

Glycogen synthase kinase-3 β may contribute to neuroprotective effects of *Sargassum oligocystum* against amyloid-beta in neuronal SH-SY5Y cells

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ABSTRACT

Glycogen synthase kinase (GSK)-3 β mediates amyloid-beta (A β) and oxidative stress-induced neurotoxicity in neurodegenerative disorders. Natural products with antioxidant activity, such as *Sargassum* (S.) *oligocystum* may modulate GSK-3 β enzyme and protect against A β -induced neurotoxicity. Therefore, we aimed to assess the neuroprotective effects of a methanolic extract of S. *oligocystum* against A β -induced neurotoxicity in the SH-SY5Y cells and the contribution of GSK-3 β inhibition to the neuroprotective effects of the S. *oligocystum* extract. SH-SY5Y neuroblastoma cells were seeded in 96 well plates and incubated with A β (20 μ M) and the methanolic extract of S. *oligocystum* (40, 50, and 70 μ g/ml) for 24h. We measured cell viability using the MTT assay. Western blot method was used to measure the expression of the GSK-3 β and phosphorylated (p)-GSK-3 β protein levels. The data were analyzed using one-way analysis of variance (ANOVA) followed by the LSD test.

Amyloid-beta (20 μ M) reduced neuronal cell viability compared with the control group. Addition of S. *oligocystum* extract at concentrations of 40, 50 and 70 μ g/ml decreased the neurotoxic effects of A β . The extract of S. *oligocystum* at a concentration of 70 μ g/ml also decreased the effects of A β on the GSK-3 β protein level. The pGSK-3 β protein levels in the S. *oligocystum* groups (40 and 70 μ g/ml) plus A β were lower than the A β -treated group. The methanolic extract of S. *oligocystum* protected SH-SY5Y cells from A β -induced neurotoxicity. The attenuation of the GSK-3 β protein level may contribute to the neuroprotective effects of S. *oligocystum* extract.

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Keywords

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INTRODUCTION

Alzheimer's disease (AD) is a progressive neurological disorder and the leading cause of dementia in the old ages. The deposition of intra-neuronal fibrillary tangles and extra-neuronal amyloid-beta (A β) aggregates in the brain are the hallmarks of AD [1]. Accordingly, A β aggregation activates neuronal signaling systems and induces an inflammatory response, oxidative stress, and apoptosis in the brain regions

responsible for cognitive functions [2]. Glycogen synthase kinase-3 β (GSK-3 β) is an enzyme with essential functions in inflammation, oxidative stress, and neuronal apoptosis. This enzyme substantially contributes to the A β -induced neurotoxicity [3].

Embi and his colleagues discovered the GSK-3 protein as an enzyme responsible for glycogen metabolism [4]. Cur-

rently, it has been considered as a regulator of several cellular functions, including cellular metabolism and apoptosis [5]. GSK-3 has two isoforms, including GSK-3 α and GSK-3 β , though GSK-3 β is the enzyme primarily involved in the neurodegenerative disorders [6]. GSK-3 β phosphorylates the tau protein and links the A β -induced tau phosphorylation and neurodegeneration [7]. This enzyme enhances pro-apoptotic protein expression and inhibits the anti-apoptotic proteins [8]. GSK-3 β mediates the A β and oxidative stress-induced neurotoxicity in the process of neurodegenerative disorders [9].

Considering GSK-3 β roles in the AD, many groups tried to find GSK-3 β inhibitors as the potential treatment for neurodegenerative disorders [10]. Some studies have reported that GSK-3 β inhibitor VIII and lithium chloride suppresses A β -induced neuronal death [8, 10]. Moreover, GSK-3 β inhibitors II or VIII have reduced oxidative stress neuronal apoptosis in differentiated PC12 cells [9]. Therefore, the inhibitory effects of the antioxidant agents on the GSK-3 β may ameliorate A β -induced neuronal cell death.

