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Cytotoxic Effect and Insulin-Like Characteristic of *Peganum harmala*: An *in vitro* Study

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ABSTRACT

Background & Objective: *Peganum harmala* has emerged as a promising anti-diabetic medicine. There is no study regarding the impact of *P. harmala* concentration on the insulin secretion, C-peptide secretion, and glucose uptake. We investigated the effect of different concentrations of methanolic extracts of *P. harmala* seed and leaf on insulin and C-peptide secretion, and glucose uptake.

Materials & Methods: After the cell passaging, pancreatic carcinoma cell line (PANC-1) and HT1080 were treated with different concentrations of seed and leaf extract of *P. harmala*, harmine, and ghrelin agonists. The MTT was employed to assess the cell survival at the selective doses, and using a spectrophotometer, the absorbance was determined at 570 nm. After 72-h treatment, the insulin and C-peptide secretion were measured by ELISA. To measure the intracellular glucose concentrations after treating muscle carcinoma cell lines, glucose oxidase method was utilized.

Results: *P. harmala* seed and leaf extracts increased the secretion of insulin and C-peptide in a dose-dependent manner compared to ghrelin and harmine. These extracts increased the intracellular glucose concentration at high doses (150 and 1500 µg/ml for the seed and leaf extract, respectively) of HT1080 cell line. However, their high concentration was toxic and reduced the cell survival. The methanolic extracts of seed showed a higher insulin (17.5-fold) and C-peptide (7.8-fold) secretion compared to the leaf methanolic extracts.

Conclusion: Due to the presence of β-carbolines, the *P. harmala* seed extract has toxicity and affects insulin secretion and C-peptide uptake secretion at lower concentrations than those of leaf extract.

Keywords: Carcinoma, C-peptide, Peganum, Insulin

Introduction

Diabetes, a metabolic disorder caused by the high blood sugar, consists of two types, including type 1 and 2 diabetes. The former occurs when body loses its capacity to produce sufficient insulin in the pancreas, and the latter arises from mistakenly attacks of body's immune system to the cells. Type 1 diabetes is basically an autoimmune disease in which human body lose its ability to process glucose owing to the death of beta cells, a key factor in producing insulin, by the immune system (1). As an anabolic hormone, insulin converts glucose from carbohydrate-containing foods into energy necessary for body functions (2). Accordingly, in the absence of insulin, glucose accumulates in the bloodstream, thereby damaging the nerves and blood vessels in the eyes, kidneys, and heart (1). Insulin is typically prescribed for patients with type 1 diabetes, but its multiple injections have several drawbacks such as severe insulin resistance (3) and hypoglycaemia (4), which comes from excessive insulin dose. Thus, it is necessary to look for new medications with the minimum adverse effect and maximum efficiency.

Various herbal medicines and plant extracts (leaf, seed, stem, etc.) have been applied as a treatment for those who suffer from diabetes. Today, the demand for medicinal plants has dramatically been rising so that in developing countries, many traditional healthcare systems have initiated the effective use of medicinal plants (5-7). According to the ethnobotanical databases, more than 800 plants have been discovered, and their antihyperglycemic properties have been tested on various animal models and used as a traditional medicine for diabetes therapy (8-13).

Peganum harmala, a member of zygophylla1ceae family, has widely been applied in varied pharmacological therapies by virtue of its immunomodulatory, antidiabetic, antimicrobial, antithrombotic, antitumor, analgesic and diuretic characteristics, as well as for its cardiovascular,

carminative, emmenagogic, gastrointestinal, osteogenic and galactagogue activities (14). This wild-growing plant contains considerable amounts of alkaloids, especially β-carbolines, which are distributed differently in various parts of the plant. The seeds of *P. harmala* have the highest concentration of β-carbolines (15). Harmaline, harmine, harmalol, harmol, and tetrahydroharmine are known as the main β-carbolines alkaloids present in *P. harmala* extracts. Many studies have used β-carboline alkaloids extracted from the *P. harmala* as the antidepressant, hallucinogenic, and antileishmanial agents (16). Therefore, it is necessary to find the optimum concentration of *P. harmala* for insulin secretion in order to be used for the treatment of diabetes.

