BIOLOGICAL AND PHYTOCHEMICAL STUDIES ON SOME TRADITIONAL ANTI-DIABETIC PLANTS

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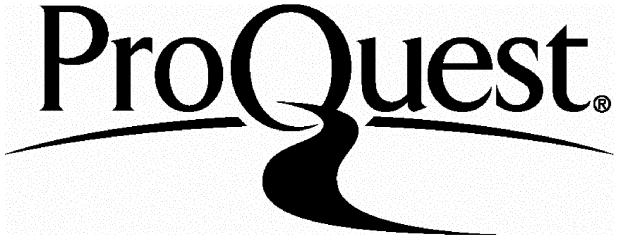

ABSTRACT

This current study aims to investigate the effect of selected medicinal plants on anti-diabetic activity, focusing on potential inhibitory effects on intestinal glucose absorption and a stimulatory effect on insulin secretion from pancreatic β-cells using two different *in vitro* models, brush border membrane vesicles (BBMV) and an insulin secreting cell line - RINm5F cells. Plants used in the studies were selected based on their reputations as anti-diabetic remedies in some countries with varying degrees of scientific evidence to support their traditional use.

Investigation of the effect of the aqueous extracts of the selected plants revealed four plants which were capable of causing marked inhibition of glucose uptake into BBMV. These plants were *Lycium chinensis* (Solanaceae), *Piper longum* (Piperaceae), *Pterocarpus marsupium* (Leguminosae) and *Salacia reticulata* (Celastraceae). The results suggested that the inhibition observed in these cases was not associated with glucose in the extracts which could interfere with the assay, as the extracts appeared to contain negligible amount of glucose. There was a possibility that these plants contained active constituents that were capable of inhibiting a transport activity of SGLT1, a transporter of glucose across the brush border. This is the first study to report the effect of these plants on this model. Bioassay-guided fractionation of *L. chinensis* afforded the isolation of a pure compound which, by means of NMR and Mass spectrometry, was identified as sitosterol glucoside.

Investigation of the effect of aqueous extracts of the selected plants on RINm5F cells revealed two plants which were capable of stimulating insulin secretion. These plants were *Anemarrhena asphodeloides* (Liliaceae) and a member of the genus *Parvatia* (Lardizabalaceae). The effect of these plants did not appear to be associated with cell membrane damage as judged by lack of appreciable LDH leakage from the cells after exposure to the extracts. However, no further study was conducted on *Parvatia spp.*, since the identity of this plant was ambiguous. Bioassay guided fractionation of *A. asphodeloides* methanolic extract which was found to have the most potent activity resulted in the isolation of 4 compounds i.e. mangiferin, mangiferin glucoside, timosaponin AIII and timosaponin BI. Of these, mangiferin was able to stimulate the secretion of insulin from both RINm5F cells

and rat islets. The effect of mangiferin was found to be potentiated by the presence of nutrient, suggesting that the compound may have potential advantages for use in the treatment of NIDDM as it would cause greater insulin release following a meal than in basal conditions. It appeared that the stimulation of insulin release caused by mangiferin was associated neither to PKA nor PKC pathway in the secretory response. Mangiferin glucoside was found to cause a significant secretion of insulin from RINm5F cells but not in rat islets. Unlike mangiferin, the effect of mangiferin glucoside did not appear to be potentiated by the presence of nutrient. Timosaponin AIII was found to be a very potent insulin secretagogue, the compound as low as 1-8 μg/ml was found to cause a significant release of insulin from RINm5F cells without damaging cell membranes. In contrast to timosaponin AIII, timosaponin BI was found to have no effect on insulin secretion from RINm5F cells. Besides these four compounds, two other compounds were also isolated from non active fraction of methanolic *A. asphodeloides* extract i.e. *cis* hinokiresinol and 4′-methyl-*cis*-hinokiresinol.

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ABBREVIATION AND SYMBOLS

ADP	Adenosine diphosphate
cAMP	Cyclic adenosine-3', 5'- monophosphate
ATP	Adenosine triphosphate
BBMV	Brush border membrane vesicles
BMI	Body mass index
BSA	Bovine serum albumin
$CHCl_3$	Chloroform
CKK	Cholecystokinin
Co A	Coenzyme A
COSY	Correlation spectroscopy
DAG	Diacylglycerol
DMEM	Dulbecco's modification Eagle's medium
DMSO	Dimethyl sulphoxide
EtOH	Ethanol
FCPD	Fibrocalculous pancreatic diabetes
FPG	Fasting glucose plasma
GDM	Gestational diabetes mellitus
GIP	Gastric inhibitory polypeptide
GLP	Glucagon-like peptide
HLA	Human leukocyte group A
hr	Hour
Hz	Hertz
IDDM	Insulin dependent diabetes mellitus
i.p.	Intraperitoneal administration
IP_3	Inositol 1, 4, 5- triphosphates
i.v.	Intravenous administration
J	Nuclear spin-spin coupling constant (in Hz)
kg	Kilogram
L	Litre
LDH	Lactate dehydrogenase
M	Molar
M^+	Molecular ion
MeOH	Methanol

ml	Millilitre
mM	Millimolar
MS	Mass spectroscopy
m/z	Mass to charge ratio
nM	Nanomolar
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NEFA	Non esterified fatty acid
NMR	Nuclear magnetic resonance spectroscopy
OGTT	Oral glucose tolerance test
PBS	Phosphate buffered saline
PDPD	Protein deficient pancreatic diabetes
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol-12-myristate, 13-acetate
p.o.	Oral administration
Rp-cAMPs	[R-isomer]-cyclic 3′, 5′ monophosphorothioate
SD	Standard deviation
SEM	Standard error of mean
STZ	Streptozotocin
TPA	Phorbol 12-*O*-tetradecanoylphorbol 13-acetate
UDP	Uridine diphosphate
VIP	Vasoactive intestinal polypeptide
μg	Microgram
μM	Micromolar
δ	Chemical shift (in ppm)

DETAILS OF PUBLICATIONS

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In vitro bioassay guided fractionation of selected anti-diabetic plants at Kew-King's meeting, December 1, 1999

Poster presentations

Effects of extracts of Lupinus species, *Alternanthera halimifolia* and *Xanthorrhiza simplicissima* on insulin secretion *in vitro* at "2000 years of natural Product research - Past, present, Future" Amsterdam, the Netherlands, July 26-30, 1999

Potential anti-diabetic effects of *Anemarrhena asphodeloides* rhizome: a study in RINm5F cells at "2000 years of natural Product research - Past, present, Future" Amsterdam, the Netherlands, July 26-30, 1999

In vitro screening of medicinal plants for potential anti-diabetic effects at 135th British Pharmaceutical Conference, Eastbourne, UK, September 8-11, 1998

Investigation of traditional anti-diabetic remedies for inhibition of intestinal glucose absorption and stimulation of pancreatic insulin secretion at 39th Annual meeting of the American Society of Pharmacognosy, Orlando, July 19-24, 1998

In vitro screening of medicinal plants for potential anti-diabetic effects at "Medicines from Nature", Royal Society of Medicine, London, June 9-10, 1998

A comparative study of Aesculus hippocastanum and Aesculus indica at 134th British Pharmaceutical Conference, Scarborough, UK, September 15-18, 1997

In vitro screening of reputed anti-diabetic plants for potential inhibitory effects on glucose uptake from the intestine at International Symposium on Bioassay Methods in Natural Product research and Drug Development, Uppsala University, Sweden, August 24-27, 1997

CHAPTER 1

GENERAL INTRODUCTION

1.1 Diabetes mellitus

Diabetes mellitus is the name given to a group of chronic disorders of metabolism caused by an absolute or relative deficiency of insulin. It is characterised by hyperglycaemia in the fasting and/or postprandial state. When present for prolonged periods, diabetes is complicated by the development of microvascular diseases (e.g. retinopathy, neuropathy and nephropathy) and macrovascular diseases (e.g. coronary artery disease, peripheral vascular disease and stroke). Diabetes is now a leading cause of adult blindness, amputation, renal failure and heart attacks (Foster, 1989).

A diagnosis of diabetes is made on the basis of patients' clinical symptoms including polyuria, polydipsia, polyphagia and glycosuria associated with a high fasting blood or plasma glucose level. Diabetes is diagnosed with unequivocal results when the random venous plasma glucose level is greater than 15 mmol/l (Keen and Barnes, 1997). The difficulty arises when the fasting glucose level falls within 'uncertain range' (8.0-15.0 mmol/l for random venous plasma level). In this case a standardised blood glucose test such as oral glucose tolerance test (OGTT) may be required to confirm the diagnosis. In OGTT, blood glucose concentration is determined while fasting and also at 2 hours after oral administration of 75 g glucose. The diagnostic criterion for diabetes, based on OGTT results proposed by WHO is shown in Table 1.1 (Keen and Barnes, 1997). A positive diagnosis is made when the fasting venous plasma glucose level is greater than 7.8 mmol/l and the level is greater than 11.1 mmol/l two hours after glucose load. Impaired glucose tolerance is diagnosed when the fasting venous blood glucose level is less than 7.8 mmol/l and the level is between 7.8-11.0 mmol/l two hours after glucose load.

Glucose homeostasis is controlled primarily by the hormone insulin. Insulin is synthesized and secreted from β-cells of islets of Langerhans which form 1-4% of the pancreatic mass. Islets contain several types of endocrine and paracrine cells; 60-80% are insulin-secreting β-cells; 15-20% are glucagon-secreting α-cells; 15-20% are pancreatic polypeptide (PP)-secreting F-cells; 5-10% are somatostatin-secreting D-cells; a small number of D-, EC- and δ-cells contain bombesin and vasoactive intestinal polypeptide (VIP), substance P and gastrin, respectively (Felig and Berglund, 1994).

Table 1.1: Values of glucose level used as a diagnostic criteria for diabetes and impaired glucose tolerance

	Glucose level (mmol/l)			
	Plasma		Whole blood	
	Venous	Capillary	Venous	Capillary
Normal				
Fasting value	< 7.8			
2 h after glucose load	< 7.8			
Impaired glucose tolerance				
Fasting value	< 7.8	< 7.8	< 6.7	< 6.7
2 h after glucose load	7.8-11.0	8.9-12.1	6.7-9.9	7.8-11.0
Diabetes mellitus				
Fasting value	≥ 7.8	≥ 7.8	≥ 6.7	≥ 6.7
2 h after glucose load	≥ 11.1	≥ 12.2	≥ 10.0	≥ 11.1

Insulin is initially synthesized in the polysomes of rough endoplasmic reticulum as preproinsulin which is rapidly converted to a polypeptide, proinsulin, within minutes of its synthesis (Felig and Bergman, 1994). Proinsulin (Fig. 1.1) is converted to insulin in Golgi apparatus by enzymatic cleavage of the connecting peptide (C-peptide). The length of C-peptide in higher vertebrates varies between 26-38 residues, that in human consists of 35 amino acids (Bailyes *et al.* 1992). Insulin is stored in secretory granules as a hexamer made up of three dimers with 2 zinc ions. Each insulin molecule consists of 51 amino acid residues with a molecular weight of 5808 Da. The molecule consists of two polypeptide chains designated A (21 amino acid residues) and B (30 amino acid residues) and joined by two disulphide bonds with an additional disulphide bond between the sixth and the eleventh amino acid residues of the A chain (Wood and Gill, 1997). Insulin is highly conserved throughout nature; human insulin differs from porcine by only one amino acid (B 30) and from bovine only by three amino acids (A8, A10, B30) (Wood and Gill, 1997). Splitting of A and B chain by oxidation or reduction of the disulphide bridges results in complete loss of biological activity (Felig and Bergman, 1994).

Insulin secretion occurs typically in a biphasic pattern. An abrupt increase of plasma glucose level elicits a rapid and transient burst of insulin secretion known as the first or acute phase of insulin response; the first phase persists for 2-5 minutes. The second phase of insulin secretion increases slowly but progressively and continues for the duration of the exposure to high glucose level. It has been thought that the pulsatile secretion of insulin may help to prevent down-regulation of insulin receptors on its target tissues, thereby maintaining insulin sensitivity (Matthews and Clark, 1997). The first phase of insulin secretion is believed to be related to a release of insulin granules which are adjacent to the cell membrane. The second phase response is thought to be related to pre-formed granules and newly synthesised insulin. In normal and healthy subjects, fasting plasma insulin level is ~ 5-15 μU/ml which rises to 50-100 μU/ml after a meal or glucose load.

C-peptide and proinsulin are also released from the secretory granules alongside insulin. In normal subjects, the amount of proinsulin released from the granules is approximately 2-5% of total insulin released in normal subjects. Increased proinsulin secretion indicates either decreased clearance of proinsulin and/or abnormal conversion of proinsulin to insulin (Kitabachi *et al.* 1990). Although, proinsulin has

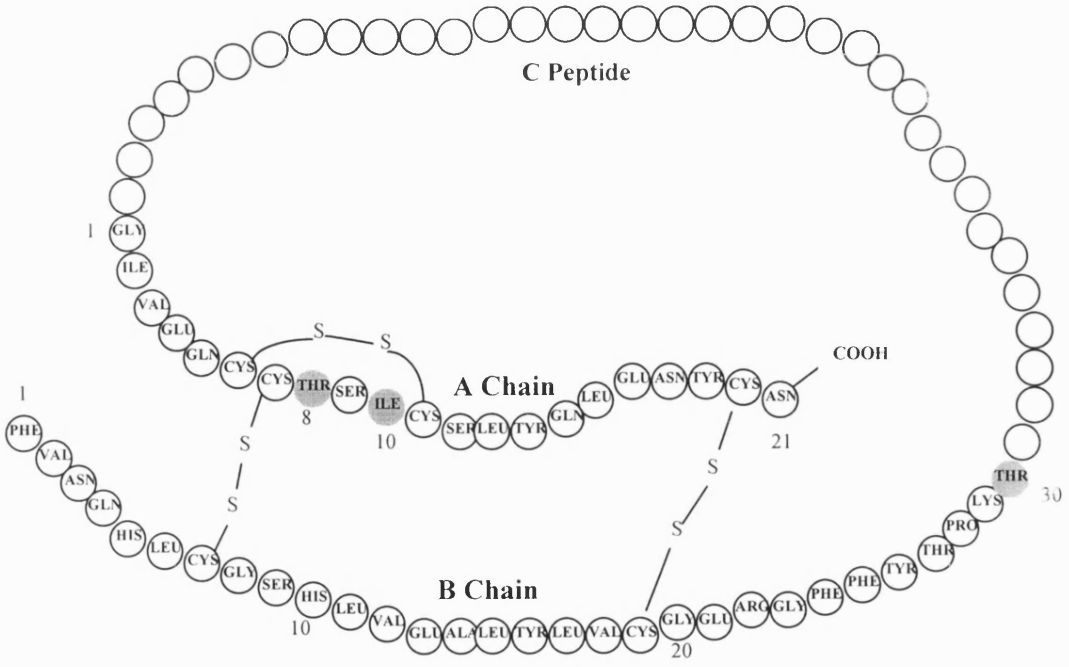


Fig. 1.1 : Amino acid sequence of human proinsulin

insulin-like effects *in vivo*, its activity is only 8-15% that of insulin (Kitabachi *et al.* 1990). C-peptide does not appear to have any physiological activity (Kitabachi *et al.* 1990).

Diabetes can be classified into six categories according to World Health Organization (WHO) (Keen and Barnes, 1997):

1 Insulin-dependent diabetes mellitus (IDDM)
2 Non insulin-dependent diabetes mellitus (NIDDM)
3 Malnutrition related diabetes mellitus (MRDM)
4 Diabetes associated with other conditions and syndromes
5 Gestational diabetes
6 Impaired glucose tolerance

These forms of diabetes are described in the following sections.

1.1.1 Insulin-dependent diabetes mellitus (IDDM, type I diabetes)

Insulin-dependent diabetes mellitus (IDDM), previously known as type I diabetes or juvenile-onset diabetes is characterised by the requirement for exogenous insulin to sustain life, absence of obesity in most patients and a tendency to ketosis (Felig and Bergman, 1994). IDDM mostly occurs at an age below 40; the disease has a peak incidence of onset between 5-15 years of age (Durinovic-Belló, 1998). Only about 25% of IDDM patients develop the disease after 35 years of age (Durinovic-Belló, 1998). The classical symptoms of IDDM are polyuria, thirst, tiredness and weight loss due to fat and protein catabolism. Other minor symptoms include muscular cramps, skin infections, candidiasis and blurred vision due to osmotic changes in the lens (Jones and Gill, 1997a).

It is believed that absolute deficiency of insulin (insulinopenia) in IDDM is a consequence of autoimmune destruction of β-cells. Evidence in support of the role of autoimmunity in the pathogenesis of IDDM is derived from a number of observations including the infiltration of mononuclear cells into β-cells (insulitis) (Robinson and Johnston, 1993) and the presence of autoantibodies to the islet cells e.g. islet cell antibody (ICA), islet cell surface antibody (ICSA), and insulin autoantibodies (IAA) in the serum of newly diagnosed patients. Although a genetic determinant is necessary in

the pathogenesis of IDDM, it has been shown that the genetic factor alone is insufficient to cause IDDM; an environmental factor is required to trigger pathogenic events (Unger and Foster, 1998).

Susceptibility to IDDM appears to be genetically determined, at least in part, by human leukocyte antigen (HLA) genes, particularly the D-region. HLA genes lie within major histocompatibility complex (MHC) region on the short arm of the sixth chromosome. The genes encode HLA molecules which are cell-surface glycoproteins and have a key role as immune regulators. The HLA molecules are classified into 3 subgroups i.e. class I, class II and class III. Class II molecules are normally expressed only on certain cells of immune system e.g. B lymphocytes, macrophages and some activated T lymphocytes. The major function of class II molecules is to bind and present the processed antigens (self or foreign) to T helper lymphocytes which recognise the antigens only when they are presented by a cell expressing the same class II molecules (Bain *et al.* 1997). According to Botazzo *et al.* (1985), the autoimmune process in IDDM is initiated by an aberrant expression of HLA class II molecules on β-cells which is thought to be, at least partially, induced by cytokines, particularly γ-interferon, released as a consequence of cell injury caused by viral or other infections (Foster, 1989). A number of viruses have been implicated in the development of IDDM including Coxsackie B4, mumps, rubella, cytomegalovirus, retrovirus, Epstein-Barr, varicella zoster, measles, polio, influenza and hepatitis A (Yoon, 1997). In genetically predisposed individuals, the aberrant expression of class II molecules on β-cells allows the presentation of self antigens to immune systems in such way that the antigens are recognised as foreign antigens which leads to the autoimmune destruction of β-cells. Alternatively, the autoimmune process has been postulated to be a consequence of cross-reactive response between autoantigens and viral antigens which share the same amino acid sequence; the process is known as molecular mimicry (Baker, 1997). A number of β-cell autoantigens have been implicated in the pathogenesis of IDDM including insulin, glutamic acid decarboxylase (GAD) and tyrosine phosphatase IA2 (Durinovic-Belló, 1998). However, these concepts are not yet conclusive and remain to be defined.

The pathology of the pancreas in IDDM is characterised by atrophy of the islets (Robinson and Johnston, 1993). The total number of β-cells in the pancreas markedly decrease to less than 10% of normal, whereas the number of alpha and delta cells is normal. Islets from patients with long term diabetes are usually devoid of β-cells (Christie, 1992). One of the prominent causes of morbidity and mortality in IDDM patients is diabetic ketoacidosis (DKA) which is characterised by hyperketonaemia and metabolic acidosis. The diagnosis of DKA is based on a high blood ketone body (acetoacetate and 3-hydroxybutyrate) concentration (> 5mmol/l) and/or a low plasma bicarbonate concentration (≤ 15 mmol/l) (Krentz and Nattrass, 1997). DKA is initiated by an excessive production of hepatic ketone bodies (acetoacetate, acetone and β-hydroxybutyrate) which results from disturbed lipid metabolism induced by severe insulin deficiency and an excessive level of glucagon. The major symptoms of DKA are nausea and vomiting, abdominal pain, deep and rapid respiration (Kussmaul breathing), and dehydration. The excessive production of acetone is also reflected by the characteristic fruity odour on the patients' breath.

1.1.2 Non insulin-dependent diabetes mellitus (NIDDM, maturity onset diabetes, type 2 diabetes)

NIDDM is a complex association between deficient insulin secretion and defects in the ability of insulin to exert its effect on peripheral tissues (insulin resistance). The level of circulating insulin in established NIDDM patients may be normal, low or high when considered in absolute terms (μU/ml plasma). However when compared with insulin levels of non diabetic subjects whose blood glucose is purposely raised to match the diabetic values, the level of insulin in NIDDM appears to be significantly lower. (Robinson and Johnston, 1993). NIDDM mostly occurs in middle-aged or elderly individuals and is generally associated with obesity (especially abdominal obesity) and insulin resistance. The disease accounts for around three quarters of all patients with diabetes (Brown, 1998). Unlike IDDM, the metabolic breakdown and overproduction of ketone bodies (ketoacidosis) are uncommon in NIDDM; signs of autoimmune damage are not seen in NIDDM islets. The precise cause of NIDDM is still unclear. It appears that the aetiological factor of NIDDM is

genetic rather than environmental, as suggested by high rate of concordance (60-100%) of NIDDM in identical twins (Foster, 1989). Despite extensive studies on the genetic determinant of NIDDM, precise genetic factors involved in the pathogenesis of NIDDM and an universal genetic mutation responsible for the development of NIDDM in all ethnic group have not yet been elucidated.

The loss of insulin release in response to glucose is a major defect observed in NIDDM (Felig and Bergman, 1994). In addition, the pulsatile secretion which is a characteristic feature of normal insulin secretion is also impaired in NIDDM (Matthews and Clark, 1997). The precise mechanism responsible for defective insulin secretion in NIDDM is still unclear. The suggested causes responsible for β-cell dysfunction include genetic defects of the specific proteins involved in the insulin secretion signalling pathway, the accumulation of islet amyloid and sustained hyperglycaemia i.e. glucose toxicity (Matthews and Clark, 1997). A typical feature of NIDDM pancreas is the formation of fine insoluble amyloid fibrils between islet cells and capillaries (Westermark, 1996). The islet amyloid fibril is formed from a polymer of amylin (also known as islet amyloid polypeptide (IAPP)), a 37 amino acid peptide which is a normal secretory product of pancreatic β-cells and is co-secreted together with insulin. According to a high prevalence (~90%) of islet amyloid in NIDDM (Kahn *et al.* 1999) and the absence of islet amyloid accumulation in normoglycaemic subjects (Westermark, 1996), attention has been given to the role of islet amyloid in the pathogenesis of NIDDM. A number of studies have shown an association between the islet amyloid and the death of β-cells (Lorenzo *et al.* 1996). One hypothesis proposed to explain this event is that the formation of islet amyloid fibrils may replace islet cells, leading to a reduction of β-cell mass and an alteration of β-cell function (Kahn *et al.* 1999). To date, pathophysiological importance of islet amyloid in NIDDM is not conclusive and remains to be proven. It is still unclear whether the formation of islet amyloid precedes the onset of glucose intolerance or if it is formed secondarily after the onset of diabetes. Further study in this area is therefore necessary.

This class of diabetes (NIDDM) also includes 'maturity onset diabetes in the young' (MODY) which is always associated with autosomal dominant inheritance. The genetic mutation responsible for MODY is thought to be associated with chromosome

7p which encodes the enzyme glucokinase or non-glucokinase-related chromosome i.e. chromosome 20q or chromosome 12q in which the encoded gene has not yet been identified (Hattersley and Clark, 1997).

1.1.2.1 Insulin resistance

The term 'insulin resistance' refers to the disorder where the ability of tissue to respond to glucose-lowering and other actions of endogenous or exogenous insulin are impaired. The sites of insulin resistance are skeletal muscle, liver and adipocytes. Insulin resistance can lead to defects in disposal of glucose in skeletal muscle, an overproduction of hepatic glucose, and impaired adipose tissue lypolysis which subsequently result in the generation of non-esterified fatty acids (NEFA). The role of muscle as a major important site for insulin resistance has been suggested in a number (DeFronzo *et al.* 1985; Beck-Nielsen, 1989), but not all studies (Gerich, 1991; Reaven, 1995). The major causes of insulin resistance are thought to be functional abnormalities at the cellular level such as the presence of circulating anti-insulin antibodies, structural abnormality of the insulin receptor, a diminished number of insulin receptors, abnormality of the intracellular pathway of insulin action, particularly defective tyrosine kinase activity and/or a decrease in the number of glucose transporters in muscle and adipose tissue (Robinson and Johnston, 1993).

It is still controversial whether insulin resistance or β-cell dysfunction has a primary role in pathogenesis of NIDDM. The role of insulin resistance as the initial defect in the development of NIDDM has been postulated in a number of studies (Taylor *et al.* 1994; Ferrannini, 1998), but not all (Cerasi, 1995). The observation that insulin resistance is demonstrated in diabetes-prone subjects (e.g. first degree relatives of NIDDM patients and high risk ethnic groups such as Pima Indians) even before the disease is diagnosed suggests that insulin resistance is an early defect in NIDDM (Alberti and Zimmet 1998,). However it has been demonstrated that, without impaired insulin secretion, insulin resistance alone is insufficient to induce the development of overt hyperglycaemia as seen in NIDDM patients (Felig and Bergman, 1994). A strong support to the latter concept arises from the study in obese non-diabetic subjects (Cerasi, 1995). Despite the fact that insulin resistance is dominant in virtually all obese

subjects, there are substantial numbers of these subjects who retain normal glucose tolerance (Cerasi, 1995).

The scheme in Figure 1.2 is a hypothetical sequence demonstrating one of the possible interactions between insulin resistance and β-cell dysfunction in the development of NIDDM. In the early stages of NIDDM, β-cells respond to an increased plasma glucose level by increasing insulin secretory capacity in an attempt to maintain normoglycaemia. This ultimately leads to hyperinsulinaemia (Jones and Gill, 1997b). In turn, a prolonged increase in insulin secretion could lead to exhaustion of β-cells. As diabetes progresses, it appears that β-cells can no longer secrete enough insulin to overcome the defect in insulin action (DeFronzo *et al* 1992). Overt hyperglycaemia (fasting glucose level > 140 mg/dl) would develop when maximal insulin secretory capacity of β-cells is exceeded (Matthews and Clark, 1997). This is reflected by a decline in the level of circulating insulin (Yki-Järvinen and Williams, 1997).

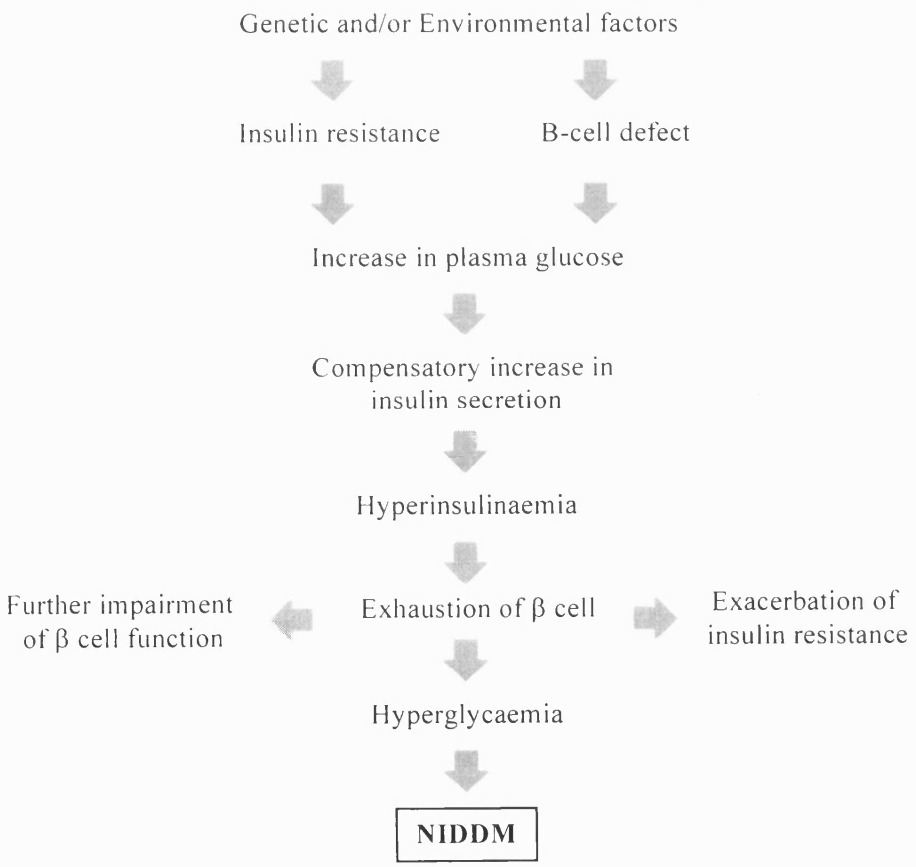


Fig. 1.2: Hypothetical scheme for the development of NIDDM (modified from Gerich, (1988) and Jones and Gill, (1977b))

1.1.3 Malnutrition related diabetes mellitus (MRDM)

MRDM generally occurs in tropical, developing countries in which young diabetics often present with a history of nutritional deficiency. MRDM can be further classified into 1) fibrocalculous pancreatic diabetes (FCPD) and 2) protein-deficient pancreatic diabetes (PDPD).

Fibrocalculous pancreatic diabetes is characterised by stone formation in pancreatic duct (pancreatic calculi) and extensive fibrosis of the pancreas (Hattori and Zimmet, 1992). The majority of patients is diagnosed at the ages of 20-40, however onset in childhood and at older age is not uncommon (Mohan *et al.* 1998). Patients with FCPD usually present with severe abdominal pain, extreme decrease in muscle mass, sunken eyes and painless bilateral parotid gland enlargement (Mohan and Premalatha, 1997). Despite marked hyperglycaemia, ketoacidosis is absent in FCPD patients. The prevalence of microvascular disease in FCPD is similar to those seen in IDDM and NIDDM (Mohan and Premalatha, 1997). The precise aetiopathogenic mechanism for FCPD is still unclear. Aetiological factors which are thought to be related to the development of FCPD include protein-calorie malnutrition, genetic susceptibility, antioxidant deficiency and dietary toxins, possibly cyanogenic glycosides, linamarin and lotustralin, derived from tuber cassava (Mohan *et al.* 1998).

Protein-deficient pancreatic diabetes, also known as 'J-type diabetes' as it was first reported from Jamaica, is difficult to diagnose because of the lack of specific clinical features (Mohan and Premalatha, 1997). In addition to marked hyperglycaemia, the disorder is generally associated with extreme degrees of emaciation, ketosis resistance and insulin resistance whereas pancreatic calcification and fibrosis are absent (Hattori and Zimmet, 1992). The patients do not respond to sulphonylureas and require a large amount of insulin to control glycaemia (Hattori and Zimmet, 1992).

1.1.4 Gestational diabetes mellitus (GDM)

Gestational diabetes mellitus is defined as diabetes which first presents during pregnancy, thus excluding diabetic women who become pregnant. GDM is found in about 3% of pregnancies in Western Europe (Harris and Zimmet, 1992). According to WHO, GDM is diagnosed in pregnant women when plasma glucose level at 120 minutes after 75-g oral glucose tolerance test is greater than 7.8 mmol/l (Girling

and Dornhorst, 1997). GDM is usually associated with an increased risk of perinatal complications and foetal macrosomia (Harris and Zimmet, 1992). There is evidence that GDM is a major determinant for the later development of diabetes in the child (Dornhorst and Beard, 1993). The cause of GDM is not completely understood, it is thought to be due to complex metabolic and hormonal changes (Harris and Zimmet, 1992). The majority of women with GDM revert to normoglycaemia after the delivery, but there is an increased risk of developing IDDM or NIDDM (Girling and Dornhorst, 1997).

1.1.5 Diabetes associated with other conditions and syndromes

This class consists of various types of diabetes which are secondary to other conditions and syndromes. These syndromes include 1) pancreatic disease, 2) endocrinopathies e.g. cushing's syndrome, acromegaly, phaeochromocytoma 3) drug-induced or chemical-induced diabetes e.g. nicotinic acid, glucocorticoids, thyroid hormone, α- and β-adrenergic agonists, thiazides 4) abnormalities of insulin or its receptor e.g. insulinopathies, receptor defects and 5) certain genetic syndromes e.g. Wolfram syndrome, myotonic dystrophy, lipoatrophy and cystic fibrosis (Dornhorst and Beard, 1993; Keen and Barnes, 1997).

1.1.6 Impaired glucose tolerance (IGT)

Impaired glucose tolerance is a term used to define a stage of impaired glucose regulation where fasting plasma glucose is normal i.e. less than 7.8 mmol/l (venous plasma glucose level) whereas glucose tolerance is outside the normal range i.e. > 7.8 mmol/l (Table 1.1) (Keen and Barnes, 1997). It is considered as a metabolic state intermediate between normal glucose homeostasis and diabetes. Individuals with IGT are at higher risk for the development of diabetes and/or cardiovascular disease than normal subjects (Alberti and Zimmet, 1998).

1.2 Management of diabetes mellitus

1.2.1 Management of insulin dependent diabetes mellitus (IDDM)

Insulin therapy has a dominant role in a treatment of IDDM. Patients require regular administration of insulin not only to prevent metabolic breakdown but also to

prevent coma and death. Insulin commercially available for clinical use can be classified into 3 categories according to duration of action (Table 1.2). In 1996, the newest insulin product 'Insulin Lispro' has been introduced. Insulin Lispro (Humalog®, Lily) is a synthetic insulin analogue produced by changing 2 amino acids on the insulin molecule. Proline at the position B28 is replaced by lysine and lysine at the position B29 is replaced by proline (Fig. 1.1). Its onset after subcutaneous injection is about 15 minutes, and it has a duration of action of 4-5 hours (Burge and Schade, 1997).

Insulin regimen needs to be specified for individual patients depending on age, life style and severity of the disease. Intermediate-acting insulin (NPH or lente) is usually used in a combination with short-acting insulin (regular insulin), the mixture is given to patients twice daily, generally in the morning and before dinner or at bedtime. A major disadvantage of this regimen is an inflexibility in daily life of the patients since it is assumed that the patients should wake and have their meals at the same time each day. The use of long-acting insulin in combination with regular insulin may offer more flexibility to patients. In this regimen, long-acting insulin given at bedtime is used to ensure a basal level of insulin throughout 24 hours, and the short-acting insulin is given separately before each meal.

Table 1.2: Insulin preparations (modified from Burge and Schade, (1997))

	Peak (h)	Duration (h)
Short-acting insulin		
Regular	2-4	5-8
Intermediate-acting insulin		
Insulin zinc suspension amorphous (Semilente)	2-4	12-16
Insulin zinc suspension (Lente)	4-8	30
Globulin zinc insulin	6-8	18-24
Isophane insulin suspension and Neutral Protamine Hagedorn (NPH)	5-7	13-20
Long-acting insulin		
Protamine zinc insulin	12-16	36+
Insulin zinc suspension crystalline (ultralente)	8-10	30-36

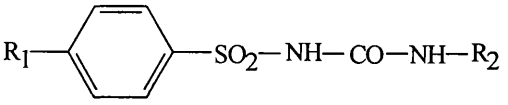
1.2.2 Management of non-insulin dependent diabetes mellitus (NIDDM)

The goal of NIDDM management is to maintain blood glucose level within the range associated with few diabetic complications i.e. HbA_{1c} below 7% and fasting plasma glucose (FPG) below 7.8 mmol/l (Riddle, 1997). The initial management of NIDDM should include diet and exercise. Drug therapy should be considered only when diet and exercise fail to maintain normal blood glucose level (Scheen and Lefèbvre, 1998). The selection of pharmacological therapy is generally based on patients' clinical characteristics (e.g. degree of hyperglycaemia, body weight, age, renal function) and pharmacological properties of the drugs (e.g. mode of action, adverse effects and safety profile) (Scheen and Lefèbvre, 1998). Oral anti-diabetic agents which have been extensively used in the treatment of NIDDM include sulphonylureas and biguanides. Thiazolidinediones, α-glucosidase inhibitors and benzoic derivative are more recent introductions.

1.2.2.1 Sulphonylureas

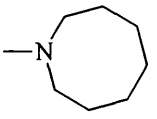
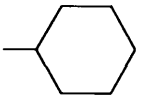
Sulphonylureas have been used in the treatment of diabetes since the late 1950s. They are the most widely used oral hypoglycaemic agents. The main pharmacological effect of sulphonylureas is to stimulate insulin secretion from β-cells (for mechanism of action see section 5.2.1).

Two generations of the compounds have been developed; they differ in terms of chemical structure and potency. The first generation compounds (e.g. tolbutamide and chlorpropamide, Fig. 1.3) have an aliphatic side chain. For the second generation compounds such as glibenclamide, glipizide and glimepiride (Fig. 1.3), the aliphatic side chain of tolbutamide has been replaced by a cyclohexyl group, and the benzene ring is also substituted with additional cyclic structures (Felig and Bergman, 1994). Those alterations lead to higher binding affinity for pancreatic β-cell membranes of second generation agents which is reflected by more potent activity and less adverse effects (Nelson *et al.* 1992). Second generation agents are now preferred to first generation agents. (Scheen and Lefèbvre, 1998). The use of individual sulphonylureas is determined by rate of onset, duration of action, mode of metabolism and excretion and their side effect profile (Lebovitz, 1992). Rapid onset drugs increase the rate of



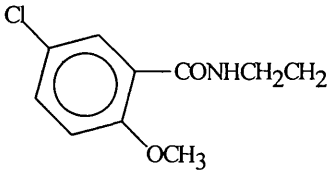
Sulphonylurea

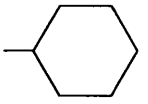
First generation agents

	R_1	R_2
Tolbutamide	CH_3-	$-(CH_2)_3CH_3$
Chlorpropamide	Cl-	$-(CH_2)_2CH_3$
Tolazamide	CH_3-	
Acetohexamide	CH_3CO-	

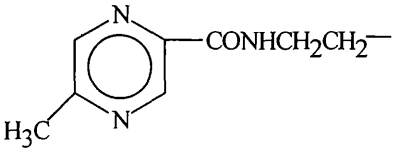
Second generation agents

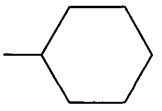
Glibenclamide (Glyburide)



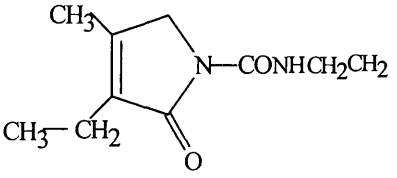


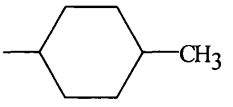
Glipizide



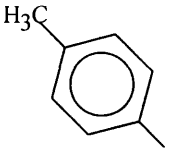


Glimepiride





Glicazide



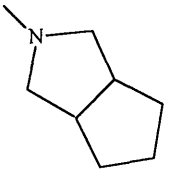


Fig. 1.3: Structure of sulphonylurea derivatives

insulin release, and short duration drugs decrease the risk of delayed hypoglycaemia (Lefèbvre and Scheen, 1992; Melander, 1996). Thus, the use of rapid onset and short-acting sulphonylureas can minimise the risk of chronic hyperinsulinaemia (Lefèbvre and Scheen, 1992).

Sulphonylureas are not absorbed until reaching duodenum, and this process is delayed by food intake. Thus, they should be given at least 30 minutes before a meal (Melander, 1996; Scheen and Lefèbvre, 1998). The dose of sulphonylureas should not be intensified if the initial dose is insufficient to control plasma glucose level since a continuous exposure of high doses of sulphonylureas may result in a downregulation of β-cell sensitivity (Melander, 1996). Instead, combined oral therapy with other oral anti-diabetic agents should be considered (Melander, 1996). Sulphonylureas should be avoided in obese patients and in patients with hepatic and renal diseases because the compounds are metabolised in liver and excreted in urine (Zimmerman, 1997). Long term treatment with sulphonylureas may induce desensitisation to these agents, possibly because of down regulation (Zimmerman, 1997) as mentioned earlier.

The major adverse effect of sulphonylureas is hypoglycaemia, particularly with chlorpropamide and glibenclamide (Scheen and Lefèbvre, 1998). Hypoglycaemia can be exacerbated by interaction with numerous drugs e.g. aspirin, sulphonamides, phenylbutazone, dicoumarol, clofibrate, monoamine oxidase inhibitors, NSAID and tricyclic antidepressants (Lefèbvre and Scheen, 1992).

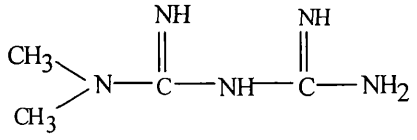
1.2.2.2 Biguanides

Metformin (Dimethyl biguanide, Fig. 1.4) is derived from the guanidine compound, galegine, which is an active compound isolated from *Galega officinalis* Linn. (Leguminosae), a plant used in Western traditional medicine as an anti-diabetic remedy (Marles and Farnsworth, 1995). Metformin is the only biguanide currently available for clinical use. Buformin and phenformin have been abandoned in most countries because of their association with lactic acidosis. Although lactic acidosis is rare with metformin, metformin should not be administered to patients at risk of lactic acidosis such as those with kidney or liver disease, cardiorespiratory insufficiency or

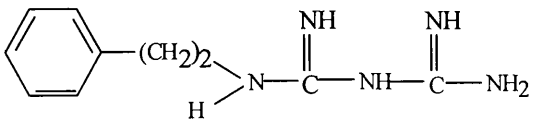
those who abuse alcohol (Lebovitz, 1992). The other side effects of metformin include anorexia, nausea, abdominal discomfort and diarrhoea (Lebovitz, 1992).

Although metformin has been used for over 30 years, the precise cellular effect of the drug on target sites is still unclear (Dunn and Peters, 1995). Metformin is known to work by decreasing hepatic glucose output, enhancing basal and insulin-stimulated glucose disposal and delaying gastrointestinal glucose absorption (Dunn and Peters, 1995). These actions of metformin lead to an improvement of insulin sensitivity. The improvement of peripheral glucose disposal is thought to be attributable to increase in non-oxidative glucose metabolism including glycogenesis, conversion of glucose to lactate and formation of triglycerides (Dunn and Peters, 1995). Metformin is found to reduce hyperinsulinaemia and promote weight loss (Scheen, 1997), thus it has been considered as the first line drug for obese diabetic patients (Scheen, 1997) and patients with insulin resistance syndrome (Scheen and Lefèbvre, 1998).

At the beginning of treatment, the recommended dose of metformin is 500 mg once or twice daily with or after meals (Dunn and Peters, 1995; Bell and Hadden, 1997). Patients can tolerate up to 2-2.5 g daily without gastrointestinal discomfort if the drug is given with cautious dose increase (Bell and Hadden, 1997). Metformin should be avoided in patients with decreased renal and liver function due to the risk of lactic acidosis (Scheen and Lefèbvre, 1998). Metformin, unlike sulphonylureas, is not associated with hypoglycaemia. The combination therapy of metformin and sulphonylureas has been proven to be more effective than monotherapy with either group of drugs (Davidson and Peters, 1997). In general, the combination would be used if the oral single treatment is inadequate to achieve the glycaemic control.



Metformin



Phenformin

Fig. 1.4: Structure of biguanides

1.2.2.3 α-Glucosidase inhibitors

Acarbose (Glucobay®, Bayer, Fig. 1.5) is the only α-glucosidase inhibitor currently available for clinical use. Other members of this class including miglitol and voglibose (Fig. 1.5) are currently under clinical development (Feinglos and Bethel, 1999). Acarbose is a reversible, competitive inhibitor of brush border α-glucosidase (mainly glucoamylase, sucrase and maltose) as well as pancreatic amylase (Bailey *et al.* 1997b). The anti-hyperglycaemic effect of α-glucosidase inhibitors is owing to their ability to interfere with the intestinal breakdown of carbohydrates into absorbable monosaccharides. This is achieved by binding with carbohydrate-binding regions of α-glucosidase enzyme, and thus competing with the binding of oligosaccharides (Lebovitz, 1997). This action of acarbose results in incomplete digestion of carbohydrate, which ultimately leads to a reduction in postprandial rise of blood glucose and insulin levels (Scheen and Lefèbvre, 1998). Acarbose is preferable in NIDDM patients in whom postprandial hyperglycaemia is greater than fasting hyperglycaemia (Lebovitz, 1997). Acarbose does not appear to have any systemic

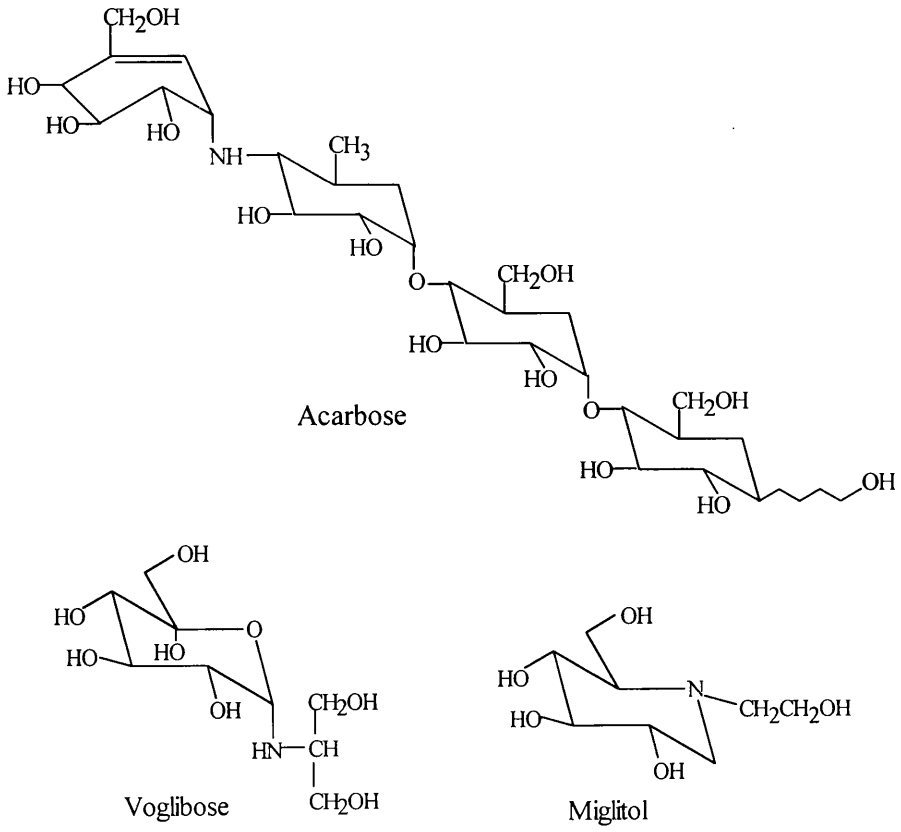


Fig 1.5: Structure of some α-glucosidase inhibitors

effect (Bailey *et al.* 1997b). The maximum effects of acarbose are achieved when it is given at a dose of 100 mg three times a day (Lebovitz, 1997). For the best glycaemic response, acarbose should be given 15 minutes before the initiation of meals (Bell and Hadden, 1997).

The major adverse effect of acarbose is gastrointestinal intolerance e.g. flatulence, diarrhoea and mild abdominal pain. Acarbose should be initially given at the minimal dose, and gradually increased to its maximum effective dose in order to limit gastrointestinal intolerance (Feinglos and Bethel, 1999).

1.2.2.4 Thiazolidinediones

Thiazolidinediones are a new class of oral anti-diabetic drugs that work by enhancing certain effects of insulin on glucose and lipid metabolism at target tissues (Plosker and Faulds, 1999). Thiazolidinediones are believed to exert their effects, at least in part, by binding to peroxisome proliferator-activated receptor gamma (PPARγ) (Day, 1999), which leads to the increased expression of a number of genes encoding proteins involved in glucose and lipid metabolism (Henry, 1997). In consequence, this results in an improvement of insulin sensitivity which is reflected by a reduction of plasma glucose.

The major concern with thiazolidinediones is the risk of serious hepatic dysfunction (Plosker and Faulds, 1999). Currently, no thiazolidinediones is available for clinical use. Troglitazone (Rezulin®, Fig. 1.6) and the other two thiazolidinediones i.e. englitazone and ciglitazone are abandoned from the market due to their severe side effect on hepatocytes (Scheen, 1997). Other drugs in this class including pioglitazone, darglitazone, and rosiglitazone are under clinical development (Scheen, 1997; Feinglos and Bethel, 1999).

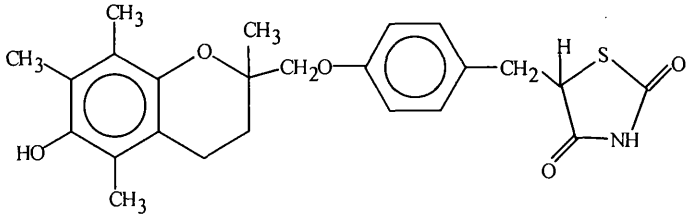


Fig. 1.6: Structure of troglitazone

Troglitazone is reported to have multiple therapeutic effects including suppression of hepatic gluconeogenesis, stimulation of GLUT1 and GLUT4 expression and translocation into the plasma membrane of skeletal muscle and fat, and lowering of triglyceride and NEFA levels (Henry, 1997; Plosker and Faulds, 1999). The action of troglitazone is dependent on the presence of insulin, confirmed by the observation that the best glycaemic control produced by troglitazone was achieved in patient who still secreted substantial amounts of insulin, whereas the patients who had extremely low basal insulin level did not respond to troglitazone (Plosker and Faulds, 1999). Troglitazone when given as monotherapy was not associated with hypoglycaemia because the agent did not stimulate insulin secretion (Lipkin, 1999). The combination therapy of troglitazone with sulphonylureas, metformin or insulin appears to further improve the glycaemic control than monotherapy with these agents (Day, 1999; Plosker and Faulds, 1999).

1.2.2.5 Benzoic acid derivatives

Agents derived from benzoic acid are novel class of insulin secretagogues which have recently been developed. Agents in this class are thought to work via the mechanism similar to those of sulphonylureas i.e. regulating of ATP-sensitive K^+ channel but via different binding site from sulphonylurea receptor (Scheen, 1997; Lewis, 1998). The agent is claimed to be glucose-sensitive secretagogue as its effect was reported to be more potent in the presence of stimulatory glucose concentration (Owens, 1998). The only agent in this class which is currently available for clinical use is repaglinide (Fig. 1.7, NovoNorm®, Novo Nordisk), a derivative of carbamoylmethyl benzoic acid. The starting dose of repaglinide in patients who have never been treated with anti-diabetic agents is 0.5 mg before each meal. For those who have previously been on medication for diabetes, a higher dose of 1-2 mg before each meal is recommended (Lewis, 1998). The rapid onset (15-30 minutes) and short duration of action (half life < 1 hour) of repaglinide may offer better prandial control over sulphonylureas, since the hypoglycaemic effect is likely to occur immediately after meals, unlike sulphonylureas whose hypoglycaemic effect may occur hours after meal (Owens, 1998). Repaglinide can be substituted for metformin in patients with gastrointestinal side effects without worsening glycaemic control. Repaglinide has been shown to be more

effective than glibenclamide in reducing postprandial glycaemia and has been shown to maintain better glycaemic control than glipizide in long term studies (Owens, 1998).

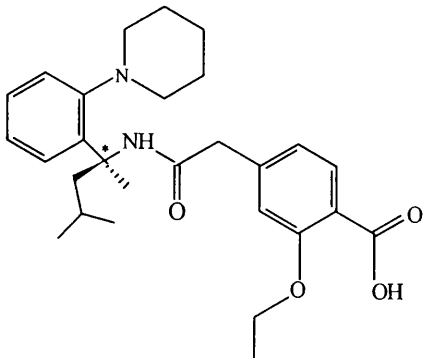


Fig. 1.7 : Structure of repaglinide

To date no serious adverse effect of repaglinide has been reported. However, repaglinide is not recommended in patients with hepatic impairment because the agent is metabolized primarily in liver (Lewis, 1998). Repaglinide is excreted mainly via the bile, thus it can be used in patients with renal impairment (Lewis, 1998). Other benzoic derivatives which are under clinical development include meglitinide, KAD-1229 and A-4166 (Fig. 1.8).

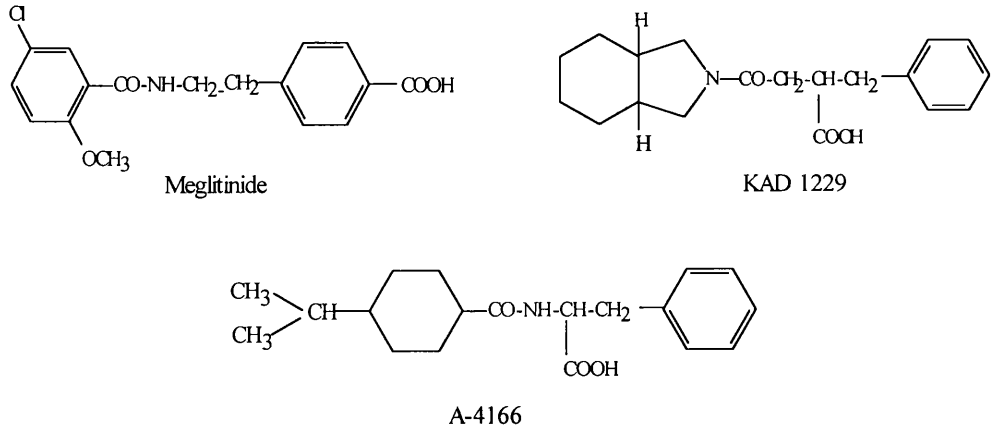


Fig. 1.8 : Structure of benzoic acid derivatives which are under clinical development

1.2.2.6 Therapeutic strategy in NIDDM

From a practical point of view, the selection of an oral agent for initial drug therapy is made by considering the level of plasma glucose (Fig. 1.9) (Riddle, 1997; Scheen and Lefèbvre, 1998). Patients with a fasting plasma glucose level below 7.8

mmol/l should be treated with those agents which are unlikely to cause hypoglycaemia i.e. metformin or acarbose (Riddle, 1997; Scheen and Lefèbvre, 1998). Metformin would be preferred in patients with mainly fasting hyperglycaemia (i.e. a capillary glucose level before breakfast is higher than the level before dinner). Acarbose is the first choice for the patients with postprandial hyperglycaemia (i.e. higher capillary glucose level before dinner than that before breakfast). If the patients have fasting plasma glucose level between 7.8-11.1 mmol/l, sulphonylureas would be the drug of choice for those who are mild to moderately overweight, whereas metformin would be preferred in patients who are more obese (body mass index > 30). In severely hyperglycaemic patients (fasting plasma glucose > 11.1 mmol/l), sulphonylureas would be the first line of choice.

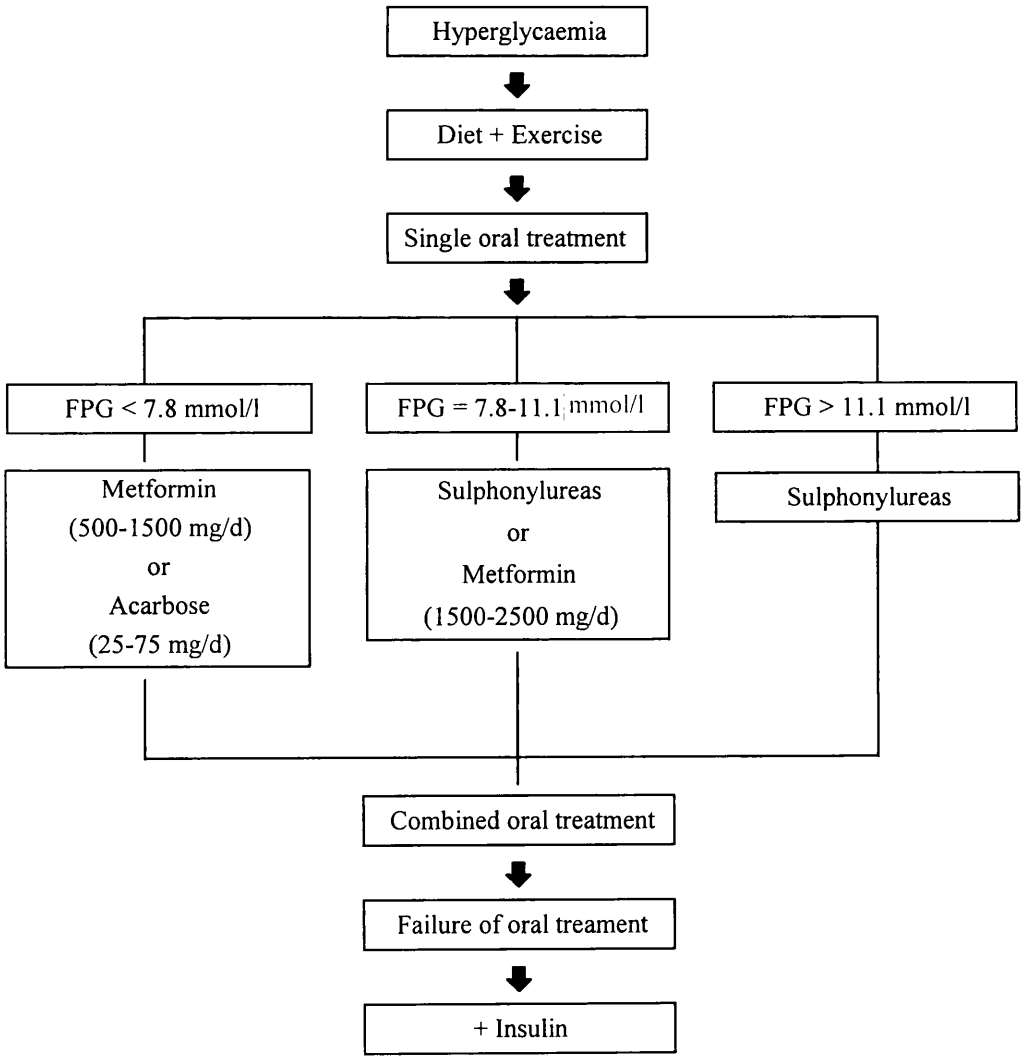


Fig. 1.9: A guide to selection of oral anti-diabetic agents for NIDDM (modified from Riddle, 1997). (FPG: Fasting plasma glucose)

Combined therapy would be considered only when a single oral agent treatment failed to maintain normal blood glucose level (Riddle, 1997; Scheen and Lefèbvre, 1998). In general, when using combined therapy, the initial drug is continued at the current dose in use, and the second drug is added at the recommended starting dose (Scheen and Lefèbvre, 1998). Many combinations of the different classes of oral agents have been suggested (Scheen and Lefèbvre, 1998), the combination of sulphonylureas and metformin has been the most widely used. The advantage offered by combined oral treatment lies in the different modes of action of the two agents (Riddle, 1997), resulting in an additive effect. Thus, a greater therapeutic effect can be achieved with moderate doses thereby reducing the adverse effects of the two agents. Insulin would be included in the therapeutic regimen only when combined oral agents failed to maintain normal blood glucose levels. At the beginning of combined therapy with insulin, intermediate-acting insulin may be given as a single dose at bedtime. For obese individuals (BMI > 30), 70/30 (NPH or lente/regular) insulin before dinner may be more effective (Riddle, 1997). If blood glucose remains greater than 11 mmol/l during the day, morning intermediate-acting insulin can be added to the therapy (Lipkin, 1999).

1.3 Candidates for novel anti-diabetic agents for the management of NIDDM

It is a difficult task to find new oral anti-diabetic agents that have both superiority over the standard drugs and minimal adverse effects. In the last 40 years, only two new types of anti-diabetic drugs i.e. acarbose and troglitazone have been introduced for clinical use (section 1.2.2). An extensive research has been taking place and a number of new agents have been developed in the last decade as potential novel oral anti-diabetic drugs (Table 1.3).

Excessive increase in the level of VLDL triglycerides and non esterified fatty acid (NEFA), which probably contributes to insulin resistance, is one of the characteristic features in IDDM and NIDDM (Bailey *et al.* 1997b). According to the glucose-fatty acid cycle proposed by Randle *et al.* 1963, glucose and NEFA can be used on a reciprocal basis as metabolic fuels by skeletal muscle and liver. Hence,

Table 1.3: Candidates for the development of new oral anti-diabetic agents

Agents	Action	Ref
Agents increasing insulin secretion		
S-22068	Blocking K^+_{ATP} channel	(1)
Agents delaying carbohydrate digestion		
Pramlintide (amylin analogue)	Reduction of gastric emptying	(2)
Agents reducing insulin resistance		
Nicotinic acid and Acipimox	Inhibition of lipolysis	(3)
Phenylisopropyladenosine (PLA)	Inhibition of lipolysis	(4)
SDZ WAG 994 (adenosine A1 receptor agonist)	Inhibition of lipolysis	(5)
Fibric acid derivatives e.g. Bezafibrate, Gemfibrozil	Stimulation of lipoprotein lipase	(6)
Etomoxir, Clomoxir and Methylpalmoxirate	Inhibition of CPT-I	(7)
PPIB	Inhibition of CPT-I	(8)
SDZ CPI 975*	Inhibition of liver CPT-I	(5)
SDZ 51-641	Inhibition of CPT-II	(5)
Aetiocholanolones	Suppression of hepatic gluconeogenesis. Increasing hepatic glucose oxidation	(3)
MTP-1307	Suppression of hepatic gluconeogenesis Increasing glucose oxidation in adipose tissue	(3, 9)
LY 177507	Inhibition of glycogen phosphorylase Activation of glycogen synthase	(1)
Vanadium compounds	Stimulation of glucose uptake and oxidation in adipose cells and skeletal muscle Stimulation of glycogen synthesis Inhibition of hepatic gluconeogenesis	(10)

* in phase 1 clinical trial, CPT: carnitine palmitoyl transferase

References: (1) Brigand *et al.* (1999); (2) Moyses *et al.* (1996); (3) Bailey *et al.* (1997b); (4) Reaven, (1988); (5) Rachman and Turner, (1995); (6) Goa *et al.* (1996); (7) Foley, (1992); (8) Haeckel *et al.* (1990); (9) Ogawa *et al.* (1992); (10) Brichard and Henquin, (1995)

increase in NEFA can inhibit the utilisation of glucose in skeletal muscle and liver. Intervention of lipid metabolism is therefore an alternative approach by which an improvement of glucose homeostasis may be achieved (Scheen, 1997). During the past 10 years, attention has been given to an attempt to develop agents that can reduce NEFA level released from adipose tissue for use in the treatment of diabetes. The agents which have been developed in this category include bezafibrate, gemfibrozil, SDZ WAG 994, and acipimox (Fig. 1.11). Bezafibrate and gemfibrozil which are fibric acid derivatives act, at least in part, by stimulating the activity of lipoprotein lipase, thereby promoting the catabolism of triglyceride-rich lipoprotein (Goa *et al.* 1996).

Inhibition of fatty acid oxidation is postulated as another therapeutic approach in the treatment of diabetes. Inhibition of fatty acid oxidation can be achieved by a number of mechanisms, one of which is by inhibiting translocation of fatty acid into mitochondria where the oxidation occurs. This process is dependent on the prior transfer of the acyl group from long chain acyl-CoA molecules to carnitine in a reaction catalysed by carnitine palmitoyl-transferase 1 (CPT-1). Intervention of fatty acid oxidation by inhibiting the activity of CPT-1 may therefore have beneficial on glycaemic control. To date, the agents in this class which have been developed include etomoxir, clomoxir, PPIB (Fig 1.12), and SDZ CPI 975. SDZ 51-641 is another agent that also act by inhibiting CPT activity, but its site of action differs from etomoxir, clomoxir and PPIB. SDZ 51-641 works by inhibiting the activity of CPT-II which, in mitochondria matrix, catalyses the transfer of the acyl group back to CoA (Rachman and Turner, 1995).

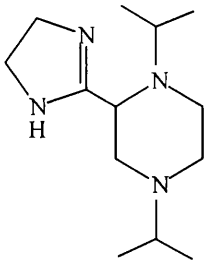


Fig. 1.10: Structure of S22068

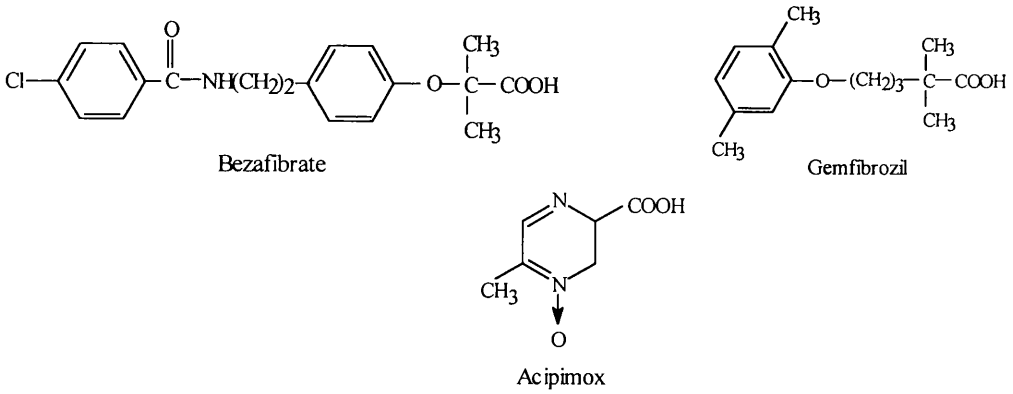


Fig. 1.11: Structure of some agents that act by lowering NEFA concentration

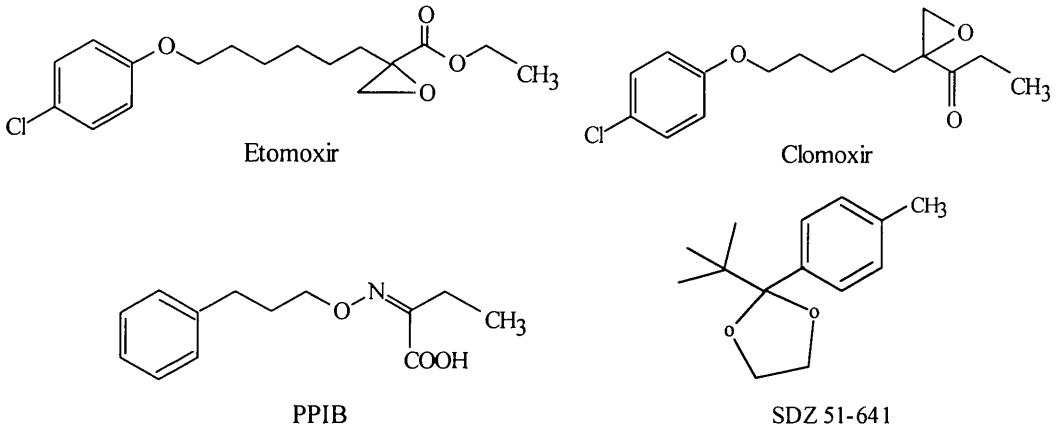


Fig. 1.12: Structure of agents that act by inhibiting CPT

A number of agents which work by inhibiting hepatic glucose production have also been developed, these included aetiocholanolone, MTP-1397 and LY 177507 (Fig. 1.13).

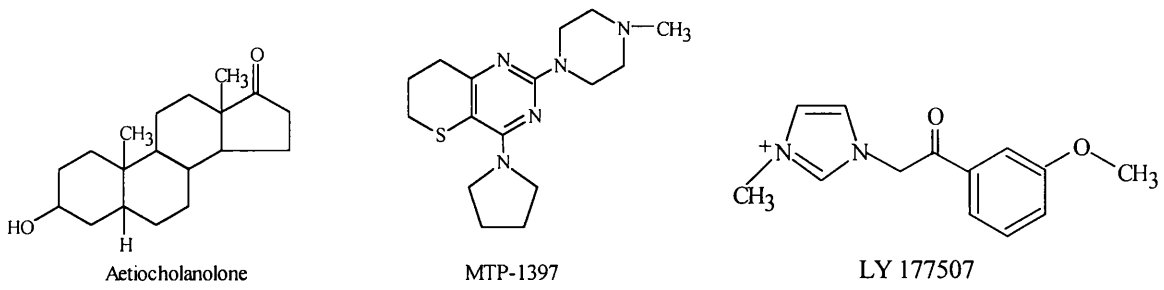


Fig. 1.13: Structure of agents that act by inhibiting hepatic glucose production

To date none of these agents (Table 1.3, Fig. 1.10-1.13) has become available for clinical use, only SDZ CPI 975 is now in phase 1 clinical trial (Rachman and Turner, 1995).

Another mode of action which has recently been a focus of attention is the delaying of gastric emptying. The only agent that works via such mechanism is pramlintide. Pramlintide (previously referred to as AC 137) is an analogue of human amylin which is a peptide synthesised and secreted from β-cells of pancreas, together with insulin. The deficiency of amylin has been reported in patients with IDDM and late stage NIDDM (Kolterman, 1997). Studies on pharmacological activity of amylin revealed that it reduced an elevation of plasma glucose concentration, following glucose load (Brown *et al.* 1994; Kolterman, 1997). Pramlintide is an amylin analogue where the amino residues of amylin at position 25 (alanine), 28 (serine) and 29 (serine) are replaced with proline. The replacement with proline is found to have no effect on biological activity but cause a significant improvement in terms of solubility and stability of the molecule (Moyses *et al.* 1996). Results from preliminary studies have shown that pramlintide (30 μg or 60 μg, q.i.d.) caused a significant decrease in the level of HbA_{1c} in NIDDM patients (Thompson *et al.* 1998). Infusion of pramlintide (100 μg/h, 5 h) prevented an elevation of postprandial plasma glucose concentration in patients with NIDDM (Thompson *et al.* 1995; Moyses *et al.* 1996). In addition to the inhibitory effect on gastric emptying, the ability of pramlintide to suppress postprandial glucagon secretion was also evident (Young *et al.* 1995; Moyses *et al.* 1996). Like other peptides, pramlintide can be administered only via intravenous or subcutaneous route.

In most cases, the newly developed agents have been hampered by their undesirable adverse effects (e.g. etomoxir and vanadium), inconvenient mode of delivery (e.g. pramlintide), modest activity on glycaemic control (e.g. fibrate derivatives) and the inconsistent effects (e.g. nicotinic acid) (Rachman and Turner, 1995).

1.4 Plants as a potential treatment for diabetes

NIDDM has been estimated to affect approximately 5-7% of the population world-wide (Yki-Järvinen, 1994). The cost of providing medical care to patients with diabetes is estimated to have been $ 45 billion per year in 1992 (Kroll, 1996). The therapy for NIDDM relies largely on the use of oral anti-diabetic agents, chiefly sulphonylureas and biguanides (section 1.2.2.1 and 1.2.2.2). However, it has been shown that none of these agents are successful in achieving ideal normoglycaemia

(Marles and Farnsworth, 1995). Primary failure, in which the patients do not initially respond to oral anti-diabetic agents, occurs in approximately 15% and 5% per year in NIDDM patients receiving sulphonylureas and biguanide monotherapy, respectively (Feinglos and Bethel, 1999). In addition, the use of oral anti-diabetic agents is always hampered by their adverse effects (section 1.2.2). Due to the enormous cost of modern treatment and the limitation of the current oral anti-diabetic drugs, there is a need for new oral anti-diabetic agents which can offer a better glycaemic control with less adverse effects.

Traditional plant remedies have been suggested to be one of the major sources in providing lead compounds for drug development (Marles and Farnsworth, 1995). Plants have been used in the treatment of diabetes since ancient times, the earliest known documentation of plant treatment for diabetes is found in the Ebers Papyrus of about 1550 BC (Day, 1990). Today, medicinal plants still have an important role in the treatment of diabetes, particularly in developing countries where most people have limited resources and thus do not have an access to the modern treatment. Medicinal plants may exert their anti-diabetic effect through several mechanisms including 1) inhibition of glucose absorption from intestine, 2) stimulation of insulin secretion from β-cells of pancreas, 3) insulin sensitisation, 4) insulin-like effects on target tissues and 5) potentiation of insulin action on target tissues.

Plants with known anti-diabetic activity have been reviewed in a number of publications (Handa *et al.* 1989; Ivorra *et al.* 1989; Rahman and Zaman 1989; Day, 1990; Marles and Farnsworth, 1995, Ernst, 1998, Hakim *et al.* 1998; Perez *et al.* 1998; Lamba *et al.* 2000). Results obtained from scientific studies on the anti-diabetic activity of these traditional plants are encouraging, since 81% of traditional anti-diabetic plants tested has been reported to be active (Marles and Farnsworth, 1995). To date, more than 800 species in the plant kingdom and a large variety of compounds isolated from these plants have been identified as potential treatments for diabetes (Perez *et al.* 1998).

This current review focuses on anti-diabetic plants used in traditional Indian (Ayurveda; Table 1.4) and Chinese medicine systems (Table 1.5). The literature survey was conducted using BIDS, Embase and MEDLINE. Among these plants, *Gymnema sylvestre* and *Momordica charantia* appear to be the most studied. The activity of these

two plants has been reviewed elsewhere (Handa *et al.* 1989, Marles and Farnsworth, 1995, Raman and Lau, 1996). Plants from Leguminosae family were found to be the most frequently cited for use as anti-diabetic remedies. Since Leguminosae is a very large and widely distributed family, the large number of species reported to have the anti-diabetic activity may be coincidental. Plant species listed in Table 1.4 and Table 1.5 have been claimed to have folklore reputation as anti-diabetic remedies; but in most cases the activity has not yet been scientifically evaluated. For those whose activities have been scientifically established, there have been only few whose modes of action and active constituents have been identified without ambiguity. The survey showed that a number of hypoglycaemic glycans have been isolated from these plants, including aconitans (from *Aconitum carmichaeli*), coixans (from C*oix lachryma-jobi*), dioscorans (from *Dioscorea japonica*), ephedrans (from *Ephedra distachya*) and lithospermans (from *Lithospermum erythorrhizon*). In most cases, the anti-diabetic effects of these glycans were observed in normal and/or diabetic animals after intraperitoneal administration. The relevance of this data to their traditional use (usually orally administration) is therefore uncertain. The data on plant selected for this study have been reviewed more extensively in Chapter 2.

Table 1.4 : Plants used in Ayurvedic medicine as anti-diabetic remedies

Plant family and species	Part used	Activity (if tested)	Dose and Route*	Active constituents*	Ref
Acanthaceae					
Barleria cristata Linn.	Whole plant	+ Normal animals			(1, 2)
Barleria prionitis Linn.	Bark, leaves				(1)
	Root	+ Normal animals			(2)
Justicia adhatoda Linn.	Leaves, root				(1)
Adiantaceae					
Adiantum incisum Forssk.	Whole plant				(1)
Alangiaceae					
Alangium salvifolium Wang.	Leaves	+ Normal animals	p.o.		(1, 2)
Amaranthaceae					
Aerva sanguinolenta Blume.†	Whole plant				(1)
Amaryliaceae					
Curculigo orchioides Gaertn.	Seed				(1)
Anacardiaceae					
Memecylon umbellatum Burm.	Leaves	+ Normal and alloxan-treated mice	p.o., 250 mg/kg		(1, 3)
Semecarpus anacardium Linn.	Fruit	+ Normal animals	p.o.		(1, 2)
Apiaceae					
Cuminum nigrum Linn.	Seed	+ Normal and alloxan-treated rabbits	p.o., 2-4 g/kg		(4)
		Normal and alloxan-treated dogs	2 g/kg		(5)
Apocynaceae					
Alstonia scholaris† Br.	Whole plant	+ Normal animals			(1, 2)

*Information given where available, †also used in traditional Chinese medicine; Activity, +: active

References: (1) Rahman and Zaman (1989); (2) Marles and Farnsworth (1995); (3) Almalraj and Ignacimuthu (1998a); (4) Akhtar and Ali (1985); (5) Qureshi *et al.* (1988)

Plant family and species	Part used	Activity (if tested)	Dose and Route*	Active constituents*	Ref
Catharanthus roseus G.Don.†	Leaves	+ Normal rat, + STZ-diabetic rat			(1)
Holarrhena antidysenterica Wall.	Fruit	+ Normal animals	p.o.		(1, 2)
Ichnocarpus frutescens Linn.	Flower				(3)
Lochnera rosea Reichb.	Leaves	+ Normal rabbits	p.o.		(4)
Araceae					
Scindapsus officinalis Roxb.	Stem				(1)
Asclepiadaceae					
Gymnema hirsuta Wall.	Leaves				(5)
Gymnema sylvetre R. Br.	Leaves, root	+ NIDDM patients, ↓ HbA_{1c}, ↑ Insulin secretion	p.o., 0.4 g/d (18-20 months)		(6, 7)
Hemidesmus indicus Schultes.	Leaves	+ IDDM and NIDDM rats			(8,9)
Bambaceae					
Bombax ceiba Linn.	Fruit, heartwood				(10)
Ceiba pentandra Gaertn.	Root				(10)
Eriodendron anfractuosum DC.					(8)
Berberidaceae					
Berberis aristata DC.	Root	+ Normal rats			(11)
Betulaceae					
Alnus nepalensis D.Don.	Stem bark				(1)

*Information given where available, †also used in traditional Chinese medicine; Activity, +: active; ↑: Increase; ↓: Decrease; HbA_{1c}: Hemoglobin A_{1c}

References: (1) Rahman and Zaman (1989); (2) Marles and Farnsworth (1995); (3) Bhandary *et al.* (1995); (4) Mukerji (1957); (5) Alam *et al.* (1990); (6) Chopra *et al.* (1956); (7) Baskaran *et al.* (1990); (8) Singh (1994); (9) Rokeya *et al.* (1997); (10) Khan and Singh (1996); (11) Kapoor (1990)

Plant family and species	Part used	Activity (if tested)	Dose and Route*	Active constituents*	Ref
Bignoniaceae					
Campsis grandiflora K.Schum.†	Flower				(1)
Heterophragma quadriloculare K. Schum.	Aerial part	+ Normal animals			(2)
Pithecolobium saman Benth.	Leaves				(2)
Stereospermum suaveolens DC.	Root	+ Normal animals	p.o.		(2, 3)
Buseraceae					
Boswellia serata Roxb.	Fruit, root	+ Normal rats			(1)
Cactaceae					
Opuntia species	Nodal pod, sap	(See Chapter 2)			(2)
Celastraceae					
Salacia oblonga Wall.	Root bark	+ Normal rats			(4)
Cochlospermaceae					
Cochlospermum religiosum Linn.					(5)
Combretaceae					
Terminalia belerica Roxb.†	Fruit				(2)
Terminalia chebula Retz.	Fruit, stem bark	+ Normal animals	p.o.		(3, 6)
Compositae					
Artemisia pallens Wall.	Aerial	+ Glucose-loaded rats	p.o.		(7)
		+ Alloxan-induced diabetic rats			
Inula racemosa Hook.	Root	+ Normal rats, ↑ glycogen	p.o., 0.4 g/kg		(8)
Pulicaria foliolosa	Whole plant				(2)

*Information given where available, †also used in traditional Chinese medicine; Activity, +: active; ↑: Increase

References: (1) Kapoor (1990); (2) Rahman and Zaman (1989); (3) Marles and Farnsworth (1995); (4) Augusti *et al.* (1995); (5) Khan and Singh (1996); (6) Chopra *et al.* (1956); (7) Subramoniam *et al.* (1996); (8) Chaturvedi *et al.* (1995)

Plant family and species	Part used	Activity (if tested)	Dose and Route*	Active constituents*	Ref
Saussurea lappa C.B.Clarke.	Root	+ Normal rats	p.o., 400 mg/kg		(1)
		↑ Glycogen			(2)
Siegesbeckia orientalis Linn.	Whole plant				(3)
Sphaeranthus indicus Linn.	Fruit	+ Normal animals			(3)
Xanthium strumarium Linn.†	Root, seed				(3, 4)
Convolvulaceae					
Convolvulus microphyllus Sieb.	Whole plant				(5, 6)
Ipomoea digitata Linn.					(1)
Rivea cuneata Wight.	Leaves	+ Alloxan-induced diabetic animals			(7)
Costaceae					
Costus speciosus Smith.	Rhizome	+ Glucose loaded rats			(8)
Cruciferae					
Lepidium apetalum Willd.					(3)
Cucurbitaceae					
Bryonia epigaea Rottl.		+ STZ-induced diabetic animals			(3, 9)
Coccinia indica Wight & Arn.	Whole plant	(See Chapter 2)			(10)
Lagenaria siceraria Standl.†	Seed				(11)
Momordica charantia Linn.	Fruit juice	+ NIDDM patients			(12)
Momordica cymbalaria Hook.	Fruit				(13)
Trichosanthes bracte 'a Voigt.					(3)

* Information given where available, †also used in traditional Chinese medicine; Activity, +: active; ↑: Increase;

References: (1) Chaturvedi *et al.* (1995); (2) Chaturvedi *et al.* (1993); (3) Rahman and Zaman (1989); (4) Handa *et al.* (1989); (5) Alam *et al.* (1990); (6) Singh and Ali (1989); (7) Chopra *et al.* (1956); (8) Mosihuzzaman *et al.* (1994); (9) Marles and Farnsworth, (1995); (10) Kapoor (1990); (11) Khan and Singh (1996); (12) Ernst (1998); (13) Kameswara Rao *et al.* (1999)

Plant family and species	Part used	Activity (if tested)	Dose and Route*	Active constituents*	Ref
Cyperaceae					
Cyperus rotundus Linn.	Rhizome	(See Chapter 2)			(1)
Kyllinga triceps Rottb.					(2)
Ebernaceae					
Diospyros melanoxylon Roxb.	Leaves, seed				(2)
Diospyros paregrina Gurke.	Stem bark				(3)
Elaeocarpaceae					
Elaeocarpus ganitrus Roxb.	Stem bark	+ Normal animals	p.o.		(3, 4)
Ericaceae					
Agapetes sikkimensis Airyshan.	Whole plant				(3)
	Aerial part	+ Normal animals	p.o.		(4)
Vaccinium lashenaultii Wight.	Aerial part	+ Normal animals	p.o.		(4)
	Whole plant (exc. root)				(3)
Euphorbiaceae					
Glochidion hohenackeri Bedd.	Aerial part	+ Normal animals			(4)
Mallotus philippinensis Muell. & Arg.	Fruit	+ Normal animals	p.o.		(3, 4)
Phyllanthus emblica H.B.K.	Leaves	+ Normal and alloxan-treated mice		Fagasterol	(5)
Phyllanthus fraterus Webster.	Leaves	+ Normal rabbits			(6)
		- Alloxan-induced diabetic rabbits			
Phyllanthus ·llowianus Mueller.	Bark, leaves, twig	Normal animals	i.v.		(3, 4)
Fagaceae					
Quercus species	Stem bark				(3)

* Information given where available; Activity, +: active; - : inactive

References: (1) Chopra *et al* (1956); (2) Khan and Singh (1996); (3) Rahman and Zaman (1989); (4) Marles and Farnsworth (1995); (5) Perez *et al* (1998); (6) Ramakrishnan *et al.* (1982)

Plant family and species	Part used	Activity (if tested)	Dose and Route*	Active constituents*	Ref
Fumariaceae					
Fumaria parviflora Lamk.	Aerial	+ Normal rabbits	p.o., 1-3 g/kg		(1, 2)
		- Alloxan-induced diabetic rabbits			
Gentianaceae					
Canscora decussata Schult.					(3)
Swertia chirayita Buch. & Ham.	Whole plant	(See Chapter 2)			
Gramineae					
Bambusa bambos Linn.	Leaves	+ Alloxan-induced diabetic rabbits			(4)
Bambusa dendrocalamus	Leaves	+ Normal and alloxan-induced diabetic animals			(3)
Hordeum vulgare Linn.	Root				(3)
Labiatae					
Calamintha umbrosa Fisch. & May.	Whole plant				(3)
Ocimum sanctum Linn.	Leaves, whole plant	+ Normal, glucose-loaded and STZ-induced diabetic animals, ↓ glucose absorption	p.o.		(5, 6, 7)
Teucrium polium Linn.		- Glucose loaded rats	p.o., 1.6 g/kg		(3, 8)
		+ Normal and STZ-diabetic rats	p.o.		(9)
Lauraceae					
Cinnamomum obtusifolia Nees.†					(3)
Cinnamomum tamala T.Nees & Ebern	Whole plant				(3)
	Root, stem bark	+ Diabetic animals	p.o.		(5)

* Information given where available; †also used in traditional Chinese medicine; Activity, +: active; - : inactive; ↓: Decrease;

References: (1) Akhtar *et al.* (1984); (2) Ivorra *et al.* (1989); (3) Rahman and Zaman (1989); (4) Kapoor (1990); (5) Marles and Farnsworth (1995); (6) Hakim *et al.* (1998); (7) Chattopadhyay, (1993); (8) Konuklugil *et al.* (1997); (9) Gharaibeh *et al.* (1988)

Plant family and species	Part used	Activity (if tested)	Dose and Route*	Active constituents*	Ref
Leguminosae					
Acacia arabica Willd.	Bark				(1)
	Seed	+ Fed rats			(2)
		+ Normal rabbit	2-4 mg/kg		(3)
		- Alloxan-induced diabetic rabbit			
Acacia ferruginea DC.	Stem bark				(4)
Acacia modesta Wall.	Seed	+ Fed rats			(2)
Albizzia molucca	Seed	+ Normal animals	p.o.		(4)
		- Alloxan-induced diabetic animals			
Bauhinia retusa Roxb.	Seed	+ Normal and alloxan-induced diabetic animals			(4)
Bauhinia variegata Linn.†	Flowers	+ Normal animals			(4, 5)
Butea frondosa Roxb.	Leaves				(6)
Butea monosperma Kuntz.	Leaves				(7)
Caesalpinia bonducella Fleming.	Seed	+ Normal rats	p.o., 0.1-0.5 g/kg		(8)
		+ STZ-induced diabetic rats	p.o., 0.1 g/kg		
		+ Normal and alloxan-induced diabetic rabbits	p.o., 0.5-1.5 g/kg		(9)
Cajanus cajan Millsp.	Seed	+ Normal and alloxan-treated mice			(4, 10)
Caranga brevispina Royle.	Whole plant				(4)
	Aerial part	+ Normal animals	p.o.		(5)

* Information given where available, †also used in traditional Chinese medicine; Activity, +: active; - : inactive

References: (1) Chopra *et al.* (1956); (2) Handa *et al.* (1989); (3) Wadood *et al.* (1989); (4) Rahman and Zaman (1989); (5) Marles and Farnsworth (1995); (6) Khan and Singh (1996); (7) Kapoor (1990); (8) Sharma *et al.* (1997), (9) Rao *et al.* (1994b); (10) Almalraj and Ignacinuthu (1998b)

Plant family and species	Part Used	Activity (if tested)	Dose and Route*	Active constituents*	Ref
Cassia alata Linn.	Leaves	+ STZ-induced diabetic mice			(1)
Cassia auriculata Linn.	Seed; flower, leaves	+ Normal and alloxan-treated animals			(2, 3)
Cassia fistula Linn.	Pod, fruit, seed, stem bark				(3, 4)
Cassia sophora Linn.	Bark, seed				(3)
Cyamopsis tetragonoloba Taub.	Fruit	+ Normal rabbits	20 g/kg		(5, 6)
	Seed	+ Glucose-loaded fasting rabbits	20 g/kg		(6)
		+ Alloxan-treated animals	40 g/kg		(6)
		↓ Glucose absorption in healthy subjects	p.o., 20-25 g/day (5-7 days)	Guar gum	(6, 7)
Erythrina indica Lam.	Root bark				(3)
Erythrina suberosa Roxb.	Stem bark	+ Normal	p.o.		(2, 8)
Galega officinalis Linn.		+ Alloxan-treated rabbits, ↑ Hepatic glycogen, ↓ Glucose absorption in Caco-2	p.o.	Galegin	(8, 9) (10)
Lablab purourea Sweet.	Pod	+ Alloxan-treated animals			(2)
Leucaena leucocephala Sic.	Seed	+ Diabetic rats			(2, 11)
	Leaves	+ Adrenaline-treated and pancreatecomized animals			
Macrotyloma uniflorum Lam.	Seed				(2)
Mucuna pruriens Linn.	Fruit				(2)
	Seed	+ Normal rats			

* Information given where available; Activity, +: active; ↓: Decrease; ↑: Increase

References: (1) Palanichamy *et al.* (1988); (2) Rahman and Zaman. (1989); (3) Chopra *et al.* (1956); (4) Khan and Singh (1996); (5) Pillai *et al.* (1980); (6) Ivorra *et al.* (1989); (7) Trinick *et al.* (1986); (8) Marles and Farnsworth (1995); (9) Lamba *et al.* (2000); (10) Neef *et al.* (1996); (11) Handa *et al.* (1989)

Plant family and species	Part Used	Activity (if tested)	Dose and Route*	Active constituents*	Ref
Pongamia pinnata Merrill.					(1)
Pterocarpus marsupium Roxb.	Heartwood	(See Chapter 2)			(2)
Pterocarpus santalinus Linn.	Seed				(2)
Tephorsia purpurea Pers.	Seed	+Normal and alloxan-treated rabbits	p.o.	Lupeol	(3, 4)
Liliaceae					
Allium cepa Linn.	Bulb	+ Healthy subjects, ↑ Insulin level	p.o., 2.5 mg/kg (essential oil)		(5, 6)
		+ Diabetic rabbits	10 mg/kg	Diphenylamine, S-methylmethionine (SMCS)	(7)
		+ Normal and alloxan-induced diabetic animals, + NIDDM patients		Allicin, Allyl propyl disulfide	(7)
Scilla indica Baker					(8)
Urginia indica Kunth.	Bulb	+ Normal animals	p.o.		(1, 4)
Loganiaceae					
Strychnos nux-vomica Linn.					(8)
Strychnos potatorum Linn.	Pulp				(8)
Lyrthraceae					
Lagerstroemia speciosa Linn.	Bark, fruit, leaves, root, seed	+ Alloxan-induced diabetic animals			(1, 4)
Magnoliaceae					
Michelia champaca Linn.	Stem bark	+ Normal animals	p.o.		(2, 3)

*Information given where available; Activity, +: active; ↑: Increase

References: (1) Rahman and Zaman (1989); (2) Kapoor (1990); (3) Rahman *et al.* (1985); (4) Marles and Farnsworth (1995); (5) Augusti and Benaim (1975); (6) Ernst (1998); (7) Perez *et al.* (1998); (8) Chopra *et al.* (1956)

Plant family and species	Part Used	Activity (if tested)	Dose and Route*	Active constituents*	Ref
Malvaceae					
Hibiscus rosa-sinensis Linn.	Flowers				(1)
Sida alnifolia Linn.	Whole plant				(2)
Meliaceae					
Azadirachta indica† A. Juss.	Leaves	(See Chapter 2)			(2)
Menispermaceae					
Stephania glabra Miers.	Root	+ Normal animals	p.o.		(2, 3)
Tinospora cordifolia Miers.	Whole Plant	(See Chapter 2)			(4)
Moraceae					
Ficus bengalensis Linn.	Bark	(See Chapter 2)			(4, 5)
Ficus glomerata Linn.	Bark	+ Normal and alloxan-treated animals	p.o.		(2)
Ficus religiosa Linn.	Bark	+ Normal and alloxan-induced diabetic animals		β-sitosterol 3-β-D-glucoside	(2)
	Root, root bark	+ Alloxan-induced diabetic animals	p.o.	Daucosterol	(3)
Ficus virens Ait.	Fruit				(6)
Musaceae					
Ensete superbum Cheesman.	Seed				(2)
Ephedra species					
Musa sapientum Linn.	Flower	+ Alloxan-induced diabetic rats, + Glucose-loaded diabetic rats, ↓ HbA_{1c}	p.o., 0.15-0.25 g/kg, 30 days		(2)

*Information given where available; †also used in traditional Chinese medicine; Activity, +: active; ↓: Decrease

References: (1) Rahman and Zaman, (1989) (2) Marles and Farnsworth. (1995); (3) Virendra, (1995); (4) Chopra *et al.* (1956); (5) Kapoor, (1990); (6) Alam *et al.* (1990)

Plant family and species	Part Used	Activity (if tested)	Dose and Route*	Active constituents*	Ref
Musa sapientum (continued)	Flower	↑ Hepatic glycogen	20 mg/kg	Pectin	(1, 2)
	Fruit	+ Normal rabbits	p.o., 0.5-1.5 g/kg		(2)
		- Alloxan-treated rabbits			
Myrtaceae					
Eribotrya japonica Lind.	Leaves	+ Normal rabbit	p.o., 0.1-0.2 g/kg		(3, 4)
		- Alloxan-treated rabbits			
		+ Normal mice		Polyhydroxylated	(2)
		↓ Glycosuria		triterpenoids and	
				sesquiterpene glycoside	
Eucalyptus globulus Labill.†	Leaves				(5)
Eugenia jambolana Lam.	Seed	+ Normal and alloxan-treated rabbits	p.o., 2-4 g/kg		(5, 6)
	Leaves	+ Alloxan-induced diabetic rats	p.o., 2.5-5 g/kg (6 weeks)		
Psidium guajava Linn.†	Fruit juice	+ Normal mice	i.p., 1 g/kg		(7)
		- Alloxan-induced diabetic mice			
	Leaves	+ Alloxan-induced diabetic animals			(8)
Nymphaeaceae					
Nelumbo nucifera Gaertn.	Rhizome	+ Normal rats	p.o., 0.1-0.6 g/kg		(9)
	Rhizome	+ Glucose-loaded and STZ- induced diabetic rats	p.o., 0.4 g/kg		(9)
	Rhizome	↑ Action of exogenous insulin	p.o., 0.4 g/kg		(9)
Nymphaea lotus Linn.	Root	+ Normal animals	p.o.		(10)

*Information given where available; †also used in traditional Chinese medicine; Activity, +: active; - : inactive; ↓: Decrease; ↑: Increase

References:; (1) Pari and Maheswari (1999); (2) Perez *et al.* (1998); (3) Rao *et al.* (1994a); (4) Noreen *et al.* (1988); (5) Ivorra *et al.* (1989); (6) Prince *et al.* (1998); (7) Chen and Yang (1984); (8) Rahman and Zaman (1989); (9) Mukherjee *et al.* (1997); (10) Marles and Farnsworth (1995)

Plant family and species	Part Used	Activity (if tested)	Dose and Route*	Active constituents*	Ref
Orchidaceae					
Orchis mascula Linn.	Root				(1)
Orobanchaceae					
Cistanche tubulosa R.Wight.	Whole plant	+ Normal animals	p.o.		(2, 3)
Palmae					
Lodoicea sechellarum Comm & Labill.	Kernel, milk				(1)
Pedaliaceae					
Sesamum indicum Linn.	Leaves				(4)
Pinaceae					
Abies pindrow Royle.	Whole plant	+ Normal animals	p.o.		(2, 3)
Pinus roxburghii Sargent.	Bark, root	+ Normal animals	p.o.		(2)
Piperaceae					
Piper cubeba Linn.†					(3)
Piper longum Linn.†	Whole plant	(See Chapter 2)			(5)
Ranunculaceae					
Aconitum ferox Wall.	Root				(6)
Rhamnaceae					
Zizyphus rugosa Lam.	Bark	+ Normal rabbits		Kaempferol-3-*O*-rhamnoside, quercetin-3-*O*-rhamnoside	(3, 6)
Rosaceae					
Malus pumila Mill.		+ Diabetic animals			(3)

*Information given where available; †also used in traditional Chinese medicine; Activity, +: active

References: (1) Chopra *et al.* (1956); (2) Marles and Farnsworth (1995); (3) Rahman and Zaman (1989); (4) Singh and Ali (1989); (5) Kapoor (1990); (6) Perez *et al.* (1998)

Plant family and species	Part Used	Activity (if tested)	Dose and Route*	Active constituents*	Ref
Pyrus communis Linn.†					(1)
Prunus persica Batsch.	Leaves				(1)
Rosa corymbifera Borkh.					(1)
Rubiaceae					
Hedyotis biflora Roth.	Whole plant	+ Alloxan-induced diabetic rabbits			(2, 3)
Morinda citrifolia Linn. †	Leaves				(1)
Rubia cordifolia Linn.	Whole plant (exc. root)				(4)
	Aerial part	+ Normal animals	p.o.		(5)
Rutaceae					
Aegle marmelos Correa.	Leaves	+ Glucose-loaded animals			(1)
		Improve functional state of pancreatic β-cells			
Murraya koenigii Spreng.	Leaves	(See Chapter 2)			(1)
Ruta albiflora	Whole plant				(1)
Zanthoxylum armatum DC.	Leaves				(6)
Samydaceae					
Casearia esculenta Roxb.	Root				(1)
Sapindaceae					
Sapindus mukorossi Gaertn.	Seed				(7)
Scrophulariaceae					
Kickxia ramosissima Janchen.					(1)
Mazus surculosus D.Don.	Whole plant	+ Normal animals			(1)

*Information given where available; †also used in traditional Chinese medicine; Activity, +: active; - : inactive

References: (1) Rahman and Zaman (1989); (2) Dechatiwongse *et al.* (1983); (3) Ivorra *et al.* (1989); (4) Chopra *et al.* (1956); (5) Marles and Farnsworth (1995); (6) Alam *et al.* (1990); (7) Khan and Singh (1996)

Plant family and species	Part Used	Activity (if tested)	Dose and Route*	Active constituents*	Ref
Pedicularis rhinanthoides Schrenk.	Whole plant (exc. root)	+ Normal animals			(1, 2)
Scoparia dulcis Linn.	Leaves, stem	+ Diabetic-treated patients			(1, 2)
				Amellin	(3)
Sterculiaceae					
Ambroma augusta Linn.					(4)
Guazuma tomentosa Kunth.					(4)
Helicteres isora Linn.	Root bark				(4, 5)
Heritiera minor Lamk.	Aerial part	+ Normal animals			(2)
Styraceae					
Symplocos thaefolia Buch. & Ham.	Leaves, root				(1)
Tiliaceae					
Grewia asiatica Linn.	Stem bark	+ Pancreatectomised rabbits			(1, 6)
	Leaves	+ Alloxan-treated and pancreatectomised animals	p.o.		(4)
Verbenaceae					
Clerodendron infortunatum Linn.	Whole plant	+ Normal animals	p.o.		(1, 4)
Clerodendron phlomidis Linn.	Whole plant	+ Healthy and diabetic subjects			(7)
		+ Adrenaline-induced diabetic rabbits	p.o.		(6, 8)
		+ Alloxan-induced diabetic rats			
		+ Diabetic patients, ↓ Glycosuria	p.o., 15-30 g/day		

*Information given where available; Activity, +: active; ↓: Decrease

References: (1) Rahman and Zaman (1989); (2) Marles and Farnsworth (1995); (3) Mukerji (1957); (4) Chopra *et al.* (1956); (5) Kapoor (1990); (6) Handa *et al.* (1989); (7) Hakim *et al.* (1998); (8) Chaturvedi *et al.* (1984)

Plant family and species	Part Used	Activity (if tested)	Dose and Route*	Active constituents*	Ref
Gmelina arborea Roxb.	Stem bark, wood of stem				(1)
Premna obtusifolia R. Br.	Root	+ Normal animals			(2, 3)
Violaceae					
Hybanthus enneaspermus					(4)
Vitaceae					
Leea crispa Linn.	Aerial part	+ Normal animal			(2)
	Whole plant (exc. root)				(1)
Leea indica Merrill.	Leaves	+ Normal animals	p.o.		(1, 2)
Zingiberaceae					
Alpinia galanga Willd.	Rhizome				(5)
Amomum subulatum Roxb.	Rhizome	+ Normal animals	p.o.		(1, 2)
Curcuma longa Linn.†	Rhizome	(See Chapter 2)			(5)
Zygophyllaceae					
Tribulus terrestris Linn.	Fruit				(6)

*Information given where available; †also used in traditional Chinese medicine; Activity, +: active

References: (1) Rahman and Zaman, (1989); (2) Marles and Farnsworth (1995); (3) Kapoor (1990); (4) Satapathy and Brahmam (1994); (5) Chopra *et al.* (1956); (6) Khan and Singh (1996)

Table 1.5: Plants used in Chinese medicine as anti-diabetic remedies

	Part used	Activity (if tested)	Dose and Route*	Active constituents*	Ref
Alismataceae					
Alisma orientalis Juzep.	Tuber	(See Chapter 2)			(1)
Alisma plantgo-aquatica Linn.	Leaves				(2)
Anacardiaceae					
Rhus chinensis Mill.					(3)
Araceae					
Typhonium giganteum Engl.	Gall				(2)
Araliaceae					
Aralia elata Seem.		+ Alloxan-induced diabetic rabbits	p.o., 0.8 g/kg, 6 days p.o., 0.2 g/kg, 14 days		(4)
Aralia elata Seem.	Bark, Root cortex			Elastoside A, E, G, H and I, Stipleanoside R	(5)
Panax ginseng C.A.Meyer.	Rhizome	(See Chapter2)			(2)
Tetrapanax paoyriferus K.Koch.	Stem				(2)
Aristolochiaceae					
Aristolochia manchurensis Komarov	Bark, stem	+ Normal animals			(2, 6)
Berberidaceae					
Epimedium brevicornum Maxim.	Whole plant				(7)
Epimedium grandiflorum Morr.					(8)
Epimedium sagittatum Maxim.	Whole plant, aerial part				(1)

*Information given where available; Activity. +: active

References: (1) Chang and But (1986b); (2) Duke and Ayensu (1985a); (3) Rahman and Zaman (1989); (4) Lee *et al.* (1988); (5) Yoshikawa *et al.* (1996); (6) Marles and Farnsworth (1995); (7) Chang and But (1986a); (8) Sanae *et al.* (1996)

Plant family and species	Part used	Activity (if tested)	Dose and Route*	Active constituents*	Ref
Boraginaceae					
Lithospermum erythorrhizon Sieb.	Root	+ Normal mice	i.p., 1 g/kg		(1)
		+ Normal mice	i.p., 10-100 mg/kg	Lithosperman A, B and C	
		+ Alloxan-treated mice (lithosperman A)	i.p., 10-30 mg/kg		
		↑ Glucose oxidation in inverted gut	250 μg/ml		(2)
Campanulaceae					
Codonopsis pilosula Nannf.	Root				(3)
Platycodon grandiflorum A.DC.	Root	(See Chapter 2)			(4)
Celasteraceae					
Salacia chinensis Linn.	Root				(3)
Compositae					
Atractylodes chinensis Koidz.	Rhizome				(5)
Atractylodes lancea Thunb.	Rhizome	(See Chapter 2)			(5)
Atractylodes macrocephala Koidz.	Rhizome	(See Chapter 2)			(5)
Xanthium sibiricum Patrin.	Seed				(5)
Convovulaceae					
Calystegia japonica Choisy.	Flower				(6)
Cornaceae					
Cornus officinalis Sieb & Succ.	Fruit				(5)
Cucurbitaceae					
Benincasa hispida Cogn.	Whole plant	(See Chapter 2)			(6)
Trichosanthes cucurmeroides Maxim.	Fruit				(3)

*Information given where available: Activity, +: active; ↑: Increase

References: (1) Konno *et al.* (1985b); (2) Perl and Hikino (1989); (3) Duke and Ayensu (1985a); (4) Chang and But (1986b); (5) Chang and But (1986a); (6) Rahman and Zaman (1989;

Plant family and species	Part used	Activity (if tested)	Dose and Route*	Active constituents*	Ref
Boraginaceae					
Lithospermum erythorrhizon Sieb.	Root	+ Normal mice	i.p., 1 g/kg		(1)
		+ Normal mice	i.p., 10-100 mg/kg	Lithosperman A, B and C	
		+ Alloxan-treated mice (lithosperman A)	i.p., 10-30 mg/kg		
		↑ Glucose oxidation in inverted gut	250 μg/ml		(2)
Campanulaceae					
Codonopsis pilosula Nannf.	Root				(3)
Platycodon grandiflorum A.DC.	Root	(See Chapter 2)			(4)
Celasteraceae					
Salacia chinensis Linn.	Root				(3)
Compositae					
Atractylodes chinensis Koidz.	Rhizome				(5)
Atractylodes lancea Thunb.	Rhizome	(See Chapter 2)			(5)
Atractylodes macrocephala Koidz.	Rhizome	(See Chapter 2)			(5)
Xanthium sibiricum Patrin.	Seed				(5)
Convovulaceae					
Calystegia japonica Choisy.	Flower				(6)
Cornaceae					
Cornus officinalis Sieb & Succ.	Fruit				(5)
Cucurbitaceae					
Benincasa hispida Cogn.	Whole plant	(See Chapter 2)			(6)
Trichosanthes cucurmeroides Maxim.	Fruit				(3)

*Information given where available: Activity, +: active; ↑: Increase

References: (1) Konno *et al.* (1985b); (2) Perl and Hikino (1989); (3) Duke and Ayensu (1985a); (4) Chang and But (1986b); (5) Chang and But (1986a); (6) Rahman and Zaman (1989;

Plant family and species	Part used	Activity (if tested)	Dose and Route*	Active constituents*	Ref
Trichosanthes kirilowii Maxim.		(See Chapter 2)			(1)
Dioscoraceae					
Dioscorea japonica Thunb.	Rhizome	+ Normal mice		Dioscorans A, B, C, D, E and F	(2)
Dioscorea opposita Thunb.					(3)
Ephedraceae					
Ephedra distachya Auct.	Whole plant	+ Normal mice	i.p., 10-100 mg/kg	Ephedran A, B, C, D and E	(4)
		+ Alloxan-treated mice (ephedran A)			
Coix lachryma-jobi Linn.	Seed	+ Normal mice	i.p., 10-100 mg/kg	Coixan A, B and C	(5)
		+ Alloxan-treated mice (coixan A)	i.p., 10-100 mg/kg		
Hordeum vulgare Linn.	Fibrous hair, sprout				(6)
Labiatae					
Salvia miltiorrhiza Bunge.	Root	(See Chapter 2)			(6)
Lauraceae					
Cinnamomum louveiri Nees.					(6)
Cinnamomum vulgaris Linn.	Stem bark				(1)
Leguminosae					
Lathyrus japonica Sic.	Seed	+ Normal and alloxan-treated mice		Lathyrine	(7)
Phaseolus vulgaris Linn	Seed	+ Glucose loaded rabbits			(8)
Liliaceae					
Anemarrhena asphodeloides Bunge.	Rhizome	(See Chapter 2)			(7)

*Information given where available; Activity, +: active

References: (1) Rahman and Zaman (1989); (2) Perez et al. (1998); (3) Sanae *et al.* (1996); (4) Konno *et al.* (1985a); (5) Takahashi et al. (1986); (6) Chang and But. (1986a); (7) Duke and Ayensu, (1985b); (8) Roman-Ramos *et al.* (1991)

Plant family and species	Part used	Activity (if tested)	Dose and Route*	Active constituents*	Ref
Asparagus cochichinensis Merr.	Root				(1)
Polygonatum cirrhifolium Wall.	Rhizome				(1)
Polygonatum cyrtonema Hua.	Rhizome				(1)
Polygonatum kingianum Coll.	Rhizome				(1)
Polygonatum macropodium Turez.	Rhizome				(1)
Polygonatum odoratum Druce.	Rhizome	(See Chapter 2)			(2)
Polygonatum sibiricum Redoute.	Rhizome				(3)
Magnoliaceae					
Schisandra chinensis Baill.	Fruit				(3)
Moraceae					
Morus alba Linn.	Leaves	(See Chapter 2)			(3)
Morus australis Poir.	Root bark				(4)
Polyporaceae					
Ganoderma lucidum Karsten.	Fruit body	(See Chapter 2)			(4)
Ranunculaceae					
Aconitum carmichaeli Dexb.	Root	+ Normal mice	i.p., 10-100 mg/kg	Aconitans A, B, C and D	(5)
		+ Normal, glucose-loaded and alloxan-treated mice (aconitan A)	i.p., 30-100 mg/kg		(6)
		↑ Glucose oxidation in inverted gut	250 μg/ml		(6)
Coptis chinensis Franch.		(See Chapter 2)			(2)
Rosaceae					
Agrimona pilosa Ledeb.	Leaves				(2)

*Information given where available; Activity, +: active; ↑: Increase

References: (1) Duke and Ayensu (1985b); (2) Chang and But (1986a); (3) Chang and But (1986b); (4) Rahman and Zaman (1989); (5) Hikino *et al.* (1989b); (6) Perez et al. (1998)

Plant family and species	Part used	Activity (if tested)	Dose and Route*	Active constituents*	Ref
Scrophulariaceae					
Rehmannia glutinosa Libosch.	Rhizome	(See Chapter 2)			(1)
Scrophularia buergeriana Miq.	Root				(1)
Scrophularia ningpoensis Hemsl.	Root				(1)
Stachyuraceae					
Stachyurus himalaicus Hook.					(2)
Solanaceae					
Lycium barbarum Linn.	Fruit				(3)
Lycium chinensis Mill.	Fruit, root bark	(See Chapter 2)			(3)

*Information given where available; Activity, +: active

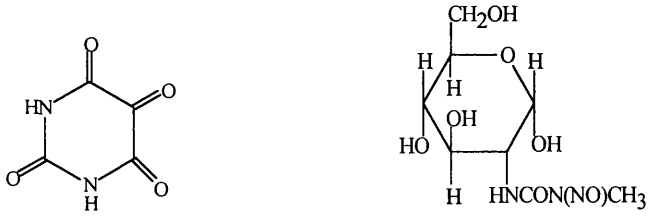
References: (1) Chang and But, (1986a); (2) Rahman and Zaman (1989); (3) Chang and But, (1986b)

1.5 Experimental models to investigate hypoglycaemic activity of plants

To isolate compounds from plant extracts which have promising anti-diabetic activity, a systematic approach should be employed for testing maximum numbers of samples in bioassays which are predictive of their therapeutic efficacy. Laboratory techniques to investigate the hypoglycaemic activity of plant extracts and their active constituents can be divided into two main classes: *in vivo* and *in vitro* techniques.

1.5.1 *In vivo models*

Animals models of diabetes consist of either transgenic models whose diabetes is genetically predisposed or models which are induced experimentally by drugs (e.g. 2,4-dinitrophenol and diazoxide), hormones (e.g. epinephrine, glucagon, corticotropin, somatotropin and anterior pituitary extract), chemical toxins and viral infection. The chemical toxins which have been extensively used to induce diabetes in animals include alloxan, streptozotocin (STZ) and, to a lesser extent, zinc chelator compounds (e.g. dithizone and 8-hydroxyquinoline).



Alloxan Streptozotocin

Fig 1.14: Structure of alloxan and streptozotocin

Alloxan (Fig. 1.14) was reported to cause various deleterious effect on β-cells including disruption of the cell membrane (Mordes and Rossini, 1981), inhibition of glucokinase (Lenzen and Panten, 1988a), inhibition of glycolytic flux and pyruvate oxidation (Borg *et al.* 1979). Its half maximum inhibition of glucokinase in isolated pancreatic islets was observed at a concentration of 200-300 μmol/l (Lenzen and Panten, 1988a). The mechanism of action of alloxan is thought, at least in part, to be due to the formation of free radicals which are possibly generated by the reduction-reoxidation cycle of alloxan (Bone and Gwilliam, 1997). The accumulation of superoxide and OH radicals has been postulated to lead to a fragmentation of DNA which results in a

stimulation of poly (ADP-ribose) synthase, an enzyme involved in DNA repair (Bone and Gwilliam, 1997, Plosker and Faulds, 1999). Increase in the activity of this enzyme subsequently leads to a depletion of NAD which in turn results in impaired β-cell metabolism and thus cell necrosis (Bone and Gwilliam, 1997). Administration of alloxan has been reported to be most effective by intravenous injection at a dose of 140-180 mg/kg (Williamson *et al.* 1996). The different degrees of severity of diabetes can be produced by varying the dose of alloxan; these can be defined according to fasting blood glucose level e.g. in rabbits moderate diabetes is defined as a fasting blood glucose level of 180-250 mg/ml and severe diabetes as a fasting blood glucose level greater than 250 mg/ml (Williamson *et al.* 1996).

The mechanisms of action of streptozotocin (Fig 1.14) is thought to be similar to alloxan. Streptozotocin is found to be more effective than alloxan in some species e.g. guinea pigs and Syrian hamsters. Streptozotocin is generally used to induce diabetes in mice, rats and dogs at either a single large dose (50-100 mg/kg) or as multiple smaller doses (Bone and Gwilliam, 1997). It has been suggested that diabetes developed in the latter case is more gradual and appears to have an autoimmune, rather than toxic basis (Bone and Gwilliam, 1997). Low dosage of streptozotocin and alloxan is occasionally used to induce NIDDM in animals. However, the appropriate dosage is difficult to achieve without causing either gradual recovery or development into IDDM (Bailey and Flatt, 1997a). Alternatively, the animal will develop NIDDM later in life if streptozotocin or alloxan is given to the neonate during the first week of life, (Bailey and Flatt, 1997a). It was reported that intravenous injection of streptozotocin (100 mg/kg) on the first day after birth will destroy most of the β-cells, followed by gradual but incomplete regeneration (Portha *et al.* 1974).

The BB (BioBreeding) rat and NOD (non-obese-diabetic) mouse are animal models with spontaneous diabetes widely used as models for human IDDM. The spontaneous IDDM that occurs in these models is closely related to human IDDM, as they share important immunological characteristics with human IDDM (Rabinovitch, 1998). NOD mouse and BB rat have been considered as the best currently available rodent models of human IDDM (Bone and Gwilliam, 1997). The features of spontaneous IDDM developed in other species including monkeys (*Macaca nigra*), dogs (the

Keeshond), the Chinese hamster, certain colonies of guinea pigs and the New Zealand white rabbit are less closely related to human diabetes (Bone and Gwilliam, 1997). Animal models have been proved to be a powerful tool for studying diabetes, however no single model is sufficient to describe the pathogenesis and represent IDDM in human (Lohmann, 1998). In addition, the pathogenesis of diabetes may be different in the different species (Rabinovitch, 1998) and there are some characteristics of animal models which do not apply to IDDM subjects e.g. lymphopenia in BB rats. Therefore, the results obtained from animal models should be interpreted with care (Lohmann, 1998).

