School of Environmental Biology

Cardioactive Compounds from the Australian Plant Genus, *Eremophila* (Myoporaceae)

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DECLARATION

I declare that all work presented in this thesis is that of myself alone unless otherwise acknowleded. The contents of this thesis have not been submitted previously, in whole or in part, in respect of any other academic award.

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Abstract

For over 40 000 years, the Australian Aboriginal people relied on native plants as a source of medicinal agents. Although they employed a wide range of species for this purpose, some of the most commonly used were *Eremophila* species (Myoporaceae). In particular, *E. alternifolia* was considered the 'number one medicine'. Reportedly, infusions of the leaves were used both internally and externally as an analgesic, decongestant and expectorant. This was believed to be useful in the treatment of colds, influenza, fevers and headaches, as well as for sterilising septic wounds and for promoting general well being.

A methanolic extract of *E. alternifolia* leaves was tested on Langendorff rat hearts and was shown to mediate significant increases in heart rate (chronotropism), contractile force (inotropism) and coronary perfusion rate (CPR). With similar responses to those induced by adrenaline, it seemed likely that the active constituents of *E. alternifolia* leaves may have acted through adrenergic receptors. Challenging the extract with α- and β-adrenergic receptor antagonists did not, however, significantly reduce the size of responses. The active compound acted through a different mechanism.

The identity of the active constituent in *E. alternifolia* leaves was revealed and shown to be a known phenylethanoid glycoside called verbascoside. Also known as acteoside, this compound's identity was confirmed by comparison of its spectral parameters with those described in the literature and by comparative tlc behaviour with that of standard samples. Its effects on the Langendorff rat heart were similar to those induced by the methanolic extract, but were more pronounced and did not act through adrenergic receptors or slow calcium channels . Repeatedly, 1 ml of 1 mM verbascoside dose-dependently increased

chronotropism, inotropism and CPR in Langendorff rat hearts by increasing intracellular levels of the second messenger, cyclic, 3',5'-adenosine monophosphate (cAMP). The increase in cAMP production occurred in response to an increase in prostacyclin. Significant increases in this hormone-like compound were detected in hearts treated with verbascoside. The calcium channel blocker, verapamil, did not significantly diminish the effects of verbascoside.

Another of the highly prized medicinal agents employed by the Aboriginal people was *E. longifolia*, a plant of sacred and mystical significance. Infusions made from the leaves were prepared for eye washes, as counter-irritants, for treating headaches and for skin and body washes. Pharmacological testing of the methanolic extract of leaves indicated that one or more active compounds with a biphasic effect on the Langendorff rat heart were responsible. The major active constituent was identified as an iridoid glucoside called geniposidic acid. One ml of 1 mM geniposidic acid significantly inhibited chronotropism, inotropism and CPR. The compound did not, however, induce a biphasic effect. This was later rationalised by the small amounts of verbascoside that co-occur in the extract.

Four similar iridoid glucosides that had been isolated from three other *Eremophila* species were tested for their effects on Langendorff rat hearts. Melampyroside (from *E. pantonii*), verminoside (*E. ionantha*), ferruloylajugol (*E. pantonii*) and catalpol (*E. maculata* subsp *brevifolia*) all significantly altered myocardial activity in the isolated rat heart preparations. Melampyroside and verminoside were predominantly stimulatory, while ferruloylajugol was biphasic and catalpol only inhibitory. The effects of these iridoids is discussed in comparison with geniposidic acid and other known cardioactive iridoid glucosides. The results suggest that the effects exhibited by these compounds is a more common phenomenon than was previously realised.

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GLOSSARY OF ABBREVIATIONS

ACE	angiotensin converting enzyme
5'-AMP.....	5'-adenosine monophosphate
ANP	atrial natriuretic peptide
ATP	adenosine triphosphate
α-AR	alpha adrenergic receptor
β-AR	beta adrenergic receptor
bpm	beats per minute
C	Controls
Ca^{++}	calcium ions
cAMP	cyclic 3',5'-adenosine monophosphate
CHD	coronary heart disease
Chronotropism.....	heart rate
CG	cardiac glycoside
CPR	coronary perfusion rate
CV	coronary vessels
D	di-methyl verbascoside
EC_{50}	dose causing 50 % excitation
fmol/ tube..	fico mol per tube
g	grams
GDP	guanasine diphosphate
Gs	stimulatory G protein
GTP	guanasine triphosphate
HDL	high density lipoproteins
HF	heart failure

IP	prostaglandin I receptors
LDL	low density lipoproteins
MeOH	methanol
ml/min	milli litres per minute
mg	milli gram
mM	millimola
nM	nanomola
nmol/g DW	nano mol per gram dry weight
nm	nano metre
Na^+	sodium ions
Na^+/K^+-ATPase...	sodium/potassium adenosine triphosphatase
NMR	nuclear magnetic resonance
NSB	non specific binding
NSW	New South Wales
NT	Norther Territory of Australia
OD	optical density
OH	hydroxy functional group
Pa	phentolamine
pa	per annum
$\%B/B_o$	percentage bound
PDE III.....	phosphodiesterase III enzyme
pg	pico grams
PGH_2	prostaglandin H_2
PGI_2	prostaglandin I_2 (prostacyclin)
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C

pmol/g	pico mol per gram
Pr	propranolol
RPM	revolutions per minute
SA	South Australia
S.E.M.....	standard error of means
spp	species
tlc	thin layer chromatography
µM	micro mole
UWA	University of Western Australia
Va	verapamil
WA	Western Australia
w/v	weight per volume
6-keto-$PGF_{1\alpha}$	6-keto prostaglandin

GLOSSARY OF MEDICAL AND BOTANICAL TERMS

Abortifacient	promotes abortions
Acteoside	synonym for verbascoside
Adrenergic receptor	glycoprotein in cell membranes that responds to adrenaline-like compounds
Agonist	stimulant
Ameliorated	improved; made better
Analgesic	pain killer
Antagonist	inhibitor or blocker
Anticoagulant	prevents blood clotting
Antipyretic	reduces fever
Arrhythmias	uncoordinated heart beats
Arrhythmogenic	generates arrhythmias
Attenuated	decreased
Auricle	synonym for atrium of the heart
Axil	the angle between two different parts of a plant
Axillary	adjective of axil
Calyx	the outermost part of the flower
Cardioactive	has an effect on the heart
Cardiotonic	strengthens heart activity
Catecholamine	a hormone, such as adrenaline, that acts on certain cells, eg. heart cells

Cholinergic receptor	glycoprotein in cell membranes that responds to acetylcholine-like compounds
Chronotropism.....	pertaining to heart rate
Decongestant	relieves congestion in the mucous membrane
Diastole	heart during relaxation
Diuretic	increasing urine flow
Drupe	fruit with one or two seeds enclosed in a stony-like structure
Epinephrine	synonym for adrenaline
Eremophila	a plant genus in the Myoporaceae family
Expectorant	promoter of mucus production
Exserted	protruding beyond and enclosure
Glabrous	without hairs
Globular	three dimensional; spherical
Haemorrhaging....	excessive bleeding
Hemolysis	destruction of red blood cells
Hepatotoxic	toxic to the liver
Histaminergic receptor.....	glycoprotein in cell membranes that responds to histamine-like compounds
Hypercholesterolemia	high cholesterol levels
Hypertension	high blood pressure
Hypotension	low blood pressure
Immunomodulating	immune system-regulating
Immunosuppressive	suppresses the immune system

Inotropism	pertaining to contractile force
Isoproterenol	β-adrenergic stimulant
Kusaginin	synonym for verbascoside
Lactating	secreting milk
Lanceolate	spear-like
Ligand	an ion or molecule that forms complex compounds
Leukocytes	white blood cells
Microsomes	tiny "protein factories" within all cells
Myocardial	pertaining to heart muscle
Neanderthal	*Homo sapiens neanderthalensis*
Normotensive	normal blood pressure
Nucleotide	a compound consisting of sugar, phosphoric acid and a base
Oblanceolate	spear-shaped with rounded edges
Ornithophilous.....	bird pollinated
Ostia	an opening
Ovoid	oval in shape
Pedicel	the stalk of individual flowers
Pendulous	drooped; hanging downwards
Petiolate	pertaining to the stalk of the leaf
Peritoneal	pertaining to the thin membrane that lines the walls of the gastrointestinal tract

Phentolamine	synthetic α-adrenergic antagonist
Phenylephrine	synthetic α-adrenergic agonist
Phosphorylates.....	adds a phosphate group
Poultice	hot herbs applied to the skin for treatment
Prophylactic	protecting from disease
Propranolol	synthetic β-adrenergic antagonist
Sessile	without a stalk
Stenosis	constriction of a passage or blood vessel
Sub-terete	almost circular in cross section
Succulent	fleshy; fruity
Symptomatic	indicative; a sign
Systole.....	heart during contraction
Terete	circular in cross section
Ulcerated	damage to soft tissue
Vasoconstriction...	constriction of blood vessels
Vasodilation	dilation of blood vessels
Verapamil	calcium channel blocker
Warty	warts on exterior

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CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1

General Introduction

1.1 Diseases of the heart

Despite an increase in the number of diseases in western industrialised countries, diseases of the heart are still considered one of the major causes of death. Thousands of people from all strata of the population die each day from heart disorders. In the US alone, cardiovascular diseases are responsible for more deaths than all other causes combined (Spencer, 1995). To help prevent or retard the progression of heart diseases, a variety of drugs have been developed. Medicines that have evolved over the last two or three decades have been useful in prolonging life and reducing suffering, but have not lived up to expectations in the advanced stages of disease.

Diseases of the heart, such as coronary heart disease (CHD), heart failure (HF) and hypertension (high blood pressure), have consistently been the focus of attention of practitioners of contemporary medicine. These three interrelated diseases are responsible for most deaths related to heart disorders. The widely accepted view for the genesis of CHD suggests that the build up of fats on arterial walls induces the formation of fibrous tissue called an atheroma. When ulcerated, the atheroma attracts platelets and gives rise to a blood clot, thus occluding the coronary arteries. Increases in serum levels of cholesterol (hypercholesterolemia) are thought to play a major role in the development of atheromas. A strong correlation exists between an increase in low density lipoproteins (LDL) and fibrous tissue formation. High levels of high density lipoproteins (HDL), on the other hand, are beneficial (Watkins *et al.*, 1985). If left untreated, CHD may lead to permanent damage of the heart (ischaemia) and may result in angina pectoris or myocardial infarction.

Angina pectoris is a severely painful condition onset by exercise, emotion, cold or eating. It occurs in response to a narrowing or obstruction of at least one or more of the coronary arteries. This alone accounts for 90 % of all instances of angina (Zoll *et al.*, 1951). The other 10 % arise from causes such as narrowing of the coronary ostia, aortic stenosis and severe pulmonary hypertension. In 1879, William Murrell established the use of sublingual nitroglycerine in treating angina. Since then, organic nitrates have been successfully employed for the rapid alleviation of the substernal pain caused by the condition. Nitrovasodilators relax the smooth muscle, especially those in the arteries and veins, thus decreasing left and right ventricular end-diastolic pressures. Symptomatic and prophylactic treatment of angina has also been achieved through the use of calcium channel and β-adrenergic blockers and anticoagulants, as have compounds that reduce myocardial oxygen consumption (Mason *et al.*, 1969; Goldstein and Eptsein, 1972).

Myocardial infarction (heart attack), in contrast, is more painful and prolonged than angina pectoris. Physical exertion and emotional crisis (Rahe *et al.*, 1974) are the major precipitating causes, but it can occur suddenly and without exertion (Lepeschkin, 1960). Myocardial infarction is therefore a more serious condition and often manifests itself as sudden death, especially in chronic cases. In the past, the use of anticoagulants has been shown to significantly reduce mortality in sufferers of acute myocardial infarction (Wright, 1971). Drugs such as heparin prevent the formation of thrombin (clotting factor) (Jacques, 1965). Others, eg. the coumarin and indandione derivatives, mediate essentially the same pharmacological effects. However, anticoagulant therapy may cause haemorrhaging, which can occur even when levels of the drugs appear to be within safe limits (Pastor *et al.*, 1962). In acute cases, disruptions to heart rhythm may also develop and lead to other complications.

Abnormal heart rhythm or cardiac arrhythmias are alterations in conductivity, automaticity or both in the heart (Hoffmann *et al.*, 1966; Cranefield *et al.*, 1973). In severe cases, a premature systole during the vulnerable phase of ventricular systole (during the brief

period of 0.06 sec.), gives rise to a series of repetitive responses in the nature of supraventricular arrhythmias (atrial fibrillation) (Wiggers and Wegria, 1940). First induced experimentally in 1850 by Ludwig and Hoffa, fibrillation has been extensively studied and is now reasonably well understood. Technically, it is described as the lack of coordination in the heart. If victims of fibrillation are not treated immediately, heart failure or cardiac arrest may soon follow. A number of antiarrhythmic drugs and other substances are available for treatment of arrhythmias.

Although common, the use of antiarrhythmic agents is limited. Most have a very low therapeutic index, often resulting in more severe complications than those they were meant to treat. Depressed contractility, impaired impulse conduction and depressed impulse formation are some of the side effects associated with overdoses of these compounds. A more common treatment is to apply an extrinsic electrical discharge across the chest to instantly depolarise all cardiac fibres and allow the sino-atrial (SA) node, which has greater automaticity, to assume control of the heart (Zoll *et al.*, 1956). This treatment is useful in all forms of arrhythmias, but long-term results have been disappointing (Resnekov, 1973). Most patients suffer a relapse within months.

Hypertension (high blood pressure) is also one of the major risk factors in cardiovascular disease (Luscher *et al.*, 1996). The severity of hypertension in adults is defined by Freis (1974) based on the following systolic and diastolic blood pressure readings: borderline hypertension = 140/90 to 160/100 mm Hg; mild hypertension = 150/90 to 160/104 mm Hg; moderate hypertension = 150/105 to 160/114 mm Hg and severe hypertension = > 160/130 mm Hg. There are two categories of hypertension: essential hypertension and secondary hypertension. Essential, or primary hypertension, accounts for 90 % of all cases of chronic diastolic hypertension. The other 10 % are accounted for by secondary hypertension. Death from aneurisms (ruptured blood vessels) in vital organs and from heart or kidney failure are common in severe or prolonged cases of high blood pressure. Alpha- and β-blockers, calcium channel blockers, angiotensin converting enzyme (ACE)

inhibitors and other antihypertensives are the cornerstone for the treatment of the disorder and have in the past halved the mortality rate in sufferers of hypertension (Dunea, 1971).

When left untreated, most diseases of the heart will degenerate into heart failure. Heart failure (HF) is the condition in which part or all of the myocardium fails and does not provide sufficient cardiac output. The most successful treatment to date involves the use of cardiac glycosides (CG). Cardiac glycosides are common throughout the plant kingdom and much has been written about their cardiotonic effects (see Repke *et al.*, 1995). In brief, these compounds increase cardiac contractility by binding to and inhibiting the enzymatic equivalent of the Na^+/K^+-pump, Na^+/K^+-ATPase (for a review of the "microscopic" mechanism of Na^+/ K^+-ATPase inhibition, refer to Repke *et al.*, 1995). Despite their wide-spread use, the narrow margin between the therapeutic and toxic doses of CG are of major concern to the clinician (Smith, 1988). Phosphodiesterase (PDE III) inhibitors have therefore recently emerged as potential replacements for CG (Cruickshank, 1993), but have not satisfied all the criteria for optimally challenging HF (Yusuf *et al.*, 1992). The search for more effective compounds for HF and all other diseases of the heart continues to attract much attention, with many researchers turning to natural products.

1.2 Natural products

Natural products have a long-standing association with the treatment of heart disease. The use of digoxin, a steroid glycoside from *Digitalis lanata*, dates back to 1785 (Withering, 1785) and is still routinely used in the treatment of HF (Yusuf *et al.*, 1992). Despite that, plant-derived drugs have not always been popular. In the past, most investigators have opted for synthetic compounds and have generally neglected what nature had to offer (Petkov, 1979). However, a turning point was reached when the antihypertensive properties of reserpine, a natural product extracted from *Rauwolfia serpentina* Benth., were realised. This lead to an increase in the search for and the use of natural products in

contemporary medicine. In addition, plants are a source of a wide variety of new and interesting model compounds that may serve as starting points for the development of future drugs. The natural assimilation of simple compounds into more complex compounds is far more superior than current synthetic chemical works (Petkov, 1979). Plant extracts may also contain several compounds acting synergistically to produce a more complete and balanced treatment. The plant world is therefore a potential and inexhaustible source of novel biologically active compounds and can no longer be ignored.

The search for useful plant-derived therapeutic agents can be achieved in three ways. Plants can be chosen at random from within areas of high plant biodiversity and endemism and then tested in known bioassays. This "trial-and-error" approach is usually very expensive and time consuming and is therefore not practical. A more effective approach would be to use chemotaxonomy as a guide. Researchers can concentrate their efforts on plant families and genera known to produce certain classes of compounds, eg. cardiac glycosides from the family Scrophulariaceae. Although a more practical approach, this method is still largely "trial-and-error". The last and most successful method is to tap into traditional knowledge (ethnopharmacology). This not only points the researcher to the plant as other sources of bioactive natural products, but also offers some insight into what they may be useful for.

1.3 Ethnopharmacology

Ethnopharmacology is a multidisciplinary study involving both the *natural sciences* (chemistry, medicine, botany and pharmacology) and the *humanities* (history, linguistics and ethnology). Aimed primarily at investigating age-old practices and cultures, it affords a better understanding of the therapeutical basis of traditional healing methods and, because of this, is a useful approach for the search of novel medicines (Svendson and

Scheffer, 1982; Samuelson, 1989). Ethnopharmacology also helps preserve traditional medical practices and knowledge and promotes the use of proven medicinals in nations that have to import modern medicines (Elizabetsky, 1991). This helps minimise the drain of hard currency in such countries (Gerez and Pedrosa, 1987).

Despite this, ethnopharmacology has, in the past, enjoyed only a marginal status (Elizabetsky and Shanley, 1994). The lack of interest was attributed to a combination of factors, of which poor interdisciplinary training programs, inadequate funding and poorly defined research methodologies were largely to blame for. Furthermore, research into ethnopharmacology is not easy. The collection and cultivation of useful plants, for example, are two of its problems, as are the accurate identification of species, the selection of the appropriate testing procedures and obtaining funding for such projects. And there is also the dilemma of ethically questionable purposes. Scientists are often accused of appropriating traditional knowledge and stealing materials for their own profit or professional advancement (Elizabetsky, 1991). In very few cases have host nations and native people received a significant share of any royalties. Posey (1990) suggests that legislation be passed to secure intellectual property rights for indegenous people.

Ethnopharmacology therefore stands at a crossroads (Plotkin, 1992). On the one hand, traditional methods and knowledge stand to disappear, along with any new and unknown cures and medicines. While on the other hand, there is the possibility of greater awareness, resulting in novel and more efficient medicines. This appears to be the road ethnopharmacology is now taking, and is largely due to the involvement of bodies such as the World Health Organisation (WHO) and pharmaceutical companies, both of which have realised that modern medicine is no panacea (Akerele, 1993; Elizabetsky and Shanley, 1994).

The first major step towards popularising ethnopharmacology was taken at the 1977 General Assembly of the WHO. At that meeting, the development of research and training

in the use of traditional medicines was encouraged and promoted. Since then, there has been renewed enthusiasm for ethnopharmacology by physicians, clinicians, other researchers and learning institutions. The 'ethno' sciences are now taught at various undergraduate and graduate levels at a variety of universities all over the world (Babulka and Piróth, 1996) and continue to generate much interest.

1.4 Ethnobotany

Ethnobotany, like ethnopharmacology, is a multidisciplinary science, with the difference that it deals specifically with herbal medicines. The use of plants for medicinal purposes dates back to Neanderthal times (Plotkin, 1992). Found near the grave of a 50 000 year old Neanderthal male, in a cave in north eastern Iraq (Shanidar Cave), were the remains of eight different species of plants, seven of which are still used for medicinal purposes. Other important archaeological sites have also shown that medicinal and edible plants were indispensable and formed the cornerstone of many great civilisations. For example, the *Pen Tsao* pharmacopoeia by the Chinese Emperor and herbalist, Shen Nung, compiled in 2800 BC, lists over 350 species of medicinal plants, while the Assyrians used at least 250 plants for their practices. In Babylonia, King Hammurabi had his pharmacopoeia engraved on stone pillars along with a code of practices. This is one of the most extensive of the early records.

The Egyptians also carved their records on stone. Carvings, listing medicinal plants brought back from Syria in 1500 BC, were found in the Temple of Karnak (Plotkin, 1992). Although this is one of the better known listings of Egyptian medicinal plants, the most famous of all is the scrolls of Ebers papyrus of 1550 BC. Measuring one by 67 feet, the scrolls were purchased by the famous German Egyptologist, Georg Ebers, from an Arab who claimed to have found them in the Necropolis outside Thebes. The scrolls

feature 877 prescriptions and formulas for diagnosing and treating a variety of illnesses. A number of chants were included with various prescriptions in case they failed.

Ancient Greek medicine, in contrast, was practised in large temples built in honour of Asclepias - God of Healing (Asclepias's snake-entwined staff has since become recognised as the symbol of medicine). All treatments in ancient Greece were performed by physicians, known as the sons of Asclepias, and were religious events often involving incantations, fasting and bathing (Low *et al.*, 1994). It was not until Hippocrates, who is considered the father of modern medicine, that healing was moved from the realms of mystery and religion into that of science. Hippocrates (cc 400 BC) believed that the four elements, fire, water, earth and air were represented in the human body by yellow bile (attributed to choleric), black bile (to melancholy), phlegm (to phlegmatic) and blood (to sanguine). It was widely believed that illnesses resulted in people whose balance of the humours was upset and could only be restored by bleeding, vomiting or sweating. Hippocrates also recognised the potential of using plants to treat illnesses. His treatise contains between 300 and 400 medicinal plants.

With the fall of Ancient Greece, many Greek philosophers and physicians moved to Rome, one of whom was Dioscorides. Born in the first century AD, Dioscorides became one of the most influential writers from ancient times. As a physician in the Roman army, he searched the countryside for medicinal plants, compiling them in his famous treatise, *De Materia Medica*. More than 600 plants, 35 animal products and 90 minerals were listed in it with advice on cultivation, harvesting, drug preparation and use. For centuries, *De Materia Medica* proved to be one of the most widely consulted herbals and was equalled only by Celcus's *De Medicina*. Arabic and Persian translations of the *De Materia Medica* formed the basis of later Moslem herbals, while a Latin translation was incorporated into herbals prepared by John Gerard and Nicholas Culpepper. Unfortunately, most of the original text was destroyed during the barbarian invasions. A number of Indian manuscripts also disappeared in similar fashion.

The earliest known recording of Indian medicinal plants is in the songs and poems of the Vedas, in which the first reference to snakeroot (*Rauwolfia serpentina*) appears (Plotkin, 1992). Reportedly, parts of the plant were used as tranquillisers and for disorders of the circulation. Its major active constituent is reserpine. The Aztecs and Incas also had extensive records and manuscripts dedicated entirely to traditional medical practices, but very few records of their erudition exist today. Most of these were destroyed along with the Aztec and Inca civilisations.

1.5 Traditional Australian Aboriginal Medicine

For over 40 000 years, the Australian Aboriginal people used plants for most of their medical practices. During their nomadic travels, these very competent and observant botanists (Stack, 1988) discovered a wide variety of medicinal and cultural uses for the local flora (Reid, 1979). New plants were encountered on a regular basis and were immediately tested for their effect in treating the illnesses that afflicted the Aborigines. The sick, frail and elderly may have served as test subjects when new plants were discovered (Barr *pers-comm.*). Then, once established as a useful species, information relating to the plant was passed onto the next generation of 'medicine men' by word of mouth. The Aboriginal people kept no written accounts of their erudition, the implementation of which gradually declined with the arrival of European colonisers. The lack of interest in traditional healing methods and medicinal plants may have been the result of the dissolving influence of modern western healing practices and drugs.

Some information was salvaged, however, and has since been annotated in Aboriginal pharmacopoeias. The majority of the Aboriginal plant preparations were used externally as skin washes (Barr, 1988, 1993; Latz, 1982, 1995; Low, 1990). Infusions and decoctions for internal use were, to a lesser degree, also common. Reportedly, the latter

were taken as analgesics, expectorants and abortifacients, as well as for treating colds, influenza and headaches (Barr, 1988, 1993; Low, 1990). Internal complaints were considered to be spiritual in nature, and were largely treated by spiritual means through the agency of powerful 'medicine men'. It therefore comes as no surprise that only approximately 10% of Aboriginal medicines were taken internally (Latz, 1995). Of these, twelve are known to be taken for disorders of the heart (Table 1.1). Lassack and McCarthy (1983) warn that some of these may have actually been used to treat indigestion and had no affect on the heart.

Table 1.1. Plant species used by the Aboriginal people for treating disorders of the heart.

Family	Species	Indication	Reference
Atherospermataceae	*Atherosperma moschatum*	Heart sedative	Maiden (1889)
	Daphnandra micrantha	Heart disease	Lassack and McCarthy (1983)
Euphorbiaceae	*Euphorbia drummondii*	Chest pains	Barr *et al.* (1993)
Fabaceae	*Templetonia egena*	Arrhythmias	Merck (1960)
Myoporaceae	*Eremophila gilesii*	Chest pains	Cleland and Tindale (1959)
	E. dalyana	Chest pains	Latz (1982)
	E. freelingi	Chest pains	Cleland and Tindale (1959)
	E. gilesii	Chest pains	Cleland and Tindale (1959)
Myrtaceae	*Eucalyptus dichromophloia*	Heart disease	Lassack and McCarthy (1983)
	E. terminalis	Heart disease	Reid (1979)
Scrophulariaceae	*Gratiola pedunculata*	Slows heart	Lassack and McCarthy (1993)
Tiliaceae	*Grewia retusifolia*	Heart disease	Scarlett *et al.* (1982)

The plant parts most commonly used in the preparation of bush medicines were leaves and bark (Reid, 1979; Scarlett *et al.*, 1982; Low, 1990). Preparation of plant material usually consisted of an initial grinding, bruising or maceration process, followed by extraction with decoctions and infusions. Inland Aborigines, who did not have access to boiling implements, carried out their extractions by soaking the plant material overnight in

water. Those living in coastal regions used old baler shells to boil plant material in, thus minimising the time needed to prepare medicines (Barr, 1988).

1.6 Aims of the study

The aims of this study were to investigate *Eremophila* species traditionally used by the Aboriginal people of Australia for novel cardioactive compounds, to determine their effects on the Langendorff rat heart and to partly describe the mode of action of one of these compounds. In doing so, this study has helped fulfil some of the objectives of the ethnopharmacologist whose aims according to Bruhn and Helmstedt (1980) include:

> "To rescue and document an important cultural heritage before it is lost, and to investigate and evaluate the agents employed".

A number of hypotheses were tested during this project, all of which were developed as the work progressed. Initially, the following null and alternative hypotheses were tested:

Ho: The extracts from five *Eremophila* species tested exhibit cardioactive properties.

Ha: None of the extracts prepared from five *Eremophila* species tested exhibit cardioactive properties.

1.6.1 Thesis Organisation

A detailed description of the plants used and the sites they were collected from is incorporated into chapter 2. All experimental work is described in five experimental chapters, starting with a detailed comparative description in chapter 3 of the effects mediated by the crude methanolic leaf extracts of *E. alternifolia* and *E. longifolia* on the

Langendorff rat heart. In chapter 4, the effects mediated by the active constituents from these two species are discussed together and again compared to show their opposite effects on rat hearts. Verbascoside, a phenylethanoid glycoside from *E. alternifolia* leaves, and its influence on the production of intracellular cAMP and prostacyclin levels in the rat heart, are discussed in chapters 5 and 6, respectively. Chapter 7 is a mini review of the effects of iridoid glucosides from three other species of *Eremophila* compared with those from other plant genera and families. Chapter 8 (general discussion) is a synthesis of this work. A list of publications resulting from this thesis can be found in Appendix 1.

CHAPTER 2

SPECIES DESCRIPTION, COLLECTION SITES AND BIOLOGICAL ACTIVITIES OF *EREMOPHILA* SPECIES

CHAPTER 2

Species Description, Collection Sites and Biological Activities of *Eremophila* Species.

2.1 Myoporaceae Family

The Myoporaceae R.Br family consists of six genera distributed throughout the south-west Pacific, China, Japan, Hawaii, Mauritius, Rodriguez, the West Indies and Australia (Chinnock, 1982; Richmond and Ghisalberti, 1994). The largest of the six Myoporaceae genera is the *Eremophila* genus with approximately 315 recognised species and sub species, all of which are endemic to Australia (Richmond *pers. comm.*, 1997). *Myoporum* is the second largest genus with 32 species, followed by *Diocirea* with four species. *Bontia*, *Calamphoreus* and *Glycosystis* each have one species (Richmond, 1993b).

2.1.1 The *Eremophila* Genus

Eremophila is a genus with a widespread distribution throughout Australia (Everist, 1974). It occurs both along the coastal margins and the hinterland, but is virtually absent from tropical zones (Chinnock, 1982). Of the 315 recognised species, approximately 220 occur in Western Australia (WA) (Richmond *pers. comm*, 1997). Most of these are found in the Eremaean Botanical Province (Fig. 2.1) (Chinnock, 1991 - unpublished data).

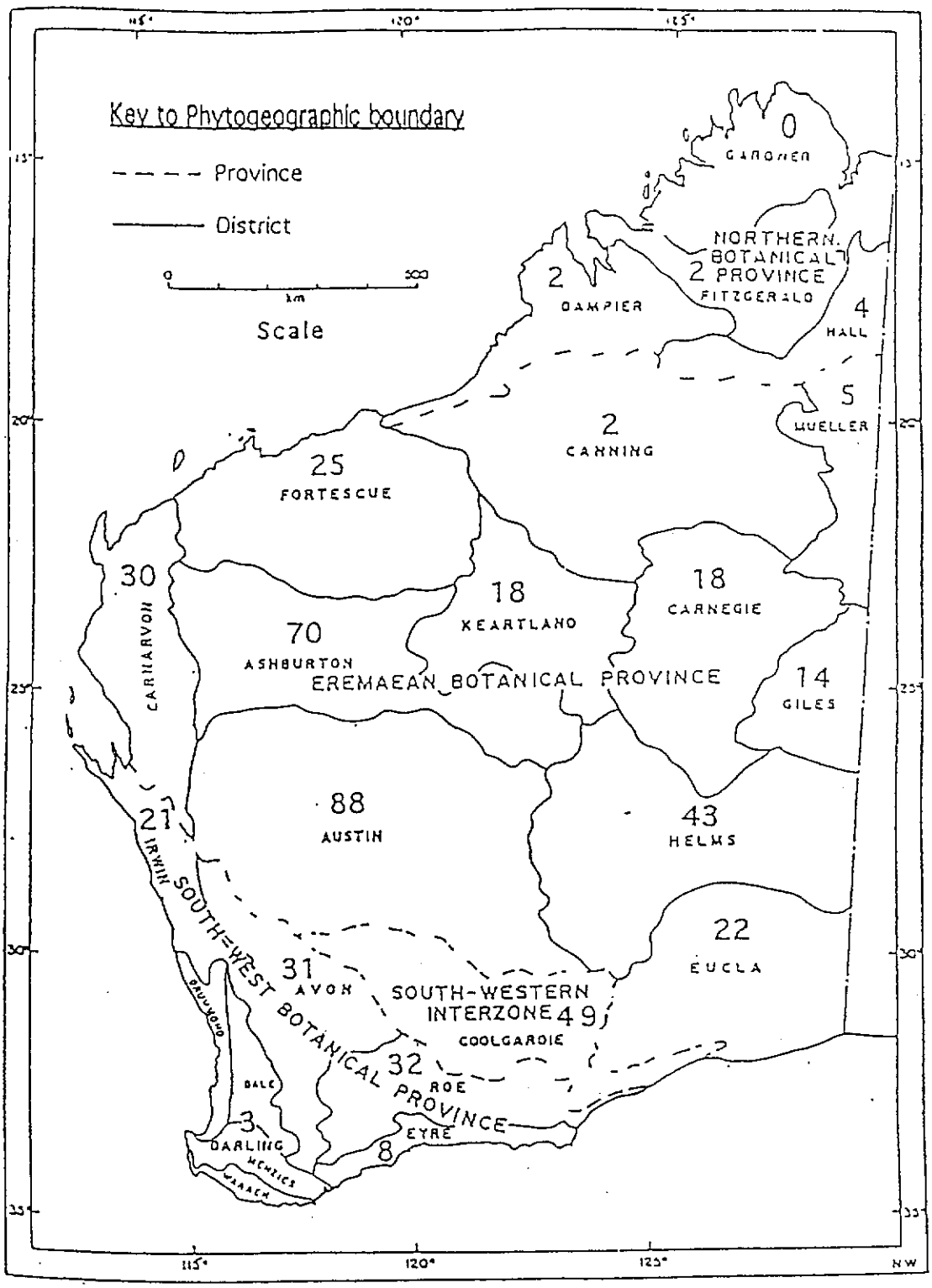


Fig. 2.1. The botanical provinces of Western Australia (after Beard, 1980). The numbers in each of the phytogeographic regions refers to the number of *Eremophila* species present in that region (Chinnock, 1991-unpublished data).

