Ex-vivo vasodilatory activity and possible mechanisms of action of distillated seed extract from *Lupinus albus L.* ("Gibto Arekei") in isolated guinea pig thoracic aorta

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A Thesis Paper Submitted to the Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health Sciences, in Partial Fulfillment for the requirements of the Degree of Master of Science in Pharmacology

Addis Ababa University
Addis Ababa, Ethiopia

October, 2021

Addis Ababa University

School of Graduate Studies

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Abstract

Ex-vivo vasodilatory activity and possible mechanisms of action of distillated seed extract from *Lupinus albus L.* seeds ("Gibto Arekei") in isolated guinea pig thoracic aorta

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Addis Ababa University, 2021

Hypertension is a major health problem globally necessitating exploration for new preventive and therapeutic tools. The community in North Western Ethiopia uses a local alcoholic beverage from *Lupinus albus L.* seed called "gebto arekei" for the treatment of hypertension. Although it has been extensively proven as an antihypertensive preparation, it lacks scientific evidence. Thus, the aim of this study was to investigate *ex-vivo* vasorelaxation activity and possible mechanisms of *Lupinus albus L.* distillated seed extract in isolated guinea pig thoracic aorta.

Vasorelaxation effects of different concentrations of extract (4-32µg/ml) was investigated in isolated thoracic aorta strips of guinea pigs. Vasorelaxation response was evaluated by ex-vivo isometric tension recording method.

The extract induced vasorelaxation response in both endothelium intact and endothelium denuded strips as well as strips pre-incubated with NG nitro-L-arginine methyl ester and methylene blue in concentration-dependent manner. The extract showed synergistic activity when combined with sodium nitroprusside. Determination of calcium concentration responses revealed significant inhibition of contraction. The concentration that caused maximal relaxation and 50% maximal relaxation was found to be 4.74µg/ml and 3.80µg/ml respectively in epinephrine pre-contracted endothelium intact strips where as it was 21µg/ml and 7.01µg/ml in similar strips pre-contracted by potassium chloride.

Hence, popular usage of *Lupinus albus L.* seed preparation as a phytomedicine in the treatment of hypertension would be related to its effect through endothelial nitric oxide synthase-nitric oxide-cyclic guanosine monophosphate pathway and direct inhibitory effect on voltage dependent calcium channels. The findings shown that the preparation is endowed with vasorelaxation activity, providing evidence for its traditional use in the treatment of hypertension.

Key words: *Lupinus albus L.*, "Gebto arekei", Vasorelaxation, Aorta strips, Endothelium

Acknowledgment

First thanks to God, and I would like to express my gratitude to Addis Ababa University Health Science College, School of Pharmacy and Department of Pharmacology and Clinical Pharmacy who gave me the chance to learn in the institution and shared their experience and knowledge without any limit. In addition I highly value the commitment of Hawassa University College of Medicine and Health Sciences for their willingness and sponsorship support. I would like to thank Ethiopian Public Health Institute, Traditional and Modern Medicine Directorate who allowed me to work in their laboratory facilities freely and for their cooperation.

Thanks to my advisor Dr. Workineh Shibeshi who was giving me many valuable idea from the beginning to the end as well as his valuable comments, suggestions, follow up and unreserved guidance and support.

Then my appreciation is to Miss Frehiwot Teka, Mr. Worku Gemechu, Mr. Aby Abebe, and Mr. Samuel Woldekidan all from Ethiopian Public Health Institute, Traditional and Modern Medicine Directorate for their technical and material support. Finally, I like to thank my beloved wife Mrs Yeshi Asaye for her help so as me to focus fully on my work and for sharing her experience.

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List of Abbreviations

ACC	American college of cardiology
AHA	American heart association
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BKca	Large conductance calcium dependent potassium channel
BP	Blood pressure
CaM	Calcium calmodulin
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CO	Cardiac output
COX	Cyclooxygenase
CV	Cardiovascular
CVD	Cardiovascular disease
CVS	Cardiovascular system
DBP	Diastolic blood pressure
DM	Diabetes mellitus
DW	Distilled water
ECs	Endothelial cells
EDHF	Endothelium derived hyperpolarizing factor
eNOS	Endothelial nitric oxide synthase
EP	Epinephrine
EPHI	Ethiopian Public Health Institute
ER	Endoplasmic reticulum
GPCRs	G-protein coupled receptors
GTP	Guanosine triphosphate
IP_3	Inositol-3,4,5-triphosphate
K_{ATP}	ATP sensitive potassium channels
KCL	Potassium chloride
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
NO	Nitric oxide
NOS	Nitric oxide synthase
PGI	Prostaglandin I
PKC	Phosphokinase C
PMCA	Plasm membrane calcium ATPase
PVR	Peripheral vascular resistance
SBP	Systolic blood pressure
SNP	Sodium nitroprusside
SPSS	Statistical package for social science
SR	Sarcoplasmic reticulum
VDCCs	Voltage dependent calcium channels
VSMCs	Vascular smooth muscle cells

1. Introduction

1.1 Epidemiology of hypertension

Hypertension is defined as persistently elevated arterial blood pressure (BP) categorized as normal (<120 systolic and <80 mmHg diastolic), elevated (120-129 systolic and <80 mmHg diastolic), stage 1 (130-139 systolic or 80-89 mmHg diastolic) and stage 2 (≥140 systolic or ≥90 mmHg diastolic) confirmed by two or more readings (averaged) made on at least two separate occasions that include office and out-of-office measurements (1). BP is the force applied by circulating blood against the walls of the body's arteries (2). Hypertension is a chronic elevation of BP that, in the long-term, causes end-organ damage and results in increased risk of morbidity and mortality (3,4). Essential hypertension, or hypertension of unknown cause, accounts for more than 90% of cases of hypertension while secondary hypertension is responsible for the rest (5).

The main function of circulation is to supply oxygen and nutrients to tissues and organs while eliminating metabolic byproducts. Thus, the animal organism consists complex vascular networks made up of blood vessels, including arteries, arterioles, capillaries, venules, and veins to carry out these function (6).

Hypertension increases the risks of cardiac, renal, cerebral, and other diseases and it is a major cause of premature death globally, with upwards of 1 in 4 men and 1 in 5 women; over a billion people having the condition in 2015 from which fewer than 1 in 5 people with hypertension have the condition under control according to data from the Global Burden of Disease survey. The report further indicated 7.8 million deaths in 2015 were directly attributable to SBP of ≥140 mmHg and that 10.7 million deaths were due to a DBP of ≥110 mmHg (7). The impact of hypertension is felt disproportionately in low and middle income countries, where two thirds of cases were found, largely due to increased risk factors in those populations in recent decades (8).

For example, almost one-third of Jordanian adults were found to be hypertensive and only one-third of those who were on anti-hypertensive medications had controlled BP indicating gaps in the management of hypertension (9).

High SBP is also the leading cause of death in Africa as well. It resulted in about 900,000 (10% of the total deaths on the continent) in 2016 and has raised by 82% since 1990 (10). It has been suggested that the prevalence of cardiovascular disease (CVD) and hypertension is increasing

rapidly in Sub-Saharan Africa (11). Sever hypertension was found 14.1% in this region. Among participants with severe hypertension, about 28.9% were either undiagnosed or untreated (12).

The prevalence of hypertension in urban dwelling adults in Nigeria was found to be 55% according to the American College of Cardiology (ACC)/American Heart Association (AHA) 2017 guideline. Over half of the adult population in Lagos Nigerian is classified to have hypertension by the recent guideline (13). In Kenya, the overall age-standardized prevalence of hypertension was 24.5%, among those individuals with hypertension, only 15.6% were aware of their elevated BP. Again from those who were aware, only 26.9% of them were on treatment and 51.7% among those on treatment had achieved their BP control (14).

Nevertheless there is no well documented data on national prevalence of hypertension in Ethiopia, only few studies conducted at different parts of Ethiopia and at the capital Addis Ababa showed an increased prevalence of hypertension. Ethiopians have been facing the consequences of epidemiologic, demographic, economic and nutritional transitions which favor the chronic diseases epidemic. Some of the major contributing factors for the prevalence of non-communicable diseases in Ethiopia include the acceptance of western lifestyle in urban areas particularly a more sedentary way of life, increased cigarette smoking, employment in manufacturing industries, greater stress, and consumption of more refined diet and increasing life expectancy in cities (8,15).

A meta-analysis conducted in 2015 showed that the prevalence of hypertension among Ethiopian population was found 19.6%. Subgroup analyses indicated that the prevalence of hypertension is higher in the urban population (23.7 %) than rural and urban combined (14.7 %) (15). A hospital based cross-sectional study conducted in Addis Ababa reported that the prevalence of hypertension as 34.7% (16). Where as a community based study in Dire Dawa in eastern Ethiopia reported the prevalence of hypertension 24.43%. Majority of adults were not aware of their elevated BP level (17). It was reported that 12.5% individuals had hypertension in Debre Markos Town (18).

1.2 Pathophysiology of Hypertension

Because BP is related to the cardiac output (CO) and peripheral vascular resistance (PVR) by the equation BP = CO × PVR, increases in either CO or PVR should produce hypertension (19). In most cases, elevated BP is associated with an overall increase in resistance to flow of blood

through arterioles, whereas CO is usually normal. Investigations of autonomic nervous system function, baroreceptor reflexes, the renin-angiotensin-aldosterone system, and the kidney have failed to identify a single abnormality as the cause of increased PVR in essential hypertension (20). It appears, therefore, that elevated BP is usually caused by a combination of multi-factorial abnormalities. Epidemiologic evidences points out to genetic factors, psychological stress, and environmental and dietary factors as contributing to the development of hypertension (7). Endothelial dysfunction, increased vascular reactivity, and vascular remodeling are causes of BP elevation while increased vascular stiffness contributes to isolated systolic hypertension in the elderly (21).

Essential or primary hypertension can't be cured but it can be controlled. Fewer than 10% of patients have secondary hypertension where either a pathological condition or a drug (or other product) is responsible for elevating BP (19).

Contraction of vascular smooth muscle cells (VSMCs) occur through an electromechanical and chemical-mechanical coupling, or a combination of both (6). The first mechanism of contraction is initiated by a change in the membrane potential, while the second type is mediated by G-protein-coupled receptors (GPCRs) and the subsequent generation of second messengers; however, both mechanisms lead to an increase in cytosolic Ca^{2+} dependent activation of the myosin light chain kinase (MLCK) and actin-myosin cross-bridges associated with structural changes and force generation (22).

1.3 Current management strategies and future perspectives

Although a number of classes of antihypertensive agents target the vascular smooth muscle [α blockers, angiotensin converting enzyme inhibitors, angiotensin receptor blockers, calcium channel blockers], until recently, there was little experimental evidence consistent with the regulation of vascular tone being an important factor for the molecular mechanism that produces hypertension (2,3).

The ACC/AHA and WHO recommends that patients adopt non-pharmacological interventions such as weight control, healthy habits with a reduced intake of sodium in the diet, and active physical exercise in the early stage of hypertension (1). The optimal treatment of hypertension significantly reduces the rate of myocardial infarction, stroke, heart failure and chronic kidney

disease (3). But this exogenous intervention with antihypertensive drugs interferes with the body's natural self-hemodynamic control mechanisms.

Numerous synthetic anti-hypertensive drugs have become available in the market through research owing to the rising prevalence and severity of hypertension throughout the world (23). This synthetic agents mainly target kidney tubule transporters and exchangers, angiotensin receptors, angiotensin converting enzyme II receptors and aldosterone and aldosterone receptors (24). However, this targets are associated with sever adverse effects and the risk of resistant hypertension is high (25). The prevalence of resistant hypertension has increased with the development of new guidelines, obesity, a high-sodium, low-fiber diet, excess alcohol intake, physical inactivity, obstructive sleep apnea, use of cocaine, amphetamines, non-steroidal anti-inflammatory drugs, oral contraceptive hormones, adrenal steroid hormones, sympathomimetic drugs (nasal decongestants and diet pills), erythropoietin, licorice, herbal supplements such as ephedra, progressive renal insufficiency, and inadequate diuretic therapy (26).

Treatment of hypertension with anti-hypertensive synthetic drugs is associated with side effects such as dizziness, nausea, stomach problems, impotence, fatigue, insomnia, loss of appetite and many others (27). It also increases the risk of developing new diseases which worsen the situation and result in sub-optimal control of BP (28). Consequently, many scientific studies suggest different life style changes and use of appropriate phyto-medicines in the treatment of high BP due to the various side effects that come with the use of different types of conventional anti-hypertensive drugs (29). The economic burden of long-term medication can also lead to non-compliance in therapy. Regardless of their availability, people who live in many developing countries are forced to frequently opt to and rely on traditional medicine which mainly uses medicinal plants due to their low socio-economic levels, high cost and harmful side effects of conventional medicines (30). This all could explain why more and more people prefer non-drug therapies to manage this condition (8). In recent years, several studies have reported associations between specific dietary ingredients improving BP (31).

Recently many different new targets to treat hypertension are being proposed. The pathophysiology of hypertension is thought to involve brain renin-angiotensin system hyperactivity. Angiotensin III, a key effector peptide in the brain renin-angiotensin system, provided tonic stimulatory control over BP in hypertensive rats. Aminopeptidase A, the enzyme

responsible for generating brain angiotensin III, constitutes a potential therapeutic target for hypertension treatment (32).

Angiotensin-converting enzyme 2/angiotensin-(1-7)/Mas receptor axis (33), vasoactive intestinal peptide receptor, intestinal sodium hydrogen exchanger 3 (NHE_3) (26), endothelin receptor, nitric oxide pathway, vaccines, gastrointestinal microbiota, leptin, sodium-glucose cotransporter 2 (24) and dopamine β hydroxylase (34) are recently identified potential new therapeutic targets in the treatment of hypertension. For the treatment of systemic hypertension, pharmacological intervention in NO-cGMP signaling is a well-explored but unexploited option (35).

2. Literature review

2.1 Cellular interactions in maintenance of vascular physiology

Endothelial cells (ECs) constitute the internal structure of the entire circulatory system (36). Although it is only a delicate single layer of cells lining blood vessels, the vascular endothelium is a highly complex organ with multiple paracrine, endocrine and autocrine functions that regulate biological homeostasis (37). They control immune responses, control blood coagulation states, serve as an interface adjusting blood-tissue permeability, contribute to angiogenesis and vessel repair, and modulate blood vessel tone (38).

Functional ECs can release vasodilators such as nitric oxide (NO) (39), prostacyclins, kinins, and endothelium derived hyperpolarizing factors (EDHF); vasoconstrictors such as endothelin-1, prostaglandin H_2 (PGH_2) and reactive oxygen species. Among endothelium-derived vasodilators, NO occupies a central position since changes in the release of endothelial NO plays a central part in the perturbation of vascular homeostasis and in the development of endothelial dysfunction associated with various cardiovascular disorders (40). NO and prostacyclin (PGI) are continuously released by ECs and the tonic release of NO plays an important role in the control of basal vascular tone (41).

