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IN VITRO ANTITUMOR ACTIVITY OF SILVER NANOPARTICLES BIOSYNTHESIZED BY MARINE ALGAE

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Cancer is a mortal disease needs a potent therapy with low side effects. So, the aim of this study is to evaluate the antitumor efficiency of different concentrations of silver nanoparticles (AgNPs) biosynthesized by seaweeds (*Ulva fasciata, Corallina elongate, Gelidium crinale, Laurencia obtusa, Cystoseira myrica* and *Turbinaria turbinata*) on Ehrlich Ascites Carcinoma (EAC) *in vitro*. The most cytotoxic silver nanoparticles synthesized by one of our studied algae would be examined by TEM, SEM, EDAX, X-ray and FT-IR to determine their characterizations. Silver nanoparticles were synthesized by the reduction of silver nitrate in the algal aqueous extracts . The green synthesis of AgNPs through algal extracts was indicated by a colour change and UV spectroscopy. Different concentrations of AgNPs were used to study their cytotoxic activity against EAC *in vitro*. Trypan blue exclusion test showed a decrease in EAC cells viability by increasing AgNPs concentration in all tested algae. The maximum inhibition percentage of EAC cells were (94 and 99%) with 98µg/ml AgNPs biosynthesized by *Ulva fasciata* and *Turbinaria turbinata* respectively. Silver nanoparticles biosynthesized by *Turbinaria turbinata* were the most cytotoxic against EAC *in vitro*. Characterization of the biosynthesized AgNPs by *Turbinaria turbinata* confirmed biosynthesis of AgNPs with spherical shape and size varying from 8-16 nm.

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Keywords: Seaweeds; *Turbinaria turbinata*; Ehrlich Ascites Carcinoma (EAC); *in vitro*; Silver nanoparticles (AgNPs).

1. Introduction

Nanotechnology is defined as a research for design, synthesis, and manipulation of the particles structures conducted at the nanoscale (from 1 to 100 nanometers) in science, engineering and technology. The new branch nanobiotechnology is used for generating nano-sized particles with specific biological or mechanical functions by combination of biological, physical and chemical principles [1]. One of the biological application of nanobiotechnology that it is a new way of diagnosis, treating and dealing with diseases such as human cancers [2]. Ultimately, the use of nanomedicine will allow simultaneous drug delivery, wound healing and tumor cell targeting in a unique manner [3].

Chemical and physical properties silver metal reflux the important role of its nanoparticles in human health, so it is necessary to investigate formation, stability, and sedimentation of AgNPs [4]. Silver nanoparticles are being extensively used in medicine for its therapeutic values. Recently, AgNPs have been reported to be efficient anti-tumor agents because silver nanoparticles had the ability to induce apoptosis by caspase signaling [5]. Chemical synthesis of nanoparticles has many flaws in using toxic solvents and production hazard by-products, on the contrary, the biomolecules involved in the green synthesis of nanoparticles are less toxic and acting as functionalizing ligands, so green synthesis of nanoparticles is more suitable than chemical synthesis [6].

Several biological systems such as microorganisms, marine organisms, micro-fluids, and plant have been used as reducing agents for the green synthesis of silver nanoparticles [7].

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Moreover Green synthesis of silver nanoparticles by algae showed more advantageous over other biological processes by bacteria and fungi, because it is more suitable for large scale production of silver nanoparticle with various size and shapes and it eliminates the cell culture maintaining process [8].

This work aimed to study the cytotoxic effect of biologically synthesized AgNPs prepared by biological (green) techniques using *Ulva fasciata , Corallina elongate, Gelidium crinale, Laurencia obtusa, Cystoseira myrica,* and *Turbinaria turbinata in vitro* against EAC. The most cytotoxic silver nanoparticles biosynthesized by one of our studied algae would be examined in details by their characterization.

2. Material and Methods

2.1. Algae collection

Marine algae *Ulva fasciata , Corallina elongate* and *Gelidium crinale* were collected manually from Abu-Qir coast, Alexandria, Egypt at May 2013. *Laurencia obtusa, Cystoseira myrica and Turbinaria turbinata* were collected at May, 2014 from shallow water beside the shore of Red Sea, Safaga, Egypt. Marine algae were identified by Taylor [9].

