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SCREENING OF NATURAL PIGMENTS FROM SEAWEED *KAPPAPHYCUS ALVAREZII* AND ITS EFFICACY IN PHARMACEUTICAL PRODUCTS

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ABSTRACT: This paper focused on research on seaweed contains good nutritional source of protein, carbohydrate, pigments, vitamins, and antioxidants and has low lipid content. The source of natural substances has considered more efficient bio-molecules in building immunity and improving resistance to cancer development, prevent infections were analyzed. Classical biochemical methods were applied for extraction of pigments and other related components from red algae; subsequently, quantities of each substance were determined and discussed its potential properties. Considerable attention has been focused on pigments-based biomaterials attributes antioxidant, antitumor, and immune-modulating properties due to their multi-functional qualities. The fresh solvent extracts of *K. alvarezii* was found to be rich in total anthocyanin 17 mg/g.f.wt content and vitamin C 21 mg/g.f.wt. Furthermore, the efficient combination treatment with anthocyanin plus vitamin C resulted in better therapeutic efficacy than carboplatin drug against Dalton's lymphoma tumor. The tumor cell growth was arrested due to the administration of anthocyanin plus vitamin C **(**500µg/kg body wt. i.p) to tumor-bearing mice; thereby the efficiency of biomolecules was confirmed. The western blot analysis explained GST expression appears to have antitumor potential to treat ascites Dalton's lymphoma in the animal model. *Kappaphycus alvarezii* is considered a source of unique natural products applied in pharmaceutical formulations and makes them suitable biomaterials for drug delivery, and its anti-tumor properties might help develop functional pharmaceutical products in the future.

INTRODUCTION: Marine polysaccharides are biopolymers extracted from sea organisms. Red and brown algae are considered seaweeds; the source of carbohydrates are alginate, agar, and carrageenan, extracted from selected genera and species of brown (Phaeophyceae) and red (Rhodophyceae) seaweeds [1].

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The global value of marine hydrocolloids is expected to increase their applications in various industries because they are cheap, natural, environmentally friendly, biocompatible, non-toxic, and versatile in properties.

Algae are small, unicellular or multicellular, autotrophic, colorful and generally grow in water and they may be either eukaryotic or prokaryotic. The pigment content in algae is a specific feature of each species [2]. Pigments from natural sources are gaining more importance mainly due to health and environmental issues. Production of pigments from algae has several advantages such as being cheaper

and easy to culture and employed simple extraction method, for higher yields also frequently available raw materials can be used and no seasonal variations [3]. The status of algal applications in aquaculture, food, special chemicals, and environmental applications has been reviewed [4].

Marine resources are today a renewable source of various compounds such as pigments poly-saccharides are used in the pharmaceutical, medical, cosmetic and food fields. *Kappaphycus alvarezii*, seaweeds are cultivated for commercial preparation phycocolloids such as agar, algin, and carrageenan, besides food, food additives, fodder, and biofertilizers, source of enzymes, pigments, nutraceuticals, drugs, antibiotics, *etc*. [5]. Commonly used synthetic pigments act as allergens, irritants and also express the carcinogenic impact on human beings [6].

Natural pigments are sustainable sources of colorants than synthetic materials. Chlorophyll as a food colorant is found to exhibit anti-mutagenic properties. Red pigment phycoerythrin is extracted from red algae (Rhodophyta). β -carotene pigment is used mainly as a food colorant that imparts a yellow-orange color, also popularly known as a nutraceutical additive, because of rich in vitamin A. Synthetic pigments are used in food, cosmetics, beverages, nutraceutical, and pharmaceutical industries. Since synthetic pigments have harmful effects to overcome, natural pigments become an attractive option in the application of food industries. Biomaterials from *Kappaphycus alvarezii* have also revealed a range of bioactive applications that have been studied, including their role as antioxidant, antiviral, antibacterial, antihyperlipidemic, anti-coagulant, antitumor, and immunomodulatory agents, enabling them to be used in the biomedical field as potential pharmaceutical formulations for the treatment of various diseases. The objective of the present study has to standardize the extraction of pigments from marine red algae *Kappaphycus alvarezii* by using classical biochemical techniques to determine the quantity of pigments and analyze their efficacy in pharmaceutical applications.