In contrast, with a few exceptions, systemic BP remains unchanged if the synthesis of the vasodilator PGI is blocked by cyclooxygenase (COX) inhibitors although it is also continuously released as indicated by its tonic effects on platelet cyclic adenosine monophosphate (cAMP) (42,43). One possible explanation for the lack of a pressure increase following COX inhibition would be that the attenuation of PGI production is compensated for by the enhanced release of another vasodilator, NO (43,44).

NO is a simple diatomic molecule with important vasodilator, anti-thrombotic and anti-proliferative activity. It is made by the constitutively expressed enzyme endothelial nitric oxide synthase (eNOS) from L-arginin in the EC layer and signals to underlying VSMCs, where it elicits hyperpolarization and relaxation primarily in a cyclic guanosine mono phosphate (cGMP) dependent manner (45).

Most endothelial functions are dependent to various extents on changes in intracellular calcium concentration. In particular, store-operated Ca^{2+} entry has been shown to regulate many of endothelial functions (38).

In ECs, agonists such as bradykinin, angiotensin II and acetylcholine, when occupying their specific receptors on the cell membrane, couple with and activate a guanosine nucleotide-binding protein, which subsequently activates phospholipase C-β_1 in a Ca -independent manner (46). Phospholipase C-β_1 then hydrolyses the lipid precursor phosphatidylinositol-4,5-biphosphate (PIP_2) to yield inositol 3,4,5 triphosphate (IP_3) and diacylglycerol (DAG) (38,46).

Once formed, IP_3 binds to its specific receptors inositol 3,4,5 triphosphate receptor (IP_3R) on the surface of the endoplasmic reticulum (ER) and activates IP_3R Ca^{2+} release channels, which depletes internal Ca^{2+} stores and results in a transient increase in intracellular calcium concentration (47). This increase in intracellular Ca^{2+} concentration results in activation of calcium calmodulin (CaM) and leads to activation of eNOS which catalyzes the production of NO from L-arginine (6).

NO diffuses in to VSMCs mainly through gap junctions and directly activates soluble guanylate cyclase, leading to the formation of cGMP from guanosine triphosphate (GTP) (40,48). In VSMCs, the formed cGMP activates large-conductance Ca^{2+} activated K^+ channels (BKca) and ATP-sensitive K^+ channel (K_{ATP}); leading to the control of membrane potential which determine the activity of voltage dependent calcium channels (VDCCs). VDCCs are the main gateway for Ca^{2+} entry into the cells (48). Thus, the closing of K^+ channels result in membrane depolarization, allowing the activation of VDCCs and entry of extracellular Ca^{2+}, which can be accompanied by the release of Ca^{2+} from intracellular Ca^{2+} stores such as sarcoplasmic reticulum (SR) and mitochondria. The result is an increase in the concentration of cytosolic Ca^{2+}, allowing VSMC contraction and, therefore, vasoconstriction (6).

On the other hand, the opening of K^+ channels causes membrane hyperpolarization and the closing of VDCCs. The concentration of cytosolic Ca^{2+} is reduced by the plasma membrane Ca^{2+}-ATPase (PMCA) and Na/Ca exchanger (NCX), which transport Ca^{2+} outside the cell, while the Ca^{2+}ATPase in the sarcoplasmic reticulum and mitochondria recover Ca^{2+} into these intracellular stores (48). Here, it results in a decrease in concentration of cytosolic Ca^{2+}, causing VSMC relaxation and consequently, vasodilation (45).

The endothelium also mediates hyperpolarization of VSMCs via an NO-independent pathway, which increases potassium conductance and subsequent propagation of depolarization of VSMCs, to maintain vasodilator tone (49). It is well recognized that endothelium derived hyperpolarization factor (EDHF) can compensate for loss of NO-mediated vasodilator tone, particularly in the microcirculation, and this appears important when NO bioavailability is reduced (50).

Prostacyclin (PGI2), derived by the action of the COX system, is another endothelium-derived vasodilator that acts independently of NO. Though it may contribute to some of the other regulatory roles of the endothelium, it appears to have a more limited role in the maintenance of vasodilator tone in humans (50).

Dysfunctional endothelium is observed when there is an imbalance between NO production and consumption, favoring consumption in the presence of reduced production. Such a pathologic state creates favorable conditions for platelet and leukocyte activation and adhesion, as well as the activation of cytokines that increase the permeability of the vessel wall to oxidized lipoproteins and inflammation mediators, finally resulting in structural damage of the arterial wall with smooth muscle cell proliferation and atherosclerotic plaque formation (51).

2.2 Smooth muscle cell control of vascular tone

Calcium is believed to provoke the phosphorylation of myosin, resulting in myosin-actin interaction, splitting of adenosine triphosphate (ATP), and shortening or tension generation (52). Cytosolic Ca^{2+} is increased through Ca^{2+} release from intracellular stores (SR) as well as entry from the extracellular space through Ca^{2+} channels (receptor-operated Ca^{2+}channels) (53). In the VSMCs, agonists such as norepinephrine, EP and phenylephrine acting on GPCRs ($G_{q/11}$), activate the phospholipase C (PLC) pathway generating IP_3 and DAG (6,53). IP3 binds to its receptors in the sarcoplasmic reticulum (SR), releasing Ca^{2+} into the cytosol; simultaneously, DAG stimulates protein kinase C (PKC), which phosphorylates various substrates including to L-type VDCCs allowing Ca^{2+} entry into the cytosol (52,53).The rise in intracellular Ca^{2+} allows it to bind calmodulin (CaM,) which leads to the activation of the myosin light-chain kinase (MLCK), which phosphorylates myosin light chain number 20 (MLC_{20}) producing actin-myosin coupling and vasoconstriction. Similarly, PKC phosphorylates and triggers CPI_{17}; a smooth

muscle-specific protein kinase inhibitor of MLCP, preventing MLCK and maintaining contraction (6).

In addition to the Ca^{2+} dependent activation of MLCK, the state of myosin light chain (MLC) phosphorylation is further regulated by MLCP which removes the high-energy phosphate from the light chain of myosin to promote smooth muscle relaxation (53).

Thus, vasodilation of VSMCs is due to the decrease in cytosolic Ca^{2+}, causing its dissociation from the CaM, MLCK deactivation, and MLCP-mediated MLC_{20} dephosphorylation, resulting in the detachment of the actin-myosin interaction. Lastly, Ca^{2+} output occurs via PMCA, and Ca^{2+} entry into SR occurs via the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) **Figure 1** (6).

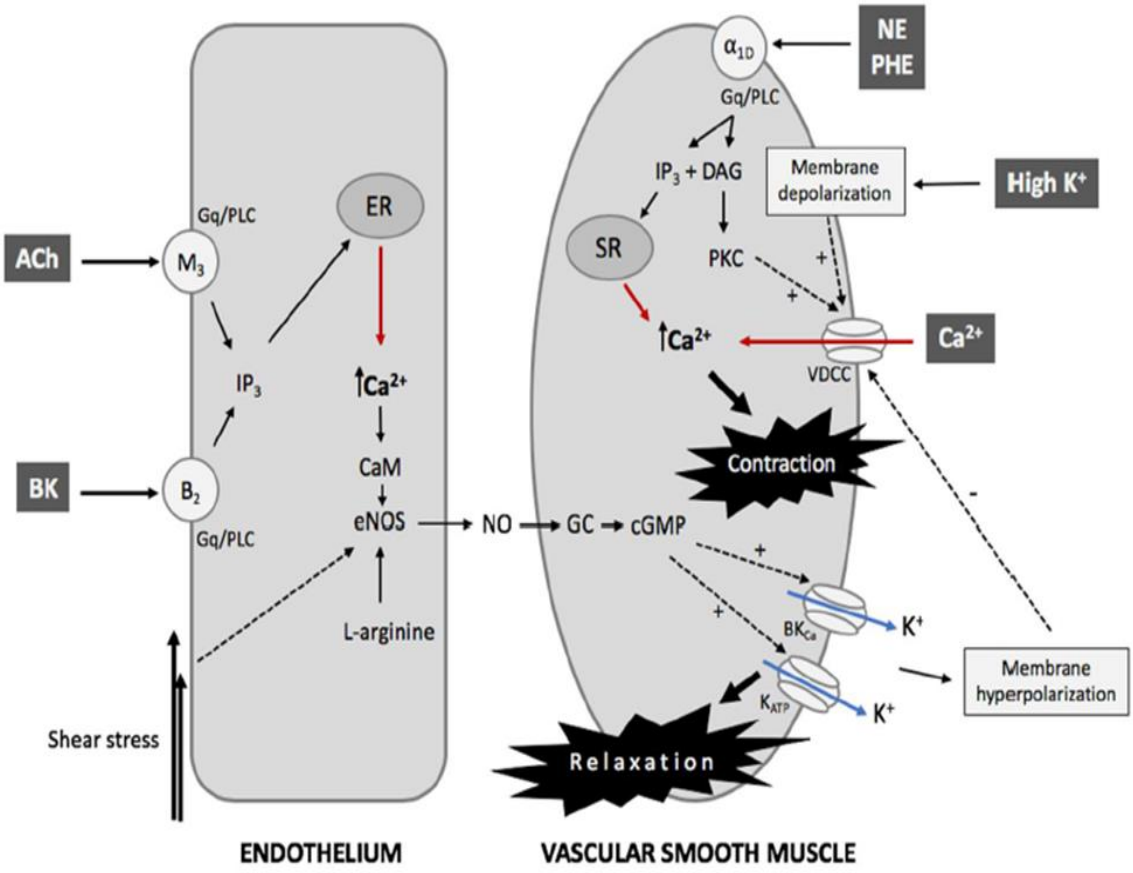

Figure 1: Interaction between endothelium and vascular smooth muscle cells in the aorta. The figure is a simplified representation showing the main component determining aortic contractibility associated with the isolated guinea pig aorta (IGPA) model used the bioactivity of phyto-chemicals. ER: endoplasmic reticulum; SR: sarcoplasmic reticulum; ACh: acetylcholine; BK: bradykinin; PHE: phenylephrine; NE: norepinephrine; M3: muscarinic type-3 acetylcholine receptor; B_2: bradykinin type-2 receptor; α1D: alpha-1D-adrenergic receptor; Gq/PLC: Gq-protein/phospholipase C; IP3: inositol 1,4,5-trisphosphate; DAG: diacylglycerol; PKC: protein kinase C; CaM: calmodulin; eNOS: endothelial nitric oxide synthase; NO: nitric oxide; GC: guanylate cyclase; cGMP: cyclic guanosine monophosphate; BKca: large-conductance Ca^{2+}-activated K^+ channel; K_{ATP}: ATP-sensitive K^+ channel; VDCC: voltage-dependent Ca^{2+} channel (6,48).

2.3 Vascular dysfunction

Vascular alterations including wall remodeling and endothelial dysfunction are common features in several forms of clinical and experimental hypertension (54). Notably, the presence of multiple risk factors is able to determine a progressive worsening of endothelial dysfunction (52).

The mechanisms controlling vascular tone are multifaceted and appear largely impaired in different pathological conditions. Hypercholesterolemia (55), atherosclerosis (56), ischemia and reperfusion (57), acute and chronic hypertension (58), congestive heart failure (59) and diabetes mellitus (DM) (60) are associated with abnormal endothelium-dependent vascular relaxation and increased vascular reactivity to different vasoactive substances, leading to vasospasm and tissue ischemia.

Nitric Oxide pathway is an important mechanism responsible for hypertension due to endothelial dysfunction. Endothelial lining of blood vessel is important for vascular health as it protects against hypertension and atherosclerosis. There are continuous evidences that inhibition of NO by superoxide anion and other ROS due to oxidative stress leads to hypertension and atherosclerosis (19). Endothelial dysfunction, defined as reduced vasodilating response to endothelial stimuli by the VSMCs, is observed in the presence of major CV risk factors, including aging, menopause, smoking, DM, hypercholesterolemia and hypertension (3).

In hypertension, it is found that there is endothelial dysfunction characterized by impaired release of endothelial-derived relaxing factors (NO, EDHF) and enhanced release of endothelial-derived constricting, pro-inflammatory, pro-thrombotic, and growth factors. Thus, the NO pathway is thought to be one of the most important regulatory mechanisms that protects against hypertension, and NO deficiency is thought to contribute in the development of hypertension (4). There is evidence of a positive association between the degree of endothelial dysfunction and the severity of hypertension (2).

Uncontrolled Endothelial Cell response is also involved in many disease processes; including pulmonary hypertension, sepsis and inflammatory syndromes. These diseases are related to endothelial injury, dysfunction and activation (46). There has been an exponential growth in knowledge on the role of endothelial cells in the modulation of underlying smooth muscle tone in response to pharmacological agents, physiological stimuli and disease (61). The major underlying mechanism for endothelial dysfunction seen in hypertension is the decrease in the

availability of NO, a consequence of increased oxidative stress in these patients (50). Endothelial dysfunction has been implicated in many CVDs, which renders modulation of endothelial functions as a promising therapeutic target (38).

2.4 Traditional medicine use in hypertension

Plants are commonly used in many indigenous systems of medicine for therapeutic purposes and are increasingly becoming popular in modern society as alternatives to synthetic medicines (27). Thus, in many countries around the world, patients frequently consume phyto-medicine to improve the therapeutic effect of conventional drugs. The interest in herbal medicine can be elucidated by the growing amount of information showing that many phytochemicals, particularly polyphenolic compounds, have healthy properties including benefits for CV health (6).

Herbal medicine is generally cheaper, accessible or readily available and more culturally acceptable to many for the reason that they cause less side effects than some synthetic drugs (62). Nowadays it is estimated that about 80% of people in developing countries still relays on traditional medicine based largely on species of plants and animals for their primary health care. Herbal medicines are currently in demand and their popularity is increasing day to day (27). Studies indicate that hypertensive patients use phyto-medicine predominantly to cure or slow progression of disease (29).

2.5 *Lupinus albus L.* (Fabaceae)

Lupinus albus belongs to genus Lupinus (Genisteae or Fabaceae) the third largest family, is an herbaceous hairy annual plant, native to the Mediterranean Regions **Figure 2** (63). Lupines are highly valued as animal feed but have limited use as human food; yet the seeds are reported to be a rich source of protein and oil. There are also claims that the seeds are rich in dietary fiber and beneficial phytochemicals (64). Lupine flour is added to pasta, crisps, bread and emulsified meat products to increase nutritional value, aroma as well as modify the texture of the end products. In addition, protein isolate produced from lupine seeds is utilized for milk and meat imitation products. Lupine seeds are consumed as a snack after they are soaked in water, scalded and de-hulled (Middle East) and used to produce pickle in Europe (65).

Figure 2: *Lupinus albus L.* seed (left) and the green plant with flower (right) (66)

Lupinus albus (white lupine) is cultivated in Ethiopia in Gondar, Gojjam, Arsi, Harrargeh and Shewa regions locally known as '*Gebto*' (in Amharic), is used as roasted bean '*kolo'* and to prepare alcoholic drink '*katikala*' known locally as *"Gebto Arekei"* which is used in traditional medicine for treatment of hypertension; and other food products especially in the northwestern part of the country, after debittering by roasting and soaking the seeds in a river/spring water for 3-7 days (63, 65).

