Investigation of traditional Australian Aboriginal and Indian Ayurvedic medicinal plants for their role in management of type 2 diabetes

A Thesis submitted for the degree of Doctor of Philosophy

By

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I dedicate this thesis to my dear parents and daughter for being a source of inspiration and for their unconditional love to me.
Abstract

Diabetes mellitus is not a single disease but is a group of metabolic diseases affecting a large number of people globally. It is mainly characterized by hyperglycemia due to insufficient production of insulin. Plants have been the main source of medicines since ancient times and are the richest source of natural compounds. Plants continue to provide new chemical entities for the development of drugs against various diseases like cancer, diabetes, inflammation, hypertension and neurodegeneration. In this project, the bioactivity of extracts of seven Australian Aboriginal and nineteen Indian Ayurvedic plants were studied. The main aim of the project was to evaluate these plant extracts for their potential to help in the management of type 2 diabetes and related complications.

Antimicrobial screening of the plant extracts revealed that the Australian Aboriginal extracts were active against only Gram-positive bacteria whereas Indian Ayurvedic extracts showed a broad spectrum of activity against Gram-positive and Gram-negative bacteria. The antioxidant activity of *Acacia ligulata* was found to be strongest with an IC$_{50}$ of $6.98$ µg/ml against DPPH. *Acacia kempeana* showed strongest activity against ABTS with IC$_{50}$ of $8.86$ µg/ml. Among the Indian plant extracts, *Bacopa moneirrei* and *Andrographis paniculata* showed good free radical scavenging activity with IC$_{50}$ of $14.95$ µg/ml each in the DPPH assay and $23.56$ µg/ml and $41.65$ µg/ml, respectively, in the ABTS assay. Cytotoxicity studies revealed that *Acacia kempeana* and *Acacia tetragonophylla* were found to have good activity against HeLa cancer cells, suggesting their potential use as anti-cancer agents.

The main focus of the study was to investigate the ability of the plant extracts to inhibit enzymes responsible for carbohydrate digestion (thus controlling hyperglycemia), specifically α-amylase and α-glucosidase. Extracts of *Santalum spicatum* (IC$_{50}$ 5.43 µg/ml), *Chlorophytum borivilianum* (IC$_{50}$ 4.17 µg/ml), *Plumbago zeylenica* (IC$_{50}$ 4.36 µg/ml), *Solanum nigrum* (IC$_{50}$ 4.87 µg/ml) and *Pterocarpus marsupium* (IC$_{50}$ 6.98 µg/ml) were particularly active against α-amylase enzyme, while *Beyeria leshnaultii* (IC$_{50}$ 0.48 µg/ml), *Chlorophytum borivilianum* (IC$_{50}$ 0.58 µg/ml) and *Mucuna pruriens* (IC$_{50}$ 0.80 µg/ml) showed potent activity against α-glucosidase enzyme. The IC$_{50}$ value was compared with positive control acarbose. The same plant extracts were also screened against angiotensin converting enzyme (ACE-I) and the results were compared
with the positive control, captopril, however none of the extracts were found be comparable with captopril against this enzyme.

Twelve plant extracts were selected and further evaluated for their antidiabetic activity against murine adipocytes to investigate glucose uptake and adipogenesis in cultured cells i.e. 3T3-L1 adipocytes. At 100 µg/ml, *Acacia kempeana* and *Curculigo orchioides* enhanced basal glucose uptake into differentiated 3T3-L1 cells by 19 % and enhanced insulin-stimulated uptake by 45 % and 48 %, respectively. The plants were compared to the positive control, rosiglitazone. Among the plant extracts examined, *Acacia tetragonophylla* reduced lipid accumulation by 82 % whereas *Beyeria leshnaultii*, *Euphorbia drummondii* and *Curculigo orchioides* were found to significantly reduce lipid accumulation by 73 %, 65 % and 31 %, respectively, in 3T3-L1 adipocytes, thus found to be helpful in reducing adipogenesis. The Australian Aboriginal plants have been traditionally used for general illnesses but have no documented use for diabetes, thus this is the first study to report their potential use in the management of type 2 diabetes. The plants showing potent activity may be developed into novel and safe treatments for type 2 diabetes and related complications.
Acknowledgements

On this gracious occasion, first of all, I bow to the Almighty without whose blessings this work could have never been blossomed and completed.

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Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma, in any University, college or any other educational institute. To the best of my knowledge, this thesis contains no material previously published or written by another person except where due reference is made in the text of the thesis.

Signature of Candidate: .................................................................

Date: .............................................................................................
List of Publications

Following is a list of publications and conference presentations arising from the work contained in this thesis.

Book Chapter


Refereed journal publications


- Shah, R., **Gulati, V.** and Palombo, E.A. Pharmacological properties of guggulsterones, the major active components of gum guggul. *Phytotherapy Research* 2012, 26:1594-1605.

Conference presentations

- Inhibition of alpha-amylase and alpha-glucosidase enzymes by medicinal plant extracts: potential application in the management of Type 2 diabetes at ADS-ADEA (*Australian Diabetes Society- Australian Diabetes Educators Association*), Adelaide, Australia, Aug. – 2009 (Poster).

- Enzymatic inhibition, antioxidant and antibacterial activity of medicinal plant extracts: potential application in the management of Type 2 Diabetes.
WorldPharma 2010, 16th World Congress of Basic and Clinical Pharmacology, Copenhagen, Denmark, July 2010 (Poster).

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<td>2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonate</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ACE-I</td>
<td>Angiotensin converting enzyme</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>AMPK</td>
<td>Adenosine mono phosphate kinase</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<td>A549</td>
<td>Lung adenocarcinoma</td>
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<tr>
<td>2-NBDG</td>
<td>2-[N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-d-glucose</td>
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<tr>
<td>3T3-L1</td>
<td>Fibroblast cell line</td>
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<tr>
<td>BC</td>
<td>Before Christ</td>
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<td>BDA</td>
<td>Broth dilution assays</td>
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<td>BHA</td>
<td>Butylated hydroxyanisole</td>
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<tr>
<td>BHT</td>
<td>Butylated hydroxyl toluene</td>
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<tr>
<td>CAM</td>
<td>Complementary and Alternative Medicine</td>
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<tr>
<td>dH₂O</td>
<td>Distilled water</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Media</td>
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<tr>
<td>DMEM/F12</td>
<td>Dulbecco’s modified Eagle medium/Ham’s nutrient mixture F12</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DNJ</td>
<td>Deoxynojirimycin</td>
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<td>DPPH</td>
<td>1, 1-diphenyl-2-picrylhydrazyl</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>EGCG</td>
<td>Epigallocatechin-3-gallate</td>
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<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
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<tr>
<td>FRAP</td>
<td>Ferric reducing antioxidant power</td>
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<tr>
<td>GAE</td>
<td>Gallic acid equivalent</td>
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<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
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<td>HCl</td>
<td>Hydrochloric acid</td>
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<td>HeLa</td>
<td>Human ovarian carcinoma</td>
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<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<td>IAA</td>
<td>Indoleacetic acid</td>
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IBMX 3-isobutyl-1-methylxanthine
IC_{50} 50% inhibitory concentration
IDDM Insulin dependent diabetes mellitus
IPA Isopropyl alcohol
KCl Potassium chloride
KH2PO4 Potassium dihydrogenphosphate
LPO Lipid peroxidation
MTT 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
MDCK Madin-darby canine kidney epithelial cells
MIC Minimum inhibitory concentration
MBC Minimum bactericidal concentration
NA Nutrient agar
NaCl Sodium chloride
Na2CO3 Sodium bicarbonate
NaH2PO4 Sodium dihydrogen phosphate
Na2HPO4 Disodium orthophosphate
NaOH Sodium hydroxide
NaNO2 Sodium Nitrite
NB Nutrient broth
NCI National Cancer Institute
NIDDM Non-insulin dependent diabetes mellitus
NIH National Institute of Health
OD Optical density
OST Oral saccharinity tolerance
PBS Phosphate-buffered saline
PDA Potato dextrose agar
PDB Potato dextrose broth
RAAS Renin angiotensin aldosterone system
ROS Reactive oxygen species
RNS Reactive nitrogen species
rpm Revolutions per minute
RT Room temperature
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>RTZ</td>
<td>Roziglitazone</td>
</tr>
<tr>
<td>SD model</td>
<td>Spring Dawley rat model</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error mean</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TFC</td>
<td>Total flavonoid content</td>
</tr>
<tr>
<td>TPC</td>
<td>Total phenolic content</td>
</tr>
<tr>
<td>TZD</td>
<td>Thiazolidinedione</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>QE</td>
<td>Quercetin equivalent</td>
</tr>
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<td>WHO</td>
<td>World Health Organization</td>
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Chapter 1. Literature Review
1.1. Abstract

Diabetes mellitus is a metabolic syndrome resulting from low levels of insulin. The increasing worldwide incidence of diabetes mellitus in adults constitutes a major global public health burden. The World Health Organization (W.H.O.) estimates that currently more than 180 million people worldwide have diabetes.

Plants have been the main source of medicines since ancient times. Despite tremendous advances in medicinal chemistry, synthetic drugs have not provided cures to many diseases due to their adverse side effects or diminution in response after prolonged use. Recently, there has been a renewed interest in traditional medicines with the belief that plant-derived drugs may be less toxic and safer than synthetic drugs. With respect to diabetes, numerous studies have indicated that plant-derived chemicals can be useful in the therapeutic treatment of diabetes. However, before the development of therapeutic insulin, diet was (and still is) the main method of treatment and modern treatment focuses on a combination of drugs and diet. Dietary measures included the use of traditional medicines mainly derived from plants. While drugs will continue to be an important part of diabetes therapy, the mass of evidence available in the literature regarding the medicinal properties of vegetables, fruits and other herbs, suggests that diet (including herbal medicines) should not be ignored or neglected.

This review will focus on recent examples of traditional medicines and foods that have been validated by scientific evaluation as having promising activity for the prevention and/or treatment of diabetes. Intriguing questions that await further elucidation include how plants, plant-derived molecules and diet can be used in the future to complement current treatment strategies for diabetes.
1.2. **General introduction**

Diabetes mellitus is a metabolic syndrome which results from low levels of insulin when β cells of the pancreas are not able to secrete sufficient insulin. The symptoms of diabetes are hyperglycemia (high blood glucose), polyuria (increase in urine production), polydipsia (increased thirst), blurred vision, lethargy and weight loss. The increasing worldwide incidence of diabetes mellitus in adults constitutes a global public health burden. It is predicted that by 2030, India, China and United States will have largest number of people with diabetes (Wild et al., 2004, Frode and Medeiros, 2008). The World Health Organization (W.H.O.) estimates that more than 180 million people worldwide currently have diabetes and this figure is likely to be more than double by 2030. In 2005, an estimated 1.1 million people died from diabetes related illnesses and almost 80% of diabetes deaths occurred in low and middle-income countries. Almost half of diabetes deaths occur in people under the age of 70 years; 55% of diabetes deaths are in women and the W.H.O. projects that diabetes deaths will increase by more than 50% in the next 10 years without urgent action. Most notably, diabetes deaths are projected to increase by over 80% in upper-middle income countries between 2006 and 2015 (WHO, 2012).

1.2.1. **Symptoms and related complications**

Over time, diabetes can damage the heart, blood vessels, eyes, kidneys, and nerves. The major complications related to diabetes are *diabetic retinopathy*, a cause of blindness which results from long-term accumulated damage to the small blood vessels in the retina, and *diabetic neuropathy*, the destruction of nerves as a result of diabetes with common symptoms of tingling, pain, numbness, or weakness in the feet and hands. Combined with reduced blood flow, neuropathy in the feet increases the chance of *foot ulcers* and eventual *limb amputation*. Diabetes is among the leading causes of kidney failure and increases the risk of *heart disease and stroke*. Ten to twenty percent of people with diabetes die of kidney failure and fifty percent of people with diabetes die of cardiovascular disease (primarily heart disease and stroke). The overall risk of dying from these conditions among people with diabetes is at least double the risk of their peers without diabetes (Organisation, 2008).
1.3. **Types of diabetes**

Diabetes is categorized into; Type 1 – Insulin Dependent Diabetes Mellitus (IDDM) – which is an autoimmune destruction of pancreatic β cells; Type 2 – Non-Insulin Dependent Diabetes Mellitus (NIDDM) – which is characterized by insulin resistance in target tissues; and gestational diabetes which occurs during pregnancy (Borch-Johnsen, 2007).

In Type 1 diabetes, there is a loss of insulin secreting β cells of the Islets of Langerhans (in the pancreas) which causes deficiency of insulin. The main cause of this β-cell loss is autoimmune attack by T-cells. The principal treatment is replacement of insulin (Garg, 2008).

Type 2 diabetes is caused by reduced insulin sensitivity due to increased glucose levels in the blood. Hyperglycaemia can be rectified by medications that improve insulin sensitivity or decrease glucose production by the liver. Hyperglycaemia can be treated by increasing physical activity, decreasing carbohydrate intake, selection of proper diet, modification of life-style and losing weight (Clemens et al., 2004).

Gestational diabetes, although temporary, increases the risk of developing Type 2 diabetes later in life. Although insulin injections are sometimes necessary, this type of diabetes is also commonly treated by life-style changes such as moderate physical activity and diet (Kim et al., 2002).

The principal clinical features of diabetes mellitus were described by Hindu scholars as long ago as about 1500 BC as a condition featuring polydipsia, polyuria and the production of urine which was sweet enough to attract flies and ants (JDRF, 2004). The current focus of drug discovery research in diabetes includes the exploration of alternative medicines, discovery of new synthetic antidiabetic agents as well as isolation of active compounds from plants which have been the source of traditional herbal medicines and have been documented and described for their antidiabetic properties in ancient texts such as Ayurveda. The W.H.O. has recommended that alternative medicines should be investigated and explored for discovery of new drugs for the treatment of diabetes mellitus (Saxena and Vikram, 2004).
1.3.1. Current therapies for diabetes

Currently available therapies for diabetes include insulin and various oral antidiabetic agents such as sulfonylureas, biguanides, α-glucosidase inhibitors, α-amylase inhibitors and glinides, all of which can be used as monotherapies or in combination to achieve better glycemic regulation (Nathan et al., 2006). The medications available in the market, and their side effects, are as follows and are summarized in Figure 1:

**Metformin**, is the only biguanide available to most of the world and its major effect is to decrease hepatic glucose output and lower fasting glycemia. It is generally well tolerated, with the most common adverse effects being gastrointestinal symptoms.

**Sulfonylureas** lower glycemia by increasing insulin secretion. The major adverse effect is hypoglycemia, while weight gain is also a common concern.

**Glinides**, like the sulfonylureas, also stimulate insulin secretion but bind to a different site within the sulfonylurea receptor and have a shorter half-life than the sulfonylureas and therefore must be administered more frequently. The glinides have a similar risk for weight gain as the sulfonylureas, but hypoglycemia may be less frequent (nateglinide) than with some sulfonylureas.

**Enzyme inhibitors** lower the rate of digestion of polysaccharides in the proximal small intestine, primarily lowering postprandial glucose levels without causing hypoglycemia. Since carbohydrates are absorbed more distally, malabsorption and weight loss are ameliorated, however, increased delivery of carbohydrate to the colon commonly results in gas production and gastrointestinal symptoms.

**Thiazolidinediones (TZD)** or **glitazones** are peroxisome proliferator–activated receptor γ modulators which increase the sensitivity of muscle, fat, and liver to endogenous and exogenous insulin (“insulin sensitizers”). The most common adverse effects with TZDs are weight gain and fluid retention.
Figure 1 - Digestion of food and mechanisms of currently available therapies, and their side effects, for diabetes.
The alternative to the above oral hypoglycemic agents is injectable Insulin which is the oldest among the currently available medications, initially developed to treat Type 1 diabetic patients for whom it is lifesaving. Insulin is the most effective in lowering glycemia. It has beneficial effects on triglyceride and cholesterol levels but is also associated with weight gain (Nathan et al., 2006).

Although oral hypoglycemic agents and insulin play important roles in the treatment of diabetes by controlling hyperglycemia, these have serious side effects which may cause other diabetic complications and most of the medicines available in the market are associated with the adverse consequences of hypoglycemia or weight gain (Grover et al., 2002). Thus, treatment of diabetes without any side effects is still a challenge (Jung et al., 2006b).

It is clear from the Figure 1 that enzyme inhibitors such as diet and herbs rich in polyphenolic compounds have bearable side effects as compared to other oral hypoglycemic agents.

When selecting an appropriate therapy for Type 2 diabetes, factors such as other co-existing medical conditions (high blood pressure and elevated cholesterol), adverse effects of that therapy, contraindications to therapy (Grundy et al., 2005), issues which may affect compliance (timing of medication, frequency of dosing, over-dosing etc.) and cost to the patient and the healthcare system should be considered alongside the magnitude of change in blood sugar control that each medication will provide. Moreover, the relatively complication-free option of diet and life-style change should be considered (Garg, 2008).

### 1.3.2. Management of diabetes with diet

An important research area is the discovery and development of more effective and safer antidiabetic agents. In this context, medicinal plants and diet continue to play an important role in the treatment of diabetes, particularly in developing countries where most people have limited resources and do not have access to modern treatment (Saxena and Vikram, 2004). A recent survey of the frequency of use of complementary and alternative medicine (CAM) in diabetes patients found that most of the patients using CAM are...
better educated, born in cities, live in large families and were suffering from diabetes for longer duration. The survey’s definition of CAM included herbal preparations (garden thyme, pomegranate syrup, stinging nettle, dog-rose, chervil, cinnamon and bitter almond), acupuncture and meditation. Further, it was reported that more than half of the subjects who were using CAM experienced beneficial effects (Suleyman Ceylan, 2009).

Many plants and their active chemical compounds have demonstrated activity in the treatment of Type 2 diabetes and various other disorders. Diet has long been the keystone in the treatment of diabetes and various other diseases. The Ebers Papyrus prescribed in 1550 BC that a diet rich in wheatgerm and ochra has glucose-lowering efficacy (Day, 2005). Diet and lifestyle play an important role in the management of several diseases, including diabetes. Before the introduction of the therapeutic use of insulin, diet was the main form of treatment and dietary measures included the use of traditional medicines mainly derived from plants (Swanston-Flatt et al., 1991).

The ancient Indian medical system of Ayurveda, which is based on scientific principles, has also described diabetes under the name madhumeha, stating it to be mainly influenced by dietary factors such as excessive eating of sugary, acidic or salty food, certain non-vegetarian foodstuffs, and life-style factors such as lack of exercise, overindulgence in sleep, sedentary habits, lack of cleanliness and “suppression of natural urges”. Current studies have confirmed that there is increased risk of developing Type 2 diabetes from lack of exercise and sedentary life-style (Manyam, 2004). Many studies have confirmed the benefits of medicinal plants with hypoglycemic effects in the management of diabetes mellitus. The effects of these plants may delay the development of diabetic complications and correct metabolic abnormalities. Moreover, during the past few years, some of the new bioactive drugs isolated from plants showed antidiabetic activity with more efficacy than oral hypoglycemic agents used in clinical therapy (Bnouham et al., 2006).

Dietary management of diabetes includes consumption of food, spices, fruits, vegetables, traditional medicines and herbs. The diet should provide adequate amounts of vitamins, minerals, carbohydrates, fats and proteins. Diet which enhance glycemic control are high in fibre, low to moderate in fats and moderate in biological value proteins such as legumes, beans, vegetables, soy and other plant-based proteins which our body can
digest, absorb and easily utilize. A decrease of calorie intake in diabetic patients helps in weight loss. Diets rich in fibre and containing 60% carbohydrates improve blood sugar and lipid levels. Thus, dietary modification is the first line of therapy for diabetic patients. Dietary strategies normalize blood glucose and lipoprotein levels to reduce morbidity and mortality caused by the disturbance of carbohydrate and lipoprotein metabolism in diabetes mellitus. These goals can be achieved by considering the quantity and quality of diets according to the clinical conditions of an individual (Garg, 2008). Some examples of dietary management of diabetes which have been evaluated scientifically are described below:

A considerable number of human and animal experiments have been carried out to evaluate the efficacy of common spices and natural food adjuncts for several physiological effects such as antidiabetic, digestive stimulant, cholesterol lowering, anticarcinogenic, anti-inflammatory, antioxidant and anti-lithogenic potential. Several common spices such as fenugreek (Trigonella foenumgraecum) were studied on diabetic and normal rats, mice, rabbits and dogs. Subsequent human clinical trials confirmed that fenugreek possess beneficial hypoglycemic potential. Garlic (Allium sativum), onion (Allium cepa), cumin (Cuminum cyminum) and turmeric (Curcuma longa) are some other spices with beneficial anti-diabetic properties (based on animal studies). Experimental data with above spices indicated that dosages of 25-50 grams of fenugreek seeds, 5-6 garlic cloves, 1 onion bulb, and 1 gram of turmeric powder incorporated into the daily diet of diabetics were effective as a support therapy in the prevention and management of diabetes and related complications such as hypertension and obesity. The mechanisms of action are recognized as stimulation of the pancreas to secrete insulin, interference with dietary glucose absorption and insulin sparing action of bioactive compounds. Ginger (Zingiber officinale), curry leaf (Murraya koenigii), mustard (Brassica nigra) and coriander (Coriandrum sativum) also improved glucose tolerance in experimental diabetic animals (Srinivasan, 2005).

Apart from serving as flavouring agents, spices can also be used in the management of certain metabolic disorders such as diabetes. Rhus coriaria L., also called sumac, and Bunium persicum Boiss, also known as black Persian cumin, are two spices used as condiments, particularly in Iran and Afghanistan. The methanolic, ethyl acetate and n-
hexane extracts of both spices have been studied for their ability to inhibit the enzyme \( \alpha \)-amylase. The ethyl acetate extract of \textit{Rhus coriaria} fruits showed significant \( \alpha \)-amylase inhibitory activity and thus has the potential to be used in the management of diabetes (Giancarlo et al., 2006). Various samples of fruit-enriched yoghurts have been tested for diabetes and hypertension management. Dairy and soy yoghurt enriched with strawberry, blueberry and peach were screened, \textit{in vitro}, for total phenolic content, antioxidant activity, \( \alpha \)-glucosidase inhibition, \( \alpha \)-amylase inhibition and the angiotensin converting enzyme-I (ACE-I) inhibition. Soy yoghurt enriched with blueberry showed the highest antioxidant activity, phenolic content, \( \alpha \)-glucosidase inhibition and \( \alpha \)-amylase inhibition. The results indicated that enrichment of yoghurts with fruit phytochemicals, e.g. blueberries showed high health functional value in terms of Type II diabetes management. Soy yoghurt, enriched with blueberries, appeared to be the best food system in the management of diabetes and its long term complications (Apostolidis et al., 2006).

Different types of cheese – cheddar, feta and Roquefort have been shown to be beneficial in the treatment of Type 2 diabetes, and inhibited \( \alpha \)-glucosidase, \( \alpha \)-amylase and ACE-I. All samples of cheese showed very high ACE-I inhibition. They were also enriched with cranberries and showed highest activity against \( \alpha \)-glucosidase and \( \alpha \)-amylase. Therefore, cheeses enriched with cranberries have promising anti-diabetic potential such that enrichment with herbs and fruit phytochemicals enhanced functional value of cheese in relation to Type 2 diabetes management (Apostolidis et al., 2007).

The aqueous extracts of some American foods (fresh green pepper, string beans, baby spinach, broccoli sprouts, red pepper, fresh carrot, romaine lettuce, red grape, tomato and basil leaves, Graham crackers, Chips Ahoy, cookies and Wheat Thins crackers) and Asian foods (powdered Asian spices fenugreek, mustard, ginger, cinnamon, turmeric, fennel powder, cardamom powder, fresh eggplant, coccinia, bittergourd, small brinjal, ginger, mustard and fresh carrot) have been screened using \textit{in vitro} enzymatic assays. Overall, Asian foods were found to be more active than the American foods and it was suggested that antioxidant activity was associated with amylase inhibition and phenolic–phenolic synergies may be involved in the food extract enzyme-inhibition mechanism. The results from experiments showed that common vegetables and spices contained significant antidiabetic activity \textit{in vitro}, as well as anti-ACE activity, and sug-
gested that dietary modification to include these types of foods along with balancing carbohydrate intake throughout the day may represent a promising strategy to help control postprandial hyperglycemia through modulation of carbohydrate absorption. Dietary α-amylase and α-glucosidase inhibitors from common foods are potentially safer, therefore, may be a preferred alternative for the reduction of carbohydrate absorption and control of blood glucose (McCue et al., 2005).