MATERIALS AND METHODS:

Collection of Seaweed Samples: *Kappaphycus alvarezii* collected from the Mandapam region in Ramanathapuram District Tamil Nadu in a living sample. The obtained seaweed has to be cleaned completely before using for the experiment. The seaweed was washed under running tap water to eliminate unwanted foreign particles. The collected sample identified by a scientist at Central Salt and Marine Chemicals Research Institute (CSMCRI), Mandapam and voucher specimen KA/UCP/02/2013 was used. The samples were maintained in the department for all research experiments.

Extracts of Pigments Samples: The sample fresh red algae (*K. alvarezii*) were weighed (5g) then washed using flowing water. The algae were cut by scissors and minced by using a blender, resulting in algae pulp. The pulps were extracted using two solvents of Acetone and methanol by the method described as [7].

Pigments from samples were extracted in organic solvents, and it was kept for 48 h at room temperature and mixed at regular intervals. After 48 h the sample homogenized in solvent was filtered using Whatman filter paper No. 3 to prepare crude filtrate for further experiments.

Estimation of Pigments: Total chlorophyll was estimated according to the method of Arnon [8] Carotenoid content was determined by [9] modified methods; the carotenoid content was calculated by the formula as follows: 7.6 [E480-1.49 E510], and quantitative analysis on the amount β-carotene was performed according to the modified procedure [10]. The extraction of anthocyanins was performed according to Liang *et al*. with some modification [11] 20 mL methanol with 5% (v/v) formic acid was added into 100 mL Erlenmeyer flasks that contained 1 g of *K. alvarezii* fresh sample. Anthocyanins were extracted at 30°C for 30 min in a dark environment also were repeated five times for collection of required volumes of the extract solution, then concentrated under vacuum at 30°C using a rotary evaporator until dryness.

The dry extraction was resolved in 2% formic acid in distilled water about 1 mL of extracted solution was strained through a 0.45 μm millipore filter for further experimental analysis. Total anthocyanins were determined by measuring the A_{530} and A_{657} of the aqueous phase using a spectrophotometer (DU-

530, Beckman). By subtracting the A_{657} from the A_{530}, the relative amount of anthocyanin was calculated [12]. Vitamin C was analyzed by the dichlorophenol indophenols method by Chinoy and Singh [13]. The antioxidant activity of the extracts was estimated using ß-carotene was performed according to the modified procedure [14].

Microwave Extraction of Phycoerythrin was estimated by Sharif *et al.* 2014 [15].

Experimental design: Animal experiments were carried out following the guidelines of the animal ethics committee of the Institute. For each experimental group, 4-5 Inbred Swiss Albino mice aged 10-12 weeks (25-30g) were maintained in the Laboratory. Dalton's Lymphoma ascites were obtained from Amala Cancer Institute, Trisshur, Kerala. Ascites Dalton's lymphoma tumor was induced *in-vivo* by intraperitoneal (i.p) transplantation of $1X10^6$ tumor cells per animal (0.25 volumes, in phosphate-buffered saline, PBS). PBS was prepared by adding 0.15 M NaCl to 0.01 M sodium phosphate buffer, pH 7.4. Since tumor-transplanted animals usually survived for 18-20 days and were allowed to grow with tumors. Experimental animals were divided into three treatment groups involving 60 mice of 4 batches containing 15 mice each and injected as follows:

Batch 1: Animals received normal saline and were used as control (saline 10ml /kg of body wt.) intraperitoneal (i.p) injection (n=5).

Batch 2: Animals were induced tumor with Dalton's lymphoma ascites and allowed to grow with tumor without treatment the batch 2 served as normal.

Batch 3: Animals received a single dose of drug carboplatin (500µg/kg body wt. i.p) was administrated to tumor-bearing mice on the 8th day of tumor growth (maintained as experimental group 1) (Preparation of doses: Carboplatin dose (10 mg /ml was dissolved in 5% dextrose in water and use immediately).

Batch 4: Animals who received a combination dose of Anthocyanin vitamin C (500µg/kg body wt.i.p) were administered to tumor-bearing mice as single-dose administration and maintained as experimental group 2.