It is the most important traditional crop mostly produced and consumed by smallholder farmers in North Western Ethiopia for its grain and soil fertility values (63, 64).

Other non-pharmacological uses of *Lupinus albus* include ornamentation, erosion control and soil stabilization, pest control, rotation crop and fence. Lupine containing alkaloids such as sparteine and anagrain has important place in pharmaceutical industries and cosmetics as well as ecto-parasite control (64).

Lupinus albus seed powder caused a profound hypoglycemic action in normal and diabetic rabbits (67). The γ-conglutin; protein contained in lupine seeds, has particular interest owing to its demonstrated ability to control glycaemia via interaction and binding with insulin and its insulin-mimetic properties. A patent, which awaits commercial exploitation, has been granted for the pharmacological use of γ-conglutin for the treatment of type II diabetes (68). In a study, 12 weeks of *Lupinus albus* use significantly decrease glycated hemoglobin, plasma total cholesterol,

low density lipoprotein cholesterol, triglycerides, and alanine aminotransferase activity (69,70). The aqueous extract of *Lupinus albus* has hypoglycemic and hypolipidemic effect by increasing insulin level and decreasing insulin resistance. In addition, it ameliorate most complications of diabetes (71).

The industrial transformation of lupine seed utilization from feed to food has recently increased the scientific interest to explore its phytochemical composition and biological activities. Lupine seeds contain significant amounts of polyphenols, carotenoids, phytosterols, tocopherols, alkaloids and peptides with antioxidant, antimicrobial, anticarcinogenic and anti-inflammatory activities. Among polyphenols, genistein and their derivatives (isoflavones) are of great importance because of their phytoestrogenic potential (72).

Lupine protein isolates have a variety of beneficial health-related properties, such as bile acid binding, angiotensin I converting enzyme inhibition and radical scavenging activity (73). It also got United States Patent Application for its content of anti-sticking compounds (74).

Edible seeds, such as edible beans and cereal grains, have been reported to possess many health benefits. Moreover, recent studies suggest that many fermented edible seeds and their products contain enhanced bioactive components and exhibit various bioactivities (75). Possible mechanisms for this include effects on oxidative stress and NO metabolism. Although regular consumption of lupine enriched bread can lower BP, results do not support for the hypothesis that this was via effects on oxidative stress or vascular function (76). NO and L-[14C]citrulline were synthesized in lupine roots and nodules in an L-arginine-dependent manner (77-79).

A diet enriched in lupine kernel flour can lower BP, but mechanisms responsible are unclear. It was found that lupine protein can attenuate the development of hypertension, and improve vascular function in a salt-sensitive rat model of hypertension. The mechanisms are uncertain, but may be linked to components in lupine such as the polyphenols, protein, and or L-arginine (76).

Two-week treatment of lupine protein isolate using the Goto-Kakizaki rat model, decreased SBP by 18.6 mmHg than control. It was explained that attenuation of the hypertension may be due to improved vascular function observed in the lupine groups with respect to the control group (80). Lupine protein is rich in L-arginine, containing approximately 10% by weight. Increased L-arginine intake can augment NO status and thus contribute to lower BP. Thus, lupine protein may benefit BP lowering via effects on NO metabolism (76).

Small quantity of *Lupinus albus L.* seed and fruit is grounded with water, filtered and the resultant juice is given orally in the morning for the treatment of hypertension in Gondar, Ethiopia (81,82). In Menz Gera Midir District, North Shewa Zone, Amhara Regional State it is soaked with water for 3-5 days, the water is decanted, and eaten or prepared in the form of alcohol and drunk (30).

The local community in North Western Ethiopia uses *"Gebto arekei"* as a locally made antihypertensive medicinal preparation (64). *"Gebto arekei"* is obtained by distilling a fermented brew prepared in the same way as other cereal based alcoholic beverages except that in this case seeds of *Lupinus albus L.* is used as one of the ingredients (63).

A study indicated that a residue from *Lupinus albus L.* distillated seed extract (fermented medicinal preparation) *("Gebto Arekei")* caused a dose-dependent drop in BP in anesthetized normotensive guinea-pigs (64). The reduction in BP was found to have rapid and sustained phases. The sustained phase had slow recovery period. The ED_{50} for the mean BP was found to be 14.86 mg/kg. The percentage change in BP variables measured during acute phase was slightly greater than that of sustained phase although there are not yet direct experimental explanations on the basis of these results. However, it is unknown if hypotensive effect was associated with components of lupine from "Gebto Arekei" or the ethanol from it or their interaction. The mechanism of hypotensive effect is also unknown.

A completely unexplored area is the possible role of polyphenols on the hypotensive effects exerted by lupine diets; in fact, numerous polyphenols have major role in the hypotensive activity of different plant extracts (76,80,83).

2.6 Traditional preparation of "Gebto Arekei"

"Gebto Arekei" is a popular traditional medicinal preparation used in the treatment of hypertension in North Western parts of Ethiopia. It is prepared by using "gebto" (*Lupinus albus L.*) seeds as a responsible medicinal ingredient. Its preparation is as follows;

Clay pot container (ensira) is washed with water and leaves of "grawa" (*Vernonia amygdalina*) and water for three times. The cleaned clay container (pot or ensira) is inverted over smoking wood fragments of "weyra" (*Olea europaea*) for about 15 minutes. This is expected to remove sensitive microorganisms to wood smoke and adds the desired flavor to the product. "Bikil" (malt), the source of amylase is prepared by moistening *Lupinus albus L.* ("gebto") seeds in a

suitable container and left to germinate for about five days and then sun dried. The dried malt is grinded by using traditional mortar and pestle (mukecha and zenazena) to a fine powder. Leafs of gesho plant (*Rhamnus prinoides*) is sun dried and grinded as well. Enkuro; which is prepared by separately toasting "gebto" flour on a metal pan with sprinkling water on it during toasting until it turns dark brown. Alternatively, enkuro can be replaced by kita (a baked form of "gebto" flour). The enkuro or broken small pieces of kita is allowed to cool. Enkuro or kita is dissolved in sufficient amount of water in to the clay pot. Then gesho which is mixed with malt powder in 1:2 ratio and dissolved in water in separate container and stayed for about three days is transferred in to the clay pot containing enkuro or kita and water to give a mixture of free flowing consistency and tightly sealed and allowed to stay aside to ferment for about five days. This fermentation product is known as "yerekei-tinsis". After five days of fermentation process, "gebto arekei" is obtained by distillation of "yerkei tinsis" by using traditionally made materials.

2.7 Statement of the problem

Cardiovascular diseases (CVDs) are the major cause of morbidity and mortality globally necessitating investigation for new preventive and therapeutic approaches. A previous study reported that both lupine (compound isolated from *Lupinus albus L.*) and soy protein treatment normalized the impaired vasocontraction observed in the NaCl-treated GK rats, but only the lupine treatment improved the severely diminished vascular function (84). In addition, residue from *Lupinus albus L.* distillated seed extract caused a dose dependent decrease in blood pressure in anaesthetized normotensive guinea pigs (64). However, the underlying mechanism for the improved vascular function following the *Lupinus albus L.* protein treatment as well as reduction in blood pressure up on administration of *Lupinus albus L.* distillated seed extract is not yet understood. Furthermore, despite its widespread traditional use as an antihypertensive preparation, there is no clear understanding on how *Lupinus albus L.* seed preparations are used for the management of hypertension along with its pharmacological mechanisms, which requires careful investigation to determine whether it is mediated through endothelium and/or smooth muscle of the blood vessels. Understanding of direct or indirect effects of medicinal plant extracts on blood vessels is very important. For example, a medicinal preparation where its mechanism of action is through endothelium will provide no benefit if given to patients with endothelial dysfunction. Therefore, this study was aimed to prove the mechanism how *Lupinus albus L.* distillated seed extract improves vascular function. Hence it will verify the rationality of traditional use of this plant preparation for hypertension and its potential in the development of new therapeutic drug entities.

3. Objectives

3.1 General objective

- To evaluate vasorelaxation activity and investigate mechanisms of relaxation of *Lupinus albus L.* distillated seed extract (Gibto Arekei) in isolated guinea pig thoracic aorta.

3.2 Specific Objectives

- To determine the role of eNOS-NO-cGMP pathway in vasorelaxation response induced by distillated seed extract from *Lupinus albus L.*; "Gibto Arekei".
- To investigate the effect of distillated seed extract from *Lupinus albus L.* "Gibto Arekei" in receptor operated calcium channel.
- To investigate the effect of distillated seed extract from *Lupinus albus L.* "Gibto Arekei" in voltage operated calcium channel.
- To determine the concentration response curves of distillated seed extract from *Lupinus albus L* "Gibto Arekei" and estimate the concentration that causes maximal response (E_{max}) and 50% of maximal response (EC_{50}).

4. Materials and methods

4.1 Chemicals and reagents

The chemicals and reagents used include distilled water, calcium chloride (Riedel-de Haen, Seelze, Germany), magnesium sulfate (Riedel-de Haen, Seelze, Germany), sodium chloride (PARK scientific limited Northampton UK), acetylcholine chloride (Sigma-Aldrich Co.), methylene blue, NG nitro-L- arginine methyl ester, potassium chloride (all from PARK scientific limited Northampton UK), nifedipine, sodium nitroprusside and epinephrine (SIGMA-ALDRICH Germany) all are analytical grade quality.

4.2 Experimental animals

The experiments were performed in four (two male and two female) adult guinea pigs (*Cavia porcellus*) aged 2-3 months and weighing 350-400 grams. The guinea pigs were bred and acquired from Ethiopian Public Health Institute (EPHI). All animals used for this study were kept in standard animal cages and kept under laboratory conditions of temperature (22°C±3°C), relative humidity (40% - 70%), and 12-hour day/night and had free access to food (standard cabbage and pellet diet) and water. The animals were maintained at the above specified laboratory conditions by transferring them from their usual living rooms to the controlled rooms 2-3 days before use for the experiment. They were acclimatized in the experimentation room by allowing them to stay for thirty minutes with intermittent gentle rubbing with hands around their head and neck areas. All procedures were according to the National Guide for the Care and Use of Laboratory Animals (85). Ethics approval for this study was obtained from Ethical review board of school of Pharmacy, college of health sciences, Addis Ababa University.

4.3 Study material preparation and extraction

Fresh *Lupinus albus L.* distillated seed preparation ("Gebto arekei") along with the seed was brought from Gojjam; Denbecha Town 350 km North West of Addis Ababa in July 2020. The plant material was authenticated by a taxonomist, at College of Natural and computational Sciences of Addis Ababa University (AAU) and a voucher number (YL001) was obtained and deposited for future reference.

The concentrate from "gebto arekei" was obtained after evaporation of the fresh ethanol under reduced pressure of -7mmHg using rotary evaporator at a temperature of 40℃. Then the concentrate was lyophilized at a temperature of -40℃ for 12 days (**Figure 3)**.

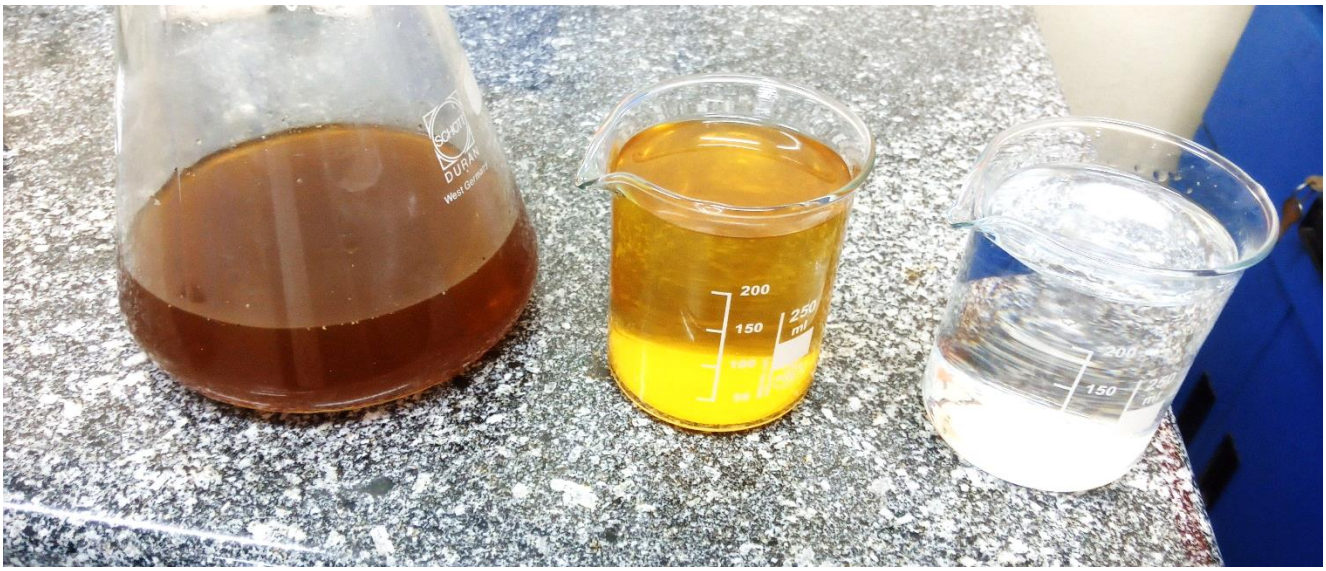

Figure 3: *Lupinus albus L.* distillated seed preparation (middle), after concentration by rotary evaporator (left) and condensed vapor of the preparation (right)

The weight was measured by using beam balance and the percent yield was calculated by weighting the initial volume of ethanol preparation used for evaporation and the extraction yield was as follows:

A total of 13 liters (13,000ml) of *Lupinus albus L.* distillated seed preparation was evaporated to get net 109.5 gram of final extract. It was found that the net weight of 13,000ml of *Lupinus albus L.* distillated seed preparation was 12,457.25 gram.

$$\text{Hence; } \%yield = \frac{weight\ of\ actual\ final\ dried\ extract}{dry\ weight\ of\ initial\ ethanol\ preparation\ used\ for\ evaporation}\ x\ 100$$

$$= \frac{109.5\ gram}{12{,}457.25\ gram} x100$$

$$= 0.88\%$$

Hence, each 100 gram dry weight of *Lupinus albus L.* distillated seed preparation consisted about 0.88 gram of dry extract.

The dried extract was grinded using mortar and pestle and transferred to refrigerator set at $4C^0$ until used for the subsequent experimental procedures (Annex I). The extract was freshly suspended in distilled water (DW) before use in experiments.