Although there are some investigations regarding the antidiabetic effects of *P. harmala*, no comprehensive study has been conducted on the dose response of the methanolic extracts of *P. harmala* (leaf and seed) on the insulin and C-peptide secretion and glucose uptake in two cell lines, PANC-1 and HT1080. Therefore, the present study examined the impact of the methanolic extract concentration on the insulin secretion, C-peptide secretion, and glucose uptake to find the optimum concentration of the methanolic extract. Moreover, the pure harmine and ghrelin agonist were compared to show the efficiency of the methanolic extract of *P. harmala* leaf and seed. The present research provides new insights into pharmaceutical studies because first, the most effective parts of the *P. harmala* plant, i.e. leaf and seed, were investigated to be considered in the treatment strategies for diabetes. Second, given the toxicity of the *P. harmala*, the exact oral dose is determined. Third and most importantly, whether the use of the plant extract is more effective or its extracted substance harmine.

Materials and Methods

Specimens and materials

P. harmala was collected from areas near Zahedan city, southeast of Iran, in spring. The *P. harmala* specimens were approved and allocated the herbarium number 107208 by the Central Herbarium of Iran (Tehran).

Pure harmine and ghrelin agonist were acquired from Sigma-Aldrich (USA). The commercial kits for insulin, C-peptide, and glucose measurements were procured from Monobind Inc. (USA), Mercodia **(**Sweden**)**, and MAK263 Sigma (USA), respectively. Myoblast (HT1080) and pancreatic carcinoma cell lines (PANC-1) were purchased from Iranian Biological Resource Center (IBRC; Tehran).

Assays and instruments

Anthocyanins and flavonoids were evaluated by Shinoda test. Ferric chloride and Borntrager tests were employed to verify the presence of tannins and to check the presence of anthraquinones, respectrively. The MTT assay was performed to measure the viability of the myoblast and pancreatic carcinoma cell lines to find the *in vitro* impact of the methanolic extracts on the insulin and C-peptide secretion and glucose uptake (17). ELISA reader (Hyperion, MPR4^{++}, Germany) was utilized for insulin, C-peptide, and as-prepared assays. Glucose oxidase (GOx) kits Pars Azmoon, Iran) were employed to measure the GOx activity after 24 hours. To quantitatively analyze the extracts, we applied high performance liquid chromatography (HPLC; KNAUER, Germany) in which aC_{18} column, isopropyl alcohol, acetonitrile, water, and formic acid (100:100:300:3 ratio) were used as the mobile phase (1 ml/min). Chromatograms were produced in 330 nm.

Extraction process

Collected plants were washed several times with water and dried out at room temperature. Following the isolation of leaves and seeds of *P. harmala* samples, their methanolic extracts were extracted through the soxhlet extractor. The extraction process was performed by mixing 500 ml of methanol with the as-collected samples in the aforesaid extractor. After 4 hours, the extracts were gathered and stored in a sealed vial at 4 °C until use. Alkaloids were also extracted according to the standard protocol and confirmed by Wagner reagent (18).

Cell culture stage

Preparation of PANC-1 culture medium

PANC-1 cells were cultured in a DMEM medium (containing 10% fetal bovine serum), supplemented with 2 mM of l-glutamine, 50 U/mL of penicillin, and 50 µg/mL of streptomycin at 37°C and in 6%/94% CO_2.

Preparation of PANC-1 cell culture from frozen cells

The frozen culture medium was immersed in hot water at 37°C. The unfrozen medium was removed from flask under a sterile hood. Subsequently, the cell monolayer was washed with 5 ml of PBS, and the solution was eliminated by aspiration; this step was repeated twice. After washing with PBS, fresh DMED/F12 containing 1% penicillin/streptomycin and 10% FBS was gently added. The quality of the cells was examined morphologically using an inverted microscope. Following the addition of 1.5% glucose to the medium, the cells were finally treated with the extracts, harmin, and ghrelin agonist.

