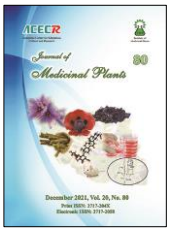
J. Med. Plants 2021; 20(80): 60-68

Institute of Medicinal Plants

Journal of Medicinal Plants

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Research Article

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

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ARTICLE INFO

Keywords:
Hyperglycemia
C. colocynthis
PC-12 cells
MTT assay
Fractionation
Neurotoxicity

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

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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doi: 10.52547/jmp.20.80.60
Received 16 May 2021; Received in revised form 28 November 2021; Accepted 29 December 2021

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[DOR: 20.1001.1.2717204.2021.20.80.3.0]
[DOI: 10.52547/jmp.20.80.60]

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 ºC. 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 ºC 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 ºC in a 90 % humid

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atmosphere containing 5 % CO_2. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 5 % (v/v) fetal bovine serum (heat-inactivated), 100 units/ml penicillin, and 100 µg/ml streptomycin. The cells were subcultured twice a week by gentle scraping.

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 ºC under 5 % CO_2 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 (IC_{50}) 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 10^3 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

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

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

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72 h. So, we could find the best concentration (IC_{50}) 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*

	Methanol Extract	Hexane Fraction	Chloroform Fraction	Ethyl acetate Fraction	Butanol Fraction	Aqueous Residue
Yield (%)	11.47	1.5	37	3	20.5	38