4.4 Evaluation of ex-vivo vasodilatory activity

The *ex-vivo* vasodilatory activity was investigated in isolated whole, spirally cut thoracic aorta strips of guinea pigs with and without endothelium using the method of ex-vivo isometric tension force recording according to Vogel (87). The guinea pigs were sacrificed by gentle cervical dislocation and the thoracic cavity was opened. The descending thoracic aorta was immediately removed and placed in Krebs-Henseleit Buffer (KHB) (118.4 NaCl, 4.7 KCl, 1.2 MgSO4×7H2O, 2.2 KH2PO4, 1.3 CaCl2, 24.9 NaHCO3, 11.1 glucose, all in mM at pH 7.4) physiological solution maintained at 37°C. Excess adherent fat and connective tissue was removed gently by surgical blade and heparinized capillary tubes. Each aorta was cut spirally in to strips using heparinized capillary tube with plastic sealing to ~2 mm wide and 5 mm long (88). The whole, spirally cut strips were then directly mounted in to an organ bath of total volume 35ml containing 31 mL physiological solution maintained at pH 7.4. The physiological solution was made to pass through a warm water jacket to maintain its temperature and continuously aerated with carbogen (95% O_2 and 5% CO_2) gas at a pH of 7.4. The pH of the buffer was checked every hour of aeration with carbogen (89). The strips were made to stabilize for 1 hour during which the physiological solution was changed every 15 minutes (90).

Experiments were carried out on aortic strips with intact (E^+) and denuded (E^-) endothelium. For experiments on intact endothelium, the presence and functional integrity of the endothelium was tested by administration of 1μM acetylcholine to pre-contracted aorta by 80mM potassium chloride, where the vasorelaxation of strips was noted if endothelium is functional (87,91).

In contrast, the experiment was performed on denuded endothelium by removing the endothelial cells from strips by gently rubbing the intimal surface with a moist Whatman filter paper No. 1 for 30-60 seconds. The success of removing endothelium was validated by using 1μM acetylcholine, which normally relaxes aortic strips with intact endothelium, but had no such effect on strips with destroyed endothelium pre-contracted by 80mM potassium chloride (92). Strips were considered to contain a viable endothelium when acetylcholine induced relaxations

exceeding 60-80% of pre-contraction, and were considered to be endothelium free when acetylcholine was failed to cause relaxation above 60% (93).

The aortic strips were then equilibrated for 1 hour during which the physiological solution was changed every 15 minutes and its pH was checked simultaneously. Then, the Grass 7D polygraph was calibrated under a resting tension of 1g. After 15 minutes of stabilization period, contraction of entire spirally cut thoracic aorta strips were induced by adding 80mM potassium chloride (KCl) or 1μM epinephrine (EP) in a drop wise fashion until 100% contraction of thoracic aorta strips was achieved in the presence or absence of specific antagonists.

For the experiments in the presence of specific antagonists (NG nitro-L-arginine methyl ester (L-NAME) or methylene blue (MB)), the spirally cut aorta strips were pre-incubated with 1ml of 100μM L-NAME and 1ml of 10μM MB for about 20 minutes prior to the administration of epinephrine (EP) (94).

In addition, endothelium denuded strips were made to stabilize in normal KHB solution and placed in calcium free KHB solution containing 0.1mM EDTA for 30 min. Then the solution was substituted by potassium rich and calcium free KHB solution containing 50mM KCl, 50.58mM NaCl, 3.1mM $MgSO_4$, 23.8mM $NaHCO_3$, 1.26mM KH_2PO_4, 11.1mM glucose and 0.1mM EDTA. After an incubation period of 1 hour, concentration response curves for calcium (control) was obtained at organ bath calcium ion concentrations of 1mM, 2mM and 3mM. The tissue was then washed by overflowing physiological fluid and stabilized in normal KHB followed by replacement of calcium free and potassium rich KHB solution. Finally, the tissue was pre-treated with highest (60μg/ml) of extract for 1 hour. Then, the calcium response curves were reconstructed for the extract (95). Similar procedure was conducted for standard (nifedipine) at a concentration of 0.03mM.

Preliminary tests were conducted for two purposes; firstly, to assure whether the aorta strip contractions were actually caused by the contracting agents (potassium chloride and epinephrine). In this case it was confirmed by using 1ml and 0.5ml DW to counter compare with KCl and EP respectively (result not presented here). The use of DW in the absence of contracting agents resulted in no contractile response from the strips. Secondly; to set the optimal extract concentration for the subsequent experimental procedure. The response to the extract was tested at much lower extract concentrations; 0.031mg/ml and 0.063mg/ml for both EP and KCl pre-contracted thoracic aorta strips (results not presented here). It was observed that the response was

non-steady and erratic at such lower concentrations. Thus, for the subsequent tests the concentration of *Lupinus albus L.* distillated seed extract was adjusted so that the lowest test concentration was set at 0.125mg/ml.

To achieve these concentrations, 100mg of extract was measured and dissolved in to 25ml of distilled water and shaken vigorously for about a minute until uniform solution of light yellowish brown color was obtained. This resulted in 4mg/ml of stock solution of *Lupinus albus L.* distillated seed extract. The stock solution (4mg/ml) of extract in distilled water was serially diluted by half until the required lowest concentrations; 0.031mg/ml and 0.063mg/ml for the pilot tests and 0.125mg/ml, 0.25mg/ml, 0.5mg/ml and 1.000mg/ml for experimental groups.

Then 1ml of extract in DW was added cumulatively from the lowest concentration (0.125mg/ml) to an organ bath with total capacity to contain 35ml but containing 31ml of physiological solution maintained at 37℃. Hence the organ bath concentration of *Lupinus albus L.* distillated seed extract was calculated by dividing the extract contained in 1ml of extract solution to total volume (DW that contained the extract and physiological fluid in the organ bath) which becomes 32ml. The organ bath concentration then becomes 0.125mg divided by 32ml resulting in 0.004mg/ml (4µg/ml) for the lowest test dose. By doubling the initial solution concentrations to 0.25mg/ml, 0.5mg/ml and 1mg/ml, the subsequent organ bath concentrations became 8µg/ml, 16µg/ml and 32µg/ml respectively.

After a contraction plateau was attained (100% contraction was achieved) using contracting agents (EP, KCl); 1ml of extract solution was administered cumulatively every 15 minutes at ascending concentrations of 0.125mg/ml, 0.25mg/ml, 0.5mg/ml and 1mg/ml into an organ bath containing 31ml resulting in an organ bath concentrations; 4µg/ml, 8µg/ml, 16µg/ml and 32µg/ml respectively. For the determination of possible synergistic effect through NO-cGMP pathway, both the extract and sodium nitroprusside (SNP) were dissolved together in one to one ratio to give the above concentrations (0.125mg/ml, 0.25mg/ml, 0.5mg/ml and 1mg/ml) and administered to pre-contracted strips. Finally, tension responses of the tissues were detected with transducers and recorded isometrically (87,96). Epinephrine was taken as a preferred standard of contracting agent throughout the experiment after relaxation response to the extract was found to be superior to potassium chloride. The experiment was conducted in four replicates (thoracic aortas from four guinea pigs; two male and two female under each experiment and control groups) and the data represented the mean value of four measurements (97).

At the completion of the experiment, relaxation which is a measure of inhibition of contraction in whole spirally cut aorta strips pre-contracted by 80mM KCl or 1μM EP in endothelium intact or endothelium denuded strips in the presence or absence of specific antagonists were determined using a measurement before and after extract and extract with SNP administration and calculated using the formula as (98):

$$\%\, Relaxation = \frac{Tc - Tt}{Tc} \times 100$$

Where;

*T*c: tension due to contracting agents

*T*t: tension after adding test extract

Aortas from four guinea pigs were used to ensure adequate biological variability (99).

To determine concentration response curves for calcium, percent response of contraction was analyzed.

4.5 Statistical analysis

All experimental data were expressed as mean values of %relaxation (inhibition of contraction) ± standard error of mean (SEM) after analysis by bio-statistical interpretation using SPSS windows Version 22 statistical packages through a one-way ANOVA followed by Tukey's test for multiple comparisons of the mean differences and responses of different drugs and extract. Independent T-test was used to compare level of significance between two groups. The values were presented as percent inhibition of contraction (% relaxation), taking the KCl and EP induced contraction before administration of extract and drugs as 100%. Statistical significance of *P*-value <0.05 was considered significant. Maximal response (E_{Max}) and concentration that caused 50% of maximal response (EC_{50}) was obtained by nonlinear regression with a four-parameter logistic equation (also known as the Hill equation) by using *Dr Fit* software version 1.042, 2015.

$$\hat{Y} = a + \frac{(b-a)}{\left[1+\left(\frac{c}{X}\right)^{d}\right]}$$

Where, $\hat{\mathbf{Y}}$= expected response at concentration **X**

a= minimum asymptote or the response when there is no extract (dosage is zero)

b= maximum asymptote or the stabilized response for an infinite dosage

c= dosage at which 50% of response (that is, the response half way between the minimum response asymptote "**a"** and the maximum response asymptote "**b"**) which also denotes the point of inflection in the dosage-response curve, and is referred EC_{50}

d= the slope at the steepest part of the curve, also known as the Hill slope

Tabulation format of rows, dose response format from 0-100 with Standard Hill curve fitting using parameters by weighting standard deviation for goodness of fit and fitting algorism of simplex constrained was applied.

5. Results

A drop wise addition of 1ml of 80mM potassium chloride (KCl) or 0.5ml of 1µM epinephrine (EP) to descending thoracic aorta strips of guinea pigs maintained in organ bath attached to isometric transducers of Grass 7D Polygraph (model 7P1G SERIAL 9L38P6 U.S.A) successfully caused 100% contraction within 15 minutes in both endothelium intact and endothelium denuded strips. The contraction response of the strips to 1µM EP (non-selective adrenergic receptor agonist) was more rapid than to 80mM KCl (voltage dependent calcium channel activator). This rapid response to EP sometimes resulted in stacking of transducers due to excessive contraction force of the guinea pig thoracic aorta strips. In these occasions the strips were washed out by overflowing physiological fluid maintained at 37℃ and re-stabilized for another 1 hour during which the organ bath fluid was changed and pH was checked every 15 minutes. Care was taken to avoid this effect by adding EP in a drop -wise fashion.

Within group analysis of mean % maximum relaxation response to extract in endothelium intact strips pre-contracted by KCl showed significant increase in relaxation response at each increase in organ bath concentration ($p<0.001$ between no extract and 4µg/ml and between 4µg/ml 8µg/ml; $p<0.05$ between 8µg/ml and 16µg/ml; $p<0.001$ between 16µg/ml and 32µg/ml). Inter group analysis indicated significantly higher relaxation response in EP pre-contracted strips at the lower concentrations ($p<0.001$) than to that of aorta strips pre-contracted by 80mM KCl (**Table 1)**.

Table 1: Vasorelaxation effects of *Lupinus albus L.* distillated seed extract in endothelium intact and denuded strips in EP compared to intact strips in KCl.

Extract Concentration (µg/ml)	**Relaxation (%)**		
	Extract in 80mM KCl pre-contracted in endo-intact	Extract in 1µM EP pre-contracted in endo-intact	Extract in 1µM EP pre-contracted in endo-denuded
4	27.50±1.44^{3a}	62.50±3.23	10.50±2.10^{3a2b}
8	56.25±4.73^{3a}	100.00±0.00	28.25±3.50^{3a3b}
16	83.75±1.25^{3a}	100.00±0.00	49.5±2.10^{3a3b}
32	100.00±0.00	100.00±0.00	61.00±2.27^{3a3b}

Data are expressed as mean ± SEM (n=4); KCl: potassium chloride, EP: epinephrine, endo-intact: endothelium intact, endo-denuded: endothelium denuded. ^{1}=p<0.05, ^{2}=p<0.01, ^{3}=p<0.001. a=Compared to extract in EP pre-contracted in endo-intact, b=Compared to extract in KCl pre-contracted in endo-intact.

It is understandable that the relaxation response of endothelium intact aorta strips pre-contracted by 1µM EP to the extract was very rapid and highly pronounced than the relaxation response of aorta strips pre-contracted by 80mM KCl which was slow and steady i.e contraction of endothelium intact aorta strips induced by EP was reversed at a very small concentration of extract **(Figure 4)**.

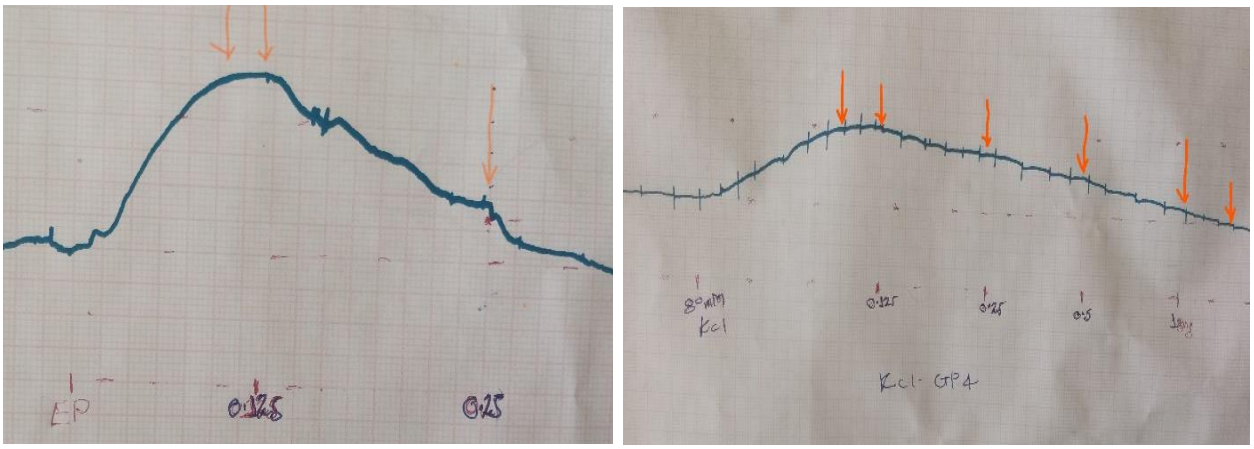

Figure 4: Relaxation response of endothelium intact aorta strips to *Lupinus albus L.* distillated seed extract pre-contracted by EP; left and KCl right. EP: Epinephrine, KCl: Potassium chloride.

The concentration of extract that caused 25% of relaxation response in aorta strips pre-contracted by 80mM KCl was 3.67µg/ml which is a concentration very similar to cause EC_{50} in strips pre-contracted by EP. The extract elicited 50%, 75% and 90% of response in endothelium intact strips pre-contracted by KCl at a concentrations of 7.01µg/ml, 12.3µg/ml and 21µg/ml respectively showing that response of endothelium intact strips pre-contracted by KCl was lower when compared to intact strips pre-contracted by EP.

In contrast aorta strips pre-contracted by 80mM KCl started to respond to extract at much lower concentration in comparison to EP pre-contracted strips which required a specific threshold extract concentration. The extract concentration of 21µg/ml caused 90% of relaxation response in endothelium intact aorta strips pre-contracted by 80mM KCl which was about five fold higher concentration to that of concentration required in EP (4.74µg/ml). The concentration of 7.01µg/ml produced half maximum response (EC_{50}) in strips pre-contracted by KCl which was about twice higher when compared to concentration of extract that was needed to induce EC_{50} in strips pre-contracted by EP (3.8µg/ml). These differences in relaxation responses between endothelium intact strips pre-contracted by 80mM KCl and 1µM EP showed increased sensitivity of strips to extract in EP pre-contracted than KCl **(Figure 5)**.

