Anticancer effect of *Dunaliella salina* under stress and normal conditions against skin carcinoma cell line A431 in vitro

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Abstract

Dunaliella salina, a green microalga, has been consumed as food and medicine for a long time.The anti-oxidant and anticancer effects are related to more production of β- carotene under stress conditions compared with normal circumstances.The scope of this study was survey of anticancer effect of the ethanol extract of *Dunaliella salina* algae under stress(EDSS) and normal (EDSN) conditions on death rate of skin carcinoma cell line A431 by MTT [3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide] method in vitro. Our results showed that the β-carotene amount in stress situation was 1.46 times more than in normal condition.The cytotoxic effect of EDSS exposed to 6.25, 12.5, 25, 50, 100 µg/ml in 6, 24, 48 h which was performed by MTT assay showed that there was a significant difference in the various time except in 6 h in both conditions.The results of compared death rate of A431cells exposed to various concentrations of EDSS and EDSN presented significant difference in 6, 24, and 48 h after exposure. The LC_{50} of EDSS in 24 and 48 h were 15.4 and 0.4 µg/ml , respectively and 478.6 and 46.4 µg/ml for EDSN.

Keywords: *Dunaliella salina*, Anticancer, A431 cell line, MTT assay, β-carotene

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Introduction

Pharmacological researches regarding natural materials of marine organisms which contain anticancer properties have always been of concern to marine scientists and other related fields have always been concerned of pharmacological researches regarding natural materials of marine organisms which contain anticancer properties. Recently remarkable researches have been done on use of microalgae such as *Dunaliella* (Alejandaro et al., 2003). Algae and plants have some curative of material for synthetic drug (Bechelli et al., 2011; Moghadasi et al., 2011).The significant intracellular components of *Dunaliella* are carotenoids, glycerol, protein and vitamins(Farouk et al., 2002).The major part of these components has antioxidant properties (Gibbs and Duffus, 1975; Farouk et al., 2002). *Dunaliella salina* is extreme halotolerant unicellular and motile green algae (Chen and Jiang, 2011, Moghadasi et al., 2011).This alga has a unique property of certain physiological responses. It has produced more glycerol under stress conditions such as high salinity, intensity of light and limitation of nutrient especially phosphorus and nitrogen. A lot of researches were done for cartenoides especially β-carotene and also Tocopherol and Ascorbic acid (Farouk et al., 2002; Takagi et al.; 2006, Raja et al., 2007b; Chen and Jiang, 2011; Moghadasi et al., 2011). β-carotene was plentifully available in *Dunaliella* which has significant effects on decreasing the risk of lung, esophagus, pancreas, stomach, breast, skin, colon , and ovary cancers (Poppel and Goldbohm, 1995; Farouk et al., 2002; Chidambara et al., 2005; Raja et al., 2007a; Raja et al., 2007b). Prevention of heart and chronic disease and malignant tumors , increase of cell division of lymphocytes , extending the immunology response and neoplastic transformations and growth control are the other characteristics of this substance (Wald et al.,1988; Stryker et al.,1990; Knekt et al., 1990; Challem, 1997; Buiatti, 1997; Chidambara et al., 2005; Raja et al., 2007a). Extracts of *Dunaliella sp.* showed that its antioxidant components can significantly inhibit induced fibrosarcoma and lung cancer in mouse and lung cancer human cell line A549 and skin cancer, (Greenberg et al., 1990, Popple and Goldbohm, 1995, Ming et al., 2008).This research focused on the effect of EDSN and EDSS on squamous cell carcinoma cell line A431.This cancer is related to common skin cancer it is and almost responsible for 20% of the malignant skin tumors.The significant cell characteristic of this cancer is wide cells which look like scales of fish. This cancer is also called Epidermoide.