Preparation of HT1080 culture medium

HT1080 culture medium (100 ml), containing 50 ml of DMEM, 2 mM of L-glutamine,4.5 g/l of glucose, 110 mg/l sodium-pyruvate, 40 ml of 10% (v/v) heat-inactivated FBS, and 10 ml of DMSO, was kept in 4 °C.

Preparation of HT1080 cell culture from frozen cells

At first, 10 ml of DMEM full growth medium was added to a 15-ml falcon tube, and then placed in a hot water bath at 37 °C for 1-2 minutes to unfreeze. After the tube was taken out of the hot water bath, its surface was sterilized with 70% ethanol at room temperature. Cell suspension was added to the falcon tube containing 10 ml of growth medium, which was then transferred to a

centrifuge at 200 rpm at room temperature for three minutes, to collect the cells. The culture medium was finally removed under aspiration conditions. Thereafter, 5 ml of culture medium was transferred to the falcon tube, gently pipetted, transferred to T-75 flasks containing 10 ml of fresh culture medium and placed in a CO_2 incubator 5% at 37 ° C. All the cells were examined daily to reach 70-80% confluence. After the confluency of the cells and the addition of 1 U of regular insulin to the medium, the cells were treated with the seed and leaf extract and insulin. The contents of the tubes were increased by one unit of regular insulin. In the end, glucose levels of all the flasks were measured.

MTT viability test

To determine the *in vitro* antiproliferative abilities of the methanolic extracts of the leaf and seed in various doses against the cell lines PANC-1 and HT1080, the MTT assay was conducted by placing a number of 30×10^3 cells per well in 24-well plates, along with the desired amount of treatment solutions, while keeping them at 37 °C and 5 % CO_2 for 24 h. Afterwards, 100 µl of MTT stock solution (5 mg of MTT powder in 1 mL of phosphate buffer saline) was added to each well and further incubated for 4 h. After this time period, MTT solution was replaced with DMSO (10%), and absorbance spectra were recorded at 570 nm by a spectrophotometer (Epoch™ 2 microplate; BioTek Instruments, USA) after 15-min incubation. The cell survival percentage was calculated based on the following equation **(19)**.

Cell survival (%) = Treated sample absorbance/Control sample absorbance × 100 (1)

IC_{50} was determined as the concentration of the treatments in which more than 50% of the cells was survived. To prepare different treatment solutions, 1 ml of water/methanol was diluted with the culture medium containing various amounts of dried extracts. Doses were selected by the logarithmic method, and dose ranges were chosen as described elsewhere **(19)**. The concentrations dissolved in the treatment solution were as follows: 0.015, 0.15, 1.5, and 15, and 150 µg/ml for the seed extract, 0.15, 1.5, 15, 150, and 1500 µg/ml for the leaf extracts, 500, 50, 5, 0.5, and 0.05 µg/ml for ghrelin, and 0.01, 0.1, 1, 10, and 100 µg/ml for harmine **(20, 21)**. Of note, the controls were comprised of only culture medium and FBS.

Statistical analysis

The One-Way ANOVA, Tukey's post hoc test, and SPSS 20 and GraphPad Prism (v. 6.0; GraphPad Software, Inc, La Jolla, CA) were used to analyze the results of tests. All the results were repeated three times and reported as means ± standard deviation. The level of statistical significance was considered as $p \leq 0.05$.

Ethical statement

The protocol for animal care and use was approved by the Ethics Committee of the Islamic Azad University, Science and Research Branch, Tehran, Iran (IR.IAU.SRB.REC.1396.40).

Results

HPLC study

HPLC was performed to measure the β-carboline-type alkaloids (harmalol, harmol, harmane, harmaline, and harmine) of *P. harmala* seed and leaf extracts (Table 1) **(22)**. The results of HPLC displayed that the methanolic extract of seed had 2.93% harmine, while for the leaf, it was 0.055%.

