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ORIGINAL ARTICLE

Experimental evaluation of a polyherbal formulation (Tetraherbs): antidiabetic efficacy in rats

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Abstract

Cinnamon (*Cinnamomum zeylanicum*), fenugreek (*Trigonella foenum-graecum*), shallot (*Allium hirtifolium* Boiss), and clove (*Syzygium aromaticum*) are well-known and widely used medicinal plants in the world. There is some experimentally proven evidence demonstrated the beneficial effects of these herbs on metabolic disorders like diabetes. Despite the fact that there are limited studies in the field of herb-herb interactions, some of the diabetic patients prefer to use these herbs in a combination form. The aim of this study was to evaluate the possible effects of an equal mixture of these plants (named Tetraherbs) in streptozotocin (STZ)-induced diabetic rats. Severe diabetic rats (FBS above 350 mg/dL) were treated orally with ethanolic extracts of cinnamon (EEC), fenugreek (EEF), shallot (EES), and clove (EECL) separately at the dose of 75 mg/kg, or in equal combination formula (Tetraherbs) at doses of 100–300 mg/kg once per day for 28 days. At the end of the study, their effects on glucose levels, plasma lipids, liver enzymes activity, and histology of pancreas were evaluated. The blood glucose lowering activity, as well as pancreatic β cell regeneration of Tetraherbs, was significantly higher than the plants when they used separately. However, there was no significant difference in lipid-lowering and hepatoprotective potentials of the herbs whether used singly or in combination. In conclusion, the results of the present study provide evidence show combination form of the studied plants increased their glucose-lowering activity by positive interaction.

Keywords Cinnamon · Fenugreek · Shallot · Clove · Tetraherbs · Diabetes

Introduction

Diabetes mellitus is defined as a group of chronic metabolic disorders characterized by persistent hyperglycemia resulting from a complete or relative lack of insulin secretion or action (Cole and Kramer 2016). The evidence demonstrated that persistent hyperglycemia may cause long-term complications including microvascular, macrovascular, and neuropathic alterations (Hami et al. 2016; Hassanzadeh-Taheri et al. 2016; Lotfi et al. 2016; Zarezadeh et al. 2017). According to the latest report of International Diabetes Federation, globally 8.8% of people aged 20–79 years had diabetes and approximately 5 million died from the disease in 2015, equivalent to one death every 6 seconds. If these trends continue, 642 million people, or one adult in ten, will have diabetes by 2040 (Ogurtsova et al. 2017).

The main strategies for the prevention and management of diabetes are lifestyle modifications including nutritional changes, physical activity, and use of different glucose-lowering medications (Gillies et al. 2007). However, despite these approaches, failure in control of glucose hemostasis occurs frequently among diabetic patients (Kokil et al. 2015). Moreover, due to some reasons such as popular belief about side effects of oral hypoglycemic drugs, and their low efficacy in managing all aspects of diabetes, scientists encouraged to explore and evaluate new methods to control the disease and its complications (Ekor and Pistelli 2013; Abtahi-Evari et al. 2017; Hassanzadeh-Taheri et al. 2018a).

In more recent years, the concepts of complementary and alternative medications (CAMs) have expanded worldwide tremendously. Among the multiple categories of CAMs, herbal medicine and nutraceuticals gained more popularity

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(Pandey et al. 2004). According to Iranian traditional medicine, the first step for treatment of any kind of disease is a lifestyle change, and the second step is the more advanced treatments such as using drugs or surgery. In sequence, recommended treatments often consist of proper diet, drugs (from single plants or mineral), followed by the combination of different herbs (Dabaghian et al. 2012). There are several polyherbal formulas for the treatment of diabetes in Iranian traditional medicine. Some of the medicinal herbs such as cinnamon bark (*Cinnamomum zeylanicum*), fenugreek seed (*Trigonella foenum-graecum*), shallot bulb (*Allium hirtifolium* Boiss), and clove flower bud (*Syzygium aromaticum*) are frequently used in different antidiabetic polyherbal formulas (Medagama and Bandara 2014; Katiyar et al. 2015; Yadav et al. 2015).

The hypoglycemic effects of cinnamon and fenugreek have been proposed by some molecular mechanisms such as decreasing gluconeogenesis, antioxidant effects, increasing glucose uptake, and direct insulin-like activities (Raju et al. 2001; Lu et al. 2012). Also, it is suggested that clove has antidiabetic potential through insulin-like activity, decreasing expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6 phosphatase, and human peroxisome proliferator-activated receptor (PPAR)-γ ligand-binding activity (Kuroda et al. 2012). Moreover, there are some scientific reports regarding hypoglycemic effects of shallot in animal models (Mahmoodi et al. 2013a).

