Research Article

Effect of the Sargassum angustifolium Extract on Methamphetamine-Induced Cytotoxicity in SH-SY5Y Cells

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This study aimed to assess the effect of the Sargassum angustifolium extract in methamphetamine-induced SH-SY5Y cells death. The brown algae S. angustifolium was extracted with 80% ethanol. The SH-SY5Y cells were treated with different concentrations of methamphetamine to measure IC50. The MTT test was used to assess the toxic effect of the S. angustifolium extract in SH-SY5Y cells. SH-SY5Y cells’ survival was measured while cells were treated with different concentrations of methamphetamine and S. angustifolium extract simultaneously. A specific kit measured intracellular ROS levels. Western blot analysis evaluated the expression of cytochrome C and Bax/Bcl2 ratio. The results showed that 5 mM methamphetamine approximately killed 50% of the cells, so it is considered IC50. The MTT test showed no toxicity effect for the S. angustifolium extract. 80, 160, 320, and 640 μg/ml of S. angustifolium extract prevented the occurrence of methamphetamine toxic effects in SH-SY5Y cells after 24 hours. Moreover, the S. angustifolium extract decreased ROS levels and cytochrome C release and reduced Bax/Bcl2 ratio in cells treated by methamphetamine. On the whole, it seems that the S. angustifolium hydroalcoholic extract has the potential to increase cell survival through in vitro antioxidant and antiapoptotic activities.

1. Introduction

Signs of using herbal remedies can be found in ethnomedicine, current supplements, and medications. [1,2]. Due to the possibility of fraud in medicinal plants and variation of therapeutic agents concentration based on provider, season, and different variables, using concentrated herbal extracts is necessary. [3]. In 1893, methamphetamine (MA), n-methyl-1-phenylpropane-2-amine, was synthesized in Japan to treat idiopathic insomnia, attention deficit hyperactivity disorder, and narcolepsy [4]. Nowadays, MA abuse has become a major concern all over the world [5] because of its effects on central nervous system stimulation and causing euphoria, amplifying emotion, increment in alertness, alteration in self-esteem, and also increment sexuality [6]. It is shown that MA has a neurodegenerative effect on human brain and also can cause cardiomyopathy, myocardial problems, and respiratory failure [5]. MA-induced toxicity mechanisms involve several complex pathways; MA dramatically increases the production of reactive oxygen species (ROS) such as hydroxyl radicals (OH⁻), hydrogen peroxide (H₂O₂), and superoxide ions (O₂⁻) by increasing the oxidation of dopamine [7]. In addition, numerous studies have shown that impaired mitochondrial metabolism plays a crucial role in MA-induced dopaminergic neurotoxicity by inhibiting the Krebs cycle and electron transfer chain and helping to induce oxidative stress. These effects lead to an imbalance between oxidants and antioxidants in nerve cells [8–11]. Moreover, gene expression analysis, in-vitro and in-vivo studies have revealed that MA can affect the apoptotic gene expression and cause cell death [5,12].

Sargassum is a genus of brown algae in the order Fucales that is widely distributed in the temperate and tropical oceans of the world [13,14]. It is claimed that different algae have had cytotoxic, antioxidant, antibacterial, antiviral, and
antitoxin activities [15]. Some studies reported the antioxidant capacity of different species of Sargassum in an in-vitro model [16–18]. It is suggested that antioxidants compounds protecting nerve cells against MA-induced oxidative stress by reducing free radical production, maintaining glutathione (GSH) homeostasis, and inducing HO• expression [19]. Numerous studies have shown that the algae extract contains compounds such as amino acids, alkaloids, gallic acid, fatty acids, phenols, polysulfides, steroids, and aromatic compounds which have antitumor roles [20,21]. Namvar et al. reported that the S. ilicifolium methanolic extract could significantly affect human cancer cell lines while it does not have any toxicity effect on normal cells [22]. Also, Harada et al. reported selective cytotoxic activity of 47 species of algae in L1210 cells [23]. Moreover, anti-inflammatory effects of alginic acid [24], furans [25], and sargachromanol G [26] from S. wightii, S. vulgare, and S. siliquastrum have been revealed.