Analysis after Treatment: The treatment was started from the 2nd day after the tumor inoculation and continued for 16 days; body weight of animals was noted daily in all groups during the treatment period. After treatment, animals were kept to check the survival time of DLA bearing mice, subsequently, animals were sacrificed at specific time intervals such as 5th, 11th and 16th days for the experimental design by anesthesia and organs tissue were isolated (liver, blood, ascites tumor) frozen in liquid nitrogen and stored at - 80° C until biochemical analysis could be completed. The parameters such as survival time, packed cell volume, body weight, and hematological parameters like RBC count and WBC count were studied during the experiment period. According to the dose and treatment schedule used, a single dose was administered to tumor-bearing mice who were killed by cervical dislocation, and liver, blood, ascites tumors were collected. Bone marrow cells were prepared from humerus and femur by flushing in PBS with a hypodermic syringe and by centrifugation (3000 g, 10 min) to collect. Ascites tumor centrifuged (3000 g, 10 min) to separate Dalton's lymphoma cell pellet, and the ascites supernatant of treated tissues was collected and used for further experiment Superoxide anion scavenging activity.

Enzyme Assays: SOD activity was determined at room temperature according to the method of [16]. The assay of Glutathione peroxides was determined by the method of [17]. Glutathione reductase (GR) activity was determined by the method of [18]. Glutathione was estimated in tissues by the method of [19]. GST activity was measured in the supernatant fractions according to the method of [20].

Western Blotting: Cells were harvested in ice-cold Ham's medium and washed with ice-cold phosphate-buffered saline (PBS). The cell pellets were lysed in Proteo JET Mammalian Cell lysis reagentcrude [21] extracts (40µg protein per lane) were analyzed by 15% SDS-PAGE [22]. Proteins were transferred electrophoretically to nitrocellulose filters (for 3 h at1A) using an immunoblot transfer apparatus. After transfer, the nitrocellulose was incubated for 1 h at room temperature in 3% (w/v) BSA in Tris-buffered saline (TBS; 500mM NaCl and 20mM Tris-HCl pH 7.5) to block nonspecific binding. The blot was

incubated overnight at 4°C with 3% (w/v) BSA in TBS containing antiserum at a dilution of 1:500. After three 15 min washes with TBS containing 0.1% BSA and 0.2% Nonidet P40, the blot was incubated for 1 h at room temperature with peroxidase-conjugated goat anti (mouse immunoglobulin) diluted at 1:1000 in 3% BSA in TBS. The blot was again washed three times with TBS containing 0.1% BSA and 0.2% Nonidet P40. Antibodies were visualized using a chem.-luminescence detection system.

RESULTS AND DISCUSSION: *Kappaphycus alvarezii* is tough fleshy marine red algae (seaweed), it may be loosely attached to broken coral or floating, sometimes in large, moving mats. Typically occurs in water 3 to 50 feet deep, the largest tropical red algae with a high growth rate (can double in biomass in 15 - 30 days). Seaweeds are autotrophic organisms capable of producing many compounds of interest **Fig. 1.**

FIG. 1: FRESH MARINE RED ALGAE *KAPPAPHYCUS ALVAREZII*

For a long time, seaweeds have been seen as a great nutritional resource, it has been reported that edible seaweeds are rich in carbohydrates, proteins, dietary fibers and low lipids contents. Moreover, have plenty of bioactive molecules that can be applied in nutraceutical, pharmaceutical and cosmetic areas. Therefore, measurement of bioactive substances has many applications in food and medical field. Present work focuses to prepare bio-molecules from fresh *K. alvarezii* using classical biochemical techniques and its products revealed antioxidants properties. In the study, seaweed based natural products are quantitatively determined in extracted samples by using spectro-photometry. The estimated amounts revealed maximum anthocyanin17 mg/g.f.wt and vitamin C 21 mg/g.f.wt were reported in **Fig. 2.** Based on the analysis anthocyanin and vitamin C has observed the most abundant among different biomolecules in the nutritional profile. The quantity of different biomolecules can vary due to difference in species, location, seasonal temperature, and age of harvest [23]. The seaweed is relatively low cost; these renewable resources have aroused great interest in the pharmaceutical, biomedical, cosmetic, and food industries. *Kappaphycus alvarezii* was used as a whole-food supplement to attenuate the development of rats fed a high-carbohydrate, high-fat diet that mimics symptoms of human metabolic syndrome, including central obesity, hypertension, dyslipidemia, and impaired glucose tolerance, coupled with the cardiovascular and liver complications of metabolic syndrome [24].

