%)	HCT(10 mg)	CZ(1mg)	CZ (10 mg)	CZ (100 mg)
4	HCT(10 mg)	CZ (10 mg)	CZ (100 mg)	NaCl (0.9%)	CZ (1 mg)
7	CZ (1mg)	NaCl (0.9%)	CZ (10 mg)	CZ (100 mg)	HCT(10 mg)
10	CZ (100 mg)	CZ (1 mg)	HCT (10 mg)	CZ (1mg)	NaCl (0.9%)
13	CZ (10 mg)	CZ (100 mg)	NaCl (0.9%)	HCT (10 mg)	CZ (10mg)

CZ: Aqueous extract of *Croton zambesicus* ; HCT: Hydrochlorothiazide

Urine samples were collected in plain bottles after the first and second hour. The volume and pH of the collected urine were measured.

3.6 Effect of *Aqueous extract of Croton zambesicus* on Isolated Guinea Pig Atrium and Rabbit Heart

3.6.1 Effect of *Aqueous extract of Croton zambesicus* on isolated right atrium of guinea pig

Guinea pigs of either sex (300-500 g) were killed by cervical dislocation. The heart was removed and the right atrium isolated. One end of the right atrium was tied to a tissue clamp and the other end was attached to the transducer connected to a microdynanometer. The tissue was mounted in Ringer-Locke solution maintained at 32 ^{o}C and continuously aerated with oxygen. The tissue was washed every 5 minutes and allowed to equilibrate for 20 minutes. After obtaining control readings, graded concentrations of the extract (0.1-2.5 mg/ml) were added cumulatively to the organ bath and responses recorded. The tone of contraction of the atrium was measured as the distance between the baseline and peak of contraction while the rate of contraction of the atrium was measured as the distance between contractions.

3.6.2 Effect of aqueous leaf extract of *Croton zambesicus* on isolated perfused rabbit heart preparation (Langendorff)

A rabbit was weighed and heparin sodium (100 I.U.) was injected into the ear vein to prevent coagulation of blood. The rabbit was killed by cervical dislocation and the chest was opened with an incision along the left and right lateral aspect of the rib cage. The diaphragm was cut and the heart exposed. The aorta, vena cavae and pulmonary vessels were cut and the heart was transferred into a petri dish containing Ringer-Locke solution. A small spring clip was attached to the ventricle and a thread was connected from spring levers to the clip to record the heart contractions. The heart was then mounted on the aorta for retrograde perfusion with Ringer locke solution continuously aerated with oxygen at 37 ^{o}C and a pressure of 70 mmHg. The heart was allowed to equilibrate for 10 minutes before drug administration. Drugs were added to the preparation by injection via a rubber tubing into the perfusion fluid.
A concentration-response curve for isoprenaline (0.1 nM-0.1 µM) was obtained and the concentration that produced submaximal response was used for the study. The effect AECZ 0.64 mg/ml and 6.4 mg/ml on isoprenaline (10 nM)-induced contraction of the isolated perfused heart was recorded.

3.7 Effect of Aqueous Leaf Extract of *Croton zambesicus*, n-Butanol and Aqueous Fractions on Spontaneous Contraction of Isolated Rabbit Ileum

A rabbit was weighed and killed by cervical dislocation. The abdomen was cut open, the caecum lifted forward and the ileo-ceacal junction identified. The ileum was cut at this point and transferred to a petri dish containing Tyrode's solution. The mesentery was removed and the ileum cut into pieces of 1-1.5 cm length. A piece was fixed to a tissue clamp with a thread and placed in a 10 ml organ bath with Tyrode solution at 37 ^{o}C being aerated with air. The other end was fixed to an isotonic transducer for measurement of changes in isotonic tension. The ileum was washed at 5 minutes interval and allowed to equilibrate for 20 minutes between each drug administration. Graded concentrations (0.08-6.4 mg/ml) of AECZ were administered and responses recorded. This protocol was repeated for the n-butanol and aqueous fraction.

3.8 Effect of Aqueous extract of *Croton zambesicus* on Angiotensin II (Ang II)-Induced Contraction of Rat Ileum

A rat was killed by cervical dislocation and the abdomen was cut open, the caecum lifted forward and the ileo-ceacal junction identified. The ileum was cut at this point and transferred immediately to a petri dish containing Tyrode solution. After removal of the mesentery, the ileum was cut into pieces of 1.5 cm length. A piece was fixed to a tissue clamp with a thread and placed in 10 ml organ bath containing Tyrode solution at 37 ^{o} C and aerated with air. The tissue was allowed to stabilize for 20 minutes. A cumulative concentration-response for Ang II (0.01 nM-1 µM) was obtained. The tissue was washed three times every 15 minutes and allowed to equilibrate for 45 minutes to allow full recovery of receptors. The tissue was then pretreated for three minutes with AECZ and another cumulative concentration-response curve for Ang II was obtained. This protocol was repeated for AECZ concentrations of (0.08-6.4 mg/ml) and completed with a cumulative concentration curve of Ang II alone.

3.9 Effect of Aqueous Extract of *Croton zambesicus* on ACh-Induced Contraction of Rat Ileum

The rat ileum was isolated and mounted as described above. A submaximal contraction was induced by 1 µM ACh. After two control responses to ACh were obtained, the tissue was pretreated with AECZ for 3 minutes, and another response to ACh was obtained. This protocol was repeated for AECZ concentrations (0.08 to 6.4 mg/ml) and responses were recorded. The ileum was washed at 5 minutes interval and allowed to equilibrate for 30 minutes between the administrations of each extract concentration.

3.10 Effect of Aqueous leaf Extract of *Croton zambesicus* on $CaCl_2$-Induced Contraction of Rat Ileum

The rat ileum was isolated and mounted as described above. The tissue was bathed for 15 minutes in Ca^{2+} free Tyrode solution. Two cumulative concentration-response curves for $CaCl_2$ (1-30 mM) were obtained. The ileum was pretreated for 3 minutes, and then a third cumulative concentration-response curve for $CaCl_2$ was obtained.

The maximal contraction obtained with the control concentration-response curve for $CaCl_2$ was taken as 100 %, and responses to the AECZ were calculated as a function of this value.

3.11 Effect of Aqueous Leaf extract of *Croton zambesicus* on KCl-Induced Contraction of Rat Ileum

Potassium chloride (KCl) 80 mM was used to depolarize the piece of ileum. When the contraction produced by KCl was sustained, graded concentrations (0.08-0.64 mg/ml) of the AECZ were added cumulatively and response was recorded (Gilani *et al*., 2010).

3.12 Determination of Angiotensin Converting Enzyme (ACE) Inhibitory Activity of Aqueous extract of *Croton zambesicus* in Rat Ileum

Angiotensin Converting Enzyme (ACE) inhibitory activity of the extract was determined using the method described by Vogel (2002). A piece of rat ileum was isolated and mounted as described above for the rabbit ileum. A cumulative concentration-response curve for bradykinin (0.1 nM-0.1 µM) was obtained. This was repeated twice for reproducibility. The tissue was preincubated with the AECZ for 3 minutes then, another concentration-response curve for bradykinin was obtained. This was repeated for different concentrations of AECZ (0.08-0.64 mg/ml). The ileum was washed at 5 minutes interval and allowed to equilibrate for 30 minutes between treatments.

3.12.1 Determination of percentage ACE inhibition of aqueous extract of *Croton zambesicus* by spectrophotometric assay

The ACE inhibitory activity of the extract was measured according to the methods of Holmquist *et al*., (1979) and Lee *et al*., (2003). The lung extract as a source of ACE was prepared from the lung of albino rat which was cut into pieces and homogenized using a potter tube. The homogenate was centrifuged and the supernatant was used for the assay. The ACE substrate, *N*-(3-[2-furyl]acryloyl]Phe-Gly-Gly) (FAPGG) was used as a read out. The following protocol was used for the assay:

a. The aqueous extract of *Croton zambesicus* (AECZ) was weighed, dissolved in deionized water and different concentrations of the extract prepared.

b. The extract solution was centrifuged and the supernatant was used for the assay.

c. The substrate FAPGG was dissolved in 50 mM Tris-HCl buffer (pH 7.5) containing 0.3 M NaCl. The substrate solution and ACE solution (homogenized lung) were incubated with different dilutions of plant extract for two minutes at room temperature.

d. The absorbance of test solutions were read at 345 nm for 5 minutes at room temperature.

e. Deionized water was used as negative control (deionized water, substrate and ACE solution) and lisinopril used as positive control.

f. The amount of cleaved product, (FAP from FAPGG) by ACE was measured and used to calculate ACE activity.

g. ACE inhibitory activity was expressed as percent ACE inhibition and calculated using the formular:

$$\% \text{ ACE inhibition} = [1- (\Delta A_{inhibitor} \div \Delta A_{control})] \times 100\%.$$

$$\Delta A = A_1 - A_2 \div T_1 - T_2$$

A= absorbance T= time $\Delta A_{inhibitor}$ = change in absorbance extract

ΔA= change in absorbance T_1= time zero $A_{control}$ = deionized water

A_1= absorbance at time zero T_2= 5 minutes

A_2= absorbance after 5 minutes

3.13 Pharmacological Studies on the Column Subfractions of *Croton zambesicus* Leaf extract

3.13.1 Determination of effect of pooled column subfractions on ACh and Ang II-induced contraction of rat ileum

The effect of pooled column subfractions (PCF) 11-13, 15-26, 28 (1), 28 crystal, 29, 30-36, 46-51 and 52-53 on ACh and Ang II-induced contraction of rat ileum was determined using the method described for the crude extract.

3.13.2 Determination of effect of column subfractions on blood pressure in anaesthetized normotensive cats

The effect of pooled column subfractions (PCF) 11-13, 29, and 30-36 on blood pressure of normotensive cat was determined.

3.14 Statistical Analysis

Data obtained were expressed as mean ± standard error of mean (SEM). Graphs were plotted using Microsoft Excel and analyzed using GraphPad Prism 6, Software. Statistical analysis of difference between control and treated groups was carried out using one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test for multiple comparism. Statistical significance was taken at $P < 0.05$.

CHAPTER FOUR

4.0 RESULTS

4.1 Preparation of Aqueous Leaf Extract and Column Fractions of *Croton zambesicus*

The crude aqueous leaf extract of *Croton zambesicus* obtained was dark brown in colour with a percentage yield of 12.2 % w/w.

4.1.1 Percentage recovery of pooled column fractions from n-butanol fraction of aqueous extract of *Croton zambesicus*

A total of 14 column fractions, referred to as pooled column subfractions (PCF) 10, 11-13, 14, 15-26, 27, 28(1), 28(2), 28(3), 28 crystal, 29, 30-36, 40-45, 46-51 and 52-53 were obtained (Figure 4.1). The weight of the column subfractions are shown in (Appendix I).

4.1.2 Phytochemical screening of aqueous leaf extract of *Croton zambesicus* and column fractions

Preliminary phytochemical analysis of the aqueous leaf extract of *Croton zambesicus* (AECZ) and column fractions revealed the presence of flavonoids, tannins, saponins, triterpenes and cardiac glycosides (Table 4.1).

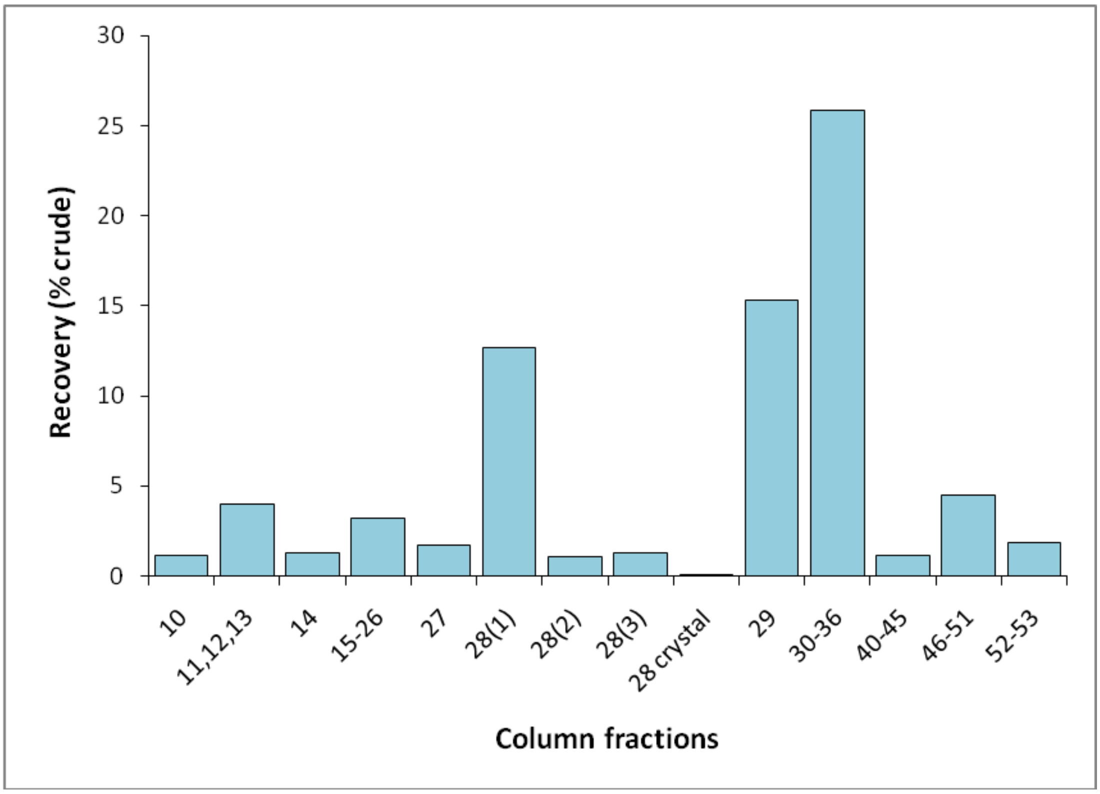

Figure 4.1: Percentage recoveries of column fractions of aqueous leaf extract of *Croton zambesicus*

Table 4.1: Phytochemical Screening of Aqueous Leaf Extract of *Croton zambesicus* and Selected Column Fractions

Secondary Metabolites	Aqueous Extract	PCF11-13	PCF 28	PCF 29	PCF30-31
Carbohydrate	+	-	-	-	-
Anthraquinones	-	-	-	-	-
Saponins	+	-	+	+	+
Flavonoids	+	+	+	+	+
Tannins	+	-	+	+	+
Alkaloids	+	+	+	+	+
Cardiac glycoside	+	+	-	-	-
Triterpenes and Steroids	+	-	+	+	+

(+) present **(-)** absent, PCF: pooled column fraction

4.2 Acute Toxicity Study of Aqueous Leaf Extract of *Croton zambesicus* in Rats and Mice

No sign of toxicity was observed and all the animals during the two phases of the toxicity study. The animals were active and alive during the study. The oral median lethal dose (LD_{50}) value in rats and mice was calculated to be greater than 5000 mg/kg.

4.3 Effect of *Aqueous extract of Croton zambesicus* on Blood Pressure in Anaesthetized Normotensive Cat

The AECZ at doses of 1 and 5 mg/kg administered *i.v.* produced no significant effect on the blood pressure of normotensive cat. However at 10 and 20 mg/kg, the extract significantly (P<0.01 and P<0.001) reduced blood pressure of anaesthetized normotensive cat compared to control. Maximal blood pressure reduction was obtained at 20 mg/kg of the AECZ. There was no significant difference in blood pressure reduction at 20 mg/kg compared to 40 and 80 mg/kg of AECZ (Figure 4.2). There was no difference in blood pressure reduction at 40 mg/kg compared to 80 mg/kg.