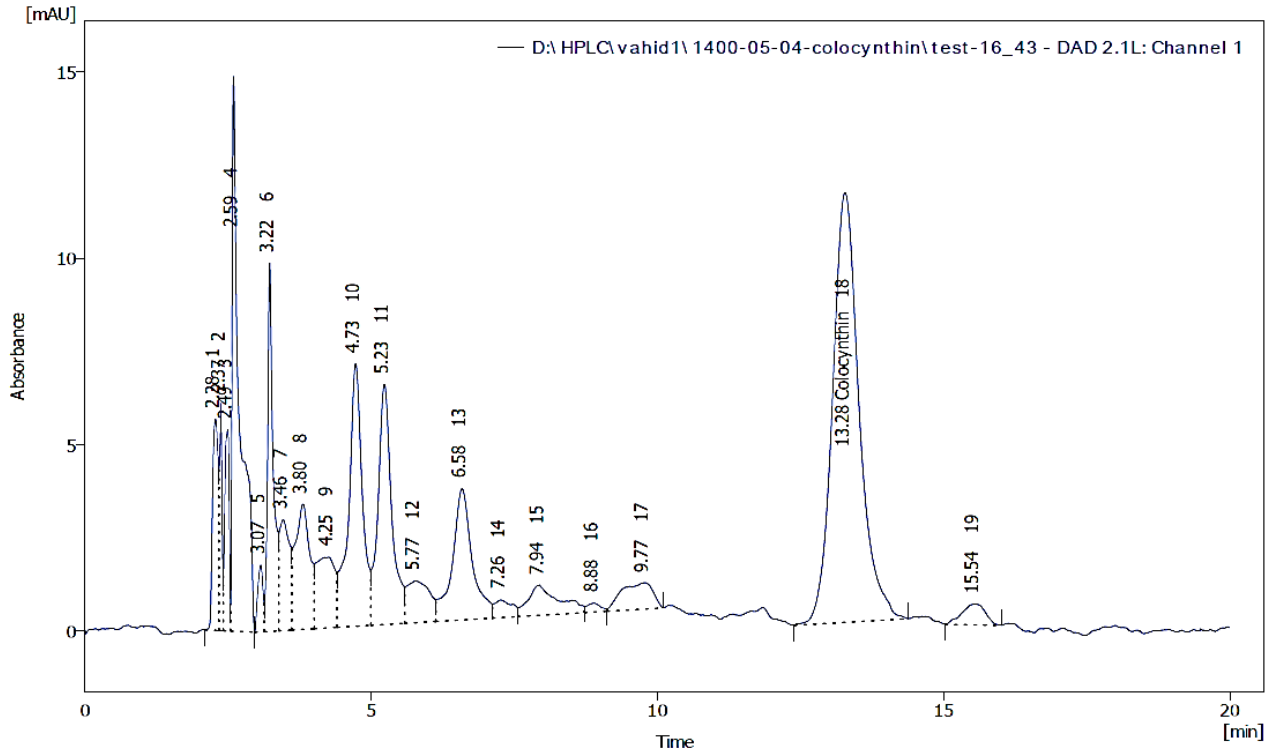


Fig. 1. HPLC Chromatogram of fruit pulp extract of *C. colocynthis*

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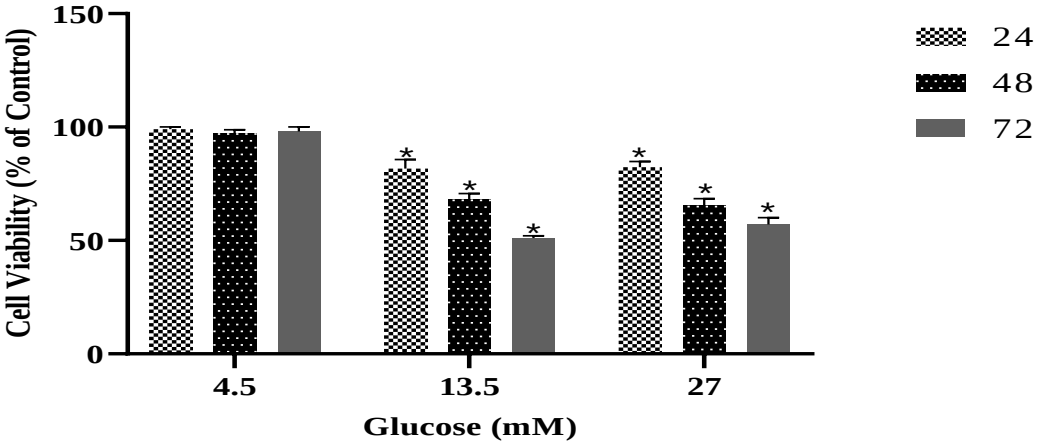


Fig. 2. Effects of glucose concentration and culture time on PC-12 cell viability. Data are presented as mean ± SEM (*n* = 4). The difference between the control group and the treatment group with different concentrations of glucose after 24, 48, and 72 h. significant level: ∗ P < 0.001.

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).

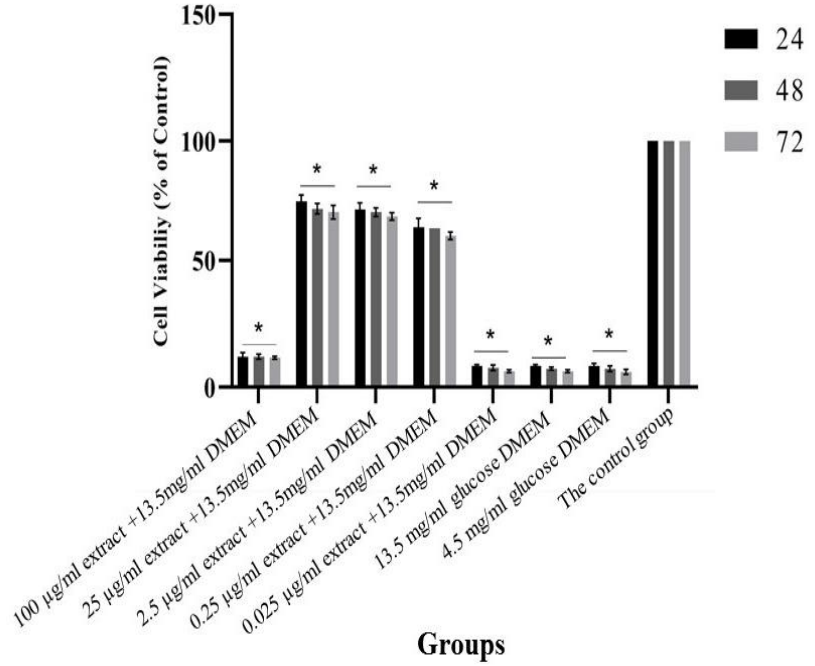


Fig. 3. Protective effect of total extract against glucose in PC-12 cells after at 24, 48, and 72 h time intervals. Cell viability was measured by MTT assay. Results are reported as the mean S.E.M (n = 4). ∗ P < 0.001 compared to controls.

