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Chemical identification, antioxidant, cholinesterase inhibitory, and cytotoxic properties of fucoidan extracted from Persian Gulf *Sargassum angustifolium*

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Abstract

Marine macroalgal sulfated fucose-containing polysaccharides, like fucoidan, have drawn significant attention due to their biotechnological potentials, such as anti-cancer, antioxidant, and anti-cholinesterase activities. The fucoidan derived from brown macroalgae *Sargassum angustifolium* species (FSA) was investigated for its cytotoxic effects and alterations in cell proliferation, and cell cycle-related gene expression in the present study occurred on NB4 cell line. The results showed that FSA would induce *p53*, *p21*, pro-apoptotic genes and increase expression of the *p15* gene as a cell arrest marker. Also, FSA inhibited the anti-apoptotic effect of the *Bcl-2* gene and decreased *dnmt-1* gene expression. FSA significantly exhibited potent 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (p<0.05) with an IC_{50} value of 0.157 mg/mL and showed moderate anti-acetylcholinesterase activity with an IC_{50} value of 1.20 μg/mL. These results indicated the potential of FSA for the development of therapeutic or preventive agents of cancer and Alzheimer's disease mainly through cytotoxic effect and AChE (acetylcholinesterase) inhibition as well as additional antioxidant capacities.

Key words: fucoidan, *Sargassum angustifolium*, cytotoxicity, antioxidant, anti-cholinesterase

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1 Introduction

Natural products, including plants, microbes, and marine animals, are primary resources for treating human diseases (Harvey, 2008; Ratcliffe et al., 2011). Seaweeds or marine algae are found to be an important source of therapeutics and various biological products. Polysaccharides exhibited numerous applications with different health benefits and great potential applications in many sectors such as nutraceutical, pharmaceutical, and functional food industries (Molinski et al., 2009; Martins et al., 2014). Fucoidan, natural sulfated polysaccharide (poly-L-fucopyranose), isolated from cell walls of brown seaweeds, is an important food source that possesses immunomodulatory, anti-tumor, anti-viral, antioxidant, and anti-cholinesterase inhibitory activities in cognitive diseases (Atashrazm et al., 2015; Gao et al., 2012; Wang et al., 2019; Fitton et al., 2015; Kandasamy et al., 2015). Acute Myeloid Leukemia (AML), a type of cancer associated with the proliferation of bone marrow, colonization of cells, and abnormal differentiation (Adams and Nassiri, 2015), was classified according to the World Health Organization classification of blood and lymphoid tumors (Sobin and Fleming, 1997). The AML studies had been performed to identify the principles of genetic organization that control the onset and progression of disease (Lindsley et al., 2015). Acute Promyelocytic Leukemia (APL) was found to be associated with coagulation disorders following expression of Alpha Trans Retinoic Acid (ATRA) receptor protein fusion in AML, the neoplastic proliferation of promyelocytic phenotype bone marrow cells, and blast cells translocation mutations (Warrell et al., 1991, 1994; Grimwade et al., 2000; Redner, 2002; Kakizuka et al., 1991). Patients confront multiple laboratory disorders, including D-Dimer, fibrinogen depletion, and prolonged bleeding time (Kakizuka et al., 1991). Many chemical drugs have been used as anti-leukemia agents in recent decades, especially against APL (Barbui et al., 1998). One of the most potent APL treatment drugs is arsenic trioxide (As_2O_3), which cannot induce apoptosis in low doses, but might induce adverse effects in high doses in patients (Shen et al., 2004). Some other

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studies had shown the ability of ATRA in the differentiation of abnormal promyelocytes to mature granulocytes as well as reduction of relapse incidence with the addition of ATRA to chemotherapy protocol in APL (Chen et al., 1991; Castaigne et al., 1990; Fenaux, 1999; Degos et al., 1990; Bernard et al., 1973). Also, ATRA was treated coagulation disorder in some patients with ATRA syndrome without causing aplasia (Warrell et al., 1991, 1994; Frankel et al., 1992). Anyway, the medications used for this type of leukemia have not been effective in all patients according to numerous side effects. Design of the novel drugs with high effectiveness in reducing chemotherapy and radiotherapy-induced side effects from natural products as well as a description of the possible mechanisms of action can improve the prevention treatment strategies for dealing with diseases such as APL (Zhang et al., 2018; Castaigne et al., 1990; Chen et al., 1991; Bernard et al., 1973). Various species of *Sargassum* (Sargassaceae), tropical brown macroalgae (seaweed), have been found to possess antitumor, anti-cholinesterase as well as antioxidant activities (Yende et al., 2014; Hu et al., 2016; Natarajan et al., 2009). The Persian Gulf, especially around the seashores of Bushehr and Hormozgan provinces in the southern parts of Iran, has potent sources of marine seaweeds with a wide range of biological activities (Ahmady-Asbchin and Mohammadi, 2011). The pharmaceutical and nutritional properties of the Persian Gulf seaweeds are entirely unknown (Sohrabipour and Rabiei, 1999, 2007; Vaziri Zadeh et al., 2012). In the present study, fucoidan extracted from the *Sargassum angustifolium* (FSA), brown algae of the Persian Gulf, was isolated and characterized. Then the cytotoxic activity of FSA on reduction of NB4 (human acute myeloid leukemia cells) cell death in APL was investigated. Also, antioxidant and anti-cholinesterase inhibition activities of FSA were assessed.

