Pharmacological Effects of *Hypoxis hemerocallidea* Fisch. & C. A. Mey. (Hypoxidaceae) Corm ("African Potato") Aqueous Extract on Some Mammalian Extra-Vascular Smooth Muscles *in vitro*

Submitted in Partial Fulfilment of the Requirements for the Award of Master of Pharmacology Degree in the School of Pharmacy and Pharmacology, University of KwaZulu-Natal, Durban 4000, South Africa

By

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DEDICATION

I sincerely dedicate this thesis to my family, without whom this would have not been possible. I convey my deepest gratitude to Professor and Doctor Mubangizi for the love, care and patience with which they have enabled me to be what I am, and for the invaluable opportunities they have provided me that have stimulated my growth. I would also like to thank my grandmother, Mrs. Juliana Munderi, Dr. Paula Munderi and Ms. Evangelista Kabami for being my pillar of strength, an unending source of wisdom and love, and for inspiring every step that I have taken in my life.

I thank God for being with me since I took my very first breath, and for blessing me generously, above all. I thank Him for blessing me with an incredibly loving, caring and understanding fiancé, Mr. Farai Razano.

To all my friends, thank you so much for being there and for your patience. God bless!

DECLARATION

I, **AGATHA NYINAWUMUNTU,** declare that the study on 'Pharmacological Effects of *Hypoxis hemerocallidea* Fisch. & C.A. Mey. (Hypoxidaceae) Corm ("African Potato") Aqueous Extract on Some Mammalian Extra-Vascular Smooth Muscles *in vitro*' is my work. All sources used throughout the study have been clearly referenced.

Signature:.....

Signed at DURBAN on this 9th day of APRIL 2009.

ACKNOWLEDGMENTS

I would like to thank my supervisor, Professor J.A.O. Ojewole, for his assistance and guidance. My sincere gratitude also goes to Ms. Kogi Moodley for her technical assistance. I would also like to thank the staff of Biomedical Resource Unit for their continued assistance. Lastly, I would like to thank my family for their unending support.

ABSTRACT

Extracts of *Hypoxis hemerocallidea* corm (African potato) are commonly used by some traditional health practitioners in KwaZulu-Natal Province of South Africa for an array of human ailments. This study was, therefore, undertaken to investigate the GIT spasmolytic, bronchospasmolytic, uterolytic and vasa deferentia relaxant effects of *Hypoxis hemerocallidea* corm aqueous extract. Respectively, these effects were determined on both naïve and spasmogen-evoked contractions of the guinea-pig and rat isolated ileum, trachea, uterine horns and the vas deferens *in vitro*. Healthy, young adult, male and female Dunkin-Hartley guinea-pigs (300-400g) and Wistar rats (250-350g) were used in this study. The isolated tissues were prepared and mounted in Ugo Basile organ-baths under normal physiological conditions. After an equilibration period of 30-45 minutes, the isolated smooth tissue segments were challenged with graded concentrations of *Hypoxis hemerocallidea* corm aqueous extract, and/or reference drugs. Changes in tension developed by the muscle preparations (relaxations and contractions) were recorded isometrically by means of Ugo Basile's force-displacement transducers and pen-writing 'Gemini' recorders. Relatively low to high concentrations of *Hypoxis hemerocallidea* corm aqueous extract (APE, 25-400 mg/ml) produced dose-dependent and significant ($p<0.05$) relaxations of the guinea-pig ileum, and the uterine horns taken from non-pregnant rats, as well as on spasmogen-provoked contractions of stilboesterol-primed, oestrogen-dominated, non-pregnant rats in a concentration-related manner. Potassium chloride (40 mM)-induced contractions of uterine horns, ACh (0.1-3.2 μg/ml)-induced increases in the amplitude of contractions of the guinea-pig ileum, as well as noradrenaline (0.2-1.6 μg/ml)-induced increases in the amplitude of contractions of the male rat isolated vasa diferentia, were significantly ($p<0.05$-0.001) reduced or abolished by bath-applied APE (25-400 mg/ml). Relatively low to high concentrations of the extract (25-400 mg/ml) caused concentration-dependent increases in the relaxations of the guinea-pig isolated tracheal smooth muscles. Inhibitions of ACh (0.1-3.2 μg/ml)-induced contractions of the guinea-pig isolated ileum probably suggests possession of antidiarrhoeal activity of APE. Results of this study show pronounced relaxant effects of *Hypoxis hemerocallidea* corm aqueous extract on guinea-pig vas deferens. The study also lends pharmacological credence to the folkloric, ethnomedical uses of APE as a natural antenatal remedy for threatening abortions, as an antidiarrhoeal remedy, and as a bronchorelaxant. The precise mechanisms of APE action on the smooth muscles could not be established in the present study. However, the uterolytic action of the corm's extract is unlikely to be mediated via β_2-adrenoceptor stimulation, but probably mediated through a *non-specific* spasmolytic mechanism.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ABBREVIATIONS OF STANDARD MEASUREMENT UNITS AND TERMS

APE	aqueous "African Potato" extract
SR	sarcoplasmic reticulum
PB	pentobarbitone
K-H	Krebs-Henseleit
ACh	acetylcholine
KCl	potassium chloride
mg	milligram
g	gram
cm	centimetre
ml	millilitre
°C	degree Celcius
mM	milliMolar

CHAPTER 1

1. INTRODUCTION

There is a growing global interest in the use of herbs and medicinal plants as sources of affordable, safe and effective medicines for the management, control or treatment of human diseases. Erasto *et al.* (2005) stated that the apparent reversal of trend from Western to herbal medicines is partly due to the fact that synthetic drugs have always shown adverse reactions and other undesirable side-effects. Natural products are believed to be safer, more harmonious with nature, and cheaper.

According to the World Health Organization (WHO, 1978), phytomedicines are defined as herbal preparations produced by subjecting plant materials to extraction, fractionation, purification, concentration or other physical or biological processes. These preparations may be produced for immediate consumption or as basis for other herbal products. Such plant products may contain recipient or inert ingredients, in addition to the active ingredients.

WHO (2001) also defined traditional medicine as the sum-total of knowledge or practices, whether explicable or inexplicable, used in diagnosing, preventing or eliminating a physical, mental or social disease, which may rely exclusively on past experience or observation handed down from generation to generation, either verbally or in writing.

Despite the limited knowledge with regard to safety, efficacy or toxicity of traditional medicines, a large number of rural African natives still use them to a great extent. Traditional practitioners are consulted much more often by these people, than those practicing Western medicine. In the wake of HIV/AIDS pandemic, Homsy *et al.* (2004) argued that people living with HIV/AIDS depend on, and choose traditional healers for psychosocial counselling and healthcare. During the Pan African Conference held in

Uganda (2004), it was mentioned that if the prevention and care efforts geared towards tackling HIV/AIDS did not engage African traditional healers, they would effectively miss out 80% of the African people that depend on and chose traditional healers for psychosocial counselling and health care. The majority of the African people, according to World Health Organisation, rely on traditional medicine for their primary healthcare needs as opposed to Western medicines. This is so because "African traditional medicine is characterised by a holistic approach to the spirit-mind-body concept of health, embracing people, animals, plants and inanimate objects, in an inseparable whole form, from which all beings derive their living and healing forces" (Homsy *et al.*, 2004).

Herbal medicines are used as primary treatment for HIV/AIDS and for HIV-related problems. "African Potato" is one of the African herbals that have played a major role in relation to this pandemic, especially here in South Africa. The herb went as far as being recommended by the Ministry of Health in South Africa for use in HIV, in conjunction with anti-retroviral treatment (Mills *et al.*, 2005).

The South African healthcare community is currently using *Hypoxis hemerocallidea* as an immunostimulant for patients with HIV/AIDS. Here in South Africa, *Hypoxis hemerocallidea* is being grown commercially, with controlled harvesting and preparation. *Hypoxis hemerocallidea* is popularly known as "African Potato" in English and "inkomfe" in isiZulu language. Other common names used for the herb in South Africa include; magic muthi, yellow stars, star lilly, streetwise, afril patat, ilabatheka, sterblom, gigbol, lotsane, olikalatsa.

This perennial herb is widely distributed in the grasslands of southern Africa, and extensively used in South African Traditional Medicine for the treatment, management and/or control of an array of human disorders. The tuberous rootstock (i. e., the corm) of the herb is the morphological part of the plant that is often used for medicinal purposes. Decoctions and infusions of the plant's corm are often used to treat dizziness, bladder disorders and insanity, usually given to weak children as a tonic, and the juice is often

applied to burns.

Other traditional uses of the plant's corm include treatment of testicular tumours, prostate hypertrophy and urinary infections (Van Wyk *et al.*, 2002). According to Zibula and Ojewole (2000), the corm of the plant has been christianised 'African potato' by the African natives of South Africa because of its physical resemblance to the Irish potato.

Hypoxis hemerocallidea is a "wonder" and miracle "cure all" herb; used for the alleviation of many immune-related ailments, such as wounds, colds, flu, arthritis, tumour and cancer (Dietzch *et al.*, 1998) and HIV/AIDS (Erasto *et al.*, 2005). The bitter-tasting plant is also said to be used in a variety of conditions, including haemorrhage, diabetes mellitus and prostate problems. The plant was used widely (in form of boiled tea) during the Civil War in Mozambique (1976-1992) by both soldiers and civilians to quickly replace lost blood and also used in conjunction with other plants to combat "bad blood" in patients with diabetes mellitus.

In South Africa, members of the Shangaan tribe used the plant, in combination with other plants, for endometriosis and pre-menstrual syndrome; and the root-stock as one of the ingredients of the infusion taken as an "internal parasitic" and purgative.
Hypoxis hemerocallidea is also used as a remedy for vomiting, loss of appetite, abdominal pains and fevers, treatment of delirium etc (http://www.natural standard.net/;© 2008).

The corm is dark-brown or black on the outside, and yellow within, when freshly cut (Van Wyk *et al.*, 2002). Figure 1 below shows the appearance of the corm. Fresh corms are crushed and the extract taken orally on a daily basis for very long periods of time (Mills *et al.*, 2005; Erasto *et al.*, 2005). The growing public interest and awareness of natural medicines have led the pharmaceutical industry and academic researchers to pay more attention to this medicinal plant.

Fig. 1. Picture of the *Hypoxis hemerocallidea* corm [Family: Hypoxidaceae] (Adapted from van Wyk *et al.*, 2002)

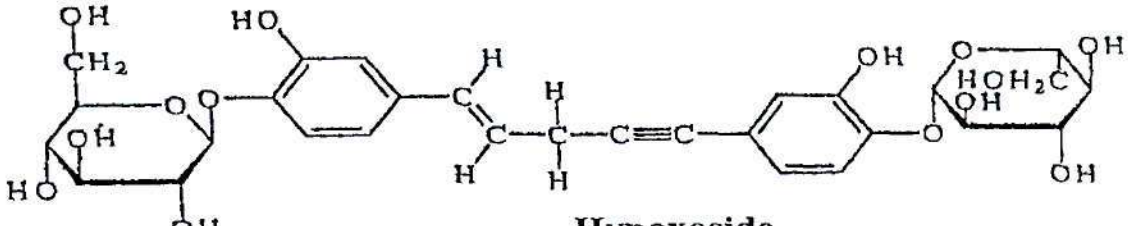
Several studies have been done on this "wonder plant", in relation to its fate in the body. In a particular study carried out to assess the toxicity of hypoxoside taken orally by 24 patients with lung cancer, some of the results indicated the following; patient survival for an average of 4 months with progression of their tumours and metastases, a small percentage of survival for more than 1 year. In this study, no toxic effects, in clinical examinations or biochemical or haematological measurements were found, that could be ascribed to ingestion of hypoxoside. However, minor cases of anxiety, drug intolerance, nausea, vomiting, diarrhoea, were noted (Albretcht *et al.*, 1995).

Also in another study, after oral ingestion of the hypoxoside by humans (methanol extract), no hypoxoside or rooperol appeared in the serum, and only rooperol was present in the

faeces. The serum and urine contained at least 3 phase II metabolite peaks. Selective enzyme hydrolysis showed that they represent the diglucoronide, disulfate and glucuronide; sulfate conjugates of all three rooperol analogues (Kruger *et al.*, 1994). Furthermore, Albrecht *et al.*, 1995, reported absence of hypoxoside or rooperi in the serum. Only phase II biotransformation products, sulphates and glucuronides, were present in the portal blood and bile. It was noted that after oral ingestion of hypoxide, metabolite concentrations can reach relatively high concentrations.

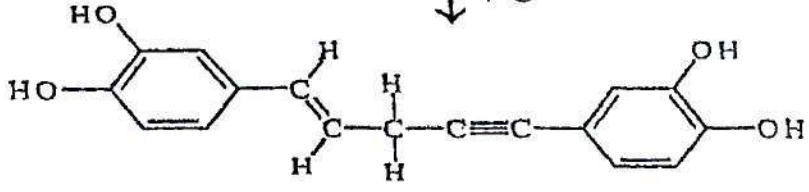
These studies highlight the gaps that still need to be filled, for example, determination of the plant's toxicity after oral ingestion.

Besides all the claims, no satisfactory clinical trials of efficacy exist, and there is definite potential for drug interactions with anti-retroviral (ARV) drugs; one area where extreme miracles are claimed. The therapeutic effects of "African potato" have been strongly ascribed to the sterols, stanols, sterolins and norlignan glycoside, hypoxoside, present in the corm (Drews *et al.*, 1984; Albrecht *et al.*, 1995; Bouic *et al.*, 1996; Nair *et al.*, 2007). The anti-HIV together with the anti-cancer and anti-inflammatory activities of this herb are ascribed to rooperol, the aglycone of hypoxoside, which is the 4,4-diglucoside (Van Wyk *et al.*, 2002, Dietzsch *et al.*, 1999, Mills *et al.*, 2005), the sterols and sterolins. The corm is also known to contain stigmastanol and other sterolins. Ojewole (2000; 2003; 2005; 2006) reported various pharmacological actions of the corm in different experimental animal paradigms, ascribed the pharmacological effects to the chemical compounds of the corm. The long-term use of the sterols and sterolins derived from "African potato" is not advisable due to its immuno-suppressive effect (Mills *et al.*, 2005). Chemical structures of the three best known and established constituents of the corm, hypoxoside, rooperol and β-sitosterol, are shown in Fig. 2.

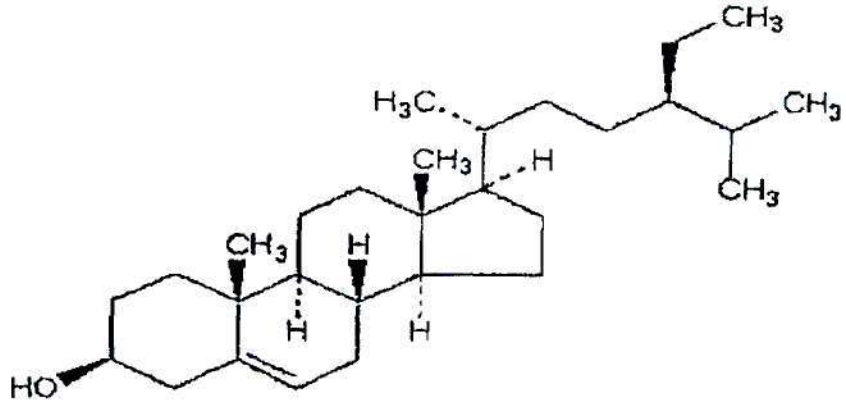


Hypoxoside

β-glucosidase



Rooperol



β-Sitosterol

Fig. 2. Structures of hypoxoside, rooperol and β-sitosterol. The biologically-inactive norligan diglucoside, hypoxide, is deconjugated and converted by β-glucosidase enzyme to form the biologically-active aglycone, rooperol.

Earlier studies have indicated that traditional botanical remedies can be valuable for treating human diseases. Potential risks and carcinogenic activities arising from long-term use of such remedies have, however, not been fully investigated. "The use of some traditional medicines has resulted in several cases of acute toxicity, leading to increased morbidity and mortality", Reid *et al.* (2006). According to Taylor *et al.* (2003), of the plants used in their study, which were recognised by traditional healers as toxic, several were found to be genotoxic and potentially dangerous. Limited data in literature exists with regard to the safety and efficacy of plant-derived medicinal products in South Africa (Johnson *et al.*, 2007).

In an effort to establish scientific basis for the claimed traditional uses of some of the frequently used medicinal plants, their chemical constituents and pharmacological activities have been recently investigated (Drewes *et al.*, 1984; Ojewole, 2001; 2002; 2003; 2004; 2006).

Extensive literature search has shown a paucity of biomedical literature on smooth muscle effects of "African Potato". This apparent 'gap' stimulated my interest in investigating the effects of "African Potato" on mammalian smooth muscles *in vitro*. The core aim of this study, therefore, was to examine the pharmacological effects (i. e., contractile or relaxant effects) of *Hypoxis hemerocallidea* corm aqueous extract solution on isolated smooth muscles of mammalian experimental animals (namely, rats and guinea-pigs).

The use of traditional medicines during pregnancy still plays a crucial role in the lives of the people living in the rural areas where modern healthcare facilities are often lacking or none existent. In these societies, pregnancy is often associated with a number of taboos and rituals to ensure successful confinement and births of healthy children (Sewram *et al.*, 1998; 2000). Pregnant mothers consume an array of plant concoctions, as antenatal remedies. These are formulated as powders, infusions, extract, and soaps; taken to induce or accelerate labour, prevention of threatening abortions, and to ensure successful confinements or deliveries.

"African Potato", the "cure all", "Zifuzonke" in isiZulu, is commonly applied (in addition to other herbal remedies used) for all these proclaimed activities; most especially for the management of threatening or premature abortions. Part of this study was, therefore, to investigate the uterolytic effects of "African Potato" aqueous extract in mammalian experimental animal paradigms *in vitro*.