Sargassaceae is a family of brown algae with broad distribution in tropical and subtropical seas [11]. Several reports have documented a potent antioxidant activity of Sargassaceae species in the peripheral tissues [12, 13]. The natural habitat of *Sargassum oligocystum* (*S. oligocystum*), a member of Sargassaceae family, is the coastal waters of the Persian Gulf [14]. Previous studies have shown that *S. oligocystum* has antibacterial, anticancer and anticonvulsant activity [14-16]. Importantly, in a study documented in 2017, we showed that *S. oligocystum* exerted potent antioxidant activity in the mouse brain [15]. Some evidence has shown the beneficial effects of Sargassum family on the neuronal cells. Therefore, in this study, we aimed to evaluate the neuroprotective effects of the methanolic extract of *S. oligocystum* against A β -induced neurotoxicity in the SH-SY5Y cells. Moreover, we tried to explore the contribution of GSK-3 β inhibition to the neuroprotective effects of the *S. oligocystum* extract.

MATERIALS AND METHODS

Materials and reagents

Human SH-SY5Y neuroblastoma cells (Pasteur Institute, Iran) were used in this study. Dulbecco's Modified Eagle's Medium and Ham's Nutrient Mixture F-12 (DMEM/F-12), fetal bovine serum (FBS), and Penicillin-Streptomycin from Gibco® life technologies™ (USA) were used for cell culture. Amyloid- β 25-35 (Sigma-Aldrich, USA), the anti-actin, anti-GSK-3 β , and anti-phosphorylated (p)-GSK-3 β antibodies (Cell Signaling Technology®, USA) were prepared.

Neuronal Cell Culture

The cells were seeded at a density of 1×10^5 cells/well in the 96-well plates and maintained in a mixture of (1:1) DMEM/F-12, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin. The plates were kept in a humidified atmosphere of 95% air and 5 % CO₂ at 37°C.

Preparation of methanolic extract of *S. Oligocystum*

The *S. oligocystum* is a native alga of the Persian Gulf. After the alga was collected, it was washed with distilled water and dried at room temperature. Then, the powdered alga was soaked in methanol in a ratio of 1:10 and homogenized. We separated the supernatant using a Whatman filter paper No. 1. After centrifugation for 10 min at 4000 rpm and 4°C, a rotary evaporator removed the solvent. The methanolic extract was maintained in the refrigerator (4 °C).

Amyloid- β 25-35 Preparation

Amyloid- β 25-35 was dissolved in sterile distilled water at a concentration of 2 μ g/ μ l and maintained at -70°C until use. After the preparation of 20 μ M solution, A β 25-35 was incubated for 4 days at 37 °C to induce the aggregation process.

Treatment

We dissolved the methanolic extract of *S. oligocystum* in the phosphate-buffered saline (PBS). An appropriate concentration of A β and *S. oligocystum* were determined in a pilot study. On the day of treatment, the culture media were replaced with the serum-free media, and then A β 23-35 (20 μ M), the *S. oligocystum* extract (40, 50 and 70 μ g/ml), or both of them were added.

Cell Viability Assay

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reagent at a concentration of 5mg/ml was added to the cell culture media at 24h after the treatments. The culture media were removed after 4h and the precipitate was dissolved in 100 μ l of dimethyl sulfoxide (DMSO). The absorbance at a 570 nm was measured using a microplate reader (Synergy HT, Biotek®) to determine the cell viability.

Total protein determination

The SH-SY5Y cells were cultured in the 6-well plates at a density of 10^6 cells/ml to analyze the protein content. Two ml of cell suspension was added to each plate. After incubation with *S. oligocystum* extract and A β , the cells were harvested by centrifuging at $14000 \times g$ for 5 min. The cells lysed using the radio-immunoprecipitation assay lysis (RIPA) buffer containing protease and phosphatase inhibitor cocktail. The cell lysates were centrifuged at 13000 rpm for 25 min at 4°C to remove the insoluble debris. The supernatant was used to determine the total protein level. Total protein was measured by Lowry method.