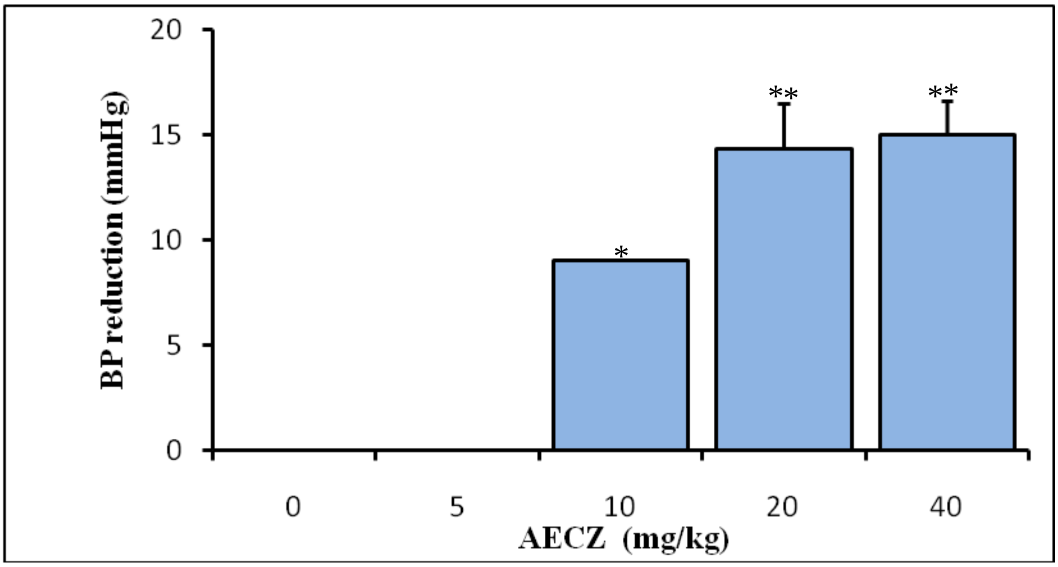

Figure 4.2: The effect of aqueous extract of *Croton zambesicus* on blood pressure in anaesthetized normotensive cat. n=3, **P*<0.01 and*P*<0.001 vs control AECZ: Aqueous extract of *Croton zambesicus***

4.4 The effect of Aqueous extract of *Croton zambesicus* (AECZ) on Urine Output in Rats

The AECZ (1 and 10 mg/kg) produced significant ($P<0.05$ and $P<0.001$) increase in urine output compared to control. There was no significant difference in urine output by 10 mg/kg when compared to 100 mg/kg of the extract. Hydrochlorothiazide (HCT) 10 mg/kg produced a significant ($P<0.05$) increase in urine output compared to untreated control. In addition there was a significant ($P<0.01$) increase in urine output by AECZ (10 mg/kg) compared to HCT 10 mg/kg (Figure 4.3).

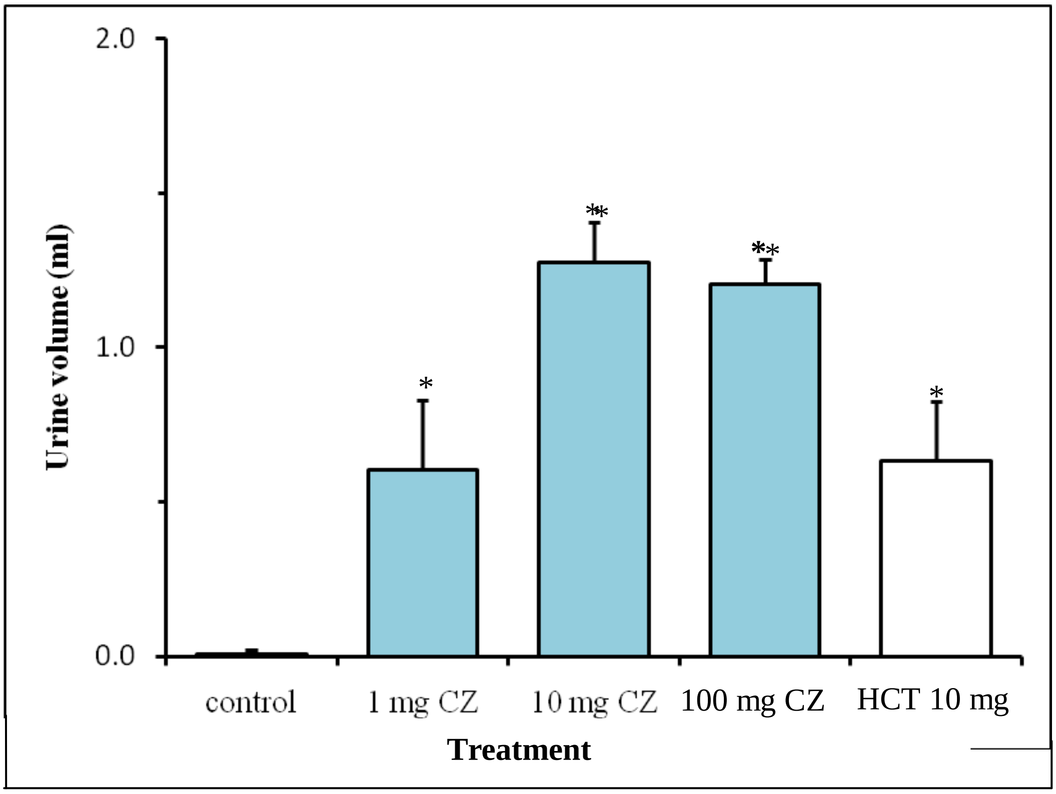

Figure 4.3: The effect of aqueous extract of *Croton zambesicus* on urine output in rats n=10, **P*<0.05, ***P*<0.001 vs control CZ: aqueous extract of *Croton zambesicus* HCT: hydrochlorothiazide

4.5 The Effect of Aqueous Extract of *Croton zambesicus* on Isolated Guinea pig ileum

The AECZ 2.5 mg/ml produced a significant (P<0.05) reduction of the tone and rate of spontaneous contraction of isolated right atrium of guinea pig (Figures 4.4 and 4.5).

4.6 The Effect of Aqueous Leaf extract of *Croton zambesicus* on Isolated Perfused Rabbit Heart

Aqueous leaf extract of *Croton zambesicus* at concentration of 0.64 mg/ml increased the tone of isoprenaline-induced contraction of isolated rabbit heart. Higher concentrations of the extract (6.4 mg/ml) produced a non significant (P>0.05) inhibition of isoprenaline-induced contraction (Figure 4.6).

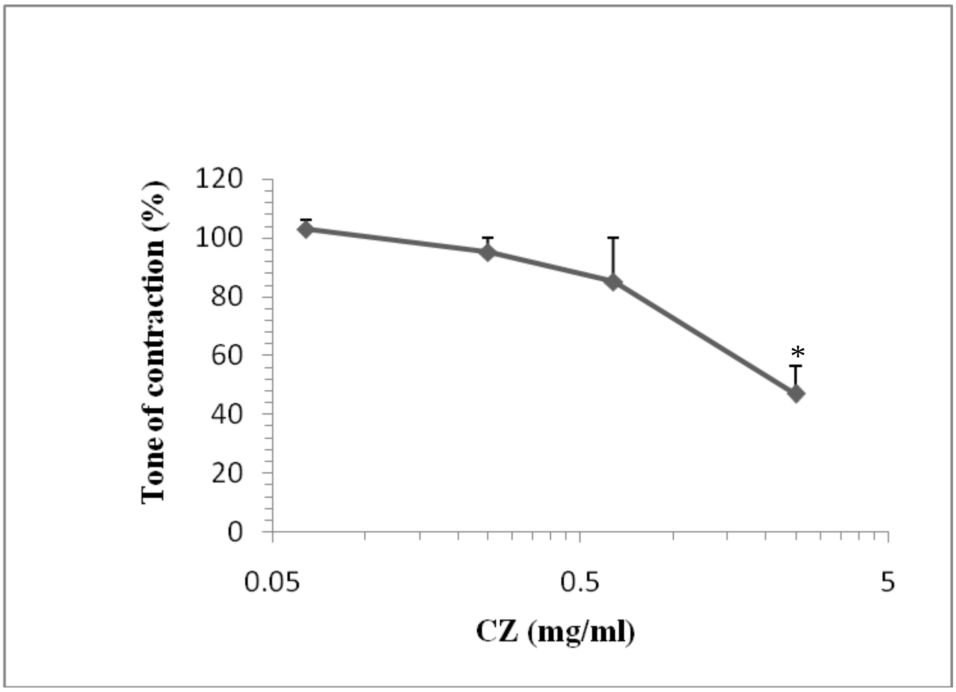

Figure 4.4: The effect of aqueous extract of *Croton zambesicus* on tone of contraction of right atrium of guinea pig n=3, ****P*<0.05 vs control
CZ: *Croton zambesicus*

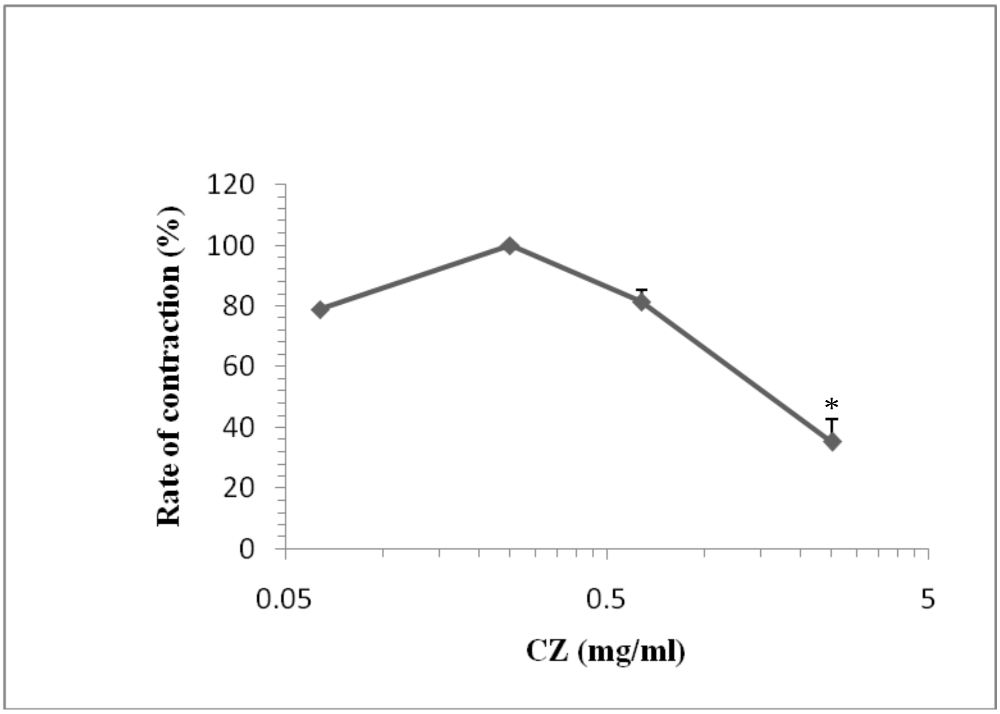

Figure 4.5: The effect of aqueous extract of *Croton zambesicus* on rate of contraction of right atrium of guinea pig n=3, **P*<0.05 vs control
CZ: *Croton zambesicus*

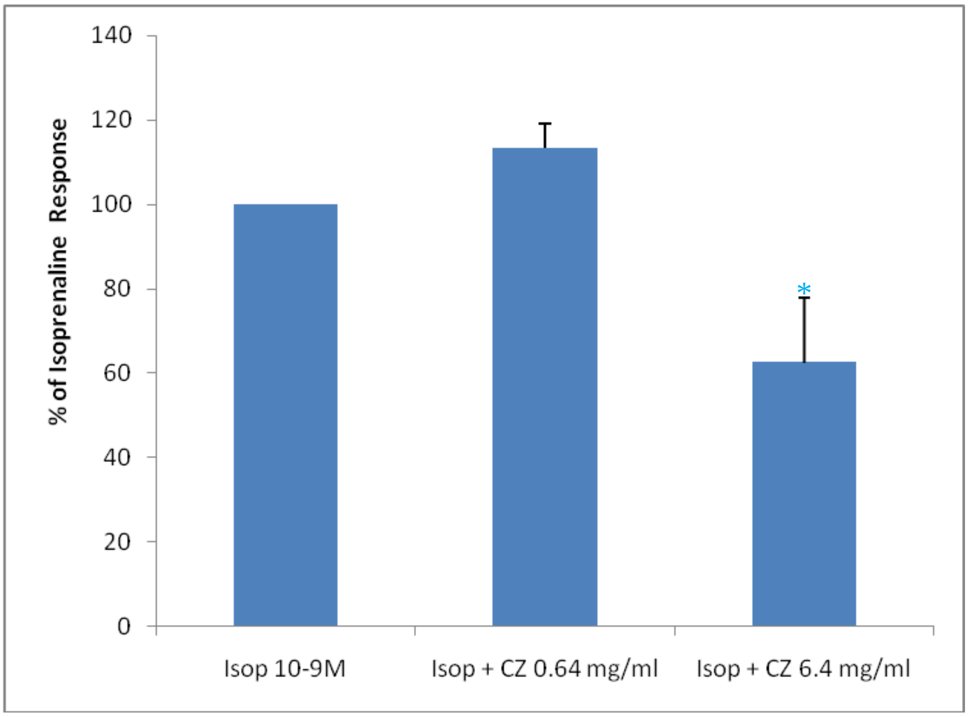

Figure 4.6: Effect of aqueous extract of *Croton zambesicus* on isoprenaline-induced contraction of isolated perfused rabbit heart n=3.
Isop: isoprenaline, CZ: *Croton zambesicus* aqueous extract

4.7 The Effect of Aqueous extract of *Croton zambesicus* on Spontaneous Contractions of Rabbit Ileum

The AECZ at concentrations of 0.08-0.64 mg/ml produced a transient increase in contraction of the rabbit ileum, while at concentrations of 3.2 and 6.4 mg/ml the extract produced a significant (P<0.001) inhibition of the spontaneous contraction of the rabbit ileum. There was a rapid recovery of the ileum to baseline contraction after washing (Figure 4.7).

4.8 The Effect of n-Butanol and Aqueous Fraction of *Croton zambesicus* on Spontaneous Contraction of Rabbit Ileum.

The fractions were screened for smooth muscle relaxant effect on rabbit ileum and n-butanol fraction was found to be most active.

The n-butanol fraction (1.6-6.4 mg/ml) significantly (P<0.001) inhibited the spontaneous contraction of the rabbit ileum. In contrast, the aqueous fraction of the extract at the same concentrations produced an increase in spontaneous contraction of the rabbit ileum (Figure 4.8).