2 Materials and methods

2.1 *Collection of Sargassum angustifolium*

Sargassum angustifolium was collected during summer from the intertidal zone of the Persian Gulf. The macroalgae were identified by the department of biotechnology, Persian Gulf Algae Development Technology, located in Bushehr province. The seaweed sample was washed with fresh water, then air-dried to obtain the dry biomass, and stored in plastic bags.

2.2 *Extraction of fucoidan*

Extraction was carried out as described by Sellimi et al. (2014), Benslima et al. (2021), Bahramzadeh et al. (2019), Borazjani et al. (2018) and Vaziri Zadeh et al. (2012) with slight modifications. Briefly, *S. angustifolium* powder was de-pigmented using 95% ethanol. The sample was suspended in a solution of 90% ethanol which included 10% formaldehyde for 8 h at 30℃ under constant stirring (200 r/min). This was done to polymerize the phenolic metabolites. Solvent filtered off, and the powder was extracted three times in succession at room temperature with 95% ethanol to remove excess formaldehyde, pigments, lipids, and other interfering phenolic metabolites. The remaining ethanol was evaporated, and the sample was brought into total dryness. Depigmented powders were air-dried and then treated in 1 L 0.1 mol/L HCl (pH=3) for 2 h at 60℃ under constant stirring (250 r/min) for the extraction. The mixture was cooled at room temperature and then centrifuged for 20 min at 4 000× *g* at 4℃ in a centrifuge. The resulting extract was mixed with 2 volumes of absolute ethanol and left for 12 h at 4℃. In addition, the fucoidan collected in the pellet by centrifugation (16 000× *g* for 10 min, 4℃) was redissolved in distilled water and dialyzed using 14 kDa cutoff dialysis membrane from Sigma-Aldrich (St. Louis, MO, USA). The supernatant was lyophilized and used for further analysis.

2.3 *Monosaccharide composition analysis*

A quantitative determination of the monosaccharide composition of FSA was performed by High-Performance Liquid Chromatography (HPLC) method using the isocratic HPLC (Knauer, Berlin, Germany) method. The HPLC system was equipped with a Knauer smart line RI detector and a Eurospher column (4.6 mm×250 mm, 5 μm, Knauer). The extract was passed through a 0.45 μm filter (Millipore, Westboro, MA, USA) before being injected into the HPLC. Before HPLC analysis, the fucoidan (6 mg) was hydrolyzed for 90 min in 2 mol/L Trifluoroacetic acid (TFA) at 120℃. After removing TFA, the hydrolyzed polysaccharide sample was injected into the HPLC system. A 90:10 *v/v* mixture of acetonitrile and deionized water was used as the mobile phase at a 2 mL/min flow rate. The resolution peaks were recorded on the HPLC chart according to the retention times of standards. The monosaccharide standard was L-fucose, galactose, mannose, glucose, and glucuronic acid (Farvin and Jacobsen, 2013).

2.4 *Fourier-transform infrared (FTIR) spectroscopy*

For structure identification analysis of FSA, Fourier transforms infrared spectroscopy (PerkinElmer FT-IR, model Spectrum RXI, Texas, USA) technique was used. Two milligrams of sample was ground with KBr until a fine particle size was produced. The signals automatically were collected using 60 scans over the range of 400–4 000 cm^{-1} at a resolution of 32 cm^{-1} and were compared to a background spectrum collected from the KBr alone at room temperature (Borazjani et al., 2018).

2.5 *Cytotoxicity of the sample*

2.5.1 *Maintenance of NB4 cell line*

The NB4 were purchased from the hematology department of Bushehr University of Medical Sciences of Iran (Bushehr) and were maintained in RPMI-1640 medium (Bioidea, Tehran, Iran) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS; Gibco, Carlsbad, USA), 100 U/mL penicillin and 100 mg/mL streptomycin, and incubated at 37℃ in a humidified 5% CO_2 incubator.

