RESEARCH ARTICLE

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The Effect of Sargassum Angustifolium-derived AgNPs on Apoptosis-associated Genes in Acute Lymphoblastic Leukemia Cells

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Abstract

Introduction: Acute lymphoblastic leukemia (ALL) is among the most prevalent child cancers. Moreover, chemotherapy and bone marrow transplantation have failed to secure the survival of patients in some cases. Various researches have revealed that glycogen synthase kinase 3 (GSK-3) inhibitors can contain the growth of some cancers. Furthermore, inositol trisphosphate receptor (IP3R) exists in all cell types and is implicated in metastasis. The application of organic, natural substances offers new prospects for the treatment of this condition. Accordingly, the aim of this study was to examine the silver nanoparticles synthesized from Sargassum Angustifolium on the expression of IP3 and GSK receptors in ALL Jurkat cells. **Methods:** We isolated Sargassum Angustifolium extract and mixed it with silver nanoparticles (NPs) and treated the cells with the mixture. The changes in the expression of *GSKα*, IP3R3 and GSKβ genes in the Jurkat cell line were studied. In this research, quantitative mRNA expression of the target gene was measured using a real-time polymerase chain reaction (PCR). Hypoxanthine phosphoribosyltransferase (HPRT) genes were studied as the internal control. The experiments were replicated 3 times. Data analysis was performed through one-way ANOVA and t-test. The significance level was considered less than 0.05. **Results:** The results of this study revealed that different concentrations of the extracts significantly decreased the expression levels of *GSKα*, IP3R3 and GSKβ gene in Jurkat cells compared to control groups. The combination of algae extract and AgNPs was consistently the most effective group. **Conclusion:** silver nanoparticles synthesized from sargassum algae in the Persian Gulf could be utilized to treat ALL cancers and even other tumors.

Keywords: Acute lymphoblastic leukemia- Inositol trisphosphate- Seaweed- Silver nanoparticles- glycogen synthase

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Introduction

Acute lymphoblastic leukemia (ALL) is a B or T lymphoblasts malignancy marked by uncontrolled proliferation of abnormal, immature lymphocytes (Terwilliger and Abdul-Hay, 2017). This type of leukemia is the most prevalent malignant disorder in children(Den Boer et al., 2009; Kantarjian et al., 2017). For children suffering from ALL, potential cancer treatment modalities are surgery, chemotherapy, steroid therapy, radiation therapy, immunotherapy (Ghaffari et al., 2019), and intensive combination treatments, i.e. bone marrow/stem cell transplant and growth factors (Eldar-Finkelman, 2002; GhaffariKhalili et al., 2021; Hooper et al., 2008). Most of the anticancer drugs that are utilized in the clinic have cytotoxicity, meaning they are toxic to normal cells. Also, bone marrow transplantation has not always been a successful treatment method. Various researches have revealed that glycogen synthase kinase 3 (GSK-3) inhibitors can contain the growth of some cancers (Duda et al., 2020). Furthermore, one of the factors examined amongst the many biomarkers whose levels have been compared with normal and leukemic cells is the level of the inositol trisphosphate receptor (IP3R). Its receptor exists in all cell types on the surface of a smooth endoplasmic reticulum that is a calcium homeostasis regulator and is implicated in metastasis. Likewise, many studies have recorded the role of calcium in cell death and autophagy (Duda et al., 2020; Hooper et al., 2008).There is a variety of natural products that influence the activity of *GSK*-3 and inositol trisphosphate and that might be viewed as a potential cancer treatment alternative approach with less cytotoxicity than that of chemotherapy drugs (Duda et al., 2020; Hooper et al., 2008).

