Research Article

Protective effect of *Citrullus colocynthis* (L.) Schard. fruit extract on high glucose-induced neurotoxicity in PC-12 cells

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**ABSTRACT**

**Background:** Diabetic neuropathy is a consequence of chronic hyperglycemia that leads to neural damage over time. The fruits of *Citrullus colocynthis* (L.) Schard. (*C. colocynthis*) have long been used in Iranian traditional medicine for treatment of diabetes. **Objective:** In this study, we investigated the protective effects of *C. colocynthis* against high glucose-induced neurotoxicity in PC-12 cells as a suitable *in vitro* model for neuronal functions. **Methods:** The seedless dry fruit powder was extracted with methanol and then dried by rotary evapoartion. Later the obtain extract was fractionated as hexane, chloroform, ethyl acetate, and n-butanol fractions and aqueous residue. HPLC method was used for qualitative analysis of colocynthin in *C. colocynthis* pulp. The PC-12 cell viability of total extract and fractions of *C. colocynthis* were evaluated by means of dose and time in high glucose medium in PC-12 cells as a suitable culture model for studying neuronal functions through MTT assay. **Results:** The PC-12 cell viability significantly decreased in wells containing high glucose, compared with normal glucose treated cells. *C. colocynthis* extract treatment significantly enhanced the cells viability under toxic high glucose condition in a dose and time depending manner. Methanol extract of *C. colocynthis* exhibited a protective effect against high glucose-induced cytotoxicity in PC-12 cells. **Conclusion:** *C. colocynthis* has neuroprotective properties against high glucose condition *in vitro*.

1. **Introduction**

The main characteristic of diabetes mellitus is increase in blood sugar level due to insufficiency in insulin secretion by pancreas beta-cells or presence of insulin resistance at the cellular level [1]. Chronic hyperglycemia can cause damage to almost all the body nerve called neuropathy, affecting the motor, sensory or autonomic functions. Developments in diabetes are leading to increasing importance of investigating the potential beneficial effects of traditional medicine in the treatment and prevention of complications of diabetes mellitus. *Citrullus colocynthis* fruit has been used in traditional medicine for treatment of diabetes and its complications. In the present study, the protective effects of *C. colocynthis* against high glucose-induced neurotoxicity in PC-12 cells were investigated.

Abbreviations: MTT, 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide; HPLC, High performance liquid chromatography; EDTA, Ethylenediamine tetraacetic acid

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The effect of Citrullus colocynthis H. Fallah Huseini, et al

nerves. This condition results in undesired and destructive impacts on the digestive system, urinary tract, sexual function, blood vessels, heart, and eyes [2]. It was well established that in hyperglycemia, overproduction of free radicals leads to metabolic dysfunction which allows reactive oxygen species to attack and damage proteins, lipids, and nucleic acids and eventually cause cellular and tissue damage [3]. There is evidence that increased oxygen free radicals due to hyperglycemia, is associated with the development of apoptosis in nerve cells and consequently neuropathy [3, 4]. Given that, antioxidant therapy may be a proper rational to improve neural protection, particularly in diabetic subjects [5]. Up to date, many studies confirmed the significance of herbal remedies with antioxidant properties in prevention or control of diabetes [6, 7].

*C. colocynthis* (L.) Schard. (Cucurbitaceae), also known as bitter apple, is a medicinal plant traditionally used for diabetes treatment in Iran [8]. Experimental studies have reported its anti-diabetic and antioxidant properties [9 - 11]. The anti-diabetic and antioxidant effects of *C. colocynthis* were also observed in clinical trials [12, 13]. In order to provide evidence on mechanism of action of *C. colocynthis* in diabetic condition in neuronal cells, the protective effect of the methanolic extract and fractions of the fruits of *C. colocynthis* was investigated in high glucose treated PC-12 cells.

