Effects of the alkaloid present in the ethyl acetate: hexane (1:4) fraction of *Crinum macowanii* on the isolated perfused rat heart

Angela Gakii Njagi

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By

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Mini thesis submitted in partial fulfillment of the requirements for the degree of Magister Pharmaceuticae in the School of Pharmacy, Department of Pharmacology, at the University of the Western Cape.

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January 14th 2004

Dedication

To my

Parents Seraphin and Lucy Njagi

Brothers Victor and Simon

Sister Christine

Lovely God-daughters Kanana and N'kirote

My mentor Lawrence Murugu

Effects of the alkaloid present in the ethyl acetate: hexane (1:4) fraction of *Crinum macowanii* on the isolated perfused rat heart

Key Words

Traditional medicine

Cardiovascular

Heart

Medicinal herbs

Isolated perfused heart

Wister Albino rats

Plant extract

Alkaloids

In vitro

Crinum macowanii

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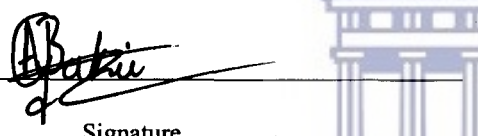
Abstract

Crinum macowanii (CM) is used in traditional medicine in the treatment of various diseases including ischemic heart disease, rheumatic fever, cancer and skin diseases. The aqueous extract of CM bulbs was found have a positive inotropic effect similar to the one of adrenaline in normotensive rats. After the extraction of CM bulbs four fractions were collected, (1:4), (2:3), (3:2) and (4:1), from ethyl actetate: hexane as an eluent. The (1:4) fraction was extracted further using PLC and the major band used for the experiments. Structure elucidation was further carried out on the major band isolated and a new alkaloid was identified from the bulbs of CM. The major aim of the study was to test the alkaloid isolated on the "double sided" working heart system. The parameters to be assessed were coronary flow (Qe), aortic output (Qa), cardiac output (CO), systolic and diastolic pressure (SP/DP), pulse pressure and heart rate (Hr). Wistar rats weighing between 250-350g were used. The hearts were isolated and perfused using Krebs Henseleit solution on the "double sided" working heart system. The parameters were monitored through a pressure transducer connected to a power lab and a computer. The Qe, Qa, CO, SP/DP, Pulse pressure and Hr reduced significantly when lycorinone (the proposed name given to the new alkaloid extracted from *Crinium macowanii*) was used at the concentrations of 0.005µg and 0.05µg. Further studies are recommended for the verification of the mechanism of action of lycorinone (negative chronotropic and negative inotropic effects).

Declaration

I declare that *Effects of the alkaloid present in the ethyl acetate: hexane (1:4) fraction of Crinum macowanii on the isolated perfused rat heart* is my work, that it has not been submitted for any degree or examination in any university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Angela Gakii Njagi



Signature

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List of abbreviations and symbols used

ATP	denosine Triphosphate
$C_{12}H_{11}N_2O_3$	gives the nuclear mass accurate to 5 decimal places.
Conc.	concentrated
CO_2	carbon dioxide
$CHCl_3$	chloroform
^{13}C nmr	^{13}C nuclear magnetic resonance
CO	cardiac output
d	doublet
DP	diastolic pressure
Et_2O	ether
EtOAc	ethyl acetate
^1H nmr	^1H nuclear magnetic resonance
HCL	hydrochloric acid
HRMS	high-resolution mass spectrum
Hr	heart rate
IR	Infra red spectrum
MgSO4	magnesium sulphate
NH_3	ammonia
O_2	oxygen
ppm	parts per million
PLC	preparative layer chromatography

RF	relative factor (the distance moved by the compound divided by the distance moved by the mobile phase)
s	singlet multiplicity of a signal.
SD	standard deviation
SP	systolic pressure
t	triplet
TLC	thin layer chromatography
q	quadruplet
Qe	coronary flow
Qa	aortic output
WJ in	water jacket in
WJ out	water jacket out
J	coupling constant and is measured in H3 (cycles per second). Gives an indication of the relative interaction between adjacent hydrogen atoms.
ν (mew)	infra spectroscopy reciprocal centimeters cm^{-1}
δ_H	chemical shift of a proton and gives a relative position of a signal to an internal standard viz $CHCl_3$
δ_C	chemical shift of a C nucleus

Chapter 1

1.1 Introduction and literature review

Traditional medicine is an integral part of South African cultural life, a position that is unlikely to change to any significant degree in the years to come. According to Zhang X. (2000), the World Health Organization (WHO) states that about 60% of the world's population depends on traditional medicine. Duncan *et al* (1999) estimate that 12 - 15 million of them live in the Southern African countries and depend on herbal medicines obtained from as many as 700 indigenous plant species. A better understanding into the mechanism of action of the traditional medicines used is imperative for the development of better, safer and more effective remedies.

There is little or no scientific evidence as to the mode of action of most of the traditional remedies currently in use. Foxglove, a medicinal herb used in the 17th century, for decades was indicated in the treatment of mental illness and other illnesses, while in actual fact it had no effect on the former. Marullaz, D. P. (2002) discusses Withering's carefully recorded clinical observations that led to clinical guidelines for the therapeutic use of foxglove. This is a clear indication of the need to scientifically identify, quantify and evaluate the active ingredients in the medicinal plants being used as alternative remedies.

In an aim to address this issue, there is an emerging growth in the field of research appertaining to traditional medicine. In Africa especially, there is a great need to evaluate these remedies since the majority of the population first consult the traditional healer. It is

when he fails that they consult a physician. Most of the people do not earn enough to enable them to buy conventional remedies. This leads to an increase in the number of people suffering from lifestyle related illnesses such as hypertension seeking the help of traditional healers who are much cheaper. The World Health Organization (WHO) and the Organization of African Unity (OAU), which later became the African Union (AU), encourage the incorporation of traditional medicines into the primary health care system (Noumi, E. *et al,* 1999). This further emphasizes the need for the benefit risk ratio to be evaluated for the different plants to be included in the treatments.

Crinum macowanii is used in traditional medicine for the treatment of ischemic heart disease, rheumatic fever, cancer and skin diseases (Elgorashi, E.E. *et al* 2001; Elgorashi, E.E. *et al* 2002; Elgorashi, E.E. *et al* 2003). Previous research carried out by Mugabo *et al* (2000) on the plant aqueous extract using the Langendorff system, showed that the plant extract has a positive inotropic effect. The study was carried out using the crude extract of the plant. The results collected left the need for further research to isolate and screen the plant for its active ingredient(s).

The current study was carried out using a specific alkaloid contained in the ethyl acetate: hexane (1:4) chromatographic fraction as specified by Nair *et al* (2000) in *Crinum macowanii*. After the extraction and fractionation, the alkaloid was used on the working heart system to test for its activity.

Chapter one will deal with the various medicinal plants that are used in traditional medicine. Special emphasis will be laid on the plant remedies that have an effect on the

cardiovascular system. This chapter shall also look at the plant, *Crinum macowanii*, and the rationale behind its selection (especially the selection of the alkaloids). Chapter two shall highlight the normal physiology of the heart with special emphasis on its relationship to the method used for the evaluation of the selected alkaloid. In particular this chapter shall relate the various functions of the heart to the "double sided" working heart system, and how it was adapted for the use from the *in vitro*.

In chapter 3 the methods used in the study shall be looked at. First the extraction, isolation and purification methods of the alkaloids shall be looked at. The various methods of structure elucidation shall be highlighted in this section. The actual method of testing the alkaloid shall be explained and the process preceding the evaluation shall be described. Chapter four illustrates and discusses the results obtained from the structure elucidation of the alkaloid under study. It also illustrates and discusses the various results that were obtained from the evaluation of the alkaloid using the "double sided" working heart system. Chapter five looks at the conclusions and recommendations from the study.

1.2 Literature review

In a bid to curb the errors that are associated with the administration of traditional medicines, there has been a surge of research in the field of traditional medicine. Research has tried to combine traditional medicine and ultramodern or conventional research techniques in order to try and validate the claims placed on traditional remedies by traditional healers to find cures for diseases that have no treatment in contemporary medicine. As a result some claims have been validated while others have been refuted. In

most instances the traditional healer uses the trial and error method of treatment and sometimes this may prove to be fatal. The conventional way of testing the drugs is to first use laboratory experiments after which there is a clinical trial period followed by the drug either being approved or rejected. This chapter shall explore the *in-vivo* type of experiments of the cardiovascular system. In addition it shall look at traditional medicinal plants and the type of research that has been carried out to validate the claims placed on them.

Various papers have been published on the research that has been carried out using traditional medicine in cardiovascular diseases. They have highlighted the concern that the traditional remedies when used concomitantly with conventional drugs have a potential to cause herb-drug interactions that may cause fatalities [Awang, D. V. C. and Fugh-Berman, A. (2002); Mahady, B. (2002); Scott, G. N. and Gary, E.W. (2002); Thor, G. and Terryberry, J. B. S. (2001); Villegas, F *et al* (2001)]. Additionally the interactions caused can either be direct whereby the prescribed drug and the herb interact in a manner that increases or decreases the pharmacological action of the pharmaceutical. In addition, effects not related to the physiological action of the pharmaceutical can occur. Indirect interaction can occur when adulterants present in the herb interact with the pharmaceutical causing untoward effects. In particular, Awang, D. V. C. and Fugh-Berman, A. (2002), Scott, G. N. and Gary, W. E. (2002) and Villegas, F *et al* (2001) discuss in detail the interactions caused by the concomitant use of herbal remedies and the contemporary drugs used in cardiovascular diseases.

Patients who are on traditional medication often do not tell their physician that they are taking other forms of remedies for the same ailments as those that they are seeking treatment for. As a result, the physician prescribes medication that could potentially interact with the remedy being taken. In some instances the physician is informed of the remedy being taken, but due to the lack of guidelines pertaining to the herbal drug being used, the physician is unable to advise the patient on the proper use of the traditional medicine.

Villegas, F *et al* (2001) discusses the use of the tetrandrine, a vasoactive alkaloid, purified from the roots of *Stephania tetrandra.* It is claimed to treat ailments such as hypertension, inflammation and angina pectoris. He further states that the therapeutic effect of this plant is due to its vasodilatory properties. The paper goes on to claim that this property is due to the blocking of the calcium–channels. Drug interactions and side effects of tetrandrine have also been reported. Due to its calcium-channel affinity, tetrandrine can potentially compete with other calcium-channel blockers. It partially inhibits the action of verapamil and completely inhibits diltiazem. Studies carried out in rats have shown that tetrandrine decreases the systolic, diastolic, and mean blood pressures. At higher doses it can cause death by cardiac suppression.

All is not doom and gloom for there are very many therapies that have been discovered from traditional remedies. For instance, *digitalis purpurea (foxglove)* has been developed for the treatment of heart failure. The effects of this drug were known since early in 1500 BC. In the 19th century it was used to treat oedema. Currently it is used to treat heart failure. It is marketed as digitalis, digitoxin and digoxin (Thor, G. and Terryberry, J. B.

S., 2001). Another example is amiodarone (Bishop's weed). It was developed from an Egyptian plant *ammi visnaga,* found in the Mediterranean and Middle East, where it was used to relive renal colic. The active ingredient, khellin, was first extracted in 1879 and its mode of action was identified as being a muscle relaxant especially for the uterine muscle. The crude extracts were found to have a vasodilatory effect on the coronary arteries. From these beginnings, amiodarone was developed in 1961 (Thor, G. and Terryberry, J. B. S., 2001). These are some examples of medicines that are in use today and that were initially traditional remedies.

In total, approximately 13,000 plant species are known to have been used as drugs throughout the world, and approximately 25% of contemporary *material medica* is derived from plants and used either as pure compounds (e.g. morphine a narcotic analgesic, codeine an anti-tussive and chemotherapeutic agents such as vincristine and vinblastine) or as teas and extracts (Kutchan M. T., *et al* 2000). Plant extracts have also served as models for modern synthetic drugs such as atropine for tropicamide, quinine for chloroquine and cocaine for procaine and tetracaine. In fact, active plant extract screening programs continue to result in new drug discoveries.

Crinum macowanii is a source for traditional remedies used in Southern African countries for the treatment of various ailments. Research using *Crinum macowanii* has varied from its anti-cancer properties to its use in the treatment of viral infections such as HIV. The main reason for choosing this plant for the current study is based on previous studies carried out using the crude extract to study its effects on the heart. The results of these studies showed that the plant extract had a positive inotropic effect (Mugabo, P., *et al,*

2000). This left a need for further research in order to identify the active components in *Crinum macowanii*.

1.3 *Crinum macowanii*

1.3.1 The plant

Crinums are large, showy plants with umbels of lily like flowers. They are found in tropical and sub-tropical regions throughout the world, where for centuries they have been used traditionally to cure ailments and disease (Fennell, C. W. and van Staden, J., 2001). They are members of the Amaryllidaceae family and are sometimes prescribed for the same medicinal purpose. *Crinum macowanii* is a species that is used to treat a variety of ailments.

Figure 1: *Crinum macowanii* plant in full bloom as adapted from Medicinal plants of Southern Africa (van Wyk, B.B., *et al* 2000)

1.3.2 General description

Crinum macowanii (Zulu-*umduze*) has a large bulb of about 200mm in diameter and has long sharp-shaped leaves radiating from it. The leaf margins are undulating and the tips end abruptly. The wide shaped flowers with black anthers are characteristic of the species (van Wyk, B.B., *et al* 2000). The plant is indigenous to Southern Africa (Elgorashi, E. E. *et al* 2001, Elgorashi, E. E. *et al* 2002, Elgorashi, E. E. *et al* 2003).

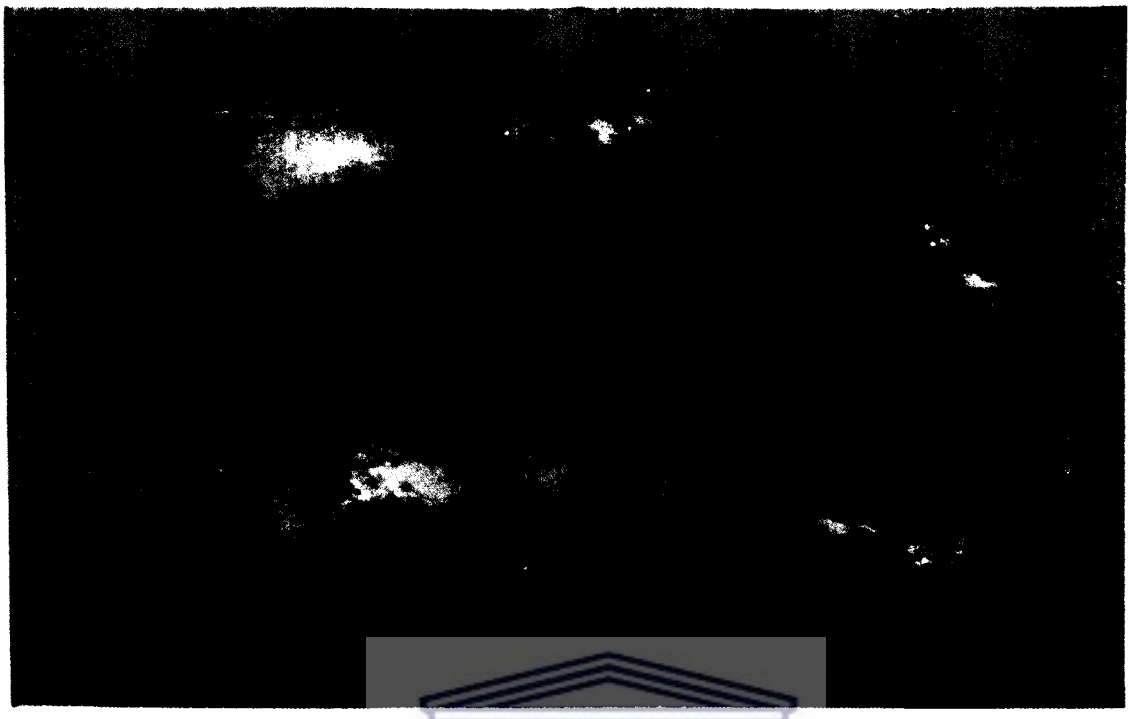


Figure 2: ***Crinum macowanii*** **bulbs as adapted from Medicinal plants of Southern African (van Wyk, B.B.,** ***et al*** **2000).**

1.3.3 Pharmacological effects

Crinum macowanii has a wide variety of uses in various parts of Southern Africa. But most significant is the finding that several of its pharmacological effects such as anti-tumor, hypotensive, analgesic activity and anti-microbial activity may be attributed to the alkaloids present (van Wyk, B.B., *et al* 2000). More recently the plants in the Amaryllidaceae family, (including *Crinum macowanii*), were reported to have pharmacological activity against HIV (van Wyk, B.B., *et al* 2000; Pham, L.H., *et al* 1998; Viladomat, F., *et al* 1996; Wu, T.S., *et al* 1996).

1.4 Alkaloids in general

Alkaloids belong to a broad category of nitrogen containing secondary metabolites. This class of molecules has historically been defined as naturally occurring substances that are not vital to the organism that produces them. Originally, alkaloids were thought to be unique to the plant kingdom; in recent times however they have been detected in some animals, e.g., in the toxic secretions of the fire ants, ladybirds and toads. Their major occurrence is still in flowering plants, and 40% of all plant families have at least one alkaloid-bearing member. Their distribution is decidedly uneven: they may be universal in some families (e.g. Papaveraceae), common in others (e.g. Amaryllidaceae, Rutaceae), and rare yet in others (Umbelliferae). They can be present throughout the plant or, alternatively, restricted to certain parts such as the root or bark. The concentration can vary from a small fraction of 0.1% to as much as 12% of the dry weight. Alkaloids have traditionally been of interest only due to their pronounced and various physiological activities in animals and humans.

Alkaloids are nitrogenous compounds that constitute pharmacologically active "basic principles" of predominantly, although not exclusively, flowering plants. Since the identification of the first alkaloid, morphine, from the poppy, *Papaver somniferum*, by Serturner in 1806, approximately 10,000 alkaloids have been isolated and their structures elucidated (Kutchan M. T., *et al* 2000). Historically, the use of alkaloid-containing plant extracts as potions, medicines, and poisons can be traced back almost to the start of civilisation. Some examples include Socrates death in 399 B.C by consumption of coniine-containing hemlock (conium *meculatum*) and Cleopatra used atropine-containing compounds to dilate her pupils and thereby look more alluring.

An interesting story is told of the discovery of the effect of adrenaline one of the most common alkaloids which is found in almost all mammals. The story goes that a certain Dr. Oliver was a practising physician who used his family for experiments. He subjected his young son to a series of experiments where he measured the diameter of the radial artery after injecting extracts from various animal glands under the skin. The experiments showed a drastic increase in the blood pressure. This led to the isolation and synthesis of adrenaline in the early 1910. From here the effects of adrenaline were recorded (Laurence, D.R., and Bennett, P.N., 1980). Adrenaline is used today in emergencies in cases of anaphylactic shock and cardiac arrest.

1.4.1 Location of alkaloids in plants

Alkaloids may occur in every part of the plant, but usually one or more parts of the plant have a higher content than others. Bark, leaves and fruits are often rich in alkaloids, which may occur in particular tissues of cells. Thus, bark (e.g. Chincona alkaloids), leaves [e.g. Datura (tropane alkaloids)], and fruits (e.g. Opium alkaloids) are typical sources. It is also important to note that the site in the plant containing the most alkaloid is not necessarily the place where the alkaloid is formed since they are end products of metabolism.

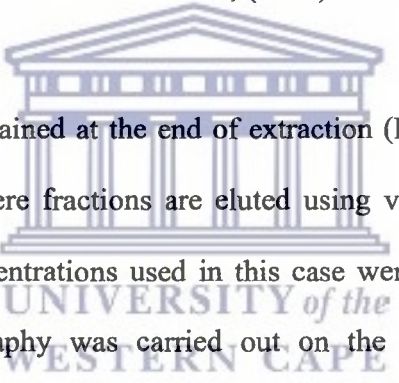
1.4.2 Role of alkaloids in plants

Little is known about alkaloid role in plants because they are secondary metabolites. Samuelson G., (1992) uses the term secondary metabolite to denote substances that are formed in plants but that do not participate in metabolic processes that are necessary for

the life and development of the plant. Most plant metabolites that are used medicinally are secondary metabolites (e.g. alkaloids).

1.4.3 Isolation of alkaloids

Alkaloids are among the natural plant products that have been studied more extensively than any other products isolated from plants because they are relatively easy to extract. The method of extraction is based on the fact that acidic alkaloid salts are readily soluble in water but not in organic solvents, whereas the reverse is true of alkaloid bases. In Figure 3 is a schematic diagram summarizing the general extraction of alkaloids according to Nair J., *et al* (2000) and Samuelson G., (1992).