To evaluate the claimed traditional use of *Hypoxis hemerrocallidea* in the treatment of asthma and other respiratory disorders, the broncholytic effect of *Hypoxis hemerocallidea* was examined using the rat isolated tracheal smooth muscle. Antispasmodic effects of the plant were also determined using the guinea-pig ileum smooth muscle. This study also involved determination of the pharmacological effects of *Hypoxis hemerocallidea* on the rat vas deferens.

This study, therefore, involved determination of the *in vitro* effects of *Hypoxis hemerrocallidea* aqueous extract on the extra-vascular smooth muscles of the respiratory system (trachea), reproductive system (uterus and the vas deferens) and the gastro-intestinal system (ileum) in mammalian experimental animal paradigms *in vitro*.

1.2 AIMS AND OBJECTIVES

1.2.1 AIM

The aim of this study was to investigate the pharmacological effects of *Hypoxis hemerocallidea* Fisch. & C.A. Mey. (Hypoxidaceae) corm ("African Potato") aqueous extract (APE) on some mammalian extra-vascular smooth muscles *in vitro.*

1.2.2 OBJECTIVES

The objectives of the study were to examine the pharmacological effects of *Hypoxis hemerocallidea* on the following extra-vascular smooth muscles *in vitro.*

(a) examine the uterolytic effects of APE on spontaneously-contracting isolated uterine horns of non-pregnant rats; as well as on acetylcholine and potassium chloride-induced contractions of uterine horns isolated from stilboesterol-primed, oestrogen-dominated, non-pregnant rats;

(b) examine the broncho-relaxant effects of APE on isolated guinea-pig tracheal muscles;

(c) examine the relaxant effects of APE on adrenaline-induced contractions of the guinea-pig vas deferens.

CHAPTER 2

2. SMOOTH MUSCLE PHYSIOLOGY

2.1 FUNCTIONS OF SMOOTH MUSCLE.

The smooth muscle is responsible for the contractility of hollow organs. Smooth muscles around blood vessels regulate blood flow through the vital organs. In the digestive and urinary tract systems, rings of smooth muscles, known sphincters, regulate the movement of materials along the internal passage ways. When made to contract, smooth muscles shorten, thereby propelling the luminal contents of the organs.

Table 1 outlines some important functions of smooth muscle bundles in different body systems:

BODY SYSTEM	FUNCTION
Respiratory	Smooth muscle contraction or relaxation alters the diameters of the respiratory passageways, and changes the resistance to airflow.
Cardiovascular	Smooth muscles encircling blood vessels control the distribution of blood, and regulate blood pressure.
Integumentary	Smooth muscles around blood vessels regulate the flow of blood to the superficial dermis; smooth muscles of the errector pili elevate hairs.

Digestive	Extensive layers of smooth muscle in the walls of the digestive tract play an essential role in moving materials along the tract. Smooth muscle in the walls of the gallbladder contract to eject bile into the digestive tract.
Urinary	Smooth muscle tissue in the walls of small blood vessels alters the rate of filtration at the kidneys. Layers of smooth muscle in the walls of the ureters transport urine to the urinary bladder; the contraction of the smooth muscle in the wall of the urinary bladder forces urine out of the body.
Reproductive	Layers of smooth muscle help move semen along the reproductive tract in males, and cause the ejection of glandular secretions from the accessory glands into the reproductive tract. In females, layers of smooth muscle help move oocytes (and sperm) along the reproductive tract, and contraction of the smooth muscle in the walls of the uterus expels the fetus at delivery.

Table. 1. Functions of smooth muscle bundles in body systems (http://cwx.prenhall.com/; © 1999-2000).

Smooth muscle cell structure differs greatly from that of skeletal muscle cells, although it can develop isometric force per cross-sectional area that is equal to that of the skeletal muscle (file://E:\SMOOTH MUSCLE.htm. The speed of smooth muscle contraction is, however, only a fraction of that of skeletal muscle. This slow speed of smooth muscle contraction, in relation to skeletal muscle, can be attributed to lack of well-developed T-tubules in smooth muscle. As a result of this, excitation of smooth muscle cell membranes is not quickly transmitted to the interior of the cell. This inefficiency is counteracted by the inherent small size of smooth muscle cells. Their size allows the events that lead to the increase in cytosolic free calcium, necessary for activation of the contractile mechanism, to occur rapidly enough (Johnson, 2003).

Smooth muscles around blood vessels regulate blood flow through the vital organs. In the digestive and urinary systems, rings of smooth muscle, known as the sphincters, regulate the movement of materials along the internal passage ways. When made to contract, the smooth muscle cells shorten, thereby propelling the luminal contents of the organ, or the cell shortening varies the diameter of a tube to regulate the flow of its contents.

2.2 SMOOTH MUSCLE STRUCTURE, INNERVATION AND STIMULATION.

Smooth muscles lack visible cross striations/banding pattern found in cardiac and skeletal muscles. The cells of smooth muscles range from 5 to 10 μm in diameter, and from 300 to 200 μm in length. Each cell is spindle-shaped and has a single, centrally- located nucleus (http://cwx.prenhall.com/; © 1999-2000).

Smooth muscle cells are of two categories; the single-unit (visceral) smooth muscle cells and the multi-unit smooth muscle cells.

◆**Single unit smooth muscles**: The cells are arranged in dense sheets or layers. A single-unit smooth muscle has pace-maker regions where contractions are generated spontaneously and rhythmically. The fibres appear to run almost parallel, but are densely and irregularly packed together most often, so that the narrower portion of one fibre lies against the wider portion of its neighbour. The initial stimulus may be the activation of a motor neurone that contacts one of the muscle cells in the region. Within each layer, adjacent muscle cells are connected by gap junctions (the plasma membranes of two neighbouring fibres which act as low resistance pathways for the rapid spread of electrical signals through the tissue. Owing to this form of connection, whenever one muscle cell contracts, the electrical impulse that triggered the contraction travels to adjacent smooth muscle cells. The contraction travels in a wave that soon

involves every smooth muscle cell in the region. The fibres contract in unison, that is, the single-unit smooth muscle is synctical.

In intestinal smooth muscle cells, for example, membranes of adjacent smooth muscle cells make intimate contact with one another, such that low resistance electrical pathways exist (connected by gap junctions). Excitation of one cell quickly spreads (an increase in the intensity of contraction is due to enhanced excitation-contraction coupling), and a group of cells will contract in unison.

◆**Multi-unit smooth muscles**: These muscle cells are innervated by sympathetic and parasympathetic nerve fibres, and respond independently from each other, upon nerve stimulation. Nerve fibres have no interconnecting bridges, and are interspersed with connective tissue fibres. When a nerve impulse arrives at the terminal of the sympathetic nerve fibres, neurotransmitters released, such as noradrenaline, will reach and activate the effector cells by diffusion. Active cells that are touched by the nerve endings will then communicate with the others, via gap junctions, to form a functional unit.

Multi-unit smooth muscle cells resemble skeletal muscle fibres and cardiac muscle cells in such a way that, neural activity produces an action potential that is propagated over the sarcolemma. In these muscle cells, an increase in the intensity of stimulation is accompanied by an increase in force, as more and more cells become active. However, contractions of these muscle cells are not as strong and pronounced as those of skeletal and cardiac muscle cells. Multi-unit muscle cells regulate a number of organs, such as the diameter of the pupil (located in the iris of the eye), along portions of the male reproductive tract (for example, the vas deferens), the walls of large arteries, and in the errector pili muscles of the skin. Multi-unit smooth muscle cells do not typically occur in the digestive tract.

The structural organisation of single-unit and multi-unit smooth muscle cells is shown in Fig.3.

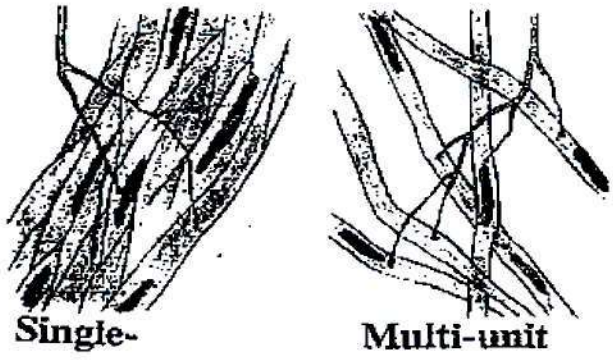


Fig. 3. Diagram showing the structure of single-unit and multi-unit smooth muscle fibres (file://E:\SMOOTH MUSCLE.htm).

Smooth muscle cells primarily receive neural innervation from the Autonomic Nervous System. Response to innervation of a smooth muscle is either a depolarisation or hyper-polarisation. Thus, integration occurs at the level of a smooth muscle cell, and not just within the central nervous system, as with skeletal muscle (Johnson, 2003). A skeletal muscle, on the other hand, is under the control of the somatic nervous system.

According to Johnson (2003), mild stimulation of an excitatory nerve leads to a small depolarisation, but no change in tension, whereas stronger stimulations lead to development of an action potential, and then a contraction. Furthermore, weak stimulation of an inhibitory nerve leads to a membrane hyper-polarisation, but no change in resting tension. Stronger stimulations of the nerve, however, lead to a larger hyper-polarisation and relaxation. Additionally, the contractile state of smooth muscle is controlled by hormones, autocrine/paracrine agents, and other local chemical signals.

2.3 LENGTH-TENSION RELATIONSHIPS IN SMOOTH MUSCLE CELLS.

According to Benoit *et al.* (1997), tension generation and muscle shortening are a consequence of phosphorylation of myosin by a calmodulin-dependent kinase, which increases the affinity of myosin, thereby promoting cross-bridge formation. Dephosphorylations of myosin by phosphatases are required to return actin and myosin to their normal low affinity states.

In smooth muscle cells, the thick and thin filaments are not organised into sarcomeres, but scattered. As a result of this arrangement, tension development and resting length in smooth muscles, are not directly related. A stretched smooth muscle quickly adapts to its new length and retains the ability to contract on demand. This ability to function over a wide range of length is known as "plasticity". About four times greater than skeletal muscle, smooth muscles can contract over a wide range of lengths. This unique ability of smooth muscles especially comes into play in regions such as the digestive system (for example, the stomach) where great changes in volume occur.

In some smooth muscles, especially those of large blood vessels, a quick stretch is followed by a temporary increase in wall tension. However, this increase in tension is followed by an almost immediate relaxation towards the original wall tension; referred to as stress relaxation. Reverse stress-relaxation occurs when the muscle is allowed to shorten and return to its original wall tension; for example, on removal of an external tensional stress. This ability of smooth muscles to undergo such changes is of vital importance. For example, in large blood vessels, this kind of response allows accommodation of different blood volumes by the larger blood vessels, while maintaining transmural pressure nearly constant.

Despite the lack of sarcomere organisation, smooth muscle contractions can be just as powerful as those of skeletal muscles. Smooth muscle cells also often undergo sustained tetanic contractions, as experienced by skeletal muscle.

2.4 SMOTH MUSCLE CONTRACTION:

A muscle contraction occurs when a muscle fibre generates tension through the action of actin and myosin cross-bridge cycling. It, therefore, refers to the generation of tension by muscle fibres, with the help of motor neurons (Wikipedia, 2008). Voluntary muscle contractions are initiated in the brain and involuntary reflexes by the spinal cord.

2.4.1 Examples of the types of smooth muscle contraction:

The action of binding/coupling of vasoconstrictor agonists with the membrane receptors is known to involve action of membrane-associated guanosine, 5'-troposphate (GTP) binding proteins, stimulation of phosphatidyl inositol turnover, and activation of protein kinase C. These events lead to the opening of calcium channels on the cell membrane and sarcoplasmic reticulum. L-type calcium channels on the sarcolemma are activated by voltage as well as phosphorylation. Calcium release channels on the sarcoplasmic reticulum predominantly respond to elevation in inositol triphosphate (IP3). Protein kinase C can promote vascular smooth muscle contraction even in the absence of increased cytoslic calcium levels. However, both the calcium dependent and the calcium independent protein kinase C pathways do require G-protein activation (Benoit *et al.*, 1997). The release of calcium from intracellular stores and endoplasmic reticulum is one of the key transduction mechanisms in the regulation of numerous cellular functions, including contractility, protein synthesis and turnover, hormone secretion, proliferation and activation (Yusufi *et al.*, 2002).

Calcium sensitisation is observed not only in vascular but also in other visceral smooth muscle tissues, including airway smooth muscles. In this case, calcium sensitivity is referred to as the increase in smooth muscle tension and/or phosphorylation of 20-kD regulatory light chain of myosin (MLC20) at a constant calcium concentration. Receptor dependent G-protein mediated calcium sensitisation was found to occur in canine trachea (Yoshi *et al.*, 1999).

Stimulation of smooth muscle nerves causes membrane depolarisation; just like in skeletal muscles. Excitation, the electrochemical event occurring at the membrane, is followed by the mechanical event, contraction. The excitation-contraction coupling event in smooth muscle is termed "electro-mechanical coupling". This is made possible by the permeation of calcium from the extracellular space into the intracellular water of smooth muscles. An increase in free intracellular calcium can result from either increased influx of calcium into the cell through calcium channels, or by release of calcium from internal stores (for example, the sarcoplasmic reticulum; SR). Once in the sarcoplasm, calcium ions interact with calmodulin, a calcium binding protein. Calcium-calmodulin activates myosin light chain kinase (MLCK), an enzyme that is capable of phosphorylating myosin light chains (MLC) in the presence of ATP (Johnson, 2003; Benoit *et al.*, 1997). Myosin light chains are 20-kD regulatory subunits found on myosin heads. MLC phosphorylation leads to cross-bridge formation between the myosin heads and the actin filaments, and hence, smooth muscle contraction.

The concentration of intracellular calcium depends upon the balance between the calcium that enters the cells, the calcium that is released from intracellular storage sites (e.g., SR), and removal of calcium either back into storage sites, or out of the cell. Calcium is re-sequestered by the SR by an ATP-dependent calcium pump. Calcium is removed from the cell to the external environment by either an ATP-dependent calcium pump, or by the sodium-calcium exchanger. In skeletal and cardiac muscles, on the other hand, contraction is triggered by the binding of calcium ions to troponin. However, one should note that smooth muscles (and cardiac muscle); do not contain enough calcium to fully activate the contractile proteins, as is observed in striated muscle which consists of well developed sarcoplasmic reticuli. Varying rates of Ca^{2+} influx and efflux make it possible for there to be moment-to-moment net gains and losses of calcium available to initiate a contraction, in smooth muscle cells (Johnson, 2003).

The mechanism for smooth muscle contraction is shown in Fig. 4 below.

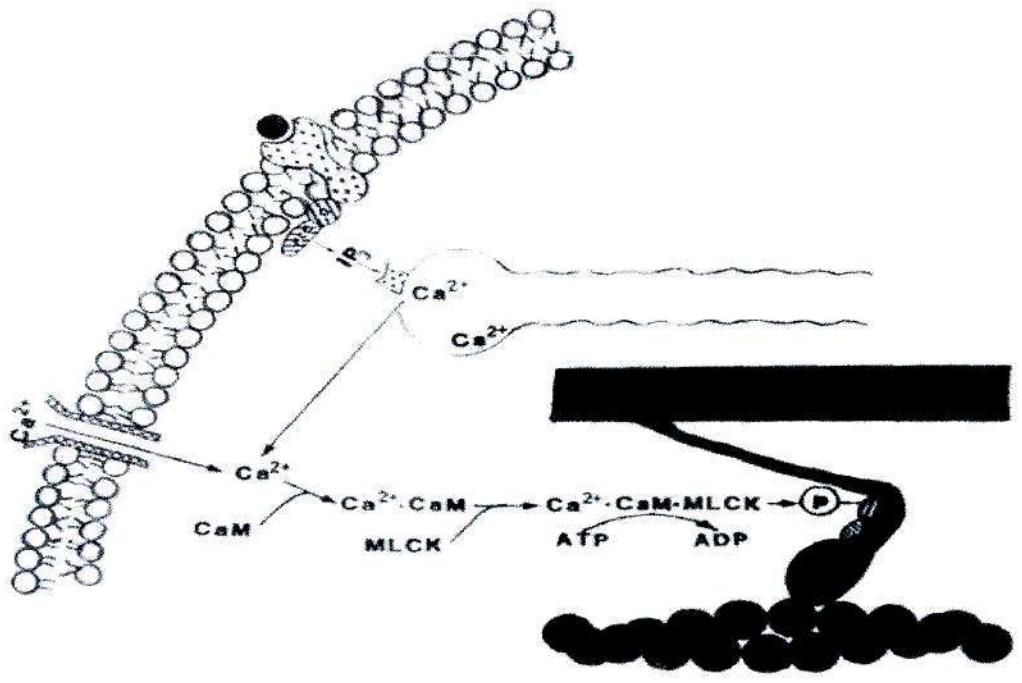


Fig. 4. Mechanism of calcium stimulation of vascular smooth muscle contraction. Contraction is initiated by the increase of Ca^{2+} in the myoplasm (file://E:\SMOOTH MUSCLE.htm).

The sequence of events leading to the contractile effect is described below.

- Ca^{2+} may enter from the extracellular fluid through channels in the plasmalemma. These channels open, when the muscle is electrically stimulated, or the plasmalemma is depolarized by excess K^{+}.
- Due to agonist-induced receptor activation, Ca^{2+} may be released from the sarcoplasmic reticulum (SR). In this pathway, the activated receptor interacts with a G-protein (G) which in turn, activates phospholipase C (PLC). The activated PLC hydrolyzes phosphatidyl inositol bisphosphate; one product of the hydrolysis is inositol 1, 4, 5-trisphosphate (IP_3). IP_3 binds to its receptor on the surface of SR, this

opens Ca^{2+} channels, and Ca^{2+} from SR enters the myoplasm.

- Ca^{2+} combines with calmodulin (CaM) and the Ca^{2+}-CaM complex activates MLCK, which in turn phosphorylates the light chains. The phosphorylated myosin filament combines with the actin filament and the muscle contracts.