Materials and methods

Culture of alga

The initial sample of *Dunaliella salina* was isolated and purified from Hoz-Soltan Lake located in the north-east of Qom in Iran. *D.salina* alga was cultured in modified Janson medium.The condition of normal phase was done with a 15% saltiness, pH 7.5, intensity glow light 100 µmol photons $m^{-2}s^{-1}$ (Takagi et al., 2006; Garcia et al., 2007) and a 35% stress condition of high saltiness, pH 8.2, and

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intensity glow light 4000 µmol photons $m^{-2}s^{-1}$ (Farouk et al., 2002; Moghadasi et al., 2011) 12 h in a dark and 12 h in a light place. To prepare the dried powder of *D.salina* the harvested samples were centrifuged (eppendorf model 5810R) at 3500 rpm for 10 minutes and after desalination by the phosphate buffered saline (PBS) they were freeze dried via the dehydration mechanism (Farouk et al., 2002; Fazeli et al., 2006; Raja et al., 2007a; Garcia et al., 2007).

Extraction

Extraction was done using 30g of *D.salina* powder, both conditions with pure ethanol at room temperature in the dark place (Raja et al., 2007a; Ming et al., 2008). After 48 h, the samples were strained and dried in rotary evaporation (Heidolph WB2000) at 40˚c and 90 rpm. Dried EDSN and EDSS were storage at -80˚c in the dark (Raja et al., 2007a; Ming et al., 2008).

HPLC analysis

The obtained extracts of EDSN and EDSS in both normal and stress conditions were analyzed by high performance liquid chromatography (HPLC) [young Lin Acme 9000, South Korea, column: Lichrosphare Rp 100 C_{18} with particle size of 4µ (25 cm of 4.6 cm) at a wave length of 40 nm via DAD/UV. The mobile phase was a Aceto nitril: methanol: dichloromethan (10:20:70,v/v/v) (Raquel et al., 2006).

Cell culture

Human squamous cell carcinoma cell line (A431) was purchased from *Pasture Institute* of *Iran*. A431 cells extended cultured in complete tissue culture medium contained RPMI 1640 (Royal Park Memorial Institute), FBS (Fetal bovine serum) 10% , Penicillin 100 u/ml, Streptomycin 100 µg/ml incubated at 37° C, 5% CO_2 , and 80% humidity (Ming et al.,2008).

Cytotoxic assay

MTT assay was performed in the A431 cells to measure the cytotoxicity of EDSS and EDSN. In living cells, the mitochondrial metabolism of 3-(4,5-dimethylthiazol-2-y1)-2,diphenyltetrazolium bromide (MTT) salt into formazan took place and the amount of produced formazan was correlated with the number of viable cells present.The A431cell was seeded in the 96 well plates at 1x 10^4 cells/well in RPMI medium supplemented with 10% FBS (Shokrgozar et al., 2007; Ming et al., 2008). After 24 h, cells were washed with PBS and then exposed to 5 concentrations (6.25, 12.5, 25, 50, 100 µg/ml) of EDSN and EDSS. After 6, 24 and 48 h , the number of viable cells was determined. RPMI alone was applied for negative control and RPMI medium with various concentrations of extract were added in wells without cells for estimating error. For every extract exposed toA431 cells 3 wells were applied.The assessment was performed at least in triplicates (Ming et al., 2008). MTT (5mg/ml in PBS) was added to each well (10µl/90µl medium) and the plate was incubated at 37 ˚c for 2h. Cells were then spun in a centrifuge at 300 rpm for 5 min and the medium was carefully aspirated. A 100 µl isopropanol alcohol

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acetic acid 4% (v/v) was added to each well.The plates were placed at room temperature for 10 minutes and the absorbance at visible region was measured for A431 cells which were exposed to extracts in 6, 24 and 48h on an Elisa reader (Ming et al., 2008). The percentage of cell death was calculated according to the formula below (Ghazanfary et al., 2006; Shokrgozar et al., 2007):

$$\text{Death percentage} : \frac{\text{Mean assay absorption test}}{\text{Mean negative control absorption}} \times 100$$

Statistical analysis

The *SPSS* 16 software package was used to analyze the raw data.Therefore, the mean slope scale of results was calculated. The one way ANOVA test was performed to identify the significant differences between data. Also, the LC_{50} values of EDSN and EDSS exposed to the A431 cell were determined by Probit value analysis from SPSS software (Shokrgozar et al., 2007, Ming et al., 2008).