The aim of this study was to evaluate the effect of S. angustifolium hydroethanolic extract on MA-induced cytotoxicity in the SH-SY5Y dopaminergic cell line.

2. Methods

2.1. Preparation of the S. angustifolium Extract. The S. angustifolium algae were collected in December 2018 from the “Lengeh” port of Iran. They were identified by Dr. Askari, faculty member of the department of biology at the Shahid Bahonar University of Kerman. The samples were completely air-dried and pulverized by an electric mill. The dried S. angustifolium algae was soaked in 80% ethanol for 24 h and the extract was obtained by a maceration method. The extract solvent was omitted with a rotary evaporator at 40°C and material-dried in a 40°C oven. After dissolving the extract in dimethyl sulfoxide (DMSO), a solution with a concentration of 250 mg/ml was made. This solution was diluted in the sterile cell culture medium. The final hydroethanolic extract concentration was determined 3000 μg/ml.

2.2. Cells and Cell Culture. The SH-SY5Y cell line was obtained from Pasteur Cell Bank of Iran. SH-SY5Y cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) medium containing glucose with 10% fetal bovine serum, penicillin (100 μg/ml) and streptomycin (100 μg/ml). The cells were incubated for 24 h at 37°C in a humidified 5% CO₂ environment.

2.3. MTT Assay. Living cells convert the MTT, a yellow dye, into formazan crystals, a purple dye, and determine the cellular metabolic activity [27]. SH-SY5Y cells were cultured in 96-well plates. SH-SY5Y cells were exposed to 20, 40, 80, 160, 320, 640 μg/ml of S. angustifolium extract to assess the toxicity effect. To find the proper concentration of MA, the SH-SY5Y cells were treated with different concentrations of MA, and IC₅₀ was determined. To evaluate the protective effect of the S. angustifolium extract on MA-induced toxicity, the cells were exposed simultaneously to 5 mM of MA and the different doses of the S. angustifolium hydroethanolic extract (20, 40, 80, 160, 320 and 640 μg/ml). The light absorption of the purple dye was read by the ELISA at a wavelength of 490 nm.

2.4. Intracellular ROS Levels. The Human reactive oxygen species (ROS) ELISA Kit (Cat. No: MBS2515781) was used to measure ROS. This ELISA kit uses the Sandwich-ELISA principle. The micro-ELISA plate pre-coated with an antibody specific to Human ROS was used. Cells were seeded onto 96-well culture plates. 100 μl of each sample (standard working solution, control group, cells treated with five mM-MA, cells treated with five mM-MA and doses of 80 and 160 μg of the S. angustifolium extract) were added to plate wells and combined with 50 μl of Biotin-Conjugate (primary antibody), then incubated for 60 min in 37°C. The supernatant was drained, and the wells were washed 3 times for 10 seconds. One hundred μl of Streptavidin-HRP solution (secondary antibody conjugated to Horse radish peroxidase) was added to all wells and incubated at 37°C for 30 min. The wells were washed three times in 10 seconds. One hundred μl of TMB substrate (tetramethylbenzidine, substrate-dye solution) was added to all wells and incubated for 15 minutes at 37°C in the dark. The absorbance was measured by ELISA-Reader at a wavelength of 450 nm.

2.5. Protein Extraction and Western Blot Analysis. To assess the ability of MA to induce apoptosis, Bax/Bcl2 ratio and cytochrome C were evaluated. The treated SH-SY5Y cells were lysed with TNE buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1 mM (PMSE) followed by centrifugation at 13,000 rpm for 4 min at 10°C. The supernatant was collected. Equal amounts of protein (20 μl at a concentration of 40 μg) were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with nonfat milk powder (with a concentration of 2% in TBST solution) and washed three times with Tris-buffered saline containing 0.1% TBST. After washing, the membranes were incubated at 4°C overnight with the following appropriate antibodies: Cytochrome c (1: 1,000), Bax (1: 1,000), and Bcl-2 (1: 1,000) and washed three times with TBST, and incubated secondary antibody; goat anti-mouse (1: 15,000) and washed 3 times with TBST. The membranes were visualized via enhanced chemiluminescence (ECL Advance Kit).