According to Webb, (2003), elevation of calcium concentration within the cell is transient, and the contractile response is maintained by the calcium-sensitising mechanism brought about by Rho kinase. This mechanism is said to be activated at the same time as the phospholipase C is activated, and involves the activation of the small GTP-binding protein RhoA. The nature of the activation is said to involve the guanine nucleotide exchange factor (RhoGEF), and migration of RhoA to the plasma membrane. RhoA increases Rho kinase activity upon activation, resulting in inhibition of myosin phosphatase. This inhibition then promotes the contractile state, since the light chain of myosin cannot be dephosphorylated. The state of light chain phosphorylation is said to be further regulated by MLC phosphatase, which removes the high-energy phosphate from the light chain of myosin to promote smooth muscle relaxation. When phosphorylated, the myosin-binding subunit of MLC phosphatase, inhibits the enzymatic activity of MLC phosphatase, thus allowing the light chain of myosin to remain phosphorylated, and hence, promoting contraction.

2.4.1.1 Changes in load or length.

Smooth muscles also develop tonic and phasic contractions in response to changes in load or length, just as skeletal muscles do. Regardless of the stimulus, smooth muscle cells use cross-bridge cycling between actin and myosin to develop force, and calcium ions serve to

initiate muscle contraction.

2.4.1.2 Pharmaco-mechanical coupling.

Pharmaco-mechanical coupling occurs in smooth muscles, eliciting contraction. This mechanism is independent of the membrane potential change; and is based on receptor activation by chemicals (for example, drugs) or hormones, response to local concentrations of oxygen or carbon-dioxide, or physical factors such as extreme stretching or irritations. This results in muscle cell contraction or relaxation. Mechanical events of smooth muscle in hollow organs serves two major roles; force development and muscle shortening, as well as tonic contractions, maintain organ dimensions against the imposed load .

2.4.1.3 Signal Transduction

The binding of an agonist (e.g. norepinephrine or oxytocin) to the surface receptor of a smooth muscle induces a signal that spreads from the outside to the inside of the plasma membrane, and activates several effectors that ultimately initiate contraction (file://E:\SMOOTH MUSCLE.htm). There are three components of this system, as discussed below (1. Inositol 1, 4, 5-trisphosphate; 2. G-proteins and 3. Phosphoinositide-specific phospholipase C)

◆ Inositol 1,4,5-trisphosphate

The inositol ring contains six hydroxyl residues; most of them can be phosphorylated by specific kinases. Inositol 1-monophosphate is the constituent of phosphatidylinositol (PI) one of the phospholipids in animal cell membranes. PI 4-kinase and PI (4) P 5-kinase sequentially phosphorylate PI to generate PI (4) P and PI (4, 5) P2, respectively. Inside the cell membrane resides a phosphoinositide specific phospholipase C, one of its hydrolytic product is inositol 1, 4, 5-trisphosphate (IP_3).

◆ G-proteins

The guanine nucleotide binding proteins (G-proteins) are heterotrimers consisting of a-, b- and g-subunits. The a-subunits appear to be most diverse, and are believed to be responsible for the specificity of the interaction of different G-proteins with their effectors. In the basal state, the a-subunit contains bound GDP (Guanine Di Phosphate), and association of a- band g-subunits is highly favoured, keeping the G-protein in the inactive form. Stimulation of the G-protein occurs when it binds GTP (Guanine Tri-Phosphate) rather than GDP. Receptors interact most efficiently with the heterotrimeric form of the G-protein, and accelerate activation by increasing the rate of dissociation of GDP, and enhancing the association of GTP. Activation of G-protein coupled receptor results in the dissociation of heterotrimeric G-proteins into a-subunits and bg-dimers. Finally, the G-protein a-subunit has an intrinsic hydrolytic activity that slowly converts GTP to GDP, and returns the G-protein to its inactive form.

◆ Phosphoinositide-specific phospholipase C

This term refers to a family of enzymes, all specific for the phosphoinositide moiety of the phosphatidylinositol, but differing in their specificity, depending on the number of phosphoryl groups on the inositol ring. The b-, g- and d-isoforms of PI-phospholipase C (PI-PLC) show the greatest specificity for the trisphosphorylated phospholipid ($PIP_{2)}$). There are two basic mechanisms by which agonists activate PIP_2 hydrolysis. In the case of hormones, neurotransmitters, and certain other agonists, the signal is transduced to b-isozymes of PI-PLC. The most common pathway for PI-PLCb-isoform activation is initiated by stimulation of α_1-adrenergic receptor (α_1-R) with norepinephrine (NE), and involves Ga_q-proteins. The activation of PI-PLC-b isoforms, is initiated by acetylcholine (ACh) stimulation of M_2-muscarinic receptor (M_2-R), and is mediated by the b g-subunit of the pertussis toxin-sensitive G-protein (G_I). Concerning the other basic activating mechanism, for example, in the case of growth factors, activation of their receptors results in enhanced tyrosine kinase activity. Activation of PI-PLC-g isoforms, initiated by the binding of epidermal growth factor (EGF) to its receptors, and executed by the tyrosine

phosphorylation (YP) of PI-PLC-g. In all three examples described above, the activated PI-PLC hydrolyzes PIP_2 to form the messengers, IP_3 and diacylglycerol (DAG). IP_3 releases Ca^{2+} from sarcoplasmic reticulum and thereby initiates smooth muscle contraction. DAG activates protein kinase C. However, the exact result of this activation is not known at the cellular level.

2.5 SMOOTH MUSCLE RELAXATION

2.5.1 Vascular relaxation mechanisms:

Factors that contract vascular smooth muscles increase blood flow, hence organ perfusion. The balance between vascular relaxation and contraction is maintained in the resting organ such that resistance to blood flow and organ perfusion are maintained relatively constant (Benoit *et al.*, 1997).

Relaxation of smooth muscles occur either as a result of removal of the contractile stimulus, or by direct action of a substance that stimulates inhibition of the contractile mechanism. According to Benoit *et al.* (1997), the act of relaxation requires a decrease in the concentration of intracellular calcium as well as an increase in myosin light chain-phosphatase activity; which decreases the rate of myosin phosphorylation.

2.5.1.1 Effect of decreased intracellular concentrations of calcium.

Decreased concentrations of intracellular calcium, an activator for smooth muscle contraction, elicit muscle cell relaxation. This calcium deficiency may be brought about by any of the following mechanisms:

- Removal of cytosolic calcium

This involves activity of the sarcoplasmic reticulum and the plasma membrane. Sarcoplamic

reticular calcium binding proteins contribute to a decreased intracellular level. Examples of these sarcoplasmic reticular calcium binding proteins in smooth muscles include calsequestrin and calreticulin (Webb, 2003). ATP hydrolysis in the sarcoplasmic reticulum results into calcium uptake. The sarcoplasmic reticular Ca^{2+}, Mg-ATPase, when phosphorylated, bind two calcium ions, which are then translocated to the luminal side of the sarcoplasmic reticulum, and released. Magnesium is necessary for the activity of this enzyme. The enzyme binds to the catalytic site of the ATPase to mediate the reaction. This enzyme can, however, be inhibited by various pharmacological agents such as cyclopiazonic acid and vanadate (Webb, 2003).

The Ca, Mg-ATPases are also located in the plasma membrane, and contribute to the reduction of calcium concentrations in the muscle cells. Plasma membrane enzyme Ca, Mg-ATPase is uniquely different from the same enzyme in the sarcoplasmic reticulum. It was observed in a study of Benoit *et al.* (1997), that the sarcoplasmic reticulum calcium pump was sensitive to stimulation by cAMP, whereas the plasma membrane-pump was not. This Ca-Mg-ATPase enzyme possesses an auto-inhibitory domain that can be bound by calmodulin, causing stimulation of the plasma membrane calcium pump.

- Sodium/calcium exchangers

These are located on plasma membrane, and aid in decreasing the intracellular calcium concentrations. The exchanger is a low affinity antiporter, and is closely coupled to intracellular calcium levels; and can be inhibited by amiloride and guanidine, for example. Inhibition of the receptor-operated and voltage-operated calcium channels located in the plasma membrane can lead to smooth muscle relaxation. This is so because these channels are important in calcium influx and smooth muscle contraction. Calcium channel antagonists can also be employed to elicit the relaxant effects. These include, benzodiazepines and dihydropyridine; which bind to distinct receptors on the channel protein, and inhibit calcium entry in smooth muscle.

Figure 5 below shows the mechanism of smooth muscle relaxation.

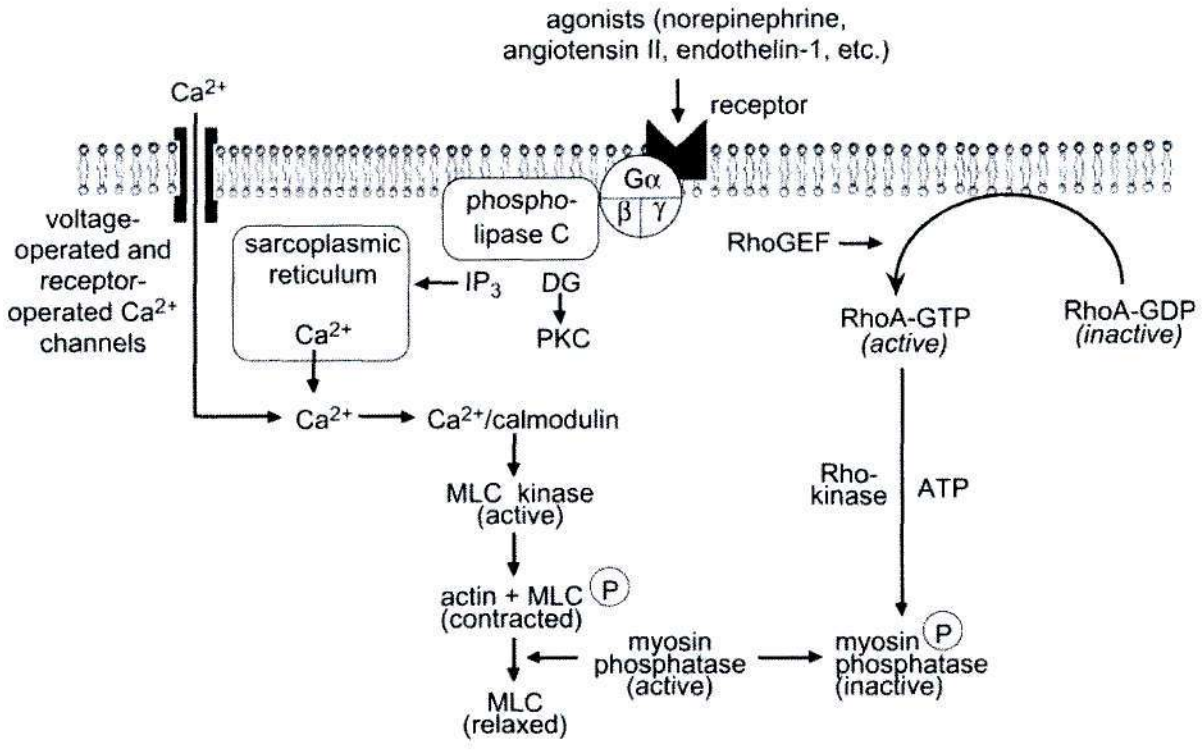


Fig. 5. Relaxation mechanism of smooth muscle. Smooth muscle relaxation occurs either as a result of removal of the contractile stimulus or by the direct action of a substance that stimulates inhibition of the contractile mechanism. Regardless, the process of relaxation requires a decreased intracellular Ca^{2+}, concentration and increased MLC phosphatase activity. The sarcoplasmic reticulum and the plasma membrane contain Ca, Mg-ATPases that remove Ca^{2+}, from the cytosol(Webb, 2003).

2.5.1.2 Mechanism of cAMP-dependent and cGMP-dependent vasodilation (Receptor-operated vasodilation)

According to Benoit *et al.* (1997), *in vitro* studies indicate that phosphorylation of myosin kinase via a cAM-dependent pathways decreases the affinity of the myosin kinase for the calcium-clamodurin kinase that is responsible for the phosphorylation of myosin. The result

is a decreased sensitivity of the smooth muscle contractile machinery. The sarcoplasmic reticulum calcium pump was found to be sensitive to stimulation by cAMP, whereas the plasma membrane pump was not. Also, cAMP dependent-protein kinases phosphorylate and prevent the opening of dihydropyridine sensitive calcium channels. This action is said to limit the rise in calcium, in response to vasoconstrictor agonists. Benoit *et al.* (1997), therefore, suggested that collectively, this results into a decreased myosin phosphorylation and/or decreased intracellular calcium mediated cAMP dependent vasodilation.

In vascular smooth muscles, the actions of cGMP are largely mediated by cGMP-dependent kinases. As stated by Benoit *et al.* (1997), reports indicate that:

i) the cGMP dependent kinases depress the rise in cytosolic calcium that occurs in response to vasoconstrictor agonists such as angiogenesis II.

ii) the action of cGMP on smooth muscle is via calcium-ATPase activity, and can be increased by cGMP-dependent protein kinases. In this case, the net effect of increased cGMP-dependent kinase is to enhance calcium pump activity.

A combination of these two actions lowers intracellular calcium, and mediates cGMP-dependent relaxation.

These mediators lead to smooth muscle relaxation induced by the binding pharmaco-therapeutic agonists.

CHAPTER 3

3 MATERIALS AND METHODS

Ethical considerations

Experimental protocols and procedures used in this study were approved by the Animal Ethics Committee of the University of KwaZulu-Natal, Durban 4000, South Africa; and conform to the "*Guide to the Care and use of Laboratory Animals in Research and Teaching*" [published by the Ethics Committee of the University of KwaZulu-Natal, Durban 4000, South Africa].

3.1 PLANT MATERIALS

Newly-harvested *Hypoxis hemerocallidea* corms were purchased from Africa herbal market on West Street in Durban, South Africa, between November 2007 and May 2008. A voucher specimen of the plant has been kept in the University's Botany Herbarium.

3.1.1 Preparation of *Hypoxis hemerocallidea* extract.

Two kilograms (2 kg) of "African Potato" corms were neatly peeled and washed with distilled water. These were then cut into small pieces to make it easier during the blending process (in a Waring commercial blender). The cut pieces were weighed again, before blending. The wet mass was taken and found to be 1772 g (88%).

Hot distilled water (3 litres) at 70-80°C was added to the finely blended corm and left standing for 24 hours in a 5-litre conical flask with occasional shaking. The flask was kept

air-tightly sealed and shaken from time-to-time to enhance the dissolution process of sap into the distilled water. After 24 hours, the supernatant was filtered from the mixture using a vacuum filter. Under reduced pressure in a rotary evaporator (see Fig. 6) at a temperature of 55±1 °C, the supernatant, in small volumes at a time, was concentrated to dryness. The resultant crude extract was left in the freezer over night. The dark-brown crystals of the extract were then scraped from the evaporation flask. These were then lightly ground to give a crystalline powder of 'African Potato' aqueous extract (APE). 154 g of powder were obtained, giving a final yield of 15 % of the powdery extract.

Aliquot portions of the crude extract were weighed, without further purification, and dissolved in distilled water (at room temperature), on each day of my experiments.

The figure below shows the rotary evaporator.

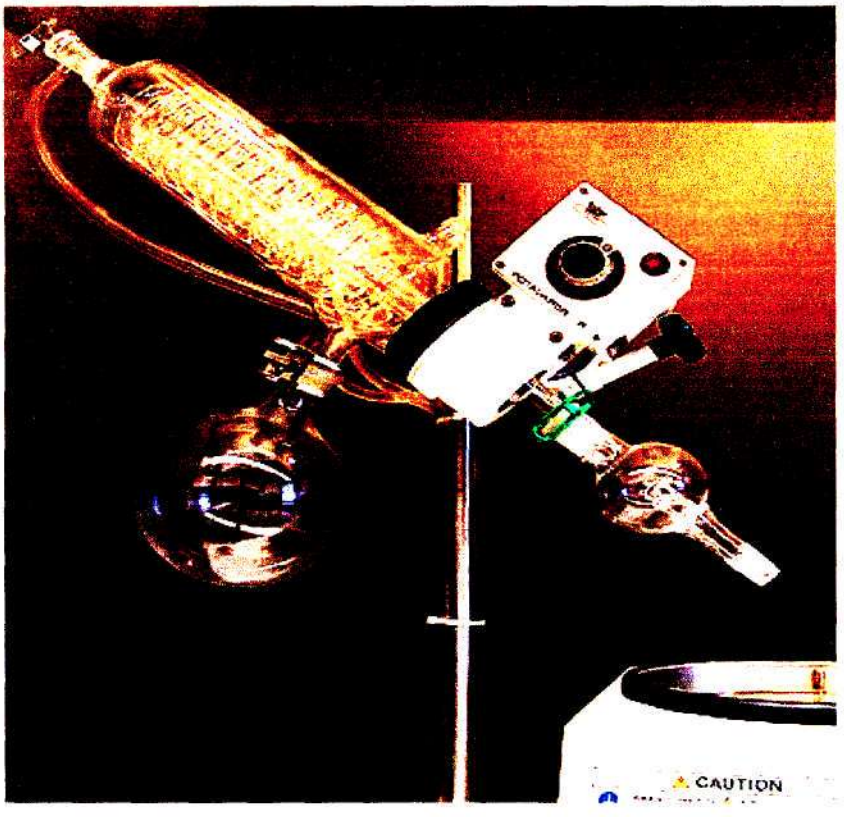


Fig. 6. Picture of a Rotary Evaporator (http://www.chem.ubc.ac/courseware)

3.2 ANIMAL MATERIAL: TISSUE PREPARATION AND METHODOLOGY.

Animals used:

Healthy, young adult, non-pregnant female Wistar rats (250-350g) and Dunkin-Hartley guinea-pigs (300-400g) were used. The animals were kept and maintained under controlled laboratory conditions of temperature, humidity and light. They were allowed free access to food (standard pellet diet) and drinking tap water *ad libitum*. The following procedures

were followed, prior to smooth muscle isolation, from the respective animals employed in this study.