Due to different mechanisms of action and inconsistent reports of hypoglycemic activity of each plant, the aim of the present study was to investigate the probable synergistic or toxic effects of an equal mixture of ethanolic extracts of these four plants named here Tetraherbs on blood glucose, plasma lipids, liver enzymes, and pancreas histology in streptozotocin-induced diabetic rats.

Materials and methods

Preparation of plant extracts

The inner bark of cinnamon, the seed of fenugreek, the bulb of shallot, and the flower buds of clove were purchased from the traditional market in Birjand, Iran. The genus and species of the plants were authenticated by an expert botanist (Department of Botany, University of Birjand, Iran). The plants' parts were washed, dried in shade and room temperature, powdered, and then stored in a cool and dry condition until use.

To prepare ethanolic extracts, a 100 g of each powdered plants organs was macerated in 1000 mL of 80% ethanol with constant stirring for 48 h at room temperature. Afterwards, the mixture was passed through a filter paper (Blue Ribbon, Grade 589, Germany), concentrated under a vacuum evaporator, and lyophilized using a freeze-dryer (Dena Vacuum Industry, model FD-5005-BT, Iran) (Hassanzadeh-Taheri et al. 2018b).

Diabetes induction

All of the animal experiments were approved and conducted in accordance with the guide for the laboratory animals' care and usage of Birjand University of Medical Sciences, Birjand, Iran (IR.BUMS.1394.373).

Two-month-old male Wistar rats were housed in a temperature-controlled room (25 °C) with a 12-h dark/light cycle and free access to commercial laboratory animal food and tap water during the study period. Diabetes was induced by a single intraperitoneal (IP) injection of streptozotocin (STZ) (Sigma, 55 mg/kg dissolved in citrate buffer, pH 4.5) in the 14-h fasted rats (Vafaei-Nezhad et al. 2016). Age-matched control animals received an IP injection of vehicle (citrate buffer). After 72 h of the injection, animals with plasma glucose concentration above than 350 mg/dL were considered as severe diabetic (Fard et al. 2015).

Experimental design

All diabetic animals were left untreated for 4 weeks to develop a complicated form of diabetes (Davidson et al. 2011). Diabetic rats were randomly divided into nine equal ($n = 8$) groups while eight age-matched healthy rats were allocated as a normal control group. All groups were orally treated once per day with an equal volume of solutions (1 mL) for consecutive 28 days. Normal control group (NC) and diabetic model group (DM) were treated with saline 0.9%. Metformin (Merck, dissolved in saline) was administrated as a reference drug to another diabetic group at the dose of 500 mg/kg (MET500) (Waisundara et al. 2009). The seven remained diabetic groups were treated with ethanolic extracts of cinnamon (EEC), fenugreek (EEF), shallot (EES), and clove (EECL) at the dose of 75 mg/kg singly, or in equal combination (Tetraherbs) at the doses of 100 mg/kg (Tetraherbs 100), 200 mg/kg (Tetraherbs 200), and 300 mg/kg (Tetraherbs 300), respectively.

Biochemical analysis

At 29th day and after 24 h of the last treatment, the rats were anesthetized by IP injection of ketamine: xylazine (65:10 mg/kg) (Hassanzadeh-Taheri et al. 2018) and immediately their blood specimens were collected. The blood samples were centrifuged (3000*g* for 10 min) and the plasma collected. Fasting blood glucose and lipid profile including total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-c), and low-density lipoprotein cholesterol (LDL-c) were assessed and liver enzymes including aspartate

aminotransferase (AST) and alanine aminotransferase (ALT) were evaluated using Pars Azmoon standard kits (Iran) and Roche Hitachi 912, auto-analyzer (Japan).

Oral glucose tolerance test in diabetic rats

In a complementary study, 30 diabetic and 6 healthy rats were divided into other six equal groups. Oral glucose tolerance test (OGTT) was carried out as follows:

Group I - Normal control received 0.9% saline
Group II - Diabetic control received 0.9% saline
Group III- Diabetic rats received metformin (500 mg/kg)
Group IV - Diabetic rats treated with 100 mg/kg of the Tetraherbs
Group V - Diabetic rats treated with 200 mg/kg of the Tetraherbs
Group VI- Diabetic rats treated with 300 mg/kg of the Tetraherbs

Thirty minutes after treatment with the solvent, reference drug (MET) and Tetraherbs, an oral glucose solution (Sigma, US) was administered at the dose of 3 g/kg (Ghiravani et al. 2016). Blood glucose was measured before and at 30, 60, 90, and 120 minutes after oral glucose loading using a glucometer (Accu-Check Active, Roche).

