

**ULVA FASCIATA NANOPARTICLES CHARACTERIZATION AND ITS ANTICANCER ACTIVITY**Abirami R. G.<sup>1\*</sup> and Kowsalya S.<sup>2</sup>Department of Food Science and Nutrition, Avinashilingam University for Women,  
Coimbatore- 641043, Tamil Nadu, India.Article Received on  
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Author****Abirami R. G.**Department of Food  
Science and Nutrition,  
Avinashilingam  
University for Women  
Coimbatore- 641043,  
Tamil Nadu, India.**ABSTRACT**

Seaweeds are non-flowering marine macro algae which are grown in wide spectra. The edible green seaweed *Ulva fasciata* shows potent therapeutic value and it is used as nanoparticle agent in cancer therapy. Synthesis of biological nanoparticles is physiologically toxic free with more effective in the treatment of cancer. Hence *Ulva fasciata* was developed as nanoparticles to treat against DAL cancer cell line in albino mice. *Ulva fasciata* nanoparticles were developed in low cost and most potential method and studied for its characterization like size, morphology and active compounds using SEM, FTIR and AFM. After confirmation of nanoparticles were exploring for its anticancer activity against cancer cells, The characterization study showed that the developed nanoparticles are spherical in shape with polydispersed and

few clusters were seen in the SEM micrograph. AFM image shows the particles were in the range of 50 to 190nm. FT-IR image depicts majority of compounds were phenols and amines. Those nanoparticles significantly increased the life span of cancer treated mice, decrease the cancer cell count and reverse the liver functional enzymes level thus it's proved an efficient anticancer activity against cancer cells. Thus it shows potent anticancer activity when compared to its extract and the techniques used here was less expensive, low-energy way to make stable and all-natural nanoparticles.

**KEYWORDS:** *Ulva fasciata*, Nanoparticles, Characterization, SEM, AFM, FTIR.**INTRODUCTION**

A green chemistry synthetic route has been used for both silver and gold nanoparticles synthesis. Among the nanoparticles biological organism, some microorganisms such as

bacteria, algae, fungi, and yeast have been exploited for nanoparticles synthesis. Several plant biomass or plant extracts have been successfully used for extracellular biosynthesis of silver and gold nanoparticles. At present, nutrient nanoparticles are made using high-energy machines that break food material into tiny particles, which are then stabilized with surfactants. But costly machines and high energy demands make this process quite expensive. A less expensive, low-energy way to make stable, all-natural nanoparticles, it would be a big benefit to the food industry, but it's very difficult to accomplish with all-natural ingredients.<sup>[1]</sup> All natural food-grade nanoparticles with different sizes, electrical properties, chemical compositions and digestibility for different applications in the food and beverage industries. Many companies utilizing nanotechnology in nutritional sciences, has marketed a new product called nanoceuticals which is a colloid (or emulsion) of particles of less than 5 nm in diameter. These products will scavenge free radicals, increase hydration and balance the body's pH. They developed nanoclusters, a nanosize powder combined with nutritional supplements. When consumed, it enhances the absorption of nutrients. Nanoceutical Delivery System (NDS) of dietary supplements, resulting in increased-bioavailability compared with gastrointestinal absorption.<sup>[2]</sup> A number of chemical companies are researching additives which are easily absorbed by the body and can increase product shelf life. Biodelivery Sciences International have developed nanocochleates, which are 50 nm coiled nanoparticles and can be used to deliver nutrients such as vitamins, lycopene, and omega fatty acids more efficiently to cells, without affecting the colour or taste of food. All these new developments will make the concept a reality and these are expected to offer many different potential benefits including drug delivery, cancer therapy, improved cognitive functions and antiaging benefits.<sup>[3]</sup>

Among the marine sources, the macroalgae (seaweeds) occupy a significant place as a source of biomedical compounds. The compounds derived from macroalgae are reported to have a broad range of biological activities such as antibacterial, anticoagulant, anticancer and antifouling activity. Seaweeds have been used since ancient times and have an exclusive place in traditional medicine of maritime nation as aesthetics, and antibiotics in the treatment of cough, wounds, gout, goiter, hypertension, venereal diseases, cancer and a variety of other sickness.<sup>[4]</sup> Hence the green algae *Ulva fasciata*- edible seaweed consumed as a food in oriental countries and the same is under exploited in India, which was consider as a nutraceutical dense marine plant acts as a nanoceutical in the cancer therapy. The developed nanoparticle was studied for its characters by using Fourier Transform Infrared Spectroscopy

(FTIR), Scanning Electron Microscope and Atomic Force Microscopy (AFM). Based on the results it was evaluated for anticancer activity on albino mice. Most of the studies in marine seaweeds projecting as a excellent source of developing biosynthesis of metal nanoparticles like gold and silver etc., this kind of nanoceutical work in seaweed was scarce., hence the need for the study.

