Oral hypoglycaemic activity of *Ipomoea aquatica* Forsk. and its active constituents

By

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PhD 2002
DECLARATION BY CANDIDATE

The work described in this thesis, was carried out by me; under the supervision of Prof. E. R. Jansz and Prof. (Mrs.) S.M.D.N. Wickramasinghe (Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura) and a report on this has not been submitted in whole or in part to any University for another Degree/Diploma.

30.12.2002

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DECLARATION BY SUPERVISORS

We certify that the above statement made by the candidate is true and that this thesis is suitable for submission to the University for the purpose of evaluation.

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Oral hypoglycaemic activity of *Ipomoea aquatica* Forsk.

and its active constituents

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Thesis submitted to the University of Sri Jayewardenepura for the award of the Degree of Doctor of Philosophy in Biochemistry on 'Oral hypoglycaemic activity of *Ipomoea aquatica* Forsk., and its active constituents'

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TO ALL MY TEACHERS
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</tr>
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<td>ALP</td>
<td>Alkaline phosphatase</td>
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<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
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<td>ALX</td>
<td>Alloxan monohydrate</td>
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<td>ANOVA</td>
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<td>Dextro</td>
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<td>DM</td>
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<td>DP</td>
<td>Dipeptidyl peptidase</td>
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<td>ELIZA</td>
<td>Enzyme linked immunosorbent assay</td>
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<td>Forsk.</td>
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<td>γ GT</td>
<td>Gamma glutamyl transpeptidase</td>
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<td>GDM</td>
<td>Gestational diabetes mellitus</td>
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<td>GIP</td>
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<td>HSL</td>
<td>Hormone sensitive lipase</td>
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<td>IDDM</td>
<td>Insulin dependent diabetes mellitus</td>
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<td>IDF</td>
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<td>IGT</td>
<td>Impaired glucose tolerance</td>
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**ABSTRACT**

*Ipomoea aquatica* Forsk. (Convolvulaceae) is a common green leafy vegetable, which has been in human consumption since antiquity. According to the indigenous medicinal system, the plant is supposed to possess an insulin-like principle. A study was done to determine the oral hypoglycaemic activity of the plant in healthy and diabetic Wistar rats as well as Type II diabetic patients. Activity directed fractionation was also carried out. A single as well as multiple doses of the aqueous, whole extract effectively reduced serum glucose concentration of healthy Wistar rats subjected to a glucose challenge. Multiple doses of the fresh, edible portion of the plant exerted a statistically significant oral hypoglycaemic effect in streptozotocin and alloxan-induced diabetic Wistar rats. The optimal dose in the rats was 3.4 g/kg and the optimal time of activity was 2 h after the administration of the extract.

The oral hypoglycaemic activity exerted by the plant was comparable to that of tolbutamide. This hypoglycaemic effect was significantly higher than the effect of the soluble and insoluble dietary fibre extracted from *I. aquatica*. The active constituents were contained in the ethanol extract of the fresh, edible portion.

The results showed that the long-term consumption of *I. aquatica* has no possible toxicity on the liver and kidney. Toxicity studies which were carried out for 8 weeks did not show any increase in the levels of key hepatic enzymes viz; alkaline phosphatase, alanine amino transferase, aspartate amino transferase and γ-glutamyl transpeptidase. Nevertheless, there was a significant reduction in the serum alkaline phosphatase level of the Test group when
compared with the Control. Uric acids levels in the Test and Control groups were not significantly different from each other indicating there was no possible renal damage. Glucose challenge studies with Type II diabetics showed a significant reduction in the serum glucose levels 2 h post glucose load when administered the aqueous, whole extract. Fractionation of the ethanol extract by gel filtration chromatography with Sephadex G25 yielded 2 oral hypoglycaemic fractions when tested on rats. When further purified, the active fraction appeared to contain flavonoids. These flavonoids comprising flavones and flavanols were found to separate into 5 sub bands on preparative TLC out of which 4 were oral hypoglycaemic in rats. MPLC on with a solvent gradient of, hexane → chloroform → ethylacetate → methanol and water with a dilution factor of 6, yielded MPLC 1 with methanol and water in the ratio of 99.168: 0.832, while MPLC II was eluted with ethyl acetate and methanol in the ratio of 93.7: 6.3. Infra red spectroscopy of the 2 compounds along with standard indicated that MPLC I was a flavone glycoside while MPLC II was a flavanol glycoside. Enzyme hydrolysis and TLC of the compounds showed the presence of glucose and rhamnose in MPLC I and rhamnose only in MPLC II The sugar moieties were necessary for the oral hypoglycaemic activity of MPLC II as shown by the inactivity of the aglycone of the compound. The infra red spectra of the aglycone indicate the presence of a tri hydroxy flavone. Studies directed at the mechanism of action showed that the extract enhanced the absorption of glucose in the intestine in the rats; and at the same time removed the absorbed glucose efficiently from circulation. The extract has enhanced the uptake of glucose by the peripheral tissues. The extract lowered the serum insulin levels of the Type II diabetics subjected to a glucose challenge, indicating that the extract may have increased the receptor sensitivity of insulin. The mechanism of action of the plant extract may be by enhancing the tissue uptake of glucose, which could be mediated via an increase in the sensitization of the receptors for insulin.
1. INTRODUCTION

1.1 General introduction

1.1.1 The importance of herbal remedies in the treatment of diseases

Indigenous medicinal systems still play a major role in the treatment of various types of diseases, in areas where Western medicine is scarcely available. Many of the world's countries have inherited a wealth of folklore medicine, which seem to have served well for their purposes to date. The Asian, African and the Latin American regions have their own medicinal practice which comprise the treatment of ailments with plant based materials mainly with little involvement of animal based materials.

Sri Lanka is being a small island in the Indian Ocean, is no exception regarding the rich nature of the country's indigenous medicinal system, which is mostly based on Ayurveda. Besides, the country is home to a wealth of natural flora and fauna, which could have many potential benefits if manipulated properly.

Even in the affluent Western societies a new trend has recently developed where herbal remedies have become much sought after. The trendsetters have made it a point that this new trend towards nature based medicines will have great commercial value, where the pharmaceutical industries are concerned. Several commercially available drugs have their roots arising from traditional medicine; e.g. cardiotonic steroid, digitalis from foxglove is a classic example.
1.1.2 Diabetes mellitus (DM); a brief history

Diabetes mellitus is a group of syndromes characterized by hyperglycaemia; altered metabolism of lipids, carbohydrates, and proteins; and an increased risk of complications from vascular disease. It is the commonest type of metabolic derangement, which affects and debilitates millions of people from all walks of life the world over.

The ancient Indian physicians and surgeons like Charaka and Sushruta had discussed this condition as far back as in the 1200 BC and 10 BC (Buddhadasa, 1862). Celsus from the West and later Avicenna from the Middle East had documented detailed accounts of this condition in the early period (Wickramanayake, 1998). Our knowledge with regard to DM has a great stimulus from the epoch making discovery of insulin by Banting, Best, Macleod and Collip in the year 1921. Few events in the history of medicine are more dramatic than the discovery of insulin, which allowed the previously fatal disorder of insulin dependent DM (IDDM) to be treated. This not only helped physicians to treat their patients effectively but also to know more about the pathogenesis and pathology of this condition.

Allopathy has not succeeded in finding a cure for this condition so far. The modern treatment of the disease comprises the use of insulin or oral hypoglycaemic agents to control hyperglycaemia and to prevent possible complications. The introduction of oral anti-diabetic agents in 1955 had given further stimulus to the study of these patients in greater detail. The high prevalence and long-term complications of DM have forced intense search for new oral hypoglycaemic agents in recent years.
1.2  Medicinal plants in the treatment of diabetes mellitus

The traditional use of medicinal plants for the treatment of DM greatly declined in
developed countries after the introduction of insulin. Many of them however have
remained as an alternative to conventional therapy in under-developed areas where
insulin is not readily available and accessible. In the last few years, serious attempts
have been made to develop new, easy to use, non-parental forms of insulin but these
have not yielded very effective results. On the other hand, there is a growing demand
for new oral anti-diabetic drugs to serve as an alternative therapy in non-insulin
dependent DM (NIDDM). The plant kingdom is a wide field to look for effective oral
hypoglycaemics. Many plants reported to be useful for the treatment of DM in the
Ayurvedic system of medicine have been tested for their hypoglycaemic activity with
experimental animals and humans (Tissera and Thabrew, 2001)

1.3  Sri Lankan scenario

Sri Lanka is home to a wealth of unique plant and animal species. Its population, while
possessing great biological richness, is burdened by economic poverty. Due to
inaccessibility and the prohibitive costs of Western medicine, many Sri Lankans still
depend on traditional Ayurveda medicine to meet their health care needs. The
expansion of the knowledge of Sri Lankan medicinal plants and the local production of
pharmaceuticals based on the derivatives of such plants, could offer an affordable
alternative to Western medicine. Much of the ethnobotanical knowledge and medicinal
plants in Sri Lanka is in danger of being lost. Increasing degradation and cultivation of
secondary forests coupled with deforestation and slash and burn agriculture in primary
forests, is reducing the abundance of known medicinal plants, limiting the potential for new discoveries. Furthermore, ethnobotanical knowledge is not always passed down by word of mouth from one generation to the next, so the need to record and utilize the current wealth of ethnobotanical knowledge remains important for ethnopharmacological purposes.

1.4 *Ipomoea aquatica* Forsk. (Convolvulaceae)

1.4.1 General description

Synonyms

*Ipomoea reptans* (L.) Poir

*Convolvulus reptans* L.

Common English names

Morning glory

Water convolvulus

Water spinach

Swamp cabbage

Chinese: Kankong

Sinhala: Kankun

Tamil: Vallal / vellai keerai

The first historical record of *Ipomoea aquatica* is of its cultivation as a vegetable during the Chin Dynasty about 300 AD (Stephens, 1994). Its ancient origin is Southeast Asia and India.
Figure 1.1  *Ipomoea aquatica* Forsk., the wet type

Figure 1.2  Mature *I. aquatica* with flowers
The plant is a semi-aquatic vine producing long shoots, which trail over the water or mud, rooting freely at the nodes. Leaves variable, commonly oblong-lanceolate, the base hastate or truncate, commonly 5-15 cm long and 2-10 cm wide, on long petioles 3-10 cm long; flowers several in cymes, peduncles to nearly 20 cm, pedicles 2-6 cm; calyx-lobes obtuse, ovate-oblong, just under 1 cm long; corolla usually 4-5 cm long, pinkish-violet, darker in the throat (rarely nearly white); fruit to 1 cm long; seeds finely pubescent (Stephens, 1994). A four-seeded pod follows flowering. At least two varieties are cultivated; the wet, succulent form and the dry, more slender form, which requires plenty of water. The growth is very rapid and over growth could block waterways.

1.4.2 Studies on *I. aquatica*

*I. aquatica* had been used traditionally in many parts of the world to treat various disease conditions. A decoction made of the leaves that had been left overnight to wilt had been used in traditional Somali medicine to treat enteritis (Samuelsson *et al.*, 1992). In the North–East Haryana of India, the plant is used for the treatment of eye diseases whereas in the Uttar Pradesh of India it is used to treat liver complaints (Jain and Verma, 1981).

*I. aquatica* at present is considered a threat to the water bodies in Florida in the United States of America and is labeled as a noxious weed. The farmers are required to obtain a permit to grow the plant for the food market (Stephens, 2001).

A few scientific studies regarding some medicinal aspects of this plant had been carried out. The oral hypoglycaemic activity of a large dose of the plant material on type II diabetics, had been studied by Garcia (1955). The dose used had been 300 g/d and the
patient compliance had been poor. The *in vitro* inhibition of the prostaglandin and leukotriene biosynthesis; by 2 derivatives of N-trans and N-cis-Feruloyltyramines, isolated from *I. aquatica* had been tested by Tseng et al, (1992). These compounds had exerted significant inhibitory effects on prostaglandin synthetase and arachidonate 5-lipoxygenase.

Kameoka *et al.*, (1992) have investigated the essential oil composition of *I. aquatica*. This team has identified 58 volatile components of which 49% had been triterpenoids. The main components isolated had been phytol, palmitic acid, (Z)-3-hexen-1-ol, a-humulene, n-hexacosane and bis (2-ethyl-hexyl) sebacate.

The cyanide liberating activity of the plant had been studied by Jansz *et al.*, (1973). The plant when grown in contaminated water is capable of concentrating metals such as iron (Fe), copper (Cu), manganese (Mn), chromium (Cr), lead (Pb) and mercury (Hg). Rai and Sinha (2001) have reported that in the district of Lucknow, India the water bodies where *I. aquatica* is grown, are contaminated with Cu, Cr, Fe, Mn and Pb. However the concentrations of metals have decreased significantly after boiling. This reduction is of significance since it would prevent toxicity after ingestion. As reported by Lin *et al.*, (2001), the plant had not concentrated the fungicide epoxiconazole used in paddy cultivation.

*I. batatas* (sweet potato), which is a closely related species of *I. aquatica*; is widely consumed as a vegetable. Sweet potato has been shown to reduce the blood sugar levels as well as the serum LDL-cholesterol levels in type II diabetics (Bernhard *et al.*, 2002).
1.5 Scope of the study

Many Sri Lankans still follow the traditional system of medicine and therefore heavily rely on herbal remedies for the treatment of diabetes mellitus. According to the indigenous system of medicine in Sri Lanka, *I. aquatica* is supposed to possess, an insulin-like principle (Jayaweera, 1982). It was thought desirable to validate this folklore scientifically. The present study was undertaken with several objectives in mind.

The main objective was to determine whether this plant exerts an oral hypoglycaemic activity in a normal and diabetic animal model as well as in diabetic patients.

The specific objectives were to determine:

A) in the healthy, Wistar rats,

I. the oral hypoglycaemic activity of a single dose on fasting blood sugar level as well as the effect on glucose challenge

II. the optimal dose and the optimal time of action

III. the oral hypoglycaemic activity of multiple doses on fasting blood sugar level as well as the effect on glucose challenge

IV. the comparisons between the oral hypoglycaemic activity of *I. aquatica* and tolbutamide after a glucose challenge

V. the effect of the long term administration of the fresh plant material on the key hepatic enzymes (alanine aminotransferase, aspartate amino transferase, alkaline phosphatase and gamma glutamyl transpeptidase) and the uric acid levels
VI. the effect of the long term administration of the fresh plant material on the fasting blood sugar and the glycated haemoglobin levels

B) in the diabetic, Wistar rats,

I. the oral hypoglycaemic activity of the fresh plant material on the fasting blood sugar levels as well as on glucose challenge, in the alloxan-induced diabetic rat

II. the oral hypoglycaemic activity of the fresh plant material on the fasting blood sugar levels of the streptozotocin-induced diabetic rats

III. effect of feeding the plant material on the body weights of diabetic rats

C) in the Type II diabetic subjects,

I. the effect of the aqueous extract on the blood glucose concentrations after a glucose challenge

II. the effect of the aqueous extract on the insulin concentrations after a glucose challenge

D) a suitable solvent for the extraction of active compounds

E) the possible mechanisms of action

F) the type of active compound by,

I. gel filtration chromatography

II. thin layer chromatography

III. medium pressure liquid chromatography

IV. enzyme hydrolysis

V. infra red spectroscopy
2. LITERATURE REVIEW

2.1 Early concepts of diabetes mellitus

(Buddhadasa, 1962; Wickramanayake, 1998 and Tissera and Thabrew, 2001)

Diabetes mellitus has been recorded as ‘Aasttravam’ in the Atharveda, one of the four ‘Vedic’ records, which are the oldest literature in the world. This term recognizes the disease to cause excessive urination. Charaka and Sushrutha referred to this condition as ‘madhumeha’, which was classified according to aetiology, body constitution, predominance of bodily humour (‘doshas’) and whether the condition was curable or incurable.

They had clearly stated that hereditary factors played an important role in the development of this condition. In the aetiology, they had mentioned two important factors; namely dietary indiscretion and sedentary habits with lack of exercise, which were responsible for the incidence of the disease. They stated that all the bodily humours get disturbed affecting the fatty tissue mainly, causing derangement and excretion through urine. The symptoms of the disease were described as follows.

1. Increased quantity and sweetness of mouth
2. Honey like urine
3. Increased sweetness of whole body
4. Excessive thirst
5. Excessive hunger
6. Lassitude
7. Constipation
Non-insulin dependant diabetes was described as ‘apathyanimittaja prameha’ (acquired diabetes) and insulin dependant diabetes was described as ‘sahaja prameha’ (inherited diabetes). Patients with ‘sahaja prameha’ were emaciated with loss of appetite and excessive thirst whereas the patients with ‘apathyanimittaja prameha’ were obese. Administration of many bitter drugs and sufficient amount of physical activity with strict dietary control were suggested as treatment. For the obese patients, purification and desaturation therapy was carried out and for the lean and weak patients, pacification and saturation therapy was given.

2.2 Diabetes, the modern concepts

Most patients can be classified clinically as having either insulin dependent diabetes mellitus (IDDM or Type I) or non-insulin dependent diabetes mellitus (NIDDM or Type II). Diabetes mellitus is associated with certain other conditions or syndromes as well and therefore, could be classified into a few different types.

2.2.1 Insulin-dependent diabetes mellitus (IDDM) / Type I

Since this type usually occurs in young individuals, it was previously called juvenile diabetes. It can, however, start at any age.

Genetic determinants seem important for the onset in most patients, with environmental factors a close second. The inheritance of IDDM is polygenic. More than 50% of heritability is contributed by Human Leukocyte Antigen (HLA) class II genes. Abnormal immune responses to viral infections with Coxsackie B4, reo virus type 1, Mengo virus, rubella virus etc. play an aetiologic role. Population studies have shown
that introduction of cow's milk before the age of 2-3 m, is associated with the production of antibodies to bovine serum albumin and an increased risk of developing IDDM (Edwards, 1995).

2.2.2 Non-insulin dependent diabetes mellitus (NIDDM)/ Type II

This type can become recognizable at any age, although it can be asymptomatic for years and thus usually presents in patients over 40 years of age. Often only the complications seen after years of having diabetes; like neuropathy and cataracts, cause a diagnosis to be made.

The resistance of target receptors for insulin is a common feature in NIDDM. The patients have to hyper-secrete insulin to achieve metabolic control which eventually leads to beta cell exhaustion.

The genetic basis of NIDDM seems even stronger than that of IDDM, and it is aggravated by environmental factors. The risk for a monozygotic twin is almost 100%. Most of the NIDDM patients (60 to 90%) in the Western world are obese, which should be seen as an indicator for sub-classification of the type II diabetic. Symptoms are usually (at least partly) alleviated by weight loss.

2.2.3 Other types of diabetes mellitus

Diabetes forms part of certain other conditions and syndromes, whether obviously aetiologically related or not. This class can be subdivided according to known or suspected aetiological relationships, where diabetes may be secondary to,

1. pancreatic disease (neonatal or later on in life),
2. hormonal abnormalities which may have either hypoinsulinaemia or hyperinsulinaemia as a consequence,
3. the administration of certain hormones, drugs and chemical agents, of which oral contraceptives, tricyclic antidepressants and marijuana are but a few,
4. insulin receptor abnormalities, either in the number of receptors or their affinity for insulin, or even because of the presence of antibodies to receptors (with or without associated immune disorders),
5. certain genetic syndromes, e.g. metabolic disorders, insulin resistance, hereditary muscle disorders and cytogenic disorders like Down's syndrome and
6. other types, of which diabetes associated with malnourished populations is the most prominent example.

2.2.4 Gestational diabetes mellitus (GDM)

There are two ways in which Diabetes Mellitus and pregnancy can occur simultaneously. One is where a previously diagnosed diabetic female becomes pregnant, which involves certain risks for both mother and child. The other is in Gestational Diabetes, when a pregnant lady becomes diabetic for the first time, at some stage during pregnancy, because of the pregnancy and most commonly only for the duration of the pregnancy.

During a normal pregnancy, the presence of placental hormones, induce the pancreas to produce increased concentrations of insulin and therefore the plasma insulin levels are
high. This phenomenon put an additional burden on the pancreas, which becomes exhausted and functions sub-optimally in genetically predisposed females, thus leading to hyperglycaemia. Macrosomic babies (birth weight > 4.0 kg), which may cause problems at partus, are a common feature with diabetic pregnancies.