The inhibitory effects of polyphenol components of berries on various digestive enzymes have been studied and it was found that anthocyanins inhibit α-glucosidase and reduce blood glucose levels after ingestion of meals rich in starch, and they may therefore control hyperglycemia. Ellagitannins, present in berries inhibit α-amylase activity. Raspberries and strawberries contain high amounts of ellagitannins and anthocyanins. Berry polyphenols such as flavonols, anthocyanidins, ellagitannins and proanthocyanidins can inhibit protease enzyme which could, in turn, affect protein digestion in the gastrointestinal tract. Proanthocyanidins can inhibit gastrointestinal lipase activity which helps in the control of obesity by reducing fat digestion. Polyphenol components in berries, fruits and other vegetables provide health benefits by inhibition of these digestive enzymes thus providing an alternative to pharmaceutical and nutraceutical treatment for non-insulin dependent diabetes and obesity (McDougall and Stewart, 2005).

The National Diabetes Education Program of the National Institutes of Health (NIH) recommends that eggplant should be included in the diet for the management of Type II diabetes. The phenolic-enriched antioxidant activity and α-glucosidase inhibitory potential might help to reduce hyperglycemia-induced pathogenesis. This was tested experimentally in vitro by extracting four varieties (Purple, White, Graffiti, Italian) of fresh and well-ripened eggplant (Solanum melongena), with water and screened for activity using α-amylase, α-glucosidase and ACE-I inhibition, DPPH and total phenolic assays. The results indicated that phenolic-enriched extracts had high α-glucosidase inhibitory activity, moderate antioxidant activity and moderate to high ACE-I inhibitory activity. Eggplant may control glucose absorption and decrease the risk of related hypertension because of its high fibre, phenolic compounds and low soluble carbohydrate content. Inhibition of these enzymes provides a strong biochemical basis for management of
Type 2 diabetes by controlling glucose absorption and associated hypertension. The phenolic antioxidant-enriched dietary strategy also has the potential to reduce cellular oxidation stress which is also related to diabetes (Kwon et al., 2008a).

In an in vitro epididymal fat cell assay, tea has been shown to increase insulin activity. Black, green, oolong and herbal teas all increased insulin activity with the insulin potentiating activity of green and oolong teas considered to be due to epigallocatechin gallate. The other compounds responsible for enhancing insulin activities are epicatechin gallate, tannins and theaflavins (Anderson and Polansky, 2002). These data were supported by in vitro enzyme inhibition analysis of other phenolic phytochemicals including the four types of tea (green tea, oolong tea, black tea and white tea) and several varieties of red and white wine. The aqueous extract of black tea showed the highest α-glucosidase inhibition followed by white and oolong tea. Red wine had high α-glucosidase inhibition compared to white wine and the inhibitory activity was correlated to phenolic content, antioxidant activity and phenolic profile of the extracts. These extracts showed less α-amylase inhibition which indicates the potential to overcome the side effects of undigested starch, and thus may have benefits for the management of hyperglycemia (Kwon et al., 2008b). Routine consumption of green tea has been reported as showing beneficial effects on various metabolic disorders such as Type 2 diabetes, obesity and cardiovascular risks because of its catechin (specifically EGCG (−)-epigallocatechin-3-gallate) content in various in vitro and animal studies (Thielecke and Boschmann, 2009).

Varieties of pumpkin (Cucurbita pepo), maize (Zea mays) and beans (Glycine max, Vigna angularis, Canavalia spp., Cicer arietinum, and Canavalia ensiformis) have been screened using in vitro enzyme (α-glucosidase, α-amylase and ACE-I) inhibition assays. Round orange and spotted orange green pumpkin extracts had the highest potential for glucosidase and ACE-I inhibition and help in reducing hyperglycemia and associated complications linked to cellular oxidation stress and hypertension (Kwon et al., 2007a).

Aqueous extracts of nine types of pepper, Capsicum annum, (green, red, orange, yellow, cubanelle, red sweet, yellow sweet, long hot and jalapeno) were investigated for inhibitory activities against α-glucosidase, α-amylase and ACE-I. Green, red sweet, long hot and yellow sweet had high inhibitory activity against α-glucosidase from both rat intestine and yeast; red sweet possessed highest α-amylase activity and yellow pepper had
the highest ACE-I inhibitory activity followed by cubanelle, red and red sweet. Some peppers showed high α-glucosidase with low α-amylase activity which could be a good dietary strategy to control glucose absorption without the side effects of undigested starch. This study indicated that peppers are rich in phenolic phytochemicals and have high free radical scavenging-linked antioxidant activity. These foods have the potential to reduce hyperglycemia-induced vascular complications and tissue damage resulting from oxidation and help reduce hyperglycemia and related long term complications of diabetes (e.g. hypertension) (Kwon et al., 2007c).

Legumes, including soybeans, chickpeas, lentils, kidney beans, cannellini beans, soybeans, berlotti beans, baked beans and peanuts all reduced the risk of developing Type II diabetes as they are low in fat, high in fibre, are a good source of protein and have low glycemic index. Animal studies of obesity and diabetes showed soybeans reduced serum insulin and insulin resistance, while a study of middle aged Chinese women has also shown that consumption of legumes, in particular soybeans, was inversely associated with the risk of Type 2 diabetes (Villegas et al., 2008). Alpha-amylase inhibitor (α-AI) has been isolated and purified from kidney beans (Phaseolus vulgaris L. cv Tender-green). Two isoforms, α-AI1 and α-AI1', of 43 kDa have been isolated showing a difference in their isoelectric point and neutral sugar content. The major isoform, α-AI1, inhibited human and porcine pancreatic α-amylase (PPA) but not bacterial or fungal α-amylase enzymes (Le Berre-Anton et al., 1997). Douchi, a fermented soybean Chinese food, has been screened for α-glucosidase inhibition and found to possess significant α-glucosidase inhibitory activity. Douchi fermented with A. oryzae had strong inhibition as compared to the same food fermented with other fungi such as A. elegans and R. arrhizus (Chen et al., 2007). Genistein, an isoflavone isolated from soybeans, is a potent α-glucosidase inhibitor (Lee and Lee, 2001).

1.3.3. Management of diabetes with plants

According to ethnobotanical information, more than 800 plants are used as traditional remedies in one or other form for the treatment of diabetes (Alarcon-Aguilara et al., 1998). Many different moieties, chemical groups and chemical constituents with therapeutic efficacy have been isolated and purified from plants which were traditionally
used to treat disease. One should note that metformin, the single most prescribed agent for the treatment of diabetes, originated from herbal medicine (Bailey and Day, 2004, Day, 2005) and was derived from galegine. Experimental and clinical evaluations of galegine, isolated from *Galega officinalis*, provided the pharmacological and chemical basis for the subsequent discovery of metformin (Howlett and Bailey, 2007, Bailey and Day, 2004). 1- Deoxynojirimycin (DNJ), a potent α-glucosidase inhibitor which helps in prevention of diabetes, was isolated from the water extract of leaves of mulberry trees (*Morus alba* L.) (Asano et al., 1994).

The folk medicines used for the treatment and prevention of diabetes include garlic, onion, ginseng, bitter melon, fenugreek, *Gymnema sylvestre*, *Pterocarpus marsupium* and other plants containing flavonoid compounds, bilberry, *Aloe vera*, and holly. The active ingredients derived from plants used for antidiabetic preparations have been identified, and potentially beneficial effects on the rate of food ingestion, glucose transport, potentiation of insulin release, inhibition of insulin clearance, insulin-mimetic effects, reduced gluconeogenesis, and β-cell protection have been attributed to these agents (Dey et al., 2002). Some plants, such as *G. sylvestre*, *M. charantia* and *P. marsupium*, may also help in regeneration of β-cells in the pancreas, which is an important discovery because none of the conventional oral hypoglycemic agents shows this action (Saxena and Vikram, 2004).

The anti-diabetic potential of ten plants, agrimony (*Agrimony eupatoria*), coriander (*Coriandrum sativum*), eucalyptus (*Eucalyptus globulus*), juniper (*Juniperus communis*), Lucerne (*Medicago sativa*), avocado (*Persea americana*), elder (*Sambucus nigra*), nettle (*Urtica dioica*), mushroom (*Agaricus campestris*) and mistletoe (*Viscum album*) were evaluated by an *in vitro* dialysis model of glucose movement. The glucose movement was decreased by more than 50% by agrimony and avocado. It was found that agrimony and avocado have the ability to inhibit glucose diffusion and represent potential dietary supplements that could be useful for allowing flexibility in meal planning for management of Type 2 diabetes (Gallagher et al., 2003). In an *in vivo* study, the aqueous and methanolic leaf extracts of avocado resulted in a reduction in plasma glucose level, total cholesterol and LDL-cholesterol levels in albino rats (Brai et al., 2007). The methanolic extract of the flowering part of pomegranate (*Punica granatum* Linn.)
was evaluated by \textit{in vivo} and \textit{in vitro} diabetes assays. The extract was shown to decrease plasma glucose levels and possess potent inhibitory activity against $\alpha$-glucosidase. It was suggested that it could improve postprandial hyperglycemia during treatment of Type 2 diabetes and obesity (Li et al., 2005). The inhibitory activity of anthocyanins and tannins was proved by removing the anthocyanin and tannin fractions from the berries and it was shown that tannins were related to amylase inhibition while anthocyanins were responsible for glucosidase inhibition (McDougall et al., 2005).

In another study, a new natural $\alpha$-glucosidase inhibitor from red wine vinegar (made by the fermentation of storage root paste of purple fleshed sweet potato, \textit{Ipomea batata}) was identified as caffeoylsophorose. The compound was tested against $\alpha$-glucosidase and studied in Sprague Dawley rats; the experiments demonstrated that caffeoylsophorose suppressed the increased postprandial blood glucose level achieved by inhibition of maltase (Matsui et al., 2004).

Clonal herbs of family Lamiaceae were evaluated for the management of diabetes and hypertension. Water extracts of clonal lines of rosemary \textit{Rosmarinus officinalis}, lemon balm (\textit{Melissa officinalis}), sage (\textit{Salvia officinalis}), chocolate mint (\textit{Mentha piperata}) and oregano (\textit{Origanum vulgare}) were screened using enzymatic inhibition assays and oregano showed the greatest $\alpha$-glucosidase inhibition activity, followed by chocolate mint and lemon balm. Clonal lines of rosemary also showed significant $\alpha$-glucosidase inhibition. ACE-I inhibition activity was greatest in rosemary followed by lemon balm and oregano (Kwon et al., 2006).

Other nutraceutical compounds which reduce the risk of diabetes are found in diets rich in fibres, legumes, coffee (chlorogenic acid), barley malt, biotin, magnesium, chromium picolinate, calcium/vitamin D, bitter melon and cinnamon extracts (McCarty, 2005). Hot water extracts of coffee seeds showed significant inhibition against both the enzymes $\alpha$-glucosidase and $\alpha$-amylase and reduced postprandial hyperglycemia (results assessed by \textit{in vivo} assays on Wistar rats using the Oral Saccharinity tolerance test (OST) (Zheng et al., 2007). Consumption of foodstuffs which are digested at slow rates is a good strategy to manage diabetes and its related complications of obesity and hypertension. Grains which are rich in $\beta$-glucans, such as Prowashonupana (a cultivar of barley that is less digestible than regular barley) are good for diabetic patients. Both bar-
ley varieties have been studied for their digestion and absorption and it was found that absorption of Prowashonupana was lower compared to barley (Lifschez et al., 2002).

Whole wheat seeds, partially decorticated wheat (belila), fenugreek seed powder, fenugreek germinated seeds, lupine, chickpeas and composite biscuits of whole wheat/fenugreek and whole wheat/chickpea have also shown beneficial effect in diabetes patients. It has been reported that daily consumption of whole grain foods and legumes in many forms improves glucose tolerance and serum insulin levels in diabetic patients (Ghallas et al., 2008). The phenolic compounds of finger millet or ragi (ELEusine coracana L.) from the seed coat have been screened against α-glucosidase and pancreatic amylase and found to exhibit strong inhibition against both enzymes (Shobana et al., 2009).

Bitter gourd (Momordica charantia L.) is consumed as a vegetable and herbal medicine in various parts of the world is believed to prevent and help in the management of diabetes and its related complications. It has been proven (by cell culture and glucose uptake assay) that the hypoglycemic potential of bitter gourd was due to activation of AMP-activated protein kinase (Cheng et al., 2008). Leaves of Tamarindus indicus showed 90% inhibition of α-amylase (Funke and Melzig, 2006). The by-products of pineapple (Ananas cosmosus) processing (i.e. remaining pulp, peels and skin) are rich in phenolic compounds, soluble sugars and high in fibre. After being dried, ground and mixed with organic soy bean flour in a ratio of 1:1 and 9:1 and bio-processed with Rhizpus oligosporus for 12 days, the 9:1 mixture showed the highest level of α-amylase inhibition after 2 days of R. oligosphorous growth (Correia et al., 2004b).

Other plants which have been reported as helping to decrease hyperglycemia are the rind of bitter cucumber (Citrullus colocolythis Schard), roots of Anthocleista voglii, fruits of Eugia jambolana, seeds of Malabar kola (Garcinia kola), leaf extract of Mangifera indica, flowers and fruits of Musa sapientum Kuntze, leaves of olive (Oleae europea L.), seeds of Cajanus cajan Millsp., leaves of mulberry (Morus alba L.), Eriobotrya japonica Lind., leaves of Atrocarpus heterophyllus Lam., leaves of Camellia sinensis L., husk of isphagula (Plantago ovate), bitter gourd (Momordica Charantia), Ivy gourd (Coccinia indica), leaves of mustard (Brassica juncea), cinnamon (Cinnamomi cassia), Beta vulgaris var. Cicla L., Aegle marmelose (Bnouham et al., 2006).

An extract of pine bark and needle showed inhibition against salivary α-amylase and yeast α-glucosidase enzymes and significantly reduced postprandial glucose level (Kim et al., 2005). The astringent extract of chestnut skin (Tsujita et al., 2008), extract of *Pycnanthus angolensis* fruits (Tchinda et al., 2008), ethanolic extract of *Butea monosperma* (Somani et al., 2006), leaf extract of kiwi fruit (Shirosaki et al., 2008), seed kernel of *Syzigium cumini* (Shinde et al., 2008), leaves of guava (*Psidium guajava* Linn.) (Shen et al., 2008) have all shown good hypoglycemic potential.
1.4. Phytochemicals with Anti-Diabetic Activities

A number of bioactive compounds have been isolated from plants which are potent α-glucosidase and/or α-amylase inhibitors and show good antidiabetic properties. The main phytochemicals with reported antidiabetic activities are flavonoids, polyphenolic compounds, tannins, glycosides, alkaloids and terpenoids. The active phytochemicals were isolated, purified and scientifically validated for antidiabetic action by in vitro or in vivo experiments.

1.4.1. Flavonoids and Polyphenolic compounds

Luteolin isolated from *Lonicera japonica*, amentoflavone isolated from the leaves of *Ginkgo biloba*, luteolin-7-O-glucoside isolated from *Salix gracilistyla* and daidzein isolated from soybeans are all natural flavonoids which show strong inhibitory activity against α-glucosidase and α-amylase; with luteolin exhibiting greater activity than acarbose (Kim et al., 2000). Similarly, hydnocarpin, luteolin and isohydnocarpin isolated from acetone extracts of seed hulls of *Hydnocarpus wightiana* Blume were screened against yeast α-glucosidase and it was found that luteolin showed the strongest inhibitory activity; isohydnocarpin was also a potent inhibitor while hydnocarpin was a mild inhibitor (Reddy et al., 2005). Quercetin 3-O-β-D-xylopyranosyl (1″ → 2″)-β-D-galactopyranoside and (-)-lyoniresinol 3-O-β-D-glucopyranoside isolated from the leaves of *Alstonia scholaris*, also known as Devil tree, is a traditional Thai medicinal plant. Quercetin 3-O-β-D-xylopyranosyl (1″ → 2″)-β-D-galactopyranoside was found to possess maltase inhibitory activity and (-)-lyoniresinol 3-O-β-D-glucopyranoside showed significant inhibition against both the sucrase and maltase activities of α-glucosidase (Jong-Anurakkun et al., 2007).

Crude 50% methanolic extracts of rhizomes of *Berginia ciliata*, a Nepalese medicinal plant used to treat several diseases, showed significant inhibitory activity against rat intestinal α-glucosidase and porcine pancreatic α-amylase. This extract was fractionated for the isolation of novel active compounds which were further screened for activity against the same enzymes. (-)-3-O-galloylepicatechin and (-)-3-O-galloylcatechin were isolated as potent antidiabetic compounds which showed dose-dependent enzyme inhi-
bition (Bhandari et al., 2008). Chebulanin, chebulagic acid and chebulinic acid isolated from *Terminalia chebula* have been shown to possess potent inhibitory activity against \( \alpha \)-glucosidase (Gao et al., 2007). Furthermore, chebulagic acid from *Terminalia chebula* was later shown to have good antidiabetic activity (Gao et al., 2008b).

*Tussilago farfara* L. is a common plant in China used in folk medicine. The aqueous methanolic extract of flower buds of this plant and the isolated compounds 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and rutin showed good maltase inhibitory activity of rat intestinal \( \alpha \)-glucosidase, and thus may help in reduction of postprandial hyperglycemia (Gao et al., 2008a).

Flour made from unripe banana (*Musa paradisiaca* L.) can be used to make pasta or spaghetti with increased undigestible carbohydrates and increase the antioxidant content. Green, unripe banana is rich in proanthocyanidins, polyphenolic compounds and vitamins and thus possesses significant antioxidant activity. Moreover, the flour is considered to be of low glycemic index food, high in resistant starch and non-starch polysaccharides and possesses slow carbohydrate absorption which could be a good strategy to prevent diabetes (Ovando-Martinez et al., 2009). An aqueous extract of unripe plantain (*Musa paradisiaca*) was shown to possess hypoglycemic activity, as it reduced glucose levels in normal and alloxan-induced diabetic rats (Oloyede, 2008).

MC 2-1-5, a water soluble peptide purified from *Momordica charantia* L. Var. Abbreviata Ser., has significant hypoglycemic potential. When studied in alloxan-induced diabetic mice, it significantly reduced the blood glucose level (Yuan et al., 2008). The hot water extract of chamomile, *Matricaria chamomilla* L., and the isolated compounds, esculetin and quercetin, help in prevention of hyperglycemia and the reduction of diabetic complications in diabetes patients. It was suggested that daily consumption of chamomile tea can prevent hyperglycemia and diabetic complications (Kato et al., 2008).

Six groups of flavonoids – flavones, flavonol, flavanone, isoflavone, flavan-3-ol, and anthocyanidins (Figure 2) – were screened for inhibitory activity on \( \alpha \)-amylase and \( \alpha \)-glucosidase enzymes and the chemical structures responsible (structural activity relationship) for these activities were evaluated. The basic structure of flavonoids consists
of benzopyran (A & C rings) and a phenyl group (B ring). The six groups of flavonoid are classified on the basis of variation in the C ring and linkage between the benzopyran and phenyl groups. Inhibitory activities of 4-hydroxylated, 4,5-dihydroxylated and 3,4,5-trihydroxylated flavonoids in the same flavonoid group were compared and it was found that activity was increased with increase in number of hydroxyl group on the B ring. The inhibitory activity was found to be increased by the unsaturated C ring, 3-OH, 4-CO, linkage of B ring at position 3 and hydroxyl substitution on the B ring. For example, 2,3-double bond (isoflavone, flavones, and flavonol > flavanone and flavan-3-ol), 5-OH of flavonol or isoflavone (quercetin > fisetin; genistein > daidzein), linkage of the B ring at the 3 position (genistein > apigenin) and hydroxyl substitution on the B ring increased the inhibitory activity (genistein > luteolin). It was found that A, B and C rings structures were related to inhibitory activity (Tadera et al., 2006).

1.4.2. Glycosides

Chrysophanol-8-O-β-D-glucopyranoside and chrysophanol anthraquinones from Rhus-barb rhizome showed good antidiabetic properties (Lee and Sohn, 2008). Rhaponticin and rhein isolated from Rhei Rhizoma improved glucose tolerance by inhibiting α-glucosylactylase activity, increasing insulin sensitivity and delaying carbohydrate digestion in STZ-induced diabetic mice. In vitro studies also showed improvement in insulin sensitivity (Choi et al., 2006). Dolichandroside A, a new phenylpropanoid glycoside isolated from Dolichandrone falcate Seem, is a novel α-glucosidase inhibitor, while saponarin II, isolated from the same plant, is a very effective α-glucosidase inhibitor having the same potency as acarbose (Aparna et al., 2008). Dendrobium chrysotoxum Lindl. is a traditional Chinese herb. Polysaccharides isolated from this plant have been found to significantly reduce blood glucose levels in alloxan-induced diabetic mice as well as having good antioxidant activity (Zhao et al., 2007). Lupinoside isolated from Pueria tuberosa helps in prevention of palmitate-induced impairment of insulin (Dey et al., 2007).
1.4.3. Alkaloids

*Adhatoda vasica* Nees, a common Indian Ayurvedic plant, was screened for activity against α-glucosidase and α-amylase. The aqueous methanolic extract of its leaves showed high sucrase inhibitory activity and enzyme assay-guided fractionation led to discovery of vasicine and vasicinol. Both the compounds showed high sucrase inhibitory activity by reversible inhibition of sucrose hydrolyzing activity of rat intestinal α-glucosidase. The enzymatic inhibition of α-glucosidase was studied for the first time in this plant, although it is well known for other pharmacological activities (Gao et al., 2008c). Two new active compounds, uniflorines A and B, have been isolated from the leaves of *Eugenia uniflora* L. and showed reduction in plasma glucose levels in sucrose tolerance tests on mice and inhibited α-glucosidase enzyme (Matsumura et al., 2000).
1.4.4. Essential oils

A mixture of oleanolic acid and ursolic acid in a ratio of 2:1 isolated from *Phyllanthus amarus* was screened for α-amylase inhibition and found to exhibit significant inhibitory activity (Ali et al., 2006). Roselle tea extract is made from the dried flowers of *Hibiscus sabdariffa* Linn. and is a popular beverage in Thailand. Hibiscus acid and its 6-methyl esters isolated from a Roselle tea extract showed significant inhibition of porcine pancreatic amylase (Hansawasdi et al., 2000). The essential oils from the wood of *Juniper oxycedrus* showed good activity against α-amylase, while the wood and berries of the same plant possessed significant antioxidant activity (Loizzo et al., 2007). A diterpenoid, andrographolide, isolated from the ethanolic extract of *Andrographis paniculata* (Burman.f.) Nees (Acanthaceae) showed significant α-glucosidase inhibition (Subramanian et al., 2008a). Swietenine, a tetranortriterpenoid, isolated from *Swietenia macrophylla* seeds showed significant in vivo hypoglycemic and hypolipidemic activity in Type 2 diabetic rats (Dewanjee et al., 2009).

Two new compounds, 7’-(3’, 4’-dihydroxyphenyl)-N-((4-methoxyphenyl) ethyl) propenamide and 7’-(4’-hydroxy, 3’-methoxyphenyl)-N-((4-butylphenyl) ethyl) propenamide, isolated from *Cuscuta reflexa* Roxb. showed strong inhibition for α-glucosidase (Anis et al., 2002). Pipataline, pellitorine, sesamine, brachystamide B and guineensine were isolated from *Piper longum* by bio-activity (α-glucosidase enzyme inhibition) guided fractionation and found to possess potent inhibitory activity (Pullela et al., 2006).

From the above scientific evaluation of phyochemicals, it is clearly seen that the majority of foods traditionally used to reduce hyperglycemia and related disorders (e.g. obesity) are rich in polyphenolic compounds and flavonoids.

In this project, seven Australian Aboriginal plant extracts and nineteen Indian Ayurvedic plant extracts were studied. The main aim was to evaluate Australian Aboriginal plants for potential use in the management of type 2 diabetes. The Australian Aboriginal plants have been used for general illness but have never been used for diabetes, thus this is the first study to investigate their use in the management of type 2 diabetes. The In-
Ayurvedic plants were studied, by contrast, because of their long held holistic medicinal benefit.

