Acknowledgment

Before all, all praise is to "ALLAH" alone, the god of all the worlds. Who have given me the strength to accomplish this work.

I would like to extend my thanks, respect and appreciation to my supervisors Prof. Dr. Khulood W. Al-Samarrae who suggested the idea of the study and Dr. Shahlaa M. Salih, for their efforts to make things clearly and simply, and throughout my thesis writing period.

Also, I would like to express my thanks to the Head of Biotechnology Department Prof. Dr. Radhim M. Ibrahim for his support.

I would like to thank staff of Biotechnology Department / College of Science / Al-Nahrain University for their assistance.

Waheed for his guidance and Mr. Ibrahim and Mis. Orooba Nadhum, for their assistance.

Very much thanks to all staff of Jenin clinical laboratory especially to Dr. Waleed Al-obaidy and the others for their assistance.

Very much thanks to Mis. Rafal shakeeb, the researcher at the Biotechnology Research Center, Al-Nahrain University for her assistance.

I am very grateful to Professor Dr. Ali Al-Mosawy in the Biology Department / College of Science / Baghdad University for his assistance.

- **Conclusions**

1. Different active compounds were detected in the aqueous and methanolic extract of *T. polium* includes phenols, flavonoids, tannins, saponins, glycosides and terpenes.

2. Presence of rutin flavonoid in both extracts and quercetin flavonoid in the methanolic extract.

3. Both aqueous and methanol extract of *T. polium* have a hypoglycemic activity in both normal and alloxan-induced diabetic mice in a dose-dependant manner.

4. [illegible] effect at low doses for short-term administration.

5. [illegible] extract did not affect liver [illegible] doses.

6. Both extracts showed immunostimulating properties and this was obvious by their effects on total and absolute leucocyte count and total immunoglobulin's level.

- **Recommendations**

1. Isolation and purification of active compounds of *T. polium* and investigate its hypoglycemic activity to detect whether the hypoglycemic activity of plant is due to specific active compound(s) or as a result of a synergistic effect of different compounds.

2. Investigate insulinotropic activity of *T. polium* to ensure the effect of plant extract on insulin action and secretion.

3. Investigate the antioxidant activity of plant and ensure its effect on enzymatic systems responsible for free radical generation and on free radical scavenging enzymes.

4. Histopathological study on the effect of *T. polium* on pancreas as well as major organs of glucose metabolism includes muscle and adipose tissue.

5. *In vitro* study on the effect of plant extract on isolated Langerhans islets and the pharmacological effect and cytotoxicity of *T. polium* on liver cell lines.

Committee Certification

We, the examining committee certify that we have read this thesis entitled "**Study the effect of *Teucreum polium* L. aerial parts extracts on normal and alloxan-induced diabetic mice**" and examined the student " Mushtaq Mufleh Khazeem " in its contents and that in our opinion, it is accepted for the Degree of Master of Science in Biotechnology.

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1.1 Introduction

Herbal medicine is a growing area of health care that demands attention. Plants have played a significant role in maintaining human health and improving quality of human life for thousands of years, and have served human as valuable components of medicines (Al-Mudhaffar, 2009). World Health Organization (WHO) estimated that around 80% of the earth's inhabitants rely on traditional medicine for their primary health care needs, and most of this therapy involves the use of plant extracts or their active components (Al-Ali, 2008). This is reasoned by the fact that medicinal plants have advantages (low cost and less side effects) over the conventionally used drugs, which are expensive and known to have harmful side effects (Ateyyat *et al*., 2009).

Diabetes mellitus is a metabolic disorder featured by hyperglycemia and alteration in carbohydrate, fat, and protein metabolism associated with absolute or relative deficiency of insulin (Sumwa and Suryawarshi, 2001). Although numerous oral hypoglycemic drugs exist alongside taking insulin, still there is no promising therapy to cure diabetes (Sumwa and Suryawarshi, 2001).

Diabetes mellitus is now taking place as a serious health care problem in the 21st century. The number of people suffering from diabetes is expecting to increase from 150 millions actually to 220 millions in 2010 and 300 millions in 2030. This explosive increase has already imposed a huge burden on health-care systems and this will continue to increase in the future (Eddouks *et al*., 2009).

Over the last few decades the reputation of herbal remedies has increased globally due to its therapeutic efficacy and safety. In recent years,

numerous traditional medicinal plants were tested for their antidiabetic potential in the experimental animals (Srivastava *et al*., 1993).

There are more than 1200 plants species worldwide that are used in the treatment of diabetes mellitus and a substantial number of plants have shown effective hypoglycemic activity after laboratory test. The medicinal plants provide a useful source of oral hypoglycemic compounds for the development of new pharmaceutical leads as well as a dietary supplement to existing therapies (Pandey, 2010).

One of these traditional hypoglycemic herbs is *Teucrium polium* (Gharaibeh, *et al*., 1988; Baluchnejadmojarad, *et al*., 2005) which belongs to Labiatae family. The aqueous extract of the dried aerial parts of *T. polium* is used traditionally to treat diabetes in Southern Iran and some middle east populations.

Aims of the Study

For the reasons mentioned above the present study was designed to investigate the positive and negative effects of aqueous extract of *T. polium* as a hypoglycemic drug for treatment of diabetes mellitus using normal and experimental alloxan-induced diabetic mice as animal model for study. Parameters of evaluation were:

- Chemical analysis of *T. polium* extracts include chemical detection of phenols, flavonoids, tannins, glycosides, saponins, terpenes, and steroids in addition to detection of some flavonoids using thin layer chromatography technique.
- Biochemical assay to (some enzymes associated with liver function include (GOT, GPT, and ALP), Blood glucose level, and total serum protein).

- Histopathological study to determine histopathological effect of *Teucrium polium* extract on liver of treated mice.
- Immunological aspects include (Total and absolute counts of leucocytes, and total serum immunoglobulin level).

1.2 Literature Review

1.2.1 *Teucrium polium* L.

1.2.1.1 Common Names and Taxonomy

Arabic: Ja'ada

English: Mountain germander, Cat thyme, Hulwort. (Sincich, 2002).

- Kingdom: Plantae
- Subdivision : Tracheophyta
- Superdivision : Spermatophyta
- Division : Magnoliophyta
- Class : Magnoliopsida
- subclass: Asteridae
- Order: Lamiales
- Family: Labiatae
- Genus: *Teucrium*
- Species: *Teucrium polium* L.

1.2.1.2 Plant Distribution

T. polium L. is a wild-growing flowering plant belonging to the family Labiatae and is found abundantly in south western Asia, Europe and North

Africa (Abu Sitta *et al.*, 2009). It is most common in Mediterranean climates and the Middle East (Moghtader, 2009). In Iraq, the plant is found all over the country especially in the northern regions as authenticated by (Dr. Ali Al-Mosawi, Department of Biology, College of Science, University of Baghdad).

1.2.1.3 Plant Description

T. Polium is herbs, shrubs, or sub shrubs. An unusual feature of this genus compared with other members of Labiatae is that the flowers completely lack the upper lip of the corolla. Its flowers are small and range from pink to white. This plant is a dwarf, pubescent, aromatic shrub possessing oval leaves with enrolled margins and dense head of white flowers (Moghtader, 2009).

Figure 1.1: Field picture of *Teucrium polium* L. (Tutin *et al.*, 1993).

1.2.1.4 Chemical Constituents

The genus *Teucrium* is one of the richest sources of clerodane diterpenes, and the new natural products are conveniently reviewed periodically (Menichini *et al.*, 2009). More than 220 of diterpenoids with a

neo-clerodane skeleton have been isolated from the genus *Teucrium* (Henchiri *et al*., 2009). Phytochemical investigations had shown that *T. polium* contains various compounds, such as iridoids, flavonoids and diterpenoids (Eskandary *et al.*, 2007). Plants of this genus are rich in essential oils, mainly monoterpene, sesquiterpene hydrocarbons and oxygenated sesquiterpenes. (Menichini *et al*., 2009). Triterpenoids and steroids were also isolated from these plants (Henchiri *et al*., 2009). Plants belonging to the genus *Teucrium* have been shown to contain different classes of compounds such as fatty acid esters and polyphenolics. Number of flavonoids that have been isolated from *T. polium* species include cirsimaritin, cirsilol, cirsilineol, 5-hydroxy-6,7,3',4'-tetramethoxyflavone, salvigenin, apigenin 5-galloylglucoside, apigenin-7-glucoside, vicenin-2- and luteolin-7-glucoside (Hasani *et al*., 2007).

1.2.1.5 Folkloric Medicinal Uses

[illegible] over [illegible] of physiological conditions, inflammations, diabetes and rheumatism (Menichini *et al*., 2009). It is also used in folk medicine for various purposes such as anti-inflammatory, anti-nociceptive, anti-bacterial, anti-hypertensive and anti-hyperlipedemic (Ardestani and Yazdanparast, 2007). *T. polium* is one of the most fragrant plants in saudi Arabia; it has been used as febrifuge, stomachic, vermifuge. Infusion of tender parts is used in stomach and intestinal troubles. The plant is also used in a steam bath in colds and fevers (Atiqur Rahman *et al*., 2004). It has been used in the treatment of diabetis, hepatitis, hemorroid and stomach pain in Turkish folk medicine (Bedir *et al.*, 1999). In Jordanian traditional medicine, the tea preparation of the aerial parts of the plant is used for the treatment of abdominal colic, headache, diabetes and as an astringent (Abu Sitta *et al.*, 2009). *T. polium* is used in Iranian folk medicine for treating many

diseases such as abdominal pain, indigestion, common cold, diabetes, and urogenital diseases (Rajabalian, 2008).

1.2.1.6 Biological Potentials and Pharmaceutical Applications

T. polium has many biological and pharmacological activities. A summary of some of these potentials and applications is outlined in the following:

- **Hypoglycemic and insolinotropic activities:** The hypoglycemic activity of an aqueous decoction of plant aerial parts was tested in normoglycemic and streptozotocin-hyperglycemic rats. Results indicate that this extract caused significant reductions in blood glucose [illegible] intraperitoneal administration (Ghanaibeh *et al*., 1998). The high insulinotropic and antihyperglycemic activity of *T. polium* crude [illegible] and isola[illegible] evaluated (Eshaeli and Yazdanparast, 2004). Other study demonstrated the effects of the aqueous extract of the aerial parts of *T. polium* on oral glucose tolerance test of normoglycemic and streptozocin-induced diabetic rats and the number of pancreatic islets in the diabetic form. In diabetic animals, the aqueous extract caused a significant reduction in the level of serum glucose during oral glucose tolerance tests and the number of pancreatic islets per unit area significantly increased (Mahmood *et al*., 2003).

- **Anti-oxidant and free radical scavenging activity:** Many of the therapeutic benefit of *T. polium* are often attributed to their antioxidant and free radical scavenging properties. In this regard, methanol extract

of *T. polium* protects red blood cells (RBCs) against lipid peroxidation induced by 10 mM hydrogen peroxide (Suboh *et al*., 2004). Another study demonstrated that extracts of *T. polium* prepared using different organic solvents (diethyl ether, ethyl acetate and n-butanol) were effective inhibitors of β-carotene oxidation (Kadifkova-Panovska *et al*., 2005). Aqueous extracts prepared from the foliage of *T. polium* suppressed iron (Fe_2)-induced lipid peroxidation in rat liver homogenates to the same extent as Trolox, the water soluble analogue of vitamin E (Ljubuncic *et al*., 2005). Other researcher study reported that adding fertilizer caused a significant concentration-dependent increase in antioxidant activity of the cultivated *T. polium* compared with the wild-type (Azaizeh *et al*., 2005). In another studies, the diethyl ether, ethyl acetate and *n*-butanol extracts from *Teucrium* species were shown to possess significant free radical scavenging, hydroxyl radical scavenging activities [illegible] inhibit lipid peroxidation in [illegible] al., 2007).

- **Hepatoprotective activities:** Hepatoprotective activity of the ethyl acetate extract of *T. polium* L. was investigated using rats with CCl_4-induced liver damage. This study has demonstrated a potential liver protective effect of the *T. Polium* ethyl acetate extract **(**Panovska *et al*., 2007).

- **Analgesic activities:** The antivisceral pain effects of *T. polium* was investigated by (Abdolahi *et al*., 2003). This study demonstrated the effects of *T. Polium* total extract and its essential oil on the writhing test in mice. Results showed that visceral analgesic effects of *T. polium*

extract compete considerably with those of indomethacin and hyoscine . This study confirms the antivisceral pain properties of *T. polium* and suggests a good place for it in antispasmodic therapies in human.

1.2.2 Diabetes mellitus

The term diabetes mellitus describes a metabolic disorder of multiple etiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both. The effects of diabetes mellitus include long–term damage, dysfunction and failure of various organs. Diabetes mellitus may present with characteristic symptoms such as thirst, polyuria, blurring of vision, and weight loss (Scobie, 2007).

There are two types of diabetes, namely type 1 and type 2. Type 1 diabetes accounts for 5-10% of diabetes. Type 2 is the most common form of the disease, accounting for 90-95% of diabetes. Type 1 diabetes is characterized by deficiency of insulin production and requires daily administration of insulin. The former was previously known as insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes. Type 1 diabetes is due to absolute insulin deficiency and associated with a usually juvenile onset, a tendency to ketosis and diabetic ketoacidosis, and an absolute need for insulin treatment (Scobie, 2007).

Type 2 diabetes mellitus is a complex disease mainly characterized by impaired insulin action and/or insulin secretion. Hyperglycemia is the result of multiple defects in insulin action and glucose sensing. In type 2 diabetes, the ability of insulin to stimulate glucose uptake in muscle and fat is impaired, suppression of glucose production in liver and lipolysis in adipose tissue is impaired, whereas glucose fails to stimulate insulin secretion and suppress its own production in liver and uptake in muscle. Patients with type 2 diabetes have fasting hyperglycemia, which is greatly determined by the magnitude of

the increase in hepatic glucose production. This increase is largely accounted for by a marked enhancement in the rate of gluconeogenesis despite elevated insulin levels (Prodi and Obici, 2006).