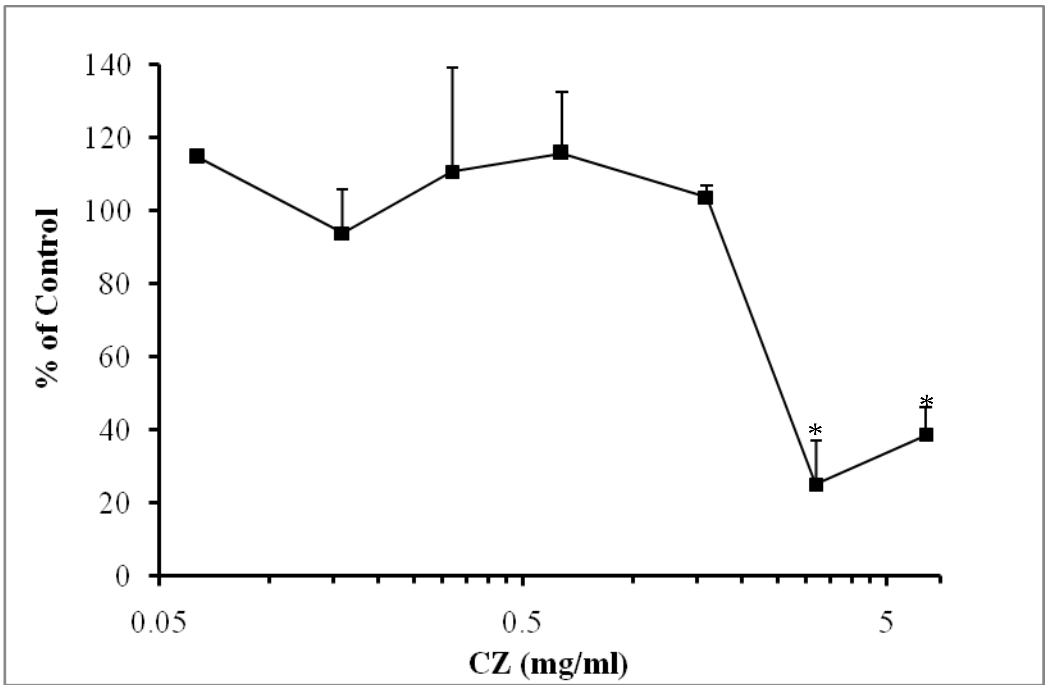

Figure 4.7: The Effect of aqueous extract of *Croton zambesicus* on spontaneous contraction of the rabbit ileum
n=5 **P* < 0.001 vs control, CZ: *Croton zambasicus* aqueous extract

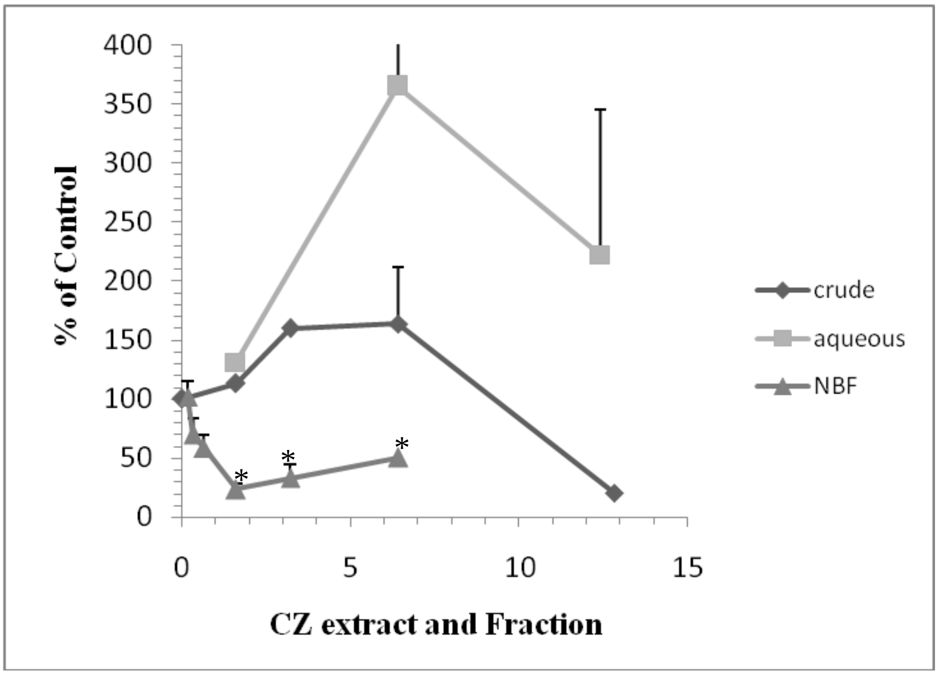

Figure 4.8: The effect of n-butanol and aqueous fractions on spontaneous contraction of rabbit ileum
n=3 ****P*<0.001 vs control CZ:*****Croton zambesicus*** **aqueous extract**

4.9 The Effect of *Aqueous extract of Croton zambesicus* on Angiotensin II (Ang II)-Induced Contraction of rat Ileum

The aqueous extract of *Croton zambesicus* (0.16-6.4 mg/ml) produced a concentration-dependent inhibition of Ang II-induced contraction of the rat ileum. The extract (0.64 and 6.4 mg/ml) produced significant (P<0.01 and P<0.001) inhibition (Figure 4.9). The inhibition was biphasic, starting at a concentration of 0.16 mg/ml with a flat slope, and complete inhibition at 6.4 mg/ml. The IC_{50} of the extract was 0.50±0.03 mg/ml (Figure 4.10). In addition, valsartan an AT_1 receptor antagonist at a concentration of 10 nM significantly (P<0.001) inhibited Ang II-induced contraction of the rat ileum (Figure 4.11).

4.10 The Effect of *Aqueous extract of Croton zambesicus* on Acetylcholine (ACh)-Induced Contraction of Rat Ileum

Aqueous extract of *Croton zambesicus* at concentrations 0.08-0.64 mg/ml had no significant effect on ACh-induced contraction of the ileum. Higher concentrations of the extract (3.2 and 6.4 mg/ml) significantly (P<0.01 and P<0.001) inhibited ACh-induced contraction of the rat ileum with an IC_{50} of 2.3±0.05 mg/ml (Fig 4.12).

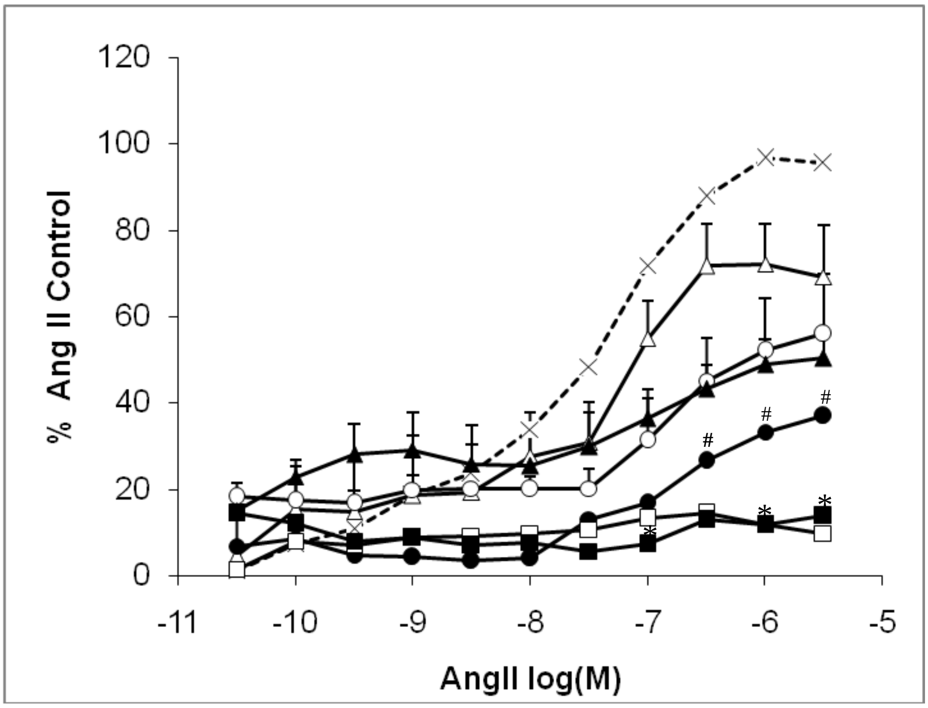

Figure 4.9: The effect of aqueous extract of *Croton zambesicus* on Ang II-induced contraction of rat ileum n=5 $^{\#}$*P*<0.01 and **P*<0.001 vs control. X Ang II, Δ AECZ 0.08 mg/ml, ▲AECZ 0.16 mg/ml, O AECZ 0.32 mg/ml, ●AECZ 0.64 mg/ml, □ AECZ 1.6 mg/ml, ■ AECZ 6.4 mg/ml AECZ: Aqueous extract of *Croton zambesicus*

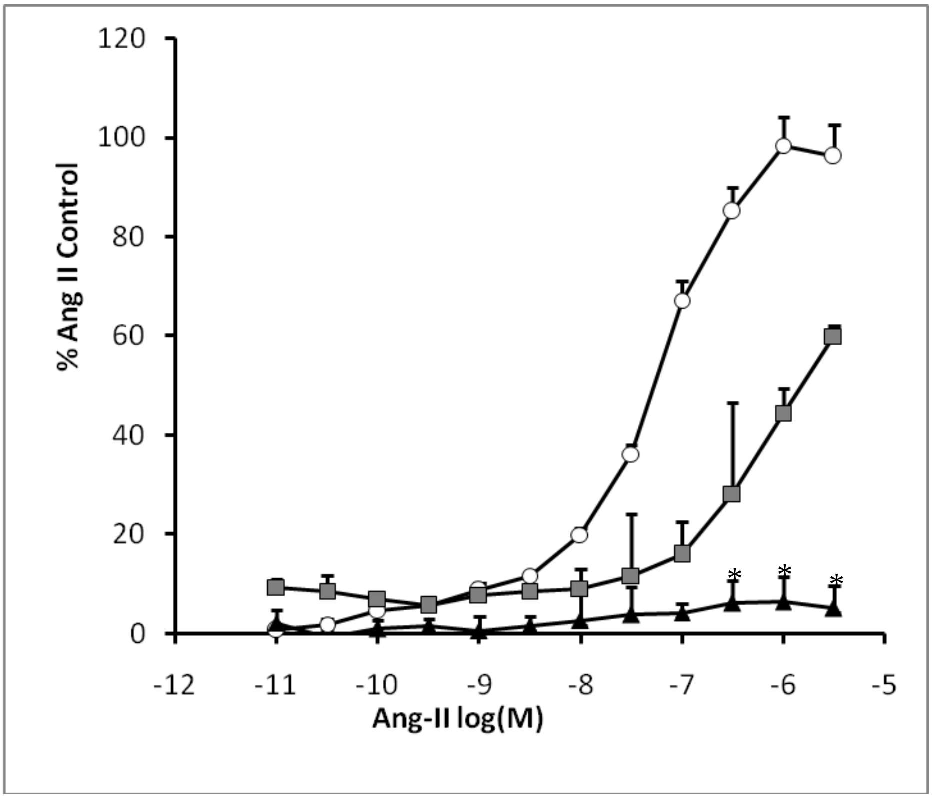

Figure 4.10: The effect of valsartan on Ang II-induced contraction of rat ileum n=5, ****P*<0.001 vs control. O Ang II, ■ valsartan 1 nM, ▲ valsartan 10 nM.

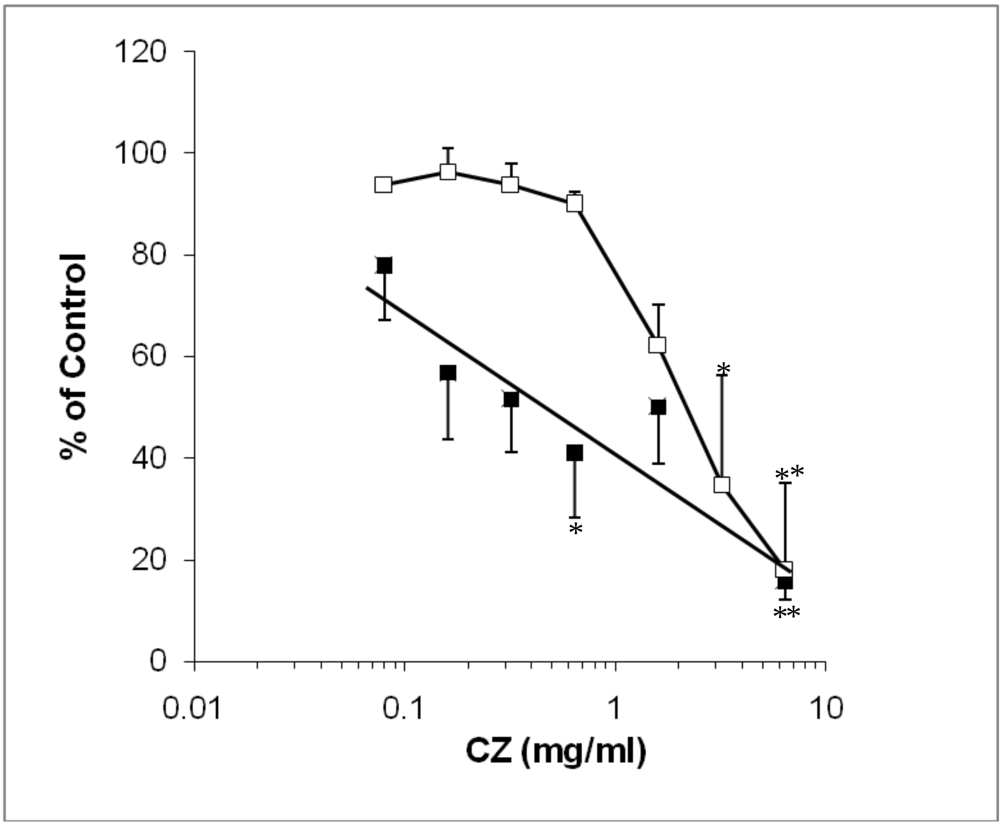

Figure 4.11: The effect of aqueous extract of *Croton zambesicus* on Ang II and ACh-induced contraction of rat ileum n=5, *P<0.01 and **P<0.001 vs control.
■ Ang II, □ ACh CZ: *Croton zambasicus* extract

4.11 The Effect of Aqueous leaf Extract of *Croton zambesicus* on $CaCl_2$-Induced Contraction of Rat Ileum

The aqueous extract of *Croton zambesicus* at concentrations of 0.16-0.64 mg/ml had no significant inhibitory effect on calcium chloride-induced contraction of the rat ileum. However at concentrations of 1.6-6.40 mg/ml the extract significantly ($P<0.001$) inhibited calcium chloride-induced contraction with an IC_{50} = 2.7±0.1 mg/ml (Figure 4.12)

4.12 The Effect of Aqueous extract of *Croton zambesicus* on KCl-Induced Contraction of Rat Ileum

The aqueous extract of *Croton zambesicus* (0.32 and 0.64 mg/ml) significantly ($P<0.01$ and $P<0.001$), inhibited KCl-induced contraction of the rat ileum with an IC_{50} of 0.3±0.01 mg/ml (Figure 4.13).

