School of Pharmacy

# Screening *Teucrium polium* for glucose lowering activity in a rat model

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**Thesis title: Screening *Teucrium polium* for glucose lowering activity in a rat model.**

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The contents of the thesis are being used for a publication and a basis for further research. Thus the restriction of the thesis is requested for 3 years.

## ANITA (BOSSI) IRENG

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ADH | Adrenocoticoid hormone |
| ANOVA | Analysis of variance |
| B.P | British Pharmacopeia |
| β-cells | Beta-cells |
| Ca | Calcium |
| Cr | Chromium |
| Cu | Copper |
| DM | Diabetes Mellitus |
| DMSO | Dimethyl Sulphate oxide |
| $Fe^{2+}$- | Iron |
| GAE/g | Gallic Acid Equivalents |
| GI tract | Gastro Intestinal Tract |
| HCl | Hydrochloric Acid |
| i.p | Intraperitoneal |
| i.v | Intravenous |
| IDDM | Insulin Demandant Diabètes Mellitus |
| IGT | Impaired Glucose Tolerance |
| K | Potassium |
| KCl | Potassium chloride |
| KX | Ketamine/Xylazine |

| | |
|---|---|
| $LD_{50}$ | Median Lethal Dose |
| LDL | Low Density Lipoproteins |
| Mg | Magnesium |
| NaCl | Sodium Chloride |
| NaOH | Sodium Hydroxide |
| NSAIDS | Nonsteroidal anti-inflammatory drugs |
| PBS | Phosphate Buffered Saline |
| S.E.M | Standard Mean Error |
| STD | Standard Deviations |
| STZ | Streptozotocin |
| TAE | Tannic Acid Equivalent |
| U/kg | Units per kilogram |
| UV | Ultraviolet |
| WHO | World Health Organisation |
| Zn | Zinc |

# ABSTRACT

*Teucrium polium* is a herb, abundant in the Mediterranean region and used in folk medicine to treat Type II *diabetes mellitus*. Limited studies investigating the anti-diabetic effects of *T. polium* in animal models have reported a range of outcomes. The main aim of the current research was to evaluate the claimed acute hypoglycaemic effects of *T. polium in vivo*. However, it was necessary to develop a reliable animal model that allows the assessment of the hypoglycaemic potential of the herb extract in comparison to other drugs currently on the market. Normoglycemic Wistar rats (190g-232g) were fasted for 18h, anesthetized with ketamine (75mg/kg) and xylazine (10mg/kg) and maintained under anesthetic using subsequent low dose ketamine/xylazine for the duration of the experimental procedure. Each rat was injected with either *T. Polium extract*, Metformin, *Humulin R* or vehicle (PBS). Blood glucose was obtained from the tail vein and measured using a Glucometer. The results indicate that *T. polium* significantly ($p<0.01$) reduces blood glucose in normoglycemic rats as compared to vehicle and the initial effect is as powerful as insulin. In conclusion, we established a relatively easy *in vivo* protocol which assesses and compares the acute hypoglycemic potential of extracts and pure compounds. The data indicates that *T. polium* extract reduces blood glucose concentrations *in vivo* similarly to insulin.

# 1. INTRODUCTION

## 1.1 BACKGROUND

Diabetes Mellitus is one of the most serious health issues in the world and is increasing at an alarming rate. The disease affects about 5-7% of the population. [1] According to the World Health Organization (WHO) report in 2009, there are more than 220 million people worldwide with diabetes [2-4] and this number is estimated to reach about 230 million cases in 2020. [2, 5-7] In 2000, the WHO recorded 3.2 million and in 2005 1.1 million deaths from the disorder, most of whom were from low and middle income countries. [3] Although the number of deaths due to diabetes was lower during this period, the figures provided by WHO indicated many recorded deaths due to the complications of diabetes.

Diabetes mellitus is a chronic metabolic disorder resulting from defects in insulin secretion, insulin action or both. [8-9] These occur when the pancreas does not produce insulin, or when the body cannot use the insulin it produces effectively, and this leads to increased blood glucose concentration (hyperglycemia) which over time causes serious damage to many body systems as well as disturbing carbohydrate, fat and protein metabolism. [5, 7, 10-13] Diabetes and its complications have a significant impact on the economy of each country, affecting individuals, families and health systems. [2-3] The WHO estimates reveal that between 2006-2015, it would cost a total of $100 billion to treat diabetes and its complications. [8]

There are different types of Diabetes mellitus but the two main types are Type I and Type II Diabetes mellitus. Type I (previously known as insulin-dependent or juvenile-onset diabetes) is caused by destruction of pancreatic β-cells islets which results in absolute insulin deficiency and patients often require long-term insulin therapy for their continual well-being. [9] Type I diabetes is low in frequency compared to type II and usually presents in children and young adults with the common symptoms of diabetes including excessive thirst and/or hunger and frequent trips to the toilet but also sudden weight loss. Type II diabetes (formerly known as non-insulin dependent or mature-onset) accounts for most (~90%) cases worldwide. [10-14] Type II is caused by ineffective use of insulin in the body. It often results from excessive

body weight and physical inactivity. Until recently Type II diabetes was only seen in adults but it is now seen in children as young as two years of age because of the epidemic of obesity. [9] Drug discoveries for Type II diabetes is the basis of this current research work.

Several classes of oral anti-diabetic agents are available for the treatment of Type II diabetes; however they appear to be effective for a certain period and ultimately fail to control blood glucose levels leaving most type II diabetes patients to eventually require insulin therapy. Oral anti-diabetic agents are useful at different stages in correcting insulin resistance and inadequate insulin secretion but often fail to control hyperglycaemia due to progressive loss of pancreatic β-cells function. Therefore, insulin therapy is frequently necessary in type II diabetes as it is the most potent glucose lowering agent aimed at reducing the long-term complications of diabetes but has its drawbacks. [5, 15] The shortcomings of oral anti-diabetic agents and insulin have resulted in pressure for new and effective therapeutics which have led to several investigations into anti-diabetic herbal agents. Traditional medicine was described by WHO as a means to achieve total health care coverage of the world's population. [2] Throughout the ages, humans have relied on nature for their basic needs including medicines. Natural products and their derivatives represent more than 50% of all the drugs in clinical use in the world today. Potential drug plant species are still undiscovered in nature. These could continue to provide natural products with compounds as starting points for the development of new drugs. The potential for finding more compounds is still enormous and more research is still needed to find drugs with pharmaceutical potential.

*Teucrium polium* is a dwarf shrub which grows mostly in the Mediterranean region. In folk medicine, the tea preparations of the aerial parts of the plant are used for the treatment of abdominal colic, headaches, diabetes and as an astringent. [16,17,18] In experimental animal models, it has been reported that the aqueous extract of the plant exhibits antispasmodic, anorexic, anti-diabetic and hypolipidemic effects. [5, 16-21, 23-25] Most of these effects have been related to its constituents especially the flavonoids, terpenoids and volatile oils. [22,26-31] This research project aims to develop an *in vivo* animal model for the assessment of plants and other products with glucose lowering reputations as well as investigate the acute effects of *T. polium* in comparison with known drugs (insulin and metformin) using the animal model developed.

## 1.7 TYPE II DIABETES

Type II diabetes, previously known as adult-onset or non-insulin dependent diabetes is a heterogeneous disorder. It comprises three metabolic abnormalities: impaired insulin secretion in response to glucose, increased hepatic glucose production and decreased insulin-dependent glucose uptake in the peripheral tissues. [5, 12,19] Insulin resistance is characterised by a subnormal response to a given concentration of insulin. [9] The condition progresses from an early stage of insulin resistance to mild postprandial hyperglycaemia to frank diabetes requiring oral hypoglycaemic agents. The mild postprandial stage has been considered a useful marker for patients who are at risk of developing type II diabetes. [10] Normally there is a progressive stage from impaired glucose tolerance (IGT) to type II diabetes which is marked by a decrease in β-cell function leading to a decline in insulin secretion. The failure of the β-cell to compensate for insulin resistance with hyperinsulinemia marks the beginning of Type II diabetes. [10, 32]

The symptoms are marked by hyperglycemia and may include polydipsia, polyuria, nocturia, weight loss and also blurred vision. [12,33,34,35] Early diagnosis of type II diabetes is accomplished through blood testing and measurements from either whole blood or serum. Diagnosis includes; a fasting plasma glucose level at or above 126 mg/dl or 7.0 mmol/L, plasma glucose at or above 200mg/dl or 11.1 mmol/L two hours after a 75g oral glucose load in a glucose tolerance test, or a random plasma glucose measurement above 126mg/dL or 11.1 mmol/L. [8,10,32] Over time, diabetes can lead to long-term complications such as increased risk of heart disease and strokes, diabetic retinopathy causing blindness, kidney failure, and even diabetic neuropathy resulting in damage to the nerves. [2, 35]

Type II diabetes also has a strong genetic component (although no single gene has been found for diabetes) frequently found in certain families and ethnic minority groups such as Australian Aborigines, Pacific Islanders, American Indians, African Americans and also Hispanics. [2,9,10,32,34,35] The condition is usually present in adults over the age of 30, however occurrence of the disorder is increasing in children and adolescents. [4] Overweight and obesity are major risk factors for type II diabetes. [9]

### 1.2.1 MANAGMENT OF TYPE II DIABETES

The management of type II diabetes mainly involves controlling blood glucose levels through weight management by proper exercise and diet, blood pressure reduction thereby reducing other complications like cardiovascular diseases and also control via the use oral hypoglycemic agents.

#### 1.2.1.1 DIET AND EXERCISE

Regular exercise and physical activity are important components of the prevention and management of type II diabetes mellitus. [10-14] If diet and exercise fail to control blood glucose at the desired level, an oral hypoglycemic agent is prescribed. Often in middle aged people with long standing habitual poor diet, a change in their diets is often difficult to maintain and implement. [10]

#### 1.2.1.2 ORAL HYPOGLYCEMIC AGENTS

Oral hypoglycemic agents are mainly used in the treatment of type II diabetes and include five distinct classes. These include the sulfonylureas, biguanides, thiazolidedione, glucosidase inhibitors and meglitidines. They act using various mechanisms as shown in Table 1.2.1.2. [8] However, many of these anti-diabetic drugs have their drawbacks and side effects as stated in Table 1.2.1.2. Some of the drawbacks of oral hypoglycemic agents are that they often require combination of drugs with an increased potential for adverse effects, they often fail to control hyperglycemia due to progressive loss of pancreatic β-cell function and therefore lead to insulin treatment being required.

TABLE 1.2.1.2. SUMMARY OF ORAL HYPOGLYCEMIC AGENTS [8, 12, 10, 35, 36]

| Hypoglycaemic agents | Mechanism of action and side effects |
|---|---|
| Sulfonylureas: | Stimulate $\beta$-cells in the pancreas to produce more insulin<br>• have several unwanted effects<br>• *Drawbacks:* Risk of hypoglycaemia, weight gain and increased cardiovascular defects<br>• *Failures:* Treatment failures reported in patients with lowest $\beta$-cells function. Also, loss of effectiveness over the years in patients |
| Biguanides<br>e.g. Metformin | • Metformin is the only available biguanide and remains the first line drug therapy for patients with type 2 diabetes mellitus<br>• Biguanides act by lowering fasting levels of insulin in plasma in hyperinsulinemia. Thus they reduce gluconeogenesis in the liver, and, as a result, reduce the level of glucose in the blood. Biguanides also tend to make the cells of the body more willing to absorb glucose already present in the blood stream, and there again reducing the level of glucose in the plasma.<br>• Metformin is the less toxic and more potent oral glucose-lowering agent developed from a plant (*Galega officianalis*)<br>• *Drawbacks:* GI tract side effects and high cost. Can also accumulate in the kidney and cause lactic acidosis if other mechanisms have induced renal failure. |
| Thiazolidedione, also known as glitazones (eg Rosiglitazone) | • Increases the sensitivity of muscles and other tissues to insulin. It improves insulin sensitivity. Enhance glucose up-take and oxidation in muscles and adipose tissues.<br>• *Drawback:* Slow onset of action and leads to weight gain. Also issues with liver toxicity and high cost. Rosiglitazone was also placed under selling restrictions in the US due to its cardiovascular effects. |
| Glucosidase inhibitors:<br>e.g.Acarbose, Miglitol | • Inhibits $\alpha$-glucosidase in the gut wall which reduce the release of glucose for carbohydrates and hence, the amount of glucose available for absorption.<br>• *Drawback:* GI side effects and high cost. They are effective only in the early stages of impaired glucose tolerance. |
| Meglitidines<br>e.g.Repaglinide, neteglinide | • Repaglinide lowers blood glucose by binding to receptors on the pancreatic cells and thus stimulates the release of insulin.<br>• Drawback: High cost |

### 1.2.1.3. INSULIN

Insulin therapy plays an important role in diabetes mellitus and remains the most potent medication for lowering blood glucose concentrations in patients with type II diabetes. [8, 32] Although insulin is essential for the treatment of type I diabetes, it can be combined with oral therapy for the management of type II diabetes and up to 27% of patients ultimately require chronic insulin treatment. Insulin therapy is aimed at mimicking nature (limiting postprandial hyperglycemia). [32] Many insulin preparations are available and are grouped according to their duration of action. These are available as short and rapid acting insulin preparations, intermediate-acting insulin, long-acting insulin preparations, and premixed insulin preparations. [32] Although still effective, insulin has its drawbacks. The main undesirable effect of insulin is hypoglycaemia which is common and can cause brain damage and even death. [35] Insulin injections can be painful and inconvenient causing diabetic patients to desire other alternatives. [5, 6, 12, 15]

Despite the introduction of new anti-diabetic agents and insulin preparations, efforts to develop better management for type II diabetics have been disappointing and the control of blood glucose levels remains unsatisfactory. [36] Also, the cost of treatment with oral antidiabetic drugs has been expensive.

