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Anti-Proliferative and Apoptotic Efficacies of Ulvan Polysaccharides against Different Types of Carcinoma Cells *In Vitro* and *In Vivo*

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Abstract

Objective: This study was designed to assess the anti-proliferative and apoptotic effects of ulvan polysaccharides *in vivo* using Ehrlich ascites carcinoma (EAC)-bearing mice model and *in vitro* using hepatocarcinoma cell lines (HepG2) and colon carcinoma cell lines (HCT116).

Methods: *In vivo*, ulvan polysaccharide was orally administered to EAC-bearing mice at dose level of 100 mg/kg b. w. for 2 weeks beginning from the 1st day of EAC-intraperitoneal transplantation and compared with a control given a vehicle. The expressed anti-apoptotic protein Bcl2, proapoptotic mediator p53 and DNA fragmentation marker TdT were detected by TUNEL assay. Plasma and ascites total sialic acid was determined. *In vitro*, the anti-tumor effect of ulvan polysaccharide against HepG2 and HCT116 was tested at 0, 12.5, 25, 50 and 100 µg/ml and IC_{50} was determined.

Results: The data revealed that EAC-aliquot volume, EAC-total and alive cell numbers were potentially decreased while dead cell number and percent were profoundly increased as a result of treatment with ulvan polysaccharide. The EAC-cells exhibited phenotypic signs of apoptosis after treatment with ulvan polysaccharide.

The expression of proapoptotic and cell cycle arrest protein p53 in cytoplasm and nuclei and the amount of TdT in the nuclei of EAC-cells in mice treated with ulvan polysaccharide were remarkably increased while the anti-apoptotic protein Bcl-2 expression was decreased.

The treatment of EAC-bearing mice with ulvan polysaccharides successfully decreased plasma and ascites total sialic acid level.

In vitro, the ulvan polysaccharide induced potential anti-proliferative and anti-tumor cytotoxic effects against EAC-cells, hepatoma (HepG2) and colon carcinoma (HCT116) human cell line.

Conclusion: Taken together, this study may provide evidence for the anti-tumor effects of ulvan polysaccharides which may be mediated by induction of apoptosis and suppression of cell division.

Keywords: Anti-proliferative effects; Apoptosis; Ulvan polysaccharide; EAC; Sialic acid; HepG2; HCT116

Introduction

Since the increase in the use of synthetic chemicals in cancer therapy has led to many side effects and undesirable hazards, there is a worldwide trend to go back to natural resources, which are therapeutically effective, culturally acceptable and economically within the reach of the poor people [1]. Thus, the search for new anticarcinogenic agents from algae and plants is a target of research by many investigators.

Polysaccharides from different plants and algae were reported to have anti-proliferative effects on different types of cancer *in vivo* and *in vitro* by various authors. Fedorov et al. [2] reported that numerous polysaccharides isolated from different marine organisms have been evaluated to have promising anticancer and cancer preventive properties through mechanisms of action, including inhibition of tumor cell proliferation, induction of apoptosis, inhibition of angiogenesis, *etc.* Itoh et al. [3] reported that marine algal polysaccharide from *Sargassum thunbergi* markedly inhibited the growth of Ehrlich ascites carcinoma with no sign of toxicity in mice. Xiaoying et al. [4] demonstrated that *Acanthopanax giraldii* polysaccharide inhibited the growth of gastric cancer cells (SGC-7901). Ren et al. [5] stated that funoran, an algal polysaccharide from *Gloiopeltis tenax*, significantly suppressed the growth of Ehrlich ascites carcinoma (a spontaneous murine mammary adenocarcinoma adapted to ascites form according to Arican and Arican [6]), Meth-A fibrosacoma, and Sarcoma-180 tumors. It was also proved by that *Astragalus membranaceus* and *Radix hedysari* polysaccharides inhibited the growth of mouse transplanted tumor, Sarcoma 180 and ascites hepatoma, but have no effect on tumor cells *in vitro* [7].

The anti-tumor activity of polysaccharide isolated from *Ulva lactuca*, a marine alga widely distributed at Egyptian sea shores was rarely investigated. Only one available publication revealed that ulvan sulphated polysaccharide has anti-proliferative effects on tumoral colonic epithelial cells (HCT-29) and Caco-2 cells [8].