2.2. Preparation of algal aqueous extracts

Fresh seaweed samples were collected in polyethene bags. Marine algae were cleaned thoroughly by rinsing with tap water to remove stones and extraneous matters. Then cut into small pieces and washing with sterile distilled water. The cleaned macro-algae were shade dried at room temperature for 4 days. The shade-dried thalli were powdered and aqueous extract was prepared.

3.3. Rapid synthesis of AgNPs

One gm of seaweed powder was extracted with water and filtered. 90ml of 1mM $AgNO_3$ solution were added to 10 mL of algal thallus extracts slowly with magnetic stirring for even coating of silver and subjected to heating at 30°C for 40 min for reduction of Ag+ ions. The reduction of pure Ag+ ions was monitored by color changing from pale yellow to red (Fig.1). This green synthesis was the modified method followed by Devi and Bhimba [10].

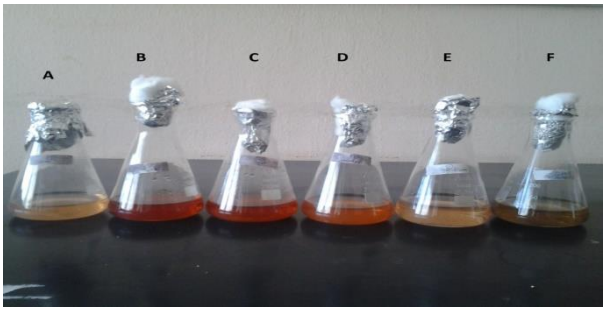

Fig. 1. Color change of silver nanoparticles biosynthesized by macro algae: A) Ulva fasciata B) Turbinaria turbinata C) Cystoseira myrica D) Laurencia obtuse E) Gelidium crinale F) Corallina elongate

3.4. Characterization of silver nanoparticles biosynthesized by marine algae

3.4.1. UV–Vis spectra analysis

The reduction of silver ions Ag^+ in aqueous extracts of marine algae and the formation of AgNPs was monitored by measuring the UV-Vis spectra. UV-Vis spectroscopy analysis of silver nanoparticles produced were carried out as a function of bio-reduction time at a wavelength of 100- 700 nm on Ultra violet-Visible spectroscopy (T80+UV/VIS Spectrometer) at Genetic Engineering and Biotechnology Research Institute (GEBRI), Egypt.

3.5. In vitro assessment of anti tumor activity of green synthesized silver nanoparticles

The antitumor activity of these AgNPs synthesized by algal extracts was determined *in vitro* against Ehrlich Ascites Carcinoma (EAC) cell line which was kindly purchased from the National Cancer Institute ,Cairo University, Egypt. 1ml of freshly ascitic fluid which was drawn from an albino mice bearing 7-14 days-old ascitic tumor (EAC) was diluted with 9ml normal saline in sterile test tube. EAC cells were thereafter propagated in GEBRI laboratories by weekly intraperitoneal injection of 0.2 ml ($1x10^6$ EAC cells) of EAC suspension into three mice to ensure that the ascitic fluid would still propagated.

Appropriates dilutions of Ag nanoparticles were tested as antitumor activity *in vitro* by trypan blue exclusion method reported by Freshney [11], as followed:

1. Prepare 0.4% solution of trypan blue in buffered isotonic salt solution, pH 7.2 to 7.3 (i.e., phosphate-buffered saline).
2. Add 0.1 mL of trypan blue stock solution to 1 mL of cells suspension after addition of AgNPs, and incubated for 15mins.
3. Load a hemocytometer and examine immediately under a microscope at low magnification.
4. Count the number of blue staining cells (non-viable cells) and the total number of cells by:

(No. of non-viable cells x 100) / total cells

This method determines the effect of different concentrations of AgNPs for regression of tumors cells.

