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The uterotonic mechanisms of the herbal oxytocics

Clivia miniata and *Agapanthus africanus*

Denise Joy Hall Veale

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree

of

Doctor of Philosophy

Johannesburg, 2000

* This thesis has been approved for the degree of Doctor of Philosophy in Medicine by the University of the Witwatersrand, Johannesburg

DECLARATION

I, Denise Joy Hall Veale, declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

.....Veale.....

24..... day of November 2000

DEDICATION

To my husband Duncan and to my children

– you are my life and inspiration.

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS STUDY

PUBLICATIONS:

1. Veale DJH, Havlik I, Katsoulis L, Kaido T, Arangies NS, Oliver DW, Dekker, T, Brookes KB, Doudoukina OV. The pharmacological assessment of herbal oxytocics used in South African traditional medicine. Biomarkers and Environment 1998;2(3) :42-45.

2. Veale DJH, Havlik I, Oliver DW, Dekker T. Pharmacological effects of *Agapanthus africanus* on the isolated rat uterus. J Ethnopharmacol. 1999;66: 257-262.

3. Veale DJH, Oliver DW, Havlik I. The effects of herbal oxytocics on the isolated "stripped" myometrium model. Life Sciences 2000; 67:1381-1388.

4. Veale DJH, Havlik I, Oliver DW , du Preez JL Comparison between functional and biochemical effects of two herbal oxytocics on the rat myometrium. Phytomedicine 2000; 7 Suppl II:14.

5. Veale DJH, du Preez JL, Havlik I, Oliver DW. Discrimination between the functional and biochemical effects of two herbal oxytocics on the rat myometrium. J Pharmacy and Pharmacology 2001. In press.

PAPERS SUBMITTED TO JOURNALS FOR CONSIDERATION FOR PUBLICATION:

1. Phytotherapy Research

   Veale DJH, Oliver DW, du Preez J, Dekker TG, Havlik I. Evaluation of the bioequivalence of leaf and rhizome extracts of two *Agapanthus* species used a oxytocics in South Africa.

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   Veale DJH, Havlik I, Katsoulis L, Kaido T, Arangies NS, Oliver DW, Dekker, T, Brookes KB and Doudoukina OV.

   The pharmacological assessment of herbal oxytocics used in South African traditional medicine.

2. 3rd International Congress on Phytomedicine, Munich, Germany – October 11-13, 2000:

   Veale DJH, Havlik I, Oliver DW and du Preez JL

   Comparison between functional and biochemical effects of two herbal oxytocics on the rat myometrium.

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   Veale DJH, Havlik I and Oliver DW.

   The isolated "stripped" myometrium model and herbal oxytocics.

ABSTRACT

In the spirit of the World Health Organisation directives to Member states to scientifically evaluate herbal medicines so that consumers and health care providers can be provided with up-to-date and authoritative information on the beneficial properties, and possible harmful effects of all herbal medicines, the aim of this study was to investigate extracts of *Agapanthus africanus* and *Clivia miniata,* as phytomedicines using WHO guidelines as a reference outline. *Agapanthus africanus* and *Clivia miniata* have been the most often cited herbal oxytocics used in South African traditional medicine and are among the 5 most often used by traditional healers in areas in Kwazulu Natal to treat prolonged labour. *A. praecox* is reported to be used during parturition as well as *A. africanus.* These plant species were botanically identified, "fingerprint" chromatograms of extracts of the plants were made and basic toxicity potential (LC_{50}) was assessed.

Pharmacological equivalence in the isolated rat uterus preparation was verified for leaf and rhizome/root extracts of *A. africanus* and *A. praecox* and evidence of chemical similarity suggests a common core chemical composition responsible for uterotonic activity. Pharmacological effects of *A. africanus* on receptor systems in the isolated rat whole uterus and the mechanism/s of initiating contraction in the uterine smooth muscle cell were investigated. *Agapanthus* was shown to activate uterine contraction by activation of uterine prostaglandin synthesis and stimulation of muscarinic cholinergic receptors. This activity was similar to that already established for *C. miniata* in the same experimental model.

Concentration-response curves constructed from dose-related responses of the isolated rat "stripped" myometrium preparations and detection of prostaglandin synthesis in rat endometrial tissue effectively unmasked differences in the spectrum of uterotonic activity of *Clivia* and *Agapanthus*. Stimulation of prostaglandin synthesis in endometrial as well as myometrial tissue appears to be the major reason for the uterotonic activity of *Agapanthus*. Activation of muscarinic and serotonergic receptors seems to play a facilitatory role. Evidence points to a dual role for the uterotonic activity of *Clivia.* It appears to act on myometrial tissue to cause contraction by an undefined mechanism and on endometrial tissue to promote prostaglandin synthesis. *Clivia* acts on uterine 5-HT_2-receptors as a partial agonist in similar way to ergometrine. The uterotonic effects of both herbal remedies rely partially on calcium influx through voltage gated membrane channels but *Clivia* activity is less dependent on the availability of extracellular calcium.

Biochemical studies showed that both plant species were able to significantly stimulate myometrial phosphoinositide metabolism The effects of *Agapanthus* were much greater than the effects of *Clivia* and this could be regarded as the major uterotonic signal transduction mechanism for *Agapanthus.*

In conclusion, this study has identified various proposed mechanisms of uterotonic activity for the phytomedicines, *A. africanus* and *C. miniata.* Identification of similar uterotonic modes of action to ergometrine and $PGF_{2\alpha}$ indicates that these herbal medicines used as oxytocic agents have the potential to cause uterine hyperstimulation

with associated foetal and maternal adverse effects and their use in traditional medicine should be discouraged. The results of this investigation should be reported to traditional healers as a warning of the possible adverse effects associated with the use of the plants as ethnomedicines during parturition.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
AC	adenylate cyclase
ACh	acetylcholine
ASW	artificial sea water
ATP	adenosine triphosphate
Ca-ATPase	calcium adenosine triphosphatase
Ca,Mg-ATPase	calcium and magnesium adenosine triphosphatase
$[Ca^{2+}]_i$	concentration of cytoplasmic calcium
cAMP	3′,5′-cyclic adenosine monophosphate
CM	circular muscle of myometrium
DAG	diacylglyerol
GMP	guanosine 5′-monophosphate
G protein	guanine nucleotide-binding regulatory protein
GTP	guanosine 5′-triphosphate
HPLC	high pressure liquid chromatography
IP_3	D-myoinositol-1,4,5-trisphosphate
KRB	mammalian Krebs Ringer Bicarbonate buffer
LM	longitudinal muscle of myometrium
MLC	myosin light chain
MLCK	myosin light-chain kinase
OT	oxytocin
PBS	phosphate buffered saline

PIP_2	phosphatidylinositol 4,5-bisphosphate
PLA	phospholipase A_2
PLC	phospholipase C
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGI_2	prostaglandin I_2
PGs	prostaglandins
PK-C	protein kinase C
ROC	receptor operated channel
SR	sarcoplasmic reticulum
TLC	thin layer chromatography
VOC	voltage operated channel

GLOSSARY OF TERMS

Augmentation of labour - usually initiated when a dysfunctional labour pattern has been established

Bilateral lumbar sympathectomy - transection or interruption in the lumbar region of the sympathetic nervous pathway

Bioequivalence - having the same strength and similar bioavailablity in the same dosage form as another specimen of a given drug substance

Braxton Hicks contractions - light, usually painless, irregular uterine contractions during pregnancy, gradually increasing in intensity and frequency and becoming more rhythmic during the late 3rd trimester

Cervix uteri - the cervix connects the uterus to the vagina.

Corpus uteri - the body of the uterus

Foetal meconium passage - passage of meconium from the foetus into the amniotic fluid; can result from foetal distress and

hypoxia which could be caused by uterine hyperstimulation.

Fundus - the part of the corpus of the uterus above the entry-points of the uterine tubes; the highest and deepest part of the uterus

***Imbelekisane* -** a herbal medicine used in extreme cases of prolonged and difficult labour

***Imbiza* -** collective term for purgative "cleansing" medicines said to facilitate pregnancy by preparing the uterus to accept a foetus

***Impeko* -** a herbal decoction

Induction of labour - stimulation of uterine contractions before the rupture of membranes.

***Inembe* -** a potent oxytocic often used as an abortifacient

***Inyanga* -** a specialist in herbal medicine

Isangoma - traditional diviner and diagnostician

Isicakathi - herbal medicine used during pregnancy by the Xhosa people in the belief that it will ensure an easy childbirth and healthy child and that the child does not develop bowel trouble.

Isichoco - a herbal infusion

Isihlambezo - various liquid herbal mixtures taken during the last trimester of pregnancy in order to promote a favourable course of pregnancy and facilitate a quick and uncomplicated labour

Isthmus of the cervix - the upper third of the cervix which forms the narrowing between the corpus and the cervix and becomes the lower uterine segment of the pregnant uterus

MAS - meconium aspiration syndrome

Oxytocic - an agent that hastens the evacuation of the uterus by stimulating contractions of the myometrium

Tachysystolic contraction - a frequency of more than five contractions per ten-minute period

Umsekelo - a remedy used specifically to prevent miscarriages or premature birth

Umthandazi - a faith healer

Uterine hyperstimulation - an exaggerated uterine response accompanied by late foetal heart rate decelerations or foetal tachycardia > 160 beats per minute.

Uterine hypertonia - a contraction with a duration of > 90 sec

Uterotonic - an agent that increases the tonus of the uterine muscle

CHAPTER 1

INTRODUCTION

1.0 INTRODUCTION

1.1 Uterine smooth muscle contraction

The mechanisms involved in the initiation of labour and uterine contraction are very complex, and are not yet fully understood. The pregnant uterus performs two very different functions – for most of the pregnancy it is in a relaxed state and then, at the time of parturition, it has to contract regularly and forcibly in order to expel the foetus through the birth canal. The trigger or "uterotonin" responsible for the transition from relaxation to the onset of labour is still unknown (1).

1.1.1 The anatomy of the uterus

The human uterus has three parts: the corpus or main body, the cervix that connects the uterus to the vagina and the isthmus that forms the narrowing between the corpus and the cervix. The fundus is the upper, thickest part of the corpus. The corpus of the mammalian uterus comprises an outer myometrial and an inner endometrial layer (2). The myometrium is the muscular layer of the uterus and is very thick and heavily vascularized and the blood vessels run between the interstices of the muscle fibers. These act as a "tourniquet" when they contract, controlling bleeding from the uterus (3). The concentration of myometrial cells is the greatest in the fundus. The endometrium consists of two layers, the superficial stratum functionalis and the inner stratum basalis, which contain glands, blood vessels and lymphatics in loose connective tissue with a lining of

epithelial cells (2). Uterine myometrial cells are responsible for the contraction of the uterus, whereas the endometrial cells are secretory and non-contractile, i.e. they differ functionally. The endometrium is immediately adjacent to the myometrium and therefore endometrial secretory products such as prostaglandins diffuse into the myometrial tissue without entering the vascular system (4). The myometrium is responsible for the powerful expulsive contractions during parturition and causes the mechanical compression of the uterine vasculature that prevents bleeding following the detachment of the placenta during parturition (2). Prostaglandins are synthesised in both tissues, but mainly in the endometrium (i.e. the "decidua" of pregnancy). The myometrium is not a uniform muscle – it consists of two types of smooth muscle - circular (CM) and longitudinal (LM). The two muscles have a different origin, structure and function and the contraction patterns are different (fig. 1.1). In animals with a bipartite uterus, e.g. the rat, the outer longitudinal and inner circular muscle are more easily separated than in the human uterus (5). The response to oxytocin has been found to be more potent in the longitudinal layer than in the circular layer (6, 7).

The uterus is innervated by both the sympathetic and the parasympathetic nervous systems, the former by postganglionic fibers from the inferior mesenteric and hypogastric ganglia, and the latter by way of the pelvic nerve. Uterine contractions are involuntary, and for the most part are independent of extrauterine control. Denervation causes little change in uterine motor activity (3, 8). The intensity and duration of each contraction is usually greatest in the fundal zone, followed by the mid zone, and finally, least of all, the lower zone. The dominance of the fundal region allows the expulsion of the foetus. If

the intensity and duration of contractions was to be the same in all three zones, the pressure gradient would be absent and labour would not progress (3).

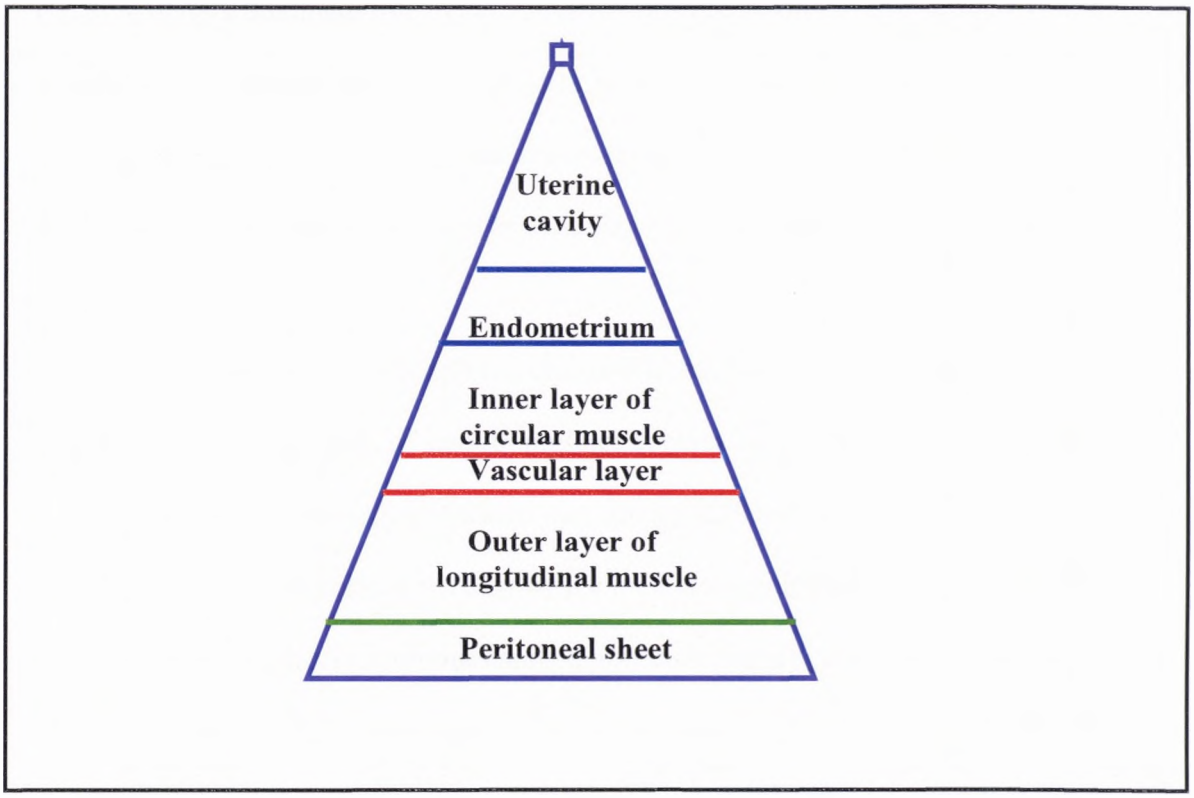


Figure 1.1. Schematic representation of a transversal section of human uterus (modified from 39).

1.1.2 The physiology of uterine contraction

Uterine smooth muscle contraction has been reviewed by Lopés Bernal, Europe-Finner, Phaneuf & Watson (1), Nichols & Ruffolo (2), Fuchs & Fuchs (9), Carsten & Miller (10) and Van Breemen & Saida (11).

1.1.2.1 The role of calcium and second messengers in the contraction of the uterus

Calcium plays a dominant role in the activation of smooth muscle contractile proteins. Contractility in smooth muscle is controlled primarily by the concentration of cytoplasmic calcium ($[Ca^{2+}]_i$) and the extent of myosin light-chain phosphorylation. Increases in $[Ca^{2+}]_i$ can be achieved by two major mechanisms:

1. Ca^{2+} entry into the cell through ion channels in the plasma membrane:
   The plasma membranes of smooth muscles contain a great variety of ion channels, the distribution and properties of which vary among different tissues. Some of these ion channels can be regulated not only by the membrane potential (voltage-operated) or by the direct action of hormones and neurotransmitters (ligand- or receptor-operated), but also by secondary messengers released by various agonists e.g. Ca^{2+} released from the sarcoplasmic reticulum (SR) can activate Ca^{2+}-gated K^+ and Cl^- channels (12). The opening of Ca^{2+}-gated channels can result in hyperpolarisation (K^+-channels), depolarisation (Cl^-- channels), or no change (opposite effects cancelling) in membrane potential (12). Similarly, arachidonic acid (AA) release by excitatory agonists can modulate K^+-channel activity and inhibit Ca^{2+} influx through voltage gated Ca^{2+}-channels. Ligand-gated channels are directly regulated by agonists, but are non-selective and can conduct several ions, mostly Na^+. It is unlikely that Ca^{2+} influx through these channels makes a significant contribution to excitation-contraction coupling, but the depolarisation due to the opening of ligand-gated

channels may cause significant Ca^{2+} influx by opening voltage-gated Ca^{2+}-channels (12).

The calcium concentration outside the cell in the interstitial fluid is 10,000-fold higher than that inside the cell. Calcium permeability is regulated by voltage operated calcium channels (VOCs) and receptor operated channels (ROCs) under the control of membrane potential and agonists. Verapamil, nifedipine and diltiazem are known as "calcium channel blockers" because they inhibit smooth muscle contraction by binding to the cell membrane within the voltage operated "L"-type membrane calcium channel.

2. Mobilising Ca^{2+} from the sarcoplasmic reticulum (SR):

   This is controlled by second messengers. The SR of the smooth muscle cell stores calcium in relaxation and is particularly abundant in the pregnant or oestrogen-treated uterus. It is the organelle primarily responsible for the physiological regulation of cytoplasmic calcium. The binding of hormones or agonists (first messengers) to specific receptors at the cell membrane surface (that are linked to guanine nucleotide-binding regulatory proteins (G proteins)) leads to a short-lived increase in the concentration of certain intracellular signalling molecules called second messengers. The major second messengers are 3′,5′-cyclic adenosine monophosphate (cAMP), 3′,5′-cyclic guanosine monophosphate (cGMP), D-myoinositol-1,4,5-trisphosphate (IP_3), diacylglycerol (DAG) and calcium. Receptor binding causes conformational

changes responsible for the activation of the G protein and initiation of signalling pathways to activate the final effector system.

G_q-protein activation of phosphoinositide-specific phospholipase C (PLC) initiates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to IP_3 and DAG (13). IP_3 is a second messenger that controls many cellular processes by generating internal Ca^{2+} signals. It operates via SR receptors whose molecular and physiological properties closely resemble those activated by the Ca^{2+} mobilising plant alkaloid, ryanodine (13). IP_3- and ryanodine- receptors act as Ca^{2+}-channels in the SR (12, 13). The polar IP_3 diffuses through the cytoplasm to the interior of the cell and combines with its specific receptor on the SR to stimulate the release of stored calcium from the SR and increase the cytosolic calcium concentration. IP_3 is then broken down stepwise to inositol by phosphatases which are stimulated by DAG (13). Inositol is then used in the cell membrane for the resynthesis of phophatidylinositol. DAG activates protein kinase C (PK-C), which phosphorylates various proteins and also modulates the activity of VOCs. Schrey, Cornford, Read & Steer (14) showed that the arachidonate-rich DAG is further hydrolysed to release free AA in human pregnant myometrium and decidua, thereby increasing the availability of the precursor for PG synthesis.

1.1.2.2 Activation of contraction and relaxation

Uterine myometrial smooth muscle has a high degree of spontaneous electrical and contractile activity. Contraction is initiated by membrane potential spike activity produced by waves of spontaneous membrane depolarisation triggered by pacemaker cells (2). The rise and fall in intracellular free Ca^{2+} are, respectively, the principal mechanisms that initiate contraction and relaxation in smooth muscle. Electromechanical coupling operates through changes in surface membrane potential and the effect on cytoplasmic $[Ca^{2+}]$. The resting membrane potential of smooth muscle is negative (-40 to –70 mV, depending on cell type) relative to the extracellular space. More positive potentials (depolarisation) can open voltage-operated Ca^{2+}-channels (VOCs), causing Ca^{2+} influx to trigger contraction. A change to a more negative membrane potential (e.g. drugs that open adenosine triphosphate (ATP)-sensitive K^{+}-channels) causes relaxation by inhibiting action potentials and reducing the open-state probability of Ca^{2+}-channels (12). Pharmacomechanical coupling is the other physiologically important mechanism of excitation-contraction coupling in smooth muscle and operates through multiple cellular signalling mechanisms that can change the level of force without necessarily changing membrane potential. The major mechanisms of pharmacomechanical coupling are Ca^{2+} release by IP_3 generated by the phosphatidylinositol cascade and modulation of the sensitivity to Ca^{2+} of myosin light chain (MLC) phosphorylation (12).

Extracellular calcium enters the myometrial cells through membrane calcium channels during the electrical spike activity. This amount of calcium is insufficient to cause direct

contraction, but this short burst of calcium entry triggers the release of much larger amounts of calcium from the SR. In uterine smooth muscle contraction, when $[Ca^{2+}]_i$ increases to 10^{-6} M, calcium binds to the protein, calmodulin, to form a calcium-calmodulin complex which activates the enzyme, myosin light-chain kinase (MLCK). MLCK phosphorylates MLC which is necessary for the interaction with actin (13). The force generating interaction between the myosin and actin filaments occurs and the muscle contracts without changing filament length, but the muscle as a whole changes its length. Smooth muscle is characterized by its ability to shorten to less than 50% and lengthen to more than twice its resting length and its greater force and low velocity of contraction when compared to skeletal muscle (10). When $[Ca^{2+}]_i$ decreases to 10^{-7} - 10^{-8} M, calmodulin dissociates from MLCK. The activity of MLCK is modulated by a cAMP-dependent protein kinase which catalyzes the phosphorylation of MLCK. The phosphorylated MLCK has a weak affinity for the calcium calmodulin complex and becomes inactive. Dephosphorylation of myosin by myosin light-chain phosphatase follows and the muscle relaxes (10). Figure 1.2 illustrates the signal transduction mechanisms proposed for the myometrial cell.

The spread of electrical activity from cell to cell is facilitated by gap junctions, which are low resistance pathways that connect cells. The gap junctions and the frequency of pacemaker potential generation are regulated by ovarian hormones, with oestrogens being excitatory and progesterone being inhibitory (2). The number of gap junctions is normally very low until the onset of labour and then the number and size increase.

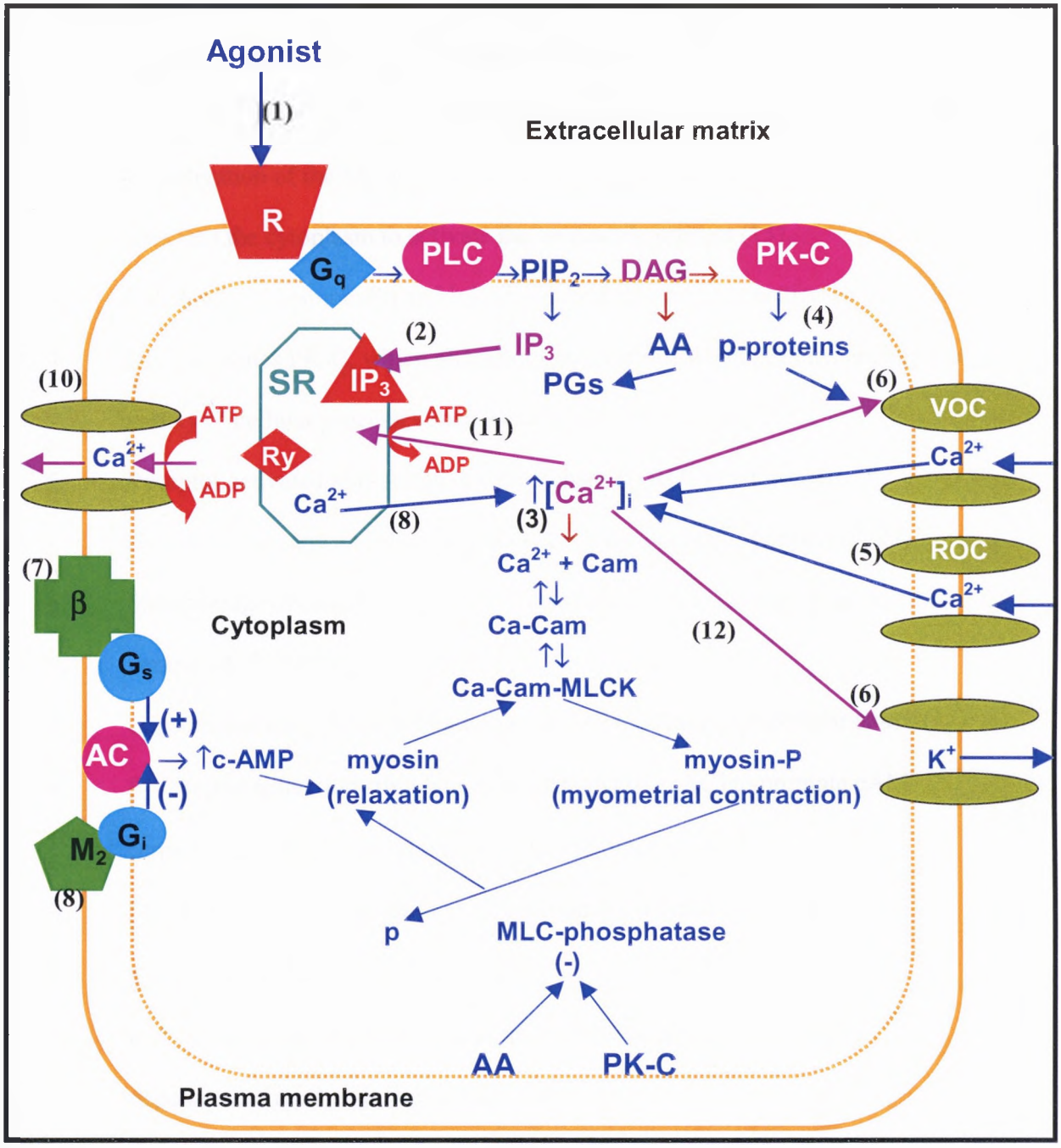


Figure 1.2. Schematic representation of signal transduction in a myometrial smooth muscle cell.

Key to figure 1.2.

1. Agonist activation of OT-, EP_1-, EP_3-, FP-, M_3- or 5-HT_2- receptors stimulates PIP_2 hydrolysis, resulting in the formation of the 2nd messengers IP_3 and DAG.
2. IP_3 activation of the SR IP_3-receptor promotes the release of Ca^{2+} from storage sites into the cytoplasm to activate the contractile process (13).
3. Ca^{2+} binds to calmodulin and initiates the myometrial contraction process (10).
4. DAG activates PK-C, which phosphorylates various target proteins leading to a variety of cellular processes such as activation of VOCs (7, 29).
5. Receptor activated non-selective cation channel (ROC) that triggers depolarisation and an influx of Ca^{2+} via voltage operated channels (7, 29).
6. Slow voltage operated "L"-type Ca^{2+} channels (VOCs) that can be blocked by verapamil (7, 10, 29)
7. Capacitative Ca^{2+} influx activated by Ca^{2+} release from intracellular stores (15).
8. β_2-receptor activation stimulates adenylate cyclase (AC) to promote cAMP formation which causes relaxation of the myometrium.
9. M_2-receptor activation inhibits AC and thereby inhibits the increase in cAMP elicited by β_2-receptor activation (31).
10. Ca^{2+} activated K^+ channels restore the membrane potential following depolarisation and an influx of Ca^{2+} (29).
11. Ca^{2+}- ATPase activated Ca^{2+}-efflux pump that pumps Ca^{2+} back into the extracellular space (10).
12. Ca^{2+}- ATPase activated Ca^{2+}-influx pump responsible for the influx of Ca^{2+} back into the SR (10).

During pregnancy the placenta produces large quantities of progesterone and myometrial activity is reduced. In the final weeks of pregnancy, oestrogen production increases and progestogen production decreases (2). Oestrogen and progesterone therefore play a facilitatory role in the initiation of labour. The stimulation of uterine muscle during labour is believed to result from an interaction of oxytocin (OT) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Evidence suggests that OT is most important for the initial phase of labour whereas increased synthesis of $PGF_{2\alpha}$ is essential for the progression of labour (9). The role of prostaglandin E_2 (PGE_2) is unclear, but it may play an important role in the ripening of the cervix which, in turn, is essential for successful parturition. Figure 1.3 illustrates the interplay between oestrogen, progesterone, oxytocin, $PGF_{2\alpha}$ and PGE_2 in the initiation and progression of labour (2).

Contractions in human myometrium are phasic and therefore mechanisms exist for the rapid lowering of $[Ca^{2+}]_i$ by extrusion of Ca^{2+} from the cells and by Ca^{2+} reuptake into the SR. Relaxation of the uterus can be achieved by lowering $[Ca^{2+}]_i$ but also by lowering the sensitivity of MLCK to Ca^{2+}, a process stimulated by the cyclic nucleotides, cyclic AMP and cyclic GMP (11). Guanine nucleotides are involved in modifying the cAMP system by receptor communication with G proteins in the cell membrane. G_s enhances the activity of adenylate cyclase (AC), the enzyme that catalyzes cAMP synthesis and G_i inhibits the enzyme. Guanosine 5'-triphosphate (GTP)-liganded adenylate cyclase is the active form and the hydrolysis of GTP to GDP is the main mechanism of inactivation. The calcium that entered the cell for contraction must

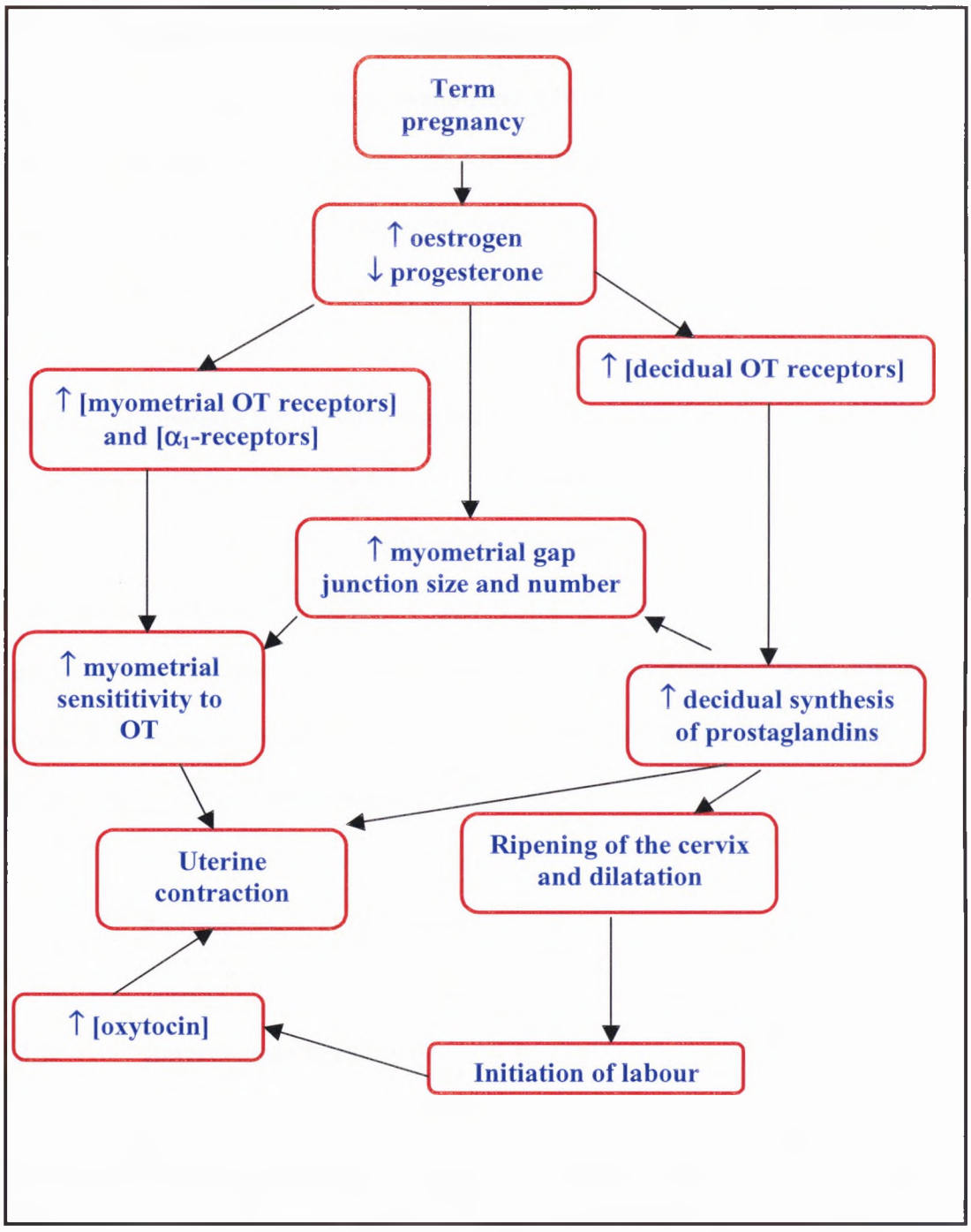


Figure 1.3. Schematic representation of the effects of heterologous regulation by female gonadal hormones in the initiation of labour (modified from 2).

eventually be pumped out of the cell and the energy for this process is mediated by the calcium and magnesium adenosine triphosphatases (Ca,Mg-ATPase). These enzymes require calcium and magnesium for activation and split ATP. The ATPase of the cell membrane is stimulated by calmodulin. Ca,Mg-ATPases are also present in the SR membrane, but differ from the ATPase of the cell membrane in that they are not stimulated by calmodulin.

Berridge (13) proposed a regenerative process of calcium induced Ca^{2+} release in waves or oscillations. Philippe (15) showed that phasic contractions of the rat myometrium were due to cycles of depletion and refill of Ca^{2+} storage sites by both IP_3-sensitive Ca^{2+} release and $Ca^{2+}{}_i$-induced Ca^{2+} release ("capacitative" Ca^{2+} influx). He proposed that the cycles of depletion and refill of the calcium storage pools caused cytosolic calcium oscillations, resulting in the cyclic activation of calmodulin, MLCK and contractile proteins and phasic myometrial contractions.

1.1.3 Pharmacology of the uterus

1.1.3.1 Heterologous regulation

The contractile response of the uterus to agonists is modulated by the gonadal steroids, oestrogen and progesterone (fig 1.3). The modification of the response of a tissue to one agent by another hormone is termed heterologous regulation (16). This modulatory effect could be due to changing the concentration or affinity of agonist receptors or post-

receptor effects such as the ability of oestrogen to increase contractile proteins and gap junctions which facilitates response to all agents which cause myometrial contractions.

1.1.3.2 Oxytocin

Oxytocin (OT), a polypeptide neurohypophyseal hormone synthesized in the supraoptic and paraventricular nuclei of the hypothalamus, is the most specific and potent known endogenous uterotonic agent. The dominant effect of oestrogen at term causes an increase in the number of oxytocin receptors in both the myometrium and decidua (17). The levels of receptor rise from late pregnancy to a maximum during early labour (a 100- to 200-fold increase in concentration). This is paralleled by an increase in uterine sensitivity to oxytocin during the last trimester. The increased receptor concentration lowers the threshold for stimulation and endogenous OT may be responsible for Braxton Hicks contractions. The onset of parturition is preceded by a marked increase in oxytocin receptor concentrations in the myometrium and the formation of gap junctions between myometrial cells. The development of these two biomolecular events may have an important role to play in the initiation of labour. It has been proposed that a premature increase in the density of myometrial oxytocin receptors could provoke preterm labour by sensitizing the uterus to relatively unchanged circulating levels of OT. OT-receptor antagonists eg atosiban decrease uterine activity significantly in women with threatening preterm labour but their ability to prevent the onset of labour is not established (1). Progesterone administration prevents the oestrogen-induced increase in OT-receptor concentration in rabbit uterus (18). OT stimulates decidual PG synthesis in the term

uterus and PGs in turn can cause uterine contractions, induce cervical ripening, and sensitize the myometrium to OT (17). Arterial OT concentration in the umbilical cord exceeds that of venous blood and this difference increases significantly when labour starts. The foetus also secretes OT, which may cross the placenta and stimulate myometrial contractions or reach the decidua by diffusion and stimulate PG production after being excreted with foetal urine to the amniotic fluid (19).

OT stimulates contractility in human myometrium, both *in vivo* and *in vitro*, and is widely used alone or in combination with prostaglandins for the induction and augmentation of labour (1). It is also used alone, or in combination with ergometrine in the 3rd stage of labour, to prevent or control post-partum haemorrhage. Although the circular layer of the myometrium may have more physiological importance for delivery, the response to OT has been found to be more potent in the longitudinal layer of the pregnant rat myometrium. OT-receptors exist at higher concentration in the distal part of the uterine horn than in the proximal part (7).

OT has a dual action in the uterus - it acts on myometrial oxytocin receptors (OT_{1a}) to directly cause uterine contraction and on another subtype of oxytocin receptors in the endometrium (OT_{1b}) to stimulate prostaglandin synthesis and release (20). The prostaglandins would then diffuse across into the adjacent myometrium and potentiate the OT-induced myometrial contractions as proposed by Fuchs (4). The oxytocin-induced uterotonic and prostaglandin releasing responses have been shown to be mediated by binding to specific cell surface receptors, resulting in the activation of a G protein-

coupled phospholipase C to hydrolyse PIP_2 to IP_3 and DAG (20). Qian, Wang & Sanborn (21) have identified the G protein as belonging to the $G\alpha_{q/11}$ class. The activation of this mechanism leads to an increase in free cytosolic calcium, the second messenger system for uterine contractions, and to the activation of phospholipase A_2 (PLA_2), releasing free arachidonic acid for the synthesis of prostaglandins. In the endometrial/decidual cell, the activation of the phosphoinositol pathway and generation of inositol triphosphate and diacylglycerol causes an increase in intracellular Ca^{2+} and free arachidonic acid, the precursor for prostaglandin synthesis and release.

The myometrial response to OT is biphasic – the first phase is due to a transient increase in cytosolic Ca^{2+} due to increased IP_3 production induced by activation of OT-receptors and the second phase is due to enhanced calcium entry from the extracellular environment. The OT-generated IP_3 induction of Ca^{2+} release from intracellular Ca^{2+} stores occurs even in the absence of extracellular Ca^{2+}. Sakai & Uchida (22) reported that OT produced sustained contractions in oestrogen-dominated rat myometrium in Ca^{2+} -free medium and Molnár & Hertelendy (23) confirmed this in human myometrial cells. The exact mechanism of OT promotion of Ca^{2+} influx into the cell has not yet been completely clarified. Arnaudeau, Leprêtre & Mironneau (24) postulate that OT enhances Ca^{2+} influx by stimulating voltage-dependent Ca^{2+} channels and/or by opening receptor-mediated Ca^{2+} channels. Shimamura, Kusaka & Sperelakis (7) showed that oxytocin induced an influx of Ca^{2+} mediated by receptor-activated nonselective cation channels in late pregnant rat myometrial cells. In addition to promotion of Ca^{2+} influx and Ca^{2+}

release from the SR, OT inhibits both the plasma membrane and SR Ca^{2-} pumps by reducing either the maximal velocity or the Ca^{2+} affinity of the pumps (24).

1.1. 3.3 Prostaglandins

The myometrium also contracts in response to prostaglandin stimulation. Unlike OT, which is a circulating hormone, prostaglandins (PGs) originate at or near their site of action, i.e. in the endometrial and myometrial cells. PGs are rapidly synthesized in uterine cells from arachidonic acid via the cyclo-oxygenase pathway and are then most probably released into the uterine circulation by facilitated diffusion (25). The events that lead to the formation of PGs in human parturition begin in the foetal membranes (amnion and chorion) and also in the decidua. PG production is the greatest in the amnion, followed by the chorion, and finally, the decidua. The first step is the release of free arachidonic acid (AA) which is the obligatory precursor of the PGs of the "2" series. AA is not stored in human tissues, but human foetal membranes accumulate AA in esterified form in the form of glycerophospholipids. The major pathway of AA release is by phospholipase A_2 (PLA) action which breaks down glycerophospholipids in the "2" position. The end products of hydrolysis are lysoglycerophospholipid and free fatty acid. Lysoglycerophospholipids are cytolytic agents and they break down cell membranes and lysosomal membranes inside the cell. The lysosomes also contain phospholipase and its release accelerates the release of AA and PG synthesis from preformed glycerophospholipids in the foetal membranes. $PGF2_\alpha$ and PGE_2 are subsequently formed from AA in a multi-step process and the first step is catalysed by

the enzyme cyclooxygenase. The intermediate products, endoperoxide and thromboxane also contract the uterus. Arachidonic acid is metabolized in the amnion to PGE_2 and in decidua to PGE_2 and PGF_{2a}. PGI_2 (synthesized preferentially in myometrium) has an inhibitory effect on contractions, i.e. promotes uterine quiescence (26). A secondary pathway of AA mobilisation is via the hydrolysis of PIP_2 to form IP_3 and DAG, as previously described. Oestrogen is thought to be the stimulus for increased PG synthesis in the foetal membranes at the onset of human parturition. PG production by the decidua is markedly enhanced during labour as a result of increased decidual OT_{1b}-receptor density and stimulation by OT.

$PGF_{2\alpha}$ and PGE_2 promote uterine smooth muscle contraction by interaction with prostaglandin receptors in the plasma membrane of myometrial cells of humans, rats and other species (27). The principal site of uterine PG synthesis is generally believed to be the endometrium/decidua but the pregnant myometrium also synthesizes a significant amount of PG. Human myometrium expresses prostaglandin EP-, FP- and IP-receptors (1). Prostaglandin FP-receptors are positively coupled to PLC and PLA_2 but there is no evidence for increased receptor density or increased coupling to PLC during pregnancy or parturition. Nevertheless, successful parturition is always accompanied by increased $PGF_{2\alpha}$ production by decidualized endometrial and probably myometrial cells (1). Different subtypes of prostanoid EP-receptors exist in human myometrium. The EP_1- and EP_3-receptors are stimulatory via PLC activation whereas EP_2-receptors are inhibitory via stimulation of adenylate cyclase (1). PGE_2 interacts with EP_1- and EP_3-receptors to cause myometrial contraction and EP_2-receptors to produce relaxation.

$PGF_{2\alpha}$ interacts with EP_1-, EP_3- and FP-receptors to cause contraction and stimulates contraction in both the pregnant and non-pregnant state. On the other hand, PGE_2 causes relaxation of the non-pregnant uterus and contraction of the pregnant uterus at low concentrations, but relaxation at high concentrations. The relaxant effect of high concentrations of PGE_2 may be due to an interaction with the uterine PG IP-receptor, which mediates relaxation, rather than to interaction with the EP_2-receptor (2). In some circumstances, preterm labour appears to be a consequence of the premature release of PGs and other inflammatory mediators in the decidua/foetal membranes area, triggered by infection (chorioamnionitis) (1). The density of myometrial PG receptors does not increase towards term, but there is the possibility of a shift in PG signalling from inhibitory to stimulatory pathways at the onset of labour in addition to the increase in myometrial excitability (1, 2).

The mechanism by which PGs raise $[Ca^{2+}]_i$ is uncertain. Molnár & Hertelendy (23) provided evidence that in isolated human myometrial cells, $PGF_{2\alpha}$ and oxytocin trigger an increase in $[Ca^{2+}]_i$ by different mechanisms. The action of $PGF_{2\alpha}$ was found to depend on extracellular Ca^{2+} at a "physiological" concentration of 100 nM and could only increase total inositol phosphate production at a 100 times higher concentration (i.e. a "pharmacological" concentration of 10 μM). This effect could be suppressed by verapamil, indicating that it acts primarily by promoting Ca^{2+} entry. In contrast, the potencies of OT to raise total inositol phosphate levels and $[Ca^{2+}]_i$ were similar and independent of extracellular Ca^{2+} and could not be suppressed by verapamil, confirming activation of the G-protein dependent phospholipase-C-IP_3-Ca^{2-} signal-transducing

pathway, causing intracellular Ca^{2+} release, which is complemented by the influx of extracellular Ca^{2+}.

Goureau, Tanfin, Marc & Harbon (28) studied the effects of different natural and synthetic PGs analysed systematically in the same preparation at the level of functional and biochemical (second messenger generation) responses in the rat myometrium. They provided evidence that PGs are able to interact with multiple signal transduction pathways coupled to specific G proteins via distinct types of receptors in the rat myometrium. They identified inhibitory IP-receptors and stimulatory FP-, EP_1- and EP_3-receptors in the the oestrogen-treated rat myometrium and in the 21 day pregnant rat myometrium. FP- and EP_1-receptors were linked to the contractile effect of $PGF_{2\alpha}$ and PGE_2 (concentration > μM) and the EP_3-receptors mediated uterine contraction caused by PGE_2 (concentration < μM).

The endometrium is the major site of prostaglandin production in the rat. PGs released from the intact uterus by OT in the rat are thought to modulate the uterotonic effects of this hormone. Therefore, when the endometrium is present, PGs released from this tissue may mask the direct effects of oxytocin on the myometrium (6). Expression of G proteins changes in rat myometrium during gestation. The expression and/or function of PG receptors may also be the target from permissive regulation, linked to hormonal status during gestation. The differential coupling of PG receptors in the pregnant rat myometrium may be of physiological relevance. Inhibition of adenylate cyclase, stimulation of PLC and activation of a putative ionic channel pathway would all lead to

an enhancement of the contractile activity of the uterus, which is of crucial importance during delivery.

1.1.3.4 The autonomic nervous system

The activity of the uterus may be modulated by the autonomic nervous system (both adrenergic and cholinergic nerves are present) but since labour appears to be normal in paraplegic women and in women with bilateral lumbar sympathectomy, this appears to be of limited physiological importance (1). This could be due to the fact that the uterine corpus and fundus are depleted of neurotransmitters as pregnancy progresses and they are replenished sometime after delivery. The cervix, however, has a rich supply of both adrenergic and polypeptinergic nerves and remains innervated during pregnancy.

1.1.3.4.1 The muscarinic cholinergic system

Muscarinic cholinergic receptors are expressed in smooth muscle throughout the body and play an essential role in maintaining the contractile state of this muscle. Muscarinic agonists cause contraction of the uterus and other smooth muscle as well as relaxation of smooth muscle in other tissues. Five subtypes of muscarinic receptor have been identified (M_1 - M_5) and more than one subtype of muscarinic receptor may be expressed in the smooth muscle of various tissues (29). Binding studies have established that M_2-receptors are the major subtype present in most smooth muscle-containing tissues with the exception of the iris of the eye (major receptor subtype is M_3) and comprise about

80% of the total receptor density in the smooth muscle of the gastrointestinal tract, the trachea, the bladder and the uterus. The less abundant M_3-receptor mediates the contractile response in these tissues (29).

The uneven numbered M_1, M_3 and M_5 subtypes interact with G proteins of the G_q family to mediate the stimulation of PIP_2 hydrolysis with the formation of the two intracellular messengers IP_3 and DAG. These second messengers lead respectively to Ca^{2+} release from the SR and activation of protein phosphorylation, implicated in the initiation and maintenance of smooth muscle contraction. The even numbered M_2 and M_4 subtypes interact with G proteins of the G_i family to mediate the inhibition cAMP accumulation by inhibiting basal adenylate cyclase activity. M_2- and M_4-receptors also mediate the activation of inwardly rectified potassium channels and the inhibition of neuronal calcium channels by direct coupling to G_i and G_o respectively.

The balance between the relaxant or contractile state of smooth muscle depends on the prevailing parasympathetic and sympathetic drive. The role of M_2- and M_3-receptors in contraction and the influence of the prevailing sympathetic tone on the relative contributions of these subtypes have been shown to be complex (30). M_2-receptors have been found to inhibit β-adrenoceptor stimulated adenylate cyclase activity in the ileum. It appears that contraction is mediated directly by M_3-receptors and indirectly by M_2-receptors as a result of inhibition of relaxation caused by adrenoceptor stimulation. M_2-receptors do not play a role when there is no prevailing relaxant tone. Activation of the parasympathetic nervous system therefore appears to be associated with a modulatory

role over the level of smooth muscle tone in addition to a direct control of contraction. The physiological implications are therefore that M_2-receptors may mediate the dominant parasympathetic control over smooth muscle tone under conditions of high sympathetic activity or where M_3-receptors are dysfunctional or agonist concentrations are low. Figure 1.4 illustrates this proposed mechanism (31) which has been confirmed by Sawyer & Ehlert (32). It has also been shown that M_2-receptors modulate contraction in the absence of sympathetic stimulation by activation of an inward depolarising current that is nonselective to monovalent and divalent cations (30, 32). The effects of M_2-receptor modulation have not yet been defined in the uterus.