Increasing evidence from both experimental and clinical studies suggested that oxidative stress plays a major role in the pathogenesis of diabetes mellitus and free radicals are formed disproportionately in diabetes by glucose oxidation, non-enzymatic glycation of protein and the subsequent oxidative degradation of glycated proteins (Maritim *et al*., 2003; Mehta *et al*., 2006) .

Abnormally high levels of free radicals and the simultaneous decline of antioxidant defense mechanisms may lead to damage of cellular organelles and enzymes (El Naggar *et al*., 2005).

1.2.3. Diabetes and oxidative stress

During diabetes, persistent hyperglycemia causes a significant increase in the production of free radicals especially [illegible] from glucose auto-oxidation [illegible] *et al*., 1999; Bonnefont Rousselot *et al*., 2000; Robertson, 2004).

Free radicals may play an important role in the causation and complications of diabetes mellitus (Mohamed *et al*., 1999).

Free radicals are generated as by-products of normal cellular metabolism; however, several conditions are known to disturb the balance between ROS production and cellular defense mechanisms. The increase in the level of ROS in diabetes could be attributed to their higher production and/or lower destruction by non-enzymatic and enzymatic such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) whose activities contribute to eliminate superoxide, hydrogen peroxide and hydroxyl radicals (Soto *et al*., 2003).

Levels of these antioxidant enzymes critically influence the susceptibility of various tissues to oxidative stress and are associated with the development of complications in diabetes. Also this is particularly relevant and dangerous for the beta islet, which is among those tissues that have the lowest levels of intrinsic antioxidant defenses (West, 2000; Robertson, 2004).

In diabetes mellitus, alterations in the endogenous free radical scavenging defense mechanisms may lead to ineffective scavenging of reactive oxygen species, resulting in oxidative damage and tissue injury. In recent years, much attention has been focused on the role of oxidative stress, and it has been reported that oxidative stress may constitute the key and common event in the pathogenesis of secondary diabetic complications (Ceriello, 2000).

1.2.4 Alloxan

The majority of studies published in the field of ethnopharmacology between 1996 and 2006 employed two models, Streptozotocin (69 %) and alloxan (31 %) are by far the most frequently used drugs and this model has been useful for the study of multiple aspects of the disease. Both drugs exert their diabetogenic action when they are administered parenterally: intravenously, intraperitoneally or subcutaneously. The dose of these agents required for inducing diabetes depends on the animal species, route of administration and nutritional status (Fröde and Medeiros, 2008).

The cytotoxic action of these diabetogenic agents is mediated by reactive oxygen species, but both drugs differ in their mechanism of action (Federiuk *et al*., 2004; Lei *et al*., 2005).

Alloxan is a toxic glucose analogue, which selectively destroys insulin-producing cells in the pancreas when administered to rodents and many other animal species. This causes an insulin-dependent diabetes mellitus (called "Alloxan Diabetes") in these animals, with characteristics similar to type 1 diabetes in humans.

Alloxan is selectively toxic to insulin-producing pancreatic beta cells because it preferentially accumulates in beta cells through uptake via the GLUT2 glucose transporter (Lenzen, 2008).

The unique capacity of alloxan to selectively destroy the pancreatic beta cells was first described by Dunn and McLetchie in [illegible] It has been suggested that alloxan induces the production of H_2O_2 and of some free radicals, [illegible] first [illegible] beta cells (Lukacova *et al*., 2008).

Alloxan and the product of its reduction, dialuric acid, establish a redox cycle with the formation of superoxide radicals. These radicals undergo dismutation to hydrogen peroxide with a simultaneous massive increase in cytosolic calcium concentration, which causes rapid destruction of pancreatic β-cell. The range of the diabetogenic dose of alloxan is quite narrow and even light overdosing may be generally toxic and may cause the loss of many animals. This loss is likely attributed to kidney tubular cell necrotic toxicity, in particular when too high doses of alloxan are administered (Fröde and Medeiros, 2008).

1.2.5 Diabetes and blood glucose level

Carbohydrates considered to be the main source blood sugar in addition to the interconversion of fructose, galactose, and mannose into glucose inside liver before releasing into the blood stream. In addition, glycogenolysis in liver and muscle cells represent another source of blood glucose. Also, glucose can be synthesized from non- carbohydrates sources such as amino acids, glycerol and lactic acids by gluconeogenesis which primerly occurred in liver. Insulin hormone has three mechanisms to lower blood glucose; stimulate glucose oxidation in the sensitive tissues such as brain, liver, and muscle and stimulate uptake of (5% of glucose) by liver and converted it into glycogen and conversion of (30-40 % of glucose) into storage lipid. Glucose entry into adipose and muscle tissues, white blood cells, pituitary and lactic glands and pancreatic beta cells need to insulin which stimulate its entry and effect the following metabolisms inside these tissues. While glucose entry into brain, kidney, erythrocytes, and re

Mark elevation in blood glucose level result in glucosurea (glucose presence in urine) and affect osmotic pressure of cells and result in excessive urination (polyurea) and finlly dehydration and which lead to increasing water drinking (polydipsia) (Edwards *et al*., 1995)

Blood glucose level considered to be the main factor in insulin secretion process because of its direct effect on pancreatic beta cells. It had been suggested two mechanisms; presence of specific glucose receptors on beta cells stimulates insulin secretion. The second mechanisms suggested that one or more metabolite of glucose metabolism inside beta cells which might stimulate insulin biosynthesis and secretion (Mariot *et al*., 1998).

Diabetes decrease glucose uptake by liver because of reduction of hepatic glucokinase activity which play a role in the regulation of glucose levels (Matschinsky *et al*., 1998).

In addition, absence of insulin result in decreasing of glycogenesis by lowering glycogen synthetase activity and increasing of glycogenolysis by increasing activity of glycogen phosphorelase. As a result, liver cells compensate glucose deficiency by gluconeogenesis which increased as a result of glucagon and cortisol hormons. On the other hand, diabetes direct body cells to use lipids as a source of energy, leading to increasing lipolysis of lipids into fatty acids and glycerol which enter glucose biosynthesis pathway. This leads to increasing ketone bodies formation and its accumulation in blood cause ketonemia or ketoacidosis and increasing secretion of ketone bodies in urine and by breached with fruits like or acetone smell (Pamela and Richard, 2005).

glucose release through the stimulation of glycogenolysis and gluconeogenesis, while insulin counteracts these hepatic effects of glucagon and facilitates the entry of glucose into other peripheral tissues (Robert *et al*., 1984).

If the body is unable to regulate glucose levels appropriately, blood glucose levels will become elevated and have a negative effect on health. Diabetes or insulin resistance syndrome result in elevated glucose levels. Increased glucose levels can lead to micro vascular disease and increase the risk of cardiovascular disease, amputations, blindness, kidney disease and others. The American Diabetes Association defines a fasting plasma glucose level of < 100 mg/dl as normal, and a level of 100-126 mg/dl as impaired

fasting glucose, for those without diabetes (Guerrero-Romero and Rodriguez-Moran, 2006).

1.2.6 Diabetes and serum protein level

Serum contains over one hundred individual proteins, each with a specific set of functions and subject to specific variation in concentration under different pathologic conditions (Alper, 1974).

Serum proteins can be divided into two major types: albumin and globulin which in turn divided into α1, α2, β, and γ globulins and the latter represent total immunoglobulins (Pamela and Richard, 2005). Majority of albumin synthesis occur in liver while 60-80% of globulin synthsis occur in liver and the remaining in the lymphatic system (Guyton, 1986). Total serum protein normal range is (6-7.8 g/dl), 53% albumin (3.2-4.5 g/dl) and 44% globulin (2.3-3.5 g/dl) (Henry, 1984).

Serum proteins play very important [illegible] products of the synthetic function of the liver as reported by White (1996).

Serum proteins have several functions; albumin play a role in maintaining osmotic pressure of plasma and transport some dissolved metabolites, while some of globulins transport dissolved materials in addition to major function of gamma type in immunity (Pamela and Richard, 2005).

It had been reported that insulin inhibit proteins catabolism and in the same time increase amino acids entry into muscle cells via active transport of amino acids includes alanine, valine, lucine, and glycine causing increasing of protein anabolism (Ganong, 1993).

One of diabetes complications are diabetic nephropathy which increase protein secretion through urine (protein urea) (Edwards *et al.*, 1995). The

major proteins are immunoglobulins followed by albumins (Morano *et al*., 1994). This reasoned by increasing permeability in capillary blood vessels of kidney (Ganong, 1993).

1.2.7 Liver function enzymes

1.2.7.1 Aspartate Amino- and Alanine Amino-Transferases

Enzymes are mostly proteins that act as biological catalysts to speed up the rates at which chemical reactions occur by lowering the activation energy, and therefore without assistance of enzymes most of the chemical reactions of metabolism would barely proceed at all (Atlas, 1995).

In this regard, transamination means the process of transferring an amino group from an amino acid to a keto-acid. Enzymes which catalyze this type of reaction are named transaminases, and the most important transferases in the diagnosis of a hepatic damage are aspartate amino-transferase (AST) and alanine amino-transferase (ALT) (Chari

different drugs or chemicals in inducing hepatic damages and the role of medicinal plants in reducing these effects. Therefore, the evaluations of AST and ALT levels are important in assessing the degree of hepatic damages (Kokdil *et al*., 2005; Sanmugapriya and Venkataraman, 2006).

1.2.7.2 Serum Alkaline Phosphataes

Alkaline phosphatase (ALP) is an enzyme found in all tissues, and its function is to catalyze the hydrolysis of phosphate esters in an alkaline environment; resulting in the formation of an organic radical and inorganic phosphate (Reichling and Kaplan, 1988). Tissues with particularly high

concentrations of ALP include liver, bile ducts, placenta, kidneys and bones, and in a lower amount in intestines and leukocytes (Kenton *et al*., 2001).

Damaged or diseased tissues release the enzyme into the blood, so serum ALP measurements can be abnormal in many conditions, including bone disease and liver disease. Serum ALP is also increased in some normal circumstances (for example, during normal bone growth) or in response to a variety of drugs (Friedman *et al*., 1996).

1.2.8 Immunological Parameters

1.2.8.1 Total and Absolute Counts of Leucocytes

Leucocytes were considered as the active cells in carrying out the specific and non-specific immune response. Their study may give a general picture about the function of the immune system (Roitt *et al*., 2001).

Leucocytes are formed in the bone marrow from a stem cell through two cell lineage; myeloid and lymphoid, which give rise to the five types of leucocytes (neutrophils, lymphocytes, monocytes, eosinophils and basophils), and each type of these cells is commenced to carry out an immune function (Roitt *et al*., 2001).

The neutrophils are mainly involved in the innate immune system to carry out phagocytosis, while lymphocytes represent the humoral and cellular arms of specific immunity. Monocytes are involved in carrying out phagocytosis, but they are also professional antigen presenting cells. Eosinophils are involved in allergic and inflammatory reactions, as well as, parasitic infections. Basophils release histamine, heparin and some pharmacological mediators of immunological reactions (Richard and Pamela, 2008).

Due to these diverse immunological functions, the normal counts of leucocytes (total and absolute) can be deviated by infections, radiations, environmental pollutants, drugs and products of medicinal plants (Ad'hiah *et al*., 2008).

1.2.8.2 Total immunoglobulin level

Immunoglobulins are glycoprotein molecules, which are produced by plasma cells in response to an immunogen and function as antibodies. The immunoglobulins can be divided into five different classes based on differences in the amino acid sequences in the constant region of the heavy chains. They are IgG, IgM, IgA, IgE and IgD. The immunoglobulins derive their name from the finding that when antibody containing serum is placed in an electrical field, the antibodies migrate with the globulin protein (Kohl *et al*., 2001).

It can specifically react with one or a few closely related antigens. Antigen binding is the primary function of antibodies and can result in the protection of the host. However, often the binding of an antibody to an antigen has no direct biological effect; rather, the significant biological effects are a consequence of secondary "effector functions" of antibodies (Mayer, 2008).

Since the introduction of moving-boundary electrophoresis by (Tiselius, 1937) and the subsequent use of zone electrophoresis, serum proteins have been fractionated on the basis of their electrical charge into five classical fractions: albumin, alpha1, alpha2, beta, and gamma proteins. Approximately fifteen serum proteins have been studied extensively because they may be measured easily (Ritzmann and Daniels, 1979; 1982).

