Original article

Protective Effects of Cinnamic Acid Against Hyperglycemia Induced Oxidative Stress and Inflammation in HepG2 Cells

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Abstract

Background: Cinnamic acid, a phenylpropanoid acid, has been investigated as a potential alternative therapy for diabetes and its complications in some studies.

Methods: In the first stage, the viability of HepG2 cells at different concentrations of glucose and CA was assessed by MTT assay. Oxidative stress markers) CAT, GPx, GSH, and MDA) were measured spectrophotometrically. After RNA extraction, the effect of different concentrations of CA on the expression of DPP4 and inflammatory factors (IL-6, NF- κB) in HepG2 cells was assessed using real-time PCR.

Results: In HepG2 cells, CA increased catalase and glutathione peroxidase activity and GSH production in a dose-dependent manner in the presence of high glucose concentrations, with the greatest effect seen at a concentration of 75 mg/ml. Also, it reduced the amount of MDA in high-glucose HepG2 cells. Furthermore, CA decreased the expression of DPP4, NF- κB, and IL-6 genes in HepG2 cells in the presence of high glucose levels.

Conclusions: The results of our study indicated that CA reduced hyperglycemia-induced complications in HepG2 cells by decreasing inflammatory gene expression, including IL-6 and NF- κB and inhibiting the expression of DPP4, and limiting oxidative stress.

Keywords: Cinnamic acid, Diabetes, HepG2 cells, Hyperglycemia ss, Oxidative stre.

Introduction

Cinnamic acid, a naturally occurring aromatic fatty acid, is considered a plant hormone, regulating cell growth and differentiation (Auxins). Cinnamic acid is the product of phenylalanine deamination utilized by the phenylalanine ammonia-lyase enzyme (1). This compound is reported to have multiple biological activities including antioxidant, anti-inflammatory, hepatoprotective, antityrosinase, antidiabetic activities, anti-malarial and anti-cancer properties (2-8).

Diabetes is a metabolic disorder of the endocrine system with multisystem complications, which is known as a leading cause of morbidity worldwide. The prevalence of diabetes mellitus is considered to extend over the following decades, which presents this disease as a significant public health concern; thus, searching for better treatment is vital (9).

A higher risk of atherosclerosis, kidney, and nerve damage has been noted in diabetic patients(10). Autoxidation reactions of glucose

adducts to protein, unsaturated lipids in plasma and membrane proteins that undergo autoxidation, hypertension, and impaired free radical scavenger and inhibitory systems induce free radicals, which then provoke various complications in diabetic patients (11). Enzymatic antioxidant defenses such as catalase (CAT) and glutathione peroxidase (GPx) counteract these effects. Reactive oxygen species lead to the peroxidation of membrane lipids and the production of malondialdehyde (MDA), and tissue damage (12).

Glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide 1 (1-GLP) are both incretins released after eating, which can increase insulin secretion. Dipeptidyl peptidase-4 is a membrane-bound peptidase from the family of prolyl oligopeptidase enzymes that degrades GLP-1 and GIP, which can pose a concern in diabetic patients; therefore, researchers aim to find DPP-4 inhibitors (13).

Inflammatory response contributes to insulin resistance in diabetic patients. Several pro-inflammatory factors, such as tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6), are produced in adipose tissue macrophages (14). TNF-α reduces insulin resistance by decreasing tyrosine kinase activity. In addition, TNF-α inhibits insulin signaling and disrupts its (15,16). secretion However, the tyrosine phosphorylation of IR substrate (IRS)-1 is reduced by IL-6 (17). NF- κB is associated with the transcription of most pro-inflammatory molecules, including TNF-α; IL-6, Homocysteine, which eventually leads to beta-cell dysfunction and apoptosis during diabetes (18,19).

Hyperglycemia contributes to the development of insulin resistance by producing reactive oxygen species (ROS) and upregulating chronic inflammation factors (20). The HepG2 cells have been defined as a cell line for *in vitro* investigation of hyperglycemia (21).

In recent years, there have been multiple studies investigating the role of cinnamic acid in the prevention, control and treatment of diabetes and its complications. However, the mechanism of effectiveness of this natural substance has not yet been entirely determined (22).

Despite numerous studies on cinnamic acid's anti-inflammatory and antioxidant properties, the efficiency of these properties in hyperglycemic conditions is still unclear (23-25). To the best of our knowledge, this is the first research to explore the mechanism of effectiveness of cinnamic acid as a possible anti-diabetic agent in the HepG2 cell line under hyperglycemic conditions.

Hence, the present study was designed to investigate the effects of cinnamic acid as a therapeutic potential on reducing oxidative stress, expression of inflammatory factors, and inhibition of DPP-4 in HepG2 cell line in the presence of high glucose concentration.

