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Evaluation of *In Vivo* Antihypertensive and *Ex Vivo* Vasodepressor Activity of 80% Methanol Leaf Extract of *Otostegia integrifolia* Benth. (Lamiacae) in Rats

By: Abel Degu

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A Thesis Submitted to the Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health Sciences, Addis Ababa University in Partial Fulfillment of the Requirements for the Master of Science Degree in Pharmacology.

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This is to certify that the thesis prepared by Abel Degu, entitled: Evaluation of *In Vivo* Antihypertensive and *Ex Vivo* Vasodepressor Activity of 80% Methanol Leaf Extract of *Otostegia integrifolia* Benth. (Lamiacae) in Rats and submitted in partial fulfilment of the requirements for the degree of Master of Science in 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 80% Methanol leaf extract of *Otostegia integrifolia* Benth (Lamiacae) in Rats**.**

Abel Degu

Addis Ababa University, 2018

Prevalence of hypertension is upsurging in the past three or four decades and it is considered as one of the major causes of morbidity and mortality via predisposing to various cardiovascular and cerebrovascular disorders. On top of that, the change in lifestyle across the globe makes the disease to be looming around on everyone. Thus, the scientific world is looking for a drug having novel mechanism of action with least side effects. To this end, there is no better inception point apart from medicinal plants which have been the major source of drug molecules with unique pharmacologic activity. In this study, the hydro-alcoholic leaf extract of *Otostegia integrifolia* was investigated for its *in vivo* antihypertensive activity using the fructose induced hypertension model and *ex vivo* vasodepressor activity was also assessed on thoracic aortic rings of rats. Preliminary mechanistic studies were also performed using agonists and antagonists as well as by construction of concentration-response curves. The *in vivo* result indicated that the extract at doses of 250, 500, and 1000 mg/kg produced a significant antihypertensive activity ($p < 0.05$) in all BP measurement parameters; systolic blood pressure (SBP, $p < 0.05$), diastolic blood pressure (DBP, $p < 0.01$) and mean arterial blood pressure (MAP, $p< 0.001$) at day 5, 10 and 15 compared to the negative controls. The extract also produced relaxation of aortic rings precontracted with high K^+ (80mM) in a concentration-dependent manner. The relaxation was significant at concentrations ranging from 0.5mg/ml to 2.5mg/ml ($p< 0.001$). Vasorelaxant activity of the extract was not mediated through cholinergic, prostanoid, histamine, ATP-dependent K^+ channel, sarcoplasmic reticulum stored Ca^{2+} and endothelium-dependent pathways. However, the extract shifted the Ca^{2+} concentration-response curve to the right and blocking of the NO/cGMP pathway only yielded 45% relaxation, suggesting that vasorelaxation could possibly be mediated via calcium channel blockade and involvement of the NO/cGMP pathway.

Key words: Hypertension, *in vivo, ex vivo, Otostegia integrifolia*, antihypertensive, vasodepressor activity

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List of Abbreviations/Acronyms

ACC	American College of Cardiology
ACEI	Angiotensin converting enzyme inhibitors
AHA	American Heart Association
Ang-II	Angiotensin II
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
ARBs	Angiotensin II receptor blockers
BP	Blood pressure
CCBs	Calcium channel blockers
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
CRCs	Concentration-response curves
DAG	Diacylglycerol
DALY	Disability-adjusted life years
DBP	Diastolic blood pressure
EDTA	Ethylenediaminetetraacetic acid
EDHF	Endothelium-derived hyperpolarizing factor
EDRF	Endothelium-derived relaxing factor
eNOS	Endothelial NO synthase
ESC	European Society of Cardiology
ET	Endothelin

ETA	Endothelin-A receptor
ETB	Endothelin-B receptor
HF	Heart failure
HTN	Hypertension
IP3	Inositol triphosphate
JNC	Joint national committee
LDL	Low density lipoproteins
MAP	Mean arterial blood pressure
NCD	Non-communicable diseases
NHE	Sodium hydrogen exchanger
NO	Nitric oxide
PAT	Putative anion transporter
PGs	Prostaglandins
RAAS	Renin-angiotensin-aldosterone system
SBP	Systolic blood pressure
SEM	Standard error of a mean
SNP	Single nucleotide polymorphism
SNS	Sympathetic nervous system
WHO	World Health Organization

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1. Introduction

1.1. Overview of hypertension

Perfusion of the various organs, tissues and different body parts through blood is possible, only if it has adequate driving force. In this case, the driving force is blood pressure (BP). BP is created by the force of blood pushing against the walls of blood vessels (arteries) as it is pumped by the heart. This BP sometimes may get elevated due to various reasons, leading to hypertension (HTN). HTN is dubbed as one of the commonest non-communicable disease (NCD) affecting human beings in the 21st century. Though the cutoff point varies on different guidelines, it is simply characterized by a systolic BP (SBP) greater than or equal to 140 mmHg and/or diastolic BP (DBP) greater than or equal to 90 mmHg (Kearney *et al.*, 2004). It remains one of the contributors to cardiovascular disease and death (James *et al.*, 2014).

According to 2014 Evidence-Based Guideline for the Management of High BP in Adults and the Report From the Panel Members Appointed to the Eighth Joint National Committee (JNC 8), if the BP of an individual with SBP $\geq$ 150 mm Hg and/or DBP $\geq$ 90 mm Hg will be deemed hypertensive. It requires also to be treated with the appropriate pharmacotherapy with the goal of lowering the SBP and DBP below 140 and 90 mm Hg respectively for adults age $<$ 60. On the other hand, for adults age above 60 the goal is to lower the SBP and DBP to 150 and 90 mm Hg respectively (James *et al.*, 2014).

According to the American College of Cardiology/American Heart Association (ACC/AHA) Task Force on Clinical Practice as it is described in Table 1, an individual will be deemed hypertensive if the SBP and/or DBP is greater than or equal to 130/80 mmHg. In addition, there is also one additional grouping called elevated or high normal BP, whose range is 120-129 and less than 80 mm Hg for SBP and DBP respectively (Carey and Whelton, 2018;Whelton *et al.*, 2017a).

Apart from this categorization, there are also hypertensive emergency and hypertensive urgency under severe HTN. Though there is no clearly defined BP cutoff point, according to some literature they put forward a cutoff BP range 20% above stage-2 HTN but with regard to hypertensive urgency, it is defined as severe HTN without acute target organ dysfunction (Lurbe *et al.*, 2016;Lai *et al.*, 2017).

Moreover, there are also other hypertensive diseases during pregnancy encompassing chronic HTN, gestational HTN, and pre-eclampsia. Where chronic HTN is defined as hypertension when SBP ≥140mmHg or DBP ≥ 90mmHg at the booking visit or before 20weeks' gestation, or if the patient is already taking antihypertensive medication. However, gestational HTN is defined as hypertension presenting after 20 weeks' gestation without significant proteinuria. On the other hand, pre-eclampsia is defined as hypertension presenting after 20 weeks' gestation with significant proteinuria (Leslie and Collis, 2015;Motha and Jayasundara, 2015)

Table 1: Categories of BP in Adults (ACC/AHA) (Carey and Whelton, 2018)

BP Category	**SBP**		**DBP**
Normal	<120 mm Hg	And	<80 mm Hg
Elevated	120–129 mm Hg	And	<80 mm Hg
Hypertension			
Stage 1	130–139 mm Hg	*Or*	80–89 mm Hg
Stage 2	≥140 mm Hg	*Or*	≥ 90 mm Hg

1.2. Classification of hypertension

1.2.1 Primary hypertension

Primary or essential HTN, unlike the secondary ones, does not have a sole attributable factor linked etiologically to the disorder. It accounts for nearly 90% of the total cases of HTN. Since it is difficult to determine the etiology, the term essential HTN is employed. It is recognized that primary hypertension happens to occur due to multifaceted disorders associated with the interplay of many genetic, environmental, and behavioral factors. Provided the multifaceted nature of BP homeostasis any change in BP as, for example, one due to a mutation, is likely to be compensated by feedback, or some other control mechanisms, in an effort to return BP to normal. It is only when the balance between the factor(s) that tend to increase BP and those that try to normalize it is sufficiently disturbed or when the compensatory mechanisms fail to counteract the perturbation, that results in essential HTN (Bolívar, 2013).

1.2.2. Secondary hypertension

Secondary hypertension is a rare form of HTN and it is defined as an elevated systemic BP where a single or multiple attributable etiologies can be ascribed for its advent. Only around 5-10% of patients suffering from systemic HTN have a secondary form of HTN, whereas the vast majority has essential (idiopathic or primary) HTN. Various conditions such as primary aldosteronism, renal artery stenosis, renal parenchymal disease, pheochromocytoma, Cushing's syndrome, hypothyroidism/hyperthyroidism, coarctation of the aorta, obstructive sleep apnoea and medication such as oral contraceptives, nonsteroidal anti-inflammatory drugs, steroids, sympathomimetic drugs (decongestants, diet pills), and illicit drugs (cocaine, amphetamines…etc.) may result in the occurrence of secondary HTN (Puar *et al.*, 2016;Rimoldi *et al.*, 2013). In this type of HTN removing or managing the underlying condition is crucial for subsequent controlling and resolving the disease (Freihage *et al.*, 2008).

1.3. Epidemiology of hypertension

HTN is a global pandemic according to World Health Organization (WHO) 2013 report, around 40% of adults aged 25 and above had been diagnosed with HTN in 2014 globally (Prince *et al.*, 2015). Moreover, HTN is also considered as one of the most common risk factors for various cardiovascular disorders. Globally, cardiovascular diseases account for approximately 17 million deaths a year, nearly one-third of the total. Of these, complications of HTN account for 9.4 million deaths worldwide every year. HTN is also responsible for at least 45% of deaths due to heart disease, and 51% of deaths due to stroke (Organization, 2013;Ibrahim and Damasceno, 2012).

The prevalence of HTN, as the pattern shows has been steadily increasing for the past 3 decades. The increment is even dramatic that in 1980 peoples leaving with HTN were 600 million but this number further increased in 2008 to 1 billion and the prevalence is expected to rise to 1.56 billion by 2025 (Joffres *et al.*, 2013). Furthermore, by 2025, approximately 75% of all hypertensive patients are expected to be residing in developing countries (Organization, 2013;Mills *et al.*, 2015).

In a worldwide conducted study, though there is some level of uncertainty in some estimates, the prevalence of increased SBP (≥110-115 and ≥140mmHg) up surged markedly between 1990 and 2015, with a proportional increase in disability-adjusted life years (DALY) and mortality

associated with increased SBP. Predictions made based on this sample suggests that in 2015, an estimated 3.5 billion adults had SBP of at least 110 to 115 mm Hg and 874 million adults had SBP of 140mmHg or higher (Forouzanfar *et al.*, 2017;Huffman and Lloyd-Jones, 2017).

The prevalence of HTN in Africa is even higher than the global prevalence. According to WHO 2013 report, 46% of adults aged 25 and above live with the condition, while the lowest prevalence is found in the Americas which stood at 35% (Ogah and Rayner, 2013). The prevalence of HTN in Western Europe is also around 40% which is higher than the Americas but still lower than Africa (Ogah *et al.*, 2012). The notable fact is also that, the pattern of diseases in sub-Saharan Africa is changing in contrast to what is believed in the past, with NCD responsible for about 22% of the total deaths in the region in 2000 (Guwatudde *et al.*, 2015;Adeloye and Basquill, 2014).