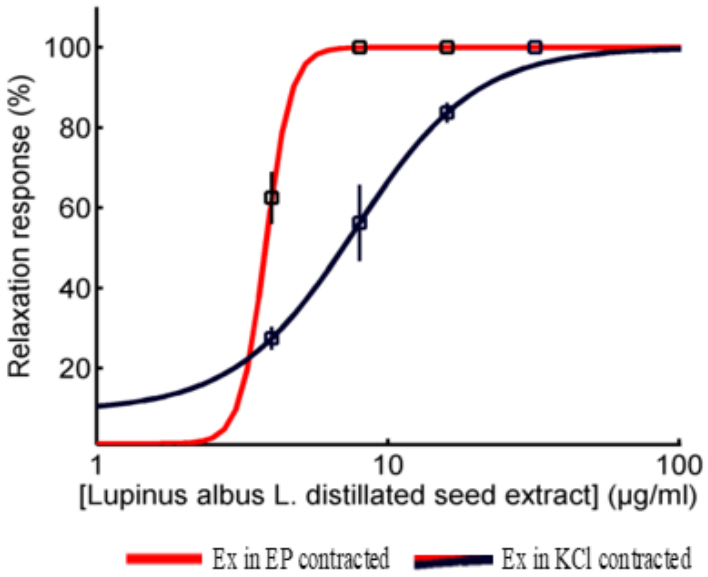

Figure 5: Concentration-response curve of *Lupinus albus L.* distillated seed extract in endothelium intact strips pre-contracted by EP in comparison to strips pre-contracted by KCl. Ex: extract, EP: epinephrine, KCl: potassium chloride.

Mechanical removal of endothelial layer triggered mean % relaxation response of up to 11.25% in strips pre-contracted by EP at the lowest concentration (4µg/ml). But up on gradual increase in extract concentration, it resulted in corresponding increase in relaxation response similar to endothelium intact strips. However, these concentration dependent increments in relaxation response of strips in endothelium intact and endothelium denuded categories were not at equivalent rate or at least in a comparable manner.

Within group comparisons between different concentration levels indicated significant ($p<0.05$ between 4µg/ml and 8µg/ml and 16µg/ml and 32µg/ml; $p<0.001$ between 8µg/ml and 16µg/ml) change in relaxation response in endothelium denuded strips. Unlike endothelium intact strips pre-contracted by EP, the extract failed to cause complete reversal of contraction in endothelium denuded aorta strips pre-contracted by EP even at the highest cumulative test concentration (60µg/ml). Hence complete relaxation response was not achieved in endothelium denuded strips though the removal of endothelium didn't prevent the strips from causing significant relaxation responses.

Hence, mechanical removal of endothelial layer from the aorta strips impaired the relaxation response of EP pre-contracted strips to extract. If the difference in concentration-response curves

between endothelium intact and endothelium denuded strips pre-contracted by 1µM EP were compared, endothelium denuded strips require significantly ($p<0.001$) higher level of extract concentration to bring about a specified relaxation response. In endothelium denuded strips it was 7.22µg/ml (3.39µg/ml in intact) of extract concentration that caused 25% of relaxation response. On the other hand, the concentration that was required to induce 50% of responses in endothelium denuded strips was 16.3µg/ml (3.8µg/ml in intact) which is significantly ($p<0.001$) different. Though endothelium denuded strips pre-contracted by EP were incapable of producing 75% of relaxation response. Rather produced a maximum relaxation response of only 66% at a concentration as high as 224µg/ml (by extrapolation) **(Figure 6)**.

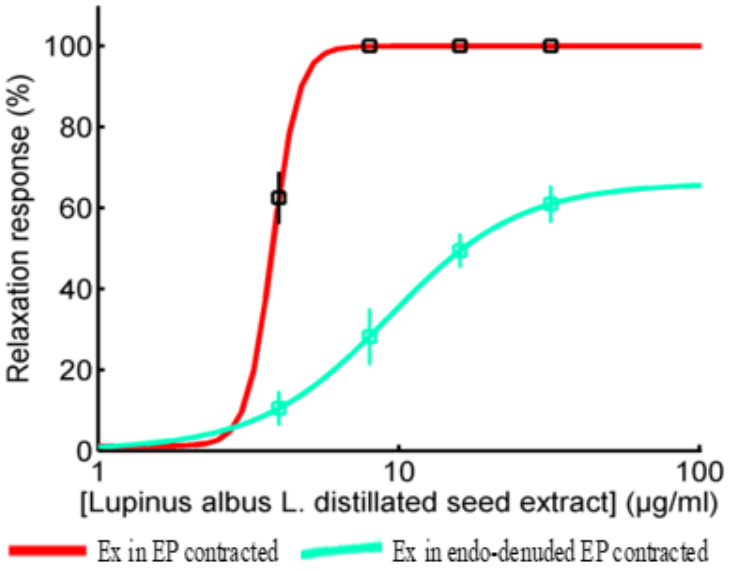

Figure 6: Concentration-response curves of *Lupinus albus L.* distillated seed extract in endothelium intact strips pre-contracted by 1µM EP in comparison to endothelium denuded. Ex: extract, endo-intact: endothelium intact strips, endo-denuded: endothelium denuded strips.

Generally observing from mean %relaxation response, it is clear that distillated seed extract of *Lupinus albus L.* when combined with SNP produced the highest relaxation response in endothelium intact strips pre-contracted by EP (88.75%) at the lowest concentration which is superior to response of similarly contacted strips to extract alone (62.5%). Combination of extract with SNP and extract alone caused complete inhibition of aortic strip contraction at a

cumulative concentration of 12µg/ml (4µg/ml plus 8µg/ml) in EP pre-contracted endothelium intact strips while it was 28µg/ml in standard **(Table 2)**.

Table 1: Vasorelaxation effects of *Lupinus albus L.* distillated seed extract in endothelium intact strips pre-contracted by EP compared to combination of extract with SNP.

Extract Concentration (µg/ml)	**Relaxation (%)**	
	Extract in 1µM EP pre-contracted	Extract with SNP in 1µM EP pre-contracted
4	62.50±3.23[3]	88.75±8.54
8	100.00±0.00	100.00±0.00
16	100.00±0.00	100.00±0.00
32	100.00±0.00	100.00±0.00

Data are expressed as mean ± SEM (n=4); EP: epinephrine, SNP: sodiumnitroprusside. [3]=$p<0.001$.

The % relaxation response in strips pre-contracted by 1µM EP to combination of extract and SNP and extract alone groups was significantly different. It is evident that the relaxation response was significantly higher ($p<0.001$) to combination of extract and SNP (88.75%) at a concentration of 4µg/ml in comparison to extract alone (62.5%). As both combination of extract with SNP and extract alone resulted in complete reversal of contracted strips, it was not possible to compare the differences between them at concentrations beyond 4µg/ml.

Comparison of concentration-response curves in endothelium intact strips of guinea pig thoracic aorta pre-contracted by 1µM EP indicated the extract when combined with SNP caused highest relaxation response at much lower concentrations than any other test groups. Extract in combination with SNP induced 90% of relaxation response at a concentration of 4.05µg/ml while the concentration that caused 90% of relaxation response in extract alone was 4.74µg/ml. Additionally, an extract concentrations of 3.39µg/ml, 3.80µg/ml and 4.24µg/ml of extract bring about relaxation responses of 25%, 50% (EC_{50}) and 75% respectively which is relatively lower responses when compared to those relaxation responses obtained by combining the extract with SNP. From these two concentration response changes, similar increase in concentration of

extract and extract combined with SNP didn't trigger equivalent relaxation response in endothelium intact strips pre-contracted by EP. The strips tended to respond to the combination of extract with SNP at lower concentrations than to extract alone. But as the concentration of extract alone and extract in combination with SNP increases at equivalent rate, the relaxation responses became closer **(Figure 7)**.

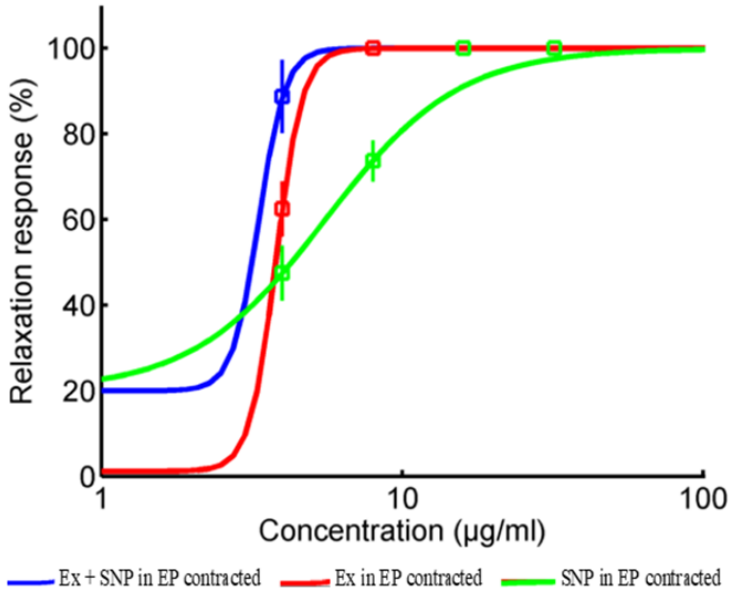

Figure 7: Concentration-response curves of *Lupinus albus L.* distillated seed extract in endothelium intact strips pre-contracted by 1µM EP in comparison to combined effect with sodiumnitroprusside. EP: epinephrine, Ex: extract, SNP: sodium nitroprusside.

Due to increased response of endothelium intact strips pre-contracted by EP to very small changes in concentration of extract in combination with SNP, increase in concentrations of both extract alone and extract in combination of SNP resulted in shifting of concentration-response curve to the right (more flattened) for the extract alone. Hence, a subtle increase in concentration of extract in combination with SNP tended to cause extensive changes in relaxation response.

Pre-incubated endothelium intact strips with L-NAME (endothelial nitric oxide synthase inhibitor) prior to contraction by EP resulted in as low as 6.25% of relaxation response at 4µg/ml (**Table 3)**. The response to extract alone and extract in combination with SNP in endothelium intact strips pre-contracted by EP was significantly higher ($p<0.001$ against L-NAME incubated, MB incubated and endothelium denuded at each change in concentration. Within group analysis in endothelium intact strips pre-incubated with MB provided significant ($p<0.001$) relaxation

response at each increase in extract concentrations even though it failed to cause complete inhibition of contraction at all concentration levels which is similar to endothelium denuded strips.

Table 3: Vasorelaxation effects of extract in endothelium intact strips pre-contracted by EP in the presence and absence of antagonists.

Extract Concentration (µg/ml)	**Relaxation (%)**		
	Extract in 1µM EP pre-contracted	Extract in 100µM L-NAME pre-incubated then 1µM EP pre-contracted	Extract in 10µM MB pre-incubated then 1µM EP pre-contracted
4	62.50±3.23	6.25±2.39[3a]	13.00±1.23[3a]
8	100.00±0.00	10.00±4.65[3a1b]	25.75±2.18[3a]
16	100.00±0.00	16.25±3.15[3a3b]	44.25±2.18[3a]
32	100.00±0.00	25.00±3.54[3a3b]	61.50±1.56[3a]

Data are expressed as mean ± SEM (n=4); EP: epinephrine, L-NAME: NG nitro-L-arginine methyl ester, MB: methylene blue. [1]=p<0.05, [2]=p<0.01, [3]=p<0.001. a=Compared to extract in EP contracted, b=Compared to extract in MB pre-incubated and EP contracted

Similar to endothelium denuded strips and endothelium intact strips pre-incubated with MB, the extract again didn't fully reverse pre-induced contractions in strips pre-incubated with L-NAME. One of the major difference of endothelium intact strips pre-incubated with L-NAME to those endothelium denuded strip group as well as endothelium intact strips pre-incubated with MB was that strips under L-NAME pre-incubated category didn't bring about significant relaxation response between consecutive changes in concentrations. But when the comparison becomes between two non-consecutive concentrations, the response becomes significant. For example, when the relaxation response at a concentration of 4µg/ml is compared to response at 32µg/ml it becomes significant ($p<0.001$) while it is not significantly different when it is compared between 4µg/ml and 8µg/ml. At lowest extract concentration (4µg/ml), there was no significant difference in relaxation response between endothelium intact strips pre-incubated with L-NAME and

endothelium denuded strips. But when the concentration was increased at the same rate for both group of strips, the difference become significantly ($p<0.01$) at 8µg/ml and ($p<0.001$) at 16µg/ml and 32µg/ml. Similar differences were also observed when endothelium intact strips pre-incubated with L-NAME was compared to endothelium intact strips pre-incubated with MB in which the extract caused no significant difference at the concentration of 4µg/ml but significant ($p<0.05$) at 8µg/ml and ($p<0.001$) at 16µg/ml and 32µg/ml.

Although the significance level at the lowest concentration (4µg/ml) was different when endothelium intact strips pre incubated with L-NAME and MB as well as endothelium denuded strips were compared to endothelium intact strips pre-contracted by KCl, the result was strongly significant ($p<0.001$) at all higher concentrations.

Pre-incubation of endothelium intact guinea pig thoracic aorta strips with 100µM L-NAME and methylene blue (MB) before induction of contraction by 1µM EP profoundly decreased ($p<0.001$) relaxation response of intact strips. The extract caused 25% of relaxation responses in 100µM L-NAME pre-incubated intact strips at 32µg/ml (3.39µg/ml in absence). However, it failed to cause relaxation response beyond 47%. In other words endothelium intact strips pre-incubated with L-NAME before induction of contraction by EP were unable to trigger relaxation response more than 47% irrespective of increasing the extract concentration to infinity **(Figure 8)**.

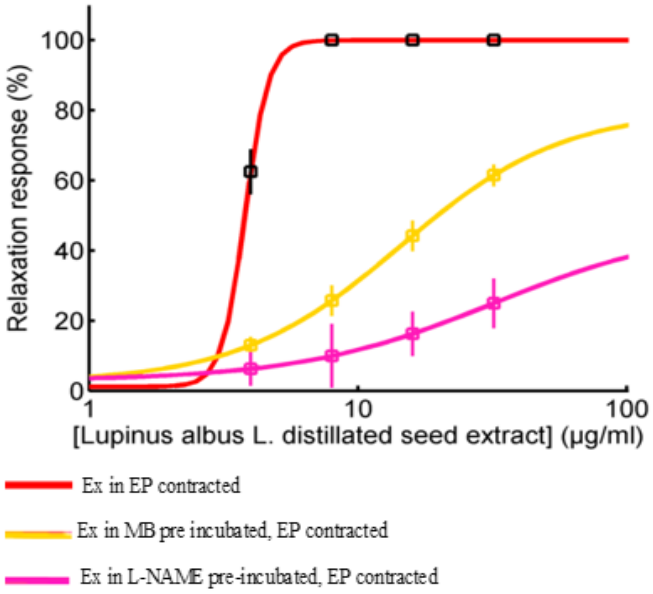

Figure 8: Concentration-response curves of *Lupinus albus L.* distillated seed extract in endothelium intact strips pre-contracted by 1µM EP in comparison to strips pre-incubated with antagonists. Ex: extract, L-NAME: NG-Nitro-L- arginine methyl ester, MB: methylene blue.

