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Hypoglycemic effects of Marrubium vulgare (*Rubia*) in experimentally induced autoimmune Diabetes Mellitus

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Abstract

Cytokines and nitric oxide (NO) are involved in the Pathogenesis of autoimmune diabetes mellitus (DM) Type1. The aim of this study is to investigate the effectiveness of *Marrbium vulgare L* in autoimmune DM. Autoimmune DM-Type1 was induced in adult male albino mice by co-administration of cyclosporine A and multiple low doses of streptozotocin. Diabetic mice were treated daily with 2, 2 and 1mg/ml doses of *Marrbium vulgare L* (methanol, water and buthanol extract) respectively for 28 days. Blood glucose level (BGL), serum insulin level, lipid profile and pancreatic levels of tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and (NO) were measured. The results showed that *Marrbium vulgare L* treatment resulted in a significant decrease in the BGL and the pancreatic levels of IFN-γ and NO compared to diabetic mice and significant decrease in total cholesterol, LDL cholesterol, VLDL cholesterol, triglycerides. The serum insulin level and HDL cholesterol level were significantly increased after *Marrbium vulgare L* treatment compared to diabetic mice. These results suggest that *Marrbium vulgare L* has a beneficial effect in the treatment of autoimmune diabetes, an effect that seemed to be a secondary consequence of its anti-inflammatory.

Key-Words: Autoimmune diabetes, *Marrubium vulgare L*, cytokines, Streptozotocin, Antioxidant

Introduction

Diabetes mellitus is a metabolic disease, characterized by hyperglycemia together with impaired metabolism of glucose and other energy-yielding fuels, such as lipids and proteins (Scheen, 1997). This metabolic disorder is the result of a deficiency in insulin secretion or a resistance to insulin action, or both (Vinik *AI and Vinik E*, 2003). More than 220 million people worldwide have diabetes and this number is likely to more than double by the year of 2030 (Ahmed *et al.*, 2011). Diabetic patients also exhibit oxidative stress, which leads to lipid peroxidation and tissue damage including retinopathy, nephropathy, and coronary heart disease (CHD) (Lyons, 1991; Wolffe *et al.*, 1991). Dyslipidemia or hyperlipidemia is also involved in the development of cardiovascular complications, which are a major cause of morbidity and mortality (Reasner, 2008).
Herbal medicines are widely used all over the world. They are often perceived as being natural and therefore harmless.

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Many herbal remedies individually or in combination with different formulations such as leaf, powder, pastes, decoction, infusion, etc. had been recommended to treat various diseases. Many, if not most of medicinal plants contain flavonoids, such compounds has been associated with several beneficial effects such as antioxidants which consider to be a fundamental property important for life (Halliwell and Gutteridge, 2010).
Some herbal alternatives are proven to provide symptomatic relief and assist in the prevention of secondary complications of the disease. Some herbs have also been proven to help in the regeneration of B-cells and in overcoming resistance. In addition to maintain normal blood sugar level. However, the hypoglycemic effects of some herbal extracts have been confirmed in human and animal models of diabetes and conventional drugs have been derived from the active molecules of these medicinal plants. Plants provide a potential source of hypoglycemic drugs and are widely used in several traditional systems of medicine to prevent diabetes. Several medicinal plants have been investigated for their beneficial use in different types of diabetes. The effects of these plants may delay the development of diabetic complications and correct the metabolic abnormalities

using variety of mechanisms (Edwan *et al*., 2008). *Marrubium vulgare L*. (Lamiaceae), commonly known as ''Horehound'', is a widespread Mediterranean plant used infolk medicine to cure a variety of diseases, and is widely distributed in Libya. M. vulgare has been widely used in Mexican traditional medicine for the treatment of DM (Herrera *et al*., 2004), and its hypoglycemic effect was demonstrated during the study of the hypoglycemic effect of some Mexican and Brazilian plants (Roman *et al*., 1998; Novaes *et al*., 2001). The plant is reported to possess vasorelaxant (El-Bardai *et al*., 2003), antihypertensive (El-Bardai *et al*., 2004), analgesic (De Souza *et al*., 2009), anti-inflammatory (Sahpaz *et al*., 2002), and antioxidant properties (Weel *et al*., 1999). The aim of this study was to investigate the hypoglycemic, anti-inflammatory and antidyslipidemic effects of the three extracts (methanol, water and butanol) of the whole parts of M. vulgare in streptozotocin-induced diabetic mice. Also, the effect of this herb on normal mice was studied during 4 weeks of treatment.

Material and Methods

Plant material: The whole plant of *M.Vulgare L* was purchased from supermarket in Benghazi Libya (2010).

Chemicals: All the following chemicals were purchased from Sigma Co., USA ((Sodium citrate buffer (ph 4.5), Streptozotocin (STZ), Tetramethylene diamine (TEMED), Vanadium (III) chloride (VCl_3), Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1 % naphthyl ethylenediamine dihydrochloride in water), Butanol, Methanol, Formalin-fixed (10%).All other used chemicals were of high analytical grade.

Experimental Animals: A male albino mouse, aged 1-2 months, weighing 20-30 g, purchased from the animal house of the Benghazi University Faculty of medicine Benghazi, Libya were used in this study; Mice were fed on commercial standard pellet diet and allowed free access to tap water.

Kits: Tumor necrosis factor-alpha (TNF-α) EASIA kit (Biosource, Belgium), Interferon-gamma (IFN-γ) EASIA kit (Biosource, Belgium), Insulin kit (Abbott Laboratories, USA).

Drugs: Cyclosporine A (Sandimmune injection, Sandoz, Switzerland). It is diluted with normal saline and kept at $4^{o}C$.

Preparation of test herb: The whole plant of *M.Vulgare L* was powdered separately using 40 mesh Willey mill to insure their complete homogeneity.

Marrubium vulgare L **extracts:** The air dried material (20g) extracted by percolation with boiling water, methanol, butanol and acetone. The extracts were filtered concentrated under vacuum.

Induction of autoimmune diabetes: Mice were injected daily with cyclosporine A (CsA, 22 mg/kg/day, S.C.) for 2 weeks prior to and simultaneously with multiple low doses of streptozotocin (MLD STZ, 45 mg/kg/day, I.P.) for another 5 consecutive days **(Wright et al., 1995; Fraser *et al*., 1997)**. STZ was dissolved in 0.1M sodium citrate buffer (PH 4.5) just before use. Mice were allowed free access to diet and water. Blood glucose level was measured every other day; diabetes mellitus was defined when the fasting blood glucose level (B.G.L.) reached more than 11.1 mmol/l (200 mg/dl) at two consecutive readings taken every other day **(Papaccio *et al*., 1999)**.

Experimental design:

The curative effect of different treatments on diabetic mice: In this experiment, a total of 75 mice were used. 15 mice were taken daily normal saline and served as negative control (-ve) for 4 weeks "**group1".** The other mice were subjected to the induction of experimental hyperglycemia for 19 days as described before. The hyperglycemic mice (60 mice) were divided randomly into equal 4sub-groups (15 mice each) as follows: **Group 2 :** Mice were served as hyperglycemic animals (+ve), n=15. **Group 3:** Mice were injected daily with Methanol extract of *Marrubium vulgare L* (2mg/ml) for 28 days, n=15. **Group 4:** Mice were injected daily with Water extract of *Marrubium vulgare L* (2mg/ml) for 28 days, n=15. **Group 5:** Mice were injected daily with Butanol extract of *Marrubium vulgare L* (1mg/ml) for 28 days, n=15.

Biochemical analysis:

Determination of blood glucose level: The blood glucose level was determined by enzymatic colorimetric method **(Trinder, 1969)**.

Determination of Serum Insulin Level: The insulin level in serum samples was determined using the microparticle enzyme immunoassay (MEIA), Abbott AxSYM®system.

Determination of serum lipid profile: Serum total cholesterol(TC), triglycerides(TG) and HDL-cholesterol (HDL-C) were determined spectrophotometrically ,using commercial kits. Low density lipoprotein (LDL and VLDL) were calculated by using **finely (1978)** . .

Determination of TNF- α and IFN-γ content In Pancreatic Tissue: The Pancreatic level of TNF- α and IFN-γ were determined by means of ELISA kits using monoclonal antibodies specific for TNF- α and IFN-γ respectively. Samples were run in duplicate

according to the manufacturer's instructions. Concentrations of cytokines were determined from standard curves using purified recombinant cytokines provided with the kits.

