AN INVESTIGATION OF PLANTS USED IN SOUTH AFRICA FOR THE TREATMENT OF HYPERTENSION

BY

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Submitted in fulfilment of the
requirements for the degree of
Master of Science

in the

Department of Botany
University of Natal
Pietermaritzburg

1998

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ABSTRACT

In most countries, as many as 15 to 25% of the adult population have raised blood pressure. People with hypertension, and even those with mild elevation of blood pressure, are at an increased risk of cardiovascular disease, whether or not the symptoms are present. The risk of serious cardiovascular disease varies greatly among individuals and is also determined by a variety of concomitant risk factors other than the level of blood pressure.

Hypertension develops as a result of disturbances of the body's blood pressure regulating system. The biological activity of the renin-angiotensin systems results from a series of specific enzymatic cleavages leading to the generation of angiotensin II, a potent vasoconstrictor.

In the treatment of hypertension, inhibition of the angiotensin converting enzyme is established as one modern therapeutic principle. Angiotensin converting enzyme inhibitors act by inhibiting the conversion of angiotensin I to angiotensin II.

The *in vitro* assay, developed by ELBL and WAGNER (1991) for the detection of angiotensin converting enzyme inhibitors in plant extracts was successfully established during this study. Plants used by traditional healers in South Africa for the treatment of high blood pressure were investigated for their anti-hypertensive properties, utilizing the established angiotensin converting enzyme assay.

Twenty plants were investigated for their angiotensin converting enzyme

inhibitory activity. The highest inhibition (97%) was obtained by *Adenopodia spicata* leaves. A further seven plants exhibited an inhibition greater than 70% and five more over 50%.

Plants exhibiting inhibition levels greater than 50% were further tested for the presence of tannins in order to eliminate possible false positives.

The leaves of *Tulbaghia violacea* were chosen for bioassay-guided fractionation in an attempt to isolate the active compound(s).

Serial extractions were made of ground *Tulbaghia violacea* leaves using polar to non-polar solvents to establish the solvent giving optimum extraction of the active compound(s). Distilled H_2O was selected as the extractant and a bulk extract was performed on 0.7 kg ground leaves. The extracted residue was partitioned against butanol, fractionated using cation exchange resin chromatography, Sephadex ® LH-20 and high performance liquid chromatography. Fractions collected after each purification step were assayed using the angiotensin converting enzyme assay. Fractions exhibiting high levels of angiotensin converting enzyme inhibition were selected for further purification. The active fraction from the final high performance liquid chromatography step used in this study requires further attention in order to purify and identify the active compound(s). The chromatographic and chemical properties of the compound(s) present in the isolated active fraction are discussed.

PREFACE

I hereby declare that unless specifically indicated,
this project is the result of my own investigation.

Andrew Cameron Duncan

December 1998

ACKNOWLEDGEMENTS

I would like to thank the following people for their contributions towards this project:

My supervisor, Professor J. van Staden and my co-supervisor Dr A. K. Jäger for their support, valuable discussions and sound advise throughout the last two years.

Dr J. F. Finnie for his contribution during the research meetings over the last two years.

Professor N. D. Emslie of the Natal University Chemistry Department for his input in the synthesis of dansyltriglycine.

The Foundation for Research and Development for their financial support for the duration of this study.

Dr S. Zschocke for helping me with certain chemistry problems.

Dr P. D. Hare and Mr P. N. Hills for proof reading the final draft of this thesis.

Finally I would like to thank my parents for their unfailing support, understanding and encouragement during my studies.

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ABBREVIATIONS

ACE	-	Angiotensin Converting Enzyme
AT1	-	Type 1 angiotensin receptor
AT2	-	Type 2 angiotensin receptor
CS	-	Captopril stock solution
Dansyl	-	5-*N,N*-dimethylaminonaphtalene-1-sulfonyl
dH_2O	-	Distilled water
HEPES	-	*N*-[2-Hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]
HPLC	-	High Performance Liquid Chromatography
IC_{50}	-	Concentration of inhibitor producing 50% inhibition of enzyme
ID	-	Internal diameter
Na_2EDTA	-	Ethylenediaminetetraacetic acid disodium salt
®	-	Registered trademark
RAS	-	Renin-Angiotensin System
TLC	-	Thin Layer Chromatography
UV	-	Ultra violet

CHAPTER 1

LITERATURE REVIEW

1.1 Role of Plants in Health Care

In most countries, as many as 15 to 25% of the adult population have raised blood pressure. Cardiovascular disease causes the greatest mortality of humans in the USA and remains a major health problem world wide (YANG, MERRILL, THOMPSON, ROBBILLARD and SIGMUND, 1994). People with hypertension, and even those with mild elevation of blood pressure, are at an increased risk of cardiovascular disease, whether or not the symptoms are present.

In America, 90% of the population suffering from a form of cardiovascular disease suffer from hypertension (YANG, MERRILL, THOMPSON, ROBBILLARD and SIGMUND, 1994). The risk of serious cardiovascular disease varies greatly among individuals and is also determined by a variety of concomitant risk factors other than the level of blood pressure (ZANCHETTI, CHALMERS, ARAKAWA, GYARFAS, HAMLET, HANNSSON, JULUIS, MACMAHON, MANCIA, MÉNARD, OMEA, REID and SAFAR, 1993).

It is thought that about 80% of the 5 200 million people of the world live in developing countries. The World Health Organization estimates that about 80% of these people rely almost exclusively on traditional medicine for their

primary health care needs. Since medicinal plants are the "backbone" of traditional medicine, this means that more than 3 300 million people in the developing countries utilize medicinal plants on a regular basis (FARNSWORTH, 1994). With up to 500 000 species of higher plants known to exist on our planet, common sense dictates that many more useful drugs remain to be discovered from this source .

Traditional medicine is an integral part of South African cultural life, a position that is unlikely to change to any significant degree during the years to come (BRANDT, OSUCH, MATHIBE and TSIPA, 1995). It is estimated that between 12 and 15 million South Africans still depend on traditional herbal medicines from as many as 700 indigenous plant species (MEYER, AFOLAYAN, TAYLOR and ENGELBRECHT, 1996).

People suffering from hypertension are typically treated for the duration of their lives. With the soaring prices of Western medicines already beyond the grasp of large proportions of the world's population, it would seem that the demand for low-cost effective remedies would be on the increase. It is thus essential that traditionally used plants be scientifically investigated and evaluated as to their future role in the health care systems of the world. By studying traditional medicines, many conditions that have previously been incurable may soon be treated with low-cost medicines.

1.2 Hypertension

Despite many years of research into the physiological mechanism regulating blood pressure, the primary causative determinants of hypertension remain elusive. Hypertension develops as a result of disturbances of the body's blood pressure regulating system. The level of the blood pressure is determined by

the diameter of the small arteries and arterioles (resistance vessels) and by the cardiac output. The latter can change owing to (a) inotropic effects on the heart itself; (b) alterations in blood volume and body electrolyte composition due, for example, to changes in renal function, and (c) through the effects on the venous capacity. During normal circulatory homeostasis the heart, kidney, vascular diameter and the veins are all influenced by (a) the activity of the autonomic nervous system; (b) various circulating hormones; and (c) a number of local hormones (KORNER,1982).

1.3 The Renin-Angiotensin System

By exploring the means by which normotension is maintained by the renin-angiotensin system (RAS), an understanding into how the treatment of hypertension using angiotensin converting enzyme (ACE) inhibitors can be achieved. The best known functions of angiotensin II are its contributions to cardiovascular and renal homeostasis. In its classic definition, the RAS maintains blood pressure through angiotensin II (LEE, BÖHM, PAUL and GANTEN, 1993). It is a complex and mixed enzyme-hormonal system that plays an important role in blood pressure regulation and fluid and electrolyte homeostasis (KANG, LANDAU, EBERHARDT and FRISHMAN, 1994).

1.3.1 Historical Overview

Renin was discovered in 1898 (TIGERSTEDT and BERGMAN, 1898). GOLDBLATT, LYNCH, HANZAL and SUMMERVILLE (1934) demonstrated that constriction of a renal artery in dogs could induce hypertension. MERRILL, WILLIAMS and HARRISON (1938) reported a pressor/vasoconstrictor response to the injection of an alcohol extract of rat renal cortex in anaesthetised rats. The effect was independent of the presence of various

organs and was evident in the isolated rat leg. Also in 1938, FASCIDO, HOUSSAY and TAQUINI induced a rapid and substantial rise in arterial pressure by grafting an ischemic kidney into the neck of adrenalectomized and nephrectomized dogs.

Thus by the end of the 1930's it was clear that an ischemic kidney could release a pressor substance. The vasoactive end product, which had a more rapid pressor action, was discovered almost simultaneously by two groups. The name given to this active octapeptide was angiotensin I. The roles of angiotensin and ACE in the formation of angiotensin II were defined. Angiotensin II became available for infusion upon the elucidation of its amino acid sequence in 1956 and its synthesis in 1957. In the late 1950's and early 1960's, it became clear that aldosterone secretion could be simulated by angiotensin II. In the 1960's and 1970's, angiotensin II was considered by many to be a major secretagogue of aldosterone (NICHOLLS, CHARLES, CROZIER, ESPINER, IKRAM, RADEMAKER, RICHARDS and YANDLE, 1994).

HABER, KOERNER, PAGE, KLIMAN and PURNODE (1969) described a radioimmunoassy method for quantifying renin activity in plasma. The assay of the activity and concentration of renin improved the awareness and understanding of the circulating RAS under physiological and patho-physical circumstances (NICHOLLS, CHARLES, CROZIER, ESPINER, IKRAM, RADEMAKER, RICHARDS and YANDLE, 1994).

More of a stumbling block, was, and still is, the accurate determination of circulating levels of angiotensin II. Few laboratories today can claim to measure angiotensin II levels in plasma with great accuracy and precision (NUSSBERGER and BRUNNER, 1993). By the 1970's, it became evident that

through angiotensin II, the RAS could induce vasoconstriction and stimulate aldosterone secretion.

1.3.2 Outline of the Renin-Angiotensin System

The biological activity of the renin-angiotensin systems results from a series of specific enzymatic cleavages of polypeptide precursors leading to the generation of angiotensin II and related peptides (Figure 1). Renin, a glycoprotein, initiates this sequence by cleaving the leu^{10}-val^{11} bond of the globular protein angiotensin, to liberate angiotensin I. Renin occurs mainly in the kidneys where it is stored in intracellular granules of the juxta glomerular cells (LIN and FRISHMAN, 1996). Its release appears to be in response to a variety of normal or abnormal phenomena that reduce arterial blood pressure, renal perfusion, or sodium chloride load in the distal renal tube (DAVIS and FREEMAN, 1976). In the second step, angiotensin I is cleaved by vascular endothelial ACE, and a dipeptide unit is split off to give the physiologically active octapeptide angiotensin II (LIN and FRISHMAN, 1996). Angiotensin II is destroyed rapidly by the enzymes grouped as angiotensinases, its half life in humans being 1-2 minutes. The angiotensinases include an aminopeptidase that removes the asparagine residue from the N-terminal of angiotensin II resulting in angiotensin III. The metabolism of angiotensin III yields several inactive by-products (GREENWALD and BECKER, 1994).