## 1.8 MEDICINAL PLANTS AND DRUG DISCOVERY

Medicinal plants have formed the basis of sophisticated traditional medicinal systems that have existed for thousands of years and continue to provide some alternative medicines. [4] Herbal medicines include herbs (crude materials), herbal materials (fresh juices, gums, oils, resins, and dry powders) and herbal preparations (extracts, tinctures, and fatty oils). [8]

Drug development from medicinal plants includes a range of methods of analysis. Collection of various plant species is carefully conducted and identification and preparation of extracts from the plant material allowed them to be screened for any pharmacological properties. The process continues on to isolation and characterisation of active compounds. Despite providing essential evidence of potential new drugs, the future of actually placing the drug on the market faces many challenges. [37-39]

Over 20,000 medicinal plants were inventoried by the World Health Organisation (WHO). From these, an estimated 250 species have been analysed to identify their bioactive chemical properties. [2, 38] Many of these plants have anti-diabetic properties. There are several mechanisms of actions that have been proposed for the actions of herbal medicines as listed in Table 1.3. [8]

TABLE 1.3. POSTULATED MECHANISM OF ACTION OF HERBAL MEDICINES BASED ON KNOWN MECHANISMS OF ACTION

| KNOWN MECHANISM OF ACTION | REFERENCE |
|---|---|
| • Stimulation of insulin secretion from β-cells of islets | *Pulok et al 2006* [40] |
| • Inhibition of insulin degradation processes | *Pulok et al 2006* [40] |
| • Inhibition in renal glucose reabsorption | *Eddouks M et al 2002* [41] |
| • Provision of certain necessary elements like Ca, Zn, Mg, and Cu for the β- cells. | *Mohamed B et al 2006* [42] |
| • Regeneration and/or repair of pancreatic β- cells | *Mohamed B et al 2006* [42] |
| • Stimulation of glycogenesis and hepatic glycolysis | *Miura T et al 2001* [43] |
| • Protective effect on the destruction of β-cells | *Kim MJ et al 2003* [44] |
| • Improvement in digestion along with reduction in blood sugar and urea | *Krishnan SH et al 1968* [45] |
| • Prevention of pathological conversion of starch to glucose | *Sepha GS et al 1956* [46] |
| • Inhibition of β - galactocidase and α-glucocidase | *Sharma AK et al 1990* [47] |
| • Cortisol lowering activities | *Gholap S et al 2004* [48] |
| • Inhibition of alpha-amylase | *Heidari R et al 2005* [49] |
| • Prevention of oxidative stress that is possibly involved in pancreatic β-cells dysfunction found in diabetes | *Hideaki K et al 2005* [93] |
| • Increase in the size and number of β-cells in the islets of Langerhans | *Mohamed B et al 2006* [42] |

## 1.9 *T. POLIUM L*

### 1.4.1 DESCRIPTION AND GEOGRAPHICAL DISTRIBUTION

Among the many plants used and identified as having potential to reduce blood glucose level is the plant *T. polium* as shown in Figure 1.4.1. (c)

**a**

**b**

**c.**

Figure 1.4.1 Images of *T. polium L*; **a.** Stems and fibres **b.** Aerial parts **c.** Powdered plant material

*T. polium* is a herb native to the Mediterranean region and has been used for many decades in folk medicine. [16-21,50-51] Commonly known as Mountain, Felty or Poly Germander, the herb comes from the genus *T.* and family *Lamiaceae.* [22] The vernacular names include *Ja'ada, neghda, neftah* and *misk al jinn.* [52] T. polium comes from a group of shrubs which are usually aromatic. There are about 260 species of Teucrium are located worldwide but they are mostly abundant in the Mediterranean region. Its flowers are small and range from pink to white with oval and marginated leaves. The plant grows to about 0.4 meters in height in September and flowers from July to September at various times of the year depending on where it is grown. The plant requires moist soil, good drainage and full exposure to sunlight to grow. [18]

There is evidence of efficacy of the plant used by traditional medicinal practitioners to treat a number of diseases, including type II diabetes. The plant has been credited with several pharmacological properties some of which were evaluated in animal models. However, very limited studies have been conducted to assess its hypoglycemic effect with inconsistent outcomes. The plant is suggested to act as an insulin mimetic agent, imitating the actions of insulin. [19] Further, neither the active components nor the mechanisms of action of the anti-diabetic properties of *T. polium* have been established. It has been proposed that the plant could be acting either by 1) supplying β-cells with the necessary elements like copper, magnesium, and calcium which stimulate the release of insulin [8, 9, 14] 2) reducing the actions of insulinase, an enzyme which catalyses the hydrolysis of insulin [8] or 3) acting on glucose homeostasis by decreasing the level of glucagon and/or reducing peripheral use of glucose. [53] [54]

The few published studies on the plant suggest its potential to reduce blood sugar levels. [14,16, 17,19,20,21,51] Garaibeh et al [16] reported that the plant extract caused a significant reduction in blood glucose concentration 4 hours after i.v. administration and 24 hours after i.p. administration using diabetic and non-diabetic rat models. Esmaeili et al [17], and Yazdanparast et al [21] also reported a significant decrease in blood glucose concentration in diabetic rat models after 6 weeks of consecutive oral treatment with an ethanolic extract of *T. polium*. However, there are conflicting scientific reports regarding its antidiabetic properties. This research project will confirm or refute the glucose lowering effects of *T. polium* in an animal model.

### 1.4.2 PROPERTIES AND MEDICINAL USES OF *T. POLIUM*

*T. polium* has been used in folk medicine for many years. The herb is consumed as a tea and also used as a spice for cooking. Researchers have been attracted by the folk reputation of this herb which has been reported to have many medicinal properties including: anti-diuretic, antipyretic, antispasmodic, antinociceptive, anticholinergic, hypoglycaemic, anti-inflammatory, anti-rheumatic, anti-bacterial, anti-convulsant, anti-pyretic, anti-ulcer, anorexic, anti-thrombotic, anti-spasmolytic, anti-viral, anti-oxidant properties. It also is used for gastrointestinal tract disorders. [16-17, 19, 21-26, 55] A recent study has also suggested that the plant has great benefits for the treatment of human metastatic prostate cancer. [27] Further studies are needed to refute or validate the many claims of medicinal values of *T. polium.*

There have been few case reports about the possible toxicological actions of *T. polium*. One of the cases reported the use of T. polium by a 37 year old Greek female who developed liver failure after 10 consecutive days of consuming a herbal tea comprising *T. polium*. [57] In this case, Mazokopakis et al [57] did not specify what species of the plant was consumed which contributed to her complications. Another similar report was also from a Greek female who consumed the plant extract daily to control her cholesterol levels. After consumption, her liver biopsies showed signs of hepatitis. [20] It is reported that confusions were encountered over which species of the plant, either *T. chamaedrys* and *T. capitatum was* consumed. These species have been *reported* to cause hepatoxicity but it is also conceivable that the herbal remedy was interacting with other medications the patients were taking. [31] These reports contradict the common use of *T. polium* in the Palestinian region to treat liver diseases and that the aqueous extract of *T. polium* was not toxic to cultured hepatic cells when the cells were exposed for 24 hours to 1mg/mL of *T. polium* extract.

### 1.4.3 CONSTITUENTS OF *T. POLIUM* WITH ANTI-DIABETIC PROPERTIES

*T. polium* has been reported to possess potent antioxidant activity which is attributed to its constituents, especially phenolic compounds. [60] Phenolic compounds include such compounds as flavonoids, tannins and phenolic acids. These compounds appear to have strong antioxidant properties in vitro. [56] The positive effects of these antioxidant components come from their ability to inhibit lipid peroxidation and chelate redox-active metals. Many diseases affecting humans are caused by free radicals or reactive oxygen species. Antioxidants obtained from many medicinal plant species are proposed to protect the body by scavenging free radicals and thus offer many health benefits. [60, 61]

Flavonoids in *T. polium* constitute over half of the naturally occurring phenolic compounds, present in the seeds, fruits, barks and flowers of the plant. [63] In a study by Ansari et al it appears that flavonoids in the plant extract from *T. polium* contribute to the increased insulin secretion from the β-cells of the pancreas. [19] Quercetin has also gained attention in the scientific community, after it was reported to have hypoglycaemic effects in rat models. Coldiron et al [63] attributed the reversal of some of the complications of diabetes to the inhibition of the enzyme aldose reductase by quercetin. Aldose reductase is an enzyme that plays a role in converting glucose to sorbitol in the body. Secondary problems such as retinopathy, neuropathy and cataracts are caused when sorbitol builds up in the body as seen in diabetes. [63] Thus quercetin could be beneficial in the nutritional managements of diabetes, but clinical studies are still to confirm this. [63] Vessel et al have also reported that quercetin at a dose of 10-50mg/kg was capable of normalising blood glucose levels and significantly reducing serum cholesterol and LDL concentrations. [28]

Terpenoids also have been reported in *T. polium*. Terpenoids, also known as isoprenoids are a large class of naturally occurring organic chemicals. These chemicals have antifungal, anti-tumour, and also antimicrobial. *Maria et al* 1989 reported on an acetone extract discovery of 3 new diterpenoids in a species of *T. polium* which are Teuvincertins A, B, and C. [95] Kawashty et al also reported that *T. polium* contained neo-clerodane diterpenoids and the flavonoids cirsiliol and cirsimaritin. [30] Two other terpenoids, auropolin and capitation were

also discovered by Ansari et al [19] during their study. Their study found that these two terpenoids had decreased blood glucose levels in STZ rat models. However it is interesting to note that some terpenoids are only found in *T. polium L* and no other species. Two of these Terpenoids are auropolin and capitatin. *Ansari et al* 2003 did an in vivo study and found these two Terpenoids decreased blood sugar. [19]

Iridoids are another interesting group of compounds identified in *T. polium*. They are a class of secondary metabolites occurring in many medicinal plants that are believed to exert hypoglycaemic effects. [22,29,30] Apart from these constituents, *T. polium* also contains many essential oils and also metallic substances such as potassium, zinc, calcium and chromium, which could also be found beneficial in normalising blood glucose levels. [22,29,30]

Tannins, which act as defensive compounds that counteract bacteria and fungi have also been located in the plant and. Tannins are usually divided into hydrolyzable tannins and condensed tannins. [96, 97]

### 1.4.4 PREVIOUS STUDIES ON THE ANTI-DIABETIC EFFECTS OF *T. POLIUM*

Although some animal studies [16,17,20,21,25,51] and one human study showed significant decreases in blood glucose level after treatment with *T. polium*. [16,17,20,21,25,51] There were studies by Afifi et al [18] and Konukugil et al [24] which showed ineffectiveness of *T. polium* in treated animals.

Table 1.4.4 is a summary of 5 studies reviewed in relation to this current study. These studies were all based on *T. polium* and show differences in study design, study duration and outcomes as well as the number of animals used to achieve a statistically significant result in blood glucose reduction. Although a majority of these studies have shown significant results in changing blood glucose reduction while some have not, one must question all factors leading to the outcome of results obtained by each study.

Firstly there were several issues with the study design of these studies (Table 1.4.4). The use of STZ-induced diabetic rats was common as models of diabetes to observe the effects of the

## TABLE 1.4.4 Animal Studies on the glucose lowering potential of *T. polium*

| Reference | Study design | Results |
|---|---|---|
| *Garaibeh et al, 1988* [16] | Male Fisher rats (200-250g) used (STZ induced diabetes)<br>Animals equally distributed into 3 groups;<br>**1. Oral**: 1 mL (equivalent to 0.04g of dry starting material. Drinking water replaced by 4% decoction of *T. polium*, control animals received tap water. Blood sampling conducted after 24 hrs.<br>**2. I.V.** 1 mL (equivalent to 0.2 g of dry staring material). Fasted for 4 hrs. Received 5 mL (20% w/v) of plant decoction through tail vein. Control received 5 mL of normal saline. Blood sampling taken before & 4 h after treatment.<br>**3. i.p.** 1 mL (equivalent to 0.2g of dry starting material. Animals received 5mL (20%) of plant decoction. Control animals received 5 mL normal saline). Blood sampling taken 24 hrs later<br>Blood glucose measured with the Trinder's glucose method. | Decrease in blood glucose in hyperglycaemic rats (20.5%) & normal rats (3.4%) but the decrease in both instances was not significant because normal rats consumed less of plant decoction. i.p. administration of plant decoction resulted in significant decrease in blood glucose in both normal & hyperglycaemic rats ($p< 0.05$). Overall: decrease blood glucose 4hrs after i.v. , 24 hrs after i.p. |
| *Konukugil et al, 1997* [24] | Healthy male Wistar rats (200 ± 20g) used (randomised into 3 groups). Fasted for 18 hrs prior to experimentation. All treatments were administered by oral gavages from 6.6g of dry plant material boiled in 50mL. 50% glucose solution was administered immediately after each treatment.<br>**Group 1**: *T. polium* decoction (12 mL/kg rat ) *n=10*<br>**Group 2**: Gliclazide (3.4 mg/kg) *n=10*<br>**Group 3**: Control (12 mL/kg distilled water) *n=10*<br>Blood sampling monitored for 5 hr at 60 min intervals obtained by clipping the toenails & measured using B-Glucose Photometer | Slight decrease in mean glycaemia, not statistically significant when compared with control group. Animals tt with plant extract show stat. sig compared to control ($p<0.01$) after 4 hrs |
| *Zal et al, 2001* [20] | Male Sprague-Dawley rats (200-220g) used. Dry plant material used at a concentration of 1g of plant extract/mL.<br>Animals were divided into 4 groups;<br>**Group 1:** STZ induced rats, received 1 mL of aqueous extract twice daily by oral gavages *n=5*<br>**Group 2**: STZ induced rats received ad lib feeding only *n=5*<br>**Group 3:** Normal rats received 1 mL aqueous extract once a day for 10 days *n=5*<br>**Group 4:** Normal rats (normal control) received ad lib feeding only *n=5*<br>Blood glucose was measured using a glucometer on day 0, 1, 4, 8, 12 in STZ induced rats & day 0, 2, 6, 10 in normal rats | In normal rats, administration of plant extract for 10 days had no significant effect on blood glucose reduction.<br>In diabetic rats, the twice daily treatment of plant extract resulted in highly significant reduction in blood glucose level ($p< 0.001$) after 24 hrs. |
| *Esmaeili et al, 2004* [17] | Male Albino rats (200-250g) used. Dry plant extract was used in a conc. of 0.5g/mL administered through oral gavages. Animals were groups into 3 groups;<br>**Group 1:** STZ induced (control) received distilled water for 6 weeks *n=8*<br>**Group 2**: STZ induced diabetic rats received 1mL/rat of plant extract (0.5g/kg) *by oral gavages for 6 weeks n=12*<br>**Group 3:** Healthy Control received distilled water *n=8*<br>Blood glucose was measured weekly using a Glucometer kit | After 6 weeks, rats treated with *T. polium* had significant reduction in blood glucose levels ($p<0.05$) lower than the control, a reduction of 64% was observed. |
| *Ardestani et al, 2008* [23] | Male Albino rats (200-250g) were used. The dose of dry plant extract used was 0.5g dry plant powder/ mL by gavages. Animals were grouped into 3 groups; **Group 1**: Normal control received citrate buffer solution *n=8*<br>**Group 2:** STZ induced diabetic; untreated, **Group 3;** STZ induced diabetic; treated with 1 mL per rat of plant powder (0.5g/kg) by gavages for 30 days *n=7.* Blood glucose level was determined every 10 days using a glucose oxidase kit. | After 30 days, the blood glucose level of the diabetic rats treated with plant extract was significantly lower ($p<0.05$) than the diabetic group. |

plants on blood glucose in comparison with normoglycemic rats controls. Thus, whilst the STZ model can be very useful for measuring the effect of agents on lowering blood glucose levels under some diabetic-like circumstances, the administration of STZ can cause death to some animals due to massive islets β-cells necrosis and consequent sudden release of insulin. [28, 64] STZ is a broad spectrum antibiotic that actually damages the pancreatic islets. It is characterised by selective destruction of pancreatic islets β-cells, causing insulin deficiency, hyperglycaemia, polydipsia and polyuria. [65] These are all features of human type I diabetes mellitus. Consequently, glucose loading doses are often administered to avoid this after an STZ injection. [64] Konukugil et al [24] administered 50% glucose solution after administration of each plant extract to normoglycemic rats. The other studies included in Table 1.4.4 showed the administration of the plant extract alone but did not specify the administration of glucose solution. Normoglycemic rat models were also used as a comparative against STZ rats and mostly as controls. Konukugil et al [24] was the only study to use normoglycemic rats alone and also use a reference drug (gliclazide) at a low dose (Table 1.4.4).