Determination of NO (Measured As Nitrite/Nitrate) in Pancreatic Tissue: The NO content of the pancreas was determined as nitrite and nitrate by spectrophotometry according to previously described method (**Miranda *et al.*, 2001**).

Histopathological Examination of the Pancreas

Hematoxylin and Eosin (H&E) staining technique:

At the end of the experiment period, pancreas was excised, washed with normal saline and the fixed in 10% neutral formalin for 24 hr. Sections were embedded in paraffin wax, serially sectioned (5 um), stained with (H&E), then mounted in Canada balsam for examination by a light microscope.

Statistical analysis of the data: The results obtained were statistically analysed according to the methods described by **Chase (1967)**. The probability "P" was deduced from table of "t" test according to the degree of freedom.

Results and Discussion

Induction of hyperglycemia

During the induction of hyperglycemia (mice were injected daily with cyclosporine A (CsA, 22 mg/kg/day, S.C.) for 2 weeks prior to and simultaneously with multiple low doses of streptozotocin (MLDSTZ, 45 mg/kg/day, I.P.) for another 5 consecutive days. Table (1) illustrated the values of different parameters under investigation before and after induction of hyperglycemia. Blood Glucose was very highly significant increased by 400%.Serum insulin was very highly significant decreased by 86% after induction of hyperglycemia. The levels of Serum total, triglycerides, total cholesterol, HDL, LDL, and VLDL cholesterol did not induce any significant change after induction of hyperglycemia. Induction of hyperglycemia causes increase of IFN-γ, TNF-α and Nitric oxide by (223.6%, 494.5% and 80%), respectively.

Histopathological studies.

Positive control (Diabetic mice)

Pancreatic sections stained by (H & E) and taken from positive control diabetic mice showed the cells lining serous acini undergo sever vascular degenerative change. The islets of langerhans (IL) showed sever involution, may reach to their absence in their location between serous acini (Sa), so this area between serous acini (Sa) in some fields under light microscope appeared devoid from cells and vascular architecture figs. (1, 2, 3, 4).
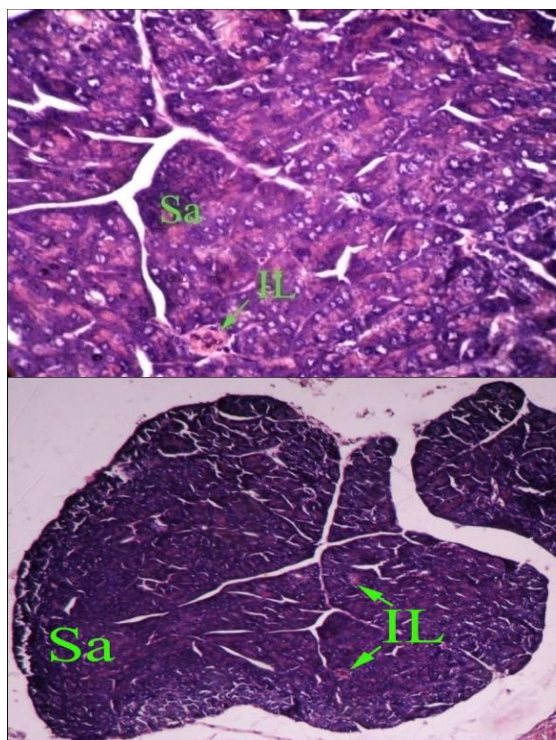

Fig. (1&2). A Photolight micrograph of pancreatic tissue hyperglycemic mice showing Pancreas Consisted of-The exocrine portion, containing serous acini (Sa). Some areas of the serous acini showing necrosis of its cells (N). The endocrine portion: represented by the islet's of langerhans (IL). Note the involution of the islet's of langerhans and represented only by pale staining regions that either contain few number of cells or completely devoid of cells-vacular degeneration of cells forming that acini (Sa) involution and atrophy of islet of langerhans(IL). Stain (H&E, X: 100).
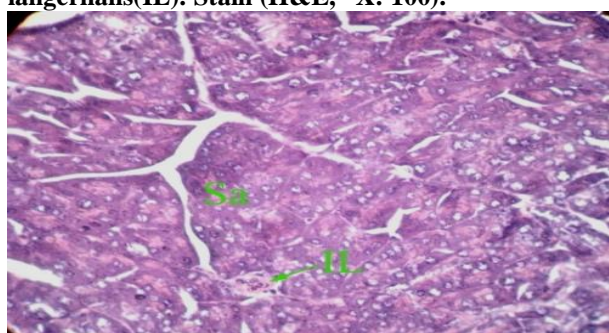

Fig. (3). A photolight micrograph of pancreatic tissue hyperglycemic mice showing in High mgnification of the privious figure showing atrophy of involution of islet of langerhans(IL) numerous vacular degeneration of serous acini (Sa) and Note the involution of the islet's of langerhans and

represented only by pale staining regions that contain few number of cells. Stain (H&E, X: 1000).
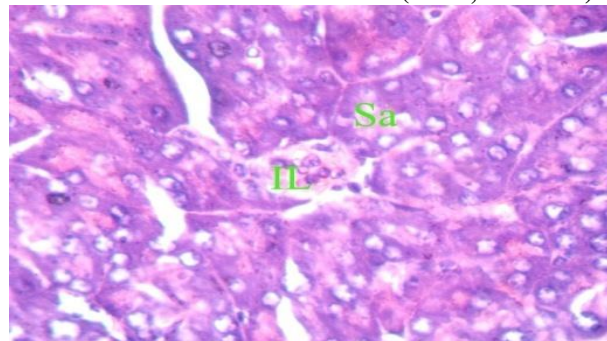

Fig. (4). A photolight micrograph of pancreatic tissue hyperglycemic mice showing the endocrine portion: represented by the islets of langerhans (IL). Note the involution of the islet's of langerhans and represented only by pale staining regions containing few number of cells Stain (H&E, X: 1000).

Curative effect of different treatments on hyperglycemic mice

Curative effect of different treatments on BL. glucose and S. insulin

All does of *Marrubium vulgare L* extracts, Methanol, Water and Butanol were very highly significant decreased the level of bl. glucose by 64.9%, 69.7% and 74.2%compared with the (-ve) control group. Also all groups of different treatments were significant increased the level of serum insulin by 259%, 496% and 622%, respectively (Table 2).

Curative effect of different treatments on lipid profile.

The effect of *Marrubium vulgare L* extracts, Methanol ,Water and Butanol (2, 2,1mg/ml.), respectively on serum total cholesterol, HDL- cholesterol, LDL cholesterol, VLDL cholesterol and triglycerides in hyperglycemic mice is present in Table (2) . All parameters were decreased . Except HDL- cholesterol was increased by 17.9%, 22.8% and 32.7% for *Marrubium vulgare L* extracts, Methanol ,Water and Butanol (2, 2,1mg/ml.), respectively Also this table indicated that serum total cholesterol was significantly decreased in all treated groups by 8.43%, 14.3% and21.4%, respectively. All groups of different treatments were significant decreased the level of serum LDL cholesterol by (16.4%, 23.3% and 36.6%), respectively Also the levels of serum VLDL cholesterol were significant decreased in all groups of different treatments by (16.4%, 23.3% and 36.6%), respectively .Serum triglycerides in the all groups of different treatments were significant decreased by (16.5%, 33.5% and 41.5%), respectively .

Curative effect of different treatments on Pancreatic IFN-γ, TNF-α and Nitric oxide (NO) content.

Table (3) revealed the effect of different treatments on Pancreatic IFN-γ, TNF-α and Nitric oxide (NO) content. This effect was highly appreciated after 4 weeks of treatment. *Marrubium vulgare L* extracts, Methanol, Water and Butanol (2, 2,1mg/ml) were significantly decrease the activity of IFN-γ by (28.1%, 48.2% and 43.1% respectively), TNF-α by (36.5%, 45.6% and 50.3% respectively), Nitric oxide (NO) by (66.8%, 58.9% and 30.6% respectively).

Histopathological studies:

Positive treatment methanol extract: Approximately similar to positive treatment water extract with slightly hyperplastic cells in the islet of langerhans (IL) with normal serous acini (Sa) fig. (5).

Positive treatment water extract: In positive diabetic animals received water extract of Marrubium vulgare L, the islets of langerhans showed hyperplasia in their cells which represented by pale staining area in between serous acini with slightly increase vasculature in this area. In addition, the serous acini (Sa) appeared looks like normal in the stained pancreatic sections figs. (6, 7, 8).