Genetically based animal models of NIDDM can be classified into 3 groups according to severity of hyperglycaemia (Bailey and Flatt, 1997a) i.e.

1) NIDDM animals with severe hyperglycaemia e.g. *db/db* mice, sand rats (*Psammomys obesus*), spiny mouse (*Acolys cahirinus*), Rhesus monkeys (*Macana mukatta*), Otsuka Long-Evans Tokhushima fatty (OLETF) rats, BBZ/*Wor* rat and Chinese hamsters
2) NIDDM animals with moderate hyperglycaemia e.g. *ob/ob* mice, yellow obese mice, KK mice, NZO mice, Paul Bailey black (PBB/Ld) mice, ZDF rat, Wistar Kyoto (WKY) fatty rat, spontaneous hypertensive corpulent (SHR/N-*cp*) rat and Wellesley hybrid mice
3) animals with impaired glucose tolerance e.g. fatty Zucker *fa/fa* rat

With any of these animal models, hypoglycaemic or anti-hyperglycaemic activity of the plant extracts can be assessed by means of measuring the level of blood glucose and insulin, and the utilisation of glucose in liver. The investigation is generally performed by treating the animals with extracts or purified compounds. After a certain period, blood sample is taken and examined for the level of glucose and insulin. Blood glucose can be determined spectrophotometrically using *ortho*-toluidine reagent (Akhtar and Ali, 1985), hexokinase (Beppu *et al.* 1993) or glucose oxidase methods (Ajabnoor, 1990). Insulin level can be determined by radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) (Webster *et al.* 1990). To investigate the effect of plant extracts on hepatic utilisation of glucose, liver is excised and measured for the content of glycogen and/or the activity of enzymes involved in the metabolism of glucose. For example, the effects of plant extracts e.g. from *Aconitum carmichaeli* (Hikino *et al.* 1989b), *Coccinia indica* (Presanna Kumar *et al.* 1993) and *Ganoderma lucidum* (Hikino

et al. 1989a) have been studied on a number of hepatic enzymes including glucokinase, hexokinase, glucose-6-phosphatase, glucose-6-phosphate dehydrogenase, phosphofructo-kinase, glycogen syntheses and phosphorylase using liver homogenates obtained from rodents pretreated with plant materials.

A major drawback of *in vivo* systems arises from the required extensive use of animals which is both costly and time-consuming, and raises ethical issues. Although *in vivo* systems can give valuable information of hypoglycaemic activity, they reveal relatively little about mechanisms of action. An additional disadvantage of *in vivo* systems is that they are not suitable for rapid throughput experiments involving large numbers of samples. These limitations of *in vivo* models led to the development of various *in vitro* systems in an attempt to establish techniques which can identify the mechanisms of action and allow a rapid throughput experiments to be performed in a limited time.

1.5.2 *In vitro models*

As insulin secretion and responsiveness to insulin are abnormal in diabetes, the development of new agents for treating diabetes has been focused on agents that can alleviate these defects and control blood glucose levels. A number of *in vitro* models have been developed in an attempt to discover compounds possessing these particular effects. These *in vitro* models can be arranged into 4 groups i.e.

1) models to study carbohydrate digesting enzymes
2) models to study glucose uptake from small intestine
3) models to study insulin secretion from β-cells of pancreas
4) models to study glucose utilisation at target tissues e.g. liver, skeletal muscle or adipocytes
5) models to study insulin mimetic effect at insulin receptor

1.5.2.1 Model to study inhibition of α-glucosidase enzyme

α-Glucosidase is a collective term referring to enzymes involved in the breakdown of α-linkages of disaccharides and oligosaccharides into glucose; these enzymes include glucoamylase, maltase, isomaltase, sucrase and trehalase. The inhibition of the activity of these enzymes will slow down the digestion of carbohydrate and hence

the absorption of glucose from the intestine. This, in turn, is beneficial in reducing postprandial hyperglycaemia. A search for compounds that can inhibit α-glucosidase is therefore regarded as one of the therapeutic approaches for developing novel anti-diabetic agents. The recently introduced drug, Acarbose (section 1.2.2.3), is an example of an agent acting by this mechanism (Lebovitz, 1992).

The inhibitory effect of plant extracts on α-glucosidase is assessed *in vitro* by determining a decrease in the amount of glucose liberated from molecules of substrate after incubation with the enzyme. α-Glucosidase enzyme used in the study is generally prepared from rat small intestinal brush border membrane (Kessler *et al.* 1978). The choice of substrate depends on the enzyme of interest; if the activity of sucrase is to be determined, sucrose will be used as the substrate, whereas maltose and isomaltose will be used in the assay for the investigation on the activities of maltase and isomaltase, respectively. Generally, the enzyme is incubated with the substrate and plant extracts for 10-30 minutes at 37°C, the reaction is then stopped by the addition of sodium carbonate (0.2-1 M) (Tsujii *et al.* 1996; Shibano *et al.* 1997). The amount of glucose liberated at the end of the reaction can be determined colorimetrically using glucose oxidase method (Bergmeyer and Bernt, 1963).

Studies on the effect of plant extracts in a search for α-glucosidase inhibitors resulted in the isolation of a number of compounds, including kotalanol from *Salacia reticulata* with a potent inhibitory effect on sucrase and maltase (Yoshikawa *et al.* 1998a), myrciacitrin, myrciaphenone, myricitrin, desmanthin-I and guaijaverin from *Myrcia multiflora* (Yoshikawa *et al.* 1998b), 1-deoxynojirimycin, α-homonojirimycin and 7-*O*-β-*D*-glucopyranosyl-α-homonojirimycin from *Commelina communis* (Kim *et al.* 1999).

1.5.2.2 Models to study inhibition of intestinal glucose uptake

Models developed for studying intestinal absorption can be arranged into two main groups i.e. the models prepared from whole small intestine and those prepared from isolated tissues or cells.

Everted segments and everted sacs are simplified systems regularly used for studying common intestinal absorption. Both models are prepared from whole small

intestine everted onto a glass rod with the mucosal face in bathing solution. The amount of substances absorbed into the sacs is quantified by means of radioactive-labelled substrates (Wood and Lawrence, 1991). The inverted gut has been used by a number of workers to study the effect of plant extracts on glucose uptake, for example: Meir and Yaniv, (1985) used inverted gut to study the effect of *Momordica charantia*; Perl and Hikino, (1989) used inverted gut to study the effect of a number of glycans, including ganoderan A, ganoderan B, aconitan A and lithosperman A.

The major drawbacks of these models are related to short-term viability of the tissues (maximum viability of 3 hours) (Wood and Lawrence, 1991). Additionally, the use of these systems alone can not provide conclusive information on the mechanism of absorption. More complicated system i.e. isolated membrane preparations have therefore been developed to study mechanism of absorption. These models include brush border membrane vesicles (BBMV, chapter 3) and basolateral membrane vesicles (BLMV, chapter 3). The advantages of these systems lie on the stability of the preparations, small sample volumes, and the lack of the unstirred mucus and water layer which are the main barriers to intestinal absorption. The knowledge of intestinal amino acid and sugar transport have been greatly expanded owing to these models (Audus *et al.* 1990).

Over the last decade, techniques to culture mature enterocyte monolayers as models for drug absorption have been extensively developed in an attempt to overcome the problems such as rapid degradation of animal tissues and the variations due to differences between species and animals which usually occurs when intestinal tissues and isolated membrane preparations are used. However, the attempt has met with little success (Hilgers *et al.* 1990). It has been reported that when the intestinal epithelial cells were cultured as a monolayer, they underwent transformation into a cell type different from the initial cells (Audus *et al.* 1990). To date the use of cell monolayers in intestinal absorption study exploits cells derived from human colon carcinoma cell lines e.g. Caco-2, HT-29, SW 116, LS 174T, SW-480 (Audus *et al.* 1990). Among these cells, Caco-2 cells, established by Fogh *et al.* (1977), have received a great deal of attention, and are widely used as a model for intestinal transport and function studies (Artursson, 1990; Hilgers *et al.* 1990). Despite its colonic origin, Caco-2 have been reported to undergo spontaneous enterocytic differentiation in culture (Audus *et al.* 1990). The cells have been reported to have morphological (e.g. polarized and columnar cells and the presence

of microvilli) and biochemical properties (e.g. the distribution of brush border enzymes) which are much more intestinal than colonic (Hilgers *et al.* 1990). Caco-2 cells have been used to study the transport of various substances including bile acids, glucose, salicylic acid, β blockers and testosterone (Hilgers *et al.* 1990; Wilson, 1990). However, the use of Caco-2 cells in the area of natural product research is not yet extensive. A few examples are the studies by Neef *et al.* (1996) and Nishioka *et al.* (1998). In these studies, the cells were used to investigate the effects of galegine, isolated from *Galega officinalis* (Neef *et al.* 1996) and baicalein, a flavone isolated from *Scutellaria baicalensis*, on intestinal glucose absorption (Nishioka *et al.* 1998).

HT-29 cell lines which also originate from colon cell lines have been reported to have a lower degree of enterocytic differentiation compared to Caco-2 cells (Audus *et al.* 1990). The application of HT-29 in the study of intestinal absorption is therefore less frequent than those of Caco-2 cells. HT29-H and HT29-18-C_1 are subclones derived from HT-29 (Phillips *et al.* 1988). HT29-H cells are different from the other cell lines in that they can secrete mucin molecules and produce a mucus gel layer which is present in human intestinal epithelium (Wikman *et al.* 1993). This layer is thought to be a rate limiting barrier to drug absorption (Wikman *et al.* 1993). Results of studies absorption in these cells may be therefore closer to the *in vivo* situation.

1.5.2.3 Models to study insulin secretion from β-cells of the pancreas

A number of *in vitro* models have been developed for studying the secretion of insulin. These include perfused pancreas, intact isolated islets, purified β-cells and insulin-secreting cell lines. Their uses are mainly restricted to study the role of second messengers in the signalling pathway of insulin secretion. Perfused pancreas, isolated islets and purified β-cells are prepared from sacrificed animals (usually mice and rats). Isolation of islets of Langerhans involves collagenase digestion and purification from exocrine tissues. Islet isolation from rodents yields a maximum of several hundreds of islets (Poitout *et al.* 1996a). The following are some examples of studies in the area of natural products that have employed the use of isolated islets. Pancreatic islets isolated from rats were used by Suzuki and Hikino, (1989a) to study the effect of panaxan B, a glycan from *Panax ginseng*, on insulin secretion. Ahmad *et al.* (1991b) used islets prepared from rats to study the effect of *Pterocarpus marsupium* on insulin secretion and the

accumulated level of cAMP. Rat islets were used in the study by Hii and Howell, (1985) to study the effects of catechin, chrysin, epicatechin, naringenin and quercetin on Ca^{2+} handling.

Although a larger number of islets can be obtained from large animal pancreata, isolation and purification techniques are time consuming and require experienced staff (Poitout *et al.* 1996a). The techniques to purify primary β-cells are equally complicated and require special techniques such as fluorescent-activated cell sorting to differentiate between β-cells and the other cells of islets of Langerhans (Poitout *et al.* 1996a). In addition, primary β-cells do not proliferate in culture, thus they are very difficult to maintain in culture for a long period without special techniques. Owing to the these limitations, the use of intact islets or primary β-cells is not suitable for use in rapid throughput experiments.

A number of insulin-secreting cell lines have been developed in an attempt to establish cell lines that retain the characteristic features of β-cells. The cell lines are transformed using different techniques such as irradiation, viral transformation, and transgenic technology (Poitout *et al.* 1996a). They may therefore be different from primary β-cells in terms of their behaviour and responsiveness to insulin secretagogues (for review see Poitout *et al.* 1996a). The most widely used β-cell lines are RINm5F, HIT-T15, βTC, MIN6, INS-1 and BRIN-BD11 cells (Poitout *et al.* 1996a). Their application in natural product area is not yet extensive. Examples include the use of HIT-T15 cells to study the effect of *Tinospora crispa* extract on insulin secretion (Noor *et al.* 1989). BRIN-BD11 have been used in a number of studies, examples are the investigations on the effect of *Coriandum sativum* (Gray and Flatt, 1999) and *Medicago sativum* (Gray and Flatt, 1997) on insulin secretion. The advantage of β-cell lines is that they can be used in a rapid throughput experiment which is exceedingly difficult to carry out using islets isolated from sacrificed animals. This therefore minimises the number of animals used in the experiment. However, it has to be borne in mind that as these cells are a transformation of pancreatic β-cells, some characteristic features of β-cells may not be faithfully represented by the cell lines (Persaud, 1999).

1.5.2.4 Models to study glucose utilisation in the liver

As the poor utilisation of glucose at insulin target tissues is one of the major defects in diabetes, agents that can alleviate this defect should proven to be useful for the treatment of diabetes. Over the last two decades, a number of *in vitro* systems have been developed for studying the pathway of glucose metabolism and the effect of hormones or drugs on enzymes involved in hepatic glucose metabolism (e.g. glucokinase, glucose-6-phosphatase, phosphofructokinase, glycogen synthetase and phosphorylase). *In vitro* systems which have been developed for studying hepatic glucose metabolism mostly use tissues taken from rodents. These models are 1) liver slices or homogenates, 2) perfused liver, 3) hepatocyte suspensions, 4) hepatocyte monolayer cultures, 5) hepatocytes in co-culture with epithelial cells and 6) periportal and perivenous hepatocyte suspensions or cultures (Agius, 1987).

Earlier studies of hepatic glycolysis and gluconeogenesis were mostly performed using crude liver preparations such as liver slices, homogenates and subcellular fractions. The use of crude liver preparations is always hampered by the limitations of substrate and oxygen diffusion (Agius, 1987). The perfused liver is the system most closely representing physiological conditions. However, the use of perfused liver is limited by short-term viability and limited availability of the tissues from a single preparation. The use of perfused liver has therefore been replaced with isolated hepatocytes maintained in suspension or culture. In 1969, Berry & Friend (1969) developed techniques for isolation of high yields of viable parenchymal hepatocytes using two-step collagenase perfusion. Isolated hepatocytes maintained in suspension have been widely used over the past two decades for studying hormone binding and acute effect of substrates or drugs on liver metabolism (Agius, 1987), examples included studies on the effect of glucagon on phosphoenolpyruvate carboxykinase synthesis (Salavert and Iynedjian, 1982) and the effect of glucose, insulin and glucagon on glycogen metabolism (Seglen, 1973). The disadvantage of this system is that the cells are in a catabolic state of protein and glycogen turnover and they are not suitable for long term studies because of their short term viability. These limitations led to the development of monolayer culture of parenchymal hepatocytes.

By means of suitable culturing, isolated hepatocytes can recover from a catabolic state and can be maintained for long term study for several days. This system has been

widely used in a number of studies, for examples the studies of extrapancreatic action of anti-diabetic drugs e.g. gliquidone (Rinniger *et al.* 1984) and glyburide (Fleig *et al.* 1984b), studies of acute and long term effects of hormone and drugs on enzyme induction (Castano *et al.* 1979, Fleig *et al.* 1984a) and studies on the glycogen synthesis pathway (Newgard *et al.* 1983, Spence and Koudelka, 1985; Bismut and Plas, 1989). The drawback of monolayer culture of hepatocytes is that the cells tend to gradually lose some characteristic functions of the liver e.g. loss of glycogen content, albumin production, gluconeogenesis and ketogenesis during the culture.

Techniques to co-culture parenchymal hepatocytes with dividing liver epithelial cells have been developed by Guguen-Guillouzo *et al.* (1983) to overcome the problem of functional loss in prolonged cultured parenchymal hepatocytes. By using co-culture techniques, cells can be maintained for several weeks without losing functional properties (Agius, 1987). Since the study of glucose metabolism is more complex with parenchymal and epithelial co-culture, its application in this area is not extensive. Isolated periportal and perivenous hepatocytes are another preparation which has been developed and widely used, in particular for studying metabolic differences between the periportal zone where the enzymes involved in gluconeogenesis and glycogenolysis are located and perivenous zone where enzymes involved in glycolysis, glycogenesis and ketogenesis are located (Agius *et al.* 1990).

More recently, hepatoma cell lines from rat (e.g. FTO-2B and H4IIE) and humans (e.g. HepG2 and Hep3B) have been established and widely used in a number of studies to investigate the effect of anti-diabetic drugs and hormones on liver metabolism, for example, H4IIE was used to study the effect of metformin on glycogen synthesis (Purrello *et al.* 1988), the inhibitory effect of tolbutamide on phosphoenolpyruvate carboxykinase activity (Agius *et al.* 1990) and the effect of insulin on glycogen synthase activity (Okubo *et al.* 1993). HepG2 cells were used to study the effect of troglitazone on glycogen synthesis and gluconeogenesis (Day, 1999). The use of these cell lines with natural products has not been reported to date.

1.5.2.5 Models to study glucose utilisation in muscle and adipocytes

Utilisation of glucose in skeletal muscle and adipocytes is another insulin-sensitive action occasionally determined when the hypoglycaemic activity of plant extracts

is assessed. Abdominal muscle taken from mice has been used to study the effect of plant extracts such as *Medicago sativum* (Gray and Flatt, 1997) and *Agrimony eupatoria* (Gray and Flatt, 1998) on the uptake of glucose, oxidative glucose metabolism and incorporation of glucose into glycogen. Rat hemi-diaphragm was used by Chattopadhyay *et al.* (1993) to study the effect of *Azadirachta indica* on muscle glycogen synthesis in an attempt to identify a mechanism of anti-diabetic action of the plant (Chattopadhyay *et al* 1993).

Adipocytes isolated from rat epididymal adipose tissue were used to study the effect of *Ganoderma lucidum* extract on adrenaline-induced lipolysis and lipogenesis from glucose (Kimura *et al.* 1988), and the effect of ganoderan B isolated from *Ganoderma lucidum* (Hikino *et al.* 1989a) and the effect of aconitan A isolated from *Aconitum carmichaeli* (Hikino *et al.* 1989b) on the binding of insulin to adipocytes.

1.5.2.6 Models to study insulin-mimetic effects at the insulin receptor

Another approach which has been recently introduced and suggested to have a great deal of potential in the development of new oral anti-diabetic agent is a search for an agent which is capable of interacting with and activating insulin receptor, thereby acting as an insulin mimic in a variety of biochemical cascades. Based on this approach, Zhang *et al.* (1999) have developed a cell-based screening assay with Chinese hamster ovary cells that overexpress the human insulin receptor. In their study, the cells were incubated with the test compound for 20 minutes, insulin receptor was then purified and assayed for activity of tyrosine kinase, which is a protein believed to be involved in the phosphorylation cascade after the binding of insulin to its receptor. Using this assay, they have discovered an agent from a fungus, *Pseudomassaria sp.*, which is capable of inducing tyrosyl protein phosphorylation; the effect has been shown to be selective to insulin receptor (Zhang *et al.* 1999). This agent is known as L-783,281 (Fig. 1.15).

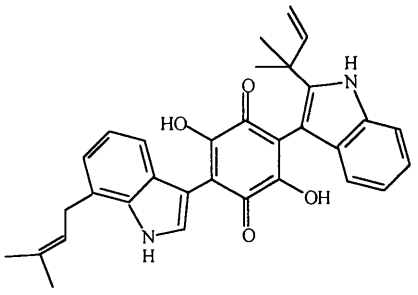


Fig. 1.15: Structure of L-783,281

Investigation of the pharmacological activity of this compound revealed that L-783,281 (25 mg/kg, p.o.) caused a marked reduction of blood glucose in *db/db* and *ob/ob* mice. The discovery of L-783,281 might prove to be an important step for the development of a new type of oral anti-diabetic agent from a natural source.

1.6 Aim of the present study

The overall aim of the current study is to investigate biological effects of selected medicinal plants traditionally used as anti-diabetic remedies by using *in vitro* models in order to discover potential new lead compounds for the treatment of diabetes.

The specific objectives of this study are

a) to select plants for the study based on their traditional uses documented in the literature

b) to set up a range of *in vitro* assays in an attempt to investigate the effect of the selected plants on specific mechanisms of anti-diabetic activity. These are to include models based on potential effects on
   - intestinal glucose absorption
   - pancreatic insulin secretion
   - insulin target tissues

c) to test the extracts of selected plants on a range of *in vitro* models based on the activities described above

d) to isolate constituents which are responsible for the effects of the active plants using bioassay-guided fractionation

e) to elucidate structures of the isolated compounds by means of spectroscopic method including mass spectrometry, and ^{1}H, ^{13}C and two dimensional NMR spectroscopy

f) to investigate the mechanisms of action of any active compounds isolated

The following chapters describe these investigations in detail.

CHAPTER 2

SELECTION OF
MEDICINAL PLANTS FOR THE STUDY

2.1 Criteria for selecting the plants used in the study

Plants selected for this study were among those which have been quoted in traditional remedies for the treatment of diabetes, according to Indian Materia Medica (Chopra *et al.* 1956), Handbook of Ayurvedic Medicinal Plants (Kapoor, 1990), Medicinal plant of China (Duke and Ayensu, 1985a & 1985b) and Pharmacology and Applications of Chinese Materia Medica (Chang and But, 1986a & 1986b). A literature search for scientific support of the anti-diabetic activity of these plants was conducted using Bath Information and Data Service (BIDS), Embase and MEDLINE (1980-1999, keywords: anti-diabetic, hypoglycaemic, anti-hyperglycaemic and plant genus). The detail of literature survey is presented in section 2.2.

It has been suggested that selection of plants for investigation should be based on the following criteria (Marles and Farnsworth, 1995):

1) traditional use as anti-diabetic remedies in one or more countries
2) experimentally determined hypoglycaemic activity
3) lack of detailed information on hypoglycaemic constituents
4) experimental evidence for low toxicity
5) availability of the plants

The plants selected for this study consisted of 31 plant species from 30 genera and 24 families, and are listed in Table 2.1. The criteria suggested by Marles and Farnsworth, (1995) were followed to some extent. The two primary criteria were that the plants were used in traditional anti-diabetic remedies and that the material was readily available from commercial sources (see section 2.3). A detailed literature survey was conducted on each selected species, results of which are summarised in section 2.2. Compliance with criteria (2) and (3) of Marles and Farnsworth, (1995) varied. Plants such as *Benincasa hispida*, *Cyperus rotundus*, *Lycium chinensis*, *Polygonatum odoratum*, *Salvia miltiorrhiza* and *Typhonium giganteum* have been quoted as remedies for diabetes in Ayurvedic or Chinese medicines, but have no conclusive scientific data to support their uses. Further studies to evaluate the use of these plants in diabetes are therefore necessary. The selection of *Benincasa hispida* (Cucurbitaceae) was also based on the fact that there

have been a number of plants from Cucurbitaceae family e.g. *Bryonia alba*, *Momordica charantia* and *Trichosanthes dioica* which have strong scientific support to be beneficial for diabetes. In addition, *Benincasa hispida* has also been quoted as anti-diabetic remedy in traditional Chinese medicine, the study of this plant is therefore of interest.

Plants in genus *Atractylodes* e.g. *A. japonicus*, *A. lancea* and *A. macrocephala* have been quoted in traditional Chinese medicine for diabetes and studies on the hypoglycaemic effect of *A. japonicus* (Konno *et al.* 1985d) have shown encouraging results. However, unlike *A. japonicus*, *A. lancea* and *A. macrocephala* still lack scientific data to support their uses as traditional anti-diabetic remedies and thus were selected for the study. For some plants such as *Bixa orellana*, *Murraya koenigii, Ophiopogon japonicus, Opuntia spp.*, *Piper longum* and *Platycodon grandiflorum*, the anti-diabetic activity of the crude extracts has been scientifically evaluated, but modes of action and constituents responsible for the activities of these extracts have not been identified. Among the selected plants, there were a few plants whose hypoglycaemic compounds have been identified (e.g. *Coptis chinensis*, *Curcuma longa*, *Morus alba*, *Swertia spp.* and *Trichosanthes kirilowii*), but the precise mechanisms of action of these compounds are still unknown. Further study is therefore necessary not only to identify modes of action of these plants but also to discover other active compounds which may be present.

Table 2.1: Plants selected for the study

	Part Used	Used in*
Alismataceae		
Alisma orientalis Juzep.	Rhizome	TCM
Araceae		
Typhonium giganteum Engl.	Rhizome	TCM
Araliaceae		
Panax ginseng C.A. Meyer	Root	TCM
Bixaceae		
Bixa orellana Linn.	Seed	Ayurveda

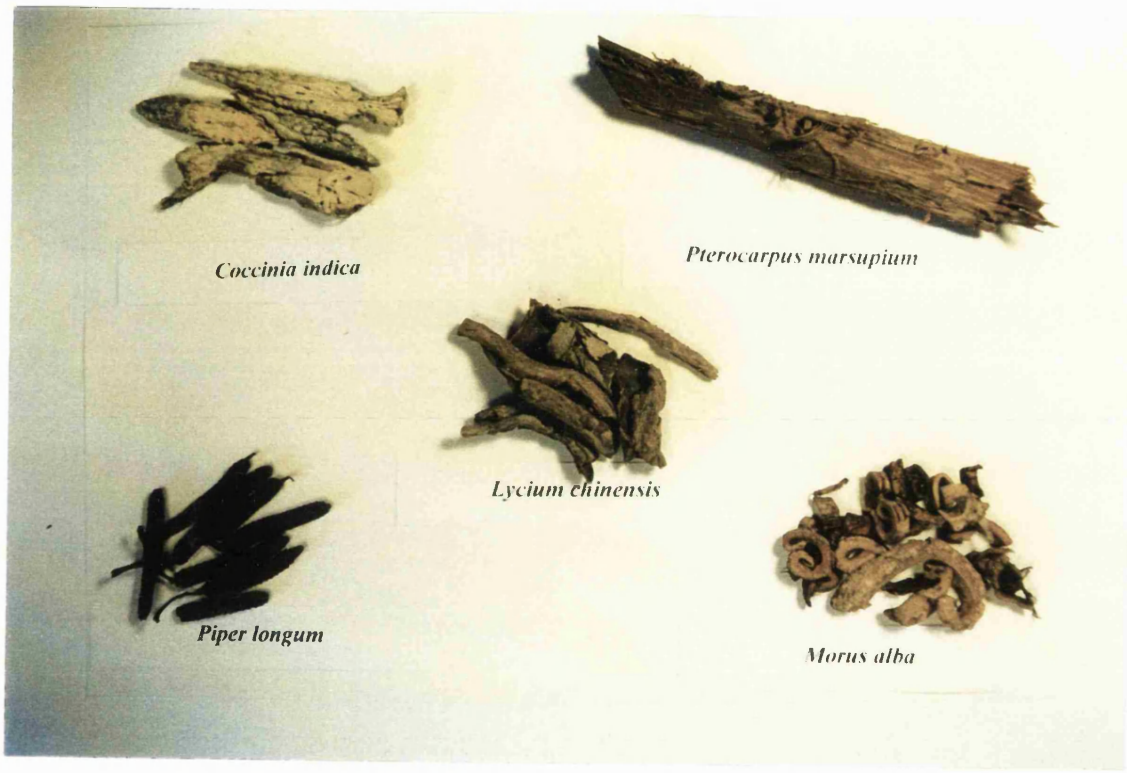


Fig. 2.1: Some of the plants selected for the study

Table 2.1 (continued)

	Part Used	Used in*
Cactaceae		
Opuntia spp.	Stem	Ayurveda
Campanulaceae		
Platycodon grandiflorum A. DC.	Root	TCM
Celastraceae		
Salacia reticulata Wight	Stem	Ayurveda
Compositae		
Atractylodes lancea Thunb.	Rhizome	TCM
Atractylodes macrocephala Koidz.	Rhizome	TCM
Cucurbitaceae		
Benincasa hispida Cogn.	Seed	TCM
Coccinia indica Wight & Arn.	Stem	Ayurveda
Trichosanthes kirilowii Maxim.	Root	TCM
Cyperaceae		
Cyperus rotundus Linn.	Root	Ayurveda
Gentianaceae		
Swertia spp.	Whole Plant	Ayurveda & TCM
Labiatae		
Salvia miltiorrhiza Bunge.	Root	TCM
Leguminosae		
Pterocarpus marsupium Linn.	Wood	Ayurveda
Liliaceae		
Anemarrhena asphodeloides Bunge.	Rhizome	TCM
Ophiopogon japonicus Thunb.	Root	TCM
Polygonatum odoratum Druce.	Rhizome	TCM
Meliaceae		
Azadirachta indica A. Juss.	Leaves	Ayurveda
Menispermaceae		
Tinospora cordifolia Miers.	Stem	Ayurveda
Moraceae		
Ficus bengalensis Linn.	Bark	Ayurveda
Morus alba Linn.	Bark	TCM

Table 2.1 (continued)

	Part Used	Used in*
Piperaceae		
Piper longum Linn.	Fruit	Ayurveda & TCM
Polyporaceae		
Ganoderma lucidum Karsten.	Fruit Body	TCM
Ranunculaceae		
Coptis chinensis Franch.	Rhizome	TCM
Rutaceae		
Murraya koenigii Spreng.	Leaves	Ayurveda
Scrophularicaea		
Rehmannia glutinosa Libosch.	Root	TCM
Scrophularia ningpoensis Hemsl.	Root	TCM
Solanaceae		
Lycium chinensis Mill.	Bark	TCM
Zingiberaceae		
Curcuma longa Linn.	Rhizome	Ayurveda

*Ethnopharmacological use in traditional medicine. TCM: traditional Chinese medicine

2.2 Literature survey on the effect of selected plants on anti-diabetic activity

A detailed literature search was carried out on each of the plants listed in Table 2.1 as described in section 2.1. The results are presented in this section.

2.2.1 Alismataceae

Alisma orientalis Juzep.

The tuber of *Alisma orientalis* is known as "Zexie" in Chinese. It has been used in traditional Chinese medicine for the treatment of bladder distension, diabetes, dropsy, fever, gonorrhoea, kidney stones, oedema, urinary infection and vertigo (Duke and Ayensu, 1985a; Chang and But, 1986b).

Alisol A, a triterpene isolated from *A. orientalis*, (Fig. 2.2) and its derivatives, were reported to have hypoglycaemic activity. The dose and route of administration were not specified in the review (Ling-Hua and Pei-Gen, 1993). Mechanism of action of these compounds remains to be defined.

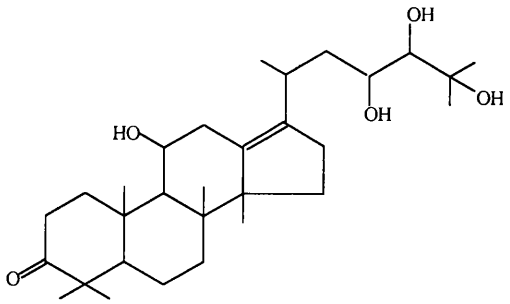


Fig. 2.2: Structure of Alisol A

2.2.2 Araceae

***Typhonium giganteum* Engl.**

Typhonium giganteum is known as "Du Jiao Lian" in Chinese. The root has been used in Chinese folklore medicines as an antispasmodic, carminative and expectorant, and to treat diabetes, headache, paralysis, rheumatism and stroke (Duke and Ayensu, 1985a). No further information relating this plant to diabetes was found in BIDS and MEDLINE (1980-1999).

2.2.3 Araliaceae

***Panax ginseng* C.A. Meyer**

Panax ginseng rhizome is commonly known in Chinese as "Ren Shen" (man root). Ginseng root has been used in folklore medicines in China, Japan and Korea since ancient times to treat various disorders including atherosclerosis, cough, diabetes, hypertension and nausea and as an antiseptic and carminative (Duke and Ayensu, 1985a; Yokozawa *et al.* 1985).

The anti-diabetic effects of *P. ginseng* have been extensively studied by a number of workers. An aqueous methanolic extract of *P. ginseng* (10 g/kg, i.p.) was reported to significantly lower blood glucose level in normal mice (Konno *et al.* 1984). The crude polysaccharide fraction of *P. ginseng* (100 mg/kg, i.p.) also

significantly lowered the level of plasma glucose 7 hours after administration to normal mice (Konno *et al.* 1985c; Oshima *et al.* 1985).

Bioassay-directed fractionation of the crude polysaccharide fractions afforded the isolation of a number of hypoglycaemic glycans i.e. panaxan A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P, Q, R, S, T, U (Konno *et al.* 1984; Konno *et al.* 1985c; Oshima *et al.* 1985; Tomoda *et al.* 1985). These glycans (3-100 mg/kg, i.p.) were found to be effective in reducing the level of blood glucose in normal mice to different extents. The effect of panaxan J, L, Q and R in normal mice was very weak, since the activities were reported only at a high dose of 100 mg/kg (i.p.) for panaxan J, L and R, and 300 mg/kg for panaxan Q. Moreover, the activities of these glycans were shown to be less potent than crude glycan fractions tested at the same dose (100 mg/kg). Investigation of the effects of panaxan A, B, J and U in alloxan-diabetic mice showed that panaxan A and B at a low dose of 10-30 mg/kg (i.p.) caused a significant reduction in blood glucose level, whereas panaxan J and U were effective only at a higher dose of 100 mg/kg (i.p.) (Konno *et al.* 1985c). Both intravenous and intraperitoneal administrations of panaxan B (10 mg/kg) were also found to have a hypoglycaemic effect in spontaneous diabetic mice (C57BL/Ksj-db/db strain) (Suzuki and Hikino, 1989b).

The mechanisms of hypoglycaemic activity of panaxan A and B have been extensively studied (Suzuki and Hikino, 1989a; Suzuki and Hikino, 1989b). It was reported that the activity of panaxan A and B was not related to an increase in a conversion of glucose to glucose-6-phosphate, since panaxan A and B (30 mg/kg, i.p.) had no effect on hexokinase and glucokinase enzymes in normal mice (Suzuki and Hikino, 1989b). The activity of the two glycans (30 mg/kg, i.p.) also did not appear to be associated with the promotion of glucose conversion into glycogen, since panaxan A had no effect on glycogen content (Suzuki and Hikino, 1989a) whereas panaxan B depleted glycogen content in normal mice (Suzuki and Hikino, 1989b). The depletion of glycogen induced by panaxan B was attributed to an increase in the activity of phosphorylase a, and a decrease in the activity of glycogen synthase (Suzuki and Hikino, 1989b). The activities of glucose-6-phosphate dehydrogenase and phosphofructokinase in normal mice were demonstrated to be increased by panaxan A

and panaxan B (Suzuki and Hikino, 1989b), suggesting that these two glycans may work, at least in part, by stimulating glycolysis and pentose phosphate pathway.

Panaxan B (30 mg/kg, i.p.) but not panaxan A was found to increase the level of plasma insulin in normal, moderately alloxan-diabetic, and glucose-loaded mice (Suzuki and Hikino, 1989b). In addition, rate of insulin release from the islets isolated from rats that had been injected with panaxan B (10 mg/kg, i.p.) in response to 3.3 mM glucose was found to be higher than that from islets of control (Suzuki and Hikino, 1989b). However, the study on the direct effect of panaxan B on isolated islets, where rat islets were incubated with the compound (30 μg/ml) in the presence of 3.3 mM glucose for 1 hour revealed that panaxan B did not have stimulatory effect on insulin secretion (Suzuki and Hikino, 1989b). The negative result obtained from the latter study may be due simply to the fact that the dose of panaxan B used in the study was very low (30 μg/ml). Since the compound was tested only at a single dose of 30 μg/ml, it is insufficient to draw any conclusion on the activity of panaxan B regarding the direct effect on insulin secretion from the islets. A definite conclusion can be made only when the compound is tested at a range of concentrations.

A number of saponins have been isolated from *P. ginseng* roots including ginsenosides Rb_1, Rb_2, Rc, Rg_1 and Rg_2 (for review on their pharmacological activities see Ng and Yeung, (1985)).

Administration of ginsenoside Rb_2 (10 mg/rat/day, i.p., 6 days) was reported to lower blood glucose level in STZ-induced diabetic rats (Yokozawa *et al.* 1985). Treatment with ginsenoside Rb_2 (10 mg/rat/day, i.p., 6 days) also significantly increased hepatic glycogen in STZ-induced diabetic rats, this is concordant with a decrease in hepatic glucose-6-phosphatase activity and increase in glucokinase activity (Yokozawa *et al.* 1985). This led to a suggestion that hypoglycaemic activity of ginsenoside Rb_2 was, at least in part, owing to its ability to enhance glucose utilisation in liver.

Ginsenoside Rg_1 (30 mg/kg/day, 3 days, i.p.), a saponin isolated from *P. ginseng* roots, enhanced insulin binding and increased number of insulin receptors in membranes of liver and brain (Tchilian *et al.* 1991). Thus both glycan and saponin components of *P. ginseng* have been linked to hypoglycaemic effects. However in

all these studies, the compounds were administered by intraperitoneal injection, and it is not known if similar effects would be observed on oral administration.

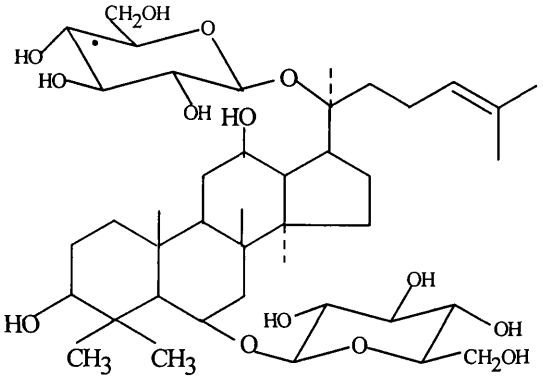


Fig. 2.3: Structure of ginsenoside Rg_1

2.2.4 Bixaceae

***Bixa orellana* Linn**

Bixa orellana is also known as 'annatto'. The plant has been used in Ayurveda as a remedy for diabetes, dysentery, gonorrhoea, jaundice and snake-bite (Chopra *et al.* 1956). The oil soluble portion of the chloroform extract of *B. orellana* seed when administered orally at doses of 1 and 2 g, was found to inhibit an elevation of plasma glucose in glucose-challenged dogs. The anti-hyperglycaemic effect observed in this animal model was reported to be accompanied by a decrease in insulin level, suggesting that the anti-diabetic effect of *B. orellana* was unlikely to be related to stimulation of insulin secretion (Morrison, 1985).

2.2.5 Cactaceae

Opuntia spp.

A number of species in the genus *Opuntia* have been quoted as traditional remedies for the treatment of diabetes (Rahman and Zaman, 1989), including *O. dillenni*, *O. ficus indica*, *O. lindheimeri*, and *O. streptacantha*. A number of studies have given support to the claims of these plants regarding their anti-diabetic effect. However, active principles of these plants are still unknown.

***Opuntia dillenii* (Ker-Gawl) Haw.** (Syn. *Cactus indicus*)

The plant is native to America and was introduced to India by the Portuguese. The fruit has been used in Ayurvedic medicine as an expectorant for the treatment of asthma and spasmodic cough. The ripe fruit has been used to treat gonorrhoea (Nadkarni and Nadkarni, 1982).

It was reported that the fruit juice of *O. dilennii* (5 ml/kg, p.o.) significantly lowered levels of blood glucose in orally glucose-loaded normal and diabetic rabbits, the glucose-lowering effect observed in the normal rabbits did not appear to be related to stimulation of insulin secretion since the levels of insulin remained unchanged (Perfumi and Tacconi, 1996). However, the juice (5 ml/kg, p.o.) failed to exert its effect in normal rabbits intravenously loaded with glucose (Perfumi and Tacconi, 1996). Both short term (5 ml/kg, p.o., single dose) and long term (5 ml/kg, p.o., 7 days) treatments with *O. dillenni* juice also failed to reduce blood glucose levels in normal and alloxan-induced diabetic rabbits (Perfumi and Tacconi, 1996). These led to a suggestion that the juice possibly exerted its effect via an inhibition of intestinal glucose absorption.

***Opuntia ficus indica* Miller**

A study of the effect of *O. ficus indica* stem in STZ-induced diabetic rats showed that *O. ficus indica* significantly reduced blood glucose levels when given intravenously (0.5 mg/ml) but failed to exert its effect when given orally (2 mg/ml) (Enigbokan *et al.* 1996). In contrast to Enigbokan's results, it was reported that the oral intake of *O. ficus indica* stem extract (500 g) markedly lowered blood glucose level in patients with NIDDM (Frati *et al.* 1990). It was also reported that the intake of 100 g of broiled stem of *O. ficus indica* prior to oral glucose load could inhibit an elevation of blood glucose levels in healthy human (Frati-Munari *et al.* 1986). The discrepancy was possibly owing to the differences in experimental models, and preparation and doses of the extracts used in the studies.

***Opuntia lindheimeri* Englm**

Administration of *O. lindheimeri* stem extract both orally (2 mg/ml) and intravenously (0.5 mg/ml) was reported to decrease blood glucose levels in STZ-

induced diabetic mice (Enigbokan *et al.* 1996). Mechanism of action and active principle of this plant remain to be assessed.

***Opuntia streptacantha* Lemaire**

Oral administration of the sap of fresh *O. streptacantha* stems (5 ml/kg) was reported to significantly lower blood glucose levels in normal anaesthetised dogs, glucose loaded rabbits and rats, but failed to exert an effect in normal and pancreatectomized rabbits (Ibañez-Camacho and Roman-Ramos, 1979; Ibañez-Camacho *et al.* 1983). The failure to exert an effect in pancreatectomized rabbits has led to a suggestion that the effect of *O. streptacantha* may partly be due to its pancreatic effect, and thus required the presence of intact pancreatic tissue. The discrepancy in the effects observed in normal dogs and normal rabbits was possibly related to the differences in animal species. Studies in NIDDM patients showed that oral administration of broiled stem of *O. streptacantha* (500 g) significantly reduced blood glucose levels in NIDDM subjects but had no effect on healthy patients. The mechanism of action of this plant has not been assessed (reviewed by Ernst, 1998).

2.2.6 Campanulaceae

***Platycodon grandiflorum* (Jacq) A. DC.**

Platycodon grandiflorum root is commonly known as kikio root and balloon flower and as "Jie Geng" in Chinese. Root of *P. grandiflorum* has been used in Chinese medicine as anti-asthmatic, astringent, carminative, expectorant, anti-diabetic, and sedative agent (Duke and Ayensu, 1985a). Aqueous and ethanol extracts (200 mg/kg, p.o.) from *P. grandiflorum* root were reported to show hypoglycaemic effect in normal rabbits. The extracts at a higher dose of 500 mg/kg (p.o., 4 days) were reported to lower the level of blood glucose in alloxan-diabetic rabbits (Chang and But, 1986a). To date, no reports were found of any isolated anti-diabetic compounds from this plant.

2.2.7 Celastraceae

***Salacia reticulata* Wight.**

Salacia reticulata, also known as "Kotala himbutu" in Singhalase, has been used as a remedy for diabetes in Ayurvedic system of Indian traditional

medicine. Aqueous extract from root bark of this plant when given orally at a dose of 2.5 g/kg (Karunanayake *et al.* 1984) and 5 g/kg (Serasinghe *et al.* 1990) was reported to significantly lower blood glucose levels in normal and STZ-induced diabetic rats, respectively.

Kotalanol and salacinol (Fig. 2.4) isolated from the aqueous extract of *S. reticulata* roots and stems were reported to have potent inhibitory activity against intestinal α-glucosidase *in vitro* (Yoshikawa *et al.* 1998a). Half maximum effect (IC_{50}) of kotalanol were 0.58 μg/ml to sucrase, 2.8 μg/ml to maltase and 1.9 μg/ml to isomaltase (Yoshikawa *et al.* 1998a). The inhibitory effect of kotalanol on sucrase and isomaltose was reported to be more potent than that of acarbose (IC_{50}: 1.1 and 100 μg/ml, respectively) and that on sucrase and maltose was more potent than salacinol (IC_{50}: 0.84 and 3.2 μg/ml, respectively) (Yoshikawa *et al.* 1998a). *In vivo* effect of these compounds has not yet been assessed.

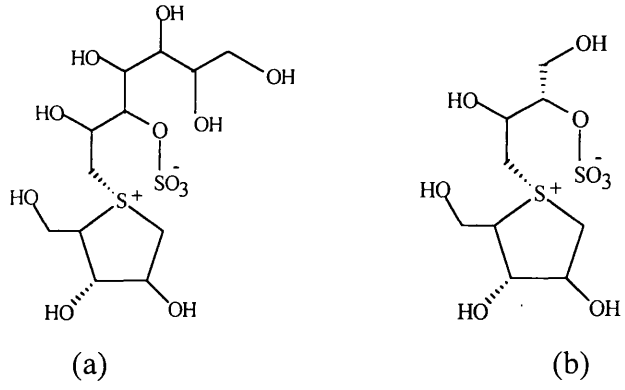


Fig. 2.4 : Structure of (a) Katalanol and (b) Salacinol

2.2.8 Compositae

A number of plants in genus *Atractylodes* have been reported to have hypoglycaemic effect, including *A. japonica*, *A. lancea* and *A. macrocephala*. The water extract of *A. japonica* rhizome was reported to have hypoglycaemic effect in normal mice when given intraperitoneally at a dose of 5 g/kg, and atractan A, B and C were claimed to be active principles of this plant (Konno *et al.* 1985d). However, the effect of these compounds on an oral administration has not been assessed. Unlike *A. japonica*, the anti-diabetic effect of *A. lancea* and *A. macrocephala* has not been extensively investigated.

***Atractylodes lancea* Thunb.**

Atractylodes lancea is known as "Cang Zhu" in Chinese. Rhizome of *A. lancea* has been used in Chinese medicines for the treatment of rheumatism, abdominal distension, edema and mild diarrhoea (Chang and But, 1986b). Its rhizome was also reported to be effective as anti-diabetic, diuretic and also for the treatment of malaria (Chang and But, 1986b). No report was found of any study on anti-diabetic effect of this plant.

***Atractylodes macrocephala* Koidz.**

Atractylodes macrocephala rhizome is known as "Bai Zhu" in Chinese. The rhizome has been widely used in traditional Chinese medicines to treat bronchitis, chronic diarrhoea, oedema, gastroenteritis, jaundice, and vertigo (Chang and But, 1986b). The rhizome was also reported to be effective as diuretic, anti-diabetic, and anti-coagulant (Chang and But, 1986b). However, its effect on blood glucose is still not conclusive. While it was reported that subcutaneous injection of the extract significantly reduced blood glucose levels in normal rabbit (dose was not specified), some workers reported that the extract failed to show this effect (Chang and But, 1986b). Thus further scientific support for the use of this plants is therefore necessary.

2.2.9 Cucurbitaceae

***Benincasa hispida* Cogn.** (Syn. *Benincasa cerifera* Savi.)

Benincasa hispida is commonly known as "ash gourd" and "Dong Gua Zi" in Chinese. Its fruit and seed have been used in folklore medicine to treat tumour, diabetes, dropsy and painful wounds and also used as laxative and diuretic (Duke and Ayensu, 1985a). No report was found of any scientific studies linking this plant to diabetes.

***Coccinia indica* Wight & Arn.** (Syn. *Coccinia grandis* Voight., *Coccinia cordifolia* Cogn.)

Coccinia indica has been used in Ayurvedic medicines against a number of diseases including diabetes, gonorrhoea, skin disease and tongue sores (Mukherjee *et al.* 1988). In Ayurvedic medicine for the treatment of diabetes, the plant

was reported to be effective when dried fruit powder of *C. indica* was given to patients at a dose of 10 g three times a day (Alam *et al.* 1990).

Anti-diabetic activity of various parts of *C. indica* has been reported in a number of studies. The ethanolic extract of the whole plant (250 mg/kg, p.o.) was reported to be active in normal and diabetic fasting rats, but failed to exert its effect in fed normal rats (Mukherjee *et al.* 1988). An administration of dried fruit powder along with diet for 45 days was reported to significantly lower blood glucose level in normal mice (Presanna Kumar *et al.* 1993). Dried powdered leaves caused a reduction of blood glucose level in alloxan-induced diabetic dogs, however it failed to exert an effect in normal dogs (Ivorra *et al.* 1989). The ethanolic extract of *C. indica* root (250 mg/kg, p.o.) had no hypoglycaemic effect in both normal and diabetic rats (Mukherjee *et al.* 1988). In humans, a study of the effect of *C. indica* in double-blinded clinical trials revealed that there was a significant improvement in glucose tolerance in patients with maturity-onset diabetes who received tablets (dose was not specified) prepared from homogenised and freeze-dried leaves of *C. indica* twice daily for 6 weeks (Azad Khan *et al.* 1980).

Regarding its mode of action, it was reported that the administration of 15% dried fruit powder along with diet for 45 days could increase the content of hepatic glycogen in normal mice (Day, 1990; Presanna Kumar *et al.* 1993), suggesting that the effect of *C. indica* fruit was possibly attributed to enhanced glycogen synthesis in the liver.

Despite the encouraging results from a number of studies on the anti-diabetic effect of *C. indica* crude extract, the investigation on the active constituents from this plant is not yet extensive. Pectin is an active compound isolated from *C. indica* which has received a great deal of attention. It was reported that normal rats which received pectin (p.o.) isolated from aqueous extract of *C. indica* fruit along with the diet had a lower fasting blood glucose compared to the control (Presanna Kumar *et al.* 1993). The study on mode of action revealed that pectin increased the incorporation rate of glucose into glycogen, increased the activity of hepatic hexokinase and glycogen synthase, decreased the activity of glycogen phosphorylase and increased hepatic glycogen content in normal mice (Presanna Kumar *et al.*

1993), suggesting that the effect of pectin was owing to its ability to promote glycogen synthesis as well as inhibit glycogen breakdown in liver. It is not clear if this effect was a direct one on the liver or mediated by other effect e.g. stimulation of insulin secretion.

Another compound isolated from *C. indica* and claimed to have anti-diabetic activity was a water soluble and dialyzable alkaloidal principle (reviewed by Handa *et al.* (1989)). The results of the study and the identity of this compound were not given in this review.

***Trichosanthes kirilowii* Maxim.**

Trichosanthes kirilowii, commonly known as Chinese snakegourd, is also known as "Tian Hua Fen" in Chinese. Its root has been used in traditional Oriental medicine as a decoction for bronchitis, congestion, constipation, diabetes and fever (Duke and Ayensu, 1985a). It has been reported that the root of *T. kirilowii* together with *Coptis chinensis* are the most frequently used remedies for diabetes (Duke and Ayensu, 1985a) in traditional Chinese medicine.

A glycan fraction (200 mg/kg, i.p.) isolated from aqueous extract of *T. kirilowii* roots was reported to lower blood glucose level in normal mice (Hikino *et al.* 1989c). A number of glycans were isolated from this fraction, including trichosan A, B, C, D and E. Intraperitoneal administration of trichosan A, B, C and E (10-100 mg/kg) but not trichosan D was reported to have dose-dependent hypoglycaemic effects in normal mice 7 hours after administration (Hikino *et al.* 1989c). Further investigation of the activity trichosan A demonstrated that trichosan A (10-100 mg/kg, i.p.) also caused a significant reduction of plasma glucose level in alloxan-induced diabetic mice. (Hikino *et al.* 1989c). However, it is still unclear if the similar effect would be observed on oral administration, since the compounds were investigated only by intraperitoneal administration.

2.2.10 Cyperaceae

***Cyperus rotundus* Linn.**

Cyperus rotundus is also known as nut grass in English, "Korehijar" in Hindi and "Hiang Fou" in Chinese. Its root has been used in Ayurveda as a remedy

to treat diarrhoea, diabetes, dysentery, gastric and intestinal disorders (Chopra *et al.* 1956). In folklore Chinese medicine, it has been used as a remedy for chest pains, digestive disorders, diarrhoea, irregular menstruation, tuberculosis and tumour (Duke and Ayensu, 1985a; Chang and But, 1986a). To date, no report was found of any scientific studies linking this plant to diabetes.

2.2.11 Gentianaceae

***Swertia chirayita* Karst (*S. chirata*)**

The hexane soluble fraction of an ethanolic extract of *S. chirayita* (250 mg/kg, p.o.) was reported to significantly reduce blood glucose level in fed and glucose-loaded rats, but had no effect on fasted rats (Chandra Sekar *et al.* 1987), suggesting that the extract may work by inhibiting intestinal glucose absorption. The xanthone, swerchirin (50 mg/kg, p.o.), isolated from the hexane fraction was reported to lower plasma glucose level in fed and glucose-loaded rats (Bajpai *et al.* 1991). In contrast to the crude extract, swerchirin (50 mg/kg, p.o.) was also reported to have hypoglycaemic effect in fasted rats (Bajpai *et al.* 1991).

In diabetic models, it was reported that swerchirin (50 mg/kg, p.o.) caused a significant reduction of plasma glucose in STZ-induced moderately diabetic rats (fasting plasma glucose < 200 mg/dl), but failed to exert the effect in severely STZ-induced diabetic rats (plasma glucose > 350 mg/dl) of which their β-cells no longer functioned normally (Saxena *et al.* 1991). In agreement with this study, it was demonstrated that the effect of swerchirin was owing to its ability to stimulate insulin release from islets of Langerhans (Saxena *et al.* 1993).

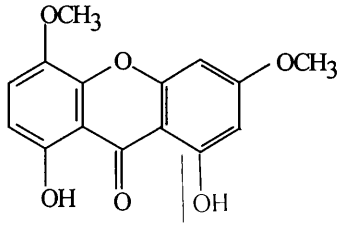


Fig. 2.5: Structure of Swerchirin

***Swertia japonica* Makino**

Swertia japonica has been widely used in traditional Oriental medicines for the treatment of gastrointestinal disorders. Ethanolic extract (400 mg/kg/day, i.p., 2 days) of *S. japonica* (whole plant) was reported to lower blood glucose level in STZ-induced diabetic rats, the extract showed more pronounced hypoglycaemic effect than tolbutamide (200 mg/kg) and an ethanolic extract of *S. chirayita* at the same dose (Basnet *et al.* 1994a). A number of xanthones isolated from the ethyl acetate soluble fraction of the ethanolic extract of this plant have been tested for anti-diabetic effect, including bellidifolin, methylbellidifolin, swertianin and methyl-swertianin (Fig. 2.6).

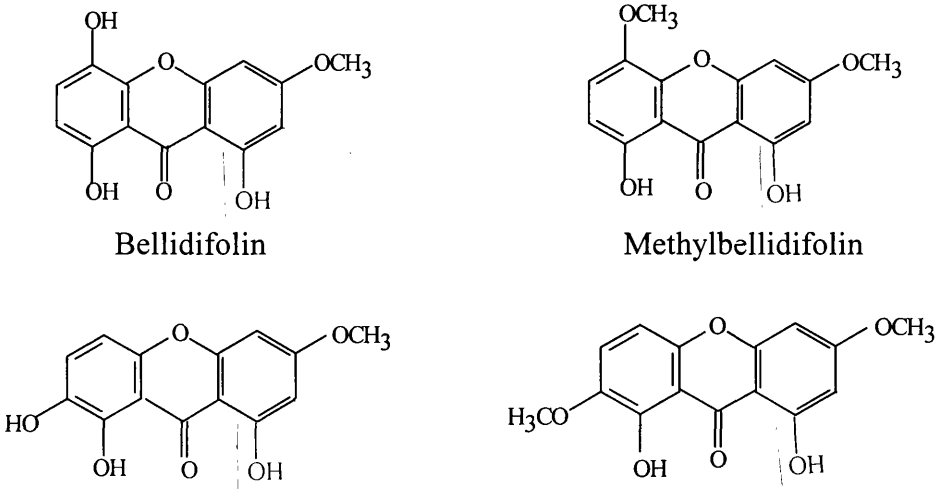


Fig. 2.6: Structure of xanthones isolated from *Swertia japonica*

Bellidifolin was reported to have glucose-lowering effect in STZ-induced diabetic rats when given intraperitoneally and orally at the doses of 50 mg/kg (twice daily for three days) and 50-100 mg/kg (single dose), respectively (Basnet *et al.* 1994b). Similarly to bellidifolin, oral administration of methylbellidifolin (50 mg/kg) was reported to significantly reduce the level of plasma glucose in STZ-induced diabetic rats (Basnet *et al.* 1994b). However, the activity of methylbellidifolin was reported to be less potent than that of bellidifolin at the same dose (Basnet *et al.* 1994b). In contrast to bellidifolin and methylbellidifolin, swertianin and methylswertianin (50 mg/kg, p.o.) had no glucose lowering effect in

STZ-induced diabetic rats (Basnet *et al.* 1994b), suggesting that the substitution pattern on the ring was an important feature in activity.

2.2.12 Labiatae (Lamiaceae)

Salvia miltiorrhiza

Salvia miltiorrhiza is commonly known as red-root sage or "Dan Shen" in Chinese. The root has been used in traditional Chinese medicine to treat angina pectoris, abdominal pain, arthritis, diabetes, hepatitis, hypertension, irregular menstruation, palpitation and insomnia (Duke and Ayensu, 1985b; Chang and But, 1986b). However, no scientific information to support its use as an anti-diabetic remedy was found.

2.2.13 Leguminosae

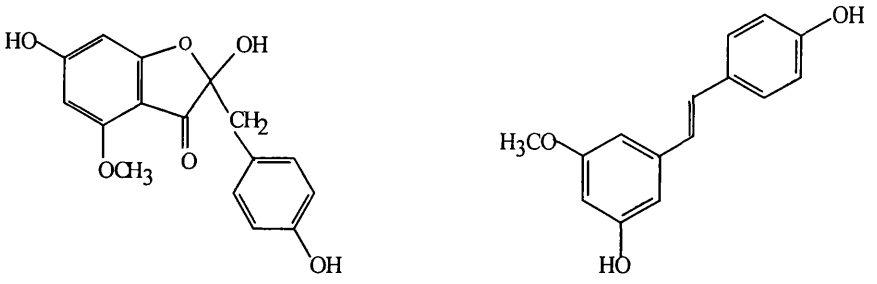
***Pterocarpus marsupium* Roxb.**

Pterocarpus marsupium is also known as "Bijasal" in Hindi. It has been used in Ayurvedic medicine as the remedy for bruises, diabetes, diarrhoea and skin disease (Kapoor, 1990). In Ayurvedic medicines, a decoction of *P. marsupium* heartwood was given with ground pepper for 30 days for the treatment of diabetes (Alam *et al.* 1990). Reportedly, oral administration of ethyl acetate soluble part of ethanolic (250 mg/kg, 5 days) and aqueous extracts (10g/kg, 10 days) of *P. marsupium* heartwood significantly reduced plasma glucose level in alloxan-induced diabetic rats (Handa *et al.* 1989; Ahmad *et al.* 1991a). The effect of the ethanolic extract was reported to be corresponded with an increase in insulin level and an increase in an incorporation of leucine into proinsulin (Ahmad *et al.* 1991a). Additionally, the ethanolic extract was also found to inhibit an elevation of blood glucose level in orally glucose loaded alloxan-induced diabetic rats (Ahmad *et al.* 1991a). According to these results, glucose lowering effect of the ethanolic extract appeared to be owing, at least in part, to two different mechanisms i.e. inhibition of intestinal glucose absorption and stimulation of insulin secretion from pancreas. It was demonstrated in a human study that oral administration of 2-4 g extract/day for 12 weeks resulted in a significant reduction in fasting blood glucose, postprandial glucose

and HbA_{1C} (glycosylated hemoglobin) levels in newly-diagnosed NIDDM patients (Seshiah *et al.* 1998).

Compounds with reputed anti-diabetic activity that have been isolated from *P. marsupium* extracts include phenolic compounds from heartwood i.e. marsupin, pterostilbene and flavonoid from bark i.e. (-)-epicatechin (Fig. 2.7 & 2.8).

Manickam *et al.* (1997) reported that marsupin and pterostilbene (Fig. 2.7) isolated from *P. marsupium* heartwood significantly reduced blood glucose level in STZ-induced diabetic rats when given at a dose of 20 mg/kg (i.p.) for 3 days, but failed to show an effect in normal rats (Manickam *et al.* 1997). The effect of these compounds given orally has not been assessed.



(a) (b)

Fig. 2.7: Structure of (a) Marsupin and (b) Pterostilbene

A study of the anti-diabetic effect of (-) epicatechin by Chakrabortty *et al.* (1981) has brought enormous attention to this compound. They claimed that (-)-epicatechin can reverse pancreatic damage of alloxan and hence prevent normal rats from alloxan-induced hyperglycaemia. Strong support to their claim arose from the finding that (-) epicatechin when given to diabetic rats at a dose of 60 mg/kg/day, i.p. 24 hours after alloxan injection could significantly reduce blood glucose level within 24 hours after the administration of epicatechin (Chakrabortty *et al.* 1981). Moreover, it was found that blood glucose level returned to normal on the 4^{th} and 5^{th} day after the treatment with epicatechin (Chakrabortty *et al.* 1981).

The effect of (-)-epicatechin was claimed to be due to enhanced regeneration of β-cells. A hypoglycaemic effect of (-)-epicatechin was also observed in diabetic rabbits, although a higher dose and a longer time were required (100 mg/kg, i.p., 15-20 days) (Chakrabortty *et al.* 1982). The effect of (-)-epicatechin was

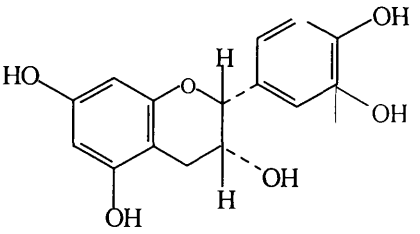


Fig. 2.8: Structure of (-) Epicatechin

not observed when the compound (90 mg/kg/day, i.p., 2 days) was given to alloxan rats 3 days after alloxan injection (Sheehans and Zemaitis, 1983). The conclusion drawn from these studies was that epicatechin was effective when given prior to or beginning within 24 hours after alloxan administration (Sheehans and Zemaitis, 1983). In agreement with these studies, it was demonstrated that epicatechin (1 mM) was capable of stimulating insulin secretion from rat islets in the presence of both 2 mM and 20 mM glucose (Hii and Howell, 1984). The stimulatory effect of (-)-epicatechin on insulin secretion from the islets was reported to be ATP-dependent, since the stimulation was abolished in the presence of 2,4-dinitrophenol (Hii and Howell, 1984). It has been postulated that (-)-epicatechin may exert its effect on insulin secretion via increasing Ca^{2+} uptake and inhibiting its efflux into the islets (Hii and Howell, 1985), increasing cAMP level (Ahmad *et al.* 1991b), and/or promoting conversion of proinsulin to insulin (Ahmad *et al* 1991b).

However, the effect of (-)-epicatechin on reversing the diabetic state was questioned by Kolb *et al.* (1982), since their repeat of Chakrabortty's experiment was not successful. Although a decrease in blood glucose level was observed in Kolb's study, the decrease was not significantly different from that observed in control (Kolb *et al.* 1982). It was found that there was no difference in the level of blood glucose between control and (-)-epicatechin-treated rats even when (-)-epicatechin (60 mg/kg/day, i.p., 6 days) was given 24 hours before alloxan injection (Kolb *et al.* 1982). The factors contributing to the conflict between the results of Chakrabortty' s study and those of Kolb *et al.* (1982) are not known. Thus, further study on the effect of epicatechin in alloxan diabetic rats is necessary. Further, since all the studies on epicatechin involved parenteral administration, the role of the compound in the oral effects seen with *P. marsupium* extracts is not known.

2.2.14 Liliaceae

***Anemarrhena asphodeloides* Bunge.**

Anemarrhena asphodeloides is known as "Zhi Mu" in Chinese. Its traditional use and the reported studies on anti-diabetic effects are discussed fully in Chapter 6.

***Ophiopogon japonicus* Thunb.**

Ophiopogon japonicus (lilyturf) is known as "Mai Men Dong" in Chinese. *O. japonicus* rhizome has been used in traditional medicine for bronchitis, cough, cold, diabetes, stress and tuberculosis (Duke and Ayensu, 1985b; Kako *et al.* 1995). A butanol extract of *O. japonicus* rhizome (100 mg/kg, i.p.) was reported to reduce plasma glucose level in normal and STZ-induced diabetic mice 4 hour after administration (Kako *et al.* 1995). The effect of *O. japonicus* observed in normal mice did not appear to be related to increased insulin secretion, since the level of insulin remained unchanged (Kako *et al.* 1995). The extract (100 mg/kg, i.p.) was also reported to have an anti-hyperglycaemic effect in epinephrine-induced hyperglycaemic mice (Kako *et al.* 1995). The effect in epinephrine-treated mice was accompanied by an increase in hepatic glycogen content (Kako *et al.* 1995), suggesting that the butanol extract of *O. japonicus* may work, at least in part, via promoting glycogen synthesis in liver. However, the mechanism of action by which *O. japonicus* extract lowered the glucose level in normal and other animal models of diabetes (STZ-diabetic and glucose-loaded mice) has not been assessed. Since, the extract was administered by intraperitoneal injection in these studies, it is not know if similar effect would be observed on oral administration.

***Polygonatum odoratum* Druce.**

Polygonatum odoratum is commonly known as aromatic Solomon's seal or "Yu Zhu" in Chinese. The rhizome has been prescribed in traditional Chinese medicine for anaemia, arthritis, cough, diabetes, fever, influenza, palpitation and rheumatism and also used as sedative and diuretic (Duke and Ayensu, 1985b; Chang and But, 1986b). A number of pharmacological activities of *P. odoratum* rhizome have been reported including the effect on blood glucose level. It was reported that

intraperitoneal administration (dose was not specified) of the rhizome extract could inhibit an increase in blood glucose level in rats loaded with glucose and rats treated with alloxan (Chang and But, 1986b). Further study is still needed to assess the activity of this plant and its mode of action.

2.2.15 Meliaceae

***Azadirachta indica* A. Juss.** (Syn. *Melia azadirachta* Linn.)

Azadirachta indica, also known as 'Neem', is an indigenous plant widely available in India. *A. indica* has been used in Ayurvedic medicine as a remedy for diabetes, headaches, skin disease and rheumatism (Chopra *et al.* 1956). In Ayurvedic medicines for diabetes, powdered young leaves of neem were mixed with *Piper nigrum* Linn. and *Zingiber officinalis* Linn. in equal ratio to prepare small tablets which was given to patients twice daily (Alam *et al.* 1990).

The anti-diabetic effect of various parts of *A. indica* has been demonstrated in a number of scientific studies. Oil (200 mg/rat, p.o.) extracted from *A. indica* seeds was found to lower the level of blood glucose in normal, as well as mildly and severely diabetic rats (Dixit *et al.* 1986). To date, no further report was found of any study investigating the mechanism of action and active constituents of *A. indica* seed oil. A greater attention has been given to *A. indica* leaves of which the anti-diabetic effect was evident in a number of studies. Anti-hyperglycaemic effect of the water soluble portion of an alcoholic extract of *A. indica* leaves was demonstrated in glucose-loaded and adrenaline-infused rats (Chattopadhyay and Maitra, 1993). Also, the aqueous/alcoholic leaf extract at a dose of 500 mg/kg (i.p) was reported to lower blood glucose level in STZ-induced diabetic rats (Chakrabortty *et al.* 1989) and the extract at a lower dose of 50-400 mg/kg (p.o.) was reported to be active in normal and severely diabetic rats (Chattopadhyay, 1999a). Long term treatment of the aqueous extract of *A. indica* leaves (300 mg/kg/day. p.o., 7 days) was reported to have glucose-lowering effect in normal rats (El-Hawary and Kholief, 1990).

Bajaj and Srinivasan (1999) reported that the aqueous extract from the leaves (1 g/kg/day, p.o., 6 weeks) showed hypoglycaemic activity in mildly and severely STZ-diabetic animals. In contrast to these studies, El-Hawary and Kholief

(1990) demonstrated that the extract (300 mg/kg/day, p.o., 7 days) of *A. indica* leaves failed to lower blood glucose level in alloxan-induced diabetic rats. The discrepancy between the results may be due to the differences in doses of the extracts, the duration of treatment and animal models used in the two studies, the higher dose (1 g/kg/day) and longer duration of treatment were proven to be more effective.

A study on the mechanism of anti-diabetic actions of *A. indica* extract revealed that the aqueous/alcoholic extract (500 mg/kg, p.o.) did not promote the synthesis of hepatic glycogen in normal and glucose-loaded rats (Chattopadhyay *et al.* 1993). *In vitro* study using rat hemidiaphragm showed that the aqueous/alcoholic extract (25 mg/ml) failed to stimulate the production of muscle glycogen (Chattopadhyay *et al.* 1993). These results suggested that the promotion of glycogen synthesis in muscle and liver did not contribute to the activity of the aqueous/alcoholic extract of *A. indica*. It has been shown in the study by Chattopadhyay, (1996) that the ethanolic extract of *A. indica* leaves (200 mg/kg/day, p.o., 7 days) significantly inhibited the effect of epinephrine on glycogen depletion in normal and diabetic rabbits, suggesting that the extract may work by inhibiting glycogenolytic pathway. In addition, it has been suggested that the effect of the aqueous/alcoholic extract was also owing to its ability to potentiate glucose-induced insulin secretion, since the extract (25 mg/ml) was found to significantly stimulate glucose-induced insulin secretion from pancreatic islets and also prevent the inhibitory effect of serotonin on glucose-mediated insulin release (Chattopadhyay, 1999b).

Studies on the active constituents of *A. indica* are not yet extensive. Nimbidin, a bitter principle isolated from *A. indica* when given at a dose of 200 mg/kg was reported to lower the level of plasma glucose 5 hours after administration (Handa *et al.* 1989). The mode of action of this compound is still unknown.

2.2.16 Menispermaceae

***Tinospora cordifolia* Miers.**

Tinospora cordifolia is known as "Gilo" in Hindi. It has been used in Ayurvedic medicine as a remedy for bronchitis, diabetes, diarrhoea, dysentery, fever, gonorrhoea, rheumatism, skin disease and urinary disease (Kapoor, 1990). Aqueous, methanol and chloroform extracts of the plant when given orally at doses of 50-200

mg/kg were reported to lower the level of plasma glucose in normal and alloxan-induced diabetic rabbits (Wadood *et al.* 1992).

1,2-Substituted pyrrolidines (unspecified dose and route of administration) isolated from *T. cordifolia* were found to have hypoglycaemic activity in rabbits (Perez *et al.* 1998).

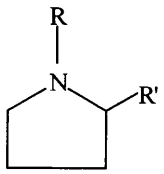


Fig. 2.9: Structure of 1,2-Substituted pyrrolidines

2.2.17 Moraceae

***Ficus bengalensis* Linn.** (Syn. *F. indica* Linn)

Ficus bengalensis is also known as banyan tree in India. Its use as traditional anti-diabetic remedy is well known in the Himalayan region. The plant has been used in the Ayurvedic system for bruises, diabetes, diarrhoea, dysentery, gonorrhoea and rheumatism (Kapoor, 1990). Aqueous extract from *F. bengalensis* bark (40 mg/rat, p.o., 5 days) was reported to have significant hypoglycaemic effect in normal rats without affecting hepatic glycogen content (Achrekar *et al.* 1991). It was reported that the decreased blood glucose level was accompanied by an increase in plasma insulin level (Achrekar *et al.* 1991), suggesting that the effect of *F. bengalensis* was, at least in part, attributed to a stimulation of insulin release from pancreatic β-cells. In agreement with *in vivo* studies, it was reported that the aqueous *F. bengalensis* extract (0.5 mg/ml) significantly increased insulin secretion from islets isolated from both normal and diabetic rats (Achrekar *et al.* 1991).

A number of compounds with anti-diabetic activity have been isolated from this plant including bengalenoside, phytosterolin, dimethoxy derivative of leucocyanidin 3-O-β-D-galactosyl cellobioside, 5,7 dimethyl ether of leucopelargonidin 3-O-α-L-rhamnoside and leucodelphinidin derivative.

A number of studies have been carried out to investigate the anti-diabetic effect of leucopelargonidin 3-O-α-L-rhamnoside and leucocyanidin-3-O-β-D-galactosyl cellobioside (Cherian and Augusti, 1995). Both short term (250 mg/kg,

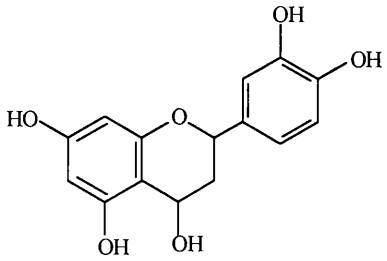


Fig. 2.10 : Structure of Leucocyanidin

p.o.) and long term (100 mg/kg/day, 1 month, p.o.) treatments with leucopelargonidin were found to significantly reduce the level of blood glucose in moderately alloxan-diabetic rats (Cherian and Augusti, 1995). Short term treatment with leucopelargonin at a lower dose of 100 mg/kg (p.o.) was also reported to be effective in normal and alloxan-diabetic dogs (Augusti *et al.* 1994). This effect has been suggested to be primarily due to its stimulatory effect on insulin secretion from residual β-cells based on the observation that the treatment with leucopelargonidin (100 and 250 mg/kg, p.o.) increased the level of plasma insulin in moderately alloxan-diabetic animals (Cherian and Augusti, 1995). It should be noted that the effect of leucopelargonidin (100 mg/kg, p.o.) on insulin secretion was similar to those observed in crude extract (40 mg/rat). In addition, leucopelargonidin was also reported to have an effect on hepatic glucose utilisation; it was found to increase activity of hexokinase and decrease activity of glucose-6-phosphatase (Cherian and Augusti, 1995). Regarding a toxicity, it was reported that administration of leucopelargonidin did not show any toxic effect even at a high dose of 1.8 g/kg in experimental animals (Augusti *et al.* 1994).

Treatment with leucocyanidin (250 mg/kg, p.o.) was reported to reduce the level of blood glucose in both normal and moderately alloxan-diabetic rats (Kumar and Augusti, 1989). No significant change in the level of plasma insulin was observed in normal rats treated with leucocyanidin (250 mg/kg, p.o.), whereas in diabetic rats a significant increase in the level of insulin was observed (Kumar and Augusti, 1989). This suggested that the stimulatory effect on insulin secretion of leucocyanidin was glucose dependent, and in cases where the level of blood glucose was normal, leucocyanidin worked via the mechanisms other than the stimulation of insulin release.

A leucodelphinidin derivative isolated from *F. bengalensis* bark at a dose of 250 mg/kg (unspecified route) was reported to lower blood glucose level in normal, glucose loaded and alloxan-diabetic rats (Geetha *et al.* 1994). The effect in normal and alloxan-diabetic rats was reported to be comparable to that of glibenclamide (2 mg/kg) (Geetha *et al.* 1994).

Phytosterolin (25 mg/kg) was reported to have glucose-lowering effect in both oral and intravenous administration (Handa *et al.* 1989). However, the effect appeared to be more potent when the compound was given intravenously since the lower dose of 5-7.5 mg/kg was required compared to the oral administration (25 mg/kg) (Handa *et al.* 1989).

***Morus alba* Linn.**

Morus alba (white mulberry) is known as Sang Bai Pi in Chinese and Sohaku-hi in Japanese. *M. alba* root bark has been used in traditional Oriental medicine for anti-inflammatory, anti-diabetic, antipyretic, antitussive, cough and diuretic purposes (Hikino *et al.* 1985b; Chen *et al.* 1995).

An aqueous methanolic extract of *M. alba* root barks was reported to reduce the level of plasma glucose when given (i.p.) to normal mice at a dose of 20 g/kg (Hikino *et al.* 1985b). Moran A (10-30 mg/kg, i.p.), a glycan isolated from *M. alba*, lowered the plasma glucose level in normal mice; the compound has been found to be more effective in alloxan-induced diabetic mice since the compound at the same dose (10-30 mg/kg, i.p.) was found to cause a greater percentage of plasma glucose reduction (Hikino *et al.* 1985b). In addition, while moran A at a dose of 3 mg/kg (i.p.) had no effect on plasma glucose level in normal mice, it was found to have a significant glucose lowering effect in alloxan-diabetic mice (Hikino *et al.* 1985b). This suggested that the effect of moran A was potentiated by the presence of moderately high blood glucose level. The precise mechanism of action of moran A is still unknown. However, in these studies moran A was administered by intraperitoneal injection, it is therefore unknown if similar effect would be observed in oral administration. Another compound with anti-diabetic activity which was isolated from *M. alba* was an alkaloid moranoline (Fig. 2.11). Moranoline was demonstrated to have potent inhibitory effect on intestinal α-glucosidase but showed

a weak inhibition on β-glucosidase, glucoamylase and α-amylase (Marles and Farnsworth, 1995).

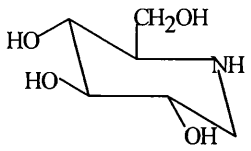


Fig. 2.11: Structure of Moranoline

2.2.18 Polyporaceae

***Ganoderma lucidum* Karst.**

Ganoderma lucidum is commonly known as "Ling zhi" in Chinese and "Reishi" in Japanese. *G. lucidum* has been widely used in traditional Oriental medicine as a remedy for the treatment of arthritis, arteriosclerosis, bronchitis, diabetes, hypertension and hyperlipemia (Kimura *et al.* 1988). The study on anti-hyperglycaemic effect of aqueous and ethanolic extracts of *G. lucidum* revealed that the aqueous extract (50 mg/rat, p.o.) but not the ethanolic extract (30 mg/rat, p.o.) inhibited an elevation of plasma glucose in glucose-challenged rats (Kimura *et al.* 1988). Treatment with the aqueous extract (50 mg/rat, p.o.) was also found to increase the level of plasma insulin in glucose-challenged rats 20 minutes after glucose infusion (Kimura *et al.* 1988). However, these effects on glucose and insulin level of the aqueous extract did not appear to be related, since the decrease in glucose level was observed while plasma insulin was at a low level (i.e. prior to the elevation of insulin). The aqueous extract (50 mg/rat, p.o.) was also found to inhibit adrenaline-induced lipolysis in rat adipocytes and prevent the elevation of blood glucose in adrenaline-infused rats without affecting the level of insulin (Kimura *et al.* 1988). These results suggested a direct insulin-like effect of components of the extract. The study in normal mice showed that aqueous extract of *G. lucidum* (10 g/kg, i.p.) caused a significant reduction in blood glucose level in normal mice (Hikino *et al.* 1985a).

A number of glycans have been isolated from *G. lucidum* and tested for anti-diabetic activity, including ganoderan A, B and C. The hypoglycaemic effect of ganoderan A (10, 30 and 100 mg/kg, i.p.) and ganoderan B (30 and 100 mg/kg, i.p.)

has been reported in both normal and alloxan-diabetic mice (Hikino *et al.* 1985a). In addition, ganoderan B at 30 mg/kg (i.p.) was also capable of inhibiting an elevation of plasma glucose in glucose-loaded mice (Hikino *et al.* 1989a). The effect of ganoderan B (100 mg/kg, i.p.) in normal mice has been thought, at least in part, to be owing to the promotion of hepatic glucose utilisation as suggested by the increase in the activities of hepatic glucokinase and phosphofructokinase as well as the decrease in the activities of glucose-6-phosphatase (Hikino *et al.* 1989a). However, it has been suggested that the effect on glucose utilisation of ganoderan B was not related to an incorporation of glucose into glycogen molecules, since the treatment with ganoderan B (100 mg/kg, i.p.) resulted in a depletion of hepatic glycogen content (Hikino *et al.* 1989a). In contrast to the crude extract, ganoderan B (100 mg/kg, i.p.) was reported to increase plasma insulin level in normal mice (Hikino *et al.* 1989a). An *in vitro* study reported by Perl and Hikino, (1989) showed that ganoderan A and B (250-300 μg/ml) were found to be effective in promoting glucose oxidation in inverted intestinal fragments. According to these results, it appeared that panaxan B may work by stimulating insulin secretion, increasing hepatic glucose utilization and increasing glucose oxidation in intestine. However, in these studies the glycans were administered by intraperitoneal injection, thus the contribution of these glycans to the observed oral effect of *G. lucidum* is still not known.

A guanidine derivative isolated from *G. lucidum* was reported to lower the plasma glucose level in normal and alloxan-induced diabetic mice (dose and route were not specified in this review (Perez *et al.* 1998)).

2.2.19 Piperaceae

Fruit of *Piper cubeba* Linn. has been quoted in traditional Chinese medicine for various disorders including amnesia, bronchitis, epilespsy, headache, diabetes and gonorrhoea (Chang and But, 1986a). However, this plant was not available for the study. Thus, the study was conducted in a plant from the same genus i.e. *Piper longum* whose hypoglycaemic effect was evident in a few studies but its mode of action and active principle have not been identified.

***Piper longum* Linn.**

Piper longum (long pepper) is known as "Pipal" in Hindi and "Bi Bo" in Chinese. It has been used in Ayurvedic medicine for antisepsis, asthma, cough, chronic bronchitis, indigestion, rheumatism and snakebite (Kapoor, 1990). The fruit of *P. longum* has been used in traditional Chinese medicine for cholera, dysentery and headache, and also used as carminative, stomachic and analgesic (Duke and Ayensu, 1985b). The study on anti-diabetic effect of this plant showed that oral administration (150 mg) of *P. longum* fruit extract (50% EtOH) caused significant reduction of blood glucose level in normal, and mild to severe hyperglycaemic rats 3 hours after the administration (Purohit and Daradka, 1999). No report was found of any study investigating mechanism of action and active principles, to date.

2.2.20 Ranunculaceae

***Coptis chinensis* Franch.**

Coptis chinensis is commonly known as Chinese goldthread and "Huang Lian" in Chinese. Root of *Coptis chinensis* has been used in folklore Chinese medicine with *Gardinia* and *Scutellaria* for fever. Its decoction has been used for anxiety, cancer, cramps and diarrhoea, oedema, insomnia, nausea and stomach ache (Duke and Ayensu, 1985b). The whole plant has been prescribed for diabetes and hemorrhoids (Duke and Ayensu, 1985b). However, the part of *C. chinensis* used in the current study was rhizome because the whole plant which has been quoted in anti-diabetic remedy was not commercially available.

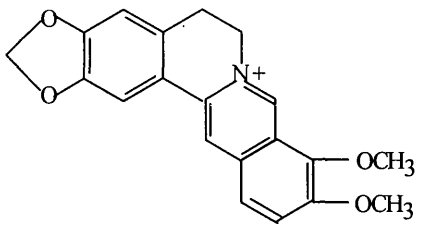


Fig. 2.12: Structure of Berberine

Berberine (Fig. 2.12) was claimed to be active constituent of *C. chinensis*. It was reported that berberine had a hypoglycaemic effect in normal, alloxan-induced diabetic and diabetic KK mice. The compound administered intra-

peritoneally also inhibited an elevation of plasma glucose induced by adrenaline in normal mice (Perez *et al.* 1998). Additionally, long term treatment (15 days) with berberine improved the glucose tolerance in KK mice (dose and route were not specified) (Ling-Hua and Pei-Gen, 1993).

2.2.21 Rutaceae

***Murraya koenigii* Spring.**

Murraya koenigii is also known as curry leaf tree. It has been used in Ayurvedic medicine as a remedy for bruises, diarrhoea, diabetes and eruptions (Chopra *et al.* 1956). Oral administration of the aqueous extract of *M. koenigii* leaves (dose was not specified) reduced blood glucose levels in normal and alloxan-induced diabetic dogs. The effect was prolonged over 8 hours in alloxan-treated dogs. In contrast, a 50% ethanolic extract of the roots (route was not specified) failed to exhibit the hypoglycaemic effect (Handa *et al.* 1989). No report was found of any study investigating mechanism of action and active principles.

2.2.22 Scrophulariaceae

***Rehmannia glutinosa* Gaertn.**

Rehmannia glutinosa is commonly known as Chinese fox glove or "Gan Di Huang" in Chinese. *R. glutinosa* leaves have been used for bruises, eczema and psoriasis. The decoction from rhizome has been used in traditional Chinese medicine for anaemia, bleeding, cancer, constipation, cough, diabetes, dizziness, fever, hepatitis, oral ulcer, restlessness and wheezing (Duke and Ayensu, 1985b).

Two compounds with anti-diabetic activity were isolated from this plant i.e. catalpol and rehmannioside D (Ling-Hua and Pei-Gen, 1993)

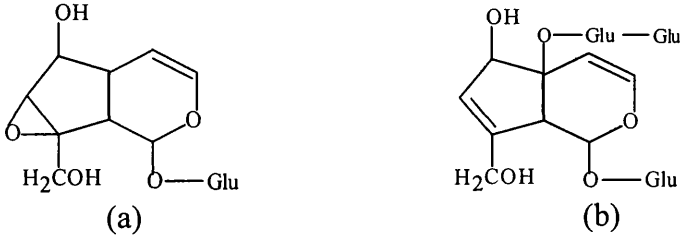


Fig. 2.13: Structure of (a) Catalpol and (b) Rehmannioside D

***Scrophularia ningpoensis* Hemsl.**

Scrophularia ningpoensis (black figwort) is known as "Xuanshen" in Chinese. Rhizome of this plant has been used in folklore medicine as a remedy for anxiety, cancer, constipation, fever, diabetes, rash and sore throat (Duke and Ayensu, 1985b; Chang and But, 1986b).

To date, the study on anti-diabetic effect of this plant is still not extensive. Mechanism of action and active constituents of the plant have not yet been assessed. Among a few studies, it was demonstrated that the extract at 5 g/kg (s.c.) caused a slight decrease of blood glucose level (Chang and But, 1986b).

2.2.23 Solanaceae

***Lycium chinensis* Mill.**

The bark of *L. chinensis* is known as Di Gu Pi in Chinese. The ethnopharmacology of *L. chinensis* is described in detail in Chapter 4.

2.2.24 Zingiberaceae

***Curcuma longa* Linn.** (Syn. *C. domestica* Valeton.)

Curcuma longa is commonly known as turmeric, "Haldi" in Hindi and "Jiang Huang" in Chinese. It has been used in traditional remedies for cough, diabetes, fever, jaundice, liver disorders and urinary disease (Kapoor, 1990). In Ayurvedic medicines for the treatment of diabetes, powdered rhizome of turmeric was mixed with fruit juice of *Phyllanthus emblica* Linn. and prescribed at a dose of 5 g twice a day for 1 week (Alam *et al.* 1990).

The glucose-lowering effect of *C. longa* ethanolic extract was reported when given at a dose of 5-10 mg/rat to normal, mildly and severely diabetic rats (Day, 1990).

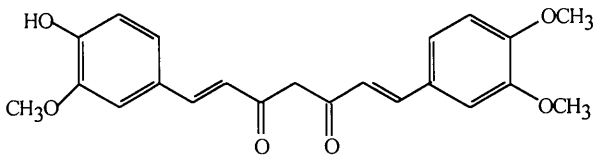


Fig. 2.14: Structure of Curcumin

Oral administration of curcumin (Fig. 2.14), a phenylethylene isolated from *C. longa*, was reported to decrease total blood cholesterol, triglycerides and phospholipids in STZ-induced diabetic rats (Babu and Srinivasan, 1997).

2.3 Sources of plant materials

Plant materials used in the project were a gift from Cipla Ltd. (Mumbai Central, Mumbai, India), or purchased from East & West Herbs Ltd. (Langstan Priory Mews, Kingham, Oxfordshire UK), Indian Herbs (Europe) Ltd. (Kingdown House, Priddy, Wells, Somerset, UK), Institute of Chinese medicine (London, UK), The Food centre (Turnpike Lane, London) and Choa Krom Per (Chakrawat road, Bangkok, Thailand). Batches were checked for homogeneity, and a representative voucher specimen prepared for storage. These are stored in the plant collections at the Pharmacognosy Research Laboratory (King's College, London).

Table 2.2: Sources of plants selected for the study

Nominal identity	Part Used	Sources	Voucher Number
Alismataceae			
Alisma orientalis Juzep.	Rhizome	East & West Herbs	SSAO
Araceae			
Typhonium giganteum Engl.	Rhizome	East & West Herbs	SSTG
Araliaceae			
Panax ginseng C.A. Meyer.	Root	East & West Herbs	SSPGi
Bixaceae			
Bixa orellana Linn.	Seed	Cipla Ltd.	SSBO
Cactaceae			
Opuntia spp.	Stem	Cipla Ltd.	SSOp
Campanulaceae			
Platycodon grandiflorum A. DC.	Root	East & West Herbs	SSPGr
Celastraceae			
Salacia reticulata Wight.	Stem	Cipla Ltd.	SSSR
Compositae			
Atractylodes lancea Thunb.	Rhizome	East & West Herbs	SSAL

Table 2.2 (continued)

Nominal identity	Part Used	Sources	Voucher Number
Atractylodes macrocephala Koidz.	Rhizome	East & West Herbs	SSAM
Cucurbitaceae			
Benincasa hispida Cogn.	Seed	East & West Herbs	SSBH
Coccinia indica Wight & Arn.	Stem	Thailand	SSCI
Trichosanthes kirilowii	Root	East & West Herbs	SSTK
Cyperaceae			
Cyperus rotundus Linn.	Root	East & West Herbs	SSCR
Gentianaceae			
Swertia spp.	Whole Plant	Cipla Ltd.	SSSw
Labiatae			
Salvia miltiorrhiza Bunge.	Root	East & West Herbs	SSSM
Leguminosae			
Pterocarpus marsupium Linn.	Wood	Cipla Ltd.	SSPM
Liliaceae			
Anemarrhena asphodeloides Bunge.	Rhizome	East & West Herbs	SSAA1
		Institute of Chinese Medicine	SSAA2
Ophiopogon japonicus Thunb.	Root	East & West Herbs	SSOJ
Polygonatum odoratum Druce.	Rhizome	East & West Herbs	SSPO
Meliaceae			
Azadirachta indica A. Juss.	Leaves	Cipla Ltd.	SSAI
Menispermaceae			
Tinospora cordifolia Miers.	Stem	Indian Herbs	SSTC
Moraceae			
Ficus bengalensis Linn.	Bark	Cipla Ltd.	SSFB
Morus alba Linn.	Bark	East & West Herbs	SSMA
Piperaceae			
Piper longum Linn.	Fruit	East & West Herbs	SSPL
Polyporaceae			
Ganoderma lucidum Karsten.	Fruit Body	East & West Herbs	SSGL

Table 2.2 (continued)

Nominal identity	Part Used	Sources	Voucher Number
Ranunculaceae			
Coptis chinensis Franch.	Rhizome	East & West Herbs	SSCC
Rutaceae			
Murraya koenigii Spreng.	Leaves	The Food Centre	SSMK
Scrophularicaea			
Rehmannia glutinosa Libosch.	Root	East & West Herbs	SSRG
Scrophularia ningpoensis Hemsl.	Root	East & West Herbs	SSSN
Solanaceae			
Lycium chinensis Mill.	Bark	East & West Herbs	SSLC1
		Institute of	SSLC2
		Chinese Medicine	
Zingiberaceae			
Curcuma longa Linn.	Rhizome	East & West Herbs	SSCL

2.4 Authentication of plant materials

Plants purchased from East & West Herbs were authenticated at the company by Mr. Shouming Zhong (Pharmacognosist). Plants purchased from Indian herbs was authenticated at the company by Mr. G.P. Kimoti (Botanist). *Bixa orellana* and *Azadirachta indica* were inspected against authenticated material available at King's College London. *Coccinia indica* was inspected by Dr. N Kaewpradub (lecturer in Pharmacognosy, Prince of Songkla University, Thailand). Some of the plants purchased from Cipla Ltd. i.e. *Opuntia spp.*, *Salacia reticulata* and *Pterocarpus marsupium* were sent for authentication to the Royal Botanic Gardens, Kew (RBGK, Surrey, UK). Material supplied as *Pterocarpus marsupium* was reported to match reference material of *P. marsupium* wood and other species of *Pterocarpus*. However, it was not possible on the basis of samples supplied to distinguish between the different species of *Pterocarpus*. In the case of *Salacia reticulata*, it was reported that the sample was a good match with reference material of *Salacia* species, but it could possibly derive from the member of another genus in the same family (Celastraceae).

The authentication process revealed that the plant material which was supplied as *Opuntia spp.* stem did not have characteristic features that matched the nominal species. In fact the material consisted of root not stem and it was a very close match with root of *Parvatia* species (Family Lardizabalaceae). However, it was not possible to entirely rule out other members of the Lardizabalaceae, of which reference material was not available for the authentication, or members of the Menispermaceae to which Lardizabalaceae is closely allied and rather similar in anatomical structure. The identity of this plant is therefore still unknown. *Murraya koenigii*, a well known condiment (curry leaves) purchased from The Food centre (London) was authentication by Dr. A. Raman (lecturer in Pharmacognosy, King's College London).

Table 2.3: Results of the authentication of plant materials

Nominal identity	Inspected by	Results of inspection
Alisma orientalis Juzep.	SM Zhong	Identity confirmed
Anemarrhena asphodeloides Bunge.	SM Zhong	Identity confirmed
Atractylodes lancea Thunb.	SM Zhong	Identity confirmed
Atractylodes macrocephala Koidz.	SM Zhong	Identity confirmed
Azadirachta indica A. Juss.	S Srijayanta	Identity confirmed
Benincasa hispida Cogn.	SM Zhong	Identity confirmed
Bixa orellana Linn.	S Srijayanta	Identity confirmed
Coccinia indica Wight & Arn.	N Kaewpradub	Identity confirmed
Coptis chinensis Franch.	SM Zhong	Identity confirmed
Curcuma longa Linn.	SM Zhong	Identity confirmed
Cyperus rotundus Linn.	SM Zhong	Identity confirmed
Ficus bengalensis Linn.		Identity not confirmed
Ganoderma lucidum Karsten.	SM Zhong	Identity confirmed
Lycium chinensis Mill.	SM Zhong	Identity confirmed
Morus alba Linn.	SM Zhong	Identity confirmed
Murraya koenigii Spreng.	A Raman	Identity confirmed
Ophiopogon japonicus Thunb.	SM Zhong	Identity confirmed

Table 2.3 (continued)

Nominal identity	Inspected by	Results of inspection
Opuntia spp.	RBGK	Matched reference *Parvatia spp.* root and other members of the Lardizabalaceae
Panax ginseng C.A. Meyer.	SM Zhong	Identity confirmed
Piper longum Linn.	SM Zhong	Identity confirmed
Platycodon grandiflorum A. DC.	SM Zhong	Identity confirmed
Polygonatum odoratum Druce.	SM Zhong	Identity confirmed
Pterocarpus marsupium Linn.	RBGK	Matched reference *Pterocarpus* wood and other species of *Pterocarpus*
Rehmannia glutinosa Libosch.	SM Zhong	Identity confirmed
Salvia miltiorrhiza Bunge.	SM Zhong	Identity confirmed
Salacia reticulata Wight.	RBGK	Good matched with reference *Salacia* species
Scrophularia ningpoensis Hemsl.	SM Zhong	Identity confirmed
Swertia spp.		Identity not confirmed
Tinospora cordifolia Miers.	GP Kimoti	Identity confirmed
Trichosanthes kirilowii Maxim.	SM Zhong	Identity confirmed
Typhonium giganteum Engl.	SM Zhong	Identity confirmed

2.5 Preparation of plant extracts

2.5.1 Introduction

Plants were sequentially extracted using different solvents, starting from less polar solvent and changing to more polar solvents. This was to separate constituents in the plant material according to their polarities.

2.5.2 Materials and methods

Hexane and ethanol extracts of the plants were obtained using the same plant material, whereas aqueous extract was prepared from a different material.

Dried and powdered plant material (20 g) was extracted with hexane (100 ml) at room temperature overnight. The hexane extract was evaporated to dryness under negative pressure. The plant material after the extraction was dried and further extracted with absolute ethanol (100 ml) at room temperature overnight. The ethanolic extract was evaporated to dryness under negative pressure. Yields of hexane and ethanol extracts are given in Table 2.4.

Aqueous extract was obtained by boiling a different portion of the powdered plant material (20 g) with distilled water (100 ml) under reflux for 1 hour. The decoction was cooled and freeze-dried until dried residue was obtained. Percentage of the residue obtained from the extraction is showed in Table 2.4. The resultant residue was used for the screening on BBMV (Chapter 3) and RINm5F cells (Chapter 5). Although prepared, the hexane and ethanol extracts were not used due to time constraints. They are available for further experiments.

2.5.3 Results and discussion

Percentage yield of the extracts was found to be varied among the plants and also the extracts from the same plants extracted using different solvents. The percentage yield of water extracts was found to be high in a number of the plants such as *Atractylodes lancea*, *Atractylodes macrocephala*, *Ophiopogon japonicus*, *Panax ginseng*, *Platycodon grandiflorum*, *Polygonatum odoratum* and *Scrophularia ningpoensis*, this was probably due to the presence of polysaccharides and proteins. The content of hexane extracts obtained from some of the plants were found to be negligible, this included *Anemarrhena asphodeloides*, *Ophiopogon japonicus* and *Rehmannia glutinosa*, whereas some of the plants e.g. *Benincasa hispida* appeared to contain very fatty components as judged by the proportion of hexane extract.

Table 2.4: Yield of hexane, ethanol and aqueous extracts of plant materials

Tested Plants*	% Yield		
	Aqueous	Hexane	Ethanol
Alismataceae			
Alisma orientalis	15.89	0.39	2.57
Araceae			
Typhonium giganteum	16.89	3.35	0.44

Table 2.4 (Continued)

Tested Plants*	% Yield		
	Aqueous	Hexane	Ethanol
Araliaceae			
Panax ginseng	34.36	0.11	2.13
Bixaceae			
Bixa orellana	13.92	1.57	3.23
Campanulaceae			
Platycodon grandiflorum	25.33	0.48	6.46
Celastraceae			
Salacia reticulata	4.00	0.79	1.55
Compositae			
Atractylodes lancea	34.64	5.73	7.93
Atractylodes macrocephala	31.58	1.77	10.37
Cucurbitaceae			
Benincasa hispida	10.43	17.31	2.79
Coccinia indica	10.67	0.27	1.07
Trichosanthes kirilowii	4.71	7.45	0.82
Cyperaceae			
Cyperus rotundus	6.73	1.54	2.65
Gentianaceae			
Swertia spp.	5.69	1.76	4.09
Labiatae			
Salvia miltiorrhiza	15.79	0.27	3.93
Lardizabalaceae			
Parvatia spp.	5.86	0.97	1.85
Leguminosae			
Pterocarpus marsupium	7.22	0.56	3.49
Liliaceae			
Anemarrhena asphodeloides	17.31	0.004	17.73
Ophiopogon japonicus	33.10	0.004	1.78
Polygonatum odoratum	25.59	0.18	9.11
Meliaceae			
Azadirachta indica	17.41	2.16	4.89

Table 2.4 (Continued)

Tested Plants*	% Yield		
	Aqueous	Hexane	Ethanol
Menispermaceae			
Tinospora cordifolia	6.87	0.32	1.90
Moraceae			
Ficus bengalensis	5.71	0.84	2.78
Morus alba	11.56	1.09	12.84
Piperaceae			
Piper longum	2.44	4.17	3.49
Polyporaceae			
Ganoderma lucidum	2.50	0.31	1.50
Ranunculaceae			
Coptis chinensis	16.75	0.26	6.11
Rutaceae			
Murraya koenigii	19.34	3.21	3.25
Scrophulariaceae			
Rehmannia glutinosa	2.98	0.008	5.62
Scrophularia ningpoensis	29.30	0.30	4.62
Solanaceae			
Lycium chinensis	3.51	0.27	0.67
Zingiberaceae			
Curcuma longa	7.63	3.48	4.28

* Nominal identity given. See Table 2.3 for authentication

2.6 Conclusion

The plants used in the study were selected based, to some extent, on the criteria suggested by Marles and Farnsworth, (1995). The selected plants (Table 2.1) were those used in traditional treatment for diabetes either in Ayurvedic or Chinese medicine. In some cases, despite the claims in folklore medicine the anti-diabetic activity of the plants has not been scientifically assessed, these included *Benincasa hispida*, *Cyperus rotundus*, *Salvia miltiorrhiza* and *Typhonium giganteum*. The plants selected for the study also consisted of some whose anti-diabetic effect has been well established in animal models, but the information on mode of action is

still limited. These included *Alisma orientalis*, *Tinospora cordifolia*, *Piper longum*, *Coptis chinensis* and *Rehmannia glutinosa*. On the other hand, some plants lacked the information on the active principles which are responsible for their anti-diabetic activity e.g. *Bixa orellana*, *Coccinia indica*, *Murraya koenigii*, *Ophiopogon japonicus*, *Piper longum* and *Platycodon grandiflorum*. A number of glycans were claimed to be responsible for the anti-diabetic of some of the selected plants such as anemaran from *Anemarrhena asphodeloides*, ganoderan from *Ganoderma lucidum*, moran from *Morus alba*, panaxan from *Panax ginseng* and trichosan from *Trichosanthes kirilowii*, however the relevance of the effect of these glycans needs to be interpreted with caution, since the effect of these compounds which were observed mostly by parenteral administration may not be related to the effect of the crude drug given orally.

Sources of the plant material used in this study are given in Table 2.2 and the results of the authentication process are given in Table 2.3. In most cases, the identity of the plants were confirmed, however there were a few for which the identity was uncertain i.e. those obtained from Cipla Ltd. as *Ficus bengalensis*, *Opuntia spp.*, *Pterocarpus marsupium*, *Salacia reticulata* and *Swertia spp.*. Inspection of plant identity by the Royal Botanic Garden, Kew revealed that the specimen provided as *P. marsupium* and *S. reticulata* matched with the reference *Pterocarpus* species and *Salacia* species, respectively. However, based on the specimen provided it was not possible to distinguish these plants from the others in the same genus. *Opuntia spp.* did not appear to match reference *Opuntia* species, in fact the specimen closely matched *Parvatia spp.* (Lardizabalaceae). The samples obtained as *Ficus bengalensis* and *Swertia spp.* were sent to Royal Botanic Gardens, Kew, but the "*Swertia*" material was unsuitable for accurate identification, and no reference samples of *F. bengalensis* were available for comparison.

The extracts of the selected plants were prepared using water, hexane and ethanol. Yield of the these extracts of the selected plants is summarised in Table 2.4. Due to time and resource constraints, only the water extracts were eventually examined in the bioassays. The other extracts are available in storage for further research work.

CHAPTER 3

BIOLOGICAL STUDIES OF SELECTED PLANTS ON INTESTINAL GLUCOSE UPTAKE

3.1 Mechanism of glucose transport *in vivo*

One method by which postprandial hyperglycaemia may be reduced in diabetic patients is to control the intestinal absorption of glucose into the blood stream (section 1.2.2). Glucose, one of the final products of carbohydrate digestion, is absorbed from the intestinal lumen into blood stream by mature enterocytes lining the upper one third of the intestinal villi (Wright, 1993). Mature enterocytes (Fig. 3.1) are derived from stem cells present in the crypts of Lieberkühn; the crypt cells differentiate into mature enterocytes as they migrate toward the tip of the villi (Thorens, 1996). For glucose to be absorbed, the molecule has to be transported against its concentration gradient across brush border membranes into the enterocytes. Once inside the cell, glucose diffuses across the intracellular compartment to basolateral membranes where it is transported into the interstitial fluid by a facilitated glucose transporter (Levin, 1994).

In 1962, Crane proposed that the uphill transport of glucose into the enterocytes occurs via a Na^+/glucose-carrier complex located at brush border membranes, the process being driven by the transmembrane Na^+ gradient (Crane, 1962). Owing to a large body of support from studies using isolated brush border membranes, this hypothesis is now widely accepted. The transport of glucose across the brush border membranes takes place via the Na^+/glucose co-transport carrier known as SGLT1 (Philpott *et al.* 1992) which is a high affinity glucose transporter with K_m of 0.35 mM for glucose (Thorens, 1996). The energy required for the uphill transport of glucose is provided by the co-transport of sodium, and the energy is stored in the form of ion electrochemical potential gradients across the cell membrane (Wright *et al.* 1998b).

SGLT1 is found in all segments of the small intestine (Takata *et al.* 1993). It is located in the mature enterocytes lining at the upper region of the villus (Wright, 1993), no SGLT1 found in the crypt cells (Philpott *et al.* 1992). Human SGLT1 is a 664 amino acid protein with a predicted molecular weight of 70-75 kDa (Wright *et al.* 1992). The amino acid residues are formed into 14 membrane-spanning domains (Fig. 3.2) with N- and C-terminal domains facing extracellularly

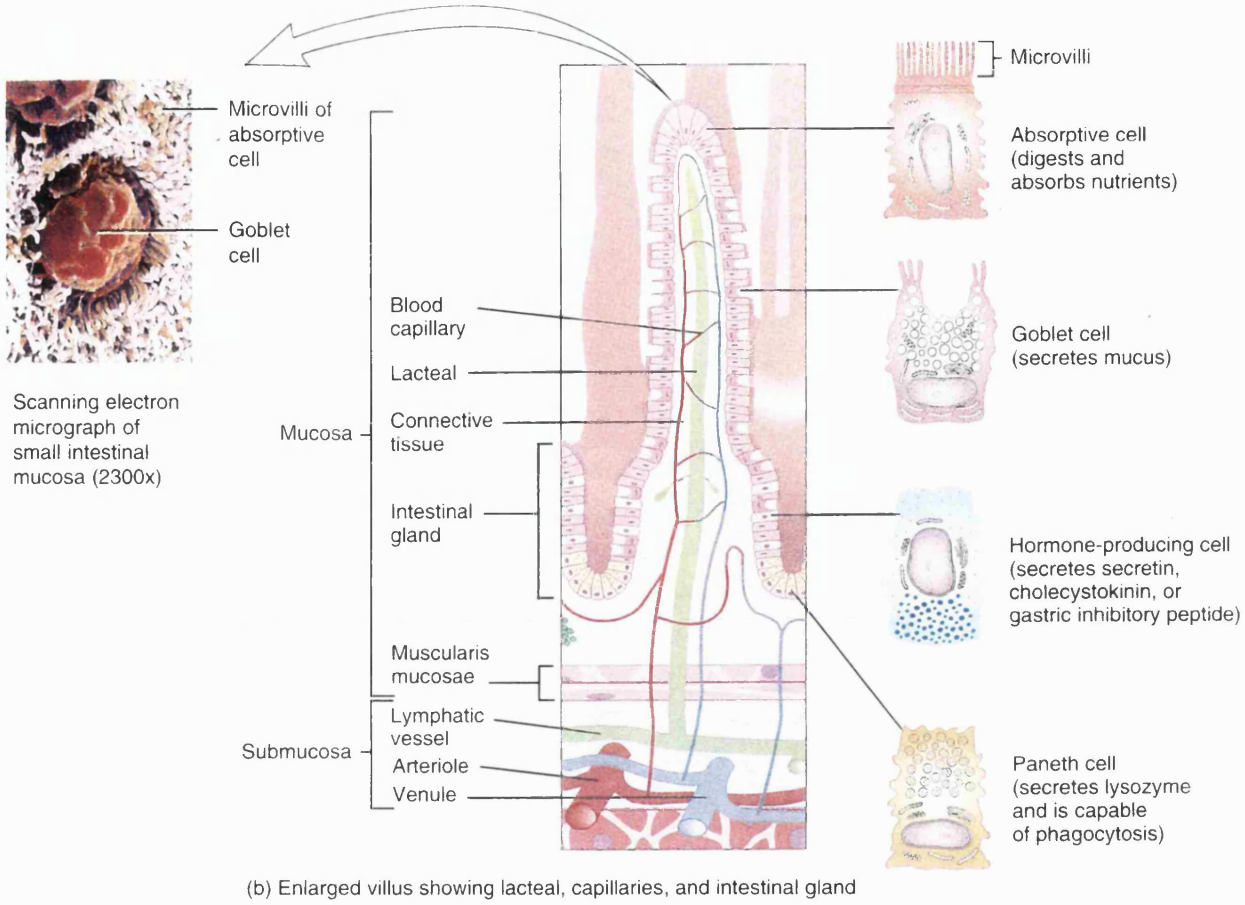


Fig. 3.1: Sectional view of the intestinal mucosa showing villi and microvilli

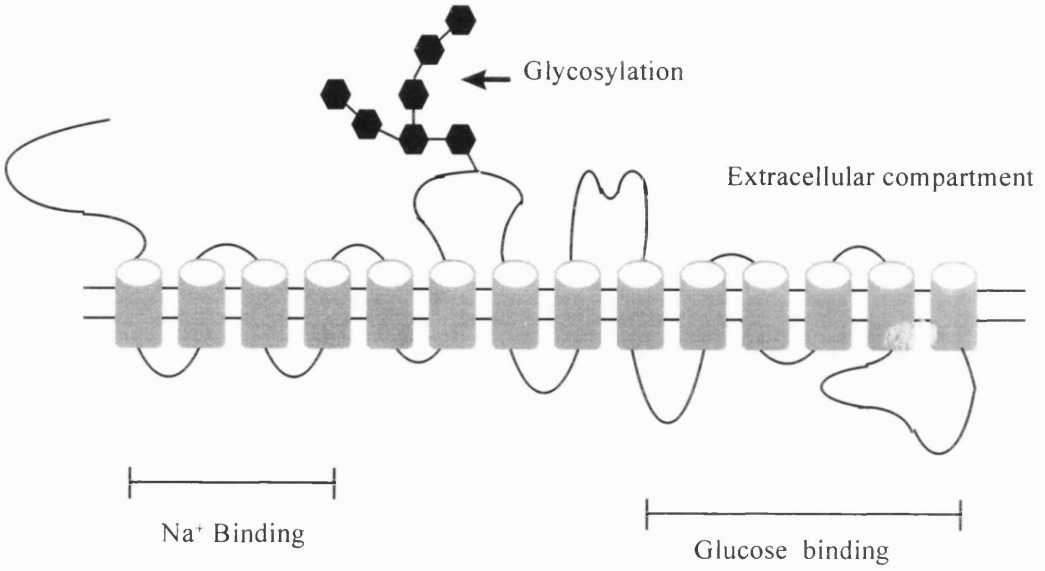


Fig. 3.2: Proposed secondary structural model of Na^+/glucose co-transporter SGLT1 (modified from Wright *et al.* 1998)

(Wright, 1998a). Each domain is composed of 21 residues arranged in an α-helix (Wright *et al.* 1991). Recently, it has been proposed that the binding of glucose to SGLT1 occurs through the C-terminal domains (helices 10-13) whereas Na^+ binding occurs through the N-terminal (helices 1-4) (Wright *et al.* 1998b). N-Glycosylation at asparagine 248 does not appear to be critical for the transport activity of SGLT1 (Wright *et al.* 1992). SGLT1 can transport a range of sugars with different affinities i.e. D-glucose > D-galactose > α-methyl-D-glucopyranoside > 3-O-methylgluco-pyranoside >>> L-glucose, 2-deoxy-glucopyranoside (Hediger and Rhoads, 1994).

The absorption of glucose across brush border membranes is Na^+-dependent (Fig. 3.3) since glucose is unable to bind to the transporter in the absence of Na^+. The binding of Na^+ to the transporter produces a conformational change which allows glucose to bind to the transporter (Wright, 1993). Transport of each molecule of glucose is coupled to the co-transport of two sodium ions (Hediger and Rhoads, 1994). After binding to glucose, the transporter undergoes another conformational change to present the substrate to the intracellular face of the membrane. Once inside the enterocytes, Na^+ is released from the transporter due to the low concentration of Na^+ in the cells (Wright, 1993). The dissociation of Na^+ from the transporter leads to a decreased affinity for glucose, and subsequent release

of glucose from the binding site to the cytoplasm. The transporter then reorients to its original state (Wright, 1993). The accumulated Na^+ is transported out of the enterocytes by basolaterally located Na^+-K^+ ATPase enzyme in order to maintain a low intracellular concentration of Na^+ (Fig. 3.3).

A defect in brush border membrane SGLT1 can lead to an autosomal recessive disease known as glucose-galactose malabsorption (Wright *et al.* 1992; Levin, 1994). Patients suffering from this disorder cannot absorb glucose and galactose but the absorption of fructose and xylose is still normal (Wright, 1993). A number of studies suggest that the functional loss of SGLT1 is due to a change in amino acid at position 28 from aspartate to an asparagine (Wright, 1993; Levin, 1994); aspartate 28 may have a role in maintaining the tertiary structure of SGLT1 (Wright *et al.* 1992). The normal absorption of fructose observed in patients with glucose-galactose malabsorption indicates that fructose is transported into the enterocytes via a different transporter. It is now well established that fructose is

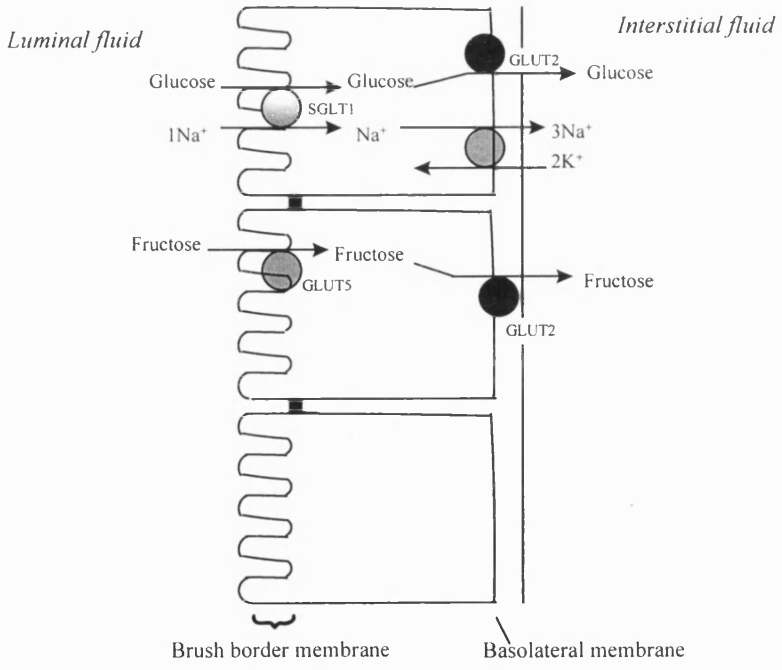


Fig. 3.3: A model for sugar transport across the enterocyte showing the brush border SGLT1 and GLUT5 transporter and the basolateral sugar transporter GLUT2 (modified from Thorens, 1993a).

transported into brush border membranes via facilitated fructose transporter known as GLUT5 (Fig. 3.3) (Mueckler, 1994; Thorens, 1996).