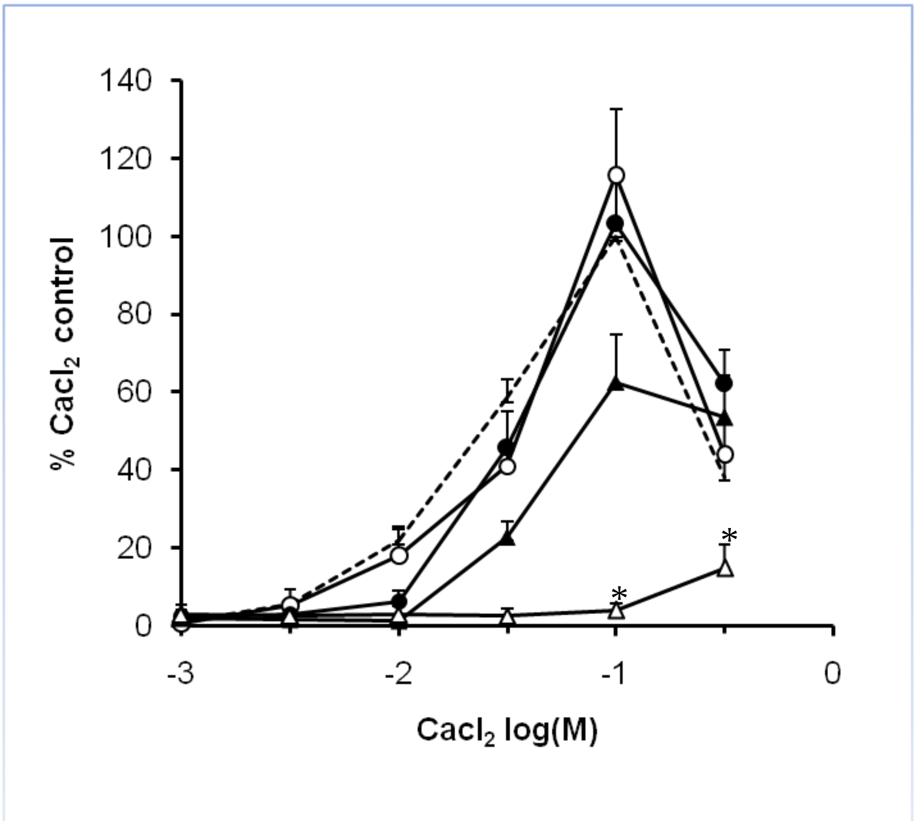

Figure 4.12: The effect of aqueous extract of *Croton zambesicus* on $Cacl_2$-induced contraction of rat ileum n=5, **P*<0.001 vs control. --- control, O AECZ 0.16 mg/ml, ●AECZ 0.64 mg/ml ▲ AECZ 1.6 mg/ml Δ AECZ 6.4 mg/ml, AECZ: aqueous extract of *Croton zambasicus*

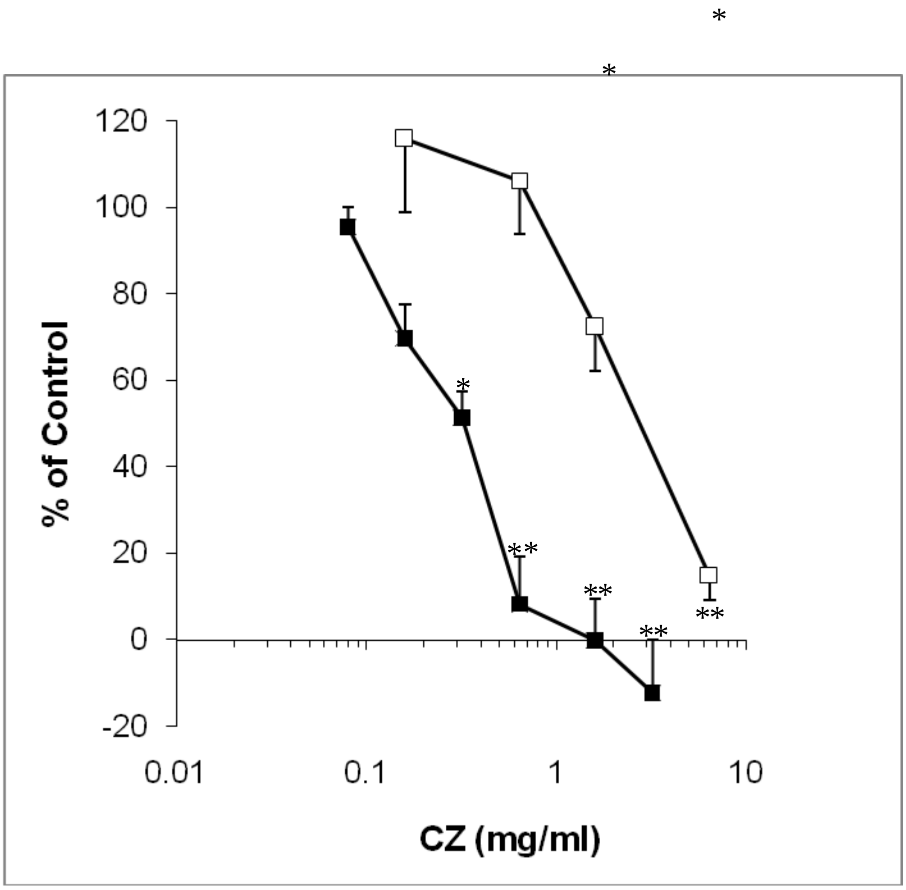

Figure 4.13: The effect of aqueous extract of *Croton zambesicus* on $CaCl_2$ and KCl-induced contraction of rat ileum
n=5, *P<0.01 and$P$<0.001 vs control. ■ KCl, □ $CaCl_2$**
CZ: *Croton zambasicus*

4.13 Angiotensin Converting Enzyme (ACE) Inhibitory Activity

4.13.1 Effect of aqueous leaf extract of *Croton zambesicus* on bradykinin-induced contraction of rat ileum

The aqueous extract (0.08-0.32 mg/ml) had no significant effect on bradykinin-induced contraction, while at concentration of 0.64 mg/ml the extract significantly (P<0.05) inhibited bradykinin-induced contraction of the rat ileum. Aqueous extract of *Croton zambesicus* had no ACE inhibitory activity (Figure 4.14).

4.13.2 Effect of aqueous leaf extract of *Croton zambesicus* on UV absorption of ACE substrate (FAPGG)

There was a decrease in UV absorption in time by aqueous extract of *Croton zambesicus* (0.15-15 mg/ml) due to conversion of ACE substrate, FAPGG to FAP. In contrast, Lisinopril an ACE inhibitor, at a concentration of 10 µM did not break down FAPGG and hence there was no decrease in UV absorption in time (Figure 4.15).

4.13.3 Effect of aqueous leaf extract of *Croton zambesicus* on ACE activity

The extract at concentrations of 0.15 and 15 mg/ml had no effect on ACE. While lisinopril 10 µM produced 100 % inhibition of ACE (Figure 4.16).

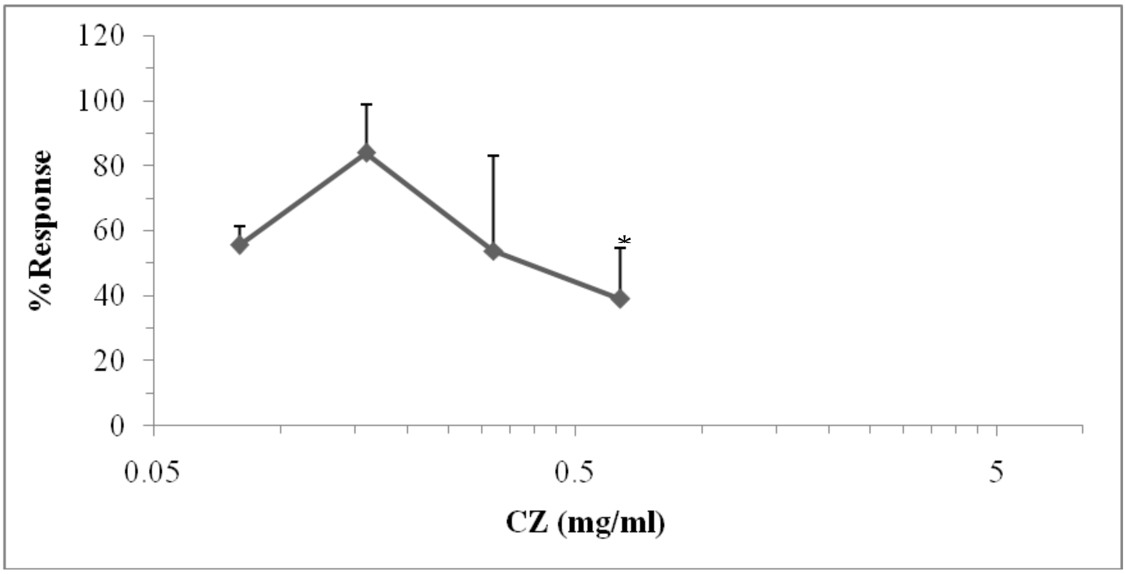

Figure 4.14: Effect of aqueous extract of Croton zambesicus on bradykinin-induced contraction of rat ileum
n=5, ****P*<0.05 vs control**

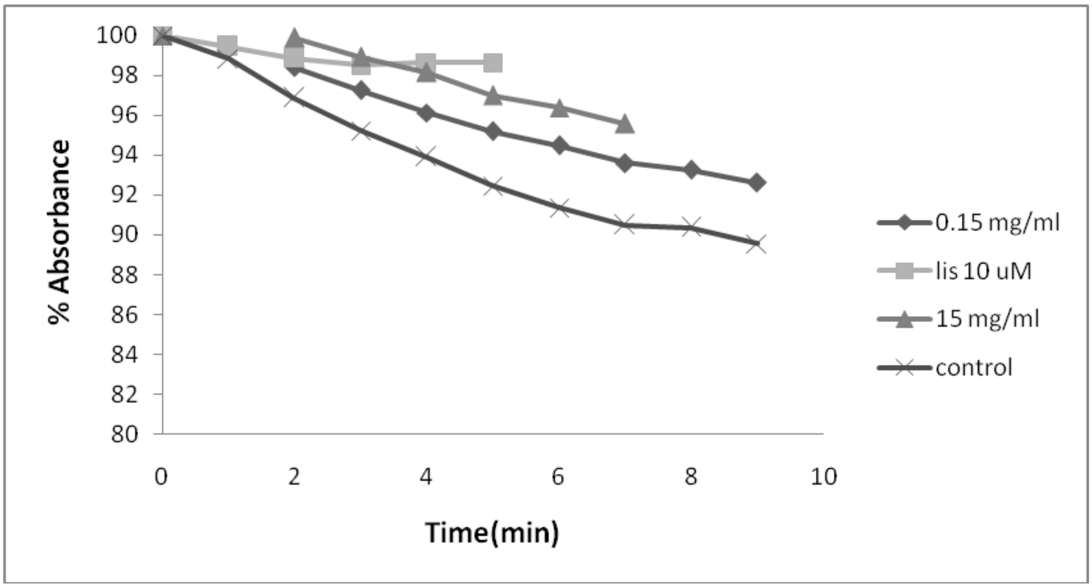

Figure 4.15: Effect of aqueous extract of *Croton zambesicus* on UV absorption by ACE substrate (FAPGG)
CZ *Croton zambasicus* extract, lis: lisinopril

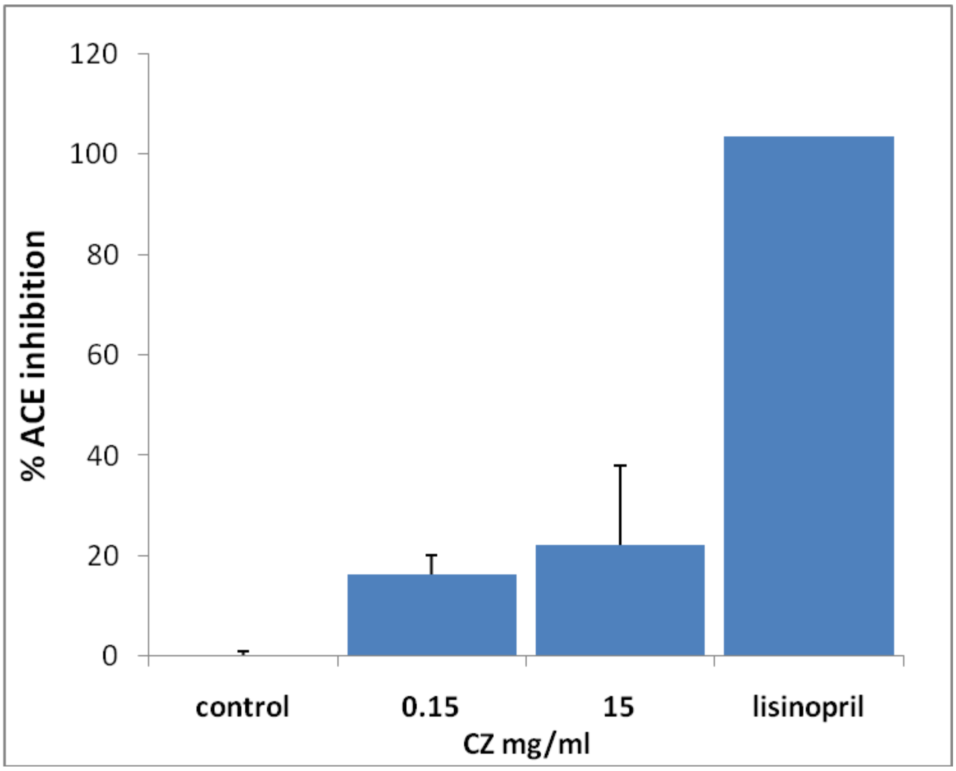

Figure 4.16: Percentage ACE inhibitory activity of *Croton zambesicus* aqueous extract n=2, CZ: *Croton zambesicus*

4.14 Effect of Pooled Column Subfractions on Isolated Rat Ileum and Cat Blood Pressure

4.14.1 Effect of Pooled column subfractions (PCF) on ACh and Ang II-induced contraction of rat ileum

PCF11-13, 29 and 30-36 had no significant inhibitory effect on Ang II-induced contraction but produced a significant (*P*<0.05) reduction in ACh-induced contraction of rat ileum (Figure 4.17, 4.19 and 4.20). PCF 28 crystal had no significant inhibitory effect on both ACh and Ang II-induced contractions of rat ileum (Figure 4.18).

PCF11-13, (28)1 and 29 produced an increase in Ang II-induced contraction or the rat ileum. Furthermore, the inhibitory effect of the pooled column fractions on both Ang II and ACh-induced contractions less compared to the aqueous extract of *Croton zambesicus* (Figure 4.21).

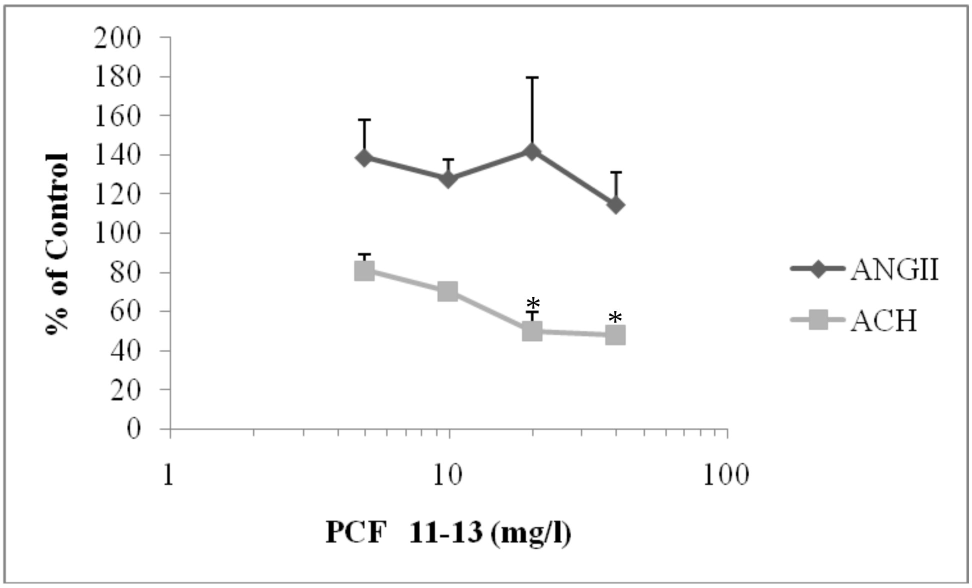

Figure 4.17: Effect of PCF 11-13 on Ang II and ACh-induced contraction of rat ileum
n=4, **P*<0.05 vs control PCF: Pooled column fraction

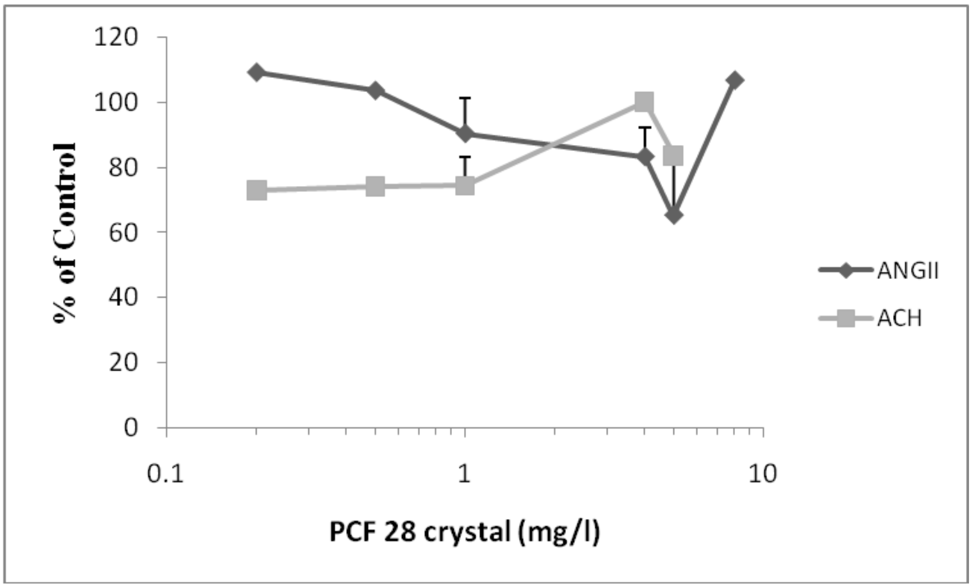

Figure 4.18: Effect of PCF 28 crystal on Ang II and ACh-induced Contraction of rat ileum
n=4 PCF: Pooled column fraction