## MATERIALS AND METHODS

### Preparation of Extract

*Ulva fasciata* was freshly collected from Gulf of Mannar region, cleaned, washed thoroughly with tap water and then with distilled water and dried under shade for further analysis. Powdered *Ulva fasciata* (100g) were dipped into 20 volumes of distilled water and kept at room temperature for 2 hrs, then homogenized and refluxed at 100<sup>0</sup>C for two hours. After cooling, the resulting material was centrifuged at 10,000 g for 15 min. The supernatant was collected and centrifuged again at 8000rpm for 15min to obtain a clarified mixture. The pooled extract was filtered using Whatmann no.1 filter paper, the filtrate was further filtrated through 0.6µm sized filters.

### Development and characterization study

#### Development of Nanoparticles from *Ulva fasciata*

Ultrasonication is a highly system-specific dispersion procedure, involving a variety of concomitant complex physicochemical interactions that can result in cluster breakdown or further agglomeration, as well as other effects including chemical reactions.<sup>[13]</sup> The sample was redissolved in ethanol with a designed concentration and the solution was sonicated by probe sonication technique 10 to 30 minutes at maximum amplitude level (61.0µm). Finally; the solvent mixture was totally removed under vaccum. The sonicated mixture was dried in a freeze dryer and stored at 4°C.

#### Characterization of developed nanoparticles

The nanoparticles of *Ulva fasciata* thus prepared were characterized by using the following techniques.

#### Scanning Electron Microscopy

The sample surface topography, composition and other properties such as electrical conductivity were studied in the SEM. The extract was finely dried into powder form by using spray dryer. The powdered sample was carefully mounted on an aluminium stub using

double stick carbon tape. Samples were then introduced into the chamber of the sputter cortex and coated with a very thin film of gold palladium before SEM (Cambridge Instruments - Stereoscan 360) examination, to know the structure of developed nanoparticles.

#### **Atomic Force Microscopy**

AFM provides a 3D profile of the surface on a nanoscale, by measuring forces between a sharp probe (<10nm) and surface at very short distance (0 to 10nm probe sample separation). The AFM consists of cantilever with a sharp tip at its end that is used to scan the specimen surface. Typically the deflection was measured using a laser spot reflected from the top surface of the cantilever into an array of photodiodes. A small volume of sample was extend on fine cleaned glass cover slip surface mounted on the AFM stub and was dried with nitrogen flow at room temperature. Images were obtained in tapping mode using silicon probe cantilever of 125  $\mu\text{m}$  length, resonance frequency 209 - 286kHz, spring constant 20 - 80 $\text{nm}^{-1}$  minimum of five images for each sample were obtained with AFM and analysed to ensure reproductive results.

#### **Fourier Transform Infrared Spectroscopy (FT-IR)**

FTIR has made dispersive infrared spectrometers all but obsolete (except sometimes in the near infrared). The functional groups present in the developed seaweed nanoparticles were analysed using FTIR Nicolet Avatar 660 (Nicolet, USA) samples were lyophilized, gently mixed with 300mg of KBr powder and compressed into disc at a force of 10KN for 2min using a manual tablet presser. To obtain good signal they were taken in the range of 400 to 4000 $\text{cm}^{-1}$  and the resolution was kept as 4 $\text{cm}^{-1}$ .

#### **Anticancer activity of *Ulva fasciata* nanoparticles**

##### **Selection of Animals and inoculation of cancer**

Healthy adult male Swiss albino mice, weighing 20-25g. They were fed a standard diet and water *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC). Dalton's Ascites Lymphoma (DAL) cells were supplied by Amala Cancer Research Centre, Trissur - Kerala, India. Ascitic fluid was strained from cancer-bearing mice at the log phase (days 7-8 of cancer bearing) of the cancer cells. Each animal received 0.2ml of cancer cell suspension containing  $1 \times 10^6$  cancer cells (i.p).

