Cuscuta epithymum Murr. crude extract pre-conditioning inhibits cell apoptosis in glutamate-induced cytotoxic condition

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Abstract

Cuscuta epithymum Murr. *(C. epithymum)*, as a herbal medication, has shown anticancerous effects in some of in vitro studies, but its neuroprotective and anti-apoptotic possible effects have not been considered in research. Here, we aimed to show the protective effects of *C. epithymum* seeds crude extract and different fractions (n-hexane, dichloromethane, and methanol) on rat glioblastoma cells (C6) in L-glutamate exposure. Initially, the total phenolic content of *C. epithymum* crude extract and the fractions (all produced by maceration method) was determined. Subsequently, C6 cells were pre-treated with various doses of crude extract and fractions 24 hours before L-glutamate exposure. Likewise, C6 cells were treated with the same extract and fractions concentrations 24 hours after exposure L-glutamate. Besides, flow cytometry testing was used to prove that *C. epithymum* extract protects C6 cells from glutamate toxicity, as evidenced by morphological features, cell viability, and oxidative stress markers such as malondialdehyde (MDA) and superoxide dismutase (SODs). The findings suggested that crude extract has concentration-depended toxicity. It has the highest antioxidant activity to significantly increase C6 cell viability in cytotoxic conditions and probably plays the neuroprotective role in reducing cell death by decreasing MDA levels and increasing SODs levels in cells, showing lipid peroxidation prevention and free radical scavenging, respectively. In conclusion, these results suggest the protective role for *C. epithymum* crude extract against oxidative stress-induced damage. It showed propective attribute in our in vitro study ,although, further research is needed to prove the neuroprotective effects in in vivo oxidative conditions.

Introduction

In the central nervous system (CNS), glutamic acid or L-glutamate is a significant excitatory transmitter and a neuronal development mediator (1). Glutamate receptors are expressed on the surface of brain cells. As a crucial uptake system, Glutamate transporters inhibit the over-activation of these receptors by continuously eliminating glutamate from the extracellular fluid in the brain (1). Excessive activation of glutamate receptors disrupts Ca^{2+} homeostasis and promotes nitric oxide synthesis, free radicals production, and programmed cell death (2, 3). Besides, excessive extracellular L-glutamate inhibits cystine uptake and blocks glutathione synthesis (4). Subsequently, the failure of anti-oxidant defense in cells results in oxidative stress that plays a significant role, along with Ca^{2+} elevated levels, in triggering necrosis and apoptosis in cells. Numerous cellular processes have been found to be involved in the extracellular glutamate pathologically increase, such as enhanced vesicle exocytosis and decreased glutamate uptake by excitatory amino acid transporters (EAATs) (5).

Glial cells, as non-neuronal cells, are the most diffused cell type in the CNS and PNS (Peripheral Nervous System) that regulate homeostasis as well as supporting and maintaining neurons (6). Glial cells express glutamate receptors and have a crucial role in sequestering neuronal-released glutamate through Na^{+}-dependent transporters (7). Recently, several studies have shown that, in addition to neurons, excitotoxicity and sustained activation of ionotropic glutamate receptors can lead to glial cell death (8). It

has been proved that some CNS disorders, such as Alzheimer's disease, Parkinson's disease, cerebral ischemia, and other diseases, result from excessive excitatory activation of L-glutamate (9).

"Medicinal plants" refers to a diverse range of plants that exhibit therapeutic pharmacological properties. Recently, the modern pharmaceutical industry revolution has led to paying much attention to the development and use of herbal medicines. Based on a World Health Organization (WHO) report, 60% of therapeutic medications are derived from natural sources, and different surveys in various countries have shown that the number of people consuming natural drugs is increasing (WHO Traditional Medicine Strategy 2014–2023) (10). The Cuscuta genus belonging to the Convolvulaceae plant family has been used in the majority of countries to treat or alleviate a wide variety of disorders, including headache, drooping spirits, epilepsy, nervous and mental disorders, hallucination, amnesia, influenza, fevers, ulcers, and chronic diseases such as diabetes, spleen, liver, and joint dysfunctions, spasms, and paralysis for many decades (11). *Cuscuta epithymum (C. epithymum)* is one of the Cuscuta species named in various countries as Kashout, Pittimo, Aftimoon, crops' parasite, among others. *C. epithymum* is a parasitic plant with several secondary metabolites such as glycosides, saponins, tannins, quercetin, steroids, and kaempferol (12). Recent studies attribute some main biological and pharmacological activities to *C. epithymum*, such as anti-oxidant, anti-bacterial, anti-fungal, anti-convulsant, urease inhibition, and cytotoxicity (12). Zhen et al. reported that *Cuscuta chinesis* protects PC12 cells against oxidative stress. The underlying mechanisms may be scavenging reactive oxygen species (ROS) and enhancing the anti-oxidant enzyme activity (13). So far, few research have been conducted to show the beneficial, biological, and pharmacological aspects of *C. epithymum* under standard conditions. In this study, we have investigated the protective effects of *C. epithymum* crude extract and fractions in in vitro condition.

