Elucidation of the signalling pathway by the erectogenic bark of 'Loozi Luna Kasiika' in Zambia

By

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The University of Zambia has approved this dissertation of Mukubesa Andrew N as partial fulfillment of the requirements for the award of the Degree of Master of Science in Biochemistry.

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Summary

The barks of the tree known as Loozi Luna Kasiika (LLK) are widely used as a sexual stimulant for treatment of erectile dysfunction. The biochemical pathway responsible for the physiological response was unknown and the goal was to explore the possible signalling pathway that may be involved. Although all along it has been widely used to boost erectile capacity, findings from the study could help to bring out other potential benefits of LLK previously unknown. An experimental study was undertaken to analyse the effects of LLK extracts on the levels of second messenger molecules 3', 5'-cyclic Guanosine monophosphate (cGMP) in platelets, and also phytochemical analysis performed to find some compounds that brings about the effects. LLK extracts showed to contain flavonoids, alkaloids and glycosides which are assumed to contribute to its mechanism of action. It was found that LLK inhibits phosphodiesterase (PDE) enzymes and increased the levels of cGMP in the cell from an average of 3.2828 ± 0.0069 pmol/10^9 platelets to 4.0273 ± 0.0056 pmol/10^9 platelets. In contrast, LLK showed low effect on the NO synthesis. LLK prevents the hydrolysis of cGMP by inhibiting PDEs, hence increases the levels of cGMP in a cell, thereby making it a potent vasodilator.

Table of contents

Content **Page**

List of abbreviations

ANT	Arterial Natriuretic Peptide
cAMP	Adenine 3', 5'-Cyclic Monophosphate
cGMP	Guanosine 3', 5'-Cyclic Monophosphate
CREB	cAMP Response Element Binding Proteins
ED	Erectile Dysfunction
EDRF	Endothelial Derived Relaxant Factor
LLK	Loozi Luna Kasiika
MLC	Myosin Light Chain
MLCK	Myosin Light Chain Kinase
NANC	Non Adrenergic Non Cholinergic
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
PDE	Phosphodiesterase
PKG	cGMP-Dependent Protein Kinase
pRB	Retinoblastoma Protein
SBA	Sulindae Benzylamine
IBMX	3-isobutyl-1-methylxanthine
SNAP	S-Nitroso-N-acetylpenicillamines
sGC	soluble Guanylyl cyclase

1.0 Introduction

1.1 Background

Plant products have been used for their various properties as either nutritional supplement and as alternative medicine. They are called herbs if they are used for therapeutic purpose, which can be any part of the tree including roots, stem, leaves, flowers and/or seeds. The uses of herbs have recently increased to improve the health of people, treating disorders and prevent life threatening diseases (Agyare et al, 2009). Herbal medicine possess therapeutic effects and this is seen from an estimation by WHO that, about 80% of the population from developing countries uses herbal remedies to meet their primary health care needs (Jadeja et al, 2011). Herbal medicine is coupled to primary health care in China, Ethiopia and Argentina (Akinyemi et al, 2005). Some drugs used in the conventional medicine are synthesized to mimic plant compounds whilst some are just extracts from natural plants, and these have saved and still serve as primary material in about 25% of pharmaceutical products.

Amongst primary health care problems that are addressed by the herbs is erectile dysfunction (ED), which is treated with different types of herbs. It has been reported that ED can be treated by a herb from a tree called *Tribulus terrestris. Tribulus terrestris* has been studied widely, and has been used in folk medicine for wide range of conditions including nervous disorders and sexual dysfunction in southern Europe, Southern Asia, Africa, and Australia. It has also been used in China and India to treat liver, kidney, urinary and cardiovascular disorders (Do et al, 2012). This tells us that some herbs can be used to treat different conditions. Another erectogenic herb that is widely studied is from

Yohimbe tree, *Pausinystalia yohimbe.* It contains indole alkaloids, yohimbine and corynatheine. This tree is native to Congo, Cameroon, Gabon and southwest Nigeria. Research shows that this herb has adverse effects on liver and kidney (Yakubu et al, 2003). These erectogenic herbs may contain compounds that can inhibit or activate enzymes. Another herb from *Butea frondosa* Koenig ex Roxb, has a compound that inhibits Rho-kinase 2 enzymes. It is a deciduous tree commonly found in India and traditionally used in India and China in various medical conditions (Sumanta et al, 2013).

Erectogenic herbs are also used in Zambia and one such is Loozi luna kasiika (LLK). LLK is the bark of a plant which is commonly found in the Gwembe valley at a place known as Kunakasiika. It is widely used as a sexual stimulant (Viagra) in Zambia. The biochemical signalling pathway that it activates to enhance penile erection is unknown, but there is a strong possibility that the mechanism of action is similar to other Viagra such as Sildenafil or Yohimbe. The properties of Viagra have been shown to be beneficial in other conditions. If indeed the mechanism of action of this herb is similar to that of Viagra then it may also potentially have the same beneficial effects. Viagra is known to affect NO/cGMP signalling pathway which is very important in the normal physiological life process of cells. Viagra is seen to increase the levels of endothelial derived relaxant factors (EDRF). An EDRF is a molecule that causes smooth muscle relaxation and it is synthesised in endothelium cells, it produces it effects through the cGMP signalling pathway (Oelze et al, 2000). One of EDRF is NO (Moncada et al, 1991), it either activates proteins through nitrosylation reaction or works through activation of guanylyl cyclase by binding to its heme moiety, the activated guanylyl cyclase (sGC) synthesises cGMP from GTP which phosphorylates cGMP dependent

protein kinase to cause smooth muscle relaxation. For this property it is used to treat erectile dysfunction (ED), to some extent it has been shown that it can be used also to prevent turmoregenesis.

Some viagra belong to the family of phosphodiesterase (PDE) inhibitors and others are NO donors. NO is released from nerve endings and endothelial cells through stimulation, and NO is absorbed by the cells of smooth muscles. NO is synthesised from an amino acid called L-arginine by nitric oxide synthase (NOS), NOS needs cofactors such as Oxygen, tetrahydrobiopterin, NADPH and Flavin adenine nucleotide. There are three different types of NOS type I is also called neural NOS (nNOS), whist type II and III also called inducible NOS (iNOS) and constitutive NOS (cNOS) respectively. Neural NOS is one of the main transmitters in peripheral nervous system. The body normally produces NO through cNOS under normal basal condition , cNOS is calcium calmodulin dependent, and can be activated through two ways, flow dependent NO formation and receptor stimulated NO formation. NO is also produced from the nerve endings (non-adrenergic non cholinergic (NANC) by iNOS in autonomic nerves that innervates many specialised tissue, iNOS is not calcium dependent and can be stimulated by lipopolysaccharides, cytokine and interleukins.

The response in the effecter cell is dependent on the cGMP binding mortifs in the target protein (Eukijung and Ji Man Park, 2003). Hence the produced NO diffuses into vascular endothelium and smooth muscles where it binds to the iron of heme moiety in guanylyl cyclase, this activate the enzyme guanylyl cyclase which dephosphorylate GTP to cGMP (Guazzi, 2008). Cyclic GMP regulate smooth muscles through various ways which include independent calcium activation of MLCP (Lee and Katazwa, 1997), inhibition of

calcium influx (Cohen et al, 1999), decrease calcium concentration, activation of potassium channel and stimulation of cGMP-dependent protein kinase through its serine/threonine that activate myosin light chain phosphatase (Francis et al, 1999), which is the enzyme responsible for dephosphorylation of myosin light chain (MLC). Smooth muscle tone is controlled by cellular calcium that activates calcium/calmodulin(CaM)-dependent enzyme myosin light chain kinase(MLCK) and this cause phosphorylation of MLC. Cyclic GMP activates cGMP-dependent protein kinase (PKG), which effect relaxation in smooth muscle through different mechanisms which include decrease in levels of cellular calcium through enhanced calcium outflow or decreased inositol triphosphate receptor-mediated calcium, or through MLC dephosphorylation MLCK. MLC phosphorylation contributes to cross-bridge formation between the myosin heads and the actin filaments hence contraction but when MLC is dephosphorylated relaxation take place by the destabilizing of cross bridge formation.

The level of cGMP contributes greatly to the relaxation of smooth muscle and also causes blood vessel dilatation. There are two main factors that raise cGMP levels in the cell; the increase in conversion of GTP to cGMP by guanylyl cyclase and the reduction in hydrolysis of cGMP. The increase of cGMP concentration by more than two fold might increase the normal basal function of the body to their optimum levels. The reaction of hydrolysis is catalysed by an enzyme called phosphodiesterase (PDE), therefore inhibition of this enzyme will cause an increase in the concentration of cGMP (Ghofrain et al, 2006).