**Grouping of animals and parameters for analysis**

All the animals in three groups were injected with DAL cells ( $1 \times 10^6$  cells/mouse) intraperitoneally, and the remaining one group from the normal control group.

Group I- normal control.

Group II-cancer control. (Group I & II- standard diet and water)

Group III- Standard drug (5- flurouracil at 20 mg/kg body weight)

Group IV- *Ulva fasciata* nanoparticle (100mg/kg of body weight)

seaweed nanoparticle was dissolved in DMSO which was used as a dosage regimen. The compound treatment was given after the 24 hrs of inoculation, once daily for 14 days. 24hours of last dose and 18 hour of fasting, all animals in each group were sacrificed and the blood was withdrawn by retro orbital plexus method. The following parameters were analysed. Physical characteristics like body weight, Life span (%), Cancer Cell Count, haematological parameters like WBC count, RBC count, Hb content, Platelet count (pentra-120 Automated Haematology Analyzer) and liver functional enzymes like AST, ALP, ALT (Modified IFCC/UV kinetic method) were assessed.

**Percentage increase in life span (ILS)**

The mortality was recorded by monitoring the mice on cancer growth and percentage increase in life span (ILS %) was calculated by the following formulae.

$$\%ILS = \frac{\text{Life span of treated group}}{\text{Life span of control group}} - 1 \times 100$$

**Assessment of Cancer Cell Count**

The fluid (0.1ml) from the peritoneal cavity of each mouse was withdrawn and diluted with 0.8 ml of ice cold ordinary saline or sterilized Phosphate Buffer Solution(PBS) and 0.1 ml of trypan blue (0.1mg/ml) and total number of the living cells were counted using haemocytometer (improved Neubauer chamber).

Total number of cells per ml = average no of cells  $\times$  dilution factor  $2 \times 10^4$

**Statistical Analysis**

The results were interpreted and articulated as mean  $\pm$  S.E.M. All the parameters were subjected to statistical analysis by one way Analysis of Variance to determine the significant variation between the groups. Derived parameters were analyzed by Graphpad Prism

software. All Pairwise Multiple Comparison Procedures by Student-Newman-Keuls Method at significant difference  $p < 0.05$ .

## RESULTS AND DISCUSSION

### Scanning Electron Microscopy (SEM) analysis

The SEM image (Figure 1) showing the developed nanoparticles of *Ulva fasciata* confirmed the nanostructures. The SEM micrographs of nanoparticle obtained in the filtrate showed that they are spherical and triangle shape randomly dispersed as few clusters of the nanoparticles were observed on the preferential region of the surface. The size of the particles was small and was found to be in range of 100nm to 200nm. From the Figure 1, it can be seen that particles are uniformly distributed over the surface. According to Rajesh *et al.*<sup>[5]</sup> biosynthesis of silver nanoparticles using *Ulva fasciata* showed that bionanoparticle were crystalline in nature, spherical in shape and poly-dispersed with size ranging from 28 to 41 nm. This results coincide with the results of present study.

### Atomic Force Microscopy (AFM) analysis

The morphology of the developed nanoparticles was characterized by AFM. Figures 2 shows the AFM image of *Ulva fasciata* nanoparticle prepared by ultrasonication method. The samples of AFM was prepared in ethanol and then deposited on sheet of mica. The particles were spherical in shape and they had smooth surfaces. The diameter of the particles was about  $190 \pm 5$  nm. From figure 2 the results shows two and three dimensional view of sample surface over a  $2 \times 2 \mu\text{m}$  scan, it was observed that majority of the particle size fell in the range of 49 to 190 nm. A gradual increase of the sonication time gradually decreases the particle size with interesting morphology and increased surface area which is similar to the results of present study.<sup>[6]</sup> Therefore, from the figure 2, it is confirmed that particle size is in nanometer range. From the study, it is evident that by adopting the standardized technique, it is feasible to prepare the nanoparticles to target the cancer cells.