The rat uterus, particularly the tubal and cervical regions, receives dense cholinergic innervation, which supplies both the secretory glands and smooth muscle (33) and stimulation of cholinergic neurons causes contraction of the uterus. Both the endometrium and myometrium receive cholinergic innervation. Oestrogen and progesterone do not appear to affect the concentration or sensitivity of muscarinic receptors in the uterus. The stimulation of myometrial muscarinic M_3-receptors by agonists such as ACh and carbachol causes contraction of the uterus. This effect can be blocked by a non-selective muscarinic competitive antagonist such as atropine. Marc, Leiber & Harbon (34) found that muscarinic as well as oxytocin receptor activation in guinea pig myometrium was coupled to an enhanced phosphoinositide metabolism. Both oxytocin and carbachol produced the same maximal contractile response but the maximum capacity of oxytocin to generate inositol triphosphate was 2 to 3 times greater than carbachol. Varol, Hadjiconstantinou, Zuspan & Neff (35) provided evidence that

activation of rat uterine muscarinic M_3-receptors resulted in contraction of the myometrium and enhanced PIP_2 turnover. Carbachol-stimulated PIP_2 turnover in the myometrium was most active in the upper third of the uterus and in the fallopian tubes, consistent with the distribution of cholinergic nerves in the rat uterus. Contraction of the guinea-pig uterus was found to be mediated by M_2-receptors (30). Pennefather, Gillman & Mitchelson (36) identified that the M_2-receptor as the predominant receptor type in the rat uterus, but concluded that the muscarinic receptor subtypes responsible for the contraction and second messenger production in the rat uterus had not yet been fully identified. Cholinergic nerves disappear from the uterine body with advancing pregnancy and there is a resultant decrease in muscarinic receptors (35). Acetylcholine has oxytocic properties but is not indicated for use in pregnancy (37). The use of the non-selective antimuscarinic agent, cimetropium, as a tocolytic agent in premature labour is presently under investigation (38).

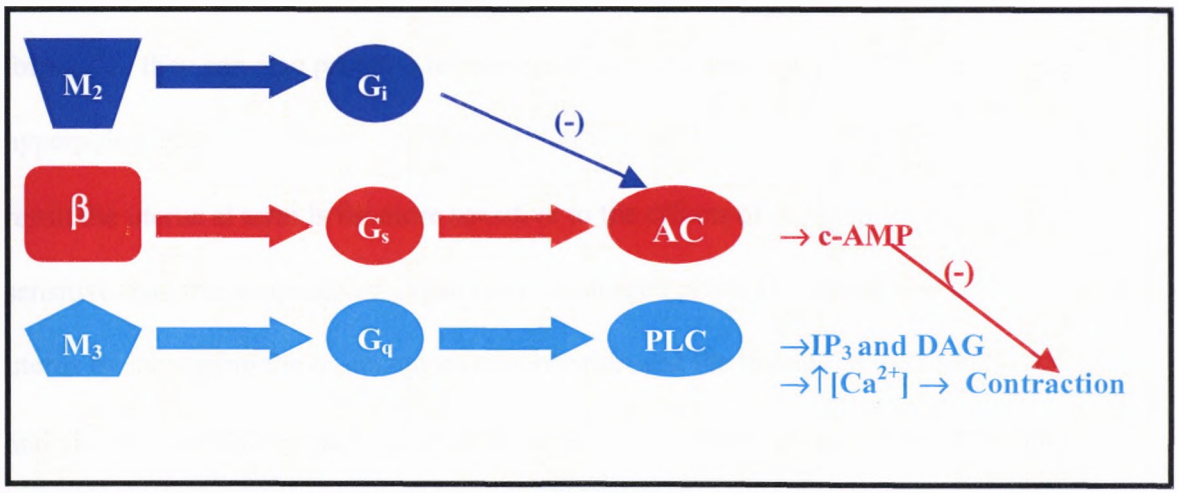


Figure 1.4. M_2-receptor modulation of M_3-receptor mediated contractile activity (modified from 31).

1.1.3.4.2 The adrenergic system

The response of the uterus to catecholamines is modulated by adrenergic receptors located on the plasma membrane of myometrial cells. Adrenoceptor agents have complex effects on myometrial contractility. Stimulation of α-adrenoceptors increases the frequency and intensity of contractions while β-adrenoceptor stimulation decreases either spontaneous or induced uterine contractility (39). Four adrenergic receptor subtypes (α_1, α_2, β_1 and β_2) have been shown to be present in pregnant human myometrium (40). Myometrial contraction is mediated by agonist stimulation of α_1-adrenoceptors via G_q-protein coupling and activation of the phosphoinositide pathway as well as by α_2-receptor coupling with G_i-protein causing inhibition of AC activation and a resultant decrease in cAMP formation. In contrast, β_2-adrenoceptor agonists relax the uterus by coupling with G_s-protein to activate AC and stimulate cAMP production (41). Although agonists at α_2-receptors favour contractility by inhibiting cAMP formation, they can also promote relaxation by activating K^+ channels leading to hyperpolarisation (1). There is an increase in the number of α_1-receptors at term and as a result the uterus at term is far more sensitive to the effects of α_1-agonists and far more sensitive than the nonpregnant organ (42). Oestrogen promotes contraction in rabbit uterus by increasing the α_1-receptor concentration and the linkage of the receptor to PLC, and also by uncoupling the β-receptor from AC. Conversely, progesterone promotes relaxation via β-receptors by uncoupling α_2-receptors from the inhibition of AC (16).

Tuross, Mahatani & Marshall (6) showed that the two muscle layers of the myometrium appeared to have different adrenoceptor populations. In the rat, there is a relative dominance of alpha-adrenoceptors in CM and of beta-receptors in LM. This difference is most prominent in early and mid-pregnancy. At term, there is an increase in beta-receptor activity in CM so that the beta-inhibitory effects of adrenergic amines are similar in both muscle layers. A predominance of beta-adrenoceptor-mediated responses in the LM as compared to the CM was also found in uteri from estrogen/progesterone-primed, non-pregnant guinea pigs. Breuiller, Rouot, Leroy, Blot, Kaplan & Ferré (39) showed that, in both the CM and LM of human myometrium near term, 65% of the β-receptors in were of the β_2 subtype. In the 35th week of pregnancy the concentration of β-receptors in the CM was 50% higher than in CM and that at term there was a decrease in β–receptor concentration in both muscle layers of the myometrium. Litime, Pointis, Breuiller, Cabrol & Ferré (43) found that adrenergic agonists did not stimulate AC in the CM or LM layers of term pregnant human myometrium and postulated that this was due to modifications in the coupling mechanism between β_2-receptors, G_s-proteins and AC. The concentration of α_2-receptors was found to be much higher in CM than in LM in human 38-40 week-pregnant uterus (44). The clinical use of adrenergic agents in pregnancy is restricted to the use of β_2-agonists such as hexoprenaline and salbutamol as tocolytic agents in premature labour.

1.1.3.5 Ergometrine

The ergot alkaloids are derivatives of 6-methylergoline and form a class of compounds with diverse pharmacological properties that include interactions with 5-HT-receptors, dopamine receptors, α-adrenoceptors and uterine smooth muscle (2, 42, 45). Some of the ergot alkaloids are selective for only one of the receptors and others act at two or more receptors. All the natural ergot alkaloids contract the uterus. This effect appears to be most closely associated with agonist (or partial agonist) effects at 5-HT_2-receptors as well as activity at α_1-receptors (which become more dominant at term) (42). The 5-HT_2-receptor is responsible for contraction of the rat uterus and whereas the oestrogenized rat uterus is exquisitely sensitive to the contractile effect of 5-HT, the human uterus is unresponsive. Ergometrine is superior to the other ergot alkaloids as a uterotonic agent due to its rapid onset of action and also because it is more selective and therefore less toxic. Ergotamine acts as a partial agonist on vascular α_1-receptors as well as on 5-HT_2- receptors. Ergometrine, however, acts mainly on 5-HT_2-receptors with only slight partial agonist activity on vascular α_1-receptors (3, 2, 45). Methysergide is a non-competitive antagonist at 5-HT_2- receptors.

In functional studies on the isolated rat uterus, Hollingsworth, Edwards & Miller (46), found that ergometrine acted as a partial agonist on 5-HT-receptors at lower concentrations (0,1-1 μM) and a competitive antagonist at higher concentrations (10 μM), indicating partial agonist activity. The spasmogenic action was often inconsistent and not concentration related. In a further study, Hollingsworth, Dascombe, Price &

Acton (47) showed that ergometrine did not act directly on 5-HT_2-receptors, but appeared to act as a partial agonist at an allosteric site. Schrey, Cornford, Read & Steer (48) have shown that ergometrine (3 μM) stimulates phosphoinositide metabolism in pregnant human myometrium and that this effect could be blocked by the α_1-antagonist, prazosin. Ergometrine has also been shown to increase the production of PGE_2 and $PGF_{2\alpha}$ in human gestational myometrium (49).

At low concentrations, ergometrine increases the force and frequency of uterine contraction followed by normal relaxation. A characteristic feature of high concentrations is a marked increase in tonus with tetanic contractions (2, 3). The non-pregnant uterus responds to the effect of the ergot alkaloids, but sensitivity increases during pregnancy. Ergometrine is not used to induce labour in obstetric practice because of its ability to produce a prolonged sustained contraction of the uterus. This effect is however used to advantage in the prevention and treatment of postpartum haemorrhage where the sustained contraction causes mechanical compression of the uterine vasculature and controls bleeding (2). Both ergotamine and ergometrine have been linked to teratogenic effects (50).

1.1.3.6 Other miscellaneous receptors related to uterine contraction

The potent vasoconstrictor, endothelin-1 (ET-1), has also been shown to contract human and rat uterine tissue, and because of its occurrence in the amniotic fluid and uterine tissues, has been suggested to play a role in the regulation of parturition (50). ET-1

Table 1.1 Receptors and signalling mechanisms linked to uterine smooth muscle activity (modified from 1 & 52).

Receptor	Mechanism	Effect
Adrenoceptors		
α_1	activate PLC	contraction
	activate VOCs	contraction
α_2	inhibit adenylate cyclase (AC)	contraction
α_2	inhibit VOCs	relaxation
α_2	activate Ca^{2+}-dependent K^+ channels	relaxation
β_2	activate AC	relaxation
β_2	close Ca^{2+} channels	relaxation
Peptide hormones		
OT_{1a}	activate PLC	contraction
OT_{1b}	activate PLC/DAG → PG synthesis	contraction
Vasopressin V_{1A}	activate PLC	contraction
Endothelin ET_A	activate PLC	contraction
ET_A	inhibit AC	contraction
Angiotensin AT_1	activate PLC, DAG/PK-C	contraction
AT_1	inhibit AC	contraction
AT_1	stimulate VOCs	contraction
Prostanoids		
DP	activate AC	relaxation
EP_1	activate PLC	contraction
EP_1	PLC independent influx of $Ca^{2+}{}_e$	contraction
EP_2	activate AC	relaxation
EP_3	activate PLC	contraction
EP_3	PLC independent influx of $Ca^{2+}{}_e$	contraction
EP_3	inhibit AC	contraction
FP	activate PLC	contraction
FP	activate phospholipase A_2	contraction
IP	activate AC	relaxation
TP	activate PLC	contraction
Muscarinic		
M_1 and M_3	activate PLC, DAG/PK-C	contraction
M_1 and M_3	inhibit AC	contraction
M_2	inhibit AC	indirect contraction
M_2	open inwardly rectifying K^+ channels	relaxation
M_2	inhibition of Ca^{2+} channels	relaxation
Others		
Bradykinin B_2	activate PLC	contraction
B_2	inhibit AC	contraction
Histamine H_1	activate PLC	contraction
H_2	activate AC	relaxation
5-HT_2	activate PLC	contraction
5-HT_2	inhibit AC	contraction

activates the inositol phospholipid cycle and raises $[Ca^{2+}]_i$ in myometrial cells and there is evidence that, in the absence of extracellular calcium, it has no effect on either $[Ca^{2+}]_i$ or myometrial contraction. Bradykinin, angiotensin and vasopressin receptors have also been identified in uterine tissue and linked to uterine contraction (1). Table 1.1 summarizes the different receptor types linked to the activity of uterine smooth muscle (1, 52).

1.1.3.7 Clinical use of oxytocic agents in modern obstetrics in South Africa

The oxytocic agents approved for clinical use in modern obstetrics to induce or augment labour or control post-partum bleeding are: oxytocin, $PGF_{2\alpha}$, PGE_2 and ergometrine. The effects of these drugs on contraction of the pregnant uterus have been well documented. OT is the preferred pharmacological agent for the induction and augmentation of labour because of its high specificity in contracting the uterus. It is also used for the prevention and control of postpartum haemorrhage. The onset of action by IV administration is immediate and the duration of action is short. At an appropriate dosage, OT increases the frequency and amplitude of contraction without raising uterine tonus. The dosage of OT is very closely monitored because of the risk of producing uterine hypertonus at higher dosages (3). In addition to the risk of hyperstimulation of the uterus, other adverse effects of high doses of OT include uterine rupture, water intoxication and an increased incidence of neonatal jaundice (53). Side effects seen at normal dosage include nausea and vomiting and cardiac arrhythmias (54).

$PGF_{2\alpha}$(dinoprost) and PGE_2 (dinoprostone) are the only PGs registered in South Africa for the induction of labour (54). These prostaglandins, like OT, cause contraction of the uterus followed by relaxation, i.e. phasic contractions. They are less suited for the initiation of labour because of their nonspecific action. They may contract other smooth muscles such as those of the intestinal tract resulting in severe nausea, vomiting and diarrhoea and $PGF_{2\alpha}$ is contraindicated in asthmatic patients because of its bronchoconstrictory effects. An important side-effect associated with these drugs, is the rupture of the posterior wall of the uterus at the cervico-isthmic junction. Dinoprostone is used as an intravaginal or intracervical gel for cervical ripening and induction of labour. Oral administration is seldom used because of unpleasant side effects. Dinoprost is administered either intra- or extra-amniotically. The intravenous route is seldom used because of side effects (54). Concurrent use with OT must be carefully monitored because of the risk of uterine hypertonia, uterine rupture or cervical laceration.

The use of ergometrine is restricted to the 3rd stage of labour only due its potential to cause sustained tetanic uterine contraction that could last for up to an hour. It is administered at the end of the second stage of labour, as the head is crowned and prior to delivery of the placenta for the prevention and control of postpartum haemorrhage (54). Oral and IM routes are preferred because IV administration is associated with a greater risk of adverse effects such as hypertension, nausea, vomiting, blurred vision, headache and convulsions (54). Ergometrine is less likely to cause gangrene of the extremities than ergotamine. Hypertension and moderate to severe heart disease are regarded as contraindications to ergometrine, and in these cases, OT is used.

1.2 The example of ergot

The introduction to this thesis would be incomplete without a summary of the history of the obstetric use of ergot. It serves as an excellent example of the complexity of the process involved in converting a crude traditional herbal medicine to a modern pharmaceutical preparation and the importance of the identification of potential toxicity. Ergot is produced from the sclerotium of *Claviceps purpurea*, a fungus that grows on rye and other grasses of Europe and North America. It was responsible for epidemics of "ergotism" (Holy Fire, St Anthony's Fire) that occurred frequently during the Middle Ages and was first described around 600 BC as a "noxious pustule in the ear of grain" (55). Ergotism was caused by eating bread made from rye contaminated with the fungus. Two distinct types of ergotism exist – gangrenous (acute) and convulsive (chronic). "Gangrenous" ergotism is characterized by abortion, gangrene and spontaneous amputation of limbs and "convulsive" ergotism by hallucinations, agonizing burning sensations in the limbs and convulsions (55). Animals suffered as well as man and pregnant sows littered before their time (56).

Ergot was recorded as an obstetrical herbal remedy as early as 1582 by Lonicer and Kreuterbach and had been used by midwives long before it was recognized by the medical profession. Lonicer and Kreuterbach stated that the ergot spurs in diseased rye "... are held to be a special medicine for women in labor and for the purpose of awakening the pains three of the spurs are swallowed" (56). This precise dose, defined so long ago, equates to 0,5 mg of ergometrine – the exact dose of pure alkaloid now used

in obstetric practice (56). Paulizky in 1787 was the first to describe it in a medical journal as *pulvis ad partum* (powder to bring forth labor) and it was prescribed by both midwives and physicians alike (55). In 1808, Dr John Stearns described the effect of a decoction of ergot on the progression of labour in a letter to a friend as: "the violent and most incessant action which it induces in the uterus precludes the possibility of turning … you will be surprised with the suddenness of its operation; it is, therefore, necessary to be completely ready before you give the medicine". The letter was published and marked the introduction of ergot into orthodox medicine (56). Medical practitioners, however, failed to heed his advice and the medicine was incorrectly administered. Hosack, in 1824, linked the use of ergot to the increased incidence of stillbirths due to uterine rupture and maternal death and proposed that it should be renamed the *pulvis ad mortem* (powder to bring forth death) (55). The use of ergot as an oxytocic to induce or augment labour had virtually ceased by the end of the 19th century.

Van Dongen & De Groot (55) have described the laborious process of the chemical isolation of the various ergot alkaloids. Wiggers (1895), in Germany, was the first person to try to analyse and isolate the active constituents of ergot but was unsuccessful. Tanret (1875), in France, described the first pure alkaloid isolated from ergot. It was called ergotinine and was pharmacologically inert. Sollmann and Brown (1905) studied the circulatory effects in mammals after intravenous and intramuscular injections of crude ergot but the results were varied because of the differences in preparations and therefore dosage. The immediate hypertensive response to adrenaline was found to be decreased by ergot. Barger and Carr (1906) obtained two impure fractions from

ergotinine and the uterotonic activity was attributed to a single alkaloid, ergotoxine. It was later established that the l-forms of the ergot alkaloids (designated by the suffix –ine) are pharmacologically active and the d-forms (designated by the suffix –inine) are inactive (57). Spiro and Stoll isolated ergotamine in 1918. Both ergotoxine and ergotamine were very complex molecules and it was later shown that ergot preparations contain a group of large-moleculed alkaloids which are able to form complexes with one another. Ergotoxine was found to be such a complex (56).

The uterotonic effects of extract of ergot were monitored externally in 1927 and it was found to be pharmacologically inert. Ergotamine and ergotoxine were then considered reliable uterine stimulants useful after delivery to prevent postpartum haemorrhage because of their prolonged action. The onset of action was, however delayed – 4 to 10 min after intravenous administration, 20 min after intramuscular injection and 35 min or longer after oral administration (55). Moir and Dale (1932) then identified uterotonic activity in an old pharmacopoeial preparation (liquid extract of ergot) using a technique for recording intra-uterine pressure. Liquid extract of ergot had been condemned by most pharmacologists on the premise that, as an aqueous extract, it could not contain any of the known alkaloids (56). The results were dramatic – an oxytocic effect was observed within 4 min of oral administration and contractions were more frequent and more regular than with the other alkaloids (55). Dudley (1935) isolated the water-soluble alkaloid, ergometrine, which, in a dose of 0,5 mg produced uterine contractions identical to those produced by the aqueous extract of ergot. The onset of action of ergometrine administered IM or IV was 4 and 2 min, respectively. Within a month, three

other groups isolated the identical alkaloid and named it ergotocin, ergostetrin and ergobasine respectively. It is currently known as ergometrine in the U.K. and ergonovine in the U.S.A. It has been used in obstetrics to prevent postpartum haemorrhage since 1935. To date, the following compounds have been isolated from ergot – acetylcholine, agmatine, amino-sulphonic acid, betaine, cadaverine, choline, ergochrysin, ergoclavine, ergocornine, ergocristine, ergokryptine, ergoflavin, ergometrine, ergosterol, ergotamine, ergotaminine, ergothioneine, ergotinine, ergotoxine, guanosine, histamine, histidine, iso-amylamine, iso-leucine, leucine, putrescine, secalonic acid, secale cornutum, sensibamine, trimethylamine, tyramine, tyrosine, uracil, and valine (55).

The example of ergot serves as an excellent illustration of the necessity for thorough investigation of the safety and efficacy of herbal medicines used for obstetric purposes. Attempts to isolate active uterotonic principles should be exhaustive and only after a comprehensive investigation of all the possible pharmacological, biochemical and toxic events related to the oxytocic activity of the crude ethnic preparation. To be presumptive in the identification of active principles could have disastrous effects.

Other plant-derived alkaloids with uterotonic activity were reviewed by Reynolds (58) (table 1.2). Of these alkaloids, sparteine and quinine have been used therapeutically as oxytocic agents for the induction of labour (57, 59). These drugs are no longer used in obstetrics because of their toxicity potential.

Table 1.2. Alkaloids with uterine stimulant activity (58). ***Used as a clinical oxytocic in modern obstetrics; ** previously used as clinical oxytocics.

Ergot alkaloids
***Ergometrine
Ergotamine
Ergotoxine
(Ergocornine
Ergocristine
Ergokryptine)

****Sparteine and related alkaloids**
d-Lupanine
Lupinine
Trilupine
Cytisine
Nicotine

Cinchona alkaloids
**Quinine
Hydroquinine
Quinidine
Cinchonine
Cinchonidine
Quinamine

Isoquinoline alkaloids
Adlumine
Bicuculline
Corlumine
Berberine
Hydrastine
Hydrastinine
Nandinine
Protropine
Cryptopine
Salsoline
Sanguinaria alkaloids
umbellatine

Erythrophleum alkaloids
Erythrophleine
Cassaine
Cassaidine
Coumingine
Coumingidine
Coumingaine
Homophleine

Senecio alkaloids
Platyphylline
Seneciphylline
Integerrimine
Jacobine
Longilobine
Isatidine
Sceleratine
Retronecine
Ridelline
Pterophine
Retrorsine

Fritillaria alkaloids
Fritimine
Verticine
Fritillarine
Verticilline

Rauwolfia alkaloids
Ajmaline
Ajmalicine
Ajmalinine
Serpentine
Serpentinine

Lycopodium alkaloids
Lycopodine
Complanatine
Obscurine
Anotine

Miscellaneous alkaloids
Muscarine
Arecoline
Pilocarpine
Physostigmine
Alstonia alkaloids
Calycanthine
Cocaine
Colchicine
Dendrobine
Ephedrine
Gambirine
Gelsemine
Gelsemicine
Harmaline
Harmine
Hordenine
Ibogaine
Lunamarine
Mitrinermine
Paipunine
Sinomenine
Strychinine
Veratrine

1.3 South African Traditional Medicine in pregnancy and parturition

1.3.1 Current status of South African traditional medicine

There are about 200 000 traditional healers in South Africa and up to 80% of the black population consult them before going to a professional nurse or medical doctor. Modern South Africa recognizes the importance of the traditional healer and traditional herbal medicines are recognized by some Medical Aid Schemes as a treatment option in place of registered pharmaceutical drugs. The South African government is to pass legislation by the end of this year to regulate Traditional Medicine by the establishment of a Traditional Medicine Council. Abdool Karim, Ziqubu-Page & Arendse (60) prepared a report on the current status of traditional medicine in South Africa for the South African Medical Research Council. This report deals with the potential for a health care partnership between African traditional healers and biomedical personnel and the following facts emerged as highlights:

1. The traditional healer is acknowledged as an integral part of all African cultures and communities and has enormous influence with the patient, his family and the community.
2. There is growing interest throughout the world in the traditional healer as a resource for community based health care delivery – China is a leader in this field.

3. There are four types of traditional healer in South Africa, namely the *Inyanga* (a specialist in herbal medicine), the *Isangoma* (traditional diviner and diagnostician), the *Umthandazi* (faith healer) and the traditional birth attendant.
4. Herbal medicine is the most common therapeutic method used by African traditional healers and the types of medication can be classified into 3 categories:
    a. Preventative and prophylactic medication
    b. Treatments for ailments
    c. Medications used to destroy the power in others
5. Attempts have been made to formalise and standardise the training of traditional healers in Africa and the Academy of Traditional Healers in Zimbabwe was established in 1982.
6. The University of Cape Town has undertaken an extensive project (TRAMED) to document all the traditional medicines derived from indigenous medicinal plants, study their safety and clinical efficacy, identify and isolate the therapeutically active ingredients, and establish a national database. The ultimate goal is to set up a herbal pharmacopoeia that will be available and accessible to all concerned with health care delivery in South Africa.

1.3.2 South African traditional herbal remedies used in pregnancy and parturition

Traditional medicine recognizes the value of antenatal medication and therefore herbal remedies used in pregnancy and childbirth are numerous. Traditional birth attendants

deliver 60% of all babies in South Africa (60). They are usually elderly women who have been midwives for many years and are highly respected for their obstetric and ritual expertise. They often provide the herbal medicines used in parturition. Veale (61) and Varga & Veale (62) have reviewed the traditional use of pregnancy-related remedies extensively. A literature survey (63) revealed that 57 different plant species were used in traditional herbal remedies by black South African women during pregnancy and childbirth. The traditional use of pregnancy-related herbal medicines has also been chronicled throughout the world – Africa, Australia, China, Japan, India, Melanesia, Hawaii, North America, South America, the United Kingdom (U.K.), U.S.A and Europe (62).

In South Africa, pregnancy-related herbal medicines are known collectively as *isihlambezo* which refers to various liquid herbal mixtures taken during the last trimester in order to promote a favourable course of pregnancy and facilitate a quick and uncomplicated labour. It is prescribed by the traditional healers as a tonic to be drunk daily from mid-pregnancy until and including delivery and is occasionally prescribed as a vaginal douche (62). There are several other types of pregnancy-related herbal medicines that are often confused with *isihlambezo*. *Imbelekisane* is a herbal medicine used in extreme cases of prolonged and difficult labour and is administered as a drink or occasionally as an enema. *Inembe* is a potent oxytocic often used as an abortifacient. *Imbelekisane* and *inembe* are considered dangerous medicines by the traditional healers (62). *Imbiza* is another category of medicine and is a collective term for purgative "cleansing" medicines said to facilitate pregnancy by preparing the uterus to accept a

foetus. It is prescribed for women at any stage in pregnancy and its ingredients and prescription are different from *isihlambezo*. *Umsekelo* is a third type of remedy used specifically to prevent miscarriages or premature birth (61).

A survey conducted amongst Zulu women during 9 months' fieldwork in rural and urban areas of KwaZulu Natal revealed that 93,6% of respondents thought the use of *isihlambezo* amongst women in their communities was a popular practice. Nearly 90% of the women felt *isihlambezo* was an important and helpful part of self-care during pregnancy and 87% favoured mixing *isihlambezo* and clinic-based antenatal treatment (62).

Plants cited in relevant literature to be used for their oxytocic activity, i.e. to induce or augment labour, expel the placenta and/or prevent post-partum haemorrhage were: *Agapanthus africanus, Callilepis laureola, Clivia miniata, Combretum erythrophyllum, Gladiolus serriceovillosus, Grewia occidentalis, Gunnera perpensa, Pentanisia prunelloides, Plantago major, Rhoicissus cuneifolia, Typha capensis* and *Vernonia neocorymbosa.* Ethnopharmaclogical interviews conducted in Zulu with 45 traditional healers in rural and urban KwaZulu Natal areas revealed that *Agapanthus, Clivia, Combretum, Gunnera and Pentanisia* were by far the most popular treatment for prolonged labour (62).

Veale (61) concentrated on one of the plants reported most often in the literature as being used as a traditional oxytocic (63) and investigated the pharmacological effects of *Clivia*

miniata on receptor systems in isolated rat whole uterus and ileum. The *Clivia* extract was found to initiate smooth muscle contraction in both the uterus and ileum (64, 65). The investigation was then continued to include the pharmacological screening of some of the other plants reported to be used most often to aid prolonged labour. Aqueous extracts of *Agapanthus africanus*, *Gunnera perpensa* and *Pentanisia prunelloides* were subjected to basic pharmacological screening tests on isolated smooth muscle and were all found to be capable of initiating smooth muscle contraction in isolated rat uterus and ileum preparations. *Agapanthus africanus* was found to be the most potent uterotonic agent (66). It has therefore been established that *Clivia miniata* and *Agapanthus africanus* extracts are the most effective uterotonic agents of the plant species tested and are also the most often quoted in the literature as being used as oxytocics. Uterotonic activity has also been identified in *Combretum krausii* and *Rhoicissus tridentata* extracts (67, 68, 69).

These herbal remedies used in pregnancy and childbirth are made either from one specific species or combinations of various plant species and are usually prepared as infusions or decoctions. The actual method of preparation of a herbal decoction or infusion varies and in many cases the patient prepares the medicine at home. Sofowora (70) describes the general preparation of traditional medicines in Africa and states that an "infusion " is prepared by pouring boiling water on a specified quantity of plant material and allowing the mixture to stand for 10-15 min. A "decoction" is prepared by placing the plant parts in cold water, bringing it to the boil, simmering for about 15 min or longer (up to 1 hr) and allowing the mixture to stand for a further 15 min. This description does

not differ significantly from the methods used in the United Kingdom and Europe for the preparation of herbal medicines, except that the African preparation of a decoction allows the plant material to be in contact with the menstruum for a longer period – i.e. it is likely to be more concentrated (71, 72). Gumede (73), a trained medical practitioner with a great interest in South African traditional medicine, described the preparation of South African traditional medicines as follows:

> *Isichoco* (an infusion) – a fresh mixture of roots, barks or leaves are crushed and mixed with cold water and then the solid matter is filtered off.
> *Impeko* (a decoction) – medicines are prepared as for an infusion but the mixture is boiled or heated just to boiling

Pujol (74), who worked as a herbalist amongst the Zulu, and studied Zulu and Xhosa "muti" preparations, described a methodology similar to that of Sofowora (70):

> Infusion - the dried or fresh herb or powdered root or bark is placed in a cup or pot and boiling water is poured over it. The mixture is then strained thoroughly and covered with a lid. It is left to stand for a few minutes and then drunk like tea.
> Decoction – the plant material is placed in a pot and boiled for a limited period.

These examples serve as confirmation of the unpredictability of the exact concentration of active principles contained in traditionally prepared herbal extracts. This has

important toxicological implications. Other factors such as harvesting plant material from different geographical areas and harvesting in different seasons can only compound the problem.

1.4 Toxicology

Drug administration to pregnant women is strictly regulated and monitored in first world medicine. The FDA classifies the safety of drugs for use during pregnancy in Categories A, B, C, D and X as a guideline to prescribers. A select few drugs are used in obstetrics and dosage is carefully monitored to avoid hyperstimulation of the uterus and both foetal and maternal toxicity. The use of herbal remedies by midwives in the U.S.A., the United Kingdom and Europe is well documented and precise recipes and cautionary statements regarding prescription and potential toxicity are given (72, 75, 76)

1.4.1 Toxicity of South African traditional herbal remedies used during pregnancy and parturition

Very little has been published regarding the pharmacology or toxicity of the South African herbal remedies used in the last trimester of pregnancy and during labour. Many South African women take these medicines and as yet there is a tragic lack of scientific proof of efficacy or safety. The potential toxicity of South African traditional herbal remedies used in pregnancy has been reviewed (61, 62) and the following toxicity profiles identified:

1. The use of *isihlambezo* by women in Gauteng has been associated with an increased incidence of foetal meconium passage (77). Foetal meconium passage can result from foetal distress and hypoxia which could be caused by uterine hyperstimulation. Meconium-stained amniotic fluid is associated with foetal meconium aspiration syndrome (MAS) which is potentially fatal. Morris & Mdlalose (78) have associated *isihlambezo* use with low birth weight in Kwazulu Natal.
2. Broster (79) relates the taking of large quantities of *imbelekisane* to cases of unexplained foetal death due to premature birth in the Transkei. Larsen, Msane & Mohnke (80) tested a Zulu *imbelekisane* infusion on isolated rat uterus and found it caused uterine hypertonia.
3. In addition to these effects related to the specific remedies used in pregnancy, many of the ingredients used to prepare the remedies are known to be poisonous. 16 plant species identified as being used as traditional remedies taken during pregnancy and parturition have been recorded as causing poisoning (63, 81). Of these, *Impila* (*Callilepis laureola*) is one of the most potentially lethal traditional herbal medicines known in South Africa and has caused many fatalities due to the hepatic and renal toxicity it causes..

An oxytocic is defined as an agent that hastens the evacuation of the uterus by stimulating contractions of the myometrium (82). The major toxic effect produced by the inappropriate use of an oxytocic agent is uterine hyperstimulation. Uterine hyperstimulation is defined by Curtis, Evens & Resnick (83) as an exaggerated uterine

response accompanied by late foetal heart rate decelerations or foetal tachycardia > 160 beats per minute. An exaggerated uterine response, in turn, is defined as hypertonic (a contraction with a duration of > 90 sec) or tachysystolic (a frequency of more than five contractions per ten-minute period). During normal contractions, uterine blood flow into the intervillous spaces is temporarily decreased. As the contraction subsides, full flow is restored, with maximal oxygen available to the foetus. If hyperstimulation occurs due to administration of an oxytocic agent, there is insufficient recovery of oxygenation between contractions and a reduction of maternal blood flow through the placenta. The altered perfusion of the placenta can culminate in derangement of the foetal acid-base basis. Although there is no exact time or duration linked with foetal asphyxia, the longer the hyperstimulation occurs, the more likely and more severe the degree of foetal hypoxia. (53). Sustained hyperstimulation could result in foetal death and maternal death due to rupture of the uterus. The potential for teratogenicity in the herbal medicines used during pregnancy is likely to be negligible as herbal *isihlambezo* is usually only taken from about mid-pregnancy and particularly in the last trimester.

Qualified medical herbalists in first world countries are aware of herbal remedies contraindicated for use in pregnancy and obstetrics and these are well documented (72). This is not however, the case in Africa and thorough investigation and documentation of the uterotonic activity and potential toxicity of the herbal oxytocics used in traditional medicine is essential.

1.4.2 Toxicity tests

The concentrations of active principles and/or toxins in the oxytocic herbal remedies used by South African women are difficult to quantify for reasons already stated. However, by using the concentration of plant extract necessary to stimulate uterine contractions as a guideline, some indication of potential toxicity could be obtained by determining LC_{50} values. The World Health Organization (WHO) research guidelines for evaluating the safety and efficacy of herbal medicines (84) advocates that non-clinical toxicological studies related to assessing the safety of herbal medicines should include an acute toxicity test. At least two animal species should be used for these tests – one rodent species and the other non-rodent. There is a severe limitation to the type of toxicity tests available to South African researchers. Acute toxicity studies on animals are not approved by the Animal Ethics Committee of the University of the Witwatersrand and other South African academic institutions (85).

1.5 Regulatory

The evaluation of the efficacy and safety of South African traditional herbal remedies presents an enormous challenge for the future. Gericke (86) in his overview of the regulation and control of traditional herbal medicines details the recognition by the World Health Organization of the importance of herbal medicines and its encouragement of Member states to develop activities in promoting the use and manufacture of herbal medicines that could be added to the national list of drugs and substituted for some that have to be purchased from abroad. The Traditional Medicines Programme of the WHO

has its policy basis in a number of resolutions adopted by the World Health Assembly and the Regional Committees. Resolutions specific to the promotion of the utilisation of herbal medicines include the following:

1. In 1978 the 31st World Health Assembly adopted a resolution (WHA31.33) that called on the Director-General to compile and periodically update a therapeutic classification of medicinal plants, related to the therapeutic classification of all drugs.
2. Resolution WHA 40.33 of 1987 urged member states to initiate comprehensive programmes for the identification, evaluation, preparation, cultivation and conservation of medicinal plants used in traditional medicine.
3. Resolution WHA 42.43 of 1989 urged member states to make a systematic inventory and assessment (pre-clinical and clinical) of the medicinal plants used by traditional practitioners and by the population.

As a result of the WHO's promotion of traditional medicine, countries have been seeking the assistance of the WHO in identifying safe and effective herbal medicines for use in national health care systems. The objective of the WHO guidelines for the assessment of herbal medicines (87) was to define basic criteria for the evaluation of quality, safety, and efficacy of herbal medicines and thereby to assist national regulatory authorities, scientific organizations and manufacturers to undertake an assessment of the

documentation/submission/dossiers in respect to all such products. The WHO (88) defines *"herbal medicines"* as follows:

Finished, labelled, medicinal products that contain as active ingredients aerial or underground parts of plants, or other plant material, or combinations thereof; whether in the crude state or as plant preparations. Plant material includes juices, gums, fatty oils, essential oils and any other substance of this nature. Herbal medicines may contain excipients in addition to the active ingredients. Medicines containing plant material, **combined with chemically defined active substances including chemically defined, isolated constituents of plants,** are not considered to be herbal medicines.

1.5.1 Identification and quality assessment of herbal preparations

The use of herbal medicines in traditional medicine is mainly based on experience and tradition. The WHO recognizes this and in the Guidelines for the Assessment of Herbal Medicines, the extent of traditional use is taken into account in the assessment of efficacy of remedies for the treatment of minor disorders and for non-specific indications (88). The European Union has taken an exemplary stand in standardizing the herbal remedies traditionally used in Europe and the United Kingdom. ESCOP, the European Scientific Cooperative on Phytotherapy, was founded in 1989 in order to regulate the assessment of safety and efficacy of herbal medicines on a European level. To date ESCOP, in collaboration with the European Pharmacopoeia, has published 60 monographs on the efficacy and safety of medicinal plants and their preparations. Further monographs and revisions of the older monographs are currently being developed (89). The WHO

published the first Model Monographs for Herbal Medicinal Products for Primary Health Care in 1999. These included monographs for Aloe, Ginseng, Garlic, Ginger and Valerian. The WHO monographs are comparable to the ESCOP monographs but are more detailed regarding quality control (89). In future, the pharmaceutical industry in Europe will be forced to comply with the ESCOP monographs in the manufacture and marketing of herbal remedies. Extensive clinical investigation of the efficacy of the European traditional herbal remedies is also underway.

The determination of the safety and efficacy of traditional herbal medicines throughout the world should be mandatory, but in many 3rd World countries such as South Africa, this is not the case. The WHO (87) recommends that pharmacological, toxicological and clinical trials should be undertaken to determine safety and efficacy. The identity and quality of the plant material must be determined in order to ensure the reliability and repeatability of research on herbal medicines. The guidelines for the quality specifications of plant materials and preparations include the following:

1. Name of the plant material in Latin, native languages and English whenever applicable.
2. Scientific name of the plant with reference to the authors and the family to which it belongs.
3. Part of the plant used and its condition (such as fresh aerial parts, dried root and rhizome, sliced or decorticated).
4. Time and method of collection, preliminary preparation and drying.

5. A brief description of the distribution and habitat of the plant; growing wild or cultivated. If more than one plant species is concerned, their differences should be indicated. Drawings or photographs of the plants should be provided.
6. Characterising compounds of the plant material, which may also be the biologically or therapeutically active principle, should be quantified and described.
7. Method of preparation – solvent used, time and temperature of extraction and concentration.
8. Authenticity – a description of the macroscopic, microscopic and sensory characteristics of the plant should be provided, including drawings or photographs. A description should be provided of the physical or chemical tests done to identify the plant substances and a chromatogram of the active fraction or characterising compound should be provided. If this is not possible, it should be sufficient to identify a characteristic mixture of substances ("fingerprint") of the plant material.

Bauer & Tittel (90) discuss the importance of establishing the identity and quality of plant materials used in pharmacological and toxicity studies in order to validate the significance and reproducibility of the investigation. Variables that affect the concentration and presence of natural chemical constituents in plants include factors such as: origin; growth conditions; date of harvest; existence of chemocultivars and drying and storage conditions after harvest.

As long as the plant material is purchased from commercial sources, the possibility of confusion and adulteration must be taken into consideration. In South Africa, the herbal

medicine trade relies on "collectors" who usually have no formal botanical training and plants are harvested from many different locations directly from the indigenous environment. Nichols (91) refers to the wide use of *Clivia* in South African traditional medicine and the fact that the species is now under threat, not only because of harvesting for the herbal medicine trade, but also due to loss of habitat due to agriculture and urbanisation. The natural systems of Durban, KwaZulu Natal, have been reduced from 100% in 1850 to 5% functioning in 1994. Of the remaining 5%, only 1% is now totally functional.

Extraction conditions such as the polarity of the solvent, the method of extraction and the chemical instability of the constituents may also influence the quality of the extract. As a result of all these variables, several scientific journals now require that the chemical composition of a herbal preparation needs to be characterised and/or standardised before any claims can be made regarding efficacy and toxicity. Bauer & Tittel (90) make recommendations on what the minimum characterisation of plant extracts should comprise when publishing data on their activity and herbal preparations are classified as follows:

1. Extracts or preparations in which the active principles are known
2. Extracts or preparations in which the active principles are not known or not finally clarified – this includes plants that have not been investigated previously and also plants which have been well studied but whose active principle is not yet clearly defined

3. Mixed (multi) preparations containing several raw drugs or extracts

Recommendations regarding category 2 herbal extracts are that lead compounds are usually used for chemical characterisation. The compounds may not be related to activity but should be typical chemical markers of the extract. In most cases a "fingerprint" chromatogram of the extract or preparation obtained by HPLC, GC or TLC, is the most appropriate way to document quality. Even if not all or none of the separated constituents can be identified, the fingerprint chromatogram together with the detailed method of extraction represent a fairly good documentation of the quality of the extract. A fingerprint chromatogram of the lipophilic and/or polar fraction of a plant extract may be obtained from almost every plant by using HPLC and gradient elution and an unspecific detection wavelength (close to 200 nm).

1.6 Bioequivalence

Bioequivalence is defined as "having the same strength and similar bioavailablity in the same dosage form as another specimen of a given drug substance" (82) and implies the same therapeutic or pharmacodynamic effect. Many examples exist in herbal medicine of a lack of bioequivalence between the different parts of a plant and between different species within a genus (71, 92, 93). Genera of medicinal plants that show a species difference in activity and therapeutic indication include: *Alchimilla, Allium, Althaea, Artemisia, Juniperus, Pimpinella, Polygonum, Rhamnus, Ruta, Sambucus, Thymus, Vaccinium,* and *Vinca*. A gynaecological illustration of this is the *Ruta* genus - a tea

made from *Ruta hortensis* is taken to relieve the cramps and excessive bleeding of dysmenorrhoea. In contrast, a tea made of *Ruta graveolens* leaves is used both as an emmenagogue to promote and regulate menstruation and to induce abortion (92).

In modern phytomedicine, only biopharmaceutical in-vitro and pharmacodynamic / pharmacokinetic in-vivo equivalence is an acceptable guarantee of identical clinical effects. A plant extract is defined as a multi-component mixture obtained by extraction of specific parts of the plant and each extract exhibits a specific qualitative and quantitative spectrum of content corresponding to the active ingredients in each case. If the active principle/s is/are known, pharmacokinetic studies may be done to establish bioequivalence. Otherwise, evidence of equivalence can be provided by evidence of identical pharmacodynamic effects (94).

1.7 Oxytocic herbal remedies used in South African traditional medicine

It has been established that *Clivia miniata* and *Agapanthus africanus* extracts exhibit uterotonic activity on the isolated rat uterus preparation and are also the most often quoted in the literature as being used as oxytocics. *Clivia miniata* is a member of the Amaryllidaceae family whereas *Agapanthus africanus* is a member of the Alliaceae family (previously known as Liliaceae) and has also been classified as Amaryllidaceae and Agapanthaceae (93, 95). Extracts of both these plant species are used in South African traditional medicine as oxytocic agents to induce and augment labour. The

respective phytochemical profiles of these plants are likely to be quite different, yet extracts of both species are able to produce phasic contractions of the isolated rat whole uterus, followed by tonic contractions at higher doses, in a similar way to oxytocin.

1.7.1 *Agapanthus africanus*

South African traditional healers use the *Agapanthus* plant in various herbal remedies to treat pregnant women (63, 96). *Agapanthus* is recorded as being used in all the medicines prescribed specifically for pregnant women, namely, *isihlambezo, imbelekisane* and *inembe. Isicakathi* is one of the most important medicines used by the Xhosa people in the belief that it will ensure an easy childbirth and healthy child and that the child does not develop bowel trouble. Of all the *Isicakathi* used, *Agapanthus* is the only one used by pregnant women. It is taken as both an ante- and postnatal medication and is also administered to the neonate (93, 97). Structured open-ended ethnopharmacological interviews with 45 traditional healers in rural and urban KwaZulu Natal revealed the *Agapanthus* was one of 5 plants used most often to treat prolonged labour (62). The interviews also confirmed that *Agapanthus africanus* was frequently used to treat constipation in pregnancy. These antenatal medicines are prepared as infusions or decoctions and in the Transkei, the Mpondo women grow the plant in water and drink some of the water daily from the fourth or fifth month of pregnancy (79, 98). Although specific details regarding the actual preparation of these medicines is not consistent in the ethnomedical and ethnobotanical literature, it appears that the rhizome and roots (i.e."root") of the *Agapanthus* sp. is most often used. Traditional healers believe that

medicines prepared from the "roots" of a plant are "stronger" than those prepared from leaves (73). The collection of rhizomes and roots of indigenous plants for sale in the herbal medicine trade in South Africa has resulted in the virtual extinction of some species.

Agapanthus africanus (L.) Hoffmg. (Alliaceae) is known as *uhlakahla* or *ubani* in Zulu, *isicakathi* in Xhosa, *leta-la-phofu* in Sotho, "African lily" in English and "Bloulelie" in Afrikaans (93, 96). In a preliminary pharmacological screening, an aqueous extract of *A. africanus* leaves was found to stimulate smooth muscle directly and to augment the initial response of the uterus to oxytocin and acetylcholine (66). A submaximal concentration of the extract initiated a tonic uterine response with superimposed phasic contractions.

Extensive literature surveys revealed that both the *Agapanthus africanus* (L.) Hoffmg. plant and the more common garden species, *Agapanthus praecox* Willd. subsp. *orientalis* (Leighton) Leighton, family Alliaceae, are used by South African traditional healers in various herbal remedies to treat pregnant women (63, 96). *A. africanus* and *A. praecox* plants are very similar in appearance, the only real difference being in the size and intensity of colour of the flowers and the length of the peduncle (99). In other words, when the plants are not in flower, they appear identical and it is therefore quite possible to confuse the species when collecting material for ethnomedical use. A very strong possiblity must also exist that the species have been confused in the original ethnographical / ethnobotanical descriptions.

Both *A. africanus* and *A. praecox* have been included in a list of potentially toxic plants used in Zulu and Xhosa medicine (81) and it is known that the sap causes severe ulceration of the mouth (63). No data on toxicity assays of these plant species have been reported to date.

Chemical extraction and isolation procedures have identified several saponins and sapogenins in *Agapanthus africanus* rhizomes (100, 101). The hydrolysis of saponins in the acidic environment of normal digestive processes would result in conversion to the steroidal sapogenins which could then be absorbed and produce a pharmacological effect.

1.7.2 *Clivia miniata*

Clivia miniata (Lindl.) Regel is known in Zulu as *umayimi*, English as the "Bush lily" or "St John's lily" and in Afrikaans as "Boslelie" (93). An infusion of *Clivia* leaves or the whole plant is taken by Xhosa and Zulu women during pregnancy to augment or induce labour (74, 93, 96, 98, 102, 103, 104, 105). *Clivia miniata* is also reported to have been used as a remedy for impotence and barrenness (98,104) A weak tea made of the chopped roots has been used for decades by African tribes to treat febrile conditions and to ease childbirth (91). Once the baby is safely born, the young mother is given a drink of this tea to help her milk flow. Should she have difficulty in feeding the newborn, this treatment is continued until the lactation process is functioning well (91, 98). Oxytocin has a similar effect on the stimulation of lactation.

Veale (61) found that an aqueous extract of *Clivia miniata* leaves could initiate smooth muscle contraction in both the isolated rat uterus and ileum. The antiviral properties of leaf and root extracts of *Clivia miniata* Regel against Herpes simplex, Semliki forest, polio, Coxsackie and measles viruses in Vero cells has been shown to be due to lycorine (106). The antiviral effect is most probably due to the inhibition of protein synthesis at the step of peptide bond formation. Lycorine was also found to exhibit consistent *in vitro* activity against 3 flaviviruses (Japanese encephalitis, yellow fever and dengue viruses) but had no antiviral effect on HIV type 1. Toxicity occurred at concentrations within 10-fold of the viral inhibitory concentrations (IC_{50}) in uninfected cells (107).

Many alkaloids have been isolated from *Clivia miniata* Regel to date (108, 109) and one of these, lycorine, is the most widespread alkaloid found in the Amaryllidaceae family. All the Amaryllidaceae alkaloids are of the isoquinoline type and uterine stimulant activity has previously been identified in members of this family (table 1.2, 58).

The toxicity of *Clivia miniata* is well documented (63, 93). All plant parts are recorded as being toxic but Van Wyk, Van Oudtshoorn & Gericke (93) specifically warn against the use of the rhizomes in traditional remedies due to the high concentration of alkaloids, especially the compound lycorine, contained in the rhizome. Lycorine is found in *Clivia* at levels of up to 0,4% of dry weight and causes salivation, vomiting and diarrhoea at low doses and paralysis and collapse at high doses. Wildman (110) reported on the cytotoxicity of lycorine.

1.8 Overview of the thesis – objectives and aims

This thesis was undertaken in the spirit of the WHO directives to Member states to scientifically evaluate herbal medicines so that consumers and health care providers can be provided with up-to-date and authoritative information on the beneficial properties, and possible harmful effects, of all herbal medicines. The objective of the thesis was to determine the uterotonic mechanisms of action and investigate the safety and efficacy of extracts of *Agapanthus africanus* and *Clivia miniata*, two South African plant species used in traditional medicine as herbal oxytocic remedies. The WHO "Guidelines for the Assessment of Herbal Medicines" (88) will serve as a guide.

The aims of the study were to:

- To establish whether the phytochemical profiles of extracts of these oxytocic herbal medicines from different botanical families differed.
- To establish fingerprint chromatograms for the standardisation of the herbal extracts
- To determine the toxicity potential (LC_{50} values) for the herbal extracts using both the brine shrimp and MTT toxicity assays.
- To determine the pharmacological equivalence of leaf and rhizome extracts of *A. africanus* and *A. praecox.*
- To determine the pharmacological activity of *Agapanthus africanus* on the isolated whole rat uterus preparation .