Angiotensin II is a potent constrictor of vascular smooth muscle and stimulates aldosterone biosynthesis in the adrenal zone glomerulosa. It also has well defined effects on the adrenal medulla, sympathetic ganglia, Na^+ handling by the kidney and perhaps central nervous actions on blood pressure control, thirst and salt appetite. Many of these actions seem to be related to blood pressure maintenance, both by direct vascular effects and via body salt and

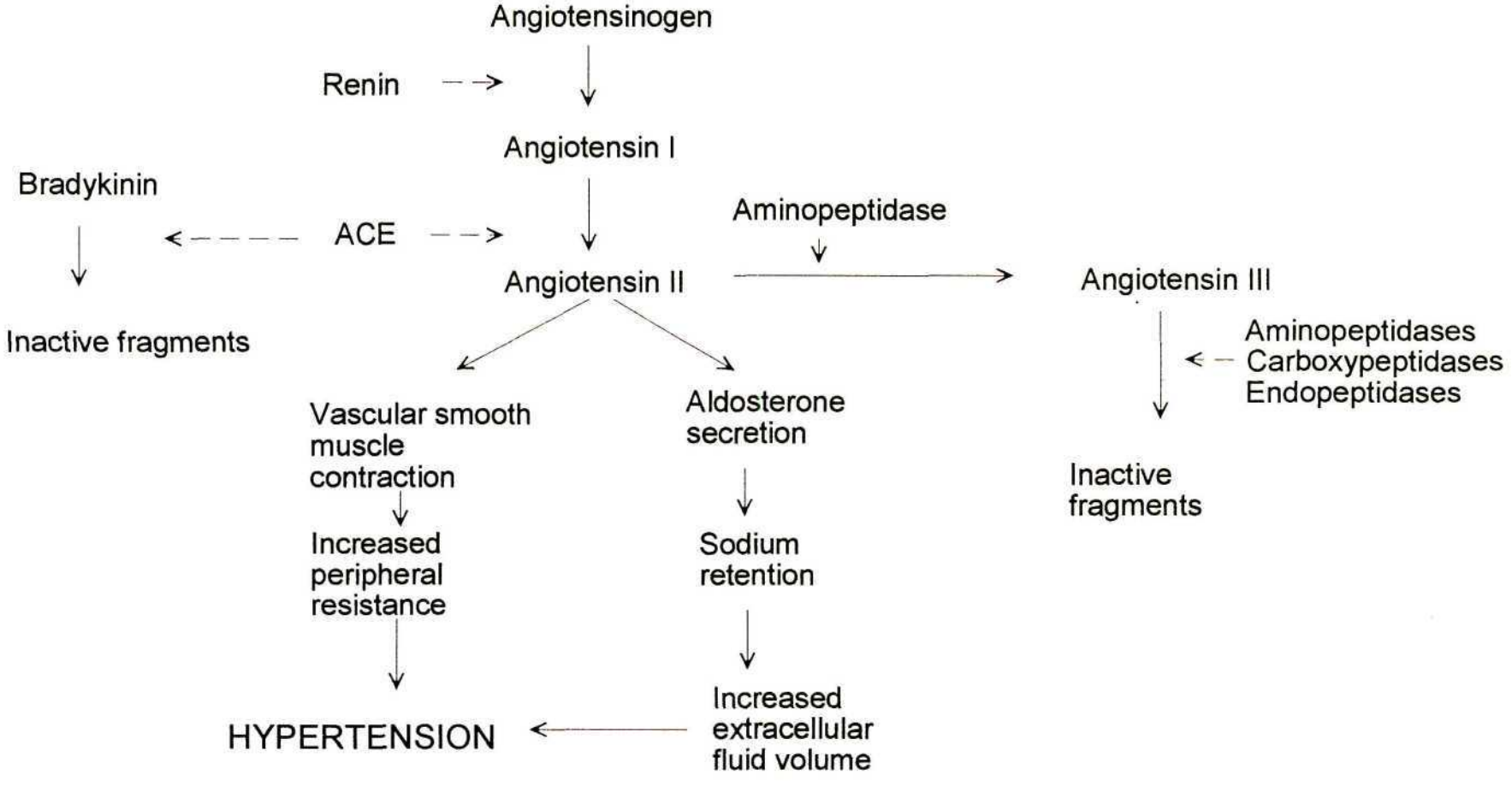

Figure 1: The renin angiotensin system . The release and physiological effects of angiotensin II and its degradation . Adapted from KELLOW (1994) and HANSEN, NYMAN, WAGENER SMITT, ADSERSEN, GUGIKSEN, RAJASEKHARAN, and PUSHPANGADAN (1995) .

water homeostasis. Aldosterone is a potent mineralocorticoid which promotes Na^+ retention and K^+ loss from epithelia (MENDELSOHN, 1982).

1.3.3 Molecular Biology of the Renin-Angiotensin System

Historically, the RAS system has been viewed as a systemic one, that being the different components have been derived from different organs and delivered to the site of action by the circulatory system (GRIENDLING, MURPHY and ALEXANDER, 1993).

Conceptionally, two kinds of RAS's exist (DZAU, 1988)(Figure 2): (a) a circulating endocrine RAS responsible for short-term cardiorenal homeostasis; and (b) local renin-angiotensin systems present in many local tissues where these systems exert autocrine and paracrine influences on local tissue function which is influenced by the intrinsic tissue renin-angiotensin system.

Recently, using molecular and biochemical approaches to angiotensin physiology, the possibility that there are distinct local RAS's with different mechanisms of regulation has been proposed. Local RAS's have been proposed in the vasculature, brain, heart and kidney, but conclusive evidence for the existence of all components of the system in physiologically relevant amounts and relationships remain elusive. It is thought that this localisation of the RAS may serve as a mechanism for limiting the actions of angiotensin II to a specific organ or physiological event (GRIENDLING, MURPHY and ALEXANDER, 1993).

Indirect evidence for a local RAS has been derived from whole animal, isolated organ and tissue culture studies. Evidence against the existence of

a complete local RAS is based largely on the observation that a bi-lateral nephrectamy caused a dramatic decease in arterial renin activity, suggesting that the renin- like activity associated with the vascular wall is derived from uptake of circulatory renin of renal origin (GRIENDLING, MURPHY and ALEXANDER, 1993). In support of this, it has been demonstrated using molecular biological techniques, that arterial tissues posses low levels of renin mRNA, with it being totally absent in other studies. Finally, it is unclear whether the angiotensinogen mRNA present in the vasculature is associated with smooth muscle and endothelium or merely with adherent adipose tissue (GRIENDLING, MURPHY and ALEXANDER, 1993).

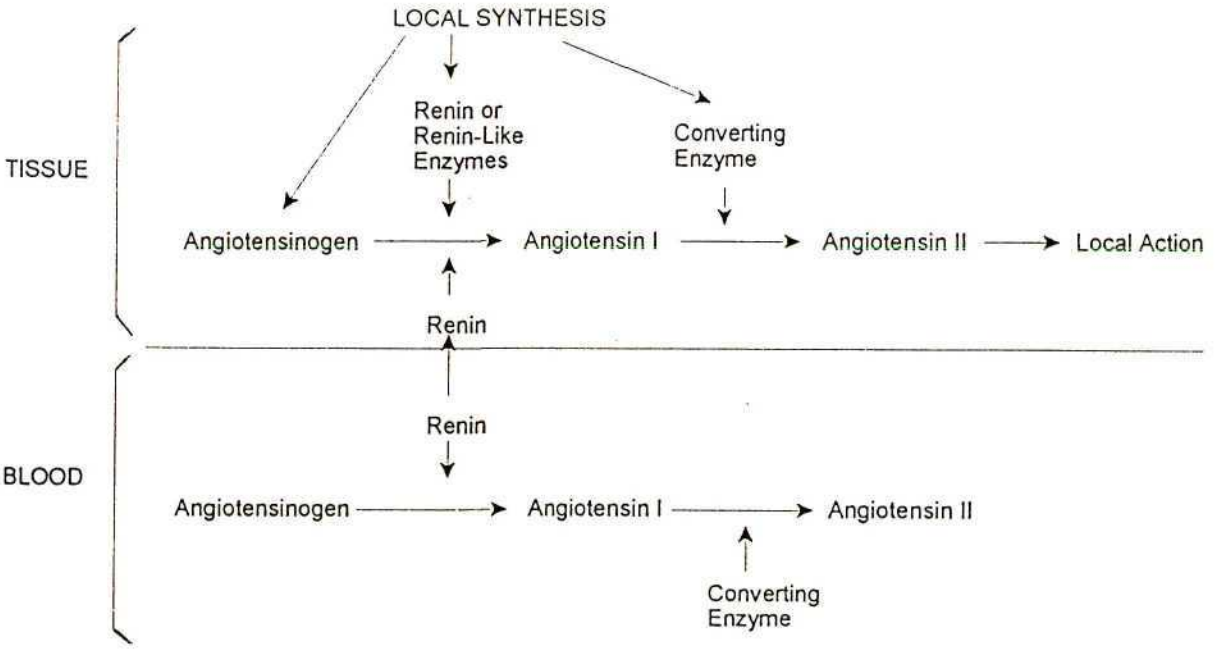

Figure 2: Circulating renin-angiotensin system vs. local renin-angiotensin system: interactions and possible angiotensin generation sites (DANSER, 1996).

It is hypothesised that angiotensin II produced locally influences local tissue function and structure such as vascular tone, cardiac contractibility and mass, renal hemodynamics and sodium handling (LEE, BÖHM, PAUL and GANTEN, 1993). This has been confirmed by studies which demonstrated the beneficial effects of ACE with respect to their anti-hypertensive, antiproliferative and cardioprotective impact, which is apparently independent of converting enzyme inhibition in plasma (LEE, BÖHM, PAUL and GANTEN, 1993).

Differentially regulated gene expression within several tissue - RAS's has been demonstrated in various animal models of hypertension. These data may provide further insight into the regulation of the RAS on a tissue level and may help clarify the physiological significance of the tissue RAS (LEE, BÖHM, PAUL and GANTEN, 1993).

1.3.4 Molecular Genetical Approach

Many studies have been carried out on the population distribution and hereditary nature of hypertension since the first reliable measurements of blood pressure were made. Blood pressure is a quantitative trait, which varies continuously throughout the whole population. The regulation of blood pressure is controlled by a variety of mechanisms that involve several genetic loci and environmental factors (CORVOL, JEUNEMAITRE, CHARRU, KOTELEVTSEV and SOUBRIER, 1995). Not much is known about how these genes regulate blood pressure in humans, and exactly which genes are actually involved in hypertension or the contributions that the environment makes with respect to the genetic regulation.

A number of studies have shown that both genetic predisposition and environmental factors govern individual blood pressure levels. The heritable

component of blood pressure has been documented in familial and twin studies. The evidence suggests that approximately 30% of the variable blood pressure is hereditary and 50% can be attributed to the environmental influences (WARD, 1990). This makes it complicated when trying to unravel the fundamentals of this disease, as it is influenced by both genetic and environmental factors.

Through molecular genetic studies, the contributions made by the RAS genes to blood pressure variance in animals and humans can be evaluated. Studies that have been carried out, conclude that it is possible that neither renin nor the ACE genes contribute to a large extent to genetic hypertension, at least in humans. They could, however, be involved in cases in humans that are yet to be defined. Molecular variants of the angiotensinogen gene constitute inherited predispositions to essential hypertension in humans, and are likely to be involved in some cases of pregnancy-induced hypertension. An ACE gene polymorphism associated with an increase in plasma and tissular ACE levels appears to be a strong marker of coronary and cardiac disease (CORVOL, JEUNEMAITRE, CHARRU, KOTELEVTSEV and SOUBRIER, 1995). The genetic approach will lead to an understanding of genomic risk factors in hypertension and may identify control systems, which are presently unknown, which will lead to a new approach to the prevention of cardiovascular death.

1.4 Blockage of the Renin-Angiotensin System

It's evident when observing the RAS, that blocking or inhibition of enzymes at specific points in the pathway would result in the reduction or removal of angiotensin II and hence improve the ability to treat and understand hypertension caused by this system.

1.4.1 Sites at which the RAS may be Blocked

Theoretically , there are a number of sites at which the RAS might be blocked (Table 1). So far no direct inhibitors of renin biosynthesis or renin maturation are available. The inhibition of renin secretion by β -adrenoceptor blockers is incomplete. Furthermore, since β -blockers have many more actions beyond renin suppression, responses elicited by them cannot be taken accurately to reflect inhibition of the RAS (NICHOLLS, CHARLES, CROZIER, ESPINER, IKRAM, RADEMAKER, RICHARDS and YANDLE, 1994).

Table 1: Reaction sites available for inhibition of the renin-angiotensin system (NICHOLLS, CHARLES, CROZIER, ESPINER, IKRAM, RADEMAKER, RICHARDS and YANDLE, 1994).

Step in the Pathway	Agent
Renin biosynthesis	Genetic-message blockers (undiscovered)
Renin secretion	β -adrenergic blockers (non-specific)
Renin maturation	Pro-renin processing-enzyme inhibitors (undiscovered)
Renin activity	Antibodies Peptide and peptide mimetic renin inhibitors
ACE	Captopril, enalapril and lisinopril
Ang II receptor	Saralasin and similar peptides Imidazole derivatives (losartan)

Efforts have and are continuously being made to improve the quality of the drugs available on the market. As can be seen in the above table, there are three sites at which drugs have been directed in order to block the RAS, these being renin, ACE and angiotensin II receptor inhibitors. There is much debate

as to the site which offers the greater control of the RAS system with the presence of minimal side effects.