Silver nanoparticle technology has wide applications in forming a new generation of cancer treatments. Nanoparticles could be obtained from many sources, namely, microorganisms and plants. Some metabolites

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extracted from algae might influence cell cycle proliferation, apoptosis, and arrest through various mechanisms, some of which include the proliferation of natural killer (NK) cells, activation of the nonspecific immune system, cell growth inhibition, angiogenesis, and induction of apoptosis (GhaffariTorabi-Rahvar et al., 2021). Likewise, some of the metabolites separated from algae induce cell cycle proliferation, apoptosis, and arrest via diverse mechanisms. Some of these mechanisms involve proliferated NK cells, activated nonspecific immune systems, inhibited cell growth, angiogenesis, and induced apoptosis (Ahmed et al., 2023). Sargassum angustifolium is a brown seaweed scattered in the Persian Gulf of Iran and is a vital source of protein, carbohydrates, vitamins, minerals, and antioxidants (Morais et al., 2022).

The fucoidan obtained from different types of algae have anti-tumor, anti-inflammatory, anti-viral, antimicrobial, wound healing, and antioxidant qualities have been previously confirmed (Li et al., 2022). Iran enjoys wide coastlines along the Persian Gulf and the Sea of Oman. Although there are considerable amounts of seaweed in the area, few studies have been performed on their biological activity.

Since ALL commonly occurs in children, and moreover, chemotherapy and transplantation failed to guarantee the survival of patients in some cases, the application of organic material is viewed as a new approach to the treatment of these patients. Thus, the aim of this study was to examine the impact of nanoparticles synthesized from Sargassum Angustifolium on the expression of IP3 and *GSK*.

Materials and Methods

Drug Preparation

S. Angustifolium-mediated silver NPs was first synthesized at Bushehr Persian Gulf Marine Biology Institute. The extract of Sargassum Angustifolium was mixed 3mM silver nitrate in a 1:10 ratio. The mixture was subsequently sonicated for an hour via an ultrasonic bath. The product was further purified with multiple centrifugations and then characterized with transmission electron microscope (TEM). Before treatment with cells, AgNPs were filtered through a 0.4 µm syringe filter and used to make serial dilutions of varying concentrations with fetal bovine serum (FBS). The production of silver nanoparticles Sargassum Angustifolium using marine algae was done by extracellular biological method. The results obtained from UV-TEN, VIS, FT-IR spectroscopy from our lab confirmed the biological production of silver nanoparticles using the tested algae. After adding Ag-NP to algae extract, the color of the resulting mixture changes from yellowish brown to dark brown (Hosseinpouri et al., 2022; Obeidi et al., 2020). Our study follows the standard AIMRDA standard reporting recommendation(Ahmad et al., 2022).

Cell Culture and Growth Synchronization

We used Jurkat cell line in this study in the laboratory of Bushehr University of Medical Sciences in the spring of 2019. The study was approved and authorized by the mentioned university's Ethics Committee (Code: 01/1274). The cells were taken from Pasteur Institute of Iran and the routine mycoplasma test was conducted to make sure cells were uninfected. The cells were transferred to 25 cm^2 flask containing 4 ml of 10% FBS, and after disinfecting the flask with 70% alcohol, the flask was transferred to an incubator at 37 degrees Celsius and 5% CO_2, following sterile instructions. For 2 days, the cells were kept in the incubator to grow, and after 2 days, the first exchange of flask medium was done. We checked the flasks under a microscope every day to get information about the conditions and morphology of the cells. The culture media containing 10% FBS plus 100mg/ml of penicillin/streptomycin was added to the flask and incubation at 37 degrees and 5% CO_2. Incubation in these conditions continued until the cells reached a certain density so that they could be transferred to a new flask. The culture medium was RPMI (Gibco, USA) plus 2 g sodium bicarbonate, 2 g HEPES, and 10% of bovine serum. We then proceeded to synchronize cells' growth phases. Before treating cells with the drug, all cells must be in the same growth phase. To do so, after passaging, serum-free culture medium was added to them. With these conditions, the cells survived and the absence of FBS (Gibco, USA) in the culture medium caused the cells to no longer spontaneously grow and all were in the same phase. The time required for the incubation of cells with different drug dilutions was 24, 48 and 72 hours. After the end of the incubation time, the desired cell culture plate was removed from the incubator and examined. The MTT (Merck KGaA kit, Germany) method was employed to achieve the optimal concentration.