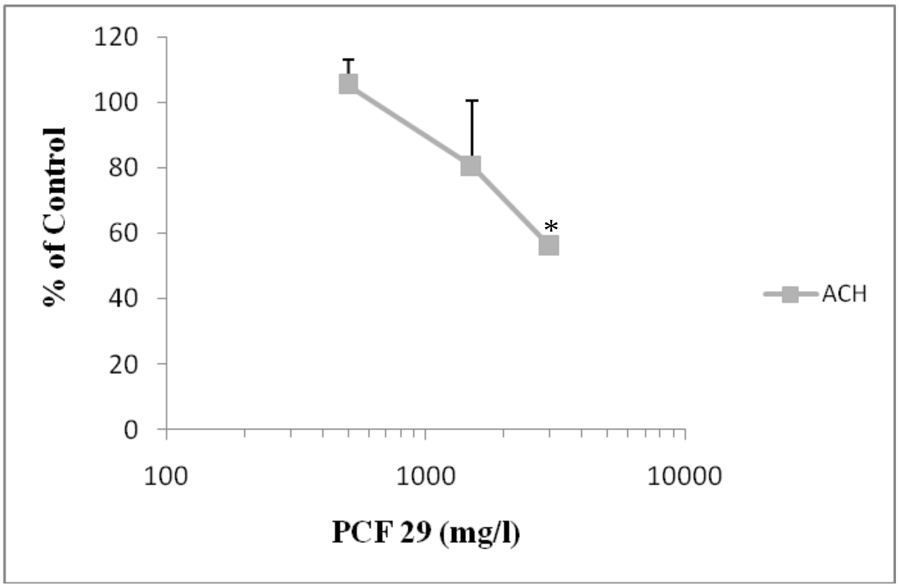

Figure 4.19: Effect of PCF 29 ACh-induced contraction of rat ileum.
n=4 ****P*<0.05 vs control**
PCF: Pooled column fraction

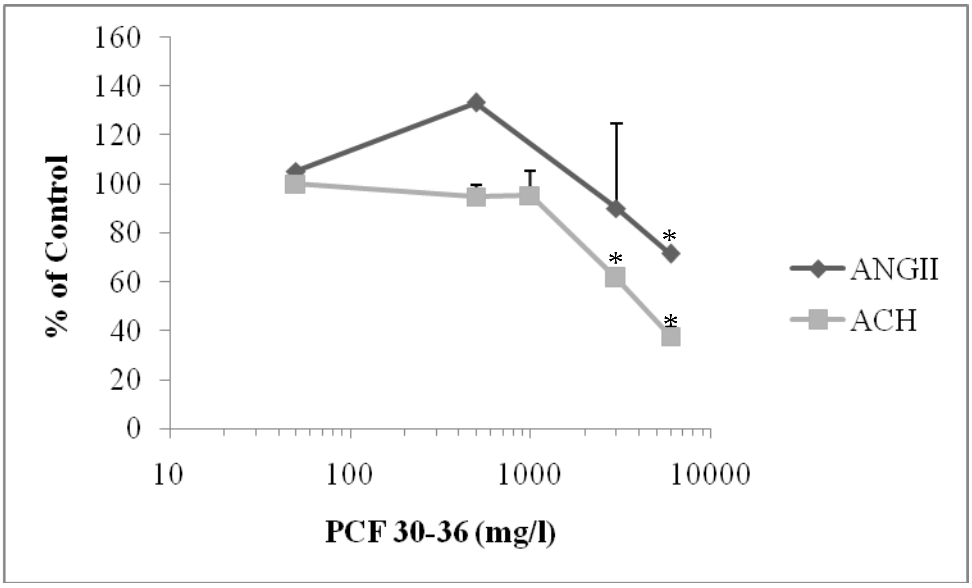

Figure 4.20: Effect of PCF 30-36 on ACh and Ang II-induced contraction of rat ileum
n=4, **P*<0.05 vs control
PCF: Pooled column fraction

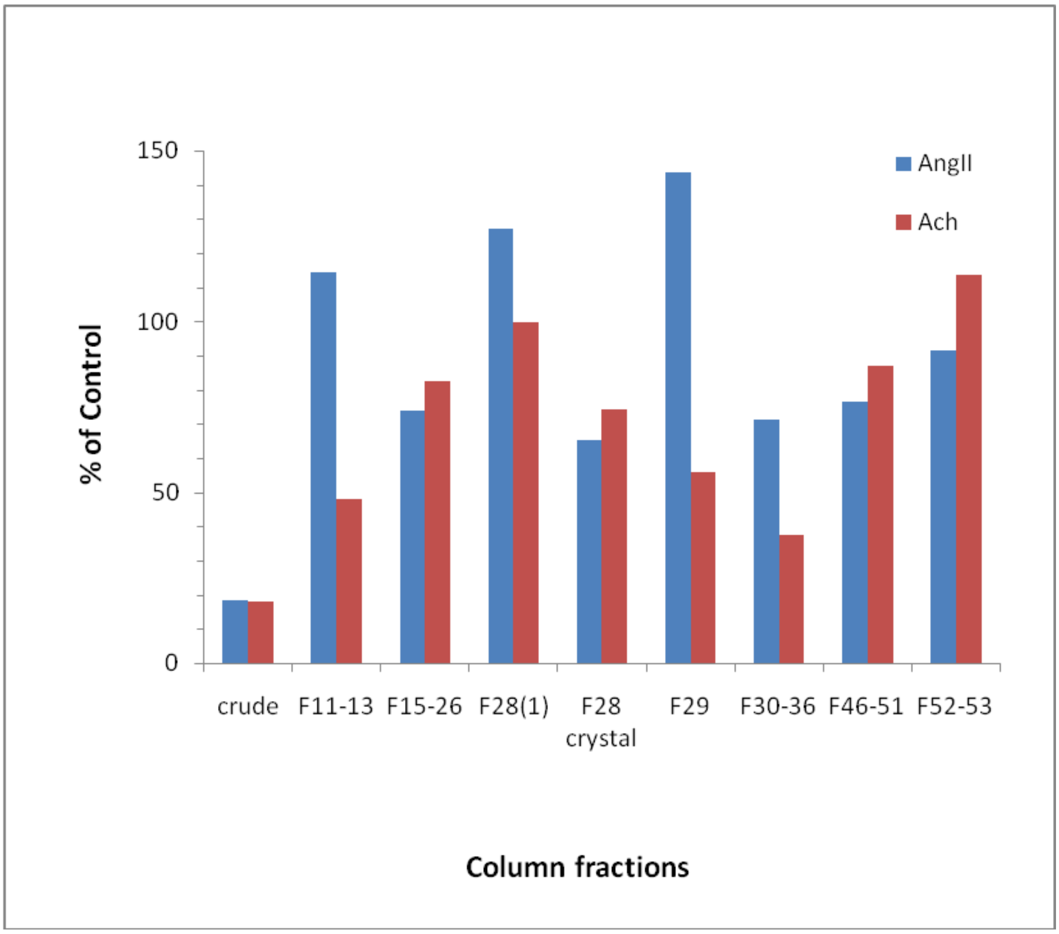

Figure 4.21: **The effect of crude extract and column subfractions of *Croton zambesicus* on ACh and AngII-induced contractions of rat ileum**

4.14.2 Effect of pooled column subfractions (PCF) of a*queous extract of Croton zambesicus* on blood pressur e in anaesthetized normotensive cat

PCF 11-13 produced a reduction in BP at the highest dose tested (Figure 4.22), while PCF 29, and 30-36 did not cause reduction in BP at the doses tested (Figures 4.23 and 4.24).

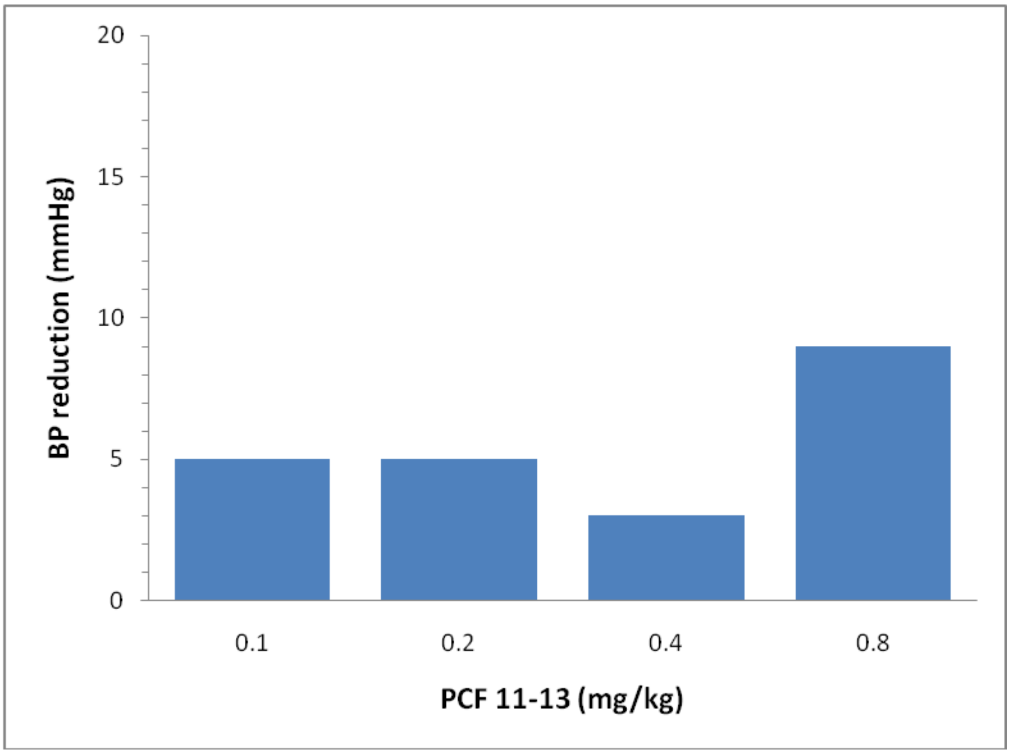

Figure 4.22: Effect of PCF 11-13 on blood pressure in anaesthetized normotensive cat, n=1 PCF: Pooled column fraction

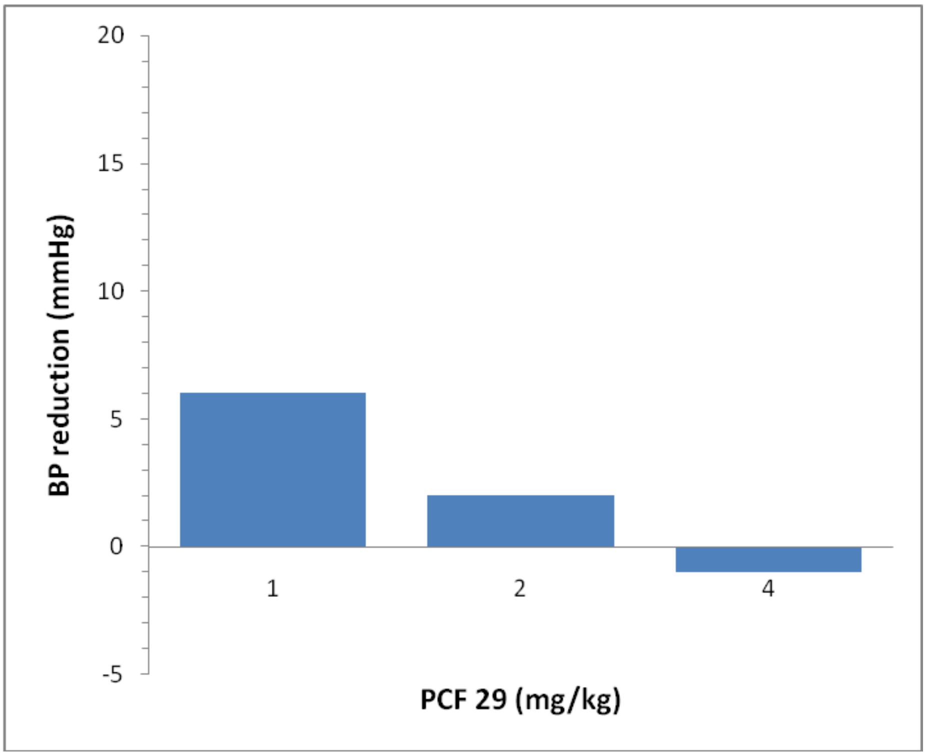

Figure 4.23: Effect of PCF 29 on blood pressure of anaesthetized normotensive cat n=1 PCF: Pooled column fraction

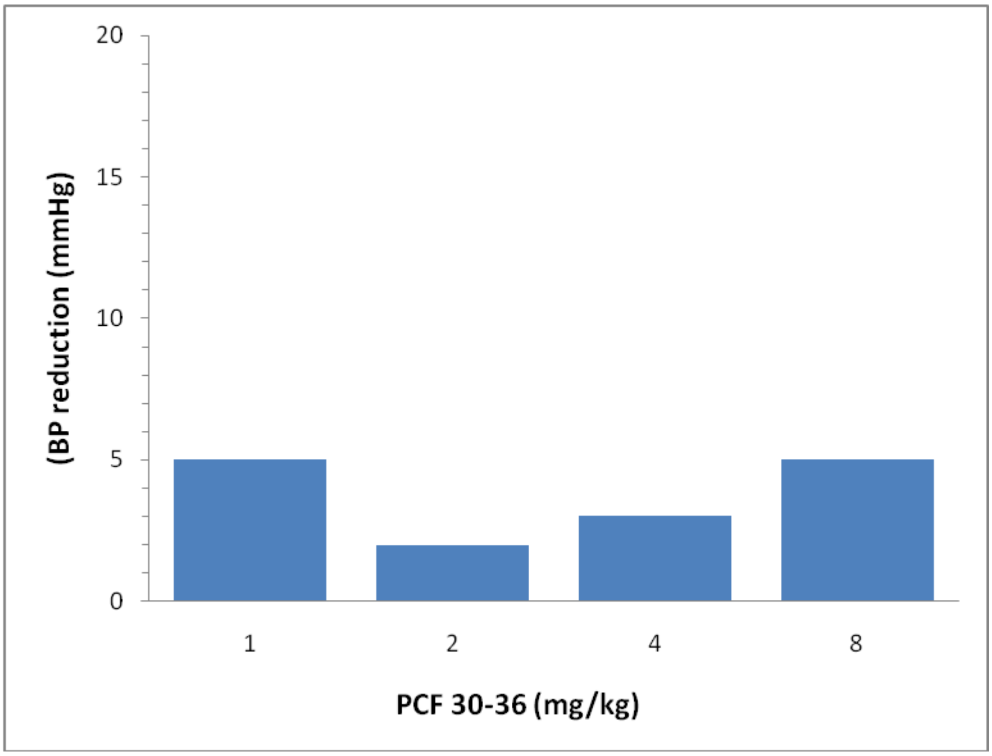

Figure 4.24: Effect of PCF 30-36 on blood pressure of anaesthetized normotensive cat n=1 PCF: Pooled column fraction

CHAPTER FIVE

5.0 DISCUSSION

Traditional medicines of proven safety and efficacy contribute to the goal of ensuring majority of people have access to health care (WHO, 2013b). *Croton zambesicus* was selected for this study based on its ethnomedicinal use in the management of cardiovascular disorders such as hypertension (Adjanohaun, 1989).