The crude mixture, which is obtained at the end of extraction (Figure 3), is fractionated by column chromatography where fractions are eluted using various concentrations of ethyl acetate: hexane. The concentrations used in this case were (1:4), (2:3), (3:2) and (4:1). Thin layer chromatography was carried out on the (1:4) fraction and two compounds were observed. They were separated using preparative layer chromatography (PLC). The major pure compound was then used for the studies.

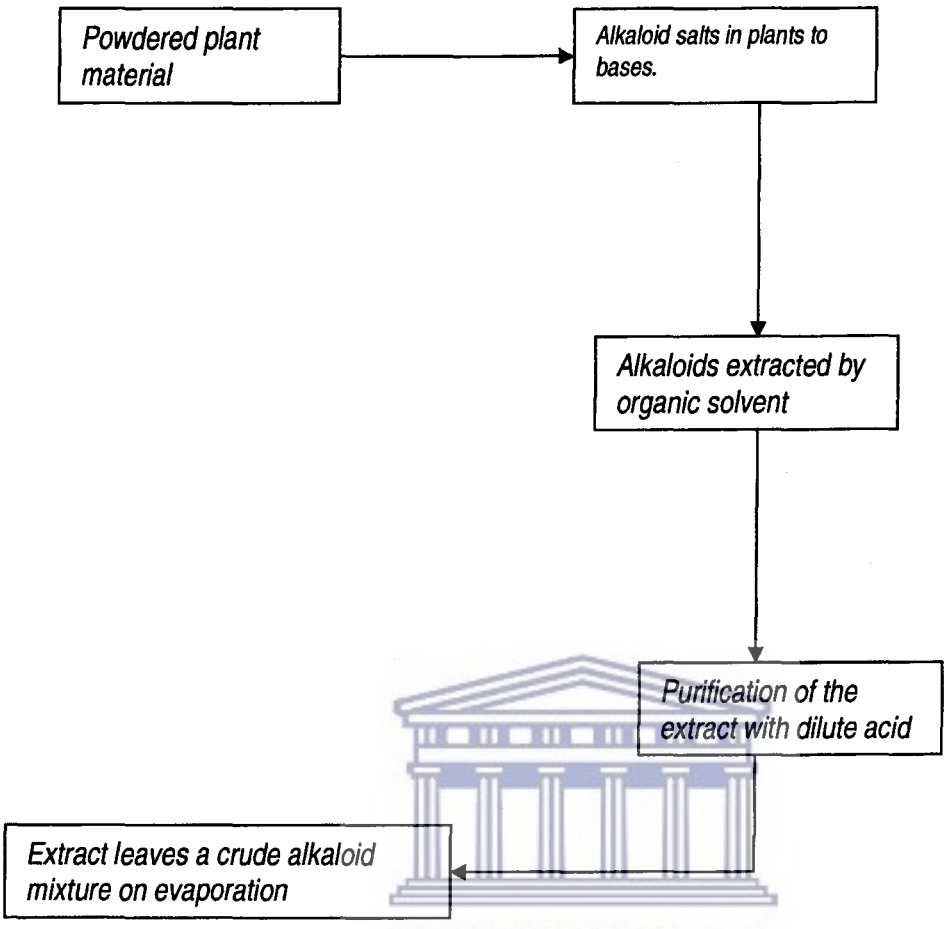
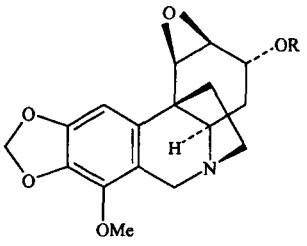


Figure 3: Schematic presentation of the general extraction process of alkaloids from the powdered plant material to the crude alkaloid mixture.

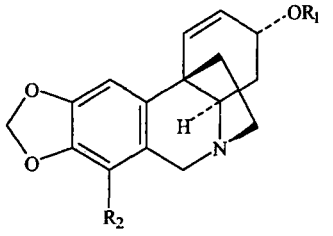
1.4.4 Alkaloids extracted from *Crinum macowanii*

There are several alkaloids that are common to the genus *Crinum*. These are lycorine, crinine and powerlline. Lycorine, crinine and powerlline were isolated by Nair *et al* (2000) together with eight other alkaloids among which a new alkaloid called macowine was reported. The alkaloids isolated were lycorine, powerlline, crinine, macowine, krepowine, buphanidrine, crinamidine, undulatine, cherylline, 4a-dehydroxycrinamabine and 1-epideacetylbowdendine. The structures of these alkaloids are as follows:



Crinamidine R=H

Undulatine R=Me

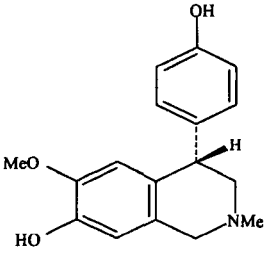


Crinine R_1=R_2=H

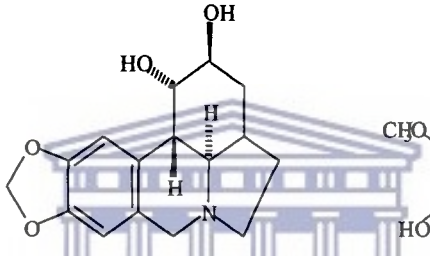
Krepowine R_1=Ac, R_2=H

Powerlline R_1=H, R_2=OMe

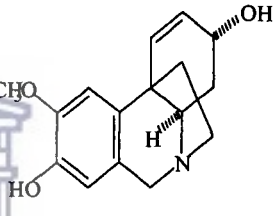
BuphanidrineR_1=Me, R_2=OMe



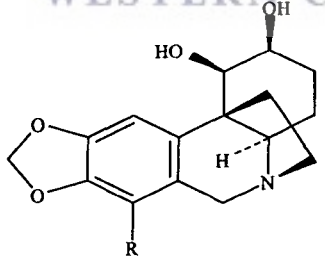
Cherylline



Lycorine



Macowine



1-Epideacetylbowdendine R=OMe

4-Dehydroxycrinamabine R=H

Figure 4: Structure of the eleven alkaloids extracted from *Crinum macowanii* by Nair *et al* (2000)

1.4.5 Classification of alkaloids

Alkaloids can be classified in a variety of ways. The classification suggested by Samuelson G., 1992, is based on several factors: a) the ring system contained in their structure, b) the alkaloids of natural origin that have an effect on the heart either as a therapeutic agent or as a side effect of the drug. For the purpose of this study the classification that will be used is the one based on alkaloids having an effect on the heart.

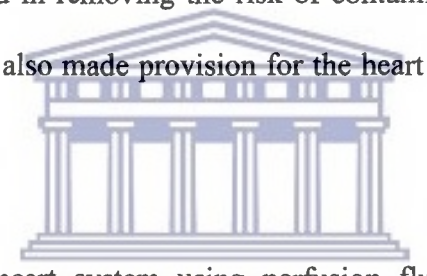
Alkaloids, generally, possess pharmacological properties of altering the function of the heart by either increasing the heart rate or decreasing it. Manske R. (1955) suggests that such a property is usually incidental to another more important pharmacological action or is but a side effect (as seen with quinine and quinidine) exhibited with high dosage. In this study only one of the alkaloids present in *Crinum macowanii* will be evaluated.

1.5 Aims and Objectives

The crude aqueous extract of *Crinum macowanii* has an effect on the isolated perfused rat heart (Mugabo,P., *et al* 2000). Almost all alkaloids known to man have an effect on the heart. The aims of this project were to extract and identify an alkaloid present in *Crinum macowanii* and to assess its effect on the isolated perfused rat heart. To realize these aims the following objectives were set:

The first objective was the identification and collection of the plant. Then the extraction and purification of the major alkaloid present in the (1:4) ethyl acetate: hexane eluent of the plant.

The next objective was the setting up of a "double sided" working heart system. Neely et al (1967) first described the use of the working heart system. This system works in a way similar to the *in-vivo* (i.e. the heart pumps the blood throughout the body) model where the left ventricle pumps the perfusion fluid throughout the system. This was the method of choice due to it being as close to the *in-vivo* environment as possible. The system described was a single sided system. This means that the system did not make allowances for the heart to be studied while the drug was being perfused. The experiments needed to study the heart during the perfusion of the drug and recovery period hence the need to add a different side that was identical to the first in order to study the heart while the drug was being perfused. This aided in removing the risk of contamination of the glassware with the drug under study and also made provision for the heart readings to be recorded throughout the experiment.



The testing of the working heart system using perfusion fluid and a known drug (adrenaline) was empirical so as to establish the absence of any errors that may arise due to various reasons. This was done using perfusion fluid and the exclusion criteria were set according to Sutherland, F. and Hearse, J., (1999).

The last objective was to test the purified compound on the heart using the working heart system.

1.6 Hypothesis

It was hypothesized that the active compound isolated from the *Crinum macowanii* bulbs had a positive inotropic effect on the isolated perfused rat heart. Based on previous studies by Mugabo *et al* (2000).

The next chapter describes the normal physiology of the heart in relation to the working heart system. In addition, the principles behind choosing this method of pharmacological testing will be highlighted.

Chapter 2

2.0 The heart and the "double sided" working heart system

2.1 Introduction

This chapter shall deal with the normal function of the heart in relation to the "double sided" working heart system. The "double sided" working heart system is set up to mimic the heart *in-situ*. The normal physiology shall be looked at in light of the parameters to be assessed and how they have been "compensated" for in the set up of the system. The "double sided" working heart system was adapted from the working heart system that was described by Neely, J.R., *et al* (1967). A need for modification of the system was realised when the system described by Neely, J. R., *et al* (1967) fell short of the requirements for the experiment. The result was an addition system of another working heart system in order to enable the heart to be observed under the working heart conditions while infusing the active substance (drug/extract). Traditionally the drug would be infused under Langendorrf. The recovery of the heart would then be recorded under working heart.

2.2 The heart

The heart as a pump is essential for the maintenance of a continuous blood supply throughout the body. The heart can be likened to two pumps in one, where the right side of the heart receives blood from the body and pumps blood to the lungs and the blood returns to the left side of the heart where the blood is pumped back to the body. The heart of a healthy 70kg male pumps approximately 5L of blood per minute, while in the average rat of about 300g the heart volume is about 1.2mls. For most people the heart

pumps for about 75 years. During exercise the amount of blood pumped by the heart increases several times above normal.

2.2.1 Function of the heart

The heart has various functions during its maintenance of homeostasis. These functions include:

1. Generating blood pressure
2. Routing blood (i.e. venous or arterial blood)
3. Ensuring one way blood flow
4. Regulating blood supply.

2.2.2 Circulation of blood through the myocardium

The heart has a coronary division that runs at an angle around the heart separating the atria from the ventricles. Two more divisions exist, that run from the first groove inferiorly, and separates the right and the left ventricles. In a healthy heart the division is covered by fat and as a result is not seen unless the fat is removed. The major arteries that supply the myocardium with blood lie within this groove on the surface of the heart. The coronary arteries originate just above the point where the aorta leaves the heart and lie within the coronary groove. Most of the myocardium receives blood from more than one arterial branch.

The two major arteries that supply the myocardium with blood are the right and left coronary arteries. They exit the aorta just above the point where the aorta leaves the heart and thus lie within the coronary sulcus. The right coronary artery is usually smaller

because it does not supply a large area of the myocardium with blood. A major branch of the left artery is the anterior intraventricular artery that supplies blood to most of the anterior parts of the heart. The left marginal artery branches form the left coronary artery and supplies the lateral wall of the left ventricle. The right coronary artery extends to the posterior part of the heart. The right marginal artery is a larger branch of the right coronary artery and supplies the lateral wall of the right ventricle. The posterior interventricular artery supplies the posterior and inferior part of the heart with blood.

Figure 5: Diagram of arteries that supply the heart. Adapted from Seeley, R.R., *et al* 2000.

The major vein that drains the myocardium on the left side is the great cardiac vein. A small cardiac vein drains the right side of the myocardium. These veins meet toward the posterior part of the coronary sinus, which in turn empties in the right atrium. In the case

of the working heart system a cut is made on the muscle of the right ventricle to drain the fluid that would otherwise accumulate in the right ventricle with nowhere to go (see section on working heart system below).

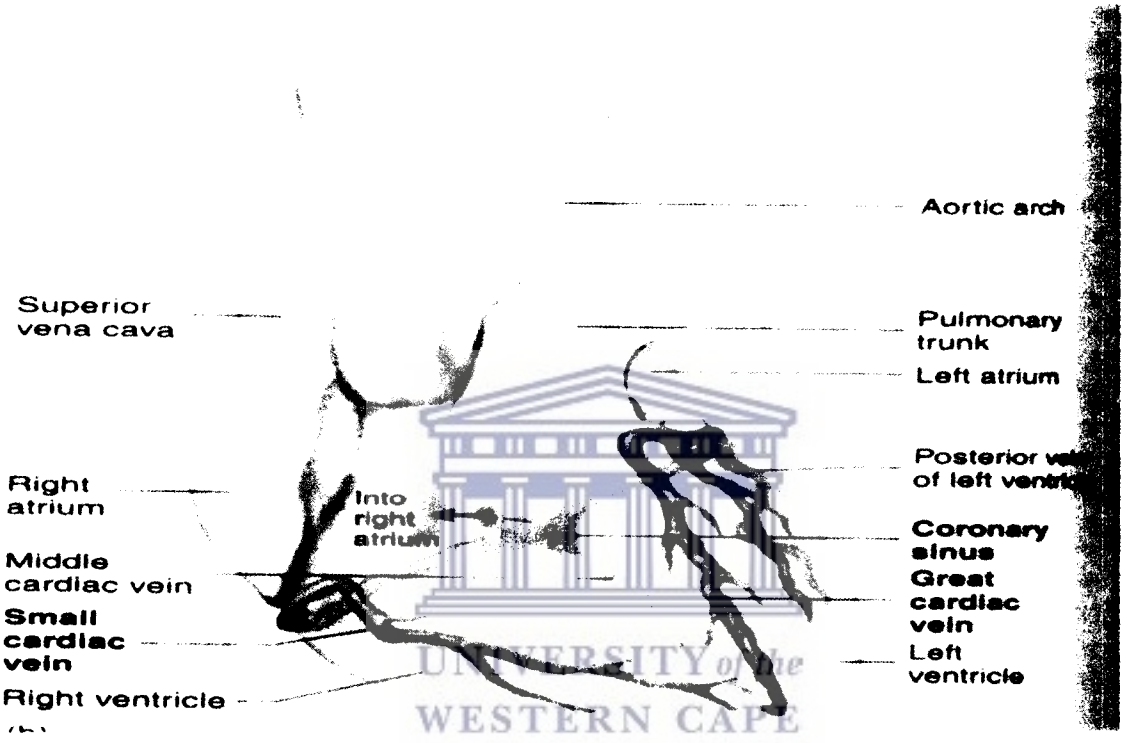


Figure 6: Diagrammatic representation of the veins draining the myocardium of deoxygenated blood (Seeley, R.R., *et al* 2000).

2.2.3 Heart chambers and valves

Right and left atria: The right atrium has three openings from the superior vena cava and the inferior vena cava and the coronary sinus. The superior and inferior vena cava receives venous blood from the body while the coronary sinus receives venous blood from the heart. The left atrium has four openings from the four pulmonary veins. These

bring arterial blood from the lungs into the atrium. The two atria are separated from each other by the interatrial septum.

Right and left ventricles: The atria open into the ventricles through the atrioventricular (AV) canals. Each ventricle has one large outflow route near the midline of the heart. The right ventricle opens into the pulmonary trunk. The left ventricle opens into the aorta. The two ventricles are separated from each other by the interventricular septum.

2.2.4 Atrioventricular and semilunar valves

Each atrioventricular canal has an atrioventricular valve (AV), which is made up of flaps. The valves allow blood to flow from the atria into the ventricles but prevent blood from flowing back into the atria. The valve between the right atrium and the right ventricle has three flaps and is called the tricuspid valve. The valve between the left atrium and left ventricle has two flaps and is called the bicuspid valve or mitral valve.

When the ventricles contract they prevent the valves from opening into the atria by pulling the valve muscles closed. Blood flow from the atrium into the ventricle pushes the valves open and blood passes through. When the ventricle contracts the blood pushes the valves back towards the atrium and the canal is closed due to the valves meeting and forming a seal.

The valve within the aorta is called the aortic semilunar valve while that in the pulmonary trunk is called the pulmonary semilunar valve. Each of the valves consists of three pocket like semilunar flaps. The three flaps meet at the centre of the artery to block the blood

flow. Blood flowing out of the ventricles forces the flaps open but when the blood flows back from the aorta into the pockets of the flaps it causes them to meet in the centre of the aorta or pulmonary trunk thus closing them and keeping blood from flowing back into the ventricles.

2.2.5 Route of blood flow through the heart

Blood flow through the heart should be discussed one side at a time. But it is important to remember that both sides of the heart contract at approximately the same time. Blood from the systemic circulation enters the heart via the superior vena cava into the right atrium. It then pushes open the tricuspid valve and enters the right ventricle during the relaxation phase of the heart. The ventricle then contracts pushing the valve closed and forcing the pulmonary semilunar valves open and blood then flows into the pulmonary trunk through the pulmonary arteries into the lungs. From the lungs the blood enters the heart through the pulmonary veins into the left atrium and the blood forces the bicuspid valve to open hence the blood then enters the left ventricle. The left ventricle then contracts and forces the aortic semilunar valves open and blood leaves the heart through the aorta into the systemic circulation.

Figure 7: Cross-section of the heart showing the blood flow through the various chambers of the heart (Seeley, R.R., *et al* 2000).

The circulatory system in the rat heart is very much the same as in most mammals and this is one of the reasons given for it being the species of choice. The working heart system is a model that is made to mimic the normal body circulation. In the case of the double-sided working heart system the aorta is cannulated onto the system and perfusion fluid leaves the heart via it to the bubble trap set at 100cm H_2O (afterload) to simulate the pressure in the normal aorta of a human which is 120 mmHg (70kg adult male) and in a 300g rat is between 116-145mmHg (Livius, V., *et al* 2000). The perfusion fluid enters the left atrium via the pulmonary vein, which is cannulated, and the bubble trap set at 15cm H_2O. This is set to simulate the 80mmHg in the average 70kg male adult while in the 300g-rat heart it simulates the 76-97mmHg (Livius, V., *et al* 2000), which is very close to that of the adult male.

2.2.6 The cardiac cycle

Cardiac cycle is a term that is used to refer to the repetitive pumping process that begins with the onset of cardiac muscle contraction and ends with the beginning of the next contraction. The changes in pressure within the various chambers of the heart are responsible for blood flow because blood moves from areas of high pressure to areas of low pressure. Duration of the cardiac cycle varies among individuals and also during various stages of an individual's lifetime. For example, the cardiac cycle of an athlete could be as long as 1 second while that of a normal individual could be approximately 0.7-0.8 seconds.

Systole means to contract while diastole means to dilate. Arterial systole refers to the contraction of the myocardium while arterial diastole refers to the relaxation of the arterial myocardium. Ventricular systole and ventricular diastole refer to the contraction and relaxation of the ventricular myocardium respectively. When the terms systole and diastole are used without reference to the specific chambers they mean ventricular systole and diastole.

Exactly what happens during the cardiac cycle?

Before a systole begins both the atria and the ventricles are relaxed and the atrio-ventricular (AV) valves are open. When the systole begins the ventricles contract and this forces the AV valves to close and blood flows into the atrium. The pressure increases as contraction continues but no blood flows into the ventricles because the mitral valve is closed. The ventricular pressure rises to exceed the pressure in the pulmonary trunk and aorta (80mmHg in human and 76-97mmHg in a 300gm rat). During the ejection period

(120mmHg in a 70kg adult male and 116-145mmHg in a 300g rat) the semilunar valves are pushed open and blood flows from the ventricles into the arteries. The ventricles relax and the diastolic period begins. The ventricular pressure drops below the pressure in the pulmonary trunk and aorta. The AV valves open and blood flows into the ventricles from the relaxed atria.