Experimental Section

2.1. Extract preparation

2.1.1. Methanol crude extract

C. epithymum (Cuscutaceae) seeds were obtained from the Medicinal Plants Store (Tehran, April 2021). F.Mojab taxonomically identified the plant at the School of Pharmacy, Shahid Beheshti University of Medical Sciences, Iran, and registered with herbarium code SBMU-8236. The extraction process was performed by the maceration method. Briefly, the seeds were grounded and soaked for 24 hours in methanol (Merck, Darmstadt, Germany) as a solvent so that it completely covered the grounded seeds. After 24 hours, the top solvent was collected, and the new solvent was poured on the seeds. The procedure was repeated three times and during the procedure seeds and methanol were shaken. Finally, the collected solvent in three days was concentrated in an oven at 40 ℃.

2.1.2. Fractions of extract

C. epithymum seeds were macerated in n-hexane (Merck, Darmstadt, Germany) for 48 h at room temperature. The extraction procedure was entirely similar to crude extraction. After the third collection,

the extraction was dried at room temperature for 24 hours to ensure that all the hexane had been completely removed. The residual plant was macerated further in two other solvents, dichloromethane (Merck, Darmstadt, Germany) and methanol, respectively, in the same manner as n-hexane. Using a rotary evaporator, the solvent was removed under a vacuum at 45 ℃. The fractions obtained from each solvent were kept at 4 ℃ for the subsequent study.

2.2. Total phenolic contents measurement

We used the Folin-Ciocalteu analysis method to evaluate the total phenolic content (TPC) in the crude methanolic extract and methanol, n-hexane, and dichloromethane fractions. Folin-Ciocalteu solution (Merck, Darmstadt, Germany) was utilized as a reagent, and gallic acid (Merck, Darmstadt, Germany) was the reference phenolic compound. 1 ml of different concentrations of gallic acid, including 0, 10, 20, 40, 60, 80, and 100 g/ml, was combined with 5 ml of diluted Folin-Ciocalteu and incubated at room temperature for 10 minutes. 4 ml of sodium carbonate solution (75 mg/mL) was added and incubated at room temperature for 30 minutes. A spectrophotometer was used to determine the absorbance of each standard and sample at 765 nm. For TPC evaluation in samples, 1 ml of the extract with a 400 µg/ml concentration was used, and by using the standard curve of gallic acid as a reference, TPC was determined. The data is presented as the average of three independently collected measurements.

2.3. Cell culture

C6 glial cells (rat glioma cell line) were purchased from Pastor Institute (Tehran, Iran) and cultured in 25-cm^2 culture flasks in DMEM/F12 supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin-streptomycin (Biosera, France)in a humidified atmosphere of 5% CO_2 at 37°C. Cells grown to 80–90% confluency were used to performing the following experiments.

2.3.1. Determination of L-glutamate cytotoxicity

The cells were sub-cultured in 96-well plates at a density of 8×10^3 cells/well and incubated for 24 h. Adherent cells were exposed to different concentrations of L-glutamate (0.312-20 mM) for 24 h. The morphology of C6 cells treated with various concentrations of L-glutamate was observed using an inverted light microscope (Olympus, Japan), and the cell viability was evaluated using the 2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Sigma, Germany). Following 24 h incubation, cells were incubated at 37 °C for an additional 4 hours after adding MTT solution at a final concentration of 0.5 mg/ml. 100 µL of DMSO was added to dissolve the formazan crystals after removing the medium. The plate was shaken for 10 minutes, and the absorbances of the samples were measured at 570 nm by an ELISA reader (BioTek,USA). For all experiments, glutamate was dissolved in DMEM/F12 at final concentrations of 0.312, 0.625, 1.25, 2.5, 5, 10, and 20 mM.

Acridine Orange and Propidium Iodide (AOPI) staining was applied to determine the apoptosis percentage of C6 cells treated with various concentrations of L-glutamate (Merck, Darmstadt, Germany). For this purpose, 24 h after L-glutamate exposure, the medium was completely removed, and 30 µl of AOPI

reagent (Nexcelom Bioscience, Massachusetts, United States) was added to each well. Cells were incubated with AOPI dye for 45 minutes at 37 °C and observed under a fluorescent microscope (Nikon digital camera, USA).

2.3.2. Determination of *C. epithymum* crude extract and fractions cytotoxicity

The cells were sub-cultured in 96-well plates at a density of 8×10^3 cells/well and incubated for 24 h. Adherent cells were exposed to different concentrations (9.37, 19, 37, 75, 150, and 300 µg/ml) of *C. epithymum* crude extract and fractions. The crude extract and fractions were diluted in 100 µL of ethanol (70%) and then in 5 ml of DMEM/F12 to prepare 300 µg/ml. The other concentrations were prepared from 300 µg/ml solution by serial dilution. After 24 h of adding *C. epithymum* crude extract and also fractions into plates, the MTT assay was performed as previously described. The remaining 96-well plates were incubated at 37°C to assess cell viability after 48 and 72 h. The morphology of cells was observed using an inverted microscope.