3.6. Characterization of the most cytotoxic AgNPs biosynthesized by one of the marine algae

3.6.1.Transmission Electron Microscope

Characterization of the size, shape and the silver nanoparticles state of assembly was monitored by using Transmission Electron Microscopic (TEM) analysis (JEOL JEM-2100) at National Research Center (NRC), Egypt. Samples for TEM studies were prepared by placing two drops of the silver nanoparticle solutions onto carbon-coated TEM grids. Then the film on the TEM grid was dried prior to measurement.

3.6.2. Scanning Electron Microscopy

Surface morphology, size and distribution of silver nanoparticles in the solution were investigated at NRC using JEOL JSM-6100 Scanning Electron Microscope with EDAX detector system operated at an applied potential of 15 kV and was adapted prior to recording.

3.6.3. X-ray diffraction

The prepared sample of silver nanoparticles was analysized by placing three drops of the silver nanoparticle solution onto microscopic slide for 2 hours at 35^o C till being air dried. The dried sample of AgNPs was analysized at NRC by XRD diffractometer (JED-2300T) Cu-Kα X-rays of wavelength 1.54060 Å and data were taken for the range of 5° to 80° with a step of 0.026°. X-ray generator was operated at a voltage of 45kv and current of 30mA with Cu kal radiation at 2θ angle.

3.6.4. Energy Dispersive X-ray Spectroscopy (EDX)

Energy Dispersive X-ray Spectroscopy measurement of silver nanoparticles Energy-dispersive X-ray (EDX) analysis was carried out at NCR using JEOL JSM-6100 Scanning Electron Microscope which operated at 15 kV and was adapted to determine the elemental composition of the sample.

3.6.5. Fourier-transform infrared (FTIR)

Identification of the biomolecules in seaweed aqueous extract responsible for the reduction of the silver ions to form nanoparticles and also capping the silver nanoparticles was monitored at

NRC by using the FT-IR spectrometer (FT/IR-6100 type A) and the spectra were recorded in the wavelength interval of 4000 to 400nm-1.

4. Results

4.1. UV-Vis spectroscopy of biosynthesized AgNPs by different marine algae

UV-Vis spectroscopy of the silver nanoparticles were characterized by one of the most widely used technique Jain and Pradeep [12]. A single peak was observed at 428 nm, which corresponded to plasmon excitation of silver nanoparticles (Fig. 2), indicating the presence of silver nanoparticles. Several researchers have observed absorption of a broad peak of colloidal silver in solution between 400 and 450 nm, which is assigned to surface plasmon excitation of the metal nanoparticles [13].