1.4.2 Renin Inhibition

The search for compounds which block the formation of renin, has involved the development of four classes of compounds, namely renin antibodies (antisera, monoclonal antibodies, Fab fragments), synthetic derivatives of the prosegment of the renin precursor, pepstatin analogs and angiotensinogen analogs.

The renin antibodies were the first class of renin inhibitors developed, which acted directly against the enzyme. Although shown to be effective through lowering of the blood pressure and plasma renin activity, this immunological approach is limited because the antibodies are orally inactive as a result of their inability to be absorbed intact from the gastrointestinal tract. With repeated intravenous administration, the antibodies can induce antigenic reactions (LIN and FRISHMAN, 1996).

The next class of renin inhibitors developed were based on structure-activity relations. These inhibitors would bind tightly to renin and would not be cleaved by renin proteases or any other proteases. From this principal, the third class of renin-inhibiting drugs were modelled on the activity of pepstatin, a natural pentapeptide that universally inhibits aspartyl protease enzymes with a high inhibitory potency for pepsin and a low inhibitory activity for renin. These analogs, however, exhibited poor specificity and affinity for human renin, and this has limited their experimental and potential clinical use (LIN and FRISHMAN, 1996).

The fourth class of renin inhibitors, the angiotensinogen (substrate) analogues holds the greatest promise. By replacing specific bonds within the human angiotensinogen molecule, potent inhibitors of renin have been produced. Extensive studies have been made of the interaction of the active sites and subsites of renin with angiotensinogen analogs incorporating statin as well as other transition state analogs, replaced at various substrate analog subsites.

Renin inhibition has potential therapeutic efficacy in the treatment of renovascular hypertension and congestive heart failure. However, renin inhibition may also demonstrate clinical utility in a number of other clinical scenarios. Conditions such as stroke, renal trauma and acute closure of renal artery grafts are all associated with high plasma renin levels (LIN and FRISHMAN, 1996). Significant adverse reactions have not yet been observed. Further research for the development of an agent with good oral bio-availability for patient treatment is being carried out.

1.4.3 Angiotensin Converting Enzyme Inhibition

Angiotensin converting enzyme inhibitors act by inhibiting the conversion of angiotensin I to angiotensin II. On the basis of chemical structure, they can be divided into three distinct groups; the sulfhydryl-containing agents (e.g. captopril), the non-sulfhydryl-containing agents and the phosphinic acid derivatives (e.g. fosinopril). Captopril and fosinopril bind to the zinc ion in the ACE molecule through their sulfhydryl and phosphinic acid moieties respectively, whereas the other agents bind to the zinc ion through carboxyl residues (KELLOW, 1994).

Angiotensin converting enzyme inhibitors are considered effective and safe for the treatment of hypertension; their anti-hypertensive effect being enhanced

by a low salt diet. Their pharmacological effects are vasodilation, increased sodium excretion, diuresis and lowering of blood pressure (ABRAMS, DAVIES and FERGUSON, 1984). They reduce the mortality in patients with heart failure and prevent progressive post-infarct heart failure. They are also well tolerated, and have a good safety profile. There is little doubt that ACE inhibition does enhance the action of bradykinin. There are theoretical grounds linking this to the development of cough and some preliminary evidence relating this to angio-oedema (SUNMAN and SEVER, 1993). ACE inhibitors are not safe in pregnancy, and as soon as pregnancy is determined, an immediate switch must be made (OPIE, 1996) (ii).

Recently ELBL and WAGNER (1991) introduced an *in vitro* assay for the detection of ACE inhibitors in plant extracts. Angiotensin converting enzyme is believed to be the rate-limiting step in angiotensin II formation by the RAS. This method is based on the ACE-catalysed cleavage of the chromophore-fluorophore labelled substrate, dansyltriglycine, into dansylglycine, which is quantitatively measured by HPLC (Figure 3). By utilizing this technique, plant species that are used in traditional medicines to treat hypertension can be effectively screened, and their level of ACE inhibition determined.

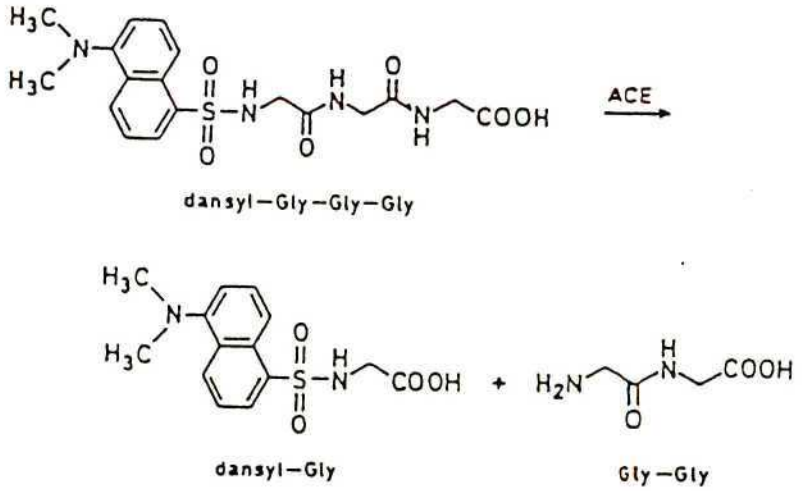

Figure 3: Dansyltrigylcine is cleaved by ACE into dansylglycine, which can be quantified by HPLC (ELBL and WAGNER, 1991).

1.4.4 Angiotensin II Inhibition

Angiotensin II receptor antagonists have been developed (VELASQUEZ, 1996). Angiotensin receptors can be placed into two distinct subtypes. These are the type 1 angiotensin receptor (AT1) and type 2 angiotensin receptor (AT2). Both of these receptor subtypes are apparently found in all tissues, in varying proportions (KANG, LANDAU, EBERHARDT and FRISHMAN, 1994). The AT1 receptor is found in virtually all vascular tissue, and is the only type present in the rat liver and spleen. The AT2 subtypes exhibit different distributions in other tissues.

The first blockage achieved in man was through the use of peptide analogues of angiotensin II. Saralasin is a partial antagonist and required intravenous infusion (NICHOLLS, CHARLES, CROZIER, ESPINER, IKRAM, RADEMAKER, RICHARDS and YANDLE, 1994). Saralasin's clinical usefulness as a long term anti-hypertensive agent is limited by its short half-life, significant agnostic properties and lack of oral bio-availability (KANG, LANDAU, EBERHARDT and FRISHMAN, 1994).

Several new non-peptide imidazole angiotensin II receptor antagonists that have potential anti-hypertensive properties have been developed. These novel compounds have a high affinity for AT1 and have no agnostic activity (VELASQUEZ, 1996). These compounds are also non-peptides, and can thus be taken orally. Losartan was the first antagonist of this type to become available for clinical use and has been the most extensively studied. It selectively inhibits angiotensin II binding in animal and human tissues, and blocks the pressor effects of exogenously administered angiotensin II in animals (VELASQUEZ, 1996).

Many more angiotensin II antagonists are undergoing clinical trails and are in various stages of development. The availability of non-peptide angiotensin II receptor blockers have provided an alternative in the treatment of hypertension. Current data suggest that these receptor blockers may be useful as initial monotherapy for mild to moderate hypertension, or in patients whose blood pressure is not controlled with thiazide therapy (VELASQUEZ, 1996).

1.5 Feasibility of Research

There is debate as to whether it is economically feasible to successfully introduce renin inhibitors into the cardiovascular market due to the fact that ACE inhibitors are becoming generic and increasingly cheaper. Angiotensin converting enzyme inhibitors are also considered by many to be the best treatment for hypertension and congestive heart failure. With the success of ACE inhibitors and the impending success of AT2 antagonists, would it be possible for renin inhibitors to demonstrate their cost effectiveness? Unless companies are prepared to respond to the challenge and take risks, ACE inhibitors, probably in conjunction with angiotensin II receptor antagonists, will remain the state of the art drugs for the treatment of hypertension and many other cardiovascular diseases (WOOD and CLOSE, 1996).

1.6 Hypertension in South Africa

SEEDAT (1996) estimated that in the early 1990's , six million cases of hypertension were documented in South Africa. Mortality related to hypertension has for many decades occurred most commonly in the urban South African black community (STEYN, FOURIE, LOMBARD, KATZENELLENBOGEN, BOURNE and JOOSTE, 1996). Ten percent of the mortalities reported on the death certificates from Johannesburg's city health

department in 1970, were cardiovascular deaths, mainly due to hypertension (SEFTEL, JOHNSON and MULLER, 1980).

Surveys carried out among urban black South Africans reflect high rates of hypertension. SEFTEL, JOHNSON and MULLER (1980) reported that hypertension, defined as a diastolic blood pressure above 90 mmHg, occurred in about 33% of both gender groups in Johannesburg blacks.

SEEDAT, SEEDAT and HACKLAND (1982) reported that in Durban, 23% of males and 27% of females had a blood pressure of over 160/95 mmHg compared with the 9% of males and 10% of females living in rural areas. These studies were all carried out in the 1970's and 1980s.

MOLLENTZE, MOORE, STEYN, JOUBERT, STEYN, OOSTHUIZEN and WEICH (1995) have shown that the difference between the incidence of hypertension in urban and rural South African blacks has become less pronounced during the 1990's. In a study conducted in a semi-rural area, QwaQwa, the rate of hypertension was 29%, compared with 30.3% in Mangauga, a black township outside Bloemfontein. These results could be due to the fact that rural communities are disappearing throughout South Africa and that the factors influencing the levels of hypertension can now be found in the semi-rural and urban settings. This could be due to the migration of rural people to the urban areas.

Comparison of hypertensive and normotensive participants in the study carried out by STEYN, FOURIE, LOMBARD, KATZENELLENBOGEN, BOURNE and JOOSTE (1996) confirmed that factors such as alcohol use and obesity were associated with hypertension. They also found that in some cases there was an indication that participants who were of a higher socio-economic standing

were more frequently hypertensive than those who were not. Such hypertension phenomenon has also been found in urbanised migrant Indian communities (STEYN, FOURIE, LOMBARD, KATZENELLENBOGEN, BOURNE and JOOSTE, 1996). If this increased rate of hypertension is not addressed, the occurrence of strokes and heart attacks will become as common in the urban black South Africans, as they already are in the other population groups.

There is a racial difference in the prevalence of hypertension, but whether it is due to genetics or other factors remains to be determined. There is a statistical difference in the prevalence of hypertension between Zulus, Whites and Indians of Durban. The blacks in the Transvaal also have a high prevalence of hypertension, as does the "mixed" race group in the Cape (SEEDAT, 1996).

1.7 Guidelines for the Management of Mild Hypertension

When managing hypertension, the present guidelines concentrate on mild hypertension, as this condition often presents a diagnostic and therapeutic problem, and there is clear evidence in the benefit of treating more severe hypertension. Patients suffering from hypertension differ with respect to age, blood pressure elevation, organ damage and concomitant risk factors and diseases and they live in societies in which cardiovascular risk and economic resources differ widely.

1.8 The Blood Pressure Limits

A guideline used to determine cases of hypertension is that of the use of blood pressure limits. The basic concept is that blood pressure elevation

results in risks, which increase as blood pressure rises. This is particularly well established in the case of strokes, although it also holds for heart diseases (OPIE, 1996) (i). It has been suggested that simple diastolic and systolic blood pressure limits be utilised to be used as a guideline in determining patients with respect to risk categories (Table 2).

Table 2: A simplified approach to hypertension (OPIE, 1996) (i).

DBP* range (mmHg)	SBP** range (mmHg)	Proposed Therapy
90-100	140-170	Lifestyle; drugs for high risk
100-115	170-200	Recheck blood pressure; drugs
115+ (confirmed)	200+	Drugs, refer
130+ (confirmed) Suspect malignant hypertension	No correlate	Urgent referral

* Diastolic blood pressure ** Systolic blood pressure

The higher the blood pressure values, the more essential it is for the patient to be issued with drugs and earlier referral. In cases when malignant hypertension is suspected, same day referral is required (OPIE, 1996) (i). Furthermore, patients with increased blood pressure levels, whether diastolic, systolic, or both, are advised to modify their lifestyle.

When dealing with hypertension, it is imperative that treatment commence as soon as possible. With the never-ending flow of rural inhabitants into urban areas, along with the ever increasing levels of stress present in a modern

lifestyle, drugs used in the treatment of hypertension must be developed that must be as effective as those already on the market, but at more competitive prices.