2.3.4. Effects of *C. epithymum* fractions and crude extract treatment in L-glutamate induced cytotoxicity

C6 cells were seeded in 96-well plates at 8×10^3 cell density in each well. C6 cells were pre-treated with various concentrations (9.37, 19, 37, 75, and 300 µg/mL) of *C. epithymum* crude extract for 24 h before exposure to L-glutamate. After 24 h, the medium was changed by a fresh culture medium containing L-glutamate (0.312, 2.5, and 10 mM) and incubated for 24 h. In the negative control and positive control groups, cells were treated with ethanol (70%) and L-glutamate, respectively. Finally, an MTT assay was performed. Each independent experiment was repeated three times. In parallel to this experiment, post-treatment of C6 cells with *C. epithymum* was performed. This way, C6 cells were first treated with L-glutamate (0.312, 2.5, and 10 mM) for 24 h. A fresh culture medium containing different concentrations of *C. epithymum* crude extract (9.37, 19, 37, 75, and 300 µg/mL) was added, and the cell viability was evaluated in the same procedure. Three concentrations (19, 37, and 75 µg/mL) were selected for next experiments to show the effects of *C. epithymum* treatment in L-glutamate-induced cytotoxicity.

2.3.5. Measurement of superoxide dismutases (SODs)activity

SODs are essential enzymes protecting cells against oxidative damage by reducing superoxide anion free radicals (O2-). SODs activity was measured in cells treated with L-glutamate and *C. epithymum* crude extract. C6 cells were grown in 25 cm^2 flasks for pre-treatment and post-treatment with 19, 37, and 75 g/ml crude extract and 10 mM glutamate to measure SODs activity. Using a rubber policeman, cells were removed from the flasks 24 hours after the last treatment. Two million cells were required for the test. The pellet was sonicated for 30 seconds at a medium power (10 s of sonication plus 10 s of rest; 3 times) before being centrifuged at 12000 g for 5 minutes at 4 ° C. The supernatant was separated and placed on ice. According to the kit protocol (TPR Innovative, Iran), samples and working solution (chromogenic reagent and SODS assay buffer) were added to 96-well plates. Eventually, the SODS enzyme solution was added and incubated for 20 minutes. After 20 minutes, a multi-mode reader read the absorbance at a 440–460 nm wavelength (BioTek, USA).

2.3.6. Malondialdehyde (MDA) assay

Malondialdehyde (MDA) is the most common reactive aldehyde produced during lipid peroxidation and an oxidative stress indicator. The thiobarbituric acid reactive substances (TBARS) were measured to determine the products of lipid peroxidation. 24 hours after the last treatment, the cells were trypsinized for the MDA test. According to the kit (TPR Innovative, Iran), one million cells were required in 1 ml of PBS buffer (pH: 7.8). First, 10 µL of butylated hydroxytoluene (BHT) was added to the cells. Cells were then placed on ice and sonicated with a medium power sonicator for 30 seconds. Detergent was added to all samples and incubated at 37 ˚C. Then, the chromogenic solution containing thiobarbituric acid, acetic acid, and alkali was added to the samples and placed in boiling water for an hour. After one hour, they were placed on ice for 10 minutes, then centrifuged at 10,000 g for 10 minutes at 4 °C. The supernatant was transferred to the 96-cell plate, and the absorbance of the tests was measured using a multi-mode reader at a wavelength of 530-540 nm. Protein concentrations were measured in samples by the multi-mode reader at 280 nm.

2.3.7. Effects of *C. epithymum* crude extract on the apoptosis of C6 cells in L-glutamate-induced damage condition

At the end of the final 24 h of cells incubation with *C. epithymum* crude extract and L-glutamate, C6 pre-treated and post-treated cells were harvested and centrifuged at 1500 rpm for 5 minutes. For determining the percentage of the apoptotic cells, Annexin V-FITC (fluorescein isothiocyanate)/PI (propidium iodide) (Biolegend, USA) co-staining method was used. For this purpose, 500 µL of 1X binding buffer was added to the cell pellet, and the sample was divided into tubes. 5 µL of Annexin V-FITC and 3 µL of PI were added to labeled tubes and then incubated at 4 °C for 15 minutes in the dark. Then the specific fluorescence of cells was detected using a BD FACS Calibur (BD Biosciences, San Jose, CA, USA) and the outcomes were evaluated by FlowJo software.

2.3.8. Effects of *C. epithymum* crude extract on the C6 cell cycle in L-glutamate-induced damage condition

At the end of the final 24 h of cells incubation with *C. epithymum* crude extract and L-glutamate, C6 pre-treated and post-treated cells were harvested and centrifuged at 1500 rpm for 5 minutes. Then 50 µL of cold PBS was added to the cells and gently vortexed. The cells were resuspended, fixed with cold 70% ethanol, and vortexed gently. After fixation by ethanol, the cells were washed with PBS and centrifuged at 1500 rpm for 5 minutes. The pellet was resuspended in 1ml of PI master mix containing 40 µL of PI (1 mg/ml), 950 µL of PBS, and 10 µl of RNase (DNase free, 10 mg/ml) and incubated for a half-hour at room temperature. Finally, the cells were analyzed by flow cytometry (BD FACSCalibur, BD Biosciences, USA), and the outcomes were evaluated by FlowJo software.