Histology

Immediately after blood collection, the animals were transcardially perfused with cold 0.9% saline, followed by a fixative solution containing 4% paraformaldehyde and 0.1 M glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Their pancreases were rapidly dissected, post-fixed in the same fixative solution for 48 h at 4 °C. The tissues were processed by routine histological methods and embedded in paraffin blocks. The specimens were sectioned (5-um thickness) using a sliding microtome (Leitz, Italy), mounted on poly-L-lysine coated slides, and stained with Hematoxylin and Eosin (H-E) dyes. For each rat, three random slides (9 sections) were analyzed under a light microscope (UPLAN FI, Japan) in a blinded manner. The morphometric parameters of pancreatic islets including the number of islets, area, and diameters of islets as well as the number of cells per islet were measured quantitatively by using Image J software (1.44p; National Institute of Health, USA).

Statistical analysis

Data were expressed as mean ± SEM. Experimental groups were compared using the ANOVA variance analysis test, and a post-hoc analysis using Tukey's test was performed. Values of $p < 0.05$ were considered statistically significant. SPSS for Windows (SPSS, Chicago, IL, USA) was used to perform all statistical analyses.

Results

Hypoglycemic activity

In diabetic rats, STZ injection caused 4-fold increase in blood glucose levels. Before starting the treatments, all the animals had well-developed signs of diabetes (polyuria, polyphagia, polydipsia, and weight loss). Table 1 shows the changes in body weight as well as fasting blood glucose (FBG) levels in all studied groups.

Table 1 Effects of single administration of ethanolic extracts of cinnamon (EEC), fenugreek (EEF), shallot (EES), and clove (EECL) or their equal combination (Tetraherbs) on body weight and fasting blood glucose levels in STZ-induced diabetic rats

Groups (mg/kg)	Body weight (g)		Fasting blood glucose (mg/dL)	
	1st day	28th day	1st day	28th day
Normal control	203.00 ± 1.59††	253.00 ± 5.87††	94.50 ± 1.45††	95.87 ± 1.04††
Diabetic model	147.25 ± 5.11**	151.37 ± 7.11**	497.75 ± 18.61**	497.62 ± 48.39**
Metformin (500)	149.00 ± 4.15**	161.87 ± 6.80**	505.87 ± 28.22**	111.62 ± 16.09††
EEC (75)	143.50 ± 7.21**	155.00 ± 13.56**	469.37 ± 55.62**	237.75 ± 49.56** ††
EEF (75)	154.62 ± 19.82**	147.62 ± 13.52**	501.12 ± 87.47**	306.75 ± 41.93** ††
EES (75)	149.25 ± 21.31**	160.25 ± 20.98**	439.37 ± 71.77**	271.50 ± 45.19** ††
EECL (75)	149.62 ± 11.09**	159.25 ± 13.51**	483.50 ± 91.24**	304.37 ± 21.50** ††
Tetraherbs (100)	151.37 ± 7.11**	153.25 ± 5.32**	442.87 ± 27.79**	250.75 ± 61.74* ††
Tetraherbs (200)	145.25 ± 1.67**	137.50 ± 2.63**	502.00 ± 21.98**	262.62 ± 67.83* ††
Tetraherbs (300)	152.25 ± 4.15**	160.18 ± 7.36**	463.50 ± 29.44**	144.75 ± 36.32††

The data present the mean ± S.D. ($n = 8$). In experimental groups, significantly different at *$p < 0.05$, **$p < 0.001$ when compared to normal control group and †$p < 0.05$, ††$p < 0.001$ when compared to the diabetic model group in each studied time points

The hypoglycemic activity of Tetraherbs was assessed by measuring FBG levels in initial and the last day of the treatments. As shown in Table 1, the FBG concentration of STZ-induced diabetic rats was significantly higher ($p < 0.001$) than the NC group in both 1st and 28th days of study. Moreover, before treatment (at 1st day), the FBG levels of the nine diabetic groups (DM, MET, EEC, EEF, EES, EECL, Tetraherbs 100, 200, and 300) were almost identical and there was no significant difference between them ($p > 0.05$). However, at the end of the study, all the investigations significantly decreased ($p < 0.001$) FBG levels in diabetic animals when compared to the DM group. Both of Metformin and Tetraherbs (300 mg/kg) exhibited the highest glucose-lowering activity in diabetic animals significantly more than the other investigations. There was no significant difference between MET500 and Tetraherbs 300 in FBG level (111.62 ± 16.09, 144.75 ± 36.32, respectively).

