Ex vivo **vasodilatory activity of aqueous crude extract of** ***Thymus schimperi*** **leaves in isolated thoracic aorta of Guinea Pigs**

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This is to certify that thesis prepared by Tadesse Alemayehu, entitled *Ex vivo* vasodilatory activity of aqueous crude extract of *Thymus schimperi* leaves in isolated thoracic aorta of Guinea Pigs and submitted in partial fulfillment of the requirements for the Degree of Masters complies with the regulations of the University and meets the accepted standards.

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External examiner_____ signature _____date_____

Chair of department _____signature _____date_____

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List of Abbreviations & Acronyms

Ach	Acetylcholine
ARBs	Angiotensin receptor blockers
ATP	Adenosine triphosphate
AQ	Aqueous
CCBs	Calcium channel blockers
CNS	Central nervous system
CVD	Cardiovascular diseases
CM	Calmodulin
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanine monophosphate
DBP	Diastolic blood pressure
E^-	Without endothelium
E^+	With endothelium
EC_{50}	Effective concentration - 50
EDH	Endothelium-derived hyperpolarization
EDHF	Endothelium derived hyperpolarizing factor
EPI	Epinephrine
GLYB	Glybenclamide
GPCR	G-protein coupled receptor
HIST	Histamine
HTN	Hypertension
IP_3	Inositol1, 4, 5-triphosphate
KCl	Potassium chloride
MB	Methylene blue
MBP	Mean blood pressure
MLC	Myosin light chain
MLCK	Myosin light-chain kinase
MLCP	Myosin light chain phosphatase
NO	Nitric oxide

PLC	Phospholipase C
SBP	Systolic blood pressure
SR	Sarcoplasmic reticulum
TB	Tuberculosis
VSM	Vascular smooth muscle

Abstract

***Ex vivo* vasodilatory activity of aqueous crude extract of *Thymus schimperi* leaves in isolated thoracic aorta of Guinea Pigs**

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Addis Ababa University, 2018

Hypertension remains a major health problem globally because of its impact on mortality and morbidity. It is defined as a persistent increase of systemic blood pressure. Blood pressure is created by the force of blood pushing against the walls of blood vessels as it is pumped by the heart. It is a multiplication product of cardiac output and peripheral vascular resistance. The goal of any antihypertensive therapy is to bring a reduction in either or both of these parameters, preferably peripheral vascular resistance. *T. schimperi* is one of the medicinal plants used in Ethiopia for treatment of hypertension and other diseases. But it lacks apposite pharmacological investigation for its vasodilatory effect. Thus, the aim of this study was to evaluate *in vitro* vasodilatory effects of aqueous crude extract of *T. schimperi* leaves in isolated thoracic aorta of guinea pigs using different contracting agents such as potassium chloride, epinephrine, methyline blue, glibenclamide, acetylcholine and histamine. Furthermore, the possible mechanism (s) of relaxation was also assessed. In the study guinea pig was sacrificed by gentle cervical dislocation and the thoracic aorta ring was removed, cut spirally and mounted in an organ bath containing 37^{o}C maintained Kreb's Henseleit physiological solution aerated with 95% O_2 and 5% CO_2 for experiment. The extract showed a relaxation effect in isolated thoracic aorta of guinea pigs pre-contracted by potassium chloride, epinephrine, glibenclamide, acetylcholine and histamine in dose dependent manner. But the extract failed to show significant relaxation effect of aorta pre-contracted by methyline blue on intact endothelium as compared to potassium chloride (intact and denuded endothelium), epinephrine, glibenclamide, acetylcholine and histamine (P<0.001). High doses of AQ crude extract have shown substantial vasodilatory activity against HIST and Ach-induced contractions of guinea pig thoracic aorta on denuded endothelium. In conclusion , the mechanisms of action of the extracts may be through blockage of both receptor-operated and L-type voltage dependent Ca^{2+} channels, activation of ATP-sensitive potassium channel, blockage of H_1 and M_3 receptors.

Keywords: *Thymus schimperi*, *Ex vivo* study, aortic strips, endothelium, vasodilatory effect, guinea pig, plant extract

1. Introduction

1.1 Background

Hypertension has been defined by the World Health Organization as a persistent increase of systemic blood pressure > 140 mm Hg systolic or > 90 mm Hg diastolic, or both. Systemic hypertension is one of the most prevalent and serious causes of coronary artery and myocardial disease globally (Alamgeer et al., 2015).

Hypertension can be classified as essential (primary) and secondary. Essential hypertension is the one in which the underlined cause is unknown; while secondary hypertension results from conditions, such as kidney disease or certain tumors (especially of the adrenal gland) (Chobanian et al., 2003).

Hypertension remains a major health problem globally because of its mortality and morbidity (Alamgeer et al., 2015). It is the most important modifiable risk factor for cardiovascular, cerebrovascular and renal disease (Guteta et al., 2015). It has been named the "silent killer," as it is asymptomatic (Geleta et al., 2015).

Hypertension is a leading cause of cardio vascular disorder such as myocardial infarction and stroke worldwide (Geleta et al., 2015). It is the most common cardiovascular disease including myocardium infract, renal insufficiency, stroke (Sakat et al., 2009; James et al., 2014) atherosclerosis, coronary artery disease, heart failure, and dissecting aneurysm of aorta (Sakat et al., 2009).

Hypertension leads to complications with considerable morbidity and mortality which is responsible for at least 45% of deaths due to heart disease and 51% of deaths due to stroke. Virtually every organ system of the body receiving circulatory flow is at risk for complications stemming from hypertension. Despite extensive research in the past several decades, hypertension-related mortality continues to increase worldwide (Du et al., 2016). Cardiovascular diseases account for 12 million deaths, annually worldwide and are known to be number one group of 'killer disease' (Sakat et al., 2009). About 80% of the global burden of cardio vascular death occurs in low and middle income countries. This is nearly as many deaths as caused by

HIV, malaria, and TB. This makes hypertension the single most important cause of morbidity and mortality globally and highlights the urgent need of action to address the problem. Hypertension was almost non-existent in African adult societies in the first half of the twentieth century, the prevalence increased significantly over the past two to three decades to more than 40% (about 80 million) and projections based on current epidemiological data suggest that this figure will rise to 150 million by 2025. WHO projects that over the next 10 years Africa will experience the largest increase in death rates from cardiovascular diseases (CVD) and therefore the negative economic impact of CVD will be more felt on the continent (Geleta et al., 2016**).**

In 2008, worldwide, approximately 40% of adults aged 25 and above had been diagnosed with hypertension; the number of people with the condition rose from 600 million in 1980 to 1 billion in 2008. Hypertension affects about 1 billion people globally **(**Haji et al., 2016**;** Guteta et al., 2015; Talha et al., 2011; Raj et al., 2015**)** and accounts for approximately 7.1 million deaths each year (Haji et al., 2016**;** Alamgeer et al., 2015**;** Abera et al., 2014), i.e., 13% of all deaths globally (Abera et al., 2014). Hypertension is prevalent not only among developed nations but also in developing countries (Haji et al., 2016); it is reported to be the fourth contributor to premature mortality in developed countries and the seventh in developing countries (Talha et al., 2011; Raj, 2015). It is 1.5 to 2.0 times more common in patients with diabetes than without diabetes (Haji et al., 2016).

In sub Saharan Africa countries like Ethiopia, published information on the prevalence of hypertension is sparse. From the studies done in Ethiopia, Kenya, Nigeria and Tanzania, the reported prevalence of hypertension ranged from 10.1% in Southern Ethiopia to 23.7% in Tanzania. Previous reports from Ethiopia on prevalence of hypertension were as high as 31.5% and 28.9% among males and females respectively in Addis Ababa (Guteta et al., 2015). Although there are shortages of extensive data, 10.5% of the Ethiopian population has been estimated to have HTN. Approximately 30% of adults in Addis Ababa have BP above 140/90mmHg or reported use of antihypertensive medications (Abera et al., 2014).

According to Guteta et al. (2015), one in four adults aged 18 and above (30.2% in male, 21.2% in females) has hypertension in Addis Ababa where the population is young with a mean age of

36.9 years. Moreover, the prevalence of prehypertension in this population is 47.3% which is quite alarming. The prevalence of hypertension in this study was significantly higher in men than women. It increased significantly with increasing age by a prevalence of about 10% each decade of life; 6.2% in those in 18-24 age groups and 59% in 65 years and above which may be explained by the increasing arterial stiffness with increasing age (Guteta et al., 2015).

1.2 Pathophysiology of hypertension

Blood pressure is created by the force of blood pushing against the walls of blood vessels (arteries) as it is pumped by the heart. It is a multiplication product of cardiac output and peripheral vascular resistance. Cardiac output (CO) and peripheral vascular resistance (PVR) are determined by interaction of multiple factors like genetic, environmental, and demographic factors. Hence, being a function of these variables, the BP level of an individual is indeed a complex characteristic. Cardiac output is mainly dependent on blood volume and PVR is determined chiefly by neural and hormonal factors acting at the level of the arterioles (Raj et al., 2015).

More than 90% of cases of hypertension do not have a clear cause. Hypertension clusters in families and results from a complex interaction of genetic and environmental factors. The hypertension-related genes identified to date regulate renal salt and water handling. Major pathophysiologic mechanisms of hypertension include activation of the sympathetic nervous system and renin–angiotensin–aldosterone system. Many pathophysiologic factors have been implicated in the genesis of essential hypertension like: increased sympathetic nervous system activity; overproduction of sodium-retaining hormones and vasoconstrictors; long-term high sodium intake; inadequate dietary intake of potassium and calcium; increased or inappropriate Renin secretion with resultant increased production of Angiotensin II and A1ldosterone; deficiencies of vasodilators, such as prostacyclin, nitric oxide (NO), and the natriuretic peptides; diabetes mellitus; insulin resistance; obesity; increased activity of vascular growth factors; alterations in adrenergic receptors that influence heart rate, inotropic properties of the heart, and vascular tone; and altered cellular ion transport (Oparil et al., 2003).

Vascular endothelial dysfunction, which is characterized by an uncoupling between the release of endothelial factors such as nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarization (EDH), as well as effects on endothelium-dependent contractile mechanisms, and the associated change in vascular smooth muscle tone (Luna-Vázquez et al., 2013**).** Endothelial dysfunction, increased vascular reactivity, and vascular remodeling may be the causes, rather than consequences, of blood pressure elevation; increased vascular stiffness contributes to isolated systolic hypertension in the elderly (Geleta et al**.,** 2016**)**. This requires the heart to work harder than normal to circulate blood through blood vessels **(**Alamgee et al., 2015**).** Chronic elevation of arterial pressure (hypertension) is a consequence of the vascular endothelium lesion, reduction of vasodilator (nitric oxide, prostacyclin) and predominance of vasoconstrictor (endothelin) endothelial mediators (Mesa, 2014).