Table 1. Chemical composition of the methanolic extracts of *P. harmala* leaf and seed determined by HPLC analysis

Extract type	Seed (%)	Leaf (%)
Extraction	29.7	31.7
Alkaloid		
Harmalol	0.12	0.026
Harmol	0.02	---
Harmane	0.029	0.011
Harmaline	3.8	0.05
Harmine	2.93	0.055
Total flavonoid contents (mg QE/mg extract)	16.63	25.84
Total phenolic contents (mg GAE/mg extract)	30.46	32.84

MTT assay

The results of MTT assay for the methanolic extracts of the leaf and seed in various doses against the cell lines PANC-1 and HT1080 are shown in Figure 1a and 1b, respectively. Based on the results, the survival rate of both cell lines decreased with increasing the concentration of seed and leaf extracts, as well as harmine and ghrelin agonist. At doses 150 µg/ml and 1500 µg/ml, the survival rate of cells treated with the *P. harmala* leaf extract significantly reduced

($p \leq 0.001$). This reduction result was also observed in harmine-treated cells (at 100 µg/ml) and ghrelin agonist-treated cells (at 50 and 500 µg/ml), with the p value of ≤0.001. The survival rate of cells treated with 150 µg/ml of the seed extract significantly diminished ($p \leq 0.001$). However, the IC_{50} of the *P. harmala* seed extract at doses of 1.5 and 15 µg/ml did not show any significant difference in the survival of cells. At IC_{50} of 15, 10, and 5 µg/ml, more than half of cells treated with the leaf extract, harmine, and ghrelin agonist survived, respectively. Besides, a meaningful difference was found in the survival of cells treated with 0.5 and 0.05 µg/ml of ghrelin agonist ($p \leq 0.01$).