Effect of Tetraherbs on OGTT

A graph of blood glucose levels (mg/dL) versus time (min) was constructed, and the area under the curve (AUC) was calculated according to the trapezoidal rule. The AUCs of each group were compared with the DM group, to represent glucose utilization by tissues.

Figure 1 illustrates the blood glucose levels of STZ-induced diabetic and non-diabetic rats. The diabetic groups had a significant elevation in blood glucose level throughout the total measurement period (120 min) compared to the NC group.

The AUC of each group was compared with the DM group. The results are presented in Table 2. Compared to the DM group, MET ($p = 0.007$), Tetraherbs 200 ($p = 0.04$), and Tetraherbs 300 ($p = 0.014$) could significantly decrease AUC in diabetic rats. These reductions were 54.87, 33.49, and 54.94%, respectively. Treatment with Tetraherbs at the dose of 100 mg/kg could not effectively (13.43%; $p = 0.32$) reduce AUC.

Hypolipidemic activity

At the end of the study, diabetic rats had significantly elevated plasma TG, TC, and LDL-c levels compared to the NC group ($p < 0.001$, each). However, there was no significant difference in HDL-c levels between the DM and NC groups.

In diabetic animals, the mean of TG concentration was about 62% higher than normal rats (65.00 ± 13.69 vs. 40.33 ± 3.16, $p = 0.003$). Treatment with EEC (46.16 ± 2.31, $p = 0.04$) and Tetraherbs 200 (44.57 ± 10.50, $p = 0.019$) decreased TG elevation in diabetic rats significantly.

In the DM group, the TC level was about 2-fold higher than the NC group (133.33 ± 25.64 vs. 55.66 ± 13.27, $p < 0.001$). All the investigations could decrease TC elevation in diabetic rats significantly ($p < 0.05$). There was no significant difference in TC-lowering activity between the herbs whether used singly or in combination.

Similar to cholesterol, LDL-c levels increased about 2-fold in diabetic rats compared to the NC group (59.66 ± 21.25 vs. 28.83 ± 4.21, $p < 0.001$). All the herbs significantly decreased LDL-c levels in diabetic rats when used singly. However, their combination (Tetraherbs) only at the dose of 200 mg/kg could ameliorate LDL-c levels in diabetic rats significantly ($p < 0.001$) (Table 3).

Liver enzymes activity

The effects of extracts on AST and ALT levels in diabetic rats are shown in Table 4. Compared to the NC group, plasma levels of AST and ALT significantly increased ($p < 0.001$) in diabetic rats. Diabetic animals had 130% greater AST levels than normal animals (260.83 ± 38.81 vs. 113.16 ± 16.33, $p < 0.001$). While all the investigations decreased the elevated AST levels in diabetic rats significantly ($p < 0.05$), the AST

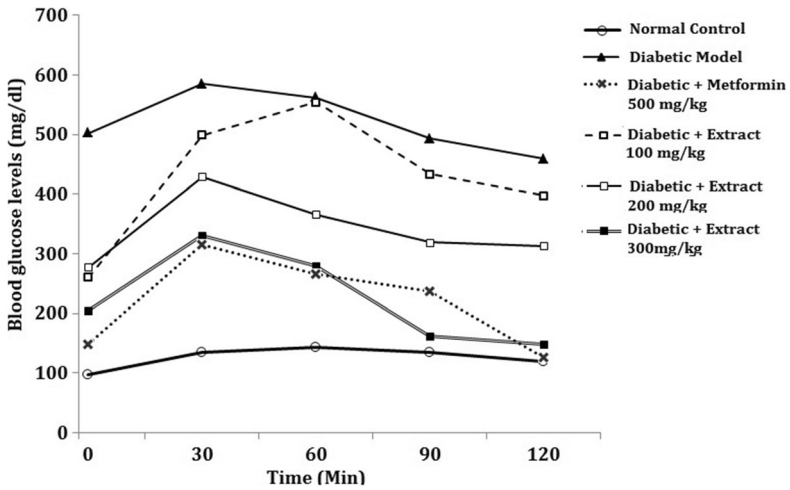

Figure 1 Changes in blood glucose level (mg/dL) during the oral glucose tolerance test (OGTT) of diabetic rats treated with different investigations for 4 weeks. Values are expressed as mean ± S.D. ($n = 6$)

Table 2 Total area under the curve (AUC) after glucose loading in diabetic rats pretreated with the tested extracts or metformin for 4 weeks

Groups (mg/kg)	Total AUC (mg/dL min)	Reduction (%)
Normal control	15,617.50 ± 227.53	–
Diabetic model	63,597.50 ± 971.37	0.00
Metformin (500)	28,697.50 ± 864.65††	54.87
Tetraherbs (100)	55,052.50 ± 928.35	13.43
Tetraherbs (200)	42,292.50 ± 3039.96†	33.49
Tetraherbs (300)	28,650.00 ± 916.31†	54.94

The data present the mean ± SEM ($n = 6$). In experimental groups, significantly different at †$p < 0.05$, ††$p < 0.001$ when compared to the diabetic model group

levels of the MET and EES groups were still remained higher than the NC group.