### Analysis of biochemical nature of developed particles by FT-IR

FTIR analysis was used for the characterization of the extract and the resulting nanoparticles. The strong broad band seen at 3617, 2060, 1650 and  $1400\text{cm}^{-1}$  were assigned to the stretching vibrations of primary and secondary amines, respectively (Figure 3). From the spectrum, it is evident that peaks such as hydroxyl, amino and C-H groups obtained near  $3600\text{-}3000\text{cm}^{-1}$  OH group of the monomeric hydrogen bond with corresponding phenol rings. C=C ring stretching was observed at  $2060\text{ cm}^{-1}$  and the spectra showed sharp and strong

absorption band at  $1,650\text{ cm}^{-1}$  assigned to the stretching vibration of (NH) C=O group. These peaks occurred in  $3409$ ,  $2388$  and  $1652\text{ cm}^{-1}$  assigned due to photochemical such as alginic acid, flavanoids, tannins, gallic acid and other phenols.<sup>[7,8]</sup> These soluble elements could have acted as reduction and stabilizing agents preventing the aggregation of nanoparticles in solution.



Figure 1 SEM image of *Ulva fasciata* nanoparticles

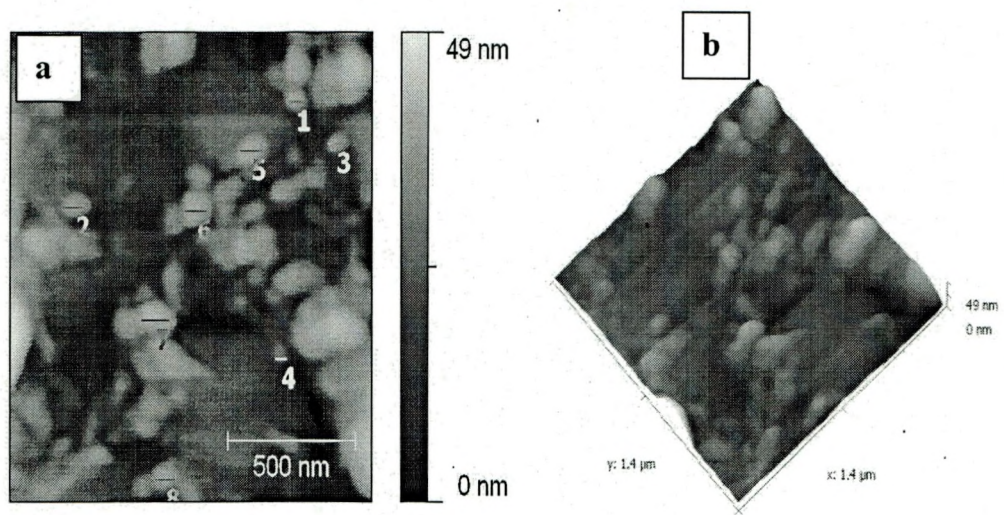


Figure 2 AFM image of *Ulva fasciata* nanoparticles a) 2D image b) 3D image

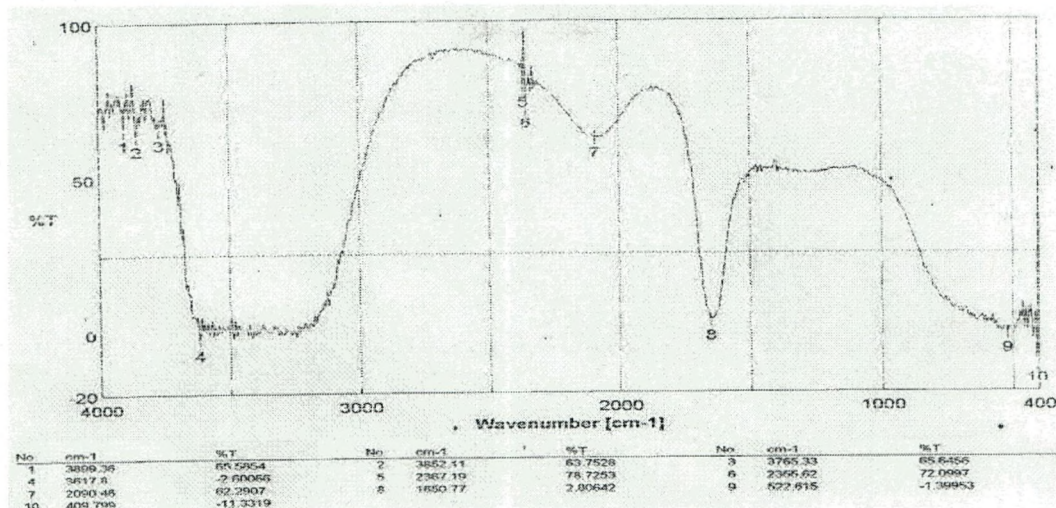


Figure 3 FTIR spectra of *Ulva fasciata* nanoparticles

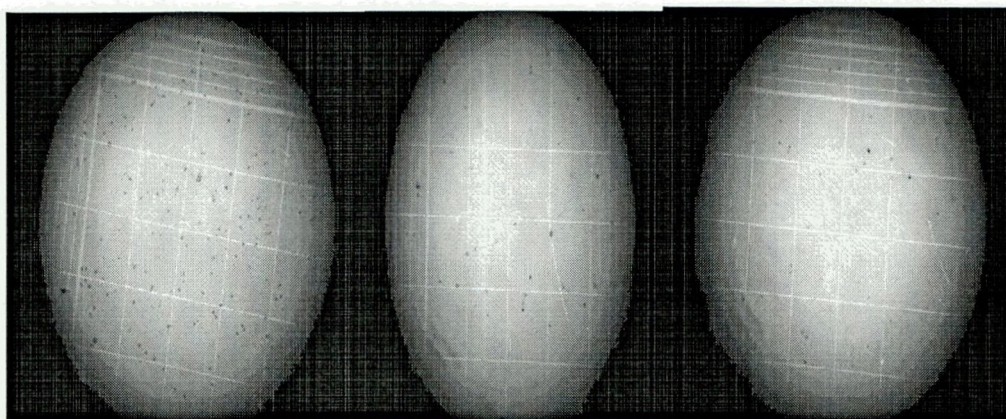


Figure 4a

Figure 4b

Figure 4c

4a. Cancer control 4b. positive control 4c. *Ulva fasciata* nanoparticle

