THE EFFECTS OF *STEGANOTAENIA ARALIACAE* EXTRACT ON CONTRACTILE FUNCTION OF ISOLATED RAT ILEUM

BY

PHARAOH HAMAMBULU

A dissertation submitted to the University of Zambia in partial fulfillment of the requirements of the degree of Master of Science in Human Physiology

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DECLARATION

I, **PHARAOH HAMAMBULU,** do hereby declare that this dissertation is a result of my own original work done in accordance with the guidelines for dissertations for the University of Zambia. Furthermore, I declare that this work has not been accepted in any previous application for award of any qualification in any other institution (University of Zambia inclusive) and that all sources of information have been specifically acknowledged by means of acceptable referencing in accordance with the University of Zambia regulations.

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APPROVAL

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ABSTRACT

A small tree, *Steganotaenia araliacae* is used as a medicine in local traditional settings in Zambia to initiate and augment parturition although very little is known and documented about its physiological and pharmacological effects. *S. araliacae* extract (SAE^c) has been observed to cause significant contractions of the uterine smooth muscle in rats but its effect on non-uterine smooth muscle is not known. The aim of this work was to establish the effects of *S. aaraliacae* extract (SAE^c) on the contractility of isolated rat ileum smooth muscle. Effects of acetylcholine, a reference agonist and antagonists that include atropine (AT), indomethacin (IND), mepyramine (MPN), ondansetron (OND) and nifedipine (NF) in the presence and absence of SAE^c were also investigated. Our findings showed that SAE^c increased the contractile strength of isolated rat ileum in a dose-response manner but had no significant effects on the frequency of the spontaneous contractions. Pre-treating the tissue with AT, IND, MPN or OND did not inhibit the contractile effects of SAE^c while pre-treating the tissue with NF significantly inhibited its contractile effects by 100% ($p<0.05$). These findings indicate that SAE^c causes contractions of isolated rat ileum by probable activation of calcium channels by either a direct or an indirect mechanism. Therefore, abdominal cramps could be induced if higher than normal pharmacological doses of SAE^c are taken during induction or facilitation of parturition. However, these undesirable intestinal contractions could possibly be controlled with nifedipine.

Keywords: Rat ileum smooth muscle, *Steganotaenia araliacae* extract agonist, nifedipine antagonist

DEDICATION

This Thesis is dedicated to:

My 'Step'Mother, Susan Shamoya-Hamambulu,

For the unconditional love, support and education she gave me

My Late 'Biological' Mother, Neless Ng'andu,

For love and support she gave me (MHSRP)

My Father, brothers and sisters

For making everything possible

My Friends

For being there for me in times of happiness and difficulties

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ABBREVIATIONS

ACh	Acetylcholine
ADP	Adenosine diphosphate
ATP	Adenosine Triphosphate
ANOVA	Analysis of variance
cAMP	Cyclic adenosine monophophate
GPCRs	G protein coupled receptors
SAE	*Steganotaenia araliacae extract*
AT	Atropine
MPN	Mepyramine
NF	Nifedipine
IND	Indomethacin
OND	Ondansetron
SEM	Standard error of the mean
SERCA	Sarcoplasmic reticulum calcium pump
UNZA	The University of Zambia
UNZABREC	UNZA Biomedical Research and Ethics committee
UNZASOM	University of Zambia School of Medicine

CHAPTER ONE

INTRODUCTION

1.1 Background

There are several drugs that are currently used to induce or augment labour. They are used in certain circumstances when the woman's labour has not started or when spontaneous uterine contractions have failed to result in progressive cervical dilatation or descent of the foetus respectively. Uterine stimulants can be used to augment existing uterine contractions, to increase their frequency, duration and strength, when labour is not progressing well. The three notable uterotonic drugs used most frequently are the oxytocin, prostaglandins, and ergot alkaloids (PATH, 2008).

Traditional medicine is in the same manner as complementary medicine used during antenatal period for various reasons including augmenting labor. Among the commonly used herbal medicines in pregnancy include ginger, cranberry, raspberry, echinacea and chamomile (Kennedy *et al*., 2013), *Agapanthus africanus* and *Gunnera perpensa* (Mkize *et al*., 2012) and *S. Araliacae* (Lwiindi *et al.*, 2015). The common reasons given for the use of traditional medicines during pregnancy are promotion of maternal health, foetal well-being, quick uncomplicated labor (Mkize *et al.*, 2012), induction of labour, removal of retained products of conception and management of post-partum bleeding (Lwiindi *et al.*, 2015). Although many herbs are used during pregnancy and around parturition time, the chemistry, pharmacology and undesirable effects of their extracts are not well documented. Lack of this information becomes a risk to both maternal and foetal health e.g. uterine rapture and foetal death respectively.

Herbal medicines can affect the function of gastrointestinal system by affecting the contractility of its smooth muscle in a similar manner they do on uterine smooth muscle. The uterine and gastrointestinal smooth muscles are under the same category of smooth muscle and therefore have similar contractile properties (Watras, 2007).The small intestines (includes the ileum) is where the vast majority of digestion and absorption of nutrients take place (Rhodes *et al.*, 2013). Therefore, any chemical substance that can affect its contractility may result in impairment of its function and may cause abdominal discomfort e.g. abdominal pain, indigestion and diarrhoea.

This study will bring out information concerning the possible side effects and safety of *S. Araliacae* extract on the function of the ileum as it is used to induce or facilitate labour.

1.2 Global perspective

Herbal medicines have been in use for a long time. The first documented records of herbal medicine use date back 5,000 years in China. India's Ayurvedic traditional medicine is thought to be more than 5,000 years old and herbal medicines remain an essential component of its practice. According to The World Health Organization, 80% of the population in the world use herbs with developing world having rates as high as 95% (Rivera *et al*., 2013).

In China, traditional medicine accounts for around 40% of all health care delivered. In Chile 71% of the population, and in Colombia 40% of the population, have used such medicine.
In India, 65% of the population in rural areas uses *Ayurveda* and medicinal plants to help meet their primary health care needs. The World Health Organization reports that the use of traditional, complementary and alternative medicines is becoming more popular. In that study, it was estimated that at least 48% of people in Australia, 31% in Belgium, 70% in Canada, 49% in France and 42% in the United States of America have used herbal medicines before(WHO, 2003).Use of herbs during pregnancy and postpartum period in various parts of the world is documented. A study conducted in 23 countries picked at random estimated that 28.9% of women use herbs during pregnancy and postpartum period. In that study, Russia had the highest score (69%) while Western Europe and Australia was at 51.8% and 43.8% respectively (Kennedy *et al*., 2013). In Malaysia a recent study concerning the use of herbal medicines during pregnancy and post-partum period estimated that 13.9% of women ingest herbs for reasons such as improving the mother and child's health (Teoh *et al*., 2013).

1.3 African perspective

Africa has a long history of use of herbal medicines. African traditional medicines used to be the dominant medical system available to millions of people in both rural and urban communities. It was the only source of medical care for a greater proportion of the population.

Traditional medicine has been used by Africans for the prevention, diagnosis and treatment of social, mental and physical ailments of different origins before and even after the advent of conventional medicine. In Ghana, Mali, Nigeria and Zambia, the first line of treatment for 60% of children with high fever resulting from malaria is the use of herbal medicines at home (WHO, 2003). Traditional medicine is not only a vital source of health care, but also an important source of income for many communities. Many communities are still involved in collection, domestication, cultivation and management of medicinal plant resources. This economic activity supports many indigenous peoples and local communities (Abbott *et al*., 2014).

A study conducted in Uganda estimated that 21% of pregnant women use herbs (Nyeko *et al*., 2016). Another study in Uganda estimates use of herbal medicine for day to day health care at 90%, with 80% of childbirths conducted at home using herbal remedies to enhance labour (Kamatenesi-Mugisha and Oryem-Origa, 2006). In Malawi a recent study reported that 94% of women eat maize porridge to hasten labour while the rest drink tea for the same purpose (Maliwichi-Nyirenda and Maliwichi, 2010).

1.4 National perspective

In Zambia the use of traditional herbal medicines by pregnant women is high. It is estimated that more than half (70%) of women depend on traditional herbal medicine for primary health care. There are several beliefs among pregnant women regarding diet during pregnancy. A study conducted by M'soka and colleagues (M'soka *et al*., 2015) at Chawama Clinic in Lusaka reported several beliefs concerning diet in pregnant women. According to that study some women believed that eating sugar cane during pregnancy caused the baby to have rough, dry and cracking skin and eating Okra (*Abelmoschus Esculentus)* causes the child to salivate.

About 66.4% agreed that herbs could assist during difficult labour by shortening the labor period and 72.4% agreed that cleansing with herbs after a miscarriage was necessary in order to prevent illness in the family. A more recent study in Chongwe and Chawama communities in Lusaka province of Zambia estimated that 32% of pregnant women have used traditional herbal medicine before. Of the 32% of these women 99% of them have used it to accelerate labour

(Maluma *et al*., 2017). That study investigated some of the common local plants that pregnant women use prior, during and after parturition.

1.5 Statement of the problem

The herbal *S. Araliacae* cold water extract (SAE^c) is used by traditional birth attendants in Zambia to start and facilitate labour (Lwiindi *et al*., 2015).A recent study revealed that SAE^c causes contractions of isolated rat uterine muscle in rat models (Goma et al., 2017). Although SAE^c has been shown to increase contractility of isolated rat uterine smooth muscle, its effects on the function of the gastrointestinal system and several organ systems is still not clear. More work need to be done to investigate the effects of this plant to reveal possible clinical implications of its use and toxicity.