- To establish the methodology and technique of measuring the contractile response of the isolated "stripped" rat myometrium model and to compare the pharmacological activity of *Agapanthus africanus* and *Clivia miniata* in this model .
- To establish the methodology and technique of the measurement of agonist-induced tritium labelled inositol phosphate accumulation in the rat stripped myometrium model and to determine the effects of *Agapanthus africanus* and *Clivia miniata* in this model compared to standard uterotonic agents.
- To establish the methodology and technique of the measurement of agonist-induced prostaglandin synthesis in rat endometrial tissue and to determine the effects of *Agapanthus africanus* and *Clivia miniata* extracts in this model..
- To establish the methodology and technique of culturing rat myometrial cells in order to investigate the viability of this model compared to isolated organs.
- To establish whether these oxytocic herbal medicines from different botanical families (i.e. *Agapanthus africanus* and *Clivia miniata*) differ, both functionally and biochemically, in their mechanisms of initiating contraction in the rat uterine smooth muscle cell compared to standard oxytocic agents.
- To establish whether or not the ethnomedical administration of decoctions of the plants has the potential to cause hyperstimulation of the uterus and related toxicity.

In 1996 the Department of Health held an Essential National Health Research Conference which identified a list of 46 Priority Health Problems. Included in this list of research priorities was: Traditional Medicine, Safe Motherhood, Perinatal Mortality, Clinical and Experimental Research and Women's Health. This thesis covers aspects of each of these

5 priorities (>10% of the priority list) which serves to illustrate the importance of a study of this nature to the National Health Policy. The investigation of the uterotonic mechanisms and potential toxicity of both *Agapanthus* and *Clivia* therefore meets the requirements of priority research in these fields.

CHAPTER 2

METHODS, RESULTS AND DISCUSSION

Introduction:

The aims and objectives of this study were to investigate and describe many diverse aspects of ethnopharmacological research and as a result a variety of experimental procedures were employed. This chapter is therefore divided into Sections A to H and each section covers a different experimental procedure or technique and includes a review of the objectives of the experimental procedure, the methodology used, results obtained and a discussion of the results.

2.1 SECTION A – BOTANICAL

2.1.1 Review

Agapanthus africanus (L.) Hoffmg. family Alliaceae, is known as *uhlakahla* or *ubani* in Zulu, *isicakathi* in Xhosa, *leta-la-phofu* in Sotho, "African lily" in English and "Bloulelie" in Afrikaans (93, 96). *Agapanthus praecox* Willd. subsp. *orientalis* (Leighton) Leighton, family Alliaceae, is known as *ubani* in Zulu, and "Bloulelie" in Afrikaans (93, 96). The genus *Agapanthus* is purely South African. It was first introduced into cultivation in England from the Cape Town area in about 1692 (103). Both species are found widely distributed along the southern and eastern coastline of South Africa (93). These perennial herbs have narrow strap-like leaves and cylindrical, branched rhizomes bearing many fleshy roots (93, 96). The flowers are characteristically borne in a dense cluster (umbel) on a long slender stalk (peduncle) (93). *A. africanus* and *A. praecox* plants are very similar in appearance, the only real difference being in the size and intensity of colour of the flowers and the length of the peduncle. *A. africanus* flowers (fig. 2.1) are deep blue to purplish in colour whereas *A. praecox* flowers (fig.2.2) are a pale to medium blue in colour and the umbel is much larger. The length of the peduncle of *A. africanus* is 30 – 50 cm and that of *A. praecox* is 60 –100 cm (99).

Clivia miniata (Lindl.) Regel, family Amaryllidaceae, is known in Zulu as *umayimi*, English as the "Bush lily" or "St John's lily" and in Afrikaans as "Boslelie" (93). This beautiful, evergreen herb-like plant grows wild in shady ravines from Haga-Haga in the

eastern Cape, along the coastal forests of the Transkei and KwaZulu-Natal and in moist shady places in the eastern Transvaal. It has been known as a pot plant locally and overseas for more than a century. The flower is lily-like, with umbels of dark orangy pink flowers and appears during spring (fig. 2.4). The flowers are followed by bright green and then red berries. The straplike leaves are thick and dark green. The roots are fleshy, juicy and almost corm-like (91).



Figure 2.1. The flower of *Agapanthus africanus* (L.) Hoffmg.



Figure 2.2. The flower of *Agapanthus praecox* Willd. subsp. *orientalis* (Leighton) Leighton.



Figure 2.3. Whole *Agapanthus africanus* plant,



Figure 2.4. The flowers of *Clivia miniata* (Lindl.) Regel.



Figure 2.5. Whole *Clivia miniata* plant.



Figure 2.6. *Clivia miniata plants (centre) for sale as umayimi in the Faraday market, Johannesburg, Gauteng.*

2.1.2 Collection and identification of plant material

Agapanthus africanus (L.) Hoffmg. and *Agapanthus praecox* Willd. subsp. *orientalis* (Leighton) Leighton, family Alliaceae, plants were collected by Dr H. Halgendorff (National Botanical Institute) and cultivated in a Sandton, (Gauteng, South Africa) garden in order to ensure a controlled source of plant material. The plants were authenticated and voucher specimens prepared by Ms R. Reddy (C.E. Moss Herbarium, University of the Witwatersrand). (*Agapanthus africanus* voucher no. H2H45 and *Agapanthus praecox* voucher no. J85312).

Clivia miniata (Lindl.) Regel, family Amaryllidaceae, plants were cultivated in a garden in Sandton (Gauteng, South Africa) in order to ensure a controlled source of plant material for the duration of the study. The plants were authenticated and voucher specimens prepared by Ms R. Reddy (C.E. Moss Herbarium, University of the Witwatersrand – voucher no. J85052).

Whole plants or leaves were always harvested in the early morning at the end of the month of April. This ensured that there would be no seasonal variation in the concentration of active components in the plant extracts for the duration of the study.

2.1.3 Preparation of extracts for pharmacological and toxicological assays

The aqueous plant extracts were prepared as aqueous decoctions according to the method of Sofowora (70). The leaves and rhizomes/roots were washed, dried, cut into small pieces, weighed and then simmered in deionised water for 30 min. The extraction procedure therefore closely resembled the ethnic method of preparation of a decoction (73). The extracts were filtered and frozen. The aqueous filtrates were lyophilized and weighed and the extract yield was calculated relative to the wet starting material. *A. africanus*: leaves 2,6% w/w, rhizome/roots 1% w/w. *A. praecox*: leaves 2,8% w/w, rhizome/roots 1,6% w/w. *Clivia miniata* leaves: 0,76% w/w, roots 5,8% w/w. The "wet" weight of one *A. africanus* leaf was approximately 10 g and of one *C. miniata* leaf was about 20 g.

2.1.4 Preparation of extracts for chemical identification and isolation purposes

2.1.4.1 Ethanolic extracts

Ethanolic extracts for chemical identification and isolation purposes were prepared from the fresh leaves and rhizomes with roots of *A. africanus* and *A. praecox* and fresh leaves and roots of *C. miniata*. The fresh material was washed and chopped up into small pieces, weighed and frozen. The frozen material was then finely divided in a blender with 80% ethanol in water and macerated for 4 hrs. The finely divided macerated

material was placed in the thimble of a Soxhlet apparatus and extracted with 80% ethanol in water until the marc was exhausted. The extract was reduced in volume under vacuum in a Rotavapor at 60°C such that 1 ml of extract was equivalent to 1 g wet plant material. The extracts were acidified with 1% HCl to pH 3 and were then filtered and frozen until required.

2.1.4.2 Flash chromatography fractions

The ethanolic plant extracts were further separated using the vacuum liquid chromatography method of Pelletier, Chockshi & Desai (111). A 20 ml sample of each extract was applied to a 50 g silica gel G-HR (TLC grade, Machery Nagel) column (70 mm x 30 mm) and gradient elution with chloroform, acetone and methanol was carried out. The fractions were collected and the solvents evaporated off. The residues were resolubilised in 1 ml ethanol and dilutions were made such that the tissue samples were not exposed to concentrations of > 1% ethanol. Fractions collected were numbered as follows: fr. 1 (chloroform (100)), fr. 2 (chloroform (50) : acetone (50)), fr. 3 (acetone (100)), fr. 4 (acetone (50) : methanol (50)) and fr. 5 (methanol (100)).

2.1.4.3 Alkaloidal extraction fractions

The leaves of *Agapanthus africanus* and *Clivia miniata* were subjected to a crude alkaloidal extraction process using the method of Campbell, Nair, Gammon, Bastida , Codina, Viladomat, Smith & Albrecht (112) and Ieven, Vlietinck, Van den Berghe & Totte (106) with modifications. Ethanolic extracts were made as described above and were acidified to pH 4. Each extract was then extracted 3 times with diethylether to remove neutral material. The remaining aqueous acidic extract was then extracted 3 times with chloroform to yield fraction A (chloroform soluble alkaloid fraction). The remaining aqueous acidic extract was then basified to pH 8-9 and extracted 3 times with chloroform to yield fraction B (aqueous basic fraction) and fraction C (residual alkaloid fraction). The precipitate that formed after basification was filtered off and washed in chloroform, dissolved in 10% hot acetic acid , basified again and the precipitate collected (fraction D). Solvents were evaporated off the fractions and the residues were than stored at -20°C until required.

2.1.4.4 Sapogenin extraction fraction

Leaves of *Agapanthus africanus* and *Clivia miniata* were also subjected to a crude sapogenin extraction process using the method of González, Freire, Francisco, Salazar & Suárez (100) with modifications. Ethanolic extracts were prepared as before and were then defatted by extraction with benzene:ethanol (50:50). Concentrated HCl was added to the aqueous ethanolic solution until it was 2 M. The acidic extract was refluxed for 2

hrs at 75°C and the acid insoluble residue was then poured into water followed by neutralization with 5% w/v ammonia solution. The excess water was evaporated off the brown precipitate and this suspension was then extracted with benzene. The benzene was evaporated off and the residue (sapogenin fraction) was stored at -20°C until required.

2.2 SECTION B – CHEMICAL ANALYSIS

2.2.1 Review of the chemical compounds already identified in the plant species

It was not the aim of this study to identify new chemical constituents in the plant extracts, but to characterise the extracts used in the pharmacological and biochemical studies for future identification purposes. Chemical extraction and isolation procedures have already identified several saponins and sapogenins in *Agapanthus africanus* rhizomes, namely: sitosterol, yuccagenin, agapanthagenin, 7-dehydroagapanthagenin, 8(14) – dehydroagapanthagenin and 9(11)-dehydroagapanthagenin (100, 101). It was not possible to obtain samples of these isolated compounds for comparitive or characterisation purposes in this study.

The following alkaloids have been isolated from *Clivia miniata* Regel to date: caranine, clivacetine, clivatine, cliviahaksine, clivialine, cliviamartine, cliviasindhine, cliviasine, clividine, clivimine, clivisyaline, clivojuline, clivonidine, clivonine, haemanthamine, hippeastrine, lycorine and miniatine (108, 109). Lycorine is the most widespread alkaloid found in the Amaryllidaceae family. It is quite abundant in the *Crinum, Galanthus, Clivia, Nerine* and *Sternbergia* genera and is present in minor amounts in most species of *Boophone* and *Haemanthus* (110). Viladomat, Bastida, Codina, Nair & Campbell (109) have classified the Amaryllidaceae alkaloid structures of the *Clivia* alkaloids as the lycorine type (lycorine, caranine, cliviasindhine), haemanthamine type

(haemanthamine), miscellaneous (cliviahaksine, clivojuline, clivialine) and the homolycorine type (all the others). All the Amaryllidaceae alkaloids are of the isoquinoline type. *Cyrtanthus and Scadoxus* sp. are used in South African traditional medicine as *isihlambezo* and *inembe* (63) and contain the alkaloids lycorine, haemanthamine and hippeastrine in common with *Clivia* (109). They are all members of the tribe *Haemanthae.* *Crinum bulbispermum* is also used but has only lycorine in common with *Clivia.* A very small sample of lycorine was obtained from Dr Bill Campbell, University of Cape Town, and was used as a standard where possible. The toxicology of lycorine is discussed in Section D.

2.2.2 Methodology

2.2.2.1 Spectral analysis of the *Agapanthus africanus* and *Agapanthus praecox* leaf and rhizome/root extracts for the pharmacological equivalence study

Spectral investigations (i.e. FT infrared and ^{13}C NMR) were performed on the lyophilized aqueous extracts of *Agapanthus africanus* and *Agapanthus praecox* leaf and rhizome/roots in order to compare them for the pharmacological equivalence study.

^{13}C NMR spectra of all the extracts were recorded at 30°C in D_2O at 75.462 MHz on a Varian Gemini 300 broadband NMR spectrometer, locked on deuterium and referenced to

the carbonyl signal of acetone d_6 at 206.0 ppm. The KBr disc technique was used to record infrared spectra on a Shimadzu FTIR 4200 spectrophotometer.

2.2.2.2 Chromatographic analysis of the plant extracts

2.2.2.2.1 HPLC analysis of ethanolic extracts of leaves and rhizome/roots of *A. africanus*, *A. praecox* and *C. miniata*

A comparative liquid chromatographic analysis was performed on all the ethanolic plant extracts and some isolated fractions to assess the relative content of some of the constituents and to obtain "fingerprint" chromatograms for identification and characterisation purposes (90). A Hewlett Packard 1090 HPLC system was used with a ternary gradient pump, autosampler, diode array UV detector and HP Chemstation v 06.04.02 software. A C18 reversed phase column was used for the separation (Luna C18(2), 5 µm, 250 x 4.6 mm, Phenomenex, Torrance, CA), with gradient elution from 0% acetonitrile to 50% acetonitrile in water after 15 minutes. The flow rate was 1 ml/min with UV detection at 205 nm, and an injection volume of 5 µl.

2.2.2.2.2 TLC detection of alkaloids, saponins and sapogenins in chemical extracts of *Agapanthus africanus* and *Clivia miniata* leaves

Samples were dissolved in 1 ml of ethanol or chloroform and applied to 20 x 20 cm glass TLC plates precoated with silica gel 60-F254 (Merck). Application started 1,5 cm from

the lower end of the plate and 5 µl of each sample was applied. Run length was 100 mm and pre-equilibrated rectangular glass TLC tanks were used. Results were reported as hRf-values – the distance in mm from starting point to spot centre when a run length of 100 mm was used (115).

The presence of alkaloids was determined using the solvent systems of Jeffs, Mueller, Abou-Donia, Seif el-Din & Campau (114) (System AI – chloroform (80): methanol (20)) and Döpke (115) (System AII - chloroform (40): ethyl acetate (40): methanol (20)). Detection was by means of UV (quenching of fluorescence in UV-254 and blue or yellow fluorescence in UV-365) and Dragendorff reagent (brown or orange zones appear immediately on spraying). Yohimbine (Sigma) was used as a standard.

The presence of saponins was detected by the foaming of the aqueous plant extracts on shaking and also by TLC detection using the solvent system of Wagner, Bladt & Zgainski (116) (System SP1- chloroform (64): methanol (50): water (10)) with detection by spaying with vanillin-sulphuric acid reagent and heating at 110°C for ten minutes (saponins form mainly blue or blue-violet and sometimes yellowish zones). Strophanthin (Sigma) was used as a standard.

Sapogenin detection was by means of the solvent system described by Harborne (117) (System SGI – acetone (80): hexane (20)) with detection by spraying the plates with antimony chloride solution and heating for 10 min at 110°C (red/yellow and blue/violet spots). Diosgenin (Sigma) was used as the standard.

2.2.2.3 Content of inorganic ions in the leaf extracts of *C. miniata* and *A. africanus*

The concentrations of Na^+, K^+ and Ca^{2+} in 10 mg/ml samples of the aqueous extracts of *Agapanthus africanus* leaf and *Clivia miniata* leaf were determined using inductively coupled plasma optical spectroscopy. An ARL 34000 ICP instrument was used consisting of inductively coupled argon plasma operating at 27 MHZ with direct reading on a 29-channel spectrophotometer using the method of Yousefi & Rama (113).

2.2.3 Results

2.2.3.1 Results of the spectral analysis

The FT infrared spectra [KBr disc, main absorptions at 3400 (broad), 2926, 1744, 1645-1617 (broad), 1427 (broad), 1105-1028 (broad) and 640-535 (broad) cm^{-1}] and the ^{13}C NMR spectra [main signals in D_2O at 103.9, 96.1, 92.3, 76.1, 75.9, 74.3, 72.9, 71.7, 69.8 and 60.9 ppm] of the leaf extracts of *A. africanus* and *A. praecox* were respectively virtually identical.

The rhizome extracts of *A. africanus* and *A. praecox* also were found to exhibit similar spectral properties. The main signals observed in the ^{13}C NMR spectra of the leaf extracts were also found to be present in the spectra (D_2O) of the rhizome extracts. However, due to additional signals a more complex pattern was obtained in the region

104 to 60 ppm for the root extracts which indicated that the rhizome extracts were less pure than the leaf extracts. The infrared spectra [KBr disc, main absorptions at 3412 (broad), 2926, 1645-1616 (broad), 1456-1427 (broad), 1039 (broad) and 670-515 (broad) cm^{-1}] of the rhizome extracts showed a good overlay comparability and resembled those of the leaf extracts although they were not identical.

2.2.3.2 Results of the HPLC analysis

2.2.3.2.1 "Fingerprint" chromatograms of ethanolic extracts of *Agapanthus africanus* and *Agapanthus praecox* leaves and rhizome/roots

The HPLC chromatograms (fig. 2.7) showed that the extracts obtained from *A. africanus* and *A. praecox* leaf and rhizome/root extracts were very similar, suggesting that the same compounds are present in both plants. The relative intensities of some of the peaks differed between different extracts, suggesting a quantitative difference between different extracts.

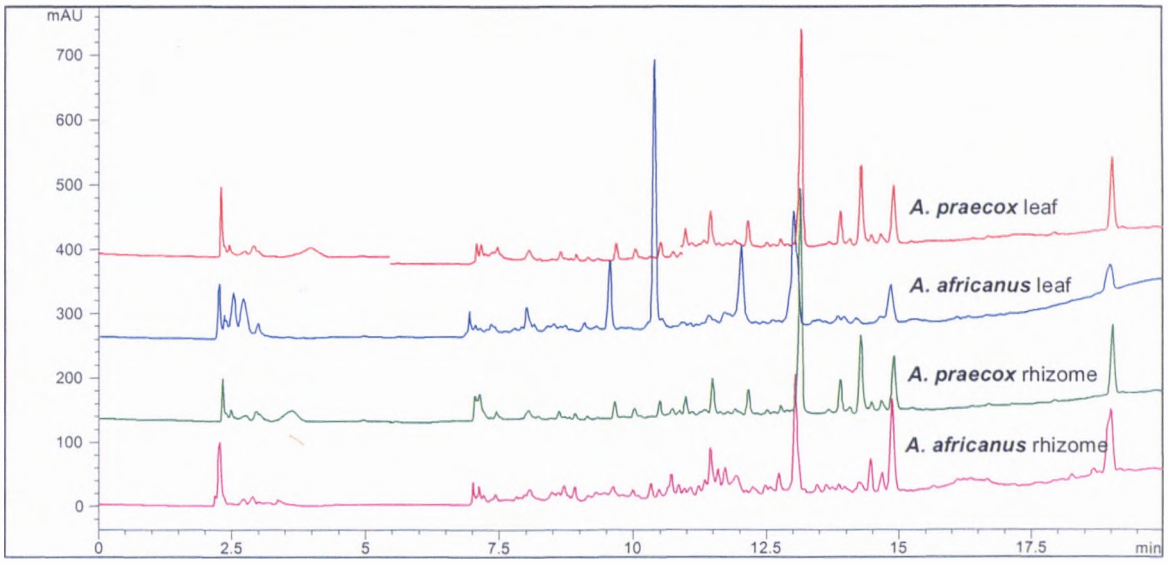


Figure 2.7. Overlays of HPLC profiles obtained from ethanolic extracts of fresh *A. africanus* leaves and rhizome/roots and *A. praecox* leaves and rhizome/roots. Separation parameters: Column: C18 reversed phase (Luna C18(2)), 5µm, 250 x 4.6 mm, Phenomenex, Torrance, CA); solvents: water and acetonitrile; gradient elution from 0% acetonitrile to 50% acetonitrile in water after 15 minutes; flow rate: 1,0 ml/min; detection: 205 nm; injection volume: 5 µl.

2.2.3.2.2 "Fingerprint" chromatograms of ethanolic *Clivia miniata* leaf and root extracts

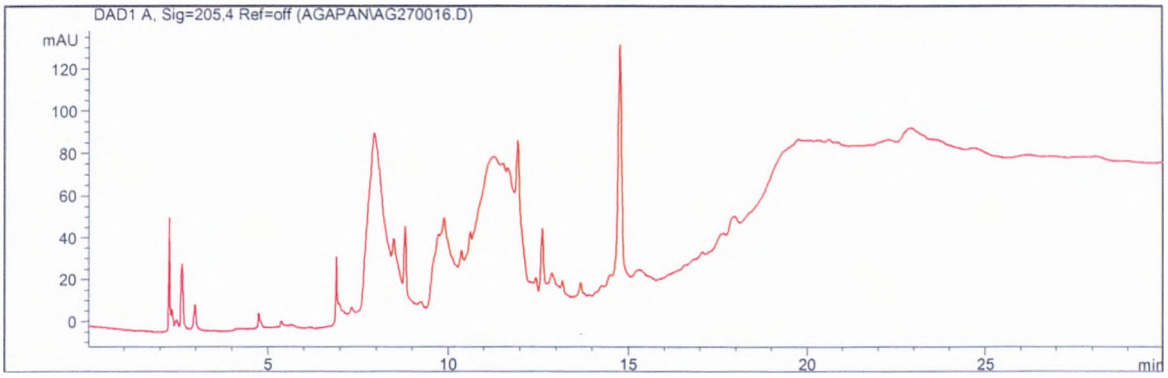


Figure 2.8. HPLC profile of constituents in the ethanolic extract of fresh *Clivia miniata* leaves.

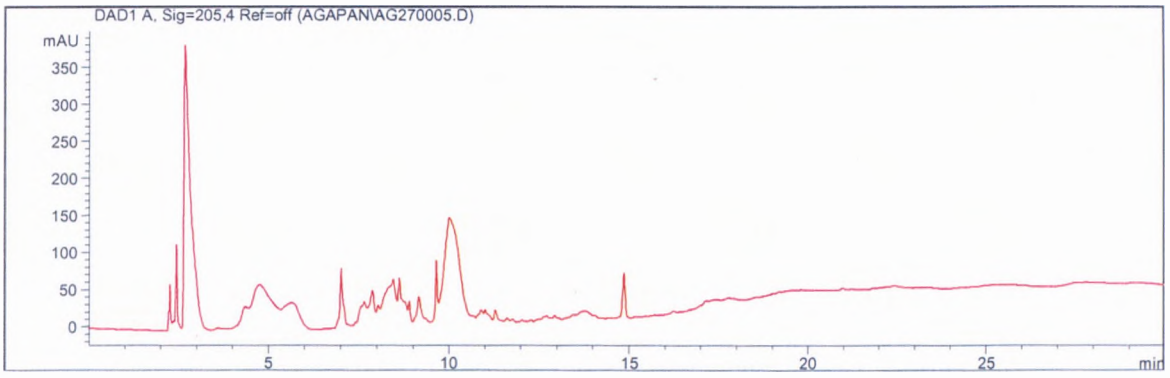


Figure 2.9. HPLC profile of constituents in the ethanolic extract of fresh *Clivia miniata* rhizomes.

2.2.4.2.3 Chromatograms of active fractions of *Agapanthus africanus* and *Clivia miniata* leaves isolated by flash chromatography

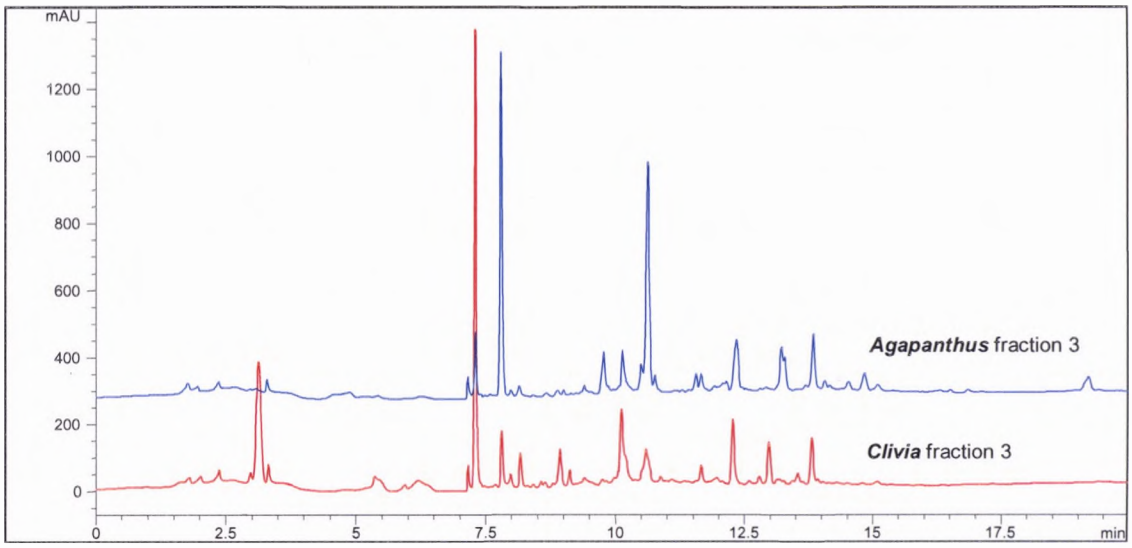


Figure 2.10. Overlaid HPLC profiles of the constituents in isolated active fractions of *Agapanthus africanus* leaf extract and *Clivia miniata* leaf extract obtained as described in Section 2.1.4.2 (activity fig. 2.45). Separation parameters: Column: C18 reversed phase (Luna C18(2)), 5 µm, 250 x 4.6 mm, Phenomenex, Torrance, CA); solvents: water and acetonitrile; gradient elution from 0% acetonitrile to 50% acetonitrile in water after 15 minutes; flow rate: 1,0 ml/min; detection: 205 nm; injection volume: 5 µl.

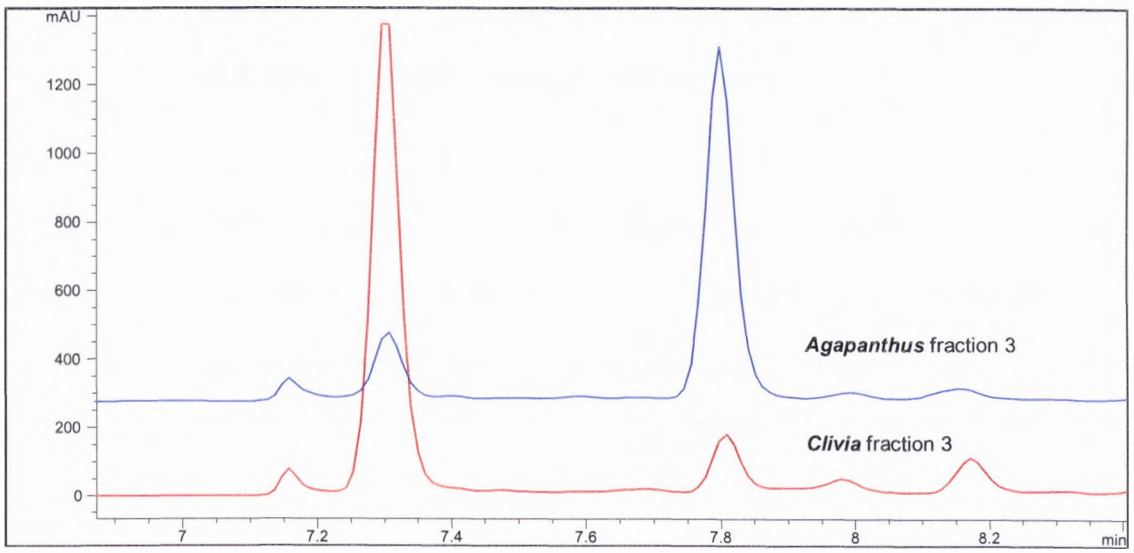


Figure 2.11. Zoomed-in chromatogram of the major peaks in the isolated acetone fractions of *Agapanthus africanus* and *Clivia miniata* (see section 2.1.4.2).

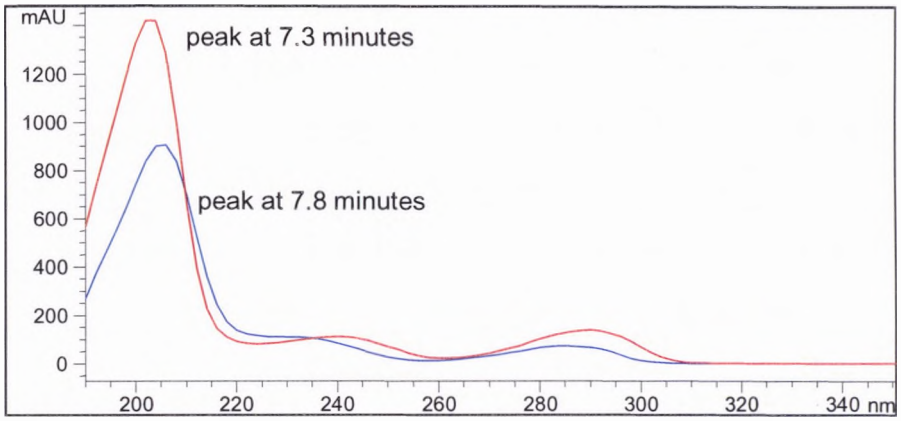


Figure 2.12. The UV spectra of the peaks eluting at 7.3 and 7.8 minutes, showing the similarity in absorption spectra, suggesting that the peaks may be due to structurally similar compounds. The spectra of the peaks with corresponding retention times in the two extracts, had exactly the same spectrum, suggesting that the same compounds were present in both the *Clivia* and *Agapanthus* extracts, but in different concentrations.

2.2.3.3 Detection of alkaloids, saponins and sapogenins in chemical fractions of *A. africanus* and *C. miniata* leaf extracts

Table 2.1. Detection of alkaloids, saponins and sapogenins in chemical fractions of *A. africanus* and *C. miniata* leaf extracts using standard TLC solvent and detection systems. Results are reported as hRf-values.

Chemical fraction	**Alkaloid AI**	**Alkaloid AII**	**Saponin SPI**	**Sapogenin SGI**
Clivia acidified ethanolic extract	15,25,50,52, 72,82,87	9,14,27,69, 77		
Clivia flash fr.3 (acetone)	12,15,50	9	54,60,73	
Clivia flash fr.5 (methanol)	11,20,40, 50		28,54,60	
Clivia alkaloid fr. C	12,50,90		65	
Clivia alkaloid fr. D	15,47,88	28	28,54,60,65	
Clivia sapogenin extract				75
Lycorine standard	50	36		
Agapanthus flash fr.1($CHCl_3$)			50,76,85,90	
Agapanthus flash fr.3 (acetone)	43		50,63,68,75, 80,85,90	
Agapanthus flash fr. 5(MeOH)			44,50,68,76	
Agapanthus alkaloid fr. C			83	
Agapanthus sapogenin extract				68,74,81,87

Alkaloids, saponins and sapogenins were detected in the various chemical fractions of *A. africanus* and *C. miniata* leaf extracts (table 2.1). The hRf-value for the lycorine standard in System AII was the same as that reported by Döpke (115). Alkaloids with hRf values of 15 and/or 50 were common to *Clivia* ethanolic extract, flash fr. 3, flash fr. 5, alkaloid fr. C and alkaloid fr. D. The value for the lycorine standard in this system was also 50, indicating its presence in all the *Clivia* fractions except D. Saponins with hRf-values of 28, 54 or 60 were common to *Clivia* flash fr. 3, flash fr. 5 and alkaloidal fr D. Saponins with hRf values of 50, 85 or 90 were common to *Agapanthus* flash fr. 1, 3 and 5.

2.2.3.4 Content of inorganic ions

The concentrations of K^+ and Ca^{2+} ions in the *A. africanus* and *C. miniata* aqueous leaf extracts was found to be insufficient to cause uterine contraction (table 2.2).

Table 2.2. Concentration of inorganic ions (mM) in plant extracts.

Inorganic ions	**K^+**	**Ca^{2+}**	**Na^+**
Agapanthus africanus	4,4	1,3	0,3
Clivia miniata	1,3	4,6	6,8

2.2.4 Discussion

The spectral investigations (i.e. FT infrared and ^{13}C NMR) have shown that the leaf extracts of *A. africanus* and *A. praecox* are respectively virtually identical. It can thus be concluded with a high degree of confidence that these extract samples are similar with respect to chemical composition or structure. In fact, the simplicity of the ^{13}C NMR spectra suggest that the aqueous leaf extracts are reasonably pure. The aqueous rhizome extracts of *A. africanus* and *A. praecox* also exhibited similar spectral properties and can therefore be regarded as relatively similar in chemical composition or structure. They appear to be more complex and less pure than the leaf extracts but the infrared overlay comparability was good, indicating that the rhizome extracts resembled the leaf extracts, although they were not identical to one another.

The chromatographic results indicate that for both these *Agapanthus* species, the major components with respect to area under the curves, present in the leaves and rhizome elute between 10 and 20 minutes under the current chromatographic conditions and system. The observation that all the component quantities for both the leaf and the rhizome of *A. praecox* show similar retention times clearly suggests that these plant parts of *A. praecox* contain similar chemical profiles. This observation was further supported by comparing the UV spectra of each component. Seven major components eluted at 11.5, 12.2, 13.2, 13.9, 14.4, 14.9 and 19 min. Inspection of the UV spectra of the components of *A. africanus* rhizome and leaf suggest that the major components are of similar chemical nature and are also present in *A. praecox* rhizome and leaf extracts. It is therefore clear

from these chromatograms and UV spectra that there are core components that are present in both the rhizomes and leaves of these *Agapanthus* species.

HPLC chromatographic analysis of the isolated active fractions of *Clivia* and *Agapanthus* (acetone flash chromatography fractions) indicate common peaks at retention times of 7,3 and 7,8 minutes (figs. 2.10 and 2.11). Spectral evidence (fig. 2.12) shows that the fractions most probably contain the same substances but at different concentrations.

TLC screening provided evidence of alkaloids, saponins and sapogenins in the *C. miniata* and *A. africanus* leaf extracts. As could be predicted, the presence of lycorine was noted in some of the *Clivia* extracts. The TLC screening of a crude "sapogenin" extract of *Clivia* leaves provided surprising evidence of the presence of a previously unreported sapogenin in this plant species. However, all the aqueous plant extracts used in this study produced foam on agitation, which is indicative of the presence of saponins.

It was established that the concentrations of K^+ and Ca^{2+} ions in leaf extracts of *C. miniata* and *A. africanus* were insufficient to initiate spontaneous contractions of the uterus.

2.3 SECTION C - STATISTICAL EVALUATION OF RESULTS

In the following Toxicology section (Section D), the Brine shrimp lethality assay results were expressed as % survival at each concentration compared to control values. LC_{50} values were calculated from the results expressed as sigmoidal curves obtained by nonlinear regression using GraphPad Prism (version 1.0). In the MTT toxicity study LC_{50} values were also calculated from the results expressed as sigmoidal curves obtained by nonlinear regression using GraphPad Prism (version 1.0). Results are expressed as the mean ± S.E.M

All the isolated organ results in Section E of this study were evaluated using sigmoidal curves generated by nonlinear regression using GraphPad Prism (version 1.0). The N value refers to the number of rats used per experiment. Results are expressed as the mean ± S.E.M.

The results of the biochemistry experiments measuring [^{3}H]inositol phosphate accumulation in Section F are expressed as the mean ± S.E.M. The significance of differences between two means was evaluated by means of the Student's *t* test using GraphPad Instat 2 (* $p < 0,05$; ** $p < 0,005$; *** $p < 0,0005$).

2.4 SECTION D – TOXICOLOGY

2.4.1 Review

Wilman (110) and Gabriellsen, Monath, Huggins & Kefauver (107) have reported on the toxicity of lycorine, an alkaloid found in *Clivia miniata*. Aqueous solutions of lycorine hydrochloride (0,1-1%) caused inhibition of the metaphase stage, contraction and irregular distribution of the chromosomes, tripolar mitoses and chromatin bridges in the sprouting root tips of *Vicia faba* L. (110). Lycorine (10 μg/g) administered to rats caused a loss of body weight, subcutaneous or submucosal haemorrhages, defects in dental enamel and a decrease in ascorbic acid content of certain tissues. Ascorbic acid supplements caused the symptoms to disappear and therefore lycorine toxicity has been linked to inhibition of ascorbic acid synthesis, probably due to a decreased ability of the liver to oxidatively degrade tyrosine. The testes and ovaries of immature rats showed a 50% decrease in weight after 3 days of treatment with lycorine. Cell division occurred only in the secondary spermatocytes and many of the cells were multinucleated and of large size. Cell division in the spermatogonia or primary spermatocytes was not observed, and no spermatid cells were present in the treated animals although development had reached the spematozoon stage in the untreated animals. In the ovaries, follicles were fewer and smaller than in the controls. The rat livers and spleens showed a relative increase in weight. The arrangement of hepatic cells was abnormal and evidence of fatty degeneration and haemorrhage was present. These effects were much less marked in mature animals and it can be surmised that the greatest effect of

lycorine in on immature cells. Repeated subcutaneous injection of lycorine (0,01-1mg/100g) in rats caused marked granulocytic leucopenia and a decrease in the number of erythrocytes of peripheral blood. Histologic examination of femoral bone marrow showed depression of cellular activity. In view of this review, the fact that *Clivia miniata* is used in herbal remedies during pregnancy gives cause for grave concern regarding the potential for toxicity in the foetus.

Gabriellsen et al (107) determined the TC_{50} (cytotoxicity in uninfected cells – the drug concentration (μg/ml) that reduces cell numbers and their metabolic activity by 50% as compared to the viability of uninfected control cells in duplicate test wells in the MTT assay). All in vitro MTT assay results represented an average of 2-6 individual test results using Vero cells (LLCMK2). The TC_{50} was in the region of 2.5 μg/ml.

A literature search of botanical toxicology identified the suitability of a well-documented toxicity test, known as the "Brine Shrimp" bioassay for determination of LC_{50} values in plant extracts. A tiny invertebrate marine crustacean, *Artemia salina* (brine shrimp), has been used as general bioassay tool (lethality assay) in order to examine pharmacological activities in plant extracts which may be manifested as toxicity towards the newly hatched nauplii. (118). Brine shrimp eggs hatch with 48 hrs of being place in a brine solution, providing large numbers of larvae (nauplii). The nauplii are exposed to various concentrations of test substance and survivors are counted after 24 hrs. Percentage deaths at each dose are recorded and these data can then be used to estimate median lethal concentration values (LC_{50}) for comparison of potencies. Although often ill defined in

terms of mechanisms of toxicity, this bioassay has none the less allowed for the rapid and economical screening of a variety of samples for biological activity e.g. pesticide residues, mycotoxins, stream pollutants, anaesthetics, dinoflagellate toxins, morphine-like compounds, oil dispersants and superoxide-mediated toxicity. The brine shrimp assay measures general cellular toxicity and is considered a general toxicity assay, not specific for any particular physiological action. In comparison to mammalian systems, brine shrimp do not possess the necessary cytochrome P-450 enzymes required for the metabolic activation or detoxification of certain chemicals and they exhibit differences in purine metabolism (119). The brine shrimp bioassay has been successfully used to detect biologically active compounds that inhibit protein synthesis within mammalian cells, as well as those that affect RNA polymerase and ATPase systems (119). The limitations of this assay include the testing of compounds with low water solubility (the assay is performed in a salt water solution) and the question of LD_{50} calculations (120). Because the shrimp are not actually being dosed but are merely being exposed to the test substance in the medium (it is an immersion assay), the results can only realistically be expressed as LC_{50} values instead of LD_{50} values.

The Brine Shrimp assay is limited in that it uses an invertebrate marine organism as the biological model. Many biological assays require the measurement of surviving and/or proliferating mammalian cells. This can be achieved by several methods, including counting mammalian cells that include or exclude a dye. The MTT-microculture tetrazolium salt assay according to Mosmann (121) is a rapid colorimetric assay for cellular growth and survival that utilizes the multiwell scanning spectrophotometer

(ELIZA reader) to measure large numbers of samples with a high degree of precision. The MTT test is based on the tetrazolium salt, MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, and is a versatile and quantitative assay. It has the advantage of avoiding washing steps that would increase processing time and sample variation. The ideal colorimetric assay for viable cells should utilize a colourless substrate that is modified to a coloured product by any living cell, but not by dead cells or tissue culture medium. The tetrazolium salts are suitable candidates for this purpose, since they measure the activity of NADH-generating dehydrogenase enzymes found in the mitochondria of metabolically active cells. The tetrazolium ring is cleaved in active mitochondria, and therefore the reaction occurs only in living cells. The conversion of the tetrazolium compound to a blue formazan product is monitored by a shift in the absorbance, and a linear relationship exists between cell number and the amount of formazan product produced. Viable cells take up MTT and conversion to the formazan product results in a colour change in the wells from yellow to purple-brown which is measured as a shift in absorbance. This assay is a growth assay and does not differentiate between cytostatic and cytocidal effects on cells (122). One aspect that must be kept in mind is that the MTT assay depends both on the number of cells present and on the mitochondrial activity per cell. Thus, although for many cell types the MTT and [^{3}H]thymidine uptake assays give similar results, other cells may give a reduced sensitivity with MTT (123). Another limitation of the MTT test is that normal cell contaminants may also reduce the dye and it is essential that sufficient time is allowed for cell death and loss of dehydrogenase activity to occur. The use of a colorimetric assay for cell growth and survival, however, performed in microtitre trays in conjugation with

multichannel pipettors and an automatic scanning spectrophotometer offers major advantages in speed, simplicity, cost and safety over conventional assays including the uptake of radiolabelled compounds (eg. [^{3}H]thymidine assay).

2.4.2 Methodology

2.4.2.1 Brine shrimp bioassay

Solutions of lyophilized aqueous extracts of *Clivia miniata* roots and leaves, *Agapanthus praecox* leaves and rhizomes (with roots) and *Agapanthus africanus* leaves and rhizomes (with roots) were made up and were then centrifuged. The supernatants were filtered and adjusted to pH 7,4 by dropwise addition of either 1 M HCl or 1 M NaOH solutions.

The brine shrimp assay was performed basically as per Meyer, Ferrigni, Putnam, Jacobsen, Nichols & McLaughlin. (118) with a few modificatons. Brine shimp eggs (Wilgo) (ca. 50 mg) were sprinkled into a shallow rectangular dish (22 x 32 cm) filled with artificial sea water which was prepared with a commercial salt mixture (Instant Oceans, Aquarium Systems Inc.) and double-distilled water. The dish was loosely covered and left at ambient temperature. The eggs hatched after ca. 48 hours and the dish was uncovered and a light source was provided at one end. After 24 hours, the phototropic nauplii were collected by pipette from the lighted side. The nauplii could be counted macroscopically in the pipette tip against a lighted background and 1ml of ASW

containing 5 to 10 shrimp were transferred to each glass sample vial using disposable pipette tips.

Four to five concentrations of each test extract and ASW controls were added to the vials (5 replicates per treatment) and the volume made up to 5 ml with ASW. A drop of dry yeast suspension (3 mg in 5 ml ASW) was added as food to each vial. The vials were maintained under illumination at ambient temperature. Survivors were counted, with the aid of a 3 x magnifying glass, after 6, 24 and 48 hours, and the percent deaths at each dose and control were determined. The 24 hr counts were the most useful. The evaluation was performed by counting "viable" vs "nonviable" shrimp in each vial. An individual was considered "nonviable" whether dead or not, if it did not change its position by controlled forward motion in the vial within about 10 s (there was normally little ambiguity, because healthy nauplii are quite active).

Results were expressed as % survival at each concentration compared to control values. LC_{50} values were calculated from the results expressed as sigmoidal curves obtained by nonlinear regression using GraphPad Prism (version 1.0).

2.4.2.2 Colorimetric MTT (tetrazolium) cytotoxicity bioassay on a human kidney epithelial cell line

The method of Mosmann (121) was followed, with modifications, to evaluate cellular viability of a Graham human renal epithelial cell line in the presence of the aqueous plant

extracts. Solutions of lyophilized aqueous extracts of *Clivia miniata* roots and leaves, *Agapanthus praecox* leaves and rhizomes (with roots) and *Agapanthus africanus* leaves and rhizomes (with roots) were made up and were then centrifuged. The supernatants were filtered and adjusted to pH 7,4 by dropwise addition of either 1 M HCl or 1 M NaOH solutions. The osmolality of the extracts was then adjusted to a value of 300 ± 5 mOsmols using the Roebling automated micro-osmometer (type 12/12DR) and sterile double distilled water (most mammalian cells can tolerate an osmolality of 260 to 350 mOsm/kg (Sigma Cell Culture Catalogue 1998). All further dilutions were make from these stock solutions using phosphate-buffered saline (PBS) (Highveld Biological (Pty) Ltd.) and all the plant extract solutions were then sterilized by filtration. The test solutions were stored at -20°C. Each plant extract was tested in a minimum of 5 concentrations in order to obtain a representative dose response curve. A volume of 10 µl of each concentration of the plant extracts was then pipetted into 96-well microtitre plates (replicates of 8 wells per concentration per plate). Each determination was performed in at least two separate plates.

Single cell suspensions of human kidney epithelial cells (Graham cell line 293, Highveld Biological (Pty) Ltd.) were obtained by trypsinization of monolayer cultures. The cells were subcultured once weekly and the growth medium used was HAM F10 (Highveld Biological (Pty) Ltd.) supplemented with 5% heat inactivated foetal calf serum (FCS) (Highveld Biological (Pty) Ltd.) and 0,1% gentamicin (Highveld Biological (Pty) Ltd.). The growth medium was changed every 2nd day. Cell counts were performed using the trypan blue exclusion method and a haemocytometer. The cell suspension was diluted

with medium to a concentration of $1x10^6$ cells/ml. The cell suspension (90 µl per well) was added to each well of the 96-well microtitre plates containing the plant extracts and a row of 8 wells serving as a positive control on each plate. A row (replicate of 8) of wells in each plate served as a negative control and contained only 10 µl of PBS and 90 µl of growth medium. The cell suspension was incubated with the plant extracts in an incubator at 37°C plus carbogen (5% CO_2 in oxygen) for a period of either 1 or 24 hrs (i.e. continuous exposure to plant extracts).

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma) was dissolved in PBS (5 mg/ml) and was then filtered to sterilize it and remove any insoluble residue that might be present. This stock solution was then stored protected from light at 4° C for a maximum of 2 weeks (35). The stock MTT solution (20 µl per 100 µl) medium was added to all the wells in the assay. The plates were then incubated at 37°C for a further 4 hrs. A volume of 100 µl of the medium was then aspirated out of each well and 100 µl of dimethyl sulphoxide (DMSO) was added to stop the reaction and solubilize the formazan crystals (121). The plates were read immediately.

The results were obtained by placing the plates in a scanning multiwell spectrophotometer (Labsystems, iEMS Reader MF). The plates were first shaken and then absorbance was measured 540 nm and 690 nm. Neither formazan nor MTT absorb at the reference wavelength of 690 nm and these values were subtracted from the corresponding well value at 540 nm to correct for artefacts such as scratches at the bottom of the wells (122). All results represent the average of a minimum of 16 wells.

The mean negative control value in each plate was subtracted from all results to correct for "background" interference due to PBS and medium composition. Absorbance from plant extract treated cells was then corrected against the untreated positive control absorbance values and surviving fraction/fractional absorbance was expressed as % cellular viability (survival). LC_{50} values were then calculated from the results expressed as sigmoidal curves obtained by nonlinear regression using GraphPad Prism (version 1.0).

2.4.3 Toxicity tests - results and discussion

The results of the Brine Shrimp lethality assay indicated that *Clivia miniata* was more toxic than *Agapanthus* (table 2.3). This confirms the reported toxicity of Clivia and is most probably due to the lycorine component (93,107, 110). *Clivia* leaf and rhizome extracts exhibited similar toxicity. The *A. praecox* extracts were found to be more toxic than the *A. africanus* extracts and *A. africanus* leaf extract was the least toxic with an LC_{50} value of 2,39 mg/ml. *Agapanthus* rhizome/root extracts were more toxic than the leaf extracts. The 24 hr counts were the most useful. Immersion for 6 hrs did not give meaningful results as all the extracts were relatively non-toxic after this exposure time. Toxicity measured after immersion for 24 hrs and 48 hrs respectively did not differ significantly.

The LC_{50} values calculated from the results of the MTT 24 hr toxicity tests (table 2.4) differed from the Brine Shrimp 24 hr lethality assay values. Results of the *C. miniata* extracts differed the most significantly, with non-lethal results in the MTT tests compared

to LC_{50} values of 0,42 mg/ml (leaf extract) and 0,49 mg/ml (root extract) in the Brine Shrimp test (figs. 2.13 and 2.14). The only extracts with similar LC_{50} values for both types of toxicity test were the *A. africanus* and *A. praecox* rhizome/root extracts (figs. 2.15 and 2.16).

Table 2.3. Median lethal concentration values (LC_{50}) of aqueous extracts of *Clivia miniata* leaves and roots, *Agapanthus africanus* leaves and rhizomes and *Agapanthus praecox* leaves and rhizomes to 1 day old brine shrimp nauplii (*Artemia salina*).

Extract	**LC_{50} (mg/ml)**	**95% Confidence Intervals**
C. miniata leaves	O,42	(0,32 to 0,55)
C. miniata roots	0,49	(0.17 to 1,37)
A. africanus leaves	2,39	(1,49 to 3,83)
A. africanus rhizomes/roots	0,90	(0,26 to 3,17)
A. praecox leaves	0,52	(0,11 to 2,41)
A. praecox rhizomes/roots	0,46	(0,13 to 1,63)

Table 2.4. Median lethal concentration values (LC_{50}) of aqueous extracts of *Clivia miniata* leaves and roots, *Agapanthus africanus* leaves and rhizomes and *Agapanthus praecox* leaves and rhizomes to a human renal epithelial cell line (Graham cells).