Secondly, the different studies use different animal numbers and they obtained statistically significant results in blood glucose reduction using different means. Garaibeh et al [16] was one of the first studies conducted on the plant and they had used different animal numbers (*n*) in each group according to the route of administration of the plant. The result of the use of normoglycemic rats (*n=5*) and hyperglycemic rats (*n=17*) in the oral route was shown clearly to not be statistically significant. The results of i.v. administration showed the animal numbers for normoglycemic (*n=9)* and hyperglycemic (*n=7*) were seen as significant. Ardestani et al [23] used 7 rats as hyperglycaemic models and Esmaeili et al [17] used 12 in the hyperglycaemic treated rats, and have also shown similar results during their study with animal numbers. Zal et al [20] was the only study from the group that used *n=5, a lesser* number but only observed a significant reduction in the diabetic treated rats and not the normoglycemic untreated rats.

Thirdly the durations of these studies were a concern. As stated in Table 1.4.4, many of procedures were conducted from 5 hours to 30 days. The designs were done according to the type of animal model used and for how long the study was to progress in order to get significant results. This is because the models needed to have induced diabetes and monitored

over time to see the effect of the plant extract on blood glucose. Konukugil et al [24] conducted their study in 5 hours only because they had used normoglycemic rats. However, longer studies would place stress on the animals, some animals would die during the process and these would also affect the results of the study. Stress is one factor that can lead to adrenaline rush causing an acute rise in blood glucose. It must also be considered that none of these studies had used any form of anaesthesia to allow calm and reduce pain of any sort in the rat model. One would presume that the animals were awake during the whole procedure, and these would make the animals restless during handling, indirectly causing pain and placing a tremendous amount of stress on the animals. Consider the study by Konukugil et al [24] who conducted their blood sampling by clipping the toenails of the animals to collect blood without administering any form of anaesthesia to reduce pain. The form of blood sampling would add an enormous amount of stress on the animals and this would affect the blood glucose level. Thus the outcome of results from such studies would also be questioned.

Finally, as shown in Table 1.4.4, the administration of the test substances and control (PBS) were through different routes. Garaibeh et al [16] used the i.v, i.p and oral route by gavages. As for Konuklugil et al [24] all animals received the extract through oral gavages. Zal et al [20] administered the extract by oral gavages to one group of STZ rats, while the group of STZ rats received ad lib feeding. The result is blood glucose reduction were different because those animals receiving the plant extract by ad lib feeding had encountered the bitter taste of the plant extract. *T. polium* has a bitter taste and could lead to less of the extract being consumed if they were receiving it on a daily basis. This was clearly demonstrated in the study by Garaibeh et al [16] who replaced normal drinking water with a 4% decoction of the plant extract. They also observed that the results were different because the animals had consumed less of the decoction. Their results indicated that administration through the oral route showed a decrease in blood glucose which was not significant in both normoglycemic and hyperglycemic rats.

## 1.10 ANIMAL MODELS TO STUDY TYPE II DIABETES

Animal models have been used extensively to investigate the *in-vivo* efficacy, mode of action and side effects of anti-diabetic plants and their active constituents. This *in-vivo* approach has steadily helped to identify lead compounds and eventually develop approved drugs. [39] Different studies of anti-diabetic plants have used animal models despite the lack of dose standardization, plant environment selection or enough information on the toxicological effects of the plants, and have confirmed the activities of each plant. However, it is critical that the animal model represent the human disease as closely as possible**.** [39, 64]

The majority of studies published in the area of screening plants for antidiabetic activity employ pharmacologically induced animal models. Streptozotocin (STZ) and alloxan induced diabetic models frequently have been used to study type II diabetes because both these drugs exert their actions when they are administered intravenously, intraperitoneally or subcutaneously. [65] However STZ can cause renal injury, oxidative stress, inflammation and endothelial dysfunction. [64] The destruction of the pancreatic cells by STZ is associated with a huge release of insulin which makes animals more susceptible to severe hypoglycaemia. Animal models are sometimes fed with glucose solutions following treatment with these drugs and the fasting period for the studies is usually 8-12 hrs. [64]

Non-diabetic normoglycemic animal models also have been considered in several studies with known antidiabetic drugs and plant extracts. [64] This also was conducted and incorporated into several studies to see the anti-diabetic effects of herbal medicine. *T. polium* is a herb for which its antidiabetic effects were observed using both non-diabetic and diabetic rat models. [7, 11, 20, 67, 68]

## 1.6 THE PRESENT STUDY

The key purpose of this study is to either refute or verify the claimed *in vivo* hypoglycemic effect of *T. polium*. Extracts of *T. poli*um were carefully extracted and prepared. An animal model utilising non-diabetic, normoglycemic rats was established and carefully optimised to enable blood glucose levels to be reliably monitored over time in response to various treatments. The study design incorporated various controls that further validated the technique and outcomes of the observed with *T. polium* extracts. This study is unique in that its design allowed for the comparison of the acute effects of *T. polium* to known hypoglycaemic medications while eliminating the effects of adrenalin, and thus minimising the number of animals sacrificed to reach statistical significance.

# 2.0 EXPERIMENTAL METHODS

## 2.4 PLANT EXTRACTION

### 2.4.1 MATERIALS

#### 2.1.1.1. PLANT MATERIAL INVESTIGATED

The dry plant material from *T. polium* was purchased from Jordan, authenticated by a Jordanian *Botanist* at the University of Jordan and brought into Australia from Jordan under quarantine. The extract was prepared from the aerial parts of *T. polium* and assessed for glucose lowering activity in a rat model.

#### 2.1.2.2. PLANT EXTRACTION

Methanol was used as the solvent of extraction. Equipment included a Soxhlet extractor, a rotary evaporator (Buchi Rotavapor R-200) with water bath, a vacuum oven and nitrogen gas supplied by (Comet $^{TM}$ CIGWELD). Other miscellaneous materials included a weighing balance, laboratory glass apparatus, mortar and pestle, extraction thimbles, an electric heater, and pH indicator strips.

#### 2.1.2.3. PHENOLIC TESTING

The phenolic test for phenols was conducted using the spectrophotometer and the following chemicals and materials were used to prepare assays for the phenolic test: Tannic acid B.P ($C_{76}H_{52}O_{46}$ ), Batch No: 536, Biolab (Aust) Ltd; sodium carbonate anhydrous ($Na_2C0_3$), Batch No: 244955, Biolab (Aust) Ltd; sodium hydrogen carbonate (s*odium bi carbonate*) ($NaHCO_3$), M.W 84.01, Batch No: (10) 234574, Chem suppl: Folin Denis' reagent, Batch No: 47742, Sigma –Aldrich Chemicals; and methanol.

#### 2.1.1.4 PHOSPHATE BUFFERED SALINE

Sodium chloride (NaCl), Batch No: (10) 233149, was supplied by Chem Supply, 0.1 % DMSO (dimethyl sulphoxide: Anala ®) was supplied by VWR International Ltd, and Phosphate Buffered saline was freshly prepared from potassium chloride (KCl), Batch No: 245699, Biolab (Aust) Ltd, di-sodium hydrogen orthophosphate (anhydrous) ($Na_2HPO_4$), Batch No: 23659, Merck Pty Ltd, potassium dihydrogen orthophosphate ($KH_2PO_4$), Batch No: 26023, Merck Pty Lt, and sodium hydroxide solution.

### 2.1.2 METHODS

#### 2.1.2.1 PLANT EXTRACTION (PRELIMINARY AND OPTIMIZED)

A preliminary extraction method was initially developed after reviewing the literature. This method was then used to conduct several extractions using a range of methanol concentrations under similar extraction conditions. Initially 5g of dry plant material was used in each plant extraction using 250 mL of different aqueous methanol concentrations (50%, 70%, 80%, 90%, and 100%) at 80-100º C for 4 hours. These extractions were conducted using the Soxhlet extraction apparatus. The outcomes of these extractions were analysed and an optimal methanol concentration that extracted the highest yield by weight from the dry plant material was selected. After preliminary trials, the amount of dry plant material was reduced to 2.5g and aqueous methanol (90% concentration) was used to extract from the aerial parts of *T. polium*. The plant material (2.5g) was placed inside the extraction thimble in the Soxhlet extractor containing the solvent of extraction (200 mL). Thus the following optimized extraction procedure was conducted for extraction from *T. polium* for administration into the rat model.

The extraction was carried out at 80-100º C. This was repeated two to three times within a 3 hour extraction time. The extracted concentrate containing the plant extract was then cooled and the non-soluble portion of the plant material remaining in the extraction thimble and was discarded. The concentrate was reduced in volume in the rotary evaporator set at 45±1 ºC for 30-45 minutes. The extract was then removed, made to volume and divided into 20 aliquots

all of equal volumes (2 mL each) to give an approximate extract from 125mg of dry plant material in each vial enabling the delivery of extract from 100mg of dry plant on dissolution in vehicle. The vials were placed in a vacuum oven set 45±1 ºC for 3 hours to remove excess moisture. After drying they were purged with nitrogen and stored at 4 ºC for further investigation and analysis.

### 2.1.2.2 ANALYSIS OF TOTAL PHENOLICS

Total phenol quantification was measured according to the method published by the Association of Official Analytical Chemists [Official Methods of Analysis, 1990]. [94] This method is a colourimetric assay which uses Folin-Denis reagent and allows the estimation of phenolic compounds at 760nm in relation to a tannic acid standard curve and is a widely used technique in other studies [60, 61] Briefly the assays were carried out as follows: 0.2 mL of tannic acid standards and the diluted (1:20) extract were mixed in with 0.25 mL Folin-Denis reagent and 0.5 mL of sodium carbonate solution and the volume was then made up to 5 mL with Milli-Q water. The tubes were incubated for 30 minutes at room temperature with intermittent shaking. The absorbance of the resulting blue colour solution was measured at a wavelength of 760nm on the Spectrophotometer (UV mini 1240: Shimadzu). Quantitative measurements were preformed based on the standard calibration curve of five points: 0 µL, 100 µL, 200 µL, 300 µL, 400 µL and 500 µL of tannic acid in Milli-Q water. The total phenolic content of the sample was expressed as tannic acid equivalent (TAE), which reflected the phenolic content, i.e. the amount of tannic acid (mg) in 1g of dry plant material using a standard curve. The procedure was conducted on freshly prepared and thereafter on unused freshly thawed samples on days 0, 15, 30, 45, 60 and 90, i.e. before and during the storage period of the plant extract.

*Preparing phosphate buffered saline (PBS)*

A stock solution of 10XPBS was prepared to give NaCl (1.37mM), KCl (27mM), $Na_2HPO_4$ (100mM), and $KH_2PO_4$ (18mM). The solution was autoclaved and stored on the shelf. Fresh preparations were diluted 10 fold monthly for use and the pH checked to be 7.4. This final

1XPBS was sterilised by filtration (through sterile 0.45 micron pore size filter) and stored (in sterile containers) at 4ºC.

#### 2.1.2.3 SOLUBILITY OF THE PLANT EXTRACT

The solubility of the plant extract was assessed in three vehicles: 0.9% saline, 0.1% DMSO and (PBS). One mL of each vehicle was added to each vial of plant extract. All samples were visualised to assess solubility.

The pH of the plant extract in each medium was assessed by using pH indicator strips. The pH of the plant was noted in each medium (~3) and this was modified to ~7.4 using 2.5mM sodium hydroxide solution.

#### 2.1.2.4 EXTRACT STORAGE

The plant extracts were purged under nitrogen to reduce oxidative degradation and stored at 4°C until needed. Total phenolic assessments of random samples were carried out throughout the storage and testing period to estimate degradation.

## 2.2 ANIMAL STUDY

### 2.2.1 MATERIALS AND EQUIPTMENT

The insulin preparation used was *Humulin R* (100 Units) which was supplied by Diabetes Australia from Eli Lilly and Company. Metformin1, 1-Dimethylbiguande Hydrochloride, ($NH_2C$ (=NH) NHC (=NH) N $(CH_3)_2$.HCl) 97% was purchased from Sigma–Aldrich. The anaesthetic used was a combination of ketamine (20mL/mL) and xylazine (100mg/mL) sourced from Troy Laboratories Ltd. Other materials and equipment used in the animal study included: Accu-chek *Go* Glucometer, Accu-chek *Go* Glucometer strips, a warming box, and

other miscellaneous items. PBS was prepared according to section 2.1.2.2 and autoclaved before use.

## 2.2.2 METHODS AND PROCEDURES

### 2.2.2.1. EVALUATING THE HYPOGLYCAEMIC EFFECTS *IN VIVO*

To evaluate the hypoglycaemic activity of the plant extract and other reference test compounds, the animal model by Schäffer et al [68] was adopted with modifications. Animal experimentation was approved (Approval No: R10/2009) by the Animal Ethics Committee at Curtin University. Male Wistar rats (180-190g) were purchased from the Animal Resource Centre at Murdoch University for the purpose of this study and were housed at the Animal Facility at Curtin University. All animals were maintained in accordance with the guidelines of the Animal Ethics Committee. The rats were fed normal laboratory food, allowed free access to water and were caged mostly in individual cages but sometimes two rats per cage. All animals were housed under standard conditions and temperature (20±1ºC), with a regular 12 hr dark and 12 hr light cycle. Daily monitoring for any signs of agitation, fighting, distress or abnormal drop in weight was performed. The body weights also were measured on a daily basis allowing frequent handling of the rats to reduce nervousness, induce calmness and reduce aggressiveness.

The study was designed to assess the glucose-lowering activity of *T. polium* in vivo in comparison with other known glucose lowering drugs. The current study was carried out on healthy normoglycemic Wistar rats housed for a 3-day acclimatisation period in the Curtin Animal facility. All animals were well cared for and housed under standard conditions: temperature (20±1ºC), with a regular 12 hr dark and 12 hr light cycle. Food and water was supplied daily and the beddings were changed as necessary but also just before the start of the 18 hour fast prior to experimentation. During their acclimatisation periods, the rats were observed to have gained weight, about 10g per day on average, weighing in the range of 190-232g. This slight gain in weight did not affect their inclusion into the study and anaesthetic dosing was adjusted for their weight on the day. During the initial testings, the animals were

observed to have eaten their own faeces during the fasting period due to hunger which is common and therefore to minimise any effect this would have on the blood glucose results, the animals always had their caged beddings changed before the start of the fast and monitored closely.

In the current study, the animals were randomly allocated upon arrival at the animal facility into one of four treatment groups in order to reduce bias in the selection for the study. Many of the studies discussed earlier (figure 1.4.4) on *T. polium* had not included the random allocation process and did not specify in their study the type of method used in allocating the animals to the different treatment groups. The study was designed with four treatment groups and each substance solubilized in 0.2mL PBS, was injected once: Group 1 (vehicle) given 0.2 mL of PBS; Group 2 (Plant extract) given an equivalent to total extract from 100mg of dry plant material solubilised in 0.2mL PBS; Group 3 (Metformin), given 20mg in 0.2 mL of PBS; Group 4 (insulin), given 0.1 Units in 0.2 mL of PBS. Two mL of the combination of ketamine (75mg/kg) and xylazine (10mg/kg) in PBS were administered intraperitoneally to the rat models 45 minutes prior to measuring the baseline readings and 50 minutes prior to injecting the test substances. An additional 1 mL/kg (0.2 mL per 200g rat) injection was administered at 40 minute intervals after the initial anaesthetic dose. Each test substance was administered intravenously through the tail vein 5 minutes after taking the baseline blood glucose measurement and blood sampling (10µL blood samples) continued over 3.5 hours in 10 timed intervals (0, 10, 20, 30,45, 60, 90, 120, 150, 180, and 210 minutes) as illustrated in Figure 2.2.2.1. During the whole procedure the animals remained under anaesthesia and were monitored for normal breathing and for any signs of pain or distress. After the completion of each procedure, the animals were sacrificed using cervical dislocation while still under anaesthetic.