Glucose is transported out of the enterocytes across the basolateral membrane via facilitated sodium-independent transporter (GLUT2). The unique feature of GLUT2 is that it has a relatively high K_m for glucose (~15-20 mM) which allows the rate of glucose transport across basolateral membrane to increase when the intracellular glucose concentration increases above normal value i.e. 5 mM (Thorens, 1993a). GLUT2 can transport a range of sugars including glucose, galactose, mannose and fructose (Thorens, 1996). Similarly to SGLT1, GLUT2 is found only in the mature enterocytes (Thorens, 1993a). Besides the basolateral membrane of intestinal epithelial cells, GLUT2 is also present in hepatocytes, pancreatic β-cells and the proximal tubule of the kidney nephron (Mueckler, 1994).

There is also evidence suggesting that intestinal glucose absorption partly occurs through the tight junction (paracellular pathway) (Philpott *et al.* 1992), it has been suggested that the activation of the Na^+-dependent glucose transporter on brush border membranes triggers the opening of the tight junction and the subsequent uptake of glucose (Philpott *et al.* 1992). However, the physiological significance of the tight junction in the uptake of glucose *in vivo* still remains to be defined. There are strong bodies of evidence suggesting that food in the lumen has significant effect on the expression of SGLT1 (Philpott *et al.* 1992; Wright, 1993). An increased amount of dietary carbohydrate can markedly increase the number of SGLT1 (Philpott *et al.* 1992) and its protein level (Wright, 1993) in the brush border membranes.

3.2 SGLT1 and Diabetes

Hyperglycaemia in the postprandial state is one of the metabolic abnormalities in diabetic patients. The main determinant of postprandial glucose levels appears to be related to an influx of glucose from the gut. Thus, the approaches to normalise postprandial blood glucose level in diabetic patients are to minimise amount of glucose absorbed from the gut. This can be achieved via a number of mechanisms such as inhibiting carbohydrate digestion or inhibiting a

transport activity of glucose transporters. There is an evidence that a number of functionally mature SGLT1 is increased in diabetic state in mice (Ferraris *et al.* 1993) and STZ-induced diabetic rats (Thorens, 1996), this in turn can lead to enhanced glucose absorption after meal and a worsening postprandial hyperglycaemia. Thus, control of SGLT1 activity might be another way to prevent excessive glucose uptake (Thorens, 1996), and hence hyperglycaemia.

This chapter describes experiments conducted to investigate the effect of selected plants (Table 2.3) on the uptake of glucose via SGLT1, using brush border membrane vesicles (BBMV) prepared from microvilli of rabbit small intestine.

3.3 Preparation and evaluation of brush border membrane vesicles (BBMV)

3.3.1 Introduction

Brush border membrane vesicles were first prepared from tips of microvilli of rat small intestine. BBMV were originally used in studies of D-glucose transport to confirm Crane's Na^+ gradient hypothesis (Hopfer *et al.* 1973). BBMV have since been successfully used in various absorption studies across intestinal membranes including the absorption of amino acids (Stevens *et al.* 1984), dipeptides, xenobiotics and β carotene (Moore *et al.* 1996). BBMV have been used in studies of drug absorption almost exclusively to study the absorption of β-lactams and cephalosporins (Wood and Lawrence, 1991). The advantages of BBMV over other complex absorption models are that they are free of organelles, endogenous substrates and metabolic enzymes, thus they can be used to study the absorption of substrates which would be metabolized by intact epithelium (Wood and Lawrence, 1991). In addition, BBMV can be used to study the transport of substrates across the brush border membrane independently from the basolateral plasma membrane (Hopfer *et al.* 1973).

The ability of BBMV to transport glucose across the membrane is well established (Hopfer *et al.* 1973; Hopfer *et al.* 1976; reviewed by Murer and Kinne, 1980). It is believed that the uptake of glucose by the vesicles is due to the transport

into an intravesicular space rather than binding to the membrane surface (Hopfer *et al.* 1973). The characteristics of glucose transport in BBMV were consistent with those observed in the intact intestinal tissues (Hopfer *et al.* 1976) as they were both inhibited by phloridzin and stimulated by sodium ion (Hopfer *et al.* 1973).

In this study the effects of 31 selected plant extracts on glucose uptake into BBMV were studied. BBMV were prepared according to the method known as 'differential precipitation' described by Schitmz *et al.* (1973) and Hopfer *et al.* (1976). Rabbit is the animal of choice because the tissues and vesicles can be stored frozen without losing transport activity whereas the vesicles made from rat small intestine are susceptible to degradation after freezing (Wood, 1991).

3.3.2 Materials and stock buffer solutions

Unless specified, chemicals were purchased from Sigma-Aldrich Co. Ltd. (Irvine, UK). N-2-Hydroxyethypiperazine-N′-2-ethanesulphonic acid (HEPES) and tris-(hydroxymethyl)-methylamine were purchased from BDH Laboratory Supplies (England). Sodium thiocyanate was purchased from Hopkin & Williams Ltd. (England). Mannitol was purchased from ICN Biochemicals Inc. (Ohio). D-[6-^{3}H] Glucose was purchased from Amersham Pharmacia Biotech (UK) Ltd. Nitro-cellulose filter (0.45 μM) was purchased from Millipore Corporation, Bedford (USA). Ready-Protein Scintillant was purchased from Beckman Instruments, Inc. (USA).

Reagents used in the preparation of BBMV are listed as follows.

Buffer 1

10 mM D-Mannitol

2 mM N-2-Hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES)

Adjust to pH 7.4 with 0.2 M Tris-(hydromethyl)-methylamine

Buffer 2

100 mM D-Mannitol

0.1 mM $MgSO_4$. $7H_2O$

2 mM HEPES

Adjust to pH 7.4 with 0.2 M Tris-(hydromethyl)-methylamine

Buffer 3

300 mM D-Mannitol

0.1 mM $MgSO_4.7H_2O$

10 mM HEPES

Adjust to pH 7.4 with 0.2 M Tris-(hydromethyl)-methylamine

Glucose incubation buffer (×1 concentration)*

100 mM Sodium thiocyanate (NaSCN)

100 mM Mannitol

10 mM HEPES

Adjust to pH 7.4 with 0.2 M Tris-(hydromethyl)-methylamine

*In some cases solution was prepared containing the same substances twice the above concentrations (" ×2 concentration").

Stop Buffer

200 mM NaCl

10 mM HEPES

250 μM Phloridzin

Adjust to pH 7.4 with 0.2 M Tris-(hydromethyl)-methylamine

3.3.3 Preparation of BBMV from rabbit intestine

Method used for BBMV preparation was summarised as a flow chart shown in Figure 3.4. A New Zealand white rabbit was anaesthetised by an intravenous injection with sodium pentobarbitone (60 mg/kg). The abdomen was opened and the small intestine was dissected from the pyloric sphincter to the ileo-caecal junction. The mesentary was separated from the intestine. The intestine was then cleaned in 200 ml ice-cold KCl solution (154 mM), the remaining mesentary and fat were removed and the contents of the intestine were squeezed out. The cleaned small intestine was cut longitudinally and washed thoroughly with ice-cold KCl solution, followed by blotting with tissue paper. The small intestine was then snap frozen in liquid nitrogen to minimize the differential freezing rates of the intra- and extracellular water that could lead to osmotic imbalance across the membranes

and tissues damage (Wood, 1991). The small intestine was stored in liquid nitrogen until use.

When required, the frozen small intestine was thawed in approximately 200 ml of Buffer 1 at room temperature for approximately 20 minutes and cut into small pieces (1×1 cm). The intestine in Buffer 1 was then placed on ice and subjected to vibration for 1 minute with a VIBRO-mixer (Chemap AG, Switzerland) at maximum speed. After homogenisation, the mixture was left on ice for another minute and repeatedly homogenised with the VIBRO mixer for another 30 seconds at maximum speed. The resulting mixture was filtered using a Buchner funnel under negative pressure to remove connective tissues and muscles. The supernatant was collected and diluted to 300 ml with Buffer 1, $MgCl_2$ (0.61 g; final concentration 0.01 mM) was then added to the supernatant to precipitate intracellular organelles and basolateral membrane. The solution was allowed to stand on ice for 20 minutes with occasional stirring. The solution was then centrifuged at 4 °C, 3015g for 15 minutes to spin down intracellular organelles, basolateral membrane and other debris. The precipitate was then discarded.

The supernatant which contained brush border membrane was centrifuged at 4 °C, 34858g for 30 minutes. The supernatant after the second centrifugation was discarded and the pellets were resuspended in Buffer 2. The resulting mixture was then centrifuged at 4 °C, 34858g for 40 minutes. The supernatant was discarded and the remaining pellets were collected. The pellets were resuspended in Buffer 3 (1 ml), the volume of the resulting mixture was doubled with Buffer 3. The diluted mixture was then passed through a 25 gauge needle (5 times) to form vesicles. Aliquots of 0.26 ml were transferred to cryotubes. The vesicles in the cryotubes were snap frozen and stored in liquid nitrogen. The vesicles were stored in 300 mM mannitol (Buffer 3) to maintain osmolarity. In liquid nitrogen, the viability of the vesicles can be retained for several months (Wood, 1991). This method produces spherical vesicles with diameter of 100-150 nm (Stevens *et al.* 1984). Approximately 15-20 aliquots of 0.26 ml were obtained from each intestine.

Small intestine was dissected from freshly killed white New Zealand rabbit and separated from the mesentary

↓

The intestine was washed thoroughly with 200-ml ice cold KCl solution (154 mM) and blotted dry with tissue paper

↓

The intestine was snap frozen and stored in liquid nitrogen until use

↓

Frozen intestine was thawed in Buffer 1 at room temperature, cut into small pieces and subjected to homogenisation

↓

The solution was filtered under negative pressure, the supernatant was made up to 300 ml with Buffer 1, added with $MgCl_2$ (0.61 g) and stirred for 20 minutes

↓

The solution was centrifuged at 4 °C, 3015g, for 15 minutes

↓

The pellet was discarded, and the supernatant was centrifuged at 4 °C, 34858g, 30 minutes

↓

The pellet was resuspended in Buffer 2 and centrifuged at 4 °C, 34858g, for 40 minutes

↓

The pellet was resuspended in 1 ml of Buffer 3, diluted (1:1) with Buffer 3 and passed 5 times through a 25 gauge needle.

↓

The solution was aliquoted in 0.26 ml volumes and snap frozen in liquid nitrogen until use.

Fig. 3.4: Flow chart for the preparation of BBMV

3.3.4 Determination of profile of glucose uptake into BBMV

Each batch of BBMV was examined for viability and ability to transport and accumulate glucose before being used in the study on the effect of plant extracts. The examination was performed by measuring the amount of glucose accumulated in the vesicles at intervals over an hour period, using a technique known as 'rapid filtration technique' developed by Hopfer *et al.* (1973).

The uptake experiment was performed using glucose incubation solution containing 0.1 mM glucose and D-[6-^{3}H] glucose (0.296 MBq/ml) prepared in glucose incubation buffer (×1 concentration). Sodium thiocyanate (NaSCN) was present in the incubation buffer to produce a sodium gradient, NaSCN was selected because it has been reported to produce the most effective Na^+ gradient compared to NaCl (Wood, 1991). The ability of vesicles to accumulate glucose was affected when NaSCN was replaced with NaCl (Wood, 1991).

Each BBMV aliquot (0.26 ml) was reconstituted in Buffer 3 (0.44 ml). Uptake was initiated by addition of the reconstituted BBMV (20 µl) to 0.1 mM glucose incubation solution (40 µl), and incubated at 25 °C in a water bath. The incubation was terminated at 7 different time points i.e. 0, 10, 20, 40, 60, 300 and 3600 seconds, by addition of ice-cold stop buffer (1 ml) containing 250 µM phloridzin. In the case of the uptake at 0 second, the order in which the solution was added was slightly different from the uptake at other time points. The stop solution (1 ml) was added to glucose solution prior to the addition of BBMV. All solutions were then filtered through a pre-wetted nitrocellulose filter (0.45 µm) using a Millipore filtration unit under negative pressure. Due to the ability of cellulose nitrate to adsorb protein, BBMV would be retained on the filter and thus separated from the solution. The filter was washed 3 times with the ice-cold stop buffer (1ml each), and transferred to a scintillation vial. The filter was dissolved in 3.5 ml Ready-Protein® scintillant and counted for 3 minutes by liquid scintillation counter (LKB Wallac 1209 Rackbeta). The uptake at each time point was investigated with 6 separate observations.

To assess non specific binding of radiolabelled glucose to the filters, the uptake was performed as described earlier, but BBMV was omitted from the

reaction. Glucose incubation solution (0.1 mM glucose, 40 µl) was incubated with 20 µl water at 25 °C for each time point. At the end of incubation period, the reaction was stopped by the addition of the ice-cold stop buffer (1 ml). The solution was filtered, the filter was then washed, dissolved in scintillation liquid and counted for radioactivity as described earlier in this section The experiment was performed in duplicate for each time point. The obtained values represented the radiolabelled glucose which non-specifically bound to the filters. The values of binding to filter was subtracted from the values obtained from the experiment conducted with BBMV. Profile of glucose was constructed by plotting the activity (cpm) of glucose transported into BBMV against time (seconds).

3.3.5 Protein assay of BBMV

Protein content of BBMV suspension was assayed according to the method described by Bradford, (1976). BBMV (0.26 ml) in Buffer 3 (0.44 ml) was diluted 1:10, 1:50 and 1:100 in water. The BBMV suspension (100 µl) was added with coomasie blue reagent (1.9 ml), intensity of colour developed in a reaction between protein and coomasie blue was measured by means of colorimetric method at λ 595 nm, each dilution of BBMV was assayed with 3 replications. Protein content of BBMV was extrapolated from a calibration curve constructed from various concentrations of bovine serum albumin (BSA, 0-0.5 mg/ml), each concentration of BSA was assayed with 3 replications. Uptake of glucose/mg protein was calculated from the following formula:

$$\frac{\text{Mean of glucose uptake (cpm)} \times \text{Amount of glucose (pmole)}}{\text{Mean of total activity} \times \text{Protein content (mg/ml)} \times \text{Volume of BBMV (ml)}}$$

Amount of glucose added	: 4000 pmole (40 µl of 0.01 mM glucose)
Mean total activity	: Mean of total activity of radiolabelled glucose added
Volume of BBMV	: 0.02 ml

Coomasie blue dye was prepared by dissolving 10 mg Coomasie Brilliant Blue in 5 ml ethanol (95%), followed by the addition of 10 ml phosphoric acid (85 %). The volume was then adjusted to 100 ml with distilled water.

3.3.6 Results and discussion

As shown in Figure 3.5, the glucose uptake profile showed not only time of maximum uptake but also the magnitude of glucose accumulation. Investigation of the time-profile of glucose uptake into BBMV revealed that the maximum uptake usually occurred between 10-20 seconds after the reaction was initiated. Twenty second was chosen as the incubation time for all the uptake experiments for the sake of convenience, since this would allow enough time to get the stop buffer ready before the end of the incubation. The magnitude of glucose uptake in the profiles varied from batches to batches. The amount of glucose transported into the vesicles ranged from 4,200-12,000 cpm, and variation was observed even when the content of protein was taken into account (Table 3.1). This variation probably reflected differences in the stability of the preparation on different occasion. This implied that the comparisons in term of the amount of glucose uptake (pmole/mg protein) obtained from different experiments would not be appropriate. Thus, it was more practical to express the experimental results as percentage of uptake (or inhibition) which was calculated against values of control from each experiment so that the results from two different experiments could be compared.

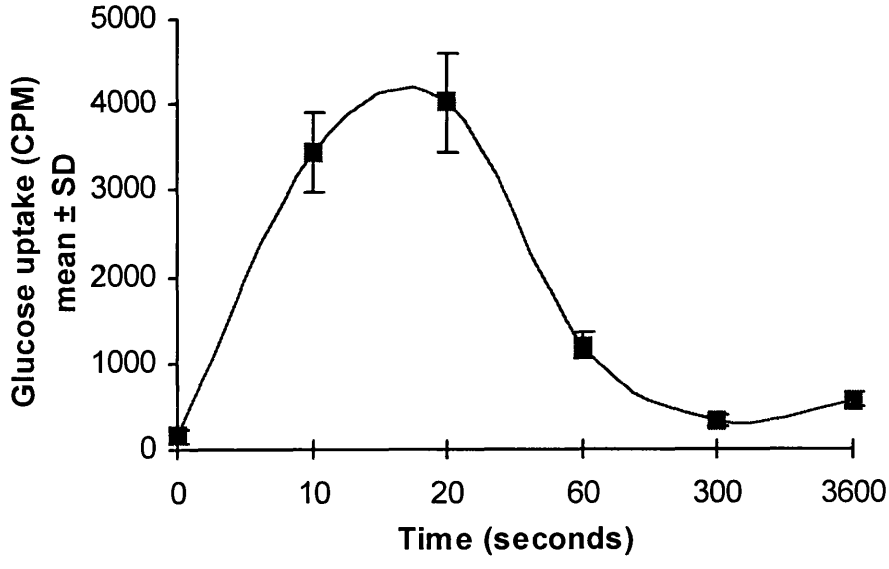


Fig. 3.5: Profile of glucose uptake into rabbit intestinal BBMV

Results are expressed as mean of glucose uptake (cpm) ± SD (corrected for non specific glucose binding). Similar results were obtained from 9 separate preparations of BBMV.

Table 3.1: Content of protein and maximum uptake of glucose into 4 different batches of BBMV

Batch	Protein content (mg/ml)	Maximum uptake* (cpm)	Uptake (mmole)/mg protein**
1	8.87	4799.37	355.75
2	7.40	4221.76	426.42
3	6.92	7421.17	697.68
4	5.93	12116.52	2005.86
5	5.70	11761.93	1504.36

* Uptake at 20 seconds. Results were taken from 5 different batches of BBMV.

**Calculated from $\frac{\text{Mean of glucose uptake (cpm)} \times \text{Amount of glucose (pmole)}}{\text{Mean of total activity} \times \text{Protein content (mg/ml)} \times \text{Volume of BBMV (ml)}}$

The extent to which glucose accumulated in the vesicles did not appear to be a major concern provided that the magnitude of the uptake was not loo low (the uptake at 20 seconds was greater than 2500 cpm) and the profile was found to be "overshoot pattern" i.e. producing a peak of glucose accumulation which was a major determinant of vesicle viability. The vesicles would be discarded if the profile failed to show the overshoot pattern since it meant that the vesicles were not able to transport and/or accumulate glucose. The overshoot of glucose uptake observed in the profile (Fig. 3.5) was due to rapid uptake of Na^+ from the incubation medium which subsequently drove glucose uptake into the vesicles. In an *in vivo* system, Na^+ would be exported out of the enterocytes via Na^+/K^+-ATPase in basolateral membrane and glucose would be passively exported via GLUT2 (section 3.1), leaving the cell with low Na^+ concentration. However, these conditions were not always prevalent in BBMV where the uptake mainly depended upon Na^+ being able to diffuse back out of the vesicles in order to maintain the gradients required to drive glucose into the vesicles. Due to the lack of basolateral membrane and hence Na^+ recycling, Na^+ transported into BBMV was retained and accumulated inside the vesicles, resulting in higher concentration of Na^+ in the vesicles compared to the bathing medium. This resulted in the inhibition of both Na^+ uptake and subsequent glucose influx into the vesicles, leading to a decline of glucose accumulation after

the maximum uptake to equilibrium level (Wood, 1991). At equilibrium, the graph represents the balance between glucose influx (via Na^+ co-transporter and diffusion) and the efflux of glucose.

3.4 Method validation using phloridzin

3.4.1 Introduction

It is well established that transport activity of SGLT1 can be competitively inhibited by plant-derived glycoside, phloridzin (Fig. 3.6). Inhibitory effect of phloridzin (syn. phlorizin) has been suggested to be owing to its competition with glucose for the same membrane binding site (Alvarado, 1967). The affinity of phloridzin to the transporter was reported to be more than 100-fold higher than that of D-glucose (Koepsell *et al.* 1990). The membrane binding site of the transporter is believed to consist of two separate regions, known as sugar- and phenol-binding sites (Alvarado, 1967). High affinity of phloridzin to the transporter is primarily due to the binding of its D-glucose moiety to the sugar binding region and the binding of its aglycone, phloretin, to the phenol binding site (Koepsell *et al.* 1990). Several lines of evidence suggest that it is intramolecular hydrogen bonding that fixes the molecule of phloridzin in an appropriate position which favours the association with the membrane binding sites (Alvarado, 1967). Main points of interaction between the phloridzin molecule and the membrane binding site are suggested to involve the oxygen at B ring of phloretin and the -OH at C-2 of glucose, however additional interactions at other groups probably also occur (Alvarado, 1967).

In this study, phloridzin at various doses was used to evaluate the method used in the uptake experiment and the ability to detect inhibition of transport activity

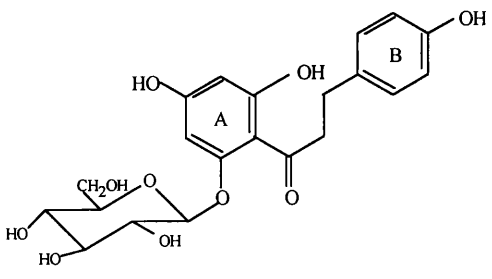


Fig. 3.6: Structure of phloridzin

of the glucose transporter present in BBMV prepared according to the method described earlier (section 3.3.3)

3.4.2 Materials and methods

The uptake experiment was performed by incubating different concentrations of phloridzin (10, 100, 200, 400 and 800 µM, 20 µl) with BBMV and 0.2 mM glucose incubation solution which was prepared in glucose incubation buffer (×2 concentration) in the presence of radiolabelled glucose (0.296 MBq/ml) to give final concentrations of phloridzin of 3.3, 33.3, 66.7, 133.3 and 266.7 mM. The reaction was initiated by the addition of 20 µl BBMV (reconstituted in Buffer 3) to a mixture of the glucose solution (20 µl) and either aqueous solution of phloridzin (20 µl) or water (20 µl, in case of control). The mixture was incubated at 25 °C for 20 seconds which was the time where maximum uptake was observed in most of the BBMV batches (section 3.3.6). As described earlier (section 3.3.4), at the end of incubation period ice-cold stop buffer (1 ml) was added to the mixture to stop the reaction. The solution was then passed through a 0.45 µm nitrocellulose filter. The filters were washed with 3 ml of the stop buffer, transferred to a vial and dissolved in scintillation liquid. Radioactivity was counted for 3 minutes. Uptake was investigated with 6 observations for each concentration of phloridzin. The results were corrected by subtracting from the test values the corresponding values obtained for binding to filter (BF) in which BBMV was omitted from the reaction and replaced with 20 µl water (section 3.3.4). The glucose uptake values (mean ± SD) were expressed as % control which was calculated using the following formula:

$$\frac{(\text{Glucose uptake}_{\text{sample}}\ (\text{cpm}) - \text{BF})}{(\text{Glucose uptake}_{\text{control}}\ (\text{cpm}) - \text{BF})} \times 100$$

3.4.3 Results and discussion

The finding that phloridzin produced a dose dependent inhibitory effect in BBMV (Fig. 3.7) gives a strong support to the method used in the uptake experiment and the validity of this model. The IC_{50} of phloridzin observed in this study was 110 µM. Studies by Brot-laroche *et al.* (1987) on the effect of phloridzin on glucose transport activity in brush border membranes of guinea pigs using α-

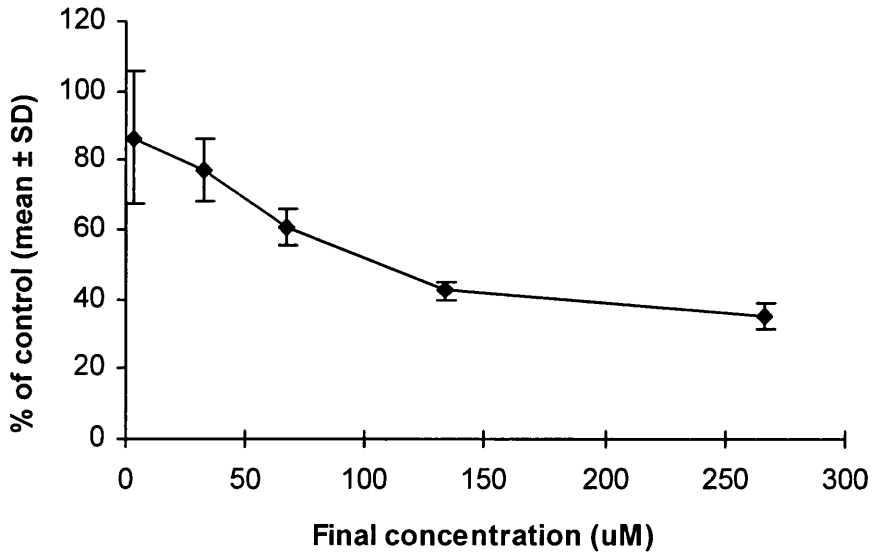


Fig. 3.7: Inhibition of glucose uptake by phloridzin (final concentration: 3-266 μM).
Results are means of %control ± SD. Each concentration of phloridzin was assayed with 6 data points.

methyl glucoside as a substrate reported K_i of phloridzin as 18 μM. In the current study, K_i of phloridzin calculated from a graph plotted 1/V against phloridzin concentration (where V = glucose uptake) was found to be 170 μM. This difference may possibly be due to differences in animal species used in the studies as the current experiment was conducted using BBMV prepared from rabbits, the use of a glucose analogue by Brot-laroche *et al.* (1987), as well as other differences in terms of experimental methodologies. No literature IC_{50} value for inhibition of rabbit intestinal glucose uptake into BBMV by phloridzin was found.

3.5 Investigation of the effect of plant extracts on glucose uptake into BBMV

3.5.1 Introduction

The studies described in this section aimed to investigate the effect of aqueous extracts of the 31 selected plants (Table 2.3) on intestinal glucose uptake into BBMV prepared as described in section 3.3.3.

3.5.1 Materials and methods

Selected plant materials and their sources were shown in Table 2.2. The results of botanical examination of these plants were shown in Table 2.3. Aqueous extracts of plant materials were prepared as described in section 2.5.2. To test on BBMV, dried plant extracts were reconstituted in distilled water to give a concentration of 5 mg/ml (i.e. the final concentration of 1.67 mg/ml in the reaction).

The effect of plant extracts on glucose uptake was investigated using the methods described in section 3.4.2 with a minor modification. Glucose incubation solution (0.2 mM glucose, 20 µl) prepared in glucose incubation buffer (×2 concentration) containing radiolabelled glucose (0.296 MBq/ml) was incubated at 25 °C for 20 seconds with BBMV (20 µl) reconstituted in Buffer 3 and reconstituted aqueous extracts of plant material (20 µl). The mixture was then filtered, the filters were washed, transferred to vials containing scintillation liquid and counted for radioactivity as described in section 3.3.4. The uptake was performed with 4 observations for each plant extract. The results were corrected by subtracting the values obtained from 'binding to filter' (n = 2) for each plant extract as described in section 3.4.2. Glucose uptake (mean ± SEM) was expressed as % of control obtained using water (20 µl) instead of plant extract (section 3.4.2).

3.5.3 Results and discussion

The results of testing the plant extracts on glucose uptake into BBMV are given in Table 3.2. The investigation revealed that the extracts of several plants including *Typhonium giganteum*, *Bixa orellana*, *Atractylodes macrocephala*, *Benincasa hispida*, *Cyperus rotundus*, *Ophiopogon japonicus*, *Ficus bengalensis* and *Curcuma longa* at a final concentration of 1.67 mg/ml had no inhibitory effect on the uptake of glucose into BBMV. The aqueous extracts of *B. hispida*, *B. orellana* and *O. japonicus* were in fact found to increase the uptake of glucose into BBMV, possibly by alterations in ion concentrations (e.g. sodium concentration).

At a final concentration of 1.67 mg/ml, 23 plant extracts were found to have apparent inhibitory effect on uptake of glucose into BBMV (Table 3.2). Among these, the aqueous extract of *Morus alba* bark demonstrated the most potent

inhibitory effect i.e. 86.6 % inhibition (13.4% uptake). Additionally, there were 4 plants of which the inhibition was found to be greater than 70% (i.e. < 30% uptake). These plants were *Azadirachta indica* (28.1% uptake), *Coptis chinensis* (28.4 %), *Parvatia spp.* (28.9%) and *Rehmannia glutinosa* (29.4%).

Table 3.2: Effect of the aqueous plant extracts on glucose uptake

Tested Plants♦	Glucose uptake (% of control) (Mean±SEM)
Alismataceae	
Alisma orientalis	38.0±7.0***
Araceae	
Typhonium giganteum	84.9±6.7
Araliaceae	
Panax ginseng	32.4±3.0***
Bixaceae	
Bixa orellana	124.7±5.8
Campanulaceae	
Platycodon grandiflorum	44.9±0.9***
Celastraceae	
Salacia reticulata	65.9±1.0**
Compositae	
Atractylodes lancea	42.2±0.4***
Atractylodes macrocephala	97.7±5.4
Cucurbitaceae	
Benincasa hispida	131.4±6.5
Coccinia indica	41.7±0.1***
Trichosanthes kirilowii	68.3±9.2***
Cyperaceae	
Cyperus rotundus	79.4±5.3
Gentianaceae	
Swertia spp.	59.8±7.1**
Labiatae	
Salvia miltiorrhiza	66.2±1.8**
Lardizabalaceae	
Parvatia spp.	28.9±5.0***
Leguminosae	
Pterocarpus marsupium	64.1±5.4**

Table 3.2 (continued)

Tested Plants♦	Glucose uptake (% of control) (Mean ± SEM)
Liliaceae	
Anemarrhena asphodeloides	32.6±2.6***
Ophiopogon japonicus	123.8±7.3
Polygonatum odoratum	36.4±1.5***
Meliaceae	
Azadirachta indica	28.1±1.9***
Menispermaceae	
Tinospora cordifolia	74.3±6.4*
Moraceae	
Ficus bengalensis	74.2±2.4
Morus alba	13.4±0.2***
Piperaceae	
Piper longum	52.0±3.1***
Polyporaceae	
Ganoderma lucidum	44.3±7.8***
Ranunculaceae	
Coptis chinensis	28.4±6.8***
Rutaceae	
Murraya koenigii	41.4±1.6***
Scrophulariceae	
Rehmannia glutinosa	29.4±5.0***
Scrophularia ningpoensis	39.6±2.3**
Solanaceae	
Lycium chinensis	49.9±8.4***
Zingiberaceae	
Curcuma longa	89.8±3.3

♦Nominal identities given. For details of authentication see Table 2.3

*P<0.05, **P<0.01, ***P<0.001. Data are means of % control from two separate experiments. n = 4 (each experiment).

Since more than two-thirds of the extracts were found to have positive results, it was suspected that the inhibition observed in most cases in this preliminary study might be artefactual, most probably due to the presence of glucose in the extracts. To prove this assumption, a further study was conducted to

investigate the effect of added glucose on the uptake of radiolabelled glucose into BBMV (section 3.6).

It was found in the experiments that the amount of glucose uptake into BBMV obtained for control incubation varied among different batches of BBMV. The values was found to range from 1450 to 3810 cpm. This wide variation may possibly due to a difference in the transport ability of different BBMV batches prepared from different animals on different occasions.

3.6 Effect of added cold glucose and fructose on the uptake of radiolabelled glucose into BBMV

3.6.1 Introduction

Since glucose is the most abundant sugar found in plants, it was expected that its presence in the extracts might interfere with the uptake of radiolabelled glucose into BBMV. The interference would probably be due to a competition between cold (unlabelled) and radiolabelled glucose for the same binding sites on the transporters, ultimately leading to less uptake of radiolabelled glucose into BBMV than it should be and hence artefactual positive results. To determine the extent to which extra added glucose could affect the observed the uptake of radiolabelled glucose, the uptake experiment was performed in the presence of various concentrations of cold glucose added as the 'test sample'.

3.6.2 Materials and methods

Investigation of the effect of extra cold glucose on the uptake of radiolabelled glucose was performed as described in section 3.5.2 with minor modification. Glucose solution (0.2 mM glucose, 20 µl) prepared in glucose solution buffer (×2 concentration) containing radiolabelled glucose (0.296 MBq/ml) was incubated for 20 seconds with reconstituted BBMV (20 µl) in the presence of 20 µl of different concentrations of unlabelled glucose at the final concentration of 0.02, 0.04, 0.10, 0.30 and 0.67 mM (which was 1/3 of original concentrations) instead of plant extracts. Each concentration of glucose was investigated with 4 observations.

BBMV retained on the nitrocellulose filter was counted for radioactivity for 3 minutes. The values were corrected with the mean values of 'binding to filters' which was assayed in duplicate for each concentration of glucose (section 3.4.2). The results were expressed as % of control (mean ± SEM).

The effect of added fructose on the uptake of radiolabelled glucose was also investigated at concentrations of 0.067, 0.67 and 6.7 mM (final concentration) using the method as described above.

3.6.3 Results and discussion

Investigation of the effect of extra added glucose on the uptake of radiolabelled glucose revealed that the presence of additional cold glucose, even at the concentration as low as 0.02 mM (final concentration), could significantly decrease the uptake of radiolabelled glucose into BBMV. The uptake of glucose was reduced by 20% upon the addition of 0.02 mM extra glucose (Fig. 3.8). As expected, the inhibition became greater when the concentration of the cold glucose in the incubation mixture was increased. At the highest concentration tested i.e. 0.67 mM, cold glucose was found to inhibit the uptake of the radiolabelled glucose by 90% (Fig. 3.8). Unlike glucose, the presence of added fructose did not affect the uptake of radiolabelled glucose (Fig. 3.9). It was found that there was no significant difference between the uptake of glucose when fructose was added and that of control where no fructose was present. The presence of additional fructose even at the final concentration as high as 6.7 mM did not appear to inhibit the uptake of glucose into BBMV (Fig. 3.9). This suggested that fructose was transported into the vesicles via the transporter different from glucose. This finding is consistent with the transport of fructose *in vivo* which was suggested in literature to take place via GLUT5 not SGLT1 (Mueckler, 1994) (see section 3.1).

As expected, results indicated that the presence of cold glucose but not fructose, could interfere with the uptake of radiolabelled glucose. This implied a potential interference on the uptake by plant extract glucose. It was also believed that glucose present in the plant extracts was probably the cause of false positive results. Further investigation on the role of plant extract glucose on the uptake of

radiolabelled glucose into BBMV was therefore conducted (section 3.7 and 3.8) to evaluate the results obtained from the studies (Table 3.2).

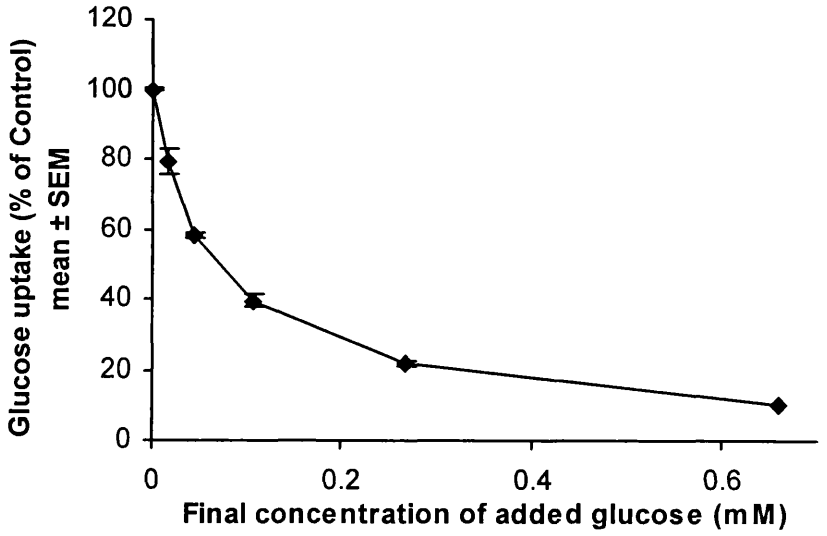


Fig. 3.8: Effect of added glucose on radiolabelled glucose uptake into BBMV.

Results are means of %control ± SEM taken from 4 data points. Glucose uptake of control was 4518.33 ± 34.42 cpm (mean ± SEM).

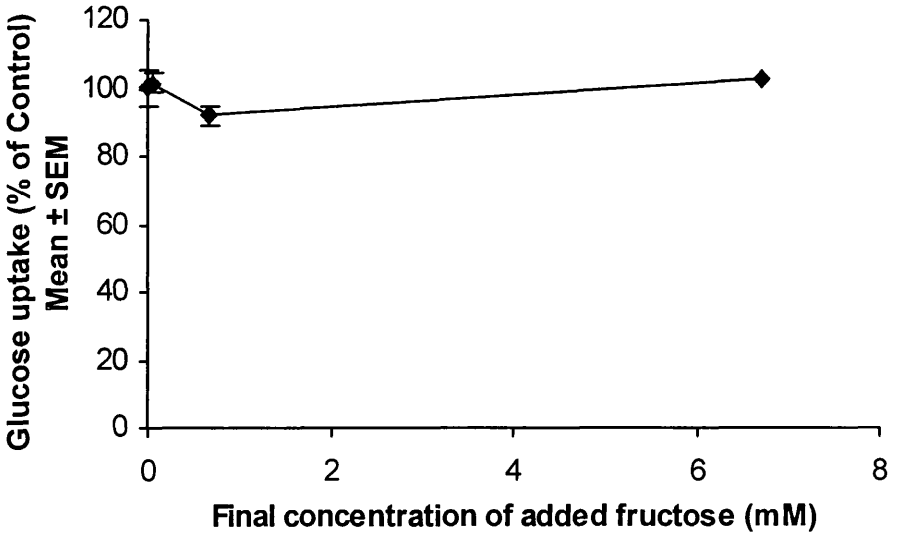


Fig. 3.9: Effect of added fructose on radiolabelled glucose uptake into BBMV.

Results are means of %control ± SEM taken from 4 data points. Glucose uptake of the control was 2134.39 ± 168.31 cpm (mean ± SEM).

3.7 Semi-quantification of glucose content in plant extracts

3.7.1 Introduction

The experiment described in this section was conducted to quantify the amount of glucose in the extracts found to have significant inhibition on the glucose uptake into BBMV, in order to determine whether or not the inhibition observed in the majority of the tested plant extracts (Table 3.2) was associated with additional glucose from the extracts. It was expected that a pronounced inhibition would be observed in the plants which contained a considerable amount of glucose.

The influence of which the plant extract glucose had on the uptake of labelled glucose was assessed based on the glucose content of the plants in conjunction with the effect of additional glucose on the uptake of labelled glucose (Fig 3.8). A number of approaches to determine the glucose content in plant extracts were considered, including enzymatic method by glucose oxidase and thin layer chromatography (TLC). In the glucose oxidase method, amount of glucose is determined from the intensity (at 405 nm) of the end product which is formed according to the following reaction.

$$\text{Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{o-Dianisidine} \xrightarrow{\text{Peroxidase}} \underset{\text{(Brown)}}{\text{Oxidized o-Dianisidine}}$$

The drawback of glucose oxidase method in this case was owing to the fact that the measurement is based on colorimetric method. Thus, it may not be suitable for plant extracts which, in most cases, have intense colour which can interfere with the determination. Quantification using TLC therefore appeared to be more appropriate in this case, since the content of glucose in the extracts could be determined without any interference by colour of the extracts.

3.7.2 Materials and methods

Unless specified, chemicals were purchased from Sigma-Aldrich Co. Ltd. (Irvine, UK). Silica GF_{254} TLC plate were purchased from Merck KGaA (Germany).

The content of glucose in the extracts was semi-quantified using thin layer chromatography (TLC) on silica gel GF_{254} coated with 0.1 M boric acid. This was achieved by spraying the plate with 0.1 M boric acid and allowing to dry at room temperature. Plant extracts (60 µl) were applied onto the TLC plate at a concentration of 5 mg/ml. The chromatogram was developed in ethyl acetate: propanol: water (65:35:20) and visualised by spraying with aniline-diphenylamine spray reagent, followed by heating on a thermoplate at 110 °C for 5-10 minutes (Lewis and Smith, 1969). The spray reagent was prepared by dissolving diphenylamine (2 mg), in acetone (80 ml), followed by an addition of aniline (2 ml) and adjusting to 100 ml with 95% ortho-phosphoric acid. The content of glucose in the extracts was determined approximately by visually comparing the intensity of the zones with those of glucose standard solutions (60 µl) at various concentrations (0.05, 0.1, 0.2, 0.25, 0.5, 1 and 2 mM) chromatographed on the same plate. The content of glucose (Table 3.3) was expressed as mM of glucose in incubation medium which was 1/3 of the value in the plant extract solution since 20 µl of extract was made up to a final volume of 60 µl (in the uptake experiment).

The content of glucose in the extracts (Table 3.3) together with the magnitude of inhibition caused by different concentrations of extra added glucose (Fig. 3.8) were then used to predict the percentage inhibition associated mainly to plant extract glucose. By comparing the predicted inhibition with the actual inhibition observed in the experiments (section 3.5.3), it could be determined whether the inhibition observed could possibly be due to other active compounds in the extracts. If the experimental inhibition was found to be greater than the predicted inhibition of which the determination was based on the influence of glucose in the extracts alone, the inhibition was likely to be due to active compounds in the extracts, other than glucose, that could inhibit the transport activity of the SGLT1.

3.7.3 Results and discussion

Table 3.3 shows the approximate content of glucose in the extracts determined using TLC, percentage inhibition predicted from added cold glucose and the actual inhibition observed in the experiments (section 3.5).

Table 3.3: Content of glucose in plant extracts found to have apparent inhibitory effect on the uptake of glucose, and the predicted and experimental inhibition caused by these extracts

Tested plants†	% Inhibition		
	mM of glucose in final concentration♣	Predicted*	Experimental (Mean ± SEM)
Alismataceae			
Alisma orientalis	0.17-0.33	~ 75%	62.0 ± 7***
Araliaceae			
Panax ginseng	< 0.07	~ 50%	67.6±3.0***
Campanulaceae			
Platycodon grandiflorum	0.08-0.17	~ 60%	55.1±0.9***
Celastraceae			
Salacia reticulata	0.02	< 20%	34.1±5.0***
Compositae			
Atractylodes lancea	0.08-0.17	~ 60%	57.8±0.4***
Cucurbitaceae			
Coccinia indica	~ 0.07	~ 50%	58.3±0.1***
Trichosanthes kirilowii	~ 0.07	~ 50%	31.7±9.2***
Gentianaceae			
Swertia spp.	~ 0.17	~ 70%	40.2±2.3**
Labiatae			
Salvia miltiorrhiza	~ 0.33	~80%	33.8±0.9**
Lardizabalaceae			
Parvatia spp.	0.08-0.17	~ 60%	71.1±5.0***
Leguminosae			
Pterocarpus marsupium	< 0.016	< 20%	35.9± 5.4***
Liliaceae			
Anemarrhena asphodeloides	~ 0.33	~ 80%	67.4 ± 2.6***
Polygonatum odoratum	0.08-0.17	~60 %	63.6±1.5***
Meliaceae			
Azadirachta indica	~ 0.33	~ 80%	71.9±1.9***
Menispermaceae			
Tinospora cordifolia	0.033	~ 40%	25.7±7.1**
Moraceae			
Morus alba	> 0.67	~ 90%	86.6±0.2***

Table 3.3 (continued)

Tested plants†	% Inhibition		
	mM of glucose in final concentration♣	Predicted*	Experimental (Mean ± SEM)
Piperaceae			
Piper longum	< 0.016	< 20 %	48.0 ±3.1***
Polyporaceae			
Ganoderma lucidum	~ 0.07	~ 50%	55.7±7.8***
Ranunculaceae			
Coptis chinensis	~ 0.08	~ 55%	71.6±6.8***
Rutaceae			
Murraya koenigii	0.07- 0.08	~ 50%	58.6±1.6***
Scrophulariceae			
Rehmannia glutinosa	0.08-0.17	~ 60%	70.6±5.4**
Scrophularia ningpoensis	0.17-0.33	~ 75%	60.4±1.8**
Solanaceae			
Lycium chinensis	< 0.016	< 20 %	53.1±8.4***

†Nominal identity is given, for authentication see section 2.3. ♣Expressed as the content of glucose in the final incubation medium which is 1/3 of the value in the plant extract at 5 mg/ml; *Extrapolated from the inhibition caused by extra added glucose at different concentration (Fig. 3.8).

It was found that the detection limit of glucose on TLC using the system described above was 0.05 mM (60 μl of standard glucose solution). If the extracts were found on TLC to contain this amount of glucose, it meant that there would be 0.0167 mM of extra glucose added to the incubating medium (i.e. diluted 3 times by other solutions in the assay) which, as suggested by Figure 3.8, would lead to the predicted inhibition of 20%.

Semi-quantification of glucose content in the extracts possessing significant inhibitory effect revealed 4 plants which contained negligible amount of glucose (not detectable on TLC, i.e. less than 0.05 mM in the extracts) i.e. *Lycium chinensis*, *Piper longum*, *Pterocarpus marsupium* and *Salacia reticulata*. This suggested that the amount of plant extract glucose added to the final incubation medium in these cases was less than 0.017 mM. If the effect observed in the 4 plants was due solely to plant extract glucose, it was expected that the inhibition observed

with these plants would be approximately 20% or less. However, this was not the case for these 4 extracts since the inhibition demonstrated by these plants was much greater than 20%; the observed inhibitions were 53%, 48%, 36% and 34% for *L. chinensis*, *P. longum*, *P. marsupium* and *S. reticulata*, respectively. The greater inhibitions demonstrated by these 4 extracts compared to those predicted (Table 3.3) indicated that the extracts potentially contained compounds which were capable of inhibiting the transport activity of SGLT1.

Besides these 4 plants, most of the extracts were found to contain a considerable amount of glucose (Table 3.3). The most potent extract, *Morus alba*, at final concentration of 1.67 mg/ml was found to contain glucose as high as 0.67 mM in the incubation medium. It was suggested, according to the predicted inhibition due to the extra added glucose (Fig. 3.8), that glucose at the concentration of 0.67 mM would approximately inhibit the uptake by 90% which was very similar to that observed in the uptake experiments with the extract of *M. alba* of which the actual inhibition observed in the experiment was 86%. In most of the extracts, the predicted values and those observed in the uptake experiments were found to be very close (Table 3.3), suggesting that the inhibition observed in the extracts containing considerable amount of glucose was likely to be due to the effect of plant extract glucose. However there were a few plants of which the inhibition observed in the experiments was nevertheless greater than the predicted values. They were *Coptis chinensis*, *Parvatia spp.* and *Rehmannia glutinosa*. These plants may therefore contain other active compounds that were capable of inhibiting intestinal uptake of glucose. To evaluate the inhibitory effect of these plants, the effect of sugar-free fractions of these extracts on BBMV was further investigated (section 3.8).

In some cases e.g. *Alisma orientalis*, *Trichosanthes kirilowii*, *Swertia spp.*, *Salvia miltiorrhiza*, *Anemarrhena asphodeloides* and *Scrophularia ningpoensis*, the observed percentage inhibition was less than the predicted values. These extracts may have contained Na^+ ion or other agents that promoted glucose uptake into the vesicles.

3.8 Desugaring process

3.8.1 Introduction

The studies described in this section were conducted to examine the effect of some of the plants whose experimental inhibitions were found to be greater than those predicted in order to determine whether the inhibitory effect observed in these extracts was owing to plant extract glucose or other active constituents. An approach taken in this study was to remove sugars from the extracts and investigate the effect of the sugar-free fractions on BBMV. The extracts selected for desugaring were *Morus alba*, *Coptis chinensis*, *Parvatia spp.* and *Rehmannia glutinosa*. *Morus alba* was selected because of its most potent inhibitory effect. *Coptis chinensis*, *Parvatia spp.* and *Rehmannia glutinosa* were selected because the greater inhibition was observed in the uptake experiments than would be predicted from the content of glucose in these extracts (section 3.7.3).

Desugaring process was conducted using Sephadex G-10. Sephadex G-10 (bead diameter: 40-120 μM (dry), 55-166 μM (wet)) is a gel suitable for size exclusion chromatography in which compounds are separated according to their sizes and molecular weights. Therefore, glucose which is a relatively small molecule compared to many other compounds in the extracts would spend longer time in the column and, thus, elute last.

3.8.2 Materials and methods

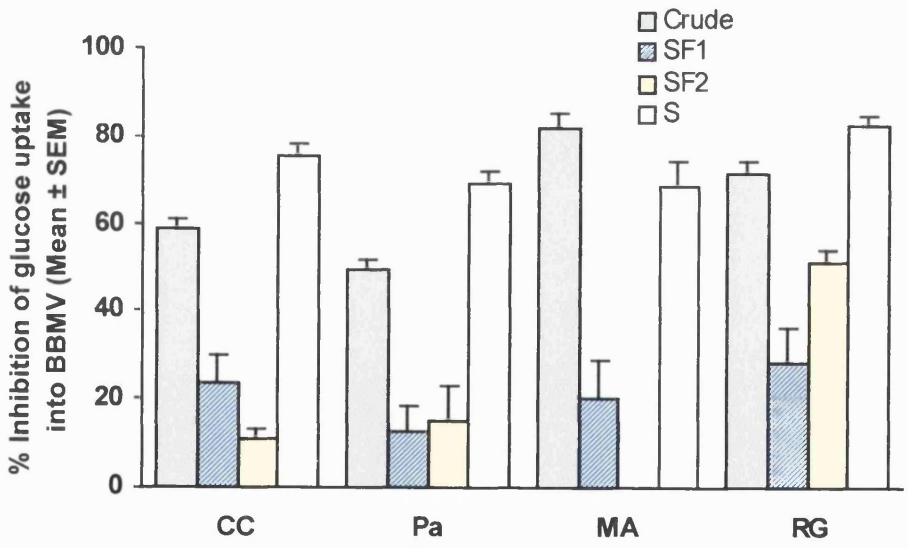
Unless specified, chemicals were purchased from Sigma-Aldrich Co. Ltd. (Irvine, UK). Glucose was removed from the selected plant extracts by passing the extracts through a Sephadex G-10 column. Dried plant extracts were reconstituted in distilled water (100 mg/ml), and 2 ml were applied to the column (2 × 15 cm). The column was eluted with distilled water, and 2-ml fractions were collected. The fractions eluted from the column were examined for the presence of glucose using TLC and the systems described in section (3.7.2). The fractions were pooled to yield two sugar-free fractions (SF1 and SF2) eluting before and after the sugars, respectively and a sugar containing fraction (S). The pooled fractions were evaporated

to dryness using a freeze dryer. The residue of the three fractions were reconstituted (5 mg/ml) in distilled water and tested on BBMV as described in section 3.5.2.

3.8.3 Results and discussion

By means of column chromatography on Sephadex G-10, sugars can be removed from crude extracts of the plants. Volume of water used in the elution of SF1 (sugar-free fraction eluted before glucose) was about 20 ml, the elution volume was approximately 25-35 ml for S (sugar containing fraction) and approximately 35-50 ml for SF2 (sugar free fraction eluted after glucose).

The investigation of the effect of glucose-containing and glucose-free fractions of *M. alba* showed that at the same concentration of 1.67 mg/ml (final concentration) glucose-free fraction was found to be much less active (20.3% inhibition) compared to crude extract (81.6% inhibition) and the sugar-containing fraction (68.5% inhibition) (Fig. 3.10). The marked loss of the activity after glucose was removed from *M. alba* extract suggested that the inhibition observed in crude



CC: *Coptis chinensis*; Pa: *Parvatia spp.*
MA: *Morus alba*; RG: *Rehmannia glutinosa*

Fig. 3.10: Effect of some plants before and after desugaring on glucose uptake

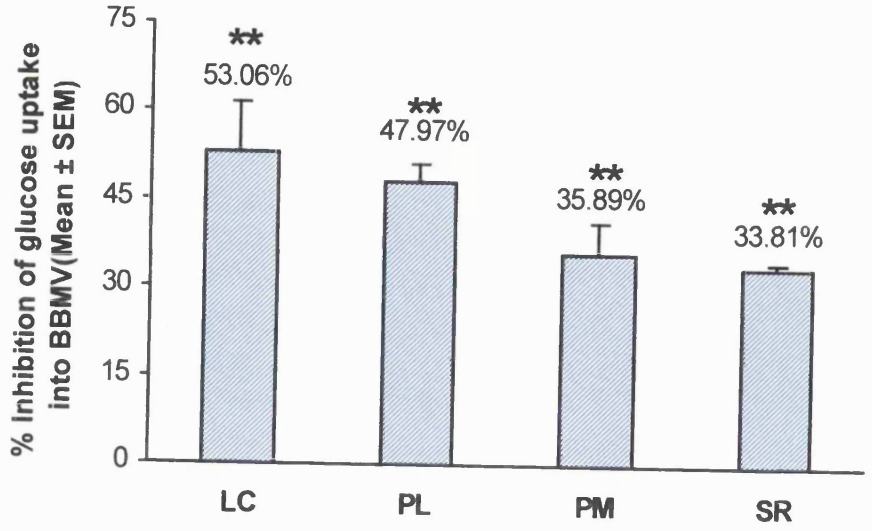
SF1: Fractions eluted from column before glucose; SF2: Fractions eluted from column after glucose; S: Sugar-containing fraction. Results are means of % inhibition ± SEM taken from 4 data points. Values of glucose uptake observed with control (water) was in a range of 1400-2800 cpm.

extract was solely due to glucose present in the extract. Similar results were also found in *C. chinensis* and *Parvatia spp.* extracts in which pronounced inhibitory effects were observed only in glucose-containing fractions of these plants. These extracts had virtually lost their activity after glucose was removed (Fig. 3.10), again suggesting that no other active compounds was involved. The weak activity observed in SF1 and SF2 for these three plants may have been due to the presence of residual glucose at below the detection limit on TLC. In case of *R. glutinosa*, the SF2 fraction did show around 50% inhibition of glucose uptake, although no glucose was detected. Thus *R. glutinosa* may contain some weak inhibitors of SGLT1.

3.9 General discussion and conclusion

Of the 31 plants tested, many showed artefactual inhibition of glucose uptake into BBMV, owing to glucose present in the extract (section 3.7.3). According to semi-quantification of glucose content in the extracts (section 3.7), TLC chromatogram revealed 4 plants which had negligible amounts of glucose (< 0.017 mM in the final solution) i.e. *Lycium chinensis*, *Piper longum*, *Pterocarpus marsupium* and *Salacia reticulata* but showed marked inhibition of glucose uptake into BBMV compared to control (Fig. 3.11). If glucose was solely responsible for the effect of these plants, the inhibition would be expected to be less than 20%. However, the inhibitory effect of these plants observed in the uptake experiments (Table 3.2) was found to be far greater than 20%. The inhibition observed in *Lycium chinensis*, *Piper longum*, *Pterocarpus marsupium* and *Salacia reticulata* was 53%, 48%, 36% and 34%, respectively (Fig. 3.11). This suggested that the inhibition was mainly not associated with glucose in the extracts, but due to other active compounds in these plants.

Since the most potent effect was found in the aqueous extract of *Lycium chinensis*, the plant was selected for further studies which involved fractionation and isolation of compounds responsible for the activity. These studies are described in Chapter 4.



LC: *Lycium chinensis*; PL: *Piper longum*

PM: *Pterocarpus marsupium*, SR: *Salacia reticulata*

Fig. 3.11: Effect on the glucose uptake into BBMV of the extracts containing low glucose content.

Results are means of % inhibition ± SEM from two separate experiments. The extracts were assayed with 4 replicates in each experiment. ** $p < 0.01$.

According to the literature survey (section 2.2), anti-diabetic activity of the crude extracts of *L. chinensis*, *P. longum*, *P. marsupium* and *S. reticulata* was also evident in *in vivo* models (section 2.2). In cases of *L. chinensis*, *P. longum and S. reticulata*, studies on their mechanism of action are still not extensive and none of these plant extracts has previously been reported to have an inhibitory effect on intestinal glucose absorption or improve oral glucose tolerance. In contrast, there were a number of studies focusing on anti-diabetic effect of *P. marsupium*, however attention was mostly given to the effect of epicatechin isolated from this plant on β-cells of pancreas. Despite extensive studies, reports on the effect of epicatechin are still controversial (section 2.2) and the precise mechanism of this compound remains to be clarified. In agreement with this current study, one of the studies showed that the ethyl acetate soluble part of an ethanolic extract of *P. marsupium* heartwood (250 mg/kg, p.o.) was able to inhibit an elevation of glucose level in

orally glucose-loaded diabetic rats (Ahmad *et al.* 1991a), suggesting that the extract may work, at least in part, by inhibiting intestinal glucose absorption.

It is feasible to assume that extracts which were able to prevent an elevation of glucose level in orally glucose-loaded animals possibly worked, at least in part, by inhibiting intestinal glucose absorption. According to literature survey (section 2.2), besides *P. marsupium*, other plant extracts which were found to exert glucose lowering effect in such models included *Azadirachta indica*, *Bixa orellana*, *Ganoderma lucidum*, *Polygonatum odoratum* and *Swertia chirayita* (section 2.2). However it was found, in the current study, that none of these plants inhibited glucose uptake into BBMV, suggesting that the effect observed in glucose-loaded animals of these plants was possibly owing to mechanisms other than inhibition of glucose transport activity of SGLT1.

CHAPTER 4

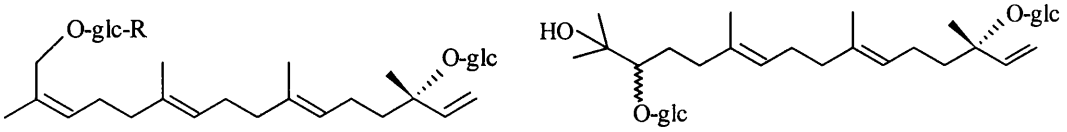
EFFECT OF *LYCIUM CHINENSIS* EXTRACTS ON INTESTINAL GLUCOSE ABSORPTION

4.1 Ethnopharmacology of *Lycium chinensis* Mill.

Lycium chinensis, commonly known as Chinese wolfberry, Chinese matrimony vine and box thorn, is a plant in suborder Solanineae, and family Solanaceae. The genus *Lycium* has been reported to consist approximately of 80-90 plant species (Evans, 1996). Different parts of *Lycium chinensis* have been used in traditional Chinese medicines for a number of disorders. Extracts from fruits and leaves of this plant have been quoted in traditional remedies for the treatment of atherosclerosis, diabetes, hypertension, liver and kidney disorders, night blindness and pulmonary tuberculosis (Duke and Ayensu, 1985b; Kim *et al.* 199/b). Dried root bark has been traditionally used as a tonic and reported to have hypotensive, hypoglycaemic and anti-pyretic activity in experimental animals (Funayama *et al.* 1995).

Anti-diabetic activity of the plant has not yet been extensively studied. Among a few studies, a study by Kim *et al.* (1994) reported that leaves and root bark of *L. chinensis* were found to have glucose-lowering effect in STZ-induced diabetic mice. To date, the precise mechanism by which the extract lowered blood glucose level and the compounds with anti-diabetic activity from this plant have not been identified.

Regarding phytochemical studies, compounds which were isolated from *L. chinensis* included acyclic diterpene glycosides (Fig. 4.1), cerebosides (Fig. 4.2), cyclic peptides, dipeptide (Fig. 4.3) and spermine alkaloids (Fig. 4.4). However, none of these compounds has been reported to have anti-diabetic activity.

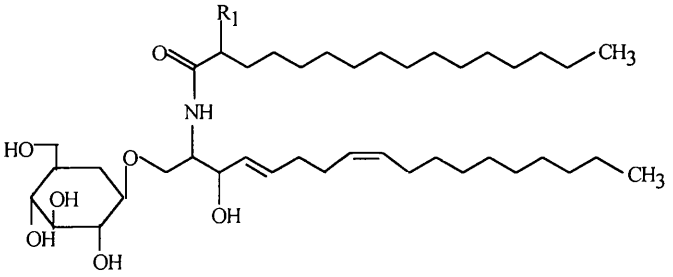


Lyciumoside I : R = H
Lyciumoside II : R = Glucose

Lyciumoside III

Fig. 4.1: Structure of Lyciumosides (acyclic diterpene glycosides isolated from *L. chinensis* root bark (Yahara *et al.* 1993))

Two cerebrosides isolated from fruits of *L. chinensis* i.e. 1-*O*-*β*-D-glucopyranosyl-(2*S*, 3*R*, 4*E*, 8*Z*)-2-*N*-palmitoyloctadecasphinga-4,8-dienine (Fig. 4.2a) and 1-*O*-*β*-D-glucopyranosyl-(2*S*, 3*R*, 4*E*, 8*Z*)-2-*N*-(2′-hydroxypalmitoyl) octadecasphinga-4,8-dienine (Fig. 4.2b) were reported to have antihepatotoxic activity against CCl_4-induced injury (Kim *et al.* 1997a).

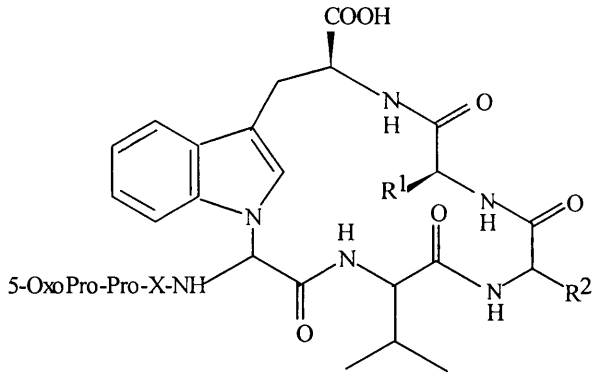


4.2 (a) $R_1 = H$

4.2 (b) $R_1 = OH$

Fig. 4.2: Structures of cerebrosides isolated from *L. chinensis* fruit

Lyciumin A and B (Fig. 4.3), cyclic peptides isolated from root bark of *L. chinensis*, were reported to have activity against angiotensin I-converting enzyme (ACE). Other cyclic peptides isolated from this plant included lyciumin C and D (Yahara *et al.* 1993).



Lyciumin A	X = Tyr	$R^1 = CH_2OH$	$R^2 = H$
B	X = Trp	$R^1 = CH_2OH$	$R^2 = H$
C	X = Tyr	$R^1 = CH_2OH$	$R^2 = CH_2Ph$
D	X = Tyr	$R^1 = CH(CH_3)CH_2CH_3$	$R^2 = H$

Fig. 4.3: Structure of lyciumin isolated from *L. chinensis*

One of dipeptides reported to be isolated from root bark of *L. chinensis* was lyciumamide (Fig. 4.4) (Noguchi *et al.* 1984). No pharmacological activities of this compound has been reported on the basis of literature survey using EMBASE and MEDLINE (1980-1999).

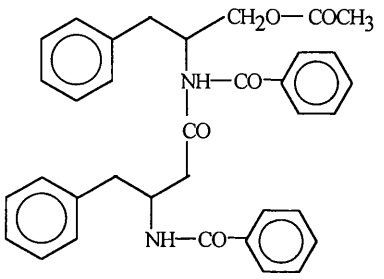


Fig 4.4: Structure of Lyciumamide

Kukoamine A and kukoamine B (Fig. 4.5) were spermine alkaloids isolated from root bark of *L. chinensis*. Kukoamine A was reported to be a hypotensive principle of this plant (Funayama *et al.* 1995).

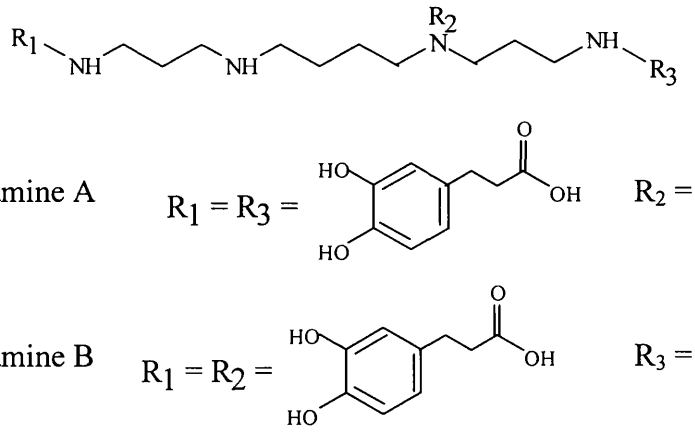


Fig. 4.5: Structure of Kukoamine

4.2 Effect of *Lycium chinensis* extracts on BBMV

4.2.1 Introduction

Among the 4 plants i.e. *Lycium chinensis, Pterocarpus marsupium, Piper longum*, and *Salacia reticulata* of which inhibitory effect on the uptake of glucose into BBMV was believed to be due to active compounds other than glucose (Table 3.3), *Lycium chinensis* was found to be the most potent extract. The initial studies

had employed the water extract of *Lycium chinensis* (section 3.5). In this study, the activity of other extracts of *Lycium chinensis* which included less polar and polar extracts i.e. hexane, chloroform and methanol extracts were investigated, since they may contain different constituents to the aqueous extract and/or a different content of the same constituents, which could provide more potent activity on intestinal glucose absorption.

4.2.2 Materials and methods

Unless specified all chemicals used in phytochemical studies were purchased from Sigma-Aldrich Co. Ltd. (Irvine, UK). Dried bark of *L. chinensis* was purchased from East-West Herbs (Kingham, UK) and The Institute of Chinese Medicine (London, UK). Silica GF_{254} TLC plate was purchased from Merck KGaA (Germany). Organic solvents were purchased from BDH Laboratory Supplies (England).

Dried bark of *Lycium chinensis* (1 kg) was sequentially extracted in a Soxhlet apparatus with hexane, chloroform and methanol (2.5 L, each) for 6-7 hours. The extracts were evaporated to dryness under negative pressure using a rotary evaporator to yield hexane, chloroform and methanol extracts. Water extract of *Lycium chinensis* was prepared as described in section 2.5.2.

The hexane, chloroform, methanol and water extracts were reconstituted in Tween 20 and diluted with distilled water to give a final concentration of 0.3% Tween 20 and 1.67 mg/ml extracts. The extracts were then tested on BBMV as described in section 3.5.2. The uptake of glucose into BBMV obtained with 0.3% Tween was regarded as 100% uptake and used as the control.

4.2.3 Results and discussion

The sequential extraction of dried *L. chinensis* bark resulted in an isolation of hexane, chloroform and methanol extracts with the yield of 4.50 g (0.45%), 4.50 g (0.45%) and 24.48 g (2.45%), respectively.

The investigation of the effect of hexane, chloroform ethanol and aqueous extracts (1.67 mg/ml final concentration) of *Lycium chinensis* showed that hexane extract was the least active extract by which the uptake was inhibited by

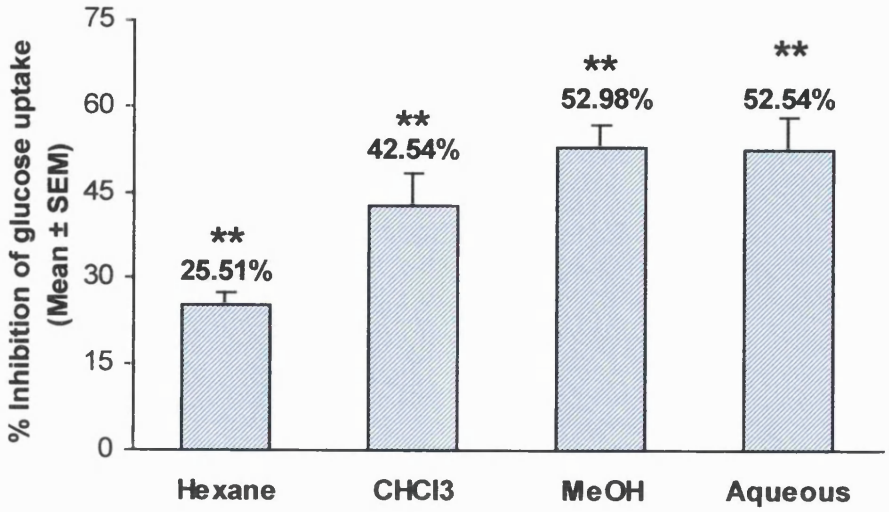


Fig. 4.6: Effect of hexane, chloroform, methanol and aqueous extracts of *Lycium chinensis* on glucose uptake into BBMV

Results are mean of %inhibition from two separate experiments ± SEM. n=4 (each experiment). Glucose uptake of control (Mean ± SEM) was 3546.64 ± 703.62 cpm. **P< 0.01 VS control.

25%. Chloroform extract was found to inhibit the uptake of glucose by 42 %, and the most potent effect was observed in methanol and aqueous extracts (Fig. 4.6). The inhibition observed in the two extracts were found to be very similar i.e. the inhibition was 52.98 % in methanol extract and 52.54 % in aqueous extract. Thus for further studies either of these two extracts would be the choice, however methanol extract was selected due to convenience in term of phytochemical work; being relatively less polar than the aqueous extract, it could be fractionated more readily on normal phase silica column.

4.3 Bioassay-guided fractionation of methanol extract of *Lycium chinensis*

4.3.1 Introduction

The methanolic *L. chinensis* extract was subjected to further studies which involved fractionation and isolation of compounds responsible for the activity observed in the extract. The methanol extract was subjected to further phytochemical investigation by means of column chromatography on silica gel. In

this case the silica gel formed a stationary phase which is relatively high in polarity compared to the mobile phase. With silica column, the separation of compounds was owing to an ability of silica gel to bind to compounds with relative high polarity, allowing low polarity compounds to be isolated from compounds with higher polarity. Silica column is always initiated with relatively low polarity solvent and changed to solvents with higher polarity. Based on this strategy, compounds which are less polar will be eluted first, polar compounds will bind to silica gel and elute with more polar solvent.

4.3.2 Materials and method

Unless specified, all chemicals were purchased from Sigma-Aldrich Co. Ltd. (Irvine, UK). Silica gel for column chromatography and Silica GF_{254} TLC plate were purchased from Merck KGaA (Germany).

4.3.2.1 Column packing

Silica column was packed by mixing the desired amount of silica gel with an appropriate solvent (100 g of silica in approximately 200 ml of the solvent) which was the first solvent used in the chromatography. The slurry of silica gel was then poured into a glass column. The column was tapped gently to promote uniform settling of the gel. Excess solvent was allowed to drain out, but the solvent level was always kept above the level of the gel to avoid column cracking. The slurry was added until a column of desired height was obtained. The column was then washed with additional amounts of the initial development solvent, after which the solvent was drained leaving only small volume of solvent on top of column.

For sample application, the extract was reconstituted in appropriate solvent which may be different from the first solvent used in the chromatography, and then mixed with an appropriate small amount of dry silica gel. The mixture was allow to stand at room temperature to dry to yield a free flowing powder. This was then slowly introduced to the top of column as a narrow zone with the aid of a funnel. The column was gently tapped to aid the settling down of the mixture of extract and silica gel. Thin layer of cotton wool was then put on top of the column to avoid disturbance of column

packing during an elution procedure. Solvent was introduced to the column using a Pasteur pipette and the elution commended.

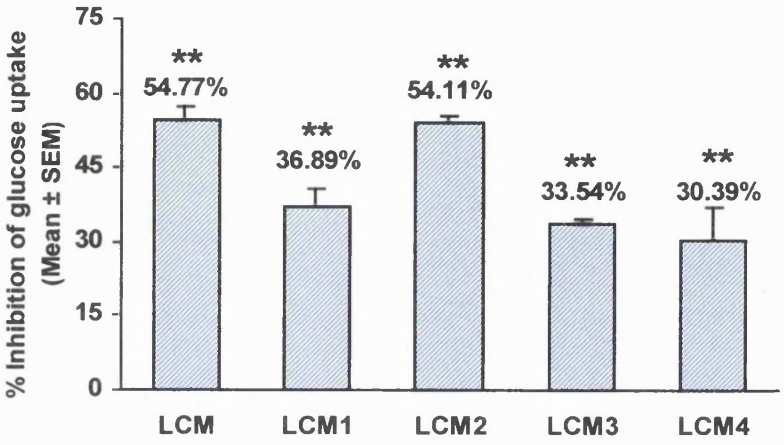
4.3.2.2 Fractionation and investigation of the activity of methanol extracts of *Lycium chinensis*

The methanol extract (20 g) of *Lycium chinensis* bark prepared as described in section 4.2.2 was subjected to chromatography on silica gel column (4 × 25 cm), and eluted with a gradient of chloroform and methanol. The elution was initiated with chloroform (100%), the proportion of methanol in the mobile phase was gradually increased to 10%, 30%, 50% and finally 70% (~1 L of each solvent mixture). Each fraction (100 ml) was collected and examined on TLC using silica gel GF_{254} as a stationary phase and a solution of ethyl acetate, propanol and water (13:7:5) as the mobile phase. The chromatogram was visualised under UV (254 and 365 nm) and by spraying with anisaldehyde spray reagent which was prepared by adding 0.5 ml of anisaldehyde to 10 ml glacial acetic acid, followed by the addition of 85 ml of methanol and 5 ml of concentrated sulphuric acid. Fractions with similar profile on TLC were pooled. The pooled fractions were evaporated to dryness under a negative pressure. To test on BBMV, the residues from each fraction were reconstituted in Tween 20 to give a final concentration of 1.67 mg/ml extracts and 0.3% Tween 20 which was also used as a control. The investigation on the effect of these fractions on glucose uptake into BBMV was performed according to the method described in section 3.5.2.

4.3.3 Results and discussion

Column chromatography of the methanol extract (LCM) on silica gel resulted in the separation of 4 fractions which were named LCM1, LCM2, LCM3 and LCM4. The yield of these fractions was 1.04 g (5.2%), 3.94 g (19.7%), 4.14 g (20.7%) and 2.18 g (10.9%) for LCM1, LCM2, LCM3 and LCM4, respectively. The effect of the 4 fractions (LCM1-LCM4) on BBMV is shown in Figure 4.7.

The investigation of the effect of the 4 fractions on BBMV showed that LCM1, LCM3 and LCM4 tested at 1.67 mg/ml (final concentration) were less active than crude methanol extract (LCM) at the same concentration. LCM2 was found to



LCM: Crude methanol extract

LCM1-LCM4: Fractions eluted from silica column

Fig. 4.7: Effect of methanol fractions of *L. chinensis* on glucose uptake into BBMV

Results are expressed as %inhibition of glucose uptake (Mean ± SEM). n = 4. Glucose uptake of control was 4357.17 ± 269.33 (mean ± SEM) cpm. **p < 0.01 VS control.

be the most active fractions among the four. The effect of LCM2 (54.11% inhibition) was comparable to that of crude methanol extract (54.77% inhibition). If the compounds in this fraction solely contributed to the activity of crude methanol extract, it was expected that the activity of LCM2 should be more potent than crude methanol extract since the fraction contained less compounds and was thus more concentrated. The comparable effects between that of LCM2 and that of crude methanol extract suggested that the activity of crude methanol extract was not entirely attributed to the compounds in LCM2. In addition, it was found that all the fractions (LCM1-4) have inhibitory effect on glucose uptake to some extent and these inhibitory effects were significantly different from the control (0.3% Tween 20), suggesting that all fractions contained compounds of slightly different polarity that could inhibit the uptake of glucose to different extent. Thus the effect observed in crude methanol extract was likely to be an addition or synergistic effect of more than one compounds.

Since the most potent effect was observed in LCM2, this fraction was selected for further phytochemical study.

4.4 Isolation of compounds from LCM2

4.4.1 Introduction

Isolation of compounds from LCM2 was conducted using column chromatography on silica gel (see section 4.3) and Sephadex LH20.

Sephadex is produced by cross-linking polysaccharide dextran with epichlorohydrin to produce a highly porous, sponge-like gel when swollen in water or organic solvent. Sizes of the pores in the gel is determined by an amount of water absorbed per gram which is, in turn, influenced by the degree of cross-linking (Sherma, 1972). When using Sephadex, the separation depends on the different abilities of components to enter pores of the stationary phase. At a given flow rate, very large molecules would spend less time in the stationary phase and move through the chromatographic bed quickly, whereas small molecular compounds would enter the pores of the stationary phase and hence spend more time in the column. On the basis of this mechanism, compounds with largest molecule will elute from the column first and the smallest molecule will elute last. The type of Sephadex used in this study was LH20 series (diameter: dry bead 18-111 μM, in methanol 27-163 μM) which is a lipophilic derivative of Sephadex that can be used in gel filtration chromatography in polar organic solvents .

4.4.2 Materials and methods

Unless specified, all chemicals used in phytochemical studies were purchased from Sigma-Aldrich Co. Ltd. (Irvine, UK). Silica gel for column chromatography were purchased from Merck KGaA (Germany).

4.4.2.1 Column packing

For the method of packing silica column see section 4.3.2.1. To pack Sephadex LH20 column, the gel was left to stand in an appropriate volume of the solvent to be used in the elution (50 g of dry beads in 200 ml of solvent) overnight to swell the beads. The slurry of Sephadex gel was slowly poured into a glass column and the solvent was drained to allow the gel to pack evenly, but the level of solvent was always kept above the level of the gel to avoid cracking. Once settled, the gel was washed with additional amount of the same solvent. To apply the extract onto the

column, the level of the solvent at the top first centimetre of the gel was lowered, the extract reconstituted in a minimal volume of solvent which was the same as that used for the elution was then introduced to the column using Pasteur pipette. Solvent was added to the column using Pasteur pipette and elution commenced.

4.4.2.2 Isolation of compounds

LCM2 was first fractionated using silica gel chromatography (Fig. 4.8). LCM2 (3.4 g) reconstituted in methanol was applied onto a silica column (2.5 × 20 cm) as described in section 4.3.2.1. The column was initially eluted with $CHCl_3$:MeOH (9:1, 500 ml), the proportion of MeOH in mobile phase was gradually increased to 20%, 25%, 30% and finally 40% (1 L each). Fractions eluting from the column were collected at volumes of 100 ml each. It was found in some of the fractions eluted with 90% $CHCl_3$ that there was white residue left after evaporation of the solvent. These fractions were pooled, the residue were then recrystalised by dissolving in methanol and the addition of a minimal volume of chloroform, and allowing to stand at 4 °C overnight. The residue obtained from

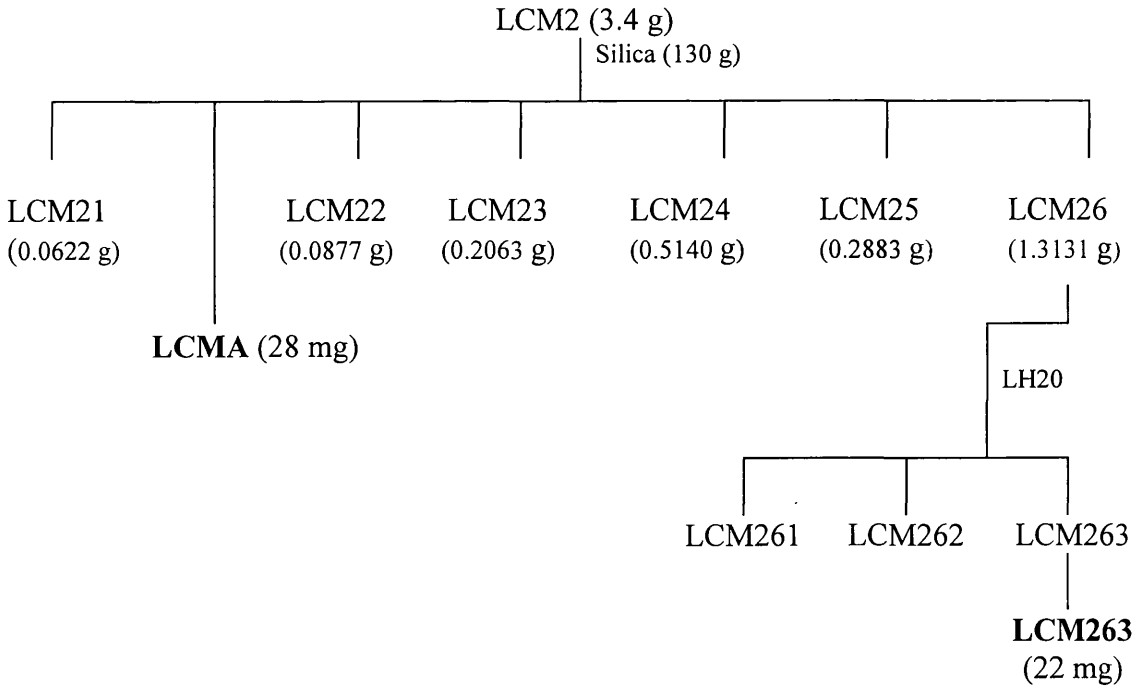


Fig. 4.8: Flow chart for fractionation and isolation of compounds from *L. chinensis* bark

recrystalisation was then washed with a mixture of $CHCl_3$:hexane (1:1). This resulted in the isolation of a pure compound which, on TLC, reacted with anisaldehyde reagent giving purple colour. The compound was named LCMA.

The remaining fractions eluted from the silica column were pooled into 6 fractions i.e. LCM21-26 according to their similarities in silica TLC chromatographic profile using $CHCl_3$:MeOH (8:2) as a mobile phase and visualisation under UV, followed by spraying with anisaldehyde reagent. LCM26 (~1 g) which was obtained with the highest yield was further fractionated on Sephadex LH-20 (1.5×25 cm) column using ethanol and water (8:2, ~700 ml) as a mobile phase, Fractions of 25-30 ml volume were collected. The fractions were examined on TLC using $CHCl_3$:MeOH (6:4) as mobile phase and visualised under UV, followed by spraying with anisaldehyde reagent. Those with similar profile were pooled into 3 fractions i.e. LCM261-263. After evaporation of solvent, LCM263 was found to contain a white residue. This residue was recrystalised by dissolving in methanol (~10 ml) followed by an addition of a few drops of chloroform, and allowing to stand overnight at 4 °C. Examination of the purity of this compound on TLC using the system as described above revealed one spot under UV (365 nm). This compound was named LCM263.

4.4.3 Results and discussion

Fractionation of LCM2 by silica gel and Sephadex LH20 resulted in an isolation of two compounds i.e. LCMA (28 mg) and LCM263 (22 mg). Details of structure elucidation of the two compounds are given in section 4.5.

4.5 Structure elucidation of LCMA and LCM263

4.5.1 Introduction

The structure of compounds isolated from methanolic *L. chinensis* extract (LCMA and LCM263) was elucidated by various types of spectroscopy, including nuclear magnetic resonance (NMR), mass spectrometry (MS), and to lesser extent, infrared spectroscopy (IR) and ultraviolet spectroscopy (UV).

In the application of nuclear magnetic resonance (NMR), structure is elucidated based on an ability of a molecule to absorb electromagnetic radiation in radio-frequency region. The absorption spectrum, which is a function of certain nuclei in the molecule, is known to be a characteristic feature of the compounds. A number of nuclei such as ^{19}F, ^{29}Si, ^{13}C, ^{1}H, ^{15}N and ^{31}P can be used in NMR for identification of organic compounds. The most extensive use lies in the use of proton (^{1}H) and carbon (^{13}C) nuclei. These two nuclei have a nuclear spin (I) of ½, thus in an applied magnetic field the nuclei can orient themselves in 2 ($2I$ + 1) ways i.e. a low energy orientation aligned with the applied magnetic field and a high energy orientation opposed to the applied field (Williams and Fleming, 1995).

When a radio frequency signal is applied to the system, nuclei in the low energy state will be promoted to high state energy if the radiofrequency applied matches the frequency at which the nuclei naturally precess in a given magnetic field (Williams and Fleming, 1995). The frequency at which each chemically distinct nucleus in a molecule comes into resonance in a given magnetic field is dependent on the environment experienced by each nucleus which is influenced by other nuclei in the neighbourhood. In practice, NMR is usually conducted with the addition of internal standard and a spectrum is expressed as the differences of the frequency (in Hz) of the peaks from an internal standard. The chemical shift (δ) scale is defined by:

$$\delta = \nu_s \text{ (Hz)} - \nu_{TMS} \text{ (Hz)/operating frequency (MHz)}$$

Internal standard which is most frequently used is tetramethylsilane (TMS). TMS is appropriate because it is chemically inert, volatile, soluble in most organic solvents, and it has only single sharp signal which comes to resonance at higher field than almost all carbon atoms in organic structures (Williams and Fleming, 1995). The sample size required for ^{13}C spectrum is about 50-100 mg, and for ^{1}H spectra about 1-10 mg dependent on sensitivity of an instrument. Choice of solvent for dissolving investigated compounds is dependent mainly on the solubility of compounds. However a solvent which does not give rise to signals in the NMR spectrum is preferable. The most commonly used solvents in NMR are $CDCl_3$, DMSO-d_6, CD_3OD and pyridine-d_5.

Besides proton (^{1}H), and carbon (^{13}C) nuclear magnetic resonance, various two dimensional NMR techniques have been developed and became powerful tools for structure elucidation of organic compounds, these include ^{1}H-^{1}H shift correlation spectroscopy (COSY), ^{1}H-^{13}C COSY (HMQC), long range ^{1}H-^{13}C COSY (Heteronuclear Multiple Bond Connectivity, HMBC), Total Correlation Spectroscopy (TOCSY) and Nuclear Overhauser effect Spectroscopy (NOESY).

Mass spectrometric data, in a conjunction with NMR DEPT (Distortionless Enhancement by Polarization Transfer), is a powerful tool in an establishment of the molecular weight of a molecule. In mass spectroscopy, compound is introduced into an ionization chamber and induced to vaporize into gas phase. The compound in gas phase is then bombarded at right angles to generate molecular ions and/or fragment ions, ions produced in this process are forced through accelerating slit where ions are separated according to their mass to charge ratio (*m/z*). The techniques commonly used to induce ionization are fast atom bombardment (FAB), electron impact (EI) and chemical ionisation (CI). With EIMS, the molecule is bombarded and ionized by means of a 70 eV electron giving rise to extensive fragmentation. The main disadvantage of EIMS is a frequent absence of molecular ion, and this can be overcome by the use of CI and FABMS. In an application of CIMS, molecular weight information is obtained from protonation of sample molecules by CH_5^+, whereas in FABMS molecules are bombarded by a beam of fast xenon atoms.

Another technique commonly used in structure elucidation of compounds is infrared spectroscopy, since each molecule has a unique infrared absorption spectrum. In infrared spectroscopy, the structural identification is based on the absorption of infrared radiation by different functional groups in a molecule at different regions. The molecule is exposed to infrared radiation of frequencies in a range from 10,000-100 cm^{-1}. The radiation is absorbed and converted by an organic molecule into energy of molecular vibration. The most useful range of IR in structure elucidation of organic molecules lies on a narrow range of 4000-625 cm^{-1}. Many functional groups in a molecule can be identified by their vibration frequencies in IR spectrum.

Ultraviolet (UV) spectrometry is one of the powerful tools available for determining the structure of organic compounds. The ability of an organic compound

to absorb ultraviolet and visible radiation is dependent on its electronic transition. The absorption results in an elevation of electron from orbitals in the ground state to higher-energy orbitals in an excited states which gives rise to readily measured and informative spectra. Organic compounds containing double or triple bonds generally exhibit useful absorption peaks in the accessible ultraviolet region which are characteristics of the functional groups. Molar extinction coefficient (ε) of a compound was calculated from the following formula:

$$\varepsilon = A/bc$$

A: Maximum absorption

b: path length of radiation (cm)

c: concentration mol/L

Degree of unsaturation which corresponds to number of double bonds and close ring in a molecule was calculated according to the following formula:

$$\text{Degree of unsaturation} = \text{No. of carbon atom} - \frac{(\text{No. of hydrogen atom} - 1)}{2} + 1$$

In this current study, structures of LCMA and LCM263 (section 4.4) were elucidated based on data obtained from NMR (^{1}H, ^{13}C spectra and COSY) and mass spectrometry, and to lesser extent, infrared spectroscopy and ultraviolet spectroscopy.

4.5.2 Materials and methods

Experiments on NMR were conducted using 400 MHz (^{1}H) and 100 MHz (^{13}C) on a Bruker AC-300 instrument equipped with standard Bruker software by Ms Jane Hawkes, NMR section Chemistry, Department, King's College University of London. Fast atom bombardment mass spectra (FABMS) and electron impact mass spectra (EIMS) were conducted using AutoSpec FAB^{+} and a Perkin-Elmer SCIX API-1 mass spectrometer, respectively at ULIRS Chromatography/Mass Spectrometry Service, Chemistry Department, King's College, University of London.

High resolution FABMS was conducted using a Zab-SE VG Analytical mass spectrometer at School of Pharmacy, University of London. Infrared (IR) spectroscopy were conducted using a Perkin-Elmer spectrophotometer (model 1605 FTIR) in potassium bromide (KBr). UV spectra were run in methanol using a Perkin-

Elmer spectrophotometer (model UV/VIS Lambda 2) at Pharmacy Department, King's College London.

The process of structure elucidation and identification followed the pattern below:

a) molecular formula was determined based on MS and NMR data
b) general type of structure was postulated based on the type of signal observed in the ^{13}C and ^{1}H spectra
c) literature on *Lycium chinensis* was examined to investigate possible identities for the compounds by a comparison of data obtained in this experiments with those reported in literature
d) the spectrum obtained in our experiments were elucidated to confirm that it matched the proposed structure.

4.5.3 Results

4.5.3.1 Structure elucidation of LCMA as sitosterol glucoside

UV, MS and NMR Spectra data for LCMA was given in Appendix 1.

LCMA was obtained as white amorphous powder. UV spectrum of LCMA in methanol exhibited maximum absorption at 204.2 nm with molar extinction coefficient of 1546.75. The compound appeared to have a molecular formula of $C_{35}H_{60}O_6$ on the basis of its MS and NMR data. FABMS exhibited a peak at 397 (23 %) which corresponded to the loss of a sugar moiety $[M-179]^+$. However, molecular peak at *m/z* 576 was not observed in FABMS.

Carbon and proton NMR spectra of LCMA are given in Table 4.1 and 4.2, respectively. It appeared that ^{1}H and ^{13}C NMR spectra of LCMA were very similar to those reported in literature for sitosterol (Table 4.1 and 4.2) (Goad and Akihisa, 1997). However, ^{1}H and ^{13}C NMR data suggested the presence of one sugar moiety in LCMA, indicating that LCMA was a glycoside of sitosterol.

^{1}H NMR spectrum of LCMA in CD_3OD exhibited two singlet signals at 0.70 and 1.03, three doublet signals at δ 0.82 (J = 6.5 Hz), 0.85 (J - 7.0 Hz), and 0.94 (J = 6.4 Hz) and one triplet signal at δ 0.85 (J = 8.5 Hz) due to steroidal methyl

groups. The assignment of these steroidal methyl protons was based on the values reported in literature for sitosterol (Goad and Akihisa, 1997). The two signals at δ 0.70 and 1.03 were appropriate to methyl groups attached to quaternary carbon since the signals did not couple to other proton and appeared in the spectra as singlets, the proton at 0.70 was therefore assigned as H-18 and δ 1.03 as H-19. The doublet signals were assigned as H-21 (δ 0.94), H-26 (δ 0.85) and H-27 (δ 0.82). The triplet signal at δ 0.85 was assignable to H-29, the signal was spilt into triplet due to the coupling with the methylene protons at H-28.

An olefinic proton was revealed on ^{1}H NMR spectrum as a broad doublet at δ 5.37 (H-6) with a coupling constant of 5.0 Hz. The assignment of this proton was based on its correlation in one bond ^{1}H-^{13}C COSY spectrum with carbon signal at δ 122.36 which was assigned as C-6 (see below). The spectrum revealed one doublet signal at δ 4.40 (J = 7.8 Hz) attributable to the anomeric proton of sugar moiety. The coupling constant of 7.8 indicated that the sugar moiety was in β configuration, the sugar moiety was assigned as β glucopyranosyl based on values reported in literature (Table 4.2) (Phoung *et al.* 1994).

^{13}C NMR spectrum in CD_3OD revealed 14 methine carbons, 12 methylene carbons, 6 methyl carbons and 3 quaternary carbons in accordance with sitosterol glucoside. Signals of carbon (each CH_3) in the upfield region of 12.02-19.49 were appropriate to steroidal methyl groups, these signals were assigned based on values reported in literature for sitosterol (Table 4.1) (Goad and Akihisa, 1997). Three signals were observed in the field lower than 100 ppm i.e. the signals at δ. 101.36 (CH), 122.36 (CH) and 140.55 (quaternary carbon). The signal at 101.36 was assignable to an anomeric carbon (C-1′) based on the C-H correlation in the one bond ^{1}H-^{13}C COSY spectrum of this carbon signal with the anomeric proton of sugar moiety which came into resonance at δ 4.40 (J = 7.8 Hz) in ^{1}H NMR (Table 4.1).

Signal of carbon in downfield region of δ 79.35 was appropriate to signal of carbon attached to an oxygen atom. Additionally, this carbon signal was found in long range ^{1}H-^{13}C COSY spectrum to correlate with the anomeric proton, and its corresponding proton (δ 3.58-3.63) was found in ^{1}H-^{1}H COSY spectrum to correlate with proton signals at δ 1.5-2.5, the latter information suggested that the carbon

belonged to aglycone rather than sugar moieties of which the proton signals normally appear at the downfield region of δ 3-4. Taking these information together, the carbon at δ 79.35 was assigned as C-3, the position where the sugar moiety is attached to the aglycone. Consequently, the proton at δ 3.58-3.63 (br m, J = 6.8, 11.2 Hz) which exhibited a correlation with this carbon signal (δ 79.35, C-3) in one bond ^{1}H-^{13}C COSY spectrum was assigned as H-3. Based on a correlation of H-3 (δ 3.58-3.63) and proton signal at 2.40-2.43, the latter signal was assigned as H-4 and the carbon (δ 38.90) which this proton attached to, according to one bond ^{1}H-^{13}C COSY spectrum, was assigned as C-4. Moreover, H-3 also showed a correlation (^{1}H-^{1}H COSY) with proton in a region of 1.86-2.04, leading to an assignment of the latter as H-2 and carbon (δ 29.81) of which this proton attached to as C-2.

The signals at δ 122.36 (CH) and 140.55 (C) indicated the presence of olefinic carbons. Additionally, the carbon signal at δ 140.55 was found in ^{1}H-^{13}C long range COSY spectrum to exhibit a three bond correlation with proton signal at δ 1.03 (*s*, 3H) which was assigned as H-19, leading to an assignment of the carbon signals at δ 140.55 as C-5, and another olefinic carbon at δ 122.36 as C-6. Assignment of quaternary carbon at 36.94 as C-10 was based on the correlation of this carbon to H-19 (δ 1.03) in long range ^{1}H-^{13}C COSY spectrum.

Long range correlation (^{1}H-^{13}C COSY) revealed three bond correlation between the methyl proton at δ 0.70 which was assigned as H-18 and the carbon signals at δ 40.00 (CH_2), and 56.28 (CH), allowing the assignment of these carbon signals as C-12 and C-17, respectively. Moreover, the same proton (δ 0.70, H-18) showed a two bond correlation with carbon signal at 42.55 (C) which was therefore assignable to C-13. The signals of carbon at δ 34.17 (CH_2) and 36.40 (CH) were assigned as C-22 and C-20 based on their long range correlation with the proton at δ 0.94 (H-21). The chemical shift of C-2, C-3 and C-4 of LCMA was found to be slightly different from those reported in literature for sitosterol (Goad and Akihisa, 1997), carbons at position 2 and 4 came into resonance at further upfield region compared to those reported for sitosterol whereas C-3 came into resonance at further downfield region. This was possibly due to an influence of sugar moiety and the different solvent used.

According to the proposed molecular formula ($C_{35}H_{60}O_6$), LCMA was found to have degree of unsaturation of 6 which matched the structure of sitosterol glucoside which comprised of 5 closed rings and one double bond. The structure of LCMA was therefore established as sitosterol glucoside.

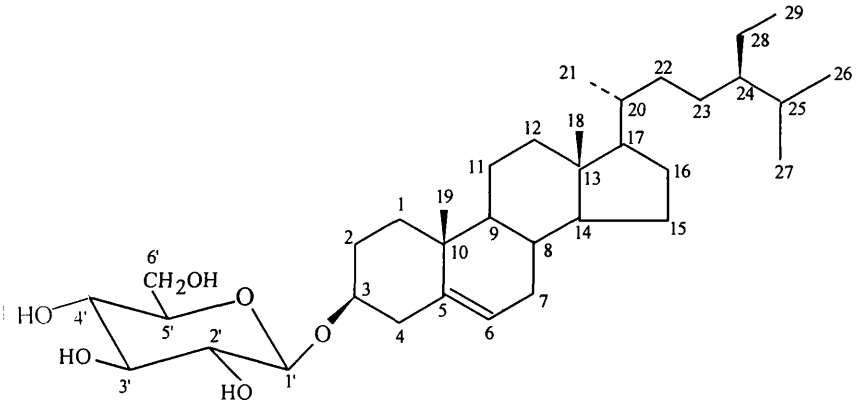


Fig. 4.9: Structure of LCMA (sitosterol glucoside)

Table 4.1: ^{1}H NMR spectrum data of LCMA in CD_3OD

Proton	δ (ppm)*	Reported Value
H-1	1.86-2.04	
	1.05-1.25	
H-2	1.86-2.04	
H-3	3.58-3.63 (*m*, *J* = 6.8, 11.2 Hz)	3.52 (*tt*, *J* = 5.1, 11.7)[a]
H-4	2.40-2.43	
H-5	-	
H-6	5.37 (*br d*, *J* = 5.0 Hz)	5.35 (*br d*, *J* = 5.1)[a]
H-7	1.86-2.04[a]	
H-8	1.47-1.70[a]	
H-9	Overlapped with H-21	
H-11	1.47-1.70	
H-12	1.05-1.25	
H-14	Overlapped with H-19	
H-15	1.47-1.70	
H-16	1.86-2.04	
H-17	1.05-1.25	

Table 4.1 (continued)

Proton	δ (ppm)*	Reported Value
H-18	0.70 (*s*)	0.68 (s)[a]
H-19	1.03 (*s*)	1.00 (s)[a]
H-20	1.47-1.70	
H-21	0.94 (*d*, *J* = 6.4 Hz)	0.92 (*d*, *J* = 6.6)[a]
H-22	Overlapped with H-19	
H-23	1.05-1.25	
H-24	Overlapped with H-21	
H-25	1.47-1.70	
H-26	0.85 (*d*, *J* = 7.0 Hz)	0.83(*d*, *J* =7.3)[a]
H-27	0.82 (*d*, *J* = 6.5 Hz)	0.81 (*d*, *J* =7.3)[a]
H-28	1.27-1.33	
H-29	0.85 (*t*, *J* = 8.5 Hz)	0.84 (*t*, *J* =7.6)[a]
3-*O* Glucoside		
H-1′	4.40 (*d*, *J* = 7.8 Hz)	4.41 (*J* = 7.6)[b]
H-2′	3.21 (*dd*, *J* = 7.8, 8.6 Hz)	3.24 (*J* = 7.3)[b]
H-3′	3.37 - 3.42(*dd*, *J* = 6.2, 8.6Hz)	3.43 (*J* = 6.7)[b]
H-4′	3.37-3.42	3.45 (*J* = 9.2)[b]
H-5′	3.28-3.30	3.3 (*J* = 4.8)[b]
H-6′	3.72 (*dd*, *J* = 5.1, 11.9 Hz)	3.76 (*J* = 3.0)[b]
	3.86 (*dd*, *J* = 2.4, 11.9 Hz)	3.85 (*J* = 12.0)[b]

*Range within which signal lay has been given in cases where overlapping signals were found. [a]Values reported for sitosterol in $CDCl_3$ (Goad and Akihisa, 1997), [b]Values reported for glucoside of sitosterol in $CDCl_3/CD_3OD$ (Phoung *et al.* 1994)

Table 4.2: ^{13}C NMR spectrum data of LCMA

Carbon	δ ^{13}C (ppm)	DEPT	Reported Value*
C-1	37.51	CH_2	37.27
C-2	29.81	CH_2	31.64
C-3	79.35	CH	71.77
C-4	38.90	CH_2	42.29

Table 4.2 (continued)

Carbon	δ ^{13}C (ppm)	DEPT	Reported Value*
C-5	140.55	C	140.76
C-6	122.36	CH	121.69
C-7	32.17	CH_2	31.92
C-8	32.13	CH	31.92
C-9	50.44	CH	50.15
C-10	36.94	C	36.51
C-11	21.30	CH_2	21.10
C-12	40.00	CH_2	39.80
C-13	42.55	C	42.33
C-14	57.00	CH	56.78
C-15	24.52	CH_2	24.31
C-16	28.49	CH_2	28.25
C-17	56.28	CH	56.08
C-18	12.02	CH_3	11.89
C-19	19.49	CH_3	19.39
C-20	36.40	CH	36.16
C-21	18.94	CH_3	18.80
C-22	34.17	CH_2	33.96
C-23	26.23	CH_2	26.11
C-24	46.09	CH	45.85
C-25	29.35	CH	29.18
C-26	19.13	CH_3	19.05
C-27	19.94	CH_3	19.82
C-28	23.27	CH_2	23.08
C-29	12.09	CH_3	11.99
O-3 Glucose			
C-1′	101.36	CH	
C-2′	73.81	CH	
C-3′	76.73	CH	

Table 4.2 (continued)

Carbon	δ ^{13}C (ppm)	DEPT	Reported Value*
C-4′	70.45	CH	
C-5′	76.12	CH	
C-6′	62.05	CH_2	

* Values reported for sitosterol in $CDCl_3$ (Goad and Akihisa, 1997)

4.5.3.2 Structure elucidation of LCM263

UV, MS and NMR spectrum of LCM263 are given in Appendix 2. High resolution FABMS of LCM263 revealed fragment peaks at 763 (25 %), 442 (43 %), 420 (50 %) and 329 (100%). UV spectrum of LCM263 in methanol showed a maximum absorption at 290 (Band II) and 323 (Band I) nm. The compound underwent bathochromic shift of the 323 nm band to 358 nm with the addition of $AlCl_3$ (1 %), the bathochromic shift was reversed by an addition of 3N HCl. The bathochromic shift upon the addition of $AlCl_3$ is known to be a characteristic feature of a flavonoid-type compound with free hydroxyl group at C-3 or C-5, as a result of a formation between 4-keto-3-hydroxyl or 4-keto-5-hydroxyl with $AlCl_3$ (Markham, 1989). Ortho dihydroxyl groups also give bathochromic shift with $AlCl_3$, but can be distinguished from the former by the instability in 3N HCl. The data obtained from UV spectrum of LCM263 indicated that the compound might be a flavonoid with *o*-dihydroxyl groups.

However, ^1H NMR spectrum of LCM263 revealed that besides three signals at δ 2.50, 3.37 and 7.34 which were likely to be the signals of solvents ($CDCl_3$ and DMSO-*d6*), no other signal was found in the ^1H spectrum despite it being present at high concentration. It was unclear why the compound exhibited in UV spectrum the characteristic shift of flavonoid upon the addition of $AlCl_3$, but was unable to be detected in ^1H NMR. One possibility was that LCM263 was a mixture rather than a single compound which contained trace amount of flavonoid but the main constituent of this mixture might not have been able to give NMR signal (e.g. some inorganic compounds). The trace amount of flavonoid gave rise to absorption in UV spectrum but was not enough to be detected in NMR.

4.6 General discussion and conclusion

Fractionation and isolation of compounds from active methanolic extract of *Lycium chinensis* resulted in an isolation of one pure compound i.e. sitosterol glucoside and one component whose identity was unknown. It was planned to investigate the effect of sitosterol glucoside on intestinal glucose uptake into BBMV. However, due to inadequate facilities and a restriction upon the use of animal in new campus (Franklin-Wilkins Building) at the time of this work, it was not possible to set up the experiment and test the compound on BBMV.

A link between β-sitosterol glucoside and anti-diabetic activity has previously been demonstrated in the studies by Ambike and Rajarama-Rao, (1967), Ivorra *et al.* (1988) and Ivorra *et al.* (1990) of which the compound was reported to be active constituent of *Ficus religiosa* (Moraceae) and *Centaurea seridis* (Compositae). In the study by Ambike and Rajarama-Rao (1967), the effect of sitosterol glucoside was evident in normal rabbits which reached a maximum when given at 25 mg/kg orally and at 5-7.5 mg/kg intravenously. Regarding the mechanism of action of sitosterol glucoside, it was showed that oral treatment with the compound was associated with increased insulin level in normal and hyperglycaemic rats (Ivorra *et al.* 1988). Moreover, sitosterol glucoside was also reported to improve glucose tolerance in glucose-loaded animals (Ivorra *et al.* 1988), suggesting the potential effect of sitosterol glucoside as inhibitor of intestinal glucose absorption.

Besides stiosterol glucoside, a number of phytosterols have previously been related to anti-diabetic effect in some of medicinal plants. Fagasterol isolated from *Phyllanthus emblica* (Euphorbiaceae) was reported to elicit anti-diabetic effect in normal and alloxan-induced diabetic mice (Cuellar and Estevez, 1980). Anti-diabetic effect of β-sitosterol isolated from *Coffea arabica* (Rubiaceae) was evident in normal and diabetic mice (Sampaio *et al.* 1979). A mixture of glucosides of sitosterol and stigmastadienol was reported to be active constituents of *Momordica charantia* (Lotlikar and Rajarama Rao, 1966).

CHAPTER 5

BIOLOGICAL STUDIES OF SELECTED PLANTS ON INSULIN SECRETION

5.1 Regulation of insulin secretion

The unique feature of β-cells is that its signal transduction for nutrient-induced insulin secretion begins with the metabolism of major secretagogues instead of their interaction with specific cell surface receptors as found in excitatory events of other tissues (Ashcroft and Ashcroft, 1992). The biological molecules which are capable of initiating insulin release include glucose, leucine, glyceraldehyde and the methyl ester of succinic acid (MacDonald and Fahien, 1988; MacDonald, 1990). Among them, glucose is the most potent physiological insulin secretagogue. Indeed, glucose is the only nutrient secretagogue which is capable of initiating the release of insulin at concentrations within its physiological range (Prentki *et al.* 1997).

Glucose is taken up into β-cells by a low affinity facilitative glucose transporter known as GLUT2 (Mueckler, 1994), and then phosphorylated to glucose-6-phosphate by both a high affinity hexokinase (K_m ~ 0.05 mM) and a low affinity glucokinase (K_m ~ 5-10 mM), also known as hexokinase IV or hexokinase D, (Ashcroft and Ashcroft, 1992). Hexokinase appears to be saturated with substrates at all physiological glucose concentrations due to its high affinity (Ashcroft and Ashcroft, 1992) whereas the activity of glucokinase varies with normal physiological fluctuations in glucose concentration. Thus, the role of hexokinase in glucose phosphorylation in β-cells is expected to be much less significant than that of glucokinase whose role is well accepted as a rate-limiting glucose sensor for glucose-induced insulin secretion (Matchinsky, 1990). Unlike hexokinase, the activity of glucokinase is not inhibited by glucose-6-phosphate (Lenzen and Panten, 1988b; Lenzen, 1992). Glucose-6-phosphate is then processed by glycolysis (Emden-Myerhof pathway) and oxidative phosphorylation in mitochondria (Krebs cycle or tricarboxylic acid (TCA)) (Fig. 5.1). Glycolysis and mitochondrial metabolism of glucose lead to multiple alterations in metabolic states, including stimulation of malonyl coenzyme A-synthesis (Fig. 5.1) (Corkey *et al.* 1989; Prentki *et al.* 1997), changes in the ratio of ATP/ADP and redox state (conversion of NADP to NADPH), and generation of phospholipid metabolites (MacDonald, 1990; Holz and Habener, 1992).

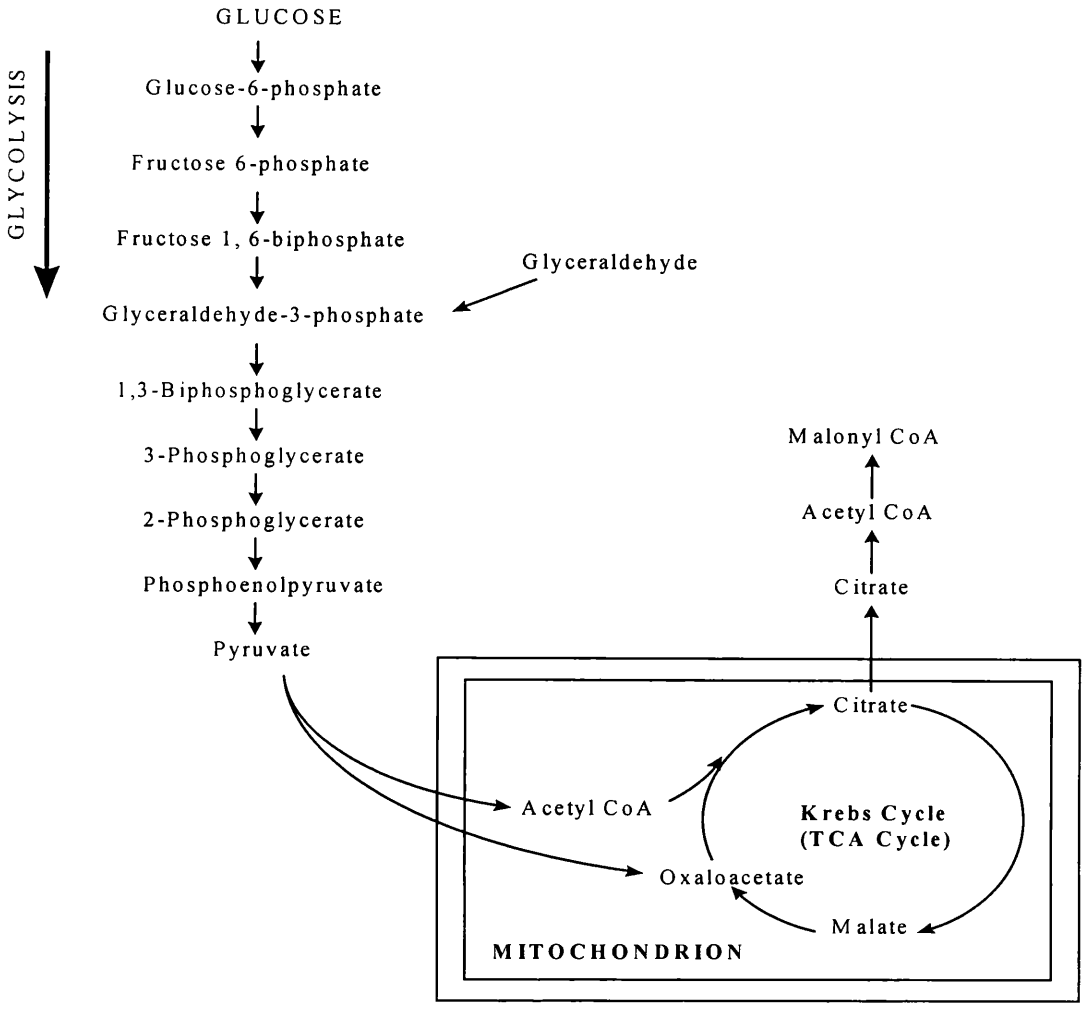


Fig. 5.1: Cellular metabolism of glucose via glycolytic pathway and Krebs cycle

The observation that glyceraldehyde, which enters glycolysis pathway at the level of triose phosphates (Fig. 5.1), is a potent insulin secretagogue suggests that the first part of glycolytic pathway (from glucose transport to the triose phosphate level) may not be critical in generating signals required for glucose-induced insulin release (MacDonald and Fahien, 1988; MacDonald, 1990). On the other hand, mitochondrial metabolism appears to be of importance in the signal transduction of glucose-induced insulin release, since an inhibition of oxidative phosphorylation has been reported to abolish the action of glucose completely (MacDonald, 1990). However, it has been demonstrated that mitochondrial metabolism alone is not sufficient to account for the action of glucose, since pyruvate which is a final metabolite of glycolysis and thus the first metabolite to enter mitochondrial metabolism, fails to stimulate insulin secretion (MacDonald,

1990). It is, therefore, likely that glycolysis has functional roles in insulin secretion beyond substrate supply for the Krebs cycle. An interaction between extramitochondrial signals produced by the glycolytic pathway and intra-mitochondrial signal is therefore required for a normal secretory response to glucose (MacDonald, 1990; Prentki *et al.* 1997). Interestingly, the interaction of extramitochondrial events with intramitochondrial events does not appear to be critical for secretory response to other secretagogues such as leucine and methyl ester of succinic acid (MacDonald, 1990), since both compounds are still capable of stimulating insulin secretion (~ 50% as insulinotropic as glucose) despite the fact that both are metabolized entirely inside mitochondria (MacDonald and Fahien, 1988; MacDonald, 1990).