Many of the plants screened here have been used as food or food supplements, suggesting that they are safe to take orally. Seeds and gums of *Acacia* species are edible and, as this plant grows in harsh environments, it is commonly known as “dead finish”, *Santalum lanceolatum* (SL) has sweet fruits which are eaten fresh and the decoction of the inner bark of *S. spicatum* (SS) is taken orally to relieve coughs (Anonymous, 1988). Fruits of *Eugenia jambolana* (EJ), called blackberries in English, are eaten fresh, are rich in polyphenols, are widely distributed in India and are known to reduce glucose (Sagrawat et al., 2006). Seeds of *Mucuna pruriens* (MP), also known as velvet beans, are cooked or can be eaten raw (Taylor, 2005) and in Central America the roasted and ground seeds are used as a substitute for coffee (Sathiyarayananan and Arulmozhi, 2007). Tuberous roots of *Curculigo orchioides* (CO) are eaten to maintain vitality, strength and have aphrodisiac effects (Chauhan and Dixit, 2007). Tribal people of West Bengal eat *Boerrhaavia diffusa* (BD) as a vegetable, while in the Assam state of India, this plant is also cooked and eaten (Awasthi and Verma, 2006). Bulbs of *Allium sativum* (AS) has been used as a food and spice in many countries (Pittler and Ernst, 2007). Ripe fruits of *Solanum nigrum* (SN) are used in Africa for bed wetting in children and the whole plant is used in India as food (Jain et al., 2011).

Table 1 and Table 2 shows the ethnobotanical uses of the plants used in this study.
### 1.5. Brief overview of plants used in this study

<table>
<thead>
<tr>
<th>Plant name</th>
<th>Family</th>
<th>Part used</th>
<th>Traditional Uses</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acacia kempeana F. Muell.</em></td>
<td>Mimosaceae</td>
<td>Leaves</td>
<td>Chest infection, severe cold and general sickness</td>
<td>(Palombo and Semple, 2001a, O’Connell et al., 1983)</td>
</tr>
<tr>
<td><em>Acacia tetragonophylla F. Muell.</em></td>
<td>Mimosaceae</td>
<td>Stem</td>
<td>Cough, treatment of circumcision wounds and dysentery</td>
<td>(Reid and Betts, 1979)</td>
</tr>
<tr>
<td><em>Acacia ligulata Cunn. ex Benth.</em></td>
<td>Mimosaceae</td>
<td>Leaves</td>
<td>Cough, cold and chest infection</td>
<td>(Webb, 1959, O’Connell et al., 1983, Latz, 1995)</td>
</tr>
<tr>
<td><em>Beyeria leschenaultii</em> (DC.) Baillon</td>
<td>Euphorbiaceae</td>
<td>Leaves and stem</td>
<td>General sickness and fever</td>
<td>(Webb, 1959)</td>
</tr>
<tr>
<td><em>Euphorbia drummondii</em> Boiss.</td>
<td>Euphorbiaceae</td>
<td>Whole plant</td>
<td>Skin sores, genital sores, fever and dysentery</td>
<td>(Palombo and Semple, 2001a)</td>
</tr>
<tr>
<td><em>Santalum lanceolatum</em> R. Br.</td>
<td>Santalaceae</td>
<td>Leaves</td>
<td>Cold, malaise, sore throat, venereal diseases and painful urination</td>
<td>(Palombo and Semple, 2001a)</td>
</tr>
<tr>
<td><em>Santalum spicatum</em> (R. Br.) A. DC.</td>
<td>Santalaceae</td>
<td>Leaves</td>
<td>Cough</td>
<td>(Webb, 1959)</td>
</tr>
</tbody>
</table>

Table 1 - Ethnobotanical uses of Australian Aboriginal plants
<table>
<thead>
<tr>
<th>Plant name</th>
<th>Family</th>
<th>Part used</th>
<th>Traditional Uses</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Andrographis paniculata</em> Nees.</td>
<td>Acanthaceae</td>
<td>Herb</td>
<td>Warts, Febrifuge, tonic, anthelmintic, useful in debility, dysentery and dyspepsia</td>
<td>(Ram et al., 2004; Ahmad et al., 1998)</td>
</tr>
<tr>
<td><em>Anacyclus pyrethrum</em> DC.</td>
<td>Compositae</td>
<td>Roots</td>
<td>Aphrodisiac, rejuvenation, rubefacient, tooth ache, apnoea. sciatica, paralysis,</td>
<td>(Sharma et al., 2009, Gautam et al., 2011)</td>
</tr>
<tr>
<td><em>Allium sativum</em></td>
<td>Liliaceae</td>
<td>Bulbs</td>
<td>Cough and fever</td>
<td>(Ahmad et al., 1998)</td>
</tr>
<tr>
<td><em>Bacopa monnieri</em></td>
<td>Scrophulariaceae</td>
<td>Herb</td>
<td>Leaves of plant are used to treat epilepsy, insanity and other nervous disorders</td>
<td>(Dabur et al., 2008)</td>
</tr>
<tr>
<td><em>Boswellia serrata</em> Roxb.</td>
<td>Bruseraceae</td>
<td>Oleo-gum-resin</td>
<td>Diarrhoea, dysentry, urinary disorders, gonorrhoea and bronchitis</td>
<td>(Kumar et al., 2006)</td>
</tr>
<tr>
<td><em>Chlorophytum borivilianum</em></td>
<td>Liliaceae</td>
<td>Tubers</td>
<td>Roots are used to treat diarrhoea and dysentery and also used as demulcent and galactogogue</td>
<td>(Dabur et al., 2008)</td>
</tr>
<tr>
<td><em>Commiphora mukul</em> Hook.</td>
<td>Bruseraceae</td>
<td>Resin</td>
<td>Antiseptic, urinary disorders, skin diseases, gonorrhoea, nasal catarrh, bronchitis, Astringent, antisepic, expectorant, carminative, enriches the blood and used for scalp lesions</td>
<td>(Kumar et al., 2006, Ahmad et al., 1998)</td>
</tr>
<tr>
<td><em>Convolvulus pluricaulis</em> Choisy</td>
<td>Convolvulaceae</td>
<td>Herb</td>
<td>Nervine tonic, memory enhancer, anthelmintic, dysentery, brain and hair tonic, cures skin ailments</td>
<td>(Sethiya and Mishra, 2010)</td>
</tr>
<tr>
<td>Plant Name</td>
<td>Family</td>
<td>Part</td>
<td>Uses</td>
<td>References</td>
</tr>
<tr>
<td>------------------------------------</td>
<td>-----------------</td>
<td>--------------</td>
<td>------------------------------------------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Curculigo orchioides Gaertn.</td>
<td>Amaryllidaceae</td>
<td>Rhizomes</td>
<td>Demulcent, diuretic, aphrodisiac, asthma and jaundice</td>
<td>(Chauhan and Dixit, 2007, Madhavan et al., 2007)</td>
</tr>
<tr>
<td>Eugenia jambolana Lam.</td>
<td>Myrtaceae</td>
<td>Seeds</td>
<td>Bronchitis, asthma, sore throat, diabetes, dysentery, antibacterial and antioxidant</td>
<td>(Sagrawat et al., 2006, Grover et al., 2000, Teixeira et al., 1997)</td>
</tr>
<tr>
<td>Mucuna pruriens Linn.</td>
<td>Fabaceae</td>
<td>Seeds</td>
<td>Antiparkinson, hypoglycemic, hypo-cholesterolemic, antioxidant, antitumour and antimicrobial</td>
<td>(Katzenschlager et al., 2004, Sathiyanarayanan and Arulmozhi, 2007)</td>
</tr>
<tr>
<td>Nardostachys jatamansi DC.</td>
<td>Valerianaceae</td>
<td>Roots</td>
<td>Cholera, flatulence and leprosy</td>
<td>(Kumar et al., 2006)</td>
</tr>
<tr>
<td>Plumbago zeylenica Linn.</td>
<td>Plumbaginaceae</td>
<td>Roots</td>
<td>Skin disease, Skin diseases, diarrhoea, piles and leprosy</td>
<td>(Kumar et al., 2006, Ahmad et al., 1998)</td>
</tr>
<tr>
<td>Pterocarpus marsupium Roxb.</td>
<td>Fabaceae</td>
<td>Wood</td>
<td>Antidiabetic, anticataract, cardiotonic and hepatoprotective</td>
<td>(Devgun et al., 2010, Abesundara et al., 2004)</td>
</tr>
<tr>
<td>Puereria tuberosa DC.</td>
<td>Fabaceae</td>
<td>Tuberous roots</td>
<td>Aphrodisiac, antirheumatic, diuretic, demulcent, galactagogue and purgative</td>
<td>(Sumalatha Gindi, 2010)</td>
</tr>
<tr>
<td>Solanum nigrum Linn.</td>
<td>Solanaceae</td>
<td>Fruits</td>
<td>Wound healing, antioxidant, antibacterial, anticancer and CNS depressant</td>
<td>(Mohamed Saleem et al., 2009)</td>
</tr>
<tr>
<td>Tinospora cordifolia Willd.</td>
<td>Menispermeace</td>
<td>Stem</td>
<td>Fever and jaundice</td>
<td>(Ahmad et al., 1998)</td>
</tr>
<tr>
<td>Valeriana wallichii</td>
<td>Valerianaceae</td>
<td>Rhizomes</td>
<td>Anti-spasmodic, stimulant, carminative, antiseptic and sedative,</td>
<td>(Jawaid et al., 2011)</td>
</tr>
<tr>
<td>-------------------</td>
<td>--------------</td>
<td>----------</td>
<td>---------------------------------------------------------------</td>
<td>-----------------------</td>
</tr>
</tbody>
</table>

Table 2 - Ethnobotanical uses of Indian Ayurvedic plants
Natural inhibitors from dietary sources have shown lower inhibitory effects against α-amylase activity and stronger inhibitory activity against α-glucosidase, which are beneficial properties that can reduce postprandial hyperglycemia with minimal side effects (McCue et al., 2005).

Lifestyle modifications and proper diet management are also important factors in the treatment and prevention of diabetes mellitus and its related complications. Diabetes patients should include wholegrain products, vegetables, fruits, low fat milk, food high in fibre, meat products and other appropriate sources of proteins, soft margarines and vegetable oils rich in monounsaturated fatty acids and foods with low glycemic index in their diets (Mann, 2002).

Nuts and peanuts are beneficial in maintaining glucose and insulin homeostasis. Omega-3-fatty acids and regular consumption of fish helps in diabetes by reducing the chances of developing cardiovascular diseases and cinnamon may also have some affect in reducing blood glucose (Rudkowska, 2009). Exercise and physical activity are the other important factors to help manage diabetes by increasing energy expenditure, as physical inactivity and a sedentary lifestyle are associated with metabolic disorders such as diabetes, obesity and other cardiovascular disorders (Astrup, 2007).

Therefore, the research on plants can lead to the identification of metabolites that promote health, reduce the risk of chronic diseases and lead to the developmental tools to assay the health-promoting effects of these metabolites which will positively contribute in preventing the social and economic burdens of chronic disease globally (Martin et al., 2011).

Hence, in the present investigation, attempts have been made to study the traditional plants as antidiabetic agents.

The overall objective of the studies in this thesis were to determine the potential use of Australian Aboriginal plant and Indian Ayurvedic plant extracts in the management of type 2 diabetes and related disorders.
1.6. **The specific aims**

- To investigate the antibacterial and antifungal activity in Australian Aboriginal and Indian Ayurvedic plant extracts against two Gram-negative and two Gram-positive bacteria and yeast.
- To determine the inhibitory concentration (IC$_{50}$) antioxidant activity of Australian Aboriginal and Indian Ayurvedic plant extracts against free radicals 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 2,2’-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS) and ferric reducing power assay (FRAP). The total phenolic and flavonoid contents were also determined for the same plant extracts.
- To determine the activity of Australian Aboriginal and Indian Ayurvedic plant extracts against $\alpha$-amylase and $\alpha$-glucosidase, both of which are involved in the metabolism of carbohydrates, and angiotensin converting enzyme-I, which is linked to hypertension.
- To investigate the mechanism of action and possible role of selected plant extracts in the management of type 2 diabetes by measuring glucose uptake and role in adipogenesis using the 3T3-L1 cell lines. Cytotoxicity was also measured in 3T3-L1, MDCK, HeLa and A549 cells.
Chapter 2. Antimicrobial activity of plants
2.1. Abstract

The antimicrobial potential of 26 plant extracts was evaluated against four bacteria (*Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*) and a yeast (*Candida albicans*) using the disk diffusion assay. Ten extracts, *Andrographis paniculata* (AP), *Convolvulus plauricaulis* (CP), *Mucuna pruriens* (MP), *Plumbago zeylenica* (PZ), *Pterocarpus marsupium* (PM), *Curculigo orchioides* (CO), *Eugenia jambolana* (EJ), *Valeriana wallichii* (VW), *Allium sativum* (AS) and *Nardostachys jatamansi* (NJ), showed broad spectrum activity against all the four bacterial strains used. Most of the plant extracts showed varied levels of antimicrobial activity against one or more test bacteria. Anticandidal activity was detected in only seven plant extracts. The lowest concentration of the extract which inhibited any visual microbial growth was considered to be the minimum inhibitory concentration (MIC). Minimum bactericidal concentration (MBC) was also determined. *Acacia kempeana* (AK) and *Santalum lanceolatum* (SL) showed mild activity against *B. cereus*. None of the Australian Aboriginal plants showed any activity against other bacteria.
2.2. **Introduction**

Medicinal plants produce bioactive molecules which react with other organisms in the environment to help inhibition of bacterial or fungal growth. The substances that can inhibit pathogens and are less toxic to host cells are considered candidates for developing new antimicrobial drugs (Sengul et al., 2009).

Infectious diseases are the major cause of death worldwide with more than 50% of the deaths occurring in tropical countries according to the World Health Organization (WHO) (Rice et al., 2000). Treatment of such diseases is complicated due to resistance in microorganisms to the commonly used antibiotics and the low income of the population in the developing countries. Therefore, about 80% of the world’s population remains dependent on plant-based drugs. Scientific experiments involving the antimicrobial properties of plants were first documented in the late 19th century with many plants now having been shown to produce antimicrobial and bactericidal compounds which have been utilized as effective treatments for infectious diseases (Shah et al., 2004). There is a need, however, for many more avenues to fight diabetes, propelling increased research into the discovery of new therapeutic agents based on herbal medicines (Kuete et al., 2011).

Patients with diabetes are more susceptible to infections, particularly soft tissue infections like diabetic foot ulcers (mainly caused by group A *Streptococcus, Staphylococcus aureus*, aerobic Gram-positive cocci, Gram-negative rods and anerobes). Urinary tract infections are mainly due to *Candida albicans*. Invasive (malignant) otitis media, which is an uncommon but life-threatening infection, is almost exclusively due to *Pseudomonas aeruginosa* in diabetic patients. Since neutrophil function, chemotaxis and phagocytosis are depressed in people with diabetes, bactericidal activity and cell-mediated immunity is also compromised. Thus, it is a good strategy to manage diabetes in a holistic fashion with plants which show good enzyme inhibitory, antioxidant and anti-microbial activities (Joshi et al., 1999). Therefore, the aim of this study was to screen traditional Australian Aboriginal plant and Indian Ayurvedic plant extracts to determine the potential against various bacteria and yeast.
2.2.1. Chapter Aims

The experiments described in this chapter were designed to evaluate the antimicrobial activity of plant extracts against

- Two Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*)
- Two Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*)
- Yeast (*Candida albicans*).
2.3. **Materials and methods**

All the plants were extracted in ethanol. Plant materials used in this study were as follows:

<table>
<thead>
<tr>
<th>Plant name</th>
<th>Codes used</th>
<th>Common name</th>
<th>Family</th>
<th>Part used</th>
<th>Voucher number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acacia kempeana</em> F. Muell.</td>
<td>AK</td>
<td>Witchetty Bush</td>
<td>Mimosaceae</td>
<td>Bark and leaves</td>
<td>AD99732178</td>
</tr>
<tr>
<td><em>Acacia tetragonophylla</em> F. Muell.</td>
<td>AT</td>
<td>Dead finish</td>
<td>Mimosaceae</td>
<td>Stem and leaves</td>
<td>AD99732184</td>
</tr>
<tr>
<td><em>Acacia ligulata</em> Cunn. ex Benth.</td>
<td>AL</td>
<td>Umbrella bush</td>
<td>Mimosaceae</td>
<td>Bark and leaves</td>
<td>AD99732187</td>
</tr>
<tr>
<td><em>Beyeria leschenaultii</em> (DC.) Baillon</td>
<td>BL</td>
<td>Turpentine bush</td>
<td>Euphorbiaceae</td>
<td>Leaves and stem</td>
<td>AD99732200</td>
</tr>
<tr>
<td><em>Euphorbia drummondii</em> Boiss.</td>
<td>ED</td>
<td>Caustic weed</td>
<td>Euphorbiaceae</td>
<td>Whole plant</td>
<td>AD99732190</td>
</tr>
<tr>
<td><em>Santalum lanceolatum</em> R. Br.</td>
<td>SL</td>
<td>Northern sandal-wood</td>
<td>Santalaceae</td>
<td>Bark, stem and leaves</td>
<td>AD99732181</td>
</tr>
<tr>
<td><em>Santalum spicatum</em> (R. Br.) A. DC.</td>
<td>SS</td>
<td>Australian sandal-wood</td>
<td>Santalaceae</td>
<td>Bark</td>
<td>AD99732191</td>
</tr>
</tbody>
</table>

Table 3 - Australian Aboriginal plants used in this study
<table>
<thead>
<tr>
<th>Plant name</th>
<th>Codes used</th>
<th>Common name</th>
<th>Family</th>
<th>Part used</th>
<th>Voucher number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Andrographis paniculata</em> Nees.</td>
<td>AP</td>
<td>Kalmegh</td>
<td>Acanthaceae</td>
<td>Herb</td>
<td>PROM/AP-25</td>
</tr>
<tr>
<td><em>Anacyclus pyrethrum</em> DC.</td>
<td>Ana.</td>
<td>Akarkara</td>
<td>Compositae</td>
<td>Roots</td>
<td>PROM/ANPY-56</td>
</tr>
<tr>
<td><em>Allium sativum</em></td>
<td>AS</td>
<td>Garlic</td>
<td>Liliaceae</td>
<td>Bulbs</td>
<td>PROM/AS-10</td>
</tr>
<tr>
<td><em>Bacopa monnieri</em></td>
<td>BM</td>
<td>Brahmi</td>
<td>Scrophulariaceae</td>
<td>Herb</td>
<td>PROM/BRM-12</td>
</tr>
<tr>
<td><em>Boerhaavia diffusa</em> Linn.</td>
<td>BD</td>
<td>Punarnava</td>
<td>Nyctaginaceae</td>
<td>Herb</td>
<td>PROM/BD-43</td>
</tr>
<tr>
<td><em>Boswellia serrata</em> Roxb.</td>
<td>BS</td>
<td>Shallaki</td>
<td>Bruseraceae</td>
<td>Oleo-gum-resin</td>
<td>PROM/BSW-02</td>
</tr>
<tr>
<td><em>Chlorophytum borivilianum</em></td>
<td>CB</td>
<td>Safed musli</td>
<td>Liliaceae</td>
<td>Tubers</td>
<td>PROM/CHBR-09</td>
</tr>
<tr>
<td><em>Commiphora mukul</em> Hook</td>
<td>CM</td>
<td>Guggul</td>
<td>Bruseraceae</td>
<td>Resin</td>
<td>PROM/CMKL-10</td>
</tr>
<tr>
<td><em>Convolvulus pluricaulis</em> Choisy</td>
<td>CP</td>
<td>Shankpushpi</td>
<td>Convolvulaceae</td>
<td>Herb</td>
<td>PROM/CP-06</td>
</tr>
<tr>
<td><em>Curculigo orchioides</em> Gaertn.</td>
<td>CO</td>
<td>Kali musli</td>
<td>Amaryllidaceae</td>
<td>Rhizomes</td>
<td>PROM/CUOR-15</td>
</tr>
<tr>
<td><em>Eugenia jambolana</em> Lam.</td>
<td>EJ</td>
<td>Jamun</td>
<td>Myrtaceae</td>
<td>Seeds</td>
<td>PROM/EUJA-10</td>
</tr>
<tr>
<td><em>Mucuna pruriens</em> Linn.</td>
<td>MP</td>
<td>konch</td>
<td>Fabaceae</td>
<td>Seeds</td>
<td>PROM/MUPR-05</td>
</tr>
<tr>
<td><em>Nardostachys jatamansi</em> DC.</td>
<td>NJ</td>
<td>Jatamansi</td>
<td>Valerianaceae</td>
<td>Roots</td>
<td>PROM/NAJT-03</td>
</tr>
<tr>
<td><em>Plumbago zeyleica</em> Linn.</td>
<td>PZ</td>
<td>Chitrak</td>
<td>Plumbaginaceae</td>
<td>Roots</td>
<td>PROM/PZ-13</td>
</tr>
<tr>
<td><em>Pterocarpus marsupium</em> Roxb.</td>
<td>PM</td>
<td>Vijayasaar</td>
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<td>Fabaceae</td>
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<td>VW</td>
<td>Tagar</td>
<td>Valerianaceae</td>
<td>Rhizomes</td>
<td>PROM/VAWA-21</td>
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</tbody>
</table>

Table 4 - Indian Ayurvedic plants used in this study
2.3.1. Preparation of extracts

Five grams of Indian plant powders were soaked overnight in 50 ml absolute ethanol and filtered with Whatmann filter paper No. 1. The filtrates were concentrated in vacuo in a rotary evaporator at 55 °C and reconstituted in ethanol at 250 μg/μl. These stock solutions were used to make working solutions of various concentrations.

Australian Aboriginal plants were obtained in solution of ethanol and used as such.

2.3.2. Media used

Materials and media were sterilized in a Siltex 250D autoclave (Siltex, Australia) at 121 °C for 20 minutes prior to use. Aliquots of agar were prepared in 20 ml McCartney bottles and aliquots of broth were prepared in 5 ml plastic vials and stored at room temperature. Circular sterile petri dishes (80 mm diameter, Techno-Plas, Australia) were used for the agar diffusion assays. Microtitre (96 well) round bottom plates were used in the broth dilution assays.

2.3.3. Anti-bacterial assay

Two Gram-positive bacteria, Bacillus subtilis (ATCC 6051) and Staphylococcus aureus (ATCC 12600), and two Gram-negative bacteria (Escherichia coli (ATCC 11775), Pseudomonas aeruginosa (ATCC 10145) were used. The cultures were part of a collection located at Swinburne University of Technology, were maintained on nutrient agar slants and recovered by culturing in nutrient broth for 18 hours at 37 ºC. The disk diffusion method was used to determine the antibacterial activity of plants (Kalemba and Kunicka, 2003). The bacterial cultures were plated over nutrient agar plates with sterile swab sticks. The plates were dried and sterile discs were dipped in the plant extracts (250 mg/ml) then placed onto the centre of nutrient agar plates. The plates were incubated for 18 hours at 37 ºC. Control plates were also prepared in which ethanol was applied to discs instead of plant extracts. The diameters of zones of inhibition were measured after incubation and the results were compared with the control.
Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were also determined for the plant extracts that showed antibacterial activity. The initial concentration was 250 mg/ml, two-fold serial dilutions were prepared in 96 well microplates and each well was inoculated with overnight bacterial cultures. The microplates were incubated at 37 ºC for 18 hours. The visual turbidity was compared with the control well which did not have the plant extract. The MIC was determined as the lowest concentration which completely inhibited bacterial growth. The MBC was also determined by sub-culturing the samples from the wells of microplate with no visible growth onto nutrient agar plates. The lowest concentration of the extract which did not show any visible growth was recorded as the MBC (Chandrasekaran and Venkatesalu, 2004).

2.3.4. Anti-fungal assay

The fungal strain *Candida albicans* was used to evaluate the antifungal activity of plant extracts. *Cadida albicans* (FRR5580) was cultured in Potato Dextrose Broth (PDB, Difco) with an incubation at 30 ºC for 48 hours. The culture was part of a collection located at Swinburne University of Technology and was maintained on Potato Dextrose Agar (PDA, Difco) slants.

The anti-fungal assay used was similar to that described for the antibacterial assay. A sterile cotton-tipped swab was dipped into a PDB fungal suspension of *Candida albicans* and streaked in two directions across PDA plates. The plates were dried for 2-3 min, and subsequently, pre-sterilised discs (6mm) were placed onto the agar surface and 5 µl of the plant extracts were individually added to the discs. The seeded plates were incubated at 30 ºC for 48 hours. Control plates were also prepared in which ethanol was applied to discs instead of plant extracts. The diameters of zones of inhibition were measured after incubation and the results were compared with the control.
2.4. **Results and Discussion**

The anti-microbial activity of 26 ethanolic plant extracts was investigated against four common bacteria and yeast.