There are three major groups of PDE, Class I, class II and Class III (Francis et al, 2000), class I is comprised of all 11 isoenzyme of PDE characterised in the human body that

work on the signalling pathway of cGMP and cAMP. PDE 1, 2, 3,10 and 11 function on both cGMP and cAMP pathways, whilst PDE5, 6 and 9 function on cGMP only and PDE 4, 7 and 8 work on cAMP pathway only (Makhlouf et al, 2006; Mostafa et al, 2008). Class II are enzymes from fungi and class III are found in prokaryotes such as Eschericia coli (Richter, 2002).

Most of class I PDEs are dimeric and they have conserved catalytic site at their carboxyl terminal to their regulatory domain comprised of approximately 270 amino acids (Motafa, 2008), therefore their activation offers self-inhibitory. PDE use the hydroxyl to offer a nucleic attack on the 3' position of the nucleotide by cleaving the phosphodiester bond between phosphorous and oxygen atoms, this is influenced by metal bound metal binding motif, PDE 5 uses zinc ion as the metal cofactor. The regulatory site differs from PDE to PDE and offers the site for dimerization, phosphorylation and modulatory function (Puzzo et al, 2008)

Some PDEs are specific for cGMP such as PDE 5 and is responsible for hydrolysis of cGMP in the pathway that leads to penile vascular smooth muscle relaxation and vessel dilatation which in turn lead to penile erection as blood flow through the vessel (Seftel, 2004). It is also involved in other physiologic functions such as coronary tone regulation (Senzaki et al, 2001). PDE is inhibited by many competitive cyclic nucleotide analogues which compete for the catalytic site as some show competitive kinetics to cGMP in the catalytic site. Different nucleotides analogues are used as inhibitors for different PDEs depending on their structures.

1.2 Statement of the problem.

Reproductive health care is the second most prevalent health problem on the African continent, which has led to an increase in affected persons seeking local Viagra, LLK inclusive. Due to increased demand of Viagra, many traditional healers and Viagra sellers have found their way on the street selling these herbs. This poses serious risks to consumers as the products of traditional healers are not regulated and quality controlled. To make it worse, local herbs and their related products sold on the street are not tested and certified fit for use as drugs. Most of drugs have side effects, and through testing of drugs some combination of drugs are allowed to reduce the side effects which can either be short term or long term effects whilst some are prohibited. Therefore herbs are not exceptional from producing side effects (Otieno J N et al, 2014), hence knowing the mechanism of action could give some understanding on the kind of side effect that might harbour the users.

The mechanism of action of LLK in treatment of erectile dysfunction was unknown and the main aim was to work out the possible signalling pathway activated by this herb in treatment of erectile dysfunction. This was to help know other possible use of LLK in other medical indications.

1.3 Justification

There are many drugs and herbs on the market, but there is also the rise in resistance against these medicines hence calling for more new and effective medicines. Therefore due to some properties of erectogenic herbs that affects the levels of second messengers and or affect PDEs, they could be used in other clinical condition such as hypertension, cancer etc other than treating ED only. Knowing the effects of the LLK on the levels of cGMP and the inhibition pattern on PDEs could greatly help predict the short term and long term effects on the users and abusers of it.

For a drug or herb to be approved safe for use, their pharmacokinetics and pharmacodynamics property needs to be investigated. Therefore if LLK was to be used in the primary health care to treat ED and other conditions, its properties needed to be identified and understood to assure safety, quality and efficacy.

1.4 Literature review

Hebal medicines have been used traditionally and conventionally, there is a rise in the use of herbs as a result of their low side effects as compared to the synthetic drugs and their effective treatment (Sinclair, 1998). Despite their efficacy, they lack credible evidence to be used in the mainstream of medical therapy (Albersen et al, (2011) because they are not well studied and approved, including some herbs shown to affect PDE5 such as erectogenics which still need validation (Lue and Broderick, 2007). The identification and isolation of bioactive compounds leads to the increase in knowledge and understanding of how the herbs work (Sinclair, 1998). Therefore a thorough analysis is needed for the better understanding on how erectogenic herbs work.

Treatment of some conditions is dependent upon the aetiology, some cases of ED are as a result of abuse of herbs or drugs such alcohol, cocaine, marijuana and other drugs (Lue and Broderick, 2007). Therefore to treat ED, a full medical history is needed and full properties of the drug/herb to be used must be known (Albersen et al, 2011). Many people in developing countries use herbs prescribed by themselves, friends and /or traditional healers without knowing the cause of their illnesses and without regulating the dose. Deeper understanding is required before the use of every drug, hence knowing the mechanism of action of LLK will greatly help to predict the effects of the drugs whether they can be used to treat other condition.

A drug that affects multiple pathways can contribute to the existing problem or cause some other clinical condition (Albersen et al, 2011). The over dose of some erectogenic herbs which inhibits PDEs can cause hypotension, which is not the anticipated results.

This can be regulated by knowing the inhibition power of the herb on the PDEs or the similarity of the substrate and the inhibitors. The other way is to know the levels of the second messenger cGMP produced as a result of taking the eretogenic herb. The extent to which the signalling molecules are produced determines the duration of the reaction. The synthetic rate of signalling molecules (cGMP) is also proportional to NO that is affected by some erectogenic substances and herbs. According to studies, PDEs controls the duration and the amplitude of cyclic nucleotide signalling by hydrolyticaly cleaving phosphoester bond from cGMP to produce GMP (Puzzo et al, 2008). cGMP activates cGMP dependent protein kinase (PKG). PKG have three domains, N-terminal, regulatory domain which contain the cGMP binding site and catalytic domain that has MgATP and peptide binding site (Pfeifer et al, 1999). The catalytic domain transfers the phosphate group between the ATP and serine/threonine of the target protein. The binding of cGMP to the regulatory site activates cGMP dependent protein kinase by changing the conformation of the site to allow phosphorylation of the target protein (Hofmann et al, 2000). PKG phosphorylate vascular stimulated phosphoprotein (Walter and Gambaryan, 2009) at the serine 239, the phosphorylation is linked to reduction in association between VASP, F-actin and actin (Benz et al, 2009). The synthesis of cGMP and activation of PKG occurs faster than the effects of these phosphorylation reactions. Cawley et al shows that the rise of cGMP levels occurs after an average of 50 seconds, then PDEs and VASP are phosphorylated within seconds (Rybalkin et al, 2002) and specifically in purkinje cells it takes an approximately 20minutes (Shimizu-Albergine et al, 2003).

Studies have shown that erectogenic herbs can be widely used in other medical conditions, they can also be used in combination with other drugs to treat some diseases.

NO stimulating agents and phosphodiesterase 5 inhibitors have increased options for treating a number of clinical conditions especially in patients with ED (Craig et al, 1998) and it is being used for treatment of renal insufficiency, cancer, inflammation and pulmonary hypertension (Savas et al, 2010). It has been reported that PDE inhibitors such as Sildenafil can be used to treat cardiac diseases and also can be used to prevent cardiac and myocyte dysfunction. Vasoconstriction is known to be a hallmark in chronic heart failure as a result of defective NO release that compromises the cGMP signalling pathway which helps regulate the vascular tone. It has been reported that defective NO is major factors which contributes to chronic heart failure (CHF) (Guazzi, 2008). This is because there is endothelial dysfunction resulting in low synthesis of NO which has been shown to be reduced in CHF patients. This shows that there could be an association between the NO/cGMP signalling pathway and the treatment of blood circulatory problems, of which some have little treatment option such as hypertension (Michelakis et al, 2002) and in most cases the aetiology is not clearly understood (Ghofrani, 2002).PDE5A can also be used as a modulator for contraction and relaxation of cardiac muscle (Nagayama et al, 2009). PDE inhibition improves memory (Rutten et al, 2005). Its therefore NO/cGMP signalling transduction system has significantly shown potential in treatment of cognitive disorders, and studies suggest that PDE5A inhibitors can be used in the treatment of Alzheimer's disease (Puzzo et al, 2009).

Side effects: Unextracted and not well processed herbs may contain one or more chemicals elements that give it more than one mechanism of action or that give it multiple target sites. Some Viagra are not specific inhibitors for specific PDE, sildenafil has been noticed to affect PDE 6 beside PDE5, and this causes colour vision disturbance

which was noticed in about 11% of men using it (Nathan et al, 2001), also Tadafil inhibits PDE6 and PDE 11. PDE6 is a photoreceptor-specific enzyme found in rod and cone in the eye and it is cardinal in the transduction of light, the outer segment of rod has rhodopsin, PDE and transducin, the PDE increases the concentration of cGMP that could cause activation of sodium channels and depolarization of the membrane (Dong et al, 2013). Most of drugs have side effects, and PDE inhibitors and vasodilating agents have shown to cause flushing, headache, dyspepsia and rhinitis.