2.5.2 *Cell viability assay*

The NB4 cells (5×10^3 cells/well) were seeded at the 96-well plate in triplicate and after 12 h, were treated with 100 μL of various concentrations of FSA (62 μg/mL, 125 μg/mL, 250 μg/mL, 500 μg/mL, 1 000 μg/mL, and 2 000 μg/mL) for 24–72 h incubation time. The cell viability was measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Biotech, Tehran, Iran). After incubation time, 100 μL of MTT (5 mg/mL in phosphate-buffered saline (PBS)) was added to each well and then incubated at 37℃ for an additional 3 h. The supernatant was aspirated, and 100 μL DMSO (Merck, Darmstadt, Germany) was added to dissolve formazan crystals. The absorbance was measured by a microplate reader (BioTech, Munich, Germany) at 570 nm (Borazjani et al., 2018). The results were calculated as percentages of the growth inhibition by the following equation:

$$\text{Cell viability} = (\text{ODtest}/\text{ODcontrol}) \times 100\%. \quad (1)$$

2.6 *Quantification of mRNA level of apoptosis-related genes through real-time PCR*

Total RNA extraction and cyclic DNA (cDNA) synthesis examined cells (5×10^4 cells/well) were treated with FSA (62.5 μg/mL) for 24 h using a Takara Primescript RT reagent kit (Cat. No. RR037Q, Tokyo, Japan). Total RNA was transcribed reversely into cDNA using a YTA Real-Time kit protocol (YT2551, Iran). Primer sequences of *HPRT*, *Dnmt1*, *p15*, *p21*, *p53*, and *Bcl-2*, as well as the reference gene, were listed in Table 1. The mRNA levels of Real-time quantitative PCR (qRT-PCR) were conducted with the Corbett RotorGene 3000 (Sydney, Australia), using SYBR Green I Master (Cat. No. RR820Q, Takara, Tokyo, Japan) master mix. qRT-PCR was performed in a reaction according to the manufacturer's instructions. Briefly, the main protocols were described as follows: (1) activation at 37℃ for 15 min; (2) denaturation at 85℃ for 5 s; (3) annealing/extension at 4℃ for 5 min and agarose gel electrophoresis for evaluation of purity of isolated DNA by determining the spectrophotometric absorbance of the samples using a UV-DOC Spectrophotometer (BioRAD). All samples and control were run in triplicate. The qRT-PCR data were analyzed by a comparative threshold (C_t) method. The fold inductions of the samples were compared with the untreated samples. *HPRT* was an internal reference gene. The C_t cycle was determined the expression level in control cells and NB4 cells treated with FSA for 24 h. The relative expression levels were measured by the following formula: $\Delta\Delta C_t$=ΔC_t (treated)–ΔC_t (control). The expression levels were presented as *n*-fold differences relative to the calibrator. The value was used to plot the expression of apoptotic genes utilizing the expression of $2^{-\Delta\Delta C_t}$.

2.7 *Assessment of human peripheral blood mononuclear cells (PBMCs) proliferation ability*

Following the Declaration of Helsinki, 10 mL of fresh and normal citrated blood was obtained from healthy donors who gave written consent to Bushehr University of Medical Sciences (Jenny et al., 2011). According to Lonza Lymphocyte Separation Protocol (INST-17-829-2 09/11), Peripheral blood mononuclear cells (PBMCs) were isolated with Ficoll-Hypaque gradient centrifugation (Sigma, Burlington, MA, USA) (Nauseef, 2014) and mixed with an equal volume of RPMI-1640 medium containing 10% FBS. The PBMCs obtained were divided to expose to the different concentrations of FSA with the MTT assay. The cells were counted with a hemocytometer slide and counted at 0.2×10^6 for every 96 well plates and treated for 24 h, 48 h, and 72 h incubation in an atmosphere of 5% CO_2 at 37℃, with concentrations of FSA (62.5 μg/mL, 125 μg/mL, 250 μg/mL, 500 μg/mL, 1 000 μg/mL and 2 000 μg/mL). In each of the incubations, the MTT solution was added to each well. After 3 h incubation, dimethyl sulfoxide was added, followed by shaking for 10 min to dissolve the formazan crystals. Then plates were put on a microplate reader (BioTek, Germany) to evaluate optical absorption at 570 nm.

2.8 *Lytic effects of FSA on red blood cells (RBCs)*

To evaluate the hemolytic effect of FSA, 5 mL of fresh and normal human citrated blood sample was prepared by the hematology laboratory of Bushehr University of Medical Sciences. According to Devi et al. (2014) blood was prepared and treated with 62.5 μg/mL, 125 μg/mL, 250 μg/mL, 500 μg/mL, 1 000 μg/mL, 2 000 μg/mL concentrations of FSA. Triton X-100, a known hemolytic agent, was used as positive and normal saline as a negative control. The supernatant of each sample was extracted and transferred to 96 well plates. Then, their optical absorption was read by an ELISA reader (BioTek, Munich, Germany) at 570 nm. The absorption was calculated according to the below formula, and the amount of RBC lysis was investigated.

$$\text{Hemolysis}(\%) = \frac{\text{absorbance of sample} - \text{absorbance of negative control}}{\text{absorbance of positive control} - \text{absorbance of negative control}}. \quad (2)$$

2.9 *Radical scavenging activity*

2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of FSA was determined according to the method proposed by Palanisamy et al. (2017) and Yamaguchi et al. (1998). Briefly, 100 μL of the sample concentration in a range of 0.5 mg/mL to 8 mg/mL was mixed with 400 μL of 0.1 mmol/L DPPH in methyl alcohol and incubated in the dark place. After 30 min, the absorbance of the sample was measured at 517 nm using a UV–Vis spectrometer. Positive control was ascorbic acid, and 0.1 mmol/L DPPH in methyl alcohol was used as blank. The following equation calculated the percentage of DPPH scavenging activity,

$$\text{Scavenging activity} = (A_0 \text{ sample} - A_1 \text{ control})/A_0 \text{ sample} \times 100\%, \quad (3)$$

where A_1 control represents the absorbance of the methanol DPPH solution without the samples, and the A_0 sample is the absorbance of the methanol DPPH solution with the tested samples.