It is well accepted that the stimulation of insulin secretion by glucose ultimately results in the closure of ATP-regulated potassium channels in the membrane of β-cells (Ashcroft and Ashcroft, 1992). The generation of ATP which is the main regulator of the K^+ channels by glucose metabolism has been hypothesised as a key event that links glucose utilization to more distal events in the secretory sequence (Ashcroft and Rorsman, 1990). The closure of K^+ channels leads to a reduction of K^+ efflux and subsequent depolarisation of membrane potential from an initial resting value of -70 mV to a value approximately of -30 mV (Holz and Habener, 1992). The depolarization of the cell membrane triggers an opening of voltage-dependent Ca^{2+} channels (VDCC) and subsequent entry of Ca^{2+} into the cells. The importance of Ca^{2+} influx in insulin secretion has been underlined by the observation that the release of insulin evoked by glucose and other secretagogues was completely abolished when extracellular Ca^{2+} was omitted from incubating medium (Prentki and Matchinsky, 1987; Howell *et al.* 1994). The increased intracellular level of Ca^{2+} is believed to trigger a discharge of insulin granules (Fig. 5.2) by activating the microtubule-microfilament system for exocytosis. Ca^{2+} also acts as a messenger to activate other transducing systems, including adenylate cyclase, protein kinase C (PKC) and phospholipase C (PLC) (Fig. 5.2; for review see Zawalich and Rasmussen, 1990).

Nonetheless, it remains uncertain whether ATP is the only glucose-derived signalling molecule which accounts for the action of glucose (Prentki *et al.* 1997). Other candidates proposed as metabolic coupling factors are malonyl CoA and long chain acyl CoA esters (Prentki, 1999). More evidence is required to confirm the physiological significance of these candidates.

Recently, it has been suggested that a simple elevation of Ca^{2+} may not be sufficient to fully account for nutrient-induced insulin secretion, since the increase in intracellular Ca^{2+} induced either by Ca^{2+} ionophores or KCl-induced depolarization does not fully reproduce typical biphasic response as seen with glucose (Komatsu *et al.* 1997). Additionally, it was reported that insulin secretion induced by K^+ which maximally increases intracellular Ca^{2+} was quantitatively and qualitatively different from that induced by glucose. Secretion in response to K^+ was reported to be transient whereas that observed with glucose was sustained (Hedeskov, 1980; Prentki *et al.* 1997). In 1992, there were a number of papers demonstrating the existence of a glucose signalling pathway which is K^+_{ATP} channel-independent and does not increase intracellular Ca^{2+} (Best and Yale, 1992; Gembal *et al.* 1992, Salo *et al.* 1992). The studies demonstrated that even in the situation when the closure of K^+_{ATP} channel was not possible due to the effect of diazoxide (an agent that selectively opens K^+_{ATP} channel) glucose was still able to amplify the effect of high K^+ (30 mM) on insulin secretion (Best and Yale, 1992; Gembal *et al.* 1992, Salo *et al.* 1992). It has been proposed that the newly identified pathway possibly acts by amplifying the triggering signal induced by glucose, thereby working in synergy with the classic pathway (K^+_{ATP}- and Ca^{2+}-dependent). While the K^+_{ATP} channel-dependent pathway provides an elevation of Ca^{2+}, K^+_{ATP} channel-independent pathway augments the response to increased Ca^{2+} (Komatsu *et al.* 1997). To date, there is still a number of questions regarding this newly identified pathway which remains to be resolved. Further studies on the physiological significance of the pathway are still necessary. The role of individual mediators in the signal transduction of insulin secretory pathway are discussed in the following sections.

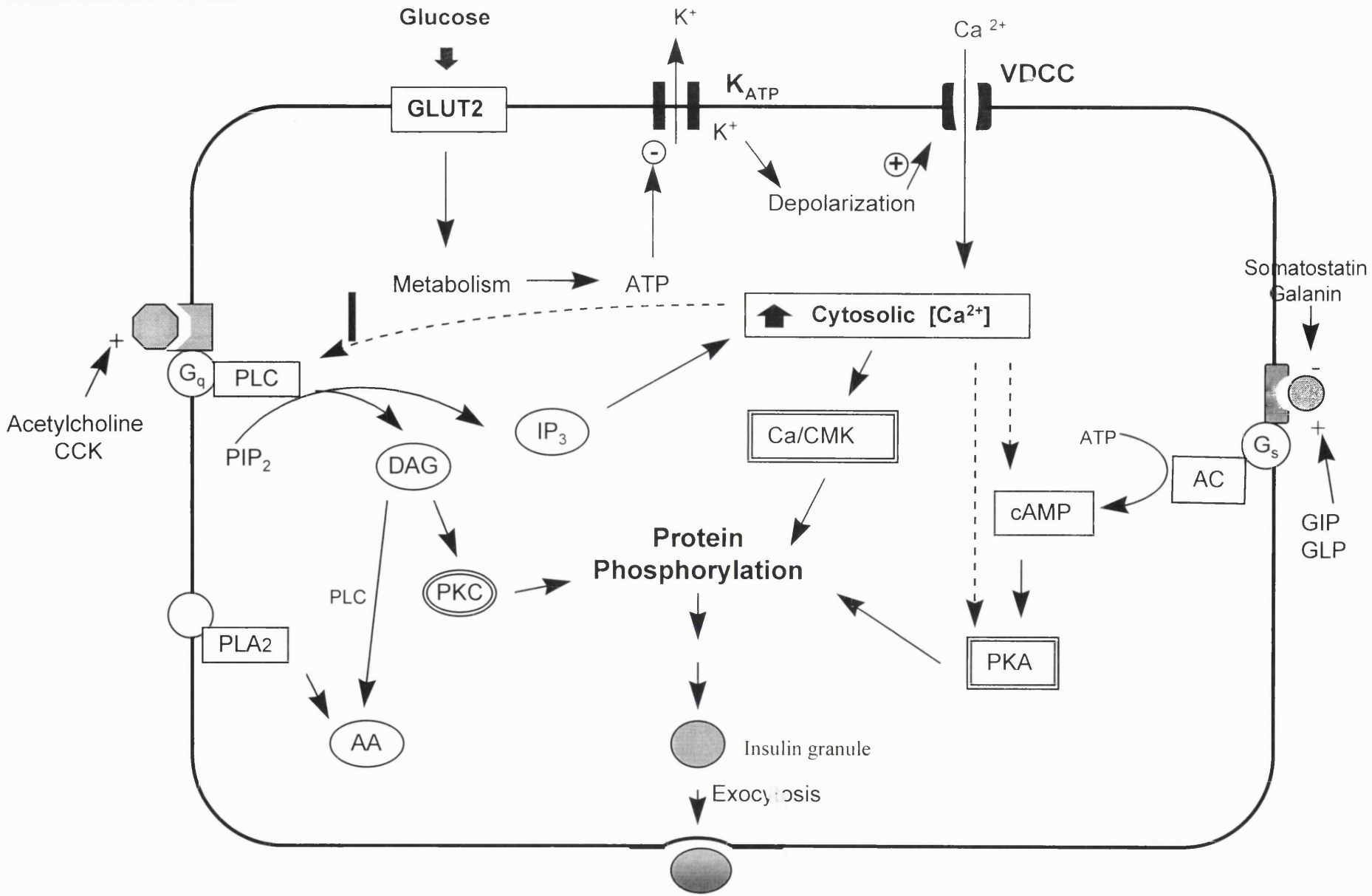


Fig. 5.1: Schematic of intracellular signal transduction pathway of insulin secretion in the pancreatic β cell

5.1.1 Role of cyclic -3', 5'- adenosine monophosphate (cAMP) and protein kinase A (PKA)

It has long been known that exposure of islets to a stimulatory concentration of glucose and other secretagogues such as dihydroxyacetone and glyceraldehyde causes a modest but significant increase in intracellular content of cAMP (Grill and Cerasi, 1973; Sharp, 1979). Although the mechanism by which glucose promotes the accumulation of cAMP has not been fully clarified, it is clear that the elevation of cAMP by glucose is secondary to increased Ca^{2+} level, since glucose failed to increase cAMP level in the absence of extracellular Ca^{2+} (Charles *et al.* 1975; Hughes and Ashcroft, 1992). It has been proposed that intracellular Ca^{2+} in combination with calmodulin possibly promotes the accumulation of cAMP by co-ordinate control of adenylate cyclase and phosphodiesterase enzymes which are key enzymes in synthesis and breakdown of cAMP, respectively (Prentki and Matschinsky, 1987). This hypothesis is widely accepted, however it may not be the only mechanism involved; there is a possibility that other mechanisms may participate in glucose-induced accumulation of cAMP (reviewed by Hughes and Ashcroft, 1992).

It is well established that agents which are capable of increasing cAMP in pancreatic β-cells such as forskolin (adenylate cyclase activator) and isobutylmethylxanthine (IBMX, phosphodiesterase inhibitor) can amplify the secretion of insulin induced by stimulatory glucose concentration (Fig. 5.2). In addition, there are a number of gut hormones e.g. gastric inhibitory polypeptide (GIP) and glucagon-like peptide (GLP) which are able to enhance the formation of cAMP in β-cells and thereby amplifying the effect of glucose on insulin secretion.

These findings have brought enormous attention to the role of cAMP in the tranducing pathway of insulin secretion. Its role as second messenger in other signalling systems is well established, it has long been known that cAMP acts as second messenger by activating cAMP-dependent protein kinase, known as protein kinase A (PKA), leading to a subsequent cascade phosphorylation of protein substrates, which in the case of insulin secretion would probably be those involved in the secretory process. Over the last 2 decades, there has been a large number of

studies attempting to investigate the participation of cAMP and PKA in the transduction of insulin secretion (for review see Zawalich and Rasmussen, 1990; Hughes and Ashcroft, 1992).

It was reported that in the absence of stimulatory concentration of glucose increased intracellular cAMP level induced by non-nutrients secretagogues (e.g. forskolin, IBMX, GIP, and GLP) alone is not sufficient to initiate insulin secretion (reviewed by Prentki and Matschinsky, 1987; Hughes and Ashcroft, 1992), suggesting that cAMP acts as a potentiator rather than a primary trigger in the secretory process. Regarding the mechanism which underlies the amplification effect of cAMP on glucose-induced insulin secretion, the hypothesis which has obtained the most attention is the one demonstrating that activation of PKA by cAMP potentiates insulin secretion primarily by sensitization of the secretory machinery to Ca^{2+}, in other words by lowering the dependence of glucose-induced insulin secretion on extracellular Ca^{2+} (Malaisse *et al.* 1984; Phang *et al.* 1984; Hill *et al.* 1987; Hughes *et al.* 1987). However, studies using cells pre-incubated with high calcium concentration (Ca^{2+}-insensitive cells) has brought this hypothesis to question (Jones *et al.* 1992; Howell *et al.* 1994). It was reported that in the condition where β-cells (Ca^{2+}-insensitive cells) were unable to respond to calcium, they were still capable of responding to cAMP, indicating that the effect of cAMP was owing to other mechanisms rather than increasing the sensitivity of cells to Ca^{2+} (Howell *et al.* 1994).

More recently, the physiological importance of PKA activation in glucose-induced insulin secretion has also been brought to question. Studies using [R-isomer]-cyclic 3′, 5′ monophosphorothioate (Rp-cAMPs) and myristoylated (*myr*) peptide, membrane permeant inhibitors of the activity of PKA on phosphorylation and secretory process of pancreatic islets demonstrated that pre-incubation of islets with Rp-cAMPs (Persaud *et al.* 1990) and myristoylated peptide (Harris *et al.* 1997b) abolished insulin secretion in response to forskolin and cAMP but had no effect on glucose- and Ca^{2+}-induced insulin secretion, leading to a suggestion that the elevation of cAMP and thereby activation of PKA may not be essential to glucose-stimulated insulin secretion (Persaud *et al.* 1990).

While it is clear that non-nutrient secretagogues such as forskolin, IBMX, GIP and GLP use the PKA pathway to enhance secretory response to glucose (Jones, 1998a), to date it is still unclear whether PKA has any important roles in glucose-induced insulin secretion. More studies are therefore necessary to clarify the extent to which cAMP and PKA activation participate in the regulation of insulin secretion.

5.1.2 Role of phospholipid turnover

It has been well established that, concomitant with increased insulin secretion, stimulation of β-cells with nutrient stimuli also results in a transient increase in diacylglycerol (DAG) and inositol 1, 4, 5- triphosphates (IP_3) (Fig. 5.2) (Laychock, 1983; Best and Malaisse, 1984). The production of IP_3 and DAG is thought to be at least in part mediated by the breakdown of plasma membrane phospholipid known as phosphatidylinositol biphospate (PIP_2) via an activation of phospholipase C (PLC) enzyme. Additionally, in the presence of stimulatory concentrations of glucose, agents which are capable of activating PLC such as muscarinic agonists (e.g. acetylcholine), its analogue (e.g. carbachol) and neuropeptides (e.g. cholecystokinin (CKK-8S)) are also reported to augment insulin secretion (Prentki and Matschinsky, 1987; Garcia *et al.* 1988, Kelley *et al.* 1995). The finding that promotion of IP_3 and DAG accumulation in response to glucose and other agonists in concomitant with insulin secretion has led to a suggestion that IP_3 and DAG may play important role in the signalling pathway of insulin secretion.

Roles of IP_3 and DAG as second messengers have long been recognised. As a second messenger, IP_3 is known to exert its effect upon the membranes of the endoplasmic reticulum to cause a movement of Ca^{2+} into the cytosol, resulting in increased cytosolic Ca^{2+} concentration. The role of DAG as a second messenger is thought to be owing to its ability to activate a particular protein kinase known as protein kinase C (PKC) (Vander *et al.* 1997). These effects have been implicated as possible functions of IP_3 and DAG in transduction of insulin secretion.

During last 20 years, enormous attentions have been given to study the physiological involvement of IP_3, DAG and phospholipid turnover in the secretory machinery of insulin release. However, there are still a number of questions

regarding the roles of PKC in glucose-induced insulin secretion which remain to be resolved.

5.1.2.1 Inositol triphosphate (IP_3) as second messenger

It was reported that incubation of islets with glucose and carbachol simultaneously leads to an additive increase of IP_3 production (Morgan *et al.* 1985; Morgan and Montague, 1992). Several lines of evidences have suggested that the two agonists stimulate phospholipid hydrolysis by using different mechanisms (Prentki and Matschinsky, 1987; Kelley *et al.* 1995). The effect of glucose on IP_3 production has been suggested to be secondary to an elevation of intracellular Ca^{2+} and is believed to be a consequence of glucose metabolism (reviewed by Prentki and Matschinsky, 1987; Morgan and Montague, 1992). In contrast to glucose, the formation of IP_3 induced by neurotransmitters is not dependent on extracellular Ca^{2+}, and it is likely to result from binding between the agonist and specific receptor on β-cell membrane, rather than resulting from metabolic events as observed with glucose (Biden *et al.* 1987; reviewed by Morgan and Montague, 1992).

The role of IP_3 as second messenger in mobilization of intracellular $Ca^{2+}$is well established in other signalling systems; it is therefore feasible to assume that IP_3 produced in response to glucose has important role in mobilizing intracellular Ca^{2+} and hence increasing cytosolic Ca^{2+} as necessary for insulin secretion in β-cells (Biden *et al.* 1984). However, there are several lines of evidences which do not support this hypothesis. Several studies demonstrated that the magnitude of IP_3 generation in the secretory process and subsequent increases in cytosolic Ca^{2+} were modest and transient, therefore this is not sufficient to account for the amplification of glucose-induced insulin secretion (Morgan *et al.* 1985).

Despite two decades of intensive research efforts, the precise participation of IP_3 in glucose-induced insulin release has not yet been clarified. The majority of evidence indicates that IP_3 may have only a minor role in glucose-induced insulin secretion, but may have an important role in insulin secretion regulated by muscarinic agonists and some neurotransmitters .

5.1.2.2 Diacylglycerol (DAG) as second messenger

Diacylglycerol (DAG) formed in the islets can be synthesised by two major pathways i.e. via the breakdown of phospholipids (Biden *et al.* 1987) and via *de novo* synthesis (Peter-Reisch *et al.* 1988; Wolheim and Regazzi, 1990). DAG produced by the two pathways is different in terms of fatty acid composition (Morgan and Montague, 1992). The observation that DAG produced in response to neurotransmitters contained mainly unsaturated fatty acid (i.e. arachidonic acid) suggests that the production is primarily via phospholipid breakdown (Peter-Reisch *et al.* 1988). In contrast, the stimulation of β-cells by glucose leads primarily to *de novo* synthesis of DAG which is enriched in palmitic acid (Dunlop and Larkins, 1985). It has been suggested that DAG synthesised by these two pathways had different locations and also different abilities to activate PKC (Wolheim and Regazzi, 1990).

In the last decade, the role of DAG as a second messenger in insulin signal transduction has been extensively studied by several groups (reviewed by Prentki and Matschinsky, 1987; Morgan and Montague, 1992). Major interest has been focused on a possible role of DAG as PKC activator and the participation of PKC on insulin secretory process. Despite enormous efforts, the role of PKC in insulin secretion is still controversial. Several lines of evidence (Ganesan *et al.* 1990; Calle *et al.* 1992; Zawalich and Zawalich, 1996), but not all (Hii *et al.* 1987; Easom *et al.* 1989; Easom *et al.* 1990; Howell *et al.* 1990; Wolf *et al.* 1990) have supported the involvement of PKC in glucose-induced insulin secretion.

The contribution of PKC to the process of insulin secretion has been studied using several approaches. One of the approaches, which has been widely used by a number of workers, is by investigating the effect which PKC inhibitors had on insulin secretion. A number of inhibitors which have been used included clomiphene, polymyxin B, polyamines, staurosporine, and H-7 (1-5(iso-quinolinylsulfonyl)-2-methylpiperazine). However, the results obtained from PKC inhibitors did not clarify the role of PKC in secretory response to glucose. In fact they added more confusion to the issue, mainly because the results obtained from these PKC inhibitors were highly controversial. It was reported that an inhibition of

PKC by polymyxin B (Stutchfield *et al.* 1986), polyamines (putrescine and spermidine) (Thams *et al.* 1986) and staurosporine (Zawalich *et al.* 1991) but not by H-7 (Niki *et al.* 1985; Metz, 1988) abolished the effect of glucose on insulin secretion. The discrepancy among these results has been suggested to be due to the differences in the specificity of the compounds to PKC (Metz, 1988). The use of more specific PKC inhibitors (such as a derivative of staurosporine, Ro 31-8220, and Gö 6976) revealed that Ro 31 8220 which was claimed to inhibit all isoforms of PKC partially inhibited glucose-induced insulin secretion whereas Gö 6976 which was selective for conventional Ca^{2+}/DAG-dependent isoforms (i.e. α, β and γ) had no effect on glucose-induced insulin secretion (Harris *et al.* 1996a). These results led them to a suggestion that glucose may work via isoforms of PKC which were not inhibited by Gö 6976 (Harris *et al.* 1996a).

More clear cut results were obtained from studies using PKC-depleted β-cells. It was demonstrated that down regulation of PKC induced by prolonged exposure of the islets with PKC-activating phorbol ester (e.g. phorbol 12-myristate, 13-acetate, PMA) did not impair secretory response of pancreatic islets to glucose, Ca^{2+} and forskolin but abolished the secretion in response to the PKC activator, PMA (Hii *et al.* 1987; Metz, 1988; Howell *et al.* 1990), leading to a suggestion that PKC activation may not play an obligatory role in the signal transduction pathway of glucose-induced insulin secretion. However, it has been argued that pre-treatment with PMA may downregulate only particular families of PKC enzymes (α-, δ-, ε-isoforms of PKC), leaving others (ζ-, ι/λ-, μ- isoforms) still able to be activated by glucose (Kariya and Takai, 1987; Jones and Persaud, 1998b). If this is the case, there is possibility that the effect on insulin secretion of glucose is mediated, at least in part, by activation of ζ-, ι/λ-, and/or μ-isoforms of PKC which are not affected by prolonged exposure with PMA (Harris *et al.* 1997a; Jones and Persaud, 1998b). However, physiological effect of glucose on these isoforms of PKC remains to be defined.

To date, the role of PKC in glucose-induced insulin secretion is still the subject of debate, and more detailed studies are required to establish its involvement in glucose-induced insulin secretion. Nevertheless, from the majority of

accumulated publications it can be concluded with confidence that the receptor-mediated non-nutrient secretagogues such as muscarinic agonists and some neuropeptides enhanced insulin secretion primarily through the activation of PKC (particularly α-, δ-, ε-isoforms). However, PKC activation did not appear to have an obligatory role in the signal transduction pathway of glucose-induced insulin secretion whose major determinant signal is cytosolic Ca^{2+} (for review see Metz, 1988; Wolheim and Regazzi, 1990; Persaud *et al.* 1992).

5.1.2.3 Arachidonic acid as second messenger

Another consequence of glucose-induced phospholipid turnover is a release of arachidonic acid and its metabolites i.e. cyclo-oxygenase (prostaglandins) and lipoxygenase (12-HPETE and 12-HETE) products (Prentki and Matschinsky, 1987; Morgan and Montague, 1992).

The involvement of arachidonic acid in insulin secretion has been studied by several groups of workers (Band *et al.* 1993; Konrad *et al.* 1994; for review see Jones and Persaud, 1993 and Turk *et al.* 1993). Increased islet content of arachidonic acid in response to glucose and carbachol in concomitance with increased insulin secretion was reported in several studies (Laychock, 1982; Dunlop and Larkins, 1984; Konrad *et al.* 1992). Inhibition of arachidonic acid synthesis was reported to result in an inhibition of glucose- and carbachol-induced insulin secretion (Konrad *et al.* 1994). These findings led to an implication that arachidonic acid may have an important role in the regulation of the secretory pathway of insulin. Additionally, exogenous arachidonic acid is reported to be able to stimulate insulin secretion in the presence of sub-stimulatory concentrations of glucose (Band *et al.* 1993), suggesting its role as an initiator rather than potentiator of insulin secretion (for review see Jones and Persaud, 1993).

The mechanism by which insulin secretagogues induce the accumulation of arachidonic acid is not yet fully defined. It has been suggested that arachidonic acid can be generated in response to insulin secretagogues via two major routes (Fig. 5.3): 1) via activation of phospholipase A_2 (PLA_2) resulting directly in hydrolysis of arachidonic acid from phospholipid and 2) via activation of phospholipase resulting in a release of DAG from phospholipid, followed by the activation of diacylglycerol

lipase (Konrad *et al.* 1994) (Fig. 5.3). However, the contribution of either pathway to glucose-induced arachidonic accumulation has not yet been clarified.

To date, the precise role and involvement of arachidonic acid in the secretory process of insulin are still unclear, there is a possibility that arachidonic acid may act as a fusogen to destabilize the membranes and promote fusion between plasma membrane and granule membrane during exocytosis (Jones and Persaud, 1993). Nevertheless further studies are still required to clarify this effect of arachidonic acid.

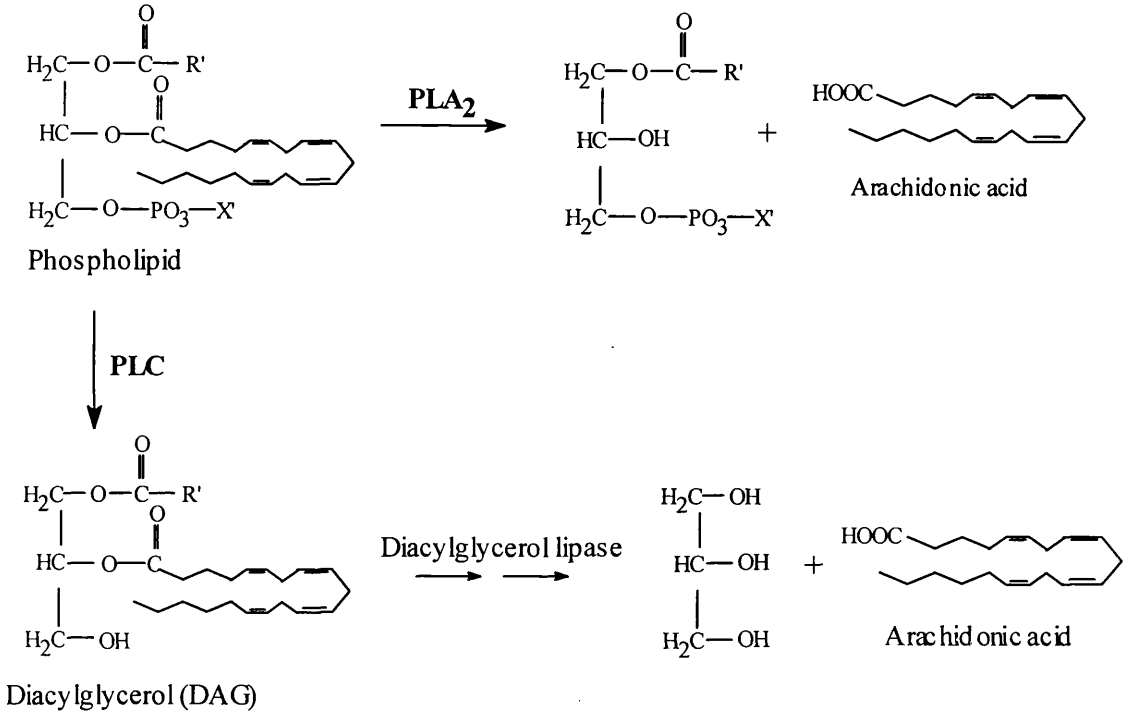


Fig. 5.3: Schematic of arachidonic acid synthesis in pancreatic β-cells

5.1.3 General overview

Over the last two decades significant advances have been made in understanding some aspects of signal transduction in the insulin secretion pathway. However, to date, the mechanism whereby glucose regulates insulin secretion is not fully elucidated. Regarding the large body of evidence, it appears that intracellular Ca^{2+} is a major determinant signalling species for glucose induced insulin secretion. Besides Ca^{2+}, other molecules have also been implicated in the regulation of insulin release, which include cAMP, IP_3, DAG and arachidonic acid. However, the roles of

these molecules in the transduction pathway in response to glucose are still ambiguous and discrepancy among the results still persists. However, it is likely that cAMP and DAG do not have an obligatory role in glucose-induced insulin secretion but may be of importance in the secretion induced by other insulin secretagogues e.g. muscarinic agonists and neuropeptides. PKA and PKC similarly do not appear to have an obligatory role in glucose and Ca^{2+} induced insulin secretion. More studies in this area are still required to clarify the roles of these molecules in physiological control of insulin secretion from pancreatic β-cells.

5.2 Agents increasing insulin secretion

A number of insulin secretagogues have been developed in an attempt to search for agents that could restore secretory response of β-cells in diabetic patients. The agents that have been developed can be divided into 2 categories: 1) initiators of insulin secretion and 2) potentiators of insulin secretion.

5.2.1 Initiators of insulin secretion

The term "initiator" refers to an agent that can stimulate insulin secretion on its own. Sulphonylureas are the most well known drugs of this category (section 1.2.2.1). These agents work by binding to a specific receptor on β-cell membrane. This receptor is known as sulphonylurea receptor, SUR-1, which is a subunit of the ATP-sensitive K^+ (K^+_{ATP}) channel itself (Aguilar-Bryan et al. 1998). The interaction of sulphonylureas with the receptor results in the closure of K^+_{ATP} channel, subsequent depolarization of the β-cell membrane and influx of Ca^{2+} into β-cells, leading to insulin secretion (Bailey *et al.* 1997b).

5.2.2 Potentiators of insulin secretion

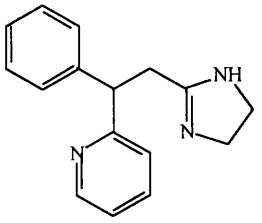
Agents in this class are those that can enhance nutrient-induced insulin secretion but can not stimulate the secretion on their own. These include some benzoic acid derivatives, glucagon-like peptide (GLP1) and α_2 adrenoceptors antagonists

Benzoic acid derivatives which have been developed for the treatment of diabetes include repaglinide (Fig. 1.7), meglitinide and KAD-1229 (Bailey *et al.* 1997b). These agents have been reported to work by closing K^+_{ATP} channel, similar

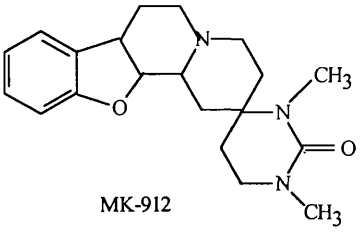
to sulphonylureas (Melander, 1996). The only benzoic acid derivative which is currently available for clinical use in UK is repaglinide (section 1.2.2.5). Repaglinide has been introduced as glucose-sensitive insulin secretagogue; the compound is believed to possess a number of therapeutic advantages over classical sulphonylureas (section 1.2.2.5). A comparison study on an insulinotropic effect of repaglinide and glibenclamide in islets showed that both compounds shifted a glucose dose response curve toward the left, but it was reported that repaglinide was five times more potent than glibenclamide (Fuhlendorff *et al.* 1998). The study also revealed that repaglinide did not stimulate insulin secretion from islets in the complete absence of glucose; the stimulation by repaglinide is confined to intermediate concentrations of glucose i.e. 3-10 mM (Fuhlendorff *et al.* 1998).

GLP1 is thought to enhance insulin secretion, at least in part, via a receptor linked to adenylate cyclase resulting in an increase in cAMP level (Thorens and Waeber, 1993) (section 5.1). The major drawbacks of GLP-1 are its short term action and its inconvenient mode of delivery, as parenteral administration of the peptide is required (Scheen, 1997).

The finding that inhibition of α_2 adrenoceptors on β-cells could lead to a potentiation of nutrient-induced insulin secretion has led to another approach to develop α_2 adrenoceptor antagonists; agents in this class include midaglizole and MK-912 (Fig. 5.4). However, the therapeutic use of α_2-adrenoceptor antagonists is hampered by a significant increase in blood pressure (Rachman and Turner, 1995).



Midaglizole



MK-912

Fig. 5.4: Structure of α_2-adrenoceptor antagonists

5.3 Aim of the present study

The studies described in this chapter aimed to investigate the effect of selected plants (section 2.1) on insulin secretion from β-cells using insulin-secreting

cell lines. The term insulin-secreting cell line refers to an endocrine cell which is able to proliferate in culture and to synthesise, process and secrete insulin. A number of cell lines have been developed by a number of different methods, resulting in cell lines with different characteristics and varying ability to respond to stimulants (see section 1.5.2.3). The selection of the cell lines depends on physiological properties of the cell lines and the nature of the studies. Over the past 20 years, insulin-secreting cell lines have been used in wide range of studies including the area of natural product research (Noor *et al.* 1989; Gray and Flatt, 1999).

5.4 General methods

Insulin secreting cell lines (βTC3, βTC6 and RINm5F cells) were kindly provided by Dr. P.M. Jones and Dr. S.J. Persaud (Cellular and Molecular Endocrinology Group, Biomedical Sciences Division, King's College London). Unless specified, chemicals and reagents were analytical grade and purchased from Sigma Chemical Co. (Sigma-Aldrich Co. Ltd, Irvine, UK). Glucose-free RPMI 1640 medium was purchased from ICN (Costa Mesa, CA), tissue culture labware and 96-well microtitre plates from Costar (Cambridge, MA, USA). Radio-labelled iodine, ^{125}I (sodium iodide), was purchased from Amersham International (Amersham, Bucks, UK). Polystyrene tubes (64 × 111 mm) for RIA were purchased from L.I.P. (Equipment & Services) Ltd (UK).

5.4.1 Maintenance and subculture of insulin secreting cell lines

a) Maintenance of the cell lines

Cells were grown as monolayers in plastic tissue culture flasks (162 cm^3) and maintained at 37 °C in a humidified incubator at an atmosphere of 95% air/5% CO_2 (BOC Limited, Surrey UK) in 20 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 2 mM glutamine, 200 U/ml penicillin and 0.2 mg/ml streptomycin. Serum was supplemented to culture media to provide the cells with essential growth factor and to enhance cell attachment, the major components in the serum include protein, polypeptide-

hormones, nutrients and minerals. Glutamine was added as an energy and carbon source for the cells.

b) Subculture of the cell lines

The culture medium was renewed with fresh DMEM (10% FCS, 2 mM glutamine, 200 U/ml penicillin and 0.2 mg/ml streptomycin) every 4-5 days. When grown to confluence, cells were sub-cultured to new flasks by trypsinisation as follows. The medium was removed and the cells were washed with phosphate buffer saline (PBS) to remove all trace of FCS and any loose or dead cells. A sufficient volume of trypsin/EDTA solution (~ 7 ml) was then added to dissociate the cell monolayer and detach cells from flasks. The cells were left to stand in trypsin/EDTA solution for 2-3 minutes at room temperature, DMEM (10% FCS, 2 mM glutamine, 200 U/ml penicillin and 0.2 mg/ml streptomycin) was then added to stop the reaction of trypsin. The cell suspension was transferred to a sterile tube and centrifuged at 1000g for 3-4 minutes to spin down any loose cells. DMEM was then aspirated and the pellets were thoroughly resuspended in fresh medium (10 ml) and transferred to new culture flasks (5 ml each). DMEM (10 ml) was then added to each flask, and the cells maintained in DMEM as described above.

5.4.2 Insulin secretion experiment

Gey & Gey Buffer

Gey & Gey buffer was prepared containing the following substances:

	g/L
Sodium chloride	13.0
Sodium bicarbonate	4.54
Potassium chloride	0.74
Magnesium chloride	0.42
Sodium phosphate diphasic	0.18
Magnesium sulphate heptahydrate	0.14
Potassium dihydrogen orthophospate	0.06

This stock buffer was kept at 4 °C. Gey & Gey buffer used in secretion experiment was the stock buffer diluted (1:1) with deionised water. The solution

was gassed with 5% CO_2/95% air to adjust to pH 7.4. Calcium chloride and bovine serum albumin were then added to give final concentrations of 2 mM and 0.5 mg/ml, respectively.

Borate Buffer

	g/L
Boric acid	8.25
Sodium hydroxide	2.70
Sodium azide	1.00
EDTA	3.72

The components were dissolved with 950 ml of distilled water and adjusted to pH 8.0 with concentrated HCl. The volume was adjusted to 1 L with distilled water and then 1 g of BSA was added.

Cells which had grown to confluence were harvested by trypsinisation as described in section 5.4.1(b). After centrifugation, the pellets were resuspended in DMEM (4 ml). Cell suspension (10 µl) was transferred to a small vial and diluted 1 in 5 with 0.4% trypan blue to identify dead cells. The suspension in 0.4% trypan blue (10 µl) was then transferred to a haemocytometer (Weber, England) to count the living cells i.e. those did not take up the dye. The cells were counted in 4 chambers under a microscope at ×10 magnification. The number of cells/ml in the cell suspension was calculated from a mean of the 4 counts using the following formula:

$$\text{Number of cells/ml} = \text{Mean cell count} \times \text{dilution factor (5)} \times 10^4$$

The cells were then added with DMEM (10% FCS, 2 mM glutamine, 200 U/ml penicillin and 0.2 mg/ml streptomycin) to give an appropriate cell density. The cells in DMEM were seeded to 96-well plates at 200 µl/well and incubated at 37 °C in 95% air/5% CO_2 for 48 hours to allow adhesion of the cells to the plate.

After 48 hours, the medium was removed by inverting the plate onto tissue paper to minimise the number of cells lost during the washing step. The medium was replaced with 200 µl of glucose-free RPMI 1640 containing 0.5 mg/ml bovine serum albumin (BSA). The cells were incubated in glucose-free RPMI 1640

for 2 hours at 37 °C in 95% air/5% CO_2. At the end of the pre-incubation period, the medium was removed and replaced with 200 µl of Gey & Gey buffer (control) or buffer containing test solutions. Each extract was assayed at each concentration in 6-8 separate well. The cells were incubated with buffer or test solution for 1 hour at 37 °C in 95% air/5% CO_2. At the end of incubation, the 96-well plate was centrifuged at 4 °C, 1000g for 5 minutes. The supernatant from each well was transferred to 1.5-ml Eppendorf tubes and then diluted (1:1) with borate buffer. The diluted solution was kept at -20 °C until use for radioimmunoassay (section 5.4.3).

5.4.3 Measurement of insulin secretion using radioimmunoassay (RIA)

5.4.3.1 Introduction

Radioimmunoassay (RIA) was performed according to the method described by Jones *et al.* (1988). Three major components of insulin RIA are

(1) anti-insulin antibody

(2) radiolabelled-insulin (iodinated insulin, ^{125}I)

(3) unlabelled insulin (insulin standard or insulin secreted from the cells)

At equilibrium, the antibody binding sites were occupied either by radiolabelled or unlabelled insulin. Radiolabelled and unlabelled insulin in the reaction compete for the binding site of antibody, thus the more unlabelled insulin (sample) present in the reaction, the less radiolabelled insulin binds to the antibody.

$$[Ab] + [I] + [I^*] \leftrightarrow [Ab\text{-}I] + [Ab\text{-}I^*] + [I] + [I^*]$$

Ab: Antibody

I: Unlabelled insulin

I*: Iodinated insulin (^{125}I)

The separation of antibody bound insulin fractions and free fractions relied on the different nature of antibody and antigen. Antibody, which is a large protein molecule, was separated from the much smaller molecule of antigen insulin by precipitation. The proportion of radiolabelled insulin bound fraction [Ab-I*] was determined by means of measuring radioactivity of the precipitate using a γ counter.

5.4.3.2 Radioimmunoassay reagents

Iodinated insulin

^{125}I-Insulin was prepared by Dr. P.M. Jones (Cellular and Molecular Endocrinology Group, Biomedical Sciences Division, King's College London) according to the method described by Salacinski *et al.* (1981). The technique involved an oxidation of a radioisotope of iodide (I^-) to I^+ using iodogen which is a large polymer with oxidative site as solid phase oxidising agent. I^+ then spontaneously incorporated into the phenolic groups of tyrosine residues within insulin molecules to form iodinated-insulin. Bovine insulin stock and NaI were added to iodogen, and incubated at room temperature for 20-30 minutes. The iodination reaction was terminated by an addition of BSA-free phosphate buffer. The fractions of unincoporated iodine and iodinated insulin were separated by means of size exclusion chromatography using Sephadex G50. Stocks of iodinated insulin (~10×10^6 cpm/ml) were kept at 4 °C until use.

Anti-insulin antibody

Antibody was raised against bovine insulin of Hartley guinea-pigs by Dr. P.M. Jones (Cellular and Molecular Endocrinology Group, Biomedical Sciences Division, King's College London) according to the method of Hurn and Chantler, (1980) with slight modification. Guinea-pigs were used because endogenous insulin of this species is most dissimilar in structure to that of other mammalian species.

Anti-insulin antibody was stored as 1:1000 dilution of serum. The antibody diluted 1 in 20 with borate buffer was used in the assay in which the antibody was further diluted (100 µl) to 300 µl (Table 5.1) to give a final concentration of 1: 60,000.

Phosphate buffered saline (PBS)

Dulbecco's phosphate buffered saline powder (95.5 g) was dissolved in 10 L deionised water.

Precipitant

γ-Globulin (2 mg/ml) was dissolved in PBS and an equal volume of 30% polyethylene glycol (PEG) was added. γ-Globulin was added to aid precipitation of

the bound antibody fraction. Tween 20 was added to give a final concentration of 0.05% v/v to reduce non-specific binding.

5.4.3.3 Procedure for the assay

The assay was set as described in Table 5.1 using polystyrene round bottom tubes (64 × 111 mm). The tubes labelled 'Total' which contained only ^{125}I-insulin were prepared in order to measure the radioactivity of ^{125}I-insulin used in the assay. The tubes labelled 'non-specific binding' (NSB) which contained ^{125}I-insulin (100 µl) and borate buffer (200 µl) but no antibody were designed to measure the amount of ^{125}I-insulin bound non specifically to sites other than antibody binding sites. All readings were corrected with this value. The tubes labelled 'maximum binding' (MB) which contained ^{125}I-insulin (100 µl), antibody (100 µl) and borate buffer (100 µl) but no unlabelled insulin was set to measure the proportion of ^{125}I-insulin bound to antibody without the competition between unlabelled and iodinated insulin, this was therefore 100% binding of ^{125}I-insulin to antibody. Standards were prepared by serial dilution (1 in 2) of unlabelled rat insulin (10 ng/ml) with borate buffer to give sample concentrations of 0.08-10 ng/ml for the assay. The standards (100 µl) were assayed in triplicate. Antibody (100 µl) was added to every tube except those labelled "NB" to give a final antibody concentration of 1:60,000. ^{125}I-Insulin (100 µl) was added to every tubes to give a final activity of 10,000 cpm/tube.

Supernatant (100 µl) containing insulin secreted from the cells exposed to buffer or test solutions (section 5.4.2) was diluted (1:1) with borate buffer. The resulting solution (100 µl) was mixed with radiolabelled insulin (100 µl) and

Table 5.1: Tubes set up for RIA assay

	Volume (µl)			
	^{125}I-Insulin	Antibody	Borate Buffer	Unlabelled Insulin
Total	100	-	-	-
NSB	100	-	200	-
MB	100	100	100	-
Samples/Standard	100	100	-	100

antibody (100 µl). Supernatant obtained from secretion experiment from each well was assayed in duplicate. Standard and sample tubes were then left at 4 °C for 48-72 hours to reach an equilibrium. Complexes of insulin and antibody were precipitated out from the reaction by addition of the precipitant (1ml) to all the tubes except those labelled 'total', followed by centrifugation (Jouan CR422, France) at 4 °C, 1000g, for 30 minutes. The supernatant was removed from all the centrifuged tubes by aspiration. Tubes containing the ^{125}I-insulin-antibody complexes were counted for radioactivity for 60 seconds using a Packard Cobra II™ gamma counter. All readings were corrected by subtracting with values obtained from 'non specific binding' (NSB). For all sample (insulin standard or supernatant from cell experiments), the % insulin binding was calculated using the formula (this was done by RIA Smart data handling package):

$$\frac{\text{Counts (cpm) for sample -NSB}}{\text{Counts (cpm) for MB-NSB}} \times 100$$

Content of insulin secreted from the cells was determined by extrapolating from a calibration curve plotted as Logit of percentage maximum binding against the log of standard insulin concentration (calculated by the RIA Smart data handling package) (Fig. 5.6). Mean of the duplicate was taken as values of insulin secretion for a particular well, the values were rejected if the percentage error between the duplicate readings from the same well was more than 50%. The results from cell secretion experiments were expressed as ng of insulin secreted/10^6 cells/hr (mean ± SEM) and statistically analysed using ANOVA, followed by Dunnett multiple comparison test. Differences between experimental and control values were considered significant at $P < 0.05$.

Quantification of RIA is based on a competition between a limited amount of radiolabelled-insulin and unlabelled insulin for a limited amount of antibody binding site. Thus, the fraction of radiolabelled-insulin bound to antibody varies inversely with the amount of unlabelled insulin (standard insulin or insulin secreted from the cells) present in the reaction (Fig. 5.5). Graph in Figure 5.5 was converted to a linear graph by plotting as Logit of percentage maximum binding against the log

of standard insulin concentration (Fig. 5.6). The content of insulin expressed in all experiments was calculated from graphs such as that in Figure 5.6.

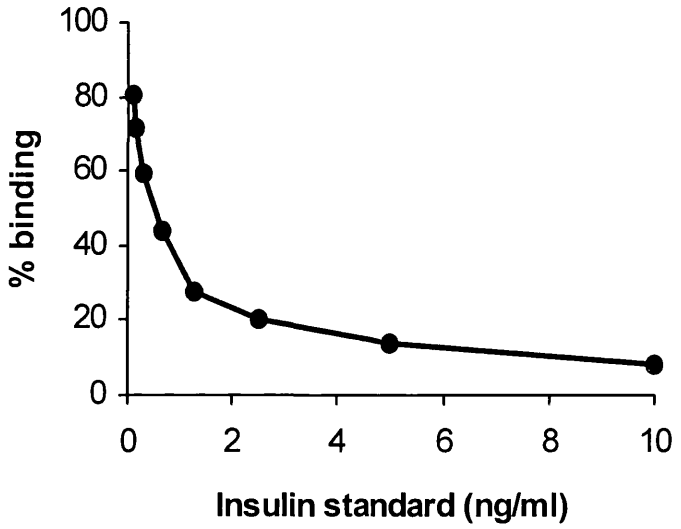
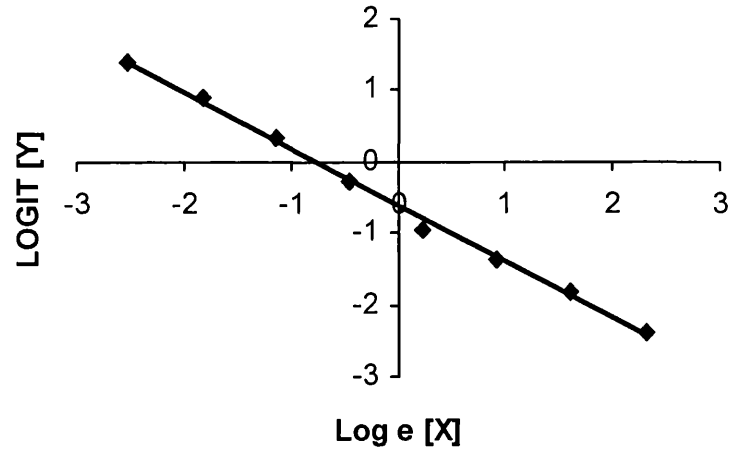


Fig.5.5: Standard curve of an insulin RIA plotted as percentage binding against unlabelled standard insulin concentration



$$\text{Logit Y} = \ln \left[\frac{(B\text{-}NS)/(B_0\text{-}NS)}{1\text{-}((B\text{-}NS)/(B_0\text{-}NS))} \right]$$

B_0 = cpm of maximum binding of iodinated insulin to anti-insulin antibody

B = cpm bound for each concentration of unlabelled standard insulin

NS = cpm of non-specific binding of iodinated insulin

Fig.5.6: Standard curve of an insulin RIA plotted as Logit of % binding against Log of standard insulin concentration

5.5 Selection of insulin secreting cell lines for the study

5.5.1 Introduction

To select the cell lines for studying the effects of plant extracts on insulin secretion, the responsiveness of three different cell lines i.e. RINm5F (passage 69), βTC3 (passage 6) and βTC6 (passage 7) to various known secretagogues was investigated. The response was quantified by means of insulin secreted after incubation of the cells with the secretagogues. It was originally hoped to investigate also the response of HIT-T15 cells, but these were not available at the time of these experiments.

RIN cells were first established by Chick *et al.* (1973) from a pancreatic tumour induced in rats by x ray-irradiation. Tumours developed in irradiated rats were then transplanted under the skin of 6-week old rats. Secondary tumours were then grown *in vivo* by serial transplantation into rats (RINr cells) or mice (RINm cells) (Poitout *et al.* 1996a). βTC3 and βTC6 (*b*eta *t*umour *c*ell) were developed by fusion of recombinant rat insulin II /SV40 genes into fertilized mice embryos, which were then transplanted into the oviduct of pseudo-pregnant mice. The offspring of these mice spontaneously developed β-cell tumours at 10-20 weeks of age. Tumours were then excised and grown in cultures, giving rise to βTC lines (Poitout *et al.* 1996b).

5.5.2 Materials and methods

Cell lines were maintained and subcultured as described under General Methods (section 5.4.1). Cells which had been grown to confluence were trypsinised, seeded to 96-well microtitre plates (section 5.4.2) at a density of 150,000 cells/well for RINm5F cells and 100,000 cells/well for βTC3 and βTC6 cells and incubated at 37 °C in 95% air/5% CO_2. After 48 hours, the cells were pre-incubated with RPMI containing no glucose for 2 hours, followed by incubation with Gey & Gey buffer (control) or various stimulants (Table 5.2) at 37 °C in an atmosphere of 95% air/5% CO_2 for 1 hour. The 96-well plate was then centrifuged at 4 °C, 1000g for 5 minutes. The supernatant from each well (150 µl) was transferred to Eppendorf tubes and diluted with borate buffer (150 µl). The diluted supernatant

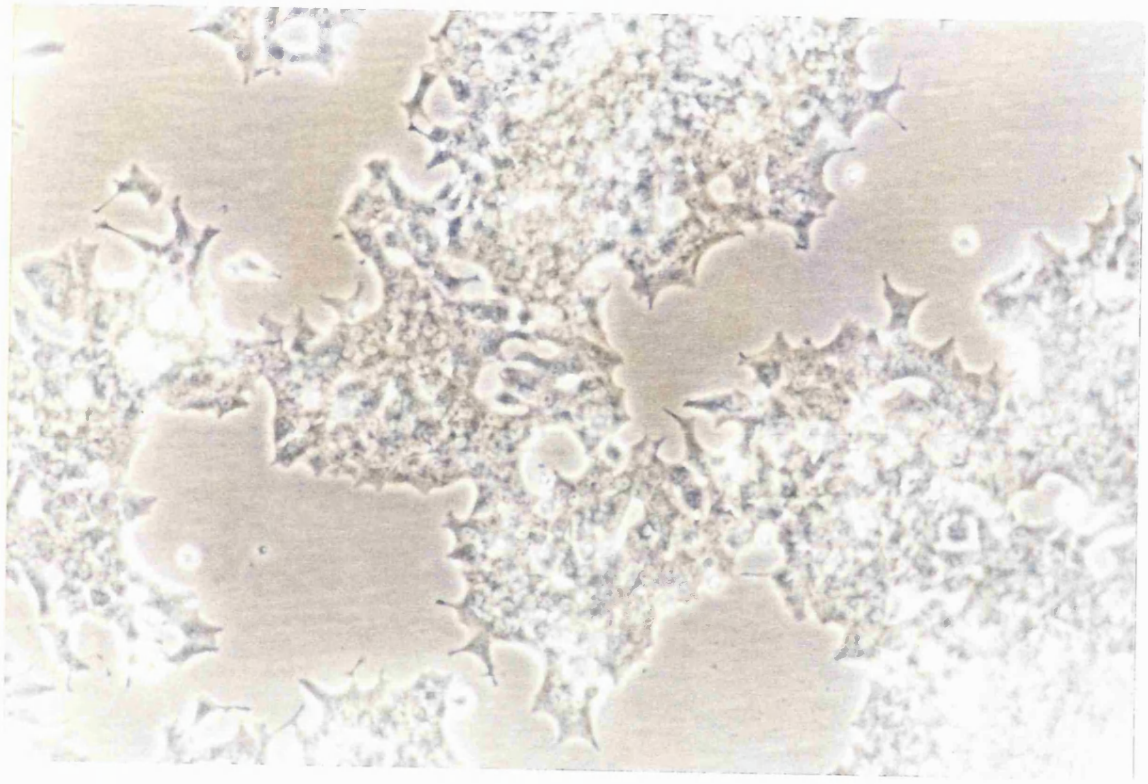


Fig. 5.7: RINm5F cells (× 200)

(100 µl) was quantified for the content of insulin in duplicate using radio-immunoassay (RIA) as described in section 5.4.3.3. The content of insulin was calculated from a graph plotted as Logit of percentage maximum binding against log of standard insulin concentration (Fig. 5.6). Results were expressed as ng/10^6 cells/hr (mean ± SEM) and statistically analysed using ANOVA.

Table 5.2: Known insulin secretagogues used in the experiment

	Secretagogues
A	Gey & Gey solution (0.5 mg/ml BSA, no glucose)
B	20 mM glucose or 10 mM glyceraldehyde*
C	20 mM glucose or 10 mM glyceraldehyde* + 10 µM forskolin + 100 µM isobutylmethylxanthine (IBMX)
D	20 mM glucose or 10 mM glyceraldehyde* + 500 nM phorbol-12-myristate, 13 acetate (PMA)
E	20 mM glucose or 10 mM glyceraldehyde* + 500 µM carbachol
F	10 mM ketoisocaproic acid (KIC)

*In the case of RINm5F cells, 10 mM glyceraldehyde was used instead of 20 mM glucose (as used in βTC) because RINm5F cells are not glucose responsive.

5.5.3 Results and discussion

Results from secretion experiments are shown in Figure 5.8 and 5.9. Originally, the study was aimed to investigate the ability of 4 different widely-used cell lines to respond to the known stimulants (Table 5.2). However due to a lack of availability of HIT-T15 cells, the study was conducted with only 3 cell lines i.e. βTC3, βTC6 and RINm5F cells.

A greater number of RINm5F cells (150,000 cells/well) were used compared to those of βTC3 and βTC6 (100,000 cells/well) because RINm5F cells have been reported to contain low insulin content (Lenzen, 1992). RINm5F cells do not respond to glucose over the range of 2.8-33.4 mM (Praz *et al.* 1983, reviewed by Persaud, 1999). Loss of insulin secretory responsiveness to glucose in RINm5F cells is believed to be owing to the lack of glucokinase activity (Lenzen, 1992).

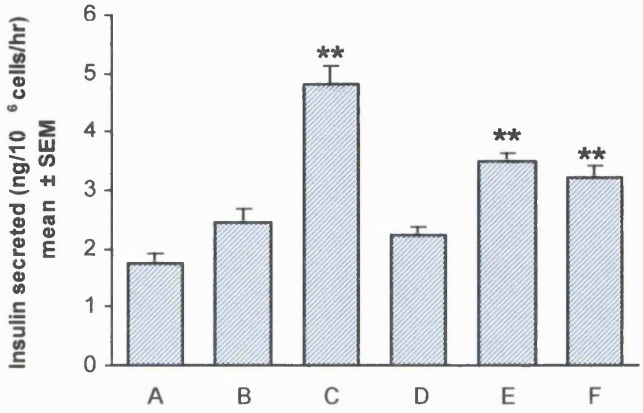


Fig. 5.8: Insulin secreted from RINm5F cells (p 69) in response to known secretagogues

Results are expressed as mean of insulin secretion (ng/10^6 cells/hr) ± SEM from two separate experiments. Each sample was investigated with 8 replicates. **P < 0.01 VS 10 mM glyceraldehyde

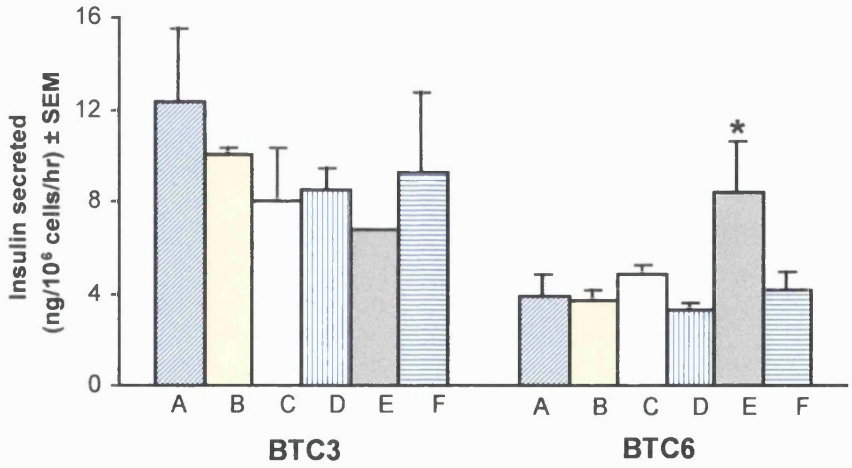


Fig. 5.9: Insulin secreted from βTC3 (p 6) and βTC6 (p 7) in response to known secretagogues

Results are expressed as mean of insulin secretion (ng/10^6 cells/hr) ± SEM from two separate experiments. Each sample was investigated with 8 replicates. **P < 0.01 VS 20 mM glucose

A Gey & Gey solution (0.5 mg/ml BSA, no glucose)
B 20 mM glucose (βTC) or 10 mM glyceraldehyde (RINm5F cell)
C (B) + 10 μM forskolin +100 μM IBMX
D (B) + 500 nM PMA
E (B) + 500 μM carbachol
F 10 mM KIC

Glyceraldehyde was therefore used instead of glucose to stimulate insulin secretion in RINm5F cells.

Although the amount of insulin secreted from RINm5F cells (1.73 ng/10^6 cells/hr for basal secretion and 2.45 ng/10^6 cells/hr in response to 10 mM glyceraldehyde) was less compared to that secreted from βTC3 (12.35 ng/10^6 cells/hr for basal insulin secretion and 11.25 ng/10^6 cells/hr in response to 20 mM glucose) and βTC6 (3.89 ng/10^6 cells/hr for basal secretion and 3.65 ng/10^6 cells/hr in response to 20 mM glucose), RINm5Fcells but not βTC3 and βTC6 were found to significantly respond to some of the secretagogues (Fig. 5.8 & 5.9). The secretion of insulin in RINm5F cells was slightly increased by 1.41 fold in response to 10 mM glyceraldehyde, however this did not reach statistical significance. RINm5F cells showed a significant increase in insulin secretion in response to 10 mM KIC (analogue of leucine), and 10 mM glyceraldehyde supplemented with secretagogues such as forskolin (activator of adenylate cyclase) and IBMX (inhibitor of phosphodiesterase), and carbachol (Fig. 5.8). However, it was found that the secretion of insulin from RINm5F cells was not affected by PMA in this experiment (Fig. 5.8), this was probably an atypical results (Dr. S. Persaud, personal communication). It has long been known that forskolin and IBMX augment nutrient-induced insulin secretion via the activation of PKA (section 5.1.1.) Carbachol is known to act via the activation of hydrolysis of PLC which generates two important second messengers, IP_3 and DAG (section 5.1.2). KIC is an analogue of the amino acid, leucine which is known to stimulate insulin secretion using a mechanism similar to glucose (section 5.1). The response of RINm5F cells to these secretagogues suggested that pathways important for regulating insulin secretion were fully functional.

Surprisingly, it was found that neither βTC3, βTC6 nor RINm5F cells responded to nutrient at stimulatory concentrations (20 mM glucose/10 mM glyceraldehyde). In the case of βTC3 cells, the cells also respond to neither forskolin and IBMX, PMA, carbachol nor KIC (Fig. 5.9). The secretion of insulin from βTC6 was significantly increased in response to carbachol, but the cells failed to respond to either 20 mM glucose, forskolin and IBMX, PMA or KIC.

The inability of βTC3 and βTC6 to markedly respond to these secretagogues suggested that RINm5F cells would be more appropriate for the examination of plant extracts. Another advantage of RINm5F arises from the fact that the cells are not glucose-responsive, therefore insulin secretion induced by glucose present in the plant extracts can be avoided.

5.6 Effect of sulphonylureas and sparteine on insulin secretion from RINm5F cells

5.6.1 Introduction

The secretory response of RINm5F cells to known insulin secretagogoues i.e. two sulphonylureas and the alkaloid, sparteine, was investigated in this experiment. The effect of sulphonylureas on insulin secretion is believed to be primarily due to the ability of the agents to close K^+_{ATP} channel (section 5.2.1). Two sulphonylureas investigated in this study were tolbutamide and glibenclamide.

Sparteine is an alkaloid isolated from *Lupinus spp.* Sparteine is well known for its stimulatory effect on insulin secretion; its effect is believed to be, at least in part, related to an inhibition of K^+_{ATP} channel, as suggested by a study in HIT-T15 cells (Ashcroft *et al.* 1991).

5.6.2 Materials and methods

Cells were maintained as described in section 5.4.1 and the insulin secretion experiment was performed with a cell density of 150,000 cells/well as described in section 5.4.2. Forty eight hours after seeding, the medium was replaced and cells were pre-incubated with glucose-free RPMI 1640 for 1 hour at an atmosphere of 95% air/5% CO_2. Tolbutamide, glibenclamide and sparteine were dissolved at the test concentrations in 2% DMSO which was also used as control. In case of tolbutamide, the agent was also tested in the presence of 10 mM glyceraldehyde. Each sample was investigated with 6 observations. At the end of incubation, supernatant (150 µl) taken from 96-well plate was diluted (1:1) with borate buffer. The diluted supernatant (100 µl) was then measured for the content of insulin using the method described in section 5.4.3.3. Significance of the response

between experimental samples and control was statistically analysed by ANOVA. The results were expressed as relative insulin secretion (% of control) which was calculated using the following formula:

$$\frac{\text{Insulin secretion}_{\text{sample}}\ (\text{ng}/10^6\ \text{cells/hr})}{\text{Insulin secretion}_{\text{control}}\ (\text{ng}/10^6\ \text{cells/hr})} \times 100$$

5.6.3 Results and discussion

Investigation of the effect of tolbutamide on RINm5F cells (Fig. 5.10 and 5.11) revealed that tolbutamide at concentrations of 0.2-3 mM failed to stimulate insulin secretion. The loss of responsiveness of RINm5F cells cultured in this study to tolbutamide may reflect the differences in term of functional characteristics between RINm5F cells (which are insulinoma transformed cells) and primary β-cells. Similarly, the cells also failed to respond to glibenclamide (0.25-2 mM) (Fig. 5.12). However, it was found that the cells responded to tolbutamide (0.38-3 mM) when glyceraldehyde was present in the incubating medium (Fig. 5.11), suggesting that in order for the cells to respond to tolbutamide, the secretory pathway of the cells needed the presence of stimulatory concentration of nutrient to trigger the secretory process. This may not be the case *in vivo* where tolbutamide is known to initiate insulin secretion even in the presence of sub-stimulatory concentration of glucose (Zimmerman, 1997). The effect of glibenclamide in the presence of nutrient was not investigated. Nonetheless, it was reported elsewhere that glibenclamide (0.01-1 mM) was able to potentiate the effect of glucose (5-15 mM) on insulin secretion from rat islets (Fuhlendorff *et al.* 1998).

Although the cells had lost their ability to respond to tolbutamide in the presence of sub-stimulatory concentration of nutrient, major pathways involved in the regulation of insulin secretion were found to be fully functional as judged by the response of the cells to forskolin + IBMX, carbachol and KIC (Fig. 5.8), indicating a validity of the cell lines for further studies. It is therefore possible that any active compounds detected in this programme would not be working by a sulphonylurea type mechanism. However, it must be borne in mind that not only RIN cells but most of the transformed cells including HIT, βTC, MIN6 and INS cells had also lost

some of the functional characteristics of primary β-cells to some extent (Poitout *et al.* 1996a), thus the results obtained from the transformed cells need to be interpreted with caution.

Stimulatory effect of sparteine on insulin secretion was evident in a number of studies. It was reported that sparteine was able to increase basal and nutrient-induced insulin secretion in normal human (Sgambato *et al.* 1987). In accord with the insulinotropic effect of sparteine reported in the literature (Sgambato *et al.* 1987; Paolisso *et al.* 1988), it was found in the current study that sparteine (0.1 and 0.16 mg/ml) initiated the secretion of insulin from RINm5F cells (Fig. 5.13). However, increasing the concentrations of sparteine above 0.16 mg/ml did not enhance the effect of the compound on insulin secretion. In fact it was found that the secretion of insulin decreased with increasing doses of sparteine, suggesting that inhibitory or even toxic effects were operational at higher concentrations. The response of the cells to sparteine again suggested the existence of functional regulated pathway of insulin secretion in RINm5F cells.

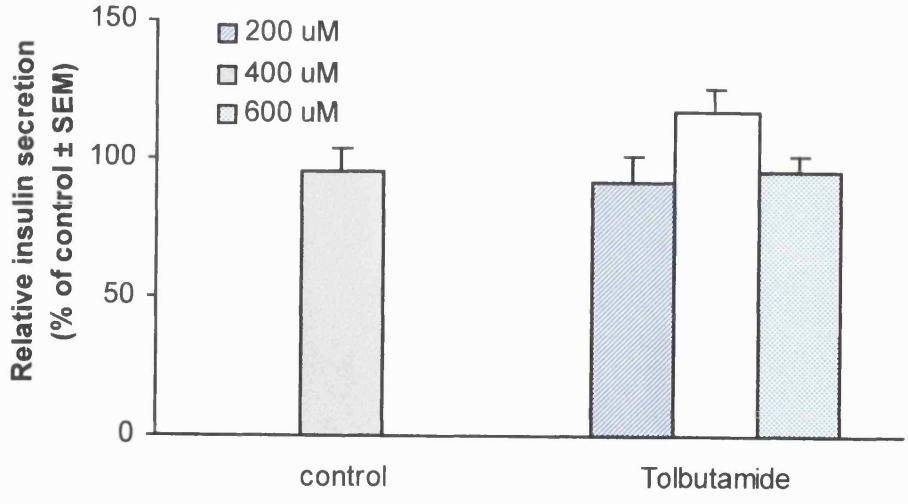


Fig. 5.10: Effect of tolbutamide on insulin secretion from RINm5F cells

Results are expressed as mean of relative insulin secretion ± SEM. Mean value for control (2% DMSO) was 2.05 ± 025 ng/10^6 cells/hr. Each sample was investigated with 6 observations.

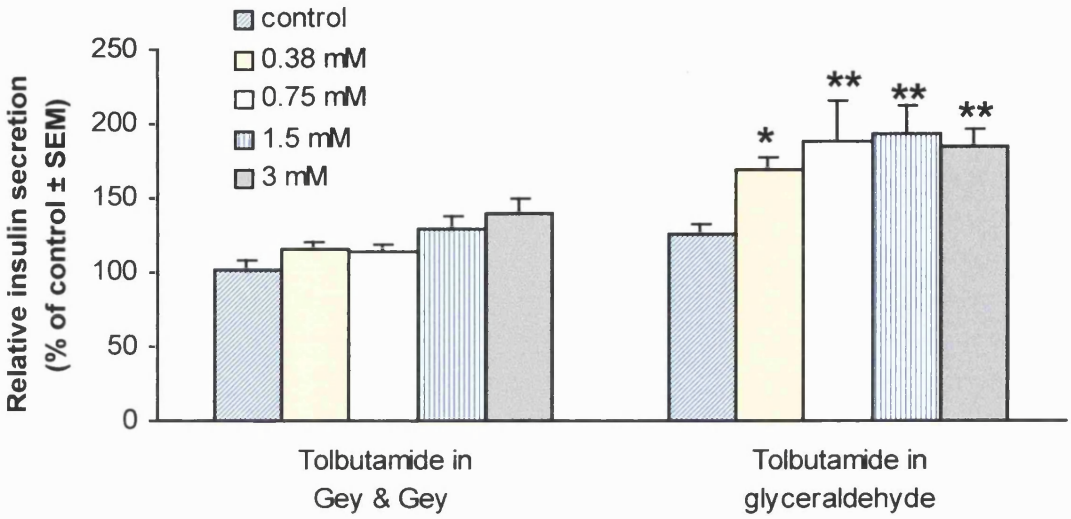


Fig. 5.11: Effect of tolbutamide on insulin secretion from RINm5F cells in the absence and presence of 10 mM glyceraldehyde

Results are expressed as mean of relative insulin secretion ± SEM. Each sample was investigated with 6 replicates. Mean values was 1.24 ± 0.06 ng/10^6 cells/hr for control (2% DMSO), and 1.59 ± 0.10 ng/10^6 cells/hr for 10 mM glyceraldehyde in 2% DMSO. **P < 0.01, *P < 0.05 VS 10 mM glyceraldehyde

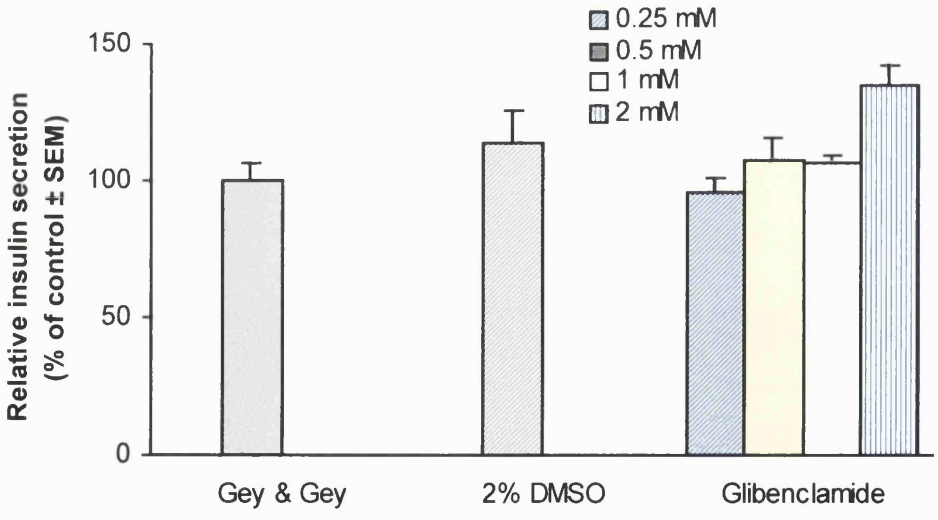


Fig. 5.12: Effect of glibenclamide on insulin secretion from RINm5F cells

Results are expressed as mean of relative insulin secretion ± SEM. Mean value for basal insulin secretion (Gey & Gey buffer) was 4.13 ± 0.28 ng/10^6 cells/hr. Each sample was investigated with 6 observations.

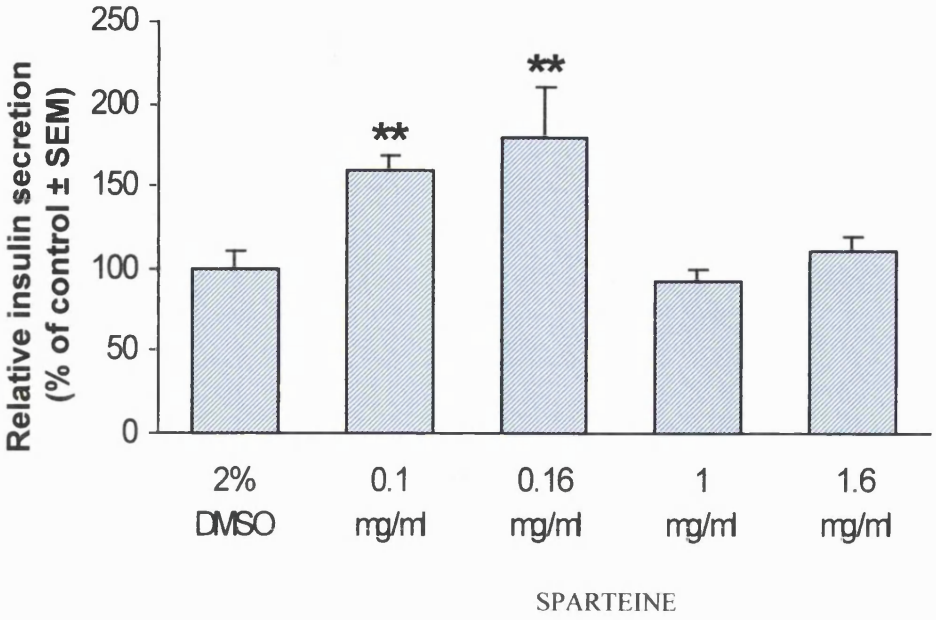


Fig. 5.13: Effect of sparteine on insulin secretion from RINm5F cells

Results are expressed as mean of relative insulin secretion ± SEM from 2 individual experiments. Mean value of basal insulin secretion was 2.05 ± 0.013 ng/10^6 cells/hr. Each sample was investigated with 6 observations. **P < 0.01 VS control.

5.7 Investigation of the effect of plant extracts on RINm5F cells

5.7.1 Introduction

It is well established that major disorders in diabetic patients are consequences of a defect in insulin secretion (section 1.1). The use of available insulin-stimulating agents (i.e. sulphonylureas) is always hampered by side effects and ineffectiveness in long term treatment (section 1.2.2.1). Thus, agents that could improve the secretory response with greater effectiveness and fewer toxic effects than sulphonylureas could prove to be a valuable treatment for diabetes. Over the past decade, medicinal plants have been proven to be a promising source for novel anti-diabetic agents (Marles and Farnsworth, 1995). This study was conducted to investigate the effect of 31 selected plants on insulin secretion on RINm5F cells.

5.7.2 Materials and methods

Plants selected for the studies and their sources are listed in Table 2.2. The aqueous extracts of these plants were prepared according to method described in

section 2.5. The dried extracts were reconstituted in Gey & Gey buffer to give a final concentration of 1.0 mg/ml. Insulin secretion experiments and the measurement of insulin secreted in response to the plant extracts was conducted using the method described in section 5.4.2. and 5.4.3.3. Briefly, RINm5F cells were seeded into 96-well plates at a density of 150,000 cells/well. Forty eight hours after seeding, the cells were pre-incubated with glucose-free RPMI 1640. After removing RPMI, RINm5F cells were incubated with plant extracts (1 mg/ml) for 1 hour (95% air/5% CO_2). Each extract was investigated with 6 observations. At the end of incubation, supernatant (150 µl) taken from 96-well plate was diluted (1:1) with borate buffer. The diluted supernatant (100 µl) was then measured for the content of insulin using the method described in section 5.4.3.3. Significance of the response between experimental samples and control was statistically analysed by ANOVA.

5.7.3 Results and discussion

Screening of the aqueous extracts of the 31 plants (Table 5.3) revealed that at a concentration of 1 mg/ml, there were 3 plants i.e. *Anemarrhena asphodeloides* (Liliaceae), *Platycodon grandiflorum* (Campanulaceae) and *Parvatia spp.* (Lardizabalaceae) that induced a significant release of insulin from RINm5F cells (Fig. 5.14). *Parvatia spp.* was only a possible identity of this plant, the true identity was still unknown (section 2.3). The plant was purchased as *Opuntia spp.*, however inspection for authentication revealed that its features did not match *Opuntia spp.* and the closest match of this plant was *Parvatia spp.* (see section 2.4 for results of authentication)

Of the three plants, the most potent effect was observed in the aqueous extract of *Anemarrhena asphodeloides* which was capable of increasing the secretion of insulin by 2.68 fold compared to control. Besides *A. asphodeloides,* two other plants from Liliaceae family were also selected for the studies, these plants were *Ophiopogon japonicus* and *Polygonatum odoratum.* In contrast to *Anemarrhena asphodeloides*, the two plants were found to have no stimulatory effect on insulin secretion (Table 5.3). The aqueous extracts of *Platycodon grandiflorum* and *Parvatia spp.* were found to increase the secretion of insulin by 2.15 and 1.74 fold, respectively (Table 5.3 and Fig. 5.14).

Further, extracts of *A. asphodeloides*, *Parvatia spp.* and *P. grandiflorum* were investigated for effects on cell integrity in order to determine whether the increases in insulin release were due simply to a detrimental effect of the plants on cell membrane integrity, or due to the effect of plant extracts on insulin secretion regulating pathway.

Table 5.3: Effect of the aqueous extracts of selected plants on insulin secretion

Tested Plants*	Relative insulin secretion (Mean ± SEM)†
Alismataceae	
Alisma orientalis	1.65 ± 0.07
Araceae	
Typhonium giganteum	1.69 ± 0.24
Araliaceae	
Panax ginseng	1.26 ± 0.01
Bixaceae	
Bixa orellana	1.74 ± 0.28
Campanulaceae	
Platycodon grandiflorum	2.15 ± 0.29**
Celastraceae	
Salacia reticulata	0.70 ± 0.13
Compositae	
Atractylodes lancea	1.57 ± 0.40
Atractylodes macrocephala	1.39 ± 0.07
Cucurbitaceae	
Benincasa hispida	1.75 ± 0.17
Coccinia indica	1.18 ± 0.09
Trichosanthes kirilowii	1.25 ± 0.18
Cyperaceae	
Cyperus rotundus	0.62 ± 0.10
Gentianaceae	
Swertia spp.	0.88 ± 0.04
Labiatae	
Salvia miltiorrhiza	1.35 ± 0.05
Lardizabalaceae	
Parvatia spp	1.74 ± 0.12 **

Table 5.3 (Continued)

Tested Plant*	Relative insulin secretion (Mean ± SEM)†
Leguminosae	
Pterocarpus marsupium	1.22 ± 0.22
Liliaceae	
Anemarrhena asphodeloides	2.68 ± 0.44**
Ophiopogon japonicus	1.23 ± 0.16
Polygonatum odoratum	1.42 ± 0.23
Meliaceae	
Azadirachta indica	1.47 ± 0.26
Menispermaceae	
Tinospora cordifolia	1.12 ± 0.06
Moraceae	
Ficus bengalensis	0.33 ± 0.05**
Morus alba	0.80 ± 0.08
Piperaceae	
Piper longum	1.15 ± 0.07
Polyporaceae	
Ganoderma lucidum	0.68 ± 0.12
Ranunculaceae	
Coptis chinensis	1.13 ± 0.19
Rutaceae	
Murraya koenigii	0.78 ± 0.13
Scrophulariaceae	
Rehmannia glutinosa	0.64 ± 0.07
Scrophularia ningpoensis	0.83 ± 0.11
Solanaceae	
Lycium chinensis	0.92 ± 0.06
Zingiberaceae	
Curcuma longa	0.65 ± 0.08

*Nominal identities given. For results of authentication details see Table 2.3. ** $P < 0.01$ VS Gey & Gey buffer. †Results are expressed as mean of insulin secretion relative to control (Gey & Gey buffer) ± SEM from 2 separate experiments conducted with 6 replications for each sample. Control values in these experiments ranged from 0.53 to 1.87 ng/10^6 cells/hr.

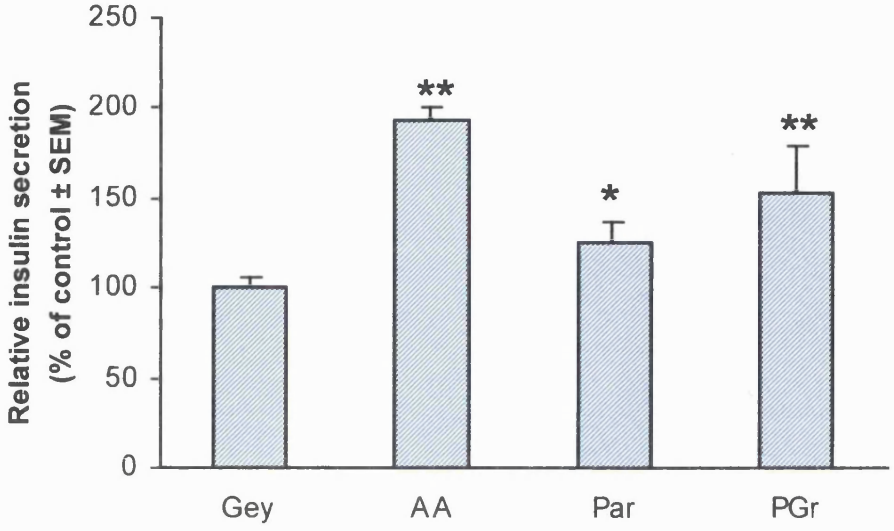


Fig. 5.14: Stimulatory effect on insulin secretion of the active plant extracts

AA: *Anemarrhena asphodeloides*, Pa: *Parvatia spp.*; PGr: *Platycodon grandiflorum*.

Results are expressed as mean of relative insulin secretion ± SEM from two separate experiments conducted with 6 replications for each sample. **P< 0.01, *P<0.05 VS control.

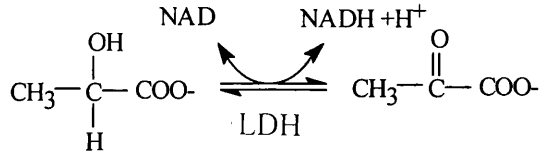
5.8 Investigation of the damaging effect of plant extracts on RINm5F cell membrane

5.8.1 Introduction

Assessment of cell integrity plays a very important role in an interpretation of the assay results in this study. Many assays have been developed to determine the integrity and viability of the cells, for examples a measurement of lactate dehydrogenase (LDH) leakage, and those using dye such as MTT, neutral red (NR) and sulforhodamine B (SRB) (Skehan *et al.* 1990; Zhang *et al.* 1990; Sepp *et al.* 1996). The choice of assay depends on the nature and aim of the study. In this study, the toxicity test was considered to be of importance, since it was an indicator whether the release of insulin was due to toxic effect of plant extracts on cell membrane integrity or due to an effect on regulating secretory pathways.

In the present study, cell integrity was assessed by measurement of lactate dehydrogenase (LDH, L-lactate: NAD oxidoreductase, EC 1.1.1.27) activity leaked from the cells into the assay medium. LDH is a common cytosolic enzyme present in all eukaryotic cells. The function of LDH in the cells is to catalyse the reaction of pyruvate to L-lactate in the presence of NADH (Bergmeyer and Bernt, 1974).

The equilibrium is far on the side of lactate and NAD^+ at neutral pH. The determination of LDH activity is based on the reaction below. Due to the fact that NADH but not NAD^+ can absorb UV light at 340 nm, LDH activity can be measured spectrophotometrically by monitoring the decrease in the optical density at 340 nm due to depletion of NADH on addition of pyruvate. The initial slope is proportional to the concentration of LDH in the reaction.



Lactate Pyruvate

Measurement of LDH activity in the assay medium was performed following exposure of the cells to those extracts which were found to cause a significant release of insulin from RINm5F cells above control rate. A significant increase in the release of insulin together with an increase in LDH activity in the assay medium is an indication of changes in cell integrity, suggesting that the insulin is released as a result of an alteration of cell membrane permeability rather than an effect on regulation pathway.

5.8.2 Materials and methods

Unless specified, chemicals and reagents were purchased from Sigma Chemical Co. (Sigma Aldrich Co. Ltd., Irvine, UK)

After the cells were exposed to plant extracts (section 5.7.2), the supernatant (175 µl) was diluted with borate buffer, 200 µl was utilised for determination of content of insulin released using RIA (see section ..4.3) and the remaining was taken for measuring an activity of LDH leaked from the cells. The diluted supernatant (50 µl) was transferred to another 96-well plate. Freshly-

prepared NADH solution (65 mM, 1 µl) was added to the supernatant, followed by the addition of 100 µl of pyruvic acid (0.62 mM) to give a final concentration of 0.43 mM NADH and 0.41 mM pyruvate. The optical density (340 nm) was measured as rapidly as possible using a plate reader (Anthos Labtec HT3) after the addition of pyruvate to the reaction.

The optical density was measured every 1 minute for the first 5 minutes, and then every 5 minutes until no further change in the optical density was observed. Total releasable intracellular LDH activity was determined by exposing the cells to 0.2% Triton X-100 in the same manner as treatment with plant extracts (section 5.7.2). For each sample, the assay was carried out using the supernatant from 3 different wells and each sample was determined in duplicate. The extent of LDH leakage was determined from the rate of decrease in the optical density at 340 nm (OD 340) over a period of time from a graph constructed by plotting the optical density against time. Decreasing optical density i.e. slope of the graph represented rate of oxidation of NADH, therefore the content of LDH present in the assay medium. The results were expressed as percentage of leakage of LDH, compared to cells exposed to 0.2% Triton X-100 which was regarded as 100% LDH leakage.

In order to confirm that the method would be able to detect LDH present in the samples, the activity of commercially available LDH (final concentrations of 18, 36 and 92 mU) was measured using the method described above. For the 3 extracts identified as stimulating insulin secretion (section 5.7.3), the secretion experiment was repeated and 50 µl of the diluted supernatant (section 5.7.2) was also taken to determine LDH activity as described above.

5.8.3 Results and discussion

As shown in Figure 5.15, the initial rate of decrease in optical density varies according to the amount of LDH in the reaction. The more LDH in the reaction, the greater the slope (Fig. 5.16). This result confirmed that LDH in the cells could be detected by this method and that the slope was proportional to LDH concentration.

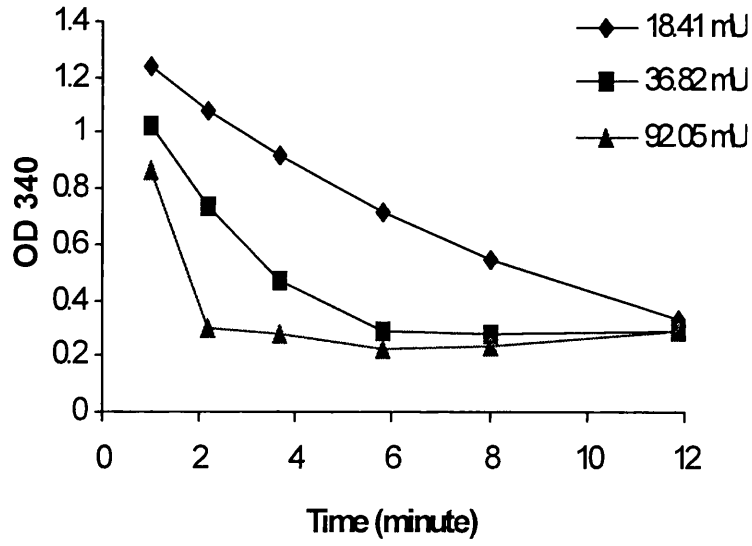


Fig. 5.15: Activity of commercial LDH

Results are expressed as mean of optical density from 3 different wells. Each sample was assayed in duplicate.

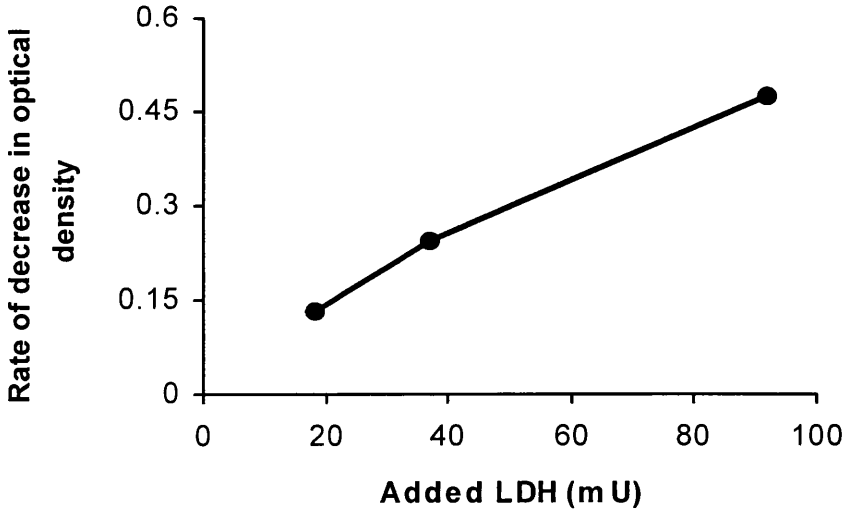


Fig. 5.16: Changes in optical density against amount of commercial LDH added to the reaction (calculated from data presented in Fig. 5.15)

The investigation of the effect of *Anemarrhena asphodeloides*, *Platycodon grandiflorum* and *Parvatia spp.* on LDH leakage revealed that, at a concentration of 1 mg/ml, the aqueous extract of *P. grandiflorum* caused a marked leakage of LDH (41.92 % of Triton X-100) from RINm5F cells after 1-hour incubation, whereas the extracts of *A. asphodeloides* and *Parvatia spp.* caused the leakage of only 8.79 % and 7.80 %, respectively (Fig. 5.17). LDH leakage in response to *A. asphodeloides* and *Parvatia spp.* was found be very close to the control (9.48%), suggesting that these two extracts did not have a detrimental effect on the membrane of RINm5F cells. In contrast, it was clearly demonstrated that exposure of the cells to *P. grandiflorum* extract caused significant changes in cell integrity as judged by marked leakage of LDH, suggesting that the release of insulin from RINm5F cells in response to *P. grandiflorum* extract was due to a toxic effect of the plant extract on cell membranes. In contrast, it appeared that the release of insulin in response to the extracts of *A. asphodeloides* and *Parvatia spp.* was not associated with cell

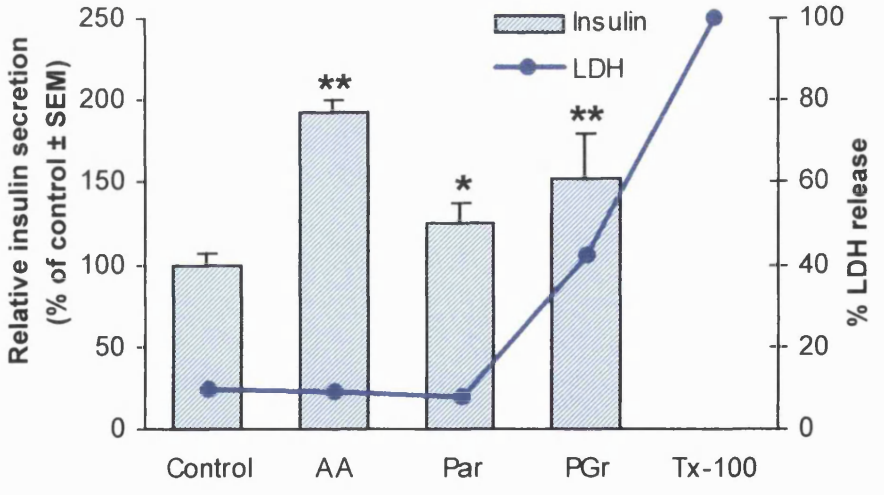


Fig. 5.17: Effect of the three active plant extracts on insulin secretion and LDH leakage from RINm5F cells

AA: *Anemarrhena asphodeloides*, PGr: *Platycodon grandiflorum*; Par: *Parvatia spp.* Results are expressed as mean of relative insulin secretion ± SEM with 6 replications for each sample. LDH leakage is expressed relative to the values obtained from cells exposed to 0.2 % Triton X-100. Changes in OD(340) per minute was 0.2395 ± 0.07 for Triton X-100 (mean from two separated experiments ± SEM conducted with 3 replications), **P< 0.01, *P<0.05 VS control.

membrane damage, suggesting that the aqueous extracts of these two plants may have an effect on regulating pathway of insulin secretion.

Due to its having the most potent effect of the plant tested, the extract of *A. asphodeloides* was selected for further investigation which involved bioassay-guided fractionation and phytochemical investigation (Chapter 6) in order to isolate the constituents responsible for the effect of this plant. Although *Parvatia spp.* was also found to have stimulatory effect on insulin secretion without damaging the cell membrane, no further investigation was carried out on this plant due to the ambiguity of its identity (see section 2.3 for authentication of plant materials).

Anti-diabetic activity of *Anemarrhena asphodeloides* has been reported in a number of *in vivo* studies (Takahashi *et al.* 1985; Hsu, 1992; Nakashima *et al.* 1993). Aqueous extract of *A. asphodeloides* (100 mg/kg, i.p.) was reported to reduce plasma glucose level in fasted alloxan- (Nakashima *et al.* 1993) and STZ-induced (Hsu, 1992) diabetic rats. In normal rats, the effect of dried extracts from rhizome of *A. asphodeloides* was observed at the doses of 500 mg/kg (i.p.) and 2.5 g/kg (Takahashi *et al.* 1985; Hsu, 1992), but not at a lower dose of 100 mg/kg (i.p.) (Hsu, 1992). The ability of *A. asphodeloides* to lower blood glucose level in both normal and experimental diabetic animals with pancreatic damage has led to an implication that its activity was possibly owing to both pancreatic and extrapancreatic effects. Results in this current study have given a strong support to pancreatic activity of this plant. Extrapancreatic activity of *A. asphodeloides* , if present, was less unlikely to be due to the inhibitory effect on intestinal glucose absorption, since it was found that the extract had no effect on glucose uptake into BBMV (Chapter 3). In addition, the *in vivo* effects were reported in basal glycaemia and not glucose tolerance.

A literature survey of the selected 31 plants showed that neither *A. asphodeloides* nor *Parvatia spp.* extract has previously been reported to have initiation effect on insulin secretion. In fact, to date, there has been no report on the effect of *Parvatia spp.* on hypoglycaemic activity. According to the survey (section 2.2), the selected plants which have been reported to exert their effects, at least in part, via stimulation of insulin release were *Azadirachta indica*, *Ficus bengalensis* and *Pterocarpus marsupium*, and ganoderan B isolated from *Ganoderma lucidum*.

The hypoglycaemic effect of *A. indica* was reported to be owing to the potentiation of glucose-induced insulin secretion. However, it was found in this current study that an aqueous extract of *A. indica* had no effect on insulin secretion from RINm5F cells. This may be due to the fact that the extract was tested in the absence of nutrient. It was also reported that leucopelargonin (100 and 250 mg/kg, p.o.) isolated from *F. bengalensis* was shown to increase insulin level in alloxan-treated animals (Cherian and Augusti, 1995). Epicatechin from *P. marsupium* was reported to increase insulin secretion from islets (Hii and Howell, 1984, for literature survey on the activity of these plants see section 2.2). Ganoderan B was reported to stimulate insulin secretion when given intraperitoneally to normal mice at 100 mg/kg (Hikino *et al.* 1989a). However, in this current study neither of these plants was found to stimulate the secretion of insulin from RINm5F cells. This may be the due to the fact that the extracts were tested at a dose containing lower compound concentrations compared to the concentrations of pure compounds tested in the above studies or differences between the experimental models used. It is noteworthy that in the study by Hikino *et al.* (1989a) the effect of ganoderan B was observed when the compounds was given intraperitoneally which may not be related to the effect of oral administration.

In agreement with the results observed *in vivo* which demonstrated that the anti-diabetic effect of *Bixa orellana* (Morrison, 1985) and *Ophiopogon japonicus* (Kako *et al.* 1995) was unlikely to be related to stimulation of insulin secretion, it was found that these two plants had no stimulatory effect on insulin secretion from RINm5F cells, suggesting that the *in vivo* anti-diabetic effect observed in these two plants was probably due to mechanisms other than stimulation of insulin secretion.

CHAPTER 6

EFFECT OF EXTRACTS AND COMPOUNDS FROM *ANEMARRHENA ASPHODELOIDES* ON INSULIN SECRETION

6.1 Ethnopharmacology and phytochemistry of *Anemarrhena asphodeloides* Bunge.

Anemarrhena asphodeloides is a member of Liliaceae family and Liliiflorae order. Rhizomes of this plant have been prescribed in traditional Chinese medicine as an antipyretic, expectorant, laxative, sedative, anti-inflammatory and diuretic (Duke and Ayensu, 1985b). It has also been used for bronchitis, influenza, pneumonia, scarlet fever, tuberculosis and typhoid (Duke and Ayensu, 1985b). *A. asphodeloides* rhizome was also found to have antimicrobial activity, inhibit platelet aggregation, inhibit carcinoma and decrease radiation injury (Ma *et al.* 1997). However, the most frequent use of *A. asphodeloides* is for the treatment of diabetes (Takahashi *et al.* 1985; Ma *et al.* 1997).

The rhizomes have been used in Kampo (Japanese) medicine in a combination with *Glycyrrhiza glabra* root, *Panax ginseng* root, gypsum fiber and rice to treat diabetes (Nakashima *et al.* 1993). This combination is known as Byakko-ka-ninjin-to (BN). BN (500 mg/kg, i.p.) was reported to reduce the level of plasma glucose in KK-A^y and alloxan-induced diabetic mice (Kimura *et al.* 1999). It was found that each crude drug tested at doses equivalent to those present in BN, except rice, could exhibit anti-diabetic effect by themselves in KK-A^y mice (Kimura *et al.* 1999). Additionally, it was demonstrated that intraperitoneal injection of extracts of *A. asphodeloides* rhizomes (170 mg/kg), ginseng roots (90 mg/kg) and liquorice (80 mg/kg) lowered blood glucose level in KK-A^y mice to the same extent as BN tested at the equivalent doses (Kimura *et al.* 1996). *Anemarrhena asphodeloides* is also one of the active ingredients in two other preparations of traditional Japanese medicine known as Seishin-kanro-to (SK) and Shokatsu-Cha (Xiao-Ke-Ca) (Miura *et al.* 1996; Sanae *et al.* 1996). A study on anti-diabetic effect of SK, which consists of 13 plant extracts, revealed that the extract (1.7 g/kg, p.o.) caused a significant reduction of blood glucose in KK-A^y mice and also inhibited an elevation of blood glucose level in glucose-loaded mice (Miura *et al.* 1996). It was suggested that the anti-diabetic effect of SK was primarily due to a synergistic effect of *Anemarrhena asphodeloides* and *Rehmannia glutinosa* (Miura *et al.* 1996). Pharmacological study on the effect of Shokatsu-Cha which is composed of 8 crude

drugs showed that an administration of the extract (3 g/kg, p.o.) to STZ-induced diabetic mice for 10 days could inhibit an elevation of blood glucose level (Sanae *et al.* 1996). However, the active ingredients in this formulation have not been identified.

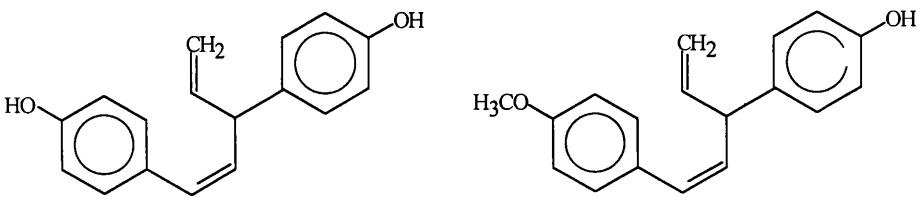
Compounds reported to be isolated from *A. asphodeloides* include glycans, lignans, saponins and xanthones. The activities of these compounds are revealed briefly here.

6.1.1 Glycans from *Anemarrhena asphodeloides*

Glycans isolated from this plant included anemarans A, B, C and D (Takahashi *et al.* 1985). A study on the pharmacological effect of these glycans revealed that anemaran A, B, C and D (10-100 mg/kg, i.p.) significantly lowered plasma glucose levels in normal mice at 7 hours after administration. Anemaran C which is the main glycan in the extract was also found to reduce the level of plasma glucose when given to alloxan-induced diabetic mice at 100 mg/kg (i.p.) (Takahashi *et al.* 1985). It was noteworthy that anti-diabetic effects of these glycans were evidenced in normal and/or diabetic animals after intraperitoneal administration. The relevance of this data to their traditional use (usually oral) is therefore limited.

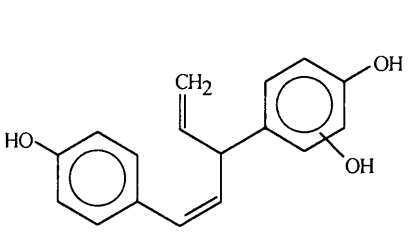
6.1.2 Lignans from *Anemarrhena asphodeloides*

Four lignans reported to be present in this plant include *cis*-hinokiresinol, 4′-methyl-*cis*-hinokiresinol, oxy-*cis*-hinokiresinol and 1,3-di-*p*-hydroxyphenyl-4-penten-1-one (Nikaido *et al.* 1981; Jeong *et al.* 1999) (Fig. 6.1). An investigation of the inhibitory effect on phosphodiesterase *in vitro* showed that aqueous extract of *A. asphodeloides* was capable of inhibiting the effect of beef heart phosphodiesterase, and the compounds which were claimed to be responsible for the effect were *cis* hinokiresinol and oxy-*cis*-hinokiresinol (Nikaido *et al.* 1981).

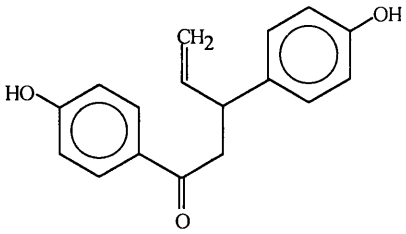


Cis-Hinokiresinol 4′-Methyl-*cis*-hinokiresinol

Fig. 6.1: Structure of lignans isolated from *Anemarrhena asphodeloides*



Oxy-*cis*-Hinokiresinol



1,3-di-*p*-Hydroxyphenyl-4-penten-1-one

Fig. 6.1 (continued): Structure of lignans isolated from *Anemarrhena asphodeloides*

6.1.3 Saponins from *Anemarrhena asphodeloides*

The rhizome of *Anemarrhena asphodeloides* is reported to contain about 6% saponins (Chang and But, 1986a), of which sarsasapogenin, timosaponin AIII, B, BI, BII and F, anemarrhenasaponin I, Ia, II and III, anemarsaponin F and G, aspidistrin, markagenin 3-*O*-*β*-D glucopyranosyl-(1→2)-galactopyranoside, degalactotigonin, diuranthoside A, and 3-*O*-β-lycotetraosyl yamogenin have been isolated (Kawassaki and Yamauchi, 1963; Nagumo *et al.* 1991; Nakashima *et al.* 1993; Saito *et al.* 1994; Ma *et al.* 1997; Meng *et al.* 1999). Structures of these saponins are shown in Figure 6.2 and 6.3. Among them, sarsasapogenin (Fig. 6.2) was reported to be main aglycone in this plant (Chang and But, 1986a).

A number of these saponins have been reported to have inhibitory effects on platelet aggregation, including anemarrhenasaponin I and Ia, timosaponin AIII, B, BI, BII and BIII in human and animal models (Ma *et al.* 1997; Zhang *et al.* 1999a). The results have led to a suggestion that these saponins might be useful as antithrombotic therapeutic agents in post-myocardial infarction patients (Zhang *et al.* 1999a).

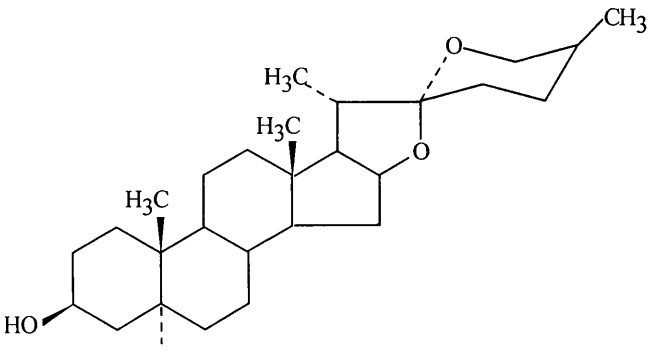
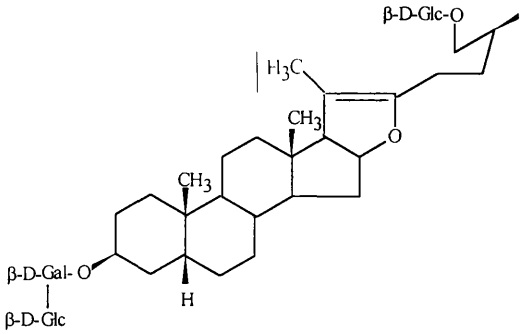
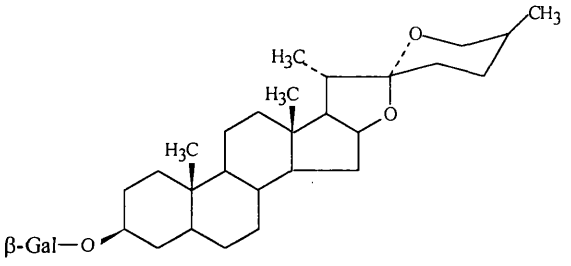


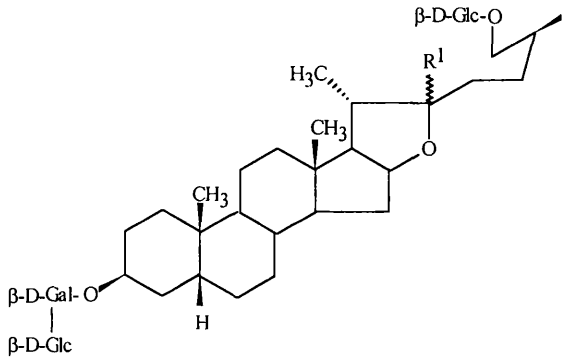
Fig. 6.2: Structure of sarsasapogenin



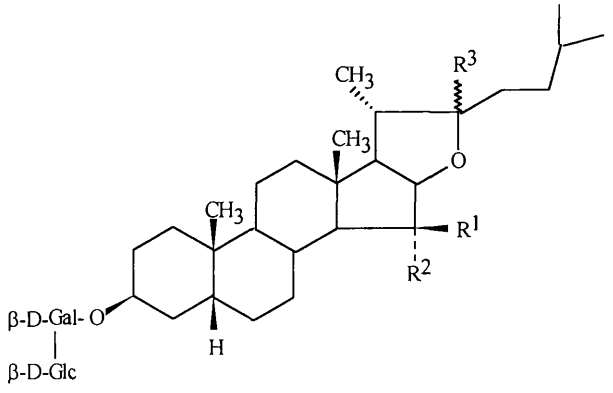
Timosaponin B
(Psuedoprototimosaponin AIII)



Timosaponin AI

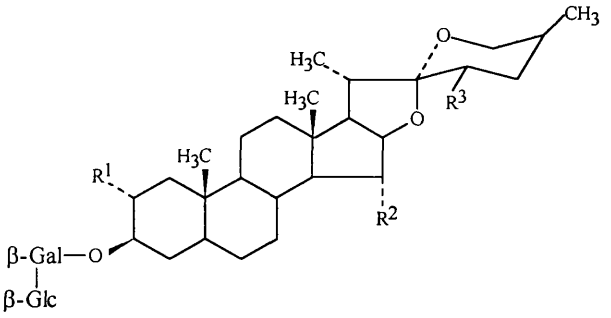


Timosaponin BI	$R^1 = OCH_3$
Timosaponin BII (prototimosaponin AIII)	$R^1 = OH$

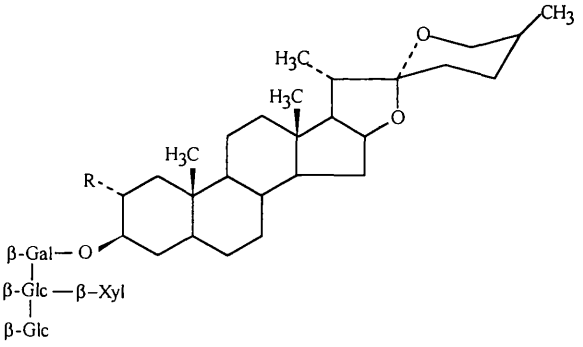


Anemarrhenasaponin I	: $R^1 = H$,	$R^2 = OH$	$R^3 = OH$
Anemarrhenasaponin Ia	: $R^1 = H$,	$R^2 = OH$	$R^3 = OCH_3$
Anemarrhenasaponin II	: $R^1 = OH$	$R^2 = H$	$R^3 = OH$

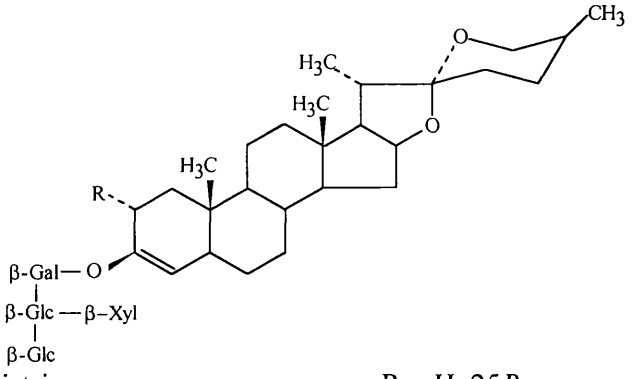
Fig. 6.3: Structures of saponins isolated from *Anemarrhena asphodeloides*



Anemarrhenasaponin III	R^1 = H	R^2 = OH	R^3 = H
Markagenin 3-*O*-*β*-D glucopyranosyl-(1→2)-galactopyranoside	R^1= β-OH	R^2 = H	R^3= H
Timosaponin AII	R^1 = H	R^2 = H	R^3 = H
Timosaponin F	R^1 = H	R^2 = OH	R^3 = OH



Anemarsaponin F	R = OH, 25*S*
Degalactotigonin	R = H, 25*R*
Diuranthoside	R = H, 25*S*



Aspidistrin	R = H, 25*R*
3-*O*-*β*-Lycotetraosyl yamogenin	R = H, 25*S*
Anemarsaponin G	R = OH, 25*S*

Fig. 6.3 (continued): Structures of saponins isolated from *A asphodeloides*

Compounds such as anemarrhenasaponin I, anemarrhenasaponin Ia, timosaponin B and timosaponin BIII were reported to significantly suppress a generation of superoxide induced by both N-formyl-methionyl-leucyl-phenylalanine (fMLP) and arachidonic acid *in vitro* (Meng *et al.* 1999; Zhang *et al.* 1999b). Timosaponin BII was found to suppress the generation of superoxide induced by fMLP but not arachidonic

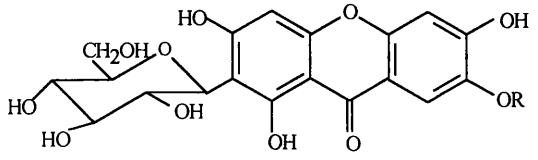
acid and the opposite effect was reported in timosaponin AIII (Meng *et al.* 1999; Zhang *et al.* 1999b), suggesting the potential effect of these compounds as anti-inflammatory agents (Meng *et al.* 1999).

A study on the effect of *Anemarrhena asphodeloides* extract on cytotoxicity against human tumour cell lines by Lee *et al.* (1995) showed that timosaponin AIII caused a significant cell growth inhibition in a number of cell lines including A-549, SK-OV-3, SK-MEL-2, XF498 and HCT15, whereas the aglycone, sarsasapogenin, had no effect on any of these cell lines, suggesting that the presence of the sugar moieties was important for activity.

6.1.4 Xanthones from *Anemarrhena asphodeloides*

Two xanthones which have been isolated from *Anemarrhena asphodeloides* included mangiferin and mangiferin glucoside (Ichiki *et al.* 1998).

Mangiferin is widely distributed in nature, it has been isolated from a number of plant families including Anacardiaceae, Iridaceae, Convovulaceae, Gentianaceae, Guttiferae, Leguminosae, Liliaceae and Polypodiaceae. Pharmacological activities of mangiferin which have been reported included inhibitory effects on platelet thromboxane and inositolphosphate formation in rabbit platelets (Teng *et al.* 1989), antioxidant activity (Sato *et al.* 1992), anti-viral activity against herpes simplex virus (HSV-2) plaque formation in HeLa cells *in vitro* (Zhu *et al.* 1993) and antitumour activity against ascitic fibrosarcoma in mice (Guha *et al.* 1996).



Mangiferin: R = H

Mangiferin -7-*O*-*β*- glucoside: R = Glucose

Fig. 6.4: Structure of xanthones isolated from *Anemarrhena asphodeloides*

6.2 Aim of the present study

The work described in this chapter aimed to investigate the effects of fractions and compounds isolated from crude extract of *Anemarrhena asphodeloides* which was the most potent extract observed in the screening on RINm5F cells (Chapter 5).

6.3 Effect of solvent on insulin secretion from RINm5F cells

6.3.1 Introduction

The initial studies had employed a water extract of *Anemarrhena asphodeloides* (section 5.7) in order to mimic the traditional method of consuming this herb. The insulin secretagogue activity of other extracts of *Anemarrhena asphodeloides* which included less polar and polar extracts were then investigated. To prepare the extracts, an appropriate solvent was required to reconstitute the extracts. Buffer was obviously not suitable for dissolving less polar extracts such as hexane and chloroform. Solvents such as dimethyl sulphoxide (DMSO) and ethanol would therefore be more appropriate in these cases. However, the solvent used in the studies should not interfere with the assay and, on the other hand, should be able to completely dissolve the extracts. Thus, the effect of two solvents i.e. DMSO and ethanol on RINm5F cells was investigated to determine the appropriate solvent and the maximum concentration that would be suitable for the assay.

6.3.2 Materials and methods

Unless specified, chemicals were purchased from Sigma-Aldrich Co. Ltd. (Irvine, UK). Ethanol was purchased from BDH Laboratory Supplies (England). RINm5F cells were kindly provided by Dr. P.M. Jones and Dr. S.J. Persaud (Cellular and Molecular Endocrinology Group, Biomedical Sciences Division, King's College London).

The effect of DMSO (2-10% in Gey & Gey buffer) and ethanol (2-8% in Gey & Gey buffer) was investigated on RINm5F cells using the method described in section 5.4.2. The supernatant taken from cells after exposure to the solvents for 1 hour was diluted with borate buffer (1:1) and measured for the content of insulin release using radioimmunoassay as described in section 5.4.3.3.

6.3.3 Results and discussion

Investigation of the effect on RINm5F cells of DMSO and ethanol showed that DMSO at concentrations of 6-10% caused a significant release of insulin, whereas the release in response to 2 and 4% DMSO was found to be not

significantly different from the control (Fig. 6.5). The significant release of insulin observed at higher concentrations of DMSO may indicate a detrimental effect that the solvent had on cell membrane integrity. Interestingly, ethanol at the concentrations tested (2-8%) did not caused a marked release of insulin, in fact the solvent at 6-8% markedly inhibited insulin secretion from RINm5F cells, implying that ethanol had a toxic effect on the cells other than membrane damage and possibly interfered with the regulating pathway of insulin secretion.

DMSO (2%) was selected to be used in the further studies, since it was a less selective solvent and no significant difference was observed in terms of the amount of insulin release between DMSO (2%) and control (Gey & Gey buffer).

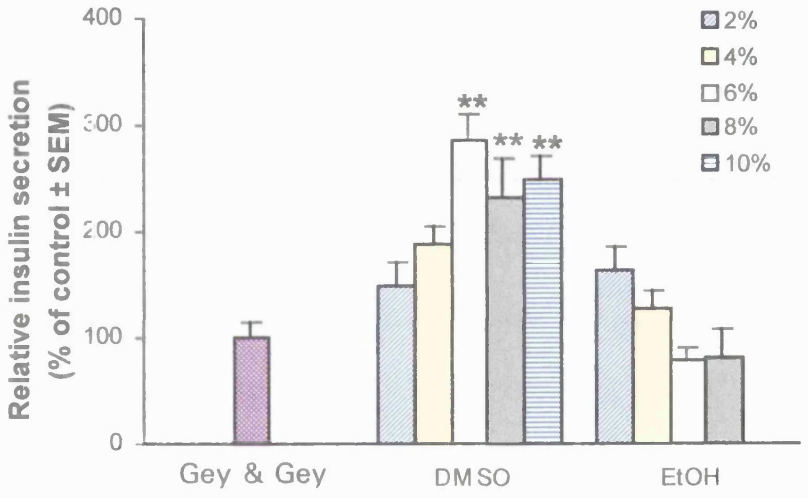


Fig. 6.5: Effect of DMSO and ethanol on insulin release from RINm5F cells

Results are expressed as relative insulin secretion (mean ± SEM) compared to control. n = 6.**P < 0.01 VS control. Basal insulin secretion (control) was 2.63 ± 0.34 ng/10^6 cells/hr.