Positive treatment butanol extract : In stained paraffin pancreatic section with (H&E) from diabetic mice treated by butanol extract of Marrubium vulgare L, the pancreatic tissues showed normal appearance of pancreatic on serous acini (Sa), on the other hand, the islets of langerhans (IL) showed cells than those in positive treatment with water extract with slightly of vasculature. In addition to mononuclear cells are present, few number in islets of langerhans figs. (9,10, 11).
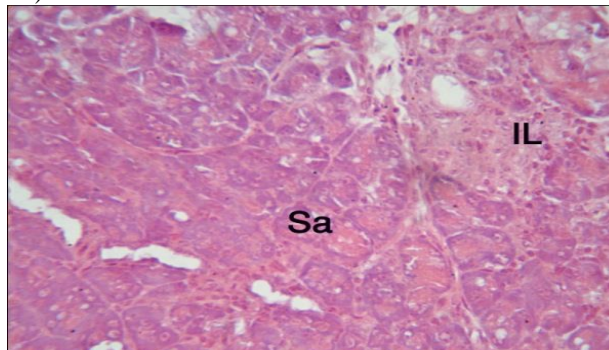

Fig. (5). A photolight micrograph of pancreatic tissue positive treatment methanol extract of Marrubium vulgare L showing Serous acini (Sa), increase in the number of the cells that exist in the islet of langerhans (IL) after treatment. Stain (H&E, X: 1000).
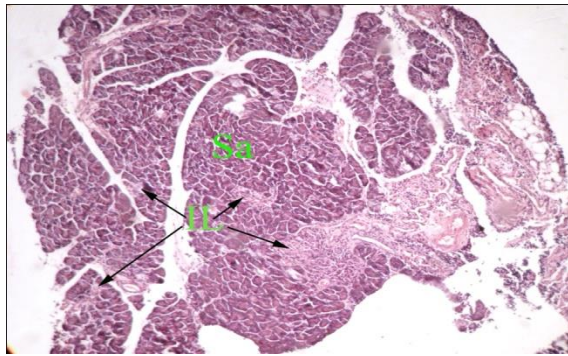
Fig. (6). A photolight micrograph of pancreatic tissue positive treatment water extract of Marrubium vulgare L showing Pancreas Consisted of: the exocrine portion, containing serous acini (Sa). The endocrine portion: represented by the islet's of langerhanse (IL). Note hyperplasia of the islets of langerhanse and represented only by pale staining regions that contain numerous numbers of cells. Note increased vasculature (IL) the arrows in the areas of islets of langerhanse and hyperplasia of islet of langerhans (IL) and normal appeared of (Sa). Stain (H&E, X: 100).
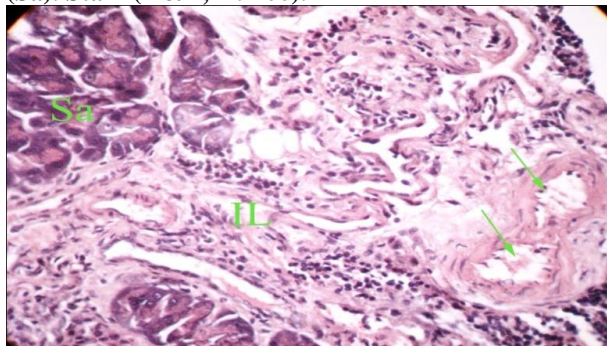

Fig. (7). A photolight micrograph of pancreatic tissue positive treatment water extract of Marrubium vulgare L showing high magnification of previous fig (40) showed:- 1. Increase valuation of islets langerhans (IL) explained by arrows. 2. Hyperplasic of islets langerhans (IL). 3. Normal appeared of serous acini (Sa). Stain (H&E, X: 1000).
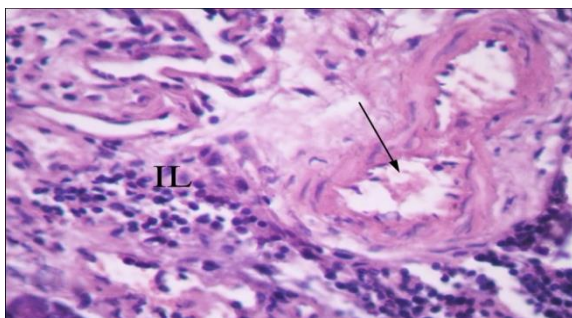

Fig. (8). A photolight micrograph of pancreatic tissue positive treatment water extract of Marrubium vulgare L showing higher magnification of previous fig (41) showed Increase valuation of islets langerhans (IL) explained by arrows. Stain (H&E, X: 1000).
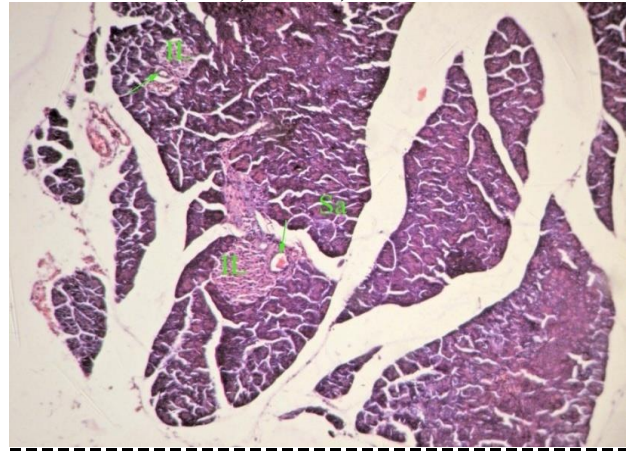

Fig. (9). A photolight micrograph of pancreatic tissue positive treatment butanol extract of Marrubium vulgare L showing Pancreas Consisted of the exocrine portion , containing serous acini (Sa). Some areas of the serous acini showing necrosis of its cells (N). The endocrine portion: represented by the islet's of langerhanse (IL). Note moderate hyperplasia of the islet's of langerhanse and represented only by pale staining regions that contain large number of cells Note increased vasculature (arrows) in the areas of islet's of langerhanse. Stain (H&E, X: 1000).
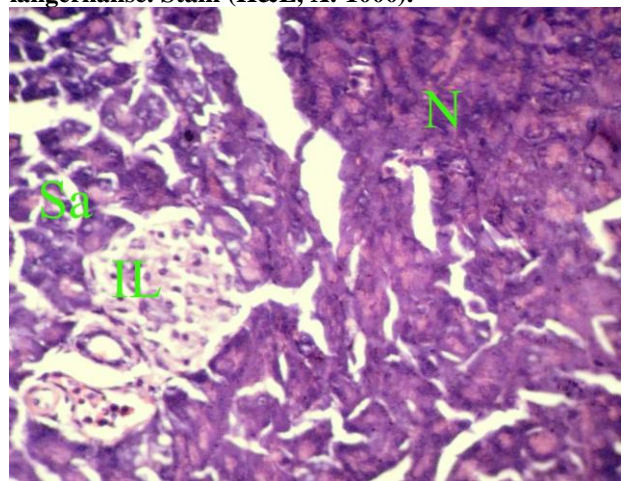

Fig. (10).A photolight micrograph of pancreatic tissue positive treatment butanol extract of Marrubium vulgare L showing moderate hyperplasia of islet's of langerhanse (IL) and increase vasculation of (IL). Stain (H&E, X: 100).
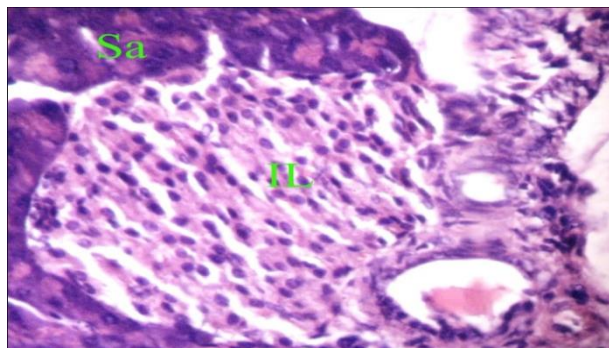
Fig. (11). A photolight micrograph of pancreatic tissue positive treatment butanol extract of Marrubium vulgare L showing higher magnification of previous fig (44) showed moderate hyperplasia of islet's of langerhanse (IL) and increase vasculation of islet's of langerhanse (IL). Stain (H&E, X: 1000).