1.9 Aims and Objectives of this Study

The aims of this project were to establish and optimise the *in vitro* assay developed by ELBL and WAGNER (1991) for the detection of ACE inhibitors. Plants used by traditional healers in South Africa for the treatment of high blood pressure were investigated for their potential anti-hypertensive properties, utilizing the ACE assay. Plants that exhibit high levels of ACE inhibitory activities were tested to determine their level of tannin content. This was carried as tannins interacting with the ACE enzyme assay could cause false positive results. A plant exhibiting low or no tannin content and high ACE inhibitory activity was subjected to bulk extraction and an attempt was made to isolate the active compound(s) using bioassay-guided fractionation.

By investigating the ACE-inhibitory activity of plants in traditional use, this project formed part of an initiative for the assessment of herbal medicines as safe and efficient remedies for treatment of hypertension, evaluated according to methods employed in Western medicine.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

All chemicals and reagents needed for the project were purchased from Sigma. Solvents used were obtained from Saarchem, South Africa; Univar, South Africa; BDH Laboratory Suppliers, England; Merck and Associated Chemical Enterprises, South Africa.

2.2 Plant Material

All plants were collected from Natal, South Africa during January and February 1997. The exact sources are shown in Table 3. The plants collected were chosen following literature searches (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM, 1996) and voucher specimens were deposited at the Herbarium, Department of Botany, University of Natal, Pietermaritzburg (Table 3).

After collection, barks and roots were immediately oven dried at 50 °C for 96 hours. All other plant parts were oven dried at 50 °C for 48 hours. The dried material was subsequently ground and stored in the dark in air tight containers at room temperature until extraction.

2.2.1 Extraction of Plant Material

Plant material was ground to a fine powder using a homogenizer. Half of the ground plant material (200 mg) was extracted in 5 ml ethanol and the other half in 5 ml water for 60 minutes using a sonicator. Ethanolic and aqueous extracts were performed for each plant part. The extracted liquid was passed through a funnel lined with Whatman No.1 filter paper to filter off all debris. The liquid was collected and re-filtered through a DynaGard 0.22 μm filter. The liquid was placed in pre-weighed pill vials and evaporated to dryness using a fan. The pill vials were then re-weighed and the weight of the residues was determined.

2.2.2 Preparation of Plant Extracts for ACE Assay

Solutions of inhibitors extracted in water were made by dissolving 1 mg of test compound in 1 ml HEPES assay buffer (Appendix 1). Inhibitors extracted using ethanol, were dissolved in HEPES assay buffer containing 10% ethanol. The same applied for all other organic solvents used in the extraction process. The final concentration was 0.33 mg of test compound in 1 ml assay volume.

Table 3: Plants collected for screening for the presence of angiotensin converting enzyme (ACE) inhibitors.

Plant Species	Plant part	Collection site	Voucher specimen number	Uses
Adenopodia spicata (E. Mey.) Presl.	l, r	S	Duncan1UN	m
Adiantum capillus-veneris L.	l	A	Duncan2UN	k

Plant Species	Plant part	Collection site	Voucher specimen number	Uses
Agapanthus africanus (L.) Hoffmg.	l, r	A	Duncan3UN	m
Agave americana L.	l	A	Duncan4UN	f, m
Catha edulis (Vahl) Forssk. ex Endl.	l	A	Duncan5UN	f, h, m
Cannabis sativa L.	l	S	Duncan6UN	hd, m
Clausena anisata (Willd.) Hook. f. ex Brnth.	l	B	Duncan7UN	h, k, m
Dietes iridioides (L.) Sweet ex Klatt	l, r	A	Duncan8UN	fd, m
Drimia elata Jacq.	l, bu	S	Duncan9UN	f, m
Drimia robusta Bak.	l, bu, r	S	Duncan10UN	-
Dombeya rotundifolia (Hochst.) Planch. var. *rotundifolia*	l, ba	A	Duncan11UN	f, m
Ekebergia capensis Sparrm	l	B	Duncan12UN	h, m
Hypoxis colchicifolia Bak.	l, r	A	Duncan13UN	m
Mesembryanthemum L.	l, s	A	Duncan14UN	f, m, p
Protorhus longifolia (Bernh.) Engl.	l	B	Duncan15UN	f, m
Rhus chirindensis Bak. f.	l, ba	B	Duncan16UN	h, m
Sclerocarya birrea (A. Rich.) Hochst. Subsp. *caffra* (Sond.) Kokwaro	l	B	Duncan17UN	fd, h, k, m, p

Plant Species	Plant part	Collection site	Voucher specimen number	Uses
Stangeria eriopus (Kunze) Baill.	l	B	Duncan18UN	f, m
Tulbaghia violacea Harv.	l, r	B	Duncan19UN	f, k
Turraea floribunda Hochst.	l	B	Duncan20UN	m, p

Key: ba: bark r: roots
s: stem l: leaves
bu: bulbs UN: University of Natal, Pietermaritzburg, Herbarium
A: Botany Department, University of Natal, Pietermaritzburg
B: Botanical Gardens, Pietermaritzburg
S: Silverglen Nursery, Durban
f: Gastro-intestinal
fd: Includes uses for diarrhoea/dysentery
h: Debility - tonics, blood purifiers, liver complaints, anthrax infection
hd: diabetes
k: Febrile - colds, fever, influenza, malaria
m: Cardiac - heart, blood pressure
p: Swellings, growths, warts/ haemorrhoids, scrofula, dropsy

2.3 Synthesis of Dansyltriglycine

In order to prepare the substrate for the ACE assay, triglycine was dansylated by combining solution A [250 mg triglycine in 25 ml of a 2% (w/v) $KHCO_3$] and solution B (500 mg dansyl chloride dissolved in 25 ml acetone). The mixture was stirred for 1 hour at room temperature. The mixture was evaporated to dryness by freeze-drying 2 ml aliquots which had been frozen in liquid nitrogen. The synthesized compounds were then dissolved in 40 ml methanol

and the solution stored at -20 °C. Half of this solution was applied to a 450 x 25 mm glass column filled with 60 g silica powder (Merck Silica gel 60, 0.063-0.2 mm) suspended in ethyl acetate/iso-propanol/water (v/v ; 4:2:1.2). Fractions of 1.5 ml were collected. Ten μl of each fraction was run on 0.25 mm thin layer chromatography (TLC) plates (Merck) using the same eluent [ethyl acetate/iso-propanol/water (v/v; 4:2:1.2)] in order to acertain the exact location of the dansyltriglycine. The resulting fractions were then combined, frozen in liquid nitrogen and freeze dried. Stock solution was prepared by dissolving 1.66 mg dansyltriglycine in 500 μl HEPES assay buffer and stored at -20 °C until further use. The working solution was prepared by diluting the stock solution to a final concentration of 100 μg/ml (0.236 mM) in HEPES assay buffer. Fresh working solution was prepared before each assay.

2.4 Buffers

HEPES assay buffer and the elution buffer for HPLC, sodium phosphate buffer, were prepared according to the protocols in Appendix 1.

2.5 Angiotensin Converting Enzyme

Five units of angiotensin converting enzyme (ACE) was dissolved in 12.5 ml demineralized and glass distilled water (1 unit/2500 μl). Aliquots of 0.3 units (750 μl) of this solution were distributed into 7 ml glass vials and lyophilized. After lyophilization the enzyme was stored at -20 °C.

When performing the enzyme assay, 0.3 units of enzyme was dissolved in 750 μl HEPES assay buffer.

2.6 Sensitivity of the Test System as Measured with Captopril

In order to test the sensitivity of the system, the IC_{50} value for the reference inhibitor, captopril, was measured. A 57.6 X 10^{-9} M (in 75 μl assay) stock solution (CS) of captopril was prepared. Working solutions from the stock solution were prepared (Table 4).

Table 4: Captopril working solutions prepared for the ACE enzyme assay.

Name	Captopril (nM - in assay)
Cap1	57.6
Cap2	28.8
Cap3	14.4
Cap4	7.2
Cap5	3.6

The percentage inhibition of each of the above captopril working solutions was determined using the ACE assay described in Section 2.8. From the inhibition results the IC_{50} of captopril was determined.

2.7 Stopper Solution

Stopper solution (0.1 N $Na_2EDTA{\cdot}2H_2O$) was prepared according to the protocol in Appendix 1.

2.8 Enzyme Assay

Twenty five μl of inhibitor solution or HEPES assay buffer (control) were added to a 1.5 ml microfuge tube, mixed with 25 μl of enzyme solution and incubated at 37 °C for 5 minutes. The reaction was started by the addition of 25 μl of substrate. After 30 minutes of incubation at 37 °C, the reaction was terminated by the addition of 50 μl 0.1 N Na_2EDTA.

For each assay, the blank was replicated twice and the average used for the subsequent calculations.

2.9 Separation of Substrate (Dansyltriglycine) and Product (Dansylglycine) using High Performance Liquid Chromatography

High Performance Liquid Chromatography separations were carried out using a Varian 5000 instrument linked to a Spectra System UV3000HR detector using a UV wavelength of 254 nm. Separations were performed using an analytical Hypersil 5 ODS column which was 25 cm in length and had a 4 mm internal diameter (ID).

Acetonitrile and 0.01M phosphate buffer (pH 7.0) were used as the mobile phase. Isocratic elution with 85% phosphate buffer and 15% acetonitrile separated the dansyltriglycine from the dansylglycine within 20 minutes at a flow rate of 1.2 ml min^{-1}. All solvents used were HPLC grade.

2.10 Quantitative Analysis

It was established that the percentage conversion of the substrate by the enzyme was an accurate method to determine the percentage inhibition of the

samples. The following formula was used to calculate the percentage conversion of the substrate:

$$\% \text{ Conversion} = \frac{\text{Peak Area (dansylglycine peak)}}{\text{Peak Area (dansyltriglycine + dansylglycine peaks)}} \times 100$$

This method assumed that the blank (without the presence on any inhibitors) would have the highest percentage of the substrate converted. A sample exhibiting high inhibition levels would have a low percentage of the substrate converted. Using the following equation, the percentage inhibition of the sample could be calculated:

$$\% \text{ Inhibition} = \left[1 - \frac{\% \text{ conversion (sample)}}{\% \text{ conversion (blank)}}\right] \times 100$$

2.11 Gelatin-Salt Block Test for Detection of Tannins

Plant extract from 200 mg of plant material was dissolved in 4 ml 50 °C distilled water and allowed to cool. Three to four drops of an aqueous 10% (w/v) NaCl solution was added to the cooled extract to "salt out" any non-tannin compounds, thereby eliminating any false-positive tests for tannins. The resulting solution was filtered and 1 ml of the filtrate was placed into four shallow white porcelain wells.

Well one served as a control and no reagent was added. Well two had 4 to 5 drops of a 1 % gelatin solution added and the mixture was observed for the formation of precipitate. Well three had 4 to 5 drops of a solution containing 1 % gelatin and 10 % NaCl added, and the mixture was observed for the formation of precipitate. Well four had 3 to 4 drops of a 10 % ferric chloride ($FeCl_3$) solution added, and the mixture was observed for colour produced

and/or precipitate formation.

2.12 Serial Extraction of *Tulbaghia violacea* Leaves

In order to determine which solvent to use in the bulk extraction of the *Tulbaghia violacea* leaves, finely ground leaves (9 g) from *Tulbaghia violacea* were placed in a Soxhlet thimble and extracted with 150 ml hexane using a Soxhlet extraction apparatus. The hexane was heated to boiling point after which extraction took place for 2 hours. The extracted liquid was passed through a Millipore filter (0.44 μm). The solvent was removed at 37 °C under vacuum, using a rotary evaporator. The residue was then transferred to a pre-weighed pill vial. The above process was repeated using ethyl acetate, ethanol and distilled water.

2.13 Bulk Extraction of the *Tulbaghia violacea* Leaves

Ground leaves of *Tulbaghia violacea* (0.7 kg) were extracted in 10 l of distilled water by sonication. The extracted liquid was dried down to 500 ml in front of a fan. The liquid residue was stored at 4 °C.

2.14 Solvent Partitioning of Bulk Extract Residue

The compounds extracted during the bulk extraction were partitioned against a wide range of solvents in order to further separate the active compounds. Twenty ml of the water fraction was partitioned with either hexane, diethyl ether or butanol. It was established that the active compounds were extracted using butanol. The compounds extracted in the bulk extract were diluted to 1 l and partitioned against 1 l butanol. This process was repeated five times to yield 5 l of butanol containing the active compounds. The butanol was removed under reduced pressure, using a rotary evaporator. The liquid

residue was stored at -20 °C until further use.