Use in people with conditions: ED is a common problem in patients with diabetes mellitus as compared to the normal males of the same age group. NO levels in the serum of diabetes mellitus patients is lower as compared to non-diabetic patients with levels being 43.83 ± 11.3 μmoles/L and 58.85 ± 12.8 μmoles/L respectively (Ghosh et al, 2011).PDE inhibitors has shown to improve the condition where 41% satisfaction rate on ED was reported in patients with diabetes mellitus (Goldstein et al, 2002).The problem of ED is also higher in Parkinsonism and is estimated to affect 60% of patients as compared to a similar population in where the prevalence is 37.5% (Sanger et al, 1989)

Treatment of cancer: NO plays a role in tumour development and progression, it is associated to angiogenesis and it has two separate roles of activation and inhibition in the proliferation of tumour cells (Fukumura et al, 2006). This has been reported by Sanyal et al, that exogenous NO enhances human cariocarcinoma (JEG-3 cells) and prevents the differentiation of cytotrophoblast to syncytial cells. Cancerous cells express low levels of cGMP as compared to non-cancerous cells (Thompson et al, 2000), this results into lower levels of PKG in neoplastic cells (Hou et al, 2006). Cyclic GMP function through activation of PKG and the activation is dependent on expression of PDE and hydrolysis

of cGMP by PDE. The increase in cGMP/PKG concentration in the cell produces anticancer. This has been demonstrated in the treatment of colon tumours, where sulindae benzylamine (SBA) is used to increase the levels of cGMP in the cells (Whitt et al, 2012). It has also been noted that PDE5 expression is more in various human carcinoma such as urinary bladder cancer (Piazza et al, 2001; Pusztai et al, 2003; Spoto et al, 2003). This is suggestive of PDEs contributing to tumourigenesis activities and their inhibition can help prevent and produce anticancer effects.

Raised concentration of cGMP and PKG causes apoptosis and arrest the growth of cells, this is shown in breast cancer MCF-7 and MDA-MB-468 cells, where it show the inhibition action of growth of breast cancer via induced apoptosis (Fallahian et al, 2011). Increased levels of cGMP also causes inhibition of cell proliferation, increased cGMP also increases intracellular phosphorylated/activated PKG which induces cell apoptosis, and PDE has shown tolerability profile in patients and that they can be used as chemo-sensitizers (Koiri et al, 2013). Hence PDE inhibiting agents and enzymes that degrades cGMP can be used as targets for new, safe and efficacious method to treat tumours (Whitt et al, 2012).

Cell proliferation: It was seen that cGMP plays a physiological function of the cell proliferation and cell maturation, this was observed in the reduced levels of cGMP during the nuclear maturation of rat oocyte (Tornell et al, 1990). cGMP acts through inhibiting cyclin D1 and activating cdk2 and 4 to suppress cell proliferation (Fukumoto et al, 1999) A report shows enhanced proliferation of lung fibroblast, in which the levels of cyclins A, D and E are raised, also enhanced phosphorylation of retinoblastoma protein (pRB) and decreased expression of cyclin dependent kinases inhibitors (Chen et al, 2003).

Raised concentration NO promotes cell proliferation (Plachta et al, 2003), it supports the transition from G1 to S phase in the nuclei and low cellular NO concentration promotes transition from G2 to M phase in the apical location of cellular nuclei (Traister et al, 2002). NO removing agents arrest cell cycle at the late S or G2/M phase and this reduces cell proliferation (Janssen et al, 1998). NO stimulates cell maturation which are important in wound healing and formation of cell receptors, it also increase collagen formation, cross linking of collagen fibers through proline to reduce scaring.

Effects of NO/cGMP/PKG pathway on metabolism: PDE5 and PDE 11 have been detected in the human adipose cells and in increased levels during their differentiation. Activated PKG increases 3T3-L1 preadipocyte differentiation and activities of aromatase (Aversa et al, 2011). Adipose tissues also contain cGMP and cAMP which plays a role in their differentiation (Hemmrich et al, 2010; lipolysis Lafontan et al, 2008). Increased inhibition of PDE5 shows increased lipid droplet in the adipocytes and increased expression of peroxisome proliferator activated receptor- γ (PPAR-γ). The similar results were seen by Haas, B. et al, where they knock out PKG gene and there was reduction lipid droplet as well as expression of PPARγ.

PKG is necessary for activation of CREB through phosphorylation, which binds to cAMP responsive elements (CREs) which is important in adipogenesis (Reusch et al, 2000). PDE5 inhibition cause the release of rennin, hence this can be used as an antihypertensive (Chiu et al, 2002) and rennin-angiotensin-aldosterone pathway can be used as a control way to liver cirrhosis and ascites (Thiesson et al, 2005). And according to the study carried out by Ghoul R et al, they found that sildenafil administration prevent retention of water (Ghaul et al, 2007). NO has a role in insulin induced effects on glucose (Duplain et

al, 2001) this is also seen in diabetic humans whose eNOS is reduced (Kashyap et al, 2005). The increase in the levels of cGMP increases the levels of phosphorylation of transcription factors, this occurs through the transfer of phosphate group in subunits of effectors and other molecules such as cAMP response element binding proteins (CREB) are activated (Puzzo et al, 2009). Cyclic GMP/PKG pathway is a new potential area to develop new treatment for metabolic diseases such as Obesity (Pfeifer et al, 2013). PDE5 inhibition has been reported to have efficacy as chemo-sensitizers. The ABC transporters whose over expression has been the cause of multi-drug resistance in cancer cells were found that they are new target of PDE5 inhibitors and these have been shown to enhance the action of various anticancer drugs (Chen et al, 2012; Sun et al, 2012). Therefore it is very cardinal to study the properties of LLK.

PDE has a functional role in spermatogenesis (Pomara et al, 2004). This was seen in dogs which were administered with larger doses of tadalafil that showed germinal epithelium atrophy and Oligospermia (Cialiss et al, 2005).

NO is a reactive oxygen species, and excess ROS can produce peroxidative damage to cells (Garg, 2011), despite it being important in the treatment of ED, and ED cases are estimated to reach 322 million around the globe by the year 2025 (Ayta et al, 1999). NO has control on neurotransmission by raising cGMP concentration to phosphorylate ion channels important for transmission of nerve signals. When PKG is activated it causes phosphorylation of gap junction and the phosphorylation of calcium and potassium causes decrease calcium influx and potassium outflow respectively, this reduces the concentration of calcium below 500nm which causes relaxation (Stankeviciuse et al, 2003).

Some studies show that penile fibrosis is caused by pathophysiological over production of plasminogen activator inhibitor 1, TGF β1, and Reactive oxygen species that can cause myofibroblast activation and production of collagen (Egydio and Kuehhas, 2013). Penile fibrosis contributes to ED, and PDE 5 inhibitors have anti-fibrotic roles (Ferrini et al, 2007)

Research Question

1. How does LLK cause smooth muscle relaxation?
2. What are its effects on the levels of second messenger cGMP?

2.1 **Objectives**

2.1.1 General Objectives

1. To elucidate the signalling pathway used by LLK to cause smooth muscle relaxation

2.1.2 Specific Objective

1. To determine the effects of LLK on the levels of cGMP in the cell
2. To determine the effect of LLK on Phosphodiesterase enzymes

3.0 Methodology

3.1 Study design: An Experimental study was undertaken to analyse the effects of LLK on the levels of cGMP in the cell

3.2 Study site: The research was done at the University of Zambia and University Teaching Hospital laboratories (UTH) in Lusaka.

3.3 Study population

3.3.1 Sample size: A convenient sample size of 9 sets for 7 drugs/chemicals (Total 63), were used in the experimental study.

3.4 Material and Methods

3.4.1 Materials

Materials and Reagents used were; gloves, thermo scientific weighing scale, pipettes and pipette tips(20 µL, 200 µL, and 1000µL), 15mL test tube, test tube rack, Colorimetric microplate reader, 5 mL heparined syringes, 20ga needles, 96 well plate, Orbital shaker, 1M HCl, 1.50mL microcentrifuge tubes (eppendorf), 50mL falcon tubes, automated soxhlet, 100mL conical flask, EDTA vacutainers, glacial acetic acid, Ferric chloride, concentrated sulphuric acid, Hager's reagent (saturated picric acid), Ferric chloride, microscope, Beckman centrifuge. 50µM SNAP, 0.50mM IBMX and the cGMP ELISA test kit were procured from abcam.

3.4.2 Sample collection and preparation

The LLK plant samples were collected from Gwembe with the help of a knowledgeable local person who identified the tree from the bush. The barks of the tree were collected and placed in sample transport bag which was placed in the cooler box and transported to the laboratory. Further processing and extraction of LLK was done at the University of Zambia, School of medicine, School of veterinary medicine and UTH Food and Drug.