2.10 *Determination of acetylcholinesterase (AChE) inhibitory activity*

The AChE inhibitory activity of the samples was measured by 96-well microplate colorimetric assay based on the modified method developed by Adhami et al. (2011) and Ellman et al. (1961). One hundred and twenty-five microliters of 3 mmol/L 5,5-dithiobis-2-nitrobenzoic acid (DTNB), 25 μL of 15 mmol/L acetylthiocholine iodide (ATCI) (Sigma-Aldrich Corporation, St. Louis, MO, USA), and 50 μL of Tris-HCl buffer (pH 8.0) were added to a 96 well plate followed by 25 μL of a sample (different concentrations (0.01 μg/mL to 100 μg/mL)) of FSA dissolved in

Table 1. Sequences of primers used in qRT-PCR

Base pair	Forward (5′–3′)	Reverse (5′–3′)	Gene
111	TGGACAGGACTGAACGTCTTG	CCAGCAGGTCAGCAAAGAATTTA	*HPRT*
90	CGGTGGGGTCATTTGTGTG	CGGTTCAGGTACTCAGTCATCC	*Bcl-2*
118	ACTCATCCGATTTGGCTCTTTC	CCTAGCCCCAGGATTACAAG	*Dnmt1*
82	GATGTGCAAGCGACGACAGA	GAGCAAAGGCCAGCATCCT	*p15*
130	CCTCTCACTGTCTTGTACCCT	GCGTTTGGAGTGGTAGAAATCT	*p21*
121	TAACAGTTCCTGCATGGGCGGC	AGGACAGGCACAAACACGCACC	*p53*

10% DMSO/buffer. The absorbance was measured by a microplate reader (Synergy MX Biotek, San Leandro, CA, USA) at 405 nm five times every 13 s. Then 25 μL of AChE (0.22 U/mL in buffer solution) was added, and after 10 min incubation, the absorbance was measured again at the same wavelength eight times every 13 s. Physostigmine and sample solvent (10% DMSO in buffer) were used as positive control and blank. The percentage of inhibition was calculated as below formula:

$$\text{AChE inhibitory} = 100\% - (\Delta_{SM}/\Delta_{BL}) \times 100\%, \quad (4)$$

where Δ_{SM} is difference of sample absorption before and after adding enzyme (AChE); Δ_{BL} is difference of blank absorption before and after adding enzyme (AChE).

2.11 *Statistical analysis*

One-way analysis of variance (ANOVA) was used for MTT data analysis. The student's *T*-test was used for the qRT-PCR study to compare the differences between various treatment groups. All statistical analyses were performed using SPSS 21.0 package (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.01 (San Diego, CA, USA). The data were presented as mean±SD, and 95% confident levels (p<0.05) were statistically significant.

3 Results

3.1 *Monosaccharide composition analysis results*

Overall, sugar analysis showed that fucose was the major sugar with significant glucose and little mannose, galactose, and uronic acids, respectively (Table 2). Other monosaccharides such as rhamnose and xylose were not detected. Fucoidan polymers in the literature have demonstrated considerable variations in their monosaccharide compositions. In general, the chemical composition of fucoidan polymers is significantly different depending on species, anatomical regions, growing conditions, and extraction procedures.

3.2 *FTIR spectroscopy results*

FTIR results indicated that FSA exhibited typical absorption bands of the fucoidan spectrum. The guluronic units produced a band in the range of approximately 11 025 cm^{-1}, and the mannuronic units created a band of about 1 100 cm^{-1}. Therefore, the FTIR spectrum can compare the guluronic to manoronic acid concentration ratios using their two peak intensities ratios. Bands of 1 022 cm^{-1} and 1 074 cm^{-1} represented the units of guluronic acid and mannuronic acid, respectively. Peaks between 1 034 cm^{-1} and 1 041 cm^{-1} belonged to the sulfate groups. These bands are characteristics of sulfated polysaccharides. The strong band at 1 400 cm^{-1} represented the deformed CH_2 groups. C-O-C and C-O-H tensile states represented bands in the regions of 1 125–1 250 cm^{-1} and 1 000–1 025 cm^{-1}, respectively.

Table 2. Sugar analysis of the polysaccharide from *Sargassum angustifolium*

Sugar	Composition/%
Fucose	53±2.0
Galactose	0.2±0.9
Mannose	0.3±0.7
Glucose	12.6±0.4
Uronic acids	0.36±0.5

Note: Each value represents the percentage of monosaccharides obtained in the fraction.