A) Wistar rats

The rats were euthanized by exposing them to an overdose of halothane. The rats were thereafter quickly placed on a dissecting table, and their abdomen opened by mid-line incision. Tubular segments (3-4cm long) of the uterine horns or vas deferens were then isolated; as described in detail by Domer (1971).

B) Guinea -pigs.

The guinea-pigs were euthenased with 0.6 ml of sodium pentobarbitone (PB) injection given via the cardiac route. The euthanased guinea-pigs were then placed on a dissecting table. The abdomen was opened, and small tubular pieces/segments (3-4 cm long of the ileum, trachea or vasa deferentia) were quickly removed.

3.2.1 ISOLATED UTERINE HORNS

Evaluation of the Uterolytic Activity of APE

Animal experiments were performed following the guidelines as described by Domer, (1977).

The uterus has inherent myogenic contractile activity. Bearing this in mind, therefore, both naive and primed uteri were used during the procedures undertaken. Since the oestrus stage of the rats could not be determined, virgin rats were treated with di-ethyl stilboesterol (Bower *et al.*, 1999). A dose of 0.1mg / kg was administered subcutaneously, 18-24 hours

before the animal was euthenased. Female rats treated with diethyl stilboesterol were oestrogen dominated. This treatment is advantageous in the sense that it abolished the rhythmic, myogenic contractions of an untreated rat uterus, as well as increasing the sensitivity of the tissue to agonist drugs.

Tissue preparation

Two segments from the uterine horns were removed from each rat (approximately 3-4 cm in length) and cleaned. Any fat and connective tissue was trimmed and the horns quickly removed from the rat by cutting off both ends.

The uterine horns were suspended vertically (similar to their natural anatomical hanging position) in 65ml Ugo Basile organ-baths, containing De Jalon's solution, of the following composition (g/litre): NaCl 9.0, KCl 0.42, $NaHCO_3$ 0.5, $CaCl_2$ $.2H_20$ 0.06, and glucose 0.5; pH 7.4. The physiological solution was maintained at a temperature of 32±1°c and continuously bubbled with carbogen (mixture of 95% oxygen and 5% carbon-dioxide). The tissues were suspended under an applied tension of 1g (Domer, 1971; Perez *et al.*, 2008) connected to force-displacement transducers. An equilibration period of 30-45 minutes was allowed, during which time the tissues were washed at regular intervals of 15 minutes (Bower *et al.*, 1999).

The isolated muscles were challenged with step-wise, graded doses of "African Potato" aqueous extract (APE), or selected reference drugs. Extract/drug-induced contractile or relaxant responses were recorded by means of Ugo Basile pen writing recorders. Extract or drug concentrations were repeated (where necessary) after washing out the previous extract/drug concentrations 3-5 times, and the tissue allowed to rest for 5-10 minutes, or until when the tissues returned to baseline tones. Each experiment was repeated 5-6 times, using new uterine horns (Perez *et al.*, 2008).

Two uterine horn strips from the same animal, one used as extract-treated 'test' and the other used as a distilled water-treated 'control' preparation, were always setup each time. This was done to account for the differences in tissue sensitivity.

Drugs used

The following drugs were used as references: acetylcholine chloride, potassium chloride, oxytocin, oxotremorine and noradrenaline. Drug concentrations were made using distilled water on each day of the experiments. Drug concentrations quoted in the text refer to final organ-bath concentrations.

The following protocol was followed during the experimental procedures:

3.2.1.1 Effects of APE on naïve female rat uterine horns

The uterolytic effects of graded concentrations of APE (25-400 mg/ml) were examined on naïve female rat uterine horns in the absence, and in the presence, of some agonist drugs.

3.2.1.2 Effects of APE on uterine horns of primed female rats.

The uterolyic effects of APE (25-400 mg/ml) on uterine horns of stilboesterol-primed female rats were also investigated in the absence, and in the presence, of some agonist drugs.

3.2.2 RAT ISOLATED VAS DEFERENS.

Experimental procedure

Healthy, young adult male rats (250-300 g) were used in this study. Both ends of each vas deferens were tied, and the tissues were subsequently isolated, mounted and connected to force displacement transducers, and suspended horizontally in Ugo Basile organ-baths containing K-H physiological solution of composition (g/litre) : NaCl, 6.92; KCl, 0.34; NaH_2PO_4, 0.15; $NaHCO_3$, 2.1; $MgCl_2$, 0.11; $CaCl_2$, 0.26; and Glucose, 1.00.

Each vas deferens tissue was subjected to a 1 gram tension, and the bathing (K-H) fluid was

continuously bubbled with carbogen.

3.2.2.1 Determination of the effects of APE on vas deferens smooth muscle.

Effects of graded concentrations of APE (25-400 mg/ml) were examined on rat isolated vasa deferentia in the absence, and in the presence, of some agonist drugs.

3.2.3 GUINEA-PIG ISOLATED TRACHEAL SMOOTH MUSCLE

Determination of the broncho-dilatory effect of APE

Healthy, male Dunkin-Hartley guinea-pigs weighing 300-350 g were used in this study.

Tissue Preparation

The protocol outlined by Foster (1960) and Ojewole (1977) was used for the preparation of the tracheal smooth muscle. Guinea-pigs used were starved over-night but allowed free access to water. They were then euthenased with 0.6 ml of sodium pentobarbitone (PB) injection; given via the cardiac route. Euthanasia was preceded with an intra-muscular injection of ketamine/xylazine combination.

The chest of each animal was opened, the entire trachea removed and quickly transferred to a Petri-dish containing warm Krebs-Henseleit physiological solution of composition (g/litre): NaCl 6.92, KCl 0.34, NaH_2PO_4 0.15, $NaHCO_3$ 2.1, $MgCl_2$ 0.11, $CaCl_2$ 0.26, and Glucose 1.00. pH adjusted to 7.4. This solution was continuously bubbled with carbogen.

After removal of the excess connective tissue and fat, the trachea was cut in a spiral manner, length-wise, through the cartilage. Each end was tied with a thread and connected to the transducer, under a resting tension of 1 g. The tissues were mounted, at a temperature of 37 °C, in 65 ml Ugo Basile organ-baths containing the Krebs-Henseleit physiological solution which was continuously aerated with carbogen.

The upper part of the tissue was tied with a thread and connected to a transducer, while the lower one was tied to a stationary wire/tube that aerated the physiological solution.

Experimental procedure

The tissues were allowed an equilibration period of 45 minutes, during which time the bathing physiological solution was changed every 15 minutes, before being challenged with the APE, or any other agonist drug used (Boskabady *et al.*, 2004; Bower *et al.*,1999).

Two muscle preparations from the same animal were used at a time; one used as the extract-treated 'test', and the other as distilled water-treated 'control' preparation.

3.2.3.1 Determination of the effects of APE on tracheal smooth muscle.

The broncholytic effects of graded concentrations of APE (25-400 mg/ml) were investigated on guinea-pig isolated tracheal smooth muscle preparations in the absence, and in the presence, of drugs such as aminophylline, nitroprusside and isoprenaline. APE concentrations (25-400 mg/ml) were added cumulatively to the bath fluid.

3.2.3.2 Determination of the effects of APE on tracheal muscle, pre-contracted with oxotremorine.

The relaxant effects of APE on the sub-maximal muscle tensions developed by the Spasmogen, oxotremorine (a muscalinic agonist) were investigated by addition of graded,

step-wise concentrations of APE (25-400 mg/ml).

The APE concentrations were added to the bath solution after the peak contractile effects of the spasmogen had been attained.

In each case (for each dose of APE added), when the maximal relaxation to each of the graded concentrations of APE had been attained, the tissue was washed-out with fresh physiological solution, 3 to 5 times, and then left to recover (20-30 minutes) and return to drug treatment base line level. Only after attaining the original base-line was the tissue further challenged with a subsequent dose of the spasmogen /APE.

Changes in tension developed by the muscle preparation (contraction/relaxation) were recorded isometrically by means of Ugo Basile force-displacement transducers and pen-writing 'Gemini' recorders (model 7070).

3.2.4 GASTRO-INTESTINAL TRACT SMOOTH MUSCLE

Determination of GIT spasmolytic effects of APE

Healthy, male and female Dunkin-Hartley guinea-pigs weighing 300-350 g were used in this study.

The guinea-pigs were starved over-night, prior to the experiments.

Tissue preparation:

Tubular segments (3-4cm long) of the ileum were removed from euthanased guinea-pigs, according to the procedure described by Domer (1971).

Pieces of the ileum, (3-4 cm long) were pierced through the lumen and threads were tied at both ends for vertical suspension in organ-baths containing K-H physiological solution at a temperature of 34 °C under a 1g tension each.

Experimental procedure:

3.2.4.1 Determination of the effects of APE on gastro-intestinal tract smooth muscle:

The effects of APE (25-400 mg/ml) were examined on isolated ileal tissues of the guine-pig in the absence, and in the presence, of some agonist drugs.

Each added APE concentration was left in contact with the tissue until a maximum relaxation effect was observed and thereafter washed out with physiological solution (5 to 6 times). The next dose was then added after a resting period of 20 minutes.

3.2.4.2 Determination of the effects of APE on ileum tissue pre-contracted with acetycholine (ACh).

After attaining graded concentration-response effect to ACh alone, the tissue was left to rest for about 3 minutes. The lowest concentration of APE was then added to the tissue and left in contact for 3 minutes. Concentration-response effect to the agonist, same as performed previously in the same tissue, was then repeated in presence of increasing concentrations of APE. The period of time between each additions of APE followed by the agonist was 3 minutes (APE was left in contact with the tissue for 3 minutes before addition of the respective concentrations of the agonist). The tissue was left to rest for 10-30 minutes after being washed, before the next concentration-response effect was determined.

3.3 DATA ANALYSIS

For all the tissues employed, the experimental data obtained are presented as means (± SEM). Data obtained from distilled water-treated 'control' tissues were used as baseline values. The differences between the data obtained with the plant's extract and the reference drug-treated, 'test' and 'control', smooth muscle preparations were subjected to two-way analysis of variance (ANOVA; 95% confidence interval), followed by Bonferroni *post-hoc* tests (Graph Pad Prism, version 5; 2007). In all cases, values of $P \leq 0.05$ were taken to imply statistical significance.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Reproductive Smooth Muscles

4.1.1 THE UTERUS

4.1.1.1 Effects of APE on uterine horn smooth muscles:

APE (25-400 mg/ml)-induced decreases in the spasmogen-provoked uterine muscle tones were considered as inhibitory effects of APE. The inhibitory effects of "African Potato" aqueous extract on the sub-maximal muscle tensions developed by each of the spasmogens used were investigated by sequential additions of step-wise, graded concentrations of APE (25-400 mg/ml) to the bath-fluid. In all cases, after the maximal relaxation to each concentration of APE had been achieved, the uterine horn muscle was washed out 4-5 times with fresh de Jalon's physiological solution, and then left to recover for 10-20 minutes and return to pre-drug treatment baseline level, before it was contracted again, with any of the standard spasmogens.

When the uterine horn smooth muscles were left in contact with APE for a relatively long period of time (0.5-5 minutes), a brief initial potentiation of the amplitude of muscle contractions was observed. The extract, however, relaxed the muscle almost immediately thereafter. After attaining the maximum relaxation, the extract was washed-out with physiological solution and left to recover.

The complete recovery obtained from the inhibitory effect of the extract (APE) probably excludes the possibility of toxic effects of the extract, at the employed concentrations, on rat isolated uterine smooth muscles.

The figure 7 below shows the effect of a high dose of *Hypoxis hemerocallidea* corm

aqueous extract (APE, 400 mg/ml) on an isolated uterine horn smooth muscle.

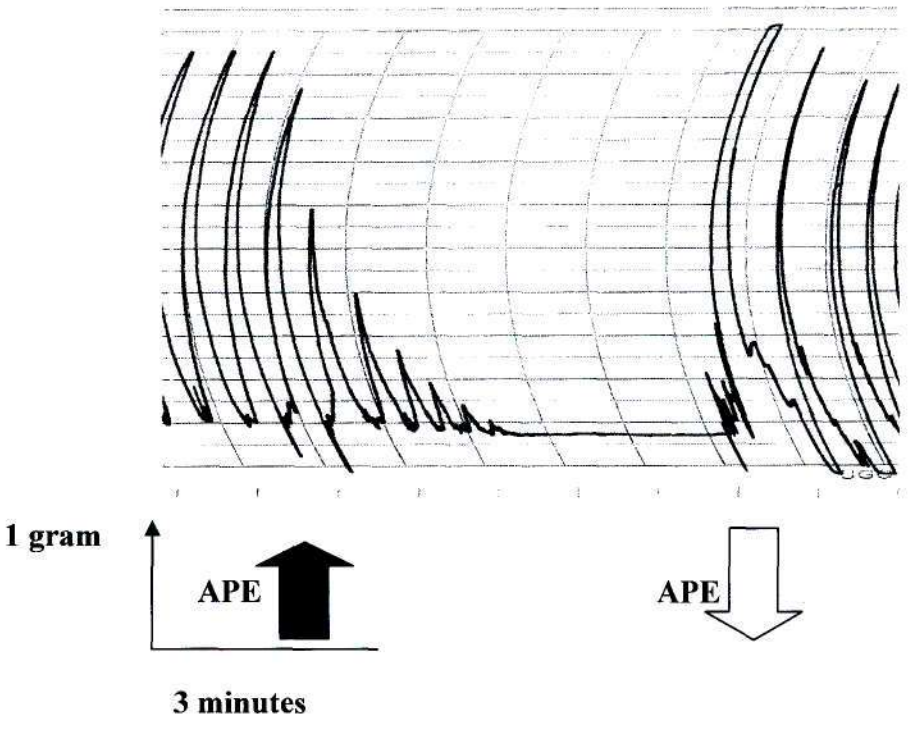


Fig. 7. Relaxant effect of APE (400 mg/ml) on a non-pregnant Wistar rat isolated uterine horn. APE was added to the bath-fluid at the left-hand-side solid upward-pointing arrow. The extract was washed-out at the right-hand-side downward–pointing open arrow.

The lowest concentration of APE (25 mg/ml) produced the highest percentage of contractile amplitude, while the highest concentration (400 mg/ml) completely inhibited the uterine contractions.

APE versus ACh-induced uterine contractions

APE potentiated the amplitude of uterine smooth muscle contractions induced by ACh on

addition of cumulative concentrations of ACh (0.02-0.12 μg/ml). After wash-out, the tissue returned to its normal spontaneous contractions, at the same amplitude as before it was challenged with ACh and APE. The contractile effect of ACh (0.02-0.12 μg/ml) was found to be concentration-dependent. The initial increase in tissue contractile amplitude induced by ACh was inhibited by addition of APE; when APE was left in contact with the tissue for a period of 3 minutes. (see Fig.8).

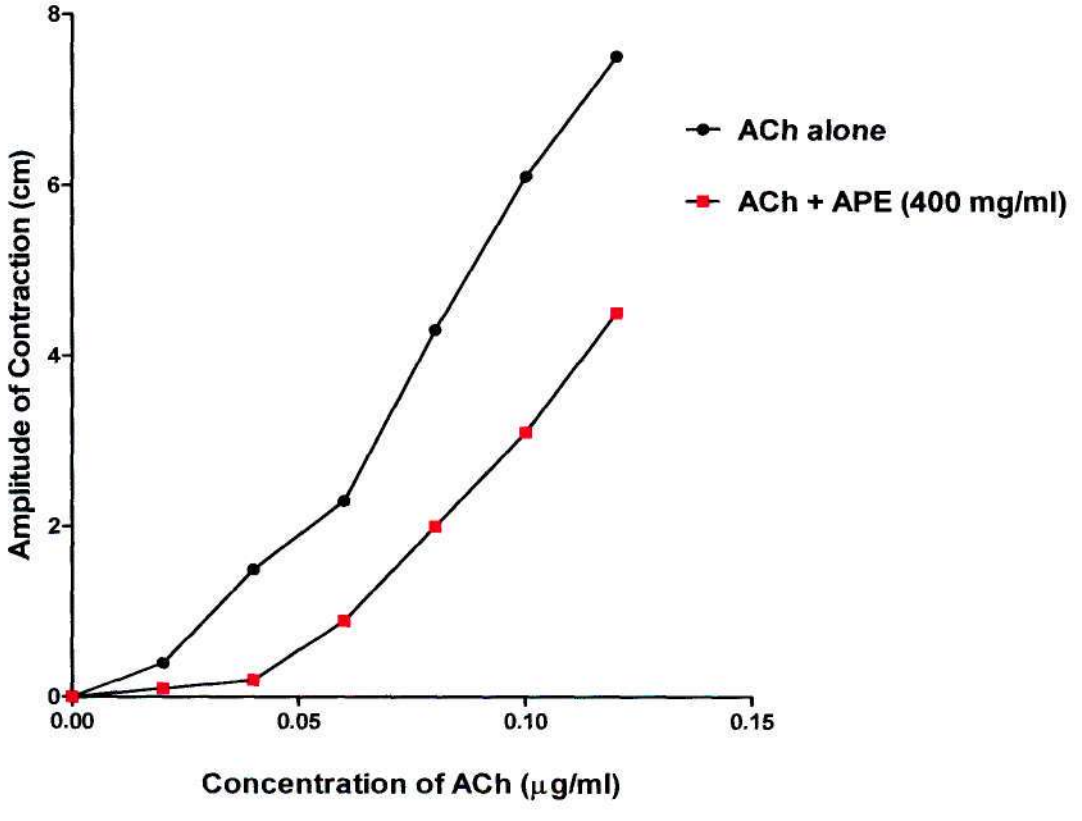


Fig. 8. Comparative contractile effects of APE on isolated uterine horns of a non-pregnant rat, in the absence, and in the presence of ACh. Each point on the graph represents the mean (±SEM) of 6 observations. The amplitude of contraction was measured in centimetres, from the baseline point.