ALT levels of diabetic animals significantly elevated (2.5-fold) compared to the NC group (132.50 ± 11.79 vs. 49.33 ± 5.71, $p < 0.001$). Like AST, all the investigations could ameliorate elevated ALT levels in diabetic rats when compared to the DM group. However, only EEF and Tetraherbs at the doses of 200 mg/kg could normalize ALT levels in diabetic rats.

Pancreas histology

Figure 2 illustrates the pancreatic sections of the studied groups. Figure 2a demonstrates a normal Langerhans' islet with normal appearance. However, sections of the diabetic groups showed the damaged islet, represented by a reduction in the size, shrinkage, marked reduction in cell density, and remaining cells showed small degenerated features such as dark and pycnotic nuclei, and also fibrosis and vacuolation were observed in the islets (Fig. 2b). Only single treatment of EEC showed some signs of the islet improvement (Fig. 2d). Treatment with Tetraherbs in a dose-dependent manner exhibited a slight improvement in size, cell density, and fibrosis of Langerhans' islets in diabetic rats compared to the DM group (Fig. 2h–k). The results of the quantitative morphometric analysis are presented in Table 5. Tetraherbs in a dose-dependent manner significantly improved Langerhans' islet cell density, islets number, islets area, and diameter in diabetic rats compared to the DM group.

Discussion

In the present study, STZ-induced diabetic rats were treated orally with the ethanolic extract of cinnamon (EEC), fenugreek (EEF), shallot (EES), and clove (EECL) singly at the dose of 75 mg/kg or in equal combination together (Tetraherbs) at the three doses (100–300 mg/kg) for 28 consecutive days. At the end of the study, their effects on glucose levels, plasma lipids, liver enzymes activity, and pancreas histology were evaluated. The blood glucose-lowering activity, as well as pancreatic β cell regeneration of combination formula, was significantly higher than the plants used singly. However, there was no significant difference in lipid-lowering and hepatoprotective activities of the herbs whether used singly or in combination.

In the most of experimental studies, interventions are performed routinely in subacute form (Shukla et al. 2011). In rodents, subacute investigation refers to repeated exposures for 1 month or less, and chronic interventional assay refers to repeated exposures for more than 3 months (Hassanzadeh-Taheri et al. 2018b). Therefore, in the present study, we performed the investigation during 28 days to find the effective dose and also long-term effects.

In diabetic rats, STZ caused significant hyperglycemia, weight loss, and histological degeneration of pancreas as shown in previous reports (Mythili et al. 2004). Although, Tetraherbs dose-dependently decreased the elevated FBG levels in diabetic rats, its effect was lower than metformin at

Table 3 Effects of single administration of ethanolic extracts of cinnamon (EEC), fenugreek (EEF), shallot (EES), and clove (EECL) or their equal combination (Tetraherbs) on lipid profiles of diabetic rats

Groups (mg/kg)	TG (mg/dL)	TC (mg/dL)	LDL-c (mg/dL)	HDL-c (mg/dL)
Normal control	40.33 ± 3.16††	55.66 ± 13.27††	28.83 ± 4.21††	16.16 ± 0.47
Diabetic model	65.00 ± 13.69	133.33 ± 25.64	59.66 ± 21.25	17.33 ± 3.72
Metformin (500)	70.42 ± 17.43	73.42 ± 12.42†	38.71 ± 4.57 †	17.85 ± 2.73
EEC (75)	46.16 ± 2.31	58.83 ± 5.26††	24.33 ± 8.82††	17.33 ± 3.20
EEF (75)	51.33 ± 2.80	54.00 ± 7.04††	36.16 ± 3.60††	16.28 ± 2.08
EES (75)	45.33 ± 4.58†	63.50 ± 7.09††	27.16 ± 3.12††	16.33 ± 1.03
EECL (75)	59.16 ± 4.57	77.50 ± 5.89†	33.66 ± 3.01††	16.23 ± 1.36
Tetraherbs (100)	50.16 ± 4.70	73.50 ± 10.22†	45.66 ± 1.96	15.66 ± 1.03
Tetraherbs (200)	44.57 ± 10.50†	61.42 ± 11.77††	30.42 ± 5.59††	15.85 ± 2.03
Tetraherbs (300)	49.16 ± 15.03	68.33 ± 13.35††	46.50 ± 5.68	19.33 ± 3.26