A Glucose Tolerance Test usually points out the need for diet or insulin therapy. Oral hypoglycaemic agents cannot be used in pregnant diabetic patients. Even after insulin treatment for GDM, most pregnant women return to normal glucose tolerance after delivery. If this is not the case, they have acquired clinical diabetes. From the previously gestational diabetics, 80 % acquire overt DM within the next 25 years and they should monitor the blood sugar levels periodically.

2.2.5 Impaired glucose tolerance (IGT)

For the diagnosis of IGT an oral glucose tolerance test is essential. The criteria for this classification lie between those for normal subjects and diabetics. Consequently, the seriousness of the disorder is intermediate between normal and diabetic, with some clinical complications being completely absent while others, especially cardiovascular abnormalities, commonly present. Thus, IGT may have prognostic implications that should not be overlooked, especially in seemingly healthy individuals. Like overt diabetes, IGT can be linked to numerous disorders and obesity. Patients with IGT do not necessarily proceed to develop clinical diabetes; many return to normal glucose tolerance for no apparent reason while others stay in the IGT class for many years.
2.3 Complications of diabetes mellitus

The major tissues affected by diabetes, the retina, the kidney and the nerves, are all freely permeable to glucose. Thus, an increase in blood glucose concentration leads to an elevation of the intracellular accumulation of both glucose and its subsequent metabolic products. The proposed mechanisms by which hyperglycaemia may lead to micro vascular and neurologic complications include the increased accumulation of polyols through the aldose reductase pathway and of advanced glycosylation end products (Charles et al., 1995).

2.3.1 Retinopathy

Diabetic retinopathy is the most important cause of visual impairment in persons under 60 y of age. The risk of proliferative retinopathy in the 20 y after diagnosis is higher for patients with IDDM than for those with NIDDM. The aldose reductase inhibitors; ponalrestat and sorbinil do not prevent the progression of early diabetic retinopathy. The treatment of diabetic retinopathy is most efficacious when started before any vision is lost. The prevention of further deterioration of vision rather than the improvement is a more likely result of treatment (Kohner et al., 1990 and Klein et al., 1992).

2.3.2 Nephropathy

Diabetic nephropathy is a leading cause of end stage renal disease and accounts for about one tenth of all new cases. Hyperglycaemia causes intraglomerular hypertension and renal hyperperfusion (Christiansen et al., 1981; Hostetter et al, 1982 and McMurray et al., 2002). Increased glomerular pressure results in the deposition of protein in the
mesangium, ultimately leading to glomerulosclerosis and renal failure. Nonenzymatic glycosylation as well as the lipoprotein abnormalities may also contribute to diabetic nephropathy. Anti hypertensive therapy combined with slow protein diet could decrease the rate at which diabetic nephropathy progresses (Bojestig et al., 1994). McMurray et al (2002), have reported that patient education regarding the disease significantly improved the outcome of in patients with diabetic nephropathy.

2.3.3 Neuropathy

Neuropathy in the diabetic is one of the most common complications and has myriad clinical presentations. The extent and the intensity of the functional and anatomical abnormalities parallel the degree and duration of hyperglycaemia. Acute hyperglycaemia reduces nerve function. Chronic hyperglycaemia is associated with the loss of myelinated and unmyelinated nerve fibres, Wallerian degeneration and blunted nerve fibre production.

Formation of sorbitol and advanced glycated end products, play a significant role in the pathophysiology. Aldose reductase inhibitors and tricyclic anti depressants are used in the therapy (Alberti and Gries, 1988).

Duration-related cognitive impairment is an increasingly recognized complication of type I diabetes (Li et al., 2002). Hippocampal abnormalities have been observed in the spontaneously type I diabetic rat. Li et al state that, a duration-related apoptosis-induced neuronal loss occurs in type I diabetes associated with cognitive impairment. The data also suggest that this is at least in part related to impaired insulin and insulin-like-growth factor activities.
2.4 Treatment of diabetes mellitus

Treatment of diabetes comprises either the administration of insulin or oral hypoglycaemic agents in conjunction with dietary control and physical exercise.

2.4.1 Dietary control

The diet advocated to a diabetic patient should be easily available, cheap, as close and similar to the normal diet as possible and also should contain a large amount of fibre.

1. The caloric intake must be calculated carefully approximately 1400-1800 kcal daily for an elderly person, 1800-3000 kcal/day for a young active adult.

2. Carbohydrate intake should be at least 100 g per day to avoid ketosis. However the patient should avoid an intake exceeding more than 300 g of carbohydrates.

3. Protein content should also be adequate; about 60-110 g daily.

4. Alcohol in excess can result in lactic acidosis. Alcohol can induce a disulfiram type of reaction in those taking sulphonylureas. Abstinence is encouraged.

5. Aspartame and similar kinds of sweetening agents are preferred over sugar for diabetics.

6. Salt intake should not increase 6 g daily.

7. Fat intake should always be reduced.

8. Physical exercise:

   Exercise should always be encouraged in diabetics essentially, since it enhances the insulin action. Exercise has a synergistic effect with oral hypoglycaemic agents (Tang and Reed 2001). However, strenuous exercise is better avoided.
2.4.2 Anti-diabetic drugs

Current oral treatment options can be subdivided into the hypoglycaemic drugs such as sulphonylureas and benzoic acid derivatives and antihyperglycaemic drugs such as biguanides, α-glucosidase inhibitors and thiazolidinediones.

2.4.2.1 Sulphonylureas

All sulphonylureas increase insulin secretion and enhance insulin activity. According to the time of evolution, these exist in three generations. Second- and third-generation sulphonylureas more readily penetrate cell membranes, than do first-generation agents because of enhanced lipid solubility and at the same time featuring a greater selective binding capacity (Melander, 1987 and Campbell 1998). Sulphonylureas stimulate insulin release from the pancreatic cells, displaying a more pronounced action in the presence of glucose (Hellman and Taljedal 1975). These drugs do so by inhibiting an adenosine triphosphate-dependent potassium channel, which results in cell membrane depolarization and leads to calcium influx and release of stored insulin from secretory granules within the cell (Bressler and Johnson, 1997).

Sulphonylureas also decrease hepatic insulin clearance, resulting in increased serum insulin concentrations (Goldfine, 1984 and Groop, 1992). Increased circulating insulin levels feed back to suppress hepatic glucose production (DeFronzo, 1999).

*In vitro* data suggest sulphonylureas indirectly decrease peripheral insulin resistance and enhance its action, although the clinical significance of these effects is questionable (Goldfine, 1984 and Groop, 1992).

Pharmacokinetic data for the sulphonylureas are presented in Table 2.1 (Taylor, 1972; Moses *et al.*, 1973; Scott and Pfoffenbarger, 1979; Spiller, 1998 and Harrower, 2000).
The principal toxicity associated with sulphonylureas is hypoglycaemia. The prolonged duration of action, hepatic metabolism, and renal excretion of active parent compound or metabolite (with selected agents) have implications with regard to sulphonylurea-related hypoglycaemia. Overdoses generally occur as intentional attempts or accidental ingestions, and most accidental ingestions involve children. In addition, drug interactions can cause profound hypoglycaemia (Table 2.2, Shumack et al., 1991).

Other drugs may enhance or attenuate the hypoglycaemic effect of the sulphonylureas (Table 2.2). Enhancement of effect may result from competition for binding sites on plasma proteins, hepatic metabolic inhibition, or impairment of renal excretion. On the other hand, attenuation of the hypoglycaemic effect of sulphonylureas may result from drug interactions, leading to a decrease in digestive absorption or induction of liver metabolism (Marchetti and Navalesi, 1989 and Scheen and Lefebvre, 1995).

2.4.2.ii Biguanides

These drugs act by inhibiting hepatic gluconeogenesis and increasing glucose utilization by the muscle. Biguanides decrease serum cholesterol and triglycerides and also reduce glucose absorption by the gut. Biguanides prevent insulin degradation and interfere with protein binding of insulin. Three biguanides; metformin, phenformin, and buformin have initially been used for the treatment of type II DM, but only metformin remains in wide use today (Spiller, 1998). Phenformin was taken off the market in the United States and Europe in 1976 because of its association with lactic acidosis.

Metformin is indicated either as monotherapy or in combination with a sulphonylurea. Sulphonylureas and metformin cause a similar decrease in fasting blood glucose levels in diabetic subjects, but whereas the sulphonylureas generally cause weight gain,
metformin does not (Gan et al., 1992; Bailey, 1996; Pearlman et al., 1996; Kwong and Brubacher, 1998 and Yki-Jarvinen et al., 1999). Metformin decreases hepatic production and intestinal absorption of glucose in addition to decreasing the oxidation of fatty acids. Metformin treatment significantly increases AMP-activated protein kinase (AMPK) α-2 activity in the skeletal muscle, and this is associated with increased phosphorylation of AMPK on Threonine 172 and decreased acetyl-CoA carboxylase-2 activity (Musi et al., 2002).

Moreover, metformin increases insulin sensitivity, thereby decreasing the insulin resistance that is often a problem in patients with type II DM (Melander, 1987; Bailey 1996 and Guthrie 1997). It decreases the blood glucose level of diabetic patients but not that of non-diabetic patients (Brown et al., 1998). As such, it is an antihyperglycaemic agent and not a hypoglycaemic agent, as are the sulphonylureas (Spiller, 1998 and Bailey, 1996). Metformin undergoes virtually no hepatic metabolism and is 90 to 100 %, excreted by the kidneys. The pharmacokinetics differ from those of phenformin, which undergoes metabolism by the liver (Table 2.3).

Lactic acidosis is the most serious adverse effect linked to the biguanides, although the link is much stronger with phenformin than with metformin (Gan et al., 1992; Melander, 1996; Pearlman et al., 1996 and Spiller 1998). The lactic acidosis is characterized as type B (aerobic lactic acidosis), which is attributable to enhanced metabolic production of lactate; this is in contradiction to type A, which is caused by tissue hypoxia and thus termed anaerobic lactic acidosis (Gan et al., 1992 and Kwong and Brubacher, 1998). Signs and symptoms are nonspecific, including nausea, vomiting, diarrhoea, epigastric pain, anorexia, tachypnoea, and lethargy.
Malabsorption of vitamin B₁₂ and folate occurs with long-term treatment, although it usually does not lead to anaemia (Bailey, 1996; Melander, 1996 and Guthrie, 1997). The interference to the absorption of vitamin B₁₂ can be reversed by increased Ca²⁺ intake (Bauman et al., 2000).

2.4.2.iii Alpha glucosidase inhibitors

There are three, α-glucosidase inhibitors: acarbose was released first, miglitol has recently been marketed, and voglibose is not yet widely available. Although they can be used as monotherapy for type II diabetes mellitus, these antihyperglycaemic drugs are frequently used in combination with sulphonylureas or insulin (Hanefield, 1998 and Spiller, 1998).

These agents competitively and reversibly inhibit α-glucosidase, an intestinal brush border hydrolase enzyme. This leads to a post-prandial decrease in carbohydrate absorption because complex dietary polysaccharides are not broken down into absorbable monosaccharides. As a result, there is a decrease in hyperinsulinism and in hepatic triglyceride synthesis. Lactose absorption is not affected because lactase is a β-galactosidase (Andrade et al., 1996; Yee and Fong, 1996; Carrascosa et al., 1997).

As might be expected, the side effect profile of the α-glucosidase inhibitors is predominantly gastrointestinal because of their limited absorption. The undigested sugars may lead to bloating, flatulence, diarrhoea and abdominal pain (Coniff and Krol, 1997; Diaz-Gutierrez et al., 1998; Fujimoto et al., 1998).
<table>
<thead>
<tr>
<th>Generation</th>
<th>Generic Name</th>
<th>Time to Peak (h)</th>
<th>Half-life (h)</th>
<th>Duration of Action (h)</th>
<th>Metabolism</th>
<th>Renal Excretion of Active Metabolite</th>
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<tr>
<td>First</td>
<td>Chlorpropamide</td>
<td>2–7</td>
<td>36</td>
<td>60</td>
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<td>Yes*</td>
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<td>Tolbutamide</td>
<td>3–4</td>
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<td>6–12</td>
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<tr>
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<td>Acetohexamide</td>
<td>3</td>
<td>4–6</td>
<td>12–18</td>
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<td>4–8</td>
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<tr>
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<td>1–3</td>
<td>7</td>
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</tr>
<tr>
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<td>7</td>
<td>24</td>
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<tr>
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<tr>
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<td>5–9</td>
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*Parent drug undergoes prolonged excretion.
Table 2.2  Drug-drug interactions: first- and second-generation sulphonylureas.

<table>
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<tr>
<th>Sulphonylurea</th>
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<th>Mechanism</th>
<th>Effect</th>
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<td></td>
<td>Allopurinol</td>
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<td>Rifampin</td>
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<td>Glipizide</td>
<td>Salicylates, clofibrate</td>
<td>Displace from proteins</td>
<td>Hypoglycaemia</td>
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<td>Glipizide cont.</td>
<td>Trimethoprim-sulphamethoxazole, Miconazole Cholestyramine Rifampin H₂ blockers</td>
<td>Inconsistent/ unclear Absorption Hepatic metabolism Hepatic metabolism</td>
<td>Hypoglycaemia Pogatsa et al., 1985 Hypoglycaemia Hypoglycaemia Hypoglycaemia</td>
<td>Glyburide H₂ blockers Trimethoprim-sulphamethoxazole Ciprofloxacin Rifampin</td>
</tr>
</tbody>
</table>
Table 2.3 Pharmacokinetics of nonsulphonylurea antidiabetic agents: biguanides, α-glucosidase inhibitors, thiazolidinediones and benzoic acid derivatives

<table>
<thead>
<tr>
<th>Generic Name</th>
<th>Time to Peak (h)</th>
<th>Half-life (h)</th>
<th>Duration &gt;3-4 wk</th>
<th>Metabolism</th>
<th>Renal Excretion of Active Metabolite</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metformin</td>
<td>2-3</td>
<td>1-5</td>
<td>&gt;3-4 wk</td>
<td>Insignificant</td>
<td>Yes* hepatic</td>
<td>Bailey, 1996</td>
</tr>
<tr>
<td>Acarbose</td>
<td>1-2</td>
<td>2</td>
<td>4 h</td>
<td>Intestinal</td>
<td>Yes†</td>
<td>Hanefield, 1998</td>
</tr>
<tr>
<td>Miglitol</td>
<td>2-3</td>
<td>2</td>
<td>4 h</td>
<td>Intestinal</td>
<td>Yes</td>
<td>Spiller, 1998</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>1-2</td>
<td>3-4</td>
<td>&gt;3-4 wk</td>
<td>Hepatic</td>
<td>No</td>
<td>Giotin et al., 1998;</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>1-2</td>
<td>3-7</td>
<td>&gt;3-4 wk</td>
<td>Hepatic</td>
<td>No</td>
<td>Imura, 1998</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>1</td>
<td>1</td>
<td>4-6 h</td>
<td>Hepatic</td>
<td>No</td>
<td>Wolffenbuttel et al., 1993</td>
</tr>
</tbody>
</table>

* Parent drug excreted >90% unchanged in the urine.
† Pharmacologic effects not dependent on systemic absorption.
‡ Fraction (2%) of drug absorbed is excreted unchanged in the urine.
2.4.2.iv Thiazolidinediones

There are 2 drugs from this class currently in the market in the United States: rosiglitazone and pioglitazone. Troglitazone, the first of the thiazolidinediones in the market, received much recent public and professional scrutiny because of a link with serious, and at times fatal, hepatic dysfunction. It was withdrawn from the market in 2000 (Al-Salman et al., 2000; Forman et al., 2000 and Fried et al., 2000).

The thiazolidinediones enhance the effect of insulin in skeletal muscle, adipose, and hepatic tissues without increasing pancreatic secretion of insulin. They seem to bind to peroxisomal proliferator-activated receptors, changing insulin-dependent gene expression in the liver; the exact mechanism remains elusive. The thiazolidinediones decrease blood glucose levels in diabetic subjects, variably lower triglycerides, and have a mild, clinically insignificant, antihypertensive effect caused by decreasing insulin levels (Giotin et al., 1998; Imura, 1998; Neuschwander-Tetri et al., 1998).

Rosiglitazone and pioglitazone are rapidly absorbed. Both agents are greater than 99% protein bound. They undergo extensive hepatic metabolism, with metabolites being excreted in the urine and faeces (Table 2.3). They are not recommended for use in patients with hepatic disease but require no dosage adjustment in individuals with renal impairment. Both drugs can be taken without regard to meals.

The thiazolidinediones are generally very well tolerated. Both rosiglitazone and pioglitazone may reinstate ovulation in premenopausal women who have not been ovulating (Vella et al., 1998; Watkins and Whitmcomb, 1998). They also should be used with caution in patients with congestive heart failure because of a propensity to increase the circulating plasma volume, which may lead to oedema. Ethinyl oestradiol/norethindrone plasma levels are reportedly decreased by pioglitazone,
leading to a loss of contraceptive effect. Ketoconazole may inhibit the metabolism of pioglitazone, thereby increasing the effect of the latter (Herrine and Choudhary, 1999; Misbin, 1999).

2.4.2. Benzoic acid derivatives

Repaglinide is the first nonsulphonylurea oral hypoglycaemic agent in the market. It is indicated either as monotherapy or in combination with metformin. Repaglinide binds to the adenosine triphosphate–sensitive potassium channels on pancreatic cells at a receptor different from that of the sulphonylureas (Wolffenbuttel et al., 1993, Guay, 1998). However, it decreases insulin levels, whereas the sulphonylureas do not, and an extra pancreatic effect leading to increased insulin sensitivity has been postulated. Comparative clinical trials have shown that mild-to-moderate hypoglycaemia occurred in approximately 16% of patients taking repaglinide, as opposed to 20% of those taking glyburide and 19% of those taking glipizide (Spiller, 1998 and Nattrass, 2000). There have been no reports of repaglinide overdose and toxicity. It is expected that hypoglycaemia would occur in cases of overdose as with the sulphonylureas.

2.4.3 Insulin therapy

Insulin treatment has been in the practice since 1922. While the discovery of insulin was one of the greatest medical achievements of the 20th century, a cure rather than a treatment for diabetes mellitus is yet to be found.

Since 1982, most of the newly approved insulin preparations have been produced by recombinant DNA technology, which allows the production of complete human insulin.
Recombinant human insulin has, for the most part, replaced animal-derived insulin, such as pork and beef insulin, which had been in use in the earlier times. More recently, insulin products called "insulin analogues" have been produced so that the structure differs slightly from human insulin (by one or two amino acids) to change onset and peak of action.

There are many different kinds of insulin, which are grouped into four main categories; based on the onset, the peak and the duration of insulin action.

Table 2.4 lists the different types of insulin preparations available today. Onset, peak, and duration of action are approximate for each insulin product, as there may be variability depending on each individual, the injection site, and the individual's exercise programme.

2.4.4 New targets for diabetic therapy

Current strategies to treat diabetes include reducing insulin resistance using glitazones, supplementing insulin supplies with exogenous insulin, increasing endogenous insulin production with sulphonylureas and meglitinides, reducing hepatic glucose production through biguanides, and limiting post prandial glucose absorption with alpha-glucosidase inhibitors. In all of these areas, new generations of small molecules are being investigated which exhibit improved efficacy and safety profiles (Evans and Krentz, 1999).

Promising biological targets are emerging as antidiabetic tools, for which some examples are given below.
Insulin sensitizers including protein tyrosine phosphatase-1B (PTP-1B) and glycogen synthase kinase 3 (GSK3)

Inhibitors of gluconeogenesis like pyruvate dehydrogenase kinase (PDH) inhibitors

Lipolysis inhibitors

Fat oxidation including carnitine palmitoyltransferase (CPT) I and II inhibitors

Energy expenditure by means of β-3-adrenoceptor agonists

Also important are alternative routes of glucose disposal such as Na⁺-glucose co-transporter inhibitors, combination therapies, and the treatment of diabetic complications (e.g. retinopathy, nephropathy, and neuropathy). With many new opportunities for drug discovery, the prospects are excellent for development of innovative therapies to effectively manage diabetes and prevent its long-term complications.

Novel classes of rapid-acting secretagogues under evaluation include the morphilinoguanide BTS 67582 and the meglitinides; mitiglinide and senaglinide. Succinate ester derivatives represent a potential novel approach to improving β-cell function through enhancement of insulin biosynthesis and secretion.