The name *Eremophila* is derived from the Greek words *eremos* and *phileo*, meaning 'desert' and 'to love', respectively (Sharr, 1988). This preference for areas of low rainfall and high temperatures has lead to an abundance of the genus in the semi-arid and arid zones of Australia (Elliot and Jones, 1984). Many species are tolerant to droughts, fire and grazing, as well as to light frost, and are often found flourishing in poor soils (Latz, 1982; 1995; Elliot and Jones, 1984). Combined with their poor palatability to live stock, *Eremophila* species are often referred to as 'poverty bush' (Gardner, 1990). The name 'emu bush' is also commonly used and is derived from the fact that emus (*Dromaius novahollandiae*) feed on the fleshy fruits of some species, eg. *E. maculata* (Richmond, 1993b).

To cope with the harsh environments in which they occur, *Eremophila* species have developed a number of adaptations. The leaves of many species, for example, exude a lacquer-like substance that protects them from hot, dry winds (eg. *E. fraseri*). A dense, hairy covering on the leaves of other species reflects the sun's rays and help reduce water loss. As a result, many species can survive from 1 - 2 years without rain (Elliot and Jones, 1984). The hardy nature of these *Eremophila* species has made them useful in the revegetation of old mine sites (Richmond, 1993a).

One of the distinguishing features of *Eremophila* species is their flowers. *Eremophila* flowers are tubular and usually consist of an upper lip with two lobes and a lower lip with three lobes (plate 2.1). They are so spectacular that many *Eremophila* species have recently attracted the attention of horticulturists (Richmond, 1993a). Most colours of the visible spectrum are represented, with those in the blue end being the most prevalent (Elliot and Jones, 1984). Spotted exteriors and interiors are common. *Eremophila* calyx lobes are variable and may enlarge after flowering and can be cream, green or purple in colour. Four exserted or non-exserted stamens are present. Fruits are non-splitting, drupe-like or rarely

druped and may be glabrous, hairy or scaly. Otherwise they are smooth or ribbed. The fruits are rarely winged.

Plate 2.1. *Eremophila alternifolia* flower and leaves

2.1.2 *Eremophila alternifolia*

Eremophila alternifolia (plates 2.1 and 2.2), commonly known as the Native Honeysuckle, is described by Elliot and Jones (1984) as a small to medium shrub (Bindon, 1996). Its leaves, which are from 1-5 cm by approximately 0.1 cm, are linear to subterete and are glabrous with an acute and hooked apex. The tubular flowers are from 2.5-3 cm with spotted interiors and glabrous exteriors; the colour is pink to carmine, but sometimes white or yellow. These flowers are extremely attractive to honeyeater birds (ornithophilous). This species is

commonly found growing on skeletal soils and in red loams that are well drained and heavy. It can tolerate light frosts, but may be damaged and killed by severe frosts. Its distribution is mainly in the southern half of Australia (Fig. 2.2). It grows abundantly in South Australia (SA), but is also common in the eastern Goldfields of WA and to a much lesser degree in the Northern Territory (NT) and in eastern-New South Wales (NSW).

Plate 2.2. *Eremophila alternifolia* in its natural habitat (Jaurdi Nature Reserve).

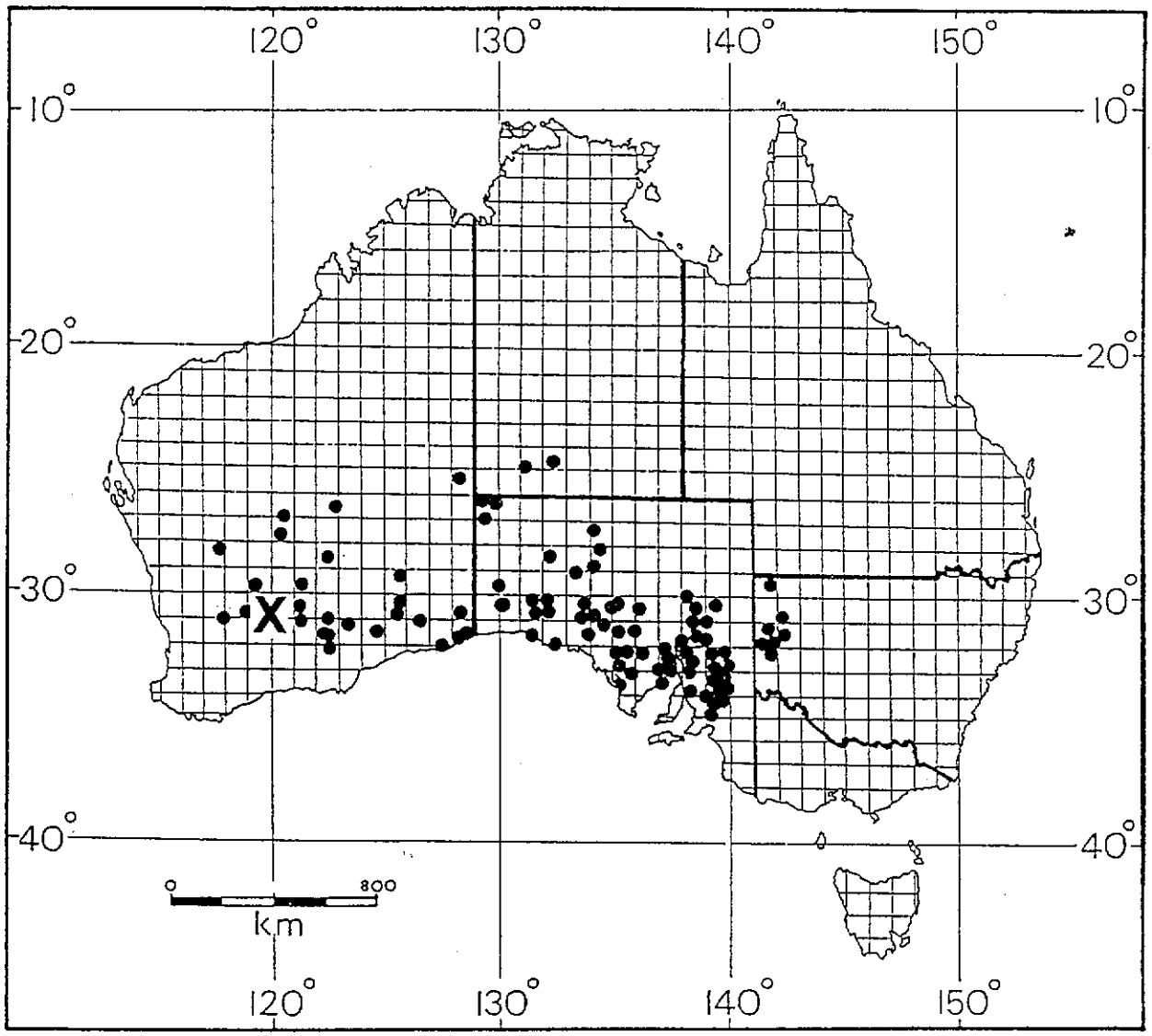


Fig. 2.2. Distribution map of *E. alternifolia* (courtesy of Dr R. J.Chinnock - unpublished data, 1997). The collection site for this species is marked with an X.

2.1.3 *Eremophila longifolia*

Eremophila longifolia (plates 2.3 and 2.4) is described as a small shrub to tree with rough, dark-grey bark (Elliot and Jones, 1984; Bindon, 1996). Branches are pendulous, streaked and hairy. The leaves, which measure up to 0.8 cm wide, and from 3 - 20 cm long and linear to linear-lanceolate, are often alternately arranged on the branches. They are usually dull-green in colour, pendulous and hairy. The margins are entire with a pointed or hooked apex. Flowers are tubular and approximately 3 cm long. Colours range from pink to reddish-brown or brick red. The exteriors are hairy with blunt, constricted lobes. There are 1 - 3 pedicels per axil and the stamens are exserted. The calyx lobes are triangular in shape, hairy and about 0.8 cm long. Fruits are ovoid to globular, succulent, glabrous and 1 cm long. This species occurs mostly on limestone soils in inland Australia where it is widely distributed (Fig. 2.3). The species does well in the semi-arid and arid zones of Australia, but will also grow in temperate zones. It is the most widely distributed of the *Eremophila* species (Chinnock *pers. comm.*, 1997). *Eremophila longifolia* is commonly referred to as 'Berrigan bush', 'Emu Bush' or 'Dogwood'.

Plate 2.3. *Eremophila longifolia* leaves (photo courtesy of Dr G.S. Richmond).

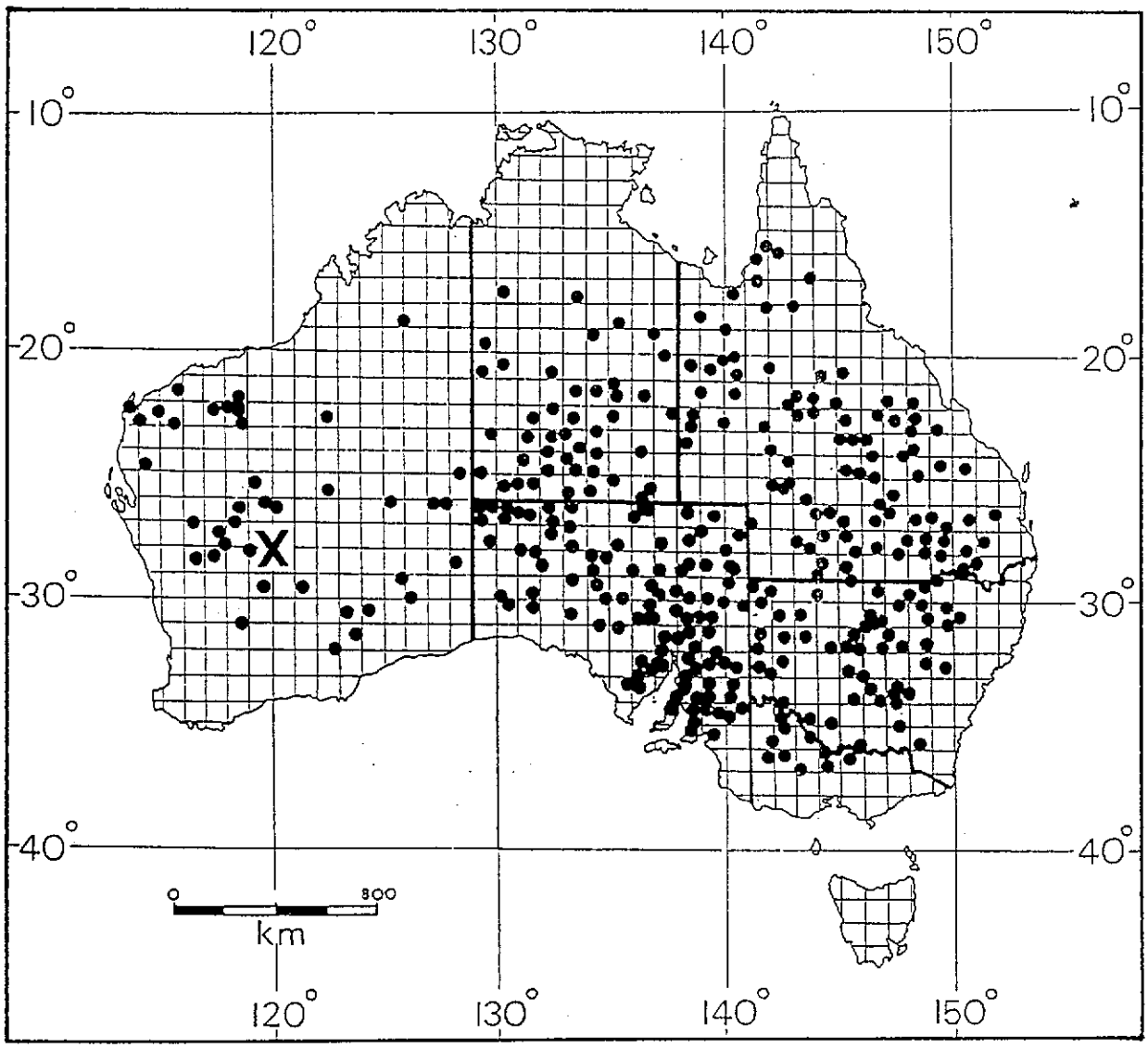


Fig. 2.3. Distribution map of *Eremophila longifolia* (courtesy of Dr R. J. Chinnock - unpublished data, 1997). The collection site for this species is marked with an X.

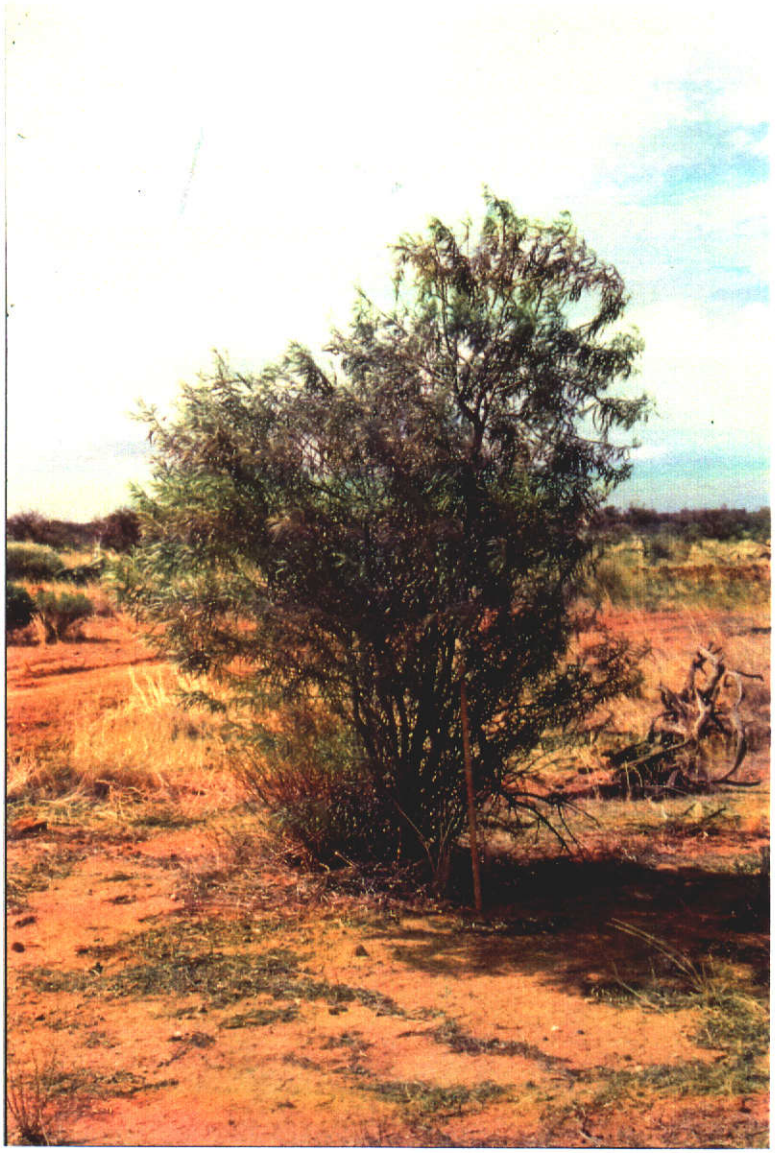


Plate 2.4. *Eremophila longifolia* in its natural habitat (photo courtesy of Dr G. S. Richmond).

2.1.4 *Eremophila maculata* subsp *brevifolia*

Eremophila maculata subsp *brevifolia* (Ker-Gawler) F. Muell is a small, dense shrub with rigid branches and hairy leaves (Elliot and Jones, 1984) (plate 2.5). Arranged alternately, the oblanceolate or petiolate leaves are grey to green in colour and measure from 0.5 - 5 cm by 1 cm. They usually have a pointed apex and have margins throughout. The flowers are tubular and approximately 2.5 cm in length. Colours range from white to deep pale pink with spotted interiors. Red, purple, yellow and orange coloured flowers are also common. The

exteriors of the flowers are glabrous with pointed lobes and are solitary, axillary and on S-shaped pedicels 2.5 cm long. Stamens are exserted. Calyx lobes measure 1 cm in length, are pointed and glabrous. The fruits are generally globular, 2 cm long, glabrous and often green or purple in colour. This species occurs naturally on heavy clay-loams which are often flooded (Fig. 2.4). It can grow in shady areas, but prefers full sun light.

Plate 2.5. *Eremophila maculata*

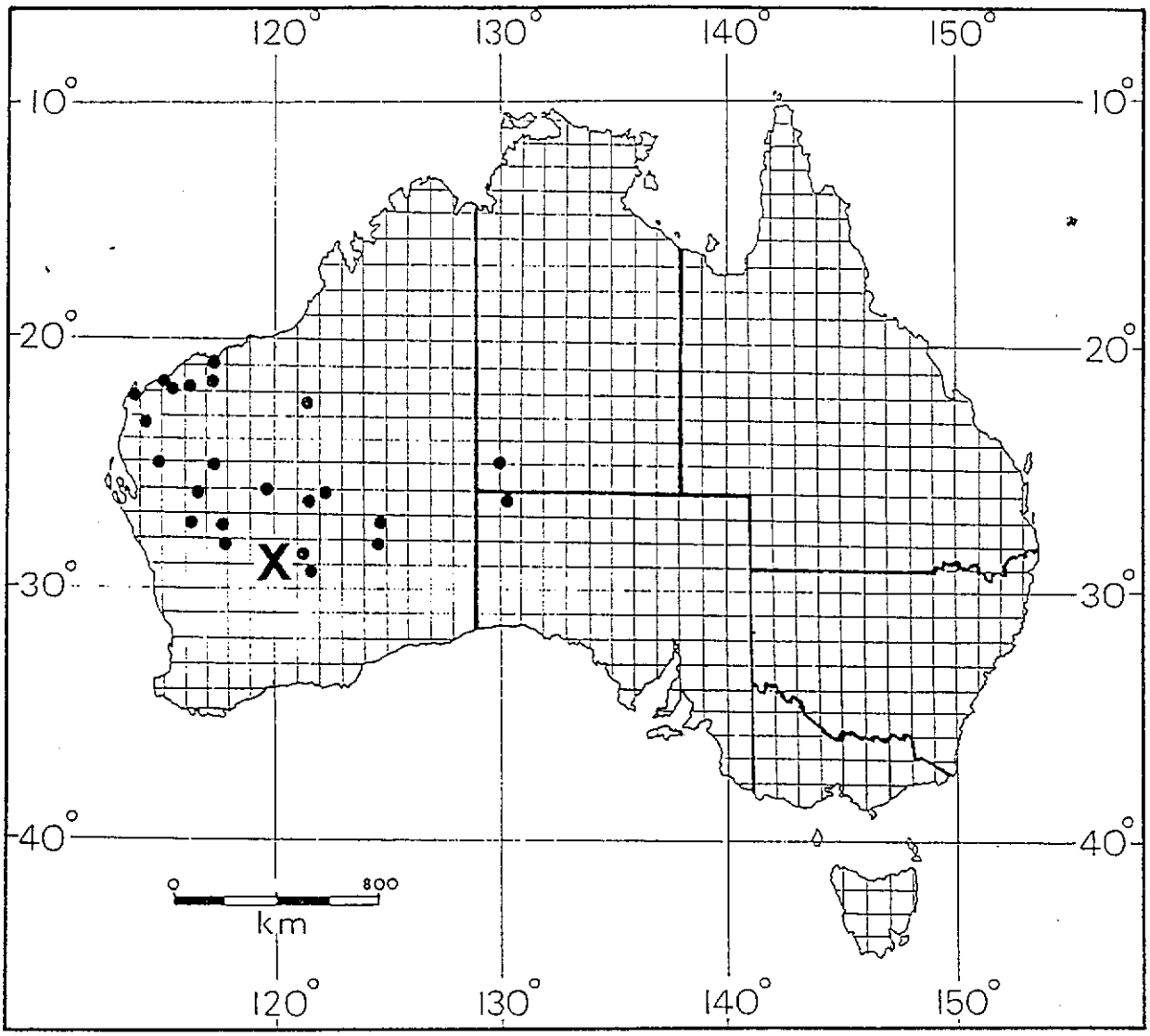


Fig. 2.4. Distribution map of *E. maculata* (courtesy of Dr R. J. Chinnock - unpublished data, 1997). The collection site for this species is marked with an X.

2.1.5 *Eremophila ionantha*

Eremophila ionantha Diels, or violet-flowered *Eremophila*, is described by Elliot and Jones (1984) as a small, dwarf shrub with many erect branches (plate 2.6). Typically, its leaves are long and narrow, measuring 2.4 cm by 0.1 cm. They are arranged oppositely near the ends of branchlets and alternate on other parts. They are linear, almost terete, mainly erect, glabrous, incurved and warty. The flowers are tubular, up to 2 cm long, glabrous on the exterior and pale blue to purple in colour. The lobes are pointed, solitary, axillary and on curved pedicels. The calyx lobes are also pointed. The fruits are ovoid to acute, hairy and about 0.7 cm long. This species grows abundantly in the Eastern Goldfields (Fig. 2.5) where it is a woodland understorey species. It prefers well-drained red loams.

Plate 2.6. *Eremophila ionantha* (photo courtesy of Dr G. S. Richmond).

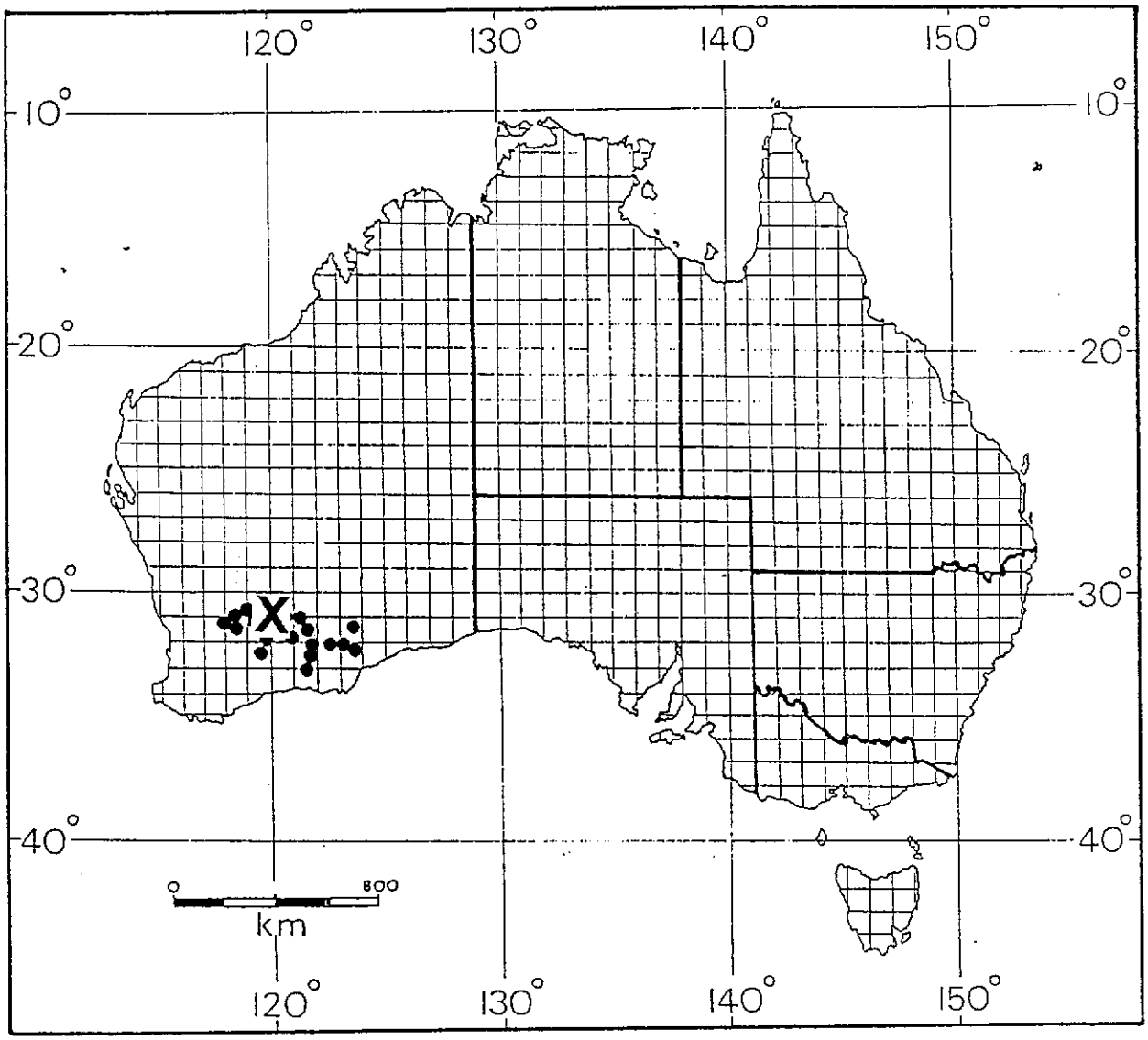


Fig. 2.5. Distribution map of *E. ionantha* (courtesy of Dr R. J. Chinnock - unpublished data, 1997). The collection site for this species is marked with an X.

2.1.6 *Eremophila pantonii*

Eremophila pantonii F. Muell., commonly referred to as 'Broombush', is a small to medium shrub with erect, glabrous and warty branches (Elliot and Jones, 1984; plate 2.7). Its alternate, hairy, warty and linear leaves measure 1.0 - 2.5 cm by 0.2 cm. The margins of the leaves are complete, apex pointed and hooked. Like most other *Eremophila* species, the flowers of *E. pantonii* are tubular and about 2 cm long. The colours are lavender to violet. The exteriors of the flowers are hairy, have pointed lobes, are solitary, axillary, have constricted bases and are sessile near branchlets. Its profuse stamens are not exserted. It has small calyx lobes, which are hairy and over-lapping. Fruits are ovoid in shape, roughly 0.8 cm in length and are hairy. This insect-pollinated (Richmond, 1993b) species occurs mainly in the Austin and Coolgardie Districts (Fig. 2.6) and prefers to grow on red loams.

Plate 2.7. *Eremophila pantonii*

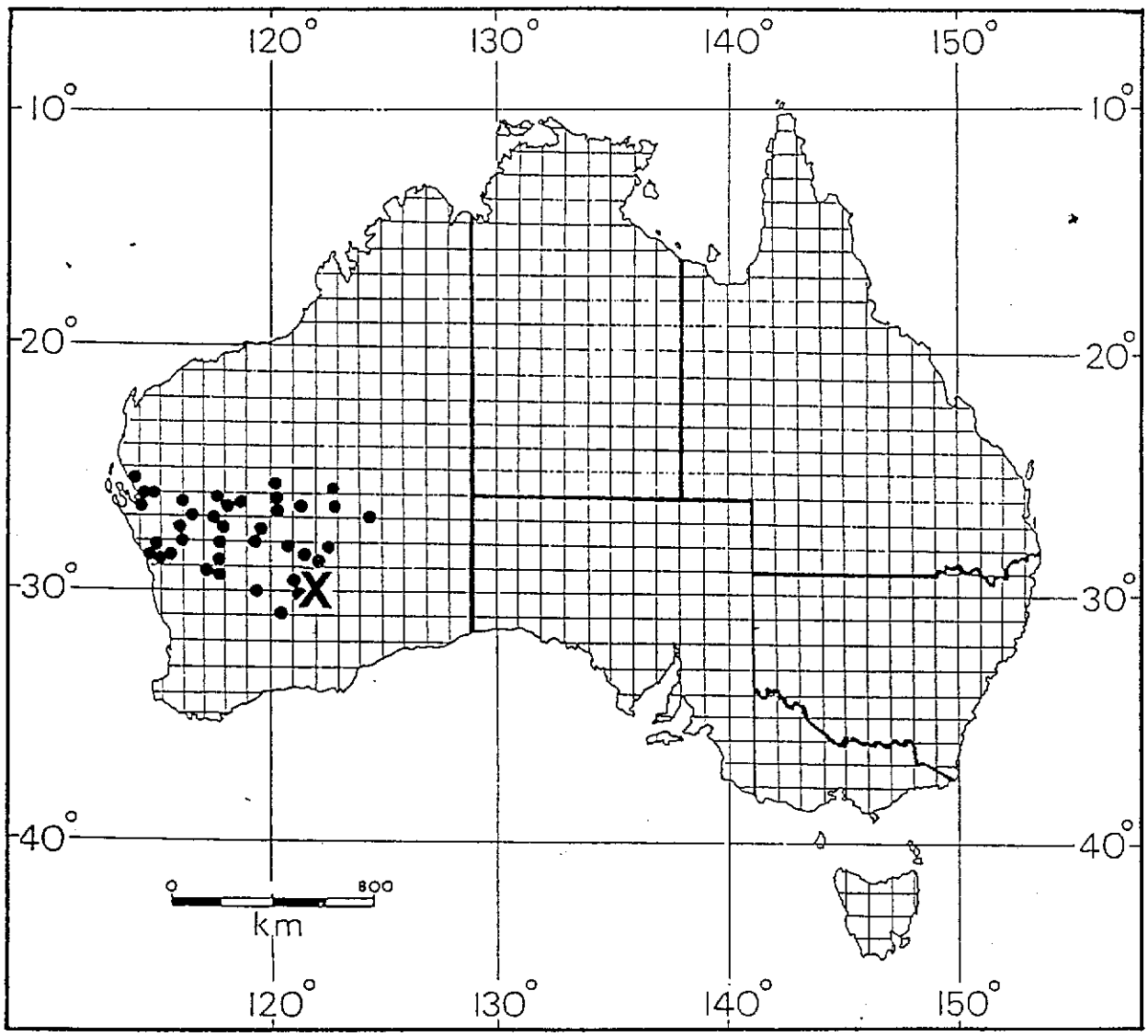


Fig. 2.6. Distribution map of *E. pantonii* (courtesy of Dr. R. J. Chinnock - unpublished data, 1997). The collection site for this species is marked with an X.

2.2 Collection Sites

Plant material of *E. alternifolia, E. longifolia, E. maculata* subsp *brevifolia, E. ionantha* and *E. pantonii* was collected from separate locations (see the distribution maps for locations of the collection sites)`. *Eremophila alternifolia* (Fig. 2.2) leaf samples (voucher No. RJC 8653) were collected in November 1993 from the Mt. Dimer region in Jaurdi Nature Reserve, approximately 100 km NE of Southern Cross, WA. *Eremophila longifolia* leaves (TI 2) were collected 24 km east of Sandstone, WA in February, 1994 (Fig. 2.3). Leaf samples of *E. maculata* subsp *brevifolia* (GSR 213) were collected 10 km south of Leonora along the Menzies Road in November, 1994 (Fig. 2.4). *Eremophila ionantha* (GSR 207) was located 1 km south of Jaurdi Station (November, 1994; Fig. 2.5). Leaves and branchlets of this species were taken. Finally, *E. pantonii* leaves (GSR 211) were collected 8.8 km south of Broad Arrow turn-off on the Kalgoorlie-Menzies Road (November, 1994; Fig. 2.6). All samples were confirmed by the Myoporaceae authority, Dr R. J. Chinnock (Botanic Gardens of Adelaide and State Herbarium) and were deposited at the South Australian State Herbarium.

2.3.1 Soil Types

The soils of each of the five collection sites have been described by Northcote *et al.* (1968), using their widely accepted codes for soil type descriptions. The collection site for *E. alternifolia* has gentle slopes with uplands on granites, gneisses and allied rocks. Narrow shallow valleys and flats traverse the gentle slopes and abrupt erosional scarps. The dominant soil types are yellow earthy sands (Uc 5.22) and sandy yellow earths of Gn 2.21 and Gn 2.22 type. Ironstone gravels (KS-Uc 4.11), (Uc 4.11) and (Uc 2.12) are also common.

The soil types of the *E. longifolia* and *E. maculata* subsp *brevifolia* collection sites are identical. Broken slopes and ridges with interspersed breakaways on gneissic granites and allied rocks dominate the area. Occasional iron stone gravels are also present in these areas. The chief soils appear to be shallow earth loams (Um 5.3) with shallow Gn 2.12 soils. These are both underlain by red-brown hardpan. A variety of soils, typically Dr 1.32, Dr 1.42 and Dr 1.73, and only 15 - 40 cm deep, occur on outwash areas around breakaways. Others include Um 5.11 and Gc 1.12 soils on calcrete platforms interspersed between shallow drainage ways around breakaways. Occasionally, Dr 2.32 soils on pediments are present. Mottled- and pallid-zone material occurs along the slopes of the breakaways.

Eremophila pantonii was collected from populations growing on alkaline and neutral red earths (Gn 2.13, Gn 2.12) in flat to undulating valley plains and pediments. Scattered gravel and Um 5.3 soils on pediments and Ug 5.38 soils occur on plains that flank ultrabasic rocks. Red-brown hardpan are common below the Um 5.3 soils. These soils are similar to those near Jaurdi Station where *E. ionantha* samples were collected. Red alkaline earths (Gn 2.13) with limestone at shallow deaths of less than 60 cm dominate the area. Some outcrops of basic rock with gentle rises of Gc 1.12 soils on gently undulating valley plains and pediments are common throughout the area. Other soil types include Ug 5.38 clays on plains flanking ultrabasic rocky outcrops. Some Um 5.41 soils are also present, but only on steeper soils.

2.4 Rainfall

The average annual rainfall for the five collection sites are summarised in Table 2.1. Since rainfall data was not available for each of the specific collection sites, data from the nearest town or sampling point was used. The long-term average rainfall for Sandstone and surrounding areas, where *E. longifolia* was collected, was 238 mm per annum (pa). This

was similar to the average rainfall for the Leonora area (*E. maculata* subsp *brevifolia* collection site), which experiences 224 mm pa. Both the Kalgoorlie and Southern Cross areas (*E. alternifolia, E. ionantha* and *E. pantonii*) have average rainfalls of 260 mm pa and 284 mm pa.

Table 2.1 Annual rainfall (mm per annum) for the four towns nearest the collection sites. Adapted from Bureau of Meteorology (1993, 1994, 1995, 1996).