### 2. Materials and Methods

#### 2.1. Extraction, fractionation and isolation

*C. colocynthis* fruits were purchased from the local market and authenticated by an expert botanist. A voucher specimen was deposited (No. PMP-3601) in the herbarium of the Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran. The fruits were washed using deionized water and dried in the shade at room temperature. The seeds were removed manually from the dried fruit pulp. The dried fruit pulp was grounded and passed through the No. 4 mesh. The plant was then weighed (450 g) and extracted with 2500 ml methanol in percolator for 24 hours. The total methanol extract was dried by rotary evaporator at 50 °C. A portion of 20 g of dried crude extract was dissolve into distilled water and then subjected to a separator funnel. Then, it was fractionated by different solvents with different polarity including hexane, chloroform, ethyl acetate, and *n*-butanol. All fractions were evaporated to dryness at 50 °C using a rotary evaporator [14].

#### 2.2. HPLC analysis of the *C. colocynthis* pulp

HPLC method was used for qualitative analysis of colocynthin in *C. colocynthis* pulp. For this purpose, powdered *C. colocynthis* pulp (100 mg) was grinded with electronic grinder. 2 ml of methanol was added and shacked slightly by vortex for 30 seconds and placed in the sonicator for 10 min. The solid material was separated by centrifugation in 6000 rpm for 10 min. A determined volume (150 μL) of clear solution was diluted with 850 μl of Millipore water, passed through 0.45 μm PTFE filter and analyzed by HPLC [14].

#### 2.3. Assessment the effect of high glucose on PC-12 cell viability MTT assay

**Cell culture:** The PC-12 cells were obtained from the Pasteur Institute of Iran (Tehran, Iran). DMEM (Dulbecco’s Modified Eagle’s medium) and FBS (fetal bovine serum) were purchased from Biosera (UK). Pen-strep and trypsin-EDTA were purchased from Gibco (UK). MTT (3-[4, 5-dimethylthiazol-2-yl] 2, 5- diphenyl tetrazolium bromide) was purchased from sigma (Germany). Cells were maintained at 37 °C in a 90 % humid...
The effect of *Citrullus colocynthis* …

H. Fallah Huseini, et al

2.4. *MTT* cell viability assay

The cell viability was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay as previously described. Cells were incubated with 5 mg/ml MTT in RPMI, at 37 °C under 5 % CO₂ for 3 h. The blue formazan reduction product, produced by the action of succinate dehydrogenase in living cells, was dissolved in 100 µl dimethylsulfoxide (DMSO), and the optical density was read at 570 nm by an ELISA microplate reader (BioTek, USA) [15]. The concentration required for 50 % inhibition of cell viability (IC₅₀) were determined by a nonlinear regression analysis and expressed in mean ± standard error of mean (SEM).

2.5. Effect of high glucose on *PC-12* cell viability

*MTT* assay

PC-12 cells seeded in appropriate condition (as mentioned) and the cells were exposed to 13.5 and 27 mM glucose for 24, 48 and 72 h and the *MTT* cell viability assay was performed accordingly. Since the optimal glucose concentration for *PC-12* cell cultures is 4.5 mg/L, we simulated *in vitro* hyperglycemia by increasing the medium glucose level up to 13.5 and 27 mg/ml (three and six fold of the optimal glucose concentration) to investigate the influence of glucose on neuronal cell death [16].

2.6. Total extract treatments

*PC-12* cells were plated at the density of 10³ per well in a 96 micro plate well. The wells were divided into 9 groups (Table 1). Based on results obtained from the previous section, we found that both the concentrations of glucose either 13.5 or 27 mg/ml result in a similar toxicity in *PC-12* cells, therefore the concentration of 13.5 mg/ml was selected for this assay.