2.15 Separation of Extracted Compounds using Cation Exchange Resin Chromatography

Forty grams of Dowex 50W-X8 was loaded into a 580 x 28 mm column. The 13.5 g of gum which had been extracted in butanol was dissolved in 20 ml dH_2O (pH 3.5) and loaded onto the column. It was firstly eluted with 80% ethanol until the elution was clear, followed by 5N ammonium hydroxide. The ammonium hydroxide was removed under reduced pressure, using a rotary evaporator. The residue was stored at -20 °C until further use.

2.16 Sephadex ® LH-20 Separation of Extracted Compounds

One hundred grams of Sephadex ® LH-20 was swollen in 10% methanol for 5 hours. It was then loaded onto a 760 x 24 mm column and allowed to settle overnight. One third of the sample (0.82 grams) was loaded and fractions of 8 ml collected over 15 minutes. The resulting fractions were then tested for ACE inhibitory activity , and the active fractions combined and brought to dryness under reduced pressure, using a rotary evaporator. The residue was stored at -20 °C until further use.

2.17 HPLC Methods

All solvents used were HPLC grade. Separations and detection took place using a Varian 500 instrument and Varian detector respectively, at a UV wavelength of 200 nm. A semi-preparative Hypersil column (250 mm X 4 mm ID) was used to separate the fractions at a flow rate of 2.5 ml min^{-1}. Two programs were used to separate the compounds:

Program1: Solvent A: dH_2O; Solvent B: Acetonitrile: elution conditions: linear gradient 100% A to 80% A over 20 minutes; isocratic 80% A for 15 minutes.

Program 2: Solvent A: dH_2O; Solvent B: Acetonitrile: elution conditions: isocratic 100% A for 20 minutes; linear gradient 100% A to 80% A over 10 minutes.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Optimisation of Assay and Conditions for Quantitative Analysis

The experimental parameters such as the NaCl and HEPES-buffer concentrations, pH, and the UV detection of dansylglycine had been optimised by ELBL and WAGNER (1991).

The HPLC method, however, had to be optimised for the equipment available in this study. According to ELBL and WAGNER (1991) the dansyltriglycine and dansylglycine could be separated within 23 minutes utilising a gradient HPLC method. HANSEN, NYMAN, WAGENER SMITT, ADSERSEN, GUDIKSEN, RAJASEKHARAN and PUSHPANGADAN (1993) described a method which could separate both the product and substrate within 7 minutes. This method was tested, but a similar separation could not be achieved. Utilising the program described in Section 2.9, the substrate, dansyltriglycine was separated from the product, dansylglycine within 20 minutes (Figure 4). Utilising an isocratic system ensured that there was no delay in injecting subsequent samples as was the case for ELBL and WAGNER (1991).

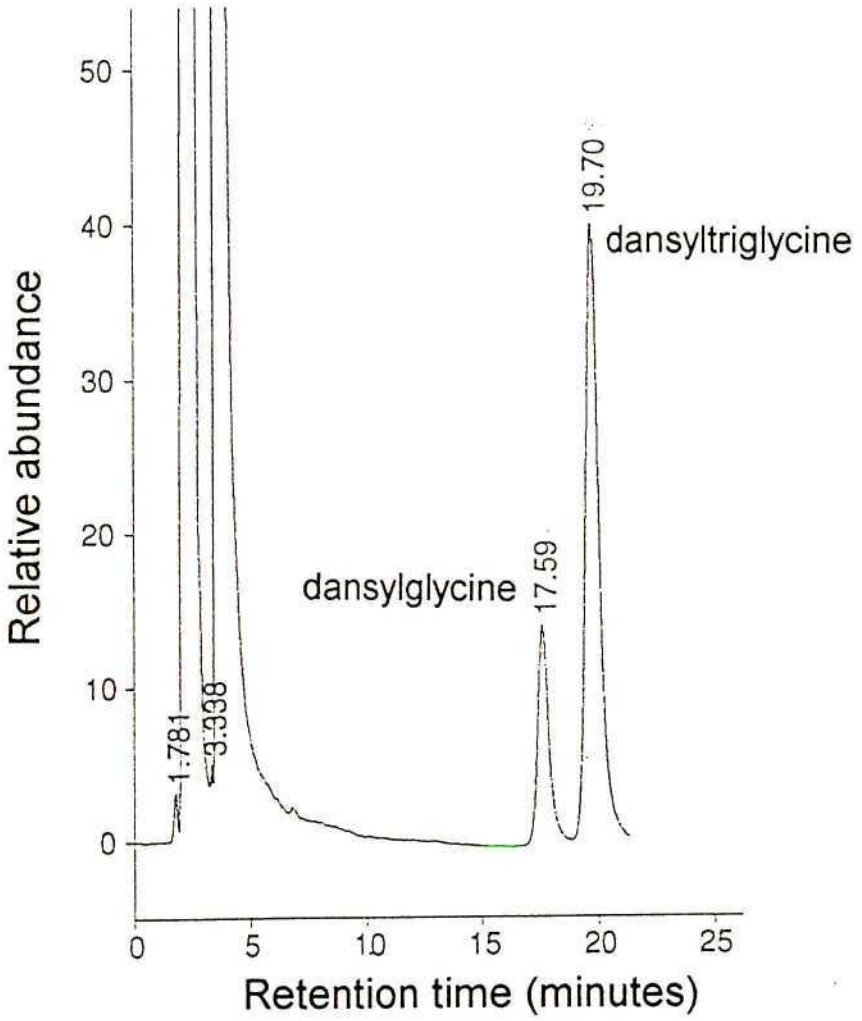

Figure 4: Chromatogram showing the HPLC separation of dansyltriglycine from dansylglycine.

3.2 Rate of Enzymatic Reaction

In order to carry out the ACE inhibitory test, the reaction must proceed at a rate that can be conveniently measured, and this rate must remain constant for a sufficient period of time. The quantity of substrate required to establish this condition must be determined under standardized conditions. Thus

variable parameters such as molarity and pH of the incubation buffer, temperature and enzyme concentration were kept constant. ELBL and WAGNER (1991) tested the time course of the enzyme reaction using three different substrate concentrations, and found a concentration of 0.236 mM to be ideal. This was the substrate concentration used for the duration of this study.

The time course action of the enzyme was determined by reacting dansyltriglycine at a concentration of 0.236 mM with the ACE enzyme and varying the incubation time. By plotting the percentage conversion of dansyltriglycine to dansylglycine against time, it was possible to see over what time period the enzymatic activity was constant. From Figure 5 it can be seen that the enzymatic rate was constant over a period of 35 minutes. Both ELBL and WAGNER (1991) and HANSEN, NYMAN, WAGENER SMITT, ADSERSEN, GUDIKSEN, RAJASEKHARAN and PUSHPANGADAN (1993) found this rate to be 35 minutes. Over this time 30% of the substrate was converted.

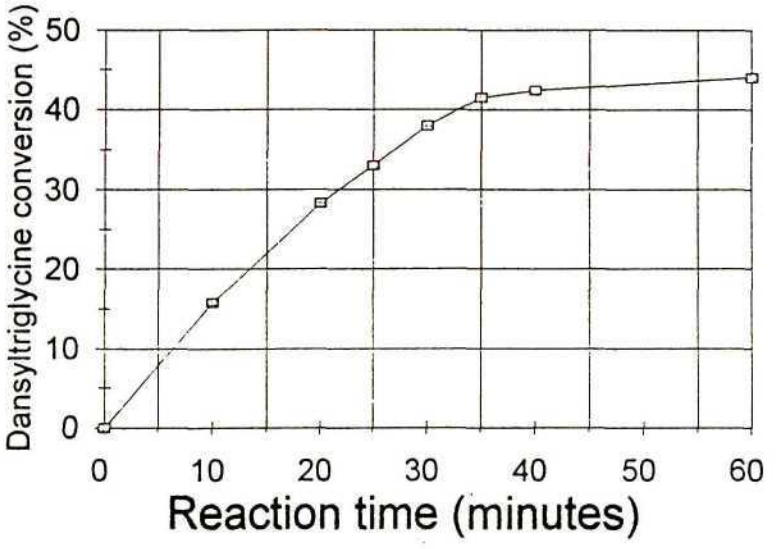

Figure 5: The time course of the ACE reaction.

The correlation coefficient for each time was calculated. According to the data in Table 5 a time of 35 minutes was chosen as the ideal incubation time.

Table 5: Correlation coefficients calculated at specific incubation times during the ACE time course assay to establish the ideal incubation time for the assay.

Incubation time (minutes)	Correlation coefficient
10	1
20	0.99549
25	0.99228
30	0.99043
35	0.98377
40	0.96194
60	0.80803

3.3 Establishing the Sensitivity of the Assay System

The IC_{50} value of an inhibitor is dependant upon inhibition mechanisms and assay conditions used and may vary from one laboratory to another, but is useful for comparing activities within a set of determinations (BUSH, 1983).

The sensitivity of the assay was tested by determination of the IC_{50} value of the competitive inhibitor captopril and comparing the values cited in the literature (Figure 6). From the data an IC_{50} value of 17.7 nM was established. HANSEN, NYMAN, WAGENER SMITT, ADSERSEN, GUDIKSEN, RAJASEKHARAN and PUSHPANGADAN (1993) found theirs to be 11 nM; ELBL and WAGNER (1991) 14 nM, while CUSHMAN, CHEUNG, SABO and ONDETTI (1977)

established a value of 23 nM. Thus, the system used in this study was not as sensitive as that used by ELBL and WAGNER (1991), but more sensitive than that used by CUSHMAN, CHEUNG, SABO and ONDETTI (1977).

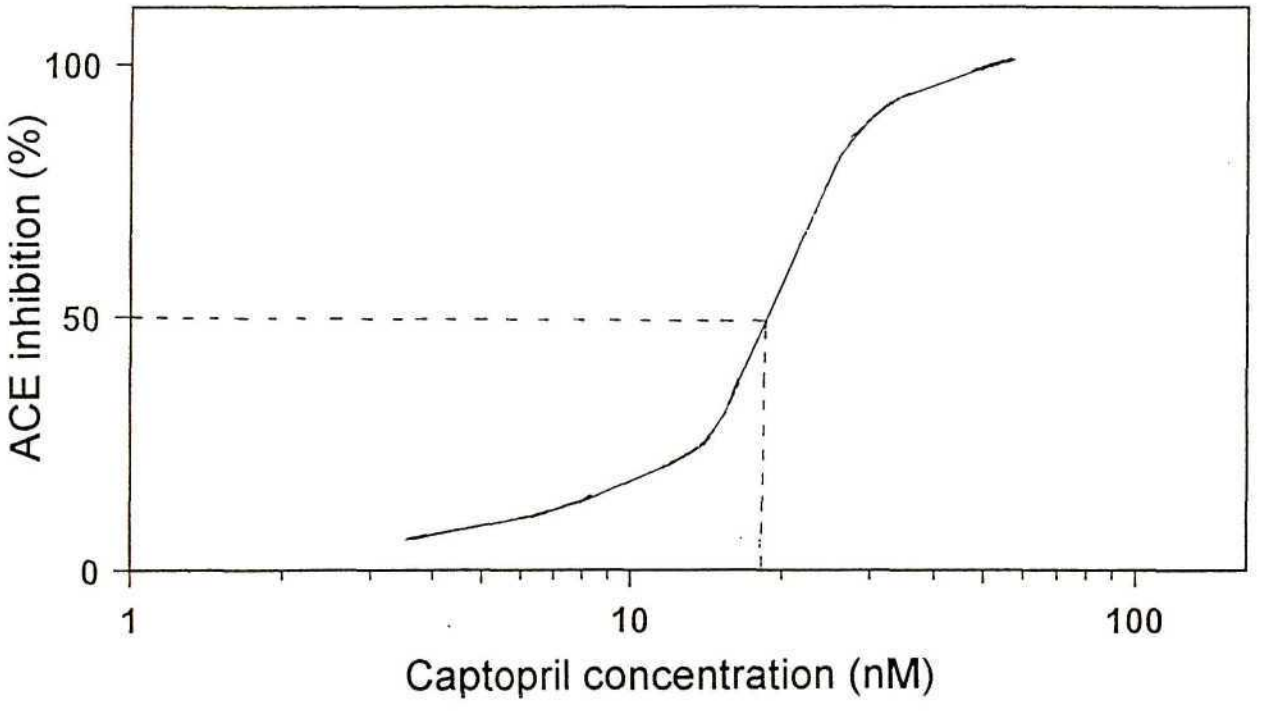

Figure 6: Inhibition of ACE by the reference inhibitor captopril at varying concentrations. The IC_{50} value was determined to be 17.9 nM.