Table 1 Life span, Body weight and Cancer Cell Count of treated groups

Treatment	Number of animals	% ILS life span	Increase in body weight (g)	Cancer cell count ml x 10 <sup>6</sup>
Group I	6	>>30 days	1.30±0.009	-
Group II	6	46%	8.60±0.18 <sup>a</sup>	2.38±2.02 <sup>b</sup>
Group III	6	92%	1.83±1.03 <sup>b</sup>	1.01±1.03 <sup>b</sup>
Group IV	6	74%	7.82±0.94 <sup>b</sup>	1.36±1.82 <sup>b</sup>

Group I – Normal Control, Group II – Cancer Control, Group III – Standard, Group VI– seaweed nanoparticle

All values are expressed as mean ± SEM for 6 rats in each group.

a – Values are significantly different from control (G<sub>1</sub>) at p<0.01

b – Values are significantly different from cancer control (G<sub>2</sub>) p< 0.05

Table 2 Hematological parameters of treated groups

TREATMENT	WBC ( $\times 10^3$ cells/mm <sup>3</sup> )	RBC ( $\times 10^6$ cells/mm <sup>3</sup> )	Hb mg/dl	Platelets Lakhs/cumm
Group I	5.23 $\pm$ 2.07	7.62 $\pm$ 0.03	12.32 $\pm$ 0.07	2.57 $\pm$ 2.24
Group II	8.29 $\pm$ 3.17 <sup>a</sup>	4.06 $\pm$ 0.09 <sup>a</sup>	5.67 $\pm$ 2.06 <sup>a</sup>	1.85 $\pm$ 1.24 <sup>a</sup>
Group III	5.68 $\pm$ 1.14 <sup>b</sup>	6.86 $\pm$ 0.04 <sup>b</sup>	10.23 $\pm$ 1.08 <sup>b</sup>	2.41 $\pm$ 1.13 <sup>b</sup>
Group IV	6.21 $\pm$ 2.18 <sup>b</sup>	5.98 $\pm$ 0.08 <sup>b</sup>	9.38 $\pm$ 0.03 <sup>b</sup>	2.32 $\pm$ 1.24 <sup>b</sup>

Group I – Normal Control, Group II – Cancer Control, Group III – Standard,

Group VI – seaweed nanoparticle

All values are expressed as mean  $\pm$  SEM for 6 rats in each group.