1.3 Risk factors for hypertension

The major identified risk factors for HTN include demographic (sex, age and residence) and Behavioral (tobacco use, alcoholism, khat chewing, low fruit and/ or vegetable intake and little physical inactivity) and biological factors. Studies have indicated that these risk factors are widespread globally. On the other hand it is shown that level of proximate demographic factors: education, occupation and income affect tobacco use, physical activity and dietary habit. Demographic factors: age, gender and residence were shown to be associated with HTN and reported factors include smoking, khat chewing, physical inactivity and low fruit and/ or vegetable intake in Ethiopia. Most of the risks are attributable to lifestyle and behavioral patterns, and can be changed. If the emergence and prevention of risk factors are left undirected, growth of the problem will continue accelerating (Birlew and Alemseged, 2015). The increasing prevalence of hypertension is attributed to population growth, ageing and behavioral risk factors, such as unhealthy diet, harmful use of alcohol, lack of physical activity, excess weight and exposure to persistent stress (Guteta et al., 2015). In most studies, the prevalence of hypertension is higher in urban areas compared with rural counter parts; this could be due to the life style, increased stress level, urbanization and due to decrease physical activity (Molla, 2015). In the last few years, life style of the Ethiopian population is changing due to urbanization and demographic transition. As a result the burden of HTN could be on the rise (Birlew and Alemseged, 2015).

1.4 Treatment of hypertension with modern drugs

As a first principle, one should always couple any drug therapy with lifestyle modifications (maintaining ideal body weight, engaging in aerobic physical exercise, eating a healthy diet low in saturated and total fats, limiting sodium intake, abstaining from any substance abuse including tobacco and reducing alcohol intake). Each of these lifestyle modifications has been shown to reduce blood pressure modestly. These modifications are inexpensive and pose very little risk (Baker, 2005).

Many antihypertensive drugs act by producing direct dilation on blood vessels (α_1-adrenegic block, release of NO in situ, calcium channel blockers, and α_2-adrenegic agonist) **(**Geleta et al., 2016**).** Clinically, various antihypertensive drugs such as Angiotensin converting enzyme inhibitors, Angiotensin receptor blockers, diuretics, Calcium channel blockers, β blockers, alpha-$_1$ blockers, central α-$_2$ agonists, non-selective α and β blockers, and direct vasodilators have been used to manage hypertension and to alleviate symptoms**.** However, the efficacy of these drugs is only 40-60% and usually two or more antihypertensive drugs from different categories are needed to be combined to achieve the optimal results **(**Haji et al., 2016; Geleta et al.**,** 2016**).** Moreover, side effects from these medications are important concerns **(**Geleta et al., 2016**).** All these ultimately increase the cost of treatment **(**Haji et al., 2016**).**

Despite prescription of several appropriate antihypertensive drugs at adequate doses and strictly following guidelines **(**Mancia et al., 2013**),** the control of blood pressure is still not achievable in a significant proportion of hypertensive patients (Monge et al., 2013). Morethan 50% of treated hypertensive patients have a BP level greater than140/90 mmHg **(**Geleta et al.**,** 2016**;** Abera et al., 2014**).** Treatment is usually lifelong. Therefore, drugs must be effective and safe over a long period of use **(**Geleta et al.**,** 2016**).** The frequent side effects of synthetic antihypertensive drugs includes dry mouth, dizziness, visual disorders, headache, cough, emotional distress, gastrointestinal disturbance, peripheral circulatory symptoms like cold hands, feet, and swollen ankles. These distressing side effects can lead to non-compliance and adversely affects health related quality of life. Hence, newer antihypertensive agents are needed to expand therapeutic options, increase treatment efficacy, decrease side effects, and enhance patient adherence **(**Haji et al., 2016**).**

Many new drugs have been introduced which may demonstrate better efficacy but posses side effects **(**Sakat et al., 2009**).** Thus there is a need to explore alternative therapies particularly from plant sources as these are cost effective and possess minimal side effects **(**Haji et al., 2016**).** Recently attention has been given towards plant and mineral preparations which are traditionally used as potential therapeutic agents in the prevention and management of cardiovascular diseases (Sakat et al., 2009**).**

1.5 Treatment of hypertension with medicinal plants

Medicinal plants are the source of traditional treatment for many diseases and ailments throughout the developing world (Debelo et al., 2015). Plants have provided effective sources of traditional medicines against many ailments since ancient times. Peoples of all continents, especially in Africa and Asia, with their diverse culture and rich plant flora, used folklore medicine for their health needs. Medicinal plants contain various pharmacologically active compounds which have useful therapeutic applications and many are utilized in drug development. About thirty percent of the drugs sold worldwide contain compounds derived from plants (Geleta et al., 2016).

Medicinal plants are used in many countries as an alternative to synthetic drugs. They are high natural source of medicinal products used in traditional medicine and chemical entities for modern drugs. According to WHO, the best source to obtain variety of drugs are medicinal plants. Medicinal value of the plant relies on the presence of different phytochemical components (tannins, alkaloids, terpenoids and phenolic compounds) that bring particular physiological effect in human body (Javed et al., 2013).

Ethiopia has high diversity of plant species (6500 to 7000 species of higher plants) making the country one of the most diverse floristic regions in the world. Most of these plant species are used in traditional medicine and often quoted as one of the six countries in the world where about 60% of plants are said to be indigenous with their healing potential. In Ethiopia, 90% of the population uses traditional medicine to meet their primary healthcare needs (Birhanu et al., 2015).

Various plant preparations have been used and claimed to have benefit for hypertension in the folk medicine. Ethiopian traditional medicine is composed of a number of specific skills, such as, use of plants, animal products and minerals as well as magic and superstition. The main body, however, is based on the use of ethno botany (Geleta et al., 2015). *Thymus schimperi* is one of such plants used in Ethiopia for treatment of hypertension (Eshetu et al., 2016).

Even though, several allopathic antihypertensive medications are available, most people living in developing countries depend on traditional medicine (Haji et al., 2016). *Thymus serrulatus* (Geleta et al., 2015), *Moringa stenopetala* (Geleta et al., 2016), *Tribulus terrestris* (Fatima et al., 2015; Mohd et al., 2012), *Borago officinalis* (Gupta and Singh, 2010), *Raphanus sativus (*Sham et al., 2012), *Passiflora edulis* (Elangovan et al., 2016), *Mammea africana* (Talha et al., 2011; Okokon and Davies, 2014), *Alpinia purpurata* (Victório et al., 2009) and *Myrtus communis* (Janbaz et al., 2013), are among some of the medicinal plants which have been reported to have antihypertensive and *in vitro* vasodilatory effects.

1.6 Mechanisms of dilation and contraction of blood vessels

Vasoconstriction is the process by which the smooth muscle of blood vessels get contracted leading to increased blood pressure. It is regulated by vasoactive substances, such as noradrenaline, angiotensin II, endothelin and thromboxane. Vasodilation is the opposite physiological process, i.e., process by which the smooth muscle of blood vessels get relaxed resulting in decreased blood pressure. One of the most prominent vasodilators is nitric oxide termed endothelium-derived relaxing factor (Versari et al., 2009).

In response to a nerve impulse or hormonal signal reaching the vascular smooth muscle cell, an influx of extracellular calcium ions occurs, increasing the intracellular calcium concentration and causing contraction. Elevation of intracellular calcium can activate the protein calmodulin. The resulting calcium-calmodulin complex can bind to MLCK, activating the enzyme. As a result, MLC become phosphorylated, and myosin can interact with actin to cause contractions. In addition, the tails of the myosins straighten out and can assemble with other myosin molecules into filaments. As the calcium levels within smooth muscle cells drop again, the MLCK is inactivated, and a second enzyme, MLCP, removes the phosphate group from the myosin light

chain. Since the dephosphorylated myosin can no longer bind to actin, the vascular smooth muscle cell relaxes (Hardin et al., 2012).

1.6.1 Mechanisms of dilation of blood vessels

The endothelium is a single-cell layer that lines all arteries and veins **(**Kaur et al., 2011**)**. It contributes to the tone of vascular smooth muscle (Kaur et al., 2011; Hamdy et al., 2001) by releasing endothelial-derived factors that relax arterial smooth muscle such as nitric oxide, prostacyclin and endothelium-derived hyperpolarizing factor **(**Luna-Vázquez et al., 2013; Jerca et al., 2002; Hamdy et al., 2001**)**, which is associated with calcium-activated potassium channel activation. The K^+ channels in vascular smooth muscle play an important role in vasodilation because the outflow of K^+ through these channels hyperpolarizes the membrane and thereby inhibits the entry of Ca^{2+}. This process eventually results in the relaxation of blood vessels (Luna-Vázquez, 2013, Jerca et al., 2002).

The vascular endothelium mediates vasodilation in response to various stimuli including shear stress and the neurotransmitter, acetylcholine (ACh) (Kaur et al., 2011; Jerca et al., 2002). In the vasculature, nitric oxide (NO) is produced from the endothelium mainly by endothelial NO synthase (eNOS), which is activated by agonists such as bradykinin and acetylcholine or by shear stress produced by the flowing blood (Li, 2012). ACh binds to muscarinic cholinergic receptors located in the endothelial cell membrane which initiates the synthesis and release of the autocoid, nitric oxide (NO) (Kaur et al., 2011). Nitric oxide is the most important vasodilator substance responsible for endothelium dependent vasodilation. After its secretion from the endothelium, it diffuses to the adjacent smooth muscle cells and stimulates the guanylate cyclase enzyme to produce cyclic guanosine 3, 5-monophosphate **(**Hamdy et al., 2001**)**. cGMP is a second messenger system that leads to activation of calcium pumps embedded in the plasma membrane and sarcoplasmic reticulum. The calcium pumps effectively lower the intracellular calcium concentration causing relaxation of VSM and dilation of the blood vessels **(**Kaur et al., 2011**).**

Cyclic nucleotides can dilate vascular smooth muscle, either by phosphorylation of myosin light chain kinase or by acting in other ways to reduce free cytosolic calcium (Duarte et al., 1993).

Endothelium-derived factors play a key role in the mechanisms of action of vasodilators. The mechanisms of action most frequently assessed in the vasodilator effects of the plant compounds were activation of the NO/cGMP pathway, blockade of Ca^{2+} channels, and activation of K^{+} channels (Luna-Vázquez, 2013).

Numerous Chinese medicinal plants, herbal preparations, or isolated compounds thereof have been shown to stimulate endothelial NO production, which is likely to be a contributing mechanism for their therapeutic effects **(**Li, 2012**).**

Most compounds that have shown vasodilatory activity are alkaloids (Luna-Vázquez, 2013), flavonoids (Luna-Vázquez, 2013; Duarte et al., 1993), terpenoids (Luna-Vázquez, 2013; Menezes et al., 2010), Glycosides (Jansacul et al., 1997) and phenolic compounds (Takahara et al., 2005; Victório et al.,2009). The classes of compounds obtained from plants according to the major mechanism(s) of action involved in their vasodilator effect are shown in Figure 1.