Western blot analysis

Equal amounts of proteins were separated by sodium dodecyl sulfate (SDS)- polyacrylamide gel electrophoresis (PAGE) and transblotted onto a polyvinylidene fluoride (PVDF) membrane. After transfer, the membrane was blocked with 5% bovine serum albumin (BSA) for 1h at the room temperature. The membrane was incubated overnight with the GSK-3 β (27C10) rabbit monoclonal antibody, p-

GSK-3 β (Ser9) antibody, and actin antibody. The membrane was washed with PBS and incubated with the Anti-rabbit IgG (HRP-linked antibody, 1:2500) (7074s, cell-signaling) at 37°C for 1h. Then the membrane was incubated in enhanced chemiluminescence (ECL) Western Blotting Substrate (enhanced chemiluminescence kit, GE Healthcare, Amersham) for 1 min at the room temperature. Finally, the membrane was scanned using the ChemiDoc™ XRS+ imaging System. The bands were analyzed by the image-J® software (version 1.8.0, Bethesda, MD, USA).

Statistical analysis

We analyzed the variables using the one-way ANOVA followed by the LSD test. $P < 0.05$ was considered statistically significant. All analyses were performed using SPSS software version 23 (SPSS, Inc.).

RESULTS

Neuroprotective effects of *S. oligocystum* against A β toxicity

The present study showed that the cell viability in various groups was different ($F(df) = 15.299(4)$, $P < 0.001$) (Fig. 1). The pairwise comparison showed that A β (20 μ M) reduced the neuronal cell viability compared with the control group ($P < 0.001$) (Fig. 1). Moreover, the addition of *S. oligo-*

cystum extract at concentrations of 40 μ g/ml ($P = 0.002$), 50 μ g/ml ($P = 0.001$) and 70 μ g/ml ($P < 0.001$) to the neurons treated with A β diminished the neurotoxic effects of A β (Fig. 1).

Glycogen synthase kinase-3 β contribution to the neuroprotective effects of *S. oligocystum* against A β toxicity

The expression of GSK-3 β and pGSK-3 β were different in the studied groups ($F(df) = 1961.09$ (5), $P < 0.001$, and $F(df) = 99.99$ (5), $P < 0.001$), respectively (Fig. 2). The GSK-3 β protein level in the A β -treated group was lower than the control group ($P < 0.001$) (Fig. 2). The GSK-3 β protein level in the *S. oligocystum* (40 and 70 μ g/ml) plus A β groups was lower compared to the A β -treated group ($P = 0.001$) (Figure 2). Moreover, the GSK-3 β protein levels in the *S. oligocystum* (40 μ g/ml) ($P < 0.001$) and (70 μ g/ml) ($P < 0.001$) groups were lower than the control group (Fig. 2).

Treatment of neuronal cells with A β decreased the pGSK-3 β protein level compared to the control group ($P < 0.001$) (Fig. 2). However, the pGSK-3 β protein level in the groups of *S. oligocystum* (40 μ g/ml) plus A β was higher than the A β -treated group ($P < 0.011$) (Fig. 2). In contrast, the pGSK-3 β in the *S. oligocystum* (70 μ g/ml) plus A β group was lower than the A β -treated group ($P < 0.014$) (Fig. 2).