Though it is difficult to obtain quality data for subsequent estimation, according to a review conducted in 2008 - 2012 an estimated number of African hypertensive individuals to be 74.7 million. The prevalence of HTN varies widely from country to country. It is projected that the number of affected individuals will increase by 68% by 2025. The possible justifications might be a mass migration of rural Africans to urban areas and rapid changes in lifestyle and risk factors account for the rising prevalence of HTN (Ogah and Rayner, 2013;Gebreselassie and Padyab, 2015).

Regarding Ethiopia like in many African countries finding accurate data source is difficult but still, there are few types of research, where one study estimate prevalence by categorizing the people based on the place of residence into urban and rural, therefore with this method the prevalence of HTN is around 23.7% and 14.7% in urban and rural parts of Ethiopia respectively (Kibret and Mesfin, 2015;Nshisso *et al.*, 2012). On another population-based prevalence study the age-adjusted prevalence of high BP, defined as SBP ≥ 140 mmHg or DBP ≥ 90 mmHg or reported use of anti-hypertensive medication, was 31.5% among males and 28.9% among females (Tesfaye *et al.*, 2009).

1.4. Regulation of normal blood pressure

The principal role of BP is to force sufficient amount of blood supply via the vascular system to the various tissues depending on the metabolic needs of the respective tissues. Subsequently, the normal BP regulation is a complex physiologic function that depends on the integrated actions of

multiple cardiovascular, renal, neural, endocrine, and local tissue control systems. The fundamental association between hemodynamics and regulation of BP is customary. For example, arterial BP, or BP, is positively related to cardiac output (heart rate × stroke volume) and total peripheral vascular resistance; with the latter being strongly influenced by sympathetically-mediated vasoconstriction (Raven and Chapleau, 2014;Chopra *et al.*, 2011).

The normal BP is regulated via interdependent mechanisms, for the sake of clustering the numerous systems, organs and chemical messengers involved in regulation. It is possible to categorize the BP control system into; local mechanisms, global neural mechanisms, and renal-endocrine system (Chopra *et al.*, 2011).

a) Local Mechanisms

The local mechanism mainly involves the endothelium and endothelium-dependent processes such as nitric oxide (NO), endothelin, prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factor (EDHF).

The first agent from the above local chemical messengers is NO. NO is also known as an endothelium-derived relaxing factor (EDRF) is a free radical gas with a very short half-life. It is released from endothelial cells in response to blood flow-induced shear stress and by activation of a number of receptors. Endothelial NO synthase (eNOS) synthesizes NO and L-citrulline from the amino acid L-arginine. In addition to vasodilatation, NO also has anti-proliferative, anti-thrombotic, leukocyte adhesion inhibition effects, and influences myocardial contractility (Luna-Vázquez *et al.*, 2013). The primary hemodynamic effect of pharmacologic NO inhibition includes an increase in systemic and pulmonary arterial BP and a parallel decrease in cardiac output. The vasoconstrictor effect of angiotensin II (Ang-II) is enhanced in the absence of NO. With this local vasodilatory action, NO regulates BP through manipulating the peripheral vascular resistance (Giles *et al.*, 2012).

Vascular endothelial cells produce endothelin 1 (ET)-1, which is one of the most potent vasoconstrictors. ET-1, ET-2, and ET-3 are members of a family of similar polypeptides but each is encoded by different genes. There are two different types of ET receptors which have been cloned, ETA and ETB. Activation of ETB receptor leads to decreased arterial pressure and natriuresis through effects on the adrenal gland, heart (negative inotropy), decreasing sympathetic activity and systemic vasodilatation. Whereas activation of ETA receptor leads to

increased arterial BP via increased sympathetic activity, positive inotropy of the heart, and systemic vasoconstriction (Rautureau and Schiffrin, 2013). There are some stimulators that do have an influence on ET-1 leading to ET-1 secretion. These include Ang-II, catecholamines, growth factors, hypoxia, insulin, oxidized low density lipoprotein (LDL), shear stress, and thrombin. On the other hand, there are also inhibitors of ET-1 secretion including NO, atrial natriuretic peptide (ANP), PGE-2, and prostacyclin. There are reports that show ET levels may be high in hypertensive patients, while there are also other studies which have reported no difference in ET levels in patients with or without HTN. ET receptor antagonists have been investigated for their use as antihypertensive agents (Hall *et al.*, 2012).

The other local chemical messenger is EDHF. The term EDHF was introduced in 1987 to describe the hypothetical factor responsible for myocyte hyperpolarization not associated with nitric oxide (EDRF) or prostacyclin. Endothelial hyperpolarization is likely due to several factors that are site- and species-specific, ultimately causing vascular smooth muscle hyperpolarization and relaxation. Although it is difficult to elucidate the exact mechanism of hyperpolarization, the proposed mechanism of EDHF is through increasing potassium (K+) conductance resulting in the subsequent hyperpolarization of vascular smooth muscle cells and relaxation (Giles *et al.*, 2012). Acetylcholine causes hyperpolarization of vascular smooth muscle in arteries with an intact endothelium but not in its absence. This hyperpolarization is mimicked by certain K+ channel agonists and is unaffected by inhibitors of nitric oxide synthase or cyclooxygenase and has been attributed to release of EDHF/s. EDHFs according to some studies are considered as epoxides or epoxyeicosatrienoic acids generated by endothelial CYP enzymes (Froemel and Fleming, 2015;Edwards *et al.*, 2010). They appear to open smooth muscle cell K+ channels, allowing K+ efflux along its chemical gradient resulting in membrane hyperpolarization. Thus, EDHF activity may be defined functionally as agonist-induced, endothelium-dependent relaxation that is not blocked by inhibitors of NO synthase or cyclooxygenase but can be inhibited, at least in part, by K+ channel blockers (Ozkor and Quyyumi, 2011).

Prostaglandins (PGs) are autacoids and a reactive product of membrane phospholipids produced normally by many cell types, including endothelial cells. The cyclooxygenase products PGI2 and PGE2 relax the vascular smooth muscle. Particularly synthesis of PGI2 is enhanced in the spontaneously hypertensive and Goldblatt hypertensive rat (Colina-Chourio *et al.*, 2000). Generally, in HTN, production of vasoactive prostanoids is selectively impaired and may

contribute to the increased systemic vascular resistance and increased incidence of thrombosis. Animal studies on the aortic rings, from aging Wistar Kyoto rats, have shown that the endothelium-dependent contractions elicited by acetylcholine most likely involve the release of PGI2 with a concomitant contribution of PGH2. Additional studies with rat aortic strips have indicated that PGI2 induces relaxation through a PGI2-PGE1 receptor (Luna-Vázquez *et al.*, 2013).

b) Global neural control

The global neural regulation of BP is fundamentally mediated through the integrated function of arterial baroreceptors and the autonomic nervous system. The arterial baroreceptors clearly provide a powerful means for moment-to-moment regulation of arterial pressure, but their role in long-term BP regulation is controversial (Chopra *et al.*, 2011). According to a study the baroreceptors are relatively unimportant in the chronic regulation of BP because they tend to reset within a few days after a change in BP. To the extent that resetting of baroreceptors occurs, this would attenuate their potency in long-term control of BP (Raven and Chapleau, 2014) .

High-pressure baroreceptors in the carotid sinus and aortic arch respond to acute elevations in systemic BP by causing a reflex vagal bradycardia that is mediated through the parasympathetic systems and inhibition of sympathetic output from the central nervous system (CNS). Low pressure cardiopulmonary receptors in the atria and ventricles likewise respond to increases in the atrial filling by causing tachycardia through inhibition of cardiac sympathetic nervous system (SNS), increasing ANP release and inhibiting vasopressin release (Chopra *et al.*, 2011).

The SNS is a major short-term and long-term controller of BP. Sympathetic vasoconstrictor fibers are distributed to almost all regions of the vasculature, heart, and kidneys, and activation of the SNS can raise BP within a few seconds by causing vasoconstriction, increased cardiac pumping capability, and increased heart rate. Conversely, sudden inhibition of SNS activity can decrease BP to as low as half normal within less than a minute. Therefore, changes in SNS activity, caused by various reflex mechanisms, CNS ischemia, or by activation of higher centers in the brain, provide powerful and rapid, moment-to-moment regulation of BP (Hall *et al.*, 2012).

c) Renal endocrine

The most powerful chronic mechanism that controls BP over weeks and months, however, is the integrated renal endocrine systems that balance the body fluid and salt homeostasis with control of arterial BP. The renin-angiotensin-aldosterone system (RAAS) is possibly the body's most powerful hormone system for regulating BP as evidenced by the effectiveness of various RAAS blockers in reducing BP in hypertensive as well as normotensive subjects. Although the RAAS has many components, its most important effects on BP regulation are exerted by Ang-II that participates in both short-term and long-term control of arterial pressure (Hall *et al.*, 2015).

Activation of the RAAS usually occurs as the compensation for conditions that cause volume depletion and/or under perfusion of the kidneys, such as sodium depletion, hemorrhage, or heart failure. In order to counterbalance, renin will be secreted from juxtaglomerular cells surrounding the renal afferent arterioles. The secreted renin enzymatically cleaves the circulating large protein called angiotensinogen to give rise to angiotensin-I which is a mild vasoconstrictor (Raven and Chapleau, 2014). Consequently, an enzyme called angiotensin converting enzyme (ACE) located in the endothelial cells cleaves angiotensin-I yielding Ang-II which is a potent vasoconstrictor. The increased formation of Ang-II in conjunction with that of aldosterone, antidiuretic hormone, and SNS helps to restore renal perfusion by causing salt and water retention, increasing vascular tone, in turn, tends to prevent reductions and maintain optimum BP (Hall *et al.*, 2012;Raven and Chapleau, 2014). On the contrary, the newer member of RAAS ACE-2 cleaves Ang-II and produce the vasodilating peptide Ang-(1–7), thereby prevent the development of HTN (Feng *et al.*, 2010).

1.5. Pathogenesis of primary hypertension

As known single or multiple etiologic agents contribute to the pathogenesis of secondary HTN, the major focus will be on primary HTN (Ettner *et al.*, 2012). Unlike the secondary one's the discrete etiology of essential HTN is not yet fully understood after a century of clinical and basic research. But it is thought that a complex interaction of multiple genetic and environmental determinants does have a hand on the pathogenesis of HTN (Ehret and Caulfield, 2013). Generally, it is possible to broadly categorize the pathogenesis factors into genetic, environmental and compensatory factors (Wang and Peng, 2013).

The involvement of genetic factors is indisputable in the development of essential HTN. Evidence shows that single nucleotide polymorphism (SNP) of various pathways on the regulation of BP control is implicated as one factor in the pathogenesis of HTN. Moreover, candidate gene studies have associated aberrations in aldosterone signaling, catecholamine pathways, ion channel regulation, and inflammatory pathways are linked with the development of HTN (Luft, 2014).

The primary DNA sequences of genes related to HTN do not change between birth and death, but the expression of related genes does change in some cells and tissues. For instance when an individual ages or progresses to a different stage of HTN, the expression of genetic factors that are directly or indirectly related to BP may change, including changes in endocrine response, cell repair mechanisms, and specific organ functions. The underlying mechanism for changes in gene expression is the main regulation of gene expression. For example, epigenetic factors can regulate somatic ACE by DNA methylation and histone acetylation. In this way, genetic factors are important and dynamic during the development and progression of HTN (Kunes *et al.*, 2012).