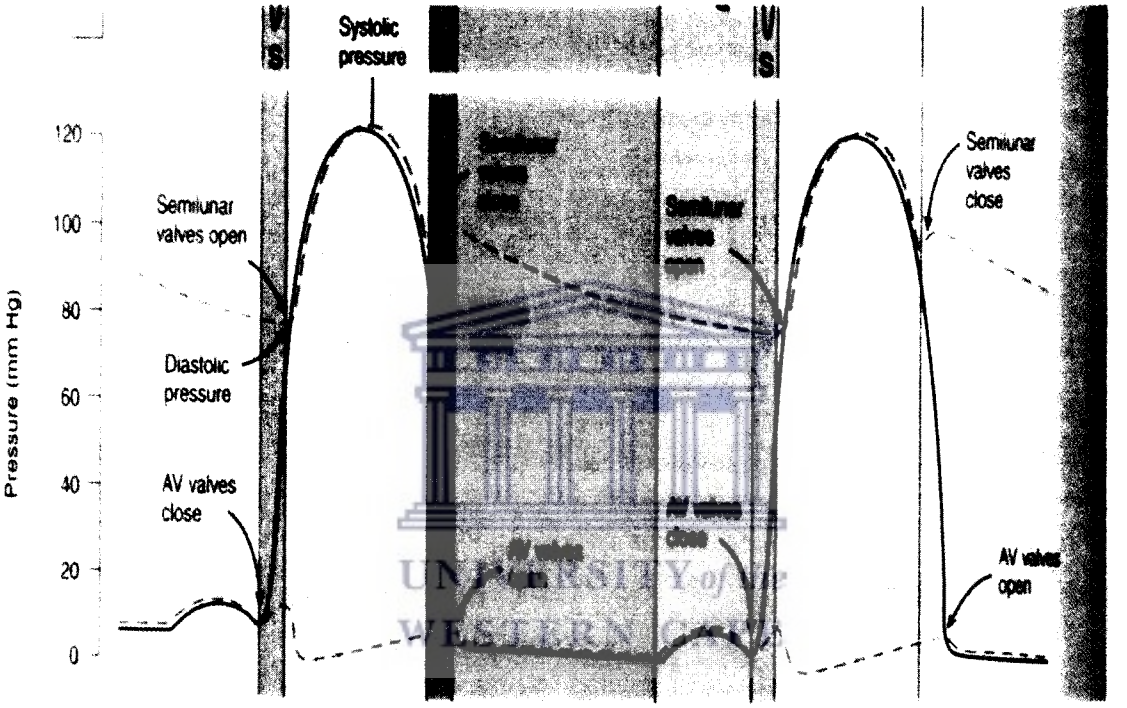


Figure 8:Illustration of what happens during the cardiac cycle. As the ventricle contracts the AV valves close and the pressure in the ventricle rises above the pressure in the aorta.

2.2.7 Aortic pressure curve

The walls of the aorta are elastic and stretch during ejection of blood from the left ventricle. The pressure in the ventricle remains higher than that in the aorta during this period. When the ventricular pressure drops the blood flows from the aorta into the ventricle due to the aorta's elasticity. The aortic semilunar valves close and the pressure within the aorta increases slightly producing a dicrotic notch in the aortic pressure curve.

Blood pressure measurements performed for clinical purposes reflect the pressure changes that occur in the aorta rather than in the left ventricle. The *aortic systolic and diastolic pressures fluctuate between 120mmHg and 80mmHg,* respectively in a 70 kg adult.

The working heart system operates on the principle described above, where the aortic pressure is measured as opposed to the left ventricular pressure. The *aortic output measured is the amount of fluid pumped by the left ventricle per minute* against the 100cm H_2O. It can be used as a measure of how efficiently the heart is working.

2.2.8 Mean arterial pressure (MAP)

This refers to the average blood pressure between systolic and diastolic pressure in the aorta. MAP is proportional to cardiac output (CO) multiplied by peripheral vascular resistance (PVR). CO can be defined as the amount of blood pumped out of the heart per minute while the peripheral resistance is described as the total resistance against which blood must be pumped. *Cardiac output is equal to heart rate multiplied by stroke volume. Heart rate being the number of times the heart beats per minute* and stroke volume is the amount of blood pumped during each heart beat (cardiac cycle).

Changes in cardiac output and peripheral vascular resistance can alter mean arterial pressure. This is a very important reference point while working with the "double sided" working heart system or the Langendorrf as an *in-vitro* preparation. What is important to remember is that in the *in-vivo* set up the veins allow for constriction or dilation depending on the pressure of the blood while in the *in-vitro* system the tubing used to

mimic the normal body vasculature is rigid and does not allow for this elasticity. Later on when the system is described in detail it will also become clear that the aorta's elasticity is 'lost' after cannulation. This is due to the aortic cannula being stiff by virtue of its metallic nature.

The elasticity of the system is allowed by the compliance chambers, which double up as bubble traps. When the system is filled up with perfusion fluid the compliance chambers are half filled to allow for the contraction/elasticity of the system. This allows for a very limited elasticity and hence the maintenance of resistance in the system similar to the *in-vivo* situation.

2.2.9 Intrinsic and extrinsic regulation

The maintenance of homeostasis is dependent on the amount of blood that is pumped by the heart and is influenced by the varying needs of the body at any given time. During exercises, for instance, cardiac output increases several times over the resting cardiac output values. Either intrinsic or extrinsic mechanisms control cardiac output. Intrinsic regulation results from the normal function characteristics of the heart and does not depend on either neuronal or hormonal regulation. It functions both *in-vivo* and *in-vitro*. On the other hand, extrinsic regulation functions only when the heart is *in-vivo* because it is governed by neuronal and hormonal control.

2.2.10 Intrinsic regulation

This is a very important aspect of the double-sided working heart system. It is due to the fact that it is an *ex-vivo* preparation and as a result it will not have the hormonal influence

experienced in the body. That is the endogenous amines will not play a role in the way the heart functions.

The amount of blood that flows into the right atrium from the veins during diastole is called venous return. The ventricular wall is stretched in comparison to the end diastolic volume. Preload is a term used to refer to the stretch in the ventricular wall. Preload is directly proportional to cardiac output. This means that an increase in cardiac output would mean that there was an increase in preload and vice versa.

The length (of the myocardium) versus tension (due to the filling and hence stretching of the myocardium) relationship exists in the myocardium. The Starlings' law of the heart is used to describe the relationship between the pumping effectiveness of the heart and the changes in preload.

Afterload is a used to refer to the pressure the left ventricle must produce to overcome the pressure in the aorta and move blood into the aorta. The pumping of the heart is not too sensitive to changes in the afterload. The aortic afterload must increase to more than 170mmHg before the pumping ability of the left ventricle can be affected.

2.2.12 Effect of oxygen and carbon dioxide

Increase in cardiac output causes an increase in blood flow through the lungs where carbon dioxide is eliminated from the body and oxygen is introduced into the blood. The reduction of carbon dioxide in the blood helps increase the pH of the blood back to its normal values.

Chemoreceptors sensitive to the blood oxygen levels are found in the carotid and aortic bodies. A dramatic reduction in blood oxygen levels activates the carotid and aortic chemoreceptors. In *in-vitro* experiments these aortic chemoreceptors do not function hence the need to keep the pH of the solution within normal range by bubbling a gaseous mixture of 95% O_2 and 5% CO_2 into the saline solution throughout the experiment.

2.2.13 Effect of temperature

Temperature plays an important role in the regulation of the heart rate. Under normal conditions the temperature of the heart does not change drastically. Small increases in the myocardial temperature cause the heart rate to increase, and a decrease in temperature has the opposite effect. In the working heart system the same principle applies where it is of paramount importance to maintain the temperature as close to normal as possible i.e. between 37.5-38.6°C. The heart as an *ex-vivo* preparation works best under these temperatures.

In setting up the system everything can be perfect, but without proper temperature monitoring it would cause the heart system to malfunction.

2.2.14 Factors affecting the coronary perfusion

Different factors affect the coronary circulation. The first and most important is the contraction of the myocardium. When the myocardium contracts, the blood vessels in the wall of the heart are compressed and as a result the blood does not flow freely through them. When the myocardium relaxes blood flow resumes. It can therefore be concluded

that coronary circulation is not continuous. Various other factors affect coronary perfusion especially when using the working heart system. They include:

a. Introduction of air bubbles in the heart due to insufficient perfusion fluid- once the air bubbles enter the ventricles they are pumped out into the arteries that supply the heart. They then circulate in the coronary arteries until they reach the microcirculatory system of the heart. Once at this point they occlude areas of the heart that are supplied by these vessels and hence ischemia results. This leads to death of the myocardium and due lack of energy and ions that are supplied by the perfusion fluid.

b. Clot formation: an occlusion in one of the arteries due to a clot formed when the heart is injured or excised without care could also result in the coronary perfusion being inhibited.

2.3 Working heart system

2.3.1 The isolated perfused heart

The working heart system is one of the two isolated perfused heart systems currently used. The isolated perfused heart is the most popular experimental model in the cardiovascular (CVS) research both in terms of quality and quantity of data it provides. It seems to be very simple, but if not well prepared it can lead to many difficulties to the researcher. Two models can be classified as the isolated perfusion models: the working heart system and the Langendorff. They both provide highly reproducible preparations that can be studied quickly and in large numbers at relatively low cost. They are ideal because they provide for the measurement of physiological, morphological and

pharmacological indices in the absence of the confounding effects of other organs, the systemic circulation and circulating neuro-hormonal factors. The heart is also denervated and as a result the vagal and sympathetic stimulation are absent in the preparation. This is an advantage in that it allows for the investigation of the heart muscle response without the influence of other neuro-transmitters in the perfusion fluid.

Despite the advantages there are a number of disadvantages that cannot be ignored since they present the researcher with challenges. The setting up of the system has to be as meticulous as possible and the measurements must be adhered to. The more experienced researcher finds it relatively easy to set up the system and adhere to specifications set for the pre-load and after-load for optimal working of the isolated heart. These settings mimic those of the *in-vitro*. The pre-load is set at 15 cm of water, while the after-load is set for 100cm of water. These two parameters are paramount for the optimal function of the isolated heart preparation. The other factors that present a problem in the setting up of the system are the cleanliness of the glassware and its maintenance. The apparatus has to be stored in 70% methanol or 4% formic acid in order to prevent bacterial growth.

The isolated perfused heart as an *ex-vivo* preparation is a constantly deteriorating preparation but is none-the-less capable of study for a few hours. The rat is the species used to supply a heart most frequently due to its availability. This species has been proven to be the most economical and surpasses other species in the ease of handling, and problems with anesthesia are few.

Of the two methods, the working heart model was the method of choice. This method has the added advantage of the researcher having a wider variety of parameters to assess and is set up to work just like the heart would work in the body, as compared to the Langendorff system whereby we have the retrograde perfusion. In addition, the Langendorff works by retrograde perfusion i.e. the ventricle does not pump the fluid as it would in the body. A balloon stretches the ventricle and hence the stretching is fixed at a constant pressure. While the working heart system works using a concept similar to the *in-vivo (i.e. the same as the way the heart would work in the body)*. The perfusion fluid enters the heart through the pulmonary vein and is pumped out through the aorta.

The biggest disadvantage of this system is that, due to the way in which it is set up, one cannot infuse two different drugs at the same time. This is because it is a continuous system, where before beginning an experiment, the system has to be filled with the perfusion fluid and the drug side intends to perfuse the drug or the sample under investigation. Once the heart is *in-situ* it is not possible to remove any fluid for risk of introducing air bubbles into the system. The air bubbles then flow into the heart and hence the pumping ability of the heart is adversely affected. It will require more force to pump the air bubble to 100cm H_2O since that is the only way the bubble will be pumped out of the system and as a result the heart tires and dies. On the other side the drug bubble may form an embolus, which can obstruct a coronary vessel. The other alternative is to introduce the sample (extract) and the control drug at the same time. The problem with this is would be the *in vitro* interaction between the two compounds. However, as far as our experiments are concerned, we did not experience any of these problems since every compound was tested alone.

2.3.2 Basic principles (set up of the system)

The main purpose of using the working heart system is to observe the way the heart behaves independent of its environment. As a result, it is of paramount importance to understand that the equipment should be up to specification. The set-up of the system should also be as meticulous as possible. This being because the heart deteriorates with time due to the fact that it is outside its natural environment.

Taking into account the fact that the heart is a continuously deteriorating organ, it is important to adhere to the measurements specified in order to optimise the conditions under which it is expected to work. For instance, setting the preload at 15-20cm of water and the afterload at 100cm of water has been shown to be the most ideal. These measurements have been shown to be as close to the physiological preload and afterload as possible. The tubing used as inlets and outlets for the perfusion fluid have also to be of a certain type. Tubing used in hospital intravenous infusion sets are the best that has been identified for this purpose. This is as a result of the width being the most idea to provide the right kind of 'vascular' resistance required within the rigid system.

Neely and Taegtmeyer first described the working heart system in the late 1960's and early 1970's. They used it to investigate the metabolic regulation of the heart and the influence of hormones on cardiac metabolism. These systems have since been used for various purposes, some of which include an investigation of the effects of various drugs in the heart. The diagram below illustrates the double-sided working heart system. The system is a modification on the one described by Neely *et al* (1967). The modification was realised when the need to observe the effect of the test drug on the myocardium,

during the administration period arose. In earlier studies, in which the Langendorff system was used, the effect of the test drug was only observed after administration, i.e. the effect during the period of administration was not recorded because there were no provisions made for it in the working heart system. In the system described by Neely *et al* (1967), the aortic pressure is measured and a reading is possible only when the heart is switched to the working heart side. To avoid contaminating the glassware with the test drug it was given using the Langendorff perfusion.

Under Langendorff the ventricle does not pump and as a result no pressure is created. The myocardium does get perfused via the coronary vessels that have their opening at the base of the aorta. Hence the need to have two identical sides in order to perfuse the drug for a given period and then give the organ time to recover while perfusing the perfusion fluid after which a second or third dose of the drug would be given (See figure 12 in the methods section).

Sutherland, F. and Hearse, J. (1999) and Neerly *et al* (1967) give very good and precise instructions how best to set up the working heart system. The double-sided system should be put up in exactly the same way. The difference being that at the aortic cannula and the pulmonary cannula there should be a three-way tap. The purpose of the tap is to allow the control of the fluid to flow from the perfusion side and the drug side. The switch from one side to the next will be dependent on the protocol of the experiment. The best protocol should be to allow the heart 10 minutes recovery time on the Langendorff and 10 minutes recovery on the 'work heart' side. The infusions protocol of the drug under investigation will depend on the researcher.

The time between infusions of the sample has been identified as 10 minutes recovery but the experimenter is as liberty to either increase or reduce the time interval as they see fit.

2.3.3 Isolated perfused model (how it works).

The working heart isolated perfused model works using anterograde perfusion. Anterograde perfusion is the perfusion of Krebs Henseleit solution passing from the left atrium to the left ventricle, which is responsible for pumping the fluid. This is referred to as the working heart because the ventricle actually performs the work of pumping the fluid. From this we get the cardiac output. The cardiac output is used as a measure of the work performed by the heart and how efficient the heart is at performing the work.

The next logical question would be: where does the heart get its energy? It is widely known that the heart can function using energy from two sources. The first being glucose and the second being triglycerides. The utilisation of glucose during glycolysis occurs under anaerobic conditions. It is responsible for 15-20% of the heart's fuel requirements. Fatty acid utilisation accounts for 60-80% of the fuel requirements. In the working heart system the fuel is supplied by the glucose in the Krebs Henseleit solution. This is because less energy is required to break down glucose to ATP.

2.3.4 Exclusion criteria for the experiments

Based on the study done by Sutherland, F. and Hearse, J., (1999), the parameters assessed for the exclusion of the experiment are listed below. The exclusion criteria are also indicated for each parameter assessed. The exclusion criteria were relevant due to the heart being *ex vivo* and it deteriorates very fast.

Coronary flow (Qe)	> 8ml/min ≤ 18ml/min
Aortic output (Qa)	≥36 ml/min

2.3.5 Perfusion solution

The Krebs Henseleit is a bicarbonate perfusion fluid. The fluid is meant to mimic the ionic content of blood or plasma and have a pH of 7.4 at 37°C. It has the following composition in mmol: sodium chloride (NaCl) 119.00; sodium bicarbonate ($NaHCO_3$) 25.00; potassium chloride (KCl) 4.75; di-hydrogen potassium phosphate (KH_2PO_4) 1.2; magnesium sulphate ($MgSO_4.7H_2O$) 0.6; sodium sulphate (Na_2SO_4) 0.6; calcium chloride ($CaCl_2.H_2O$) 1.25 and glucose 10.00. During the preparation of this solution it is important to remember that the calcium is to be added last and the pH of the solution is to be lowered by gasing with 95% O_2 and 5% CO_2. This is to reduce the risk of the calcium precipitating. Abnormally high levels of glucose are used and this is due to the inability of normal glucose levels sustaining the heart. In the normal heart, fatty acids are the most preferred source of energy. This is because for every fatty acid broken down to produce ATP 105 molecules of ATP are produced. As opposed to the 32 ATP molecules produced

per molecule of glucose. But the fatty acids are very difficult to dissolve and have a problem of foaming during bubbling and as a result glucose is preferred.

2.3.6 Species selection

In these experiments the best species for perfusion is the rat. The reason for this being the ease of handling as opposed to the mouse, which is very small and complicated to work with. The rabbit on the other hand has a big problem with anaesthesia. The rat is affordable and is easily available.

Chapter 3

3.0 Materials and methods

3.1 Materials:

All the equipment and materials used were of standard analytical grade.

3.1.1 Isolated perfused heart experiments

The equipment used in this case was:

a. Air traps Types A, B and C

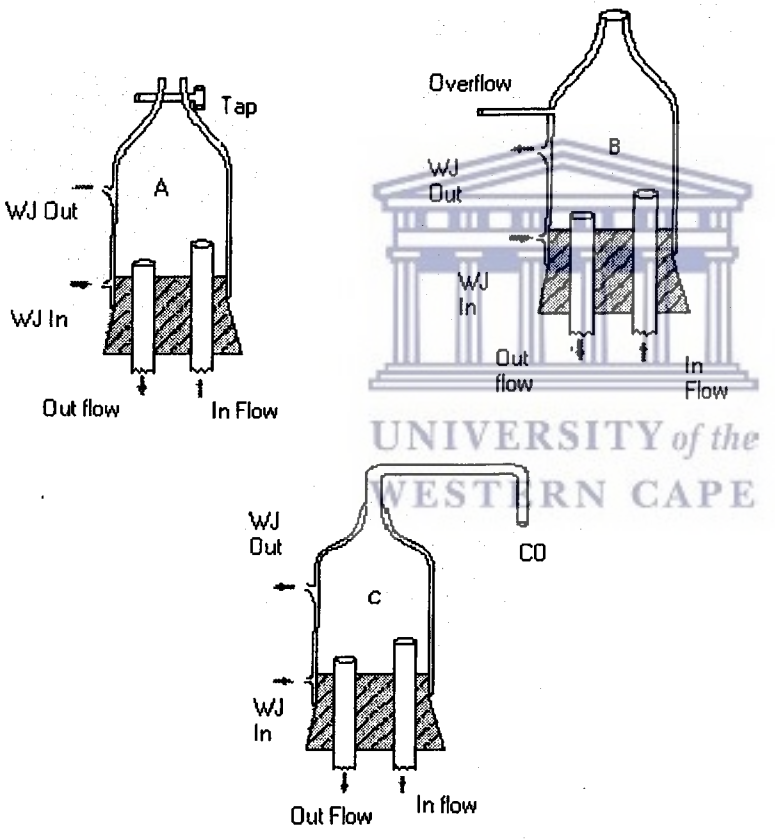


Figure 9: Diagrammatic illustration of the compliance chambers and bubble traps used. A shows the aortic compliance chamber, B represents the pulmonary vein bubble trap and C is the cardiac output compliance chamber. Inflow is the tubing that brings in the perfusion fluid to the compliance chamber while out flow drains the compliance chamber. WJ in and WJ out represent the circulation of the water jacket.

c. Type D and E glass condenser

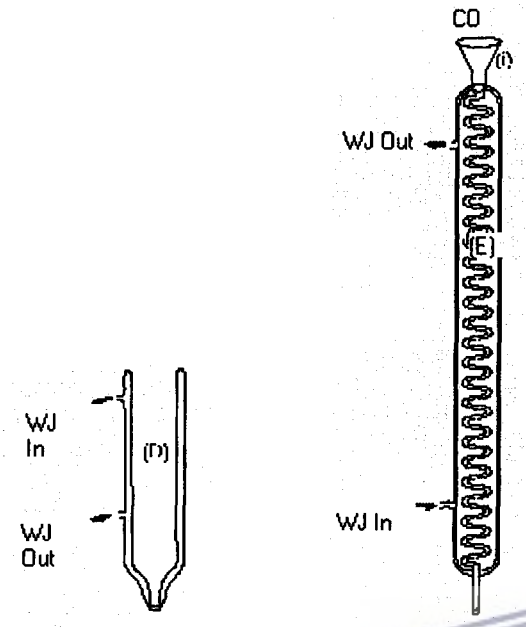


Figure 10: Illustration of the glass condenser (E) used in the working heart system to capture the cardiac output. E has a funnel at the top to help catch all perfusion fluid. D is used as a reservoir in the system. It is used as a means to warm up the perfusion fluid before it is re-circulated. This assists in maintaining the temperature within the required limits.

d. Organ chamber

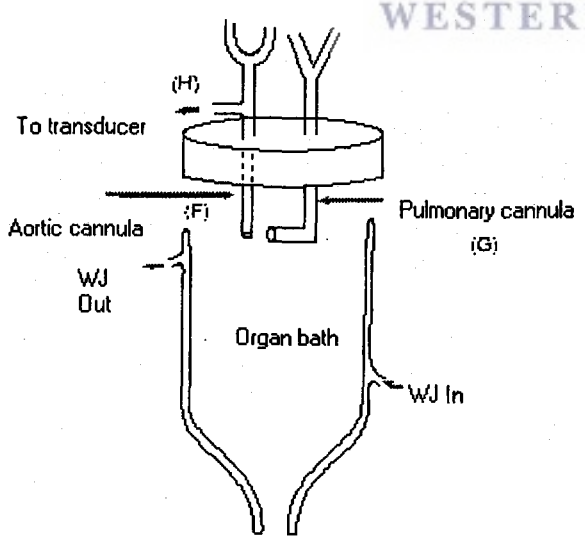


Figure 11: Chamber used to protect the heart from loss of heat during the experimentation process.

d. Blood pressure amplifier

e. PVC tubing

f. Rubber stoppers

g. Data capture analysis system Chart recorder V2 (Genotronics)

h. Circulator + water bath Thermo Haarke B3 (Lab and scientific equipment)

3.1.2 Artificial perfusion medium (Krebs-Henseleit solution)

The buffer solution has the following composition in mmol: Sodium chloride (NaCl) 119.00; Sodium bicarbonate ($NaHCO_3$) 25.00; Potassium chloride (KCl) 4.75;Di-hydrogen potassium phosphate (KH_2PO_4) 1.2; Magnesium sulphate ($MgSO_4.7H_2O$) 0.6; Sodium sulphate (Na_2SO_4) 0.6; Calcium chloride ($CaCl_2.H_2O$) 1.25 and Glucose 10.00. The chemicals used to make the buffer solution were obtained from Merck Chemicals.