Thus, this study was conducted with the previous ones to assess the effect of ulvan polysaccharide on Ehrlich ascites carcinoma (which

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corresponds to mammary adenocarcinoma in female mice) *in vivo* and *in vitro*, and hepatoma (HepG2) and colon carcinoma (HCT-116) cell lines *in vitro*. The study also extended to investigate the histological changes and immunohistochemical changes in the levels of anti-apoptotic protein Bcl-2 and apoptotic factors p53 and TdT in EAC-cells as a result of ulvan polysaccharide treatment.

Materials and Methods

Tested agent

Ulvan polysaccharide isolated from *Ulva lactuca* (sea lettuce) which was collected from Mediterranean Sea shores of Alexandria and authenticated by Dr. Ibraheem Boreey, Professor of Phycology (Algology), Botany Department, Faculty of Science, Beni-Suef University, Egypt. The polysaccharide (both sulphated and non-sulphated) was isolated by boiling the dried powdered algae for two hours in distilled water, precipitated by ethyl alcohol several times and dried (to remove any traces of alcohol) [9,10]. The polysaccharide was dissolved in hot water and left to cool, then administered orally at dose level of 100 mg/kg body weight (b. w.) / day for 2 weeks.

In vivo studies

Experimental animals and EAC-bearing model: Female virgin albino mice were obtained from Animal House, Institute of Ophthalmology, Giza, Egypt. EAC-bearing stock female mice were obtained from Cancer Biology Department, National Cancer Institute, Cairo University, Egypt. To induce EAC (which corresponds to mammary adenocarcinoma) in mice for the experimental study, 0.2 ml EAC aliquot aspirated from stock mice was added to 9.8 ml saline (dilution is 1:50) and 0.2 ml of this diluted EAC was intraperitoneally administered by syringe into each mouse.

Animal grouping: The EAC-injected mice were divided into 2 groups each of 12 animals. Mice of group 1 (control group) was administered distilled water as a vehicle in a volume equivalent to that given to treated animals. Group 2 was treated with ulvan polysaccharide at dose of 100mg/kg b. w. The treatments were orally applied by gastric gavage between 10-12 AM daily for 2 weeks beginning from the 1st day of EAC-intraperitoneal inoculation.

Animal survival: The number of animals survived in each group was detected at the end of the experiment and the survival percent in each group was calculated as follows: Survival percent = (number of survived animals/total number of animals) x 100.

Sampling and detection of EAC-volume and cell number: At the end of the experimental period, animals were sacrificed under diethyl ether anesthesia and 0.2 ml saline was intraperitoneally injected. One minute later, EAC-aliquot was aspirated by a sterile syringe into test tube. The volume of EAC-aliquot for each mouse was measured. The number of alive and dead EAC-cells was determined using trypan blue exclusion assay [11]. Alive and dead EAC-cells were counted by Neubauer haemocytometer. Briefly, 40 µl of EAC-aliquot was added to 4 ml 2% trypan blue (dissolved in 0.9% saline) and the mixture was left for 5 minutes. One drop from mixture was taken on Neubauer haemocytometer and the number of stained cells (dead cells) and non-stained cells (viable or alive cells) were counted. Total number of EAC-cells and percent of dead EAC-cells were calculated for each EAC-bearing mouse. This procedure was adopted from the methods of Freitas et al. [12] and Chandru et al. [13].

Blood samples were obtained from carotid artery of each mouse into EDTA (15%)-containing tubes under mild diethyl ether anesthesia and were centrifuged at 3000 r.p.m. for 15 minutes. The plasma was aspirated into Eppendorf tubes and kept in deep freezer at -30°C until used for plasma sialic acid determination. One ml of EAC fluid from each mouse was homogenized in 2 ml saline (0.9% NaCl) and centrifuged at 3000 r.p.m. for 15 minutes. The supernatant was aspirated into Eppendorf tubes and kept in deep freezer at -30°C until used for ascites sialic acid determination.

Part of EAC-aliquot (1 ml) from each tumor-bearing mouse was centrifuged at 3000 r.p.m. for 15 minutes and the precipitated EAC-cells were fixed in neutral buffered formalin for histological and immunohistochemical studies.

Determination of plasma and ascites sialic acid concentration: Plasma and ascites sialic acid level was determined according to the method of Warren [14]. In this method, sialic acid is oxidized into formylpyruvic acid which reacts with thiobarbituric acid to form a pink color product. The color intensity measured at 549 nm is proportional to the concentration of sialic acid in the sample.