Enhancement of nutrient-induced insulin secretion is a mechanism with several putative targets within the β-cell; potentiators of insulin secretion include glucagon-like peptide-1 and its analogues, phosphodiesterase inhibitors and the imidazoline derivatives. The amylin agonist pramlintide slows gastric emptying and suppression of glucagon secretion. Non-thiazolidinedione insulin-sensitising agents include the γ-receptor agonists and D-chiro-inositol. Insulin analogues with prolonged action and inhaled insulin preparations are also under investigation. Insulin-mimetic agents include
organic vanadium compounds. Whether newer agents will offer clinically relevant efficacy and tolerability advantages over existing therapies remains to be determined.

Hormone-sensitive lipase (HSL) is a key enzyme of lipid metabolism and its control is therefore a target in the treatment of diabetes mellitus. Cultures of the Streptomyces species 13381, have been shown to potently inhibit HSL. Ten inhibitors of HSL, termed cyclipostins, have been isolated from the mycelium of this microorganism and a further nine related compounds detected. Their structures had been characterized by 2-dimensional nuclear magnetic resonance (NMR) experiments and by mass spectrometry and had been found to comprise neutral cyclic enol phosphate esters with an additional y-lactone ring. On account of their ester-bound fatty alcohol side chain, the cyclipostins have physicochemical properties similar to those of triglycerides. The outstanding characteristic of the cyclipostins is their strong anti-HSL activity with concentrations in the nanomolar range (Vertesy et al., 2002).

Upon release into the circulation, the potent insulin secretagogues; glucose-dependent insulino-tropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are rapidly cleaved and inactivated by the enzyme, dipeptidyl peptidase IV (DP IV). Pospisilik et al., (2002) have demonstrated that DP IV inhibitors could be used to improve hepatic and peripheral insulin sensitivity and also to improve the β cell function and therefore could be used in the treatment of DM.
### Table 2.4  Common insulin preparations

<table>
<thead>
<tr>
<th>Type of Insulin</th>
<th>Onset of Action</th>
<th>Peak of Action</th>
<th>Duration of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapid-acting</td>
<td>15 min</td>
<td>30-90 min</td>
<td>3-5 h</td>
</tr>
<tr>
<td>Short-acting (Regular)</td>
<td>30-60 min</td>
<td>50-120 min</td>
<td>5-8 h</td>
</tr>
<tr>
<td>Intermediate-acting</td>
<td>1-3 h</td>
<td>8 hours</td>
<td>20 h</td>
</tr>
</tbody>
</table>

Intermediate- and short-acting mixtures: The onset, peak, and duration of action of these mixtures would reflect a composite of the intermediate and short- or rapid-acting components, with one peak of action.

| Long-acting           | 4-8 h           | 8-12 h         | 36 h              |

Source: (Food and Drug Administration, 2002)
2.5 Plants with hypoglycaemic properties

2.5.1 Sri Lankan plants of interest

Plants have been, through the ages, used as a source of drugs administered empirically or otherwise in the cure of diseases. Many of these plants, described to possess hypoglycaemic activity, have been investigated scientifically (Table 2.5). Different parts of the plants as well as the whole plant have been prescribed for the treatment of diabetes. Traditional Ayurvedic preparations include; kashayas, churnas, vatis etc. Single plant preparations as well as combinations of different plant materials have been utilized. A few common Sri Lankan plants are claimed for the anti diabetic effects.

A few common Sri Lankan plants that have been investigated for the hypoglycaemic activity are given below.

2.5.1.i *Artocarpus heterophyllus* (Moraceae)

Sinhala : Kos
Tamil : Musurapalam

An infusion of the mature leaves of this plant has been traditionally prescribed for the treatment of DM (Jayaweera, 1982). The hypoglycaemic activity of the aqueous extract of the leaves, was established in Sprague-Dawley rats by Fernando et al. (1989). The hypoglycaemic effect exerted by the extract was significantly higher than that of tolbutamide. The mechanism of action is supposed to be similar to that of sulphonylureas. The aqueous extract was also shown to be effective in reducing the blood glucose
concentration after an oral glucose tolerance test in maturity onset diabetics (Fernando et al., 1991).

There had been no adverse effects on the liver function, haematological parameters, reproductive function or the histology of the heart, lung, kidneys, intestine and the pancreas of rats.

2.5.1.ii Gymnema sylvestre (Asclepiadaceae)

Sinhala: Bin nuga
Tamil: Amuthupushpam

The hypoglycaemic effect of G. sylvestre, had been demonstrated in the alloxan-induced diabetic rabbits and dogs but not when pancreatectomized (Maskhar and Caius, 1930). Administration of the dried leaves for 24 weeks, to alloxan-induced diabetic rabbits had brought an increase in the serum insulin levels (Shanmugasunderam et al., 1981). The hypoglycaemic effect had been established in the streptozotocin-induced diabetic rats as well (Shanmugasunderam et al., 1990).

Studies have revealed that the extract was effective in reducing the fasting blood glucose levels in diabetic as well as normal individuals (Balasubramaniam et al., 1988 and Shanmugasunderam et al., 1981). Furthermore, the blood sugar levels at 2 h post glucose load were also significantly reduced by the plant extract.
Table 2.5   Medicinal plants used in Sri Lanka for the control of diabetes mellitus

<table>
<thead>
<tr>
<th>Name and family</th>
<th>Part used</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Family Acanthaceae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Hygrophila longifolia</em> (Linn.)</td>
<td>Whole plant</td>
<td>Contains alkaloids, Lupeol in the roots and Henriacontane in leaves.</td>
</tr>
<tr>
<td>(formerly <em>Asteracanthus longifolia</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S: Neeramulliya, Katu-ikiriya</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Family Anacaridaceae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Anacardium occidentale</em> (Linn.)</td>
<td>Nut</td>
<td>Pericarp of nut contains Cardol, Anacardol, Cardanol and Anacardic acid. Kernel yields oil containing linoleic, palmitic, stearic and lignoceric acid and sitosterin.</td>
</tr>
<tr>
<td><em>Spondias pinnata</em></td>
<td>Fruit</td>
<td>Fresh, ripe fruit contains 152 aromatic substances. Stem bark contains several triterpenoids.</td>
</tr>
<tr>
<td>S: Ambarella</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Mangifera indica</em></td>
<td>Kernel of seeds</td>
<td></td>
</tr>
</tbody>
</table>
Family Apocyanaceae

*Alstonia scholaris* (Linn.) Bark

Bark contains alkaloids,
- Alstonine
- Ditamine
- Echitamidine
- Echitine
- Echitenine
- Porphyrine

*Catharanthus roseus* Plant

Contains several alkaloids
- Catharanthine
- Leurosine
- Lochnerine
- Tetrahydroalstonine
- Vindoline

Family Asclepiadaceae

*Gymnema sylvestre* Leaves

Gymnemic acid present in leaves.
<table>
<thead>
<tr>
<th>Family</th>
<th>Plant Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bambusaceae</td>
<td><em>Bambusa vulgaris</em></td>
<td>Tender leaves</td>
</tr>
<tr>
<td></td>
<td>S: Una</td>
<td></td>
</tr>
<tr>
<td>Burseraceae</td>
<td><em>Canarium zeylanicum</em></td>
<td>Bark</td>
</tr>
<tr>
<td></td>
<td>S: Kekuna or Dik Kekuna</td>
<td></td>
</tr>
<tr>
<td>Celastraceae</td>
<td><em>Kokoona zelanica</em></td>
<td>Yellow inner</td>
</tr>
<tr>
<td></td>
<td>S: Wanapotu or Pothueta</td>
<td>bark</td>
</tr>
<tr>
<td>Convolvulaceae</td>
<td><em>Ipomoea aquatica</em></td>
<td>Plant, contains iron, calcium, vitamins B and C, fat and insulin like principle</td>
</tr>
<tr>
<td></td>
<td>S: Kankun</td>
<td></td>
</tr>
<tr>
<td>Cucurbitaceae</td>
<td><em>Benincasa hispida</em></td>
<td>Fruit contains a fixed oil, starch, Cucurbitine, resin, proteins myosin and vitellin, sugar and vitamins B and C.</td>
</tr>
<tr>
<td></td>
<td>S: Alu-puhul</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Coccinia grandis</em></td>
<td>Plant, contains an enzyme, hormone</td>
</tr>
</tbody>
</table>

36
<table>
<thead>
<tr>
<th>Plant Name</th>
<th>Part Used</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cucumis callosus</em></td>
<td>Seeds</td>
<td>S: Kowakka, kobowakka and traces of an alkaloid</td>
</tr>
<tr>
<td><em>Momordica charantia</em></td>
<td>Young fruit and leaves</td>
<td>S: Gon-kekiri</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E: Bitter gourd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tricosanthes cucurmerina</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whole plant, used along with other drugs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S: Dummella</td>
</tr>
<tr>
<td><strong>Family Flacourtiaceae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Caseacia zeylanica</em></td>
<td>Root</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>S: Wal-waraka</td>
</tr>
<tr>
<td><strong>Family Hippocrataceae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Salacia reticulata</em></td>
<td>Root bark and stem</td>
<td>Contains sugar derivatives</td>
</tr>
<tr>
<td></td>
<td>bark</td>
<td>S: Kothala himbutu</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Salacinol and Kotalanol.</td>
</tr>
<tr>
<td>Family Leguminosae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------------------------------------</td>
<td>--------------------</td>
<td>------------------------------------------------------------------</td>
</tr>
<tr>
<td><em>Acacia nilotica</em></td>
<td>The gum</td>
<td>Fruit contains an alkaloid</td>
</tr>
<tr>
<td>S: Babbula</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E: Indian gum Arabic</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Butea monosperma</em></td>
<td>Bark</td>
<td>Contains Tannic acid, little</td>
</tr>
<tr>
<td>S: Gas kela, kela</td>
<td></td>
<td>Gallic acid and Pyrocatechin</td>
</tr>
<tr>
<td><em>Cassia oxidentalae</em></td>
<td>Bark</td>
<td>Contains</td>
</tr>
<tr>
<td>S: Penithora</td>
<td></td>
<td>Oxymethylnanthroquinone and Malic acid.</td>
</tr>
<tr>
<td><em>Cassia sophera</em> (Linn.)</td>
<td>Roots</td>
<td>Contains cardiac glycoside</td>
</tr>
<tr>
<td>S: Ranawara</td>
<td></td>
<td>Sennapiorin. Bark contains tannins. Leaves contain Oxymethylnanthroquinone.</td>
</tr>
<tr>
<td>E: Tanner's cassia</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Caesalpnia digyna</em></td>
<td>Roots</td>
<td></td>
</tr>
<tr>
<td>S: Vakirimul</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pongamia pinnata</em></td>
<td>Flowers</td>
<td>Root, bark and stem contain an</td>
</tr>
<tr>
<td>S: Karanda, Magul karanda</td>
<td>alkaloid.</td>
<td></td>
</tr>
<tr>
<td>---------------------------</td>
<td>----------</td>
<td></td>
</tr>
<tr>
<td><em>Pterocarpus marsupium</em></td>
<td>Red latex of tree</td>
<td>Wood contains Catechin and Kinotannic acid</td>
</tr>
<tr>
<td>S: Gammalu</td>
<td>Seeds</td>
<td>Seeds contain Brucine.</td>
</tr>
<tr>
<td>E: Bastard teak</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Family Loganiaceae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Strychnos potatorium</em></td>
<td>Seeds</td>
<td></td>
</tr>
<tr>
<td>(Linn.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S: Ingini</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E: Clearing-nut tree</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Family Lythraceae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lagerstromia speciosa</em></td>
<td>Mature leaves, ripe fruits</td>
<td>Bark, fruit and leaves contain Tannin, while stem contains an alkaloid. Older leaves and mature fruits also contain an insulin-like principle.</td>
</tr>
<tr>
<td>S: Murutha</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Family Melastomaceae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Osbeckia octandra</em> and <em>Osbeckia aspera</em></td>
<td>Leaves</td>
<td></td>
</tr>
<tr>
<td>S: Heenbowitiya</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Family Menispermaceae  Stem  
*Tinospora cordifolia*  
S: Rasakinda

Family Moraceae  
*Artocarpus heterophyllus*  Mature leaves and bark  Wood contains Morin and a  
crystalline compound  
S: Kos, Herali, Waraka, Wela  
E: Jack fruit tree  
Cyanomaclurin.

*Ficus benghalensis* (Linn.)  Stem bark  Contains hypoglycaemic  
S: Nuga, Mahanuga  
E: Banyan  
glucosides and flavonoids.

*Ficus racemosa* (Linn.)  Root sap  
S: Attikka

Family Myrtaceae  
*Psidiium guajava* (Linn.)  Fruit  Leaves contain anti-bacterial  
S: Pera  
E: Guava  
substances, Avicularin and  
Guajaverin together with  
Psidiolic acid.

*Syzygium caryophylatum*  Bark and leaves
<table>
<thead>
<tr>
<th>Family</th>
<th>Plant</th>
<th>Part</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmae</td>
<td><em>Calamus rotang</em></td>
<td>Rhizome</td>
<td>S: Wewal</td>
</tr>
<tr>
<td>Plantaginaceae</td>
<td><em>Plantago erosa</em></td>
<td>Roots</td>
<td>S: Isapagol</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>E: Greater plantain</td>
</tr>
<tr>
<td>Petridaceae</td>
<td><em>Adiantum caudatum</em></td>
<td>Leaves</td>
<td>S: Thuda-wediya</td>
</tr>
<tr>
<td>Rubiaceae</td>
<td><em>Morinda citrifolia</em></td>
<td>Fruit</td>
<td>S: Ahu</td>
</tr>
<tr>
<td>Rutaceae</td>
<td><em>Citrus sinensis</em></td>
<td>Fruit juice</td>
<td></td>
</tr>
</tbody>
</table>

Ripe fruit and pulverised seed

Bark contains gallic acid and a crystalline substance, Jambosine.

Contains large amount of potassium salts and citric acid.

Leaves, root and flower buds contain glucoside, Acubin and enzymes, invertin and emulsin.

Leaves and fruit contain an alkaloid. Fruit also has volatile oil.

Juice contains -Sito-steryl-D-
<table>
<thead>
<tr>
<th>Family</th>
<th>Plant Name (Scientific)</th>
<th>Part</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solanaceae</td>
<td><em>Solanum nigrum</em> (Linn.)</td>
<td>Fruit</td>
<td>Fruit contains Solamargine, Solanidine and Solasodine</td>
</tr>
<tr>
<td>Zingiberaceae</td>
<td><em>Languas galanga</em> (Linn.)</td>
<td>Rhizome</td>
<td></td>
</tr>
</tbody>
</table>

S – Sinhalese name (local)

E – English name

Source: (Tissera and Thabrew, 2001)
2.5.1.iii Hygrophylla longifolia (Acanthaceae)

Sinhala: Neeramulliya
Tamil: Niramalli

An aqueous extract of the fresh whole perennial herb was shown to reduce the fasting blood glucose concentration and improve glucose tolerance in rats (Fernando et al., 1989 A). The effect was comparable to that of tolbutamide. The hypoglycaemic effect has been proven in normal individuals and NIDDM diabetic patients. When treated with an aqueous extract of the plant, the glycogen content in the liver and muscle were increased and the triglyceride content of the adipose tissue, was also found to be significantly increased (Fernando et al., 1998).

2.5.1.iv Lagerstromia speciosa L. (Lythraceae)

Sinhala: Murutha
Tamil: Kadali

Traditionally, a decoction of the mature leaves and ripe fruit of this plant is recommended for the treatment of DM (Jayaweera, 1982). Garcia (1956) has reported that the oral administration of tablets containing L. speciosa, significantly reduced the blood sugar levels of diabetic subjects. The dose given was equal to 5 units of insulin. A decoction of the plant significantly lowered the blood sugar levels of stress-induced hyperglycaemic subjects as well as alloxanized diabetic rats (Jacinto et al., 1965).

A hot water extract of the leaves had caused a significant reduction in the weight gain of obese female mice at the same time decreasing the total hepatic lipid content (Suzuki et al., 1999). Liu et al. (2001) have reported that an extract of L. speciosa stimulated glucose uptake and inhibited adipocyte differentiation in the cultured 3T3-L1 cells.
indicating that the extract might be useful for prevention and treatment of hyperglycaemia and obesity in Type II diabetics.

2.5.1. v Momordica charantia (Cucurbitaceae)

Sinhala: Karawila
Tamil: Pavakkai

M. charantia is a commonly available and widely consumed vegetable in Sri Lanka. It is prescribed in traditional medical systems for the treatment of diabetes. This vegetable has been subjected to extensive research regarding its ability to reduce the blood sugar levels. The powdered fruit, fruit juice, freeze dried fruit juice and the seed extract have been shown to exert a hypoglycaemic effect in diabetic animals (Karunanayake et al., 1984; Chandrasekar et al., 1989; Jungle and Navthar, 1989 and Sankaranarayan and Jolly, 1990). The hypoglycaemic effect has been shown in the Type II diabetics as well (Welihinda et al., 1986). The subcutaneous injection of ‘polypeptide P’, a polypeptide isolated from M. charantia had significantly reduced the fasting blood sugar levels of both IDDM and NIDDM patients (Baldwa et al., 1977 and Khanna et al., 1981).

The fruit juice exerts insulinomimetic effects such as stimulation of lipogenesis and increased up take of glucose into muscle in tissue preparations in vitro. Contrary to this Welihinda and Karunanayake (1986) have reported that the triacylglycerol content in the adipose tissue of the M. charantia treated rats did not differ significantly from the untreated ones. The active principle of the plant is possibly a nitrogenous steroidal glycoside (Jeevathayaparan, 1998).
2.5.1. vi *Musa sapientum* L. (Musaceae)

Sinhala : Kesel

Tamil : Vaalaimaram

Banana is the fruit of *M. sapientum* and is widely consumed either raw or ripe. The hypoglycaemic activity of the flower of this plant, has been demonstrated by Jain (1968) and Pari and Maheswari (1999). The flower is consumed as a tempered salad by Sri Lankans. The chloroform extract of the flower had produced a significant hypoglycaemic effect in alloxanized diabetic rats. It has also decreased the glycated haemoglobin level of the diabetic rats. These investigators have shown that the effect exerted by the extract was better than the hypoglycaemic effect exerted by glibenclamide.

2.5.1. vii *Nelumbo nucifera*

Sinhala : Nelum

Tamil : Thamarai

Daily doses of an extract of the rhizome of *N. nucifera* had produced hypoglycaemia and at the same time, had shown antihyperglycaemic effects on glucose challenge in the normal rats (Huralikuppi et al., 1991). In moderately diabetic rabbits, the extract had exerted a hypoglycaemic effect, which had been less than the effect exerted by phenformin and tolbutamide.

Mukherjee et al (1997) have shown that the ethanolic extract of the rhizome produced significantly reduced the blood sugar levels of normal, glucose-fed hyperglycaemic and streptozotocin-induced diabetic rats. The effect had been comparable to the effect exerted by tolbutamide.
5.1. viiii Osbeckia octandra DC (Melastomaceae)

Sinhala : Heenbovitia
Tamil : Mahthulai

Fernando (1989 B) had demonstrated that an aqueous extract of the fresh, mature leaves of *O. octandra* significantly reduced the fasting blood sugar level of Sprague-Dawley rats. The glucose tolerance of the rats also had been improved by the extract. The hypoglycaemic effect had been evident in the ethyl acetate fraction. The separated, biologically active compounds had been identified as two flavonoids and a flavonoid derivative.

2.5.1. ix Salacia reticulata Wight (Hippocrataceae)

Sinhala : Kothala himbutu

The decoction of the root bark, of *S. reticulata* had reduced the blood glucose levels of normal as well as alloxan- and streptozotocin-induced diabetic rats (Serasinghe *et al.*, 1990). The aqueous extract had improved the fasting blood sugar levels and glucose tolerance in NIDDM patients. A sucrose tolerance test had shown that the plant extract had inhibited the entry of glucose from intestine into blood. The sugar derivatives, salacinol and kotalanol are thought to exert the hypoglycaemic effect (Tissera and Thabrew, 2001). The ethanolic extract of *Salacia macrosperma*, a related species too, had exerted a significant hypoglycaemic effect in the fasted rabbits and alloxan-induced hyperglycaemic rats (Venkateswarlu, *et al.*, 1990).
2.5.1.x Syzygium cuminii (Myrtaceae)

Sinhala: Maadan
Tamil: Arugadam

The ripe fruit and the pulverized seed have been traditionally used for the treatment of DM (Jayaweera, 1982). Different doses of the aqueous, alcoholic and acetone extracts of the seeds, when administered for 15-45 days had produced significant hypoglycaemic effects in experimental rats (Singh et al., 1990). The raised serum cholesterol and urea levels in the alloxanized rats have significantly reduced to the normal levels with the administration of the acetone extract. The effect had been evident even at 2 weeks after the discontinuation of the drug.