Specifically, the following microorganisms were used to detect antimicrobial activity:

- *E. coli* is the best-known member of the normal microbiota of the human intestine and a versatile gastrointestinal pathogen,
- *P. aeruginosa* is an opportunistic pathogen which causes urinary tract infections, respiratory system infections, dermatitis and soft tissue infections,
- *B. subtilis* is representative of endospore-forming bacteria, some of which are food-borne pathogens.
- *S. aureus* is a pyrogenic bacterium known to play a significant role in invasive skin diseases including superficial and deep follicular lesions, and rapidly develops resistance to many antimicrobial agents,
- *C. albicans* was chosen for this study since it is one of the most pervasive pathogenic fungi, especially infecting immuno-compromised hosts, in which it can invade various tissues and causes serious systemic infections, including opportunistic infections in diabetic patients and those infected with HIV (Paiva et al., 2003).

Antibacterial activity was assessed by the disc diffusion method which determines the average diameter of inhibition zones of microbial growth around the disc. The Australian Aboriginal plant extracts have been previously screened for antibacterial activity and it was found that the majority of the extracts were active against *B. cereus* but none was active against the other micro-organisms tested here (Palombo and Semple, 2001).
None of the seven Australian Aboriginal plant extracts showed any activity against Gram negative bacteria (Table 5). The disc used here was of 5mm diameter and control ethanol did not give any zone of inhibition. Of the seven, only four plant extracts showed some activity against the Gram positive bacterium *B. cereus* but not against *S. aureus*. *Santalum lanceolatum* (SL) was the only plant extract which showed biofungal activity against *C. albicans* with a 16 mm zone of inhibition. Table 6 shows the zone of inhibition of Indian Ayurvedic plant extracts. Ethanol (100%) was used as a control and showed no zone of inhibition. This suggested that the solvent used for plant extraction did not contribute to the inhibitory activity of plants against the growth of bacteria.

All the Indian Ayurvedic plant extracts investigated showed activity against both the Gram-negative bacteria and both the Gram-positive bacteria, except for *Pueraria tuberosa* (PT) which did not show any activity against any tested bacteria (Table 6)

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<thead>
<tr>
<th>Plant name</th>
<th><em>E. coli</em></th>
<th><em>B. cereus</em></th>
<th><em>P. aeruginosa</em></th>
<th><em>S. aureus</em></th>
<th><em>C. albicans</em></th>
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Table 5 - Zones of inhibition by Australian Aboriginal plant extracts (diameter in mm).
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Table 6 - Zone of inhibition by Indian Ayurvedic plant extracts (diameter in mm)
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Table 7 - Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of plant extracts
Most of the extracts showed bactericidal activity at concentrations greater than the respective MIC (Table 7). However, an MBC against *Bacillus subtilis* was unable to be determined, indicating that the extracts were unable to inhibit this species, possibly due to the production of endospores.

Among all the plants screened, AS, TC, PM, AP, CP, MP, PZ, CO, EJ, VW and NJ showed broad spectrum activity against all the four bacterial strains used.

Among these plant extracts, AS, TC and CP showed the strongest inhibitory effect on the growth of *E. coli* with MIC of 1.95, 1.95 and 7.81 mg/ml and MBC of 3.90, 15.6, 15.6 mg/ml, respectively.

The MIC of AS was 3.90 mg/ml with bactericidal concentration 7.81 mg/ml against *S. aureus*. Otunola et al. (2010) found that garlic has high levels of saponins, alkaloid, tannin, carotenoids, flavonoids while steroids and cardenolides are present in trace amounts. The consumption of garlic has the potential to reduce arterial plaque and the bulb possesses antioxidant properties against skin cancer. Garlic and other spices are good sources of nutrients, mineral elements and phytochemicals which could be exploited for the development of drugs and/or nutritional supplements (Otunola et al., 2010).

It has been reported that the pharmacological actions of garlic (antimicrobial, anticancer, antioxidant, ability to reduce cardiovascular diseases and antidiabetic activity) are mainly due to allicin (diallyl-thiosulfinate), which is one of the major organo-sulfur compounds in garlic and a chemically very reactive and unstable compound (Rahman, 2007).

*In vivo* studies showed that infections induced by injecting *S. aureus, E. coli, C. albicans* or *Klebsiella pneumoniae* into immunosuppressed animals were prevented by extracts of TC (Panchabhai et al., 2008).
NJ showed an MBC of 3.90 mg/ml against *S. aureus* but the value was 62.5 mg/ml against *P. aeruginosa* and *B. subtilis*, whereas, the same plant showed very high MBC (125 mg/ml) against *E. coli* (Table 6). This is a relaxing plant with established effectiveness for mental health, mainly used for anxiety and insomnia (Samy et al., 2008). The antiseptic properties are due to essential oils present in the same plant (Jadhav et al., 2009).

PZ and AP showed an MBC of 25 mg/ml against *E. coli*, *B. subtilis* and *S. aureus* but against *P. aeruginosa* both of the plants exhibited an MBC of 12.5 mg/ml. Other studies have also reported the antimicrobial activity of both these plants. The extracts of both species exhibited maximum inhibition against *P. aeruginosa* and *S. aureus* at lower concentrations (Ram et al., 2004).

Previous studies have reported that aqueous and alcoholic extracts from roots of PZ showed antibacterial activities against *S. aureus*, *P. aeruginosa*, *B. subtilis*, *Proteus vulgaris*, and against the yeast *C. albicans*. Plumbagin exhibited relatively specific antimicrobial activity. The isolated naphthoquinone, plumbagin, could be the main constituent from roots responsible for its activity. The growth of *S. aureus* and *C. albicans* was completely inhibited. However, it was ineffective against the Gram-negative bacteria *E. coli* and *S. typhimurium*, demonstrating the specificity of plumbagin activity (Paiva et al., 2003).

**CO** showed antibacterial activity with an MIC of 7.81 mg/ml and an MBC of 62.5 mg/ml against *P. aeruginosa* in our study. Previous studies were also carried out with different extracts and showed antibacterial activities against *P. aeruginosa*, *S. aureus* and *K. pneumoniae*. Out of different extracts tested, the methanolic extract was found to be the most active against *P. aeruginosa* followed by *S. aureus* and *K. pneumonia*. The antimicrobial (and antitumor) activities were found to be due to presence of saponins, which are the glycosides present in the plant (Singh and Gupta, 2008).

**CP** showed broad spectrum activity against all the tested microorganisms in this study. This plant is traditionally used as a nerve tonic, for learning and memory, and for treatment of neurodegenerative disorders (Sethiya and Mishra, 2010). However, a study by Nautiyal et al. (2007) showed that airborne bacteria in the environment can be reduced
by medicinal smoke which is produced from natural substances. The smoke is a kind of Havana in Indian culture, *agnihotra–yagnas* to purify the environment as described in *Rigveda*—by sublimating the *havana sāmagri* (mixture of mango wood and odoriferous and medicinal herbs) in the fire accompanied by the chanting of *Vedic mantras*. The report showed that medicinal smoke emanated from *havan sāmagri* has very interesting inhibition effects on the aerial bacterial population. CP was one of the ingredients of Havana Samagri (Nautiyal et al., 2007) which explains the broad spectrum antibacterial properties of the same plant.

*PT* extract did not show any activity against any bacterial strains but showed a 10 mm zone of inhibition against *C. albicans*. The findings supported the previous work by Ratnam and Raju (2009) that ethanolic extracts of PT exhibited the least antimicrobial activity when tested against a range of Gram positive and Gram negative bacteria whereas the ethyl acetate extract showed promising results (Ratnam and Raju, 2009).

Extracts of EJ also showed broad spectrum activity against all the bacterial strains tested with an MIC of 7.81 and MBC of 62.5 mg/ml against *E. coli, P. aeruginosa* and *S. aureus* whereas the MBC was greater than 250 mg/ml against *B. subtilis*. Previous research conducted on crude methanolic seed extracts of EJ against beta-lactamase-producing drug-resistant bacteria and further fractionation and phytochemical analysis indicated that the most promising fraction revealed activity due to the presence of saponins as the active phytoconstituent (Jasmine et al., 2010).

Previous reports investigated on aqueous and methanolic extracts of BD have shown good activity against *S. aureus, K. pneumoniae, B. species, E. coli* and *P. aeruginosa* which supports the use of this plant since ancient times as a useful in the treatment of infectious diseases like diarrhoea, intestinal tract, throat and ear infections, fever and skin diseases (Girish and Satish, 2008). They have also shown anticandidial activity in the current study but only mild activity against Gram negative bacteria. Other plant extracts which showed activity against *C. albicans* were AS, BM, MP, CP and AP.
In brief, out of twenty six plant extracts screened here, seventeen plant extracts showed broad spectrum activity against different microbial strains. In classifying the antibacterial activity against Gram-positive or Gram-negative bacteria, it would generally be expected that a greater number would show activity against Gram-positive rather than Gram-negative bacteria species. However, in this study, a large number of the extracts were active against both Gram-positive and Gram-negative bacteria. The activity against both the types of bacteria may be indicative of the presence of broad spectrum antibiotic compounds or general metabolic toxins (Srinivasan et al., 2001).

In general, plant antibiotic compounds appear to be inhibitor towards Gram-positive microorganisms than Gram-negative. Even Penicillin and some of the other prominent antibiotic agents of fungal origin are moderately selective in their inhibitory action, most of them being inhibitory to Gram-positive organisms. The major component of the outer surface of Gram-negative bacteria is the lipopolysaccharide layer along with proteins and phospholipids. Thus, access of most compounds to the peptidoglycan layer of the cell wall is hindered by the outer lipopolysaccharide layer. This explains the general resistance of Gram-negative strains to the lytic action of plant extracts exhibiting activity (Kumar et al., 2006).

Most of the plant extracts showed relatively high inhibitory and bactericidal concentrations. However, the antibacterial activity of plant extracts is strain specific and depends on bioactive compounds present in the extract and this property can be expressed in different ways. Mishra et al. (2009) reported that two types of S. aureus had different responses against the extract of Andrographis paniculata. The antibacterial property of medicinal plants also depends on different parts of the plant used which contain greater or fewer antibacterial bioactive compounds. In addition, the reaction of these compounds individually or together (synergism) also influences antibacterial property of extracts (Mishra et al., 2009).

Previous literature on the plant extracts used in this study also showed variable results which may be explained by differences in the composition of bioactive compounds due to different geographic conditions and different method of extractions. For example, the antimicrobial activity of garlic powder against most bacteria was greater than for the plant or crude drug (Ross et al., 2001). The solvent use can also affect the activity. Stud-
ties have demonstrated that species such as *Helichrysum italicum* or *Phytolacca dodecandra* showed moderate activity against *E. coli* when the diethyl extract obtained after extraction of the aqueous suspension of the drug powder was tested. However, no active fractions were identified when a sequential extraction with petroleum ether, dichloromethane, dichloromethane–methanol (9:1) and methanol was used (Nostro et al., 2000). The pH of compounds in dilutions can also modify the results, for example when phenolic or carboxylic compounds are present in the extract. Not only do ionisable compounds change the activity, but it has been reported that the different effects of neutral essential oil depend on the pH. Thus, for example, anise oil had higher antifungal activity at pH 4.8 than at 6.8, while the oil of *Cedrus deudora* was most active at pH 9. The composition of the growth medium could also influence the activity of the tested extracts or compounds. The antimicrobial activity of garlic oil was found to be greater in media lacking tryptone or cysteine, which led to the hypothesis that the effects may involve sulphydryl reactivity (Rios and Recio, 2005).

This study is a preliminary evaluation of antimicrobial activity of the plant extracts. It indicates that several extracts have the potential to generate novel antimicrobial metabolites. In particular, the crude extracts demonstrating anticandidal activity could result in the discovery of novel anticandidal agents. The plants demonstrating broad spectra of activity may help in the discovery of new chemical classes of antibiotics that could serve as selective agents for the maintenance of animal or human health and provide biochemical tools for the study of infectious diseases.

### 2.5. Conclusions

The above results show that the crude extracts of *Plumbago zeylenica, Andrographis paniculata, Allium sativum, Tinospora cordifolia* and *Convolvulus pluricaulis* exhibited broad spectrum antimicrobial activity and properties that support their folkloric use in the treatment of infectious diseases. This probably explains the use of these plants by the indigenous people against a number of infections for many generations and suggests that some of the plant extracts possess compounds with antibacterial properties that can be used as antimicrobial agents for the treatment of infectious diseases caused by patho-
gens. The most active extracts can be subjected to isolation of the therapeutic antimicrobials and undergo further pharmacological evaluation.

Though the inhibitory and bactericidal concentrations obtained in this study were high, modifying the solvent and the extraction system may both influence the final results such that their efficiency in the field may be higher, or lower, than their efficiency when isolated in the laboratory. Previous literature on the plant extracts used show variable results which could be explained, for example, by differences in bioactive compounds due to the collection of plants from different geographic conditions, different method of extractions and use of solvent can modify the results. It can be concluded, therefore, that further study of medicinal plants as antimicrobial agents is warranted and may result in insights into their real value as well as validation of their traditional medicinal use.
Chapter 3. Antioxidant activity of plants
3.1. **Abstract**

The ethanolic extracts of twenty-six traditionally used Australian Aboriginal and Indian Ayurvedic plants were screened for their antioxidant free radical scavenging properties using ascorbic acid and butylated hydroxy toluene (BHT) as standard antioxidants. Free radical scavenging activity was evaluated using diphenyl picryl hydrazyl (DPPH) radical, 2,2’-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS), and ferric reducing antioxidant power (FRAP). The overall free radical scavenging activity was strongest in *Acacia ligulata*, followed by *Acacia kempeana, Beyeria leshnaultii* and *Santalum lanceolatum*. Among the Indian plant extracts, *Bacopa moneirrei, Andrographis paniculata* and *Pterocarpus marsupium* showed good free radical scavenging activity. The phenolic and flavonoid concentrations were also determined. The 50% inhibition (IC₅₀) values were also calculated for the same plant extracts. This is the first study on the antioxidant potential of Australian Aboriginal plant extracts. The Australian Aboriginal plant extracts were found to be more potent in comparison to Indian Ayurvedic plant extracts.
3.2. **Introduction**

Oxidation is the important pathway of generating free radicals and can play a vital role in diverse biological phenomenon. Oxidation, in general, plays a dual role in our body; it is essential for life but can also aggravate and damage the cells (Sen et al., 2010). Free radicals are continuously formed inside the human body when cells use oxygen to generate energy. These free radicals are reactive oxygen species (ROS) such as superoxide anion (O$_2^•$), hydroxyl radical (OH$^•$), hydrogen peroxide (H$_2$O$_2$), peroxyl (ROO$^•$) and nitric oxide (NO$^•$) and result from cellular redox processes (Pandey et al., 2010). There is evidence that ROS and reactive nitrogen species (RNS) can be damaging to cells leading to cellular dysfunction and disease. The adverse effects of free radicals were discovered in the last few decades. In low or moderate concentrations, free radicals are involved in normal physiological functions but excess production leads to oxidative stress due to the high reactivity of these radicals forming a chain reaction (Sen et al., 2010). These are generated as part of the body’s normal metabolic process and the chain reactions are usually produced in the mitochondrial respiratory chain, from atmospheric pollutants, and from transitional metal catalysts, drugs and xenobiotics. In addition, chemical mobilization of fat stores under various conditions like lactation, exercise, fever, infection and fasting, can result in increased free radicals in our body. Stress hormones secreted by the adrenal glands during excessive emotional states also give rise to free radicals (Atawodi, 2005).

Free radicals also arise due to radiation, chemicals, toxins, deep fried foods and physical stress. They cause depletion of the immune system, changes in gene expression and induce abnormal proteins (Pourmorad et al., 2009). ROS can cause great damage to cell membranes and DNA. The resulting oxidation causes membrane lipid peroxidation which in turn stimulates glycation of protein, inactivation of enzymes and alteration in the structure and function of collagen basement membranes. This, in turn, plays a role in the long-term complication of diabetes, decreased membrane fluidity and other DNA mutations that leads to cancer, cardiovascular diseases, autoimmune and neurodegenerative disorders and ageing (Subhasree et al., 2009).
When the rate of ROS production overwhelms antioxidant defence mechanisms, oxidative damage can lead to the onset of many chronic and degenerative diseases in humans. The process of oxidation can also affect foods, where it is one of the major causes of chemical spoilage, causing rancidity, deterioration of nutritional quality, colour, flavour, texture and safety of foods (Antolovich et al., 2002).

The formation of ROS is prevented by an antioxidant system containing low molecular mass antioxidants and enzymes which generate reduced (less active) forms of antioxidants. Antioxidants such as ascorbic acid, glutathione, tocopherols and ROS-interacting enzymes such as superoxide dismutase (SOD), peroxidases and catalases help to terminate the harmful chain reaction caused by free radicals. Many phenolic compounds, flavonoids, tannins and lignin precursors found in plants are potential antioxidants (Blokhina et al., 2003).

Antioxidants scavenge free radicals and prevent the damage caused by them. These compounds are endogenous as well as exogenous in nature. Beta-carotenes and some micronutrient elements such as zinc and selenium are found in food sources (Subhasree et al., 2009). Results from biological and phytochemical studies have indicated the existence of a wide range of complex and effective defence systems of antioxidants such as secondary metabolites in plants which can overcome this oxidative stress (Valyova et al., 2009). Therefore, antioxidants are those elements that deactivate and scavenge the free radicals. Antioxidants inhibit the effect of oxidants by donating the hydrogen atom or chelating metal and prevent oxidation of biomolecules (Koksal et al., 2011). Antioxidants can also be used in food products to lengthen the shelf life of the food and reducing nutritional loss and formation of harmful substances (Moein and Moein, 2010).

Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been used as food additives to prevent oxidation of lipids. However, these compounds are undesirable due to their toxic and harmful effects and are suspected of being responsible for liver damage and carcinogenesis (Nguyen and Eun, 2011, Koksal et al., 2011).

Many studies have confirmed that plants and foods rich in polyphenolic contents are effective scavengers of free radicals, thus helping to prevent diseases through their anti-
oxidant activity (Nabavi et al., 2009). Antioxidants which are present in plants, herbs and dietary sources help in preventing vascular diseases in diabetic patients (Buyukbalci, 2008). Tannins and flavonoids are secondary metabolites in plants and are considered to be the best natural source of antioxidants which prevent destruction of β-cells and diabetes-induced ROS formation (Aslan et al., 2010).

Medicinal plant parts (roots, leaves, branches/stems, barks, flowers, and fruits) are commonly rich in phenolic compounds, such as flavonoids, phenolic acids, stilbenes, tannins, coumarins, lignans and have various biological effects and antioxidant activity (Surveswaran et al., 2007). Their traditional use often indicates that their toxic affects are minimal known or can be ameliorated. There is therefore much interest in the discovery of natural antioxidants from plants that can be safe and effective (Ebrahimzadeh et al., 2009).

### 3.2.1. Chapter Aims

This study aims to evaluate the antioxidant effects of ethanolic extracts of 26 plants by evaluating free radical scavenging activity with *in vitro* assays using

- 1,1-diphenyl-2-picrylhydrazyl (DPPH)
- 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS)
- determining ferric reducing antioxidant power (FRAP)

The total phenolic and flavonoid contents of the extracts were also determined.

### 3.3. Materials and methods

The plant materials used in this study have been detailed in Table 3 and 4 in Chapter 2 on page 34-35.

All chemicals were purchased from Sigma-aldrich Australia. The chemicals used were DPPH, ABTS, Folin-Ciocalteu reagent, gallic acid, sodium bicarbonate (Na₂CO₃), sodium nitrite (NaNO₂), aluminum chloride, sodium hydroxide (NaOH), butyl hydroxyl tol-
uene (BHT), potassium persulfate, ascorbic acid, quercetin, potassium ferricyanide (III) powder, trichloroacetic acid and ferric chloride.

3.3.1. Determination of total phenolic

The total phenolic content was quantified using a modified version of the assay described by Singleton et al. (1977) using Folin-Ciocalteu reagent. 20 µl of plant sample or gallic acid (standard phenolic compound) was diluted with 1580 µl of distilled water and then mixed with 100 µl of 2N Folin-Ciocalteu reagent. The mixture was shaken and kept for 6 minutes, after which 300 µl of 5% aqueous Na$_2$CO$_3$ solution was added and mixed properly. The mixture was incubated for 2 hours at 20 ºC. The absorbance was measured for all the samples at 765 nm. A standard curve was prepared using 0 - 1000 mg/l of gallic acid. The total phenolic values were expressed in terms of gallic acid equivalents (µg/mg of dry mass). The (distilled water) was treated in the same way as the samples and the dilution factor was taken into account for the samples where dilution was performed.

3.3.2. Determination of total flavonoids

The total flavonoids were determined using a modification of the assay described by Dixit et al. (2009). Plant samples (250 µl) were diluted with 1250 µl of distilled water and 75 µl of 5% NaNO$_2$ was added to the sample together with 150 µl of 10% aluminum chloride. After mixing and incubation for 5 minutes, 500 µl of 1 M NaOH was added to the reaction mixture and a total volume of 2500 µl was made up with distilled water. Following vigorous mixing, the absorbance was measured at 510 nm. A standard calibration curve was prepared using 0 - 1000 mg/l of quercetin. Results were expressed as µg of quercetin equivalents per milligram of dry mass of the plant extract.

3.3.3. Antioxidant activity determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical inhibition

The DPPH scavenging activity was determined by an assay modified from Kwon et al. (2007b). To 150 µl of 0.1 mM DPPH in methanol, a volume of 50 µl of plant extract
was mixed and kept in the dark at room temperature for 60 minutes. After incubation, the absorbance was recorded at 490 nm. The results were compared with the negative control which contained 50 µl of ethanol instead of plant extracts. The positive controls were BHT and ascorbic acid in the concentration range 3.12 to 250 µg/ml. The antioxidant activity was expressed as a percentage (%);

Percentage inhibition =

$$\left(\frac{\text{absorbance of control 490 nm} - \text{absorbance of samples 490 nm}}{\text{absorbance of the control 490 nm}}\right) \times 100.$$
3.3.4. Antioxidant activity determined by 2, 2′-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS) radical inhibition

The free radical scavenging activity of plant extracts was also studied using ABTS radical cation. The decolorization assay is based on reduction of ABTS +• radical by plant extracts having antioxidant capacity (Dudonn et al., 2009). ABTS radical was dissolved in deionized water to make 7 mM solution and 2.45 mM potassium persulfate solution was added to the same. To produce ABTS free radical cation, the mixture was allowed to stand in the dark at room temperature for 12 to 16 hours. The free radical solution of ABTS was diluted with ethanol to an absorbance of 0.7 at 734 nm for the assay. To 990 µl of ABTS free radical solution, a volume of 10 µl of plant extract (range 1 to 1000 µg/ml) was mixed and kept in the dark at room temperature for 60 minutes. After incubation, the absorbance was recorded at 734 nm. The results were compared with the negative control which contained 10 µl of ethanol in place of plant extracts. The positive controls were BHT and ascorbic acid in concentration range 1.56 to 250 µg/ml. The antioxidant activity was expressed as percentage (%).

\[
\text{Percentage inhibition} = \left( \frac{\text{absorbance of control 734 nm} - \text{absorbance of samples 734 nm}}{\text{absorbance of the control 734 nm}} \right) \times 100.
\]

3.3.5. Ferric reducing antioxidant power assay

The ferric reducing power assay was carried out as described by Fawole (Fawole et al., 2010) using 30 µl plant extracts and standards (BHT and ascorbic acid) of different concentrations (range 25 to 1000 µg/ml) added to 96 well plates along with 40 µl of 0.2M potassium phosphate buffer (pH 7.2) and 40 µl of potassium ferricyanide (1% w/v). The reaction mixtures were incubated at 50 ºC for 20 minutes. After incubation, 40 µl of trichloroacetic acid (10% w/v), 150 µl distilled water and 30 µl of ferric chloride (0.1 % w/v) were added and the reaction mixture again incubated for 30 minutes at room temperature in the dark. Absorbance was recorded at 630 nm using a microplate reader and the positive controls were BHT and ascorbic acid whereas the negative con-
control was buffer. The absorbance of each sample was plotted against concentration and compared with the standards.

3.3.6. Statistical analysis

All samples were analyzed in triplicates. Data are presented as mean ± standard error mean (SEM). Differences were evaluated by one-way analysis of variance (ANOVA) test completed by a Bonferroni’s multicomparison test. Differences were considered significant at p < 0.001. The concentration giving 50% inhibition (IC$_{50}$) was calculated by non-linear regression with the use of GraphPad Prism Version 5.0 for Windows (GraphPad Software, San Diego, CA, USA) (www.graphpad.com). The dose–response curve was obtained by plotting the percentage inhibition versus concentration (Loizzo et al., 2008).