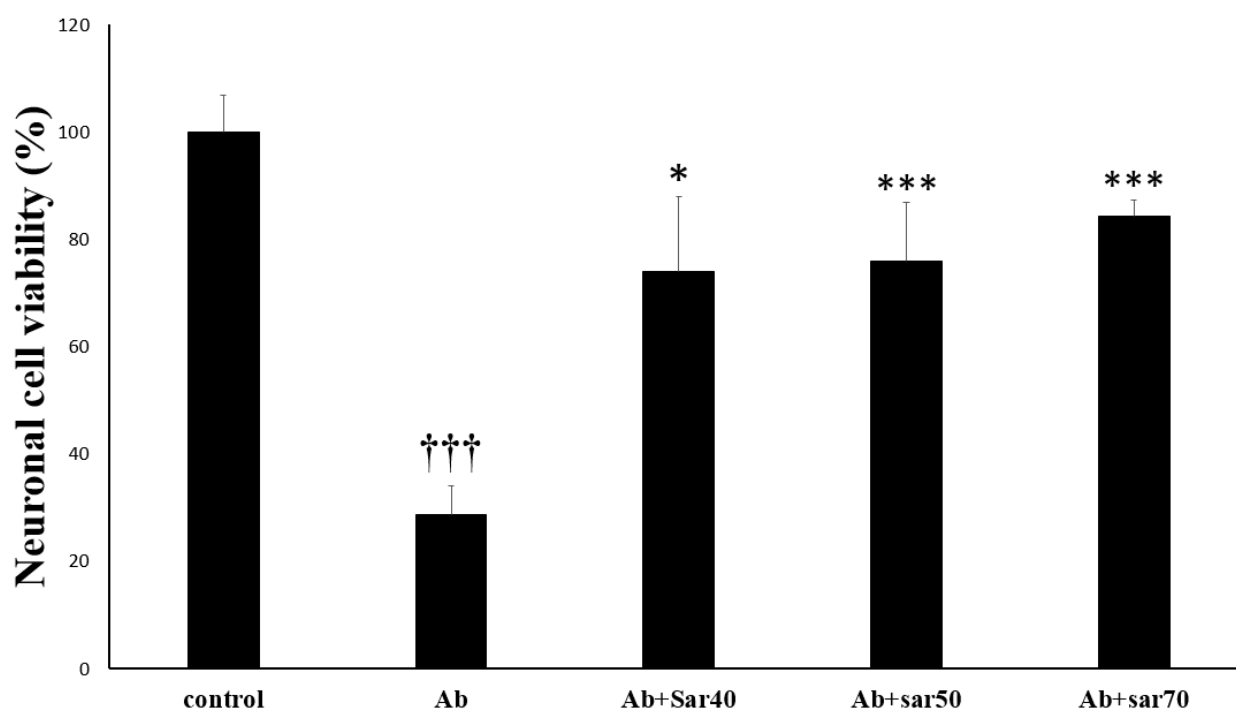


Figure 1. Neuronal viability of SH-SY5Y neuroblastoma cells treated with beta amyloid (A β) (20 μ M) and *Sargassum oligocystum* methanolic extract (40, 50, and 70 μ g/ml) for 24h. The data indicated 4 independent experiment. The cell viability was measured by the MTT test. Data were analyzed using one-way analysis of variance (ANOVA) followed by the LSD test. Data were expressed as mean + standard error of mean. $P < 0.05$ was considered statistically significant. †††: $P < 0.001$ compared to the control group, * and ***: $P < 0.05$ and $P < 0.001$ compared to the A β -treated group, respectively. A β : beta amyloid, Sar40: *sargassum oligocystum* methanolic extract 40 μ g/ml, Sar50: *sargassum oligocystum* methanolic extract 50 μ g/ml, Sar70: *sargassum oligocystum* methanolic extract 70 μ g/ml.