Induction of hyperglycemia

It is well known that the pathogenesis of type 1 diabetes mellitus (DM) involves several hypotheses where the immune system plays a major role. An infiltration of inflammatory cells into the islets either directly or through the release of inflammatory mediators such as cytokines or free radicals is responsible for β-cell destruction and death in both human type 1 diabetic subjects (**Gepts and In'tveld, 1987; Santamaria *et al*., 1992**) and animal models of autoimmune diabetes (**Mordes *et al*., 2000; Carlsson et al., 2000**).

In this study, autoimmune type 1 DM was experimentally induced by injecting the mice with CsA plus MLDSTZ. This experimentally-induced diabetes offered a number of advantages. First, the islet lesions of MLDSTZ model closely resemble those of human disease (**Herold *et al*., 1987; Itoh et al., 1993; Somoza et al., 1994**). Second, the mice involved are normal and don't have underlying immune abnormalities that may complicate the study (**Herold *et al*., 1990; Leiter and Serreze, 1991; Serreze et al., 1993**). Importantly, the onset of diabetes is controlled, so the kinetics of disease induction may be analyzed (**Herold *et al*., 1996**).

Several studies have emphasized that induction of autoimmune diabetes by MLDSTZ treatment depends on the genetic susceptibility of the animal strain (**Rossini *et al*., 1977; Maclaren *et al*., 1980**). Elias *et al*., (1994) reported that adult male albino mice were resistant to the induction of hyperglycemia by MLDSTZ.

The current study, however, clearly showed that approximately 86% of male albino mice co-treated with CsA plus MLDSTZ became diabetic after 19

days of the first dose of MLDSTZ. The serum insulin levels of the diabetic mice were significantly decreased as compared to control group. In addition, histopathological examination of the pancreatic tissue of diabetic mice examined on day 19 of the first dose of MLDSTZ revealed early vascular degeneration with mild hyalinization of pancreatic islets of langerhans. Moderate lymphocytic infiltration could be detected in the pancreatic islets.

These results were in agreement with the previous reports that emphasized the potential effect of CsA on diabetes induced by MLDSTZ (**Iwakiri *et al*., 1987; Wright *et al*., 1995**). Reported that CsA supplementation increased significantly the B.G.L. in resistant male albino mice for MLDSTZ induced diabetes.

In addition, it was found that CsA enhanced the hyperglycemia, hypoinsulinemia and β-cell destruction following MLDSTZ treatment in highly susceptible strain of CD-1 male mice (**Sestier *et al*., 1985; Iwakiri et al., 1987**).

The effect of CsA alone on the biochemical parameters (B.G.L. and serum insulin level) and histopathological findings modified during diabetes development was variable.

It was found that oral administration of 50 mg/kg of CsA to Wistar rats for 7 days caused degenerative changes in the pancreatic β-cells (**Helmchen *et al*., 1984**). The daily I.P. injection of 20 mg/kg of CsA to CD-1 mice for 10 days reduced the pancreatic insulin content to about 50% of the control mice, thereby suggesting that pancreatic β-cell function deteriorated in the presence of CsA. However, neither morphological changes in the pancreatic β-cells nor hyperglycemia have occurred (**Iwakiri *et al*., 1987**).

On the other hand, it was found that CD-1 mice that received oral CsA (50, 25 or 12.5 mg/kg/day) alone displayed glucose intolerance, insulinopenia, and high pancreatic CsA content (**Sai *et al*., 1988**).

Accordingly, the current study confirmed the potential effect of CsA on DM induced by MLDSTZ treatment. However, the discrepancy in the effect of CsA treatment alone may be due to the different doses of CsA or to the different animal species.

In the present study, the diabetes induced by CsA/MLDSTZ co-treatment may be considered type 1 DM with immune-mediated nature, due to the reduction of B.G.L. in diabetic mice below 200 mg/dl when injected with exogenous insulin. Also, the increase in the B.G.L. that occurred was parallel to the decrease in the serum insulin levels. In addition, the histopathological examination of the pancreatic tissues of diabetic mice revealed moderate insulitis

which confirm the immune-mediated component of experimental DM. In experimental models of type 1 DM, infiltration of lymphocytes into the pancreatic islets (insulitis) was recognized as the hallmark of immune-mediated disease (**Muri *et al.*, 1991; Suarez-Pinzon *et al.*, 1999**).

In animal models of type 1 DM, the involvement of free radicals in the development of the disease has been clearly demonstrated (**Ho and Bray, 1999**). Among free radicals, the role of NO in β-cell destruction has been reported (**Mandrup-Poulsen, 1996; Eizirik *et al.*, 1996; Thomas *et al.*, 2002**).

It was found that IFN-γ was required to stimulate iNOS expression and induced cellular damage of NOD mouse islets (**Thomas *et al.*, 2002**). Consistent with these findings, the combination of TNF-α and IFN-γ has been shown to reduced the viability of wild-type but not iNOS-deficient mouse islets, suggesting that cytokines-induced damage is caused by NO production (**Liu *et al.*, 2000**).

Nitric oxide also appeared to participate, in part, in cytokines induced human islet damage. Treatment of human islet with TNF-α and IFN-γ stimulated iNOS expression, nitrite production, inhibition of insulin secretion and islet cell apoptosis (**Corbett *et al.*, 1993; Delaney *et al.*, 1997; Arnush *et al.*, 1998b**). All of these effects are attenuated, but not completely prevented, by inhibition of iNOS.

In the present study, the pancreatic tissue content of NO in diabetic mice was significantly increased compared to normal mice. This was in agreement with several lines of research that suggested that NO production contributed to the cytotoxicity of MLDSTZ to pancreatic β-cells (**Corbett *et al.*, 1992; Fehsel *et al.*, 1995; Kroncke *et al.*, 1995**). In MLDSTZ-induced diabetes, it was found that the incidence of hyperglycemia was decreased when iNOS inhibitors were used (**Kolb *et al.*, 1991**). In addition, it was found that transgenic mice deficient in iNOS have reduced sensitivity to MLDSTZ-induced diabetes (**Flodstrom *et al.*, 1999**).

It may be proposed that the local increase in the pancreatic content of NO together with the elevated levels of TNF-α and IFN-γ can contribute to β-cell damage by two mechanisms. Cytokines such as TNF-α and IFN-γ may directly stimulate iNOS expression and NO production by β-cells resulting in NO-mediated β-cell damage. Alternatively, cytokines may stimulate iNOS expression and release of additional cytokines by non-endocrine cells in the islets such as macrophages and in a paracrine fashion that may mediate β-cell damage.

The colocalization of iNOS and insulin in rat and human islets treated with IL-1 or IL-1 plus IFN-γ .respectively would support the first hypothesis (**Corbett and McDaniel, 1995**).

Whereas in support of the second hypothesis, it was found that resident islet macrophages activation with TNF plus LPS would lead to production and release of IL-1 that followed by IL-1-induced iNOS expression by β-cells (**Suarez-Pinzon *et al.*, 1999**). In addition, it has been documented that depletion of macrophages by previous gadolinium chloride treatment partially inhibited the generation of free radicals induced by direct, *in vivo* administration of TNF-α and IFN-γ into the rat pancreas (**Tabatabaie *et al.*, 2003**). Therefore, it may be concluded that macrophages most likely enhanced the generation of free radicals by the β-cells through the release of additional cytokines.

Moreover, (**Takamura *et al.*, 1998**) documented that transgenic mice overexpressing iNOS in pancreatic β-cells have markedly reduced β-cell mass without infiltration of macrophages or lymphocytes, and extensive DNA strands breaks were detected in the islets. All these transgenic mice developed hypoinsulinemic diabetes and treatment with an inhibitor of iNOS, aminoguanidine prevented or delayed the development of diabetes.

Taken together, it's noteworthy how important the NO is either alone or in combination with other cytokines in the β-cell destruction and subsequent DM development.

Evaluation of *Marrubium vulgare L*

Our results revealed that *Marrubium vulgare L* had significant effect on normal mice and curative experiment. An amelioretic picture was shown in the experiments in all parameters during 4weeks of treatment. These data agreement with the results of many authors who stated that *Marrubium vulgare L* extract could act as hypoglycemic agent (**Natural Standard, 2011; Herrera-Arellano *et al.*, 2004; Mahmoud *et al.*, 2005**).

The medicinally used parts of white horehound *(Marrubium vulgare L)* are the dried leaves and flowering tops. Processed white horehound contains 0.3-1% of the bitter principle marrubiin, diterpene alcohols, alkaloids, bitter lactone, flavonoids, saponin, sterols, tannins, and vitamin C, and 0.06% of a volatile oil (**Takeda *et al.*, 2000**). The volatile oil of *Marrubium vulgare L* also contains monoterpenes. Marrubiin does not exist in the fresh plant but is formed from premarrubiin during processing (**De Jesus *et al.*, 2000**). White horehound also contains glycosides (**El Bardai *et al.*, 2004**).