Phytochemical screening of the aqueous extract of *Croton zambesicus* and column subfractions revealed the presence of flavonoids, alkaloids, terpenoids, saponins and tannins. Screening of phytochemicals in medicinal herbs is essential in the discovery of new sources of active pharmacological compounds (Kashani *et al*., 2012). Terpenoid compounds (including the monoterpenoids and diterpenoids), have been shown to produce beneficial effect on the cardiovascular system (Hipolito *et al*., 2011; Awang *et al*., 2012). Diterpenoids are among the main compounds that have been linked with cardiovascular properties such as vasorelaxant, negative inotropic, diuretic, and hypotensive activity (Alfieri *et al*., 2007; Tirapelli *et al*., 2008). Baccelli *et al*., (2007) and Martinsen *et al*., (2012) reported the vascular *smooth muscle relaxant activity of diterpenes isolated from the extract of Croton zambesicus. Flavonoids have also been reported to have antioxidant and vasodilatory activity which is beneficial in cardiovascular disorders particularly hypertension (*Pietta, 2000; Scalbert *et al*., 2005).

The aqueous extract of *Croton zambesicus* (AECZ) is relatively non toxic, which was shown by it's oral median lethal dose (LD_{50}) value of above 5000 mg/kg. The extract produced a significant (*P*<0.01) reduction in the blood pressure of anaesthetized normotensive cats. The hypotensive effect of the extract was dose-dependent and transient, lasting for a few minutes after a single i.v. administration. The acute reduction in blood pressure may be attributed to a reduction in heart rate, stroke volume and cardiac output (Armstrong *et al*., 2012) although a reduction in vascular tone cannot be excluded (Zeggwagh *et al*., 2014). The mean arterial blood pressure is a product of cardiac output and systemic vascular resistance. Changes in either cardiac output or systemic vascular resistance will affect mean arterial blood pressure (Klabunde, 2011).

Diuretics are beneficial in the management of cardiovascular disorders including hypertension. They act by inhibiting NaCl reabsorption in the distal tubule. This results in an increase in Na^+ loss, a reduction in plasma volume and reduced total peripheral resistance (Wright *et al.*, 2007). The significant increase in urine output produced by AECZ is beneficial in reducing blood pressure (Mengistu *et al*., 2012). Thiazide diuretics induce an initial decrease in intravascular volume that decreases blood pressure by lowering cardiac output. It is hypothesized that a vasodilatory affect of the thiazides complements the compensated volume depletion, leading to a sustained decrease in blood pressure (Armstrong *et al.*, 2012).

The AECZ produced a decrease in both the rate and force of contraction of isolated right atrium suggesting that a reduction in heart rate contributes to its hypotensive activity (Ojewole, 2007). In addition, a reduction in the positive chronotropic effect of isoprenaline on isolated perfused heart by AECZ seems to suggest a mechanism involving Beta-adrenoceptor blockade. Beta blockers reduce blood pressure by decreasing cardiac output via negative inotropic and chronotropic actions. When used for long term therapy, they decrease vasomotor tone with a consequent decrease in systemic vascular resistance (Armstrong *et al*., 2012). In addition, they block sympathetic nervous system stimulation of renin release by juxtaglomerular cells in the kidney (Remedica, 2004).

The effect of AECZ on spontaneous contractions of isolated rabbit ileum was determined and at a concentration of 0.64 mg/ml the extract produced a transient increase in spontaneous contraction of the rabbit ileum which may indicate an agonistic effect of the extract (Cardozo *et al*., 2005). Spontaneous contraction of both intestinal and vascular smooth muscle is regulated mainly by changes in Ca^{++} influx (Berridge, 2008). However, at a concentration of 6.4 mg/ml the extract significantly inhibited spontaneous contraction of the rabbit ileum suggesting an inhibitory effect on extracellular Ca^{++} influx.

The mechanism(s) by which the AECZ produced relaxation of smooth muscle was investigated by determining the effect of extract on agonist-induced contraction of rat ileum.

Angiotensin II (Ang II), a bioactive peptide of the renin angiotensin system (RAS) plays a critical role in cardiovascular homeostasis (Feox and Sear, 2014). It is an activator of smooth muscles, both in the intestinal wall and vasculature of rats and humans (Ewert *et al*., 2006). The AECZ produced a noncompetitive antagonism of Ang II-induced contraction, characterized by a decrease in maximal response. Inhibition of Ang II-induced contraction was obtained at concentrations of the extract lower than those inhibiting the isolated atrium, heart and rabbit preparations suggesting that the extract has a high affinity for angiotensin 1 (AT_1) receptors in the ileum. Ang II produces contraction of smooth muscle via distinct post-receptor mechanism (Guibert *et al.*, 2008). It acts on AT_1 receptors and causes vasoconstriction, which is mediated by "classical" G protein-dependent signaling pathways (Akazawa *et al.*, 2013). These activate downstream effectors including phospholipase C (PLC), phospholipase A_2 (PLA_2), and phospholipase D (PLD). Activation of PLC produces inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) resulting in increased intracellular calcium and smooth muscle cell contraction (Ushio-Fukai *et al.*, 1999). Inhibition of Ang II-induced contraction by AECZ may be linked to its binding on AT_1 receptors and consequently inhibition of G protein-dependent signaling pathways.

In the rat ileum, acetylcholine (ACh) acts on muscarinic M_3 receptors coupled to G-proteins which results in activation of IP_3, efflux of extracellular calcium and release from intracellular stores (Guibert *et al.*, 2008; Pintérova *et al.*, 2011). ACh mobilizes calcium through store dependent mechanism or activates non selective cationic channels (Komori and Bolton, 1991). Low concentrations of the AECZ had no significant inhibitory effect on ACh-induced contraction of the rat ileum. However at high concentrations, the extract inhibited the spontaneous contraction of the rabbit ileum and also significantly inhibited ACh-induced contraction of the rat ileum. This effect of the extract may be related to an inhibitory action on receptor operated calcium channels (Pintérova *et al.*, 2011) resulting in relaxation of smooth muscle of the ileum.

The role of calcium channels in the smooth muscle relaxant activity of the AECZ was investigated. $CaCl_2$-induced contraction was significantly inhibited by the same concentration (6.4 mg/ml) of extract that inhibited ACh-induced contraction. This finding suggests that the extract may inhibit contractile mechanisms involving extracellular calcium influx (Morel *et al*., 1997).

Furthermore, a significant reduction of KCl-induced contraction of the rat ileum by a ten times lower concentration of AECZ than that required to inhibit $CaCl_2$-induced contraction suggests that the extract acts on voltage-dependent calcium channels and inhibits Ca^{++} influx via voltage-dependent calcium channels (Lee *et al*., 2003 and Adaramoye *et al*., 2009). Calcium channel blockers lower blood pressure through reduction of both systemic vascular resistance and cardiac output (Armstrong *et al.*, 2012). They decrease the influx of calcium via voltage-gated L-type calcium channels in the plasma membrane. The resulting decrease in intracellular Ca^{++} concentration leads to reduced contraction of both cardiac and vascular smooth muscle cells (Armstrong *et al*., 2012). Inhibition of extracellular Ca^{++} influx by medicinal herbs in both intestinal and vascular smooth muscle has been reported (Ajagbonna *et al*., 2001 and Giliani *et al*., 2010). Baccilli *et al* (2007); Martinsen *et al* (2010) reported inhibition of KCI-induced contraction of the rat aorta by diterpenes isolated from *Croton zambesicus*.

ACE inhibitors competitively inhibit ACE leading to inhibition of angiotensin II synthesis and aldosterone. They cause a fall in blood pressure due to decrease in peripheral resistance (Jain, 2008). Bradykinin-induced contraction is potentiated by ACE inhibitors (Vogel, 2002). Inhibition of bradykinin-induced contraction of the rat ileum by AECZ implies it has no ACE inhibitory activity. This result was confirmed by evaluating ACE inhibitory activity using spectroscopy. The extract had no ACE inhibitory activity when compared with lisinopril a standard ACE inhibitor.

The n- butanol fraction had a significant inhibitory effect on the spontaneous contraction of the rabbit ileum. The n-butanol fraction at concentration of 0.64 mg/ml did not increase the contraction of rabbit ileum in contrast to AECZ. The pooled column subfractions obtained from AECZ were screened for smooth muscle relaxant activity using agonist-induced contraction in isolated tissue preparation. Three of the subfractions (F11-13, F29 and F30-36) were found to significantly inhibit ACh-induced contraction of the rat ileum with no significant effect on Ang II-induced contraction of the rat ileum. Based on this finding, fractions (F11-13, F29 and F30-36) were screened further for hypotensive activity.

Subfractions (F29 and F30-36) at the doses tested had no effect on BP in normotensive cats while subfraction F11-13 produced a reduction in blood pressure at the highest dose tested. This lower activity observed with the subfractions when compared with the AECZ may be attributed to loss of solubility, stability and synergy of the active constituents present in the crude extract. The phytochemicals responsible for activity in crude extracts often act synergistically to enhance pharmacological action of plant extracts (Lopes *et al*., 2013).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

The results obtained from this study revealed that the aqueous leaf extract of *Croton zambesicus* contains bioactive constituents that are beneficial in the management of cardiovascular disorders such as hypertension.

Preliminary phytochemical screening of extract and fractions revealed the presence of terpenoids, flavonoids, tannins, saponins and alkaloids. The AECZ was found to be safe with a median lethal dose (LD_{50}) above 5000 mg/kg p.o in rats and mice. The AECZ produced a significant reduction in blood pressure of anaesthetized normotensive cat. In addition the extract produced a significant depressant effect on the heart, mediated possibly via an inhibitory action on $Beta_1$ receptors in the heart.

Furthermore, the AECZ had significant diuretic activity, with smooth muscle relaxant activity mediated possibly via inhibition of angiotensin 1 (AT_1) receptor, and inhibition of calcium efflux via receptor and voltage gated calcium channels.

The AECZ had no angiotensin converting enzyme (ACE) inhibitory. The pooled column subfractions had lower hypotensive activity compared to the AECZ and this may be attributed to loss of solubility, stability and synergy of the active constituents present in the crude extract.

6.2 Conclusion

The results obtained from this study revealed the aqueous leaf extract of *Croton zambesicus* contains bioactive constituents with hypotensive, smooth muscle relaxant, cardiac depressant and diuretic activity. These have been found to be beneficial in the management of cardiovascular disorders.

6.3 Recommendations

Based on the findings and the limitations in this work, these recommendations are made:

- Studies on antihypertensive activity using spontaneously hypertensive rats should be carried out on the AECZ.
- Further studies on the effect of AECZ on uric acid-induced hyperuriceamia in fructose fed rats should be carried out.
- Further purification, isolation and biological evaluation of fractions and isolated compounds from the extract should be carried out.
- Sub chronic and chronic toxicological studies on AECZ should be carried out.

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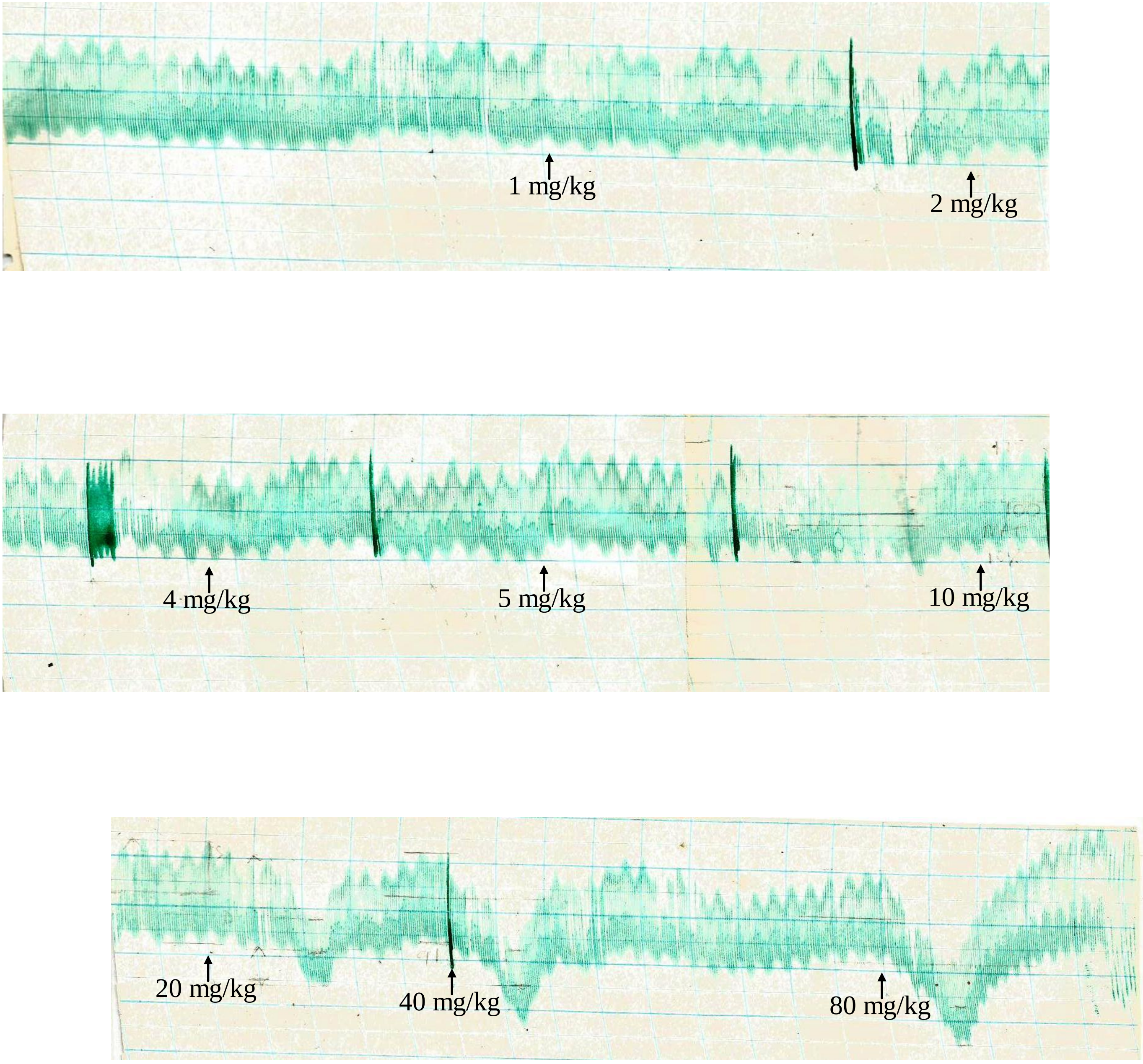
APPENDICES

Appendix I: Percentage yield of pooled column fraction of aqueous extract of *Croton zambesicus*

Fraction	Weight (mg)	% of crude extract
PCF10	39.5	1.16
PCF 11-13	135	3.97
PCF 14	43.3	1.27
PCF 15-26	110	3.24
PCF 27	58	1.17
PCF 28(1)	431	12.68
PCF 28(2)	37.4	1.10
PCF 28(3)	44	1.29
PCF 28(Crystal)	3	0.09
PCF 29	521	15.32
PCF 30-36	880	25.88



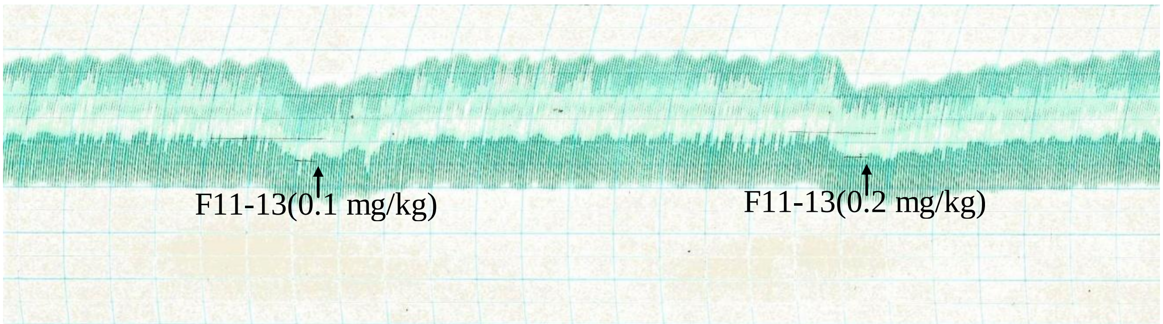
Appendix II: The effect of aqueous extract of *Croton zambesicus* (1-80 mg/kg) on blood pressure of normotensive cat