Table 1. The effective concentration of total extract against cytotoxicity of glucose

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sample name</th>
<th>Total extract (µg/ml)</th>
<th>Glucose (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A0</td>
<td>100</td>
<td>13.5</td>
</tr>
<tr>
<td>2</td>
<td>A1</td>
<td>25</td>
<td>13.5</td>
</tr>
<tr>
<td>3</td>
<td>A2</td>
<td>2.5</td>
<td>13.5</td>
</tr>
<tr>
<td>4</td>
<td>A3</td>
<td>0.25</td>
<td>13.5</td>
</tr>
<tr>
<td>5</td>
<td>A4</td>
<td>0.025</td>
<td>13.5</td>
</tr>
<tr>
<td>6</td>
<td>G1</td>
<td>0</td>
<td>13.5</td>
</tr>
<tr>
<td>7</td>
<td>G2</td>
<td>0</td>
<td>4.5</td>
</tr>
<tr>
<td>8</td>
<td>C</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

2.6. Assessment of the effect of *C. colocynthis* total extract and fractions on *PC-12* high glucose-induced cell toxicity

At first, we culture cells in normal medium. Following 70 % confluency, were switched from the standard culture (normal glucose DMEM) to the high glucose DMEM overnight (13.5 mg/ml). Based on our pilot study, we found that both the concentrations of glucose either 13.5 or 27 mg/ml result in a similar toxicity in *PC-12* cells, therefore the concentration of 13.5 mg/ml was selected for this assay. The *PC-12* cells were treated with different concentrations of the aqueous, methanol, hexane, chloroform and ethyl acetate fractions ranging from 0 to 250 µg/ml and the cell viability was investigated after 24, 48 and
The effect of *Citrullus colocynthis* …

H. Fallah Huseini, et al

72 h. So, we could find the best concentration (IC<sub>50</sub>) of every fractions which show higher protective effect against high glucose.

### 3. Results

#### 3.1. Extraction, fractionation and isolation

The yield of total methanol extract of the plant was 11.47%. The yields of the fractions in 20 g of dry methanol extract were presented in Table 2.

#### 3.2. HPLC analysis of the *C. colocynthis* pulp

The HPLC chromatogram confirmed the presence of the colocynthin peak (RT = 13.277 min) comparison with the study by Shekarchi et al (Fig. 1) [14].

#### 3.3. Effect of high glucose on PC-12 cell viability

**MTT assay**

Co-culture of PC-12 cells and 13.5 and 27 mM glucose for 24, 48 and 72 h exhibited toxicity within the cells, reaching a maximal effect after 72 h. Furthermore, we found that concentration of 13.5 and 27 mM show same cytotoxicity and therefore for following experiment we used 13.5 mM glucose for hyperglycemia condition (Fig. 2).

**Table 2.** The yields of methanol extract and fractions of *C. colocynthis*

<table>
<thead>
<tr>
<th>Methanol Extract</th>
<th>Hexane Fraction</th>
<th>Chloroform Fraction</th>
<th>Ethyl acetate Fraction</th>
<th>Butanol Fraction</th>
<th>Aqueous Residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield (%)</td>
<td>11.47</td>
<td>1.5</td>
<td>37</td>
<td>3</td>
<td>20.5</td>
</tr>
</tbody>
</table>

**Fig. 1.** HPLC Chromatogram of fruit pulp extract of *C. colocynthis*
The effect of *Citrullus colocynthis* … H. Fallah Huseini, et al

Fig. 2. Effects of glucose concentration and culture time on PC-12 cell viability. Data are presented as mean ± SEM (n = 4). The difference between the control group and the treatment group with different concentrations of glucose after 24, 48, and 72 h. significant level: *P < 0.001.

3.4. Effect of *C. colocynthis* total extract on glucose-induced cell toxicity

Co-cultivation of G2: 13.5 mg/ml glucose and PC-12 cells led to a significant cytotoxicity. Instead, co-administration of glucose and *C. colocynthis* at concentrations of 2.5 µg/ml, 0.25 µg/ml and 0.025 µg/ml significantly maintained the PC-12 cell viability and exhibited a positive neuroprotective effect against glucose-induced cell toxicity. However, *C. colocynthis* at concentrations of 100 µg/ml was not effective and the cell viability reduced below 10% (Fig. 3).