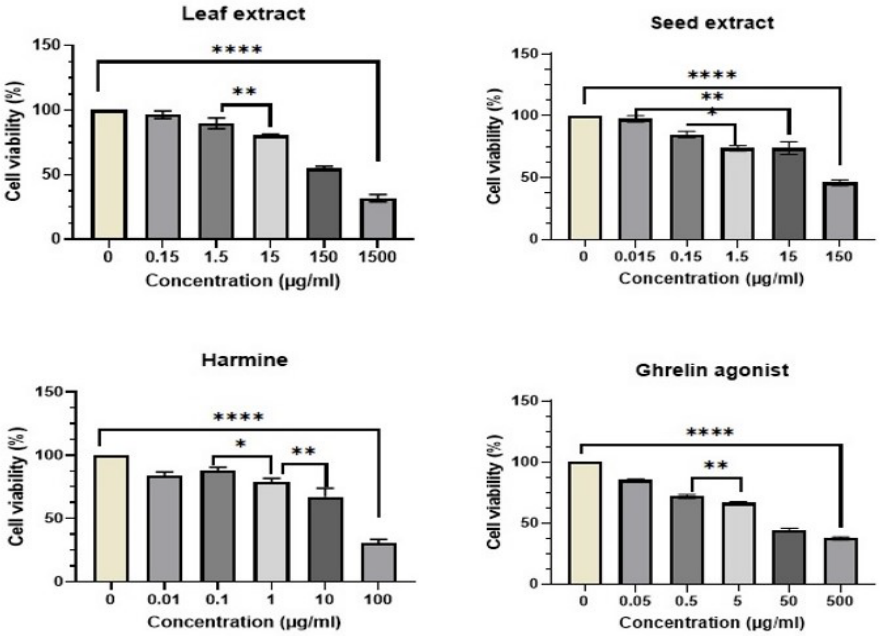

Fig. 1a

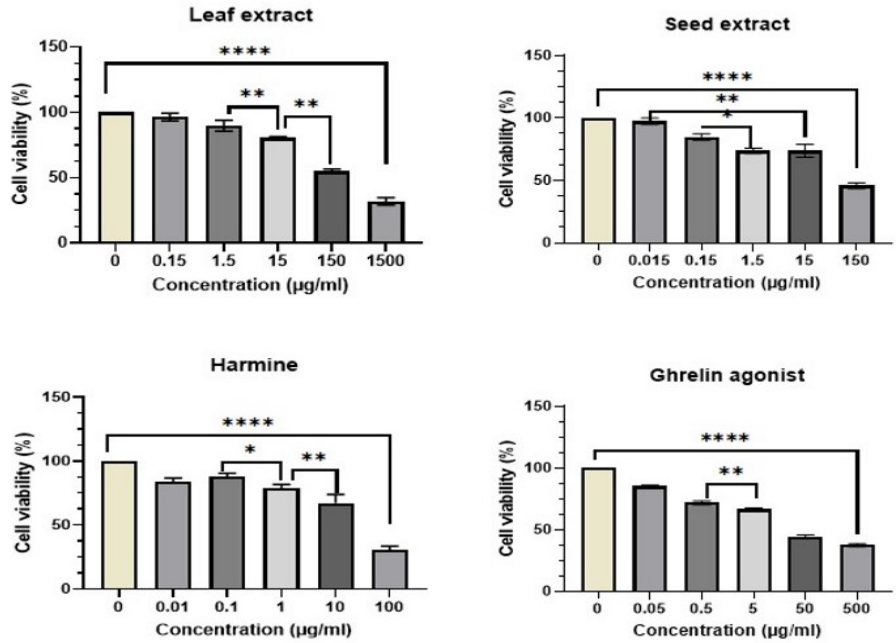

Fig. 1b

Figure 1. Cytotoxic effect of harmine, ghrelin agonist, and *P. harmala* leaf and seed methanolic extracts in PANC-1 (a) and HT1080 (b) cell lines ($^{****}p<0.001$), ($^{**}p<0.01$), ($^{*}p<0.05$). The survival rate of both cell lines decreased with increasing the concentration of seed and leaf extracts, as well as harmine and ghrelin agonist.

Insulin assay

The effect of different concentrations of the pure harmine, ghrelin agonist, and the methanolic extracts of *P. harmala* leaf and seed on the insulin secretion of PANC-1 was investigated, and its results are represented in Figure 2. There was a significant difference in the rate of insulin secreted from PANC-1 cells at the seed extract dose of 15 µg/ml ($p \leq 0.001$). However, at doses of 1.5 and 0.15, and 0.015 µg/ml, the insulin secretion was insignificant. The results also displayed that the elevation in the insulin secretion occurs only at very high doses so that insulin was secreted only at 150 µg/ml dose of the leaf extract. The insulin secretion did not show any significant difference between the cells treated with harmine (at the doses of 1 µg/ml and 10 µg/ml), but at low doses, a meaningful raise was detected ($p \leq 0.01$). In ghrelin agonist-treated cells, the rate of insulin secretion reduced; however, this reduction was not significant at the doses of 0.5,0.05, 5 and 50 µg/ml ($p \leq 0.01$).