Statistical Analysis

Each experiment was conducted at least three times, and the data were presented as the mean ± standard deviation (SD). Statistical analysis for cell viability tests, SOD, and MDA were performed using one-way ANOVA and appropriate posthoc test in GraphPad Prism 8.4.3. Two-way ANOVA and appropriate posthoc test analyzed the apoptosis and cell cycle data. The $P < 0.05$ indicated that the result was statistically significant.

Results

3.1 Yield extract and total-phenolic content

The crude and fractions extraction yield and TPC results are presented in Table 1. The yield of the crude extract was about 18.26 %, and n-hexane, dichloromethane, and methanol fractions were approximately 0.89%, 1.33%, and 28.59%, respectively. This experiment showed that the *C. epithymum* crude methanolic extract contains more amount of phenolic compound, followed by methanol fraction in comparison with other fractions.

Table 1 The extraction yield and total phenolic contents of the extract and the fractions

Fractions				
	Crude extract	n-hexane	Dichloromethane	methanol
Yield extract (%)	18.26	0.89	1.33	28.59
TPC (mg gallic acid/g extract)	55.99 ± 2.795	Undetectable	Undetectable	50.80 ± 2.969

3.2 Cytotoxicity of L-glutamate in a concentration-dependent manner

In this study, MTT assay was used to determine the viability of C6 cells in various L-glutamate concentrations exposure (0, 0.312, 0.625, 1.25, 2.5, 5, 10, and 20 mM). Results demonstrated that increased L-glutamate concentration significantly decreased the percentage of viable cells. Comparing 10 mM glutamate to the control group showed that cell viability decreased by approximately 50 percent ($P<0.01$) (Figure 1b). Moreover, the inverted and fluorescent microscope images showed apoptotic morphological and color changes in the L-glutamate-treated cells (Figure 1a, c). The existence of apoptotic cells in the treated cells was indicated by the presence of a red and bright orange stain, in contrast to the bright green coloring of the living cells. By increasing L-glutamate concentration, the proportion of green cells gradually reduced while the percentage of orange cells enhanced, indicating a transition from viable to apoptotic cells. In this study, a 10 mM concentration of L-glutamate was selected for the subsequent experiments.

3.3 Cytotoxicity of *epithymum* crude extract and fractions

In different concentrations (9.37, 19, 37, 75, 150, and 300 µg/ml), the cytotoxicity of *C. epithymum* methanolic crude extract and the n-hexane, dichloromethane, and methanol fractions were studied to choose the optimum concentration for cell treating at the subsequent steps. Results of MTT assay for the crude extract after 24, 48, and 72h incubation showed that *C. epithymum* crude extract at high concentrations decreased the cell viability (Figure 2a). It means high concentrations (75, 150, and 300 µg/ml) have a significant toxic effect on C6 cells and lead to cell death ($P<0.0001$). The concentrations lower than 75 µg/ml did not show significant toxic effects.

Moreover, comparing the toxicity of *C. epithymum* crude extract and the fractions indicated that the 9.37, 19, and 37 µg/ml concentrations of all samples did not show a significant toxic effect on the cells (Figure 2b). Dichloromethane fraction at high concentrations (75, 150, and 300 µg/ml) showed significant toxic effects similar to the crude extract. In contrast, the methanolic fraction at high concentrations indicated lower toxicity compared with the dichloromethane fraction and the crude extract. The n-hexane fraction did not show cytotoxicity at any concentration. The crude extract, dichloromethane, and methanol fraction concentrations that induced 50 percent cell death in C6 cells were 126.47, 140.97, and 218.96 µg/ml, respectively.

3.3 Effects of *epithymum* crude extract and fractions on C6 cell viability in L-glutamate-induced condition

This investigation aims to determine whether *C. epithymum* extract protects C6 cells against L-glutamate-induced damage or not. We chose 9.37, 19, and 37 µg/ml as optimum concentrations, 75 and 300 µg/ml as high concentrations of the crude extract. In pre-treatment groups, the crude extract was added to the cells 24 h prior to removing the medium and adding the fresh medium containing glutamate (0.312, 2.5, and 10 mM). In post-treatment groups, the cells were exposed to glutamate, and after 24 h, a fresh medium containing the crude extract was added to the culture medium. Then, after 24h of incubation, an MTT assay was performed. Results showed that the 0.312 and 2.5 mM L-glutamate are not suitable concentrations for the study. There were no significant changes between *C. epithymum* crude extract and L-glutamate treated groups in these concentrations in cell viability (Figure 3). But compared with 10 mM concentration L-glutamate, crude extract pre-treatment and post-treatment significantly changed cell viability ($P<0.0001$). The crude extract with 9.37, 19, 37, and 75 µg/ml concentrations significantly protected the survival of cells in L-glutamate high doses exposure. So we chose a 10 mM concentration of L-glutamate to demonstrate *C. epithymum* fractions' protective effects on C6 cells.

