Evaluation of *In Vivo* Antihypertensive and *Ex Vivo* V asodepressor Activities of the Seed Extract of *Calpurnia aurea* (Ait.) Benth.subsp. *aurea* (Fabaceae)

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This is to certify that the thesis prepared by Yohannes Getiye, entitled: *Evaluation of In Vivo Antihypertensive and Ex Vivo Vasodepressor Activities of the Seed Extract of Calpurnia aurea* (Ait.) Benthssp. *aurea* and submitted in partial fulfilment of the requirements for the degree of Master of Science (Pharmacology) complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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ABSTRACT

Evaluation of *in vivo* antihypertensive and *ex vivo* vasodepressor activities of the seed extract of *Calpurnia aurea* ssp. *aurea (Ait.) Benth***.**

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Being the most important modifiable risk factor for stroke, renal, vascular and heart diseases; hypertension is still one of the leading causes of disability, morbidity and mortality among the populace worldwide. Consequently, the continued search for alternative antihypertensive agents of natural origin, with fewer side effects but greater effectiveness, necessitated evaluation of *Calpurnia aurea* for possible antihypertensive potential. In this study, the antihypertensive property of hydro-alcoholic seed extract of *Calpurnia aurea* was assessed using *in vivo* and *ex vivo* techniques. The *in vivo* antihypertensive efficacy of the crude extracts was evaluated in normotensive and 2K1C rat model of hypertension. The crude extract caused a significant fall in systolic blood pressure (SBP), diastolic blood pressure (DBP)and mean arterial blood pressure (MABP) at the doses of 15, 30 and 45 mg/kg in normotensive anaesthetized rats ($p < 0.05$, $p < 0.01$, $p< 0.001$).The effect on DBP was greater than SBP. In the same manner with the normotensive study, the BP fell dose-dependently and significantly in renal hypertensive rats, induced by renal ischemia. The extract produced 15.4% ($p< 0.05$), 26.9% ($p< 0.01$), 33.2% ($p <0.01$) reduction in SBP at the respective doses of 15, 30 and 45 mg/kg. Similar to the normotensive result, higher DBP effect was observed; 15.1% ($p< 0.05$), 30.2% ($p< 0.01$), 36.1% ($p <0.01$) fall from the control value at the above mentioned doses. Regarding MABP; 15.2% ($p< 0.05$), 28.8% ($p <0.01$) and 34.9% ($p <0.01$) reduction was noted with 15 mg/kg, 30 mg/kg and 45 mg/kg doses of the extract, respectively. The extract also caused a dose-dependent relaxation of guinea pig aorta precontracted with KCl (80mM),at a concentration of 5–250 mg/mL, with a maximum relaxation of 92.1±0.72% ($p < 0.001$) achieved at 250 mg/mL concentration. The relaxation mechanism was found to be independent of the endothelium system, muscarinic

receptors, histamine receptors, ATP dependent K^+ channels, cyclooxygenase enzymes and cGMP/NO pathway. The relaxation may be correlated with the effect on calcium ion channels, as it shifted the Ca^{2+} dose response curve ($p< 0.05$, $p< 0.01$ and $p< 0.001$) to the right with suppression of the maximum effect by 37.4% ($p < 0.001$). In conclusion, the findings suggest that the extract had a promising antihypertensive effect most likely caused by dilation of the blood vessels through Ca^{++} channel blockade, a confirmation for the folkloric use of the plant.

Key words: Hypertension, *Calpurnia aurea*, hydro-alcoholic seed extract, *in vivo, ex vivo,* antihypertensive, vasorelaxation.

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List of Abbreviations

2K1C	2-kidney-1-clip
ACE	Angiotensin converting enzyme
ACh	Acetylcholine
ADH	Antidiuretic hormone
Ang II	Angiotensin II
ANOVA	Analysis of variance
ARBs	Angiotensin receptor blockers
AT1r	Angiotensin type I receptor
BBs	Beta blockers
BP	Blood pressure
CASA	*Calpurnia aurea* subspecies *aurea*
CCBs	Calcium channel blockers
cGMP	Cyclic guanidine monphosphate
CO	Cardiac output
CRCs	Concentration-response curves
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
EDTA	Ethylene diaminetetra-acetic acid
EPHARM	Ethiopian pharmaceutical manufacturing
ESC	European society of cardiology
ESH	European society of hypertension
HR	Heart rate
i.p.	Intraperitoneal

i.v.	Intravenous
JNC	Joint National Committee
MABP	Mean arterial blood pressure
NO	Nitric oxide
PHT	Primary hypertension
PP	Pulse pressure
PVR	Peripheral vascular resistance
RAAS	Renin-angiotensin-aldosterone system
SBP	Systolic blood pressure
SEM	Standard error of mean
SNS	Sympathetic nervous system
ssp	Subspecies
SV	Stroke volume
WHO	World health organization

1.Introduction

1.1 Clinical background of hypertension

Cardiovascular disease (CVD) is responsible for one third of global deaths and is a leading and increasing contributor to the global disease burden. One of the highly prevalent risk factor for CVD throughout the world is hypertension (Kloet *et al*., 2013).Being the most important modifiable risk factor for stroke, renal, vascular and heart diseases; hypertension is one of the leading causes of disability, morbidity and mortality among the populace (Delles and Padmanabhan, 2012; Okumura *et al.,* 2014). It is becoming an increasingly common health problem worldwide because of increasing longevity and prevalence of contributing factors such as obesity, physical inactivity and unhealthy diet (Kunes and Zicha, 2009).

High blood pressure (BP) or hypertension is defined as frequent, chronic, age-related disorder and characterized by systolic blood pressure (SBP) of 140 mmHg or greater and/or a diastolic blood pressure (DBP) of 90 mmHg or greater (Mancia *et al*., 2013). BP, like most physiologic variables, exhibits a bell-shaped distribution with no simple cut-off to demarcate "safe" from "dangerous" levels of BP (WHO, 2005). According to the Seventh Report of the United States Joint National Committee (JNC) on Prevention, Detection, Evaluation and Treatment of High BP JNC 7 criteria, normal BP, which is considered as safe, is defined as a SBP<120 mm Hg and a DBP<80 mmHg on repeated measurement (Chobanian *et al.,* 2003; Winter *et al.*, 2013).

Persons with a SBP between120-139 mmHg or DBP between 80-89 mmHg are designated as prehypertensive state (Taylor and Rahman, 2009). Hypertension is further characterized by two stages: Stage1, the milder (systolic 140-159 mmHg and/or diastolic 90-99 mmHg) and most common form of hypertension, accounts for approximately 80% of hypertension (Luehr *et al*., 2012). Stage 2 hypertension includes those with SBP ≥160 mmHg and/or DBP ≥100 mmHg. Isolated systolic hypertension is defined as SBP of ≥140 mmHg and DBP <90 mmHg (Siyad, 2011).

A more elaborated classification of BP is provided by the European Society of Hypertension and the European Society of Cardiology (ESH/ESC) (Table 1) (Tran and Giang, 2014).

Table 1: European society of hypertension/European society of cardiology classification of blood pressure for adults (Mancia *et al*., 2013; Tran and Giang, 2014)

Category	**SBP (mmHg)**		**DBP (mmHg)**
Optimal	<120	and	<80
Normal	120–129	and/or	80–84
High normal	130–139	and/or	85–89
Grade 1 hypertension (mild)	140–159	and/or	90–99
Grade 2 hypertension (moderate)	160–179	and/or	100–109
Grade 3 hypertension (severe)	≥180	and/or	≥110
Isolated systolic hypertension	≥140	and	<90

1.2 Classification of hypertension

1.2.1 Primary hypertension

Over 95% of all cases of hypertension are primary (essential or idiopathic), which is systemic hypertension of unknown cause but appears to be the result of an interplay of complex genetic and environmental factors (Tabassum and Ahmad, 2011).It may result from dys-regulation of normal homeostatic control mechanisms of BP in the absence of detectable known secondary causes (Thalgahagoda and Shenoy, 2012).This is the common type and cannot be permanently cured. However, the level of BP can be substantially reduced by drugs and non-pharmacological measurements such as sharp reduction of obesity (where present) and reduction of dietary salt (WHO, 2005; Huei *et al.*, 2010).

1.2.2 Secondary hypertension

Secondary hypertension is systemic hypertension that results from underlying, identifiable, often correctable causes and is treatable or reversible. It accounts about 5% of cases of hypertension (Garg*et al*., 2013). Different medical conditions such as renal artery obstruction, coarctation or narrowing of the aorta, thyroid dysfunction, hyperfunction and hypofunction of the adrenal cortex (Cushing syndrome and aldosteronism), pheochromocytoma as well as sleep apnea are considered as causes of secondary hypertension (Winter*et al.,* 2013).Commonly used medications such as cortisone, oral contraceptives and non-steroidal anti-inflammatory drugs can also result in such type of hypertension (Siyad, 2011; Winter *et al.*, 2013).

1.3 Epidemiology of hypertension

Hypertension is a worldwide epidemic accounting for about 6% of deaths globally (Gudina *et al.*, 2013).Overall, the prevalence of hypertension appears to be around 30–45%of the general population, with a steep increase with ageing (Mancia *et al*., 2013). Although there has been recent progress in the prevention, detection and treatment of hypertension, it persists as a major public health challenge affecting more than one quarter of the population worldwide (Okumura *et al.,* 2014). It has also been identified as the leading risk factor for mortality (7.1 million deaths a year), and is ranked third as a cause of disability-adjusted life-years (Gudina *et al.*, 2013).

World health statistics 2012 has estimated the prevalence of hypertension to be 29.2% in males and 24.8% in females (Kumar, 2013).Hypertension affects nearly 26% of the adult population worldwide and by 2025, the number of people affected by high BP is estimated to increase by 60% to 1.56 billion (Himansu *et al*., 2014).

Almost three-quarters of people with hypertension (639 million people) live in developing countries (Ibrahim and Damasceno, 2012; Himansu *et al*., 2014). When it comes to Ethiopia, the epidemiology of hypertension is not well studied, but as late as 1940,a prevalence of 1.8% was reported in rural villages (Zein and Assefa, 1986).In one

study conducted in a rural and semi-urban community of Butajira, investigators reported that 8.2% of women and 12.3% of men had prevalent hypertension (Tesfaye *et al.*, 2007).

Pauletto *et al.* (1994) reported exceedingly low prevalence estimates for hypertension in their study of semi-nomadic residents of Arssi (0.4%) and urban residents of Shashamane (3.15%). A study conducted among residents of Addis Ababa found the prevalence of hypertension among adults to be 31.5% for men and 28.9% for women (Tesfaye *et al.*, 2009).

Recent study done on prevalence of hypertension and diabetes among Ethiopian adults estimate prevalence of hypertension to be 14.9% in women and 22% in men, suggesting it is an increasing health problem in the country (Nshisso *et al.*, 2012).In addition, according to the health and health-related indicators of Ministry of Health2005 –2006, hypertension was ranked as the sixth leading cause of death among hospitalized patients (WHO, 2009)

1.4 Physiology of normal blood pressure control

The arterial BP is the product of cardiac output (CO) and peripheral vascular resistance (PVR). Elevation in either CO or PVR, or both, will contribute to an elevated BP (Thalgahagoda and Shenoy, 2012). CO depends primarily on heart rate (HR) and stroke volume (SV). HR is governed by adrenergic and cholinergic receptors under the control of sympathetic and parasympathetic stimulation, respectively (Averill and Diz, 2000). The ventricular force of contraction and the filling pressure, which is in turn determined by the intravascular fluid volume status and the venous capacitance, determines the SV (Bakris and Mensah, 2003).