Extract	**LC_{50} (mg/ml)**	**95% Confidence Intervals**
C. miniata leaves	Not applicable	
C. miniata roots	Not applicable	
A. africanus leaves	0,37	(0,14 to 0,96)
A. africanus rhizomes/roots	1,12	(0,97 to 1,3)
A. praecox leaves	1,28	(0,82 to 2,03)
A. praecox rhizomes/roots	0,52	(0,11 to 2,40)

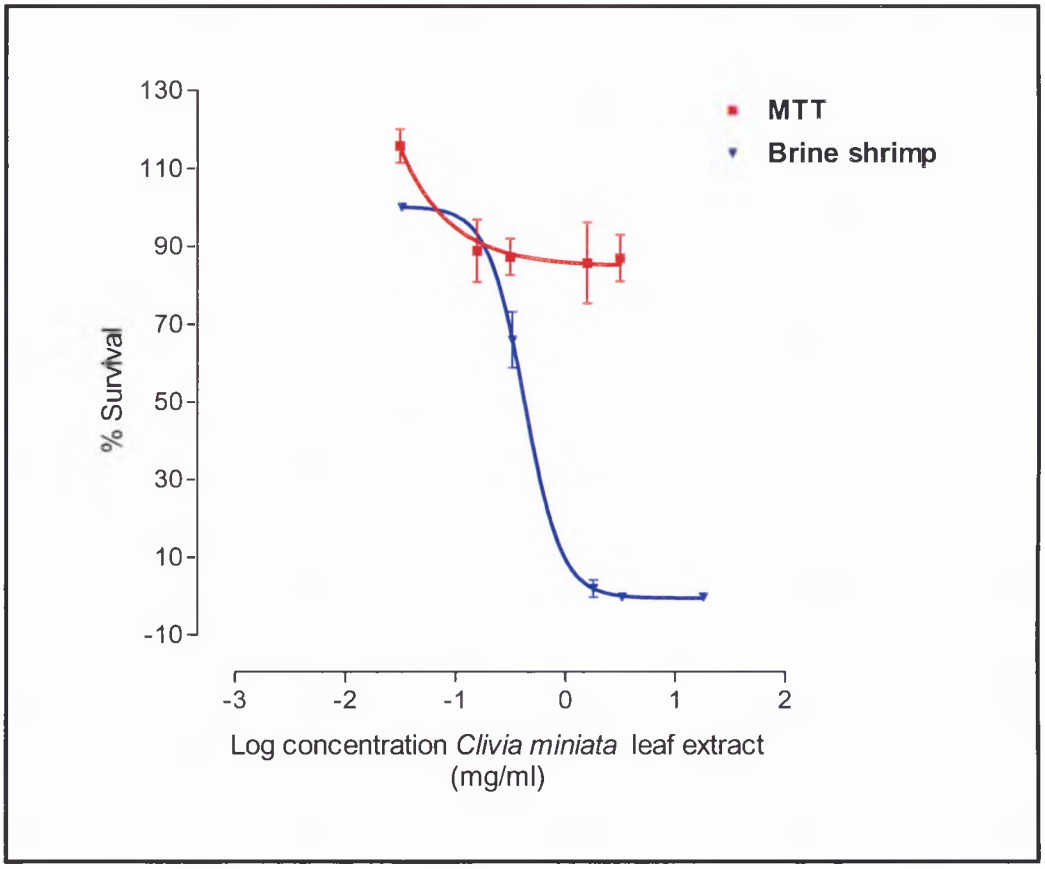


Figure 2.13. The effects of *Clivia miniata* leaf extract on % survival in the Brine Shrimp and MTT bioassays. Results are expressed as the mean and the vertical bars represent the S.E.M.

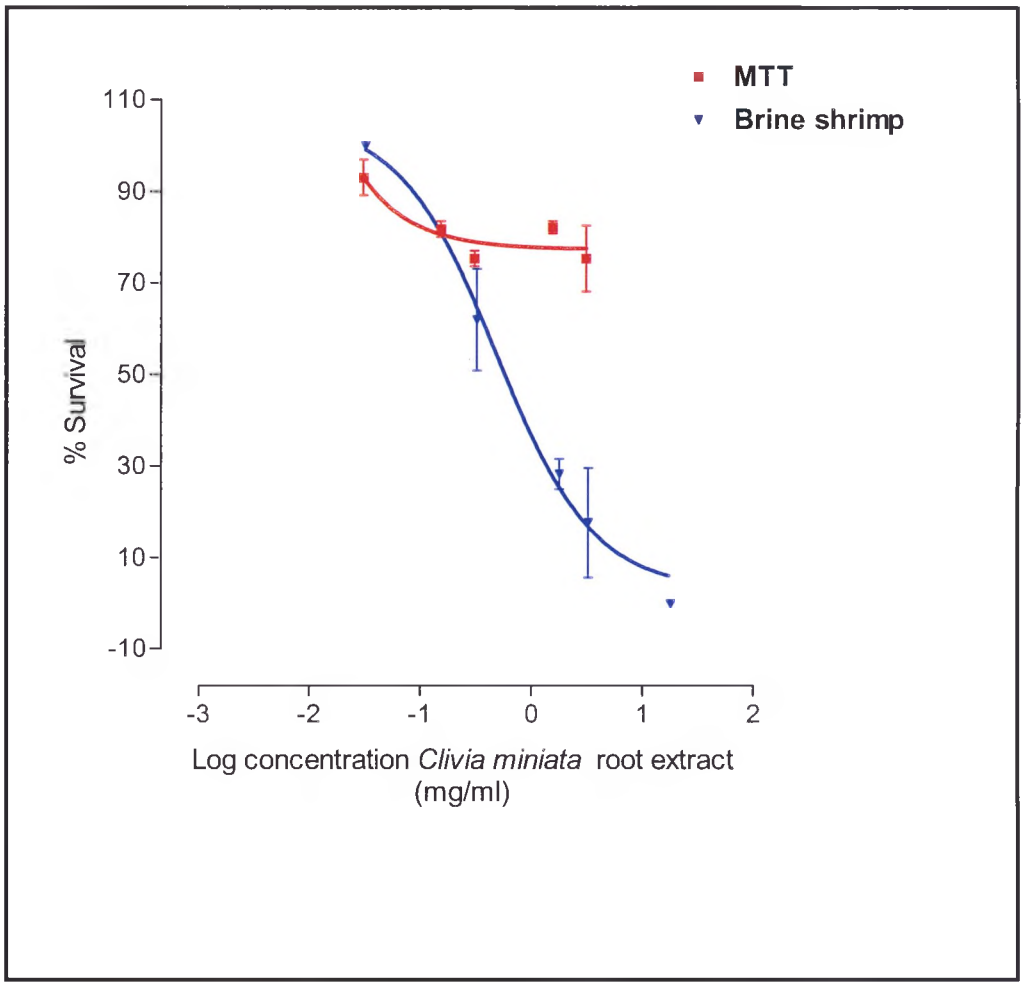


Figure 2.14. The effects of *Clivia miniata* root extract on % survival in the Brine Shrimp and MTT bioassays. Results are expressed as the mean and the vertical bars represent the S.E.M.

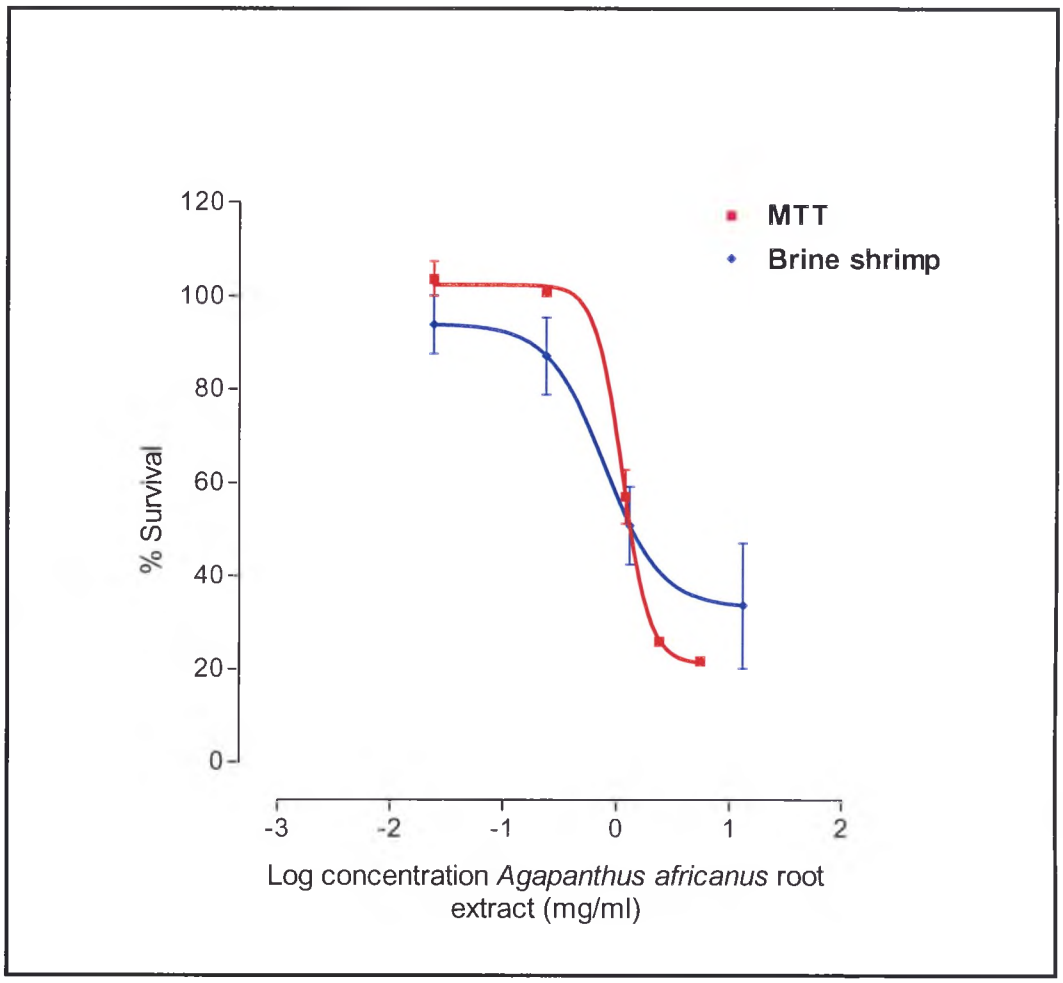


Figure 2.15. The effects of *Agapathus africanus* root extract on % survival in the Brine Shrimp and MTT bioassays. Results are expressed as the mean and the vertical bars represent the S.E.M.

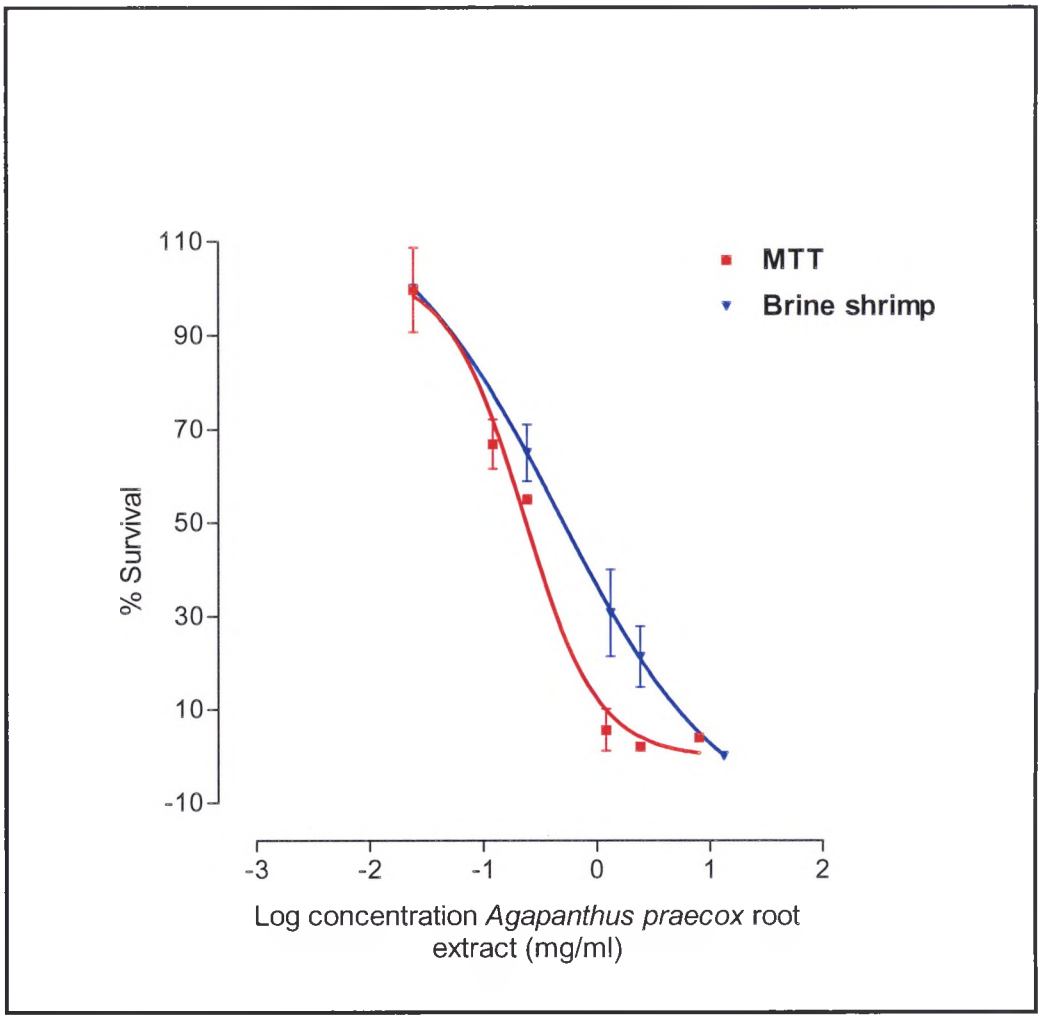


Figure 2.16. The effects of *Agapathus praecox* root extract on % survival in the Brine Shrimp and MTT bioassays. Results are expressed as the mean and the vertical bars represent the S.E.M.

The difference in the results of the two toxicity assays employed in this study illustrate the pitfalls of using only one type of toxicity assay in assessing potential toxicity of plant extracts. The MTT test measures intracellular toxicity (mitochondrial) in a human renal epithelial cell line whereas the Brine Shrimp bioassay measures general cellular toxicity to an invertebrate zoological species. LC_{50} values determined from only one type of bioassay could have potentially dangerous results if extrapolated to human dosage, as would be the case in an investigation of herbal remedies used in traditional medicine.

The results of both bioassays indicate that, with the exception of the Brine shrimp results for *A. africanus* leaf extract and MTT results for *C. miniata* root and leaf extracts and *A. praecox* leaf extracts, all the LC_{50} values are lower than the concentrations of plant extract required to augment oxytocin-induced contractions of the isolated rat uterus preparation. These findings can be interpreted as indicating that the plant extracts might have toxic effects if taken by pregnant women in high enough doses to augment uterine contractions during labour. The use of more than 3 leaves of any of the plant species examined to make a decoction would be equivalent to a potentially toxic dose.

2.5 SECTION E – PHARMACOLOGY

2.5.1 Experimental animal model

The pharmacological model selected was the isolated rat uterus preparation which is a well recognised model for the study of drugs exhibiting uterotonic activity. The approval of the Animal Ethics Committee of the University of the Witwatersrand was granted (Cert. AEC 97/5/3 mod. 99/22/3). The method of Veale (61) was followed. Virgin female Sprague-Dawley rats (250 - 350 g) were injected with stilboestrol (stilboestrol dipropionate, Maybaker) in arachis oil (0.1 mg/kg I.P.) and were then euthanazed by inhalation of CO_2 24 hrs later. It was previously established by means of vaginal smears and Shorr's stain that the oestrogen treatment brought the rats into a state of oestrus and this then mimicked the hormonal state of the uterus at the time of parturition (61).

2.5.2 The isolated "whole" uterus preparation - Methodology

The method of Veale (61) was followed. The central 1,5 cm portion of each uterine horn was dissected out and opened longitudinally. The uterine strips were maintained in 50 ml organ baths containing Tyrode's solution and were aerated with 5% CO_2 in O_2. The bath temperature was maintained at 26° C which inhibited spontaneous contractility in most cases (Kumegai, Ebashi & Takeda, 124). Isotonic contractions against a load of 1 g were recorded electronically on a Metrohm Labograph Potentiometric recorder (model E428).

The isolated organs were allowed to equilibrate for a period of at least 30 min and were frequently rinsed during this period. Once the baseline was steady, cumulative dose-response curves were constructed after the method of Van Rossum (125). The organs were allowed to rest for at least 30 minutes between drug challenges and the organ baths were well rinsed during this period. At the start of each experiment, a minimum of two reference control curves were constructed with a standard agonist prior to the first test curve and thereafter a single control curve was constructed before every test curve. The plant extracts were used as test drugs. Acetylcholine chloride (Sigma) and oxytocin (Syntocinon, Novartis) were used as standard reference agonists. Atropine sulphate (BDH) was used as a non-selective cholinergic muscarinic competitive antagonist and indomethacin (Sigma) was used as a prostaglandin synthesis inhibitor (antagonist). The organs were preincubated for 5 min with the extracts and/or atropine and for 15 min with indomethacin. The concentration range of *Agapanthus* aqueous extract used for cumulative dosage was 9×10^{-8} to $2{,}4 \times 10^{-3}$ g/ml.

2.5.2.1 Evaluation of the pharmacological equivalence of aqueous extracts of *Agapanthus africanus* and *Agapanthus praecox*

An aqueous extract of *A. africanus* leaves was shown to stimulate spontaneous contractions in the isolated rat uterus and ileum (66). The aim of this study was to investigate whether aqueous extracts of *A. praecox* exhibited similar uterotonic activity. Although decoctions, infusions and aqueous solutions of the rhizomes and roots are most commonly used by the traditional healers, leaf extracts were found to possess potent

uterotonic activity (66). Leaf extracts and rhizome/root extracts of both species were therefore examined separately in order to identify any differences in activity in extracts prepared from different parts of the plant.

2.5.2.1.1 Pharmacological equivalence – Results

All the plant extracts were able to induce spontaneous contractions in the isolated rat uterus preparation. Maximal response to cumulative dosage of the extracts was calculated relative to the maximal response achieved in each prior acetylcholine (ACh) control curve and the extracts showed similar activity to one another (table 2.5). The statistical significance of the difference between the maximal response to each extract relative to *A. africanus* leaf extract was assessed using the student's *t* test and was found to be non significant. The maximal response to oxytocin was the same as to ACh. ACh was used as the standard agonist here because oxytocin occupation of endometrial oxytocin receptors stimulates prostaglandin synthesis which could have influenced the uterine response to cumulative addition of the plant extracts.

Pretreatment of the uterus with 1mg/ml of each *Agapanthus* extract 5 minutes before cumulative dosage of ACh augmented the response to threshold doses of ACh in each case (fig. 2.17) and the maximal response to ACh was retained.

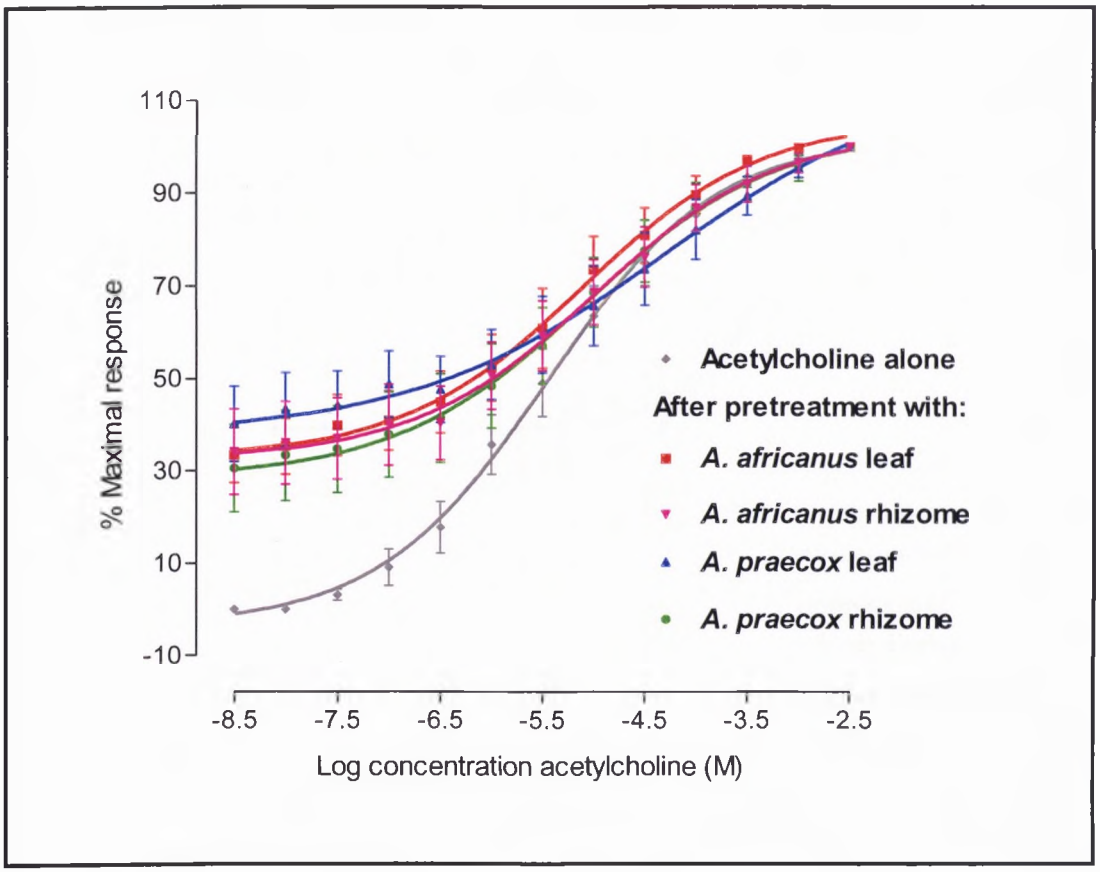


Figure 2.17. Mean cumulative dose-response curves for ACh alone (N=7) and after pretreatment with *A. praecox* leaf extract (N=6), *A. praecox* rhizome extract (N=8), *A. africanus* leaf extract (N=7) and *A. africanus* rhizome extract (N=6). Vertical bars represent the S.E.M. The pretreatment concentration for all the plant extracts was 1mg/ml and was administered 5 minutes before cumulative administration of ACh.

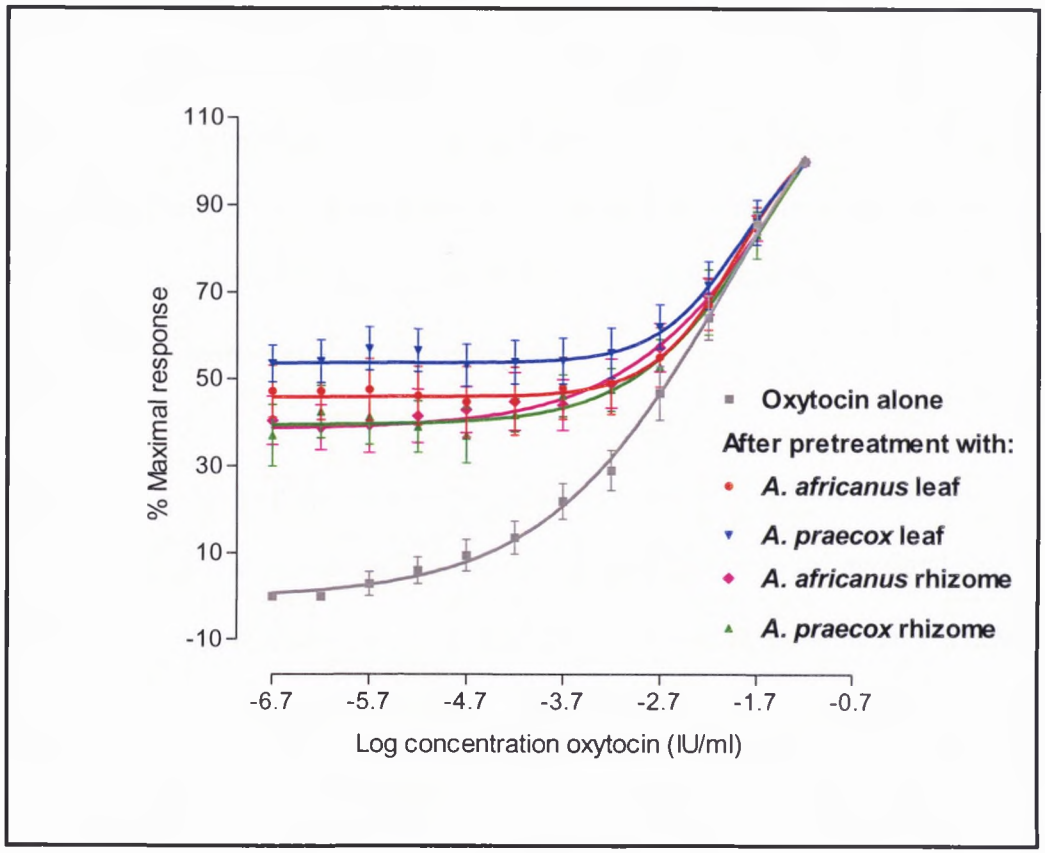


Figure 2.18. Mean cumulative dose-response curves for oxytocin alone (N=7) and after pretreatment with *A. praecox* leaf extract (N=6), *A. praecox* rhizome extract (N=6), *A. africanus* leaf extract (N=6) and *A. africanus* rhizome extract (N=6). Vertical bars represent the S.E.M. The pretreatment concentration for all the plant extracts was 1 mg/ml and was administered 5 minutes before cumulative administration of oxytocin.

Pretreatment of the uterus with 1 mg/ml of each plant extract 5 minutes before cumulative addition of oxytocin resulted in augmentation of the response to threshold doses of oxytocin in a similar fashion by each herbal extract (fig. 2.18). The maximal response to oxytocin as achieved in prior oxytocin control curves was retained. The augmentary effect of the plant extracts was greater when combined with oxytocin than with ACh. Both acetylcholine and oxytocin produced the same maximal uterine response when dosed cumulatively (table 2.5)

Table 2.5. Maximal response of the isolated rat uterus to cumulative addition of each herbal extract and oxytocin calculated relative to the maximal response in the ACh control curves. Results are expressed as the mean ± S.E.M.

	% Response	**N**
Ach	100	20
Oxytocin	99,60 ± 0,20	10
A. africanus leaf	57,92 ± 6,52	12
A. africanus root	58,20 ±10,03	7
A. praecox leaf	54,80 ± 8,73	7
A. praecox root	46,30 ± 9,07	6

2.5.2.1.2 Discussion

The ethnopharmacological evaluation of a traditional herbal remedy must rely on an appropriate pharmacological model. The isolated oestrogenized rat uterus preparation is a model well suited to pharmacological investigations of contractile activity in the uterine smooth muscle and is used extensively by reproductive pharmacologists. Basic initial pharmacological screening procedures which identify direct uterine smooth muscle activity in crude plant extracts need to be followed by more detailed studies which characterise the pharmacological activity of the phytomedicine as fully as possible. The results of these investigations can then be used to design and develop more specific techniques which can identify active principles extracted and isolated from the plants. As every ethnopharmacologist knows, a major disadvantage is that chemical extraction procedures, although essential in isolating active principles, often produce complex, insoluble fractions which are most often incompatible with the aqueous physiological salt solutions used in classic pharmacological testing procedures (126). Chemical principles so isolated are also often available for testing in very small quantities, thereby limiting the selection and extent of the pharmacological investigation even further. In this study, however, the crude aqueous extracts of *A. africanus* and *A. praecox* leaves and rhizomes/roots, which were evaluated as phytomedicines, were easily resolubilised in water and truly representative of the ethnic method of preparation.

This study has established that the crude extracts of both *A. africanus* and *A. praecox* rhizomes and leaves initiated contractions in the isolated rat uterus preparation. The

maximal response to each plant extract administered alone, cumulatively, were similar when calculated relative to maximal response in ACh control curves. Pretreatment with each individual plant extract augmented the initial response of the uterus to ACh.

The leaf and rhizome extracts of both species also augmented the initial response of the uterus to subthreshold doses of oxytocin, the most potent of the endogenous uterotonic agents, in a similar fashion, providing evidence of a possible enhancement of oxytocic action by some means during labour. The augmentation of oxytocin activity by the herbal extracts was greater than with ACh.

These results confirm the pharmacological equivalence of extracts of both leaves and of rhizomes/roots of *A. africanus* and *A. praecox* as evaluated in the isolated rat whole uterus model. Spectral and chromatographic analyses have confirmed the relative chemical similarity of extracts of the two species of *Agapanthus* and it is proposed that a common "core" chemical composition is responsible for the uterotonic activity of the extracts. Evidence has therefore been provided to theoretically validate the use of both plant species in South African traditional medicine as herbal remedies to augment labour.

2.5.2.2 Measurement of pharmacological activity of *Agapanthus africanus* leaves in the rat isolated "whole" uterus preparation

Agapanthus africanus (family Alliaceae) and *Clivia miniata* (family Amaryllidaceae) are used in South African traditional medicine to induce and augment labour. It has been shown that aqueous extracts of leaves of both species initiate contractions in isolated smooth muscle preparations - the isolated rat uterus and ileum models. A similar pharmacological effect has therefore been identified for herbal extracts from different botanical families and, by implication, with different phytochemical profiles. The pharmacological mechanisms of action of an aqueous extract of *Clivia* leaves have been investigated in the isolated rat whole uterus preparation (61) but the activity of *Agapanthus africanus* on this model has not been established. The objective of this study was therefore to examine the effects of *A. africanus* extracts on the various receptor systems in the isolated rat whole uterus preparation and compare them to those already identified for *C. miniata* extract.

2.5.2.2.1 Results

2.5.2.2.1.1 Effects of atropine

Pretreatment with atropine (20 nM) followed by cumulative addition of extract resulted in displacement of the dose-response curve to the right (fig. 2.19). Pretreatment with atropine (20 nM) and extract (1 mg/ml) before cumulative addition of acetylcholine

caused a reduction in the augmentary effect of the extract on the initial response of the organ to subthreshold doses of acetylcholine and a parallel displacement of the curve to the right with retention of maximal effect (fig. 2.20).

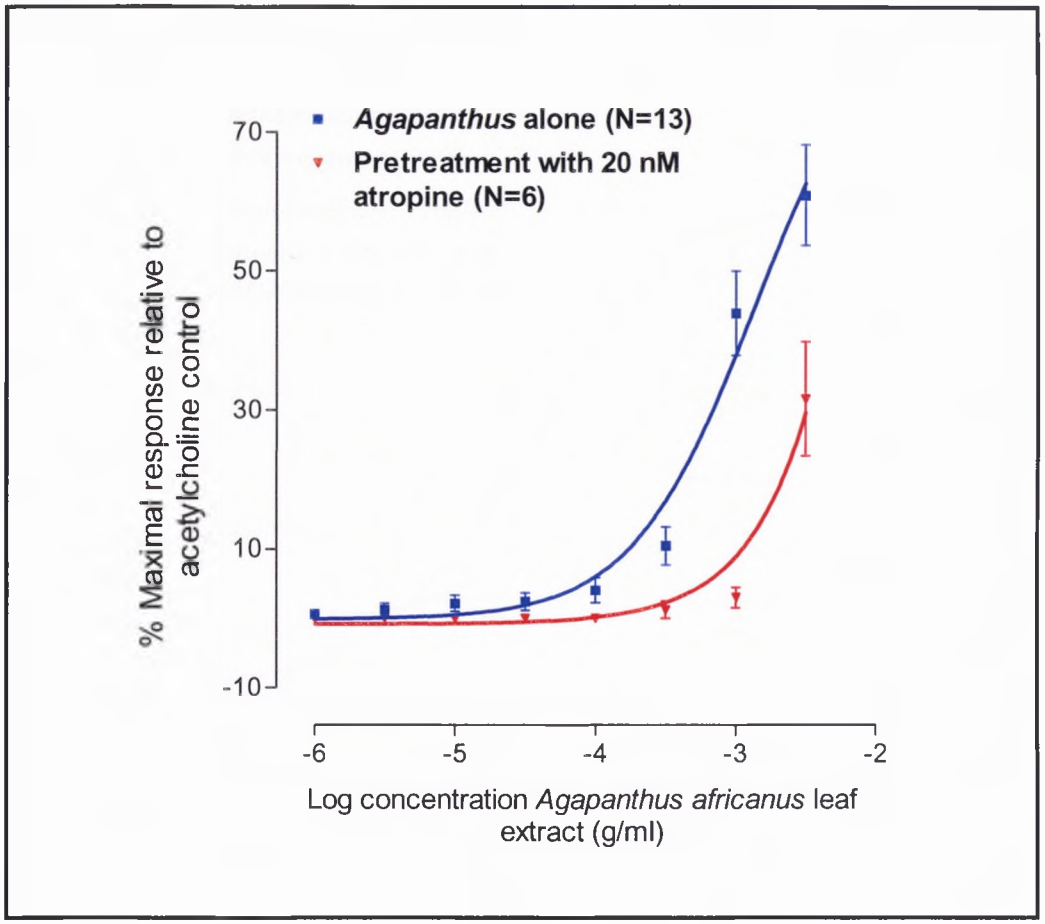


Figure 2.19. Mean cumulative dose-response curves for *Agapanthus africanus* leaf extract (N=13) alone and after pretreatment with atropine (20 nM) (N=6). Vertical bars represent the S.E.M. Response was calculated relative to the maximal response obtained in acetylcholine control curves. Atropine was administered 5 minutes before cumulative addition of plant extract.

Cumulative addition of oxytocin after pretreatment with atropine (70 nM) and extract (1 mg/ml) resulted in a reduction in the extract's augmentary effect on the initial response of the organ to subthreshold doses of oxytocin but there was no displacement of the curve and the maximal response was retained (fig. 2.21).

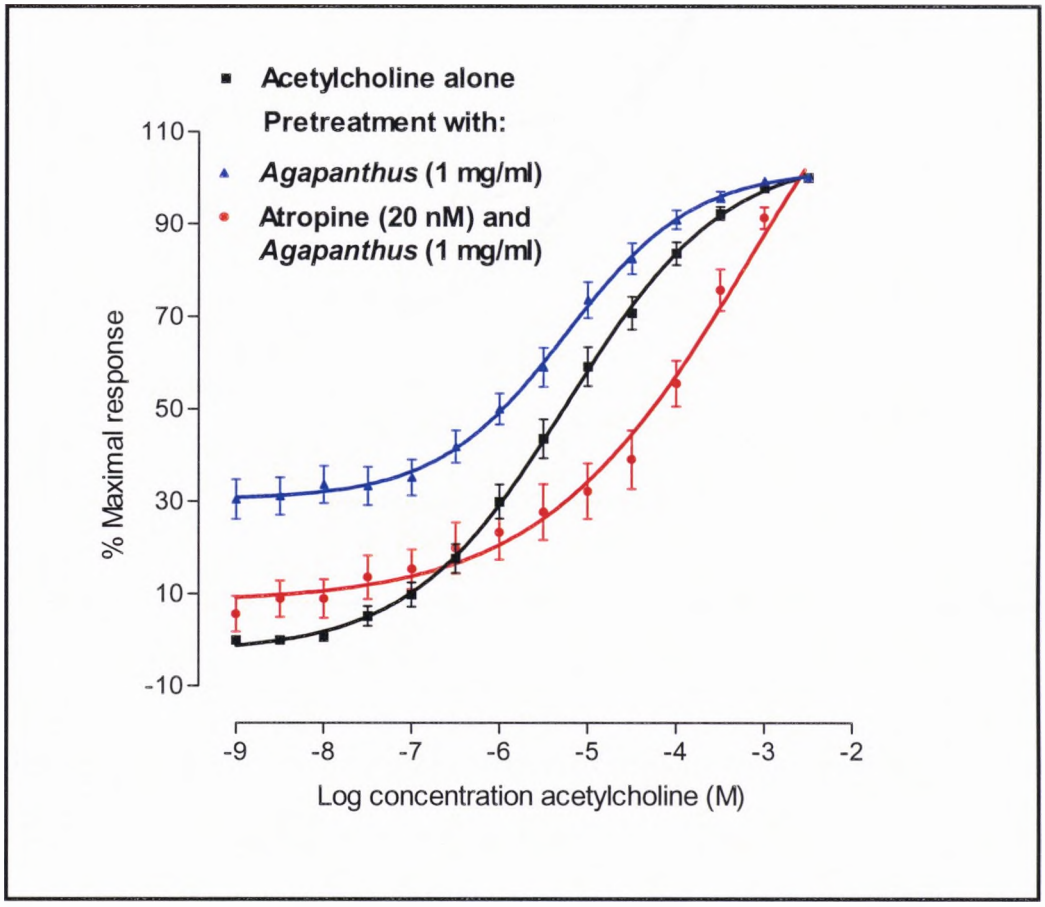


Figure 2.20. Mean cumulative dose-response curves for acetylcholine alone (N=21) or after pretreatment with *A. africanus* leaf extract (1 mg/ml) (N=17), or atropine (20 nM) and extract (N=10). Vertical bars represent the S.E.M. Atropine was administered 5 mins before the extract and the extract was administered 5 minutes before cumulative addition of acetylcholine.

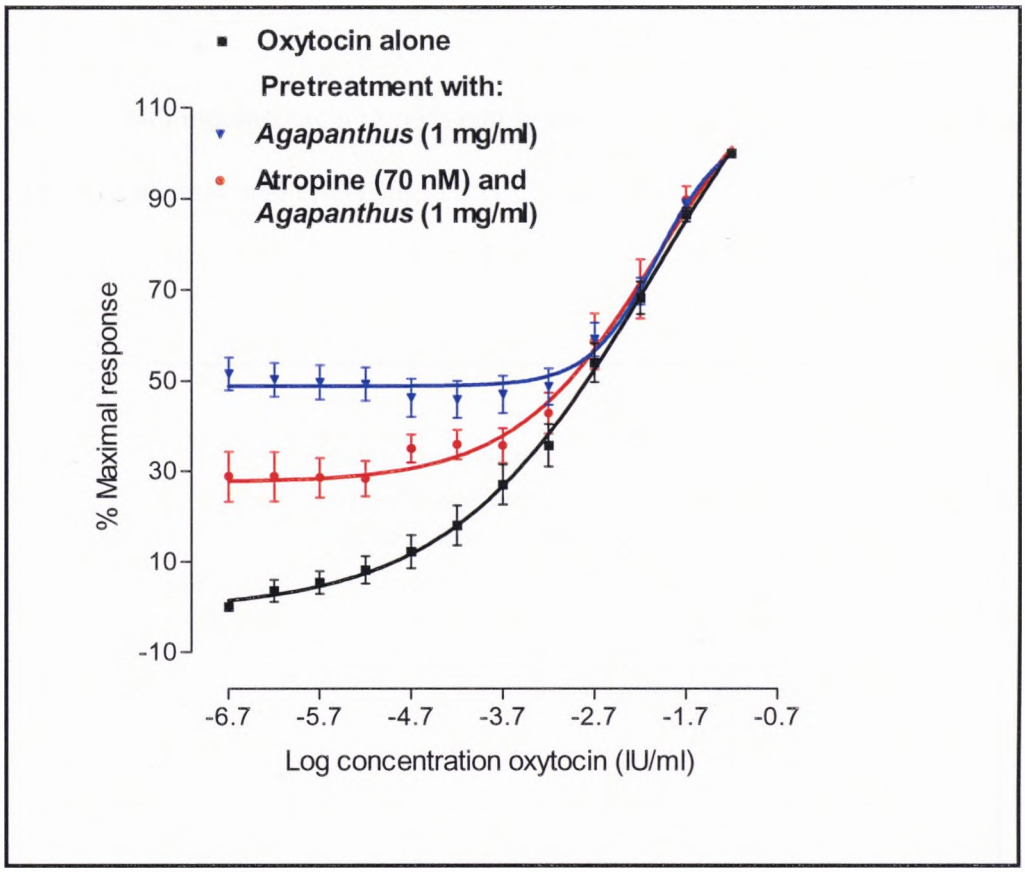


Figure 2.21. Mean cumulative dose-response curves for oxytocin alone (N=21) and after pretreatment with *Agapanthus africanus* leaf extract (1 mg/ml) (N=20) and atropine (70 nM) and extract (N=7). Vertical bars represent the S.E.M. Atropine was administered 5 minutes before the extract and the extract was administered 5 minutes before cumulative addition of oxytocin.

2.5.2.2.1.2 Effects of indomethacin

Pretreatment with indomethacin (5 μM) before cumulative addition of the extract caused a reduction of the maximal effect (fig. 2.22).

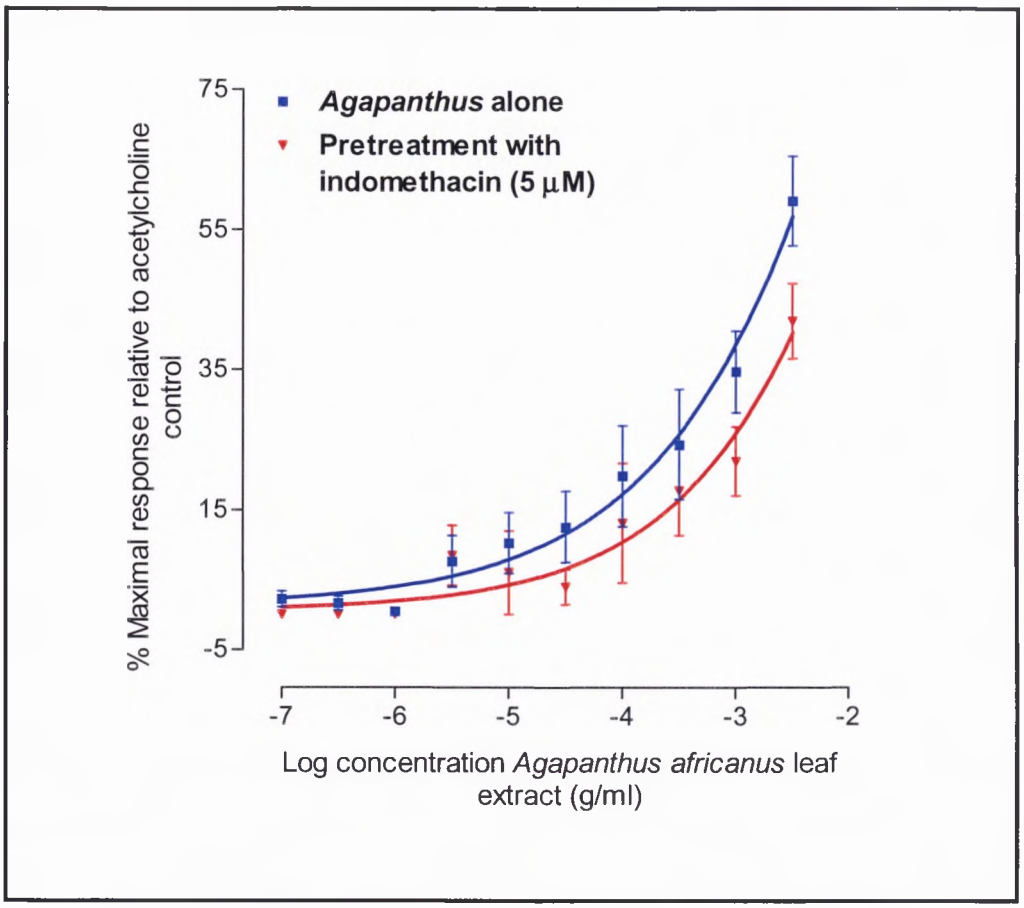


Figure 2.22. Mean cumulative dose-response curves for *Agapanthus africanus* leaf extract (N=9) alone and after pretreatment with indomethacin (5 μM) (N=9) 10 minutes before cumulative addition of plant extract. Vertical bars represent the S.E.M.

Cumulative addition of acetylcholine after pretreatment with indomethacin (5 μM) and then extract (1 mg/ml) resulted in inhibition of the initial augmentary effect of the extract on the response to subthreshold doses of acetylcholine, there was no displacement of the curve to the right and maximal response was unaffected (fig. 2.23).

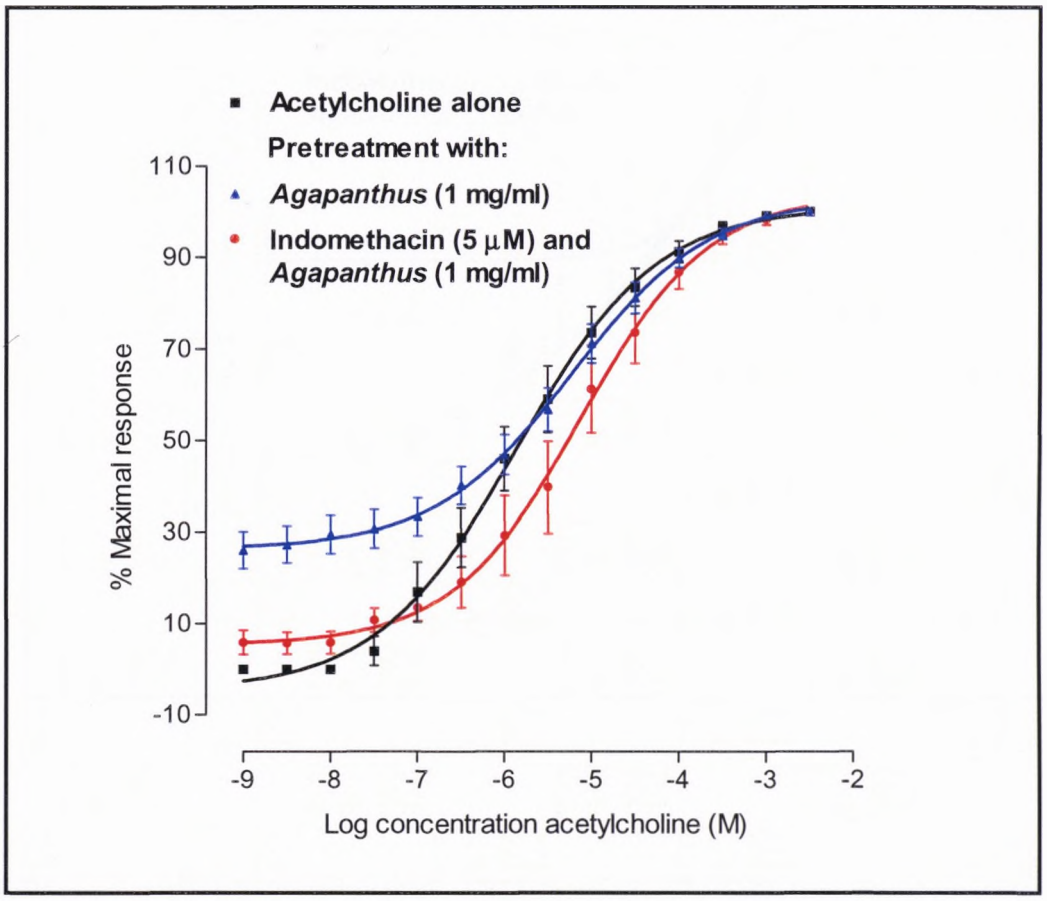


Figure 2.23. Mean cumulative dose-response curves for acetylcholine alone (N=12) and after pretreatment with *Agapanthus africanus* leaf extract (1mg/ml) (N=12) and indomethacin (5 μM) and extract (N=6). Vertical bars represent the S.E.M. Indomethacin was administered 10 minutes before the extract and the extract was administered 5 minutes before cumulative addition of acetylcholine.

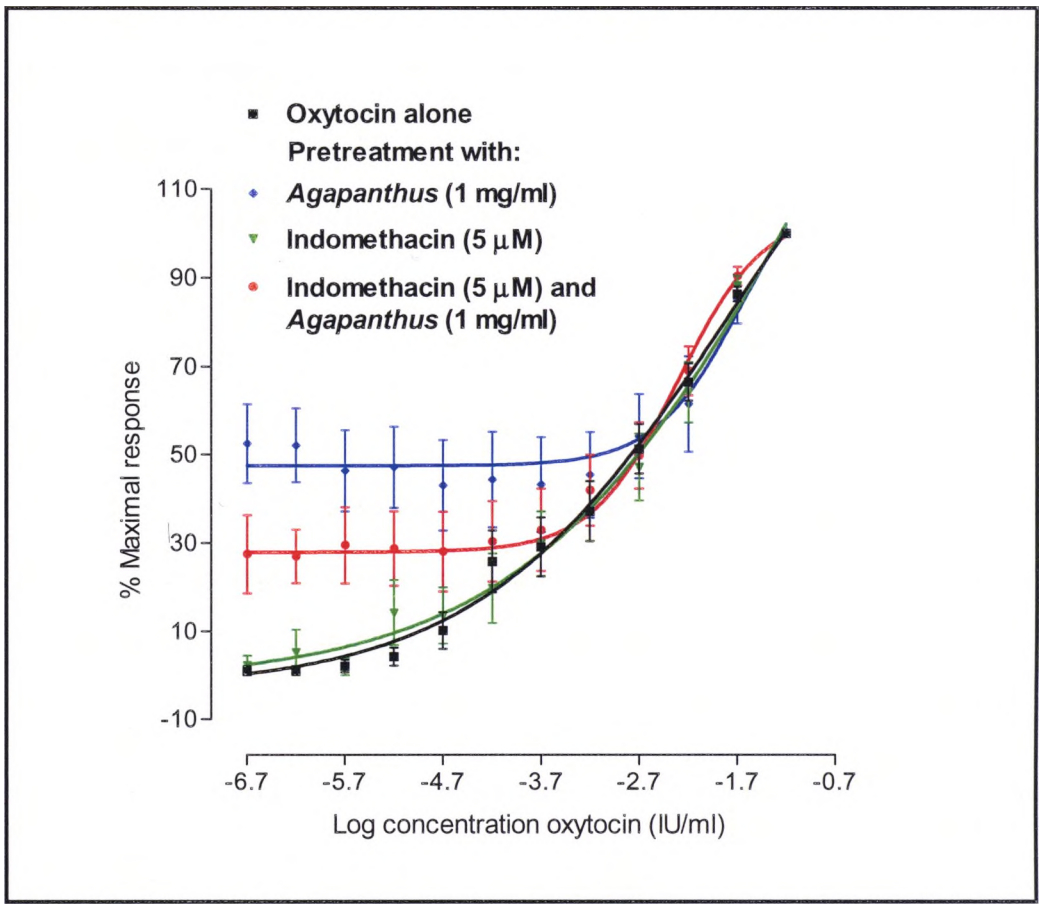


Figure 2.24. Mean cumulative dose-response curves for oxytocin alone (N=12) and after pretreatment with *Agapanthus africanus* leaf extract (1mg/ml) (N=6), indomethacin (5 µM) and extract (N=9) and indomethacin (5 µM) (N=10). Vertical bars represent the S.E.M. Indomethacin was administered 10 minutes before addition of extract or oxytocin. The extract was administered 5 minutes before cumulative addition of oxytocin.