The aqueous seed extract had antagonized the weight loss in alloxan-induced diabetic rats when administered for 6 weeks (Prince et al., 1998). The tissues had been actively protected against the free radical damage and the extract had been more effective than glibenclamide.

2.5.1.xi Tinospora cordifolia

Sinhala: Rasakinda
Tamil: Amridavalli

According to the indigenous medicinal system of Sri Lanka, T. cordifolia is supposed to be an antidiabetic drug (Jayaweera, 1982). The antioxidant activity of the aqueous extract of the root, had been reported by Prince and Menon (1999). The extract had significantly reduced the thiobarbituric reactive substances and at the same time had increased the levels of glutathione and vitamin C in alloxanized rats. (Mainzen et al., 2001) have shown that the oral administration of the aqueous extract of the roots for 6
weeks resulted in a significant reduction in thiobarbituric acid reactive substances and an increase in reduced glutathione, catalase and superoxide dismutase in alloxan diabetic rats.

Mainzen et al. (1999) had shown that the administration of the extract of *T. cordifolia* roots for 6 weeks resulted in a significant reduction in serum and tissue cholesterol, phospholipids and free fatty acids in alloxan-induced diabetic rats. The root extract has been reported to cause a significant reduction in the blood glucose levels and brain lipids in the alloxan-induced diabetic rats. The extract had further increased the haemoglobin as well the hepatic glucokinase. Hepatic glucose-6-phosphatase, serum acid and alkaline phosphatases and lactate dehydrogenase levels had been significantly reduced by the root extract.

The anti-hyperglycemic effect of aqueous and alcoholic extracts as well as lyophilized powder of plant extract had been established by Grover et al. (2000). Daily oral feeding of the aqueous extract for 40 days, had significantly decreased the plasma glucose concentrations, urine volume and the excretion of urine albumin in streptozotocin-induced diabetic mice (Grover et al., 2001).

### 2.5.2 Molecules with hypoglycaemic activity

Some of the active compounds from these hypoglycaemic plants have been chemically characterized. These compounds could be broadly categorized into a few classes (Table 2.6). These compounds could serve as potential targets for the development of novel anti diabetic agents.
2.5.3 Mechanisms of action of plant bioactive molecules

The plant compounds that have been studied exert hypoglycaemic and anti hyperglycaemic effects via different mechanisms. Some of the mechanisms have been elucidated.

2.5.3.i Insulin secretion from pancreatic β cells

Insulin is the main anti diabetic hormone and aberrations in insulin metabolism are indicated in many forms of diabetes. *In vitro* studies carried out with the fruit juice of *M. charantia* suggest that it is a potent stimulator of insulin release from isolated pancreatic cells (Welihinda *et al.*, 1982).

2.5.3.ii Inhibition of intestinal glucose absorption

Serum glucose levels could be reduced by compounds, which prevent the glucose absorption from the intestine. This effect could be mediated via the inhibition of α glucosidase. The two compounds, salacinol and kotalonol from *S. reticulata* are postulated to exert the hypoglycaemic effect via the inhibition of α glucosidase.

2.5.3.iii Activation of glycolytic enzymes

Glycolysis is a universal pathway that uses glucose and therefore plays a significant role in the elimination of blood glucose values. The activators of the key enzymes such as hexokinase, phosphofructokinase II and pyruvate kinase are valuable tools in this regard. Pectin isolated from *Coccinia indica* is an enhancer of hexokinase (Presanna-
Kumar et al., 1993). The root extract of *T. cordifolia*, had been shown to increase the levels of hepatic glucokinase (Mainzen et al., 1999).

2.5.3.iv Enhancing glycogen synthesis

Several plant extracts have been found to increase the glycogen synthesis in the liver. The extracts of *H. longifolia* and *M. charantia* increase the glycogen content in the liver and muscle significantly (Welihinda and Karunanayake, 1986; Fernando et al., 1998 and Jeevatthayaparan, 1998).

2.5.3.v Increasing glucose uptake

Compounds that increase glucose uptake by peripheral tissues decrease serum glucose concentrations. Bellidifolin from *Svertia japonica* has been shown to increase glucose uptake in cultured fibroblasts (Basnet et al., 1995). Brazilin from *Caesalpinia sappan*, stimulates the glucose transport in 3T3-L1 adipocytes isolated from mouse embryo (Kim et al., 1995).

2.6 Induction of experimental diabetes

Diabetes cannot be induced in humans due to ethical reasons. Besides, humans have a long life span, which hinders the long-term studies of the disease. The relatively very short life span of animals and the consistency of genetics, make diabetic animal models, an efficient device for the study of pathophysiology, complications, efficacy of therapy as well as the effect of external factors that affect the disease.
Table 2.6  Examples of some chemical groups of different antidiabetic plant biomolecules

<table>
<thead>
<tr>
<th>Chemical group</th>
<th>Plant</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td><em>Morus alba</em></td>
<td>Asano <em>et al.</em>, (2001)</td>
</tr>
<tr>
<td>Amino acids</td>
<td><em>Allium cepa</em></td>
<td>Sheela <em>et al.</em>, (1995)</td>
</tr>
<tr>
<td>Diterpenoids</td>
<td><em>Croton cajucara Benth.</em></td>
<td>Silva <em>et al.</em>, (2001)</td>
</tr>
<tr>
<td>Flavonoids</td>
<td><em>Phyllanthus fraternus</em></td>
<td>Huheri <em>et al.</em>, (1990)</td>
</tr>
<tr>
<td>Glucosides</td>
<td><em>Paeonia lactiflora</em></td>
<td>Hsu <em>et al.</em>, (1997)</td>
</tr>
<tr>
<td>Glycans</td>
<td><em>Oryza sativa</em></td>
<td>Tissera and Thabrew (2001)</td>
</tr>
<tr>
<td>Quinones</td>
<td><em>Salacia macroperma</em></td>
<td>Venkateswarlu, <em>et al.</em>, (1990)</td>
</tr>
<tr>
<td>Sesquiterpenoids</td>
<td><em>Psacalium decompositum</em></td>
<td>Aguilar <em>et al.</em>, (2000)</td>
</tr>
<tr>
<td>Short chain fatty acids</td>
<td><em>Blighia sapida</em></td>
<td>Tissera and Thabrew (2001)</td>
</tr>
<tr>
<td>Triterpenoids</td>
<td><em>Bumelia sartosum</em></td>
<td>Naik <em>et al.</em>, (1991)</td>
</tr>
<tr>
<td>Xanthones</td>
<td><em>Anemarrhena asphodeloides</em></td>
<td>Miura <em>et al.</em>, (2001 a and b)</td>
</tr>
</tbody>
</table>
Experimental diabetes could be induced in different animal models such as the rat, mouse, guinea pig, rabbit, dog and monkey etc. Alloxan and streptozotocin are widely employed for the induction of diabetes in animals and cyproheptadine had been used to induce hyperglycaemia in animals.

2.6.1 Alloxan (ALX)-induced diabetes
Type 1 diabetes results from irreversible damage of insulin-producing β cells. In laboratory animals, diabetes can be induced with alloxan, a 2,4,5,6-tetraoxopyrimidine. It is a potent generator of reactive oxygen species (ROS), which can mediate β-cell toxicity.
Walde et al. (2002) have reported that the glucose transporter 2 (GLUT2) and glucokinase (GK) are target molecules for ALX. Ex vivo, a gradual decrement of both GLUT2 and GK mRNA expression had been found in islets isolated from ALX-treated C57BL/6 mice. This reduction had been more pronounced for GLUT2 than for GK.

2.6.2 Induction of diabetes by streptozotocin (STZ)
Streptozotocin {2-deoxy-2-(3’-methyl-3’-nitrosoureido)—D—glucopyranose} is a potent diabetogen. Streptozotocin is capable of producing a clinical diabetic picture, which closely resembles human diabetes. (Sanchez de Medina, 1994). It exerts the diabetogenic effect by the destruction of pancreatic β cells. Injections of multiple low doses of STZ reduce GLUT2 expression (Walde et al, 2002). The potent carcinogenicity of STZ, requires strict safety measures when handling (Jeevathayaparan, 1998).
2.6.3 Cyproheptadine induced hyperglycaemia

Oral administration of cyproheptadine causes a significant increase in the nonfasting blood glucose level. The hyperglycaemia induced by cyproheptadine is reversible 24 h after the treatment has stopped (Cakici et al., 1994).

2.7 Significance of an edible plant hypoglycaemic agent

None of the available modern treatment regimes of DM is able to provide a cure for this metabolic disorder. Apart from providing just a control of the condition and minimizing complications, allopathic treatment itself causes many an undesirable effect on the patient. Many of the plant materials tested positive for the hypoglycaemic effect are not edible and need considerable preparatory manoeuvres. Great value lies therefore, on an edible plant material, which could easily be prepared for consumption. Considering all these facts, it was decided to test *I. aquatica* for the hypoglycaemic effect.
3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Water

Deionized water was used in all experiments except in the case of control medium in animal trials and the medium for the preparation of extracts. Distilled water provided the control medium for animal experiments.

3.1.2 Chemicals

3.1.2.i General chemicals

All the chemicals used were purchased from, either British Drug House, Poole, United Kingdom or P S Park Scientific Limited, Northampton, United Kingdom. Solvents used were of analytical grade.

3.1.2.ii Special Chemicals

Alloxan monohydrate, quercetin, quercitrin, streptozotocin and flavone (unsubstituted) were obtained from the Sigma Chemical Company, Saint Louis, United States of America.

3.1.2.iii Enzymes

Naringinase (from \textit{Penicillium decumbens}) and pancreatin were purchased from the Sigma Chemical Company while \(\beta\)-glucosidase (EC No. 3.2.1.21, from almonds) was from Fluka Chemie (Hirsch, Switzerland). Pepsin, purchased from British Drug House,
Poole, United Kingdom and Termamyl purchased from Novo (Denmark) were also used for the experiments.

3.1.3 Plant material

*I. aquatica* was purchased from a locally grown home garden in Gangodawila, and identified by Dr. P. Tissera of the Department of Botany, Faculty of Applied Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda, Sri Lanka by comparing with herbarium specimens. The edible portion of the plant (leaves and stems) was used for the experiments.

3.1.4 Animals

Out-bred, male, Wistar rats (parental line originating from Japan), obtained from the Medical Research Institute, Colombo 8, were used in all animal experiments. The rats were 6-8 weeks of age and weighed 195±12 g.

3.1.5 Diabetic patients

Newly diagnosed diabetic subjects (n=20) who were not on any oral hypoglycaemic agents served as diabetic patients. Informed and written consent was obtained from the patients before enrolling them for the study.
3.1.6 Chromatographic materials

3.1.6.i Gel filtration chromatography

Sephadex G-25 (Superfine 17-0031-01), purchased from Amersham Pharmacia Biotech AB, Uppsala, Sweden, was used as the medium in gel filtration chromatography.

3.1.6.ii Thin layer chromatography

Chromatography plates (100 µm thickness) for normal detection of compounds were prepared using Silica gel G254, from the Sigma Chemical Company. The preparative chromatography plates were made to thickness of 1 mm with the same silica gel using a plate spreader (Camag, United States of America).

3.1.6.iii Paper chromatography

Paper chromatography sheets (Whatman No. 1, Whatman, United Kingdom) were used for paper chromatography.

3.1.6.iv Column chromatography

For column chromatography, column silica gel (230-400 mesh, Merck, Germany) was used.
3.2 Methods

3.2.1 General methods

3.2.1.i Ethical clearance

A project protocol was submitted to the Ethics Committee of the Faculty of Medical Sciences, University of Sri Jayewardenepura and ethical clearance was obtained.

3.2.1.ii Determination of the moisture content

The method of Dean and Stark (AOAC, 1984), was used to determine the moisture content of \textit{I. aquatica}. Toluene (British Drug House, Poole, United Kingdom) was allowed to saturate overnight with distilled water. The saturated toluene (300 ml) and the shredded, fresh plant material (10 g) were added into the flask and the Dean and Stark apparatus was set up. At the end of the experiment, the volume of water collected was measured and the percentage of moisture was calculated.

The moisture content was also determined by drying to constant weight. The plant material (100 g) was kept in the oven (Galenkamp Plus, Germany), at 105\degree C until constant weight. The percentage of moisture was determined.

3.2.1.iii Housing of rats

The rats were housed in plastic rat cages in a well-ventilated room (room temperature=29±2\degree C) at the Animal House of the Faculty of Medical Sciences, University of Sri Jayewardenepura with access to food and water \textit{ad libitum}, unless specified otherwise. Feeding pellets for the rats were purchased from Vet House Private Limited, Seeduwa.
3.2.1.iv Preparation of extracts

The ethanol extract was obtained by using the Soxhlet apparatus (Cope Quickfit S2071, Germany). The fresh, edible portion of the plant (100 g) was run in the Soxhlet apparatus with ethanol as the solvent for 30x20 min cycles. Ethanol was evaporated in the rotary evaporator (Eyela N-N Series, Japan) at 40° C. The weight of the resultant aqueous product was taken. This sample was dissolved in distilled water (10 ml) to obtain the extract from alcohol and was termed as ethanolic extract.

The whole aqueous extract of *I. aquatica* was prepared by blending the edible portion of the plant (100 g) in boiled water (100 ml) in an electric blender (Sumeet Industries, India).

The aqueous filtrate was obtained by filtering the aqueous extract through a muslin cloth.

3.2.1.v Administration of extracts, glucose and tolbutamide

These preparations were administered orally to the rats via a Sondi needle.

3.2.1.vi Drawing of blood

Blood (200 μl) was drawn from the lateral tail vein under light anaesthesia with diethyl ether.

3.2.1.vii Type of animal experiments

All animal experiments were carried out as analytical interventional studies. Random sampling was carried out to divide the rats into the respective groups.
3.2.1.viii Glucose challenge

During experiments, glucose tolerance tests were performed only in order to determine the optimal time of hypoglycaemic activity. Usually, glucose challenge tests were carried out. Here, a glucose load was given orally and 90 min post glucose serum glucose values were analyzed since intermediate blood sampling is not necessary according to the World Health Organization criteria (Kumar and Clark, 1997). The glucose load administered to rats was 3.0 g/kg body weight (Farias et al., 1997).

3.2.1.ix Determination of glucose

Glucose was estimated by using DMA reagent kits employing the glucose oxidase method of Hugget and Nielson (1956). Glucose is converted to gluconic acid by glucose oxidase and at the same time the chromogen (4-amino antipyrine) is converted to the oxidized chromogen by peroxidase; the colour intensity of which is measured at 520 nm. The absorbance is directly proportional to the amount of glucose present in the sample.

The glucose oxidase reagent (2 ml) was pipetted into labeled tubes followed by the addition of sample, control or standard (0.01 ml each). The tubes were incubated for 10 min at 37°C and the absorbance was measured in the spectrophotometer (Shimadzu-UV 200, Japan), at 520 nm.

\[
\text{Glucose concentration} = \frac{\text{Absorbance of sample} \times \text{Concentration of sample (mg/dl)}}{\text{Absorbance of standard}}
\]
3.2.1.x Determination of key hepatic enzymes

Reagents kits manufactured by DMA (Unites States of America) were used to determine the serum alkaline phosphatase, alanine transaminase and aspartate transaminase whereas kits from Human Diagnostics (Germany) were used for the estimation of \( \gamma \) glutamyl transpeptidase.

Alanine aminotransferase (ALT)

The kinetic method described by Bergmeyer et al., (1986) was used for this assay. Alanine aminotransferase catalyzes the exchange of the amino group of alanine with the oxo group of 2-oxoglutarate to form pyruvate and glutamate. In the presence of lactate dehydrogenase, pyruvate reacts with NADH to form lactate and NAD\(^+\). The NADH oxidation is directly proportional to the ALT activity and is measured via an absorbance decrease at 340 nm.

For each sample, ALT reagent (2 ml) was dispensed into the cuvettes and warmed to the reaction temperature (30\(^\circ\) C). Sample (0.2 ml of unhaemolyzed serum) was added to the respective cuvettes, mixed gently and incubated for 30 sec at the reaction temperature. The decrease in absorbance was recorded in the spectrophotometer (zeroed with deionized water) at 1 min intervals for 2 min.

ALT concentration in the sample \( \{ \text{U (Units)/l} \} = \frac{\Delta A}{\min \times (-1768)} \)

Aspartate aminotransferase (AST)

The method of Wilkinson (1976) was employed for the determination of aspartate transaminase. This enzyme catalyzes the exchange of the amino group of aspartate with
the oxo group of 2-oxoglutarate to form oxaloacetate and glutamate. In the presence of malate dehydrogenase, oxaloacetate reacts with NADH to form malate and NAD\(^+\). The NADH oxidation is directly proportional to the AST activity and is measured via an absorbance decrease at 340 nm.

The testing procedure and the calculation for AST was the same as for ALT.

**Gamma glutamyl transpeptidase (γ-GT)**

The kinetic and colourimetric method of Persijn and van der Silk (1976) was used for the determination of γ-glutamyl transpeptidase levels. L-γ-glutamyl-3-carboxy-4-nitroanilide and glycyglycine are converted to L-γ-glutamyl-glycylglycine and 5-amino-2-nitro-benzoate by γ GT.

The reagent (1 ml) was pipetted into cuvettes previously warmed to the reaction temperature (30°C) followed by the addition of the sample (0.1 ml of unhaemolyzed serum). The solutions were mixed and the absorbance was read against air at 1 min intervals for 4 min in the spectrophotometer at 405 nm.

\[
\gamma \text{ GT concentration of the sample (U/l)} = \frac{\Delta A}{\text{min}} \times 1421
\]

**Alkaline phosphatase (ALP)**

Alkaline phosphatase hydrolyzes p-Nitrophenylphosphate to form p-nitrophenol and phosphate, the rate of which is directly proportional to its activity. This method was described by, Tietz et al. (1983).
For each sample, ALP reagent (3 ml) was pipetted into test tubes and warmed to reaction temperature (30° C). Clear non-haemolyzed serum (0.05 ml) was added to the tubes and incubated for 30 sec. After the incubation period, the increase in absorbance was recorded in the spectrophotometer at 405 nm, (zeroed with deionized water), at 1 min intervals for 6 min.

ALP activity of the sample (U/l) = ΔA/min x 3231

3.2.1.xi Determination of uric acid levels

The method is based on the oxidation of uric acid by uricase to allantoin, carbon dioxide and hydrogen peroxide. Hydrogen peroxide in the presence of peroxidase causes the oxidative coupling of 4-aminoantipyrine and 2-hydroxy-3,5-dichlorobenzenesulphonic acid to form a red chromogen, which is proportional to the amount of uric acid present (Fossati et al., 1980).

The test was performed by pipetting the uric acid reagent (2 ml) into tubes followed by the addition of standard or sample (0.05 ml). The tubes are mixed gently and incubated for 10 min at 37° C. The absorbance was recorded in the spectrophotometer at 520 nm.

Uric acid concentration = \( \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Concentration of standard} \)

of sample (mg/dl) Absorbance of standard

3.2.1.xii Determination of glycated haemoglobin (HbA1c) levels

Diagnostic kits from Human Diagnostics were used to assess the glycated Hb levels. Whole blood was mixed with a lyzing reagent. The haemolysate was then mixed for 5
min with a weakly binding cation exchange resin. During this time, HbA binds to the resin. A resin separator was used to remove the resin from the supernatant fluid, which contained the HbA\textsubscript{1c}. The glycated Hb, as a percentage of total Hb was determined by measuring the absorbance of the glycated Hb and of the total Hb fraction at 415 nm in comparison with a standard glycated Hb preparation carried through the test procedure.

For the preparation of the haemolysate, well mixed blood samples and standard (0.1 ml each) were pipetted into labeled test tubes followed by the addition of the lyzing reagent and mixed well. The tubes were allowed to stand for 5 min.

The following steps were carried out in order to separate the glycated Hb. The contents in the ion exchange resin tubes were mixed well to obtain a homogenous resin suspension. Haemolysate (0.1 ml each) was added to the resin tubes. Resin separators were inserted into each tube and the tubes were mixed well for 5 min. After incubation, the resin separators were pushed into the tubes until the resin is firmly packed. Each supernatant was poured into a cuvette and the absorbance was directly read against water at 415 nm.

To determine the total Hb levels, the haemolysates (0.02 ml each) were pipetted into labeled tubes followed by the addition of distilled water (5 ml). The solution was mixed thoroughly and the absorbance was recorded against water at 415 nm.