Rapid, intermediate and late-acting mechanisms govern the above physiologic determinants and maintain the arterial BP within the normal range despite significant variations in the individual parameters (Singh *et al.*, 2010).

i) Rapid-acting mechanisms

An acute increase in BP results in rapid response occurring within seconds, which involves the baroreceptor system, vagus nerve and the central vasomotorcenter of the brainstem (Bakris and Mensah, 2003).The baroreceptor reflex is generally recognized as a relatively high gain control system that provides mean arterial blood pressure (MABP) regulation of over a timeframe of seconds to minutes (Averill and Diz, 2000).

Pressure sensors located primarily in the arterial walls of carotid artery sinus and aortic arch sense changes in MABP and drive afferent neural feedback centrally in proportion to changes in MABP (Wehrwein and Joyner, 2013). When the artery is distended due to elevated pressure, there is activation of the embedded barosensitive afferent nerve endings (*i.e.*, as pressure increases there is increased vessel wall stretch and increased afferent firing) (Averill and Diz, 2000; Lohmeier, 2001). The baroreceptor sensors transduce stretch of the vessel wall into an electrical signal in the afferent nerves that is relayed to an integrating center in the brain, the medulla oblongata. The signal is processed centrally and then relayed *via* both sympathetic and parasympathetic efferent pathways to effect or organs (Kloet *et al*., 2013).The sympathetic efferent pathways are targeted to both the blood vessels and heart, while the parasympathetic efferent pathways are targeted to the pacemaker cells in the sinoatrial node of the heart, causing a reflex vagal bradycardia (Ji*et al*., 2010; Siyad, 2011).

Though the baroreceptor reflex counteracts temporary disturbances in arterial pressure, it cannot effectively regulate arterial pressure in the long-term, as it can adapt to prolonged changes in arterial pressure (Singh *et al.*, 2010; O'Donohoe and Pandit, 2013).

ii) Intermediate-acting mechanisms

Intermediate-acting responses to changes in BP occur over minutes and hours, and involves the Renin-angiotensin-aldosterone system (RAAS), antidiuretic hormone (ADH) and the renal juxtaglomerular apparatus (Singh *et al.*, 2010). Reduction in BP stimulates renin release *via* afferent arteriole baroreceptor mechanisms and increased activity of the renal sympathetic nerves as well as local macula densa mechanism (Taylor and Rahman, 2009). The released renin finally ends up with formation of angiotensin II (Ang II), a

potent vasoconstrictor, which stimulates aldosterone release thereby directly increasing proximal sodium chloride reabsorption (Izzo and Weir, 2011).ADH secretion will increase principally as a result of acute reduction in BP because of a decrease in the normal inhibitory tone from the baroreceptors to the hypothalamus and *vice versa* (Bakris and Mensah, 2003). ADH acts as part of the rapid response in the setting of acute, severe decrease in BP, at the same time part of the late-acting response during periods of prolonged hypotension (Wehrwein and Joyner, 2013).

iii) Long-acting mechanisms

The long-term efficiency in BP regulation comes from late-acting mechanisms which operate in days to weeks. Renal mechanisms determining the long-term control of BP includes pressure natriuresis and pressure diuresis (Lohmeier, 2001; O'Donohoe and Pandit, 2013). This indicates the role of the kidney in the long-term regulation of BP as none of the mechanisms discussed under short and intermediate acting BP regulation can operate effectively to regulate BP by themselves (Dorrington and Pandit, 2009). An increase in BP level beyond the normal set point leads directly to an increase in salt and water excretion (Taylor and Rahman, 2009).When the renal-body fluid feedback mechanism for arterial pressure control is impaired and body fluid volumes are elevated, large increases in atrial pressure and atrial natriuretic peptide secretion occur. These surges exert sustained natriuretic effects and chronically shift renal-pressure natriuresis to lower arterial pressures (DPhil and Sear, 2004).Thus, in the long-term, BP is regulated by changes in blood volume (Montani and Vliet, 2009).

1.5 Pathogenesis of primary hypertension

The pathogenesis of primary hypertension (PHT) is complex and somewhat poorly understood. It is thought to be the result of the interplay of multiple genetic and environmental factors (Thalgahagoda and Shenoy, 2012). In almost any of the cases, the involvement of the sympathetic nervous system (SNS), the RAAS and the kidney need to be considered with regard to pathogenesis of PHT (Siyad, 2011), though other factors, such as endothelin, nitric oxide and the kallikrein-kinin system cannot be totally excluded (DPhil and Sear, 2004).

i) The role of genes

Hypertension has been suggested to result from the additive effects of multiple variant genes acting in concert to increase BP (Tabassum and Ahmad, 2011).Though no robust association could be established between those genes and hypertension, most obvious candidate genes are components of the RAAS (e.g., the aldosterone synthase gene *CYP11B2*), salt and water handling (e.g., the adducin-α gene*ADD1)* etc (Delles and Padmanabhan, 2012). Much attention is also focusing on genetically mediated alterations in the regulation as well as expression of renal ion channels and transporters as observed in genetic diseases like liddle syndrome (Delles *et al*., 2010).From family and twin studies, heritabilities of SBP and DBP are generally estimated in the range of 15 to 40% and 15 to 30%, respectively (Kunes and Zicha, 2009). A child having a history of hypertension in both parents and who has a sibling with hypertension has a 40% to 60% chance of developing hypertension as an adult, but the risk increases to 80% if the sibling is a monozygotic twin (Bakris and Mensah, 2003; Mancia *et al*., 2013).

ii) The role of environment

In addition to genetic susceptibility, environmental determinants such as physical activity, psychosocial stress, alcohol intake, dietary factors (salt, fat), cigarette smoking and other lifestyle choices play a role in development of PHT (Kunes and Zicha, 2009; Ibrahimand Damasceno, 2012). General epidemiological data have shown the strong link between high salt intake (>4 g/day) with a high risk of hypertension (Badyal *et al*., 2003). Low potassium intake may also contribute to salt sensitivity, showing the fact that diets low in calcium or potassium are associated with a higher prevalence of hypertension(Grossman, 2010).

The pathogenesis of hypertension in the obese is complex. As it is the most prominent risk factor for expression of hypertension, human obesity and hypertension frequently coexist (Thalgahagoda and Shenoy, 2012). Activation of SNS leading to renal sodium retention seems to play a pivotal role. This increment in SNS activity is mediated by two mechanisms, hyperinsulinemia and hyperleptinemia (Parati and Esler, 2012).

Hyperinsulinemia results from peripheral resistance to insulin and is postulated to cause hypertension through increased PVR in addition to abnormal sodium handling by the kidney and increased activity of SNS (Kishi and Hirooka, 2013).Hyperleptinemia is, however, a consequence of the increased mass of adipose tissue in the obese and causes increased activation of the SNS. Activation of the RAAS also appears to play an important role in the etiology of obesity related hypertension, as it contribute to renal sodium retention directly and through aldosterone production (Silva *et al*., 2009).

An increase in the intra renal pressure due to compression on the kidney by surrounding adipose tissue could also lead to hypertension (Kloet *et al*., 2013). Hyperuricemia, hyperglycemia (insulin resistance), low socioeconomic status and increased stress are also associated with the development of high BP (Johnson *et al*., 2005).

iii) Sympathetic nervous system and hypertension

Enhanced sympathetic activity is a well-recognized hallmark of most forms of clinical hypertension. Despite years of investigations, the mechanisms for SNS over activity do remain enigmatic (Singh *et al.*, 2010; Kishi and Hirooka, 2013).Baroreflex dysfunction has been postulated and may contribute to the increased BP in some hypertensive patients. As described above, insulin resistance and hyperinsulinemia have also been hypothesized to play an important role in SNS activation and hypertension in obesity(Silva *et al.*, 2009).This is based on the evidence that insulin may have central sympathoexcitatory effects, which could be enhanced in the adrenergic drive of patients who are hypertensive (Kishi and Hirooka, 2013).

The consequences of increased SNS stimulation are increased norepinephrine level, peripheral vasoconstriction, increased HR and a resultant increase in systemic BP (Ji*et al*., 2010). This high SNS activity has also been shown to exert growth promoting effect on the myocardial tissues and plays a crucial role in mediating vascular hypertrophy, increased vascular stiffness leading to reduced arterial dispensability and increased cardiac wall thickness (Parati and Esler, 2012).Activation of renal efferent sympathetics

causes intrarenal vasoconstriction with a decrease in renal blood flow and an increase in renal vascular resistance (Lohmeier, 2001).

As the afferent renal arteriole thickens in response to the increase in BP, the arteriolar lumen becomes progressively smaller and narrower, and finally collapses (Winter *et al.*, 2013). These together with SNS mediated intra renal vasoconstriction causes renal ischemia, which in turn leads to mild tubular injury and inflammatory infiltrates in the form of T-lymphocytes and macrophages (Thalgahagoda and Shenoy, 2012). These result in local depletion of intrarenal NO, resulting in further renal arterial vasoconstriction and the development of hypertension (Johnson *et al.*, 2005; Huei *et al.*, 2010). This (ischemia) also leads to further activation of the RAAS with sodium and water retention. The ensuing rise in BP increases perfusion and negates the ischemia allowing salt handling to return to normal, albeit at a higher BP (Lohmeier, 2001).

iv) Renin-angiotensin-aldosterone system and hypertension

The role of the RAAS, being the most important mechanisms in the regulation of blood volume and pressure, in essential hypertension is complex (Taylor and Rahman, 2009). Renin is a protease released by the juxtaglomerular apparatus of the kidney and it cleaves angiotensinogen to angiotensin I, which in turn is converted to angiotensin II (Ang II) by angiotensin-converting enzyme (ACE) (Mentz *et al.*, 2013).Diminished renal perfusion pressure, reduced sodium concentration in distal renal tubular fluid and β- adrenoceptor stimulation lead to release of renin and stimulation of the RAAS (O'Donohoe and Pandit, 2013).

Angiotensin II *via* Angiotensin type I (AT1) receptors causes renal vasoconstriction, stimulation of vascular smooth muscle contraction and hypertrophy, increases cardiac contractility, stimulation of the SNS in the central and peripheral nervous system(Izzo and Weir,2011),generation of reactive oxygen species in the vasculature and facilitating the atherosclerotic process, increases thirst and vasopressin release and stimulates aldosterone synthesis, all of which can aggravate hypertension(Thalgahagoda and Shenoy, 2012).In the early phase of hypertension, the high renin levels maybe secondary

to an increased SNS activity. But, with time, hypertension may shift from a salt-resistant, renin-dependent type of hypertension to a salt-sensitive, kidney-dependent and volume-dependent hypertension (Singh *et al.*, 2010;Kloet *et al.*, 2013).

1.6 Signs and symptoms of hypertension

Hypertension has been called as "the silent killer" since uncomplicated hypertension is usually asymptomatic. Even if not totally asymptomatic, it can go unrecognized for years because overt symptoms and signs generally coincide with the onset of target organ damage (WHO, 2005). The clinical features may be due to the elevated BP itself, target organ involvement or due to underlying diseases, as in secondary hypertension. Elevated BP is associated with headache, epistaxis, dizziness, and easy fatigability (Siyad, 2011). Symptoms due to affection of target organ include left ventricular hypertrophy, arrhythmias, palpitations, blurring of vision owing to retinal changes, murmurs over neck arteries, episodes of weakness due to transient cerebral ischemia, angina pectoris and dyspnea due to cardiac failure (Cohuet and Boudier, 2006; Garg *et al.*, 2013). Furthermore, symptoms underlying disease in secondary hypertension can be edema and puffy face due to acute nephritis, weight gain and hirsutism in patients with cushing's syndrome, polyuria, polydipsia and muscular weakness secondary to hypokalemia in patients with primary aldosteronism (Labinson *et al.*, 2006; Winter *et al.*, 2013).