Materials and Methods
Cell culture and treatment
Human hepatoma cell line, HepG2, packaging cells were purchased from the Pasteur Institute of Iran. HepG2 cells were cultured in Roswell Park Memorial Institute (RPMI) medium, which included 10% fetal bovine serum (FBS) and 1.5% penicillin-streptomycin. Afterwards, the cells were incubated at 37 °C in a humidified environment of 5% CO2.
HepG2 cells were divided into eight groups:
i. Control cells,
ii. Control cells +25 mg/ml Cinnamic acid,
iii. Control cells +50 mg/ml Cinnamic acid,
iv. Control cells +75 mg/ml Cinnamic acid,
v. Cells treated with 60 mM D- glucose (high glucose),
vi. Cells treated with 60 mM D- glucose (high glucose) + 25 mg/ml Cinnamic acid,
vii. Cells treated with 60 mM D- glucose (high glucose) + 50 mg/ml Cinnamic acid,
viii. Cells treated with 60 mM D- glucose (high glucose) + 75 mg/ml Cinnamic acid.

Cell viability assay
The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the effect of glucose and cinnamic acid (Merck) on HepG2 cell viability. In brief, 3×10^4 cells/well were seeded onto 96-well plates and treated with different glucose or cinnamic acid doses. The plates were incubated for 24, 48, and 72 hours at 37 °C with 5% CO2.

Subsequently, MTT (0.5 mg/mL PBS) was added to each well and incubated for 3 hours at 37 °C. Finally, each plate absorbance was measured at 570 nm (26).

Biochemical Analysis
Measurement of CAT activity
Catalase activity was measured in accordance with previous study by measuring the decomposition rate for hydrogen peroxide (H2O2) at 240 nm (27).

Measurement of MDA
malondialdehyde (MDA) levels were measured in accordance with previous study, by assessment of Thiobarbituric acid reactive substances. The spectrophotometric measurements of absorbance were taken at 532 μm. MDA concentrations were represented as μmol/ mg-pr (28).

Measurement of GPx activity
Glutathione peroxidase activity was measured using commercial kits (Aryagen Sobhan Azma Novin (ASAN), Iran).

Measurement of GSH
Glutathione level was measured using commercial kits (Aryagen Sobhan Azma Novin (ASAN), Iran).

RNA extraction and real-time PCR
Total RNA extraction from HepG2 cell lines was performed according to the instructions of previous studies (29). The absorption of the extracted solution was then measured at 260 and 280 using NanoDrop. The purification of the RNA extraction product was investigated using agarose gel electrophoresis, and the appearance of clear 18s and 28s RNA bands was an indication of high-quality RNA. To convert RNA into cDNA, commercial kits (YTA, Yekta Tajhiz Azma, Iran) were used according to the manufacturer's instructions. For real-time PCR assessment, 2 x SYBR Green qPCR Mix (1 X), 0.2 microliters of targeted gene primers (Table 1), one microliter of cDNA template, and 8.2 microliters of RNAse-free water comprise the reaction mixture. Under the following conditions, the final reaction solution (20 L) was evaluated for proliferation: Initial denaturation stage at 95 °C for 3 minutes; then 40 cycles of denaturation for 5 seconds at 95 °C and annealing for 30 seconds at 60 °C in a Real-Time PCR System thermocycler, proceeded with a 5-minute final extension step at 50-99 °C. The products were placed onto a 1% agarose gel in 1X TBE solution, and the PCR bands were detected using an ultraviolet transilluminator.

Table 1. List of primers used in this study.

Gene	Forward (5' -3)	Reverse (5' -3)	Product size (bp)
GAPDH	CGACCACTTTGTCAAGCTCA	AGGGGTCTACATGGCAACTG	228
DPP-4	AAGATGGAACTGCTTAGTGG	TAGAGCTTCTATCCCGATGAC	226
IL-6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC	81
NF-κB	AACAGAGAGGATTTCGTTTCC G	TTTGACCTGAGGGTAAGACTTCT	104

Statistical analysis
After confirming the normality of biochemical data by the Shapirovilk test, the final data were analyzed by one-way ANOVA, and then the means were compared by Tukey post hoc test at 5% significance. The analysis of the gene expression results was normalized by the expression of the GAPDH gene as a reference gene. Samples were performed in triplicate replication. The raw data (Ct) generated by the real-time was converted to Fold change. Finally, after normalization, the final data were analyzed by one-way ANOVA and means were compared by Tukey post hoc test at 5% significance. Data analysis was performed using SPSS software (version 24).