1.6 General objective

To investigate the contractility effects of SAE^c on the isolated rat ileum

1.7 Specific objectives

i. To measure the change in isometric tension and contractile frequency of isolated rat ileum smooth muscle produced by SAE^c
ii. To determine possible mechanism of contractile action of SAE^c on isolated rat ileum smooth muscle

1.8 Study justification

Some uterotonic plants have been documented to cause harmful side effects when taken in high doses e.g. rupture of uterus (Gruber *et al*., 2010). Possible clinical implications and toxicity of *S. Araliacae* on the gastrointestinal tract is not well documented. Knowledge of the contractility effects of SAE^c on isolated rat ileum will provide some information regarding the safety of the extract on gastrointestinal system.

CHAPTER TWO

LITERATURE REVIEW

2.1 The contractile mechanism and process in smooth muscle

Muscle tissue is subdivided into striated muscle and non-striated muscle. Striated muscle includes skeletal and cardiac muscle. Non-striated muscle includes smooth muscle such as vascular, respiratory, uterine, and gastrointestinal muscles. In all muscle types, the contractile apparatus consists of two main proteins: actin and myosin. Striated muscle is so called because the regular arrangement of alternating actomyosin fibers gives it a striped appearance. This arrangement allows coordinated contraction of the whole muscle in response to neuronal stimulation through a voltage- and calcium-dependent process known as excitation–contraction coupling. The coupling enables the rapid and coordinated contraction required of skeletal muscles and the heart. Smooth muscle does not contain regular striations or undergo the same type of excitation– contraction coupling. Instead, it typically uses second messenger signaling to open intracellular channels that release the calcium ions that control the contractile apparatus (Kuo *et al*., 2015).

Smooth muscle contractility, and therefore hollow organ function, is regulated by changes in intracellular free calcium concentration. Increased intracellular free calcium concentration activates a protein calmodulin forming calcium-calmodulin complex. Calcium-calmodulin complex activates calcium-calmodulin-dependent myosin light chain kinase (MLCK) that causes phosphorylation of myosin light chains (Rhoades *et al*., 2013). Phosphorylation of myosin light chains results in an increase in myosin ATPase activity and, consequently, cross-bridge cycling and development of muscle tension (Horowitz *et al*., 1996).

There are two major mechanisms by which intracellular calcium concentration is raised in smooth muscle: (1) Entry of calcium ions from the extracellular space, and (2) Release of calcium ions from intracellular stores. Influx of extracellular Ca^{2+} is mediated by ion channels in the plasma membrane, the most prominent of which is the voltage-dependent Ca^{2+}channel (Rhoades *et al*., 2013).

Nonselective cation channels, such as transient receptor potential (TRP) channels and ionotropic purinergic (P2X) receptors, are also potentially important extracellular Ca^{2+}entry pathways in smooth muscle cells. Although a number of intracellular organelles take up and release Ca^{2+}, the sarcoplasmic reticulum (SR) represents the largest pool of releasable Ca^{2+}in smooth muscle cells (Hill-Eubanks *et al.,* 2011). Smooth muscle relaxation is brought about by mechanisms that lower cytoplasmic Ca^{2+}concentration. When the cytoplasmic Ca^{2+}concentration falls, MLCK activity is reduced because the calcium dissociates from the calcium-calmodulin complex. An enzyme called myosin light-chain phosphatase (MLCP) dephosphorylates myosin light chains. Because dephosphorylated myosin has a low affinity for actin, the reactions of the cross-bridge cycle can no longer take place and the muscle relaxes (Rhoades *et al.,* 2013). Calcium leaves the cytosol in two directions: A portion of it is returned to storage in the sarcoplasmic reticulum (SR) by sarcoplasmic reticulum Ca^{2+}- ATPase (SERCA) pump. The rest is ejected from the cell by activity of the sarcolemmal Ca^{2+}-ATPase, and by a $3Na^{+}/Ca^{2+}$ (Watras, 2007). The SERCA and sarcolemmal Ca^{2+}-ATPase are slow-acting in comparison with the fast-acting sarcoplasmic pump in skeletal muscle fiber resulting in a single smooth muscle contraction lasting longer (seconds) compared to hundredths to tenth of a second in skeletal muscle (Guyton *et al.,* 2006).

2.2 Regulation of gastrointestinal and uterine smooth muscle contraction

The contractile activity of smooth muscle is controlled by numerous factors that include hormones, autonomic nerves, pacemaker activity, and a variety of drugs. All the agents listed above cause smooth muscle contraction by increasing intracellular Ca^{2+} concentration. In contrast to skeletal or cardiac muscle, action potentials in smooth muscle are highly variable and not always needed to initiate contraction. Several agents can increase the intracellular Ca^{2+}concentration, and hence contract smooth muscle, without changing the membrane potential (Watras, 2007).

Smooth muscle in various organs in the body including gastrointestinal and uterus has an intrinsic property of spontaneous depolarization and contraction.

The presence of interstitial cells of Cajal (ICC) or ICC-like cells has been demonstrated in most visceral organs which generate spontaneous rhythmic muscle contractions, e.g. the urinary and genital system and these cells are believed to be the origin ('pacemaker') of electrical activity that cause spontaneous contractions (Allix *et al*.,2008).

The resting membrane potential of gastrointestinal and uterine smooth muscle like most other visceral smooth muscle is unstable and alternates between –20 to –65 mV. The pacemaker currents cause the cell to depolarize until membrane potential reaches threshold, triggering an action potential (Moczydlowski *et al*., 2012). Depolarization of smooth muscle opens voltage-gated Ca^{2+} channels in the sarcolemmal membrane allowing influx of calcium from extracellular fluid. The influx of Ca^{2+} causes an increase in intracellular Ca^{2+} concentration. Calcium ions that enters the smooth muscle cells through voltage-gated Ca^{2+} channels releases additional Ca^{2+} from the sarcoplasmic reticulum (Ca^{2+} induced Ca^{2+} release). The rise in intracellular Ca^{2+} concentration causes Ca^{2+} to bind to calmodulin. The Ca^{2+} -calmodulin complex binds to and activates myosin-light-chain kinase. When activated, myosin-light-chain kinase phosphorylates myosin light chain resulting in cross-bridge cycling and development of muscle tension (Costanzo, 2014).

Neural regulation of contraction of smooth muscle depends on the type of innervation and neurotransmitters released in the proximity of the nerves to the muscle cells, and the type and distribution of the neurotransmitter receptors on the muscle cell membranes. Generally smooth muscle is innervated by the autonomic nervous system (Watras, 2007). The most important neurotransmitter substances secreted by the autonomic nerves innervating smooth muscle are acetylcholine and noradrenaline. Acetylcholine is secreted by parasympathetic nerves and noradrenaline by sympathetic nerves. Acetylcholine and noradrenaline can be either excitatory or inhibitory depending on the receptor types present in target organ. Generally when acetylcholine excites a muscle fiber, noradrenaline ordinarily inhibits it and vice versa (Guyton *et al*., 2006). In the non-pregnant uterus α_1 adrenergic receptors predominate whereas in the pregnant uterus are β_2 receptors. The gastrointestinal smooth muscle has α_1, α_2 and β_2 receptors. Stimulation of uterine α_1 receptors causes contraction of non-pregnant uterus and stimulation of β_2 receptors causes relaxation of pregnant uterus (Tica *et al*., 2011).

Relaxation of gastrointestinal smooth muscle can be brought about by both α and β stimulation. Beta receptors are located directly on the smooth muscle cells and mediate relaxation via hyperpolarization and decreased spike activity. Alpha stimulation, especially α_2 cause gastrointestinal muscle relaxation indirectly by presynaptically reducing the release of acetylcholine and possibly other stimulants within the enteric nervous system.

Alpha$_1$ receptors are coupled to polyphosphoinositide hydrolysis and formation of inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG). The G proteins in the G_q family couple α_1 receptors to phospholipase C. IP_3 promotes the release of sequestered Ca^{2+} from intracellular stores, which increases the cytoplasmic concentration of free Ca^{2+} and the activation of various calcium-dependent protein kinases (Katzung, 2006). Alpha$_2$ receptors inhibit adenylyl cyclase activity and cause intracellular cyclic adenosine monophosphate (cAMP) levels to decrease. In addition to this effect, α_2 receptors utilize other signaling pathways, including opening of potassium channels allowing efflux of K+ and closing of calcium channels resulting in reduced influx of Ca^{2+}. Activation of β_2 receptors activates adenylyl cyclase and increased conversion of adenosine triphosphate (ATP) to cAMP. Although the mechanism of the smooth muscle effect is uncertain, it may involve the phosphorylation of myosin light-chain kinase to an inactive form that results in smooth muscle relaxation (Rang *et al*., 2012). The muscarinic receptors in the gastrointestinal tract are of the M_3 type (Tripathi, 2013). Muscarinic (M_3) receptors are also present in the myometrium (Tica *et al*., 2011). Muscarinic (M_3) receptors are coupled to Gαq and then to phospholipase C which release IP_3 and diacylglycerol (DAG) when activated. IP_3 and DAG triggers release of stored Ca^{2+} and activates protein kinase C (PKC). The net effect of these biochemical events is a coordinated regulation of K+, Ca^{2+}, and nonselective cation channels, which are ultimately coupled to oscillations in myoplasmic Ca^{2+} concentration. Increased myoplasmic Ca^{2+}activates the contractile proteins to generate force (Gerthoffer *et al*., 2005).