3.4 Calculation of the Percentage Inhibition of Samples Tested in the Assay

Both HANSEN, NYMAN, WAGENER SMITT, ADSERSEN, GUDIKSEN, RAJASEKHARAN and PUSHPANGADAN (1993) and ELBL and WAGNER (1991) made use of an internal standard in their assay system to quantify the amount of product formation. From this they were able to calculate the percentage inhibition of the test compounds introduced into the assay. Determining the percentage inhibition utilising this method was inconsistent,

and a new method was developed (Section 2.10) that did not necessitate the addition of an internal standard.

In order to test the variability of the new method, three replicates comprising eight samples were carried out on three different days (Table 6).

Table 6: Investigation of the percentage conversion of dansyltriglycine to dansylglycine by ACE. Means given at 95% confidence limits.

Sample	Replicate 1 Substrate conversion (%)	Replicate 2 Substrate conversion (%)	Replicate 3 Substrate conversion (%)
1	28	32	29
2	31	31	31
3	29	31	30
4	30	32	31
5	29	30	29
6	29	31	30
7	31	31	31
8	31	32	30
Average	29.75 ± 0.498	31.25 ± 0.498	30.125 ± 0.498

The data within each treatment was uniform. Although the absolute difference between the replicates is very small there is a significant difference which is due to the uniformity within each treatment.

3.5 Concentration of the Plant Extract in the Assay

A concentration of 0.33 mg ml^{-1} assay volume (as proposed by ELBL and

WAGNER, 1991) was used in the assay and only plants exhibiting an inhibition of greater than 50 % were further investigated. At this concentration, false positive results due to nonspecific interaction of ubiquitous components like chlorophyll, tannins and different acids could practically be excluded. Concentrations ranging from 0.2 mg ml^{-1} to 3.3 mg ml^{-1} have been utilised (HANSEN, NYMAN, WAGENER SMITT, ADSERSEN, GUDIKSEN, RAJASEKHARAN and PUSHPANGADAN, 1993).

3.6 Screening of Plants for ACE Inhibitory Activity

According to ABRAMS, DAVIES and FERGUSON (1984), the principal pharmacological effects of ACE inhibitors are vasodilatory, increased sodium excretion, diuresis and lowering of blood pressure. Plants chosen for this study were thus selected based on their traditional usage in the treatment of hypertension and as diuretics. Plants used for the treatment of cardiovascular diseases were also of interest for screening for ACE inhibitory activity. Results of plant extracts screened to determine their level of ACE inhibition are shown in Table 7. Both ethanolic and aqueous extracts were tested in the assay. These are the two main solvents available to traditional healers in South Africa and would thus follow their extraction methods more closely.

Table 7: Plants screened for the presence of angiotensin converting enzyme (ACE) inhibitors. Aqueous and ethanolic extracts were tested .
* Indicates inhibition levels greater than 50% that were tested for tannins in the Gelatin-Salt Block Test.

Plant Name	Plant Part	ACE Inhibition (%)	
		Water	Ethanol
Adenopodia spicata (E. Mey.) Presl	leaves*	97	72
	roots*	8	89
Adiantum capillus-veneris L.	leaves	6	13
Agapanthus africanus (L.) Hoffmg.	leaves*	63	44
	roots	37	19
Agave americana L.	leaves*	72	82
Catha edulis (Vahl) Forssk. ex Endl.	leaves*	48	82
Cannabis sativa L.	leaves	3	18
Clausena anisata (Willd.) Hook. f. ex Brnth.	leaves*	54	1
Dietes iridioides (L.) Sweet ex Klatt	leaves*	80	7
	roots	13	13
Drimia elata Jacq.	leaves	16	16
	bulbs	2	0
Drimia robusta Bak.	leaves	24	20
	bulbs	12	7
	roots	23	0
Dombeya rotundifolia (Hochst.) Planch. var. *rotundifolia*	leaves*	10	83
	bark	5	24
Ekebergia capensis Sparrm	leaves	26	37

Plant Name	Plant Part	ACE Inhibition (%)	
		Water	Ethanol
Hypoxis colchicifolia Bak.	leaves	30	37
	roots	4	15
Mesembryanthemum sp.	leaves*	90	30
	stems*	30	57
Protorhus longifolia (Bernh.) Engl.	leaves*	64	77
Rhus chirindensis Bak. f.	leaves*	0	85
	bark	31	1
Sclerocarya birrea (A. Rich.) Hochst. Subsp. *caffra* (Sond.) Kokwaro	leaves*	0	68
Stangeria eriopus (Kunze) Baill.	leaves*	55	7
Tulbaghia violacea Harv.	leaves*	72	61
	roots	49	27
Turraea floribunda Hochst.	leaves	45	0

The highest inhibition (97%) of ACE was recorded for *Adenopodia spicata* leaves. A further seven extracts exhibited inhibition greater than 70% and five over 50%. It was interesting to note that in most cases the leaves of the plants were found to be the most active.

Plants exhibiting inhibition levels of greater than 50% (marked with asterisks in Table 7) were further investigated and subjected to a literature search in order to get a composite picture of compounds that have already been isolated from them, other known uses the plant has, and a detailed report (if available) of how the plant is administrated to the patient for the treatment of hypertension. The results are presented below:

The underground caudex [swollen stem base] of *Stangeria eriopus* is used for magico-medicinal purposes by certain ethnic groups in South Africa. In a survey carried out in July 1992 at the two largest traditional medicine markets at Durban and Isipingo involving a total of 170 gatherer-vendors, the 28 people trading in *Stangeria eriopus* were asked to assess the monthly amount of plant material sold, and to comment on the range of plant uses by the customers. Twelve Zulu herbalists were also interviewed concerning plant usage. A total of 3 410 plants were sold during the month of July at both markets, indicating that plant conservation actions are urgently needed. In a biochemical study, the dried caudices of *Stangeria eriopus* plants were found to be contain 85.1% carbohydrate, 6.1% protein, 6.1% fibre, 2.4% ash and 0.3% fat. The ratio of sitosterol to stigmasterol was 4:1; the main fatty acids were palmitic, oleic, stearic and arachidic acids. Twelve amino acids were identified, including β-alanine, γ-aminobutyric acid and pyroglutamic acid. The high concentration of sodium sulphate was thought to be responsible for the efficacy of *Stangeria eriopus* as an emetic. The toxins cycasin (0.21% DW) and macrozamin were also identified in the caudex (OSBORNE, GROVE, OH, MABRY, NG and SEAWRIGHT, 1994).

Agave americana is used by the Xhosa for high blood pressure (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM, 1996). *Agave attenuata*, *Agave lophantha* and *Agave legrellianan* have been studied for molluscicide activity. In China, hecogenin and tigogenin from *Agave* spp., are used as industrial precursors for the partial synthesis of steroidal drugs (CHEN and WU, 1994).

Agapanthus africanus is an indigenous plant which is also used in traditional herbal remedies during pregnancy and childbirth (VEALE, FURMAN and OLIVER, 1992). A crude decoction was tested for pharmacological activity on the isolated rat uterus and ileum. *Agapanthus africanus* exhibited direct

smooth muscle activity on the uterus and ileum preparations. Hot root infusions of *Agapanthus africana* are taken daily as emetics for heart disease (BRYANT, 1966). Known chemical constituents are sitosterol, yuccagenin and agapanthagenin. The steroid spirostan sapogenins, 7-, 8(14)- and 9(11)-dehydroagapanthagenin have been isolated from the rhizomes (GONZALEZ, FREIRE, GARCIA, SALAZAR and SUAREZ, 1974).

Known chemical constituents isolated from *Tulbaghia violacea* include sulfhur compounds, 2,4,7-tetrathiaoctane-2.2-dioxide and 2,4,5,7-tetrathiaoctane, fifteen flavones, including the flavonols kaempferol and quercetin and several sugars and steroidal saponins (BURTON, 1990). Bacteriostatic activity has been shown in the aqueous extracts from various parts of the plant. The presence of a β-adreneric agonist with inhibitory effects on normal muscle contractions was indicated in experiments with the extracts on isolated smooth muscle preparations (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM, 1996).

Infusions made from the inner part of the rhizomes of *Dietes iridioides* are taken orally or in enemas and are used in childbirth and against hypertension (PUJOL, 1990).

Unspecified parts of *Protorhus longifolia* are used by herbalists as medicine to strengthen the heart (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM, 1996). The bark yielded 10.2 - 18% tanning material and 7% tannin (WATT and BREYER-BRANDWIJK, 1962).

Rhus chirindensis is used by herbalists as a medicine for heart complaints (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM, 1996). The bark is also used to stimulate circulation and rheumatism (PUJOL, 1990). A flavone from

the roots of *Rhus undulata* was identified as apigenin dimethylether (FOURIE and SNYCKERS, 1984).

The bark of *Sclerocarya birrea* is taken as a tea twice a day to strengthen the heart (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM, 1996). Bark decoctions are taken in West Africa to treat diarrhoea. A lyophilized decoction of material collected in Burkina Faso showed significant activity against 2 models of experimentally-induced diarrhoea (GALVEZ, ZARZUELO, CRESPO, UTRILLA, JIMINEZ, SPIESSENS and DE WITTE, 1991). Fruits are aromatic and edible, but are also reported to be potent insecticides and to have possible disinfectant properties (BRYANT, 1966). A condensed tannin isolated from the crude drug inhibited intestinal mobility; its monomer, obtained by acid hydrolysis, was identified as procyanidin C_1 (GALVEZ, ZARZUELO, CRESPO, UTRILLA, JIMINEZ, SPIESSENS and DE WITTE, 1991).

Roots of *Adenopodia spicata* are used for sharp pains in the chest (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM, 1996). Root decoctions are also taken by the Mfengu for sharp, non-tubercular pains in the chest. The roots contain saponins (WATT and BREYER-BRANDWIJK, 1962).

Emetics made from boiling a handful of leaves from *Mesembryanthemum* sp. are administered for fearful dreams believed to be symptomatic of heart weakness (BRYANT, 1966).

Wood smoke and the steam from boiling the leaves of *Clausena anisata* are used to cleanse the body internally. Such procedures are also thought to strengthen the heart. Root decoctions are taken in doses of half a wine glass twice a day for heart ailments (PUJOL, 1990). The glandular leaves are strong smelling, particularly when crushed. Many terpenoid hydrocarbons,

sequiterpenoids and fatty acids have been isolated from pericarps, roots and leaves (REISCH, ADESINA, BERGENTHAL, and HUSSAIN, 1985). The leaves of *Clausena anisata* are widely used as an insect repellent and the root has molluscicidal effects (ADESINA and ADEWUNMI, 1985).

The inner bark of *Dombeya rotundifolia* is used to treat weakness of the heart (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM, 1996). There appears to be no published information on the chemical composition of *Dombeya* species (Dictionary of Natural Products on CD-ROM, release 4:2; 1996).

3.7 Determination of False Positives

The inhibition of ACE by the plant extract could be due to many factors, such as a specific, highly active compound present in small amounts; one or many compounds with low activity present in large amounts; or unspecific inhibitory compounds like tannins interacting with the enzyme. In many laboratories, the inhibitory activity of plant extracts has been reported to be due to tannins, or a mixture of tannins and compounds with low inhibitory activity. KNUDSEN and LANGHOFF (1995) illustrated this point where in an investigation of 32 Indian plant species, 10 plants showed inhibitory activity. After the precipitation of the tannins with gelatin, only one plant showed significant activity.

The thirteen plants inhibiting ACE by 50 % or more were tested for the presence of tannins using the gelatin-salt block test described in Chapter 2. The results are presented in Table 8.