2.1 Materials

2.1.1 Apparatus and Equipments

The general laboratory apparatus and equipments which were employed in the present study were listed below:

Equipment	Company / Origin
Accu check system	Roche / Germany
Autoclave	SES little Sister / England
Centrifuge	Beckman / England
Digital camera	Mercury / China
Electrical balance	Sartorius / Germany
Electrophoresis system	Hellabio / Spain
Hemocytometer	Neubauer / Germany
Hellabio scan (Gel analyzer)	Hellabio / Spain
Incubator	Memmert / Germany
Microfuge	Eppendorf / Germany
Micropipette	Gilson / France
Microtome	Gallenkamp / England
Oven	Osaw / India
Reflotrone plus	Roche / Germany
Shaker incubator	Gallenkamp / England
Soxhlet	Electrothermol / England
Spectrophotometer	Optima / Japan
Water bath	Gallenkamp / England

2.1.2 Chemicals

The chemicals which were employed in the present study, were listed below:

Chemical Material	Company / Origin
acetic anhydride	Fluka/ Switzerland
Alloxan	BDH / England
Canada Balsam	BDH / England
Chloroform	BDH / England
Dextrose solution	ADWIC / Egypt
Eosin stain	BDH / England
$FeCl_2$	Fluka / Switzerland
Haematoxylin stain	BDH / England
Hydrochloric acid	Sigma / U.S.A.
Lead acetate (CH_3Coopb)	Fluka/ Switzerland
KH_2PO_4	BDH / England
Lieshman Stain	Merck/ Germany
Methanol	Fluka / Switzerland
Methylene blue	Fluka / Switzerland
Na_2HPO_4 (anhydrous)	Fluka/ Switzerland
Potassium hydroxide (KOH)	Fluka / Switzerland
Potassium iron cyanide	BDH / England

Quercetin Standard	Sigma / U.S.A.
Rutin Standard	Sigma / U.S.A.
Sodium hydroxide (NaOH)	Sigma / U.S.A.
sulphuric acid	Sigma / U.S.A.
Xylene	BDH / England

2.1.3 Laboratory Prepared Solutions and Reagents

i. Leucocyte diluent: The solution was prepared by adding 2 ml of glacial acetic acid to 98 ml of distilled water, in addition to a few drops of methylene blue as a color indicator (Sood, 1986).

ii. Eosins stain (1%): The stain solution was prepared by dissolving a gram of eosin yellowish powder in 100 ml of distilled water, and then the solution was filtered (Whatman filter paper No. 1) (Wywiorek and Bruce, 1975).

iii. Leishman stain: The stain solution was prepared by dissolving 100 ml of absolute methanol was filtered (Whatman filter paper No. 1) (Collee *et al*., 1996).

iv. Leishman stain buffer: The buffer was prepared by mixing together 5.447 grams of Na_2HPO_4 (anhydrous) and 4.752 grams of KH_2PO_4. Then, a gram of the mixture was dissolved in 2 liters of distilled water, and the pH was adjusted to 7.0 (Collee *et al*., 1996).

v. Haematoxylin stain: The stain solution was ready supplied by the Histopathology Department at Educational Laboratories of Medical Baghdad City.

vi. Ferric chloride solution (1%): The solution was prepared by dissolving 1gram of ferric chloride in 100 ml of distilled water (Collee *et al*., 1996).

vii. Sodium hydroxide solution: The solution was prepared by dissolving 40 mg of sodium hydroxide in 1000 ml of distilled water (Collee *et al*., 1996).

viii. Potassium hydroxide solution: It was prepared by dissolving 50 grams of potassium hydroxide in 100 ml of distilled water (Collee *et al*., 1996).

ix. potassium iron cyanide (1%): The solution was prepared by dissolving 1gram of ferric chloride in 100 ml of distilled water (Collee *et al*., 1996).

2.1.4 Laboratory Diagnostic Kits

i. ALP strips for measurement of alkaline phosphatase activity
(Roche / Germany)

ii. GOT strips for measurement of aspartate amino-transferases activity
(Roche / Germany)

iii. GPT strips for measurement of alanine amino-transferases ctivity
(Roche / Germany)

iv. Protein electrophoresis kit (Hellabio / Spain)

- **Kit Components**

3. staining solution
4. Gel blotter strips
5. protein Diluent Solution
6. Sample Templates

v. Reagent strips for urinalysis (Cybow / Korea)

vi. Sugar strips for measurement of blood glucose level (Roche / Germany)

vii. Total serum protein kit (Human / Germany)

- **Kit Components**

1. Color Reagent (RGT) consist of:

- Sodium hydroxide 200 mmol/l
- Potassium sodium tartrate 32 mmol/l
- Copper sulfate 12 mmol/l
- Potassium iodide 30 mmol/l

2. standard (STD) consist of:
   - protein 80g/l
   - sodium azide 0.095 %

2.1.5 Plant Material

The plant taxonomist Professor Dr. Ali Al-Mosawy (Department of Biology, College of Science, University of Baghdad) identified the plant *T. polium* which was purchased from local market in September 2009. The plant was left at room temperature (20-25ºC) until use.

2.1.6 Laboratory Animals

Albino mice of (Mus musculus) were employed for carrying out the experiment of the study. They were supplied from (The National Center for Drug Control and Research) and their age at the start of experiments was 2-4 months, and their weight was 25-35 grams.

Mice were kept for one week for acclimatization before being used in the experiments. They were divided into groups, and each group was housed in separate transparent plastic cages with stainless steel cover lids.

The animals were maintained at a temperature of 20-25°C and they had free excess to food (standard pellets) and water through out the experimental work.

2.2 Methods

2.2.1 Plant Extraction

The dried aerial parts of *T. polium* (leaves, stem and flowers) were powdered using a coffee grinder, and then extracted with two types of solvents (distilled water and methanol)**.** In the methanol extraction, 25 grams of the processed plant were extracted in 250 ml of the methanol (70%) using the Soxhlet apparatus. The obtained extract was then evaporated at 37°C in the incubator and the resultant crude extract was frozen at -20°C until use to prepare the required doses (Arokiyaraj *et al*., 2007)**.**

In the aqueous extraction, 25 grams of the processed plant were macerated in 250 ml of boiled distilled water for 24 hrs in shaking incubator at (40 °C). The obtained extract was then evaporated at 37°C in the incubator and the resultant crude extract was frozen at -20°C until use to prepare the required doses (Parsaee and Shafiee-Nick, 2006).

2.2.2 Chemical Analysis of Plant Extracts

2.2.2.1 Qualitative Detection of Plant Extracts

i. Phenols: Equal quantity of aqueous ferric chloride (1%) was mixed with potassium iron cyanide (1%). Equal quantity of the reagent and water or alcoholic plant extract was mixed. Appearance of blue-green color indicates a positive result (Harborne, 1984).

ii. Flavonoids: The detecting solution was prepared by mixing 10 ml of ethanol (50%) with 10 ml of potassium hydroxide (50%), and then 5 ml of this solution was added to 5 ml of the plant extract. The appearance of yellow color was an indicator of the presence of flavonoids (Jaffer *et al*., 1983).

iii. Tannins: The procedure of (Harbone, 1984) was used for the detection of tannins. In this procedure, 50 ml of each extract was equally divided into two conical flasks. For the first one, lead acetate solution (CH_3COOPb) (1%; w/v) was added and the appearance of jelly pellet was considered a positive

reaction, while for the second flask, ferric chloride solution ($FeCl_2$) (1%; w/v) was added and the appearance of blue color was an indicator for the presence of tannins.

iv. Glycosides: This method was done according to the method described by (Harbone, 1973).

Non hydrolysed extract:

Equal amount of the plant extract was mixed with Fehling reagent in the test tube, and then boiled in the water bath for about 10 minutes. The development of red precipitate indicates a positive result.

Hydrolysed extract:

Few drops of dilute HCl was added to 5ml of the aqueous extract of the plant, then boiled in water bath for 20 minutes, the acid was neutralized by NaOH solution, equal volume of Fehling reagent was added. The development of red precipitate indicates a positive result.

Saponins were detected by two methods :

- A solution of plant powder was shaken vigorously in a test tube. The formation of foams standing for a time indicate a positive result.
- Five ml solution of plant powder was added to 1-3 ml of 3% ferric chloride solution. The development of white precipitate indicate a positive result.

vi. Terpenes and steroids: one ml of a solution of plant powder participated in a few drops of chloroform, and then a drop of acetic anhydride and a drop of concentrated sulphuric acid were added, brown precipitate appered which represents the presence of terpenes. The appearance of dark blue color after few minutes indicates the presence of steroids (Al-Maisary, 1999).

2.2.2.2 Detection of Flavonoid Compounds by TLC

The procedure of Harborne (1984) was followed, in which a silica gel TLC plate (200×200×0.25mm) was activated in the oven at 110˚C for one hour, and after cooling the plate at room temperature (around 30 minutes), a marginal line was drawn on the upper and lower side of the plate with a distance of 2 centimeters from the margins. On the lower line of the plate, 25 µl of aqueous and methanol extract (100 mg/ml) and the same volume of quercetin (10 mg/kg) and rutin (10 mg/kg) standard was applied. After around 5-7 minutes, the plate was placed in a jar-closed-system containing the mobile phase (*n*-BuOH : HOAc : H_2O at a ratio 4:1:5), and the system was left for two hours at room temperature. Then plate was examined under UV light to detect the separated flavonoid compounds in the aqueous and methanol extract samples as compared to the flavonoid standard and the Rf value. The Rf value is the distance moved by the compound in the chromatography relative to the distance moved by the [illegible] the distance from the base line to the center of the substance, and this is divided by the distance between the origin and the solvent front (i.e. the distance the solvent travels).

2.2.3 Experimental Induction of Diabetes in mice

Three doses of alloxan monohydrate were evaluated for their effect in induction of experimental diabetes, 40, 60, and 100 mg/kg body weight and the effective dose was 100 mg/kg. Mice were injected intraperitoneally (within 3 minutes from dose preparation) with freshly prepared alloxan monohydrate solution at a dose of 100 mg/kg (prepared by dissolving required dose in 0.2 ml of 0.9% NaCl solution). 5% dextrose solution was administered orally to combat the immediate hypoglycemia that could occur. Blood glucose was observed 20 hrs after alloxanisation (Sushruta *et al*., 2006).

Diabetes was confirmed by testing blood glucose with dextrose strips read by Accu-Chek active system or glucose in urine by urine strips clinically used. Mice showing a blood or urine glucose concentration of less than 250 mg/100 ml were excluded (Thurston *et al*., 1974).

2.2.4 Experimental Design

The experiment was designed to assess the effects of two doses (250, 500 mg/kg) of *T. Polium* aqueous and methanol extract on the investigated parameters in normal and alloxan-induced diabetic mice. The plant extracts were given orally using gavage needle as a single dose (0.2 ml) per day and for 7 days, and then the mice were sacrificed in day 8 for laboratory assessments. Forty male mice were used in this study and divided into ten groups (four mice for each group):

• **Group I (control):** mice treated with only distilled water.

• **Group II:** normal mice treated with *T. polium* aqueous extract at a dose (500 mg/kg).

• **Group III:** normal mice treated with *T. polium* aqueous extract at a dose (250 mg/kg).

• **Groups IV:** normal mice treated with *T. polium* methanol extract at a dose (500 mg/kg).

• **Groups V:** normal mice treated with *T. polium* methanol extract at a dose (250 mg/kg).

• **Group VI (Diabetic):** alloxan-induced diabetic mice treated with only distilled water.

.• **Group VII:** alloxan-induced diabetic mice treated with *T. polium* aqueous extract at a dose (500 mg/kg).

• **Group VIII:** alloxan-induced diabetic mice treated with *T. polium* aqueous extract at a dose (250 mg/kg).

• **Group IX:** alloxan-induced diabetic mice treated with *T. polium* methanol extract at a dose (500 mg/kg).

• **Group X:** alloxan-induced diabetic mice treated with *T. polium* methanol extract at a dose (250 mg/kg).

2.2.5 Biochemical Tests

2.2.5.1 Blood Glucose Level

Blood glucose level was measured with commercially dextrose measurement strips read by Accu-Chek active system.

Sample required and testing time: Accu-Chek active meter requires 1-2 µL blood per test and the testing time is about 5 seconds.

Test Principle: On each test strip there is a test area containing sensitive chemicals. When blood is applied to this area, a chemical reaction takes place (glucose dye oxidoreductase mediate reaction) causing the colour of the test area to change. The meter registers this co

2.2.5.2 Total serum protein level

(A) Principle

The total protein was evaluated in mouse serum using a commercial kit produced by Human company based on photometric colorimetric of Biuret. In which cupric ions react with protein in alkaline solution to form a purple complex. The absorbance of this complex is proportional to the protein concentration in the sample (Weichselbaum, 1946; Josephson and Gyllensward, 1957).

(B) Assay

Two test tubes (sample and standard) were used, and the forthcoming reagents were added as shown in table 2.1.

Table 2-1: pipetting scheme for measuring total serum protein.

Pipette into cuvettes	**Reagent blank**	**Sample/(STD)**
Sample/(STD)	**---**	**20 µl**
(RGT)	**1000 µl**	**1000 µl**
Mix, incubate for 10 min. at 20-25 °C. Measure the absorbance of the sample and (STD) against the reagent blank within 30 min. (ΔA)		

(C) Calculations

protein.

2.2.5.3 Liver function tests

The activity of liver function enzymes aspartate amino-transferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were measured in the sera of mice by Reflotrone plus system (figure 2.2).

Figure 2.1 Reflotrone plus system

Test principle: After application to the test strip, the sample flows into the reaction zone, in the case of blood after separation of the erythrocytes from the plasma. In the presence of GOT, α-ketoglutarate and alanine sulfinate are converted to pyruvate and glutamate. In a second reaction step, catalyzed by pyruvate oxidase, the resulting pyruvate is cleaved into acetyl phosphate, carbon dioxide and hydrogen peroxide. In the presence of POD, the hydrogen peroxide converts an indicator into its oxidized blue form (Deneke, 1985):

$$\alpha\text{-ketoglutarate} + \text{Alanine sulfinate} \xrightarrow{\text{GOT}} \text{glutamate} + \text{pyruvate} + SO_3^{-2}$$

$$\text{pyruvate} + PO_4^{-3} + O_2 + H_2O \xrightarrow{\text{PyOD}} \text{acetyl phosphate} + H_2O_2 + CO_2$$

$$H_2O_2 + \text{indicator (red.)} \xrightarrow{\text{POD}} \text{Indicator (ox.)} + H_2O$$

Endogenous pyruvate is eliminated in a preliminary reaction. At a temperature of 37°C the formation of the dye is measured kinetically at 567 nm as a measure of the enzyme activity of GOT and the result displayed after 124 seconds.

2.2.5.3.2 ALT (GPT)

Test principle: After application to the test strip, the sample flows into the reaction zone, in the case of blood after separation of the erythrocytes from the plasma. In the presence of GPT, α-ketoglutarate and alanine are converted to pyruvate and glutamate. In a second reaction step, catalyzed by pyruvate oxidase, the resulting pyruvate is cleaved into acetyl phosphate, carbon dioxide and hydrogen peroxide. In the presence of POD, the hydrogen peroxide converts an indicator into its oxidized blue form (Deneke and Rittersdorf, 1984):

$$\alpha\text{-ketoglutarate} + \text{Alanine} \xrightarrow{GPT} \text{glutamate} + \text{pyruvate}$$

$$\text{pyruvate} + PO_4^{-3} + O_2 + H_2O \xrightarrow{PyOD} \text{acetyl phosphate} + CO_2 + H_2O_2$$

$$H_2O_2 + \text{indicator (red.)} \xrightarrow{POD} \text{Indicator (ox.)} + H_2O$$

Endogenous pyruvate is eliminated in a preliminary reaction. At a temperature of 37°C the formation of the dye is measured kinetically at 567 nm as a measure of the enzyme activity of GPT and the result displayed after 140 seconds.