Gastrointestinal and uterine smooth muscle like other visceral smooth muscle contains many receptors that can be activated by hormones and many local factors that modulate its contractile function (Watras, 2007). Hormones and local factors that stimulate gastrointestinal smooth muscle contraction and motility include adrenaline, noradrenaline, tachykinins (such as substance P), and opioid peptides such as enkephalins and dynorphins. Most excitatory agents of

intestinal motility work by stimulating increases in cytosolic Ca^{2+}, and/or inhibiting the formation of cyclic nucleotides.

Inhibitory agents that stimulate increases in smooth muscle cell cAMP include vasoactive intestinal peptide (VIP), glucagon, and peptide histidine isoleucine (PHI) or peptide leucine methionine (PHM), in humans. Nitric oxide is inhibitory because it stimulates soluble guanylate cyclase and increased cellular cGMP levels. Inhibitory agents generally work by stimulating increases in cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP), secondary messengers that appear to counteract the effects of increased cytolic Ca^{2+} in several ways (Chang *et al*., 2014).

Both gastrointestinal and uterine smooth muscles have prostanoid receptors that mediate either contraction or relaxation. PGD_2 stimulates DP prostanoid receptors and cause relaxation of both gastrointestinal and uterine smooth muscle. $PGF_2\alpha$ acts on FP prostanoid receptors in the human uterus and cause myometrial contraction. PGE_2 stimulates EP_1 receptors and cause contraction of gastrointestinal smooth muscle but causes relaxation of gastrointestinal muscle on EP_2 receptors. EP_3 receptors are present on both gastrointestinal smooth muscle and the uterus and their stimulation by PGE_2 cause contraction of both the gastrointestinal and pregnant uterine smooth muscle (Rang et al., 2012).

Histamine receptors (H_1) are present both in the myometrium and gastrointestinal smooth muscle. Stimulation of H_1 receptors by histamine or its agonists cause contraction of ileum and uterine smooth muscle. Activation of H_1 receptors on smooth muscle cells and nerve endings results in an increase in the formation of inositol 1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG) resulting in an increase in intracellular calcium and muscle contraction (Rang *et al*., 2012). The guinea pig ileum and uterus are very sensitive and contracted by histamine whereas the uterus of a rat is relaxed. The human uterus is not as sensitive to histamine compared to other visceral smooth muscle (Tripathi, 2013).

The contractile response of gastrointestinal smooth muscle may be either enhanced or inhibited by six subtypes of 5-HT receptors. The stimulatory response can occur at the nerve endings on

both longitudinal and circular smooth muscles (5-HT_4 receptors), postsynaptic cells of the enteric ganglia (5-HT_3 and 5-HT_{1P} receptors) and directly and by direct effects of 5-HT on the smooth muscle cells (5-HT_{2A} receptors in intestine, 5-HT_{2B} receptors in stomach fundus).

In the oesophagus, 5-HT acts on 5-HT_3 receptors and cause either relaxation or contraction depending on the species (Brunton *et al*., 2011). 5-HT_4 receptors are also present in the myometrium and can cause contraction of myometrium when stimulated (Rang *et al*., 2012).

Oxytocin is a peptide hormone secreted by the posterior pituitary gland. It plays a central role in the mechanisms of parturition and lactation. The uterus has oxytocin receptors which belong to G-protein coupled receptor superfamily while Gq/phospholipase C (PLC)/inositol 1, 4, 5-triphosphate (IP_3) is the main pathway via which it causes contraction of the myometrium. When activated the oxytocin receptor causes contraction of the uterus by increasing intracellular calcium through IP_3, DAG, synthesis of prostaglandins and activation of voltage -gated calcium channels in the plasma membrane (Vrachnis *et al*., 2011)

Intracellular calcium concentration is raised by either entry of calcium from the extracellular space or from intracellular stores (sarcoplasmic reticulum) as illustrated in Figure 1.

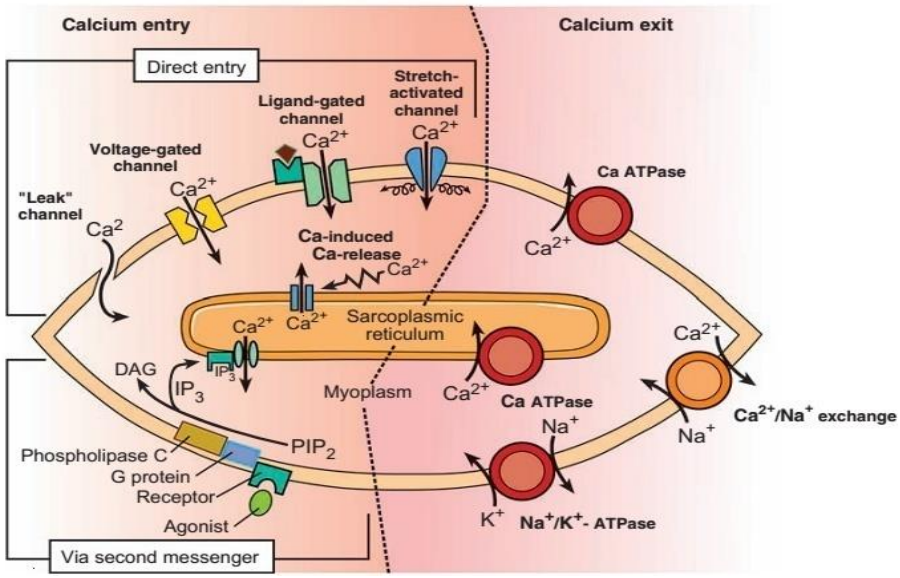


Figure 1: Major routes of calcium entry and exit from the cytoplasm of smooth muscle

Source: Rhoades *et al.*, 2013

Phosphorylation of myosin light chains by MLCK activates cross-bridge and causes muscle contraction whereas pumping of calcium from the cytosol and dephosphorylation of myosin light chains by MLCP deactivates the cross bridge and leads to muscle relaxation (Figure 2).

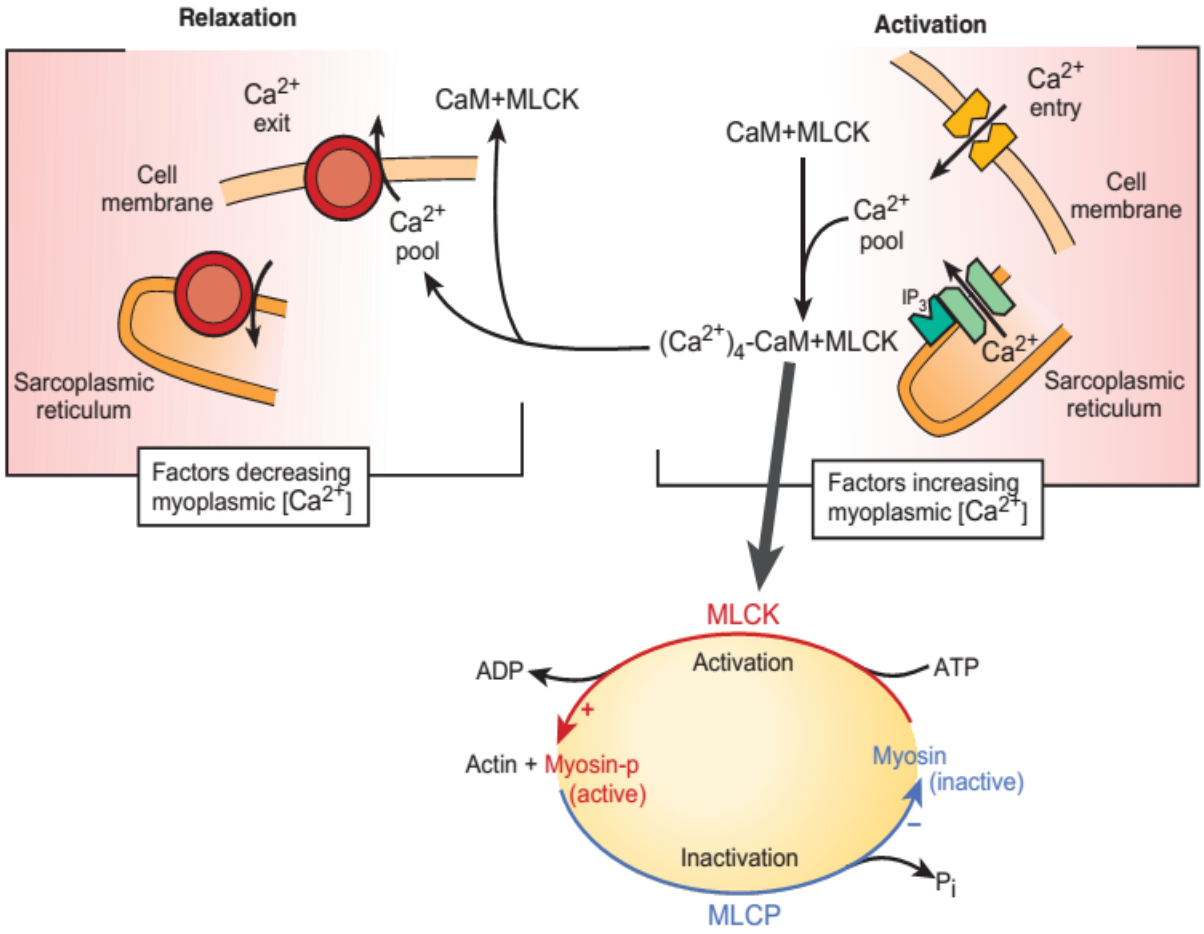


Figure 2: Reaction pathways involved in the regulation of the cross-bridge cycle in smooth muscle

A, actin; ADP, adenosine diphosphate; ATP, adenosine triphosphate; Pi, inorganic phosphate ion; CaM, Calmodulin; MLCK, Myosin light-chain kinase; MLCP, Myosin light chain phosphatase.