The data present the mean ± SEM ($n = 8$). In experimental groups, significantly different at †$p < 0.05$, ††$p < 0.001$ when compared to diabetic groups

Table 4 Effects of single administration of ethanolic extracts of cinnamon (EEC), fenugreek (EEF), shallot (EES), and clove (EECL) or their equal combination (Tetraherbs) on plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity in diabetic rats

Groups (mg/kg)	AST (IU/L)	ALT (IU/L)
Normal control	113.16 ± 16.33††	49.33 ± 5.71††
Diabetic model	260.83 ± 38.81	132.50 ± 11.79
Metformin (500)	156.28 ± 36.37 †	86.42 ± 26.61††
EEC (75)	119.83 ± 6.49††	75.83 ± 3.48††
EEF (75)	112.00 ± 10.17††	70.00 ± 14.56††
EES (75)	145.33 ± 6.40††	98.83 ± 2.04††
EECL (75)	154.62 ± 45.88†	88.00 ± 3.03††
Tetraherbs (100)	186.00 ± 9.85†	78.16 ± 13.71††
Tetraherbs (200)	150.57 ± 18.93†	69.00 ± 8.24††
Tetraherbs (300)	148.00 ± 30.43†	73.66 ± 10.03††

The data present the mean ± SEM ($n = 8$). Significantly different at †$p < 0.05$ compared to the diabetic group

the doses of 100 and 200 mg/kg. However, Tetraherbs showed significant improvement in OGTT at the doses of 200 and 300 mg/kg and this effect was similar to that of metformin in diabetic rats. These findings were inconsistent to previous studies reporting hypoglycemic effects of these medicinal plants or their active ingredients including of Cinnamaldehyde, Eugenol, Trigonelline, and Isoalliin (as a single drug) in animal models or diabetic patients (Jalal et al. 2007; Lu et al. 2012; Adefegha and Oboh 2012; Farrokhfall et al. 2014). Nevertheless, there are conflicting data about cinnamon and its main ingredients such as Cinnamaldehyde and Procyanidins. Some studies reported that cinnamon lowers glucose level by increasing glucose transporters (GLUT) in myocytes and adipocytes (Lu et al. 2012), increasing insulin secretion (Hosni et al. 2017), downregulation of gluconeogenesis gene expression (Cheng et al. 2012), and also its hypolipidemic effects were reported (Ping et al. 2010). On the other hand, there is evidence demonstrated that cinnamon did not change fasting blood glucose, cholesterol, and LDL-c levels (Akilen et al. 2012). Hypoglycemic effects of fenugreek and