Phenylpropanoid glycosides such as acteoside 1, forsythoside B 2, arenarioside 3, and ballotetroside 4 have been isolated from the aerial parts of *Marrubium vulgare L* (**Martin-Nizard *et al.,* 2004**). Sesquiterpene bitters (including marrubiin), Diterpene alcohols (marrubenol, marrubiol, peregrinol, vulgarol, phytol), flavonoids (apigenin, luteolin), small amounts of pyrrolidine alkaloids (betonicine, stachydrine), traces of volatile oil (containing alpha-pinene, sabine, camphene and p-cymol), alkaloids, ursolic and caffeic acid, tannins, saponin, mucilage, minerals (especially potassium), vitamin C, resins, wax, sterols (**Rohman *et al.,*2012**). Flavonoids, which occur in most plant species, have been shown an antibacterial, anti-inflammatory, antiallergic, antimutagenic, antiviral, antithrombotic and vasodilatory activity. The potent antioxidant activity of flavonoids may be the most important function of flavonoids. *Marrubium* flavone glycosides can be utilized in preventative and treatment of cardiovascular disease, cancer, inflammatory conditions, asthma, liver disease, cataracts and macular degeneration (**Khanavi *et al.*, 2005**).The flavonoids substances were considered to be responsible for the free radical scavenging properties of *Marrubium vulgare L* extract. Superoxide anion scavenging properties correlated to the presence of terpenes, addition to the scavenging of hydroxyl radicals were also reported by studies with pulse radiolysis or photolysis (**Patrycja and Emilia, 2008**). *Marrubiumvulgare L* extract has been effective in the treatment of disorders related to oxidative stress and is commonly used to treat peripheral arterial diseases and cerebral insufficiency *Marrubium vulgare L* extract has been described as a potent scavenger of free radicals, such as superoxide radical, hydroxyl, peroxyl radicals and NO (**Halliwell, 2008**). Moreover, the antioxidant potency of *Marrubiumvulgare L* extract appeared to be greater than that of ascorbic acid, glutathione and uric acid, and comparable with that of lipid soluble antioxidants such as retinal acetate and α- tocopherol (**Wolski *et al.*, 2007; Patrycja *et al.*, 2008**).

A considerable attention has been devoted to the role of the different natural antioxidants as inhibitors of LDL oxidation and their possible therapeutic effects to prevent hyperlipaemia and atherosclerosis. Phenylpropanoid glycosides such as acteoside 1, forsythoside B 2, arenarioside 3, and ballotetroside 4 (which can be isolated from the aerial parts of *Marrubium vulgare* L.) have been implicated as the active constituents involved in inhibition of low-density lipoprotein (LDL) oxidation (**Martin-Nizard**

et al., **2004**). The oxidation of LDL is considered by many sources to be a very important component of the development of atherosclerotic lesions (**Wolski *et al.*, 2007**). Circulating monocytes scavenge oxygen–modified LDL molecules with a very high affinity up to 10 times greater than native LDL. These monocytes / macrophages penetrate into the subendothelial space and become the first stage of atherogenesis. Antioxidants which interrupt this process can be very helpful in the process of preventing and /or treating cardiovascular disease. A number of flavonoids e.g. quercetin, which present in *Marrubium*, have been shown in in-vitro studies to inhibit the oxidation of LDL (**Natural Standard, 2011**). Oxidation of LDL is generally used as a model of the anti-lipid peroxidation activity of flavonoids, as the LDL molecule has an outer phospholipid layer similar to cell membranes. The same authors added that the mechanism by which flavonoids inhibit LDL is thought to be reduce free radical formation, protect LDL -α – tocopherol or regenerate oxidized LDL -α – tocopherol, and/or sequester metal ions which participate in oxidation reactions.

Several studies have demonstrated that treatment of animals (or isolated heart) with *Marrubium*, a multi component drug with a polyvalent action, protects against myocardial ischemia – reperfusion injury (**El Bardai *et al.*, 2001, and Martin-Nizard *et al.*, 2004**). It is generally considered that such cardioprotection is associated with the free radical scavenging properties of *Marrubium* which have been demonstrated in in-vitro experiments and which may involve its flavonoid constituents. *Marrubium* is specially used for ameliorating peripheral vascular diseases such as intermittent claudication and cerebral insufficiency. It has been reported to prevent ischemia – induced oxidation, improve cerebral blood flow (**El Bardai *et al.*, 2001**), and antagonize the action of platelet – activating factor (**Herrera-Arellano *et al.*, 2004**). In patients with type 2 diabetes treated with *Marrubium vulgare L*, cholesterol and triglyceride levels were lowered by 4.16% and 5.78%, respectively. In this regard, the antioxidant properties of *Marrubium* are possibly related to a reduced lipid peroxidation, leading to the increase in PUFAs (poly unsaturated fatty acids) observed, since these fatty acids can both regulators and targets of oxidative damage (**Herrera-Arellano *et al.*, 2004**).

Animal studies and early human studies suggest that white horehound may lower blood sugar levels. White horehound has been used for diabetes in some

countries, including Mexico. Further well-designed human trials are needed (**Natural Standard, 2011**).These actions appear to be beneficial in influencing the peripheral vascular diseases and confirmed that horehound and its major ingredient quercetin produce vasodilation via NO in the aorta of mice increasing intracellular calcium levels in the aortic endothelial cells. So horehound and quercetin can activate undefined calcium channels in endothelial cells. The resulting increase in intracellular calcium level can activate NO – synthase and enhance formation of NO. This may be involved in improvement of local circulation.

In this study, treatment of diabetic mice with *Marrubium* extract(methanol, water and butanol) (2mg/ml,2mg/ml,1mg/ml) respectively   for 28 days showed significant decrease in the pancreatic tissue content of both TNF-α and IFN-γ compared with diabetic untreated mice. These findings were supported by the previous reports which documented that *Marrubium* through activation of PPAR-γ could modulate inflammatory processes associated with several inflammatory diseases by reducing the expression of TNF-α and/or   IFN-γ (**Mahmoud *et al*., 2005; Lisa R.W.Plano *et al*., 2008**; **Blumenthal *et al*., 2000**).

In the present study, treatment of diabetic mice with *Marrubium vulgare L* extracts for 28 days caused a significant decreased in the pancreatic content of NO compared to diabetic untreated mice. This effect may be explained on the basis that PPAR-γ agonists such as *Marrubium vulgare L* have a beneficial role in inflammatory diseases through decreasing iNOS expression and NO production (**Mahmoud *et al*., 2005**)

Butanol Marrubium vulgare L extract has been found to exert potent anti-inflammatory effects in animal models of acute inflammation (carrageenan-induced pleurisy) (**De Souza *et al*., 2009**). This effect is mediated, in part, by reducing the expression of iNOS in the lungs of carrageenan-treated rats. The author also showed that *butanol Marrubium vulgare L* extract treatment reduced the development of nonseptic shock induced by zymosan in mice. *Butanol Marrubium vulgare L* extract attenuated the peritoneal exudation and the migration of polymorphonuclear cells caused by zymosan (**Blumenthal *et al*., 2000**).

It was also reported that *Marrubium vulgare L* extracted a significant vascular protective effect in hypercholesterolemic rabbits, most likely by suppressing iNOS expression and reduced superoxide and NO production in the carotid arteries of the treated rabbits (**Natural Standard, 2011**). The decreased in iNOS expression and NO production that has been reported in the *in vivo* studies were found to be associated with the decrease in the accumulation of inflammatory cells and inflammatory mediators in the site of inflammation (**Natural Standard, 2011**; **Herrera-Arellano *et al*., 2004**).

Therefore, it can be concluded that the decrease in NO level observed in diabetic mice that received *Marrubium vulgare L* extracted may be a secondary consequence of the decreased accumulation of inflammatory cells in the pancreatic islets as well as the decreased level of TNF-α and IFN-γ in the pancreatic homogenate. Overproduction of TNF-α and IFN-γ by inflammatory cells were documented to increase the expression of iNOS in β-cell and macrophages infiltrating pancreatic islets with subsequent increase in NO production (**Natural Standard, 2011**).