2.6. Statistical Analysis. Data are expressed as the mean ± standard error of the mean (SEM) and were analyzed using SPSS Statistics 23.0 software. Groups were compared using the Student-Newman–Keul’s and one-way analysis of variance (ANOVA). The significance level was set at p value < 0.05. Figures were designed using Excel 2019 software.

3. Result

3.1. Effect of Different Concentrations of MA on SH-SY5Y Cell Survival. As shown in Figure 1, the MA lethal effect was
dependent on its concentration. Since MA showed a significant toxic effect at a concentration of 5 mM and approximately killed 50% of the cells, this concentration was considered IC_{50} and was used in other stages of the experiment.

Results are expressed as the means ± standard error of the mean (SEM). ***, P < 0.001 compared with the control (untreated) group.

3.2. Effect of Different Concentrations of the S. angustifolium Extract on SH-SY5Y Cell Survival. According to the results of MTT test, the S. angustifolium extract did not show toxicity in any dose (Figure 2).

Results are expressed as the means ± standard error of the mean (SEM). No significant difference was seen between the groups treated with the S. angustifolium extract and the control (untreated) group.

3.3. Effect of Different Concentrations of the S. angustifolium Extract on SH-SY5Y Cell Survival in the Presence of MA. The results of MTT test showed that the S. angustifolium extract in doses 80, 160, 320, and 640 μg/ml prevented occurrence of MA toxic effects after 24 hours (Figure 3).

Results are expressed as the means ± standard error of the mean (SEM). ***, P < 0.001 compared with the control (untreated) group. ***P < 0.001 and **P < 0.01 compared with the MA group.

3.4. The Effect of the S. angustifolium Extract on ROS Produced by MA in SH-SY5Y Cells. According to the results (Figure 4), ROS levels in the MA-treated group significantly increased in comparison to the control group (P < 0.001). The results of ROS assay in MA-treated cells with doses of 80 and 160 μg/ml S. angustifolium extracts were significantly different from the MA group (P < 0.001) and the S. angustifolium extract could prevent the production of intracellular ROS.

Results are expressed as the means ± standard error of the mean (SEM). ***, P < 0.001 compared with the control (untreated) group. ***P < 0.001 compared with the MA group.

3.5. Protein Extraction and Western Blot Analysis. The release of cytochrome C in SH-SY5Y cells exposed to MA was significantly different from the control group (treated with normal medium) (P < 0.001). As shown in Figure 5, when cells were treated simultaneously with 5 mM MA and different doses of the S. angustifolium extract (80 and 160 μg/ml), the amount of cytochrome C was significantly reduced by the MA group. In SH-SY5Y cells treated with MA, the ratio of Bax/Bcl2 increased significantly compared to cells cultured in a normal medium (P < 0.001). When MA-treated cells received 80 and 160 μg/ml of the S. angustifolium extract, the Bax/Bcl2 ratio was significantly reduced compared to MA-treated cells (P < 0.001) (Figure 6).

β-actin served as a protein loading control. The relative level of cytochrome C was normalized with β-actin. Results are expressed as the means ± standard error of the mean (SEM). *, P < 0.05, **, P < 0.01 and ***, P < 0.001 compared with the control (untreated) group. ***P < 0.01 compared with the MA group.

The relative levels of Bax and Bcl2 were normalized with β-actin. Results are expressed as the means ± standard error of the mean (SEM). ***, P < 0.001 compared with the control (untreated) group. ***P < 0.001 compared with the MA group.

4. Discussion

Mitochondrial metabolism disturbance plays a vital role in MA-induced dopaminergic neurotoxicity by inhibiting the Krebs cycle and electron transport chain and contributing to oxidative stress [28]. Due to lipophilic and cationic structure, MA could easily enter the mitochondria. Superoxide ion and hydrogen peroxide formation have been identified as the main cause of MA-induced neurotoxicity [29,30]. This phenomenon is clinically important because it could ultimately lead to destructive neurological effects in the brain due to mitochondrial dysfunction, induction of intracellular oxidants, caspase activation, and apoptotic neuronal death [10]. In the present study, MTT assay was used to evaluate the protective effects of the S. angustifolium hydroethanolic extract on cytotoxicity induced by MA. Results demonstrated that the S. angustifolium extract in doses of 80, 160,
Figure 2: Effect of different concentrations of the *S. angustifolium* hydroethanolic extract on SH-SY5Y cell survival.