3.4.3 Preparation and extraction of LLK

To get an extract from the bark of LLK, Soxhlet extraction technique was employed. The bark of the tree from the trunk was taken and weighed, dried at room temperature, and then pounded. 100g of the powder was immersed in 50mls of water (LLKn) to taste the natural way as used by the locals. Another 100g of LLK powder was immersed in 50mls

of methanol (LLKa). The mixture was heated up to 110 °C for 25 cycles of condensation for 4 hours in an automated Soxhlet extraction system to collect the extract of interest (LLK extract solution). The LLK extract solution was used in the experiment according to the procedure below.

3.5.1 Experiment

Permission to use mice in the study was obtained from the University of Zambia Biomedical Research Ethics Committee (UNZABREC). Treatment of mice was done according to standard guidelines for use of laboratory animals (National Institute of Health, USA: Public Health Service Policy on Humane Care and Use of Laboratory Animals, 2002). Even though mice platelets and human platelets are morphologically different, physiologically they are very similar (Schmitt et al., 2001). Platelets were extracted as described elsewhere (Jirouskova et al., 2007). Briefly the mice were anesthetized with diethyl ether and blood was collected by amputation of the tail end. Blood was centrifuged to get platelet rich plasma which was then washed twice in PBS at 37°C to remove exogenous cGMP. The platelets were then suspended to 1-4 x10^9platelets/µL in Tyrode buffer. Using mice models the activity of phosphodiestrase and cGMP levels have been studied in platelets (Dangel et al., 2010, Sun et al., 2007, Gresele et al., 2011). There were seven test solutions which comprised LLKa (extracted in alcohol); LLKn (extracted in water); and a NO donor which increases levels of cGMP, S-nitroso-N-acetyl-penicillamine (SNAP) 50µM; a phosphodieaterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) 0.50mM; phosphate buffered saline (PBS) control; IBMX+LLKn; SNAP +LLKn. Three sets of 9 tubes were prepared for each test solution

and to each tube 500µL of platelet suspension was added. Each test substance was then added to each of the three sets of 9 tubes with platelets and levels of cGMP were determined by ELISA every 5 minutes from 0 minutes up to 40minutes. An internal control (INT) was also done with two set of 9 tubes in which PBS was used instead of drugs to demonstrate the fluctuations of cGMP in platelet cells. All these experiments were done at 37°C and the reactions were stopped by adding 1M of HCl.

All samples were acetylated by adding 5 µL of acetylating reagent and 50 µL of the sample was added into the protein G coated wells to which 50 µL of cyclic GMP Alkaline Phosphatase Conjugate was added. 10 µL of reconstituted cGMP antibodies were added per well and incubated for 1 hour. 10 µL of cGMP-HRP was added to each well and incubated for 1 hour and then washed 5 times with 200 µL of 1X assay buffer. To develop the colour 100 µL of HRP developer was added and incubated for 1 hour, 100µL of HCl was added to stop the reaction and the ELISA plate was read immediately according to abcam cGMP direct Immunoassay colorimetric assay kit standard protocol. The amount of cGMP-HRP bound to plate was determined by reading at OD450 nm. The intensity of OD450 nm was inversely proportional to the concentration of cGMP in samples. The kit used could detect 0.04 -10 pmol/well (0.008 - 2 µM) cGMP samples. SNAP, IBMX and the ELISA kit were procured from abcam

3.5.2 Phytochemical Tests

LLK was screened for flavonoids, alkaloids, steroids and glycosides qualitatively as done elsewhere (Hossain et al., 2013) . This is based on a specific precipitation or colour change that takes place when test solutions are mixed with specific reagents. The

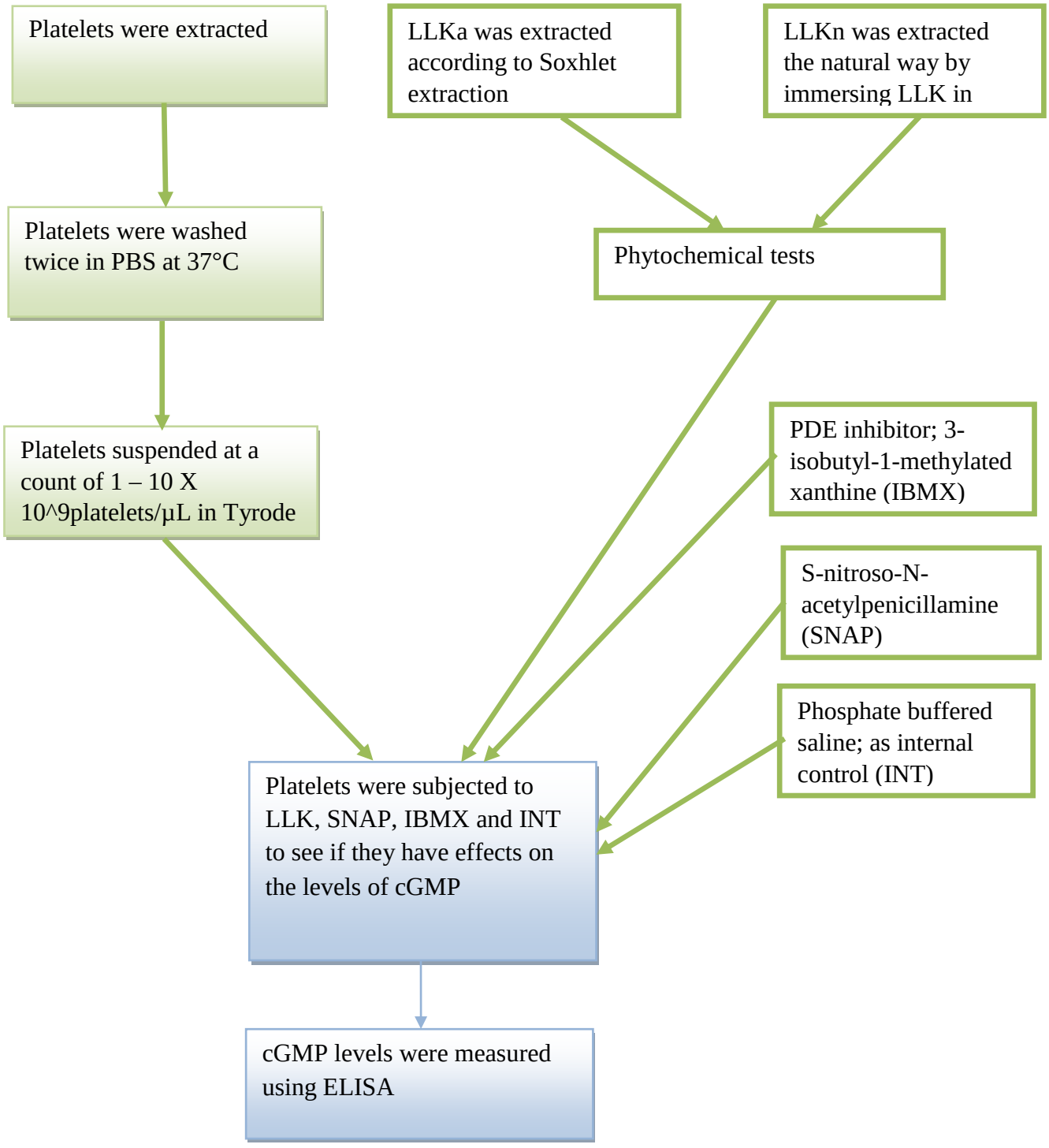
procedure was as follows; Test for Glycosides: Keller Killiani Test – Test solution was treated with few drops of glacial acetic acid and Ferric chloride solution and mixed. Concentrated sulphuric acid was added, and observed for the formation of two layers. If lower reddish brown layer and upper acetic acid layer turned bluish green then indicated a positive test for glycosides.

Test for Alkaloids: Hager's Test – Test solution was treated with few drops of Hager's reagent (saturated picric acid solution).Formation of yellow precipitate showed a positive result for the presence of alkaloids.

Test for Flavonoids: Ferric chloride test – Test extracts ware treated with few drops of Ferric chloride solution and resulted in the formation of blackish red color indicating the presence of flavonoids.

Salkowski Tests for Steroids: the extracts ware shaken with concentrated sulphuric acid and on standing would yield red colour to show presence of steroids.

3.5.3 Experiment procedure flow chart



3.6 Data analysis

Data was analysed quantitatively to know the figures and statistical software SPSS version 16 was used to calculate two independent sample t-test in order to establish the significance of in the levels of NO and cGMP. All values were expressed as the mean ± SEM (standard error of the mean). Qualitative analysis was employed to understand how the phytochemicals results affect the biochemical pathway involved as well as how valuable the information is to the society.