2.2.5.3.3 Alkaline phosphatase (ALP)

Test principle: After application to the test strip, the sample flows into the reaction zone, in the case of blood after separation of the erythrocytes from the plasma. ALP hydrolyzes o-cresolphthalein phosphate to o-cresolphthalein and transfers the phosphate group to the acceptor molecule methylglucamine. The coloured hydrolysis product o-cresolphthalein that is produced per unit of

time under alkaline conditions is directly proportional to alkaline phosphatase activity (Haenseler, 1997):

o-cresolphthalein phosphate + methylglucamine $\xrightarrow{ALP}$ o-cresolphthalein + methylglucamine phosphate

Dye formation is determined kinetically at 37°C as a measure of the enzyme activity of ALP. The result is displayed after approximately 135 seconds.

Procedure: (same for GOT, GPT, and ALP)

- The test strip was unwrapped
- Using Reflotrone pipette, the sample material was drew (sample required is 30µL) and applied as a drop to the centre of the application zone.
- Sliding cover was closed within 15 sec. and slide forward horizontally until it locks in place.
- Sliding cover was closed.
- The results are calculated automatically using a function and conversion factors that are entered into the instrument via the magnetic strip on the underside of each test strip.

2.2.6 Histopathological Study

The liver was fixed in 10% formalin for 48 hrs, and the procedure of Bancroft and Stevens (1982) was followed to prepare sections for histopathological examinations. The procedure is outlined as the following:

i. Washing: The specimen was placed in 70% ethanol overnight.

ii. Dehydration: The sample was dehydrated with ascending concentrations (50, 70, 90 and 99)% of ethanol. There were 2 hrs for each concentration.

iii. Clearing: The sample was placed in xylene for 2 hrs.

iv. Infiltration: The sample was first placed in paraffin-xylene (1:1) for 30 minutes at 57-58ºC, and then in paraffin alone for 2 hrs at 60-70ºC.

v. Embedding: The sample was embedded in pure paraffin wax (melting temperature: 60-70ºC) and left to solidified at room temperature.

vi. Sectioning: The paraffin block was sectioned (rotary microtome) at a thickness of 5µ, and then the sections were transferred to a slide covered with Mayer's albumin. The section of tissue was placed in a water bath (35-40ºC) for few seconds.

vii. Staining: The slide was first placed in xylene for 15-20 minutes, descending concentrations (90, 80 and 70)% of ethanol (two minutes for each concentration) and finally distilled water. After that, the slide was stained with haematoxylin for 10-20 min and then washed with distilled water for 5 min. Then, the slide was placed in acidic alcohol for one min and washed with distilled water. After washing, the slide was stained in eosin stain for 10-20 min (depending on the concentration of each concentration). Finally, the

viii. Mounting: The slide was mounted with a Canada balsam and covered with a cover slip. Then, the slide was examined microscopically to inspect the histopathological changes.

2.2.7 Immunological parameters

2.2.7.1 Total Leucocyte Count

Blood samples were collected by heart puncture using a disposable insulin syringe (1ml) then samples were kept in (EDTA) anticoagulation tubes. The method of Haen (1995) was followed, in which an aliquot of 0.02 ml blood was mixed with 0.38 ml of leucocyte diluent in a test tube, and left at room temperature for 3 minutes. A drop of the mixture was applied to the

surface of Neubauer chamber under the cover slip, and the chamber was left for 3 minutes to settle the cells. The leucocytes were counted in 4 large squares (each with 16 small squares), and the total count of leucocytes was obtained using the following equation:

$$\text{Total Count (cell/cu.mm.)} = \left[\frac{\text{number of cells counted}}{4}\right] \times 20 \times 10$$

2.2.7.2 Absolute Count of Leucocytes

One drop of blood was smeared on a clean slide using another slide and left for air-drying at room temperature. The smear was stained with Leishman stain for 5 minutes, buffered for 10 minutes with Leishman buffer, and finally washed with tap water. The slide was examined under the oil immersion lens (100X) (Haen, 1995). At least 100 leucocytes were examined, and the percentage of each cell type was recorded, while the absolute count of each cell type was calculated using the following equation:

$$\text{Absolute Count (cell/cu.mm.)} = \left[\frac{\text{Percentage of each cell type}}{\text{Total count of cells}}\right] \times 100$$

2.2.7.3 Total serum immunoglobulin level by protein electrophoresis

1. Principles and methodology

The (Hellabio company) agarose gels for protein electrophoresis were intended to be used for *in vitro* diagnosis and they enable the quantitative and qualitative estimation of proteins in serum and other biological fluids. The clinical use of electrophoresis in protein analysis generally is based on the simple electrophoretic separation of proteins according to their relative mobility and molecular weight, into albumin, alpha-1, alpha-2, beta-1, beta-2

and gamma globulin's in spite of knowledge that each of the classical electrophoretic zones may contain more than one major proteins.(Laurel, 1972; Jeppsson *et al*., 1979; Killingsworth, 1979).

2. Procedure

1. Serum samples were diluted to a ratio of 1:3 with protein diluent solution (20 µl + 60 µl).
2. The electrophoresis chamber was filled with adequate volume of electrophoresis buffer.
3. The agarose gel was put on the plate in a horizontal position.
4. The gel was blotted with a gel blotter strip on the sample application zone.
5. The suitable sample template was placed on the application zone.
6. The template was rubbed with forefinger so that it makes contact with
7. [illegible] µl of diluted serum was applied [illegible] and left them absorb into the gel for 1 min.
8. The excess sample was blotted with a gel blotter strip, both the sample template and gel blotter strip was gently removed and discarded.
9. The gel was placed on the gel carrier with the gel upstairs and the sample on the cathodic side, put it in the tank, the tank was connected to the power supply and run 20 min. / 120 volts.
10. The gel was completely dried with hot air (less than 75°C) and stained it for 5 min. with protein staining solution.
11. The gel was destained for 5 min. in three-destaining solution baths, subsequently.

12. The gel was dried again with hot air and the results were evaluated by Hellabio Scanner.

2.2.8 Statistical Analysis

The values of the investigated parameters were given in terms of mean ± standard error, and differences between means were assessed by analysis of variance (ANOVA), least significant difference (LSD) and Duncan test. The difference was considered significant when the probability value was equal or less than 0.05.

A further estimation was also given; it was treatment efficiency (Perez-Serrano *et al*., 1997), which was calculated according to the following equation:

$$\text{Treatment efficiency} = \frac{A-B}{B} \times 100$$

A= Treatment group (plant extracts or alloxan

The values of the body weights and blood glucose levels before and after treatment were expressed as mean ± SE, and differences between means were assessed by paired student's t-test. The difference was considered significant when the probability value was equal or less than 0.05.

All statistical analysis of investigated parameters was assessed using the computer program (SPSS v. 7.5).

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3.1 Plant Extracts

3.1.1 Percentage of Extracts

Aqueous extraction resulted in 1.48g representing 5.92% per 25g of the raw plant material. While for methanol extraction, yield was 2.16g representing 8.64% per 25g of the raw plant material.

3.1.2 Chemical Detection of Plant Extracts

Chemical detections of *T. Polium* aqueous and methanol extracts revealed that flavonoids, phenols, tannins, saponins and glycosides were detected in both extracts. Only aqueous extract was positive for terpenes. Both extracts were negative for steroids (Table 3.1). Similar findings were reported by (El-Shazly and Hussein, 2004) and (Esmaeili and Yazdanpanah, 2004).

Table 3.1: Chemical detection of secondary metabolites in aqueous and methanol extracts of *T. Polium*

Secondary Metabolite	Aqueous Extract	Methanol Extract
Phenols	+	+
Flavonoids	+	+
Tannins	+	+
Glycosides	+	+
Saponins	+	+
Terpenes	+	-
Steroids	-	-

+: Positive reaction
- : Negative reaction

3.1.3 Detection of Flavonoid Compounds by TLC

The plate was examined under UV light to detect the separated flavonoid compounds in the aqueous and methanolic extract samples as compared with standard flavonoid and the R_f values (Figure 3.1). As suggested by spots formed and R_f values of the separated extracts, the flavonoid rutin was present in both extracts. Rutin was identified and isolated from *T. Polium* by (Sharififar *et al*., 2009). While the flavonoid quercetin was present in the methanol extract only. GC-MS analysis of *T. polium* extract revealed the presence of quercetin as reported by Proestos *et al*. (2006). Quercetin was reported to be one of several flavonoids present in *T. polium* (Vessal *et al*., 2003).

Quercetin standard
Rutin standard
Aqueous extract
Methanol extract

Figure 3.1: Thin layer chromatography of aqueous and methanol extract of aerial parts *T. polium.*

3.2 General parameters and body weight of experimental mice

Experimental alloxan-induced diabetic mice exhibited all diabetes symptoms including (hyperglycemia, polydipsia, polyphagia, polyuria and glucosurea [urine sugar +++ ~ ++++]) and loss of body weight ($P \leq 0.05$). Normal mice treated with distilled water (control) showed no change in physiological activities and body weight during the period of treatment suggesting that experimental conditions (nutrition, humidity, and light) had no effect on body weight. However, *T. polium*-treated diabetic mice showed signs of recovery in body weight gains at the end of the experiment as compared to non-treated diabetic mice. *T. polium* extract had no effect on body weight in normal groups during the period of experiment (Table 3.2).

Table 3.2: Body weight of normal and alloxan-induced diabetic mice before and after treatment for 15 days with aqueous and methanol extracts of *T. polium*.

Treatment		Dose (mg/kg)	Body weight before treatment	Body weight after treatment
Normal + (distilled water)		0.0	31.3 ± 1.2	31.7 ± 0.8
Normal Mice	**Aqueous (*T. polium*)**	500	29.5 ± 0.6	28.9 ± 2.7
	Aqueous (*T. polium*)	250	32.1 ± 0.4	31.3 ± 3.2
	Methanol (*T. polium*)	500	26.2 ± 0.3	25.4 ± 2.4
	Methanol (*T. polium*)	250	23.6 ± 0.2	23.1 ± 1.1
Diabetic + (distilled water)		0.0	33.5 ± 0.9	26.2 ± 1.9*
Diabetic Mice	**Aqueous (*T. polium*)**	500	34.8 ± 0.8	31.8 ± 3.3
	Aqueous (*T. polium*)	250	31.1 ± 1.5	28.0 ± 1.7
	Methanol (*T. polium*)	500	31.0 ± 0.5	29.2 ± 0.9
	Methanol (*T. polium*)	250	24.4 ± 0.6	22.5 ± 0.9

* Significant difference ($P \leq 0.05$) between means before and after treatment for each group.

It is well known that alloxan, induces formation of superoxide radicals which dismutate to hydrogen peroxide with simultaneous massive increase in cytosolic calcium concentration resulting in a rapid destruction of β pancreatic cells (Fröde and Medeiros, 2008). This destroys a large number of β cells, resulting in a decrease in endogenous insulin release, which paves the way for the decreased use of glucose by the tissues (Qia *et al*., 2008) and leading to increase breakdown of stored carbohydrates, lipids, and proteins to compensate the deficiency of glucose. Using of carbohydrates, lipids, and proteins as a source of energy causing lose of body weight (Pamela and Richard, 2005).

Diabetic groups treated with both extracts of *T. polium* keep their weights from marked decreasing observed in the non-treated diabetic group. This might be attributed to flavonoid and/or terpenoid content in both extracts which might be responsible for potentiating insulin action and increasing glucose [illegible] and [illegible] carbohydrates, lipids and proteins (Ramesh [illegible]). These results agreed with Vessal *et. al* (2003) who demonstrated that number of pancreatic islets per unit area was increased and activity of glucokinase was elevated by *T. Polium.* Another study by (Esmaeili and Yazdanparast, 2004) showed the anti-diabetic effect of *T. polium* and insulinotropic potential had been tested by an *in vitro* investigation using pancreatic rat islets. Similar result was obtained by Yazdanparast *et. al* (2005) who reported that *T. Polium* extract probably without metabolic transformation, was able to reduce blood glucose levels through enhancing pancreatic secretion of insulin. This result came in accordance with Abdelmoaty *et. al* (2010) who reported that quercetin could prevent hyperglycemia induced by streptozotocin in rats. It was suggested that this effect was owing to its antagonistic effect to prevent decrease of pancreatic

activity of antioxidant enzymes (catalase, glutathione peroxidase and superoxide dismutase) induced by streptozotocin.

3.3 Biochemical Effects of *T. Polium* Extracts

3.3.1 Blood glucose level

The blood glucose level in normal mice treated with distilled water (control) was observed during the period of treatment which revealed that experimental conditions (nutrition, humidity, and light) did not have effect on the blood glucose level. A marked elevation in blood glucose levels was measured in non-treated diabetic group. Diabetic mice treated with both extracts exhibited a significant decrease in blood glucose level as compared to non-treated diabetic group. Only the high doses of aqueous and methanol extracts showed a significant decrease in blood glucose level in the normal mice during the period of experiment (Table 3.3).

Groups		Dose (mg/kg)	Before treatment	After treatment
Normal + (distilled water)		0.0	157.2 ± 9.3	159.2 ± 19.3
Normal Mice	**Aqueous (*T. polium*)**	500	180.7 ± 9.1	161.5 ± 12.9*
	Aqueous (*T. polium*)	250	187.5 ± 10.4	180.1 ± 7.9
	Methanol (*T. polium*)	500	185.7 ± 6.0	158.5 ± 18.7*
	Methanol (*T. polium*)	250	165.0 ± 15.2	156.2 ± 8.7
Diabetic + (distilled water)		0.0	624.7 ± 13.4	744.2 ± 36.2*
Diabetic Mice	**Aqueous (*T. polium*)**	500	582.5 ± 23.4	418.0 ± 10.1*
	Aqueous (*T. polium*)	250	560.7 ± 17.2	485.2 ± 10.4*
	Methanol (*T. polium*)	500	573.0 ± 26.0	363.0 ± 23.9*
	Methanol (*T. polium*)	250	563.5 ± 7.9	464.7 ± 4.8*

* Significant difference ($P \leq 0.05$) between means before and after treatment for each group.