3.1.3 Chemicals used for the extraction process

The chemicals used for the extraction process include: ethyl acetate (EtOAc), hexane, hydrochloric acid (HCl), chloroform ($CHCl_3$), ether (Et_2O), ammonia (NH_3), magnesium sulphate ($MgSO_4$), (these chemicals were obtained from Kimix laboratories) silica gel (Fine:35-70 mesh and Coarse:70-230 mesh) and chromatography plates TLC aluminium sheets 20x20cm (Silica gel 60 F_{254}) plates and PLC plates 20x20 (Silica gel F_{254} 1mm). The chromatography plates were from Merck

Other chemicals used include:

Pentobarbitone (Kyron Laboratories Pty Limited.)

Adrenaline (Fluka)

O_2 95%, CO_2 5% (Afrox)

3.2 Methodology

The plants were collected at Kristenbosch gardens, Cape Town, South Africa. Dr. Lyezl Van Der Walt a botanist at the gardens, authenticated the samples.

3.2.1 Extraction and isolation of alkaloids

The fresh bulbs were weighed and chopped up. The smaller pieces were then dried in an oven at 30° C for 15 days to a constant mass. The dry plant material (423.4gm) was then milled to a fine powder and soxhlet extraction of the powder using ethanol was carried out for 48 hours. Another soxhlet extraction was carried out for a further 24 hours with fresh ethanol. The extracts were combined and evaporated under reduced pressure. Water (1000ml) was added to the residue and then it was acidified using conc. HCl. The acidic phase was then extracted using $CHCl_3$. The aqueous acid solution was then basified using conc. NH_3, and the basic solution was extracted using two organic solvents viz Et_2O and $CHCl_3$. TLC was carried out on the Et_2O and the $CHCl_3$ extracts to determine which contained the alkaloids. The dried ($MgSO_4$) $CHCl_3$ extract containing the alkaloids yielded a residue (Nair, J., *et al* 2000), which was chromatographed over silica gel (70-230 mesh) and eluted with the following mobile phases:

a. EtOAc: hexane (1:4)
b. EtOAc: hexane (2:3)
c. EtOAc: hexane (3:2)
d. EtOAc: hexane (4:1)

TLC was carried out on the fractions collected to determine those that contained the same alkaloids and these were pooled and evaporated. NMR spectroscopy was employed to assist in the structural elucidation of the alkaloids.

Only the alkaloid found in the EtOAc: hexane (1:4) fraction was investigated. T.L.C. in the same solvent system indicated a major and a minor compound. P.L.C in the same solvent afforded the major compound, which was obtained as a white crystalline compound and was used in the evaluations of the *ex-vivo* heart functions as will be described.

3.2.2 Spectroscopy

In this section a very brief guideline will be given as to the spectroscopic techniques employed in order to elucidate the structure of the new alkaloid, **lycorinone.**

3.2.2.1 Infrared (IR) spectroscopy:

From the absorption bands observed in the IR spectrum, one obtains an idea of the functional groups present in the molecule, i.e. the type of reaction sites present. The position of the absorption bands measured in reciprocal centimeters i.e., cm^{-1}, and the intensity of the absorption, provide information about the functionality present in the molecule. It provides an idea of what is present in a molecule, i.e. if there is for instance an oxygen atom in the molecule that this may be an alcohol, a phenol, an aldehyde or a ketone, and if a molecule has two oxygen atoms the functionality may be a hydroxyketone, a hydroxyaldehyde, an ester or a carboxylic acid. This is due to the fact

that in IR spectroscopy, the vibrational frequencies of the bonds between atoms is measured as a function of the differences in the molecular masses and the bond strength.

The normal IR spectrum is run as a nujol mull i.e. a finely ground paste in liquid paraffin or as a solution in chloroform in a cell and has a scale starting at 4000 cm^{-1} and ending at 650 cm^{-1}. Bands in different areas of the entire spectrum provide clues as to the functional groups present. Thus an absorption band in the region of 3400 cm^{-1} indicates the presence of a hydroxyl group while a strong band in the region of 1700 cm^{-1} indicates the presence of a carbonyl group in the molecule being studied.

3.2.2.2 Nuclear magnetic resonance (NMR) spectroscopy

For the purposes of the thesis, it is sufficient to indicate that in general two nuclei are investigated since this will provide almost 90% of structural information that might be desired. The two nuclei investigated the most are Proton and Carbon-13 and are referred to as ^{1}H-nmr and ^{13}C-nmr spectroscopy. Almost all compounds derived from nature contain hydrogen and carbon and thus a study of these nuclei will assist in establishing the structures of natural products.

3.2.2.3 ^{1}H-nmr spectroscopy

In this instance the only nucleus that provides a spectrum is the proton and thus an idea about the relationship between adjacent protons and the magnetic (chemical) environment they exist in, might be deduced.

The chemical shift, viz., the position of a signal due to a particular proton relative to an internal standard indicates the nature of the proton. Thus an aromatic proton is found in the region of 6.5-8.5 ppm while that of a saturated proton occurs at 1.0-1.5 ppm i.e. in totally different regions of the spectrum scale, which is arbitrarily assigned values of 0.00-10.00 ppm. The ppm (parts per million) is the scale used in NMR spectroscopy. Thus the position of a signal, i.e. its chemical shift, indicates the nature of the magnetic (chemical) environment a particular proton occurs and thus one may assign a proton as being aromatic, olefinic, next to an oxygen, nitrogen or sulphur atom and saturated as in a primary, secondary or tertiary environment.

The nature of the signal, i.e. its multiplicity, provides information about the number of neighbouring protons. Thus, if a signal is a singlet (s), i.e. a single peak, it means there are no neighbouring protons and this gives structural information regarding this particular proton. It means one is able to associate a structural environment for this proton. If a signal is a doublet (d), i.e. a double peak, it means this proton has one neighbouring proton magnetically different to the one giving rise to the signal and thus one may then associate a certain structure to these two protons as CHCH. If the multiplicity of a signal is a triplet (t), i.e. a peak comprising three signals in the ratio 1:2:1, it means the proton being studied has two neighbouring protons different to the one in question and relates to a structure viz., $CHCH_2$.

Thus in summary at this stage, the position of the signal (chemical shift) and the multiplicity of the signal provide an indication of the nature and proton environment of the proton being studied.

The next issue to be considered is the separation between the peaks of the multiplet, which is referred to as the coupling constant and is assigned the sign *J*. The *J* values are given in Hertz (Hz), i.e. cycles per second. In essence, if a *J* value is large viz., 8-10 Hz, it means the two adjacent protons are strongly coupled and it allows one to distinguish between a cis and a trans double bond viz., cis CH=CH has *J* between 10-12 Hz while trans CH=CH has *J* between 14-19 Hz. The *J* values also allow one to distinguish between ortho, meta and para hydrogens on an aromatic ring viz *J*-ortho 6-8 Hz, *J*-meta 2-3 Hz and *J*-para 0.5-0.9 Hz. This then allows one to establish a substitution pattern on an aromatic ring as being 1,2-; 1,3- or 1,4- in the case of a di-substituted aromatic ring or 1,2,3-; 1,2,4-;1,2,5-; or 1,3,5- etc for a tri-substituted aromatic ring.

The final important point about proton N.M.R spectroscopy is the integration of the signals. The spectrometer when it integrates the area of each peak (signal) then relates this to the total number of protons in the molecule and thus one now knows exactly how many protons give rise to a particular signal. Thus, if a signal integrates for one proton then the structure must have the structural feature of a CH, while if it integrates for two protons the group must be a CH_2, and finally if the signal integrates for three protons, the group giving rise to the signal must be a CH_3.

3.2.2.4 ^{13}C-nmr spectroscopy:

In this case the carbon-13 nucleus is the only one that is observed and this provides information about the number and type of carbons the molecules contain. The way the spectrometer is set up shows each carbon atom as a singlet peak only to make the interpretation easier. The scale of ^{13}C-nmr spectroscopy ranges from 0.00-220.00 ppm

and again depending on the chemical shift of the signal, one is able to obtain structural data or confirmation about an assigned structure. Thus signals in the region of 12-35 ppm indicate saturated C atoms, a signal at 54-58 ppm indicates a C next to an oxygen, while signals in the region of 120-140 ppm indicate aromatic C atoms, while signals in the region of 190-210 ppm indicate carbonyl C atoms.

There is no integration of peaks as this is too inaccurate and neither is there any multiplicity of signals, simply single peaks and thus the positions of the signals and the number of signals provide structural information about the nature of the carbon atoms in the molecule. As a simple example, if no signals appear in the region between 14 and 60 ppm then the molecule must have all the Carbon atoms in an unsaturated structure ie aromatic, or olefinic and thus one is able to focus attention on structural types containing aromatic rings as possibilities.

3.2.2.5 High-resolution mass spectroscopy (HRMS):

The technique of mass spectroscopy provides one with information regarding the molecular ion of the molecule i.e. what the actual mass of the molecule is. This gives information as to the elemental makeup of the molecule i.e. how many carbons, hydrogens, nitrogens, oxygens etc., it contains and thus one has an idea of the molecular formula.

The usual mass spectrometers are low-resolution machines and measure the mass of molecule in whole numbers viz., one obtains a mass of 203 or 355 for the molecular ion i.e. the highest mass which relates to the molecule being studies. The molecule also gives

a fragmentation pattern which is a function of structural features in the molecule and which provides corroboration about suspected structural features one may have derived from the ^{1}H- and ^{13}C-nmr spectra.

However there is a facet about mass spectroscopy which can only be resolved by HRMS and an example will illustrate this:

The LRMS gives say a peak at m/z 26. This could be either CN or C_2H_2. The only way to differentiate between the two is to measure the mass to 5 decimal places since the isotopic abundances must also be taken into account, thus the HRMS measured for the two molecules gives the following data:

m/z for CN is 26.0031 while m/z for C_2H_2 is 26.0157. This implies that the HRMS will give an accurate and unambiguous molecular formula for an unknown molecule and this allows one to have a known starting point for a structural assignment since whatever structure one proposes, it must have all the elements and their numbers as obtained from the HRMS.

Thus for lycorinone the HRMS provided a molecular formula of $C_{16}H_9NO$ and consequently the structure assigned had to account for all these atoms.

3.2.2.6 Experimental section

Lycorinone 3: White crystals (from ethyl alcohol), m.p. 220-221°C; *v*max 3056 and 1674 cm^{-1}; δ_H 6.17 (2H, s, OCH_2O), 6.90 (1H,d, *J* 3.6, H-4), 7.48 (1H, t, *J* 7.7, H-2), 7.66 (1H, s, H-11), 7.76 (1H,d, *J* 7.7, H-3), 7.92 (1H, d, *J* 7.7,H-1), 7.98 (1H, s, H-8), and 8.05 (1H,

d, *J* 3.6, H-5); δ_C 101.8 (OCH_2O), 102.4 (C-4), 108.2 (C-11), 110.9 (C-8), 116.9 (C-3c)[a], 118.5 (C-5), 122.7 (C-3)[b], 123.7 (C-2)[b], 124.1 (C-1)[b], 125.3 (C-3a)[a], 127.0 (C-3b)[a], 128.6 (C-7a)[c], 131.8 (C-11a)[c], 148.7 (C-9), 152.7 (C-10) and 187.5 (C=0). Assignments with the same superscripts may be interchanged. HRMS: $C_{16}H_9NO_3$: requires 263.058243. Found: 263.058289.

3.3 Set up of the working heart system

Below is a diagrammatic representation of the working heart system set up as described in chapter 2.

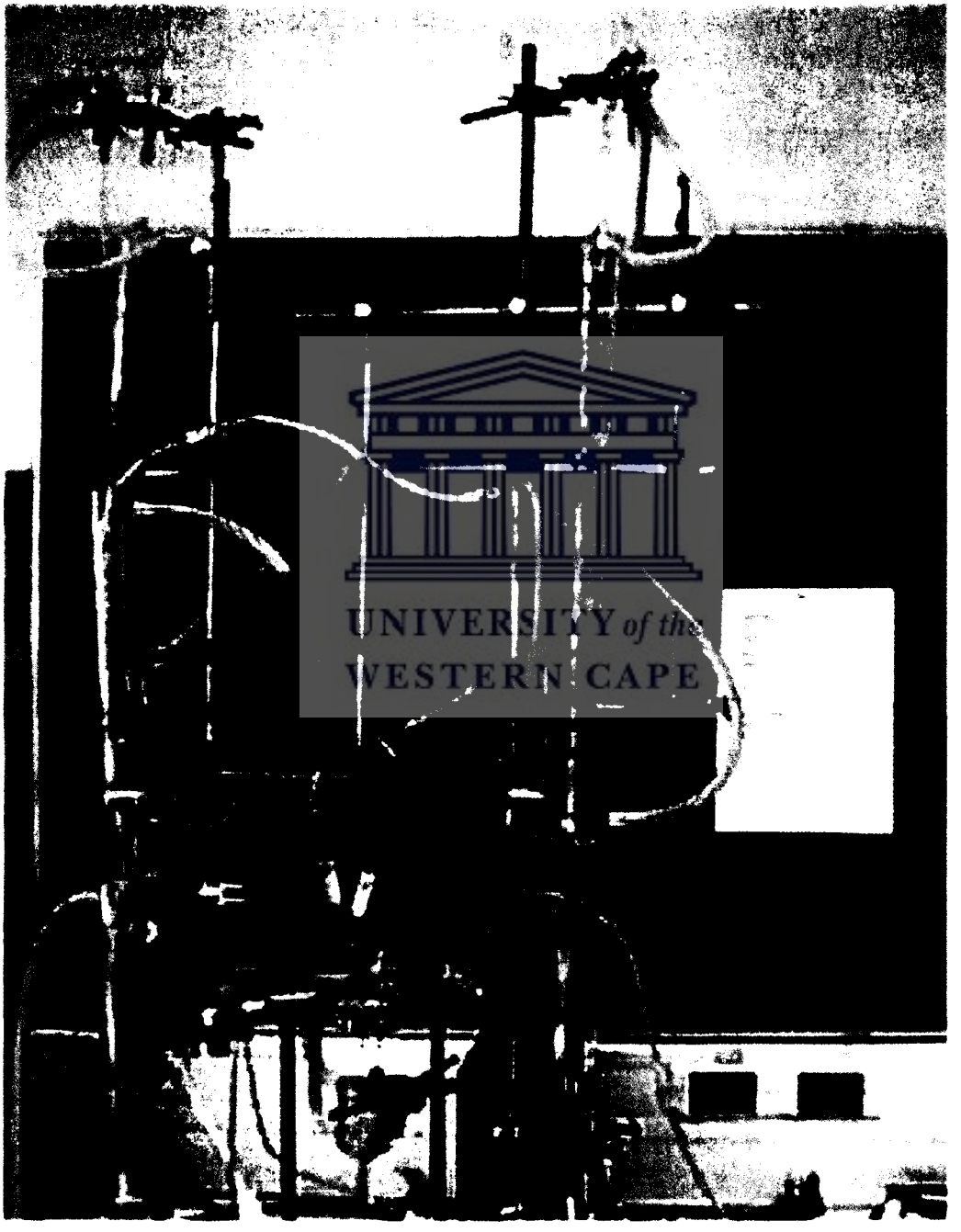


Figure 12: Diagrammatic representation of working heart system (photo by Kenechukwu Obikeze).

3.4 Animal preparation

Male Wister rats weighing between 250-350g and were less than 4 month old were used. The animals were anaesthetized using an intra-peritoneal injection of sodium pentobarbitone. The diaphragm was accessed by a trans abdominal incision and cut carefully to expose the thoracic cavity. The thorax was opened by a bilateral incision along the lower margin of the last to the first rib. The cut out section of the thoracic cage was reflected over the animals' head exposing the heart. The heart was quickly removed (less than 30 seconds) and immediately immersed in cold perfusion fluid (4°C- to reduce the risk of ischemia).

3.4.1 Cannulation and re-establishment of vascular perfusion

The aorta was eased onto the aortic cannula and secured in place using thread. Retrograde perfusion (100cm H_2O pressure) was established at this stage. Cannulation of the left atrium (through one of the pulmonary veins) with the use of the pulmonary cannula, was done and the cannula secured in place using a piece of thread. Care was taken to eliminate leaks. A pressure transducer was connected via a side arm of the aortic cannula. This transducer was connected to a computer system for recordings of the aortic pressure. A small incision was made at the base of the pulmonary artery (for temperature monitoring and to drain coronary flow) and a temperature probe was inserted into the right ventricle for continuous monitoring of the temperature.

3.4.2 Protocol of the experiments

Two protocols were used in the experimentation process. The first protocol was in the evaluation of the system and the second was during the administration of the drugs. For the evaluation of the system the following protocol was used:

LH 10min	$Wh_{perf.}$10min	Wh_{drg} 10min	$Wh_{perf.}$10min	Wh_{drg} 10min	$Wh_{perf.}$10min

Key

LH-Langendorrf (establishing reperfusion period and cannulation period)

$Wh_{perf.}$-Work heart perfusion (stabilization period)

Wh_{drg} -Work heart drug (perfusion period)

For the evaluation of the extract and the perfusion of the known drug the following protocol was used:

LH 10min	$Wh_{perf.}$10min	Wh_{drug} 5min	$Wh_{perf.}$10min	Wh_{drug} 5min	$Wh_{perf.}$10min

Key

LH-Langendorrf (establishing reperfusion period and cannulation period)

$Wh_{perf.}$-Work heart perfusion (stabilization period)

Wh_{drug} -Work heart drug (drug/sample perfusion period)

3.4.3 Parameters assessed and drugs used

The parameters that were assessed included heart rate (Hr), coronary flow (Qe), cardiac output (Co), aortic flow (Qa), systolic/diastolic pressure (SP and DP) and pulse pressure (Pu). The effects of adrenaline and an alkaloid from *Crinum macowanii* on these parameters were evaluated. Adrenaline was used because it has known effects on the

heart. It increases coronary flow, aortic output and heart rate in a dose dependent manner. It increases systolic while reducing the diastolic pressure. In higher concentrations its effects are reversed where it increases the diastolic and reduces the systolic pressure (Laurence, D.R., and Bennett, P.N., 1980; Hoffman, B.B. and Lefkowitz, R.J., 1990).