3.7 Ethics Clearance

Permission to carry out the study was obtained from the University of Zambia Biomedical Research Ethics Committee (UNZABREC) to use mice in the study. To ensure authenticity of the research, permission was also obtained from the UTH administration and all those in charge of laboratories that were to be used.

4.0 Results

Time (min)	LLKa	LLKn	SNAP	IBMX	INT	IBMX/ LLKn	SNAP/ LLKn
0	3.3006 ± 0.005	3.1907 ± 0.01965	3.2013 ± 0.00205	3.294 ± 0.01	3.22 ± 0.0155	3.3201 ± 0.01055	3.18 ± 0.011
5	3.8485 ± 0.059	3.742 ± 0.111	3.6508 ± 0.00505	3.872 ± 0.0365	3.1878 ± 0.0874	3.968 ± 0.01875	3.987 ± 0.002
10	3.8975 ± 0.079	3.845 ± 0.0765	3.709 ± 0.0305	3.985 ± 0.0295	3.2704 ± 0.0347	3.9865 ± 0.00175	4 ± 0.0115
15	3.9265 ± 0.108	3.862 ± 0.071	3.734 ± 0.00815	4.016 ± 0.02745	3.157 ± 0.0275	4.047 ± 0.022	4.036 ± 0.012
20	3.9155 ± 0.059	3.882 ± 0.07825	3.745 ± 0.006	4.046 ± 0.003	3.1919 ± 0.08745	4.16 ± 0.0065	4.099 ± 0.00305
25	3.964 ± 0.074	3.928 ± 0.05775	3.864 ± 0.0025	4.043 ± 0.0165	3.2001 ± 0.0199	4.164 ± 0.00795	4.156 ± 0.024
30	3.937 ± 0.02525	3.943 ± 0.0455	3.912 ± 0.00445	4.058 ± 0.0125	3.2331 ± 0.00345	4.1825 ± 0.00175	4.175 ± 0.006
35	4.009 ± 0.00925	4.007 ± 0.0295	4.05 ± 0.002555	4.068 ± 0.0145	3.208 ± 0.00295	4.1903 ± 0.00585	4.309 ± 0.026
40	4.0561 ± 0.021	4.041 ± 0.03065	4.13 ± 0.0385	4.076 ± 0.015	3.1996 ± 0.0044	4.2408 ± 0.0151	4.33 ± 0.0155

Table 1: the amount of cGMP detected in platelets by LLK compared to controls: SNAP-NO donor, IBMX-PDE inhibitor, INT-internal control for cGMP basal levels, and cGMP is expressed in $\mathrm{pmol}/10^9$platelet. All values are expressed as the mean ± SD, n = 3

Figure 1a: Effects of LLK, IBMX (0.50mM) and SNAP at 50µM on the level of cGMP in platelets with respect to time.

There was a slight difference between effects of SNAP and the other drugs used, on levels of cGMP in the cells. Suggesting that NO donors increases more cGMP in the cells at normal hydrolysis of cGMP by PDEs, as compared to the inhibition of PDEs at normal synthesis of cGMP by sGC in the cell, at the concentration used which was the same in all the experiment conducted throughout this study.

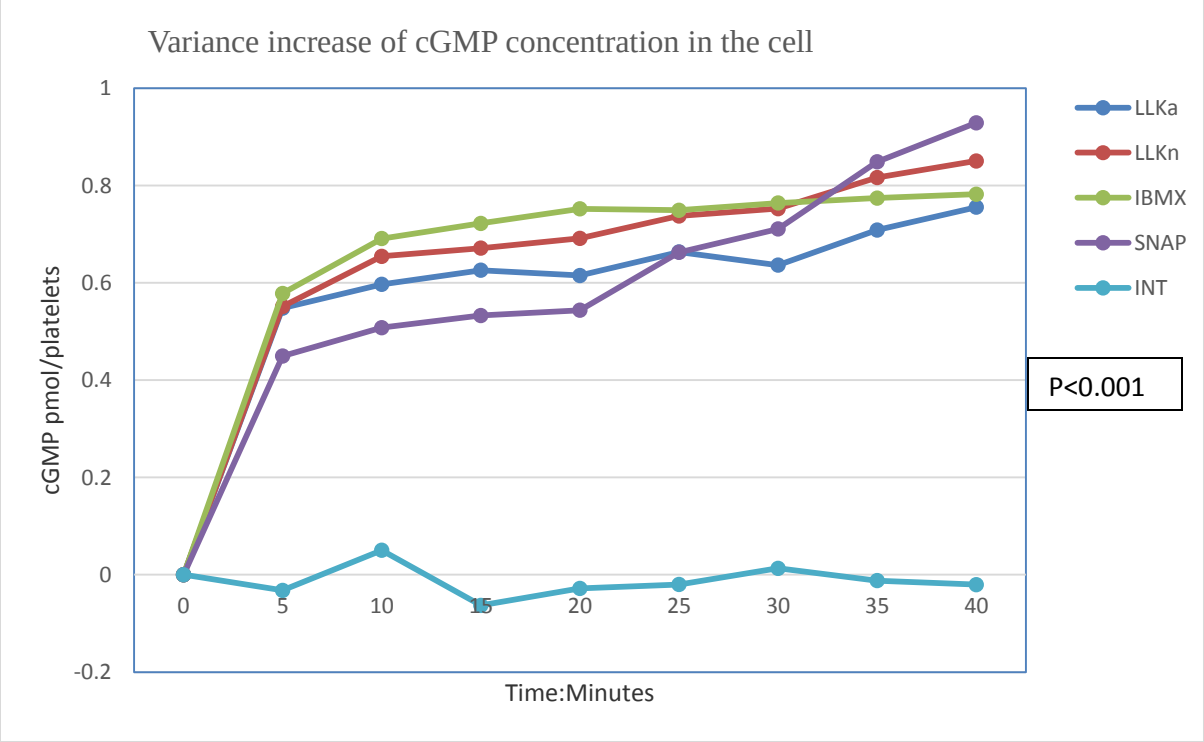


Figure 1b: shows the variance increase of cGMP in platelets, cGMP is expressed in pmol/ 10^9platelets.

Comparing LLK to IBMX and SNAP shows an increase in cGMP from an average basal level of 3.2828 ± 0.0069 pmol/10^9platelets to an average of 3.73 ± 0.0056 pmol/ 10^9platelets within the first 5 minutes. There was a steady increase in the levels of cGMP for all the compounds. SNAP is a NO donor and IBMX is a PDE inhibitor, which were used as positive controls. Also an internal control (INT) was done in which deionised water was used as a drug (Placebo) to check the fluctuation of cGMP in the cells within the experimental time. The levels of cGMP for INT ranged from 3.157 ± 0.02023 to 3.2704 ± 0.03177 pmol/10^9platelets. Comparing LLK to the two controls, LLK can be said to have effects similar to both NO donors and PDE inhibitor. But graph closely shows that LLKa and LLKn figures are more close to PDE inhibitor IBMX. LLK and IBMX showed significant increase in the first 15minutes whilst SNAP had a steady rise

in the production of cGMP. LLKa showed a reduced cGMP level in the 30th minute but rose again to higher cGMP levels. SNAP produced the highest amount of cGMP, as compared to other drugs. All the samples showed a significant increase with $p<0.001$ in LLKa, LLKn, IBMX and SNAP at 90% confidence interval taking 0.400 pmol/ 10^9platelets as a significant level that can alter the reaction in a cell.