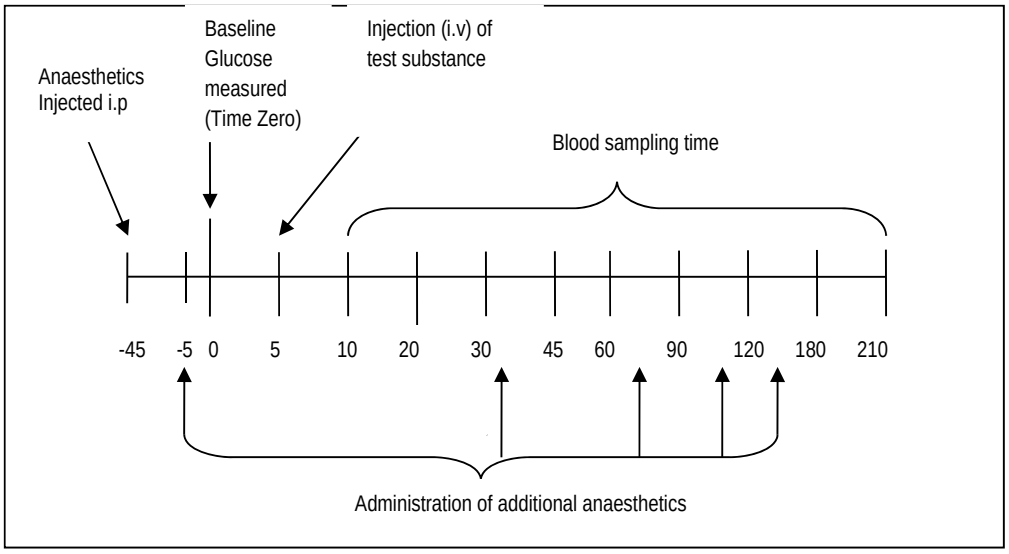


Figure 2.2.2.1 Diagrammatic representation of the final protocol developed indicating administration times of anaesthesia. Protocol development is discussed in the Discussion section (Chapter 4).

Each sample was transferred onto Accu-chek glucose strips and all readings were obtained from the Accu-chek glucometer after 5 seconds. The readings were recorded as mmol/L of blood glucose. The % change of blood glucose was calculated according to the following formula [Torres and Suarez et al [69]**]**

$$\% \text{ change of glycaemia} = (Gx - Go) / Go \times 100$$

*(Go=blood sampling before treatments Gx= blood sampling after treatment*

### 2.2.2.2. METHOD OF PREPARATION AND ADMINISTRATION OF TEST SUBSTANCES

*Preparing the plant extract dose for the rat model*

The plant extract was solubilised in 300 µL of phosphate buffered saline and a pH check was conducted on the concentrate (found to be pH ~3), 2 µL of NaOH (2.5 mM) was added to adjust to pH 7.2-7.4. This was then filtered using a size 0.8µm syringe filter. The rats then were administered intravenously with 0.2 mL of the extract in PBS.

*Preparing Humulin R for the rat model*

The concentrated Humulin R was diluted to an initial dose of 2U/kg in PBS and was then assessed in the anaesthetised rat model by an intravenous injection followed by measurement of blood glucose using the glucometer. This was then adjusted to 0.5U/kg after preliminary studies which indicated that 1U/kg was too high. The dose was diluted in PBS to give 0.1 Units of insulin in a 0.2mL injection delivered intravenously to the rats.

*Preparing metformin for the rat model*

Initially 50mg/kg was tested in the anaesthetised rat model. After initial preliminary results, this was adjusted to 100mg/kg [70, 82]. Metformin 100mg/kg was used as a standard dose in the study. A total weight of 50mg of metformin was solubilised in 0.5 mL of PBS and from the solution 0.3 mL was drawn and 0.2 mL (equivalent to 20mg of metformin) was administered to the rat model. The pH of metformin alone was 6.9 as indicated during the pH check and also indicated by Adikwu et al [71] therefore there was no need for a pH adjustment.

## 2.3 STATISTICAL ANALYSIS

The statistical analysis was established in two ways. An analysis of variance (ANOVA) [72] model was supplied at each time point as a separate and also as a repeated measure using all the data obtained. These were then adjusted for the correlation due to the multiple measurements made on each rat. A p-value was obtained for each analysis which was a comparison between the control and each test treatment as well as a p-value for each pair-wise comparison, of the mean glucose concentration at that time point. The analysis also included a term for the initial weight of the rat, so that p-values obtained involved adjustments for weight. Initially a power analysis [72] (post hoc power analysis) was conducted with a sample of 7 rats in each group.

Results are given as mean blood glucose levels ± standard error of the mean (S.E.M) and also expressed as relative percentage mean blood glucose levels (%) with glucose level at time 0 taken as 100%. *P* values of 0.05 and less were taken as significant.

# 3.0 RESULTS

## 3.1 PLANT EXTRACTION AND PHENOLIC CONTENT ANALYSIS

### 3.1.1 EXTRACTION OF *T. POLIUM* WITH METHANOL

The optimized procedure included a reduced extraction time of 3 hours reduced from the initial time of 4 hours. This was to minimise the effects of heat on the constituents of the plant, which could lead to degradation. The volume of extract obtained after the Soxhlet extraction was reduced by evaporation under vacuum giving an extract which was viscous and greenish in colour.

TABLE 3.1.1 OPTIMIZED EXTRACTION FROM 2.5G OF DRY PLANT MATERIAL USING 200 mL of 90% METHANOL CONCENTRATON AT ROOM TEMPERATURE

| Conc. Of Methanol | Extraction Yield from dry plant material (%) | Total Phenolics (mg) | pH of extract in vehicle (PBS) |
|---|---|---|---|
| 90 % | 20.4 | 17.85 | 3 |

### 3.1.2 PHENOLIC CONTENT

The total phenolic content in *T. polium* extract was assessed over time as a non-specific measure of extract decomposition during storage and the results are given in Table 3.1.2. A standard curve was run at the same time of sample analysis and these curves were averaged (Figure 3.1.1) to assess their variability over the entire period.

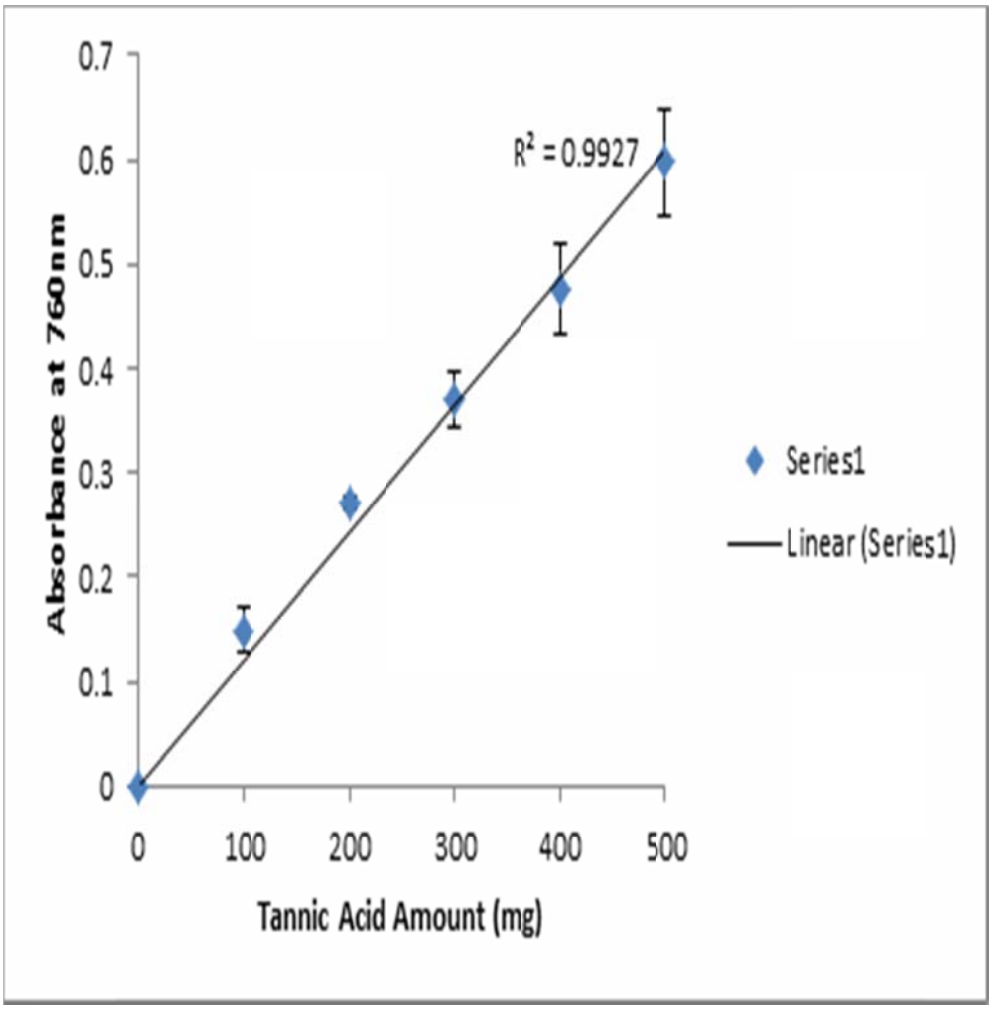


Figure 3.1.1 Mean standard curve of tannic acid concentration verses absorbance at 760nm for sample phenolic content analysis The graph is the mean of 6 curves (±SEM) *with a cumulative co-efficient of variation = 0.9927*

TABLE 3.1.2. PHENOLIC CONCENTRATION IN PLANT EXTRACT DURING STORAGE

| Storage time of plant extract (Days) | Total Phenolic content (mg) | Yield of phenolic content (%) |
|---|---|---|
| 0 | 17.85 | 7.14 |
| 15 | 16.1 | 6.44 |
| 30 | 12.25 | 4.9 |
| 45 | 14.5 | 5.8 |
| 60 | 14.25* | 5.7 |
| 90 | 13.5* | 5.4 |

*Note:* Results were calculated from individual standard curves (*Absorbance verses amount of Tannic acid*) prepared on the day of testing. (*) Indicates the total phenolic concentration in plant extracts during the period the animal trial was conducted.

Extractions were carried out using 90% methanol and solubilised in phosphate buffered saline (PBS). Testing of phenolics was conducted over 90 days but the animal trials were conducted from day 60 to day 90 only. The phenolic content was determined straight after extraction (day 0) and on 5 other days 15 to 30 days apart to estimate overall stability of the phenolic compounds extracted.

### 3.1.5 SOLUBILITY OF PREPARATION IN SUITABLE VEHICLES FOR ADMINISTRATION INTO THE ANIMAL MODEL.

The plant extract was observed to be partially soluble in 1 mL of all three vehicles. The extract solutions were filtered to remove any residual insoluble material using a sterile syringe (0.8μm) filter. Phosphate buffered saline was then chosen as the medium for administration of all test substances because it has buffering capacity in the pH 7 range and is of course safer than DMSO. Since the plant extract had a pH=3, which is very acidic compared to blood pH, it was adjusted with 2 μL of NaOH (2.5 mM) to pH=7.2-7.4.

Metformin and insulin both had a pH of 6.9-7.0 therefore there was no need for pH adjustment and they were solubilised and/or diluted in PBS for i.v. administration.

TABLE 3.1.3. THE pH LEVEL OF PLANT EXTRACT IN DIFFERENT VEHICLES

| Vehicle | pH of plant extract in vehicle |
|---|---|
| 0.9% saline | 3 |
| 0.1% DMSO in saline | 3 |
| Phosphate buffered saline | 3 |

## 3.2 ANIMAL STUDY DESIGN

The design of the study was made with reference to Garaibeh et al [16] and Schäffer et al [68] Other references also were reviewed and considered in the study design. [17, 19, 21, 23, 25, 55]

### 3.2.1 PRELIMINARY PROTOCOL DEVELOPMENT

Table 3.2.1 Preliminary data assessing the initial concentrations of the anaesthetic used, the resting time (time between initial anaesthetic administration and before baseline glucose sampling: Fig 2.2.2.1) and the doses of the different drug treatments to be tested.

Table 3.2.1 PRELIMINARY DATA OF DOSES USED IN ANIMAL STUDY AND RESTING TIME

| **Anaesthesia** | | **Dose Adjustments** | |
|---|---|---|---|
| | **Trial 1** | **Trail 2** | **Trial 3** |
| Ketamine<br><br>Xylazine | Ketamine [65]<br>100mg/kg rat<br>Xylazine [65]<br>5mg/kg rat | Ketamine<br>75mg/kg rat<br>Xylazine<br>20mg/kg rat | Ketamine<br>75mg/kg rat<br>Xylazine 10<br>mg/kg rat<br>(Effective) |
| **Resting Blood glucose level** | | | |
| Resting time before baseline reading | -15min | -30min | -45min<br>(Effective) |
| Treatments | | Dose Adjustments | |
| Insulin | 2U/kg [88] | 1 U/kg | 0.5U/kg<br>(Effective) |
| Metformin | 50mg/kg | 100mg/kg (Effective) | |
| Plant extract | 0.5g/kg rat or 100 mg/0.2mL of diluents (Effective) | | |

### 3.2.1.1 TREATMENT AND DOSES

Animals in the control group were administered PBS and no problems were observed with regard to its administration.

The insulin dosage was adjusted three times. Initially we used 2U/kg. [88] This produced a dramatic drop in blood glucose to dangerous levels. Thus the dose was reduced to 1U/kg and trialled. Again, this produced a similar effect as the previous dose. The dose was then adjusted to 0.5U/kg. This dose gave a more gradual drop in blood glucose which did not place the animals at risk of dying during the experiment (Figures 3.3.1 and 3.3.2.4).
The dose of metformin, the dose required adjustment. Initially a dose of 50mg/kg was trialled. [91,92] This dose was observed to product less effect in the normoglycemic rats. The dose was then increased in accordance to Adikwu et al[71] to 100mg/kg This also was observed and showed slight effect and was used in the animal trial as a second control because of its plant origin as a comparison to the plant extract. (Figures 3.3.1 and 3.3.2.3)

A dose was chosen to comply with previous publications. [16] A 100mg of the plant extract diluted in PBS and pH adjusted was administered to the animals which caused a drop in blood glucose level (Figures 3.3.1 and 3.3.2.2).