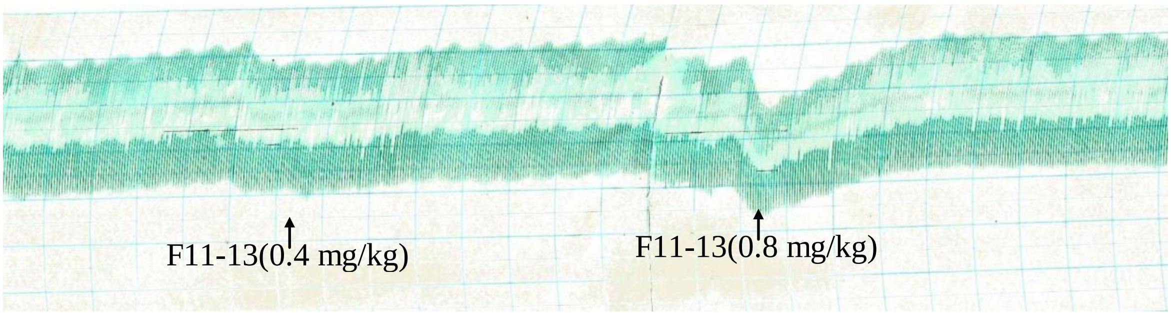
Appendix III: Effect of aqueous extract of *Croton zambesicus* on blood pressure of normotensive cats

Treatment	**BP mmHg**	**BP mmHg**	**BP mmHg**	**BP mmHg**
NaCl 0.9%	**-**	**-**	**-**	**-**
10 mg/kg	11	3	13	9
20 mg/kg	18	10	15	14.3
40 mg/kg	18	10	17	15

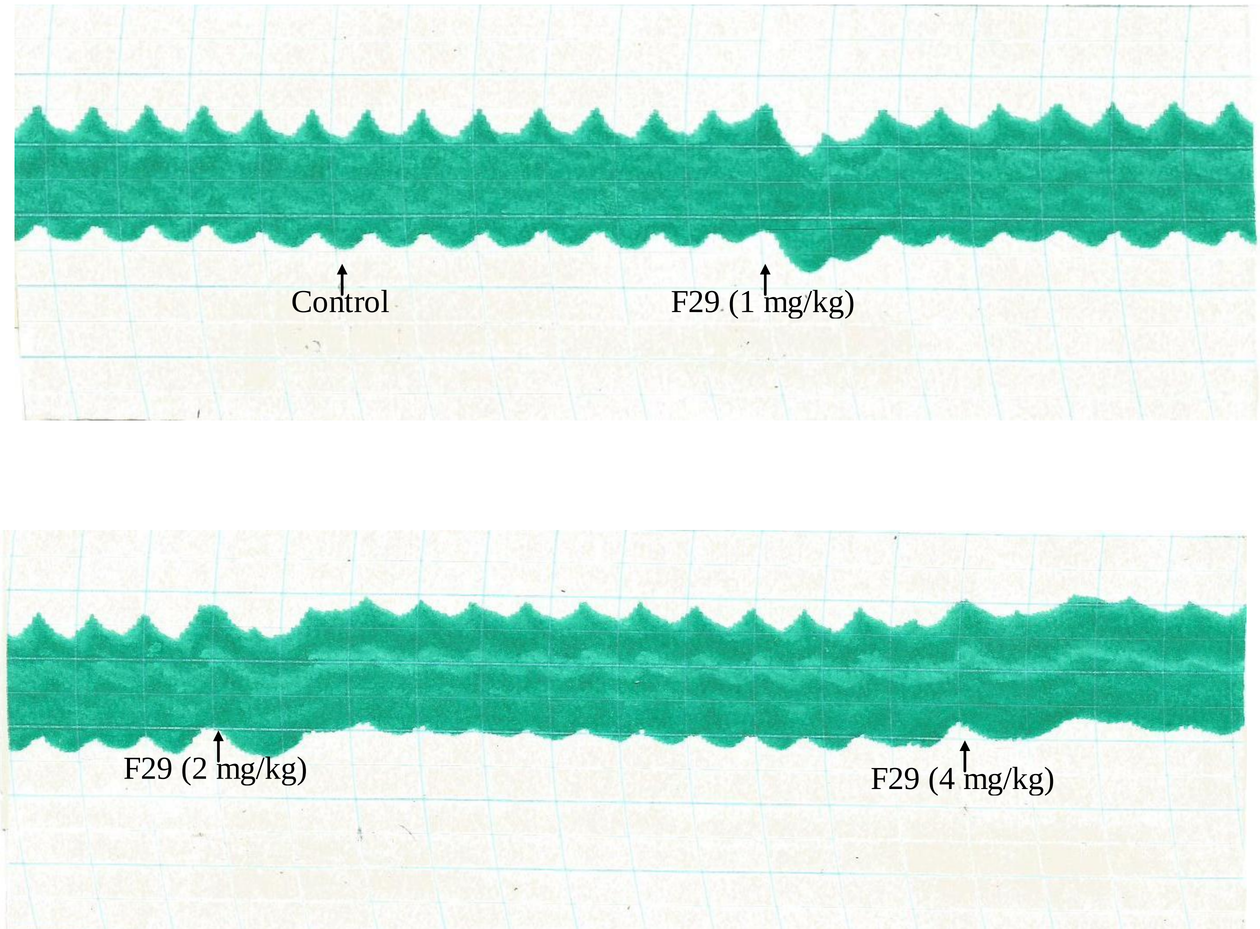
Appendix IV: Effect of aqueous extract of *Croton zambesicus* on urine output in rats

Treatment	Urine volume (ml)	Urine volume (ml)	Urine volume (ml)	Urine volume (ml)	Urine volume (ml)
NaCl 0.9% (0.2 ml)	0.050	0.000	0.000	0.000	0.000
AECZ 1 mg	0.000	0.650	0.850	1.275	0.250
AECZ 10 mg	1.075	1.750	1.350	1.110	1.100
AECZ 100 mg	1.450	1.100	1.050	1.075	1.350
Hydrochlorothiazide 10 mg	0.350	0.000	0.750	1.750	0.800

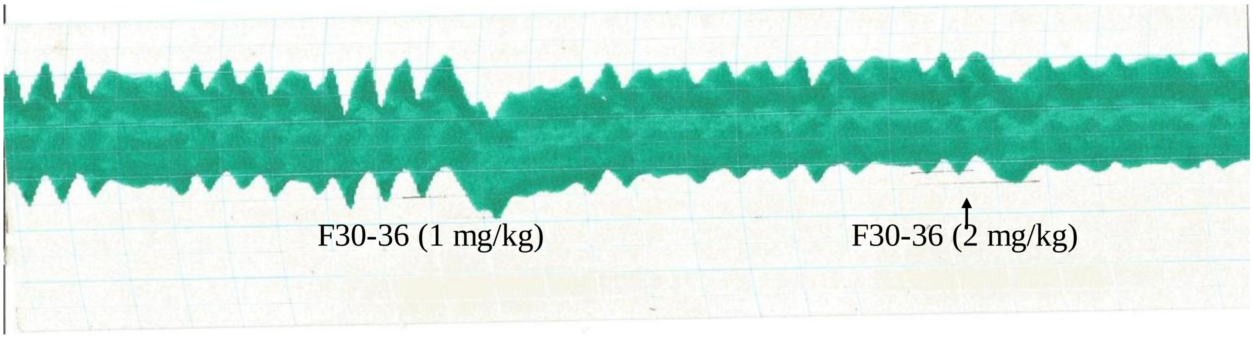


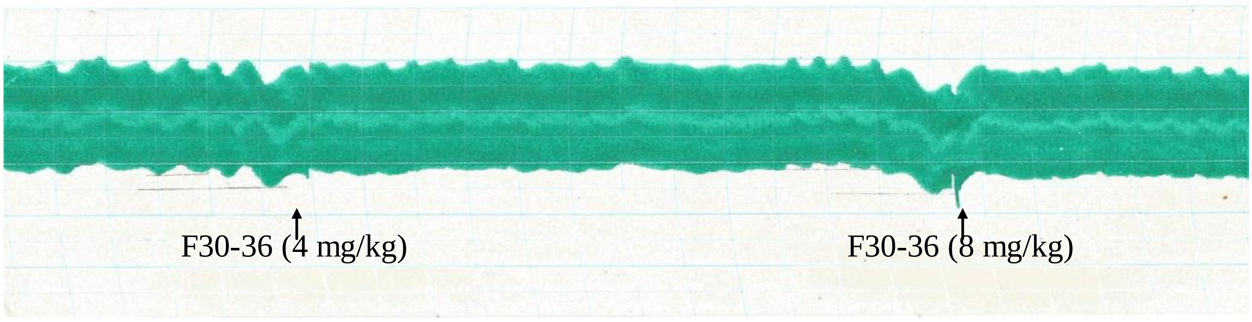


Appendix V: The effect of PCF 11-13 (0.1-0.8 mg/kg) on blood pressure of normotensive cat

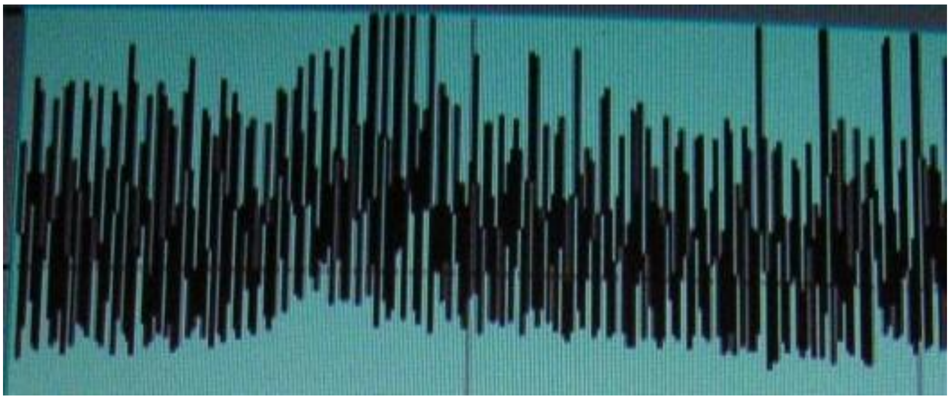


Appendix VI: Effect of PCF 29 (1-4 mg/kg) on blood pressure of normotensive cat





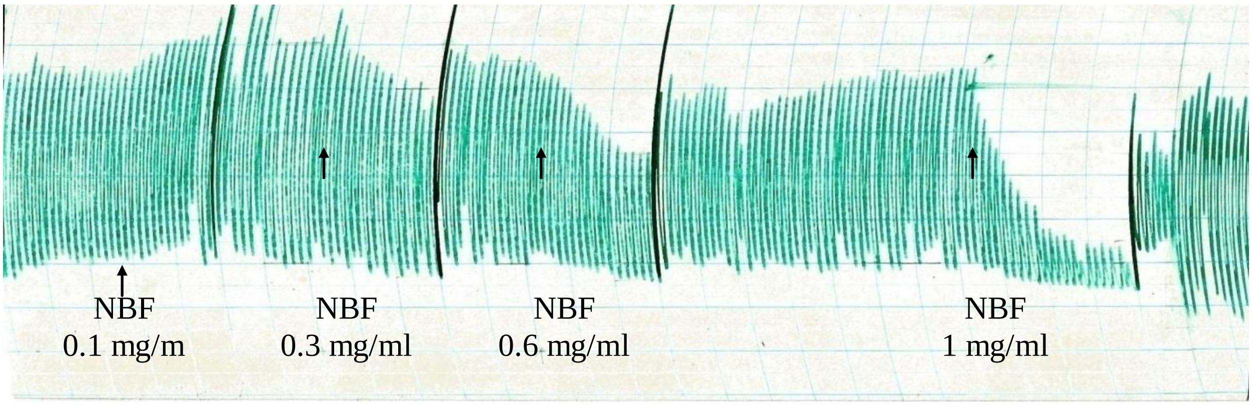
Appendix VII: Effect of PCF 30-36 (1-8 mg/kg) on blood pressure of normotensive cat

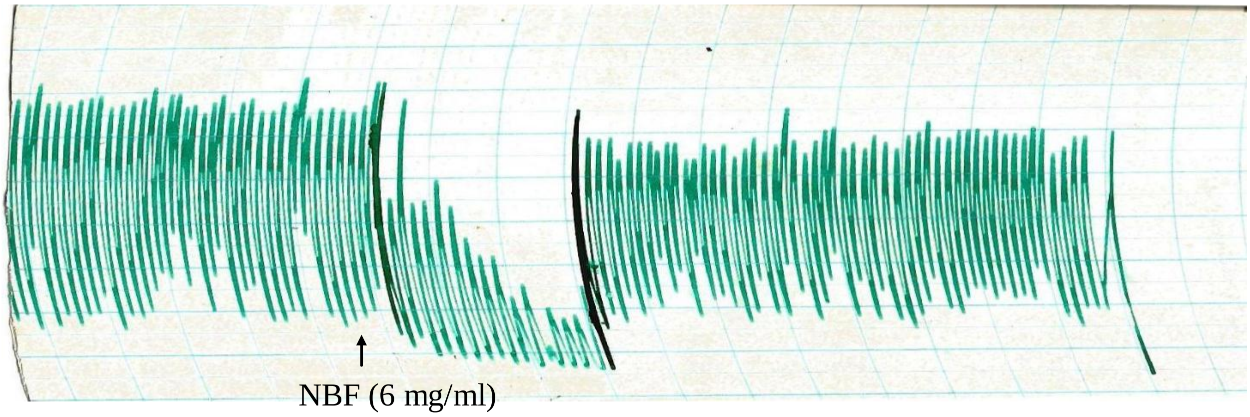


↑
CZ 0.64 mg/ml

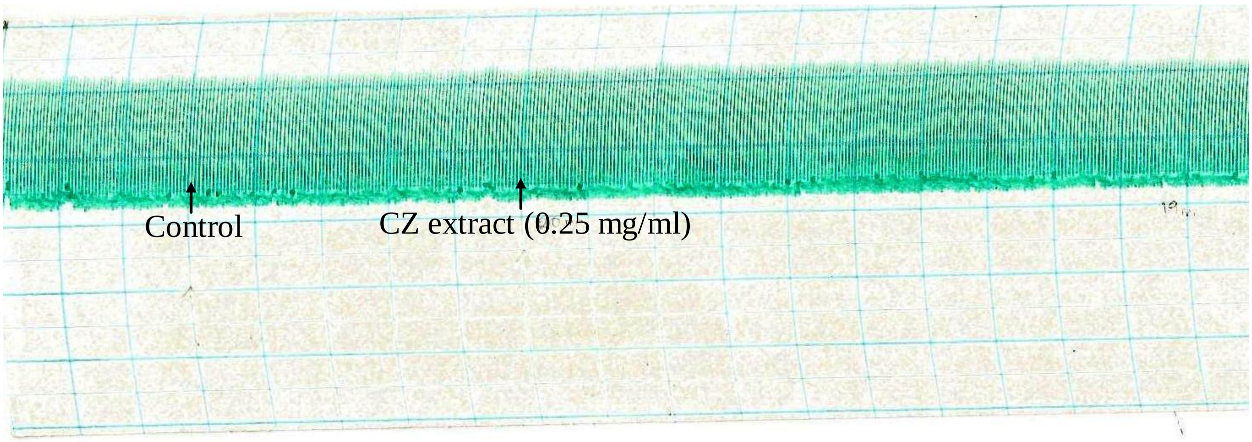
↑
CZ 6.4 mg/ml

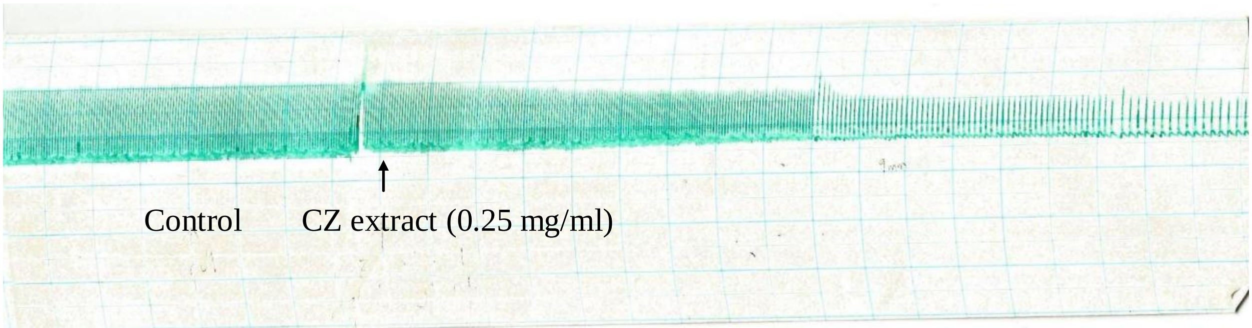
Appendix VIII: Effect of aqueous extract of *Croton zambesicus* on spontaneous contraction of isolated rabbit ileum