Moreover, epigenetic mechanisms are affected by several factors and processes including the development (*in utero* and in childhood), environmental chemicals, drugs, aging, and diet. The molecular basis of epigenetics is complex but it does not involve the modification of the basic structure of DNA. Additionally, the chromatin proteins associated with DNA may be activated or silenced (Luft, 2014). However, most epigenetic changes occur only within the course of one individual organism's lifetime, but if such a change has been caused in autosomal cells, then some epigenetic changes may get inherited from one generation to the next (Kunes and Zicha, 2009).

The other component in the pathogenesis is the role of environmental factors. As it is mentioned above these environmental factors directly or indirectly predispose individuals to the development of HTN. The environmental factors may include stress, obesity, a high sodium and low potassium diet, being physically inactive, exposure to toxins, pathogens, radiation, and chemicals are common environmental factors in the development of HTN. Because these factors can affect BP, changes in these factors during the development of HTN are important. The actual pathogenesis pathway is considered to be several, of which the probable pathways might be via

epigenetic mechanism and/or through predisposing individuals to other risk factors such as diabetes mellitus (Kunes and Zicha, 2009).

The third component in the pathogenesis of HTN is the involvement of deregulated compensatory factors. Compensatory mechanisms are very common in BP regulation. Here, compensatory factors are defined as vasoactive factors that regulate BP, the ability of the human body to regulate BP, and the process of BP regulation. For example, the RAAS system and concentration of Ang-II, a vasoconstriction polypeptide, is increased in plasma, but that of the ANP, a vasodilatory polypeptide, is also increased in plasma (Hall *et al.*, 2015). An increase in Ang-II may be part of the primary etiology of HTN, but an increase in ANP secretion (or that of any other BP-lowering agent) may be part of a compensatory mechanism that helps to lower BP. Because ANP is a powerful vasodilator and a polypeptide hormone involved in the homeostatic control of body water, sodium, potassium, and adipose tissue, it can reduce the water, sodium, and adipose loads in the circulatory system, thereby reducing BP (Adrogué and Madias, 2007). This regulation is a continuous part of the body's regulation of homeostasis. The result of this regulatory process reflects the compensatory ability of the human body (Moon, 2013).

Compensatory factors are not always beneficial to the human body. For example, when BP increases, the secretion of ANP also increases, and this decrease BP. Low BP can decrease the secretion of ANP. This is an example of a negative feedback loop (virtuous circle). However, increased BP may induce vascular smooth muscle cell proliferation, which can increase vessel stiffness and cause BP to increase further. This is a positive feedback loop (vicious cycle) (Hall *et al.*, 2012). Unfortunately, in patients with HTN, factors that promote primary HTN always appear stronger than the compensatory factors. In this way, HTN may originate from insufficient compensatory factors in affected patients. This can induce hypertension-related complications (Büssemaker *et al.*, 2010).

1.6. Clinical manifestations of hypertension

HTN is often called 'the silent killer' because it is a disease that shows no early symptoms. This is especially typical for mild to moderate primary HTN and it may remain asymptomatic for many years. The most frequent symptoms are headache, fatigue, dizziness and facial flushing. Sub-occipital pulsating headaches, occurring early in the morning and subsiding during the day, are said to be characteristic, but any type of a headache may occur. In addition, accelerated HTN

is associated with somnolence, confusion, visual disturbances, nausea, and vomiting (Sawicka *et al.*, 2011).

Moreover, other manifestations are associated with HTN induced complications due to either sustained elevations of BP, with consequent changes in the vasculature and heart or to accompanying atherosclerosis that is accelerated by long-standing HTN. Generally, HTN is a well-known risk factor that predisposes to the development of left ventricular hypertrophy, coronary flow abnormalities, systolic and diastolic dysfunction, stroke, as well as chronic kidney disease (Ilyas *et al.*, 2015). Therefore, for example, hypertensive heart disease manifests itself through the sequelae of cardiac hypertrophy and/or the symptoms and signs of coronary insufficiency. Both of these conditions may lead to ischemic events, arrhythmias, and congestive heart failure. On top of this, hypertensive patients may have angina complaints or other signs of myocardial ischemia that might be associated with, the imbalance between oxygen supply and demand and are also believed to be related to increased coronary resistance at the microvascular level (Katayama *et al.*, 2018;Sawicka *et al.*, 2011).

1.7. Management of hypertension

Management of HTN should be handled in a comprehensive manner comprising two basic approaches: prevention and treatment. Regarding preventive approaches, in general for normotensive and pre-hypertensive population, education, training, and informing people on how to improve their lifestyle habits should be put forward (Hedayati *et al.*, 2011). On the other hand, the management approach for hypertensive patients consists of both pharmacological and non-pharmacological interventions to benefit against high BP, depending on the classification of BP levels. In addition to hypertensive population, non-pharmacological interventions can be applied either as an initial treatment before drug therapy or in conjunction with pharmacological therapy (Vamvakis *et al.*, 2017).

1.7.1. Non-Pharmacological management

There are different non-pharmacological approaches which majorly target the daily habits of individuals. Among these, nutrition, salt intake, cigarette smoking, exercise, and body weight are some of the modifiable factors, to this end manipulating these factors results in a measurable reduction of BP levels (Hedayati *et al.*, 2011). According to one meta-analysis, where improved

diet reduced SBP by 5.0 mmHg and DBP by 3.7 mmHg, exercise reduced SBP by 4.6 mmHg and DBP by 2.4 mmHg (Dickinson *et al.*, 2006).

1.7.2. Pharmacological management

The overall goal of therapy in the management of HTN is to reduce BP. But having this goal alone is not enough; therefore with harnessing the BP control, the aim is to reduce HTN-associated morbidity and mortality. The majority of HTN-associated morbidity and mortality is due to the subsequent cardiovascular complications and comorbid conditions; as a result, adequate BP control does have paramount importance. In order to go through it, depending on the stages of HTN, age, and comorbid conditions monotherapy or multiple therapies with the standard pharmacotherapy will be used (Carey and Whelton, 2018).

i) Diuretics

Diuretics are indispensable drugs in the treatment of HTN (Grossman *et al.*, 2011). The European Society of Cardiology/ European Society of Hypertension (ESC/ESH) guidelines recommend that thiazide diuretics should be considered as suitable as β-blockers, calcium antagonists, ACE inhibitors, and angiotensin receptor blockers (ARB) for the initiation and maintenance of antihypertensive treatment (Whelton *et al.*, 2017b). Their mechanism of action is through inhibition of tubular reabsorption of the different ions via blocking the effect of sodium/potassium/chloride transporter, with this path they tend to reduce the cardiac output thereby helping to lower the BP (Grossman *et al.*, 2011). There are numerous diuretics that can be used for HTN of which thiazide and thiazide-like diuretics are preferred among the group. According to JNC 8 report and AHA/ACC guideline, thiazide-type diuretics are first-line medications as monotherapy or in combination with other class of antihypertensive agents, unless the comorbid condition is diabetes mellitus and/or chronic kidney disease (James *et al.*, 2014).

ii) β-blockers

Beta-blockers belong to a diverse group of drugs, which block the action of endogenous catecholamine on beta-adrenergic receptors, part of the autonomic (or sympathetic) nervous system (Ram, 2010). The autonomic nervous system has been known to play a role in BP control. The principal adrenergic receptors present in the human cardiovascular system are the

β1, β2, and α1 receptors. Beta-blockers vary in their β1/β2-adrenergic receptor selectivity and vasodilatory properties, and this diversity has given rise to their classification into first, second, and third generation. First-generation beta-blockers exercise identical affinity for β1 and β2 receptors and are thus classified as non-selective beta-blockers (e.g. propranolol). Second-generation beta-blockers are more attracted to β1 than β2 receptors and are thus termed selective beta-blockers (e.g. atenolol). The third-generation of beta-blockers are known for their intrinsic vasodilatory properties (e.g. nebivolol, carvedilol) (Mann, 2017). But the cardiovascular outcome is inferior when first-line treatment is commenced with a beta-blocker compared to other antihypertensive drug classes. Moreover, beta-blockers are less effective in reducing the incidence of stroke, and the composite of major cardiovascular outcomes including stroke, myocardial infarction, and death, compared to all drugs for treating HTN (Wiysonge *et al.*, 2017).

iii) Calcium channel blockers

There are three forms of calcium channel blockers (CCBs) based on their structure. These are dihydropyridines (e.g. nifedipine), phenylalkylamines (e.g. verapamil) and benzothiazepines (e.g. diltiazem) (Elliott and Ram, 2011). CCBs inhibit the flow of extracellular calcium through ion-specific channels. Although several types of such channels have been identified, currently available CCBs inhibit the L-type channels in humans. When inward calcium flux is inhibited, vascular smooth muscle cells relax, resulting in vasodilation and a lowering of BP (Tocci *et al.*, 2015). In cardiac muscle, contractility is reduced and the sinus pacemaker and atrioventricular conduction velocities are slowed. Moreover, in the kidney, CCBs produce natriuresis by increasing renal blood flow, dilating afferent arterioles, and increasing glomerular filtration pressure. Regarding their role in the treatment of HTN, according to JNC 8, CCBs are one of the classes of anti-hypertensive agent where they are recommended to initiate therapy as monotherapy or in conjunction with other agents for non-black and black population, however, they are more efficacious in blacks compared to non-blacks (James *et al.*, 2014;Wang *et al.*, 2017).

iv) Angiotensin-converting enzyme inhibitors

Angiotensin converting enzyme inhibitors (ACEI) (e.g. captopril) have the basic structure of 2-methyl-propyl-L-proline; they are classified into three types, chemically different depending on

the ligand group of the zinc ion: sulfhydryl, carboxyl, and phosphoryl. These structures affect directly tissue distribution and elimination pathways (ȚÂNȚU *et al.*, 2014). The mechanism of action is the marked reduction in circulating levels of Ang-II, preventing direct vasoconstriction induced by this peptide. Simultaneously, ACE is inhibited apparently variably in the vascular walls and other tissues, including the brain and the heart. Pharmacological blockade of plasma ACE appears to be less important in chronic treatment, whereas the inhibition in various tissues appears to be the main determinant of the pharmacological effects of the ACEI. Additionally, ACEI inhibit kininase II and increase bradykinin levels, stimulating the release of nitric oxide and vasoactive prostaglandins. In addition, ACEI attenuate the expected increase of SNS activity; thereby reducing the heart rate and cardiac output. ACEIs are strongly recommended to be taken in the following case since they do have morbidity as well as mortality benefit: chronic kidney disease, diabetes mellitus, and HF (Gabb *et al.*, 2016;Schmieder *et al.*, 2015).

v) Angiotensin-II receptor blockers

The RAAS has been a major target pathway for the development of antihypertensive medications. The four classes of medications that are involved in this pathway include ACEI, ARBs, aldosterone antagonists, and direct renin inhibitors. From these classes of drugs, ARBs have been in clinical use since 1995 and are known to be an effective antihypertensive agent with excellent tolerability profiles (Abraham Hazel Mae A *et al.*, 2015). The effect of ARBs mostly resembles that of ACEI; however, inhibition of ACE only partially inhibits the formation of Ang-II. Whereas ARBs can block the activities of Ang-II on angiotensin receptor regardless of whether it was created from ACE or other enzymes such as cardiac chymase. Moreover, ARBs lack the adverse effects such as cough and angioedema which are associated with accumulation of bradykinins and substance-P as a result of the blockage of ACE (Burnier *et al.*, 2013). Regarding their place in the treatment of HTN, ARBs are well tolerated as a monotherapy as well as a combination of therapy. Furthermore, the ARBs have proven mortality and morbidity benefit in comorbid conditions such as in HF and chronic renal disease, particularly when associated with type 2 diabetes (Roy *et al.*, 2012;Abraham Hazel Mae A *et al.*, 2015).

