

Effects of *Heracleum Persicum* Hydroalcoholic Extract on Insulin, Serum Anti-Oxidant Enzymes, Glucose, and Lipid Profiles in Alloxan-Induced Diabetic Rats

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What's Known

- Heracleum persicum* exhibits anti-oxidant, anti-hyperlipidemic, anti-inflammatory, and analgesic properties in vitro and in animal models.

What's New

- Oral administration of *Heracleum persicum* hydroalcoholic extract in alloxan-induced diabetic rats reduced the blood glucose level and improved insulin and IGF-I levels.

Abstract

Background: *Heracleum persicum* (*H. persicum*) is a medicinal herb used in Iranian traditional medicine for its anti-toxic property. It is commonly consumed in the form of food additives and as a medicinal herbal tonic to treat liver and kidney diseases. The present study aimed to investigate the anti-oxidant, anti-diabetic, and anti-hyperlipidemic effects of *H. persicum* hydroalcoholic extract in alloxan-induced diabetic rats.

Methods: Adult male Wistar rats (n=30) were assigned to five groups; a normal group, a diabetic control group, and three diabetic groups treated orally with 200 and 400 mg/kg of the extract and 5 mg/kg of Glibenclamide, respectively, for two weeks. Blood glucose and body weight were measured at the end of each week. On day 15, blood samples were collected to measure the levels of insulin, insulin growth factor-I (IGF-I), antioxidant markers for malondialdehyde (MDA), glutathione peroxidase (GP_x); superoxide dismutase (SOD), total antioxidant activity (TAS), total cholesterol (TC), triglycerides (TG); high-density lipoprotein cholesterol (HDL), low density lipoprotein (LDL), and very low-density lipoprotein (VLDL) using commercial kits. The data were analyzed using SPSS Software (version 22.0).

Results: Daily treatment with 400 mg/kg of the extract significantly reduced the blood glucose level (P<0.001) and improved body weight (P=0.002), insulin (P<0.001), IGF-I (P=0.024), SOD (P=0.001), GP_x (P=0.009), MDA (P<0.001), TAS (P=0.006), TG (P<0.001), HDL (P=0.023), LDL (P=0.005), and VLDL (P<0.001) compared to the diabetic control group.

Conclusion: Beneficial effects of *H. persicum* for the treatment of diabetes were confirmed.

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Introduction

Diabetes mellitus (DM) is a debilitating condition caused by impaired insulin secretion and/or action. It is characterized by hyperglycemia and dyslipidemia.¹ Diet control and physical exercise are the initial important steps to prevent DM

complications such as retinopathy, neuropathy, and cardiovascular problems. Additional steps include the administration of oral anti-diabetic drugs such as biguanides, α -glucosidase inhibitors, thiazolidinedione (TZD), glucagon-like peptide-1 (GLP-1) inhibitors, and sulfonylureas. Glibenclamide belongs to the sulfonylurea class that controls hyperglycemia by stimulating insulin secretion.^{2, 3} Due to the side effects of these drugs (e.g., liver toxicity, digestive disorders, weight gain), scientists have been interested in developing oral drugs that effectively treat hyperglycemia while suppressing the side effects.^{4, 5}

Heracleum persicum Desf. ex. Fisch (*H. persicum*) is a branched perennial plant, native to Asian countries.⁶ It is an Iranian traditional medicine used for its anti-toxic property and commonly consumed in the form of food additives and medicinal herbal tonics to treat liver and kidney diseases.⁷ Animal studies have confirmed the low toxicity of *H. persicum*. Intraperitoneal injection of mice with *H. persicum* aqueous extract (up to 1600 mg/kg) has shown no significant change in the general behavior or mortality rate.⁸ A median lethal dose of 1103 mg/kg (988.2-1245.9 mg/kg, i.p.) of *H. persicum* acetone extract in mice has also been reported.⁹ Various reports have indicated the presence of aliphatic esters, carbonyls, phenylpropenes, terpenes, flavonoids; furanocoumarins, tannins, alkaloids, microelements, proteins, and fibers in *H. persicum*.¹⁰⁻¹² Various studies have demonstrated the anti-oxidant,¹³ analgesic,¹⁴ anti-inflammatory,¹⁴ anti-diabetic (in vitro),¹³ and anti-hyperlipidemic¹⁵ properties of this plant.