1.7 Management of hypertension

1.7.1 Non-pharmacological management

Non-pharmacological approaches to the reduction of BP generally are advisable as the initial approach to treatment of patients (Luehr *et al.*, 2012).In the initial phase of hypertension (high, normal or mild hypertension), the non-pharmacological measures can lower BP in most of individuals (Mancia *et al.*, 2007). Further, these approaches will augment the effectiveness of pharmacological therapy in patients with higher level of BP(WHO, 2005).There is robust evidence to support maintenance of an ideal body weight, sodium restriction, limited alcohol consumption (Winter *et al.*,2013), regular isotonic exercise, relaxation, vegetarian diet and fruits could be appropriate lifestyle

regimens to reduce BP (He *et al*., 2000).Whereas smoking cessation advice should be given as part of an overall strategy to reduce total cardiovascular risk, it does not reduce BP *per se (*Huei *et al.*, 2010).

1.7.2 Pharmacological management

The general aim of therapy is the reduction of BP *per se* by whatever means. As arterial pressure is product of CO and PVR, it can be lowered by the action of drug on either the peripheral resistance or CO, or both (Kolck *et al.*, 2004; Siyad, 2011). The ultimate goal of antihypertensive therapy is adequate control of BP to reduce the cardiovascular and renal morbidity and mortality (Taylor and Rahman, 2009). This can be achieved using a single therapy but most commonly concurrent use of drug from different classes is a strategy for achieving effective control of BP while minimizing the dose related adverse effects (Tran and Giang, 2014).

a) Diuretics

Diuretics have remained the cornerstone of antihypertensive treatment since at least the first JNC report in 1977 and the first WHO report in 1978 and still, they were classified as the only first-choice drug to start treatment (Mancia *et al*., 2013).Being the first-line treatment of hypertension, effectively reduce BP, while at the same time decrease morbidity and mortality associated with hypertension (Sica *et al*., 2011). Diuretics in general act by inhibiting sodium–potassium chloride co-transport block the re-uptake of these ions to the plasma. This inhibition induces urinary excretion of salt and water so that this decreased volemia of the circulatory system results in BP lowering (diuresis) (Cohuet and Boudier, 2006). Thiazide-type diuretics (e.g. hydrochlorothiazide) should be used as initial therapy for most patients with hypertension, either alone or in combination with one of the other classes (Roush *et al.*, 2014).Loop diuretics and potassium sparing diuretics could also be used in certain hypertensive patients (Chen *et al*., 2009; Sica *et al*., 2011).

b) *β-blockers*

Beta blockers (BBs) are often used to block the effects of noradrenalin on the heart, inhibit the adrenergic-mediated release of renin and prevent the reflex sympathetic stimulation secondary to the use of diuretics and α-blockers (Cohuet and Boudier, 2006). However, compared to other antihypertensive agents, there was no benefit for the end points of all-cause mortality, myocardial infarction and stroke (Bangalore *et al*., 2008; Caterina and Leone, 2010). Reduction of HR with BBs therapy is also associated with increased risk of CV events, making them less effective at preventing hypertension-related CV events than other antihypertensive agents (Khan, 2009). However, vasodilatory BBs (e.g. labetalol, nebivolol, carvedilol) decrease BP largely through reducing systemic vascular resistance; also avoid the typical adverse effects of the class (Ram, 2010).

c) *Calcium channel blockers*

Dihydropyridines (e.g. amlodipine, felodipine), phenylalkylamines (e.g. verapamil) andbenzothiazepines (e.g. diltiazem) are the three types of calcium channel blockers (CCB) (Richard, 2005).At therapeutic doses, dihydropyridine CCBs are predominantly peripheral vasodilators with little direct cardiac effect. Verapamil and, to a lesser extent, diltiazem have direct cardiac effects in addition to peripheral vasodilatation (Whyte *et al*., 2011).CCBs interact with the L-type voltage-gated plasma membrane channel to produce vasodilation, restore NO availability, inhibit aldosterone production, resulting in natriuresis (Richard, 2005; WHO, 2005). Diltiazem, a non-dihydropyridine CCB, was as effective as diuretics, BBs, or both in preventing cardiovascular events like stroke (Hansson *et al.*, 2000).

d) *Angiotensin-converting enzyme inhibitors*

The most obvious manner by which ACE inhibitors (e.g. captopril, enalapril) lower BP is by reducing the circulating level of Ang II, thereby removing the direct vasoconstriction induced by this peptide (Izzo and Weir, 2011).ACE inhibitors also blunt the expected

increase in sympathetic activity seen after vasodilation. As a result, HR and CO do not increase (Bertrand, 2004). Additionally, ACE inhibitors improve endothelial dysfunction and suppress endogenous endothelin secretion, inhibition of the break down of bradykinin, with an additional contribution from kinin stimulation of NO production (Bertrand, 2004; Kloet *et al*., 2013).By having three special benefits beyond those produced by other agents, ACE inhibitors are recommended for initial first line mono therapy in hypertensives with compelling indications like: relief of heart failure, prevention of ventricular remodeling after myocardial infarction and slowing glomerulosclerosis in diabetic and other nephropathies (Khalil *et al.,* 2001; Tran and Giang, 2014)

e) ***Angiotensin receptor blockers***

The development of antagonists of AT1r has been promoted because ACE also mediates the metabolism of the bradykinin system and because Ang II synthesis may shift to alternative ACE-independent enzymatic pathways like the chymase, which could reduce the efficacy of therapy (Savarese *et al.*, 2013).Angiotensin receptor blockers (ARBs) (e.g. losartan) displace Ang II from its specific AT1 receptor, antagonizing all its effects and result in vasodilation, antiproliferation and activation of NO causing a fall in PVR with little change in HR and CO (Mentz *et al.*, 2013).The major difference between ARBs and ACE inhibitors is the absence of an increase in kinin levels. This increase may be responsible for some of the beneficial effects of ACE inhibitors and more probably is responsible for their side-effects, particularly cough (Khalil *et al*., 2001; Bakris, 2010).They improve outcomes in hypertensive patient with chronic heart failure as well as beneficial with respect to endothelial dysfunction and vascular remodeling; overall reduce cardiovascular and renal morbidity and mortality (Spinara *et al*., 2013).

f) ***Other antihypertensive agents***

Central agonists(methyldopa, guanfacine, guanabenz) by stimulating α_2-adrenoreceptors, inhibit sympathetic nerve activityand norepinephrine-mediated vasoconstriction (Huei *et al.*, 2010).α_1 blockers (doxazosin) reduce left ventricular hypertrophy, lower total peripheral resistance, improve glomerular filtration rate, relax the arterial and venous

beds (Lavie *et al.*, 2009). Direct vasodilators such as minoxidil, potassium channel activators, enter the vascular smooth muscle cells to produce direct vasodilation unmediated by other mechanisms, such as inhibiting hormonal vasoconstriction, calcium entry or blocking α-adrenergic receptors (Al-Hashimi and Thompson, 2012). Other drugs include aldosterone antagonists (Clark *et al*., 2012), antioxidants (Queisser and Schupp, 2012), renin inhibitors like aliskiren (a direct inhibitor of renin at the site of its activation), antisense gene therapy (Mancia *et al*., 2013), and NO donors (e.g. sodium nitroprusside) (Al-Hashimi and Thompson, 2012), etc.

1.7.3 Herbal medicines and hypertension

Herbs have been used as medical treatments since the beginning of civilization and some derivatives (e.g., reserpine from *Rauwolfia serpentina*)have become mainstays of human pharmacotherapy (Kagathara *et al*., 2009).Several herbs like *Moringa Oleifera*offer potential for cardiovascular conditions including hypertension, venous insufficiency, hyperlipidemia, congestive heart failure, angina pectoris, atheroschlerosis, cerebral insufficiency and arrhythmia (Valli and Giardina, 2002). Data from epidemiologic studies support the potential of dietary antioxidants and flavonoids present in several herbs to improve cardiovascular health (Adedapo *et al*., 2008). Some of this include garlic (*Allium sativum*)(Reinhart *et al*., 2008),green tea (*Camellia sinensis*)(Kuriyama *et al*., 2006)and ginseng by enhancing synthesis of NO (Han *et al*., 1998). Other herbs such as *Tribulus terrrestris, Rawolfia serpentine* (source reserpine), *Stephania tetrandra, Veratrum* species, *Evodia rutaecarpa, Foeniculum vulgare, Coscinium fenestratum, etc.*are also employed for their antihypertensive activity (Kagathara *et al*., 2009; Tabassum and Ahmad, 2011).

1.8 The experimental plant, *Calpurnia aurea* subspecies *aurea*

The genus *Calpurnia* (Fabaceae) comprises some six or seven species, which have their centre of distribution in South Africa. *Bolusunthus* and *Virgili* are closely related genera and, formerly, species of *Calpurnia* were included in the latter genus. The genus is well known to contain different alkaloids and flavonoids. *Calpurnia aurea* (Ait.) Benth. is a yellow-flowered small tree or shrub widely distributed in Africa from Cape province to

Eritrea and which also occurs in southern India (Adedapo *et al*., 2008). Three subspecies of *Calpurnia aurea* are recognized, namely subsp. (ssp.) *aurea,* which occurs in Ethiopia but it is also distributed through Zaire, Zimbabwe, Angola and West Africa. The other two include ssp. *Sylvatica,* which is found in Cape Province and ssp. *Indica,* which is confined in India.Ssp. *sylvatica* is now included into *Calpurnia aurea* ssp. *aurea* (Brehan *et al.,* 1989).

Ssp.*aurea* from Ethiopia is known locally as "*digitta"* by the Amhara people and used in traditional medicine to treat diverse medical conditions and parasitic infestation as mentioned below, both in humans and animals. It is a multi-stemmed shrub or a small graceful slender evergreen tree 2-4 m tall (Fig 1). The leaves are compound, up to 20cm long, each having 5-15 pairs of leaflets and a terminal one. The flowers are bright yellow, each about 2.5 cm long, in showy or bright hanging bunches of 8 to 30 flowers. The fruit is a thin pod drying light brown with a papery texture, 5-12 cm long and 0.8-1.9 cm wide, narrowly winged on one side containing up to 8 brownish seeds (Notten, 2005; Zorloni *et al*., 2010).A) B)

Figure 1:Photographs showing *Calpurnia aurea* ssp. *aurea* (Ait.) Benth: tree (A) and fruit containing the seed (B).

Chemical investigations of *Calpurnia aurea* have resulted in the isolation of a series of alkaloids, phenolic compounds**,** flavonoids, flavonols, and proanthocyanidins , which also founds in the genus *Calpurnia* (Adedapo *et al*., 2008). Two novel alkaloids 3β,4α,13α-trihydroxylupanine and 3β,4α-dihydroxy 13α-*O*-(2'-pyrrolylcarbonyl)- lupanine

(calpaurine) have been isolated from the leaves of Ethiopian *Calpurnia aurea* ssp. *aurea.* Two minor quinolizidine alkaloids, 4β-hydroxy-13α - *O*-(2'-pyrrolylcarbonyl)-lupanine (digittine) and 4β, 13α–dihydroxylupanine have also included in the list. Furthermore, lupinine and epilupinine, calpurmenine and calpurmenine pyrrolecarboxylic acid ester (previously found in subsp. *sylvatica* but not in subsp. *aurea*) have been isolated together with 13-hydroxylupanine, its tiglate and pyrrolecarboxylic acid esters (calpurnine), virgiline and virgiline pyrrolecarboxylic acid ester (Asres *et al*., 1986a, b; Adedapo *et al*., 2008).The main pharmacologically active compounds may be the alkaloid calpurmenin and its 13α-(2'-pyrrolecarboxylic acid) ester though phenolics compounds may also contribute for its pharmacological effects, mainly of antibacterial and antioxidant activities. The alkaloids virgiline and lupanine as well as their carboxylicesters have also been implicated for its effect like as insect attractant/repellent in addition to the above activities (Zorloni *et al*., 2010).