The Hb standard was treated exactly like a sample and the respective readings were obtained.
% Hb_{1c} in sample = \frac{\text{Absorbance of sample} \times \text{% of HbA}_{1c}}{\text{Absorbance of HbA}_{1c} \text{ standard}} \times \frac{\text{Absorbance total of HbA}_{1c} \text{ sample}}{\text{Absorbance of HbA}_{1c} \text{ standard}}

3.2.1.xiii Determination of serum insulin levels (Hsu et al., 1997)

Insulin level was determined by an Enzyme Linked Immunosorbent Assay (ELIZA) based, one-step sandwich assay with Reagent Kits (Boeringer Manheim, Germany).

Insulin in the sample binds to the anti insulin antibody coated in the tubes. The antigen-antibody complex reacts with the chromogen to give a green colour, the absorbance of which is read at 420 nm and is directly proportional to the amount of insulin present in the sample.

The samples, standards and controls (0.1 ml each) were added to the tubes coated with anti-insulin antibodies followed by the addition of the incubating solution (1 ml each) and incubated at 20–25°C for 2 h. The contents were aspirated and discarded and the tubes were rinsed with the Enzymun—Test solution. Complete aspiration of the solution was achieved within 15 min. The substrate—chromogen solution (1 ml each) was next added to the tubes and incubated for 1 h. The contents of the tubes were mixed well and the absorbance was recorded at 420 nm.

Insulin level of sample = \frac{\text{Absorbance of sample} \times \text{Concentration of standard}}{\text{Absorbance of standard}}
3.2.2 Experiments to determine the oral hypoglycaemic effect of the whole, aqueous extract and the shredded leaves of *I. aquatica* on normoglycaemic, Wistar rats

3.2.2.1 Effect of a single dose

Rats (n=20) were divided into 2 groups termed test and control with ten rats in each. After an overnight fast, venous blood (0.1 ml) was drawn for the estimation of fasting blood sugar level. The test group was given the whole extract of *I. aquatica* (1 ml each) at a dose of 3 g/kg while the control group was given distilled water (1 ml each). After 30 min, a glucose challenge was performed on both groups.

3.2.2.2 Dose curve

Five groups of rats (n=5 in each) were termed. Groups I to IV and control. After an overnight fast, four different doses (1.7, 3.4, 5.1 and 6.8 g/kg) of the whole extract (1 ml each) were given to Groups I to IV respectively while distilled water was given to the control group (1 ml each). A glucose challenge was carried out 0.5 h later. The dose that gave the optimal reduction in blood glucose levels was determined.

3.2.2.3 Time course

To determine the optimal time of activity, 2 groups of rats termed test and control (10 rats in each) were used. Fasting blood sugar levels were determined after an overnight fast. The test group received the whole extract of *I. aquatica* (1 ml each) at a dose of 3.4 g/kg, while the control received distilled water (1 ml each). After the administration of glucose, blood was drawn for the estimation of serum glucose at 1, 2 and 3 h.
3.2.2.iv Effect of multiple doses

Rats were assigned into test and control groups with 10 rats in each. The test group received 1 ml of the whole extract at a dose of 3.4 g/kg/day for one week while the control group received distilled water (1 ml each). At the end of one week, a glucose challenge was performed after the determination of fasting blood sugar levels.

3.2.2.v Comparison with tolbutamide

Rats (n=24) were divided into three groups, the test, the drug and the control with 8 rats in each, fasted overnight and blood was drawn (0.1 ml) for fasting blood glucose estimation. The test group was administered 1 ml of the whole extract orally at a dose of 3.4 g/kg body weight. Distilled water (1 ml) was orally administered to the control group. The drug group was administered tolbutamide, (obtained from the State Pharmaceutical Corporation, Sri Lanka) at a dose 15 mg/kg body weight. This dose was comparable to that of 0.5-1.5 g (mean 0.75 g), given to humans. After 0.5 h, the glucose challenge was performed.

3.2.3 Effect on diabetic, Wistar rats

3.2.3.i Streptozotocin- induced diabetic rats

Rats (n=20) were made diabetic by an intravenous injection of streptozotocin (30 mg/kg body weight) dissolved in freshly prepared sterile citrate buffer (pH 4.5). After diabetes was well established (fasting serum glucose concentration > 250 mg/dl), the surviving rats (n=12), were randomly assigned into 2 groups, termed test and control with 6 rats in each. The test group was given shredded *I. aquatica* for one week along with their
normal feed. The control group was given the normal feed only. Both groups had access to water *ad libitum*. After seven days, the rats were fasted overnight and the fasting blood samples were collected for the determination of fasting serum glucose. The body weights at the start and the end of the experiment were determined.

3.2.3.1 Alloxan-induced diabetic rats

Alloxan monohydrate, dissolved in normal sterile saline was used at a dose of 40 mg/kg to induce diabetes intravenously in 30 rats. The surviving 20 rats were divided randomly into 2 groups termed test and control (with 10 rats per group), after diabetes was well established and the experiment was carried out as in 3.2.3.1. The body weights of the rats were determined at the beginning and the end of the experimental period.

3.2.4 Effect of dietary fibre

Isolation of soluble and insoluble dietary fibre was carried out by modifying, the method of Asp and coworkers, omitting the incineration step. (Asp *et al.*, 1983). The sample (100 g of finely blended, fresh, edible portion) suspended in phosphate buffer (50 ml, pH 6.0) was incubated with Termamyl (2 ml) at 95°C for 30 min. The pH was adjusted to 1.5 and pepsin was added (1 g) followed by incubation at 37°C for 2 h. Pancreatin (1 g) was next added after the pH was adjusted to 6.8 and incubated for 2 h. Finally the pH was adjusted to 7.0 and the sample was filtered through a Buchner funnel. The filtering aid, Celite was not used for filtering. The residue, consisted of the insoluble fibre. Ethanol (500 ml) was added to the filtrate and the soluble dietary fibre was allowed to precipitate for 2 h. The sample was again filtered and the residue
2. which consisted of the soluble dietary fibre was obtained. These residues were freeze-dried and reconstituted in 8 ml of deionized water on the day of administration.

Four groups of rats were termed whole extract (WE), soluble dietary fibre (SDF), insoluble dietary fibre (IDF) and control with 8 rats in each. After an overnight fast the WE group was given the whole extract (1 ml). To the SDF and IDF groups, the suspensions of soluble and insoluble dietary fibre were given respectively (1 ml each). Distilled water (1 ml each) was administered to the control. A glucose load was given 0.5 h later and blood samples were obtained at 1.5 h after glucose loading.

3.2.5 Long term feeding of the fresh, edible portion to rats

3.2.5.i Effect on key hepatic enzymes viz., ALT, AST, ALP and γ GT levels

Rats were divided into two groups as test and control with 12 rats in each. The shredded, fresh, edible portion of the plant was given to the rats in the test group only, at a dose of 4.0 g/kg for eight weeks in addition to their normal diet and water. The control group received the normal diet and water for the same period of time. At the end of the experimental period, blood (1 ml) was drawn and the concentrations of the above parameters (ALT, AST, ALP and γ GT) were determined as described in section 3.2.1.x. The rats were also observed for any abnormal behaviour.

3.2.5.ii Effect on uric acid levels

The uric acid levels were estimated in rats described in section 3.2.5.i. The method for the estimation was given in detail in section 3.2.1.xi.
3.2.5.iii Effect on fasting blood sugar and glycated haemoglobin levels

These parameters were estimated in the same rats described in 3.2.5.i. Whole blood (100 μl) was collected into heparinized vials for the estimation of glycated Hb. The testing procedure was given in detail in section 3.2.1.xii.

3.2.6 Studies on human diabetic subjects

The study was carried out in the Family Practice Centre of the Faculty of Medical Sciences, University of Sri Jayewardenepura. Written, informed consent was obtained from the 20 patients who participated in the study. Following an overnight fast, a standard glucose challenge test (75 g glucose in 300 ml water) was performed with the experimental subjects receiving distilled water instead of the plant extract 0.5 h prior to glucose loading. Blood samples were collected before glucose loading and at 1 h and 2 h after glucose loading, for the estimation of serum glucose concentrations. The oral glucose challenge test was repeated on a subsequent day, with the same subjects receiving the extract of I. aquatica equivalent to 100 g of the fresh edible portion, 0.5 h prior to glucose loading. Glucose loading and blood sampling were performed as described above.

3.2.7 Experiments directed at the mechanism of action

3.2.7.i Determination of the serum insulin levels of the diabetic patients

Serum insulin levels were checked in the Type II diabetic patients who were included in the study described in section 3.2.6. Insulin levels in the serum were determined in the blood samples obtained 2 h post glucose load in the test and control studies. Once
collected, the serum was stored in −20°C until analysis. The analytical procedure was described in section 3.2.1.xiii.

3.2.7.ii Intestinal glucose absorption in the rats

Rats (n=10) were divided into 2 groups termed test and control with 5 in each group. After an overnight fast, the rats were subjected to a glucose challenge. It was preceded 0.5 h earlier by the administration of the whole extract of *I. aquatica* in the test group and distilled water in the control group (1 ml each). Two hours after the administration of the extract/water, the animals were euthanized with diethylether and intestines harvested. Blood was obtained by cardiac puncture for the determination of the serum glucose concentrations.

The intestines were packed in ice and transported within 30 min to the laboratory where they were opened up and the contents washed repeatedly with distilled water and made up to a final volume of 50 ml. The contents were centrifuged and the glucose concentration was determined in the supernatants.

3.2.8 Activity directed fractionation

3.2.8.ii Oral hypoglycaemic activity of the ethanol extract and the aqueous filtrate

Rats (n=36) were randomly divided into three groups; test I, test II and control with 12 rats in each. After an overnight fast, the test I group was administered the ethanol extract (1 ml each) at a dose of 3.4 g/kg body weight and test II was given the aqueous
filtrate (1 ml each). The control group was given distilled water (1 ml each). After 0.5
h, a glucose challenge was performed.

3.2.8.ii Fractionation by gel filtration chromatography

Sephadex G25 (25 g) was allowed to swell for 3 h, in 0.01 M phosphate buffer (pH 7.0).
The rotory evaporated and freeze-dried ethanol extract was dissolved in the same buffer
(1 ml). The extract was run through a glass, chromatography column (25 x 2.5 cm²),
containing Sephadex G25 using 0.01 M phosphate buffer (pH 7.0) as the eluent.
Fractions (2 ml each) were collected and pooled into five groups (fraction numbers; 1-
40, 41-70, 71-120, 121-160 and 161-200) and freeze-dried. All five fractions were
tested for the hypoglycaemic effect in rats.
The fractions were also subjected to thin layer chromatography (refer section 3.2.8.iv).

3.2.8.iii Effect of Sephadex-separated fractions on rats

Rats were divided into six groups viz., Fr1, Fr2, Fr3, Fr4, Fr5 (representing Sephadex
separated fractions) and control with 5 rats in each. After an overnight fast, respective
fractions were dissolved in distilled water and administered (1 ml each) to the five
groups (Fr1-Fr5) and phosphate buffer (pH 7.0, 1 ml each) was given to the control. A
glucose challenge followed.

3.2.8.iv Preparative thin layer chromatography with Fractions I and II

Pooled Fr I and II were freeze-dried. Chromatography plates (1 mm) were prepared
using Silica gel Gp254. The freeze-dried fractions were dissolved in distilled water and
streaked at the origin of the TLC plate. The plate was run in the solvent system of n-
butanol, ethanol and ammonia (BEN), in the ratio = 7:2:5. Spots identified by the anisaldehyde spray reagent were divided into three bands named Band 1, 2 and 3. These bands were scraped off and the contents were extracted into ethanol and the solvent was evaporated in a water bath at 80°C. The 3 residues thus obtained were reconstituted with deionized water for the animal experiments. Ortho-cyanidin test, Liebermann-Burchard test and the froth test were also performed on these compounds (refer section 3.2.9).

3.2.8 Effect of the extracted TLC bands on rats

Four groups of rats (6 in each) were named Band 1, Band 2, Band 3 and control. After an overnight fast, the groups 1, 2 and 3 were administered the respective bands, which were dissolved in distilled water, while the control was given distilled water (1 ml each). Glucose loading and blood sampling were performed.

3.2.9 Preliminary chemical tests

3.2.9.i Liebermann-Burchard test

The 3 bands (1 mg each) were dissolved in ethanol. Acetic anhydride (8 drops) was added to this followed by the addition of concentrated H₂SO₄. The colour of the solution was observed.
3.2.9.ii Ortho-cyanidin test

To alcoholic solutions of the 3 bands (1 mg each), concentrated HCl was added along the wall of the tubes. A few magnesium chips were put into each of the tubes and the colour of the froth and the solutions were observed.

3.2.9.iii Froth test

The 3 bands (1 mg each) were dissolved in 1 ml of distilled water and shaken vigorously for 5 min in test tubes. The tubes were allowed to stand and the height and the stability of the froth was observed.

3.2.10 Studies on Band I

3.2.10.i Determination of the approximate molecular weight of the active Band I

A gel column was repeated with the ethanol extract and the first 40 fractions (2 ml each) were collected. Aliquots of every other fraction, were subjected to TLC with BEN as the solvent system and sprayed with anisaldehyde spray reagent.

3.2.10.ii Testing Band I for the presence of sugars

Band I was subjected to TLC with chloroform and ethyl acetate in the ratio of 7:3. Phosphomolybdic acid spray reagent was used to detect the presence of sugars in the sample.
3.2.10.iii Preparative TLC with Band 1

Freeze-dried Band 1 was streaked on a preparative TLC plate (300 μ) and run in chloroform and ethyl acetate. The sub bands were extracted as described in 3.2.8.iv. The oral hypoglycaemic activity of the spots was tested in the rat model.

3.2.10.iv Effect of different sub bands on rats

Six groups of rats were termed sub band I, II, III, IV, V (representing extracted sub bands) and control, with 6 rats in each. The spots obtained in 3.2.10.iii, were dissolved in distilled water. The experiment was performed as described in 3.2.8.v.

3.2.10.v Medium pressure liquid chromatography

Pooled sub band 1 and 2 were separated further by MPLC. The sample was pre-adsorbed into column silica before being packed into the MPLC column (5.5 x 1.5 cm²). A solvent gradient of hexane -> chloroform -> ethylacetate -> methanol -> water with a dilution factor of 6, was used at a flow rate of 15 ml/min. Fractions (10 ml each) were collected and tested by TLC with n-butanol: glacial acetic acid: distilled water = 4:1:5, top layer (BAW), as the solvent system using 100 μm Silica gel Gp254 plates. The plates were also visualized under ultra violet light (365 nm).

The solvents were evaporated under reduced pressure and the active compounds were isolated. The compounds, MPLC 1 and MPLC 2 were obtained in sufficient quantities for further studies.
3.2.11 Attempts to elucidate the structure of compounds, MPLC 1 and MPLC 2

3.2.11.1 Determination of the class of flavonoid

Thin layer chromatography was performed on the compounds using BAW and Forestal (glacial acetic acid: concentrated HCl: distilled H₂O =10:3:30) solvent systems. The spots obtained were viewed under UV light (365 nm) and the O-cyanidin test was also performed (refer section 3.2.6.ii).

3.2.11.ii Enzyme hydrolysis

The isolated flavonoids were subjected to enzyme hydrolysis by naringinase where the isolates (50 mg each) were incubated with the enzyme (10 mg) at 37° C at pH 4.0 and 5.5. Aliquots of 5 µl were removed at 0 time, 6, 12 and 24 h after incubation and TLC was performed on them with BAW. Quercitrin, glucose and rhamnose were used as standards. The spots obtained were visualized under UV light (365 nm), prior to being sprayed using the anisaldehyde-sulphuric acid spray reagent.

3.2.11.iii Infrared spectroscopy of MPLC 1 and 2

The MPLC fractions that contained the MPLC 1 and 2 were pooled and the solvents removed in the rotary evaporator. The samples were stored in a desiccator with sulphuric acid as the dessicant until analysis. Both compounds and quercitrin (serving as the standard) were subjected separately to IR spectroscopy using a KBr disc (Shimadzu, Hyper IR, Japan). The spectra obtained were compared for the characteristic peaks.
3.2.11.iv Activity of the hydrolyzed and unhydrolyzed MPLC 1

The hydrolyzed and the unhydrolyzed MPLC 1 were tested in rats for the hypoglycaemic effect. Compound 1 (50 mg) was incubated with naringinase for 24 h for hydrolysis of the glycosidic bonds. The hydrolyzed flavone (aglycone) was extracted into ethyl acetate. The solvent was evaporated in the rotary evaporator at 40° C and the residue dissolved in distilled water. The unhydrolyzed MPLC 1 (50 mg) was also dissolved in distilled water.

Rats were divided into 3 groups termed test 1, test 2 and control with 8 rats in each. After an overnight fast, the unhydrolyzed MPLC 1 was administered to the test 1 group while hydrolyzed MPLC 1 (aglycone) was given to test 2 group. Distilled water (1 ml each) served as the control. Blood sampling and analysis was performed as earlier.

3.2.12 Identification of sugars released from the glycoside

The compound, MPLC 1 (50 mg and 10 mg respectively) was subjected to enzyme hydrolysis with:

(i) both naringinase (20 mg) and β-glucosidase (10 mg)

and

(ii) β-glucosidase (10 mg) alone.

The tubes (tube 1 with both the enzymes and tube 2 with only β-glucosidase) were incubated at 37° C and pH 5.5 for 24 h. The hydrolysate in tube 1 was extracted with 6 x 10 ml of ethyl acetate and the solvent was evaporated in the rotary evaporator. The
remaining solution of tube 1 and the hydrolysate of tube 2 were tested by TLC using BAW as the solvent system. Glucose and rhamnose were used as standards.

3.2.13 Infra red spectroscopy of the aglycone of MPLC 1

The aglycone of MPLC 1 obtained in 3.2.11.ii, was further purified by subjecting to preparative TLC, with BAW as the solvent system. The pure spot was extracted into ethyl acetate and the solvent was removed as described in 3.2.11.iv. The compound was stored in a desiccator over sulphuric acid. The aglycone and the standard flavones were subjected to IR spectroscopy (refer section 3.2.11.iii).

3.2.14 Ultra violet/visible spectroscopy of MPLC 1, MPLC 2 and quercitrin

The UV/visible spectra of MPLC 1, MPLC 2 and quercitrin in methanol were obtained using double beam, Shimadzu (UV 200, Japan) spectrophotometer with an automatic chart recorder.

3.2.15 Statistical analysis

The results are presented as mean±SEM. Analysis of variance (ANOVA) was tested in the Microsoft Excel and the results were further subjected to the Student’s t test. A p value of less than 0.05 was considered as significant.
4. RESULTS

4.1 Moisture content of *I. aquatica*

4.1.1 Dean and Stark method
The mean moisture content of the fresh plant material when subjected to the above method was 89.0±0.1%.

4.1.2 Drying to constant weight
When dried until constant weight, the moisture content of *I. aquatica* was 89.5±0.2%.

4.2 Experiments to outline the oral hypoglycaemic effect of the whole, aqueous extract and the shredded, fresh edible portion

4.2.1 Experiments on normoglycaemic, Wistar rats
These experiments were carried out to establish the oral hypoglycaemic activity of *I. aquatica* in normal rats. The optimal time as well as the dose, which exerted the highest hypoglycaemic activity was determined.

4.2.1.1 Effect of a single dose of the aqueous extract of *I. aquatica* on the serum glucose concentration in normal rats challenged with glucose
The effect of a single dose of *I. aquatica* extract on the serum glucose concentration in normal rats after a glucose challenge is given in figure 4.1. The 90 min post-glucose serum glucose concentration in the test group was 70.9±3.7 mg/dl while it was
106.8±3.6 mg/dl in the control group. There was a 33.6% reduction in the test group compared to the control and this reduction was statistically significant (p=0.0029).

4.2.1.iii Dose-response curve for *I. aquatica*

The dose-response curve is given in figures 4.2. The serum glucose concentrations with the doses of 1.7, 3.4, 5.1 and 6.8 g/kg were 92.3±4.2, 88.0±3.6, 90.0±3.5 and 103.4±2.9 mg/dl respectively. The glucose value for the control group was 122.5±4.3 mg/dl. The mean serum glucose concentrations of the different groups treated with the extract were lower than that of the control group. However, the dose, which exerted a significant hypoglycaemic effect (p=0.02913), was 3.4 g/kg. This dose was used in the other experiments.