3.4. Results

The antioxidant capacities, flavonoid and phenolic contents of 26 ethanolic extracts of Australian Aboriginal and Indian Ayurvedic medicinal plant species were systematically assessed. The results of three in vitro assays (ABTS, DPPH and FRAP) for antioxidant properties of the 26 plant samples are given in the figures and tables below.

3.4.1. Determination of total flavonoids and phenolic content

The total phenolic contents (TPC) of these samples were estimated using the standard Folin–Ciocalteu colorimetric method which is based on the chemical reduction of a reagent. It was found that TPC of the 26 plant samples varied from 0.42 to 1.47 µg of gallic acid equivalents (GAE) / mg for Australian aboriginal plant extracts whilst total flavonoid content ranged from 0.51 to 1.78 µg of quercetin equivalents (QE) / mg (Table 8). TPC was 1.04 to 30.27 µg of gallic acid equivalents (GAE) / mg and total flavonoid content ranged from 0.20 - 32.94 µg/mg quercetin equivalents for the Indian Ayurvedic plant extracts (Table 9).
<table>
<thead>
<tr>
<th>Plant name</th>
<th>TPC (µg GAE/mg extract)</th>
<th>TFC (µg QE/mg extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AK</td>
<td>1.47 ± 0.07</td>
<td>1.78 ± 0.08</td>
</tr>
<tr>
<td>AT</td>
<td>0.95 ± 0.06</td>
<td>1.51 ± 0.20</td>
</tr>
<tr>
<td>AL</td>
<td>0.56 ± 0.17</td>
<td>0.94 ± 0.07</td>
</tr>
<tr>
<td>BL</td>
<td>0.42 ± 0.03</td>
<td>1.33 ± 0.12</td>
</tr>
<tr>
<td>ED</td>
<td>0.93 ± 0.01</td>
<td>0.51 ± 0.06</td>
</tr>
<tr>
<td>SL</td>
<td>1.28 ± 0.03</td>
<td>1.35 ± 0.10</td>
</tr>
<tr>
<td>SS</td>
<td>0.87 ± 0.11</td>
<td>1.36 ± 0.06</td>
</tr>
</tbody>
</table>

Table 8 - Total phenolic and flavonoid contents of Australian Aboriginal plant extracts
<table>
<thead>
<tr>
<th>Plant name</th>
<th>TPC (µg GAE/mg extract)</th>
<th>TFC (µg QE/mg extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP</td>
<td>7.57 ± 0.12</td>
<td>8.11 ± 0.06</td>
</tr>
<tr>
<td>Ana.</td>
<td>3.09 ± 0.10</td>
<td>2.89 ± 0.23</td>
</tr>
<tr>
<td>AS</td>
<td>28.31 ± 0.22</td>
<td>23.06 ± 0.48</td>
</tr>
<tr>
<td>BM</td>
<td>5.54 ± 0.24</td>
<td>3.58 ± 0.35</td>
</tr>
<tr>
<td>BD</td>
<td>11.80 ± 0.07</td>
<td>8.52 ± 0.25</td>
</tr>
<tr>
<td>BS</td>
<td>1.90 ± 0.15</td>
<td>0.52 ± 0.46</td>
</tr>
<tr>
<td>CB</td>
<td>1.04 ± 0.14</td>
<td>0.24 ± 0.30</td>
</tr>
<tr>
<td>CM</td>
<td>20.58 ± 0.68</td>
<td>22.93 ± 15.79</td>
</tr>
<tr>
<td>CP</td>
<td>15.17 ± 0.42</td>
<td>22.17 ± 0.25</td>
</tr>
<tr>
<td>CO</td>
<td>7.76 ± 0.13</td>
<td>12.32 ± 0.32</td>
</tr>
<tr>
<td>EJ</td>
<td>6.98 ± 0.24</td>
<td>5.32 ± 0.53</td>
</tr>
<tr>
<td>MP</td>
<td>5.83 ± 0.57</td>
<td>13.25 ± 2.13</td>
</tr>
<tr>
<td>NJ</td>
<td>9.50 ± 0.22</td>
<td>9.14 ± 0.11</td>
</tr>
<tr>
<td>PZ</td>
<td>30.27 ± 0.88</td>
<td>32.93 ± 1.87</td>
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<tr>
<td>PM</td>
<td>1.61 ± 0.12</td>
<td>0.20 ± 0.33</td>
</tr>
<tr>
<td>PT</td>
<td>7.34 ± 0.19</td>
<td>8.84 ± 0.40</td>
</tr>
<tr>
<td>SN</td>
<td>2.88 ± 0.03</td>
<td>3.01 ± 0.26</td>
</tr>
<tr>
<td>TC</td>
<td>10.24 ± 0.12</td>
<td>7.96 ± 0.09</td>
</tr>
<tr>
<td>VW</td>
<td>10.12 ± 0.13</td>
<td>12.89 ± 0.58</td>
</tr>
</tbody>
</table>

*Table 9: Total phenolic and flavonoid content of Indian Ayurvedic plant extracts*
3.4.2. 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical inhibition

Antioxidant studies were carried out with the seven Australian Aboriginal and nineteen Indian Ayurvedic plant extracts. The free radicals scavenging ability of extracts were evaluated by studying the reaction of DPPH radicals with extracts using \textit{in vitro} colorimetric assays.

The IC_{50} values were calculated, IC_{50} value is defined as the concentration which shows 50\% of maximum inhibitory activity. The lesser the IC_{50} value for a compound the more effective (Kumanan et al., 2010). IC_{50} values of standard compounds, ascorbic acid and BHT, were determined as 12.37 µg/ml and 41.3 µg/ml, respectively.

DPPH is a radical, purple in colour, which is reduced to the yellow coloured diphenylpicrylhydrazine by plant extracts. This antioxidant assay is based on the reduction of alcoholic DPPH solution in the presence of hydrogen-donating antioxidant due to the formation of the non-radical form, DPPH-H (Sunil and Ignacimuthu, 2011).

Out of seven Australian aboriginal plants tested (Figure 3), extract AL showed an IC_{50} value of 6.98 µg/ml, whereas extracts AK, SL and ED had IC_{50} in the range of 14 - 15 µg/ml. Extracts BL and AT showed quite high IC_{50} as compared to other Australian plant extracts tested in this study.

Out of the 19 Indian plant extracts tested, only BM (14.95 µg/ml), AP (14.95 µg/ml) and TC (29.49 µg/ml) showed an IC_{50} value less than the control, BHT. For ease of comparison, the Indian plants were divided into three categories as based on IC_{50} values; (a) IC_{50} less than 200 µg/ml, (b) IC_{50} between 200 - 400 µg/ml and (c) IC_{50} greater than 400 µg/ml (Figure 4). The majority of the tested Indian plants had IC_{50} values greater than 200 µg/ml.
Figure 3 - DPPH radical scavenging IC$_{50}$ of Australian Aboriginal plant extracts. Each value represents the mean ± SEM of triplicates experiments. No significant difference between tested conditions P > 0.05, one-way ANOVA.
Figure 4 - DPPH radical scavenging IC$_{50}$ of Indian Ayurvedic plant extracts.

a) IC$_{50}$ value less than 200 µg/ml, b) IC$_{50}$ between 200 - 400 µg/ml and c) IC$_{50}$ more than 400 µg/ml. Each value represents the mean ± SEM of triplicates experiments. No significant difference between tested conditions P > 0.05, one-way ANOVA.
3.4.3. 2, 2′-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS) radical inhibition

The free radical scavenging capacity of plant extracts was also studied using the ABTS radical cation decolorization assay, which is based on the reduction of ABTS$^{+·}$ radical by antioxidants of the plant extracts tested. ABTS is a stable free radical, bluish green in colour. Two standard antioxidants (ascorbic acid and BHT) were used to compare the antioxidant potential. Of the seven Australian Aboriginal plants tested, AK showed significantly lower IC$_{50}$ as compared to BHT (Figure 5). AK, which had shown IC$_{50}$ of 14.07 µg/ml in the DPPH assay, showed IC$_{50}$ value of 8.86 µg/ml in this assay. AL, SL and AT showed ten, five and three fold rise, respectively in their IC$_{50}$ values of DPPH assay, whereas the remaining plants showed similar IC$_{50}$ as obtained in the DPPH assay.

IC$_{50}$ values (Figure 5) for positive controls, ascorbic acid and BHT, were found to be 30.20 and 88.24 µg/ml, respectively. Out of seven Australian Aboriginal plant extracts screened for ABTS radical scavenging activity, AK showed significantly lower IC$_{50}$ as compared to BHT. With the exception of SL and AT, the remainder of the plant extracts tested showed IC$_{50}$ value equal or less than the positive controls.

None of the Indian plant extract tested showed significant activity in comparison to selected standards. The positive outcome from the ABTS assay was that findings were consistent with IC$_{50}$ obtained by DPPH assay. Indian plants were divided into three categories a) IC$_{50}$ less than 200 mg/ml b) IC$_{50}$ between 200 to 400 mg/ml and c) IC$_{50}$ greater than 400 mg/ml.
Figure 5 - ABTS radical scavenging IC$_{50}$ of ethanolic extracts of Australian Aboriginal plants.

Each value represents the mean ± SEM of triplicates experiments.
Figure 6  - ABTS radical scavenging IC50 of ethanolic extracts of Indian Ayurvedic plants.

a) IC\textsubscript{50} values less than 200 µg/ml, b) IC\textsubscript{50} between 200 - 400 µg/ml and c) IC\textsubscript{50} more than 400 µg/ml. Each value represents the mean ± SEM of triplicates experiments. No significant difference between tested conditions P > 0.05, one-way ANOVA.

3.4.4. Ferric-reducing antioxidant power assay (FRAP)

Antioxidant activities of these plant extracts were assessed through their ability to reduce the Fe\textsuperscript{3+}/ferricyanide complex to the ferrous (Fe\textsuperscript{2+}) form. The ferrous ion was monitored by measuring the formation of Perl’s Prussian blue at 630 nm (Moyo et al., 2010). The reducing power of all the plant extracts, ascorbic acid and BHT increased with increasing concentration.

The reducing power of BHT was significantly more pronounced relative to all the Australian Aboriginal plant extracts and ascorbic acid (Figure 7)
However, the antioxidant potencies of ascorbic acid and plant extracts were comparable. AK and ED plant extracts had similar activity to BHT, thus suggesting that they have similar capacity to act as electron donors, indicating their potential to react with free radicals which they can convert to more stable products.

The phenolic content of AK and ED was 10-fold higher than that of BL extract, and a similar trend was observed in the ferric-reducing antioxidant power assay. For example, an absorbance reading of 0.6 corresponded to a dose level of approximately 100 mg/ml for AK, ED and 1000 mg/ml for BL. Thus, to achieve a similar extent of reducing power as AK and ED extracts, a 10-fold dose level is required for BL extract.

Many previous studies have reported a direct relationship between reducing power effects of plant extracts and the content of phenolic compounds and it has been postulated that high total phenol content represents the primary source of antioxidant activity (Moyo et al., 2010). Due to the high total phenolic content of PM and AS extracts, it is postulated that they represent the primary source of this antioxidant activity. The rest of extracts tested did show dose-dependent activity but not as high as BHT and ascorbic acid which could be attributed to moderate phenolic content. Overall, it was seen that most of the extracts possessed promising antioxidant activity (Figure 8).
Figure 7 - Ferric ion-reducing power effects of Australian Aboriginal plant extracts.
Figure 8 - Ferric ion-reducing power effects of Indian Ayurvedic plant extracts.

3.5. Discussion

Plants which contain high levels of phenolics are good source of antioxidants and therefore it is important to quantify the total phenolics and total flavonoids in plant extracts as they might have some beneficial effects on health (Gorinstein et al., 2004). Gallic acid and hydroxycinnamic acids are phenolic acids commonly found in plants. Phenolic acids are known to be chain-terminating antioxidant agents contributing the scavenging activity due to their hydrogen-donating capacity. Their antioxidant activity depends on the number and position of hydroxyl groups in the molecule and is also affected by glycosylation of aglycones and other groups (-NH and -SH). Previous studies have also found that there is a direct relationship between antioxidant activity and total phenolic content (Cai et al., 2004).

There is considerable evidence suggesting that plant flavonoids may be health promoting and disease-preventing dietary compounds. Of current interest is the presence of potent antioxidant flavonoids in red wine, the cause of the so-called French paradox. Despite an intake of a high-fat diet, the low incidence of coronary heart disease among the
French people has been attributed to the consumption of red wine with meals. Flavonoids have been shown in both in vitro and in vivo experimental systems over several decades to possess anti-allergic, anti-inflammatory, antiviral and anticancer properties (Leopoldini et al., 2011).

The current study has revealed that a wide range of total antioxidant activities and associated phenolic content exist among the 26 plant extracts assayed. A highly significant, positive correlation was found between antioxidant capacity and phenolic content, indicating that phenolic compounds are a major contributor to antioxidant activity in the medicinal plants. The medicinal plants with the strongest antioxidant capacity were AK, SL, BM, AP, TC, VW, PM and AS.

The Australian Aboriginal plant extracts showed very good potential for antioxidant activity and, to our knowledge; no data are currently available on antioxidant studies with these plants. However, the family Acaciaceae and other species of Acacia have been shown various pharmacological properties and also contain very high levels of carotenoids, tocopherols and sterols (Nasri et al., 2012).

Previous studies also evaluated the protective effect of Bacopa monnieri on the tissue antioxidant defense system and lipid peroxidative status in streptozotocin-induced diabetic rats. In their study, an aqueous ethanolic extract of BM was administered orally at doses 50, 125 and 250 mg/kg (body weight) once a day for 15 days to diabetic rats. Significant increase in activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) were observed along with increased levels of glutathione (GSH). The study showed significant reversal of disturbed antioxidant status and peroxidative damage which indicated that extract of BM modulates antioxidant activity and enhances the defense against ROS generated damage in diabetic rats (Kapoor et al., 2009) which supports the present study in revealing its potential to play a crucial role in defense against free radical damage.

Arabinogalactan polysaccharides (G1-4A) and (1, 4)-alpha-d-glucans extracted from the stems of Tinospora cordifolia have been shown to induce tolerance against endotoxic shock by modulating activity of cytokines and activation of macrophages. Thus, polysaccharides of the same have been shown to provide effective protection against lipid
peroxidation and metastasis. The other compounds having immunomodulatory properties are cordioside, cordiofolioside A and cordiol (Krishna et al., 2009) which might also be responsible to show antioxidant activity in the present study.

TC has been shown to be effective in killing cancer cells (cultured HeLa cells) \textit{in vitro} and inhibiting skin carcinogenesis in mice and experimental metastasis. It may offer an alternative treatment strategy for cancer in combination with gamma radiation. TC has also been reported to possess heptoprotective activity and reduces the damage induced by radiations to liver cells (Upadhyay et al., 2010).

\textit{Valeriana officinalis} has been used as a sedative for the treatment of insomnia and restlessness throughout the world since the second century and many clinical trials have reported that valerian extract decreases sleep latency, improves sleep quality and hence is useful in treating insomnia (Stevinson and Ernst, 2000). The other uses which have been reported for this plant are anxiolytic, antimicrobial, antispasmodic, analgesic, anti-inflammatory, antidepressant and neuroprotective (Chopra et al., 2010).

However, very limited data are available on \textit{Valeriana}'s antioxidant potential, even though insomnia may also occur due to oxidative stress and anxiety; therefore, it can be hypothesised that this plant exerts its pharmacological effects in treatment of insomnia via modulation of oxidative stress because of its good antioxidant potential. Furthermore, alcoholic extracts of valerian roots have been considered promising pharmacological agents against lipid peroxidation (LPO) in previous studies (Sudati et al., 2009). \textit{In vitro} models have shown PM, AP and AS to have potent antioxidant activity, presumably linked to the phenolics and flavonoids in these plants (Maruthupandian and Mohan, 2011, Banerjee et al., 2003, Akbar, 2011) which also supports the present study that demonstrated good antioxidant potential.

Many studies have revealed that intake of natural antioxidants is correlated with low incidence of cancer, heart disease, diabetes, and other diseases associated with ageing. Phenolics may inhibit carcinogenesis by affecting the molecular events in the initiation, promotion, and progression stages of free radical chain reaction (Cai et al., 2004).
It has been observed flavonols and flavones containing a catechol group in ring B are found to be highly active, with flavonols more potent than the corresponding flavones because of the presence of the 3-hydroxyl group. An additional hydroxyl group in ring B (pyrogallol group) seems to further enhance the antioxidant capacity, as exemplified by myricetin (Leopoldini et al., 2011).

3.6. **Conclusions**

In summary, this study has revealed that many of the 26 plant extracts investigated demonstrated promising antioxidant activity. This activity correlated with the presence of phenolic compounds which are known to contribute to antioxidant activity. Systematic evaluation of a large number of plants is useful for understanding their functionality and chemical constituents, and also supports the view that they can be potential sources of potent natural antioxidants. Among Australian Aboriginal plants AK, BL, ED and SS whereas, only AP and BM among Indian Ayurvedic plants were found to possess very good free radical scavenging potential as they have shown IC$_{50}$ less than 50 µg/ml against ABTS and DPPH free radicals. The plants can be further exploited and utilized for drug discovery.
Chapter 4. Inhibition of enzymes relevant to the control of hyperglycemia and other diabetes-related conditions
4.1. Abstract

The work described in this chapter addressed the hypothesis that some Indian Ayurvedic plants commonly used in the management of diabetes can reduce glucose by inhibiting key carbohydrate hydrolyzing enzymes. Extracts of these and Australian Aboriginal plants were evaluated for α-amylase, α-glucosidase and angiotensin converting enzyme inhibitory effects. Among the Australian Aboriginal plants, *Santalum spicatum* extract was found to inhibit α-amylase with IC₅₀ 5.43 µg/ml. Extracts of *Chlorophytum borivilianum*, *Plumbago zeylenica*, *Solanum nigrum* and *Pterocarpus marsupium* were found to inhibit α-amylase activity with IC₅₀ values of 4.17, 4.36, 4.87 and 5.16 µg/ml respectively whereas, the positive control (acarbose) showed IC₅₀ of 7.81 µg/ml. The Australian Aboriginal plants significantly inhibited (p < 0.001) α-glucosidase enzyme as compared to acarbose with IC₅₀ of 0.48 µg/ml for *Beyeria leshnaulti* and the other plants also exhibited significant IC₅₀ (p < 0.001) in the range of 1 to 1.83 µg/ml. Among the Indian Ayurvedic plants, *Chlorophytum borivilianum* and *Mucuna pruriens* showed significant inhibition (p < 0.001) with IC₅₀ of 0.58 and 0.80 µg/ml, respectively. However, all tested plant extracts showed very high IC₅₀ as compared to Captopril against angiotensin converting enzyme. These findings provide evidence for the hypoglycemic action of these plants and indicate that they may represent alternative therapies in the management of diabetes. The Australian Aboriginal plant extracts were found to be more potent in comparison to Indian Ayurvedic plant extracts. This is the first study of the enzyme inhibition potential of Australian Aboriginal plant extracts in the context of management of type 2 diabetes and the related condition of hypertension.
4.2. **Introduction**

Medicinal plants are not only used for the management of mild diseases such as the common cold, headaches or gastrointestinal ailments but also in the long-term treatment of chronic or incurable diseases, such as diabetes, hypertension or cancer. The antihyperglycemic activity of plants is due to their ability to restore pancreatic tissue function causing an increase in insulin output or inhibition of intestinal glucose absorption (Patel et al., 2012). Diabetes mellitus is a multi-factorial disease. It is an endocrine and metabolic disorder characterized by chronic hyperglycemia. There is a need to develop strategies for the management of diabetes to decrease its incidence and deaths from related complications. One therapeutic approach for management of type 2 diabetes is to reduce glucose absorption by inhibiting the enzymes α-amylase and α-glucosidase which are involved in degradation of starch. Various berries have been investigated for their potential use by inhibiting starch-degrading enzymes, consumption of soft fruits, Brazilian native fruits, strawberries and raspberries have been proved effective in managing type 2 diabetes. Natural sources of inhibitors of α-amylase and α-glucosidase would be beneficial to avoid potential gastrointestinal side-effects caused by commercial α-glucosidase inhibitors (Johnson et al., 2011).

Carbohydrates constitute the major part of the human diet and play a main role in the energy supply. The complex components of dietary carbohydrates must be broken down to monosaccharides since only the monomers can be absorbed from the intestinal lumen and transported into the blood. Inhibition of α-amylase and α-glucosidase can considerably prolong the overall carbohydrate digestion time and decrease the postprandial increase of blood glucose level after a mixed carbohydrate diet. This can therefore be an important strategy in the management of postprandial blood glucose levels linked to type 2 diabetes (Nickavar and Yousefian, 2011). Thus, the retardation of the α-glucosidase and α-amylase enzymes by inhibitors might be one of the most effective approaches to control type 2 diabetes. At present, the commercial enzyme inhibitors acarbose and voglibose are used either alone or in combination with insulin to control post-prandial hyperglycaemia.
The modern medicines currently available for management of diabetes exert serious side effects such as hepatotoxicity, abdominal pain, flatulence, diarrhoea and hypoglycaemia. Traditional herbal medicines have been used throughout the world for a range of diabetes and related complications. The search for improved and safe natural antidiabetic agents is underway, and the World Health Organization has also recommended the development of herbal medicines in this context (Ghosh et al., 2011).

It is important to note that there are no reports about the antidiabetic activity of Australian Aboriginal plants. Similarly, no research has been carried out to identify the targets that can confirm and support the antidiabetic potential of these plants. Hence, this investigation will evaluate the inhibitory effect of various extracts on commercially available porcine pancreatic \( \alpha \)-amylase and glucosidase. Furthermore, the \textit{in vitro} antidiabetic potential of the active plant extracts was determined in 3T3L1 cells line as described in the next chapter (Chapter V).

\section*{4.2.1. Chapter Aims}

The study aimed to evaluate the effect of ethanolic extracts of 26 plants against specific enzymes using \textit{in vitro} assays.

- Preliminary \( \alpha \)-amylase enzyme inhibition assay by disc diffusion method
- \( \alpha \)-amylase enzyme inhibition using spectrophotometric assay
- \( \alpha \)-glucosidase enzyme inhibition using spectrophotometric assay
- Angiotensin converting enzyme inhibition using High performance liquid chromatography (HPLC)

\section*{4.3. Materials and methods:}

The plant materials used in this study have been described in Table 3 and Table 4 in chapter 2 (page 34-35).

All chemicals were purchased from Sigma-Aldrich (Australia) as follows: porcine pancreatic \( \alpha \)-amylase (PPA) (EC 3.2.1.1) type VI B, 3, 5-dinitro salicylic acid reagent (DNS), acarbose, yeast \( \alpha \)-glucosidase (EC – 2328898), \( p \)-nitrophenyl \( \alpha \)-D-
glucopyranoside (p-NPG), rabbit lung (ACE-I) angiotensin converting enzyme (EC – 3.4.15.1), hippuryl-L-histidyl-L-leucine (HHL), hippuric acid and captopril.

