PHENOLIC COMPOUNDS, β-CAROTENOIDS TASK IN ANTI-OXIDANT AND APOPTOTIC ACTIVITY OF *CHLORELLA PYRENOIDOSA* FROM WASTEWATER AGAINST HT-29 TUMOR CELLS

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Abstract

Chlorella pyrenoidosa is a freshwater green alga which is generally utilized as a part of wastewater treatment prepare. The present research expects to investigate the cancer prevention agent movement and anticancer capability of the microalgae Chlorella pyrenoidosa developed from the wastewater created tremendously by the instructive foundation. Regular cancer prevention agents, including shades, phenolics, and tocopherols, were measured in methanolic concentrates of microalgae biomass. The consequences of every test were connected to the substance of regular cancer prevention agents in microalgae biomass. Phenolic mixes were found as real patrons to the cell reinforcement action in all cancer prevention agent tests while carotenoids were found to add to the 1,1-diphenyl-2-picryl-hydrazil (DPPH) radical rummaging movement, ferrous lessening power (FRAP), and ABTS-radical searching limit action. Cytotoxicity of the concentrate was controlled by Methyl thiazolyl tetrazolium (MTT) test. The tumor cells experiencing apoptosis were controlled by Hoechst 3324 fluorescence recoloring. The apoptotic cells were splendid white shaded, which speaks to they have experienced chromatin build-up and atomic fracture.

Key words: Microalgae, wastewater, antioxidant, cytotoxicity, apoptotic.

I. INTRODUCTION

Microalgae are photosynthetic organisms that grasp nitrogen (N) and phosphorus (P) during their growth and ends up in biomass production. This generated biomass can be converted into a valuable product through appropriate processing technology [1]. More importantly, a freshwater microalgae bends to adjust the N and P concentration in their biomass in relation to the environment concentration in the water [2, 3]. The main challenges in the current scenario of a Wastewater Treatment Plant (WWTP) are not only to produce reusable clean water, but also to find new technologies and implementation of such technology for supporting the welfare of the humanity [4].

Phenolics are the leading group of phytochemicals possessing the huge role of antioxidant activity in plants or plant products [5]. Nonetheless, customers are demanding a new and cheap alternative natural antioxidants obtained from reliable sources to replace the synthetic antioxidants currently used such as butylhydroxyanisole (BHA), butylhydroxytoluene and tertabutylhydroquinone, which exert toxic and carcinogenic [6].

The most commonly diagnosed cancers among both males and females are the colon rectal cancer [7]. Normal cells are not excused when cancerous cells are targeted by the anticancer drugs. Cytotoxicity and immunotoxicity of the drugs affects not only tumor development, but also aggravates patient's recovery. Discovery of new antitumor drug with low side effects from a cheap substrate will turn out to be an essential goal in pharmacological studies.

Here, we have cultivated the microalgae in the wastewater which is generated enormously by the educational institution daily. The microalgae are tested for its antioxidant scavenging activity, cell cytotoxicity level and the apoptotic ability against HT-29 human adenocarcinoma cell lines.

II. MATERIALS AND METHODS

A. Chemicals and reagents

All chemicals and reagents used in the experiments were of analytical grade and purchased from Merck, India.

B. Wastewater resource

The wastewater used for the study was obtained from the wastewater treatment plant at Sri Sivasubramaniya Nadar College of Engineering, Chennai, Tamilnadu. Prior to the experiments, pretreatment of wastewater is carried out by removing large solid particles by using filter cloth (Kimberly- Clark) and autoclaved at 121°C for 15 min. After that cooled down to room temperature, and then kept undisturbed for settling of dissolved solids at 4°C, After few days the supernatant is taken up for the further experiments.

C. Characteristics of the wastewater

The collected wastewater is used as the culture medium for the growth of microalgae; the nitrogen (N), phosphorus (P), and chemical oxygen demand (COD) were determined using the protocol specified for each test [8].The initial characteristics of the wastewater are observed in Table 1

Table 1 Initial and final characteristics of wastewater.