Results

Effect of D-glucose and Cinnamic acid on the viability of HepG2 cells

The HepG2 cells viability were not harmed by 10 mM glucose replicating normoglycemic conditions at 24 or 48 hours (97–98 percent viability) (Fig. 1). After a 24 or 48-hour incubation time, 20, 40, or 60-mM glucose did not cause toxicity in the HepG2 cells (86–99% viability). Although incubation of HepG2 cells with 70 mM glucose did not cause toxicity in 24 hours (92%), after 48 hours, it demonstrated a decrease in viability (73%) (Fig. 1).
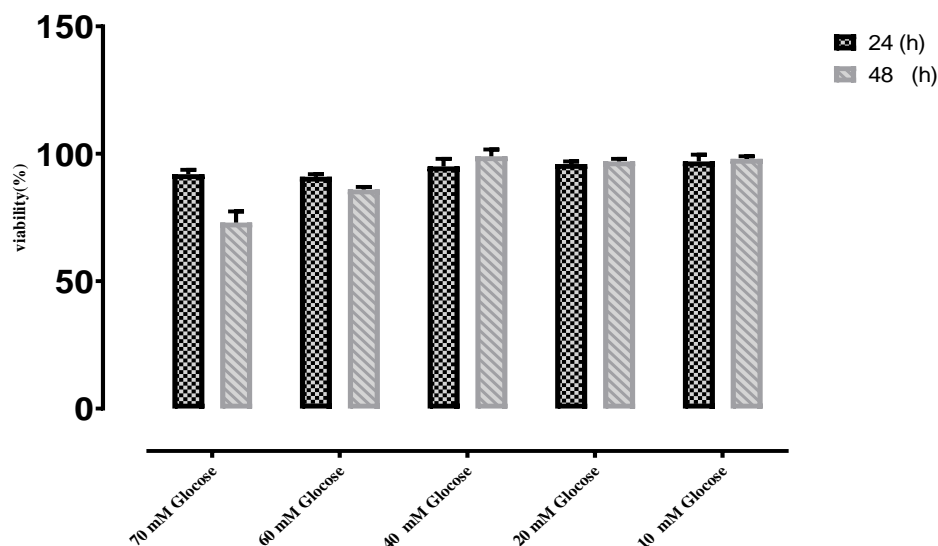

Fig. 1. Impact of glucose on the viability of HepG2 cells after 24- and 48-hours treatment and at different concentrations. The findings are presented as a percentage relative to control and are calculated as the mean (standard deviation) of three separate measurements.

Furthermore, HepG2 cell viability was not affected after incubation for 48 hours with 25, 50, 75 µg/ml of cinnamic acid at the presence or absence of 60 mM glucose (81–94% viability) (Fig. 2). The above concentration and time span were employed in future assessments to reproduce hyperglycemia-induced effects in HepG2 cells based on the reported results.
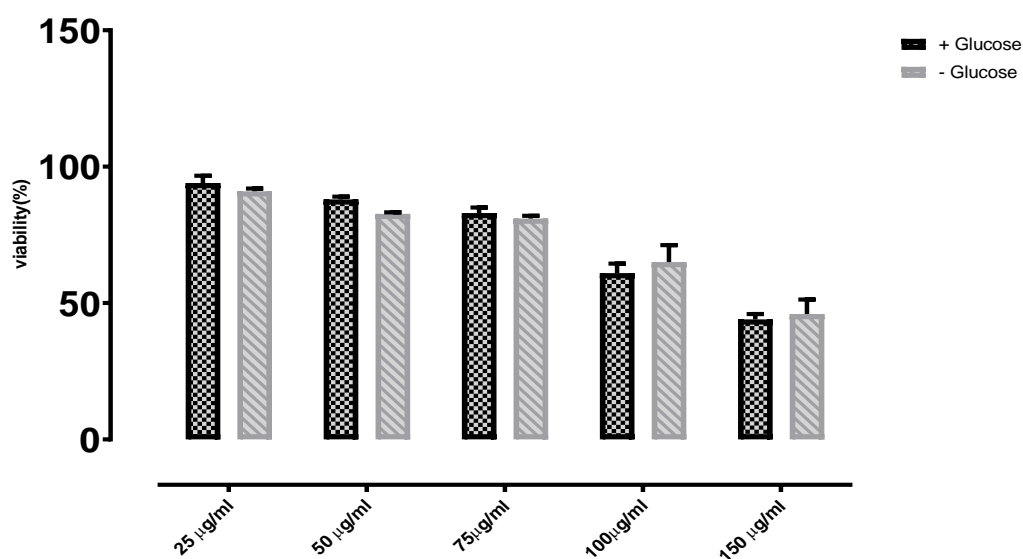

Fig. 2. Impact of different concentrations of cinnamic acid on the viability of HepG2 cells after 48 hours treatment in the presence or absence of 60 mM of glucose. The findings are presented as a percentage relative to control and are calculated as the mean (standard deviation) of three separate measurements.

Table 2. Effect of cinnamic acid on oxidative stress markers in HepG2 cells. Different letters in each row indicate a significant difference between groups.