The study done by Mugabo *et al*, (2000), showed that the crude aqueous extract of *Crinum macowanii* has a positive inotropic effect similar to the one of adrenaline on the isolated rat heart perfused using the Langendorrf system. Since the study recommended further investigations to isolate and pharmacologically screen the active ingredient(s) responsible for *Crinum macowanii* effect, it is obvious that adrenaline gets compared again with the effect of the alkaloid isolated.

3.5 Data analysis

The Qe, Qa, CO, SP and DP, pulse pressure and Hr readings taken from the control hearts were pooled together and the means, of each parameter separately, analysed statistically using the Student's test. The Qe, Qa, CO, SP and DP, pulse pressure and Hr readings taken from the 'test' (those treated with the extract and those treated with adrenaline) rats were pooled and expressed as %change (± SD).

3.6 Ethical considerations

The animals were treated according to the University of Western Cape Animal Regulations Act.

Chapter 4

4.0 Results and Discussion

4.1 Introduction

The first part of the results deals with the structure elucidation of the major alkaloid present in the (1:4) ethyl acetate: hexane extract. The isolated compound has a proposed name (lycorinone). The next part deals with the results obtained after the pharmacological screening of the compound The first section of the pharmacological screening shall illustrate and discuss the result of the evaluation of the "double sided" working heart system. Both sides of the system were tested against the exclusion criteria to reduce the errors that may have arisen as a result of the modification to the system. The second section shall look at the effects of adrenaline in different concentrations on the different parameters of the heart and the effect of the alkaloid extracted on the same.

4.2 Structure of the new alkaloid

Since it is well known that lycorine is present in *Crinum* family of amaryllidaceae (Nair *et al*, 2000), it was considered reasonable that this basic skeletal structure would be a likely candidate to be found in the current study of *Crinum macowanii*. In addition, Kobayashi, S., *et al* (1984) in their thorough investigations of *Crinum macowanii* isolated 11 alkaloids one of which was lycorine together with a new alkaloid macowine.

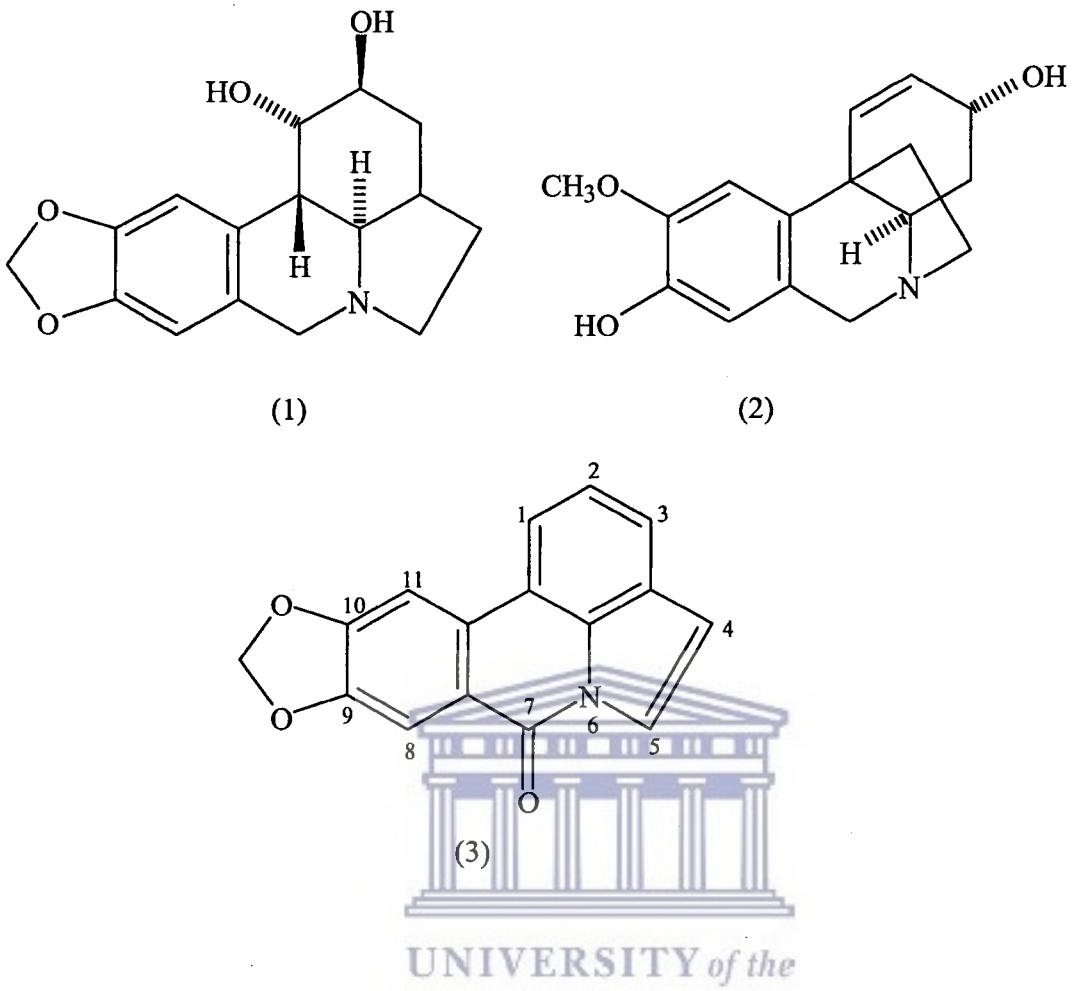


Figure 13: The structures of lycorine (1), macowine (2) and the new alkaloid lycorinone (3). The numbering of the carbons on the new compound has also been shown. The illustration aims to highlight the structural similarity of the three alkaloids.

In the current investigation, the crude extract of the bulbs of *Crinum macowanii* was eluted with a mobile phase comprising of ethyl acetate and hexane in the ratios of (1:4), (2:3), (3:2) and (4:1). Due to time constraints, only the fraction obtained from the (1:4) ratio mobile phase was investigated and the structure of the active alkaloid that was isolated is now described.

A white semi-solid material was isolated from this fraction and subjected to PLC using the same eluent as mobile phase. Two bands were observed viz., Rf 0.80 and a major band at Rf 0.45. This latter band contained a white crystalline product (from ethanol) with m.p. 220-221°C.

The High Resolution Mass Spectrum (figure 15) indicated that the molecular formula for the alkaloids was $C_{16}H_9NO_3$ (Requires: 263.058243. Found: 263.058289). An infrared (figure 14) spectrum run in dichloromethane showed strong bands at 3056 cm^{-1} for an aryl C-H stretching frequency and 1675 cm^{-1} for a carbonyl of a δ (delta) lactam ring.

The ^{1}H-n.m.r. spectrum displayed 7 protons (Figure 16) in the aromatic region with a methylene dioxy group at 6.17 ppm. From the splitting patterns and coupling constants as well as the ^{13}C-n.m.r. spectrum (Figure 18 and 19), the structure of this new alkaloid has been assigned as 3 and due to its skeletal similarity to lycorine the name lycorinone is suggested for the new alkaloid found in the bulbs of this plant.

Rationalization of the assigned structure of lycorinone to the new alkaloid and the ^{1}H-n.m.r. (Figure 16) spectrum is as follows. A 2-proton singlet at 6.17 ppm is assigned to the methylene dioxy group attached to C-9 and C-10; a 1-proton doublet at 6.90 ppm (*J* 3.6) and the corresponding 1-proton doublet at 8.05 ppm (*J* 3.6) are assigned to H-4 and H-5, respectively, with H-5 being more de-shielded due to its proximity to the anisotropic magnetic field of the C-7 carbonyl group; two 1-proton singlets at 7.66 and 7.98 ppm are assigned to H-11 and H-8, respectively, with H-8 being more deshielded due to its proximity to the C-7 carbonyl group; a 1 proton doublet at 7.76 (*J* 7.7) is assigned to H-3

while a 1-proton doublet at 7.92 (*J* 7.7) is assigned to H-1, the latter being more desheilded due to its proximity to the angular aryl ring, and finally a triplet at 7.48 ppm (*J* 7.7) assigned to H-2. COSY (figure 17) spectroscopy confirmed the connectivity patterns.

In the ^{13}C-n.m.r (figure18 and 19) spectrum the lactum C=O signal appeared at 187.5 ppm while the methylene dioxyl carbon appeared at 101.8 ppm. Signals due to C-9 and C-10 appeared at 148.7 and 152.7 ppm respectively as expected.

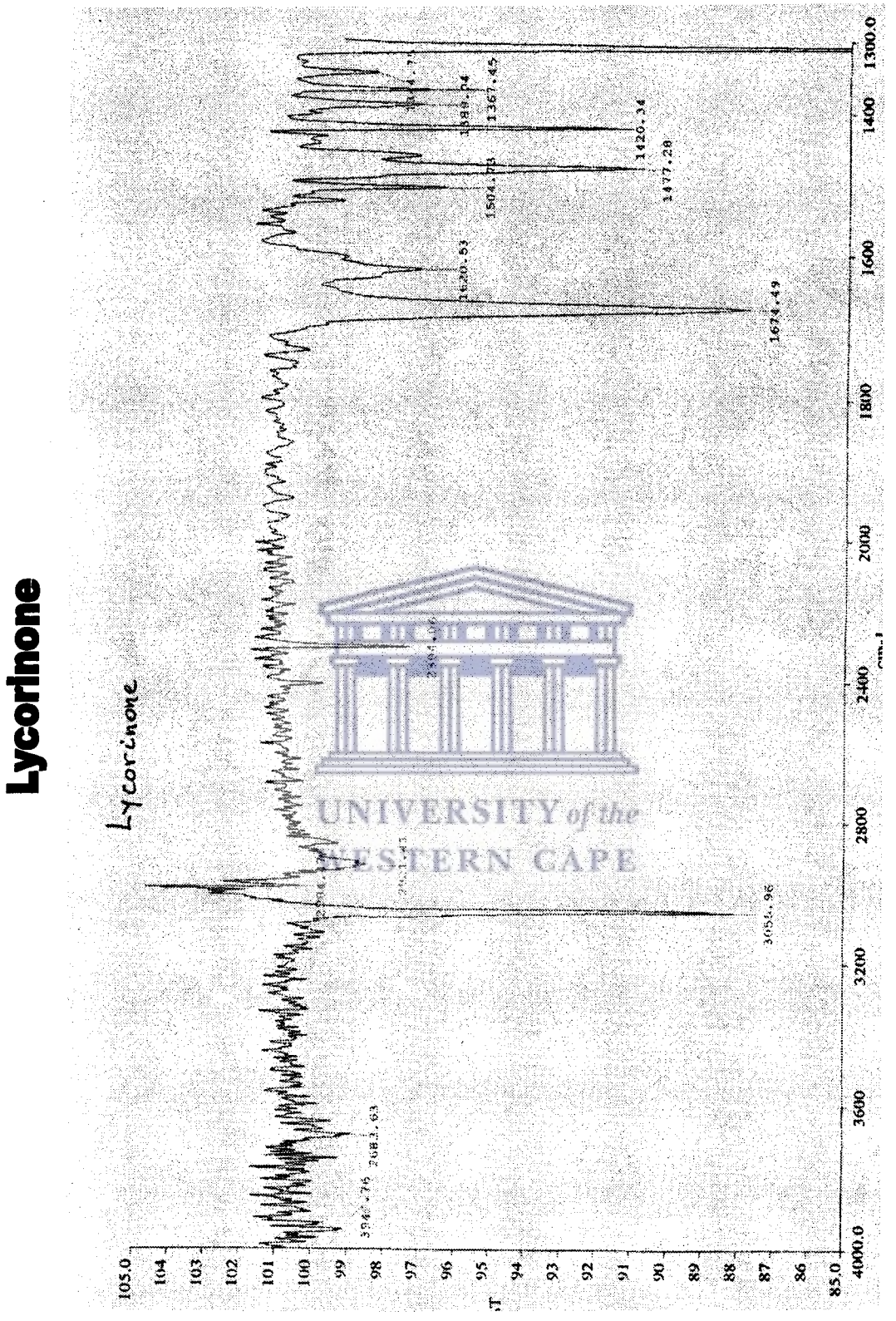


Figure 14: Infra red Spectrum of the new alkaloid lycorinone.

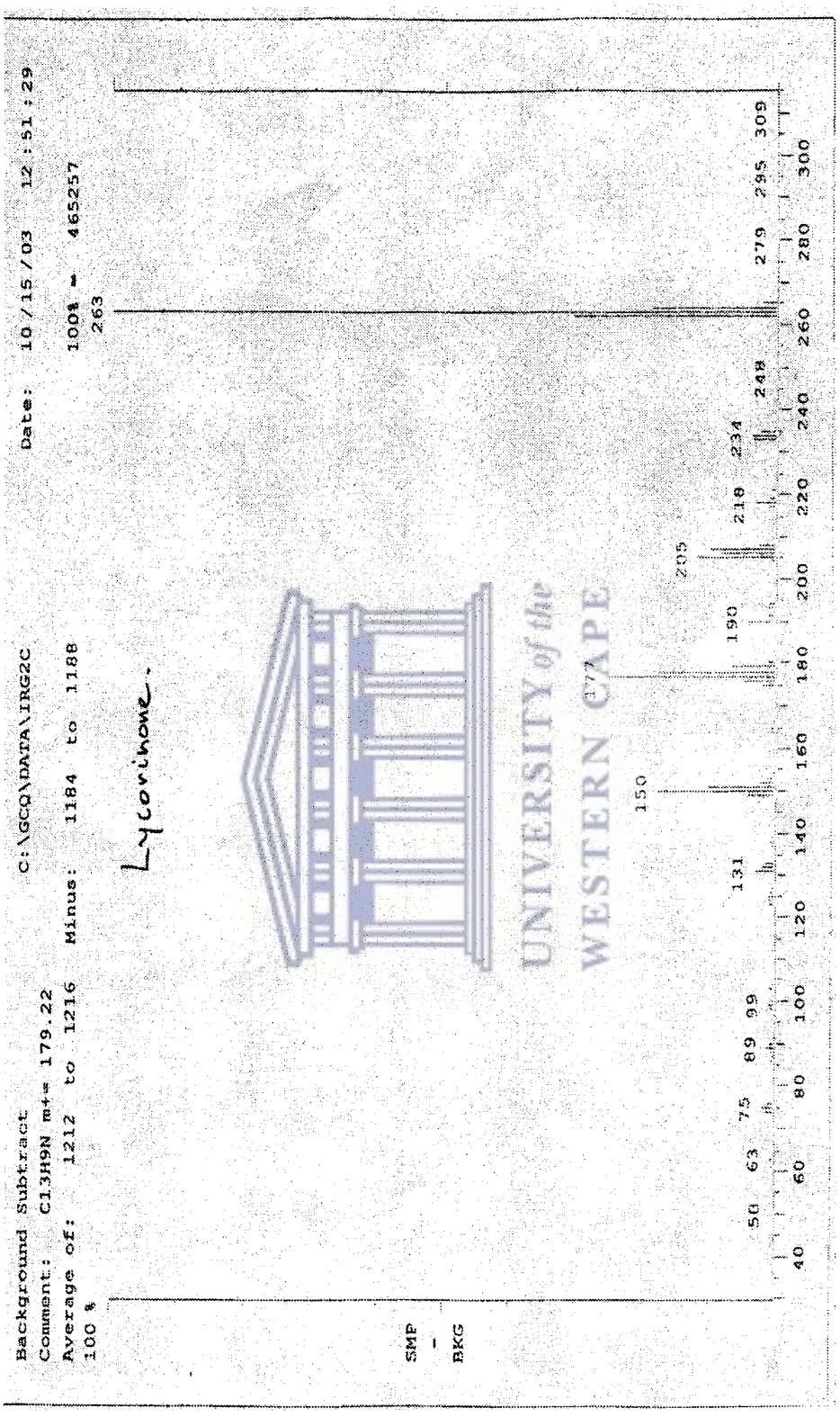


Figure 15: Low resolution mass spectrum for the new alkaloid lycorinone.