6.4 Investigation of the activity of *Anemarrhena asphodeloides* extracts on RINm5F cells

6.4.1 Introduction

From the screening of 31 selected medicinal plants on RINm5F cells (section 5.7), the aqueous extract of *Anemarrhena asphodeloides* was found to have the most potent effect on insulin secretion without causing cell membrane damage

(section 5.8.3). The investigations described in this study aimed to identify fractions responsible for the activity observed in RINm5F cells. In this study, extracts of *A. asphodeloides* including hexane, chloroform and methanol extracts were investigated on RINm5F cells, since they may contain different constituents to the aqueous extract and/or a different content of the same constituents, which could provide more potent effect on insulin secretion.

6.4.2 Materials and methods

Dried rhizome of *A. asphodeloides* was purchased from East & West Herbs (Kingham, UK) and Institute of Chinese Medicine (London, UK). Unless specified, chemicals were analytical grade and purchased from Sigma-Aldrich Co. Ltd. (Irvine, UK). Silica gel for column chromatography and Silica GF_{254} TLC plate were purchased from Merck KGaA (Germany). Organic solvents were purchased from BDH Laboratory Supplies (England).

Dried material of *A. asphodeloides* was sequentially extracted with hexane, chloroform and methanol as described in section 7.1.2.2, whereas the aqueous extract was prepared as described in section 2.5. To test on RINm5F cells, hexane, chloroform, methanol and aqueous extracts were initially dissolved in DMSO and then reconstituted in Gey & Gey buffer to give a concentration of 1 mg/ml extract and 2% DMSO. In this case, DMSO (2%) in buffer was also used as control. The effect on insulin secretion in RINm5F cells of the four extracts at 1 mg/ml was then tested on RINm5F cells using the method described in section 5.4.2. The content of LDH leakage from the cells was measured as described in section 5.8.2. The extracts which were active and not toxic were again tested at a range of concentration (0-1 mg/ml). Results were expressed as mean of relative insulin secretion (% of control) calculated using the formula described in section 5.6.2.

6.4.3 Results and discussion

Investigation of the effect of hexane, chloroform, methanol and aqueous extracts of *Anemarrhena asphodeloides* revealed that at 1 mg/ml only the methanol and aqueous extracts were capable of increasing the release of insulin from RINm5F cells compared to control (2% DMSO), whereas hexane and chloroform extracts had

no significant effect on insulin secretion (Fig. 6.6). The most potent stimulation was observed in methanol extract in which the secretion was found to be 3.6 fold greater than that of control. The stimulation observed in the aqueous extract was 2.8 fold relative to control, whereas hexane and chloroform extracts were found to stimulate the secretion only by 1.1 and 1.7 fold, respectively (Fig. 6.6).

Measurement of LDH leakage from cells exposed to *A. asphodeloides* extracts showed that the methanol and aqueous extracts caused the leakage of LDH of 9.47% and 8.3%, respectively (Fig 6.6) relative to that observed with Triton X-100 which represented maximum leakage of LDH (100% LDH leakage). The similarity in terms of the amount of LDH leakage observed in these cases and that of control (7.52%) indicated that methanol and aqueous extracts at the dose of 1 mg/ml did not damage membranes of RINm5F cells. These findings suggested that the significant secretion of insulin in response to methanol and aqueous extracts was not a consequence of cell membrane damage, and therefore owing possibly to the influence of the extracts on the regulation pathways of insulin secretion.

It was found (Fig. 6.7-6.9) that the stimulation of insulin secretion from RINm5F cells in response to methanol and aqueous extracts of *Anemarrhena asphodeloides* was dose-dependent. A significant increase of insulin secretion was observed in the cells exposed to 0.5-5 mg/ml of the aqueous extract of *A. asphodeloides* (Fig. 6.8). Significant stimulation was observed with methanol extract at the same doses and also at a lower dose of 0.25 mg/ml (Fig. 6.9) at which the effect of the aqueous extract was found to be insignificantly different from the control (Fig. 6.8). Additionally, at any dose, it was found that the effect of the methanol extracts was more potent than that of aqueous extracts (Fig. 6.8 and 6.9).

Measurement of LDH leakage in response to the aqueous extracts of *A. asphodeloides* revealed a significant release of the enzyme from cells exposed to 5 mg/ml of the extract (Fig. 6.8); the leakage of LDH observed in this case was 37.79 % of Triton X-100. In contrast, no sign of cell membrane damage was observed at the lower doses. These results suggested that the aqueous extract at 5 mg/ml (but not at the lower doses) had toxic effect on membranes of RINm5F cells and hence the release of insulin was partly due to the damages of cell membranes. Similarly to the

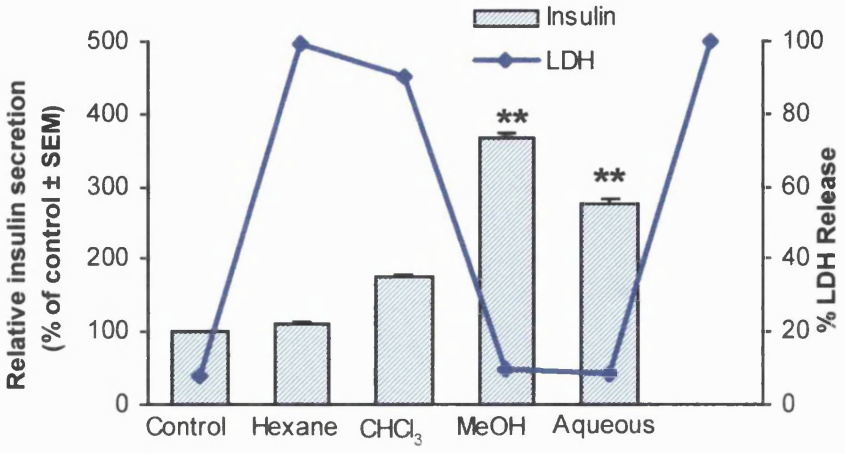


Fig. 6.6: Effect of *Anemarrhena asphodeloides* extracts on insulin secretion and LDH leakage from RINm5F cells.

Results of insulin secretion are expressed as mean ± SEM from 3 independent experiments. n = 6 (each experiment) Mean value of basal insulin release (control) was 2.03 ± 0.34 ng/10^6 cells/hr. LDH release was expressed as % leakage relative to Triton X (100 % leakage). Mean of changes in OD 340/minute observed with Triton X-100 from 2 separate experiments (n=3 each experiment) was 0.894 ± 0.01 (mean ± SEM). **P< 0.01 VS control.

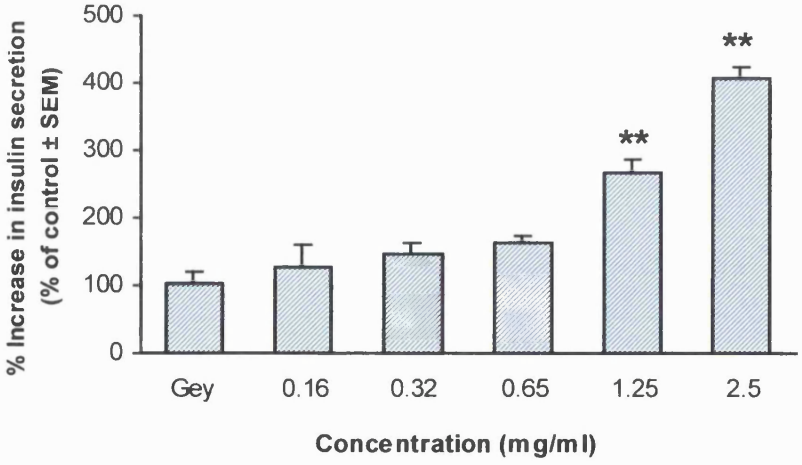


Fig. 6.7: Effect of *Anemarrhena asphodeloides* aqueous extracts on insulin release from RINm5F cells.

Results are expressed as mean ± SEM. n = 6. Mean values of basal insulin secretion (control) was 0.55 ± 0.08 ng/10^6 cells/hr. **P < 0.01 VS control.

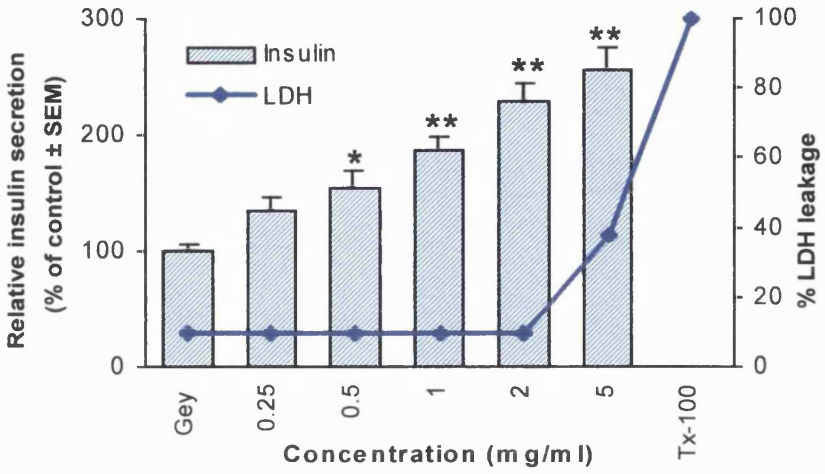


Fig. 6.8: Effect of *Anemarrhena asphodeloides* aqueous extracts on insulin release and LDH leakage from RINm5F cells.

Results of insulin secretion are expressed as means ± SEM of relative insulin secretion; each sample was assayed with 6 replicates. Mean value of basal insulin secretion (control) was 4.85 ± 0.26 ng/10^6 cells/hr. LDH release is expressed as % leakage relative to that of Triton X-100 (100% leakage). Changes in OD 340/minute observed with Triton X-100 was 0.2858 ± 0.02 (mean ± SEM). n = 3. **P< 0.01, *P < 0.05 VS control.

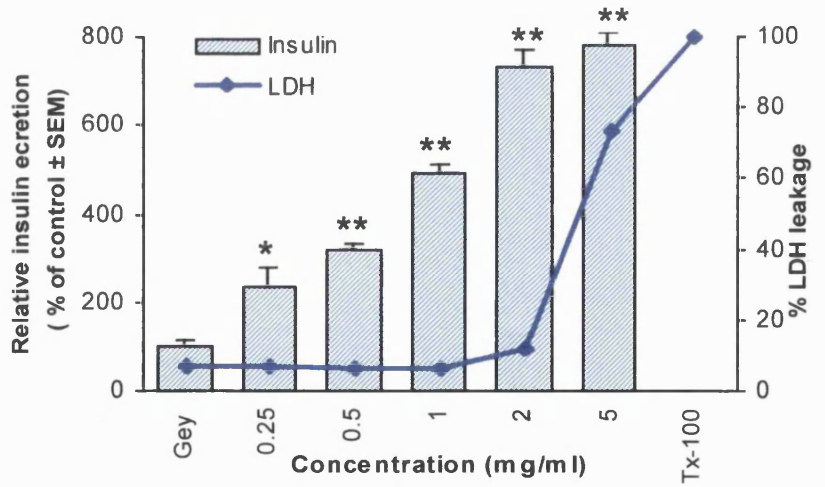


Fig. 6.9: Effect of *Anemarrhena asphodeloides* methanol extracts on insulin release and LDH leakage from RINm5F cells.

Results of insulin secretion are expressed as means ± SEM of relative insulin secretion; each sample was assayed with 6 replicates. Mean values of basal insulin secretion was 0.55 ± 0.03 ng/10^6 cells/hr. LDH release is expressed as % release relative to values obtained with Triton X-100 (100% leakage). Changes in OD 340/minute observed in Triton X-100 was 0.1232 ± 0.02 (mean ± SEM). **P < 0.01, *P < 0.05 VS control.

aqueous extract, the methanol extract was also found to have toxic effect on the cells when tested at the high dose of 5 mg/ml, as judged by a significant leakage of LDH i.e.72.99% of Triton X-100 (Fig. 6.9). According to these results, the doses of *A. asphodeloides* that caused significant release of insulin without damaging the cell membranes were 0.5-2 mg/ml for the aqueous extract and 0.25-2 mg/ml for the methanol extract. The results strongly supported the presence of compounds capable of acting on regulatory pathways of insulin secretion.

The hexane and chloroform extracts were found to cause a significant release of LDH from RINm5F cells; the leakage of LDH observed with hexane and chloroform extracts was 99.49% and 90.01%, respectively (Fig. 6.6), suggesting that the extracts had damaging effects on cell membranes of RINm5F cells. Surprisingly, the release of insulin observed in these cases was found to be only slightly affected. It was expected, in this case, that the release of insulin should also be markedly increased because the membranes had been damaged and hence content in the cells should be released into the assay medium. It was unclear why the secretion of insulin was not correlated with the leakage of LDH.

To evaluate the results observed in this study, it was further investigated whether or not the observed stimulation was artefactual results which possibly occurred if the extracts interfered with the binding of antibody and insulin in the radioimmunoassay (section 6.5).

6.5 Investigation of a possible interference in RIA by plant extracts

6.5.1 Introduction

In radioimmunoassay, false results would be inevitable if samples caused an interference in the binding between antibody and insulin. In RIA, the complex of antibody and radiolabelled insulin was measured by means of radioactivity and the content of insulin in samples was quantified by extrapolating from a calibration curve constructed by plotting % binding of radiolabelled insulin to antibody against the amount of unlabelled insulin in the assay (section 5.4.3.3). Less radiolabelled

insulin bound to antibody would be interpreted as more unlabelled insulin being present in the samples. Thus, if the binding between antibody and insulin was interfered with in any situation, the values of insulin content in such samples obtained by extrapolating from the calibration curve would be higher than they should be. If binding or precipitation of labelled insulin was enhanced for any reason, then the result would be an underestimation of insulin concentration.

This study was performed to evaluate the effect of extracts on the RIA results in order to determine the validity of the results described in section 6.4.3. In this study, known concentration of insulin was assayed by RIA in the presence of chloroform and methanol extracts, and the results were compared to those in the presence of buffer. These extracts were chosen as they had indicated low insulin release in the presence of high LDH leakage, and high insulin release, respectively.

6.5.2 Materials and methods

Radioimmunoassay was performed with varying known concentrations of insulin standard in the presence of chloroform and methanol extracts. Instead of diluting with borate buffer as usually done in RIA (section 5.4.3.3), serial dilution of insulin standard was performed by diluting with chloroform and methanol extracts (reconstituted in 2% DMSO in Gey & Gey buffer) to give the solution containing insulin at final concentration of 0-5 ng/ml and the extracts at concentration of 1 mg/ml. The assay was then performed as described in section 5.4.3.3. The solutions were added with antibody (100 µl, final concentration 1:60,000) and radiolabelled insulin (100 µl, 10,000 cpm/tube). The reaction was left at 4 °C for 48-72 hours to reach equilibrium. The content of insulin was then determined by means of radioactivity as described in section 5.4.3.3.

6.5.3 Results and discussion

It was clearly demonstrated (Fig. 6.10) that the curves of insulin bound fraction plotted against unlabelled insulin in the reaction in the presence of chloroform and methanol extracts were very similar to that of control. This suggested that the presence of chloroform and methanol extracts did not affect the binding between antibody and radiolabelled insulin. Thus, it could be concluded

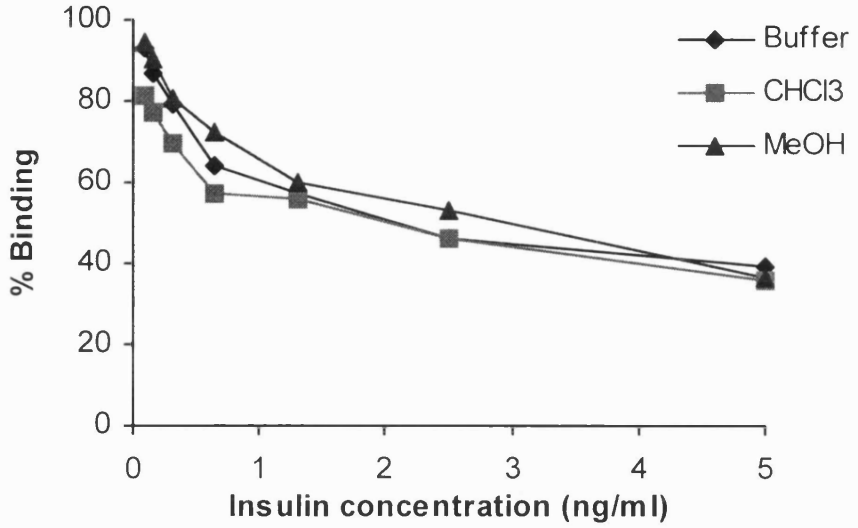


Fig. 6.10: Effect of chloroform and methanol extracts on the binding of radioactive insulin and antibody in RIA

Antibody and radioactive insulin were incubated with varying concentrations of unlabelled insulin in the presence of buffer, or buffer containing chloroform extract (1 mg/ml) or methanol extract (1 mg/ml).

with some confidence that the different insulin secretion results observed with the methanol and chloroform extracts were reliable. However, it is still unclear why the chloroform extract (which damaged cell membrane) was not accompanied by a large release of insulin. It may be that the extract also denatured the insulin in some way.

It was encouraging that the marked stimulation of insulin secretion by the methanol extract without membrane damage, was shown by these experiments to be genuine results. Further studies were undertaken on the methanol extract involving fractionation of the extracts and isolation of active compounds from the active fraction (section 6.6-6.10).

6.6 Bioassay guided fractionation of the methanol extract of *Anemarrhena asphodeloides*

6.6.1 Introduction

The most active fraction of *Anemarrhena asphodeloides* i.e. methanol extract (Fig. 6.6) was further studied in an attempt to isolate compounds which were

responsible for stimulatory effects on insulin secretion. The extract was fractionated using column chromatography, and the fractions obtained were again tested on RINm5F cells. Details of the isolation procedure are given in Chapter 7 whereas the bioassay results are given here.

6.6.2 Materials and methods

Methanol extract was further fractionated using column chromatography on silica gel (section 7.1.2.2), resulting in the separation of 5 fractions namely AAM1-AAM5. These fractions were dissolved in DMSO and then reconstituted in Gey & Gey buffer to give a final concentration of 1 mg/ml and 2% DMSO. The extracts were tested on RINm5F cells (150,000 cells/well) as described in section 6.4.2 using DMSO (2%) as the control. The supernatant (175 µl) after incubation was diluted with borate buffer (1:1); the diluted supernatant was then used for both determination of insulin content (section 5.4.3.3) and leakage of LDH (section 5.8.2).

6.6.3 Results and discussion

Of the 5 fractions separated from the methanol extract, it was found that AAM1 (1 mg/ml) had no effect on the secretion of insulin from RINm5F cells, whereas AAM4 and AAM5 slightly increased the secretion of insulin. However the effects were not significantly different from that observed in control (Fig. 6.11). Only AAM2 and AAM3 (1 mg/ml) were found to cause a significant release of insulin from RINm5F cells compared to control, and the effect of AAM2 was found to be more potent than that of AAM3. Additionally, the effect of these two fractions was found to be greater than that of crude methanol extract (AAM) since crude methanol extract was found to stimulate insulin secretion by 3.8 fold, whereas AAM2 and AAM3 increased the secretion by 7 and 4.7 fold, respectively (Fig. 6.11), suggesting that the active compounds were more concentrated in these fractions.

Measurement of LDH leakage showed that AAM1 and AAM2 caused a marked release of LDH from RINm5F cells compared to control as the leakage of LDH observed in AAM1 and AAM2 was 24.08% and 25.79%, respectively whereas

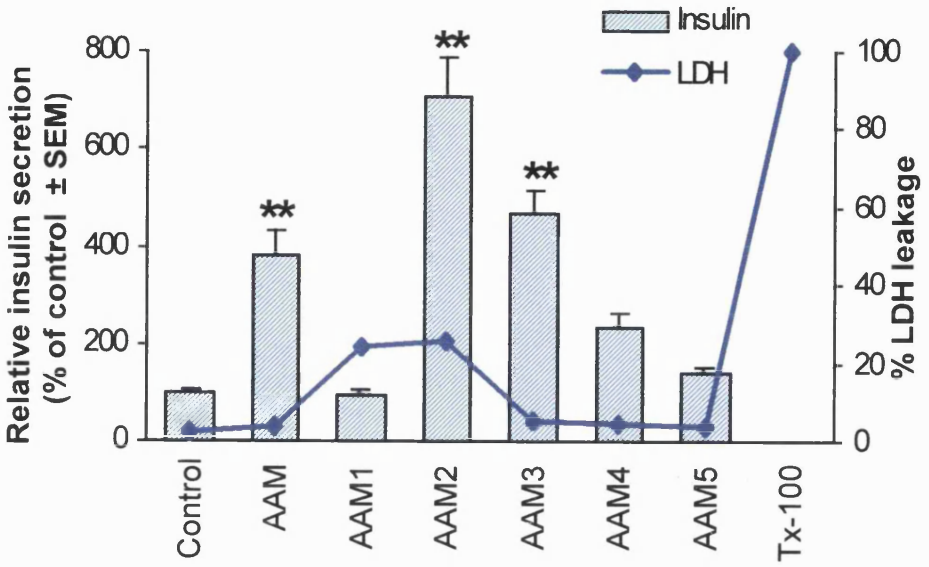


Fig. 6.11: Effect of the methanolic fractions of *Anemarrhena asphodeloides* on insulin secretion and LDH leakage from RINm5F cells

AAM: crude methanol extract; AAM1-AAM5: Fractions isolated from methanol extract

Results of insulin secretion are expressed as mean of relative insulin secretion ± SEM. Each fraction was observed with 6 replicates. Mean values of basal insulin secretion was 1.02 ± 0.13 ng/10^6 cells/hr. LDH release is expressed as % relative to those observed in 0.2% Triton X-100 (100% leakage). Changes in OD 340/minute obtained from Triton X-100 was 0.0813 ± 0.002 (mean ± SEM). n =3. **P < 0.01 VS control.

that observed in the control was 2.08% (Fig. 6.11). These results suggested that these two fractions caused damage to cell membranes, and hence were toxic to the cells to some extent. Moreover, damages of cell membrane caused by these two fractions were significantly more substantial compared to that observed in crude methanol extract (AAM) and other fractions. Marked leakage of LDH observed in AAM2 suggested that the greatly enhanced release of insulin observed in this case may partly be a consequence of cell membrane damage.

The leakage of LDH in response to AAM3 (i.e. 5.01%) was much less than those observed in AAM2 and found to be close to the control value. This suggested that the release of insulin caused by this fraction was not due to damaging effect on the cell membranes. Due to this potent effect of AAM3 on insulin secretion and negligible toxic effect on cell membrane, this fraction was selected for

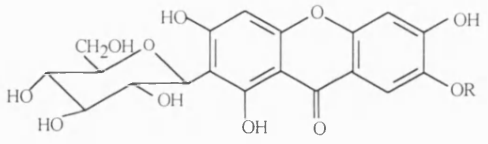
further studies which involved isolation of single compounds by means of column chromatography (section 7.1.2.2).

6.7 Investigation of the effect of compounds isolated from AAM3-fraction on insulin secretion

6.7.1 Introduction

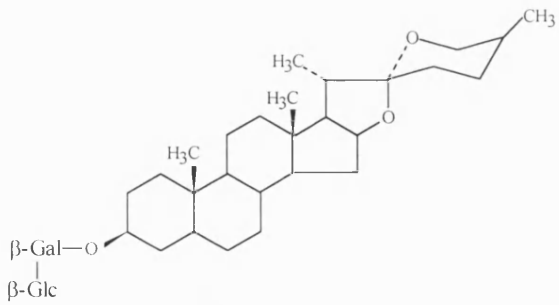
Column chromatography of AAM3 (section 7.1.2.2) resulted in the isolation of 4 pure compounds (Fig. 6.12) which included two xanthones i.e. mangiferin and mangiferin-7-glucoside, and two saponins i.e. timosaponin AIII and timosaponin BI (see section 7.2 for structure elucidation).

The isolated compounds were then tested on RINm5F cells to investigate their activity on insulin secretion.

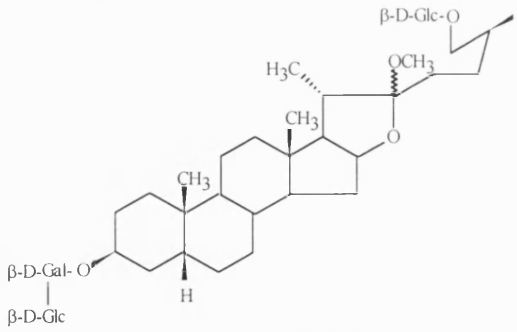


Mangiferin: R = H

Mangiferin glucoside: R= Glucose



Timosaponin AIII



Timosaponin BI

Fig 6.12: Compounds isolated from AAM3-fraction

6.7.2 Materials and methods

The stimulatory effect of the four compounds on RINm5F cells was investigated as described in section 5.7.2. Mangiferin and mangiferin glucoside were reconstituted in Gey & Gey buffer to give final concentrations of 0.08-1 mg/ml. Gey & Gey buffer was used as the control. The compounds were also tested at the same

range of concentrations in the presence of 10 mM glyceraldehyde to investigate their possible potentiation effect on insulin secretion in the presence of nutrient stimulus. In this case the compounds were reconstituted in buffer containing 10 mM glyceraldehyde to give final concentrations of 0.08-1 mg/ml; and 10 mM glyceraldehyde was used as the control. Timosaponin AIII and timosaponin BI were dissolved in DMSO and reconstituted in Gey & Gey buffer to give final concentrations of 0-1 mg/ml and 2% DMSO. The investigation for a stimulatory effect on insulin secretion was performed as described in section 5.4.

RINm5F cells (150,000 cells/well) were incubated with the compounds at 37 °C for 1 hour as described in section 5.7.2. At the end of incubation, the supernatant was taken and diluted with borate buffer (1:1), the diluted supernatant was then determined for both the content of insulin (section 5.4.3.3) and the LDH leakage (section 5.8.2).

6.7.3 Results and discussion

6.7.3.1 Effect of mangiferin on insulin and LDH leakage from RINm5F cells

Investigation of the effect of mangiferin on RINm5F cells revealed a significant release of insulin when the cells were exposed to the compound at 1 mg/ml (Fig. 6.13); the secretion was found to be 2.7 fold greater than that of control. The compound at lower doses (0.008-0.5 mg/ml) was found to have no effect on insulin secretion. Measurement of LDH leakage showed that mangiferin at 0.008-1 mg/ml did not cause a marked release of LDH from the cells (Fig. 6.14), indicating that stimulation of insulin release observed at 1 mg/ml was not a result of cell membrane damage, thus the compound possibly had an effect on the regulating pathway of insulin secretion.

To investigate the effect of mangiferin in the presence of nutrient secretagogue, glyceraldehyde (10 mM) was used instead of glucose since RINm5F cells do not respond to glucose due to the lack of glucokinase enzyme (section 5.5). Figure 6.15a shows that although glyceraldehyde (10 mM) itself did not significantly stimulate insulin secretion from RINm5F cells as would be expected, its presence

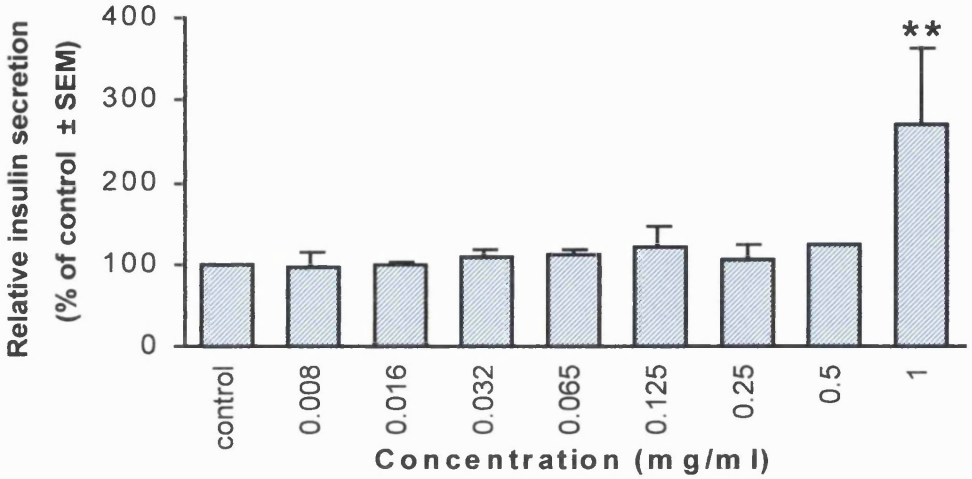


Fig. 6.13: Effect of mangiferin on insulin secretion from RINm5F cells

Results are expressed as mean of relative insulin secretion ± SEM from 2 independent experiments. n = 6 (each experiment). Mean value of basal insulin secretion (control) was 0.55 ± 0.08 ng/10^6 cells/hr. **P < 0.01 VS control.

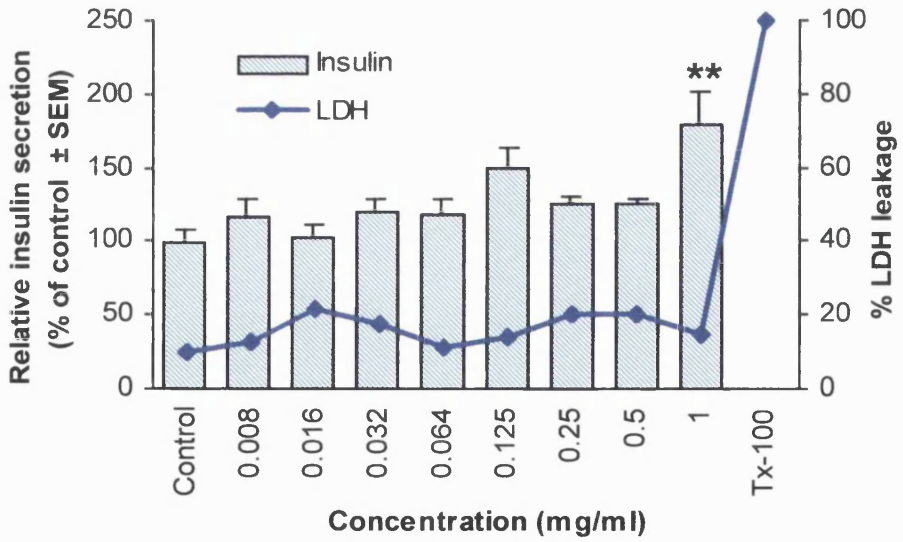
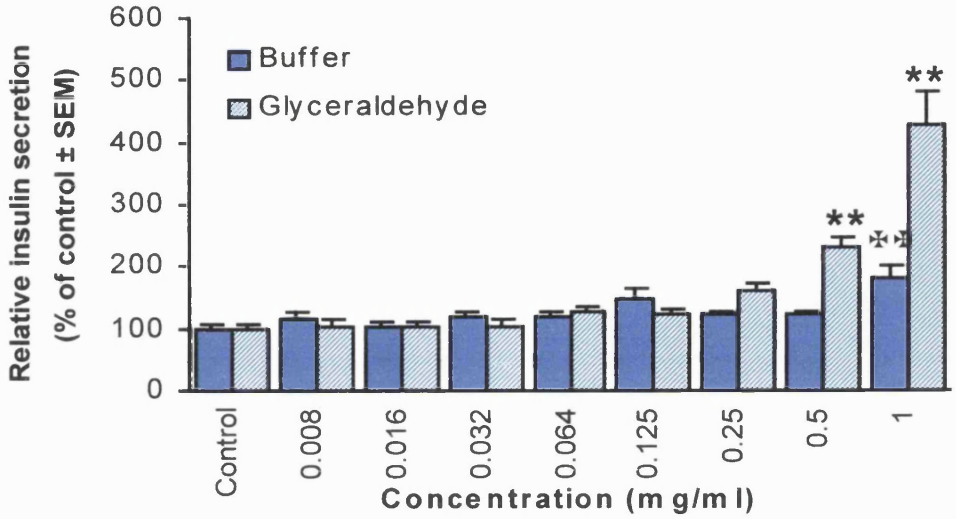
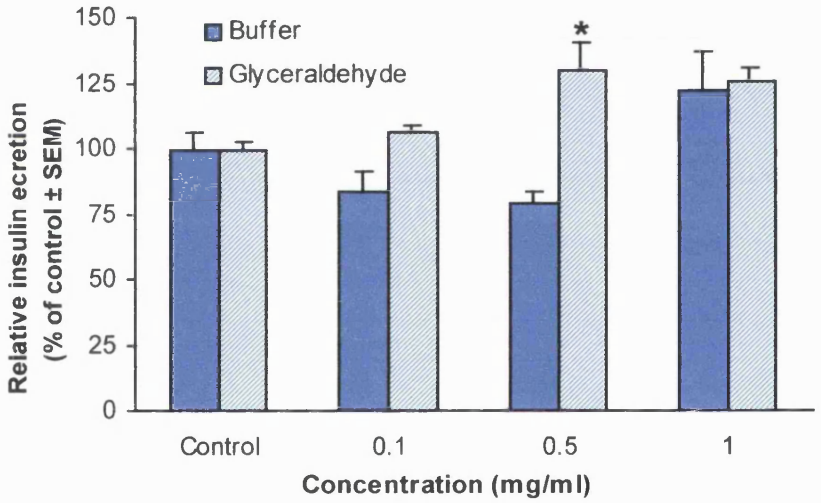


Fig. 6.14: Effect of mangiferin on insulin release and LDH leakage from RINm5F cells.

Results are expressed as mean of relative insulin secretion ± SEM. n = 6. Mean value of basal insulin secretion was 1.15 ± 0.15 ng/10^6 cells/hr. LDH release is expressed as % of LDH leakage relative to that caused by Triton X-100 (100% leakage). Changes in OD 340/minute observed with Triton X-100 was 0.0163 ± 0.0002 (mean ± SEM). n = 3. **P < 0.01 VS control.



(a)



(b)

Fig. 6.15: Effect of mangiferin on insulin secretion in RINm5F cells in the absence (buffer) or presence of 10 mM glyceraldehyde in 2 separate experiments (a) and (b)

Values are mean ± SEM relative to control i.e. Gey & Gey buffer or 10 mM glyceraldehyde. n = 6. Values of basal insulin secretion with Gey & Gey buffer were (a) 0.63 ± 0.05 and (b) 0.95 ± 0.07. ✣✣P < 0.01 VS Gey & Gey buffer; **P < 0.01, *P < 0.05 VS 10 mM Glyceraldehyde

was found to enhance the stimulatory effect of mangiferin on insulin secretion. In the presence of glyceraldehyde, mangiferin not only at 1.0 mg/ml but also at the lower dose of 0.5 mg/ml was found to significantly stimulate the secretion of insulin compared to the effect of 10 mM glyceraldehyde alone.

Figure 6.15b shows the effect of mangiferin in the absence and presence of glyceraldehyde from another experiment. Similarly to the results shown in Figure 6.15a, it was found that the effect of mangiferin at 0.5 mg/ml was significantly enhanced by the presence of 10 mM glyceraldehyde. Stimulation of insulin secretion by mangiferin (0.5 mg/ml) was found to be 1.7 fold greater in the presence of glyceraldehyde compared to those when glyceraldehyde was absent from the incubating medium (Fig. 6.15b). However, there were a few differences between the results observed in this experiment and those shown in Figure 6.15a. It was found in this experiment (Fig. 6.15b) that mangiferin at 1 mg/ml only modestly increased insulin secretion which was found to be insignificantly different from the control, and the presence of 10 mM glyceraldehyde did not enhance the effect of 1 mg/ml mangiferin as observed in the previous experiment. The differences in the secretory response to mangiferin at 1 mg/ml (Fig. 6.15a & b) may be owing to the fact that the studies were performed using the different passages of the cells

Despite the discrepancy, these results indicated that mangiferin stimulated insulin secretion and was able to enhance the effect of glyceraldehyde on insulin secretion. Thus, according to these results, it was feasible to conclude that mangiferin not only initiated insulin secretion but also augmented the effect of glyceraldehyde. The potentiation of mangiferin effect by glyceraldehyde suggested that the compound may be nutrient-sensitive insulin secretagogue. Such compounds have potential advantages for use in the treatment of NIDDM as they would cause greater insulin release following a meal than in basal conditions.

6.7.3.2 Effect of mangiferin glucoside on insulin secretion and LDH leakage from RINm5F cells

Investigation of the effect of mangiferin glucoside on RINm5F cells revealed that the compound at 0.5 and 1 mg/ml significantly increased the secretion

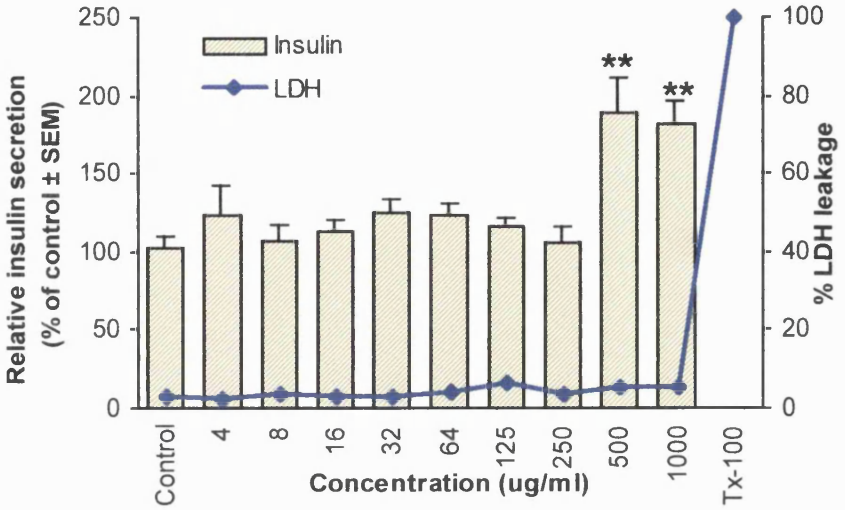


Fig. 6.16: Effect of mangiferin glucoside on insulin secretion and LDH leakage from RINm5F cells

Results of insulin secretion are expressed as mean ± SEM relative to control. n = 6. Mean value of basal insulin secretion was 0.47 ± 0.03 ng/10^6 cells/hr. LDH release is expressed as % LDH leakage in relative to that of Triton X-100 (100% leakage). Changes in OD 340/minute observed with Triton X-100 was 0.2461 ± 0.02 (mean ± SEM). n = 3.**P < 0.01 VS 10 mM glyceraldehyde.

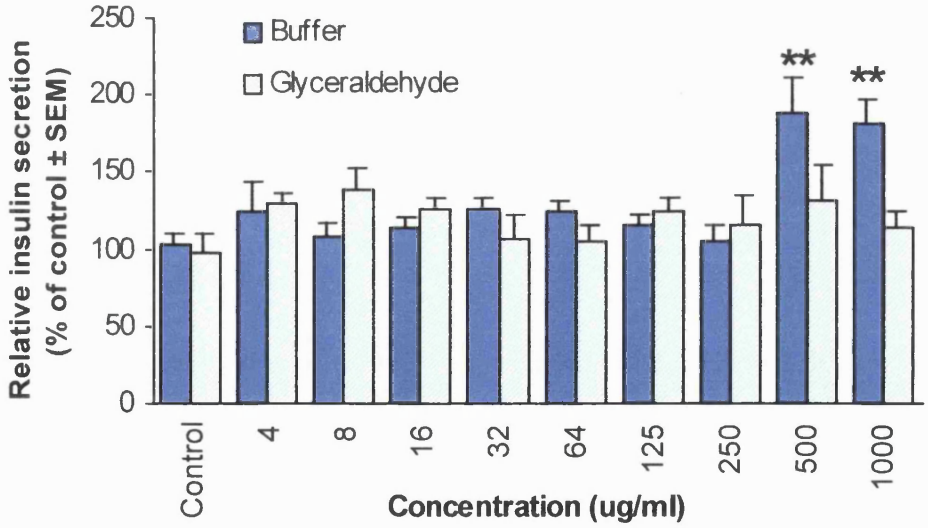


Fig. 6.17: Effect of mangiferin glucoside on insulin secretion RINm5F cells in the absence and presence of 10 mM glyceraldehyde

Results are expressed as mean ± SEM relative to control (Gey & Gey buffer). n = 6. Mean values of basal insulin secretion (control) was 0.47 ± 0.03 ng/10^6 cells/hr, and 0.47 ± 0.04 ng/10^6 cells/hr for 10 mM glyceraldehyde. **P < 0.01 VS Gey & Gey buffer.

of insulin (Fig. 6.16). The secretion of insulin was found to be significantly increased (by 1.9 and 1.8 fold, respectively) in response to 0.5 and 1 mg/ml mangiferin glucoside. The percentage of LDH leakage in response to mangiferin glucoside (0.004-1 mg/ml) was very close to those observed in control (Fig. 6.16), suggesting that the enhanced release of insulin was not a consequence of cell membrane damage. In contrast to mangiferin, the presence of glyceraldehyde did not appear to have any potentiating effect on the activity of mangiferin glucoside on insulin secretion (Fig. 6.17). In the presence of glyceraldehyde, no significant stimulation was observed at any concentrations of mangiferin glucoside, and the secreted insulin level was lower compared to secretion in response to mangiferin glucoside alone. It was unclear why the cells exposed to mangiferin glucoside and glyceraldehyde secreted lower insulin compared to those exposed to mangiferin glucoside alone.

6.7.3.3 Effect of timosaponin AIII on insulin secretion and LDH leakage from RINm5F cells

Investigation of the effect of timosaponin AIII on insulin secretion revealed that the compound at concentrations of 8-32 μg/ml caused a significant release of insulin from RINm5F cells (Fig. 6.18). The stimulation was increased by 2.31, 2.64 and 2.61 fold compared to the control (2% DMSO) in response to 8, 16 and 32 μg/ml of timosaponin AIII, respectively. However, increasing the concentration of timosaponin AIII did not increase its effect on insulin secretion. It was found that the compounds at 125-1000 μg/ml had no stimulatory effect on insulin secretion (Fig. 6.18).

Measurement of LDH leaked from the cells revealed that leakage of the enzyme caused by timosaponin AIII at 8 μg/ml was not significantly different from that of control. At the concentration of 16 μg/ml where the most potent stimulatory effect on insulin secretion was observed, it was found that the compound caused a higher leakage of LDH which was found to be statistically different from the control ($P < 0.05$) (Fig. 6.18). This suggested that the compound at this concentration may have damaged cell membranes to some extent. The results suggested that increasing concentration of the compound did not increase its effect on insulin secretion, in fact

it was found that increasing doses had increased the toxicity of the compound on cell membranes. The reduced effect on insulin secretion observed at the concentration of 32 μg/ml and higher, may possibly be due to other detrimental effects on the function of the cells. These results lead to a suggestion that the compound at 8 μg/ml was capable of stimulating insulin secretion without significantly damaging cell membrane. At higher doses of 16-32 μg/ml in which the most potent effect on insulin secretion was observed with the statistically different leakage of LDH, the release of insulin may be due mainly to cell membrane damages.

In a further experiment in which the compound was investigated at the lower doses, the stimulation caused by timosaponin AIII was also observed even at the lower doses of 1-8 μg/ml (Fig. 6.19). It was found that timosaponin AIII at 16 μg/ml and above did not stimulate insulin secretion. Measurement of LDH leakage showed that at the concentrations of 1-8 μg/ml, the compounds had no toxic effect on membranes of RINm5F cells, as judged by % leakage of LDH which was found to be similar to the control. These suggested that timosaponin AIII at 1-8 μg/ml was capable of stimulating insulin secretion without damaging cell membranes. In contrast to the secretion from the previous experiment (Fig. 6.18), the effect of timosaponin AIII at 16 μg/ml in this latter experiment (Fig. 6.19) was less than its effect at 8 μg/ml and was insignificantly different from the control. The differences in this response may be due to the fact that different passages of the cells were slightly different in terms of their responsiveness to the secretagogue.

It could be concluded from these experiments that timosaponin AIII at very low doses (1-8 μg/ml) could significantly stimulate insulin secretion from RINm5F cells without damaging cell membranes. This concentration is much lower than that required by tolbutamide, sparteine, or mangiferin and its glucoside (Fig. 5.10, 5.13, 6.13 and 6.16).

Timosaponin AIII was reported in a study by Nakashima *et al.* (1993) to lower blood glucose-level in alloxan-induced diabetic mice when given intraperitoneally at a dose of 50 mg/kg. It was noteworthy that the effect of this compound was marginal compared to the crude methanolic extract tested at the same

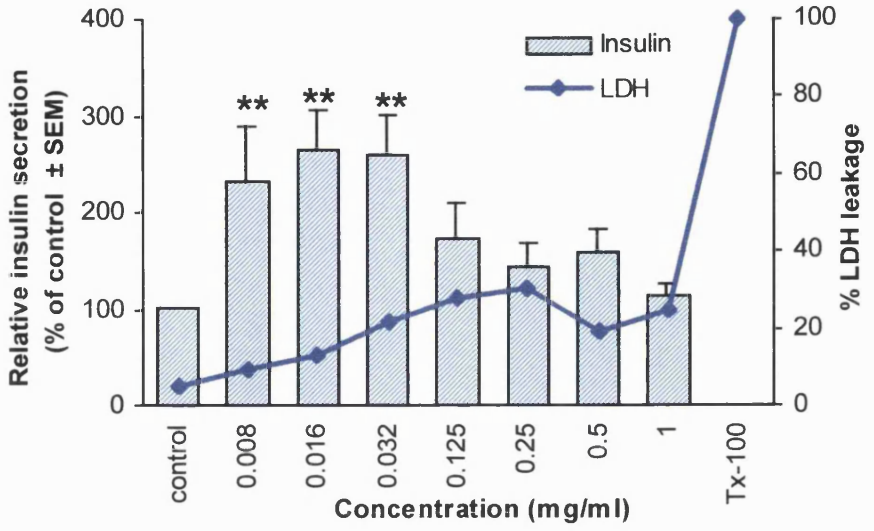


Fig. 6.18: Effect of timosaponin AIII (0.008-1 mg/ml) on insulin secretion and LDH leakage from RINm5F cells

Results of insulin secretion are expressed as mean ± SEM relative to control (2% DMSO) from 2 independent experiments (n = 6). Mean of basal insulin secretion observed with the control (2% DMSO) was 0.97 ± 0.25 ng/10^6 cells/hr. LDH release is expressed as mean of % LDH leakage relative to that of Triton X-100 (100 % leakage). n = 3. **P < 0.01 VS control.

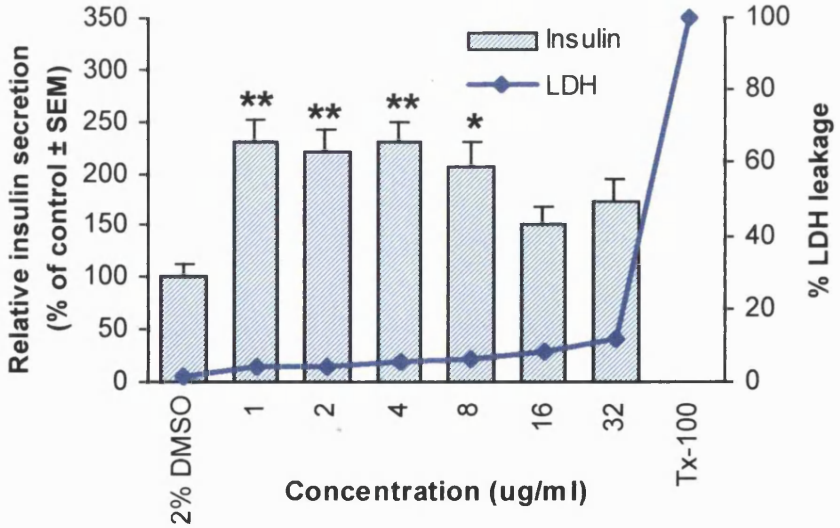


Fig. 6.19: Effect of timosaponin AIII (1-32 μg/ml) on insulin secretion and LDH leakage from RINm5F cells.

Results of insulin secretion are expressed as mean ± SEM relative to control (2% DMSO). n = 6. Mean of insulin secretion observed with 2% DMSO was 0.91 ± 0.10 ng/10^6 cells/hr. LDH release is expressed as mean of % LDH leakage relative to that of Triton X-100 (100 % leakage). n = 3. **P < 0.01 VS control.

dose (Nakashima *et al.* 1993). Although timosaponin AIII was found to have glucose-lowering effect in alloxan-induced diabetic mice, the effect of the compound on pancreas could not be excluded. However, it was clearly shown in this current study that timosaponin AIII was capable of stimulating insulin secretion. Taking both *in vivo* (Nakashima *et al.* 1993) and *in vitro* effects (Fig. 6.19) into consideration, there is a possibility that timosaponin AIII may have both pancreatic and extrapancreatic effect.

6.7.3.4 Effect of timosaponin BI on insulin secretion and LDH leakage from RINm5F cells

Despite the similarity in their structures (Fig. 6.12), the effects of timosaponin AIII and timosaponin BI were completely different in terms of insulin secretion as well as the toxicity on cell membranes (Fig. 6.18 & Fig. 6.20), as it was found that timosaponin BI at concentrations of 8-500 μg/ml did not stimulate the secretion of insulin from RINm5F cells (Fig. 6.20).

Absence of significant leakage of LDH from RINm5F cells suggested that timosaponin BI at the concentrations tested did not cause damage to cell membranes (Fig. 6.20). Interestingly, timosaponin BII (Fig. 6.3), one of the saponins isolated from *A. asphodeloides* of which the structure is very similar to timosaponin BI (Fig. 6.12) was reported to have blood glucose lowering effect in alloxan-induced diabetic mice of which the pancreas no longer functioned normally, suggesting that the hypoglycaemic effect was likely to be due to other mechanisms rather than stimulation of insulin secretion (Nakashima *et al.* 1993). In support to this finding, it was also found that timosaponin BII (1 mg/ml) had no effect on insulin secretion on rat perfused pancreas (Nakashima *et al.* 1993)

According to literature survey, saponins from *Anemarrhena asphodeloides* which have been linked to anti-diabetic effect were timosaponin B (psuedo-prototimosaponin) and timosaponin BII (prototimosaponin) (Nakashima *et al.* 1993). The compounds (50 mg/kg, i.p.) lowered plasma glucose level 6 hours after the administration to alloxan-induced diabetic mice, but had no effect on fasted

normal ddY-strain mice (Nakashima *et al.* 1993). The effect of timosaponin B and timosaponin BII were reported to be comparable to those observed in the aqueous extract of *A. asphodeloides* (100 mg/kg, i.p.). Although timosaponin B and timosaponin BII were able to lower blood glucose level in this model, considering doses of extract and the two compounds used in their study it may not be feasible to postulate that the activity of the two compounds were solely responsible for the activities observed in *A. asphodeloides* crude extract. This is because compounds were tested at relatively high doses (i.e. 50 mg/kg) compared to the tested dose (100 mg/kg) of the crude extract whose reported content of timosaponin B and timosaponin BII were less than 0.04 and 0.23 %, respectively (Nakashima *et al.* 1993). It is possible that these saponins, along with timosaponin AIII could work in an additive or synergistic manner.

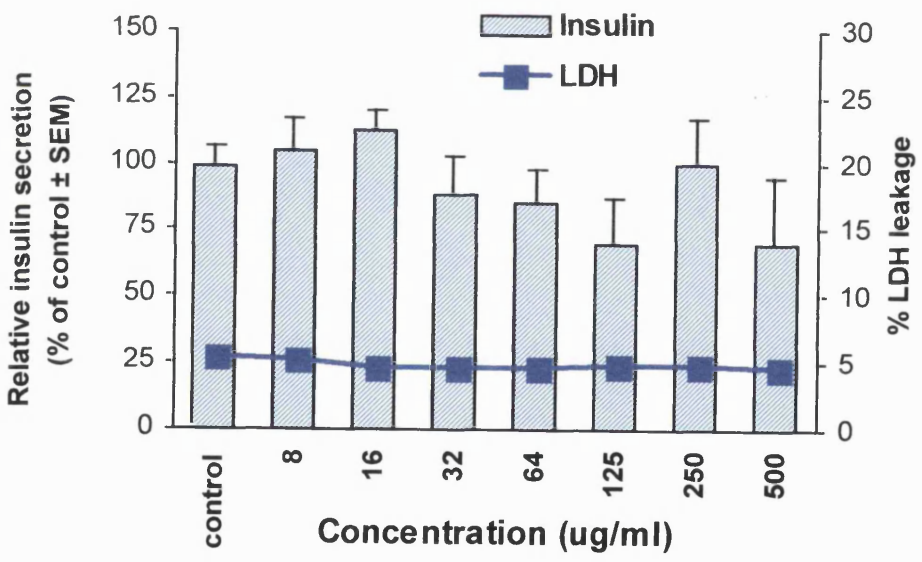


Fig. 6.20: Effect of timosaponin BI on insulin secretion and LDH leakage from RINm5F cells

Results of insulin secretion are expressed as mean ± SEM relative to control (2% DMSO). n = 6. Mean of insulin secretion observed with the control (2% DMSO) was 0.63 ± 0.05 ng/10^6 cells/hr. LDH release is expressed as mean of % LDH leakage relative to that of Triton X-100 (100 % leakage). n = 3.

6.8 Investigation of the effect of mangiferin and mangiferin glucoside on rat islets

6.8.1 Introduction

Mangiferin and its glucoside isolated from *Anemarrhena asphodeloides* which were found to have stimulatory effect on insulin secretion on RINm5F cells without damaging cell membranes (Fig. 6.14 & 6.16) were further tested on islets freshly isolated from rat pancreas in order to confirm that the activity observed in RINm5F cells was indicative of activity in a model close to the *in vivo* situation.

6.8.2 Materials and methods

Unless specified, all chemicals were purchased from Sigma-Aldrich Co. Ltd. (Irvine, UK). Mangiferin used in these studies was that isolated from *Anemarrhena asphodeloides* and in some cases, a sample purchased from Apin Chemicals Ltd. Identity of the latter was confirmed by NMR (section 7.2.3.5). Rats (Sprague-Dawley) were supplied by King's College London Animal Unit.

6.8.2.1 Isolation of islets

Islets were kindly isolated and provided by Dr. T.E. Harris (Cellular and Molecular Endocrinology Group, Biomedical Sciences Division, King's College London) using the method described by Persaud *et al.* (1990).

Male Sprague-Dawley rats were stunned by a sharp blow to the back of the head and killed by cervical dislocation. The abdomen was cut open and the bile duct was clamped. A plastic cannula was carefully inserted into the bile duct and a solution of collagenase (1 mg/ml) was injected into the pancreas using a syringe. The pancreas was then excised from the surrounding tissue and transferred to a glass vial containing Gey & Gey buffer. The pancreas was incubated in a water bath for 10 minutes at 37 °C. At the end of the incubation period, cold Gey & Gey buffer (~20 ml) was added to the vial to disperse the islets. The suspension was centrifuged at 1000g for 30 seconds and the supernatant was removed and replaced with fresh Gey & Gey buffer. The pellets were resuspended and recentrifuged at the same

speed. The pellets were transferred to a black-bottomed petri dish and Gey & Gey buffer was added. The islets were picked from the suspension under a microscope (× 10 magnification) using a sharp tipped Pasteur pipette. The picking process was repeated on the selected samples to obtain a pure population of islets.

6.8.2.2 Measurement of insulin secretion

Three freshly isolated islets were transferred by Pasteur pipette to 1.5 ml Eppendorf containing Gey & Gey buffer (600 µl) supplemented with one of the following:

1) 2 mM or 20 mM glucose
2) 2 mM or 20 mM glucose + mangiferin or mangiferin glucoside (0.25 mg/ml)
3) 2 mM or 20 mM glucose + mangiferin or mangiferin glucoside (0.5 mg/ml)
4) 2 mM or 20 mM glucose + mangiferin or mangiferin glucoside (1.0 mg/ml)

Each sample was investigated with 8 observations. The solution was gassed with 5% CO_2/95% air and incubated in a water bath (37 °C) for 1 hour. At the end of incubation period, the tubes were centrifuged at 1000g for 1 minute and 200 µl of the supernatant was transferred to another tube and diluted with 800 µl borate buffer. The samples were kept at -20 °C until the insulin content was measured by RIA (section 5.4.3.3). For the RIA, 100 µl of the diluted supernatant was mixed with antibody (100 µl) and radiolabelled insulin (100 µl), incubated at 4 °C for 48-72 hours and measured for radioactivity using a γ-counter as described in section 5.4.3.3. The results were expressed as mean of relative insulin secreted ± SEM. Significance between control and tested compounds was analysed using ANOVA or t-test as appropriate. The experiment was carried out on 4 different occasions.

6.8.3 Results and discussion

6.8.3.1 Effect of mangiferin on insulin secretion from rat islets

Results from 4 individual experiments are given in Table 6.1, the results shown in Figure 6.21 and 6.22 are percentage increase of insulin secretion of the pooled data calculated from the 4 experiments shown in Table 6.1. The secretion

of insulin from rat islets observed in the studies was found to vary in a range of 0.072-0.64 ng/islet/hr in response to 2 mM glucose, and 1.72-3.14 ng/islet/hour in response to 20 mM glucose. This wide variation was possibly due to the difference in the responsiveness between the islets isolated from different animals and on different occasions. Although the effect of mangiferin was only marginal according to the pooled results (Fig. 6.21 and 6.22), it should be noted that a significant secretion by the compound was observed in some of the individual experiments (Table 6.1). This was possibly due to wide variation between the experiments.

In the presence of 2 mM glucose, significant increase of insulin secretion induced by mangiferin was observed in some experiments but not all (Table 6.1). Significant stimulation was observed at 0.25 mg/ml (Exp I) and 0.5 mg/ml (Exp I and II). When the results from the 4 experiments were pooled and expressed as % increase of insulin (Fig. 6.21), it was shown in the graph that the compound at 0.25-1 mg/ml caused a slight increase in the secretion of insulin from the islets. The secretion was increased by 138%, 140% and 145% in response to 0.25, 0.5 and 1.0 mg/ml, respectively (Fig. 6.21). It was noteworthy that the effect of mangiferin observed in islets in this case was similar to those observed in RINm5F

Table 6.1: Effect of mangiferin on insulin secretion in rat islets in the presence of 2 and 20 mM glucose

	Insulin secreted (ng/islet/hr) ± SEM			
	Exp. I	Exp. II	Exp. III	Exp. IV
2 mM glucose (control)	0.072 ± 0.01	0.64 ± 0.10	0.44 ± 0.07	0.21 ± 0.06
+ 0.25 mg/ml mangiferin	0.14 ± 0.01**	0.91 ± 0.15	0.35 ± 0.08	0.21 ± 0.13
+ 0.50 mg/ml mangiferin	0.16 ± 0.01***	1.12 ± 0.12**	0.35 ± 0.08	0.21 ± 0.04
+ 1.0 mg/ml mangiferin	0.15 ± 0.03*	-	0.34 ± 0.12	-
20 mM glucose (control)	3.14 ± 0.29	1.72 ± 0.10	1.97 ± 0.35	2.11 ± 0.43
+ 0.25 mg/ml mangiferin	4.05 ± 0.68	1.98 ± 0.31	3.15 ± 0.42^{+}	1.69 ± 0.30
+ 0.50 mg/ml mangiferin	5.04 ± 0.50^{++}	1.11 ± 0.25	2.29 ± 0.35	1.68 ± 0.20
+ 1.0 mg/ml mangiferin	2.17 ± 0.32	-	1.36 ± 0.23	1.64 ± 0.22

Results were statistically analysed using student t-test. ***P< 0.005, **P< 0.01, * P< 0.05 VS 2 mM glucose; $^{++}$P < 0.01, $^{+}$P< 0.05 VS 20 mM glucose. In some cases, mangiferin was tested only at 0.25 and 0.5 mg/ml (Exp II and IV) due to limited availability of rat islets.

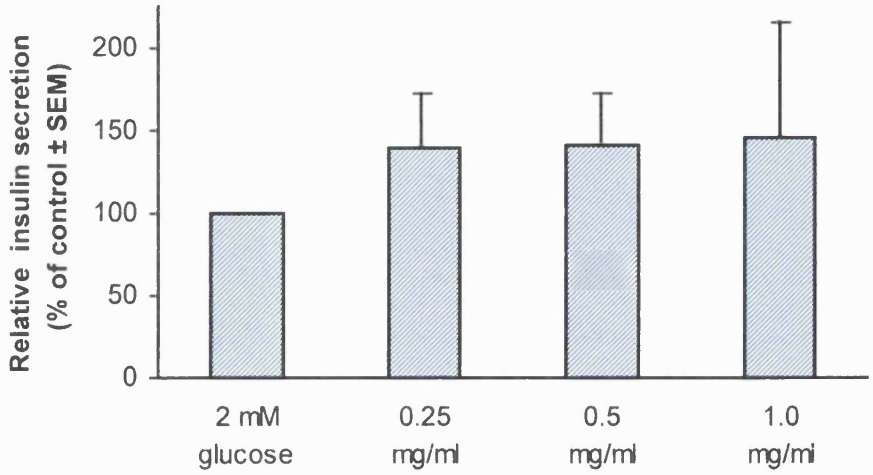


Fig. 6.21: Effect of mangiferin on insulin secretion from rat islets in the presence of substimulatory concentration of glucose (2 mM)

Results are mean ± SEM relative to control (Gey & Gey buffer containing 2 mM glucose) from 4 separate experiments. n = 8 in each experiment. Mean value of the secretion in response to 2 mM glucose was 0.34 ± 0.12 ng/islet/hr.

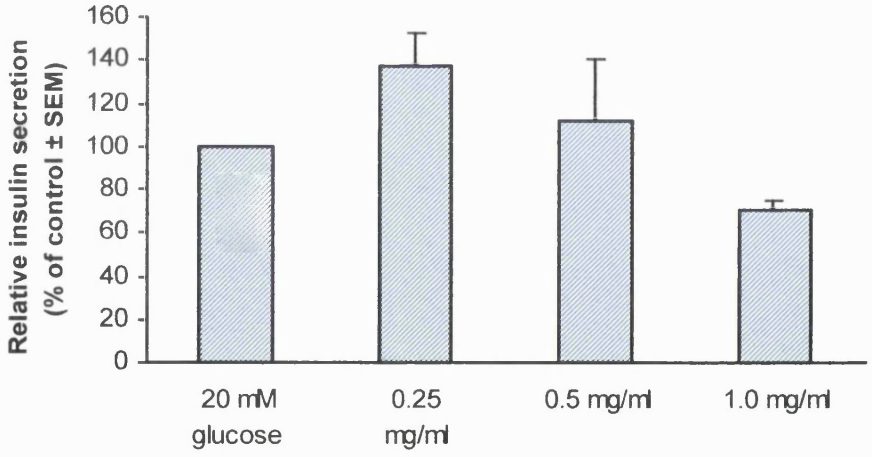


Fig. 6.22: Effect of mangiferin on insulin secretion from rat islets in the presence of stimulatory concentration of glucose (20 mM)

Results are mean ± SEM relative to control (Gey & Gey buffer containing 20 mM glucose) from 4 separate experiments. n = 8 (each experiment). Mean value of the secretion in response to 20 mM glucose was 2.23 ± 0.31 ng/islet/hr.

cells to some extent. The effects were similar in the sense that mangiferin at 1 mg/ml stimulated basal insulin secretion in both RINm5F cells (Fig. 6.13) and islets (Fig. 6.21), but the effect in RINm5F cells appeared to be more pronounced. However, whereas the compound at dose lower than 1 mg/ml was found to have no effect on insulin secretion in RINm5F cells, it caused modest increase in insulin secretion in islets.

In contrast to the results observed in RINm5F cells, the results observed in rat islets showed that the most pronounced potentiation effect of mangiferin occurred at 0.25 mg/ml (Fig. 6.22), and increasing concentrations of mangiferin did not increase its potentiation on glucose-induced insulin secretion, in fact mangiferin at 1 mg/ml was found to inhibit rather than stimulate the secretion of insulin (Fig. 6.22). Similarly to the results observed with 2 mM glucose, it was found in the presence of 20 mM glucose that the secretion of insulin was significantly enhanced by mangiferin in some experiments (Table 6.1). The significant stimulation was observed with mangiferin at a dose of 0.25 mg/ml (Exp III) and 0.50 mg/ml (Exp I). When the results from four separate experiments were pooled (Fig. 6.22), it was seen from the graph (Fig. 6.22) that the secretion of insulin was modestly increased by 0.25 and 0.50 mg/ml mangiferin by 140% and 112%, respectively.

The results obtained from the islets were in accordance with those observed in RINm5F cells in the sense that the effect of mangiferin was potentiated by the presence of nutrient in this case glucose (Fig. 6.21). However, the results were different to some extent particularly in terms of the response patterns and doses of maximum response. As shown in Fig. 6.15 (section 6.7.3.1), in RINm5F cells the potentiation effect of mangiferin was observed at the concentrations of 0.50 and 1 mg/ml (Fig. 6.15a), and the potentiation effect increased with the increasing concentration with the most pronounced effect was observed at 1 mg/ml. In contrast to the results observed in RINm5F cells, the results observed in rats islets showed that the most pronounced potentiation effect of mangiferin occurred at 0.25 mg/ml, and increasing concentrations of mangiferin did not increase its potentiation on glucose-induced insulin secretion, in fact mangiferin at 1 mg/ml was found to inhibit rather than stimulate the secretion of insulin (Fig. 6.22).

According to the results obtained in this study, it could be concluded, in agreement with the results from RINm5F cells, that mangiferin could modestly initiate insulin secretion as well as potentiate the insulin secretion induced by nutrients. Mangiferin was found to stimulate secretion from islets in further experiments using PKC and PKA inhibitors (section 6.9 and 6.10). Thus, mangiferin has potential advantages for use in the treatment of NIDDM as it would cause greater insulin release following a meal than in basal conditions. The lack of consistency of results in the different experiments may either be due to differences in the islets themselves or in the variations in the diffusion of the compound into the cells.

Mangiferin and its glucoside isolated from *A. asphodeloides* rhizomes were recently reported to have hypoglycaemic activity in KK-A^y mice (Ichiki *et al.* 1998). In their experiment, the effect of mangiferin and its glucoside (p.o., 10-90 mg/kg) on blood glucose and insulin level was determined 7 hours after administration; they reported that the compounds caused a significant reduction in blood glucose level. However, the effect of the two xanthones demonstrated in Ichiki's study was different from those observed in this current study to some extent. They have suggested that the hypoglycaemic effect of these compounds was due to their ability to increase insulin sensitivity at its target tissues. Their conclusion was based on the observation that the compounds could improve hyperinsulinemia in treated mice, as the level of plasma insulin was significantly decreased upon the treatment. This was contrary to the findings in our studies since it was found that mangiferin was capable of stimulating insulin secretion from RINm5F cells and possibly rat islets (section 6.7 and 6.8). However, in the study by Ichiki *et al.* (1998) the level of insulin was only measured 7 hours after administration of the compounds. Thus, it may not be feasible to make any comparisons with our data since changes in the level of insulin at earlier time points would have been missed. There was a possibility that insulin secreted by pancreatic β-cells in response to mangiferin and mangiferin glucoside was cleared from the circulation before the 7 hour time point when the plasma was examined. The low level of insulin observed at 7 hours was most probably due to the fact that basal glycaemia had decreased.

Besides mangiferin and mangiferin glucoside, a number of xanthones isolated from medicinal plants have been linked to anti-diabetic effects. These included swerchirin isolated from *Swertia chirayita* (Gentianaceae), and bellidifolin and methylbellidifolin isolated from *Swertia japonica* (Chapter 2). In agreement with the effect of mangiferin on insulin secretion, the effect of swerchirin was documented to be associated with pancreatic activity as it was reported that the compound was capable of stimulating insulin secretion from islets of Langerhans (Saxana *et al.* 1993).

6.8.3.2 Effect of mangiferin glucoside on insulin secretion from rat islets

Investigation of the effect of mangiferin glucoside on insulin secretion from islets revealed that the compound at 1 mg/ml did not stimulate the secretion of insulin. In fact, it was found that basal and 20 mM glucose-stimulated insulin secretion were decreased in the response to mangiferin glucoside. In order to draw any conclusion on the effect of this compound on insulin secretion, further investigation on the effect of the compound at lower doses are necessary, since it was possible that the compound at low and high doses may act differently as seen in case of mangiferin where the secretion was inhibited by the compound at 1 mg/ml but was enhanced by the compound at 0.25 and 0.5 mg/ml (Fig. 6.22). However, due

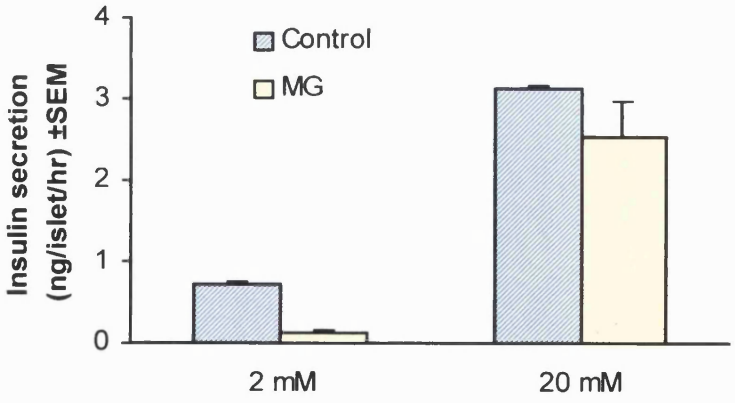


Fig. 6.23: Effect of mangiferin glucoside (1 mg/ml) on insulin secretion from rat islets in the presence of glucose (2 and 20 mM)

Results are expressed as mean ± SEM (n = 8).

to the limited supply of islets the compound was not tested at other concentrations. The only conclusion that can be drawn from this experiment was that mangiferin glucoside at 1 mg/ml had no effect on insulin secretion from rat islets. Mangiferin glucoside may be found in future work to have no effect on islets even at lower concentrations. In this case, a discrepancy between these results and that observed with RINm5F cells may be due to physiological differences between the two models or inaccessibility of the cells to the compounds.

6.9 Investigation of the effect of PKC inhibitor on mangiferin-induced insulin secretion from rat islets

6.9.1 Introduction

It has been well established that some non-nutrient secretagogues such as acetylcholine, carbachol and cholecystokinin (CCK) enhance glucose-induced insulin secretion primarily through the activation of PKC (section 5.1.2.2). This finding has underlined the physiological importance of PKC pathway in signal transduction of insulin secretion. This next study aimed to investigate mode of action by which mangiferin stimulated insulin secretion from rat islets (section 6.8). To determine whether or not the activation of PKC is involved in the observed activity, the effect of mangiferin on insulin secretion from rat islets was investigated in the presence of the PKC inhibitor, Gö 6976 which has been used widely in the studies to investigate the pathway of the insulin secretory response (Harris *et al.* 1996; Harris *et al.* 1997a).

6.9.2 Materials and methods

Unless specified, all chemicals were analytical grade and purchased from Sigma-Aldrich Co. Ltd. (Irvine, UK). Mangiferin was purchased from Apin Chemicals Ltd. (UK). Rats (Sprague-Dawley) were supplied by King's College London Animal Unit. Gö 6976 was purchased from Calbiochem (UK) Ltd. (Nottingham, UK).

Rat islets of Langerhans were obtained by collagenase digestion of the pancreas using the method described in section 6.8.2.1. Three freshly isolated islets

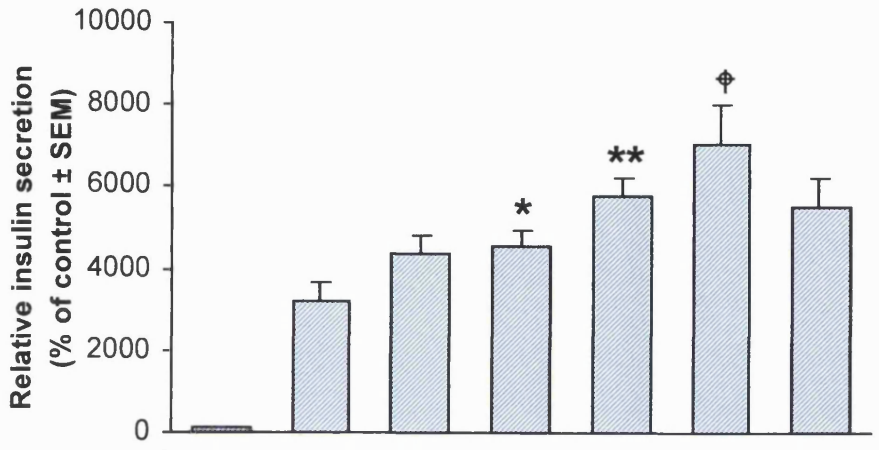
were transferred to 1.5 ml Eppendorf tubes containing Gey & Gey buffer (600 μl) supplemented with:

A) 2 mM glucose
B) 20 mM glucose
C) 20 mM glucose + 0.25 mg/ml mangiferin
D) 20 mM glucose + 0.50 mg/ml mangiferin
E) 20 mM glucose + 1 μM Gö 6976
F) 20 mM glucose + 0.25 mg/ml + 1 μM Gö 6976
G) 20 mM glucose + 0.5 mg/ml + 1 μM Gö 6976

Islets were incubated in the above solutions in a water bath at 37 °C for 1 hour. Each sample was investigated with 8 replicates. At the end of incubation, the tubes were centrifuged at 1000g for 1 minute, 200 μl of the supernatant was transferred to another tube and diluted with 800 μl borate buffer. The content of insulin in the diluted supernatant was then determined using RIA as described in section 5.4.3. The diluted supernatant (100 μl) was mixed with antibody (100 μl) and radiolabelled insulin (100 μl), and incubated at 4 °C for 48-72 hours, followed by the measurement of radioactivity using a γ counter. Results were expressed as % increase of insulin secretion in relative to control (2 mM glucose). Differences between groups were statistically compared using t-test.

6.9.3 Results and discussion

The results (Fig. 6.24) showed that mangiferin slightly increased the secretion of insulin in the presence of 20 mM glucose. The secretion was found to be 1.35 and 1.42 fold increase in response to mangiferin at 0.25 and 0.50 mg/ml, respectively compared to 20 mM glucose. In the latter case, the increase was found to be significantly different from that observed with 20 mM glucose ($P = 0.0382$), confirming the potentiation effect of mangiferin on nutrient-induced insulin secretion. Surprisingly, the incubation of the islets with Gö 6976 (group E) increased rather than decreased the secretion of insulin. The presence of Gö 6976 was found to significantly enhanced the secretion of insulin by 180% compared to control (20 mM glucose). It was unclear why the incubation of the islets with



	A	B	C	D	E	F	G	
Glucose	2	20	20	20	20	20	20	mM
Mangiferin	-	-	0.25	0.50	-	0.25	0.50	mg/ml
Gö 6976	-	-	-	-	1	1	1	μM

Fig. 6.24: Effect of Gö 6976 on mangiferin-induced insulin secretion from rat islets

Results are expressed as mean of insulin secretion ± SEM in relative to control (2 mM glucose). Mean of basal insulin secretion with 2 mM glucose was 0.08 ± 0.001 ng/10^6 cells/hr. Each sample was investigated with 8 replicates. **P < 0.01, *P < 0.05 VS 20 mM glucose (t-test); ♦P< 0.05 VS 20 mM + 0.25 mg/ml mangiferin (t-test).

Gö 6976 increased the secretion of insulin from the islets. Similar results were observed in one of the studies by Persaud *et al.* (1991) who reported the increase of first phase insulin secretion from PKC-depleted islets.

A number of studies have suggested that PKC is not necessary involved in glucose-induced insulin secretion (section 5.1.2.2). The results shown in this study that the presence of Gö 6976 did not inhibit the secretion of insulin evoked by mangiferin (0.5 mg/ml) and 20 mM glucose (group F and G) suggested that the inhibition of PKC by Gö 6976 had no influence on the effect mangiferin on nutrient-induced insulin secretion. Gö 6976 was claimed to be potent and selective PKC inhibitor which has specificity for α and βII isoforms (Ca^{2+}/DAG dependent isoforms) but not ε, ζ and τ isoforms of PKC. One of the studies using Gö 6976 showed that Gö 6976 inhibited potentiation of insulin secretion by PMA but had no effect on glucose-induced insulin secretion (Harris *et al.* 1997a). Thus, the finding

that Gö 6976 did not abolish the effect of mangiferin on glucose-induced insulin secretion did not rule out the possibility that mangiferin may work via other isoforms of PKC i.e. ε, ζ, ι/λ and μ isoforms (section 5.1.2.2). Based on the results obtained from this experiment, it could be concluded that the potentiation effect of mangiferin on glucose-induced insulin secretion was not mediated by the activation of Ca^{2+}/DAG dependent isoforms of PKC.

6.10 Investigation of the effect of a PKA inhibitor on mangiferin-induced insulin secretion from rat islets

6.10.1 Introduction

Evidence that agents such as forskolin and IBMX which enhance an accumulation of cAMP, an activator of PKA, can significantly potentiate glucose-induced insulin secretion (section 5.1.1) has led to a suggestion that PKA may be involved in a regulation pathway of insulin secretion. To determine the mechanism of action of mangiferin, the possible involvement of PKA in the secretion of insulin induced by the compound was studied using the PKA inhibitor, Rp-cAMPs.

6.10.2 Materials and methods

Rat islets of Langerhans were obtained by collagenase digestion of the pancreas using the method described in section 6.8.2.1. Three freshly isolated islets were transferred to a well of 96-well plate containing Gey & Gey buffer and centrifuged at 1000g for 5 minutes, followed by an aspiration of supernatant. The islets were then pre-incubated with 75 μl of Gey & Gey buffer containing 2 mM glucose (groups A-D) or 500 μM Rp-cAMPs (groups E-G) for 1 hour at 37 °C in an atmosphere of 95% CO_2/5% air. At the end of the pre-incubation, another 75 μl of Gey & Gey buffer supplemented with the following secretagogues was added to each well

A) 2 mM Glucose

B) 40 mM Glucose*

C) 40 mM Glucose* + 0.5 mg/ml mangiferin*

D) 40 mM glucose* + 1 mg/ml mangiferin†

E) 40 mM Glucose[*] + 500 μM Rp-cAMPs

F) 40 mM Glucose[*] + 0.5 mg/ml mangiferin* + 500 μM Rp-cAMPs

G) 40 mM Glucose[*] + 1 mg/ml mangiferin[†] + 500 μM Rp-cAMPs

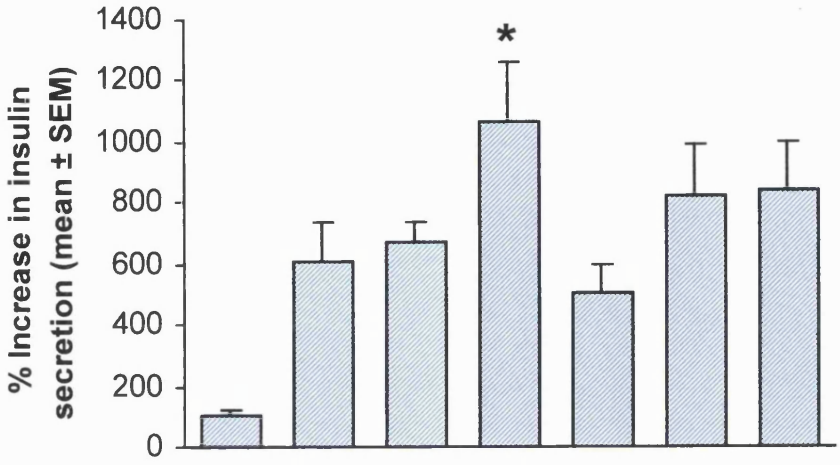
[*]Final concentration of glucose in the assay medium was 20 mM; *final concentration of mangiferin in the assay medium was 0.25 mg/ml; [†]final concentration of mangiferin in the assay medium was 0.5 mg/ml.

After incubation for 1 hour at 37 °C, islets were centrifuged at 1000g for 5 minutes, the supernatant (50 μl) was transferred to test tubes and diluted with borate buffer to 1 ml. The diluted supernatant was determined for the content of insulin as described in section 5.4.3.3. Results were expressed as % increase of insulin secretion compared to control (2 mM glucose). Difference between groups were statistically compared using t-test.

6.10.3 Results and discussion

Investigation of the effect of mangiferin in islets revealed that 20 mM glucose-induced insulin secretion was enhanced by 1.7 fold by mangiferin (0.5 mg/ml) in relative to 20 mM glucose alone as shown in Figure 6.25, confirming the potentiation effect of mangiferin on nutrient-induced insulin secretion. The presence of PKA inhibitor, Rp-cAMPs (500 μM), did not significantly inhibit the effect of glucose on the secretory response, suggesting that PKA pathway may not have an obligatory role in the regulation pathway of glucose-induced insulin secretion as suggested by a number of studies (Persaud *et al.* 1990; Harris *et al.* 1997b).

The secretory response to mangiferin (0.25 mg/ml) was modestly enhanced in the presence of Rp-cAMPs compared to the group treated with mangiferin alone, however these were considered not different by means of statistic analysis ($P = 0.4710$, t-test). A slight decrease in the content of insulin secreted (~ 20%) was observed in the islets exposed to mangiferin (0.5 mg/ml) and Rp-cAMPs compared to those exposed to mangiferin alone, however the difference between the two groups (group D and G) was again not significant ($P = 0.370$, t-test), suggesting that inhibition of PKA did not abolish the secretory response to mangiferin. According to these results, it was suggested that the potentiation effect of mangiferin on glucose-induced insulin secretion was not mediated via a PKA activating pathway.



	A	B	C	D	E	F	G	
Glucose	2	20	20	20	20	20	20	mM
Mangiferin	-	-	0.25	0.50	-	0.25	0.50	mg/ml
Rp-cAMPs	-	-	-	-	500	500	500	μM

Fig. 6.25: Effect of Rp-cAMPs on mangiferin-induced insulin secretion from rat islets

Results are expressed as mean of relative insulin secretion ± SEM compared to control. Mean of basal insulin secretion with 2 mM glucose was 0.11 ng/10^6 cells/hr ± SEM. Each sample was investigated with 8 replicates. *P < 0.05 VS 20 mM glucose (t-test).

6.11 Quantification of mangiferin content in crude methanol extract

6.11.1 Introduction

The content of mangiferin in crude methanol extract of *A. asphodeloides* was examined in order to determine the contribution by which the compound has to the effect of the crude methanol extract.

The content of mangiferin was quantified using a TLC densitometric method. The unknown concentration of mangiferin in the sample was extrapolated from a calibration curve plotted as log[peak area] against concentration of mangiferin standard.

6.11.2 Materials and methods

Unless specified, all chemicals were purchased from Sigma-Aldrich Co. Ltd. (Irvine, UK). Mangiferin standard was purchased from Apin Chemicals Ltd. (UK). Silica GF_{254} TLC plate were purchased from Merck KGaA (Germany).

Mangiferin standard solution was prepared by dissolving 4 mg of the compound in methanol (1 ml). The sample was prepared by dissolving the methanol extract of *A. asphodeloides* (7.3 mg) in 0.25 ml of methanol (final concentration 29.2 mg/ml). Prior to an application of the standards and sample, a silica TLC plate was saturated with a mobile phase ($CHCl_3$:Acetic acid:methanol:H_2O - 60:32:12:8) by leaving the plate in a TLC tank containing the mobile phase and allowing the mobile phase to run to the top of the plate to improve a chromatographic separation. After the plate was dried, the standards were applied onto the plate at the volumes of 5, 10, 15, 20 and 25 µl using calibrated microtubes. The sample was applied at a volume of 15 µl. The mobile phase was allowed to run a distance of 15 cm. The spots were visualised under UV and by spraying with anisaldehyde reagent (section 4.3.2.2). The intensity of the spots was determined using a densitometer (Shimadzu Dual Wavelength TLC scanner). The samples were determined with 2 observations. The content of mangiferin in the extract was expressed as mean percentage (% w/w).

6.11.3 Results and discussion

The quantification of the content of mangiferin revealed that a percentage yield of mangiferin (% w/w) in crude methanol extract was approximately 6.4% (Table 6.2). According to the effect on RINm5F cells, the methanol extract at the concentration of 1 mg/ml (equivalent to ~ 0.06 mg mangiferin) significantly stimulate the secretion of insulin (Fig. 6.6) whereas mangiferin at the concentration of 0.064 mg/ml failed to stimulate the insulin secretion (Fig. 6.14). The results implied that mangiferin was not the only compound in the methanol extract that can exert the effect on insulin secretion. The effect on insulin secretion of *A. asphodeloides* methanolic extract was clearly a combined effect induced by more than one compound, at least 3 compounds with hypoglycaemic activity i.e. mangiferin, mangiferin glucoside and timosaponin AIII have been identified in this study.

Table 6.2: Content of mangiferin in crude methanol extract determined by densitometric method

	Log [peak area]	Mangiferin (μg) in the sample	Mangiferin in crude methanol extract (%w/w)
Sample (438 μg)	4.3712	26.56	6.06
	4.3997	29.24	6.67
Mean			**6.37**

6.12 Discussion

The rhizome of *Anemarrhena asphodeloides* has long been used in traditional Chinese medicine for a treatment of diabetes. In agreement with reported *in vivo* effects of *A. asphodeloides* extract, the current study has given a strong scientific support for its use as anti-diabetic remedy. This study is the first to report that the effect of aqueous and methanol extracts of this plant could, at least in part, be due to the ability to stimulate insulin secretion from β-cells (section 6.4.3). Bioassay-guided fractionation of the methanol extract resulted in an isolation of 4 compounds i.e. mangiferin, mangiferin glucoside, timosaponin AIII, and timosaponin BI (Fig. 6.12, for isolation and structure elucidation of these compounds see Chapter 7). Of these compounds, timosaponin AIII, mangiferin and mangiferin glucoside, but not timosaponin BI was capable of stimulating insulin secretion from RINm5F cells without concurrent membrane damage (section 6.7.3). This is the first time that these compounds have been shown to be active in insulin secreting cells.

The known distribution of timosaponins was found to be restricted only to *A. asphodeloides*. Pharmacological activity of these compounds are summarised in section 6.1. Timosaponin AIII, B and BII have previously been linked to anti-diabetic effect of the plant (Nakashima *et al.* 1993). Despite the similarity in the structure between timosaponin AIII and timosaponin BI, the activity of the two compounds on RINm5F cells was found to be different (Fig. 6.18 and 6.20). The studies showed that timosaponin AIII had a very potent effect on the secretion in RINm5F cells as the stimulation was observed at a very low dose of 1-8 μg/ml

(section 6.7.3). In contrast, it was found that timosaponin BI had no effect on insulin secretion. It was noteworthy that timosaponin BII of which the structure differed from timosaponin BI only at one functional group (Fig. 6.3) was also reported to have no effect on insulin secretion in perfused rat pancreas (Nakashima *et al.* 1993).

Mangiferin and its glucoside were found to significantly stimulate insulin secretion from RINm5F cells (Fig. 6.15 and 6.17), the effect of mangiferin but not mangiferin glucoside was potentiated by the presence of stimulatory concentration of nutrient (10 mM glyceraldehyde). These results suggested that mangiferin has potential advantages for use in the treatment of NIDDM as it would cause greater insulin release following a meal than in basal conditions and therefore causing less adverse effect of hypoglycaemia. In agreement with the effect observed in RINm5F cells, the potentiation effect of mangiferin was also observed in rat islets where the compound was tested in the presence of 20 mM glucose (Fig. 6.22). The finding that mangiferin was also capable of stimulating insulin secretion from rat islets gave strong support to the use of RINm5F cells in this approach. It was clearly shown in this study that bioassay-guided fractionation based on RINm5F cells afforded the isolation of a compound i.e. mangiferin which was capable of exerting its activity in rat islets. Interestingly, a number of xanthones have been linked to anti-diabetic effect, one of which was swerchirin whose effect was reported to be owing to the ability to stimulate insulin secretion (Saxana *et al.* 1993).

The studies on mechanism of action of mangiferin in rat islets revealed that the stimulatory effect of the compound was related neither to PKC nor PKA pathway, since the secretion induced by mangiferin was not inhibited by PKA and PKC inhibitors (section 6.9 and 6.10). Besides activating PKC and PKA, other pathways which have been thought to be involved in stimulatory process of insulin secretion included 1) elevation of cytosolic Ca^{2+} either by increasing an entry of Ca^{2+} into β-cells or inducing a release of Ca^{2+} from the intracellular pools and 2) an effect at a distal site of secretory pathway e.g. a promotion of the fusion between plasma membrane and insulin granules. There is a possibility that mangiferin may work by one of these mechanisms, further studies are necessary to clarify the mechanism of action of mangiferin.

Mangiferin is widely distributed in nature. Besides *Anemarrhena asphodeloides* and *Mangifera indica* L. (Anacardiaceae) from which mangiferin was first isolated (Hostettmann and Wagner, 1977), the compound has been isolated in a number of plant species including *Hiptage madablota* (Finnegan *et al.* 1968), *Gentiana asclepiadea* (Goetz and Jacot-Guillarmood, 1977), *Iris florentina* (Fujita and Inoue, 1982), *Salacia reticulata* (Karunanayake and Sirimanne, 1985), *Asplenuim adiatum-nigrum* (Imperato, 1991), *Hedysarum alpinum* (Geodakyan *et al.* 1992), *Iris nigricans* (Alkalil *et al.* 1995), *Hibiscus liliastrum* (Cafferty *et al.* 1996), *Hypericum* species (Kitanov and Nedialkov, 1998), *Swertia calycina* (Bain, 1998) and *Davallia solida* (Rancon *et al.* 1999).

Among these plants, only the extracts of *Mangifera indica* (Sharma *et al.* 1997; Aderibigbe *et al.* 1999) and *Salacia reticulata* (section 2.2) (Karunanayake *et al.* 1984; Serasinghe *et al.* 1990) have been reported to have anti-diabetic effect. However, active constituents of these plants have not yet been identified. The effect of *Salacia reticulata* was also investigated in this study, but it was found to have no stimulatory effect on insulin secretion in RINm5F cells (Chapter 4). As suggested by the results in this Chapter, mangiferin was not the only active constituent of *Anemarrhena asphodeloides*, and its stimulatory effect on insulin secretion appeared to be a synergistic effect of at least 3 components i.e. mangiferin, mangiferin glucoside and timosaponin AIII. It was therefore not surprising to find that the plant which also contained mangiferin e.g. *Salacia reticulata* was inactive in this particular model, since the activity would dependent on other compositions and quantity of the compound in the extract.

Swertia calycina was reported to be one of the sources of mangiferin. It was noteworthy that plants in the same genus e.g. *Swertia japonica* and *Swertia chirayita* have been quoted as a remedy for a traditional treatment of diabetes and their activity were well documented in a number of studies (section 2.2). The results suggested that their activity was, at least in part, owing to xanthones i.e. swerchirin, bellidifolin and methylbellidifolin (section 2.2). Unlike, *S. japonica* and *S. chirayita,* no study on the anti-diabetic effect of *S. calycina* has been reported. Therefore, study on the anti-diabetic effect of this plant may provide interesting information.

Other activities of mangiferin (section 6.1) which have been reported included inhibitory action on platelet thromboxane (Teng *et al.* 1989), antioxidant activity (Sato *et al.* 1992), anti-HIV (Zhu *et al.* 1993) and antitumour activity against fibrosarcoma (Guha *et al.* 1996). Among them, its activity on antioxidant and anti-inflammatory were the most studied (see section. 6.1.4 for details).

CHAPTER 7

ISOLATION AND IDENTIFICATION OF COMPOUNDS FROM *ANEMARRHENA ASPHODELOIDES*

7.1 Fractionation and isolation of compounds from *Anemarrhena asphodeloides*

7.1.1 Introduction

Stimulation of insulin secretion observed in the aqueous extracts of *Anemarrhena asphodeloides* was indicative of the presence of biologically active compounds in this plant (Fig. 5.14 & Chapter 6). Studies described in this chapter were undertaken to isolate single purified compounds from the extract of *A. asphodeloides*. Compounds in *Anemarrhena asphodeloides* were isolated by means of column chromatography using three different stationary phases i.e. normal phase silica gel (0.04-0.063 mm), Lipophilic Sephadex LH20 (bead diameter 25-100 μ) and reverse phase silica gel.

In silica gel chromatography, the separation of compounds is owing to an ability of silica gel to adsorb compounds with relative high polarity. The stationary phase formed by silica gel is relatively high in polarity compared to the mobile phase. A silica column is always initiated with relatively low polarity solvents and elution changed sequentially to solvents with higher polarity. Based on this strategy, compounds which are less polar will be eluted first, whereas polar compounds will initially bind to silica gel and are eluted with more polar solvent. With Sephadex LH 20 column (see section 4.4.1), compounds are separated by means of their sizes. At a given flow rate, relatively small compounds will spend longer time in the gel, thus are eluted last from the column; whereas compounds with relatively large molecules will pass through the gel more quickly and are eluted from the column first.

In the case of column chromatography on reverse phase (RP) silica gel, compounds with the least polarity will spend the shortest time in the column and the compounds are eluted in order of decreasing polarity, since the stationary phase i.e. reverse phase silica is less polar than the mobile phase. This is due to the fact that the OH groups on the surface of silica have been treated with reagents e.g. organic silanes to produce bonded structures e.g. long hydrocarbon chains such as octadecane (Houghton and Raman, 1998). Reverse phase column is always eluted with solvents of decreasing polarity.

Compounds isolated from *A. asphodeloides* extracts were identified by means of spectroscopic methods including NMR and Mass Spectroscopy. Details of the instrument and approach used in structure elucidation were given in section 7.2.

7.1.2. Materials and methods

Dried rhizome of *A. asphodeloides* was purchased from East-West Herbs (Kingham, UK) and Institute Of Chinese Medicine (London, UK) (section 2.3). Details of the authentication are given in section 2.4.

Unless specified, chemicals were analytical grade and purchased from Sigma-Aldrich Co. Ltd. (Irvine, UK). Silica gel for column chromatography and Silica GF_{254} TLC plates were purchased from Merck KGaA (Germany). Organic solvents were analytical grade and purchased from BDH Laboratory Supplies (England).

7.1.2.1 Column Packing

For the method of packing of silica and Sephadex LH20 columns see section 4.3.2.1 and 4.4.2.1, respectively.

The reverse phase silica (Rp-silica) column was packed using a similar method as described in the packing of normal silica column (see above). Rp-silica was mixed with water:MeOH (9:1) and gently transferred to a column. The column was gently tapped to promote a uniform settling. The extract reconstituted in 10% methanol in water was then applied to the top of the column using a Pasteur pipette. Rp-silica consisted of very fine particles, thus the solvent was eluted from the column under negative pressure to speed up the process.

7.1.2.2 Isolation of compounds from *Anemarrhena asphodeloides*

One kilogram of dried and powdered rhizome of *A. asphodeloides* was sequentially extracted with hexane, $CHCl_3$ and MeOH (2.5 L each) in a Soxhlet extractor for 6 hours (each). The extracts were evaporated to dryness under reduced pressure using a rotary evaporator and the weight of the residues was recorded. An investigation of the effect of hexane, $CHCl_3$ and MeOH extracts on insulin secretion

in RINm5F cells revealed that MeOH extract was the most active extract and had no damaging effect on cell membranes(section 6.4.3).

Fractionation and isolation of compounds from the methanol extract of *A. asphodeloides* are described in a flow chart in Figure 7.1. The methanol extract (30 g) was subjected to column chromatography (8.5 × 20 cm.) on silica gel as described in section 4.3.2.1. The column was initiated with $CHCl_3$ (500 ml) and the proportion of MeOH in mobile phase was gradually increased to 5, 10, 15, 30 and 50% (1 L each). The eluent was collected as 150 ml-fractions and the fractions were evaporated to dryness using a rotary evaporator. All fractions eluted from the column were then reconstituted in a minimal volume of appropriate solvent and examined on silica gel TLC using the following system:

Mobile Phase : $CHCl_3$: MeOH: acetic acid: H_2O (60:32:12:8)

Visualisation : UV light at 254 and 365 nm,
followed by spraying with anisaldehyde reagent.

The TLC plate after spraying with anisaldehyde was heated on a thermoplate at 110 °C for 5-10 minutes. Fractions with similar TLC chromatographic profile were pooled, and the solvent was then evaporated under rotary evaporator. The column chromatography on silica gel afforded a separation of the methanol extract into 5 fractions which were named AAM1-AAM5.

Fractions AAM1-AAM5 were tested on RINm5F cells and AAM3 was identified as the active fraction without causing marked LDH release (section 6.6.3). AAM3 (2 g) was further chromatographed on Sephadex LH20 column (2.5 × 30 cm.) using MeOH (700 ml) as an isocratic solvent as described in section 4.4.2.1. The eluent from the column was collected as 20 ml-fractions. Each fraction was examined on TLC using $CHCl_3$: acetic acid: MeOH: H_2O (60:32:12:8) as the mobile phase as described earlier. Fractions with similar chromatographic profile were pooled to afford 3 combined fractions named A31-A33.

A31 was further chromatographed on Sephadex LH20 column (2.5 × 20 cm.) eluted with methanol (500 ml). The fractions eluted from the column were examined on TLC using $CHCl_3$: acetic acid: MeOH: H_2O (60:32:12:8) as the mobile phase as described earlier. Fractions with similar chromatographic profile were pooled to afford

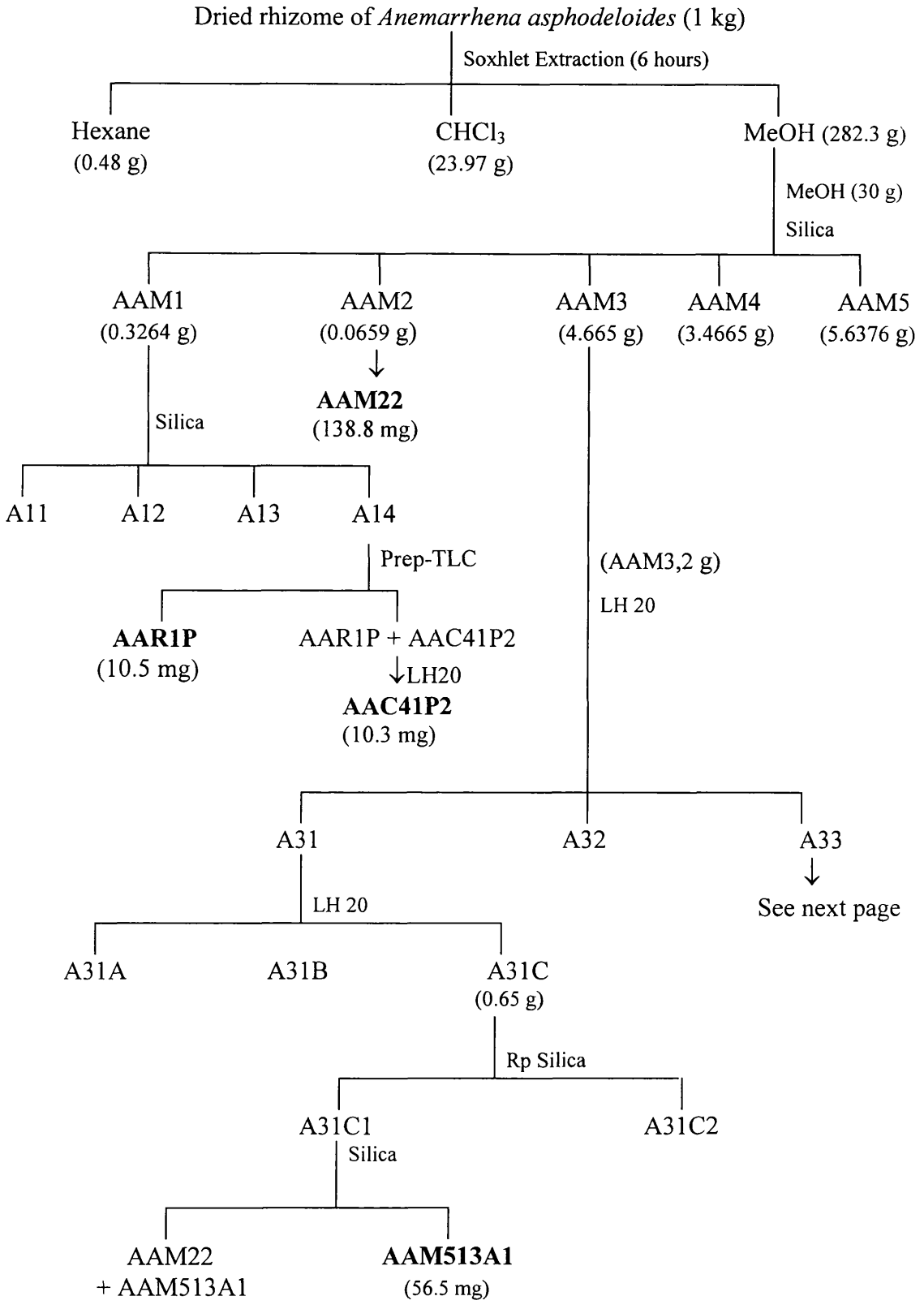


Fig. 7.1: A flow chart for fractionation and isolation of compounds from *Anemarrhena asphodeloides* rhizome

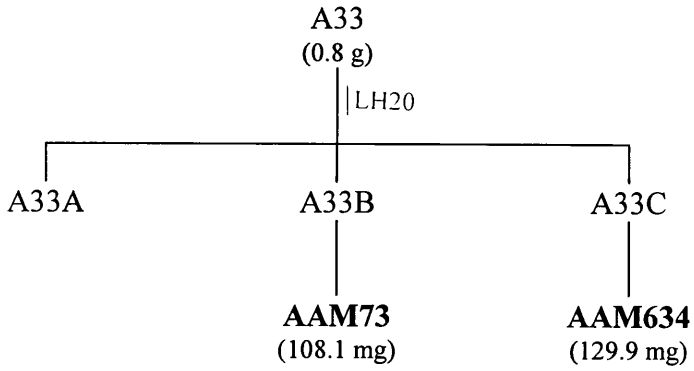


Fig. 7.1 (continued): A flow chart for fractionation and isolation of compounds from *Anemarrhena asphodeloides* rhizome

the isolation of 3 fractions named A31A-A31C. A31C (0.65 g), which according to TLC chromatogram contained one main compound and trace amounts of other compounds including AAM22 (see below), was rechromatographed on reverse phase silica column (2 × 10 cm) initiated with 10% methanol in water, gradually increasing to 20%, 30%, 40%, 50% and 70% methanol (100 ml each). The eluents were collected as 10-ml fractions. Each fraction was examined on TLC as described earlier and similar fractions were pooled, resulting in 2 main fractions (A31C1 and A31C2). The first fraction was rechromatographed on silica gel using $CHCl_3$:MeOH:H_2O (70:30:3) as mobile phase to afford the isolation of one single compound named AAM513A1 and a mixture containing AAM22 (see below) and AAM513A1. On TLC using $CHCl_3$: acetic acid: MeOH: H_2O (60:32:12:8) as the mobile phase, AAM513A1 could not be detected under UV but gave yellow colour when sprayed with anisaldehyde reagent.

A33 (0.8 g) was further purified by chromatographed on a Sephadex LH20 column (2.5 × 20 cm.) eluted with methanol (500 ml). The fractions of 15 ml was collected, each fraction was examined on TLC using the system described above. The fractions with similar profile were pooled, resulting in 3 combined fractions (A33A-A33C). A33B was rechromatographed on Sephadex LH20 (2.5 × 20 cm.) and eluted with MeOH (500 ml) to afford the isolation of a pure compound named AAM73. TLC chromatogram of this compound revealed a dark spot under UV 254 nm which gave orange colour under UV 365 nm and yellow colour when sprayed with anisaldehyde spraying reagent. It was shown on TLC that A33C contained 2 main spots, one of

which corresponded to AAM73, another gave bright orange colour under 365 nm. The two compounds were separated by subjecting the fraction to chromatography on a Sephadex LH20 column (2.5 × 15 cm), eluted with methanol (400 ml). Fractions of a volume of 5-10 ml were collected. Two compounds were isolated from this column, one of which was AAM73 and another was AAM 634.

Evaporation of the AAM2 fraction by allowing the extract to stand overnight at room temperature resulted in a precipitation of white residue. Examination of this residue on TLC using the system as described above revealed one main spot and trace amount of another constituents. The residue was then reconstituted in methanol and recrystallised by adding a few drops of chloroform and allowing to stand overnight at room temperature. The residue obtained from recrystallisation was filtered under negative pressure and washed with chloroform. Investigation of the purity of this residue on TLC revealed a single spot which gave yellow colour with anisaldehyde reagent but could not be detected under either short or long wave UV. This purified compound was named AAM22.

AAM1 was chromatographed on silica gel (2.5 × 25 cm.) initiated with $CHCl_3$ (100%), the polarity gradually increased to 20% MeOH in 5% increments (300 ml each). The fractions were examined on normal phase silica TLC using $CHCl_3$:MeOH (100:4) as a mobile phase. The chromatogram was visualised under UV and sprayed with anisaldehyde reagent. The fractions with similar profile were pooled to afford the isolation of 4 combined fractions (A11-A14).

A14 which contained two main compounds was subsequently separated on a preparative silica TLC plate (0.75 mm thickness) using hexane: ether (1:1) as a solvent system. The fraction was reconstituted in a mixture of $CHCl_3$ and methanol (1:1) and applied on the TLC plate as a 15-cm band. After the mobile phase was allowed to run for a distance of 15 cm., the plate was removed from the TLC tank and dried. The plate was then put back to the TLC tank, the mobile phase was again allowed to develop with the same distance. The repeated development was to improve the separation between the two compounds. The two main bands were scraped off from TLC plate, the compounds were recovered by shaking the scraped silica gel in $CHCl_3$:MeOH (1:1). The solvent was evaporated to dryness using rotary evaporator

under negative pressure. The residues were reconstituted in a minimal volume of $CHCl_3$:MeOH (1:1) and examined on TLC using $CHCl_3$:MeOH (100:4) as mobile phase. The residue scraped from one of the bands was revealed as a single spot which could be detected under UV (254 nm) and gave blue colour when sprayed with anisaldehyde reagent, this compound was named AAR1P (10.5 mg). The residue scraped from another band was found on TLC to be a mixture of AAR1P and another compound. The two compounds were separated by elution from Sephadex LH 20 column (2 × 20 cm) using MeOH (400 ml), resulting in two purified compounds i.e. AAR1P and another compound named AAC41P2.

7.1.3 Results and discussion

Soxhlet extraction of dried rhizome (1 kg) of *A.. asphodeloides* with different solvents afforded three extracts i.e. hexane extract (0.48 g), $CHCl_3$ extract (23.97 g) and MeOH extract (282.3 g). Fractionation and purification of methanol extracts on a series of columns using silica gel, Sephadex LH 20 and reverse phase silica gel as the stationary phase, and preparative TLC afforded the isolation of 6 compounds: AAR1P (10.5 mg), AAC41P2 (10.3 mg), AAM22 (138.8 mg), AAM513A1 (56.5 mg), AAM73 (108.1 mg) and AAM634 (129.9 mg).

On TLC AAM22 appeared to be the most abundant compounds in methanol extract of *A. asphodeloides*. The details of structure elucidation of these compounds are given in section 7.2.

7.2 Structure elucidation of the compounds isolated from the methanol extract of *Anemarrhena asphodeloides* rhizome

7.2.1 Introduction

Fractionation and isolation of compounds from the methanol extract of *Anemarrhena asphodeloides* rhizome as described in section 7.1.2.2 resulted in the purification of 6 compounds. In order to elucidate the structure of these compounds, various types of spectroscopy were conducted. These included proton nuclear magnetic resonance (^{1}H NMR), carbon nuclear magnetic resonance (^{13}C NMR), ^{1}H-^{1}H shift correlation spectroscopy (COSY), ^{1}H-^{13}C COSY (HMQC), long range ^{1}H-^{13}C

COSY (Heteronuclear Multiple Bond Connectivity, HMBC), mass spectrometry, and to a lesser extent, infrared spectroscopy and ultraviolet spectroscopy (section 4.5.1).

7.2.2 Materials and methods

^{1}H, ^{13}C and 2D NMR spectra were recorded in $CDCl_3$ for AAR1P and AAC41P2, in CD_3OD for AAM513A1, in DMSO-d_6 for AAM22 and AAM73, and in D_2O for AAM634 using tetramethylsilane (TMS) as internal standard at 400 MHz (^{1}H) and 100 MHz (^{13}C) on a Bruker AC-300 instrument equipped with standard Bruker software. The experiments were conducted by Ms Jane Hawkes, NMR section Chemistry Department, King's College, University of London for AAM2, AAM634 and AAM73, and by Mr WH Zhang, Department of Chemistry, Hong Kong University of Science and Technology, Hong Kong for AACR1P, AAC41P2 and AAM513A1.

Fast atom bombardment mass spectra (FABMS) and electron impact mass spectra (EIMS) were conducted using AutoSpec FAB^+ and a Perkin-Elmer SCIX API-1 mass spectrometer, respectively at ULIRS Chromatography/Mass Spectrometry Service, Chemistry Department King's College, University of London. High resolution FABMS was conducted using a Zab-SE VG Analytical mass spectrometer at School of Pharmacy, University of London. Infrared (IR) spectroscopy were conducted using a Perkin-Elmer spectrophotometer (model 1605 FTIR) in potassium bromide (KBr) and UV spectra were obtained using a Perkin-Elmer spectrophotometer (model UV/VIS Lambda 2) at Department of Pharmacy, King's College London.

Molar extinction coefficient (ε) of a compound was calculated from the following formula: $\varepsilon = A/bc$ (A: maximum absorption; b: path length of radiation (cm); c: concentration (mol/L))

Degree of unsaturation which corresponds to number of double bonds and close ring in a molecule was calculated according to the following formula:

$$\text{Degree of unsaturation} = \text{No. of carbon atom} - \frac{(\text{No. of hydrogen atom} - 1)}{2} + 1$$

7.2.3 Results and discussion

The process of structure elucidation and identification of compounds isolated as described above (section 7.1.2.2) followed the pattern below:

a) molecular formula was determined based on MS and NMR data
b) the general type of structure was postulated based on the type of signal observed in the ^{13}C and ^{1}H NMR spectra
c) literature survey on *Anemarrhena asphodeloides* was conducted to investigate possible identities for the compounds by a comparison of data obtained in the current study with that reported by previous workers
d) the spectra obtained in current experiments were evaluated to confirm that it matched the proposed structure.

Spectral data for the compounds discussed in this section are given in the Appendices 3-9.

7.2.3.1 Structure elucidation of AAR1P as *cis* hinokiresinol (nyasol)

UV, MS and NMR spectral data of AAR1P are given in Appendix 3.

AAR1P was isolated as brownish oily residue. UV spectrum of AAR1P in methanol gave absorption peaks at and 210 and 256 nm. The compound appeared to have a molecular formula of $C_{17}H_{16}O_2$ (MW = 252) in agreement with the molecular ion peak at *m/z* 252 $[M]^+$ in its EI mass spectrum. The EIMS also showed fragment peaks at 237 $[M-15]^+$ (32%) and 158 $[M-94]^+$ (99%), due to the loss of CH_3 and C_6H_6O respectively. The spectrum also exhibited a fragment peak at 77 (28 %) which corresponded to the fragment of C_6H_5.

NMR data and structure of AAR1P are given in Table 7.1 & 7.2, and Figure 7.2, respectively. Proton and carbon NMR spectra of AAR1P (Table 7.1 and 7.2) were found to match those reported in literature for *cis* hinokiresinol (Tsui and Brown, 1996). Proton spectrum of AAR1P in $CDCl_3$ showed signals of four pairs of aromatic protons at δ 6.77 (2H, *d*, *J* = 8.5), 6.79 (2H, *d*, *J* = 8.5), 7.07 (2H, *d*, *J* = 8.5) and 7.15 (2H, *d*, *J* = 8.5). Each signal appeared as doublet with *ortho* coupling (*J* = 8.5). Integration of each signal indicated the presence of two protons experiencing very similar environment and hence came into resonance at identical chemical shift. The signals at δ 6.77 and 6.79 were appropriate to aromatic protons attached to carbons situated adjacent to carbons bearing hydroxyl groups. These signals were therefore assignable to H-3′ & H-5′, and H-3″ & H-5″, respectively based on the values reported by Tsui and Brown, (1996) for *cis* hinokiresinol. A similar pattern of signals

was observed at δ 7.07 and 7.15. The signal at δ 7.07 was found in long range ^{1}H-^{13}C COSY spectrum to exhibit three bond correlation with carbon at δ 46.70 which was assigned as C-3 (see below), leading to an assignment of this proton signal as H-2″ and H-6″. Signal at δ 7.15 was therefore assigned as H-2′ and H-6′.

AAR1P also exhibited signals of five vinyl protons at δ 5.13 (1H, *dd*, *J* = 17.1), 5.14 (1H, *dd*, *J* = 10.0), δ 5.65 (1H, *dd*, *J* = 11.5, 10.0), 5.99 (1H, *ddd*, *J* = 17.1, 10.0, 6.4) and 6.49 (1H, *d*, *J* = 11.1). The presence of proton signal at δ 5.99 as septet with one overlapping peak, as suggested by double intensity of the middle peak, indicated that this proton was coupling to other protons of methine and methylene carbons. This signal (δ 5.99) was therefore appropriate to proton at position 4. Based on the coupling constant, the splitting pattern of H-4 appeared to arise from its coupling to proton signals at δ 4.50 (*J* = 6.4), 5.13 (*J* = 17.1) and 5.14 (*J* = 10). The signals of H-4 could have appeared as a doublet quartet (8 lines) but did not because of the overlapping of the signals which came to resonance at the same chemical shift. Based on the coupling constant and splitting pattern of the signals, proton at 4.50 was appropriate to signal of allyl proton and therefore was assigned as H-3, and protons at δ 5.13 and 5.14 were assigned as the two protons of H-5. The environment that the two protons of H-5 experiencing was different, thus they came into resonance at slightly different chemical shifts. The coupling constant of the H-5 protons with H-4 corresponded to *trans* (*J* = 17.1) and *cis* (*J* = 10), respectively. Besides coupling to the proton adjacent to them (i.e. H-4), the two protons of H-5 also coupled to each other, giving rise to multiplet signals as observed in the ^{1}H spectrum. However, the coupling constant between the two protons of H-5 could not be resolved in this spectrum. In agreement with this assignment, proton at 5.99 (H-4) was found in ^{1}H-^{1}H COSY spectrum to exhibit a correlation with proton signals at 5.13 and 5.14 (H-5). However, the correlation between H-4 and H-3 was not observed in this spectrum.