In the next step, we comprised the impact of concentrations (19, 37, and 75 µg/ml) of *C.epithymum* crude extract and three fractions on cell viability in the presence of 10 mM of L-glutamate (Figure 4). Cell viability in the L-glutamate pre-treatment and the post-treatment groups, without the intervention of extracts and fractions, was significantly decreased. However, the crude extract and the fractions significantly enhanced cell viability in L-glutamate treated groups compared to the glutamate-exposed C6 cells without extract treatment. In addition, the data suggested that the viability of cells in the pre-treatment and the post-treatment with crude extract are significantly more than the fractions-treated groups, and crude extract of *C. epithymum* inhibit the toxic effects of L-glutamate more efficiently.

3.4 Effect of *epithymum* crude extract on oxidative stress markers in cytotoxic condition

As shown in Figure 5, SOD and MDA levels were measured in the *C. epithymum* crude extract-treated cells in the presence of L-glutamate as an oxidative stress inducer. SODs levels, as an anti-oxidant enzyme, in the L-glutamate treated groups without extract intervention were significantly decreased compared with the control group ($P<0.01$). The evidence proved that pretreatment with 37 µg/ml *C. epithymum* crude extract significantly increased SODs activation against L-glutamate-induced oxidative stress ($P<0.001$) (Figure 5a). SODs levels were not significantly changed in the other concentrations of the extract. According to Figure 5b, MDA level was significantly enhanced in the L-glutamate groups, especially on the second day, compared to the control group ($P<0.0001$). All concentrations of the extract (19, 37, and 75 µg/ml) significantly decreased the MDA level compared to the L-glutamate-non-extract-treated groups ($P<0.0001$).

3.5 Effects of *epithymum* crude extract on the cell cycle and apoptosis in cytotoxic condition

To determine the proportion of apoptotic cells in pre-treated and post-treated cells with different concentrations (19, 37, and 75 µg/ml) of *C. epithymum* crude extract and L-glutamate (10 mM), we used Annexin V and PI co-staining assay. In this method, flow cytometry analysis plots indicate four regions: 1) viable cells (negative for Annexin V and PI), 2) early apoptotic cells (positive for Annexin V but negative for PI), 3) late apoptotic cells (positive for Annexin V and PI), and 4) necrotic cells (negative for Annexin V but positive for PI) (10). As shown in Figure 6, all the groups, including control and the treated cells with L-glutamate and *C. epithymum* crude extracts, indicated the apoptotic (early and late apoptotic cells' results are combined) and necrotic cells percentage after 24 h of incubation. Pre-treatment and post-treatment with 19 µg/ml ($P<0.01$), 37, and 75 µg/ml ($P<0.0001$) of the crude extract significantly reduced the percentage of the apoptotic cells in comparison with the L-glutamate treated cells. Pre-treatment and post-treatment with all concentrations (19, 37, and 75 µg/ml) of crude extract in the presence of L-glutamate not only significantly decreased the percentage of necrotic cells, but also increased the percentage of the live cells in comparison with the L-glutamate treated groups without extract ($P<0.0001$).

In addition, to cell cycle phases analysis in the pre-treated and post-treated cells, DNA content detection was conducted using the Propidium Iodide staining method. The results were presented in percentages in Figure 7. We found a reduction in S and G2 phases and an enhance in G1 phase in C6 cells at 37 and 75 g/ml concentrations, indicating that the crude extract inhibits S arrest in C6 cells.

Discussion

This study evaluated the protective effect of *C. epithymum* crude extract and fractions on C6 cells in oxidative stress condition induced by L-glutamate. The results showed that *C. epithymum* methanolic crude extract could efficiently decrease the cytotoxicity effects of L-glutamate in a concentration-dependent manner. Pre-treatment and post-treatment of the cells with low concentrations of *C. epithymum* extracts decreased dead cell percentage in the presence of L-glutamate-induced cytotoxicity

and increased cell viability. In addition, *C. epithymum* crude extract reduced MDA and enhanced SODs levels in the C6 cells.

Glutamate, an abundant excitatory neurotransmitter in mammals' central nervous system, plays a crucial role in fast synaptic transmission, neuronal development, pain perception, learning, and memory (14). Because of increased release or/and uptake disorders, the excess glutamate level has been demonstrated to stimulate significant neurotoxicity and neuronal death in brain tissue (15). The neurotoxicity can be related to the enhanced cell components damage, such as mitochondria and membrane depolarization, causing cell death and the production of reactive oxygen species (e.g., superoxide anion (O_2^-), hydroxyl radical (OH), and hydrogen peroxide (H_2O_2)) (16, 17). The stimulation of oxidative stress by glutamate has been indicated in cell lines, including C6, PC-12, oligodendroglia cells, and immature cortical neurons (17).