Source: Rhoades *et al.*, 2013

2.3 The plant *Steganotaenia Araliacea*

S. Araliacea is a small savannah tree 2-7 meters tall. It is characterized by a yellow-green or grey, rather waxy barks that peel off in papery strips or rectangles. Leaves are pinnate and crowded towards branch ends with 2-3 pairs of leaflets on a 10cm long leaf stalk that has an expanded base around the stem. It has small, green-white flowers, in rounded compound clusters at twig ends. About 3 - 7 long stalks arise together; each further bears a crown of small heads about 8 cm across. Stamens are longer than petals in male flowers. Its fruits are cream-brown, dehiscent, and flat and heart shaped. The generic name is based on Greek '*stegnas*' meaning covered and the Latin '*taenia*' meaning band. It has many Zambian local names such as *fyopola* (Chewa), *Mutobolo* (Tonga) and *Mumono yakumapili* (Luvale) (Lwiindi *et al*., 2015).

S. Araliacea occurs over a wide range of altitude, but is abundant in low-altitude woodland or on rocky outcrops. It is native to Angola, Benin, Botswana, Democratic Republic of Congo, Ethiopia, Kenya, Mozambique, Namibia, Somalia, South Africa, Sudan, Tanzania, Togo, Uganda, Zambia, and Zimbabwe (Orwa *et al*., 2009).

Phytochemical analysis of the methanol extract of the seeds of *S. Araliacae*revealed that alkaloids, flavonoids, tannins, steroids, saponins, phenols, cardenolides and coumarines are the main constituents of the extract (Demoz *et al*., 2014).

SAE[c] has been used traditionally to treat many health related conditions. The roots have been used to treat snake bites, sore throats and painful chest conditions. The tree trunk was reported to have snake deterring activity and leaves are rubbed on wounds as general disinfectant. Twigs are used in dental care as toothbrushes while bark is used as a medication for heart complications (Alemika *et al*., 2004). The bark is chewed to treat fever and when prepared by boiling for one hour, it is added to milk and administered orally to adults as a remedy for stomachache/dysentery (Orwa *et al*., 2009). Whole plant material is also used as remedy for gas in stomach. Its stem barks extract is used to quicken labor and delivery process (Maluma *et al*., 2017). The cold water extract of SAE has been demonstrated to cause significant contractions of the rat uterus (Goma *et al*., 2017).

Ethanol extract of the stem bark of *S. Araliacae* has been shown to cause relaxation of isolated rabbit jejunum in a concentration-dependent manner and significantly attenuates histamine induced contractions of the isolated guinea pig ileum (Alemika *et al*., 2004).

Although SAE^{c} has been shown to increase contractility of isolated rat uterine smooth and relax rabbit jejunum and guinea pig ileum, its effects on the function of the rat gastrointestinal system and its mechanism of action is still not clear. More work need to be done in order to document its mechanism of action, side-effects and toxic effects that could arise during its clinical use.

CHAPTER THREE

METHODOLOGY

3.1 Collection and authentication of plant materials

The fresh plant with leaves and roots of *S. Araliacea* were collected from traditional birth attendants and some other knowledgeable local people in Chongwe and Katuba townships of Lusaka province of Zambia. Some plant materials were also collected from Chikankata district of Southern province. Identification and authentication of the plant was done at The University of Zambia (UNZA), School of Natural Sciences, in the herbarium section where the sample was assigned voucher number of LL2.

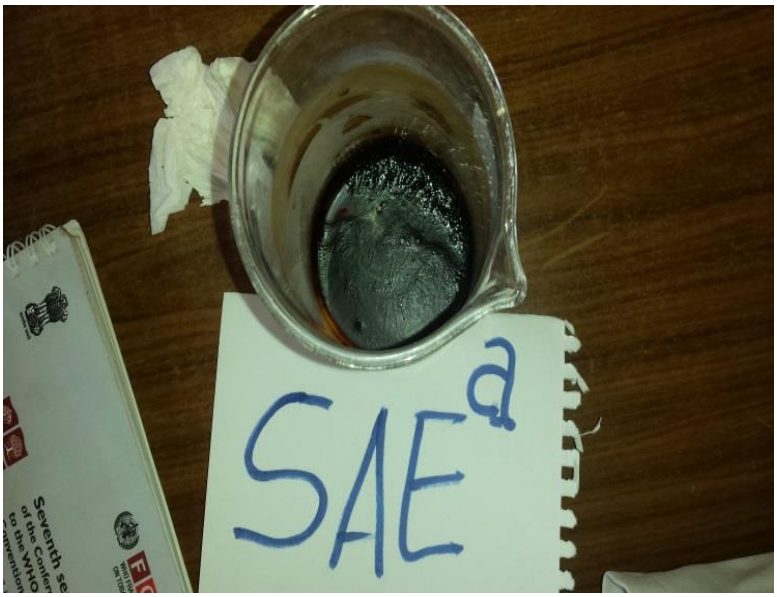
3.2 Preparation of the cold water extract of *S. Araliacea*

Fresh bark roots of *S. Araliacea* were collected and washed to remove adulterants and soil particles (Figure 3a). The barks were chopped into bits and dried in the shade for 14 days (Figure 3b). The dried root barks were further reduced to powder by pounding with mortar and pestle (Figure 3c). A 362.67g quantity of powder was weighed using an electronic balance (Model: BPS-1000-C_2-V_2, Serial No.: 453040/15, Manufacturer: MRC. Ltd) and soaked in 1800ml of distilled water for 24 hours. The mixture was sieved and squeezed through a mutton cloth to get rid of large particles. The infusion was then centrifuged at 2000 rev/min for 10 minute using a centrifuge (MFG No.:99698, CAT No.: 001472, Manufacturer: Hitachi kokico.Ltd). The supernatant was decanted and filtered using Whitman filter paper size No.1 to obtain a brown filtrate. The filtrate was concentrated to a volume of about 400ml using a laboratory hot plate (Model No: 13474, Manufacturer: Ikemoto Rikakogyo co.Ltd) and later in a hot air drying oven (Model: DG-81, Manufacturer: Yamato co. Ltd, Japan) at 60°C to complete dryness until a constant weight (27.22g) of a brown semi-solid extract was obtained and named *S. Araliacea* cold water extract (SAE^c) as shown in figure 3d. The percentage yield was 7.51% [(27.22g/362.67g) X 100%].

a) *S. araliacae* **fresh roots**

b) **Shade - dried bark roots of** *S. araliacae*

c) *S. araliacae bark r*oot powder



d) **Dark-brown semi solid extract (SAE^{c})**

Figure 3: Steps of *S. Araliacae* extract (SAE^{c}) preparation.

3.3 Experimental animals

Eight (8) female gravid albino rats of Wistar strain (*Rottus norvegicus*) weighing 180 -250 grams were selected and housed in the animal unit of the Department of Biomedical sciences, School of Medicine, University of Zambia. The animals were maintained according to standard nutritional and environmental conditions, had free access to standard feed (Bendel Feeds and Flour Mill) and water. Animal studies were conducted 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).

3.4 Drugs

Acetylcholine ($1.65x10^{-3}$M) was used as reference agonist drug with atropine sulphate ($6 x 10^{-2}$µg/ml) as its antagonist. Nifedipine ($2.9 x10^{-4}$M), Indomethacine (1.2mg/ml), mepyramine (100µg/ml) and ondasentron (136 mM) were used as antagonists to investigate the mechanism of action of SAE^{c}

3.5 Isolation and mounting of ileum segments

Animals were humanely sacrificed by a quick neck dislocation. An abdominal incision was made to expose the visceral organs and the ileum was identified. The ileum was dissected out and transferred into a beaker containing Tyrode solution at 37^0C. Intestinal contents were removed by flushing with Tyrode solution and the ileum from each rat was cut into 3 segments of 2 - 3 cm long. To each isolated ileal segment, a thread was tied fixed to a lever system fitted on the isometric transducer (Make: MLT 0210/A, Serial No.: 1003011, Manufacturer: Panlab, S.L, Spain) while the other end was fixed to a hook. The tissue was transferred and mounted in a 25ml organ bath containing Tyrode solution maintained at 37^0C and aerated with ordinary air with Sonic aquarium air pump (Model No: 9905). An initial resting tension of 1g (10mN) was applied to the mounted segments. The tissues were left to stabilize in the organ bath for 60 minutes before any drug was administered while the physiological Tyrode solution was replaced with fresh one every 15 minutes.

3.6 Effects of acetylcholine on contractile response of isolated rat ileum

At the end of the equilibration period stated above, spontaneous contractions of the isolated ileum segments from the three rats (one segment from each rat) were recorded. Thereafter acetylcholine of varying final bath concentrations ranging from 1.817 $x10^{-6}$mg/ml to 1.817 x 10^{-3}mg/ml was added in each organ bath containing one of the three ileum segments with three tissue washes between additions. The contractions caused by addition of each dose of acetylcholine were recorded on the Lab Tutor for 2 minutes. At the end of the wash period, tissues were allowed to equilibrate for 15 min before adding the next dose. Acetylcholine was used as a positive control and to confirm the viability of tissue preparation during the experiments.