Considering all these beneficial effects of *H. persicum*, an effective anti-diabetic property of the plant is expected. However, there are limited studies on the effect of *H. persicum* on the blood glucose level, lipid profile, and oxidative stress biomarkers in diabetes. Hence the present study aimed to investigate the anti-oxidant, anti-diabetic, and anti-hyperlipidemic effects of *H. persicum* hydroalcoholic extract (HPHE) in alloxan-induced diabetic rats.

Materials and Methods

The Plant and Extracts

H. persicum fruits were purchased locally from an herbal medicine market in Tehran, Iran. A voucher specimen (number: PMP-759) was identified and authenticated by a botanist at the School of Pharmacy, Tehran University of Medical Science, Tehran, Iran. Two hundred grams of the fruit powder were successively

mixed with (3×1 liter) ethanol:water (70:30) on a shaker for 2 days at room temperature. The upper liquid mixture was separated, filtered, and the resulting hydroalcoholic extracts were oven-dried at 40 °C. Then, the crude extract was stored at -20 °C for biological assays.

Animals

Adult male Wistar rats (n=30) weighing 220-260 g were obtained from the Central Animal Facility, Pasteur Institute, Tehran, Iran. The animals were fed with water and rat pellets, and acclimatized to the laboratory conditions for 1 week prior to the experiments. The rats were randomly assigned to five experimental groups (n=6 each). Each group was housed in a temperature controlled environment (24±1 °C) under a 12-hour light:dark cycle. Animal care and the experimental procedure were in accordance with the guidelines of the European Council Directive (86/609/EEC).¹⁶ The study protocol was approved by the Animal Research Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran (code: IR.TUMS.VCR.REC.1396.3020).

Alloxan-Induced Diabetes

After 16 hours of fasting, except for the rats in the control group (n=6), diabetes was induced in 24 rats by a single intraperitoneal injection of alloxan monohydrate (Sigma, USA) in the dose of 120 mg/kg body weight dissolved in normal saline. The rats were administered 20% glucose solution (w/v) to reduce mortality caused by post-alloxan hypoglycemia. Blood samples were obtained from the tail veins 72 hours post-alloxan administration and the blood glucose levels were measured using a glucometer (ARKRAY Inc, Japan). Rats with blood sugar levels above 250 mg/dl were considered diabetic and randomly divided into four diabetic groups; in accordance with a previous study.¹⁷ Including the normal control group, the five experimental groups (n=6 each) were:

1. Normal control (drinking water 5 ml/kg)
2. Diabetic control (drinking water 5 ml/kg)
3. Diabetic+Glibenclamide 5 mg/kg
4. Diabetic+HPHE 200 mg/kg
5. Diabetic+HPHE 400 mg/kg

All animals were treated daily for 2 weeks by oral gavage. On day 15, at the end of the treatment period, the animals were sacrificed under deep anesthesia using xylazine (15 mg/kg) and ketamine (80 mg/kg).¹⁸ Blood samples were directly drawn from the heart and centrifuged at 4,000 rpm for 10 minutes at 4 °C. The resulting serum was stored at -70 °C until the biochemical analysis phase.

Measurements and Assays

The measurements included body weight, blood glucose, serum insulin, insulin-like growth factor-I (IGF-I), glutathione peroxidase (GPX), superoxide dismutase (SOD), and serum lipid profile.

During the treatment period, the body weight was recorded on days 0, 7, and 14. Glucose levels were measured and blood was extracted from the rats' tail veins. Blood glucose levels were measured using standard diagnostic kits (Pars Azmun, Iran) and an automatic analyzer (Abbott, Alcyon 300, USA). Serum insulin and IGF-I levels were measured using enzyme-linked immune sorbent assay (ELISA) kits (catalog number: E0707Ra and E0709Ra, Shanghai Crystal Day Biotech Co., Ltd., China) in accordance with the manufacturer's guideline.