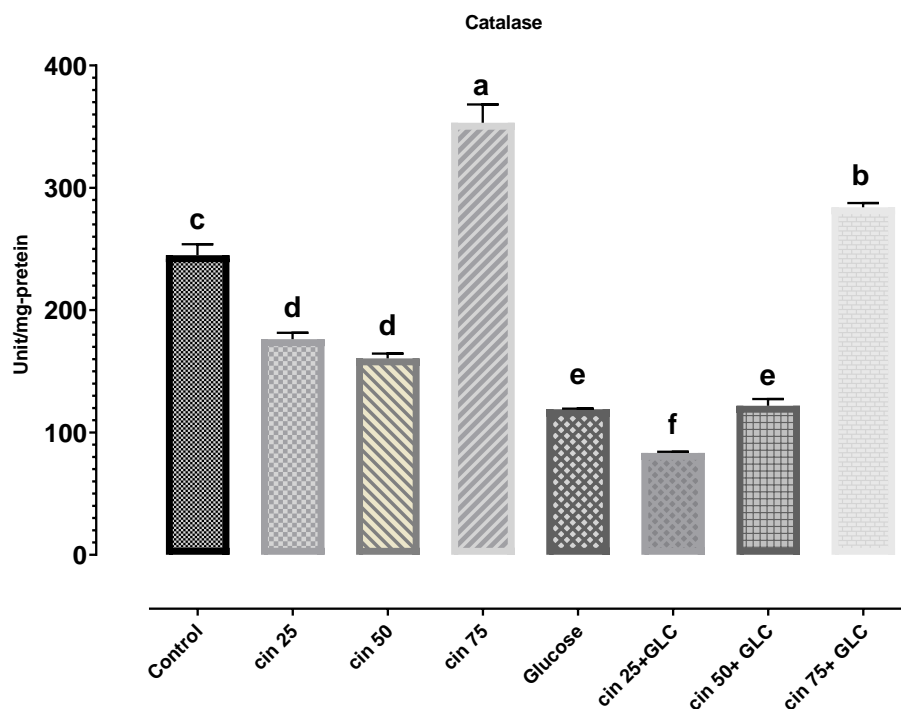| Parameter	Protein concentration ± SD								P Value
	Control	Glucose	cin 25	cin 25+GLC	cin 50	cin 50+ GLC	cin 75	cin 75+ GLC	
Catalase (unit/mg of protein)	244.85 ±18.23[e]	118.975 ±0.952[ef]	176.29 ±10.3[d]	83.27 ±2.10[f]	160.51 ± 4.16[d]	121.94 ±10.82[e]	353.1 ±30.1[a]	284.15 ±6.81[b]	<0.0001
GPX (unit/mg of protein)	560.7 ±60.9[de]	510.98 ±18.64[e]	616.8 ±37.6[cd]	552.6 ±20.7[de]	628.57 ±15.71[cd]	646.1 ±58.2[bc]	872.41 ±10.37[a]	719.50±17.2[b]	<0.0001
GSH (µmole/mg of protein)	1.88 ±0.0756[ab]	1.622 ±0.00789[c]	1.51± 0.0425[c]	1.58 ±0.01259[c]	1.58 ±0.0642[c]	1.78 ±0.0934[b]	2.01 ±0.0295[a]	1.870 ±0.0907[b]	<0.0001
MDA (µmole/mg of protein)	120.79 ±7.75[d]	285.74 ±1.87[a]	123.77± 7.2[d]	196.6 ±4.83[b]	116.63 ±6.108[d]	180.81 ±14.7[b]	112.36 ±5.32[d]	158.51 ±1.048[c]	<0.0001

Fig. 3. Impact of different concentrations of cinnamic acid on the CAT activity in HepG2 cells after 48 hours treatment in the presence or absence of 60 mM of glucose. The findings are presented as U/mg protein and are calculated as the mean (standard deviation) of three separate measurements. Different letters in each column indicate a significant difference between groups.

Catalase activity in high/normal glucose treated HepG2 cells

The catalase activity was significantly reduced by 51.4% in HepG2 cells, which received 60 mM glucose (Table 2 and Fig. 3). Although 25 and 50 µg/ml Cinnamic acid reduced catalase activity in HepG2 cells (176.29±10.33 and 160.51±4.16, respectively), 75 µg/ml CA significantly increased catalase activity by 44.21% in normal glucose cells (P< 0.05). The HEPG cells adjacent to 25 µg/ml Cinnamic acid, presented lower catalase activity compared to HEPG2 cells. However, cells receiving 50 µg/ml had a higher catalase activity compared to HEPG2 cells. The HEPG2 cells which received 75 µg/ml Cinnamic acid possessed a significantly higher catalase activity compared to HEPG2 cells (P< 0.05).
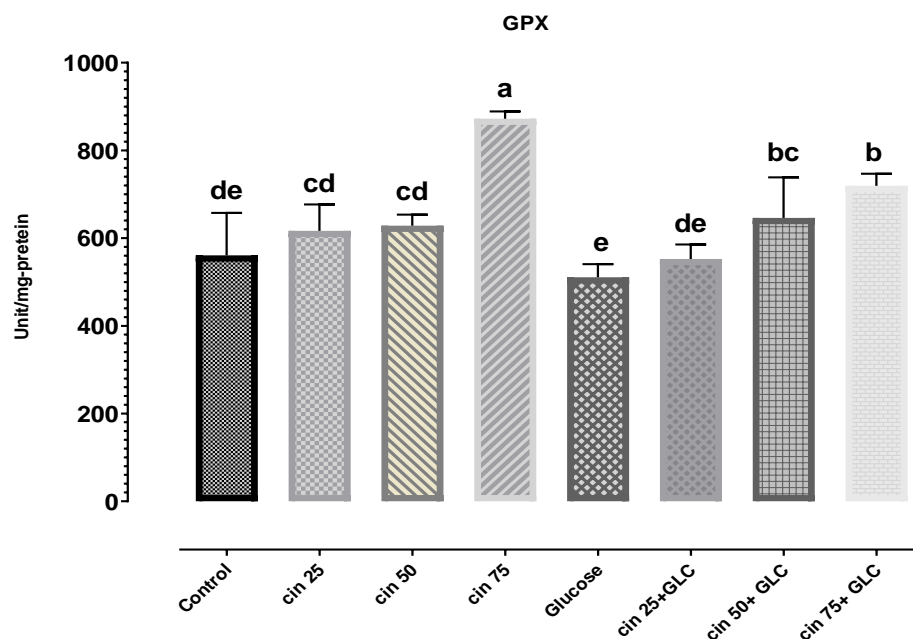
GPX

Fig. 4. Impact of different concentrations of cinnamic acid on the GPX activity in HepG2 cells after 48 hours treatment in the presence or absence of 60 mM of glucose. The findings are presented as U/mg protein and are calculated as the mean (standard deviation) of three separate measurements. Different letters in each column indicate a significant difference between groups.