Fig. 3. Protective effect of total extract against glucose in PC-12 cells after at 24, 48, and 72 h time intervals. Cell viability was measured by MTT assay. Results are reported as the mean S.E.M (n = 4). *P < 0.001 compared to controls.
3.5. The effect of *C. colocynthis* fractions on glucose-induced cell toxicity

PC-12 cells were treated with different concentrations of aqueous, methanol, hexane, chloroform and ethyl acetate fractions ranging from 0 to 250 µg/ml for 24, 48 and 72 h. Therefore, the most effective concentration of each fraction with the lowest toxicity towards PC-12 cells was selected to be used in further study against high glucose treatment (Table 3).

To elucidate the protective actions of *C. colocynthis* fractions *in vitro*, PC-12 cell viability was assessed under hyperglycemic conditions (13.5 mg/ml glucose). The protective effect of each fraction against cytotoxicity of glucose has shown in Fig. 4. The ethyl acetate and butanol fractions of *C. colocynthis* exhibited the best protective effect against glucose-induced cell toxicity as compared with that of hexane, chloroform and aqueous treated cells.

Table 3. The most effective (lowest cytotoxicity) concentration (µg/ml) of each fraction against PC-12 cells

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Aqueous</th>
<th>Hexane</th>
<th>Chloroform</th>
<th>Ethyl acetate</th>
<th>Butanol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>125.0</td>
<td>250.0</td>
<td>62.50</td>
<td>125.0</td>
<td>62.50</td>
</tr>
</tbody>
</table>

Fig. 4. The protective effect of different *C. colocynthis* fractions on PC-12 cells cultured under hyperglycemic condition. Data are presented as mean ± SEM (n = 4). Significant level: ∗ P < 0.001
The effect of *Citrullus colocynthis* ...

**4. Discussion**

In current study we have examined the protective effect of *C. colocynthis* against hyperglycemic condition in PC-12 cells by MTT cell viability assay. Aside from hypoglycemic effect of this plant, the study intended to determine whether *C. colocynthis* has a direct protective effect on nerve cells. Considering our results, the methanolic extract of *C. colocynthis* at concentrations of 2.5 µg/ml, 0.25 µg/ml, and 0.025 µg/ml significantly protected PC-12 cells against toxic effects of high glucose concentration. Treatment with butanol fraction of this plant at 62.5 µg/ml also significantly reduced the glucose-induced cell toxicity in PC-12 cells.

Mechanism of this protective effect of *C. colocynthis* against high glucose condition is not clearly understood yet. In previous studies on the experimental model of diabetes and clinical trials in type 2 diabetic patients, we have shown that *C. colocynthis* has antioxidant properties in addition to its hypoglycemic function [8, 11-12].

In this connection the protective effects of *C. colocynthis* on liver, kidneys, and pancreas tissue in STZ-induced diabetic rat reported previously [17].

Nonetheless, it is known that during high glucose treatment production of ROS, one of the major determinant of cell death, lead to apoptosis of PC-12 cells [18]. In addition it is known that in diabetes there are significant functional and metabolic disorders, as a result of hyperglycemia and oxidative stress, which leads to the intensification of violations of the structure of cell membranes [18]. It has been reported that *C. colocynthis* pulp extract possess antioxidant effects in alloxan-induced diabetic rats [19].

**5. Conclusion**

In the present study, we observed the effect of *C. colocynthis* in protecting PC-12 cells against toxic effects of high glucose concentrations. Although we could not find the protective mechanisms of *C. colocynthis* in the PC-12 cell line, there is no doubt that chronic hyperglycemia plays an important role in the development and exacerbation of diabetic neuropathy by producing ROS and oxidative stress as an important mediator in the apoptotic pathway. However the protective effects of *C. colocynthis* in the PC-12 cell line may justify its usefulness in the treatment of diabetes and some of its complications if more comprehensive studies confirm it.