Since glutamate receptor subtypes (ionotropic and metabotropic) are expressed in C6 glioblastoma cells, these cells can be a beneficial model for investigating excitotoxicity (18). Moreover, C6 glial cells provide physical and metabolic support to the survival of neurons, such as insulation and communication of neurons and transportation of nutrients and waste; hence, glial cells damage may play role in neurodegeneration process. Recent investigations show that astroglial cell damage is strongly related to many neurological disorders, including Parkinson’s disease, Alzheimer’s disease, and ischemic stroke (18). In the *in vitro* toxicity studies, depending on the culture conditions, the L-glutamate concentration ranges from 0.01 to 20 mM (19). We selected 10 mM L-glutamate in our study because, at this concentration, approximately 50% cell viability was detected and the effect of *C. epithymum* on neutralizing the cytotoxicity of L-glutamate was significant.

C. epithymum is a parasitic plant widely used in agriculture and food industries, pharmaceutical, cosmetics, and sanitary due to its fungicidal, virucidal, bactericidal, anti-parasitical, and insecticidal properties (20). Recent findings have also shown that *C. epithymum* has neuropharmacological and sedative-hypnotic effects without significant toxic influence in mice. This plant's sedative-hypnotic properties are mediated through benzodiazepine receptors (21). Due to its anti-oxidant properties, we selected this medicinal plant to evaluate its effect on L-glutamate-induced damage in C6 cells. Ganapaty et al. also reported the anti-oxidant and free radical scavenging properties of *C. epithymum* depend on the doses (22).

This study investigated the extraction yield and TPC of *C. epithymum* crude extract and the fractions. Different chemicals can be obtained in the extraction procedure due to the different solvent polarity, the various chemical composition of the plant, and the presence of interfering materials (23). Methanol showed the most yield percentage in the extraction procedure, followed by dichloromethane and n-hexane. Therefore, we can infer that the solvent with more polarity will produce a higher extraction yield. The TPC results showed that the crude methanol extract has the highest total phenolic content, suggesting that the solvent polarity influences the extraction of various phenolic chemicals from *C. epithymum*. We showed that C6 cells treated with different concentrations of L-glutamate (24 h) resulted

in cell damage and cell viability decrease. According to the results, we suggested that low concentrations of *C. epithymum* crude extract and the fractions can enhance cell survival and counteract the damaging effects of L-glutamate. Considerably, results demonstrated that the crude extract compared with the fractions had more impact on the protection and survival of cells. We used the crude extract for further tests, including anti-oxidant, cell cycle, and apoptosis assays. We also showed that *C. epithymum* crude extract and fractions at high concentrations have cytotoxic effects and reduce cell viability. Our findings were consistent with the findings of earlier studies indicating that a high concentration of *C. epithymum* extracts shows cytotoxicity and can be used for inducing apoptosis in cancer cells (24, 25).

Under oxidative stress conditions, the organism's oxidant and anti-oxidant defense mechanisms become unbalanced, leading to excessive ROS production, tissue damage, and physiological function impairment (26). In addition, oxidative stress is one of the most critical risk factors for neurodegenerative conditions and CNS disorders (27). According to previous studies, *C. epithymum* showed dose-dependent scavenging activity using DPPH, superoxide, and hydroxyl radical scavenging assays (28). Qualitative phytochemical studies on the *C. epithymum* also manifested the presence of alkaloids, triterpenoids, flavonoids, steroids, glycosides, and carbohydrates, which might be responsible for its anti-oxidant properties (28). Another electrochemical assay study demonstrated that *C. epithymum* has desirable anti-oxidant properties (20). Consistent with these studies, in our study, the anti-oxidant assays (SODs and MDA) revealed that the relative anti-oxidant ability of *C. epithymum* crude extract plays a scavenger role in C6 cells against free radicals produced in the presence of L-glutamate.

Long-term glutamate accumulation in the intercellular space stimulates neurotoxic effects. It elevates Ca levels in cells- an imbalance of electrogenic ions gradients- and triggers various intracellular cascades that lead to plasma membrane damage and neuron apoptosis (29). In our study, L-glutamate stimulates apoptosis and necroptosis in C6 cells based on the flow cytometry analyses. Pre- and post-treatment with *C. epithymum* crude extract prevented apoptosis and necroptosis induced by L-glutamate. A study in line with our results indicated that glutamate stimulates apoptosis and necroptosis in C6 cells (18).

Conclusion

Our research indicates that *C. epithymum* methanolic crude extract can protect neurons against neurotoxicity. This protective effect may be mediated by mechanisms related to anti-oxidant and anti-apoptotic activities through the removal of reactive oxygen species (ROS). Nevertheless, further research in animal models with neurodegenerative disorders is required to demonstrate the molecular mechanisms involved in *C. epithymum* treatment and its neuroprotective effects. We also require to evaluate its active component(s) and efficacy to introduce it as a protective factor.