The GPx activity was reduced in high glucose cells by 8.9% (Fig. 4). In the NG cells which received 75 µg/ml Cinnamic acid, GPx activity was induced to 872.41±10.37, which was significantly higher than control and NG cells with 25 and 50 µg/ml CA (P< 0.05) (560.7±60.9,616.8±37.6, and 628.57±15.71 respectively). The high glucose cells which received CA showed the same pattern as NG cells. The GPX activity was induced dose-dependently in HEPG2 cells which received CA (552.6±20.7, 646.1±58.2 and 719.50±17.25 respectively) and CA at 50 and 75 µg/ml showed significantly higher GPX activity by 26.4% and 40.8% compared to the HEPG2 group (P< 0.05).
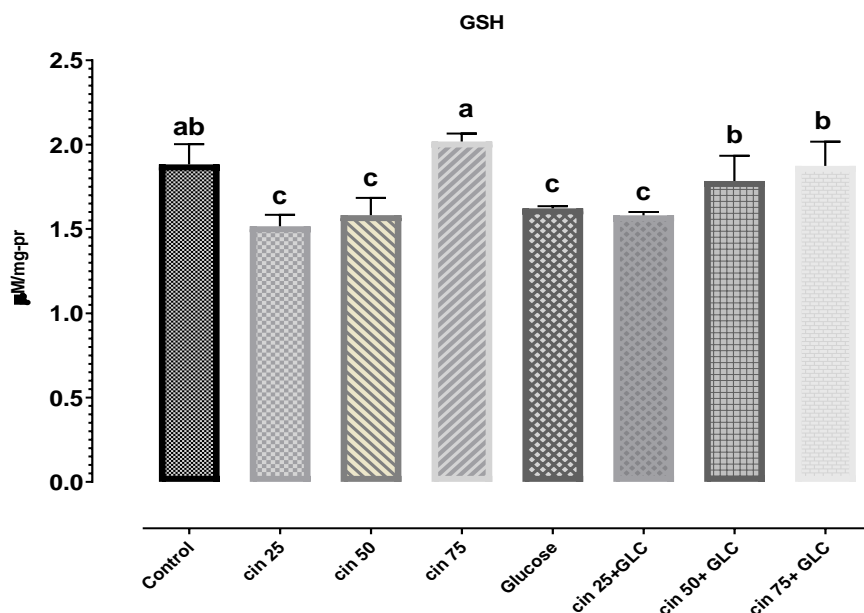
GSH

Fig. 5. Impact of different concentrations of cinnamic acid on the GSH protein in HepG2 cells after 48 hours treatment in the presence or absence of 60 mM of glucose. The findings are presented as µM/mg protein and are calculated as the mean (standard deviation) of three separate measurements. Different letters in each column indicate a significant difference between groups.

The HepG2 cells had a significantly lower amount of GSH (1.62±0.01) compared to the control group (1.88±0.07) (Fig. 5). No significant increase was observed in the level of GSH in the NG cells, which received cinnamic acid. The quantity of GSH in HEPG2 cells increased dose-dependently with cinnamic acid (1.58±0.01, 1.78±0.09, and 1.87±0.09 respectively). The GSH content of HEPG2 cells treated with cinnamic acid at 50 and 75 µg/ml was significantly higher than HEPG2 cells which received no treatments (P< 0.05).
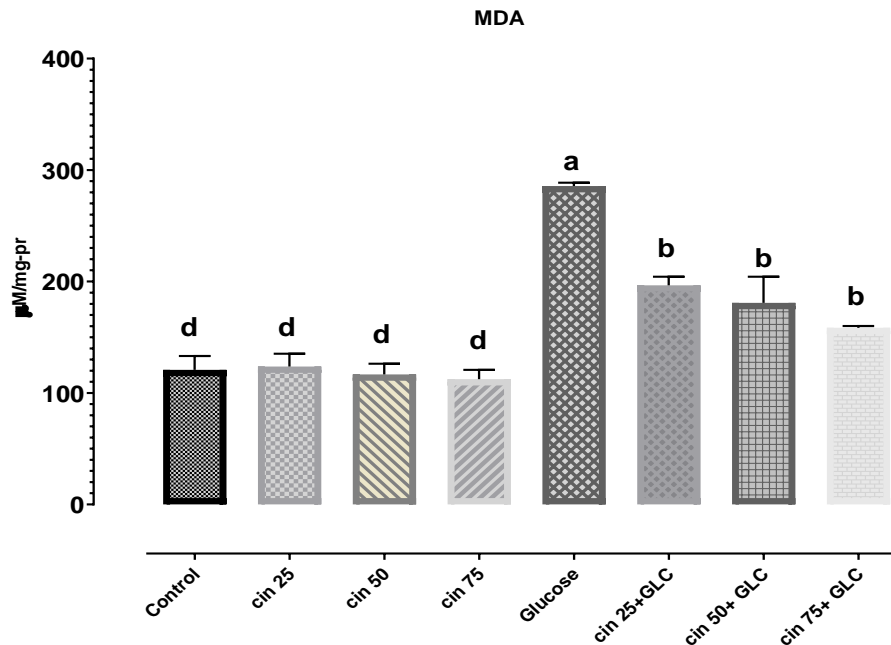