**Author contributions**

M.Z. and S.A. designed the study and analyzed the results. E.J. and R.M performed the material preparation and data collection. S.M supervised the laboratory work. F. K.S prepared extracts and performed HPLC. H.F and M.Z. drafted manuscript. All of the authors have read the final manuscript and approved the submission.

**Conflict of interest**

The authors declare that there is no conflict of interest.

**Acknowledgement**

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**References**


The effect of *Citrullus colocynthis* …


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... بهتر نشان داد. نتیجهگیری: هندوانه ابوجهل دارای خواص محافظتی عصبی در برابر شرایط بالای گلوکز در شرایط آزمایشی می‌باشد.

چکیده

مقدمه: نوروباتی دیابتی از پیامدهای هیبرکلیسی مزمن است که با گذشت زمان زنگ زده می‌شود. میوه هندوانه ابوجهل مورد بررسی قرار گرفت. این میوه از طریق از آبیاری در طب سنتی ایران برای درمان دیابت استفاده می‌شود. هندوانه ابوجهل در زمانهای مختلفی که با افزایش قند خون در بدن مواجه می‌شود، زنده ماندگی سلول‌ها را در برابر سمیت عصبی تست کرد. با استفاده از دوز و زمان در محیط مورد بررسی قرار داد. مطالعه عملکرد سنجش MTT در پالپ شگاه بهتر نشان داد.

اهداف مطالعه:

1. بررسی اثرات میوه هندوانه ابوجهل بر عملکرد سنجش MTT در پالپ شگاه به شکل گلگزی

2. بررسی اثرات میوه هندوانه ابوجهل بر عملکرد سنجش MTT در پالپ شگاه به شکل گلگزی

3. بررسی اثرات میوه هندوانه ابوجهل بر عملکرد سنجش MTT در پالپ شگاه به شکل گلگزی

4. بررسی اثرات میوه هندوانه ابوجهل بر عملکرد سنجش MTT در پالپ شگاه به شکل گلگزی

5. بررسی اثرات میوه هندوانه ابوجهل بر عملکرد سنجش MTT در پالپ شگاه به شکل گلگزی

6. بررسی اثرات میوه هندوانه ابوجهل بر عملکرد سنجش MTT در پالپ شگاه به شکل گلگزی

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10. بررسی اثرات میوه هندوانه ابوجهل بر عملکرد سنجش MTT در پالپ شگاه به شکل گلگزی

11. بررسی اثرات میوه هندوانه ابوجهل بر عملکرد سنجش MTT در پالپ شگاه به شکل گلگزی

12. بررسی اثرات میوه هندوانه ابوجهل بر عملکرد سنجش MTT در پالپ شگاه به شکل گلگزی

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15. بررسی اثرات میوه هندوانه ابوجهل بر عملکرد سنجش MTT در پالپ شگاه به شکل گلگزی

16. بررسی اثرات میوه هندوانه ابوجهل بر عملکرد سنجش MTT در پالپ شگاه به شکل گلگزی

17. بررسی اثرات میوه هندوانه ابوجهل بر عملکرد سنجش MTT در پالپ شگاه به شکل گلگزی

18. بررسی اثرات میوه هندوانه ابوجهل بر عملکرد سنجش MTT در پالپ شگاه به شکل گلگزی

19. بررسی اثرات میوه هندوانه ابوجهل بر عملکرد سنجش MTT در پالپ شگاه به شکل گلگزی

20. بررسی اثرات میوه هندوانه ابوجهل بر عملکرد سنجش MTT در پالپ شگاه به شکل گلگزی

اطلاعات مقاله

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هندوانه ابوجهل  

PC-12  

MTT

سنجش

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جهان دانشگاهی

هندوانه ابوجهل

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