Pretreatment with indomethacin (5 μM) and then extract (1 mg/ml) followed by cumulative addition of oxytocin reduced the augmentary effect of the extract on the initial response to subthreshold doses of oxytocin but there was no displacement of the curve. Indomethacin (5 μM) did not significantly affect the uterine response to cumulative addition of oxytocin alone (fig. 2.24).

2.5.2.2.2 Discussion

The uterotonic effects of the crude aqueous extract of *A. africanus* were inhibited by both atropine and indomethacin. The tonic effect of a submaximal dose of the extract (1 mg/ml) was blocked by atropine but phasic contractions continued. This indicated that one or more of the active principles in the extract possessed agonistic activity on uterine muscarinic receptors. The M_3- muscarinic receptor has been identified as the subtype responsible for the contractile activity of the rat uterus (35).

The augmentation of the uterine response to subthreshold and threshold effects of oxytocin was much more marked than with acetylcholine. The inhibition of this effect by pretreatment with indomethacin indicates that the *A. africanus* extract may promote prostaglandin synthesis in the uterus in a similar way to oxytocin. The reason for the increased augmentative effect of the extract could be due to additive synthesis of uterotonic prostaglandins.

These results have therefore identified that an aqueous extract of *A. africanus* (family Alliaceae) leaves has a similar spectrum of activity on the isolated rat whole uterus preparation as an aqueous extract of *Clivia miniata* (family Amaryllidaceae) leaves (61). Both plant extracts are able to initiate spontaneous phasic contractions of the uterus, followed by a sustained tonic contraction at higher concentrations. In addition, extracts of both plants are able to augment the initial contractile response of the uterus to ACh and oxytocin and appear to act by stimulation of M_3-receptors and promotion of prostaglandin synthesis i.e they appear to have similar uterotonic mechanisms of action in the isolated rat whole uterus preparation although they belong to different botanical families.

2.5.3 The evaluation of the uterotonic effects of herbal oxytocic agents using the isolated "stripped" myometrium preparation

The aim of this part of the study was to determine the uterotonic activity of the herbal remedies, *A. africanus* and *C. miniata* in an endometrium-free preparation (i.e. "stripped" myometrium) to determine the role of endometrial prostaglandin synthesis on the activity of the herbal extracts. The isolated "stripped" myometrium preparation was used in order to evaluate the effects of the herbal oxytocics and reference agonists and antagonists on myometrial smooth muscle without any intervention due to endometrial prostaglandin synthesis. The "whole" uterus was "stripped" of endometrium and circular muscle, leaving a myometrial preparation consisting mainly of longitudinal muscle (127, 128). The isolated myometrium (i.e. free of endometrium) has already been

established as a standard model in biochemical studies of uterine mechanisms and metabolism.

2.5.3.1 Methodology

The same animal model was used as for the isolated whole uterus experiments. The uterine horns were dissected out and opened longitudinally. The endometrium and most of the circular muscle layer were gently stripped away using an artery clamp and forceps, following the method of Hodgson & Daniel (127) with modifications. The myometrial strips (15 mm long) were then placed in organ baths using the same system described for the isolated whole uterus preparation. The myometrial strips were allowed to equilibrate for a period of about 30 min and then cumulative dose-response curves were constructed (125) at 30 min intervals using acetylcholine chloride (Sigma) as a standard agonist. When the concentration–response curve to ACh stabilized (usually after 3 consecutive control curves), the plant extracts were used as test drugs, oxytocin (Syntocinon, Novartis), 5-hydroxytryptamine creatinine sulphate.H_2O (Aldrich), ergometrine maleate (Intramed), methysergide maleate (Sigma), noradrenaline (Sigma), prostaglandin $F_{2\alpha}$ (Dinoprost, Pharmacia & Upjohn), calcium chloride (Saarchem) and potassium chloride (Saarchem) were used as agonists and atropine (atropine sulphate, BDH), indomethacin (Sigma), prazosin HCl (Sigma), verapamil HCl (Sigma), yohimbine HCl (Sigma) and EGTA (ethylene glycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid, Sigma) as antagonists. The concentration range of *Agapanthus* aqueous extract used for cumulative dosage was 9×10^{-6} to $2{,}4 \times 10^{-3}$ g/ml and it was 4×10^{-6} to $3{,}2 \times 10^{-3}$ g/ml for *Clivia*.

Administration of test drugs was only undertaken after equilibration and each subsequent test was preceded and followed by an ACh control curve. The organs were preincubated for 5 min with the extracts and/or atropine and for 15 min with indomethacin. The organs were allowed to rest for at least 30 minutes between drug challenges and the organ baths were well rinsed during this period.

The recovery period required for the myometrial preparation to respond normally to agonist stimulation was carefully monitored using matching uterine horns as time-matched controls. This was necessary because the effects of dissection trauma and potential damage to the tissue using this method might significantly affect the subsequent contractile response of the "stripped" myometrial preparation to agonist stimulation. A mean time-matched control curve was constructed and used to monitor all test and internal control curves.

2.5.3.2 Results

The stripped myometrium preparation was found to equilibrate within a period of 3 hours. ACh was found to be a reliable standard agonist which did not sensitize or desensitize the isolated organ or initiate spontaneous contractions. The stripped myometrium preparation was capable of reproducible results for several hours after the prerequisite equilibration period.

2.5.3.2.1 The effects of atropine

Atropine competitively inhibited the response of the stripped myometrium preparation to ACh, i.e. a reversible parallel shift of the dose-response curves to the right and atropine (70 nM) depressed the maximal response of the organ to ACh (fig. 2.25).

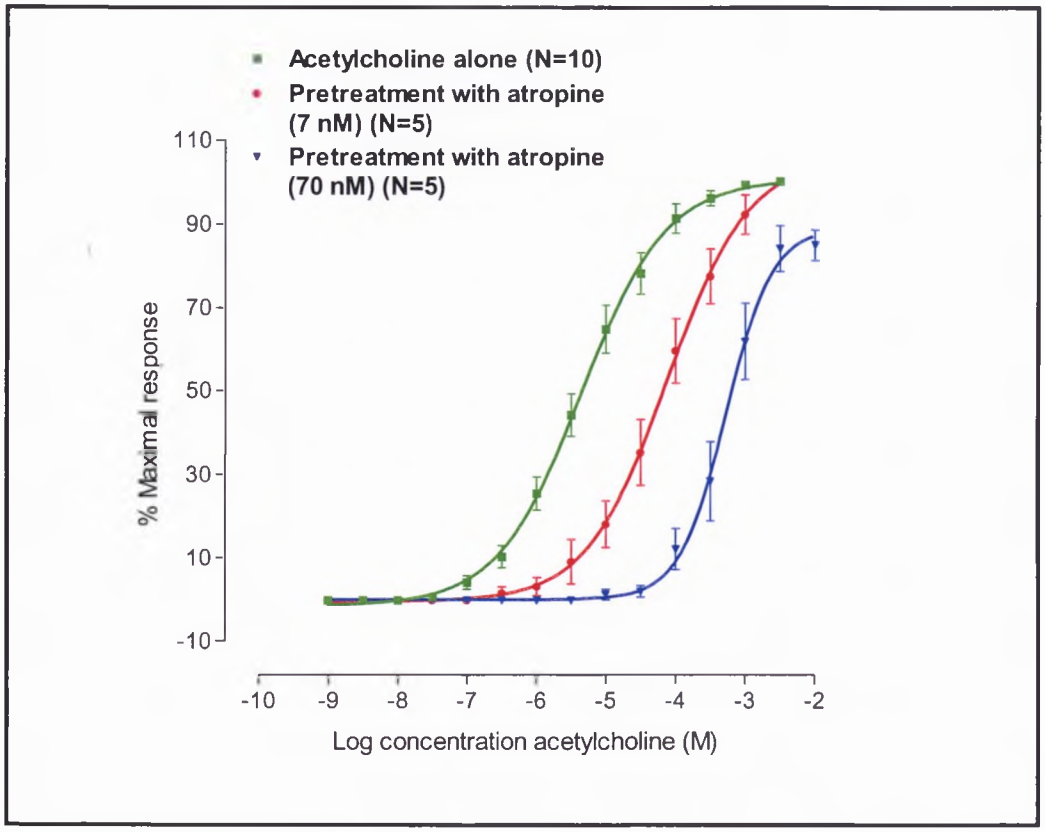


Figure 2.25. Mean concentration-response curves for acetylcholine alone and after pretreatment with atropine (7 and 70 nM) in the stripped myometrium preparation. Each point represents the mean of experiments with 5 to 10 rats and the vertical bars represent the S.E.M. Atropine was administered 5 min before cumulative addition of acetylcholine.

In this study, both *Agapanthus* extract (fig. 2.26) and *Clivia* extract (fig. 2.27) administered cumulatively were able to initiate and maintain phasic, followed by tonic contractions in the "stripped" preparation.

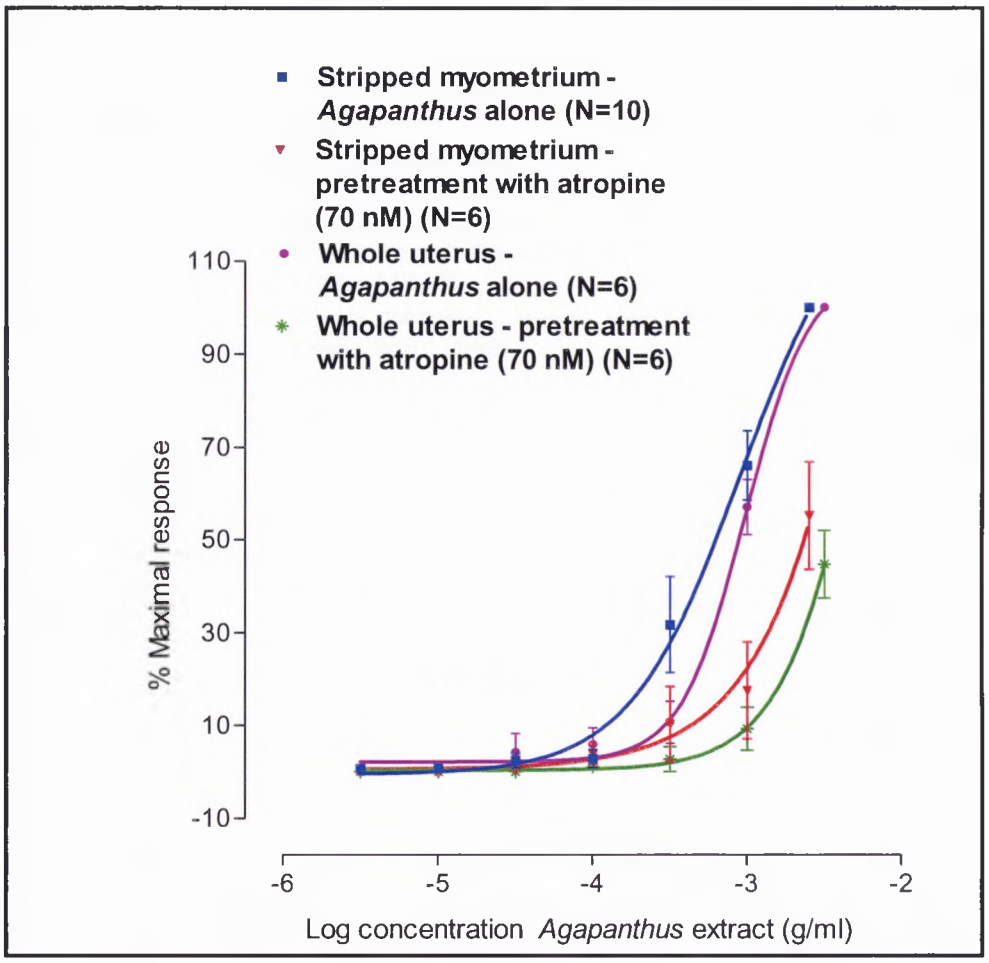


Figure 2.26. Mean concentration-response curves for *Agapanthus africanus* extract alone and after pretreatment with atropine (70 nM) in both the whole uterus and in the stripped myometrium preparations. Each point represents the mean of experiments with 6 to 10 rats and the vertical bars represent the S.E.M. Atropine was administered 5 min before cumulative addition of *Agapanthus* extract.

Atropine (70 nM) inhibited the response of the myometrium to *Agapanthus* (fig. 2.26) but did not affect the response to cumulative *Clivia* administration (fig. 2.27). Both the *Agapanthus* (fig. 2.28) and *Clivia* (fig. 2.29) extracts augmented the initial response of the endometrium-free preparation to subthreshold and threshold doses of ACh.

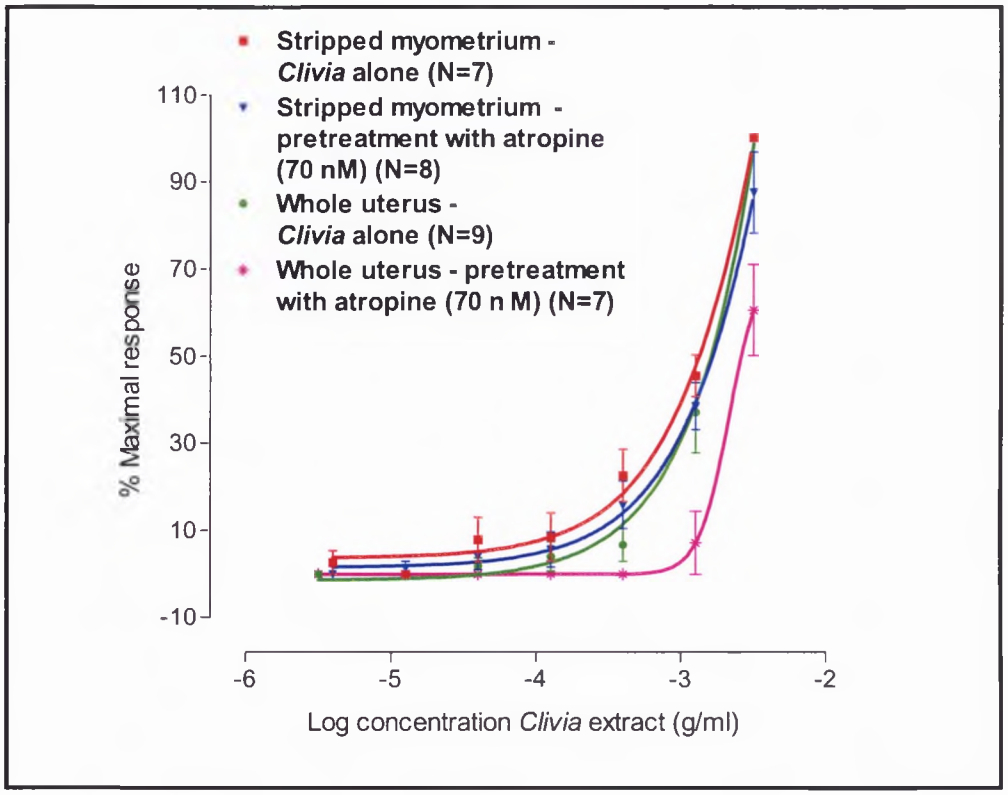


Figure 2.27. Mean concentration-response curves for *Clivia miniata* extract alone and after pretreatment with atropine (70 nM) in both the whole uterus and the stripped myometrium preparations. Atropine was administered 5 min before cumulative addition of *Clivia* extract. Each point represents the mean of experiments with 7 to 9 rats and the vertical lines indicate the S.E.M.

2.5.3.2.2 The effects of indomethacin

Preincubation with indomethacin (20 μM) significantly inhibited both the augmentary effect of *Agapanthus* extract on the response of the "stripped" preparation to ACh (fig. 2.28) and the response of the organ to cumulative addition of *Agapanthus* extract (fig. 2.32). This inhibition was reversible.

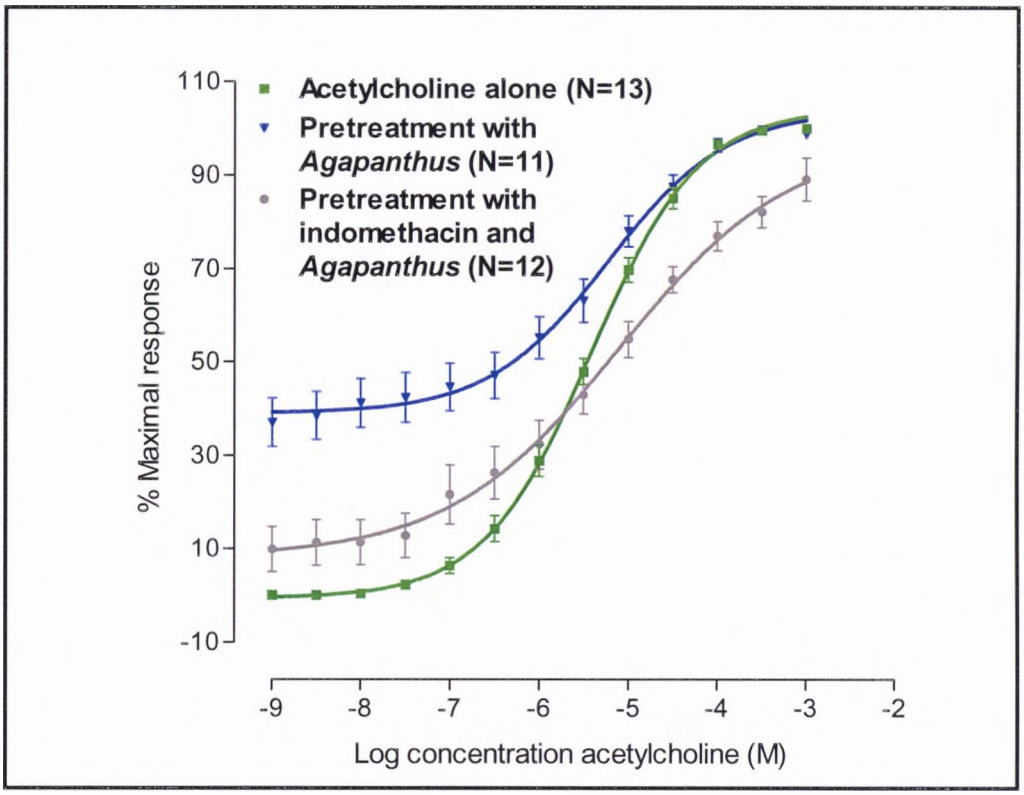


Figure 2.28. Stripped myometrium - mean concentration-response curves for ACh alone and after pretreatment with *A. africanus* leaf extract (1mg /ml) and indomethacin (20 μM) and extract. Each point represents the mean of experiments with 11 to 13 rats and the vertical bars represent the S.E.M. Indomethacin was administered 15 min before addition of extract and the extract was administered 5 min before cumulative addition of ACh.

Indomethacin (20 μM) administration did not affect the initial augmentary effect of *Clivia* on the reponse of the "stripped" myometrium to ACh (fig. 2.29).

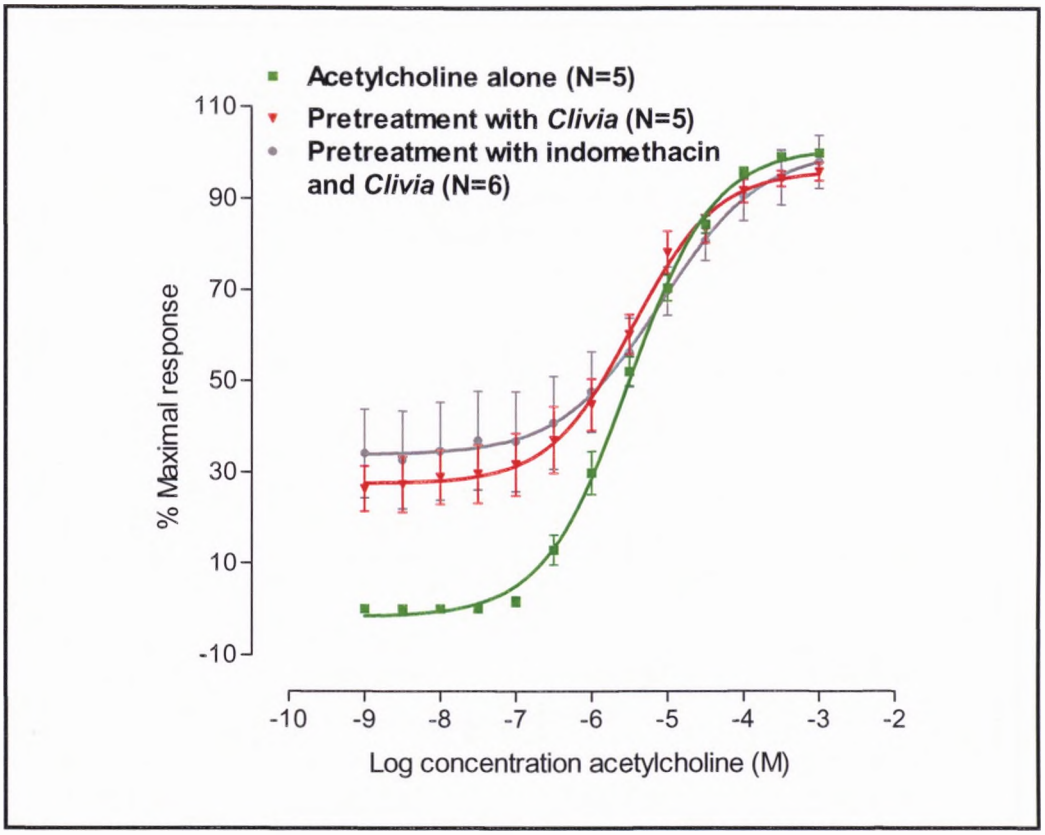


Figure 2.29. Stripped myometrium – mean concentration-response curves for acetylcholine alone and after pretreatment with *Clivia miniata* extract (2 mg/ml) and indomethacin (20 μM) and extract. Indomethacin was administered 15 min before addition of extract and the extract was administered 5 min before cumulative addition of acetylcholine. Each point represents the mean of experiments with 5 to 6 rats and the vertical bars represent the S.E.M.

Preincubation with indomethacin (20 μM) had no effect on the response of the preparation to oxytocin (fig. 2.30), ACh or *Clivia* (fig. 2.31) administered alone.

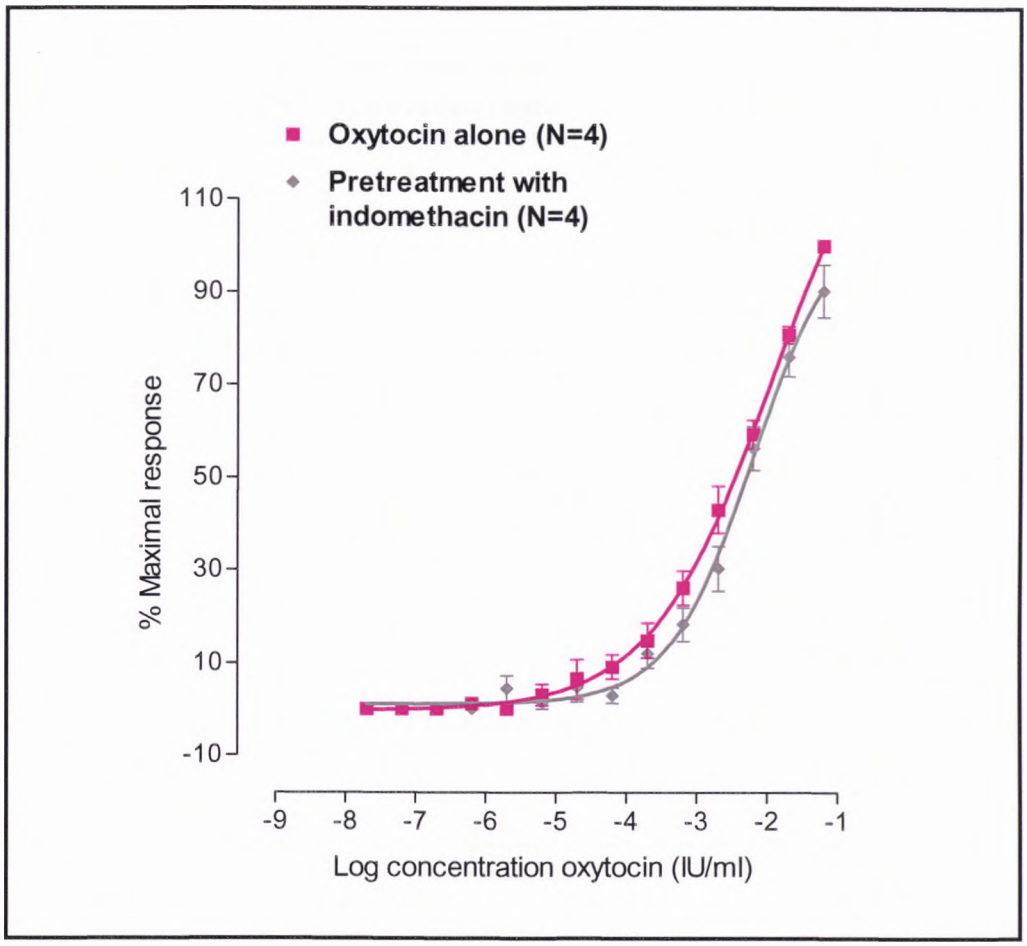


Figure 2.30. Mean concentration-response curves for oxytocin alone and after pretreatment with indomethacin (20 μM) in the stripped myometrium preparation. Indomethacin was administered 15 min before cumulative addition of oxytocin. Each point represents the mean of experiments with 4 rats and the vertical bars represent the S.E.M.

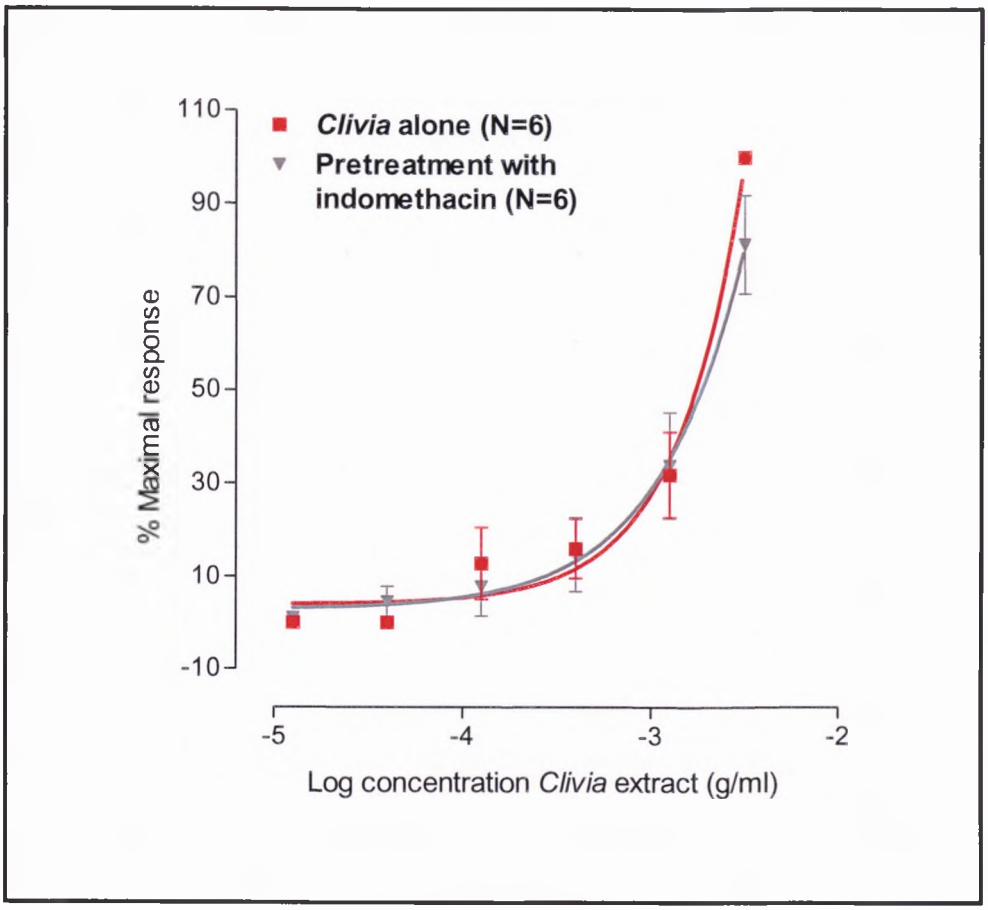


Figure 2.31. Mean concentration-response curves for *Clivia miniata* extract alone and after pretreatment with indomethacin (20 μM) in the stripped myometrium. Indomethacin was administered 15 min before cumulative addition of *Clivia* extract. Each point represents the mean of experiments with 6 rats and the vertical bars represent the S.E.M.

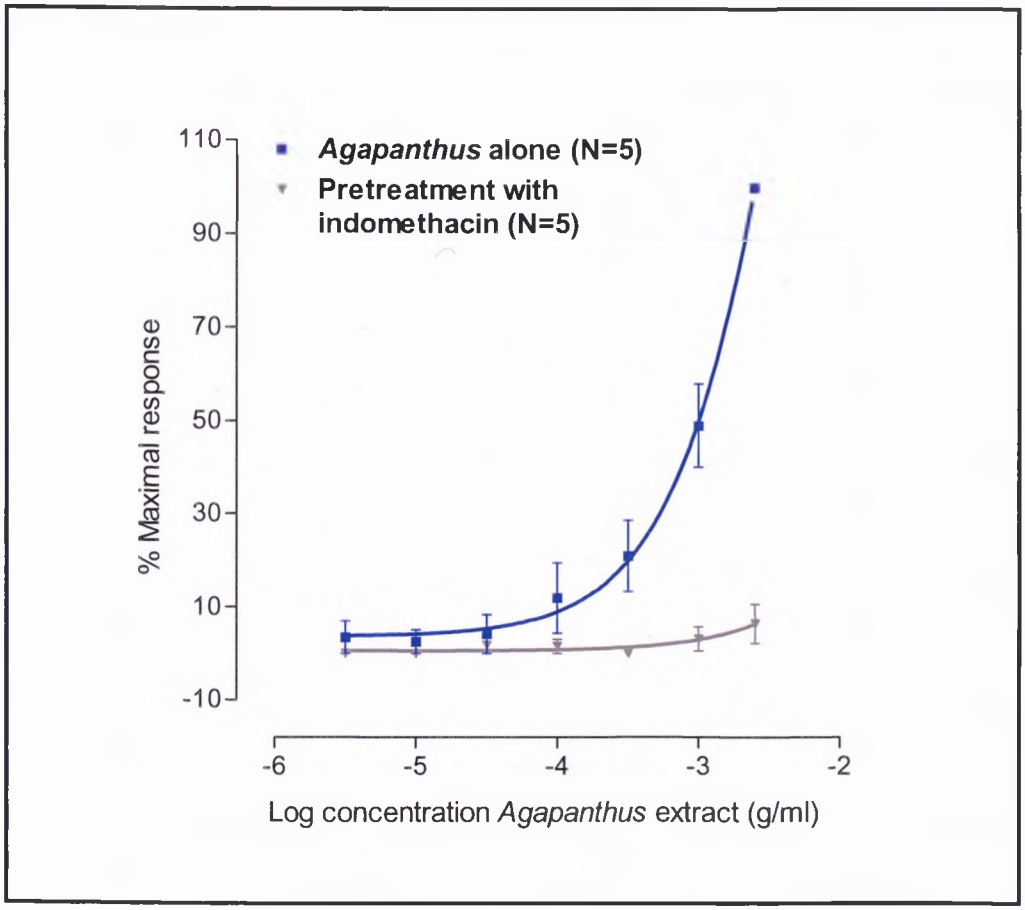


Figure 2.32. Mean concentration-response curves for *Agapanthus africanus* extract alone and after pretreatment with indomethacin (20 μM) in the stripped myometrium. Indomethacin was administered 15 min before cumulative addition of *Agapanthus* extract. Each point represents the mean of experiments with 5 rats and the vertical bars represent the S.E.M.

2.5.3.2.3 Effects on response to $PGF_{2\alpha}$

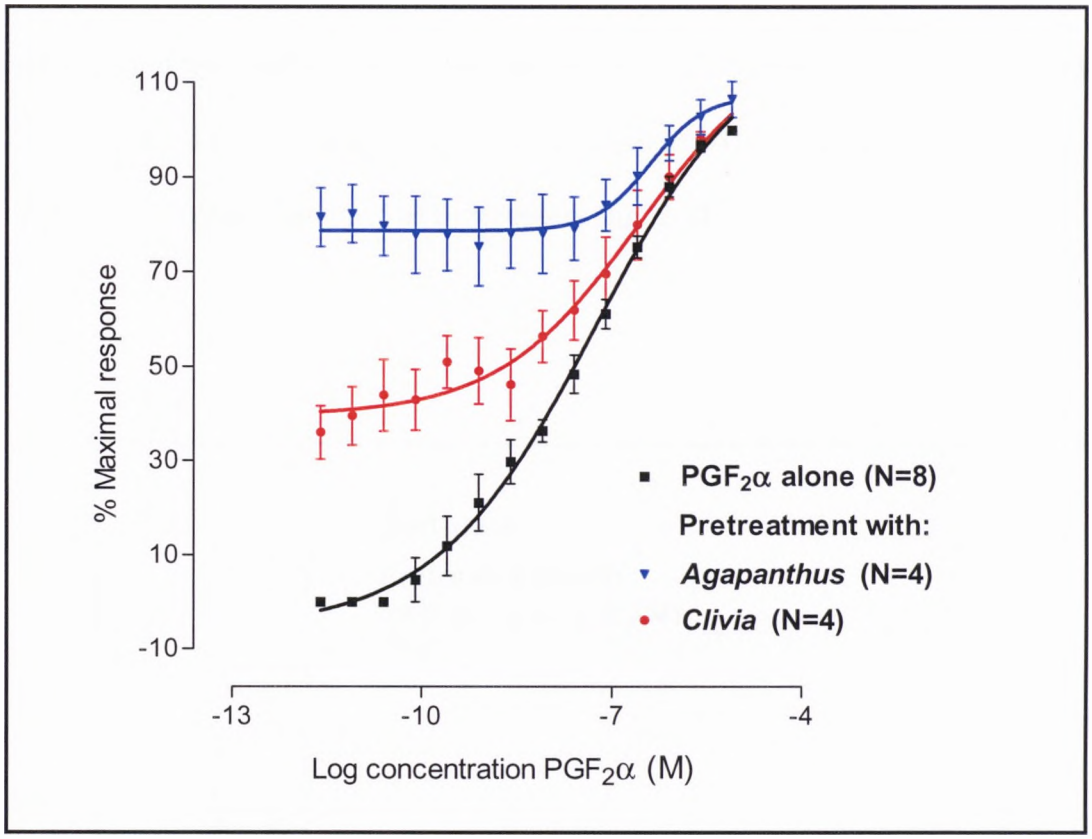


Figure 2.33. Mean concentration-response curves for $PGF_{2\alpha}$ alone and after pretreatment with either *Agapanthus africanus* extract (1 mg/ml) or *Clivia miniata* extract (2 mg/ml) in the stripped myometrium. The plant extracts were administered 5 min before cumulative addition of $PGF_{2\alpha}$.. The vertical bars represent the S.E.M.

The augmentary effect of pretreatment with *Agapanthus* on the response of the stripped myometrium to $PGF_{2\alpha}$ was much greater (ca. 80%) than the augmentary effect of *Clivia* (ca. 40%) (fig. 2.33). This confirms the additive effects of *Agapanthus* stimulation of PG synthesis on the response of the myometrium to $PGF_{2\alpha}$.

2.5.3.2.4 The effects of the herbal oxytocic agents on 5-HT_2-receptors

Pretreatment of the myometrium with methysergide (0,2 µM) before cumulative addition of 5-HT inhibited the maximal response of the organ to 5-HT, demonstrating non-competitive antagonism on the 5-HT_2-receptors (fig. 2.34).

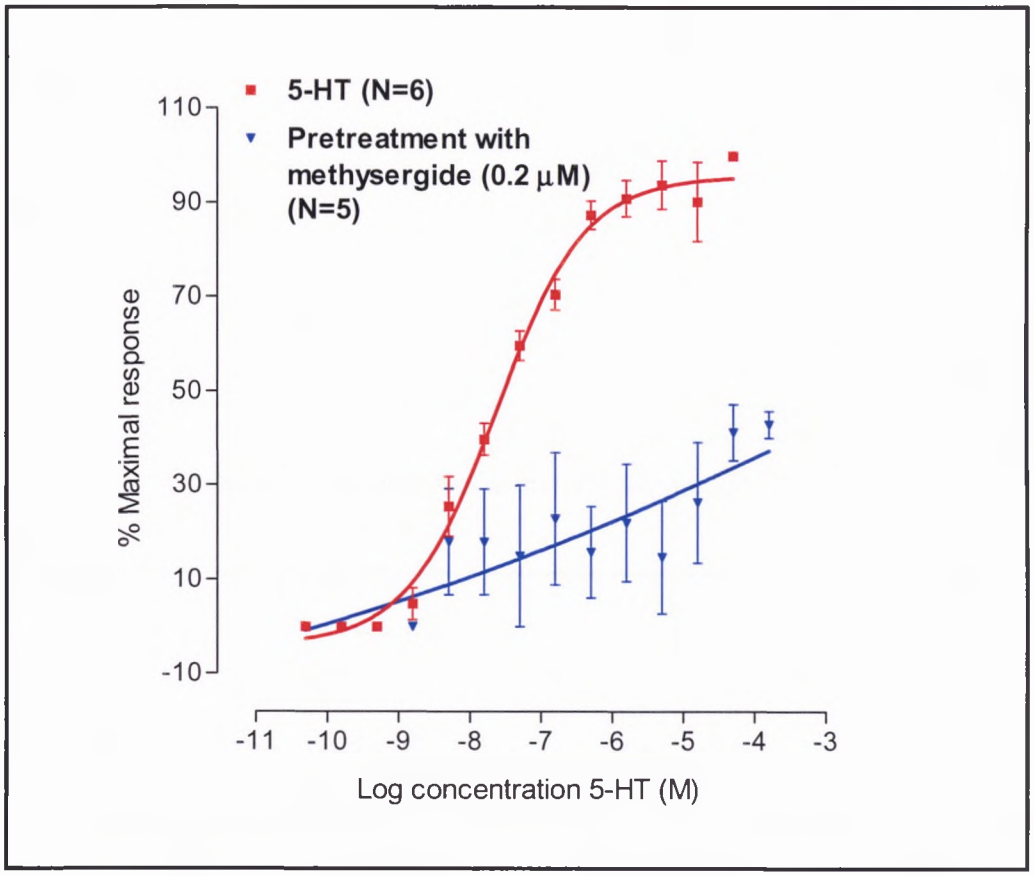


Figure 2.34. Mean concentration-response curves for 5-HT alone and after pretreatment with methysergide (0,2 µM) in the stripped myometrium. Methysergide was administered 5 min before addition of 5-HT. The vertical bars represent the S.E.M.

Pretreatment with methysergide (0,2 μM) caused a slight parallel shift of the *Clivia* concentration-response curve to the right, indicating a competitive interaction (fig. 2.35).

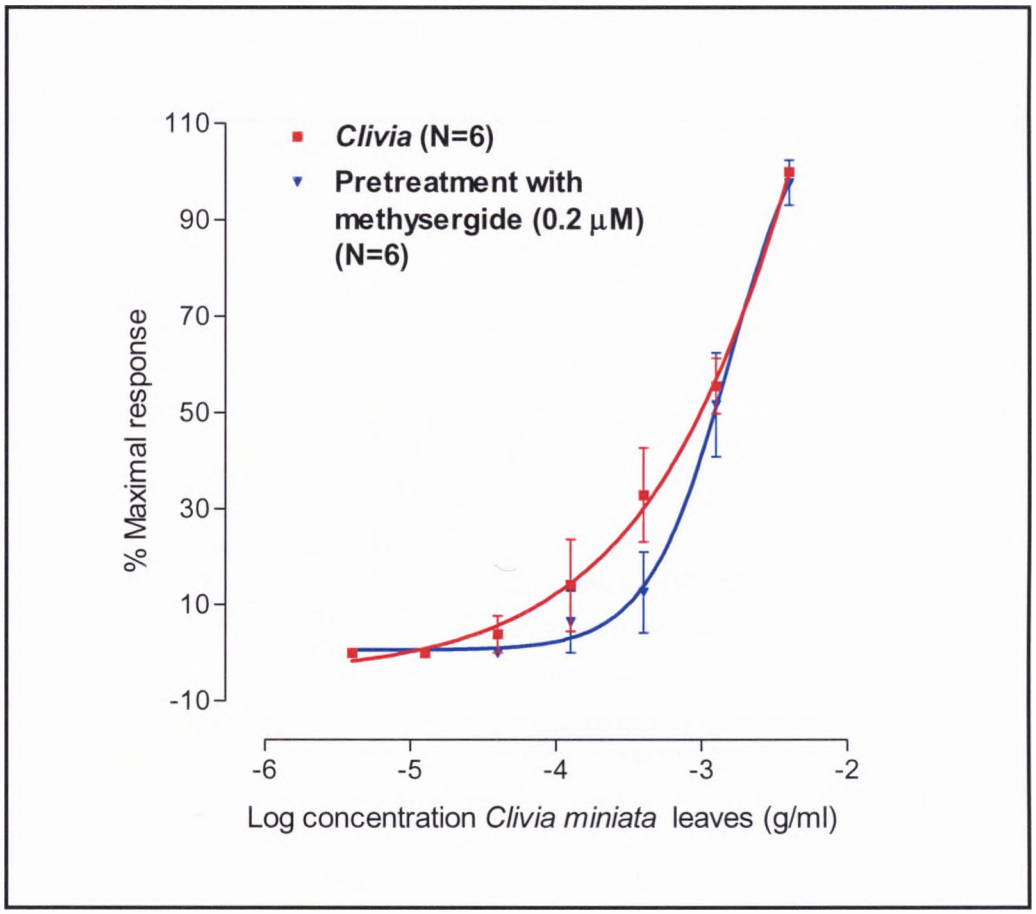


Figure 2.35. Mean concentration-response curves for *Clivia* alone and after pretreatment with methysergide (0,2 μM) in the stripped myometrium. Methysergide was administered 5 min before addition of *Clivia*. The vertical bars represent the S.E.M.

Pretreatment with *Clivia* at 40% of a submaximal dose (0,8 mg/ml) caused an initial augmentation of the myometrial response to cumulative addition of 5-HT, followed by a parallel shift of the curves to the right (fig. 2.36), indicating partial agonism. Additional pretreatment with ergometrine (1 μM) enhanced the partial agonistic effect of *Clivia*.

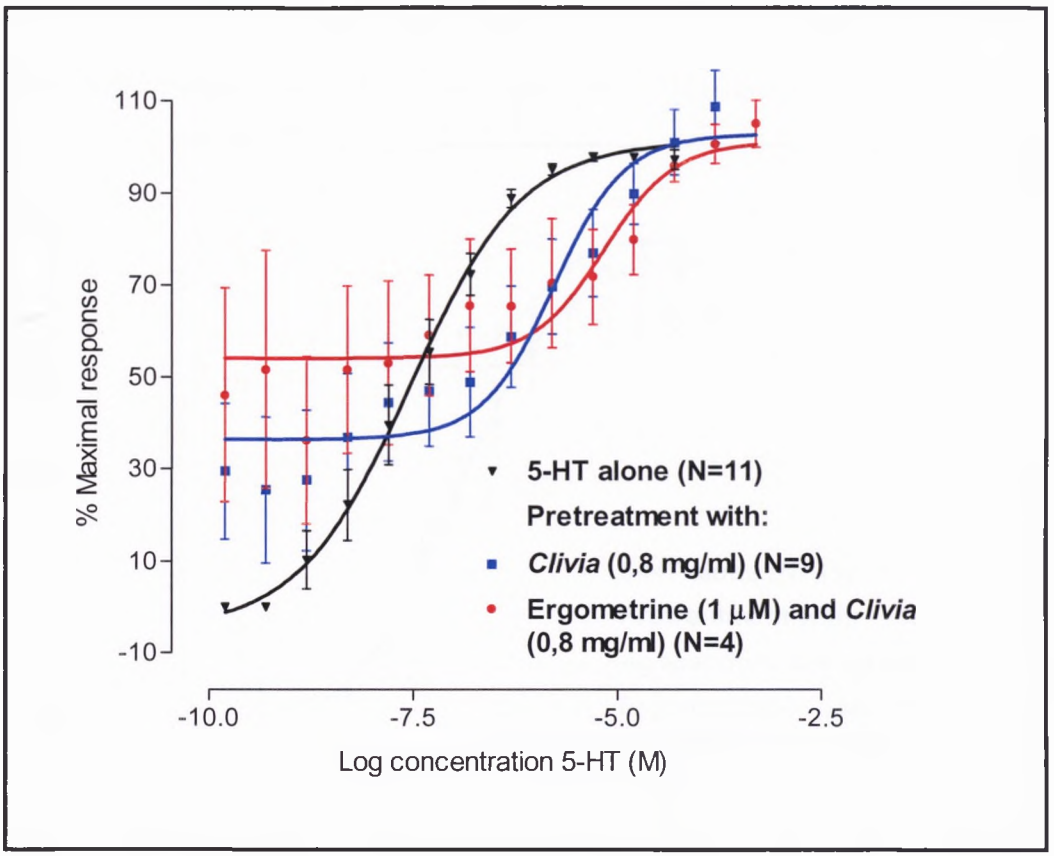


Figure 2.36. Mean concentration-response curves for 5-HT alone and after pretreatment with *Clivia* (0,8 mg/ml) or ergometrine (1 μM) and *Clivia* (0,8 mg/ml) in the stripped myometrium. Ergometrine was administered 5 min before addition of *Clivia* extract and the extract was administered 5 min before cumulative addition of 5-HT. The vertical bars represent the S.E.M.

Pretreatment with 40% of a submaximal dose of *Agapanthus* (0.4 mg/ml) augmented the response of the myometrium to cumulative addition of 5-HT and potentiated the maximal effect. Additional pretreatment with the partial agonist, ergometrine (1 μM), depressed the maximal response (fig. 2.37). Pretreatment with 0,2 μM methysergide depressed the maximal response to *A. africanus* to 64,8% (±8,9) (N=4). These results indicate that *Agapanthus* might act as an agonist on 5HT-receptors.

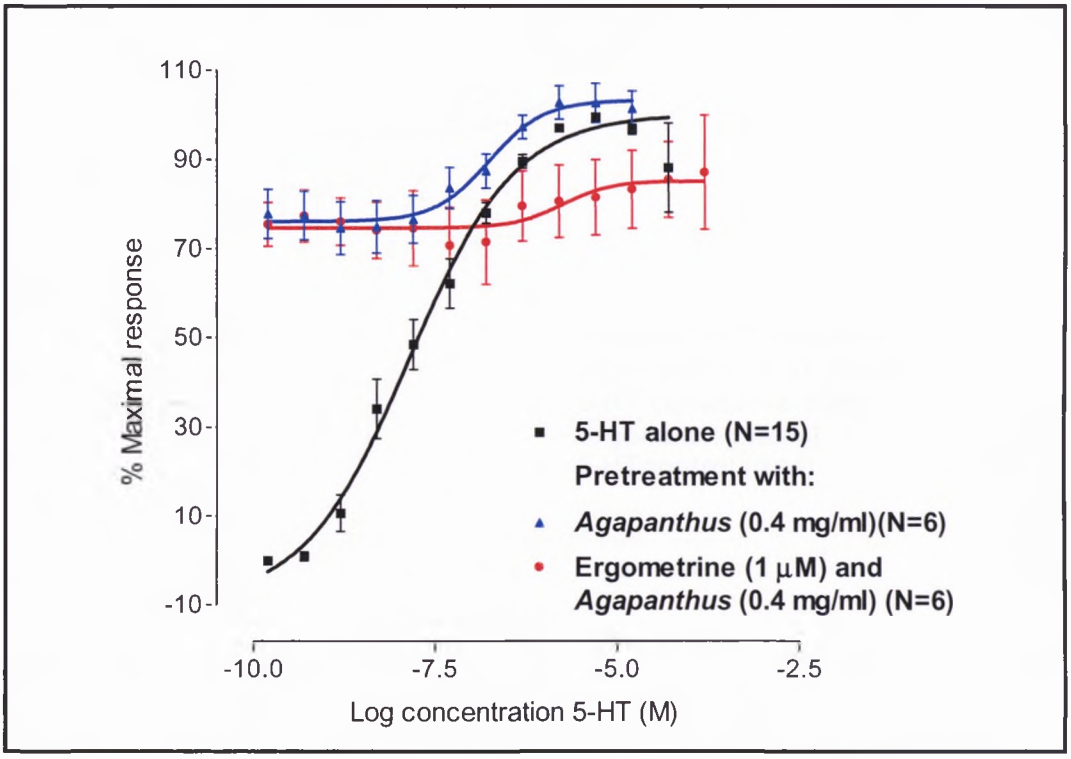


Figure 2.37. Mean concentration-response curves for 5-HT alone and after pretreatment with *Agapanthus* (0,4 mg/ml) or ergometrine (1 μM) and *Agapanthus* (0,4 mg/ml) in the stripped myometrium. Ergometrine was administered 5 min before addition of *Agapanthus* extract and the extract was administered 5 min before cumulative addition of 5-HT. The vertical bars represent the S.E.M.