4.1.1.2 Effect of APE on oestrogen-dominated female rat uterine horns.

Uterine horns obtained from rats that had been pre-treated with stilboesterol (oestrogen dominated) were always quiescent and devoid of spontaneous, rhythmic contractions. Relatively low to high concentrations of *Hypoxis hemerocallidea* corm aqueous extra (APE, 25-400 mg/ml), repeatedly relaxed the basal tones developed by the smooth muscle preparations in a concentration-dependent manner.

Low to high concentrations of the plant's extract inhibited acetylcholine (ACh)-and potassium chloride-induced contractions of the uterine horns in a concentration-dependent manner.

Spasmogen-induced tensions developed by the uterine smooth muscles were antagonised by increasing concentrations of APE. The relaxations of the tissues caused by additions of increasing concentrations of APE (100, 200 and 400 mg/ml) were concentration-related (see Figures 9 and 10).

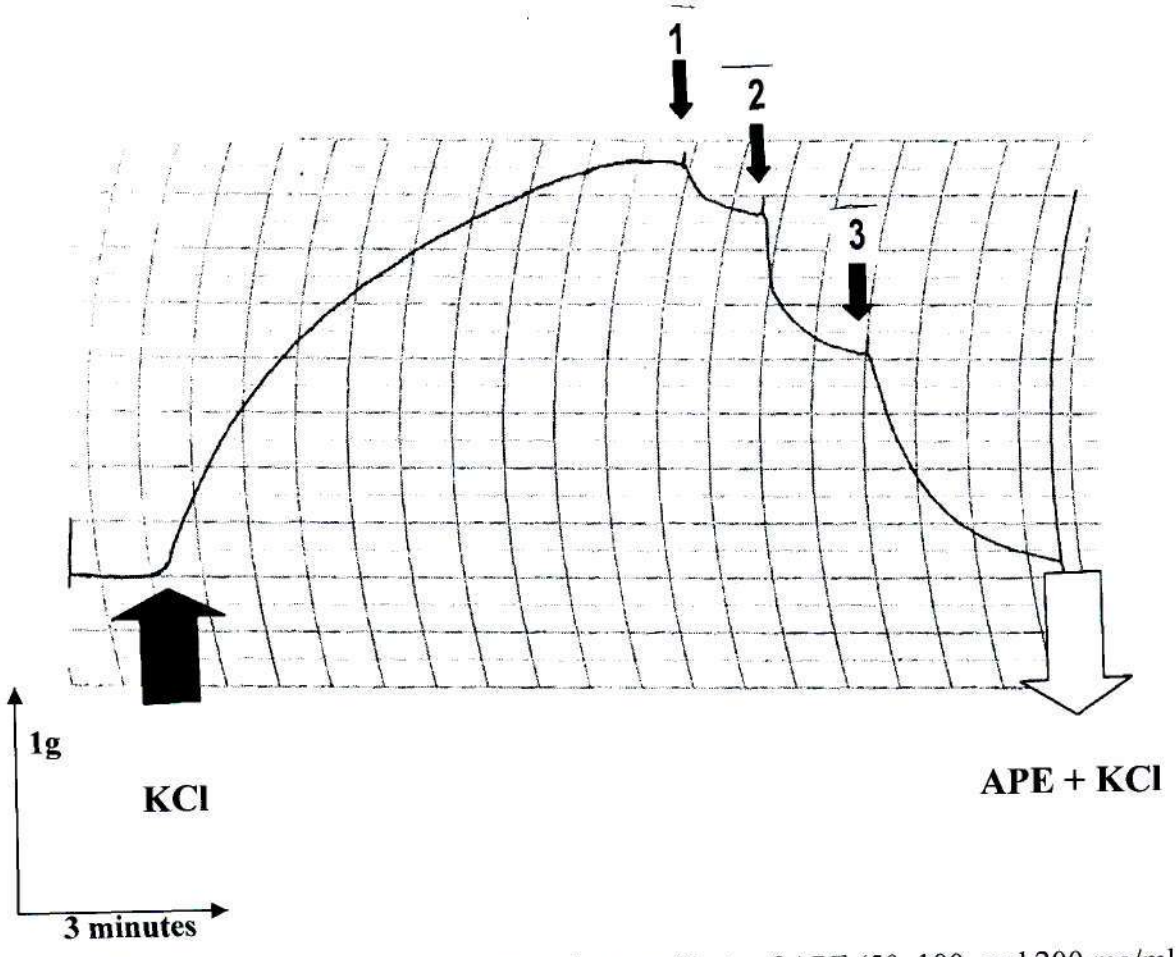


Fig. 9. Representative trace showing the relaxant effects of APE (50, 100, and 200 mg/ml, respectively) added cumulatively to the bath-fluid at the right-hand-side 1, 2 and 3 solid down-ward-pointing arrows. The primed rat isolated uterus was depolarized by addition of KCl (40 mM), at the left-hand-side solid upward-pointing arrow. Both KCl and APE were washed out 5 times at the right-hand-side open downward-pointing arrow.

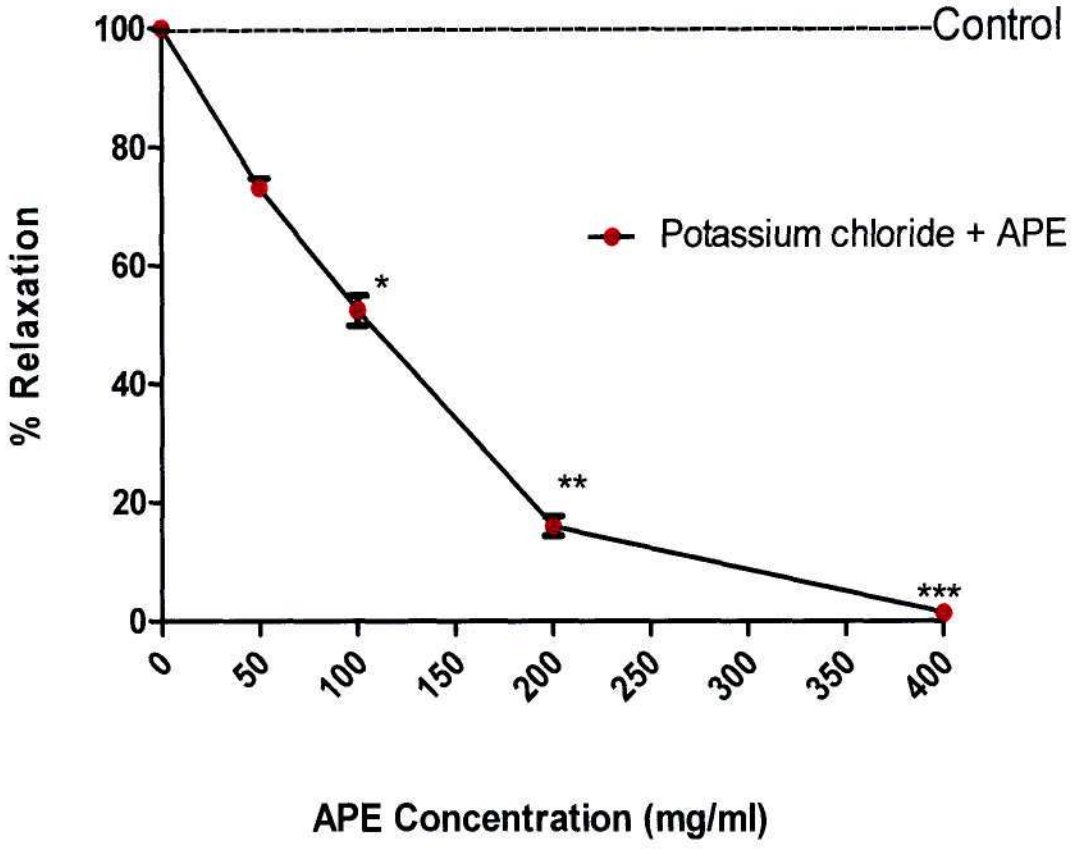


Fig. 10. Effect of increasing concentrations of APE on KCl-contracted oestrogen-dominated uterus. APE (50, 100, 200 and 400 mg/ml) were added cumulatively to the bath-fluid. Values presented are means (±SEM) of 6-8 observations. (* ($p<0.05$), ** ($p<0.1$) and *** ($p<0.001$) versus the control.

APE inhibited uterine smooth muscle contractions induced by high KCl (40 mM). APE (400 mg/ml) produced complete relaxation of the high KCl-induced contractions. This probably suggests that APE may block the influx of calcium through the L-voltage-dependent calcium channels (Perez *et al.*, 2008). This inhibition of calcium is said not to

depend on NO (nitric oxide) and/or beta-adrenergic receptors; and can be reversed by addition of calcium; which suggests, according to the investigators (Perez *et al.*, 2008) that the drug in question/used in their study, acted on L-voltage-sensitive calcium channels.

According to Gutierrez *et al.* (1998), the relaxant effect of 17α-oestradiol on rat uterus could be produced by reduction of calcium influx, and induction of polyamine synthesis by transcriptional mechanisms that do not involve oestrogenic receptors. In this same study, 17α-oestradiol relaxed KCl-induced tonic contractions in a concentration-dependent fashion. The mechanism was postulated not to be mediated by oestrogenic receptors or cAMP. The study by Revuelta *et al.* (1997) also supported these findings, stating that drugs with oestrogenic activity produce vascular and uterine smooth muscle relaxations by inhibiting calcium influx through a non-genomic mechanism of action. In this study, therefore, the uterolytic action of the corm's extract is unlikely to be mediated via β_2-adrenoceptor stimulation but probably mediated through a *non-specific* spasmolytic mechanism.

4.1.2 THE VAS DEFERENS

The vas deferens muscle is not spontaneously-active and normally contracts only in response to neural stimulation or exogenous spasmogen administration. The sympathetic nervous stimulation gives rise to an excitatory junction potential which add to each other, and when a critical value is reached, a spike is initiated. In the guinea-pig vas deferens, the spikes are all-or-none, with over-shots of 20mv (Chinoy *et al.*, 1983).

The vas deferens smooth muscle contraction is generally associated with alpha-adrenoceptor stimulation, and relaxation with beta-adrenoceptor stimulation. Alpha-receptor activation involves an increase in permeability to calcium, whereas activation of beta-receptors promote a decrease in calcium permeability and hence, relaxation. The calcium that activates noradrenaline-induced contractions originates from the loosely-bound calcium pool in extra-cellular fluid, as well as from the pool of tightly-bound

calcium (bound to calmodulin, a polypeptide which acts as an activator for norepinephrine release and muscle contraction), found intra-cellularly in the muscle fibre (Chinoy *et al.*, 1983). In the same cited study, the density of innervations of the vas deferens, the amount of calcium, sodium and potassium ions found in each, were noted to complement the physiological properties of the right and left vas deferens. It was thought probable that, the left vas deferens was more innervated, containing more of the calmodulin-bound calcium intra-cellularly, and less sodium, and hence, more responsive to graded concentrations of adrenaline and noradrenaline.

Adrenaline was used in the present study to contract the vas deferens smooth muscle, prior to addition of APE (Walland *et al*; 1997). This sympathetic agonist plays a role in the release of calcium in the smooth muscle. Noradrenaline released from the sympathetic nerves acts on α_1-adrenoceptors to increase systosolic calcium, and promote the smooth muscle contraction.

The vas deferens is an example of a multi-unit smooth muscle. It exhibits little or no spontaneous activity; and depends on nervous stimulation for activation and generation of force (neurogenic force). Many of its muscle cells are, however, in direct contact with innervating nerve fibers, and communicate with each other via gap-junctions, as described earlier in Chapter 1.

An increase in the frequency of nerve impulses promotes a temporal summation of excitatory post synaptic potentials, and hence, generation of calcium-dependent action potentials and calcium influx, hence increasing neurogenic tone. Increase in impulse frequency of nerves enhances the rate of neurotransmitter release and membrane receptor occupancy by their respective agonists.

An influx of calcium can be blocked by antagonists of noradrenaline receptors (α_1-adrenergic receptors), as well as by calcium-channel blockers (calcium antagonists), and by drugs that open potassium channels (potassium agonists) which cause hyper-polarisation and in turn, close the voltage-dependent calcium channels.

4.1.2.1 Determination of the effects of APE on vas deferens tissues, pre-contracted with adrenaline.

Adrenaline (0.2-1.6 μg/ml) induced concentration-related contractions of the rat and guinea-pig vasa deferential smooth muscles. Addition of APE (25-400 mg/ml) relaxed the muscles and antagonised adrenaline-induced contractions of the tissues. The observed effects of increasing concentrations of adrenaline, alone and in presence of the APE are shown in Figure 11.

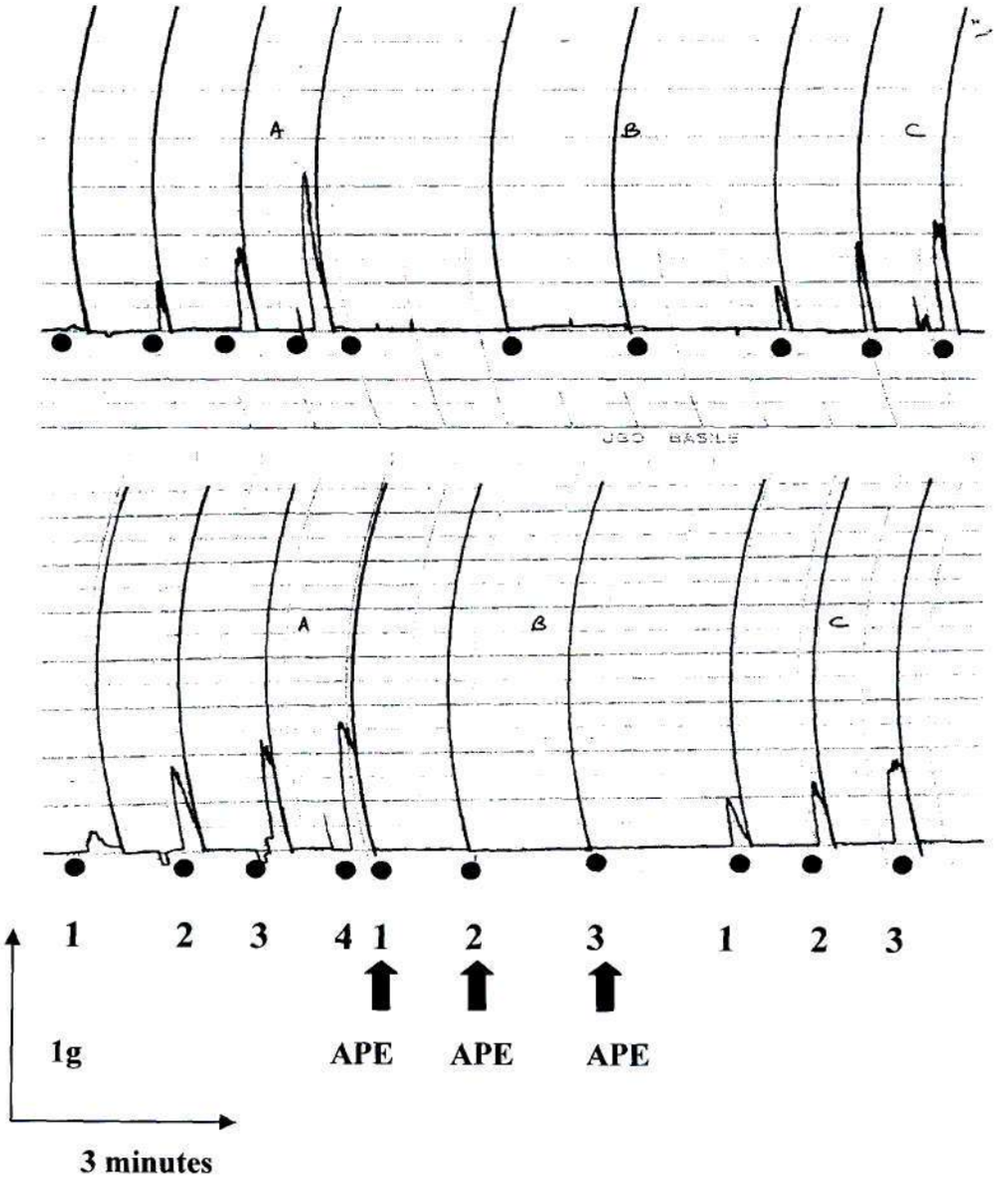


Fig. 11. Effects of increasing concentrations of adrenaline, alone (panel A), and in the presence of APE (panel B), on adrenaline-induced contractions of guinea-pig isolated vas deferens. The solid dots 1-4 indicate points of addition of adrenaline (0.2, 0.4, 0.8 and1.6 μg/ml)/and APE, 1-3 (100, 200 and 400 mg/ml), respectively. Each of the respective concentrations were left in contact with the tissue for 30 seconds, and thereafter washed out. Panel C denotes tissue recovery, after 5 tissue wash-outs.