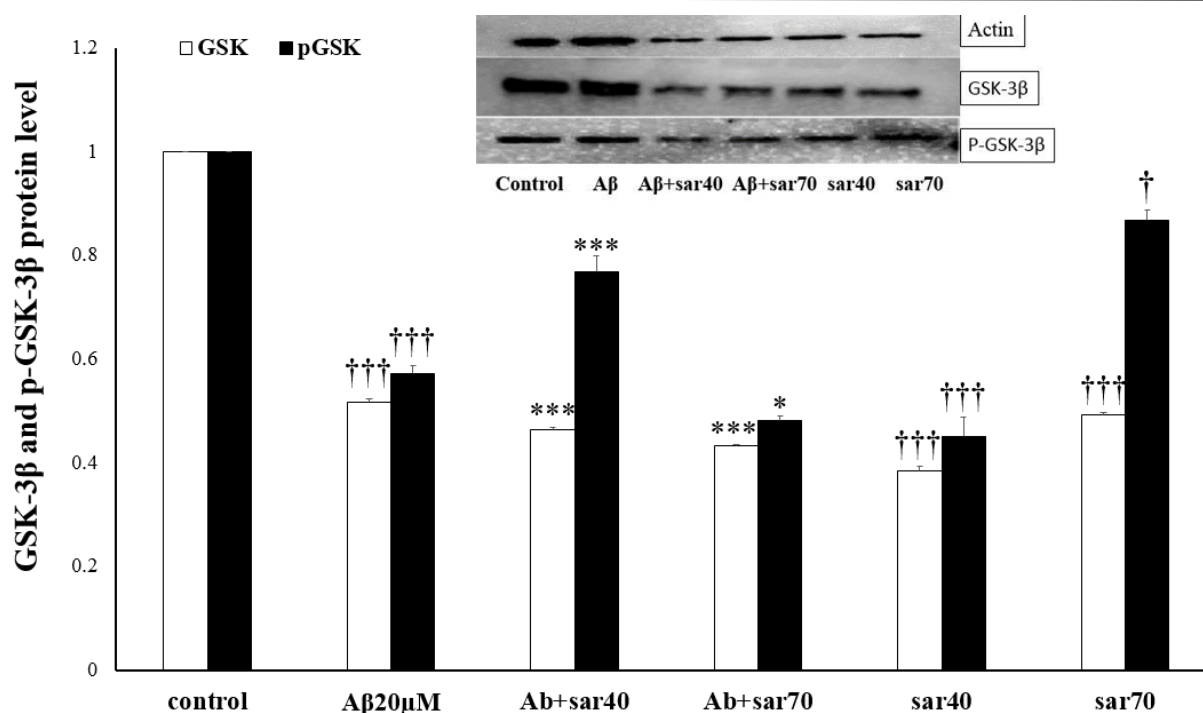


Figure 2. The glycogen synthase kinase (GSK)-3 β and phosphorylated-GSK-3 β protein levels in the SH-SY5Y neuronal cell lines were treated with amyloid-beta (A β) (20 μ M) and Sargassum *oligocystum* methanolic extract (40, 50, and 70 μ g/ml) for 24h. The protein levels were measured using western blot. The data indicated four independent experiments. Data were analyzed using one-way analysis of variance (ANOVA) followed by the LSD test. Data expressed as mean + standard error of mean. $P < 0.05$ was considered statistically significant. †††: $P < 0.001$ compared to the control group, * and ***: $P < 0.05$ and $P < 0.001$ compared to the A β -treated group, respectively. A β : amyloid-beta, Sar40: sargassum *oligocystum* methanolic extract 40 μ g/ml, Sar70: sargassum *oligocystum* methanolic extract 70 μ g/ μ l.

DISCUSSION

This study showed that the *S. oligocystum* extract prevented the A β -induced neurotoxicity in the SH-SY5Y cell lines. Some studies have shown the neuro- and glioprotective effects of Sargassum family or their derivatives. Yang et al. [17] showed that 3 extracts of *S. crassifolium* possessed a potent antioxidant effect and neuroprotective activity in PC-12 cells. Moreover, the methanolic extract of *S. muticum* produced the neuroprotective effects against 6-hydroxydopamine (6-OHDA) neurotoxicity in the SH-SY5Y cells [18]. An in vivo study in a scopolamine-induced amnesia model showed that a Sargassum species protected cerebral cortex neurons of mice and enhanced the animal's memory [19]. Furthermore, the crude extract of *S. fusiforme* enhanced memory, but it decreased the A β load in an animal model [20]. Except for the crude extract of Sargassum family, some studies have reported the neuroprotective and neuromodulatory effects of Sargassum derivatives. Jin and his colleagues [21] showed that the heteropolysaccharides from Sargassum species attenuated 6-OHDA-induced neurotoxicity in MES 23.5 cells. Moreover, sargachromenol, a compound extracted from *S. macrocarpum*, exerted the neuroprotective effects and promoted neurogenesis in PC-12 cells [22]. Fucosterol, another brown alga component, decreased

the intracellular level of A β and protected SH-SY5Y cells against A β [20]. Pheophytin-a produced a nerve growth factor (NGF)-like activity in neurons [23]. Sargaquinoic acid, another substance extracted from *S. macrocarpum*, promoted neurite outgrowth by the mediation of TrkA-dependent MAP kinases pathway [24]. Thus, the Sargassum species especially *S. oligocystum* are potential targets to produce new neuroprotective agents in the treatment of AD.