Calpurnia aurea leaves and powdered roots are used to destroy lice and to relieve itches in South Africa. Unspecified parts are used to destroy maggots and the leaves are used to treat allergic rashes, earache, rheumatism, and tick control (Zorloni *et al*., 2010). In Nigeria, the seeds are used to treat abscesses. In Ethiopia, its extracts are used in indigenous medicine as insecticides as well as for the treatment of stomach complaints, headache (Tadeg *et al.*, 2005), eye diseases, amoebic dysentery (Abebe *et al*., 2012), scabies (skin infection caused by ticks) (Giday *et al.*, 2007).Antibacterial and antioxidant activity of *Calpurnia aurea* have been reported (Adedapo *et al*., 2008) and the leaves has been used to treat bacterial dermatitis (Tadeg *et al*., 2005). Giday*et al.* (2007) mentioned the traditional use of *Calpurnia aurea* seed to treat hypertension in some parts of Shinasha, Agew-awi and Amhara peoples in northwest Ethiopia.

1.9 Rationale for the study

About 75 to 80% of the world population use herbal medicines, mainly in developing countries, for treatment of different diseases because of their better acceptability with human body and lesser side effects (Tabassum and Ahmad, 2011).One of which is

hypertension, in most cases, a chronic condition that almost always must be daily treated with anti-hypertensive drugs(Mancia *et al*., 2007).

Despite the available antihypertensive agents, which successfully reduce BP in many hypertensive subjects, numerous patients remain unresponsive to available pharmacological interventions and are left with severely high BP (Kloet *et al*., 2013).For example, hypertension is not adequately managed in approximately 30% of patients who are compliant with prescriptive therapeutics, suggesting that new agents and/or strategies to manage hypertension are still needed (Taylor and Rahman, 2009; Kloet *et al*., 2013).The number of synthetic antihypertensive agents available in clinical practice are not also effective in all cases in addition to the worldwide severity of the diseases itself (Lonn, 2004). Moreover, these agents are characterized by high cost, lack of definitive curative regimen, combinational therapy, which discourages drug adherence, many side effects and drug-drug interaction (Kagathara *et al*., 2009). That is why most of patients and even medical professionals prefer herbal medication and preventive strategies in hypertension management (Kagathara *et al*., 2009).

In the last three decades, many concerted efforts have been channeled into researching local plants with antihypertensive effect, of which some of these medicinal plants have been validated and others disproved (Tabassum and Ahmad, 2011).Therefore, those herbs, including the current experimental plant, can be source of drugs with fewer side effects and better bioavailability in the treatment of hypertension (Soncinia *et al.*, 2011).

The plant *Calpurnia aurea* ssp. *aurea* has been used to treat hypertension traditionally in some parts of Shinasha, Agew-awi and Amhara peoples in northwest Ethiopia (Giday *et al.*, 2007). Though the seed is used traditionally for the treatment of hypertension, no scientific evidence has so far been presented justifying its use. Therefore, the rationale forthis study is to assess the possible antihypertensive activity of seed extract of *Calpurnia aurea* using animal models. This would help to impart adequate and scientific justification for the traditional use of *Calpurnia aurea* as well as it can be the source of lead compounds in the treatment of hypertension.

2. Objective

2.1 General objective

To assess the antihypertensive activity of the seed extract of *Calpurnia aurea* ssp. *aurea* (Ait.) Benth. (Fabaceae) in acute hypertension induced by a 2-kidney-1-clip rat model.

2.2 Specific objectives

- To study the effect of the extract on animal model of hypertension.
- To assess the hypotensive effect of the seed extract on normotensive rats.
- To evaluate the vasorelaxant effect of the extract on isolated aorta *ex vivo*.
- To do preliminary assessment of the possible mechanism of action of the plant extract by selective receptor agonist and antagonist.

3. Materials and Methods

3.1 Materials

3.1.1 Drugs and chemicals

The following chemicals were obtained from the sources specified: distilled water, normal saline (EPHARM, Ethiopia), methanol (Carlo-Erba, France), acetylcholine atropine, indomethacin, glibenclamide, diphenhydramine and methylene blue (Sigma Chemical Company, USA). The chemicals used to make physiological salt solutions (Kreb-Henseleit solution) include: sodium chloride, potassium chloride, sodium bicarbonate, magnesiumsulphate, calcium chloride, potassium dihydrogen phosphate, glucose, ethylenediaminetetra-acetic acid (EDTA) (BDH Laboratory Supplies, England).

3.1.2 Plant collection

The seeds of *Calpurnia aurea* ssp. *aurea* (Ait.)Benth. (Fabaceae) were collected in the month of February 2013 from Addis Alem, which is found in west of Shewa zone of the Oromia region, about 46 Km west of Addis Ababa, Ethiopia. The fruit pods were wrapped with plastic sheets during transportation to avoid any possible damage to the seeds. Identification and authentication of the plant was done by a taxonomist (Melaku Wendafrash) and a voucher specimen (YG001) was deposited at the National Herbarium, Department of Biology, Addis Ababa University for future reference.

3.1.3 Experimental animals

Sprague-Dawley rats (250–300g, 8–10 weeks of age) and guinea-pigs (450–500 g) of either sex were bred at the animal house of School of Pharmacy, Addis Ababa University, as well purchased from animal unit of Ethiopian Health and Nutrition Research Institute, Addis Ababa, Ethiopia. They were provided with food and water *ad libtum* under a controlled environment (12 h light–dark cycle and temperature of 23–25°C). Before the experiment, the animals were acclimatized for a week and deprived of food for 12h

before the actual experiment but provided with water *ad libitum*. The care and handling of animals was performed following international guidelines (Garber *et al*., 2011).

3.2 Methods

3.2.1Plant extraction

The plant material was thoroughly washed with distilled water to remove dirt and soil. It was then dried at room temperature under shade and powdered. Two hundred grams of the powdered plant material was extracted by maceration at room temperature with a sufficient amount of 80% methanol. After about 72 h, it was filtered with Whatman no 1 filter paper. After filtration, the residue was re-macerated two times, each for 72 h and the filtrate obtained from the successive maceration was concentrated using a rota vapor (Buchilabortechnik AG, Switzerland) at 40^{o}C. The concentrate was then freeze dried in a lyophilizer (Gperon, Korea).The resulting extract was then transferred into a vial and kept in a desiccator layered with $CaCl_2$until further use.

3.2.2 BP measurement in anaesthetized normotensive rats

In this experiment, twenty-eight Sprague-Dawley rats were used and arterial BP was recorded according to the method described elsewhere (Gilani *et al*., 1994, 2005; Ghayur *et al*., 2005). First the animals were anaesthetized with an intraperitoneal (i.p.) injection of pentobarbital (60 mg/kg). The rats were then fixed on a supine position on a dissecting table. The absence of somatic motor reflexes in response to tail pinching or blinking in response to a low-pressure corneal stimulation was assumed to be indicative of deep anesthesia and analgesia.

A longitudinal midtracheal incision approximately 2 cm long was made in order to expose the trachea, the right jugular vein and left carotid artery. The trachea was exposed and cannulated to facilitate spontaneous respiration (Bioscience ventilator, UK, SN-1062). The arterial BP was recorded from the left carotid artery filled with heparinized saline *via* an arterial cannula (Portex cannulae, external diameter 1.02 mm, internal diameter 0.75 mm) connected to a pressure transducer. The pressure transducer was

calibrated by using "Sphygmomanometer" to standardize the pen deflection on chart paper for its equivalent pressure (1cm rise of normal saline in "Sphygmomanometer" equivalent to 1mmHg deflection). The right external jugular vein was cannulated with similar tubing to facilitate intravenous injections of the drugs and plant material. The exposed surface for the cannulation was covered with cotton wool moistened in warm saline. The rats were injected with heparinized saline (0.1 ml) to prevent blood clotting.

The temperature of the animal was maintained at 37^0C by the use of a heated table and overhead lamp. The animal was then allowed to equilibrate for at least30 min before administration of any drug or extract during which the pressure transducer was connected to BBC recorder (SN 9089935, Austria) to measure systolic and diastolic BP. Pulse pressure was then obtained by subtracting diastolic pressure from systolic pressure and MABP was also determined from the sum of DBP plus one-third of pulse width. The control value was the BP obtained before the administration of the test extract as the design was a cross over design, *i.e* .each animal served as its own control. At the end of experiments, animals were killed with an overdose of pentobarbital (over 60 mg/kg, *i.v*.).

3.2.3Induction of experimental hypertension (2K1C rat model)

To evaluate the antihypertensive effect of *Calpurnia aurea*, first acute hypertension was induced as follows. Twenty five normotensive Sprague-Dawley rats were used for induction of acute hypertension according to the method described by Vogel *et al.* (2002).First the animals were anesthetized by i.p. injection of 60 mg/kg pentobarbital sodium. The BP of the rats was measured for 30min before induction of hypertension by cannulating the left carotid artery, as described above.

An incision was then made by scalpel blade 10 mm below the sternum and 10 mm above the genitals. The left renal artery was exposed and polyvinyl chloride coated Dieffenbach clip was placed onto the artery proximal to the aorta to block blood flow. The left renal artery was occluded for 3.5–4h. Three and half hours following surgery, the animals were again anesthetized by *i.p*. injection of 30–40 mg/kg pentobarbital sodium to prevent further awakening of the animal. Then for administration of the test compound, the

external right jugular vein was cannulated by the same method mentioned under BP measurement in normotensive rats. After 3.5–4 h the renal arterial clip was removed (reperfusion). This leads to a rise in BP as a consequence of elevated plasma renin level. Within 15 min a stable hypertension is achieved (Vogel *et al.*, 2002).

3.2.4Dosing of the animal

Pilot experiments were carried out to estimate the doses to be used for the subsequent studies. Rats with normal BP (SBP around 120-125mmHg) were selected and randomly assigned into four groups of six animals each for the corresponding *i.v*. doses of 15, 30, 45 mg/kg and acetylcholine (ACh) (10^{-5}M) in normotensive study. The same grouping was also done for antihypertensive study by selecting rats with SBP>140.0 mmHg except no grouping for Ach this time. Each animal had served as its own control and the effects of treatment were compared with the pretreatment values of BP of the respective group. The extract was suspended in normal saline and diluted to be administered by *i.v.* in a maximal volume of 1ml/kg.

3.2.5 *Ex vivo* vasorelaxant activity

The *ex vivo* experiment was conducted on guinea-pig aortas according to the method described by Ghosh (1984),Gilani *et al*. (1994) and Ghayur *et al*. (2005).Twelve guinea-pigs of either sex were killed by stunning on the head. The descending thoracic aorta was then immediately removed and placed in Krebs-Henseleit solution. Excess fat and connective tissues were trimmed off and then cut spirally to make a strip of about 3 mm wide and 4 cm long. The tissue was kept moistened with Krebs-Henseleit solution (pH of 7.4)during the whole procedure and finally it was mounted in an organ bath containing 2.5 mL of the solution(composition in mM: 118.2 NaCl, 4.7 KCl, 1.2 $MgSO_4$,2.5 $CaCl_2.2H_2O$, 1.3 KH_2PO_4, 25 $NaHCO_3$ and 11.7glucose) at 37°C and aerated with oxygen.