3.7 Effects of SAE^{c} on contractile response of isolated rat ileum

To carry out this experiment, the ileum segment preparations that we had used to study effects of acetylcholine in the above experiment were used again. Tissues were washed three times each at the end of acetylcholine challenge and allowed 30 minutes to recover and return to baseline. After equilibration, SAE^{c} of varying final bath concentrations ranging from a dose one-half lower than the minimum dose that caused tissue response (1mg/ml) was added and tissue responses were recorded. The subsequent tissue responses were recorded after doubling every previous dose until maximal tissue response was recorded at 64mg/ml of SAE^{c}. Tissue responses to each dose of SAE^{c} were recorded on a Lab Tutor and compared with those for acetylcholine.

3.7.1 Drug administration

The starting dosage for acetylcholine and SAE were computed using the dilution method below;

$Concentration_1$ x $Volume_1$ = $Concentration_2$ x $Volume_2$, Where;

$Concentration_1$ = concentration of stock solution

$Volume_1$ = Volume of stock drug solution to be added to organ bath

$Concentration_2$ = Final bath concentration of drug

$Volume_2$ = Volume of the organ bath (25ml)

The starting dosage (C_2) above was archived by rearranging the dilution equation, making V_1 subject of the formula. Recordings were done for 2 minutes after addition of the required

starting dosage. Thereafter tissues were washed three (3) times and allowed appropriate recovery time (return to baseline) before a new higher dose was added. The second and subsequent doses (final bath concentrations) were computed by simply doubling the previous dose until no further change in isometric tension were recorded with any further increase in the dosage

3.8 Determination of the mechanism of action for SAE^c

In this experiment fifteen (15) isolated rat ileum segments from five (5) rats (three segments per rat) were used. The effects of atropine (4.8mg x 10^{-4}mg/ml)), indomethacin (0.036mg/ml), nifedipine ($2.9x10^{-4}$M)**,** mepyramine (1µg/ml) and Ondansetron (1µM) on the contractile activity of SAE^c were investigated in an attempt to establish the possible mechanism(s) of action of the plant extracts (SAE^a) from the observed response(s) as follows;

i. After stabilization of the isolated tissues in the organ bath, a dose of SAE^c that produced 50 % (EC50) change in isometric tension was tested on three isolated ileum tissues each from a different rat. Tissues were washed three times and allowed to return to the baseline each time before introducing a new dose. EC50 value was determined from the dose-response relationship curve obtained from the above experiment
ii. The procedure from above (i) was repeated but with incubation of a known antagonist (i.e. atropine, indomethacin, nifedipine, mepyramine and ondasentron) in the organ bath for 5 minute prior to addition of a dose of SAE^c (EC50 dose). The tissue responses were compared to those of acetylcholine in the presence atropine.

3.9 Statistical analysis

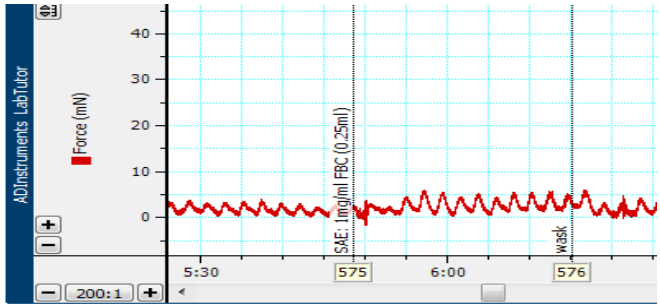
All values were expressed as the mean ± SEM (standard error of the mean) and **n** represents the number of rats from which ileum segments were obtained. Graph.Pad Prism version 5 statistical package was used for statistical analysis. The levels of significance were made using one-way ANOVA with Bonferroni's Multiple Comparison Test for comparison of means between groups. A value of $P < 0.05$ was considered significant in all cases

CHAPTER FOUR

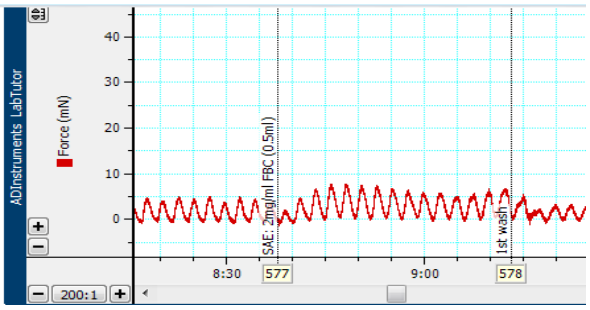
RESULTS

4.1 Effects of SAE^{c} on isolated rat ileum preparations

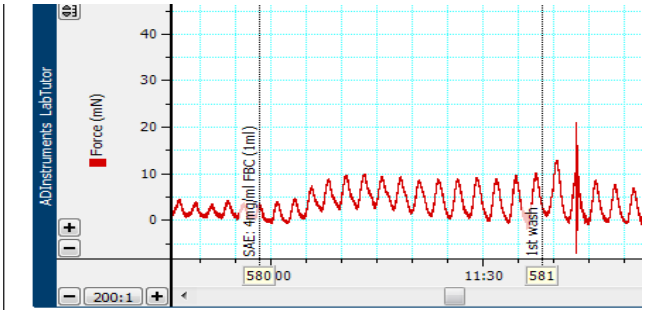
An increase in the dose of SAE^{c} from 1mg/ml to 32mg/ml resulted in a proportional increase in tissue tension as shown on the tracing in Figure 4.



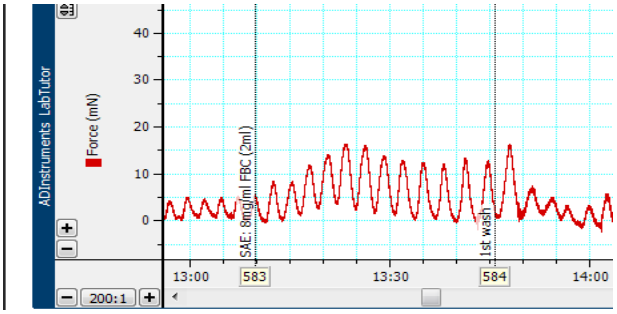
a) SAE^{c} at 1mg/ml



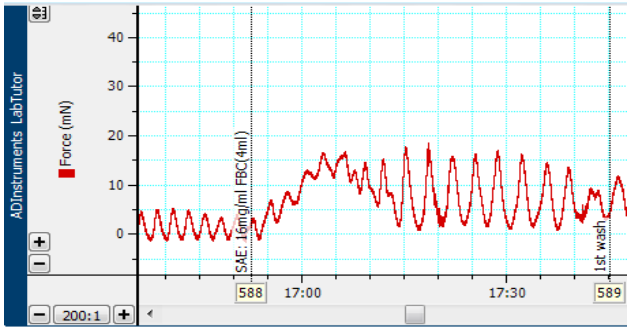
b) SAE^{c} at 2mg/ml



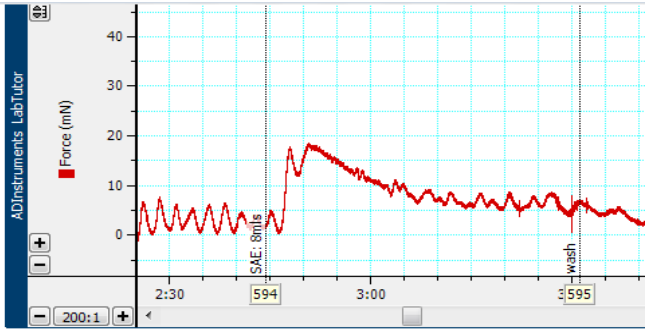
c) SAE^{c} at 4mg/ml



d) SAE^{c} at 8mg/ml



e) SAE^{c} at 16mg/ml



f) SAE^{c} at 32mg/ml

Fig 4: Tracing showing tissue response (tension) of rat ileum muscle following administration of various doses of SAE^{c} .

Increase in concentration of SAE^c form 1mg/ml to 32 mg/ml resulted in a proportional increase in tissue response. However, doses of SAE^c higher than 32mg/ml did not result in any further increase in tissue response (Table 1) implying that all the available receptors for SAE^c were saturated at 32mg/ml.

Table 1: Effects of non-cumulative concentrations of SAE^c on contractility of the isolated rat ileum (n=3)

Bath concentration of SAE^c(mg/ml)	Mean response (mN)
0	4.92
1	7.43
2	8.90
4	10.47
8	15.63
16	17.13
32	19.81
64	19.21

4.1.2 Acetylcholine and SAE^c Log dose –response curves

A plot of Log-dose of SAE^c and Ach against maximal tissue response showed a dose-response relationship. The curves were sigmoidal in shape, typical of drug-receptor interaction as illustrated in Figure 5. The curves also show that Ach was more potent than SAE^c.