3.7.1 Interpretation of Resultant Reactions

The reaction with ferric chloride has been used widely to identify phenolic

compounds, but it is non-specific and therefore of little value in distinguishing between different chemical classes in crude extracts. It gives a greenish colour with catechol derivatives (ortho dihydroxy phenols) and a blue colour with pyrogallol derivatives (3,4,5-trihydroxy phenols). If a deep blue-black colour appears, it is indicative of the presence of a 3,4,5-trihydroxy phenol, but the formation of a green colour does not necessarily indicate the absence of this group or the presence of a catechol group (ROBINSON, 1975). Thus, caution should be exercised when interpreting the results from the reaction with ferric chloride as described below:

No reaction with ferric chloride solution indicates that no tannins or phenolic compounds are present in the plant extract. A greenish-blue or greenish-black colour after the addition of ferric chloride (assuming that precipitation was noted after the gelatin-salt block test) indicates the presence of tannins of the condensed type. Bluish-black colour after the addition of ferric chloride (assuming that precipitation was noted after the gelatin-salt block test) indicates the presence of tannins of the pyrogallol type. A negative gelatin-salt block test (no precipitate), but colours produced (either greenish or blue-black) after the addition of ferric chloride solution, indicates that there were no tannins present. In these cases colour changes were induced by other phenolic plant constituents.

As can be seen from Table 8 , seven of the thirteen plants investigated produced a negative gelatin-salt block test. Out of these five, *Agave americanus* did not react with the ferric chloride and it could be concluded that its inhibiting properties were not due to the presence of tannins nor to other phenolic plant constituents. It was interesting to note that the results for plants showing positive reactions for the presence of tannins in Table 8 are in agreement with reports in the literature.

Table 8: Results obtained when plant extracts exhibiting levels greater than 50% ACE inhibitory activity were tested for the presence of tannins using the Gelatin-Salt Block Test.

Plant name	**Plant Part**	**1% Gelatin**	**1% Gelatin and 10 % NaCl**	**Ferric Chloride ($FeCl_3$)**
Adenopodia spicata	leaves roots	P. P.	P. P.	Green/Black Black/Grey
Agapanthus africanus	leaves	N.P.	N.P.	Green/Brown
Agave americana	leaves	N.P.	N.P.	No Change
Catha edulis	leaves	P.	P.	Green/Black
Clausena anisata	leaves	N.P.	N.P.	Green/Black
Dietes iridioides	leaves	N.P.	N.P.	Green/Brown
Dombeya rotundifolia	leaves	P.	P.	Brown/Black
Mesembryanthemum spp.	stems leaves	N.P. N.P.	N.P. N.P.	Orange Green/Brown
Protorhus longifolia	leaves	P.	P.	Black
Rhus chirindensis	leaves	P.	P.	Green/Black
Sclerocarya birrea	leaves	P.	P.	Green/Black
Stangeria eriopus	leaves	N.P.	N.P.	Green/Brown
Tulbaghia violacea	leaves	N.P.	N.P.	Green/Brown

Key: N.P. No precipitate P. Precipitate

The bark of *Protorhus longifolia* yields tanning material and tannin (WATT and BREYER-BRANDWIJK, 1962). Although not much is known about the chemical composition of *Rhus chirindensis*, tannins have been found in the bark and twigs of *Rhus laevigata* (WATT and BREYER-BRANDWIJK, 1962).

The gum of *Sclerocarya birrea* is rich in tannins. Tannins are also present in the leaves of the plant (GUEYE, 1973 cited in OLIVER-BEVER, 1986).

3.8 Selection of Plant for Bioassay-Guided Isolation of Active Compound(s)

It was decided to use the leaves of *Agave americanus* for further investigation. Unfortunately, when new material was obtained, its ability to inhibit ACE was vastly reduced when compared to the earlier results. Further investigation showed that plant material remaining from the first collection still exhibited high inhibition levels (Table 9). It was concluded that the most likely cause for the loss of activity was seasonal variations in secondary plant metabolites, with the active plant material being collected in summer, and the inactive plant material in winter. A seasonal variation in the ACE inhibiting properties of *Dietes iridioides* was also established. The inhibitory properties of leaves harvested in winter differed from those harvested in summer (Table 9).

Table 9: Percentage inhibition of ACE by *Agave americana* and *Dietes iridioides* leaves harvested in winter and summer.

Plant Name	Season Harvested	ACE Inhibition (%)	
		Water	Ethanol
Agave americana	Summer (January)	85	74
	Winter (June)	46	30
Dietes iridioides	Summer (January)	80	7
	Winter (June)	32	3

After further testing of plants that had displayed a negative gelatin-salt block test, it was decided that further investigations be made using *Tulbaghia violacea*, which was collected in June from the National Botanical Gardens, Pietermaritzburg.

3.9 Establishment of the Optimum Extracting Solvent

A serial extraction was carried out using hexane, ethyl acetate, ethanol and dH_2O successively (Section 2.12). This was carried out in order to determine which solvent extracted the compound(s) inhibiting ACE. The results of the assay can be seen in Table 10.

Table 10: Percentage inhibition of ACE by extracts obtained by using various solvents for the serial extraction of the leaves of *Tulbaghia violacea*.

Solvent	ACE Inhibition (%)
Hexane	3
Ethyl Acetate	12
ETOH	60
dH_20	74

As can be seen in the above Table, the aqueous extract gave the highest inhibitory result.

3.10 Testing Stability of the Aqueous Extract

The aqueous extract was boiled for 1 hour to determine its stability before the bulk extraction was made. The percentage inhibition of the resultant fraction was calculated using the ACE assay (Table 11).

Table 11: Percentage inhibition of ACE by the boiled aqueous *Tulbaghia violacea* extract.

Extract	ACE Inhibition (%)
dH_20	74
dH_20 (boiled)	71

Its was interesting to note that boiling the extract lead to an insignificant loss of inhibitory activity. It was thus concluded that the activity of the sample was not due to the presence of peptides or proteins, which would have denatured during boiling.

3.11 Bulk Extraction

Dried and finely ground *Tulbaghia violacea* leaves (0.7 kg) were extracted in 10 l dH_20 by sonication (Section 2.13). The extraction was successful, with the resulting residue exhibiting 90 % inhibition in the ACE assay.

As aqueous extracts posses certain difficulties in normally used purification techniques, it was decided to partition the aqueous extract against a range of solvents in order to establish whether or not the activity moved into an organic phase.

3.12 Solvent Partitioning of the Aqueous Extract

Portions of the bulk extract (20 ml) were partitioned against hexane, diethyl ether and butanol individually. The resultant residues were assayed in order to determine the solvent which was to be used to partition the bulk aqueous extract (Table 12).

Table 12 : Percentage inhibition of ACE by the compounds extracted using different solvents.

Partitioning Solvent	ACE Inhibition (%)
Hexane	64
Diethyl ether	62
Butanol	79

The compounds partitioning into butanol gave the highest ACE inhibition. The aqueous extract against which butanol was partitioned still exhibited ACE inhibitory activity of 70%.

The butanol fraction was taken to dryness, yielding 13.5 grams of a gum. The aqueous extract was dried under reduced pressure, and the resultant residue stored at - 20°C until further use.

3.13 Cation Exchange Resin Chromatography of the Active Butanol Fraction

The 13.5 grams of gum obtained after drying the butanol fraction was dissolved in 20 ml dH_2O (pH 3.5) and fractionated using cation exchange resin chromatography (Dowex 50W-X8). The column was washed with copious amounts of 80% ethanol. This was followed by 5N ammonium hydroxide to remove the absorbed compounds. The ethanolic and ammonium hydroxide fractions were collected, taken to dryness and assayed (Table 13).

As can be seen in Table 13, compounds exhibiting ACE inhibitory activity were limited to the ammonium fraction. Compounds present in the ethanolic fraction exhibited no ACE inhibitory activity, whilst comprising 82% of the initial butanol factions obtained. Using the cation exchange resin chromatography step thus

proved to be a particularly powerful step in removing non-active impurities.

Table 13: Percentage Inhibition of ACE by the two fractions obtained following cation exchange purification.

Fraction Assayed	ACE Inhibition (%)	Weight recovered (g)
Ethanolic wash	0	10.9
5N Ammonium hydroxide	84	2.46

3.14 Fractionation of the Ammonium Residue by Sephadex® LH-20

The ammonium residue (2.46 g) was purified by Sephadex ® LH-20 using 10% methanol as eluent. One hundred fractions were collected, and initially every fifth fraction assayed. After establishing the region of activity, all fractions within the region were assayed. Fractions 13-17 exhibited inhibition greater than 80% and were thus combined and evaporated to dryness under nitrogen leaving a dry residue of 250 mg in weight. This residue was subjected to several TLC systems. No successful separation was attained. Due to the hydrophilic nature of the compounds, it was not expected that they could be separated by normal-phase silica gel chromatography. It was thus decided to proceed directly to reverse phase HPLC.

3.15 Separation of the Active Residue using HPLC Techniques

The residue collected after the Sephadex purification step (Fractions 13-17) was re-suspended in dH_2O and fractions of 50 μl (5 mg) were fractionated by HPLC (Semi-preparative Hypersil column; 2.5 ml min^{-1} flow rate; UV wavelength 200 nm; Solvent A: dH_2O; Solvent B: Acetonitrile: elution conditions: linear gradient 100% A to 80% A over 20 minutes; isocratic 80% A for 10 minutes). The compounds were eluted off the column within 30

minutes (Figure 7).

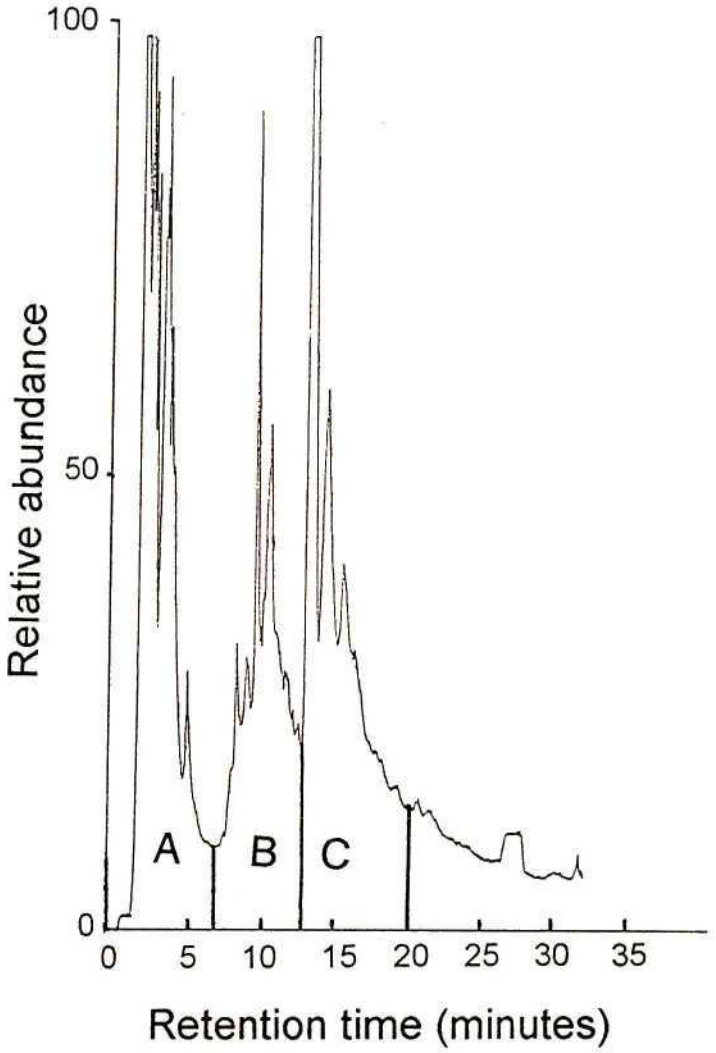

Figure 7: Chromatogram showing the separation of the active residue obtained after Sephadex purification (Fraction 13-17) by HPLC.

As can be seen in the above chromatogram, the residue yielded many UV-peaks. These were grouped together into three Fractions, namely A, B, and C. Each Fraction was collected, taken to dryness and assayed (Table 14).

Table 14: Percentage inhibition of ACE by Fractions A, B and C obtained following HPLC of the active Fraction collected after Sephadex purification (Figure 7).

HPLC Fraction	ACE Inhibition (%)
A	67
B	92
C	59

Fraction B exhibited the greatest ACE inhibition activity. It was thus decided to attempt to isolated the active compound(s) from this fraction by further HPLC.

Ten mg of the residue (Fraction B) was fractionated using HPLC (Semi-preparative Hypersil column; 2.5 ml min^{-1} flow rate; UV wavelength 200 nm; Solvent A: dH_2O; Solvent B: Acetonitrile: elution conditions: isocratic 100% A for 20 minutes; linear gradient 100% A to 80% A over 10 minutes). Fractions corresponding to Peaks 1-6 (Figure 8) were collected, taken to dryness and assayed (Table 15).