Results

Cytotoxic assessment

The effect of ethanol extracts of EDSN and EDSS response that leads to producing more β-carotene and accumulation was analyzed against A431cells. The results of this survey in three time periods of 6, 24 and 48 h and in 5 concentrations of 6.25,12.5, 25, 50 and 100 µg/ml from EDSN and EDSS are shown in Table 1. The assay technique in two processes was based on evaluating the percentage of cell death and MTT method. Figure 1 shows the mean percentage of cell death of A431 cells exposed to EDSN and EDSS in three periodic times and various concentrations. Mean percentage of cell death of A431 cells by various concentrations of EDSN in 6, 24 and 48 h showed a significant difference (0.001, 0.001, 0.511) respectively.Therefore, it could be indicated that between 24 and 48 h in concentrations of 6.25, 12.5, 25, 50 and 100 µg/ml a significant difference was observed. Also, the result of this effect in stress condition in the three time periods respectively showed significant p-value (0.001, 0001 and 0.332). Although in time of 6 h among various concentrations in normal and stress conditions a significant difference was not observed. By comparing the death rate of A431 between EDSN and EDSS a significant difference in various concentrations in time periods of 6, 24, and 48 h was found. As seen in Figure 1, although both extracts showed significant differences in the percentage of resulted death, in time period of 6 h there was a harmonized and equal increase in their death.

A431 cells without exposure to ethanol extract *D.salina* showed marker viability very well. After evalouating the A431 cells growth , these cells contained suitable separation and natural morphology (Figure 2), A431 cells exposed to ethanol extract of *D.salina* lost natural morphology and showed marker death cell so membrane cells unable were separated (Figure 3).

Evaluation LC_{50}

The LC_{50} was found from EDSN against A431 cells for 48 h was 46.4µg/ml and for EDSS at time of 24 h was 15.4 µg/ml.

HPLC analysis

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Following evaluation of the results obtained from analysis of EDSN and EDSS circumstances using HPLC, the amount of β-carotene components in EDSS was calculated 1.46 times compared to EDSN (Table 2).

Table 1: Effect of ethanol extract of EDSN and EDSS on the percentage of death rate of cell line A431 at different times

		Concentration µg/ml						
	T(h)	0	6.25	12.5	25	50	100	P-value
EDSN	6	0	*-1.9 ±16.6	2.04 ±8.6	5.59 ±12/06	6.25 ±5.3	8.97 ±10.7	0.511
	24	0	12.41±1.50	14.22 ±2.7	19.02 ±8.93	24.15±9.4	30.59±19.1	< 0.001
	48	0	28.7 ±5.67	33.66±32.3	42.5±17.15	57.72±15.12	60.71 ±29.6	< 0.001
EDSS	6	0	5.12 ± 16.8	13.87±11.25	20.08 ± 21.1	23.3 ± 24.6	26.1 ± 16.8	0.332
	24	0	34.71 ± 22.7	44.55±21.08	62.4 ± 8.6	64.34 ± 10.1	70.24 ± 8.07	< 0.001
	48	0	78.35 ± 10.6	79.1 ± 4.04	81.26 ± 6.7	84.13 ± 4.8	83.37 ± 9.5	< 0.001