The chemical shift of proton at δ 6.49 was appropriate to vinyl proton attached to carbon situated next to aromatic ring, thus this proton was assigned as H-1. The signal at a slightly higher field of δ 5.65 was assigned as H-2 based on its correlation to H-1 in ^{1}H-^{1}H COSY. In agreement of this assignment, proton at δ 5.65 was also found in ^{1}H-^{1}H COSY spectrum to correlate with H-3 (δ 4.50).

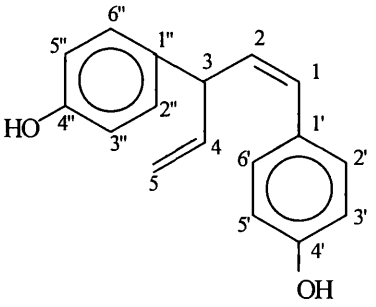


Fig. 7.2: Structure of AAR1P (*cis* Hinokiresinol, Nyasol)

Table 7.1: ^{1}H NMR spectral data of AAR1P in $CDCl_3$

Position	δ ^{1}H (ppm)	Reported values*
1	6.49 (1H, *d*, *J* = 11.5)	6.51
2	5.65 (1H, *dd*, *J* = 11.5, 10.0)	5.67
3	4.50 (1H, *dd*, *J* = 10.0, 6.4)	4.51
4	5.99 (1H, *ddd*, *J* = 17.1, 10.0, 6.4)	6.00
5	5.14 (1H, *dd*, *J* = 17.1)	5.15
	5.13 (1H, *dd*, *J* = 10.0)	5.17
2′, 6′	7.15 (2H, *d*, *J* = 8.5)	7.17
3′, 5′	6.79 (2H, *d*, *J* = 8.5)[a]	6.78
2″, 6″	7.07 (2H, *d*, *J* = 8.5)	7.10
3″, 5″	6.77 (2H, *d*, J = 8.5)[a]	6.77

*Values reported by Tsui and Brown, (1996) for *cis* hinokiresinol (in $CDCl_3$); [a]Signals might be interchangeable

^{13}C NMR spectrum in $CDCl_3$ revealed 13 resonances; four of which appeared to be double intensity (δ 114.64, 114.92, 128.24, 129.46), suggesting the presence of 17 carbons of which 4 pairs had similar environment. The ^{13}C NMR further revealed signals of one methylene carbon (δ 114.19), 12 methine carbons and 4 quaternary carbons (δ 128.43, 134.18, 154.61 and 155.23) in accordance with ^{13}C spectral data reported in literature for *cis* hinokiresinol (Tsui and Brown, 1996).

The methine carbon in the downfield region of δ 46.70 was appropriate to a non aromatic carbon. It was found in long range ^{1}H - ^{13}C COSY spectrum that this proton exhibited two bond correlation with H-2 (δ 5.65) and H-4 (δ 5.99), and three bond correlation with H-5 (δ 5.14 and 5.13) and H-1 (δ 6.49), leading to an

Table 7.2: ^{13}C spectral data for AAR1P in $CDCl_3$

Position	δ ^{1}H (ppm)	Reported values*	DEPT
1	128.20	128.6	CH
2	130.93	131.7	CH
3	46.70	46.8	CH
4	140.60	140.7	CH
5	114.19	115.0	CH_2
1′	128.43	129.8	C
2′, 6′	129.46	130.0	CH
3′, 5′	114.64	115.1	CH
4′	155.23	154.6	C
1″	134.18	135.5	C
2″, 6″	128.24	128.9	CH
3″, 5″	114.92	115.4	CH
4″	154.61	154.1	C

*Values reported by Tsui and Brown, (1996) for *cis* hinokiresinol (in $CDCl_3$)

assignment of this carbon as C-3. Signals at δ 114.19, 128.20, 130.93, and 140.60 were assigned as C-5, C-1, C-2 and C-4, respectively based on the correlation on one bond ^{1}H-^{13}C COSY spectrum of these signals to the proton signals at δ 5.13-5.14 (H-5), 6.49 (H-1), 5.65 (H-2) and 5.99 (H-4) respectively. The chemical shift of signals of quaternary carbons at δ 154.61 and 155.23 was appropriate to carbons in aromatic rings bearing hydroxyl group, these signals were assigned as C-4″ and C-4′, respectively based on values reported in literature (Tsui and Brown. 1996). The quaternary carbon at 134.18 was found to exhibit two bond correlation with proton at δ 4.50 (H-3) in long range ^{1}H-^{13}C COSY spectrum, leading to an assignment of this carbon as C-1″, and the remaining carbon signal at δ 128.43 was assigned as C-1′.

According to a molecular formula, AAR1P appeared to have degree of unsaturation of 10 which matched the structure of *cis* hinokiresinol which had 8 double bonds and 2 closed rings. Oketch-Rabah *et al.* (1997) suggested that two isomers of hinokiresinol are naturally occurring and can be distinguished by means of coupling constant on NMR spectrum, chemical shift of C-3 on NMR spectrum and λ_{max}. According to Oketch-Rabah *et al.* (1997), *cis* isomer has a coupling constant

($J_{1,2}$) of 11-12 Hz whereas *trans* isomer has a coupling constant of 16 Hz; the chemical shift of C-3 is reported to be δ 46-48 for *cis* isomer and δ 51 for *trans* isomer, and the λ_{max} for *cis* isomer is 258 nm and 264 nm for *trans* isomer. NMR and UV spectra of AAR1P showed that 1) H-1 (δ 6.49) and H-2 (δ 5.65) of the vinyl group were coupled with a coupling constant of 11.5, 2) chemical shift of C-3 is δ 46.7 and 3) λ_{max} in MeOH was 256. These findings suggested that AAR1P was indeed the *cis* isomer.

Taking all the information into account, the compound was assigned as 4-4′-hydroxy (3-ethenyl-1-propene-1,3,diyl)-bisphenol (*cis*-hinokiresinol, nyasol). The assignment was in a good agreement to that reported in the literature (Tsui and Brown, 1996).

7.2.3.2 Structure elucidation of AAC41P2 as monomethyl-*cis*-hinokiresinol

UV, MS and NMR spectra of AAC41P2 are given in Appendix 4.

NMR data and structure of AAC41P2 are given in Table 7.3 and Figure 7.3, respectively. UV spectrum of AAC41P2 in methanol gave the absorption peaks at 210 and 252 nm. AAC41P2 appeared to have a molecular formula of $C_{18}H_{18}O_2$ (MW = 266) based on its NMR and MS data. This proposed molecular formula was in accordance with a molecular ion peak at *m/z* 266 $[M]^+$ (100 %) in its EIMS. The EIMS also showed fragment peaks at 251 $[M-15]^+$ (38%), 172 $[M-94]^+$ (42%), 159 $[M-107]^+$ (100%), and 107 $[M-159]^+$ (38%), due to the loss of CH_3, C_6H_6O, C_7H_7O, and $C_{11}H_{11}O$, respectively. The peak at 77 corresponded to the fragment of C_6H_5. Similarity between ^1H and ^{13}C NMR spectra of AAC41P2 and those of AAR1P (section 7.2.3.1, Appendix 3) suggested that the two compounds shared the same skeleton. ^1H and ^{13}C NMR spectra of AAC41P2 was interpreted in comparison with the spectra of AAR1P.

Despite broad spectral signals, ^1H NMR of AAC41P2 was found to be very similar to that of AAR1P; the only difference was that AAC41P2 exhibited an extra singlet signal at δ 3.62 (3H). The chemical shift and integration of this signal was appropriate to a methoxy group. According to ^1H NMR spectra of AAC41P2, it appeared that one of the hydroxyl groups attached to aromatic rings was substituted by a methoxy group as indicated by the downfield shift of *ipso* carbon (C-4′) and the

upfield shift of *ortho* carbon (C-3′/5′, see below) compared to the spectrum of AAR1P.

^{13}C NMR spectra of AAC41P2 in $CDCl_3$ (with approximately 20% of CD_3OD) revealed 14 resonances; four of which appeared to be double intensity (δ 113.25, 114.97, 128.27, 129.42), suggesting the presence of 18 carbons including 4 pairs of equivalent protons. ^{13}C NMR spectrum further revealed one methyl carbon at δ 54.94 which was absent in the ^{13}C spectrum of AAR1P (Table 7.2) as well as one methylene carbon (δ 114.27), 12 methine carbons and 4 quaternary carbons (δ 127-

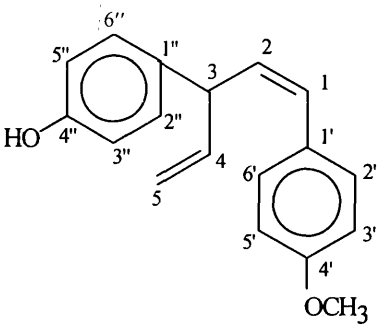


Fig. 7.3: Structure of AAC41P2 (monomethyl *cis*- hinokiresinol)

Table 7.3: ^{1}H and ^{13}C NMR spectral data of AAC41P2 in $CDCl_3$

Position	δ ^{1}H (ppm)	δ ^{13}C (ppm)	DEPT
1	6.50	127.97	CH
2	5.68	131.55	CH
3	4.48	46.68	CH
4	6.01	140.58	CH
5	5.14	114.27	CH_2
1′	-	127-128	C
2′, 6′	7.40[a]	129.42	CH
3′, 5′	7.07[b]	113.25	CH
4′	-	158.01	C
O-CH_3	3.62	54.94	CH_3
1″	-	134.07	C
2″, 6″	7.22[a]	128.27	CH
3″, 5″	6.87[b]	114.97	CH
4″	-	154.77	C

[a, b]Signals identified with the same letter might be interchangeable

128, 134.07, 154.77 and 158.01). By comparing with the ^{13}C spectrum of AAR1P (Table 7.1 and 7.2), it appeared that one quaternary carbon of AAC41P2 possibly overlapped to signals in the region of δ 127-128 and could not be resolved using this instrument.

The chemical shift of the carbon located at position 4′ was found to come into resonance at further downfield at δ 158.01 compared to C-4′ of AAR1P which was observed at 155.23. In contrast to C-4′, it was found that C-3′ and C-5′ of AAC41P2 shifted upfield to 113.25 (-1.39 ppm). These finding indicated the substitution of OH group in aromatic ring with methoxy group at C-4′. The upfield shift of *ortho* carbon and the downfield shift of *ipso* carbon are the characteristic shifts observed when OH group of aromatic ring was substituted with methoxy group (Williams and Fleming, 1995). Besides the signals at δ 113.25 and 158.01, the chemical shifts of the other carbons were very close to those observed in the spectra of AAR1P.

Similarly to AAR1P, AAC41P2 was found to have degree of unsaturation of 10 which matched the structure of monomethyl *cis*-hinokiresinol (8 double bonds and 2 closed rings).

7.2.3.3 Structure elucidation of AAM22 as timosaponin AIII

UV, IR, MS and NMR spectra of AAM22 are given in Appendix 5.

AAM22 was obtained as white amorphous powder. Its UV spectrum in methanol exhibited maximum absorption at 200 nm, indicating the absence of any conjugated chromophore. The compound was found to have a molar extinction coefficient of 543.67. Its IR (KBr) spectrum showed absorption at 3419, 2930, 1448, 1383, 1174, 1069, 985, 926, 851 and 637 cm^{-1}. Strong absorption observed in IR spectrum at 3419 (OH) and 1069 cm^{-1} was an indicative of the presence of glycosidic moiety (Mimaki *et al.* 1998). AAM22 appeared to have a molecular formula of $C_{39}H_{64}O_{13}$ (MW = 740) based on NMR and MS data. The proposed molecular formula was in a good agreement with FABMS spectrum of which the compound was found to have a molecular peak at *m/z* 763 $[M + Na]^+$ (72%) and other peaks at 741 $[M + H]^+$ (4%), 439 [aglycone + Na + H]$^+$ (6%), 413 (16%). Calculated mass of AAM22 was 740.9268 on the basis of proposed molecular formula which matched the value

obtained from FABMS high resolution spectrum (i.e. 740.46473). Melting point of AAM22 was 294.4-300°C (literature: > 300 °C (Nagumo *et al.* 1991)).

NMR spectrum of AAM22 was assigned based on a comparison with data reported in literature for *Anemarrhena* saponins (Nagumo *et al.* 1991; Saito *et al.* 1994). The spectra of AAM22 were found to match those reported for timosaponin AIII (Saito *et al.* 1994). ^{1}H and ^{13}C NMR spectra and structure of AAM22 are given in Table 7.4, Table 7.5 and Figure 7.4, respectively. Proton NMR spectrum of AAM22 in DMSO-d_6 (Table 7.4) exhibited two singlet signals at δ 0.71 and 0.90 (3H, each) due to steroidal methyl groups located at H-18 and H-19, respectively, and two doublet signals at δ 0.93 (J = 7.8) and 1.01 (J = 7.0) due to methyl groups located at H-21 and H-27, respectively. The assignment of these methyl groups was based on values reported in literature for timosaponin AIII (Saito *et al.* 1994). One doublet signal at δ 4.39 in proton spectrum (J = 7.7) indicated the presence of anomeric proton of sugar moiety. The signal of another anomeric proton of sugar moiety was found to overlap with signal of H-16 of the aglycone. The signals of two anomeric protons were clearly resolved in a spectrum run in pyridine-d_5 of which the two anomeric protons were observed at δ 4.93 (*d*, J = 7.64) and 5.29 (*d*, J = 7.67). The slight change in the chemical shift of the anomeric protons was possibly due to effect of the solvents. Configuration of sugar moiety was revealed as β-linkage, according to large J value (7 Hz).

^{13}C NMR spectrum of AAM22 in DMSO-d_6 revealed signals of 39 carbons; 19 of which were methine carbons, 13 were methylene carbons, 4 were methyl carbons i.e. δ 14.84, 16.29, 16.56 and 23.94 and 3 were quaternary carbons i.e. δ 34.87, 40.56 and 109.20 in agreement with the spectrum reported in literature for timosaponin AIII (Saito *et al.* 1994) (Table 7.5). Two methine carbons in the downfield region of δ 101.09 and 104.20 indicated the presence of oxygenated carbons (O-CH-O) corresponding to H-1 of sugar moieties. Signals of methyl carbons were assigned as C-21 (δ 14.84), C-27 (δ 16.29), C-18 (δ 16.56) and C-19 (23.94) based on values reported in literature for timosaponin AIII (Saito *et al.* 1994). The signal of quaternary carbon at δ 109.20 was appropriate to carbon at position 22 where the nuclei was deshielded by two atoms of oxygen, resulting in the resonance of this carbon in the

downfield region. The other signals of quaternary carbons at the upfield region of δ 34.87 and 40.56 were assigned as C-10 and C-13, respectively based on values reported by Saito *et al.* 1994.

The signals of methine carbons in the region of δ 68.11-80.76 were appropriate to carbons attached to oxygen atoms, 8 of which belonged to sugar moieties and two were carbons of the aglycone. It was found in ^{1}H-^{1}H COSY spectrum that proton signals at δ 3.90 and 4.23-4.28 which attached to methine carbons at δ 74.20 and 80.76, respectively on the basis of ^{1}H-^{13}C COSY spectrum exhibited a correlation with protons in the upfield region of δ 1-2 which, according to the chemical shift, appeared to be protons of aglycone rather than sugar moieties of which protons usually came into resonance at slightly lower field (δ 4-5). These findings in turn suggested that the protons at δ 3.90 and 4.23-4.28 were protons of the aglycone moiety. The presence of these protons in the downfield region indicated that they possibly attached to oxygenated methine carbons of which there were only two in the aglycone part of the molecule i.e. H-3 and H-16. The proton at δ 3.90 was found in long range ^{1}H-^{13}C COSY spectrum to exhibit three bond correlation with methine carbon at δ 101.09 which appeared to be C-1 of one of the sugar moieties based on its correlation with anomeric proton at δ 4.20, leading to an assignment of the proton at δ 3.90 as H-3 and its corresponding carbon (δ 74.20) as C-3. Consequently, the signal of anomeric proton at δ 4.20 was assigned as H-1′ and its corresponded carbon (δ 101.09) as C-1′. The proton at δ 4.23-4.28 was assigned as H-16 and its corresponding carbon (δ 80.76) as C-16. In turn, H-3 was also found in long range ^{1}H-^{13}C COSY spectrum to exhibit three bond correlation with methine carbon at 36.29, leading to an assignment of this carbon as C-5.

In ^{1}H-^{1}H COSY spectrum, H-16 was found to correlate with signals at δ 1.14-1.16, 1.64-1.68 and 1.89, suggesting the possible identity of these protons as H-15 which attached to methylene carbon and H-17 which attached to methine carbon. The signal at δ 1.64-1.68 was found in ^{1}H-^{13}C COSY spectrum to correlate with methine carbon at δ 62.22, leading to an assignment of this proton as H-17 and the carbon signal at δ 62.22 as C-17. The signals at δ 1.14-1.16 and 1.89 were found in

^{1}H-^{13}C COSY spectrum to exhibit a correlation with a methylene carbon at δ 31.77, thus the protons were assignable to the two protons of H-15. The two protons came into resonance at different chemical shift because being part of ring system, these position was fixed and they were experiencing slightly different environment of the neighbourhood. Three methylene carbons in downfield region (δ 60.57, 61.38 and 64.61) indicated the presence of carbon adjacent to oxygen atoms. The signals at δ 60.57 and 61.38 were found in ^{1}H-^{13}C COSY spectrum to correlate with proton signals in the downfield regions of δ 3-4, suggesting that they are methylene carbons of sugar moieties (C-6′/6″).

The methylene carbon at δ 64.61 was found to correlate with proton signals at δ 3.21 and 3.78. ^{1}H-^{1}H COSY spectrum revealed a correlation between proton signals in the downfield region of δ 3.21 and 3.78 and the signals in the upfield region of δ 1.64-1.68, suggesting that the former (δ 3.21 and 3.78) were part of the aglycone rather than sugar moieties. The resonance of these protons in the downfield region indicated that they were attached to oxygenated carbon, these protons were therefore assigned as H-26, leading to the assignment of the carbon signal at δ 64.61 as C-26 which was the only oxygenated methylene carbon in the aglycone. Two protons of H-26 came into resonance at slightly different chemical shift because the environment they were experiencing was different. Consequently, the protons at δ 1.64-1.68 which correlated to H-26 were assigned as H-25, and carbon attached to this proton i.e. the signal at δ 26.82 as identified on the basis of ^{1}H-^{13}C COSY spectrum was assigned as C-25. The signal of H-25 was found to overlap with signal of H-17.

The proton signal at δ 0.71 (s, H-18) showed two bond correlation with carbon signal at δ 40.56 (C-13) and three bond correlation with carbons at δ 40.02 (C-12), 56.10 (C-14) and 62.22 (C-17). Long range ^{1}H-^{13}C NMR spectrum revealed two bond correlation between proton signal at δ 0.90 (s, H-19) and carbon signal at δ 34.87, and three-bond correlation with carbon signals at δ 30.43, 36.29 and δ 39.76. These carbons were therefore assigned as C-1 (δ 30.43), C-10 (δ 34.87), C-5 (δ 36.29) and C-9 (δ 39.76). Two bond correlation between carbon signal at δ 41.92 and proton signal at δ 0.93 (s, H-21) allowed the carbon to be assigned as C-20. Carbon at

position 25 was assigned based on its correlation on long range ^{1}H-^{13}C COSY spectrum with methyl group at position 27 (δ 1.01).

Acid hydrolysis of AAM22 was conducted by dissolving the compound (30 mg) in 3 ml of 1M H_2SO_4 in H_2O -dioxane (1:1), followed by heating at 100 °C for 1 hr. After dilution with water (~10 ml), the solution was extracted twice with 20 ml of ethyl acetate. The water layer was then neutralised with aqueous $Ba(OH)_2$ solution, and centrifuged to remove precipitated $BaSO_4$. The supernatant was evaporated to dryness. The residue was then reconstituted in methanol and investigated for the presence of sugars which was conducted using TLC and compared with reference glucose, galactose and rhamnose. Investigation on silica TLC using propanol: ethyl acetate: water (65:35:20) as a mobile phase and visualised by spraying with diphenylamine reagent (section 3.7.2) revealed the presence of two sugars i.e. D-glucose and galactose in agreement with sugar moieties of timosaponin AIII reported by (Saito *et al.* 1994).

According to a molecular formula ($C_{39}H_{64}O_{13}$), AAM22 was found to have a degree of unsaturation of 8 in agreement with the structure of timosaponin AIII (8 closed rings)

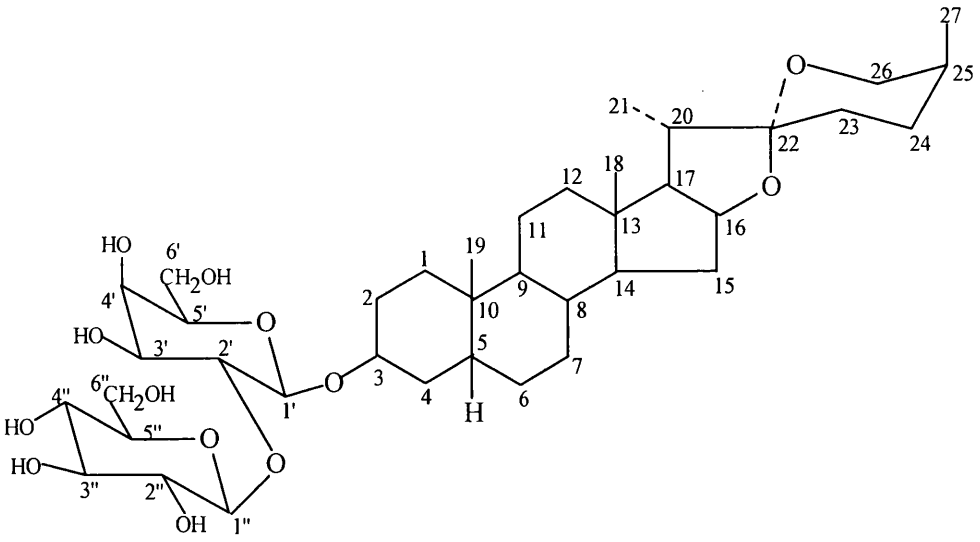


Fig.7.4: Structure of AAM22 (timosaponin AIII)

Table 7.4 : ^{1}H NMR spectrum of AAM22 in DMSO-d_6

Aglycone	δ ^{1}H (ppm)	Reported Values* (in pyridine-d_5)
H-1	1.33-1.43	-
H-2	1.02-1.08[a]	-
H-3	3.90 (br s)	4.30 (br s)
H-4	1.65-1.73	-
H-5	1.73-1.77	-
H-6	1.33-1.43[a]	-
H-7	1.51-1.57	-
H-8	1.51-1.57	-
H-9	1.33-1.43	-
H-10	-	-
H-11	1.33-1.43	-
H-12	1.64-1.68	-
H-13	-	-
H-14	1.08-1.20	-
H-15	1.14-1.16, 1.89	1.25-2.25
H-16	4.23-4.28	4.68 (*ddd*, *J*=11.6, 7.6, 0.2)
H-17	1.64-1.68	2.00-3.00
H-18	0.71 (s)	0.82 (s)
H-19	0.90 (s)	0.96 (s)
H-20	1.73-1.79	-
H-21	0.93 (*d*, *J* = 7.8)	1.15 (*d*, 7.1)
H-22	-	-
H-23	1.14-1.16	-
H-24	176-1.89	-
H-25	1.64-1.68	-
H-26	3.21 (br *d*, *J* = 10.8)	3.37 (*d*, *J*=11.0)
	3.78 (br *d*, *J* = 10.8, 1.9)	4.07 (*d*, *J*=11.0, 2.4)
H-27	1.01 (*d*, *J* = 7.0)	1.08 (*d*, *J*=7.0)
O-3 Galactose		
H-1′	4.20 (overlapped with H-16)	4.88 (*d*, *J*=7.6)
H-2′	3.57 (*dd*, 7.7, 9.6)	4.62 (*dd*, *J* = 9.5, 7.6)
H-3′	3.47-3.54	4.23 (*dd*, *J*=9.5, 3.1)

Table 7.4 (continued)

Aglycone/Sugar	δ ^{1}H (ppm)	Reported Values* (in pyridine-d_5)
H-4′	3.61-3.63	4.53 (*d*, *J* = 3.1)
H-5′	3.0-3.09	4.00 (*dd*, *J* =7.3, 6.4)
H-6′	3.38-3.54	4.32-4.48
O-3 Glucose		
H-1″	4.39 (*d*, *J* =7.7)	5.24 (*d*, *J* =7.7)
H-2″	2.96-2.99	4.03 (*dd*, *J* = 8.7, 7.7)
H-3″	3.11-3.16	4.15 (*dd*, *J* =9.1, 8.7)
H-4″	3.0-3.09	4.26 (*dd*, *J* = 9.4, 9.1)
H-5″	3.30-3.33	3.81 (*ddd*, *J* = 9.2, 4.0, 2.4)
H-6″	3.47-3.65	4.32-4.48

Signals assignment was based on ^{1}H-^{13}C COSY. *Values reported in literature (Saito *et al.* 1994).

Table 7.5: ^{13}C NMR spectrum of AAM22 in DMSO-d_6

Position	δ ^{13}C (ppm)	Reported values*	DEPT
C-1	30.43	30.9	CH_2
C-2	26.64[a]	27.0	CH_2
C-3	74.20	75.4	CH
C-4	30.26	30.9	CH_2
C-5	36.29	36.8	CH
C-6	26.53[a]	26.7	CH_2
C-7	26.27	26.7	CH_2
C-8	35.25	35.5	CH
C-9	39.76	40.2	CH
C-10	34.87	35.2	C
C-11	20.80	21.1	CH_2
C-12	40.02	40.3	CH_2
C-13	40.56	40.9	C
C-14	56.10	56.4	CH
C-15	31.77	32.1	CH_2
C-16	80.76	81.3	CH
C-17	62.22	61.9	CH
C-18	16.56	16.6	CH_3

Table 7.5 (continued)

Position	δ ^{13}C (ppm)	Reported values*	DEPT
C-19	23.94	24.0	CH_3
C-20	41.92	40.5	CH
C-21	14.84	15.9	CH_3
C-22	109.20	109.7	C
C-23	25.90[b]	26.4	CH_2
C-24	25.77[b]	26.2	CH_2
C-25	26.82	27.5	CH
C-26	64.61	65.7	CH_2
C-27	16.29	16.3	CH_3
O-3 Galactose			
C-1	101.09	102.4	CH
C-2	79.47	81.6	CH
C-3	73.55	75.1	CH
C-4	68.11	69.7	CH
C-5	77.33[c]	76.5	CH
C-6	60.57[d]	62.1	CH_2
O-3 Glucose			
C-1	104.20	105.9	CH
C-2	75.51	76.8	CH
C-3	76.49	77.8	CH
C-4	70.32	71.6	CH
C-5	75.05[c]	78.3	CH
C-6	61.38[d]	62.7	CH_2

[a-d]These values are interchangeable in each column. *Values reported in literature, spectrum run in pyridine-d_5 (Saito *et al.* 1994).

7.2.3.4 Structure elucidation of AAM513A1 as timosaponin BI

UV, IR, MS and NMR spectra of AAM513A1 are given in Appendix 6.

AAM513A1 was isolated as white amorphous powder with melting point of 229 °C. AAM513A1 was found in UV spectrum in methanol to have maximum absorption at 202 nm, suggesting the absence of conjugated chromophore. The compound had molar extinction coefficient of 618.51. IR spectrum of this compound

showed a strong absorption at 3427 (OH) and 1074 (C-O) cm^{-1}, suggesting the presence of glycosidic moieties (Mimaki *et al.* 1998), and other peaks at 2929, 1451, 1383, 1170, 892 and 636 cm^{-1}. From high resolution EI and FABMS, AAM513A1 was found to have molecular formula of $C_{45}H_{78}O_{18}$ (MW = 906) in agreement with the presence in FABMS of fragment peak at *m/z* 904 $[M-2H]^+$ (100%) and 741 $[(M-2H)-163]^+$ (15%) which corresponded to the loss of one sugar moiety, EIMS showed a fragment peak at 416 which corresponded to the loss of three sugar moieties.

^1H and ^{13}C NMR spectra of AAM513A1 are given in Table 7.6 and 7.7, respectively. The assignment of ^1H and ^{13}C NMR spectra of AAM513A1 were based on the comparison with values reported in literature for *Anemarrhena* saponins (Nagumo *et al.* 1991; Saito *et al.* 1994). NMR data of AAM513A1 were found to be very similar to those of timosaponin BII reported by Nagumo *et al.* (1991), except for the fact that AAM513A1 had one extra methoxy group which matched a structure of timosaponin BI (Nagumo *et al.* 1991) whose functional group at C-22 was substituted with methoxy group instead of hydroxyl group as seen in timosaponin BII.

^1H NMR spectrum of AAM513A1 in CD_3OD exhibited two singlet signals at δ 0.76 and 0.94 (3H, each) due to steroidal methyls located at H-18 and H-19, respectively. The spectrum also showed two doublet signals at δ 0.91 (3H, J = 6.6) and δ 0.94 (3H, J = 7.8) which were assigned as H-27 and H-21 respectively based on values reported in literature for timosaponin BII (Nagumo *et al.* 1991). One singlet signal (3H) at lower field (δ 3.09) was appropriate to a methoxy group. ^1H NMR spectrum also exhibited three doublet signals in the field lower than 4 ppm (1H, each), indicating the presence of three anomeric protons of sugar moieties; the three protons located at δ 4.18 (J = 7.8), 4.35 (J = 7.9) and 4.60 (J = 7.8), coupling constant of these anomeric protons indicated that the sugars were present in β configuration. The signals at δ 3-4 corresponded to the protons of the three sugar moieties.

^{13}C NMR revealed signals of 45 carbons, of which 24 were methine, 14 methylene, 5 methyl and 3 quaternary carbons. The downfield signals of methine carbons at δ 101.52, 104.42 and 104.52 were assigned as C-1 of sugar moieties based on their correlation with the anomeric protons in ^1H-^{13}C COSY spectrum.

Signals of methine carbon in the downfield region of 70.35-82.46 indicated the presence of carbon adjacent to oxygen atoms. Two of which appeared to be carbons of the aglycone and 12 of which appeared to be methine carbons of the three sugar moieties based on the correlation of their corresponded protons on ^{1}H-^{1}H COSY spectrum with protons in the region of δ 3-4. The carbon signal at δ 76.23 was found in ^{1}H-^{13}C COSY spectrum to exhibit a correlation with a proton in downfield region of δ 4.03. This proton, in turn, correlated in long range ^{1}H-^{13}C COSY spectrum to a carbon signal at 101.52 which appeared to be the carbon at position 1 of a sugar moiety. This finding led to a suggestion that the methine carbon at δ 76.23 was the position where one of the sugar moieties attached to aglycone, and therefore assigned as C-3. Consequently carbon at 101.52 was assignable to C-1′ of the sugar moiety and its corresponding proton (δ 4.35) as H-1′.

It was found in ^{1}H-^{13}C COSY spectrum that the signal of oxygenated methine carbon at 82.46 correlated to proton signal at δ 4.32; this proton on ^{1}H-^{1}H COSY spectrum was found to exhibit a correlation with protons in the upfield region at δ 1.68-1.69 which appeared to be proton of aglycone rather than those of sugar moieties which usually came into resonance at δ 4-5. The correlation between these two protons indicated that the signal at δ 4.32 and its corresponding carbon (δ 82.42) arose from the aglycone not the sugar moieties, leading to an assignment of this proton signal as H-16 and its corresponding carbon (δ 82.46) as C-16. The proton signal at δ 1.68-1.69 was found in ^{1}H-^{13}C COSY spectrum to be overlapping and correspond to methine carbon at δ 65.29 and also methylene carbon at 31.49, leading to an assignment of the carbon signal at δ 65.29 as C-17 and the signal at δ 31.49 as C-15.

Signals of four methylene carbons in the downfield region of δ 62.41, 62.81, 63.18 and 75.81 were appropriate to carbons adjacent to oxygen atoms. The signal of methylene carbon at 75.81 was found in long range ^{1}H-^{13}C COSY spectrum to correlate with signal of anomeric proton at δ 4.18, leading to an assignment of the carbon signal at δ 75.81 as C-26, proton signal at δ 4.18 as H-1‴ and carbon signal at δ 104.52 which corresponded to proton at δ 4.18 as C-1‴. Three other signals of methylene carbons appeared, on ^{1}H-^{1}H and ^{1}H-^{13}C COSY spectra, to be protons at the 6-position on the sugar moieties.

^{13}C NMR spectrum showed signals of methyl carbons at 16.31, 17.03, 17.52 and 24.51 which were assigned as C-21, C-18, C-27 and C-19, respectively based on a comparison with values reported in the literature (Nagumo *et al.* 1991). As expected, the signal of methoxy carbon was found in the downfield region (δ 47.72) compared to signals of other methyl groups since the nucleus was deshielded by oxygen atoms. The signals of a quaternary carbon at 113.79 was appropriate to a carbon adjacent to oxygen atoms, and thus assigned as C-22. Other signals of quaternary carbons at δ 36.15 and 42.25 were assigned as C-10 and C-13, respectively. The remaining carbon signals were assigned based on values reported in literature (Nagumo *et al.* 1991).

According to a molecular formula ($C_{39}H_{64}O_{13}$), AAM22 was found to have a degree of unsaturation of 8 in agreement with the structure of timosaponin AIII (8 closed rings)

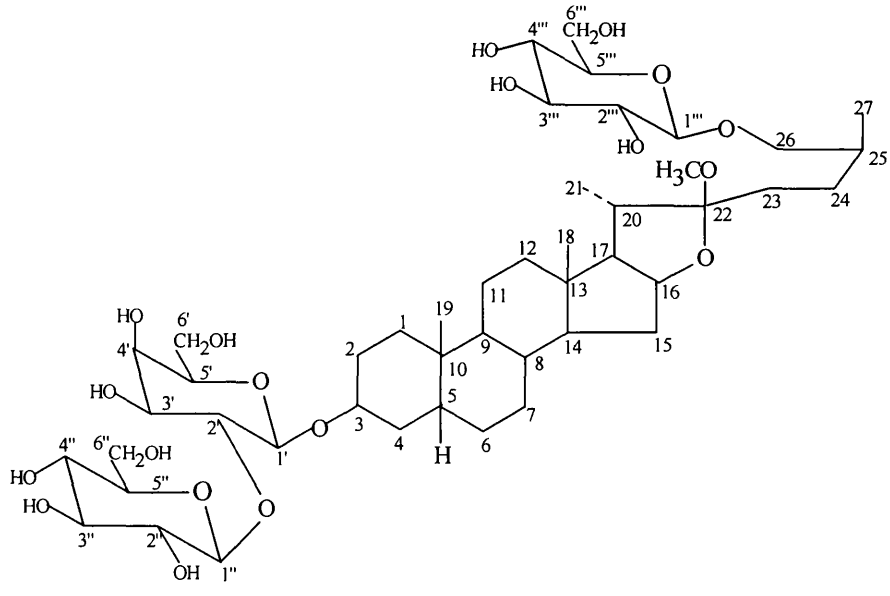


Fig.7.5: Structure of AAM513A1 (timosaponin BI)

Table 7.6 : ^{1}H NMR spectrum of AAM513A1 in CD_3OD

Aglycone	δ ^{1}H (ppm)
H-1	1.40-1.50
H-2	1.78-1.86
H-3	4.03 (br s)
H-4	1.60-1.66
H-5	1.46-1.50

Table 7.6 (continued)

Aglycone	δ ^{1}H (ppm)
H-6	1.50-1.60
H-7	1.49-1.54
H-8	1.40-1.60
H-9	1.38-1.46
H-10	-
H-11	1.38-1.46
H-12	1.38-1.46
H-13	-
H-14	1.13-1.23
H-15	1.60-1.66
H-16	4.32
H-17	1.60-1.66
H-18	0.76 (3H, s)
H-19	0.94 (3H, s)
H-20	2.10-2.12
H-21	0.94 (3H, *d*, *J* = 7.8)
H-22	-
H-23	1.86-1.90
H-24	1.13-1.23
H-25	-
H-26	3.21-3.26
H-27	0.91 (3H, *d*, *J* = 6.6)
OCH_3	3.09 (3H, s, OCH_3)
O-3 Galactose	
H-1′	4.35 (1H, *d*, *J* = 7.9)
H-2′	3.73-3.75
H-3′	3.60-3.67
H-4′	3.10-3.21[a]
H-5′	3.60-3.67
H-6′	3.60-3.67
	3.73-3.83[b]

Table 7.6 (continued)

Aglycone	δ ^{1}H (ppm)
O-3 Glucose	
H-1″	4.60 (1H, *d*, *J* = 7.8)
H-2″	3.10-3.21
H-3″	3.10-3.26[c]
H-4″	3.73-3.83[a]
H-5″	3.26-3.34[c]
H-6″	3.60-3.67[b]
	3.73-3.83
O-26 Glucose	
H-1‴	4.18 (1H, *d*, *J* = 7.8)
H-2‴	3.10-3.20
H-3‴	3.10-3.26[c]
H-4‴	3.10-3.20[a]
H-5‴	3.10-3.26[c]
H-6‴	3.60-3.67[b]

Table 7.7 : ^{13}C NMR spectrum of AAM513A1 in CD_3OD

Aglycone	δ ^{13}C (ppm)	Reported values*	DEPT
C-1	31.10	30.8	CH_2
C-2	27.81	27.0	CH_2
C-3	76.23	75.2	CH
C-4	31.49	30.9	CH_2
C-5	37.57	36.9	CH
C-6	27.72	27.0	CH_2
C-7	27.34	26.8	CH_2
C-8	36.77	35.5	CH
C-9	41.33	40.3	CH
C-10	36.15	35.3	C
C-11	22.02	21.2	CH_2
C-12	41.33	40.5	CH_2

Table 7.7 (continued)

Aglycone	$\delta^{13}C$ (ppm)	Reported values*	DEPT
C-13	42.25	41.3	C
C-14	57.63	56.5	CH
C-15	31.49	32.4	CH_2
C-16	82.46	81.2	CH
C-17	65.29	64.0	CH
C-18	17.03	16.7	CH_3
C-19	24.51	24.0	CH_3
C-20	41.41	40.7	CH
C-21	16.31	16.5	CH_3
C-22	113.79	110.6	C
C-23	32.77	37.1	CH_2
C-24	28.96	28.3	CH_2
C-25	35.10	34.4	CH
C-26	75.81	75.4	CH_2
C-27	17.52	17.5	CH_3
OCH_3	47.72		
O-3 Galactose			
C-1′	101.52	106.1	CH
C-2′	78.98	75.5	CH
C-3′	75.21	78.0	CH
C-4′	71.68[a]	717	CH
C-5′	76.38	78.3	CH
C-6′	62.41[b]	69.2	CH_2
O-3 Glucose			
C-1″	104.42	1025	CH
C-2″	76.14	81.8	CH
C-3″	77.84[c]	76.8	CH
C-4″	70.35[a]	69.9	CH
C-5″	78.10[c]	76.6	CH
C-6″	62.81[b]	62.2	CH_2
O-26 Glucose			
C-1‴	104.52	105.1	CH

Table 7.7 (continued)

Aglycone	$\delta^{13}C$ (ppm)	Reported values*	DEPT
C-2'''	75.11	75.2	CH
C-3'''	77.84[c]	78.6	CH
C-4'''	71.94[a]	71.8	CH
C-5'''	78.34[c]	78.3	CH
C-6'''	63.18[b]	62.9	CH_2

*Values reported in literature for timosaponin BII (Nagumo *et al.* 1991), spectrum run in pyridine-d_5.

[a-c]These pairs of values are interchangeable in each column

7.2.3.5 Structure elucidation of AAM73 as 1,3,6,7-tetrahydroxy-2-*C*-β-D-glucopyranosylxanthone (mangiferin)

UV, IR, MS and NMR spectra of AAM73 are given in Appendix 7.

AAM73 was obtained as yellow amorphous powder. UV spectrum of AAM73 in methanol showed four maxima at 241, 258, 316 and 367 nm which were a characteristic absorption of xanthone chromophore (Hostettmann and Hostettmann, 1989). A bathochromic shift of the 316 and 367 nm bands with increasing intensity was observed upon the addition of sodium acetate (1%), indicating that the compound was a xanthone with a free hydroxyl group at position 3 and/or 6. The bathochromic shift with $AlCl_3$ which was not destroyed by the addition of HCl suggested the presence of free hydroxyl group at position 1 and/or 8. On TLC, the compound was observed as yellow in colour under UV (254 nm), reaction of this compound with NH_3 vapour changed the colour from yellow to orange when observed under UV 254 nm. Its IR spectrum showed absorption at 3375 (OH), 1649 (α, β-unsaturated C=O), 1621, 1594, 1494, 1407, 1254, 1199, 1095 and 829 cm^{-1}. AAM73 appeared to have a molecular formula of $C_{19}H_{18}O_{11}$ (MW = 422) based on NMR and MS data, and in agreement with a molecular ion peak in FABMS at *m/z* 423 $[M+H]^+$ (5 %) and other peaks at 404 $[M-H_2O]^+$ (5%), 395 (26%), 344 (25%), 286 (100%). EIMS revealed a peak at 260 (93%) which corresponded to $[M-162]^+$, suggesting the loss of a sugar moiety.

^1H and ^{13}C NMR spectral data (Table 7.8 and Table 7.9, respectively) were found to match those obtained from reference mangiferin purchased from Apin Ltd.

(Appendix 8) and those reported in literature for mangiferin (Goetz and Jacot-Guillarmood, 1977, Fujita and Inoue, 1982). ^{1}H NMR spectrum of AAM73 showed signals of three aromatic protons (1H, s each) at δ 6.37 (1H), 6.85 (1H) and 7.37 (1H), indicating the absence of *ortho* and *meta* coupled aromatic protons. These signals were very similar to those of 1, 3, 6, 7, tetrahydroxyxanthone and could therefore be assigned as H-4 (δ 6.37), H-5 (δ 6.85) and H-8 (δ 7.37) of the xanthone nucleus (Goetz and Jacot-Guillarmood, 1977, Fujita and Inoue, 1982). ^{1}H NMR spectrum also showed one doublet at δ 4.59 (1H, J = 9.80) and other signals at 3.09-4.06 (5H, m) which were assignable to a glucopyranosyl unit. The signal at δ 4.59 (1H, *d*, J = 9.80) was assigned as an anomeric proton (H-1′); the coupling constant (J) of 9.80 suggested the presence of a glucopyranose ring with β-linkage.

^{13}C NMR spectrum was assigned in comparison with reported data (Frahm and Chaudhuri, 1979; Fujita and Inoue, 1982). ^{13}C NMR spectrum further revealed signals of one methylene (δ 61.85), 8 methine, and 10 quaternary carbons. Based on the chemical shift, three of the quaternary carbons were found to have no oxygen substitution (δ 101.64, 107.95 and 112.01) and seven had oxygen substitution (δ 144.11-179.44). The signal in the downfield region of δ 179.44 was appropriate to a

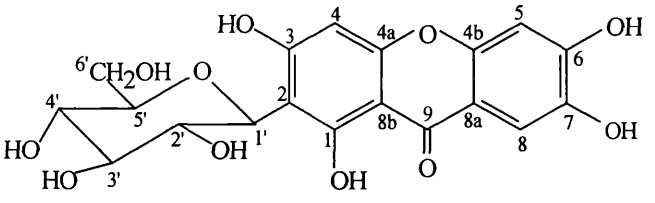


Fig. 7.6 : Structure of AAM73 (mangiferin)

Table 7.8: ^{1}H NMR spectral data of AAM73 in DMSO-d_6

	δ ^{1}H	
	AAM73	Reference Mangiferin
H-4	6.37 (1H)	6.37
H-5	6.85 (1H)	6.86
H-8	7.37 (1H)	7.37
H-1′	4.59 (1H, *d*, J = 9.80)	4.59 (1H, *d*, J = 9.83)
H-2 - H-6 (sugar moieties)	3.09-4.06 (5H, m)	3.06-4.07

Table 7.9: ^{13}C NMR spectrum data of AAM73 in DMSO-*d6*

	δ ^{13}C (ppm)			
	AAM73	Mangiferin (reference)*	Reported Values**	DEPT
C-1	162.14	162.20	161.86	C
C-2	107.95	107.95	107.68	C
C-3	164.19	164.19	163.89	C
C-4	93.63	93.65	93.48	CH
C-4b	151.15	151.15	150.89	C
C-5	102.95	102.93	102.73	CH
C-6	154.47	154.51	154.11	C
C-7	144.11	144.12	143.80	C
C-8	108.35	108.33	108.21	CH
C-8a	112.01	111.99	111.85	C
C-8b	101.64	101.63	101.43	C
C-9	179.44	179.43	179.20	C=O
C-1′	73.42	73.42		CH
C-2′	70.99	70.99		CH
C-3′	79.35	79.34		CH
C-4′	70.56	70.55		CH
C-5′	81.94	81.95		CH
C-6′	61.85	61.84		CH_2

*Values obtained from reference mangiferin purchased from Alpin Ltd. Spectrum run in DMSO-d_6

**Values reported for mangiferin in literature (Frahm and Chaudhuri, 1979)

carbonyl carbon. A substitution at C-1 and C-8 is known to have a strong influence on chemical shift of the carbonyl carbon (Frahm and Chaudhuri, 1979). If there is no substitution at C-1 and C-8 the chemical shift of the carbonyl carbon would be 175.9 ppm, mono substitution (either at 1-OH/8-OH) would cause about 4.5 ppm downfield shift (i.e. ~ 180 ppm) whereas double substitution (1, 8-OH) would cause about 6 ppm (i.e. ~ 182 ppm) downfield shift (Frahm and Chaudhuri, 1979). The chemical shift of carbonyl carbon at δ 179.44 observed in AAM73 indicated that either C-1 or C-8 of AAM73 was substituted.

Signals of quaternary carbons at δ 101.64, 107.95 and 112.01 were assigned as C-8b, C-2 and C-8a, and the signals of quaternary carbons at the downfield region of δ 144.11, 155.15, 156.57, 162.14 and 164.19 were assigned as C-7, C-4b, C-4a, C-1 and C-3 based on values reported for mangiferin in literature (Goetz and Jacot-Guillarmood, 1977, Fujita and Inoue, 1982). The ^{13}C NMR chemical shift at (δ 70.56-81.94) revealed the signals of 6 carbons of which one was methylene carbon, corresponding to a glucopyranosyl unit. The chemical shift of C-1′ was shifted upfield to δ 73.42 as compared to the literature data reported for C-1′ of *O*-glucoside xanthone (Lenherr and Mabry, 1987, Wolfender *et al.* 1991, Wu *et al.* 1998), indicating the presence of *C*-glucoside. In addition, melting point of AAM73 was found to be 274 °C which was also in agreement with that of mangiferin (reference) i.e. 272-273 °C (Fujita and Inoue, 1982). AAM73 had a calculated degree of unsaturation of 11 which matched the structure of mangiferin which comprised of 7 double bonds and 4 closed rings. Thus taking into consideration all the above data, the structure of AAM73 was established as 1,3,6,7-tetrahydroxy-2-*C*-β-D-glucopyranosylxanthone (mangiferin) in agreement with ^{1}H and ^{13}C NMR spectra reported in the literature (Fujita and Inoue, 1982) and the spectra obtained with reference mangiferin (Table 7.9, Appendix 8).

7.2.3.6 Structure elucidation of AAM634 as mangiferin-7-*O*-β-*D*-glucopyranoside

UV, IR, MS and NMR data of AAM634 are given in Appendix 9. AAM634 was isolated as yellow amorphous powder. UV spectrum of AAM634 in methanol showed four maxima at 240, 252, 315 and 357 nm which were a characteristic absorption of xanthone chromophore (Hostettmann and Hostettmann, 1989). A bathochromic shift of the band at 315 nm with increasing intensity was observed upon the addition of sodium acetate (1%), indicating a free hydroxyl group at position 3 or 6. Similarly to AAM73 (section 7.2.3.5), the compound underwent bathochromic shift with addition of $AlCl_3$ which was not destroyed by the addition of HCl, suggesting the presence of free hydroxyl group at position 1 and/or 8. Its IR spectrum showed absorption at 3416 (OH), 1648 (α, β-unsaturated C=O), 1613, 1581, 1478, 1221, 1185, 1072 and 843 cm^{-1}. AAM634 had a molecular formula of $C_{25}H_{28}O_{16}$ (MW =

584) based on NMR and MS data in agreement with a molecular ion peak in FABMS at *m/z* 585 $[M+H]^+$ (28%), and other peaks including 607 $[M+Na]^+$ (36%), 329 (36%), 307 (36%).

NMR spectral data of AAM634 in DMSO-d_6 was found to be similar to those of AAM73 (section 7.2.3.5), suggesting that the compounds had similar xanthone skeletons. The difference was that AAM634 appeared to have 2 sugar moieties instead of one as seen in AAM73. ^1H spectrum of AAM634 showed three singlet peaks of aromatic protons (1H, each) at δ 6.47, 6.97 and 7.71 which showed no *ortho* or *meta* coupling, these signals were assigned as H-4, H-5 and H-8, respectively. The spectra also exhibited 2 anomeric protons at δ 4.65 (1H, J = 7.22) and 4.82 (1H, J = 9.85) which corresponded to H-1′ and H-1″ of sugar moieties. Signals at δ 3.2-3.98 were assignable to protons at position 2-6 of sugar moieties. These suggested that the compound was a 1, 3, 6, 7-tetrahydroxyxanthone with two sugar moieties.

The ^{13}C NMR spectrum of AAM634 in DMSO-d_6 showed signals of two methylene carbons (δ 60.82, 61.49), 13 methine carbons, and 10 quaternary carbons. Two signals of methylene carbons at δ 29.51 and 29.98 appeared to be impurity peaks since these peaks had no correlation to other protons of the aglycone and sugar moieties of the compound. Three of the methine carbons i.e. δ 94.68, 103.23 and 109.42 appeared to be carbons in the aromatic rings, and were assigned as C-4, C-5 and C-8, respectively based on their correlation with the aromatic protons at δ 6.47 (H-4), 6.97 (H-5) and 7.71 (H-8), respectively in ^1H-^{13}C COSY spectrum.

Methine carbons in the region of δ 69.52-80.86 indicated the presence of non aromatic oxygenated carbons of which their corresponding protons were found in ^1H-^{13}C COSY spectrum to correlate with protons in the region of δ 3-4, suggesting that these carbons belonged to sugar moieties. Signals of three quaternary carbons at δ 101.46, 105.54 and 111.61 were appropriate to carbons in the aromatic rings with no oxygen substitution. The signal at δ 105.54 was found in long range ^1H-^{13}C COSY spectrum to correlate with anomeric proton (δ 4.65), leading to an assignment of this carbon as C-2. The correlation between carbon at δ 101.46 and H-4 (δ 6.47) in long range ^1H-^{13}C COSY spectrum led to an assignment of this carbon as C-8b. The

assignment of carbon signal at δ 111.61 as C-8a was based on its three-bond correlation on ^{1}H-^{13}C COSY spectrum with H-5 (δ 6.97).

Signals of quaternary carbons adjacent to oxygen atoms came into resonance further downfield at δ 142.84-178.66 due to the deshielding effect of oxygen. In long range ^{1}H-^{13}C-COSY, H-4 was also found to exhibit a two bond correlation with a signal of quaternary carbon at 156.55 leading to an assignment of this carbon as C-4a. The signal of H-5 was found in ^{1}H-^{13}C long range COSY spectrum to correlate with signals of quaternary carbons at 142.84, 152.17 and 153.37, leading to an assignment of these carbons as C-7, C-4b and C-6 respectively. Carbon signal at δ 163.22 was found to exhibit three bond correlation with anomeric proton (δ 4.65), leading to assignment of this carbon as C-3.

The signal at δ 178.66 corresponded to a carbonyl carbon. Similar to those observed in the spectra of AAM73, the chemical shift of the carbonyl carbon suggested the presence of 1-OH or 8-OH substitution. The carbonyl carbon was found in long range ^{1}H-^{13}C COSY spectrum to correlate with proton at 7.71, this was in agreement with the assignment of δ 7.71 as H-8. Three bond correlation between H-1″ and C-7 indicated the attachment of one of the sugar moieties to C-7 of the aglycone.

According to the molecular formula, AAM634 has 12 degree of unsaturation, which matched the structure of mangiferin glucoside (7 double bonds and 5 closed rings). Taking all the information into consideration, the structure of AAM634 was established as mangiferin 7-*O*-β-*D*-glucopyranoside.

Table 7.10: ^{1}H NMR spectrum data of AAM634 in DMSO-d_6

	δ ^{1}H (ppm)
H-4	6.47 (1H)
H-5	6.97 (1H)
H-8	7.71 (1H)
3-*C* glucose	
H-1′	4.65 (1H, *J* = 7.22)
H-2′	4.12
H-3′	3.32-3.43
H-4′	3.29-3.30

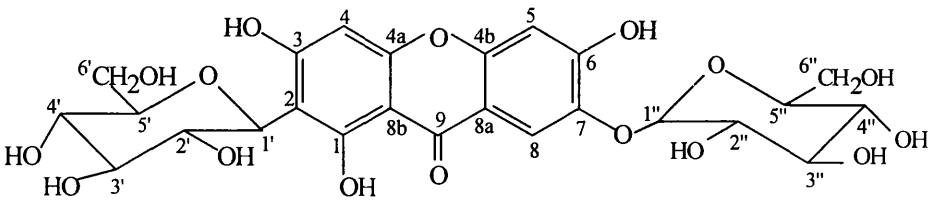


Fig. 7.7: Structure of AAM634 (mangiferin 7-*O*-β-*D*-glucopyranoside)

Table 7.10 (continued)

	δ ^{1}H (ppm)
H-5′	3.49-3.56
H-6′	3.61-3.65
	3.72-3.80
7-*O* glucose	
H-1″	4.82 (1H, J = 9.85)
H-2″	3.44-3.46
H-3″	3.29-3.30
H-4″	3.34-3.36
H-5″	3.49-3.56
H-6″	3.61-3.65
	3.72-3.80

Table 7.11: ^{13}C NMR spectral data of AAM634 in DMSO-d_6

	δ ^{13}C (ppm)	DEPT
C-1	160.88	C
C-2	105.54	C
C-3	163.22	C
C-4	94.68	CH
C-4a	156.55	C
C-4b	152.17	C
C-5	103.23	CH
C-6	153.37	C
C-7	142.84	C
C-8	109.42	CH
C-8a	111.61	C

Table 7.11 (continued)

	δ ^{13}C (ppm)	DEPT
C-8b	101.46	C
C-9	178.66	C=O
2-*C* Glucose		
C-1′	73.77	CH
C-2′	71.14	CH
C-3′	78.22	CH
C-4′	70.31	CH
C-5′	75.65	CH
C-6′	61.49	CH_2
7-*O* Glucose		
C-1″	101.46	CH
C-2″	73.05	CH
C-3″	80.86	CH
C-4″	69.52	CH
C-5″	76.24	CH
C-6″	60.82	CH_2

7.3 Discussion

Fractionation of methanol extract of *Anemarrhena asphodeloides* resulted in an isolation of 6 known compounds, of which two were lignans, 2 were saponins and 2 were xanthones. The compounds were identified by means of NMR and MS spectra, and, to lesser extent, UV and IR spectra. These compounds were *cis* hinokiresinol, monomethyl *cis*-hinokiresinol, timosaponin AIII, timosaponin BI, mangiferin and mangiferin glucoside. Of these the last four were isolated from the subfraction showing greater stimulating activity on insulin secretion from RINm5F cells.

Cis hinokiresinol was also reported to be present in other plants including *Asparagus cochinchinensis* (Tsui and Brown, 1996) and *Asparagus africanus* (Oketch-Rabah *et al.* 1997) which are also plants in the family Liliaceae. Mangiferin was first isolated from *Mangifera indica* L. (Anacardiaceae) (Hostettmann and Wagner, 1977). Mangiferin was also reported to be present in a number of plants in families including Iridaceae, Gentianaceae, Leguminosae, Convolvulaceae and

Celastraceae (section 6.12). In contrast to mangiferin, the reported occurrence of the timosaponins was found to be restricted to *Anemarrhena asphodeloides*, in which main constituents appeared to be saponins (Chapter 6).

The investigation of stimulatory effect on insulin secretion from RINm5F cells showed that timosaponin AIII, mangiferin and mangiferin glucoside caused a significant release of insulin without damaging cell membrane, as judged by a leakage of LDH (Chapter 6). The effect of mangiferin was also observed in rat islets, studies on mechanism of action of this compound indicated that its mechanism of action did not involve PKA and PKC pathway (Chapter 6).

CHAPTER 8

DEVELOPMENT OF A HEPATOCYTE-BASED ANTI-DIABETIC ASSAY

8.1 Action of insulin in liver

Maintenance of plasma glucose within normal limits depends on rates of glucose entry into the circulation and its uptake into tissues. Insulin is the hormone that plays an important part in maintenance of normal glucose homeostasis (section 1.1) which is achieved by its ability to control three major metabolic fuels i.e. carbohydrate, protein and fat. This process occurs in three principle tissues - liver, muscle and adipose tissue. Changes in the rates of glucose production and/or uptake of glucose in these tissues are the main determinant of plasma glucose level between meals.

After a meal when the plasma glucose level rises, insulin is secreted from the pancreas (section 1.1) into the pancreatic vein which empties into the portal vein system, so that insulin reaches the liver before entering the systemic blood supply. This emphasizes the importance of the regulation of glucose homeostasis played by the liver, which is one of a very few organs which is capable of releasing glucose for use by other tissues (Garret and Grisham, 1999). One of the most important effects of insulin is to cause most of the glucose absorbed after a meal to be stored in the liver and inhibit the release of glucose produced in the liver into circulation system. Insulin action to lower the blood glucose level via the liver can be achieved by the following mechanisms (Guyton and Hall, 1997):

- stimulation of glycogen synthesis by activating the enzyme that promotes glycogen synthesis i.e. glycogen synthase
- stimulation of glucose uptake by activating glucokinase enzyme
- stimulation of glycolytic enzymes e.g. phosphofructokinase and pyruvate kinase
- inhibition of glycogenolysis by inactivating phosphorylase which is involved in the breakdown of glycogen
- inhibition of gluconeogenesis

It has long been known that a major metabolic defect observed in many NIDDM patients is the inability of insulin to exert a normal biological response at target tissues (section 1.1.2.1). Thus one of the approaches to develop an anti-diabetic agent is to search for an agent which is capable of mimicking or potentiating these effects of insulin at the target tissues in a hope that it may

alleviate insulin resistance in NIDDM. Troglitazone and metformin (section 1.2.2.2 and 1.2.2.3) are anti-diabetic agents known to work via this mechanism which were introduced for clinical use. However, troglitazone has been currently withdrawn from the market due to its severe toxicity to the liver. Thus, there is still a need for this type of compound for clinical use in the treatment of diabetes.

During the past 20 years primary hepatocytes, isolated mostly from rodents, have been widely used to study the effects of a number of agents on the hepatic disposal of glucose. For example, Fleig *et al.* (1984b) and Rinniger *et al.* (1984) used primary hepatocytes isolated from rats to study glycogen synthesis under stimulation by glyburide (Fleig *et al.* 1984b) and gliquidone (Rinniger *et al.* 1984). However, the use of hepatocyte-based *in vitro* bioassays in the area of natural product research is much less extensive. The studies are mostly conducted *in vivo* (section 1.5.2.4) by giving plant extracts to the animals, and the end products such as enzyme activities or components synthesised in the liver are then determined from the liver excised from sacrificed animals. One example is the study by Suzuki and Hikino (1989b). They employed liver excised from mice to investigate the effect of pre-treatment with panaxan A and B, isolated from *Panax ginseng* on the activities of glucokinase, glucose-6-phosphatase, glycogen synthase and phosphorylase.

This current study aimed to develop a hepatocyte-based *in vitro* assay for an investigation of the effect of plant extracts on one of the mechanisms which plays an important part in glucose disposal i.e. glycogen synthesis.

8.2 Glycogen synthesis

One of the effects of insulin in the regulation of carbohydrate metabolism is to promote an accumulation of glycogen in liver and muscle. Glycogen is a high-molecular weight, storage polysaccharide composed of glucose units linked in 1,4 bonds, with branch points of 1,6-glucosidic linkage.

One characteristic feature of glycogen production is that a primer is needed for biogenesis. The primer is identified as a protein named glycogenin (Alonso *et al.* 1995). The attachment of the first glucosyl unit to glycogenin has been reported to be a self-glucosylating process (Fig. 8.1) in which C-1 of glucose is

attached to a tyrosine residue of glycogenin. The chain of glycogen attached to glycogenin is elongated by glycogen synthase which, by linking the hydroxy group of C-1 of glucose to the terminal C-4 of glucosyl residue of the glycogen, plays an important role in chain elongation. Biogenesis of glycogen (Fig. 8.1) begins with a phosphorylation of glucose by glucokinase to form glucose-6-phosphate which is subsequently isomerized to glucose-1-phosphate and converted to uridine diphosphate (UDP)-glucose. UDP-Glucose serves as a substrate for glycogen synthase. Once a glucosyl chain of 10-12 residues is formed, a chain of at least 6 glucosyl residues is removed and the hydroxy group of C-1 is subsequently linked to C-6 of a neighbouring shorter chain to form α[1→6] linkage, this is regulated by amylo-(1,4 → 1,6)-transglycosylate, also known as branching enzyme (Garret and Grisham, 1999).

For many years, it has been thought that glycogen is primarily synthesised directly from glucose as shown in Figure 8.1. However, in 1943 a hypothesis

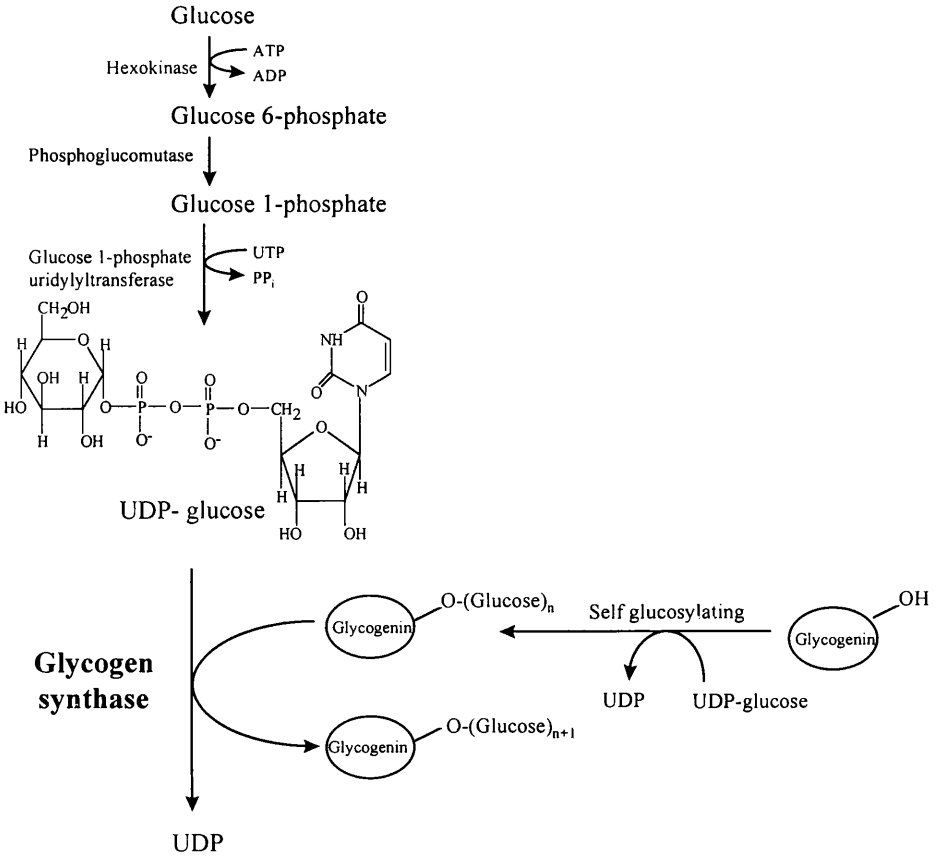


Fig. 8.1: Pathway of glycogen synthesis

suggesting that glycogen may also be synthesised from three carbon intermediate was introduced by Bollman, (1943). Owing to an enormous number of studies (reviewed by Kurland and Pilkis, 1989; Shulman and Landau, 1992), it has now been established that glycogen can be synthesised by both direct (i.e. glucose → UDP-glucose → glycogen) and indirect pathways where glucose is first converted to a three-carbon compound such as lactate or pyruvate which is thought to be generated in extrahepatic tissues e.g. gastrointestinal tract and peripheral muscle and/or in the liver (i.e. glucose → C_3 carbon intermediate → glucose-6-phosphate → glycogen, Fig. 8.2).

The contribution of the indirect pathway to the synthesis of glycogen is still controversial and the physiological significance of this pathway remains to be defined. The indirect pathway has been shown to contribute approximately 45-75%

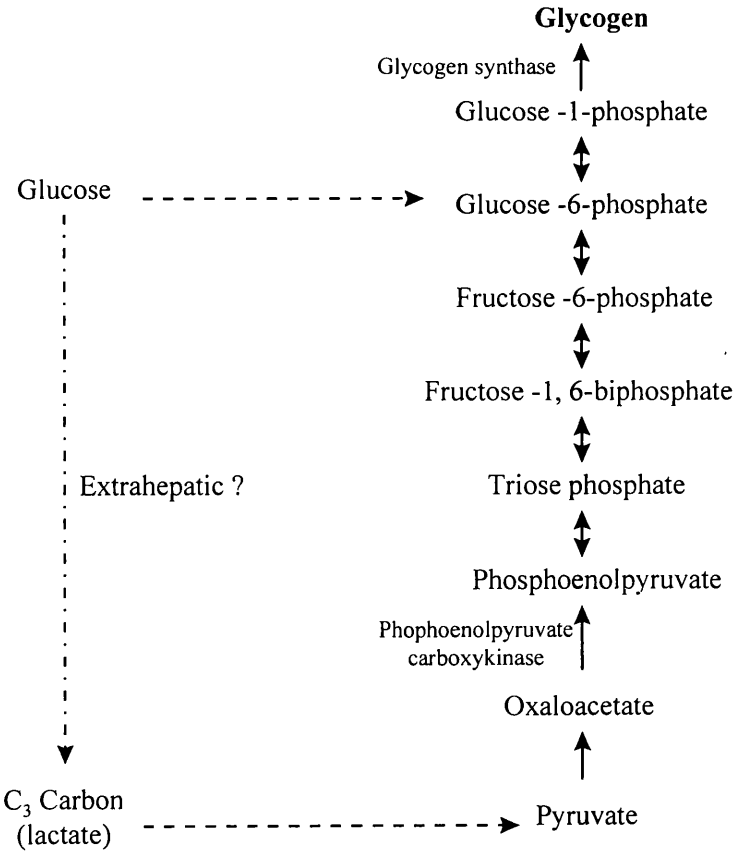


Fig. 8.2: Biogenesis pathway of glycogen from C_3 carbon intermediate (modified from Kurland and Pilkis, (1989))

to glycogen synthesis (Shulman and Landau, 1992); the pathway was found to be affected by dietary intake. Rats fed with high protein diet were reported to have approximately 20% of glycogen synthesised by the direct pathway whereas rats fed with high carbohydrate diet had 52% of their glycogen synthesised by direct pathway (Shulman and Landau, 1992).

Glycogen synthase is a key enzyme responsible for biogenesis of glycogen (Fig. 8.1, 8.2). It exists in two interconvertable forms (Fig. 8.3) i.e. synthase *b* (synthase D), a more phosphorylated form, and synthase *a* (synthase I). The conversion of synthase *a* to synthase *b* by phosphorylation results in an inactivation of the enzyme. *In vitro*, glycogen synthase can be phosphorylated by a number of enzymes including protein kinase A, phosphorylase kinase, protein kinase C, Ca^{2+}- and calmodulin-dependent protein kinase II, casein kinase 1 (CK1), casein kinase II (CK2), glycogen synthase kinase-3 (GSK-3), GSK-4 insulin-stimulated protein kinase I (ISPK-1) and AMP-stimulated protein kinase (Villar-Palasi and Guinovart, 1997; Bollen *et al.* 1998). However, the significance of these enzymes *in vivo* remains to be determined (Bollen *et al.* 1998).

It has been suggested that glucose, besides being the substrate, also has other roles in the regulation of the glycogen synthesis pathway. Gluc[illegible], by binding to phosphorylase *a* which is an enzyme responsible for a degradation of glycogen

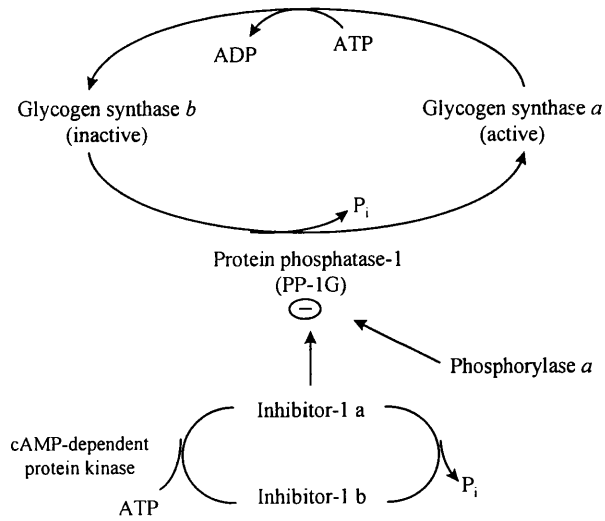


Fig. 8.3: Regulation of glycogen synthase enzyme

(Stalmans *et al.* 1997), was able to cause an inactivation of the enzyme. This subsequently results in a relieving of an inhibitory effect of phosphorylase *a* on type 1 protein phosphatase (PP-1G, Fig. 8.3). This would allow PP-1G to act as glycogen synthase phosphatase, thereby converting glycogen synthase into the active form (Stalmans *et al.* 1997). However, there were several lines of evidence which did not agree with this postulation and suggested that glucose itself cannot solely account for the activation of glycogen synthesis (Guinovart *et al.* 1997; Villar-Palasi and Guinovart, 1997), since the inactivation of phosphorylase *a* alone was not sufficient to activate glycogen synthase (Massillon *et al.* 1995; Stalmans *et al.* 1997).

On the other hand, it has been proposed that the activation of glycogen synthesis by glucose is primarily related to glucose 6-phosphate (reviewed by Villar-Palasi and Guinovart, 1997). The effect of glucose-6-phosphate on glycogen synthesis is thought to be related to its ability to promote the dephosphorylation and convert glycogen synthase into active form (Stalmans *et al.* 1997). Several lines of evidence have suggested that glucose 6-phosphate may act by activating PP-1G (Bollen *et al.* 1998), which was found to be completely abolished by phosphorylase *a*, implying that the inactivation of phosphorylase *a* is a prerequisite for glucose-6-phosphate-induced activation of glycogen synthase (Bollen *et al.* 1998).

Insulin is thought to activate glycogen synthase by promoting the dephosphorylation of glycogen synthase *a* and by activating PP-1G which is thought to be associated with the de-activation of phosphorylase *a* activity (Bollen *et al.* 1998). However, the precise mechanism, at a molecular level, by which insulin stimulates the activity of glycogen synthase remains to be defined.

8.3 Aim of the present study

This study aimed to develop a method for studying the effect of plant extracts on glucose utilization in hepatocytes, focusing on the synthesis of glycogen. An increase in glycogen synthesis was assessed by measuring the content of glycogen accumulated in the hepatocytes after incubation with an activator which, in this preliminary study, was insulin.

8.4 General methods

Unless specified, all chemicals were purchased from Sigma-Aldrich Co. Ltd. (Irvine, UK). Glucose oxidase was purchased as a diagnostic kit from Sigma. Rats (Wistar) were supplied by King's College London Biological Services Unit.

8.4.1 Reagents

Krebs & HEPES solution

300 ml of the solution contains

NaCl	2.09	g
KCl	0.1071	g
KH_2PO_4	0.0486	g
$MgSO_4.H_2O$	0.0880	g
$CaCl_2.2H_2O$	0.1153	g
$NaHCO_3$	0.6300	g
HEPES	0.90	g

The solution was freshly prepared by dissolving NaCl, KCl, KH_2PO_4, $MgSO_4$, $CaCl_2$ and $NaHCO_3$ in ultrapure water (150 ml). The resulting solution was then gassed with carbogen (95% O_2/5% CO_2) for 5-10 minutes. Ultrapure water (150 ml) was then added to the solution, followed by the addition of HEPES and gassing with carbogen. A portion of the final solution (100 ml) was removed and added with bovine albumin (1 g), the solution was labelled K+H+Alb. The remaining (200 ml) was labelled K+ H. Both of the solutions were then adjusted to pH 7.4.

Hank solution

One litre of the solution contains

NaCl	8.0	g
KCl	0.4	g
$MgSO_4.7H_2O$	0.2	g
$Na_2HPO_4.H_2O$	0.06	g
KH_2PO_4	0.06	g
$NaHCO_3$	2.10	g
HEPES	3.0	g

The solution was prepared by dissolving all the components in ultrapure water (1 L), followed by gassing with carbogen for 2-3 minutes. The resulting solution (600 ml) was removed and EGTA (136.8 mg) and albumin (4 g) added. This solution was labelled Hank I. Another 200 ml of Hank solution was supplemented with $CaCl_2$ (5.88 %, 2 ml) and collagenase (50 mg), this was labelled Hank II. Hank I and Hank II were then adjusted to pH 7.4.

William E medium

The medium (500 ml) was supplemented with:

Foetal calf serum	25 ml
Insulin (0.1 μM)	5 ml
Hydrocortisone hemisuccinate (0.1 mM)	5 ml
Glutamine (2 mM)	5 ml
Gentamicin (10 mg/ml)	3 ml

8.4.2 Isolation of hepatocytes

Isolation of hepatocytes was kindly performed by Dr. C.J. Waterfield (Biochemical Toxicology Group, Pharmacy Department, King's College London). Hepatocytes were isolated from liver of male Wistar rats (approximate weight 180 g-250 g) using the collagenase perfusion method described by Moldeus *et al.* (1978). Rats were sedated by injecting them intraperitoneally with Hypnorm/Hypnoval (1:1:2 water, 3.33 ml/kg); the abdominal cavity of the sedated rats was then cut opened longitudinally. The liver was perfused with Hank I buffer by passing the buffer through a cannula inserted into the portal vein. When it began to swell and started to blanch, the liver was removed from the animals and transferred into Hank I buffer with the cannula still attached. The perfusion was continued for another 5 minutes. The liver was then perfused for approximately 8 minutes with Hank II containing collagenase which was added just before the start of the second perfusion. The digestion with collagenase was stopped when the liver had began to lose its resilience. The perfused liver was then transferred to a dish containing K+H+Alb. The capsules of the liver were then broken to allow the hepatocytes to be released. The suspension was then filtered and transferred to centrifuge tubes. The resulting filtrate was centrifuged (ALC, model: PK 121R) at 400 rpm at room

temperature for 3 minutes. The supernatant was then aspirated, and replaced with 50-60 ml of William E medium. The pellets were resuspended in the medium by gently shaking back and forth. The suspension was again centrifuged, and the supernatant was discarded. The washing with William E medium was repeated.

Viability of the hepatocytes was determined using Trypan Blue exclusion method. Cell suspension was diluted 10 times with 0.4% trypan blue. The number of live cells (those excluding the dye) and dead cells which were blue (those taking up the dye) were counted using a haemocytometer. Percentage of cell viability was calculated from (No. of live cells/Total No. of cells) × 100. The cells used in the experiment should have an initial percentage viability not less than 70%.

8.4.3 Maintenance of cell monolayer

The suspension of hepatocytes (section 8.4.2) was diluted to a final density of 5×10^5 cells/ml with William E medium. The cells were cultured as a monolayer in 6-well plate in William E medium at a density of 1×10^6 cells/well (2 ml/well) at 37 °C in an atmosphere of 95% air/5% CO_2 for 4 hours. The cells after this pre-incubation were used in the assay for glycogen content (section 8.4.4).

8.4.4 Quantification of glycogen synthesised in hepatocytes

The hepatocytes were maintained as described in section 8.4.3. After pre-incubation for 4 hours, the medium was replaced with 2 ml/well of Modified Eagle's Medium (MEM) supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 1 mg/ml albumin and 100 nM dexamethasone. The hepatocytes were incubated at 37 °C for 24 hours, followed by replacement with MEM supplemented with 20 mM glucose, 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 1 mg/ml albumin with or without various concentrations of insulin.

There have been a number of methods developed to measure glycogen production in hepatocytes. These methods differed in terms of the techniques used to extract glycogen from cell homogenates and the methods used to determine glycogen content. In general, the cells were first solubilized to allow the release of glycogen, this can be achieved either by 1) treating the cells with 5.35 M KOH

(Good *et al.* 1933) or 3% $HClO_4$ (Salhanick *et al.* 1989), 2) subjecting to sonication (Vu *et al.* 1998) or 3) subjecting to freezing and thawing (Vu *et al.* 1998). Glycogen released from the cells upon the above processes can be separated from cell homogenates by 1) precipitation with 66% ethanol (Fleig *et al.* 1984b) or 2) adsorption onto 31ET Whatman filter paper, followed by precipitation with ethanol (66%) (Sølling and Esmann, 1975). After separation from cell homogenates, glycogen is usually quantified by means of radioactivity or a colorimetric method. In the former case, glycogen is quantified based on the amount of radiolabelled glucose incorporated into glycogen. It is noteworthy that, if tritium labelled glucose is used, it should be D-[3-^{3}H]glucose rather than D-[6-^{3}H]glucose, since the hydrogen at position-6 could be partially lost during the conversion of glucose to glycogen in the indirect pathway (section 8.2) (Tosh *et al.* 1994). In the colorimetric method, the quantification is based on the amount of glucose liberated from glycogen molecules which are hydrolysed using α-amyloglucosidase (Plomp *et al.* 1990; Vu *et al.* 1998). Glucose liberated from glycogen molecules is quantified using enzymatic methods by employing enzymes such as glucokinase/glucose-6-phosphate dehydrogenase (Fleig *et al.* 1985) or glucose oxidase (Bergmeyer and Bernt, 1963).

In this present study, the content of glycogen synthesised was quantified according to the method described by Gómez-Lechón *et al.* (1996) with slight modification. The method was selected because of its less complicated processes compared to the others (Sølling and Esmann, 1975; Bernaert *et al.* 1977). Frozen hepatocytes in the wells were subjected to a freeze and thaw cycle (twice). Glycogen was then hydrolysed to glucose by the addition of 300 µl amyloglucosidase enzyme (4 U/ml) prepared in 0.2 mM sodium acetate buffer (pH 4.8) to each well to give a final concentration at 1200 mU/well of amyloglucosidase enzyme. The cells were then incubated with amyloglucosidase for 2 hours at 40 °C. Blanks were performed by incubating the cells with 0.2 mM sodium acetate buffer (200 µl) containing no amyloglucosidase. This value represented free glucose in the reaction and had to be subtracted from the total glucose present after the hydrolysis. At the end of the incubation period, 40 µl of the incubated suspension was transferred to a 96-well plate and neutralised with 10 µl of 0.25 mM NaOH. The content of glucose liberated

from glycogen was determined using a colorimetric glucose oxidase method which was based on the following reaction:

$$\text{Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{o-Dianisidine} \xrightarrow{\text{Peroxidase}} \text{Oxidized o-Dianisidine (Brown)}$$

A solution of glucose oxidase enzyme (5 U/ml) was prepared according to the method described by Sigma. The enzyme solution was prepared by adding the contents of one capsule of the enzyme which contains 500 units of glucose oxidase and 100 Purpurogalin units of peroxidase (horseradish) to 100 ml distilled water. The enzyme solution (100 ml) was then mixed with 1.6 ml colour reagent solution containing *o*-dianisidine dihydrochloride (2.5 mg/ml).

The combined enzyme-colour reagent solution (200 µl) was added to 96 well plates containing cell hydrolysate. The plates were kept in the dark at room temperature for 45 minutes. The amount of glucose in the reaction was quantified by measuring the intensity of the colour produced at 450 nm using a microplate reader (Anthos Labtec HT3). A calibration curve was constructed using various concentrations (8, 16, 32, 62.5 and 125 µg/ml, 40 µl) of glucose solutions prepared in 0.2 mM sodium acetate and treating them with glucose oxidase as described above.

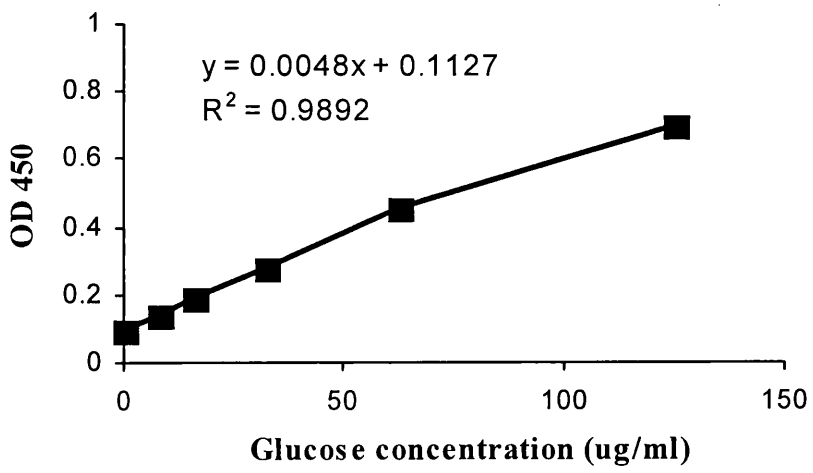


Fig. 8.4: Calibration curve used for a determination of glycogen synthesis (n= 3)

8.4.5 Protein Assay

Protein content of cell lysate obtained by freeze and thaw was determined using the method of Bradford, (1976) with slight modification. The reagent used in the determination was prepared by dissolving 10 mg of Coomasie brilliant blue in 95% ethanol (5 ml), followed by the addition of 85% phosphoric acid (10 ml). The solution was then adjusted to 100 ml with distilled water.

Cell lysate (40 µl) after incubating with amyloglucosidase enzyme (section 8.4.4) was transferred to another 96-well plate, mixed with 200 µl of the reagent and incubated at room temperature for 30 minutes. At the end of incubation, absorbance was measured at 595 nm. Albumin (0-80 µg/ml) was used as a standard solution to obtain a calibration curve, this was carried out by measuring the absorbance resulting from the reaction of albumin standards (40 µl) with 200 µl of the reagent.

8.5 Preliminary study on the effect of insulin on glycogen synthesis

8.5.1 Introduction

Glycogen synthesised by hepatocytes after incubation with insulin (section 8.4.4) was quantified by a colorimetric method based assay in which the glycogen molecules were hydrolysed using amyloglucosidase enzyme. Glycogen content was assayed by means of glucose released from glycogen molecules, which was then assayed by glucose oxidase method.

8.5.2 Materials and methods

Hepatocytes were isolated and maintained in culture as described in section 8.4.2 and 8.4.3, respectively. The cells after pre-incubation in MEM for 4 hours as described in section 8.4.4 were incubated with MEM supplemented with 20 mM glucose, 2 mM glutamine, 100 U/ml penicillin, 100 mg/m streptomycin, 1 mg/ml albumin with or without insulin (1 and 100 nM) at 37 °C, 95% air/5% CO_2 for 24 hours. Cells incubated in the absence of insulin were used as the control. The experiment was performed with four replicates for each concentration of insulin and

two replicates for blank. The hepatocytes were then washed with cold PBS, and all the liquid was removed. The cells were then subjected to freezing at -80 °C, and the frozen cells were used in the assay for the amount of glycogen and protein content as described in section 8.4.4 and 8.4.5, respectively.

8.5.3 Results and discussion

In this experiment, the amount of glycogen produced by hepatocytes was examined by determining the amount of glucose generated from the hydrolysis with amyloglucosidase (section 8.4.4). Table 8.1 shows the results from an experiment investigating the effect of insulin on the production of glycogen by hepatocytes maintained in culture for 24 hours. It can be seen clearly from Table 8.1 that insulin at 1 and 100 nM caused marked increase in an accumulation of glycogen compared to control.

Table 8.1: Effect of insulin on glycogen production

	Amount of glucose (μg/ml)	Protein (mg/ml)	Amount of glucose (nM) /mg protein
Control	8.52 ± 5.45	6.44 ± 0.14	7.3 ± 4.7
10 nM insulin	23.98 ± 13.18	4.65 ± 0.20	28.7 ± 15.7
100 nM insulin	33.78 ± 14.57	5.58 ± 0.29	33.6 ± 14.5

Results were expressed as mean ± SEM. n=3.

Unfortunately, repeats of this experiment using the same protocol (results from two separate experiments are shown in Table 8.2) failed to produce results consistent with those observed earlier (Table 8.1). In two repeated experiments (Table 8.2), the profiles of glycogen production were inconsistent and insulin failed to exhibit a dose dependent effect on the production of glycogen. Negative values were obtained in the cases when the amount of free glucose (blanks) was apparently found to be higher than the amount of glucose generated from the hydrolysis with amyloglucosidase. Inconsistency in the amount of glucose produced under the experimental conditions can possibly be explained by one of these assumptions: 1) the generation of glucose from glycogen was incomplete (provided that the cells were able to efficiently produce glycogen) or 2) the cells themselves failed to

produce glycogen due to a defect at the cellular level of synthesis pathway which possibly resulted from unsuitable culturing conditions. The former assumptions were examined in the next experiments (section 8.6 and 8.7).

Table 8.2: Effect of insulin on glycogen production (repeats of the first experiment)

	Amount of glucose produced (nM)/ mg protein	
	Exp. I	Exp. II
Control	125.75 ± 174.02	138.4 ± 131.6
0.01 nM	82.40 ± 36.34	204.8 ± 90.0
0.1 nM	19.03 ± 32.24	110.2 ± 110.0
1.0 nM	-41.90 ± 28.56	-11.1 ± 10.9
10 nM	60.35 ± 0.78	189.2 ± 50
100 nM	-470.90 ± 92.20	106.8 ± 59.0

8.6 Examination of the hydrolysis conditions used in the measurement of glycogen content

8.6.1 Introduction

The inconsistent results observed in section 8.5.3 may be explained by incomplete generation of glucose from glycogen molecules which was expected if 1) the experimental condition was not suitable for amyloglucosidase to exert its effect and/or 2) freezing and thawing, the method used to obtain cell lysates was inefficient to release the accumulated glycogen from the cells. This experiment was conducted to examine the first assumption.

8.6.2 Materials and method

To examine whether or not the experimental condition (section 8.4.4) was suitable for amyloglucosidase to exert its effect, the ability of the enzyme to hydrolyse commercial purified glycogen (Sigma) and hence generate glucose was examined using the same experimental conditions used with cell lysate (i.e. glycogen was incubated with amyloglucosidase at 40 °C, for 2 hours (section 8.4.4).

Glycogen in 0.2 mM sodium acetate buffer at concentrations of 0.08, 0.16 and 0.40 mg/ml (100 µl/well) was added to 12-well plate, followed by the addition of amyloglucosidase (0-7500 mU/ml, 200 µl) to give the final concentrations of glycogen of 0.027, 0.054 and 0.135 mg/ml and the concentrations of amyloglucosidase enzyme of 6.7, 33.3, 166.7, 666.7 and 1000 mU/well. Glycogen was then incubated with the enzyme using the condition described in section 8.4.4, and the amount of glucose generated in the reaction was quantified using glucose oxidase method (section 8.4.4). Blank was performed in the same manner but glycogen at each concentration was incubated with 0.2 mM sodium acetate buffer containing no amyloglucosidase. Each concentration of glycogen was examined with 4 replicates, and 2 replicates for blank.

In addition, the activity of amyloglucosidase enzyme used in the experiments (section 8.5.2) was also investigated in comparison with a new batch of the enzyme to determine whether or not the enzyme had lost its activity after 1 month storage at 4°C, since the loss of enzyme activity was another possibility of incomplete generation of glucose from glycogen molecules. The enzymes at the concentration of 1200 mU/ml was incubated with various concentrations of glycogen (0.017-0.136 mg/ml) using the conditions described in section 8.4.4. The amount of glucose generated from glycogen was determined using glucose oxidase method.

8.6.3 Results and discussion

The results (Fig. 8.5) showed that the amount of glucose produced from the hydrolysis of glycogen depended on the amount of enzyme present in the reaction. In this case where glycogen up to the concentration of 0.13 mg/ml was used, the maximum effect of the enzyme was observed at the dose of 33.3 mU/well which was far lower than those used in the experiments with hepatocytes (section 8.5.2) in which the enzyme at the concentration of 1200 mU/well was used; such concentration was chosen based on the method described by Gómez-Lechón *et al.* (1996).

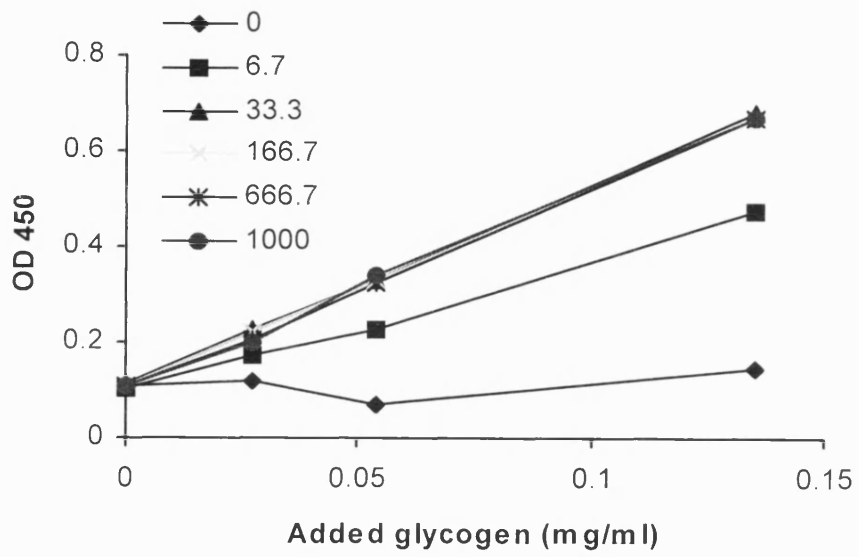


Fig. 8.5: Effect of amyloglucosidase at various concentrations on commercial glycogen

OD450 is representative of the amount of glucose generated

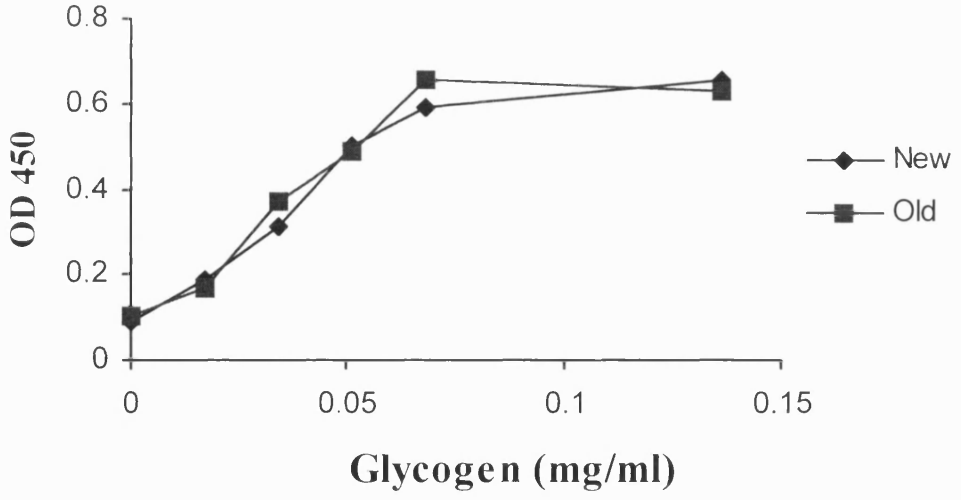


Fig. 8.6: Effect of two different batches of amyloglucosidase on glycogen hydrolysis

OD450 is representative of the amount of glucose generated

The results obtained with this experiment clearly suggested that under these condition amyloglucosidase was able to hydrolyse the molecule of glycogen and generate glucose as the end product. This therefore indicated that the failure in the hepatocytes experiments (section 8.5.3) was not due to the unsuitability of the glycogen hydrolysis condition. In addition, it was also found that increasing the

incubation time to 3 hours did not cause much difference in terms of glucose generated.

The activity of a new batch of amyloglucosidase was also examined and compared with that of the old batch which was a month old. The results (Fig. 8.6) showed that there was no difference in terms of activity between the two batches, indicating that the failure observed in the two repeated experiments (Table 8.2) was not due to the loss in the enzyme activity.

8.7 Liberation of glycogen from hepatocytes by a treatment with alkali

8.7.1 Introduction

Since it has been shown that the failure to observe an effect of insulin on glycogen synthesis (section 8.5.3) was not due to an inappropriate hydrolysis conditions; another possibility was that, by using freeze and thaw method, glycogen accumulated was not efficiently released from the cells. To improve the method used to liberate cell lysate from hepatocytes, treatment with alkali was used instead of freezing and thawing. The experiment was performed according to the method of Passonneau, (1975) with slight modifications.

8.7.2 Materials and methods

Frozen hepatocytes obtained from the experiment performed as described in section 8.5.2 were mixed with 0.33 M KOH (300 µl) and incubated at room temperature for 30 minutes. The cell lysate (275 µl) was transferred to Eppendorf tubes and incubated at 85 °C for another 40 minutes, followed by the addition of 50% acetic acid (15 µl) to adjust to pH 4.8. The resulting cell lysate (40 µl) was analysed for the content of free glucose and the values obtained from this determination represented the amount of free glucose. All readings were corrected with this value. The remainder of the cell lysate (240 µl) was subjected to hydrolysis as described in section 8.4.4 with amyloglucosidase (60 µl, 6 U/well) to give a final concentration of 1200 mU amyloglucosidase/well. The resulting solution from the

hydrolysis was examined for the amount of glucose generated by colorimetric method using glucose oxidase enzyme as described in section 8.4.4.

8.7.3 Results and discussion

Treatment with alkali was thought to be more rigorous compared to freezing and thawing, and hence expected to cause a complete lysis of the cells and thoroughly release all the contents including glycogen from the cells. Figure 8.7 showed the amount of glucose generated by amyloglucosidase from the cell lysate formerly treated with 0.33 N KOH in response to various concentrations of insulin. The results were found to be very similar to those obtained from the cells subjected to freezing and thawing. The similarity lay in the finding that glycogen produced by the cells in response to insulin was non-patterned and did not appear to be dose dependent. This suggested that the inconsistent results (Table 8.2) was not due to the inefficiency of freeze and thaw method to release from the cells since no improvement was obtained even when a more vigorous method was used. According to the results, there was a possibility that the failure to produce a plausible results in both cases was due to the fact that the cells were unable to efficiently synthesize glycogen under the condition used in this experiment, rather than defects in the quantification method.

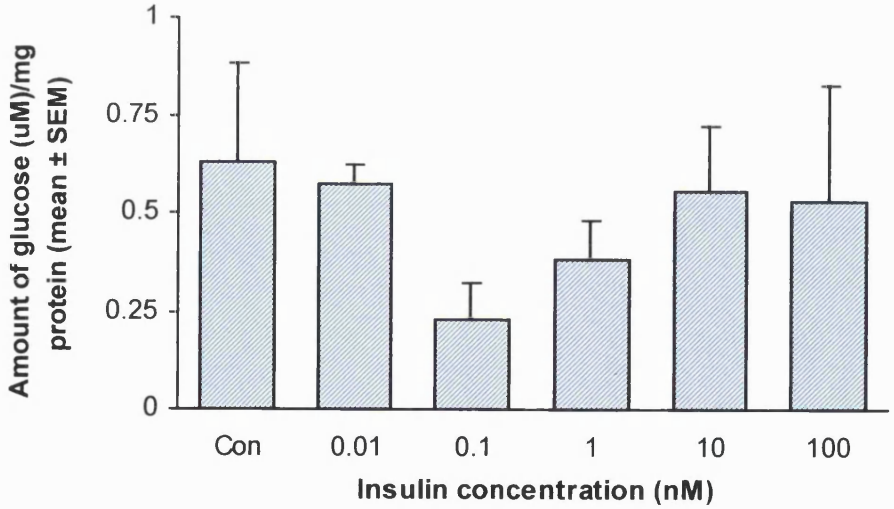


Fig. 8.7: Glycogen produced from cells formerly treated with alkali

Results are expressed as mean ± SEM. Each sample was investigated with4 replicates.

8.8 General discussion

The effect of glycogen synthesis is one of the mechanisms by which insulin enhances glucose disposal and hence regulates glucose homeostasis. This present study aimed to develop an *in vitro* based assay using primary hepatocytes for examining insulinomimetic effect of plant extracts on the synthesis of glycogen. To validate the method, preliminary studies (section 8.5.2) were performed using various concentrations of insulin. The content of glycogen produced by the cells was assessed by means of glucose liberated which was determined using glucose oxidase method. Results from one of the experiments (Table 8.1) showed that the treatment of the cells with insulin (1 and 100 nM) markedly enhanced the synthesis of glycogen in dose dependent manner. However, repeats of this experiments were unsuccessful (Table 8.2). It was observed in two individual experiments that the cells failed to appropriately respond to insulin. The amount of synthesised glycogen was not related to insulin concentration in dose dependent manner. Further investigation suggested that the failure was not due either to unsuitability hydrolysis conditions or the inefficiency of freeze and thaw method to release glycogen from the cells (section 8.6 and 8.7). These studies could not be pursued further due to loss of access to appropriate facility due to relocation of the department.

Further study on the development of this method is therefore necessary to establish a validated method which gives an appropriate results upon the stimulation with insulin, and validated method will further be used to investigate the effect of plant extracts on the production of glycogen.

CHAPTER 9

GENERAL DISCUSSION AND CONCLUSION

General discussion and conclusion

Diabetes mellitus is a chronic disorder of metabolism which results in hyperglycaemia; it is characterized by an absolute or relative deficiency of insulin. Diabetes is now a leading cause of adult blindness and a major cause of renal failure, gangrene, myocardial infarction and stroke (section 1.1.1 and 1.1.2). The treatment of diabetes currently relies on the use of insulin, sulphonylureas and biguanides, and is hampered by the adverse effects of these drugs and unmet therapeutic needs such as treatment of insulin resistance (section 1.2). Therefore there is still a need for new oral anti-diabetic agent which offers a better glycaemic control but has less adverse effect than the agents available in the market.

9.1 Overview of the findings of this study

This current study aims to investigate the effect of selected medicinal plants on anti-diabetic activity, focusing on a potential inhibitory effects on intestinal glucose absorption and a stimulatory effect on insulin secretion from pancreatic β-cells. The former was studied using an *in vitro* model namely rabbit intestinal brush border membrane vesicles (BBMV), whereas the latter was studied using rat insulinoma cell lines, RINm5F cells. In order to select the plants for the study, a literature survey on plants with anti-diabetic activities was conducted using Bath Information and Data Service (BIDS), Embase and MEDLINE (1980-1997, keywords: anti-diabetic, hypoglycaemic, anti-hyperglycaemic and plant genus). The selection of particular plants for this study was based on their reputations as anti-diabetic remedies in some countries with varying degrees of scientific evidence to support their traditional use (section 2.1). Identity of the selected plants was evaluated either by the source companies (East & West Herbs or Indian Herbs) or by the Royal Botanical Gardens, Kew (UK). This botanical examination revealed one plant whose identity did not match the nominal identity i.e. plant purchased as *Opuntia spp.* (section 2.4). The closest match of this plant as suggested by botanical examination was *Parvatia spp.*

Validity of BBMV for studying intestinal glucose uptake was assessed using phloridzin, a compound known to inhibit transport activity of Na^+/glucose cotransporter (SGLT1) (section 3.4). The use of BBMV for studying the activity of

plant extracts needed cautious interpretation, since it was found that extra added glucose present in the samples could interfere with the investigation and result in artefactual positive results (section 3.6). An ideal approach is to subject all the extracts to desugaring process (section 3.8) prior to the investigation on BBMV. This would enable the actual effect of the extracts to be examined. However, due to a large number of samples employed in this study it was not feasible to perform desugaring process for every extract. Thus, the approach taken in this study was to determine content of glucose in the extract and correlate the content of glucose to the observed percentage inhibition (section 3.7). Using this approach it was revealed that the effects observed in 80% of the extracts were primarily due to an interference caused by glucose in the extracts (section 3.7).

Of 31 plants tested, only four plants caused a marked inhibition of glucose uptake but at the same time contained negligible amount of glucose. These plants were *Lycium chinensis* (Solanaceae), *Piper longum* (Piperaceae), *Pterocarpus marsupium* (Leguminosae) and *Salacia reticulata* (Celastraceae), the results suggested that these plants contained constituents other than glucose that were capable of inhibiting labelled glucose uptake into BBMV (section 3.7). Among these plants, *Lycium chinensis* was found to have the most potent effect. In term of future study to confirm the effect observed in BBMV, a study on the relevant effects of *L. chinensis*, *P. longum*, *P. marsupium* and *S. reticulata* in *in vivo* model would be of interesting and an appropriate model would be an investigation for the effect on glucose tolerance using orally loaded glucose animals.

Despite the quotations in folklore medicines, *L. chinensis*, *P. longum* and *S. reticulata* had very little scientific evidence to support their uses as anti-diabetic remedies (section 2.2). This study is the first to report the effect of *L. chinensis*, *P. longum* and *S. reticulata* on the inhibition of intestinal glucose uptake in this model, suggesting that the glucose lowering effect of these extracts is, at least in part, due to inhibition of glucose absorption. In case of *P. marsupium*, there have been a number of studies which had been published on the effect of this plant, however they did not provide clear cut results on mode of action of this plant. A suggested, but controversial, effect was the activity on insulin secretion (section 2.4). The extract

of *P. marsupium* in agreement with results observed in this study was also suggested in a study by Ahmad *et al.* (1991a) to have inhibitory effect on intestinal glucose absorption, as judged by a marked glucose lowering effect of the extract in orally glucose loaded rats. It was encouraging to find that the use of *in vitro* assay based on BBMV afforded the identification of a plant extract which was also found to exert relevant effect *in vivo.* The results in this current study and those reported in the study by Ahmad *et al.* (1991a) strongly suggested that the glucose lowering effect of *P. marsupium* may be, at least in part, due to the inhibition of intestinal glucose absorption.

Bioassay-guided fractionation of a methanol extract of *Lycium chinensis* afforded the isolation of a compound which was identified as sitosterol glucoside (section 4.5). Although the study aimed to investigate the effect of sitosterol glucoside on BBMV, this study was not possible due to a lack of facilities arising from department relocation. A link between β-sitosterol glucoside and anti-diabetic activity has previously been demonstrated in the studies by Ambike and Rajarama-Rao, (1967), Ivorra *et al.* (1988) and Ivorra *et al.* 1990 of which the compound was reported to be active constituent of *Ficus religiosa* (Moraceae) and *Centaurea seridis* (Compositae). Interestingly, in the study by Ivorra *et al.* (1988) sitosterol glucoside was demonstrated to have anti-hyperglycaemic activity in orally glucose loaded rats, suggesting the potential effect of sitosterol glucoside as an inhibitor of intestinal glucose absorption. A second substance isolated from *L. chinensis* was found to give UV spectrum changes similar to that expected for flavonoids. However no NMR spectrum could be obtained for this compound probably due to a high content of inorganic impurities (section 4.5.3.2).

Investigation of the effect of the selected plants on insulin secretion was conducted using insulin-secreting cells called RINm5F cells. RINm5F cells were not glucose responsive due to the lack of glucokinase enzyme, but they were reported to respond to glyceraldehyde, a metabolite of glucose in the glycolytic pathway. However, it was found in a number of experiments in this study that glyceraldehyde had only marginal effect on insulin secretion from RINm5F cells (Chapter 5). Nevertheless, it was found that the cells were able to respond to sparteine (section

5.6.3) and, in the presence of glyceraldehyde, to forskolin, carbachol and KIC (section 5.5.3). The response to these secretagogues suggested the existence of the functional regulation pathways of insulin secretion.

The results obtained from initial insulin secretion experiments needed to be interpreted with caution, since damage to the cell membrane caused by the extracts also led to significant release of insulin from the cells, as observed in the extract of *Platycodon grandiflorum* (section 5.8.3). The determination of LDH leakage from the cells therefore played a very important role in the interpretation of the experiments on insulin secretion, since it provided valuable information on the effect of plant extracts on cell membrane. By means of LDH leakage, plants which damaged the cell membranes could be distinguished from those which had a genuine effect on insulin secretion. The current study clearly demonstrated that the aqueous *Anemarrhena asphodeloides* (Liliaceae) and *Parvatia spp.* (Lardizabalaceae, possible identity) extracts significantly stimulated the secretion of insulin from RINm5F cells without damaging the cell membrane as judged by leakage of LDH (section 5.8.3). Due to an uncertainty in the identity of *Parvatia spp.*, no further investigations were conducted with this plant.

There have been a number of studies (Takahashi *et al.* 1985; Hsu, 1992; Nakashima *et al.* 1993) conducted to investigate anti-diabetic effect of *Anemarrhena asphodeloides,* a plant well known in traditional Chinese medicine for its anti-diabetic effect. These studies, in an agreement with the current study, demonstrated a blood-glucose lowering effect of this plant. The majority of these studies was performed *in vivo* which provided limited information on mode of action. In fact, none of these studies had clearly clarified the mechanism of action by which *A. asphodeloides* extract exerted its glucose-lowering activity. It has been reported that *A. asphodeloides* was able to exert glucose-lowering effect in normal animals (Takahashi *et al.* 1985; Hsu, 1992). The study by Nakashima *et al.* (1993) had suggested that the effect of aqueous *A. asphodeloides* extract was unlikely to relate to stimulation of insulin secretion. The postulation was made based on the findings that the extract of *A. asphodeloides* was found active in alloxan-induced diabetic animals whose pancreas were believed to no longer functioned normally. However in cases of chemically induced diabetes, there is always a possibility of residual

pancreatic activity. Thus it may not be feasible to rule out the possibility that the activity of *A. asphodeloides* was owing to its pancreatic effect. In fact, it has been clearly demonstrated for the first time in this current study that *A. asphodeloides* extract had a stimulatory effect on insulin secretion from RINm5F cells, and this may, partly, give an explanation to glucose-lowering effect of the crude extract of this plant observed *in vivo*.

Three compounds present in *A. asphodeloides* which were capable of stimulating insulin secretion from RINm5F cells were isolated and identified in this study (Chapter 6). These compounds were mangiferin, mangiferin glucoside and timosaponin AIII. These compounds have not previously been reported to have this effect. Besides these three compounds, phytochemical study on *A. asphodeloides* also afforded the isolation of two lignans i.e. *cis*-hinokiresinol and 4′-methyl-*cis*-hinokiresinol, and another saponin i.e. timosaponin BI from *A. asphodeloides* methanol extract (Chapter 7).

Investigation of the effect of mangiferin on insulin secretion showed that the compound was able to significantly stimulate insulin secretion from RINm5F cells (section 6.7.3.1) and was also found to be active in islets (section 6.8.3.1) although the effect was more marginal compared to those observed in RINm5F cells. The effect of mangiferin was found to be enhanced in RINm5F cells in the presence of 10 mM glyceraldehyde. Similarly, an enhanced effect was also observed with the compound in the presence of 20 mM glucose in islets. It is encouraging to observe that a compound isolated based on *in vitro* bioassay was also able to exert a similar effect on primary β-cells. The effect of mangiferin observed in RINm5F cells and islets suggested that the compound may have potential advantages for use in the treatment of NIDDM as it would cause greater insulin release following a meal than in basal conditions. Investigation of the mechanisms by which mangiferin potentiated glucose-induced insulin secretion using Gö 6976 (PKC inhibitor) and Rp-cAMPs (PKA inhibitor) has shown that inhibition of PKC and PKA by the above agents did not reduce the effect of mangiferin on insulin secretion (section 6.9 and 6.10). The results suggested that mangiferin may work by a mechanism other that PKC and PKA activation. There is a possibility that mangiferin may work by

increasing an influx of Ca^{2+} into the cells or by regulating the downstream process of secretory pathway e.g. a fusion of insulin granules to β-cell membranes.

Mangiferin glucoside (0.5 and 1 mg/ml) on the other hand was found to stimulate insulin secretion from RINm5F cells (section 6.7.3.2), but failed to show an effect in rat islets (section 6.8.3.2). It is noteworthy that mangiferin glucoside is more polar than mangiferin, possibly giving it more difficulty in crossing cell membranes. Further, it is possible that for such compounds, access to cells in cultures is easier than access to β-cells in islets making their effects more marked in the cell line.

It needs to be borne in mind that RINm5F cells are β-cells which had been transformed from normal β-cells in order for the cells to grow in culture. Thus, some features of these cells may be different from primary β-cells in islets. It is also necessary to point out that while RINm5F cells are purified and transformed β-cells, islets were an aggregation of endocrine cells composed not only of β-cells but also other cells including α-, F-, D-, EC- and δ-cells (section 1.1), thus some biological properties and physiological response of the cell lines may not closely mimic those in intact islets. Despite the limitation of using cell lines, they are a more useful means of screening plant extracts than islets. Studies using rat islets are not ideal for investigating large number of samples since the isolation of islets is time consuming and need trained expertise, thus the use of cell line is probably more appropriate particularly for preliminary screening which involved a large number of samples.

The effect of mangiferin and its glycoside has been studied *in vivo* in KK-A^y mice by Ichiki *et al.* (1998). The conclusions of their study both agreed with and apparently contradicted those observed in this current study. The observed hypoglycaemic effect would be as expected for administration of all insulin secretagogues. However, they had suggested that hypoglycaemic effect of the two compounds was due to their ability to reduce a requirement for insulin at target tissues. This postulation was made based on the findings that the compounds lowered the level of plasma insulin. However plasma insulin was only determined 7 hours after administration of the compounds. Therefore it is quite possible that the study had missed out an increase in the level of plasma insulin at our earlier time point, and the

level of insulin determined in this particular study might not reflect the actual influence of the compounds on insulin secretion. Additionally, the discrepancy between the current study (section 6.7.3.1) and that conducted by Ichiki *et al.* (1998) may simply be due to the fact that the studies were conducted in different models, since the study by Ichiki *et al.* (1998) was performed in diabetic KK-A^y mice whose prominent features were hyperglycaemia, and defects in insulin secretion accompanied by hyperinsulinemia and insulin resistance (Bailey and Flatt, 1997) whereas the current study was performed *in vitro* models which reflected the features of normal animals (i.e. RINm5F cells and rat islets).

A number of saponins have been isolated from *Anemarrhena asphodeloides* (Kawassaki and Yamauchi, 1963; Nagumo *et al.* 1991; Nakashima *et al.* 1993; Saito *et al.* 1994; Ma *et al.* 1997; Meng *et al.* 1999). Three of these saponins i.e. timosaponin AIII, timosaponin B (psuedoprototimosaponin AIII) and timosaponin BII (prototimosaponin AIII) have previously been investigated for anti-diabetic activity and were reported to lower blood glucose level when administration to alloxan-diabetic mice at the dose of 50 mg/kg (i.p.) (Nakashima *et al.* 1993). It should be noted that the effect of timosaponin AIII was reported to be marginal compared to the other two saponins and the crude extract (Nakashima *et al.* 1993).

To date, modes of action of these compounds had not been identified. The current study has shown that timosaponin AIII was capable of stimulating insulin secretion from RINm5F cells (section 6.7.3.3). Since saponins are known to have hemolytic effect, it would be expected that the compound may have a damaging effect on cell membrane. However this was the case only at the concentration higher than 16 μg/ml (section 6.7.3.3). Timosaponin AIII at lower doses of 1-8 μg/ml did not damage cell membranes, as judged by the content of LDH leaked from the cells (section 6.7.3.3), but these concentrations were able to markedly stimulate insulin from RINm5F cells, suggesting its potential role as a potent, novel, insulin secretagogue. This current study is the first to report the effect of timosaponin AIII on insulin secretion. Interestingly another saponin isolated in this study, timosaponin BI (Fig. 6.3) was found to have no effect on insulin secretion from RINm5F cells (section 6.7.3.4). In agreement with these findings, it was also reported that

timosaponin BII whose structure was also a five-membered ring similar to timosaponin BI but differing from timosaponin BI only at one functional group, also failed to stimulate insulin secretion from perfused pancreas and normal mice (Nakashima *et al.* 1993). The activity of timosaponin AIII and the lack of activity in timosaponin BI and timosaponin BII observed in our studies and that of others suggests the possibility that the six-membered ring as seen in the structure of timosaponin AIII may be important for the effect of timosaponins on insulin secretion. It was encouraging to note that activities noted for compound in an *in vitro* cell based screening programme were agreement with results observed *in vivo*.