Collection Site	*1993*	*1994*	*1995*	*1996*
Southern Cross	317.7	204.0	475.6	416.2
Sandstone	215.8	165.4	511.0	347.2
Kalgoorlie	326.2	245.2	479.8	235.2
Leonora	285.1	107.3	444.4	337.7

2.5 Biological Activities of *Eremophila* Species

A number of interesting biological activities have been discovered for several *Eremophila* species. Richmond (1993), Ghisalberti (1994) and Richmond and Ghisalberti (1995) have reviewed these, a summary of which can be found in Table 2.2. Despite being listed together, there is little or no evidence to suggest that there is any correlation between the compounds listed and the medicinal uses for the corresponding species. Ghisalberti (1994) suggests that the information be treated as a starting point from which to obtain empirical data.

Table 2.2. Medicinal uses and biologically active compounds isolated from them (adapted from Richmond, 1993; Ghisalberti, 1994 and Richmond and Ghisalberti, 1995).

Species	Purpose	Active Compounds
E. alternifolia	Deep sleep (Tindale, 1937). Colds, influenza, fever and headaches (Smith,1991). Septic wounds (Bowen, 1975). Internal pain (Barr, 1988).	Fenchone, limonene and a unidentified monoterpene (Barr, 1988). Camphor and furanoid sesquiterpenes (Sutherland and Rodwell, 1989).
E. binonifolia	Laxative and purgative (Bowen, 1975).	Mannitol and verbascoside (Ghisalberti unpublished data).
E. cuneifolia	Colds (Lassack and McCarthy, 1983).	Myrcene, spathulenol, β-eudesmol, elmelol and 10,11-dehydonagaione (Ghisalberti *et al.*, 1984; Ghisalberti and Loh, 1994). Mannitol, verbascoside and geniposidic acid (Ghisalberti and Loh, 1994). Four diterpenoid acids (Croft *et al.*, 1984; Ghisalberti *et al.*, 1984).
E. dalyana	Chest pains (Lassack and McCarthy (1983). Body wash for scabies (O'Connell *et al.*,	Elemol, α- and β-eudesmol, cineole and the lignans 3,6-bis-(3,4-dimethoxyphenyl)-

	1983).	tetrahydro-1*H*,3*H*- furo(3,4*c*)furan-1,4-diol (Ghisalberti *et al.*, 1987).
E. duttonii		α -pinene (Barr, 1988).
	Sores, cuts, influenza and eye/ear sores (Smith, 1991). Sore throat (Barr, 1988).	Serrulatane diterpenes and flavones (Croft *et al.*, 1981; Tippet and Massy-Westropp, 1993).
E. elderi		Unknown
	Bedding, head rest and colds (O'Connell *et al.*, 1983).	
E. fraseri		Cembranoids, eremanes and flavones (Jefferies *et al.*, 1962; Carroll *et al.*, 1985; Ghisalberti *et al.*, 1986). Mannitol (Jefferies *et al.*, 1962).
	Colds, toothache and rheumatism (Lassack and McCarthy, 1983).	
E. freelingii		α -pinene (Barr, 1988).
	Headaches and chest pains (Cleland and Tindale, 1959). Antiseptic wash for sores (Smith, 1991). Colds and general well-being (Latz, 1982). Aches and pains (Low, 1990). Antidiarrhoea (Barr, 1988). Headaches (Lassack and McCarthy, 1983). Promote rest (Meggitt, 1962).	Freelingyne and several analogues (Massy-Westropp *et al.*, 1966; Ingham *et al.*, 1974; Knight and Pattenden, 1975). Eremolactone (Birch *et al.*, 1963).
E. gilesii		Modified bisabolene diterpenes (Forster *et*

	Headaches and chest pain (Cleland and Tindale, 1959). Sores (Tynan, 1979).	*al.*, 1993). 19-acetoxy-3,15-epoxycembr-11-en-19-oic acid (Ghisalberti *et al.*, 1994).
E. gilesii	Colds, and general well-being (Latz, 1982) Promote rest (Latz, 1982).	5 α -hydoxyviscida-3,14-dien-20-oic acid and 16-hydroxyerema-5,17-dien-19-oic acid (Ghisalberti unpublished data).
E. goodwinii	Purgative (Latz, 1982).	Unknown
E. latrobei	Body wash for scabies (Cleland and Tindale, 1959). Smoke treatment for babies (Meggitt, 1962). Colds and influenza (Smith, 1991).	Ngaione (Birch *et al.*, 1954; Hegarty *et al.*, 1970). Myoporone (Blackburne *et al.*, 1972). Biflorin (Forster *et al.*, 1986).
E. longifolia	Body wash (Silberbauer, 1971). Smoke treatment for mothers and babies (Cleland and Johnston, 1993, 1937). Eye wash (O'Connell *et al.*, 1983). Colds and headaches (Spencer and Gillen, 1969). Counter-irritant (Tynan, 1979).	Safrole and methyleugenol (Della and Jefferies, 1961). α -pinene (Barr, 1988).
E. maculata	Colds (Cunningham *et al.*, 1982).	Prunasin (Finnemore and Cox, 1929; Everist, 1981). Myoporone and its 10,11-dehydro analogue (Blackburne *et al.*, 1972).

E. mitchelii	General medicinal purposes (Low, 1990).	Eremophilone (Bradfield *et al.*, 1932). Eremophilone analogues (Bates and Paknikar, 1966; Massy-Westropp and Reynolds, 1966; Chetty *et al.*, 1969; Lewis *et al.*, 1982).
E. mitchelii		
E. neglecta	General well-being (Latz, 1982).	Uknown.
E. paisleyi	Wash for scabies (Latz, 1982).	Spathulenol and 8,20-dihydoxyserrulat-14-en-19-oic acid (Ghisalberti, 1994b).
E. sturtii	Bachaches (Silberbauer, 1971). Bachaches (Bowen, 1975). Sores and cuts (Smith, 1991). Head colds, sore ayes and antidiarrhoea (Barr, 1988).	Elemol, α- and β-eudesmol, α -pinene and other terpenes not yet identified (Barr, 1988).

CHAPTER 3

THE EFFECTS OF CRUDE *EREMOPHILA* EXTRACTS ON LANGENDORFF RAT HEART PREPARATIONS

CHAPTER 3

The effects of crude *Eremophila* extracts on Langendorff rat heart preparations

3.1 Introduction

The Australian Aboriginal people were generally nomadic and periodically moved from one place to another in search of food. It seems reasonable to suggest, therefore, that these people would have targeted a variety of plants with a broad spectrum of medicinal uses rather than carry several species with them. Because of their wide distribution, *Eremophila* species were well known to, and utilised by, the Aborigines. At least 18 of the 315 recognised species were used for medicinal purposes (Richmond, 1993a; Richmond and Ghisalberti, 1994), of which 50% were used in preparations meant to be taken internally (Ghisalberti, 1994a).

Although much has been written on the medicinal uses of Australian flora by the Aboriginal people (Meggit, 1962; Reid and Betts, 1979; Cribb and Cribb, 1981, 1982; Lassack and McCarthy, 1983; O'Connell *et al.*, 1983; Low, 1990), few attempts have been made to list the active constituents. Lassack and McCarthy (1983) were the first to prepare such a list, but with limited information. A more detailed treatise was prepared by Barr (1988, 1993). Later, Ghisalberti (1994a) reported on the phytochemistry and ethnopharmacology of *Eremophila* species. Many of the species listed in the review appear to possess compounds which may be useful as medicinal agents. Two species, *E. alternifolia* and *E. longifolia*, both of which were of great significance to the Aboriginal people, were investigated for novel cardioactive compounds.

3.1.1 *Eremophila alternifolia*

Eremophila alternifolia R.Br (Native Honeysuckle) features prominently in the pharmacopoeias of the Australian Aboriginal people (Richmond, 1993a; Ghisalberti, 1994) and was termed the "number one medicine" by some tribes (Barr, 1988). Its importance to these people can be inferred from the fact that it was one of few species in which the leaves were harvested, dried and carried around in case of need (Low, 1990). Reportedly, decoctions were used for colds and influenza, as well as for alleviating pain caused by headaches (Smith, 1991). The leaves were chopped and mashed, put into grass and then tied to the head as a poultice to cure headaches (Goddard and Kalotas, 1985). Infusions were drunk as promoters of sleep (Tindale, 1937) and were considered to induce pleasant dreams (Latz, 1995). Freshly picked leaves were rubbed on external wounds for cleansing (Bowen, 1975). In addition, *E. alternifolia* was used as an analgesic, decongestant and for general well-being (Richmond, 1993a; Ghisalberti, 1994a; Richmond and Ghisalberti, 1994).

3.1.2 *Eremophila longifolia*

Eremophila longifolia F. Muell. (berrigan bush) was not only of importance as a medicinal agent, but was also of sacred and mystical significance to the Aboriginal people (Richmond, 1993). Its medicinal uses include the burning of *E. longifolia* twigs (`smoke treatment') near trenches, in which newly born babies and their lactating mothers lay, to induce lactation in the mother and to strengthen the babies (Chewings, 1936; Latz, 1982). Decoctions of the leaves were used as eye washes (O'Connell *et al.*, 1983), as counter-irritants (Richmond, 1993a) and for skin and body washes (Silberbauer, 1971). An infusion of the leaves was prepared for colds and headaches (Spencer and Gillen, 1969). However, Barr (1988) warns that the internal use of *E. longifolia* infusions is considered dangerous.

Crude methanolic (MeOH) extracts of *E. alternifolia* and *E. longifolia leaves* were tested on Langendorff rat hearts to determine their ability to alter heart rate (chronotropism), contractile force (inotropism) and coronary perfusion rate (CPR). The terms heart rate and chronotropism are synonymous, as are contractile force and inotropism, and will be used interchangeably throughout this thesis.

3.2 Materials and Methods

The collection of all plant material was previously described in chapter 2. *Eremophila alternifolia* and *E. longifolia* leaves, collected in 1993 and 1994, were dried in an oven at 40°C for 48 hrs and then ground up to a fine powder using a vegetative grinder (Dietz-Motoren KG, Eleckromotorenfabrik, West Germany, 220 v, type WRB 80 C12Q SIL). *Eremophila alternifolia* leaf powder (28.1 g) was successively extracted with hexane (0.48 g; 1.7% dry weight) and then filtered to leave behind a residue. The residue was then extracted with dichloromethane (0.61 g; 2.2% dry weight), followed by methanol (1.80 g; 6.4% dry weight) and then water (0.80 g; 2.9% dry weight) to generate three more fractions. All extractions were performed overnight. *Eremophila longifolia* powdered leaves (106 g) were extracted in the same way with hexane (1.8 g, 1.7% of dry weight), dichloromethane (0.7 g, 0.6%), methanol (40 g, 39%) and water (11.6 g, 11%).

3.2.1 Isolated Heart Preparation

Isolated Langendorff rat hearts were prepared from 50 young male and female albino normotensive Wistar rats of body mass between 400-600 g. The animals were fed and given water *ad libitum* until they were sacrificed by spinal dislocation. The hearts were rapidly excised, freed of adhering tissue and immediately mounted intact on the Langendorff heart apparatus (Fig. 3.1) and perfused retrogradely with a modified Krebs-

Henseleit solution. This technique and all experiments were approved by the Animal Ethics and Experimentation Committee (approval No. R15/93).

3.2.2 Perfusate

The hearts were perfused with a Krebs-Henseleit (Krebs and Henseleit, 1932) solution (pH 7.36) modified according to Alexander *et al.* (1987). The solution was kept at a constant temperature of 37°C and constant pressure of 70 cm of water and was aerated with a 95% O_2 and 5% CO_2 gas mixture (Carbogen) 20 minutes prior to and during the experiment. The Krebs-Henseleit solution consisted of the following (mM): NaCl, 118.0; KCl, 4.7; $MgCl_2.6H_2O$, 0.5; $NaHCO_3$, 25.0; NaH_2PO_4, 1.0; Glucose, 10.0 and $CaCl_2.2H_2O$, 2.2.

3.2.3 Drugs and Extracts

Phentolamine methane sulphonate (α-adrenergic blocker), propranolol hydrochloride (β-adrenergic blocker), phenylephrine hydrochloride (α-adrenergic agonist) and isoproterenol (β-adrenergic agonist), all purchased from Sigma Chemical Company®, were used. Stock solutions of 10^{-2} M were prepared in deionised water and kept frozen to avoid oxidation. All further dilutions were prepared daily (as they were required) and kept in a dark, cool place until used. All drugs and extracts were administered through a polyethylene cannula in 1 ml/min retrograde perfusions. The freshly prepared solutions were introduced into the system only after the hearts had stabilised (usually 30 minutes after being mounted onto the Langendorff apparatus).

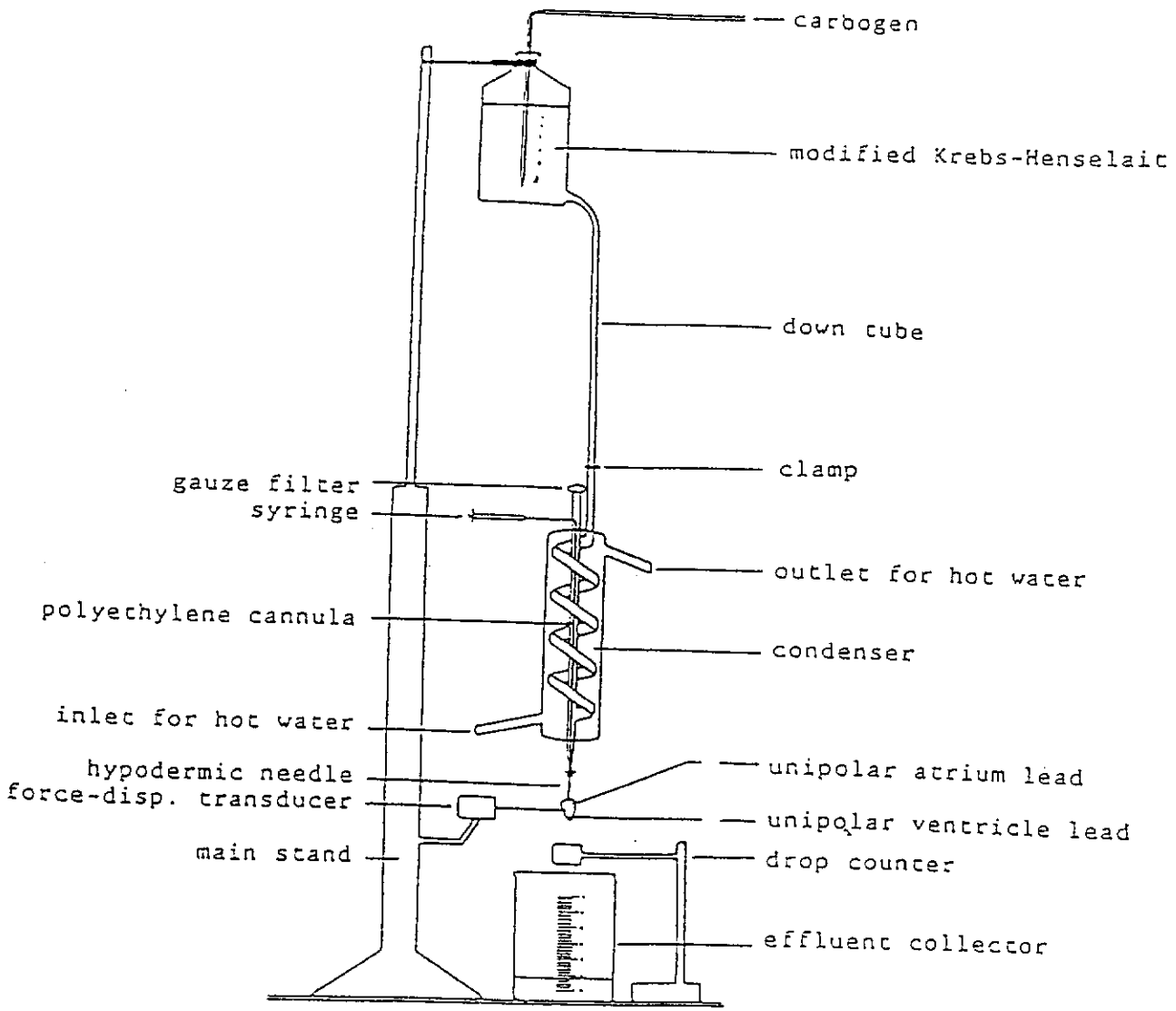


Fig. 3.1. The Langendorff rat heart bioassay.

3.2.4 Recordings

Heart rates were monitored throughout each experiment by electrocardiograms recorded by micro-electrodes attached to the right and left atrial appendage and to the left ventricular wall of the isolated heart. The electrodes were directed through to an analog-to-digital converter (MacLab) and recorded on a Macintosh computer. Contractile force was measured with a Nihon Kohden Kogyo Co. Ltd. force-displacement transducer, model SB-IT-H. The transducer was coupled with the MacLab and computer set-up and attached perpendicular to the heart by a small hook and thread. The transducer was calibrated prior to each experiment. Coronary effluent was measured by a drop counter constructed in the laboratory.

3.2.5 Data Analysis

At least six experiments were conducted for each treatment. Paired Student's *t*-tests were performed on the data. Probabilities of less than 0.05 were considered statistically significant. Where possible, actual P values are given. Otherwise, $P < 0.05$ is indicated. All results are represented as means ± S.E.M.

3.3 Results

3.3.1 Eremophila alternifolia

The administration of a single injection of one ml of crude methanolic (MeOH) *E. alternifolia* leaf extract (8 g/L injected at the rate of one ml/min; preliminary results revealed that this dose was sufficient to mediate a significant response) induced a biphasic response in chronotropism and inotropism in Langendorff rat hearts. Heart rate was significantly reduced immediately following the introduction of the extract (from 195 ± 8 bpm to 168 ± 11 bpm; 14% decrease) (Fig. 3.2a). Within a minute, however, it significantly ($P < 0.05$) increased to 230 ± 8 bpm (19% increase). The increased chronotropism coincided with the commencement of a decrease in inotropism. It was gradually restored to 195 ± 8 bpm after an average time period of 12 minutes. The initial response in inotropism consisted of an increase from 1.03 ± 0.04 g to 1.30 ± 0.07 g (26% increase), followed by a decrease of 14 % (down to 0.88 ± 0.07 g (Fig. 3.2b). The response occurred within a minute and lasted less than a minute. Where arrhythmias were observed (38% of experiments), they occurred during this phase. The ensuing decrease in force persisted for an average of 13 minutes, during which time it gradually returned to the initial contractile force.

A sharp, significant ($P < 0.05$) increase in coronary perfusion rate also occurred concurrently with the decrease in contractile force (Fig. 3.2c). The average increase of 37%, which started at 9.73 ± 0.29 ml/min, increased to 13.32 ± 0.83 ml/min. After an average period of 10 minutes, it had returned to normal.

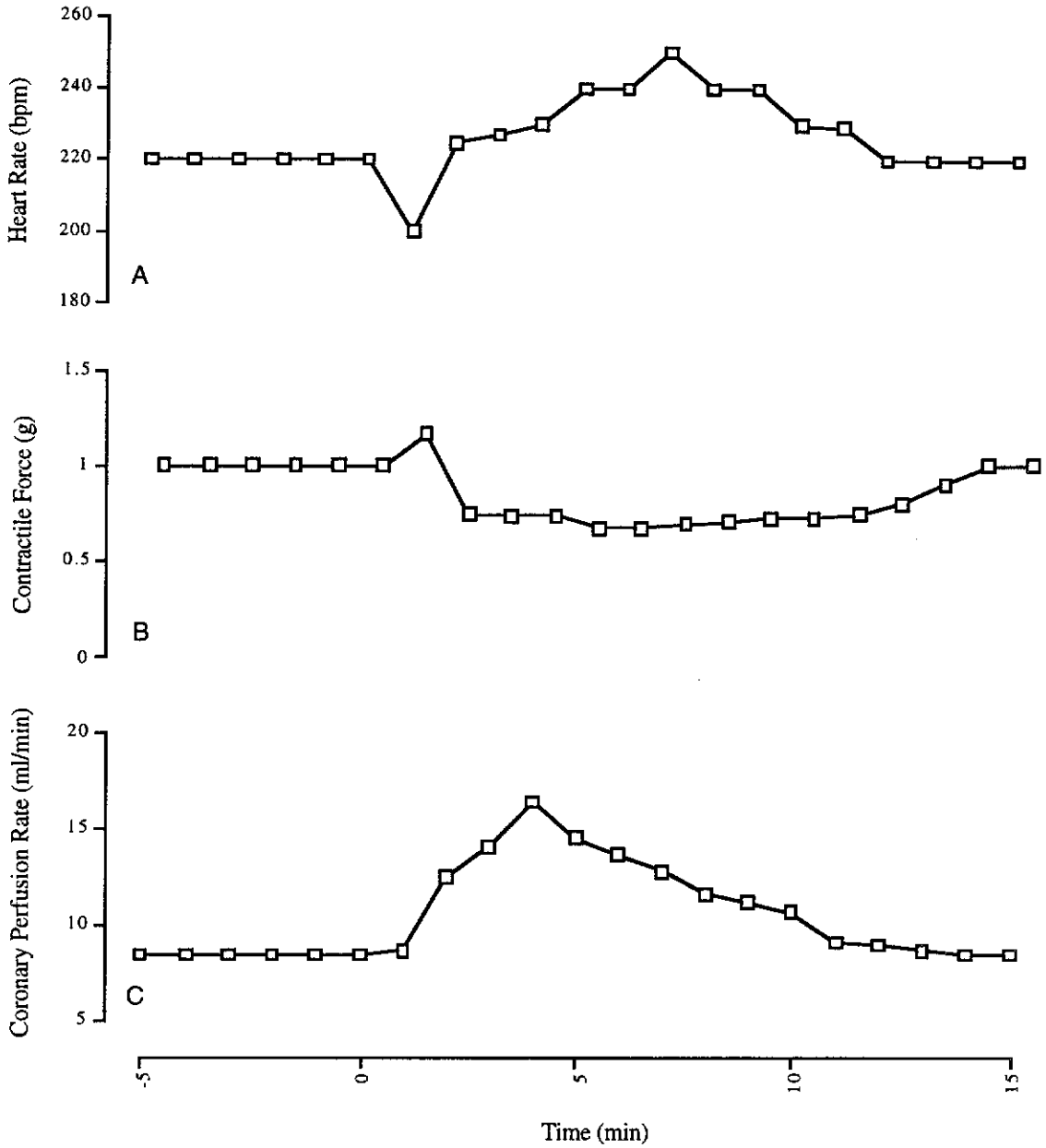


Fig. 3.2 (A) Dynamics of changes in heart rate (bpm); (B) changes in contractile force (g); (C) changes in coronary perfusion rate (ml/min) mediated by the MeOH extract of *E. alternifolia* leaves.

3.3.2 Adrenergic Receptor Blockage

Propranolol (β-adrenergic blocker) did not in any way diminish the dynamics of the chronotropic, inotropic and CPR effects of the MeOH extract. A dose of 1 μM, which was enough to completely block the effects of isoproterenol (1 μM), did not abolish the effects mediated by the extract. Heart rate, which was initially reduced during the positive inotropic phase, increased within a minute from 166 ± 10 bpm to 192 ± 13 bpm (14% increase). The increase in chronotropism lasted for a period of 15 minutes. Positive inotropism (29%) was followed by negative inotropism (10%) (Fig. 3.4), simultaneously with positive chronotropism (16%) (Fig. 3.3) and increased coronary perfusion (9%) (Fig. 3.5). Contractile force increased from 1.06 ± 0.11 g to 1.37 ± 0.20 g in the first minute and then decreased to 0.95 ± 0.11 g, progressively returning to the initial force of contraction within 12 minutes.. Coronary perfusion increased from 8.75 ± 0.21 ml/min. to 12.62 ± 1.21 (44% increase), slowly decreasing over 12 minutes. Arrhythmias were generated in 38% of all experiments where the extract was administered in the presence of propranolol. These results did not significantly differ ($P < 0.05$) to those obtained for the extract when administered in the absence of blockers.

Phentolamine (one μM), like propranolol, did not alter the effects of the MeOH extract. Heart rate again initially decreased during the positive inotropic phase (19%) (Fig. 3.3), but increased within a minute to 203 ± 23 bpm (from the initial rate of 161 ± 11 bpm; 26% increase). Heart rate was restored to normal within 12 minutes. Contractile force increased from 1.08 ± 0.19 g to 1.23 ± 0.22 g (14% increase) initially but decreased to 0.90 ± 0.06 g (17%). The reduction in contractile force lasted for an average of 15 minutes (Fig. 3.4). Coronary perfusion also increased (Fig. 3.5). The initial rate of 8.94 ± 0.73 ml/min. rose to 11.66 ± 1.20 ml/min. (30% increase), but slowly decreased over 12 minutes. No arrhythmias were observed when the extract was tested in the presence of phentolamine (α-adrenergic blocker). *T*-tests performed on these data, except those of

arrhythmogenic activity, suggest that the increases observed were not significantly different ($P < 0.05$) to those induced by the extract when in the absence of blockers.

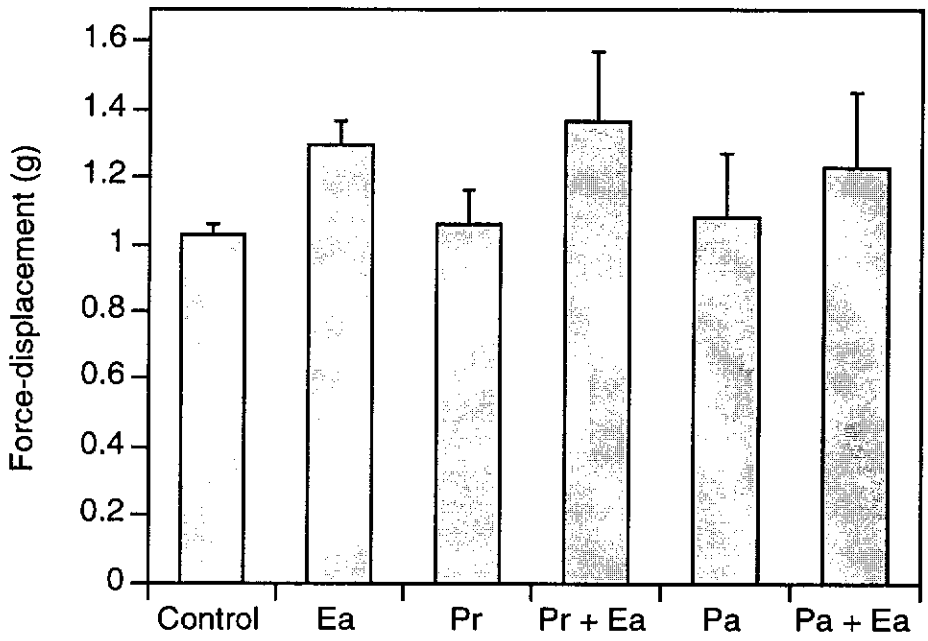


Fig. 3.3. Effects of the MeOH extract of *E. alternifolia* leaves on Contraction Force (g) (mean averages and S.E.M). Key to graph: Ea = *E. alternifolia* MeOH extract; Pr = propranolol; Pa = phentolamine.

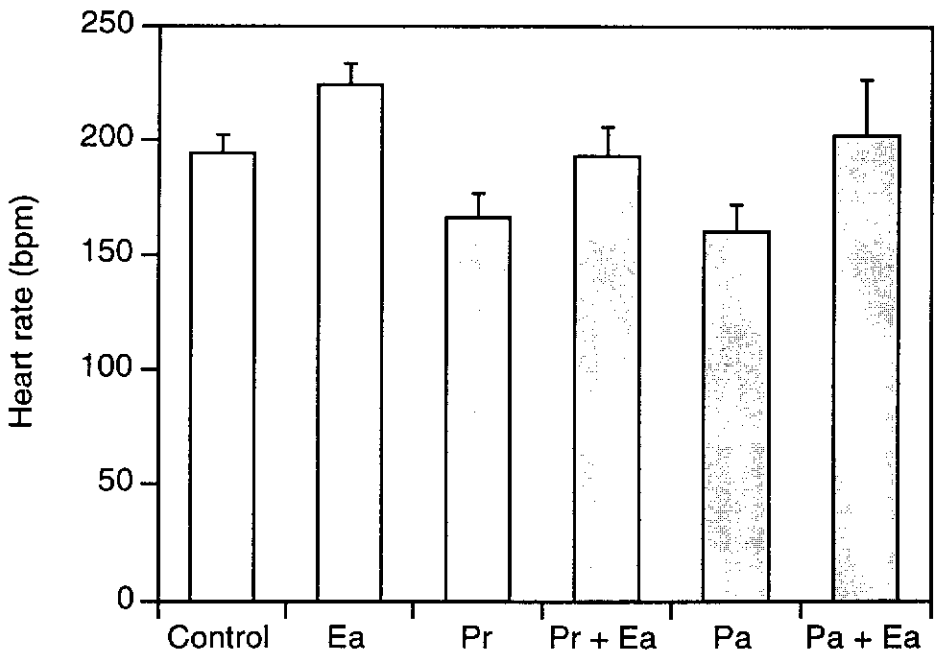


Fig. 3.4. Effects of the MeOH extract of *E. alternifolia* leaves on heart rate (mean averages and S.E.M). Key to graph: Ea = *E. alternifolia* MeOH extract; Pr = propranolol; Pa = phentolamine

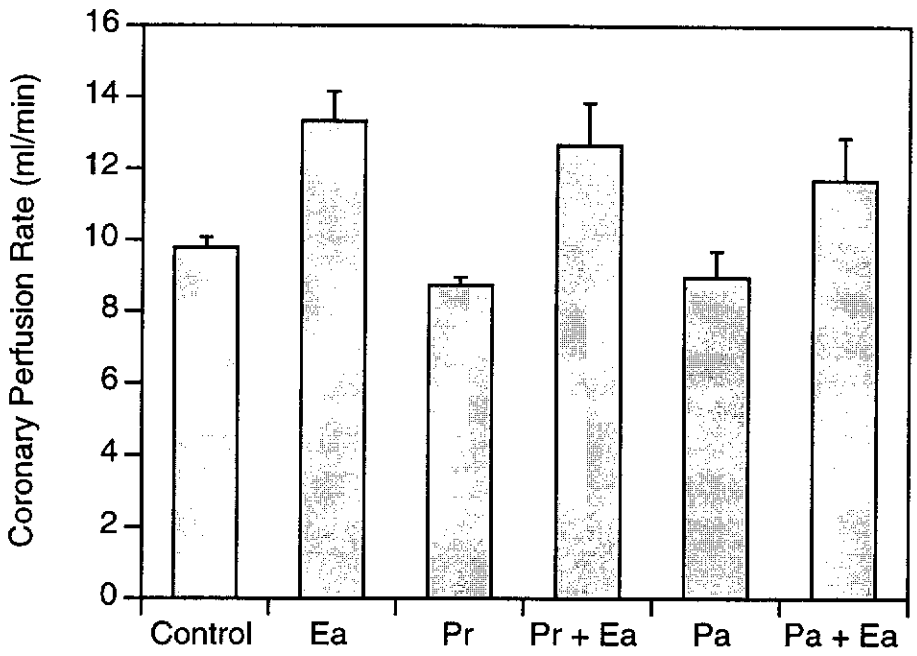


Fig. 3.5. Effects of the MeOH extract of *E. alternifolia* leaves on coronary perfusion rate (mean averages and S.E.M). Key to graph: Ea = *E. alternifolia* MeOH extract; Pr = propranolol; Pa = phentolamine).

3.3.3 *Eremophila longifolia*

The MeOH extract of *E. longifolia* leaves had the opposite effect to those mediated by the MeOH extract of *E. alternifolia* leaves. Instead of an increase in the three parameters monitored, ie. chronotropism, inotropism and CPR, there were significant decreases (Fig. 3.6). The major effect observed was on CPR (Fig. 3.6c). The initial perfusion rate of 8.6 ± 1.7 ml/min. decreased rapidly to 6.2 ± 1.0 ml/min (P = 0.040). This 27.9 % reduction in CPR manifested itself at the same time as a 26.9 % decrease in contractile force (1.03 ± 0.08 to 0.75 ± 0.04 g; P = 0.042) (Fig. 3.6b) and a 12.0 % fall in heart rate (Fig. 3.6a). An initial rate of contraction of 215 ± 8 bpm fell to 189 ± 6 bpm (P = 0.031). Within a minute, there was a partial recovery in all three parameters, all of which were soon followed by further decreases from which the hearts did not recover.

Since it was decided that a detailed analysis be conducted on only one species, viz. *E. alternifolia*, the MeOH extract of *E. longifolia* leaves was not tested in the presence of adrenergic or other blockers. However, in a preliminary study involving adrenergic antagonists, the effects of the extract were not blocked.