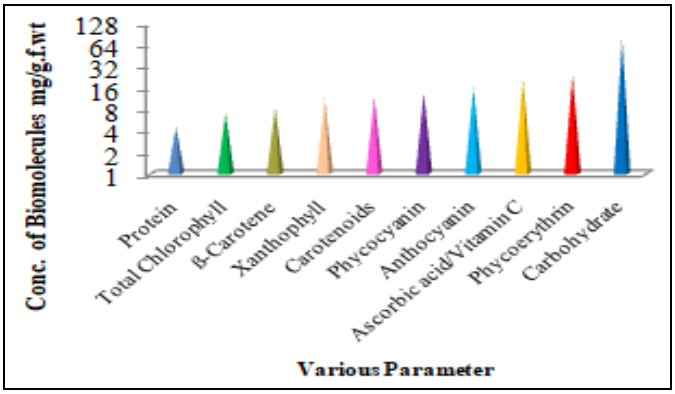

FIG. 2: ESTIMATION OF BIOMOLECULES IN K. ALVAREZII EXTRACTS SHOWED PREPARATION OF NATURAL BIO-MOLECULES THROUGH CLASSICAL BIOCHEMICAL TECHNIQUES TO GET A HIGH YIELD. X-INDICATES THE VARIOUS PARAMETERS OF EXTRACTED COMPOUNDS; Y-INDICATES QUANTITY OF RESPECTIVE NUTRIENTS FOUND IN THE SEAWEED EXTRACTS OF FRESH SAMPLES. RESULTS WERE REPRESENTED FIVE REPLICATED EXPERIMENTS

Fig. 3 Results revealed natural substances has remarkable ability to treat tumor conditions might be confirmed by analysis of parameters such as packed cell volume positively correlated with changes in bodyweight of animal which reflected survival of the animals. Haematological studies showed an increased WBC and altered state of HB levels along with RBC.

To investigate the efficiency of bioactive component administration to animals were induced tumor with Dalton's lymphoma ascites (details given methods). The impact of treatment confirmed

that the Anthocyanin plus vitamin C treated group was more effective in exhibiting the antitumor activity against DLA cells. The body weight differences after treatment were calculated by observing the weight gain on 7^{h} day after cancer induction. Whereas the tumor inhibition was calculated by estimating the packed cell volume of DLA, treated groups showed better tumor inhibition in the presence of biomolecules by arresting proliferation and inducing apoptosis of tumor cells. Animal survival was calculated and expressed as a Percentage increase in life span. Body weights of tumor animals were determined individually during treatment, and the differences were calculated from three replicates of each group. A combination of anthocyanin and vitamin C was found to both inhibit the growth of ascites tumor transformed cells and reduce tumor size. The animal survival was calculated from the formula

$$T - C / C \times 100$$

Where, T= Number of days treated animal survived=Number of days control animal survived. The mortality of animals was noted and represented in terms of the percentage increase in life span (%ILS). Body weights of animals were given in **Fig. 3** which had explained body wt. increased (g) and the body wt. difference represents in % denoted life span of the animal.