In this currently study, the selected plants were studied *in vitro* on two different modes of action including an inhibition of intestinal glucose uptake and a stimulation of insulin secretion. For the plants which were found to be inactive in these models, it did not mean that these plants are ineffective as anti-diabetic remedies since these plants may work via mechanisms different from those studied in this project. In conclusion, the results give some scientific support to the use of *Anemarrhena asphodeloides*, *Lycium chinensis, Piper longum, Pterocarpus marsupium* and *Salacia reticulata* in folklore medicine for the treatment of diabetes. The results presented in this study also suggested, for the first time, that the effect of *Lycium chinensis, Piper longum and Salacia reticulata* may be due to the inhibition of intestinal glucose uptake, and the activity of *Anemarrhena asphodeloides* extract due to the stimulation of insulin secretion from β-cells. Additionally, three compounds i.e. mangiferin, mangiferin glucoside and timosaponin AIII were isolated and identified to contribute to the activity of *Anemarrhena asphodeloides* observed in RINm5F cells. This was in agreement with the quantification of the content of mangiferin in an aqueous extract of *A asphodeloides* which showed that mangiferin, with respect to its proportion in the extract, was not the only active constituents in the plant.

This thesis also reported some initial attempts to set up a model for measuring glycogen content in hepatocytes. However these preliminary studies were not successful, the production of glycogen by the cells was not consistent and failed

to elicit a dose dependent upon the stimulation of insulin. This work could not be pursued further due to disruption to animal facilities at that stage of the project.

The results presented in this current study illustrate the use of *in vitro* bioassays to guide the isolation of compounds with specific activities. By using *in vitro* based assays, the results not only supported the traditional claims as the anti-diabetic remedy of some of the plants but was also able to identify their potential mechanisms of action. *In vitro* models were also more useful for large scale experiments of these nature which would have been extremely difficult to carry out *in vivo*. Their use therefore minimised a number of animals used in the experiments.

9.2 Future work

Regarding the results observed in this current study, the following is a suggestion of further study as an extension of this PhD project:

a) Investigation of the effect of sitosterol glucoside on BBMV and the effects of *Lycium chinensis* and sitosterol glucoside *in vivo*

Regarding the study on the effect of plant extracts on BBMV, sitosterol glucoside was isolated from the active fraction of *Lycium chinensis*. Unfortunately, study on the effect of this compound on BBMV was not possible at the time due to the lack of the appropriate facility. The investigation of the effect of sitosterol glucoside on BBMV will possibly provide interesting information which may explain the effect of *Lycium chinensis* observed in BBMV. Sitosterol and its glucoside are widely distributed in nature and may explain the activity of a number of anti-diabetic plants.

To confirm the results observed in this study, it is advisable that the effect of *Lycium chinensis* and sitosterol glucoside should also be tested *in vivo*. The *in vivo* model which is most appropriate for this study will be glucose tolerance test using orally glucose-loaded animals.

b) Investigation of the mechanism of action of mangiferin

As suggested by the current study that the effect of mangiferin on insulin secretion was not related either to protein kinase A (PKA) or protein kinase C (PKC) pathway, further study in islets to identify the mechanism of action of this compound may therefore provide interesting information. There is a possibility that the compound may work by increasing an entry of Ca^{2+} into β-cells. The effect of mangiferin on the Ca^{2+} influx can be investigated by direct measurement of Ca^{2+} influx into the cells. If mangiferin is found to have no effect on the entry of extracellular Ca^{2+} into the cells, it suggests that mangiferin may work by other downstream mechanisms e.g. a fusion of insulin granules to the cell membranes.

c) Investigation of the effect of timosaponin AIII in rat islets

To confirm the stimulatory effect of timosaponin AIII which was one of the active compounds isolated from *Anemarrhena asphodeloides* observed in RINm5F cells, the compound should also be examined in rat islets. It is also interesting to examine whether or not timosaponin AIII is a nutrient sensitive secretagogue. The answer to this question can be obtained by investigating the effect of timosaponin AIII on the secretion of insulin in the presence of nutrient (glyceraldehyde for the experiment in RINm5F cells and glucose for islets).

d) Investigation on the effect of *cis* hinokiresinol on insulin secretion

Although this compound was isolated from non active fraction of methanol extract, there was a study reported that the compound was capable of inhibiting the effect phosphodiesterase extracted from heart (Nikaido *et al.* 1981). Phosphodiesterase plays an important role in controlling the level of cAMP in cells. An inhibition on the activity of phosphodiesterase is believed to prevent the breakdown of cAMP, resulting in an increase in cellular cAMP level. It has been suggested in numerous studies that agents such as forskolin and IBMX which are capable of increasing cAMP level in β-cells are potentiators of insulin secretion. Thus, it is interesting to investigate the effect of *cis* hinokiresinol on RINm5F cells

or islets, as there has been no previous study reporting its effect on insulin secretion.

e) Development of a hepatocyte based model to determine of the effect of plant extracts on glycogen production

This current study described an initial attempt to develop a method for determination of glycogen synthesis in hepatocytes. The future study would be to establish a protocol which is able to produce a valid and consistent result. Once established, the method would then be further used for examining the effect of the selected plant extract. The use of hepatoma cell line e.g. H4IIE may be more useful and practical than primary hepatocytes; the advantage lies on more abundant supply of the cell lines compared to primary hepatocytes for which their isolation needs trained expertise to obtain a high yield of viable cells.

f) Investigation of the potential insulinomimetic effect of selected plants

Another area which has been a focus of attention is to search for agents that are capable of enhancing utilisation of glucose at insulin target tissues such as liver, muscle and adipocytes. Further investigation of selected plants on this area may be prove to be fruitful and may lead to the discovery of novel naturally-occurring agents with insulin-like effects. Various approaches have been taken to search for an agent that is capable of promoting the utilisation of glucose at the target tissues (section 1.5.2.4 and 1.5.2.5), these include:

- study on the enzymes involved in the synthesis of hepatic glycogen e.g. hepatic glycogen synthetase and hepatic phosphorylase.
- study on the activities of enzymes involved in glycolytic pathway e.g. glucokinase and hexokinase
- study on the activities of enzymes involved in gluconeogenesis e.g. glucose-6-phosphatase and phosphofructokinase

9.3 Conclusion

In conclusion, this study has demonstrated the utility of *in vitro* bioassays for the isolation of compounds with relevant biological activities from plants

selected using an ethnopharmacological approach. Compounds have been isolated and identified with the potential ability to inhibit intestinal glucose uptake of to stimulate insulin secretion. Both of these activities would be useful in NIDDM therapy.

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APPENDICES

Appendix 1: UV, MS and NMR data for LCMA (sitosterol glucoside)

1.1) UV spectrum in methanol

1.2) Fast atom bombardment mass spectrum

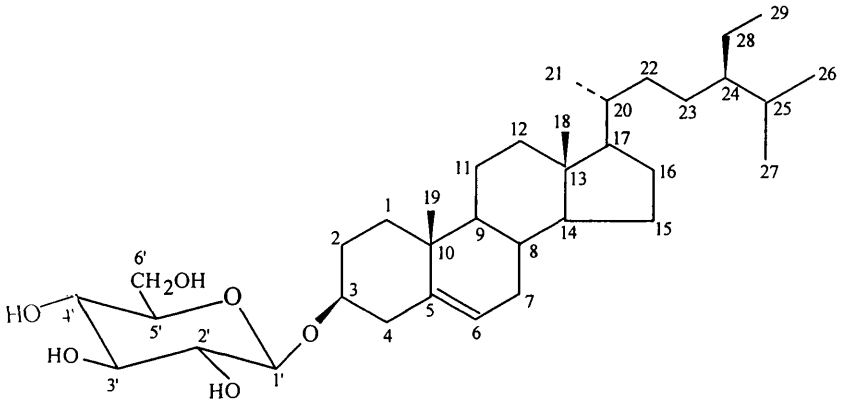
1.3) ^{1}H-400 MHz NMR spectrum in CD_3OD

1.4) ^{13}C-100 MHz NMR spectrum (DEPT) in CD_3OD

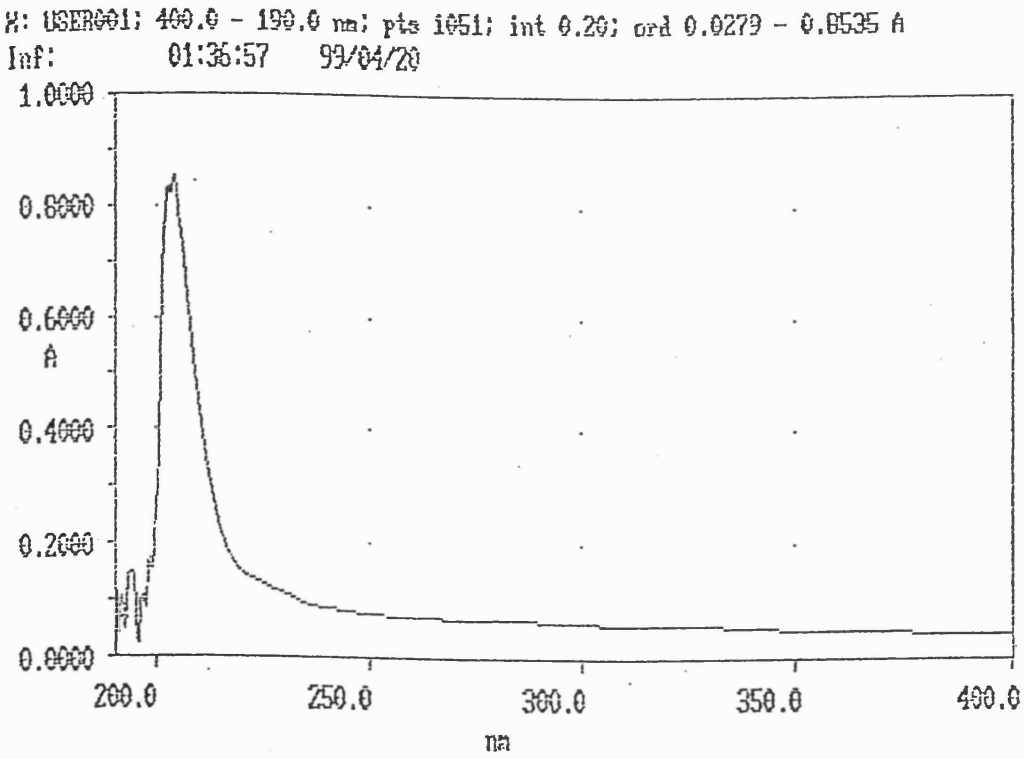
1.5) ^{1}H-^{1}H COSY spectrum in CD_3OD

1.6) ^{1}H-^{13}C one bond correlation spectrum in CD_3OD

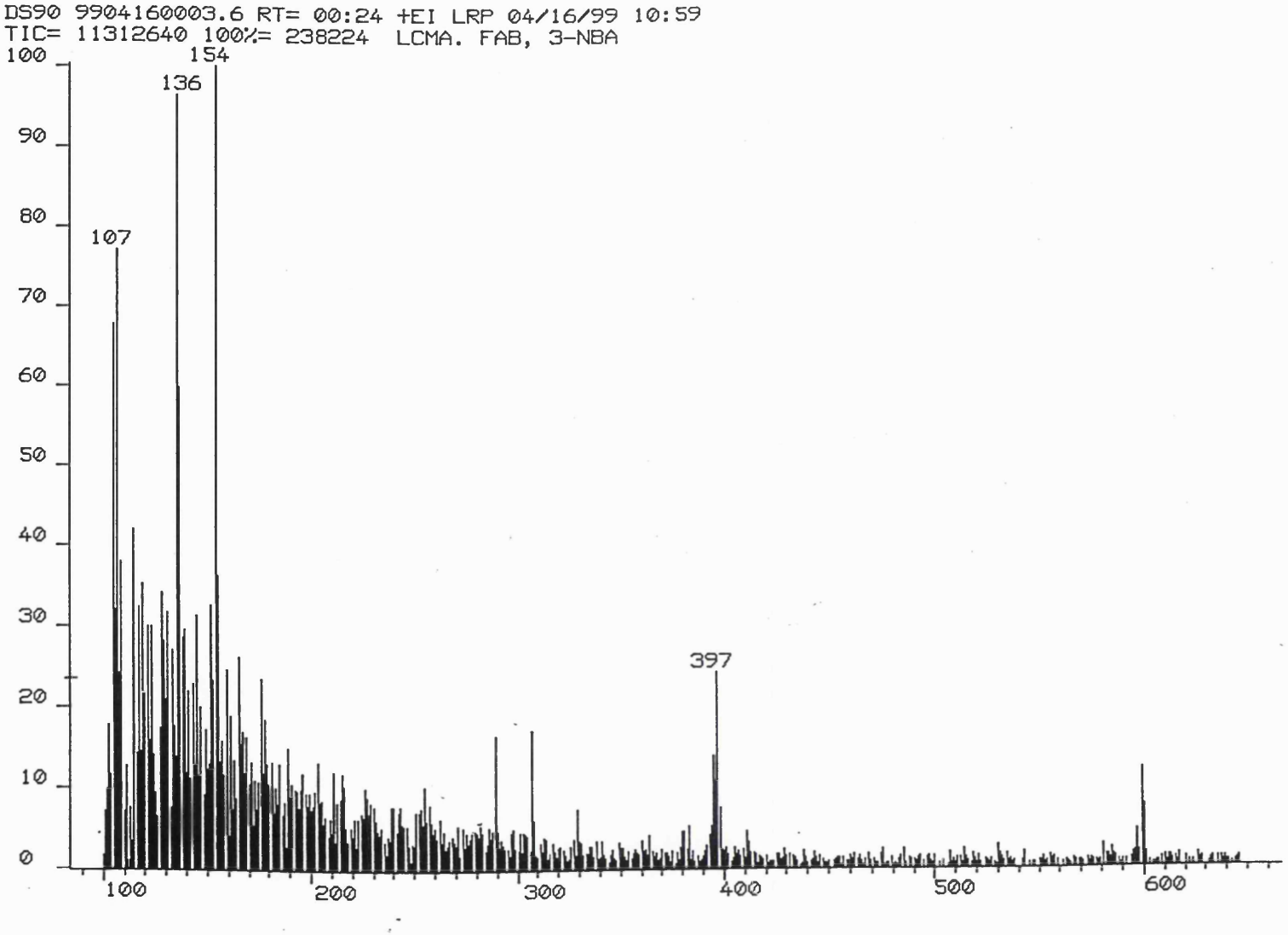
1.7) ^{1}H-^{13}C long range correlation spectrum in CD_3OD



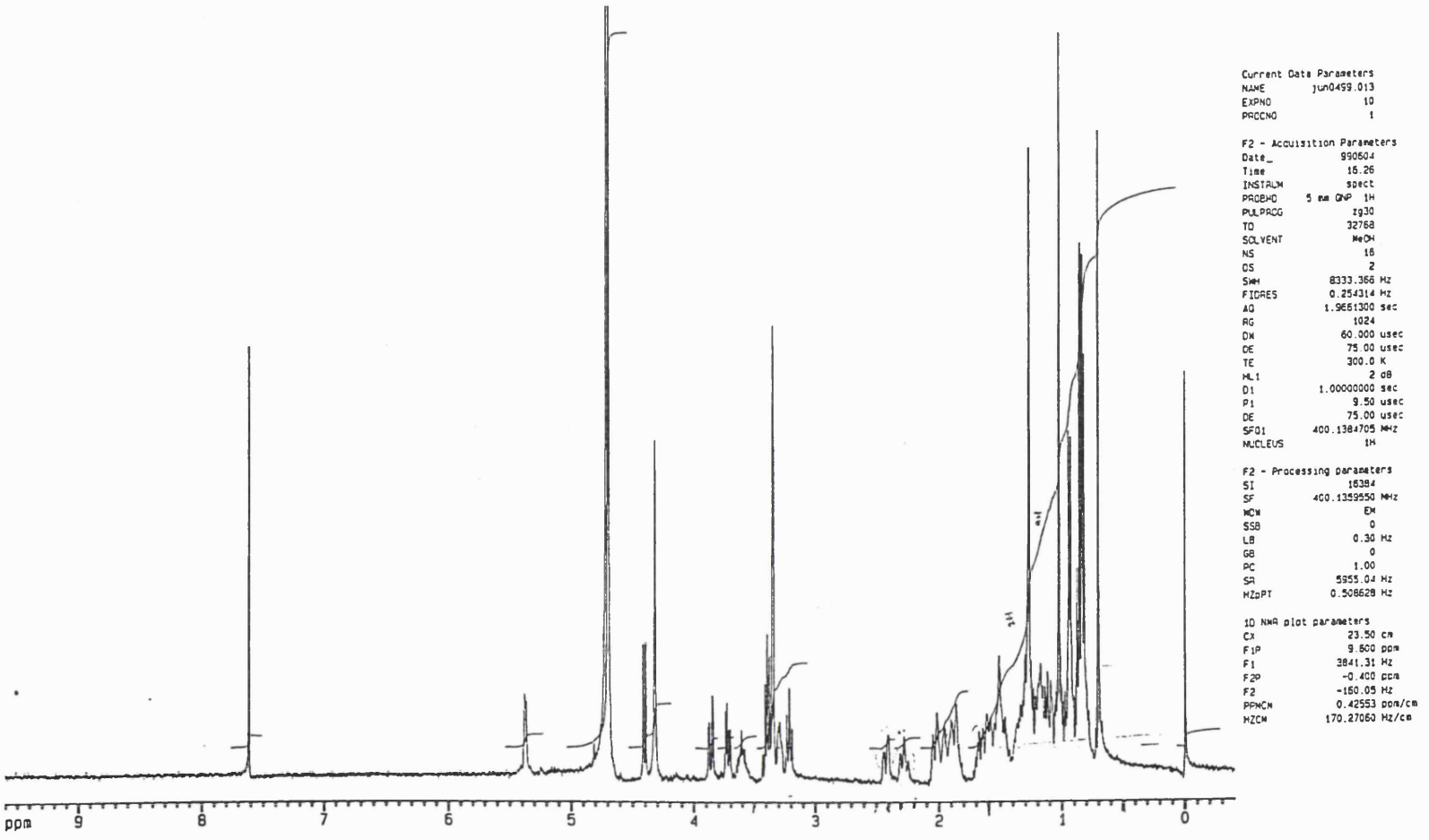
LCMA (sitosterol glucoside)



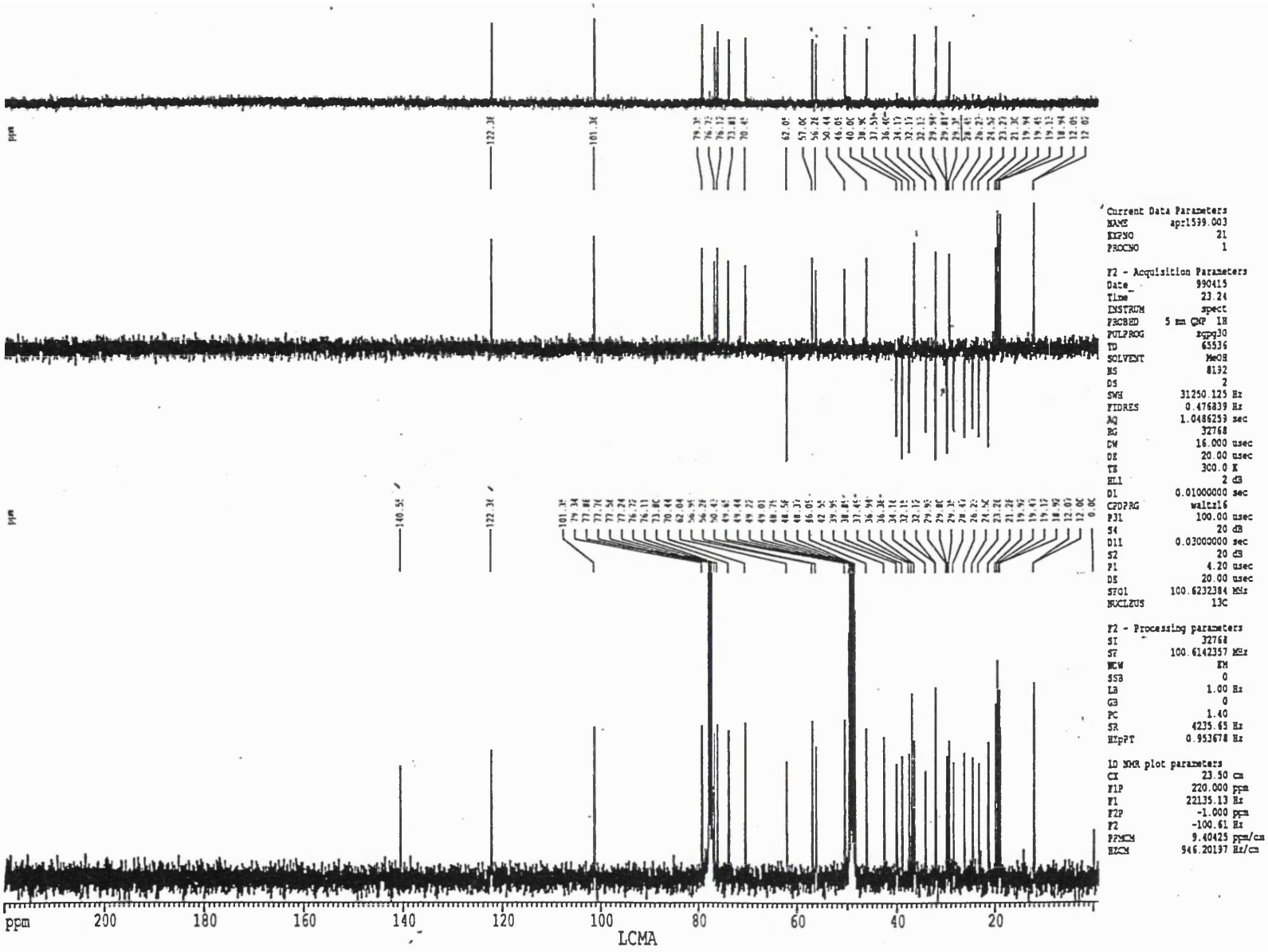
UV spectrum of LCMA (sitosterol glucoside) in methanol



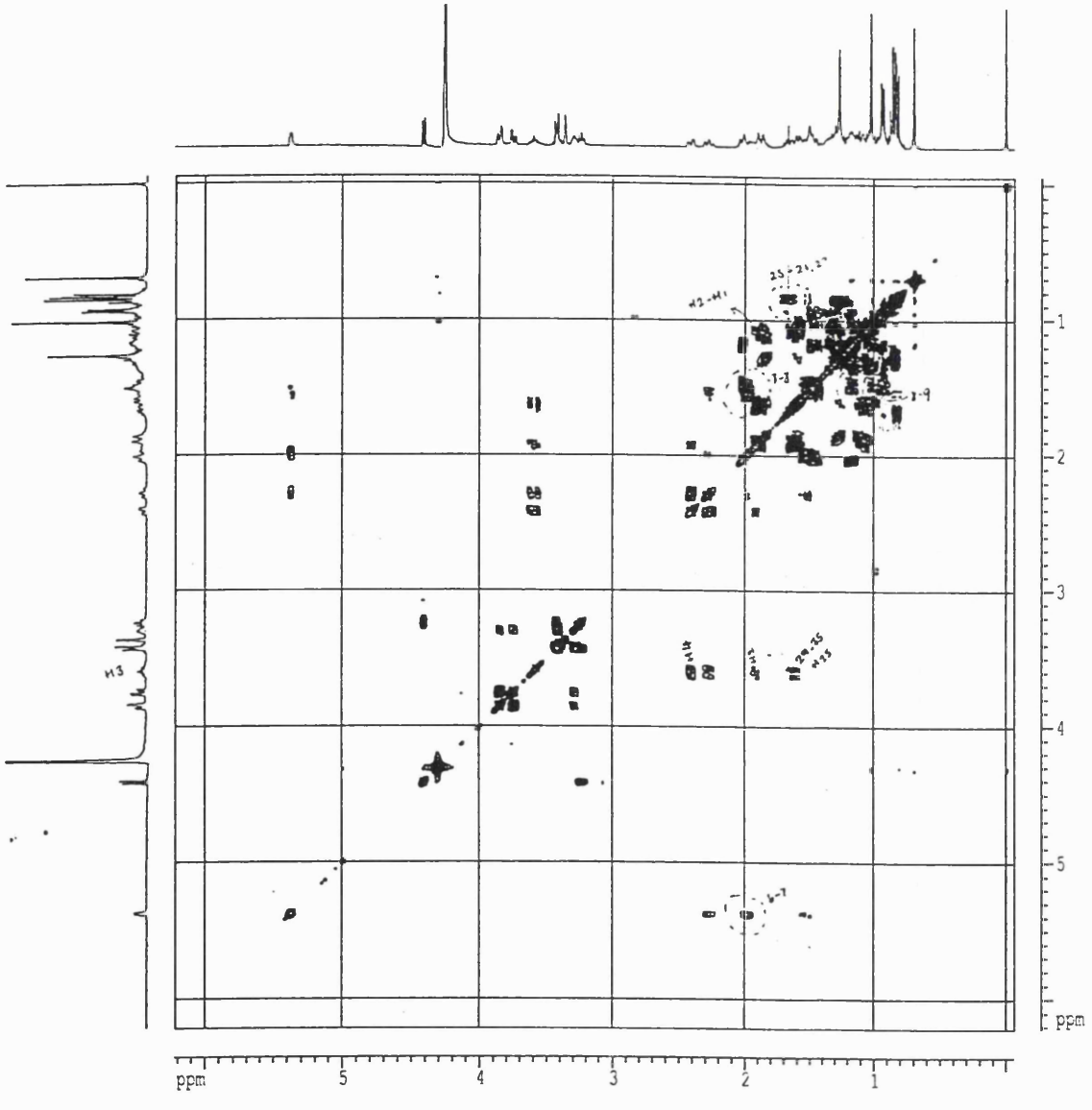
Fast atom bombardment mass spectrum of LCMA (sitosterol glucoside)



^{1}H NMR spectrum of LCMA (sitosterol glucoside) in CD_3OD



^{13}C NMR spectrum of LCMA (sitosterol glucoside) in CD_3OD



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AQ 0.4074620 sec
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GB 0

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CX1 15.00 cm
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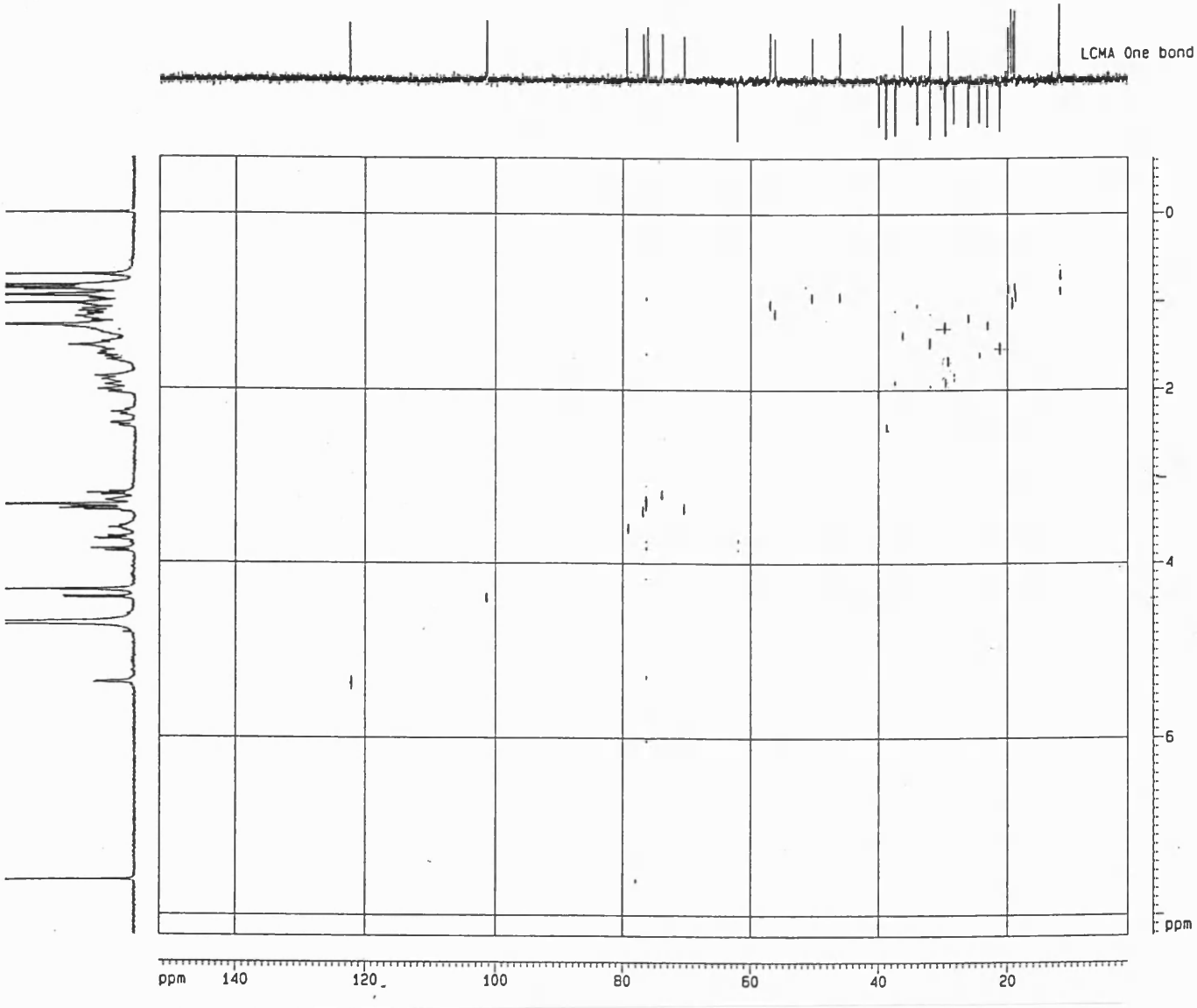
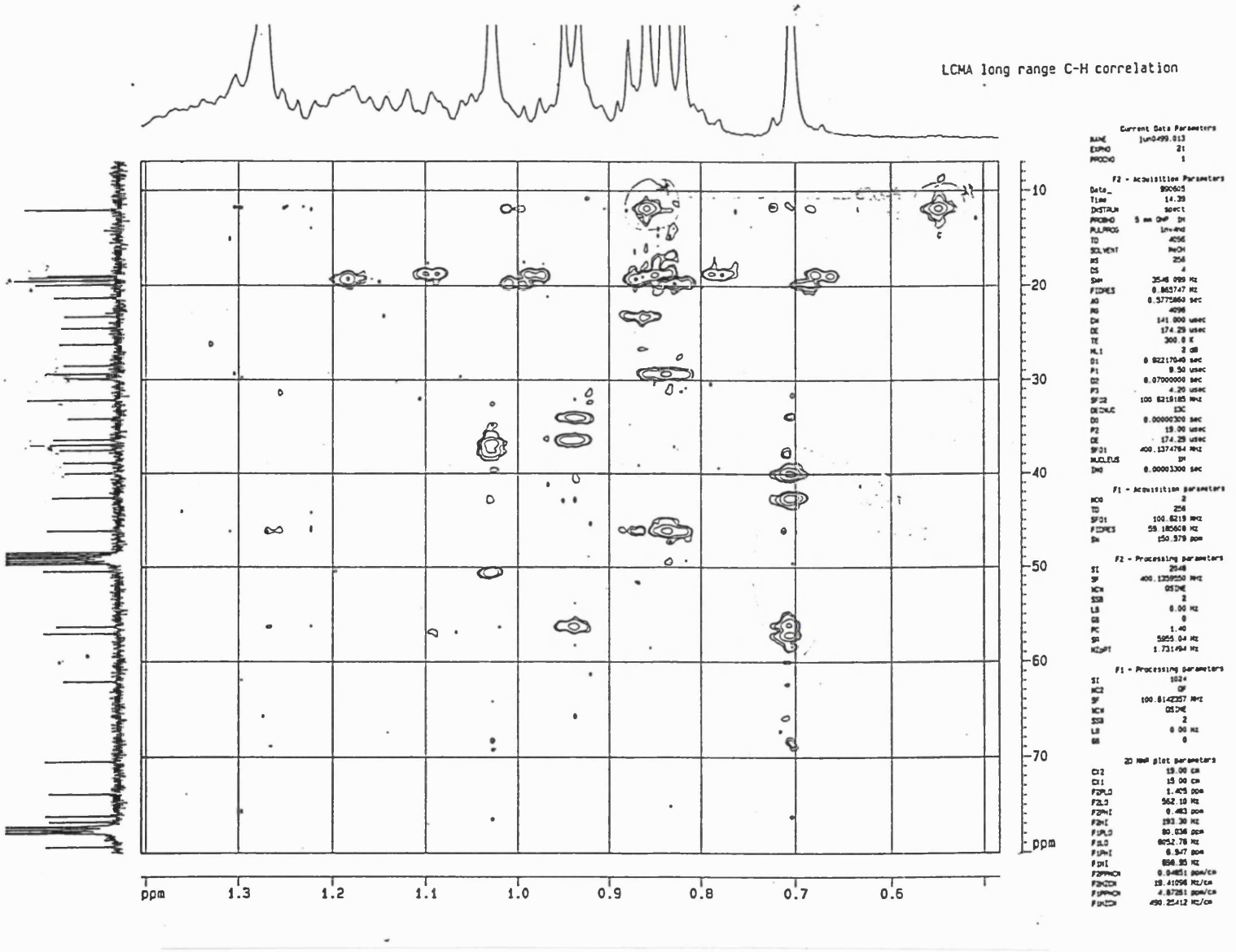
F2HI -24.23 Hz
F1PLO 6.219 ppm
F1LO 2488.33 Hz
F1PHI -0.061 ppm
F1HI -24.23 Hz
F2PPMCM 0.41862 ppm/cm
F2HZCM 167.50420 Hz/cm
F1PPMCM 0.41862 ppm/cm
F1HZCM 167.50420 Hz/cm

^{1}H-^{1}H COSY spectrum of LCMA (sitosterol glucoside) in CD_3OD



^{1}H-^{13}C one bond correlation spectrum of LCMA (sitosterol glucoside) in CD_3OD



^{1}H-^{13}C long range correlation spectrum of LCMA (sitosterol glucoside) in CD_3OD

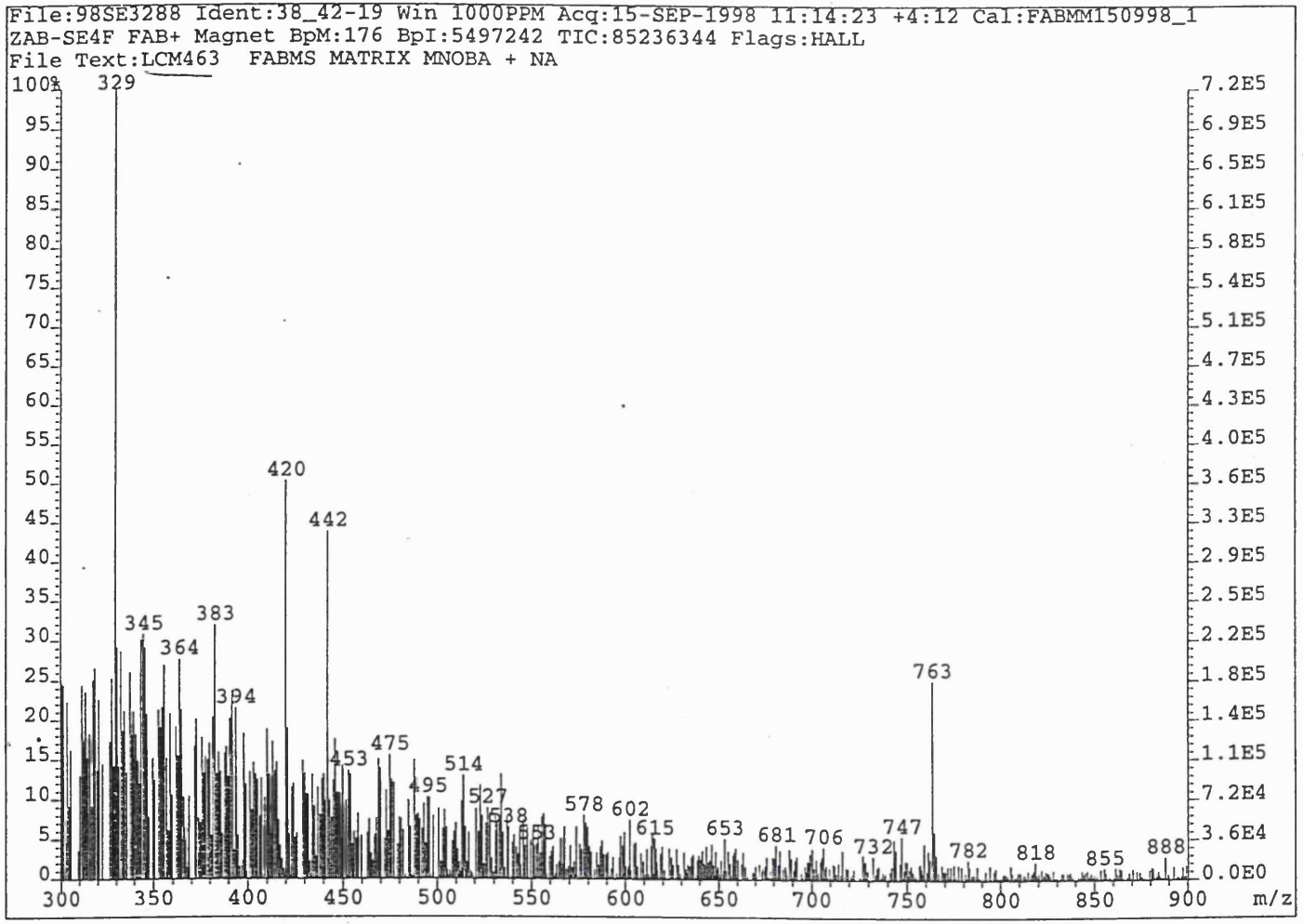
Appendix 2: UV, MS and NMR data for LCM263

2.1) UV spectrum in methanol

2.2) Fast atom bombardment mass spectrum

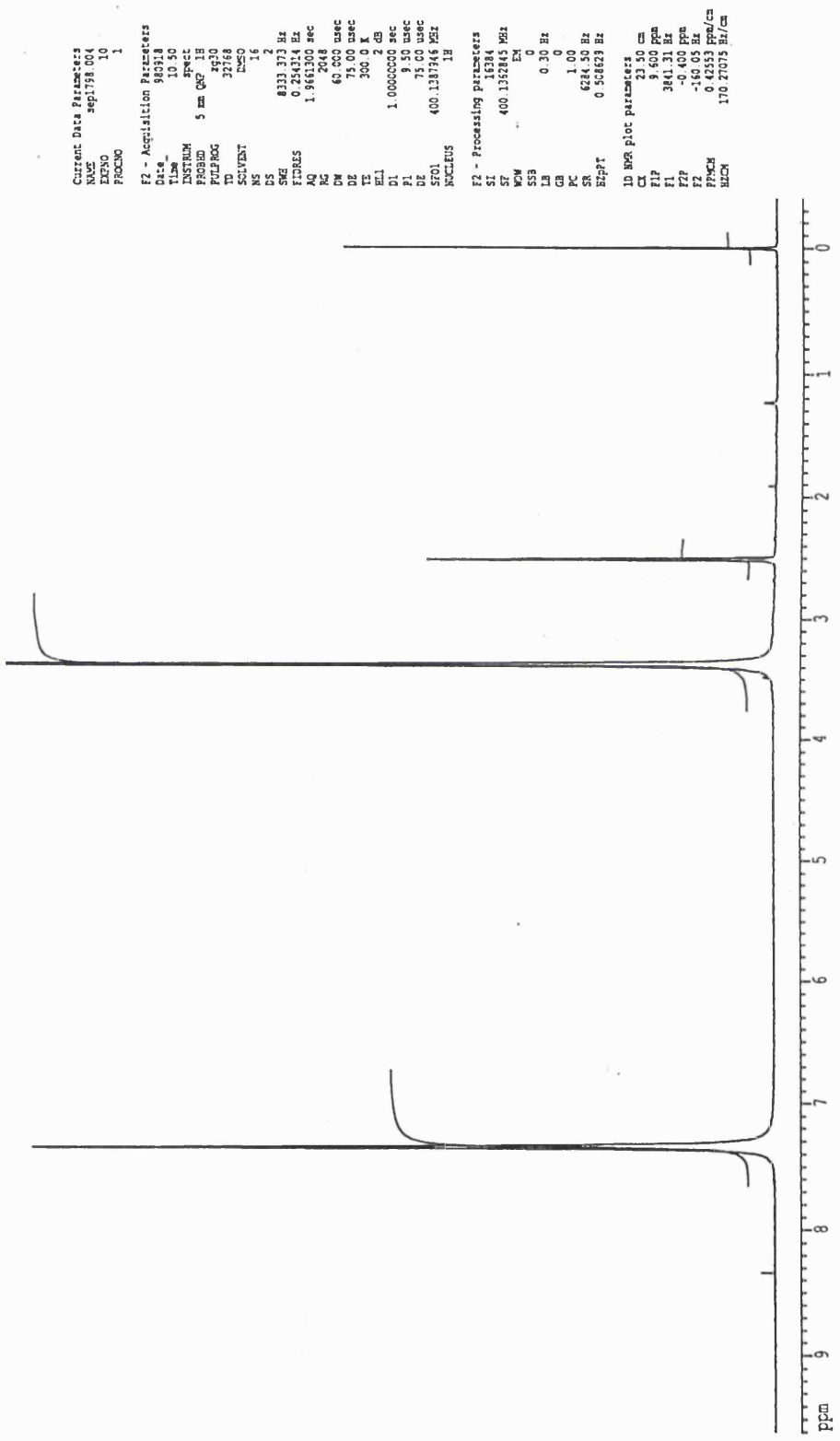
2.3) ^{1}H-400 MHz NMR spectrum in CD_3OD

UV spectrum of LCM263 in methanol



Fast atom bombardment mass spectrum of LCM263

Appendix 2.3



^{1}H NMR spectrum of LCM263 in CD_3OD

Appendix 3: UV, MS and NMR data for AAR1P (*cis* hinokiresinol)

3.1) UV spectrum in methanol

3.2) Electron impact mass spectrum

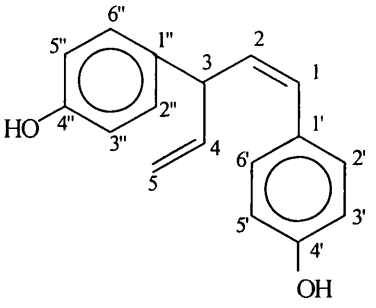
3.3) ^{1}H-400 MHz NMR spectrum in $CDCl_3$

3.4) ^{13}C-100 MHz NMR spectrum (DEPT) in $CDCl_3$

3.5) ^{1}H-^{1}H COSY spectrum in $CDCl_3$

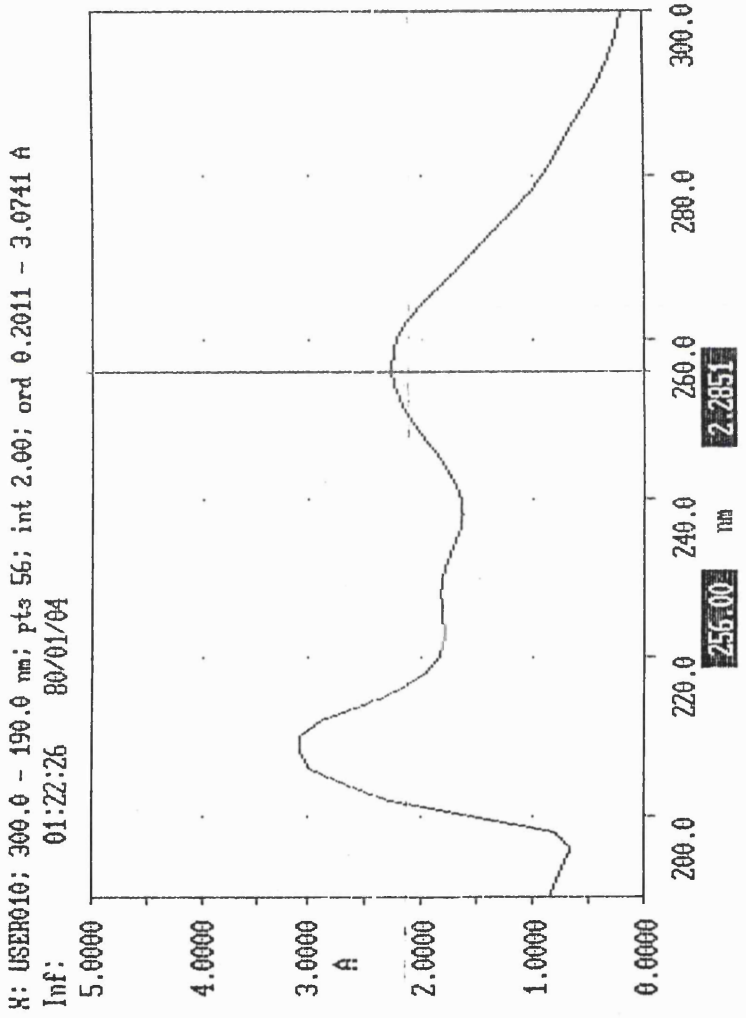
3.6) ^{1}H-^{13}C one bond correlation spectrum in $CDCl_3$

3.7) ^{1}H-^{13}C long range correlation spectrum in $CDCl_3$

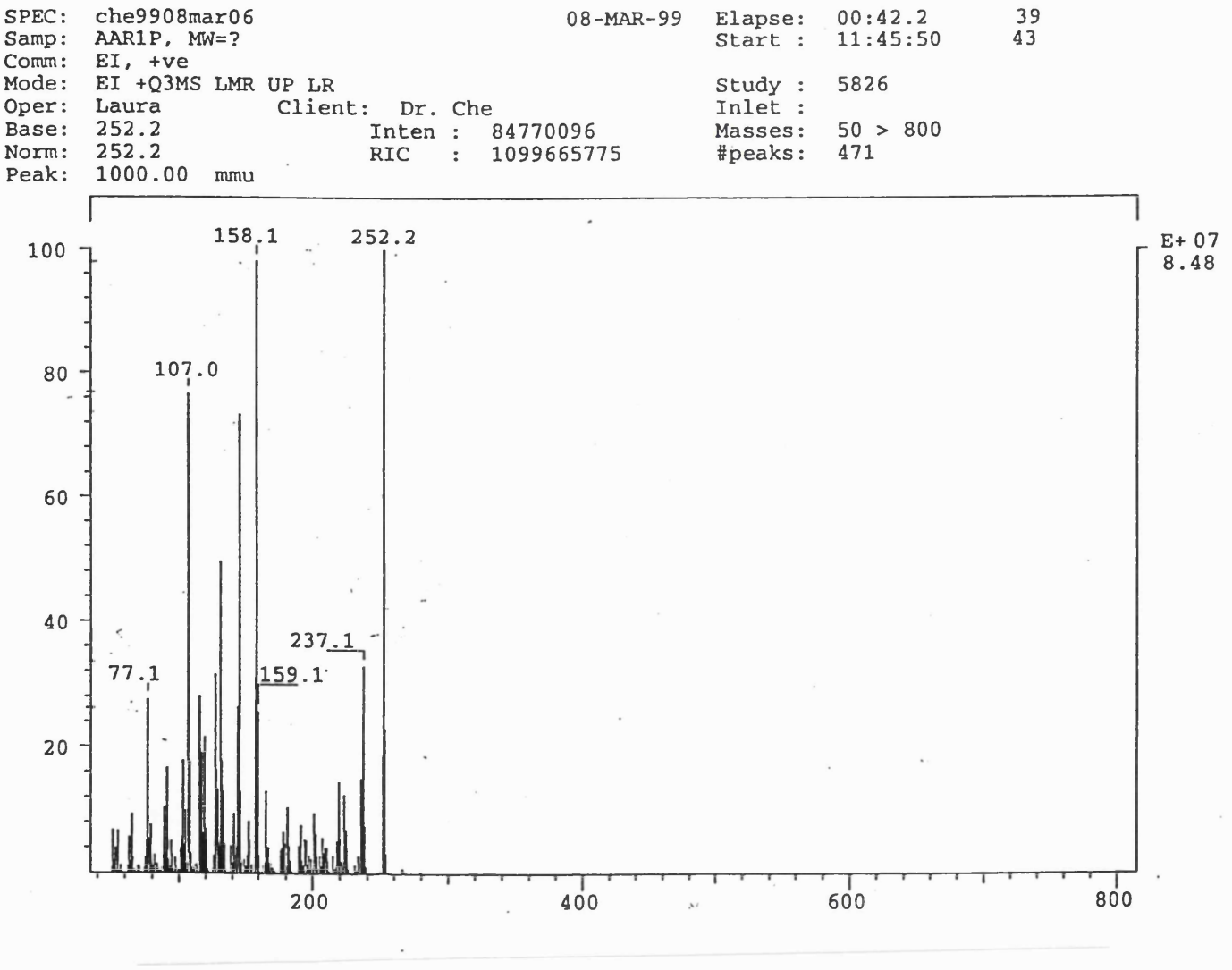


AAR1P (*cis* Hinokiresinol, Nyasol)

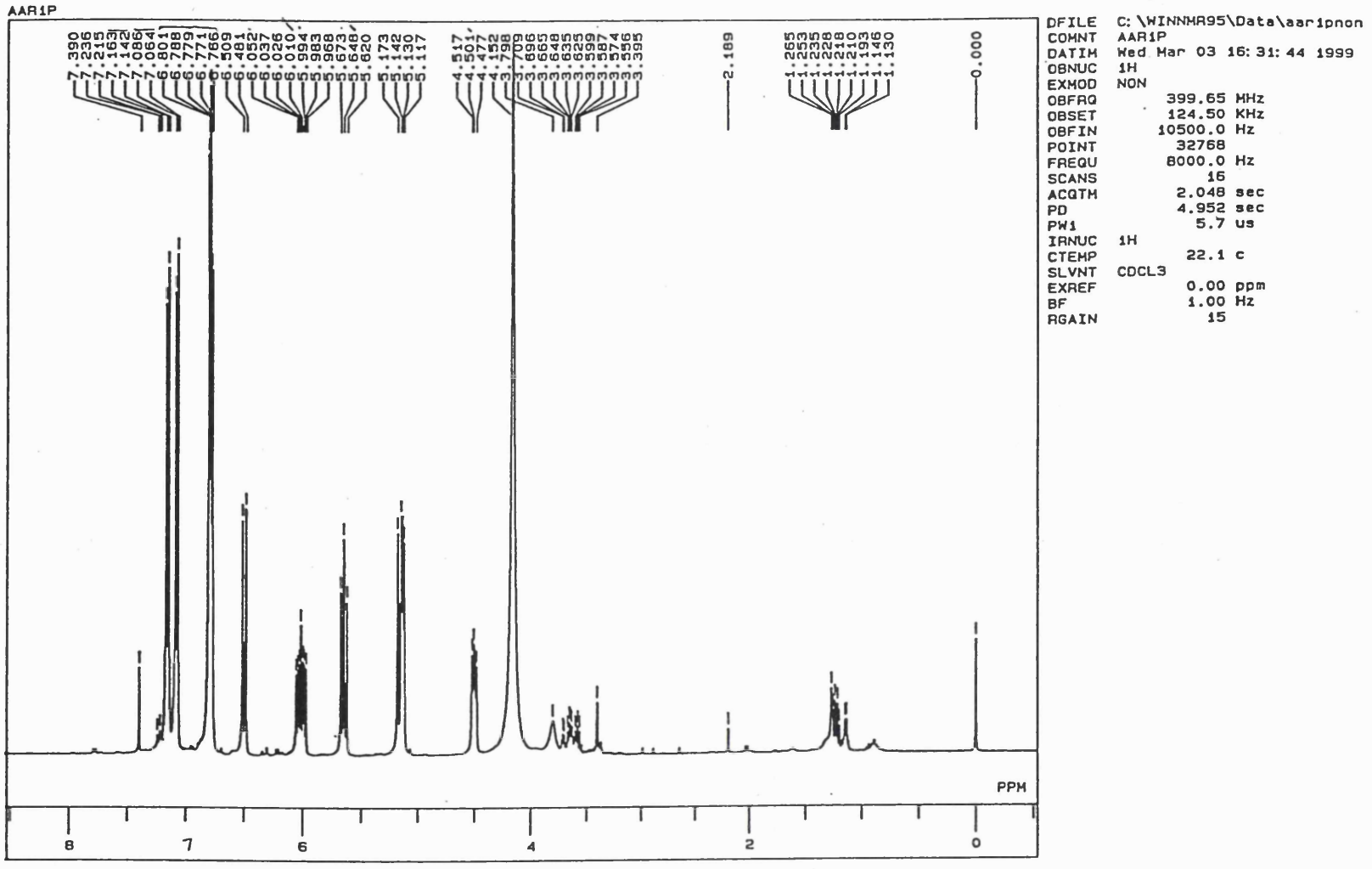
Appendix 3.1



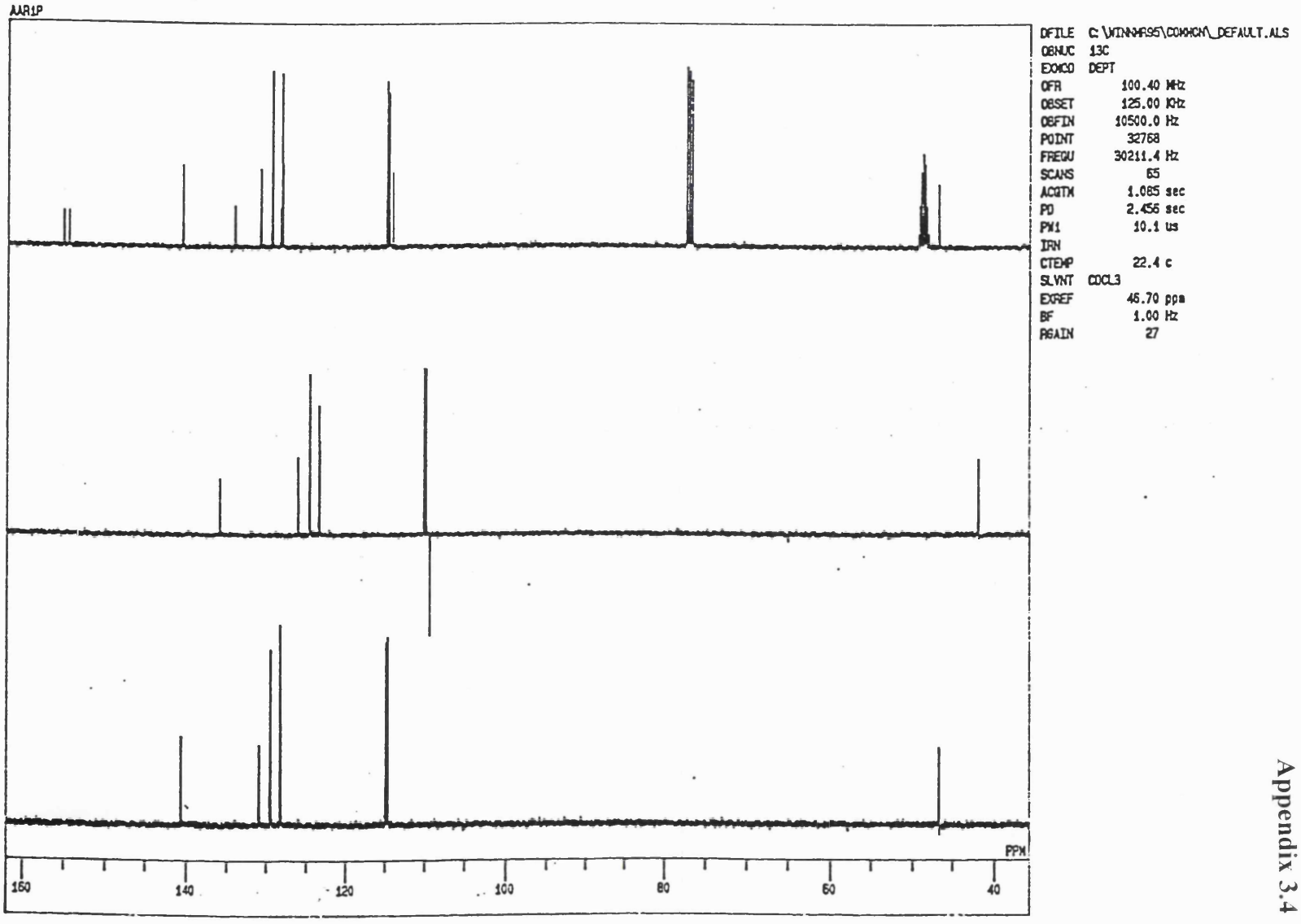
UV spectrum of AAR1P (*cis* hinokiresinol) in methanol



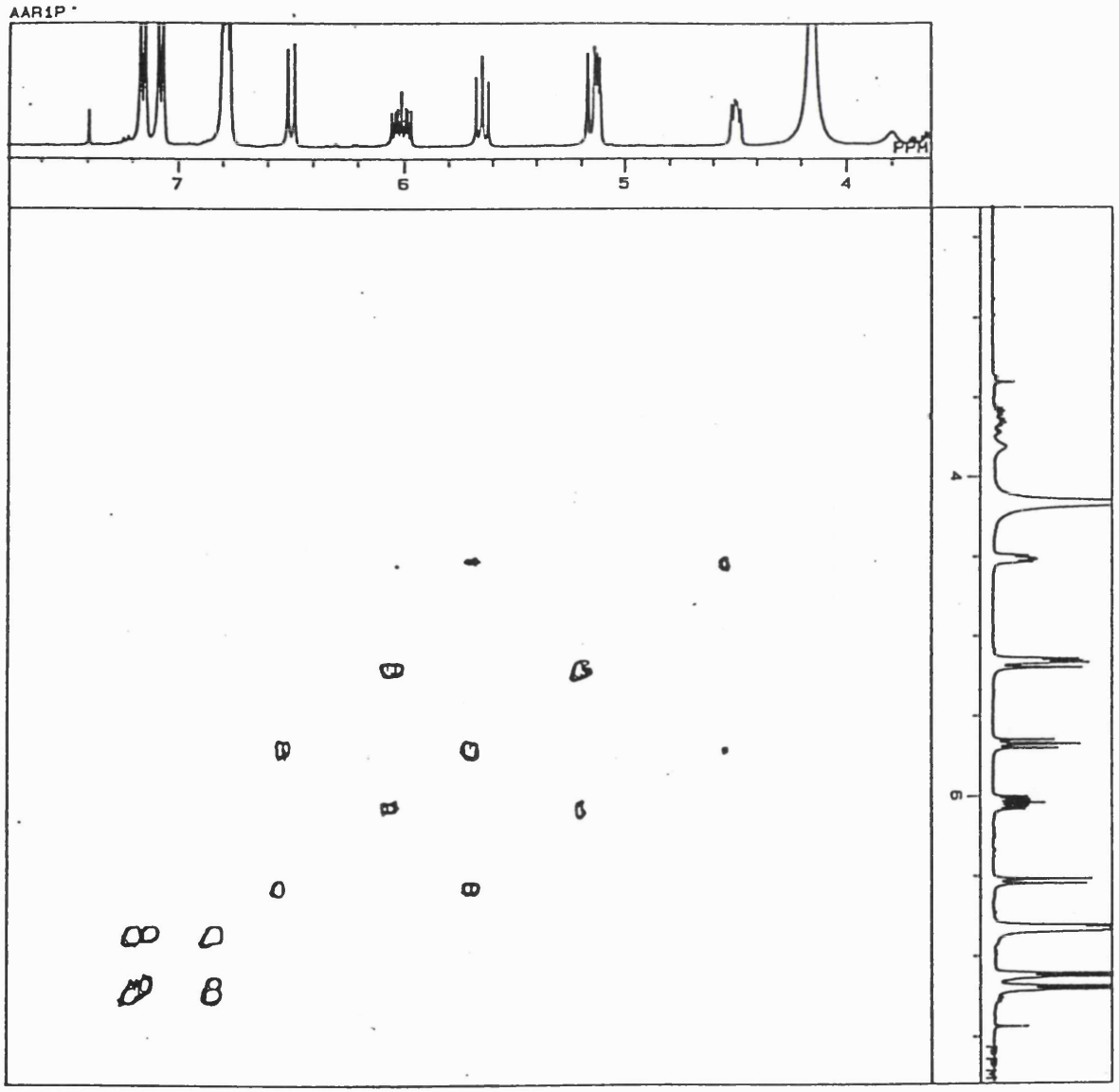
Electron impact mass spectrum of AAR1P (*cis* hinokiresinol)



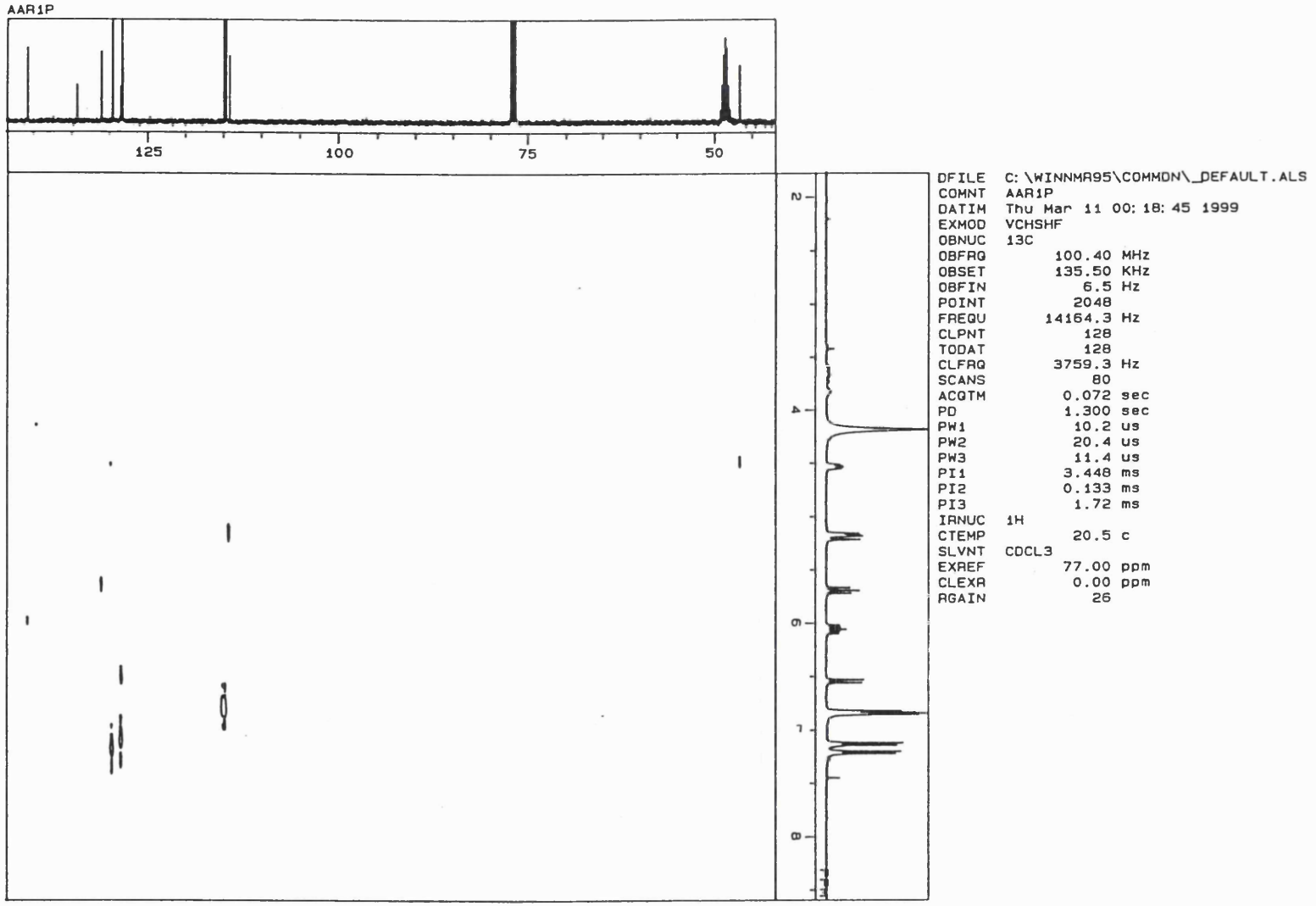
^{1}H NMR spectrum of AAR1P (*cis* hinokiresinol) in $CDCl_3$



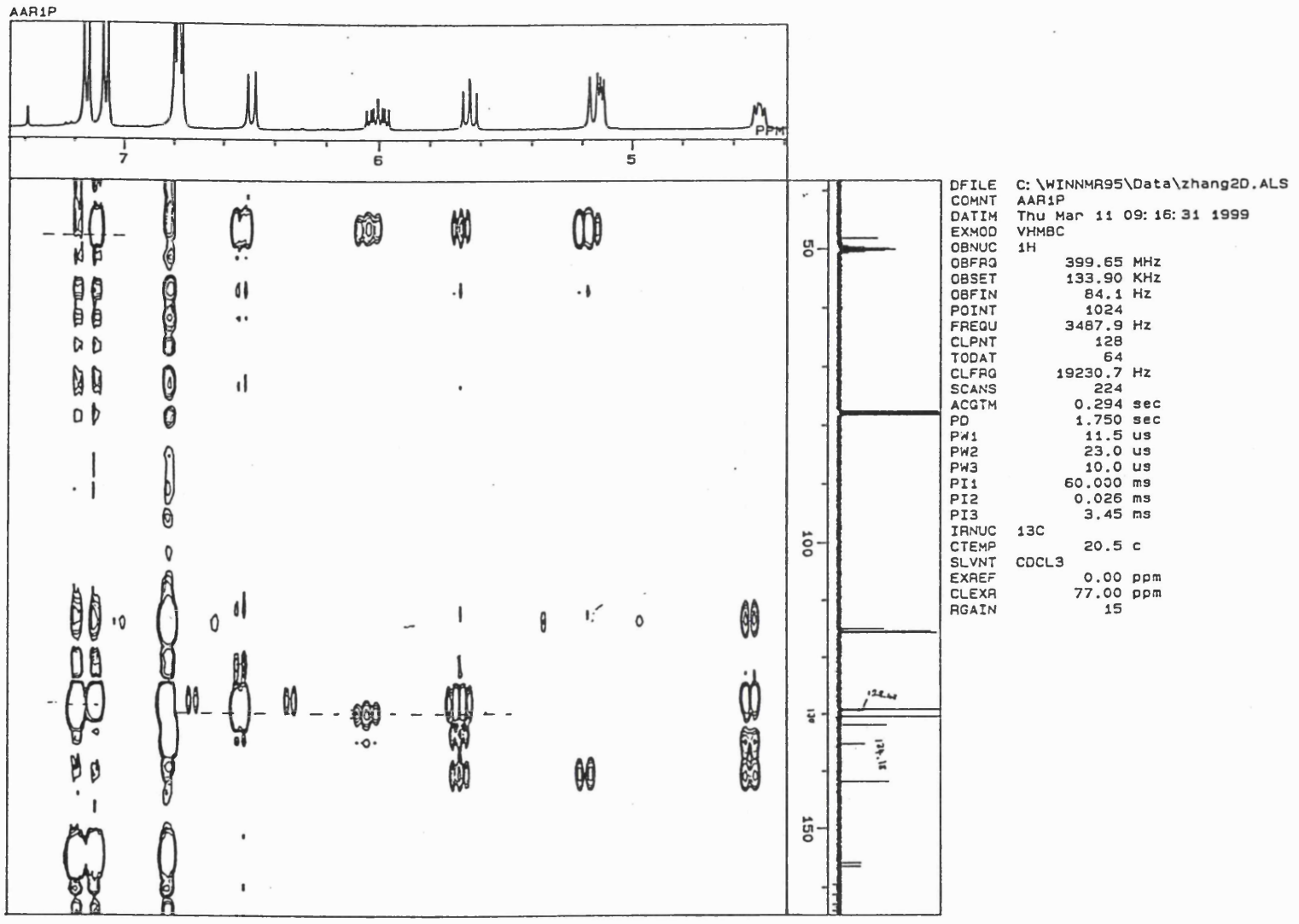
^{13}C NMR spectrum of AAR1P (*cis* hinokiresinol) in $CDCl_3$



^{1}H-^{1}H COSY spectrum of AAR1P (*cis* hinokiresinol) in $CDCl_3$



^{1}H-^{13}C one bond correlation spectrum of AAR1P (*cis* hinokiresinol) in $CDCl_3$



^{1}H-^{13}C long range correlation spectrum of AAR1P (*cis* hinokiresinol) in $CDCl_3$

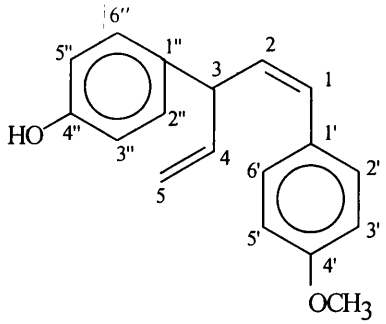
Appendix 4: UV, MS and NMR data for AAC41P2 (monometyl *cis* hinokiresinol)

4.1) UV spectrum in methanol

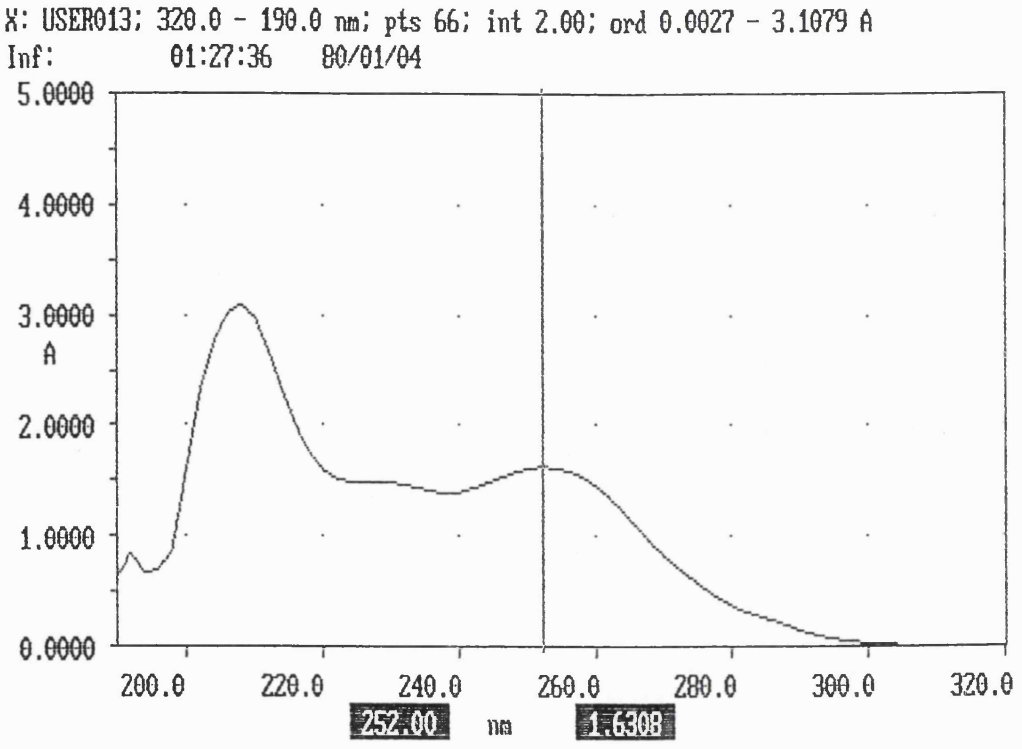
4.2) Electron impact mass spectrum

4.3) ^{1}H- 400 MHz NMR spectrum in $CDCl_3$

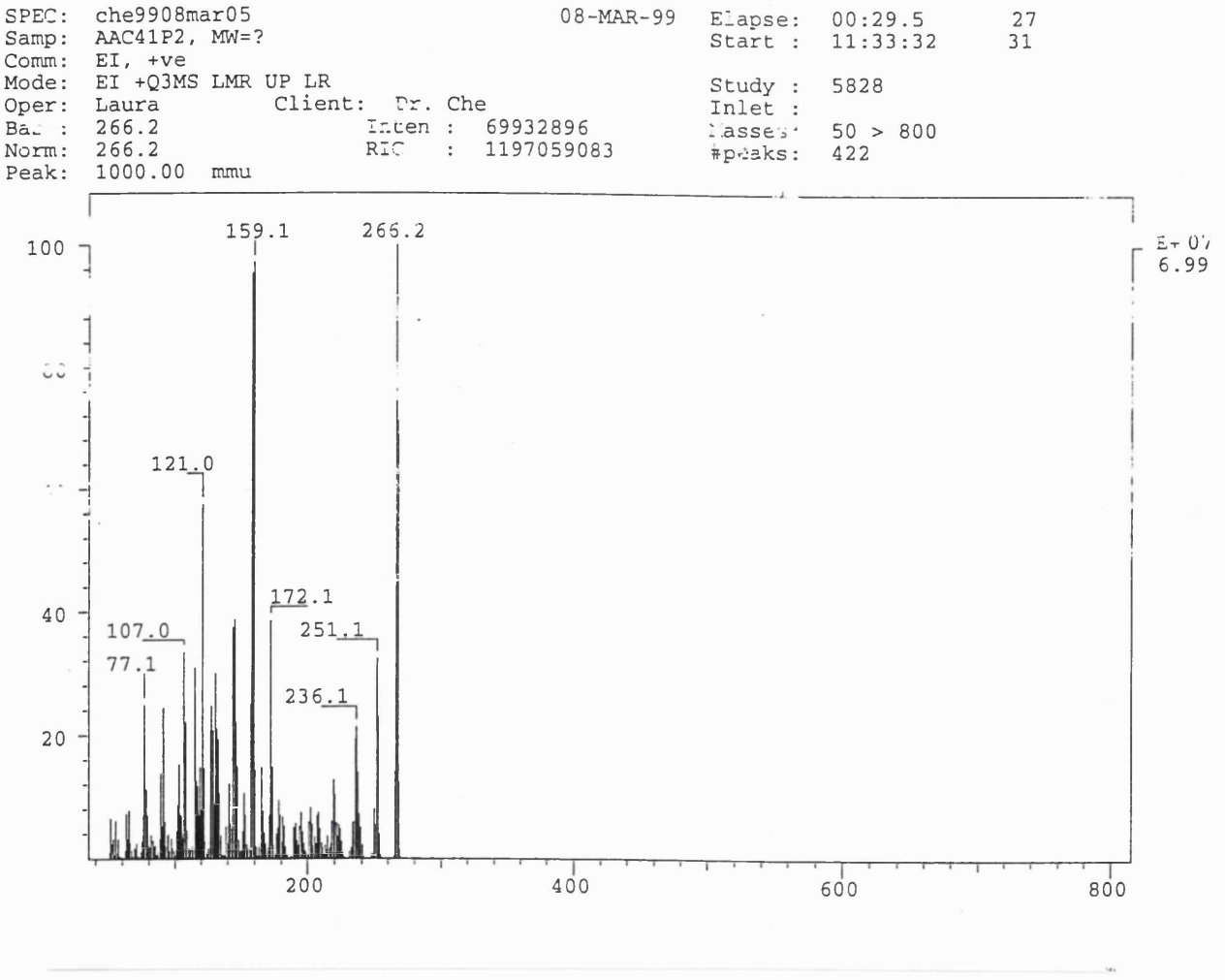
4.4) ^{13}C-100 MHz NMR spectrum (DEPT) in $CDCl_3$



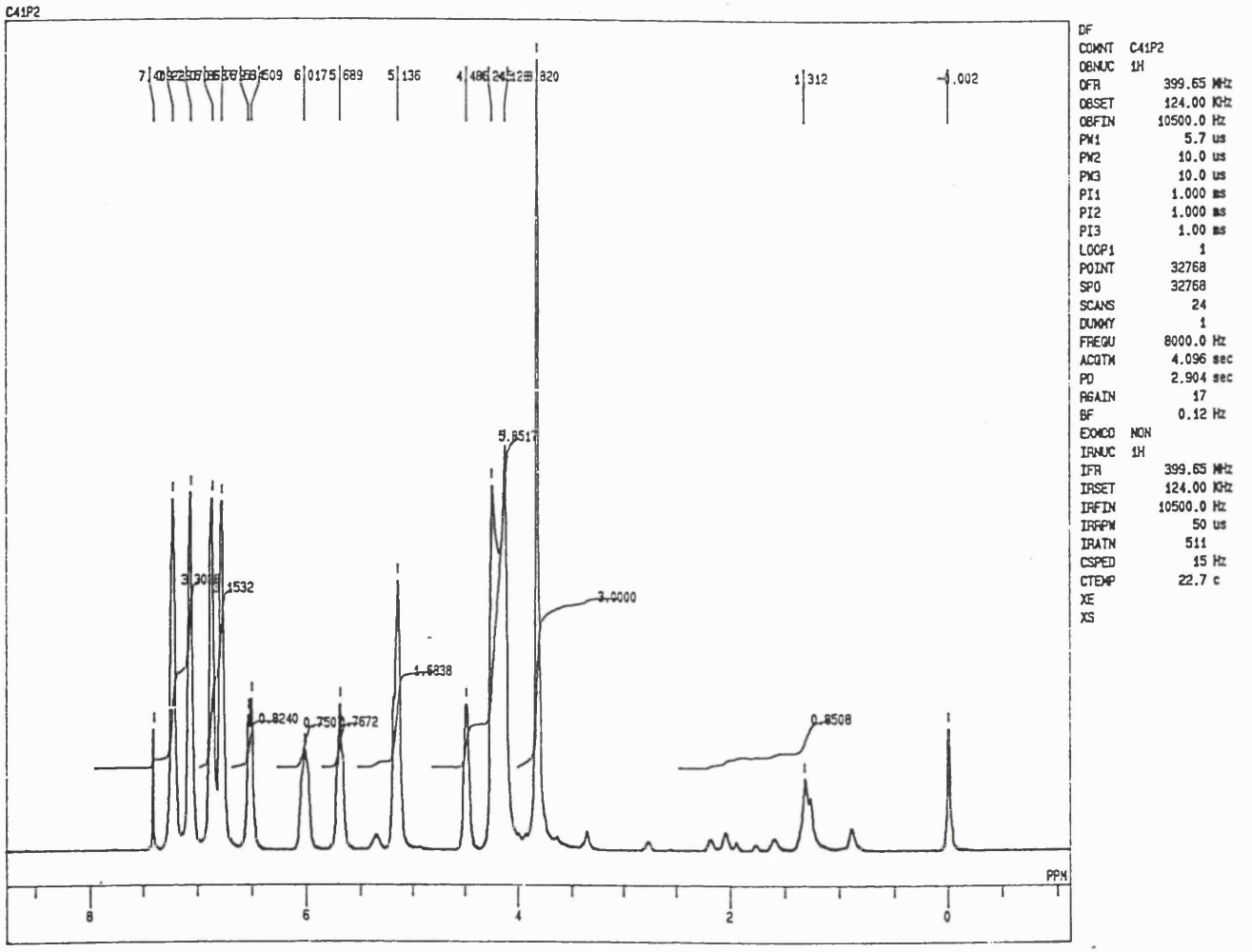
AAC41P2 (monomethyl *cis*- hinokiresinol)



UV spectrum of AAC41P2 (4′methyl *cis* hinokiresinol) in methanol

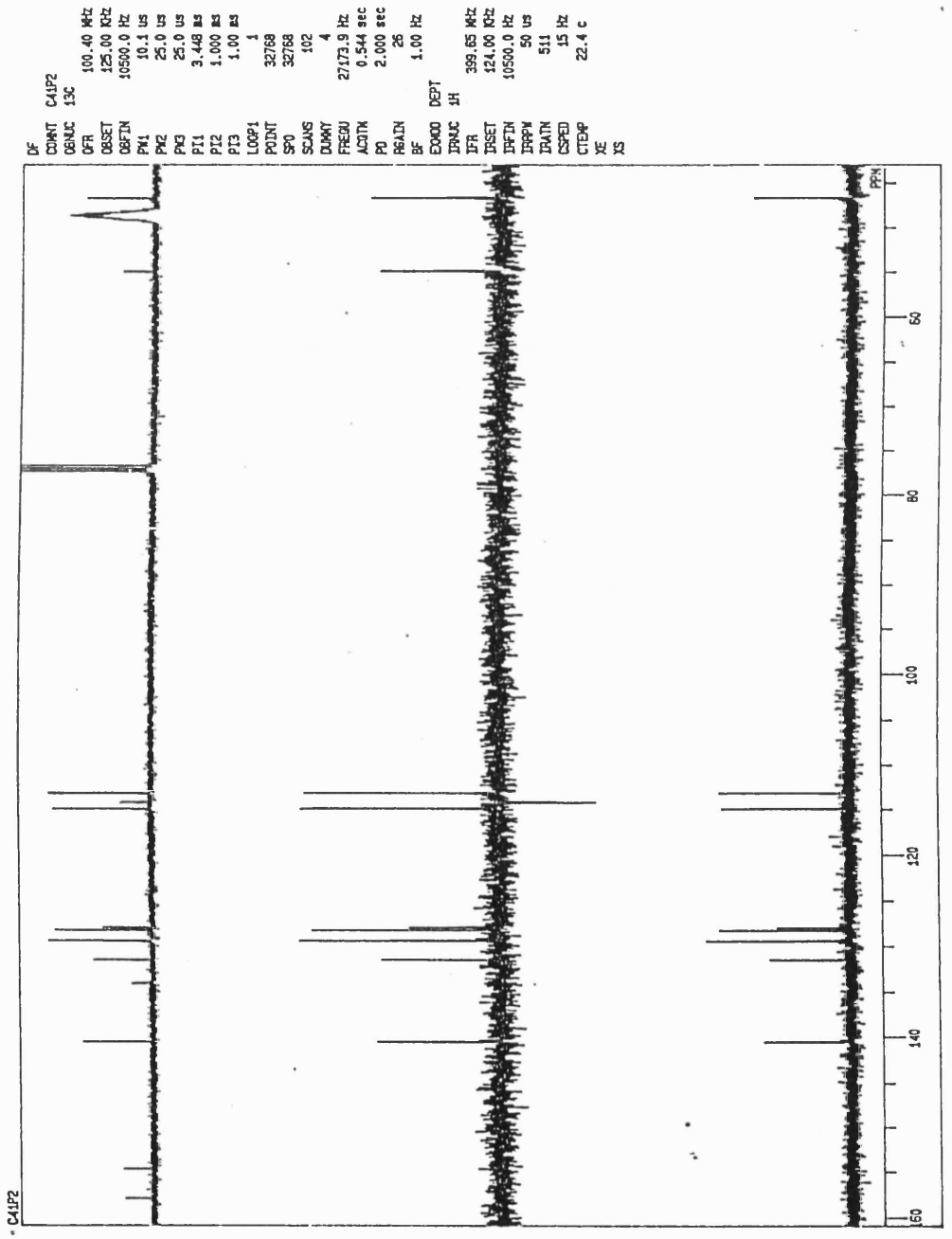


Electron impact mass spectrum of AAC41P2 (4′methyl *cis* hinokiresinol)



^{1}H NMR spectrum of AAC41P2 (4′methyl *cis* hinokiresinol)in $CDCl_3$

Appendix 4.4



^{13}C NMR spectrum of AAC41P2 (4′methyl *cis* hinokiresinol) in $CDCl_3$

Appendix 5: UV, IR, MS and NMR data for AAM22 (timosaponin AIII)

5.1) UV spectrum in methanol

5.2) IR spectrum

5.3) Fast atom bombardment mass spectrum

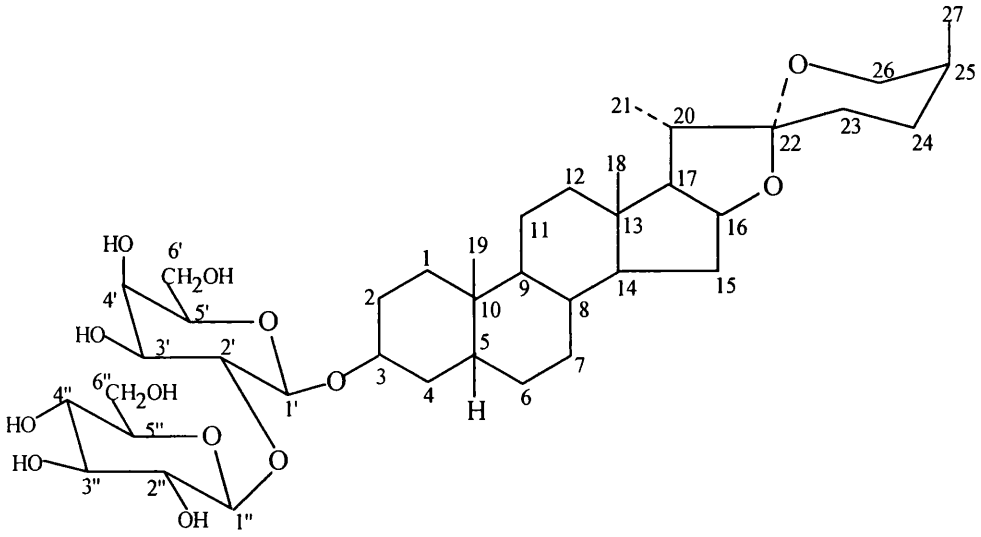
5.4) ^{1}H-400 MHz NMR spectrum in DMSO-d_6

5.5) ^{1}H-400 MHz NMR spectrum in pyridine-d_5

5.6) ^{13}C-100 MHz NMR spectrum (DEPT) in DMSO-d_6

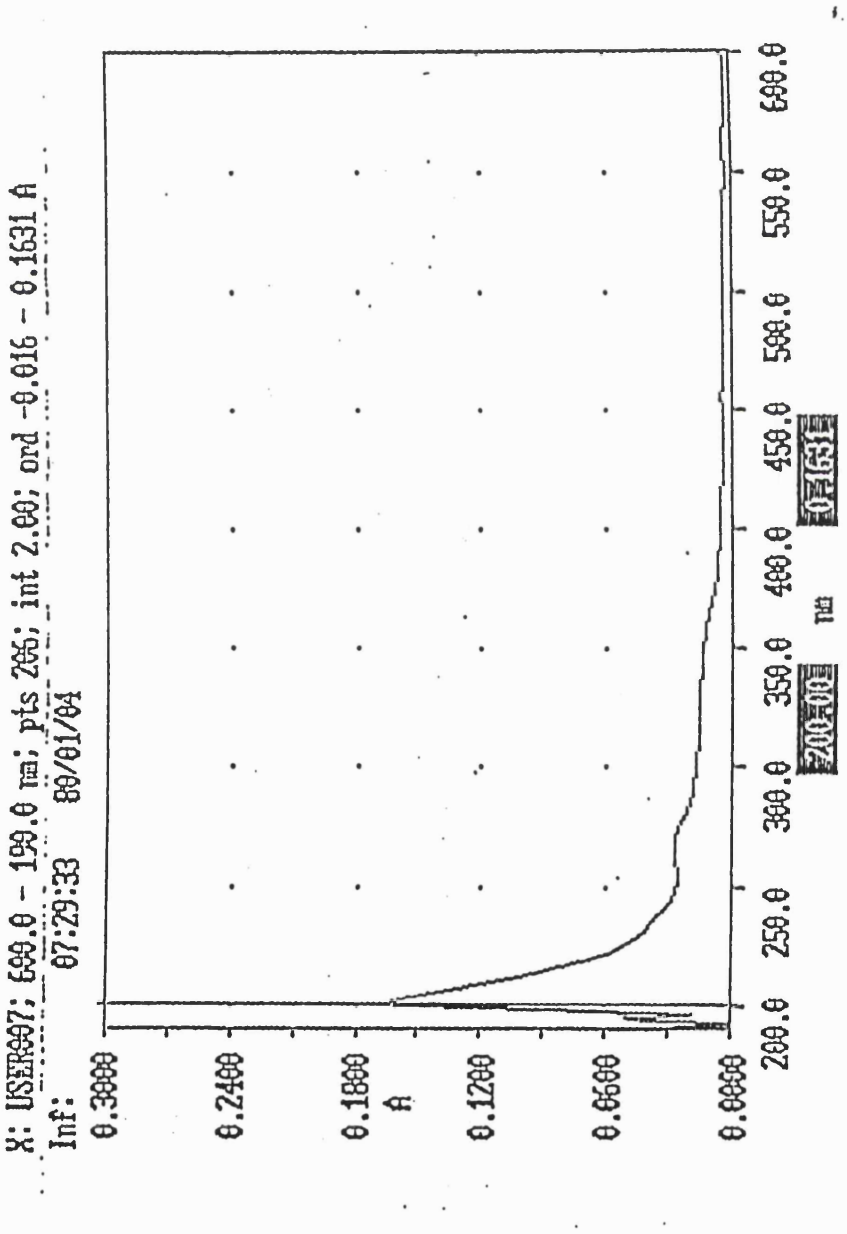
5.7) ^{1}H-^{1}H COSY spectrum in DMSO- d_6

5.8) ^{1}H-^{13}C one bond correlation spectrum in DMSO- d_6

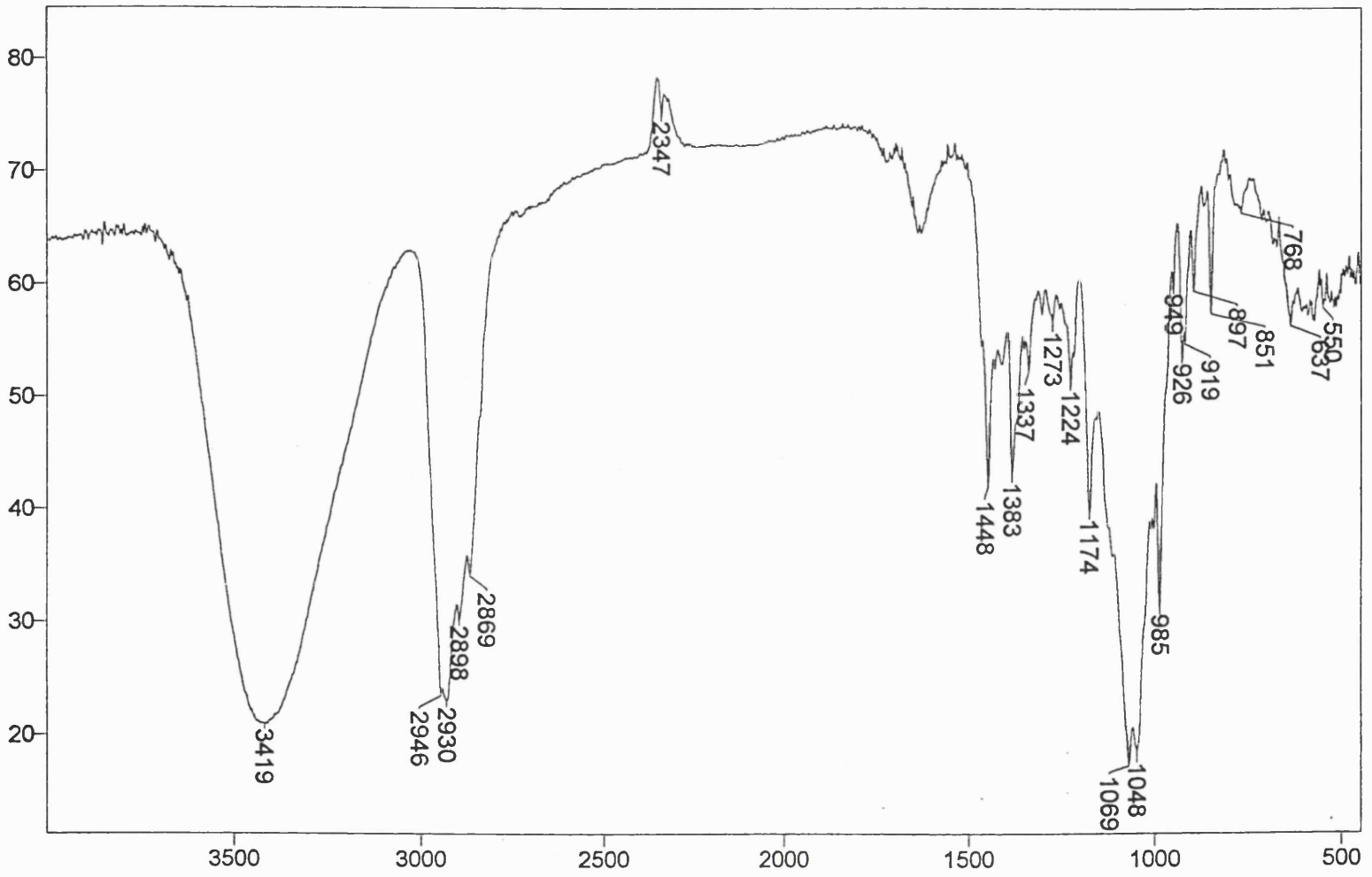


AAM22 (timosaponin AIII)

Appendix 5.1



UV spectrum of AAM22 (timosaponin AIII) in methanol



Transmittance / Wavenumber (cm-1)

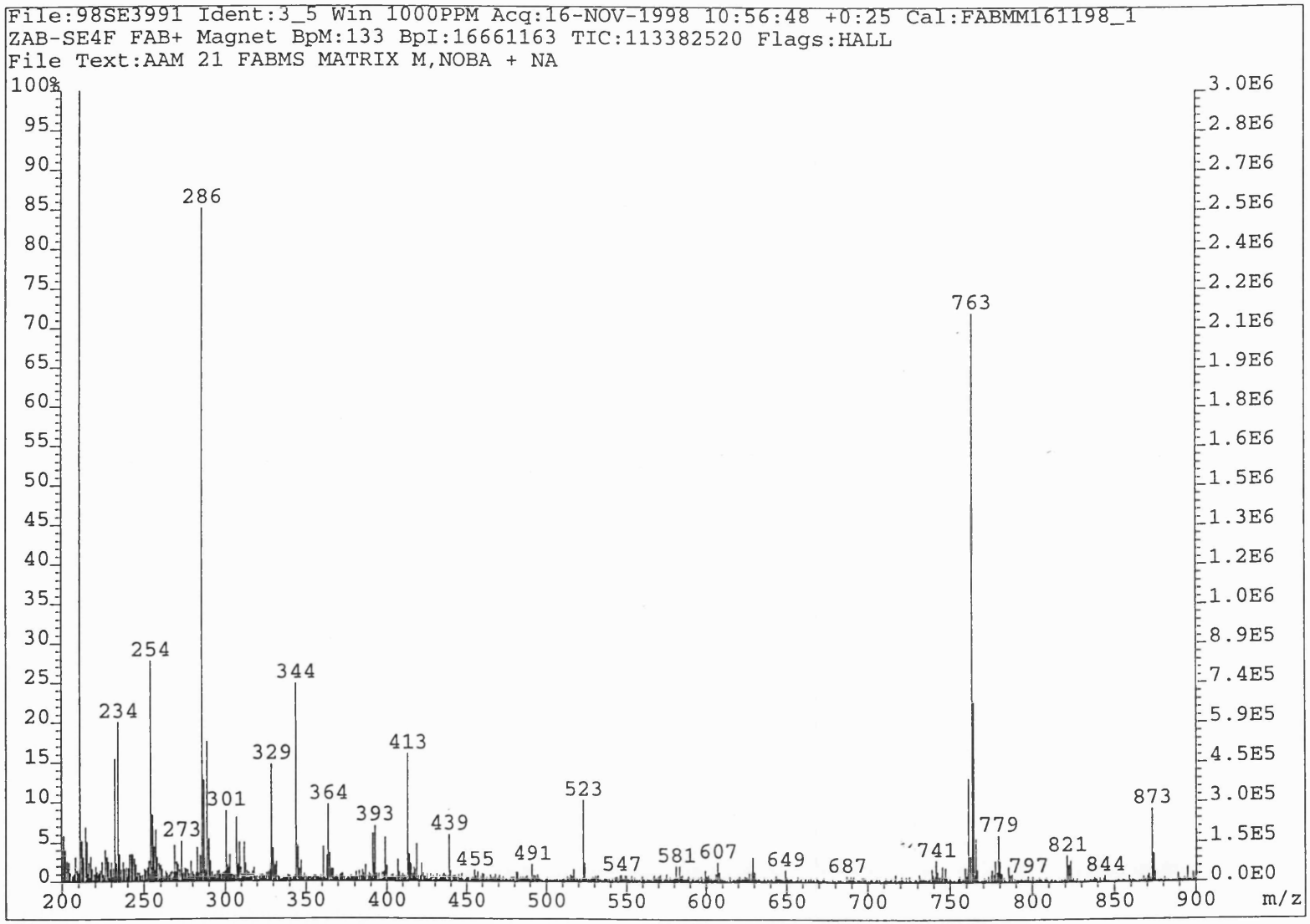
File # 1 = AAM22

pink

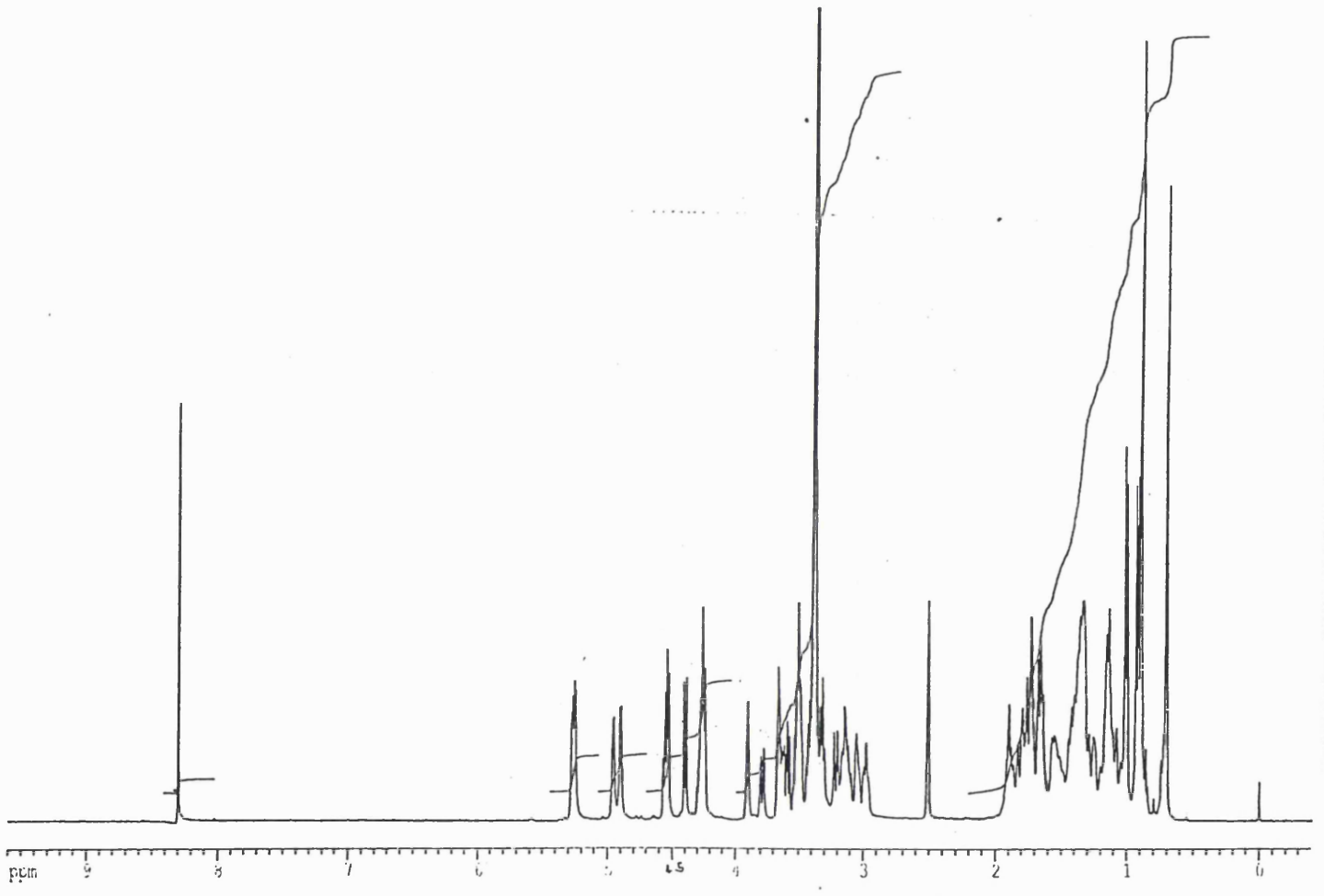
Paged X-Zoom CURSOR

13/04/99 14:31 Res=4 cm-1

IR spectrum of AAM22 (timosaponin AIII)



Fast atom bombardment mass spectrum of AAM22 (timosaponin AIII)

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PROCNO                  1

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DE                75.00 usec
TE                300.0 K
HL1                    2 dB
D1          1.00000000 sec
P1                 9.50 usec
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SFO1       400.1387946 MHz
NUCLEUS              1H

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SR              6285.00 Hz
HZPPT          0.508629 Hz