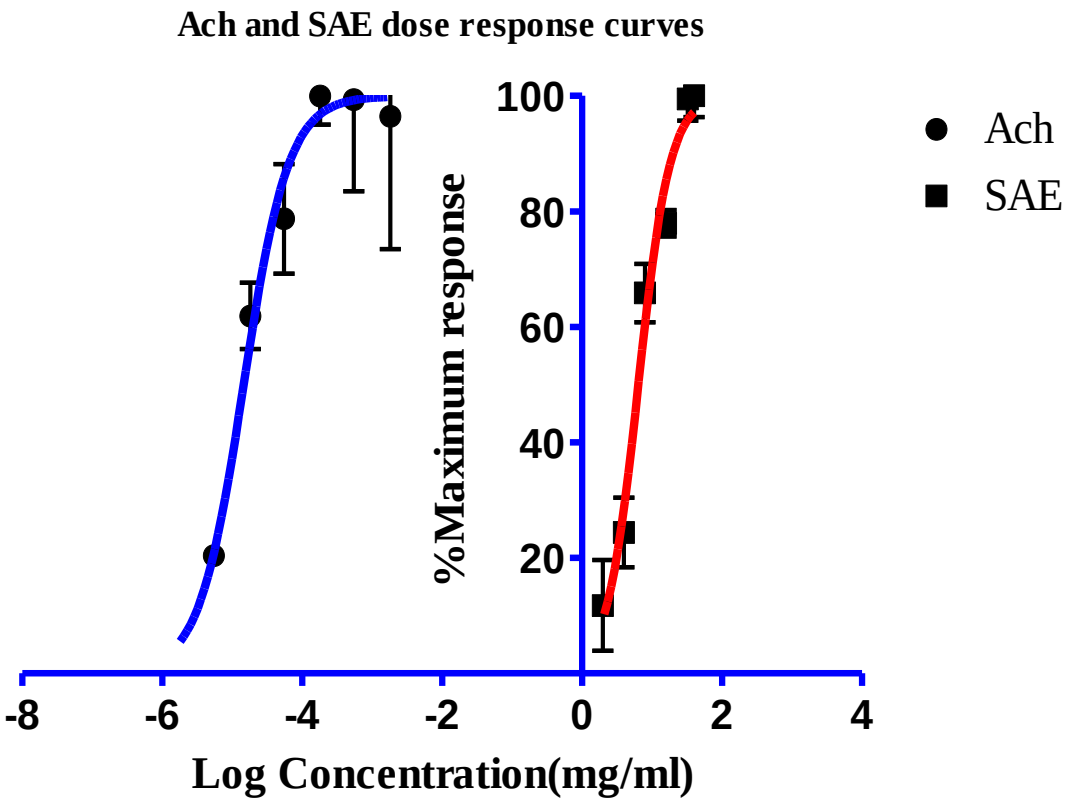


Figure 5: Dose - response curve for SAE and Ach. Each point is the mean ± sem (n=3).

EC50 values for Ach and SAE^{c} shown in Table 2 were obtained from dose response curves above and were used to determine the mechanism of action of SAE^{c}.

Table 2: EC50 values of Ach and SAE^{c} on the contractile response isolated rat ileum

Stimulating drug	Ach	SAE^{c}
EC50 mg/ml	5×10^{-5}	8

4.2 Effects of atropine on the contractile response of isolated rat ileum produced by SAE^{c} and Ach

Atropine physiologically inhibited the ileum contractions produced by acetylcholine by about 55% ($p<0.05$) from average of 11.642mN to 5.264mN. The same dose of atropine did no inhibit the contractions caused by SAE^{c} ($p>0.05$) (Table 3 and Figure 6).

Table 3: Contractile responses of rat ileum to Ach and SAE^{c} in the presence and absence of atropine

Rat	Baseline	AT alone	Ach alone	SAE alone	Ach + AT	SAE + AT
1	5.419	5.419	11.89	12.73	4.725	14.439
2	4.676	4.679	11.501	14.389	6.034	14.958
3	6.5041	6.508	11.534	13.836	5.034	14.286
Mean tension (mN)	**5.533**	**5.535**	**11.642**	**13.652**	**5.264**	**14.561**

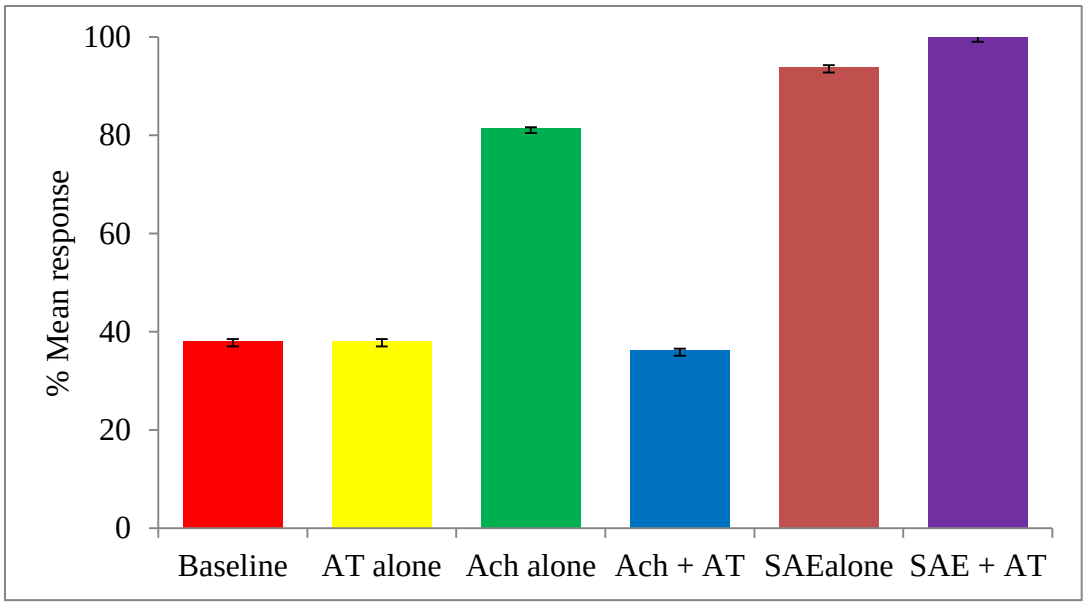


Figure 6: Effects of atropine (4.8mg x 10^{-4}mg/ml) on responses produced by Ach (5 x 10^{-5} mg/ml and SAE^{c} (8mg/ml) on isolated rat ileum. Each column represents the mean ± sem (n = 3 rats).

4.3 Effects of indomethacin on the contractile response of isolated rat ileum produced by SAE^{c} and Ach

Indomethacin did not inhibit the ileum contractions caused by either SAE^{c} or Acetylcholine ($p>0.05$) as shown in Table 4 and Figure 7.

Table 4: Contractile responses of the rat ileum to Ach and SAE^{c} in the presence and absence of indomethacin

Rat	Baseline	IND alone	Ach alone	SAE alone	Ach + IND	SAE + IND
1	7.057	5.88	11.89	12.73	11.632	13.517
2	6.194	5.593	11.501	14.389	11.783	13.541
3	7.188	5.107	11.534	13.836	11.457	13.129
Mean response (mN)	**6.813**	**5.527**	**11.642**	**13.652**	**11.624**	**13.396**

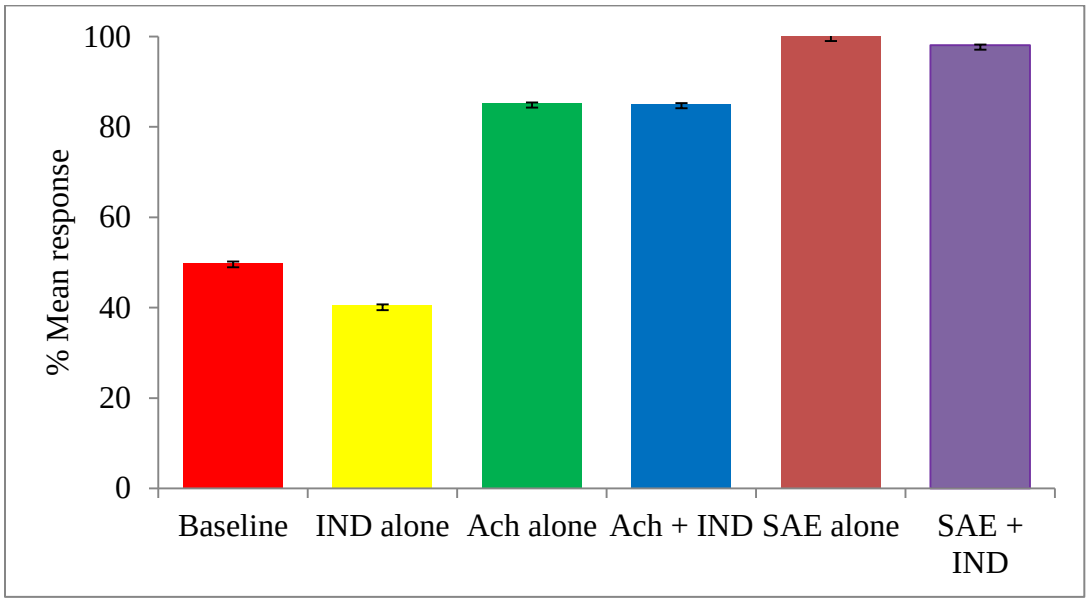


Figure 7: Effects of indomethacin (0.036mg/ml) on responses produced by acetylcholine (5×10^{-5} mg/ml and SAE (16mg/ml). Each column represents the mean ± sem (n = 3 rats).

4.4 Effects of nifedipine on the contractile response produced by SAE^{c} and Ach

Nifedipine inhibited the contractions caused by both acetylcholine and SAE^{c} by 100% ($p < 0.05$) from 11.642mN to 0mN for acetylcholine and from 13.652mN to 0mn for SAE^{c} (Table 5 and Figure 8). This observation indicated involvement of calcium channels in the contractile response produced by both Ach and SAE^{c}.