4.3.1. Alpha-amylase enzyme inhibition

The α-amylase inhibitory assay was modified from Correia et al. (2004a). Twenty µl of porcine pancreatic α-amylase solution (EC 3.2.1.1; equivalent to 3000 U in 50 mM phosphate buffer, pH 6.9) were mixed with 15 µl of plant extract and incubated at 37 ºC for 45 minutes. After incubation, the plant - enzyme mixture was applied to a sterile paper disc and placed onto the centre of petri plates containing medium consisting of 1% (w/v) agar and 1% (w/v) starch in distilled water. Plates were allowed to stand for 3 days at 25 ºC then stained with iodine and allowed to stand for 15 min. The diameter of the clear zone was measured and used to calculate the amylase inhibitory activity. As a control, the enzyme was mixed with the solvent in which the plants were extracted (ethanol) and applied onto the sterile disc. Results were expressed as:

\[
\text{Percentage inhibition} = \left( \frac{\text{diameter of control} - \text{diameter of samples}}{\text{diameter of the control}} \right) \times 100
\]

4.3.2. Alpha-amylase enzyme inhibition by quantitative starch hydrolysis

The α-amylase inhibitory activity was determined (Kwon et al., 2008a) using porcine pancreatic α-amylase (EC 3.2.1.1) type VI B. To 125 µl of plant extracts at different concentrations (range 1.56 to 500 µg/ml), α-amylase solution (0.5 mg/ml in 0.02M sodium phosphate buffer) was mixed and the reaction mixture was pre-incubated for 10 minutes at room temperature. After pre-incubation, 25 µl of 1% starch solution was added every 5 seconds for a total of 125 µl. The reaction mixture was again incubated for 10 minutes at room temperature. The reaction was terminated by adding 250 µl of 3, 5-dinitro salicylic acid reagent. The tubes were placed in a boiling water bath for 5 minutes and then cooled at room temperature. The reaction mixture was diluted by adding 5000 µl of distilled water. The generation of maltose was quantified by measuring the absorbance at 540 nm of 3-amino-5-nitrosalicylic acid (from reduction of 3, 5-
dinitrosalicylic acid (Loizzo et al., 2008) using a UV-visible spectrophotometer. The control was buffer treated in the same way as plant samples. The standard used was acarbose (concentration range 1.56 to 500 µg/ml). Results were expressed as:

Percentage inhibition

\[
\text{Percentage inhibition} = \left( \frac{\text{absorbance of control at } 540 \text{ nm} - \text{absorbance of samples at } 540 \text{ nm}}{\text{absorbance of the control at } 540 \text{ nm}} \right) \times 100
\]

4.3.3. Alpha-glucosidase enzyme inhibition

The α-glucosidase inhibition assay was modified from Apostolidis et al. (2007) using yeast α-glucosidase (EC – 2328898). A volume of 25 µl of plant extract (range 0.35 to 100 µg/ml) was mixed with 50 µl of α-glucosidase enzyme (0.1 U/ml in 0.1 M potassium phosphate buffer solution, pH 6.9) in 96 well plates and incubated at 37 ºC for 30 minutes. After pre-incubation, 25 µl of 5 mM pNPG in 0.1M phosphate buffer were added to each well and the reaction mixture was incubated again at 37 ºC for 30 minutes. Thirty µl of 0.1 M sodium carbonate solution were added to the above reaction mixture and incubated again for 20 minutes at 37 ºC. Before and after incubation, the absorbance was measured at 405 nm and compared to the control that contained 25 µl of buffer solution instead of plant extract. The standard used was acarbose (concentration range 0.35 to 100 µg/ml). The α-glucosidase activity was determined by measuring release of p-nitrophenol from p-nitrophenyl α-D-glucopyranoside (Hogan et al., 2010).
The α-glucosidase inhibitory activity was expressed as:

Percentage inhibition

\[
\text{Percentage inhibition} = \left( \frac{\text{absorbance of control at } 405 \text{ nm} - \text{absorbance of samples at } 405 \text{ nm}}{\text{absorbance of the control at } 405 \text{ nm}} \right) \times 100
\]

4.3.4. Angiotensin converting enzyme inhibition

The angiotensin converting enzyme (ACE) inhibition activity was determined (PEGG et al., 2007) using rabbit lung angiotensin converting enzyme (EC – 3.4.15.1). A volume of 100 µl of plant extract (range 50 to 1000 µg/ml) was mixed with 100 µl of ACE (0.25 U/ml in 0.05 M sodium borate buffer solution, pH 8.2) and 100 µl of 5 mM hippuryl-L-histidyl-L-leucine (HHL) and incubated at 37 °C for 30 minutes without mixing. After pre-incubation, the reaction was removed from the incubator, mixed and again incubated at 37 °C for 30 minutes. The reaction was terminated by adding 300 µl of 1 M hydrochloric acid. The liberated hippuric acid was detected and quantified by UHPLC (Shimadzu). The negative control used was buffer solution instead of plant extract. The standard used was the commercially available ACE inhibitor, captopril. Ten µl of sample was injected by auto sampler. The calibration curve of hippuric acid was constructed using 0.5, 0.4, 0.3, 0.2 and 0.1 mmol/l solutions made from a 2mmol/l stock solution. An isocratic method was used to detect the hippuric acid peak. The mobile phase used was 6% acetonitrile in 0.025 M KH₂PO₄ (pH 3) with a flow rate 0.6 ml/min. The column used was a ResteK Ultra II® C18 1.9 micron, 100 X 2.1 mm (Catalog No. 9604212).

The ACE inhibitory activity was expressed as:

\[
\text{Percentage inhibition} = \left( \frac{\text{peak area of control} - \text{peak area of samples}}{\text{peak area of the control}} \right) \times 100
\]
4.3.5. Statistical analysis

All samples were analyzed in triplicates. Data are presented as mean ± standard error mean (SEM). Differences were evaluated by one-way analysis of variance (ANOVA) test completed by a Bonferroni’s multicomparison test. Differences were considered significant at \( p < 0.001 \). The concentration giving 50% inhibition (IC$_{50}$) was calculated by non-linear regression with the use of GraphPad Prism Version 5.0 for Windows (GraphPad Software, San Diego, CA, USA) (www.graphpad.com).
4.4. Results

4.4.1. Alpha-amylase enzyme inhibition by disc diffusion

![Image of disc diffusion assay](image)

Figure 9 - Disc diffusion assay of α-amylase for starch hydrolysis by plant extracts.
Where 9 a) is the negative control i.e. ethanol mixed with α-amylase enzyme where the starch was hydrolysed as is evident after staining with iodine solution. Fig. 9 b) shows complete inhibition of α-amylase i.e. the starch was not hydrolysed by the plant extract. Fig. 9 c) shows the partial inhibition of α-amylase enzyme by the plant extract.
The percentage inhibition of $\alpha$-amylase by plant extracts is shown in Table 10 and Table 11 for Australian Aboriginal and Indian Ayurvedic plant extracts, respectively. All of the Australian Aboriginal plant extracts showed complete inhibition of $\alpha$-amylase such that no hydrolysis of starch was evident.

<table>
<thead>
<tr>
<th>Plant name</th>
<th>Concentration (mg/ml)</th>
<th>Percent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AK</td>
<td>321</td>
<td>100</td>
</tr>
<tr>
<td>AT</td>
<td>43</td>
<td>100</td>
</tr>
<tr>
<td>AL</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>BL</td>
<td>242</td>
<td>100</td>
</tr>
<tr>
<td>ED</td>
<td>188</td>
<td>100</td>
</tr>
<tr>
<td>SL</td>
<td>326</td>
<td>100</td>
</tr>
<tr>
<td>SS</td>
<td>120</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 10 - Inhibition of $\alpha$-amylase by Australian Aboriginal plant extracts.

The Australian plant extracts were received in random concentrations and used as such for this disk diffusion assay since this was the qualitative assay.
<table>
<thead>
<tr>
<th>Plant name</th>
<th>Percent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>EJ</td>
<td>100</td>
</tr>
<tr>
<td>CO</td>
<td>100</td>
</tr>
<tr>
<td>VW</td>
<td>57.8</td>
</tr>
<tr>
<td>CP</td>
<td>55.6</td>
</tr>
<tr>
<td>PM</td>
<td>51.42</td>
</tr>
<tr>
<td>MP</td>
<td>51.42</td>
</tr>
<tr>
<td>CM</td>
<td>51.42</td>
</tr>
<tr>
<td>BD</td>
<td>48.57</td>
</tr>
<tr>
<td>NJ</td>
<td>45.71</td>
</tr>
<tr>
<td>AP</td>
<td>45.71</td>
</tr>
<tr>
<td>SN</td>
<td>42.85</td>
</tr>
<tr>
<td>TC</td>
<td>42.85</td>
</tr>
<tr>
<td>CB</td>
<td>35.45</td>
</tr>
<tr>
<td>PZ</td>
<td>28.57</td>
</tr>
<tr>
<td>BS</td>
<td>28.57</td>
</tr>
<tr>
<td>AS</td>
<td>27.28</td>
</tr>
<tr>
<td>PT</td>
<td>22.85</td>
</tr>
<tr>
<td>Ana.</td>
<td>20</td>
</tr>
<tr>
<td>BM</td>
<td>14.28</td>
</tr>
</tbody>
</table>

Table 11 - Inhibition of $\alpha$-amylase by Indian Ayurvedic plant extracts.
Among the Ayurvedic Indian plant extracts, which were all tested at 250 mg/ml, only EJ and CO showed complete inhibition and considered to be highly potent.

**Extracts with 50 – 75 % inhibitory effect**

In this study, the ethanolic extracts of VW, CP, CM, MP and PM extracts showed partial inhibition. The plants which showed percentage inhibition in range of 50-75 % are considered to be moderately potent.

**Extracts with 25 – 50 % inhibitory effect**

The extracts of BD, NJ, AP, SN, TC, CB, PZ, BS and AS showed amylase inhibitory activity of 25 - 50 % and are thus considered to have lower potency.

**Extracts with less than 25 % inhibitory effect**

PT, Ana. and BM showed percentage inhibition of 22.85%, 20% and 14.28%, respectively, among the 26 plant extracts screened. These plant extracts are considered to have minimal inhibitory effect on the amylase enzyme (Ahmad Gholamhoseinian, 2008).

While the majority of the extracts demonstrated potent amylase inhibiting activity, some of the Australian plant extracts were particularly active (AL and AT) and showed activity at concentrations lower than those of the other extracts.

**4.4.2. Alpha-amylase activity determined by spectrophotometric assay**

Much research has focused on glucosidase inhibitors to control hyperglycemia, but many forms of starch are also digested as rapidly as glucose absorption (Ghavami et al., 2001). Slowing the digestion and breakdown of starch may have beneficial effects on insulin resistance and glycemic index control in people with diabetes. Of the seven Australian Aboriginal plants screened for inhibitory activity against α- amylase (Figure 10), no significant differences were observed compared to acarbose. However, the plant extract SS showed a particularly low IC₅₀ value of 5.43 µg/ml while AL, BL and SL showed IC₅₀ values of 11.52, 13.27 and 13.41 µg/ml respectively, which compare favourably with acarbose.
Figure 10 - Inhibitory activity of Australian Aboriginal plants against α- amylase.
Each value represents the mean ± SEM of triplicate experiments. No significant difference between tested conditions $p > 0.05$, one-way ANOVA.

Most of the herbs used in traditional Indian medicine for the treatment of diabetes mellitus are known to contain rosmarinic acid and quercetin. Therefore, the health benefits of these herbs against diabetes mellitus may be due to the amylase-inhibiting activity of the phenolic compounds (McCue and Shetty, 2004). Hence, 19 Indian Ayurvedic plant extracts were screened for α-amylase inhibitory activity (Figure 11). Five of the selected plants showed an $IC_{50}$ value less than 10 µg/ml, of which extracts CB, PZ, SN, PM and BS showed $IC_{50}$ value of 4.17, 4.36, 4.87, 5.16 and 7.25 µg/ml respectively (Figure 11 a).
4.4.3. Glucosidase inhibition assay

Glucosidases of the intestine are known to play key roles in carbohydrate digestion and inhibitors against these enzymes will be effective in retarding glucose absorption to suppress post prandial hyperglycemia (PPHG). Thus working on the same principle, the seven Australian Aboriginal and 19 Indian Ayurvedic plants were investigated for their inhibitory potential of glucosidase enzyme. From Figure 12, it can be observed that all of the selected Australian plants showed significantly lower IC$_{50}$ as compared to acarbose. BL extract showed an IC$_{50}$ of 0.48 µg/ml whereas the rest of the plants showed values of between 1 to 2 µg/ml.

Out of the 19 Indian Ayurvedic plants tested, extracts CB and MP showed IC$_{50}$ of 0.58 and 0.80 µg/ml, respectively, whereas PM, PZ, BD and TC showed IC$_{50}$ values of 1.06, 1.16, 1.72 and 1.86 µg/ml respectively (Figure 13). Figure 13 b shows that plant extract
SN, EJ, BS, AP, PT, NJ and BM showed similar IC\textsubscript{50} to acarbose and highest IC\textsubscript{50} against glucosidase enzyme was shown by CO at 10 µg/ml.

![Figure 12 - Inhibitory activity of Australian Aboriginal plants against α-glucosidase.](image)

Each value represents the mean ± SEM of triplicate experiments. **** p < 0.001 indicate a significant difference between acarbose and plant extracts, one-way ANOVA post-hoc Bonferroni’s multiple comparison test.
Figure 13 - Inhibitory activity of Indian Ayurvedic plants against α-glucosidase.
a) IC$_{50}$ less than 2 µg/ml b) IC$_{50}$ between 2 – 6 µg/ml c) IC$_{50}$ greater than 6 µg/ml. Each value represents the mean ± SEM of triplicate experiments. **** p < 0.001 indicate a significant difference between acarbose and plant extracts, one-way ANOVA post-hoc Bonferroni’s multiple comparison test.

4.4.4. ACE inhibition assay

Angiotensin converting enzyme (ACE) is a key component in the renin angiotensin aldosterone system (RAAS) which regulates blood pressure. As the over expression of RAAS is associated with vascular hypertension, ACE inhibition has become a major target for the control of hypertension. New alternatives have been explored extensively as replacements of current therapeutic drugs to minimise the side effects of dizziness, coughing and angioneuretic edema related to these drugs. Most of the research have been targeted at bioactive compounds from natural resources such as peptides, anthocyanins, flavonols and triterpenes (Balasuriya and Rupasinghe, 2011).

The objective of this experiment was to assess whether the selected plant extracts have the potential to be used as ACE inhibitors. Of the seven Australian Aboriginal plants
screened for ACE inhibitory potential, none of extracts showed significant activity as compared to the commercially available ACE inhibitor, captopril (Figure 14). Captopril showed an IC$_{50}$ value of 12.31 µg/ml and plant extracts AK, AL and ED showed five times the IC$_{50}$ of this positive control. The remaining plant extracts showed nearly ten-fold higher IC$_{50}$ values compared to captopril. Flavonoids are the largest group of polyphenolic compounds found in higher plants. Thus, working on the same hypothesis, 19 Indian Ayurvedic plants (Figure 15) were investigated for potential in management of hypertension by inhibiting ACE. Of the 19 plant extracts screened, only seven showed IC$_{50}$ value less than 200 µg/ml but none of them were as effective as captopril.

![Figure 14 - Inhibitory activity of Australian Aboriginal plants against ACE.](image)

Each value represents the mean ± SEM of triplicate experiments. No significant difference between tested conditions p > 0.05, one-way ANOVA.
Figure 15 - Inhibitory activity of Indian Ayurvedic plants against ACE.
Plants were divided into three categories based on their IC\textsubscript{50} values: (a) IC\textsubscript{50} value less than 200 µg/ml; (b) IC\textsubscript{50} value between 200 – 400 µg/ml; (c) IC\textsubscript{50} value greater than 400 µg; (c) IC\textsubscript{50} value greater than 400 µg/ml. Each value represents the mean ± SEM of triplicate experiments. No significant difference between tested conditions p > 0.05, one-way ANOVA.

4.5. **Discussion**

In the present study, medicinal plant extracts were investigated for their possible application in the control of type 2 diabetes and hypertension. Specifically, the extracts were tested for their ability to inhibit the enzymes amylase, glucosidase and angiotensin converting enzyme - I. This investigation is a novel approach to identify the agents involved in lowering the glycaemic index and controlling of post-prandial hyperglycemia. In this study, the detailed biochemical basis of the potential antidiabetic activity was presented which involved assessing the inhibition of enzymes involved in carbohydrate
metabolism, which are the key players in the development of type 2 diabetes, and an enzyme related to the associated condition of hypertension.

The data obtained showed that the different plant extracts exhibited variable inhibitory effects on α-amylase activity in vitro (Figures 10 and 11). In this study, SS showed an IC$_{50}$ of 5.43 μg/ml. This plant bears sweet fruits which are eaten fresh and the decoction of the inner bark is taken orally to relieve cough (Anonymous, 1988). The other Australian Aboriginal plant extracts tested here (AL, BL and SL) were found to inhibit α-amylase with an IC$_{50}$ less than 15 μg/ml. Seeds and gums of Acacia species are edible and, as this plant grows in harsh environments, it is commonly known as “dead finish”. Many of the Australian Aboriginal plants screened here have been used as either food or food supplements, suggesting that they are safe to be consumed. (Anonymous, 1988). The natural occurring compounds in the plants such as flavonoids, alkaloids, triterpenoids, saponins, steroidal glycosides, amino acids have been reported to possess hypo-glycaemic action (Narender et al., 2011). BL contains several di- and triterpenoids (Baddeley et al., 1964) which might have contributed towards inhibition of α-glucosidase (IC$_{50}$ of 0.48 μg/ml).

Among the Indian Ayurvedic plant extracts, CB was a good inhibitor against porcine pancreatic amylase as well as glucosidase with IC$_{50}$ of 4.17 μg/ml and 0.58 μg/ml (Figure 11 a and 13 a), respectively. Previous studies detected the antidiabetic activity of aqueous extracts of Chlorophytum borivilianum roots in streptozotocin (STZ)-induced hyperglycemia in rats (Mujeeb, 2009). Among all the species of Chlorophytum present in India, CB produces the highest yield of roots along with the highest saponin content. Several steroidal sapogenins and glycosides have been isolated with various pharmacological actions (Kaushik, 2005). These possess great aphrodisiac and adaptogenic actions and are hence considered as alternatives to Viagra (Thakur et al., 2009).

MP inhibited glucosidase with IC$_{50}$ 0.80 μg/ml whereas it showed IC$_{50}$ of 25.10 μg/ml against α-amylase. The powder of MP seeds is a rich source of dietary fibre and contains both organic and inorganic minerals. The aqueous extract is reported to significantly reduce blood glucose in STZ-induced diabetic rats after 21 days of daily oral administration (Bhaskar et al., 2008). The current study also indicated its potential in in-
hibitation of carbohydrate hydrolyzing enzymes. It also contains L-dopa and hence can be used in management of Parkinson’s diseases. The clinical efficacy of the plant was assessed in a double-blind randomised clinical trial and was compared with standard L-dopa/carbidopa (LD/CD). The rapid onset of action demonstrated in the study suggested that this natural source of L-dopa might possess advantages over conventional L-dopa preparations in the long-term management of Parkinson’s disease (Katzenschlager et al., 2004b).

PM is one of the important widely used plants in traditional Indian Ayurvedic medicine for the treatment of diabetes mellitus (Maruthupandian and Mohan, 2011). The heart wood of *Pterocarpus* has been used for many years to treat diabetes. Traditionally, people would soak water overnight in mugs made up of heart wood and drink the same water (known as Beeja wood water) next morning to manage diabetes. The presence of pterostilbene, marsupin and other phenolics known to be present in PM might be contributing to the hypoglycaemic effect of the plant (Devgun et al., 2010) which might be the reason of inhibition of amylase and glucosidase in our study with IC\(_{50}\) 5.16 and 1.06 \(\mu\)g/ml respectively.

PZ possessed amylase inhibition at IC\(_{50}\) 4.36 \(\mu\)g/ml and glucosidase inhibition with an IC\(_{50}\) of 1.16 \(\mu\)g/ml in our study. It is reported to have hypoglycaemic action by increasing hepatic hexokinase activity and decreasing hepatic glucose-6-phosphatase as previously studied in oral administration of an ethanolic extract (100, 200 mg/kg) in STZ-induced diabetic rats. (Zarmouh et al., 2010).

The findings of the current study indicated that all of the Australian Aboriginal plant extracts showed potent amylase and glucosidase inhibitory activity and one of the mechanisms by which these extracts could exhibit a hypoglycemic effect would be by inhibition of these carbohydrates-hydrolysing enzymes leading to retardation of starch hydrolysis, eventually lowering post-prandial hyperglycemia (PPHG). Since, this is the first study on anti-hyperglycemic activity of these plants, the nature of the phytochemicals responsible for enzyme inhibition and the active principles need to be isolated, characterized and further tested through *in vitro* and *in vivo* studies. The Indian Ayurvedic plant extracts also showed potent amylase and glucosidase inhibition. Though many
of the plants traditionally have been used to treat diabetes, limited information was available on their mechanism of action.

ACE plays an important role in the renin–angiotensin system, which regulates arterial blood pressure as well as salt and water balance. In the cardiovascular system, ACE converts angiotensin I to angiotensin II, a potent vasoconstrictor, and degrades bradykinin, a vasodilator. Angiotensin II also stimulates the synthesis and release of aldosterone, which increases blood pressure by promoting sodium retention in the distal tubules. Therefore, inhibition of ACE by drugs such as captopril and natural ACE inhibitory peptides has been shown to result in an antihypertensive effect. Lifestyle modifications and diet therapy are two of the most important tools for effective lowering of blood pressure. Moreover, even small decreases in blood pressure result in significantly lower risks for cardiovascular diseases. A higher dietary protein intake, a diet rich in fruits, vegetables and low-fat dairy products, is associated with an effective reduction in blood pressure (Vermeirssen et al., 2004). Hypertension is one of the long-term complications of diabetes, therefore inhibition of this enzyme is considered a useful therapeutic approach in the treatment of high blood pressure in both diabetic and non-diabetic patients (Kwon et al., 2007c).

In the current study, none of the plant extracts showed ACE inhibitory activity comparable to captopril. Previous studies have shown that flavonoids possess higher IC$_{50}$ values for ACE when compared with antihypertensive drugs, as most flavonoids are found to be competitive inhibitors of ACE (Balasuriya and Rupasinghe, 2011). Therefore, further studies using animal models are required to confirm their ACE inhibitory properties.

It has been observed in previous studies that enzymatic inhibition is the basis for the management of type 2 diabetes by controlling glucose absorption and decreasing the risk of hypertension. This is due to the presence of high fibre and phenolic content (Kwon et al., 2008a). Plants have a wide array of phytochemicals that are known to produce a large variety of pharmacological activities. In type 2 diabetes, excessive hepatic glycogenolysis and gluconeogenesis are associated with underutilization of glucose by tissues causing hyperglycemia. The glucosidase inhibitors inhibit α-1,6-glucosidase of glycogen-debranching enzymes in the liver and reduce the glycogenolyt-
ic rate which increases the glycogen stores in the liver. Hence, inhibition of these enzymes decreases the blood glucose levels in diabetic patient (as a short-term effect) and shows a small reduction in haemoglobin A1c level (as a long-term effect) (Bhat et al., 2011b).

Plant extracts are known to be potent enzyme inhibitors due to their rich phenolic content that bind to the reactive sites of enzymes, thus altering the catalytic activity. It has been suggested that the mechanism of inhibition of α-amylase may occur through the direct blockage of the active centre at several subsites of the enzyme (Ghosh et al., 2011). A main drawback with acarbose is the development of side effects including abdominal distention and flatulence which might be due excessive inhibition of pancreatic α-amylase resulting in the abnormal bacterial fermentation of undigested carbohydrates in the colon by the anaerobic microbial community. This would increase the concentration of gases and butyrate produced in the colon (Kwon et al., 2007c). These side effects are well-tolerated in comparison to the other oral hypoglycaemic agents that can cause severe hypoglycaemia and weight gain.