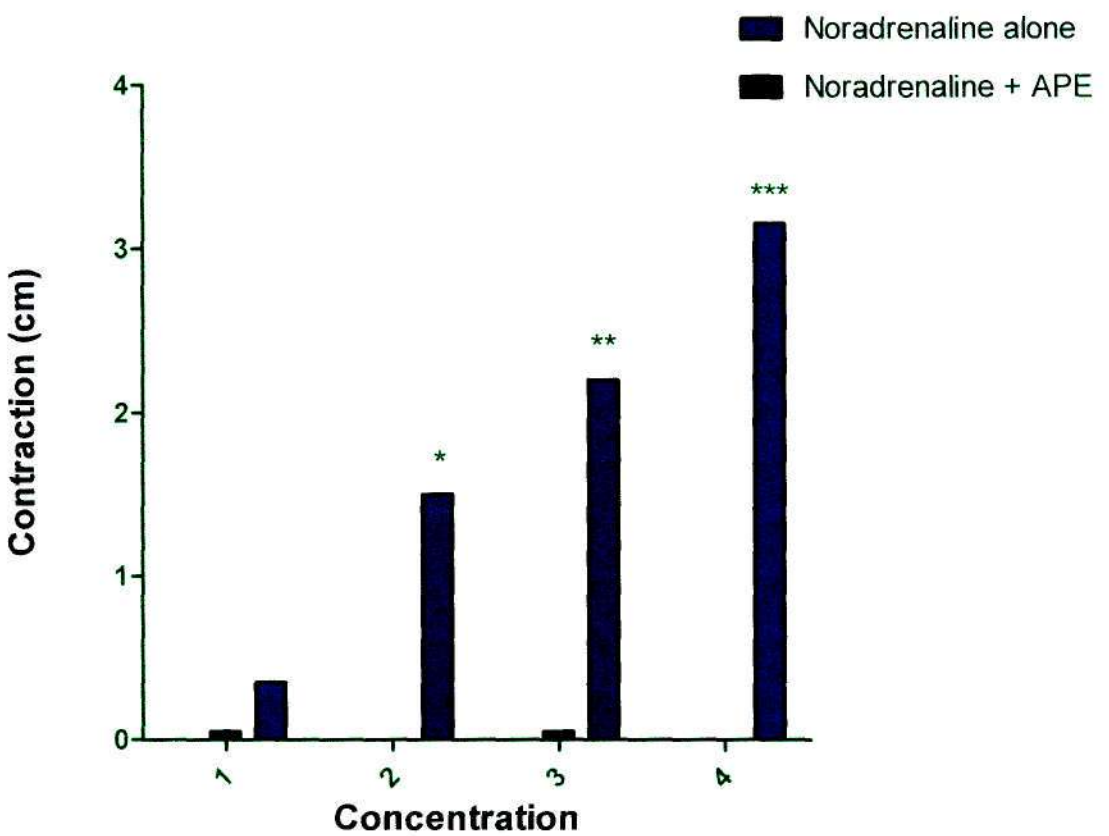


Fig. 12. Inhibitory effects of APE on noradrenaline-induced contractions of the vas deferens. Points 1 to 4 represent concentrations of noradrenaline (0.2, 0.4, 0.8, 1.6 μg/ml) and APE (50, 100, 200 and 400 mg/ml), respectively. Each value is a mean (±SEM) of 6-8 observations. * ($p<0.05$), ** ($p<0.1$) and *** ($p<0.001$).

Table 2 shows the data analysed to give Figure 11 above. The analysis was done using 2-way ANOVA, followed by Bonferroni *post-hoc* test.

Source of Variation	% of total variation	P value
Interaction	19.45	0.0035
Column Factor	57.24	P<0.0001
Row Factor	18.50	0.0041

Source of Variation	P value summary	Significant?
Interaction	**	Yes
Column Factor	***	Yes
Row Factor	**	Yes

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	3	4.283	1.428	10.77
Column Factor	1	12.60	12.60	95.11
Row Factor	3	4.073	1.358	10.25
Residual	8	1.060	0.1325	

Number of missing values 8

Bonferroni posttests :

Epinephrine vs Epinephrine + Extract

Row Factor	Epinephrine	Epinephrine + Extract	Difference	95% CI of diff.
Row 1	0.3500	0.0500	-0.3000	-1.638 to 1.038
Row 2	1.500	0.0000	-1.500	-2.838 to -0.1617
Row 3	2.200	0.0500	-2.150	-3.488 to -0.8117
Row 4	3.150	0.0000	-3.150	-4.488 to -1.8

Row Factor	Difference	t	P value	Summary
Row 1	-0.3000	0.8242	P > 0.05	ns
Row 2	-1.500	4.121	P < 0.05	*
Row 3	-2.150	5.907	P<0.01	**
Row 4	-3.150	8.654	P<0.001	***

Table. 2. Data analysed to give Figure 11 (2-way ANOVA, followed by Bonferroni *post-hoc* test). Inhibitory effects of APE on adrenaline–induced contractions of the vas deferens.

The inhibitory effects of APE on adrenaline-induced contractions of the vas deferens are thought to be due to antagonism of α_1-adrenergic receptors.

4.2 Respiratory Smooth Muscle

4.2.1 Effects of APE on guinea-pig isolated tracheal smooth muscles

Agonist-receptor interactions regulate airway smooth muscle tone through activation of guanine nucleotide binding proteins (G-proteins), which are coupled to second messenger pathways that mediate changes in the tissue's contractile state. According to Hakon *et al.* (1998), with respect to airway smooth muscle contraction, receptor activation elicits phosphatidylinositol turnover that results in the formation of the second messengers, 1, 2-diacylglyserol, which activates protein kinase C (PKC), and inositol 1, 4, 5-triphosphate, which binds to its intracellular receptor to mobilise intracellular calcium. Both the mobilisation of calcium and activation of PKC play critical roles in initiating and acutely modulating the intensity and duration of the airway smooth muscle contraction.

Bronchodilator-agonist mediated receptor activation is typically coupled to an enhanced accumulation of the second messenger, adenosine 3', 5', -cyclic monophosphate (cAMP) which, through activation of cAMP-dependent protein kinase, induces posphorylation of specific proteins, leading to airway smooth muscle relaxation.

In a study by Yoshii *et al.* (1999), relaxation of intact trachea was attributed to selective inhibition of the protein rho/ROCK (rho- associated coil-forming protein kinase) pathway by Y-27632. Calcium sensitisation through this pathway was thought to play a role in the sustained phase of airway smooth muscle contraction, including human airway smooth muscle. Inhibition of this rho/ROCK signalling was also thought to become the new strategy to resolve airflow limitations in diseases such as bronchial asthma.

APE (25-400 mg/ml) relaxed guinea-pig isolated tracheal smooth muscles. The muscles

were then contracted with oxotremorine (1μg/ml). Figure 12 shows typical traces obtained.

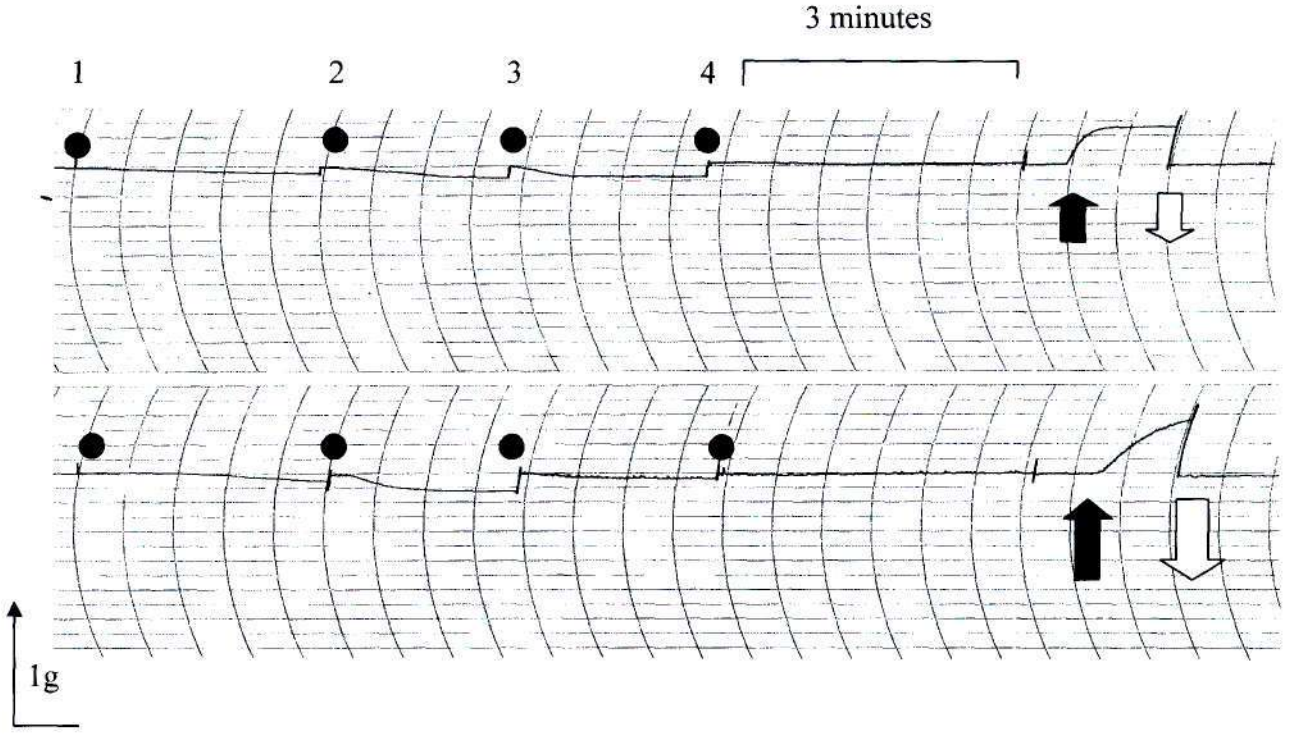


Fig. 13. Comparative relaxant effects of APE (25-400 mg/ml) on two different isolated tracheal tissues of the guinea-pig. The solid dots 1 to 4 indicate APE, 25, 50, 100 and 200 mg/ml, respectively, exogenously added to the bath-fluid. The upward-pointing solid arrows indicate the points of addition of oxotremorine (1μg/ml). The tissues were washed-out 5 times at the downward-pointing right-hand side arrows.

The results in this study indicate that APE, like isoprenaline, aminophylline, nitroprusside, inhibited and relaxed spasmogen-evoked contractions of guinea-pig isolated tracheal muscle preparations in a concentration dependent manner. This observation suggests that APE has a bronchodilatory property. The exact mechanism of the bronchospasmolytic action APE in this regard is not known; but is likely to be due to stimulation of the $beta_2$-adrenoceptors that are abundant in the bronchial smooth muscle. According to Ojewole and Nyinawumuntu, 2008 (unpublished observations), this hypothesis is strengthened by the

following observations:

i) aminophylline and nitroprusside produced pharmacological effects that are similar to those of APE on guinea-pig tracheal muscle preparations, and

ii) concentrations of propranolol which markedly inhibited or completely abolished the bronchospasmolytic effect of isoprenaline did not affect the bronchospasmolytic action of APE.

In the same study, it was also observed that APE inhibited the spasmogenic action of potassium on the tracheal smooth muscle preparations. This observation probably suggests that the plant extract is unlikely to produce its bronchodilatory effects through a specific receptor, but rather, through a non-specific bronchospasmolytic mechanism.

4.3 Gastro Intestinal Tract Smooth Muscle.

4.3.1 Effects of APE on guinea-pig isolated ileal smooth muscles.

The contractile pattern of the guinea-pig isolated ileum was first determined. The ileum was found to possess its own myogenic contractions. These myogenic contractions of the ileum were inhibited or abolished by APE (25-400 mg/ml). On washing out the APE, the tissue completely recovered (see Figure 14)

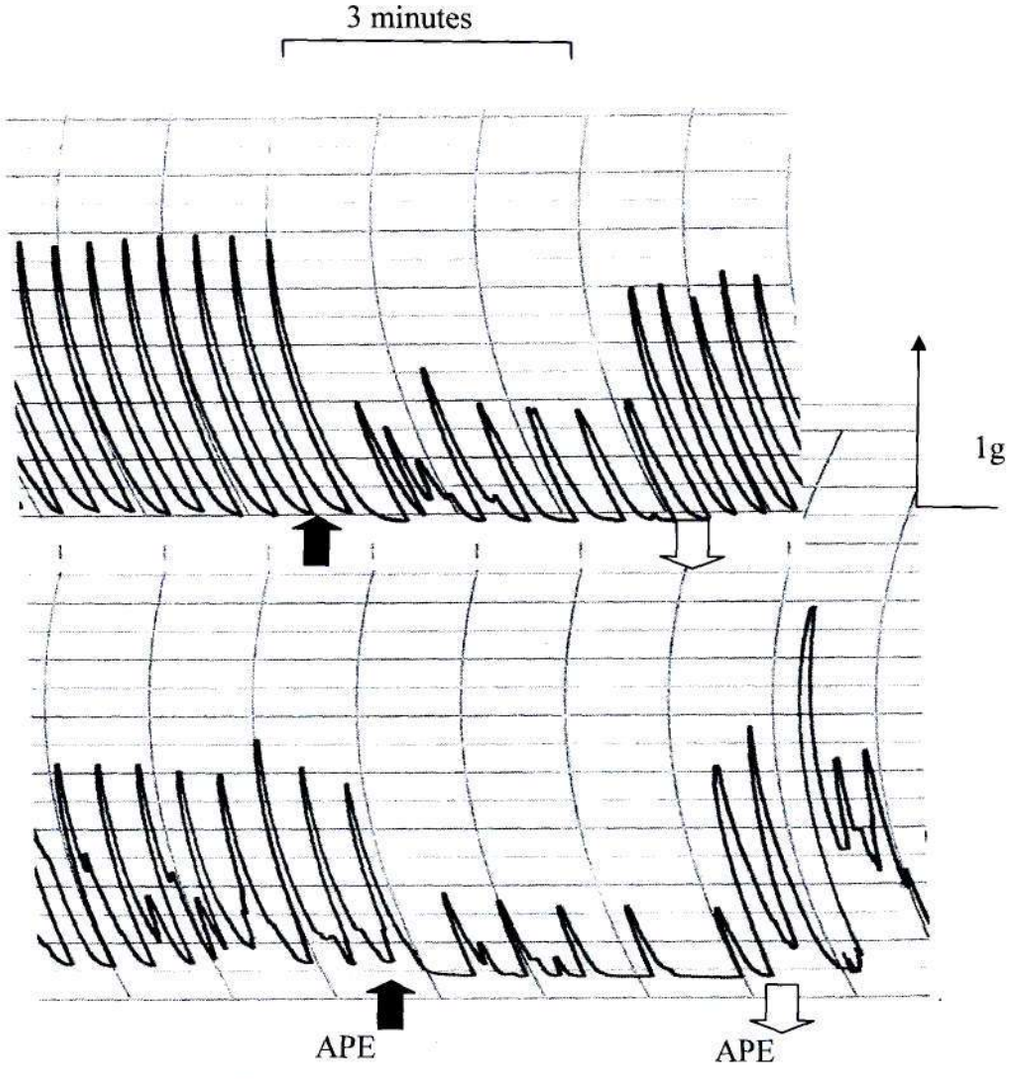


Fig. 14. Relaxant effects of single concentrations of APE (100 and 300 mg/ml) on two different pieces of guinea-pig isolated ileum. The concentrations of APE were sequentially added to the bath-fluid at the left-hand-side solid upward-pointing arrows, left in contact with the tissue for 3 minutes, and then washed out at the right-hand-side downward-pointing arrows.

4.3.2 Effects of APE on ACh pre-contracted ileal smooth muscles.

Contractions of the ileal muscles provoked by the ACh were inhibited or abolished in a concentration-related manner by APE (25-400 mg/ml). Figure 14 shows a typical trace

obtained in response to graded concentrations of ACh (0.1-3.2 μg/ml) in the absence (panel A), and in the presence, of APE (200 mg/ml), (panel B).

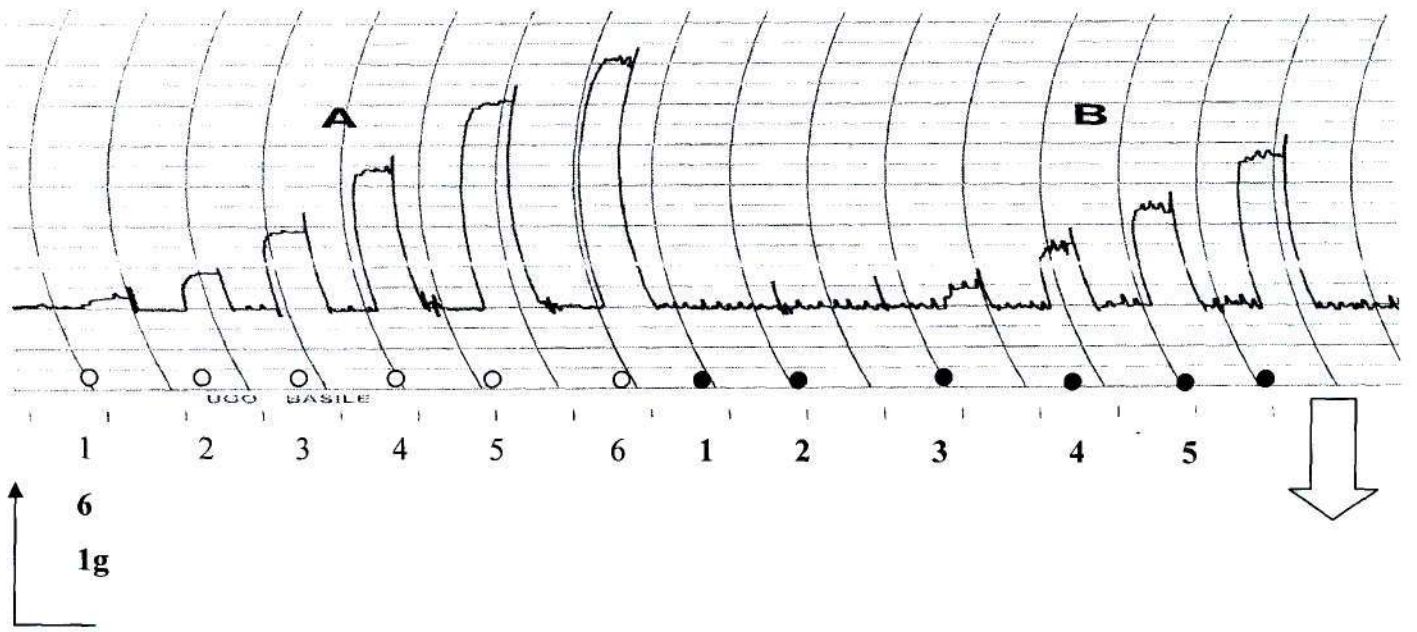


Fig. 15. Effect APE (200 mg/ml) on ACh-induced contractions of guinea-pig isolated ileum. Contractions of the tissue were induced by sequential additions of increasing concentrations of ACh (0.1-3.2 μg/ml, respectively) to the bath-fluid at the clear dots 1-6 (panel A). The solid dots 1-6 (panel B) indicate additions of both APE (200 mg/ml) and ACh (0.1-3.2 μg/ml, respectively) as in panel A.