Declarations

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Authors' contributions

This article is the first part of the Ph.D thesis by M.P. at Shahid Beheshti University of Medical Sciences, Tehran, Iran. H.Z. designed and supervised the in vitro section, and F.M. supervised the extraction section of the study. M.P. designed and carried out all laboratory jobs, including extract preparation, 2D cell culture, assays, imaging, data analysis, and manuscript writing. Z.N. helped with data analysis and editing the manuscript, and V.N. helped with data analysis. R.Gh., M.F., and M.S. consulted in the study design.

Availability of data and materials

Most of the data are included in this published article and further data are available from the corresponding author on request.

Ethics approval and consent to participate

All methods were performed in accordance with the relevant guidelines and regulations (IR.SBMU.RETECH.REC.1401.104).

Consent for Publication

Not applicable.

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Competing interests

The authors and this study do not present any kind of conflict of interests.

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Figures

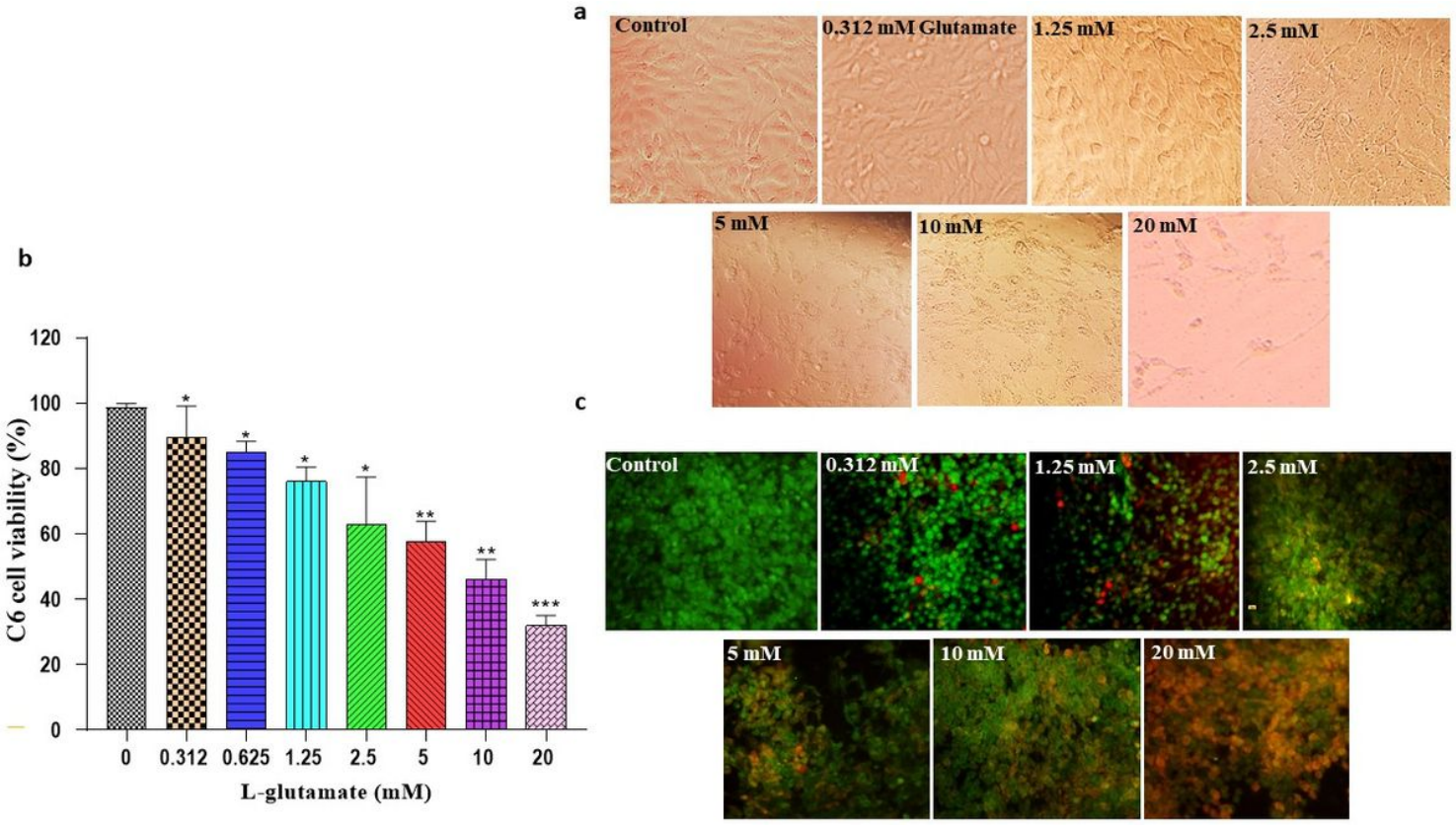

Figure 1

Effects of L-glutamate on C6 cell viability. a) Observation of cells at the inverted microscope with a 20X b) MTT assay showed that cell survival is dependent on glutamate concentration. AO/PI results were compatible with MTT assay. Increased L-glutamate concentration led to significantly decreased cell viability. The data are expressed as mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001. c) AOPI fluorescence images of C6 cells in the presence of different glutamate concentrations.