Figure 3: Effect of different concentrations of the *S. angustifolium* extract on SH-SY5Y cell survival in the presence of MA.

Figure 4: Effect of the *S. angustifolium* extract on ROS produced by MA in SH-SY5Y cells.
320, and 640 could prevent the occurrence of MA toxic effects.

MA leads to the formation of intracellular ROS by altering intracellular calcium signaling and weakening antioxidant defenses [31]. Following the use of antioxidants, increasing the expression of Cu-Zn-SOD and MnSOD (superoxidases) and decreasing the amount of ROS, MA-induced toxicity is attenuated [32]. In the present study, the results of ROS assay in cells treated simultaneously with MA and 80 and 160 μg/ml of S. angustifolium extract showed that the S. angustifolium extract significantly inhibited intracellular ROS production. The effect of different species of Sargassum on reducing intracellular ROS production has been reported previously [24,33]. According to studies,
Sargassum contains terpenoids that have biological activities such as cytotoxicity in cancer cells and antioxidant activity [15,34]. In addition, the brown algae of the genus Sargassum have unique secondary metabolites such as plastoquinone [35], chromanols [36], polysaccharides [37], and fecosterol [38], which have been introduced as a good option for protection against free radicals. A study by Babakhani et al. reported that the phenolic compounds of S. angustifolium include gallic acid, protocatechuic acid, gentisic acid, and hydroxybenzoic acid which makes it seem that this alga is a rich source of antioxidant compounds [39]. Numerous studies have shown that fucoidan, stypoldione, terpenes, sterols, fatty acids, and phenolic compounds have great potential for anticancer and cytotoxic activities [40,41]. Due to the fact that the S. angustifolium extract contains large amounts of phenolic compounds, which is among the most important antioxidants [42], it is possible that the protective effect and cell viability of S. angustifolium hydroethanolic extract be related to its phenolic compounds.

MA-induced neurodegeneration associated with mitochondrial apoptosis [43]. The presence of reactive oxygen and nitrogen species indicates an increase in the expression of apoptotic promoting proteins: Bax, Bad, and Bid as well as a decrease in expression of antiapoptotic proteins: Bcl-2 and Bcl-XL [44], and increased Bax/Bcl-2 ratio indicates release of cytochrome C from mitochondria [45]. Subsequently, cytochrome C binds with apoptotic protease-activating factor 1 (Apaf-1), and in the execution phase of apoptosis, activates the initiator caspase [6,46,47]. According to the abovementioned facts, oxidative stress caused by MA is one of the main factors in inducing apoptosis. Inhibition of these factors plays an important role in preventing the progression of apoptosis.

The present study results showed that the S. angustifolium extract has a significant effect on reducing the Bcl2/Bax ratio and reducing the release of cytochrome C in SH-SY5Y cells exposed simultaneously to 5mM-MA and 80 and 160μg/ml of S. angustifolium extract. Therefore, decreased expression of apoptotic proteins could be considered as one of the possible mechanisms of the S. angustifolium extract in reducing apoptosis. In addition, the anti-inflammatory activity of Sargassum species extract has been reported through inhibition of COX-2, TNF-α, IL-6, PGE2, and inhibition of NF-xB nuclear translocation [24,38]. Anti-inflammatory compounds, via inhibition of the expression of inflammatory mediators and apoptosis proteins, play a role in controlling their function [48]. So, according to the abovementioned statements, one of the possible mechanisms of S. angustifolium extract in reducing the expression of apoptotic proteins could probably be the inhibition of inflammatory factors.

5. Conclusion

The S. angustifolium hydroethanolic extract decreased MA-induced toxicity in the SH-SY5Y cells line by reducing intracellular ROS, cytochrome C, and Bax to Bcl2 ratio, and significantly increased SH-SY5Y cells survival in the current in vitro study. Further studies are needed to identify the most effective and potent compounds in the S. angustifolium extract which play a key role in cellular protection.

Data Availability

The data used to support the findings of this study are available from the corresponding author on request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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