vi) Miscellaneous antihypertensive agents

Miscellaneous agents comprises; alpha-1 blockers (prazosin, terazosin, doxazosin) blocks α-1 adrenergic receptors thereby inhibits vasoconstriction induced by catecholamines (Taraphder,

2014), centrally acting α-2 agonist (Guanfacine, Guanabenz, Methyldopa, Clonidine) methyldopa metabolized to a-methyl norepinephrine where the metabolite stimulates the α-2 receptor whereas the others simply stimulate α-2 receptors, direct vasodilators (hydralazine, minoxidil,) hydralazine inhibits 1,4,5 triphosphate inositol (IP3)-induced release of calcium, minoxidil mechanism is via ATP-modulated potassium channels in the vascular smooth muscle cells, allowing potassium efflux and smooth muscle relaxation (Cohn *et al.*, 2011). There are also nitrates and sodium nitroprusside where they may be viewed as nitric oxide donors since their vascular effects appear to be related to the generation of nitric oxide gas as a consequence of their metabolic breakdown. Aliskiren approved in 2007 as new drug where it works as a direct inhibitor of renin which is a product of this type of investigation These miscellaneous agents do have a place in the treatment of HTN such as in pregnancy e.g. methyldopa as well as in hypertensive crisis such as the vasodilator hydralazine (McComb *et al.*, 2016).

vii) Newer and Future agents

Numerous molecules are undergoing preclinical and clinical investigation for HTN treatment. The molecules under investigation targets novel cell-signaling pathways and pathophysiological mechanisms (Şen *et al.*, 2013). Some of the targets that are under investigation are:

a) Vasopeptidase inhibitors they target in addition to ACE, two other zinc metalloproteinases neprilysin (also called neutral endopeptidase) and endothelin-converting enzyme. Combined inhibitions of these three enzymes aimed to not only improve BP control in patients with HTN, particularly those with resistant HTN help also in other comorbid conditions (Laurent *et al.*, 2012). Dual inhibitors of ACE and neprilysin such as Samapatrilat, Gemopatrilat, Fasidotril, MDL-100240, Z-13752A (von Lueder *et al.*, 2014) are under investigation of which particularly Omapatrilat has been highly investigated and found out to be promising in salt-sensitive HTN though the risk of angioedema is high(Vardeny *et al.*, 2014).
b) The other one is endothelin antagonists which work via blocking the effect of ET-1. ET-1 is a potent endothelium-derived vasoconstrictor peptide that acts through ETA receptors, and in particular, mediates vasoconstriction and inflammation (Oparil and Schmieder, 2015). From this class of agents Bosentan, Ambrisentan and Macintentan were approved, where liver dysfunction is the most common adverse effect (Emoto, 2017).

c) Natriuretic peptide receptor agonists are being developed as an alternative approach to inhibiting the degradation of endogenous natriuretic peptides for the treatment of HF and refractory or resistant HTN (Burnier *et al.*, 2013).
d) Intestinal Na+/H+ Exchanger 3 inhibitor where excessive sodium intake and impaired sodium excretion plays an important role in the pathogenesis of HTN and its complications, including HF and chronic kidney disease. Furthermore, there are also other targets such as dopamine β-hydroxylase, aldosterone synthase inhibitors, and many others are in the pipeline (Oparil and Schmieder, 2015).

1.7.3. Herbs in hypertension

The use of traditional medicines that may include plants, animals, microorganisms, and marine organisms dates back to antiquity, for such claim there are several lines of evidence such as fossil records and ancient scriptures that have lived yet to tell the story (Antwi-Baffour *et al.*, 2014). It has been estimated that around 80% of the world population use traditional medicines. Furthermore, according to WHO more than 65% of the global public still heavily relies on traditional medicines as a primary health care (SERIAL and ORGANIZA, 2010). Likewise, many in Africa are also dependent on traditional medicines for both communicable as well as non-communicable diseases prevention and/or treatment. Since Ethiopia is part of Africa, the situation is not also different, where according to demographic distribution of the people, majority of the public live in rural areas and it is heavily reliant on traditional medicine for health care (Burton *et al.*, 2015;Abdullahi, 2011).

According to studies, there are hundreds of plant species that has been used in complementary and alternative medicine as antihypertensive agent across the globe (Piero *et al.*, 2012). However, of all, a small portion of them have passed through rigorous scientific testing and investigation. Moreover, via exploiting this untapped resource, as history has shown, there is a probability of discovering a molecule having strong pharmacological activities with a novel mechanism of action. Since Ethiopia is blessed with extraordinary and rich varieties of endemic flora and fauna, it is imperative to look for medicinal plants with superior activity (Mertens *et al.*, 2016). With this direction, there has been researches conducted on plants that have a traditional claim for treatment of HTN. Some of them are; *Calpurnia aurea*(Getiye *et al.*, 2016), *Moringa stenopetala* (Geleta *et al.*, 2016), *Thymus serrulatus* (Geleta *et al.*, 2015), *Syzygium*

guineense (Ayele *et al.*, 2010), and *Ajuga remota* Benth (Lamiaceae) (Hailu and Engidawork, 2014).

1.8. Overview of the experimental plant, *Otostegia integrifolia* Benth

Otostegia integrifolia Benth (Figure 1) is commonly known with the vernacular name of "Tunjite" in Amharic (Kidane *et al.*, 2013). This plant belongs to the family Lamiaceae (Labiatae) which comprises 236 genera and more than 7,000 species, the largest family of the order Lamiales distributed nearly worldwide, and many species are cultivated for their fragrant leaves and attractive flowers. The family is particularly important to humans for flavor, fragrance, or medicinal properties (Britannica)**.** The genus *Otostegia* (Lamiaceae) consists of about 15 species. It is endemic to the northern part of tropical Africa and South-western and Central Asia. Five species of this genus have been reported to occur in the flora of Ethiopia including *Otostegia integrifloia* (Endale *et al.*, 2013).

Otostegia integrifolia Benth is an erect perennial shrub, much branched and spiny, 1-3 m. There are usually 4 spiny stipules at the leaf nodes. The leaves are long oval, mealy grey-green, variable in size, 1-3 cm, young leaves are silky. The flowers arise in whorls of 6, falling quickly. The flower is strongly 2-lipped, the upper lip oblong green-white, hairy, the lower orange-yellow in the center with pale side lobes. The greenish calyx has a small upper lip but the lower lip soon enlarges, becomes stiff and papery, pale white-yellow-brown, with well-marked veins. The fruits are small nutlets within the calyx**.** The plant grows in the wild but is also cultivated in gardens. It grows on montane bushlands and woodlands overgrazed slopes at altitudes ranging from 1, 300 to 2, 800 m. The plant is endemic to Ethiopia, Eritrea, and Yemen (Endale *et al.*, 2013).

Figure 1: Photograph showing *Otostegia integrifolia* Benth shrub during collection.

Investigation of the constituents of *Otostegia* species revealed that Thymol, c-terpinene, and p-cymene were reported as major constituents of the essential oil of *O. fruticose*. Furthermore, it comprises labdane diterpenes such asotostegin15- epiotostegin, preleoheterin, leoheterin, leopersin, 15-epi-leopersi, ballonigrin, vulgarol, 8-O acetylharpagide (Tesso and König, 2004). Particularly *Otostegia integrifolia* contains a total of 40 constituents including monoterpenes, sesquiterpenes, and diterpenes. However, five of the chemical compounds namely axinyssene, otostegindiol, pretostegindiol, pentatriacontane, and stigmasterol are observed to be the potential sources of medicine, to treat various illnesses (Sadeghi *et al.*, 2014).

1.9. Rationale for the study

HTN is one of the major causes of cardiovascular-related morbidity and mortality in the public, and the potential for acquiring the disease is still looming around on everybody. Setting aside other cardiovascular disorders, HTN prevalence is increasing at higher rate, where both developed as well as developing nations are suffering from it (Kearney *et al.*, 2004). According to World Hypertension League and the International Society of Hypertension 2014 policy statement, HTN was the cause for an estimated 9.4 million deaths and 162 million years of life lost in 2010. Moreover, HTN is the cause of 50% of heart disease, stroke, and HF; 18% of deaths overall and more than 40% of deaths in people with diabetes. The other fact is that

approximately 4 in 10 adults older than 25 years have HTN, and in many countries, another 1in 5 have prehypertension (Campbell *et al.*, 2014). Besides, more than two-thirds of these group of peoples reside in developing nation (Bromfield and Muntner, 2013).

Despite the available anti-hypertensive agents have managed to achieve optimal BP in the majority of patients, still achieving optimal BP control and reducing the potential of acquiring other cardiovascular complications are far from the target in some group of patients. The possible rationale for such claim can be attributed to various problems. Even if the other factors were made insignificant, the gap in medication-related problems is still high. Particularly elderly patients, patients with comorbid conditions, patients on polypharmacy and resistant hypertension (Yaxley and Thambar, 2015) are not able to bear the side effects of the medications. Furthermore, this may end up in poor adherence and in the long run maintaining adequate BP control would be difficult and finally, treatment failure will ensue (Campese and Schneider, 2010).

Due to the problems stated above and also, the incessant urge and endeavor of mankind to meet the ongoing demand, it is difficult only to rely on the existing or conventional medications, and this obliges us to look for viable alternatives (Kooti *et al.*, 2016). Traditional medicine has been used since prehistoric times, the cores of which are plants. Therefore, the best way to start the search for alternatives with traditional medicines (Yuan *et al.*, 2016). Ethiopia is blessed with huge biodiversity, so does with the ethnobotanical data, which offers the opportunity to scientifically test the plant constituents that have pharmacological activities towards certain disease or ailments (Araya *et al.*, 2015). The herb *Otostegia integrifolia*, according to Andemariam *et al*, (2010) has been used for the treatment of HTN traditionally by Eritrean as well as peoples in the northern part of Ethiopia for decades (Araya *et al.*, 2015;Andemariam, 2010). Thus, the purpose of this study is to verify whether the plant of interest possesses the claimed antihypertensive activity using *in vivo* and *ex vivo* models.

2. Objectives

2.1. General objective

- To assess the antihypertensive activity of the leaf extract of *Otostegia integrifolia* Benth (Lamiaceae) in rat model of fructose-induced hypertension.

2.2. Specific objectives

- To assess the *in-vivo* anti-hypertensive effect of the extract in rats.
- To evaluate the vasorelaxant effect of the extract on isolated aorta *ex vivo*.
- To perform a preliminary assessment of the possible mechanism of action of the plant extract using selective agonists and antagonists.

3. Materials and Methods

3.1. Materials

3.1.1. Drugs and chemicals

The main chemicals used for the experiment include distilled water, methanol, fructose (LOBA Chemie laboratory reagents and fine chemicals, India), acetylcholine, atropine, indomethacin, glibenclamide, diphenhydramine, methylene blue, phenylephrine, and nifedipine (BDH Laboratory Supplies, England). The chemicals used to make physiological salt solutions (Kreb-Henseleit solution) include sodium chloride, potassium chloride, sodium bicarbonate, magnesium sulfate, calcium chloride, potassium dihydrogen phosphate, glucose, and ethylenediaminetetraacetic acid (EDTA) (Sigma Chemical Company, USA). All reagents were of analytical grade.

3.1.2. Plant collection

The leaf of *Otostegia integrifolia* Benth. (Lamiaceae) were collected in the month of December 2016 from Tulu Dimtu, which is found in East Shewa zone of the Oromia region, about 29 km southeast of Addis Ababa, Ethiopia. Identification & authentication of the plant specimens was done by a taxonomist and a voucher specimen (001) was deposited at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University, for future reference.