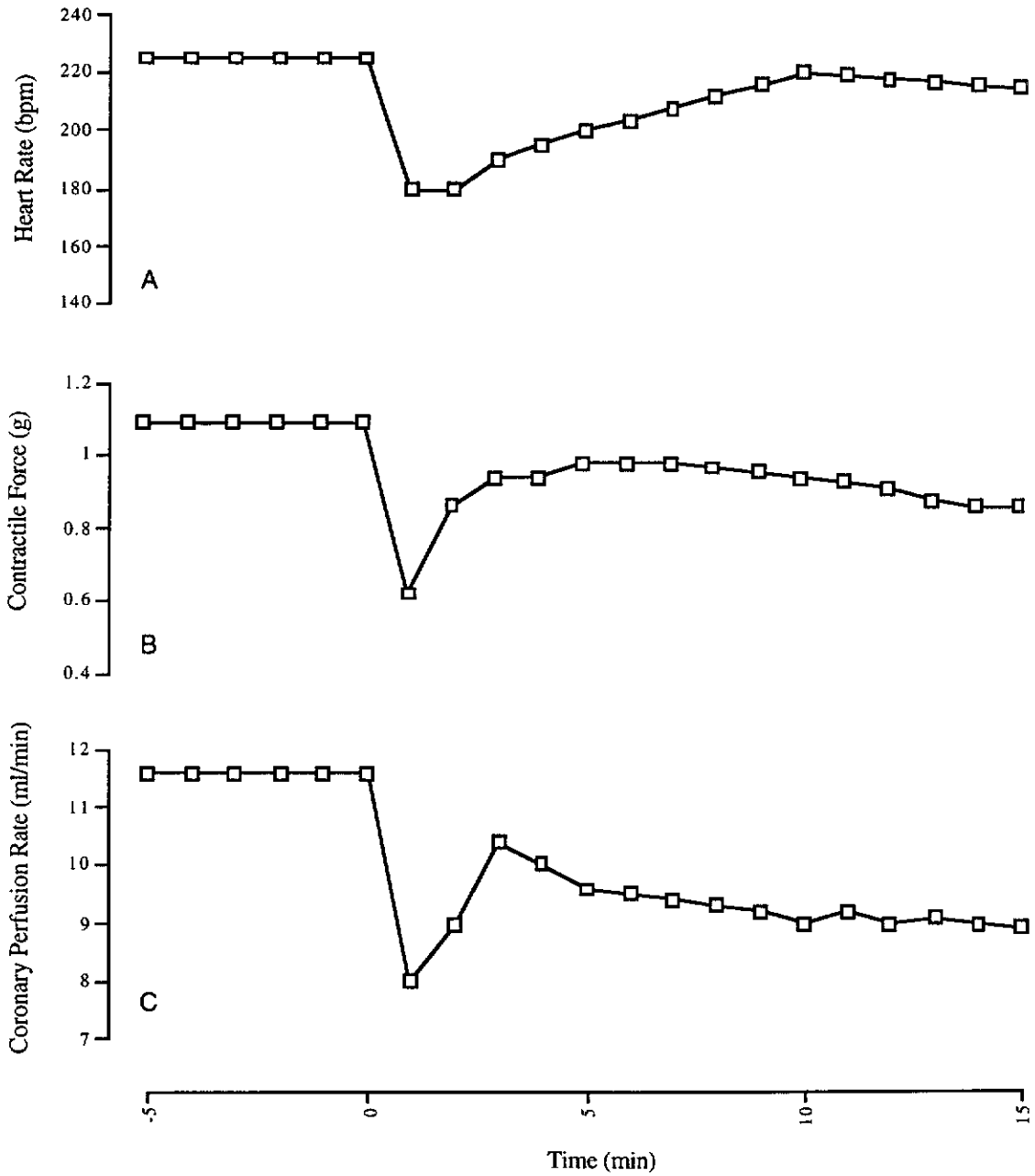


Fig. 3.6. (A) Dynamics of changes in heart rate (bpm); (B) changes in contractile force (g); (C) changes in coronary perfusion rate (ml/min) mediated by the MeOH extract of *E. longifolia* leaves. Please note: this graph is a representative of one trial only.

3.3.4 Active constituents

Before a bioassay-guided chemical fractionation was performed to identify the active constituents, two compounds previously isolated from other *Eremophila* species, and suspected (Ghisalberti, *pers. comm.*) of occurring in *E. alternifolia* and *E. longifolia*, were tested. Verbascoside (Fig. 3.7a), previously isolated from the callus tissue of *E. denticulata, E. decipiens, E. clarkii, E. drummondii, E. glabra and E. viscida* (Dell *et al.*, 1989), later proved to be the active constituent in *E. alternifolia* leaves. Geniposidic acid (Fig. 3.7b), previously from *E. cuneifolia* (Ghisalberti, 1994b), was the active principle in *E. longifolia* leaves. Small traces of verbascoside were also detected in *E. longifolia* leaves.

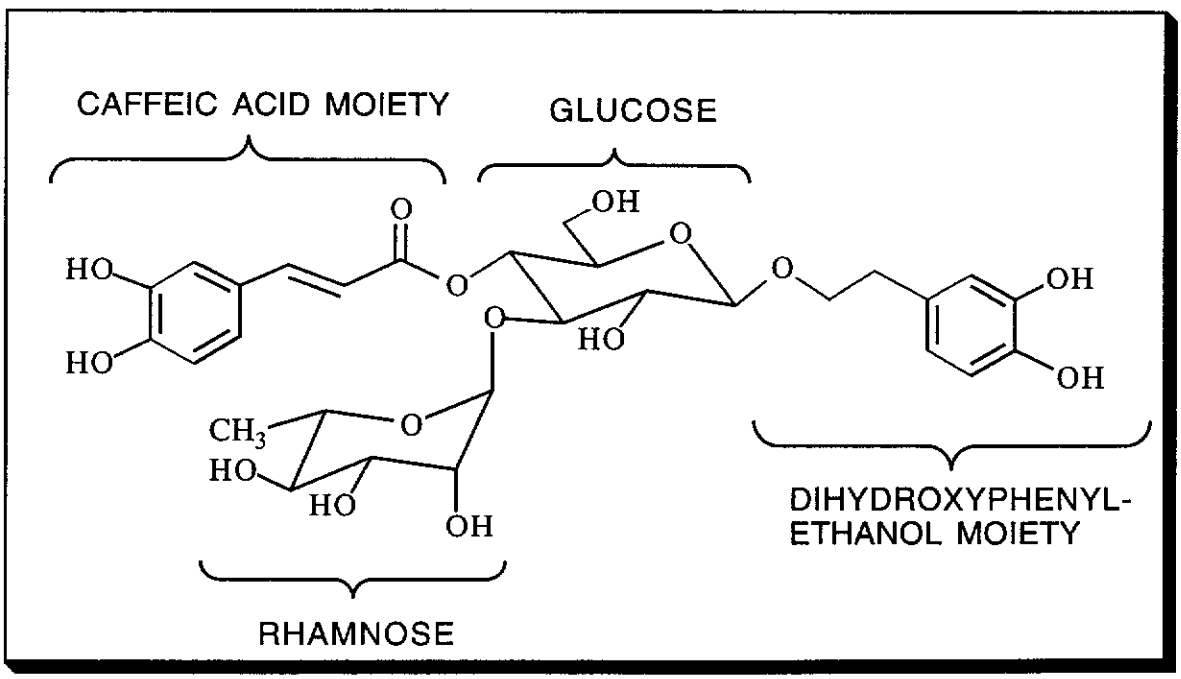


Fig. 3.7a. Chemical structure of verbascoside (from *E. alternifolia* leaves)

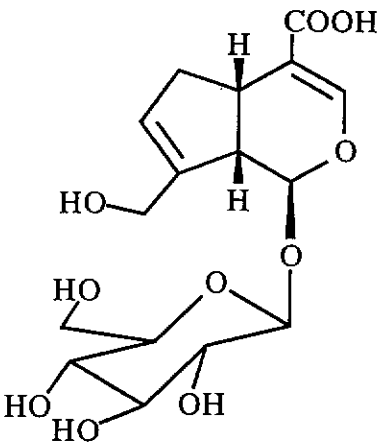


Fig. 3.7b. Chemical structure of geniposidic acid (from *E. longifolia* leaves)

Tests conducted on a previously isolated sample of verbascoside (slightly oxidised) had a significant effect on Langendorff rat hearts. A 1 ml injection of 1 mM verbascoside increased heart rate from 192 ± 10 bpm to 239 ± 11 bpm, which was a significant rise (P = 0.0049) of 24.5 %. Contractile force increased by only 5.3 % (P = 0.030). The initial contractile force of 0.94 ± 0.05 g increased to 0.99 ± 0.06 g. Coronary perfusion rate was also significantly increased (P = 0.020). Within a minute, CPR increased from 6.3 ± 0.4 ml/min. to 9.3 ± 0.3 ml/min. This represented an increase of 47.6 %. With the exception of positive inotropism, which lasted for less than two minutes, all effects lasted for approximately 12 minutes. A small but insignificant decrease in all three parameters was noted prior to the increases. Verbascoside, therefore, did not appear to be responsible for the biphasic nature of the *E. alternifolia* leaf extract.

A pure sample of geniposidic acid had a significant inhibitory effect on Langendorff rat hearts (see chapter 4). Chronotropism, inotropism and CPR were immediately reduced following the introduction of the compound. There were, however, no increases in these parameters, therefore suggesting that the stimulatory effect noticed in the MeOH extract of *E. longifolia* leaves were also mediated by another compound.

3.4 Discussion

The extract obtained from the leaves of *E. alternifolia* with methanol mediated an initial positive inotropic effect followed by an immediate decrease. The decrease occurred simultaneously with increased chronotropism and coronary perfusion rate. All effects lasted less than 15 minutes, after which the isolated rat hearts made a full recovery and returned to their initial force and rates of contraction and to their initial coronary perfusion rates. Preliminary tests with the aqueous extract of *E. alternifolia* leaves and aqueous extract of the bark resulted in identical responses to those induced by the MeOH extract of the leaves. All these responses are similar to the dynamics associated with the catecholamine, epinephrine (adrenaline).

Comparisons with data obtained by Shavit *et al.* (1986), Alexander *et al.* (1987), Pennacchio (1992) and many others (see Jenkinson, 1973 for a review) suggest that the effects of the crude *E. alternifolia* leaf extract were similar, although not as pronounced, as those of epinephrine. A single 1 ml injection of the hormone (1 ml of 1 μM), when introduced to isolated rat hearts, induced a significant increase in contractile force (40%), followed by a reduction in force coinciding with increased chronotropism (50%) (Pennacchio, 1992). No data was available for comparisons in CPR. It seemed likely then that a compound or compounds catecholamine-like in nature was the active constituent. However, the inability of phentolamine and propranolol to block these effects suggested otherwise.

The results with α- and β-adrenergic blockers showed that the MeOH extract does not exert its effects through the adrenergic receptors of the isolated rat heart. The α-adrenergic blocker phentolamine, widely used in clinical medicine, did not abolish any of the inotropic or chronotropic effects induced by the extract. Moreover, the increase in coronary perfusion was unaffected by the blocker. Propranolol, a non-specific clinical β-blocker, was also unable to reduce the effects caused by the extract. Both blockers did,

however, completely antagonise the effects of phenylephrine (α-adrenergic agonist), in the case of phentolamine, and isoproterenol (β-adrenergic agonist) in the case of propranolol.

Studies with the MeOH extract of *E. alternifolia* leaves on isolated rat gut indicated that several compounds, possibly acting through a number of different receptor types, accounted for most of the inhibitory effects in isolated rat duodenal preparations (Walker, 1996). Walker (1996) suggested that approximately 70 % of the activity could be accounted for by cholinergic and adrenergic compounds. Cholinergic receptors were not blocked in tests with Langendorff rat hearts on the basis that cholinergic agonists inhibit myocardial activity. Adrenergic agonists were also excluded (see above), suggesting that the active compound(s) acted through other receptors or mechanisms.

Using the Langendorff rat heart bioassay as a guide, the cardioactive constituent in MeOH extract of *E. alternifolia* leaves was identified. A phenylethanoid compound known as verbascoside was shown to be responsible for the cardioactivity manifested by the extract. The slightly oxidised sample of verbascoside used in the preliminary tests (and later with pure samples; see chapter 4) suggested that the phenylethanoid significantly increased chronotropism, inotropism and CPR in Langendorff rat hearts and therefore warranted further testing. Verbascoside (syn: acteoside, kusaginin) was first isolated in 1924 from the *Orobanche rapum-genistae* and was given the name orobanchin (Bridel and Charaux, 1924). A compound with similar moieties was later isolated in 1963 from *Verbascum sinuatum* (Scarpati and Delle Monache, 1963), but the identity of verbascoside remained a mystery until it was shown by Andary *et al.* (1980) to be identical to acteoside (Fig. 3.7a). The isolation and purification of verbascoside, as well as a description of its effects on Langendorff rat hearts are discussed in chapter 4.

Similarly, the effects mediated by the *E. longifolia* leaf extract were also biphasic, but opposite to the effects of *E. alternifolia* leaves. Significant decreases in chronotropism,

inotropism and CPR were immediately followed by partial recoveries and then further decreases. This suggested that more than one active compound was present in the extract. Geniposidic acid, a member of the large iridoid glucoside group of compounds (El-Naggar and Beal, 1980), was identified as one of the active principles. First isolated from *Genipa americana* (Guarnaccia *et al.*, 1972), geniposidic acid (Fig. 3.7b) is a member of the iridoid glucoside family and has a more limited distribution than verbascoside (El-Naggar and Beal, 1980). A small quantity of verbascoside was also detected in *E. longifolia* leaves and may account for the partial positive responses that succeeded the initial reductions in the three parameters monitored. The extraction of geniposidic acid and its effects on the Langendorff rat heart are discussed in chapter 4.

CHAPTER 4

THE EFFECTS OF VERBASCOSIDE AND GENIPOSIDIC ACID ON LANGENDORFF RAT HEARTS

CHAPTER 4

The effects of verbascoside and geniposidic acid on Langendorff rat hearts

4.1 Introduction

Verbascoside, which should not be mistaken for the two iridoids, verbascoside A and B (Boros and Stermitz, 1990), is represented in several plant families (Jiménez and Riguera, 1994) and has been shown to display an interesting spectrum of biological activity. An ethyl acetate fraction, obtained from whole *Cistanche phelypaea* plants and known to contain verbascoside, was shown to have an analgesic, antipyretic and diuretic effect on rats, as well as inhibit rabbit intestinal activity (Melek *et al.*, 1993). Verbascoside is also an inhibitor of protein kinase C (Herbert *et al.*, 1991), rabbit lens aldolase reductase (Kohda *et al.*, 1989) and lipid peroxidation (Zhou and Zheng, 1991). Furthermore, it exhibits immunomodulating (Molnar *et al.*, 1989) and immunosuppressive properties (Endo & Hikino, 1982; Sasaki *et al.*, 1989), and apparently potentiates the anti-tremor effects of L-DOPA (Andary *et al.*, 1980). Verbascoside had no effect on blood pressure when injected intravenously into rats and did not mediate any chronotropic or inotropic effect on the artificially stimulated isolated heart auricle of a rat (Andary *et al.*, 1980). Conversely, Ahmad *et al.* (1995) reported that verbascoside was responsible for mediating a significant drop in blood pressure in Wistar rats.

Verbascoside also appears to display interesting *in vivo* activity against murine P-388 (PS) lymphocytic leukemia (Pettit *et al.*, 1990). Other useful biological activities include analgesic activity (Andary *et al.*, 1980; Andary *et al.*, 1982), protection against oxidative haemolysis (Li *et al.*, 1993); it has antibacterial (*Escherichia coli*) properties and acts against the Aujeszky virus (Molnar *et al.*, 1989). Houghton and Hikino (1989) reported anti-hepatotoxic activity with extracts obtained from fresh leaves of *Buddleja globosa* and

B. officinalis and the roots of *B. americana.* They claim that the activity was possibly mediated by verbascoside and echinactoside, but don't exclude flavonoid compounds. In contrast, geniposidic acid had no previously known cardioactivities.

Having identified verbascoside as the active constituent in *E. alternifolia* leaves and geniposidic acid in *E. longifolia* leaves (chapter 3), their effects on the Langendorff rat heart were analysed and compared. In addition, verbascoside was administered at different doses for dose-response curves and tested against adrenergic and calcium channel blockers.

4.2 Materials and Methods

Isolated heart preparation, perfusate, data recording and data analysis were identical to those used in Chapter 3. At least six animals were used in each test (Animal Ethics and Experimentation Committee approval No. was R43/94). The extraction of verbascoside and geniposidic acid was carried out under the guidance of Yana M. Syah (Chem. Dept. UWA). The following is an account of the extraction and fractionation procedures.

4.2.1 Extraction of Plant Material and Fractionation of Extracts

a) *E. alternifolia.* Powdered leaves (85 g) of the plant were successively extracted into four fractions with light petroleum (4.7 g, 5.5% of dry weight), dichloromethane (4.0 g, 4.7%), methanol (20 g, 23.5%) and water (9.3 g, 10.9%). A portion (15 ml) of the total aqueous extract (500 ml) was fractionated into three fractions by chromatography on a polyamide column (15 g, 120-150 mesh, Koch-Light); fraction 1 (eluted with water): 240 mg; fraction 2 (eluted with 50% aqueous methanol): 40 mg; fraction 3 (eluted with methanol): 10 mg.

A portion of the methanol extract (5 g) was subjected to vacuum liquid chromatography (120 g Silicic acid 100 mesh; Mallinckrodt). Discontinuous gradient elution from 10% methanol-dichloromethane to methanol afforded 19 fractions which were grouped on the basis of thin layer chromatography (tlc) (Kieselgel 60 F_{254} aluminium sheets; Merck) into three main fractions; fraction 1: 500 mg; fraction 2: 38 mg; fraction 3: 115 mg. Further chromatography of fraction 1 gave 85 mg of a mixture of verbascoside and mannitol which exhibited cardioactivity.

b) *E. longifolia*. Powdered leaves (106 g) of the plant were successively extracted with light petroleum (1.8 g, 1.7% of dry weight), dichloromethane (0.7 g, 0.6%), methanol (40 g, 39%) and water (11.6 g, 11%). A portion (6 g) of the methanol extract was fractionated into methanol-ether soluble (1.9 g) and insoluble components (3.7 g). The latter fraction was subjected to vacuum liquid chromatography (120 g Silicic acid 100 mesh; Mallinckrodt) with gradient elution from 20% dichloromethane-methanol to methanol. Analysis of the fractions by tlc showed one fraction (1.6 g) to be mainly one component. A portion of this (470 mg) was purified by further chromatography to yield a fraction (180 mg) which contained geniposidic acid and small traces of verbascoside.

4.2.2 Analysis of Chromatography Fractions

^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were obtained for methanol or D_2O solutions using a Bruker AMX 500 or a Bruker AM 300 spectrometer. The identity of all compounds was confirmed by comparison of their spectral parameters with those described in the literature and by comparative tlc behaviour with that of standard samples. Interpretation of the spectra was carried out by Yana M. Syah (Chem. Dept. UWA).

4.2.3 Test Compounds

Pure verbascoside (MW = 624.594) and geniposidic acid (MW = 374.344) were administered through a polyethylene cannula in 1 ml/min retrograde perfusions of 1 mM concentration. Verbascoside was also tested over a wide range of doses for dose-response analysis. The freshly prepared solutions were introduced into the system only after the hearts had stabilised following mounting onto the Langendorff apparatus (usually 30 minutes). Rat hearts used in calcium channel blockade testing were constantly retrogradely perfused with verapamil at a dose of 10^{-9} M. This was the maximum permissible dose without too much loss in heart rate, contractile force and CPR. Verapamil hydrochloride was purchased from the Sigma Chemical Co.

4.3 Results

4.3.1 Verbascoside

Verbascoside and geniposidic acid (Fig. 4.1a and Fig. 4.8, respectively) were tested separately for their effect on the Langendorff rat heart (Tables 4.1 and 4.2). Following the administration of 1 ml of 1 mM verbascoside (Fig. 4.2), there were significant dose-dependent increases in chronotropism (Fig 4.2a), inotropism (Fig. 4.2b) and CPR (Fig. 4.2c), all of which occurred within the first two minutes. These effects lasted for several minutes except positive inotropism, which lasted less than a minute. The average increase in chronotropism was as high as 43 % (Fig. 4.2a). From an initial rate of 192 ± 11 beats per minute (bpm), it significantly (P = 0.001) increased to 275 ± 9 bpm. The effect lasted for a period of 10 minutes, the heart beat progressively returning to normal within that time and dropping slightly after that. Contractile force (Fig. 4.3) significantly (P = 0.016) increased from 1.15 ± 0.10 g to 1.32 ± 0.14 g (18%) in the first minute but returned to the initial value by the second minute (Fig. 4.2b). Following the positive inotropic phase

there was a gradual decline in contractile force, which is a common occurrence in the Langendorff rat heart bioassay. The most pronounced effect mediated by verbascoside was an increase in CPR (Fig. 4.2c). The initial rate of 8.2 ± 0.6 ml/min significantly (P = 0.002) rose to 14.3 ± 1.1 ml/min (74%). This increase was the longest lasting effect of the three recorded, taking an average of 15 minutes to return to the initial rate.

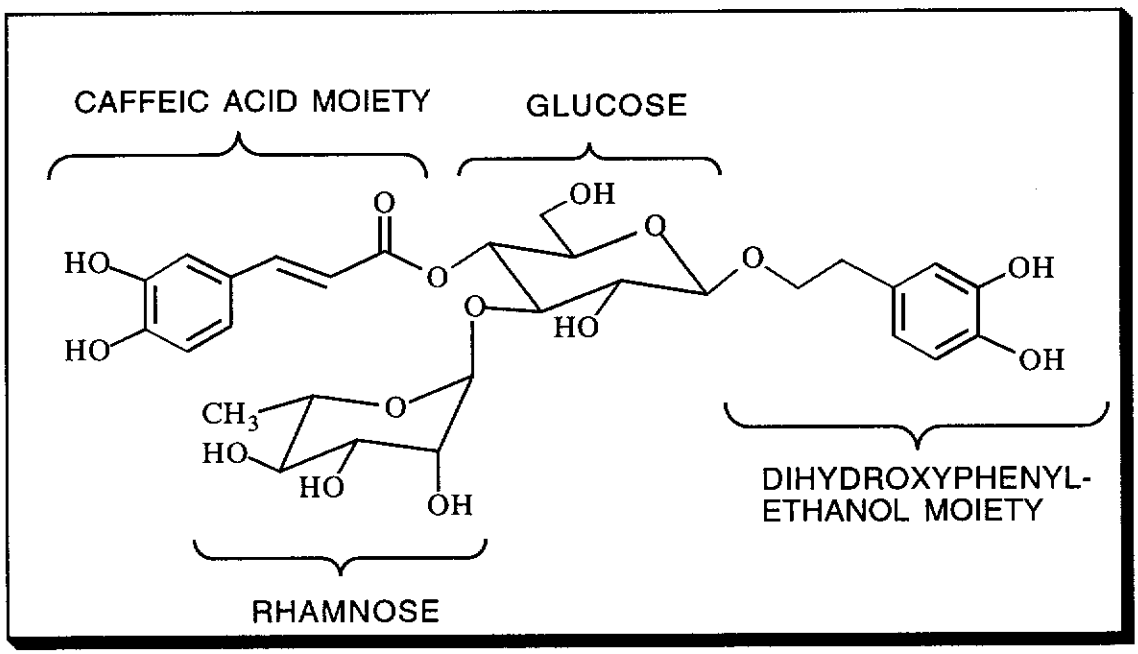


Fig. 4.1a. Chemical structure of verbascoside.

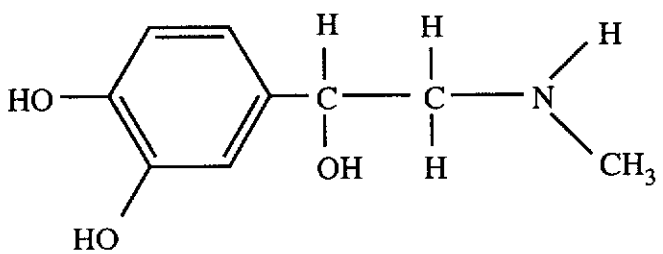


Fig. 4.1b. Chemical structure of adrenaline (epinephrine)

Table 4.1. Effects of verbascoside (1 ml of 1 mM) on Langendorff rat hearts (mean ± S.E.M).

Time (min)	*Heart Rate (bpm)*	*Force (g)*	*CPR (ml/min)*
-4	192 ± 11	1.15 ± 0.10	8.2 ± 0.6
-3	192 ± 11	1.15 ± 0.10	8.2 ± 0.6
-2	192 ± 11	1.15 ± 0.10	8.2 ± 0.6
-1	192 ± 11	1.15 ± 0.10	8.2 ± 0.6
0 (Start)	192 ± 11	1.15 ± 0.10	8.2 ± 0.6
1	237 ± 15	1.32 ± 0.14	14.3 ± 1.1
2	275 ± 9	1.14 ± 0.15	12.8 ± 1.0
3	237 ± 11	1.03 ± 0.14	12.5 ± 1.0
4	229 ± 16	1.12 ± 0.11	11.5 ± 0.8
5	216 ± 12	1.12 ± 0.11	10.9 ± 1.0
10	188 ± 8	0.98 ± 0.15	9.2 ± 0.8
15	187 ± 5	0.94 ± 0.15	8.2 ± 0.5

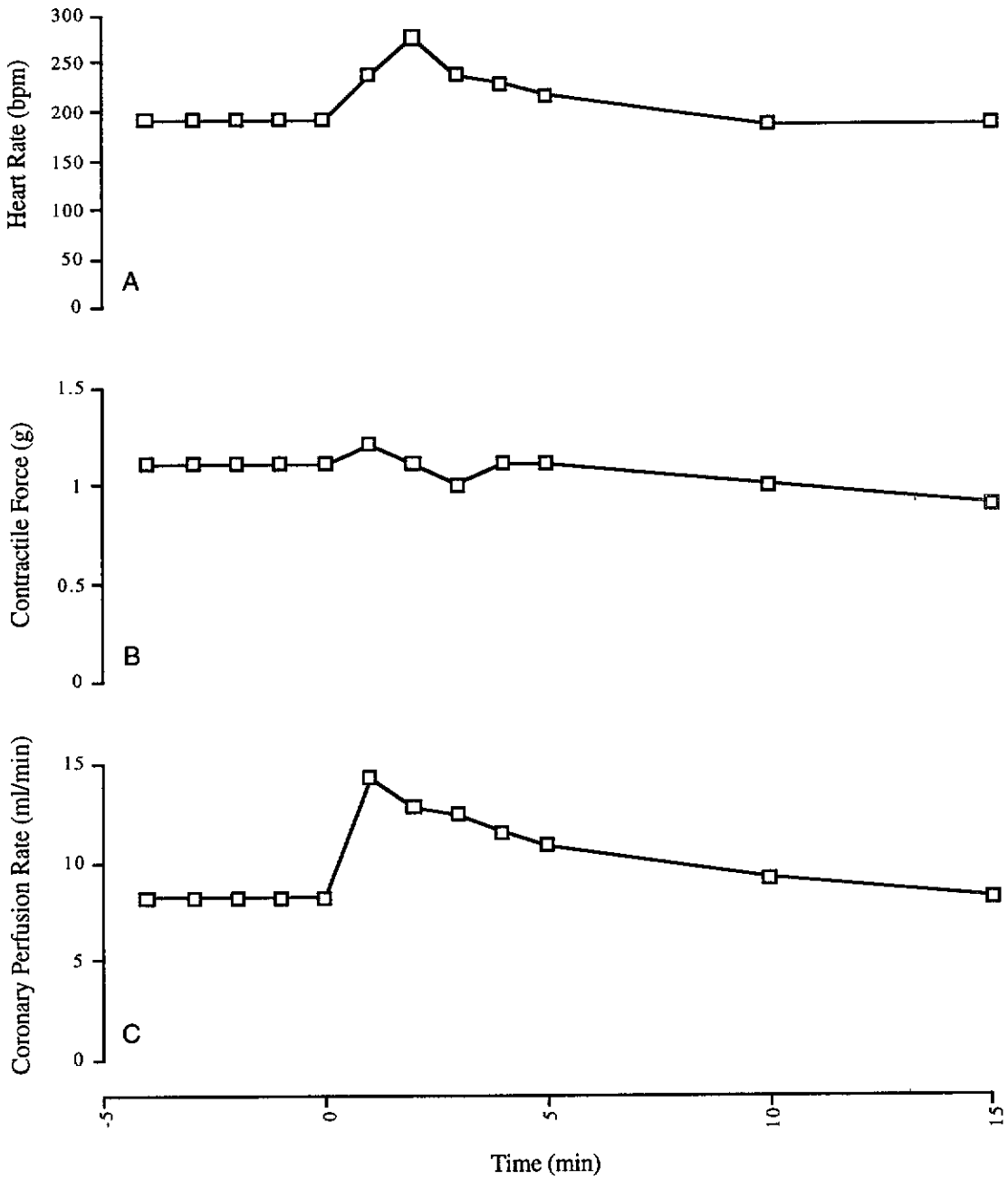


Fig. 4.2. (A) changes in chronotropism (bpm); (B) changes in inotropism (g); (C) changes in coronary perfusion rate (ml/min) after 1 ml of 1 mM verbascoside was administered to Langendorff rat hearts.

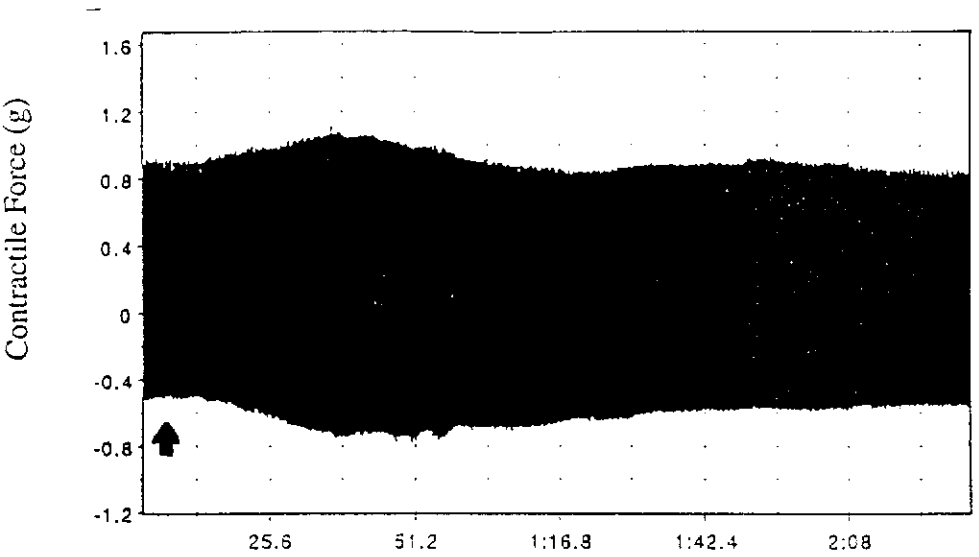


Fig. 4.3. Effects of verbascoside on contractile force.

4.3.1.1 *Dose-response Curves for Verbascoside*

At lower doses, the increases in chronotropism, inotropism and CPR were as follows. There were no significant changes in hearts treated with 1 ml of 1 μM verbascoside. Those treated with 1 ml of 10 μM exhibited a 9.5 % increase in chronotropism, a 4.7 % increase in inotropism and a 16.0 % increase in CPR. The initial average heart rate in these tests, which was 273 ± 22 bpm, increased to 299 ± 10 bpm. This increase was not significant according to the *t*-test (P = 0.233). Contractile force significantly increased from 1.07 ± 0.07 g to 1.12 ± 0.09 g (P = 0.041). Coronary perfusion rate was initially 10.4 + 2.0 ml/min., but soon after significantly rose to 12.0 ± 2.1 ml/min (P = 0.047). Heart rate increased by 16.3 % in hearts treated with 1 ml of 0.1 mM verbascoside. The initial rate of 257 ± 19 bpm rose to 299 ± 23 bpm (P = 0.029). Contractile force increased by 12.2 %, from 0.98 ± 0.02 g to 1.10 ± 0.07 g (P = 0.032). Finally, CPR increased

from 8.2 ± 0.9 ml/min. to 13.2 ± 1.7 ml/min (61.0 % increase; P = 0.030). The EC_{50} values (dose causing 50% excitation) derived from the three dose-response curves (Fig. 4.4a, 4.4b and 4.4c) were 0.33 mM for heart rate, 30.9 µM for contractile force and 29.5 µM for CPR. Attempts to increase the dose beyond 1 ml of 1 mM resulted in complete stoppage of all myocardial activity.

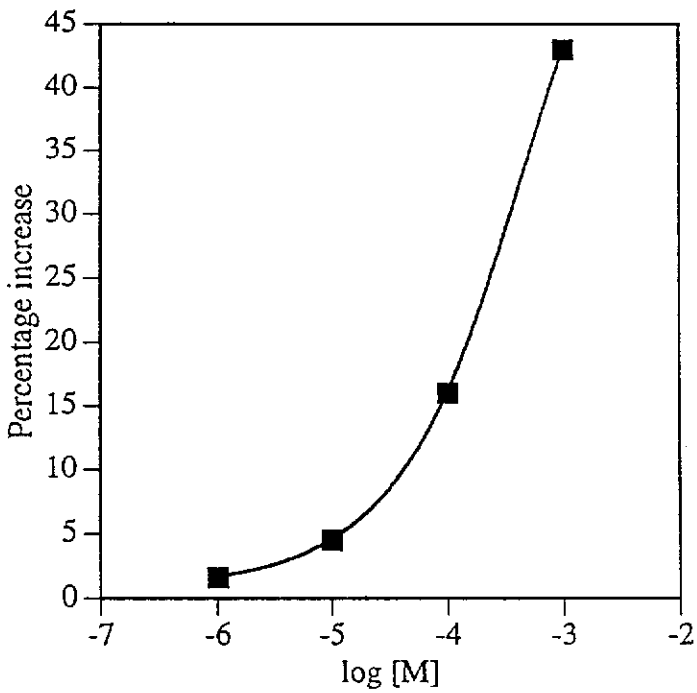


Fig. 4.4a. Dose-response curve for the effects of different doses of verbascoside on heart rate. Attempts to increase the dose beyond 1 ml of 1 mM caused complete stoppage of all myocardial activity.