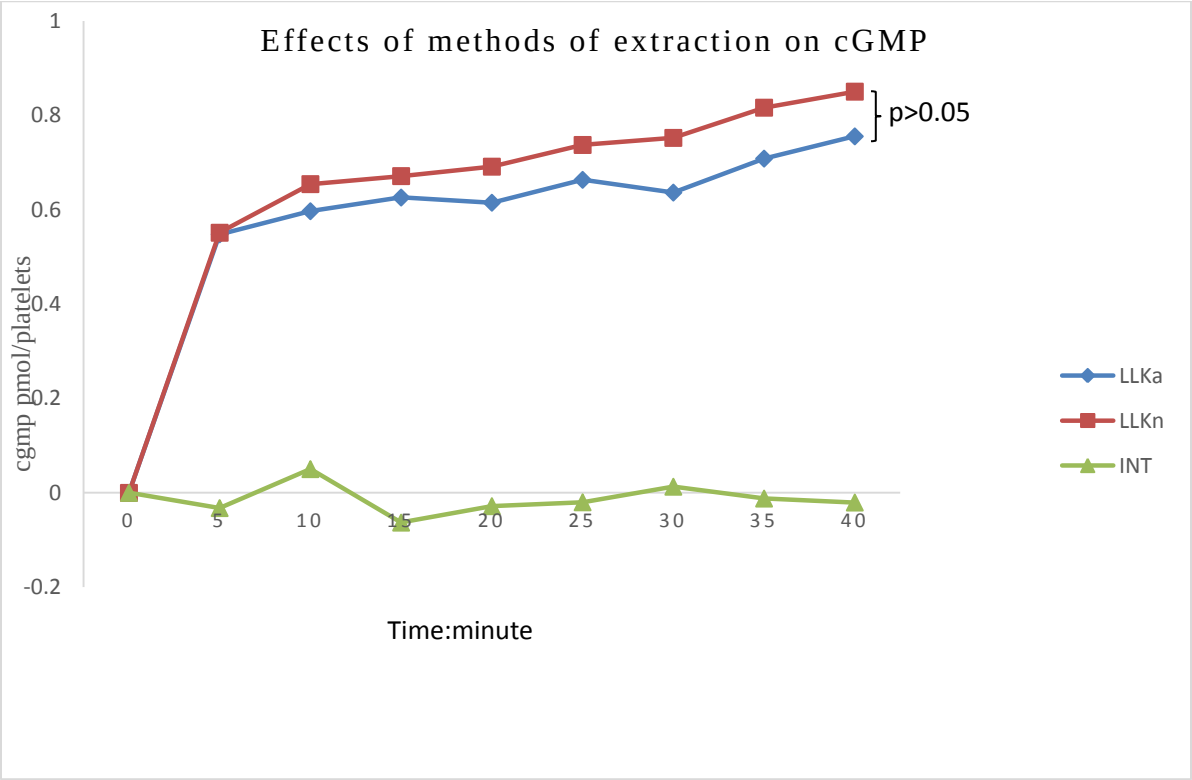


Figure 2: shows the effect of methanol extracted herb (LLKa) and distilled water extracted herb (LLKn) and the variance increase in the level of cGMP respectively

Distilled water extracted herb showed to increase cGMP levels more than methanol extracted. To compare the water and methanol extracted LLK, comparison made based on the fluctuations of cGMP in a cell (INT) and by taking 0.20pmol/10^9platelets as a significant difference by a stimulating agent. We clearly saw the difference in the levels

of cGMP despite being not statistically significant, which could possibly arise from a difference in concentrations of active ingredient in the two drugs. Methanol could have some little effect on the potency of LLK as it could be seen that the levels of cGMP was higher after 5 minutes of exposure to platelets. We couldn't measure the concentration of both LLKa and LLKn as they showed loss of some properties upon heating to concentrate. Despite both having been extracted from same mass of the herb and in equal volumes of solvents the difference was seen. Therefore it can be said that LLK is better extracted in water as compared to methanol despite both showing significant increase with $p<0.001$.

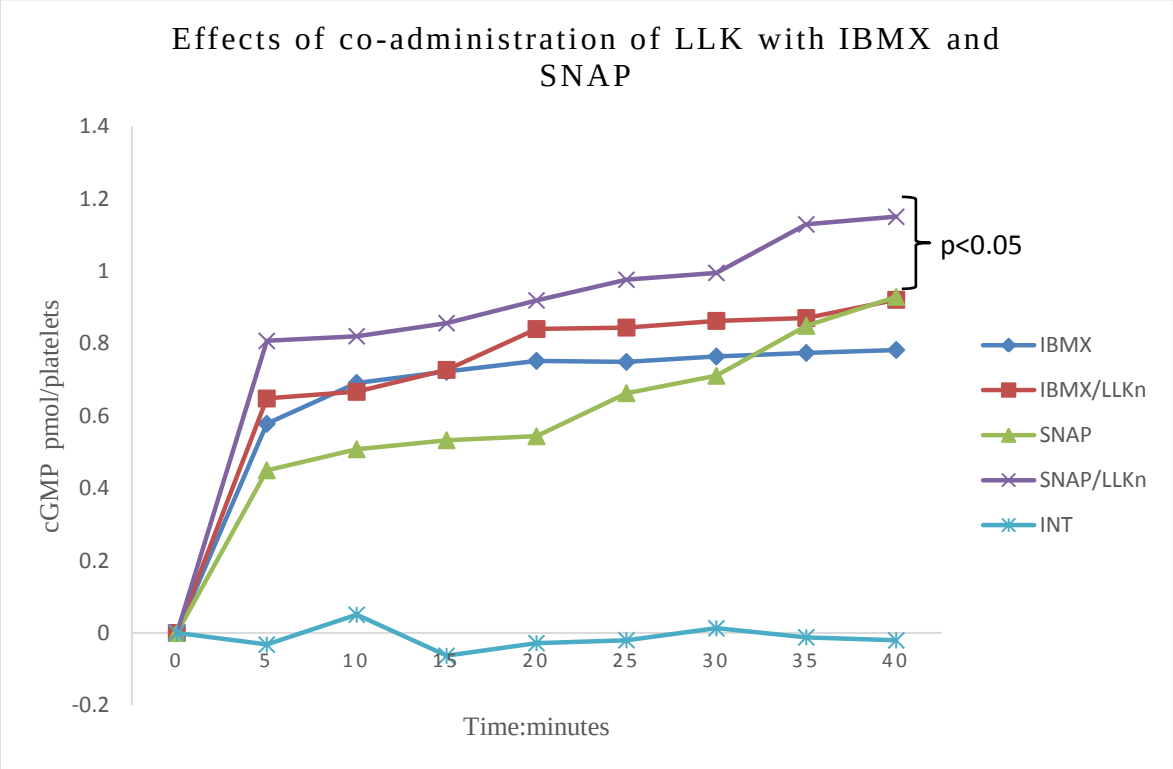


Figure 3: Effects of co-treatment/administration SNAP with LLK and IBMX with LLK on cGMP in platelets taking $0.20 pmol/10^9$platelets as significant difference between drugs effect.

If LLK was a NO donor then the results for SNAP/LLK were supposed to be slightly higher than that of SNAP and if LLK was a PDE inhibitor then results were supposed to be higher compared to SNAP alone. There is a significant difference between SNAP and SNAP/LLK and there is more cGMP produced in SNAP/LLK than any drug or combination used. Therefore there could be higher synthesis of cGMP in SNAP/LLK, as a result of higher rate of activation of either NOS or sGC by NO donors of which LLK could have contributed. The other possibility could be that, there was reduced hydrolysis of cGMP as a result of PDE inhibition of which could have been brought by only LLK.

But when these results are compared to IBMX and IBMX/LLK, which was prepared (IBMX/LLK,) the same way as SNAP/LLK, We found that the amount of cGMP produced in IBMX/LLK was higher as compared to IBMX only, meaning there could be some activation of NOS enzyme that caused NO production that activated sGC for more cGMP production and or less hydrolysis of cGMP.