GPx and SOD activities in the plasma samples were determined using Randox test kits (Randox, UK). GP_x activity was determined with the method developed by Paglia and Valentine (1967) based on the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) by glutathione reductase.¹⁹ The SOD activity was assayed using the method developed by Crosti and colleagues, based on the inhibitory effect of SOD in the production of O₂⁻ anions by the xanthine/xanthine oxidase system.²⁰

Assays were performed to determine the total anti-oxidant status (TAS) and malondialdehyde (MDA) activities. TAS was determined in serum samples using the chromogenic method with Randox kits (Randox, UK).²¹ MDA activity was measured in serum samples using a commercial kit (Randox, UK) based on the thiobarbituric acid (TBA) method.²²

The measurement of lipid profile included

total cholesterol (TC), triglycerides (TG), and high-density lipoprotein cholesterol (HDL). These were determined using commercial kits in accordance with the manufacturer's instructions (Diasys, Germany). The Friedewald formula was used to estimate the serum low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) levels (VLDL= TG/5; LDL=TC-[HDL+VLDL]).²³

Statistical Analysis

The data were analyzed using SPSS Software (version 22.0). The one-way ANOVA and post hoc test were used to determine statistically significant differences between the groups. Data were expressed as mean±SEM and P<0.05 was considered statistically significant.

Results

The effect of HPHE on body weight is shown in figure 1. After 1 and 2 weeks repeated administration of HPHE, a significant decrease in body weight was observed in the diabetic control group compared to the normal control group (P=0.007, 10.78%; P=0.002, 14.32%; respectively). A considerable improvement in the body weight was also observed in the diabetic rats (groups 3, 4, and 5) compared to the diabetic control group on day 7 (P=0.029, 10.69%; P=0.032, 11.64%; and P=0.017, 10.83%; respectively) and on day 14 (P=0.013, 13.81%; P<0.001, 19.24%; and P=0.002, 15.5%; respectively). No side effects of HPHE were observed in the animals.

The effect of HPHE on the level of blood glucose in alloxan-induced diabetic rats is shown in figure 2. Intraperitoneal injection of

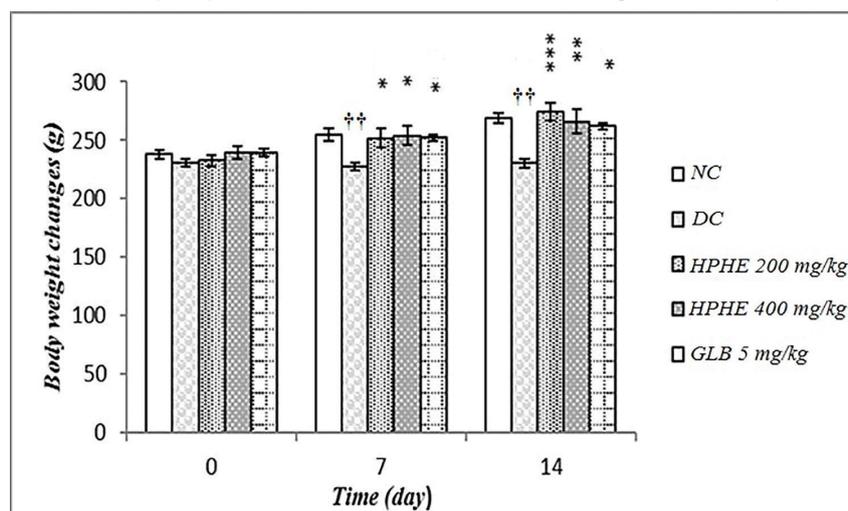


Figure 1: Repeated administration of HPHE improved the body weight of alloxan-induced diabetic rats (mean±SEM, n=6). ††P<0.01 significant compared to Normal control, †††P<0.001, ††P<0.01 significant compared to Diabetic control. HPHE: *Heraclium persicum* hydroalcoholic extract; NC: Normal control; DC: Diabetic control; HHP: Hydroalcoholic extract of *H. persicum*; GLB: Glibenclamide; mg/kg: milligram/kilogram of animal weight; Body weight changes(g): Body weight changes (gram)