Lycorinone

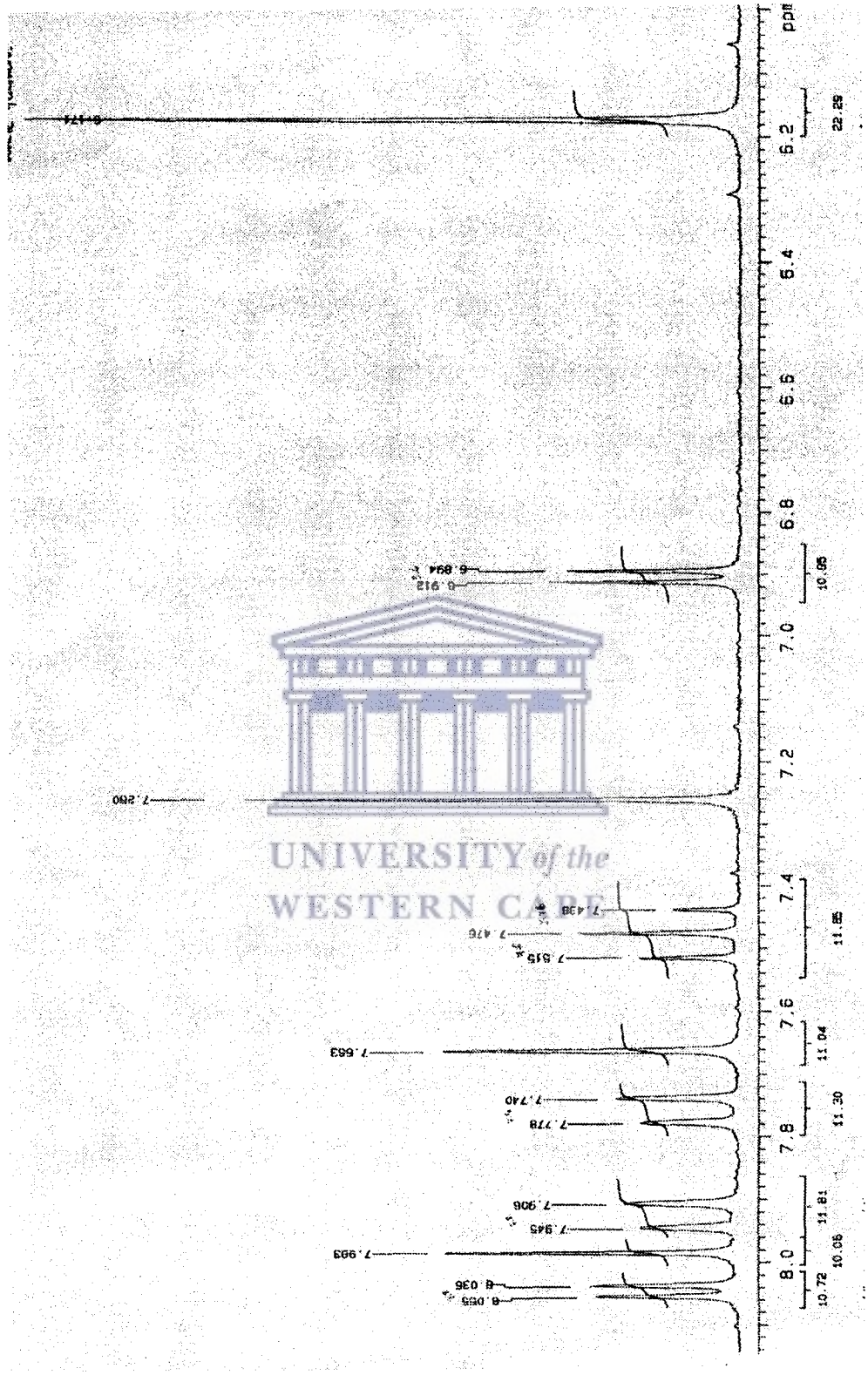


Figure 16: ^{1}**H-n.m.r spectrum of the new alkaloid lycorinone**

Lycorinone

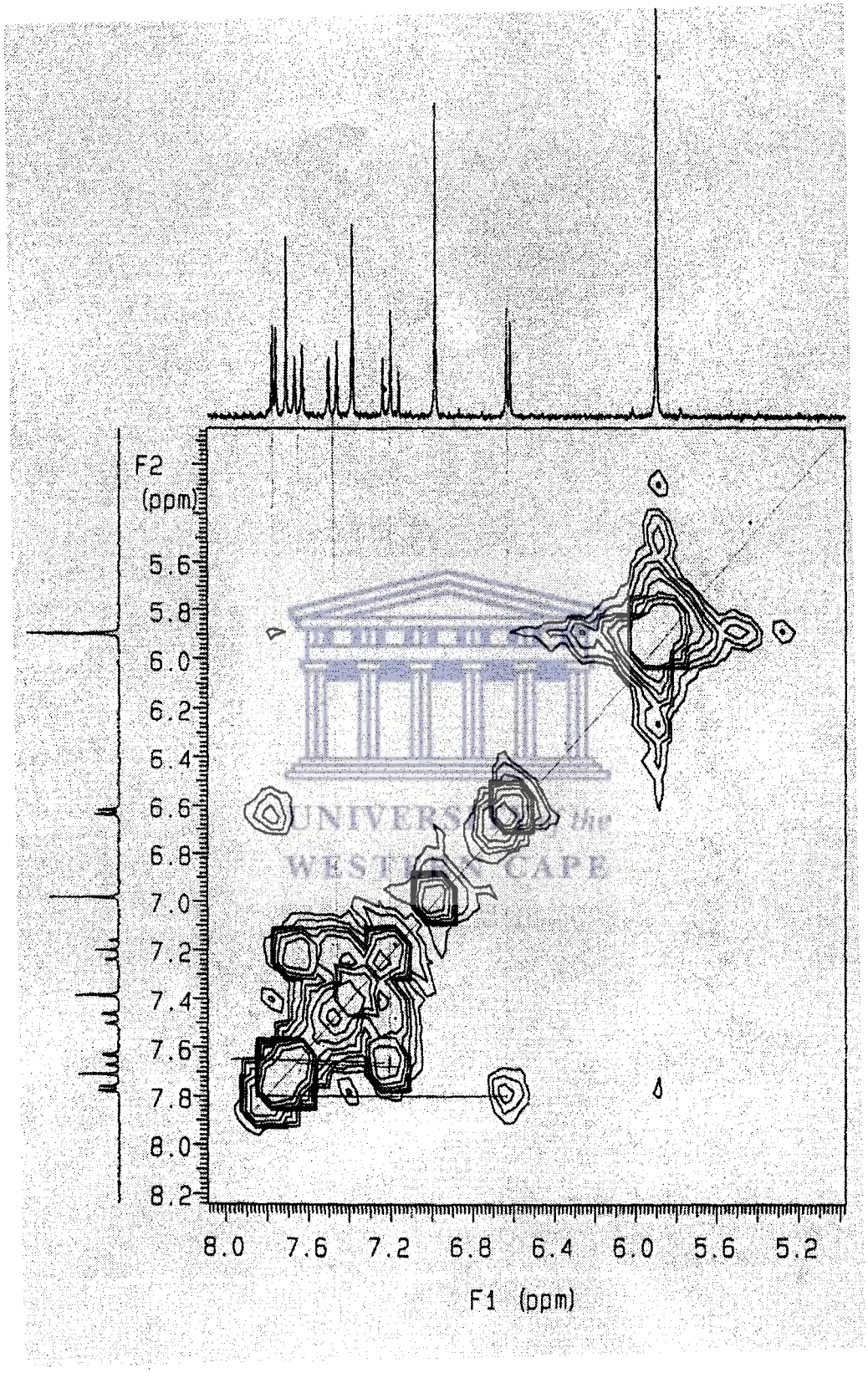


Figure 17: COSY spectrum of the new alkaloid lycorinone .

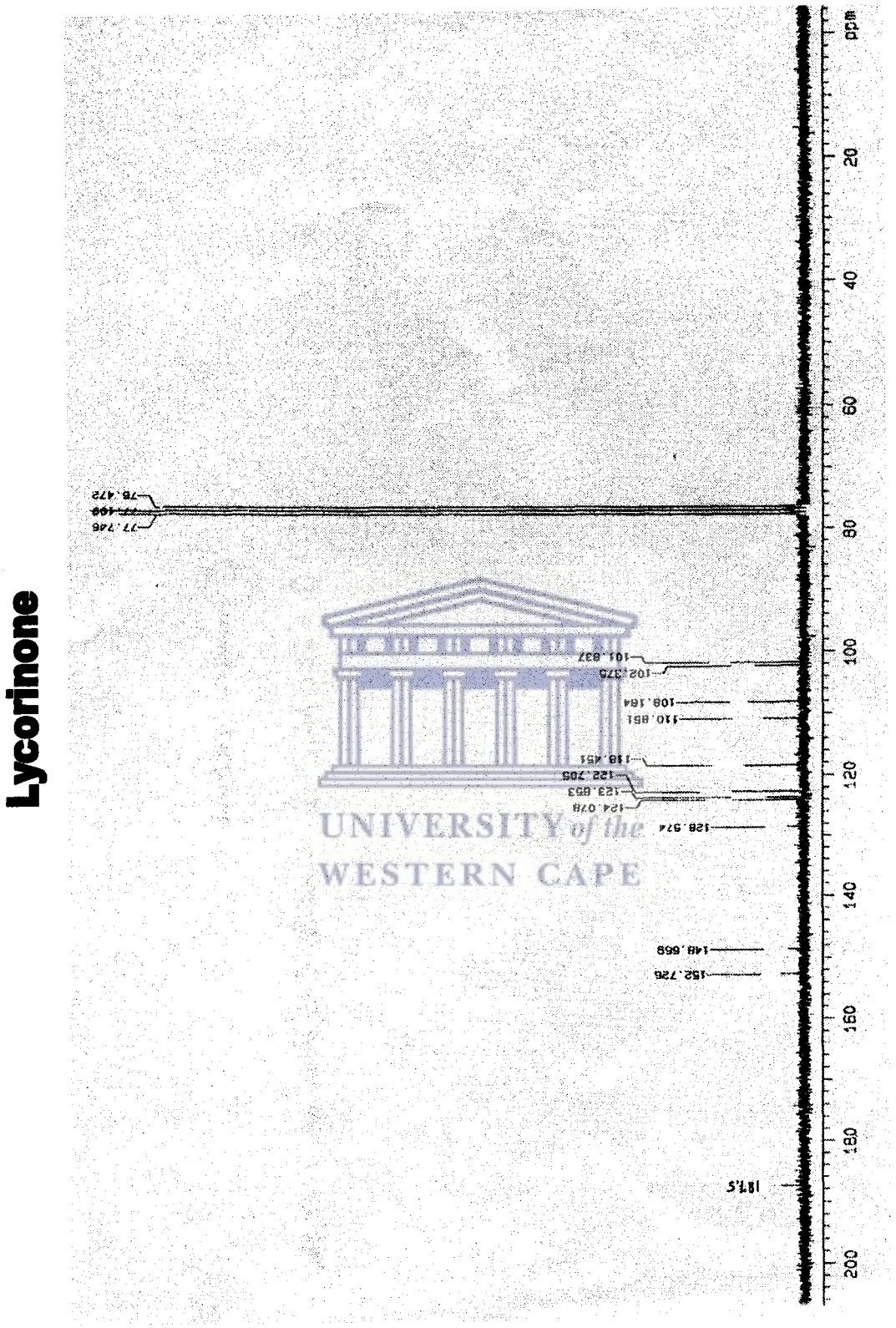


Figure 18: ^{13}C-n.m.r spectrum of the new alkaloid lycorinone. 0-200ppm to show the C=0 at 187.5 ppm

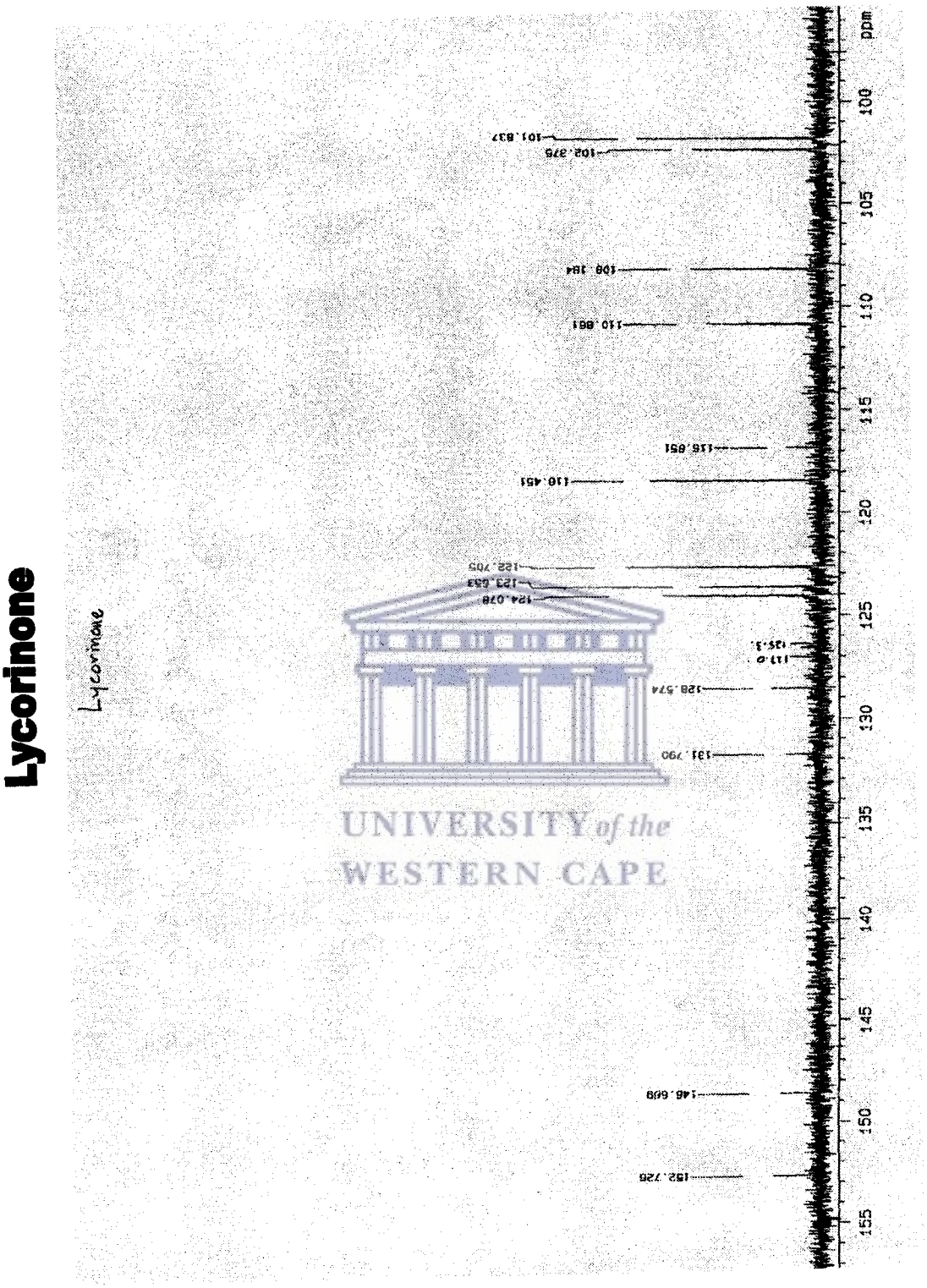


Figure 19: ^{13}C-n.m.r spectrum of the new alkaloid lycorinone expanded to show all the C-atoms of the molecule.

4.3 Evaluation of the "double sided" working heart system

In the "single sided" working heart system the active substance is perfused using the Langendorff system. The switch in the system allows for the perfusion fluid to flow in one direction i.e. retrograde perfusion. This does not allow for the ventricle to contract and therefore there is no pressure that is created and hence there is no aortic pressure that can be read. The result is that during the perfusion of the active substance only the myocardium is perfused and hence the effect of the active substance can only be measured after the system has been switched to the working heart system.

To overcome this, the "double sided" working heart system was set up. Due the fact that this was a new adaptation, the evaluation of the system to check if both sides were identical was paramount. Perfusion fluid was used as the medium of choice in order to test the viability of the heart and how long the heart would stay viable in the system.

Various parameters were assessed using the "double sided" working heart system. These parameters include:

a. Coronary flow (Qe)- section 4.2.1

b. Aortic output (Qa)-section 4.2.1

c. Cardiac output (Co)-section 4.2.1

d. Systolic and diastolic pressures (SP and DP respectively)-section 4.2.2

e. Pulse pressure (Pu)-section 4.2.2

f. Heart rate (Hr)-section 4.2.3

4.3.1 Control readings for coronary flow, aortic output and cardiac output

Using the perfusion fluid the viability of 8 hearts was tested by taking the readings of the coronary flow (Qe), aortic output (Qa) and cardiac output (CO). The parameters were assessed after 10 minutes of perfusion on each side i.e. work heart perfusion and work heart drug (see protocol). The results (Figure 20) showed that there was no statistically significant difference during the switching from the Whp to Whd and visa versa. The *p* values for the Qe, Qa and CO were 0.426, 0.1692 and 0.301 respectively. They were all above p=0.05.

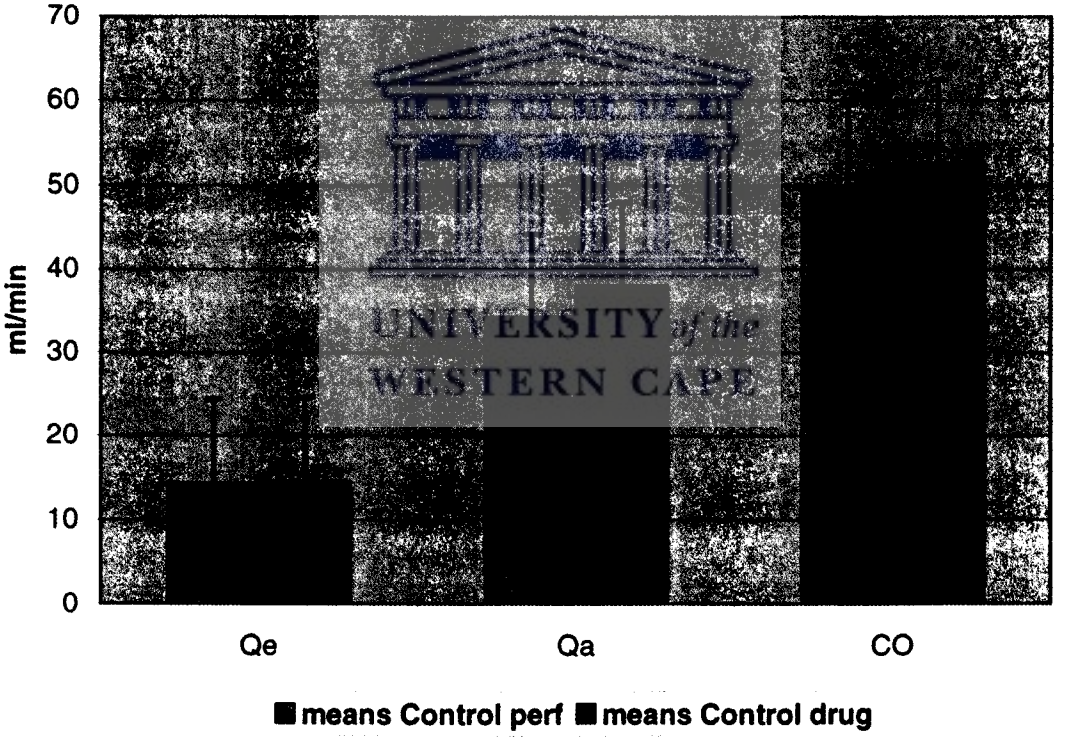


Figure 20:Changes in Qe, Qa, and CO during the switch from work heart perfusion to work heart drug.

The *p* values indicated that both sides of the system were almost identical and as a result the margin of difference between the readings on both sides would not affect the results that would be obtained using the system. Qe and CO are very important readings that are

used to check the viability of the heart. The exclusion criteria are set based on these two parameters where the Qe in either side should be between 8-18ml/min and the CO should be between 36-80ml/min.

4.3.2 Control readings for the systolic/diastolic and pulse pressure

The systolic and diastolic pressures readings were dependent on the exclusion criterion being met. The results obtained (Figure 21) using a sample size of 8 hearts showed that there was no significant increase or drop in systolic and diastolic pressures after the switch from one side to the other. The p values for these were 0.295 and 0.177, respectively. The pulse pressure is the difference between the systolic and diastolic pressures and it follows that there will be no significant change in the two sides (p=0.190).

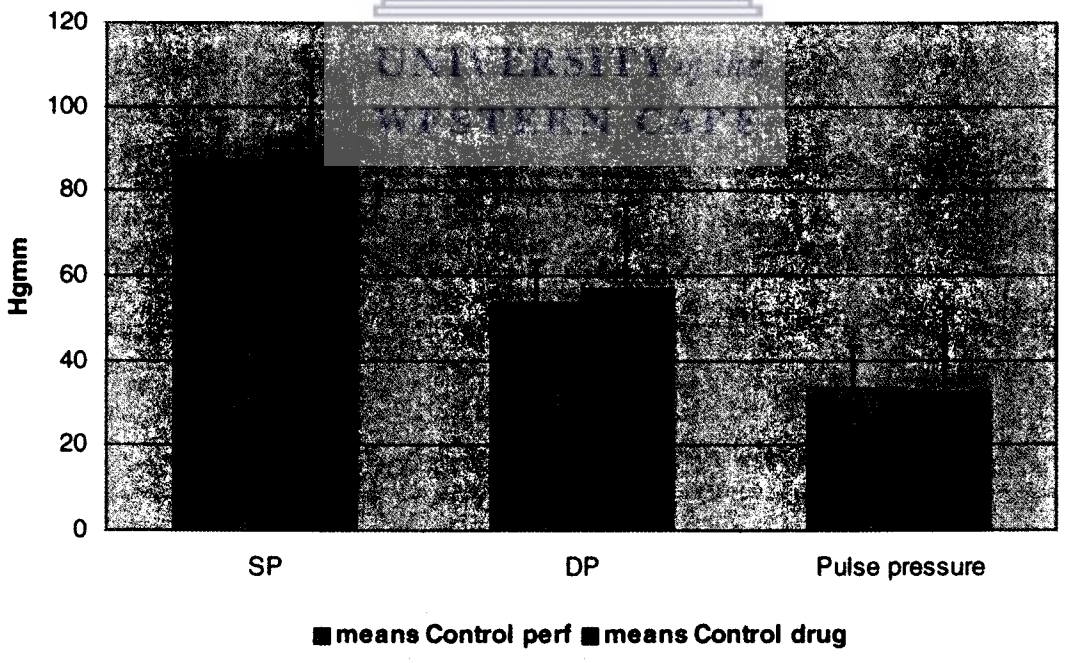


Figure 21: Systolic/diastolic and pulse pressures as a result of switching from the perfusion to the drug side and back.

4.3.3 Control readings for the heart rate

The heart rate readings did not vary significantly (p=0.338) during the switch. Figure 22 illustrates the means of the heart rate for the two sides of the system. The means of the heart rates taken from 8 hearts ranged between 232 and 234.5 beats/min in the work heart perfusion and work heart drug. These fell within the recommended range of readings for heart rate in the work heart perfusion.

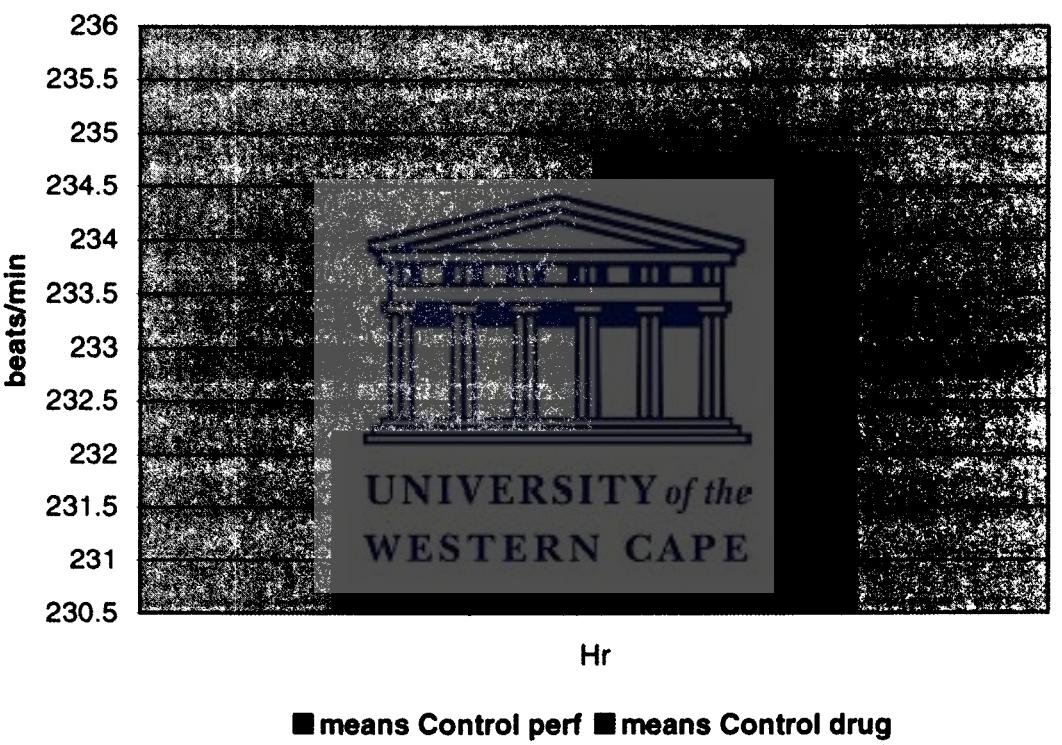


Figure 22: Illustration of the heart rate after switching from work heart perfusion to work heart drug side of the system. The readings were taken at the end of a 10-minute perfusion fluid period.