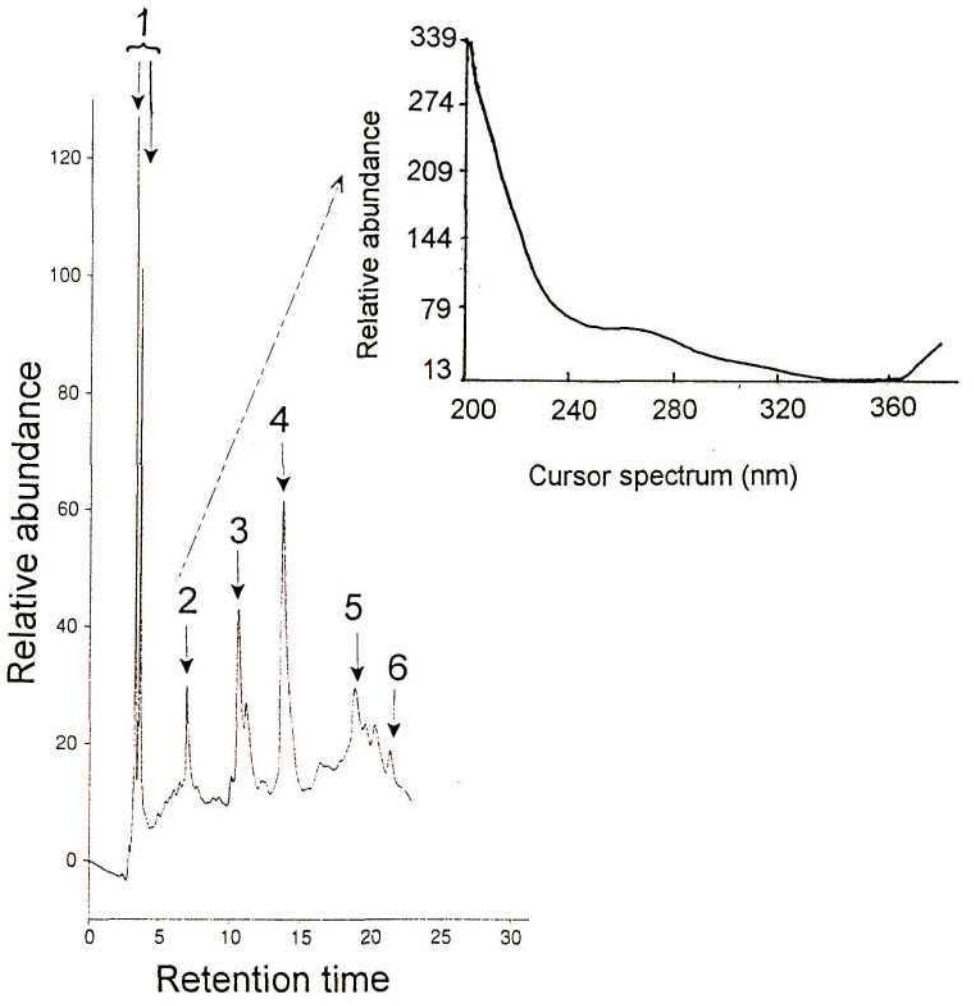

Figure 8: Chromatogram showing the separation of Fraction B by HPLC and the UV spectra of Fraction 2, the most active fraction.

Table 15: Percentage inhibition of ACE by Fractions 1 to 6 collected after fractionation of Fraction B by HPLC.

Peak Number on Chromatogram (Figure 8)	ACE Inhibition (%)
1	50
2	52
3	14
4	42
5	0
6	7

As can be seen from Table 15, Fractions 1 and 2 exhibited significant levels of ACE inhibition, although not as high as the levels in Fraction B. This could be due to the synergistic nature of the compounds that were separated.

A diagramatic representation of the procedures used to fractionate the residue from the bulk extract up to this point is summarized in Figure 9.

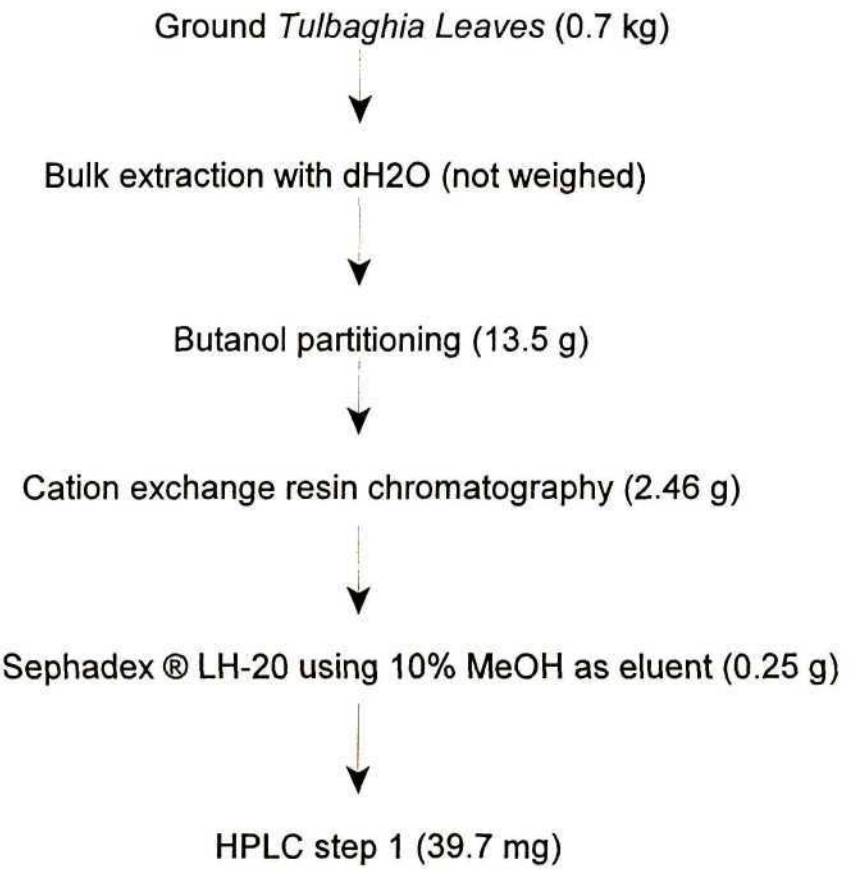

Figure 9: Flow diagram illustrating the purification steps and the amount of active fraction recovered after each step.

Due to time constraints, the remainder of Fraction B was not fractionated into Fractions 1 and 2. Further purification will take place, but the results won't be presented in this study.

3.16 ACE inhibitory Compounds Isolated from Plants in Previous Studies and Chemical Properties of Fraction 2

Angiotensin converting enzyme inhibitors were first isolated from plants in the mid 1980's. Since then, approximately 82 compounds exhibiting ACE inhibitory activity have been isolated, comprising mainly of proanthocyanindins, flavonoids, terpenes, peptides and amino acids. Hydrolysable tannins, phenylpropanes, xanthones, fatty acids and alkaloids are represented, but not to the same extent (HANSEN, 1995). In an attempt to get a better idea about the compound(s) causing ACE inhibition in this study, the chemical properties of Fraction 2 (Section 3.15) are discussed.

The active fraction is hydrophilic. After boiling for 1 hour no loss in ACE inhibitory activity was noted. It does not exhibit any fluorescing properties when applied on a TLC plate and observed under 254 and 365 nm. Analysis of TLC plates using anisaldehyde and 20% H_2SO4 as spray reagents gave no results. Amino acids were tested for by spoting the fraction on a TLC plate and spraying with ninhydrin spray reagent. The results were negative. Tannins were tested for using the gelatin-salt block test. The results were negative. Poly-phenolic compounds were tested for using $FeCl_3$. This test was also negative. The fraction was eluted off a reverse phase HPLC column with H_2O as the mobile phase within 7 minutes confirming its hydrophilic nature. Using the characteristics discussed above, groups of naturally-occurring compounds have been eliminated.

Both simple and furano coumarins as well as flavonoids have been eliminated as these compound groups always fluoresce at 365 nm. Xanthones have been eliminated on the basis that their distinctive UV spectrum does not match the one obtained for the active region (Figure 8). Steroids and fatty acids are not hydrophilic and thus would not be eluted off a reverse phase column using H_2O

as the mobile phase. Proteins and peptides have already been discounted due to the fact that the activity of the fraction is not lost through boiling (Section 3.10). Amino acids , tannins and poly- phenolic compounds were eliminated through specific tests.

Groups of natural compounds which could not be eliminated were saponin glycosides, polyacetylenes, chromene glycosides or other low molecular weight glycosides. This is due to the hydrophilic nature of the glycosides and their UV spectra, which is similar to that of the active fraction.

CHAPTER 4

CONCLUSIONS

Twenty plants that are routinely used by Zulu traditional healers for the treatment of hypertension were assayed to determine their levels of angiotensin converting enzyme (ACE) inhibitory activity. Out of the twenty plants assayed, eight exhibited ACE inhibitory levels greater than 70% with a further five over 50%, giving a 65 % hit rate. Both aqueous and ethanolic extracts exhibited high levels of ACE inhibition. These two extractants would be the most freely available to traditional healers, and thus most extensively used. Other plants used by traditional healers could not be obtained, and thus there is still a wealth of plants to be tested for their ACE inhibitory activity.

The presence of a strong ACE inhibitory activity does, however, not necessarily imply that the species could provide powerful anti-hypertensive drugs. As discussed by WAGNER, ELBL, LOTTER and GUINEA (1991), some flavonoids may show an *in vitro* activity due to the generation of chelate complexes within the active centre of ACE. It must be remembered that if the plant exhibits low levels of ACE inhibitory activity, that it may act on a different mechanism causing hypertension and could thus be used as a anti-hypertensive agent.

The ACE bioassay, which was set up during this study, yielded results that were constant and reproducible. This makes further research into ACE inhibitory compounds more feasible.

The separation techniques used in this study proved to be very efficient in the fractionation of compounds from the original sample. The results obtained using the techniques were very reproducible. This would aid further attempts to isolate the active compound(s) from *Tulbaghia violacea* in the future.

It was unfortunate that due to time constraints the compound(s) extracted from *Tulbaghia violacea* that inhibited ACE were not isolated. Steps that still require attention in future are further HPLC separation and GCMS analysis, after which the isolated compounds could undergo structural elucidation a task beyond the scope of this M.Sc project.

Other plants that showed promise were *Agave americana* and *Dietes iridioides*. It was unfortunate that they both showed seasonal variation with respect to their inhibitory activity and could not be further investigated in this study. Further studies into ACE inhibitory compounds from plants could thus start with these two promising plants.

Within the wealth of plants in South Africa, there lies an untapped reservoir of potentially useful chemical compounds, unique templates that could serve as starting points for analog preparation by chemists, or as interesting tools that can be applied to achieve a better understanding of biological processes involved in hypertension.

The process of isolating pharmacologically-active compounds from plants in their pure form is often long, troublesome and requires a multi-disciplinary approach. Projects need to run concurrently to test toxicity of active compounds as well as to make the results available to the traditional healers, without whose knowledge studies such as this would take many more years to complete.

FARNSWORTH (1984) summed it up as follows: The discovery of useful drugs from plants will be best carried out through a concentrated multi-disciplinary attack on the plant kingdom by means of collaboration, good planning, imaginative and creative thinking, dedication, a touch of luck, and a great deal of serendipity.

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APPENDIX 1

HEPES Assay Buffer

HEPES-NaOH (50 mM) (11.92 g) and 17.52g NaCl (300 mM) were added to 900 ml deionised distilled water and the pH adjusted to 8.0 with NaOH. The final volume was made up to 1 l.

Elution Buffer for HPLC

Stock solution A was prepared by dissolving 15.601 g (0.1 M) of monobasic sodium phosphate (NaH_2PO_4) in distilled water to a total volume of 1 l. Stock solution B was prepared by dissolving 14.196 g (0.1M) of dibasic sodium phosphate (Na_2HPO_4) in distilled water to a total volume of 1 l. By mixing 50 ml of A and 50 ml of B and diluting to a total of 1 l a phosphate buffer (0.01M) of pH 7.0 was prepared. The buffer was then filtered through a 0.22 μm filter.

Stopper Solution

$Na_2EDTA.2H_2O$ (3.72 g) was dissolved in 100 ml distilled water (0.1 N).