RNA Extraction cDNA Synthesis

It is important to extract RNA with the right quantity and quality for gene expression analysis with Real Time PCR. One of the most important factors in producing good quality cDNA is to obtain total RNA with proper purity and integrity. After extracting the RNA of the cells with Thermo FisherTM RNA Extraction Kit (by the column method and based on the kit brochure), we checked the quality of the RNA by electrophoresis on agarose gel containing ethidium bromide. The Reverse Transcriptase enzyme was utilized on the RNA sequence to produce the DNA strand that represents the same RNA sequence. Measuring the amount of cDNA synthesized can express the same amount of RNA produced or the expression of the desired gene.

Real-Time PCR

This study employed the Addibo kit (South Korea) and Applied Biosystem to implement Real-Time PCR and quantitatively measure target gene mRNA. PCR conditions were optimized for 2MgCl concentration in the 5-1 µM range. In order to proliferate each gene fragment, a specific primer pair was picked for each gene, which constituted forward and reverse primers (Table 1).

Statistical Analysis

The trials were conducted three times. Descriptive statistics methods (Tables, frequency distribution, indexes,

and graphs) were used to describe the data. Data analysis was applied using ANOVA (Graphpad prism7 software Inc., San diego, CA, USA and t-test. The significance level was considered less than 0.05.

Results

Changes in IP3R3, GSKβ, GSKα gene expression in Jurkat and control groups

As shown by the one-way ANOVA (Graphpad prism7 software Inc., San diego, CA, USA) test, the changes in *GSKα* gene expression in the Jurkat cell line were significant, and a two-by-two comparison was made with the Jurkat control group (Figure 1). According to our results, only the nanoparticle plus algae group at maximum concentration was able to statistically significantly decrease the expression of *GSKα* (P= 0.0097). This group at half the concentration (JUR-Ag-50) and maximum algae concentration (JUR-Alga-Max) were also able to decrease *GSKα* expression compared to Jurkat control group, but the difference was not significant.

Likewise, the one-way ANOVA test showed that the changes in *GSKβ* gene expression in the Jurkat cell line group were statistically significant (Figure 2). The JUR-Ag-Max group had a significant decrease in *GSKβ* gene expression compared to the Jurkat control group (p= 0.0002). The respective significant increase (p=0.0001) and decrease (p=0.0053) of JUR-Ag-50 and JUR-Alga-Max groups were also observed.

Based on the one-way ANOVA test, changes in *IP3R3* gene expression in Jurkat cells were statistically significant in almost all the groups which could

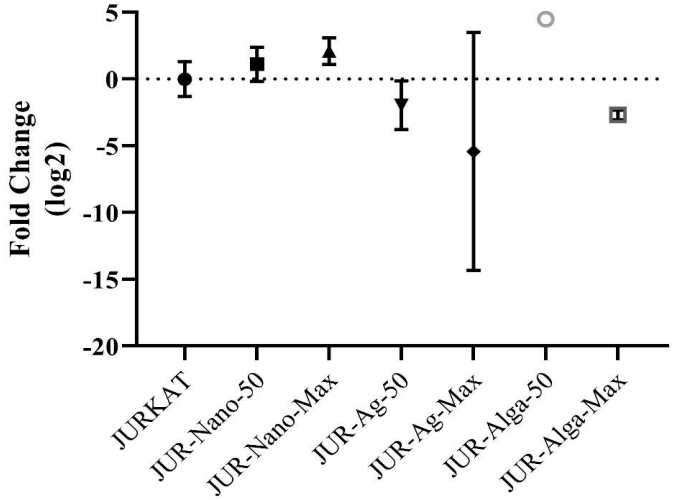

Figure 1. Changes in *GSKα* Gene Expression in Jurkat Cell Line. The nanoparticles alone (JUR-Nano-50 and JUR-Nano-Max) slightly increased GSKα expression while algae alone (JUR-Alga-50 and JUR-Alga-Max) changes *GSKα* expression as well. Only the combined group at maximum concentration statistically significantly decreased *GSKα* expression compared to Jurkat control group.