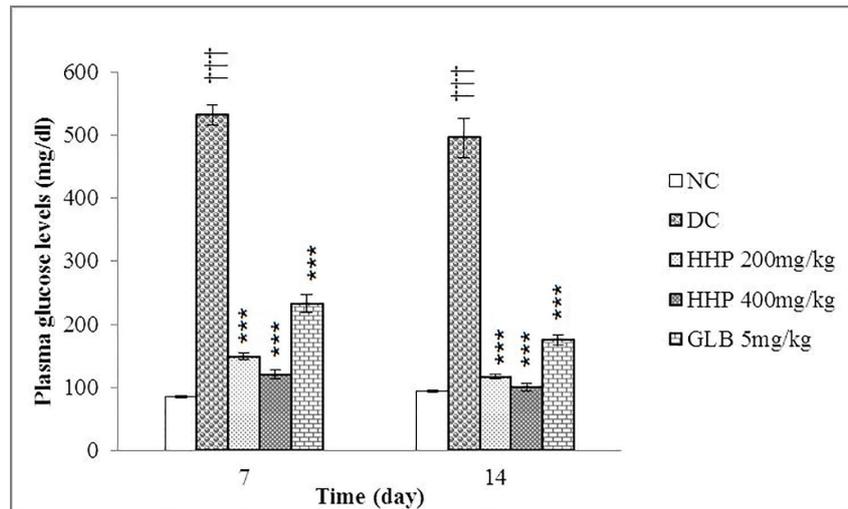


Figure 2: Daily administration of HPHE reduced the blood sugar level in alloxan-induced diabetic rats (mean \pm SEM, n=6). $^{+++}P<0.001$ compared to Normal control, $^{***}P<0.001$ significant compared to Diabetic control. HPHE: *Heracleum persicum* hydroalcoholic extract; NC: Normal control; DC: Diabetic control; HHP: Hydroalcoholic extract of *H. persicum*; GLB: Glibenclamide; mg/kg: milligram per kilogram of animal body weight; Plasma glucose level (mg/dl): Plasma glucose level (milligrams per deciliter).

alloxan significantly increased the blood glucose level in comparison with normal control rats ($P<0.001$, 178.16%). Daily treatment with HPHE (200 and 400 mg/kg) and Glibenclamide (5 mg/kg) significantly decreased the blood sugar level to near-normal values compared to the diabetic control rats on day 7 ($P<0.001$, 71.95%; $P<0.001$, 77.37%; and $P<0.001$, 56.22%; respectively) and day 14 ($P<0.001$, 76.27%; $P<0.001$, 79.80%; and $P<0.001$, 64.61%; respectively). On day 7, the potency of the hypoglycemic effect in the groups receiving HPHE (200 and 400 mg/kg) was higher than that of the group receiving Glibenclamide ($P<0.001$). While on day 14, there was a significant difference in the level of blood glucose between the groups receiving HPHE 400 mg/kg and the group receiving Glibenclamide ($P=0.01$). However, the difference between the group receiving HPHE 200 mg/kg and Glibenclamide was not significant ($P=0.06$). The difference between the groups receiving HPHE 200 mg/kg and HPHE 400 mg/kg was not significant on days 7 and 14.

As shown in table 1, there was a statistically significant decrease in the serum insulin and IGF-I levels in the diabetic rats compared to normal control rats ($P=0.009$, 33.70% and $P<0.001$, 27.74%, respectively). During the 2-week treatment, HPHE 400 mg/kg and Glibenclamide significantly increased the insulin ($P=0.009$ and $P<0.001$, respectively) and IGF-I levels ($P=0.024$ and $P=0.003$, respectively) compared to the levels in the diabetic control rats. At the end of the experiment, treatment with HPHE 400 mg/kg and Glibenclamide resulted in a significant increase in the insulin (15.78% and 30.52%, respectively) and IGF-I levels (20.07%

and 25.62%, respectively) compared to the diabetic control rats. There was a significant difference in insulin level between the HPHE 400 mg/kg and Glibenclamide groups ($P=0.01$). However, there was no significant difference in the serum insulin and IGF-I levels between the HPHE 200 mg/kg group and the diabetic control rats ($P=0.55$ and $P=0.99$, respectively).