Depending on our results it is clear that oral administration of both extracts of *T. polium* showed hypoglycemic effect on normal and diabetic mice. This result agreed with Iriadam *et. al* (2006) who reported the hypoglycemic action of *T. polium* extract suggest more than possible mechanism of action. One of which the potentiation of insulin action released from pancreatic β-cells.

It had been found that the hypoglycemic effects of the aerial parts of *T. polium* may be due to its content of flavonoids and/or terpenoids (Zal *et. al* 2001). Other researchers suggested that the hypoglycemic activity was due to the presence of several flavonoids in *T. polium*. One such flavonoid with hypoglycemic effects in diabetic animals was quercetin. They reported that quercetin might exert its effects on insulin release from rat islets of Langerhans via changes in Ca^{++} metabolisms (Vessal *et al*., 2003).

Similar findings were reported by Vessal *et al* (2003) who reported that quercetin [illegible] the activity of [illegible] responsible for islet cell generation and possibly [illegible] insulin release and/or they might have insulin-like properties.

Other researchers reported that the therapeutic action of flavonoids is due to their antioxidant activity by various mechanisms, e.g., by scavenging or quenching free radicals, by chelating metal ions, or by inhibiting enzymatic systems responsible for free radical generation (Bláha *et al*., 2004; Dias *et al*., 2005).

Now, it is widely accepted that dietary polyphenolics such as flavonoids might play an important role in protecting the body against chronic diseases, such as diabetes mellitus (Knekt *et al*. 2002).

Similar observation was obtained by Jelodar *et. al* (2005) who suggested that the hypoglycemic effect of plants might be due to the presence of insulin-like substances, stimulation of β-cells to produce more insulin, high

level of fiber which interferes with carbohydrate absorption or the regenerative effect on pancreatic tissue.

3.3.2 Liver Function Enzymes

3.3.2.1 Aspartate and Alanine Amino-Transferases (ALT and AST)

The non-treated diabetic mice showed a significant increase in the activity of AST (447.0 IU/L) as compared to control (290 IU/L) with treatment efficiency (+54.1%), while the normal groups treated with both extracts of *T. polium* did not show a significant change in the activity of AST as compared with control group. Diabetic mice treated with both extracts also showed no significant change in the activity of AST as compared with non-treated diabetic group (Table 3.4).

Table 3.4: AST activity in normal and alloxan-induced diabetic mice treated with aqueous and methanolic extracts of *T. polium*

	Treatment	Dose (mg/kg)	Mean ± SE (IU/L)	Treatment efficiency (%)
Normal Mice	Diabetic + (distilled water)	0.0	290 ± 2.0[a]	-
	Aqueous (*T. polium*)	500	269.25 ± 40.4[b]	- 7.1
	Aqueous (*T. polium*)	250	259.75 ± 20.9[b]	- 10.4
	Methanolic (*T. polium*)	500	262.5 ± 11.5[b]	- 9.4
	Methanolic (*T. polium*)	250	265 ± 27.0[b]	- 8.6
Diabetic + (distilled water)		0.0	447 ± 2.0[a]	+ 54.1
Diabetic Mice	Aqueous (*T. polium*)	500	401 ± 48.9[a]	+ 38.2
	Aqueous (*T. polium*)	250	370.2 ± 1.4[a]	+ 27.6
	Methanolic (*T. polium*)	500	399.25 ± 29.6[a]	+ 37.6
	Methanolic (*T. polium*)	250	432 ± 6.5[a]	+ 48.9

*Different letters: Significant difference ($P \leq 0.05$) between means of column.

The non-treated diabetic group showed a significant increase in the activity of ALT (98.0 IU/L) as compared to control (50.0 IU/L) with treatment efficiency (+96%), whereas the normal groups treated with both extracts of *T. polium* did not show significant change in the activity of ALT as compared to control.

The diabetic groups treated with both extracts also showed no significant change in the activity of ALT as compared with non-treated diabetic group (98.0 IU/L) (Table 3.5).

Table 3.5: ALT activity in normal and alloxan-induced diabetic mice treated with aqueous and methanol extracts of *T. Polium.*

Treatment		Dose	Mean ± SE*	Treatment
Normal + (distilled water)		0.0	50.0 ± 4.6[b]	
Normal Mice	**Aqueous (*T. polium*)**	500	51.5 ± 4.9[b]	+ 3
	Aqueous (*T. polium*)	250	52.1 ± 4.8[b]	+ 4.2
	Methanolic (*T. polium*)	500	50.4 ± 9.2[b]	+ 0.8
	Methanolic (*T. polium*)	250	49.7 ± 5.2[b]	- 4.6
Diabetic + (distilled water)		0.0	98.0 ± 6.1[a]	+ 96
Diabetic Mice	**Aqueous (*T. polium*)**	500	83.2 ± 2.1[a]	+ 66.4
	Aqueous (*T. polium*)	250	87.9 ± 5.8[a]	+ 75.8
	Methanolic (*T. polium*)	500	80.7 ± 21.3[a]	+ 61.4
	Methanolic (*T. polium*)	250	81.0 ± 3.8[a]	+ 62.0

*Different letters: Significant difference ($P \leq 0.05$) between means of column.

The present study revealed that *T. polium* extracts at the given doses had no deleterious effect on liver function enzymes in normal mice and this was accepted with results of Shahraki *et. al*. (2007) who found that there was no increase in liver enzyme activity in mice fed with *T. polium* extracts at a

dose (50 mg/kg) for a month. Other study by Iriadam *et. al*. (2006) proved that oral administration of *T. polium* aerial parts extract at a dose (82 mg/kg) in Streptozotocin (STZ)-induced diabetic rabbits did not cause any acute toxicity or behavioral changes and no change was noticed in ALT and AST activity.

A similar observation stated by Khleifat *et. al*. (2002) who reported that intraperitoneal administration of *T. polium* extract for a short period and/or at low doses had no hepatotoxic effect, while long term administration caused hepatotoxic effect. Other researchers showed that hepatotoxicity of *T. polium* was due to long administration period at high doses (Rasekh *et al*., 2005). Abu Sitta *et. al*. (2009) reported that high doses applied were responsible for hepatotoxicity. Results indicated that there was a significant increase in AST and ALT activity in alloxan-induced diabetic mice. This result matched with obtained by Qia *et. al*. (2008) who showed that a significant increase in the [illegible] of hepatic damage [illegible] Alkaline Phosphatase (ALP)

The non-treated diabetic group showed a significant increase in ALP activity (252.0 IU/L) as compared to control (176.2 IU/L) with treatment efficiency (+43.0%), while the normal groups treated with both extracts of *T. polium* did not show significant change in the activity of ALP as compared to control. All the diabetic groups treated with both extracts showed a significant decrease in the activity of ALP as compared with non-treated diabetic group and much more significant decrease activity was observed in the groups treated with high dose of methanol and aqueous extracts (179.0 and 180.0 IU/L respectively) as compared with non-treated diabetic group (252.0 IU/L) with treatment efficiency (1.6 and 2.3) % respectively (Table 3.6).

Table 3.6: ALP activity in normal and alloxan-induced diabetic mice treated with aqueous and methanol extracts of *T. Polium.*

Treatment Groups		Dose (mg/kg)	Mean ± SE* (IU/L)	Treatment efficiency (%)
Normal + (distilled water)		0.0	176.2 ± 24.0^{d}	
Normal Mice	**Aqueous (*T. polium*)**	500	176.0 ± 13.2^{d}	- 1.1
	Aqueous (*T. polium*)	250	171.5 ± 21.2^{d}	- 2.6
	Methanolic (*T. polium*)	500	174.5 ± 25.9^{d}	- 1.0
	Methanolic (*T. polium*)	250	174.7 ± 14.1^{d}	- 0.8
Diabetic + (distilled water)		0.0	252.0 ± 5.0^{a}	+ 43.0
Diabetic Mice	**Aqueous (*T. polium*)**	500	180.2 ± 7.0^{bcd}	+ 2.3
	Aqueous (*T. polium*)	250	190.5 ± 4.0^{b}	+ 8.1
	Methanolic (*T. polium*)	500	179.0 ± 13.3^{bcd}	+ 1.6
	Methanolic (*T. polium*)	250	188.1 ± 26.4^{bc}	+ 6.9

*Different letters: Significant difference ($P \leq 0.05$) between means of column.

activities of alloxan-induced diabetic mice showed a statistical increase. This suggested that a significant increase in the ALP was due to damage effect of alloxan (Qia *et al*., 2008).

T. polium extracts did not have a significant effect on serum alkaline phosphatase activity in normal groups, whereas *T. polium* extracts caused a significant decrease in serum alkaline phosphatase activity in diabetic groups. this might be attributed to flavonoids content of *T. polium.*

It is known that flavonoids are a group of polyphenolic compounds with known hepatoprotective properties that include free radical scavenging,

anti inflammatory action in addition to inhibition of hydrolytic and oxidative enzymes (Hewawasam *et al*., 2003).

3.3.3 Total Serum Protein (TSP)

The non-treated diabetic group showed a significant decrease in TSP levels (44.5 g/L) as compared to control (57.25 g/L) with treatment efficiency (- 22.2%). All the diabetic groups treated with both extracts keep their TSP values from marked decreasing observed in the non-treated diabetic group while normal groups treated with both extracts showed a slight decreasing in TSP levels.

Results showed a much more significant decrease in the group treated with high dose of aqueous extract (54.5 g/L) with treatment efficiency (- 4.8%) (Table 3.7).

Table 3.7: TSP level in normal and alloxan-induced diabetic mice treated with aqueous and methanolic extracts of *T. Polium*

Groups		Dose (mg/kg)	TSP (g/L)	Treatment efficiency (%)
Normal + (distilled water)		0.0	57.25 ± 4.5^{a}	
Normal Mice	**Aqueous (*T. polium*)**	500	54.5 ± 4.9^{a}	- 4.8
	Aqueous (*T. polium*)	250	55.0 ± 5.9^{a}	- 3.9
	Methanolic (*T. polium*)	500	54.25 ± 4.7^{a}	- 5.2
	Methanolic (*T. polium*)	250	54.0 ± 5.4^{a}	- 5.6
Diabetic + (distilled water)		0.0	44.5 ± 2.0^{b}	- 22.2
Diabetic Mice	**Aqueous (*T. polium*)**	500	52.0 ± 3.1^{ab}	- 9.1
	Aqueous (*T. polium*)	250	50.0 ± 5.5^{ab}	- 12.6
	Methanolic (*T. polium*)	500	49.25 ± 1.7^{ab}	- 13.9
	Methanolic (*T. polium*)	250	50.5 ± 5.0^{ab}	- 11.8

*Different letters: Significant difference ($P \leq 0.05$) between means of column.

TSP levels in the normal groups treated with both extracts showed no significant change during period of experiment. This finding agreed with results of others (Khleifat *et al*., 2002) and (Rasekh *et al*., 2005).

The non-treated diabetic group showed a significant decrease in TSP values, this might be due to effect of diabetes which affect protein metabolism by reducing protein biosynthesis inside cells, and loss of insulin (which considered to be an important factor in controlling protein biosynthesis and in the same time inhibitory factor for gluconeogenesis in liver) causing marked elevation of glucose level upon protein biosynthesis. This is accompanied with accumulation of blood amino acids without using it in protein biosynthesis (Pamela and Richard, 2005).

However, *T. polium*-treated diabetic mice showed significant recovery in serum protein levels at the end of experiment compared to non-treated diabetic mice. This might be attributed to [illegible] *T. polium* which antagonize the effect of insulin deficiency as reported by (Esmaeili and Yazdanparast, 2004).

3.4 Histopathological Effects of *T. polium* on mice liver

The normal appearance of liver section was observed in normal mice (figure 3.2). While experimentally alloxan-induced diabetic mice showed necrosis and degeneration of hepatocyte cells with aggregation of chronic inflammatory cells (Figure 3.3). Liver section of normal mice treated with high dose (500 mg/kg) of aqueous extract showed a mild effect represented by mild necrosis and degeneration of hepatocyte cells with mild chronic inflammatory cells (Figure 3.4). Low dose of aqueous extract caused exiguous effect represented by normal structure appearance of liver with slight dilatation of sinusoid (Figure 3.5).

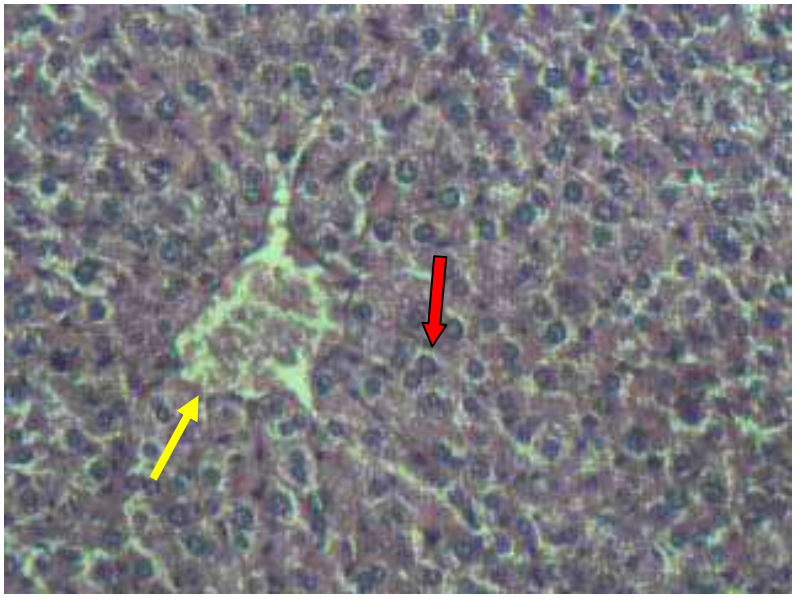


Figure 3.2: A liver section of mouse treated with distilled water (control) showed normal structure appearance of hepatocyte cells arrangement (→) around Central vein (↑) (100x).