Histological and immunohistochemical investigations: The fixed samples were transferred to the Department of Pathology, National Cancer Institute for processing, blocking, sectioning and staining with haematoxylin and eosin or mounting on +ve slides for immunhistochemical investigations. Sections mounted onto positive-charged slides (Fisher Scientific, Pittsburgh, PA) were used to detect the Bcl-2 and p53 reactivity or apoptotic cells using the TUNEL assay [15]. The TdT-mediated dUTP nick-end labelling (TUNEL) assay was performed using a kit (*in situ* cell death detection kit, Roche Molecular Biochemicals, Manheim, Germany) according to the protocol provided by the manufacturer, while Bcl-2 and p53 reactivity were determined following Hua and Ya-wei method [16]. Briefly, before the incubation with antibodies, endogenous peroxidase activity was quenched, slides washed and then incubated in a blocking solution of hydrogen peroxide 1% in methanol, in darkness for 15 min. Antigen retrieval occurred with citrate buffer 10 mM, pH = 6.0. After cooling, sections were rinsed in tap water and then phosphate buffer saline 1M. Primary mouse antibodies for Bcl-2 (DakoCytomation, USA) or p53 (Lab Vision Corporation, 47777 Warm Springs Blvd. Fremont CA, 94539 USA) diluted 1:150 and 1:100, respectively in PBS, were applied for 1 hour at 37°C. Secondary biotinylated antibody diluted 1:100 and 1: 200 in PBS was applied for a period of 30 minutes at 37°C. Streptavidin-biotin or avidin-biotin peroxidase (ABC/ HRP) was applied for 10 minutes at room temperature. Bound antibody complex was visualized by the reaction of 3, 3`-diaminobenzidine (DAB) substrate and counter stained with haematoxylin. Secondary biotinylated antibody, ABC/ HRP and DAB were obtained from Zymed Laboratories, Invitrogen Immunoprotection, 561 Eccles Avenue, South San Francisco, California, USA. Haematoxylin was obtained from Sigma Chemical Compay, USA.

In vitro studies

Antitumor cytotoxicity against EAC-cells: The viability of cells as a result of 3 different concentrations (25, 50 and 100 µg/ml) of ulvan polysaccharide was tested by trypan blue exclusion assay [17]. Briefly, EAC-cells at concentration 2.5 x 10^5 cells/ml suspended in phosphate buffer saline were incubated at 37 °C for 2 hours in the presence of 3 different concentrations of the compound. At the end of incubation period, equal volume of trypan blue solution was added to sample cells, then the stained cells (dead cells) and unstained cells (alive cells) were counted using Neubauer haemocytometer. The percent of dead cells for each test was calculated.

HepG2 an HCT116 cell lines (cell culture): Hepatoma (HepG2) and colon carcinoma (HCT116) cell lines were obtained from the American Type Culture Collection (ATCC, Minisota, U.S.A.) and the testing of the cytotoxic effect was performed in the Pharmacology Unit, Cancer Biology Department, National Cancer Institute, Cairo University, Egypt. Cells were maintained in DMEM (Dulbeco's Modified Eagle's Medium) while HCT116 were cultured in Mc Coy's medium. Media were supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 units/ml penicillin G sodium, 100 units/ml streptomycin sulphate and 250 ng/ml amphotericin B. Cells were maintained at 37°C in humidified air containing 5% CO_2. All culture media was obtained from Cambrex BioScience (Copenhagen, Denmark). All chemicals were obtained from Sigma/Aldrich Company, USA.

Anti-proliferation assay: Cytotoxicity and anti-proliferative activity of ulvan polysaccharide against HepG2 or HCT116 cell line was measured by MMT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) cell viability assay. MTT assay is based on the ability of active mitochondrial enzyme of living cells to cleave the tetrazolium rings of yellow MTT and form dark blue insoluble formazan crystals which are largely impermeable through cell membrane, resulting in its accumulation within healthy cells. Solubilization of the cells results in the liberation of crystals, which are then solubilized. The number of viable cells is directly proportional to the level of soluble formazan dark blue color. The extent of reduction of MTT was quantified by measuring the absorbance at 570 nm [18]. Briefly, Monolayer HepG2 or HCT116 cells (0.5×10^5/well) plated in a flat bottom 96-multiwell microplate were treated with 20 µl of ulvan polysaccharide solution to reach final concentrations of 0, 12.5, 25, 50 and 100 µg/ml, and then incubated for 20 hours at 37°C in atmosphere of 5% CO_2. At the end of the incubation period, media were removed and 40 µl MTT solutions (5 mg of MTT in 0.9% NaCl)/well were added and incubated for an additional 4 hours. MTT crystals were solubilized by adding 180 µl of acidified isopropanol (0.04N HCl in absolute isopropanol)/well and plate was shaked at room temperature. The absorbance was measured at 570 nm using micoplate ELISA reader. Triplicate repeats were performed for each concentration and mean and standard deviation were calculated. The relation between the percentage of relative viability compared with cells treated with a vehicle and concentration of ulvan polysaccharide was plotted. In addition, the half maximal inhibitory concentration (IC_{50}) was calculated from the dose response curve.