The broadband at 3 424 cm^{-1} was related to the O-H tensile vibration. The 2 926 cm^{-1} and 1 652 cm^{-1} were related to C-H tensile vibration and O-C-O asymmetric tensile carboxylate. This finding was in support of the conclusions from Mathlouti and Koening in 1986. They reported that the absorption at 1 438 cm^{-1} was a sign of modified C-OH vibration with the participation of symmetric tensile vibration of the carboxylate group. The bands generated at 1 022 cm^{-1} and 1 074 cm^{-1} could be attributed to the C-O tensile vibration and the pyranose's C-O and C-C tensile vibration. The anomeric region (750–950 cm^{-1}) was the most widely discussed carbohydrate. The band at 947 cm^{-1} indicated uronic acid residues by C-O tensile vibration. The weak band at 873 cm^{-1} showed the vibration signal of C-H deformation of the β-mannuronic acid residues. The other weak band at 2 138 cm^{-1} was the characteristic of the mannuronic acid residues.

Further signals at 1 669 cm^{-1} to 800 cm^{-1} indicated the vibration of the asymmetric α–L glucopyranuronic ring and sulfated groups, respectively.

3.3 *Cytotoxicity results*

As shown in Fig. 1, reduction of the concentration of FSA had decreased the inhibitory effect, therefore, at 62 μg/mL, the inhibitory effect reached to (40±0.024) μg/mL within 72 h, while its inhibitory effect after 48 h decreased to (37±0.116) μg/mL. These results indicated that FSA inhibited the survival of NB4 cells in a dose and time-dependent manner.

3.4 *Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*

The changes in expression levels of apoptosis-related genes from cancer cells after treatment with FSA were shown in Fig. 2. Gene expression analysis using GraphPad Prism (*T*-test, unpaired) revealed that the expression of *p53*, *p21*, *p15*, *Dnmt1*, and *Bcl-2* genes was affected by 62 μg/mL FSA treatment after 24 h. The gene expression of pro-apoptotic genes *p53*, *p21*, *p15*, and *Dnmt1*, responsible for DNA methylation and anti-apoptotic *Bcl-2*, was decreased (p<0.000 1).

3.5 *Effect of FSA on correlation of p53, p21, p15, Dnmt1, Bcl-2 genes*

In the correlation analysis of genes (Fig. 3), it was found that the expression of tumor suppressor genes under the influence of FSA was significantly increased, and the expression of anti-apoptosis-inducible genes was decreased considerably. The experi-

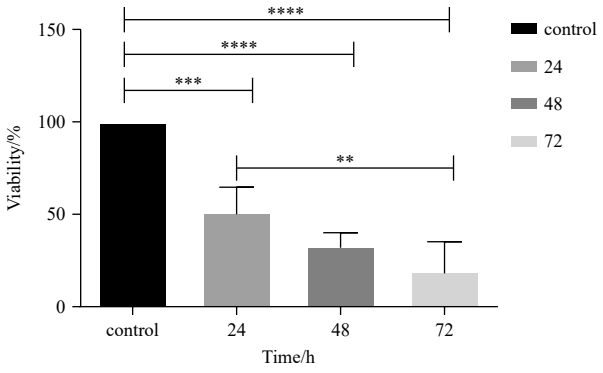

Fig. 1. Survival of NB4 cell viability under fucoidan treatment with concentrations of 62 μg/mL, 125 μg/mL, 250 μg/mL, 500 μg/mL, 1 000 μg/mL and 2 000 μg/mL after 24 h, 48 h and 72 h incubation (**p<0.01, ***p<0.005 and ****p<0.000 5).

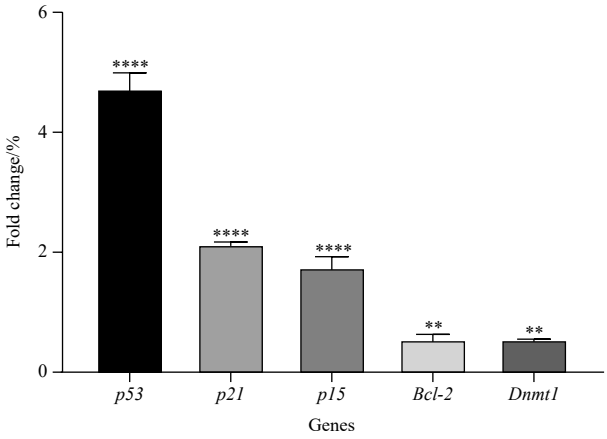

Fig. 2. Real-time PCR diagram of fucoidan extracted from *Sargassum angustifolium* (**p<0.01 and ****p<0.000 5).