Fig. 6. Impact of different concentrations of cinnamic acid on the MDA protein in HepG2 cells after 48 hours treatment in the presence or absence of 60 mM of glucose. The findings are presented as µM/mg protein and are calculated as the mean (standard deviation) of three separate measurements. Different letters in each column indicate a significant difference between groups.

After 48 hr treatment, malondialdehyde level as a marker of lipid peroxidation was significantly elevated in HepG2 cells (285.74±1.87) compared to the control group (120.79±7.75) (P< 0.05) (Fig. 6). Cinnamic acid exhibited no significant increase or decrease in the level of MDA in NG cells. However, in HepG2 cells treated with 60 mM D - glucose, cinnamic acid presented a significant dose-dependent decrease in the MDA level compared to HEPG cells with no cinnamic supplement (P< 0.05) (196.6±4.83, 180.81±14.76, and 158.51±1.04, respectively). Cinnamic acid at 75 µg/ml demonstrates the highest activity with a 44.5% lower MDA level compared to the glucose group.

Table 3. Effect of cinnamic acid on the expression of DPP4 and inflammatory cytokines in HepG2 cells. Different letters in each row indicate a significant difference between groups.

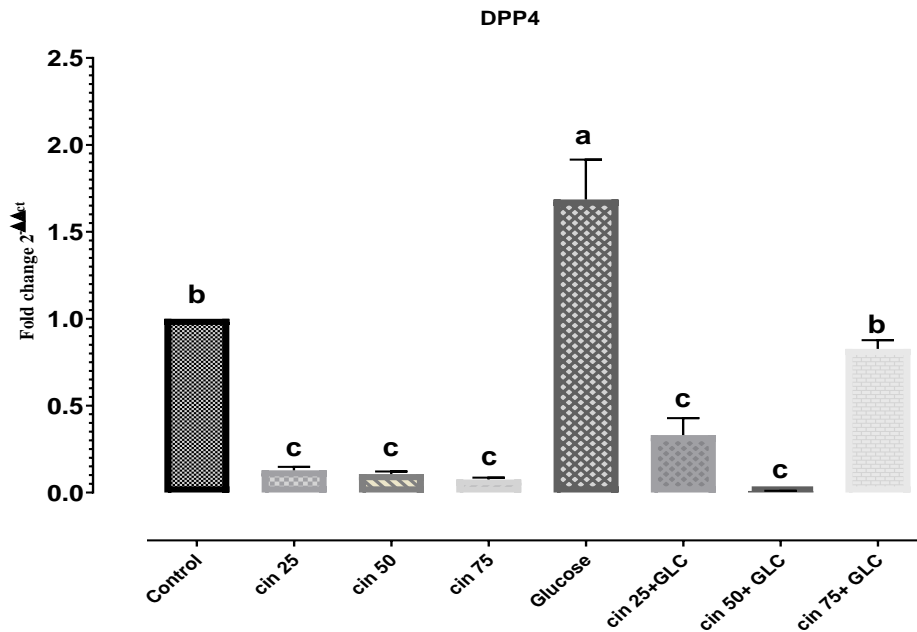| Gene	Group								P-Value
	Control	Glucose	cin 25	cin 25+GLC	cin 50	cin 50+ GLC	cin 75	cin 75+ GLC	
DPP4	1[b]	1.686[a]	0.1282[c]	0.3312[c]	0.1070[c]	0.0091[c]	0.0754[c]	0.8267[b]	<0.0001
NF- κB	1[b]	2.3623[a]	0.6676[bc]	0.6899[bc]	0.4026[c]	0.6017[bc]	0.2415[c]	0.5101[bc]	<0.0001
IL-6	1[d]	3.281[a]	0.4256[e]	2.257[b]	0.6470[de]	1.536[c]	0.5387[e]	0.6200[e]	<0.0001

DPP4

Fig. 7. Impact of different concentrations of cinnamic acid on the DPP4 gene expression in HepG2 cells after 48 hours treatment in the presence or absence of 60 mM of glucose. The findings are presented as Fold change 2-ΔΔCt compared to the control group. Different letters in each column indicate a significant difference between groups.