When ergometrine and *Clivia* extract were administered alone to maximal response followed immediately by cumulative addition of 5-HT to maximal response, the partial agonist effect of both ergometrine and *Clivia* on the myometrial 5-HT_2-receptors was clearly demonstrated (figs. 2.38 and 2.39).

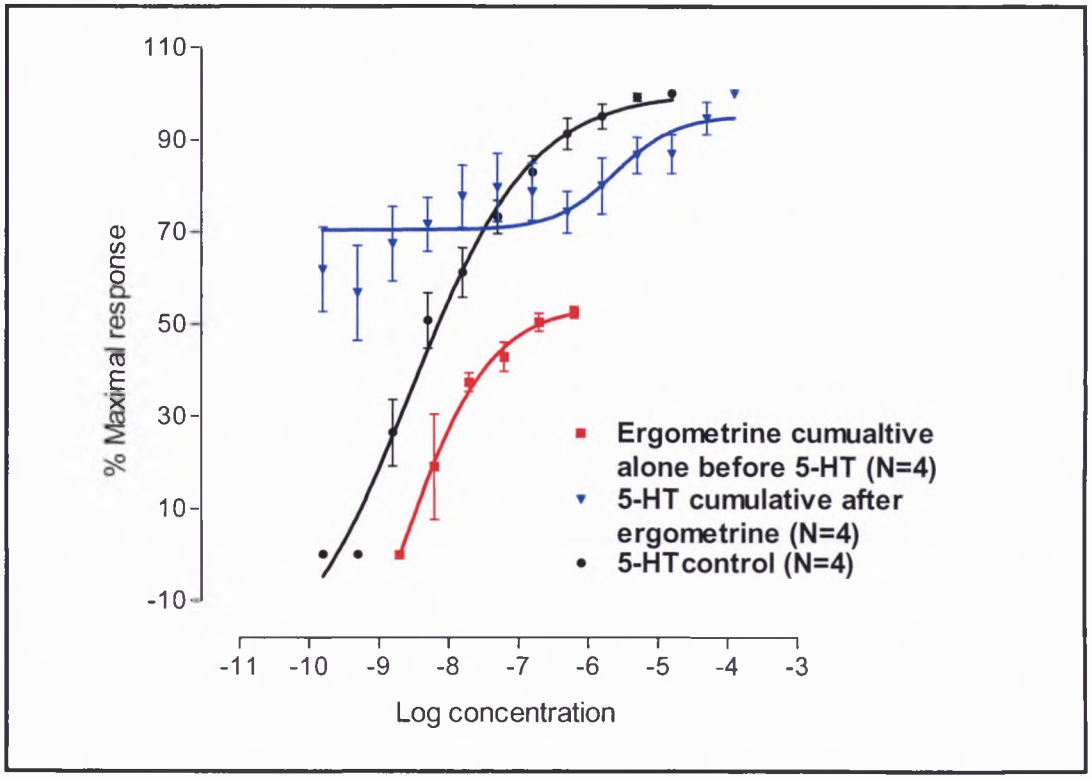


Figure 2.38. Mean concentration-response curves for 5-HT alone and then after pretreatment with ergometrine administered cumulatively until maximal response. The vertical bars represent the S.E.M.

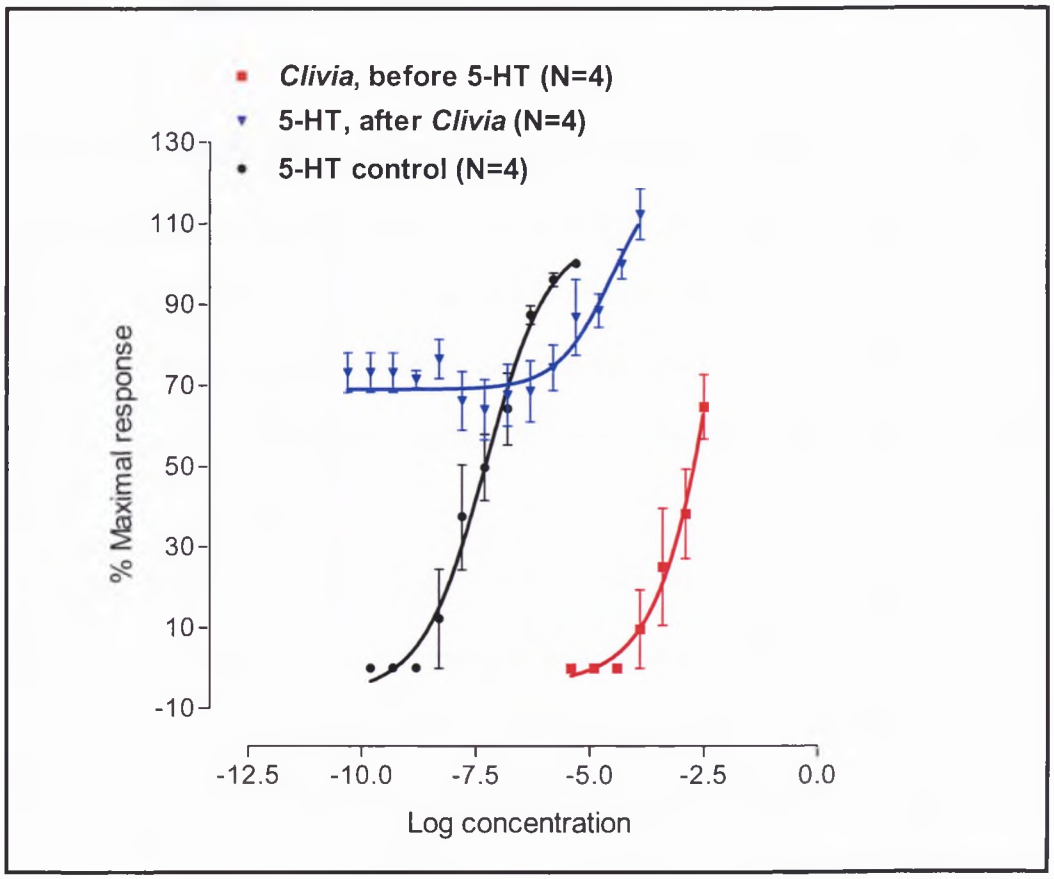


Figure 2.39. Mean concentration-response curves for 5-HT alone and then after pretreatment with *Clivia miniata* leaf extract administered cumulatively until maximal response. The vertical bars represent the S.E.M.

2.5.3.2.5 The effects of prazosin on the response to *Clivia* and ergometrine

The administration of prazosin (10 μM) had no effect on the response of the myometrium to cumulative administration of *Clivia* or to ergometrine. This indicates that these agents appear not to act on the α_1-receptor system in the oestrogenized rat myometrium.

2.5.3.2.6 The role of calcium on the contractile response of the rat myometrium

Pretreatment with the calcium channel blocker, verapamil (1 μM), depressed the maximal contractile response of the isolated oestrogenized rat myometrium to cumulative administration of the plant extracts as well as other agonists (table 2.6). These results indicate that all the agonists tested depended to various degrees on calcium influx into the myometrial cell via the voltage operated "L"-type membrane calcium channels (VOCs).

Table 2.6. The effects of blockade of the voltage operated "L"-type calcium channel in the stripped myometrium by verapamil (1 μM). The % maximal response to cumulative administration of each agonist after pretreatment with verapamil for 5 minutes was calculated relative to the maximal response of the agonist administered alone. N represents the number of rats.

Agonist	**% Maximal response**	**S.E.M.**	**N**
Agapanthus africanus	16,8%	2.6	6
Acetylcholine	42,0%	10,0	4
Calcium	58,0%	5,4	4
Clivia miniata	19,7%	12,5	6
Ergometrine	12,3%	4,0	6
$PGF_{2\alpha}$	50,2%	13,3	4
Potassium	27,8%	5,3	4

The effects of calcium sequestration of the extracellular calcium available to the myometrial preparation in the Tyrode solution by preincubation with EGTA (2 mM) was determined. The augmentary effects of submaximal doses of both *Agapanthus* and *Clivia* on the response of the myometrium to cumulative addition of ACh were reduced. In the case of *Agapanthus*, the augmentary effect was almost abolished (fig. 2.40), whereas the inhibition of the augmentary effect of *Clivia* was less marked (fig. 2.41). Deprivation of extracellular calcium reduced the maximal response to ACh administered alone to ca. 23% and the maximal response after pretreatment with *Agapanthus* to ca. 82% (fig. 2.40). Pretreatment with *Clivia* prevented the depression of the maximal response to ACh (fig. 2.41).

These results indicate that ACh, *Agapanthus* and *Clivia* depend on the availability of extracellular calcium for their contractile activity. It has already been shown that the contractile activity of these agents depends partially on calcium influx via VOCs. The contractile activity of *Clivia* appears much less dependent on extracellular calcium than that of *Agapanthus* and ACh, indicating a difference in mechanisms of raising intracellular calcium concentration to bring about myometrial contraction.

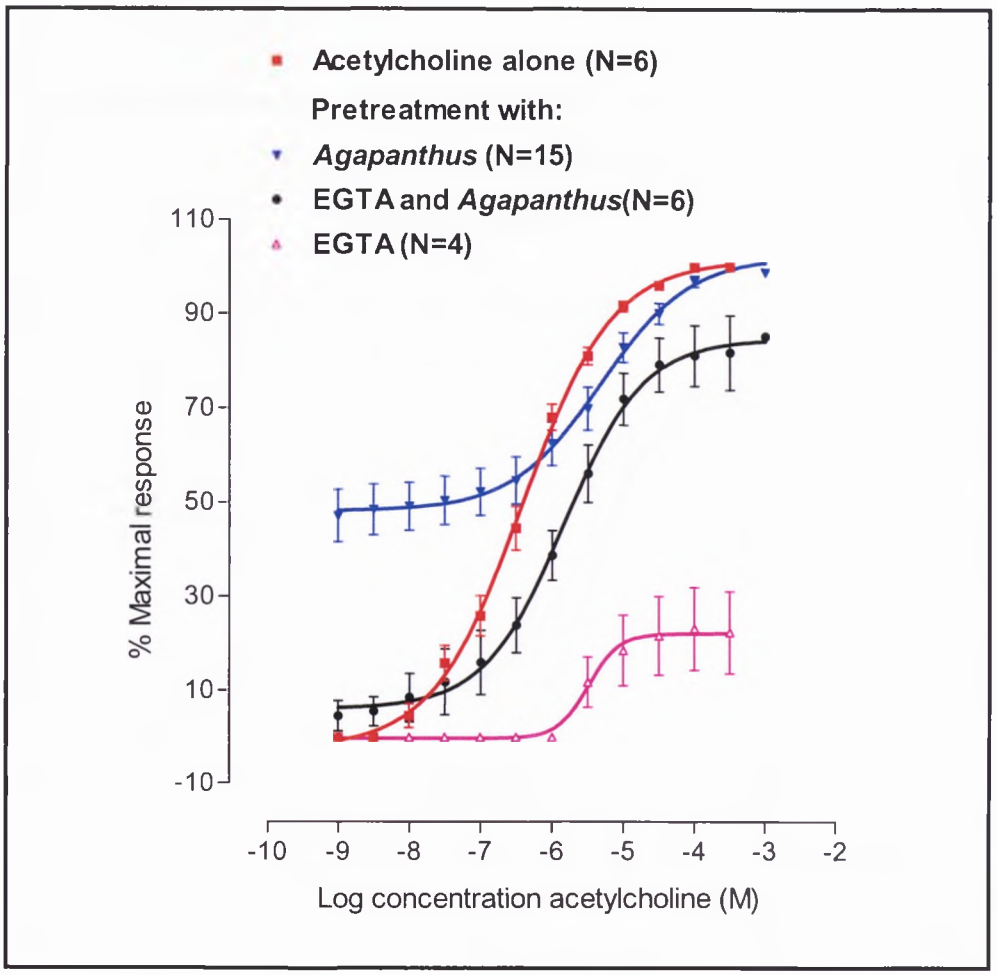


Figure 2.40. Mean concentration-response curves for ACh alone and after pretreatment with *Agapanthus* (1 mg/ml) or EGTA (2 mM) and *Agapanthus* (1 mg/ml) or EGTA (2 mM) alone in the stripped myometrium. EGTA was administered 10 min before addition of *Agapanthus* extract or ACh and the extract was administered 5 min before cumulative addition of ACh. The vertical bars represent the S.E.M.

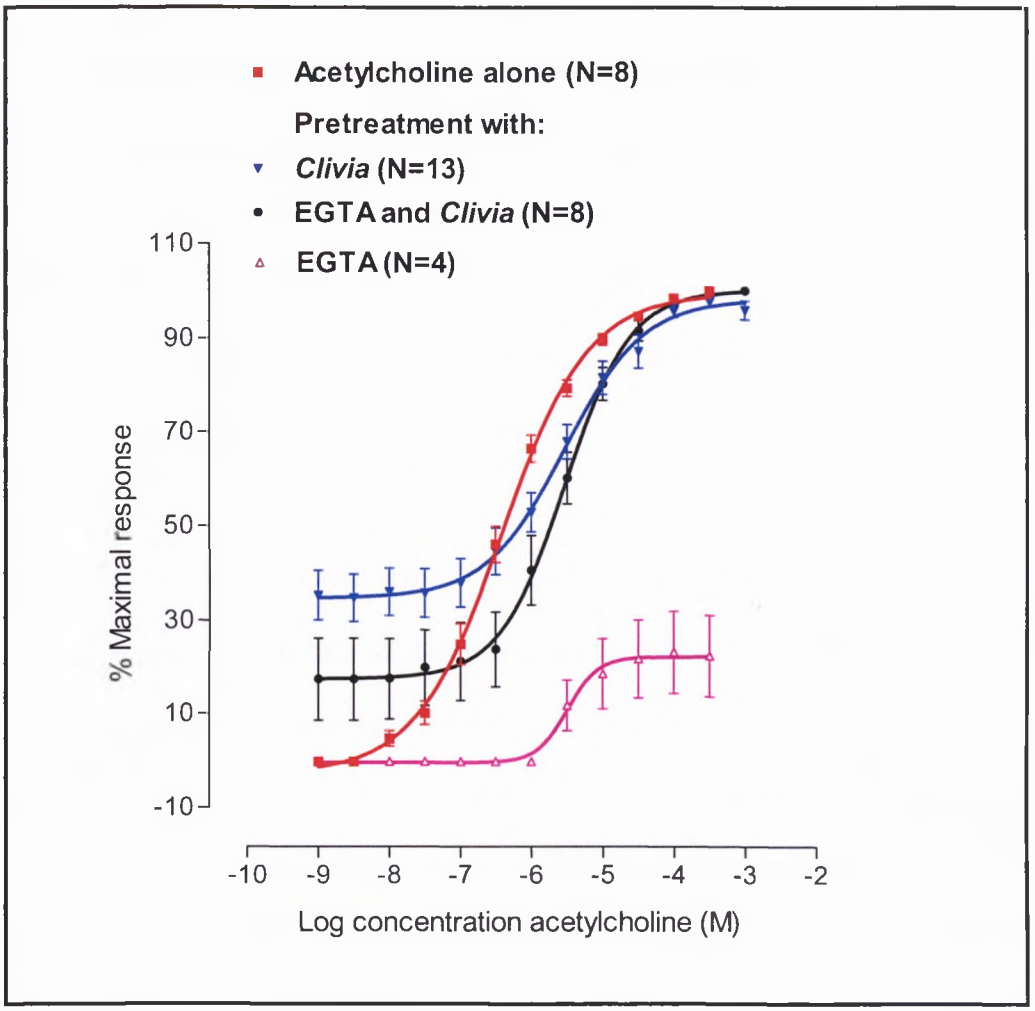


Figure 2.41. Mean concentration-response curves for ACh alone and after pretreatment with *Clivia* (2 mg/ml) or EGTA (2 mM) and *Clivia* (2 mg/ml) or EGTA (2 mM) alone in the stripped myometrium. EGTA was administered 10 min before addition of *Clivia* extract or ACh and the extract was administered 5 min before cumulative addition of ACh. The vertical bars represent the S.E.M.

2.5.3.3 The isolated "stripped" myometrium – Discussion

The anatomical structure of the uterus is complex and the contractile myometrial cells and secretory endometrial cells differ functionally. In addition, the myometrium is not a uniform muscle – it consists of two types of smooth muscle, circular and longitudinal, having a different origin. structure and function and the contraction patterns are different in the two muscles (5). The response to oxytocin has been found to be more potent in the longitudinal layer than in the circular layer (6).

The myometrium contracts in response to $PGF_{2\alpha}$ and PGE_2 stimulation. Indomethacin (5 μM) was found to specifically inhibit prostaglandin synthesis in tissue homogenates (129). The rat uterus also receives cholinergic innervation and stimulation of myometrial muscarinic M_3-receptors by agonists such as ACh causes contraction of the uterus. This effect can be blocked by a non-selective muscarinic competitive antagonist such as atropine (35). Marc et al (34) found that muscarinic as well as oxytocin receptor activation in guinea pig myometrium was coupled to an enhanced phosphoinositide metabolism. Both oxytocin and carbachol produced the same maximal contractile response but the maximum capacity of oxytocin to generate inositol triphosphate was 2 to 3 times greater than carbachol. Parry, Okwuasaba, Ekpenyong & Ashraf (130) mention the possibility that *Olax* herbal extract could release prostaglandins in smooth muscle, which through a "permissive" effect could modulate the responses of the herbal extract on muscarinic receptors. Calixto, Pizzolatti, & Yunes (131), refer to reports of prostaglandin release in the guinea pig ileum resulting in stimulation of the release of

ACh. These "permissive" effects could possibly also apply to uterine smooth muscle. Figure 2.42 is a schematic representation of some of the relevant factors regarding anatomical arrangement and receptor systems pertinent to the concepts of receptor activation of myometrial muscle cells relative to whole uterus as investigated in this study.

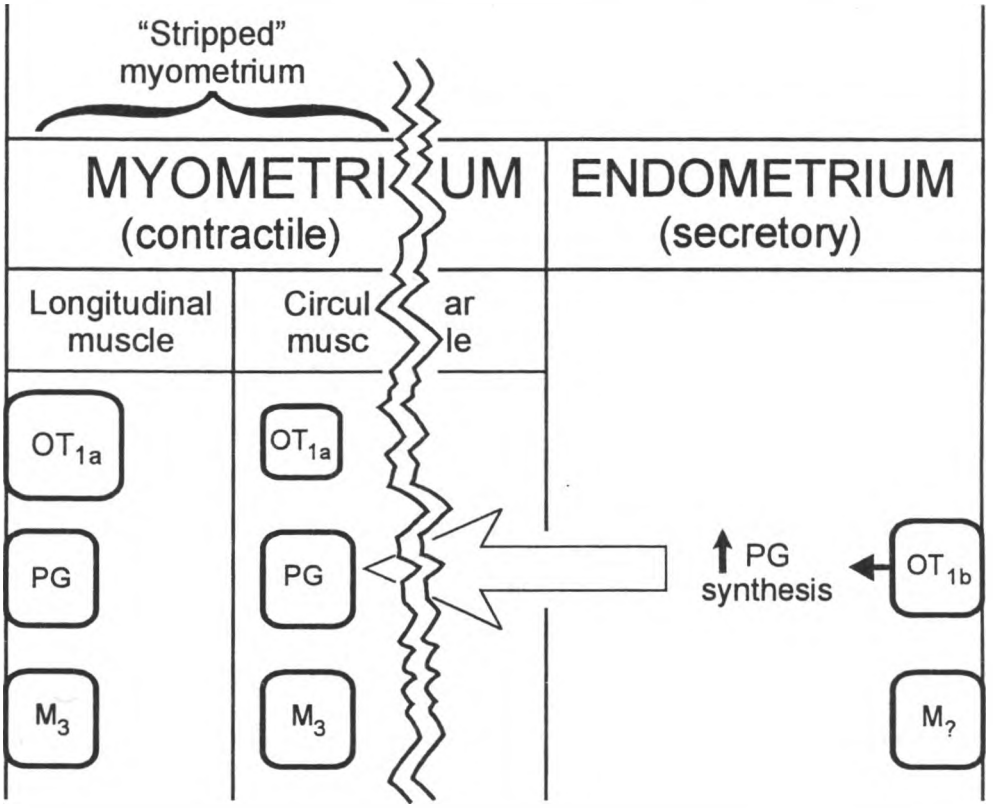


Figure 2.42. Schematic representation of the origin of the "stripped" myometrium preparation relative to the whole uterus. Receptor systems pertinent to the present study are represented as follows: OT (oxytocin); PG (prostaglandin); M (muscarinic).

Although *Clivia miniata* (Amaryllidaceae) and *Agapanthus africanus* (Alliaceae) belong to different botanical families and could therefore be expected to have different phytochemical profiles, the uterotonic effects of extracts of both plants on the rat isolated whole uterus have been shown to be similar and to augment the response to oxytocin (64, 65, 132). The augmentary effects of both extracts on the initial response of the whole organ to cumulative dosage of ACh could be reduced by pretreatment with indomethacin (5 μM), indicating that stimulation of prostaglandin synthesis could contribute to the uterotonic activity. Conversely, pretreatment with indomethacin did not significantly inhibit the augmentary effects of *Clivia* or *Agapanthus* on the response of the whole uterus to oxytocin. The activity of both extracts on the whole uterus could be inhibited by atropine (70 nM) (figs. 2.26 and 2.27).

In this study, the results have clearly shown that both the *Agapanthus* and *Clivia* extracts were able to directly initiate and maintain contractions in the "stripped" preparation. The herbal extracts augmented the initial response of the endometrium-free preparation to ACh as was previously observed in the whole uterus preparation (64, 132). Atropine (70 nM) administration did not change the response to *Clivia* extract in the myometrium but inhibited the contractile response to *Agapanthus,* suggesting that muscarinic receptor stimulation in the uterus is a component of the action of *Agapanthus* in the myometrium, but is seemingly of less importance with *Clivia.* This finding is in contrast to the previous observation that atropine inhibited the activity of both *Clivia* and *Agapanthus* in the whole uterus preparation (65, 132).

Indomethacin (20 μM) pretreatment did not affect the response of the myometrium to cumulative dosage of ACh, oxytocin or *Clivia* extract alone or the augmentary effects of *Clivia* to the response to acetylcholine. This concentration of indomethacin was higher than that used in the whole uterus studies (5 μM). Preincubation with indomethacin, however, significantly inhibited the augmentary effects of *Agapanthus* extract on the myometrial response to ACh and cumulative dose-response curves constructed with *Agapanthus* extract. These findings indicate that synthesis of prostaglandins plays an important role in the uterotonic activity of *Agapanthus*, whereas the contribution to the myometrial activity of *Clivia* is insignificant. In the whole uterus studies, the augmentary effects of both the *Agapanthus* and *Clivia* extracts on the response of the whole uterus to oxytocin could not be effectively inhibited by indomethacin blockade. We postulate that the organ was most probably responding to the additive effect of prostaglandin synthesis in endometrial cells due to oxytocin stimulation. This additive effect was not present when the whole uterus was pretreated with indomethacin and herbal extract prior to acetylcholine administration, and the augmentary effects of both *Clivia* and *Agapanthus* were reduced (65, 132).

The marked inhibition of *Agapanthus* activity by indomethacin masked any muscarinic activity. This could possibly be a result of blockade of a "permissive" modulating effect by prostaglandins on muscarinic effects in the myometrium as referred to by Parry et al (130) and Calixto at al (131) in the ileum. The "stripped" myometrium preparation has clearly identified that *Clivia* has a dual action in the uterus – a direct contractile effect on myometrial cells with no effect on prostaglandin synthesis, whereas it does appear to

stimulate some prostaglandin synthesis in the whole uterus. The apparent loss of muscarinic activity by *Clivia* in the myometrium could be due to a greater sensitivity to its effects on another receptor system in the stripped myometrium preparation (which consists mainly of longitudinal muscle). Tuross et al (6) have shown that the response to oxytocin was more potent in the longitudinal layer than in the circular muscle layer. The possibility therefore exists that the active principle/s in the *Clivia* extract could activate or modulate a major system responsible for uterine contraction such as oxytocin receptors or other systems such as serotonergic or adrenergic receptors, or act via a yet unknown mechanism. It has therefore been shown that in the "stripped" myometrium, unlike *Agapanthus*, the contractile activity of *Clivia* is independent of promotion of prostaglandin synthesis or muscarinic cholinergic stimulation.

Serotonin (5-HT) is known to be able to regulate uterine contractile activity in mammals (133). The rat is highly sensitive to the effects of 5-HT during oestrus or pregnancy and the sensitivity to 5-HT can be increased by pretreatment with oestrogen. This increase in sensitivity is believed to be due to an oestrogen-induced increase in uterine 5-HT-receptors (133). The effects of *Agapanthus* and *Clivia* on the 5-HT_2-receptor system in this model appear to differ. The maximal response to *Agapanthus* is depressed by the non-competitive antagonist methysergide, indicating that the plant extract acts as an agonist on myometrial 5-HT_2-receptors. The marked augmentary effect of *Agapanthus* on the response of the myometrium to threshold concentrations of 5-HT could be due to the multiple additive effects of the herbal extract on 5-HT_2-, PG- and muscarinic receptors.. *Clivia* in contrast, appears to interact with the 5-HT_2-receptors in this model

in the same fashion as ergometrine, i.e. as a partial agonist. Veale (61) found that pretreatment of the isolated whole rat uterus with an aqueous extract of *Clivia miniata* leaves caused a parallel shift of the 5-HT concentration curves to the right, indication of a competitive antagonism. The results now obtained with the stripped myometrium model indicate partial agonism. These conflicting "partial agonism/antagonism" results have been found with ergometrine (46, 47) on various preparations of rat uterus. The actual significance of activation of 5-HT_2-receptors in the initiation or maintenance of labour is unknown and is regarded as insignificant. The importance of the identification of a similar mechanism of action for *Clivia* and ergometrine on the myometrium lies in the fact that the specific uterotonic mechanism of action of ergometrine, a potent oxytocic, has not yet been elucidated. This study has now provided some evidence that *Clivia* is able to activate a receptor system in a similar manner to ergometrine and therefore its uterotonic mechanism of action may also be linked. It is believed that ergometrine may act as a partial agonist on 5-HT_2-receptors in an allosteric fashion (47) and this may apply to *Clivia* as well.

The role of calcium in the contractile activity of the plant extracts appears to differentiate their mechanisms of action even further. The contractile activity of both herbal remedies depends partially on calcium influx via VOCs (7, 29). This could be an indication that they activate myometrial ROCs, triggering depolarisation and an influx of calcium through VOCs. The blockade of VOCs by verapamil would inhibit the end result of the activation of ROCs by the herbal extracts. In addition, these results have shown that although both *Agapanthus* and *Clivia* depend on the availability of extracellular calcium

for their contractile activity, it is more critical to *Agapanthus* activity than to that of *Clivia*. Oxytocin activity has also been found to be reduced, but not totally inhibited by extracellular calcium depletion whereas the uterotonic activity of ACh is dependent on extracellular calcium.

In conclusion therefore, these results illustrate that the "stripped" myometrium model has successfully "unmasked" differences in the mechanism of action of two herbal oxytocics which appeared to act by similar mechanisms in the whole uterus model. The study confirms that the uterotonic effects of the herbal remedies are retained in the "stripped" myometrium preparation. The possibility exists that *Clivia miniata* may act on the myometrium in a similar way to oxytocin i.e. direct stimulation of smooth muscle contraction. *Agapanthus africanus* activity however, has been shown to be dependent on the stimulation of prostaglandin synthesis which may involve muscarinic receptor activation. This study has also provided valuable confirmation of the viability of the contractile response of the "stripped" myometrium preparation to standard agonists and has established critical equilibration conditions.

2.6 SECTION F - BIOCHEMISTRY

2.6.1 Measurement of the accumulation of inositol phosphates as an index of agonist-induced phosphoinositide hydrolysis in myometrial smooth muscle

Contractile activity in myometrium as well as other smooth muscle is determined by the concentration of intracellular free Ca^{2+}. Intracellular Ca^{2+} mobilization can result from the activation of cell surface receptors which are coupled to the hydrolysis of phosphoinositides by PLC. The degradation of phosphatidylinositol 4,5-biphosphate (PIP_2) leads to the formation and release of two intracellular messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG), into the cell cytoplasm. IP_3 then stimulates the release of stored intracellular Ca^{2+} from IP_3-sensitive stores in the SR. It has been shown that the uterotonic action of OT, $PGF_{2\alpha}$, and agonists acting on M_3-muscarinic and α_1-adrenoceptors is mediated by the activation of PLC, resulting in the accumulation of IP_3 (27, 34, 35, 134).

Inositol phospholipid breakdown can be conveniently monitored by following the accumulation of [^{3}H]inositol phosphates in the presence of lithium to provide a sensitive assay for studying agonist induced phospholipid turnover in smooth muscle (134). The aim of this study was therefore to examine the uterotonic mechanism of action of *Clivia* and *Agapanthus* more comprehensively by determining the effect of the extracts on phosphoinositide metabolism, and to relate the level of activity to that of the clinically

used oxytocics. The herbal extracts and a series of uterotonic agonists were examined for their ability to stimulate accumulation of [^{3}H]-labelled inositol phosphates in rat myometrial strips. The maximal contractile response of the isolated rat myometrium to uterotonic agonists and the herbal extracts was also determined in order to relate contractile activity to the level of accumulation of inositol phosphates.

2.6.2 Methodology - Measurement of the accumulation of [^{3}H]inositol phosphates in the "stripped" myometrium

Virgin female Sprague-Dawley rats (300 g) were injected with stilboestrol in arachis oil (0.1 mg/kg IP) and were then euthanazed by inhalation of CO_2 24 hours later. The uterine horns were dissected out and opened longitudinally and the myometrium was separated from the endometrium as described in section 2.5.3.1 (127). The endometrium obtained by the "stripping" of the myometrium contained some circular muscle (127). The "whole" uterus preparation was also used in the initial phase of this study.

The method of Marc, et al., (34) was followed with modifications. Initially, the individual response to acetylcholine (ACh) by strips of whole uterus, myometrium and endometrium was determined. Thereafter only myometrial strips were used. Strips of tissue (50 – 100 mg) were placed in 5 ml KRB buffer (pH 7.4) after dissection and were then blotted and weighed. A tissue strip from the matching uterine horn of each rat was used as a basal control in every experiment. Each strip was then individually incubated with 12 μCi of myo-[2-^{3}H]inositol (Amersham) in 1 ml Krebs Ringer Bicarbonate (KRB)

buffer in a shaking water bath at 30°C (aerated with 95% O_2 plus 5% CO_2). Incubation continued for 3.5 hrs by which time the incorporation of [^{3}H] into inositol lipids should have reached a plateau (34).

The tissue strips were then rinsed 3 times with 20 ml KRB, and transferred to 1,5 ml fresh buffer and allowed to equilibrate for 20 min before transfer to 1,0 ml fresh buffer containing 10 mM LiCl. Lithium was added to inhibit inositol monophosphatase in order to enhance the accumulation of the inositol phosphates and prevent metabolism to inositol – i.e. quantitative "trapping" of inositol phosphates in response to receptor activation (135).

After 10 min, the tissue was transferred to fresh buffer containing either 10 mM LiCl alone for the control strips or the specific concentration of agonist to be tested. Incubation was continued for a further 10 min. Reactions were stopped by immersing the tissue in 1 ml ice-cold 10% (w/v) trichloroacetic acid (TCA) and followed by snap freezing in liquid nitrogen and storage at –70°C. Each frozen strip was allowed to thaw slightly and was removed from the TCA solution. It was then minced finely with scissors and pulverized in liquid nitrogen in a plastic vial. The chilled TCA solution was added to the pulverised tissue in the vial, followed by homogenization (Ultra-Turrax with N10 shaft, Janke & Kunkel) on ice (2 x 20 sec). The suspension was poured into a clean eppendorf tube (A) and stored on ice. The vial was rinsed by adding 0,5 ml ice-cold 10 % TCA followed by homogenization (2 x 20 sec) and the suspension was poured into

another clean eppendorf tube (B). Tubes A and B were then centrifuged at 10,000 x *g*, 4°C for 10 minutes (136) to remove the TCA-precipitable proteins and lipids (137).

The supernatant in tube A was decanted into eppendorf tube C and stored on ice. The supernatant in tube B was decanted into tube A, the pellet was resuspended, followed by centrifugation. The final supernatant was added to that in tube C followed by snap freezing in liquid nitrogen and storage at –70°C. The TCA supernatants which contained the water-soluble inositol metabolites were extracted 4 times with 4 ml of diethyl ether on ice to remove the TCA. The extract was then neutralized with KOH (1,25 M) and was snap frozen in liquid nitrogen and stored at –70°C. When needed, the extract was allowed to thaw and was applied to a column (10 x 25 mm) of Dowex AG1-X8 formate form, 200-400 mesh (Bio-Rad). The columns were 2,5 ml polypropylene syringes stoppered with a glass wool plug and filled with a slurry of swollen resin in deionized water to the 2,5 ml mark.

Free inositol was eluted by 10 ml of water and glycerophosphoinositol by 10 ml of 5 mM sodium tetraborate and 60 mM ammonium formate. Inositol monophosphate (IP_1), inositol biphosphate (IP_2) and inositol triphosphate (IP_3) were then eluted successively by 10 ml of 0,1 M formic acid and 0,2 M ammonium formate; and 10 ml of 0,1 M formic acid and 0,5 M ammonium formate; and 10 ml of 0,1 M formic acid and 1 M ammonium formate. Each peak was collected as a single fraction and 2,5 ml of each fraction was taken for determination of radioactivity in 7,5 ml Ultima Gold scintillation fluid

(Packard). Results were expressed as cpm/mg tissue per elution or as a percentage of total [^{3}H]inositol phosphate accumulation compared to control.

2.6.3 Results

2.6.3.1 Evaluation of the response of whole uterus, "stripped" myometrium and endometrium to stimulation of phosphoinositide hydrolysis

This study employed the measurement of [^{3}H]inositol phosphates as an index of phosphoinositide hydrolysis in rat myometrium. Initial experiments comparing whole uterus, myometrium and endometrium indicated that the myometrial preparation was significantly more sensitive to ACh stimulation of phosphoinositide hydrolysis than the whole uterus preparation (table 2.7). The myometrium was therefore used as the standard preparation in all the experiments that followed in order to eliminate the effects of spontaneous release of prostaglandins from the endometrium (138). ACh has proved to be a very reliable standard agonist in the isolated "stripped" myometrium preparation, capable of reproducible results and not inducing spontaneous contractions or sensitizing or desensitizing the preparation (138).

Table 2.7. The effect of acetylcholine (20 mM) on the accumulation of [^{3}H]inositol phosphates (% of basal) in whole uterus, myometrium and endometrium respectively. Results are expressed as the mean ± S.E.M. and statistical significance was assessed using the student's *t* test. The response by myometrial tissue was significantly different to whole uterus (* $p < 0.05$).

	Total [^{3}H]inositol phosphates (% of basal)	**N**
Whole uterus	215,65 ± 36,48	3
Myometrium	528,98 ±100,23	3 *
Endometrium	258,55 ± 82,26	3

2.6.3.2 Measurement of [^{3}H]inositol phosphate accumulation in the myometrium in response to stimulation by agonists and plant extracts

It was found that the herbal extracts of *Agapanthus africanus* and *Clivia miniata* caused an accumulation of [^{3}H]inositol phosphates, particularly inositol monophosphate as would be expected after lithium inhibition of inositol monophosphatase (table 2.8). The standard uterotonic agonists used as control reference agents also stimulated inositol phosphate accumulation, as has been shown before (34, 14, 23). The rank order of increase in accumulation of total [^{3}H]inositol phosphates above basal due to the individual agents was as follows: oxytocin > *Agapanthus* > $PGF_{2\alpha}$ > 5-HT > ACh >

Clivia > ergometrine (fig. 2.43). Basal values were measured as the response by a myometrial strip from a matching uterine horn in the absence of agonist or extract.

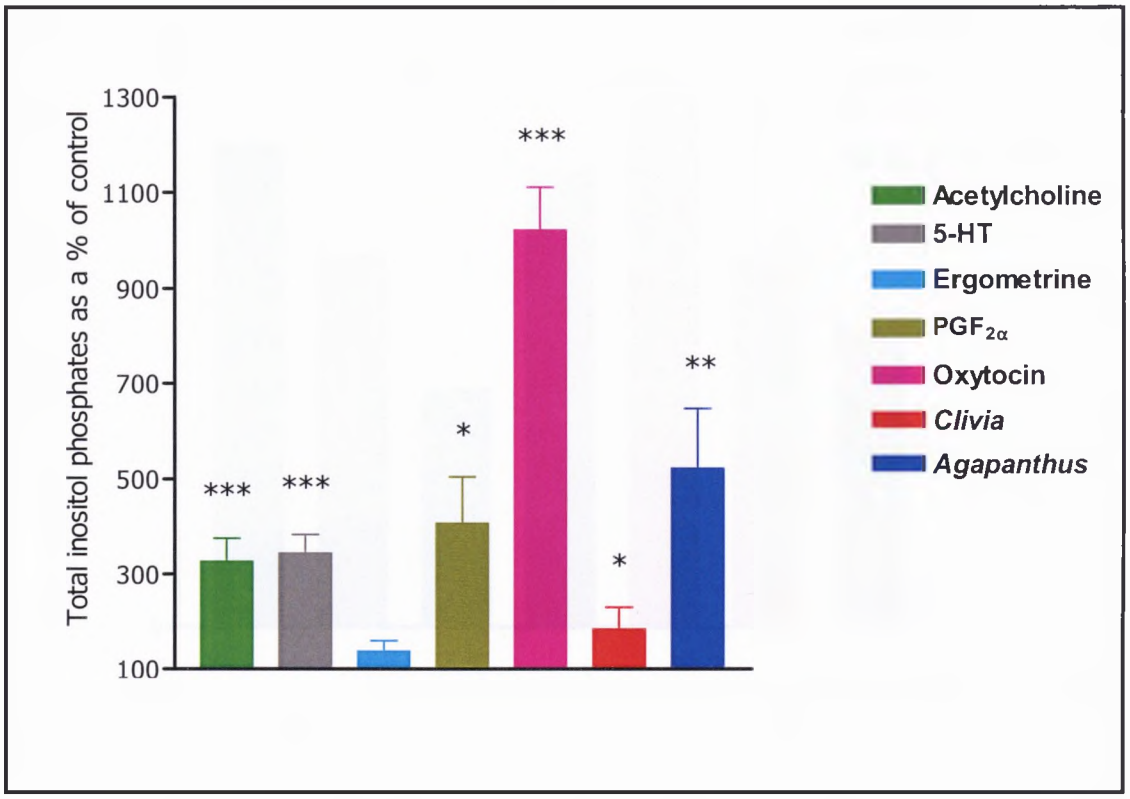


Figure 2.43. Total [^{3}H]inositol phosphates accumulated in the rat myometrium in response to stimulation by : ACh (20 mM, N=8); Oxytocin (0,6 µM, N=6); $PGF_{2\alpha}$ (70 µM, N=5); 5-HT (125 µM, N=6); ergometrine (1 µM, N=5); *Clivia* aqueous extract (40 mg/ml, N=4) and *Agapanthus* aqeuous extract (30 mg/ml, N=4). All results are expressed as percent increase over control values from the same experiment. Results are expressed as the mean ± S.E.M. and N represents the number of rats.

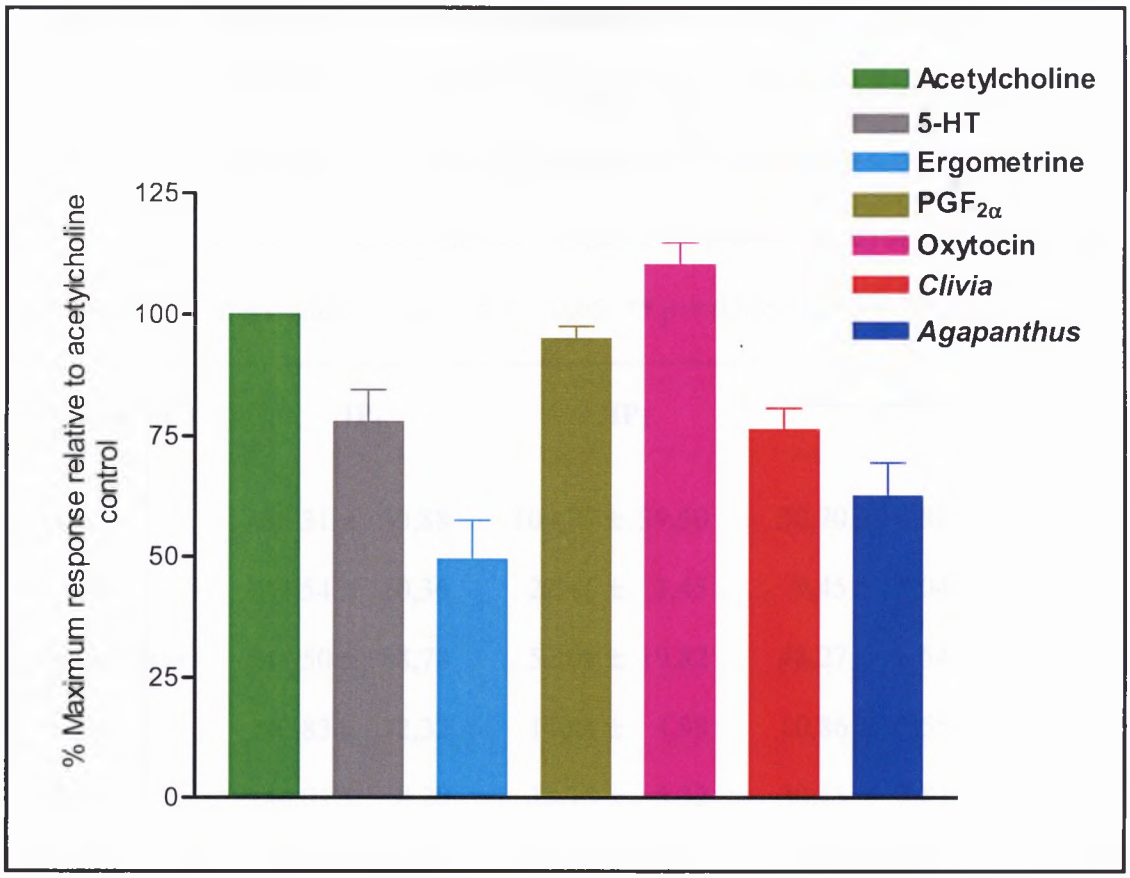


Figure 2.44. Maximal contractile response of the isolated rat myometrium in response to cumulative addition of oxytocin (N=10); $PGF_{2\alpha}$ (N=12); 5-HT (N=12); ergometrine (N=9); *Clivia* aqueous extract (N=22) and *Agapanthus* aqueous extract (N=29) relative to the maximal response to cumulative addition of ACh. Results are expressed as the mean ± S.E.M.

Table 2.8. Effect of agonists on accumulation of [^{3}H]inositol metabolites in rat myometrium (cpm/mg myometrial tissue). The results are expressed as the mean ± S.E.M. and the statistical significance (total [^{3}H]inositol phosphate accumulation compared to control tissue) was assessed using the student's *t* test. (* $p < 0,05$; ** $p < 0,005$; *** $p < 0,0005$).

Treatment	**IP_1**	**IP_2**	**IP_3**	**N**
ACh	430,31 ± 50,88	104,37 ± 39,60	30,70 ± 4,88	8 ***
Control	144,54 ± 10,36	22,46 ± 3,45	9,45 ± 1,04	
Agapanthus	544,50 ± 88,78	51,08 ± 19,82	48,27 ± 8.54	4 **
Control	90,83 ± 12,32	14,84 ± 4,98	10,86 ± 1,55	
Clivia	144,01 ± 8,20	15,15 ± 5,32	30,15 ± 2,81	4 *
Control	91,78 ± 15,86	15,68 ± 6,34	11,64 ± 1,72	
Ergometrine	163,29 ± 35,88	42,73 ± 10,68	15,75 ± 2,37	5 **NS**
Control	120,75 ± 10,39	42,96 ± 12,55	11,29 ± 0,42	
5-HT	448,66 ± 100,76	218,27 ± 31,86	60,15 ± 11,98	6 ***
Control	158,10 ± 15,22	42,64 ± 3,90	14,92 ± 1,34	
Oxytocin	804,33 ± 114,17	106,49 ± 9,93	74,78 ± 14,59	6 ***
Control	77,64 ± 8,55	9,01 ± 1,00	9,31 ± 0,90	
$PGF_{2\alpha}$	424,14 ± 118,57	141,34 ± 53,73	28,00 ± 5,97	5 *
Control	112,93 ± 18,62	26,10 ± 4,73	12,80 ± 0,99	

When the maximal contractile response of the myometrium to cumulative addition of the agonists and aqueous herbal extracts was compared relative to the maximal contractile response of the myometrium to cumulative addition of ACh, the rank order of response was different: oxytocin > ACh >$PGF_{2\alpha}$ > 5-HT ≈ *Clivia* > *Agapanthus* > ergometrine (fig. 2.44). As found by others (34, 129), agonists were able to stimulate phosphoinositide hydrolysis at much higher concentrations than those required for maximal activation of contraction in the rat myometrium – indicating that a small elevation of IP_3 would be sufficient to maximally activate contraction (table 2.9). It was noted, however, that ergometrine was an exception – there was no enhancement of phosphoinositide metabolism in response to 100 μM ergometrine. No correlation could be found between concentration of agonist, measurement of stimulation of phosphoinositide (PIP_2) metabolism and maximal contractile response. An *Agapanthus* concentration 12,5 x greater than that required for maximal contractile response produced a 5,2 x increase in total [^{3}H]inositol phosphate accumulation, whereas in the case of *Clivia*, the increase was only 1,8 x (table 2.9).

Crude fractionation of the ethanolic extracts of both *Clivia* and *Agapanthus* yielded active fractions (fraction 3, eluted by acetone) able to stimulate phosphoinositide hydrolysis (fig. 2.45). The *Agapanthus* faction was more potent than the *Clivia* fraction. Clivia fr. 3 and *Agapanthus* fr. 3 directly stimulated contractile responses in the isolated rat myometrium that were 23,09% (± 5,08) and 21,24% (± 6,00) of the maximal response to acetylcholine respectively (N = 4) in a similar proportion compared to the contractile effects of the crude aqueous extracts (table 2.9).

Table 2.9. Discrimination between biochemical and functional response: Stimulation of [^{3}H]inositol phosphate accumulation (% of basal) compared to % maximum contractile response relative to maximal contractile response to cumulative addition of ACh in the rat myometrium.

	Biochemical	**Response**	**Functional**	**Response**
Treatment	**Concn.**	**Increased [IPs]**	**Bath concn.**	**% Response**
ACh	(20 mM)	3,3 x	1 mM	100,0
Agapanthus	(30 mg/ml)	5,2 x	2,4 mg/ml	62,6
Clivia	(40 mg/ml)	1,8 x	3,2 mg/ml	76,3
Ergometrine	(1μM)	1,4 x	1,9 μM	49,4
5-HT	(125 μM)	3,4 x	5 μM	79,9
Oxytocin	(0,6 μM)	10,2 x	0,1 μM	110,3
$PGF_{2\alpha}$	(70 μM)	4,1 x	7,9 μM	95,0

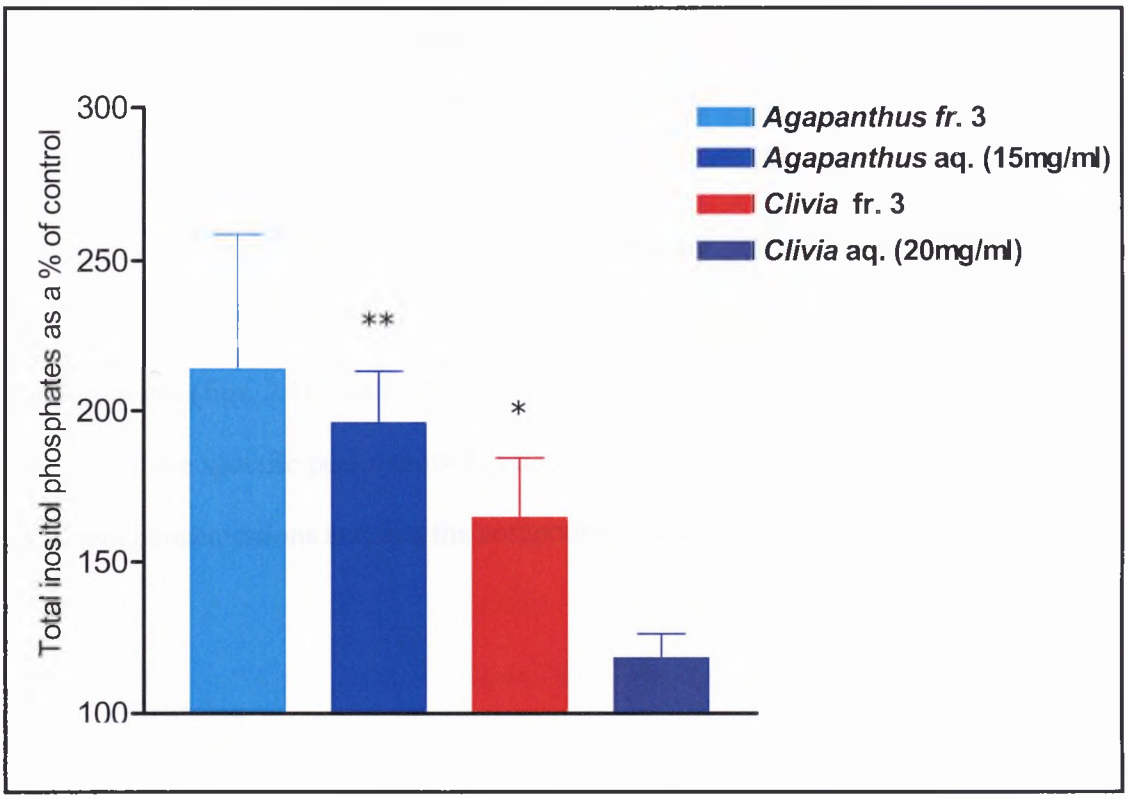


Figure 2.45. Total [^{3}H]inositol phosphates accumulated in the rat myometrium in response to stimulation by : *Agapanthus* fr. 3 (N=6); *Agapanthus* aqueous extract (15 mg/ml, N=6), *Clivia* fr. 3 (N=6) and *Clivia* aqueous extract (20 mg/ml, N=6). All results are expressed as percent increase over control values from the same experiment. Results are expressed as the mean ± S.E.M. and N represents the number of rats.