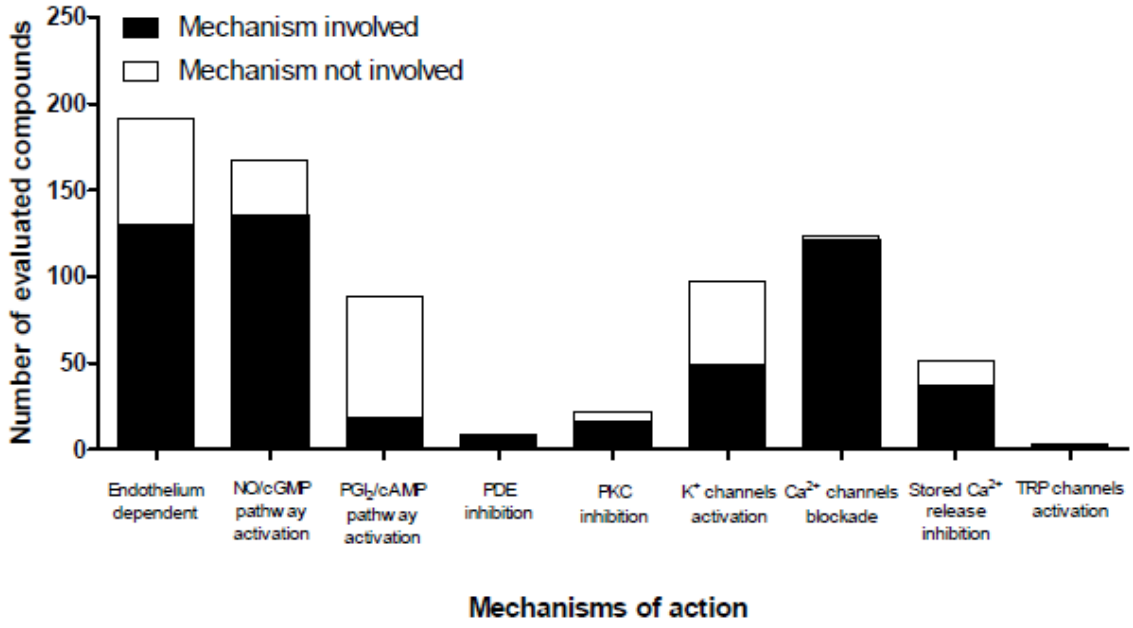

Figure 1**.** Classification of compounds obtained from plants according to the main mechanism(s) of action involved in their vasodilator effect (Luna-Vázquez, 2013).

1.6.2 Mechanisms of contraction of blood vessels

The mechanism of vascular smooth muscle contraction involves the participation of different signal transduction pathways, all of which converge to increase cytoplasmic Ca^{2+} concentrations. The concentration of this cation increases both by extracellular Ca^{2+} entering through voltage operated Ca^{2+} channels (VOCCs) and receptor-operated Ca^{2+} channels (ROCCs), and by the release of Ca^{2+} from intracellular stores. Therefore, the mechanisms of action associated with vasodilating agents that decrease intracellular Ca^{2+} concentration involve blocking VOCCs and ROCCs or inhibiting release of this cation from intracellular stores.

The mechanism of vascular smooth muscle contraction evokes the phosphorylation of myosin light chain by increasing intracellular Ca^{2+} concentration. Additionally, decreasing myosin light chain phosphatase increases the sensitivity to Ca^{2+} (Luna-Vázquez, 2013). PKC (protein kinase C) has been proposed to play a key role in maintenance of tonic contractions of vascular smooth muscle although the mechanism involved is not well defined **(**Duarte et al., 1993**).** PKC has been found in high concentrations in vascular smooth muscle and can be activated by diacylglicerol (Luna-Vázquez, 2013).

Agents such as potassium chloride (KCl), epinephrine (EPI), methylene blue (MB) glibenclamide (GLIB), histamine (HIST), and acetylcholine (ACH) induce vascular contraction through different mechanisms.

KCl bypasses GPCRs and induced contractions are the result of an increased Ca^{2+} influx through voltage stimulated type-L Ca^{2+} channels **(**Duarte et al., 1993**)**, which results in increasing cytosolic-free Ca^{2+} ($[Ca^{2+}]i$), Ca^{2+}-calmodulin-dependent MLCK activation, and MLC phosphorylation and contraction (Ratz, 2005). EPI also activates smooth muscle by a highly reproducible and relatively "simple" mechanism involving activation of receptor operated Ca^{2+} channels that leads to increase in cytosolic-free Ca^{2+} ($[Ca^{2+}]i$), MLC kinase activation, and MLC phosphorylation and contraction (Waugh, 1962). MB inhibits guanylate cyclase enzyme and decreases the synthesis of cGMP, thereby preventing MLC dephosphorylation and contraction. In addition, it might interact directly with endothelium-derived relaxing factor and cause inhibition of the endothelium-independent relaxation (Martin, 1985). On the other hand, GLIB

inhibits the ATP-sensitive potassium channel opening that causes contraction as shown in Figure 2 (Nakanishi, 1993).

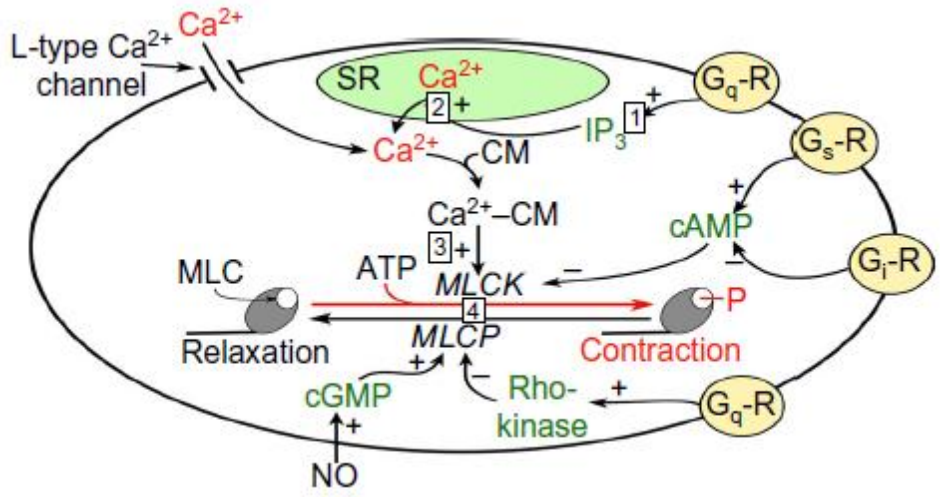

Figure 2. Mechanism of contraction by contracting agents.

Notes: 1, the contracting agents bypass GPCR and activate smooth muscle; 2, activation of receptor-operated Ca^{2+} channels that lead to increment in cytosolic free Ca^{2+} ($[Ca^{2+}]i$); 3, Ca^{2+}-calmodulin-dependent MLC kinase activation; 4, MLC phosphorylation and contraction (Klabunde, 2011).

Four subclasses of G protein-coupled receptors (H_1, H_2, H_3 and H_4) mediate the actions of histamine. In general, activation of H_1 receptors on vascular smooth muscle results in vasoconstriction while H_2-receptor activation mediates vasodilation. H_3 receptors are described as modulators of histamine synthesis and release in the CNS, therefore, primarily function in modulation of neurotransmitter. H_4 receptors possess a limited expression; they are expressed in haemopoetic cells, involved in immunne response and are targets of particular interest in immunomodulatory therapies. Histamine-induced contraction of rabbit aorta is dependent to a large extent upon Ca^{2+} entry from the extracellular space. (Ebeigbe and Talabi, 2014).

Histamine could activate H_1 histaminergic receptors on the endothelium and in turn release EDRF in an autocrine fashion or, by activating histaminergic receptors on vascular smooth muscle, cause a contraction. Histamine released can therefore potentially be considered an endothelium-derived contracting factor (Versari et al., 2009).

In general, the effect of histamine on a specific regional vasculature could be best described as a result of its multiple effects on smooth muscle and the lining endothelium. H_1 and H_2 receptors on vascular smooth muscle mediate direct constriction and relaxation, respectively, while endothelial H_1 receptors promote vasorelaxation via release of endothelial-derived relaxing factor (EDRF: i.e. nitric oxide) and/or prostacyclin. It is supposed that the transmembrane signaling mechanisms are involved in the different effects of histamine on vascular smooth muscles (Vodenicharov A, 2012).

Findings suggest that the contraction is mediated by histaminergic H_1, receptors, while H_2, receptors located in smooth muscle are involved in the amine-induced relaxation. Removal of endothelium abolished the relaxant response of human coronary arteries to substance P or low concentrations of histamine (Toda, 1987). Pretreatment with NO synthase inhibitor, L-NG nitroarginine methyl ester (L-NAME, 0.3 mM, 60 min), or removal of the endothelium completely inhibit histamine-induced vascular relaxation (Ashina et al., 2015).

In the human artery, histamine appears to act on H_1, receptors in endothelium and produce relaxing factor(s), and on H_1, and H_2 receptors in smooth muscle, which elicit contractions and relaxations, respectively. Histamine-induced contractions in human conduit coronary arteries were potentiated by impairment of endothelium and blockade of H_2 receptors. The possible mechanisms of histamine action in human coronary arteries are depicted in Figure 2.

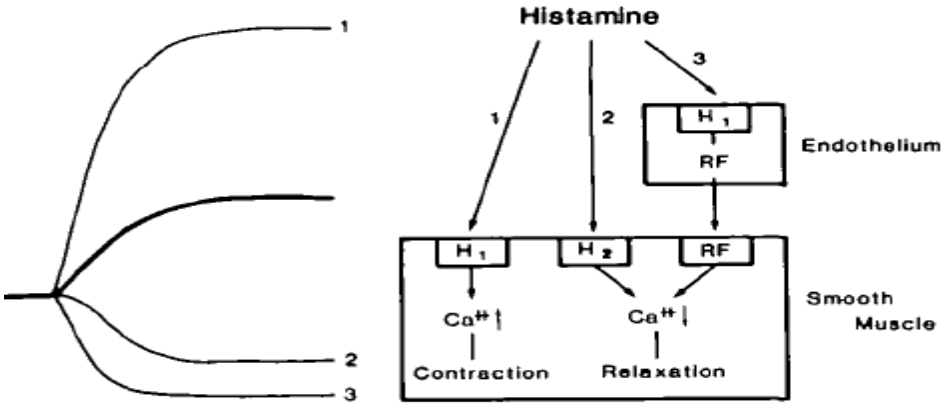

Figure 3. Schematic presentation of possible mechanisms of histamine action in human coronary arteries. Squares in endothelium and smooth muscle represent histaminergic H_1, and H_2 and

relaxing factor receptors. Possible responses mediated by different sites of action, 1 to 3 in right side, are demonstrated in the left side. Heavy line represents observed response to hislamine. RF, relaxing factor (Toda, 1987).

Acetylcholine (Ach) is an endothelium dependent vasodilator; the peak relaxation to ACh is used as an indicator for the presence of functioning endothelium (Ddaugirdas and Nnawab, 1987).

In 1980, Furchgott and Zawadzki, established the necessity of an intact vascular endothelium in order to obtain a vasodilatation induced by acetylcholine. From this moment, vascular endothelium demonstrated to have an active role in the vascular homeostasis regulation. Later, these scientists pointed out an endothelial relaxation factor EDHF liberated by intact endothelium in the presence of acetylcholine. When the endothelium is impaired, acetylcholine produces constriction as a result of the vasoconstrictor effects on the smooth muscle, which aren't counteracted. This was identified as nitric oxide, because both have similar chemical and biological properties (Suciu, 2009).

Acetylcholine by acting on autonomic ganglia stimulates the release of acetylcholine and catecholamines from postganglionic parasympathetic and sympathetic nerve terminals respectively. It also causes the release of adrenaline by adrenal medulla (Dow University of Health Sciences, 2014).