Table 5: Contractile responses of rat ileum to Ach and SAE^{c} in the presence and absence of nifedipine

Rat	Baseline	NF alone	Ach alone	SAE alone	Ach+ NF	SAE + NF
1	4.186	0	11.89	12.73	0	0
2	5.565	0	11.501	14.389	0	0
3	4.834	0	11.534	13.836	0	0
Mean response (mN)	**4.862**	**0.000**	**11.642**	**13.652**	**0.000**	**0.000**

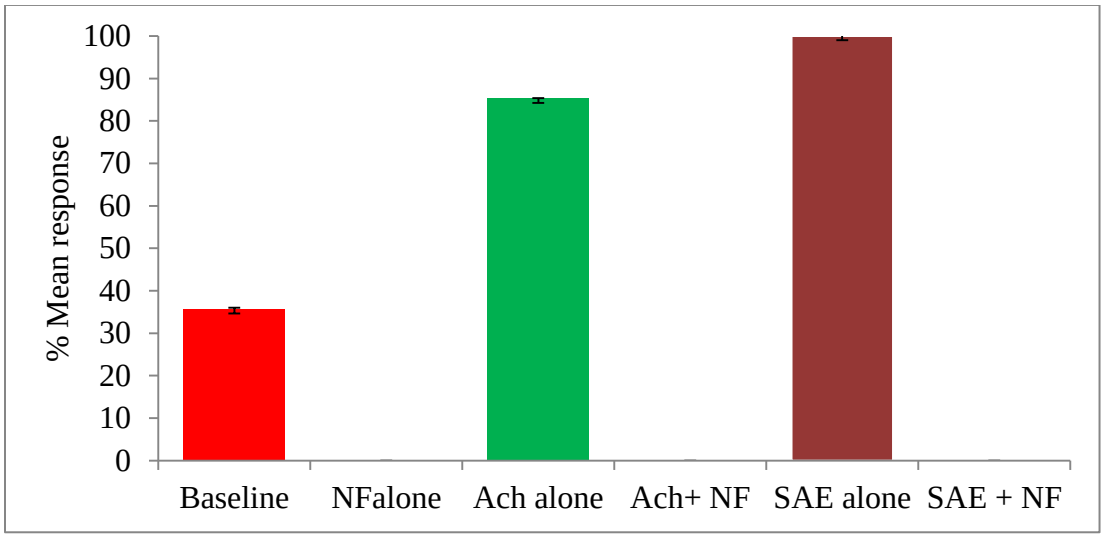


Figure 8: Effects of nifedipine (2.9×10^{-4}M) on responses produced by Ach (5×10^{-5} mg/ml and SAE^{c} (8mg/ml) on isolated rat ileum. Each column represents the mean ± sem (n = 3 rats).

4.5 Effects of mepyramine on the contractile response produced by and SAE^c and Ach

Mepyramine (1µg/ml) did not inhibit the contractions caused by either acetylcholine or SAE^c ($p>0.05$) indicating none involvement of H_1 receptors in the contractile response produced by Ach and SAE^c (Table 6 and Figure 9).

Table 6: Contractile responses of rat ileum to Ach and SAE^c in the presence and absence of mepyramine

Rat	Baseline	MPN alone	Ach alone	SAE alone	Ach+ MPN	SAE+ MPN
1	4.186	6.39	11.89	12.73	12.087	14.964
2	5.565	5.543	11.501	14.389	11.675	13.342
3	4.83	5.731	11.534	13.836	11.945	13.734
Mean	**4.860**	**5.888**	**11.642**	**13.652**	**11.902**	**14.013**

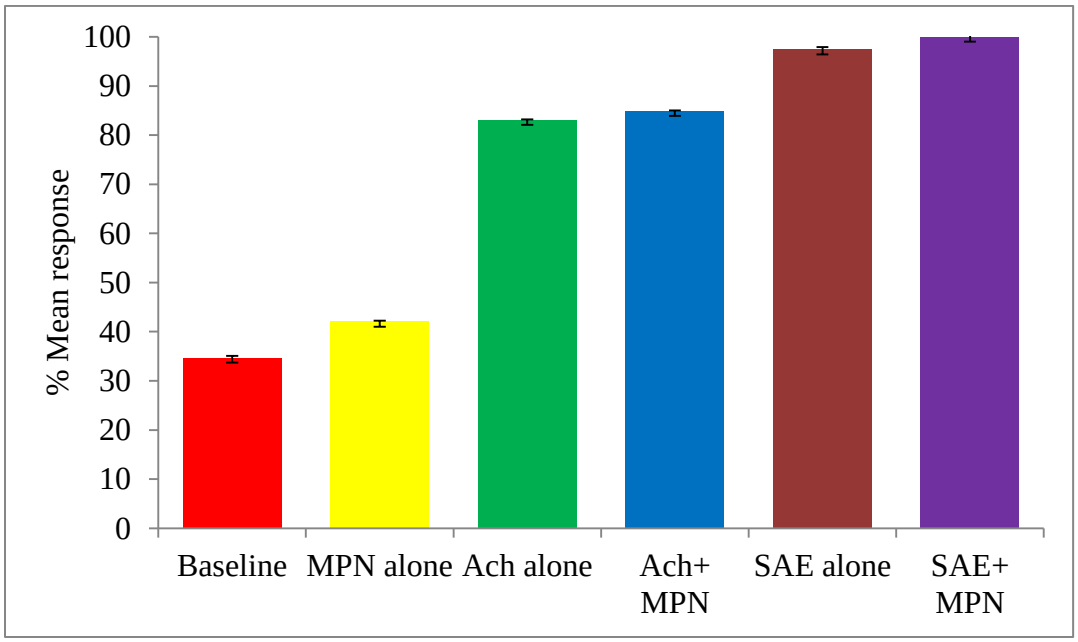


Figure 9: Effects of mepyramine (1µg/ml) on responses produced by Ach (5×10^{-5} mg/ml and SAE (8mg/ml) on isolated rat ileum. Each column represents the mean ± sem (n = 3 rats).

4.6 Effects of ondansetron on the contractile response produced by and SAE^c and Ach

Ondansetron (1μM) did not inhibit the contractions caused by either acetylcholine or SAE^c ($p>0.05$) indicating none involvement of 5-HT_3 receptors in the contractile response produced by Ach and SAE^c (Table 7 and Figure 10).

Table 7: Contractile responses of rat ileum to Ach and SAE^c in the presence and absence of ondansetron

Rat	Baseline	OND alone	Ach alone	SAE alone	OND + Ach	OND + SAE
1	6.681	6.076	11.89	12.73	11.976	13.054
2	7.268	6.063	11.501	14.389	11.362	14.873
3	6.518	7.617	11.534	13.836	11.067	13.065
Mean response(mN)	**6.822**	**6.585**	**11.642**	**13.652**	**11.468**	**13.664**

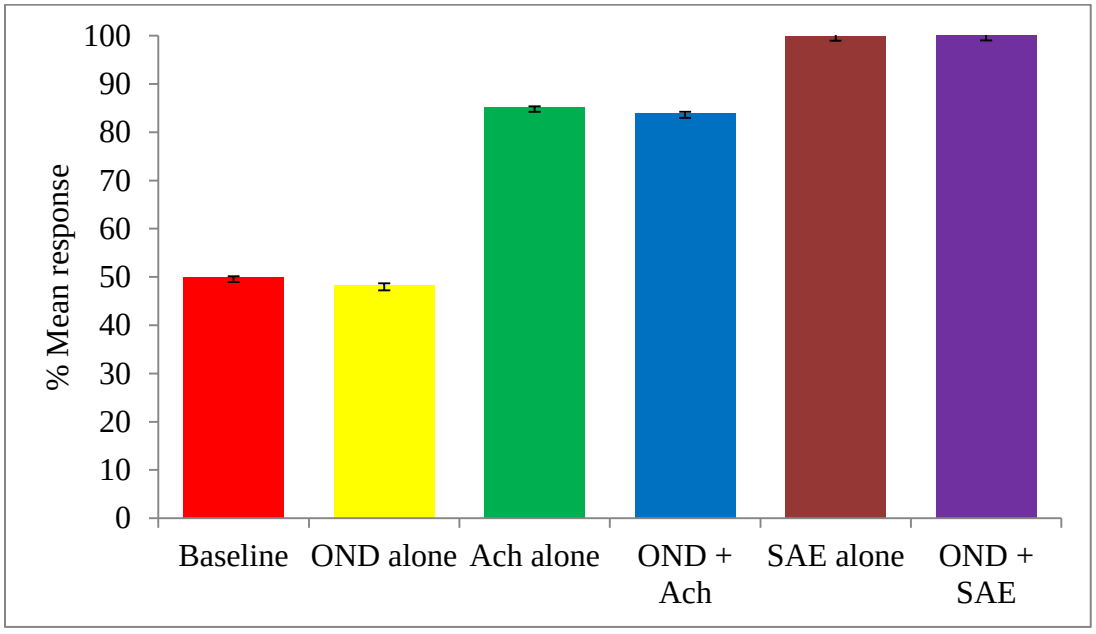


Figure 10: Effects of Ondansetron (1μM) on responses produced by Ach (5×10^{-5} mg/ml and SAE (8mg/ml) on isolated rat ileum. Each column represents the mean ± sem (n = 3 rats).

CHAPTER FIVE

DISCUSSION

5.1 Effects of hormones and neurotransmitters on ileum function

The small intestine is the site where most digestion and absorption of nutrients take place (Berne *et al*., 2007). The ileum is special because it is site where absorption of vitamin B_{12} and bile acids take place (Rhoades *et al*., 2013). It secretes large quantities of bicarbonate ions that neutralize the acids produced by bacteria in the large intestine (Guyton *et al*., 2006). Literature has documented that vitamin B_{12} deficiency is common during pregnancy and is associated with adverse outcomes for the mother and infant (Finkelstein *et al*., 2015). The motor (contractile) activity of the intestine affects the process of digestion and absorption of nutrients. It enables mixing of chyme with digestive secretions, expose it to absorptive surface and propel it towards the aboral direction (Boeckxstaens *et al*., 2016). Disorders of motility and contractility can impair the intestinal function and can result in diarrhoea, abdominal cramps, and constipation and nutrient deficiencies due to malabsorption (Hasler *et al.*, 2015). There are many chemical substances that affect the motor activity of the small intestine and hence digestion and absorption of nutrients. They include hormones, neurotransmitters, paracrines, and drugs (Rang *et al*., 2012). The extract of *S. Araliacae* was demonstrated by a recent study to increase the contractility of the rat uterus (Goma *et al*., 2017). The contractile mechanism and regulation of uterine and intestinal smooth muscle is similar in many respects (Berne *et al*., 2007). It is possible that the same extract can affect the contractile activity of the ileum and therefore alter its function because. It was therefore important for us to investigate whether or not the extract of *S. Araliacae* has an effect on the contractile function of the ileum to predict its side effects and safety when taken for the purpose of induction and facilitation of labour.