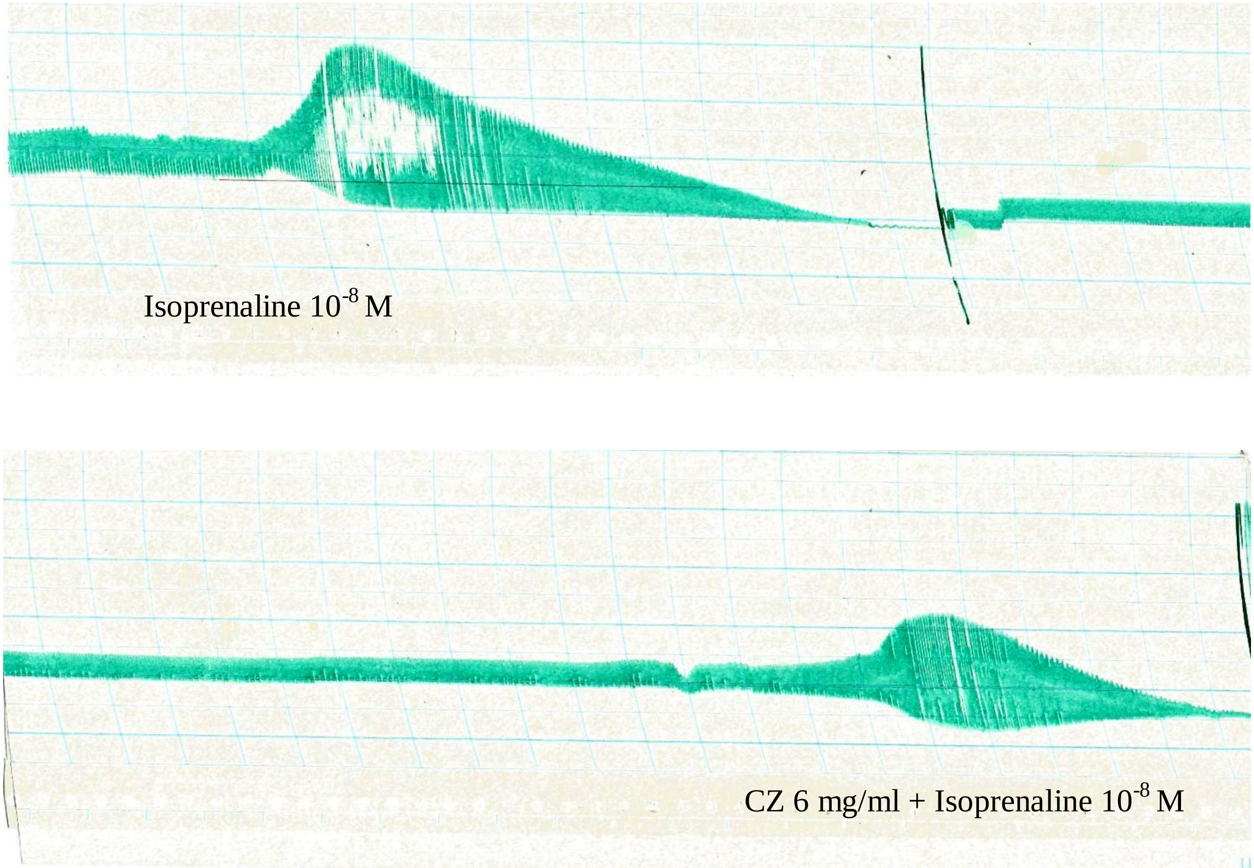


Appendix IX: Effect of n-butanol fraction on spontaneous contraction of isolated rabbit ileum

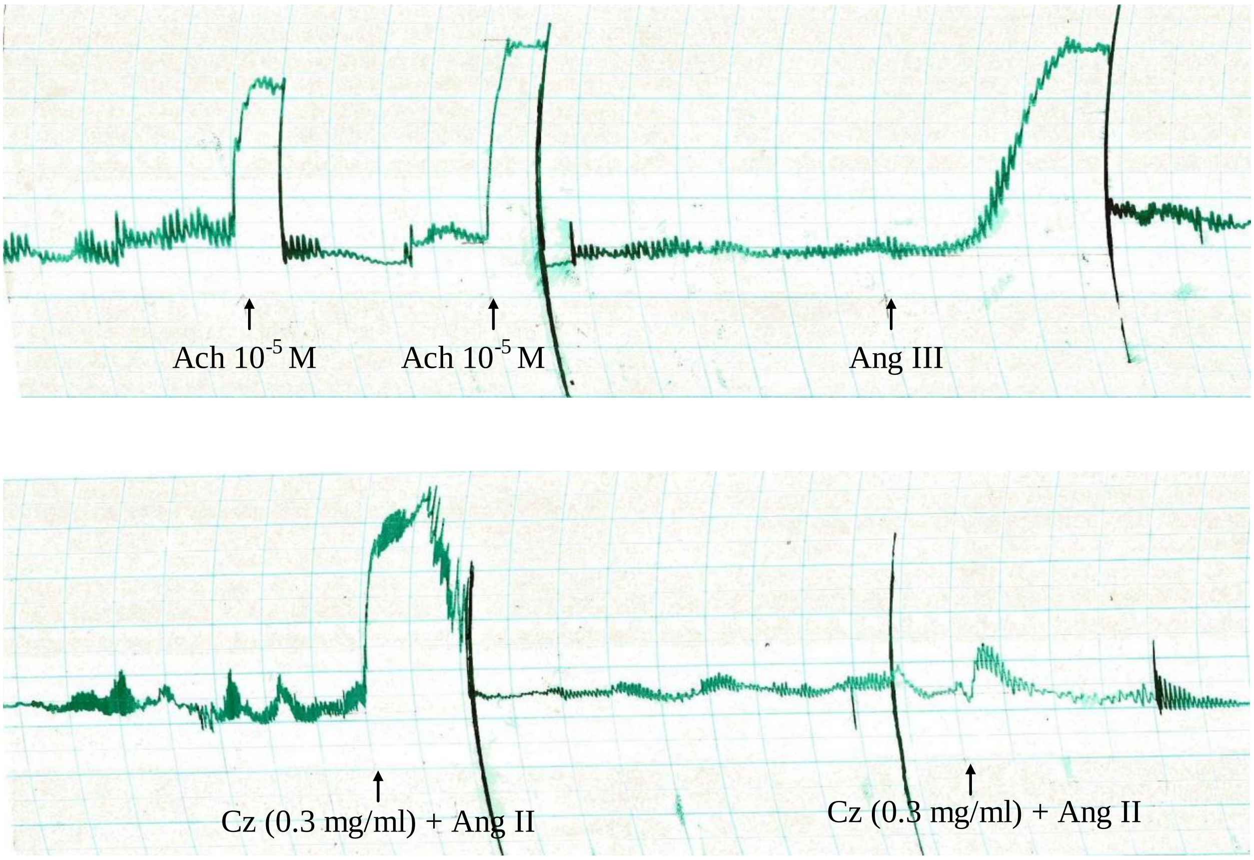




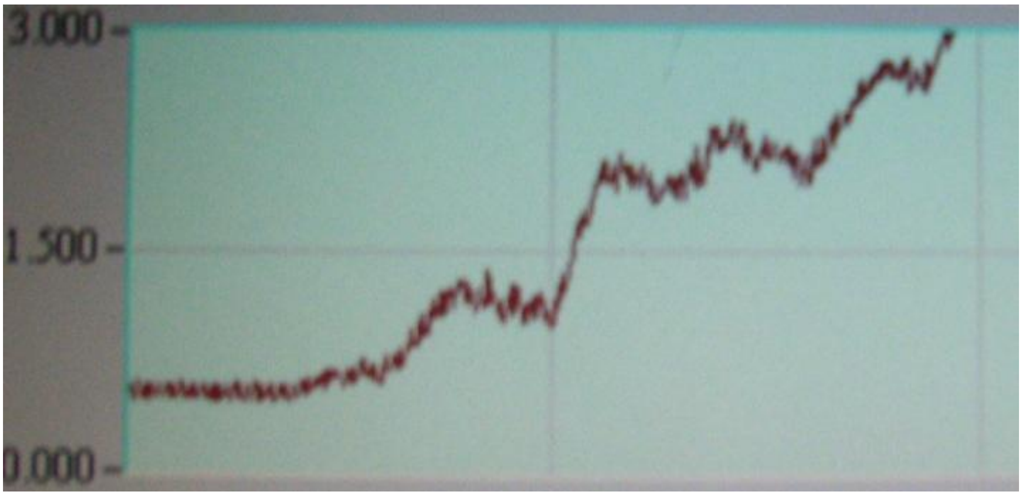
Appendix X: Effect of aqueous extract of *Croton zambesicus* on isolated right atrium of guinea pig atrium



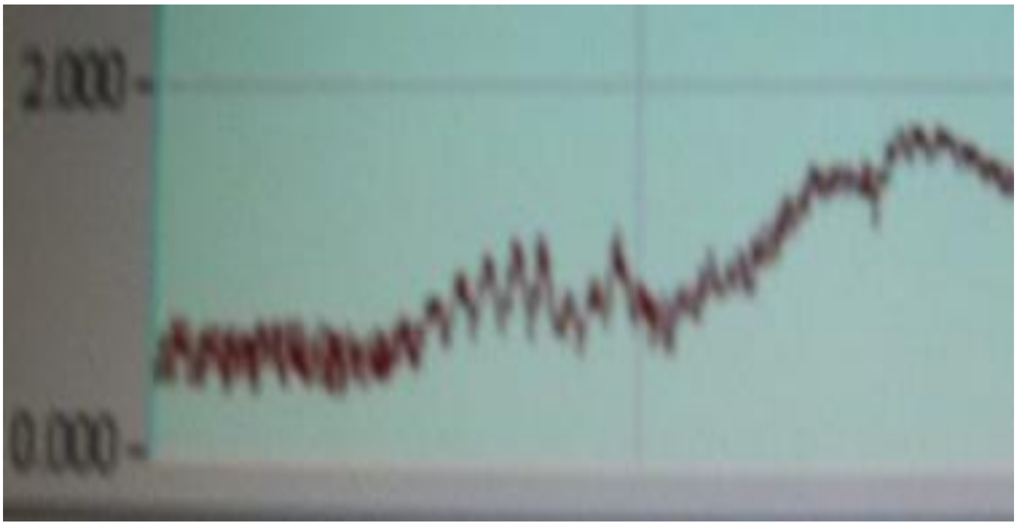
Appendix XI: Effect of aqueous extract of *Croton zambesicus* on isoprenaline-induced contraction of isolated rabbit heart.



Appendix XII: Effect of aqueous extract of *Croton zambesicus* on Ang II-induced contraction of rat ileum

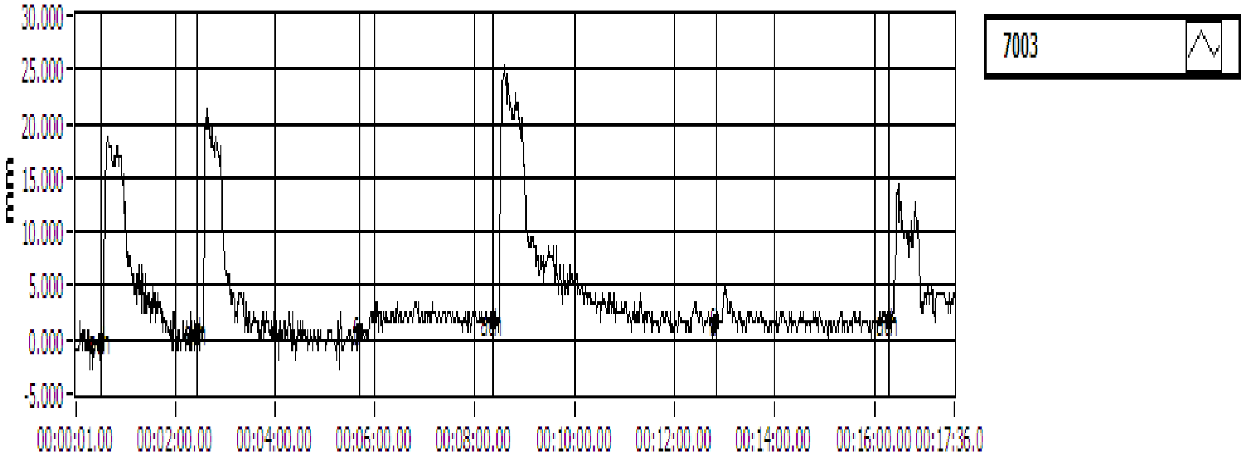


A. Cummulative response curve of $CaCl_2$ (1-30 mM) in rat ileum



B. CZ extract 0.08 mg/ml and $Cacl_2$ (1-30 mM)

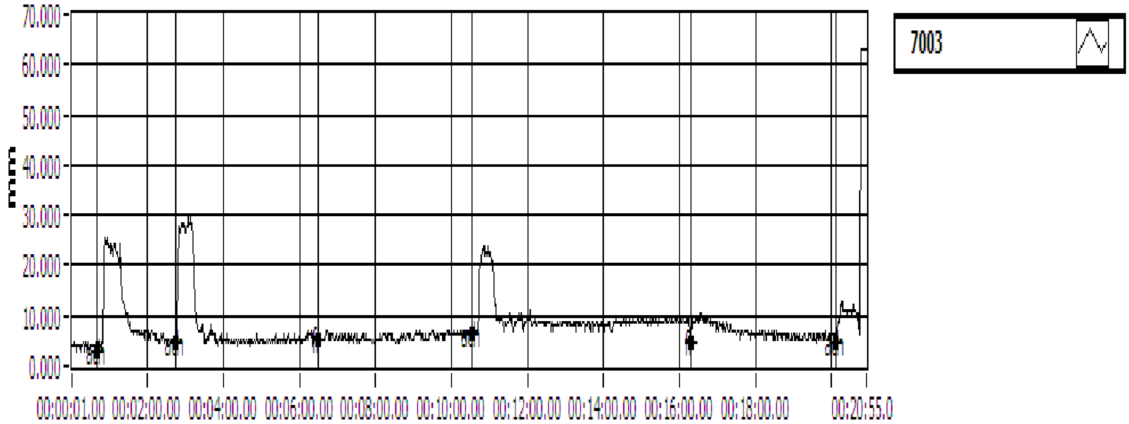
Appendix XIII: Effect of aqueous extract of *Croton zambesicus* on calcium chloride-induced contractions of rat ileum



↑ ↑

ACh 1 µM F29 0.5 mg+ACh 1 µM 3mg/ml+ACh 1 µM

Effect of F29 (0.5and 3 mg/ml) on ACh-induced contraction



Effect of F 30-36 (1 and 6 mg/ml) on ACh-induced contraction of rat ileum

Appendix XIV: Effect of PCF of aqueous extract of *Croton zambesicus* on ACh-induced contraction of rat ileum