The effect of HPHE on GPx and SOD activities is shown in table 2. The activity of GPx and SOD enzymes significantly decreased in the alloxan-induced diabetic rats compared to the normal control rats ($P=0.005$, 20.14%; and $P=0.015$, 17.18%; respectively). On day 14, treatment with HPHE (200 and 400 mg/kg) significantly increased SOD activity compared to that of the diabetic control rats ($P=1.00$, 6.55%; and $P=0.001$, 27.79%; respectively). The effect of HPHE 400 mg/kg was more potent than Glibenclamide in increasing SOD activity ($P=0.001$ and $P=0.788$, respectively). After 2 weeks of daily administration, there was a non-significant difference between GPx activity of the rats treated with HPHE 400 mg/kg compared to the diabetic control rats ($P=0.009$). Whereas the administration of HPHE 200 mg/kg resulted in a significant increase in GPx activity compared to the diabetic control rats ($P=0.069$). No significant difference in GPx activity was observed between the HPHE groups (200 and 400 mg/kg).

As shown in table 2, the TAS level decreased markedly in diabetic rats treated with HPHE compared to that of the normal control rats ($P<0.001$, 28.08%). However, after 2 weeks of treatment with HPHE 400 mg/kg, the TAS level returned to near-normal levels in diabetic rats compared to the diabetic control rats

Table 1: Effects of HPHE on insulin and insulin-like growth factor-I in the serum of alloxan-induced diabetic rats (mean±SEM, n=6)

Treatment	Normal control	Diabetic control	<i>H. persicum</i>	<i>H. persicum</i>	Glibenclamide
Dose	Distilled water (5 mL/kg)	Distilled water (5 mL/kg)	200 mg/kg	400 mg/kg	5 mg/kg
Serum insulin (uIU/mL)	14.33±0.35	9.5±0.36 †††	10.1±0.17	11±0.126 †††	12.4±0.3 †††
Serum IGF-1 (ng/mL)	179.3±4.83	129.55±5.16 †††	133.06±5.33	150.56±5.48 *	162.75±8.68 †††

†††P<0.001 significant compared to Normal control, *P<0.05, †††P<0.001 significant compared to Diabetic control. HPHE: *Heracleum persicum* hydroalcoholic extract; The one-way ANOVA and post hoc test were used to determine statistically significant differences between the groups

Table 2: Effects of HPHE on the stress oxidative markers in the serum of alloxan-induced diabetic rats (mean±SEM, n=6)

Treatment	Normal control	Diabetic control	<i>H. persicum</i>	<i>H. persicum</i>	Glibenclamide
Dose	Distilled water (5 mL/kg)	Distilled water (5 mL/kg)	200 mg/kg	400 mg/kg	5 mg/kg
SOD (U/gHb)	1562±55.92	1293.58±14.05 †	1378.42±36.52 †	1653.17±97.85 ††	1312.65±58.61
GP _x (U/gHb)	60.66±2.34	48.44±0.98 ††	56.09±2.01	59.85±2.28 ††	60.55±2.25 ††
MDA (nmol/ml)	1.2±0.06	2.18±0.09 †††	2.1±0.06	1.13±0.08 †††	2.01±0.07
TAS (mmol/L)	0.86±0.04	0.64±0.03 †††	0.72±0.03 †	0.8±0.02 †	0.69±0.01

†P<0.05, ††P<0.01, †††P<0.001 significant compared to Normal control, †P<0.05, ††P<0.01, †††P<0.001 significant compared to Diabetic control. The one-way ANOVA and post hoc test were used to determine statistically significant differences between the groups. HPHE: *Heracleum persicum* hydroalcoholic extract; SOD: Superoxide dismutase; GP_x: glutathione peroxidase; MDA: Malondialdehyde; TAS: Total antioxidant status