Similarly, pre-incubation of endothelium intact strips with 10µM MB prior to contraction by EP reduced the relaxation response to the extract as well. The extract caused 25%, 50% and 75% of relaxation responses in 10µM MB pre-incubated endothelium intact strips at 7.75µg/ml (3.39µg/ml in without MB), 19.7µg/ml (3.8µg/ml in without MB) and 89.5µg/ml (4.24µg/ml in without MB) respectively although it didn't cause relaxation response more than 80%.

When the concentration-response curve to extract in endothelium intact strips pre-incubated with 100µM L-NAME is compared to response curve in similar strips pre-incubated with 10µM MB, the extract triggered greater response in strips pre-incubated with 10µM MB than strips pre-incubated with 100µM L-NAME. For example, endothelium intact strips pre-incubated with 10µM MB induced the relaxation response of 25% at the concentration of 7.75µg/ml whereas a concentration of as high as 32µg/ml was needed to trigger the same response in similar strips pre-incubated with 100µM L-NAME. Additionally, endothelium intact strips pre-incubated with 10µM MB caused maximum response of up to 80%, however the highest response caused by similar strips pre-incubated with 100µM L-NAME was only 47%.

In endothelium denuded group of strips, a concentration of 7.22µg/ml caused 25% of relaxation response which is not different to those concentration required (7.75µg/ml) to bring about the

same relaxation response in endothelium intact strips pre-incubated with 10µM MB. It was a concentration of 16.3µg/ml that resulted in 50% of relaxation response in endothelium denuded strips pre-contracted by EP which is lower concentration (19.8µg/ml) than in the case of intact aorta strips pre-incubated with 10µM MB pre-contracted by EP.

Above findings were strengthened by analysis of endothelium denuded thoracic aorta strips in potassium reach but calcium free solution and grudual increase of Ca^{++}. These provided further evidences regarding extracts effect on calcium channels. In control group strips, some level of contractile response was observed even in the absence of Ca^{++} in physiological solution. But such contractions were not detected in strips pre-incubated with extract. Comulative increase in Ca^{++} concentration caused increase in contractile response in control and test strips althogh in the test strips the rate of change in contractile response was different and even the strips didn't attain complete level of contraction **(Figure 9)**.

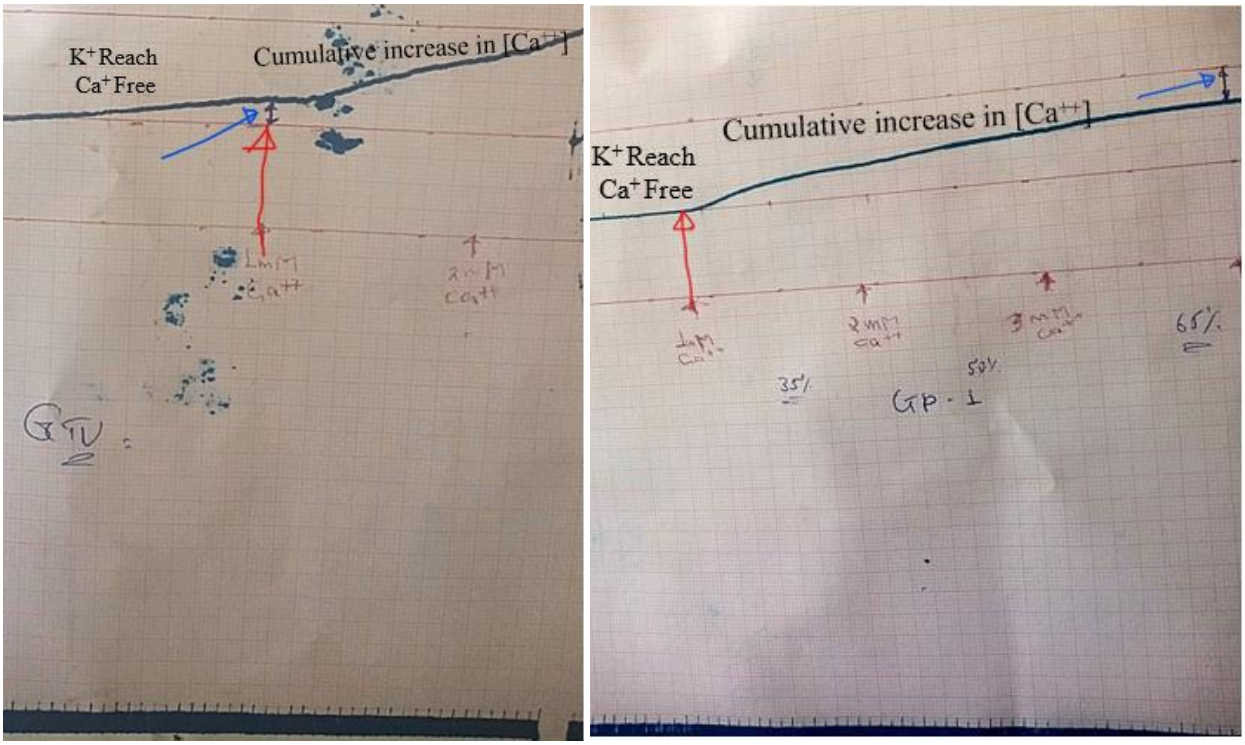

Figure 9: Change in contractile response of endothelium denuded aorta strips to cumulative increase in [Ca^{++}] in the absence of *Lupinus albus L.* distillated seed extract (left) and after incubated with extract (right).

Comparison of calcium concentration-response curves further showed presence of extract resulted in significant reduction in contractile response. Mean maximum contraction of 47.5%, 78.75% and 100% was recorded at a calcium concentration of 1mM, 2mM and 3mM respectively in the absence of extract while it was 28.75%, 52.5% and 65% in strips pre-incubated with maximum cumulative concentration of extract (60µg/ml). These results were significant at calcium concentration of 1mM ($p<0.05$), 2mM ($p<0.01$) and 3mM ($p<0.001$). Though, the standard didn't cause significant inhibition of contraction than extract at the lowest calcium concentration (1mM), it becomes significant at 2mM ($p<0.05$) and 3mM ($p<0.001$). From calcium concentration-response curves of control, extract and standard (nifedipine); it is observable that the contractile response in the presence of extract is increased when calcium concentration rises. As calcium concentration upsurges for both test and standard, the response curve for standard is shifted to the right (flattened) indicating superior inhibition of contraction in standard. Contractile response in test strips becomes strongly significant as the concentration of calcium increases. The nature of calcium concentration-response curves for both control and test is nearly similar except significant reduction in contraction with extract (**Figure 10)**.

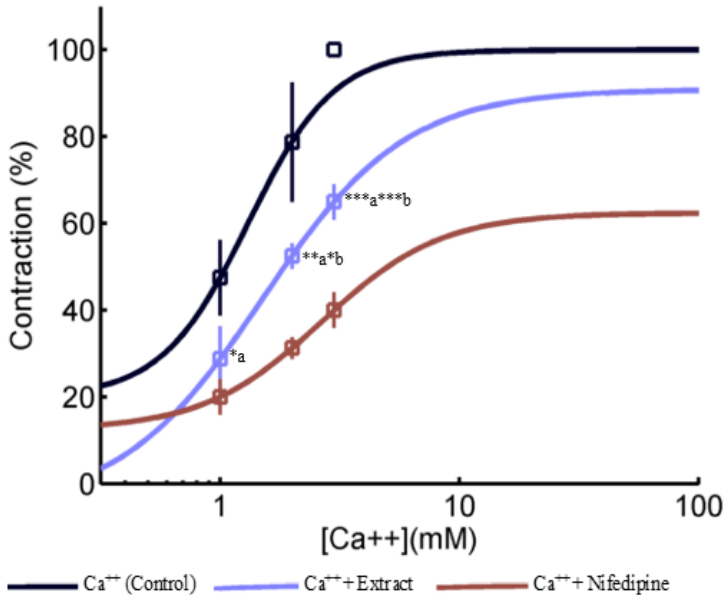

Figure 10: Differences in calcium concentration-response curves between control, *Lupinus albus L.* distillated seed extract and standard. a= compared to control, b= compared to standard. *: $p<0.05$, ** : <0.01, ***: $p<0.001$.

6. Discussion

This study focused on investigation of vasodilation effect and underling mechanisms of distillated seed extract from *Lupinus albus L*. The study showed for the first time that the distillated seed extract induced concentration dependent vasorelaxation effects in guinea pig thoracic aorta strips as evidenced from relaxation responses of endothelium intact and endothelium denuded strips pre-contracted by EP and KCl at different concentration ranges with the presence and absence of specific antagonists; L-NAME and MB.

Superior relaxation response of endothelium intact strips pre-contracted by EP to combination of extract with SNP shows possible synergistic activity of extract with SNP. An important question here is; how is the extract causing the observed synergistic activity in relaxation response. It leads to an assumption that the extract might have additional pharmacological mechanisms working together to induce the relaxation response explained as follows;

Previous phytochemical investigations carried out on *Lupinus albus L.* seed extracts reported presence of secondary metabolites such as polyphenols, alkaloids, tannins, flavonoids, saponins, coumarins, ketonic terpenoids and phytosterols with identified bioactivities especially on CV system (100,101). Generally, the greatest relaxation responses detected in intact strips pre-contracted by EP might be related to extract's combination effect through NOS-NO-cGMP pathway as well as inhibition of α_1-adrenergic receptors which are linked to diverse intracellular cascades one of which is inhibition of VDCCs (6). Since SNP is a known pharmacological agent that cause vasorelaxation through liberation of NO, the observed increase in vasorelaxation response when combined with the extract may indicate the extract effect through enhancing synthesis of endothelial NO and inhibiting calcium channels (103). This statement is more corroborated by significant reduction in relaxation response to extract when the eNOS was blocked by L-NAME.

Although removal of endothelium from strips and pre-treatment of strips with L-NAME and MB profoundly reduce relaxation response, it didn't completely prevent the strips from triggering concentration dependent changes. Furthermore, calcium concentration-response curves provided positive evidence that the extract has inhibitory effect up on calcium channels (53).

Significant ($p<0.001$) fall in relaxation response of strips up on removal of endothelium prior to contraction by EP confirms the important role of endothelium in relaxation response to extract (104). Failure of the extract to cause complete relaxation response in endothelium denuded

strips, while inducing significant concentration dependent relaxation response denotes the presence of other physiological pathways that are involving in relaxation response as shown from results of calcium concertation-response curves (105). Significant ($p<0.001$) reduction in relaxation response in endothelium intact strips pre-incubated with MB further strengthens the evidence that the presence of functional endothelium was mandatory for most of the relaxation response of strips to the extract (106).

A study that used aorta from rats treated with red wine polyphenolic compounds reported increased endothelium-dependent relaxation to acetylcholine and that it was related to increased endothelial NO activity and involved a mechanism sensitive to superoxide anion scavengers (107). But other study aimed at investigating the effects of a *Lupinus albus L.* enriched diet on oxidative stress and factors influencing vascular function in overweight subjects found that although regular consumption of lupine enriched bread can lower BP, these results did not support the hypothesis that this was via effects on oxidative stress (76). Hence, polyphenols in *Lupinus albus L.* seed preparations may be more likely associated with effects through eNOS-NO-cGMP pathway (108). In endothelium intact strips pre-incubated with L-NAME, the extract failed to induce significant relaxation response at all consecutive concentrations but observation from the nature of concentration-response curve and significant differences between non-consecutive test concentration levels may provide some hint that even in intact strips pre-incubated with L-NAME, there is no complete inhibition of relaxation response (109).

Endothelium intact aorta strips pre-contracted by KCl responded significantly ($p<0.001$) to extract in concentration dependent manner ($p<0.001$) at all consecutive test concentrations. Hence activation of VDCCs reduced relaxation response of endothelium intact strips to the extract but did not prevent the extract to achieve complete relaxation response indicating major vasorelaxation effect of extract as through eNOS-NO-cGMP pathway (110). The response was limited at lower extract concentrations in KCl pre-contracted strips and become more pronounced when the concentration of the extract rises. This clearly shows that vasoconstriction caused by activation of VDCCs can be reversed by further increasing the concentration of the extract which might be the result of competitive advantage to the molecules in the extract as a result of their higher concentration at channel sites. Thus, the presence of KCl in physiological solution (activation of VDCCs) only interferes with the effect of distillated seed extract rather than completely blocking it signifying the major effect of extract through eNOS-NO-cGMP

pathway than VDCCs. This assumption is also evidenced by inhibition in contractile force when denuded strips were pre-incubated with extract before cumulative increase in Ca^{++} concentration. A class of tetracyclic terpenes synthesized and evaluated for inhibitory activity of VDCCs was shown to inhibit voltage-activated Ca^{2+} currents but didn't affect Na^{+} or K^{+} currents in dorsal root ganglion cells (111). Hence, terpenoids from distillated seed preparations of *Lupinus albus L.* could be associated with the extracts effect through VDCCs.

These generally point to extract certainly a vasodilator through eNOS-NO-cGMP pathway and through calcium channels. A study reported that vasorelaxant effects of most of the flavonoids involved in part the release of NO and prostaglandins from the endothelium as well as inhibition of Ca^{+2} influx and release of Ca^{+2} from intracellular stores (112). Flavonoids mediate their antihypertensive effects through increasing NO bioavailability, reducing endothelial cell oxidative stress or modulating vascular ion channel activity (113). The antihypertensive effects of flavonoids extracted from Spergularia purpurea Pers. studied in both normotensive and spontaneously hypertensive conscious rats by daily oral administration of the flavonoid mixture (5mg/kg for 1 week) exhibited a significant decrease in BP and both SBP and DBP was decreased significantly (114). The potency, structure-activity relationship and mechanism of vasorelaxation of a series of flavonoids representing different subclasses examined in the isolated rat aorta showed that most flavonoids induced concentration dependent relaxant effects against 80mM KCl and 0.1 μM phenylephrine induced contractions with a greater inhibition of the responses to the α_1 adrenoceptor agonist (112). Accordingly, flavonoids present in the *Lupinus albus L.* seed preparations could probably be responsible for both effects through eNOS-NO-cGMP pathway and VDCCs.