Some major vasoactive mediators; nitric oxide, EDHF and prostanoids are supplied by endothelial cells upon stimulation of their muscarinic receptors (Ockenga et al., 2013). When endothelium is damaged, acetylcholine directly acts on vascular smooth muscles and produces vasoconstriction (Dow University of Health Sciences, 2014).

The muscarinic receptors belong to the G-protein coupled receptors (GPCR) family. In mammals, at least five different muscarinic acetylcholine receptor subtypes (mAChRs; m_1 through m_5) are known to be widely expressed and distributed in different tissues from different species. They mediate distinct physiological functions according to their location and receptor subtype *(*Brass D, 2010).

The functions of the different muscarinic receptor subtypes in the non-neuronal cholinergic system are diverse, depending on the distribution of single receptor subtypes in different tissues. Cells expressing mAChRs can be found virtually everywhere in the body. They are present e.g., in the epithelial layer of the airways, the skin, the immune system, the urinary bladder, reproductive organs, connective tissue, muscles, tendons and vascular endothelial cells (Ockenga et al., 2013). M_1 muscarinic receptors mediate slow EPSP (excitatory postsynaptic potential) at the ganglion in the postganglionic nerve, and are common in exocrine glands and in the CNS. They are predominantly found acting via G proteins of class G_q. M_2 muscarinic receptors are expressed in the heart, where they act to slow the heart rate down to normal sinus rhythm by slowing the speed of depolarization after stimulatory actions of the parasympathetic nervous system. They also reduce contractile forces of the atrial cardiac muscle, and reduce conduction velocity of the atrioventricular node (AV node). M_2 muscarinic receptors act via G proteins of class G_i. M_3 muscarinic receptors are located in the smooth muscles of the blood vessels, as well as in the lungs. M_3 receptors act via G proteins of class G_q. M_4 muscarinic receptors are found mainly in the CNS. These receptors act via G proteins of class G_i. The M_5 muscarinic receptors distribution is not well known. Like the M_1 and M_3 receptors, M_5 receptors are coupled with G proteins of class G_q (Brass, 2010).

The M_1, M_3 and M_5 receptor subtypes preferentially couple to $G_{q/11}$, whereas M_2 and M_4 receptors couple to $G_{i/o}$. Depending on the class of the G protein involved, different downstream effectors are activated in a cell upon receptor stimulation. Through their linkage to $G_{q/11}$, M_1, M_3 and M_5 receptors predominantly activate PLC via α-subunit. The activation of PLC results in production of diacylglycerol (DAG), which is generated together with inositol trisphosphate (IP_3) upon the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by PLC. This path facilitates the mobilization of intracellular Ca^{2+} and activation of the protein kinase C (PKC). On the other hand, $M_2$and M_4 receptors mainly inhibit the adenylyl cyclase through their corresponding G proteins, leading to a decrease in cAMP levels (Ockenga et al., 2013).

Bioactive chemicals from plant extract and herbal medicine exert this effect they influence on one or more of these pathways. Among plants known to have vasorelaxant effect is thymus.

1.7 Distribution of thyme in Ethiopia

Thymus species are among the medicinal plants commonly used in Ethiopia (Debelo et al., 2016; Dejene et al., 1998**).** The genius Thymus includes about 350 species worldwide (Debelo et al., 2016; Dejene et al., 1998**;** Asfaw et al., 2000) and is distributed widely in temperate zones **(**Hailemariam and Emire, 2013; Debelo et al., 2016; Dejene et al., 1998**;** Asfaw et al., 2000**).** Ethiopia has considerably abundant Lamiaceae family herb growing at different regions and possesses a variety of the wild growing species of this family. Many species belonging to different genera of the family Lamiaceae have been reported to be found in different parts of the country. The two species, *T. schimperi Ronniger* and *T. serrulatus Hochst.ex Benth*, are the endemic species represented in Ethiopia while *T. vulgaris* is a species, native to Southern Europe. *Thymus schimperi* is wild growing species of thyme and comparatively well-known in Central, Eastern and Northern Ethiopia. Wild thyme of *T. Schimperi* is harvested and dried by people living close to the town of Dinsho and near Menz (North Showa), put in plastic bags and sold to travelers on buses. (Hailemariam and Emire, 2013).

One of the plants used for hypertension in Ethiopia is *T. schimperi Ronniger*. *T. schimperi Ronniger* locally known as 'Tossign' (Amharic) is endemic to the Ethiopian highlands, growing on edges of roads, in open grassland, on bare rocks and on slopes, between 2200-4000 m altitudes. It is perennial herb, woody at the base and 5- 40 cm high **(**Haji et al., 2016; Geleta et al., 2015**).**

1.8 Traditional uses and chemical composition of thyme

The leaves of Thymus are used in Ethiopia as spices to flavor a wide range of food products as well as medicines **(**Debelo et al., 2016; Dejene et al., 1998**).** They are extensively used by local people as food preservatives, cure for various ailments and food flavoring and seasonings (Nasir et al., 2015).

In the Ethiopian traditional medicine the plant has many medicinal applications, such as for the treatment of inflammation, spasm, thrombosis, mental illness, eye disease, toothache, leprosy, lung TB, acne, ascariasis, pain, to wash skin and used as mouth wash. Thyme is prepared as infusion to treat spasmodic cough, laryngitis, bronchitis, urinary infections, renal diseases,

hypertension, and *Tinea capitis*. It is also used as a decongestant, to reduce flatulence and to fight parasites. External uses of thyme include preparations to wash skin wounds or infections **(**Debelo et al., 2016). In Ethiopia the leaf of *T. schimperi* used in traditional medicine for the treatment of headache, cough, stomachache, earache, liver disease, gonorrhea **(**Asfaw et al., 2000**),** fungal infections, bacterial infections (Pagiotti et al., 2010; Asfaw et al., 2000), urinary retention and hypertension, and is reported to show a diuretic activity with increased ionic content of urine in rats (Eshetu et al., 2016).

The ethanol extract or essential oil of thyme has carminative action **(**Debelo et al., 2016), a significant rate of antifungal and antimicrobial activities with strong lipid peroxidation inhibitory and high hydroxyl radical scavenging activities (Geleta et al., 2015)**,** in perfumery and in the food industry. The oil is claimed to possess antiseptic, antifungal and vermifuge properties and is thus used for medicinal purposes (Asfaw et al., 2000).

Thymus contains about 2.5% but not less than 1.0% of volatile oil. The composition of the volatile oil fluctuates depending on the chemotype under consideration. The principal components of Thymus are thymol and carvacrol (up to 64% of oil), along with linalool, p-cymol, cymene, hymene, α- pinene, apigenin, luteolin, and 6-hydroxyluteolin glycosides, as well as di-, tri- and tetra-methoxylated flavones **(**Debelo et al., 2016).

The volatile oils from *T. schimperi* have been examined by means of gas chromatography and gas chromatography-mass spectrophotometery and the main constituents of the essential oils of *T. schimperi* from four regions such as Bale, Gonder, Shewa, and Wollo were identified as p-cymene (9-23%), γ-terpinene (8-17%), thymol (6-38%) and carvacrol (5-63%) **(**Haji et al., 2016**).**

Figure 4. Photograph of the *Thymus schimpery*

2. Objectives

2.1. General objective

To evaluate *Ex vivo* vasodilatory activity of aqueous crude extract of *Thymus schimperi* leaves in isolated thoracic aorta of Guinea Pigs

2.2 Specific objectives

- To evaluate the effect of aqueous crude extract of *T. schimperi* leaves on Potassium Chloride induced contraction
- To evaluate the effect of aqueous crude extract of *T. schimperi* leaves on Epinephrine induced contraction
- To evaluate the effect of aqueous crude extract of *T. schimperi* leaves on Methylene blue induced contraction

- To evaluate the effect of aqueous crude extract of *T. schimperi* leaves on Glibenclamide induced contraction
- To evaluate the effect of aqueous crude extract of *T. schimperi* leaves on acetylcholine induced contraction
- To evaluate the effect of aqueous crude extract of *T. schimperi* leaves on histamine induced contraction
- To evaluate the effect of aqueous crude extract of *T. schimperi* leaves on intact and denuded endothelium
- To determine the possible mechanism (s) of relaxation from aqueous crude extract of *T. schimperi* leaves
- To carry out phytochemical screening of aqueous crude extract of *T. schimperi* leaves

3. Materials and Methods

3.1 Materials

3.1.1 Drugs, Chemicals and reagents

Epinephrine (lot no: 111K1610; Sigma-Aldrich Co., St Louis, MO, USA), methylene blue (lot no: 073K3413; Sigma-Aldrich Co.), acetylcholine chloride (lot no: 12134/1; Sigma-Aldrich Co.), histamine (lot no: A019367301; Acros Organics, New Jeresy, USA), glibenclamide (lot no: 53917; Remedica, Limassol, Cyprus), d-glucose anhydrous (lot no: GL2863; Eurostar Scientific Limited, Liverpool, UK), potassium chloride (lot no: 8114/86; Park Scientific Limited, Northampton, UK), potassium phosphate (lot no: 46F-0522; Sigma chemical company, MO 63178 USA), sodium hydrogen carbonate (lot no: 205- 633-8; Eurostar Scientific Limited), calcium chloride (lot no: 1501; Allied Chemical, Camden, NJ, USA), magnesium sulfate (lot no: 400290; The British Drug Houses Limited, UK), sodium chloride (lot no: 108278; Riedel-de Haen, Seelze, Germany), n-butanol (Merck, Germany), and dichloromethane (Chromasolr, UK) were used in the study. All the drugs, chemicals and reagents used complied with the required standard and were of analytical grade.

3.1.2 Instruments and apparatus

Balance (Mettler Toledo, Seoul, South Korea), Whatman filter paper number 1 (Whatman International Ltd, Maidstone, UK), orbital shaker (VWR DS-500; The LabWorld Group, Boston, MA, USA), rota vapor (Rotavapor R-bb210/215 B-490; Buchi, Flawil, Switzerland), water bath (DVE-Kottermann, D-3162; Uetze-Hanigsen/W, Berlin, Germany), lyophilizer/freeze dry system (Labconco, 12L Console Freeze Dry 230v-60 (7754040; Freeze Dry System, Labconco, Kansas City, MO, USA), and grass polygraph (Model 7E; Diversified Equipment Company Inc., Alexandria, VA, USA) were used in the expriment.

3.1.3 Plant materials

The fresh leaves of *Thymus schimperi* were collected from south eastern Ethiopia around Dinsho, about 400 km far from Addis Ababa. The plant material was authenticated by a botanist in the Directorate of Traditional and Modern Drug Research, Ethiopian Public Health Institute and a voucher number HH-001was deposited in the herbarium for future reference.

3.1.4 Experimental animals

The experiments were performed on adult, healthy guinea pigs (*Cavia porcellus*) weighing between 350-400 g. The guinea pigs were bred and obtained from the animal house of the Ethiopian Public Health Institute. The animals used for this study were kept in standard animal cages and maintained under laboratory conditions of temperature (22 ± 3°C), relative humidity (40-70%) and 12 hour day-12 hour night and had free access to food and water *ad libitum* for acclimatization and experimentation. The animals were treated humanely throughout the study period and were kept in a well controlled area according to the guideline for use and care of animals (National Research Council, 2011).