Table 3: Effects of HPHE on the lipid profile in the serum of alloxan-induced diabetic rats (mean±SEM, n=6)

Treatment	Normal control	Diabetic control	<i>H. persicum</i>	<i>H. persicum</i>	Glibenclamide
Dose	Distilled water (5 mL/kg)	Distilled water (5 mL/kg)	200 mg/kg	400 mg/kg	5 mg/kg
TC (mg/dl)	48.5±0.83	57±2.2 †	55.33±3.20	52.33±1.08	55.83±1.65
TG (mg/dl)	30.16±1.27	56.83±0.82 †††	21±1.16 †††	41.83±1.79 †††	50.33±1.84 ††
HDL (mg/dl)	29.58±1.32	23.75±0.48 †	32.33±5.04 ††	30.83±1.18 †	29.16±2.32 †
LDL (mg/dl)	12.96±2.15	21.88±2.43 ††	18.8±1.83	13.01±0.52 ††	16.6±2.28
VLDL (mg/dl)	6.03±0.35	11.36±0.16 †††	4.2±0.23 †††	8.36±0.35 †††	10.06±0.36 ††

†P<0.05, ††P<0.01, †††P<0.001 significant compared to Normal control, †P<0.05, ††P<0.01, †††P<0.001 significant compared to Diabetic control. The one-way ANOVA and post hoc test were used to determine statistically significant differences between the groups. HPHE: *Heracleum persicum* hydroalcoholic extract; TC: Total cholesterol; TG: Triglyceride; HDL: High density lipoprotein; LDL: Low density lipoprotein; VLDL: Very low density lipoprotein

(P=0.006, 25%). Whereas the administration of Glibenclamide did not significantly increase the TAS activity in diabetic rats compared to the diabetic control rats (P=0.665, 1.18%). The administration of alloxan significantly increased the MDA level in the serum of diabetic rats compared to normal rats (P<0.001, 81.66%). Repeated administration of HPHE 400 mg/kg revealed a significant decrease in the mean MDA level of plasma samples compared to the diabetic control rats (P<0.001, 48.16%). Whereas the administered Glibenclamide supplement showed no significant improvement in the mean MDA level compared to the diabetic rats (P=0.471, 7.79%).

The effect of HPHE on the lipid profile (TG, TC, LDL, HDL) is shown in table 3. Administration of alloxan showed a significant elevation in TG (P<0.001, 88.42%), LDL cholesterol (P=0.005, 68.82%), and VLDL (P<0.001, 88.39%) compared to the normal

control rats. Whereas those of TC (P=0.061) and HDL (P=0.081) were changed insignificantly. On day 14, treatment with HPHE (400 mg/kg) caused a significant decrease in TG (P<0.001, 24.39%), LDL cholesterol (P=0.005, 40.53%), VLDL (P<0.001, 26.40%), and a significant increase in HDL cholesterol (P=0.023, 29.8%) compared to that of the diabetic control group. Furthermore, administration of Glibenclamide for 14 days caused a significant decrease in TG (P<0.001, 11.43%), but no significant improvement in VLDL (P=0.081), TC (P=1.00), LDL cholesterol (P=0.082), and HDL (P=0.119) levels was observed in comparison with the diabetic control rats.

Discussion

The effects of *H. persicum* fruit extract on blood glucose, serum lipids, anti-oxidant markers, insulin, IGF-I levels, and body weight were

evaluated in alloxan-induced diabetic rats. Alloxan produces free radicals that have a destructive effect on the β -cells of the pancreatic islets, resulting in a decrease in insulin secretion.²⁴ The results showed that a 2-week treatment of diabetic rats with HPHE led to a significant decrease in the blood glucose level. The potency of the hypoglycemic effect in diabetic rats receiving HPHE (200 or 400 mg/kg), in a time- and dose-dependent manner, was higher than in the group receiving Glibenclamide (67.45% and 77.38% versus 52.43%, respectively). Moreover, the blood glucose levels of the treated rats were similar to those of the healthy non-diabetic group.