S.No	Parameter	Before treatment	After treatment	% Reduction
1	pH	7.8	7.1	NA
2	Suspended solids (gL^{-1})	143	21	85.32
3	COD (mgL^{-1})	1460	132	90.96
4	Total nitrogen (mgL^{-1})	572	15	97.38
5	Total phosphorus (mgL^{-1})	12.1	0.2	98.35

D. Micro algae culturing and harvesting

Chlorella pyrenoidosa was cultivated heterotrophically using sterile f/2 medium [9]. The microalgae was cultivated in polythene bags of 200 L capacity, agitated bubbling air (1 $v/m/ml^{-1}$ min^{-1}) under 5000 lux illuminated white fluorescent bulb for 12:12 h light and dark condition for 20 days. Growth was monitored by measuring the optical density at 550 nm. When the culture reached stationary phase, the biomass was harvested by centrifugation at 8500 rpm for 10 min to get thick algal paste. Then the micro algal paste was rinsed with distilled water to remove residual salts and oven dried.

E. Sample preparation

A precisely weighed ~1 g of freeze dried microalgae powder was extracted sequentially with 100 ml of different solvents (water, methanol, acetone and hexane) at room temperature (20°C). Extracts were concentrated (Rotary evaporator, Instruments, Germany).

F. Determination of total phenolic content (TPC)

TPC was assessed by the Folin–Ciocalteau strategy depicted by [10]. To 200 μL of algal concentrates at 1mg/mL were included 1.4 mL refined (distilled) water, 100 mL Folin–Ciocalteau reagent and 300 mL sodium carbonate (20%). After 30 min hatching at room temperature oblivious, absorbance was perused at 760 nm in a microplate reader. TPC was computed in view of the standard bend of gallic acid at a focus scope of (0.005–0.500) mg/mL, and the outcomes are communicated in milligrams (mg) of gallic acid proportionate/g separate.

HPLC analysis

Phenolic acids were analyzed using a Water HPLC system (Perkin Elmer, USA) consisting of a pump and system controller, sample processor and photo-diode array detector. Phenolic acids separation was done by a reversed phase PRP-1. The mobile phase comprised of 3.1% methanol (v/v) in 20 mmol/L K2HPO4 titrated to pH 9.5 with 1 mol/L KOH. The mobile phase was delivered at a rate of 0.7 mL/min, and samples (10 μL) were introduced into the column using an auto sampler. The detection was monitored at 300 nm.

III. DETERMINATION OF ANTIOXIDANT ACTIVITY

A. Total antioxidant activity

Total antioxidant activity of the algal extract was determined by the method [11].. Briefly, 300 μl of sample was mixed with 3.0 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Reaction mixture was incubated at 95°C for 90 min in water bath. Absorbance was measured at 695 nm. All determinations were performed in triplicate. Total

antioxidant activity is expressed as number of equivalents to ascorbic acid.

B. Ferric reducing antioxidant power assay

Ferric reducing efficiency of algal extract was determined by the method [12]. Briefly, 1 ml of extract was mixed with 2.5ml of 0.2M phosphate buffer, pH 6.6 and 2.5ml of 1% potassium ferric cyanide and kept at 50ºC for 20 min in water bath. After incubation period, 2.5 ml of 10% Trichloroacetic acid was added and centrifuged at 650 rpm for 10 min. 2.5 ml of supernatant was mixed with 2.5 ml milli-Q water and 0.5 ml of $FeCl_3$ (0.1%). Absorbance was read at 700 nm. Ferric reducing antioxidant Power is expressed as the number of equivalents of ascorbic acid.

C. DPPH radical scavenging assay

The scavenging effect of algal extract for DPPH radical was determined by the method [13]. Briefly, 2 ml of extracts of different concentration was added to 2 ml of DPPH methanolic solution. The mixture was vortexed for 1 min and kept undisturbed at room temperature for 30 min in the dark environment. The absorbance was measured at 517 nm. The scavenging effect (%) was calculated by using the Eq.1

$$\text{Scavenging activity}\,\% = \left(1 - \frac{OD_{Treated}}{OD_{Control}}\right) \times 100 \quad (1)$$