In conclusion, in the present study the experimentally-induced diabetes mellitus by CsA/MLDSTZ co-treatment is clearly type 1 autoimmune diabetes. Our findings extend the list of known synergistic actions of TNF-α and IFN-γ, in general and on type 1 DM development as the levels of both cytokines were elevated significantly in the pancreases of diabetic mice. The increase in TNF-α and IFN-γ was accompanied with significant elevation of NO level in the pancreatic tissue. In addition, the differential expression of some proteins between the pancreases of normal and diabetic mice which may play a role in the pathogenesis of type 1 DM was reported.

Marrubium vulgare L extracts was found to be effective in treatment of type 1 diabetes mellitus. The anti diabetic effect of *Marrubium vulgare L* extracts seemed to be a secondary consequence of its anti inflammatory effects, as *Marrubium vulgare L* extracts treatment resulted in significant decrease in the elevated level of TNF-α, IFN-γ and NO.

In general, to use this plant extracts as safe curative agent, more studies should be carried out to know all the active / inactive components and their mechanism of actions weither synergesic or antagonist using different doses from these extracts and another types of experimental animals for a long period in order to judgment if these extracts could be use as safe agents or not in human therapy.

Table 1: Arithmetic mean values ± S.D and % changes from the corresponding control of different biochemical parameters of serum and tissues before and after induction of hyperglycemia in male albino mice

Parameters		Before induction of hyperglycemia	After induction of hyperglycemia	% change
Bl. Glucose (mg /dl)		84.2± 7.90	421 ± 8.39***	400 ↑
S. insulin (mu/ml)		19.6±3.02	2.74±1.44***	86 ↓
S. T.chol.(mg /dl)		159.1± 6.16	171 ± 12.9†	7.48 ↑
S.LDL-chol.(mg /dl)		93.2± 3.8	110 ± 7.36†	18.0↑
S.HDL-chol.(mg /dl)		49.2 ± 2.45	41.3 ± 0.42†	16.1 ↓
R.R (T/HDL-chol.)		3.23 ± 0.11	4.14 ± 0.41†	28.2 ↑
S. Triglyc.(mg /dl)		83.7± 7.26	98.7 ± 8.51†	17.9 ↑
S.V1LDL-chol.(mg /dl)		16.7± 2.04	19.7± 3.26†	18.0↑
IFN-γ (PG/ML)	tissues	119.6± 8.74	387± 8.54***	223.6↑
TNF-α (PG/ML)		2.00± 0.09	11.89± 0.11†	494.5↑
Nitric oxide (IU/ML)		22.0± 1.12	39.6± 5.33**	80↑

† Insignificant difference from the corresponding control at P > 0.1, * Significant difference from the corresponding control at P < 0.05, ** Highly sig. difference from the corresponding control at P < 0.01, *** Very highly sig. difference from the corresponding control at P < 0.001,↓ Decrease,↑ Increase

Table 2: The curative effect of different treatments on Bl. Glucose (mg/dl ± S.D) , S. Insulin (mu/ml ± S.D) ,S. lipids (mg/ dl± S.D) and % variation from the corresponding control after induction of hyperglycemia as well as during 4 weeks of treatment in male albino mice

Animal groups	Item	Time intervals (Wk's)		% var.
		Before treatment	After treatment	
Control	Bl. Glucose	84.2±8.12	86.4±4.67 †	2.61↑
	S. Insulin (mu/ml)	21.6±0.19	21.2±0.16†	1.85↓
	S. T.C	165.7±4.23	163.7±8.54 †	1.21↓
	S. T.G	83.7±7.00	85.9±4.38 †	2.62↑
	S. HDL.C	45.3±1.62	46.6±4.60†	2.87↑
	S. LDL.C	103.7±4.23	99.9±6.01†	3.66↓
	S. VLDL.C	16.7±7.00	17.2±4.38 †	3.0↑
positive control	Bl. Glucose	428.6±6.04	442±3.21†	3.27↑
	S. Insulin (mu/ml)	3.22±0.61	2.74±0.29†	14.9↓
	S. T.C	179.9±14.4	188.5±9.50†	4.78↑
	S. T.G	99.6±6.15	98.7±12.1†	0.90↓
	S. HDL.C	39.2±2.58	37.0±0.41†	5.61↓
	S. LDL.C	120.8±3.72	131.8±3.79*	9.10↑
	S. VLDL.C	19.9±6.15	19.7±12.1†	1.0↓
Methanol extract of Marrubium vulgare L(2mg/ml.)	Bl. Glucose	416±7.39	146±11.5***	64.9↓
	S. Insulin (mu/ml)	3.12±0.53	11.2±2.02***	259↑
	S. T.C	170.9±17.1	156.5±4.25*	8.43↓
	S. T.G	97.2±13.9	81.0±9.28†	16.7↓
	S. HDL.C	39.7±2.36	46.8±3.04*	17.9↑

	S. LDL.C	111.8±4.00	93.5±3.22*	16.4↓
	S. VLDL.C	19.4±13.9	16.2±9.28*	16.5↓
Water extract of Marrubium vulgare L(2mg/ml.)	Bl. Glucose	422±5.67	128±5.52***	69.7↓
	S. Insulin (mu/ml)	3.24±0.99	19.3±4.62***	496↑
	S. T.C	179.6±12.0	153.9±9.70*	14.3↓
	S. T.G	98.4±9.36	65.5±9.48*	33.4↓
	S. HDL.C	39.4±2.06	48.4±2.74*	22.8↑
	S. LDL.C	120.5±3.99	92.4±4.05**	23.3↓
	S. VLDL.C	19.7±9.36	13.1±9.48**	33.5↓
Butanol extract of Marrubium vulgare L(1mg/ml.)	Bl. Glucose	419±4.53	108±7.23***	74.2↓
	S. Insulin (mu/ml)	3.16±0.87	22.8±2.98***	622↑
	S. T.C	176.4±7.39	138.6±8.83**	21.4↓
	S. T.G	100.2±9.42	58.7±10.1**	70.7↓
	S. HDL.C	40.1±3.49	53.2±2.53**	32.7↑
	S. LDL.C	116.3±6.01	73.7±3.21***	36.6↓
	S. VLDL.C	20.0±9.42	11.7±10.1**	41.5↓

† Insignificant difference from the corresponding control at P > 0.1: * Significant difference from the corresponding control at P < 0.05: ** Highly sig. difference from the corresponding control at P < 0.01: *** Very highly sig. difference from the corresponding control at P < 0.001:↑ Increase:↓ Decrease.

Table 3: The curative effect of different treatments on Pancreatic IFN-γ, TNF-α content (PG/ML ± S.D) , Nitric oxide (NO) content (IU/ML ± S.D) and % variation from the corresponding control after induction of hyperglycemia as well as during 4 weeks of treatment in male albino mice

Animal groups	Item	Time intervals (Wk's)		
		Before treatment	**After treatment**	**% var.**
Control	IFN-γ	119±2.52	121±2.78†	1.68↑
	TNF-α	1.98±0.01	2.00± 0.02†	1.01↑
	Nitric oxide	21.9±1.33	19.8±1.27†	9.6↓
positive control	IFN-γ	389±8.94	392±7.02†	0.77↑
	TNF-α	11.02±0.06	10.90±0.10†	1.08↓
	Nitric oxide	39.4±1.16	38.4±1.55†	2.5↓
Methanol extract of Marrubium vulgare L(2mg/ml.)	IFN-γ	377±5.95	271±4.32***	28.1↓
	TNF-α	10.89±0.10	6.91± 0.07***	36.5↓
	Nitric oxide	37.3±1.32	12.4±1.28***	66.8↓
Water extract of Marrubium vulgare L(2mg/ml.)	IFN-γ	382±6.03	198±6.25***	48.2↓
	TNF-α	11.01±0.13	5.99±0.09***	45.6↓
	Nitric oxide	37.5±1.12	15.4±2.19***	58.9↓
Butanol extract of Marrubium vulgare L(1mg/ml.)	IFN-γ	390±6.12	222±4.99***	43.1↓
	TNF-α	10.66±0.04	5.300±0.02***	50.3↓
	Nitric oxide	39.5±1.34	27.4±1.55***	30.6↓

† Insignificant difference from the corresponding control at P > 0.1: * Significant difference from the corresponding control at P < 0.05: ** Highly sig. difference from the corresponding control at P < 0.01: *** Very highly sig. difference from the corresponding control at P < 0.001:↑ Increase:↓ Decrease.

References

1. **Ahmed A. Elberry, Fathalla M. Harraz, Salah A. Ghareib, Salah A. Gabr, Ayman A. Nagy, Essam Abdel-Sattar(2011):** Methanolic extract of Marrubium vulgare ameliorates hyperglycemia and dyslipidemia in streptozotocin-induced diabetic rats. Int J Diab Mellitus, doi:10.1016 \ j.ijdm. 2011.01.004.