3.1.3. Experimental animals

Sprague-Dawley rats (250–300g, 6–8 weeks of age) of either sex were obtained from the animal house of School of Pharmacy and animal unit of the Ethiopian Public Health Institute, Addis Ababa, Ethiopia. They were provided with standard pellet 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 with the tail cuff instrument and to the environment. The care and handling of animals were in line with international guidelines (NRCotN, 2011).

3.2. Methods

3.2.1. Plant extraction

The plant material was thoroughly washed with tap water to remove dirt and soil. The leaves of the plant were allowed to dry at room temperature under shade and powdered using mortar and

pestle. Five hundred grams of air-dried and powdered plant materials were extracted by cold maceration technique for three consecutive days at room temperature with 80% methanol to get the crude hydroalcoholic extract. The procedure was repeated twice by adding another fresh solvent to the marcs. The resulting liquid extract was then filtered with Whatman no 1 filter paper & concentrated using rotavapor (Buchilabortechnik AG, Switzerland) at 40^{o}C under reduced pressure and the concentrated extract was freeze-dried using a lyophilizer (Heto Power Dry LL3000 freeze-dryer;, USA). The color of the dried extract was yellowish brown, particularly of apricot type. It was hygroscopic shiny powder and a percentage weight yield of 16.6% was obtained. The resulting extract was then transferred into a vial and kept in a desiccator layered with $CaCl_2$ until further use.

3.2.2. Blood pressure measurement using Tail-Cuff method

SBP and DBP were measured by employing a tail-cuff method using model 179 BP analyzer (IITC Life Sciences, California, US) which is a non-invasive BP recorder. The rats were placed in the restrainer and kept in a scanner for 30 min to get warmed to about 32-34°C of tail temperature prior to obtaining pressure measurements. When they become relaxed and calmed, the cuff was placed and inflated to a pressure well above the expected SBP (200mmHg) on the base of the tail to occlude the blood flow which is analogous to sphygmomanometry in humans. A transducer was placed close to the cuff which measured the pulse rate upon deflation. The non-invasive BP sensor was utilized to monitor the BP and an average of three readings was taken for each rat and a total of four measurements were taken (Vogel and Vogel, 2013;Geleta *et al.,* 2016).

3.2.3. Induction of experimental hypertension

There are various methods for inducing experimental HTN in this case the dietary fructose induced HTN model was employed. According to a study conducted by Hwang *et al*.,(1987) and many other studies the mechanism of fructose induced HTN showed that it is concentration and time dependent (Abdulla *et al.,* 2011;Hwang *et al.,* 1987).

There is uncertainty regarding the exact mechanisms by which fructose consumption produces an increased BP. But several possibilities have been proposed that includes increased SNS activity, elevated circulating catecholamine, enhanced RAAS activity and Ang II levels, increased sodium reabsorption, impaired endothelium-dependent relaxation, and increased secretion of endothelin-

1. Therefore, the mechanisms by which excess fructose increases BP fall into three broad categories: increased salt absorption, endothelial dysfunction and stimulation of the SNS. All these pathogenesis pathways are preceded and causally linked to insulin resistance and compensatory hyperinsulinemia (Klein and Kiat, 2015).

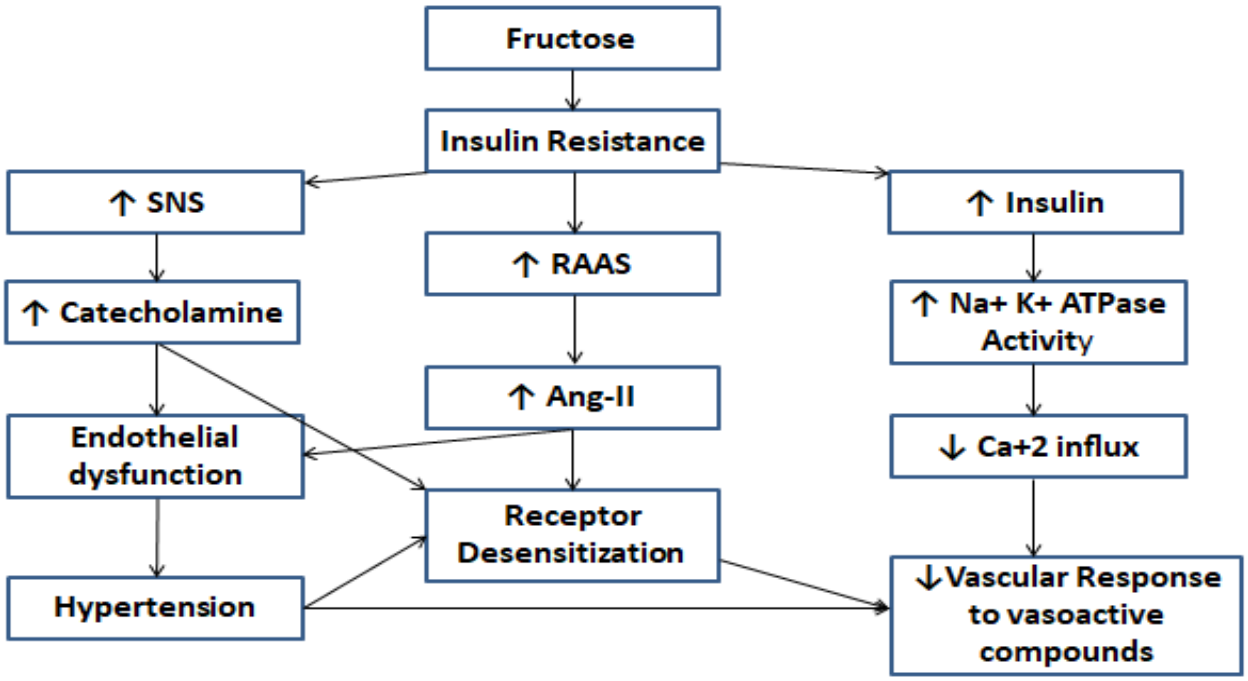

Figure 2**:** Proposed mechanisms that may explain the decreased vascular responses to vasoactive compounds in the fructose-fed rats. ↑ and ↓ stand for increase and decrease, respectively (Abdulla *et al.*, 2011).

3.2.4. Grouping and dosing of animals

A total of 36 Male Sprague-Dawley rats were used for the *in vivo* experiment, which were randomly assigned into five groups that drank the HTN inducing agent 66% w/v D-fructose along with the corresponding chemical/extract assigned to each group, and a normal control group. The group included normal control rats (NCR) that received distilled water *ad libitum*, negative control rats received 66% w/v D-Fructose (DF66) *ad libitum*. Positive control rats received Captopril 20 mg/kg/day (CAP20) with 66% w/v D-Fructose *ad libitum* and treatment group rats received 250 mg/kg (OI250), 500 mg/kg (OI500), and 1000 mg/kg (OI1000) of the

extract with 66% w/v D-Fructose *ad libitum* for 15 days (Geleta *et al.*, 2016). All doses were administered per oral route where the maximum volume being 2.5ml.

The doses were selected based on acute oral toxicity study that had been done on the experimental plant *Otostegia integrifolia* as per the internationally accepted protocol using OECD guidelines 423 by Endale *et al.* (2013). The toxicity of hydroalcoholic leaves extract of *O. integrifolia* was done with the dose of 2 g/kg and 5 g/kg and no sign of toxicity or mortality was observed. Thus, prescreening was carried out to select the appropriate dose for the subsequent experiment. The lower doses of the 2 g/kg limit dose, particularly 100mg/kg and 200mg/kg didn't exhibit significant BP reducing tendency during prescreening but the 400mg/kg did. On the other hand, from 5 g/kg limit test the higher doses, 500mg/kg and 1000mg/kg showed significant BP lowering activity. For such reason, the latter high limit dose was used for the *in vivo* antihypertensive study. Subsequently, SBP, MAP, and DBP were measured on day 0 of the experiment before inducing HTN and recorded as a basal BP (D0). BP was then measured every 5 days in 15 days. The SBP and MAP were read from the pulse tracings and DBP was calculated using the following formula DBP = (3MAP-SBP)/2. In addition, every measurement was made in triplicate and the average value was reported (Ayele *et al.*, 2010).

3.2.5. *Ex vivo* vasorelaxant activity

The *ex vivo* experiment was conducted on Sprague Dawley rats aortas according to the method described elsewhere (Vogel and Vogel, 2013). Fifteen Sprague Dawley rats of either sex were sacrificed by cervical dislocation. 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 then kept moistened with Krebs-Henseleit solution (pH of 7.4) during the whole procedure and finally mounted in an organ bath containing 30 mL of the solution (composition in mM: 118.2 NaCl, 4.7 KCl, 1.2 MgSO4, 2.5 CaCl2.2H2O, 1.3 KH2PO4, 25 NaHCO3 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 the 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.

Once a contraction plateau was achieved, the 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 (Grass model 7E polygraph).

3.2.6. Evaluation of possible mechanism of vasorelaxation

The possible mechanism(s) of vasorelaxation produced by the hydroalcoholic leaf extract of *Otostegia integrifolia* were partly studied using different agonists and antagonists. To check the involvement of cholinergic and prostanoid (PGI2) effects, the tissue was preincubated with atropine (antimuscarinic) and indomethacin (non-selective inhibitor of cyclooxygenases), respectively, for 15 min before adding the test substance. The possible role of NO/cyclic guanosine monophosphate (cGMP) pathway was also studied by preincubating the aorta with methylene blue (cGMP pathway blocker). Any involvement of ATP dependent K+ channel and histamine was studied by preincubating the tissue with respective inhibitors, glibenclamide, and diphenhydramine (Ayele *et al.*, 2010).

To investigate the role of the endothelium, the procedure was carried out in endothelium-denuded aorta. The endothelium lining of the aortic rings was removed mechanically by gently rubbing the intimal surface of the aortal strip with a moist wooden stick for approximately 30sec. Denudation of endothelium was assessed by determining acetylcholine elicited relaxation in aortic rings precontracted with high K^+. The extract was then tested for its ability to relax the contractions induced with high K^+. In addition, to assess the role of sarcoplasmic reticulum sequestered calcium the tissue was preincubated with phenylephrine (Gilani *et al.*, 1994).

Another series of experiments were performed in order to determine the inhibitory effect of *Otostegia integrifolia* on the extracellular Ca2+- entry-induced responses. To confirm calcium channel blocking (CCB) activity, concentration-response curves (CRCs) of Ca2+ were constructed (Gilani *et al.*, 1994). For this purpose, the tissue was stabilized in normal Kreb's solution and then placed in Ca2+free Kreb's solution, containing EDTA (0.1 mM) for 30 min to remove Ca2+ from the tissues. This solution was further replaced with K+ rich and Ca2+ free Kreb's solution, having the following composition (mM): KCl 50, NaCl 50.58, MgSO4 3.1, NaHCO3 23.8, KH2PO4 1.26, glucose 11.1 and EDTA 0.1. Following an incubation period of 1 h, control CRCs of Ca2+ was obtained. Following construction of CRCs of Ca2+, the tissue was

then pre-treated with the extract for 50–60 min for the possible CCB effect. Finally, the Ca2+ CRCs were reconstructed in the presence of the highest concentration of the test material. In all experiments, the endothelium was removed by gently rubbing the luminal surface (Getiye *et al.*, 2016).