4.4 Pharmacological screening of lycorinone

In this evaluation a switch was made from work heart perfusion to work heart drug side. The perfusion time for the control drug (adrenaline) and the sample (lycorinone) was 5 minutes of each and a recovery time of 10 minutes was allowed in each case. Two

perfusions, of the sample drug and control drug, were made per heart due to the viability of the heart being only 1hour. The experiments were performed on a sample size of seven hearts (n=7) for the perfusion fluid infusion, three hearts (n = 3) for lycorinone at 5.0μg/ml, four hearts (n = 4) for lycorinone at 0.5μg/ml and seven hearts (n=7) for adrenaline.

4.4.1 Effect of adrenaline and lycorinone on the heart

4.4.1.1 Effect on coronary flow (Qe)

Adrenaline

Using adrenaline on the "double sided" working heart system was to further validate the use of the system and to compare the results with those found using the extracted compound. The results obtained with adrenaline at different concentrations, in the "double sided" working heart system, are shown in Figure 16. As the concentration increases the flow increases until it reaches its peak effect and then it drops at high doses. This is evident at the doses used - 0.01μg/ml (2.01% ± 0.184), 0.1μg/ml (9.99% ± 0.218) and 1.0μg/ml (12.52% ± 0.276). At high doses (100.0μg/ml) there is a drop in the percentage increase and hence 7.00% ± 0.123 (see Figure 23). Adrenaline is non-specific, and acts on both α and β receptors as an agonist. At very high concentrations the coronary flow drops due to the vasodilatory effects of adrenaline at very high concentrations, hence the drop at 100μg/ml (see Figure 23). With low doses of adrenaline the α_2 receptor effects predominate hence vasodilation. The α_1 receptor effect predominates at high concentrations and there is vasoconstriction hence the drop in the coronary flow (Laurence, D.R., and Bennett, P.N., 1980).

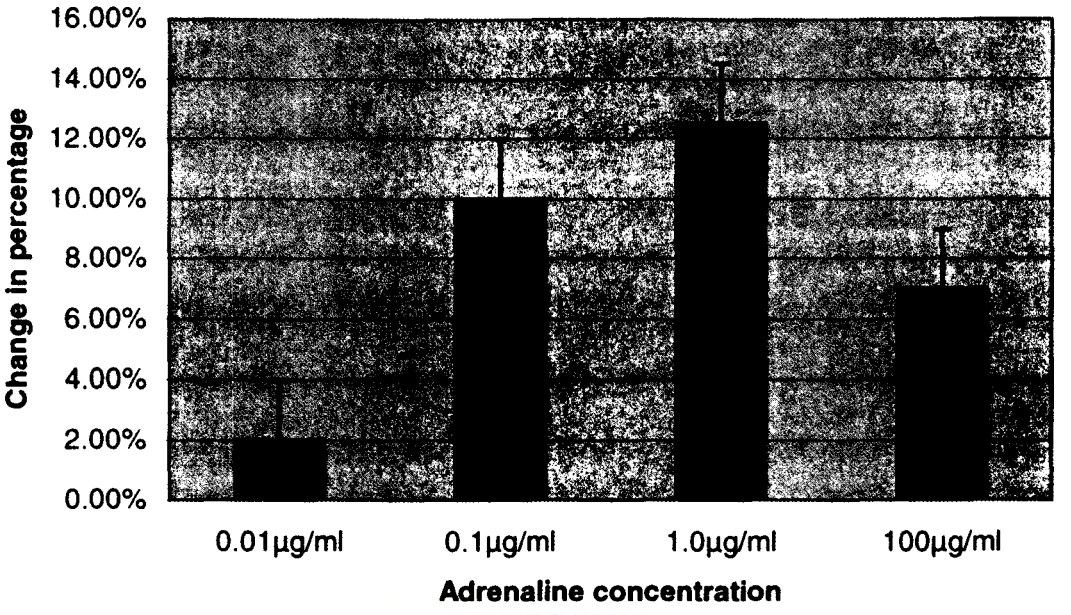


Figure 23: Effects of adrenaline on the coronary flow with increasing concentrations.

The coronary blood flow is enhanced by adrenaline. The increase occurs even with doses that do not increase aortic blood pressure (see Figure 29). This increase occurs due to an increase in the duration of the diastole (relaxation), which is offset by decreased blood flow during the systole (contraction) due to the forceful contraction of the myocardium hence the coronary vessels are compressed. The coronary flow may further be increased due to a metabolic dilator effect. The increase in the strength of myocardial contraction and O_2 consumption of the myocardium leads to vasodilation due to the released of adenosine from the cardiac myocytes (Hoffman, B.B. and Lefkowitz, R.J., 1990). This cancels the direct effect of adrenaline on the α adrenoceptors that results in vasodilation of the coronary vessels (Hoffman, B.B. and Lefkowitz, R.J., 1990; Laurence, D.R., and Bennett, P.N., 1980).

Lycorinone

Lycorinone reduces the coronary flow. At the highest concentration of 5.0µg/ml there is a reduction (48.83% ± 0.172) in the coronary flow. At a lower concentration of 0.5µg/ml the percentage (15.82% ± 0.109) change is less marked (see Figure 24).

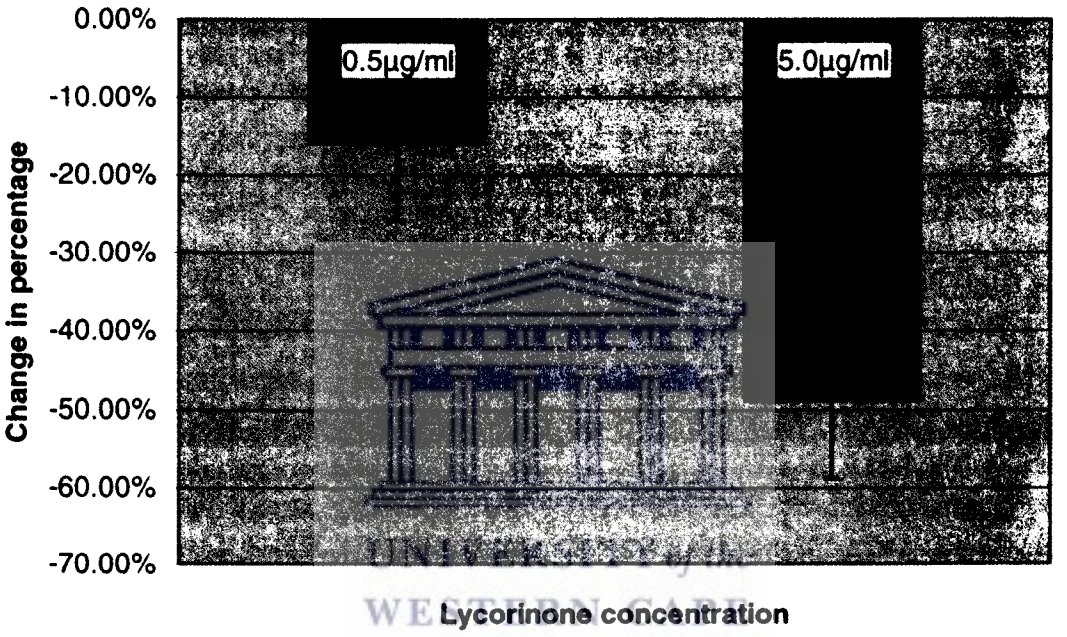


Figure 24: Lycorinone effects on the coronary flow at different concentrations i.e. 5.0µg/ml and 0.5 µg/ml.

The concentration of lycorinone used here is 5 times higher than that of adrenaline. The effects observed could be due to the vasodilatory effects of the compound and hence the total reduction of coronary flow. Lapadiformine, an alkaloid isolated from *Clavelina lepadiformis*, showed similar results in the cardiovascular system. It reduced blood flow and this was attributed to its vasodilatory effect (Juge, M., *et al*, 2001). These effects can further be compared to the effects of adrenaline at very high doses. The reason for the

comparison being that adrenaline is a natural alkaloid that is produced in animals and has effects that are known and recorded (Hoffman, B.B. and Lefkowitz, R.J., 1990; Laurence, D.R., and Bennett, P.N., 1980). Yew alkaloids especially the most potent of them (Taxine B) have been reported to reduce coronary flow as well (Alloatti, G., *et al* 1996).

4.4.1.2 Effect on the aortic output (Qa)

Adrenaline

Adrenaline increases the aortic output by 25.44% ± 0.028 at 0.01μg/ml, 28.82% ± 0.165 at 0.1μg/ml, 32.00% ± 0.102 at 1.0μg/ml and 54.53% ± 0.511 at 100.0μg/ml concentrations (Figure 25). Adrenaline is a non-specific agonist, that works on the α and β receptors. The β_1 adrenoceptors are mainly found in the muscle of the heart, hence the increase in the strength and rate of contraction in the myocardium, while the α_1 adrenoceptors are found in the coronary vessels (Laurence, D.R., and Bennett, P.N., 1980).

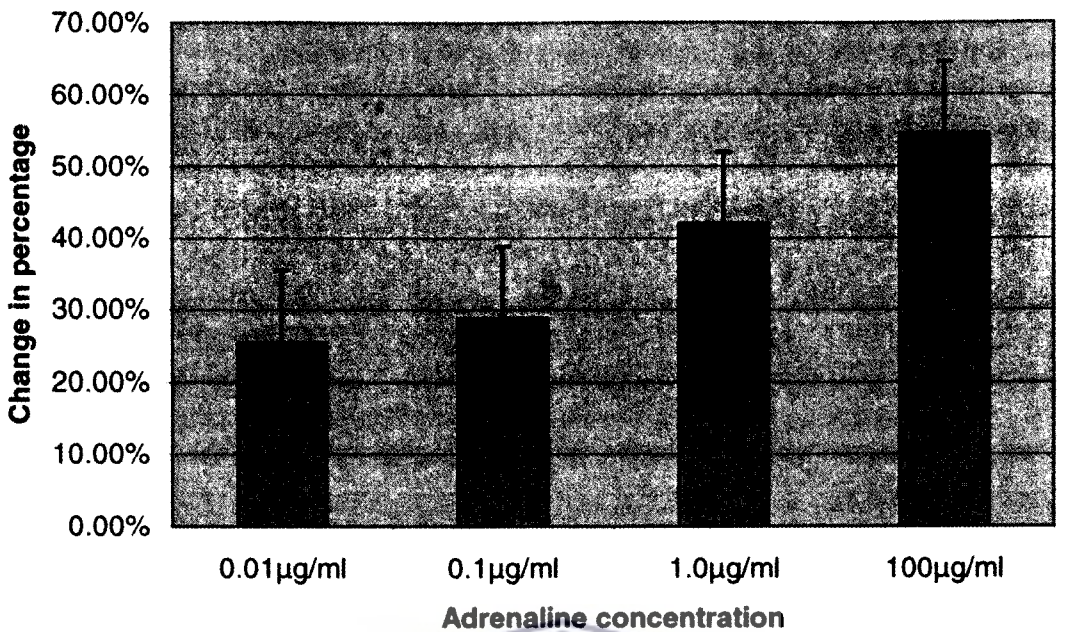


Figure 25: Adrenaline effects on aortic output at different concentrations.

The aortic output of the heart is increased due to the increase in the strength of the myocardial contraction. Increase in oxygen consumption also leads to an increase in the production of adenosine from the myocardium that is responsible for the energy producing metabolic processes in the myocardium.

Lycorinone

Lycorinone has a negative effect on the aortic output. At 0.5µg/ml the percentage change is 1.38% ± 0.180 and at 5.0µg/ml there is a 100% ± 0.000 reduction in the aortic output.

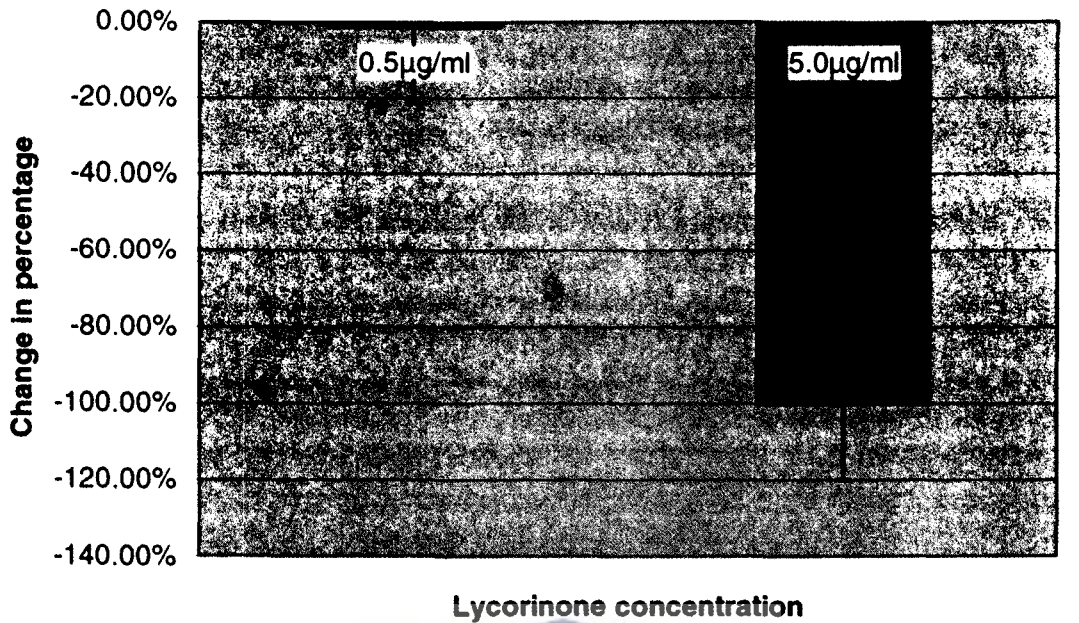


Figure 26: Lycorinone effects on the aortic output.

The aortic output drops to zero at the concentration of 5µg/ml and drops by about 1.38% ± 0.180 at 0.5µg/ml (Figure 26). In the isolated perfused heart experiments were carried out using yew alkaloids and they showed negative inotropic effect and atrio-ventricular conduction block (Alloatti, G., *et al* 1995). Taxine B, a potent compound in this group reduced cardiac contractility (Alloatti, G., *et al* 1996). These results were similar to what was obtained using lycorinone in the above experiments.

4.4.1.3 Effects on the cardiac output (Co)

Adrenaline

The cardiac output is the amount of blood ejected at each stroke of the heart. This is governed by both the coronary flow and aortic output (sum total of both parameters). When adrenaline was used at the concentrations of 0.01µg/ml, 0.1µg/ml, 1.0µg/ml and

100 µg/ml there was an increase of the cardiac output of 19.31% ± 0.048, 23.80% ± 0.155 and 32.00% ± 0.139, respectively (Figure 27).

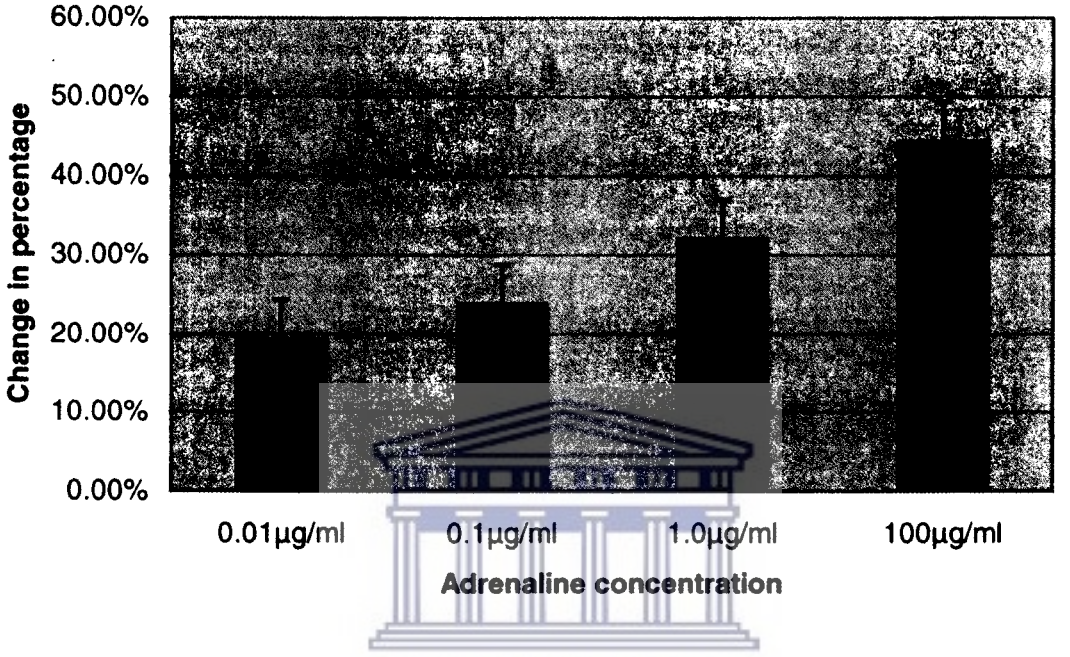


Figure 27: Adrenaline effect on cardiac output at different concentrations. There is a general increase in cardiac output as the concentration increases.

Cardiac output being the sum total of coronary flow and aortic output rises or falls in direct proportion to the rise or fall of these two parameters. Due to the increase in coronary flow and aortic output as a result of the administration of adrenaline, the cardiac output also rises in direct proportion to this. The dual effect on the myocardium, increased force of contraction, and the increase in coronary flow are the cause of the gradual increase in cardiac output as the concentration of adrenaline increases.

Lycorinone

There is a decrease in the cardiac output. The cardiac output decreases by 14.35% ± 0.067 at 0.5µg/ml and by 89.13% ± 0.039 at 5.0µg/ml. The effect is seen in Figure 28.

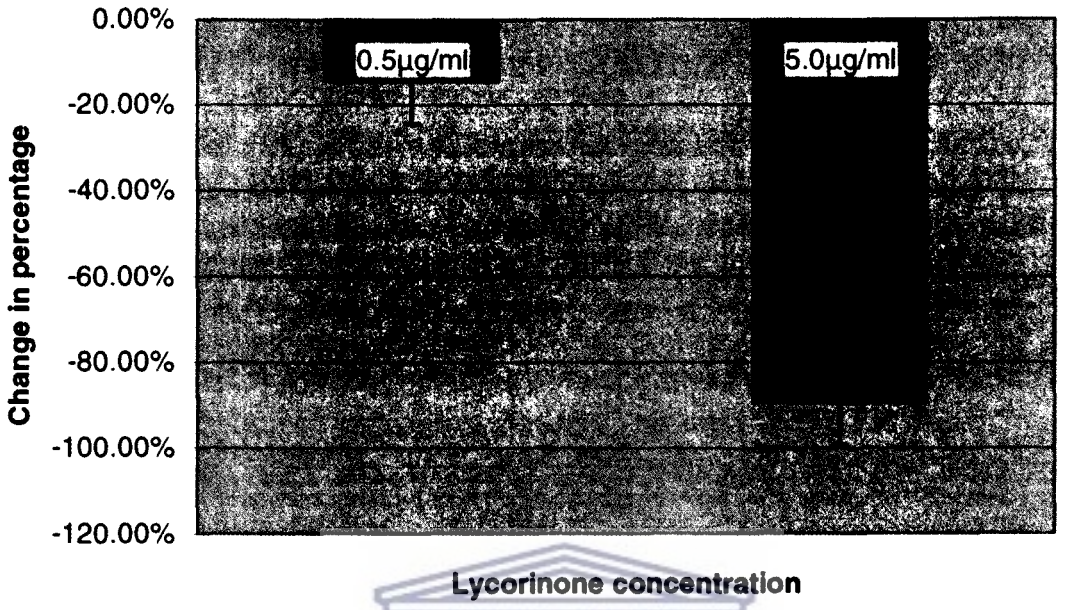


Figure 28: Lycorinone effect on the cardiac output.