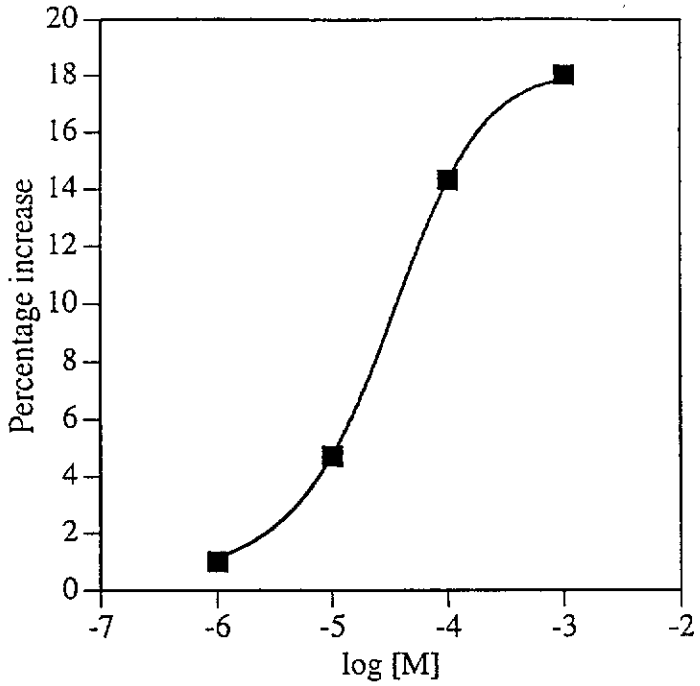


Fig. 4.4b. Dose-response curve for the effects of different doses (1 ml) of verbascoside on contractile force.

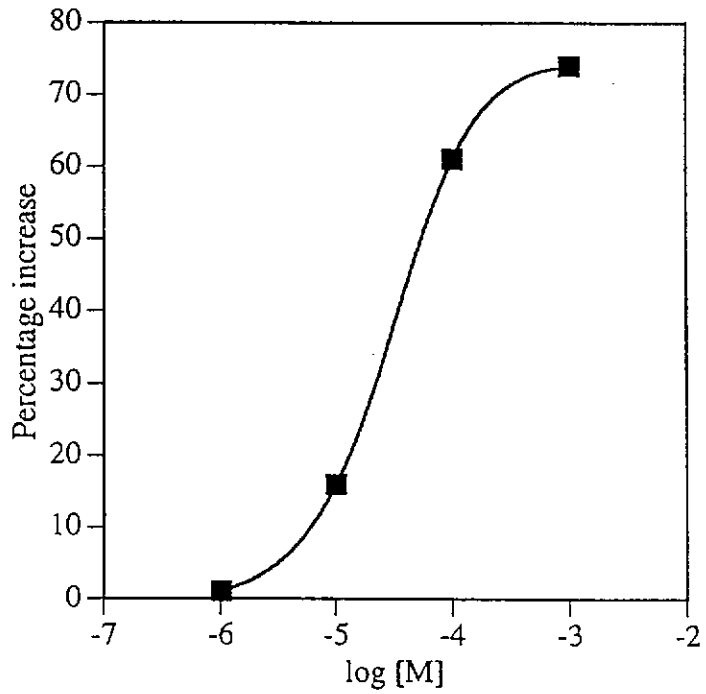


Fig. 4.4c. Dose-response curve for the effects of different doses (1 ml) of verbascoside on CPR.

4.3.1.2 *Adrenergic Receptor Blockage*

Since the caffeic acid moiety of verbascoside (Fig. 4.1a) bears a resemblance to the adrenergic agonist, adrenaline (Fig. 4.1b), verbascoside was tested in the presence of alpha (α) and beta (β) adrenergic antagonists (blockers). In chapter 3 it was shown that the cardioactive effects of the methanolic extract of *E. alternifolia* leaves on Langendorff rat hearts was not blocked by propranolol (β-blocker) or phentolamine (α-blocker). It was therefore hypothesised that the effects of verbascoside on Langendorff rat hearts would not be blocked by either of these antagonists.

In the presence of 1 μM propranolol, a dose high enough to block 1 ml of 1 μM isoproterenol, the effects of verbascoside were not blocked (Fig. 4.5a, 4.5b and 4.5c). An initial heart rate of 193 ± 21 bpm significantly (P = 0.003) rose to 260 ± 15 bpm (34.7 % increase). Coronary perfusion was also significantly increased (P = 0.0003). Initially 7.2 ± 0.7 ml/min., the rate at which coronary effluent was expelled rose to 13.0 ± 1.2 ml/min (80.6 % increase). Due to a faulty force-transducer, contractile force was not measured for experiments with propranolol.

When tested against the competitive α-adrenergic blocker, phentolamine (1 μM), verbascoside significantly increased chronotropism (P = 0.010), inotropism (P = 0.016) and CPR (P = 0.016). The initial heart rate of 185 ± 34 bpm was increased to 253 ± 33 bpm (36.6 %). The increase in positive inotropism was by 9.4 %. Starting at 0.96 ± 0.08 g, the force of contraction increased to 1.05 ± 0.07 g. Finally, CPR was increased from 8.5 ± 1.2 ml/min. to 14.3 ± 0.6 ml/min. (68.2 %). The effects of verbascoside on Langendorff rat hearts treated with phentolamine, in a dose high enough to completely block a 1 μM phenylephrine (α-adrenergic agonist), were not significantly blocked.

4.3.1.3 *Calcium Channel Blockage*

The widely used calcium channel blocker, verapamil, was also used in tests involving 1 ml of 1 mM verbascoside (Fig. 4.5a, 4.5b and 4.5c). Verapamil was added to the Krebs-Henseleit solution and continuously retrogradely perfused into the rat hearts. Previous experience with the blocker suggested that the 10^{-9} M was the maximum permissible dose without too much loss in heart rate, contractile force and CPR. Following the introduction of verapamil, Langendorff rat hearts experienced a 19.6 % decrease in heart rate. The initial rate of 271 ± 17 bpm significantly dropped to 218 ± 21 bpm (P = 0.019). The decreases in contractile force and CPR were greater. Contractile force was significantly (P = 0.020) reduced from 1.01 ± 0.03 g to 0.61 ± 0.05 g (39.6 %). The decrease in force was the deciding factor when determining the maximum permissible dose of verapamil. Coronary perfusion rate was significantly (P = 0.034) reduced from 10.1 ± 2.0 ml/min. to 7.9 ± 1.7 ml/min. (21.8 % decrease). Once the effects of verapamil had stabilised, the Langendorff rat hearts were treated with 1 ml of 1 mM verbascoside. In the presence of verapamil, verbascoside mediated a 46.8 % increase in chronotropism (Fig. 4.5a, Fig. 4.5b and Fig. 4.5c). At a verapamil-reduced heart rate of 218 ± 21 bpm, heart rate significantly increased to 320 ± 10 bpm (P = 0.023). The increase in CPR was also significant. After verapamil had reduced CPR to 7.9 ± 1.7 ml/min., verbascoside significantly increased it to 13.8 ± 2.2 ml/min. (94.4 % increase; P = 0.020). There was a small, but insignificant (P = 0.053) increase in inotropism. Contractile force rose from 0.61 ± 0.05 g to 0.65 ± 0.05 g.

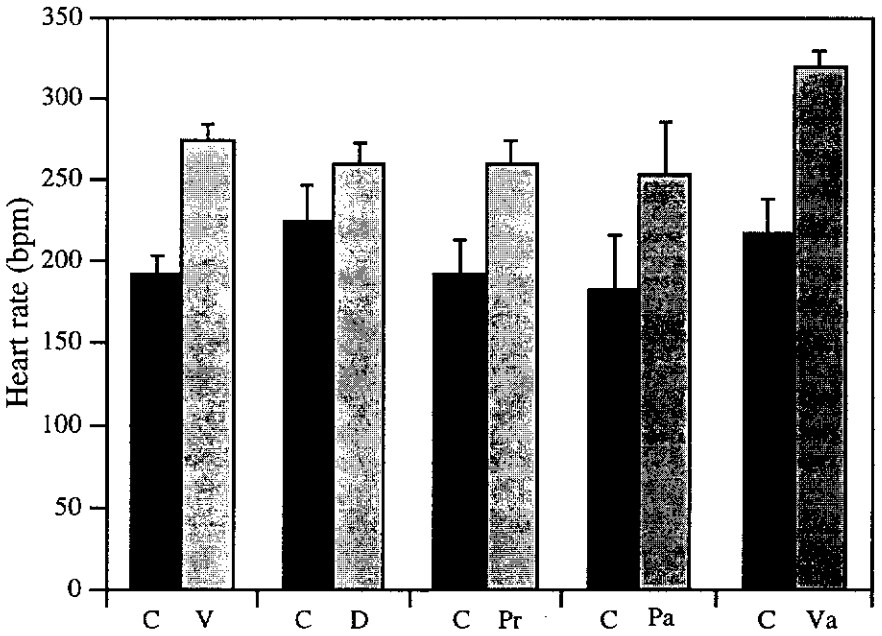


Fig. 4.5a. The effects of verbascoside (V) on heart rate when in the presence of propranolol (Pr), phentolamine (Pa) and verapamil (Va), compared to controls (C) and dimethyl verbascoside (D).

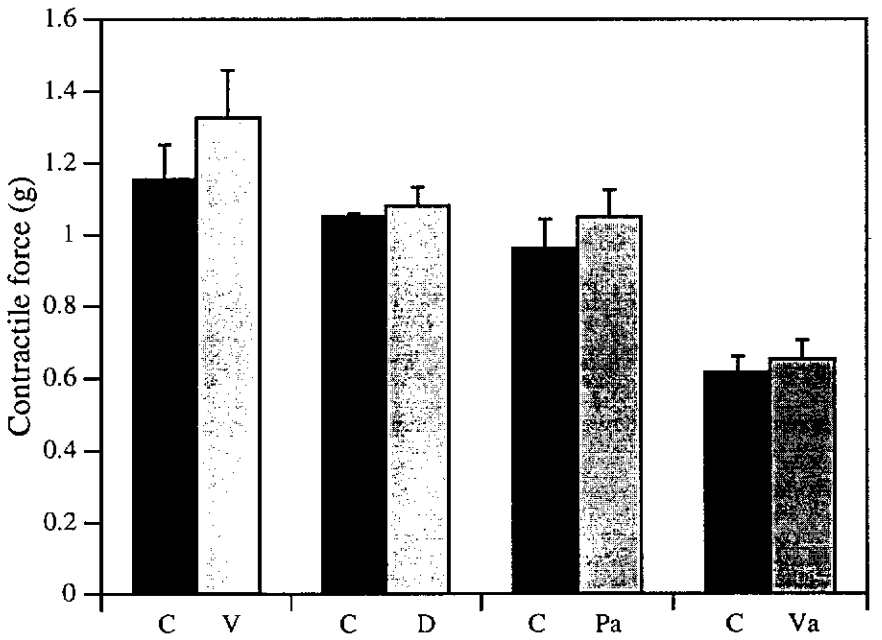


Fig. 4.5b. The effects of verbascoside (V) on contractile force when in the presence of phentolamine (Pa) and verapamil (Va), compared to controls (C) and dimethyl verbascoside (D).

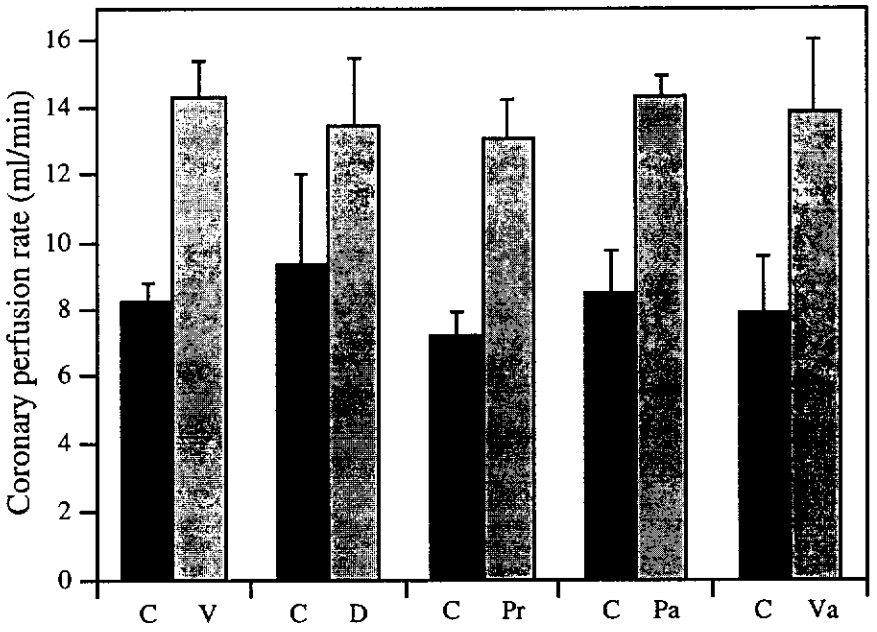


Fig. 4.5c. The effects of verbascoside (V) on CPR when in the presence of propranolol (Pr), phentolamine (Pa) and verapamil (Va), compared to controls (C) and dimethyl verbascoside (D).

4.3.2 *Dimethyl Verbascoside*

A similar and related compound to verbascoside was isolated from the leaves of *Lantana camara* and shown in five experiments to have a similar effect to verbascoside on Langendorff rat hearts. Dimethyl verbascoside (Fig. 4.6; MW = 652.648) was found to occur in the leaves of *L. camara* when this species was looked at as an alternative source for verbascoside. Herbert *et al.* (1991) showed that *L. camara*, a weed species in WA, yielded reasonably high quantities of verbascoside. Dimethyl verbascoside, like verbascoside, significantly (P = 0.024) increased chronotropism from 224 ± 24 bpm to 260 ± 13 bpm (16.1 % increase) (Fig. 4.5a, Fig. 4.5b, Fig. 4.5c; and Fig. 4.7). Inotropism was increased slightly (2.9 %), but was not significant. From an initial contractile force of 1.05 ± 0.01 g, it increased to 1.08 ± 0.05 and gradually decreased. Coronary perfusion rate was significantly (P = 0.044) increased from 9.3 ± 2.7 ml/min to 13.4 ± 2.1 ml/min (44.1 %). The effects lasted for an average period of two minutes before fading to normal over a period of 12 - 15 minutes.

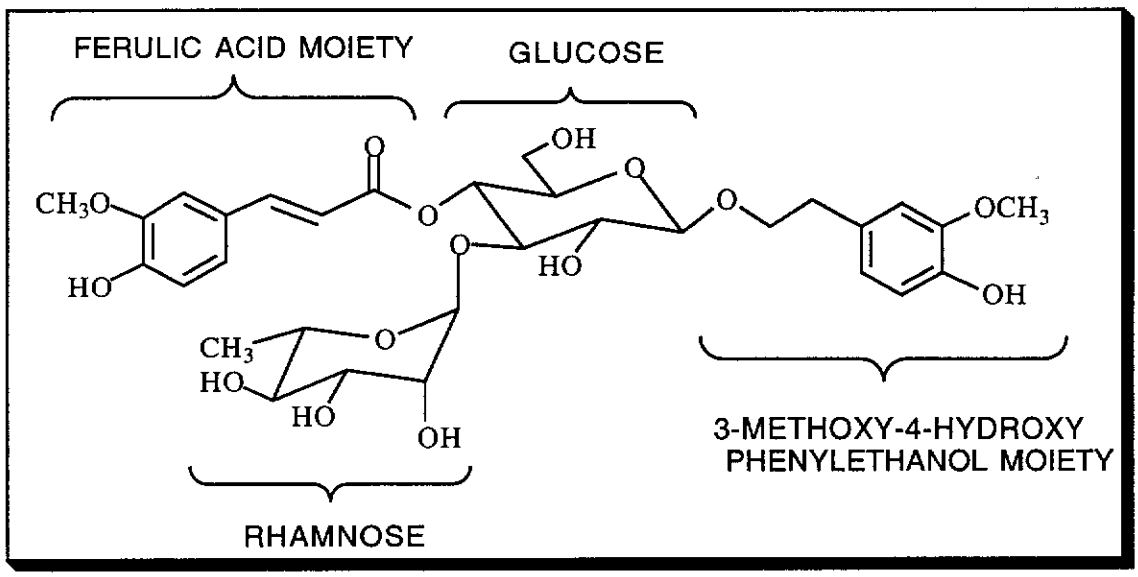


Fig. 4.6. Chemical structure of dimethyl verbascoside (from *Lantana camara* leaves). Note the substitution of one hydroxyl group for a methoxyl group on the caffeic acid and methyl phenylethanol moieties.

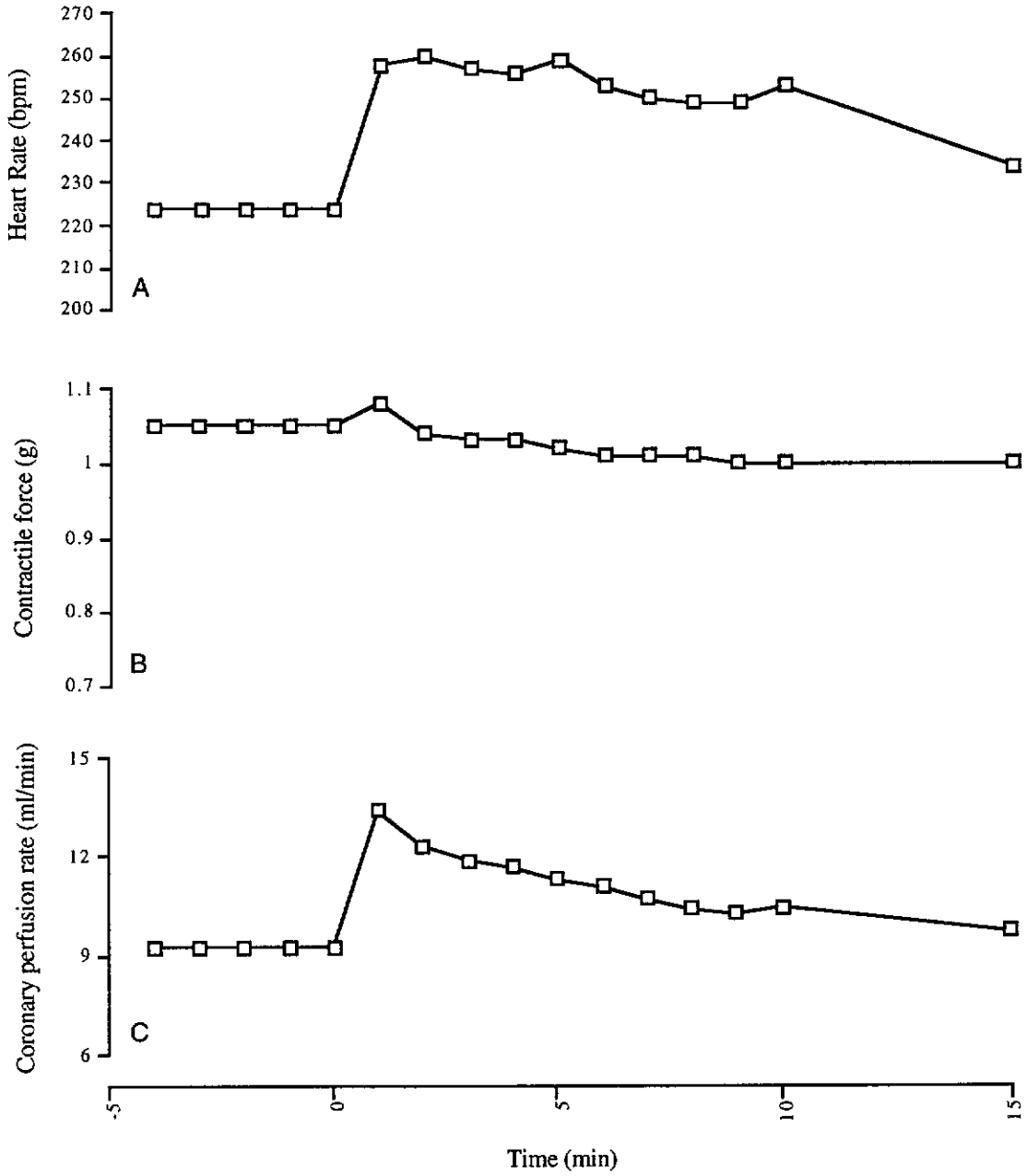


Fig. 4.7. (A) changes in chronotropism (bpm); (B) changes in inotropism (g); (C) changes in coronary perfusion rate (ml/min) after 1 ml of 1 mM dimethyl verbascoside was administered to Langendorff rat hearts.

4.3.3 Geniposidic Acid

With geniposidic acid (Fig. 4.8), at doses of 1 ml of 1 mM solution, the initial contraction force significantly (P = 0.002) decreased from 1.08 ± 0.05 g to 0.65 ± 0.10 g (40% decrease). Although there was a slight recovery from this effect, it did not return to its starting point (Fig. 4.9b). The changes in chronotropism were similar to those of inotropism (Fig. 4.9a). Starting at 247 ± 10 bpm, the heart rate significantly (P = 0.005) decreased to 214 ± 17 bpm (13%) two minutes after geniposidic acid was introduced. During this period CPR also decreased by 6%. After three minutes, the CPR increased slightly (7%) and then decreased again (43 %). By five minutes, the initial CPR of 8.6 ± 1.1 ml/min was reduced significantly (P = 0.004) to 4.9 ± 0.9 ml/min (Fig. 4.9c).

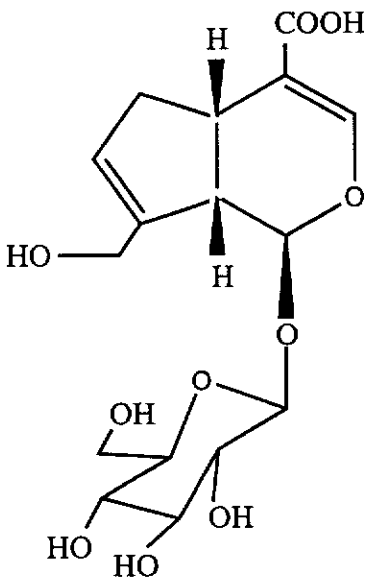


Fig. 4.8. Chemical structure of geniposidic acid

Table 4.2. Effects of geniposidic acid on Langendorff rat hearts (mean ± S.E.M).

Time (min)	*Heart Rate (bpm)*	*Force (g)*	*CPR (ml/min)*
-4	247 ± 10	1.08 ± 0.05	8.6 ± 1.1
-3	247 ± 10	1.08 ± 0.05	8.6 ± 1.1
-2	247 ± 10	1.08 ± 0.05	8.6 ± 1.1
-1	247 ± 10	1.08 ± 0.05	8.6 ± 1.1
0 (Start)	247 ± 10	1.08 ± 0.05	8.6 ± 1.1
1	222 ± 11	0.65 ± 0.10	8.1 ± 1.1
2	214 ± 17	0.78 ± 0.11	8.3 ± 1.2
3	222 ± 17	0.85 ± 0.12	9.2 ± 1.3
4	230 ± 14	0.89 ± 0.11	9.1 ± 1.5
5	233 ± 14	0.94 ± 0.12	4.9 ± 0.9
10	229 ± 15	0.86 ± 0.12	5.2 ± 1.2
15	229 ± 15	0.84 ± 0.11	7.6 ± 1.4

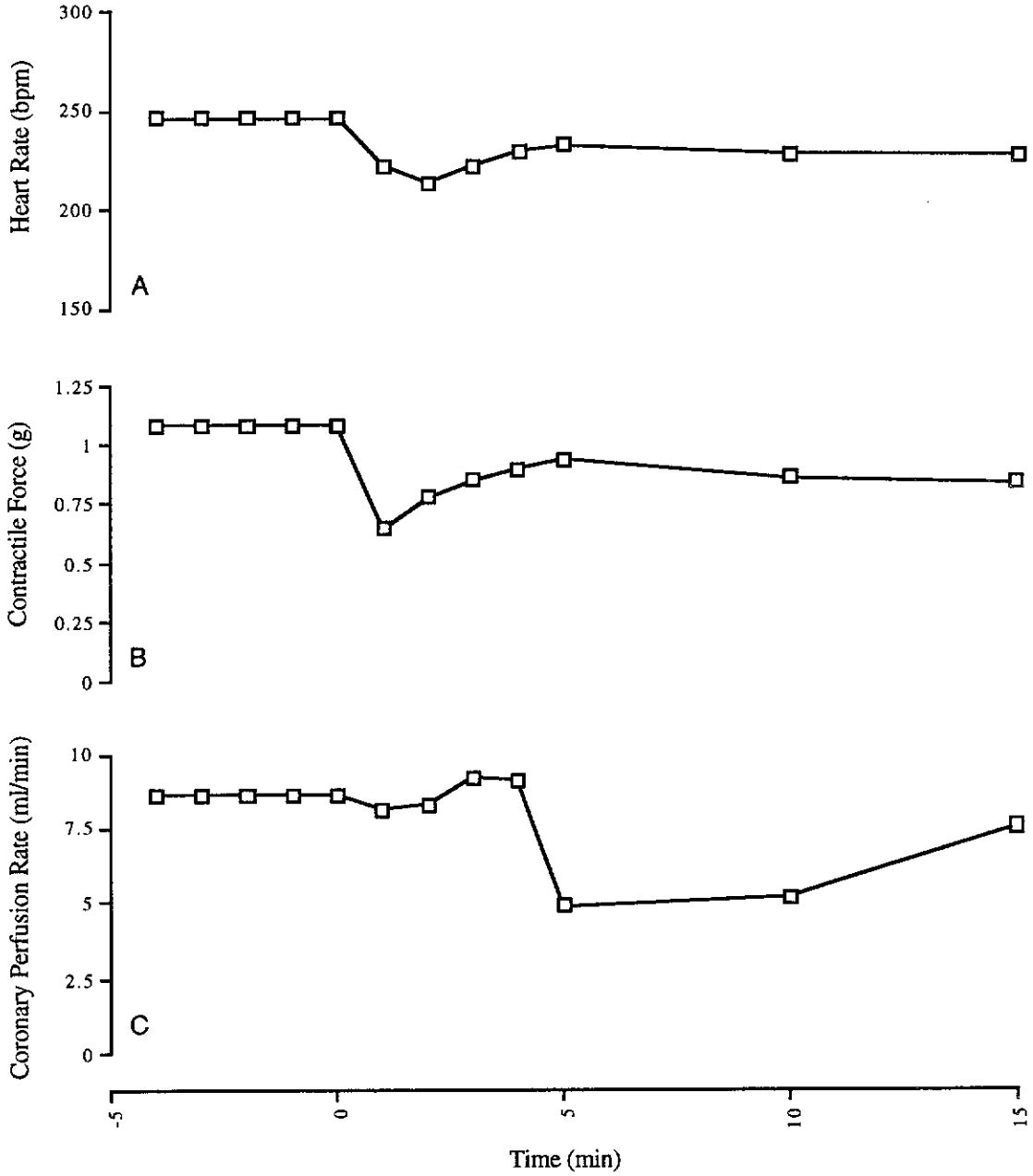


Fig. 4.9. (A) changes in chronotropism (bpm); (B) changes in inotropism (g); (C) changes in coronary perfusion rate (ml/min) after 1 ml of 1 mM geniposidic acid was administered to Langendorff rat hearts.

4.4 Discussion

4.4.1 Verbascoside

Verbascoside mediated dose-dependent increases in activity in Langendorff rat hearts. Significant increases in chronotropism, inotropism and CPR all occurred within two minutes of introducing the compound into the system and generally lasted for about 15 minutes. The dose-response curve for chronotropism suggested that higher doses would have induced even greater increases in this parameter. However, all attempts to further increase the dose beyond 1 ml of 1 mM were detrimental to the rat hearts. Hearts treated with doses greater than 1 ml of 1 mM remained in a constant state of contraction (systole), from which they did not recover. This indicates that 1 ml of 1 mM is the maximum permissible dose of verbascoside for the Langendorff rat heart bioassay.

When treated with verbascoside, Langendorff rat hearts exhibited a massive increase in CPR. One minute after 1 ml of 1 mM verbascoside was injected into the retrograde perfusion of Langendorff rat hearts, there was a 74 % increase in CPR. The increase indicated that there was significant vasodilation in the coronary arteries. In a whole animal bioassay, there would be a similar response in other blood vessels, resulting in a decrease in systemic vascular resistance and, thus, in blood pressure. From this rationalisation, it seems reasonable to suggest that the large verbascoside-induced drop in rat blood pressure, reported by Ahmad *et al.* (1995), was the result of significant vasodilation in the blood vessels of the cardiovascular system of Wistar rats. Verbascoside is therefore a potential antihypertensive agent.

Verbascoside is also a potent stimulator of the sinoatrial node (heart pacemaker). Heart rate rose by an average of 42 % immediately after verbascoside was administered. Surprisingly, this contradicts claims made by Andary *et al.* (1980) who suggested that

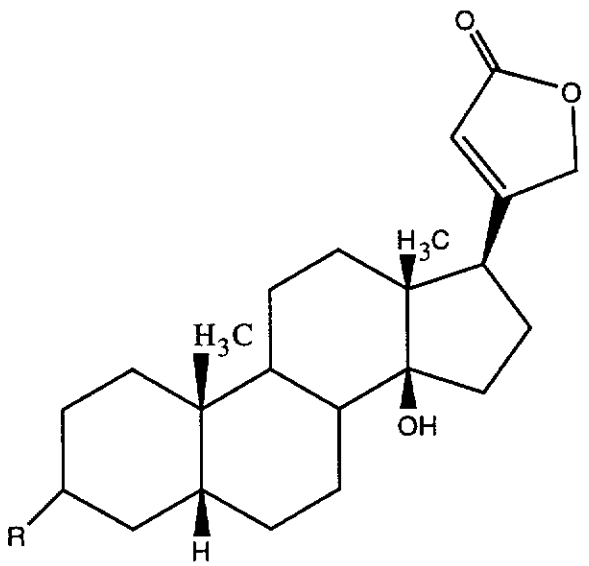
verbascoside had no significant effect on chronotropism in isolated rat auricle. It is not known why it was not affected in those tests. However, the dose used may have been too low. The only reference to dose made by Andary *et al.* (1980) was to a "large dose" specifically aimed at blocking (antagonising; inhibiting) the effects of isoproterenol - a potent β-adrenergic (β-AR) agonist (stimulator). In Langendorff rat hearts, doses of 1 ml of 0.01 mM, which are considered large in comparison to most other agonists, did not significantly increase heart rate (see results section of this chapter). It was not clear whether an agonistic (stimulatory) effect was searched for or suspected by Andary *et al.* (1980) and may have been overlooked.

It was interesting to note, however, that Andary *et al.* (1980) were unable to block the effects of isoproterenol when administered in the presence of verbascoside. Similarly, Ahmad *et al.* (1995) reported that verbascoside did not block the effects of noradrenaline (potent β–AR neurotransmitter), nor was it blocked by phentolamine (α–AR). In Langendorff rat hearts, tests with both α- and β-AR antagonists showed that verbascoside does not act through adrenergic receptors. The effects of verbascoside were not affected by the widely used clinical adrenergic antagonists, propranolol (β–AR) and phentolamine. All tests were performed on Langendorff rat hearts treated with sufficient doses of the adrenergic antagonists to completely block the effects of isoproterenol (β–AR agonist) and phenylephrine (α–AR agonist).

In support of these claims, it can be seen from the chemical structure of verbascoside that it lacks certain key chemical functional groups with which to act through adrenergic receptors. Adrenergic agonists bind with extracellular adrenergic receptors (glycoproteins) embedded in the phospholipid bilayer of cells. On contact with the receptor, the catecholamine is anchored in place by an amino acid in the cell receptor (aspartic acid), which is located in the 113th position of the receptor (Strader *et al.*, 1988; Strader *et al.*, 1989a). Critical to the anchoring process is the presence of a protonated amine group in the catecholamine, which verbascoside does not possess (see Figs. 4.1a and 4.1b).

Once anchored, the compound is orientated so that the two hydroxy groups of the catecholamine's benzene ring, located on positions 3 and 4, come in contact and bind with the two amino acids, serine 204 and 207, respectively (Strader *et al.* 1989b). However, whether or not the catechol moiety is necessary is not certain. Ephedrine, extracted from the branches of *Ephedra trifurca* (Ephedraceae) and widely used in Asia and America (Weiner, 1980), stimulates both α- and β-adrenergic receptors to increase heart rate and cardiac output, but does not possess the catechol moiety (Hoffman and Lefkowitz, 1991).

Likewise, the involvement of the Na^+-K^+-ATPase enzyme was also eliminated on the basis that the chemical structure of verbascoside does not conform with the basic requirements for inhibition of this enzyme. In order to satisfy the requirements, compounds must be comprised of three parts, consisting of a steroidal nucleus, a sugar moiety and a lactone ring attached at C 17 (Fig. 4.10). Cardiotonic activity is thought to involve the lactone ring, which must be unsaturated, the β-hydroxy group at C 14 and the *cis*-fusion of the A/B ring junctures (Ross and Brain, 1977). Verbascoside does not possess any of these important moieties and therefore does not satisfy any of the criteria for inhibition of the Na^+-K^+-ATPase enzyme.



R = tridigitoxosyloxy

Fig. 4.10. Digitoxin. Adapted from Repke *et al.* (1995).