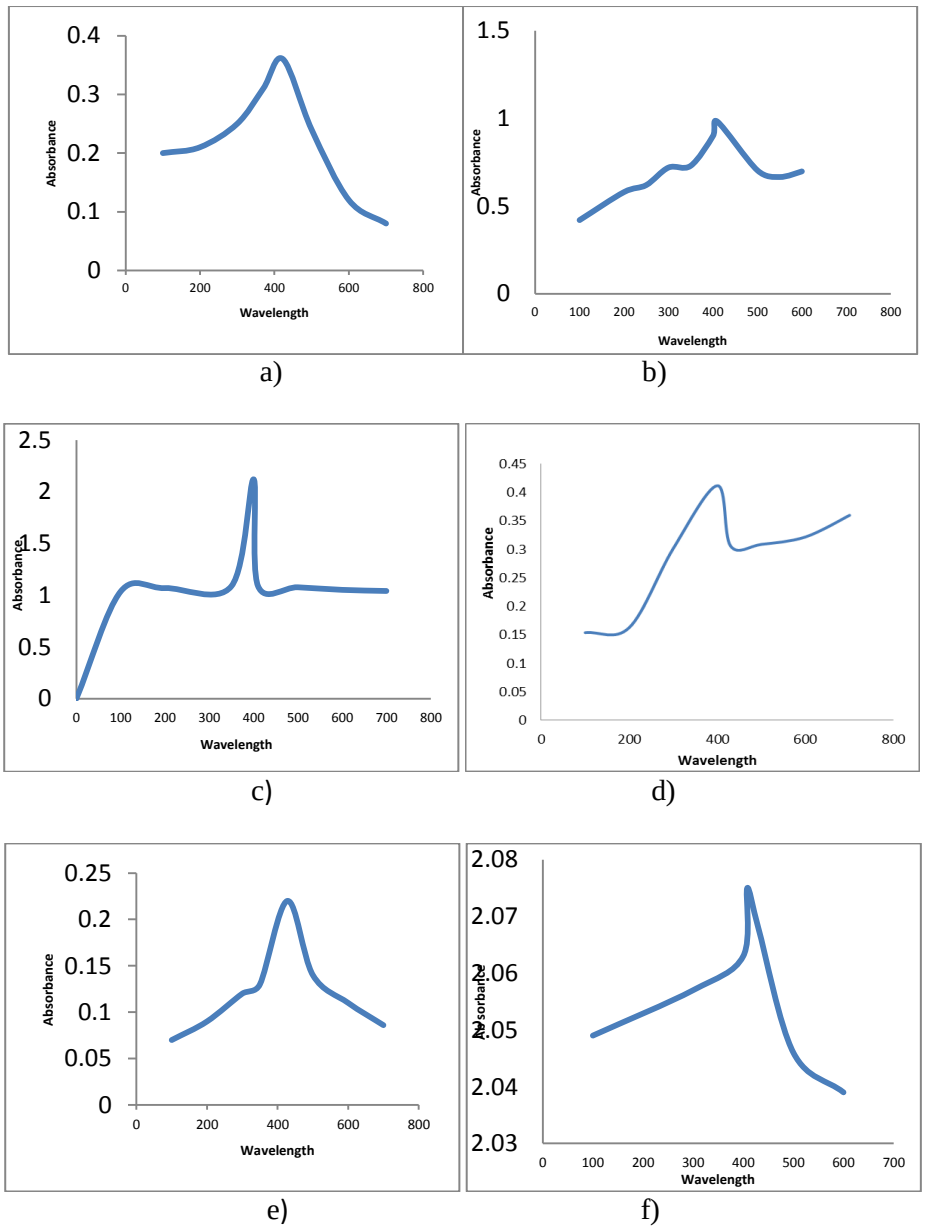

Fig. 2. UV-Vis absorption spectra of silver nanoparticles biosynthesized by: A) Ulva fasciata B) Cystoseira myrica C) Gelidium crinale D) Laurencia obtuse E) Turbinaria turbinata F) Corallina elongate.

4.2. Antitumor activity of AgNPs biosynthesized by different marine algae

The effect of Different concentrations of AgNPs synthesized by different marine algae, *Ulva fasciata , Cystoseira myrica, Gelidium crinale, Laurencia obtusa, Turbinaria turbinata and Corallina elongate* were investigated on Ehrlich Ascites Carcinoma (EAC) cell line viability as shown in (Tab. 1). In general, results showed a decrease in EAC cells viability by increasing AgNPs concentration in all studied algae .The different concentrations (42,56, 69,85 and 98 µg/ml) of green synthesized AgNPs inhibited the proliferation of EAC cells. The maximum percentage of inhibition of EAC cells were (94 and 99%) with 98 µg/ml AgNPs synthesized by *Ulva fasciata* and *Turbinaria turbinata* respectively.

Table 1. percentage of In vitro cytotoxic effect of different concentrations of AgNPs biosynthesized by the collected marine algae on the viability of (EAC).

Conc. of AgNPs µg/ml	**Percentage of cytotoxic activity against EAC %**					
	Turbinaria turbinata	*Ulva fasciata*	*Cystoseiramyrica*	*Gelidiumcrinale*	*Laurenciaobtusa*	*Corallina elongate*
42	67	62	53	46	55	41
56	77	69	60	54	62	48
69	85	79	69	61	71	57
85	92	87	75	70	78	66
98	99	94	83	81	86	74

In vitro results showed that silver nanoparticle biosynthesized by *Turbinaria turbinata* had the most cytotoxic activity than other studied marine algae, so we are going to discuss their characterization.