Results of calcium concentration-response curves were additional evidences that indicate involvement of VDCCs in the vasorelaxation process. The presence of maximum cumulative concentration of extract significantly inhibited the contractile response indicating the extract activity in blocking the influx of extracellular Ca^{++}. The extract effect is not only limited to blocking the influx of extracellular Ca^{++} but also inhibits release of Ca^{++} from intracellular storage sites as observed from lack of contractile response in extract incubated strips in the absence of calcium in the physiological fluid. Nevertheless, inhibition of contractile response due to extract is not as pronounced as the extracts ability to reverse EP pre-contracted intact strips. This can be justified as the extract had superior effect through eNOS-NO-cGMP pathway than

VDCCs. Four new diterpenoids scapanacins isolated from Chinese liverwort (*Scapania carinthiaca J.B. Jack ex Lindb*) revealed that the vasodilatation effect was mediated through inhibition of Ca^{2+} influx via VDCCs, and this Ca^{2+} channel blocking effect was also confirmed by inhibiting the extracellular Ca^{2+} influx in mouse aortic VSMC line with very little decrease of the relaxant activity on endothelium-denuded mesenteric arterioles with nor-epinephrine which suggested the vasodilatation as mainly produced by inhibiting Ca^{2+} induced contraction of smooth muscle cells (115).

Altogether, the extract's effect in reversing arterial contractile responses could possibly be due to two reasons. Firstly, as *Lupinus albus L.* distillated seed extract is a crude extract; it may consist multiple secondary phyto-chemicals that may have effects on CV system such as polyphenols (83), flavonoids (113) and terpenoids (116). As a result, presence of multiple secondary metabolites may result in multiple corresponding pharmacologic relaxation mechanisms. For example, a phytochemical from a polyphenol group may have effect through eNOS-NO-cGMP pathway while other chemical entity from a flavonoid group acting through calcium channel (117). Secondly, a single phytochemical entity may have two or more pharmacologic sites of action. For instance, a single phytochemical entity from a polyphenol group may have effect via both eNOS-NO-cGMP and calcium channels (85,107) which requires further investigation through phytochemical screening and isolation.

The mechanism of vascular smooth muscle contraction involves different signal transduction pathways, all of which come together to increase in cytosolic calcium ion concentration (6). Cytosolic Ca^{2+} is increased through Ca^{2+} release from intracellular stores (SR) as well as entry from the extracellular space through Ca^{2+} channels (receptor-operated Ca^{2+}channels or voltage dependent calcium channels) (53). Hence profound relaxation response to extract observed in endothelium intact strips pre-contracted by EP to extract would be due to inhibition of adrenergic receptors and resultant multiple associated intracellular cascades in addition to eNOS-NO-cGMP pathway (118).

K^+ channels, through the control of membrane potential, determine the activity of VDCCs, which are the main gateway for Ca^{2+} entry into the cells (6).

Thus, highest relaxation response that was observed at much lower concentration in endothelium intact strips pre-contracted by EP than strips in any other category can be an indicative of either high level of potency or presence of multiple pharmacological synergistic mechanisms. By

observing the nature of concentration-response curve of endothelium intact strips, the strips started to respond to the combination of extract with SNP at much lower concentration than to the extract alone. As the concentration of extract and extract in combination with SNP increases at similar rate, the response to the combination as well as extract alone increases at rapid rate but becomes closer at higher concentrations. This could be explained in terms of slower involvement of smooth muscle cells in responding to the extract. Furthermore, this also shows endothelium intact strips pre-contracted by EP were more sensitive to a subtle changes in concentration of extract after once the threshold concentration was achieved may be owing to inhibition of multiple intracellular cascades related to α-adrenergic receptors.

The slope of concentration-response curve of extract in endothelium intact strips pre-contracted by KCl is more flattened when compared to the slope of concentration-response curve of extract in endothelium intact strips pre-contracted by EP. This is again taken as an indication for the presence of some level of inhibition of relaxation in strips pre-contracted by KCl. Similarly the slope of concentration-response curves in endothelium intact strips pre-incubated with L-NAME and MB as well as mechanical removal of endothelial layer is further flattened confirming the high level of inhibition in relaxation response in the absence of or nonfunctional endothelium.

Phytochemical analysis results show that fermented *Lupinus albus L.* seed products consisted mainly polyphenols, flavonoids, ketonic terpenoids and phytosterol with anoids. When phytochemical composition of seed extract of *Lupinus albus L* were compared to fermented seed extracts, secondary metabolites present in one was absent in another and vice versa except some. This might dictate the effect of germination, fermentation and distillation processes on phytochemical constituent of the plant material or the effect of temperature on some thermo-labile components (73,114). A study reported germination of seeds affected chemical composition of seeds due to synthesis, degradation and transformation of biomolecules for the development of the plant (119). Germination of lupine seeds was found to increase the total phenolic contents (120). A study conducted on *Lupinus albus* found that isoflavones content was increased after germination with the highest content occurring at day 2-4 (121). Another study that investigated the effect of traditional processing methods on phytochemical contents of *Lupinus albus L*. grown in Debretabor and Denbecha reported profound reduction in anti-nutritional factors such as alkaloids, tannins and phytic acids after roasting, soaking, and cooking (122). Although there were no detail chemical investigations carried out on distillated seed

preparations, the level of these water soluble toxic phytochemicals especially alkaloids are expected to decrease and or disappear due to harsh treatment conditions during distillation process.

All secondary phytochemical compounds identified in *Lupinus albus L.* seed extracts are well known for their effects on CV system (107,114, 123, 124) which also have pharmacological activities in reducing BP.

A comparison of antioxidant capacities and phenolic compound profiles of wild and cultivated *Lupinus albus L.* seeds previously showed total phenolic compounds of lupine seeds ranged from 4.36 to 7.24mg gallic acid equivalent/g dry matter. The dominant phenolics of all genotypes were p-coumaric acid derivatives and antioxidant assays of wild lupine extracts were similar to or lower than those of the cultivated variety (120). A systematic review and meta-analysis of randomized controlled trials that investigated the effects of phytosterol supplementation on BP found that it can decrease both SBP and DBP and dose response analysis revealed phytosterol intake changed SBP significantly based on treatment dose in nonlinear fashion (125).

Both oxygenated and non-oxygenated terpenes exhibit vasorelaxation activity (126). A significant dose-dependent hypotensive effect was observed following auraptene (a monoterpene coumarin from *Citrus spp*) injection. The mean arterial BP lowering effect of auraptene was found to be significantly lower than that of nifedipine (116). Chronic administration of auraptene also significantly reduced the mean systolic BP in desoxycorticosterone acetate salt treated rats in a dose and time dependent manner.

A study examined for vasorelaxant effects of three purified tannins by acetone and butyl alcohol from *Geum japonicum Thunberg (Rosaceae)* in isolated rat thoracic aorta rings at a cumulative concentration of 30 µg/ml potently relaxed phenylephrine contracted aortic rings by 73±5% and 80±7%, respectively, without affecting the resting tension of these vessels. But removal of the vascular endothelium and inhibition of eNOS with L-NAME both abolished the vasorelaxant effects of the *Geum japonicum* extracts (123).

Intravenous administration of *Daucus carota* which is known to contain coumarin glycosides caused a dose-dependent (1-10mg/kg) fall in arterial BP in normotensive anaesthetized rats. *In-vitro* studies showed both compounds caused a dose-dependent inhibitory effect on K^+ induced contractions of rabbit aorta at similar concentrations. These results indicate that these compounds

may be acting through blockade of calcium channels and this effect may be responsible for the blood pressure lowering effect of the compounds observed in the *in-vivo* studies (124).

Over all, at the current level of study on *Lupinus albus L*. distillated seed preparation; it is not possible to assign a single group of secondary phytochemical entity to a specific pharmacological effect on guinea pig thoracic aorta strips.

The major findings of this study were; firstly, concentration-response (vasorelaxation) of *Lupinus albus L*. distillated seed extract was mainly dependent on functional endothelium the effect of which is inhibited significantly by mechanically removing the endothelium and pre-incubation of intact aorta strips with L-NAME and MB. Secondly, *Lupinus albus L*. distillated seed extract also induced minor concentration dependent vasorelaxation response which is mediated through VDCCs as observed from significant reduction in relaxation response in aorta strips pre-contracted by KCl and slight concentration dependent relaxation response in endothelium denuded aorta strips as well as L-NAME and MB pre-incubated strips in addition to results from calcium concentration response curves. Thirdly, the concentration that caused maximal relaxation response and 50% maximal relaxation was estimated to be 4.74µg/ml and 3.80µg/ml respectively in EP pre-contracted endothelium intact strips where as it was 21µg/ml and 7.01µg/ml in similar strips pre-contracted by KCl.

This finding supports the traditional use of *Lupinus albus L.* distillated seed preparation ("Gebto arekei") in the treatment of hypertension owing to its effect through eNOS-NO-cGMP and VDCCs.

Drugs that directly target the enzymes of eNOS-NO-cGMP pathway are currently the most promising compounds, but none of these compounds are under investigation as a treatment option for systemic hypertension. In addition to improving vascular function, eNOS-NO-cGMP signaling pathway is also known to reduce BP through other mechanisms such as decreasing renin release, increasing natriuresis through modulation of various sodium transporters, for example; sodium-hydrogen exchanger, epithelial sodium channel, and Na-K-Cl cotransporter-2 in the renal tubules (35). Though this study identified major vasorelaxation effects of *Lupinus albus L.* distillated seed preparation, it didn't explain about underlying factors that were responsible for stimulation of endothelial nitric oxide synthase and subsequent increase in NO production. Moreover, it didn't identified a single or multiple phytochemical constituent responsible for observed vasorelaxation effects.

7. Conclusion

Lupinus albus L. distillated seed extract significantly reversed the guinea pig thoracic aorta strips pre-contracted by a high concentration of KCl (80 mM) and EP (1µM) indicating opening of potassium channels through eNOS-NO-cGMP pathway and subsequent inhibition of voltage dependent calcium channels. Most of the vasorelaxation effects of *Lupinus albus L*. distillated seed extract was inhibited by mechanical removal of the endothelium and incubation of strips with L-NAME and MB implying major role of eNOS-NO-cGMP pathway in relaxation response.

Taken together, the results demonstrated that the *Lupinus albus L*. distillated seed extract was able to induce endothelium-dependent vasorelaxation as well as minor vasorelaxation through direct inhibition of VDCCs in isolated guinea pig thoracic aorta strips.

Based on the findings obtained in this study, it may be an important step for validation of the popular usage of *Lupinus albus L*. distillated seed extract as phyto-medicine in the treatment of hypertension.

8. Recommendations

Further investigations should be carried out in order to identify the type of phytochemical constituents of *Lupinus albus L.* distillated seed preparation responsible for a particular vasorelaxation effect along with the type of endothelial receptor interactions (acetyl choline, PGI_2 etc). Additionally, both the efficacy and safety for acute and chronic usage of the preparation remain to be investigated.

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Annexes

I. Observations during extraction process of *Lupinus albus L.* distillated seed preparation (“Gebto arekei”)

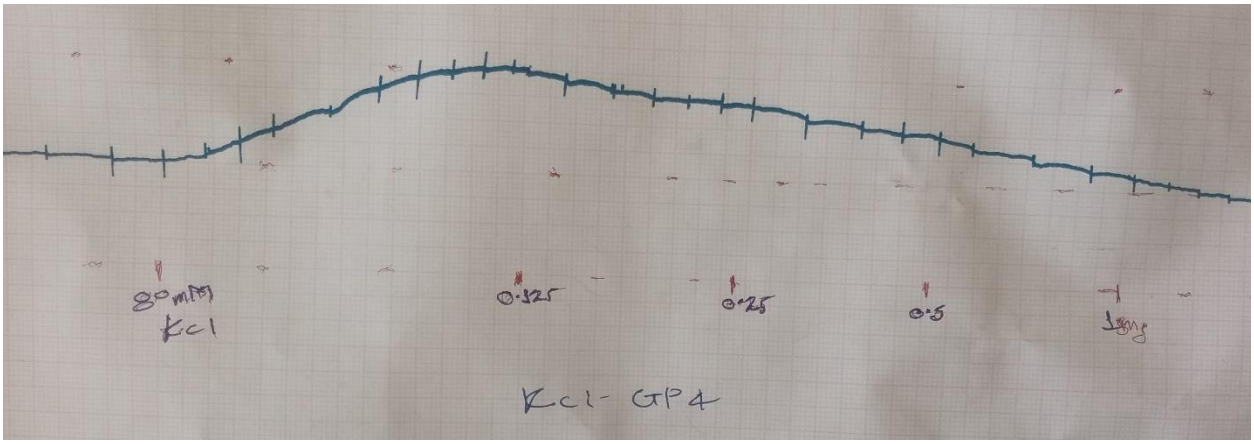
View of *Lupinus albus L.* distillated seed preparation in its container at the beginning of the extraction (concentration) process (left) and appearance of un-concentrated (beaker) versus concentrated (Erlenmeyer flask).

Final concentrated *Lupinus albus L. distillated* seed preparation (“Gebto arekei”) concentrate before introduced in to liyophilyzer for drying.

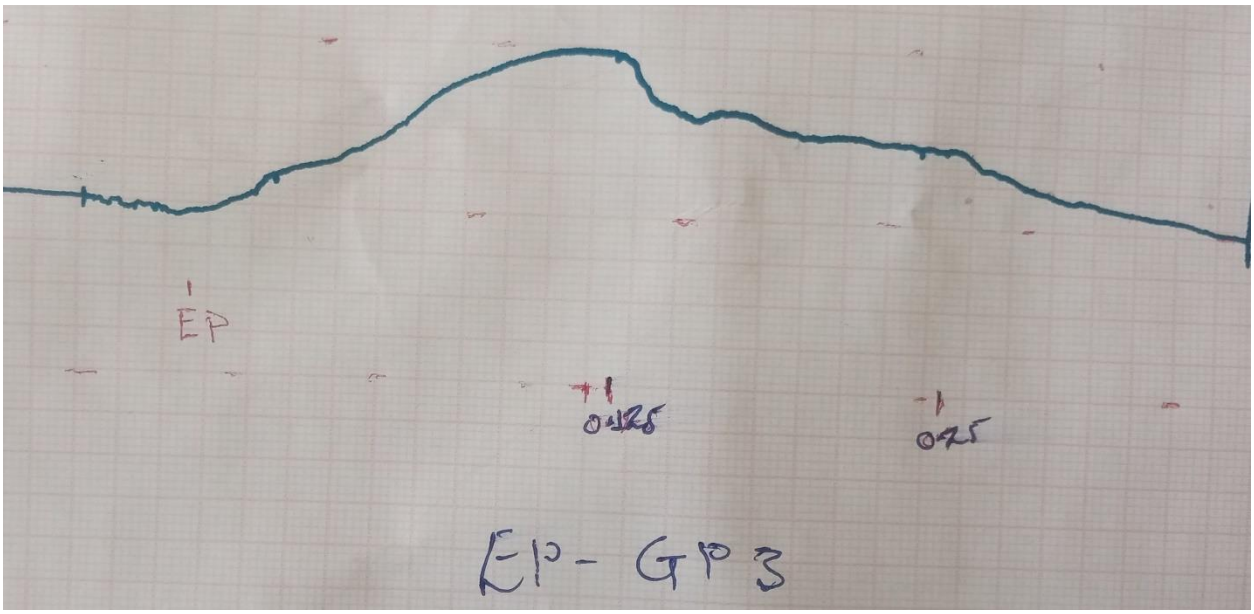
Dried extract of *Lupinus albus L. distillated* seed extract obtained after lyophilyzation (left), dried and grinded final product (right).