### 3.2.1.2 ANAESTHESIA

The anaesthetic used in the study was a combination of ketamine and xylazine. Ketamine and xylazine can either be used in combination or alone as anaesthetics. [66] In the current study, a combination was used. This was to avoid the side effects (tremor, muscle rigidity) of ketamine if it were to be used alone. Initially ketamine 100mg/kg and xylazine 5mg/kg were trialled. [66,74] However, the sleep time (unconsciousness) before recovery was long, about one hour and the animals were also constantly passing urine after the anaesthetic dose was administered resulting in an extended time to respond to painful stimuli. The combination of 100mg/kg ketamine and 10mg/kg xylazine induces acute hyperglycemia at 20 minutes which tapers off in fasted rats.[73] Therefore the anaesthetic dose was to be adjusted to achieve

anaesthesia initially to keep the animals under anaesthesia with minimal effects on blood glucose levels. Considering these results and the individual effects of ketamine and xylazine, the dose of ketamine was reduced to 75mg/kg and the xylazine was increased to 20mg/kg. This is because ketamine produces sedation and immobilization but its side effects include tremor and muscle rigidity [73] which was observed during the initial trials in the rat at 100mg/kg. Xylazine, which produces more sedation and muscle relaxation, was increased to 20mg/kg but this was found to prolong the sleep time (unconsciousness) to 45-50 minutes in the current study. Xylazine affects the antidiuretic hormone (ADH) secretion and glucose homeostasis and thus causes a slight rise in blood glucose[74]. Also a dose of xylazine higher than 20mg/kg had resulted in pulmonary edema observed in some animals. [74]

The dose regimen was finally adjusted to deliver 75mg/kg ketamine and 10mg/kg xylazine. The sleep time was about 40 minutes allowing for four additional anaesthetic doses to be administered every ~40 minutes. These times were also adjusted slightly according to the responsiveness of the individual rats. This is because during the initial preliminary work with the rats, the animals responded well and were all starting to wake up after about 40 minutes from the time of the administration of the anaesthesia. The recovery period after each procedure took effect within 3 minutes. This initial work allowed for the recovery period since this was required in order to determine when re-dosing was necessary. The protocol finally appeared very suitable and convenient for the study with minimal effects on blood glucose levels (Figure 3.3.2.1).

### 3.2.1.3 RESTING PERIOD

Determining the resting period before baseline readings was another task. Initially -15 minutes (15 minutes before baseline reading) was trialled. [68] This did not appear long enough in our laboratory because the initial handling and administration of the anaesthetic appeared to have unsettled the rats (adrenalin rising and/or insulin dropping) [73] which affected the initial blood glucose levels. Therefore the time was gradually extended eventually reaching 45 minutes prior to administration of vehicle. This time was found to be suitable allowing for the initial rise in blood glucose level to come back to normal and stabilise. This meant that

40 minutes after the initial intraperitoneal administration of the anaesthetic a top up anaesthetic injection was given and then 5 minutes later the first blood glucose measurement was taken (Figure 2.2.2.1). This appeared to give enough time for the animals to stabilise with regards to any hormonal effects that might have affected their initial blood glucose levels.

### 3.2.2 FINAL STUDY DESIGN & PROCEDURE

The animals used were all healthy normoglycemic rats but as they would have experienced some anxiety due to handling prior to anaesthesia, a suitable resting period before baseline reading was necessary to normalise their blood glucose levels before starting with the treatments. This was established to be 45 minutes (Section 3.2.1.3). The whole procedure was conducted in 210 minutes at 10 timed intervals therefore the animals were maintained under anaesthesia throughout this period. The combination dose of ketamine 75mg/kg and xylazine 10mg/kg were administered at 40 minutes intervals which kept the animals sleeping. This was also suitable in reducing stress upon the animals. The number of animals used in the current study was also minimised and still achieve statistical significance in the results. The animals numbers used were suitable because in all four treatment groups, a maximum number of 8 rats were used in three of the groups and 6 rats in one of the groups. This number of animals produced statistical significance in the results. The use of only normoglycemic rats in the entire study was a success making this model simple and less expensive. Also this model developed can be used to study an acute effect over short period of time, which is vital for screening many medicinal plants.

In the current study, a random allocation of the animals upon arrival was carried out in order to reduce bias in the selection and the study. Many of the studies mentioned (on *T. polium)* had not included the random allocation process and did not specify in their study the type of method used in allocating the animals to the different treatment groups. Our current study was carried out on healthy normoglycemic Wistar rats weighing in the range of 190-232g and housed for a 3-day acclimatisation period in the Curtin Animal facility. Food and water were supplied daily and the beddings were changed as necessary but also just before the start of the

18 hour fast. During their acclimatisation periods, the rats were observed to have gained weight, about 10g per day on average. This slight gain in weight did not affect the average weight set for each rat to be included in the study because upon arrival at the facility, the animals were record to have lost weight during travel. During the fast, the animals were observed to have eaten their own faeces due to hunger and also which is common (*coprohagia).* Therefore to minimise any effect this would have on the blood glucose results, the animals always had their caged beddings changed before the start of the fast and monitored closely.

The procedures developed for the testing of *T. polium* in normoglycemic rats was convenient for the study. All animals were weighed before each procedure and were all within the weight range to be included on the day of the procedure. All test substances were prepared daily for the procedure and the animals were handled before intravenous administration of the anaesthetic in order to reduce some stress. After the administration of the anaesthetics, the animals went to sleep within 3 minutes of receiving their doses. Set doses were calculated for each animal depending on the weight and all were observed to remain calm during the procedure. The procedure time was appropriate with a resting period of -45 minutes before blood sampling at time zero. During this time the animals were kept asleep with another additional dose administered (half the dose of ketamine 75mg/xylazine 10mg /kg) at -5 minutes before time zero (Figure 2.2.2.1).

## 3.3 EFFECT OF *T. POLIUM* ON BLOOD GLUCOSE LEVEL

According to the data on Figure 3.3.1 and Table 3.3.1, the average baseline blood glucose level show variations amongst the four treatment groups. These differences in the baseline readings (*time=0*) were adjusted and accounted for in the statistical analysis. The blood glucose levels for each rat were calculated as change from baseline levels.

The results show (Figure 3.3.1) that the blood glucose levels after administration of plant extract, insulin and metformin as compared to the vehicle (PBS). Animals receiving the plant extract and insulin differed from the control from 20 minutes after receiving their treatments.

Both plant extract and insulin significantly ($p<0.001$) reduced blood glucose levels as compared to the vehicle (PBS). After 30 minutes, the plant extract and insulin still showed a statistically significant ($p<0.001$) drop in blood glucose level as compared to the vehicle (PBS). However after 30 minutes into treatment, results for the plant extract were not statistically significantly different when compared to insulin, but were statistically significantly different from metformin ($p<0.05$). This indicated that the plant caused an acute effect in reducing the blood glucose levels. The blood glucose level in animals treated with plant extract showed a much higher rate of decrease than metformin. Those that received metformin treatments had shown a greater drop in their blood glucose from baseline as compared to the control but this was not statistically significant.

### 3.3.1 EFFECT OF TEST COMPOUND ON BLOOD GLUCOSE LEVELS IN NORMOGLYCEMIC RATS

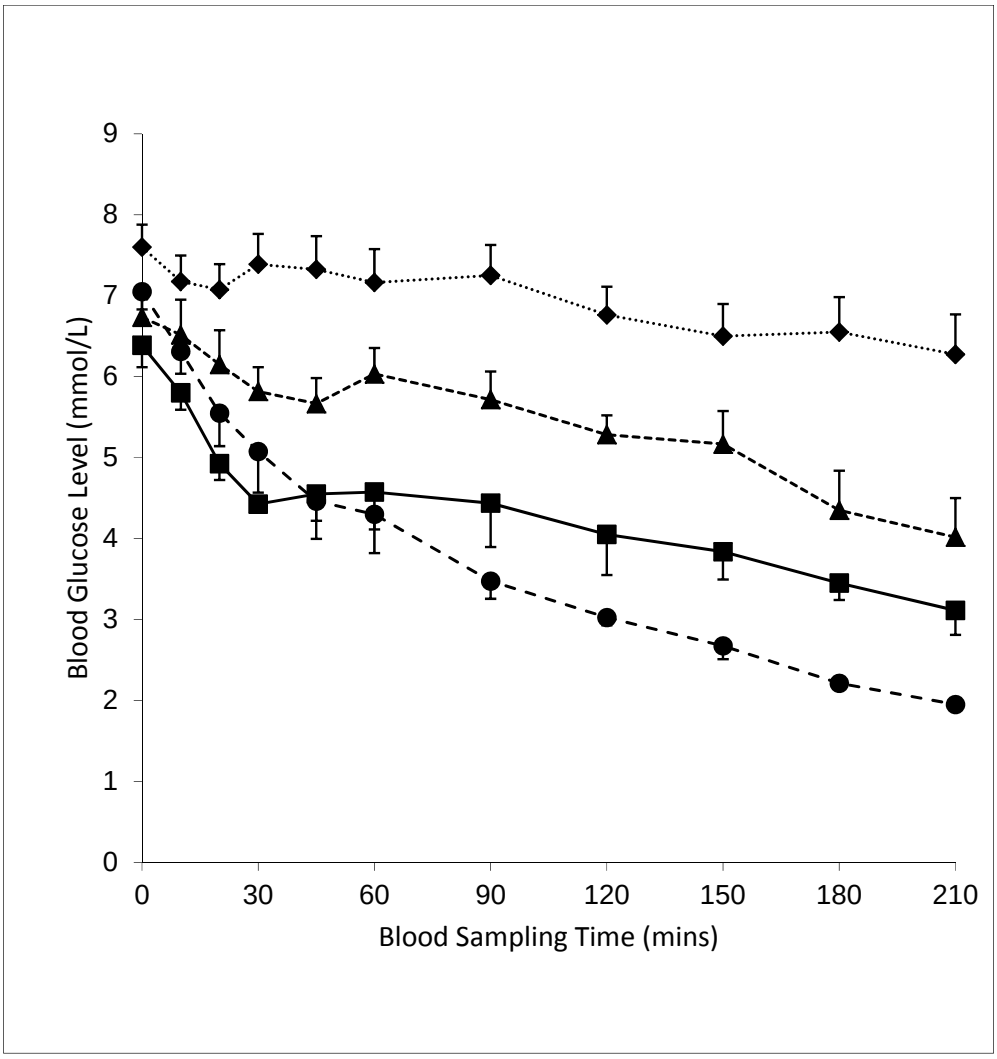


Figure 3.3.1 Blood glucose level after i.v administration of 0.2 mL of vehicle (PBS, *n=8*, ◆), metformin in vehicle (*n=6*, ▲), plant extract in vehicle (*n=8*, ■) and insulin in vehicle (*n=8*, ●) to anesthetized Wistar rats. Results are expressed as mean ±SEM.

A separate Analysis of Variance (ANOVA) [72] revealed that after 45 minutes, a less significant effect was observed between the plant extract and metformin when the effect of insulin diverged. This is shown in Table 3.3.1. The figures shown are the raw unadjusted mean blood glucose levels at each time points. The pairwise comparison of the means was performed at each time point by conducting an Analysis of Variance (ANOVA) on the change from baseline glucose for each animal. The data shows that all pairwise comparisons of these adjusted means were statistically significant.

Comparisons of the average values of blood glucose levels in the rats were analysed as described in the methods (Chapter 2, section 2.3) addressing differences from the baseline. However statistical analyses collected from all data points and using a repeated measure of ANOVA revealed that statistical significance was observed between each pair of treatments as shown in Table 3.3.1. A statistically significant difference of $p<0.01$ was observed between the plant extract and vehicle and between insulin and the vehicle. Metformin was not statistically significantly different as compared to the vehicle ($p>0.08$).

Table 3.3.1: The effect of *Teucrium polium* extract on blood glucose levels within 3 hours after intravenous administration

| Experimental Group | Dose in 0.2mL of PBS (i.v.) | Average weight (g) [range] | Mean fasting blood glucose levels (mmol/L) | mean blood Glucose Levels (mmol/L) | | | | Average drop in blood glucose from baseline |
|---|---|---|---|---|---|---|---|---|
| | | | | 30 min | 60 min | 120 min | 180 min | |
| Vehicle *(n=8)* | PBS | 208.12 [190-232] | 7.61 | 7.38 | 7.16 | 6.76 | 6.55 | -0.68 |
| Metformin *(n=6)* | 20mg/ rat | 199.33 [190-215] | 6.75 | $5.81^{cd}$ | $6.03^{cd}$ | $5.28^{d}$ | $4.35^{d}$ | -1.14 |
| Plant Extract *(n=8)* | 100mg / rat | 214.62 [200-230] | 6.38 | $4.4^{ab}$ | $4.57^{b}$ | $4.05^{b}$ | $3.45^{b}$ | -2.09 |
| Insulin *(n=8)* | 0.1 Unit/ rat | 220.5 [210-232] | 7.03 | $5.07^{a}$ | $4.3^{a}$ | $3.02^{ab}$ | $2.21^{ab}$ | -3.16 |

**The figures in the table are the raw (unadjusted) mean blood glucose levels at each time point. Pairwise comparison of these means was performed at each time point by conducting an ANOVA on the change from baseline glucose for each rat (*p-values* noted below): Note that all pairwise comparisons of these adjusted (for the baseline weight of each rat) means are statistically significant (p≤0.01 in all cases).adjusted.**
**[a] Significantly different from the vehicle (*p<0.001,*) [b] Significantly different from the vehicle (*p<0.01),* [c] Significantly different from plant extract (*p<0.05),* [d] Significantly different from insulin *(p<0.01)***
**In addition, a Repeated Measures ANOVA was performed on the change from baseline glucose at all time points, (adjusting for baseline weight), and the 'adjusted average' changes from baseline (last column) were obtained as the Least Squares Means from that analysis.**

### 3.3.2 COMPARISON OF PLANT EXTRACTS AGAINST CONTROL, INSULIN AND METFORMIN

Percentage change of glycaemia from baseline was calculated, using the formula in Chapter 2, section 2.2.2.1, for control plant extract, insulin, and metformin. The percentage change in blood glucose level in those animals treated with the control was about 5% in the first 30 minutes and 2.5% at 60 minutes from time zero. This was maintained throughout the procedure. (Figure 3.3.2.1)

The plot for the percentage change in blood glucose in those treated with the plant extract is shown in Figure 3.3.2.2. The percentage change in blood glucose was about 30% in the first 30 minutes and 25% in the next 60 minutes. This was then decreased to 40% at 210 minutes. The highest significant ($p<0.001$) hypoglycaemic effect of the plant extract was observed at 30min, 60min, 90min and 180mins. This indicates that the extracts from the plant *T. polium* significantly lowered the blood glucose level after an intravenous dose to normoglycemic rat models in the fasting state. The plot for metformin (Figure 3.3.2.3) shows the percentage change in blood glucose was estimated at 15% in the first 30 minutes and 5% at 60 minutes, 15% at 90 minutes and 30% at 210 minutes. The percentage change in blood glucose within the first 30 minutes was about 15% and 20% within the first 60 minutes. A large percentage change in blood glucose was observed with insulin as there was an increase over time (Figure 3.3.2.4). In the first 30 minutes from time zero, the change was 25%. This could indicate that the plant extract had a similar effect to that of insulin within 30 minutes of intravenous administration. The percentage change was then increased to 35% in 60 minutes, 50% in 90 minutes and 70% in 210 minutes.