4.3. Characterization of AgNPs biosynthesized by Turbinaria turbinata

4.3.1 Transmission Electron Microscope

The results obtained from TEM micrograph recorded from the silver nanoparticles deposited on carbon coated copper TEM grid was shown in (Fig. 3). Silver nanoparticles micrograph showed spherical shaped, well distributed in solution and in a range of 8-16nm in size. Silver nanoparticles biosynthesized by *Ulva lactuca* showed low density dispersion and are in the range of 20-56 nm in size [10]. Characterization of silver nanoparticles by TEM has been reported by Sondi [14].

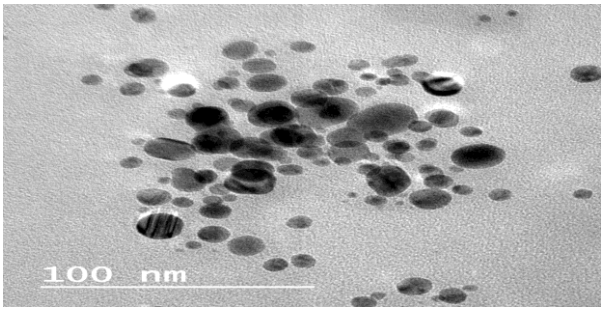

Fig. 3. Transmission Electron Microscopic image of silver nanoparticles biosynthesized b y Turbinaria turbinata.

4.3.2. Scanning Electron Microscope

The results obtained with scanning electron microscopy (SEM) showed that the silver nanoparticles have a spherical shape, low density dispersion , well distributed on the algal aqueous extract surface and were in the range varying between 10-16nm (Fig. 4).

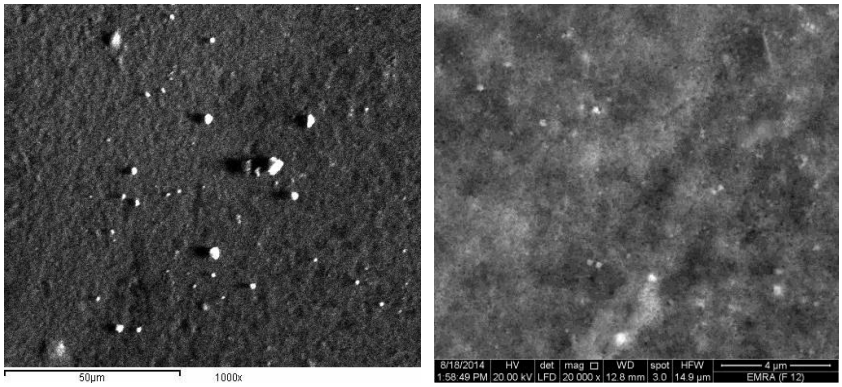

Fig. 4. Scanning Electron Microscopic images of silver nanoparticles biosynthesized by Turbinaria turbinata.

4.3.3. X-ray diffraction analysis

(Fig. 5) showed the X-ray diffraction analysis for silver nanoparticles synthesis using *Turbinaria turbinata*. The diffracted intensities from 5° to 80° at 2θ theta angles were 26.092°, 32.098°, 37.233° and 46.2° which can be indexed to the lattice planes (56.87 %), (100%), (42.4%) and (30.65 %) respectively, which confirmed the face-centred cubic structure of the formed AgNPs by aqueous extract of brown alga *Turbinaria turbinata* and indicated that the particles were crystalline. The obtained results illustrated that AgNPs formed in the solution had been oxidized to Ag_2O nanoparticles by air, during preparation of AgNPs sample for x-ray analysis. These peaks corroborated with the standard Ag_2O nanoparticles [15]. Number of weak unassigned Bragg reflections could be seen due to few perecent of salts (as chloride) in brown-marine algae or by other biomolecules as reducting agents in *Turbinaria turbinata*.

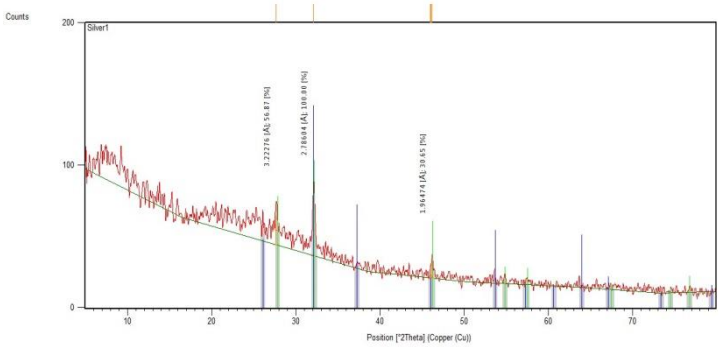

Fig. 5. X-ray diffraction analysis of silver nanoparticles biosynthesized by Turbinaria turbinata.