3.2 Methods

3.2.1 Plant material preparation and extraction

Fresh *T. schimperi* leaves were garbled, chopped, dried under shade (at room temperature), grinded to powder using mortar and pestle, and stored in a cool and dry place. Four hundred ninety eight grams of the powdered leaves of *T. schimperi* were kept in Erlenmeyer flasks and macerated with distilled water with continuous shaking on an electric shaker for 4 hours. Then,

the suspension was filtered through gauze and the filtrate was lyophilized to give an amorphous powder. The extract was collected in a vial and kept in silica gel desiccators until further use. The percentage yield was calculated to be 10% (w/w).

3.2.2 Evaluation of *ex vivo* vasodilatory activity

The *in vitro* vasodilatory study was conducted on isolated guinea pig thoracic aorta according to the methods described by Vogel (2008). The guinea pig was sacrificed by gentle cervical dislocation and the thoracic cavity was opened, and the aorta was identified. The descending thoracic aorta was then immediately removed and placed in Krebs-Henseleit physiological solution maintained at 37^0C. Excess adherent fat and connective tissue were trimmed off and cleaned; each aorta was cut spirally using heparinized capillary tube with plastic sealing to a strip of about 2 mm wide and 4 cm long. The strip was immediately mounted in an organ bath containing 20ml Krebs-Henseleit physiological solution (118.4 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4.7H_2O$, 2.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 24.9 mM $NaHCO_3$, 11.1 mM glucose, pH 7.4). The aortic strip was attached to isometric transducers connected to a polygraph and a resting tension of 1g was applied to strip. Aorta strip was mounted under this resting tension onto two 0.2 millimeters L shaped stainless steel wire hooks gently inserted into the lumen to avoid damage to the endothelium in 20 ml organ baths containing Krebs-Henseleit physiological solution and allowed to stabilize for about 1 hour before commencing an experiment during which period it was washed by overflowing every 15 minutes.

The physiological solution was allowed to pass through a warm water jacket to maintain its temperature and was continuously aerated with 95% O_2 and 5% CO_2 gas at a pH of 7.4. The pH of buffer was checked in every 60 minutes of aeration with carbogen.

Experiments were performed on aortic strip with intact (E^+) and denuded (E^-) endothelium. For the experiment on aortic strip with intact endothelium, the functional integrity of the endothelium was tested before extract and contracting agent administration by 1μM acetylcholine in the organ bath, which should result in a transient relaxation.

On the other hand, the experiment was done on denuded endothelium by removing endothelial cells from strip by gently rubbing the intimal surface with a moist wooden stick for 30-60 seconds. The effectiveness of this procedure was subsequently investigated using acetylcholine (1mM), which normally relaxed aorta strips, but had no such effect on rubbed strip precontracted with 80 mM of KCl (Nwokocha et al., 2011).

After aortic strip equilibration period of 1 hour under a resting tension of 1 g, tissue viability was confirmed by adding 80 mM KCl. Contraction of whole, spirally cut aortic strip was then induced by administering one of the following contracting agents to organ bath: (80 mM) Potassium Chloride (KCl), (1 μM) Epinephrine (EPI), (10μM) Methylene Blue (MB), (10μM) Glibenclamide (GLIB), (5 μM) Acetylcholine and (10 μM) Histamine.

Once a contraction plateau was achieved, extracts of *T.shimperi* were administered cumulatively every 15 minutes for their capacity of reducing aortic strip contraction induced and tension responses of the tissue were detected with transducers and recorded isometrically and were displayed on a Grass model 7E polygraph (Madingou et al., 2012; Vogel, 2008).

At the end of the experiment, relaxation, which is a measure of inhibition of contraction in spirally cut aortic strip precontracted with contracting agent, was determined using a measurement before and after extract administration and calculated using the following formula: (Bamidele et al., 2011).

$$\%\text{Relaxation} = \frac{(T_c - T_t)}{T_c} \times 100$$

Where, T_c stands for tension due to contracting agents, while T_t stands for tension after adding extract. In addition, EC_{50} (dose responsible for 50% of the maximal effect) for highly active extracts was determined. For each experiment, aorta from four animals were tested to ensure sufficient biological variability.

3.2.3 Phytochemical screening

The extract used for the *Ex vivo* study were subjected to phytochemical screening following methods described by Tiwari (2011), Trease and Evans (1989). The extract along with negative controls were tested for the presence of alkaloids, saponins, polyphenols, flavonoids, coumarins, terpenoids, anthraquinones, tannins, phytosterols, and glycosides.

3.2.4 Data analysis

All values were expressed as mean (%relaxation) ± standard error of the mean (SEM) and were subjected to biostatistical interpretation by statistical package for social science (SPSS) windows version 20 statistical package (IBM Corporation, Armonk, NY, USA) all the way through a one-way analysis of variance followed by post hoc test (Tukey's test) for multiple comparisons of the mean differences and responses of different drugs and extracts. Statistical significance of *P*-value <0.05 was considered as the level of significance. The software GraphPad Prism 6 Demo was used to calculate the half maximal effective concentration (EC50).

4. Result

4.1 *Ex vivo* vasodilatory activity

AQ crude extract has shown 99.23% ($EC_{50,}$ 3.81) and 84.75% ($EC_{50,}$ 3.72) relaxation on intact (*n*=4) and denuded (*n*=4) endothelium, respectively against KCl induced contraction and the vasodilatory effect of the AQ crude extract on intact endothelium was significantly (*P*<0.001) higher than that on denuded endothelium (Figure 5).

AQ crude extracts showed 99.93% ($EC_{50,}$ 3.36) relaxation against EPI induced contraction of guinea pig thoracic aorta on intact endothelium.

No significant difference was seen between the relaxation induced by *T. schimperi* AQ crude extract on endothelium-intact aortic rings precontracted by using KCl and EPI.

The crude extract (1.25-5mg/ml) showed significantly (*P*<0.001) lower relaxation against GLYB than against KCl and EPI induced contraction on intact endothelium in a concentration dependent manner.

AQ crude extract at the low and the moderate concentrations (1.25 and 2.5 mg/mL, respectively) failed to elicit vasodilatory activity. Relaxation at the highest concentration (5mg/ml), however, was significantly (P<0.001) higher than that induced by the low (1.25mg/ml) and the moderate (2.5mg/ml) concentrations against HIST-induced contractions of guinea pig thoracic aorta on denuded endothelium. Fifty percent of the maximal relaxation was achieved by a dose > 4 mg/ml. The highest concentration (5mg/ml) of AQ crude extract 97.50% (EC_{50}, 4.47) relaxation against HIST induced contraction.

Relaxation induced by AQ crude extract at the concentration of 5mg/ml was not significantly (P>0.05) different between KCl and HIST (on intact and denuded endothelium, respectively) induced contractions. AQ crude extract at the concentration of 5mg/ml showed 99.23% (EC_{50}, 3.81) against KCl and 97.50% (EC_{50}, 4.47) against HIST induced contractions. This indicates that the vasodilatory effect of the crude extract is visible at high concentration than that at the low and the moderate concentration against HIST – induced contraction on denuded endothelium.

AQ crude extract failed to show vasodilatory activity against MB induced contraction. At all tested concentrations (1.25-5 mg/mL) the crude extract showed significantly (P<0.001) lower relaxation against MB than KCl induced contraction of guinea pig thoracic aorta on intact endothelium. Percentage relaxation achieved by the highest concentration (5mg/ml) of the crude extract was 12.25. Which was the maximum relaxation achieved by the extract against MB induced contraction. At the highest concentration which showed significantly (P<0.001) lower relaxation against MB than KCl (intact and denuded endothelium), EPI, GLYB, HIST and ACH–induced contraction of guinea pig thoracic aorta (Table 2).

AQ crude extract at both the low and the moderate concentrations failed to show vasodilatory activity against ACH induced contraction on denuded endothelium.

At the lower (1.25mg/ml) and the moderate (2.5mg/ml) concentration the crude extract showed significantly (P<0.05 and P<0.001, respectively) lower relaxation against HIST than KCl induced contraction. On the contrary, relaxation by the crude extract at the highest concentration (5mg/ml) was significantly (P<0.001) higher against HIST than KCl induced contraction on denuded endothelium (Figure 7).

At the highest concentration the crude extract showed higher relaxation against KCl and HIST than ACH induced contraction on denuded endothelium (Figure 8 and 9).

Table 1. Vasodilatory activity of *T.schimperi* aqueous leaf extract on guinea-pig thoracic aorta (intact) 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**
0.50	**98.75 ± 0.4**	**1.25 ± 0.4**
1.00	**90.30 ± 0.74**	**9.70 ± 0.74**
1.25	**84.15 ± 1.63***	**15.85 ± 1.63***
2.50	**61.65 ± 2.14*****	**38.35 ± 2.14*****
5.00	**5.75 ± 0.85*****	**94.25 ± 0.85*****

*$p < 0.05$, ***$p < 0.001$($n = 4$. Results are expressed as mean ± SEM)

Table 2. Vasodilatory activity of aqueous crude extract of *T. schimperi* leaves in precontracted isolated thoracic aorta of Guinea Pigs.

		%Relaxation with contracting agents						
Substance administered	**Dose (mg/ml)**	**E^+ + KCl**	**E^- + KCl**	**E^+ + EPI**	**E^+ + GLYB**	**E^+ + MB**	**E^- + HIST**	**E^- + ACH**
AQ crude extract	1.25	21.25±1.50	12.63±1.03	22.71±2.62	5.31±0.56***	2.88±0.31***	2.25±0.14***	6.78±1.72**
	2.5	32.50±1.04	24.50±2.10	42.00±5.26	10.21±0.94***	4.88±0.43***	3.13±0.43***	13.00±0.74***
	5.0	99.23±0.47	84.75±2.32***	99.93±0.05	74.52±4.72***	12.25±0.66***	97.50±1.20	72.71±4.32***

Values are expressed as mean ± SEM (n=4). **P<0.01; ***P<0.001 compared with AQ crude (E^+ + KCl). E^+: Intact endothelium; E^-: Denuded endothelium.

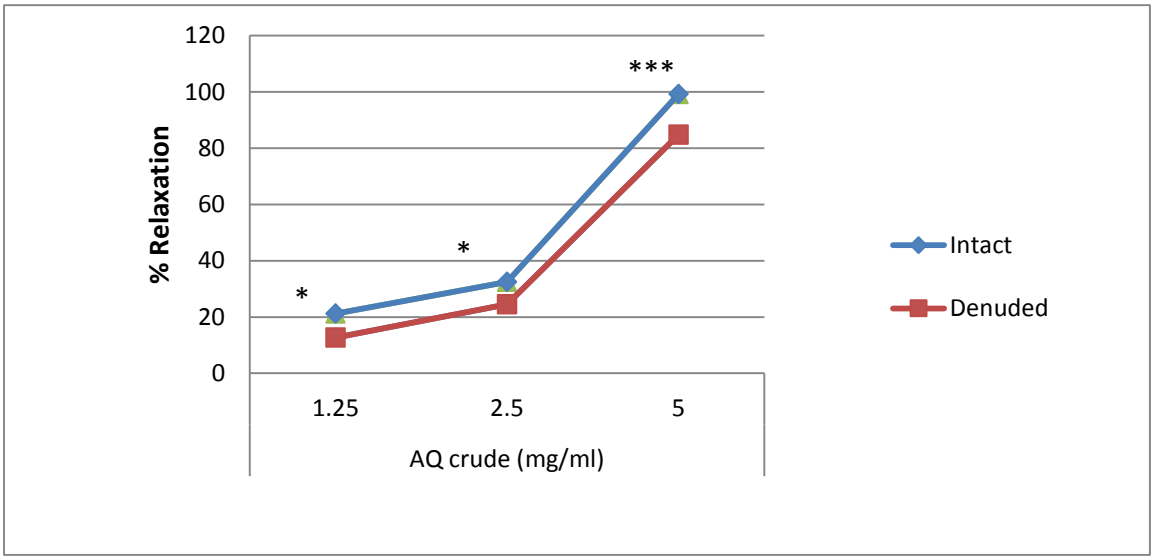

Figure 5. Dose-response curve showing the vasodilatory effect of *T. schimperi* aqueous leaf extract on intact and denuded endothelium of guinea pigs thoracic aorta precontracted by KCl (80 mmol/L).