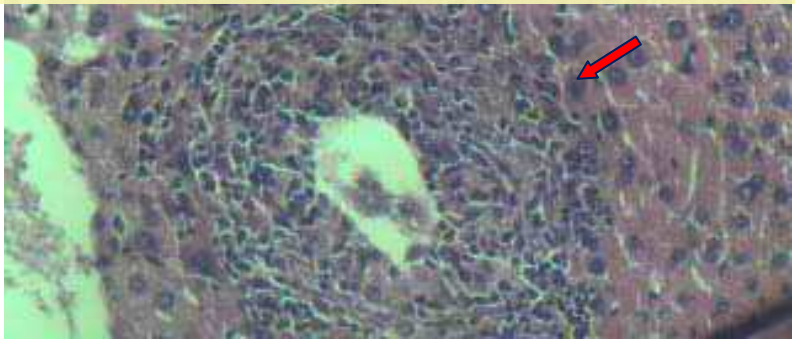


Figure 3.3: A liver section of experimental alloxan-induced diabetic mouse treated with distilled water shows necrosis and degeneration of hepatocyte cells with aggregation of chronic inflammatory cells (→) (100x).

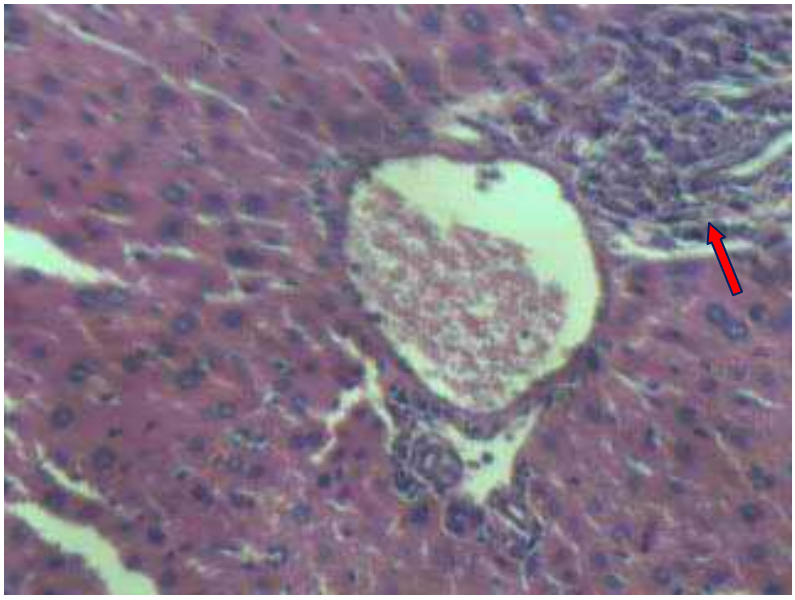


Figure 3.4: A liver section of normal mouse treated with (500 mg/kg) of *T. polium* aqueous extract showed mild necrosis and degeneration of hepatocyte cells with mild chronic inflammatory cells (⟹) (200x).

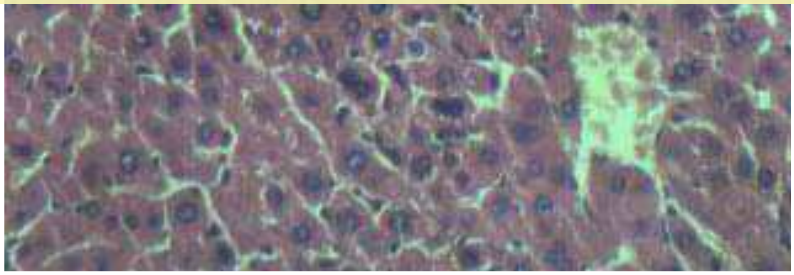
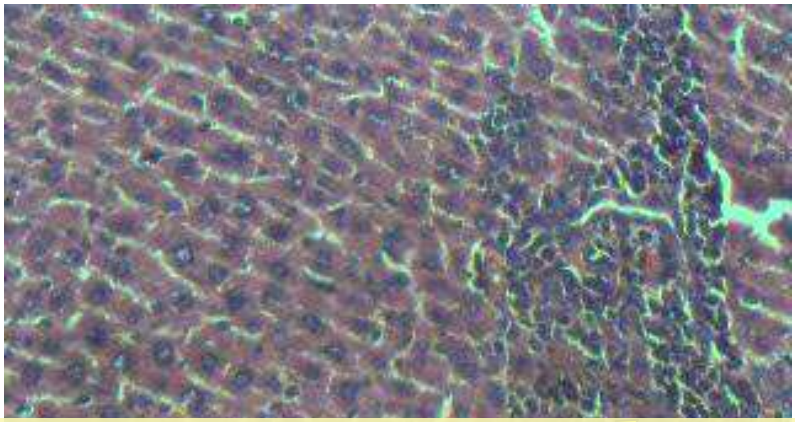


Figure 3.5: A liver section of normal mouse treated with (250 mg/kg) of *T. polium* aqueous extract showed normal structure appearance with slight dilatation of sinusoid (⟹) (100x).

With respect to the normal mice treated with methanol extract, the liver sections of mice treated with the high dose (500 mg/kg) showed a mild effect represented by mild necrosis and degeneration of hepatocyte cells with

infiltration of chronic inflammatory cells (Figure 3.6). The second dose of methanol extract (250 mg/kg) showed no effect represented by normal structure appearance of liver (Figure 3.7).



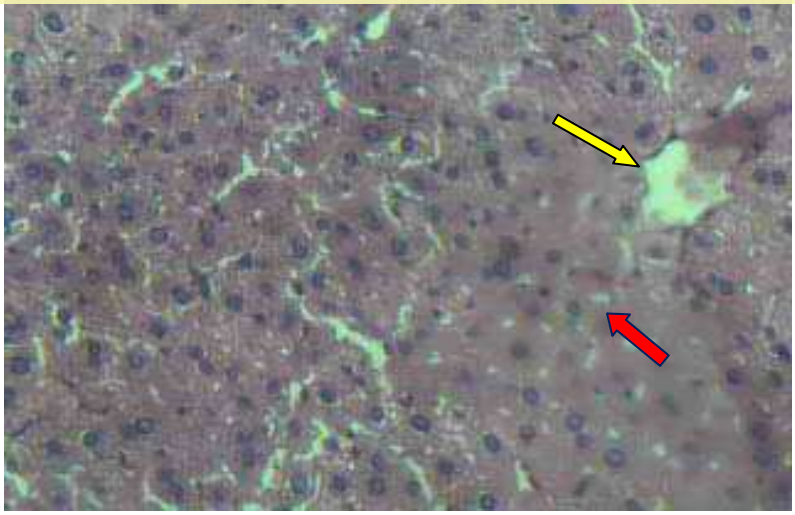


Figure 3.7: A liver section of normal mouse treated with (250 mg/kg) of *T. polium* methanol extract showed normal structure with central vein (⇨) and sheets or cord-like arrangement of hepatocytes cells (➡) (100x).

Sections of liver in diabetic mice treated with high dose of aqueous extract showed mild necrosis and degeneration of hepatocyte cells with infiltration of chronic inflammatory cells (Figure 3.8) while diabetic mice treated with low dose showed degeneration and necrosis of hepatocytes with mononuclear inflammatory cells infiltrate especially in portal area (Figure 3.9).

Diabetic mice treated with high dose of methanol extract showed mild degeneration of hepatocyte cells with few inflammatory cells infiltration and congestion (Figure 3.10). On the other hand, liver section of mice treated with low dose of methanol extract showed necrosis with degeneration and inflammatory cells infiltrate especially in portal area (Figure 3.11).

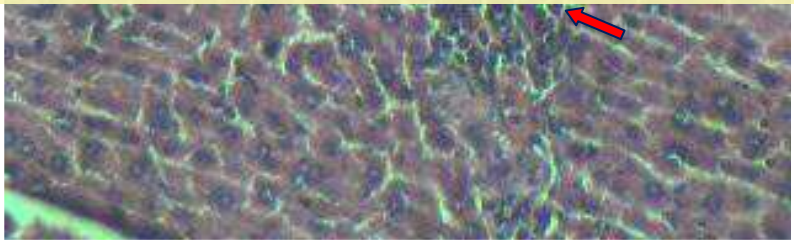


Figure 3.8: A liver section of alloxan-induced diabetic mouse treated with (500 mg/kg) of *T. polium* aqueous extract showed mild necrosis and degeneration of hepatocyte cells with infiltration of chronic inflammatory cells (➡) (100x).

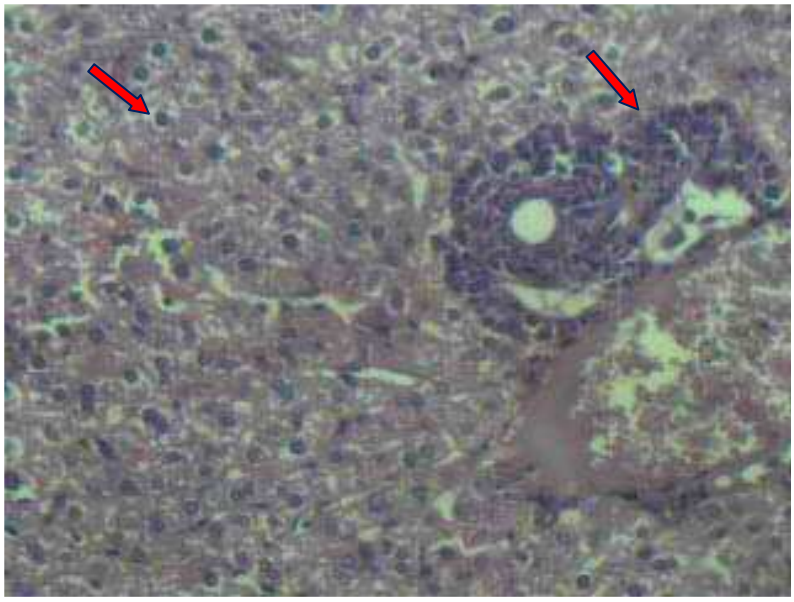


Figure 3.9: A liver section of alloxan-induced diabetic mouse treated with (250 mg/kg) of *T. polium* aqueous extract showed degeneration and necrosis of hepatocytes with mononuclear inflammatory cells infiltrate ( ) especially in portal area (100x).

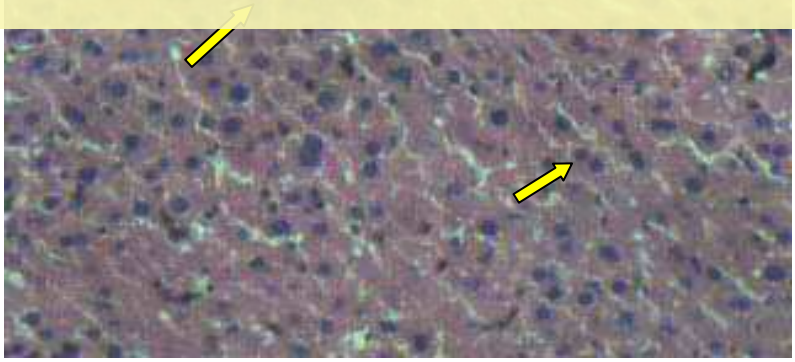


Figure 3.10: A liver section of alloxan-induced diabetic mouse treated with (500 mg/kg) of *T. polium* methanol extract showed mild degeneration of hepatocyte cells with few inflammatory cells infiltration ( ) and congestion ( ) (100x).

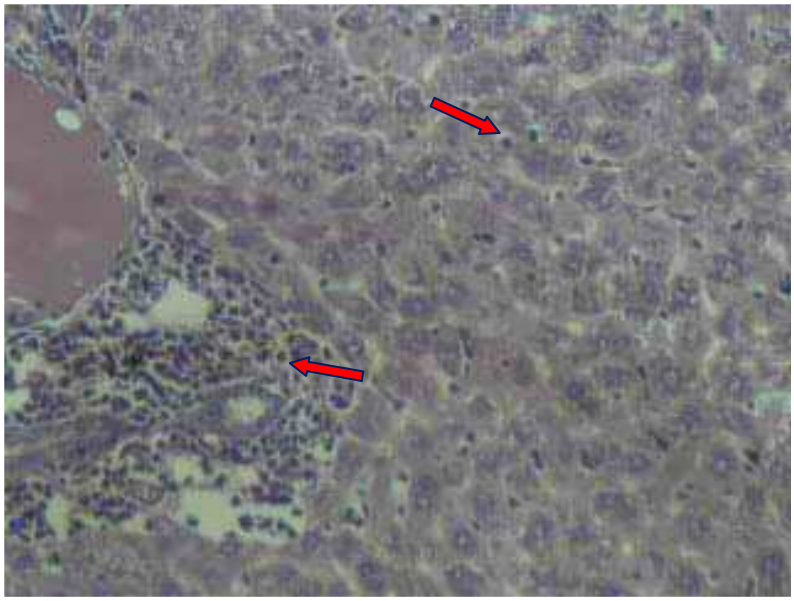


Figure 3.11: A liver section of alloxan-induced diabetic mouse treated with (250 mg/kg) of *T. polium* methanol extract showed necrosis with degeneration and inflammatory cells infiltrate (→) especially in portal area (100x).

The present study revealed that *T. polium* extracts at the given doses effect on liver sections of normal and experimental alloxan-induced diabetic mice. This result hepatotoxicity of *T. polium* reported in some studies might be due to high doses or long-term administration (Hasani-Ranjbar *et al*., 2010) as in the study of (Khleifat *et al*., 2002) which demonstrated the phytotoxic effect of the medicinal plant *T. polium* on rat liver and kidney after prolonged herbal administration. Liver section of non-treated alloxan-induced diabetic mice showed necrosis and degeneration of hepatocyte cells with aggregation of chronic inflammatory cells, these effects might be attributed to damage effect of oxidative stress caused by alloxan (Fröde and Medeiros, 2008).