A resting tension of 1g was applied to the tissue and an equilibrium period of 60-70min was allowed before addition of any drug or the test extract, during which period it was washed every 15 min. Effect of extract was first determined on the resting baseline of the

tissue to see if it had any vasoconstrictor effect. After stabilization, the aorta was contracted by addition of KCl (bath concentration of 80 mM). Once a contraction plateau was achieved, increasing concentrations of the extract dissolved in distilled water were cumulatively added and tension changes of the tissue were recorded. The effect of the extract on resting tension was tested with isometric sensors and traced using a recorder (Servogor 124 recorder, SN-CUO62631586, Austria)

3.2.6Evaluation of possible mechanism of vasorelaxation

The possible mechanism(s) of vasorelaxation produced by the hydroalcohol seed extract of *Calpurnia aurea* was partly studied using different agonists and antagonists. To check the involvement of cholinergic and prostanoid (PGI_2) effects, the tissue was preincubated with 0.1μm of atropine (antimuscarinic) and 10μm indomethacin (nonselectiveinhibitor of cyclooxygenases), respectively, for 15 min before adding the test substance. The possible role ofNO/cyclic guanidinemonphosphate (cGMP) pathway was also studied by preincubating the aorta with 1μm methylene blue (cGMP pathway blocker). Any involvement of ATP dependent K^+channel and histamine were studied by preincubatingthe tissue with respective inhibitors, glibenclamide (10μm) and diphenhydramine (10 μm) (Ayele *et al*., 2010).

To investigate the role of the endothelium, the procedure was carried out in endothelium denuded aorta (Fujimoto *et al.*, 1992: Khan and Gilani, 2008). The endothelium lining of the aortic rings was removed mechanically by gently rubbing the intimal surface of the aortal strip with blunted forceps for approximately 30sec. Denudation of endothelium was confirmed by the absence of baseline relaxation to vasodilator ACh, 0.1-0.3 μM. The extract was then tested for its ability to relax the contractions induced with high K^+ (80 mM).

Another series of experiments were performed in order to determine the inhibitory effect of *Calpurnia aurea* on the extracellularCa^{2+}- entry-induced responses (Chulia *et al*., 1996; Khan *et al*., 2012).To confirm calcium channel blocking (CCB) activity, concentration-response curves(CRCs) of Ca^{2+} were constructed. For this purpose, the tissue was stabilized in normal Kreb's solution and then placed in Ca^{2+}free Kreb's

solution, containing EDTA (0.1 mM) for 30 min to remove Ca^{2+} from the tissues. This solution was further replaced with K^+ rich and Ca^{2+}free Kreb's solution, having the following composition (mM): KCl 50, NaCl 50.58, $MgSO_4$3.1, $NaHCO_3$23.8, KH_2PO4 1.26, glucose 11.1 and EDTA 0.1.

Following an incubation period of 1 h, control CRCs of Ca^{2+} were obtained. When the control CRCs of Ca^{2+}were found, the tissue then pre-treated with the extract for 50–60 min for the possible CCB effect. Finally, the Ca^{2+}CRCs were reconstructed in presence of the highest concentration of the test material. In all experiments, the endothelium was removed by gently rubbing the luminal surface.

3.3Statistical analyses

All values in the text are expressed as mean ± standard error of mean (SEM). Test of significance was performed by the use of Student's t test for paired data and by one-way analysis of variance (ANOVA)followed by Dunnett's multiple comparison tests and all the graphing were performed using Graph Pad Prism software version 6.00 for Windows (Graph Pad Software Inc, San Diego, California, USA).*P*-values of less than 0.05 were considered statistically significant. With regard to the *ex vivo* studies, the values are expressed as the percentage contraction, taking the control high K^+ induced contraction before the application of the test extract as 100%.

4. Results

4.1 Percentage yield of extract

A yellowish brown, highly hygroscopic and shiny powdered solid was obtained from 80% methanolic seed extract of *Calpurnia aurea* ssp. *aurea*. The percentage weight yield was calculated to be 18.5%.

4.2 *In vivo* studies

4.2.1 Effect on normotensive rats

A tracing of the effect of the extract on BP of normotensive rats is depicted in Fig 2. When the hydro-alcoholic seed extract of *Calpurnia aurea* was tested for its effect on BP in anaesthetized normotensive rats, it caused a transient, dose-dependent fall in systolic, diastolic, pulse pressure and mean arterial BP (Fig 2, Table 2).

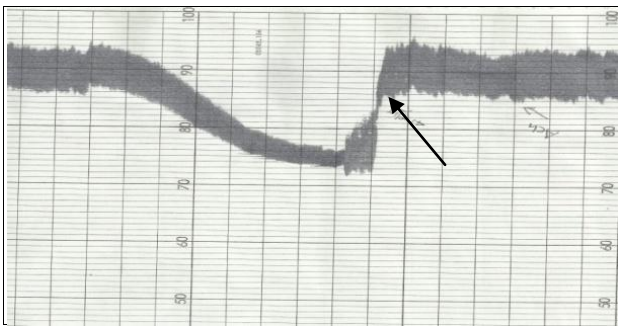

Figure 2: Typical tracing showing the effect of *Calpurnia aurea* (45mg/kg) on blood pressure of normotensive rats: (Arrow indicate the point where the extract was administered)

The seed extract of *Calpurnia aurea* caused a significant reduction in SBP by 17.8% ($p< 0.05$), 27.7% ($p< 0.01$), 37.8% ($p <0.001$) with 15 mg/kg, 30 mg/kg and 45 mg/kg doses of the extract, respectively, compared with the control value. With regard to DBP, 45 mg/kg produced the highest percentage reduction (45%, $p<0.001$) followed by 30 mg/kg (33.1%, $p<0.01$) and 15 mg/kg (22.3%, $p<0.05$). This high percentage reduction in DBP provide an evidence for the likelihood that the extract at all doses had more pronounced

effect on the diastolic than the systolic component of BP. After the hypotensive peak, SBP and DBP increased progressively and reached the initial basal value in about 10–30 min, depending on the dose. For example, 45 mg/kg produced longer hypotensive effect, about 28 min (Fig 2), as compared to 30 mg/kg and 15 mg/kg, which took about 20 and 12 min to reach to the baseline measurement, respectively. An immediate (less than 4 sec) BP reduction was observed after the administration of the extract at all doses in addition to its long duration of action.

Table 2: The effects of intravenous infusion of hydro-alcoholic seed extract of *Calpurnia aurea* on blood pressure in anaesthetized normotensive rats.

Blood pressure	Group 1		Group 2		Group 3	
	Control	15mg/Kg	Control	30mg/Kg	Control	45mg/Kg
SBP (mm Hg)	124.0±0.34	101.9±0.74*	123.2±0.71	89.1±0.73**	121.9±0.42	75.9±0.60***
DBP (mm Hg)	84.1±0.29	65.3±1.43*	83.0±0.39	55.5±0.94**	83.4±0.36	45.9±0.84***
PP (mm Hg)	39.9±0.37	36.6±1.33*	40.1±0.63	33.6±1.51**	38.6±0.62	30.0±1.05***

Results are expressed as mean ± SEM. *p< 0.05,**p < 0.01, ***p< 0.001. SBP = systolic blood pressure, DBP = diastolic blood pressure, PP = pulse pressure. Control = the BP obtained before administration of the test extract. n = 18.

The effect of 15 mg/kg on PP was once again lower than the other doses but was still significantly decreased (9.2%, p<0.05) compared to the control value. The middle (30 mg/kg) and the highest (45 mg/kg) doses in the present study produced significant decline in PP i.e., 16.2% (p <0.01), 22.2%(p <0.001), respectively, compared to control value. The data also showed that the percentage decline in PP obtained with the first two doses was not that large but the third dose produced a higher percentage reduction analogous to SBP and DBP.

In the same manner with SBP, DBP and PP; the extract of *Calpurnia aurea* caused a dose-dependent decrease in MABP compared with controls (Fig 3).Maximum reduction was noted with 45 mg/kg (42%, p<0.001), intermediate (31%, p<0.01) with 30 mg/kg and minimum (20.4%, p<0.05) with 15 mg/kg. All in all, 45mg/kg produced the highest

percent reduction in all parameters measured followed by 30 mg/kg, and 15 mg/kg consistently resulted in a relatively lower BP reduction.

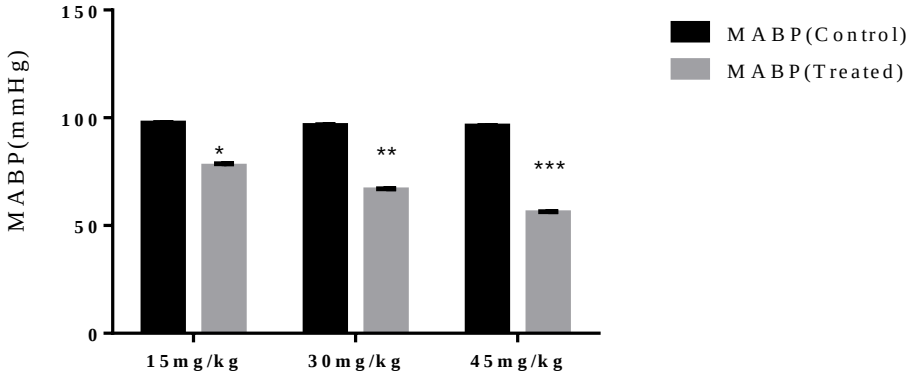

Figure 3:A bar chart representing effect of *Calpurnia aurea* on mean arterial blood pressure in anesthetized rats. (n=18, data presented as mean ± SEM. * $p< 0.05$ ** $p < 0.01$ *** $p < 0.001$ vs. control).

4.2.2 Comparative study with acetylcholine

The effect of *Calpurnia aurea* extract that produced the highest effect on MABP (45mg/kg) was compared with that of ACh (10^{-5}M) in a separate sets of experiment. The tracing obtained following administration of ACh in normotensive rats is shown in Fig 4.

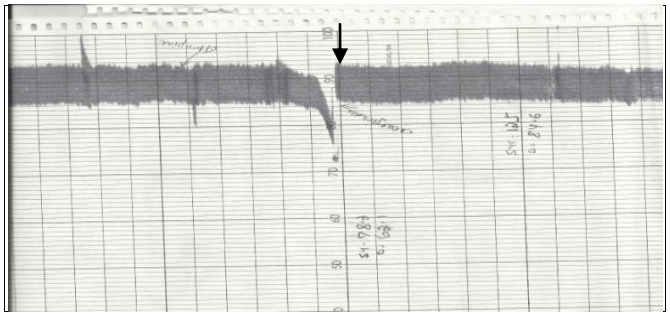

Figure 4: Effect of ACh (10^{-5}M) on blood pressure of normotensive rats. (Arrow indicate the point where the extract was administered)

The result showed that both drugs produced a highly significant decrease in MABP (p <0.001) when compared with their controls. However, no apparent difference was observed between the extract (41.7±0.31) and ACh (40.9±0.27)(Fig 5). The result also revealed that the duration of action for hypotensive activity of *Calpurnia aurea* was longer, about 30 min for45mg/kg, as compared to the 1 min action of ACh.