Results are expressed as mean ± SEM (n=4). Significant (*P<0.05; ***P<0.001) difference on intact and denuded endothelium.

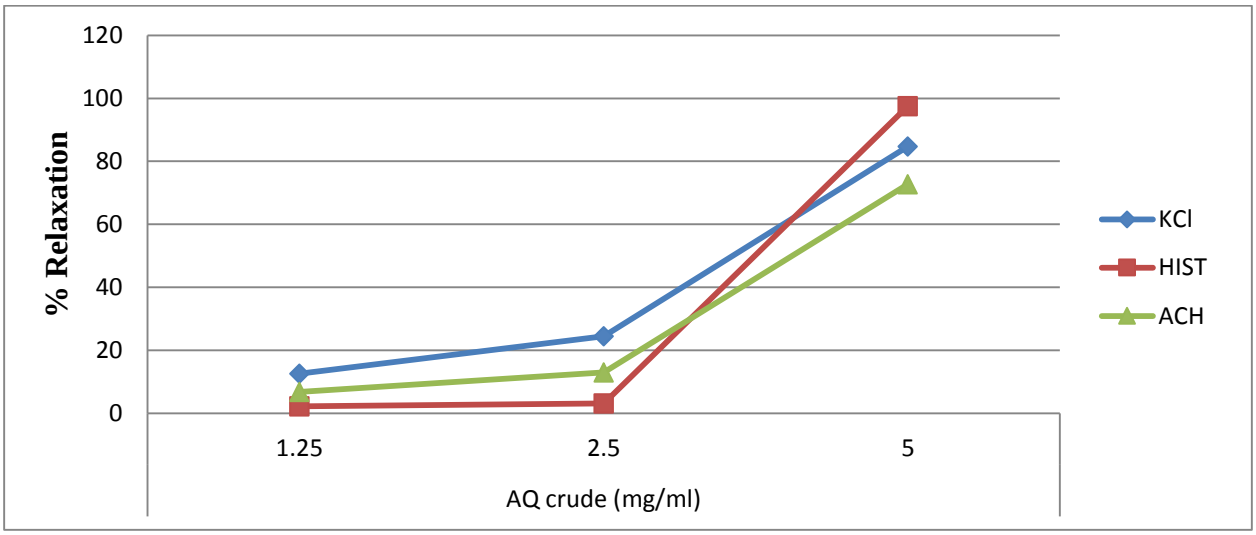

Figure 6. Dose-response curve showing the vasodilatory activity of aqueous crude extract of *T. schimperi* leaves on denuded (*n*=4) endothelium of guinea pigs thoracic aorta precontracted by KCl, HIST and ACH.

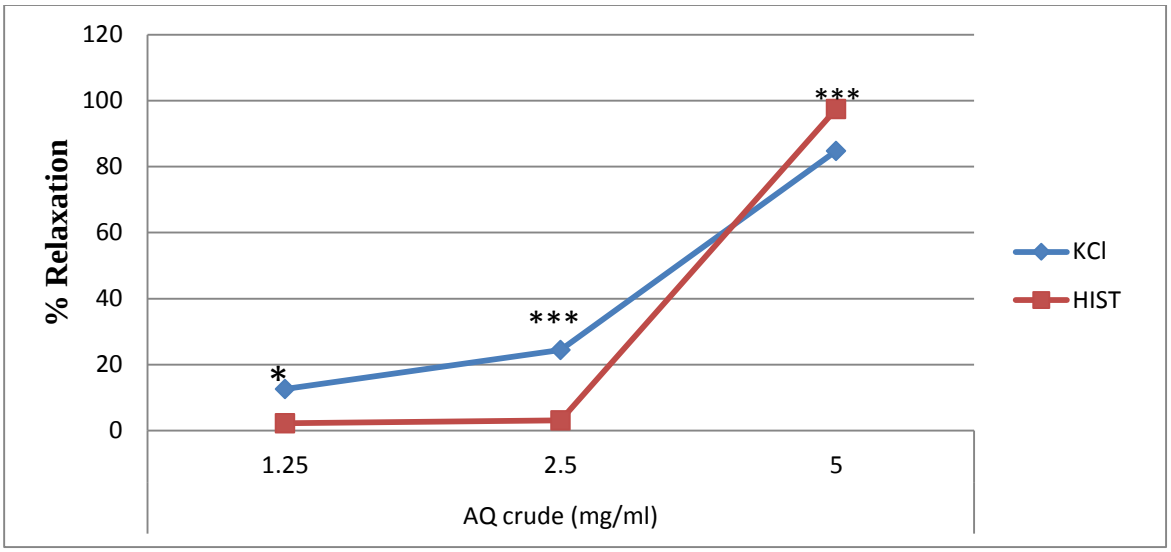

Figure 7. Dose-response curve showing the vasodilatory activity of aqueous crude extract of *T. schimperi* leaves on denuded endothelium of guinea pigs thoracic aorta precontracted by KCl and HIST.

Values are expressed as mean ± SEM (n=4). Significant (**P*<0.05; ****P*<0.001) difference between KCL and HIST contracted aorta on denuded endothelium.

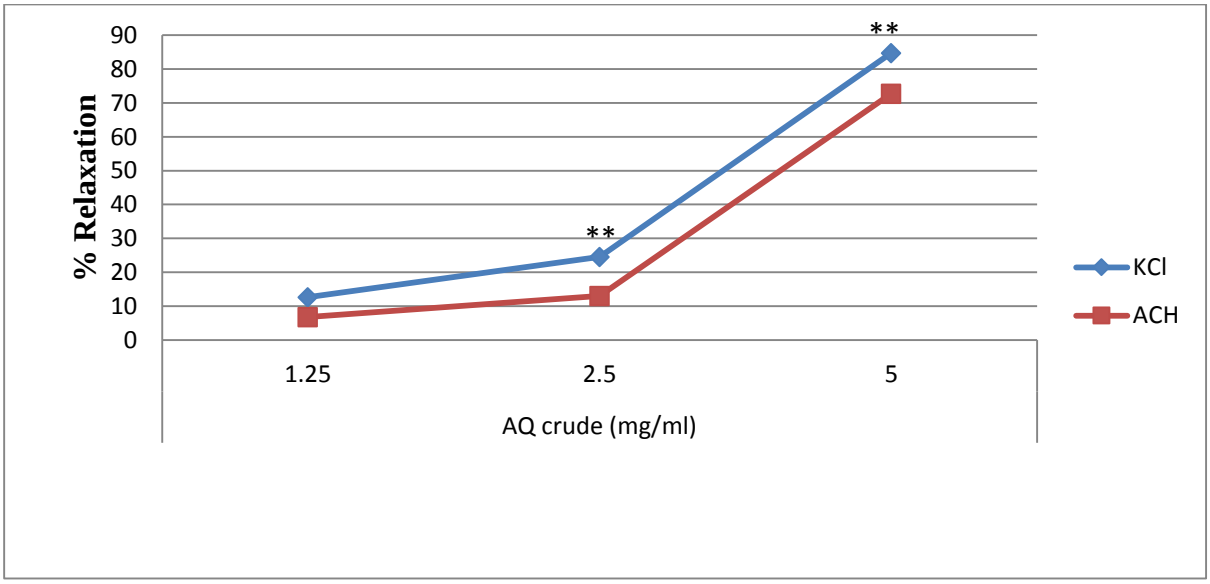

Figure 8. Dose-response curve showing the vasodilatory activity of aqueous crude extract of *T. schimperi* leaves on denuded endothelium of guinea pigs thoracic aorta precontracted by KCl and ACH.

Values are expressed as mean ± SEM (n=4). Significant (**P<0.01) difference between KCl and ACH contracted aorta on denuded endothelium.

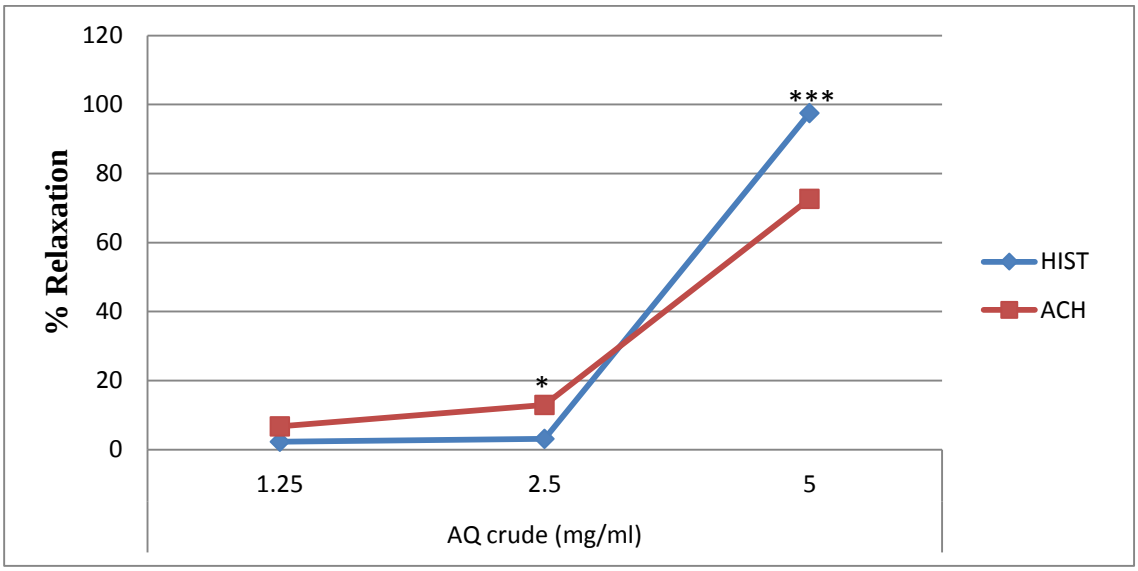

Figure 9. Dose-response curve showing the asodilatory activity of aqueous crude extract of *T. schimperi* leaves on denuded endothelium of guinea pigs thoracic aorta precontracted by HIST and ACH.

Values are expressed as mean ± SEM (n=4). Significant (*P<0.05; ***P<0.001) difference between HIST and ACH contracted aorta on denuded endothelium.