3.3. Data analysis

All results of the *in vivo* study were expressed as mean ± standard error of a mean (SEM). The data was analyzed using one-way analysis of variance (ANOVA) to look at difference between groups and also subjected to two-way ANOVA to see the effect over time followed by post-hoc test (Tuckey's and Bonferroni tests respectively). The graphing was performed using Graph Pad Prism software version 7.00 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 were expressed as percentage contraction, taking the control high K+ induced contraction before the application of the test extract as 100%.

4. Results

4.1. *In vivo* studies

4.1.1. Induction of hypertension

BP measurements obtained from animals with or without fructose ingestion is presented in Table 2.

Table 2: Blood pressure changes after induction of hypertension with 66% w/v D-fructose in rats.

BP	Group	BP Measurement Period			
		Day 0	**Day 5**	**Day 10**	**Day 15**
SBP	**NCR**	112.75 ± 3.99	109.50 ± 4.31	101.16 ± 7.2	111.41 ± 0.96
	DF66	118.66 ± 4.8	185.16 ± 6.68***	161 ± 9.25 ***	151.58 ± 4.02***
DBP	**NCR**	69.00 ± 6.91	84.00 ± 5.49	80.37 ± 6.95	78.79 ± 3.43
	DF66	89.66 ± 5.03	164.54 ± 6.69***	144.62 ±10.84***	143.58 ±4.02***
MAP	**NCR**	83.58 ± 3.80	92.50 ± 4.92	87.41 ± 7.07	89.66 ± 2.41
	DF66	99.33 ± 4.51	171.41 ± 6.41***	150.25 ±10.26***	142.91 ±4.88***

Data are expressed as mean ± SEM (n=6); Analysis was performed by one-way ANOVA SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, Mean arterial pressure; NCR, normal control rats without fructose ingestion; DF66, rats with fructose ingestion; *p<0.05; **p<0.01; ***p<0.001.

Fructose ingestion significantly increased ($p<0.001$) BP at the different point times, where BP measurements were performed. Maximum increase in the three types of BP was observed on Day 5, the increase being on average 56%, 84%, and 71%, for SBP, DBP, and MAP, respectively. Whilst the increase for SBP, DBP, and MAP on day 10 was 36%, 61%, and 50% ; that of Day 15 was 28%, 60%, and 43%, respectively.

All BP parameters were found to decrease significantly ($p<0.001$) between day 5 and 10 in the negative control group. However, no significant decrement was recorded between day 10 and 15 measurements.

4.1.2. Effect of the extract on hypertensive rats

Daily oral administration of the extract produced significant antihypertensive activity on three measurement points. SBP was significantly decreased by all doses of the extract at all time-points compared to the DF66 (Table 3). OI250 mg/kg produced a decrease by 18.7%-25.2% ($p<$ 0.01), with maximum reduction achieved on Day 15. Increasing dose increased reduction, as OI500 brought about a reduction by 24.3-45.3% ($p<$ 0.001). However, maximum effect was observed on Day 10. Further increase in dose was, however, not accompanied by a corresponding increase in reduction, as OI1000 was able to produce a reduction of 24.1-39.5% ($p<$ 0.001), with maximum effect on Day 10. CAP20 induced reduction was significantly higher ($p<0.001$) than the extract on Day 5. However, the difference worn out with increasing dose and time.

Table 3: The effect of the hydro-alcoholic extract of *Otostegia integrifolia* on systolic blood pressure in fructose induced hypertensive rat.

Dose of Substance (mg/Kg)	**Systolic BP**			
	Day 0	**Day 5**	**Day 10**	**Day 15**
DF66	118.66 ± 4.8	185.16 ± 6.68	161 ± 9.25	151.58 ± 4.02
CAP20	108.41± 2.45	98.66 ± 4.81 b^3	96.33 ± 7.91 b^3	93.00 ± 5.31 b^3
OI250	116.16± 3.49	150.66 ± 8.79 c^3 a^2	135 ± 13.17 c^1	109.75 ± 11.64 a^3
OI500	121.91± 4.76	140 ± 6.26 c^3 a^3	87.08 ±9.24 a^3 d^2	109.33 ± 3.08 a^3
OI1000	110.25± 6.13	142.41 ± 3.17c^3 a^3	96.91 ± 4.51 a^3 d^1	102.91 ± 4.17 a^3

Data are expressed as mean ± SEM (n=6); Analysis was performed by one-way ANOVA; a, Fructose vs Extract; b, Fructose vs Standard; c, Extract vs Standard; d, Extract vs Extract; DF66, rats with fructose ingestion; CAP20, Captopril 20mg/kg; OI, *Otostegia integrifolia* at 250, 500 and 1000 mg/kg; *p<0.05; **p<0.01; ***p<0.001.

A tracing of the effect of the extract on BP of hypertensive rats is depicted in Figure 3.

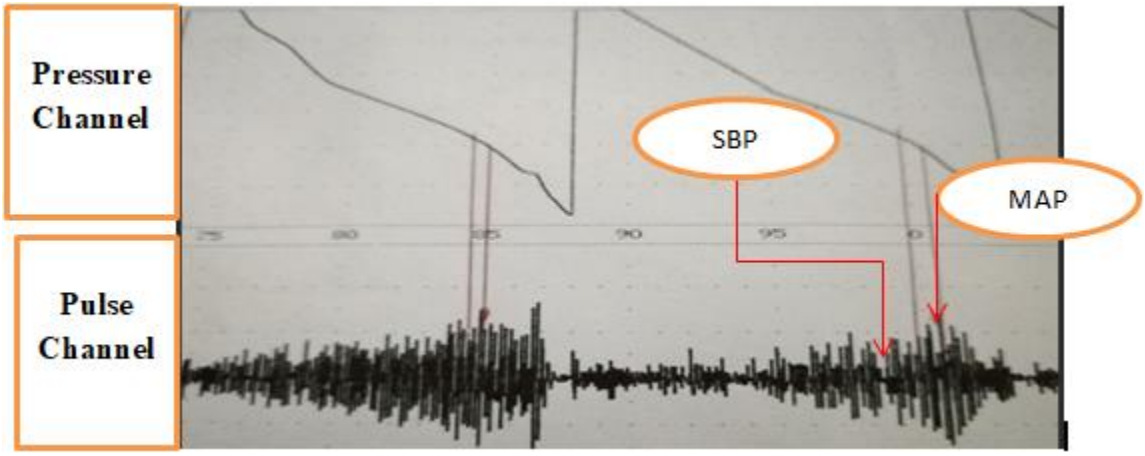

Figure 3: A representative tracing of rat blood pressure measurement showing the pressure of tail cuff and pulse.

DBP also significantly reduced by the extract at all measured days, but the significance was lost at Day 10 with OI250 (Table 4). OI250 reduced DBP by 17.1% ($p< 0.05$), 16.1%, and 34.1% ($p< 0.001$) at day 5, day 10 and day 15 respectively. The pattern obtained with OI500 and OI1000 was similar with that of SBP, with maximum reduction, 54.9% ($p< 0.001$) and 49.7% ($p< 0.001$), respectively, achieved on Day 10. CAP20 produced a significantly higher decrease on Day 5 (with all doses, p<0.001)) and on Day 10 (OI250, p<0.05) compared to the extract.

Table 4: The effect of the hydro-alcoholic extract of *Otostegia integrifolia on* diastolic blood pressure in fructose induced hypertensive rat.

Dose of Substance (mg/Kg)	**Diastolic BP**			
	Day 0	**Day 5**	**Day 10**	**Day 15**
DF66	89.66 ±5.03	164.54 ± 6.69	144.62 ± 10.84	143.58 ± 4.02
CAP20	88.54 ±4.86	74.91 ± 3.85 b^3	69.87 ± 8.07 b^3	65.25 ± 3.94 b^3
OI250	82.54 ±2.43	132.34 ± 10.16c^3a^1	119.25 ±10.83 c^1	86.50 ±11.58a^3
OI500	90.91 ±6.14	118.61 ± 6.78 c^3a^3	65.08 ± 9.24a^3 d^2	87.33 ± 2.77 a^3
OI1000	86.33 ±5.70	127.75 ± 5.37 c^3 a^2	72.79 ± 8.00a^3 d^1	85.54 ± 5.34 a^3

Data are expressed as mean ± SEM (n=6); Analysis was performed by one-way ANOVA; a, Fructose vs Extract; b, Fructose vs Standard; c, Extract vs Standard; d, Extract vs Extract; DF66, rats with fructose ingestion; CAP20, Captopril 20mg/kg; OI, *Otostegia integrifolia* at 250, 500 and 1000 mg/kg; *p<0.05; **p<0.01; ***p<0.001.

Daily oral administration of the extract produced a change in MAP that was similar to DBP. OI250, once again, failed to exhibit a decrease in MAP on Day 10, although it was able to reduce MAP by 17.2%. OI250 on Day 5 (20%, $p< 0.01$) and Day 15 (28.2%, $p< 0.01$) as well as OI500 (27-52%, $p< 0.001$) and OI1000 (22-45.3%, $p< 0.01$) on all measured days were able to reduce MAP. CAP20 also produced a similar change that was observed with DBP (Table 5).

Table 5: The effect of the hydro-alcoholic extract of *Otostegia integrifolia* on mean arterial blood pressure in fructose induced hypertensive rat.

Dose of Substance (mg/Kg)	**MAP**			
	Day 0	**Day 5**	**Day 10**	**Day 15**
DF66	99.33 ± 4.51	171.41 ± 6.41	150.25 ± 10.26	142.91 ± 4.88
CAP20	95.16 ± 3.91	82.83 ± 3.60 b^3	78.58 ± 7.39 b^3	73.50 ± 4.67 b^3
OI250	93.75 ± 1.42	137.58 ± 9.36 c^3 a^2	124.50 ± 10.89c^1	94.25 ± 11.47 a^3
OI500	101.25 ±5.63	125.70 ± 6.47 a^3 c^3	72.41 ± 9.20a^3d^2	94.66 ± 2.65 a^3
OI1000	93.41 ± 5.18	134.25 ± 4.58 a^2c^3	82.33 ± 7.26a^3d^1	91.33 ± 4.84 a^3

Data are expressed as mean ± SEM (n=6); Analysis was performed by one-way ANOVA; a, Fructose vs Extract; b, Fructose vs Standard; c, Extract vs Standard; d, Extract vs Extract; DF66, rats with fructose ingestion; CAP20, Captopril 20mg/kg; OI, *Otostegia integrifolia* at 250, 500 and 1000 mg/kg; *p<0.05; **p<0.01; ***p<0.001.

Comparison among the different dose of the extract revealed that OI500 ($p<0.01$) and OI1000 ($p<0.05$) showed a significantly higher decrease in SBP, DBP, and MAP on day 10 compared to OI250. However, no apparent change in reduction was observed between the medium and higher doses in all time-points (Table 3-5). Two--way analysis was performed to see the change in BP measurement across time. Overall, the trend over time in SBP, DBP and MAP reduction was significant ($p < 0.001$) between day 5 and 10, but lacks consistency latter on between day 10 and day 15 (Figure 4, 5 & 6).