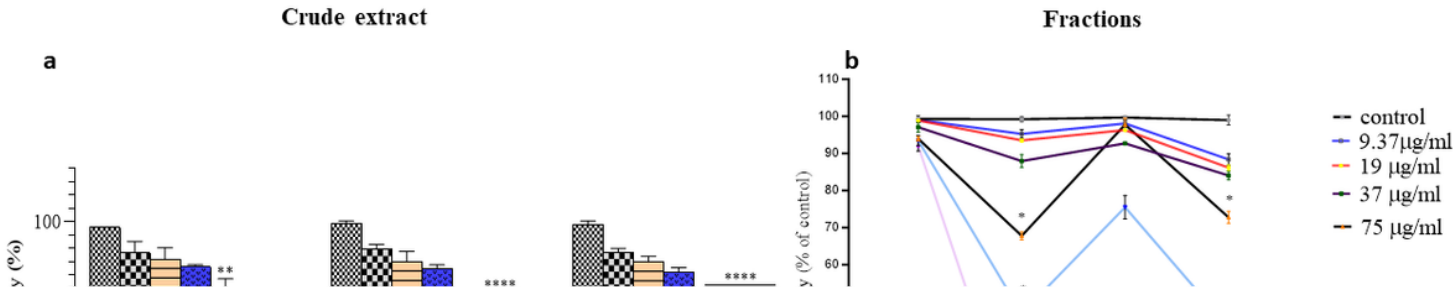

Figure 2

Effects of *C. epithymum* extract on cell survival in C6 cells. a) MTT assay of crude extract. b) Viability of cells in the presence of crude extract and fractions (average of three days results). At the high concentrations of *C. epithymum* extract and dichloromethane, and methanol fractions, cell viability significantly decreased. The data are expressed as mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

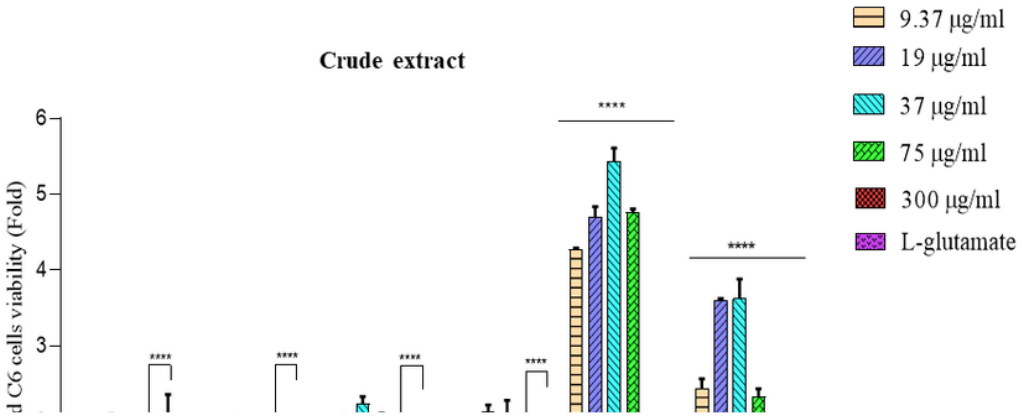

Figure 3

Viability of cells in pre-treatment and post-treatment with crude extract, in the presence of L-glutamate using MTT assay. Pre-treatment and post-treatment of the crude extract in the presence of 10 mM concentration of L-glutamate caused a significant increase in cell viability compared to the L-glutamate group. The data are expressed as mean ± standard deviation. ****P<0.0001

Figure 4

Viability of cells in pre-treatment and post-treatment with crude extract and fractions, in the presence of 10 mM L-glutamate using MTT assay. The crude extract and fraction significantly enhanced cell viability in L-glutamate treated groups. Crude extract showed more effectiveness on cell viability. The data are expressed as mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

Figure 5

Effect of *C. epithymum* on levels of oxidative stress factors in C6 Cells after L-glutamate-induced cytotoxicity. a) Superoxide dismutase (SODs) activity. Pretreatment with 37 µg/ml *C. epithymum* crude extract significantly increased SODs activation against L-glutamate-induced oxidative stress. b) Malondialdehyde (MDA) contents. All concentrations of the crude extract significantly decreased the MDA level. The data are expressed as mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

Figure 6

Effect of *C. epithymum* on apoptosis in C6 cells after L-glutamate-induced cytotoxicity. Pre-treatment and post-treatment with crude extract significantly decreased the percentage of apoptotic cells. The data are expressed as mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 as compared to control-untreated group; ##P< 0.01, ####P< 0.0001 as compared to L-glutamate-treated group.

Figure 7

Effect of *C. epithymum* on cell cycle in C6 cells after L-glutamate-induced cytotoxicity. Pre-treatment and post-treatment with crude extract at 37 and 75 g/ml have a positive effect on the cell cycle and significantly increased the population of cells in G1 and decreased the percentage of G2 and S phase cell population. The data are expressed as mean ± standard deviation. *P<0.05 as compared to the L-glutamate-treated group.