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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

	Aqueous	**Hexane**	**Chloroform**	**Ethyl acetate**	**Butanol**
Concentration (µg/ml)	125.0	250.0	62.50	125.0	62.50

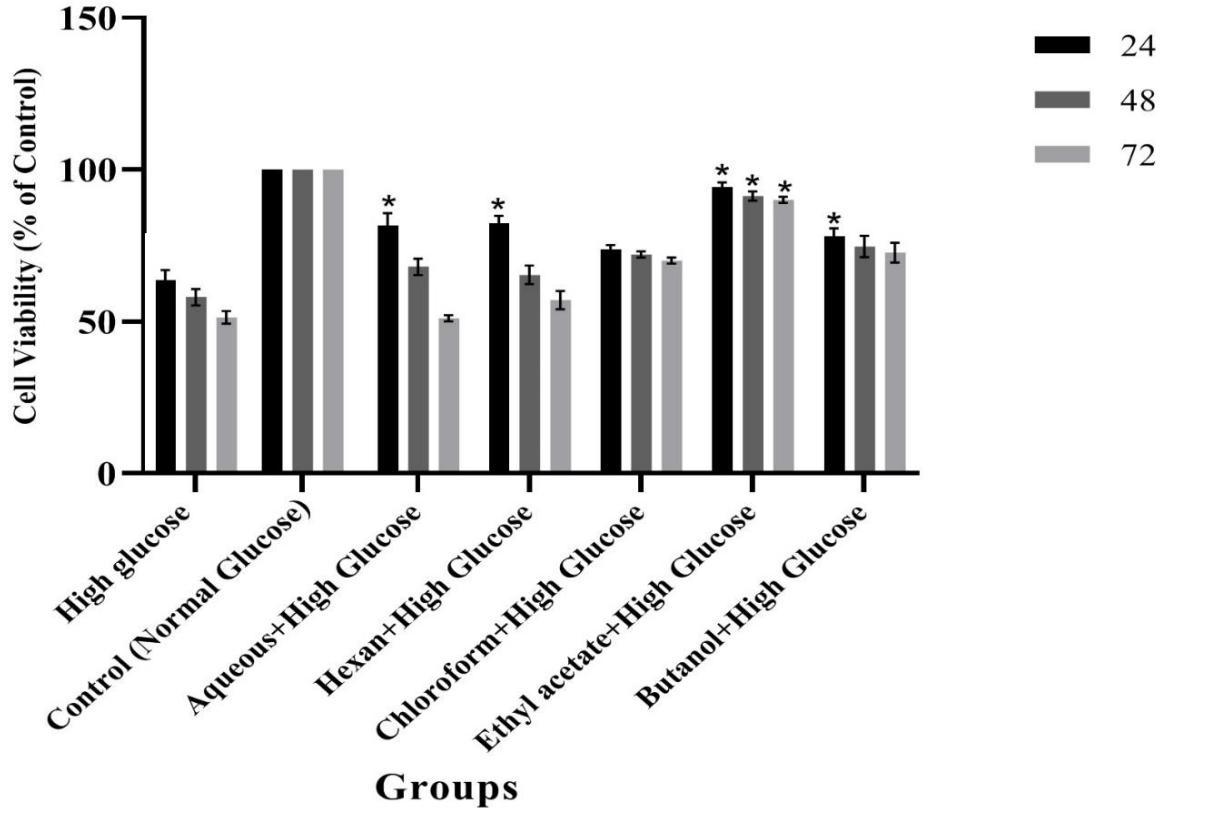


Fig. 4. The protective effect of different *C. colocynthis* fractions on PC-12 cells cultured under hyperglycemic condition. Data are presented as mean ± SEM (n = 4). Significant level: ∗ P < 0.001

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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

This study was financially supported by Medicinal Plants Research Center, Institute of Medicinal Plants, ACECR, Karaj, Iran.