Statistical analysis: Data were analyzed by one-way analysis of variance (ANOVA) followed by least significance difference (LSD) test to compare groups with each other [19]. Data were expressed as mean ± standard error (SE). Values with $p>0.05$ are not significantly different while values with $p<0.05$ and $p<0.01$ are significantly and highly significantly different respectively. F-probability expresses the general effect between groups.

Results

Effects on animal survival and mortality

As indicated in Table 1, the survival percentage of EAC-bearing mice was 83.33% (10 of 12 mice survived) after 2 weeks of EAC-intraperitoneal transplantation. As a result of treatment of EAC-bearing mice with ulvan polysaccharide, the survival percent was increased to reach 100% at the end of the experiment.

Effects on EAC-aliquot volume and cell number *in vivo*

The daily treatment of EAC-bearing mice with ulvan polysaccharide from the 1st day of EAC-cells intraperitoneal transplantation to 2 weeks produced a very highly significant depletion (F-probability; $p<0.001$) of EAC- aliquot volume, number of total and alive EAC-cells. The number and percent of dead EAC-cells, as indicated by one-way ANOVA, were respectively highly significantly ($p<0.01$) and very highly significantly ($p<0.001$) increased in EAC-bearing mice treated with ulvan polysaccharide as compared with EAC-bearing mice control. LSD test revealed that the effect of ulvan polysaccharide on all these previous variables was highly significant ($p<0.01$) (Table 1).

Effects on EAC, HepG2 and HCT116 *in vitro*

Incubation of EAC-cells (2.5×10^5/ml suspended in phosphate buffer saline) with 25, 50 and 100 µg/ml for 2 hours respectively produced 15.00 ± 2.23, 35.00 ± 2.24 and 65.00 ± 6.71 percent inhibition of cell viability *in vitro* (Table 2). The treatment of hepatoma (HepG2) and colon carcinoma (HCT116) cell lines with gradual increasing doses of ulvan polysaccharide produced a high inhibition of cell proliferation as concluded by low IC_{50} 55.56 and 22.65 µg/ml, respectively. Thus, the ulvan polysaccharide seemed to have more anti-tumor efficacy against HCT116 than HepG2 cell line. However, the highest dose (100 µg/ml) of ulvan polysaccharide induced more or less similar effect on HepG2 and HCT116. The effect on both cell lines was dose-dependent (Figure 1).

Effect on plasma and ascites sialic acid level *in vivo*

Plasma sialic acid level in EAC-bearing mice was highly significantly ($p<0.01$) decreased as a result of treatment with ulvan polysaccharides recording percentage decrease of 31.93. Similarly, the ascites sialic acid concentration was also highly significantly ($p<0.01$) decreased in EAC-bearing mice treated with ulvan polysaccharides recording percentage decrease of 16.05 as compared with EAC-bearing control (Table 3).

Table 1: Effect of ulvan polysaccharide administration on animal survival percent EAC-aliquot volume, EAC-cells number and percent of dead cells in EAC-bearing mice.

Parameter / Group	Animal survival percent	EAC-aliquot volume (ml)	Total EAC-cells number.10^7	Alive EAC-cells number.10^7	Dead EAC-cells number.10^7	Percent of dead EAC-cells
EAC-bearing mice control (n=10)	83.33	4.39 ± 0.45	116.27 ± 9.42	113.55 ± 9.36	2.73 ± 0.18	2.45 ± 0.12
EAC-bearing mice treated with ulvan polysaccharide (n=12)	100.00	2.13 ± 0.17**	68.02 ± 2.48**	64.72 ± 2.56**	3.30 ± 0.03**	4.95 ± 0.21**
F-porbability	-	P<0.001	P<0.001	P<0.001	P<0.01	P<0.001
LSD at the 5% level	-	1.037	18.74	18.69	0.36	0.54
LSD at the 1% level	-	1.420	25.55	25.49	0.49	0.73

Data are expressed as mean ± standard error (SE).
*p<0.05: difference is significant; difference between two means is higher than value of LSD at the 5% level.
**p<0.01: difference is highly significant; difference between two means is higher than value of LSD at the 5% level.