The involvement of cholinergic and histaminergic receptors was considered, but both were excluded by Ahmad *et al.* (1995) who showed that verbascoside's ability to reduce blood pressure in rats was unaffected by atropine (antimuscarinic agent) and chloropheniramine/cimetidine (antihistaminic agent). Having eliminated all known extracellular receptors capable of eliciting a positive response, slow calcium channels emerged as potential candidates. Calcium plays an important role in myocardial activity. It is generally accepted that Ca^{++} prolongs contraction in heart cells. Inhibition of the Na^{+}-K^{+}-ATPase enzyme, for example, results in a increase in the concentration of Ca^{++} ions inside myocardial (heart) cells, thus increasing activity (Repke *et al.*, 1995). Slow calcium channels were blocked with verapamil to determine their role, if any, in the positive effects mediated by verbascoside. Routinely used clinically, verapamil did not significantly diminish the increases in chronotropism and CPR mediated by verbascoside. Contractile force was also increased, but not significantly. However, it was by only a small margin that the increase was not significant ($P = 0.053$). It is possible that the rapid onset of the large chronotropic effect masked the inotropic effect. Similar occurrences have been observed in other tests with the Langendorff rat heart bioassay. The intrinsic myocardial depressant effect of verapamil, which was noted immediately following its introduction into the perfusate, is a well established effect of this compound (Francis, 1991).

Although no conclusions can be made about the structure-related cardioactivity of verbascoside, there is some evidence to suggest that subtle variations in the structure of the compound are possible without significant loss in activity. When treated with 1 ml of 1 mM of di-methyl verbascoside, Langendorff rat hearts experienced a significant increase in chronotropism and CPR. The chemical structure of di-methyl verbascoside is similar to that of verbascoside, with the difference that a OH group on the caffeic acid moiety and a OH group on the hydroxy phenylglycol moiety are substituted with methoxyl groups

(compare Fig. 4.1a and Fig. 4.6). Dimethyl verbascoside has not previously been shown to have biological activity.

4.4.2 Geniposidic Acid

Tests with pure geniposidic acid confirmed that it was the active constituent in the MeOH extract of *E. longifolia* leaves. Geniposidic acid significantly decreased heart rate, contractile force and CPR, a response similar to that noted for the MeOH extract (chapter 3). However, in tests with the MeOH extract the effects were biphasic, suggesting that more than one compound played a role in mediating the effect. Alternatively, one compound capable of two effects was responsible. Pure geniposidic acid did not appear to exhibit a biphasic effect, indicating that at least one other compound caused the positive response. Verbascoside, which also occurs in *E. longifolia* leaves, was responsible for the positive effect induced by the extract. The effects of geniposidic acid are compared in chapter 7 with similar iridoid glucosides from other *Eremophila* and plant species.

CHAPTER 5

THE EFFECTS OF VERBASCOSIDE ON INTRACELLULAR LEVELS OF CYCLIC 3',5'-ADENOSINE MONOPHOSPHATE

CHAPTER 5

The effects of verbascoside on intracellular levels of cyclic 3',5'-adenosine monophosphate

5.1 Introduction

Cardiotonic agents exist in two forms; those that affect intracellular cyclic 3',5'-adenosine monophosphate (cAMP) levels and those that act independently of this mechanism. Compounds in the cAMP-dependent class mediate cardiac contractility by increasing the levels of intracellular cAMP or by inhibiting the metabolism of this nucleotide. These include the β-adrenergic agonists, forskolin from *Coleus forskohlii* (Metzger and Linder, 1981) and the phosphodiesterase inhibitors. The cAMP-independent class of compounds, eg. cardiac glycosides, inhibit the Na^+/K^+-ATPase enzyme (Packer, 1992).

The role of cAMP is the subject of much interest in the life sciences (Brown *et al.*, 1971). Cyclic AMP plays an important role as a second messenger to regulate a number of physiological responses. It is generally accepted that production of cAMP in myocardial cells (heart cells) is increased by the activation of the adenylate cyclase enzyme (Fig. 5.1). In brief, the adenylate cyclase, enzyme converts adenosine triphosphate (ATP) into cAMP. Cyclic AMP in turn phosphorylates (adds a phosphate group) two protein kinases (first protein kinase A, which then phosphorylates protein kinase B), to induce the release of stored Ca^{++} from within the sarcoplasmic reticulum. A phosphodiesterase III enzyme then inactivates cAMP by converting it into the inert 5'-adenosine monophosphate (5'-AMP).

Increases in intracellular cAMP levels can be achieved in several ways. Compounds, such as forskolin, directly stimulate the adenylate cyclase enzyme to speed up production (Metzger and Linder, 1981). Others, the catecholamines for example, bind with

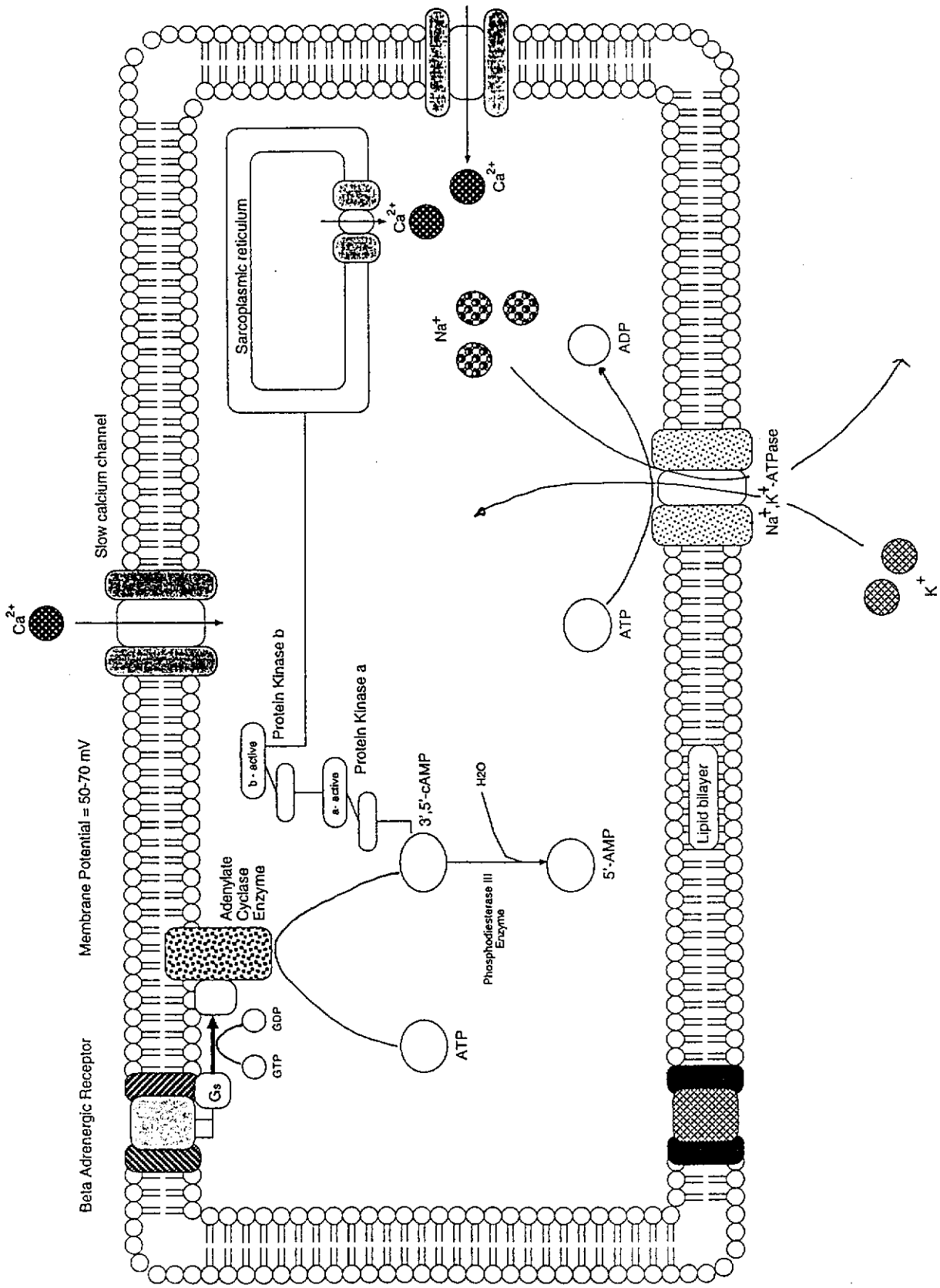


Fig. 5.1. Stimulation of heart cells by cAMP-dependent and independent mechanisms.

adrenergic receptors and directly stimulate adenylate cyclase through a protein coupled to the enzyme (Sutherland and Rall., 1960). Phosphodiesterase III inhibitors, such as amrinone, prevent the breakdown of cAMP into 5'-AMP (Cruickshank, 1993). Cyclic AMP production is also increased through the agency of prostacyclin (Moncada *et al.*, 1976). However, it is not clear how prostacyclin affects adenylate cyclase.

5.2 Materials and Methods

The collection of plant material, plant preparation, extractions, isolated rat hearts, perfusate and data recording were the same as those in Chapters 3 and 4. In all, 17 rats were used, 10 of which were controls and seven experimentals (Animal Ethics and Experimentation Committee approval No. was R7/95).

5.2.1 cAMP level analysis

All tests were conducted only after Langendorff rat hearts had reached a constant rate and force of contraction. Freshly prepared solutions of verbascoside (1 mM) were administered through a polyethylene cannula in 1 ml/minute retrograde perfusion. Each heart was allowed to reach a maximum response, after which it was immediately freeze-clamped using liquid nitrogen (-196°C), according to Brown *et al.* (1971), and stored at -15°C until analysed for cAMP levels. The control hearts were allowed to reach a constant rate of contraction before being treated with liquid nitrogen.

Frozen tissue of known mass was then homogenised in cold 6% trichloroacteic acid at 2-8°C to give a 10% (w/v) homogenate which was centrifuged at 2000 revolutions per minute (RPM) for 15 minutes. The supernatant was removed and washed five times with

water-saturated diethyl ether and then lyophilized. Just prior to the analysis, the dried extract was dissolved in assay buffer. Intracellular cAMP levels were measured using a Biotrak™ radioassay kit from Amersham International and adjusted for mass according to the following procedure:

The presence of cAMP for each sample was determined with a beta counter (in duplicate), averaged and then represented as a percentage ($\%B/B_0$: percentage bound divided by the zero standard) (table 5.1). The concentration of cAMP per tube was then calculated (fmol/tube) from an equation determined based on the regression line obtained from the standards (see results below for equation). Dividing the concentration of cAMP per tube by the mass of heart tissue used in the extraction of the nucleotide, the concentration of cAMP per gram of tissue (pmol/g) was calculated. This was then converted to the concentration of cAMP for the dry weight of the tissue samples (nmol/g DW). An average for the control hearts and experimental (treated) hearts was determined separately.

5.2.2 Statistical Analysis

At least seven hearts were used for each group. The data was analysed by *t*-tests with probabilities of less than 0.05 considered statistically significant. All results are expressed as mean ± S.E.M.

5.3 Results

In experiments for cAMP determination, the normal heart rate was 231 ± 12 beats/minute, the contractile force was 1.05 ± 0.04 g and CPR was 9.8 ± 1.5 ml/minute. The

intracellular level of cAMP was 3.0 ± 0.2 nmol/g dry weight (Table 5.1). The average count for the $B\%/B_o$ was 9739 (from beta counter).

The equation used to determine the concentration of cAMP per tube (fmol/tube) was:

$$\text{Conc. cAMP/tube} = 10^{(\%B/Bo - 157.898/ - 40.931)}$$

The introduction of verbascoside (1 ml of 1 mM) into the perfusate increased the heart rate to 327 ± 14 beats/minute (42%) and the coronary perfusion rate to 20.9 ± 2.2 ml/minute (113%). Just prior to that, the contraction force increased to 1.21 ± 0.06 g (13%). All of these responses were statistically significant ($P < 0.05$) and occurred within a minute of the compound being introduced into the system. During the increases, a significant ($P < 0.05$) rise in cAMP was observed. Cyclic adenosine monophosphate levels had risen to 55.2 ± 4.8 nmol/g dry weight (1733 %) (Table 5.1).

Table 5.1 cAMP data

Sample	1st Count	2nd Count	Aver/min	Samp. Mass	%B/Bo	fmol/tube	pmol/g	nmol/g dw
C1	16692	18806	8875	0.2259	91	43	189	3.03
C2	18375	18554	9232	0.1957	95	35	178	2.85
C3	19411	15850	8815	0.2105	91	44	210	3.37
C4	16439	17120	8390	0.2205	86	57	257	4.11
C5	19154	17277	9108	0.2241	94	37	167	2.67
C6	17471	20503	9494	0.2138	97	30	140	2.24
C7	18768	18563	9333	0.2157	96	33	152	2.44
C8	18326	18742	9267	0.2009	95	34	170	2.72
C9	19044	19444	9622	0.2025	99	28	137	2.20
C10	18692	15977	8667	0.2097	89	48	230	3.68
C11	17217	17440	8664	0.2222	89	48	217	3.48
C12	17417	17787	8801	0.2103	90	45	212	3.40
E1	18729	20155	4861	0.1126	50	435	3862	61.79
E2	21142	21138	5285	0.1265	54	340	2690	43.04
E3	20123	20220	5043	0.1231	52	391	3179	50.87
E4	20641	18572	4902	0.1578	50	425	2691	43.06
E5	18815	19016	4729	0.1091	49	469	4301	68.81
E6	18652	18513	4646	0.1751	48	492	2812	44.99
E7	17139	19497	4580	0.1112	47	511	4600	73.60

5.4 Discussion

The assay used to determine intracellular levels of cAMP in Langendorff rat hearts before and after treatment with verbascoside is based on the competition between unlabelled cAMP and a fixed amount of tritium-labelled substance that binds to a protein with a high affinity for cAMP (Brown *et al.*, 1971). The protein and cAMP complex is inversely related to unlabelled cAMP, thus enabling the amount of cAMP in the assay sample to be calculated.

The results presented here suggest that verbascoside increases chronotropism, inotropism and CPR via a cAMP-dependent mechanism. Injection of 1 ml of 1 mM verbascoside significantly increased intracellular levels of cAMP from 3.0 ± 0.1 nmol/g dry weight to 55.2 ± 4.8 nmol/g dry weight (1733 %). By comparison, forskolin (0.2 μM) increased intracellular cAMP levels from 3.0 ± 0.1 to 14.1 ± 0.9 nM/g dry weight (370 %) and epinephrine (1 μM) by 257 % (Manning *et al.*, 1985).

The increase in intracellular cAMP levels was higher than anticipated. According to Manning *et al.* (1985), there is a linear relationship between increases in intracellular cAMP levels and chronotropism. They suggested that a 17 % increase in cAMP resulted in a 1 % increase in heart rate. Using these figures, it was estimated that a 42 % verbascoside-induced increase in heart rate would require a 714 % increase in cAMP. The figure obtained in this work was 2.4 times higher (1733 %). The actual increase in cAMP levels may have been lower. When working with such minute concentrations, such as those used in the detection of the second messenger, eg 3.0 nM, there is a possibility that there may be large errors associated with the final figure. Calculating errors was not possible with the kit used.

Despite these results showing a significant increase in intracellular cAMP levels due to 1 ml of 1 mM verbascoside, they do not reveal whether the observed increases were the result of stimulation of the adenylate cyclase enzyme or by inhibition of the phosphodiesterase III enzyme (PDE III). Phosphodiesterase III breaks down the cAMP to 5'-AMP, rendering it inert. If inhibited, PDE does not carry out its function and the whole process continues.

Kitagawa *et al.* (1984) reported that verbascoside had no inhibitory effect on the phosphodiesterase III enzyme *in vitro*. It is tempting to suggest therefore that the rise in intracellular cAMP mediated by verbascoside may be the result of increases in levels of prostacyclin. In 1977, Moncada *et al.* showed that prostacyclin was both a potent vasodilator and stimulator of cAMP production. This prostanoid, which is synthesised via the arachidonic acid pathway, is spontaneously transformed into 6-keto-$PGF_{1\alpha}$. Significant quantities of 6-keto-$PGF_{1\alpha}$ were observed when 1 ml of 1 mM verbascoside was administered to rat peritoneal cell preparations (Kimura *et al.*, 1987). Also, the vasodilatory effects of prostacyclin would explain the massive increases in coronary perfusion rate (113 %), which were mediated by verbascoside in the isolated rat heart. Changes in intracellular levels of 6-keto-$PGF_{1\alpha}$ were monitored for in Langendorff rat hearts treated with 1 ml of 1 mM verbascoside (Chapter 6).

CHAPTER 6

THE EFFECTS OF VERBASCOSIDE ON INTRACELLULAR LEVELS OF PROSTACYCLIN

CHAPTER 6

The effects of verbascoside on intracellular levels of prostacyclin

6.1 Introduction

Prostacyclin was first discovered in 1976 by Moncada and his colleagues (Moncada *et al.*, 1976). Working with bovine aortic microsomes, they discovered that the novel labile substance resulted from the metabolism of endoperoxidases in the arichidonic acid pathway (Gryglewski *et al.*, 1976) (Fig. 6.1). Provisionally labelled `prostaglandin X', the substance's structure was later established and given the trivial name prostacyclin (PGI_2) (Moncada *et al.*, 1971). This highly unstable vinyl ether is generated when the cyclo-oxygenase enzyme catalyses free arachidonic acid into PGH_2 (prostaglandin H_2), which is then transformed into PGI_2 (Fig. 6.1). Prostacyclin is spontaneously hydrolysed into the more stable 6-keto-$PGF_{1\alpha}$ (6-keto-prostaglandin), a substance widely used as an index of PGI_2 formation (Änggård and Flower, 1993). Prostacyclin is a potent vasodilator of arteries and veins, inhibits platelet aggregation, stimulates the production of cAMP (Moncada *et al.*, 1976) and is found in all tissue types (Coleman *et al.*, 1990).

Whilst studying the effects of verbascoside on rat peritoneal cells and human peripheral polymorphonuclear leukocytes, Kimura *et al.* (1987) detected increases in the levels of 6-keto-$PGF_{1\alpha}$. They proposed that the verbascoside-induced PGI_2 effects in leukocytes may also be present in coronary vessels and, therefore, may play a major role in dilating those vessels. To determine whether increases in CPR and intracellular cAMP levels in Langendorff rat hearts (in response to verbascoside) were mediated by PGI_2, the measurement of levels of 6-keto-$PGF_{1\alpha}$ was considered important.

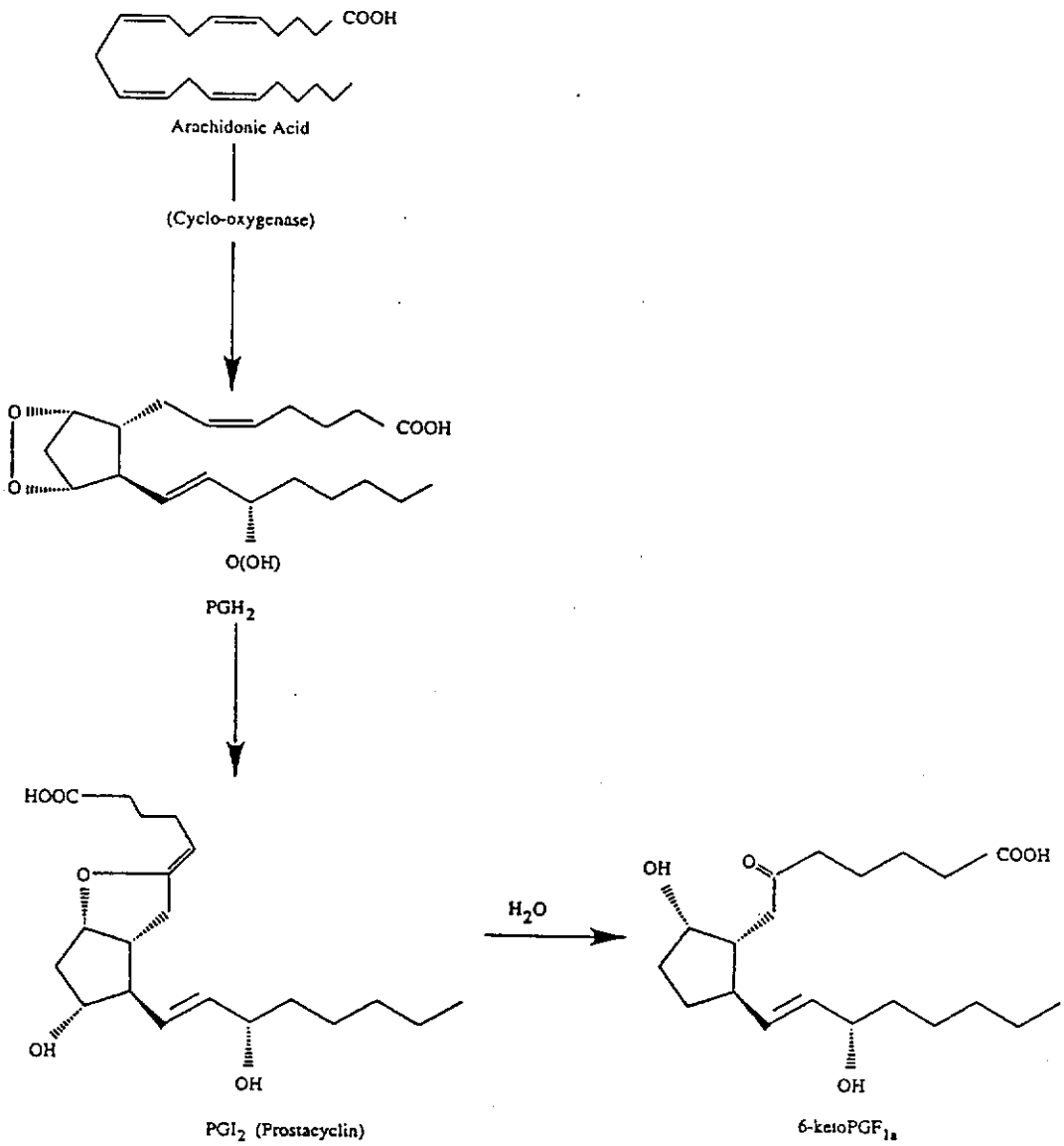


Fig. 6.1 Production of prostacyclin ($PG1_2$) via the arachidonic acid pathway.

6.2 Materials and Methods

The collection of plant material, plant preparation, extractions, isolated rat hearts, perfusate, data recording and data analysis were the same as those in Chapters 2 and 3. Ten rats were used in these experiments, five of which were for controls and five experimental (Animal Ethics and Experimentation Committee approval No. was R28/96).

6.2.1 6-keto-prostaglandin Level Analysis

A freshly prepared solution of verbascoside (1 ml of 1 mM) was administered through a polyethylene cannula in 1 ml/minute retrograde perfusions. Following the method of Grandstrom and Kindahl (1978), each stabilised heart was allowed to reach a maximum response, after which it was immediately freeze clamped using melting isopentane (pre-cooled in liquid nitrogen) and then stored at -15°C until analysed for 6-keto-$PGF_{1\alpha}$ levels. The control hearts were allowed to reach a constant rate of contraction before being immersed into melting isopentane.

Freeze-clamped tissue was homogenised in cold phosphate buffer at 2-8°C. Acetone was added to the homogenate and was shaken for two minutes. The supernatant was then transferred to a separate vial containing hexane and was shaken for two minutes before being centrifuged (2000 RPM) for five minutes at 4°C. The upper hexane layer was discarded and the remaining extract adjusted to a pH of between 3 - 4 with 1 M citric acid. Two ml of chloroform was then added to the extract and shaken for two minutes before being centrifuged for five minutes at 4°C . The upper chloroform layer was removed and the aqueous layer was re-extracted with chloroform. The two extracts were then combined and vacuum dried. Prior to the analysis, the dried extract was dissolved in assay buffer.

Intracellular 6-keto-$PGF_{1\alpha}$ levels were measured using a Biotrak™ enzyme-immunoassay kit from Amersham International. The enzyme-immunoassay used (Biotrak) is based on the competition between unlabelled 6-keto-$PGF_{1\alpha}$ and a known quantity of peroxidase labelled 6-keto-$PGF_{1\alpha}$ with a limited number of binding sites on a 6-keto-$PGF_{1\alpha}$ specific antibody. The amount of peroxidase labelled ligand bound by the antibody is inversely proportional to the concentration of added unlabelled ligand. The walls of the microtitre plate used were precoated with second antibody. Any peroxidase ligand that was bound to the antibody was immobilised onto the polystyrene microtitre walls. All unbound ligand was removed by simple washing procedures. The amount of peroxidase labelled 6-keto-$PGF_{1\alpha}$ bound to the antibody was then determined by adding tetramethylbenzidine/hydrogen peroxide substrate. This reaction was stopped 15 minutes later by the addition of 1 M sulphuric acid. The labelled 6-keto-$PGF_{1\alpha}$ present was determined using a microtitre plate photometer at 450 nm.

6.2.2 Data Analysis

All data was analysed by *t*-tests with probabilities of less than 0.05 considered statistically significant. All results are expressed as mean ± S.E.M.

6.3 Results

Langendorff rat hearts treated with 1 mM verbascoside were compared for levels of 6-keto-$PGF_{1\alpha}$ (experimentals) against those without treatment (controls). The mean average heart rate was 236 ± 20 bpm, the contractile force was 1.00 ± 0.01 g and CPR 8.3 ± 1.7 ml/min. The resting heart rates, contractile forces and CPR of the experimental hearts were also measured before being treated with verbascoside. They were 269 ± 20 bpm, 1.02 ± 0.02 g and 8.2 ± 1.2 ml/min. Following the administration of 1 ml of 1 mM verbascoside, heart rate was significantly ($P = 0.005$) increased to 311 ± 17 bpm,

contractile force to 1.19 ± 0.03 g (P = 0.0003) and CPR to 15.6 ± 1.1 ml/min (P = 0.001). These corresponded to increases of 42.0 %, 16.7 % and 90.2 %, respectively.

The percentage bound (%B/B_0) of the six standards, one zero standard (B_0) and non-specific binding standard (NSB) are listed in Table 6.1 and graphically represented in Fig. 6.2. Figure 6.3 is the same graph drawn with a logarithmic X axis. To obtain these figures, the optical densities (OD) for each standard and sample was first determined on the microtitre plate photometer. From these, the NSB optical density was subtracted and then divided by the zero standard (minus NSB). The resulting number was then multiplied by 100 to express it as a percentage as according to the following formula:

$$\%B/B_0 = \frac{\text{(standard or sample OD - NSB OD)}}{\text{(B_0 OD - NSB OD)}} \times 100$$

Table 6.1 %B/B_0 for standards

Standard	OD	OD	Average	pg/well
	1st Count	2nd Count		
2	0.606	0.608	0.607	88.8
4	0.527	0.478	0.503	69.8
8	0.430	0.451	0.441	58.6
16	0.293	0.382	0.338	39.9
32	0.304	0.275	0.290	31.3
64	0.243	0.239	0.241	22.5
NSB	0.108	0.126	0.117	0.00
B_0	0.608	0.658	0.669	100.0

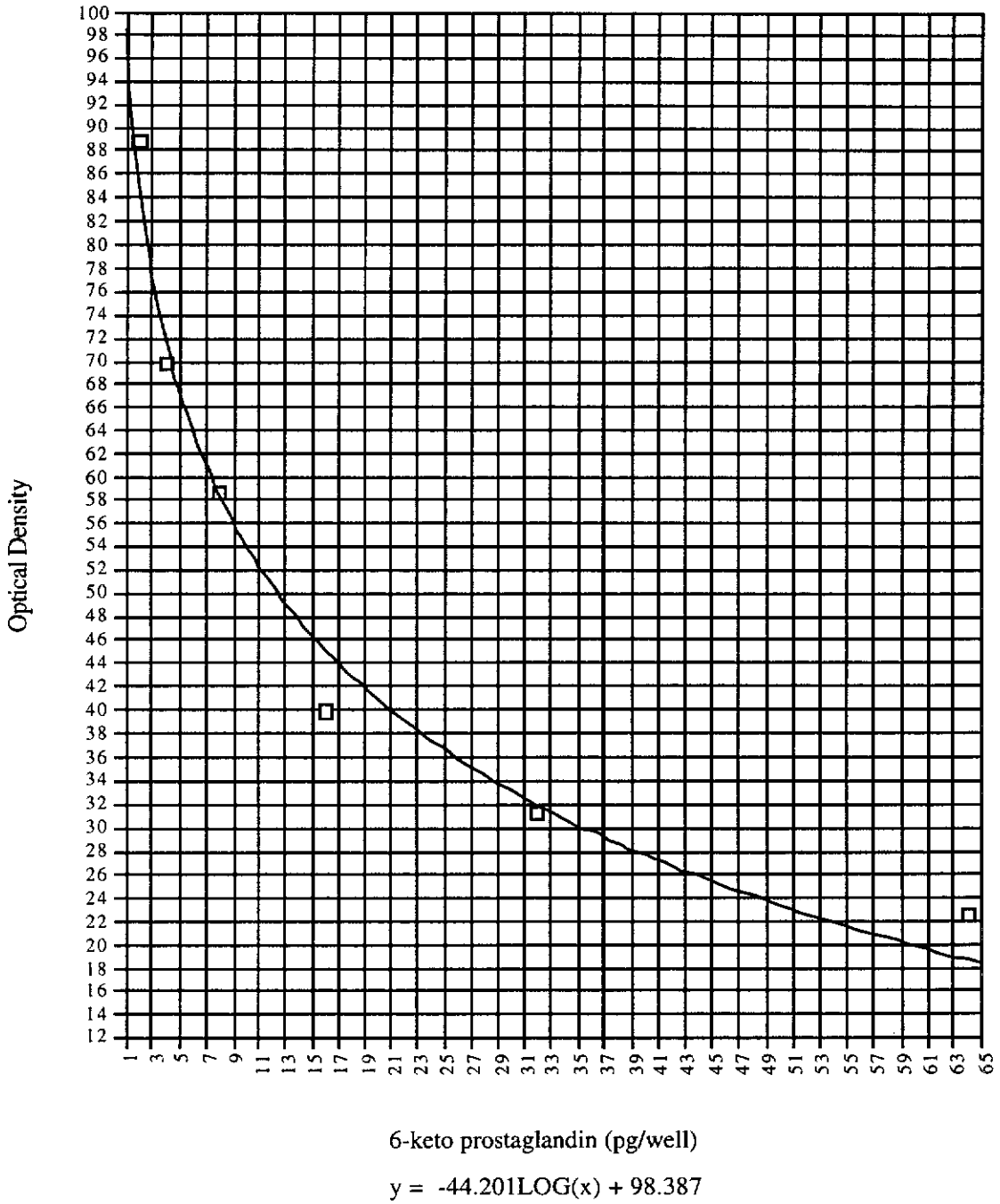


Fig. 6.2. Curve for 6-keto prostaglandin standards.

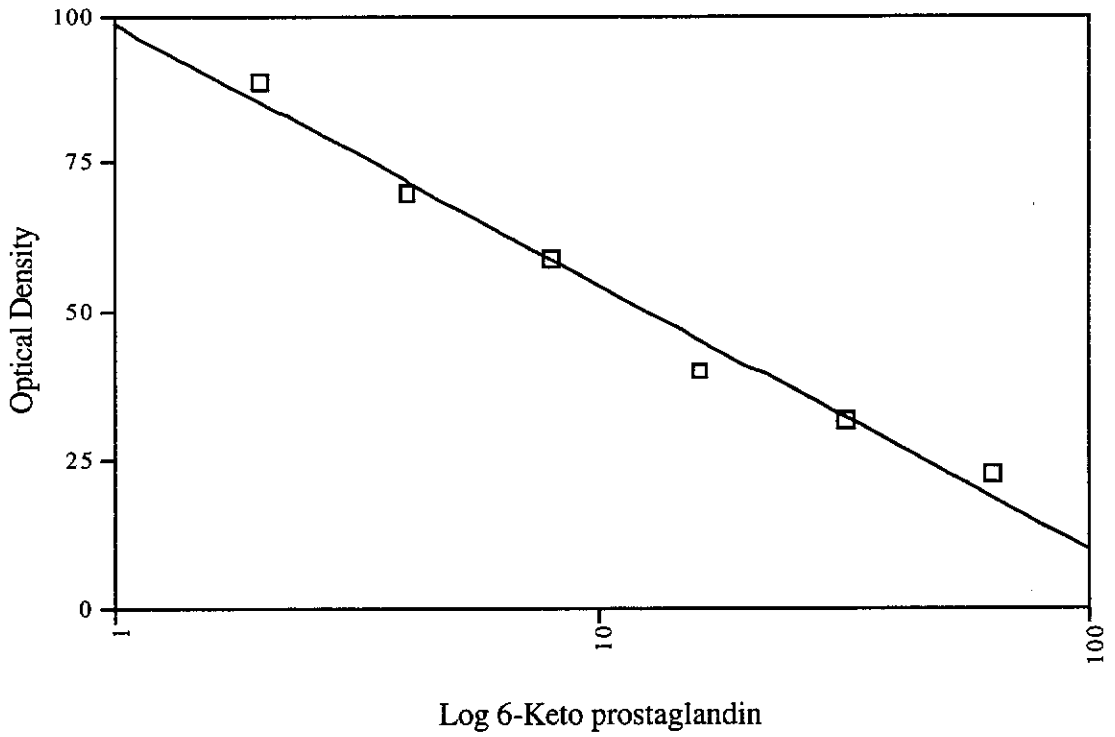


Fig. 6.3. Log. graph of 6-keto prostaglandin standards.

The values for intracellular 6-keto-$PGF_{1\alpha}$ levels for each sample were determined from the curve for standards (Fig. 6.2) and then adjusted for heart tissue mass. The results are presented in Table 6.2. The average amount of 6-keto-$PGF_{1\alpha}$ in the control hearts was found to be 103.1 ± 20.2 pg/g rat heart mass. During the maximum increase in chronotropism, inotropism and CPR the hearts were freeze-clamped and then analysed for 6-keto-$PGF_{1\alpha}$. The average quantity was 249.8 ± 42.2 pg/g heart mass. A *t*-test revealed that the 142.3% increase was significant (P = 0.020).