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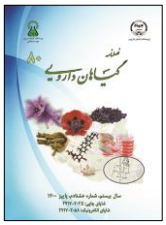
How to cite this article: Fallah Huseini H, Andalib S, Jasemi E, Khalighi-Sigaroodi F, Momtaz S, Mohammadi Savadroodbari R, Salehirad H, Ziaee M. The effect of *Citrullus colocynthis* (L.) Schard. fruit extract on high glucose-induced neurotoxicity in PC-12 cells. ***Journal of Medicinal Plants*** 2021; 20(80): 60-68.
doi: 10.52547/jmp.20.80.60

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اثر حفاظتی عصاره میوه هندوانه ابوجهل در مقابل اثرات سمیت عصبی گلوکز در رده سلول‌های PC-12

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اطلاعات مقاله

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سلول‌های PC-12
سنجش MTT
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سمیت عصبی

چکیده

مقدمه: نوروپاتی دیابتی از پیامدهای هیپرگلیسمی مزمن است که با گذشت زمان منجر به آسیب عصبی می‌شود. میوه هندوانه ابوجهل *Citrullus colocynthis* (L.) Schard. از دیرباز در طب سنتی ایران برای درمان دیابت استفاده می‌شده است. **هدف:** در این مطالعه، ما اثرات محافظتی *C. colocynthis* را در برابر سمیت عصبی ناشی از گلوکز بالا در سلول‌های PC-12 به عنوان یک مدل آزمایشگاهی مناسب برای عملکردهای عصبی مورد بررسی قرار دادیم. **روش بررسی:** میوه خشک بدون دانه با متانول عصاره‌گیری و سپس با تقطیر در خلاء خشک شد. سپس عصاره بدست آمده به فراکسیون‌های هگزانی، کلروفرمی، اتیل استاتی، بوتانول نرمال و باقیمانده آبی تقسیم شد. از کروماتوگرافی مایع با کارایی بالا برای آنالیز کیفی کولوسینتین موجود در پالپ میوه هندوانه ابوجهل استفاده شد. زنده ماندن سلول PC-12 توسط عصاره تام و فراکسیون‌های آن با استفاده از دوز و زمان در محیط قند بالا در سلول های PC-12 به عنوان یک مدل مناسب برای مطالعه عملکردهای عصبی از طریق سنجش MTT مورد بررسی قرار گرفت. **نتایج:** زنده ماندن سلول PC-12 در چاهک‌های حاوی گلوکز بالا در مقایسه با سلول‌های طبیعی تحت درمان با گلوکز به میزان قابل توجهی کاهش یافت. اما درمان با عصاره هندوانه ابوجهل بطور قابل توجهی باعث افزایش ماندگاری سلول‌ها در شرایط سمی بالای گلوکز بسته به دوز و زمان شد. عصاره متانولی هندوانه ابوجهل این اثر محافظتی را در برابر سمیت سلولی ناشی از گلوکز بالا در سلول‌های PC-12 بهتر نشان داد. **نتیجه‌گیری:** هندوانه ابوجهل دارای خواص محافظتی عصبی در برابر شرایط بالای گلوکز در شرایط آزمایشگاهی می‌باشد.

مخففها: MTT، ۳- (۵،۴- دی متیل تیازول-۲- ایل)- ۵،۲- دی فنیل تترازولیوم بروماید؛ HPLC، کروماتوگرافی مایع با کارایی بالا؛ EDTA، اتیلن دی آمین تترا استیک اسید

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تاریخ دریافت: ۲۶ اردیبهشت ۱۴۰۰؛ **تاریخ دریافت اصلاحات:** ۷ آذر ۱۴۰۰؛ **تاریخ پذیرش:** ۸ آذر ۱۴۰۰

doi: 10.52547/jmp.20.80.60

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[DOR: 20.1001.1.2717204.2021.20.80.3.0]
[DOI: 10.52547/jmp.20.80.60]