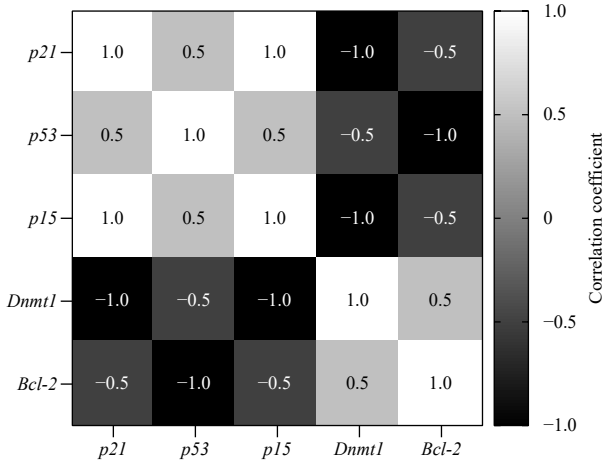

Fig. 3. Diagram of correlation analysis of apoptosis-inducing and inhibitory genes.

mental results at the mRNA level showed that *p53* significantly increased fourfold than the control. At the same time, the expression of *Dnmt1* and *Bcl-2* genes was reduced compared to control (p<0.5). According to the gene expression diagram, *p15* had the highest correlation with *p21*. After that, *p53*, *Dnmt1* and *Bcl-2* had also been shown to exhibit a correlation of 0.500, which may indicate the coexistence of these genes in the process of cell proliferation. These results can be used to investigate further the signalling pathways underlying FSA and their specificity of function.

3.6 *Evaluation of the sensitivity of gene markers to fucoidan*

The level of FSA below the Roc chart showed a 60% real positive. This indicated low sensitivity of the target genes to the FSA. The specificity of *Bcl-2, Dnmt1,* and *p53* genes was 100%, and the specificity of *p21* and *p15* genes was shown to be zero. The sensitivity of *Bcl-2, Dnmt1*, *p53* genes was 20%, 40%, and 60%, respectively, and this value for *p21* and *p15* genes was 60% and 80%, respectively (Fig. 4).

3.7 *PBMC proliferation activity*

The results showed that with increasing concentration, cell viability was changed from low to high. According to Fig. 5, PBMC survival was approximately 100% after 48 h and 72 h incubations compared to the control sample. In low concentrations, due to

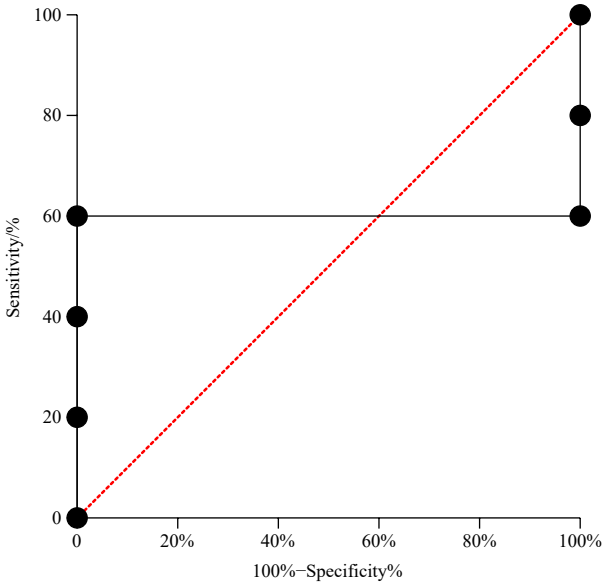

Fig. 4. Diagram of gene susceptibility evaluation to fucoidan extracted from *Sargassum angustifolium.*

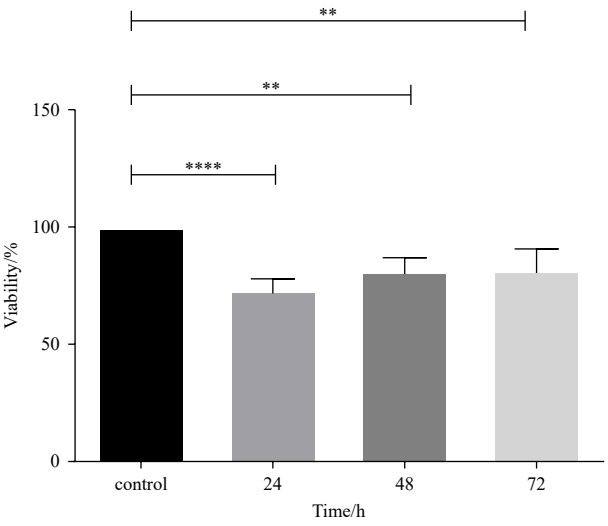

Fig. 5. Diagram of PBMC cells survival after treatment with concentrations of 2 000 μg/mL, 1 000 μg/mL, 500 μg/mL, 250 μg/mL, 125 μg/mL, 62.5 μg/mL related to fucoidan extracted from *Sargassum angustifolium* at 24h , 48 h and 72 h incubations (**p<0.01 and ****p<0.000 5).

the decrease in glucose concentration, the effect of FSA decreased. Therefore, cell viability at low concentrations was at a minimum level.