4.3.4. Energy dispersive x-ray spectroscopy analysis

Energy dispersive x-ray spectroscopy analysis was used to verify the presence of silver in the suspension. The vertical axis represents the number of counts of x-ray while the horizontal axis displays energy. The presented spectrum could clearly showed four peaks located between 1 kV and 4 kV. Silver nanoparticles showed typical absorption peak with 3 kV **(**Fig. 6). Quantitative analysis proved high silver contents (65.38%) in the sample synthesized by *Turbinaria turbinata*. Other elements were shown such as Si, Cl and Ca with percentages of 15.63, 15.27 and 4.14 % respectively (Tab. 2), may be found because of some salt or protein residue in marine algae.

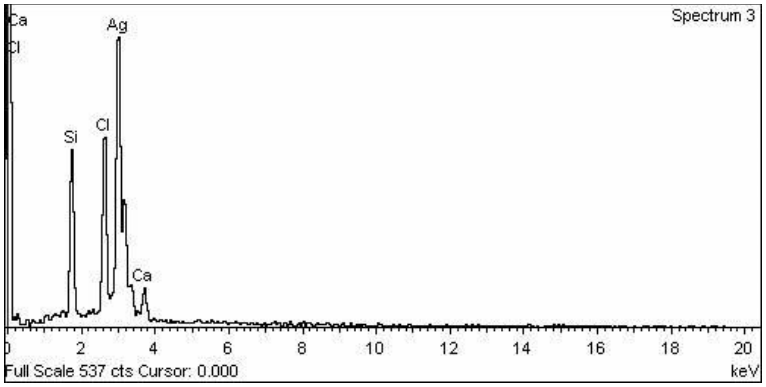

Fig. 6. Energy-dispersive X-ray analysis of AgNPs biosynthesized by Turbinaria turbinata.

Table 2. Energy-dispersive X-ray spectroscopic result showed percentage of elements in resulting suspension

Elements	Weight%
Si	15.41
Cl	15.27
Ca	4.14
Ag	65.38

4.3.5. Fourier transform infrared spectroscopy

Fourier transform infrared measurements were carried out to identify the active groups in biomolecules responsible for the stabilization and coating of the newly biosynthesized silver nanoparticles. The FTIR spectrum of silver nanoparticles are shown in (Fig. 7) which recorded from the powdered sample. The absorption peaks at 1631, 1383 cm^{-1} showed the presence of NO_2 which may be from $AgNO_3$. C-H stretching mode peaks represented at 2921, 2854 and 2315 cm^{-1}. The peak at 1631 cm^{-1} represented C=O stretching peak. The peak at 3432 cm^{-1} was assigned to OH stretching. These results may be due to phenols compound. El-Kheshen [16], reported that a prominent and very sharp peak is observed at 1384 cm^{-1} which was concluded to be due to the nitrate ions. Molecules containing NO_2 group, such as nitro compounds, nitrates and nitro amines commonly exhibited asymmetric and symmetric stretching vibrations of the NO_2 group at 1660 to 1500 and 1390 to 1260 cm^{-1} regions [17].

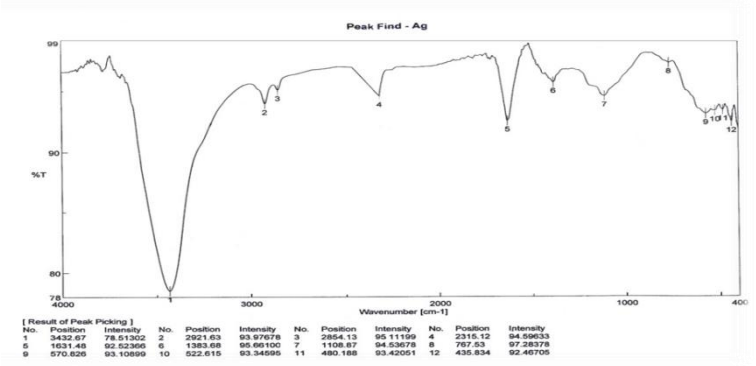

Fig. 7. Fourier transform infrared spectroscopic data of AgNPs biosynthesized by Turbinaria turbinata.