2. **Blumenthal M., Goldberg A., Brinckmann J., (eds.) (2000):** Herbal Medicine. Expanded commission E Monographs. American Botanical Council. Integrative Medicine Communications, Newton MA.

3. **Carlsson O-P, flodsrom M, and Sandler S (2000):** Islet blood flow in multiple low dose streptozotocin-treated wild-type and inducible nitric Oxide synthase-deficient mice. Endocrinology, 141: 2752-2757.

4. **Chase, C. I. (1967):** Statistical analysis of data using "t" Test according to the degree of freedom. Elementary statistical procedures. McGrow Hill. Book Comp. 9, p 140.

5. **Corbett JA, Wang JL, Sweetland MA, Lancaster JR, and McDaniel ML (1992):** IL-1 beta induces the formation of nitric oxide by beta-cells purified from rodent islets of Langerhans: Evidence for the beta-cell as a source and site of action of nitric oxide. J Clin Invest., 90: 2384-2391.

6. **Corbett JA, Sweetland MA, Wang JL, Lancaster JR, and McDaniel ML (1993):** Nitric oxide mediates cytokine-induced inhibition of insulin secretion by human islets of langerhans. Proc Natl Acad Sci USA, 90: 1731-1735.

7. **Corbett JA and McDaniel ML (1995):** Intraislet release of interleukin-1 inhibits beta cell function by inducing beta expression of inducible nitric oxide synthase. J Exp Med., 181: 516-526.

8. **De Jesus, R. A., Cechinel-Filho, V., Oliveira, A. E., and Schlemper, V. (2000):** Analysis of the antinociceptive properties of marrubiin isolated from Marrubium vulgare. *Phytomedicine*;7(2):111-115.

9. **Delaney CA, Pavlovic D, Hoorens A, Pipeleers DG, and Eizirik DL (1997):** Cytokines induce deoxyribonucleic acid strand breaks and apoptosis in human pancreatic islet cells. Endocrinology, 138: 2610-2614.

10. **De Souza MM, De Jesus RA, Cechinel-Filho V, and et al. (2009):** Analgesic profile of hydroalcoholic extract obtained from *Marrubium vulgare.Phytomedicine* ;5(2):103-107.

11. **Edwan J., Siddaheswar B.J., and Dharam C.J., (2008):** Diabetes and Herbal Medicines. *IJPT* (7):97-106.

12. **Eizirik DL, Flodstrom M, Karslen AE, and Welsh N (1996):** The harmony of the spheres: inducible nitric oxide synthase and related genes in pancreatic beta cells. Diabetologia, 39: 875-890.

13. **El Bardai S., Lyoussi B., Wibo M., Morel N., (2001):** Pharmacological evidence of hypotensive activity of Marrubium vulgare and Foeniculum vulgare in spontaneously hypertensive rat. *Clini.and Exp. Hypertension. (*23):329-343.

14. **El Bardai S, Wibo M, Hamaide MC, Lyoussi B, Quetin-Leclercq J, Morel N (2003).** Characterisation of marrubenol, a diterpane extracted from *Marrubium vulgare*, as an L-type calcium channel blocker. *Br. J. Ethnopharmacol*. 140 (7): 1211-1216.

15. **El Bardai, S., Lyoussi, B., Wibo, M., and Morel, N. (2004):** Comparative study of the antihypertensive activity of Marrubium vulgare and of the dihydropyridine calcium antagonist amlodipine in spontaneously hypertensive rat. *Clin Exp Hypertens*; 26(6):465-474.

16. **Elias D, Prigozin H, Polak N, Rapoport M, Lohse AW, and Cohen IR (1994):** Autoimmune diabetes induced by the β-cell toxin STZ: immunity to the 60-KDa heat shock protein and to insulin. Diabetes, 43: 992-998.

17. **Fehsel K, Korncke KD, and Kolb-Bachofen V (1995):** The action of NO and its role in autoimmune diabetes mellitus. Res Immunol., 146: 711-715.

18. **Finely, P. R. (1978):** Enzymatic colorimetric determination of serum total cholesterol. Clin. Chem. 24:391.

19. **Flodstorm M, Tyrberg B, Eizirik DL, and Sandler S (1999):** Reduced sensitivity of inducible nitric oxide synthase-deficient mice to multiple low-dose streptozotocin-induced diabetes. Diabetes, 48: 706-713.

20. **Fraser RB, Rowden G, Colp P, and Wright JR (1997):** Immunophenotyping of insulitis in control and essential fatty acid deficient mice

treated with multiple low-dose streptozotocin. Diabetologia, 40: 1263-1268.

21. **Gepts W and In'tveld PA (1987):** Islet morphological changes. Diabetes Metab Rev., 3: 589-572.

22. **Halliwell, B. and Gutteridge, J.M.C. (2010):** The importance of free radicals and catalytic metal ions in human diseases. Mol. ASP. Med. 8: 89.

23. **Halliwell B (2008).** Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and in vivo studies? _Arch. Biochem Biophys_. 476: 107-112.

24. **Helmchen U, Schmidt WE, Siegel EG, and Creutzfeldt W (1984):** Morphological and functional changes of pancreatic B cells in cyclosporine A-treated rats. Diabetologia, 1984: 27(3): 416-418.

25. **Herold KC, Montag A, and Fitch FW (1987):** Treatment with anti-T-lymphocytes antibodies prevents induction of insulitis in mice given multiple low doses of streptozotocin. Diabetes, 36: 796-801.

26. **Herold KC, Montag AG, Meeyer SM, Wojcikowski C, and Fitch FW (1990):** Expression of Ly-6c by T lymphocytes of NOD mice after CD3-complex stimulation. Diabetes, 39: 815.

27. **Herold KC, Vezys V, Sun Q, Viktora D, Seung E, Reiner S, and Brown D (1996):** Regulation of cytokine production during development of autoimmune diabetes induced with multiple low doses of streptozotocin. J Immunol., 156: 3521-3527.

28. **Herrera A.A., Aguilar S.L., Garc B.H., Nicasio T.P., and Tortoriello J. (2004):** Clinical trial of cecropiaobtusi folia and Marrubium vulgare leaf extracts on blood glucose and serum lipids in type 2 diabetes. _Phyto med_. (11):561-6.

29. **Ho E and Bray T (1999):** Antioxidants, NF-κB activation, and diabetogenesis. P.S.E.B.M., 222: 205-213.

30. **Itoh N, Hanafusa T, Miyazaki A, Miyagawa H, Yamagata K, Yamamoto K, Waguri M, Imagawa A, Tamura S, Inada M, Kawata S, Tarui S, Kono N, and Natsuzawa Y (1993):** Mononuclear cell infiltration and its relation to the expression of major histocompatibility complex antigens and adhesion molecules in pancreas biopsy speciments from newly diagnosed insulin-dependent diabetes mellitus patients. J Clin Invest., 92: 2313.

31. **Iwakiri R, Nagafuchi S, Kounoue E, Nakano S, Koga T, Nakayama M, Nakamura M, and Niho Y (1987):** Cyclosporin A enhances streptozotocin-induced diabetes in CD-1 mice. Experientia, 43: 324-327.

32. **Khanavi M., Ghasemian L., Motlagh E.H., Hadjiakhoondi A., Shafiee A. (2005):** Chemical composition of the essential oils of _Marrubium parviflorum_ fisch and C.A.Mey. and _Marrubium vulgare L._ from Iran. Flavor and fragrance Journal(20): 324-326.

33. **Kolb H, Kicscel U, Kroncke KD, and Kolb-Bachofen V (1991):** Suppression of low-dose streptozotozin induced diabetes in mice by administration of nitric oxide synthase inhibitor. Life Sci., 49: L213-L217.

34. **Kroncke KD, Fehsel K, Sommer A, Rodriguez ML, and Kolb-Bachofen V (1995):** Nitric oxide generation during cellular metabolization of the diabetogenic N-methyl-N-nitroso-urea streptozotozin contributes to islet cell DNA damage. Biol Chem

35. **Leiter EH and Serreze DV (1991):** Autoimmune diabetes in the nonobese diabetic mouse: suppression of immune defects by bone marrow transplantation and implications for therapy. Clin Immunol Immunopathol., 59: 323.