* Mean ± SD

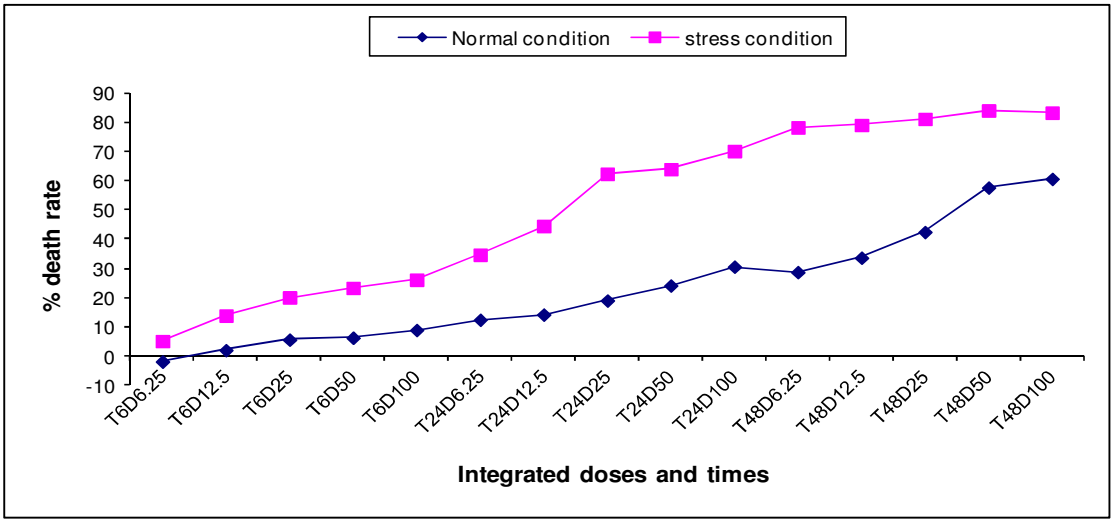
Table 2: The average of β-carotene in EDSN and EDSS by HPLC

Extraction(100g)	Concentration (ppm)	*RT(min)
Normal	**920**	**39.2**
Stress	**1352**	**39.2**
Standard βcarotene	**501**	**40.6**

*RetentionTime

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Figur 1: Comparative effect of EDSN and EDSS on percentage death rate of A431 cells in different times and cocentrations
D =Doses 6.25,12.5,25,50.100(µg/ml) T =Times 6,24,48(h)

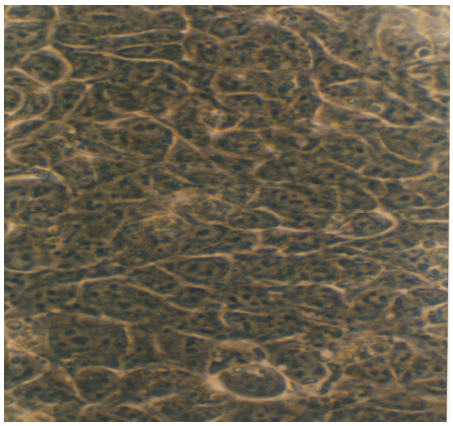


Figure 2: A431cells without exposed ethanol extract *D.salina* (× 100)

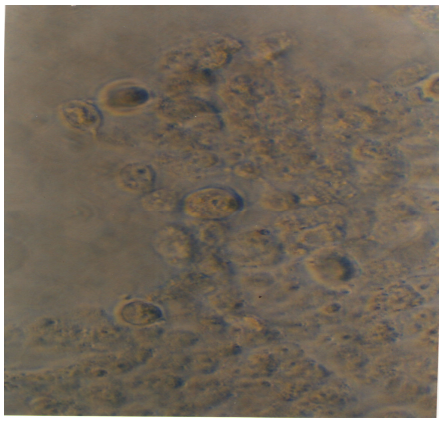


Figure 3: A431cells exposed ethanol extract *D.salina* (× 100)

Discussion

In this research, for the first time we used different amount of 6.25, 12.5, 25, 50 ,100 µg/ml ethanol extracts. In the recent years, many researches have been done for using *Dunialla*.This alga owing to have high reserve of β-carotene is valuable from economical point of views (Raja et al., 2007b, Moghadasi et al., 2011). Following several studies that applied in vitro and in vivo on animal models also subsequent the epidemiological surveys about various cancers, the antioxidant and anticancer of internal components especially existent beta carotene in this alga have proved (Chidambara et al., 2005, Raja et al., 2007a). In this research, HPLC analysis of extracted compounds showed the presence of β-carotene and revealed the comparison of the amount of this