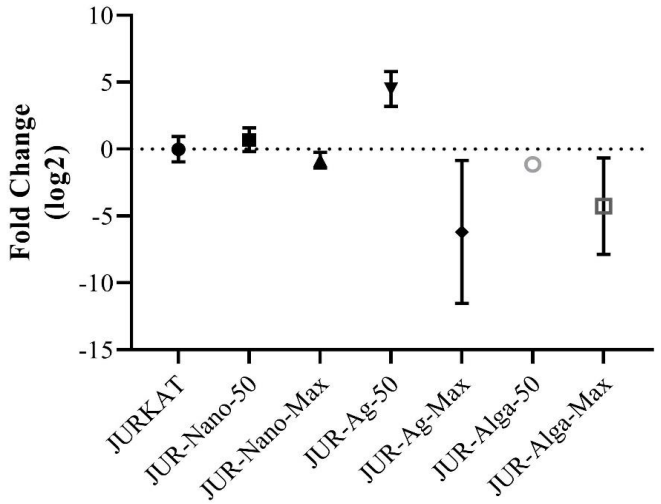

Figure 2. Changes in *GSKβ* Gene Expression in Jurkat Cell Lines. The nanoparticles alone (JUR-Nano-50 and JUR-Nano-Max) slightly changed *GSKβ* expression while algae alone (JUR-Alga-50 and JUR-Alga-Max) decreased *GSKα* expression as well (max algae concentration was significant). Also, the combined group at maximum concentration statistically significantly decreased *GSKβ* expression compared to Jurkat control group.

Table 1. The Sequence of Primers Used in PCR

Base Pair	Forward primer (5`-3`)	Reverse primer (5`-3`)	Gene name
111	CCAGCAGGTCAGCAAAGAATTTA	TGGACAGGACTGAACGTCTTGC	HPRT
95	CAGCCAGCTGACCAAACAT	GTCTCCTACATCTGTTCTCGCTA	GSK-3 α
78	CGGACTATGTTACAGTGATCTAG	GAAAGTATTGCAGGACAAGAGAT	GSK-3 β

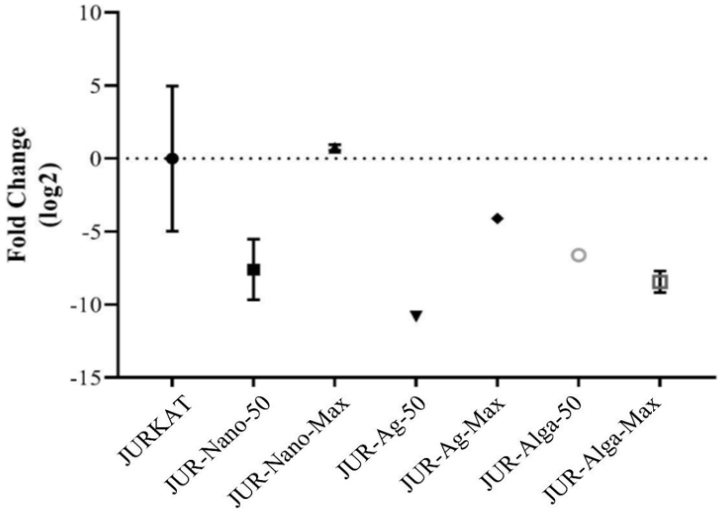

Figure 3. Changes in *IP3R3* Gene Expression in Jurkat Cell Lines. Except for nanoparticles alone at maximum concentration (JUR-Nano-Max), the rest of the groups saw a decrease in *IP3R3* expression. The most significant drop of *IP3R3* gene was in the combined group at half concentration.

pinpoint *IP3R3* as a potential target. The nanoparticle group alone (JUR-Nano-50) and the algae group alone (JUR-Alga-Max) were similarly decreased with *IP3R3* expression (Figure 3). The decreases were significant: p=0.0118 for JUR-Nano-50 and p=0.0186 for JUR-Alga-Max. The biggest decrease compared to the Jurkat control was attributed to the combined group at half the concentration (p=0.0191). No other significant changes were observed in other groups compared to the control group.