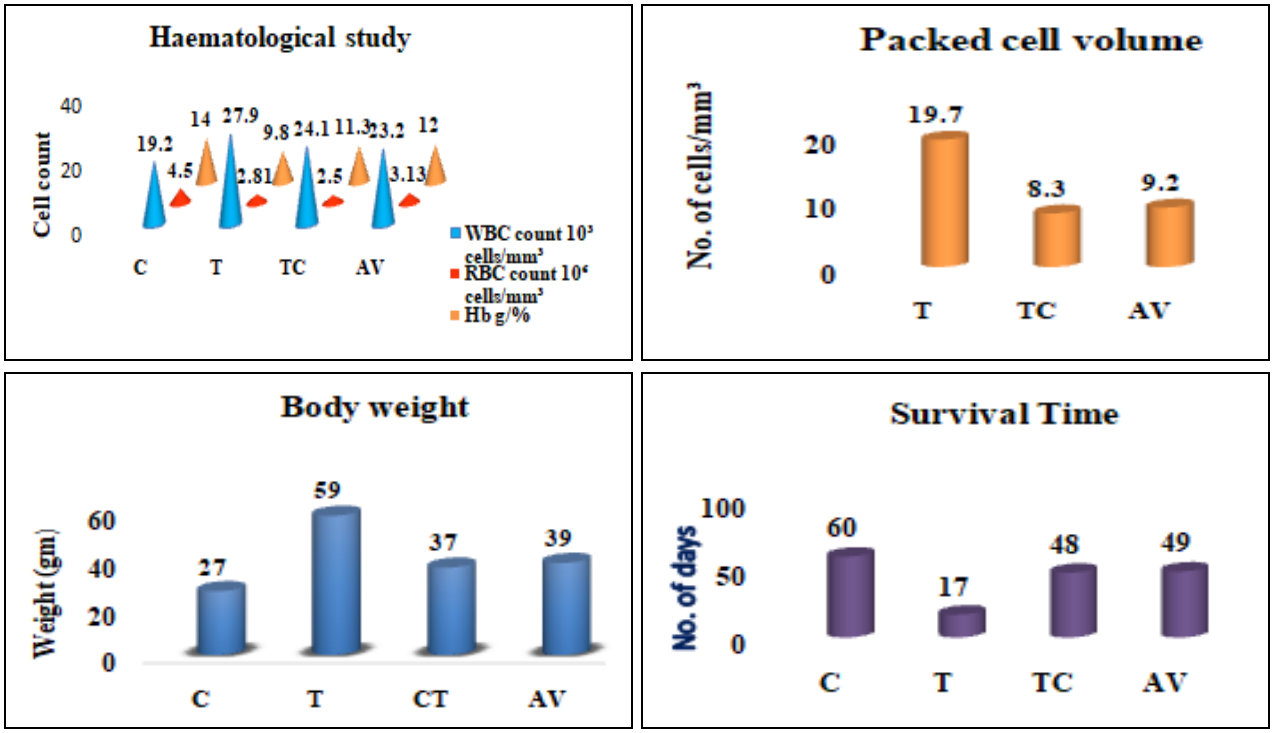

FIG. 3: ANALYSIS OF TUMOR INHIBITION. X-AXIS REPRESENTED TREATMENT GROUPS IN WHICH C-INDICATES CONTROL (NON TUMOR MICE); T-INDICATES TUMOR MICE (TUMOR INDUCED, UNTREATED); TC-INDICATES TUMOR-INDUCED MICE TREATED WITH CARBOPLATIN DOSE (10 mg /ml IP INJECTIONS; AV- INDICATES TUMOR-INDUCED MICE TREATED WITH COMBINATION DOSE OF ANTHOCYANIN PLUS VITAMIN C(500µG/KG BODY WT, I. P) EACH VALUE REPRESENTS THE MEAN N=5

Bioactive material delivered to cancer cells is directly responsible for tumor suppression at a low dose of treatment. An endogenous antioxidant enzyme such as superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase, and glutathione-S-transferase (GST) played a significant role in exerting defense against free radicals, which is generated in Dalton's lymphoma induced tumor in mice model **Fig. 4.** Cancer cells generate large amounts of hydrogen peroxide, which may contribute to their ability to mutate and damage normal tissues and facilitate tumor growth and invasion. It has been suggested that persistent oxidative stress in tumor cells could partly explain some important characteristics of cancer, such as activated proto-oncogenes, genomic instability, drug resistance, invasion and metastasis, and the resistance of many cells against oxidative stress is often associated with high intracellular levels of GSH. DLA cells exhibit a higher oxidative stress

level than normal cells, rendering tumor cells more vulnerable to raising ROS levels. Glutathione-S-transferase activity may induce a general protection state, leading to inhibition in cancer initiation in the liver and other sites such as ovarian tissue. Therefore, animal studies found it interesting to determine antioxidant scavenging enzyme activities in various tissues during ascites Dalton's lymphoma growth. Furthermore, there was a reduction of intracellular glutathione (GSH) concentration, and glutathione S-transferase (GST) activity have been reported in **Fig. 4.** GSH and GST are believed to play important roles in drug detoxification. Notwithstanding the antioxidant defense system of the cell to neutralize oxidative injury from ROS, radical linked damage of protein and DNA has been suggested to play a significant role in the inhibition or further development of tumors. It has been proved that marine algae contain unique bioactive metabolites which have tremendous antitumor potential.