a – Values are significantly different from control (Group I) at  $p < 0.01$

b – Values are significantly different from cancer control (Group II)  $p < 0.05$

Table 3 Enzyme levels of treated groups

Treatment	AST (U/L)	ALT (U/L)	ALP (U/L)
Group I	40.46 $\pm$ 2.50	28.49 $\pm$ 0.09	30.42 $\pm$ 1.80
Group II	85.65 $\pm$ 1.14 <sup>a</sup>	55.81 $\pm$ 0.38 <sup>a</sup>	78.23 $\pm$ 0.61 <sup>a</sup>
Group III	42.58 $\pm$ 2.13 <sup>b</sup>	38.28 $\pm$ 0.45 <sup>b</sup>	40.12 $\pm$ 0.50 <sup>b</sup>
Group IV	42.62 $\pm$ 1.33 <sup>b</sup>	43.25 $\pm$ 1.08 <sup>b</sup>	40.46 $\pm$ 1.28 <sup>b</sup>

Group I – Normal Control, Group II – Cancer Control, Group III – Standard,

Group VI – seaweed nanoparticle

All values are expressed as mean  $\pm$  SEM for 6 rats in each group.

a – Values are significantly different from control (G<sub>1</sub>) at  $p < 0.01$

b – Values are significantly different from cancer control (G<sub>2</sub>)  $p < 0.05$

### Effect on Cancer

The methanolic-, aqueous- extract of *Ulva fasciata* showed potent anticancer activity was evaluated in our previous research.<sup>[9]</sup> The growth of cancer cell was scrutinized and presented in terms of % increased life span (ILS), increase in body weight and cell count of *Ulva fasciata* nanoparticle in Table 1. Group II (cancer bearing mice), the mean life span of mice was found to be 46 to 92 %. Group IV (*Ulva fasciata* nanoparticles) increase in the life span about 74%. However, the average life span of Group III (5-FU-Std drug) showed 92%, indicating its potent anticancer nature. From Figure 4a,b,c, the cell count of the treated groups showed significant reduction in the cancer live cells and increase the dead cell count. The *Ulva fasciata* nanoparticle treated rats also showed similar activity when compared to the crude extracts in our previous research these changes were statistically proved. Figure 5a,b,c shows the cell count image in neubauer chamber.

The apoptosis-inducing effect of fucoxanthin on human promyelocytic leukemia HL-60 cell line has been investigated by Hosokawa *et al.*<sup>[23]</sup> who found that fucoxanthin exhibited strong antiproliferative activity and could induce apoptosis of HL-60 cells. A similar study reported that the sulphated polysaccharide from green seaweed was markedly activating macrophages and inhibit the growth of lung cancer and melanoma in mice, which was coinciding with the present report.

#### **Effect on hematological Parameters**

Haematological profile was presented in the Table 2. It shows the RBC, Hb, platelet count was decreased and WBC count significantly increased in the Group II (DAL control group) as compared to the normal control group. This elevated level of WBC clearly indicates the leukocyte against the cancer cell. The efficacy of *Ulva fasciata* nanoparticle significantly increased the Hb content, RBC, platelets and decreased the WBC count to about normal level. All these All these examinations suggest the effective anticancer nature.. However, the standard drug 5-FU shows better result in physical and haematological parameters which was well known since it is existing drug. The activity of nanoparticle tends to uphold the haematological profile to the normal level. . Reverse the above parameters is mainly due to the contribution of mineral content like iron, zinc etc of the seaweeds and high bioavailability in cancer treated mice.

Accordingly, to Dong.<sup>[12]</sup> results indicate that *Ulva lactuca* may be useful as a natural antitumor and immunostimulating agent. Using the water-soluble fraction of a methanol extract from *Ulva lactuca*, a concentration of 140 g/mL was found to inhibit 50% of the human leukemia cell line U937 in growth, In addition, NO production by a macrophage cell line (RAW 264.7) and alkaline phosphatase activity in mouse splenocytes were both stimulated with 10 µg/mL of WSM. Dose-dependent patterns were observed on all three cell-lines this result correlated with the present study.