We also extended our experiment to investigate the effect of the most effective groups in each gene (*GSKα*, *GSKβ*, and *IP3R3*) to determine if the cytotoxicity against tumor cells is also seen in normal peripheral mononuclear cells (PBMC). When PBMCs were treated with PBMC-Ag-Max (*GSKα*), PBMC-Ag-Max (*GSKβ*), and PBMC-Ag-50 (*IP3R3*), the apoptosis related genes experienced no significant changes, indicating the safety of this combination for normal healthy cells (Figure 4).

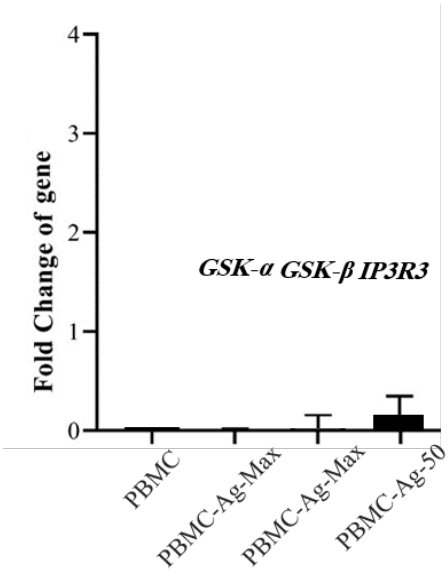

Figure 4. Changes in *GSK-α, GSK-β*, and *IP3R3* Gene Expression Compared to Untreated PBMC. When treated with the combined group (PBMC-Ag-Max), GSK genes had no significant change. And when treated with half the concentration (PBMC-Ag-50) also saw no change in *IP3R3* expression.

Discussion

Nowadays, the study of biological activities and the use of compounds obtained from marine sources have gained much attention. Marine algae of various species produce different secondary metabolites with engaging biological and physiological activities. Many studies have shown that seaweed has potential cytotoxic activity against cancer cells, which could be used instead of or in tandem with traditional novel cancer therapies (GhaffariTorabi-Rahvar et al., 2021). The aim of the current study was to examine the effect of nanoparticles synthesized from Sargassum brown microalgae on the expression of IP3 and GSK receptors in ALL cells in the cell lines. The results of this study revealed a significantly reduced expression of the said genes in the Jurkat groups in comparison with unaltered Jurkat cell line.

According to research of Ahmed et al., (2023) cretica albumin and silver nanoparticles upregulated

the in vitro *TRAIL, DR4, DR5* and *FADD* death receptor genes' expression at statistically significant levels in Hep-2 cell lines. Nano-formulations of F. cretica proved to be therapeutically important biomolecules in vitro. The hypothesized modulation of extrinsic apoptosis pathway genes through the plant nanoparticles proved novel medicinal options for effective treatment of cancer and enhancing the bioavailability of the active plant metabolites (Ahmed et al., 2023; Hooper et al., 2008). Data gathered by Abashkin et al, using multicolor flow cytometry showed that AgNPs are able to deliver (up to 90%) proapoptotic siRNA (siMCL-1) efficiently to some types of tumor cells, depending on the degree of PEGylation, the modification molecules with polyethylene glycol (PEG). Analysis of cell death showed that complexes of some AgNP variations with siMCL-1 lead to ~70% cell death in the tumor cells due to apoptosis (Duda et al., 2020). According to other studies published by Morais et al., (2022) the synthesized AgNPs in the present study revealed good characteristics and stability for administration to cancer cells. Their uptake through endocytosis led to cytotoxic effects against castration-resistant prostate cancer (CRPC) cells, revealing the potential of G-AgNPs as a future therapeutic approach to improve the management of patients with prostate cancer resistant to hormone therapy or metastatic disease. In another study by Li et al., (2022) it was shown that Saringosterol acetate (SA) significantly downregulated Bcl-xL and upregulated Bax, and cleaved PARP, and cleaved caspase 3 in a dose-dependent manner. Thus, these results suggest that SA induced mitochondria-mediated apoptosis in MCF-7 cells, making it a plausible candidate for drug development against breast cancer. In this study, SA was investigated for its anticancer effect on MCF-7 breast cancer cells.