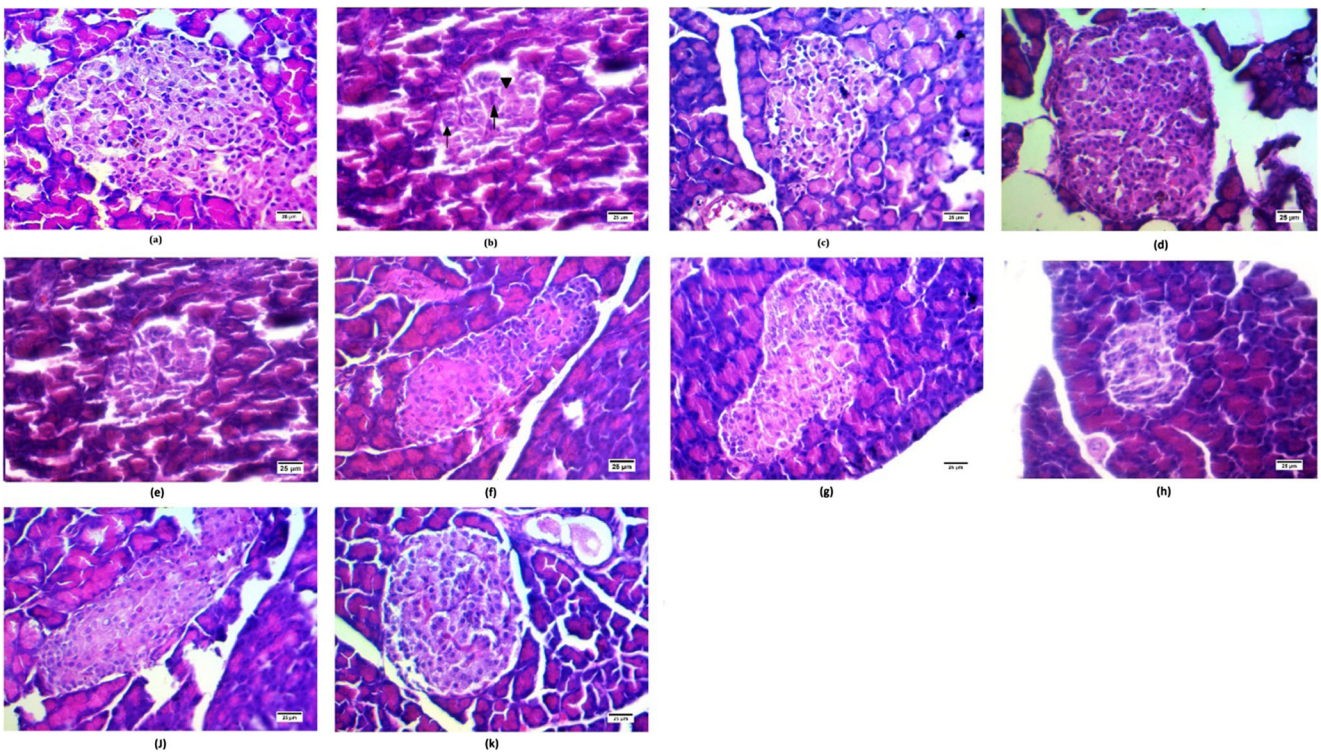

Figure 2 **a**–**k** Photomicrographs of H-E stained slides in the islets of Langerhans of each group, × 400 magnification, scale bar = 25 um. **a** Normal control rats. **b** Diabetic model rats. **c** Diabetic rats treated with metformin. **d** Diabetic rats treated with 75 mg/kg of ethanol extract of cinnamon. **e** Diabetic rats treated with ethanolic extract of fenugreek. **f** Diabetic rats treated with ethanolic extract of shallot. **g** Diabetic rats treated with ethanolic extract of clove. **h** Diabetic rats treated with 100 mg/kg of Tetraherbs. **j** Diabetic rat treated with 200 mg/kg of Tetraherbs. **k** Diabetic rat treated with 300 mg/kg of Tetraherbs. The pancreatic specimen of the diabetic model group showed an apparent decrease in cell density and fibrosis (arrowheads), and few cells looked dark and degenerated (arrows). The severity of these alterations generally reduced in metformin-treated diabetic rats. The extracts of fenugreek, shallot, and clove did not cause any significant improvement in pancreatic architecture compared to untreated diabetic rats. Tetraherbs at the doses of 100 and 200 mg/kg could not effectively ameliorate the pancreatic alterations. However, the general morphology of pancreatic islets in diabetic animals treated with 300 mg/kg of Tetraherbs was improved and showed quite a normal appearance

Table 5 Histomorphometric parameters of the pancreatic islet of different studied groups

Groups (mg/kg)	Number of islets (N/10 mm2)	Area of islets (mm2)	Diameter of islets (um)	Number of cells per islet (N/1000um2)
Normal control	21.13 ± 1.66††	0.0239 ± 0.0013††	205.73 ± 24.39††	16.32 ± 1.73††
Diabetic model	5.07 ± 0.66	0.0090 ± 0.0004	110.55 ± 22.15	4.33 ± 0.67
Metformin (500)	18.77 ± 7.11††	0.0143 ± 0.0019††	142.39 ± 24.13†	6.49 ± 0.53†
EEC (75)	13.26 ± 3.21†	0.0208 ± 0.0024††	185.50 ± 28.32††	17.20 ± 2.12††
EEF (75)	7.01 ± 2.26	0.0092 ± 0.0003	107.07 ± 13.26	7.11 ± 2.37†
EES (75)	8.61 ± 3.56	0.0138 ± 0.0092†	167.32 ± 19.42†	8.24 ± 1.23†
EECL (75)	9.93 ± 2.23†	0.0202 ± 0.0053††	151.25 ± 27.14†	7.03 ± 2.24†
Tetraherbs (100)	9.32 ± 2.37†	0.0092 ± 0.0043	90.42 ± 21.63	7.11 ± 2.42†
Tetraherbs (200)	20.13 ± 4.31††	0.0188 ± 0.0071†	178.45 ± 19.39††	10.28 ± 3.91††
Tetraherbs (300)	19.73 ± 1.06††	0.0197 ± 0.0040††	164.84 ± 15.28††	13.05 ± 2.49††

The data present the mean ± S.D. ($n = 8$). In experimental groups, significantly different at †$p < 0.05$, ††$p < 0.001$ when compared to the diabetic model group

its main ingredient Trigonelline have been investigated and several studies reported its beneficial effects on FBG, OGTT, lipids, and HBA1c (Hemoglobin A1c) in both animal and human studies by regeneration of beta cells, increasing of insulin secretion, and antioxidant effects (Deng 2012). Mechanisms of action of clove and its major ingredients including Eugenol, Eugenol acetate, and Caryophyllene have been evaluated in both in vitro and animal experiments and it has been proposed that inhibition of α-glucosidase and α-amylase, increasing muscle glycolysis, improving mitochondrial function, and especially decreasing gluconeogenesis are responsible for its beneficial effects on glucose, urea, liver enzymes, and lipid levels (Prasad et al. 2005; Jeong et al. 2014; Tu et al. 2014).