4.2.1.iii Time course of *I. aquatica*

The maximum hypoglycaemic effect was shown at 2 h after the administration of the extract as shown in figure 4.3. Table 4.1 gives the results obtained for the time course. The reduction in the Test at 2 h post extract was 31.5% as compared with the control. In all the animal experiments with glucose challenge, blood was drawn 2 h after the administration of the plant extract.
Figure 4.1 Effect of a single dose (3.4 g/kg body weight) of *I. aquatica* on the serum glucose concentrations of test (plant extract) and control (distilled water) groups of healthy, male Wistar rats (test and control=10 per group) following a glucose challenge (3g/kg body weight)

A significant reduction in the test group (*p* = 0.002) was observed when compared with the control.
Figure 4.2  Dose-response curve of *I. aquatica* on administration of the whole extract at doses of 1.7, 3.4, 5.1 and 6.8 g/kg to 4 test groups and distilled water to the control group of healthy, male Wistar rats (5 rats per group) followed ½ h later by a glucose challenge (3g/kg body weight).

A significant reduction in the blood sugar levels was observed with dose of 3.4 g/kg body weight (*p* = 0.029).
<table>
<thead>
<tr>
<th></th>
<th>Zero time</th>
<th>First hour</th>
<th>Second hour</th>
<th>Third hour</th>
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<tr>
<td></td>
<td>Test</td>
<td>Control</td>
<td>Test</td>
<td>Control</td>
</tr>
<tr>
<td>Serum glucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>concentration (mg/dl)</td>
<td>58.4±</td>
<td>59.2±</td>
<td>89.2±</td>
<td>122.7±</td>
</tr>
<tr>
<td>% decrease in Test compared with Control</td>
<td>27.1 %*</td>
<td>31.5 %**</td>
<td>15.7 %***</td>
<td></td>
</tr>
</tbody>
</table>

n=10 per group

* Significantly different from Control at p=0.002 at 1 h after administration of plant extract

** Significantly different from Control at p=0.0002 at 1 h after administration of plant extract

*** Significantly different from Control at p=0.05 at 1 h after administration of plant extract

The maximum reduction in the blood sugar levels was observed at 2 h after the administration of the whole extract of *I. aquatica*.
Figure 4.3 Time course for a single dose (3.4 g/kg body weight) of *I. aquatica* showing the serum glucose concentrations at 1, 2 and 3 h following a glucose challenge (3 g/kg body weight) on healthy, male Wistar rats (10 rats each in test and control groups).

A significant reduction (p=0.002, 0.0002 and 0.05 respectively), in the blood sugar concentration of the test group at 1, 2 and 3 h after the administration of *I. aquatica* but not with the control group administered distilled water.
4.2.1.iv Effect of multiple doses of *I. aquatica* on serum glucose levels

The fasting serum glucose concentration of the test and the control were 57.0±1.7 and 54.0±2.4 while the values after glucose challenge were 59.7±2.7 and 79.7±1.7 mg/dl in the two groups. The reduction in the test compared to the control was 25.0% with a significance of p=0.02.

Since hypoglycaemic effect in normal rats was now established, it was decided to test the extract with a known hypoglycaemic agent, namely tolbutamide.

4.2.1.v Comparison with tolbutamide

The fasting blood glucose levels of the test, drug and control groups were 52.0±0.8, 50.1±7.9 and 54.0±12.2 mg/dl respectively (figure 4.4). The Test group, which was given the aqueous extract of *I. aquatica*, had the lowest serum glucose levels followed by the tolbutamide treated group. After the glucose challenge, the mean blood glucose concentration of the *I. aquatica* treated group (68.2±14.4 mg/dl) and the tolbutamide treated group (86.0±15.18 mg/dl) were significantly lower (p=0.0001 and p=0.0001 respectively) than that of the control group treated with distilled water (130.0±16.1 mg/dl). The decrease in the blood glucose level in the test group compared to that of the control was 47.5% whereas the decrease in the drug group was 33.8%. The effect exerted by the extract was not significantly different from that of tolbutamide (p=0.29).
Figure 4.4  Comparison of the oral hypoglycaemic activity of a single dose (3.4 g/kg body weight) of *I. aquatica* with that of tolbutamide (15 mg/kg body weight) on test, drug and control groups of healthy, male Wistar rats subjected to a glucose challenge (3 g/kg body weight)

n=8 per group

The hypoglycaemic effect exerted by *I. aquatica* (47.5%) is better than that of tolbutamide (33.8%).

* p=0.0001 when compared with Control

* p=0.0001 when compared with Control
Having confirmed hypoglycaemic effect of *I. aquatica* in normoglycaemic Wistar rats, the oral hypoglycaemic effect was tested in diabetic Wistar rats to determine whether the same activity could be seen in diabetic rats as well.

### 4.2.2 Studies on diabetic rats

#### 4.2.2.i Effect on the streptozotocin-induced diabetic rats

As shown in figure 4.5, the fasting serum glucose concentration of the test group was 178.0±37.7 mg/dl while it was 346.1±4.2 mg/dl in the control group. Prior to feeding with *I. aquatica* the fasting serum glucose values were 437.2±3.6 mg/dl and 458.3±4.1 mg/dl in the test and control respectively (p=0.3859). The reduction in the test after feeding with *I. aquatica* for one week was statistically significant (p=0.0108).

The body weights at the start of the experiment were 237.0±4.0 and 241.0±9.0 g in the test and control groups respectively. At the end of the experiment, the body weight of the test group was 240.0±12.0 g whereas it was 215.0±16.0 g in the control. The prevention of the reduction in the body weight of the streptozotocin-induced diabetic rats by *I. aquatica* was significant (p=0.0003).

#### 4.2.2.ii Effect on the alloxan- diabetic rats (figure 4.6)

The mean blood glucose level of the rats before the induction of diabetes was 54.1±12.2 mg/dl. Prior to the experiment, the fasting serum glucose concentrations were 376.0±22.2 mg/dl and 393.1±19.0 mg/dl in the test and the control respectively. The fasting serum glucose concentrations at the end of one week were 63.2±10.1 mg/dl and 85.1±12.2 mg/dl where as after the glucose challenge the values were 90.2±19.3 mg/dl.
and 133.3±11 mg/dl in the test and control respectively. The fasting (p=0.002) and the post glucose (p=0.0002) serum glucose values were significantly lower in the test group when compared with the control.

The body weights of the test and control groups at the start of the experiment were 208.0±4.0 g and 202.0±12.0 g (p=0.2537) respectively while they were 209±9.0 and 191.0±16.0 g at the end of the experiment. The reduction of the body weight in the control group was significant (p=0.0147), when compared with the test (figure 4.7).

At this point it could be argued that the oral hypoglycaemic activity of *I. aquatica* could be attributed to the dietary fibre present in the plant. It was essential to check whether the effect was exerted, by the dietary fibre only or by any other component also.

### 4.3 Effect of dietary fibre on the hypoglycaemic activity

Dietary fibre, equivalent to that of a dose of *I. aquatica* was extracted by modifying the protocol of Asp *et al.*, (1983). The concentrations of soluble dietary fibre and insoluble dietary fibre in the fresh edible portion were 7.0 and 2.2% respectively.

The mean serum glucose concentration of the rats treated with SDF, IDF, the whole edible extract and the control are shown in figure 4.8. There was a 45.3% reduction with the whole extract compared with control while there were 19.0% and 13.0% reductions in the SDF and IDF treated groups, respectively when compared with the control. The ANOVA showed, F=73.835 and p=2.06 E-13.
Figure 4.5  Effect of feeding the shredded, fresh, edible portion of *I. aquatica* for 7
days on the fasting serum glucose concentrations of streptozotocin-
induced (30 mg/kg body weight) diabetic rats

n=6 per group

The fasting blood sugar level of the rats fed with the plant material is significantly
lower than that of the control group ($^t$, $p=0.0108$)
Figure 4.6: Effect of the shredded, fresh, edible portion of *I.* *aquatica* (3.4 g/lg body weight) on the fasting serum glucose concentration and at 2 h following a glucose challenge on alloxan-induced (40 mg/kg body weight) diabetic rats fed shredded *I.* *aquatica* in addition to the standard feed (test) and control (standard feed only) with 10 rats in each.

Both the fasting and the post-glucose-load glucose concentrations were significantly reduced by *I.* *aquatica*.

\* p=0.002

\*\* p=0.0002
Figure 4.7 Body weights at the start and the end of the experimental period (10 days) of diabetic, Wistar rats (induced by intra venous injection of 40 mg/kg body weight of alloxan and 30 mg/kg body weight of streptozotocin) following administration of the fresh edible portion of *I. aquatica* (3.4 g/kg body weight) to the test group in addition to the standard diet and standard diet only to the control group.

*I. aquatica* was able to antagonize the weight loss in alloxanized diabetic rats as shown by the higher weights in both the test groups when compared to the control groups.

\^p = 0.0147 when compared with the control in alloxan-induced diabetic rats

\$p = 0.0003 when compared with the control in streptozotocin induced diabetic rats
Figure 4.8 Effect of the whole extract (WE), soluble dietary fibre (SDF) and insoluble dietary fibre (IDF) of *L. aquatica* on the serum glucose concentrations of healthy, male Wistar rats, challenged with glucose. The doses used were 3.4 g/kg/body weight of WE and SDF and IDF in doses equivalent to a single dose of the WE. Distilled water was given to the control group.

n=8 per group

* p=0.00001 when compared with Control

* p=0.0001 when compared with Control

* p=0.001 when compared with Control
It was confirmed that an active compound was present in the plant. Since the activity was established in diabetic rats as well, it was decided to check the plant for any hepato-renal toxicity before embarking on studies with human subjects.

4.4 Effect of the fresh edible portion on serum analytes

4.4.1 Effect on the key hepatic enzymes viz., alkaline phosphatase, alanine aminotransferase, aspartate amino transferase and \( \gamma \) glutamyl transpeptidase (table 4.2)

The alanine aminotransferase levels in the test and the control groups were 46.4±1.0 U/l and 45.8±1.8 U/l respectively while the aspartate aminotransferase concentrations were 27.2±1.4 U/l and 29.9±4.3 U/l. The \( \gamma \) glutamyl transferase values were 4.0±0.4 U/l and 4.2±0.4 U/l in the test and the control groups respectively. There was no significant difference in the above value between the test and the control group. However there was a significant reduction (\( p = 0.04 \)), in the concentration of the alkaline phosphatase levels of the test group (68.4±7.0 U/l) compared to the control group (102.0±11.0 U/l).

4.4.2 Effect on uric acid levels

The uric acid levels of the test and control groups were 2.8±0.1 mg/dl and 2.9±0.1 mg/dl respectively. These values were not significantly different from each other.
### Table 4.2
Key hepatic enzymes and uric acid levels of Wistar rats fed with the fresh, edible portion of *I. aquatica* for 8 weeks

<table>
<thead>
<tr>
<th>Test (U/l)</th>
<th>Control (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/l)</td>
<td>27.2±1.4</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>46.4±1.0</td>
</tr>
<tr>
<td>ALP (U/l)</td>
<td>68.4±7.3$^\dagger$</td>
</tr>
<tr>
<td>$\gamma$ GT (U/l)</td>
<td>4.0±0.4</td>
</tr>
<tr>
<td>Uric acid (mg/dl)</td>
<td>2.8±0.1</td>
</tr>
</tbody>
</table>

$n=12$ per group

$^\dagger$ p=0.04 when compared with Control
4.4.3 Effect on the glycated haemoglobin levels in the rats after long-term feeding

The glycated Hb value of the test group was 3.9±0.5 % while it was 4.8±0.7 % in the control group. The reduction in the Test was significant (p=0.01) when compared with the control.

Except for the alkaline phosphatase levels, which were significantly lower (p=0.04), in the test group, there was no difference between the two groups in the parameters studied after feeding the plant material for 8 weeks. *Ipomoea aquatica* did not show any toxic effects on the liver and kidney of healthy, Wistar rats. From the foregoing tests, the plant material appeared to be safe to use in the human diabetic patients.

4.5 Studies on diabetic patients (figure 4.9)

The mean fasting serum glucose levels of the test and control groups were 149.0±2.7 mg/dl and 147.6±8.7 mg/dl respectively. (p=0.904). Percentage increases in glucose levels at 1 h were 186.3±12.1% and 198.5±11.7% in the test and control respectively.

At 2 h post glucose, the percentage increases in the serum glucose concentrations of the test and control studies were 154.6±13.0% and 184.0±15.0% respectively (figure 4.9). There was a 29.4 % decrease in the serum glucose of the diabetic patients when treated with the plant extract. A significant difference was observed in the serum glucose levels of the test and control studies at 2 h (p=0.001) and it was not significant at 1 h (p=0.09).
Figure 4.9  Effect of *I. aquatica* on the serum glucose concentrations of newly diagnosed Type II diabetic patients subjected to a glucose challenge (75g in 300 ml) following the administration of a single dose of *I. aquatica* (100 g/100 ml) to test and distilled water (100 ml) to control n=20 per group

Aqueous extract of *I. aquatica* significantly reduced the serum glucose concentrations of the Type II diabetics at 2 h after the administration of the extract (p=0.001).
4.6 Mode of action of the whole extract

4.6.1 Effect on insulin levels of Type II diabetic patients (figure 4.10)

The serum insulin levels of the Type II diabetics at 2 h post glucose load were 23.7±1.5 and 29.5±2.7 μmol/l in the test and control, respectively. There was a significant reduction (p=0.001) in the insulin level of the test study where the patients were administered an oral dose of the whole, edible extract of *I. aquatica*. The aqueous extract of *I. aquatica* may have enhanced the binding of insulin to its receptor.

4.6.2 Effect on intestinal glucose uptake in rats

The glucose concentrations of the intestinal contents after the glucose challenge were 12.7±1.3 and 29.3±2.1 mg/dl in the test and control groups respectively where as the glucose concentrations in the serum were 100.0±4.7 and 156.9±13.8 mg/dl, in the two groups respectively (figure 4.11). The reduction of glucose levels in the intestinal contents as well as the serum in the test group was statistically significant (p=0.0003 and p=0.02 respectively).

The whole extract had enhanced the absorption of glucose in the intestine as shown by the lower level of glucose in the intestinal contents of the test group. The glucose taken into the body had been effectively removed from the circulation since the concentration of glucose in the serum of the test group is significantly lower that of the control.
Effect of *I. aquatica* on the serum insulin concentrations of newly diagnosed Type II diabetic patients subjected to a glucose challenge (75g in 300 ml) following the administration of a single dose of *I. aquatica* (100 g/100 ml) to test and distilled water (100 ml) to control

n=20 per group

The serum insulin levels of type II diabetics treated with *I. aquatica*, was significantly lower than the control values (p=0.001).
Figure 4.11  Effect a single dose the whole, aqueous extract of *L. aquatica* (3.4 g/kg/body weight) on the intestinal glucose absorption of healthy, male Wistar rats subjected to a glucose challenge (3 g/kg)

n=5 per group

* p=0.02 when compared with the control group

* p=0.0003 when compared with the control group

*L. aquatica* enhanced the absorption of glucose in the intestine and at the same time, removed the absorbed glucose effectively from blood.
4.7 Activity guided fractionation

As the first step in activity directed fractionation, it was necessary to determine the type of solvent that could be used for extraction. The oral hypoglycaemic activity of the ethanol extract and the aqueous filtrate was tested on rats with distilled water as the control.

4.7.1 Oral hypoglycaemic effect of the ethanol extract and the aqueous filtrate

When subjected to a glucose challenge, the mean serum glucose concentrations of the ethanol extract, aqueous filtrate and distilled water treated groups were 71.7±5.1, 94.5±5.5 and 99.5±8.5 mg/dl respectively. The reduction given by the ethanol extract was significantly lower when compared with the control (p=0.003). The aqueous filtrate did not cause a significant hypoglycaemic effect (p=0.167).

The results clearly show that the active fraction is ethanol soluble with perhaps sparing solubility in water. The fact that the ethanol extract showed activity also clearly shows that this effect is not due to dietary fibre.

The next step was to fractionate the ethanol extract after evaporation of ethanol. Sephadex G25 was selected as the gel for gel filtration chromatography techniques.
Table 4.3  Effect of different extracts of *I. aquatica* on the blood glucose levels of Wistar rats subjected to a glucose challenge

<table>
<thead>
<tr>
<th>Group</th>
<th>Ethanol extract</th>
<th>Aqueous filtrate</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean serum glucose</td>
<td>71.7 ± 5.1*</td>
<td>94.5 ± 5.5</td>
<td>99.5 ± 8.5</td>
</tr>
<tr>
<td>concentration (mg/dl)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n=12 per group

* Significantly different from control at p=0.003

A reduction of 24.1% was observed in the group fed with the ethanol extract.
4.7.2 Effect of pooled chromatography fractions on serum glucose levels

The Sephadex fractions were pooled into 5 groups in the order of elution and termed Fraction I to fraction V.

Table 4.4 shows the effect of different chromatography fractions on serum-glucose values of healthy male Wistar rats challenged with glucose. The ANOVA showed F=5.599 and p=0.0007. Fractions I (p=0.0001) and II (p=0.009) showed a significant hypoglycaemic effect.

As it was clear that most of the hypoglycaemic activity resided in Fractions I and II, further studies were conducted with pooled, Fr I (eluent fractions 1-40) and II (eluent fractions 41-70).

4.7.3 Thin layer chromatography with Fr I and II

The pooled Fr I and II, were freeze dried. The residue on dissolving in methanol (100 μl) was subjected to TLC and spraying with anisaldehyde-sulphuric acid resulted in 7 spots (Figure 4.12). These spots were divided into three bands viz., Band I - III depending on the R_f values on the TLC.

As noted from figure 4.12, the bands did not comprise pure compounds as the spots did not give a single spot on TLC.
Table 4.4  Effect of different chromatography fractions of *L. aquatica* on blood sugar levels of glucose loaded Wistar rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Fr I</th>
<th>Fr II</th>
<th>Fr III</th>
<th>Fr IV</th>
<th>Fr V</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum glucose concentration (mg/dl)</td>
<td>78.3* ±</td>
<td>68.7** ±</td>
<td>111.6 ±</td>
<td>84.8 ±</td>
<td>102.9 ±</td>
<td>106.2 ±</td>
</tr>
<tr>
<td>n=5 per group</td>
<td>2.1</td>
<td>9.1</td>
<td>9.2</td>
<td>8.2</td>
<td>2.8</td>
<td>9.2</td>
</tr>
</tbody>
</table>

* Significantly different from Control at p=0.0001

** Significantly different from Control at p=0.009

Fr I and II contained the active compounds of *L. aquatica*. 
4.7.4 Effect of different TLC bands on the blood sugar levels of Wistar rats

Band 1 contained the largest spot at the bottom of the plate with a very low R_f value (0.05-0.36). Band 2 (R_f=0.44-0.55) comprised of the next three very close spots at the middle of the plate and band 3 (R_f=0.66-0.75) contained the three very close spots at the top of the plate.

As shown in figure 4.13, the mean serum glucose levels of rats treated with Band 1, Band 2 and Band 3 were 88.6±6.3, 94.0±10.9, and 110.2±8.9 mg/dl respectively while the control group showed a serum glucose value of 120.9±6.4 mg/dl. A significant reduction in the glucose concentration was given only by, Band 1 (p=0.0004).

Activity directed fractionation was therefore carried out only on Band 1.

4.7.5 Chemical tests on different bands

4.7.5.1 Liebermann-Burchard's test

Bands 2 and 3 showed a dark brownish yellow colour when subjected to the Liebermann- Burchard test. This colour is characteristic of triterpenoids (Fong 1967).
Figure 4.12 Diagrammatic representation of spots obtained by TLC on Fr I and Fr II obtained by gel filtration chromatography with Sephadex G_{25}, of the alcoholic extract

Solvent system = n butanol: ethanol: ammonia = 7:2:5

Spray reagent = Anisaldehyde/sulphuric acid

Band 1 comprised one large spot while Bands 2 and 3 consisted of three spots each.
Figure 4.13  Effect of different compounds contained in Band 1, Band 2 and Band 3, obtained from the preparative TLC of Fr I and FrII on the serum glucose concentrations of Wistar rats subjected to a glucose challenge (3 g/kg body weight)

n=6 per group

* Significantly different from control at p=0.0004

The active compounds are contained in Band 1.
4.7.5.ii O-cyanidin test (Table 4.5)

This test showed a dark orange colour, which indicated the presence of flavones in only band 1 (Fong et al., 1967).

4.7.5.iii Froth test

The froth test yielded a froth height of 10 mm, which was stable only up to 15 min. The reaction was observed in the tubes with bands 2 and 3 only.