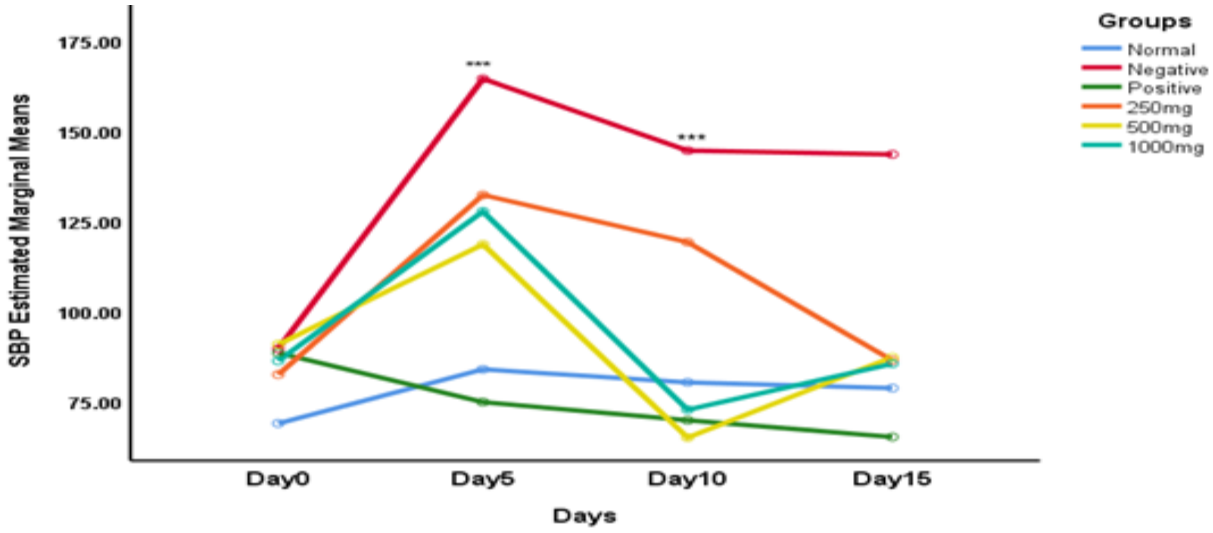

Figure 4: The effects of the different groups on systolic blood pressure across the days of measurement. (*p < 0.05, **p < 0.01 and ***p < 0.001, compared to the previous Day value)

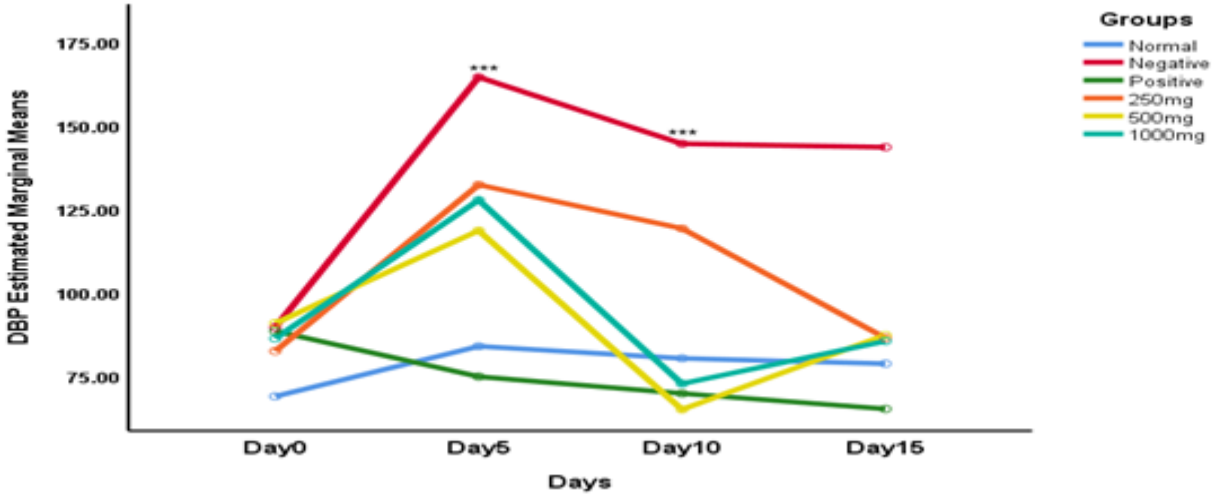

Figure 5: The effects of the different groups on diastolic blood pressure across the days of measurement. (*p < 0.05, **p < 0.01 and ***p < 0.001, compared to the previous Day value).

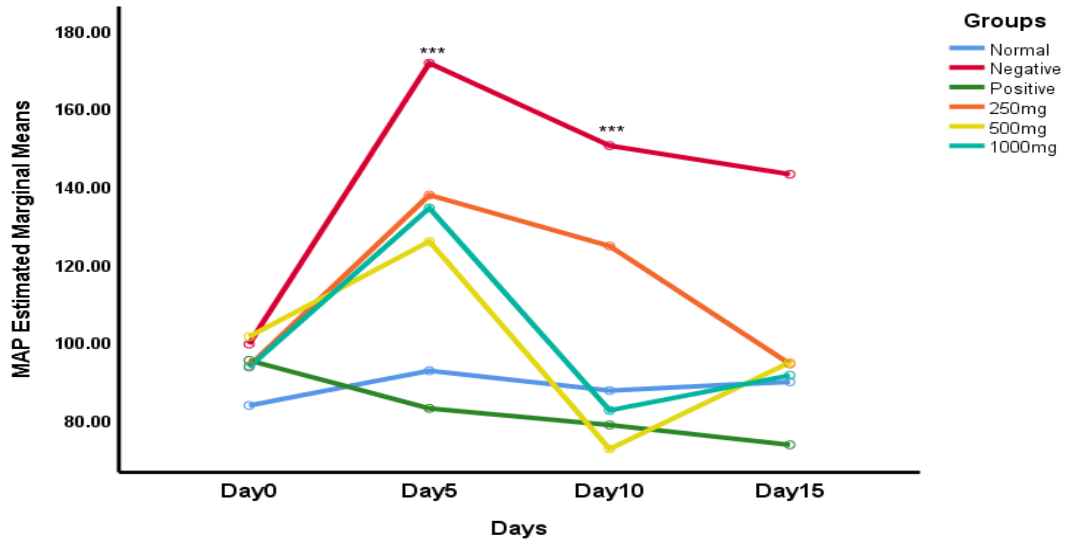

Figure 6: The effects of the different groups on mean arterial pressure across the days of measurement. (*p < 0.05, **p < 0.01 and ***p < 0.001, compared to the previous Day value).

4.2. *Ex vivo* studies

4.2.1. Vasodepressor activity

Initial assessment was performed on resting baseline aortic strips to see whether the extract possessed vasoconstrictor activity, the extract was found to be devoid of any vasoconstrictor activity. Afterwards the effect of the extract was assessed on high-K^+ (80 mM) induced contraction (Figure 7) and the extract demonstrated a concentration-dependent vasodepressor activity, with maximum relaxation attained at a cumulative concentration of 6.375mg/ml (Table 6).

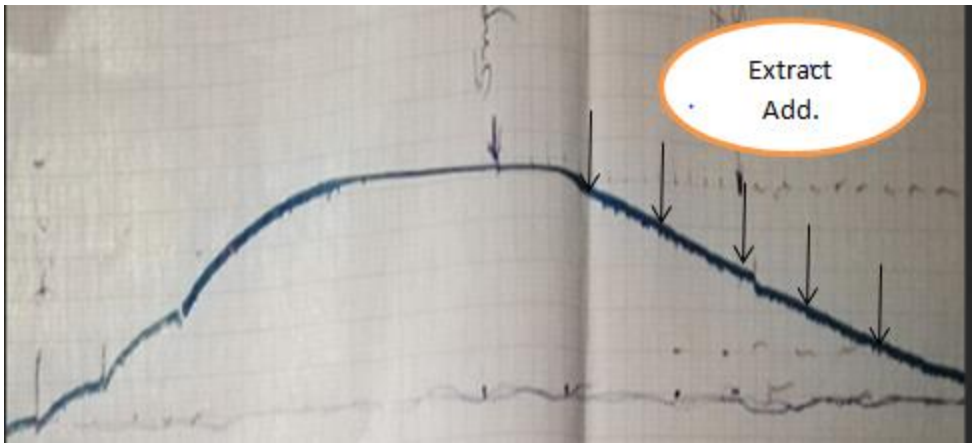

Figure 7: Typical tracing showing relaxant effect of *Otostegia integrifolia* on high K+ (80 mM)-induced contraction in isolated aorta of Sprague Dawley rat.

With lower concentrations particularly, 0.125mg/ml, and 0.25mg/ml, the observed percentages of relaxation on high K^+ (80 mM) induced contraction were not statistically significant. With subsequent increment in concentration, beginning at a dose of 0.5mg/ml, however, significant ($p < 0.001$) percentage relaxation was recorded. Relaxation began within a couple of seconds and extended to some more minutes with subsequent addition of the extract into the bath. This may indicate that the vasodepressor effect of the test substance followed both times and concentration-dependent pattern. After subsequent washout the tissue regained its activity, possibly suggesting that the effect of the extract is reversible (Table 6).

Table 6: Vasorelaxant effect of *Otostegia integrifolia* extract on Sprague Dawley rat 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
0.125	98.75 ± 0.05	1.25 ± 0.55
0.25	93.5 ± 1.98	6.5 ± 1.98
0.5	79.0 ± 2.54***	21.0 ± 2.54***
1	55.5 ± 2.61***	44.5 ± 2.61***
2	23.7 ± 2.41***	76.3 ± 2.41***
2.5	00 ± 00***	100.00 ± 00***

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

4.2.2. Possible mechanism of vasorelaxation

To find out the preliminary mechanism through which the test substance reduces BP; agonists, and antagonists of different receptors thought to be involved in the relaxation mechanism were employed. Vasodepressor effect of the extract was not affected following pre-incubation with atropine, indomethacin, glibenclamide, diphenhydramine, and phenylephrine. The effect was also not altered with removal or denudation of the endothelium from the aortic strip. However, the maximum observed relaxant effect of the extract was 45% following pre-incubation with methylene blue. Next, the involvement of calcium channel was assessed in a denuded aorta. For this purpose, a Ca^{2+} dose-response curve was constructed in Ca^{2+}-free high-K+(50 mM) medium through continuously raising Ca^{2+} concentration in the bath, so that a gradual increase in tension could be induced.

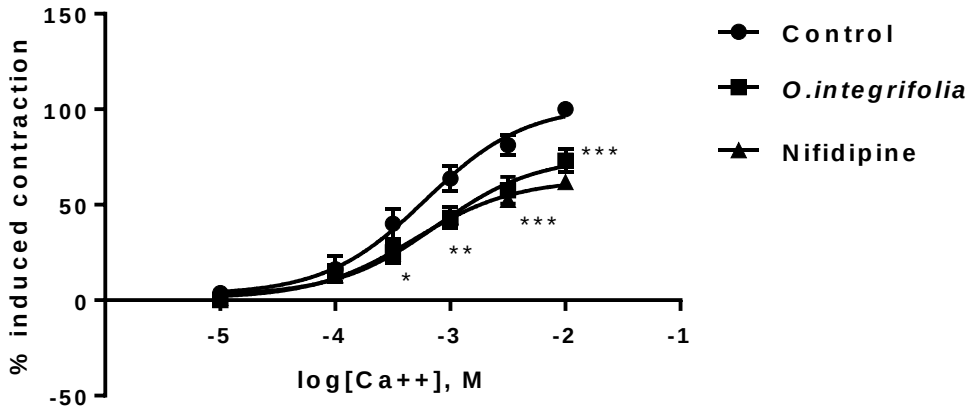

Figure 8: Concentration-response curve of Ca++ in the absence and presence of the highest concentration of crude extract of *Otostegia integrifolia* and nifedipine in isolated rat aortic ring preparations. (n = 4. *p < 0.05, **p < 0.01 and ***p < 0.001 compared to respective concentration values in the Ca++ control curve)

Pretreatment of the denuded aorta with cumulative maximum concentration (6.375mg/ml) of the extract for sixty minutes shifted the Ca^{2+} concentration-response curve ($p< 0.05$, $p< 0.01$ and $p< 0.001$) to the right with suppression of the maximum effect (Figure 8). The extract as well as the calcium channel blocker nifedipine prevented the Ca^{2+}induced maximum contraction of the aortic strips by 26.9% and 38.1%, respectively, from the control value ($p <0.001$). Furthermore, to make sure that whether the mechanism is only mediated with NO/cGMP and calcium channel pathway, the tissue was also pre-incubated with methylene blue and nifedipine, but the extract did not show any significant relaxation.