Histological and immunohistochemical effects on EAC-cells *in vivo*

As depicted in Figure 2a, c and e, EAC-cells of control mice appeared to be characterized with highly proliferated rate, abundant basophilic and dark-stained cytoplasm and moderate-sized nuclei. Many dividing cell are shown (Figure 2e). As a result of treatment with ulvan polysaccharide, the EAC-cells were decreased in number. Some cells were degenerated (Figure 2d) and many others exhibited phenotypic apoptotic signs including cell shrinkage, irregular shape,

Table 2: Effect of ulvan polysaccharide on percent inhibition of EAC-cell viability *in vitro*.

Compound	% inhibition of cell viability		
	25 µg/ml	50 µg/ml	100 µg/ml
Ulvan polysaccharide	15.00 ± 2.23	35.00 ± 2.24	65.00 ± 6.71

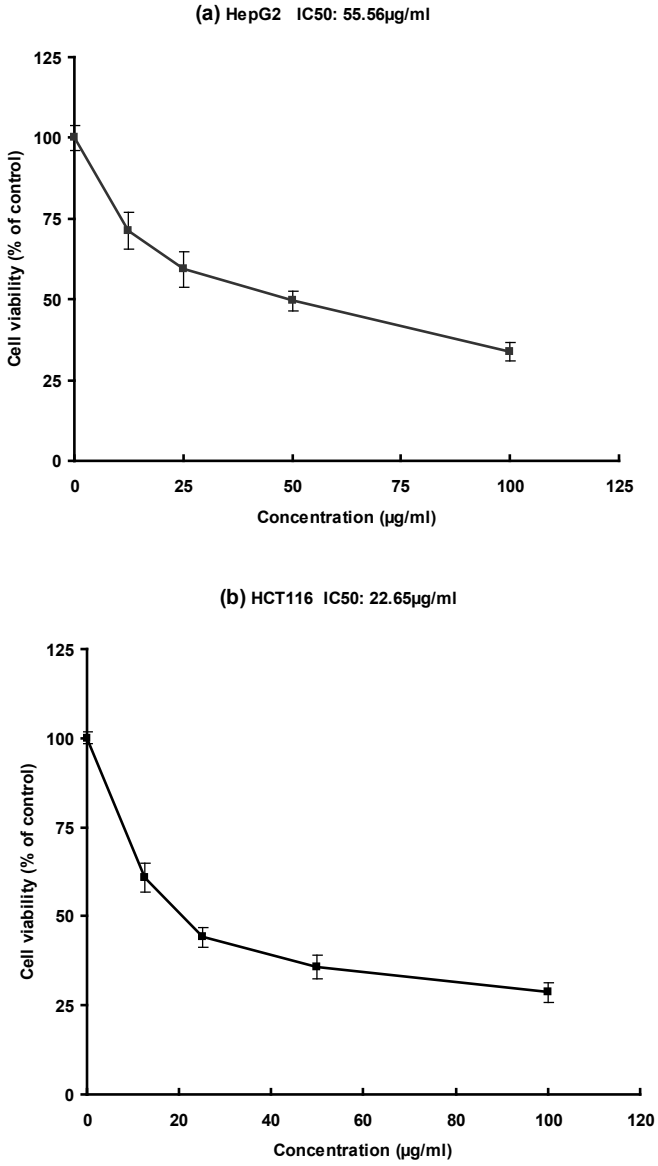

Figure 1: Anti-proliferative effects of ulvan polysaccharide against HepG2 (a) and HCT116 (b) cell lines.

Table 3: Effect of ulvan polysaccharide administration on plasma and ascites sialic acid concentration in EAC-bearing.

Parameter / Group	Plasma sialic acid concentration (mg/100ml)	Percent change	Ascites sialic acid concentration (mg/g protein)	Percent change
EAC-bearing mice control (n=10)	94.45 ± 1.48		214.56 ± 6.16	
EAC-bearing mice treated with ulvan polysaccharide (n=12)	64.80 ± 1.93**	-31.39	180.12 ± 5.65**	-16.05
F-probability	P<0.001		P<0.001	
LSD at the 5% level	5.24		17.45	
LSD at the 1% level	7.15		23.80	

Data are expressed as mean ± standard error (SE).
*p<0.05: difference is significant; difference between two means is higher than value of LSD at the 5% level.
**p<0.01: difference is highly significant; difference between two means is higher than value of LSD at the 5% level.