As it was clear that the activity resided in Band 1, studies on further purification was carried out with this band, which indicated the presence of flavonoids.

4.7.6 Studies with Band 1

It was important to know whether the active compound was a large flavonoid or a relatively small one. The appearance of the compound in the chromatography fractions were tested.

4.7.6.1 Approximate molecular weight of the active Band 1

It was observed that the steroids and saponins eluted before the elution of the flavonoids. Flavonoid spot appeared in the 23\textsuperscript{rd}-35\textsuperscript{th} fractions while the steroids and saponins were eluted in the 15\textsuperscript{th}-21\textsuperscript{st} fractions when subjected to gel filtration chromatography. Since a steroid would usually have a molecular weight of approximately 400 D, the active flavonoid should have a molecular weight of $<400$ D (figure 4.14). The elution pattern corresponded to a flavonoid with a small molecular weight flavonoid; $<400$ D.
The active band, which tested positive for flavonoids, was readily soluble in water even though water is not the best solvent to dissolve flavonoids since these are non-polar compounds. The presence of sugars attached to the flavonoid nucleus was suspected at this point and the active compound was tested for sugars.

4.7.6.ii Testing for the presence of sugars in Band 1

When sprayed with the phosphomolybdic acid spray reagent, the TLC of Band 1 run in chloroform and ethyl acetate showed grayish blue spots confirming the presence of sugars (figure 4.15). The relatively slow movement of the active compound in the solvent system could be explained here since the presence of sugar moieties would hinder the movement in a non-polar solvent. The readily soluble nature of the active compound in water could be attributed to the attached sugars with perhaps, many OH groups.

4.7.6.iii Preparative TLC with Band 1 (figure 4.16)

When preparative TLC was conducted with ethyl acetate and chloroform, 5 distinct sub bands were obtained from Band 1. The $R_f$ values of sub bands I-V are given in table 4.6.
Table 4.5  Characteristic features of different groups of flavonoids

<table>
<thead>
<tr>
<th>Class</th>
<th>Colour reaction with Mg$^{2+}$-hydrochloric acid (O-cyanidin test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthocyanins</td>
<td>Reddish pink</td>
</tr>
<tr>
<td>Flavones</td>
<td>Yellowish orange</td>
</tr>
<tr>
<td>Flavanols</td>
<td>Reddish magenta</td>
</tr>
<tr>
<td>Flavanones</td>
<td>Red, magenta, violet and blue</td>
</tr>
<tr>
<td>Isoflavones</td>
<td>Yellow</td>
</tr>
<tr>
<td>Leucoanthocyanins</td>
<td>Pink</td>
</tr>
</tbody>
</table>

Source: Fong et al., 1967

Band 1 yielded a dark orange colour indicating the presence of flavones.
Figure 4.14  Diagrammatic representation of the TLC of the active Fr I and II obtained from gel filtration chromatography with Sephadex G25, to determine the relative molecular weight of Band 1

Solvent system = n-butanol: ethanol: ammonia = 7:2:5

The flavonoid faction appeared in the 15th–35th fractions.

Yellow fluorescence observed with the flavonoid spots.
Under UV light (365 nm) After being sprayed with phosphomolybdic acid spray reagent

Figure 4.15 Diagrammatic representation of the TLC to confirm the presence of sugars in active Band I obtained by preparative TLC of Fr I and II

Solvent system = chloroform : ethylacetate = 7:3

The bluish gray spot obtained, suggested the presence of sugars.

Yellow fluorescence observed at the origin.
4.7.6.iv Oral hypoglycaemic effect of the different sub bands obtained from Band 1

When tested on the rats, 4 out of 5 sub bands showed significant oral hypoglycaemic effect after the glucose challenge (figure 4.17).

The mean serum glucose concentrations obtained were 54.2±2.3, 48.7±2.6, 74.3±9.4, 81.0±10.2, 164.2±8.1 mg/dl in the sub band I, II, III, IV and V groups, respectively while it was 146.2 ± 16.0 mg/dl in the control group. The reduction in the test groups of sub band I (p=0.00043), sub band II (p=0.000715), sub band III (p=0.002147) and sub band IV (p=0.005176) were significant when compared to the control. Sub band V did not exert such an effect (p=0.446849). The sub band, which exerted the maximal hypoglycaemic effect was sub band 2 followed by sub band 1.

When subjected to further purification by TLC, only sub band 2 appeared to be pure. Sub band 1 consisted of 2 very close spots while the other 2 active compounds comprised more than 2 spots. The second spot found in sub band 1 was found to be sub band 2, itself when purified further by TLC. It was decided to proceed with sub bands 1 and 2.
Figure 4.16  Diagrammatic representation of the preparative TLC of active Band 1
obtained by preparative TLC of Fr I and II

Solvent system used was chloroform: ethylacetate = 7: 3.

Five sub bands were obtained of which sub band I was the largest and sub band II was
the smallest.
Table 4.6  Characteristics of sub bands obtained by preparative TLC of Band I

<table>
<thead>
<tr>
<th>Sub band</th>
<th>$R_f$</th>
<th>Texture</th>
<th>Yield (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.05</td>
<td>Dark brown paste</td>
<td>0.677</td>
</tr>
<tr>
<td>2</td>
<td>0.2</td>
<td>White powder</td>
<td>0.092</td>
</tr>
<tr>
<td>3</td>
<td>0.4</td>
<td>Yellow powder</td>
<td>0.067</td>
</tr>
<tr>
<td>4</td>
<td>0.6</td>
<td>Brownish yellow powder</td>
<td>0.054</td>
</tr>
<tr>
<td>5</td>
<td>0.96</td>
<td>Light yellow powder</td>
<td>0.036</td>
</tr>
</tbody>
</table>
Figure 4.17 Effect of different sub bands obtained from preparative TLC of active Band 1 on the serum glucose concentrations of healthy, male Wistar rats subjected to a glucose challenge (3 g/kg bodyweight)

n=6 per group

\* p=0.0004 when compared with Control

\* p=0.0007 when compared with Control

\* p=0.005 when compared with Control

\* p=0.002 when compared with Control

Except for sub band 5, all the other sub bands exerted significant hypoglycaemic activity.
4.7.7 Medium pressure liquid chromatography

It was clear that a better separation technique was needed than TLC. Then MPLC was resorted to.

Sub bands I and II were separated by MPLC on the solvent system given in section 3.2.7.v. Sub band I eluted with methanol and water in the ratio of 99.168: 0.832, while sub band 2 eluted with ethyl acetate and methanol in the ratio of 93.7: 6.3. This showed that sub band I was more polar than sub band II and perhaps had more sugar residues.

On concentration of the compounds sub band I yielded the yellow compound, MPLC 1 (550 mg) while sub band 2 gave a white compound, MPLC 2 (70 mg) from 100 g of the fresh edible portion of I. aquatcia.

4.7.8 Hypoglycaemic activity and the structural characteristics of the isolates of MPLC

4.7.8.1 Determination of the class of flavonoid

The O-cyanidin test on the 2 isolates yielded brownish yellow colour in MPLC 1 and brownish red in MPLC 2. This suggests that the MPLC 1 may be a flavone while MPLC 2 may be a flavonol. (Fong et al., 1967)

When subjected to TLC with the Forestal solution, a yellow fluorescence was observed at $R_f=0.8$ with MPLC 1 where as no fluorescence was observed with MPLC 2, which appeared at $R_f=0.95$. The absence of a carbonyl group in MPLC 2 was indicated by this result.
The BAW chromatogram showed a yellow fluorescence at the origin of the plate, as well as at the spot at R = 0.45, with MPLC 1. The compound did not run higher. The reason was probably the presence of sugars as indicated on TLC or any other group attached to the flavonoid. No fluorescence was given by MPLC 2 which appeared at the top of the plate at R = 0.9 (figure 4.18).

4.7.8.ii Infrared spectroscopy of the isolates

The isolates, MPLC 1 and 2 were dried over concentrated H₂SO₄, for 21 days and subjected to IR spectroscopy. Quercitrin (flavonoid glycoside, 3,3',4',5',7-pentahydroxyflavone-3-L-rhamnoside) was used for comparison as a standard. The spectra obtained are shown in figures 4.19, 4.20 and 4.21.

As seen from the spectra, interpretation is difficult due to the presence of large Hydrogen-bonded OH moieties. This is indicative of the presence of sugar moieties. However the peaks were sharp showing that the isolates were relatively pure.

The yellow compound (MPLC 1) had a carbonyl stretching at 1634 cm⁻¹ (cf. quercitrin stretching at 1659 cm⁻¹). The white compound (MPLC 2) had no evidence of a carbonyl stretching and was probably a flavanol derivative. The finger print regions, 1000–1500 cm⁻¹ were similar enough to conclude that the standard and the unknown belonged to the same group of compounds, i.e., flavonoid glycosides.
MPLC 2 showed a higher mobility in both solvent systems while MPLC 1 moved slower.
It was next decided to study the nature of sugars by enzymatic cleavage and identification of the sugars released by comparison with standards.

This step was logical since,

(i) the removal of sugars would give a clear IR spectrum and,

(ii) it may give some idea as to the identification and positioning of the carbohydrate moieties.

4.8 Hydrolysis of the isolates

4.8.1 Hydrolysis of MPLC 1

On subjecting the MPLC 1 to naringinase and β-glucosidase, two sugars were released.

This showed the presence of free glucose and free rhamnose on hydrolysis with naringinase and glucose on hydrolysis with β-glucosidase. A fluorescent spot with a high Rf was obtained which is presumably the aglycone. The sugars released from the compounds showed lower Rf values than the standard glucose and rhamnose. An intermediate glycoside was observed with MPLC 1.

These results are interpreted as the presence of glucose and rhamnose (both in β linkage) moieties either on; (i) different OH groups or (ii) with the rhamnose linked terminally to glucose in a disaccharide moiety (see discussion).
Figure 4.19  Infra red spectrum of MPLC 1
4.8.2 Hydrolysis of MPLC 2

When subjected to cleavage by naringinase and β-glucosidase, the MPLC 2 yielded free rhamnose only indicating it to be a flavonol mono glycoside.

4.8.3 Activity of the hydrolyzed and unhydrolyzed MPLC 1 in rats

The enzyme hydrolyzed (18 mg resulting from the hydrolysis of 50 mg) and the non-hydrolyzed (50 mg), MPLC 1 was tested in rats for the oral hypoglycaemic effect. When subjected to a glucose challenge, there was a significant reduction in the blood glucose levels of the group fed with the non-hydrolyzed spot (p=0.006). There was no significant reduction in the group fed with the hydrolyzed spot (p=0.147) when compared with the control group, which was given distilled water. The mean glucose concentrations in the three groups are shown in the table 4.7.

The results indicate that the sugar moieties are necessary for the exertion of the oral hypoglycaemic effect.

4.8.4 Infrared spectroscopy of the aglycone of MPLC 1

Hydrolysis of the yellow MPLC 1 (50 mg) using naringinase yielded a yellow solid (18 mg). The IR spectrum of this aglycone (figure 4.22) was compared with those of a penta-hydroxy flavone (figure 4.23) and an unsubstituted flavone dihydrate with no OH groups (figure 4.24). A clear carbonyl group is shown at 1725 cm⁻¹ to 1664 cm⁻¹ of the penta-hydroxy flavone and 1645 cm⁻¹ of the flavone unsubstituted.

The aglycone is probably a tri-hydroxy flavone where the three OH groups are widely separated and therefore are not H-bonded. The flavone carbonyl is very much like that
of the standard. The finger print region is not very describable and it may not be 100 %
pure.

4.9 Ultra violet/visible spectra of MPLC 1, MPLC 2 and quercitrin

(figures 4.25, 4.26, 4.27 and 4.28)

Both the yellow (MPLC 1) and the white (MPLC 2) compounds showed strong UV
absorption which were similar. In the visible region, the yellow compound (MPLC 1)
had an absorbance without a peak (375-450 nm). Quercitrin showed a definite peak for
yellow at \( \lambda \) max, 400 nm where as MPLC 2 had no absorbance in the visible region.
The UV/visible absorbance peaks were comparable and this shows that conclusions of a
substituted flavone glycoside and a substituted flavonol glycoside were justifiable.
Table 4.7  Effect of the hydrolyzed and non-hydrolyzed MPLC 1 on the Serum glucose concentrations of rats subjected to a glucose challenge

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean glucose concentration (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test 1 (non-hydrolyzed spot)</td>
<td>81.1±3.3*</td>
</tr>
<tr>
<td>Test 2 (hydrolyzed spot)</td>
<td>96.0±4.3</td>
</tr>
<tr>
<td>Control</td>
<td>110.6±8.9</td>
</tr>
</tbody>
</table>

n=8 per group
* Significantly different from Control at p=0.006

Sugars are essential for the hypoglycaemic activity of *I. aquatica*
Figure 4.22 Infra red spectrum of the aglycone of MPLC 1
Figure 4.23  Infra red spectrum of the penta-hydroxy flavone
Figure 4.25  Ultra violet/visible spectrum of MPLC 1

Figure 4.26  Broad absorption given by MPLC 1 in the yellow region
Figure 4.27  Ultra violet/visible spectrum of MPLC 2

Figure 4.28  Ultra violet/visible spectrum of quercitrin
5. DISCUSSION

Many plants are reputed to have hypoglycaemic activity. This is discussed in the review of literature (Tissera and Thabrew, 2001). The documentation regarding the anti-diabetic effect of *Ipomoea aquatica* is found in the compilation of Jayaweera (Jayaweera 1982). It is stated that, according to the indigenous medicinal system of Sri Lanka, the plant is supposed to possess an insulin-like principle.

The objectives of the study were the following:

- to determine the oral hypoglycaemic activity of *I. aquatica*
- to determine whether the hypoglycaemic effect was due to dietary fibre or any other active constituent
- to determine the type of active extract
- to compare the hypoglycaemic effect with a known hypoglycaemic agent
- to determine the effect on diabetic rats as well as NIDDM patients
- to determine possible toxicity, judging by the serum enzyme and uric acid levels
- to determine the possible mode of action
- to determine some structural characteristics
Table 5.1  Examples of some commonly consumed plant materials, used in the
treatment of diabetes mellitus

<table>
<thead>
<tr>
<th>Botanical name</th>
<th>Common name</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aegle marmelos</em></td>
<td>Bael fruit</td>
<td>Ponnachan et al., (1993)</td>
</tr>
<tr>
<td><em>Allium cepa</em></td>
<td>Onion</td>
<td>Shiela et al., (1995)</td>
</tr>
<tr>
<td><em>Brassica rapa</em></td>
<td>Turnip</td>
<td>Farnsworth and Segelman (1971)</td>
</tr>
<tr>
<td><em>Capsicum frutescens</em></td>
<td>Pepper</td>
<td>Farnsworth and Segelman (1971)</td>
</tr>
<tr>
<td><em>Citrus limonum</em></td>
<td>Lemon</td>
<td>Law (1970)</td>
</tr>
<tr>
<td><em>Juniperus communis</em></td>
<td>Juniper</td>
<td>De Medina et al., (1994)</td>
</tr>
<tr>
<td><em>Lactuca sativa</em></td>
<td>Lettuce</td>
<td>Lewis (1949)</td>
</tr>
<tr>
<td><em>Pisum sativum</em></td>
<td>Pea</td>
<td>Lewis (1949)</td>
</tr>
<tr>
<td><em>Psidium guajava</em></td>
<td>Guava</td>
<td>Perry (1980)</td>
</tr>
<tr>
<td><em>Pyrus communis</em></td>
<td>Pear</td>
<td>Perry (1980)</td>
</tr>
<tr>
<td><em>Pyrus malus</em></td>
<td>Apple</td>
<td>Grieve (1976)</td>
</tr>
<tr>
<td><em>Rubus fructicosus</em></td>
<td>Blackberry</td>
<td>Farnsworth and Segelman (1971)</td>
</tr>
<tr>
<td><em>Solanum tuberosum</em></td>
<td>Potato</td>
<td>Lewis (1949)</td>
</tr>
<tr>
<td><em>Syzigium cumini</em></td>
<td>Black plum</td>
<td>Singh et al., (1990)</td>
</tr>
<tr>
<td><em>Syzigium jambos</em></td>
<td>Java plum</td>
<td>Mukerje (1953)</td>
</tr>
<tr>
<td><em>Trigonella foenum-graecum</em></td>
<td>Fenugreek</td>
<td>Abdel-Barry et al., (1997)</td>
</tr>
</tbody>
</table>
I. aquatica is an aquatic plant and contains 89% of water. The results showed that a single dose of the aqueous extract could improve glucose tolerance in healthy, Wistar rats (figure 4.1) proving it has acute hypoglycaemic activity. A reduction of 33.6% was observed in the test group when compared with the control.

Various plants that have been scientifically tested show, varying degrees of hypoglycaemic activity. For example, the percentage reduction given by a single dose of M. charantia in Sprague–Dawley rats, is 30.0%. The effect given by a particular extract depends on the dose used and the animal model tested and therefore cannot be compared with another unless the conditions are similar.

When the dose curve was determined for the plant extract, it was noted that all the doses (1.7, 3.4, 5.1 and 6.8 g/kg body weight) tested showed a reduction in the serum glucose levels but the dose that exerted a significant hypoglycaemic activity (p=0.029) was 3.4 g/kg body weight. This dose is comparable to approximately double the amount of this green leafy vegetable eaten during a meal (in the form of a tempered salad) by a 50 kg man, which is about 80 g. This approximate intake was determined by taking the mean weight of the cooked vegetable eaten by healthy adults.

The optimal time of the hypoglycaemic action was found to be 2 h (31.5% reduction in the blood sugar levels), from the time of administration of the plant extract. At 3 h after the administration also, there was a significant reduction in the blood sugar levels but the percentage reduction was lower (15.7%). The optimal time of activity of the aqueous extract of Hygrophila longifolia also has been found to be 2 h after the administration of the extract (Fernando et al., 1998) whereas with the dried root extract of Paeonia lactiflora, the maximum activity was given at 25 min after administration (Hsu et al., 1997).
The optimal time of activity being 2 h, could imply that the activity of the extract is of short duration. The long acting nature of the aqueous extract is evident with the results obtained in the multiple dose experiment. Here, the glucose challenge test was performed approximately 22 h after the administration of the last dose and still a significant reduction (25.0%), in the serum glucose levels was observed in the test group in comparison with the control. The percentage decrease in the blood sugar level of the test compared with the control, in this experiment, was lower than the reduction observed in the single dose experiment. The plant extract was not administered to the test group on the day of the experiment and it could be the reason for the above observation. The chronic hypoglycaemic effect of the shredded fresh edible portion of the plant was observed when the rats were allowed to eat the plant material at a dose of 4.0 g/kg body weight for eight weeks. The treatment resulted in a reduction of 26.1% (p=0.004) in the fasting blood sugar level of the test group in comparison to the control.

Since *I. aquatica* contains 9.2% of total fibre, it could be argued that the hypoglycaemic effect was in part due to fibre. The method of Asp *et al.*, (1983) was modified to isolate the dietary fibre where incineration at 550° C was omitted and the SDF and the IDF were collected separately. The argument that the oral hypoglycaemic activity is only due to the fibre was disproved by two ways.

Firstly, administration of the fibre isolated from *I. aquatica* equivalent to a typical dose showed lesser hypoglycaemic effect than the whole extract. There was a 45.3% reduction with the whole extract compared with control whereas only 19.0% and 13.7% reductions were observed in the SDF and IDF treated groups, respectively when compared with the control.
No reports are available where the fibre from a hypoglycaemic extract had been isolated and tested along with that extract for the hypoglycaemic effect. Different fibre components have been isolated and tested for the hypoglycaemic effect.

Presannakumar and co-workers (1993) have reported that pectin, the SDF isolated from *Coccinia indica* is the active compound in the plant, which caused a 15.0% reduction in the fasting blood sugar levels of Sprague-Dawley rats. Guar gum, which is a gel forming dietary fibre has been shown as a factor which reduces the fasting blood sugar levels significantly in the type II diabetics (Uusitupa et al., 1990).

Secondly, active principles could be extracted using ethanol and water. It therefore, became clear that a polar, organic–extractive may be the hypoglycaemic component.

The hypoglycaemic effect exerted by the whole extract of *I. aquatica* was comparable to that of tolbutamide, a sulphonylurea drug. The hypoglycaemic effect of the whole extract was higher than that of the drug. Tolbutamide caused only a 33.8% reduction in the serum glucose concentration where as *I. aquatica* showed a reduction of 47.5% when compared with the control.