II. Some randomly drawn test results from each test groups

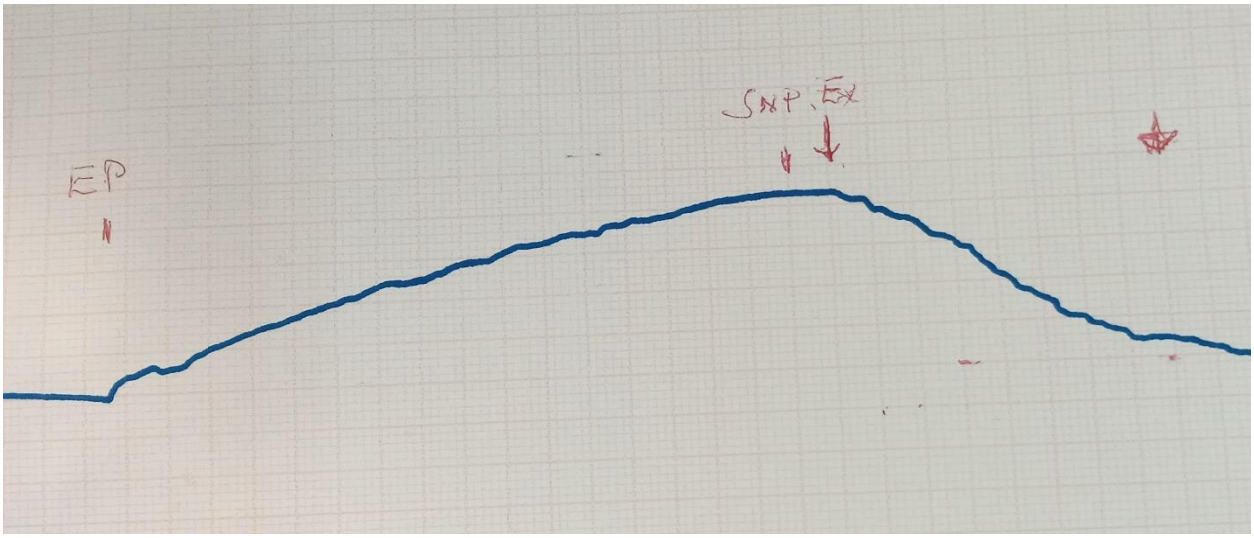
A. Ex-vivo vasodilatory activity



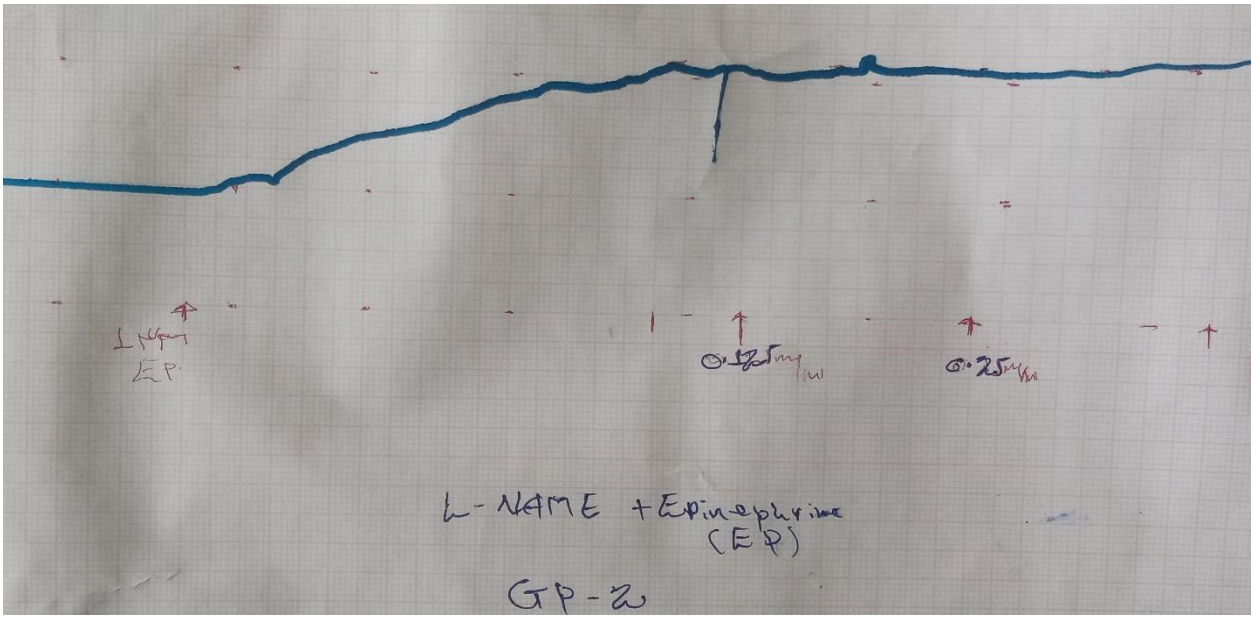
Data record from fourth guinea pig isolated thoracic aorta strip allocated under KCl group to cumulative doses of extract.



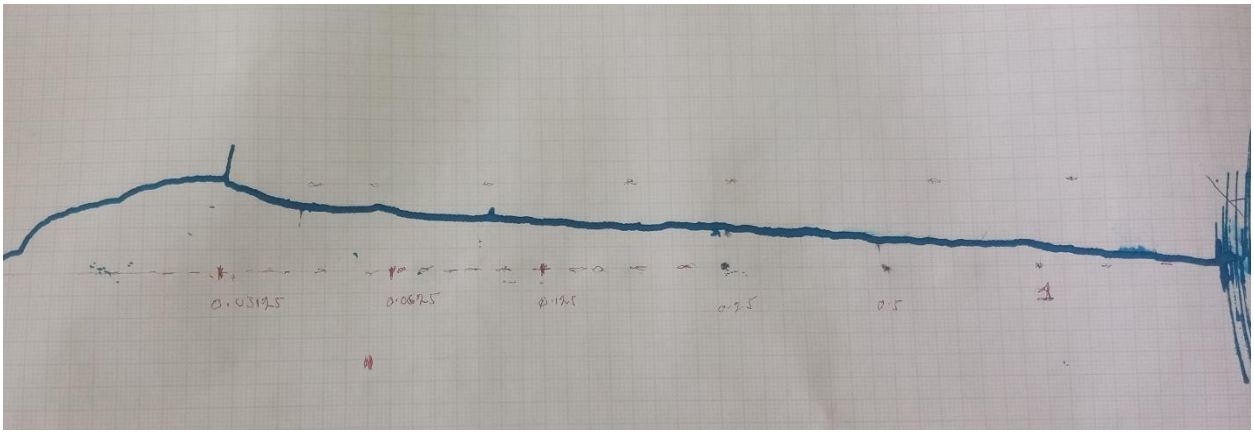
Data record from third guinea pig isolated thoracic aorta strip allocated under EP group to cumulative doses of extract.



Data record from first guinea pig isolated thoracic aorta strip allocated under EP group to cumulative doses of extract in combination with SNP in 1:1 ratio.

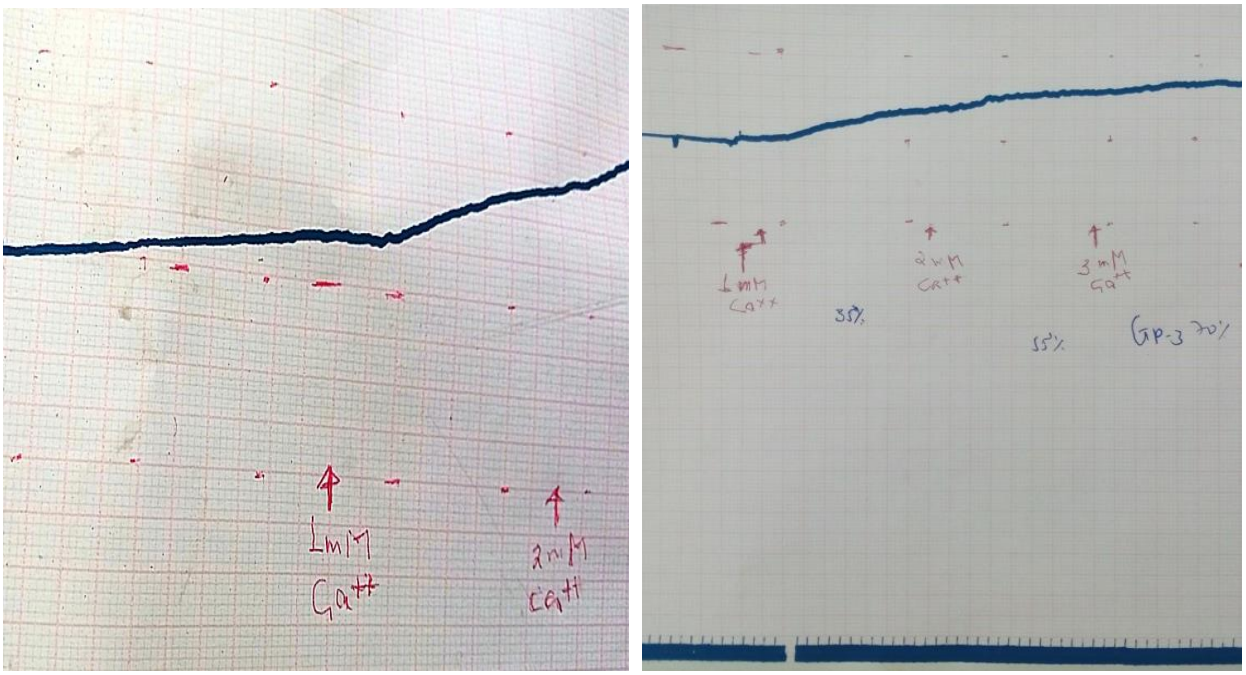


Data record from second guinea pig isolated thoracic aorta strip allocated under EP group pre-incubated with L-NAME to cumulative doses of extract.



Data record from fourth guinea pig isolated thoracic aorta strip allocated under EP group pre-incubated with MB to cumulative doses of extract.

B. Calcium concentration-response curve



Data record from fourth guinea pig under control group (left) and third guinea pig under test group (right) in calcium concentration-response curve determination.

III. Data registration tools

A. Ex-vivo vasodilatory activity

Change in tension force (% relaxation) of intact (E^+) aorta strips pre-contracted by 80mMKcl to different concentrations of distillated seed extract of *Lupinus albus L*.

GPA	Concentration of *Lupinus albus L.* distillated seed extract (µg/ml)					
	0	4	8	16	32	Remark
GP - 1	0	30	55	85	100	
GP - 2	0	25	50	80	100	
GP - 3	0	30	70	85	100	
GP - 4	0	25	50	85	100	

GPA-Ginea pig aorta

GP-ginea pig assigned under specific group

Change in tension force (% relaxation) of intact (E^+) aorta strips pre-contracted by 1µM EP to different concentrations of distillated seed extract of *Lupinus albus L.*

GPA	Concentration of *Lupinus albus L.* distillated seed extract (µg/ml)					
	0	4	8	16	32	Remark
GP - 1	0	65	100	100	100	
GP - 2	0	60	100	100	100	
GP - 3	0	55	100	100	100	
GP - 4	0	70	100	100	100	

Change in tension force (% relaxation) of intact (E^+) aorta strips pre-contracted by 1µM EP to different concentrations of distillated seed extract of *Lupinus albus L.* and SNP in 1:1 ratio.

GPA	Concentration of extract + SNP (µg/ml)					
	0	4	8	16	32	Remark
GP - 1	0	90	100	100	100	
GP - 2	0	80	100	100	100	
GP - 3	0	85	100	100	100	
GP - 4	0	100	100	100	100	

Change in tension force (% relaxation) of intact (E^+) aorta strips pre-contracted by 1µM EP to cumulative addition of SNP (positive control).

GPA	Concentration of sodiumnitroprusside (µg/ml)					
	0	4	8	16	32	Remark
GP - 1	0	40	75	100	100	
GP - 2	0	50	70	100	100	
GP - 3	0	55	80	100	100	
GP - 4	0	45	70	100	100	

Change in tension force (% relaxation) of intact (E^+) aorta strips pre-incubated with 100µM L-NAME and pre-contracted by 1µM EP to different concentrations of distillated seed extract of *Lupinus albus L.*

GPA	Concentration of *Lupinus albus L.* distillated seed extract (µg/ml)					
	0	4	8	16	32	Remark
GP - 1	0	10	20	25	35	
GP - 2	0	5	0	15	20	
GP - 3	0	0	5	10	20	
GP - 4	0	10	15	15	25	

Change in tension force (% relaxation) of intact (E^+) aorta strips pre-incubated with 10µM MB and pre-contracted by 1µM EP to different concentrations of distillated seed extract of *Lupinus albus L.*

GPA	Concentration of *Lupinus albus L.* distillated seed extract (µg/ml)					
	0	4	8	16	32	Remark
GP - 1	0	15	20	40	58	
GP - 2	0	12	30	45	60	
GP - 3	0	10	25	42	63	
GP - 4	0	15	28	50	65	

Change in tension force (% relaxation) of denuded (E^-) aorta strips pre-contracted by 1µM EP to different concentrations of distillated seed extract of *Lupinus albus L.*

GPA	Concentration of *Lupinus albus L.* distillated seed extract (µg/ml)					
	0	4	8	16	32	Remark
GP - 1	0	15	20	50	64	
GP - 2	0	5	35	45	60	
GP - 3	0	10	25	55	55	
GP - 4	0	12	33	48	65	

B. Calcium concentration-response

Change in tension force (% contraction) of denuded (E^-) aorta strips in the absence of distillated seed extract of *Lupinus albus L.* (extract).

GPA	Concentration of Ca^{++} (mM)				
	0	1	2	3	Remark
GP - 1	0	45	65	100	
GP - 2	0	40	70	100	
GP - 3	0	45	85	100	
GP - 4	0	60	95	100	

Change in tension force (% contraction) of denuded (E^-) aorta strips incubated with distillated seed extract of *Lupinus albus L.* (extract).

GPA	Concentration of Ca^{++} (mM)				
	0	1	2	3	Remark
GP - 1	0	35	55	70	
GP - 2	0	35	50	65	
GP - 3	0	25	55	65	
GP - 4	0	20	50	60	

Change in tension force (% contraction) of denuded (E^-) aorta strips incubated with nifedipine.

GPA	Concentration of Ca^{++} (mM)				
	0	1	2	3	Remark
GP - 1	0	15	30	45	
GP - 2	0	25	35	40	
GP - 3	0	20	30	40	
GP - 4	0	20	30	35	