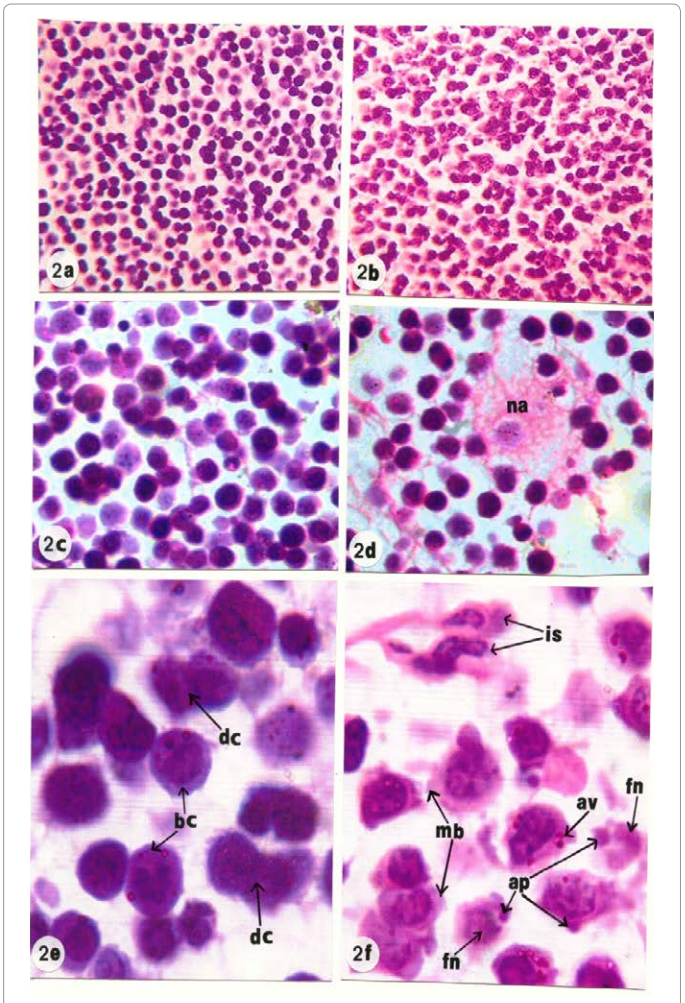

Figure 2: Photomicrographs of haematoxylin and eosin stained EAC-cells sections showing decreased number of cells, necrotic area (na) (figure 2d; x400), irregular shrinked cells (is) (figure 2b; x100 and figure 2f; x1000), plasma membrane blebbing (mb), azurophilic eosinophilic vesicle (av), fragmenting nuclei (fn) and apopoptic bodies (ap) (figure 2f) as a result of treatment of EAC-bearing mice with ulvan polysaccharide in comparison with EAC-bearing mice control which showed highly proliferating and dividing cells (dc) with abundant basophilic cytoplasm (bc) (figure 2a; x100, figure 2c; x400 and figure 2e; x1000).

plasma membrane blebbing, cytoplasmic azurophilic lytic vesicles, apoptotic bodies and fragmenting nuclei (Figure 2b, d and f).

In an attempt to unveil the mechanism of EAC-killing, the changes in anti-apoptotic protein Bcl2, proapoptotic mediator p53 and DNA fragmentation marker TdT were assessed.

As indicated by the density of brownish yellow color in cytoplasm, Bcl-2 was detectably decreased in EAC-cells of mice treated with ulvan polysaccharide (Figure 3b) as compared with the counterpart control (Figure 3a).

On the other hand, p53 in cytoplasm and nuclei and TdT in nuclei was moderately and greatly increased in EAC-cells obtained from mice treated with ulvan polysaccharide (Figures 4b and 5b) in comparison with EAC-cells of control mice (Figures 4a and 5a).

Discussion

Synthetic compounds are used to control the advanced stages of cancer, but most of these compounds exhibit normal tissue toxicity with undesirable side effects. Thus, the search for safe agents or products that may have anti-tumor efficacies should be assessed. Ulvan polysaccharide isolated from *Ulva lactuca* (sea lettuce) as natural product was tested in this study to assess its anti-proliferative and apoptotic effects.