5. Discussion

Both the *in vivo* as well as the *ex vivo* experiments revealed that the plant extract is endowed with antihypertensive activity probably partly mediated through vasorelaxaton. The plant material was traditionally prepared by boiling the leaves with water then one cup of the solution will be drunk and the leaves will be eaten. Thus, to harvest both polar and medium polar principles of the plant, the leaves of *Otostegia integrifolia* were extracted using the universal solvent 80% methanol (Kaneria *et al.*, 2012).

In the induction of HTN, consistent increase in BP was not obtained after Day 5, although the animal was hypertensive. Fructose is known to cause up-regulation of the sodium hydrogen exchanger (NHE-3) and putative anion transporter (PAT-1) that leads to salt retention; endothelial dysfunction, which appears with up-regulation of the vasoconstrictor Ang-II, hyperinsulinemia; down-regulation of eNOS; SNS activation, which is a result of insulin resistance and hyperinsulinemia with subsequent vasoconstriction and via other mechanisms (Klein and Kiat, 2015). The BP would therefore be elevated, with the early days of ingestion through the aforementioned pathways. However, compensatory mechanisms are activated with continuous ingestion that would counteract the upsurge of BP that could possibly explain the decrease in BP with time. However, one should note that despite these counter mechanism, BP increment even at day 15 was statistically significant ($p < 0.001$) compared to that of the NRC, demonstrating that fructose-induced derangement overwhelms the regulatory pathways (Madero *et al.*, 2011). The ACE inhibitor captopril was used as a positive control for the experimental groups because the RAAS system is implicated in this model of HTN, as mentioned above.

The extract produced BP reduction beginning from day 5 up to day 15, where the maximum effect was obtained on Day 10 ($p< 0.001$) particularly with OI500 and OI1000. This may be due to the combined effect of body's compensatory mechanism against fructose induced upsurge of BP as mentioned earlier and the cumulative effect of the extract. The pattern observed with OI250 appeared to be different. Firstly, maximum reduction was obtained on Day 15. Secondly, significant decrease in BP was not noted on Day 10. The likely explanation could be smaller sample size and variation among individual values has precluded statistical significance. Moreover, there was no recorded significant difference between OI500 and OI1000 possibly

suggesting that 500mg/kg could be the maximum dose and further escalation of the dose might not be accompanied by an increase in response (Macheboeuf *et al.*, 2008).

The extract showed a statistically significant reduction in all the BP parameters. However, it exerted greater BP lowering effect on DBP than SBP. Maximum reduction was achieved in DBP (55%) with OI500 than SBP (45.3%) with the same dose. Such relatively strong effect of the extract on DBP than SBP could probably occur because 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 afterload. Having effect on both SBP and DBP offers an advantage. Clinically, lowering SBP reduces the risk of coronary heart disease in elderly individuals and lowering DBP reduces the risk more in young adults. Therefore, the antihypertensive property of the extract has a paramount importance since it can lower both SBP and DBP, thereby helps to reduce the risk of various cardiovascular disorders (Messerli *et al*., 2017).

A baseline 100% smooth muscle contraction was achieved during the *ex vivo* study conducted on isolated aortic rings. Addition of high concentration of K^+ (80mM) leads to opening of voltage-gated Ca2+ channels thereby influx of extracellular Ca^{2+} occurs (Gilani *et al.*, 2005). Sprague Dawley rats aorta were selected because the *in vivo* study was conducted in rats. To verify whether the BP lowering effect was due to the vasorelaxant effect of the extract, it is imperative to use the same animal. This helps to avoid inter-species variability. It is also helpful to assess the vasorelaxant effect on K^+ induced contraction, as this helps to evaluate whether the vasorelaxation produced by the extract was endothelium-dependent or not. Finally, it also helps to scrutinize the preliminary mechanism of action of the extract (Getiye *et al.*, 2016).

At lower concentrations specifically with 0.125mg/ml and 0.25mg/ml, the observed percentages of relaxation on high K^+ (80 mM) induced contraction were not statistically significant. Later on with increasing concentration of the extract significant ($p < 0.001$) relaxation was achieved beginning at doses of 0.5mg/ml (Table 6 and Figure 7). This may indicate that the *ex vivo* vasodepressor activity or inhibition of contraction increases with dose. Similarly, in the *in vivo* study increasing the dose of the extract also produced an increase in response, as it was seen between OI250 and OI500. This similarity in response in both studies may imply that observed *in vivo* antihypertensive effect of the extract may probably mediated through vasodilation of the

blood vessel, this further lowers the peripheral vascular resistance thus finally bears the fall in the BP.

The *ex vivo* study was not only destined to investigate the extracts vasorelaxant activity. Such types of studies are also usual tools to investigate the preliminary mode of action of the substance of interest. The principle of investigation was to expose the isolated thoracic aorta to different selective agonist and antagonist drugs through which either by ruling in or ruling out the respective pathways finally helps to narrow down and probably indicate the possible mechanism of action of the extract. Accordingly, the rat aortic rings were treated with muscarinic receptor antagonist (atropine). Acetylcholine via cholinergic muscarinic receptors mediates dilation of blood vessels by stimulating the release of NO from the endothelium. The synthesized NO diffuses to the nearby vascular smooth muscle cells thus activating the enzyme guanylate cyclase thereby causing relaxation of smooth muscle by increasing the level of cGMP in the tissues (Ayele *et al.,* 2010). This pathway was, however, excluded, as incubation with atropine didn't prohibit vasodilation.

The tissue was further treated with indomethacin, which is an inhibitor of cyclooxygenase, an enzyme responsible for the synthesis of prostacyclin from arachidonic acid. Prostacyclin is another mediator released from endothelium that mediates vasodilation. This treatment did not attenuate vasodilation, suggesting that the dilation does not involve prostanoids. To check for the involvement of histamine, diphenhydramine a histamine receptor antagonist was used, but vasodilation was not affected.

On the other hand, treating the tissue with methylene blue, which blocks the NO/cGMP pathway, produced a 45% relaxation of the aortic rings, indicating the role of NO at least in part in mediating vasodilation. To assess the involvement of endothelium, the extract was also tested in endothelium denuded aortic rings contracted with K^+ (80mM), but this didn't abolish the vasorelaxation produced by the same concentration of the extract as it was used in the intact tissue. Failure to abolish vasorelxation in endothelium denuded rats is in concordance with the ligand studies using atropine and indometachin, as both agents inhibit mediators that require intact endothelium for producing their effect. However, partial blockade of relaxation with methylene blue, which blocks the NO/cGMP pathway, in the face of attenuation of relaxation in denuded rats, strongly suggests that the source of NO is not the endothelium and methylene blue

could also act by blocking neuronal NO synthases which are known to be expressed in vascular smooth muscles (Villanueva and Giulivi, 2010). This assertion is further reinforced by the observation that blockade of histamine, which is known to cause vascular hyperpermeability and vasodilation via release of NO and PGI_2 from the endothelium (Ashina *et al.*, 2015), with diphenhydramine did not abrogate vasodilation.

To rule out the involvement of subcellular calcium and K^+ channel in the relaxation, phenylephrine, an α-1 receptor agonist, and glibenclamide, an inhibitor of ATP-dependent K^+ channel were used. Phenylephrine binding to α-1 receptor leads to stimulation of phospholipase C and the synthesis of secondary messengers: 1,4,5 triphosphate inositol (IP3) and diacylglycerol (DAG). IP3 binds to the sarcoplasmic reticulum membrane IP3 receptors and causes the release of Ca^{2+} from intracellular store (Fransen *et al.*, 2015). Inhibition of ATP-dependent K^+ channel by glibenclamide is known to cause depolarization and contraction (Niazmand *et al.*, 2014). Pretreated with each agent prior to contraction by K^+ (80 mM) did not affect *Otostegia integrifolia* mediated vasodepressor activity, ruling out the role of subcellular calcium and K+ channels in the observed effect.

After ruling out the involvement of the above mentioned mechanistic agents in the plant's mediated vasorelaxation, the role of calcium channels was investigated. Pretreatment of the tissue with the extract inhibited calcium-induced contraction by 26.9% and resulted in rightward non-parallel shift of Ca^{2+}-CRCs (Figure 6). This shift was similar to the one produced by nifedepine, a dihydropyridine calcium channel blocker, albeit the effect was smaller than the comparator, suggesting that the effect partly be mediated through blocking the calcium channel.

Collectively, the mode of action of the extract may hinge on two pathways. The first is the NO/cGMP pathway, as the relaxation achieved was only 45% upon blocking this route with methylene blue. The other route is via blocking the L-type voltage-gated calcium channel, since both the extract and nifedipine similarly shifted the Ca2+-CRC to the right with varying level of depression of maximal effect. To produce further evidence that the two pathways were indeed responsible for the observed effect aortic rings were pre-incubated with the respective blockers (methylene blue and nifedipine) and contracted with high K^+ (80 mM). The extract was able to produce a negligible or insignificant vasorelaxation. This observation increases the likelihood

that vasorelaxation might principally be mediated by the NO/cGMP pathway and blocking the voltage-gated L-type calcium channel.

One of the species from the genus *Otosetgia*, *O. persica* also possesses an antihypertensive activity (Takhtfooladi *et al.*, 2016). This reinforces the results of the study and may suggest that there might be similar constituents found in these two species. Besides, the experimental plant contains numerous secondary metabolites (Tesso and König, 2004). This could be cited as a possible explanation for the antihypertensive activity and probably for more than one mechanism of action of vasorelaxation.

In the *in vivo* experiment the effect of the extract on normotensive animals was not studied. However, the effect of the extract compared to NRC didn't produce significant BP reduction; this probably may suggest lack of hypotensive effect of the extract.

6. Conclusion

The result from the *in-vivo* experiment showed that the extract significantly lowered SBP and DBP as well as MAP, indicating the extract's promising antihypertensive activity with the method employed in this experiment. The *ex vivo* experiments also indicated that the extract produced a dose-dependent vasodepressor activity probably mediated through the blockade of L-type calcium channel as well as NO/cGMP pathways. Vasorelaxation appeared to be unlikely to be mediated by the cholinergic, prostanoid, histamine, ATP-dependent K^+ channel, sarcoplasmic reticulum stored Ca^{2+} and endothelium-dependent systems. The overall investigation of the plant *Otostegia integrifolia* extract for its antihypertensive and vasodepressor activity produced a result supporting the traditional claim.

7. Recommendations

Based on the present study the following recommendations are proposed:

- Since hypertension is a chronic disease chronic toxicity study should be performed to evaluate the long-term impact of the plant extract.
- The present investigations used dietary hypertension model using fructose to assess the antihypertensive effect of the extract, supporting this finding with other models such as chronic hypertension model further verify the result obtained from this study.
- The *ex vivo* study depicted that the probable mechanism of action is mediated via blockade of calcium channel and enhancement of NO/cGMP pathway thus, detailed mechanistic investigation is worth pursuing.
- Phytochemical investigations and isolation of compounds should be conducted to identify the active principles responsible for the observed activities of the plant extract.

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