36. **LisaR.W.P., Traci P., Bradley C.B., and Cassandra L.Q., (2008):** Effects of extracts from Italian medicinal plants on planktonic growth, biofilm formation and adherence of methicillin-resistant Staphylococcus aureus. _J.of Ethnopharmacology_. (118):418-428.

37. **Liu D, Papavolic D, Chen MC, Flodstrom M, Sandler S, and Eizirik DL (2000):** Cytokines induce apoptosis in β-cells isolated from mice lacking the inducible isoform of nitric oxide synthase (iNO$^{-/-}$). Diabetes, 49: 1116-1122.

38. **Lyons TJ. (1991):** Oxidized low density lipoproteins: a role in the pathogenesis of atherosclerosis in diabetes? Diabet Med ;8:411–9.

39. **Maclaren NK, Neufeld M, McLaughlin JV, and Taylor G (1980):** Sensitization of streptozotocin-induced diabetes in mice. Diabetes, 29: 710-716.

40. **Mahmoud M., Saeed M.M., Abdolbaset G., and Farzaneh N. (2005):** Labiatae family in

folk medicine in Iran. *Iranian J. of pharmaceutical Res*. (2); 63-79.

41. **Mandrup-Poulsen T (1996):** The role of interleukin-1 in the pathogenesis of IDDM. Diabetologia, 39: 1005-1029.

42. **Martin-Nizard, F., Sahpaz, S., Kandoussi, A., Carpentier, M., Fruchart, J. C., Duriez, P., and Bailleul, F. (2004):** Natural phenylpropanoids inhibit lipoprotein-induced endothelin-1 secretion by endothelial cells. *J Pharm Pharmacol* ;56(12):1607-1611.

43. **Miranda KM, Espey MG, and Wink DA (2001):** A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric oxide: biology and chemistry, 5: 62-71.

44. **Mordes JP, Greiner DL, and Rossini AA (2000):** Animal models of autoimmune diabetes mellitus. In: Diabetes mellitus a fundamental and clinical text, Le Roith D, Taylor SI and Olefsky JM, Eds. Lippincott Williams and Wilkins, Philadelphia, USA, pp: 430-441.

45. **Muri A, Rovin BH, Lacy BE, and Schreiner GF (1991):** Macrophage-specific chemotactic lipid release by in vivo streptozotocin-administration in mouse islets. Diabetes, 40: 1459-66.

46. **Natural Standard (2011):** (www.naturalstandard.com)

47. **Novaes AP, Rossi C, Poffo C, Pretti JE, Oliveira AE, SchlemperV, et al. (2001):** Preliminary evaluation of the hypoglycemic effect of some Brazilian medicinal plants. Therapie;56:427–30.

48. **Papaccio G, Ammendole E, and Pisanti FA (1999):** Nicotinamide decreases MHC class II but not MHC class I expression and increases intercellular adhesion molecule-1 structures in non-obese diabetic mouse pancreas. J Endocrinol., 160: 389-400.

49. **Patrycja T., Emilia S., and Adam M., (2008):** Antioxidant activity of herb extracts from five medicinal plants from Lamiaceae, subfamily Lamioideae.*J.of Med.Plant Res*. Vol.2 (11):321-330.

50. **Reasner CA. (2008):** Reducing cardiovascular complications of type 2diabetes by targeting multiple risk factors. J Cardiovasc Pharmacol ;52:136–44.

51. **Rohman, A., Riyanto, S., Yuniarti, N., Saputra, W.R., Utami, R. (2012):** Antioxidant activity, total phenolic, and total

flavaonoid of extracts and fractions of red fruit (*Pandanus conoideus* Lam). *Int. Food Res. J.* **17**, 97-106.

52. **Roman, Ramos R**.,**Alarcon-Aguilar,F., Lara-Lemus,A., and Flores-Saenz,J.L.(1998):** Hypoglycemic effect of plants used in Mexico as antidiabetics. *Arch Med Res*; 23(1):59-64.

53. **Rossini AA, Appel MC, Williams RM, and Like AA (1977):** Genetic influence of the streptozotocin-induced insulitis and hyperglycemia. Diabetes, 26: 916-920.

54. **Rowan Hillson (1996):** Late Onest Diabetes. Vermilion an imprint of Ebury press; 16-17.

55. **Sahpaz, S., Garbacki, N., Tits, M., and Bailleul, F. (2002):** Isolation and pharmacological activity of phenylpropanoid esters from Marrubium vulgare. *J Ethnopharmacol* ; 79(3):389-392.

56. **Sai P, Maugendre D, Loreal O, Maurel C, and Pogu S (1988):** Effects of cyclosporin on autoimmune diabetes induced in mice by streptozotocin: beta cell-toxicity and rebound of insulitis after cessation of treatment. Diabetes Metab., 14(4): 455-62.

57. **Santamaria P, Nakhleh RE, Sutherland DER, and Barbosa JJ (1992):** Characterization of T lymphocytes infiltrating human pancreas allograft affected by isletitis and recurrent diabetes. Diabetes, 41: 53-61.

58. **Scheen JA. (1997):** Drug treatment of non-insulin dependent diabetes mellitus in the 1990s. Achievements and future development.Drugs. 54:355–68.

59. **Serreze DV, Gaskins HR, and Leiter EH (1993):** Defects in the differentiation and function of antigen presenting cells in NOD/Lt mice. J Immunol., 150: 2534.

60. **Sestier C, Odent-Pogu S, Bonneville M, Maurel C, Lang F, and Sai P (1985):** Cyclosporin enhances diabetes induced by low-dose streptozotocin treatment in mice. Immunol Lett., 10(1): 57-60.

61. **Somoza N, Vargas F, Roura-Mir C, Vives-Pi M, Fenandez Figueras MT, Ariza A, Gomis R, Bragado R, Marti M, Jaraquemada D, and Pujol-Borrell R (1994):** Pancreas in recent onset insulin-dependent diabetes mellitus. J Immunol., 153: 1360.

62. **Suarez-Pinzon W, Sorensen O, Bleackley RC, Elliott JF, Rajotte RV, and Rabinovitch A (1999):** β-cell destruction in

NOD mice correlates with Fas (CD95) expression on β-cells and proinflammatory cytokines expression in islets. Diabetes, 48: 21-28.

63. **Tabatabaie T, Vasquez-Weldon A, Moore DR, and Kotake Y (2003):** Free radicals and the pathogenesis of type 1 diabetes: β-cell cytokine-mediated free radical generation via cyclooxygenase-2. Diabetes, 52: 1994-1999.

64. **Takamura T, Kato I, Kimura N, Nakazawa T, Yonekura H, Takasawa S, and Okamoto H (1998):** Transgenic mice overexpressing type 2 nitric oxide Synthase in pancreatic β-cells develop insulin-dependant diabetes without insulitis. J Biol Chem., 273: 2493-2496.

65. **Takeda, Y., Yanagihara, K., Masuda, T., Otsuka, H., Honda, G., Takaishi, Y., Sezik, E., and Yesilada, E. (2000):** Labdane diterpenoids from Marrubium globosum ssp. globosum. *Chem Pharm Bull (Tokyo)* 48(8):1234-1235.

66. **Thomas HE, Darwiche R, Corbett JA, and Kay TW (2002):** Interleukin-1 plus gamma-interferon-induced pancreatic beta-cell dysfunction is mediated by beta-cell **n**itric oxide production. Diabetes, 51(2): 311-316.

67. **Trinder P (1969):** Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. Annals Clin Biochem., 6: 24.

68. **Vinik AI, Vinik E. (2003):** Prevention of the complications of diabetes. Am J Manage Care; 9:S63–80.

69. **Weel K.G.C., Venskutonis P.R., Pukalskas A., Gruzdiene D., Linssen J.P.H. (1999):** Antioxidant activity of horehound (Marrubium vulgare L.) grown in Lithuania European Journal of lipid science and Technology (101):395-400.

70. **Wolffe SP, Jiang ZY, Hunt JV. (1991):** Protein glycation and oxidative stress in diabetes mellitus and ageing. Free Radic Biol Med;10:339–52.

71. **Wolski T, Matosiuk D, Baj T, Ziewiec A (2007).** White horehound (*Marrubium vulgare* L.) medicinal plant with multidirectional pharmacological activity. *Post. Fitoterapii* 8: 39-45.

72. **Wright JR, Fraser RB, Kapoor S, and Cook HW (1995):** Essential fatty acid deficiency prevents multiple low-dose streptozotocin-induced diabetes in naive and cyclosporine-treated low-responder murine strains. Acta Diabetologica, 32: 125-130.