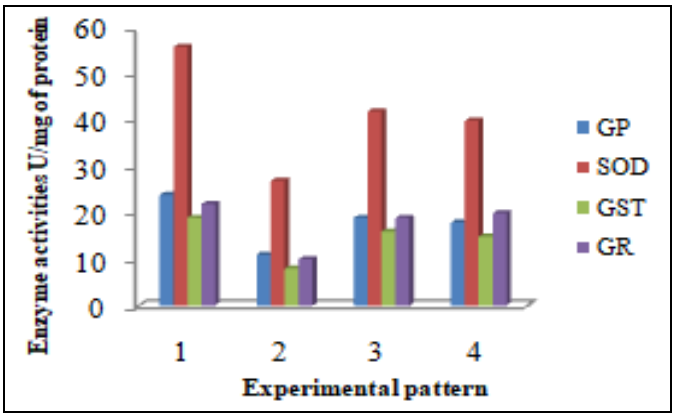

FIG. 4: ANALYSIS OF FREE RADICALS SCAVENGING ENZYMES. X-AXIS REPRESENTED TREATMENT GROUPS IN WHICH 1- INDICATES CONTROL (NON TUMOR MICE); 2-INDICATES TUMOR MICE (TUMOR INDUCED, UNTREATED); 3-INDICATES TUMOR-INDUCED MICE TREATED WITH CARBOPLATIN DOSE (10 MG /ML IP INJECTIONS; 4- INDICATES TUMOR-INDUCED MICE TREATED WITH COMBINATION DOSE OF ANTHOCYANIN PLUS VITAMIN C (500µg/kg BODY WT, I.P) EACH VALUE REPRESENTS THE MEAN N=5

There was a specific induction in GST expression, which paralleled the increase in total GST activity. GST expression on western blot analysis results in **Fig. 5** expressed pharmaceutical efficiency as inhibiting tumor cell growth by cell arrest and leading to cell apoptosis in the administration of anthocyanin plus vitamin C extracted from *Kappaphycus alvarezii*. GST enzyme remarkable expressed equal to the drug, which reflects on arresting cell proliferation ultimately confirms its effect to suppress cancer cell development, which might help to exert its antitumor activity of anthocyanin and vitamin C (500µg/kg body wt,i.p) by a decrease in expression of TNF receptor.

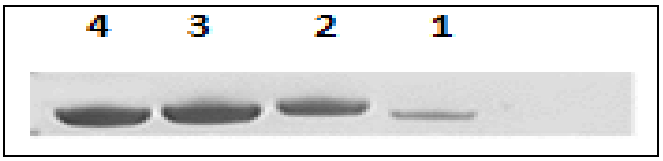

FIG. 5: GST EXPRESSION ON WESTERN BLOT ANALYSIS. THE EXPERIMENTAL TISSUE ANALYZED BY WESTERN BLOT TECHNIQUES SHOWED THE EXPRESSION OF GST. LANE 1: GST EXPRESSION IN ANIMALS INDUCED TUMOR WITH DALTON'S LYMPHOMA ASCITES [BATCH2]. LANE 2: GST EXPRESSION IN CONTROL [BATCH1]. LANE 3: INCREASED GST EXPRESSION IN TUMOR-INDUCED TREATED WITH CARBOPLATIN. LANE 4: GST EXPRESSION IN TUMOR INDUCED TREATED WITH COMBINATION DOSE OF ANTHOCYANIN PLUS VITAMIN C (500µg/kg BODY WT, I.P).

Seaweeds also contain high quantities of vitamins, protective pigments, minerals, and trace elements that are essential for the human diet and may collaborate with many approved nutritional claims (such as iron, calcium, iodine, or magnesium) relative to bone health, cognitive function, maintenance of normal metabolism, normal growth and muscle function. However, the different therapeutic applications are still in the experimental phase; there are certain limitations in the use of these sulfated polymers in the biomedical field, but the path is outlined, and currently, the development of new research focused on the use of the main components leads to these formulations behave in clinical trials, to verify their effectiveness and real potency.

CONCLUSION: The fresh seaweed extract significantly inhibited ascites Dalton's lymphoma *in-vivo* condition the natural molecules has been shown to exhibit inhibition of cell growth proliferation, thereby confirming antitumor properties. Carboplatin, one of the most effective drugs against cancer, has been approved worldwide to treat cancer patients. Biomolecules extracted from marine algae possess approximately equal impressive anticancer efforts and might support inhibit/arrest further growth of tumorous stage in

the animals also lead to decrease in expression of TNF receptors. The combination of anthocyanin plus vitamin C content is considered an active component responsible for the inhibition of ascites Dalton's lymphoma tumor cell and arrest for further growth. These combinations essentially improved possibilities for the treatment of tumors. This study clearly observed that due to their antioxidant ability, these biomolecules had gained great importance in recent decades in the food industry and pharmaceutical research.

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