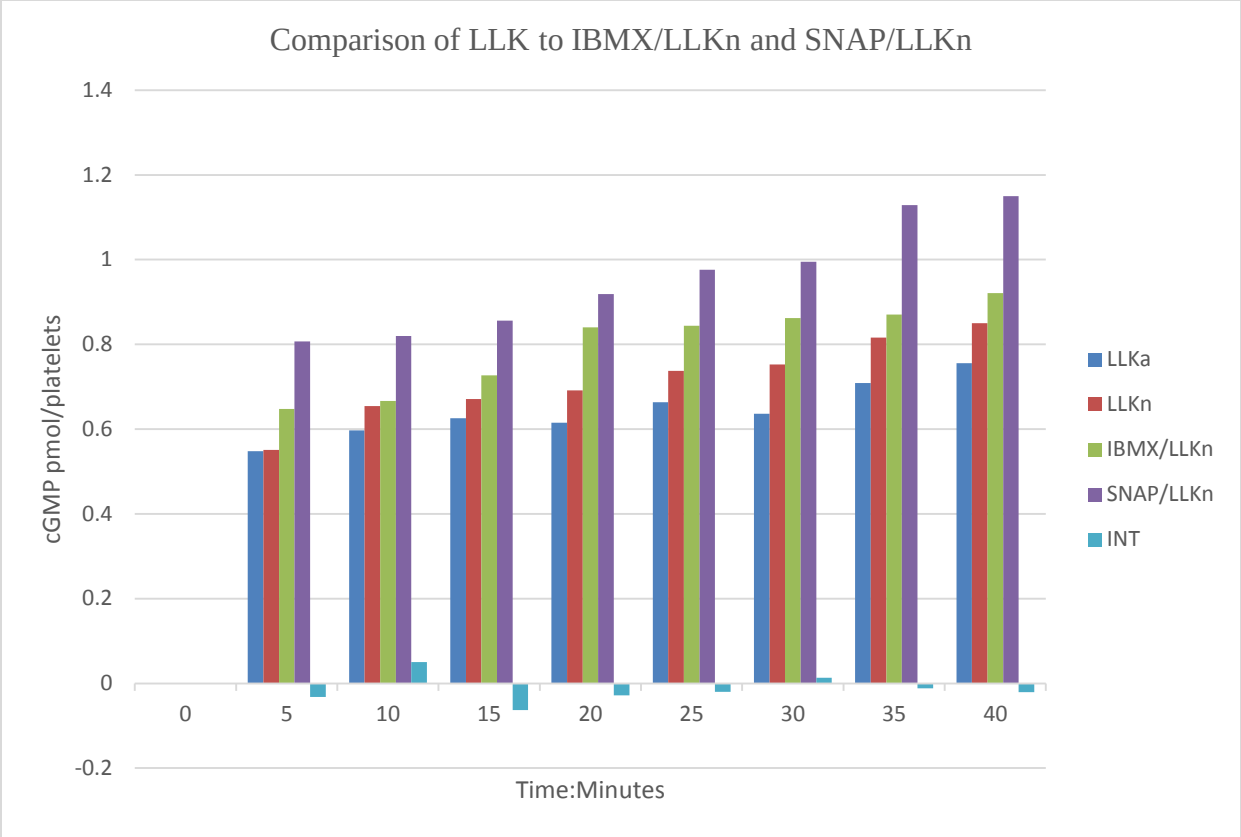


Figure 4: LLKn showed lower levels of cGMP as compared to drug combination SNAP/LLK and IBMX/LLK. Suggesting of synergic effect on cGMP accumulation in the cells and that it could be used with other NO donors and or PDE inhibitors. It also points towards LLK being a PDE inhibitor as there was more cGMP produced as compared to IBMX/LLK. The lower cGMP production in IBMX/LLK could have been brought about saturation of PDE enzymes as can be seen from the low increase of cGMP from the 20th to 40th minute. SNAP and IBMX produced less cGMP as compared to their combination with LLK signifying that there was some activation of NOS or sGC and inhibition of PDEs by LLK. If there was more cGMP conversion/synthesis in IBMX/LLK, we would have had more cGMP produced as compared to SNAP/LLK or even more. This suggest that LLK has more of inhibitory effects of PDEs than activation of either NOS or sGC.

Phytochemical reactions/tests

Phytochemicals	**Results**	
	Water Extract	Methanol Extract
Flavonoids	Positive	Positive
Alkaloids	Positive	Positive
Steroids	Negative	Negative
Glycosides	Positive	Positive

Table 1: some compounds that were detected in LLK which could possibly contributes to its effects and mechanism of action

5.0 Discussion

The extract of LLK has been found to contain a number of compounds which determines its mechanism of action. An increase in cGMP concentration was detected in platelet cells confirming that LLK had effects on cGMP signalling pathway. The increase of cGMP in a cell is affected by the concentration of active ingredient, although that of LLK was not determined. Comparing our findings to those carried out by other researchers we found higher basal level of cGMP in platelet cell. Peng et al (2004) found the levels of cGMP to range between 0.026 $pmol/10^9$platelets to 0.148 $pmol/10^9$platelets also a study carried out by Begonja et al, (2013) found lower figures. Whilst we found an average of 3.2828 ± 0.0069 $pmol/10^9$platelets to 4.0273 ± 0.0056 $pmol/10^9$platelet being the highest level of cGMP. And we detected low increase of cGMP levels for SNAP, IBMX and LLK in platelets. This could be due to different methodologies as well as the different concentration of the drugs used. Nevertheless the levels of cGMP were all within the same range for SNAP, IBMX and LLK in our study and the mechanism of action is known.

The continuous use of organic nitrates or NO producing agents may have some tolerance effects as suggested by McVeigh et al (2002). In a study they carried out, they found a significant increase in both the NO production and cGMP levels from 1.0 ± 1.17 $pmol/10^9$platelets to 2.52 ± 0.88 $pmol/10^9$platelets and 0.60 ± 0.10 $pmol/10^9$platelets to 0.89 ± 0.16 $pmol/10^9$platelets respectively. The accumulation of cGMP and cAMP as a result of constant use of organic nitrates and PDE inhibitors leads to an increase in the signalling cascade in target cells. It has been found that there is an increase in the expression of PDEs in cells as a result of constant increase of cGMP concentration. This

occurs to regulate the levels of cGMP in a cell, as the amount of cGMP does not just affect one reaction, but involves phosphorylation and activation of other molecules. Therefore, regulation of these signalling molecules is very cardinal for maintenance of normal physiology of the cell. PDEs expression levels differ from one tissue to the other and different type of PDEs exist in different types of cells. Platelets expresses three types of PDEs, type 2, 3 and 5, types 2 and 3 hydrolyses both cAMP and cGMP while type 5 hydrolyses cGMP only (Shatha H an Adnan, 2015). Therefore more than one type of tissue may be affected by the use of PDE inhibitors and the site with the most affected PDE type, have the most amplified phosphorylation or secondary effects of secondary messengers (cGMP and cAMP) (Rumi Ghoshi at al, 2009).

Phytochemical reactions reviewed that LLK contained alkaloids, flavonoids and glycosides. These compounds have effects on the signalling molecules and other biochemical reactions that occur in the human body. Most chemical's potency is concentration dependant, and the users of these compounds do not regulate the concentration because of lack of knowledge and they always want the quickest responses/effects of the herb. Alkaloids and flavonoids have contributed greatly to the reactions of LLK as many studies have suggested and some have proved them to be potential PDE inhibitors.

Alkaloids contain nitrogen, they are also found in plants as well as aquatic organisms (Rahimi et al, 2009). They have vasodilation effects and are potent inhibitors of PDEs (Silva, 2006). Many different studies have shown and demonstrated the effects of alkaloids, Aporphines alkaloids have been shown to bind to receptors in the paraventricular nucleus of hypothalamus to produce pro-erectile mechanism, and also

they are seen to react like dopamine as they are even similar in structure (saftel, 2002). Alkaloids produce its vasodilation by blocking PDEs through its structure and side group attached, some purine alkaloids have similar structures like cGMP and cAMP hence confers competitive inhibitory effects on PDEs. They have ethyl groups of xanthenes analogues on position N-1 and N-3 which have inhibitory effects on PDE 5, also alkaloids with a furoyl or a pyrimidinyl on N-2 showed some inhibitory activities on PDEs (Cao et al, 2007; Wang et al, 2002). Alkaloids such as furoquinoline with methoxyl group at C-7 or C-8 have shown high inhibitory activities on PDE5A and for those with methoxyl group at both C-7 and C-8 have less inhibitory effects on PDE5A. Despite that, the methoxyl group at C-8 of furoqunoline alkaloid has stronger inhibitory activities on PDE5A (Nam et al, 2005). The positioning of double bond on the structure of alkaloids contributes to inhibition activities on PDEs. It was seen that the existence of a double bond between C-2 and C-3, as well as the presence of hydroxyl group at C-5 and C-7 promotes inhibition of PDE 5. In a study carried by Ko et al (2004) it was observed that hydroxyl groups on C-4 has less inhibitory activities. It is through the inhibition of PDEs that they cause an increase in the levels of cGMP in cells, there in turn, phosphorylate PKG to produce vasodilation and vasorelaxation effects. Akaloids have also been shown to cause vasodilation/vasorelaxation through inhibition of Ca2+ channels, this reduces calcium influx. This was demonstrated with Curine (alkaloid) which decreased intracellular Ca2 + concentration (Medeiros et al, 2011).

Flavonoids are plant phenolic compounds, occurring virtually in all plant parts. Recently there has been an upsurge of interest in the therapeutic potential of medicinal plants which might be due to their phenolic compounds, specifically to flavonoids. LLK was

found to have flavonoids in its extract. The physiological effect of LLK may be due to the presence of these flavonoids as studies have shown that flavonoids do inhibit phosphodiesterase(Peluso, 2006, Sabphon et al., 2015). Alkaloids are a group of naturally occurring chemical compounds that contain mostly basic nitrogen atoms and they are produced by plants and other living organisms and they have a wide range of pharmacological activities. Like flavonoids alkaloids have also been shown to inhibit phosphodiesterase which in part explains their wide range of activities (Nam et al., 2005). It is likely that the observed effects of LLK are due to the presence of flavonoids and alkaloids in the extract. Flavonoids have antioxidant effects as well as antiviral activities and inhibitory growth effects on cancer cells (Middleton et al, 2000; Narayama et al, 2001). They have coronary dilation effects and have been reported to have cardioprotective effects (Huesken et al, 1995). Flavonoids affect hormones by binding 3 and 17 beta hydroxyl steroid dehydrogenases to regulate estrogen, progestin and androgen levels in humans (Naro et al, 1983). Apigenine inhibits PDEs which increases the levels of cAMP and cGMP thereby producing cardiotonic activities.