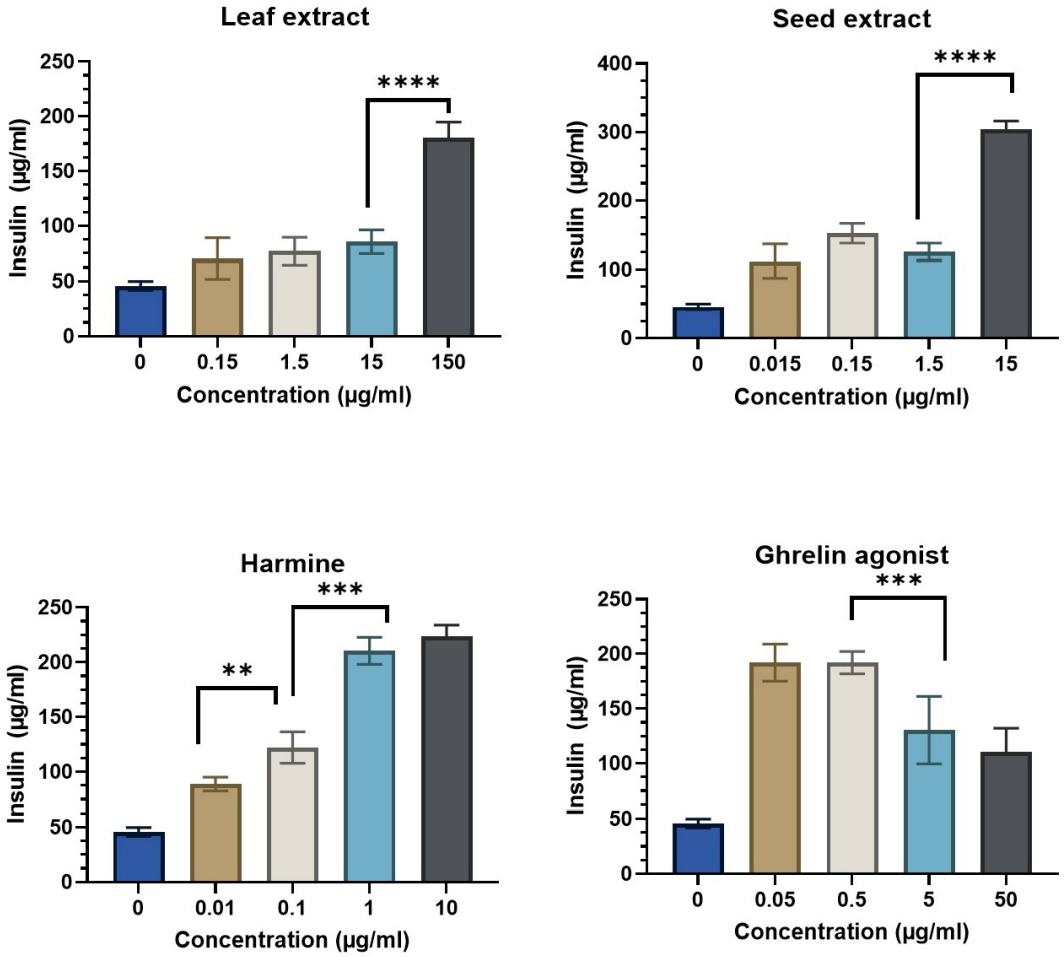

Figure 2. Insulin assay in PANC-1 with treatments of harmine, ghrelin agonist, and *P. harmala* leaf and seed methanolic extracts ($^{****}p<0.001$), ($^{***}p<0.01$). There was a significant difference in the rate of insulin secreted from PANC-1 cells at the seed extract dose of 15 µg/ml ($p \leq 0.001$). The insulin secretion increased only at very high doses (150 µg/ml) of the leaf extract.

C-peptide assay

The impact of various concentrations of the leaf and seed methanolic extracts of *P. harmala* on the C-peptide level in PANC-1 was assessed using the C-peptide assay. The results obtained from this test (Figure 3) showed that increased level of C-peptide in the PANC-1 in a dose dependent manner, from 3.19 to 6.9 µg/ml in leaf and from 1.93 to 5.08 µg/ml in seed in the presence of 0.15 and 150 µg/ml of the leaf extract and 0.015 and 15 µg/ml of the seed extract, respectively, with significant difference of $p \leq 0.01$. The results of C-peptide assay also revealed that the C-peptide level of PANC-1 in the methanolic extracts of seed was 7.8 folds higher than those of the leaf.

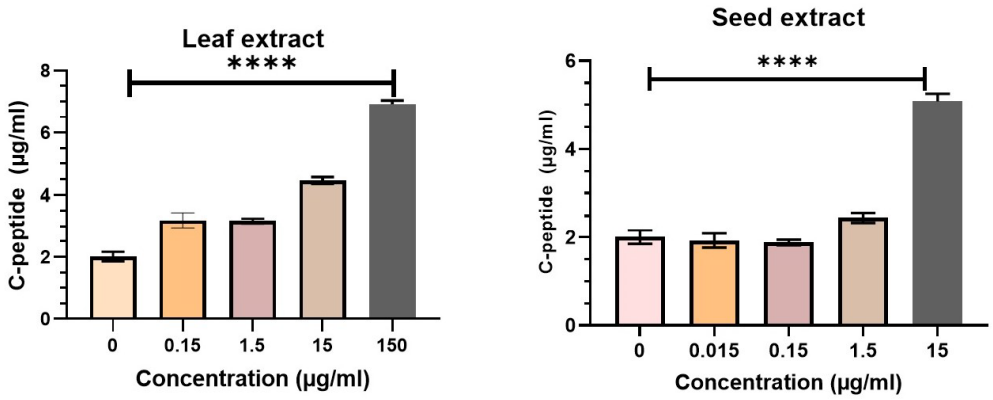

Figure 3. C-peptide assay in PANC-1 with treatments of *P. harmala* leaf and seed methanolic extracts (**$p<0.001$). The C-peptide level of PANC-1 in the methanolic extracts of seed was 7.8 folds higher than those of the leaf.**