Unlike the other plants of Tetraherbs, shallot has not investigated extensively. Phytochemical studies showed that shallot has a high amount of sulfur compounds such as Diallyl disulfide, S-allyl cysteine, and Diallyl trisulfide which, generally claimed to be responsible for antihypertensive, hypolipidemic, and antithrombotic potentials of this plant (Kubec and Dadakova 2009). One study reported that shallot lowered FBG slightly and improved OGTT while had no effects on LDL-C and HDL levels and even increased total cholesterol and urea in rat (Mahmoodi et al. 2013a).

The effects of Tetraherbs on lipid profile in diabetic rats exhibited significant ameliorating effects on TG, TC, and LDL-c particularly at the dose of 200 mg/kg; however, the other doses had no efficient effects. Generally, the drug interaction would generally produce one of three different effects: synergism, antagonism, and additive effect. The dose-response relation is a key topic in pharmacology (Gu et al. 2015). Hence, it seems that the studied plants, in high dose, have antagonistic interaction together mainly in lipid lowering-activity. Metformin slightly decreased TC and LDL-c, while it had no noticeable effects on TG elevation. These findings were inconsistent with previous studies with the same experimental design (Hule et al. 2011).

In a study conducted by Zarvandi et al., polyherbal formulation consisted of 39.1% fenugreek (main compound) could efficiently decrease FBG, TG, and LDL-c levels in diabetic patients (Zarvandi et al. 2017).

The results of histopathological examination of pancreatic islets showed that in single form of the herbs, only cinnamon could efficiently improve damaged islets. However, as expected, administration of Tetraherbs in a dose-dependent manner efficiently ameliorated pancreatic alterations. The exact mechanism by which Tetraherbs regenerated damaged islets has not elucidated in this study, but some hypothesis can be put forward. Similar to our findings, some evidence showed that phytochemicals can regenerate β cells in STZ-induced diabetic rats. Earlier studies showed that single administration of fenugreek, cinnamon, shallot, and clove could regenerate or prevent pancreatic beta cells destructions induced by Alloxan or STZ in rodents (Li et al. 2013; Mahmoodi et al. 2013b; Srinivasan et al. 2014; Joshi et al. 2015). In most of these studies, antioxidant properties were proposed as probable mechanism involved in β cell regenerations.

AST and ALT are two main aminotransferases that have leaked into circulation and served as hepatocytes injury markers. In this study, single or concurrent administration of the studied herbs decreased the elevated levels of AST and ALT in diabetic rats. The hepatoprotective effects of the single use of the plants by decreasing of ALT and AST have been reported in some previous studies (Sushma and Devasena 2010; Mbarki et al. 2017).

Recently, polyherbal formulations have become an interesting area of research and several formulations have been studied to attenuate hyperglycemia and its related complications. Some of these polyherbal formulations showed favorable results. The most prevalent plants used in these products were *Momordica charantia*, *G. sylvestre*, *T. foenum-graecum*,

Curcuma longa, and *Syzygium cumini*. However, one of the disadvantages of active ingredients of plants is low bioavailability (Kalia and Gauttam 2013). Fortunately, some of the plants such as cinnamon have convoy effects, and therefore may increase absorption of other drugs into target organs by inhibition of P-glycoproteins and p450 enzymes (Sadati et al. 2016). Hypoglycemic effects and improvement of histopathological changes in the pancreas were superior in Tetraherbs compared to single administration of each plant. These results may be due to convoy activity of the herbs involved in the Tetraherbs.

Conclusion

The evidence from this study supports the use of Tetraherbs as an antidiabetic remedy in Iranian traditional medicine. The antidiabetic activity of Tetraherbs is supported by biochemical and histopathologic analyses.

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Compliance with ethical standards

All the experiments involving animals were performed according to the guide line of the Institutional Animal Ethical Committee, Birjand University of Medical Sciences (the permit code is Ir.bums.REC.1394.373).

Conflict of interest The authors declare that they have no conflict of interest.

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