#### **Effect on liver function**

From the table 3, it was apparent that the inoculation of DAL cells caused elevated levels of total aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) in the cancer control animals (group II), when compared to the normal group mice (Group I). Group IV inverted these changes near to the normal. These changes were statistically significant when compared to the cancer control (group II). Thus the seaweed possesses secondary metabolites i.e bioactive compounds like chlorogenic acid and

gallic acid in *Ulva fasciata* plays major role in reaction against enzyme level. The management with standard drug 5- FU also gave similar results shown in the table 3. Research studies conducted on cancer in human or experimental animals will affect and alter the function of liver that will evident the enzyme level. Drastically elevated liver function enzymes of cancer inoculated mice indicated liver damage and defeat of efficient veracity of cell membranes.

### CONCLUSION

In this study nanotechnology Technique, developing a potentially nanoceutical *Ulva fasciata* as a more optimistic and potential to change the existing system of developing nanopowder in the therapeutic agent. The developed nanoparticles actively participate in the drug delivery system against the cancer cell line. They are also promising of enhancing the nutritional quality and improve absorption of active compounds in the beleaguered cell. Thus the nanoclusters, nanosize powder combined with nutritional supplements enter in to the transmucosal administration of nutritional fortification and enrichment, resulting in increased-bioavailability compared with gastrointestinal absorption was evidently examined in this study. All natural food-grade nanoparticles with diverse magnitude, size, electrical properties, chemical compositions and digestibility of dissimilar application in cancer therapy, those tiny particles give nutrients more stability and a longer projection time in the drug delivery system.

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### CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

### REFERENCES

1. David Julian McClements, at the University of Massachusetts Amherst. <http://phys.org/wire-news/96962786/food-scientist-studying-the-use-of-all-natural-nanoparticles-in.html>. 2012.

2. Food Standards Agency. A review of potential implications of nanotechnologies for regulations and risk assessment in relation to food. United Kingdom. 2006. 90
3. USDA (2003) Nanoscale Science and Engineering for Agriculture and Food Systems
4. Lipton, AP; Jose, J: Anticoagulant and immune enhancing activities of marine macroalgae explored. Spectrum - ICAR News., 2006; 12(4): 8-6.
5. Rajesh S, Patric Raja T, Rathi J M and Sagaya Raj K. Biosynthesis silver nanoparticles using *ulva fasciata* using ethyl acetate extract and its activity against *Zanthomonas campestriensis*, J.Biopest., 2012; (5):118-128
6. Majumdar, Sanhita; Devi, P. Sujatha. Synthesis of SnO<sub>2</sub> Nanoparticles Using Ultrasonication. International Conference on Advanced Nanomaterials and Nanotechnology (ICANN-2009). AIP Conference Proceedings., 2010; 1276: 1-7
7. Abirami R. G and Kowsalya S. Phytochemical screening, microbial load and antimicrobial activity of underexploited seaweeds. International Research Journal of Microbiology., 2012; 3(10): 328-332.
8. Nagarajan and Arumugam Kuppasamy: Extracellular synthesis of zinc oxide nanoparticle using seaweeds of gulf of Mannar, India. Journal of Nanobiotechnology., 2013; 11: 39.
9. Abirami RG and Kowsalya .S. Anticancer activity of methanolic and aqueous extract of *ulva fasciata* in albino mice Int J Pharm Pharm Sci., 2012; 4(2): 681-684.
10. Raj Kapoor B, Jayakar B, Muruges N. Antitumor activity of *Indigofera aspalathoides* on Ehrlich Ascites carcinoma in mice. Indian J Pharmacol., 2004; 36: 38-40.
11. Koyanagi, S., N. Tanigawa, H. Nakagawa, et al. Over sulfation of fucoidan enhances its antiangiogenic and antitumor activities. Biochem Pharmacol., 2003; 65: 173-179.
12. Dong Geun Lee, Jin-Won Hyun, Kyong Ah Kang, Jin Ok Lee, Sang Hyun Lee, Bae Jin Ha, Jong-Myung Ha, Eun Yeol Lee, *Ulya lactuca*: A potential seaweed for tumor treatment and immune stimulation Biotechnology and Bioprocess Engineering., 2004; 9(3): 236-238.
13. Taurozzi, J.S., V.A. Hackley, and M.R. Wiesner. On the Application of Ultrasonic Energy to Prepare Nanoparticle Dispersions for Environmental, Health and Safety Assessment. In Preparation. 2010.