5.2 Effects of SAE^c on contractility of rat ileum and uterine smooth muscle

Our findings showed that SAE^c increased contractility of rat ileum smooth muscle in a dose-response manner. This observation opposed the findings of a previous study where lower doses (0.1mg/ml to 3.2mg/ml) ethanol extract of SAE caused relaxation of the rabbit jejunum in a dose

effect relationship (Alemika *et al*., 2004). The discrepancy between the findings of that study and ours could be multiple. To begin with, the dosage of SAE that was used in that study was much lower (0.1mg/ml-3.2mg/ml) compared to a much higher dose that we used in our study (1mg/ml-64mg/ml). The extraction method and solvents that they used was different (ethanol extraction) compared to ours (cold water extraction). Species variation may also have resulted in this discrepancy because isolated guinea pig ileum instead of the rat ileum was used for their study. A recent study revealed that very low doses of a cold water extract of SAE (0.016mg/ml – 2.048mg/ml) increase contractility and motility of rat uterus smooth muscle in a dose response manner (Goma *et al*., 2017). This finding may indicate that SAE is more potent on the isolated rat uterus than it is on the isolated rat ileum smooth muscle because a much lower dose achieved maximum tissue response on uterus than the ileum smooth muscle. This could be that the uterus is much more sensitive to SAE^{c} than the ileum. The significant differences in the minimum dose that produces ileum and uterine smooth muscle contractions as observed by our study and uterine smooth muscle study may justify the absence of abdominal and gastrointestinal associated complaints in women who take the herb.

5.3 Rat ileum receptors and demonstration of mechanism of action of SAE^{c}

Physiological pathways controlling smooth muscle contraction involve increasing intracellular calcium concentration (Watras, 2007). Calcium entry into smooth muscle cells may be through any or a combination of all of the following ways; voltage-gated calcium channels in response to cell depolarization, calcium release from sarcoplasmic reticulum or entry of calcium from voltage independent channels (Moczydlowski *et al*., 2012). These calcium channels could be opened by stimulation of one or several receptors that include histaminergic (H_1) receptors (Izzo *et al.*, 1998), serotonergic (5-HT_3) receptors (Thompson *et al*., 2006), muscarinic (M_2 and M_3) receptors (Gerthoffer, 2005) and activation of L-type calcium channels by depolarization of the plasma membrane (Rhoades *et al*.,2013). The observed increase in contractility of rat ileum smooth muscle caused by SAE^{c} could be through activation of one or more of the pathways involved in ileum smooth muscle contraction. One aim of our study was to find the probable mechanism by which SAE^{c} caused the observed dose-response relationship on contractility of isolated rat ileum smooth muscle. This was done by pretreating the tissue with atropine, a

muscarinic receptor antagonist (Katzung *et al*., 2006), nifedipine, an L-type calcium channel blocker (Wrzos *et al*., 2004), indomethacin, a prostaglandin synthetase inhibitor (Mard *et al*., 2011), mepramine, an H_1 receptor antagonist (Rang *et al*., 2012) and ondansetron, an 5-HT_3 antagonist (Tripathi, 2013). The ileal contractions caused by SAE^c were not inhibited by atropine, indomethacin, ondansetron or mepyramine. The failure by any of these antagonists to inhibit the SAE^c induced contractions of the isolated rat ileum ($p>0.05$) confirmed none involvement of M_3, H_1, 5-HT_3 and prostanoid receptors (DP, FP, EP_1 and EP_3) in pathways mediating SAE^c - induced contractions. A study carried by Alemika, Onawunmi and Olugbade (2004) associated the relaxant effect of SAE saponin mixture on guinea pig ileum with its inhibitory effect via (H_1) receptors because SAE attenuated histamine induced contractions of guinea pig ileum by about 93% at a dose of 0.8mg/ml. However our findings did not show an association between H_1 receptor mediated contractions with those caused by SAE because blockade of these receptors by mepyramine did not inhibit SAE^c-induced contractions of the isolated rat ileum. One reason for our failure to demonstrate involvement of H_1 receptors involvement could be due to species variation. Guinea pig ileum smooth muscle is documented to be much more sensitive to histamine while that of the rat is much less sensitive (Rang *et al*., 2012). Another recent study on isolated rat uterus linked SAE induced contractions of uterine smooth muscle with oxytocin–like receptors (Lwiindi *et al*., 2014). In that study involvement of oxytocin induced uterine contractions were inhibited (44% inhibition) by salbutamol, a β_2 adrenoceptor agonist. Salbutamol is not an oxytocin receptor specific antagonist although it can physiologically inhibit the contractions caused by oxytocin though not as effective as atosiban, an oxytocin receptor specific antagonist (Tripathi, 2014). While it is possible that SAE^c-induced contractions of the isolated rat uterine muscle were mediated by oxytocin-like receptors, this observation did not confirm the mechanism of action of SAE^c on the rat uterus because a receptor specific antagonist was not used. Our findings regarding the mechanism of action of SAE^c on smooth muscle is somewhat contrary to the study on the uterus in that SAE^c was able to induce contractions of isolated rat ileum where oxytocin receptors are not expressed (Kimura *et al*., and 2003).

Pretreating the tissue with nifedipine inhibited the SAE^c induced contractions of isolated rat ileum by SAE^c by 100%. This observation suggested that SAE^c-induced contractions of isolated

rat ileum involved activation of a pathway that increases influx of Ca^{2+}through L-type calcium channels. Activation of L-type calcium channels could be through direct binding of SAE^{c} on these L- type Ca^{2+} channels, non-selective cation channels, purinergic receptors or activation of calcium channels through G-protein coupled receptors on the sarcolemma (Hill-Eubanks *et al*., 2011).

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

It can be concluded that SAE^c causes contractions of isolated rat ileum smooth muscle in a dose-response manner by probable activation of calcium channels.It is possible that SAE^c if used in high doses may cause diarrhoea or severe abdominal cramps which may be controlled by nifedipine.

6.2 Recommendations

We recommend using more specific receptor antagonist in future studies of contractile effect of SAE^c on smooth muscle to clearly demonstrate its mechanism of action. From our observations and the review of some previous studies, it is worth noting that herbal medicines may have multiple effects on function of body systems depending on the dose and toxicity of the herbal extract, so further works need to be done.

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APPENDICES

Appendix 1: Composition of Tyrode's Physiological solution

The following composition of Tyrode's Physiological solution was used to maintain the isolated rat ileum preparation

Ingredient	Quantity (g/5 liters of distilled water)
Sodium Chloride(NaCl)	40.0
Potassium Chloride(KCl)	1.0
Magnesium Chloride ($MgCl_2.\ 6H_2O$)	0.5
Sodium hydrogen carbonate($NaHCO_3$)	5.0
Sodium hydrogen phosphate (NaH_2PO_4)	0.25
Calcium Chloride ($CaCl_2.2H_2O$)	1.32
D-Glucose($C_6H_{12}O_6$)	5.0
Aerating gas	Ordinary air

Appendix 2: Apparatus

Power lab 26T

- Model: ML 856
- Manufacturer: PanLab, S.L, Spain for ADINSTRUMENTS

Bridge pod

- model : ml 301
- manufacturer: Adistruments, Australia

Teaching force transducer

- model: mlt 0210/a
- serial no. 1003011
- manufacturer: panlab, s.l, Spain, for Adistruments

Ugo basile (4050 two chamber isolated organ bath)

- Biological Research apparatus, 21025 Cornella (BGN), Italy

Aquarium air pump-sonic

- Model: 9905
- AC 230V, 50Hz, 2.9W

Laboratory hot plate

- Model No: 13474
- Manufacturer: Ikemoto Rikakogyo co.Ltd

Laboratory ware drying oven

- Model: No. DG-81
- Serial No: 206002
- Manufacturer: Manufacturer: Yamato Scientific Co. Ltd, Japan

Hitachi centrifuge

- Type: 05p-21
- MFG No: 99698
- CAT No: 001472
- Hitachi Koki Co. Ltd

Electronic balance

Model: BPS-1000-C_2-V_2

Serial No. 453040/15

Manufacturer: MRC. LTD

Appendix 3: Drugs

Acetylcholine chloride

Wako pure chemical industries LTD

Atropine sulphate

Fresenius kabi manufacturing SA (Pty) LTD

12-073/05-13

Lot: 90KDO93

Nifedipine

Licence No. 192/031

Macleods Pharmaceutical LTD, Mumbai-400 059, India

Indomethacin

Reg. No. 022/017

Code: MH/DRUGS/PD/215

JMB Pharmaceuticals P.L, Mumbai-400 080, India

Pyrilamine maleate

Sigma Chemical Company, ST.LOUIS, MO.63178, U.S.A

Ondansetron

Mfg. Lic. No. MNB/05/267

Zambia Lic. No. 065/02

Cachet Pharmaceuticals pvt. LTD

Solan, Himachal Pradesh-173 205, India