DPP4 expression was substantially increased by 1.686-fold in HepG2 cells in companies with a high level of glucose compared with control (Fig. 7) (P< 0.05). Compared to the control group, CA have significantly reduced DPP4 expression in normal glucose HepG2 cells (P< 0.05). Cells that received high amounts of glucose alongside cinnamic acids have shown to reduce DPP4 expression compared to cells which only received glucose. Cinnamic acid at 25 and 50 µg/ml had a significantly greater effect in DPP4 reduction in HepG2 cells, which received a high level of glucose (P< 0.05).
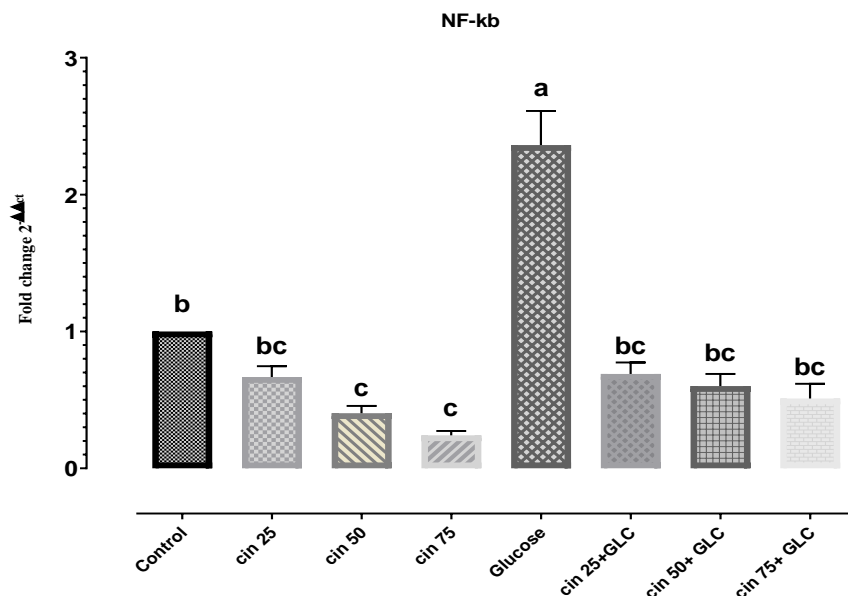
NF-kb

Fig. 8. Impact of different concentrations of cinnamic acid on the NF- κB gene expression in HepG2 cells after 48 hours treatment in the presence or absence of 60 mM of glucose. The findings are presented as Fold change 2-ΔΔCt compared to the control group. Different letters in each column indicate a significant difference between groups.

The NF- κB pathway has been activated in the presence of a high level of glucose in the cells, which causes the upregulation of inflammatory cytokines. After being exposed to high glucose levels, the NF- κB expression was significantly elevated by 2.3623-fold compared to the NG HepG2 cell line (P< 0.05) (Fig. 8). Although cells that were only exposed to cinnamic 25 µg/ml did not show a significant decrease, CA in 50 and 75 µg/ml showed a significant decrease in expression of NF- κB compared to HepG2 cell line, which had not received any supplement (P< 0.05). High glucose HepG2 cells that received cinnamic acid had significantly lower expression of NF- κB compared to cells that only received a high level of glucose (P< 0.05) (Fig. 8).
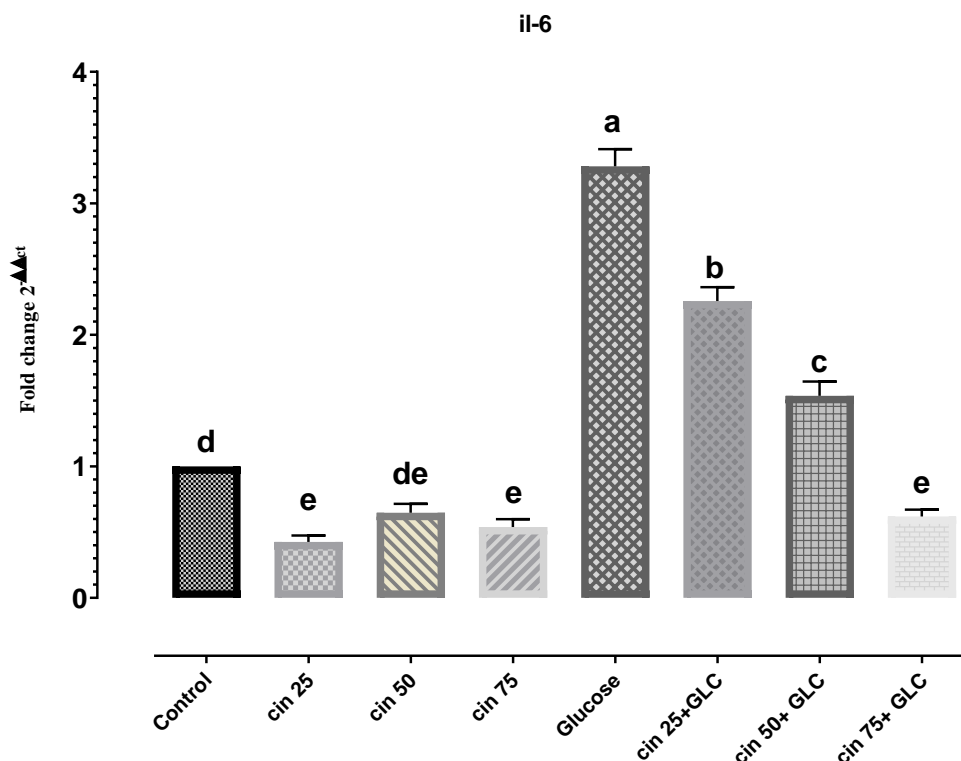

Fig. 9. Impact of different concentrations of cinnamic acid on the IL-6 gene expression in HepG2 cells after 48 hours treatment in the presence or absence of 60 mM of glucose. The findings are presented as Fold change 2-ΔΔCt compared to the control group. Different letters in each column indicate a significant difference between groups.