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component in alga extract in two conditions (normal and stress). It appeared that the amount of EDSN and EDSS was 1.46 times more than normal condition. The obtained results the from analysis of samples were similar to the research of Fazeli et al., 2006 , Raja et al., 2007 , Moghadasi et al., 2011. By considering the results of low LC_{50} in shorter time under stress condition (production of greater β-carotene), it was revealed that as an antioxidant β-carotene can repulse 1000 free radicals and it could be caused by presence of more β-carotene in the extract (Challem,1997).The performed epidemiological testimonies in USA by Peto et al., 1981 indicated that β-carotene with antioxidant properties prevented cancer in various organs of the body (Poppel and Goldbohm, 1995, Buiatti, 1997, Chidambara et al., 2005). In a study, scientists found out that β-carotene via destroying the free radicals caused decrease of protuberance and β-carotene reduce and erythema associated of with exposure to sun light (Stryker et al., 1990, Challem, 1997).The most valid proof of its anti cancer characteristics was resulted from researches on oral leukoplakia and via 5 clinical tests it was proved that β-carotene supplement can transform this disease to the normal condition (Challem, 1997).The curative substance of β-carotene has been proved to prevent various tumors in human and animals such as melanoma skin cancer, head and neck epidermoid, stomach, intestinal, and gland secretory of pancreas (Stryker et al., 1990, Buiatti, 1997). β-carotene is known as the perquisite in the production of vitamin A, correcting controls of natural reproduction, maintaining of epithelial tissue, and a chemical material preventing epithelial cancer(Raja et al., 2007a, Poppel and Goldbohm, 1995, Ming et al., 2008). Other inter cellular carotenoides of *Dunaliella* alga are alfa carotene, lycopene, lutein , zeaxanthin and cryptoxanthin which through many studies their antioxidant and anticancer properties have been recognized (Challem, 1997). Through extraction process, these carotenoides would be extracted along by β-carotene (Ming et al., 2008). Existence of these components in cell structure of the micro alga involved in our study could cause its vast application in destroying cancer cells. By initiating 24 h an intense difference between operations of these two extracts was always observed and in low concentration of 25 µg/ml for EDSS, a more significant peak was seen. In 48 h more significant differences between the two extracts was observed. Therefore, by increasing the concentration amounts of ethanol extracts of alga in EDSN and EDSS in three incubation times , the cell survival rates were decreased and effect of EDSS decreased more in cell survival rates compared to EDSN.The research of Ming et al., 2008 showed that the ethanol extract DS could cause the cell

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death of human lung cancer A549 cell line, by changing expression P21 and P53 proteins which are involved in the regulation of the cell cycle then causing the cell cycle to stop. At the same research ethanol extracts of *D.salina* inhibited growth of Human Liukemeia cell line (HL-60) with exposure to 10 µl/ml and biphenotypic B myelomonocytic leukemia cell line (MV-4-11) with exposure to 8 µl/ml (Bechelli et al., 2011). In the current research, we proved that the death percentage of A431cells would increase by adding to the incubation time and raising various concentrations of EDSN and EDSS and the increased cell death was shown further in EDSS that might be owing to its high β-carotene (Ming et al., 2008). Besides, Raja et al., 2006 demonstrated that *Dunaliella salina* extract had anticancer effects against induced fibrosarcoma in Wistar rats (Raja et al., 2007a). Also in the research done by Fujii et al., 1993 the effect of *Dunaliella bardawile* in the natural growth of udder cells and prevention of tumor cells were proved (Fujii et al., 1993). Parallel with Fujii et al, another performed research by Xulex et al., 1993 on *Dunaliella bardawil* extract represented the significant prevention of NSAR induced cancer of proventriculus in mice. Based on the obtained results from this research the concentration of 15.4 µg/ml of EDSS against stress in 24 h could kill 50% of A431 cells, this indicated that Lc_{50} of alga EDSS is lower and acted in a shorter time compared to normal condition also implying that alga extracts under stress condition was more effective to destroy A431 cells.*Dunaliella* can act as a strong chemical inhibitor and maximum accumulation of anticancer against as β-carotene in *Dunaliella salina* occurred when it was grown under combined stress conditions (Farouk et al., 2002, Fazeli et al., 2006, Hosseini and Shariati, 2009). Our results suggest that performed and extended research on molecular path way of cell dead signaling by these products and further study could be helpful for better use of *Dunaliella* extracts in both cell culture system and another in vitro study.

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