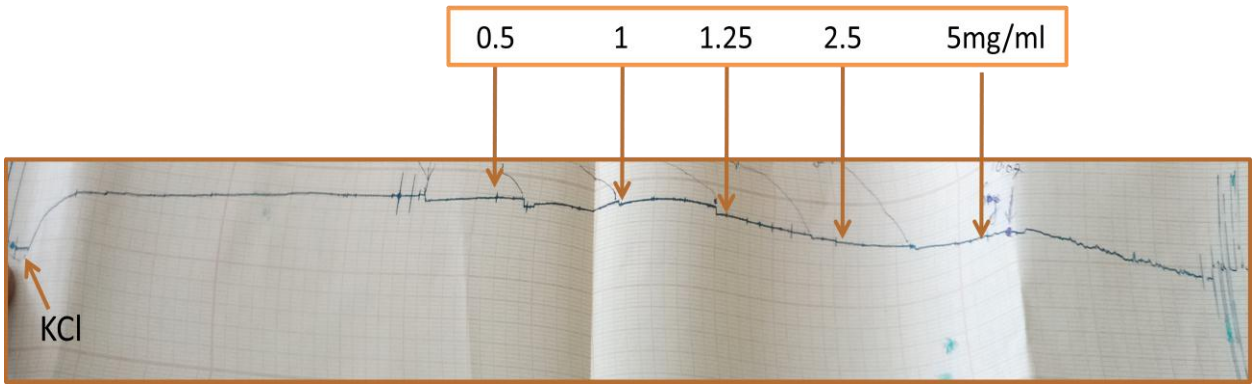

Figure 10. Vasodilatory activity of *T.schimperi* aqueous leaf extract on guinea-pig thoracic aorta (intact) precontracted with 80 mM KCl.

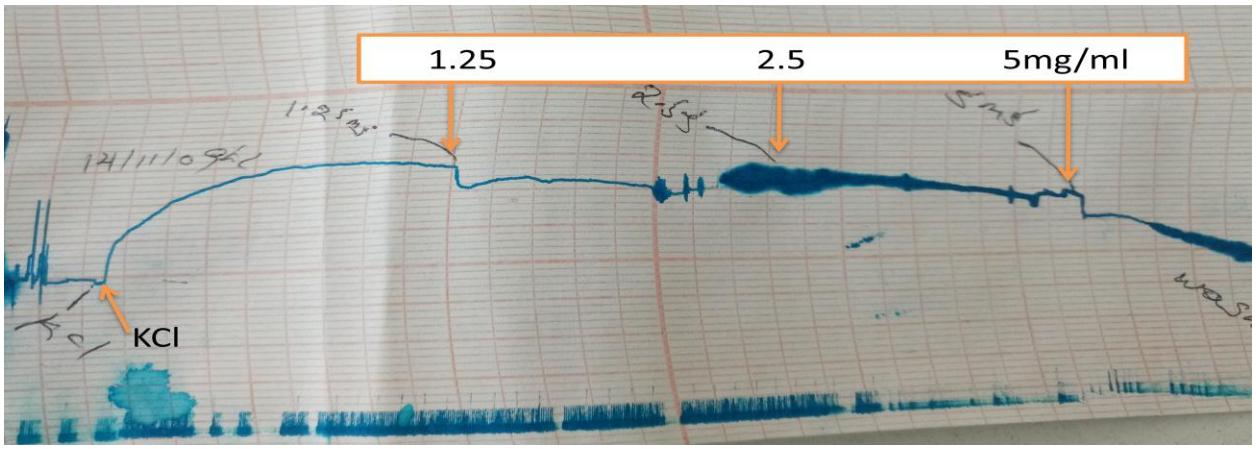

Figure 11. Vasodilatory activity of *T.schimperi* aqueous leaf extract on guinea-pig thoracic aorta (intact) precontracted with 80 mM KCl.

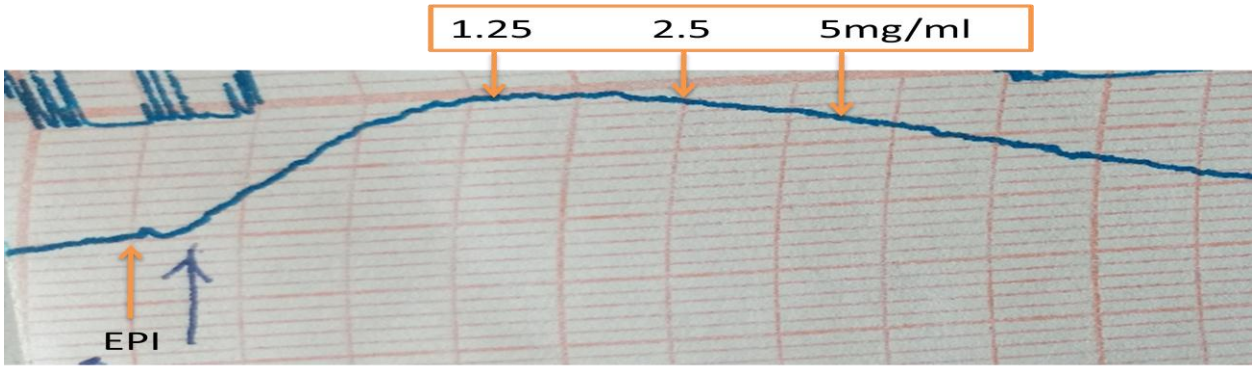

Figure 12. Vasodilatory activity of *T.schimperi* aqueous leaf extract on guinea-pig thoracic aorta (intact) precontracted with 1 µM EPI.

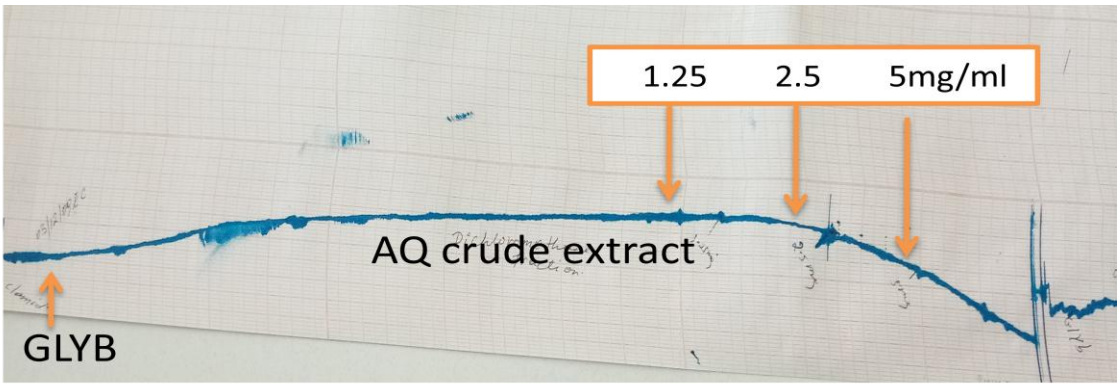

Figure 13. Vasodilatory activity of *T.schimperi* aqueous leaf extract on guinea-pig thoracic aorta (intact) precontracted with 10 µM GLYB.

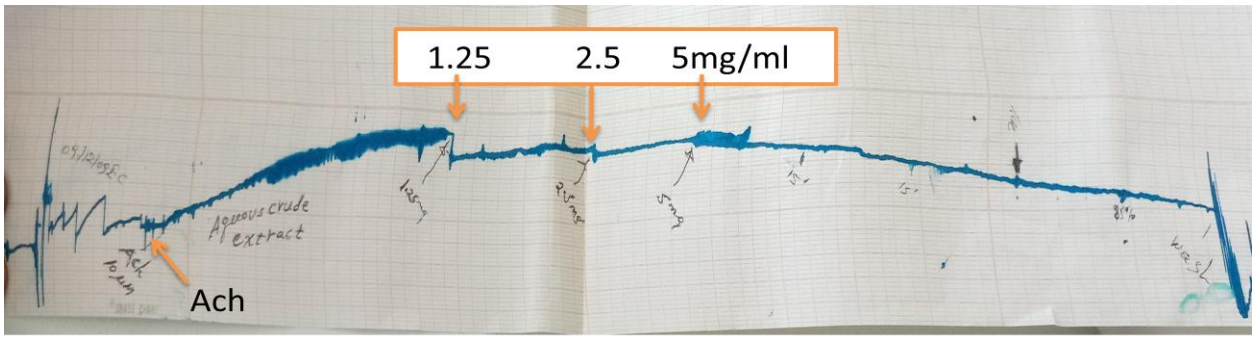

Figure 14. Vasodilatory activity of *T.schimperi* aqueous leaf extract on guinea-pig thoracic aorta (denuded) precontracted with 5 µM Ach.

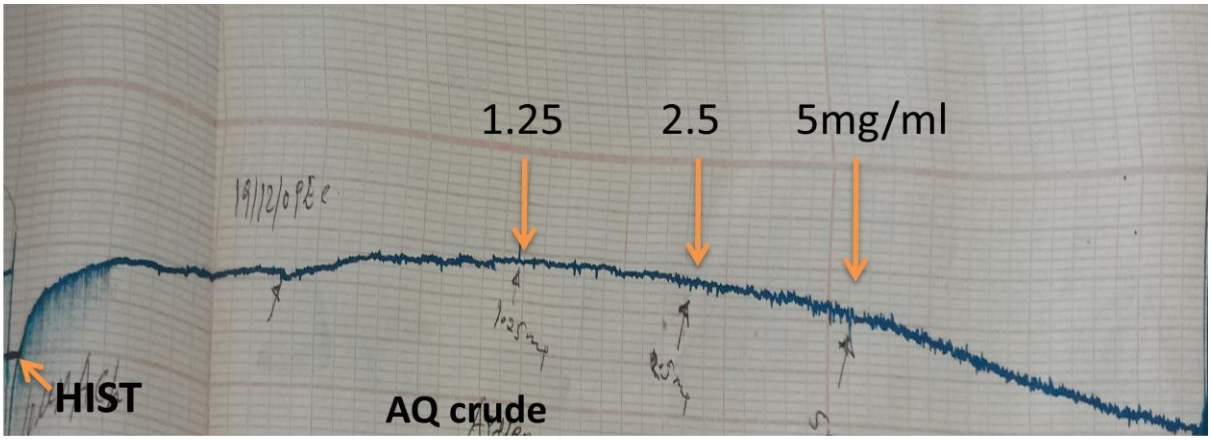

Figure 15. Vasodilatory activity of *T.schimperi* aqueous leaf extract on guinea-pig thoracic aorta (denuded) precontracted with 10 µM HIST

4.2 Phytochemical screening

Basic investigations of the extracts for their major phytocompounds is vital as the active principles of many drugs are the secondary metabolites found in plants. The various phytochemical screening tests performed on the crude extract of *T.schimperi* leave revealed the presence of different secondary metabolites. The results of the phytochemical composition of *T.schimperi* extracts are given in Table 3.

Table 3. Phytochemical screening of aqueous crude extract of *T. schimperi* leaves

Type of extract	Alkaloids	Saponins	Polyphenols	Flavonoids	Coumarins	Terpenoids	Anthraquinones	Tannins	Phytosterols	Glycosides
AQ crude	-	+	+	+	-	+	+	+	-	+

Key: + = present; - = absent

5. Discussion

5.1 *Ex vivo* vasodilatory activity

In the present study AQ crude extract and fractions of *T. schimperi* showed dose dependent relaxations of isolated whole, spirally cut aortic strips of guinea pig pre-contracted by KCl (on intact and denuded endothelium), EPI, GLIB, HIST and ACH.