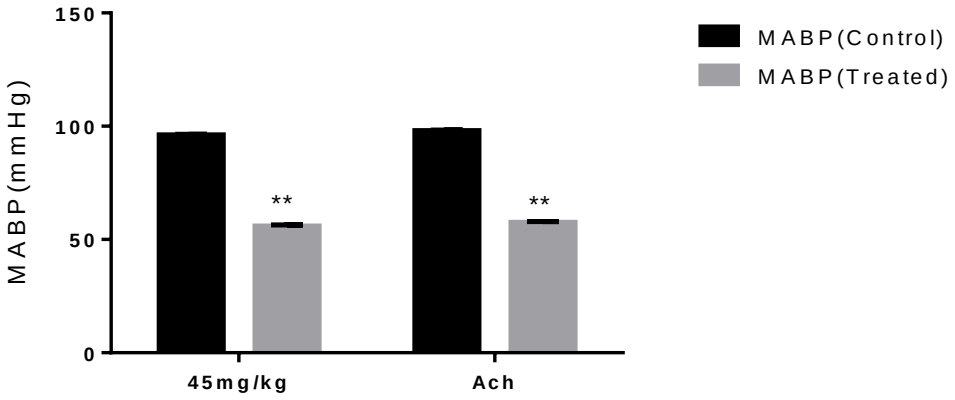

Figure 5: Comparative study between the effect of *Calpurnia aurea* (45 mg/kg) and ACh (10^{-5}M) on mean arterial blood pressure. (n= 6, mean± SEM, **p<0.001)

4.2.3 Induction of acute hypertension

A total of 25 animals were used for induction of hypertension. Out of all, about 72% of the animals had SBP of more than 140.0 mmHg during this period. Thus, those hypertensive animals were randomly assigned into three groups for the corresponding doses to evaluate effects of the extract on hypertension.

Table 3: Blood pressure changes after induction of acute hypertension by 2K1C paradigm in rats.

Blood pressure	Group 1		Group 2		Group 3	
	control	hypertensive	control	hypertensive	control	hypertensive
SBP (mm Hg)	124.1±0.34	143.7±0.95* (15.8)	124.2±0.71	142.6±0.85* (14.9)	123.9±0.32	142.9±1.24* (15.4)
DBP (mm Hg)	85.1±0.29	110.6±0.71* (29.9)	85.0±0.39	109.9±0.52* (29.3)	83.4±0.36	109.9±0.41* (31.7)
PP (mm Hg)	39.0±0.57	33.1±0.68	39.2±0.63	32.6±0.79	40.6±0.62	33.0±1.18*
MAP (mm Hg)	98.1±0.36	121.6±0.72* (23.9)	98.1±0.33	120.8±0.53* (23.1)	96.9±0.22	120.9±0.56* (24.7)

*p<0.05 (n = 18).Values in parenthesis denote the percentage increase in each parameter as compared to the control.SBP = systolic blood pressure, DBP = diastolic blood pressure, PP = pulse pressure. Control = the BP obtained before induction of hypertension.

SBP, DBP and MABP increased ($p<0.05$) significantly in renal hypertensive rats after surgical manipulation as shown in Table 3. The results revealed that the average increase following 3.5-4 h occlusion was 19 mmHg, 25.6 mmHg, and 23.4 mmHg for SBP, DBP and MABP, respectively. By contrast, PP failed to show notable increment after the surgery. The rat achieved a stable BP with in the first 15 min following the removal of the clip (Fig 6).

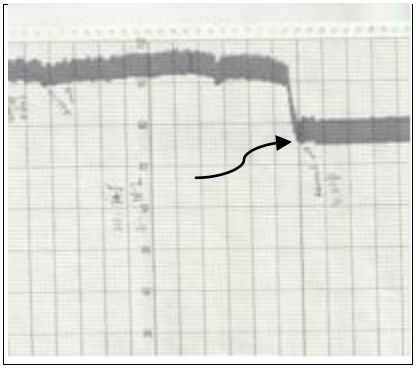

Figure 6: Tracing showing the progressive rise in blood pressure following clip removal. (Arrow indicates the time for clip removal).

4.2.4 Effect on hypertensive rats

Effects of the hydro-alcoholic extract of *Calpurnia aurea* on BP of 2K1C induced hypertensive rats are shown in Fig 7 and Table4. As it can be seen from the results, MABP, SBP and DBP reduced dose-dependently and significantly in renal hypertensive rats following the administration of the extract at different doses.

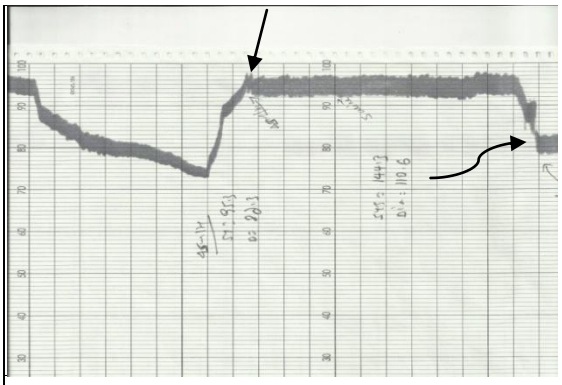

Figure 7: Tracing showing the effect of *Calpurnia aurea* (45mg/kg) on blood pressure of hypertensive rats. (Straight arrows indicate the point where the extract was administered, zigzag arrow shows the time for clip removal).

Acute *i.v.* administration of the seed extract of *Calpurnia aurea* at a dose of 15 mg/kg produced 15.4% ($p< 0.05$) reduction in SBP compared to the control value and this effect lasted for 12 min before reaching to the basal level. This is similar to the result obtained from hypotensive study in normotensive rats, as 15 mg/kg caused less percent reduction as well as 12 min of action in both cases. The systolic effect of 45 mg/kg remained for a longer period of time, about 32 min as shown in Fig 7,and produced a corresponding significant decrease (33.2%, $p <0.01$). Though 45 mg/kg produced the highest reduction in normotensive and hypertensive animals, its effect on the normotensive one took relatively shorter time, about 28 min. The middle dose (30 mg/kg) produced an intermediate effect, a fall by 26.9% ($p< 0.01$) in SBP, compared with the control value with duration of action of 24 min.

Likewise, the DBP effect of the extract in hypertensive rats was relatively lower than that of normotensive animals. The largest dose (45 mg/kg) produced a 36.1% ($p <0.01$) fall in hypertensive rats, while 15 mg/kg and30 mg/kg produced a 15.1% ($p< 0.05$) and 30.2% ($p< 0.01$) reduction, respectively. Overall, the result revealed that the extract has more pronounced effect on the DBP than SBP as it causes higher percentage reduction in DBP

especially at the highest doses. This result is consistent with what was found in hypotensive studies.

Table 4: The effects of intravenous infusion of *Calpurnia aurea* hydro-alcoholic extract on blood pressure of 2K1C induced hypertensive rat at different doses.

Blood pressure	Group 1		Group 2		Group 3	
	Control	15mg/Kg	Control	30mg/Kg	Control	45mg/Kg
SBP (mm Hg)	143.7±0.95	121.6±0.52*	142.6±0.85	104.1±0.58**	142.9±1.24	95.4±0.43**
DBP (mm Hg)	110.6±0.71	93.9±0.37*	109.9±0.52	76.8±0.66**	109.9±0.41	70.4±0.27**
MAP (mm Hg)	121.6±0.72	103.1±0.41*	120.8±0.53	85.9±0.53**	120.9±0.56	78.7±0.29**

Data are presented as mean ± SEM. (n=18), *p<0.05 **p<0.01 vs. control. SBP = systolic blood pressure, DBP = diastolic blood pressure, MABP= mean arterial blood pressure.

Regarding MABP, the result showed that the extract of *Calpurnia aurea* produced proportionally less percent reduction at 15 mg/kg, 15.2% (p< 0.05). 30 mg/kg and 45mg/kg produced larger percentage reduction, 28.8% (p <0.01) and 34.9% (p <0.01) in hypertensive animals respectively. In general, the result from normotensive and hypertensive study on SBP, DBP and MABP showed that the pressure response to *Calpurnia aurea* is more or less comparable in both normotensive and hypertensive animals, characterizing the antihypertensive activity of *Calpurnia aurea***.**

4.3 *Ex vivo* studies

4.3.1 Vasodepressor activity

The *ex vivo* vasodepressor effect of the hydro-alcoholic seeds extract of *Calpurnia aurea* was carried out on isolated descending thoracic aorta of guinea pigs. When the test was made on resting baseline contraction of guinea pig aorta, the extract did not exhibit any vasoconstrictor activity. The extract was then tested on high-K^+ (80 mM) induced contraction and the results are shown in Table 5 and in the typical tracing of Fig 8. The test substance exhibited a concentration-dependent vasorelaxant activity, with maximum activity obtained by the highest concentration used in the study (250 mg/ml).

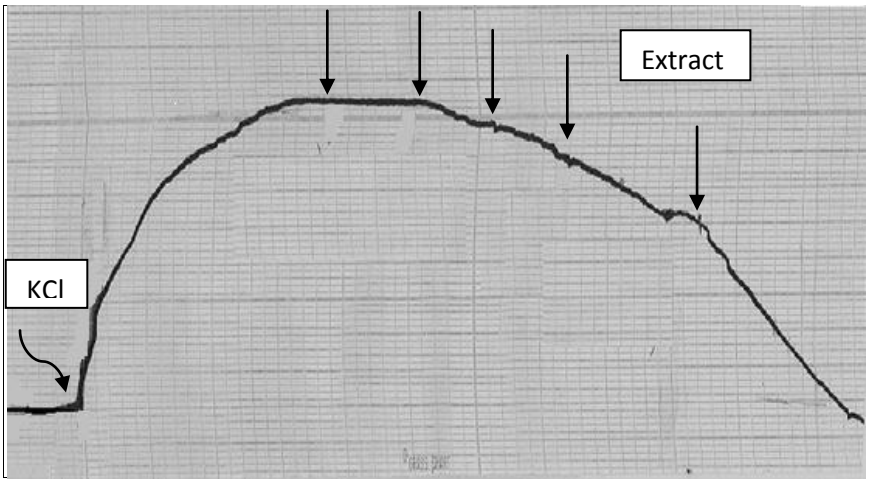

Figure 8: Typical tracing showing relaxant effect of *Calpurnia aurea* on high K+ (80 mM)-induced contraction in isolated aorta of guinea pig.

The test extract showed a statistically insignificant percent inhibitory effect on high-K^+ (80mM)induced contraction of guinea pig aorta at the smallest concentration used. However, an immediate, persistent, dose dependent and significant relaxation was observed beyond the second concentration (50mg/ml) (Table 5). The inhibition of contraction was observed within few seconds (less than 5sec) after the application of each concentration of the test extract. This indicates that the vasorelaxant effect was mainly dose dependent with little or no time dependence. The relaxant effect of the test extract was reversible as the tissue regained its spontaneous activity at least within 1-1.5 h after repeated washout.

Table 5: Vasorelaxant effect of *Calpurnia aurea* extract on guinea-pig thoracic aorta precontracted with 80 mM KCl.

Concentration (mg/mL)	% Contraction caused by KCl	% Relaxation by the extract in KCl precontracted aorta
0.00	100 ± 0.0	0.00 ± 0.00
5.00	98.8±0.05	1.2±0.05
50.00	84.2±0.37*****	16.0±0.31*****
100.00	62.4±0.41******	37.6±0.41******
150.00	31.0±0.26******	69.0±0.26******
200.00	15.5±0.41*******	84.5±0.41*******
250.00	7.9±0.71*******	92.1±0.72*******

*$p < 0.05$,**$p < 0.01$, ***$p < 0.001$($n = 10$. Results are expressed as mean ± SEM)

4.3.2 Possible mechanism of vasorelaxation

To determine how the extract lowers BP, the respective receptor agonist and antagonist were used. The result indicated that the relaxant effect of the test substance was not affected following incubation with atropine, indomethacin, methylene blue, glibenclamide or diphenhydramine. Likewise, removal of the endotheliumfrom the aortic strip did not affect the vasorelaxant effect of the extract. The effect of *Calpurnia aurea* on Ca^{2+} dose-response curve constructed in a Ca^{2+}free medium on guinea pig aortic rings is presented in Fig 9. In Ca^{2+}-free high-K^{+}(50 mM) medium, addition of increasing doses of $Ca^{2+}$to the bath induced a gradual increase in tension.