5. Discussion

Synthesis of metal and semiconductor nanoparticles through biological route offers a few advantages over the common chemical and physical procedures as it is an easy, fast and eco-friendly alternative that doesn't involve any costly instruments and hazardous chemicals as well. Marine algae contain several biologically active molecules which are used as source of food, feed and medicine (as anticancer agents). Until now, more than 2400 marine natural products have been isolated from seaweeds [18].

The present study was presented to evaluate the synthesis of AgNPs by marine macro-algae and their cytotoxic potential against EAC cell line. The cytotoxic effects of AgNPs , probably due to the fact that AgNPs may interfere with the proper functioning of cellular proteins and induce subsequent changes in cellular chemistry [19].

The organic compounds in the marine algae are responsible for reduction of silver ions into nanoparticles [20]. After exposure of the aqueous extract of seaweed to silver nitrate solution (keeping the whole reaction in a dark place to avoid excitation energy) color changed to red, thus is an indication of the synthesis of silver nanoparticles [1].

UV spectrum showed that the peaks at 400-450 nm indicated the synthesis of AgNPs. At the beginning of the reaction the band recorded low wavelength and the reaction was carried out hasty. After 48hrs of the reaction the band was at high wavelength due to aggregation of nanoparticles forming large size of nanoparticles that needed less energy and hence longer wavelength due to poly dispersion of the nanoparticles [18]. So the reaction rate is directly proportional to reaction time till 48hrs of synthesis because after 48hrs, the activity of AgNPs in the solution were stable for a period of 2 months [21].

The result showed that AgNPs biosynthesized by *Turbinaria turbinata* had the most cytotoxic activity over that formed by other tested marine algae, so we discussed its characterization deeply as followed:

The morphology of AgNPs formed by *Turbinaria turbinata* using TEM and SEM showed spherical crystalline shaped and it is known the electronical and optic properties of metal nanoparticles is depended on their physical shaped reflecting their antitumor activity [22]. Also TEM and SEM showed that the size range is 8-16nm.The size has a relative advantages since particles less than 100nm range have the capability of accumulation in tumor cells, so the cytotoxic activity of silver nanoparticles is directly proportional to their size [10].

EDX spectra results showed absorption peak at the range of 3 to 4 keV which confirmed the presence of silver nanoparticles [23]. High content of silver metal in the sample (65.38%) indicated that the *in vitro* antitumor activity was due to silver nanoparticles . Weak peaks represented Si, Cl and Ca are likely due to X ray emission from proteins\ sugars present in the seaweeds.

Fourier transform infrared spectroscopy results showed C=O group from the amino acids and proteins which was the main group in biosynthesis of metal nanoparticles by binding and capping of AgNPs to reduce agglomeration of nanoparticles after biosynthesis and to increase stabilization of the medium [24].

The cytotoxic of biosynthesized AgNPs were confirmed by *in vitro* study so in the near future we will study the anti-proliferation activity of different concentrations of silver nanoparticles biosynthesized by marine algae *in vivo*, and the toxicity of AgNPs should be evaluated.

6. Conclusions

In the present study silver nanoparticles were biosynthesized by marine macro algae, *Ulva fasciata, Corallina elongate, Gelidium crinale, Laurencia obtusa, Cystoseira myrica, and Turbinaria turbinata*. silver nanoparticles biosynthesized by *Turbinaria turbinata* were the most cytotoxic against EAC. Silver nanoparticles formed biologically by *Turbinaria turbinata* characterized by a uniform shaped and very small in size and these properties making these AgNPs having marked cytotoxic activity.

Acknowledgments

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