Blood pressure is created by the force of blood pushing against the walls of blood vessels as it is pumped by the heart. It is a multiplication product of cardiac output and peripheral vascular resistance (Raj et al., 2015). The goal of any antihypertensive therapy is to bring a reduction in either or both of these parameters, preferably peripheral vascular resistance. Many antihypertensive drugs act by producing direct dilation on blood vessels (Aziz et al., 2009).

A number of studies have been carried out to support the vasodilatory effect of many traditional plants. The previous studies show that the *in vitro* vasodilatory effect of *T. serrulatus*, antihypertensive activity of *T. vulgaris*, and *T. serpyllum* which has similar genius with *T. schimperi* (Haji et al., 2016). *T. schimperi* is used in traditional medicine for the treatment of headache, cough, stomachache, earache, liver disease, gonorrhea (Asfaw et al., 2000), fungal infections, bacterial infections (Pagiotti et al., 2010; Asfaw et al., 2000), urinary retention and hypertension. The diuretic and antihypertensive activity of the aqueous extract in rats was reported by Haji et al. (2016). However, there is no scientific report on the vasodilatory activity of the plant. In view of these facts, the present study attempted to elucidate the vasodilatory effect of the crude aqueous extract of the leaves of *T. schimperi* at different doses.

The present work is the first report on the vasodilatory effects of *T. schimperi* extract on guinea pig thoracic aorta. It is observed that the crude extract of *T. schimperi* produced a relaxation of KCl (on intact and denuded endothelium), EPI, GLIB, HIST, and ACH induced contraction in isolated whole, spirally cut aortic strips of guinea pigs in a dose-dependent manner. But the extract failed to show significant relaxation effect of aorta pre-contracted by methyline blue on intact endothelium as compared to potassium chloride (intact and denuded endothelium), epinephrine, glibenclamide, acetylcholine and histamine induced contraction ($P<0.001$). The greatest vasodilatory activity was observed at maximum tested doses of 5 mg/mL.

The percent relaxation of AQ crude extract against KCl-induced contraction was found to be greater in intact than denuded endothelium of isolated whole, spirally cut aortic strips of guinea pigs. This showed that the presence of endothelium has a contribution for increment in the percent relaxation of the precontracted aortic strips. This may be attributed to the stimulation of endothelium-derived relaxing factor and release of nitric oxide by some phytoconstituents present in the extracts. As the extracts also produced significant relaxation in denuded endothelium in KCl precontracted isolated whole, spirally cut aortic strips, they have a potential to induce endothelium-independent vasodilation.

AQ crude extract of *T. schimperi* showed lower potency against HIST (on denuded endothelium) than KCL (denuded and intact endothelium), ACH (on denuded endothelium, EPI and GLYB on intact endothelium of guinea pig thoracic aorta. This may be due to the absence of the effect of AQ crude extract components on endothelial derived relaxing factors that could be prostacycline or EDRF.

Fifty percent of the maximal relaxation was achieved at a dose > 4 mg/ml against HIST-induced contractions of guinea pig thoracic aorta on denuded endothelium. This indicates that the vasodilatory effect of the crude extract and the fractions are more visible at the highest (5mg/ml) concentration. The low (1.25mg/ml) and the moderate (2.5mg/ml) concentrations failed to show vasodilatory effect perhaps due to the low concentration of the active principle(s) present in the extracts. From these observations, one can only suggest that high doses of AQ crude extract and fractions of *T. schimperi* have substantial vasodilatory activity against HIST and ACH-induced

contractions of guinea pig thoracic aorta on denuded endothelium. One of the previous study explained that, much higher concentrations of organic calcium antgonists were required to inhibit histamine or acetylcholine induced contractions of guinea pig isolated trachea (Advenier et al., 1984). Histamine-induced contraction of rabbit aorta is dependent to a large extent on Ca^{2+} entry from the extracellular space (Ebeigbe and Talabi, 2014).

EPI activates smooth muscle by activation of receptor operated Ca^{2+} channels that leads to increase in cytosolic-free Ca^{2+} ($[Ca^{2+}]i$), MLC kinase activation, and MLC phosphorylation and contraction (Waugh, 1962). The inhibitory effects of *T. schimperi* on the contractile responses induced by α_1-agonist, EPI could, therefore, be attributed to a blockade of Ca^{2+} entry through receptor-operated Ca^{2+} channels. KCl-induced contractions are the result of an increased Ca^{2+} influx through voltage-stimulated type-L Ca^{2+} channels and are specifically inhibited by Ca^{2+} antagonists (Duarte et al, 1993). Thus, the inhibitory effects of *T. schimperi* on the contractile responses induced by high KCI could be attributed to a blockade of Ca^{2+} entry through voltage stimulated Ca^{2+} channels. The mechanism of relaxation by *T. schimperi* might, therefore, be through blockade of Ca^{2+} influx through both receptor-operated and L-type voltage dependent Ca^{2+} channels.

GLIB inhibits the ATP-sensitive potassium channel opening that causes contraction (Nakanishi, 1993). The vasodilatory mechanism of AQ crude extract and fractions of *T. schimperi* against the contractile responses induced by GLYB might, therefore, be due to activation of ATP-sensitive potassium channel. The hyperpolarizing effect of ATP-sensitive potassium channel activation may be excreted by reducing Ca^{2+} influx through voltage-dependent Ca^{2+} channels in vascular smooth muscle (Thomas et al., 1997).

Activation of H_1 receptors on vascular smooth muscle results in vasoconstriction. Histamine-induced contraction of rabbit aorta is dependent to a large extent upon Ca^{2+} entry from the extracellular space (Ebeigbe and Talabi, 2014). Thus, the inhibitory effects of *T. schimperi* on the contractile responses induced by HIST might, therefore, be through blockage of H_1 receptors on vascular smooth muscle.

When the endothelium is impaired, acetylcholine will produce constriction as a result of the vasoconstrictor effects on the smooth muscle, which cannot be counteracted. (Suciu, 2009). The muscarinic receptors belong to the G-protein coupled receptors (GPCR) family. They mediate distinct physiological functions according to their location and receptor subtype *(*Brass D, 2010). Through their linkage to $G_{q/11}$, M_1, M_3 and M_5 receptors predominantly activate PLC via α-subunit. The activation of PLC results in production of diacylglycerol (DAG), which is generated together with inositol trisphosphate (IP_3) upon the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by PLC. This path facilitates the mobilization of intracellular Ca^{2+} and activation of the protein kinase C (PKC) (Ockenga et al., 2013). PKC (protein kinase C) has been proposed to play a key role in maintenance of tonic contractions of vascular smooth muscle although the mechanism involved is not well defined **(**Duarte et al., 1993**).** PKC has been found in high concentrations in vascular smooth muscle and can be activated by diacylglicerol (Luna-Vázquez, 2013). M_3 muscarinic receptors are located in the smooth muscles of the blood vessels, as well as in the lungs. M_3 receptors act via G proteins of class G_q (Brass, 2010). The mechanism for vasodilatory effects of AQ crude extract and fractions of *T. schimperi* against the contractile responses induced by ACH might, therefore, be due to blockage of M_3 receptors, indicating release of Ca^{2+} from intracellular stores is inhibited.

The mechanism of vascular smooth muscle contraction involves the participation of different signal transduction pathways, all of which converge to increase cytoplasmic Ca^{2+} concentrations. The concentration of this cation increases both by extracellular Ca^{2+} entering through voltage operated Ca^{2+} channels (VOCCs) and receptor-operated Ca^{2+} channels, and by the release of Ca^{2+} from intracellular stores (Luna-Vázquez, 2013). Therefore, the mechanisms of action associated with vasodilating effect of *T.schimperi* may be by decreasing intracellular Ca^{2+} concentration through blockage of both VOCC and ROCCs, and inhibiting release of Ca^{2+} from intracellular stores and activation of ATP sensitive K^+ channels to a less extent.

5.2 Phytochemical screening

Aqueous crude extract of the fresh *T.schimperi* leaves were screened for the presence of different phytochemicals.

The qualitative phytochemical screening showed that the extract contain saponins, terpenoids, anthraquinones, tannins, polyphenols, glycosides and flavonoids but not alkaloids, coumarins and phytosterols..

One of the earlier studies showed the presence of alkaloids, saponins, tannins, phytosterols and polyphenols but no flavonoids, terpenoids, glycosides and anthraquinones in aqueous crude extract of *Thymus serrulatus* (Eshetu A. *et.al*, 2016).

Most compounds that have shown vasodilatory activity are flavonoids (Luna-Vázquez, 2013; Duarte et al., 1993), terpenoids (Luna-Vázquez, 2013; Menezes et al., 2010), glycosides (Jansacul et al., 1997) and phenolic compounds (Takahara et al., 2005; Victório et al.,2009).

According to the previous study, essential oils of *T. schimperi* from four regions such as Bale, Gonder, Shewa, and Wollo were identified as p-cymene (9-23%), γ-terpinene (8-17%), thymol (6-38%) and carvacrol (5-63%) (Haji et al., 2016). The phythochemical screening of aqueous crude extract and powdered leaves of *T. schimperi* showed the presence of phenols, tannins and saponins. Both the crude extract and powdered leaves, however, did not reveal presence of alkaloids, phytosterols and glycosides (Haji et al., 2016). Moreover, Acetone extract of *T.schimperi* revealed presence of anthraquinone, phenolic compounds, steroids and glycosides (Haile, 2013).

One of the earlier studies showed the presence of alkaloids, saponins, polyphenols, tannins and phytosterols in AQ crude extract and n-butanol fraction of *T. serrulatus* leaves. The same study showed the presence of saponins, flavonoids, tannins and phytosterols (Eshetu et al., 20016). Another study showed the presence of all tested metabolites (flavonoid, anthraquinone, alkaloid, saponin, terpenoid, glycoside, and tannin) in both EtOH and AQ crude extract of *M. oleifera* (Nweze et al., 2014).

The current study indicated that the fresh leaves of *T. schimperi* extract and its fractions contain different classes of secondary metabolites. The presence of these phytochemicals gave a great potential for *T. schimperi* leaves extract in producing vasodilatory effect that signifies the potential as a source of antihypertensive agent.

6. Conclusion

The results obtained from the present study, AQ crude extract of *T. schimperi* leaves exert a relaxant effect on isolated thoracic aorta of guinea pig. The extract of *T. schimperi* exhibits vasodilatory effect on EPI, KCl, GLYB, HIST, and ACH – induced contraction of isolated aorta. The possible mechanisms of action of the extracts may be through blockage of both receptor-operated and L-type voltage dependent Ca^{2+} channels, activation of ATP-sensitive potassium channel, blockage of H_1 and M_3 receptors. The ability *T. schimperi* to display such pharmacological property represents a rational explanation for the use of this medicinal plant as valuable source of vasodilator agents.

7. Recommendations

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

- The M_5 muscarinic receptors distribution is not well known, therefore, further experiment is needed to identify the presence of these receptors in vascular smooth muscle since the extract might have action on these receptors.

- Additional work need to be done on assessing other possible mechanism of action of the extract in its vasodilatory effect.

- Purification, isolation and characterization of the different phytoconstituents responsible for the *ex vivo* vasodilatory effect and exploring the exact mechanism of action are needed.

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