Glucose assay

Based on the results from glucose assay, the concentration-dependent behavior of HT1080 in glucose uptake can be observed by increasing the methanolic extract of *P. harmala* leaf and seed, which increased form 0.0107 µg/ml to 0.0533 µg/ml and from 0.019 µg/ml to 0.1167 µg/ml, respectively. More precisely, the methanolic extract of seed causes the enhancement of glucose uptake by 21.89-fold higher than the methanolic extract of leaf (Figure 4).

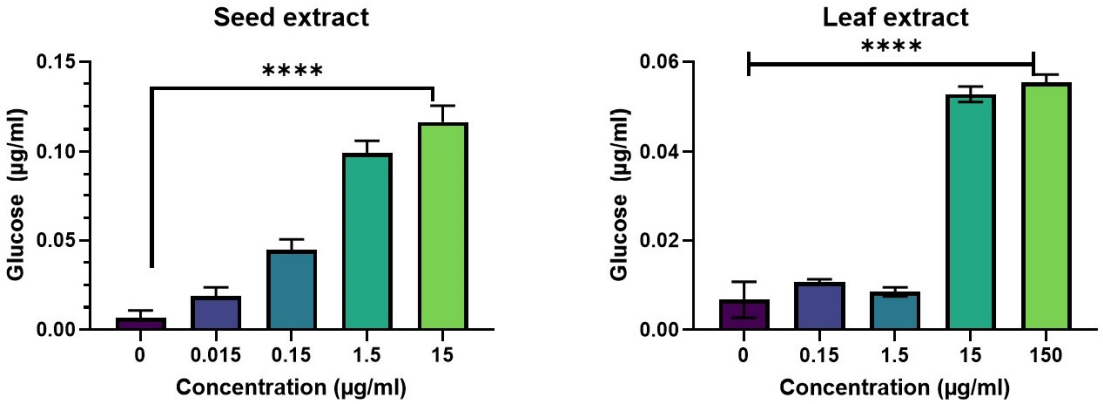

Figure 4. Glucose assay in HT1080 cell line with treatments of *P. harmala* leaf and seed methanolic extracts (**$p<0.001$). The concentration of HT1080 in glucose uptake enhanced with increasing the methanolic extract of *P. harmala* leaf and seed, form 0.0107 µg/ml to 0.0533 µg/ml and from 0.019 µg/ml to 0.1167 µg/ml, respectively. The methanolic extract of *P. harmala* seed resulted in the increased level of glucose uptake by 21.89-fold higher than the methanolic extract of leaf.**

Discussion

The present study assessed the effect of the methanolic extract concentration of *P. harmala* seed and leaf on the insulin secretion, C-peptide secretion, and glucose uptake.

The HPLC results indicated a significant difference between the concentrations of β-carboline in the seed and leaf extracts; however, seed had a higher level of β-carboline than the leaf. A former study on the anti-diabetic effect of the alcoholic extract of *P. harmala* in streptozotocin-induced diabetic rats verified anti-diabetic effects of this herb at the doses of 30 and 120 mg/kg (23). Moreover, the level of harmine in the methalonic extract of the seed was 53 times greater than in the leaf, implying that the insulin secretion by the methanolic extracts of seed is higher than the leaf. Numerous investigations have suggested that imidazoline compounds can stimulate insulin secretion by the activation of imidazoline I3 binding sites in the pancreatic β-cell.