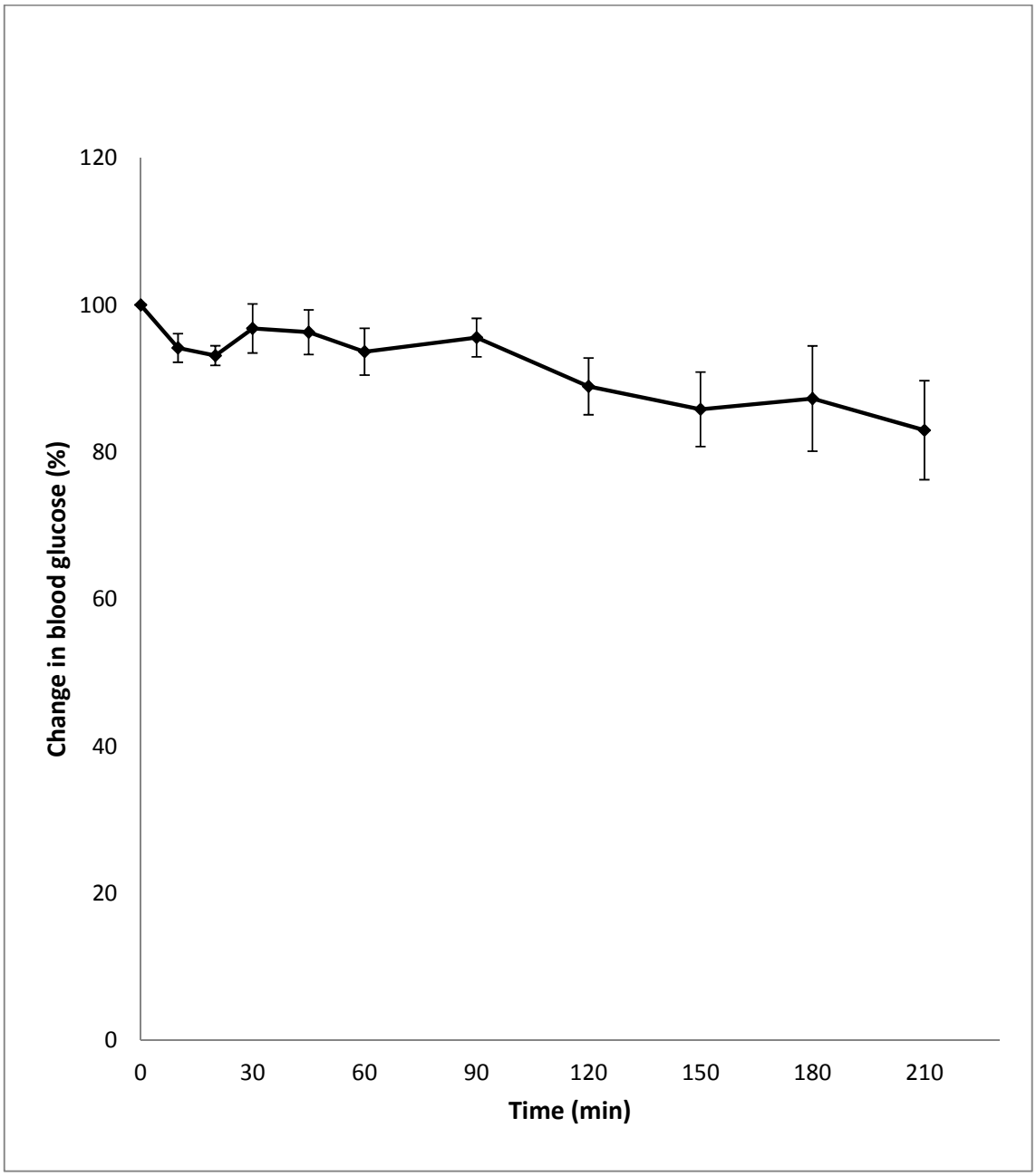


Figure 3.3.2.1 Mean (± SEM) Percentage change in blood glucose in normoglycemic rats (*n=8*) receiving the vehicle

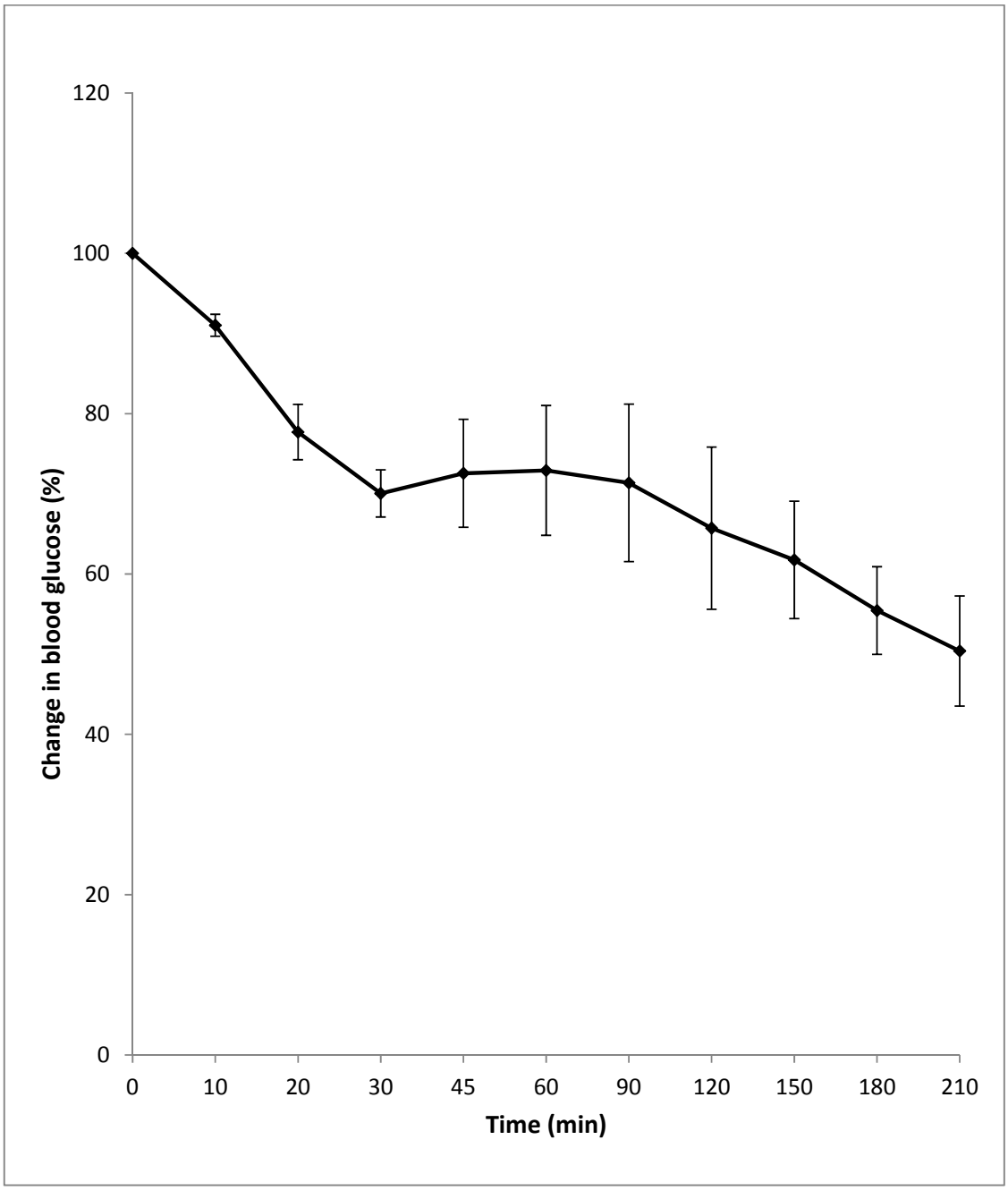


Figure 3.3.2.2 Mean (± SEM) Percentage change in blood glucose in normoglycemic rats (*n=8*) receiving the plant extract

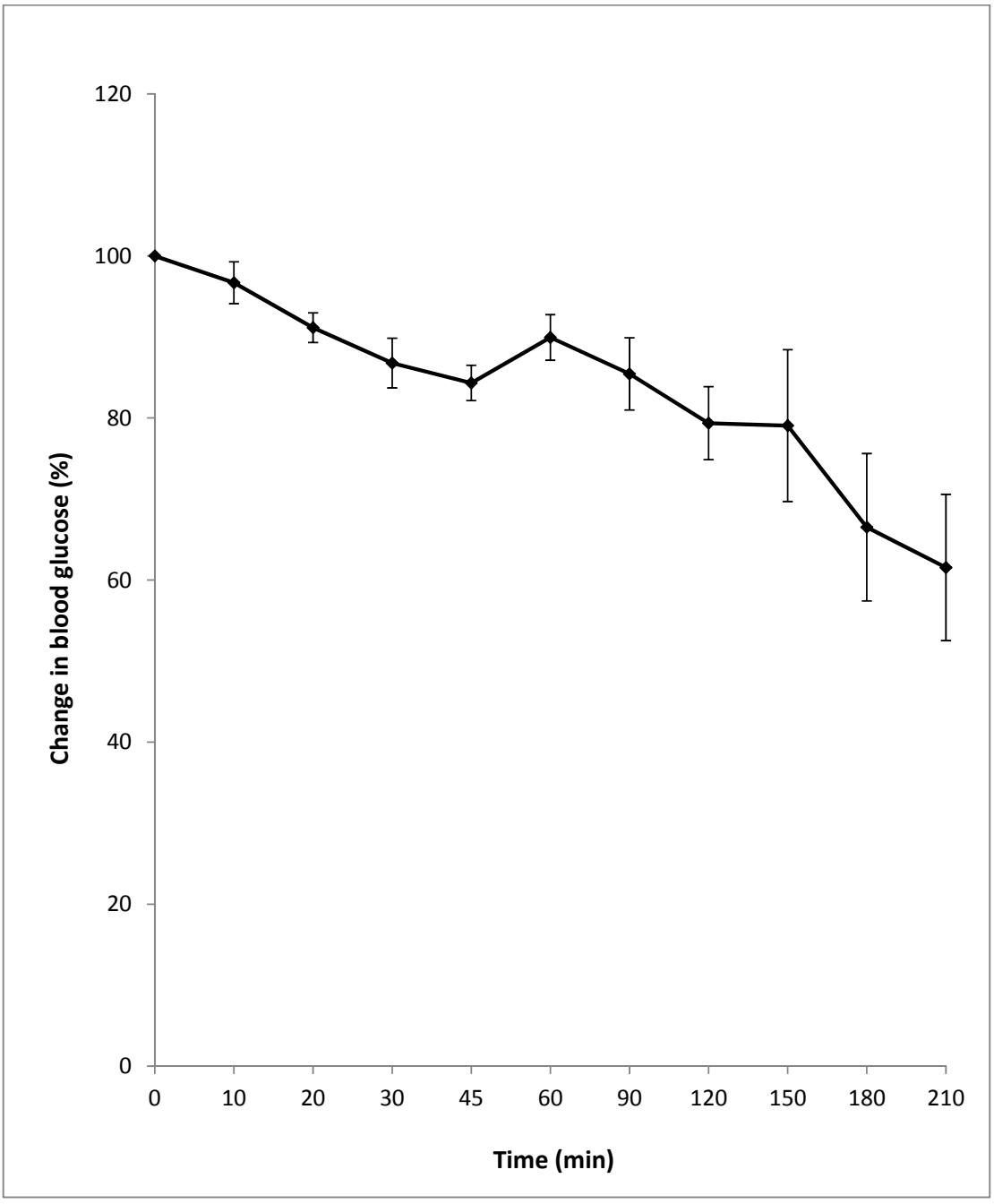


Figure 3.3.2.3 Mean (± SEM) Percentage change in blood glucose in normoglycemic rats (*n=6*) receiving metformin

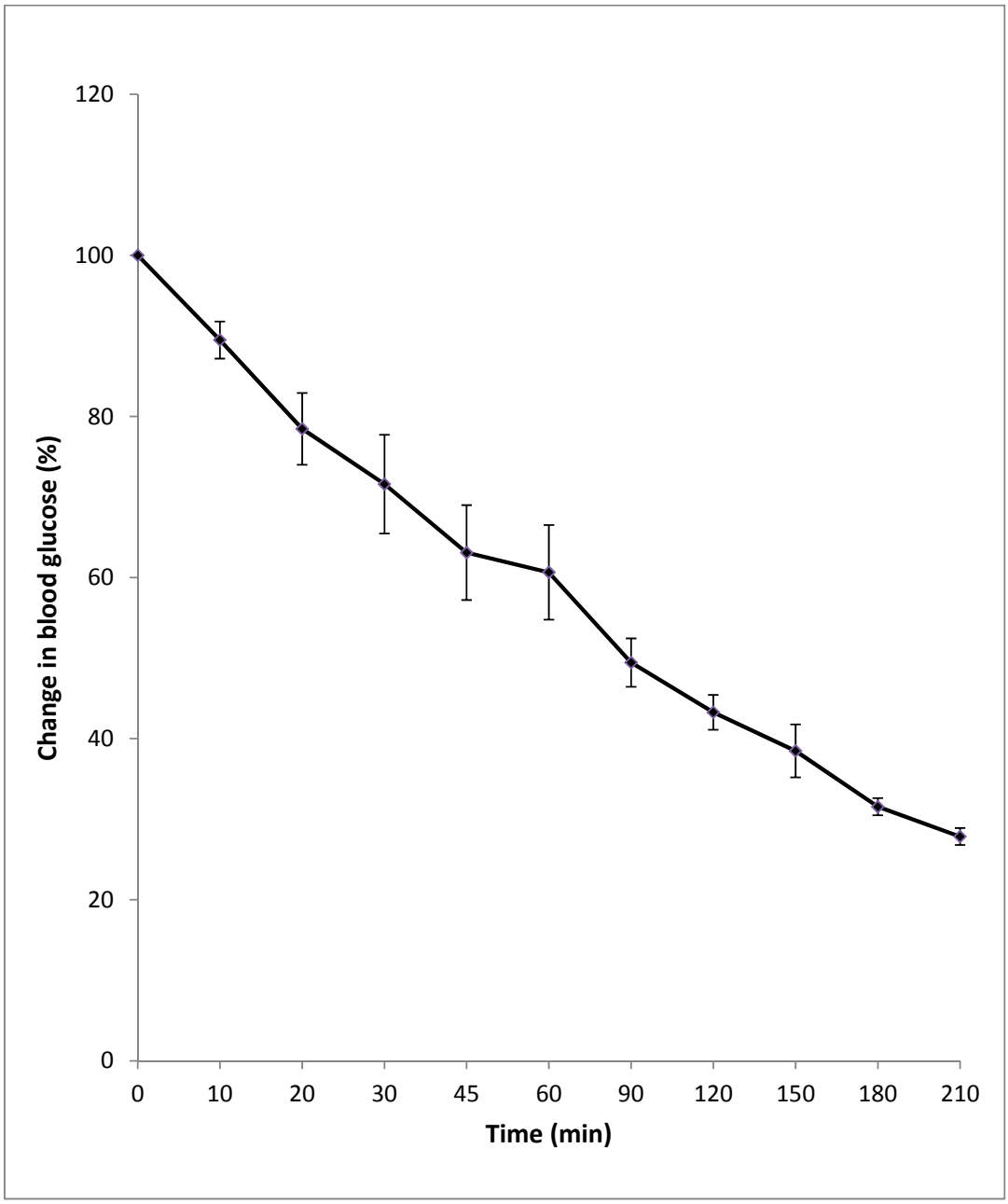


Figure 3.3.2.4 Mean (± SEM) Percentage change in blood glucose in normoglycemic rats (*n=8*) receiving insulin

# 4. 0 DISCUSSION

## 4.1 PLANT EXTRACT AND PHENOLIC CONTENT ANALYSIS

Various extraction methods have been developed and used over the decades to extract from plant materials. These range from simple extractions of decoctions obtained from the powdered plant material being boiled [18, 20, 24, 27, 51] to plant maceration in distilled water [15,55]. Other extraction methods have used alcohol (either ethanol or methanol) with the aid of the Soxhlet extraction apparatus usually followed by the rotary evaporator for concentrating the extract [17, 21, 23]. Studies on the plant *T. polium* [16, 17,18, 20, 21, 23, 24, 25, 27, 51, 55] have used various differing and modified extraction methods. These extracts were then administered directly to the experimental model either through oral gavages or i.v injection. From the many extraction procedures being conducted, water extracts and alcohol extracts, mostly ethanol and methanol (30%, 50% and 70%), were commonly used.