The anti-proliferative activity of ulvan polysaccharide *in vivo* was evaluated in terms of changes in EAC-aliquot volume, EAC- total and alive cell burdens, which were about two-fold decreased, and percent and number of dead cells that were remarkably increased in EAC-bearing mice treated with the compound as compared with the vehicle. These anti-tumor effects were concomitant with the absence of mortality between EAC-bearing mice treated the compound. These results are in accordance with Itoh et al. [2] and Ren et al. [5] who revealed that marine algal polysaccharide from *Sargassum thunbergii* and *Gloiopeltis tenax* markedly inhibited the growth of EAC with no sign of toxicity in mice. Both latter authors attributed the anti-tumor activity of this polysaccharide to the augmentation of immune responses. The present results are also in accordance with the previous publications which evidenced the anticancer effects of *Ulva lactuca* extract and polysaccharides in acetaminophen-induced carcinogenicity [10] as well as *Ulva rigida* polysaccharides against solid tumor induced in female mice by EAC cell line [20].

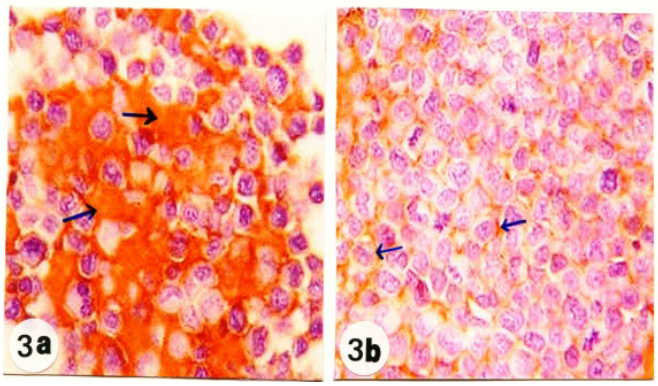

Figure 3: Photomicrographs of EAC-sections stained immunohistochemically to show the higher concentration of Bcl-2 (arrow; brownish yellow color) in the cytoplasm of EAC-cells in control mice (figure 3a; x100) as compared with mice treated with ulvan polysaccharide (figure 3b; x100).

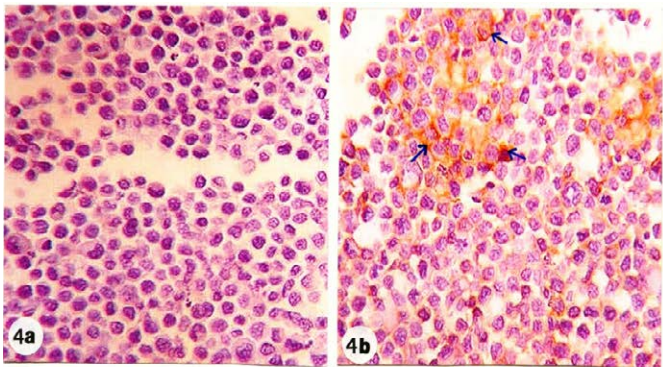

Figure 4: Photomicrographs of immunohistochemically stained EAC-sections, showing the higher amount of p53 (arrow; brownish yellow color) in the cytoplasm and nuclei of EAC-cells in mice treated with ulvan polysaccharide (figure 4b; x100) than in control mice (figure 4a; x100).

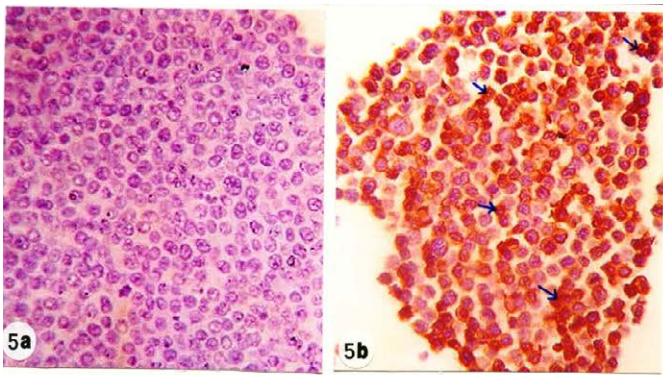

Figure 5: Photomicrographs of immunohistochemically stained EAC-sections, showing the higher amount of TdT (arrow; brownish yellow color) in the nuclei of EAC-cells in mice treated with ulvan polysaccharide (figure 5b; x100) than in control mice (figure 5a; x100).