FLavonoids have the potency to produce NO due to their structure, they have hydroxyl group on their C3' and C4' which are very important. This was seen in a study conducted by Taubert et al, (2002) that meta-positioning hydroxyl group significantly reduced the production of NO. The other two hydroxyl groups which enhances the production of NO is at C5' and C3 on ring C of flavonoids. From our findings, LLK seemed to have an effect on NOS activation as there was a difference in cGMP produced between the use of SNAP/LLK and IBMX/LLK, suggesting that there was an extra activity of sGC which should have been more activation from NO or other molecules/compound from LLK not

identified. Comparing the results of SNAP and LLK shows minor differences. Some researchers have shown that Flavonoids inhibits NOS enzymes and others have shown that they activate NOS.

The research carried out by McVeigh et al (2002) showed that Nitroglycerin/placebo co-administration in platelets increased the level of NO production from 1.0 ± 1.7 to 2.25 ± 0.88 $pmol/10^8$platelet and this saw the increase of cGMP content in platelets from 0.69 ± 0.10 to 0.89 ± 0.16 $pmol/10^9$platelet. From this we can note that 100% increase in NO production increased the level of cGMP by 0.200 $pmol/10^9$platelet. If we assume that it is true, that could mean LLK has the potential to increase the level of NO in platelets as it was seen that SNAP/LLK increased the level of cGMP up to 4.33 $pmol/10^9$platelets. This is compared to IBMX/LLK which produced 4.2408 $pmol/10^9$platelet making a difference of 0.0892 $pmol/10^9$platelet which is not quite significant but could cause a percentage of activation on a biomolecule. Meaning there was a level of NO production in SNAP/LLK as compared to IBMX/LLK. This was observed as the NO, produced more cGMP than PDE inhibition in the research conducted by Piggott et al (2006). In their research they found that the combination of NO donor and PDE inhibitor produced more cGMP. Deducing that to our findings suggest that there was some activities of NO production.

Having found that LLK has effects on cGMP levels through the inhibition of PDEs, that means it can work as IBMX which is a PDE inhibitor or as other PDE inhibitors such as Sildenafil, Tadafil etc. PDE inhibitors have been demonstrated to treat hypertension and many other conditions as mentioned in literature review. The users must be aware of side effects that are brought about by PDE inhibitors such as headache and night blindness.

Flavonoids and alkaloids are known to be compounds with most vasodilatory activities (Francisco J et al, 2013), and from our findings we can say LLK can be used to treat ED as it has always been used by the locals.

6.0 Conclusion

The study was able to demonstrate that LLK increases the levels of cGMP through an invitro experiment. The mechanism of action was most likely through the inhibition of PDEs and this property rendering it a potential source of valuable medicine for treatment of conditions like erectile dysfunction, pulmonary hypertension, peripheral vascular diseases and many others. The study also demostrated that LLK has an active ingredient which increased cGMP that has some vasodilatory effects on smooth muscles thereby able to dilate blood vessels and allow more blood to flow through leading to sustained erection. Phytochemical tests reviewed that extracts contained Akaloids, glycosides and Flavonoids.

7.0 Limitation

1. Further studies would be needed to purify the efficacious ingredients of the LLK extract as this couldn't be determined, and to investigate whether it is from Akaloids, glycosides or Flavonoids.
2. We could not test for all compounds, also we couldn't measure their levels in LLK because it showed loss of some properties upon heating to concentrate and lack of proper compound isolating machinery.
3. Scientific identification of the herb was difficult without the flower of the plant as its flowering period was outside the study period (August-September)

8.0 Recommendation

Further studies are needed to classify the alkaloids and flavonoids in LLK as well as isolation and identification of other compounds in it.

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10.0 Appendix

10.1 Work plane

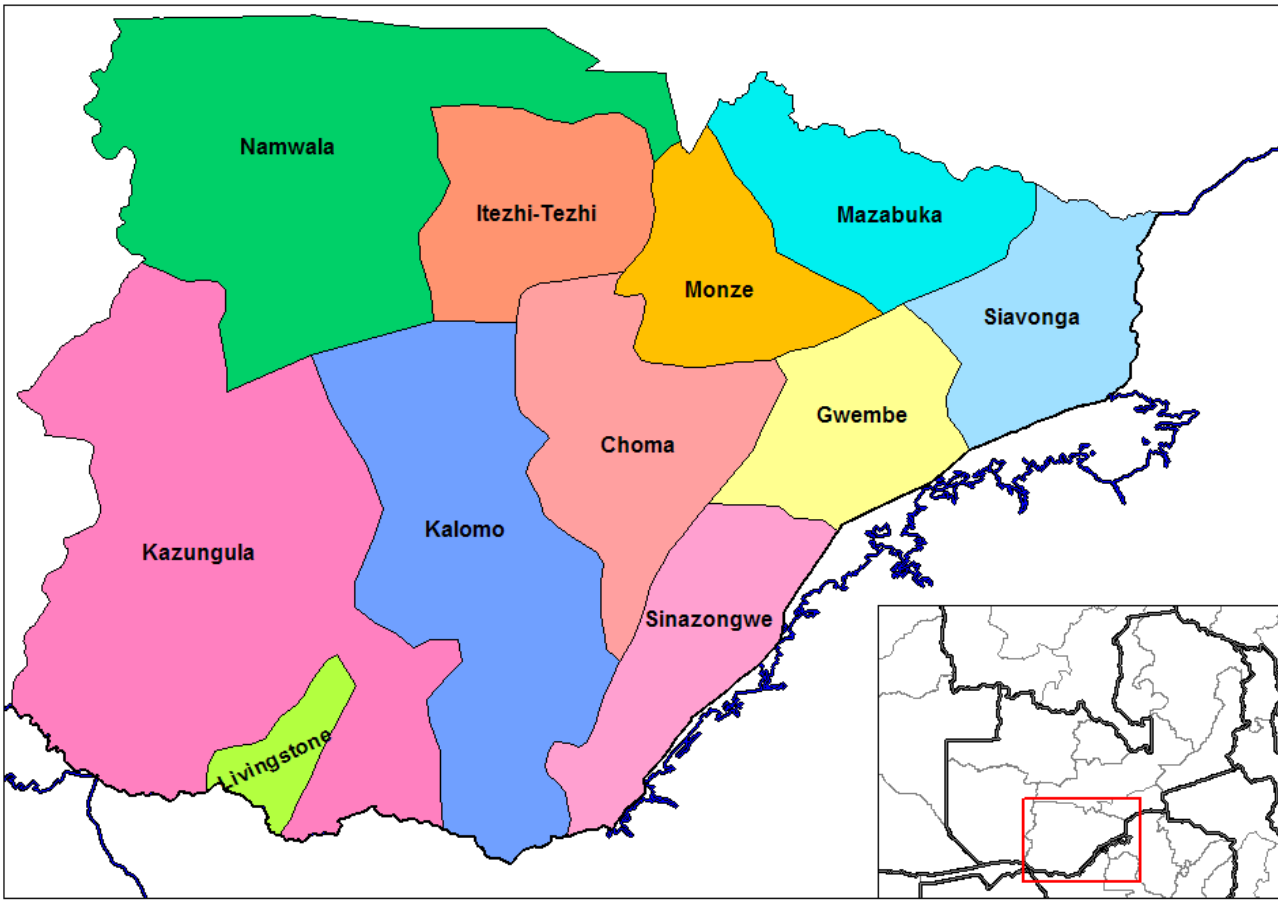
	Aug 2014	Sep 2014	Nov-Feb 2014	Mar-May 2015	June 2015	July 2015
Study identification						
Proposal writing and submission						
Proposal approval						
Ethical clearance						
Data collection and						
Data analysis						
Report writing and submission						

10.2 Budget

Quantity	Description	@(ZMW)	Amount(ZMW)
1	Ream of paper	50	50
5	Pens	2	10
5	Pencil	1	5
	Printing	300	300
5	Disposable gloves (Box)	20	100
3	cGMP Elisa test kit	9,000	27,000
	Pipette tip	500	500
	Transport	1,000	1,000
	Shipping	1,800	1,800
	Wash buffer	1,000	1,000
	Mice feed	100	100
100	Mice		500
	HCl	300	300
100	EDTA tubes	2	200
100	Centrifuge tubes	5	500
1	SNAP	1,200	1,200
1	IBMX	970	970
			35 535

Map of Southern province of Zambia from where LLK was collected

LLK was collected from Gwembe district, it is mostly used by the local in the district and surrounding districts. It is commonly found in the valley



Loozi Luna Kasika (LLK) plant

The sterm of Loozi Luna Kasika from where the barks have been collected