A review of the scientific literature shows that a significant contribution has been made regarding α-glucosidase inhibitors like acarbose, miglitol, voglibose and dexynojirimycin (discovered from roots of mulberry trees) (Kuriyama et al., 2008). These were the first drugs developed to more effectively control postprandial glucose compared to sulfonylureas and biguanides which were previously the only available oral antidiabetics. Inhibition of this enzyme can significantly decrease the postprandial increase of blood glucose after a mixed carbohydrate diet and therefore can be an important strategy in the management of hyperglycaemia (Mahomoodally et al., 2012).
4.6. **Conclusions**

Plants have been suggested as a rich, as yet unexplored, source of potentially useful antidiabetic drugs. However, only a few have been subjected to detailed scientific investigation due to a lack of mechanism-based *in vitro* assays. There are several reports on antidiabetic plants in traditional use. However, many of these plants are used for the treatment of diabetes mellitus with no mechanistic basis known. The present investigation showed that the antidiabetic property of some of the plants can be related to their enzyme inhibitory effects. Therefore, they can be considered as potential candidates for further studies aimed at isolating the enzyme inhibitors. These efforts may provide new alternative treatments for diabetes and validate the use of traditional medicinal plants having anti-diabetic activity.
Chapter 5. Assessing the Anti-Diabetic and Anti-Cancer Potential of Plant Extracts using Cell-Based Assays
5.1. **Abstract**

Plant-derived compounds have been used clinically to treat type 2 diabetes for many years a decision often made easier because they can also exert beneficial effects on various other disorders. In the present study, the possible mechanism of anti-diabetic activity of selected Australian Aboriginal and Indian Ayurvedic plant extracts (based on antioxidant and enzyme inhibition assays) was investigated by determining if they could significantly improve glucose uptake in murine 3T3-L1 adipocytes. The plants were also investigated for their role in adipogenesis. Of the seven Australian Aboriginal plant extracts tested, only *Acacia Kempeana* and *Santalum spicatum* stimulated glucose uptake in adipocytes. Among the five Indian Ayurvedic plant extracts, only *Curculigo orchioides* enhanced glucose uptake. With respect to adipogenesis, the Australian plants *Acacia tetragonophylla*, *Beyeria leshnaultii*, *Euphorbia drumondii* and the Indian plants *Pterocarpus marsupium*, *Andrographis paniculata* and *Curculigo orchioides* reduced lipid accumulation in differentiated adipocytes. Cytotoxicity studies were also carried out against two cancerous cell lines, HeLa and A549, to investigate the potential anti-cancer activities of the extracts. Extracts of *Acacia kempeana* and *Acacia tetragonophylla* showed potent and specific activity against HeLa cells suggesting they may be useful candidates for the development of chemotherapeutic agents for the treatment of cervical cancer.
5.2. **Introduction**

Type 2 diabetes has become a major health problem in both developed and developing countries. Readily-available high calorie foods and sedentary lifestyles are major factors for obesity which contribute to insulin resistance and type 2 diabetes. Insulin resistance is defined as defective insulin signalling and decreased insulin efficiency to induce glucose transport from the blood into key target cells (Boden, 1997). Obesity, mainly visceral fat, contributes to insulin resistance (Lazar, 2005). Most antidiabetic drugs promote long-term weight gain. Thus, these drugs treat one of the key symptoms, hyperglycemia, but exacerbate weight gain and obesity which further contributes to the progression of type 2 diabetes. Therefore, while these drugs are beneficial over the short-term, they are not optimal for long-term health of type 2 diabetic patients (Chan et al., 2012). The most desirable situation would be the development of new types of antidiabetic drugs that are either hypoglycaemic or anti-hyperglycemic without the side effect of promoting weight gain (Klein et al., 2007). The activity of numerous plants has been evaluated and confirmed in animal models which suggest that herbal remedies could represent culturally relevant complementary and alternative treatments, as well as serve in the search for new anti-diabetic agents (Baldea et al., 2010). Reducing obesity can slow down the rate of occurrence of type 2 diabetes (Tom Yates, 2011). The side effects associated with currently available therapies discourage correct and complete compliance to medication protocols by patients, with the concomitant progression of diabetes and its associated complications. Therefore, it is highly desirable to find new anti-diabetic agents that stimulate glucose uptake by adipose or muscle cells but, unlike thiazolidinediones or insulin, do not induce obesity or other side effects (Alonso-Castro and Salazar-Olivo, 2008). Obesity and weight gain increase the risk of type 2 diabetes and hypertension which increases the number and size of adipocytes (Kahn and Flier, 2000). The increase in adipocyte lipid content can influence adipocyte function by reducing adiponectin secretion which promotes adipocyte differentiation, insulin sensitivity and lipid accumulation *in vivo* (Yeo et al., 2011a). Low levels of circulating adiponectin have been linked to insulin resistance and an increased risk of diabetes. Secondary plant metabolites such as saponin glycosides, triterpenes and phenolic compounds have been
reported to influence adipocyte differentiation in cultured 3T3-L1 cells, a murine fibroblast cell line that is often used as a model for adipocyte metabolism (Yeo et al., 2011b).

Green and Kehinde (1975) established several cloned lines of mouse 3T3 fibroblasts which are capable of differentiating into adipocyte-like cells \textit{in vitro}. The most frequently employed adipocytes cell lines are 3T3-F442A and 3T3-L1. They were clonally isolated from Swiss 3T3 cells derived from disaggregated 17 to 19-day mouse embryos (Green and Kehinde, 1975). Cell lines have been used as model systems to understand various mechanisms of plants in animal and human health as they provide a continuous source of large numbers of cells necessary for proliferation and differentiation. The 3T3-L1 cell line was selected for this study because it displays relevant features including lipid storage and glucose homeostasis. These murine adipocytes have been used extensively to study the regulation of glucose transporters, cell proliferation and insulin signalling. During differentiation, 3T3-L1 pre-adipocytes become adipocytes with a 20-fold increase in the number of insulin receptors and acquire the ability to utilize glucose in response to insulin (Frost and Lane, 1985).

Many studies have exploited the Sprague-Dawley rat model (SD model) for \textit{in vitro} evaluation of hypoglycemic activity. This is normally time-consuming, restricted to limited animal sources and involves sacrificing of animals. Therefore, the differentiated 3T3-L1 adipocyte model (3T3-L1 model) was developed as an alternative to the SD model and is used by researchers to evaluate hypoglycaemic and anti-adipogenic effects and establish the mechanisms of action. Wu et al. (2011) screened yeast extracts for hypoglycemic activity with the 3T3-L1 model, compared results with the SD model and found that the two models were highly correlated (Wu et al., 2011).

In the search for novel treatments, attention should be given to the many traditional herbal medicines for diabetes which have been employed by various ethnic groups throughout the world. One region which contains a rich flora and fauna is Australia. However, Australian Aboriginal plants have never been screened for their use in diabetes. Therefore, in this work, the well-characterized 3T3-L1 model was used to investigate the role of Aboriginal Australian and selected Indian Ayurvedic plant ethanolic extracts for their anti-diabetic mechanisms and ability to inhibit lipid accumulation.
As all seven Australian Aboriginal plants and particular Indian plants, namely AP, BM and PM, showed good *in vitro* radical scavenging activity, the anti-cancer activity of these extracts against cervical carcinoma HeLa cells and lung adenocarcinoma A549 cells was also investigated.

### 5.2.1. Chapter Aims

The overall aim of this study was to further evaluate the anti-diabetic mechanisms of ethanolic extracts of 12 traditional medicinal plants. This was achieved by:

1. Evaluating glucose uptake in 3T3-L1 mouse pre-adipocytes
2. Assessing inhibition of lipid accumulation in 3T3-L1 mouse pre-adipocytes

In addition, cytotoxicity against MDCK cells, 3T3-L1 cells and human cancer cell lines (cervical carcinoma HeLa cells and lung adenocarcinoma A549 cells) was evaluated by establishing the cytotoxic concentrations of the extracts using MTT assays.

### 5.3. Materials and Methods

Dulbecco’s modified Eagle medium (DMEM), Dulbecco’s modified Eagle medium/Ham’s nutrient mixture F12 (DMEM/F12), fetal bovine serum (FBS), insulin, 2-[(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-d-glucose (2-NBDG), trypsin/EDTA and penicillin-streptomycin were purchased from Invitrogen Australia. Bovine serum albumin (BSA), 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, 3-(4, 5 dimethylthiazol- 2-yl)-2, 5 diphenyltetrazolium bromide (MTT), d-biotin, rosiglitazone and oil red O were obtained from Sigma-Aldrich, Australia.

#### 5.3.1. Cell lines

Madin-darby canine kidney epithelial cells (MDCK) were procured from the American Type Cell Culture (ATCC). A549, HeLa and 3T3-L1 cells were provided by Monash University, Victoria, Australia. The cells were routinely passaged as described below.
5.3.2. Passaging of cell lines

Cells were routinely cultivated as monolayers in disposable 25 cm² flasks (Corning) in DMEM supplemented with 10% (v/v) FBS, 1% (v/v), penicillin-streptomycin (10,000 U/ml penicillin and 10,000 μg/ml streptomycin in 0.85% saline) and passaged when 70-80% confluent. The media was aspirated from the confluent cells using a sterile pipette and cells were washed with approximately 5 ml sterile 1X PBS solution, which was subsequently aspirated. Trypsin/EDTA solution (2.5 ml) was added to the flask to cover the cell monolayer and the flask was incubated at 37 °C for 3 minutes to allow the cells to detach. Fresh media (3 ml) was used to resuspend the detached cells and neutralize the action of trypsin. The cell suspension was centrifuged at 200 rpm for 5 min at 20 °C. The supernatant was discarded and cell pellet was resuspended in 5 ml of fresh media. Cell counts were carried by the trypan blue dye exclusion method. Cells were seeded at a density of 150000/flask and incubated at 37 °C in 5% CO₂ atmosphere.

5.3.3. Cryopreservation of cells

To prepare cells for storage, a confluent monolayer was detached from the tissue culture flask with trypsin/EDTA solution as described above. The suspended cells were transferred to a sterile 15 ml falcon tube, centrifuged at 200 rpm for 10 min, and the supernatant aspirated with a pipette. The cell pellet was resuspended into DMEM with 80% FBS and 10% DMSO. A cell count was performed and 150000 cells per vial were aliquoted into 1.8 ml cryogenic vial using a sterile pipette. The vials were packed in cotton wool and gradually frozen at -80 °C for long term storage in liquid nitrogen.

5.3.4. MTT cytotoxicity studies

Cell viability was determined by the MTT (3-(4, 5 dimethylthiazol- 2-yl)-2, 5 diphenyl-tetrazolium bromide) method. Cells were cultured in 96-well plates (5000 cells/well) containing 100 μl medium, prior to the treatment with plant extracts or vincristine (anti-cancer drug) at 37 °C for 24 hrs. The cells were exposed to 100 μl of each test solution (containing various concentrations of plant extracts or vincristine) and incubated for a further 72 hrs at 37 °C. Plant extract (1 – 500 μg/ml) and vincristine (0.001 – 200
µg/ml) in media containing 2% FBS were freshly prepared prior to each experiment. MTT (5 mg/ml) was dissolved in PBS and the solution was freshly prepared every time, filtered through a 0.2 µm filter and used immediately. The test solutions were then removed and the cells were washed in PBS and 50 µl of media was added into each well. Then, 5 µl of MTT solution (5 mg/ml PBS) was placed into each well and incubated at 37 °C. After 4 hrs, 25 µl of cells were removed, 50 µl DMSO was added and the mixture incubated at 37 °C for 10 min. The absorbance at 540 nm was measured using a microplate reader (Bio-Rad Laboratories). Each experiment was performed three times with each sample tested in triplicate. The data represent the mean cell viability of the test solutions, calculated as a percentage of the total cell viability found with the control well (which contained no test solutions).

5.3.5. Adipocyte differentiation of 3T3-L1 cells

3T3-L1 cells (ATCC; CL-173) represent a subclone of the 3T3 cells which is able to undergo adipocyte differentiation. Cells were cultured and differentiated as described previously (Huang et al., 2006), Kühn et al., 2011) with minor amendments. 3T3-L1 cells at passage 9 or 10 were seeded in 96-well plates (5000 cells/well) for Oil red O staining and glucose uptake measurements using DMEM/F12 medium with 10% FBS. DMEM/F12 is a serum free media which is supplemented with a defined combination of nutrients, growth factors, and hormones to culture variety of cells. Two days after reaching confluence, the medium was changed to differentiation medium- DMEM/F12 + 2% FBS containing 10 µg/ml insulin, 0.5 mM (IBMX) 3-isobutyl-1-methylxanthine and 1.0 µM dexamethasone. 3T3-L1 cells when treated with a combination of dexamethasone, isobutylmethylxanthine (IBMX) and insulin adopt a rounded phenotype and within 5 days begin to accumulate lipids intracellularly in the form of lipid droplets (Wise and Green, 1979). Cells remained in the differentiation medium for four days with media replenished every 48 hrs. Thereafter, differentiation medium was replaced by DMEM/F12 + 2% FBS in which cells remained for the respective experiments.
5.3.6. Glucose uptake measurements

At day 9 of differentiation, adipocytes were incubated for 24 hrs with the respective test solutions. Ethanol was used as a negative control whereas 10 μM rosiglitazone was used as a positive control. Next day, the cells were rinsed with PBS and incubated for 60 min at 37 °C with exclusion of light in DMEM containing 80 μM of the fluorescent glucose analogue, 2-NBDG, again in the presence of the extracts for basal glucose uptake measurement. As a second positive control, cells were treated with 100nM insulin during the 2-NBDG incubation to measure the insulin-stimulated glucose uptake. The reaction of 2-NBDG uptake was terminated by washing the cells with pre-cooled PBS. The remaining fluorescence activity in the cells was measured by using fluorescence microplate reader (POLARStar Omega, BMG Labtech, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Fluorescence activity in the absence of 2-NBDG was subtracted from all values.

5.3.7. Lipid accumulation inhibition assay and Oil Red O staining of intracellular triglycerides

Lipid accumulation inhibition assay was carried out as per standard protocols with minor amendments (Fang et al., 2008). 3T3-L1 cells were differentiated into adipocyte as described above. To quantify the effect of plant extracts on lipid accumulation in 3T3-L1 cells, the cells were treated with plant extracts in DMEM supplemented with 2% FBS from day 2 till day 10 of differentiation (Roh and Jung, 2012). On day 10 of differentiation, media was removed and the cells treated with and without plant extracts were washed with PBS and fixed with 10% formalin for 30 minutes. Cells were rinsed with deionized H2O and then incubated with Oil Red O solution (0.25% w/v in 60% isopropanol) for 1 hr at room temperature. Finally, the dye retained in the 3T3-L1 cells was eluted with isopropanol and quantified by measuring the optical absorbance at 540 nm (BioRad Plate Reader). Cells were also imaged under a light microscope.
5.3.8. Statistical analysis

All samples were analysed in triplicates. Data are presented as mean ± standard error mean (SEM). The dose–response curve was obtained by plotting the percentage inhibition versus concentration (Loizzo et al., 2008). For the final evaluation of the glucose uptake assay, fluorescence activities measured for the negative control (solvent ethanol) were set to 100% and values for test extracts and positive controls were calculated accordingly. In the case of lipid inhibition assays, cells treated with inducers set to 100% and values for tested extracts were calculated accordingly. Differences were evaluated by one-way analysis of variance (ANOVA) test completed by a Bonferroni’s multicomparison test. Differences were considered significant at p < 0.001. The concentration giving 50% inhibition (IC$_{50}$) was calculated by non-linear regression with the use of GraphPad Prism Version 5.0 for Windows (GraphPad Software, San Diego, CA, USA) (www.graphpad.com).
5.4. Results

5.4.1. Cytotoxicity studies

This study examined the cytotoxicity and anti-tumour activity of seven Australian Aboriginal plant extracts. The ethanolic extracts were tested for cytotoxic effects on A549, HeLa and MDCK cells. The cytotoxicity and selectivity of the Australian Aboriginal plant extracts against the selected cancerous cell lines are summarized in. According to the standard National Cancer Institute (NCI) criteria, crude extracts possessing an IC$_{50}$ of $<30$ $\mu$g/ml are considered active against the tested cancer cells (Itharat et al., 2004). Of the seven extracts tested, only two extracts, AK and AT, showed activity according to NCI criteria with IC$_{50}$ of $13.73$ $\mu$g/ml and $27.00$ $\mu$g/ml respectively (Table 12) against HeLa cells. Vincristine, a chemotherapeutic drug used for some cancer types, had cytotoxic effects on MDCK, A549 and HeLa with values of $145.83$ $\mu$g/ml, 0.6 and 0.39 ng/ml, respectively. The five Indian Ayurvedic plant extracts were tested against selected leukemic cell lines.

None of the Indian Ayurvedic plant extracts showed promising effects (Table 13) against the cells used in this assay. The Australian Aboriginal plant extracts showed IC$_{50}$ values in the range of $158.81 - 386.95$ $\mu$g/ml and Indian plant extracts showed IC$_{50}$ values in range of $171.45 - 394.03$ $\mu$g/ml against 3T3-L1 cells. Thus, two concentrations, 10 and 100 $\mu$g/ml, were selected for evaluating the effects of the extracts on adipogenesis and glucose uptake.
<table>
<thead>
<tr>
<th>Plant name</th>
<th>3T3-L1</th>
<th>MDCK</th>
<th>A549</th>
<th>HeLa</th>
</tr>
</thead>
<tbody>
<tr>
<td>AK</td>
<td>202.36 ± 21.44</td>
<td>550.03 ± 36.20</td>
<td>75.17 ± 6.25</td>
<td>13.73 ± 1.51*</td>
</tr>
<tr>
<td>AT</td>
<td>240.70 ± 65.22</td>
<td>398.51 ± 31.26</td>
<td>298.6 ± 44.45</td>
<td>110.61 ± 35.82</td>
</tr>
<tr>
<td>AL</td>
<td>306.46 ± 65.56</td>
<td>567.38 ± 52.59</td>
<td>210.85 ± 30.82</td>
<td>27.00 ± 14.28*</td>
</tr>
<tr>
<td>BL</td>
<td>386.95 ± 56.32</td>
<td>331.33 ± 30.90</td>
<td>84.08 ± 24.37</td>
<td>229.11 ± 40.57</td>
</tr>
<tr>
<td>ED</td>
<td>256.34 ± 59.53</td>
<td>460.25 ± 20.96</td>
<td>91.26 ± 19.84</td>
<td>142.50 ± 29.27</td>
</tr>
<tr>
<td>SL</td>
<td>173.14 ± 24.86</td>
<td>406 ± 24.31</td>
<td>162.95 ± 27.34</td>
<td>186.66 ± 62.21</td>
</tr>
<tr>
<td>SS</td>
<td>158.81 ± 25.53</td>
<td>283.66 ± 12.64</td>
<td>112.28 ± 13.28</td>
<td>110.11 ± 11.91</td>
</tr>
</tbody>
</table>

Table 12 - IC$_{50}$ values (μg/ml) of Australian Aboriginal plant extracts on two cancer cell lines and the non-cancerous MDCK and 3T3-L1 cell lines. Data are expressed as mean ± SEM of independent experiment (n = 3). * denotes IC$_{50}$ less than 30 μg/ml which is considered as an active extract against cancer cells.
### Table 13 - IC\textsubscript{50} values (μg/ml) of Indian Ayurvedic plant extracts on two cancer cell lines, MDCK and 3T3-L1 cell line.

Data are expressed as mean ± SEM of independent experiment (n = 3).

<table>
<thead>
<tr>
<th>Plant name</th>
<th>3T3-L1</th>
<th>MDCK</th>
<th>A549</th>
<th>HeLa</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO</td>
<td>214.73 ± 42.89</td>
<td>403.28 ± 12.64</td>
<td>457.6 ± 65.93</td>
<td>287.61 ± 42.86</td>
</tr>
<tr>
<td>MP</td>
<td>256.34 ± 59.53</td>
<td>441.33 ± 59.12</td>
<td>301.73 ± 48.71</td>
<td>397.41 ± 87.83</td>
</tr>
<tr>
<td>PM</td>
<td>171.45 ± 17.06</td>
<td>491.95 ± 35.80</td>
<td>416.15 ± 108.93</td>
<td>380.73 ± 72.88</td>
</tr>
<tr>
<td>AP</td>
<td>352.18 ± 41.27</td>
<td>449.55 ± 23.13</td>
<td>351.31 ± 59.06</td>
<td>487.86 ± 47.64</td>
</tr>
<tr>
<td>BM</td>
<td>394.03 ± 25.95</td>
<td>540.3 ± 37.66</td>
<td>320.36 ± 22.08</td>
<td>366.05 ± 37.63</td>
</tr>
</tbody>
</table>

Table 13 - IC\textsubscript{50} values (μg/ml) of Indian Ayurvedic plant extracts on two cancer cell lines, MDCK and 3T3-L1 cell line. Data are expressed as mean ± SEM of independent experiment (n = 3).
5.4.2. Glucose uptake assay

The seven Australian Aboriginal and five Indian Ayurvedic plant extracts were all tested at 10 and 100 µg/ml to assess their impact on basal and insulin-stimulated glucose uptake into differentiated 3T3-L1 adipocytes. After incubation for 28 hrs, the Australian aboriginal (Figure 16) and Indian Ayurvedic plant (Figure 18) failed to enhance basal and insulin-stimulated glucose uptake at concentration of 10 µg/ml. However, when the same extracts were tested at 100 µg/ml, it was observed that AK, AT and SS moderately enhanced basal glucose uptake by 19, 19 and 16%, respectively, as compared to the ethanol control (Figure 17). In contrast, rosiglitazone was enhanced basal glucose uptake by nearly 43 % as compared to the control. Of the Indian Ayurvedic plant extracts tested at 100 µg/ml, only CO was able to enhance basal glucose uptake by nearly 19% as compared to control (Figure 19).

AK and SS at 100 µg/ml were able to enhance insulin-stimulated glucose uptake by 45 and 47%, respectively, which approached the 65% enhancement observed for rosiglitazone (Figure 17). AT enhanced glucose uptake in the presence of insulin by 34% which was approximately half that of rosiglitazone (Figure 17). CO was able to enhance glucose uptake by 48% in the presence of insulin (Figure 19).
Figure 16 - Effects of Australian Aboriginal plant extracts at 10 µg/ml on basal and insulin-stimulated glucose uptake in 3T3-L1 adipocytes.

Cells were treated with the individual extract for 24 hrs followed by incubation for 60 min in serum- and glucose-free media containing 80 µM 2-NBDG. Ethanol was used as a negative control, while rosiglitazone and insulin were used as positive controls. Cells received insulin only during 2-NBDG uptake. After incubation, fluorescence activity remaining in the cells was measured by a fluorescence microplate reader. Fluorescence activity in the absence of 2-NBDG was subtracted from all values. Data shown are means ± SD of at least three independent experiments performed in triplicates. Significance against ethanol control (= 100%): *** p < 0.001. Significance against ethanol + 100 nM insulin control: +++ p < 0.001.
Figure 17  - Effects of Australian Aboriginal plant extracts at 100 µg/ml on basal and insulin-stimulated glucose uptake in 3T3-L1 adipocytes.

Cells were treated with the individual extract for 24 hrs followed by incubation for 60 min in serum- and glucose-free media containing 80 µM 2-NBDG. Ethanol was used as a negative control, while rosiglitazone and insulin were used as positive controls. Cells received insulin only during 2-NBDG uptake. After incubation, fluorescence activity remaining in the cells was measured by a fluorescence microplate reader. Fluorescence activity in the absence of 2-NBDG was subtracted from all values. Data shown are means ± SD of at least three independent experiments performed in triplicates. Significance against ethanol control (= 100%): ** p < 0.01, *** p < 0.001. Significance against ethanol + 100 nM insulin control: + p < 0.05, ++ p < 0.01, +++ p < 0.001.
Figure 18 - Effects of Indian Ayurvedic plant extracts at 10 µg/ml on basal and insulin-stimulated glucose uptake in 3T3-L1 adipocytes.

Cells were treated with the individual extract for 24 h followed by incubation for 60 min in serum- and glucose-free media containing 80 µM 2-NBDG. Ethanol was used as a negative control, while rosiglitazone and insulin were used as positive controls. Cells received insulin only during 2-NBDG uptake. After incubation, fluorescence activity remaining in the cells was measured by a fluorescence microplate reader. Fluorescence activity in the absence of 2-NBDG was subtracted from all values. Data shown are means ± SD of at least three independent experiments performed in triplicates. Significance against ethanol control (= 100%): *** p < 0.001. Significance against ethanol + 100 nM insulin control: +++ p < 0.001.
Figure 19 - Effects of Indian Ayurvedic plant extracts at 100 µg/ml on basal and insulin-stimulated glucose uptake in 3T3-L1 adipocytes:

Cells were treated with the individual extract for 24 h followed by incubation for 60 min in serum- and glucose-free media containing 80 μM 2-NBDG. Ethanol was used as a negative control, while rosiglitazone and insulin were used as positive controls. Cells received insulin only during 2-NBDG uptake. After incubation, fluorescence activity remaining in the cells was measured by a fluorescence microplate reader. Fluorescence activity in the absence of 2-NBDG was subtracted from all values. Data shown are means ± SD of at least three independent experiments performed in triplicates. Significance against ethanol control (= 100%): * p < 0.05, ** p < 0.01, *** p < 0.001. Significance against ethanol + 100 nM insulin control: ++ p < 0.01, +++ p < 0.001.
5.4.3. Inhibition of lipid accumulation in 3T3-L1 cells

Adipocyte differentiation of 3T3-L1 cells is a highly-controlled process that can be induced under a hormonal cocktail of insulin, dexamethasone and IBMX (Liu et al., 2001). Intracellular lipid accumulation is commonly monitored as a general marker to indicate the extent of adipogenesis in 3T3-L1 cells (Yeo et al., 2011a). 3T3-L1 pre-adipocytes were differentiated in the presence of seven selected Australian Aboriginal plant extracts and five Indian plants extracts for 8 days.

Figure 20 - Micrographs showing effects of plant extracts on lipid accumulation

Effect of AT (b) and CO (c) extracts on fat droplet formation in 3T3-L1 cells as compared to control (a).