The results of this study revealed that the changes in *GSKβ* and *GSKα* gene expression were statistically significant in the studied Jurkat group and that they experience great drops in expression. Studies have shown that *GSKβ* inhibitors can suppress chronic lymphocytic leukemia, glioblastoma, breast and colon cancer cell lines through phosphatidylinositol 3-kinase mutations, catalytic, and apolip3-cD3 (PIK3ca) (Abrahamsson et al., 2009; Ougolkov et al., 2007). *GSK*-3b supports cell proliferation mixed lineage leukemia (MLL) using a mechanism that includes instability of p27Kip1 cyclin-dependent kinase inhibitor. Inhibition of *GSK*-3b by means of a preclinical MLL provides promising evidence of efficacy, thus recognizing *GSK*-3b as a cancer drug candidate for MLL (Wang et al., 2008). A study by Songa et al., (2010) was in line with our data supporting additional evaluation of *GSK*-3 inhibitors as promising new agents for therapeutic intervention in leukemia and require clinical evaluation in patients with leukemia. Our study could have benefitted from experimenting on other cell lines of leukemia or lymphoma. We also could have tracked the expression of *GSK* and *IP3R3* on the protein level using the western blotting technique.

In this study, the *IP3R3* gene expression changes were statistically significant, and compared with the control group, the silver nanoparticle group with IC_{50} concentration experienced a significant decrease. IP3 receptors and intracellular calcium-releasing channels play principal functions in cell death and survival processes, which if not regulated, contribute to carcinogenesis. Alterations in *IP3R3* expression have been reported in a variety of cancers, causing impaired Ca^{2+} signaling and cellular dysfunctions. *IP3R3* can suppress cancer by cell death and cellular aging, or through inducing metabolism, anabolic processes, cell cycle progression, proliferation, and invasion (Arif et al., 2014).

These studies displayed the likelihood of developing different nanoparticles by algae. Actuality, studies have revealed that biomolecules and some of the phytochemical compounds in seaweed could lessen metal ions in the form of silver nanoparticles. Likewise, these compounds play an influential role in the coating of generated nanoparticles and their stability (Arif et al., 2014). A study by Vaseghi et al., (2018) showed that *Sargassum Angustifolium* decreases cell viability in cancer. In a study by Khanavi et al., (2012) Sargassum Vulgare ethanol extract displayed cytotoxic activity against Jurkat cancer cell line after 72-hour treatments. Given the resistance of cancer cells to antitumor drugs, the discovery of new and effective anticancer compounds that have fewer side effects has led to the surge of studies by scientists in recent years conducted using biotechnology. Likewise, much attention has been given to natural compounds, including plants or seaweeds to investigate their medicinal qualities. Also, marine drugs extracted from algae have been shown to have antitumor effects. Brown algae have effective biological effects including anti-tumor (Khanavi et al., 2012).

Extracts of Gulf sargassum algae can be used to inhibit ALL cells by inhibiting the expression of the *IP3R3* and *GSK* genes. Thus, these extracts are suggested to include in the medical and pharmaceutical cycles for the treatment of lymphocytic leukemia cancers and other tumors.

Author Contribution Statement

MDB and NO conducted the experiments. GK and FF helped with obtaining the results and drafting the manuscript. SV isolated the extract. SAM analyzed the statistical, raw data. All authors helped with finalizing the draft.

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Conflict of Interest

The authors declare to have no conflict of interest

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