APE (25-400 mg/ml) antagonised ACh- and other spasmogen-inducedcontractions of the guinea-pig ileum in a concentration-related manner. The spasmolytic effect of APE is thought to be mediated via a non-specific mechanism.

Source of Variation	% of total variation	P value
Interaction	10.39	0.0035
Concentration of ACh	60.06	P<0.0001
% Contraction	14.01	P<0.0001

Source of Variation	P value summary	Significant?
Interaction	**	Yes
Concentration of ACh	***	Yes
% Contraction	***	Yes

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	6	13.9	2.32	4.01
Concentration of ACh	2	80.5	40.3	69.6
% Contraction	3	18.8	6.26	10.8
Residual	36	20.8	0.579	

Bonferroni post-tests

Concentration of ACh (0.1-3.2 μg/ml) vs Ach + Extract

% Contraction	Conc.of ACh (0.1-3.2 μg/ml)	ACh + Extract	Difference	95% CI of diff.
Row 1	2.00	0.475	-1.53	3.09 to 0.0373
Row 2	2.85	0.275	-2.58	-4.14 to -1.01
Row 3	3.88	0.400	-3.48	-5.04 to -1.91
Row 4	5.43	0.325	-5.10	-6.66 to -3.54

% Contraction	Difference	t	P value	Summary
Row 1	-1.53	2.84	P < 0.05	*
Row 2	-2.58	4.79	P<0.001	***
Row 3	-3.48	6.46	P<0.001	***
Row 4	-5.10	9.48	P<0.001	***

Table. 3. Results of two-way ANOVA analysis of data. Concentration of ACh (0.1-3.2 μg/ml) vs. ACh + Extract. These results are shown in the graph below (Fig.16).

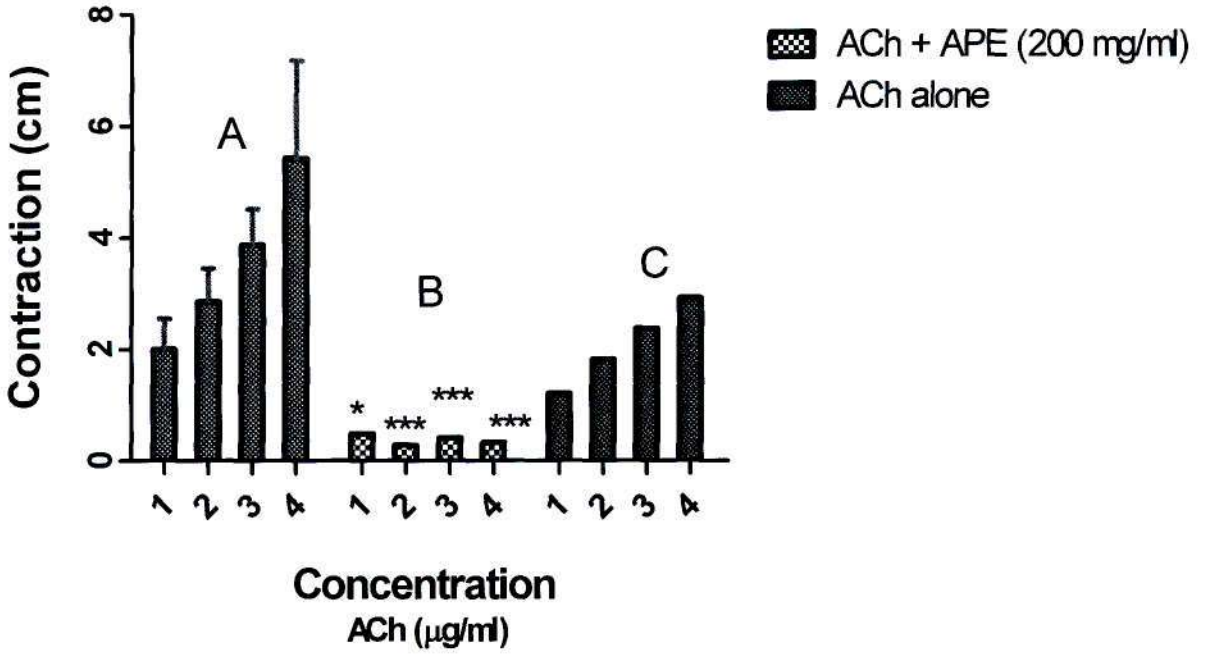


Fig. 16. Relaxant effect of APE on ACh-induced contractions of guinea-pig isolated ileum. The responses shown represent; A (ACh alone), B (ACh + APE) and C (ACh alone after wash-out). Each value is the mean (±SEM) of 6 observations. (* ($p<0.05$), ** ($p<0.1$) and *** ($p<0.001$) versus the control. Concentrations1 to 4 are represented in Table 3 (Rows 1-4).

CHAPTER 5

5 CONCLUSION AND RECOMMENDATIONS.

The results obtained in the present study support some of the folkloric claims about the therapeutic effects of "African potato". Experimental evidence obtained in the present laboratory animal study indicates that *Hypoxis hemerocallidea* corm aqueous extract (APE) possesses uterolytic, vas deferens and guinea-pig ileum smooth muscle relaxant, and bronchodilatory activities. These findings lend pharmacological support to the anecdotal, ethnomedical uses of 'African Potato' in some rural communities of South Africa.

Generally, the exact mechanisms of action of the plant's extract on the smooth muscles studied were, however, not established. I would, therefore, recommend further investigations in this regard. Such an investigation should include determination of the effects observed in this study, in presence of other known agonists and antagonists under various experimental conditions both *in vivo* and *in vitro*.

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APPENDIX I

ETHICAL CLEARANCE LETTER

UNIVERSITY OF
KWAZULU-NATAL

RESEARCH OFFICE
HOWARD COLLEGE CAMPUS
E-Mail: bawa@ukzn.ac.za
Tel.: 27-31-260 2273 Fax: 27-31-260 2384

25 June 2007

Reference: 029/07/Animal

Miss A Nyinawumuntu
School of Pharmacy and Pharmacology
University of KwaZulu-Natal
WESTVILLE CAMPUS

Dear Miss Nyinawumuntu

Ethical Approval of Research Project using Animals

I have pleasure in informing you that on recommendation of the review panel, the Animal Ethics Sub-committee of the University Ethics Committee has granted ethical approval for 2007 on the following project:

"Pharmacological effects of *Hypoxis hemerocallidea* Fisch. & C.A. Mey. Corm ("African Potato") Aqueous Extract on Mammalian Smooth and Skeletal Muscles *in vitro*."

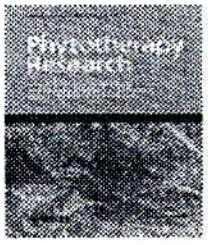
Yours sincerely

Professor Theresa HT Coetzer
Chairperson: Animal Ethics Sub-committee

Cc Registrar
Research Office
→ Head of School

APPENDIX II

ARTICLES PUBLISHED FROM THE STUDY PRESENTED IN THIS THESIS



Phytotherapy Research

Published Online: 12 Jan 2009

Research Article

Antidiarrhoeal activity of *Hypoxis hemerocallidea* Fisch. & C. A. Mey. (Hypoxidaceae) Corm ('African potato') aqueous extract in rodents

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KEYWORDS

Hypoxis hemerocallidea corm • 'African potato' • aqueous extract • antidiarrhoeal activity

ABSTRACT

This study investigated the antidiarrhoeal activity of *Hypoxis hemerocallidea* corm aqueous extract (APE) on experimentally-induced diarrhoea, gastrointestinal motility, intestinal transit and enteropooling in rodents. *H. hemerocallidea* corm aqueous extract (APE, 50-400 mg/kg, p.o.) produced dose-dependent and significant ($p < 0.05$-0.01) protection of rats and mice against castor oil-induced diarrhoea, inhibited intestinal transit and delayed gastric emptying. Like atropine (1 mg/kg, p.o.), APE (50-400 mg/kg, p.o.) produced dose-dependent and significant ($p < 0.05$-0.01) antimotility effect, and caused dose-related inhibition of castor oil-induced enteropooling in the animals. Like loperamide (10 mg/kg, p.o.), APE (50-400 mg/kg, p.o.) dose-dependently and significantly ($p < 0.05$-0.01) delayed the onset of castor oil-induced diarrhoea, decreased the frequency of defaecation and reduced the severity of diarrhoea in the rodents. Compared with control animals, APE (50-400 mg/kg, p.o.) dose-dependently and significantly ($p < 0.05$-0.01) decreased the volume of castor oil-induced intestinal fluid secretion, and reduced the number, weight and wetness of faecal droppings. APE (50-400 mg/mL) also produced concentration-related and significant ($p < 0.05$-0.01) inhibitions of the spontaneous, pendular contractions of the rabbit isolated duodenum, and attenuated acetylcholine (ACh, 0.1-5.0 μg/mL)-induced contractions of the guinea-pig isolated ileum. Although the precise mechanism of the antidiarrhoeal activity of APE could not be established, the results of this study indicate that APE possesses antidiarrhoeal activity. This finding supports the use of 'African potato' as a natural supplementary remedy for the treatment, management and/or control of diarrhoea in some rural communities of southern Africa. Copyright © 2009 John Wiley & Sons, Ltd.

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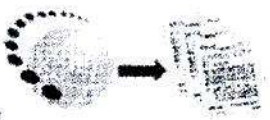

Request Reprint

Uterolytic effect of *Hypoxis hemerocallidea* Fisch. & C.A. Mey. (Hypoxidaceae) corm ['African Potato'] aqueous extract

Agatha NYINAWUMUNTU[1], Emmanuel O. AWE[1] and John A. O. OJEWOLE[1]

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Received August 6, 2008; Accepted September 9, 2008

Abstract

Extracts of *Hypoxis hemerocallidea* corm (African potato) are commonly used by some traditional health practitioners in KwaZulu-Natal Province of South Africa as natural antenatal remedy to prevent threatening or premature abortion and miscarriage, and to ensure successful confinement. In this study, we investigated the uterolytic activity of *H. hemerocallidea* corm aqueous extract on spontaneous, rhythmic contractions of uterine horns taken from pregnant rats and guinea-pigs, as well as on spasmogen-provoked contractions of stilboesterol-primed, oestrogen-dominated, non-pregnant rat and guinea-pig isolated uterine horns. Relatively low to high concentrations of *H. hemerocallidea* corm aqueous extract (APE, 25–400 mg/ml) inhibited the amplitude of the spontaneous, rhythmic contractions of, and relaxed, uterine horns isolated from pregnant rats and guinea-pigs in a concentration-related manner. Furthermore, relatively low to high concentrations of APE (25–400 mg/ml) relaxed basal tones of uterine horns taken from non-pregnant, oestrogen-dominated rats and guinea-pigs in a concentration-dependent manner. The same moderately low to high concentrations of APE (25–400 mg/ml) inhibited acetylcholine-, oxytocin-, bradykinin-, and potassium chloride (K^+)-induced contractions of oestrogen-dominated rat and guinea-pig isolated uterine horns in a concentration-related manner. Although the mechanism of uterolytic action of APE could not be established, the results of the present study lend pharmacological credence to the folkloric, ethnomedical uses of APE as a natural antenatal remedy for threatening or premature abortion, and suggest that the uterolytic action of the corm's extract is unlikely to be mediated via β_2-adrenoceptor stimulation, but probably mediated through a *non-specific* spasmolytic mechanism.

Key words: *Hypoxis hemerocallidea* corm, African Potato, aqueous extract (APE), natural antenatal remedy, threatening or premature abortion

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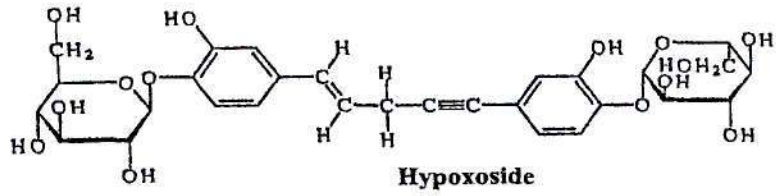
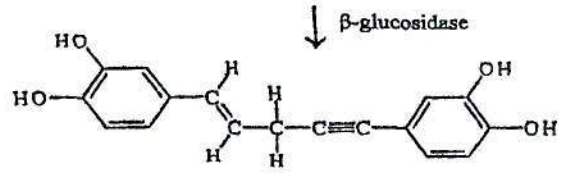
Introduction

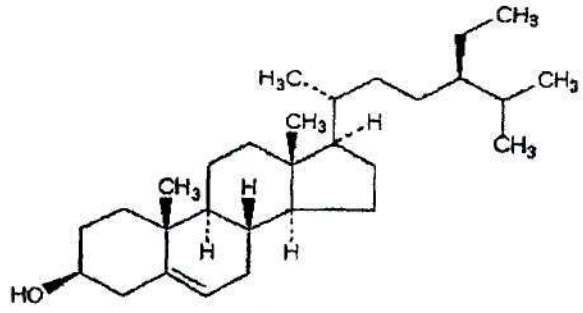
Despite the remarkable advancement made in orthodox medicine within the last few decades, available biomedical evidence indicates that in South Africa, approximately 80% of the black Africans still rely on traditional health practitioners and medicinal plants for their daily healthcare needs. The use of traditional medicines during pregnancy still plays a crucial role in the lives of the people living in rural areas where modern healthcare facilities are often lacking. In many rural African communities, pregnancy is usually accompanied by many traditional taboos and ceremonies to ensure successful confinement and births of healthy children (Sewram *et al.* 1998; 2000). In the rural communities, various morphological parts of an array of plants from diverse families and species are usually consumed by pregnant women as antenatal remedies. Such antenatal remedies are traditionally formulated as powders, extracts, infusions, decoctions, concoctions, bath soaps, and so forth, to ensure successful confinement, and/or to induce or accelerate labour at full-term. Many known medicinal plants are used as natural remedies to induce or accelerate labour (Sewram *et al.* 1998; 2000), while a few others are used to prevent threatening or premature abortion, and ensure successful confinement. Unfortunately, however, the quality, safety and efficacy of most herbal medicines and plant products used as South African traditional antenatal remedies have not been subjected to scientific scrutiny. One of such frequently-used South African antenatal medicinal plants is *Hypoxis hemerocallidea* (Fisch. & C.A. Mey.; family, Hypoxidaceae). This 'cure-all' medicinal plant of South Africa is a tuberous, perennial herb with long, strap-shaped leaves and yellow, star-shaped flowers. The broad and slightly hairy leaves of *H. hemerocallidea* are arranged one above the other to form three distinct groups of leaves spreading outwards from the centre of the plant, while the bright yellow, star-shaped flowers are borne on long, slender stalks (Van Wyk *et al.*, 2002). The tuberous rootstock (*i.e.*, the 'corm') of the herb is popularly known as '*African Potato*' in South Africa, and is widely used in South African traditional medicines as a remedy for a catalogue of human ailments. The traditional healers of South Africa have employed the corm of the plant as a "***muthi***" (isiZulu word for "***medicine***") for centuries, and now, the humble African Potato has been claimed to be a 'miracle plant medicine' in the fight against various modern and 21st century diseases of mankind. This South African 'cure-all miracle plant medicine' has been claimed to be an effective remedy against HIV/AIDS-related diseases, arthritis, yuppie flu, hypertension, diabetes mellitus, cancer, psoriasis, gastric and duodenal ulcers, tuberculosis, urinary tract infections, asthma, and some central nervous system (CNS) disorders, especially epilepsy and childhood convulsions (Watt and Breyer-Brandwijk, 1962; Hutchings, 1989; Pujol, 1990; Hutchings *et al.* 1996; Albrecht, 1995; Albrecht *et al.*, 1995; Van Wyk *et al.*, 2002). The above pharmacotherapeutic effects of African Potato have been strongly ascribed to the sterols, stanols, sterolins and norlignan glycoside, hypoxoside, present in the corm (Drewes *et al.*, 1984; Albrecht *et al.*, 1995; Bouic *et al.*, 1996; Nair *et al.*, 2007). The best-known and fully-established chemical constituents of the plant's corm are presented in Fig. 1.

The effects of some cations, anions and certain drugs on spontaneous activities of uterine smooth muscle strips taken from non-pregnant and pregnant mammals have been investigated and reported by a number of workers (Kuriyama and Suzuki, 1976; Osa and Kawarabayashi,





Rooperol



β-Sitosterol

Fig. 1. Structures of hypoxoside, rooperol and *β*-sitosterol. The biologically-inactive *norlignan diglucoside, hypoxoside, is deconjugated and converted by β-glucosidase* enzyme to form the biologically-active aglycone, rooperol.

1977; Kuriyama *et al.*, 1998). According to Kuriyama *et al.* (1998), visceral smooth muscle cells play a critical role, through changes in their contraction-relaxation cycle, in the maintenance of homeostasis in biological systems. The investigators further observed that features of the cells differed markedly from one tissue to the other, and from one species to another. The workers also noted that often, there were regional differences within a given tissue.

Using micro-electrode techniques, Kuriyama and Suzuki (1976) investigated the changes in membrane electrical properties of rat myometrium during gestation and following ovarian hormone treatments. The investigators recorded spontaneously-generated bursts of electrical activity, alternating with silent periods, from non-pregnant, pregnant and post-partum myometria, and found that membrane potential was highest during the middle stage of gestation, although the spike amplitude within a burst was not uniform. The investigators further observed that in the final stage of gestation and during parturition, the membrane potential was low, and that the spikes within a burst were of low frequency and uniform amplitude. Kuriyama and Suzuki (1976) further observed that the resting and active membrane properties of progesterone-treated myometria were similar to those seen during the middle stages of gestation.