Table 6.2. 6-keto-$PGF_{1\alpha}$ data for Langendorff rat hearts treated with 1 ml of 1 mM verbascoside.

Sample		OD		Average	pg/well	pg/g HM	Average	SEM
	1st Count	2nd Count	3rd Count	(OD)	(Graph)			
C1	0.502	0.550	0.583	0.545	3.4	83.8		
C2	0.504	0.442	0.484	0.477	5.7	189.1		
C3	0.599	0.557	0.535	0.564	2.9	94.9		
C4	0.490	0.424	0.482	0.465	2.2	73.3		
C5	0.613	0.597	0.603	0.604	2.2	73.3	103.1	20.2
V1	0.445	0.381	0.359	0.395	10.3	343		
V2	0.546	0.661	0.571	0.593	6.0	199.8		
V3	0.521	0.494	0.512	0.509	8.1	162		
V4	0.407	0.495	0.433	0.445	7.2	360		
V5	0.488	0.411	0.335	0.411	9.2	184	249.8	42.2

6.4 Discussion

Since $PG1_2$ is rapidly hydrolysed to 6-keto-$PGF_{1\alpha}$ in all tissue types (Coleman *et al.*, 1990), 6-keto-$PGF_{1\alpha}$ is commonly used to detect changes in prostacyclin (PGI_2) levels (Änggård and Flower, 1993). When Langendorff rat hearts were treated with 1 ml of 1 mM verbascoside, a significant increase in 6-keto-$PGF_{1\alpha}$ levels occurred. This suggests that the production of PGI_2 must have increased significantly in response to verbascoside. Kimura *et al.* (1987) showed that increases in the levels of 6-keto-$PGF_{1\alpha}$ and, therefore, in PGI_2 levels, were responsible for the positive effects on rat peritoneal cells and human peripheral polymorphonuclear leukocytes. The increases in 6-keto-$PGF_{1\alpha}$ occurred in response to the introduction of verbascoside.

Prostacyclin is a potent vasodilator (Moncada *et al.*, 1976) that induces elevations in coronary flow rate and oxygen uptake in Langendorff rabbit hearts (Edlund *et al.*, 1986). An increase in PGI_2 levels in Langendorff rat hearts may therefore account for the increases in CPR observed here. It is not known how PGI_2 mediates its effects, but is believed to act through a series of receptors known as IP-receptors (IP = prostaglandin type I) (Coleman and Humphrey, 1993). When stimulated, IP-receptors mediate an increase in intracellular levels of cAMP and cause vasodilation to occur. In chapter 5, it was shown that verbascoside mediated a massive increase in cAMP levels in rat heart and thus accounted for at least some of the significant increases in heart activity. However, it is not certain how the activation of IP-receptors by PGI_2 mediates the increase cAMP levels. Strange (1988) suggested that G-proteins linked to the adenylate cyclase enzyme are involved (see chapter 5 for a full description of this mechanism). In brief, G-proteins stimulate the adenylate cyclase enzyme to convert ATP to cAMP. It is also not clear how verbascoside promotes the increase in the production of PGI_2. Prostacyclin is released in response to atrial natriuretic peptide (ANP), which in turn is released when stimulated by protein kinase C (PKC) (Church *et al.*, 1994). Verbascoside inhibited PKC in rat brain

(Herbert *et al.*, 1984). Protein kinase C exists in many isoforms and may have different effects in different cell types. It is recommended that the effects of verbascoside on PKC activity in Langendorff rat hearts be determined in future studies.

Preliminary experiments with verbascoside have shown that it cannot be re-administered to the same heart until at least 30 minutes have elapsed since the initial introduction. During that time, any subsequent re-administration of verbascoside will not illicit the same response as the first administration. However, if another inotrope, eg. isoproterenol, is administered immediately following verbascoside, it will produce a significant effect. Isoproterenol is a synthetic β-adrenergic agonist that acts on adrenergic receptors embedded in the cell membrane. As mentioned previously (chapter 4), adrenergic receptors mediate an increase in cAMP levels by activating the adenylate cyclase enzyme. Adrenergic agonists, therefore, do not interfere with the production of prostacyclin or with its effects and thus act independent of this mechanism.

Although the results presented here suggest that a significant increase in 6-keto-$PGF_{1\alpha}$ levels resulted from the introduction of verbascoside, they do not reveal the true amount of 6-keto-$PGF_{1\alpha}$ in the cells. It was assumed that all or most of the 6-keto-$PGF_{1\alpha}$ was extracted during the extraction procedure. However, since this was the first study of its type, there was no literature available for a comparison. Nevertheless, the aim of the study was to show if prostacyclin levels were affected by verbascoside. Based on the results, there is sufficient evidence to support this mode of action.

CHAPTER 7

CARDIOACTIVE IRIDOID GLUCOSIDES FROM *EREMOPHILA PANTONII, E. IONANTHA* AND *E. MACULATA* SUBSP *BREVIFOLIA*

CHAPTER 7

Cardioactive iridoid glucosides from *Eremophila pantonii*, *E. ionantha* and *E. maculata* subsp *brevifolia*.

7.1 Introduction

In chapter 3 it was reported that the main active constituent in *E. longifolia* leaves was an iridoid glucoside called geniposidic acid. Geniposidic acid was shown to have an inhibitory effect on Langendorff rat hearts (chapter 4). Chronotropism, inotropism and CPR were all significantly decreased after 1 ml of 1 mM of the iridoid was introduced into the retrograde perfusion of the rat hearts. Although this was the first reported biological activity for this compound, other iridoid glucosides have been shown to exhibit biological activity including cardioactivity. Most notably amongst these is oleoeuropein, an iridoid isolated from green olives (Fig. 7.7; **10**). In addition to its hypotensive and antiarrhythmic effects, it has coronary dilating properties (Petkov and Manolov, 1978) and reduces blood pressure in Wistar rats (Ahmad *et al.*, 1995). Its hydrolysis product, elenolic acid, produced during the fermentation of brined olives, also exhibits hypotensive properties (Sticher, 1977). These and other iridoids are reviewed in the discussion of this chapter.

As part of a PhD study, Yana M. Syah, of the Chemistry Department of the University of Western Australia, recently isolated four known iridoid glucosides from three species of *Eremophila*, viz. *E. ionantha*, *E. maculata* subsp *brevifolia* and *E. pantonii*. (see chapter 2). Of these, only *E. maculata* was known to be used by the Australian Aboriginal people for medicinal purposes. The leaves were prepared as a poultice and tied around the head for

colds (Cunningham *et al.*, 1981). Interest in the phytochemistry of this species dates from as early as 1910, when it was shown to contain a poison responsible for killing stock animals. The toxic component was later identified as the cyanogenic glucoside, prunasin (Finnemore and Cox, 1929).

The effects of the four afore-mentioned iridoids were tested on the Langendorff rat heart bioassay and compared with those of other known cardioactive iridoid glucosides.

7.2. Materials and Methods

Isolated rat hearts, perfusate, data recording and data analysis were the same as those in Chapters 3 and 4. At least six hearts were used for each treatment (Animal Ethics and Experimentation Committee approval No. was R28/96). The isolation and identification of active substances was conducted by Yana M. Syah (UWA) under the supervision of Assoc. Prof. Emilio L. Ghisalberti. A description of the isolation and identification of the compounds is included below.

7.2.1. Plant Material

Collection sites for all samples were previously described in chapter 2. All plant material was dried at 40°C for 48 hrs and was ground up to a fine powder using a vegetative grinder (Dietz-Motoren KG, Eleckromotorenfabrik, West Germany, 220 v, type WRB 80 C12Q SIL).

7.2.2 Active substances in *E. pantonii*

Dried powdered leaves (100 g) were soaked overnight in 1 L of acetone. Ten g of the acetone extract was separated into seven fractions by vacuum liquid chromatography (Si gel) eluting with dichloromethane and increasing amounts of methanol. The fraction eluted with CH_2Cl_2: MeOH (9:1) was rechromatographed on Si gel to yield two compounds which were identified from their physical and spectroscopic data as melampyroside (MW = 450.441) (**1**) (65 mg) (Chauduri and Sticher, 1980) and 6-O-*trans*-ferruloylajugol (MW = 348.349) (**3**) (20 mg) (Nishimura *et al.*, 1989).

7.2.3 Active substances in *E. ionantha*

A similar extraction of *E. ionantha* afforded an 8.5 g acetone extract which was fractionated by vacuum liquid chromatography. The fraction eluted with $CHCl_3$: MeOH was further purified by column chromatography (Si gel). Elution with CH_2Cl_2: MeOH (4:1) yielded a foam-like solid which was identified as 6-O-trans-caffeoylcatalpol (**2**; verminoside; MW = 524.477) (100 mg) on the basis of its spectroscopic data (Stuppner and Wagner, 1989).

7.2.4 Active substances in *E. maculata* subsp *brevifolia*

The isolation and identification of catalpol (**4**; MW = 492.479) from this species has been described previously by Syah and Ghisalberti (1996).

7.3 Results

Melampyroside (Fig. 7.1b), verminoside (Fig. 7.2b), ferruloylajugol (Fig. 7.3b) and catalpol (Fig. 7.4b) had an immediate and significant negative inotropic effect on Langendorff rat

hearts (Table 7.1). Decreases in contractile force occurred within a minute of the introduction of the moderately high dose of 1 ml of 2.0 mM of these iridoids into the system. Less than a minute later there was an almost complete restoration of contractile force, followed by a gradual decrease from which the hearts rarely recovered. Melampyroside and verminoside mediated the largest responses in negative inotropism with decreases of 26.2 % ($P = 0.027$) and 18.2 % ($P = 0.020$), respectively (Table 7.1). The resting contractile force of hearts treated with melampyroside decreased from 1.03 ± 0.02 g to 0.76 ± 0.08 g. The initial contractile force for hearts treated with verminoside was 0.99 ± 0.02 g. This dropped to 0.81 ± 0.06 g. Ferruloylajugol reduced contractile force by 15.9 % ($P = 0.047$) and catalpol by 14.0 % ($P = 0.032$). The initial contraction forces for the two were 1.07 ± 0.02 g and 1.06 ± 0.07 g, respectively. These decreased to 0.90 ± 0.05 g for ferruloylajugol and 0.91 ± 0.09 g for catalpol.

Melampyroside and verminoside were also more active than ferruloylajugol and catalpol in altering heart rate (Table 7.1; Fig 7.1a and 7.2a, respectively). From an initial rate of 180 ± 8 bpm, the hearts treated with 1 ml of 2.0 mM melampyroside experienced a drop to 167 ± 14 bpm (not significant) and then increase ($P = 0.012$) of 16.7 % to 210 ± 10 bpm. Similarly, there was a verminoside-mediated drop in heart rate of 12.7 % ($P = 0.047$) followed by an 18.9 % rise ($P = 0.027$). The resting heart rate of 212 ± 8 bpm first decreased to 185 ± 15 bpm and then increased to 252 ± 6 bpm. The ferruloylajugol (Fig. 7.3a) and catalpol-mediated (Fig. 7.4A) decreases in heart rate were also significant as was the increase in heart rate, from 206 ± 26 bpm to 231 ± 15 bpm, mediated by ferruloylajugol (12.1 %). Catalpol did not induce a significant positive chronotropic response ($P = 0.222$) even though there did appear to be an increase in heart rate (5.1 %).

The largest of the responses was in CPR. Melampyroside (Fig. 7.1c) and verminoside (Fig. 7.2c) were the more potent of the four iridoids with increases of 41.1 % and 39.1 %,

respectively. Along with positive chronotropism, these effects lasted for approximately 15 minutes. Immediately after melampyroside was introduced into the system, there was a large increase in CPR from 5.6 ± 0.4 ml/min to 7.9 ± 0.3 ml/min, followed less than a minute later by positive chronotropism. The increase mediated by verminoside changed from 6.9 ± 0.5 ml/min to 9.6 ± 0.8 ml/min. In addition to the increase in CPR, ferruloylajugol (Fig. 7.3c) also mediated a reduction in the parameter (Table 7.1), which was from 8.1 ± 1.7 ml/min to 7.0 ± 1.8 ml/min and then to 8.8 ± 1.6 ml/min. The inhibition of CPR coincided with the decreases in contractile force and heart rate. Catalpol had no significant effect on CPR (Fig. 7.4c).

Table 7.1. Effects of iridoid glucosides on Langendorff rat hearts.

Compound (1 ml/min)	Heart Rate (bpm)			Force (g)			CPR (ml/min)		
	Control	*Decrease*	*Increase*	*Control*	*Decrease*	*Increase*	*Control*	*Decrease*	*Increase*
Melanopyroside	180 ± 8	167 ± 14 (7.2 %)	210 ± 10 (16.7 %) *	1.03 ± 0.02	0.76 ± 0.08 (26.2 %) *	----	5.6 ± 0.4	----	7.9 ± 0.3 (41.1 %) *
Verminoside	212 ± 8	185 ± 15 (12.7 %) *	252 ± 6 (18.9 %) *	0.99 ± 0.02	0.81 ± 0.06 (18.2 %) *	----	6.9 ± 0.5	----	9.6 ± 0.8 (39.1 %) *
Ferruloylajugol	206 ± 26	190 ± 24 (7.8 %) *	231 ± 15 (12.1 %) *	1.07 ± 0.02	0.90 ± 0.05 (15.9 %) *	----	8.1 ± 1.7	7.0 ± 1.8 (13.6 %) *	8.8 ± 1.6 (8.6 %) *
Geniposidic Acid (see Chapter 4)	247 x 10	214 x 17 (13.0 %) *	----	1.08 x 0.05	0.65 x 0.10 (40.0 %) *	----	8.6 x 1.1	9.2 x 1.3 (- 6.9 %)	4.9 x 0.9 (- 43.0 %) *
Catalpol	217 ± 12	198 ± 9 (8.8 %) *	228 ± 10 (5.1 %)	1.06 ± 0.07	0.91 ± 0.09 (14.2 %) *	----	9.8 ± 2.9	8.9 ± 2.4 (9.2 %)	10.2 ± 2.6 (4.1 %)

Values given are means ± SEM.
* $P < 0.05$

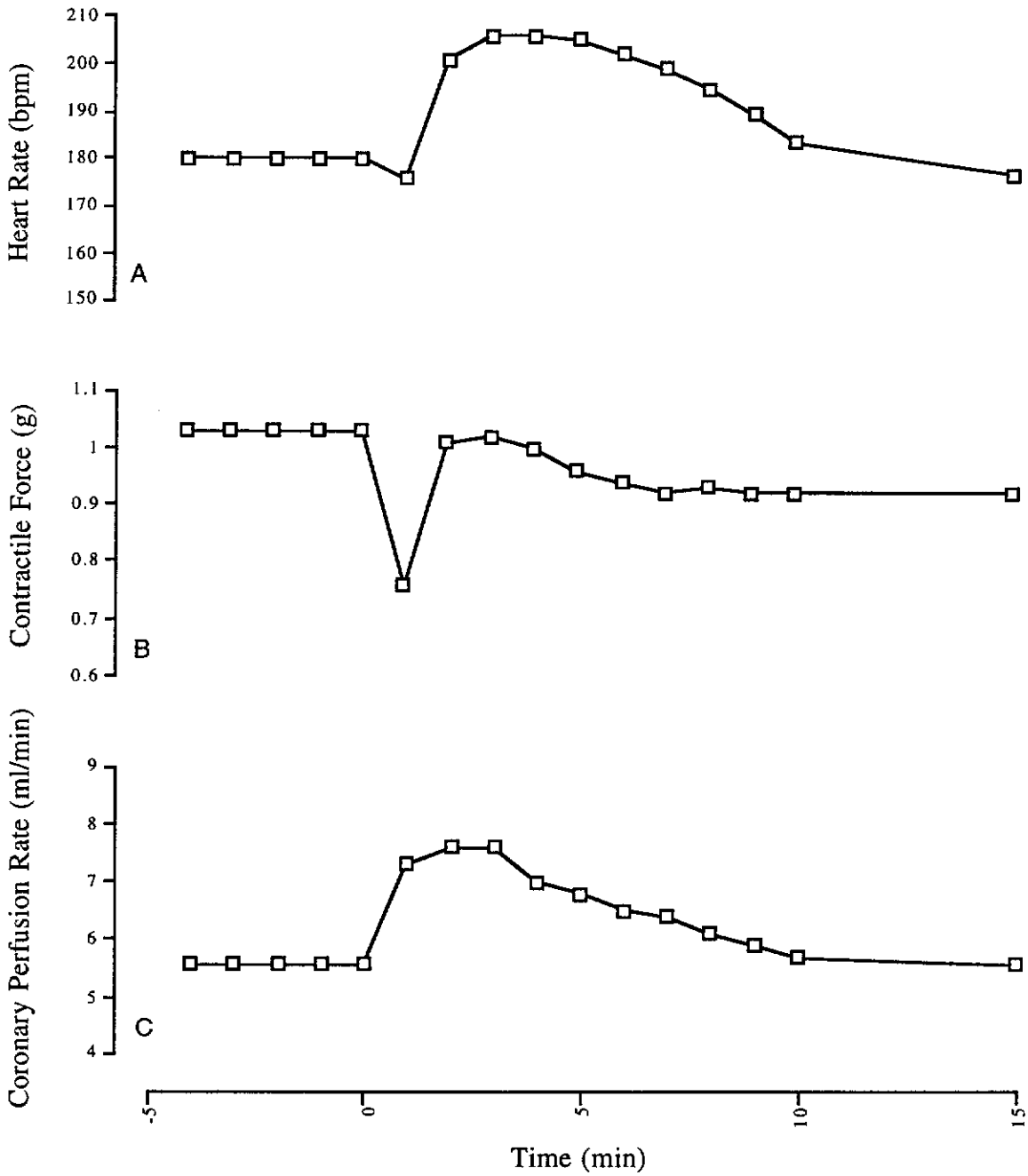


Fig. 7.1. Melampyroside; (A) changes in heart rate (bpm); (B) changes in contractile force (g); (C) changes in coronary perfusion rate (ml/min); means and S.E.M.

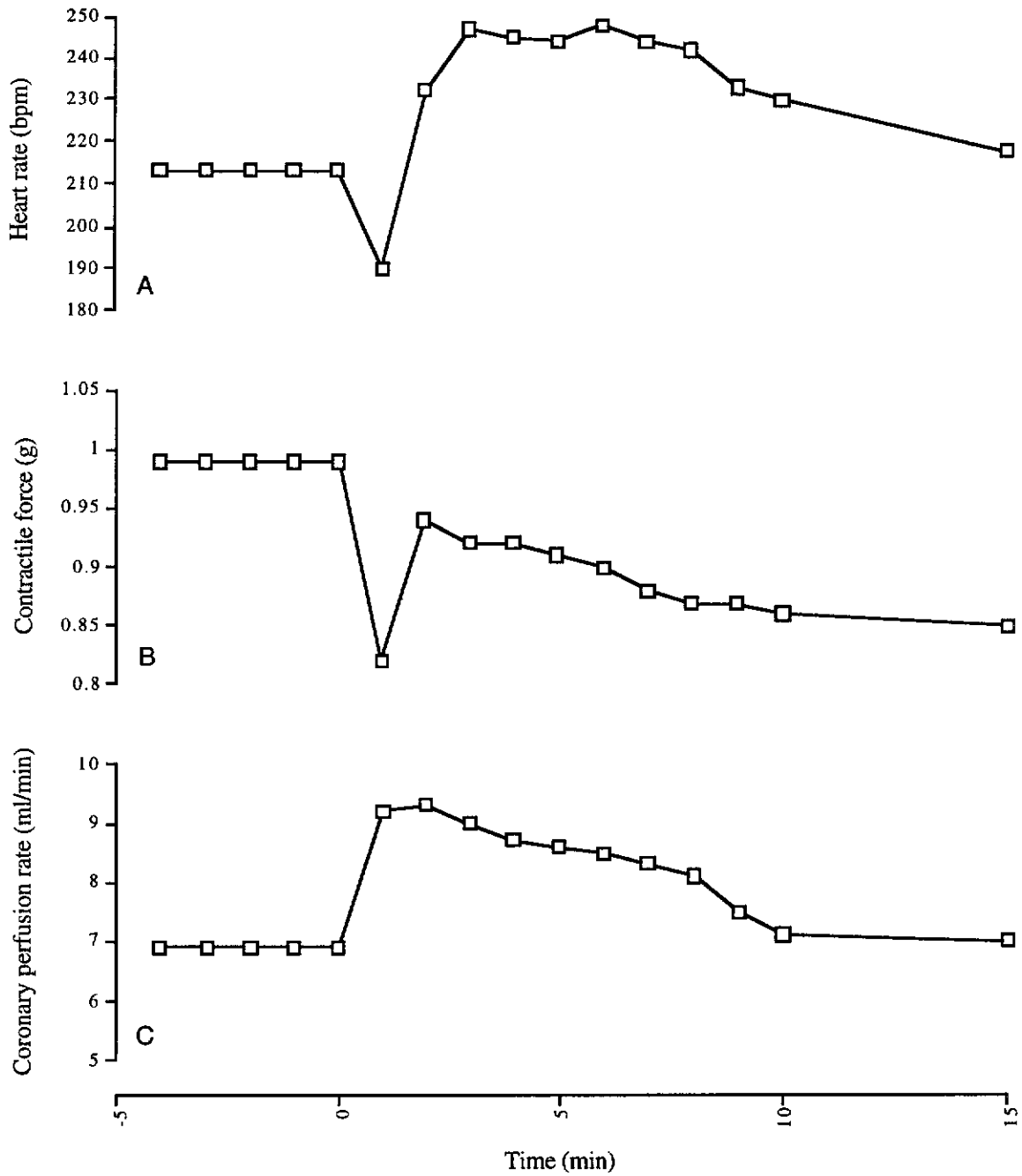


Fig. 7.2. Verminoside; (A) changes in heart rate (bpm); (B) changes in contractile force (g); (C) changes in coronary perfusion rate (ml/min); means and S.E.M.

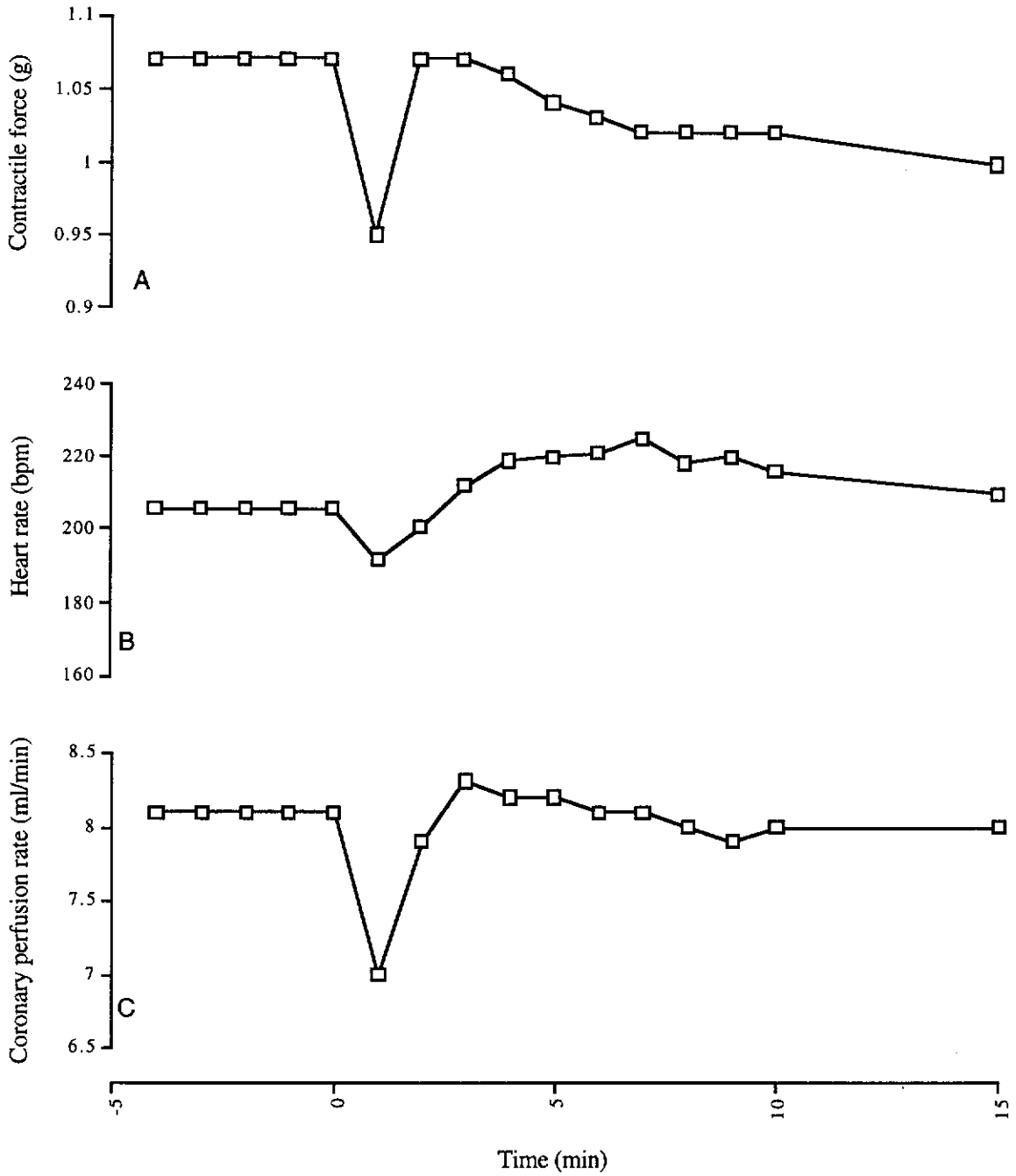


Fig. 7.3. Ferruloylajugol; (A) changes in heart rate (bpm); (B) changes in contractile force (g); (C) changes in coronary perfusion rate (ml/min); means and S.E.M.

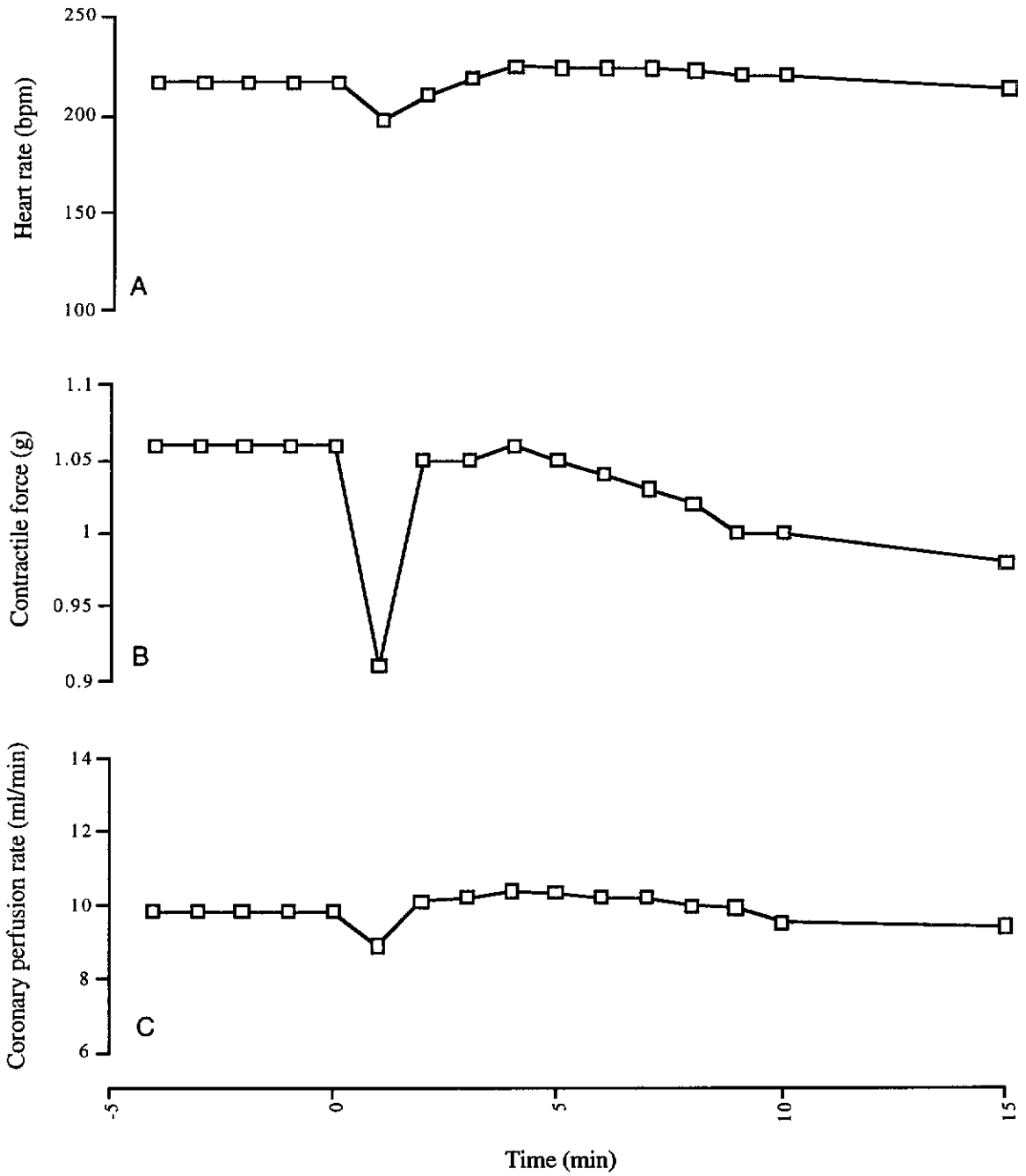


Fig. 7.4. Catalpol; (A) changes in heart rate (bpm); (B) changes in contractile force (g); (C) changes in coronary perfusion rate (ml/min); means and S.E.M.

To determine whether the effects of iridoid glucosides were dose-dependent, verminoside was tested at doses ranging from 1 ml of 0.2 μM to 1 ml of 2.0 mM (Table 7.2). In the interests of reducing the number of rats used, only verminoside was tested in dose-response experiments. All doses of verminoside except 1 ml of 2.0 μM had a significant effect on the Langendorff rat hearts, with only the increases in chronotropism and CPR being dose-dependent. The EC_{50} values (dose causing 50 % excitation) for these were 0.21 mM and 0.63 mM, respectively (Fig. 7.6 and 7.7). Preceding the increase in chronotropism, hearts treated with 1 ml of 2.0 μM, 1 ml of 0.2 mM and 1 ml of 2.0 mM verminoside also experienced a significant reduction in heart rate, which was not dose-dependent (Table 7.2). Similarly, negative inotropism did not appear to depend on the dose used. One ml of 2.0 μM verminoside, the lowest active dose, mediated a 19.2 % drop in contractile force. Successive ten-fold increases thereafter did not significantly increase the magnitude of negative inotropism (Table 7.2).

Table 7.2. Effects of different concentrations of verminoside on heart rate, contractile force and CPR on Langendorff rat hearts.

Dose (1 ml/min)	Heart Rate (bpm)			Force (g)			CPR (ml/min)		
	Control	*Decrease*	*Increase*	*Control*	*Decrease*	*Increase*	*Control*	*Decrease*	*Increase*
0.2 µM	207 ± 13	196 ± 15 (5.3 %)	213 ± 11 (2.9 %)	1.00 ± 0.01	0.91 ± 0.04 (9.0 %)	----	6.9 ± 1.3	----	7.3 ± 1.3 (5.8 %)
2.0 µM	229 ± 9	191 ± 7 (16.6 %) *	238 ± 6 (3.8 %)	1.04 ± 0.03	0.84 ± 0.08 (19.2 %) *	1.05 ± 0.03 (0.96 %)	8.3 ± 0.9	7.3 ± 1.0 (12.0 %)	9.0 ± 0.9 (8.4 %) *
0.2 mM	211 ± 8	171 ± 10 (18.9 %) *	227 ± 10 (7.6 %) *	1.01 ± 0.01	0.80 ± 0.04 (20.8 %) *	1.11 ± 0.03 (10.0 %) *	6.8 ± 0.4	6.3 ± 0.9 (7.9 %)	8.3 ± 0.4 (22.0 %) *
2.0 mM	212 ± 8	185 ± 15 (12.7 %) *	252 ± 6 (18.3 %) *	0.99 ± 0.02	0.81 ± 0.06 (18.2 %) *	----	6.9 ± 0.5	----	9.6 ± 0.8 (39.1 %) *

Values given are means ± S.E.M.
* $P < 0.05$

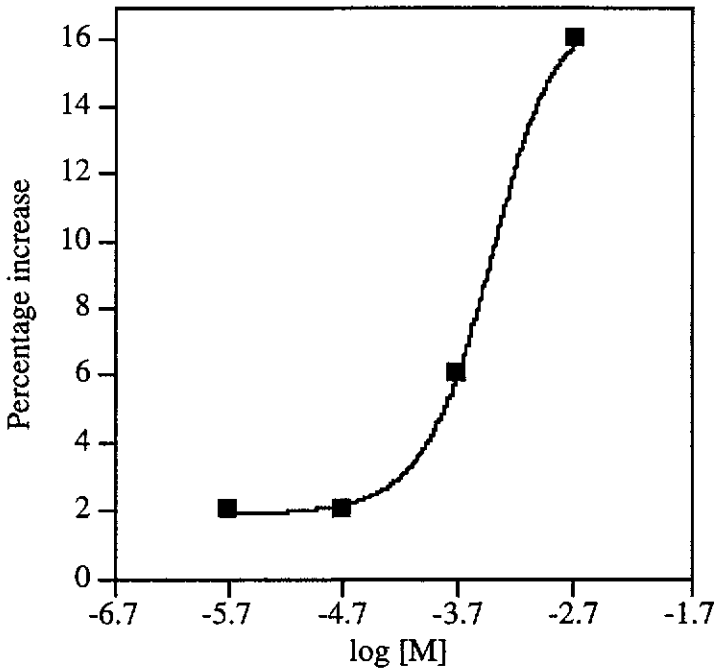


Fig. 7.5 Dose-response curve for the effects of verminoside on heart rate.