Many herbal extracts have exerted better hypoglycaemic responses than standard antidiabetic agents. Dried sap of *Aloe vera* had exerted a reduction of 45.4% in the blood sugar levels when compared with glibenclamide, which caused a reduction of only 30.0% in normal mice (Ghannam et al., 1986). When tested on healthy, Wistar rats, the polysaccharide fraction of *Psacalium decompositum* had caused a reduction of 20.0% whereas tolbutamide had reduced the 2 h post glucose serum glucose levels by 34.3% (Aguilar et al., 2000).
The shredded, edible portion of the plant was effective in reducing the fasting blood sugar levels of streptozotocin as well as alloxan–induced diabetic rats. *M. charantia* has been able to reduce the blood glucose levels in the Sprague-Dawley rats with graded diabetes in a dose dependent manner (Jeevathayaparan, 1998). Aqueous infusions of the dried leaf extract of *Bauhinia candidans*, and *Rubus ulmifolius* have exerted significant hypoglycaemic effects in streptozotocin as well as alloxan induced diabetic rats (Lemus, 1999). The two plants have not been effective in modifying blood glucosur levels of normal rats.

The plant material was able to counteract the weight loss induced by alloxan as well as streptozotocin-induced diabetic rats. *Musa sapientum* also had been able to counteract the weight loss in alloxanized diabetic rats after 30 days of administration (Pari and Maheshwari, 1999). *Ficus bengalensis* contains a leucocyanidin, which had antagonized the weight loss in alloxan-induced diabetic rats (Kumar and Augusti, 1993).

Having satisfied the criteria for the presence of a useful hypoglycaemic agent, in order to determine the safety of consumption of the plant material regularly over a period of time, a study of its toxic effects was important. After feeding the plant material for 8 weeks, all the key hepatic enzymes, viz. alanine aminotransferase, aspartate aminotransferase and γ glutamyl transpeptidase and uric acid levels were not significantly different in the test and control groups. The unexpected reduction in the alkaline phosphatase levels in the test group when compared to the control is unexplained. The specific isozymes of the enzyme have to be studied for proper interpretation.
Although, generally presumed to be safe for consumption, herbal preparations too exhibit some adverse effects on various organs. Fruit juice and the seed extract of *Momordica charantia* have been shown to elevate the γ glutamyl transpeptidase levels and the alkaline phosphatase levels significantly in normal Sprague-Dawley rats (Tennekoon et al., 1994). Both these enzymes are membrane bound and the elevation of serum γ GT levels is taken as one of the most sensitive indices of hepatic damage whereas the hepatic alkaline phosphatase isozyme levels are markedly elevated in cholestasis. Intra-peritoneal administration of an ethanolic extract of *Trigonella foemum-graecum* had produced hepatotoxicity in mice (Abdel-Bary et al., 1997). The aqueous extract of *Hygrophila longifolia* had not caused an elevation in any of the hepatic enzymes in normal Sprague-Dawley rats as reported by Fernando and co-workers (Fernando et al., 2000).

The fresh, edible portion of *I. aquatica* does not exert any toxic effects on the kidney after long-term consumption, as shown by the normal uric acid levels in the test and control groups (2.8±0.1 and 2.9±0.1 mg/dl in the test and control groups respectively). Since uric acid is eliminated via kidneys, the serum uric acid levels should be elevated in nephropathy. Some plant extracts have been reported to increase the serum urea and creatinine levels and are presumed to cause renal toxicity. The alcoholic extract of *Opuntia megacantha* had significantly elevated serum creatinine and urea levels in the diabetic rats but not in normal rats (Bwititi et al., 2000). Alkaloids isolated from *Aegle marmelos* do not cause an increase in the urea levels and therefore are considered to be non-toxic to the kidneys (Ponnachan et al., 1993).

Glycated Hb is known to be a guide as to the risk of developing diabetes. Simon and co-workers have reported that the concentration of glycated Hb had been significantly
higher in healthy, males and females with all the risk factors for diabetes such as age, obesity, family history and large new born delivery (Simon et al., 1989). These results imply that the concentration of HbA1c could be used to predict about the possibility of becoming a diabetic. In diabetics, the glycated Hb levels allow getting a better picture regarding the long-term glycaemic control and at the same time, it is useful in monitoring the effectiveness of the therapeutics used. A reduction in the HbA1c levels decreases the possible complications of DM and improves the life span of diabetics, as determined by the UK Prospective Diabetes Study (James et al., 1999). The UK Prospective Diabetes Study followed 5,000 patients over a 20 year period and concluded that for every 1.0% drop in HbA1c level, there was:

- a reduction by 25% in diabetes related deaths
- an overall reduction of 25% in diabetes related complications
- a 35% reduction in vascular side effects of the disease
- an 18% reduction in diabetes related heart attacks

In the present study, it was observed that the consumption of the shredded, fresh, edible portion of *I. aquatica* for eight weeks significantly decreased the concentration of HbA1c in healthy, Wistar rats (23.1 %) when compared with the control. Considering the fact that a reduction in HbA1c reduces the complications and improves the life span of diabetics, the plant material could become a valuable tool. Pari and Maheshwari (1999) have found that the chloroform extract of *Musa sapientum* significantly decreased the HbA1c concentration in the alloxanized diabetic Wistar rats.

Non-toxic nature of the plant material, which in any case is consumed regularly in Sri Lanka encouraged the studies on type II diabetics. When given a blended extract of the plant, Type II diabetics showed a reduction in the serum glucose levels at 2 h after a
glucose challenge while it was not significantly reduced at 1 h. The reduction in the 2 h post glucose, serum glucose levels of diabetic patients administered *I. aquatica*, was 25.0 % when compared with the control values. A decoction of *Artocarpus heterophyllus* had decreased the serum glucose levels of diabetic patients at 2 h post glucose load by 86.0 % and a decoction of *Hygrophila longifolia* had decreased the glucose levels by 25.0 % in type II diabetics (Fernando *et al.*, 1991). Although the reduction caused by *A. heterophyllus* is very high, the dose used in the patients had been 10 ml/kg body weight which would require a 50 kg man to consume 500 ml of the decoction. A similar study with the aqueous infusion of the leaves of *Zygophyllum goenhum* had caused a 13.3 % reduction at 3 h after the administration (Jaouhari *et al.*, 1999).

The studies directed at the possible mode/s of action showed that the whole, aqueous extract enhances intestinal glucose absorption. The concentrations of the glucose in the intestine as well as the serum were lower in the test group when compared with the control. The lower concentration of glucose in the intestinal contents of the test group implies that the uptake at the intestinal brush border enhanced by *I. aquatica*. At the same time, the glucose that had come into the system had disappeared from blood. These findings indicate that the aqueous extract of *I. aquatica* enhances the uptake of glucose by peripheral tissues. Plants that decrease intestinal glucose uptake include, *Morus alba* (mulberry), the hot water extract of which had shown a potent inhibitory effect on intestinal α-glucosidases (Asano *et al.*, 2001).

In the type II diabetic patients, the same extract was able to reduce the serum insulin levels, indicating that the *I. aquatica* may increase the sensitivity of the insulin receptors for insulin. Commonly, type II diabetics have an elevated level of serum
insulin due to resistance of the receptors. Another herbal preparation, *banaba* from *Lagerstromia speciosa* had significantly reduced the serum insulin levels of type II diabetics when administered for 4-8 weeks (Kakuda *et al.*, 1996). An aqueous extract from the unripe fruits of *M. charantia* had been found to be a potent stimulator of insulin from pancreatic beta cell in the obese–hyperglycaemic mice (Welihinda *et al.*, 1982). At present it is not known how *I. aquatica* affects insulin secretion.

Having established that useful hypoglycaemic action is observed from extractives of the plant it was next decided to find out what the active components were. Common chemical compounds that have been shown to exert hypoglycaemic effect are given in Table 2.5.

- The active components were extractable into ethanol, which implied that the active compounds should be polar and organic in nature. Many plant compounds have been extracted into ethanol or methanol (Huheri *et al.*, 1995 and Yoshikawa *et al.*, 1998).

- On evaporation of ethanol, it showed positive hypoglycaemic activity.

- Total extractives were 2.41 g per 100 g of the fresh edible portion (for 11.0 g of the dry weight).

- It was clear these extractives were of a complex mixture and had to undergo further separation.

- Due to its polarity, it was suspected that this was a relatively small molecule and Sephadex G25 (separating molecular weights 200 – 1000) was used to fractionate it. Five groups of eluents were collected out of which two were found to be active. As it was likely that these two fractions were mixtures of
compounds, Fr 1 and 2 were subjected to further preparative TLC. These two fractions were of a brownish yellow nature in colour and fraction three was of a very dark yellow while the fourth and fifth fractions were greenish. Freeze-dried Fraction I had the characteristic odour, which is associated with the ethanol extract of \textit{I. aquatica}.

- This resulted in active band 1 (out of 3 bands) and this band was subjected to further preparative TLC.

- Preparative TLC resulted in three sub bands, which were tested on rats as in all the activity directed tests. Four out of the five sub bands were orally hypoglycaemic in the rats.

- Two of the active sub bands (R$_f$ = 0.6 and R$_f$ = 0.8) were obtained in very small quantities and were not proceeded with.

- The other two bands: sub band II containing only one spot and the large sub band I were subjected to MPLC, using gradients of hexane $\rightarrow$ dichloromethane $\rightarrow$ ethyl acetate $\rightarrow$ methanol $\rightarrow$ water with a dilution factor of 6. Here each solvent was used pure and between two solvents, a mixture was used in the ratios, 0.78, 3.13, 6.25, 12.5, 25.0 and 50.0 \%, resulting in different mixtures.

- Two apparently pure spots were separated.

  1. yellow in colour (550 mg) from 11.0 g dry weight

  2. white in colour (70mg) from 11.0 g dry weight

At the point of obtaining three bands, bands 1, 2 and 3 had chemically tested positive for the class of compounds by O-cyanidin and Liebermann – Burchard tests. This
indicates the presence of flavonoids. The behaviour on TLC, of the purified spot with a low 
low $R_f$, suggested that they were glycosides.

Many polar compounds including flavonoids can be extracted into ethanol and methanol. Flavonoids being polyphenolic compounds can act as anti oxidants as well. Flavonoid glycosides have been found to be the active constituent in many plant extracts. Cetto et al., (2000) have isolated three active flavonoid glycosides from *Equisetum myriochaetum* where glucose had been the sugar associated with one of the compounds. Myricetin fucoside is another flavonoid glycoside, which exerts a hypoglycaemic effect by inhibiting aldose reductase (Haraguchi et al., 1998).

Bitterness had been associated with many hypoglycaemic extracts. The classic example is *M. charantia*. *Swertia japonica* is another plant with very bitter properties that had been used traditionally and which had been proven for its antidiabetic property (Basnet et al., 1994). Interestingly non of the compounds isolated from *I. aquatica* was bitter.

Infra red spectroscopy is a good method of typing flavonoids. The IR spectra of the yellow compound, white compound and authentic quercitrin showed similarities as shown below.

<table>
<thead>
<tr>
<th>Quercitrin</th>
<th>White</th>
<th>Yellow</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>quercetin</em>–3–<em>L</em>-rhamnoside</td>
<td>compound</td>
<td>compound</td>
</tr>
<tr>
<td>$2500–3600$ cm$^{-1}$</td>
<td>$2500–3600$ cm$^{-1}$</td>
<td>$2500–3600$ cm$^{-1}$</td>
</tr>
<tr>
<td>H–bonded OH (large)</td>
<td>1659 cm$^{-1}$</td>
<td>No carbonyl</td>
</tr>
</tbody>
</table>
The values for carbonyl stretching were low in both quercitrin and MPLC 1, probably as a result of H-bonding. Like quercitrin, which is a glycoside, the two unknown spots also showed large H-bonded areas, probably due to the sugars. The MPLC 2 seem to have less H-bonded OH which may indicate that the number of sugar moieties were smaller than in MPLC 1. The fingerprint region was not easily interpreted, possibly due to large contribution from sugar peaks. Presence of sharp OH groups and a distinct carbonyl indicates that there is no intra-molecular H-bonding. Both exhibited similarities, confirming that these were flavonoid glycosides. The yellow compound is a typical flavone glycoside where as the white compound could be a flavanol glycoside since no carbonyl stretching (-C=O), is involved.

Figure 5.1 Quercitrin
Flavone glycosides from many plants show potent hypoglycaemic activity (Table 2.6). One such example is the ethylacetate-soluble fraction of *Myrcia multiflora*, which contains novel flavone glucosides, which had shown potent inhibitory activities on aldose reductase and α-glucosidase (Yoshikawa *et al.*, 1998).

Up to this point, nothing was known about the position of OH' groups, their number and nature of the glycosidic moiety. This was investigated by subjecting each of the pure yellow and white compounds to:
a) naringinase hydrolysis and

b) β-glucosidase activity

The isolated flavonoids were subjected to enzyme hydrolysis by naringinase where the isolates were incubated with the enzyme at 37° C for 24 h at pH 4.0 and 5.5. Aliquots of 5 μl were removed at 0 time, 6, 12 and 24 h after incubation and checked by TLC for the presence of compounds.

Naringinase is an enzyme preparation purchased from Sigma Chemical Company, with both β-glucosidic and β-rhamnosidic activity. The yellow compound, MPLC 1 released both glucose and rhamnose, whereas only rhamnose was released from the white compound, MPLC 2. The structure of the white compound was not pursued further since it was present only in very small quantities.

Although, most of the flavone glycosides are reputed to have an α-glycosidic rhamnose (Fong et al., 1967), MPLC 2 indicated the presence of a β-anomeric carbon. The next question asked was, “What was the relationship regarding the position of the glucose and rhamnose with respect to one another?” There were three possibilities.
Figure 5.4 Possible positions of the sugars in the flavone glycoside

Hydrolysis with β-glucosidase from almonds liberated glucose ruling out structure B and therefore the rhamnose and glucose must reside on different OH groups as in structure A or are present in a diglycoside as in structure C; that is the glucose on the terminus.

This can be worked out only by detailed methylation analysis (Hakomori, 1964), for which facilities were not available.

Once the aglycone was isolated the oral hypoglycaemic activity of compound I was tested along with its aglycone for the hypoglycaemic activity in rats. The results revealed that the presence of sugars is essential for the hypoglycaemic effect of the compound as shown by the inability of the aglycone to reduce the blood sugar levels in the rats. Flavonoid glycosides, are known to be hydrolyzed by intestinal bacteria to generate corresponding aglycones (Bokkenheuser and Winter, 1988 and Haraguchi at
al., 1998). This would be only possible if the glycoside contains α-glycosidic bonds. Since the compound I was found to contain the sugars in the β-linkage, it would not be possible for it to lose the activity.

Next it was decided to investigate for more details on the aglycone. Since it was a flavone, the customary trifluoroacetic acid hydrolysis was not possible s it would result in ring opening. Naringinase was used instead of the acid for the hydrolysis. The aglycone was not extractable in dichloromethane. This was adduced as been due to high polarity involving many OH groups. This resulted in the aglycone being extracted with ethyl acetate.

In order that the aglycone is obtained pure, it was subjected to preparative TLC with BAW as the solvent system. A high Rf, fluorescent compound was obtained which was subjected to IR analysis using an unsubstituted flavone and quercetin dihydrate as standards.

Obtaining the spectra of quercetin along with the aglycone was thought appropriate since daily doses of quercetin had been effective as an anti oxidant and therefore is postulated to have anti-diabetic properties (Akagi et al., 1995). If it is the same compound that is present in I. aquatica, similar spectra would be obtained.

The main features of the IR spectra are given below.

<table>
<thead>
<tr>
<th></th>
<th>Quercetin dihydrate</th>
<th>Unsubstituted flavone</th>
<th>Aglycone</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-bonded OH'</td>
<td>3282–3411 cm⁻¹</td>
<td>3038–3058 cm⁻¹</td>
<td>2855–2955 cm⁻¹</td>
</tr>
<tr>
<td>Carbonyl stretching</td>
<td>1664 cm⁻¹</td>
<td>1645 cm⁻¹</td>
<td>1725 cm⁻¹</td>
</tr>
</tbody>
</table>
The flavone standard showed a peak at 3058 cm⁻¹, which is unexplained since it does not have an OH⁻ group. However this peak is very small and may be it is due to water. The OH⁻ stretching of quercetin dihydrate is very large indicating both intermolecular and intra-molecular H-bonds. The aglycone has relatively sharp peaks at 2855 cm⁻¹, 2923 cm⁻¹ and 2954 cm⁻¹. These sharp peaks do not indicate a normal H–bonding.

However the positions of the OH⁻ are hard to explain and it is possible that there is some chelation. If each peak corresponds to an OH⁻ group, the aglycone would have three hydroxyl groups.

The spectrum however has a clear carbonyl group at 1725 cm⁻¹, which corresponds to a six membered ring containing a carbonyl with no halogen bonding. This fits in well with the flavone.

The finger print region of the aglycone is not clearly defined from 900-3500 cm⁻¹ and this indicates that the compound is not 100 % pure and suggests that there might be some chelation. This chelation, if present could explain why this compound is more yellow than the flavones are expected to be since the presence of transition metals would confer more colour to the compound.

Elucidation of the structures of these compounds is a natural sequence of events. For this, we need H¹-NMR, C¹³-NMR, MS and a method to determine the position of the attachment of sugars and to determine whether sugars are bound to the same or different OH⁻ group.

The UV spectra of the yellow (MPLC 1) and white (MPLC 2) compounds were nearly identical from 200-325 nm showing that they have the same basic nuclear structure. The MPLC 1, however showed an absorbance around 390-450 nm.
Quercitrin showed an absorption maximum at 275 nm which is not surprising as quercitrin has five OH groups whereas the yellow MPLC 1 has only three. The stronger absorbance in the yellow region of quercitrin and MPLC 1 rules out chelation with any transition metals. This is of importance since it eliminates the presence of chromium, which would have resulted in a different interpretation of the results. The presence of chromium in the MPLC 1 was found to be < 3 parts per billion (PPB) when determined by atomic absorption flame photometry (Jayasinghe, 2002) and therefore it is evident that the oral hypoglycaemic activity of *I. aquatica* is not due to chromium. The chelation indicated in the spectra could be with a harmless metal like magnecium.

The common availability, the ease in the preparation of the aqueous extract and the non-toxic nature of the plant; are all positive aspects of *I. aquatica*, which could be consumed by diabetics to control blood sugar levels.

**SHORTCOMINGS**

1. *I. aquatica* contains a large number of flavonoids. The present study concentrated on 2 compounds; the white MPLC 2 and the yellow MPLC 1. The other compounds, which might be more potent than these two, have to be studied.

2. Complete structural elucidation of the active compounds, could not be carried out.

3. The detailed mechanism of action is yet unknown.
The studies on the precise structure as well as the studies on the exact mechanism of action could not be carried out since the required facilities were not available.

CONCLUSIONS

I. aquatica has potent acute and chronic oral hypoglycaemic as well as antihyperglycaemic activity. The oral hypoglycaemic and the antihyperglycaemic effects are evident in normal as well as alloxanized and streptozotocin induced diabetic rats. Further, the aqueous extract exerts a potent antihyperglycaemic effect on NIDDM diabetic patients when subjected to a glucose challenge. The hypoglycaemic effect of the plant extract is comparable to that of tolbutamide.

The plant material is neither hepatotoxic nor nephrotoxic, as judged by the serum levels of key hepatic enzymes and uric acid levels. The aqueous extract increases intestinal glucose absorption but enhances peripheral tissue uptake of glucose. In the NIDDM diabetics, the extract decreases the insulin levels in serum indicating that the extract may have sensitized the receptors for the binding of insulin.

The plant contains more than one active compound. The active constituents of the plant comprise flavonoids. One of the compounds is a flavone glycoside in which glucose and rhamnose are present in β-linkage. The presence of sugars is essential for the hypoglycaemic effect of the flavone glycoside. The second compound is a flavonol glycoside with no carbonyl group and rhamnose is present as the sugar moiety.

Thus, I. aquatica has the potential to be developed as a novel oral hypoglycaemic agent.
RECOMMENDATIONS FOR FOLLOW UP STUDIES

- Elucidation of the complete structure of the white compound (MPLC 2) and its mode of action.

- Spectroscopic studies on the yellow compound (MPLC 1) and its mode of action.

- Extra pancreatic action with labeled glucose and the mode of action studies.

- Isolation of the minor components that show hypoglycaemic activity and studies on their structures and mechanisms of actions.
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