The effect on cardiac output is directly proportional to the effect on coronary flow and aortic output. Due to the reduction in myocardial contractility and coronary flow the cardiac output is also reduced. These effects have been attributed to the poisonous effects of the combined activity of the alkaloids present and their corresponding cinnamates, which reduce both coronary flow and heart rate, which in turn affects the cardiac output (Alloatti, G., et al 1995). The effects of lycorinone can be linked to those of the yew alkaloids.

4.4.1.4 Effects on the contractility of the heart

Adrenaline

Adrenaline effect on the contractility of the myocardium is dose dependent. At low doses of 0.01µg/ml and 0.1µg/ml, there is an increase in systolic pressure (6.11% ±0.023,

6.83% ± 0.054 respectively) while the diastolic pressure reduced (–3.35% ± 0.015, -0.44% ± 0.003 respectively). At 1.0µg/ml the diastolic pressure increases by 12.92% ± while the systolic increases by 10.78% ±0.24. In high doses (100µg/ml) adrenaline causes a slight increase in diastolic pressure (2.20% ±0.035). The systolic pressure is increased further by this concentration of adrenaline to 12.52% ±0.035 (Figure 29).

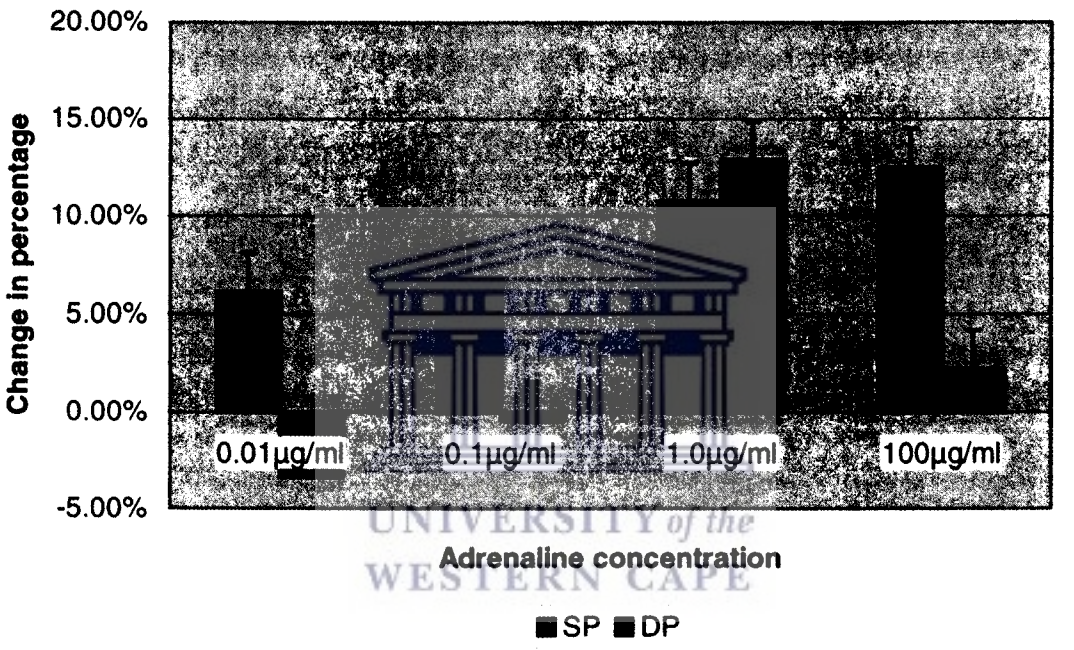


Figure 29: Adrenaline effect on systolic and diastolic pressures.

The administration of adrenaline gives a characteristic effect of an increase in the systolic pressure in direct proportion to the dose administered. The increase in systolic is greater than the increase in diastolic pressures hence the pulse pressure increases. The mechanism behind the increase in blood pressure is two fold. Firstly, the increase is due to the direct stimulation of the myocardium that increases the strength of ventricular contraction. Secondly, adrenaline causes an increase in heart rate and this also increases

the blood pressure. Low doses of adrenaline (0.1μg/kg) may cause the pressure to fall due to its depressor effect at such concentrations. In large doses the pressure falls due to the greater sensitivity of the vasodilator β_2 receptors to adrenaline as opposed to the α constrictor receptors.

Lycorinone

At the concentration of 0.5μg/ml of lycorinone there is reduction (10.03% ±0.012) in the systolic pressure while there is an increase (3.98% ± 0.076) in diastolic pressure. At a higher dose (5.0μg/ml) of lycorinone there is a further reduction in the systolic pressure (20.56% ± 0.021) and the diastolic does not increase significantly (0.58%± 0.047). This is illustrated in Figure 30.

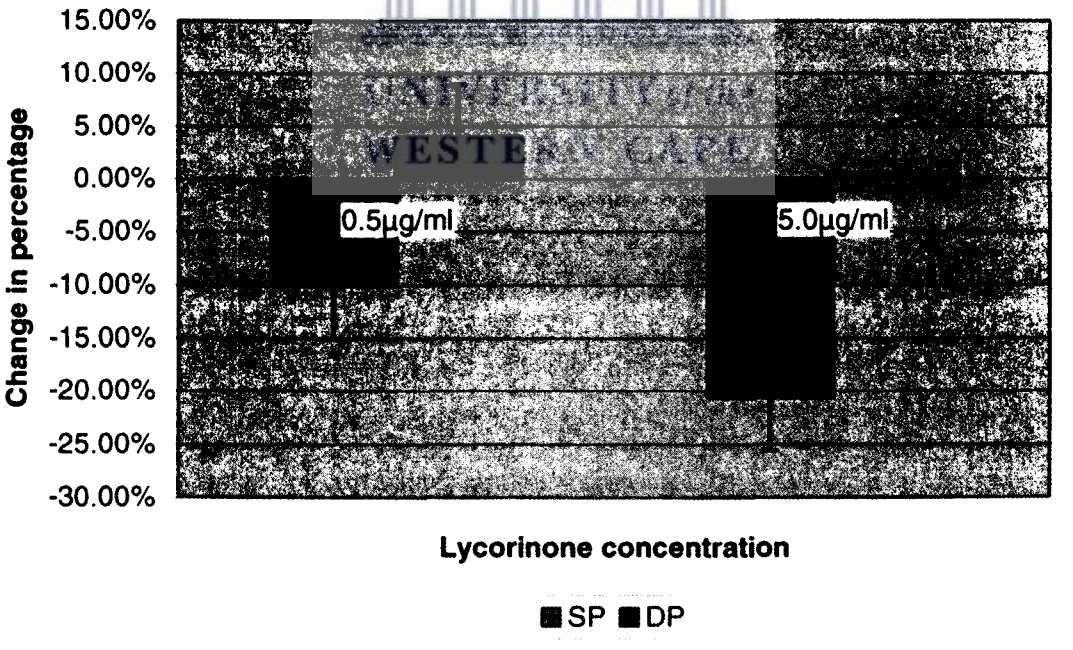


Figure 30: Lycorinone effect on systolic and diastolic pressure.

Lycorinone drops the systolic pressure and it increases the diastolic pressure. The reduction of the systolic pressure could be due to the reduction in the coronary and aortic flow. While the increase in the diastolic blood pressure could be attributed to the reduction (the length of the cycle is shortened due to incomplete contraction of the myocardium) of the cardiac cycle. Lepadiformine (an alkaloid from *Clavelina lepadiformis*) lengthens the interval in the cardiac cycle suggesting that the alkaloid prolongs the duration of the action potential (Juge, M., *et al* 2001). This could explain the effect observed with lycorinone.

4.4.1.5 Effects on pulse pressure (Pu)

Adrenaline

Pulse pressure is dependent on the systolic and diastolic pressures. It is the difference between the two parameters. At low doses adrenaline causes vasodilation and at high doses it causes vasoconstriction. Figure 31 illustrates the adrenaline effect on pulse pressure where at 0.01μg/ml there is a 23.72% ± 0.206 increase, at 0.1μg/ml the increase is 21.46% ± 0.424, at 1.0μg/ml the increase is 14.25% ± 0.058, and at 100.0μg/ml the increase is 9.95% ± 0.374.

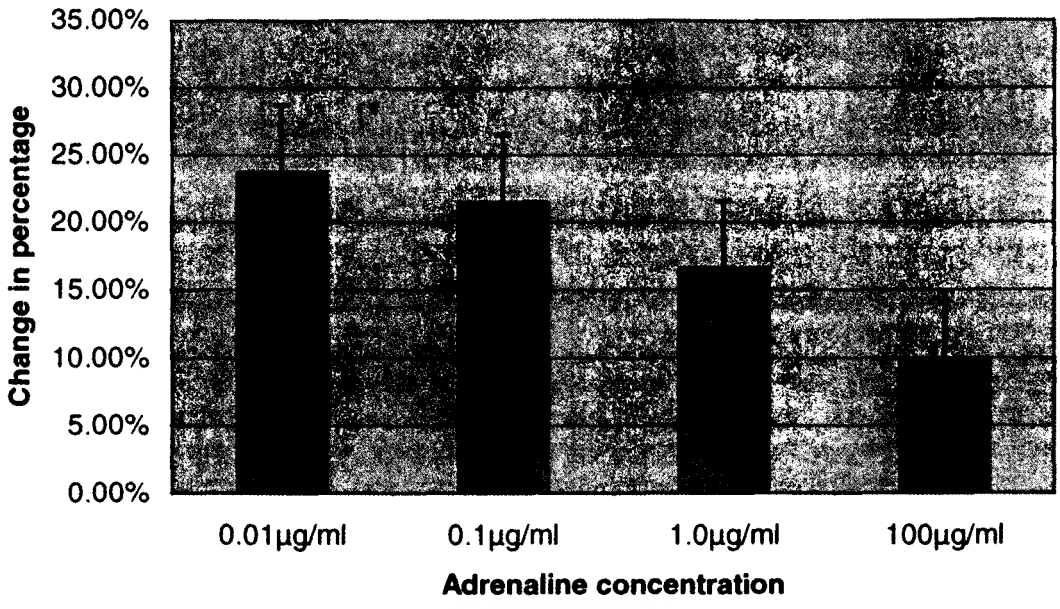


Figure 31: Adrenaline effect on pulse pressure.

There is a gradual drop in the pulse pressure as the concentration of adrenaline increases. The drop is attributed to the increase in the force and rate of myocardial contraction and hence the cardiac cycle is reduced. This means that there is a decrease in the amplitude in the cardiac cycle. Due to the existence of α_1 receptors in most areas of the heart the activation leads to a long refractory period, which strengthen the contractions of the myocardium. At high doses (100µg/ml) the drop is attributed to the vasodilator effect of adrenaline and the reduction in the force of myocardial contraction.

Lycorinone

There is a decrease in the pulse rate. At 0.5µg/ml the decrease is 26.98% ± 0.081 while at 5.0µg/ml the decrease is 57.96% ± 0.251. The decrease increases as the dose increases (Figure 32).

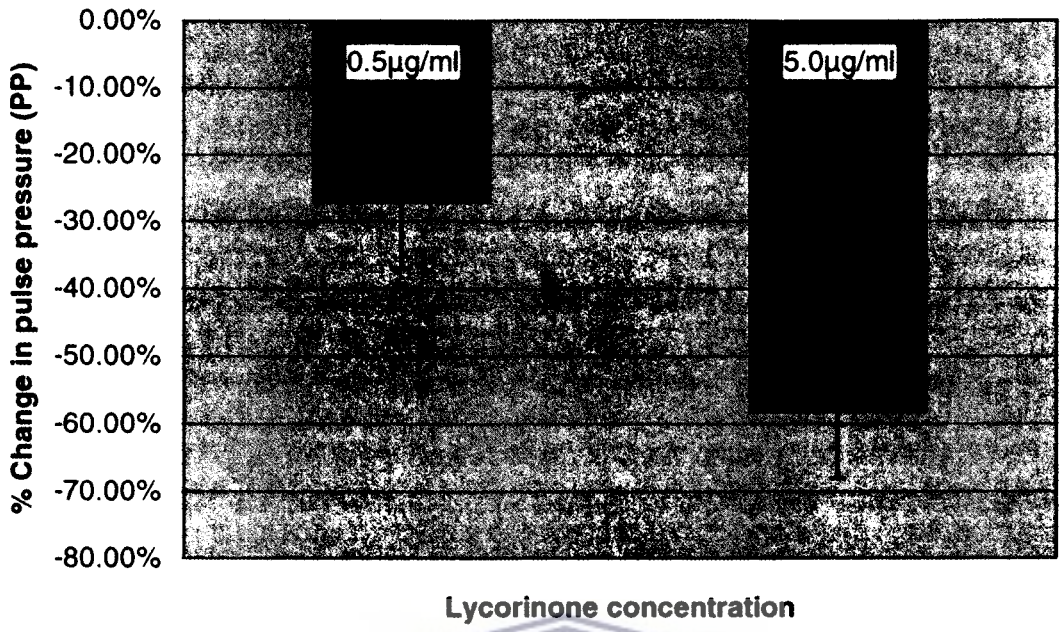


Figure 32: Lycorinone effect on pulse pressure.

These effects can be attributed to the reduction in the force and rate of myocardial contraction and the lengthening of the action potentials. This is an effect comparable to those described by Juge, M., *et al* (2001) in the paper about the alkaloid lapadiformis. As already mentioned, the pulse pressure is the difference between the systolic and diastolic pressures. Therefore the factors that affect the systolic and diastolic blood pressure will determine the pulse pressure.

4.4.1.6 Effect on the heart rate (Hr)

Adrenaline

Adrenaline increases the rate and force of myocardial contraction. At 0.01µg/ml the rate increased by 4.03% ± 0.041, at 0.1µg/ml the rate increased by 4.52% ± 0.113, at

1.0µg/ml the rate increased by 7.66% ± 0.220 and 1mg/ml drops the heart rate to 0.25% ± 0.035 (Figure 33).

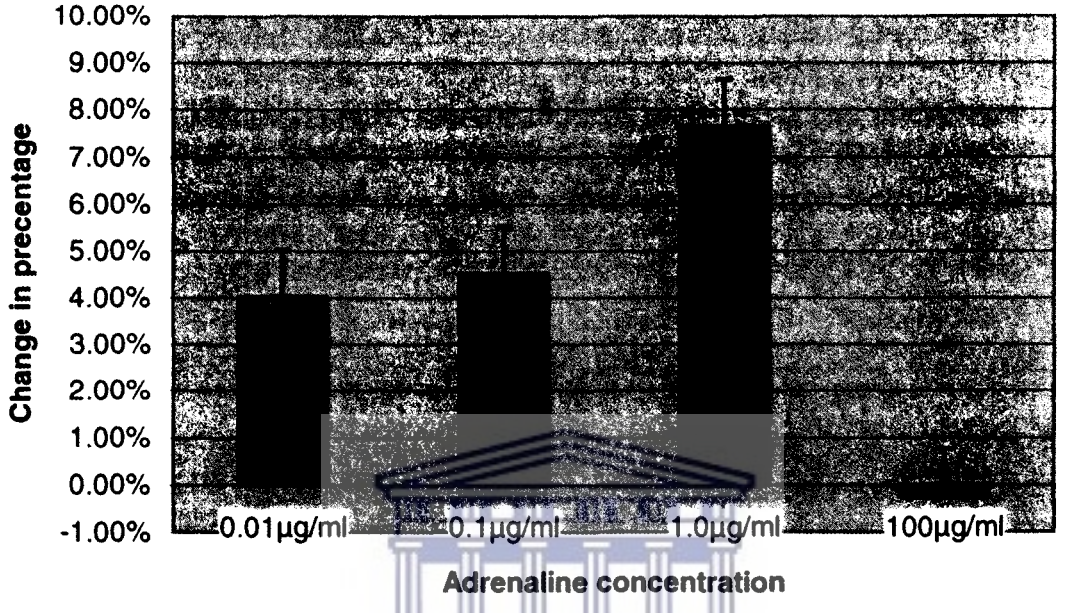


Figure 33: Adrenaline effect on heart rate.

Adrenaline stimulates the β_1 receptor of the myocardium increasing the rate and force of contraction. It also increases the oxygen consumption. The drop in the heart rate could be due to the concentration of adrenaline being too high.

Lycorinone

There is a reduction in the heart rate when lycorinone is administered. The reduction occurs even at the lower concentration but not to as great an extent as at a higher concentration. At 0.5µg/ml the reduction was 7.59% ± 0.112 while at a higher concentration of 5.0µg/ml the reduction was 36.01% ±0.266 (See Figure 34).

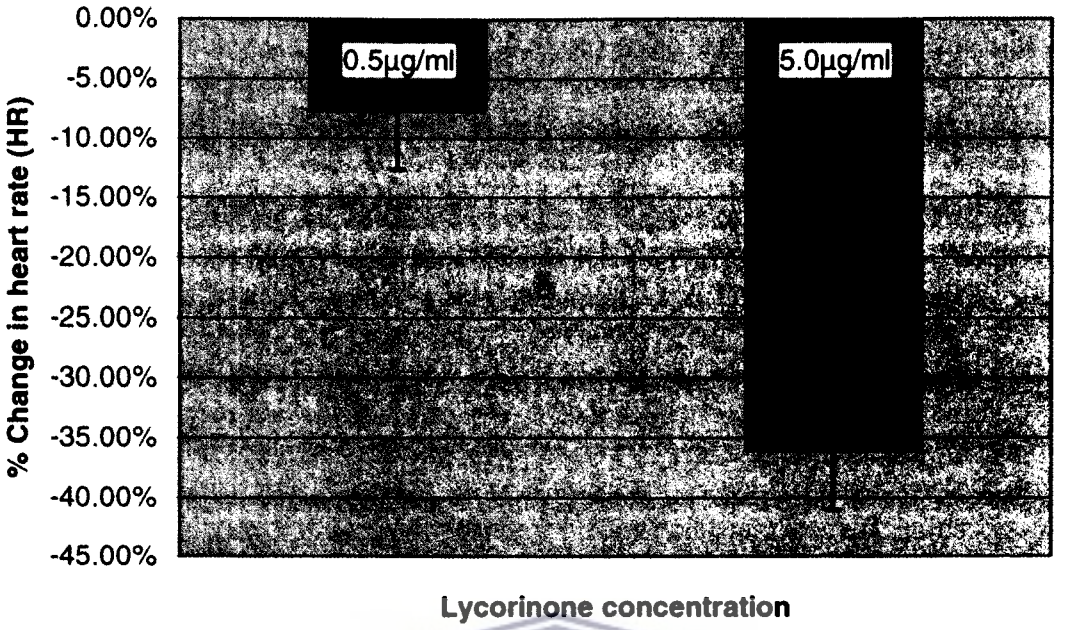


Figure 34: Lycorinone effect on heart rate.

Lycorinone causes bradycardia. It reduces the heart rate at both concentrations used and as a result it affects most of the other functions of the heart. A reduction in heart rate affects the coronary flow (by reducing the coronary blood flow) since myocardial contraction is one of the factors that affect coronary flow. The aortic output is reduced due to the hearts inability to pump the perfusion fluid. The systolic blood pressure is reduced and there is a slight rise in the diastolic blood pressure.

In conclusion, the effects obtained by lycorinone indicate that the alkaloid has the ability to have a negative chronotropic and a negative inotropic effect on the heart. This alkaloid at the concentrations used can be detrimental to the isolated perfused heart if perfused for long durations of time. But if perfused for only five minutes and if a recovery period of

10 minutes is allowed the heart recovers fully. This is an aspect of the alkaloid that needs further investigation.

Chapter 5

5.0 Conclusion and recommendations

The main aim of the study was to identify and pharmacologically screen the activity of the major alkaloid present in the EtOAc:hexane (1:4) fraction of *Crinum macowanii*. This was in a bid to validate the results obtained by Mugabo *et al* (2000) and the claims placed on the plant by the traditional healers in its use in oedema. From the results obtained in the animal model (working heart system) of this study, it may be stated that the major alkaloid, which was structurally identified for the first time and for which the name lycorinone was proposed, reduces significantly myocardial function and additionally reduces all the other parameters significantly. It can also be stated that at a lower dose the effect is reduced.

In conclusion, this project contributes not only to a scientific proof of the efficacy of Lycorinone isolated from *Crinum macowanii*, but also to the phyotchemical and pharmacological screening of the other alkaloids found in the plant. My recommendation is a suggestion for more extensive pharmacological, toxicological and phytochemical screening of this plant with a view to fully elucidate the mechanism of action and the activity of each of the alkaloids present and the safety of the plant as a medicinal remedy.

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