Other chemical fractions of *A. africanus* and *C. miniata* with positive results in the TLC screening tests were tested on this model and found to have no effect on the stimulation of phosphoinositide metabolism. These included *Clivia* flash fr.1 (chloroform) and fr.2 (methanol), *Agapanthus* flash fr.1 (chloroform) and fr.2 (methanol), *Clivia* and *Agapanthus* sapogenin fractions and the lycorine standard.

There were some similarities in the HPLC chromatograms of the acetone fractions of *Clivia* and *Agapanthus* (fig. 2.10). The most significant of these were the peaks at 7,3 and 7,8 minutes (figs. 2.10 and 2.11). The photo diode array UV spectra (fig. 2.12) taken from these specific peaks show that they probably contain the same substances, but in different concentrations and that the compounds may be structurally related.

2.6.4 Discussion

This study on the rat myometrium can be considered as the first report where the effects of herbal extracts used as oxytocics in traditional medicine have been analyzed systematically in the same preparation at the level of functional (contractile) and biochemical (i.e. second messenger generation) responses. Extracts of *Agapanthus africanus* and *Clivia miniata* stimulated phosphoinositide metabolism in the rat myometrium with increased generation of [^{3}H]inositol phosphates. The effect of *Agapanthus* was much greater than that of *Clivia*. Chromatographic analysis of the active fractions of *Clivia* and *Agapanthus* indicate common peaks at retention times of

7,3 and 7,8 minutes. Spectral evidence shows that the fractions most probably contain the same substances but at different concentrations. Although the common components cannot be directly related to potency in the active fractions, it is interesting to note that the active *Agapanthus* fraction is more potent than the *Clivia* fraction in this model, as is the case with the crude aqueous extracts. This is in contrast to the direct contractile activity of the herbal extracts in the stripped myometrium model where the crude aqueous extracts and the active fractions of both plants show similar activity. These results are the first to identify common chemical components in the active isolated chemical fractions of two uterotonic herbal medicines from different botanical families. Drewes, Horn & Mavi (139) have identified the presence of two major components from the bark of *Cryptocarya liebertiana* (family Lauraceae) that are also common to *Ocotea bullata* (family Lauraceae) bark. This result is significant because the *Cryptocarya* bark is currently being utilized by South African traditional healers as an alternative and more accessible source for their specific herbal medicines than *O. bullata* bark. The question to be posed here is – how did they identify the common medicinal modality without the aid of scientific analysis and could a similar observation have been made by traditional healers regarding the uterotonic activity of *Clivia* and *Agapanthus*? *Agapanthus* has also been classified as part of the Amaryllidaceae family by some taxonomists (12).

It has already shown that the contractile response of the myometrium to *Agapanthus* is associated with increased prostaglandin synthesis and muscarinic cholinergic activation (138) and these results appear to confirm the previous findings i.e. response to

stimulation by *Agapanthus* activates the same second messenger system as activation of prostanoid (FP)-receptors and M_3-muscarinic cholinergic receptors (27, 35).

The effect of *Clivia* on phosphoinositide metabolism was much less than its effect on myometrial contraction (table 2.9) and the same result was found with ergometrine. The mechanism of activity of *Clivia* is more difficult to define and although there appears to be some interaction with muscarinic cholinergic receptors, this plays a minor role in the myometrium (140). The *Clivia* extract (2 mg/ml) has been shown to act as an antagonist at 5-HT_2-receptors in the rat whole uterus preparation, seemingly by a competitive mechanism i.e. by shifting the concentration-response curve of 5-HT to the left (61). *Clivia* appears to act as a partial agonist on 5-HT_2- receptors in the stripped myometrium at a concentration of 0.8 mg/ml, causing an initial additive effect and then, at higher concentrations of 5-HT, induces a parallel shift of the 5-HT concentration curve to the right. Ergometrine has been shown to have a similar profile of activity on the 5-HT_2 receptor in the rat uterus. Hollingsworth et al (46) showed that ergometrine acted as a partial agonist at lower concentrations (0,1-1 μM) and a competitive antagonist at higher concentrations (10 μM). This could indicate allosteric interference on the 5-HT_2-receptor and possibly other binding sites and explain why ergometrine (100 μM) did not stimulate phosphoinositide metabolism.

This study has illustrated that the measurement of [^{3}H]inositol phosphate accumulation in the rat myometrium is a viable and reproducible bioassay for the determination of the biochemical activity of herbal extracts. The advantages of using this bioassay can be

summarized as follows: measurement of biochemical activity in the absence of interference from endometrial/decidual secretions such as prostaglandins in studies of uterine activity; indication of activation of Ca^{2+} mobilization from intracellular sources; confirmation of type of signal transduction mechanism activated by cell surface receptor occupation and correlation to receptor type activated ; and finally, the methodology allows for the bioassay of very small quantities of unknown substance as is often encountered when herbal extracts are subjected to chemical analysis and isolation procedures in order to identify active principles.

In conclusion, this study shows that extracts and chemically isolated fractions of *Agapanthus africanus* and *Clivia miniata* are able to stimulate phosphoinositide metabolism and that this can be regarded as the signal transduction mechanism for the uterotonic activity of *Agapanthus*, as is the case for oxytocin and $PGF_{2\alpha}$. The activity of *Clivia* is less marked in this model and indicates additional interaction with other biochemical systems that activate smooth muscle activity.

2.7 SECTION G - ENDOMETRIAL PROSTAGLANDIN SYNTHESIS

2.7.1 Measurement of prostaglandins in endometrial strips

It has already been established that the endometrium/decidua is the major site of PG production in the human and in the rat (1, 6). The effects of the plant extracts on the stimulation of PG synthesis in the rat endometrium were therefore investigated to determine whether there was any correlation between the evidence that *Clivia* had been shown to promote prostaglandin synthesis in the whole uterus preparation and not in the "stripped" myometrium (61, 138). *A. africanus* was shown to stimulate PG synthesis in both the isolated stripped myometrium and whole uterus preparations (132, 138) and it was therefore considered important to confirm its activity in the endometrium as well.

2.7.2 Methodology

The method of Rydzik, Terragno & Tackett (140) was followed with modifications. Endometrium (including some circular muscle) was stripped off whole uterine horns as described in the preparation of "stripped" myometrium (138). The endometrial strips were equilibrated in KRB after dissection and were then blotted and weighed. Each endometrial strip (representing a single uterine horn) was placed in a polypropylene tube containing 5 ml KRB and was incubated in a shaking water bath at 30°C (gas phase 95% O_2 plus 5% CO_2) for 1 hr. The temperature was kept 30°C to prevent the development of

spontaneous contractions which could promote prostaglandin synthesis and deplete the tissue of arachidonic acid precursors (124). An endometrial strip from a matching uterine horn was used as a control representing each experimental animal used. The strips were then placed in fresh KRB and incubated for a further 30 min to ensure equilibration of the tissue. Each endometrial strip was then transferred to an eppendorf tube containing 1 ml KRB (sample 1) and was incubated as before for 10 min. The strips were removed from control medium (sample 1) and placed in solutions of the test extracts or standard agonists in KRB or KRB alone (sample 2) and were then incubated for a further 10 min. The effects of the plant extracts, isolated fractions of the plant extracts and lycorine on prostaglandin production in endometrial tissue were compared to the effects of oxytocin, ACh, 5-HT and ergometrine. Sample 1 (control – medium only) was acidified to pH 3,5 with 0,8 M phosphoric acid, snap frozen in liquid nitrogen and stored at 70°C. Acidification was necessary to convert any prostaglandins and fatty acids in the sample to the water-insoluble non-dissociated form (140). The tissue strips were removed from sample 2 (test medium) and placed in a solution of ice cold 10% TCA (sample 3 – tissue sample). Sample 2 was acidified in the same way as sample 1. Sample 2 and sample 3 were snap frozen in liquid nitrogen and stored at -70°C until needed.

Acidic organic extraction of any prostaglandins in the acidified aqueous phase was carried out by extracting the sample with the organic solvent, ethyl acetate. Sample 1 and sample 2 were allowed to thaw and were immediately extracted 3 times in 2 volumes of ethyl acetate by centrifugation at 2000 x *g* for 7 min at 4°C and separation in a separating funnel. The organic phase was collected in 20 ml glass vials and dried under

a continuous stream of nitrogen. The residues were stored at -20°C until needed. This extraction method has been used previously to determine prostaglandin content in uterine tissue (141, 142, 143, 144). Xu, Cissel, Varghese, Whipkey, Blaha, Graeber & Keeting (145) found that ethyl acetate extraction recovered 72% of radiolabelled PGE_2 from the medium.

Each frozen tissue sample (sample 3) was allowed to thaw slightly and was then removed from the TCA solution. It was minced finely with scissors and pulverised in liquid nitrogen in a polypropylene vial. Saline (0,9%) was acidified to pH 3,5 with 0,8M phosphoric acid and 1ml was added to the pulverised tissue in the vial, followed by homogenization on ice (2 x 20 sec). The suspension was poured into a clean eppendorf tube (A) and stored on ice. The vial was rinsed by adding 0,5 ml of acidified saline followed by homogenization (2 x 20 sec) and the suspension was poured into another clean eppendorf tube (B). Tubes A and B were then centrifuged at 10,000 x *g*, 4°C for 10 minutes . The supernatant in tube A was decanted into eppendorf tube C and stored on ice. The supernatant in tube B was decanted into tube A, the pellet was resuspended and centrifugation was repeated. The final supernatant was added to that in tube C followed by snap freezing in liquid nitrogen and storage at -70°C. These samples (i.e. sample 3) were extracted with ethyl acetate and processed further in the same way as sample 1 and sample 2.

Further purification by LH-20 chromatography was then carried out to remove any other contaminants. LH-20 columns were prepared by allowing preparing a slurry of 1 g LH-

20 (Sephadex, Pharmacia) in 10 ml chloroform per column and allowing it to swell overnight. The suspension was then poured into a glass column 70 mm x 8 mm containing a glass wool plug and was allowed to settle. The columns were pre-equilibrated with chloroform. The sample residues were reconstituted in 0,5 ml chloroform-ethyl acetate (85:15) and were applied to the columns. The columns were sequentially washed with 3,5 ml chloroform-ethyl actetate (85:15) and 10 ml chloroform (column became translucent). The eicosanoid fraction was then collected with 10 ml methanol (column went white) and the methanol fractions were dried under a nitrogen and stored at -20°C until TLC separation. The rationale for the separation of the eicosanoids from impurities with a high distribution coefficient for ethyl acetate by partition chromatography using the chloroform-ethyl acetate mixture with a chloroform-rich gel as the stationary phase and ethyl acetate as the mobile phase was described by Rydzik et al (140). Using chloroform alone, the structural characteristics of eicosanoids were exploited by adsorption of their hydroxyl and carboxyl groups, by hydrogen bonding, to the glucose hydroxyls within the LH-20 gel. This removed some of the co-extracted impurities with affinity for chloroform. Finally, gel permeation with methanol alone as the mobile phase eluted the eicosanoids by molecular mass. Chloroform (10 ml) was run through each column before re-use. The methanol was evaporated off the eicosanoid fractions under N_2 gas and the residues were stored at -20°C in new glass vials until required for analysis.

The presence of $PGF_{2\alpha}$ and PGE_2 in the samples was first determined by TLC analysis. The sample residues were reconstituted in 0,25 ml ethyl acetate (144) and 20 µl of

sample was applied in aliquots of 5 µl, allowing each aliquot to dry before adding the next, and applied to 20 x 20 cm precoated Silica gel G plates (Polygram Sil G/UV 254, Machery-Nagel) (141, 142, 143, 145, 146, 147). The samples were applied 1,5 cm above the bottom edge of the plate, allowed to dry and placed in a filter-paper-lined rectangular glass tank containing the upper organic phase of the solvent system: ethyl acetate-water-2,2,4-trimethylpentane-acetic acid (22:20:10:4) (144). The solvent system was well agitated and then allowed to equilibrate in a separation funnel and then the lower aqueous phase was removed and the upper organic phase was placed in the TLC tank. The chromatography tank was allowed to equilibrate for 30 min. The plate was allowed to run at room temperature up to 10 cm from the origin and was removed from the chamber, dried and sprayed with 10% phosphomolybdic acid (Saarchem) in ethanol and heated at 120°C for 5 min after development. This resulted in the appearance of dark bluish spots on a yellow-green background. Rf values of compounds in the samples were compared to Rf values of $PGF_{2\alpha}$ and PGE_2 standards (Sigma) run concurrently. Serial dilutions were made of the standards to identify the lower range of concentration of the PGs detectable by this system.

The samples that tested qualitatively positive for the presence of $PGF_{2\alpha}$ or PGE_2 in TLC screening were then tested quantitatively for the presence of PGE_2 using the Biotrak PGE_2 enzymeimmunoassay (EIA) system. There is at present no EIA kit available for the quantative evaluation of $PGF_{2\alpha}$.. The sample residues were reconstituted in the assay buffer prior to the assay and only the control and test medium samples were evaluated. After the required incubation period with antibody, followed by rinsing, and then

incubation with the TMB substrate conjugate, 1 M H_2SO_4 was added to all the wells to halt the reaction. The optical density was then read on a scanning multiwell spectrophotometer (Labsystems, iEMS Reader MF) at 450 nm after 30 minutes.

2.7.3 Results and Discussion

The results of the test of serial dilutions of the standards identified that the lower limit for detection in this system was in the $PGF_{2\alpha}$ and PGE_2 concentration range of 125 ng. The medium samples (2) and tissue samples (3) treated with *Clivia miniata* aqueous extract and flash fr.3 ; *A. africanus* aqueous extract and flash fr.3 and oxytocin tested positive for the presence of $PGF_{2\alpha}$. Medium and tissue samples from endometrial strips treated with ACh, ergometrine, lycorine and 5-HT tested negative for the presence of $PGF_{2\alpha}$. No samples tested positive for the presence of PGE_2 and it was assumed that, if present, the concentration of PGE_2 fell below the concentration range detectable by the TLC system.

The PGE_2 EIA test standard calibration curve results are expressed in fig. 2.46. The standard curve was generated by non-linear regression using GraphPad Prism (version 1.0) and was then used to calculate the pg/well value of the samples. The EIA test revealed that submaximal and supramaximal concentrations of *Agapanthus africanus* aqueous extracts (1 mg/ml and 45 mg/ml) as well as the flash chromatography fr. 3 (acetone) promoted endometrial synthesis of PGE_2 in the rat model (fig. 2.47). A supra-maximal dosage of *Clivia miniata* (60 mg/ml) also stimulated PGE_2 synthesis whereas a

submaximal dose of OT (0,6 μM) had an insignificant effect and a submaximal dose of *Clivia* extract had no effect.

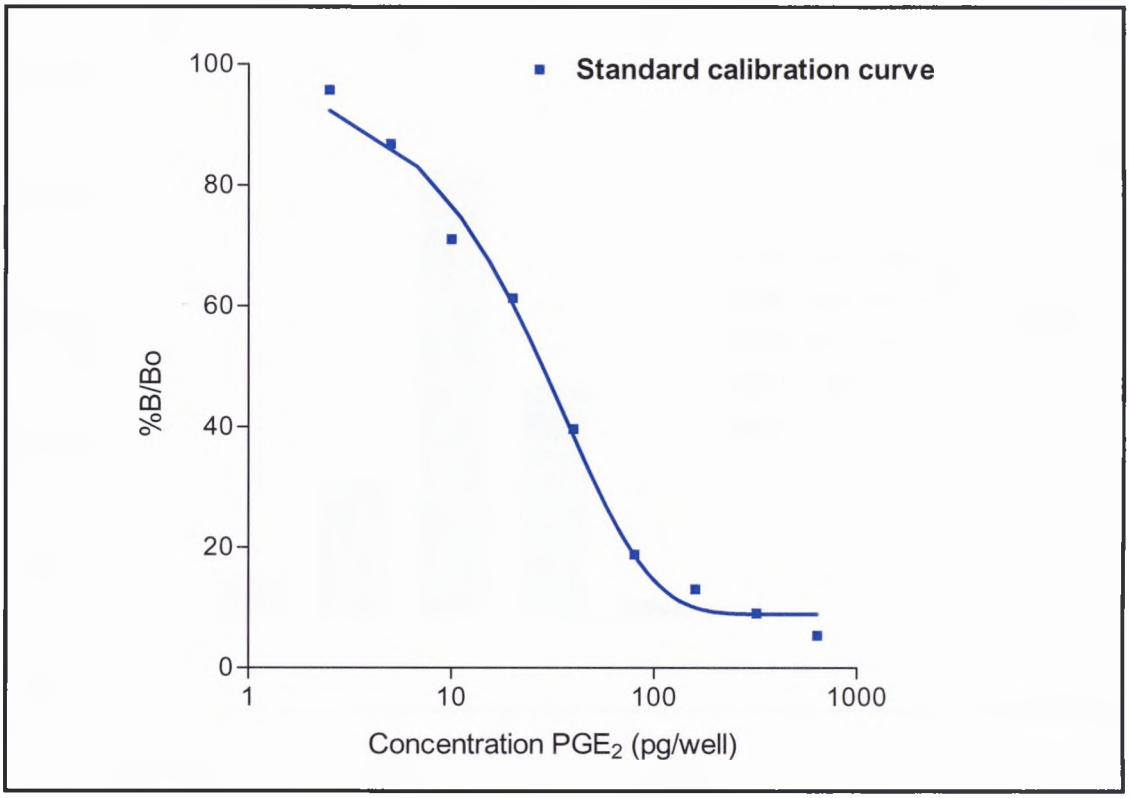


Figure 2.46. Standard calibration curve for the PGE_2 enzymeimmunoassay used to measure the release of PGE_2 into the medium after treatment of rat endometrial tissue with plant extracts and various agonists.

These results confirm that the *Clivia miniata* extract has a selective effect on the stimulation of PGs in the uterus. As already established for OT (20), it appears to stimulate endometrial PG synthesis and have no effect on stimulation of PGs in the myometrial smooth muscle. This investigation has also confirmed that *A. africanus*

extract has a stimulatory effect on endometrial as well as myometrial PG synthesis and this must be regarded as the major uterotonic mechanism of action for this herbal extract.

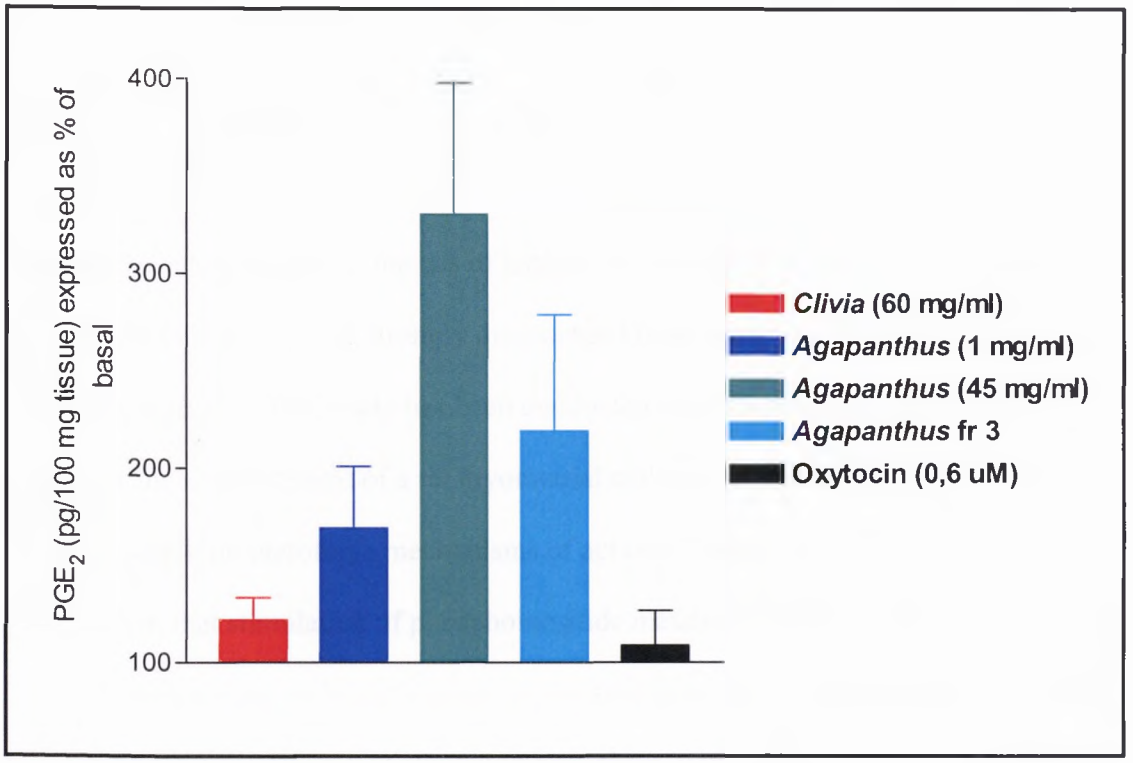


Figure 2.47. PGE_2 accumulation after stimulation of the rat endometrium by *A. africanus* aqueous leaf extract (1 mg/ml (N=2) and 45 mg/ml (N=3)) and flash fr. 3 (N=3), *C. miniata* aqueous leaf extract (60 mg/ml, N=3) and oxytocin (0,6 µM, N=3). The results are expressed as percent increase over control values for the same experiment. Results are expressed as the mean ± S.E.M.

2.8 SECTION H - TISSUE CULTURE OF RAT MYOMETRIAL CELLS

2.8.1 Review

The public outcry regarding the use of laboratory animals for research has resulted in the scientific community being strongly discouraged from using experimental animals in research projects. This study has been conducted on a laboratory rat model and therefore the establishment of a rat myometrial cell line would be the ideal solution for future research on uterotonic mechanisms of action of herbal oxytocics. It has already been shown that stimulation of phosphoinositide metabolism, PG synthesis and intracellular calcium mobilisation and regulation can be measured in a myometrial cell model (26, 136). The establishment of a continuous rat myometrial cell line model would dramatically reduce the requirement for experimental animals.

2.8.1 Methodology

Virgin Sprague Dawley rats (250-350 g) were oestrogen-primed by injection of stilboestrol in arachis oil (0.1 mg kg^{-1} IP) and were then euthanazed by inhalation of CO_2 24 hrs later. The uterine horns were dissected out and placed in a solution of Hanks Balanced Salt Solution (HBSS) (Highveld Biologicals (Pty) Ltd.) containing 10% of an antibiotic/antimycotic solution (PSF) (10,000U penicillin G; 10,000 µg streptomycin sulphate; 25 µg amphotericin B (Fungizone), Highveld Biologicals). The tissue was then

transferred to a laminar flow cabinet and was further processed using aseptic technique. Each uterine horn was opened longitudinally and endometrium and most of the circular muscle layer was gently "stripped" away from the myometrium as described previously. The myometrial strips were then placed in a solution of HBSS plus 10% PSF for 5 min. The method of Casey et al (26), with some modifications, was followed for the preparation of rat myocytes. The myometrial tissue was placed into HBSS containing 2% PSF, 2 mg/ml collagenase II (Sigma) and 0,2 mg/ml deoxyribonucleaase I (DNase I) (Boehringer Mannheim) and minced into 1 mm^3 fragments with micro scissors. The minced myometrium in digest solution was transferred to a small glass conical flask and incubated at 37°C with shaking for 60 min. The suspension was centrifuged (600 x *g* for 10 min at 4°) and the digest solution was aseptically aspirated off and replaced with a rinse solution (HBSS containing 2% PSF). Centrifugation and aspiration was repeated and the cells were rinsed a second time. The final rinse solution was aspirated off and replaced with 20 ml Dulbecco's Minimal Essential Medium (DMEM) (Highveld Biologicals (Pty) Ltd.) containing 10% heat inactivated foetal calf serum (FCS) (Highveld Biologicals (Pty) Ltd.), 2% PSF and 2% gentamicin (Highveld Biologicals (Pty) Ltd.). The cells were mixed with the medium by gentle aspiration and the cell suspension was pipetted into a 20 ml culture flask (Nunc) and incubated at 37°C under carbogen until confluence was reached (10 days). Cultured confluent cells were harvested by trypsinization and cell counts and cell viability were determined by the Trypan blue exclusion method.

2.8.3 Results and Discussion

The technique of culturing rat myometrial cells was successful. Dispersed myometrial cells grew to confluence within 10-14 days and displayed the typical characteristics of cultured myometrial cells when examined microscopically, i.e. elongated and either spindle shaped or fanned out at their ends (26). The culture could be maintained for a number of months. However, the establishment of a continuous cell line was not successful as the cells proliferated at a very slow rate after the first pass by typsinization. It was established that uteri dissected from 9 rats yielded only ± 1 million cells after trypsinization which was sufficient for just one cell suspension experiment and measurement. It is possible that establishing a confluent cell layer on glass cover slips after the first passage would be a more efficient technique than that of cell suspensions and would be worthwhile investigating in the future. The purchase of a continuous pregnant human myometrial cell line (PHM 1-41) is possible, but at prohibitive cost.

CHAPTER 3

GENERAL DISCUSSION AND CONCLUSIONS

3.1 GENERAL DISCUSSION

The aim of this ethnopharmacological study was to examine the uterotonic mechanisms of action of two herbal remedies used in South African traditional medicine as agents to induce or augment labour. A good scientific approach to ethnopharmacological investigations must be multidisciplinary and the first absolute requirement is the appropriate identification of the plant species studied. The *Agapanthus* and *Clivia* species used in this study were identified by botanists and voucher specimens were lodged in the C.E. Moss Herbarium at the University of the Witwatersrand. The next most important requirement is ensuring a continuous source of plant material of regulated quality, thereby ensuring the reproducibilty of results for the duration of the study. After botanical identification, the plant species used in this study were cultivated in a controlled environment in a garden in Sandton, Gauteng. This ensured ready accessibility of the plants for repeated collection as well as an established geographical location. Changes in geographical distribution and the season of harvesting of plant material for experimental use can significantly alter the concentration of chemical components in a plant (90, 126). The plant parts used to make the extracts for the study were harvested at a specific time of the year, namely the last two weeks of April, in order to ensure a relatively constant chemical composition. The exact method of extraction and preparation of all the extracts used in this study have been detailed in order to ensure reproducibility of results. Attention was given to employing an extraction method that closely resembled the ethnic method of preparation of the medicine. This approach ensured that the results of the study could at least be theoretically linked to the

availability of the active compounds in the ethnic dosage form. The aqueous extracts used in this study ensured the aqueous matrix utilized by most biological systems. Freeze drying and calculation of percentage yield ensured flexibility in the adjustment of concentrations as required in the various experimental models used in the study

It was not the intention of this study to identify the active uterotonic chemical principles present in the plant extracts, but to identify uterotonic mechanisms of action by means of functional and biochemical studies. Biological and pharmacological activity in a plant extract may be due to the presence of one or more components and a thorough investigation of the whole plant extract as it is traditionally used is an essential prerequisite to determination of the safety and efficacy of an ethnomedicine. The whole spectrum of pharmacological and toxicological activity in a herbal extract is most probably due to a combination of the stimulatory effects of active principles and modulation by synergistic or antagonistic effects due to other chemical components. This principle has long been recognized and is the reason for the extensive used of herbal extracts as medicinal preparations in Europe (71, 84, 89, 94, 126). However, chemical characterisation and fingerprinting of the plant extracts is essential to establish the identity and quality of the plant materials used in pharmacological and toxicity studies in order to validate the significance and reproducibility of the investigation (87, 90).

A comprehensive information base on the chemical compounds already identified in the plant species concerned was made for reference purposes. A rough TLC screening of crude alkaloidal and sapogenin extracts of *A. africanus* and *C. miniata* leaves as well as

some crude chemical fractions obtained by flash chromatography separation confirmed the presence of a number of alkaloids and saponins in *Clivia* and the presence of saponins and sapogenins in *Agapanthus*. All the aqueous extracts used in this study exhibited "foaming" on shaking which is characteristic of saponins. "Fingerprint" HPLC chromatograms were made of all the plant extracts used in this study for identification purposes.

The toxicity potential of aqueous leaf and rhizome/root extracts of *A. africanus*, *A. praecox* and *C. miniata* was evaluated. The Brine Shrimp bioassay, which measures general cellular toxicity to an invertebrate zoological species, and the MTT test, which measures intracellular toxicity in a human renal epithelial cell line, were used to determine LC_{50} values for the extracts. The results of the two types of toxicity assay differed and illustrated the importance of using a truly representative *in vitro* model, which is extremely difficult to identify and implement. The MTT test results contradicted the well-reported toxicity of *Clivia miniata* whereas its toxicity was confirmed in the Brine Shrimp assay. The leaf extracts of *Agapanthus* appeared to be the least toxic of those tested. The Brine Shrimp LC_{50} values for all the other extracts were found to be lower than the concentrations of plant extract required to augment oxytocin-induced uterine contractions in the rat model.

The selection of an appropriate pharmacological target is a primary consideration in an ethnomedical study. The ethnomedical background to the use and toxicity of the plant species examined in this study was thoroughly investigated and reported in the

introductory chapter. The validation of ethnomedical activity is a challenge because clinical evaluation at the initial stage of an investigation like this is out of the question. Pharmacological models are therefore used and *in vitro* systems are the most acceptable to the scientific community. *In vitro* efficacy must rely on the selection of a pharmacological model that relates best to the ethnomedical use of the herbal remedy. The uterus of the virgin female Sprague-Dawley rat was selected as an appropriate pharmacological target in this study on uterotonic activity. The isolated rat uterus preparation is a well-recognized and well-documented pharmacological model for this type of investigation. It must be noted, however, that the *in vitro* method of validation of ethnomedical activity is far from the human pharmacological model where the effects of the herbal remedies were first noted and reported. Although the results of this study cannot therefore be directly related to the response of the human pregnant uterus, they will serve as an indication of what could be expected in the human model. Because the use of experimental animals for research purposes is discouraged, one of the aims of this study was to investigate the viability of establishing a continuous cell culture of rat myometrial cells for future use in this type of investigation. Although it was possible to establish a rat myometrial cell culture and maintain it through several passages over a period of months, the culture proliferated very slowly after the first passage. The yield of cells was therefore very low compared to the number of rats used for the establishment of the tissue culture and was it was considered not ethically acceptable to use so many laboratory animals for one experimental measurement. Other methodologies such as using confluent cell layers on cover slips instead of cell suspensions should be investigated for future research projects.

The pharmacological equivalence of two species of *Agapanthus* reported to be used as herbal medicines to augment labour was investigated. This study has demonstrated that within the *Agapanthus* genus, crude extracts of both *A. africanus* and *A.. praecox* were able to initiate spontaneous uterine contractions and to augment the effects of oxytocin and ACh on the isolated rat uterus. The uterotonic activity of leaf extracts of both species was similar to the activity of rhizome extracts, confirming pharmacological equivalence in this model. The investigation has provided some evidence that that ethnomedical preparations of both species may be effective in the augmentation of labour and that, contrary to ethnomedical beliefs, the "root" extracts are not necessarily the most potent. The toxicity studies in the Brine Shrimp model, however, showed that *A. praecox* is more toxic than *A. africanus* and that the rhizome/root extracts are more toxic than the leaf extracts. This is an important deviation from the demonstration of the uterotonic pharmacological equivalence of the extracts and has provided evidence that the toxic principles in the extracts are probably not the same as the active uterotonic principles.

A spectral investigation of the extracts of the leaf and rhizome/root extracts of *A. africanus* and *A. praecox* was undertaken in order to establish whether there were any major differences in the chemical profiles of the extracts which could distinguish them from one another. The spectral results revealed that chemical components in the leaf extracts of both species were virtually identical and that the rhizome/root extracts of both species also closely resembled one another. The HPLC results confirmed that the leaf and rhizome/root extracts possessed similar chemical profiles in both species investigated

and that activity is most probably due to certain core components present in both leaf and rhizome/root extracts. Although the leaf extracts closely resembled the rhizome/root extracts, they were found to be less complex chemically than the rhizome extracts. This evidence, combined with the results of the toxicity tests, indicated that leaf extracts would be the most suitable for further pharmacological and biochemical studies. The necessity for removing a whole plant from the soil for preparation of the ethnomedicine has therefore been proved unnecessary and this information should be transmitted to traditional healers and would hopefully help prevent the herbal medicine trade from denuding our natural resources of the plant species concerned. The conservation aspect of this particular result may well prove to be very significant if the present massive destruction of indigenous flora in South Africa for the herbal medicine trade is considered. Traditional healers should be informed of these findings and encouraged to use leaves rather than the rhizomes of *Agapanthus africanus* plant for their medicines and also be discouraged from using the more potentially toxic *Agapanthus praecox* plant at all.

An aqueous extract of *Agapanthus africanus* leaves was then used to further investigate the effects of *Agapanthus* on uterotonic mechanisms and receptor systems in the isolated rat whole uterus preparation. It was found that the *Agapanthus* extract stimulated uterine muscarinic receptors and promoted PG synthesis in this experimental model. This was similar to the mode of action proposed for *Clivia miniata* (61) using the same experimental model. It would be anticipated that because these two plant species are members of different botanical families, their phytochemical profiles should be different.

However, these results showed that extracts of both species exhibited similar uterotonic mechanisms of action. It has been shown that members of different botanical families may share common chemical components (126, 139) and therefore it was decided to clarify the situation further by examining the effects of extracts of both species using a more sophisticated isolated organ model.

The complexity of the anatomical structure of the uterus and the functional difference between myometrial and endometrial cells has been described in the introduction to this study. The isolated "stripped" myometrium preparation differs from the whole uterus preparation in that the endometrial layer is removed by "stripping", resulting in a preparation consisting of smooth muscle only, devoid of interaction with the secretory endometrium. The endometrium is the major site of PG synthesis in the uterus and removal of this layer would reduce the possible interference of PG secretion and diffusion from the endometrium on the response of the myometrial smooth muscle to the herbal extracts. It has already been shown that OT has a dual action in the uterus and acts on OT_{1a}- receptors in the myometrium to cause contraction and on OT_{1b}-receptors in the endometrium to promote PG synthesis (20).

The results of the investigation of the activity of *A. africanus* and *C. miniata* extracts on the rat myometrium have clearly shown that the "stripped" myometrium preparation has unmasked a difference in the uterotonic mode of action of extracts of the two species. The major difference found was that promotion of PG synthesis played a dominant role in the myometrial contractile response to *Agapanthus,* whereas *Clivia* did not appear to

promote myometrial PG synthesis at all. *Agapanthus* activity in the myometrium was also linked to stimulation of M-receptors, whereas the effects of *Clivia* on this receptor system were negligible compared to the whole uterus preparation. *Clivia* was shown to act as a partial agonist on 5-HT_2-receptors in the myometrium in a similar fashion to that already established for ergometrine. *Clivia* activity in the myometrium has therefore been linked to the same pattern of uterine activity as ergometrine. The exact mode of oxytocic action of ergometrine has not yet been identified although it has been used in obstetric practice for 65 years. *Agapanthus* appeared to act as an agonist on the myometrial 5-HT_2-receptors, confirming its multiple activity on receptor systems in the rat myometrium. The role of calcium in the contractile activity of extracts of *Clivia* and *Agapanthus* differentiates their mode of action even further. The activity of both herbal extracts appears to depend on Ca^{2+} influx into the myometrial cell via VOCs, which may indicate initial activation of ROCs (7, 29). However, it has been shown that, like ACh, the contractile activity of *Agapanthus* depends largely on the availability of extracellular Ca^{2+} whereas, like OT, this requirement is less critical for *Clivia* activity.

The next phase of this study analysed the effects of the herbal oxytocics systematically in the same preparation (rat myometrium) at the level of functional (contractile) and biochemical (second messenger) responses in order to investigate their uterotonic mechanisms of action at a cellular level. Extracts of both *Clivia* and *Agapanthus* stimulated phosphoinositide metabolism with increased generation of [^{3}H]inositol phosphates but the effect of *Agapanthus* was much greater than that of *Clivia*. The rank order of stimulation was OT > *Agapanthus* > $PGF_{2\alpha}$ >5-HT > ACh > *Clivia* >

ergometrine. Activity was also identified in chemically isolated fractions of *Clivia* and *Agapanthus* where it was shown that the fractions contained 2 common components present in different concentrations and that the *Agapanthus* fraction was the most potent. It can therefore be postulated that receptor activated stimulation of phosphoinositide metabolism can be regarded as the major transduction mechanism for the contractile activity of *Agapanthus.* The identification of chemical components common to both herbal extracts confirms the complexity of all the contributory factors to the overall uterotonic effect of these herbal remedies.

Finally, it was shown that both *Agapanthus* and *Clivia* were able to promote the synthesis of both $PGF_{2\alpha}$ and PGE_2 in rat endometrial tissue. The stimulatory effect of *Agapanthus* on PGE_2 synthesis was greater than that of *Clivia* and reconfirms the important role that promotion of PG synthesis plays in its uterotonic mechanism of action. These results have also confirmed that *Clivia* appears to have a dual effect on the uterus – stimulation of myometrial contraction by an undefined mechanism and stimulation of PG synthesis in endometrial cells.

3.2 CONCLUSIONS

The main aims and objectives of this study have been achieved and various mechanisms of action responsible for the uterotonic activity of extracts of *Agapanthus africanus* and *Clivia miniata* leaves have been identified. Initial chemical analysis revealed that the phytochemical profiles of the two species were not identical although it was shown that there were chemical components common to both species in chemically isolated active fractions. *A. praecox* extracts were included in the initial investigation in order to identify pharmacological equivalence with *A. africanus*. Fingerprint chromatograms were made of all the extracts examined in the study for identification purposes.

The rank order of toxicity determined in the Brine shrimp bioassay was *C. miniata* ≥ *A. praecox* > *A. africanus*. The MTT bioassay on a human renal epithelial cell line did not detect the toxicity of *C. miniata* and this assay should therefore not be used as the sole method of predicting toxicity in this type of investigation. The process of correlating ethnopharmacological and ethnobotanical evidence provided proof that the uterotonic effects of *A. praecox* leaf and rhizome/root extracts were pharmacologically equivalent to those of *A. africanus* leaf and rhizome/root extracts on the isolated rat whole uterus. The chemical similarities in the extracts indicated common "core" components that could be responsible for uterotonic activity. The traditional use of both species in herbal remedies to augment labour could therefore be theoretically validated.

An aqueous extract of *A. africanus* leaves was shown to have similar uterotonic activity to an aqueous extract of *C. miniata* leaves on the isolated rat whole uterus preparation. Both herbal remedies directly stimulated spontaneous contractions and augmented the response to threshold concentrations of OT and ACh. The mode of action for the uterotonic activity of both herbal remedies on this model appeared to be stimulation of M-receptors and promotion of PG synthesis. The isolated "stripped" myometrium preparation and detection of stimulation of endometrial PG synthesis were effective in unmasking differences in the uterotonic action of *Agapanthus* and *Clivia*. The major reason for the uterotonic effect of *Agapanthus* was identified as the promotion of PG synthesis in both the myometrium and the endometrium and stimulation of M- and 5-HT_2-receptors is believed to play a facilitatory role. A dual role was identified for the uterotonic effect of *Clivia*. It appears to act on myometrial cells to cause contraction by an unidentified mechanism and on endometrial cells to stimulate PG synthesis. *Clivia* was also shown to act as a partial agonist on 5-HT_2-receptors in a similar way to ergometrine. It is possible that both herbal remedies could act via activation of ROCs, thereby increasing the influx of Ca^{2+} into the myometrial cell, but the activity of *Clivia* is less reliant on the availability of extracellular Ca^{2+} than *Agapanthus*. These results provide some evidence of efficacy for the ethnomedical use of these herbal remedies as oxytocic agents.

Aqueous extracts of *A. africanus* and *C. miniata* leaves and chemically isolated fractions of extracts of the plants were able to stimulate phosphoinositide synthesis in the rat myometrium. The effect of *Agapanthus* was much greater than the effect of *Clivia* and

this can be regarded as the signal transduction and second messenger system responsible for the uterotonic effects of *Agapanthus.* This study has shown that *Agapanthus* relies on the promotion of PG synthesis for its uterotonic activity and that *Clivia* has a similar pattern of activity to ergometrine and OT. OT, ergometrine and $PGF_{2\alpha}$ are all capable of causing hyperstimulation of the uterus with the potential of causing rupture of the uterus and foetal hypoxia. By implication therefore, decoctions of *A africanus* and *C. miniata* could also cause hyperstimulation of the uterus and traditional healers should be warned of the toxicity potential of the herbal extracts.

In conclusion therefore, this study has revealed the pharmacological activity of three plant species used as herbal oxytocic agents, namely *A. africanus, A. praecox* and *C. miniata.* The uterotonic mechanisms of action of *A. africanus* and *C. miniata* have largely been elucidated, indicating multiple actions at both receptor and second messenger level. The potential toxicity of these plants must be kept in mind when considering their use as ethnomedicines. The chemical fingerprinting of these extracts has successfully identified both similarities and differences in the plant species.

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APPENDIX A – Publication 1

THE PHARMACOLOGICAL ASSESSMENT OF HERBAL OXYTOCICS USED IN SOUTH AFRICAN TRADITIONAL MEDICINE

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The thalidomide tragedy made the medical community and the pharmaceutical industry aware and ultraconservative regarding the prescription of medication for pregnant women. Today, the use of any medication by pregnant women in first world countries is strictly monitored and regulated by health professionals and drug regulatory bodies. The oxytocic agents approved for use in obstetrics to induce or augment labour or control postpartum bleeding are: oxytocin, prostaglandin $F_2\alpha$, prostaglandin E2 and ergometrine. These drugs are all capable of causing uterine hyperstimulation in sufficiently high doses and ergometrine use is restricted to the 3rd stage of labour only due to the type of tonic uterine contracture it causes. The potential toxicity of these agents is well known and they are only administered by trained medical staff.

However, the situation in African countries is very different. In South Africa, the majority of black South Africans still patronize traditional healers and a large percentage of pregnant women still use herbal remedies during pregnancy and childbirth. A comprehensive literature survey undertaken at the initiation of this project revealed that at least 56 botanical species were used in ethnic herbal remedies taken by pregnant women (1). These traditional herbal remedies used during pregnancy and childbirth are known collectively as *isihlambezo*. *Isihlambezo* is taken as an antenatal tonic during the last trimester of pregnancy in the belief that it promotes a favourable course of pregnancy and facilitates a quick and uncomplicated labour. Many different plants can be used as isihlambezo ingredients and the recipes vary depending on factors such as the traditional healer consulted, the general state of health of the woman, the geographical area or the tribal community. The ingredients are boiled or infused in water and the "tea" is then taken by the spoonful or cupful. The concentration of the mixture may be increased at term in order to speed up labour. *Imbelekisane* and inembe are more specific remedies used as oxytocics in cases of prolonged and difficult labour and may only comprise one ingredient. Interviews conducted with traditional healers in Kwazulu Natal revealed that imbelekisane and inembe are regarded by them as dangerous medicines (2). *Isishlambezo* mixtures can be purchased dispensed from traditional healers and herbalist or "muti" shops and individual ingredients can be obtained from open herbal medicine markets throughout the country. In the rural areas, the ingredients for *isihlambezo* are often harvested from the local countryside by the senior women in the tribal community or traditional birth attendants.

The total lack of scientific evaluation of the efficacy or safety of these medicines has resulted in the condemnation of their use during pregnancy by health professionals in South Africa. Teratogenicity can be excluded here as these remedies are usually only used in the last trimester. However, the potential for maternal and foetal toxicity remains. Sixteen of the species identified to be used in these remedies are known to be poisonous, one of which, *Callilepis laureola* (Impila) is extremely poisonous and has been responsible for many fatalities due to hepato-renal failure (1). Other toxic effects that have been linked to the use of these medicines in pregnancy are: low neonatal birthweights (3), foetal meconium staining of amniotic fluid (4) (a sign of possible foetal distress and hypoxia) and fatal uterine rupture (5).

Uterine hyperstimulation can be defined as an exaggerated uterine response i.e. hypertonic or tachysystolic contractions with late foetal heart rate decelerations or foetal tachycardia (6). This can result in foetal hypoxia and uterine rupture. We

therefore elected to study the effects of certain plant extracts used as oxytocics on isolated smooth muscle, particularly the uterus, in order to determine whether uterine hyper-stimulation and its associated toxicity could result from ingestion of these herbal remedies.

The plant species most often cited as being used as ethnic herbal oxytocics (*inembe*) (1, 2) were selected for the study and this selection was confirmed by interviews with traditional healers (2). These plants are: *Clivia miniata* (umayimi), *Agapanthus africanus* and *A. praecox* (ubani), *Pentanisia prunelloides* (icishamlilo), *Gunnera perpensa* (ugobo), *Rhoicissus tridentata* (isinwazi) and *Combretum erythrophyllum* and *C. kraussii* (umdubu). The plants were collected, authenticated and voucher specimens were lodged with herbariums. Plants were then cultivated or harvested in a specific location where possible. Crude aqueous extracts of the plants were made according to the ethnic methods of preparation and were filtered, frozen and lyophilized. All the plants were first evaluated in this form as "herbal remedies" according to the WHO definition. Some species were later subjected to further chemical extraction and isolation procedures. The concentration of Ca^{--} and K^{-} ions in each extract was determined and found to be too low to cause direct smooth muscle contraction.

Pharmacological assessment of contractile activity was made using isolated uterus and ileum preparations dissected from euthanazed oestrogenized female Sprague Dawley rats (7). Isotonic contractions were measured and cumulative dose response curves were constructed. A control curve using a standard reference agonist (acetylcholine or oxytocin) was always constructed prior to a test curve.

Clivia miniata was the first plant to be evaluated and it was found that the aqueous leaf extracts were able to stimulate contractions of the uterus and ileum directly. *Clivia* augmented the initial response of the uterus to threshold doses of acetylcholine and oxytocin (7). Atropine inhibited the uterotonic activity of *Clivia* administered alone and also the augmentary effects of *Clivia* on the uterine response to acetylcholine and oxytocin, indicating an interaction with muscarinic cholinergic receptors in the uterus (fig.1). The effects of *Clivia* on prostaglandin synthesis in the uterus were tested using indomethacin as an antagonist and it was found to inhibit the direct effect of the extract alone at high concentrations. *Clivia* was also found to inhibit the uterine response to serotonin in a competitive manner.

Agapanthus africanus, *Pentanisia prunelloides*, and *Gunnera perpensa* were next screened for pharmacological activity and all proved to directly stimulate uterine contractions (fig.2) *Agapanthus* and *Pentanisia* were also able to stimulate contractions of the isolated ileum directly. *Agapanthus* was found to augment the uterine response to oxytocin by at least 30% more than the other two extracts (8) and was investigated further.

Because both *Agapanthus africanus* and *A. praecox* are cited as being used in isihlambezo, a comparison was first made between the uterotonic effects of the leaf and root extracts of both species, in order to confirm the accuracy of the ethnobotanical reports. It was found that the direct uterotonic effects of the 4 extracts were superimposable (fig.3). All the extracts augmented the initial uterine response to both acetylcholine and to oxytocin in a similar manner, with the augmentation of oxytocin's effect being greater than that of acetylcholine (fig. 4). Preliminary spectral investigations (IR and NMR) of these 4 crude extracts have confirmed these results - the leaf extracts were virtually identical, and the root extracts, although more complex, contained the same core chemical composition and structure as the leaf extracts.

Agapanthus africanus was then selected for further investigation and extracts of the leaves were subjected to more isolated organ studies. It was found that atropine inhibited the uterotonic response to *Agapanthus africanus* administered alone (fig.5) and also reduced the augmentary effects of the extract on the initial response to acetylcholine as well as causing parallel displacement of the acetylcholine curve to the right with retention of maximal effect. Atropine also inhibited the initial augmentary effect of the extract on the uterine response to oxytocin without any displacement of the curve or inhibition of maximal effect. These results indicated that *Agapanthus* interacts with cholinergic muscarinic receptors in the uterus in some way.