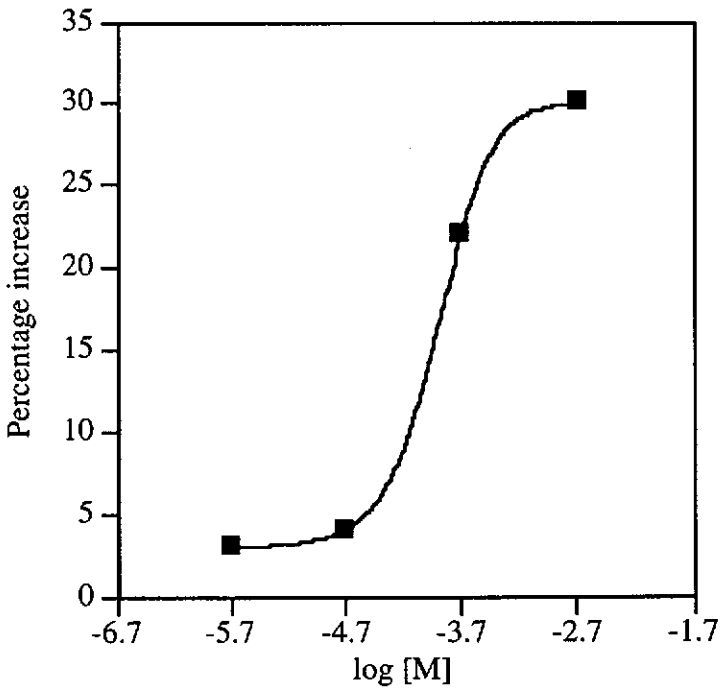


Fig. 7.6. Dose-response curve for the effects of verminoside on CPR.

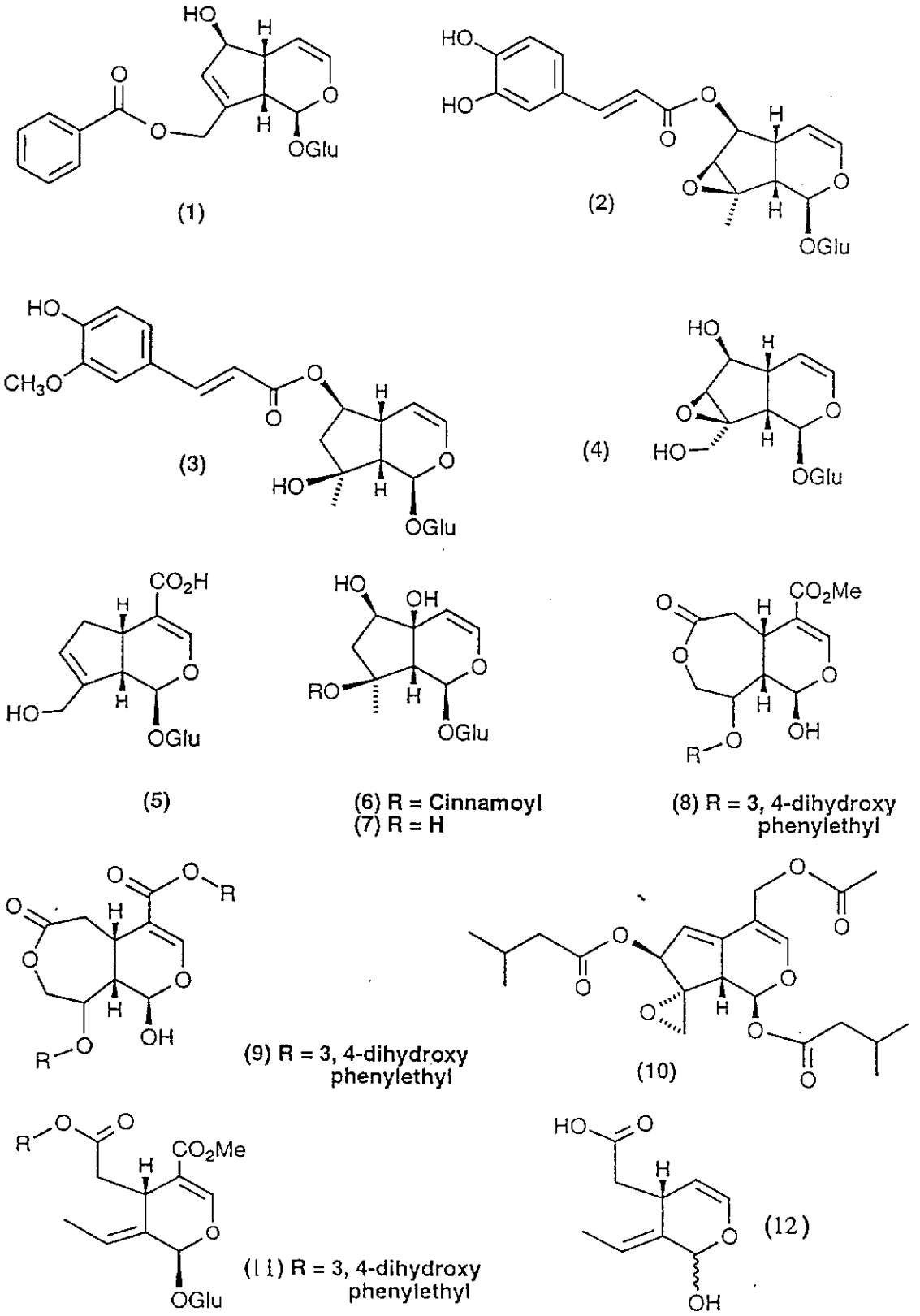


Fig. 7.7. Cardioactive iridoids

In addition to monitoring heart rate, contractile force and CPR, the number of arrhythmias generated by the iridoid glucosides was considered. Apart from the occasional extra systole, melampyroside, verminoside and catalpol did not upset the rhythm of the heart. However, 50 % of the hearts treated with ferruloylajugol were at some time arrhythmic before the effects of the compound attenuated. In most cases, small bouts of arrhythmias occurred within the first five minutes and rarely affected the hearts for more than two minutes.

7.4 Discussion

Melampyroside (**1**), verminoside (**2**), ferruloylajugol (**3**) and catalpol (**4**) all significantly altered myocardial activity in Langendorff rat hearts (Fig. 7.7). Melampyroside and verminoside were predominantly stimulatory, while ferruloylajugol was biphasic and catalpol only inhibitory. The only shared effect by all five iridoids (including geniposidic acid) was negative inotropism which did not appear to be dose-dependent. This can be rationalised by assuming that the receptors responsible for the effect require only a very small dose for inhibition to occur and are therefore saturated early. Positive inotropism was observed only for 1 ml of 0.2 mM verminoside, but 1 ml of 2.0 mM failed to induce a similar response. One possibility is that the larger positive chronotropic effect mediated by 1 ml of 2.0 mM (18.8 % as compared to 7.6 % for 1 ml of 0.2 mM) is onset before positive inotropism can manifest itself. Increases in contractile force are usually masked by the rapid onset of moderate to large increases in heart rate in Langendorff rat hearts.

Although iridoid glucosides are widely distributed in the plant kingdom (Boros and Stermitz, 1990), there are only a few examples in which the cardioactive properties have been studied. Of the five investigated here, only catalpol (**4**) was previously shown to have any

cardioactivity. Reportedly, it inhibited the human erythrocyte Na^+ pump (Lu *et al.*, 1983). The following is a summary of other studies on related iridoids.

Oleoeuropein (**10**), isolated from green olives, has hypotensive, coronary dilating and antiarrhythmic effects and is the hypotensive principle in the leaves of the olive tree (Petkov and Manolov, 1978; Ahmad *et al.*, 1995). Similarly, valtrae (**11**) increased coronary blood flow in isolated rabbit heart preparations. The presence of a glucosidic group in oleoeuropein, but not in valtrae, does not appear to play a role in the effect and may suggest that glycosides function as pro-drugs as has been suggested for other compounds (Nishibe, 1994). Jasmolactone B (**8**) and D (**9**) iridoids are also coronary vasodilators (Shen and Chen, 1989) and exhibit negative inotropic and chronotropic activities on isolated guinea pig hearts, responses which were similar to those observed for geniposidic acid on Langendorff rat hearts (chapter 4). Coronary blood flow in Langendorff rabbit heart preparations was not affected by harpagoside (**6**), which is structurally related to melampyroside. However, it did display a dose-dependent positive inotropic effect on rabbit hearts. At doses of 0.35 mg/ml, harpagoside produced a dose-dependent negative chronotropic effect (Circosta *et al.*, 1984). A qualitatively similar negative inotropic effect to melampyroside was reported for the parent compound of harpagoside, harpagide (**7**).

Also of interest is the secoiridoid oleacin (**12**), a strong inhibitor of angiotensin converting enzyme (ACE). This enzyme is responsible for acting on angiotensin 1 to produce angiotensin 2, which mediates increases in blood pressure and the retention of sodium and water. In addition, it inactivates bradykinin which affects vasodilation and natriuresis. Oleacin is structurally different to other iridoid glucosides in that it lacks the glucose portion of the molecule. Related secoiridoids, also isolated from *Olea* spp, do not exhibit the same activity as oleacin. However, the aglycones produced from the enzymatic hydrolysis of these have demonstrated similar activities (Hansen *et al.*, 1996).

For comparisons such as these to be useful, all compounds must be tested under identical conditions (Repke *et al.*, 1995). Despite this, there is some evidence to suggest that the cardioactive behaviour of the iridoids may be a more common phenomenon than is presently realised. More information is required before any conclusions on the structure-related activity can be made.

CHAPTER 8

GENERAL DISCUSSION

CHAPTER 8

General Discussion

8.1. Screening techniques

Much of what is known about the effects of biologically active compounds has been gathered from tests with simple bioassays. The use of bioassays to help identify novel compounds has gained momentum in more recent times and is now recognised as an efficient method by which to achieve this goal. As a result, a number of simple and inexpensive high through-put screening techniques have been developed and are now available for wide-scale use. Gaining importance in this field are molecular screening techniques (Cordell, 1995). A variety of automated `bench top' assays with broad ranges of bioactivity have evolved and have been adapted for the testing of a wide spectrum of biologically active compounds including those with cardioactive properties. The effects of compounds on cAMP production (chapter 5), phosphodiesterase inhibition, Na^+-K^+-ATPase inhibition, adenylate cyclase activity, ACE inhibition, platelet-aggregation factor inhibition, prostaglandin production (chapter 6) and other screens for cardiovascular activity can now be achieved quickly, routinely and cheaply.

A number of animal and organ-based assays are also available for the screening and testing of cardioactive compounds. Tests on whole animals, isolated hearts and isolated atria are common and, like molecular screening, are simple, inexpensive and effective. One of the earliest methods for determining the effects of compounds on the heart was developed in 1895 by O. Langendorff (Langendorff, 1895). This bioassay has been used repeatedly with reliable results. The advantage of using isolated hearts in such assays is that it severs the organ from the pathophysiological

effects encountered with whole animal bioassays. Separated from the influence of the sympathetic and parasympathetic nervous systems, as well as hormones and other endogenous pharmacologically active substances, Langendorff hearts give a true indication of the effects of a compound or drug on the heart without the secondary effects mediated through other systems.

It was for this reason that the Langendorff rat heart bioassay was chosen for most of the tests undertaken in this study. Furthermore, the assay is relatively cheap to run and can be used repeatedly to show the effects of a wide range of water-soluble compounds. It has been noted, however, that comparisons with other compounds and bioassays may not always be useful unless they are treated in the same manner (Repke *et al.*, 1995). Nevertheless, the Langendorff rat heart bioassay is an accurate method for identifying novel cardioactive compounds and for testing the effects of previously known cardioactive compounds.

Once identified, the mode of action of cardioactive substances can be identified by other bioassays. For example, the involvement of prostacyclin was indirectly arrived at by detecting changes in the intracellular levels of 6-keto-PGF_{1a}. Traditionally, the measurement of 6-keto-PGF_{1a} has been with radioimmunoassay techniques. Despite their accuracy, these techniques rely on radioactive substances and are therefore a potential health risk. Recent advances with enzyme-immunoassays have resulted in a decline in the use of radioimmunoassays and an increase in enzyme-immunoassays (chapter 6). These non-isotopic assays and chromogens have been shown to be equal or superior to radio-immunoassay techniques (Pradelles *et al.*, 1985). It was therefore decided that a enzyme-immunoassay be used in this study. Advances in cAMP have not progressed as fast and are still conducted using radio-immunoassay techniques (see chapter 5).

8.2. Synthesis

This is the first comprehensive ethnopharmacological study undertaken to establish novel cardioactive compounds from traditional Australian medicinal plants. Compounds isolated from five species of *Eremophila,* three of which were used extensively by the Aboriginal people, exhibited previously unknown biological activity. Verbascoside, from *E. alternifolia* leaves and geniposidic acid, from *E. longifolia* leaves, had contrasting effects on heart rate, contractile force and CPR in Langendorff rat hearts (chapter 4). Compounds related to geniposidic acid, isolated from *E. maculata* subsp *brevifolia*, *E. ionantha* and *E. pantonii*, also exhibited cardioactivity that was similar to iridoids from plants of other taxa (chapter 7). A description of the collection sites of the *Eremophila* species used, collection dates and the climate of each site was included as base-line data for future studies in which the effect of different soils and climate on the production of the active constituents can be assessed. It is well established that the quantity of compounds present in any plant may vary depending on soil type and climate.

8.2.1 *Eremophila alternifolia* and Verbascoside

The immediate effect of an 8 mg/ml dose of *E. alternifolia* MeOH leaf extract on the Langendorff rat heart was decreased chronotropism, inotropism and CPR (chapter 3). The inhibition in myocardial activity was sudden and short-lasting. Within a minute, there was a sustained and significant increase in all three parameters. However, it was not immediately apparent whether this biphasic effect was induced by one or more compounds. It was only after experiments with the active compound were conducted that this matter was resolved (see below).

Before a time-consuming bioassay-guided fractionation of the MeOH extract of *E. alternifolia* leaves was performed, a known phenylethanoid compound known for biological activity, and suspected of occurring in *E. alternifolia* leaves (Ghisalberti pers-comm), was tested. Verbascoside is represented in several plant families (Jiménez and Riguera, 1994) and has been shown to display an interesting spectrum of biological activity. The effects of verbascoside on Langendorff rat hearts was similar to those of the methanolic extract of *E. alternifolia* leaves, with two exceptions. The stimulatory effects of verbascoside were greater and there was no inhibitory or biphasic effect (see chapters 3 and 4). These findings confirm that verbascoside was the cardioactive agent responsible for some of the cardioactivity exhibited by the MeOH extract of *E. alternifolia* leaves.

In contrast, the inhibitory effect of the methanolic extract may have been induced by compounds with cholinergic activity. It is generally accepted that cholinergic agonists, such as acetylcholine, inhibit myocardial activity. Walker (1996) suggested that some of the activity mediated by the *E. alternifolia* extract in rat gut was the result of cholinergic activity. Cholinergic receptors were not blocked in the Langendorff rat heart, so it was not known whether acetylcholine-like compounds were present in the extract and, if so, whether they were responsible for the inhibitory effect. However, there was no evidence to suggest that verbascoside caused the inhibition of Langendorff rat hearts or acted through cholinergic receptors to induce the negative effect. In an extension of their study, the involvement of muscarinic receptors (a sub-type of cholinergic receptors) was excluded by Ahmad *et al.* (1995) who showed that verbascoside was not blocked by atropine (antimuscarinic agent). It seems reasonable to conclude, therefore, that verbascoside does not act through muscarinic receptors and has only a positive effect on Langendorff rat hearts.

To determine the effects of verbascoside on Langendorff rat hearts, a detailed analysis was undertaken (chapter 4). It was hoped that some conclusions about the potential

medical uses for the compound could be drawn from the results. Dose-dependent increases in chronotropism, inotropism and CPR occurred immediately following the introduction of verbascoside (1 ml of 1 mM). All responses commenced within a minute and gradually attenuated during the next 15 minutes (except for contractile force, which lasted for less than two minutes). Verbascoside is therefore both fast-acting and long-lasting. Based on the equations derived from the dose-response curves, the EC_{50} values (the dose required to produce 50 % excitation) were 0.33 mM, 30.9 µM and 29.5 µM, for chronotropism, inotropism and CPR, respectively. For the maximum positive response without complications, a dose of 1 ml of 1 mM verbascoside was the considered the most effective. This dose is consistent with doses reported for other biological activities (Herbert *et al.*, 1991). Doses greater than 1 ml of 1 mM resulted in prolonged contraction (systole), from which the hearts never recovered.

Verbascoside is therefore a potent cardiotonic and vasodilatory agent. Its ability to significantly increase CPR (by up to 113 % in some cases; see chapter 5) is evidence of its vasodilatory potential and may account for the large reductions in rat blood pressure reported by Ahmad *et al.* (1995). It is generally accepted that significant vasodilation in the cardiovascular system affects systemic vascular resistance and therefore induces hypotension. This implies that verbascoside has potential as a antihypertensive agent. However, increases in either heart rate or stroke volume will increase cardiac output and increase blood pressure. The dose administered therefore becomes critical. The dose-response curves suggest that doses lower than the most effective dose, ie. 1 ml of 0.1 mM, still mediate significant vasodilation, but with less positive chronotropism. This would appear to be a more suitable dose to use when only a small or no increase in heart rate is desired. The doses used by Ahmad *et al.* (1995) on anaesthetised Wistar rats (200 - 250 g) ranged from 1 mg/kg body mass to 10 mg/kg. Assuming that a rat with a body mass of 250 g has a total blood volume of 30 mls (Baker *et al.*, 1979), a dose of 10 mg/kg (2.5 mg in all) would correspond to

0.1 mM and thus would correlate well with the results obtained by Ahmad *et al.* (1995).

When these results were compared with those obtained by Andary *et al.* (1980; 1982), a number of similarities and discrepancies emerged. For example, it is agreed that verbascoside does not act through adrenergic receptors (see below). However, Andary *et al.* (1980; 1982) have suggested that verbascoside has no effect on the isolated rat auricle (atrium). As previously mentioned (chapter 4), it was not clear what dose Andary and his colleagues used in their tests. There is reference to a "large dose", which was specifically aimed at blocking (antagonising) the effects of isoproterenol - a potent β-adrenergic agonist (stimulator). The dose they used may not have been sufficient to stimulate the rat auricle. Doses of 1 ml of 0.1 mM or greater are required. It is also possible that an undetectable synergistic trace compound, isolated contemporaneously with verbascoside, either augments the effects of verbascoside or is responsible for them. In later studies, latter samples of verbascoside did not appear to mediate the same effect as comparable doses of earlier samples. In some cases, there was no significant activity.

In their work with G-regulators, Yoshida *et al.* (1988) reported that highly active trace substances escaped detection despite the purification of samples. No trace substances were detected in the verbascoside samples. However, if an undetectable synergistic compound is present, it must also affect dimethyl verbascoside and be common in other plant species as well. No structural damage to verbascoside was detected in the latter samples.

Andary *et al.* (1980) also reported that verbascoside did not block the effects of isoproterenol (potent β-AR agonist) and therefore could not act through β-adrenergic receptors. Similarly, Ahmad *et al.* (1995) excluded β-AR receptors on the basis that verbascoside did not block the effects of noradrenaline (β-AR agonist). Langendorff

rat hearts treated with propranolol (1 μM, which was enough to completely block the effects of 1 μM isoproterenol. At this dose, isoproterenol produced a response similar in magnitude to the 1 ml of 1 mM dose of verbascoside), showed no reduction in activity when tested with verbascoside (chapter 4). In addition, Ahmad *et al.* (1995) reported that the effects of verbascoside were not blocked by phentolamine - a potent α-AR blocker. It came as no surprise, therefore, that phentolamine did not in any way diminish the effects of verbascoside on Langendorff rat hearts. These findings confirm earlier results, when it was shown that adrenergic receptor blockers did not affect responses mediated by the MeOH *E. alternifolia* leaf extract (chapter 3).

Further evidence to exclude adrenergic receptors came from a comparison of the chemical structures of adrenergic agonists with verbascoside (chapter 4). Verbascoside lacks the amine functional group with which to anchor ligands to adrenergic receptors. The beta hydroxy functional group is also missing. The only similarities between the two classes of compounds is the aromatic ring. Verbascoside has two aromatic rings, one on each side of the molecule. Both the caffeic acid moiety and the dihydroxy phenylethanoid moiety possess one, but whether it is essential or not is debatable. Hoffman and Lefkowitz (1991) have suggested that the aromatic ring is not necessary to stimulate adrenergic receptors, even though the two hydroxy functional groups on the 3 and 4 positions of the aromatic ring are thought to be a prerequisite for activity (Strader *et al.* 1989b). In support of this, Ahmad *et al.* (1995) have suggested that the two hydroxy groups of the aromatic ring in verbascoside (they did not specify which aromatic ring) play an important role in the antihypertensive effects of verbascoside. Since no experiments on the structure-related activity of verbascoside were performed, no conclusions can be made except that adrenergic receptors are not involved in the activity.

Having eliminated adrenergic and muscarinic receptors, the involvement of other receptors was considered. Histaminergic receptors were one possibility, but were

excluded by Ahmad *et al.* (1995). Chloropheniramine/cimetidine (antihistaminergic agent) did not block the antihypertensive effects of verbascoside in rats. The involvement of the Na^+-K^+-ATPase enzyme activity was also eliminated on the basis that the chemical structure of verbascoside does not resemble cardiac glycosides and therefore does not conform with the basic requirements for inhibition of this enzyme. Calcium channels were also excluded (chapter 4).

Since many cardiotonic agents mediate their effects through a cAMP-dependent, it was decided that the effects of verbascoside on intracellular levels of cAMP be determined. Using a standard radioimmunoassay technique and kit, first developed by Brown (1971) and later modified with the help of Brown by Biotrak (Amersham International), changes in intracellular cAMP levels in Langendorff rat hearts treated with verbascoside were monitored. In response to a 1 ml of 1 mM dose of verbascoside (original stock), which induced normal increases in chronotropism, inotropism and CPR, there was a 1733 % increase in cAMP levels (chapter 5).

Cyclic 3',5'-adenosine monophosphate is present in many cell types including myocardial cells. It is generally accepted that its primary role is that of a second messenger (chapter 5). In heart cells, cAMP activates a cAMP-dependent kinase and activates it to phosphorylate (add a phosphate group) protein kinase A (PKA). This in turn is responsible for phosphorylating protein kinase B (PKB), which is responsible for the influx and release of Ca^{++}. The primary role of Ca^{++} is to prolong contraction in heart cells. Based on this premise, it seems reasonable to suggest that massive increases in cAMP would induce similar increases in Ca^{++} influx and may account for the sustained contraction noted for the very large doses of verbascoside (1 ml of 2 mM and higher). By blocking calcium channels with the widely used clinical antihypertensive agent, verapamil, implicated that the increase in cAMP levels was the result of the release of Ca^{++} by the T tubules and sarcoplasmic reticulum and that the slow calcium channels are not involved.

Despite the massive rise in cAMP levels, it was not clear how verbascoside induced the effect. Changes in the production of cAMP can be achieved in several ways. Compounds, such as forskolin, directly stimulate the adenylate cyclase enzyme to speed up production (Metzger and Linder, 1981). Others, the catecholamines for example, act on adrenergic receptors to increase the yield (Sutherland and Rall., 1960). This mechanism was excluded earlier, when the effects of verbascoside were not blocked by adrenergic receptor blockers (chapter 4). Phosphodiesterase III inhibitors, which prevent the breakdown of cAMP, were also excluded. Kitagawa *et al.* (1984) reported that verbascoside did not inhibit PDE III. Another mechanism by which cAMP production is increased is through the agency of prostacyclin (Moncada *et al.*, 1976). Kimura *et al.* (1987) showed that verbascoside affected prostacyclin levels in rat peritoneal cell preparations. The effect of verbascoside on this eiconasoid was considered and tested (chapter 6).

Langendorff rat hearts treated with verbascoside showed a significant increase in prostacyclin levels (142 %). Prostacyclin is believed to act through IP receptors to increase intracellular levels of cAMP (Coleman and Humphrey, 1993). It has also been shown to cause significant vasodilation (Moncada *et al.*, 1976). This would account for the large increases in CPR observed here (up to 113 %), which may have occurred independently of increases in intracellular cAMP levels.

In addition to its antihypertensive potential, verbascoside may also be useful in the treatment of angina pectoris. In angina pectoris, coronary blood vessels are partially occluded by the build up of artherosclerotic plaques (chapter 1). Traditionally, nitroglycerine and other nitrovasodilators have been used to dilate these blood vessels and thus increase blood flow to the heart. Verbascoside could considerably counteract the effects of an attack of angina pectoris by dilating the coronary vessels. The fact that verbascoside has an immediate and long-lasting effect on Langendorff

rat hearts would suggest that the compound could be used for the fast and prolonged treatment of angina pectoris. It has a reasonably large therapeutic index and can be used over a wide variety of doses, depending on the illness it is to be used against.

8.2.2. Iridoid Glucosides

The effects of geniposidic acid on Langendorff rat hearts, in contrast, were opposite to those induced by verbascoside. Geniposidic acid, like verbascoside, occurs in other *Eremophila* species (Ghisalberti, 1994b) and was tested after it was suspected of occurring in *E. longifolia* leaves. Once isolated and identified, only a preliminary study was carried out to determine its effects on Langendorff rat hearts. The most effective dose was determined in a preliminary study and shown to be 1 ml of 1 mM (chapter 4). Geniposidic acid significantly decreased chronotropism, inotropism and CPR, but did not exhibit the biphasic effect which was noted for the methanolic extract of *E. longifolia* leaves. The increases in myocardial activity were later explained by the presence of verbascoside, which also occurs in *E. longifolia* leaves (chapter 3).

Prior to this study, no biological activity was associated with geniposidic acid. However, cardioactivity was reported for similar compounds (chapter 7). Iridoid glucosides are widely distributed in the plant kingdom (Boros and Stermitz, 1990), some of which have been studied for their cardioactive properties. Interestingly, many are capable of coronary vasodilation. Oleoeuropein and valtrae, for example (Petkov and Manolov, 1978) are two such compounds, as are the B and D jasmolactones (Shen and Chen, 1989), harpagoside, melampyroside and verminoside (chapter 7). Oleoeuropein has also been associated with antihypertensive properties (Petkov and Manolov, 1978; Ahmad, 1985; Ahmad *et al.*, 1995). It is interesting to note that the antihypertensive effect of oleoeuropein is similar to that induced by verbascoside (Ahmad, 1985; Ahmad *et al.*, 1995). These researchers claim that both compounds

have similar structures (aromatic ring with two OH groups) and therefore attribute their antihypertensive actions to the similarities of the structures (chapter 7). Whether this alone is enough to make any conclusions about the structure-related activity is debatable. However, it is tempting to suggest that the antihypertensive properties of oleoeuropein and its ability to dilate blood vessels are linked.

Based on studies with iridoid glucosides, both here and elsewhere, it is obvious that the cardioactive effects of these compounds are a more common phenomenon than was previously thought. Five iridoids were isolated from *Eremophila* species (chapters 3, 4 and 7), of which only one was previously associated with biological activity. Reportedly, catalpol inhibited human erythrocyte Na^+ pumps (Lu *et al.*, 1983). In Langendorff rat hearts, catalpol exhibited an inhibitory effect, which was similar to the effect induced by geniposidic acid. At a dose of 1 ml of 2 mM, catalpol completely inhibited cardioactivity (chapter 7). In contrast, melampyroside, from *E. pantonii*, and verminoside, from *E. ionantha*, both had a significant positive effect on chronotropism and CPR (chapter 7). The largest of these responses was in CPR (41.1 % and 39.1 %, respectively). Interestingly, harpagoside, an iridoid structurally similar compound to melampyroside, also increases blood flow. Ferruloylajugol was biphasic and was the only iridoid tested that induced arrhythmogenic activity. All of the compounds had an inhibitory effect on inotropism, which they shared with geniposidic acid.

8.3. Conclusions

In conclusion, the presence of verbascoside in *E. alternifolia* leaves may explain why this species was so highly "prized" by the Aboriginal people. Infusions of *E. alternifolia* leaves were used to treat colds, influenza and headaches (Smith, 1991), to induce sleep (Tindale, 1937) and pleasant dreams (Latz, 1995), acted as an analgesic, decongestant and promoted general well being (Richmond, 1993a; Ghisalberti, 1994a;

Richmond and Ghisalberti, 1994). Verbascoside, in comparison, has manifested the ability to act against viruses (Aujeszky virus; Molnar *et al.*, 1989) and to relieve pain (Andary *et al.*, 1980; Andary *et al.*, 1982; Melek *et al.*, 1983). Its ability to dilate blood vessels and reduce blood pressure (Ahmad *et al.*, 1995) may offer some insight for why infusions of *E. alternifolia* leaves help induce sleep and promote general well being. It should be noted, however, that no definite correlation exists between the effects of verbascoside and the afore-mentioned treatments for herbal infusions of *E. alternifolia* leaves.

With such a broad spectrum of biological activities, it comes as a surprise that verbascoside's potential as a pharmaceutical has not been fully realised. For example, natural products with immunomodulating properties are much sought after (Labadie *et al.*, 1989), as are potent antitumour agents (Duke, 1986), bacteriocides (Caceres *et al.*, 1990) and analgesics (Elisabetsky and Castilhos, 1990). Verbascoside has exhibited all of these properties, except for antibacterial effects (see chapter 4), but has not been used clinically for any of these purposes. Tests into its viability and suitability as an antianginal and antihypertensive agent are being explored, however, and patents into its extraction and administration applied for.

Geniposidic acid's ability to inhibit myocardial activity may also help substantiate some of the Aboriginal medical claims for *E. longifolia leaves*. This species was reported to have mystical significance to the Aboriginal people (Richmond, 1993a) and was used as a counter-irritant (Richmond, 1993a), skin and body wash (Silberbauer, 1971) and as an eye wash (O'Connell *et al.*, 1983). Chewings (1936) and Latz (1982) have reported that fumes from burning twigs induced lactation in mothers and strengthened babies. Infusions of the leaves were also useful for treating colds and influenza and for treating headaches (Spencer and Gillen, 1969). Geniposidic acid is a potential inhibitor of myocardial activity and may be useful for treating conditions when heart activity needs to be reduced.

The other iridoids tested, in particular melampyroside and verminoside, which had similar effects to verbascoside, may be useful as antihypertensive agents. A similar recommendation was made by Ahmad *et al.* (1995) who suggested that the effects of verbascoside and oleoeuropein were similar in reducing blood pressure in rats. Whether those two compounds act through the same mechanisms is not known. Nor is it known if the effects of verbascoside, melampyroside and verminoside have the same mode of action. Further testing of these compounds is needed to resolve these questions.

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APPENDIX 1

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PUBLICATIONS

The following is a list of publications which have resulted from this PhD project.

Journal Articles

Ghisalberti, E.L., Pennacchio, M. and Alexander, E. (1996) Plant-derived cardioactive compounds. *International Journal of Phramacognosy* (submitted).

Pennacchio, M., Alexander, E., Ghisalberti, E.L. and Richmond, G.S. (1995). Cardioactive effects of *Eremophila alternifolia* extracts. *Journal of Ethnopharmacology* 47: 91-95

Pennacchio, M., Alexander, E., Syah, Y.M. and Ghisalberti, E. L. (1996) The effect of verbascoside on cyclic 3',5'-adenosine monophosphate levels in isolated rat heart. *European Journal of Pharmacology* 305: 169 - 171

Pennacchio, M., Syah, Y.M., Ghisalberti, E.L. and Alexander, E. (1996) Cardioactive Compounds from *Eremophila* species. *Journal of Ethnopharmacology* 53: 21 -27

Pennacchio, M., Syah, Y.M., Ghisalberti, E.L. and Alexander, E. (1997) Cardioactive iridoid glycosides from *Eremophila* species. *Phytomedicine* (in press).

Pennacchio, M., Syah, Y.M., Ghisalberti, E.L. and Alexander, E. (1997) The effects of verbascoside on 6-keto-$PGF_{1\alpha}$. *Phytomedicine* (submitted).

Conference Proceedings

Pennacchio, M., Syah, Y.M., Alexander, E. and Ghisalberti, E.L. (1996) Cardioactive compounds from species in the Australian plant genus *Eremophila* (Myoporaceae) *3rd European Colloquim in Ethnopharmacology*, Genoa, Italy May 29 - June 3, 1996. Book of Proceedings, Vol. 3 (in press).

Pennacchio, M., Syah, Y.M. Alexander, E. and Ghisalberti, E.L. (1996). Australian medicinal plants: Cardioactive compounds from *Eremophila* species. *Plants for Food and Medicine Conference,* London, July 1 - July 6, 1996 Book of Proceedings (in press).

Conference Posters

Pennacchio, M. and Alexander, E. (1993) A search for biologically active compounds in the Australian plant genus, *Eremophila* (Myoporaceae). Abstract No. 4123, pp 383 XV International Botanical Congress, Yokohama, Japan (Pacifico Yokohama). August 28 - September 3, 1993: Abstracts. The International Union of Biological Sciences (IUBS).

Richmond, G.S., Pennacchio, M., Ghisalberti, E.L. and Alexander, E. (1994) *Eremophila* in perspective. Poster presentation to the Western Australian Wildflower Festival, Kings Park and Botanic Gardens, 30 September - 4 October, 1994