Sixty minute pretreatment of denuded aorta with the highest dose (250mg/ml)of extract shifted the Ca^{2+} dose response curve ($p< 0.05$,$p< 0.01$ and $p< 0.001$)to the right with suppression of the maximum effect (Fig 9). In the Ca^{2+}-induced contraction of the aortic rings, the extract reduced the maximum contraction by 37.4% from the control value ($p <0.001$).

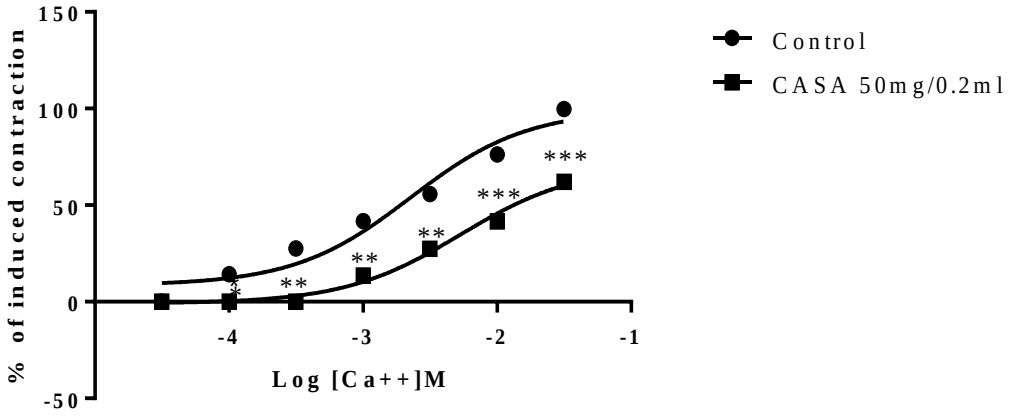

Figure 9: Concentration-response curve of Ca^{++} in the absence and presence of the highest concentration of crude extract of *Calpurnia aurea* (CASA) in isolated rat aortic ring preparations. (n = 4. *p < 0.05, **p < 0.01 and ***p < 0.001 compared to respective concentration values in the Ca^{++} control curve)

5. Discussion

Giday *et al.* (2007) mentioned the traditional use of *Calpurnia aurea* seed to treat hypertension in some parts of Shinasha, Agew-awi and Amhara peoples in northwest Ethiopia. However, there is no mentioning of the clear-cut method on how it is going to be prepared. For this reason, the seeds were extracted by a hydro-alcoholic (80% methanol)solvent, as this solvent has the ability to increase cell wall permeability, facilitating the efficient extraction of large amounts of polar and medium to low-polarity constituents (Seidel, 2006). To ensure exhaustive extraction, the seed powder was macerated for a total of 9 days and relatively larger percentage yield was obtained from the seed as compared to other parts of the plant such as the leaves (Adedapo *et al.*, 2008; Umer *et al.*, 2013)

Two purposes could be cited for the need to conduct hypotensive study on normotensive animals. One, to rule out whether the extract does have an intrinsic BP lowering effect or not so that it can be further evaluated for possible antihypertensive effect in hypertensive rats. The other reason is that to determine to what extent it can lower BP, which may give an idea about the severity of the possible hypotensive side effect, if any, that might be

observed in hypertensive study. In the present study, different doses (15 mg/kg, 30 mg/kg and 45 mg/kg) of the hydro-alcoholic extract of *Calpurnia aurea* seed were tested and found to lower SBP, DBP, PP and MABP of normotensive rat in a dose-dependent manner. The extract showed a statistically significant reduction in all the BP parameters. However, it exerted greater BP lowering effect on the DBP than on SBP. The highest dose of 45 mg/kg caused 45% fall in diastolic pressure, compared to37.8% fall in systolic pressure by the same dose. Such strong effect of the extract on DBP than SBP may occur due to the fact that the extract had strong relaxant effect at the venous bed, so that less blood would return to the heart, affecting the venous return, indicating that the test substance preferentially lower preload than after load.

It is interesting to note that this kind of BP lowering effect has been observed with isoprenaline, a β-receptor agonist with a potent vasodilating property, marked increase in CO and a greater fall in DBP than SBP (Amaechina and Omogbai, 2007). However, isoprenalin, unlike that of *Calpurnia aurea*, do not have antihypertensive effect. In fact, it has a positive chronotropic and inotropic effect when it is given to hypertensive patients, leading to a rise in CO and SBP (White *et al.,* 1998). Antihypertensive drugs such as nifedipine, a CCB with vasodilatory activity, also show this preferential DBP lowering effect (Lopez *et al.*, 1988).Yet, CCBs including nifedipine have little or no effect on the venous smooth muscle so they do not affect venous return unlike the extract.

The effect of the extract on SBP could not also be ignored as the highest dose produced 37.8% reduction and this result may be attributed to the relaxant effect of the extract on the arterioles thereby lowering PVR. Although the extract was not tested in isolated heart, it is not possible to totally exclude a cardiodepressant effect, since certain conventional vasodilators such as hydralazine and minoxidil also shared the same effect (Zwieten, 2001; Ayele *et al*., 2010). Therefore, the extract may exert relaxant effect both in venous and arteriolar bed as this effect is observed by other antihypertensive drugs such as α_1 blockers (doxazosin), though the mechanism for relaxation is different with the extract(Lavie *et al.*, 2009). Such hypotensive effect of the extract is in agreement with the findings of other studies done on different herbs such as “Gebto

Arekei"(*Lupinus Albus)*(Ambaye*et al.*, 2002), *Phyllanthus amarus* (Amaechina and Omogbai, 2007), avocado (*Persea americana*)(Ojewole *et al*., 2007), ginger (*Zingiber officinale*)(Ghayur*et al*., 2005), etc.

The hypotensive data also showed that the percentage decline in PP obtained with the first two doses, 15 (9.2%) and 30 mg/kg (16.2%), was not that large but the third dose produced a higher percentage reduction (22.2%). This may be ascribed to the greater SBP and DBP lowering effect of the highest dose,45 mg/kg, as PP is the difference between these two BP. Indeed, this further supports the fact that the hypotensive effect of the extract increases as the dose increase.

The *in vivo* study also indicate that the hypotensive effect was mainly dose-dependent with little or no time dependence as immediate BP reduction was observed (less than 4sec) after administration of each of the three doses. This may indicate the fast onset of action of the test substance after *i.v.* administration, as this notion is further reinforced by the *ex vivo* studies. Such kinds of results are also mentioned by many other studies (Gilani *et al*., 2005; Soncinia *et al*., 2011; Souzaa *et al.*, 2011). However, certain herbs such as "Gebto Arekei" (*Lupinus Albus*), unlike that of *Calpurnia aurea*, characterized by two phasic hypotensive effect, an acute phase which lasts only for a minute and a second prolonged phase, which remains for longer time (Ambaye *et al.*, 2002).

With regard to the duration of action, the result revealed that the test substance showed a relatively long lasting hypotensive effect, since sustained BP reduction was observed for atleast10 min with the lowest dose, 20 min with the middle dose but longer effect with the highest dose, about 28 min. One possibility for this could be that the active compound(s) in the extract might have a long lasting effect at the site of action as partly observed in the *ex vivo* study. A slow rate of elimination of the active principle (s) may be another reason for the sustained action. Third, the extract might have accumulated in the tissues so that it would take a longer time for elimination.

In comparative study with ACh, the reduction in MABP observed by *Calpurnia aurea* extract was as good as ACh i.e., both produced a highly significant decrease ($p<0.001$) compared with their controls. Based on this result, one may suggest that both the extract

and ACh act through the same mechanism for their hypotensive effect (Shamim and Ahmad, 2012). However, this is inconsistent with the *ex vivo* result which had shown that the cholinergic receptors do not involve in the hypotensive effect of the extract rather its calcium antagonism effect was partially responsible for its BP lowering effect. Furthermore, the duration of action for the hypotensive effect of the crude extract was longer; that is, about 10-30 min depending on the dose (Fig2) as compared to the 1 min action of ACh (Fig4). Such shorter duration of action may be the reason as to why ACh is not used as antihypertensive agent unlike that of the extract, which has promising antihypertensive activity. This result is further in line with the long duration of action of the test extract observed in the *ex vivo* experiment where the high K^+ (80mM)-induced contraction inhibition of *Calpurnia aurea* in isolated aortic tissue was removed in about 1-1.5h after repeated washout. Therefore, one may conclude that both ACh and the extract act at different site for their effect on BP.

The antihypertensive claim of the plant was evaluated by inducing acute hypertension in rats using 2K1C paradigm. This model also gives a clue whether the extract is characterized by hypotension as a possible side effect in parallel with its antihypertensive effect. Since the classical experiments of Goldblatt *et al.* (1934), there is clear evidence that the ischemia of the kidneys causes elevation of BP by activation of the RAAS. The principle can be used both for acute and chronic hypertension. In rats, acute renal hypertension is induced by clamping the left renal artery (2K1C model), more than 50%, for 4 h. After reopening of the vessel, accumulated renin is released into circulation. The protease renin catalyzes the first and rate-limiting step in the formation of angiotensin II leading to acute hypertension (Vogel *et al.*, 2002; Badyal *et al*., 2003;Grossman, 2010). The resultant hypertension at this stage is renin-angiotensin dependent as there is no salt and water retention because of the other normal kidney being intact.

The result of acute hypertension induction indicates that the rats displayed significant increment in SBP, DBP and MBP after surgical manipulation as shown in Table 3.However, insignificant increase in PP was observed in the entire three groups. This may be due to proportional rise in both SBP and DBP, since PP is the difference of these two

pressures. A closer look of the result showed that the average percentage increase in DBP was higher than that of SBP, making the MABP to become relatively higher.

SBP, DBP and MAP fell dose-dependently and significantly in renal hypertensive rats after an *i.v.* administration of the extract, suggesting that *Calpurnia aurea* possesses antihypertensive activity, which is in accordance with its medicinal use for hypertension (Giday *et al.*, 2007).The extract lowered both SBP and DBP in the present model; however, DBP appeared to be affected more than SBP, similar to the normotensive study. The highest dose of 45 mg/kg caused 36.1% fall in diastolic pressure, compared to 33.2% fall in systolic pressure by the same dose. Historically, DBP has taken precedence in hypertension management and it is an important index in terms of hypertension with 85 mm Hg defined as borderline, 95 mm Hg as hypertensive, 100 mmHg as severe hypertension in younger patient (Ambaye *et al.*, 2002; Taylor *et al.*, 2011).It is also useful as a CVD risk predictor as an increased DBP is associated with greater risk for heart attacks, strokes and kidney failure (Rosendorff *et al.*, 2007). Hence, the extract by reducing DBP, it can minimize CVD associated with hypertension as well as may be used in the treatment of moderate to sever hypertension and hypertensive emergencies just like nifedipine.