The results of MTT assay confirmed that the methanolic extracts of both leaf and seed had cytotoxic activity against PANC-1 and HT1080 cell lines. Moreover, the cytoxicity of the methanolic seed extract was 10 times higher than those of leaf. Since harmine has high toxicity, the methanolic extracts of leaf has higher cellular viability in comparison with those of seeds. The possible mechanism of the cytotoxic activity of harmine may be due to intercalating in DNA, causing the inhibition of DNA topoisomerases and accodingly cytotoxicity (24). The MTT results also

demonstrated that the low concentrations of the methanolic extract of *P. harmala* leaf and seed have low toxicity.

The results displayed that PANC-1 secretes insulin in a concentration-dependent manner in which increase in the concentrations of methanolic extracts of seed and leaf and causes the elevated insulin secretion. Due to various hormones secreted from liver, it is impossible to directly calculate the amounts of insulin secretion from peripheral insulin concentrations **(25)**. Therefore, monitoring peripheral C-peptide concentrations, which reflects changes in insulin secretion more precisely is suggested as since C-peptide, at the concentrations equal to insulin, is secreted from the pancreatic beta cell **(20-24, 26, 27)**. By comparing the insulin secretion results of leaf and seed, we found that the insulin secretion of PANC-1 in the presence of the methanolic extracts of seed is 16.83 folds higher than that of the leaf. We also observed the insulin secretion in the PANC-1 cell line in the presence of the leaf and seed extracts and pure harmine.

Comparing the amount of insulin secretion from cells treated with pure harmine and the methanolic extracts of seeds revealed that the efficiency of the seed extract in insulin secretion is very close to the pure harmine. As a result, the methanolic extracts of *P. harmala* seeds could be used as an acceptable alternative medicine to insulin secretion. The ghrelin, an appetite-stimulating hormone in humans, plays a vital role in the progression of diabetes **(25)**. Many surveys have also approved that ghrelin inhibits glucose-stimulated insulin secretion in pancreatic islets and ghrelin can directly inhibit beta cells **(28)**. These insulin static effects likely emerges from the direct effects of ghrelin on beta cells **(29)**. Hence, in this research work, ghrelin agonist was applied dose-dependently, to prove the efficiency of the methanolic extracts of *P. harmala* seed and leaf in increasing the insulin secretion. Based on the insulin secretion results, the concentration of ghrelin agonist increased with the reduction of insulin secretion, suggesting that *P. harmala* methanolic extracts have an opposite reaction toward the pancreatic carcinoma cells. Thus, harmine analogues can serve as ghrelin inhibitors in the treatment of diabetes, though more studies are needed.

Any alteration in the glucose homeostasis leads to metabolic disorders such as hypoglycemia, hyperglycemia, and diabetes mellitus **(30)**. To this end, the present study examined the impacts of varied concentrations of the methanolic extract of *P. harmala* leaf and seed on the glucose uptake of HT1080 cell line through the glucose oxidase technique **(31)**. Under resting conditions, skeletal muscle needs insulin in order to enhance the glucose uptake. After the insulin bound to its membrane receptor, it triggers a cascade of intracellular reactions, which leads to the activation of the glucose transporter 4, GLUT4. This transporter migrates to the plasma membrane and contribute to the internalization of glucose **(32)**.

Considering the fact that PPARγ is one the main factors involved in the regulation of the GLUT4, the possible mechanism of increase in intracellular glucose concentration in the presence of high concentrations of extracts is likely related to PPARγ **(33)**.

Conclusion

Our findings demonstrate that the methanolic extracts of *P. harmala* seed and leaf have ability to increment not only the insulin secretion but also the glucose uptake. The methanolic extract of *P. harmala* seed, due to the presence of β-carbolines, has lower cytotoxicity than *P. harmala* leaf extract. The methanolic extracts of *P. harmala* leaf, however, has high antioxidant, making it toxic in high concentrations. Therefore, *P. harmala* seed and leaf extracts, because of their special compounds, have the potential to be used in designing new medicine for the treatment of diabetes.

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Not applicable.

Authors' Contribution

A. S.: formal analysis, writing-original draft preparation; S. O.: data curation, supervision, writing-review and editing; RA: methodology, writing-review and editing; K. P.: data curation, writing-review and editing.

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Conflict of Interest

The authors have declared no potential conflicts of interest in researching, authoring and/or publishing this article.

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