For this study methanol was used in the extraction procedure since it was the cheaper option. Also it has a protective and preservative effect on the constituents of the medicinal plants and would evaporate and dry faster during the drying process of the extracts. [80] According to our preliminary extraction results, 90% methanol generated a higher yield from the dry plant material of *T. polium* as compared to other methanol concentrations (100%, 80%, 70% and 50% Methanol) therefore it was used as the solvent of extraction. It appears that the 10% water content assists in the extraction process allowing the release of the constituents from the plant cells into the surrounding solvent. [81]

The plant material to volume of solvent ratio is another variable that needed careful assessment in order to optimize total phenolic extraction [81]. According to the literature, certain extractions from plants are planned according to the ratio between the amount of plant to extract and the volume of solvent to be used in the extraction. [9, 67, 78] It appears that ratios of 1:4, 1:10, 1:20 and 1:50 (amount of plant material to volume of solvent) are commonly employed. [81] From the current study the volume of methanol and how much dry plant material to be use were considered and assessed carefully. Judging from the literature, [9,67,78, 81,89,] it was decided to use the 1:50 parts ratio in the current work, 5g of dry plant material to

250mL of solvent (90% methanol). Thus there was no need for large quantities of solvent of extractions; because a smaller volume of the solvent of extraction allowed a faster drying process. Just before injecting, the dried extracts were dissolved in phosphate buffered saline, pH adjusted and filtered to eliminate any particulate matter. From the three vehicles tested (Normal saline, DMSO and PBS), PBS was considered to be the most suitable (Table 3.1.3) especially since the solubility of the extract was not aided by the use of DMSO but also PBS is a better vehicle due to its osmolarity and buffering range.

One aspect that had to be adjusted for was to obtain the exact amount of extract when diluted to give equivalent doses to be administered to the animals. This dosage as used in accordance with previous literature[16] which was extracted from 0.5g plant material/kg rat. The extract obtained was divided into equal aliquots into separate vials enabling the delivery of extract from 100mg of the plant material per rat on the day of experimentation (Section 2.1.2.1). There was only one batch of extract produced in order to avoid variations between batches and thus reduce variability between doses administered to the rats. These vials were stored at 4°C in the refrigerator for single use. Before i.v. administration, the extract was pH adjusted and then filtered to be more suitable physiologically. There were no side effects observed to the plant extract.

The total phenolic content in the plant extract from *T. polium* was assessed using the Folin-Denis Method. [60] *T. polium* and many medicinal plants contain a high level of phenolic constituents which have been as attributed to playing vital roles in its many medicinal properties. A simple test was carried out to determine the level of phenolic content in the plant extracts immediately after extraction and before each use in the animal study. This was used as a mean of assessing the stability of the extract over the storage period. [60, 61, 80]

Figure 3.1.1 shows the mean standard curve of tannic acid concentration verses the absorbance at 760nm over the entire study period. The graph shows linearity with a *cumulative co-efficient of variation of 0.9927.* Table 3.1.2 indicates the phenolic concentration in the plant extract during storage and immediately after extraction. The plant extract was stored for a total of 90 days.

From day 0 to day 45, there was a slight decrease in total phenolic content within this period. However, during day 60-90, there was a small decrease (5%) which had a negligible effect on the glucose lowering properties of the plant extract. The phenolic content on day 30 could not be verified but was most likely due to an error in the assessment technique of that vial on the day or could also be a result of a pippetting error on collection of the sample on the day of the extraction or possibly a result of loss of sample during evaporation before storage.

The total phenolic content was determined and believed to contribute to the hypoglycaemic effects of the plant. [19, 90] The animal trial ran between day 60 and 90 during which time the phenolic content of the extract appeared rather stable reducing unnecessary variability in the study. The method used only gives a crude estimate of the total phenolic compounds present in plants therefore the amount of individual phenolic compounds may vary depending on the plant, with some seasonal and regional variations. We could not specify the exact stability of individual constituents of the extract during storage within the 90 days as the compound or compounds of interest is/are unknown and could be the smallest component(s) of the mixture.

Several studies have indicated the importance and role of specific phenolic substances as having "medicinal" properties. Phenolic substances are believed to cause a decrease in blood glucose level. [14, 16, 17, 18, 19, 21, 24] One of the phenolic substances identified in *T. polium* which has been tested separately and believed to possess anti-diabetic properties is quercetin. Quercetin is a flavonoid found in small quantities in *T. polium* which possesses hypoglycaemic activity. [22,28,61] Quercetin administered to STZ-induced diabetic rats at a dose of 10-15mg/kg per rat was shown to be capable of normalising blood glucose levels, augmenting liver glycogen content and significantly reducing serum cholesterol and LDL. [9, 28, 61] Analyses of specific constituents were not conducted as this was beyond the scope of the current study but this is an area for future work.

## 4.2 STUDY DESIGN FROM PRELIMINARY TO FINAL STUDY

Animal models have been used extensively in diabetic research to investigate anti-diabetic plants. In the past, and still current practice, both STZ (Streptozotocin)-induced diabetic rat models have been used for studying diabetes and anti-diabetic properties of plants and pure substances. [65] More recently animal models have been developed which are used to clarify the mechanisms for the progression of type II diabetes in humans and for screening anti-diabetic plants and substances; however, these models are expensive to purchase and require extensive work and time for their development. [64,74] One of the objectives of the current research was to develop an animal model that could be used for *in vivo* screening of plants for potential glucose lowering properties. In the current research, the use of normoglycemic rat model was made with reference to previously published animal trials screening for insulin like molecules[68] and others screening antidiabetic plants. Several issues were dealt with to choose the animal model that would suit the current study. One of the issues considered was that the rats were normoglycemic rat models, which have been included in many animal studies as controls (refer also to Table 1.4.4.); however reduced blood glucose levels on *T. polium* administration have also been reported in these rats. Many of these studies prefer the use of STZ-induced diabetic rat models, obese rat models or genetically bred models. [65, 74, 83] STZ-induced diabetic rat models are common in screening plants for antidiabetic properties. Studies using STZ rat models are usually longer studies which take up to 4-6 weeks, off which they observe the glucose-lowering effect of the plants being screened over time. In STZ rat models, there are no beta cells since they are being destroyed therefore they are better models for Type I diabetes rather than Type II diabetes. STZ destroys the pancreatic β-cells which are associated with a huge release of insulin and this makes animals more susceptible to severe hypoglycemia. That is why in several studies using STZ rats, the animals are often fed with glucose solution and are fasted for 8-12 hours to avoid hypoglycaemia [24] The drug is also toxic and is restricted to pancreatic β-cells, because it may cause renal injury. [65,70] Therefore a choice was made to use normoglycemic rats in order to observe a quick and acute effect of the plant. There were no difficulties with the use of normoglycemic rats in the current study because these bred of animals were fed normal laboratory food, they were allowed free access to water and caged in individual cages or two per cage. Those rats engaged two per cage were monitored closely because they were male

rats and aggressiveness and fighting were observed during the time of fasting. However all animals were well cared and housed under standard conditions and temperature (20±1ºC), with a regular 12 hr dark and 12 hr light cycle.

The final protocol developed was (section 2.2.2.1) a very convenient method which allowed the assessment of the plant extract for acute hypoglycaemic potential. The animals were handled frequently during their stay at the animal facility (before intravenous administration of the anaesthetic) in order to reduce some stress. All animals were weighed before each procedure and were all within the weight range to be included on the day of the procedure. All test substances were solubilized and/or diluted daily for the procedure. After the administration of the anaesthetics, the animals went to sleep within 3 minutes of receiving their doses. Set doses were calculated for each animal depending on the weight and all were observed to remain calm during the procedure. The procedure time was suitable with a resting period of -45 minutes before the blood sampling at time zero. During this time the animals were kept asleep with another additional dose administered at -5 minutes before time zero which is the baseline reading for blood glucose (Figure 2.2.2.1).

An animal model developed following procedures previously published [68] was to measure the acute effects of the plant extract on blood glucose levels. The preliminary work was necessary to establish the dosing and sampling procedure for the main trial. Factors such as the type of animal model, the number of animals, the sampling methods, the type and administration of anaesthesia, the treatments and doses used were considered with reference to the literature. Since in many preliminary reports the extract appeared to lower blood glucose levels in normoglycemic rats it appeared reasonable to reduce the complications and use a normoglycemic rat model. A type II diabetic would have the pancreas intact and thus a normoglycemic model is a better representation than an STZ induced diabetic model. A published normoglycaemic model has been successfully used to assess the acute effects of insulin. [69]

During the preliminary experiments, all procedures were adjusted including the dosages for the test substances and anesthesia. The current study design allows for the assessment of the acute effects of different substances on blood glucose levels and other blood parameters if

necessary. The reason for the use of only normoglycemic rats was to observe a quick effect of the plant extract measured over a short period of time. This model was also cheaper, easy to care for, handle and readily available. Considering the time span of the study, the use of this model was convenient and the number of rats necessary to achieve significance was kept to a minimum as blood glucose fluctuations due to hormonal changes was reduced by the use of anaesthesia.

According to the study design, the animals were to remain calm and alive throughout the procedure. The procedure included injecting a test substance and multiple tail vein bleedings over a certain time for blood glucose measurements. These would commonly cause some stress in the animals and thus would definitely affect the results of the study (elevated blood glucose levels in response to adrenalin). To avoid this issue, the use of an anesthetic was proposed. Previous similar animal work[68] used 2 mL/kg Hypnorm-Dormicum (Hypnorm-Dormicum (1.25 mg/mL Dormicum_2.5 mg/mL fluanisone_0.079 mg/mL fentanyl citrate). Originally this anaesthetic was sought; however this was difficult to obtain. Therefore ketamine, in combination with xylazine, was used as an alternative. Ketamine and xylazine combinations are commonly used in animal studies including rat models as this anesthetic combination is reliable, cheaper and more readily available.

Initially ketamine 100mg/kg [73,66] and xylazine 20mg/kg [73] were trialled. This dose combination was observed to produce a very quick sedative effect and the animals went to sleep within 2 minutes after administration. Even though the animals were relaxed in sleep, they were consistently passing urine throughout the study and the sleep time exceeded 60 minutes before the animal awaking which was not appropriate for the study design. There were also concerns with over-sedating the animals and therefore we tried re-adjusting the dose. The ketamine dose remained the same (ketamine 100mg/kg); however the xylazine dose was reduced to 10mg/kg also according to referenced dosage. [66,73] A similar effect again was observed with similar outcomes to the previous initial dose. The dose was re-adjusted and ketamine 100mg/kg was reduced to 75mg/kg to reduce its effects of tremor and rigidity as observed when using the higher doses. This dose combination was observed to be very effective: the animals went under anaesthesia in less than 3 minutes, and they remained sedated for 40 minute after which full recovery of the animals was achieved within 3min.

This indicated that the sedation was not too deep and that additional top-up anaesthetic doses was to keep the animals under anaesthesia around 40 min after the initial dose and 40 min there after (section 2.2.2.1). Ketamine (75mg/kg) and xylazine (10mg/kg) were administration by the intraperitoneal route. This was easily conducted with the assistance of an experienced animal technician. Using this combination, the doses had to be calculated to cater for the weight of each animal on the day of the procedure.

Factors such as stress and pain (due to animal handling, anesthetic administration and tail vein bleeding) were minimized with the use of this anesthetic combination since they contribute to having an acute rise in blood glucose which could greatly affect the results. As shown in Table 1.4.4, many of the studies[17, 18, 24, 25] conducted earlier on *T. polium* did not incorporate the use of any anesthesia. Previous reports[25] had inflicted pain during blood sampling by clipping the toe nails of the animals to obtain blood; however no form of anesthesia was reported to have been administered. One would expect much variability in the data introduced by stress and thus large animal numbers to achieve significance.

The baseline blood glucose level was another important factor considered in the study design. As shown in Figure 2.2.2.1, the anesthetics were administered at -45 minutes and at -5 minutes before baseline blood glucose was taken at time zero. This allowed a 40 minute sleep time for the rats. These times were trialed and observed to be suitable for the study because ketamine and xylazine takes effect on blood glucose after 20 minutes of administration causing an acute rise in blood glucose. [74] There from the study design, the baseline blood glucose was measured 5 minutes after the administration of the second dose of anesthesia. However several baseline readings were observed to be slightly higher as shown in Figure 3.3.1 and Table 3.3.1 which will be explained further in the thesis.

The administration of the test substances was also considered important in the study design. The animals were distributed into four groups each being administered the test substance through the veins in the tail. The oral route of administration was avoided because of issues such as the unpleasant taste of many medicinal plants. *T. polium* is reported to have a very bitter taste and to administer the plant through the oral route to the animals over time would cause a decrease in consumption of the expected dose as the animals would consume less of

the bitter plant extract. Two previous publication and Konuklugil et al had both administered oral gavages.[16,24] These studies also included decoctiona administered as oral gavages after an 18 hour fast, however the doses administered were lower. This was followed by the administration of 50% glucose solution immediately after each treatment. One study [16] had administered the plant decoction through three different routes and obtained statistical significance. Other studies have also used oral gavages mainly because these studies were conducted over a longer period of time and also the rats were induced diabetic. In order to avoid such issues, the intravenous route was considered because a uniform dose was administered, and the extract pH adjusted (to suit the physiology of the rat) and filtered so as not to cause any reactions leading to death of the animal model.