Exact mechanism of action of *S. oligocystum* is not completely clear. However, some studies have shown that the antioxidant activity and the manipulation of signaling systems involved in neuronal apoptosis may be relevant [21, 25]. We previously showed that a *S. oligocystum* extract produced an antioxidant activity in the mouse brain [15]. Furthermore, the methanolic extract of *S. muticum* protected SH-SY5Y cells by decreasing the peroxide free radicals and protecting mitochondria by reducing Caspase-3 activity [18]. In addition, Huang et al [26] have shown that fucoidan extracts of *S. hemiphyllum* exerted antioxidant and neuroprotective activity against 6-OHDA-induced apoptosis. Moreover, Sargassum species protected the neurons via reducing hydrogen peroxide production, repairing the impaired mitochondrial membrane's potential and the amelioration of

Caspase-3 activity [18]. Thus, the neuroprotective effects of *S. oligocystum* might be related to the antioxidant activity of this alga.

Ongoing rigorous investigations try to elucidate the pathophysiology of AD. However, the A β aggregation may start a process that changes the central nervous system (CNS) homeostasis and causes neuronal death in the brain regions responsible for memory [8]. Glycogen synthase kinase-3 β may be involved in the apoptotic mechanism of A β [3]. Thus, GSK-3 β regulation is an attractive target for the treatment of neurodegenerative disorders [9]. The GSK-3 β pathway is among intracellular pathways that have close convergence with oxidative stress-induced apoptotic death in neurons [9]. Furthermore, some GSK-3 β inhibitors suppressed oxidative stress-induced neurotoxicity by the modulation of GSK-3 β activity [9]. The *S. oligocystum* extract produced strong antioxidant effects in the CNS[15]. Therefore, the regulation of GSK-3 β activity may contribute to the neuroprotective effects of *S. oligocystum*. In the present study, the methanolic extract of *S. oligocystum* protected the neuronal cells by decreasing of GSK-3 β . It is possible to suggest that the attenuation of oxidative stress by *S. oligocystum* may suppress GSK-3 β activity in the SH-SY5Y cells. Another possibility may be the notion that *S. oligocystum* derivatives have a direct GSK-3 β inhibiting activity. Therefore, future studies by extracting specific fractions or compounds from *S. oligocystum* may help to produce a new GSK-3 β inhibitor.

Our study also showed that A β reduced the phosphorylated (p)-GSK-3 β level in the SH-SY5Y cells. Similarly, Crouch et al. [27] showed that A β decreases the p- GSK-3 β level in the SH-SY5Y cells. The interaction of A β with insulin, Wnt signaling or N-Methyl-D-Aspartate (NMDA) receptors elevated the GSK-3 β activity in the brain of patients with AD and mouse model of AD [28]. In the CNS, Akt is a downstream cascade of these signaling system that is responsible for the GSK-3 β phosphorylation and inactivation [29]. Amyloid- β oligomers exert an antagonizing activity on insulin receptors and prevent the activation of PI3 kinase/Akt [30]. Moreover, A β effects on Wnt signaling prevents the inactivation of GSK-3[31]. The hydroalcoholic extract of *S. oligocystum* exerted beneficial effects on diabetes mellitus in an animal model [32]. The finding of our study showed that methanolic extract of *S. oligocystum* increased the p-GSK-3 β protein level after treatment with A β .

The main limitation of this study may be the use of the crude extract of *S. oligocystum*. The extraction of the specific fractions of *Sargassum* may result in more favorable results. Moreover, the present study was conducted in the cell line and future studies in the animal models of AD seems necessary.

CONCLUSION

The methanolic extract of *S. oligocystum* protected SH-SY5Y cells from A β -induced neurotoxicity. The suppression of oxidative stress and the attenuation of GSK-3 β protein level were the possible mechanisms of action of *S. oligocystum* extract. Future studies on specific derivatives of this

alga may help to find new treatments for AD.

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CONFLICTS OF INTEREST

The author(s) declare(s) that there is no conflict of interest regarding the publication of this article

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