Pre-adipocytes were differentiated with 100 μg/ml of AT and CO extract treatment for 8 days, then stained with Oil Red O dye and examined using a light microscope.
Figure 20 shows reduction in lipid accumulation in adipocytes treated with selected extracts. Three Australian plant extracts, AT, BL and ED, were found to significantly reduce lipid accumulation in 3T3-L1 adipocytes, suggesting anti-obesity activity. AT was able to significantly reduce lipid accumulation by 51 and 82 % at 10 and 100 µg/ml respectively (Figure 21). Lipid accumulation was reduced by 34 and 35 % at concentration of 10 µg/ml BL and ED, respectively, and was further reduced by 74 and 65 % respectively, by the same extracts at 100 µg/ml (Figure 21). Indian Ayurvedic plants tested failed to reduce lipid accumulation at 10 µg/ml but CO, PM and AP were able to moderately reduce lipid accumulation by 31, 30 and 28 % respectively at 100 µg/ml (Figure 22).
Figure 21 - Effect of Australian Aboriginal plant extracts on Oil Red O staining in cultured 3T3-L1 adipocytes.

(A) Effect of 10 µg/ml extract and (B) Effect of 100 µg/ml extract on fat droplet formation in 3T3-L1 cells. Values are expressed as mean ± standard deviation of at least three independent experiments. Values are means ± SE (n = 3), significance against control (without plant extract) (= 100%): *** p < 0.001 and * p < 0.05.
Figure 22 - Effect of Indian Ayurvedic plant extracts on Oil Red O staining in cultured 3T3-L1 adipocytes.

(A) Effect of 10 µg/ml extract and (B) Effect of 100 µg/ml extract on fat droplet formation in 3T3-L1 cells. Values are expressed as mean ± standard deviation of at least three independent experiments. Values are means ± SE (n = 3), significance against control (without plant extract) (= 100%): * p < 0.05.
5.5. Discussion

Plants have played an important role as a source of effective anti-cancer agents, and it is important to note that over 60% of the currently used anti-cancer agents are originally derived from natural sources, including plants, marine organisms and micro-organisms. The modern scientifically based search for anti-cancer agents from plant sources started as early as the 1950s with the discovery of the alkaloids vinblastine and vincristine from *Vinca rosea* and the isolation of cytotoxic podophyllotoxins from *Podophyllum* (Ukiya et al., 2002). The phytochemicals present in plants possess strong antioxidant activities that may prevent and help cure cancer by protecting healthy cells from damage caused by the highly reactive oxygen species known as ‘free radicals’ (Schafer and Buettner, 2001). Thus, consuming a diet rich in antioxidant plant foods will provide a milieu of phytochemicals that possess health protective effects, provide therapeutic actions to all cells with low cytotoxicity and are beneficial in producing nutrient repletion to immune-compromised people (Reddy et al., 2003). Strong and consistent epidemiology evidence also indicates that a diet rich in antioxidants significantly reduces the risk of many cancers (Dai and Mumper, 2010).

In the present study, plants previously shown to display good antioxidant activity (Chapter 3) were assessed for their cytotoxicity against cancerous (HeLa, derived from a human uterus carcinoma, and A549, derived from human small lung carcinoma) and non-cancerous (MDCK, normal epithelium) cell lines. The cells were exposed to the extracts and the viability of cells was measured and expressed in terms of the relative absorbance of extract-treated cells, in comparison with control cells.

The results of cytotoxicity testing of Australian Aboriginal and Indian Ayurvedic plant extracts (Tables 1 and 2) were assessed according to the US NCI plant screening program, where a crude extract is generally considered to have *in vitro* cytotoxic activity if the IC\(_{50}\) value is less than 30 µg/ml. Among all the plant extracts screened here, two extracts, AK and AT, showed particularly potent activity with IC\(_{50}\) values of 13.73 µg/ml and 27.00 µg/ml, respectively, against HeLa cells. Other extracts showed moderate activity. None of the Indian Ayurvedic plant extracts are likely candidates for anticancer drug development as all showed IC\(_{50}\) values of more than 200 µg/ml against HeLa and
A549 cells. None of the Australian Aboriginal plant extracts had IC\textsubscript{50} values of less than 30 µg/ml against A549 cells, although AK, BL, ED, SS and SL had IC\textsubscript{50} values of less than 200 µg/ml so they have moderate anticancer activity. However, only two cell lines were tested in this study and further testing against other cancer cells may reveal additional anticancer activity.

Based on the IC\textsubscript{50} of the plant extracts, they can be divided into three groups:

1. Those with IC\textsubscript{50} values of less than 30 µg/ml can be considered as potential candidates for further development as cancer therapeutic agents; such as AK and AT against HeLa cells.
2. Those with IC\textsubscript{50} values between 30 and 200 µg/ml have moderate potential to be developed into cancer therapeutic agents.
3. Those with IC\textsubscript{50} more than 200 µg/ml are unlikely candidates for development into cancer therapeutic agents.

An important observation was that the activity against HeLa cells exhibited by extracts AK and AT was specific as no cytotoxicity was observed against the non-cancer cell line, MDCK. Therefore, these extracts may be promising candidates for the development of chemotherapeutic agents targeting cervical cancer with minimal side effects against normal cells.

The well-characterized murine pre-adipose 3T3-L1 cell line was used to investigate the mechanisms of action by which plant extracts exert their anti-diabetic effects. Since obesity is a side effect of some anti-diabetic drugs, the effects of plants on adipogenesis was also evaluated.

The impact of plant extracts on basal and insulin-stimulated glucose uptake into 3T3-L1 adipocytes was examined, using the nonradioactive method of measuring 2-NBDG uptake. Of the seven Australian Aboriginal plant extracts tested, six were able to enhance insulin-stimulated glucose uptake at a concentration of 100 µg/ml. By contrast, only AK, AT and SS were able to enhance basal glucose uptake by 19.71, 19.19 and 16.82 % respectively. It is well known that thiazolidinediones have beneficial effects on hyperglycemia in type 2 diabetes, but the molecular mechanism is still to be elucidated.
These drugs stimulate glucose uptake either by enhancing synthesis of the insulin independent (basal) glucose transporter GLUT-1 or by increasing expression or translocation of the insulin-dependent/sensitive glucose transporter GLUT-4 (Kim et al., 2007, Kühn et al., 2011).

Upon the completion of adipogenesis, spindle-shaped pre-adipocytes were transformed into round-shaped cells that accumulated lipids and acquired the metabolic mechanisms to facilitate glucose uptake in response to insulin, synthesize fatty acids, accumulate triglyceride and secrete a wide variety of hormones and cytokines (Yeo et al., 2011a). Therefore, intracellular lipid accumulation is commonly monitored as a general marker to indicate the extent of adipogenesis in 3T3-L1 cells (Avram et al., 2007). The results of this study showed that the three Australian plant extracts AT, BL and ED were able to significantly reduce lipid accumulation (by 82, 74 and 65 % respectively when tested at 100 µg/ml) in 3T3-L1 adipocytes when compared to control, suggesting anti-obesity activity which is a desirable property for an anti-diabetic drug.

Morphological observations of cells stained with Oil Red O, a lipid stain, showed a decrease in cellular lipid content in cells treated with plant extracts. Among the Indian Ayurvedic plant extracts, CO, PM and AP (31, 30 and 28 % respectively at 100 µg/ml), were able to moderately reduce lipid accumulation. Thus, these plants have the potential for the management of obesity as they inhibited adipogenesis. A number of studies have demonstrated that natural compounds like EGCG, genistein, esculetin, berberine, resveratrol, guggulsterone, capsaiacin, baicalein and procyanidins inhibited adipogenesis (Rayalam et al., 2008). 3T3-L1 cells are widely used models of adipocyte function. In vivo, excessive triglyceride accumulation by the adipocyte has been linked to an increased risk of a variety of metabolic disorders (Popovich et al., 2010). The presence of phenolic compounds, tannins, alkaloids, procyanidins and cyanogenic glycosides have been attributed to the hypoglycaemic action of various plants (Alonso-Castro and Salazar-Olivo, 2008). Tannins, catechins and epicatechins are the most active antioxidant constituents and are found to enhance the glucose uptake and inhibit adipogenesis in differentiated adipocytes (Deutschländer et al., 2011, Muthusamy et al., 2008).
5.6. **Conclusions**

The results of the current study showed that plant extract AT probably exerts its anti-diabetic properties by stimulating glucose uptake in adipocytes with significant inhibition of adipogenesis. Plant extracts AK, SS and CO were also observed to enhance basal and insulin-stimulated glucose uptake. BL, ED, MP and PM inhibited lipid accumulation but should be further studied using anti-lipase activity assays and Western blot analysis to confirm their anti-adipogenic effect. The ability of AT to enhance glucose uptake in insulin-resistant adipocytes, in addition to its anti-adipogenic effects, suggests that this extract could be useful in the treatment of type 2 diabetes. Future studies should address the molecular mechanisms by which these plants and their active compounds regulate glucose uptake by adipose and muscle tissues.

The ability of existing therapies to target various aspects of the insulin resistance syndrome induces other metabolic abnormalities, chiefly those involved in lipid metabolism. Therefore, glucose-lowering drugs with minimal adipogenic activity are desirable and this study has demonstrated that phytochemicals from traditional medicinal plants have such properties. However, the pharmaceutical significance of the present results will depend on further exploration including lead molecule identification and *in vivo* studies.
Chapter 6. General Discussion
6.1. Discussion

This chapter provides a general overview of the work presented in the thesis and its implications. It also provides possible future directions that will help in the identification of novel drug targets for the management of type 2 diabetes.

It has been estimated that 275 Australians develop diabetes every day. The 2005 Australian AusDiab Follow-up Study (Australian Diabetes, Obesity and Lifestyle Study) showed that 1.7 million Australians have diabetes but up to half of the cases of type 2 diabetes remain undiagnosed and it is estimated that by 2033 nearly 3.5 million Australians will have type 2 diabetes (Cameron et al., 2003). Therefore there is a great need to develop new drugs for diabetes. Part of this drug discovery research effort will be to identify plant species that can potentially be applied in management of type II diabetes and related complications of weight gain, hypertension and immune-suppression.

The strategy used in this thesis was to investigate the traditional plants used by Australian Aboriginal people for general health and medicinal plants utilised by Indians for the management of diabetes which are described in ancient Indian texts of Ayurveda. The initial part of this study involved screening the plants for bioactivity by testing their ability to inhibit common microbes i.e. two Gram-positive and two Gram-negative bacteria and a fungus, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*, respectively, using the disk diffusion assay. MIC and MBC were also determined. Results from disk diffusion assays with these microbes showed that many extracts exhibited broad spectrum activity. However, the MIC and MBC values indicated that the plant extracts were not very potent. Cos (Cos et al., 2006) have suggested that strict criteria should be used to assess the potential application of natural products. In the context of anti-infective agents, MIC levels less than 100 μg/ml are indicative of useful bioactivity for natural product extracts. None of the extracts showed MIC less than 100 μg/ml against any bacteria tested in our study, which was unexpected, the poor result may be simply due to natural variations in chemical components due to factors such as difference in geographic conditions of the plants. Despite the poor MIC and MBC results, most of the Indian Ayurvedic plants showed broad spectrum antimicrobial activity. However, SL and MP showed good activity against
**Candida albicans**, suggested that these extracts can be potential candidates as antifungal agents, while AS, BD, BM, CP and PT demonstrated moderate antifungal activity. The mode of extraction, solvent used for extraction, the nature of bioactive compounds and the geographical sources of plants affect the presence of bioactive compounds and thus the activity (Rios and Recio, 2005). These factors, individually or collectively, may explain the low antimicrobial activity observed for the extracts.

The antioxidant activity of the plants was evaluated against free radicals. Free radicals can damage biomolecules in our body such as lipids, nucleic acids and proteins. They also cause cellular membrane peroxidation and attract various inflammatory mediators (Leopoldini et al., 2011) as shown in **Figure 23**. Phenolic compounds and flavonoids are known to have antitumor properties, antiproliferative effects and induce apoptosis in different cancer cell lines. They are free radical scavengers, and flavonoids in particular inhibit invasion and metastasis (Čipák et al., 2003). Polyphenols are able to interact with the cytochrome P450 (CYP1) family enzymes which promote the initiation of carcinogenesis. These interactions act via two mechanisms: to inhibit the procarcinogen activation and to be a substrate for the release of inhibitors of tumour cell growth (Sawadogo et al., 2012). Several studies have described that the anticancer activity of phytochemicals is due to their antioxidant compounds such as vitamins, minerals, polyphenols, flavonoid, terpenoids, lignins, xanthones and polysaccharides (Pandey and Madhuri, 2009). Therefore, antioxidants are considered to be good source of anticancer agents. In this study, the free radical scavenging activity was strongest in AL, AK, BL and SL. Among Indian Ayurvedic plant extracts, AP, BM and PM demonstrated potent free radical scavenging activity against the diphenyl picryl hydrazyl (DPPH) radical, 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS) and ferric ions. It was therefore decided to target these plants for screening against cancer cells to investigate the anticancer activity.

Of the total twenty six plant extracts initially included in this study, only twelve plant extracts (all seven Australian Aboriginal and five Indian Ayurvedic) were selected based on their results with antioxidant and enzyme inhibition assays for further studies with cell culture experiments. The cancer cell lines used were cervical carcinoma HeLa cells and lung adenocarcinoma A549 cells. According to the US NCI plant screening
program, a crude extract is generally considered to have \textit{in vitro} cytotoxic activity if the IC$_{50}$ value is less than 30 µg/ml. Among the plant extracts screened, only two extracts, AK and AT, showed cytotoxic activity (IC$_{50}$ of 13.73 µg/ml and 27.00 µg/ml, respectively) against HeLa cells as assessed by the MTT assay. None of the other plant extracts showed IC$_{50}$ values less than 30 µg/ml against A549. However, it is possible that these extracts possess activity against other cancer cell lines. Thus, further testing is warranted.

Figure 23 - Physiological pathway representing the generation of free radicals and their contribution to various disorders which can be moderated or prevented by antioxidants found in plant species.
Phenolic components found in vegetables, fruits, spices and medicinal herbs might prevent cancer through antioxidant action and/or the modulation of several protein functions. They may inhibit carcinogenesis by affecting the molecular events in the initiation, promotion and progression stages of free radical chain reactions (Cai et al., 2004). Phenolics have also been reported to demonstrate agonism and/or antagonism of carcinogenesis-related receptors such as the arylhydrocarbon receptor, epidermal growth factor and estrogen receptor β. They modulate the secretion of protein kinases in tumour cell proliferation and induce the expression of anticarcinogenic enzymes or inhibit induction of cancer-promoting enzymes (Sakakibara et al., 2002).

The use of natural products as anticancer agents has a long history that began with folk medicine and has been incorporated into modern medicine. Several drugs currently used in chemotherapy were isolated from plant species such as alkaloids from *Vinca*, vinblastine and vincristine isolated from *Catharanthus roseus*, etoposide and teniposide, the semisynthetic derivatives of epipodophyllotoxin isolated from species of the genus *Podophyllum*, the naturally derived taxanes isolated from species of the genus *Taxus* and the semisynthetic derivatives of camptothecin, irinotecan and topotecan isolated from *Camptotheca acuminata* as detailed in the review by Costa (Costa-Lotufo et al., 2005).

The plant extracts were also assessed for their ability to inhibit enzymes involved in carbohydrate metabolism, which is an important feature of diabetes control or prevention. Enzyme inhibition activity was evaluated against starch hydrolysing enzymes α-amylase and α-glucosidase. Hydrolysis of dietary carbohydrates such as starch is the major source of glucose in the blood. This hydrolysis is carried out by a group of hydrolytic enzymes that includes pancreatic α-amylase and intestinal α-glucosidases. Therefore, the inhibition of these enzymes is believed to be a good and important strategy for management of type 2 diabetes (Apostolidis and Lee, 2010). α-glucosidase is abundantly found in the brush border of the small intestine and can delay carbohydrate digestion and absorption, and thereby diminish post-prandial hyperglycaemia (PPHG) (Tiwari et al., 2008). Recently, there had been widespread interest in these enzymes because of their potential as therapeutic targets. In particular, inhibition of α-glucosidase had been found to help control postprandial blood glucose levels in diabetic patients (Ryu et al.,
Clinical trials showed that the α-glucosidase inhibitors improved long-term glycaemic control as measured by decreased haemoglobin A1c (HbA1c) in patients with type 2 diabetes and delay the development of type 2 diabetes in patients with impaired glucose tolerance (Du et al., 2006).

The commercial inhibitors acarbose and miglitol are drugs that inhibit α-glucosidase present in the epithelium of the small intestine and have been demonstrated to decrease post-prandial hyperglycaemia and improve impaired glucose metabolism without promoting insulin secretion in NIDDM patients (Subramanian et al., 2008b). Several food components and plants have also been reported to inhibit these enzymes. Therefore, natural α-glucosidase and α-amylase inhibitors from plant sources offer an attractive strategy for the control of postprandial hyperglycaemia (Bhat et al., 2011a). Similarly, angiotensin converting enzyme (ACE) in the rennin–angiotensin system is known to increase blood pressure by the hydrolysis of the rennin-induced decapeptide, angiotensin I to octapeptide angiotensin II, a potent vasoconstrictor. This reaction stimulates aldosterone secretion in adrenal/cardiovascular tissue which promotes sodium and water retention and causes an increase in rennin generation in the kidneys (Kurtz and Pravenec, 2004). Therefore, the inhibition of ACE may prevent hypertension, other cardiovascular and renal diseases and oxidative stress-associated diseases. The synthetic ACE inhibitors are captopril, benazepril, enalapril, and lisinopril. Tannins, flavonoids, xanthones, terpenoids, peptides and caffeoylquinic acid derivatives are the natural compounds that have been reported to have anti-hypertensive action through ACE-inhibition (Jung et al., 2006a).

Extracts of CB, PZ, SN and PM were found to inhibit α-amylase activity with IC\textsubscript{50} values 4.17, 4.36, 4.87 and 5.16 µg/ml; whereas acarbose showed an IC\textsubscript{50} of 7.81 µg/ml. SS was found to inhibit α-amylase with an IC\textsubscript{50} of 5.43 µg/ml. Among the Australian plants, AL, AK, AT, BL, ED, SS and SL showed an IC\textsubscript{50} of 1.01, 1.34, 1.38, 0.48, 1.06, 0.90 and 1.83 µg/ml respectively and were significantly more potent inhibitors of α-glucosidase compared to acarbose (p < 0.001). Among the Indian Ayurvedic plants, CB and MP showed significant inhibition (p < 0.001) with IC\textsubscript{50} values of 0.58 and 0.80 µg/ml respectively against α-glucosidase. These findings provide evidence for the hypoglycaemic action of these plants and indicate that they may represent alternative ther-
apies for the management of diabetes. Interestingly, the Australian Aboriginal plant extracts were found to be more potent compared to Indian Ayurvedic plant extracts. Although the Australian plants investigated have no recorded traditional use in the treatment of diabetes, this study shows that ethno medicinal knowledge alone cannot predict the therapeutic potential of medicinal plants, especially when the ethnic group did not naturally experience the disease under investigation.

All tested plant extracts showed very high $IC_{50}$ as compared to captopril against ACE, suggesting that these could be used as preventative nutraceuticals against hypertension rather than therapeutic drugs for the treatment of the condition. Alternatively, they could perhaps help in managing the side effects by modern anti-hypertensive drugs.

The plant extracts were selected on the basis of their enzyme inhibition (Chapter 4) activity and were further evaluated by cell culture assays to assess their mode of action. The ability of the selected plant extracts to stimulate glucose uptake in 3T3-L1 adipocytes was examined and compared with that of rosiglitazone. In addition, the effects of extracts on differentiation of pre-adipocytes into adipocytes, a process induced by addition of an insulin/3-isobutyl-1-methylxanthine/dexamethasone (IS-IBMX-DEX) cocktail, were also investigated. These studies were designed to characterize the effects of extracts in 3T3-L1 cells at the cellular level and to identify extracts that may be used for prevention and treatment of obesity and NIDDM without the undesirable side effects of insulin therapy (Liu et al., 2001).

Antidiabetic drugs such as insulin or thiazolidinediones (TZD) up-regulate both glucose transport and lipid biosynthesis in adipocytes. Weight gain is a frequent side effect of insulin therapy in type 2 diabetic patients (Laville and Andreelli, 2000). Therefore, drugs with glucose-lowering activity, but lacking adipogenic activity, are highly desirable. The extracts of AK, SS and CO found to stimulate glucose uptake in mature adipocytes with moderate inhibition on adipogenesis. AT, BL and ED were found to significantly reduce lipid accumulation whereas CO, PM and AP were able to moderately reduce lipid accumulation at 100 µg/ml. Thus, an understanding of the mechanism of action will be valuable for the study, prevention and treatment of obesity, insulin resistance and type 2 diabetes (Liu et al., 2001) if these extracts were to be further investigated. The use of plants with good antidiabetic potential, which help reduce lipid accu-
mulation and have anti-oxidant activity, would be an excellent strategy to control hyp-
perglycaemia and related complications of diabetes. Strengthening the immune system
and controlling weight gain and obesity would assist in the overall management of dia-
betes and related conditions, as depicted earlier in Figure 23.

To our knowledge, this is the first study of the potential use of Australian Aboriginal in
the management of diabetes and related complications. The Australian medicinal plants
investigated in this study (SS, AL, ED, BL, AK, AT and SL) have been used for general
illness, cold, cough and pain (Palombo and Semple, 2001b). The traditional hunter-
gatherer lifestyle and diet of Aboriginal people, which was high in carbohydrates, fibre,
proteins and nutrients but low in fat and sugars, meant that cardiovascular diseases and
diabetes were not common in these people. After the settlement of Europeans, the diet
became Westernized with high sugar and fat content and the lack of essential nutrients,
vitamins, minerals, proteins and fibre. This has increased the disease risk and these dis-
orders are now prevalent in indigenous populations with the incidence of type 2 diabetes
rapidly increasing (Diamond, 2003, Gulati et al., 2012).

This thesis has evaluated the biochemical and cellular properties of a number of plant
extracts with potential application in the management, treatment and prevention of di-
betes and related conditions. Tables 14 and 15 summarize the results obtained with dif-
ferent bioassays. In addition, Figure 24 summarizes the activities of these extracts.
### Table 14 - Results summary of Australian Aboriginal plant extracts

For antibacterial assays: + denotes activity against 1 species, ++ against 1-3 species and +++ >3 species; for antioxidant and enzyme inhibition assay: + denotes IC$_{50}$ >200 µg/ml, ++ denotes IC$_{50}$ value 50-200 µg/ml and +++ IC$_{50}$ value <50 µg/ml; for glucose uptake assay: + denotes glucose uptake is stimulated by 10-30 %, ++ denotes 30 to 50 % and +++ denotes >50 %; for adipogenesis assay: + denotes lipid accumulation is reduced by 0-30 %, ++ between 30 and 60 % and +++ >60 %; for antileukemic assay: + denotes IC$_{50}$ >200 µg/ml, ++ IC$_{50}$ between 30 and 200 µg/ml and +++ IC$_{50}$ <30 µg/ml.
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Table 15 - Results summary of Indian Ayurvedic plant extracts

The symbols used are as for Table 14; n.d = not done
6.2. **Future directions**

The conclusions and recommendations outlined in this chapter relate to the original aims and outlines of the study. A summary of the study findings was included in the individual chapter.

The present study details an investigation of the bioactive properties of traditional medicinal plant extracts. This included activity against common microbes and free radical scavenging properties, however, the main focuses was the potential role in the management of hyperglycaemia. The results of this study demonstrated that many of the plants had broad-spectrum antimicrobial activity, good anti-oxidant activity and promising anti-diabetic potential. Further studies should include *in vivo* experiments to examine the effectiveness of the extracts in animal models.

On the basis of results of this study, extracts from AK (*Acacia kempeana*), AL (*Acacia ligulata*), AT (*Acacia tetragonophylla*), SS (*Santalum spicatum*), BL (*Beyeria
leschnaultii), ED (Euphorbia drumondii) and CO (Curculigo orchioides) were deemed to be the most active. Therefore, these plants should be further explored and detailed phytochemical analysis, isolation of active compounds and their characterisation through bio-activity testing should be carried out with cell culture assays and animal models. In particular, given the promising anti-diabetic potential of these plants, extracts or purified compounds should be examined in diabetes mouse models (Cheng et al., 2012, Kolb, 1987). Encouraging results would then allow the bioactive compounds to be developed as drug candidates and used in human clinical trials.

The other approach with moderately active plant extracts such as SL (Santalum lanceolatum), AP (Andrographis paniculata), BM (Bacopa monneirei), PM (Pterocarpus marsupium) and MP (Mucuna pruriens) could be to develop them into nutraceuticals.
References


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