3.8 *Fucoidan effect on RBC lysis*

RBC lysis test is an important method for assessing hemolytic properties of natural materials and evaluating their hemoglobin release in the plasma upon treatment of RBCs with substances. The effect of FSA on RBC was found that FSA did not induce RBC lyses in the mentioned concentrations compared to positive control treated with Triton X-100, implying the biocompatibility of the FSA. RBCs incubated with normal saline were used as negative control and showed no hemolysis.

3.9 *Radical scavenging activity results*

DPPH method was used to measure the antioxidant activity of FSA. According to the test, when the DPPH solution (a stable free radical) was mixed with the FSA sample, it became a donor of the hydrogen atom and a non-radical form of DPPH obtained by simultaneous change of the color from purple to yellow. The length of the reaction depends on the ability of the antioxidants to hydrogenate in the FSA. Concentrations of 0.5 μg/mL, 1 μg/mL, 2 μg/mL, 4 μg/mL, 5 μg/mL, 7 μg/mL, and 10 μg/mL of FSA were used, and 50% inhibition activity of free fucoidan radicals (IC_{50}) was between 2 μg/mL and 4 μg/mL. The antioxidant activity of FSA is related to the sulfur functional group, which refers to their ability to donate protons to free radicals. This test was repeated with mean±SD three times and was statistically significant compared with the control group (*p<0.05, **p<0.01, and ***p<0.001).

3.10 *Cholinesterase (AChE) inhibitory activity results*

According to "Amyloid Hypothesis", Cholinesterase inhibitors (ChEIs) are the most promising therapeutic agents for Alzheimer (AD) disease since they could cause progress in a β-amyloid deposition in the form of senile plaques as well as the modulation of amyloid precursor protein (APP) processing (Bolognesi et al., 2009). AChE inhibition plays a key role in enhancing the cholinergic neurotransmission in the brain and decreases the aggregation of β-amyloid peptides in AD (Hodges, 2006). Different concentrations of FSA (0.01 μg/mL, 0.1 μg/mL, 1 μg/mL, 10 μg/mL, and 100 μg/mL) were tested for AChE inhibitory activity according to the microplate assay discussed in the methods, and the results were shown in Fig. 6. AChE inhibitory activity of fucoidan (p< 0.05) compared with the control group had an IC_{50} value of (1.20±0.445) μg/mL. FSA inhibited the enzyme by 10%–20% at lower doses (0.01 μg/mL to 0.1 μg/mL). However, higher doses, i.e., 10–100 μg/mL, induced more than 50% inhibition indicated that FSA exhibited anti-cholinesterase activity in a dose-dependent manner. Physostigmine served as a positive control, and the IC_{50} value for this compound was determined as 0.98 μg/mL, which was in accordance with published values (Carpinella et al., 2010; Mukherjee et al., 2007).

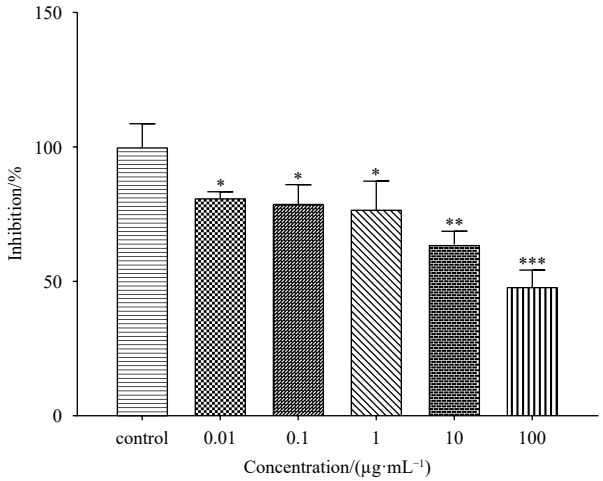

Fig. 6. Diagram of the anti-cholinesterase activity of fucoidan extracted from *sargassum angustifolium*. The anti-cholinesterase activity of fucoidan in concentrations of 0.01 μg/mL, 0.1 μg/mL, 1 μg/mL, 10 μg/mL, and 100 μg/mL compared with the control group (10% DMSO in buffer). The values are presented as mean±SEM of 3 independent experiments (n=3) and analyzed by one-way analysis of variance (ANOVA) (*p<0.05, **p<0.01 and ***p<0.001 compared with the control group).