Compared to the hypotensive effect, the extract at all doses produced relatively less percent reduction in all the BP parameters in hypertensive animals though highly significant reduction was recorded especially at higher doses. For example, 45 mg/kg produced 33.2%, 36.1%, 34.9% fall in SBP, DBP and MABP in hypertensive rats respectively but its corresponding effect on normotensive animals showed 37.8%, 45%, 42% reduction at the above respective BP parameters. Such a reduction in BP effect is common with other antihypertensive agents and this may indeed support the fact that combination therapy than monotherapy is imperative in the management of hypertension since the efficacy of a single agent will be compromised as BP rises. However, the result of antihypertensive study also showed that the duration for the BP effect of the test substance increases as the animals become hypertensive. Both 45 mg/kg and 30 mg/kg produced 32 and 24 min of antihypertensive activity, 4 min greater than their respective hypotensive effect in normotensives rats. Yet, 15mg/kg produced a 12 min of BP

reduction in both normotensive and hypertensive rats suggesting that the antihypertensive effect of the test substance increases with further increase in dose just like what was observed in hypotensive study.

Ambaye *et al.* (2002) and Holzgreve (1993) stated that whether oral or *i.v.* antihypertensive therapy is employed, the recommended BP reduction goes up to 30% and the acute reduction of BP should never exceed 50%, as a great reduction may increase the severity of ischemia or may even lead to death. Based on this fact, the test substance reduces BP parameters by less than 30% at the doses of15and 30 mg/kg which is within the 30% recommended BP reduction. However, 45mg/kg resulted in 33.2% and 36.1% fall in SBP and DBP, respectively; greater than 30% but less than the 50% recommendation. These show that still the extract could be employed for management of hypertension without severe hypotension and ischemia.

A systematic review of recent clinical practice guidelines on the diagnosis, assessment and management of hypertension by Al-Ansary *et al*. (2013) indicate that reduction of10 mm Hg in SBP and 5 mm Hg in DBP was associated with a 20% reduction of coronary heart disease and 32% reduction in stroke. These facts provide an evidence for the rational use of the plant as antihypertensive agent because let alone the highest dose, the lowest dose (15mg/kg) produced 22.1 and 16.7 mmHg reduction in SBP and DBP respectively. Furthermore, the extract at the highest dose of45 mg/kg demonstrated antihypertensive activity with the maximum decrease of MABP at 34.9% that was almost 3-fold of prazosin hydrochloride, α_1-blocker used as antihypertensive agent. Moreover, a single dose of *Calpurnia aurea*, as low as 15 mg/kg, did reduce the MABP of rats in a range of∼20 mm Hg. It has also been estimated that, in humans suffering from hypertension, a reduction of 5 mm Hg in MABP can decreases the mortality due to stroke and coronary events by 14 and 9%, respectively (Chobanian *et al*., 2003; Souzaa *et al.*, 2011), reinforcing the importance of the above findings. This further indicates that higher doses, 30 and 45 mg/kg, will have much contribution with regard to reduction of mortality due to hypertension complication as they causes ∼30 and ∼35 mmHg reduction.

The antihypertensive result also showed that none of the doses of the extract used in this experiment produced a reduction of SBP <90 and DBP<60. This may indicate that the extract of *Calpurnia aurea* is not characterized by hypotension as a possible side effect at the doses used in this study. But as the normotensive study indicates, the extract does have an intrinsic hypotensive effect which could be observed at doses greater than what was used in this experiment. Such kind of hypotensive effect has been observed by certain antihypertensive drugs like pure vasodilating agents such as the dihydropyridine class of CCBs (Chen *et al*., 2010).The same result was obtained by Ojewole *et al.* (2007) and Soncinia *et al*. (2011) who reported that the extract *Persea Americana* (avocado) and *Averrhoa carambola* produced antihypertensive effect with possible hypotension as the dose increases, respectively.

The *in vivo* antihypertensive property of crude seed extract of *Calpurnia aurea* is substantiated by *ex vivo* vasorelaxant effect on isolated aorta of guinea pigs contracted with KCl. Chulia *et al*., (1996) and Gilani *et al*., (2005) described that K^+ at high doses (>30 mM) is known to cause smooth muscle contractions through opening of voltage-dependent slow Ca^{2+} channels, thus allowing influx of extracellular Ca^{2+} causing a contractile effect. Guinea pigs aorta was selected to: a) evaluate effect of the extract on K^+-induced contractions b) determine if the vasodilator effect of the test substance is endothelium-independent or-dependent c) investigate the possible mode of action of the test drug including its effect on membrane bound voltage-operated Ca^{2+}channels.

The *ex vivo* study demonstrated that the test extract caused a dose dependent inhibition (relaxation) of K^+-induced contraction in isolated aortic preparation (Table 5and Fig 8), with the least dose being devoid of significant vasodilator activity. The largest concentration, 250 mg/mL, produced a maximum relaxation of 92.1±0.72% ($p < 0.001$) and this confirms that the extract had a prominent relaxant effect on isolated aorta contracted with KCl. The result also showed that the vasorelaxant effect, similar with that of the *in vivo* study, was mainly dose-dependent with little or no time dependence as the extract produced immediate relaxation (less than 5sec) after the application. This may in fact give a clue about the mechanism of action of the extract as mentioned below. Moreover, the tissue regained its spontaneous activity at least within 1-1.5 h after

repeated washout indicating the long and persistent relaxant effect of the extract on aorta. Therefore, it could be suggested that the BP lowering effect of the plant is ascribed to relaxation of the vascular system.

It is customary to use isolated vascular tissue preparations to investigate the possible mode of action, as the interference by the intact reflexes is not a problem (Gilani *et al*., 2005; Ayele *et al*., 2010).As mentioned earlier, isolated guinea-pig aortic preparations were used in order to explore the underlying mechanism of action of *Calpurnia aurea* extract. First, the involvements of muscarinic receptors were excluded since blocking the receptors with atropine did not abolish the vasorelaxant effect. Basically, ACh through cholinergic receptors dilates the blood vessels indirectly by stimulating the release of NO from the endothelium. Once formed, NO diffuses out of the endothelium, entering the underlying vascular smooth muscle where it binds to and activates soluble guanylate cyclase. This enzyme catalyses the conversion of guanidine triphosphate to cyclic guanidinemonphosphate (cGMP), which in turn, causes relaxation of the vascular smooth muscle cells (Gewaltig and Kojda, 2002; Ojewole *et al.*, 2007).Blocking this NO/cGMP pathway with methylene blue also did not affect the relaxant effect of the extract.

The result also indicates that the extract decreased the KCl induced contractions in endothelial denuded aortic rings as it mediate the same relaxation at similar concentration as in intact preparations. Khan *et al*., (2012) mentioned the role of the vascular endothelium in modulating vascular tone through release of mediators like NO and PGI_2 which diffuses to the cells in the vicinity to cause relaxation. So the above findings further strengthen the claim that vasodilator effect of the extract did not involve the cholinergic and NO/cGMP pathways. The roles of ATP dependent K^+ channels and cyclooxygenase, an enzyme responsible for the synthesis of prostacyclin (PGI_2), were excluded as the relaxant effect remained intact. Blocking the H_1 (histamine) receptors, which is known to have a role in receptor-mediated NO release from the endothelium in addition to muscarinic receptors, also did not affect the outcome (Siddiqi *et al.,* 2012).

The present data demonstrated that the extract might, at least in part, exert its BP lowering effect through the blockade of calcium ion channels, as evaluated by *ex vivo* study. An evidence for the likelihood that ion channels could be a target molecule for the

extract comes from the observation that the extract produced an immediate relaxation of the aortic strip, less than 5sec. It is well-known that drugs acting on ion channels often have a fast onset of action (Ayele *et al*., 2010).In fact, smooth muscle relaxation can be achieved by various mechanisms such as potassium channel opening, calcium channel blocking or receptor antagonism (Suresh *et al*., 2006). K^+ channels opening *via* activated ATP-sensitive K^+ channels hyperpolarizes the smooth muscle, which closes voltage-gated calcium channels and decreases intracellular calcium, leading to vasodilation (Al-Hashimi and Thompson, 2012). The involvement of this channel as a mechanism for vasorelaxant activity of the extract was excluded as the standard potassium channel openers such as minoxidil do not inhibit high K^+-induced contraction (Keiichi *et al*., 1997; Suresh *et al*., 2006). This notion further confirmed by the fact that preincubation of the tissue with glibenclamide, an inhibitor of ATP dependent K^+channels, did not affect vasorelaxant effect. Moreover, Wang *et al*., (2001) and Khan *et al*., (2012)depicted the fact that inhibition of K^+ (80mM)-induced contractions would indicate the involvement of L-type voltage-operated calcium channel blocking mode of vasodilatation.

Based on these facts, pre-treatment of tissues with plant extract caused rightward non-parallel shift of Ca^{++}-CRCs, constructed in Ca^{++}-free high K^+ medium with suppression of maximum contractile effect (Fig 9), similar to that produced by standard CCB like verapamil (Khan *et al*., 2012; Siddiqi *et al*., 2012).This shows that the plant extract might have the ability to block influx of extracellular Ca^{++} through voltage-dependent channels resulting vascular smooth muscle relaxation. The relaxation leads to vasodilation, causing a decrease in peripheral resistance and as a result, reduction in BP (Soncinia *et al*., 2011).In a similar study, Ghayur *et al*. (2005) described that the ability of ginger crude extract to relax K^+(80mM)induced contraction would indicate an L-type voltage-dependent calcium channel blocking(CCB) mode of vasodilation. Other herbs such as *Gentiana floribunda* (Khan *et al*., 2012),*Averrhoa carambola* (Soncinia *et al*., 2011), *Viola odorata* (Siddiqi *et al*., 2012) also mentioned to have similar activity with the current plant. Thus, the hypotensive and antihypertensive properties of the hydro-alcoholic seed extract of *Calpurnia aurea* may, at least in part, be mediated through its calcium antagonism.

6. Conclusion

The results obtained from the present investigation suggest that *i.v*. administration of hydro-alcoholic seed extract of *Calpurnia aurea* possessed a promising antihypertensive activity both *in vivo and ex vivo* models. The extract lowers the different BP parameters in normotensive anaesthetized rats suggesting for its intrinsic BP lowering effect as well as the possible hypotensive side effect as the dose increases. Its evaluation on hypertensive rats also showed that the test substance has good antihypertensive activity as it produced greater reduction in both SBP and DBP. It also exhibited a profound depressor effect on diastolic component of BP than the systolic one in both normotensive and hypertensive rats, suggesting that it might mainly affect the venous return in addition to its relaxant effect on the arterioles thereby lowering PVR. In isolated guinea-pig aortic preparations, the crude extract possessed inhibitory effect on high K^+-induced contraction which further supports its vasorelaxant effect observed in the *in vivo* study. The *ex vivo* results also suggest that the hydro-alcoholic seed extract of *Calpurnia aurea* lowers BP by a mechanism that might not involve cholinergic, prostanoids, histaminergic, ATP dependent K^+channels, NO/cGMP pathway or endothelial-dependent pathways. Perhaps, the antihypertensive action is most probably mediated through dilation of blood vessels which involves voltage dependent Ca^{++} channel blockade.

7. Suggestion for future work

Based on the findings of the present work, further investigation on the following directions is recommended:

- As the present study is focused mainly on the antihypertensive effects of *Calpurnia aurea* on experimentally induced acute hypertensive rats, further work should be done in chronic hypertensive animal models.
- Since the drug administration was *i.v.*, in our study, further research is needed to determine the reduction of BP during oral administration.
- Though blockage of influx of extracellular Ca^{++} through voltage-dependent channels was proposed as a mechanism, additional work need to be done on assessing other possible mechanism of action of the extract in its BP lowering effect.
- Detailed phytochemical screening, further fractionation and isolation of active ingredient is required to identify the exact chemical compound (s) responsible for the activities observed from the seeds in the present study.

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