The mechanism of the HPHE activity was examined by comparing the insulin levels in rats. We found that the insulin levels were elevated significantly in diabetic rats treated with the plant extract. According to previous studies, improvement in insulin level was attributed to an increased insulin sensitivity, increased β -cell mass, and repaired β -cell function.^{25, 26} Flavonoids and coumarins have shown an anti-diabetic activity due to the improved level of secretion and sensitivity of insulin and glucose uptake in insulin-sensitive tissue, improved pancreatic cell protection, and restoration of insulin signaling.²⁷⁻²⁹ Therefore, hypoglycemic and enhanced insulin effects of HPHE might be associated with the presence of flavonoids and coumarin derivatives.¹⁰ Considering the greater effect Glibenclamide has on blood insulin enhancement compared to the HPHE, it can be concluded that HPHE involves other mechanisms to reduce blood glucose levels. The action mechanism could be in the form of increasing insulin excretion, improving glucose uptake by adipose and muscle tissues, preventing glucose absorption from the intestine, and inhibiting glucose production from hepatocytes.³⁰ Recently, the inhibitory effect of ten furanocoumarins isolated from the roots of *H. persicum* against α -glucosidase has been reported.³¹ It was shown that moellendorffiline (a pimpinellin-dimer) displayed a more effective potent activity than acarbose; as an inhibitor of hydrolase enzymes such as alpha-glucosidase, which reduces blood glucose levels following the intake of hydrocarbons.³¹

IGF-I played an important role in peripheral glucose uptake by tissues and in β -cell function.³² Our results showed that the administration of HPHE over a 2 weeks period increased the IGF-I levels. In fact, the plant extract possesses anti-oxidant compounds that can prevent secondary complications of diabetes by increasing IGF-I as a major marker in metabolic disorders.¹³

Both the insulin deficiency and insulin

resistance led to a significant weight loss in diabetes.³³ The use of HPHE not only prevented weight loss but led to a significant weight gain in diabetic rats. It controlled muscle wasting and weight loss through its ability to increase the insulin level and adequate glucose uptake by tissues. It has been reported that untreated diabetic rats show significant lipid abnormalities in serum.³⁴ Our results showed that HPHE treatment had beneficial effects on hyperlipidemia. The HPHE significantly reduced TG, LDL cholesterol, VLDL, and significantly elevated the serum levels of HDL cholesterol. The hypolipidemic activity of *H. persicum* can be attributed to the improved insulin secretion or function that resulted in the stimulation of the lipoprotein lipase (LPL) activity and increased glucose intake by peripheral tissues. Our findings were confirmed in a study by Hashemi and colleagues.³⁵ Therefore, the hypolipidemic effects of HPHE may reduce the incidence of coronary heart diseases and strokes in humans.³⁶

An increase in free radicals following diabetes leads to increased lipid peroxidation and reduced superoxide dismutase, total anti-oxidant capacity, catalase, and glutathione.³⁷ Since MDA is the end product of lipid peroxidation,³⁸ it is therefore important to evaluate the anti-oxidant activity of *H. persicum*, which has been shown to produce significant protective effects against lipid peroxidation and dramatically decrease plasma MDA levels.

The results of the present study revealed a significant increase in SOD, GPx, and TAS activities in the serum after HPHE treatment. It indicated that the presence of anti-oxidant compounds in *H. persicum* extract could play an important role in reducing free radicals. The results showed that there was no significant difference in the blood glucose levels between the groups treated with HPHE 200 and 400 mg/kg on days 7 and 14. On the other hand, serum insulin, IGF-I, and lipid profile levels were only significantly affected in the HPHE 400 mg/kg group compared to the diabetic control group. The effects of the extracts on the oxidative enzyme activity also indicated that the 400 mg/kg dose had a stronger effect than 200 mg/kg.

The main limitation of the present study was the lack of identification of the effective compounds of *H. persicum* extract and the fact that histopathological analysis of the liver was not performed. These are the subject of future studies.

Conclusion

Beneficial effects of *H. persicum* on anti-oxidant enzymes, anti-hyperglycemic, and

anti-hyperlipidemic in alloxan-induced diabetic rats were confirmed.

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Conflict of Interest: None declared.

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