Metformin 100mg/kg rat was also administered to one of the groups. Several studies have indicated its use in four effective doses in animal models; 50mg/kg, 100mg/kg, 200mg/kg and 500mg/kg. [70,71,79] The lowest dose of these was included in the current study. Initially, 50mg/kg was used, however it was observed to be less effective in the normal rats and caused only a slight decrease in blood glucose. The dose then was adjusted to 100mg/kg [79] which reduced the blood glucose level but was not considered to be statistically significant. This was expected because some other studies [70,71] also have shown that metformin was not effective in normal rat models but only in diabetic rat models. The higher dose of metformin used was suitable in the rat models and also did not cause any unwanted effect. This drug was chosen as a reference drug because the drug was used in some animal studies using rat models. An example also referenced in the study was that by Konukugil et al [24] who later considered the work by Garaibeh et al [16] and included the use of an oral hypoglycemic agent (Gliclazide) as a reference drug. Metformin has been found to be effective at higher doses in reducing blood glucose in other studies and its effects were compared against that of *T. polium* extract. Another reason for the use of metformin was because of its plant origin and that it was easy to prepare and administer in the form of injection to the rat. There were no adjustments because Metformin was diluted in PBS and had a pH of 3.

*Humulin R* (insulin) was also used as a positive control to fully compare the activity of the plant against and was effective in reducing blood glucose. During animal trials different doses of insulin were used; however the dose of insulin and the type of preparation used in

our current work was in accordance with one trial. [68] which used a lower dose of human insulin 0.35U/kg (2.5nmol/kg). In the current study, difficulty was experienced during the dilution of this dose in PBS for the intravenous administration into the animal models therefore a higher dose of 1 U/kg initially was used and tested during the preliminary trials. This dose was effective in reducing blood glucose levels however it was observed in some rats that their blood glucose was reduced so low resulting in severe hypoglycaemia. There was a re-adjustment to a lower dose of 0.5 U/kg (3.6nmol/kg), slightly higher than that previously quoted in the same animal model in the literature. **[68]** This dose was found to be suitable because within the 3.5 hours of sampling time, the dose had effectively reduced blood glucose levels. There were no deaths recorded due to severe reduction in blood glucose and it was easily prepared and diluted in PBS before i.v administration. Animals in the control group were administered phosphate buffered saline and no problems were observed with regard to volume administration.

In the study design, an important aspect was the number of animals to use to actually observe a statistical significance result. To achieve statistical significance in the study, the number of animals used has to be at a minimum. The level of significance varies and the minimum numbers could range from 4-12, as is the case with the studies shown on Table 1.4.4. some of which used *n=12* STZ-induced diabetic rats treated with the plant extract and observed significant effects of the plant extract after 6 weeks of blood glucose measurements **[17]**. Konukugil et al **[24]** used *n=10* but their results were not considered significant probably because they had used a lower dose and observed the effects over 5 hours at 60 minute intervals. Other factors had also contributed to their results. The lowest animal numbers used were that by Zal et al **[20]** (*n=5*). They observed a significant reduction in blood glucose only in the STZ-induced diabetic treated rats but not in the normoglycemic treated rat models. Other studies had used *n=8* which was also used in the current study. A total of 8 normoglycemic rats treated intravenously with the plant extract had produced significant reductions in blood glucose over 3.5 hours of study.

Finally, we used normoglycemic rats to observe a quick effect of the plant extract therefore the duration of the study was designed to be conducted within 3.5hrs, unlike other studies using STZ induced rat models which were observed over longer period from days to weeks.

The current study had observed the acute effect of the plant extract within 3.5 hours, which follows the study conducted by Konuklugil et al [24] which was 5 hours. A shorter blood sampling time was chosen [68] because a quicker effect of the plant was to be observed, and this could also reduce the amount of stress inflicted on the animals.

## 4.3 HYPOGLYCEMIC EFFECT OF *T. POLIUM* EXTRACT

The present study provides statistically significant evidence that *T. polium* has hypoglycemic potential *in vivo*. Past studies were either poorly controlled or have a number of factors affecting the outcomes due the study design or methodology which have been discussed in Chapter 1. [11-18, 21-26] In the present study we aimed to verify whether this plant had significant hypoglycemic properties and thus issues with the previous studies were taken into consideration.

The current trial investigates the acute effects of *T. polium* in comparison with other reference drugs like insulin and metformin as well as vehicle. Previous trials only examined the effects of *T. polium* extract compared to vehicle. Our current work indicates that T. polium acutely decreases blood glucose levels similarly to insulin but normalises faster with some similarity to metformin and less likelihood to cause hypoglycaemia like insulin at the doses studied.

The model used is of an animal that is not experiencing fluctuations in glucose levels due to stress every time it is bled and thus smaller numbers of animals were required to achieve significance. Using normoglycemic rats is a better representation of type II diabetics than STZ induced diabetic rats since the pancreas is intact in type II diabetes but not in the STZ induced diabetic rats. Previous trials have indicated that *T. polium* can cause a decrease in blood glucose levels in normoglycemic rats and therefore this further supported us trialling such a model for our current and future work. The administration of the plant extract to the normoglycemic rats was conducted intravenously through the tail vein 5 minutes after the baseline readings were recorded. This study design was optimised to observe a quick effect of the plant extract on blood glucose reduction. The results (Figure 3.3.1 and Table 3.3.1,

Section 3) indicate that blood glucose levels started dropping immediately after intravenous administration of the plant extract to the rats. This decrease from baseline was observed as statistically significant ($p<0.001$). Even after 30 minutes, the plant extract still maintained a significant drop in blood glucose level ($p<0.001$). This was also significantly different ($p<0.05$) after metformin up to 1h. The plant had maintained an acute hypoglycaemic effect throughout the study period. Although the effect of the plant extract and insulin were not considered significant after 30 minutes, it appears that both the plant extract and insulin could be exerting similar effects initially.

An Analysis of Variance of the data conducted after 45 minutes revealed a less significant effect being observed between the plant extract and metformin when the effect of metformin diverged. The slight rise in blood glucose after administration of the plant extract within the 30-60 minutes period was probably due to stress factors as indicated earlier.

The percentage change in blood glucose for Metformin and Insulin are shown in Figure 3.3.2.3 and Figure 3.3.2.4. The percentage change in blood glucose in those animals treated only with the control is shown in Figure 3.3.2.1. The percentage change remained within the range of 7% in the first 30 minutes, 12% at 210 minutes again showing little change in blood glucose reduction throughout the procedure. The percentage change in blood glucose for each treatment as shown in Figure 2.4.2.2 indicates similar results. Blood glucose at time 0 minutes is taken as 100%.

The animals receiving the extract from *T. polium* showed a different pattern. In the first 30 minutes from time zero, there was a 30% reduction in blood glucose. This slightly decreased back to 28% in the next 30 minutes (60 minute mark). At 90 minutes, the percentage change was at 29% and then 50% at 210 minutes. This indicates a large change in blood glucose as the plant extract reduced blood glucose from 30% to 50%. Maximum reduction of 55% and 50% in blood glucose level was recorded at 180 minutes after administration of the plant extract. Comparing the activity of the plant extract to insulin, the herbal drug does not lead to severe hypoglycaemia even leading to death.

Thus statistical significance was observed between the plant extract and the control as well as the insulin and the control. Our results have clearly indicated that the crude extract of *T. polium* is able to significantly reduce blood glucose in normoglycemic rat models over a very

short period of time and its activity closely resemble that of insulin and not that of the reference drug metformin.

## 4.4 EFFECT OF INSULIN AND METFORMIN ON BLOOD GLUCOSE

Metformin was used as a references drug in the study. Metformin acts by decreasing insulin resistance and has the advantage of reducing insulin levels at the early stages of Type II diabetes. From the study, Metformin was not statistically significant as compared to the control *(p=0.0149)* even though there was a slight drop in blood glucose level within the time period (Table 3.3.1, Figure 3.3.1). Metformin treated animals also had a percentage change in blood glucose reduction from about 13 % in the first 30 minutes to 15% at 45 minutes. This slightly increased to 10% at 60 minutes and reduced to 38% at 210 minutes. The activities of metformin do not show any similarities to that of the plant extract. The change in blood glucose at the first 30 minutes from time zero was only 13% compared to the plant extract which was a 30% reduction. This was a clear indication that metformin acted differently from the plant, which could indicate that the plant could be more effective than metformin in reducing blood glucose, especially in normoglycemic rats. Several studies have also showed that metformin did not show blood glucose reduction in normal rat models but only in diabetic rat models. [71,72] Metformin's activity in normoglycemic animals is less effective compared to diabetic or STZ-induced diabetic rats. [71]

In the current study Metformin was administered at a dose of 100 mg/kg rat. Initially during the preliminary trials, 50 mg/kg rat was the dose administered to the animal models which was observed to be less effective in reducing blood glucose. This dose was appropriate and was also used in several other studies, [70, 71] however were still considered too low to observe a significant reduction in blood glucose. Metformin was also reported as not effective at low concentrations, lower than 200 mg/kg (i.e. <40 mg per rat).[70, 71] This is because larger doses are required to achieve measureable reductions of blood glucose levels in experimental animals [70]. Several studies also concluded that Metformin had no significant effect in reducing blood glucose in normal rats which could indicate that Metformin does not affect normoglycemic rats. [70,83,84]

Insulin was also used as a reference drug to compare the glucose lowering effect of the plant against. The effect of the plant extract and insulin were not considered significant after 30 minutes and this shows that both the plant extract and insulin could be exerting similar effects (Figure 3.3.1, Table 3.3.1). Animals treated with insulin showed a large percentage change in blood glucose in normoglycemic rats. In the first 30 minutes, the percentage reduction in blood glucose was ~30% (Figure 3.3.2.4). However, the percentage reduction by insulin was ~39% at 60 minutes, ~57% at 120 minutes and ~72% at 210 minutes which indicates a large percentage reduction in blood glucose. The plant extract showed similar effects to that observed with the insulin from 0 to 30 minutes but differed thereafter. The percentage reduction of blood glucose by the plant extract was ~27% at 60 minutes, ~34% at 120 minutes and ~50% at 210 minutes.

As seen from the data in Figure 3.3.1 and Table 3.3.1 of Section 3, insulin reduced blood glucose levels and maintained a level of significance as compared to the control at 30 minutes *($p<0.05$)*, 60 minutes *($p<0.05$),* 120 minutes *($p<0.01$)* and at 180 minutes *($p<0.001$)*. There was a dramatic decrease in blood glucose level and the decrease from baseline was maintained through to 210 minutes after administration. This is most likely the case because normal rats administered insulin alone would more likely exhibit a rapid dramatic decrease in blood glucose level which sometimes may require a rapid administration of glucose to prevent the animals from dying [64,65]. The rapid administration of glucose was not employed in the current research because firstly the dose was reduced after initial trials, the study duration was short and during the preliminary trials (and during the trial) the animals did not show any side effects associated with severe hypoglycemia but remained in a stable good condition. Secondly *Humulin R* takes effect rapidly within a short duration of time. [10,12,36] Finally the procedure time from administration of insulin to total blood sampling times was conducted in less than 4 hours and none of the animals suffered or died. Previously published work [68] used a lower dose of human insulin which showed a rapid decrease from baseline and after 60 minutes increased back to normal levels. Although the concentration of insulin we finally used was still some units higher than those used previously [68] the dose was still suitable because within the 3.5 hours of sampling time, the dose had effectively reduced blood glucose levels with no undesirable effects or related

to the reduction in blood glucose levels recorded. Further, the dose was easily prepared and diluted in PBS before i.v administration.

## 4.5 BASELINE VARIATIONS AND FACTORS AFFECTING STUDY

There was a great variation observed during baseline readings at time zero within the four treatment groups but this was a completely random effect and therefore this baseline shift was taken into account in the statistical analysis conducted. The fasting blood glucose levels were measured at time zero as shown in Figure 3.3.1 and Table 3.3.1 of Section 3.0. Animals in the control group had a higher average baseline glucose level (7.61mmol/L) as compared to the other three groups. In a normal untreated rat model, the baseline blood glucose level is expected to be in the range of 4.41-6.11 mmol/L (80-110mg/100 mL) [63, 65, 75]. Other published studies on animal models have also observed similar results in baseline readings. [16, 17,18,76,77] Our results indicate a slightly higher baseline reading on average. We propose several factors to be the likely cause of this, some of which were unavoidable but were minimised as much as possible such as the animals feeding on their own faeces during the fasting period. They are as follows:

Stress factors have been identified as a major influence in the variations in baseline readings. The adrenaline effect could not be avoided in such situations as animal handling, noise in the animal housing, cage movements, blood sampling techniques and even the i.p injection of the anaesthetic. All these factors inflict stress on the animals causing disturbances of the endocrine system leading to adrenaline rush and causing a rise in the blood glucose levels. [63, 65, 72] The effects of noise in the laboratory have been associated with the development of a variety of abnormalities and stress. In addition, the noise associated with normal husbandry activities has been associated with a marked increase in plasma corticosterone levels. [73]

Animal handling is another factor identified. The animal models were frequently handled before the experiment because less handling can create fear and cause aggression in the animals. Frequent handling of the animals allowed calmness and non-aggressiveness. Loud noises in the animal facility and frequent or rough movement of the rat's cages would cause

fear and stress in animals. Although this was not a factor in our animal facility, there were slight noises that may have caused nervousness in the animals.

It was considered if the administration of the anaesthetics might have an effect on the variation observed in baseline glucose levels in each treatment group. The administration of anaesthesia is a likely cause of stress. [68] The initial administration of anesthetic by i.p was identified to cause stress (leading to adrenaline rush) thus causing a rise in blood glucose. Little is known about the mechanism of hyperglycaemia induced by the ketamine/xylazine combination. Ketamine itself is a short acting anaesthetics and analgesic which produces sedation and immobilization. [72] Xylazine is a sedative and muscle relaxant which is beneficial in reducing the side effects of ketamine such as tremor and muscle rigidity. [72] Xylazine also affects the antidiuretic hormone (ADH) secretion and glucose homeostasis [73] which might contribute to effects on blood glucose levels at baseline. A study reported the effects of the use of a combination of ketamine (100 mg/kg) and xylazine (5 mg/kg) that caused an acute rise in blood glucose level in rat models. [72] Even though our dose for the anesthetic combination was slightly different from that used in the literature [72] the reports show similar effects. The published report indicates that the effects of the anesthetic combination after 20 minutes showed an acute rise in blood glucose in the normal rat model [72]. Compared to the current study a slight rise in blood glucose was observed during baseline reading and this could be a reason for the slightly higher baseline reading recorded in all treatment groups. However, if the anesthetic combination affects all the rats in the same way then this should not contribute to the baseline variation observed between treatment groups.

# 5.0 CONCLUSION

The use of traditional medicinal plants is the most common form of folk medicine and provides a wealth of resources for drug discovery.

In the current research work, the study design enabled the assessment of the hypoglycaemic potential of the plant extract with reference to insulin and metformin as well as vehicle. The animal model developed eliminates fluctuations in glucose levels caused by adrenaline release in response to i.v injection of treatment and blood sampling; thus one would expect to achieve significance with smaller animal numbers. Furthermore, using normoglycemic rats is a better representation of type II diabetics than STZ induced diabetic rats. The current results clearly indicate that blood glucose levels are significantly decreased acutely following a single intravenous administration of the plant extract in normal rats. A statistically significant difference of $p<0.01$ was observed between the plant extract and vehicle and between insulin and the vehicle. Metformin was not statistically significantly different as compared to the vehicle ($p>0.08$).

In summary, the current research has evaluated the acute hypoglycemic effects of *T. polium* showing significance in blood glucose reduction. However, further studies are still required to isolate the active ingredient(s) and to investigate the mechanism(s) of action of the plant extract and any of the individual constituents isolated.

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