4 Discussion

Acute myeloid leukemia is a horrible hematological disorder. Although many patients with AML receive common remedies, their side effects might impress survival chances (Degos et al., 1990). In decades, brown algae have been used in the health and production of medicines (Fitton et al., 2015; Choo et al., 2016; Nagamine et al., 2009; Yuan et al., 2015). Fucoidan is a sulfated polysaccharide extracted from brown algae and has been recognized as a promising medicine for preventing and treating diseases like cancer (Wei et al., 2015). First, the present study investigated the anti-cancer property extracted from native Persian Gulf algae, *Sargassum angustifolium*. The previous studies have been shown the effects of standard fucoidan inhibition activities on PC-3 cell line at a concentration of 10 μg/mL to 100 μg/mL (Boo et al., 2013), MCF-7 cell line at a concentration of 80 μg/mL to 820 μg/mL (Zhang et al., 2011) and U937 cells at a concentration of 20 μg/mL to 100 μg/mL (Park et al., 2013). The FSA extracted from the native species of *Sargassum angustifolium* had an inhibitory effect at concentrations of 62.5 μg/mL, 125 μg/mL, 250 μg/mL, 500 μg/mL, 1 000 μg/mL, and 2 000 μg/mL on the proliferation of NB4 cells. This was similar to the effects of the fucoidan inhibitors of other species. Based on the obtained results, FSA acted in a dose and time-dependent manner, inhibiting growth and inducing apoptosis. The greatest inhibitory effect of FSA was observed at a concentration of 2 000 μg/mL at 72 h when cell growth was zero. With concentration reduction, the inhibitory effect of FSA decreased. Therefore at concentration of 62 μg/mL, IC_{50} was (40±0.023 9) μg/mL and (37±0.116 1) μg/mL within 48 h and 72 h, respectively. In comparison to Jin et al. (2010), fucoidan at a concentration of 62 μg/mL caused cell death, while in the study of Jin, the concentration of 150 μg/mL resulted in cell cycle arrest within 48 h. However, the cell death rate in this study was similar to those reported by Jin et al. (2010). In the meantime, multiple gene families play a role in maintaining or induction of apoptosis (Saitoh et al., 2009). Fucoidan, as previously described, induces apoptosis by various mechanisms, including activation of caspases and activation of mitochondrial signaling pathways (Park et al., 2013, 2015). In this study, the effect of fucoidan on the expression of *p53, p21*, and *p15* pro-apoptotic genes showed that fucoidan at 62 μg/mL increased *p53*, *p21*, and *p15* genes expression within 24 h. Also, in this concentration, the expression of the *Bcl-2* gene decreased, which is an apoptotic inhibitor gene. The results indicated that the treatment of NB4 cells with FSA increased the expression of *p53* and *p21* genes that activated the mitochondrial pathway of apoptosis, and this was similar to the results of the work by Jin et al. (2010). Also, they indicated that in the NB4 cell line, fucoidan has a strong apoptotic effect. By increasing *p15* expression, cellular arrest in the G_0/G_1 phases increases, and *Bcl-2* expression reduction happens, which prevents its anti-apoptotic effect (Jin et al., 2010). In this study, the expression level of the *Dnmt1*, the responsible gene for DNA methylation, decreased, which also has an inhibitory effect on tumor suppressor genes. Yan et al. (2015) have also reported that fucoidan might suppress the expression of methylation genes. The correlation analysis of the present study showed an inverse relationship between the expression of *Dnmt1* and *p53* under the influence of FSA. In the study conducted by Wong et al., the oncogenic effect of the *Dnmt1* gene on inhibition of tumor suppressor genes in AML has been described (Wong et al., 2019). According to Fig. 3, it seems that diminished expression of *Dnmt1*

treated with FSA increased *p53* gene expression and downstream of the *p21* gene. Due to the assessment of the FSA effect on PBMC, it was found that FSA had no effect on normal cells and even stimulated normal cell growth. A study by Dinesh et al. had not reported any adverse effects of fucoidan on PBMC (Wong et al., 2019). It has also been reported by Yang and colleagues that fucoidan induced mononuclear cell maturation (Yang et al., 2008). Irhimeh indicated that oral use of fucoidan increased CD^{34+} as a marker of T-cell growth (Irhimeh et al., 2007). Since the hemolysis rate should be in the range of 10% to 100% (Devi et al.,2014), the study of the effect of fucoidan on RBC lyses of fucoidan did not cause in the specified concentrations. Further study by Veena et al. (2007) explained increasing RBC unity and decreased membrane damage due to increased oxalate. Activation and enhancement in *p53* expression indicate that fucoidan is an apoptotic component through mitochondrial pathways. Some investigations have reported the independent relation of *p53* mediated apoptosis, but as shown in the present study, fucoidan increased the expression level of *p53* in cancer cells. However, it has to be determined whether the fucoidan directly induces DNA damage in the S phase, leading to cell arrest, or whether it interferes with DNA regeneration by destroying the replication. These data indicated that the anti-cancer activity of fucoidan occurs through multiple pathways. Therefore, the fucoidan of *Sargassum angustifolium*, native to the Persian Gulf, could be suggested as a promising treatment for APL. It can be concluded that FSA was a complex molecule with a similar structure to the standard fucoidan and was capable of absorbing free radicals comparing to ascorbic acid. For the first time, the present study elucidated the anti-cholinesterase properties of the Persian Gulf brown seaweed *Sargassum angustifolium*. This study suggested that FSA had powerful AChE inhibitory activity, which may help prevent or slow down AD progress. The reason for their potential ChEI activity was plastoquinones and farnesylacetone derivatives (Choi et al., 2007; Natarajan et al., 2009). However, further research is underway to isolate the active compounds responsible for different biological activities of the native Persian Gulf *Sargassum angustifolium*.

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