Oxidative stress is known responsible for pathogenesis of various diseases like diabetes and hepatotoxicity. In most of these conditions, use of antioxidants had been beneficial in ameliorating or even reversing the disease

(Rahimi *et al*., 2005; Rezaie *et al*., 2007; Sarkhail *et al*., 2007; Chitturi and Farrell, 2008).

Treatment with both extract of *T. polium* in our study caused a slight recovery on hepatic tissue. This might be attributed to antioxidant activity of polyphenolic and flavonoid compounds.

It had been reported that Polyphenolic compounds in *T. polium* have shown strong antioxidant activity. The positive effects of these antioxidant components come from their ability to inhibit lipid peroxidation and chelate redox-active metals. There is evidence that flavonoids have anti-phosphodiesterase activity and could thus elevate intracellular levels of cyclic nucleotides (Hasani *et al*., 2007).

Antioxidant effect of *T. polium* might be due to presence of several flavonoids in *T. polium* include cirsimaritin, cirsiliol, cirsili[illegible]ol, 5-[illegible]oxy-6,7,3’,4’-tetramethoxyflavone, salvigenin, [illegible] 5-galloylglucoside, apigenin 7-glucoside, [illegible] 2- and [illegible] [illegible] (2007). It has been suggested [illegible] activity of these flavonoids include, e.g., by scavenging or quenching free radicals, by chelating metal ions, or by inhibiting enzymatic systems responsible for free radical generation (Bláha *et al*., 2004; Dias *et al*., 2005).

Recently, it has been reported that the extract of *T. polium* reduces NADPH-initiated lipid peroxidation in rat liver microsomes *in vitro* (Hasani *et al*., 2007).

In this study, the histopathological effect of *T. polium* extract on liver was associated with biochemical evaluation of liver function enzymes, since hepatocellular necrosis leads to elevation of the serum marker enzymes, which are released from the liver into blood (Shenoy *et al*., 2002), and the increased levels of AST, ALT and ALP are conventional indicators of liver injury (Achliya *et al*., 2004).

3.5 Immunological Effects of *T. Polium* Extracts

3.5.1 Total Count of Leucocytes

The non-treated diabetic mice did not show a significant change in total count of leucocytes (5725 cell/cu. mm.) as compared to control (5241.5 cell/cu. mm.) with treatment efficiency (+9.2%). In the normal mice treated with plant extracts, the two doses of aqueous extract (250 and 500 mg/kg) showed a significant increase in leucocytes count in a dose-dependent manner (9450 and 11143.7 cell/cu.mm. blood respectively) with treatment efficiency (+80.3 and +112.6)% respectively as compared to control (5241.5 cell/cu.mm. blood).

The similar effects were observed in mice treated with methanol extract and the two doses were significantly effective in increasing the total count of leucocytes in a dose-dependent manner (7744.2 and 9300 cell/cu.mm. blood respectively) with treatment efficiency (+47.7 and +77.4)% respectively as compared to control (5241.5 cell/cu.mm. blood).

In the diabetic groups, only the high doses of aqueous and methanol extracts showed a significant increase in leucocytes count (7725 and 7590 cell/cu.mm. blood respectively) with treatment efficiency (+47.3 and +44.8)% respectively as compared to control (5241.5 cell/cu.mm. blood) and non-treated diabetic group (5725 cell/cu.mm. blood) (Table 3.8).

Table 3.8: Total leucocyte count in normal and alloxan-induced diabetic mice treated with aqueous and methanol extracts of *T. polium.*

Treatment Groups		Dose (mg/kg)	Mean ± SE* (cell/cu. mm. blood)	Treatment efficiency (%)
Normal + (distilled water)		0.0	5241.5 ± 700.6^{c}	
Normal Mice	**Aqueous (*T. polium*)**	500	11143.7 ± 364.6^{a}	+ 112.6
	Aqueous (*T. polium*)	250	9450 ± 1369.7^{ab}	+ 80.3
	Methanolic (*T. polium*)	500	9300 ± 767^{ab}	+ 77.4
	Methanolic (*T. polium*)	250	7744.2 ± 479.4^{bc}	+ 47.7
Diabetic + (distilled water)		0.0	5725 ± 1010.4^{c}	+ 9.2
Diabetic Mice	**Aqueous (*T. polium*)**	500	7725 ± 1153.3^{bc}	+ 47.3
	Aqueous (*T. polium*)	250	5866.5 ± 725.2^{c}	+ 11.9
	Methanolic (*T. polium*)	500	7590 ± 147.3^{bc}	+ 44.8
	Methanolic (*T. polium*)	250	5570 ± 1019.4^{c}	+ 8.6

*Different letters: Significant difference ($P \leq 0.05$) between means of column.

3.2.4 Effect on leucocytes

Diabetic mice without treatment did not show a significant change in lymphocytes count (4302 cell/cu. mm.) as compared to control (3839.4 cell/cu. mm.) with treatment efficiency (+12 %).

The two doses of aqueous extract caused a significant increase in lymphocyte count in normal mice and in a dose-dependent manner (6666.4 and 7651.3 cell/cu.mm. blood respectively) as compared to control. and treatment efficiency was (+73.6 and +99.2)% respectively.

The methanol extract showed a similar effect on normal mice and the two doses caused a significant increase in the count of lymphocytes in a dose-dependent manner (5517.6 and 6479.8 cell/cu.mm. blood respectively) as compared to control (3839.4 cell/cu.mm. blood) with treatment efficiency (+43.7 and +68.7) % respectively.

Results showed that high dose of aqueous and methanol extract caused a significant increase in count of lymphocytes (5285 and 5337.7 cell/cu.mm. blood respectively) as compared to control (3839.4 cell/cu.mm. blood) with a statistical difference compared with non-treated diabetic group (4302 cell/cu.mm. blood). Efficiency of treatment with aqueous and methanol extract was (+37.6 and +39) % respectively (Table 3.9).

Table 3.9: Total lymphocytes count in normal and alloxan-induced diabetic mice treated with aqueous and methanol extracts of *T. polium*.

Treatment Groups		Dose (mg/kg)	Mean ± SE* (cell/cu. mm. blood)	Treatment efficiency (%)
Normal + (distilled water)		0.0	3839.4 ± 511.6[c]	
Normal Mice	**Aqueous (*T. polium*)**	500	7651.3 ± 264.1[a]	+ 99.2
	Aqueous (*T. polium*)	250	[illegible]	[illegible]
	Methanolic (*T. polium*)	500	[illegible]	[illegible]
	Methanolic (*T. polium*)	250	[illegible]	[illegible]
Diabetic Mice	[illegible]	0.0	[illegible]	[illegible]
	Aqueous (*T. polium*)	500	[illegible]	[illegible]
	Aqueous (*T. polium*)	250	4297.7 ± 536.5[c]	+ 11.9
	Methanolic (*T. polium*)	500	5337.7 ± 106.3[bc]	+ 39
	Methanolic (*T. polium*)	250	4287.2 ± 78.2[c]	+ 11.6

*Different letters: Significant difference (P ≤ 0.05) between means of column.

In monocytes count, non-treated diabetic group also did not show a significant change in count of monocytes (229.1 cell/cu. mm.) when compared with control (233.1 cell/cu. mm.) with treatment efficiency (- 1.7%).

The two doses of aqueous extract caused a significant increase in monocytes count in normal mice and their effect was a dose-dependent (440.6 and 599.4 cell/cu.mm. blood respectively) as compared to control (233.1

cell/cu.mm. blood). Treatment efficiency of both doses was (+145.1 and + 157.1) % respectively.

Normal mice treated with two doses of methanol extract showed significant increase in monocytes count (355.4 and 571.4 cell/cu.mm. blood respectively) as compared to control (233.1 cell/cu.mm. blood). Treatment efficiency of two methanol doses was (+52.4 and +145.1) % respectively.

Diabetic groups treated with high dose (500 mg/kg) of both extracts showed a significant increase in monocytes count (392 and 341.7 cell/cu.mm. blood respectively) when compared with normal control (233.1 cell/cu.mm. blood) and with non-treated diabetic group (229.1 cell/cu.mm. blood). Efficiency of treatment with both extract was (+68.1 and +46.6) % (Table 3.10).

Table 3.10: Total monocytes count in normal and alloxan induced diabetic mice treated with aqueous and methanol extracts of *T. polium*.

Group		Dose (mg/kg)	Count (cell/cu.mm. blood)	Efficiency (%)
Normal + (distilled water)		0.0	233.1 ± 34.8^{c}	
Normal Mice	**Aqueous (*T. polium*)**	500	599.4 ± 17.4^{a}	+ 157.1
	Aqueous (*T. polium*)	250	440.6 ± 59.8^{ab}	+ 89
	Methanolic (*T. polium*)	500	571.4 ± 145.5^{a}	+ 145.1
	Methanolic (*T. polium*)	250	355.4 ± 26.8^{bc}	+ 52.4
Diabetic + (distilled water)		0.0	229.1 ± 39.1^{c}	- 1.7
Diabetic Mice	**Aqueous (*T. polium*)**	500	392.0 ± 60.4^{bc}	+ 68.1
	Aqueous (*T. polium*)	250	236.1 ± 24.9^{c}	+ 1.3
	Methanolic (*T. polium*)	500	341.7 ± 9.6^{bc}	+ 46.6
	Methanolic (*T. polium*)	250	252.3 ± 14.8^{c}	+ 8.2

*Different letters: Significant difference ($P \leq 0.05$) between means of column.

Result of neutrophils count came in accordance with count of lymphocyte and monocyte. The non-treated diabetic group showed no significant change in count of neutrophils (1066.3 cell/cu. mm.) as compared to control (1053.3 cell/cu. mm.).

The two doses of both extracts caused a significant increase in neutrophils count. The count of neutrophils in normal mice treated with two doses of aqueous extract (250 and 500 mg/kg) showed a significant increase in neutrophils count in a dose-dependent manner (2135.9 and 2624.5 cell/cu.mm. blood respectively) as compared to control (1053.3 cell/cu.mm. blood). Treatment efficiency of both doses was (+102.7 and +149.1) % respectively.

Similar effect of methanol extract was noticed and both doses of methanol extract caused a significant increase in the count of neutrophils in a dose-dependent manner (1738.1 and 2157.[illegible] cell/cu.mm. blood respectively) as compared to control. Treatment efficiency of both doses was (+64.4 and +104.8) % respectively.

On the other hand, only high dose (500 mg/kg) of both extract caused a significant increase in neutrophils count in diabetic groups. Neutrophils count was (1879.3 and 1751.2 cell/cu.mm. blood) for mice treated with aqueous and methanol extracts respectively and showed a significant increase when compared with non-treated diabetic group (Table 3.11).

Table 3.11: Total neutrophils count in normal and alloxan-induced diabetic mice treated with aqueous and methanol extracts of *T. polium.*

Treatment Groups		Dose (mg/kg)	Mean ± SE* (cell/cu. mm. blood)	Treatment efficiency (%)
Normal + (distilled water)		0.0	1053.3 ± 140.9^{c}	
Normal Mice	**Aqueous (*T. polium*)**	500	2624.5 ± 98.1^{a}	+ 149.1
	Aqueous (*T. polium*)	250	2135.9 ± 301.7^{ab}	+ 102.7
	Methanolic (*T. polium*)	500	2157.3 ± 169.2^{ab}	+ 104.8
	Methanolic (*T. polium*)	250	1738.1 ± 112.5^{b}	+ 65
Diabetic + (distilled water)		0.0	1066.3 ± 193.7^{c}	+ 1.2
Diabetic Mice	**Aqueous (*T. polium*)**	500	1879.3 ± 283.1^{b}	+ 78.4
	Aqueous (*T. polium*)	250	1203.8 ± 142.4^{c}	+ 14.2
	Methanolic (*T. polium*)	500	1751.2 ± 36.8^{b}	+ 66.2

*Different letters: Significant difference (P ≤ 0.05) between means of column.

mm. respectively).

Both doses of aqueous extract had no a statistical effect on basophils and eosinophils count in normal and diabetic groups.

Methanol extract showed similar effect and both doses had no significant effect on normal and diabetic mice (Table 3.12 and 3.13).

Table 3.12: Total basophils count in normal and alloxan-induced diabetic mice treated with aqueous and methanol extracts of *T. polium.*

Treatment Groups		Dose (mg/kg)	Mean ± SE* (cell/cu. mm. blood)	Treatment efficiency (%)
Normal + (distilled water)		0.0	52.9 ± 8.6[a]	
Normal Mice	**Aqueous (*T. polium*)**	500	62.75 ± 9.8[a]	+ 18.6
	Aqueous (*T. polium*)	250	55.75 ± 11.2[a]	+ 5.4
	Methanolic (*T. polium*)	500	64.58 ± 11.7[a]	+ 22
	Methanolic (*T. polium*)	250	50.75 ± 6.7[a]	- 4
Diabetic + (distilled water)		0.0	54.5 ± 8.4[a]	+ 3
Diabetic Mice	**Aqueous (*T. polium*)**	500	64.49 ± 14.1[a]	+ 21.9
	Aqueous (*T. polium*)	250	47.9 ± 8.2[a]	- 9.4
	Methanolic (*T. polium*)	500	50.5 ± 1.2[a]	- 4.5
	Methanolic (*T. polium*)	250	60.6 ± 1.4[a]	+ 4.6

*Different letters: Significant difference (P ≤ 0.05) between means of column.

Table 3.13: Total basophils count in normal and alloxan-induced diabetic mice treated with aqueous and methanol extracts of *T. polium.*

Treatment Groups		Dose (mg/kg)	Mean ± SE* (cell/cu. mm. blood)	Treatment efficiency (%)
Normal + (distilled water)		0.0	62.7 ± 8.3[a]	
Normal Mice	**Aqueous (*T. polium*)**	500	59.25 ± 5.0[a]	- 5.5
	Aqueous (*T. polium*)	250	56.25 ± 16.0[a]	- 10.2
	Methanolic (*T. polium*)	500	59.75 ± 10.6[a]	- 4.7
	Methanolic (*T. polium*)	250	51.9 ± 6.4[a]	- 17.2
Diabetic + (distilled water)		0.0	46.4 ± 11.7[a]	- 26
Diabetic Mice	**Aqueous (*T. polium*)**	500	63.7 ± 13.3[a]	+ 1.6
	Aqueous (*T. polium*)	250	52.7 ± 14.1[a]	- 15.9
	Methanolic (*T. polium*)	500	57.0 ± 2.6[a]	- 9.1
	Methanolic (*T. polium*)	250	51.0 ± 5.7[a]	- 18.6

*Different letters: Significant difference (P ≤ 0.05) between means of column.