1D NMR plot parameters
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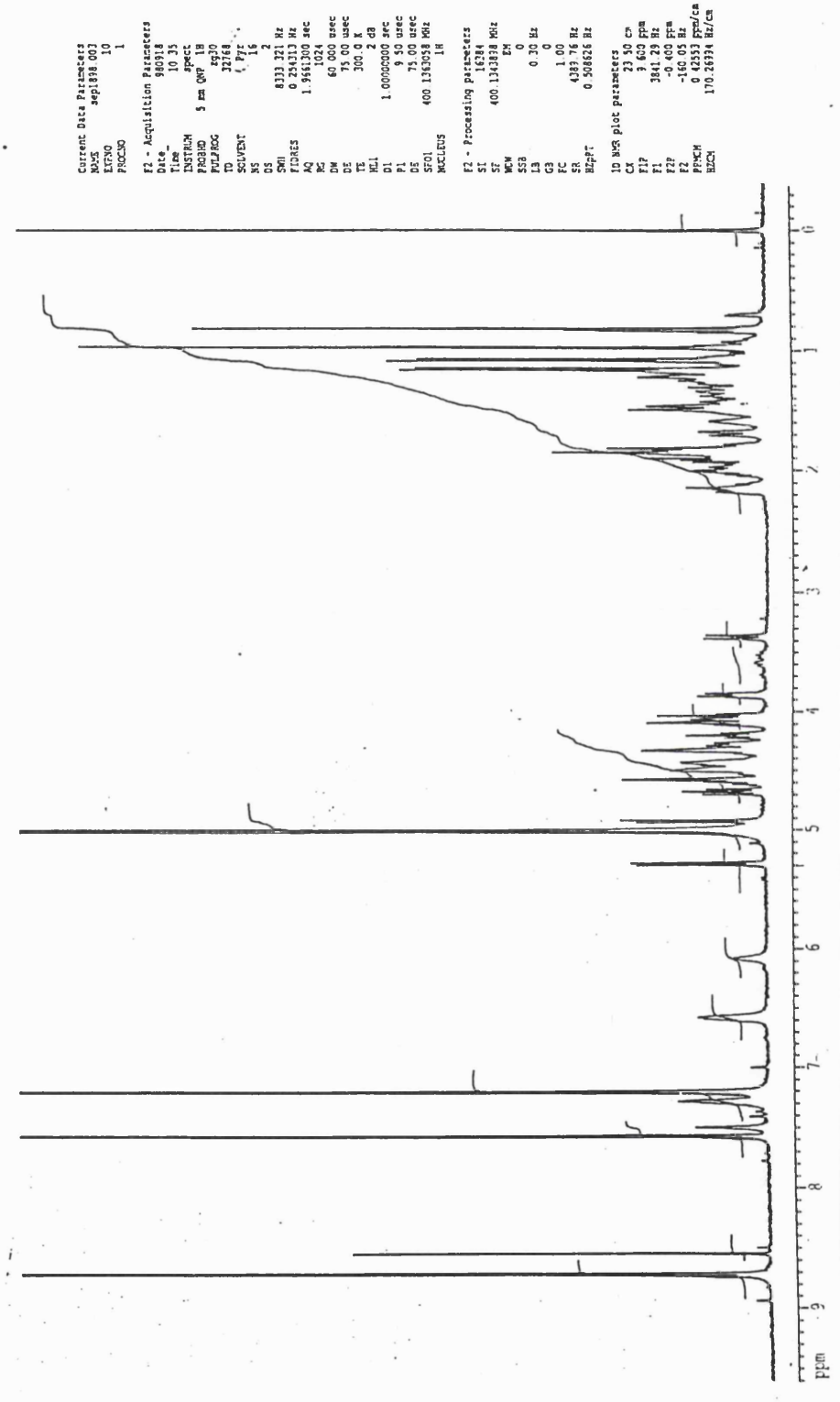
F1P                9.600 ppm
F1              3841.31 Hz
F2P               -0.400 ppm
F2              -160.05 Hz
PPMCM          0.42553 ppm/cm
HZCM          170.27075 Hz/cm
```

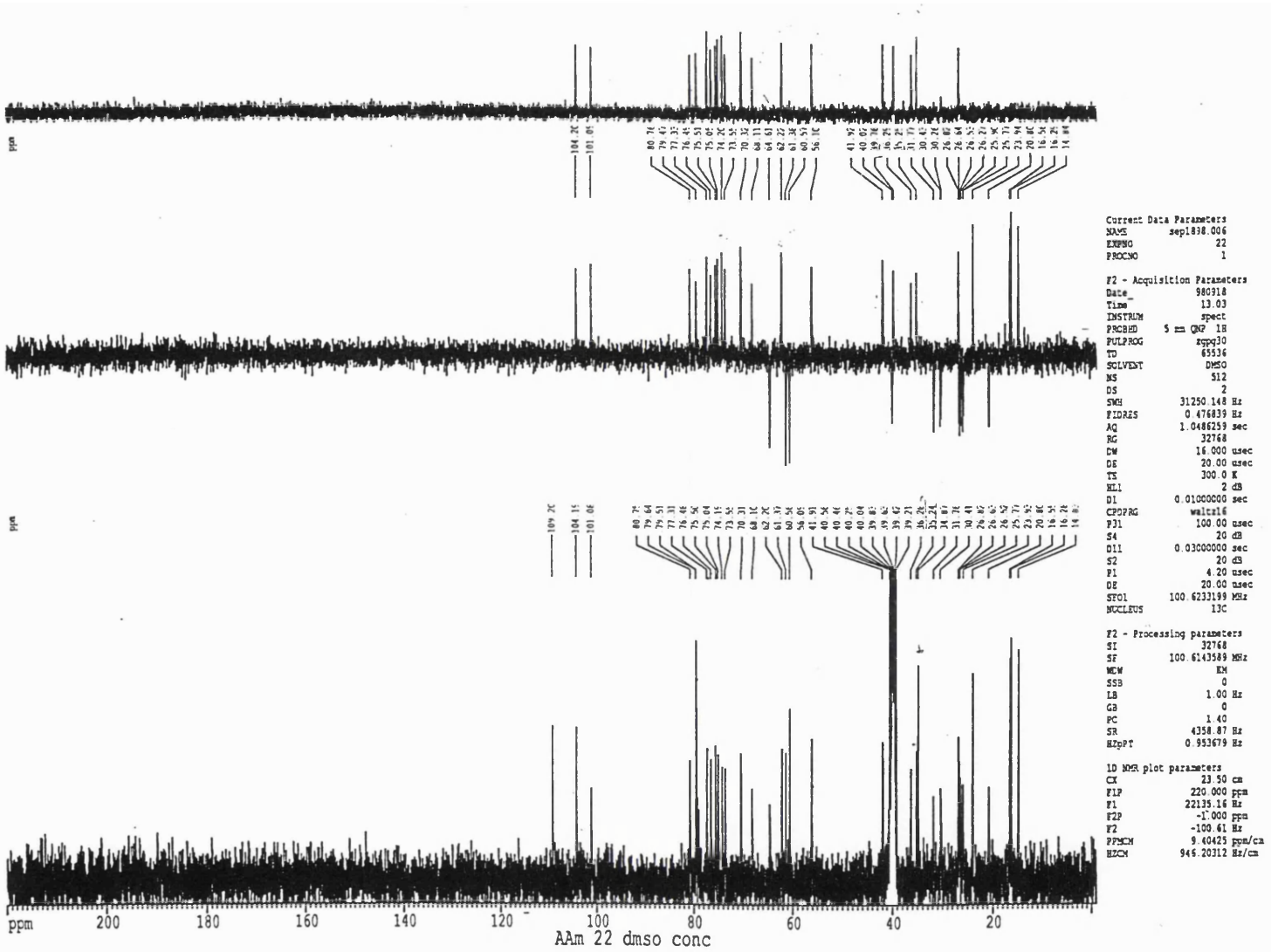
Appendix 5.4

^{1}H NMR spectrum of AAM22 (timosaponin AIII) in DMSO-d_6

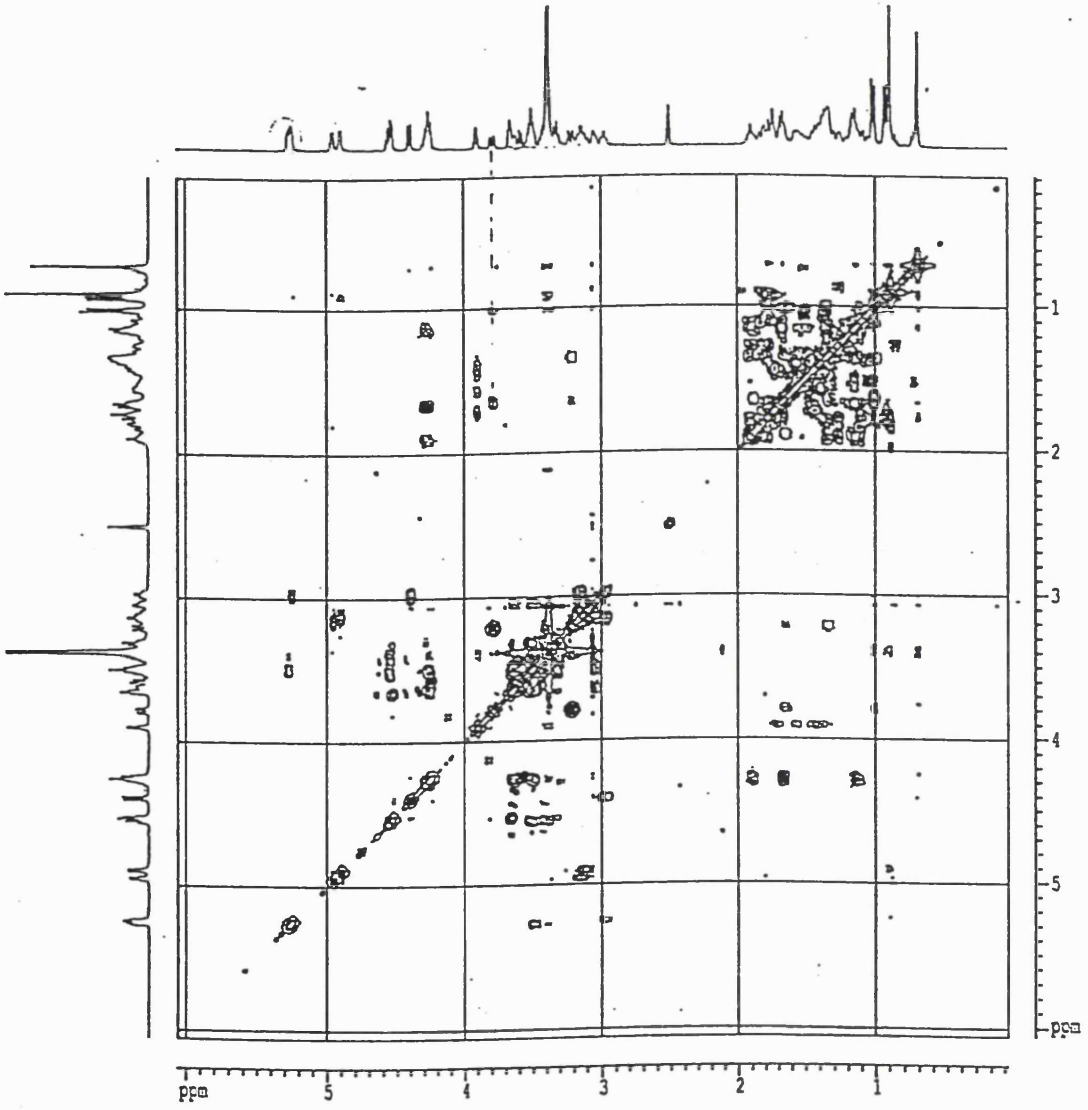
Appendix 5.5



^{1}H NMR spectrum of AAM22 (timosaponin AIII) in pyridine-d_5



^{13}C NMR spectrum of AAM22 (timosaponin AIII) in DMSO-d_6



AAM22

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RG	128
DW	209.000 usec
DE	172.86 usec
TE	300.0 K
D11	2 sec
D1	0.81977433 sec
P1	9.50 usec
D0	0.00000300 sec
DE	172.86 usec
SFO1	400.1375126 MHz
NUC1	1H
DW	0.00041800 sec

F1 - Acquisition parameters

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TD	256
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FIDRES	9.345096 Hz
SW	5.979 ppm

F2 - Processing parameters

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LB	0.00 Hz
GB	0
PC	1.40
XDIM	6295.00 Hz
MCREST	2.336274 Hz

F1 - Processing parameters

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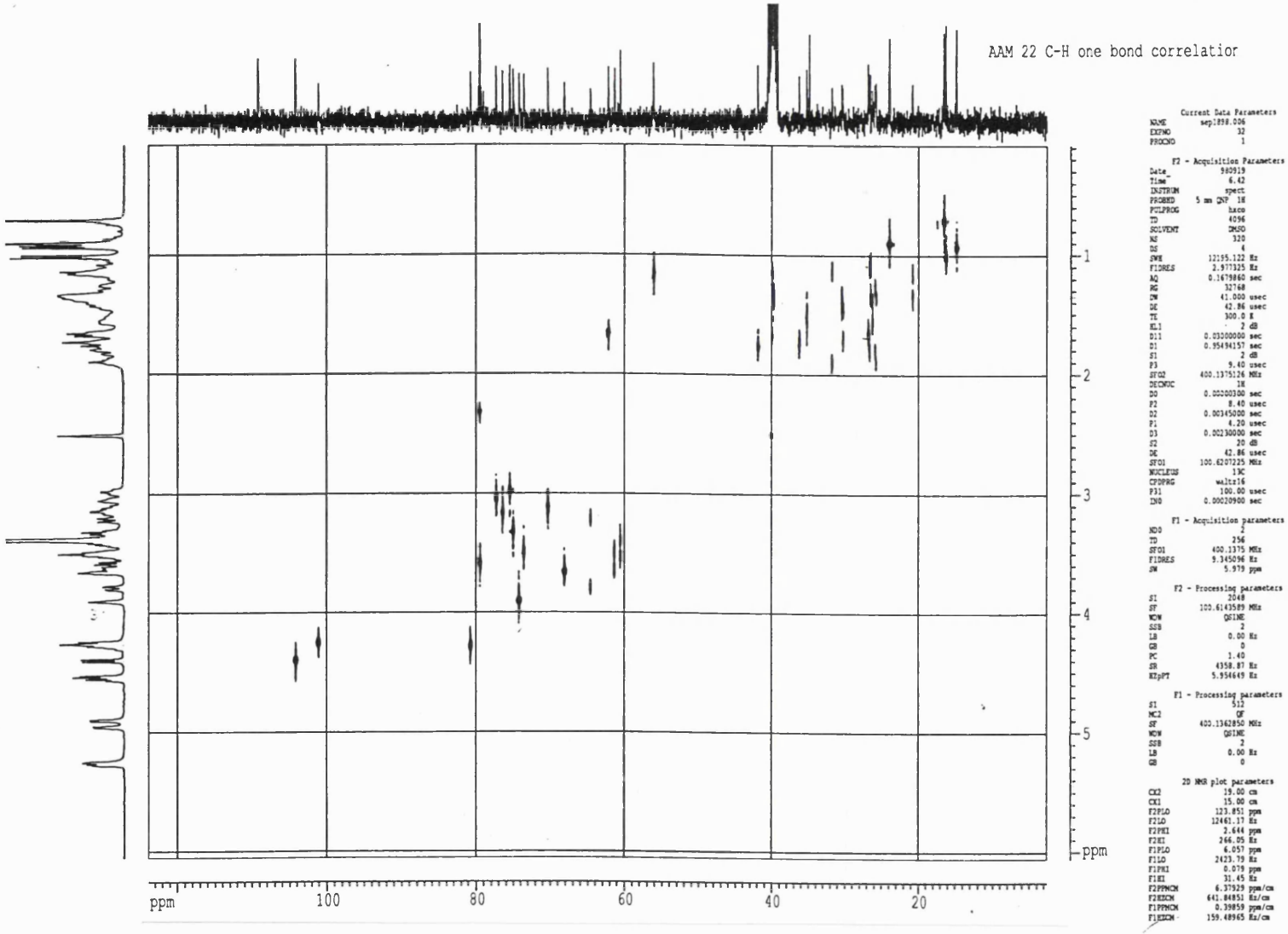
SF	400.1362850 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0

2D NMR plot parameters

CS	15.00 cm
CS1	15.00 cm
F2PPLO	6.057 ppm
F2LO	2423.79 Hz
F2PPSI	0.079 ppm
F2SI	31.45 Hz
F1PPLO	6.057 ppm
F1LO	2423.79 Hz
F1PPSI	0.079 ppm
F1SI	31.45 Hz
F2PPCM	0.39659 ppm/cm
F2HZCM	159.48917 Hz/cm
F1PPCM	0.39659 ppm/cm
F1HZCM	159.48917 Hz/cm

^{1}H-^{1}H COSY spectrum of AAM22 (timosaponin AIII) in DMSO- d_6



^{1}H-^{13}C one bond correlation spectrum of AAM22 (timosaponin AIII) in DMSO- d_6

Appendix 6: UV, IR, Ms and NMR data for AAM513A1 (timosaponin BI)

6.1) UV spectrum in methanol

6.2) IR spectrum

6.3) Fast atom bombardment mass spectrum

6.4) Electron impact mass spectrum

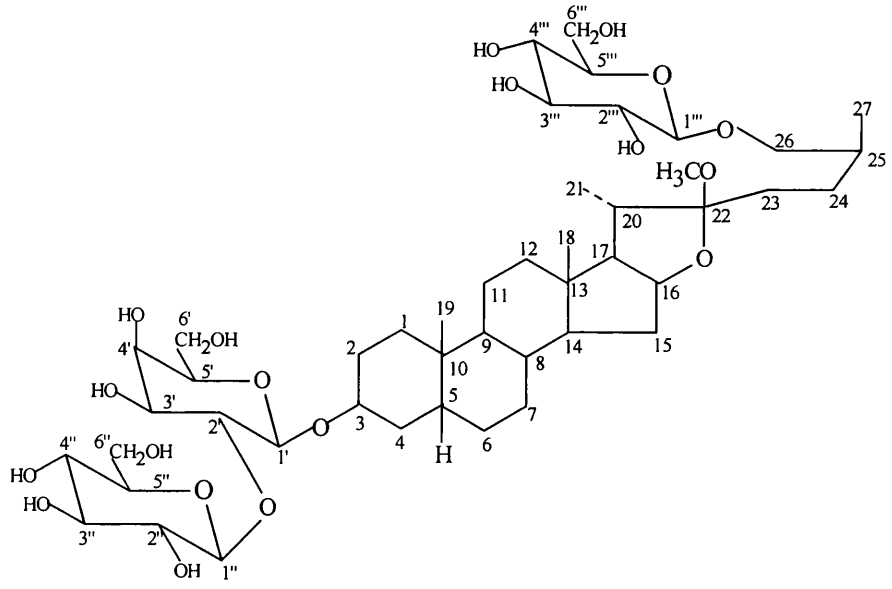
6.5) ^{1}H- 400 MHz NMR spectrum in CD_3OD

6.6) ^{13}C-100 MHz NMR spectrum (DEPT) in CD_3OD

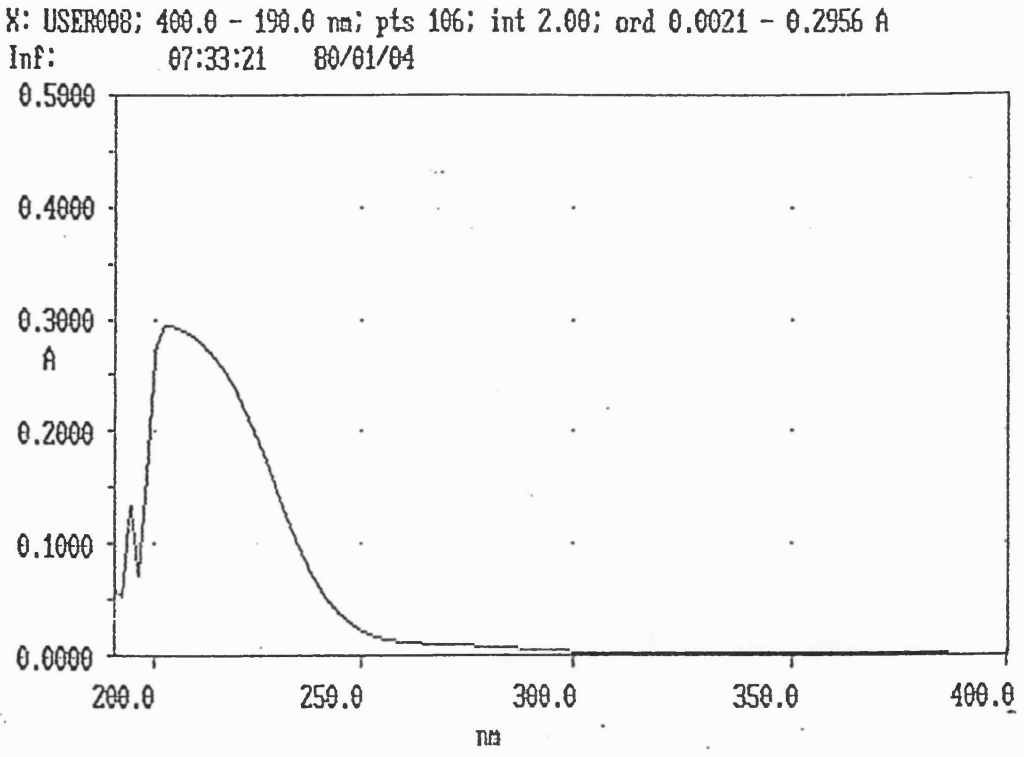
6.7) ^{1}H-^{1}H COSY spectrum in CD_3OD

6.8) ^{1}H-^{13}C one bond correlation spectrum in CD_3OD

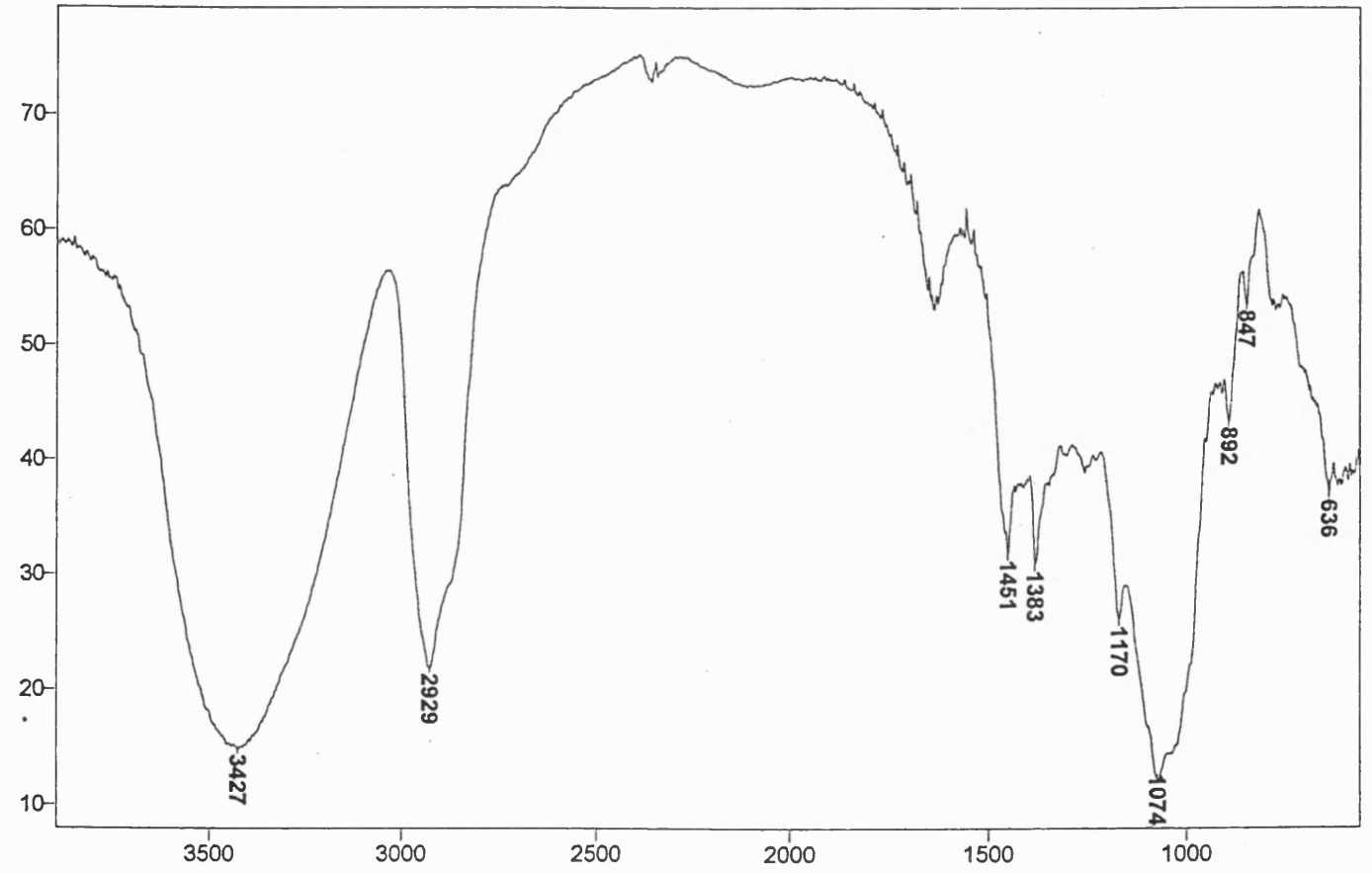
6.9) ^{1}H-^{13}C long range correlation spectrum in CD_3OD



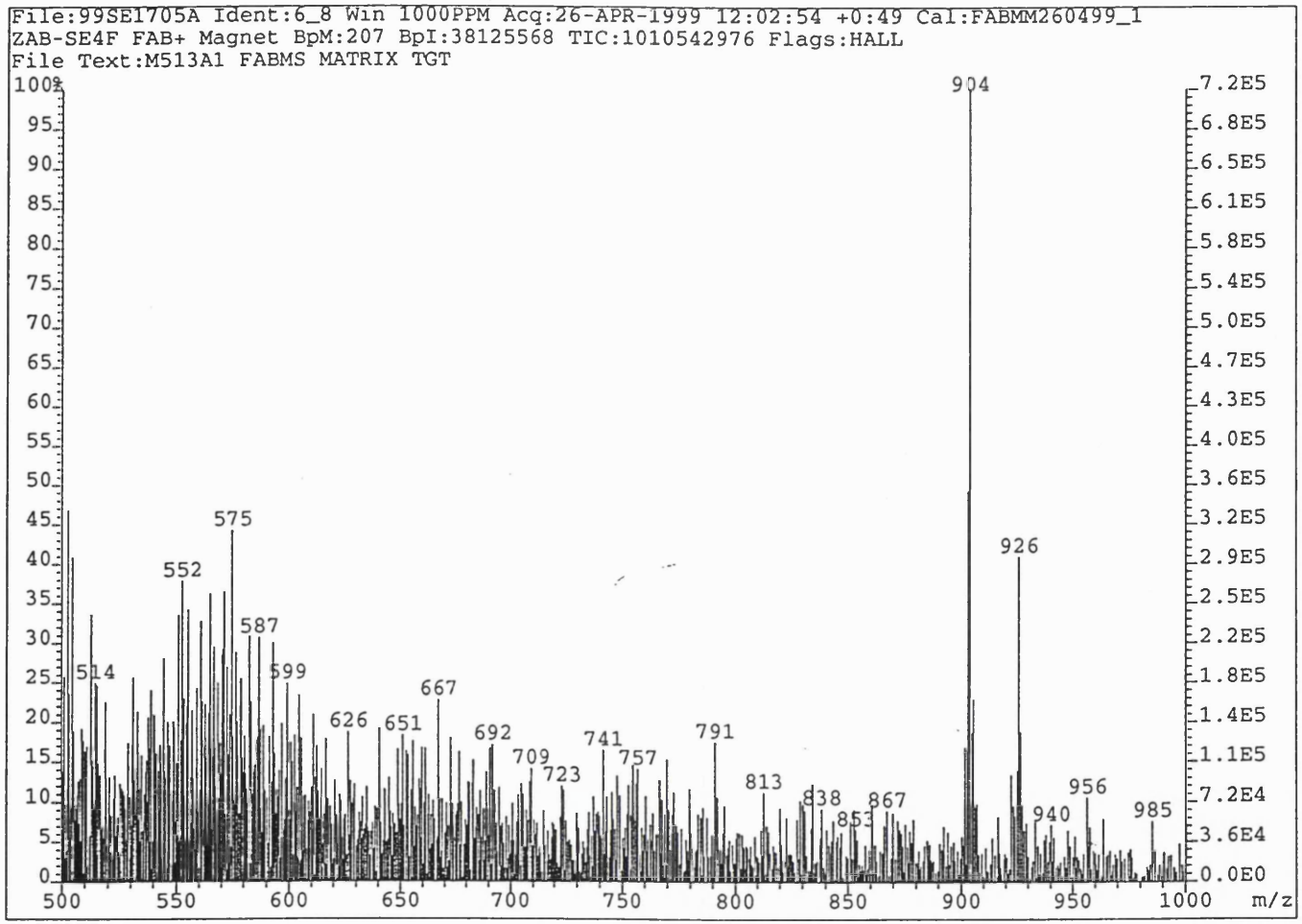
AAM513A1 (timosaponin BI)



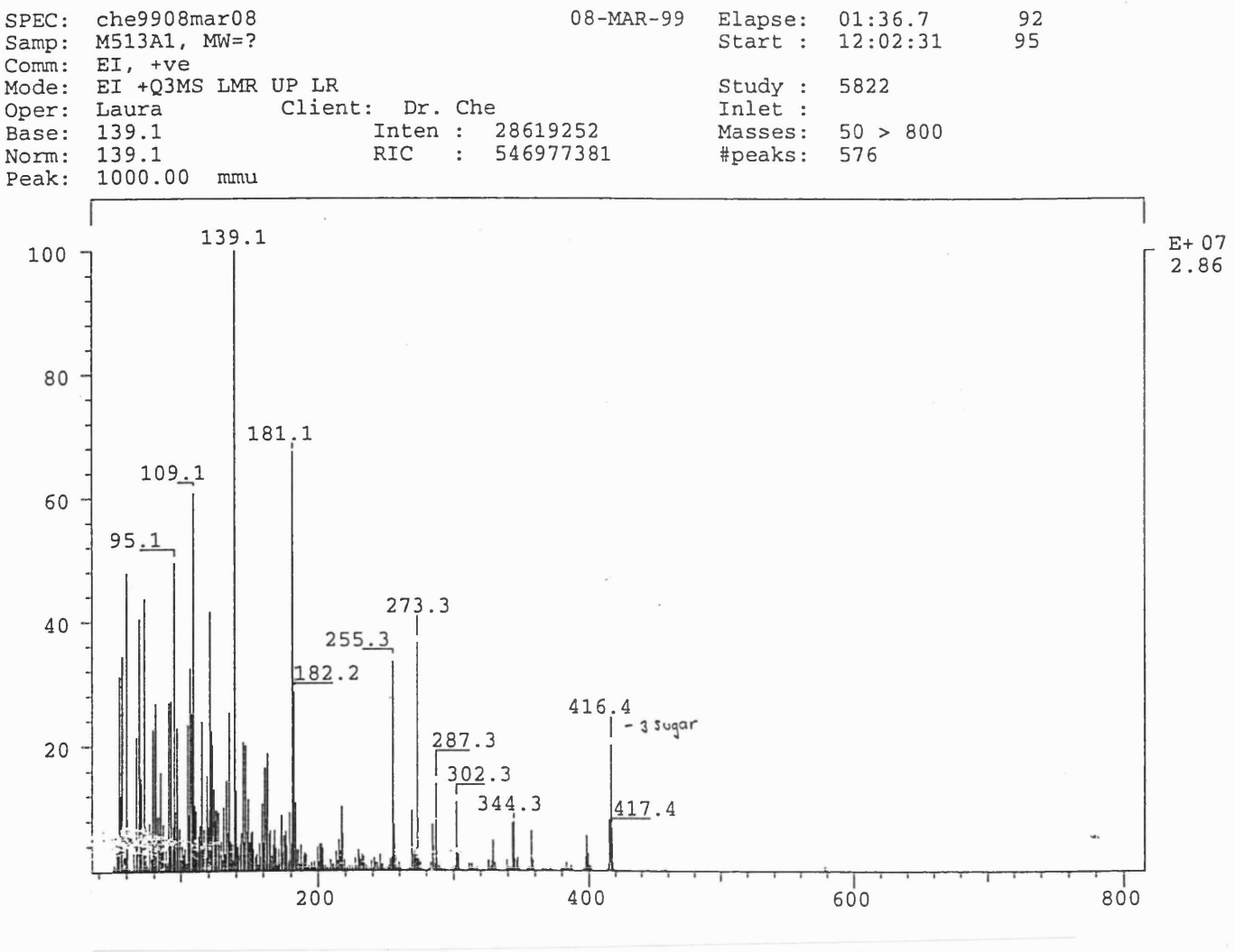
UV spectrum of AAM513A1 (timosaponin BI) in methanol



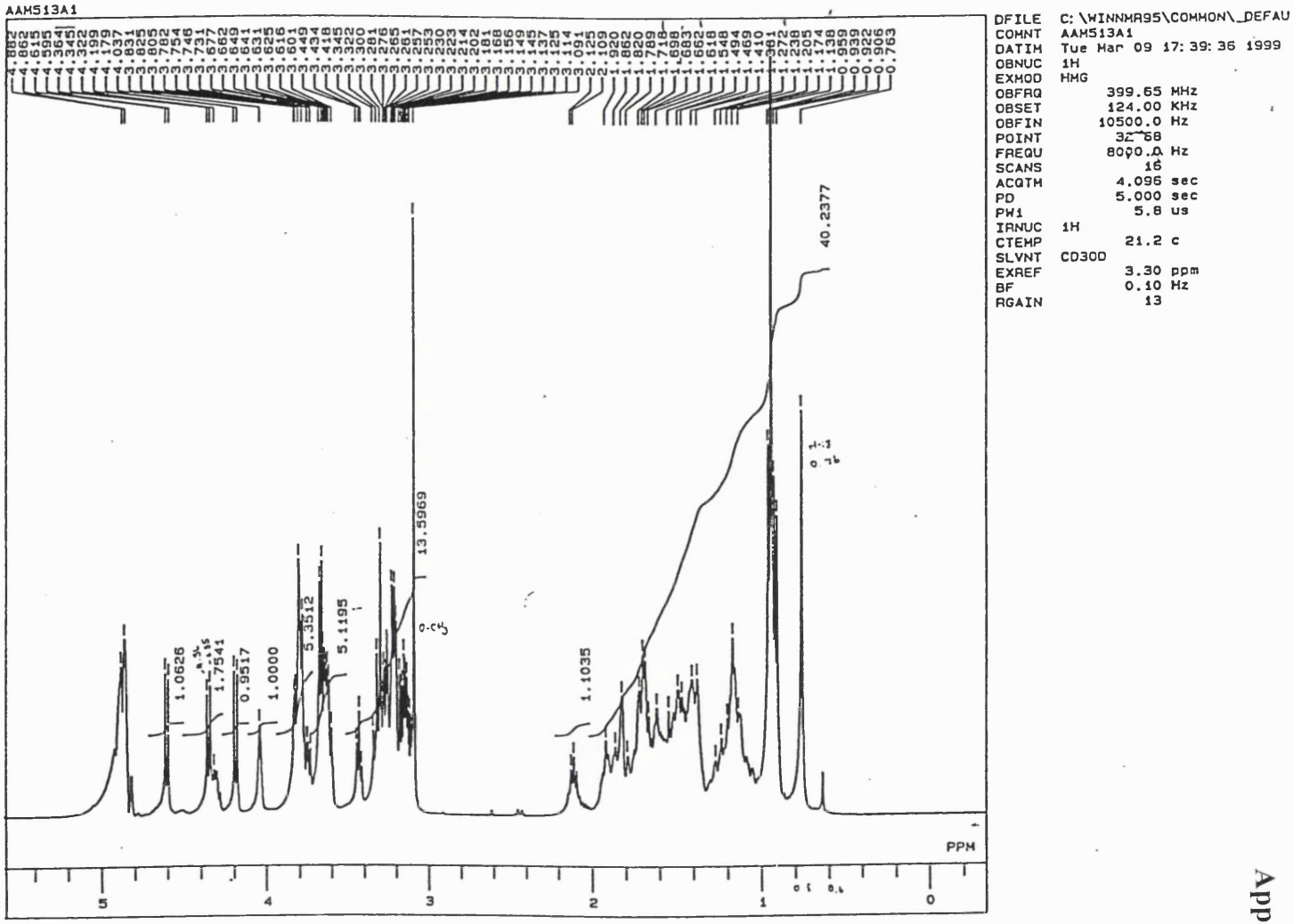
IR spectrum of AAM513A1 (timosaponin BI)



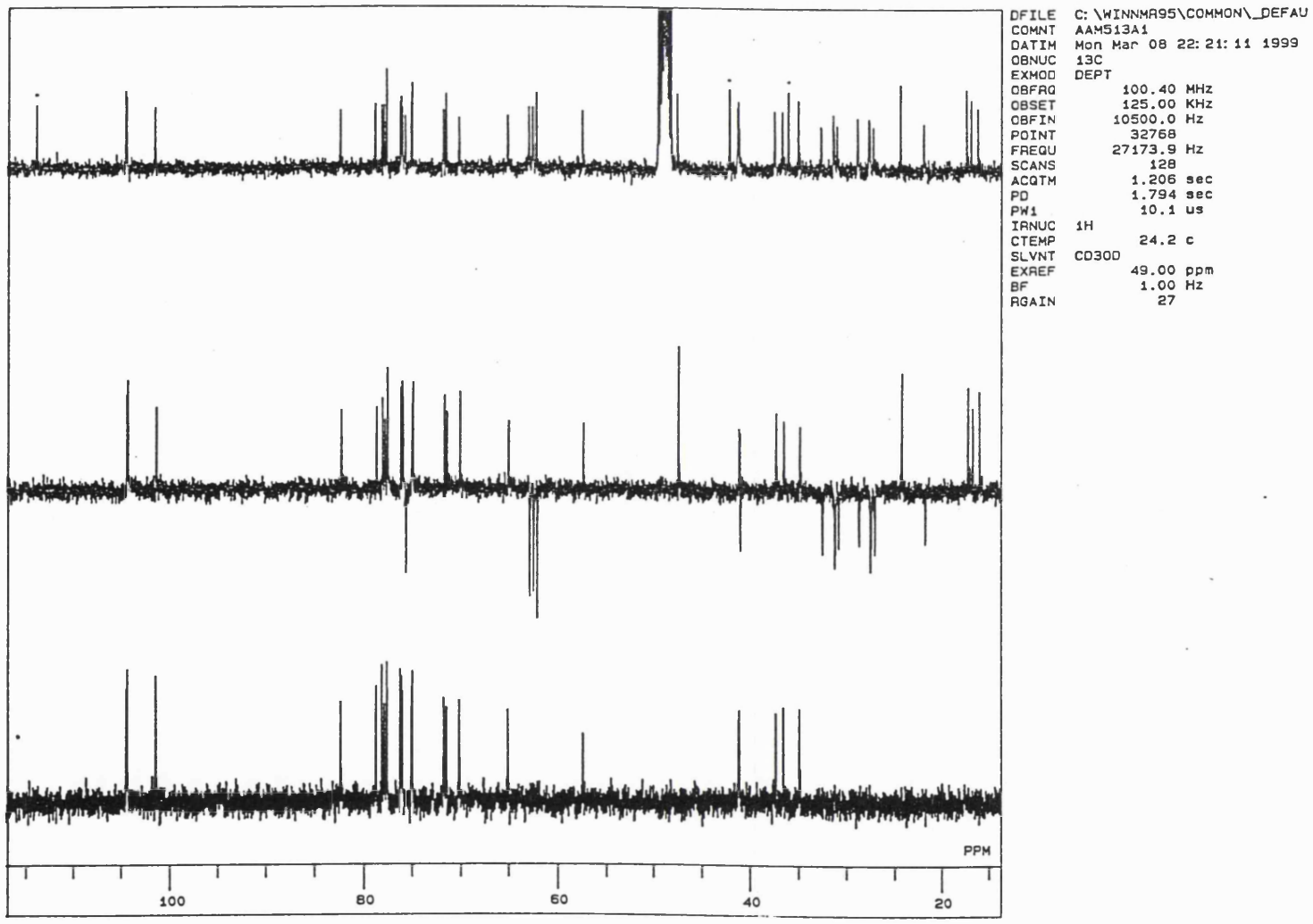
Fast atom bombardment mass spectrum of AAM513A1 (timosaponin B1)



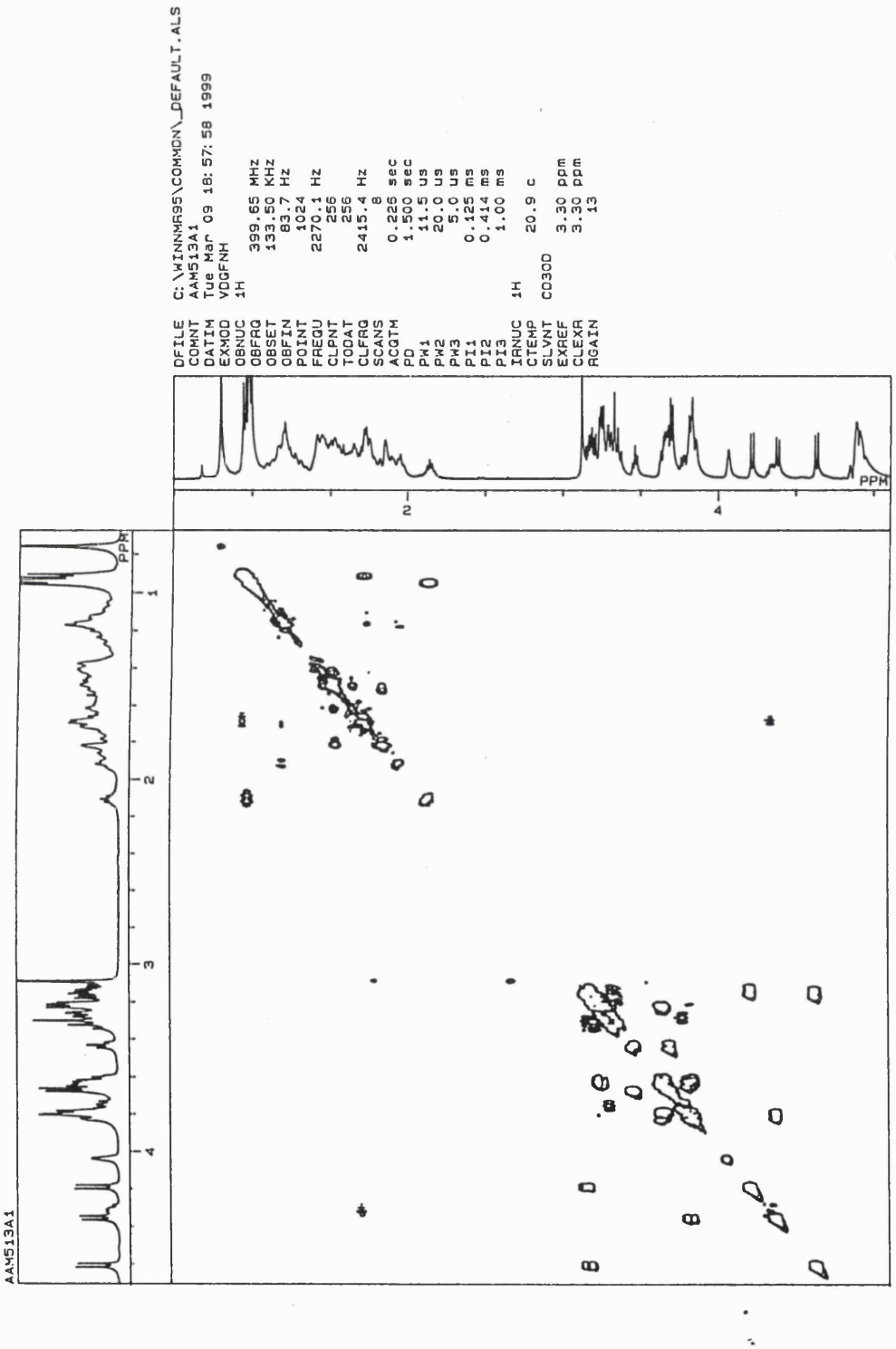
Electron bombardment mass spectrum of AAM513A1 (timosaponin BI)



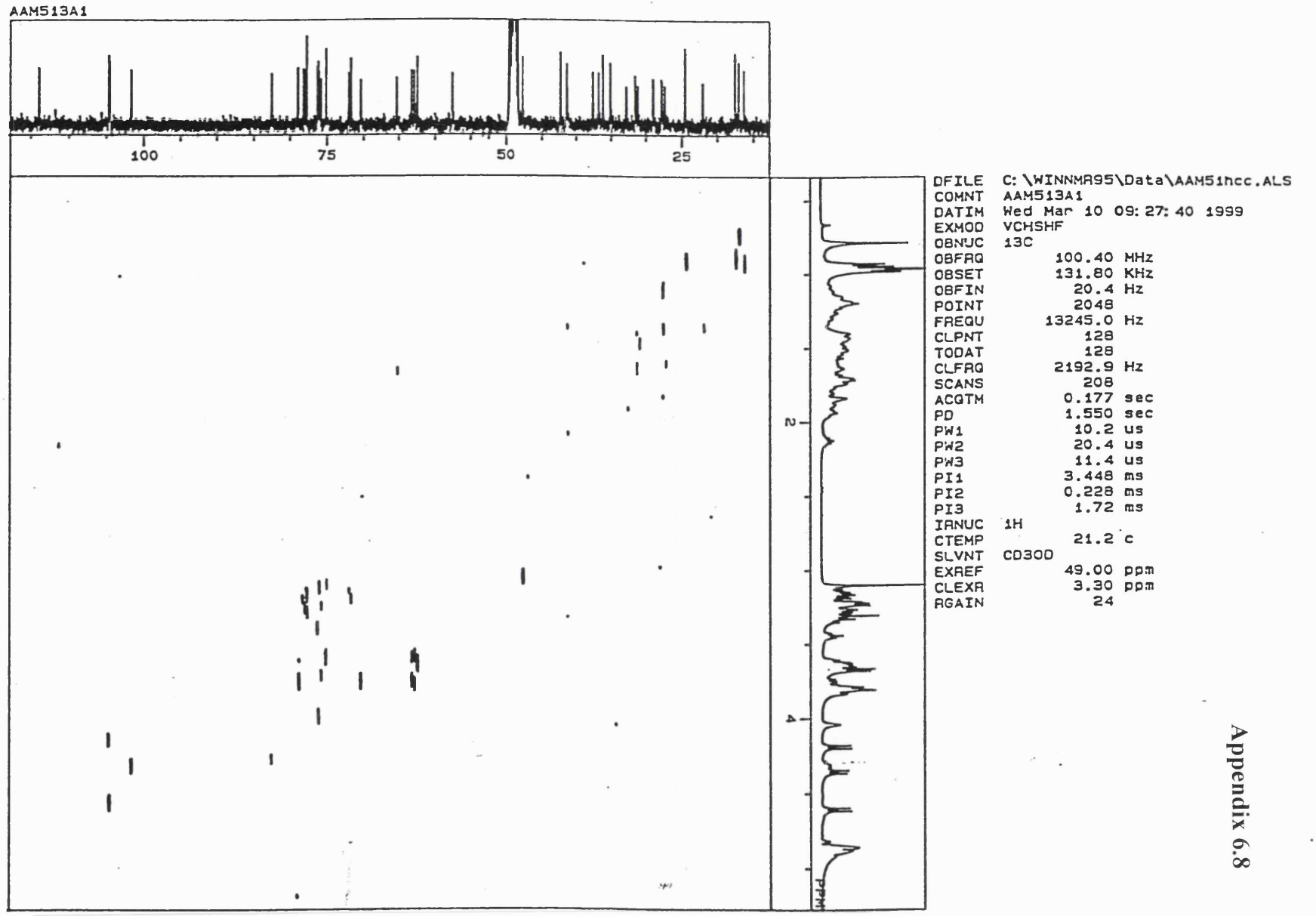
^{1}HNMR spectrum of AAM513A1 (timosaponin BI) in CD_3OD



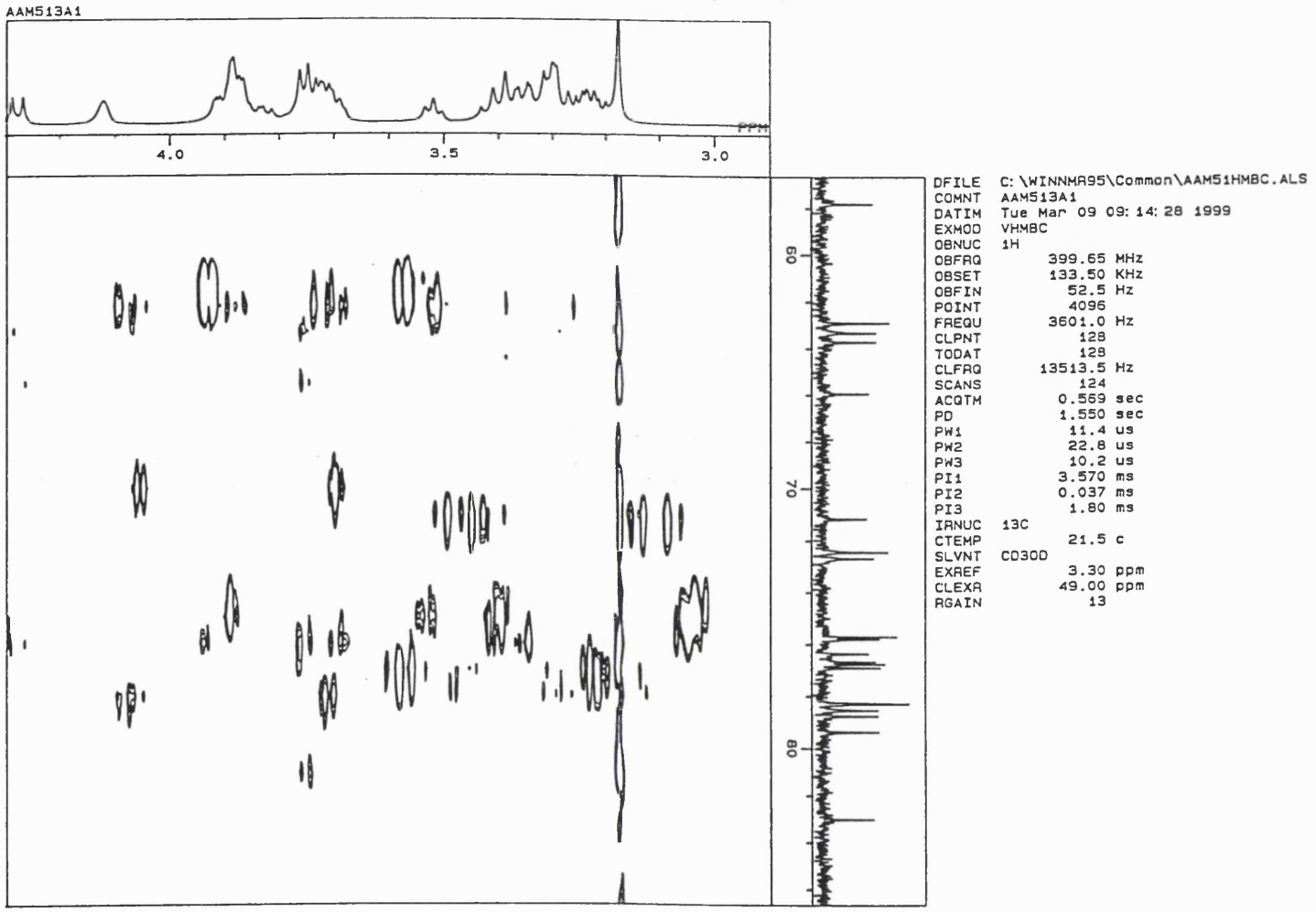
^{13}C NMR spectrum of AAM513A1 (timosaponin BI) in CD_3OD



^{1}H-^{1}H COSY spectrum of AAM513A1 (timosaponin BI) in CD_3OD



^{1}H-^{13}C one bond correlation spectrum of AAM513A1 (timosaponin BI) in CD_3OD



^{1}H-^{13}C long range correlation spectrum of AAM513A1 (timosaponin BI) in CD_3OD

Appendix 7: UV, IR, MS and NMR data for AAM73 (mangiferin)

7.1) UV spectrum in methanol

7.2) UV spectrum in methanol with the addition of $AlCl_3$ and $AlCl_3$+HCl

7.3) UV spectrum in methanol with the addition of sodium acetate

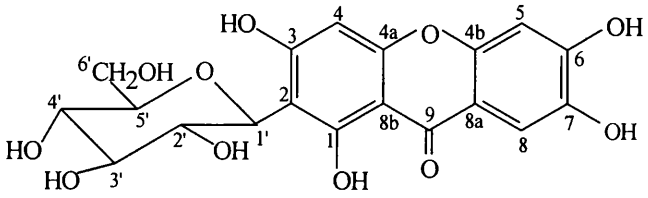
7.4) IR Spectrum

7.5) Fast atom bombardment mass spectrum

7.6) Electron impact mass spectrum

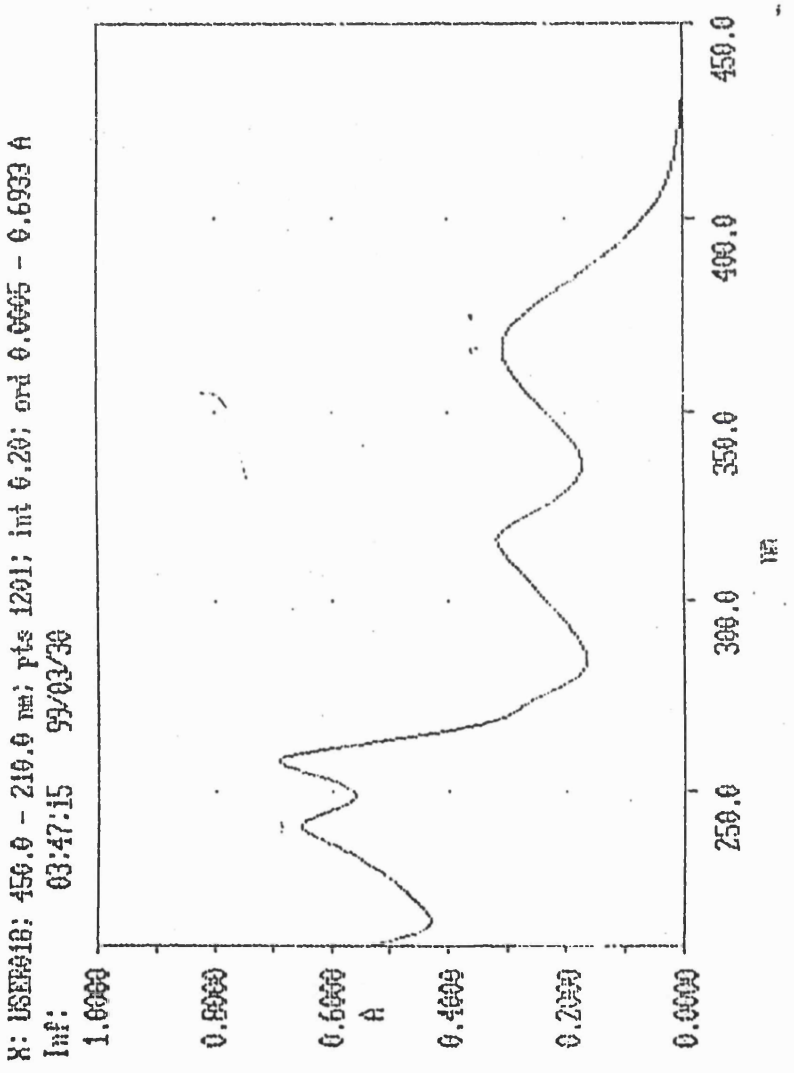
7.7) ^{1}H-400 MHz NMR spectrum in DMSO-*d6*

7.8) ^{13}C-100 MHz NMR spectrum (DEPT) in DMSO-*d6*

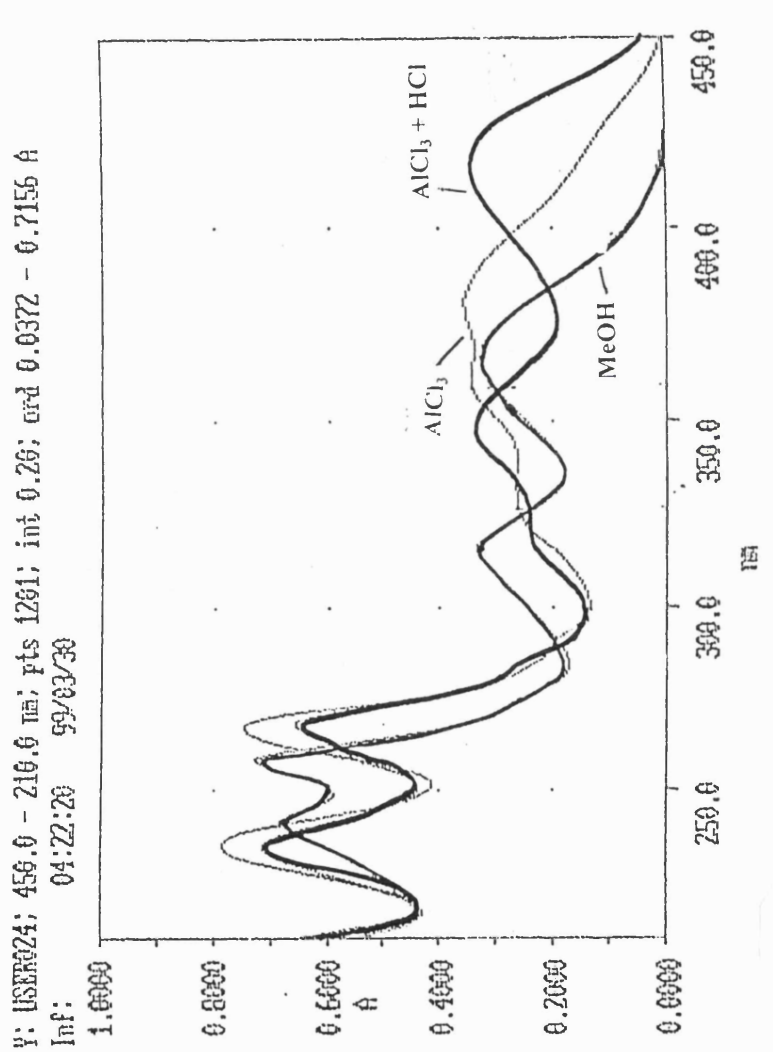


AAM73 (mangiferin)

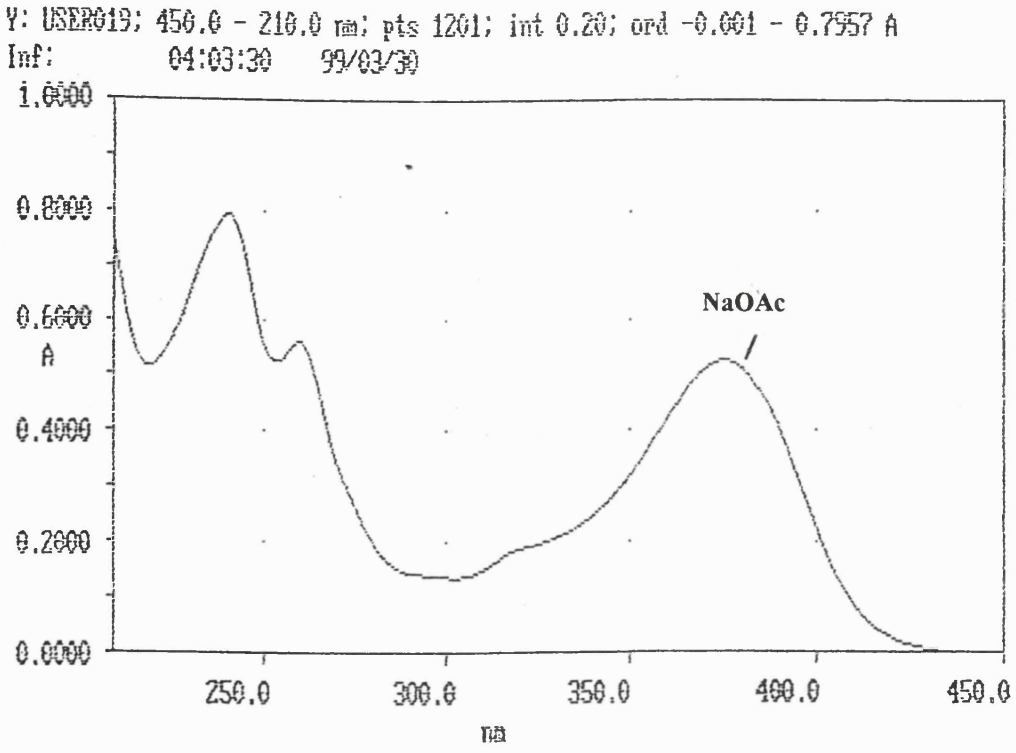
Appendix 7.1



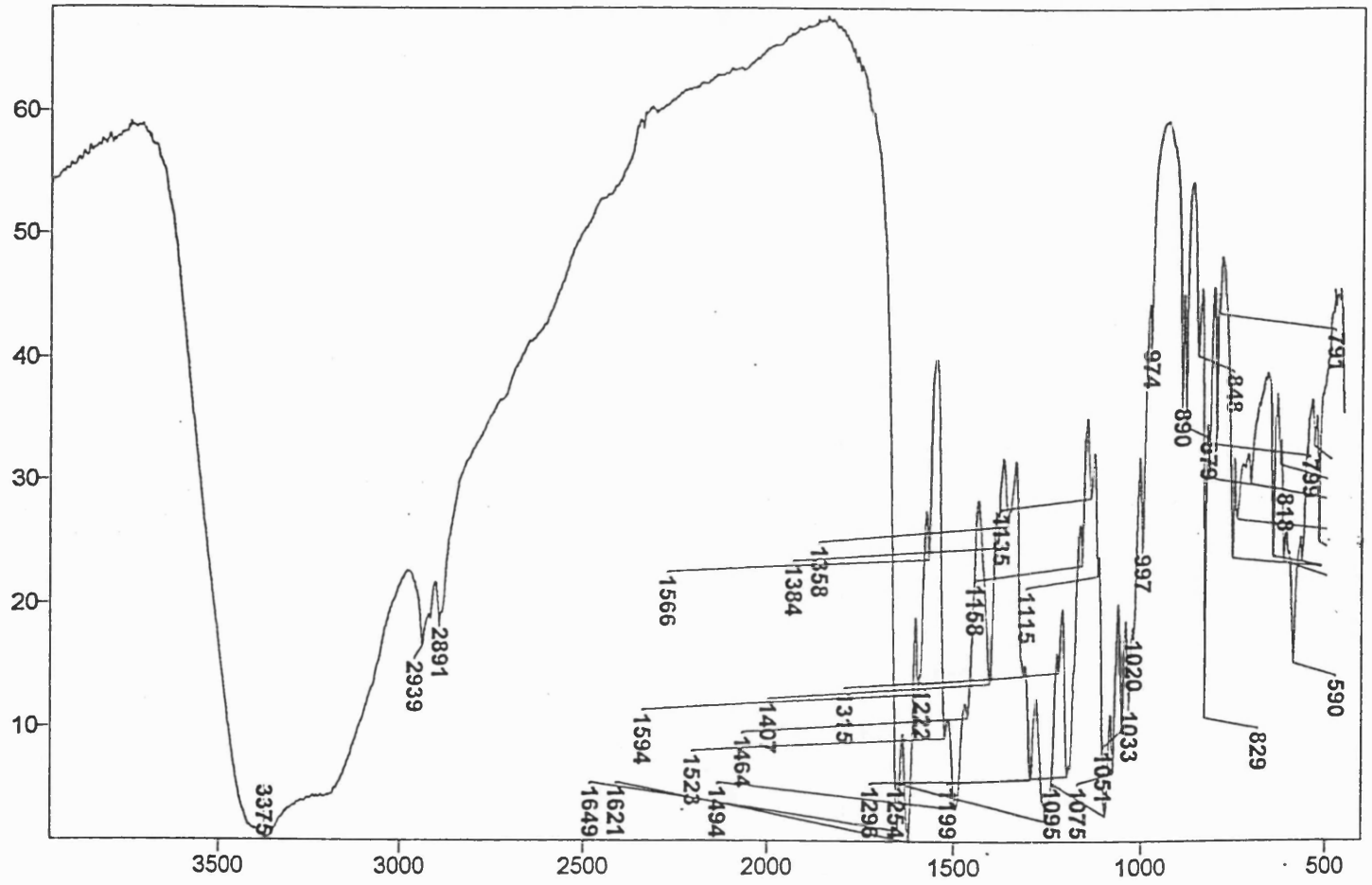
UV spectrum of AAM73 (mangiferin) in methanol



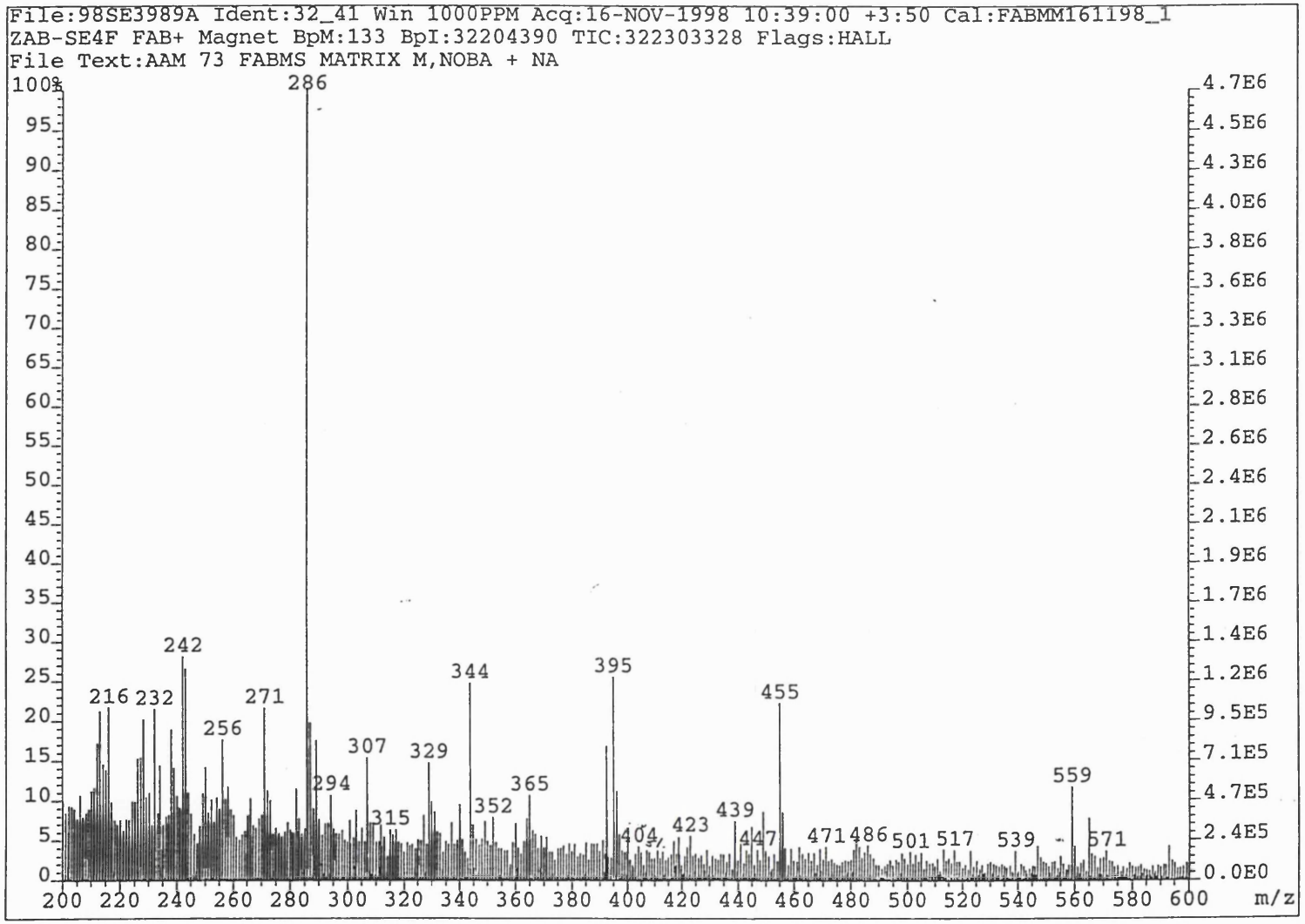
UV spectrum of AAM73 (mangiferin) in methanol with the addition of $AlCl_3$ and $AlCl_3$+HCl



UV spectrum of AAM73 (mangiferin) in methanol with the addition of sodium acetate



IR Spectrum of AAM73 (mangiferin)



Fast atom bombardment mass spectrum of AAM73 (mangiferin)

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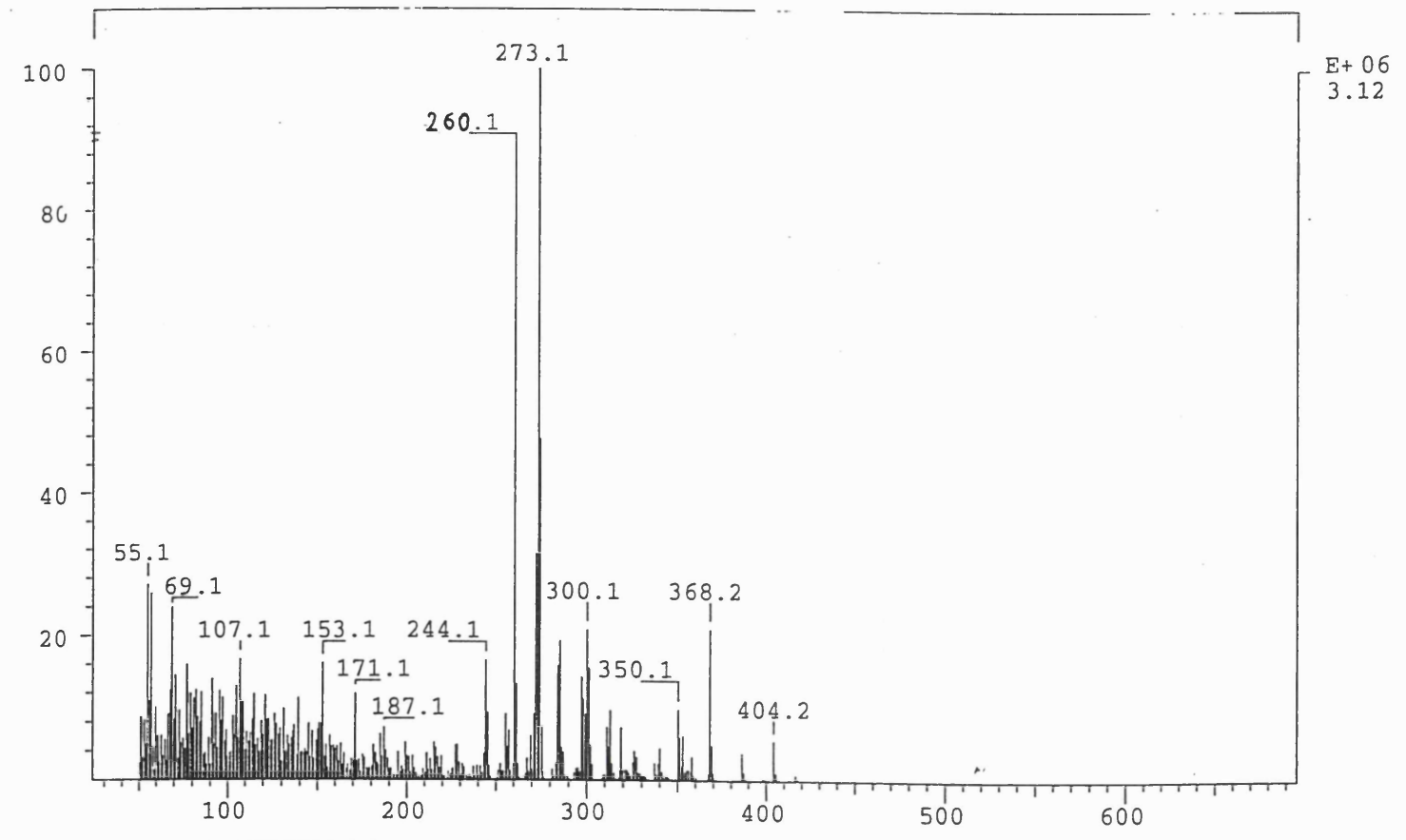
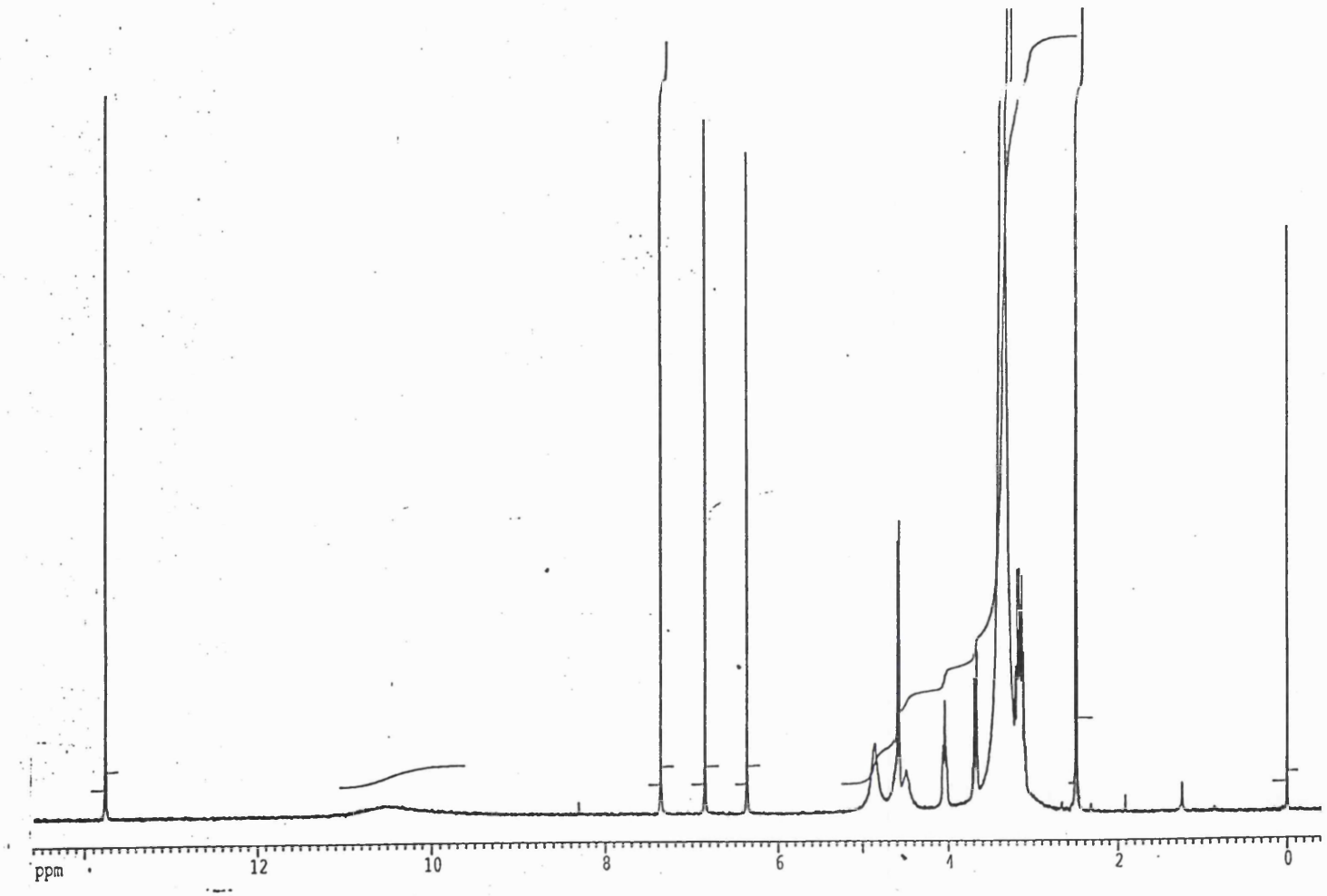
Comm: EI, +ve
Mode: EI +Q3MS LMR UP LR Study : 5819
Oper: Laura Client: Dr. Che Inlet :
Base: 273.1 Ion : 3117542 Masses: 50 > 800
Norm: 273.1 RIC : 51938522 #peaks: 437
Peak: 1000.00 mmu



Electron impact mass spectrum of AAM73 (mangiferin)



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RG	2048
DW	60.000 usec
DE	75.00 usec
TE	300.0 K
HL1	2 dB
D1	1.00000000 sec
P1	9.50 usec
DE	75.00 usec
SFO1	400.1387946 MHz
NUCLEUS	1H

F2 - Processing parameters

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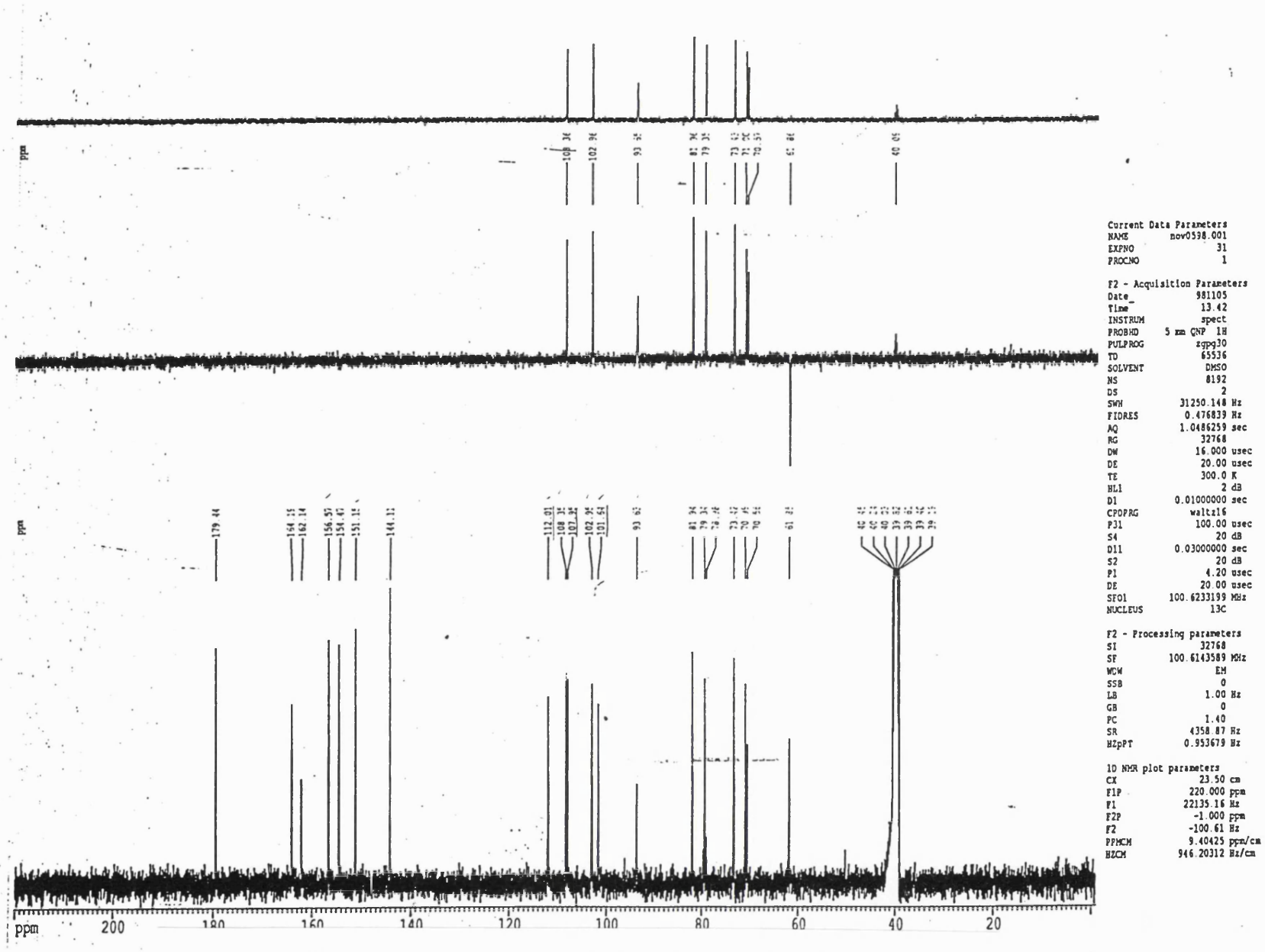
SSB	0
LB	0.30 Hz
GB	0
PC	1.00
SR	6287.14 Hz
HZPPT	0.508629 Hz

1D NMR plot parameters

CX	23.50 cm
F1P	14.600 ppm
F1	5841.99 Hz
F2P	-0.400 ppm
F2	-160.05 Hz
PPMCM	0.63830 ppm/cm
HZCM	255.40613 Hz/cm

^{1}H NMR spectrum of AAM73 (mangiferin) in DMSO-$_{d6}$

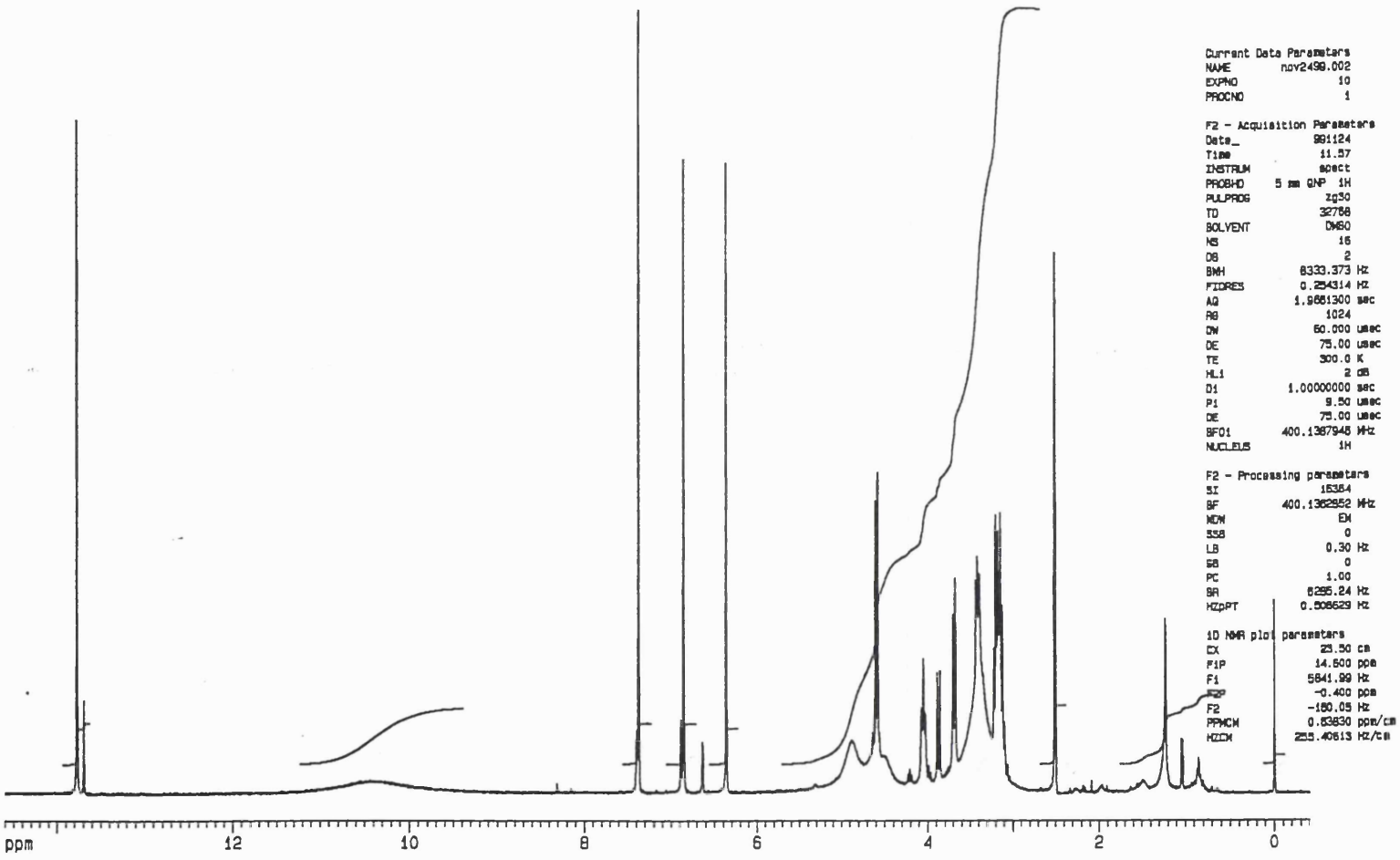


^{13}C NMR spectrum of AAM73 (mangiferin) in DMSO-*d6*

Appendix 8: NMR data for reference mangiferin (Alpin Ltd.)

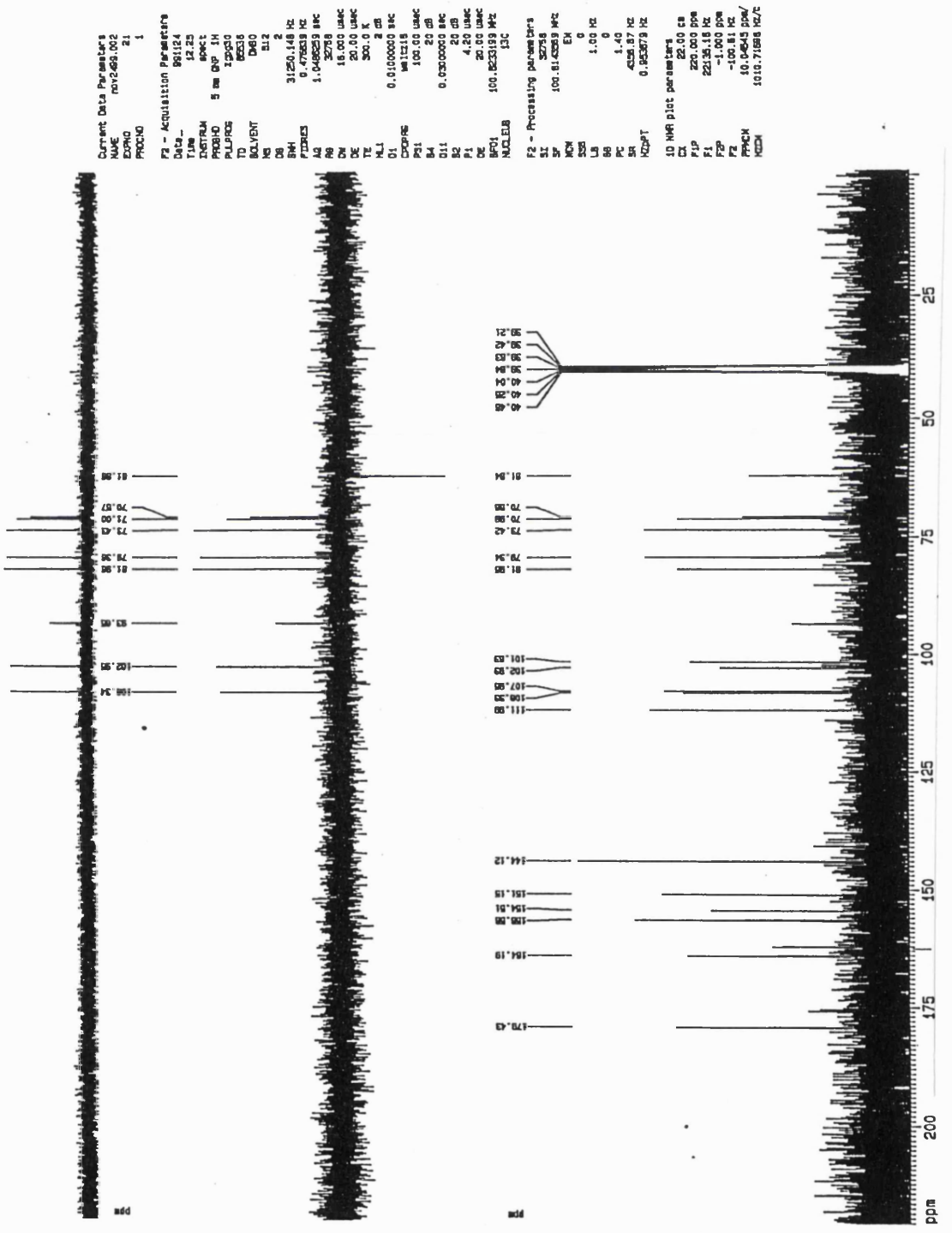
8.1) ^{1}H-400 MHz NMR spectrum in DMSO-d_6

8.2) ^{13}C-100 MHz NMR spectrum in DMSO-d_6



H- NMR spectrum of mangiferin in DMSO-d_6

Appendix 8.2



^{13}C- NMR spectrum of mangiferin in DMSO-d_6

Appendix 9: UV, IR, MS and NMR data for AAM634 (mangiferin glucoside)

9.1) UV spectrum in methanol

9.2) UV spectrum in methanol with the addition of $AlCl_3$ and $AlCl_3$+HCl

9.3) UV spectrum in methanol with the addition of sodium acetate

9.4) IR Spectrum

9.5) Fast atom bombardment mass spectrum

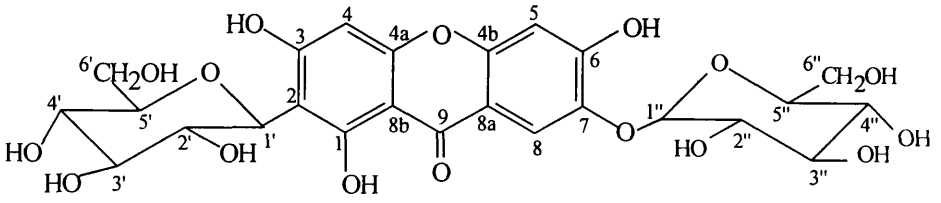
9.6) ^{1}H-400 MHz NMR spectrum in DMSO-d_6

9.7) ^{13}C-100 MHz NMR spectrum (DEPT) in DMSO-d_6

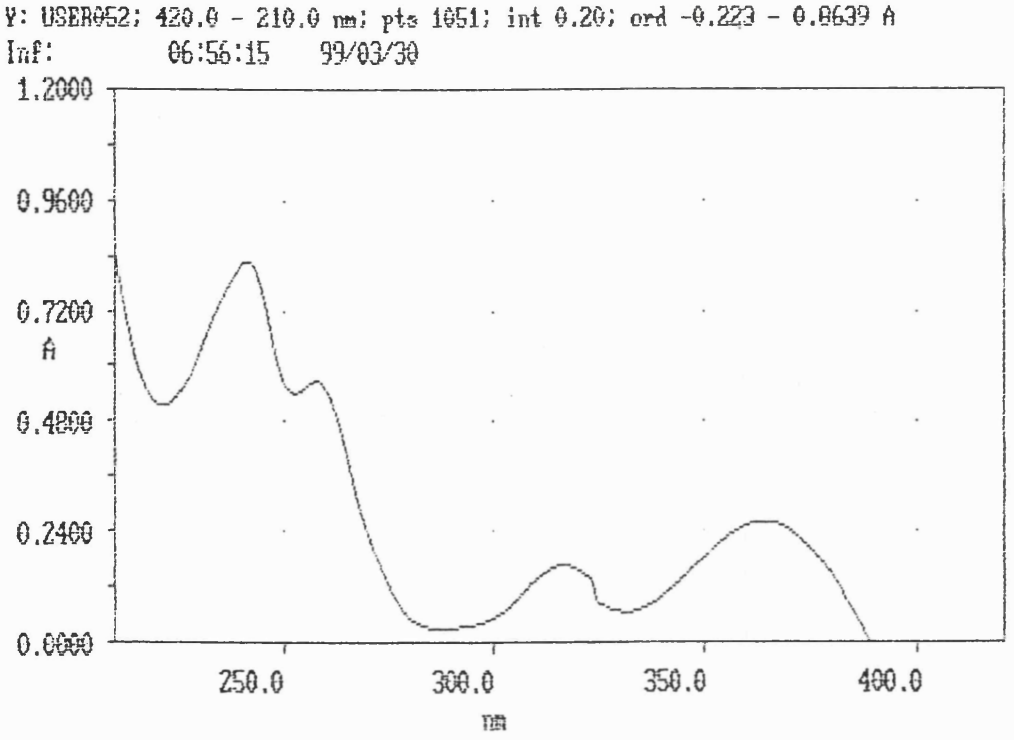
9.8) ^{1}H-^{1}H COSY spectrum in DMSO-d_6

9.9) ^{1}H-^{13}C one bond correlation spectrum in DMSO-d_6

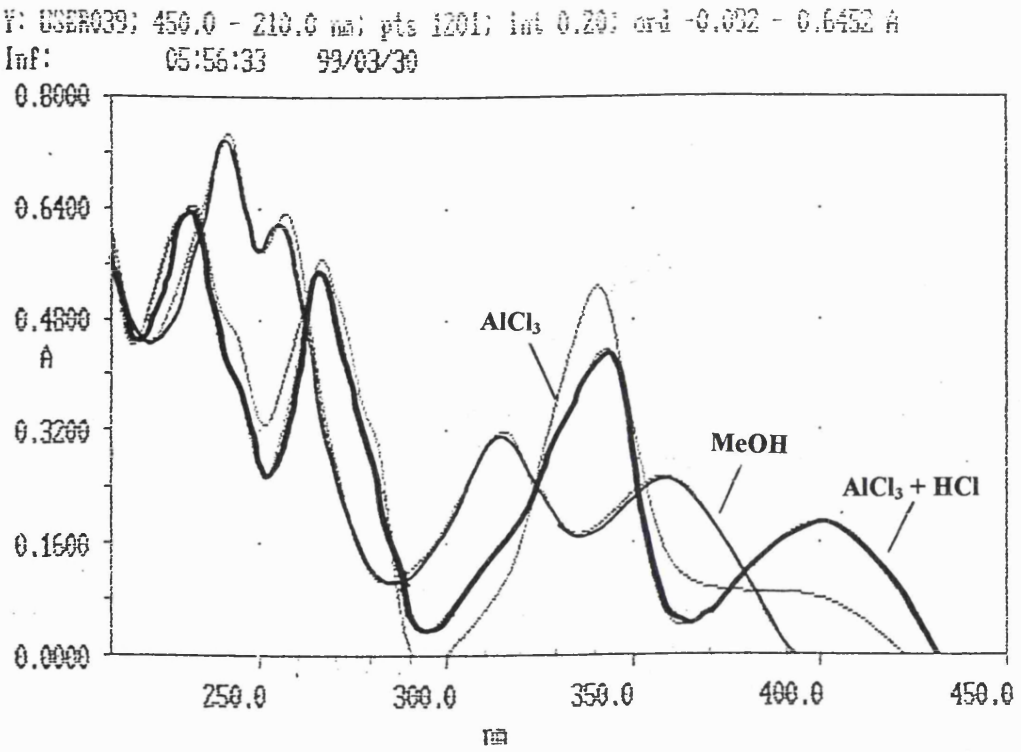
9.10) ^{1}H-^{13}C long range correlation spectrum in DMSO-d_6



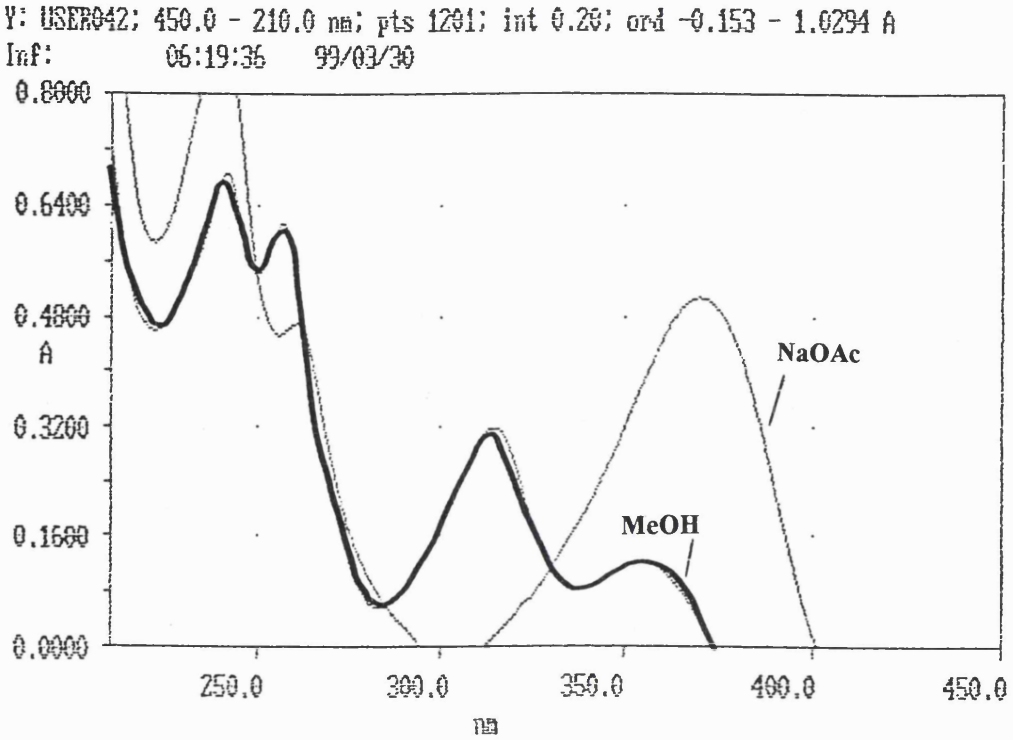
AAM634 (mangiferin 7-*O*-β-D-glucopyranoside)



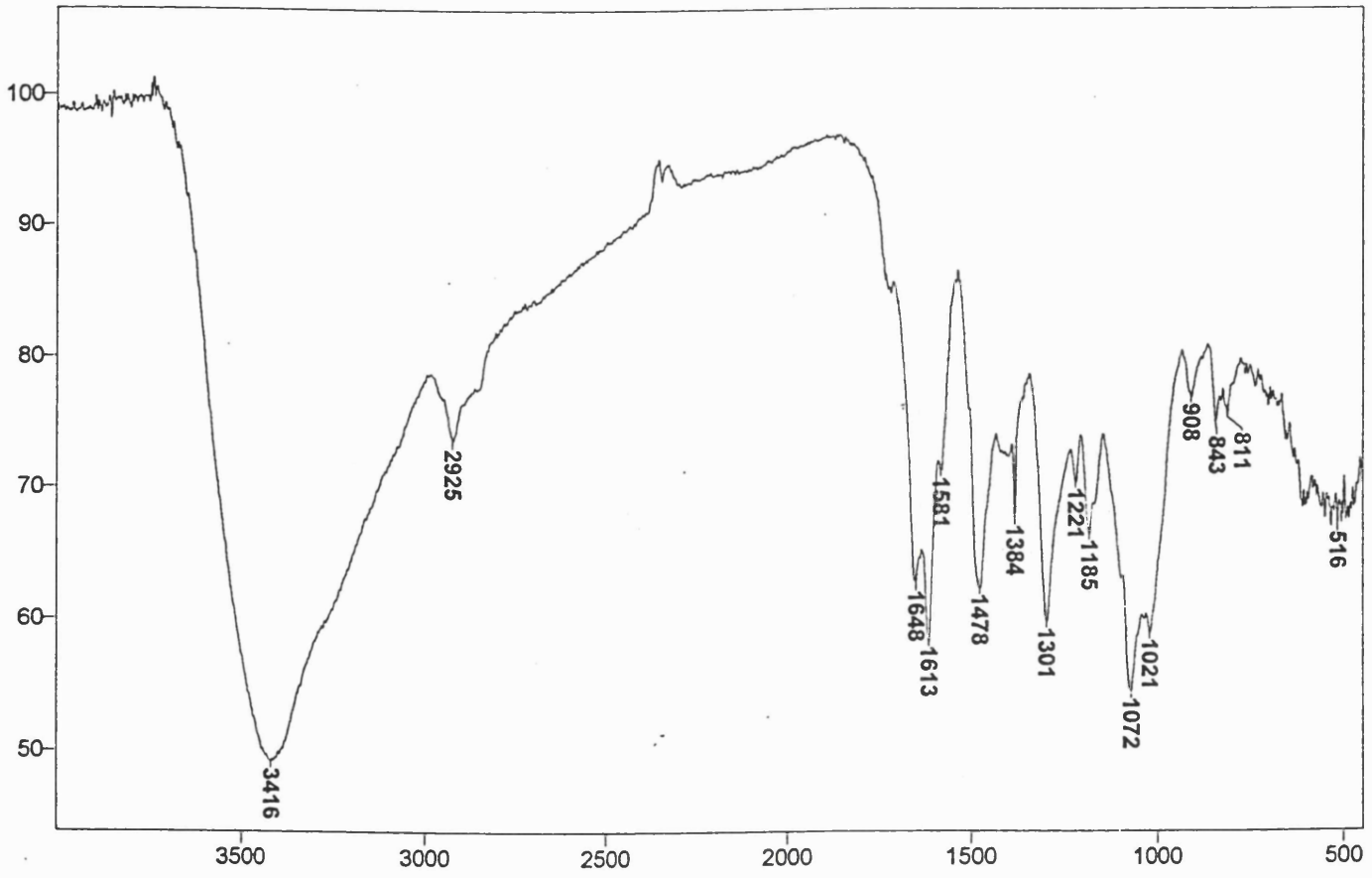
UV spectrum of AAM634 (mangiferin glucoside) in methanol



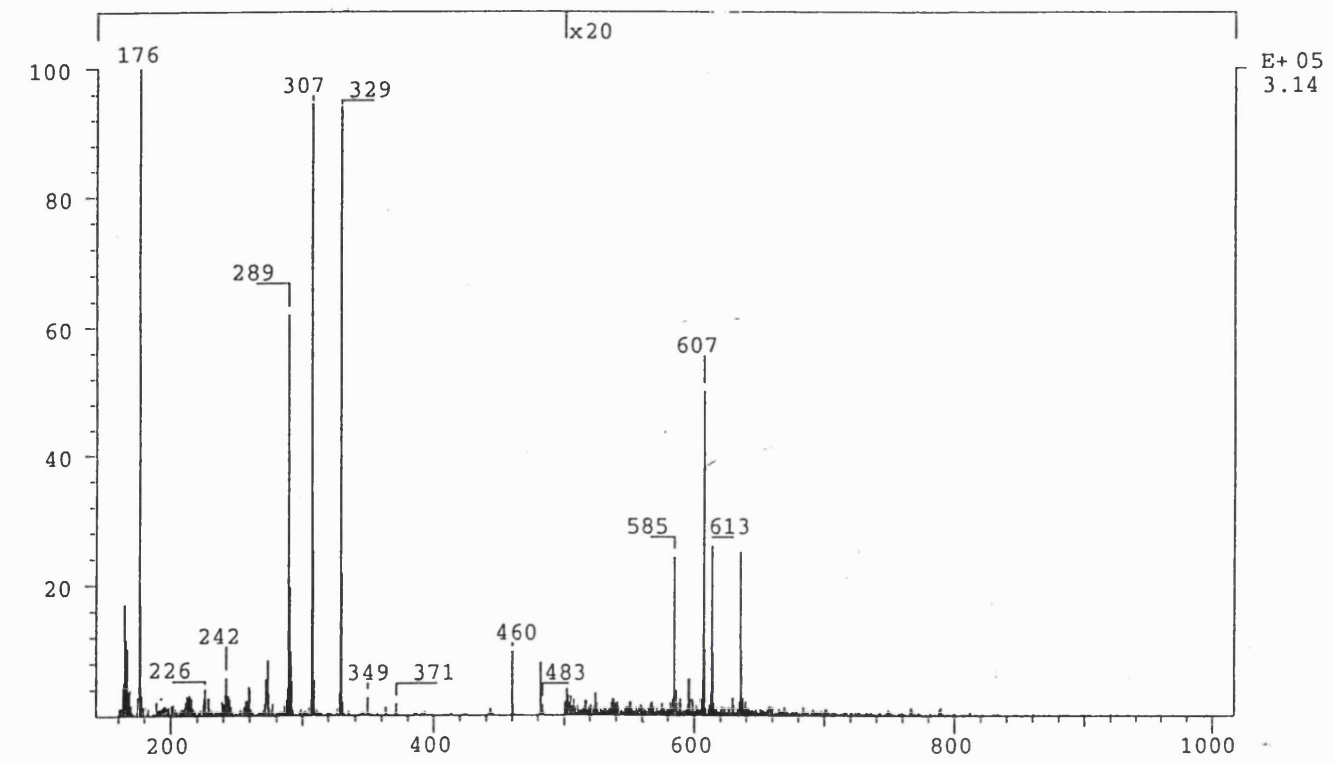
UV spectrum of AAM634 (mangiferin glucoside) in methanol with the addition of $AlCl_3$ and $AlCl_3$+HCl



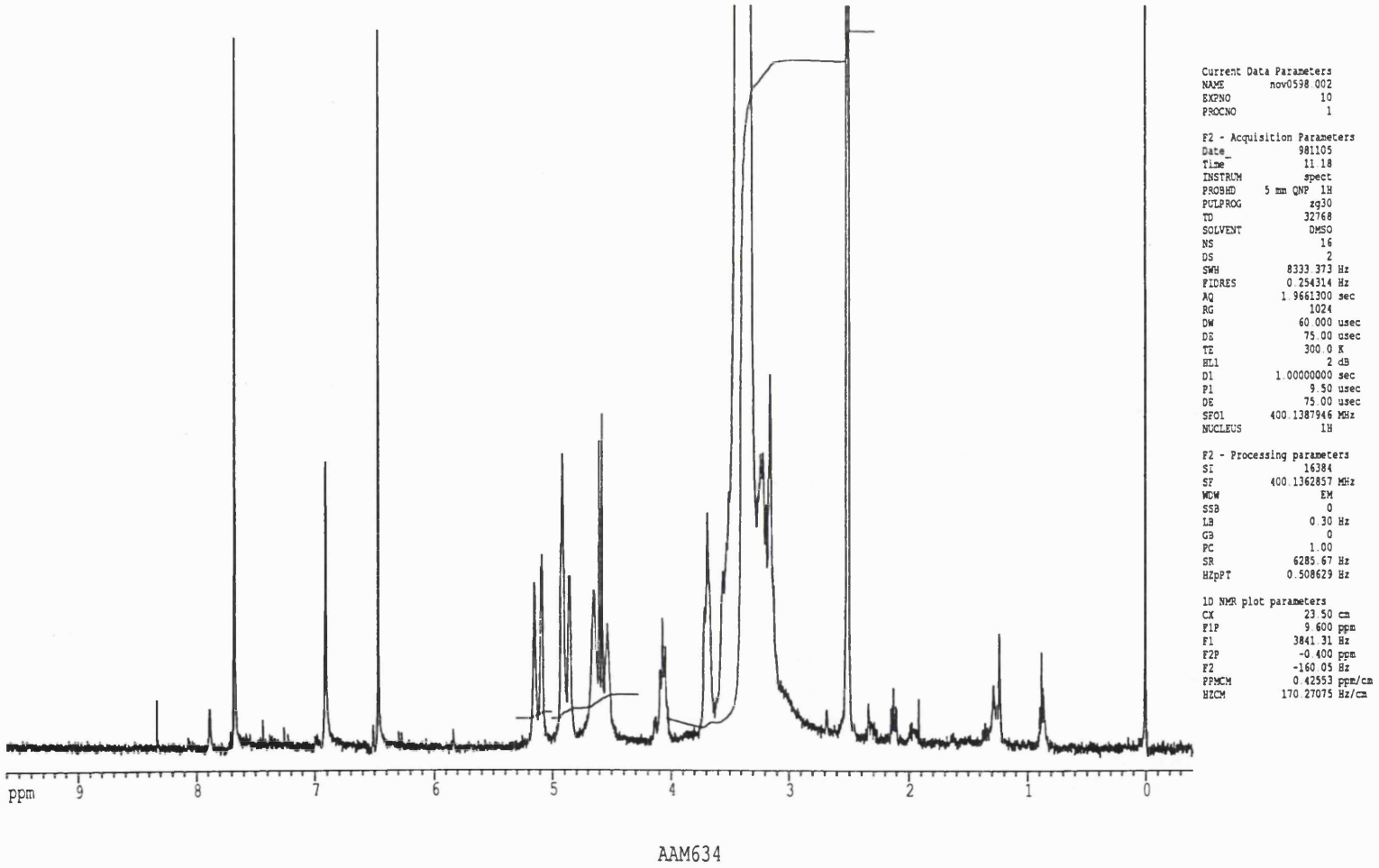
UV spectrum of AAM634 (mangiferin glucoside) in methanol with the addition of sodium acetate



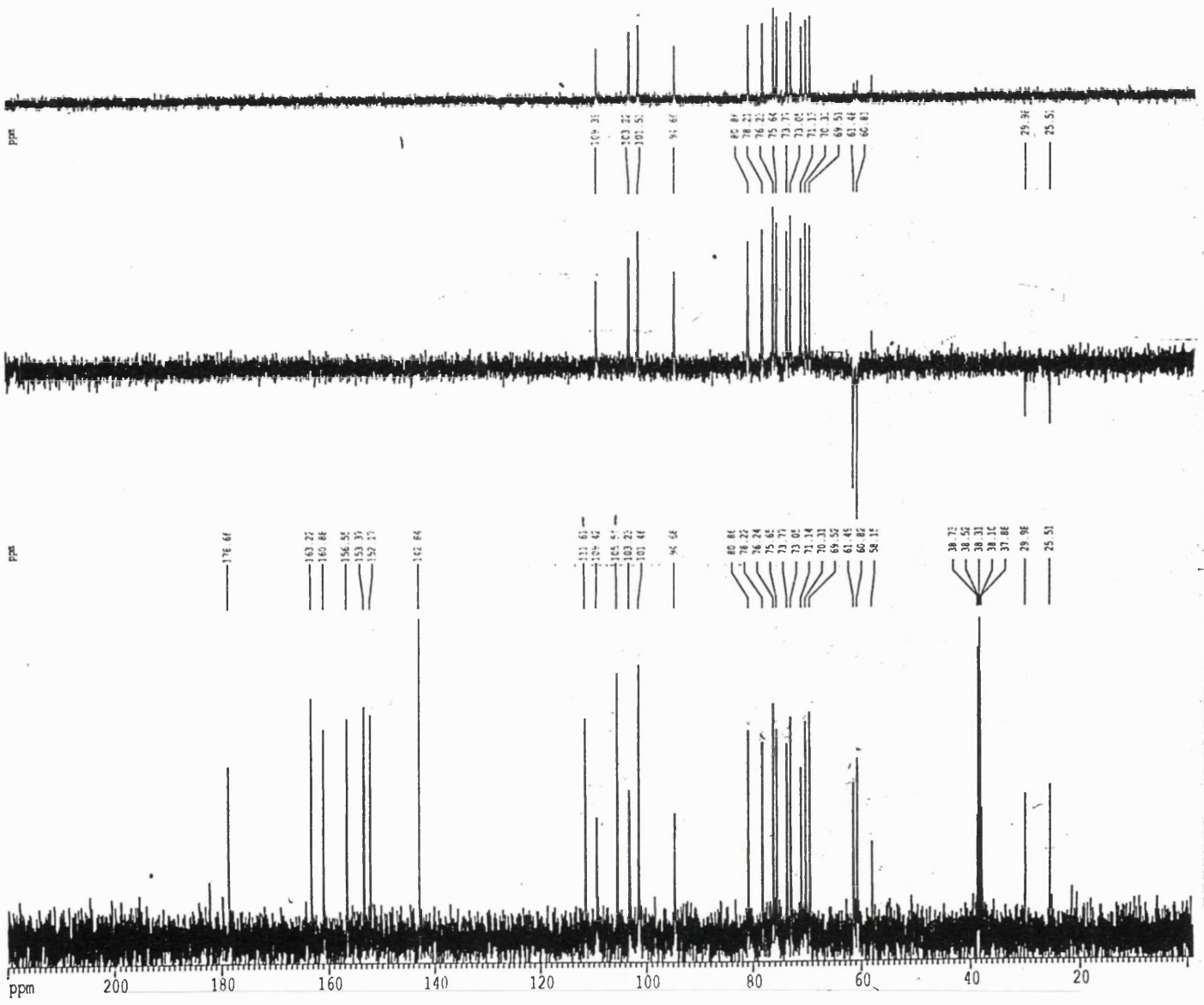
IR Spectrum of AAM634 (mangiferin glucoside)



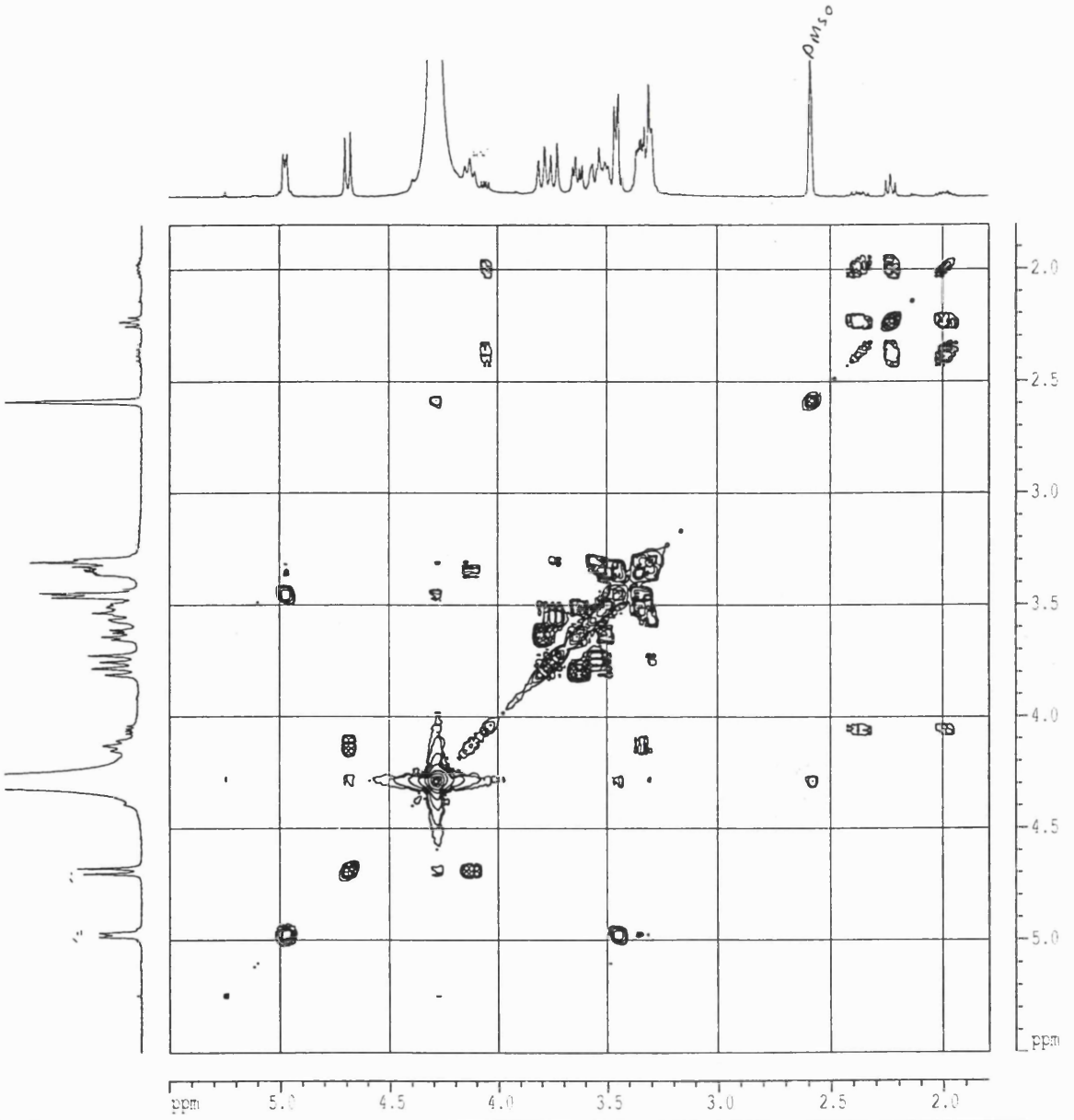
Fast atom bombardment mass spectrum of AAM634 (mangiferin glucoside)



^{1}H NMR spectrum of AAM634 (mangiferin glucoside) in DMSO-d_6



^{13}C NMR spectrum of AAM634 (mangiferin glucoside) in DMSO-d_6



AAM 6 COSY 45

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EXPNO 30
PROCNO 1

F2 - Acquisition Parameters
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Time 12.37
INSTRUM spect
PROBHD 5 mm QNP 1H
PULPROG cosy45
TD 2048
SOLVENT DMSO
NS 8
DS 4
SWH 3311.258 Hz
FIDRES 1.616825 Hz
AQ 0.3092980 sec
RG 1024
DW 151.000 usec
DE 172.86 usec
TE 300.0 K
HL1 2 dB
D1 0.93855828 sec
P1 9.50 usec
D0 0.00000300 sec
DE 172.86 usec
SFO1 400.1379671 MHz
NUCLEUS 1H
IN0 0.00030200 sec

F1 - Acquisition parameters
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TD 256
SFO1 400.138 MHz
FIDRES 12.934603 Hz
SW 8.275 ppm

F2 - Processing parameters
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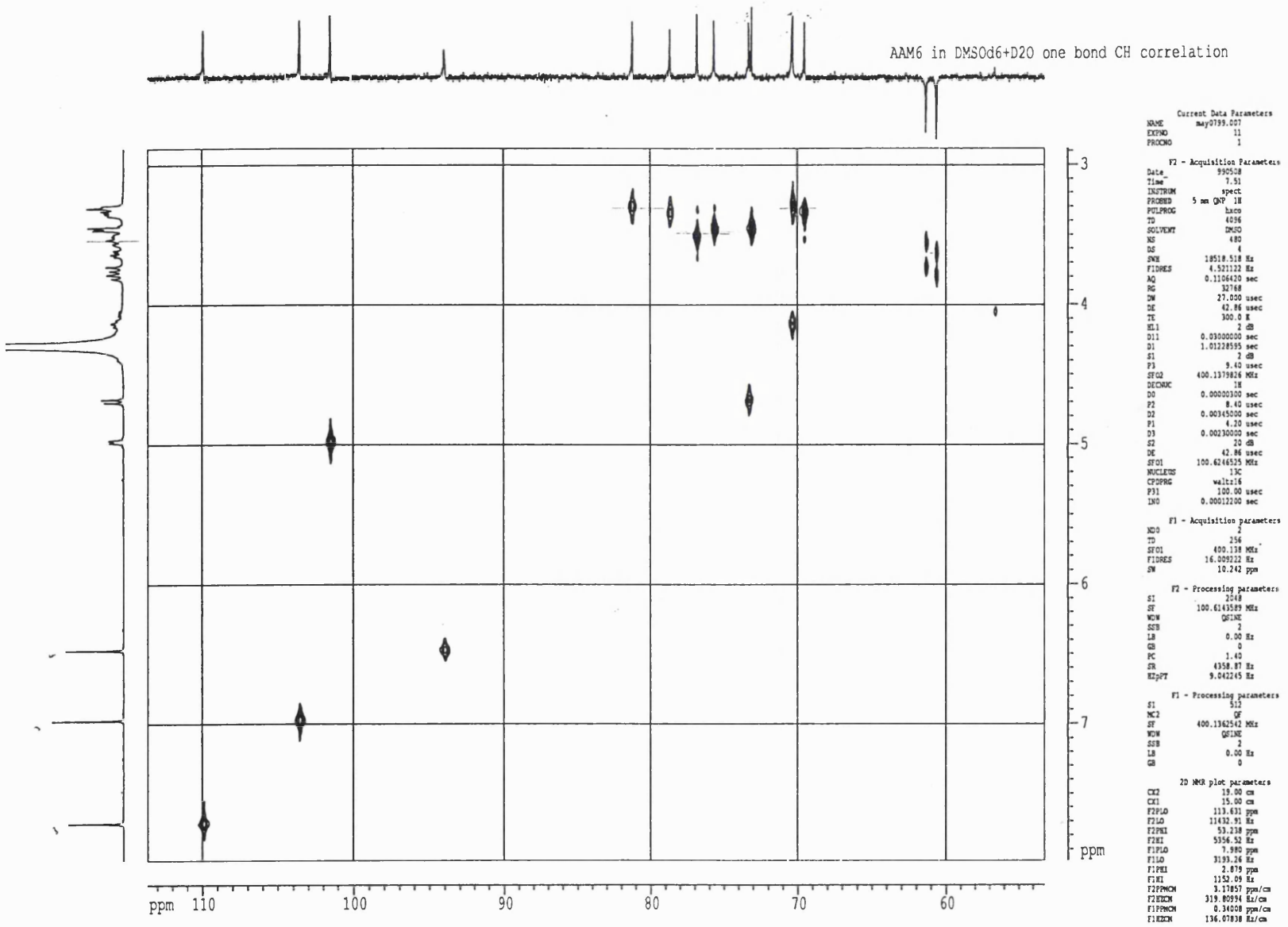
SF 400.1362551 MHz
WDW SINE
SSB 0
LB 0.00 Hz
GB 0
PC 1.40
SR 6255.13 Hz
HZpPT 3.233651 Hz

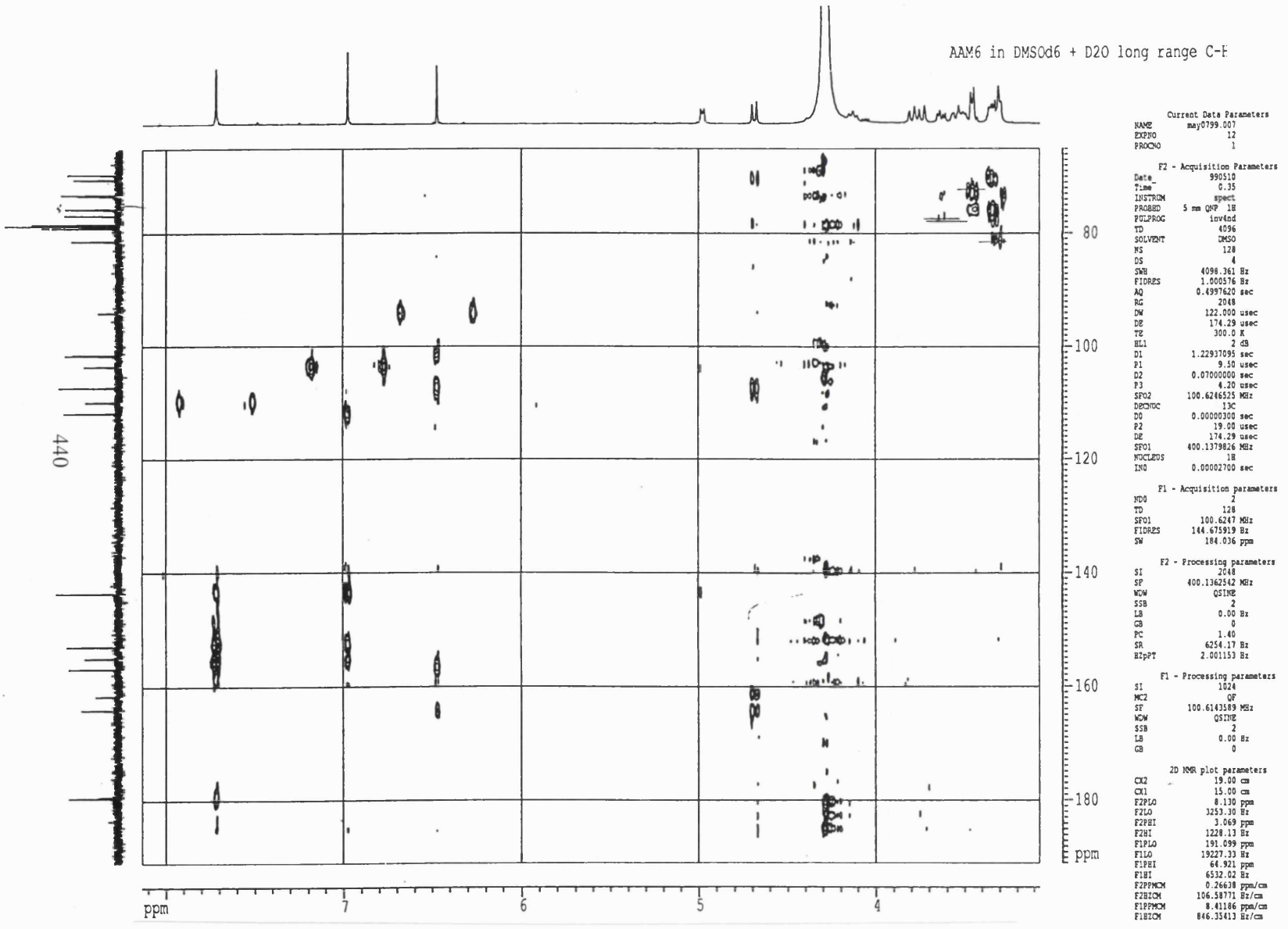
F1 - Processing parameters
SI 1024
MC2 QF
SF 400.1362551 MHz
WDW SINE
SSB 0
LB 0.00 Hz
GB 0

2D NMR plot parameters
CX2 15.00 cm
CX1 15.00 cm
F2PLO 5.500 ppm
F2LO 2200.75 Hz
F2PHI 1.800 ppm
F2HI 720.25 Hz
F1PLO 5.500 ppm
F1LO 2200.75 Hz
F1PHI 1.800 ppm
F1HI 720.25 Hz
F2PPMCM 0.24667 ppm/cm
F2HZCM 98.70027 Hz/cm
F1PPMCM 0.24667 ppm/cm
F1HZCM 98.70027 Hz/cm

^{1}H-^{1}H COSY spectrum of AAM634 (mangiferin glucoside) in DMSO-d_6



^{1}H-^{13}C one bond correlation spectrum of AAM634 (mangiferin glucoside) in DMSO-d_6



^{1}H-^{13}C long range correlation spectrum of AAM634 (mangiferin glucoside) in DMSO-d_6