Sialic acid level is considered as a marker of many pathological conditions including cancer [21]. Many reports revealed a positive correlation between sialic acid level and metastatic tumors both *in vivo* and *in vitro* [22-25]. These reports proposed that tumor cells might excrete and contain more sialyl glycans, glycoproteins, or glycolipids which contain sialic acid on their terminals [23,26].

The marked decrease of plasma and ascites sialic acid concentration in EAC-bearing mice treated with ulvan polysaccharides as compared with EAC-bearing control mice may reflect the anti-tumor effect of ulvan polysaccharides in the present study.

The present *in vivo* results of EAC were supported with *in vitro* investigations which indicated that algal polysaccharide, isolated from *Ulva lactuca*, induced anti-tumor cytotoxic and anti-proliferative potentials against EAC-cells, hepatoma (HepG2), colon carcinoma (HCT116) cell lines. The effect on HCT116 (IC_{50}= 22.65 µg/ml) seemed to be more potent than that on HepG2 (IC_{50}=55.56 µg/ml). In accordance with this study, Kaeffer et al. [8] reported that ulvan polysaccharide inhibited proliferation of colonic cancer cells HT-29 and Caco-2. In addition, Wu et al. [27] found that aqueous extracts of two algae *Spirulina* and *Chlorella*, which contain algal polysaccharides, produced anti-proliferative effects on hepatic stellate cells (HSC) and HepG2 cells *in vitro*.

It nowadays well recognized that apoptosis is a form of cell death characterized by active suicide of cells. Thus, the study of this process is a worthwhile and is of crucial role in cancer chemoprevention and therapy [21] Histological photomicrographs of sections of EAC-cells from EAC-bearing mice, in the present study, revealed that after treatment with ulvan polysaccharide, many EAC-cells exhibited many signs of apoptosis such as shrinkage, plasma membrane blebbing, cytoplasmic azurophilic lytic vesicles, apoptotic bodies and fragmenting nuclei. These changes were associated with decreased number of cell burden. Thus, the anti-proliferative effect of ulvan polysaccharide may be owing to the induction of apoptosis. To confirm this explanation based on morphologic or phenotypic signs, some mediators (p53, Bcl-2 and TdT) involved in the molecular mechanisms of apoptosis were detected by immunohistochemical techniques.

It is now well recognized that whether a cell committed to apoptosis partly depends upon the balance between proteins that mediate growth arrest and apoptosis e.g. P53, p21 and Bax and proteins that promote the cell viability e.g. Bcl-2 [28-31]. The present immunohistochemical-stains sections indicated that treatment of EAC-bearing mice with ulvan polysaccharide potentially decreased the anti-apoptotic protein Bcl-2 in cytoplasm while it profoundly increased the levels of pro-apoptotic, tumor suppressor and cell cycle arrest protein p53 in cytoplasm and nuclei of EAC-cell of mice treated with ulvan polysaccharide. It was also depicted by the current study that level of DNA fragmentation mediator was also increased in nuclei of EAC-cells obtained from mice treated with ulvan polysaccharide. Based on these results, it can be suggested that induction of apoptosis by ulvan polysaccharide is not only mediated by down-regulation of anti-apoptotic protein Bcl-2, but also induced via upregulation of pro-apoptotic and cell cycle arrest protein p53. It was previously reported that the stimulating effect on p53 probably results from the inhibition of cyclin dependent kinases [31,32]. It is also relevant to mention that in EAC, the tumor suppressor protein p53 may upregulated the proapoptotic protin Bax on one hand and/or mediate growth arrest involving p21 as a major effector on the other [28,33]. In concomitant with the result of the present study, Wu et al. [27] found that the aqueous extract of alga *Spirulina* which contains algal polysaccharide, induced an apoptosis and trigerred a cell cycle arrest of hepatic stellate cells (HSC).

In conclusion, this study indicates that (1) ulvan polysaccharide has potential anti-tumor cytotoxicity effects on EAC-cells *in vivo* and *in vitro* and on HepG2 and HCT116 human cell lines *in vitro* and (2) the anti-proliferative effect of this agent may be mediated via apoptosis and cell cycle arrest. However, further clinical studies are needed to validate the safety and usefulness of ulvan polysaccharide in the prevention and therapy of different types of cancer in human beings.

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