3.5.3 Total Immunoglobulin Level

The non-treated diabetic group showed a significant reduction in gamma-globulin percentages of total serum protein (3.27%) as compared to control (4.54%) with treatment efficiency (-27.9%).

Normal mice treated with aqueous extract exhibited a significant increase in gamma-globulin percentage in both doses (250 mg/kg and 500 mg/kg) in a dose-dependent manner (5.53 and 6.06)% respectively as compared to control (4.54%).

Gamma-globulin percentages in normal mice treated with methanol extract showed a similar result and the two doses were significantly effective in increasing gamma-globulin percentages of total serum protein (5.61 and 5.88)% respectively as compared to control (4.54%). Efficiency of treatment with aqueous and methanol extract was (+23.5 and +29.5) % respectively.

Diabetic groups treated with high dose of aqueous and methanol extracts showed gamma-globulin percentages [illegible] the non-treated diabetic and showed [illegible] globulin percentages (4.07 and 3.9) % respectively as compared to non-treated diabetic group (3.27%) (Table 3.14).

Table 3.14: Gamma-globulin percentages of total serum protein in normal and diabetic mice treated with aqueous and methanol extracts of *T. polium.*

Treatment Groups		Dose (mg/kg)	Mean ± SE* (% of Gamma-globulin)	Treatment efficiency (%)
Normal + (distilled water)		0.0	4.54 ± 0.89^{abc}	
Normal Mice	**Aqueous (*T. polium*)**	500	6.06 ± 0.21^{a}	+ 33.4
	Aqueous (*T. polium*)	250	5.53 ± 0.1^{ab}	+ 21.8
	Methanolic (*T. polium*)	500	5.88 ± 0.72^{a}	+ 29.5
	Methanolic (*T. polium*)	250	5.61 ± 0.68^{ab}	+ 23.5
Diabetic + (distilled water)		0.0	3.27 ± 2.17^{c}	- 27.9
Diabetic Mice	**Aqueous (*T. polium*)**	500	4.07 ± 0.82^{bc}	- 10.3
	Aqueous (*T. polium*)	250	3.44 ± 2.1^{c}	- 24.2
	Methanolic (*T. polium*)	500	3.9 ± 1.39^{bc}	- 14.0

*Different letters: Significant difference ($P \leq 0.05$) between means of column.

Results of immunological parameters

with the extracts of *T. polium*

system in normal and diabetic groups. The general outcome of these findings was that *T. polium* extracts might have immunostimulating constituents (Hughes, 2001).

The detected active constituents can justify these findings, especially if we consider that flavonoids, terpens, saponins and glycosides have important role as immune stimulators (Williams *et al*., 2002).

The stimulation of the immune system of mice can be explained in the ground of saponins, which are a further constituent of *T. polium* and they were detected in the present extract. It has been demonstrated that plants contain saponins can modulate the function of the immune system, due to their action in stimulating the cell-mediated immunity and activating the

production of different cytokines, especially those involved in the cellular immune response. Therefore the present effect of the plant extract can also be explained in this context (Francis *et al.*, 2002).

Also stimulation of the immune system can be explained by the presence of glycosides in the *T. polium* extract which was detected in the present study. Ugochukwu *et al.* (2002) reported that effect of some extract on the total leucocyte count could be due to the presence of glycosides. This compound has an anti-inflammatory property and so has vital effect on inflammatory processes of some pathological states such as bacterial infection, malaria and liver diseases.

These augmentations of immune responses involve total and absolute immune cells count and the increasing effect of *T. polium* leaf extract on these cells, numerically or functionally, can be explained in the view of active constituents that act either separately or synergistically enhancing the [illegible] profile of leucocytes in the peripheral blood which is a good picture about the functional status of the immune system (Hughes, 2001). This reasoned by the fact that each type of leucocytes is commenced to carry out a special immunological function in the innate and adaptive immune response, and the numerical count of these cells may correlate with their function. Treatement with both extracts causing a significant increase in lymphocyte, monocytes and neutrophils. It is known that neutrophils are important cellular component of the innate immune system, and they are involved in carrying out phagocytosis, and such function is also shared by monocytes that are also called macrophages in tissues (Herant *et al.*, 2003).

Accordingly, the enhancement of the total and absolute counts of leucocytes can be ascribed to these chemical constituents (flavonoids and tannins), which may be able to modulate the immune response through the

interaction between cytokines that were affected by the plant extracts treatment (Al-bederi, 2009).

The non-treated diabetic mice showed a significant decrease in gamma-globulin percentage of total serum protein which could be attributed to adverse effects of non-treared diabetes, since it had been reported that non-treated diabetes accompanied with increasing Ig excretion throughout the urine (Al-Mashhadany, 1999).

Diabetic mice treated with both extracts, showed sign of recovery in gamma-globulin percentages in a dose-dependent manner as compared to non-treated. On the other hand, normal mice treated with both extracts showed a significant increase in gamma-globulin percentage. The general outcome of these findings was that *T. polium* extract contains ingredients having immunostimulating properties (Hughes, 2001).

Also, this could be explained with the effect of *T. polium* extracts on [illegible] since it [illegible] diabetes could [illegible] responsible fo[illegible] the circulatory system (Roitt *et al*., 2001).

However, *T. polium*-treated diabetic mice showed signs of recovery in gamma-globulin at the end of experiment compared to the diabetic mice. This might be attributed to the reported antidiabetic activity of *T. polium* which antagonize the above complication and adverse effect of diabetes (Esmaeili and Yazdanparast, 2004).

Supervisors Certification

We, certify that this thesis entitled "**Study the effect of *Teucreum polium* L. aerial parts extracts on normal and alloxan-induced diabetic mice**" was prepared by "Mushtaq Mufleh Khazeem" under our supervision at the Biotechnology department, College of Science, Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology.

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الملخص

هدفت هذه الدراسة إلى تقييم التأثيرات المناعية (العد الكلي والمطلق لكريات الدم البيض بالإضافة إلى قياس المستوى الكلي للكلوبيولينات المناعية) والكيميائية (مستوى كلوكوز الدم، مستوى بروتينات الدم الكلي، فعالية أنزيمات وظائف الكبد الثلاثة AST, ALT, and ALP) والنسيج المرضي للكبد للمستخلصين المائي والميثانولي لنبات الجعدة على الفئران الطبيعية والفئران المصابة بالسكري المحفز بالألوكزان.

تم تحضير المستخلص المائي والكحولي للنبات وأجريت كشوفات كيميائية للتحري عن وجود الفينولات، والفلافونات، والتانينات، والكلايكوسيدات، والتربينات، والصابونينات، والستيرويدات في المستخلصين، كما حلل المستخلصان بطريقة كروماتوغرافيا الطبقه الرقيقه للكشف عن الكوارستين والروتين من انواع الفلافونات الموجودة في المستخلصين. أظهرت الكشوفات الكيميائية وجود

أنزيمات وظائف الكبد ومستوى بروتينات الدم فلم تظهر النتائج فرقا معنويا. أما التأثيرات المناعية المتمثلة بالعد الكلي والمطلق لكريات الدم البيضاء ومستوى الكلوبيولينات المناعية فقد ارتفعت ارتفاعا معنويا. كما أظهرت الفحوصات النسيجية لنسيج الكبد وجود تأثير طفيف للمستخلصين.

أبدت المجموعة المصابة بالسكري غير المعاملة بالمستخلصين أعراضا ظاهرة للمرض متمثلة بارتفاع معنوي وملحوظ لسكر الدم بالإضافة إلى (فرط التبول، العطش، انخفاض الفعالية والخمول، بالإضافة إلى انخفاض شديد في الوزن). بالنسبة إلى فعالية أنزيمات وظائف الكبد فقد ارتفعت ارتفاعا معنويا ملحوظا وأما مستوى بروتينات الدم فقد انخفض انخفاضا معنويا. أما التأثيرات المناعية فبالنسبة للعد الكلي والمطلق لكريات الدم البيضاء فلم تظهر النتائج فرقا معنويا في قيمها، وأما مستوى الكلوبيولينات المناعية فقد انخفضت انخفاضا معنويا. كما أظهرت الفحوصات النسيجية لنسيج الكبد تنخراً وتنكساً للخلايا الكبدية فضلا عن تجمع وتكتل للخلايا الالتهابية المزمنة.

أظهرت نتائج مجاميع الفئران المصابة والمعاملة بالمستخلصين أن هذه الفئران حافظت على معدل أوزانها من الانخفاض الكبير الذي لوحظ في الفئران غير المعاملة، وأما بالنسبة إلى مستوى كلوكوز الدم فقد أظهرت النتائج انخفاضا معنويا ملحوظا مقارنة مع المجموعة المصابة غير المعاملة. أما فعالية أنزيمات وظائف الكبد فبالنسبة إلى أنزيمي (AST , ALT) فلم تتغير فعاليتهما بالمقارنة مع المجموعة المصابة غير المعاملة وأما أنزيم (ALP) فقد انخفضت فعاليته انخفاضا معنويا بالمقارنة مع المجموعة المصابة غير المعاملة. بالإضافة إلى ذلك فقد لوحظ حدوث تحسن نسبي في مستوى بروتينات الدم. أما التأثيرات المناعية فبالنسبة للعد الكلي والمطلق لكريات الدم البيضاء فقد أظهرت النتائج زيادة معنوية في عدها في المجاميع المعاملة بالجرعة العالية (500 mg/kg) لكل من المستخلصين وكذلك مستوى الكلوبيولينات المناعية حيث ارتفع ارتفاعا معنويا مقارنة مع المجموعة المصابة غير المعاملة. بينما أظهرت الفحوصات النسيجية لنسيج الكبد حدوث تحسن طفيف مقارنة بالفئران المصابة غير المعاملة.

Summary

This study was conducted to evaluate the antidiabetic, hepatoprotective, and immunological effects of *Teucrium polium* L. plant extracts on normal and experimental alloxan-induced diabetic mice.

To study the effect of *T. polium*, aqueous and methanol aerial part extracts were prepared and chemical detection of phenols, flavonoids, tannins, terpenes, steroids, glycosides and saponins was carried out. Results revealed that the aqueous extract contains phenols, flavonoids, tannins, terpenes, glycosides and saponins compounds, while the methanol extract contains phenols, flavonoids, tannins, glycosides and saponins compounds. The TLC analysis of aqueous and methanol extracts showed that rutin flavonoid was present in both extracts while quercetin flavonoid was detected in the methanol extract only.

Evaluations of parameters of our study were carried out on both normal and alloxan-induced diabetic mice. Diabetes was induced by intraperitoneal injection of single dose (150 mg/kg) of freshly prepared alloxan monohydrate.

Results indicated that normal mice treated with both extracts showed no significant change in body weights and blood glucose level except those treated with high dose of both extracts since they exhibited a significant decrease ($P \leq 0.05$) in blood glucose level. No significant change was recorded in in serum aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) activities and total serum protein (TSP) level. Total, absolute count of leucocytes and total immunoglobulin (Igs) level were statistically higher in normal treated mice. Histopathological effect of both extract exhibited mild effect on liver of normal treated mice. Non-treated alloxan-induced diabetic mice exhibited all diabetes symptoms

including (hyperglycemia, polydipsia, polyphagia, polyuria and glucosurea [urine sugar +++ ~ ++++]) and loss of body weight. A significant increase in AST, ALT and ALP enzyme activities were measured. Total serum protein (TSP) level was significantly reduced as compared with healthy mice. No significant change was recorded in total and absolute count of leucocytes with a significant decrease in total immunoglobulin level. Liver sections of alloxan-induced diabetic mice showed necrosis and degeneration of hepatocyte cells with aggregation of chronic inflammatory cells.

T. polium-treated diabetic mice showed signs of recovery in body weight gains and blood glucose level at the end of experiment as compared to the non-treated diabetic mice. No significant change in AST and ALT enzyme activities was recorded, but ALP activity was statistically lower as compared to non-treated diabetic group. Mild increase in TSP level was measured. A significant increase was recorded in total and absolute count of leucocytes and [illegible] mice treated [illegible] compared to non-treated diabetic group. Mild [illegible] treated diabetic mice was noticed as compared to non-treated diabetic mice.

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List of Abbreviations

ALP	Alkaline phosphatase
ALT	Alanine transaminase
ANOVA	Analysis of Variance
AST	Aspartate transaminase
CAT	Catalase
EDTA	Ethylene Diamine Tetra Acetic acid
GOT	Glutamate Oxalate Transaminase
GPT	Glutamate Pyruvate Transaminase
GPx	Glutathione peroxidase
HOAc	Acetic acid
HPLC	High Performance Liquid Chromatography
IU	International Unit
***n*-BuOH**	*n*-butanol
POD	Peroxidase
PYR	pyruvate oxidase
RBC	red blood cells
Rf	Mobility relative to front
ROS	reactive oxygen species
Redox	Reduction oxidation cycle
SOD	Superoxide dismutase
TLC	Thin layer chromatography
TSP	Total Serum Protein

جمهورية ألعراق
وزارة ألتعليم ألعالي وألبحث ألعلمي
جامعة ألنهرين
كلية ألعلوم
قسم ألتقانة ألأحيائية

دراسة تأثير مستخلص ألأجزاء الهوائية لنبات الجعدة على الفئران الطبيعية والمصابة بالسكري المحفز بالألوكزان.

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Study the Effect of *Teucrium Polium* L. Aerial Parts Extract on Normal and Alloxan-Induced Diabetic Mice.

A thesis
Submitted to the College of Science, Al-Nahrain University as a
Partial Fulfillment of the Requirements for the Degree of
Master of Science in Biotechnology

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