IL-6 expression was significantly 3.281-fold higher in HepG2 cells treated with a high level of glucose compared to control (P< 0.05). The CA significantly reduced normal glucose cells IL-6 expression at 25 and 75 µg/ml (P< 0.05). Cinnamic acid significantly reduced IL- 6 expression in high glucose cells at 50 µg/ml compared to cells that received 25 µg/ml CA (P< 0.05). Moreover, CA at 75 µg/ml showed the best effect on reducing IL-6 expression in HepG2 cells and was significantly greater than CA at 50 µg/ml (P< 0.05) (Fig. 9).

Discussion
In recent years, dipeptidyl peptidase-IV inhibition has emerged as a promising novel therapeutic approach for diabetes management (30). Our results demonstrated a reduction in DPP4 expression in both normal and high glucose HepG2 cells treated with cinnamic acid at all concentrations. In the hyperglycemic cells, cinnamic acid at 50 mg/ml showed the highest activity. Our lab conditions may be responsible for the lack of dose-dependent activity of cinnamic acid in HEPG cells. In an

in vitro assays by Adolpho et al.'s 2013 study, cinnamic acid showed 4.4% inhibitory activity against DPP-IV at the concentration of 500 µM (31). Cinnamic acid also demonstrated −5.5 kcal/mol Docking score interaction with DPP-4 in a 2017 study (32). These results are compatible with the results of our study. The activation of the nuclear factor kappa B gene leads to the transcription of several proinflammatory genes, and it is one of the target therapies for diabetes and its complications (18).

According to a 2018 report by Ghodratollah Panahi et al., a high level of glucose induces inflammation in HepG2 cells through oxidative stress and activation of the NF-κB pathway (33). We explored NF- κB and IL-6 expressions in HepG2 cells to determine the possible molecular mechanism underlying cinnamic acid's anti-inflammatory properties. There are many studies that showed the role of cinnamic acid and others natural compounds from herbal medicins in the prevention, control and treatment of diabetes and its complications (34,35).Our research showed that cinnamic acid has the ability to significantly reduced the upregulated NF- κB gene to reduce the hyperglycemic induced inflammation. The cinnamic acid also showed the ability to reduce the NF- κB expression at 50 and 75mg/ml in NG cells. According to a 2006 report, cinnamic acid inhibits NF- KB expression in human umbilical endothelium-derived cell lines, consistent with the findings of our investigation in HepG2 cells (36).

Interleukin-6, a pro-inflammatory cytokine, has been shown to play a key role in insulin resistance in HepG2 cells (17). Cinnamic acid has been shown to inhibit LPS-induced IL-6 expression in microglia (37). In our study, IL-6 expression was elevated in HEPG cells; however, cinnamic acid effectively reduced IL-6 expression dose-dependently.NG cells treated with cinnamic acid also showed lower IL-6 express ion compared to the control group. In Song F et al. study, Cinnamic acid reduced serum levels of TNF-α and IL-6 in isoproterenol-induced acute myocardial

ischemia rats, which is consistent with our results (23).

Our findings, which were compatible with previous results, revealed that the oxidative stress induced by hyperglycemia in HepG2 cells reduced the activity of antioxidant enzymes (catalase and glutathione peroxidase), and glutathione level. Hyperglycemia also increased the level of malondialdehyde as an oxidative stress marker (38). In a study by Hatice Gül ANLAR et al., the CAT and GPX activity diabetic rats which received 50mg/kg cinnamic acid were significantly higher than diabetic rats with no treatments, which is in the same line of our study in hyperglycemic HepG2 cells. However, the results of our study demonstrated that cinnamic acid at 75 mg/ml could significantly induce the CAT activity and CA at 50 and 75 mg/ml showed an increased GPx activity compared to HEPG cells. However, the Gül ANLAR study demonstrated no significant alteration in CAT and GPx activity in CA treated rats compared to control; this may be due to a difference in the amount of cinnamic acid used.

The GSH level was also significantly higher in the diabetic rats which had received cinnamic acid compared to diabetic rats with no treatments; however, cinnamic acid did not show a significant increase in the level of GSH in rats which only received cinnamic acid compared to control, which is consistent with the result of our study in HEPG cells.

The MDA level was also significantly lowered in CA-treated diabetic rats compared to diabetic rats with no treatments, and no significant difference was observed in normal rats, which also consisted of our results (39).

Our research's major limitation is that it was conducted as an *in vitro* assay, and the CA antidiabetic mechanism must be further investigated in human body circumstances and compared to the results of this study. However, our findings in HepG2 cells demonstrated that cinnamic acid could ameliorate hyperglycemic-induced complications by lowering inflammation by inhibiting the

expression of IL-6, NF- κB and DPP4, and reducing oxidative stress.

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