The effects of *Agapanthus* on prostaglandin synthesis in the rat uterus were examined by using indomethacin as an antagonist, and it was found that indomethacin inhibited the uterine response to the extract administered alone, as well as reducing the initial augmentary effect of the extract on the uterine response to acetylcholine (fig.6) and to oxytocin. This appeared to be a clear indication that the uterotonic activity of *Agapanthus africanus* is linked to the promotion of prostaglandin synthesis. More definitive studies using the isolated "stripped" myometrium model have proved that promotion of prostaglandin synthesis is an important component of the uterotonic activity of *Agapanthus*. New models are currently being developed to examine the effects of *Clivia* and *Agapanthus* on second messenger systems in myometrial cells.

Rhoicissus tridentata has also proved to possess direct uterotonic activity and is currently in the process of being comprehensively evaluated. One of the interesting results so far achieved has been the seasonal effect on the potency of uterotonic activity. It was found that tubers harvested in the wet months of Summer and Autumn were more potent than those harvested in the drier months of Winter and Spring. Because *Rhoicissus* is poisonous (1), this result has an important toxicological significance that herbalists and collectors for herbal markets may not be aware of.

Combretum erythrophyllum and *C. kraussii* crude aqueous extracts were found to be less potent uterotonic agents than the others examined. Chemical extraction and isolation procedures have, however, revealed that 3 of the 6 water soluble fractions isolated are able to augment the initial response of the uterus to acetylcholine without depression of maximal response. The other three fractions show initial

augmementation followed by depression of the maximal response. The fractions extracted in methanol and methylene choloride, however, inhibited response.

These results are a very good illustration of the complexity of action of a "phytomedicine" - combinations of active principles having both agonistic, partial agonistic and antagonistic activity. The evaluation of a herbal medicine for safety and efficacy today relies on the chemical isolation and identification of a standardized "active principle" rather than a complex of active principles i.e. conversion of the phytomedicine to a phytopharmaceutical. In many cases the search for the standardized active principle results in a loss of pharmacological activity where it might still be a safe and efficacious "phytomedicine" if evaluated as a complex.

In conclusion, therefore, we can summarize the status of our investigation as follows: All the plants investigated were able to directly stimulate uterine contraction to varying degrees. *Clivia*, *Agapanthus* and *Rhoicissus* significantly augmented the initial response of the uterus to oxytocin and were able to produce initial phasic contractions followed by tonic contractions at higher doses. Herbal remedies containing these plants must therefore be considered to have the potential to cause uterine hyperstimulation and its associated toxicity. *Clivia*, *Agapanthus* and *Rhoicissus* appear to interact with the muscarinic cholinergic system in the uterus. The uterotonic activity of *Clivia* and *Agapanthus* is linked to the promotion of prostaglandin synthesis in the whole uterus preparation. In all cases, uterotonic activity was found in the water-soluble extracts/fractions. These results therefore substantiate the ethnic use of these plants as herbal oxytocics. Although these observations cannot be directly transposed to a human clinical condition, they are nevertheless significant.

Summary

Many South African women take traditional herbal remedies during pregnancy and childbirth and at least 56 different plant species are used. Some of these plants specifically used as oxytocic remedies were assessed for uterotonic activity and the results substantiated their efficacy as herbal oxytocics in the isolated rat uterus model.

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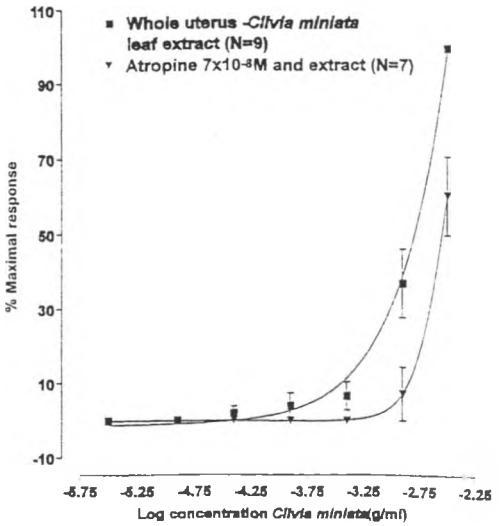


Figure 1.

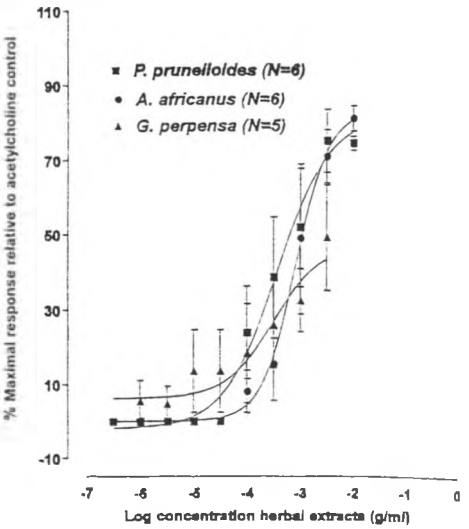


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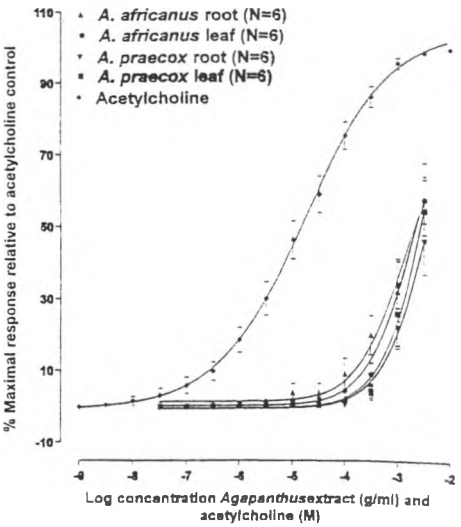


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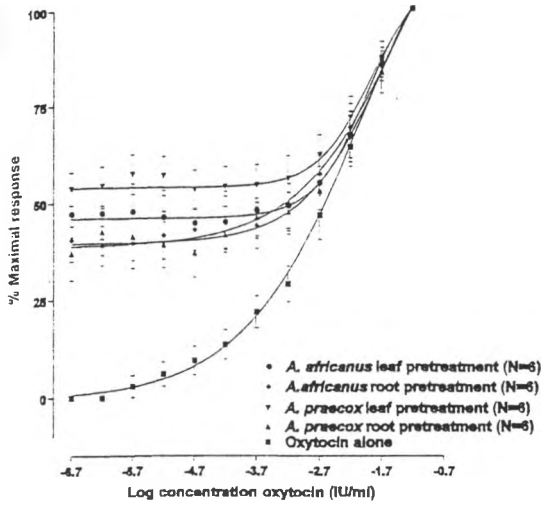


Figure 4

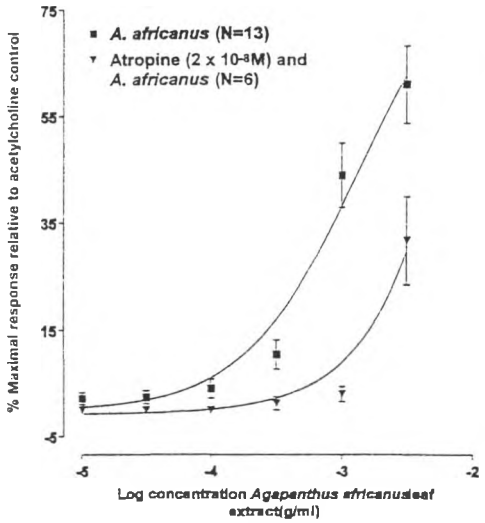


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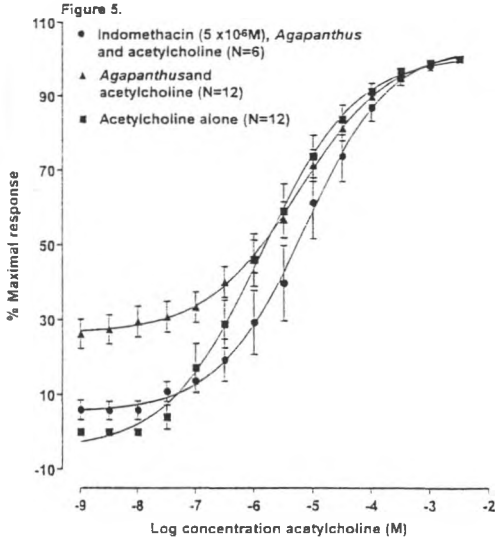


Figure 6.

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MONITORING THE SAFETY OF PHYTOTHERAPY - INTERNATIONAL PROGRESS

Ralph Edwards

The WHO Collaborating Centre for International Drug Monitoring collects information on adrverse drug reactions (ARD) from currently 50 national centres. This includes information on herbal remedies.

There are. however. problems in reporting since many ARDs are channeled through poisons information systems. Moreover. a luck of legislation controlling herbal products ingredients in many countries. plus the multiple ingredients. maker the retrieval and analysis of data difficult.

Our Centre has developed new contacts in many countries to try to improve reporting and analysis. and has set up a cooperative network to further this work. The Centre has also developed a system for classifying and handling herbal product report and their analysis.

APPENDIX B – Publication 2

ELSEVIER

Journal of Ethnopharmacology 66 (1999) 257–262

Journal of ETHNO-PHARMACOLOGY

Pharmacological effects of *Agapanthus africanus* on the isolated rat uterus

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Abstract

The *Agapanthus africanus* plant is used by South African traditional healers as a phytomedicine in herbal remedies to treat pregnancy-related ailments and to augment labour. It has already been shown that an aqueous extract of *A. africanus* causes smooth muscle contractions in the isolated uterus and ileum preparations. In the present study, the effects of an aqueous extract of *A. africanus* leaves was examined on receptor systems involved in contraction of the uterine smooth muscle in order to determine the mechanism of its pharmacological effect relevant to its ethnic use to augment labour. The extract was tested on the isolated rat uterus preparation. The aqueous extract of *A. africanus* leaves was found to exhibit agonist activity on uterine muscarinic receptors and to promote the synthesis of prostaglandins in the oestrogenized rat uterus. Some pharmacological justification for the ethnic use of *A. africanus* as a herbal oxytocic in prolonged labour has been provided. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *Agapanthus africanus*; Herbal remedies; Pregnancy; Labour; Uterotonic

1. Introduction

Agapanthus africanus (L.) Hoffmg. (Alliaceae) (*uhlakahla, ubani*) is used as a traditional medicine by South African women during pregnancy (in *isihlambezo* mixtures) and to induce or augment labour (as *inembe* or *imbelekisane*) (Veale et al., 1992; Varga and Veale, 1997). Structured open-ended ethnopharmacological interviews with 45 traditional healers in rural and urban KwaZulu Natal revealed the *Agapanthus* was one of five plants used most often to treat prolonged labour (Varga and Veale, 1997). The interviews also confirmed that *A. africanus* was frequently used to treat constipation in preg-

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nancy. These antenatal medicines are prepared as infusions or decoctions and in the Transkei, the Mpondo women grow the plant in water and drink some of the water daily from the fourth or fifth month of pregnancy (Watt and Breyer-Brandwijk, 1962; Broster, 1981). In a preliminary pharmacological screening, an aqueous extract of *A. africanus* leaves was found to stimulate smooth muscle directly and to augment the initial response of the uterus to oxytocin and acetylcholine (Kaido et al., 1997). A submaximal concentration of the extract initiated a tonic uterine response with superimposed phasic contractions. The concentration of K^+ and Ca^{2+} ions in the plant extract were determined as insufficient to cause contraction of the uterus (Kaido et al., 1997).

Oxytocin, the most potent of the endogenous uterotonic agents has a dual action in the uterus. It acts on one subtype of oxytocin receptor to cause contraction of myometrial cells and on another subtype of receptor to promote synthesis and release of prostaglandins (Chan et al., 1993). This pharmacological differentiation has been shown in both the rat uterus and pregnant human uterus. Prostaglandins are also synthesized in response to other hormonal stimuli. The rat uterus contracts in response to prostaglandin F (PGF) and prostaglandin E (PGE) (Kuriyama and Suzuki, 1976). Prostaglandins are rapidly synthesized in uterine cells from arachidonic acid via the cyclo oxygenase pathway and are then most probably released into the uterine circulation by facilitated diffusion (Smith, 1992). $PGF_{2\alpha}$ and PGE_2 promote uterine smooth muscle contraction by interaction with EP_1, EP_3 and FP receptors in the plasma membrane of humans, rats and other species. These receptors are coupled to different second messenger systems such as elevation of free intracellular Ca^{2+} (EP_1, FP) and increased phosphoinositide turnover (EP_3, FP) (Coleman et al., 1994). Indomethacin inhibits the synthesis of prostaglandins by inhibition of cyclo oxygenase and is therefore a useful tool for investigating pharmacological activity on this complex system.

The rat uterus, particularly the tubal and cervical regions, receives cholinergic innervation. Stimulation of the uterine muscarinic M_3 receptors by agonists such as acetylcholine causes contraction of the uterus and this effect is blocked by a muscarinic competitive antagonist such as atropine (Varol et al., 1989).

The present study therefore examined the uterotonic activity of the herbal extract on the isolated rat uterus with respect to its possible interactions with uterine muscarinic receptors and prostaglandin synthesis in the uterus.

2. Materials and methods

2.1. Plant material

Authenticated *A. africanus* plants were cultivated in a Lonehill, Sandton garden in order to ensure a controlled source of plant material. The plants were authenticated and voucher specimens prepared by R. Reddy (C.E. Moss Herbarium, University of the Witwatersrand-voucher no. H2H45).

2.2. Preparation of the extract

Leaves were harvested in April after good rains, thereby ensuring that the extracts made were not subject to variable seasonal effects. The leaves were washed, dried, cut into small pieces, weighed and then simmered in deionised water for 30 min. The extraction procedure therefore closely resembled the ethnic method of preparation. The aqueous filtrate was lyophilized and weighed and the extract yield was calculated relative to the wet starting material (2.9% w/w). The lyophilized crude extract was resolubilized in deionised water to the required concentrations.

2.3. Chemical instrumentation

A spectral investigation of the aqueous extract was undertaken as follows:

^{13}C NMR spectra of the extract was recorded at 30°C in D_2O at 75.462 MHz on a Varian Gemini 300 broadband NMR spectrometer, locked on deuterium and referenced to the carbonyl signal

of acetone d_6 at 206.0 ppm. The KBr disc technique was used to record infrared spectra on a Shimadzu FTIR 4200 spectrophotometer.

2.4. Animals

The pharmacological model selected was the isolated rat uterus preparation which is a well recognised model for the study of drugs exhibiting uterotonic activity. The method of Veale et al. (1989) was followed. Virgin female Sprague–Dawley rats (250 g) were injected with stilboestrol i.p. and were then euthanazed by inhalation of CO_2 24 h later.

2.5. Isolated organ preparation

The central 1.5 cm portion of each uterine horn was dissected out and opened longitudinally. The uterine strips were maintained in 50 ml organ baths containing Tyrode's solution and were aerated with 5% CO_2 in O_2. The bath temperature was maintained at 26°C to prevent development of spontaneous contractions.

2.6. Drug challenges

Isotonic contractions against a load of 1 g were recorded electronically on a Metrohm Labograph Potentiometric recorder (model E428). The organs were allowed to rest for at least 30 min between drug challenges and the organ baths were well rinsed during this period. Cumulative dose–response curves were constructed after the method of Van Rossum (1963) after a 30 min equilibration period, using acetylcholine HCl (Sigma) or oxytocin (Syntocinon, Sandoz) as standard reference agonists or *A. africanus* leaf extract. Atropine sulphate (BDH) was used as a non-specific muscarinic antagonist and indomethacin (Sigma) was used as a prostaglandin synthesis inhibitor. Initially, at least two reference control curves were constructed with a standard agonist prior to the first test curve and thereafter a single control curve was constructed before every test curve.

3. Results

3.1. Effects of atropine

Pretreatment with atropine (2×10^{-8} M) followed by cumulative addition of extract resulted in displacement of the dose-response curve to the right (Fig. 1).

Pretreatment with atropine (2×10^{-8} M) and extract (1 mg/ml) before cumulative addition of acetylcholine caused a reduction in the augmentary effect of the extract on the initial response of the organ to subthreshold doses of acetylcholine and a parallel displacement of the curve to the right with retention of maximal effect (Fig. 2).

Cumulative addition of oxytocin after pretreatment with atropine (7×10^{-8} M) and extract (1 mg/ml) resulted in a reduction in the extract's augmentary effect on the initial response of the organ to subthreshold doses of oxytocin but there was no displacement of the curve and the maximal response was retained (Fig. 3).

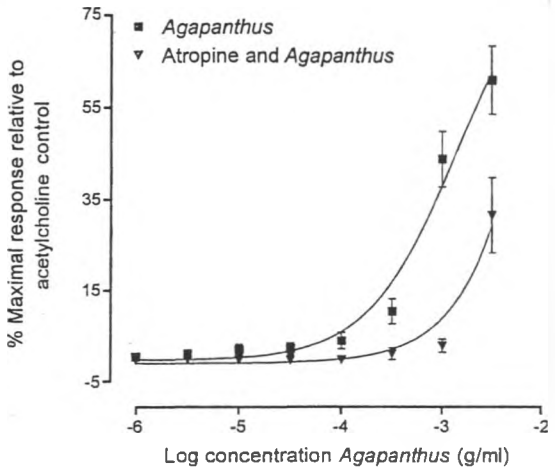


Fig. 1. Mean cumulative dose–response curves for *A. africanus* leaf extract ($n = 13$) alone and after pretreatment with atropine (2×10^{-8} M) ($n = 6$). Vertical bars represent the SEM. Response was calculated relative to the maximal response obtained in acetylcholine control curves. Atropine was administered 5 min before cumulative addition of plant extract.

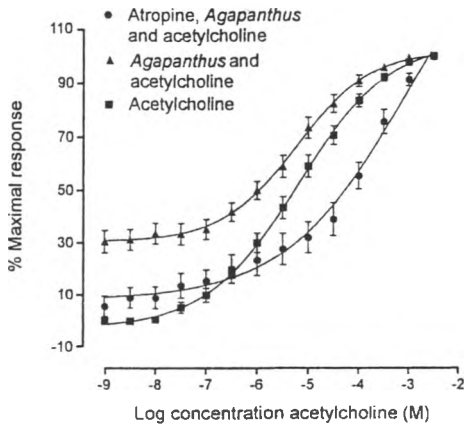


Fig. 2. Mean cumulative dose–response curves for acetylcholine alone ($n = 21$) or after pretreatment with *A. africanus* leaf extract (1 mg ml) ($n = 17$), or atropine (2×10^{-8}M) and extract ($n = 10$). Vertical bars represent the SEM. Atropine was administered 5 min before the extract and the extract was administered 5 min before cumulative addition of acetylcholine.

3.2. Effects of indomethacin

Pretreatment with indomethacin (5×10^{-6} M) before cumulative addition of the extract caused a reduction of the maximal effect (Fig. 4).

Cumulative addition of acetylcholine after pretreatment with indomethacin (5×10^{-6} M) and then extract (1 mg/ml) resulted in inhibition of the initial augmentary effect of the extract on the response to subthreshold doses of acetylcholine, there was no displacement of the curve to the right and maximal response was unaffected (Fig. 5).

Pretreatment with indomethacin (5×10^{-6} M) and then extract (1 mg/ml) followed by cumulative addition of oxytocin reduced the augmentary effect of the extract on the initial response to subthreshold doses of oxytocin but there was no displacement of the curve. Indomethacin (5×10^{-6} M) did not significantly affect the uterine response to cumulative addition of oxytocin alone (Fig. 6).

3.3. Spectral investigation

FT infrared spectrum of the *A. africanus* leaf extract: (KBr disc, main absorptions at 3400 (broad), 2926, 1744, 1645–1617 (broad), 1427 (broad), 1105–1028 (broad) and 640–535 (broad) cm^{-1}). ^{13}C NMR spectrum: (main signals in D_2O at 103.9, 96.1, 92.3, 76.1, 75.9, 74.3, 72.9, 71.7, 69.8 and 60.9 ppm)

4. Discussion

The ethnopharmacological evaluation of a traditional herbal remedy must rely on an appropriate pharmacological model. The isolated oestrogenized rat uterus preparation is a model well suited to pharmacological investigations of contractile activity in the uterine smooth muscle and is used extensively by reproductive pharmacologists. Basic initial pharmacological screening procedures which identify direct uterine smooth muscle activity in crude plant extracts need to be followed by more detailed studies which characterise the pharmacological activity of the phy-

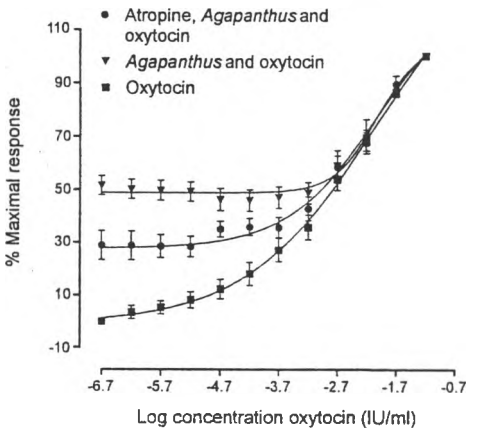


Fig. 3. Mean cumulative dose–response curves for oxytocin alone ($n = 21$) and after pretreatment with *A. africanus* leaf extract (1 mg/ml) ($n = 20$) and atropine (7×10^{-8} M) and extract ($n = 7$). Vertical bars represent the SEM. Atropine was administered 5 min before the extract and the extract was administered 5 min before cumulative addition of oxytocin.

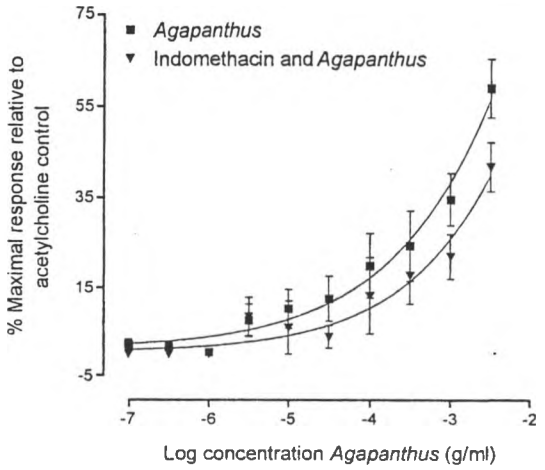


Fig. 4. Mean cumulative dose - response curves for *A. africanus* leaf extract ($n = 9$) alone and after pretreatment with indomethacin (5×10^{-6} M) ($n = 9$) 10 min before cumulative addition of plant extract. Vertical bars represent the SEM. Response was calculated relative to the maximal response obtained in acetylcholine control curves.

tomedicine as fully as possible. The results of these investigations can then be used to design and develop more specific techniques which can identify active principles extracted and isolated from the plants. As every ethnopharmacologist knows, a major disadvantage is that chemical extraction procedures, although essential in isolating active principles, often produce complex, insoluble fractions which are most often incompatible with the aqueous physiological salt solutions used in classic pharmacological testing procedures (Waller, 1993). Chemical principles so isolated are also often available for testing in very small quantities, thereby limiting the selection and extent of the pharmacological investigation even further. In this study, however, the crude aqueous extract of *A. africanus* leaves, which was evaluated as a phytomedicine, was easily resolubilised in water and truely representative of the ethnic method of preparation. Spectral investigation also revealed that the extract was in a relatively pure state.

The uterotonic effects of the crude extract of *A. africanus* were inhibited by both atropine and indomethacin. The tonic effect of a submaximal dose of the extract (1 mg/ml) was blocked by atropine but phasic contractions continued. This indicated that one or more of the active principles in the extract possessed agonistic activity on uterine muscarinic receptors which could result in the activation of receptor operated Ca^{2+} channels in smooth muscle cells.

The augmentation of the uterine response to subthreshold and threshold effects of oxytocin was much more marked than with acetylcholine. The inhibition of this effect by pretreatment with indomethacin indicates that the *A. africanus* extract may promote prostaglandin synthesis in the uterus in a similar way to oxytocin. The reason for the increased augmentative effect of the extract could be due to additive synthesis of uterotonic prostaglandins. These results illustrate the benefit of performing more detailed pharmacological studies using crude extracts prepared in the same way as used ethnomedically and can now be used as a guideline in planning more definitive pharmacological and biochemical tests.

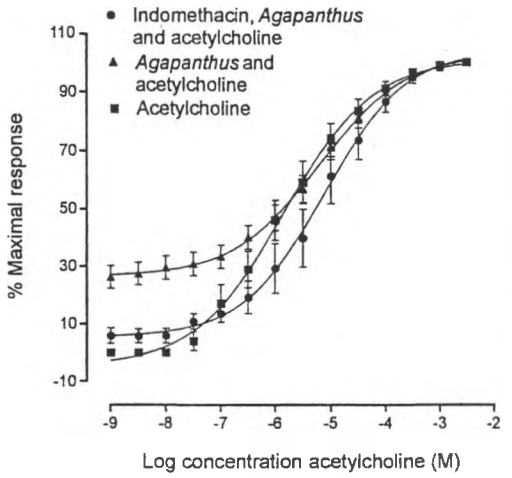


Fig. 5. Mean cumulative dose–response curves for acetylcholine alone ($n = 12$) and after pretreatment with *Agapanthus africanus* leaf extract (1 mg/ml) ($n = 12$) and indomethacin (5×10^{-6} M) and extract ($n = 6$). Vertical bars represent the SEM. Indomethacin was administered 10 min before the extract and the extract was administered 5 min before cumulative addition of acetylcholine.

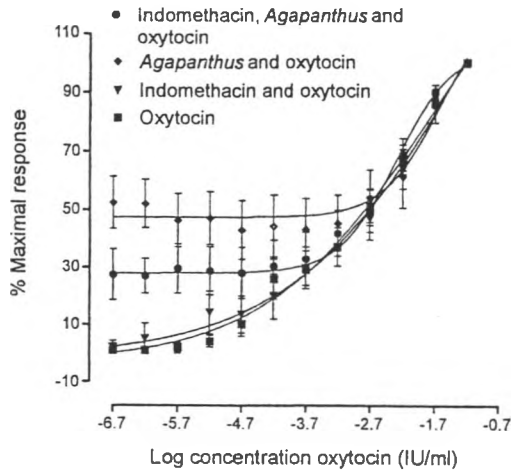


Fig. 6. Mean cumulative dose–response curves for oxytocin alone ($n = 12$) and after pretreatment with *A. africanus* leaf extract (1 mg/ml) ($n = 6$), indomethacin (5×10^{-6} M) and extract ($n = 9$) and indomethacin (5×10^{-6} M) ($n = 10$). Vertical bars represent the SEM. Indomethacin was administered 10 min before addition of extract or oxytocin. The extract was administered 5 min before cumulative addition of oxytocin.

5. Conclusion

The results of this study indicate that the crude extract of *A. africanus* leaves contained one or more active principles with agonist activity on the uterine muscarinic receptors and that the extract promoted the synthesis of prostaglandins in the uterus. This proposed mechanism of action provides further justification for the ethnic use of this phytomedicine as an oxytocic agent in the treatment of prolonged labour.

An examination of the effects of the *A. africanus* extract on second messenger systems is now being undertaken. The inhibition of the tonic effects of the extract by atropine indicate possible activity on muscarinic receptor operated Ca^{2+} channels in uterine smooth muscle cells and studies using Ca^{2+} channel antagonists are now being pursued in modified isolated uterus preparations. The effects of the extract on phosphoinositide metabolism is also under investigation. Once a more definitive pharmacological and biochemical model has been developed, the isolation and testing of active principles can be started.

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APPENDIX C – Publication 3

ELSEVIER

Life Sciences

Life Sciences 67 (2000) 1381–1388

Pharmacology letters
Accelerated communication

The effects of herbal oxytocics on the isolated "stripped" myometrium model

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Abstract

Decoctions of *Agapanthus africanus* and *Clivia miniata* are used as oxytocic agents in South African traditional herbal medicine. Aqueous extracts of *A. africanus* and *C. miniata* leaves have been shown to possess similar uterotonic activities in the isolated whole uterus preparation. The uterus however, comprises a myometrial and an endometrial layer and the activity of both oxytocin and the prostaglandins differs in these layers. The aim of this study was to determine the uterotonic activity of the herbal remedies in an endometrium-free preparation (i.e. "stripped" myometrium) and, if active, whether this effect could be related to prostaglandin synthesis or to interaction with specific receptors. The effects of the herbal extracts were tested on the isolated "stripped" rat myometrium preparation. Both herbal extracts caused a direct contractile response by the isolated tissue. Pretreatment of the myometrium with either plant extract augmented the initial response to acetylcholine. Preincubation with atropine inhibited the response to cumulative dosage of *Agapanthus* extract but had no effect on the response to *Clivia*. Indomethacin administration did not affect the response of the myometrium to cumulative dosage of acetylcholine, oxytocin or *Clivia* extract but inhibited the response to *Agapanthus* extract. These results clearly indicate that the *Agapanthus* and *Clivia* herbal extracts exhibited uterotonic activity in this model. The study illustrates that the "stripped" myometrium model has successfully differentiated between the mechanisms of action of two herbal oxytocics compared to the whole uterus preparation where their uterotonic activity was thought to be similar. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: *Agapanthus africanus*; *Clivia miniata*; Herbal remedies; Labor; Myometrium; Oxytocics; Pregnancy

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Introduction

Decoctions of *Agapanthus africanus* (uhlakahla, ubani) and *Clivia miniata* (umayime) and many other herbal remedies are used as oxytocic agents in South African traditional herbal medicine in order to induce or augment labor (1). The paucity of information on the mode of action of these phytomedicines and the potential for maternal and/or fetal toxicity is of great concern to health care professionals in this country. Aqueous extracts of *C. miniata* and *A. africanus* leaves have been shown to possess similar uterotonic activity in the rat isolated whole uterus preparation (2–4).

The mechanisms involved in the initiation of labor and uterine contraction are very complex, and are not yet fully understood. The mammalian uterus comprises an outer myometrial and an inner endometrial layer. Uterine myometrial cells are responsible for contraction of the uterus whereas the endometrial cells are secretory and non-contractile. Prostaglandins are synthesized in both tissues, but mainly in the endometrium. The endometrium is immediately adjacent to the myometrium and endometrial secretory products such as prostaglandins diffuse into the myometrial tissue without entering the vascular system (5). The myometrium is not a uniform muscle—it consists of two types of smooth muscle, circular and longitudinal, having a different origin, structure and function and the contraction patterns are different. In animals with a bipartite uterus, e.g. the rat, the outer longitudinal and inner circular muscle are more easily separated that in the human uterus (6).

Oxytocin, the most potent of the endogenous oxytocics, acts on myometrial oxytocin receptors (OT_{1a}) to directly cause uterine contraction and on endometrial oxytocin receptors (OT_{1b}) to stimulate prostaglandin synthesis and release (7). The response to oxytocin has been found to be more potent in the longitudinal layer than in the circular layer (8). The myometrium contracts in response to prostaglandin F (PGF) and prostaglandin E (PGE) stimulation. $PGF_{2\alpha}$ and PGE_2 promote uterine smooth muscle contraction by interaction with EP_1, EP_3 and FP receptors in myometrial cells of humans, rats and other species (9). The rat uterus receives cholinergic innervation and stimulation of myometrial muscarinic M_3 receptors by agonists such as acetylcholine (ACh) causes contraction of the uterus. This effect can be blocked by a non-selective muscarinic competitive antagonist such as atropine (10). Parry et al. (11) mention the possibility that *Olax gambecola* extract could release prostaglandins in smooth muscle, which through a "permissive" effect could modulate the responses of the herbal extract on muscarinic receptors. Calixto et al. (12), refer to reports of prostaglandin release in the guinea pig ileum resulting in stimulation of the release of ACh. These "permissive" effects could possibly also apply to uterine smooth muscle.

In order to evaluate the effects of the herbal oxytocics and reference agonists and antagonists on myometrium without intervention due to endometrial prostaglandin synthesis the isolated "stripped" myometrium preparation was used. The uterus was "stripped" of endometrium and circular muscle, leaving a myometrial preparation consisting mainly of longitudinal muscle (13,14). The isolated myometrium has already been established as a standard model in biochemical studies of uterine mechanisms and metabolism. The aim of this study was to determine whether the uterotonic activity of the herbal remedies was retained in an endometrium-free preparation (i.e. "stripped" myometrium) and to ascertain whether the mechanism of action could be related to prostaglandin synthesis or to possible interaction with specific receptors.

Materials and methods

Plant material and preparation of the extracts

Authenticated *Agapanthus africanus* and *Clivia miniata* plants were cultivated in a garden in Sandton (Gauteng, South Africa) in order to ensure a controlled source of plant material. The plants were authenticated and voucher specimens prepared by Ms R. Reddy (C.E. Moss Herbarium, University of the Witwatersrand—voucher nos. H2H45—*Agapanthus africanus* and J85052 *Clivia miniata*). Leaves were harvested in April and were washed, dried, cut into small pieces, weighed and then simmered in deionised water for 30 min. The extraction procedure closely resembled the ethnic method of preparation. The aqueous filtrate was lyophilized and weighed and the extract yield was calculated relative to the wet starting material (2,9% w/w *Agapanthus africanus* and 0.86% w/w *Clivia miniata*). The lyophilized crude extract was resolubilized in deionised water to the required concentrations. The herbal extracts were therefore evaluated as "phytomedicines" according to the World Health Organization definition of a herbal medicine (15).

Evaluation of pharmacological activity

Virgin female Sprague-Dawley rats (300 g) were injected with stilboestrol in arachis oil (0.1 mg kg^{-1} i.p.) and were then euthanazed by inhalation of CO_2 24 hours later. The uterine horns were dissected out and opened longitudinally. The endometrium and most of the circular muscle layer were gently stripped away (13) and the myometrial strips (15 mm long) were then placed in 50 ml jacketed organ baths filled with Tyrode solution aerated with 5% CO_2 in oxygen. The bath temperature was maintained at 26°C which inhibited spontaneous contractility in most cases (16). Isotonic contractions against a load of 1 g were recorded electronically (Metrohm Labograph Potentiometric Recorder—model E428). The myometrial strips were allowed to equilibrate for a period of about 30 min and then cumulative dose-response curves were constructed (17) at 30 min intervals using ACh as a standard agonist. When the concentration–response curve to ACh stabilized (usually after 3 consecutive curves) the plant extracts were used as test drugs, oxytocin as an agonist and indomethacin and atropine as antagonists. Administration of test drugs was only undertaken after equilibration and each subsequent test was preceded and followed by an ACh control curve. The tissues were preincubated for 5 min with the extracts and/or atropine and for 15 min with indomethacin. The tissues were allowed to rest for at least 30 minutes between drug challenges and the organ baths were well rinsed during this period. The recovery period required for the myometrial preparation to respond normally to agonist stimulation was carefully monitored using matching uterine horns as time-matched controls and a time-matched control curve was constructed and used to monitor all test and internal control curves.

Drugs

The following drugs were used: acetylcholine chloride (Sigma), oxytocin (Syntocinon, Novartis), indomethacin (Sigma), atropine sulphate (BDH) and stilboestrol (Maybaker). Indomethacin was dissolved in ethanol and stilboestrol was diluted in arachis oil.

Results

The stripped myometrium preparation was found to equilibrate within a period of 3 hours. ACh was a reliable standard agonist which did not sensitize or desensitize the isolated tissue or initiate spontaneous contractions. The preparation was capable of reproducible results for several hours after the prerequisite equilibration period.

Atropine competitively inhibited the response of the stripped myometrium preparation to ACh (Fig. 1). It caused a reversible parallel shift of the dose-response curves to the right with retention of maximum response at the lower concentration of 7nM. Atropine (70 nM) depressed the maximal response of the organ to ACh.

Both *Agapanthus* extract and *Clivia* extract exhibited direct uterotonic activity in the "stripped" myometrium and when administered cumulatively were able to initiate and maintain phasic, followed by tonic contractions (Fig. 2). Atropine (70 nM) inhibited the response of the myometrium to *Agapanthus* but did not affect the response to cumulative *Clivia* administration.

The *Agapanthus* and *Clivia* extracts augmented the initial response of the endometrium-free preparation to subthreshold and threshold doses of ACh (Fig. 3). Preincubation with indomethacin (20 μM) had no effect on the response of the preparation to ACh (Fig. 3), oxytocin or *Clivia* (Fig. 4) administered alone. Preincubation with indomethacin (20 μM) signifi-

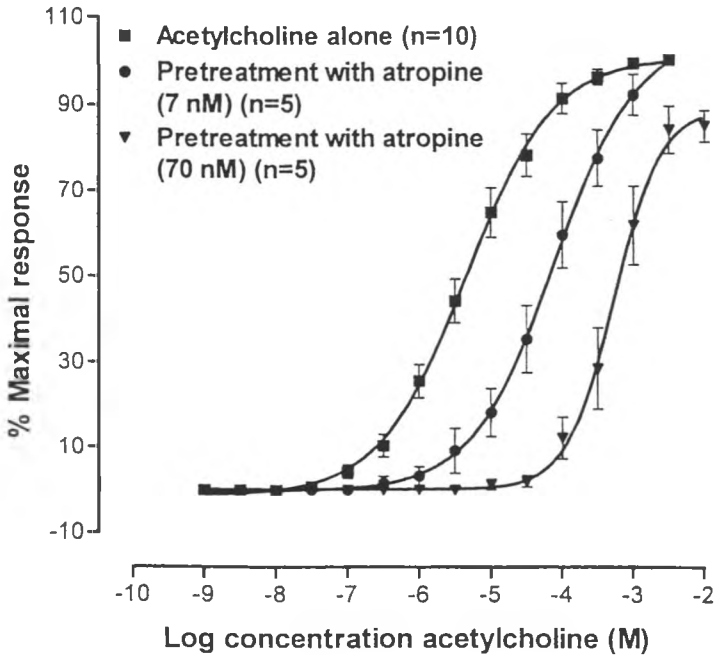


Fig. 1. Mean concentration-response curves for ACh alone and after pretreatment with atropine (7 and 70 nM) in the stripped myometrium preparation. Each point represents the mean ± S.E.M. Atropine was administered 5 min before cumulative addition of ACh.

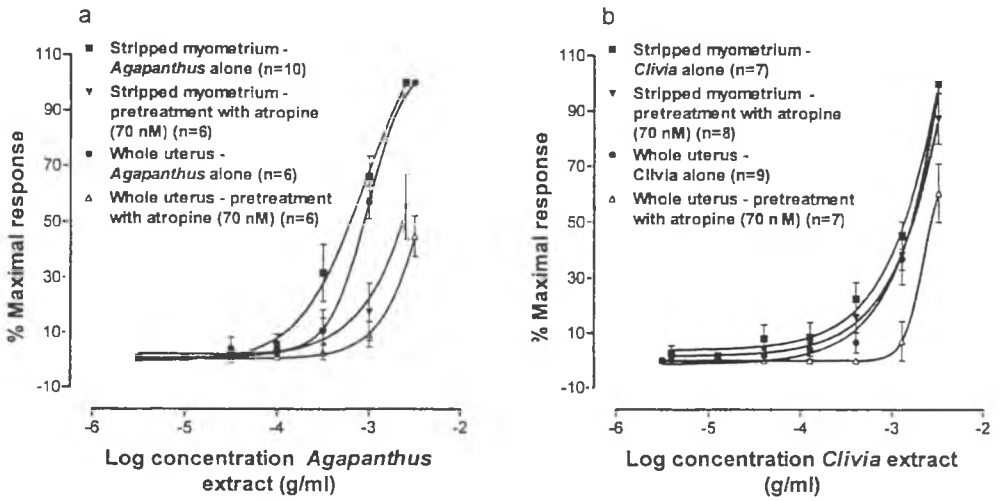


Fig. 2. Mean concentration-response curves for *Agapanthus africanus* extract (a) and for *Clivia miniata* extract (b) alone and after pretreatment with atropine (70 nM) in both the whole uterus and in the stripped myometrium preparations. Each point represents the mean ± S.E.M. Atropine was administered 5 min before cumulative addition of extract.

cantly inhibited both the augmentary effect of *Agapanthus* extract on the response to ACh (Fig. 3) and the response of the tissue to cumulative addition of *Agapanthus* extract (Fig. 4). This inhibition was reversible. Indomethacin (20 μM) administration did not affect the initial augmentary effect of *Clivia* on the reponse to ACh (Fig. 3).

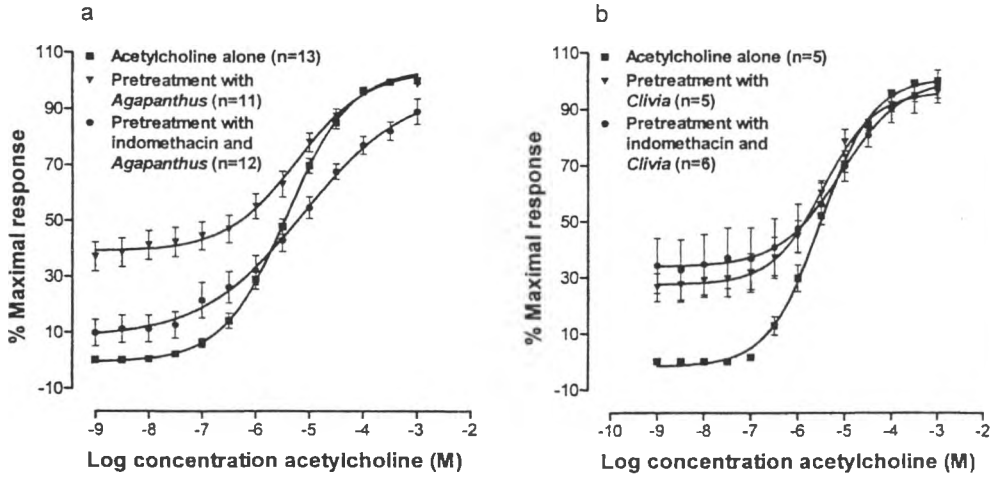


Fig. 3. Stripped myometrium - mean concentration-response curves for ACh alone and after pretreatment with (a) *Agapanthus africanus* leaf extract (1mg ml^{-1}) or (b) *Clivia miniata* extract (2 mg ml^{-1}) or indomethacin (20 μM) and/or extract. Each point represents the mean ± S.E.M. Indomethacin was administered 15 min before addition of extract and the extract was administered 5 min before cumulative addition of ACh.

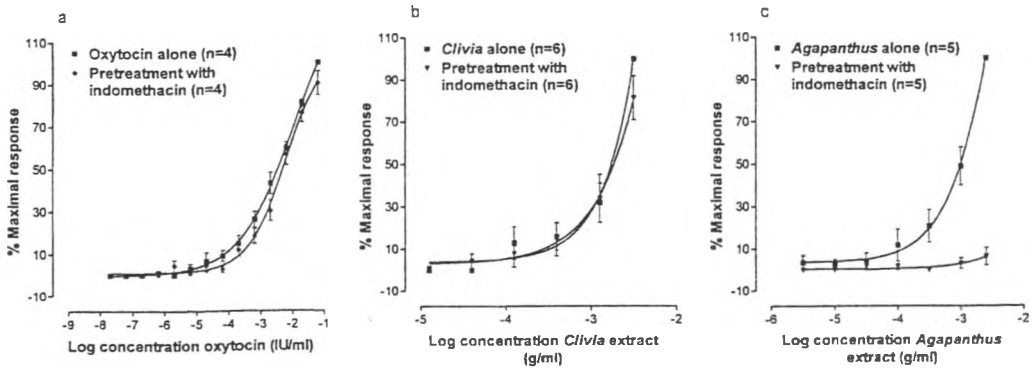


Fig. 4. Mean concentration-response curves for (a) oxytocin, (b) *Clivia miniata* or (c) *Agapanthus africanus* alone and after pretreatment with indomethacin (20 μM) in the stripped myometrium preparation. Indomethacin was administered 15 min before cumulative addition of oxytocin or extract. Each point represents the mean ± S.E.M.

Discussion

Clivia miniata (Amaryllidaceae) and *Agapanthus africanus* (Alliaceae) belong to different botanical families and could be expected to have different phytochemical profiles. The uterotonic effects of extracts of both plants on the rat isolated whole uterus however, were shown to be similar and to augment the response to oxytocin (2–4). The augmentary effects of both extracts on the initial response of the whole uterus to cumulative dosage of ACh could be reduced by pretreatment with indomethacin (5 μM), indicating that stimulation of prostaglandin synthesis could contribute to the uterotonic activity. Conversely, pretreatment with indomethacin did not significantly inhibit the augmentary effects of *Clivia* or *Agapanthus* on the response of the whole uterus to oxytocin. The activity of both extracts on the whole uterus could be inhibited by atropine (70 nM) (Fig. 2).

The results obtained in this study have shown that both *Agapanthus* and *Clivia* extracts were able to directly initiate and maintain contractions in the "stripped" preparation. The herbal extracts augmented the initial response of the endometrium-free preparation to ACh as was previously observed in the whole uterus preparation (2,4). Atropine (70 nM) pretreatment did not change the contractile response to *Clivia* extract in the myometrium but inhibited the response to *Agapanthus*. These findings suggest that muscarinic receptor stimulation is a component of *Agapanthus* action in the myometrium, but not of *Clivia* activity. This is in contrast to the previous observation that atropine inhibited the activity of both *Clivia* and *Agapanthus* in the whole uterus preparation (3,4). The apparent loss of muscarinic activity by *Clivia* in the myometrium could be due to greater sensitivity to its effects on another receptor system in the stripped myometrium preparation. This tissue consists mainly of longitudinal muscle and Tuross et al. (8) have shown that the response to oxytocin was more potent in the longitudinal layer than in the circular muscle layer. Another explanation could be that *Clivia* has an action via ACh release from the endometrium. This would be evident in the response of the whole uterus preparation and absent in the myometrial response.

Vane (18) found that indomethacin (5 μM) specifically inhibited prostaglandin synthesis in tissue homogenates. In this study, pretreatment with indomethacin (20 μM) did not affect the response of the myometrium to cumulative dosage of ACh, oxytocin or *Clivia* extract alone or the augmentary effects of *Clivia* to the response to ACh. The concentration of indomethacin was higher than used in the whole uterus studies (5 μM). In contrast, cumulative dose-response curves constructed with *Agapanthus* extract and the augmentary effects of *Agapanthus* extract on the myometrial response to ACh were significantly inhibited by preincubation with indomethacin. These findings indicate that *Agapanthus* promotes the synthesis of prostaglandins in both the myometrium and endometrium and that this plays an important role in its uterotonic activity. In the whole uterus studies, the augmentary effects of both *Agapanthus* and *Clivia* extracts on the response to oxytocin could not be effectively inhibited by indomethacin blockade (3,4). We propose that the uterus was responding to the additive effect of prostaglandin synthesis in endometrial cells due to oxytocin stimulation. This additive effect was not present when the whole uterus was pretreated with indomethacin and herbal extract prior to ACh administration, and the augmentary effects of both *Clivia* and *Agapanthus* were reduced (3,4). The marked inhibition of *Agapanthus* activity by indomethacin masked any muscarinic activity. This could possibly be a result of blockade of a "permissive" modulating effect by prostaglandins on muscarinic effects in the myometrium as referred to by Parry et al. (11) and Calixto at al. (12) in the ileum. The contractile activity of *Clivia* in "stripped" myometrium, unlike *Agapanthus*, appears independent of promotion of prostaglandin synthesis or muscarinic cholinergic stimulation. The inhibition of *Clivia* activity by indomethacin in the whole uterus indicates an indirect action via promotion of prostaglandin synthesis in the endometrium.

In conclusion, these results illustrate that the "stripped" myometrium model has successfully identified a difference in the mechanism of action of two herbal oxytocics which were thought to be similar in the whole uterus model. The study confirms that the uterotonic effects of the herbal remedies are retained in the "stripped" myometrium preparation. *Clivia miniata* appears to act on the myometrium by direct stimulation of smooth muscle contraction. *Agapanthus africanus* activity however, has been shown to be dependent on the stimulation of prostaglandin synthesis which may involve muscarinic receptor activation. This investigation has also provided valuable confirmation of the viability of the contractile response of the "stripped" myometrium preparation to standard agonists and has established critical equilibration conditions. These findings have prompted current biochemical studies.

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APPENDIX D – Publication 4

SL-15 COMPARISON BETWEEN FUNCTIONAL AND BIOCHEMICAL EFFECTS OF TWO HERBAL OXYTOCICS ON THE RAT MYOMETRIUM

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Agapanthus africanus and *Clivia miniata* are used as oxytocics in South African traditional medicine. The effects of the herbal extracts were analyzed systematically in the same preparation at the level of functional (contractile) and biochemical (second messenger generation) responses. The herbal oxytocics were compared to other uterotonic agents with regard to their ability to stimulate phosphoinositide metabolism in the rat myometrium and cause accumulation of [^{3}H]inositol phosphates (1). The maximal contractile response of the isolated rat myometrium in response to stimulation by the herbal extracts and agonists was compared to the maximal contractile response to cumulative addition of acetylcholine (2).
The rank order of intensity of stimulation of [^{3}H]inositol phosphates was: oxytocin > *Agapanthus* > $PGF_{2\alpha}$ > 5HT > acetylcholine > *Clivia* > ergometrine. This differed from the order of maximal contractile response: oxytocin > acetylcholine > $PGF_{2\alpha}$ > 5HT > *Clivia* > *Agapanthus* > ergometrine. Activity was also identified in chemical fractions of the extracts.
These results have identified that the uterotonic activity of both *Agapanthus* and *Clivia* is linked to increased turnover of phosphoinositides as a transduction mechanism. The biochemical response to *Agapanthus* was much greater than the functional response compared with *Clivia*, confirming differences in mechanisms of increasing intracellular [Ca^{++}]. The benefits of using the measurement of the stimulation of phosphoinositide metabolism as a bioassay in phytomedical research is illustrated.

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