Osa and Kawarabayashi (1977), using the double sucrose gap method, investigated the

effects of Na^+, Ca^{2+}, anions and isoprenaline on the plateau potential in circular muscles of pregnant rat myometrium, and observed that the amplitude and duration of the plateau potential increased by raising the concentration of the external Ca^{2+} to between 0.3 and 3 mM, and that the plateau potential decreased when the external Ca^{2+} concentration was further increased. The investigators also observed that the plateau potential was prolonged in low Na^+ solution, and that isoprenaline increased membrane conductance and depressed the plateau.

However, the present study was prompted by the claim of some traditional health practitioners in KwaZulu-Natal Province of South Africa that decoctions, infusions and extracts of *Hypoxis hemerocallidea* corm are effective antenatal remedies for the treatment, management and/or control of threatening or premature abortions. The aim of this study was, therefore, to investigate the uterolytic action of African Potato aqueous extract in mammalian experimental animal paradigms *in vitro*.

Matetrials and methods

Ethical considerations

The experimental protocols and procedures used in this study were approved by the Animal Ethics Committee of the University of KwaZulu-Natal, Durban 4000, South Africa; and conform to the "*Guide to the Care and Use of Animals in Research and Teaching*" [published by the Ethics Committee of the University of KwaZulu-Natal, Durban 4000, South Africa].

Animals

Healthy, young adult, pregnant and non-pregnant (normal) female Wistar rats (250–350 g) and Dunkin-Hartley guinea-pigs (300–400 g) were used. The animals were kept and maintained under laboratory conditions of temperature, humidity, and light; and were allowed free access to food (standard pellet diet) and drinking tap water *ad libitum*. Some of the non-pregnant (normal) rats and guinea-pigs were pre-treated with stilboesterol (0.2 mg/kg s.c.) 20–24 hours before use. Vaginal smears were taken immediately before sacrifice in order to ascertain that the animals were in oestrus. Pregnancy was established in mated rats and guinea-pigs by examining the animals daily for the presence of cervical plugs. The day on which cervical plug was first found was taken as 'day one' of pregnancy. Early pregnancy was regarded as days 1–8, middle gestation period was taken as days 10–14, and late pregnancy was taken to be days 16–20. All the animals were fasted for 16 hours, but still allowed free access to drinking tap water, before the commencement of our experiments.

Plant material

Fresh corms of *Hypoxis hemerocallidea* (African Potato) were purchased from a fruit kiosk along West Street in Durban, KwaZulu-Natal Province of South Africa, between June and November, 2007. The corms were identified and authenticated by the staff of Botany Department, University of KwaZulu-Natal (where a voucher specimen of the plant has been deposited). One kg of the fresh corms were washed with distilled water, cleaned, cut into smaller pieces and milled in a waring commercial blender. The milled corm was macerated in

distilled water and extracted twice, on each occasion with 2.5 l of distilled water at room temperature (26 ± 1°C) with occasional shaking for 48 hours. The combined distilled water solubles obtained were concentrated to dryness under reduced pressure in a rotary evaporator at 60 ± 1°C. Freeze-drying and solvent elimination of the crude aqueous extract gave 78 g (*i.e.*, 7.8% yield) of a dark-brown, powdery, 'African Potato' aqueous extract (APE). Without any further purification, aliquot portions of the crude extract residue (APE) thus obtained were weighed and dissolved in distilled water (at room temperature) for use on each day of our experiments.

Acute toxicity testing

The median lethal dose (LD_{50}) of APE was determined in mice by a modified method of Lorke (1983), using intraperitoneal and oral (intragastric) routes. Mice fasted for 16 hours were randomly divided into groups of 10 mice per group. The procedure described in detail earlier by Ojewole (2006) was used for the determination of the acute toxicity of the plant's extract in the mice, following intrapritoneal and oral routes.

Evaluation of uterolytic activity of APE

Each non-pregnant (normal) or pregnant animal was euthanized with halothane inhalation and bled out. Its two uterine horns were carefully cleaned free from extraneous and connective tissues, trimmed and quickly removed. Tubular segments of approximately equal lengths (2–3 cm long) were removed from the uterine horns by cutting off both ends. The two uterine horn segments thus obtained were separately suspended in 30-ml Ugo Basile's two-chambered organ-baths (model 4050) containing de Jalon's physiological solution (DJS, of composition, in g/l: NaCl, 9.0; KCl, 0.42; $NaHCO_3$, 0.5; $CaCl_2.2H_2O$, 0.06; and glucose, 0.5) maintained at 32 ± 1°C and continuously aerated with carbogen (*i.e.*, 95% O_2 + 5% CO_2 gas mixture). Each uterine horn strip was subjected to an applied resting tension of 1 g, and allowed to equilibrate for 45–60 min, during which time the bathing de Jalon's physiological solution (DJS) was changed every 15 min before it was challenged with stepwise, escalated concentrations of APE (25–400 mg/ml) alone, or sequential doses of acetylcholine (ACh), oxytocin, bradykinin or potassium chloride. Two uterine horn strips from the same animal, one used as extract- or drug-treated 'test', and the other one used as distilled water-treated 'control' preparation, were always set up at a time (in order to make allowance for changes in tissue sensitivity). The 'control' uterine horn strips were always treated with distilled water (0.1–0.5 ml) only. Sub-maximal contractions (*i.e.*, 70–80% of the maximum contractions) of the drug-treated 'test' preparations were elicited by sequential, exogenous additions of either ACh (1 μg/ml), oxytocin (0.5 μU), bradykinin (5 ng/ml), or potassium (K^+, 30 mM) to the bath fluid. Sub-maximal muscle tensions developed by the spasmogens used were similar, and approximately equal to 1.5 g. APE-induced decreases in the spasmogen-provoked muscle tensions were considered as inhibitory effects of APE. The inhibitory effects of APE on the sub-maximal muscle tensions developed by each of the spasmogens used were investigated by sequential additions of stepwise, graded concentrations of APE (25–400 mg/ml) to the bath-fluid, followed, 2–3 min later, by subsequent additions of any of the spasmogens used to the bath fluid.

In all cases, after the maximal relaxation to each of the graded concentrations of APE had

been achieved, the uterine horn muscle preparation was washed out 3–5 times with fresh de Jalon's physiological solution, and then left to recover (for 10–20 min) and return to pre-drug treatment baseline level before it was contracted again with any of the standard spasmogens. Changes in tension developed by the uterine horn preparations (contractions and/or relaxations) were recorded isometrically by means of Ugo Basile's force-displacement transducers and pen-writing 'Gemini' recorders (model 7070).

Drugs

The following reference drugs were used: acetylcholine chloride, potassium chloride (Sigma Chemical Co.), oxytocin (Parke-Davis), and bradykinin (Sandoz). All drugs were dissolved and/or diluted in distilled water on each day of our experiments. Drug concentrations quoted in the text refer to final organ-bath concentrations.

Data analysis

Experimental data obtained are presented as means (± SEM). Data obtained from distilled water-treated 'control' uterine horn muscle strips were used as baseline values. The differences between the data obtained with the plant's extract- and reference drug-treated 'test' uterine horn muscle preparations, and the data obtained with distilled water-treated 'control' uterine horn muscle strips, were subjected to one-way analysis of variance (ANOVA; 95% confidence interval, GraphPad Prism 5), followed by Dunnett's *post-hoc* test (Dunnett and Goldsmith, 1993). In all cases, values of $P \leq 0.05$ were taken to imply statistical significance.

Results

Acute toxicity study

The LD_{50} value for intraperitoneally-administered APE was found to be 1,785 ± 116 mg/kg, while the LD_{50} value for orally-administered APE was 3.72 ± 0.45 g/kg. Oral administration of APE up to 2.5 g/kg did not produce any visible toxic manifestations (*e.g.*, respiratory distress, uncoordinated muscle movements, *etc*) or mortalities in mice.

Effects of APE on uterine horns isolated from pregnant rats and guinea-pigs

Uterine horns taken from pregnant rats and guinea-pigs exhibited spontaneous, rhythmic, pendular contractions. Relatively low to high concentrations of APE (25–400 mg/ml) inhibited the amplitude, and sometimes the frequency, of the spontaneous, rhythmic contractions, and relaxed the uterine muscle preparations in a concentration-related manner. Figure 2 shows a typical trace obtained with a pregnant guinea-pig isolated uterine horn, while Fig. 3 summarizes results obtained with rat and guinea-pig uterine horns. In all cases, moderate to high concentrations of APE (200–400) always caused profound relaxations of uterine horns taken from pregnant rats and guinea-pigs in a concentration-dependent fashion (Fig. 4).

Effects of APE on uterine horns isolated from oestrogen-dominated, non-pregnant rats and guinea-pigs

Uterine horns taken from stilboesterol-primed, and oestrogen-dominated, non-gregnant

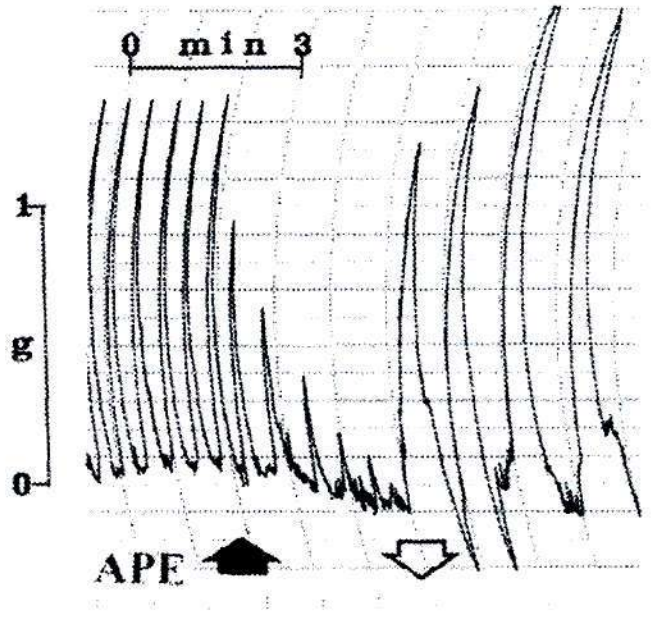


Fig. 2. Relaxant effect of APE (300 mg/ml) on a pregnant guinea-pig isolated uterine horn. APE was added to the bath-fluid at the left-hand side solid, upward-pointing arrow; and washed out 4 times at the adjacent right-hand side open, downward-pointing arrow.

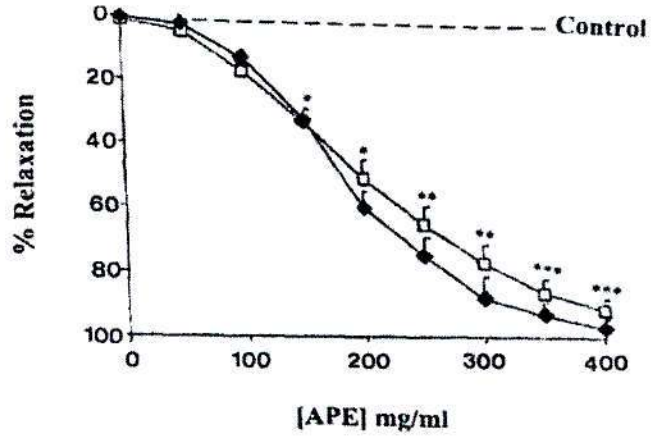


Fig. 3. Comparative relaxant effects of graded concentrations of APE on pregnant rat (□—□) and pregnant guinea-pig (◆—◆) isolated uterine horns. Each point represents the mean of 6–8 observations, while the vertical bars denote standard errors of the means (SEM). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

(normal) rats and guinea-pigs were always quiescent and devoid of spontaneous, rhythmic contractions (unlike uterine horns isolated from pregnant rats and guinea-pigs). Relatively low to high concentrations of APE (25–400 mg/ml) always relaxed the basal tones of the uterine horn muscle preparations in a concentration-dependent manner. The same low to high concentrations of APE (25–400 mg/ml) always inhibited ACh-, oxytocin-, bradykinin- or

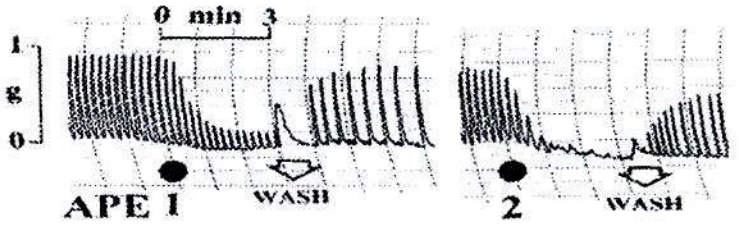


Fig. 4. Relaxant effects of graded concentrations of APE on pregnant rat isolated uterine horns. APE 1 and 2 represent 300 mg/ml and 400 mg/ml of APE, respectively, added to the bath-fluid at the left-hand side solid dots (●); and washed out 4–5 times at the adjacent right-hand side open, downward-pointing arrows.

potassium chloride-induced contractions of the oestrogen-dominated uterine horns in a concentration-related manner (data not shown).

Discussion

The LD_{50} value for intraperitoneally-administered APE was found to be 1,785 ± 116 mg/kg, while the LD_{50} value for orally-administered APE was 3.72 ± 0.45 g/kg. The gut wall and hepatic 'first pass' metabolic and elimination processes, coupled with the slow and variable degree of absorption following oral administration of APE would result in low blood (systemic) levels of the extract that would be non-toxic and non-lethal to the animals. On the contrary, the relatively rapid absorption following intraperitoneal administration of APE would result in high systemic levels of the extract, leading to toxic effects in the animals. The differences in blood concentrations of the extract following oral and intraperitoneal routes of administration would, therefore, seem to account for the differences obtained for the LD_{50} values of APE in the acute toxicity studies.

The effects of some cations, anions and certain drugs on spontaneous activities of uterine smooth muscle strips taken from non-pregnant and pregnant mammals have been investigated by a number of workers (Kuriyama and Suzuki, 1976; Osa and Kawarabayashi, 1977; Kuriyama *et al.*, 1998). Pharmacologically, relaxations of uterine smooth muscle strips taken from non-pregnant and pregnant mammals are believed to be mediated via β_2-adrenoceptor stimulation. However, the results of the present study indicate that APE possesses uterolytic activity in the mammalian experimental animals used. To the best of our knowledge, this is the first report on uterine activity of *H. hemerocallidea* corm in biomedical literature. The findings of our study are in agreement with the observations reported by Kuriyama and Suzuki (1976), Osa and Kawarabayashi (1977) and Kuriyama *et al.* (1998).

However, previous studies in our laboratories and elsewhere have reported antidiabetic, hypoglycaemic, anti-inflammatory and analgesic (Ojewole, 2002; 2006; Mahomed and Ojewole, 2003); antibacterial, anti-inflammatory and antioxidant (Steenkamp *et al.*, 2006); anti-cancer (Albrecht *et al.*, 1995); and renal (Musabayane *et al.*, 2005) effects of *APE* in various experimental animal paradigms. The above pharmacotherapeutic effects of 'African Potato' have been ascribed to the sterols, stanols, sterolins and norlignan glycoside, hypoxoside, present in the corm (Drewes *et al.*, 1984; Albrecht *et al.*, 1995; Bouic *et al.*, 1996; Nair *et al.*, 2007). The best-

known and fully-established chemical constituents of the plant's corm are presented in Fig. 1.

APE (25–400 mg/kg *p.o.*) dose-dependently and significantly (P<0.05–0.01) inhibited the amplitude (and in some cases, the frequency) of the spontaneous, rhythmic contractions of, and relaxed, pregnant rat and guinea-pig uterine horn preparations in a concentration-dependent manner. The same low to high concentrations of APE (25–400 mg/ml) also inhibited ACh-, oxytocin-, bradykinin-, or potassium chloride-induced contractions of oestrogen-dominated, non-pregnant rat and guinea-pig uterine horn preparations in a concentration-related manner (data not shown), suggesting that APE-induced uterine horn relaxations are unlikely to be mediated through β_2-adrenoceptor stimulation. Recent studies in our laboratories (Nyinawumuntu and Ojewole, 2008 - unpublished observation) have shown that APE provoked concentration-related inhibitions of the spontaneous, rhythmic, peristaltic contractions of the rabbit isolated duodenum, and relaxed the muscle. Moreover, it has been observed that APE relaxed guinea-pig isolated ileum in a concentration-related manner, and antagonized ACh-, histamine-, serotonin-, and potassium chloride (K^+)-induced contractions of the guinea-pig ileum in a concentration-dependent manner (Nyinawumuntu and Ojewole, 2008 - unpublished observation). The above observations are in consonance with the findings of the present study which suggest that APE possesses antimotility and non-specific spasmolytic effects. However, the spasmolytic effects of APE on spasmogen-induced contractions of uterine horn muscle strips taken from stilboesterol-primed, oestrogen-dominated, non-pregnant rats and guinea-pigs, like the spasmolytic effects of the plant's extract on rabbit and guinea-pig intestinal smooth muscles, would appear to be mediated through a non-specific spasmolytic mechanism. The sterols, stanols and sterolins present in *H. hemerocallidea* corm, especially rooperol and β-sitosterol (see Fig. 1), are speculated to account for the uterolytic and spasmolytic activities of APE. Further studies are, however, required to clarify this speculation. In conclusion, experimental evidence obtained in the present laboratory animal study indicates that *Hypoxis hemerocallidea* corm aqueous extract possesses uterolytic activity. This finding lends pharmacological support to the anecdotal, ethnomedical uses of 'African Potato' as a natural supplementary antenatal remedy for the management and/or control of threatening or premature abortion in some rural communities of South Africa.

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