D. Degradation of β-carotene

Degradation of β-carotene was determined by the method [14] with negligible modifications. 3 ml of emulsion (2.5 mg β-carotene and 40 mg linoleic acid were solubilized in chloroform to 400 mg Tween 40 emulsifier and concentrated using rotary evaporator) was mixed with 1 ml algal extracts and the initial absorbance was read at 470 nm. The samples were incubated at 50 °C for 3 hours and after that, the absorbance was read again at the same wavelength; the two readings were made in a microplate reader. The BHA was used as positive control at the same concentrations of algal extract. The antioxidant activity was calculated according to Eq.2

$$\text{Antioxidant activity}\,\% = \left(\frac{OD_{3\,hours}}{OD_{initial}}\right) \times 100 \quad (2)$$

IV. ANTI TUMOR ACTIVITY

A. Cell lines and culture conditions

HT-29 is human colorectal adenocarcinoma cell lines were chosen as representatives of human adenocarcinoma cell lines for this study. The cells were cultured in 50 ml cell culture flasks and 96 wells cell culture microplate by using McCoy's 5A medium supplemented with 10% FBS and 1% antibiotic and antimycotic solution and were incubated at 37°C in the presence of 5% CO_2 [15].

B. Cell viability assay

The cytotoxic ability of algal extract was assessed by MTT (3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyltetrazolium bromide) assay. The cells ($5x10^3$ cells / well) were plated in 96-wells microplate and were treated with different concentration of algal extract (10, 20, 30, 40 and 50 μg/ml for 24 and 48 hours). Negative control was treated with DMSO. After the treatment, media were removed and 20μl of MTT (5mg/ml) were added to all wells and incubated for 4hrs at 37°C in dark and the formazan crystals were dissolved by adding 100μl of DMSO [16]. The plate was shacked at room temperature, followed by photometric determination at 570 nm. The percentage of cell viability inhibition was calculated using Eq.3

$$\text{Cell viability inhibition} = \left(1 - \frac{OD_{Treated}}{OD_{Control}}\right) \times 100 \quad (3)$$

C. Fluorescence microscopy analysis

Fluorescence microscopy analysis was done to determine the apoptosis in HT29 cells. 2.5 lakh cells / well were seeded into 6 well plates and incubated at 37°C with 5% CO_2.The cells were treated with different concentrations of algal extracts (100-400 μg/ml) for 24 hours and 48 hours. After treatment, cell suspensions alone taken and cells were stained with Hoechst3324 (5μg/ml) and visualized under fluorescence microscope.

V. RESULTS AND DISCUSSION

A. Microalgae growth

Growth profile of microalgae cultivated from the wastewater was elaborated in Fig.1. No lag phase was observed in the microalgae growth curve, suggesting that this freshwater microalga have adapted well in this wastewater. This volumetric biomass yield was moreover higher or similar to that achieved in batch cultivation in precedent studies using municipal, industrial or poultry breeding wastewater [17].

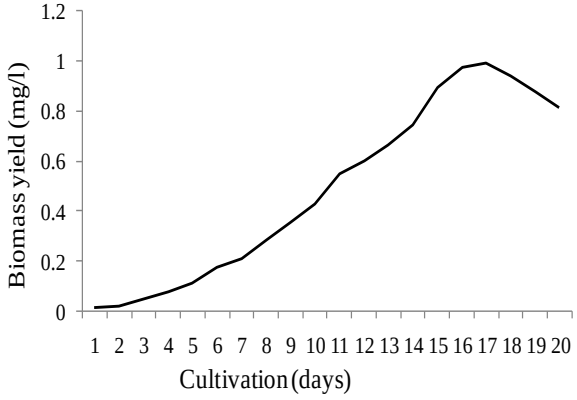

Fig.1 Algal biomass yield profile

B. Characteristics of wastewater after biomass recovery

Microalgae can play an important role in biological processes coupled with bioremediation of wastewaters due to its competence to assimilate organic compounds and nutrients. Table.1. elaborates the characteristics of wastewater after biomass recovery. Wastewater with elevated concentrations of Phosphorus, Nitrogen, COD and TSS when discharged into the water bodies, leads to contamination of water bodies. To prevent this dilemma, our results show that microalgae treatment is economically viable process for treating the municipal waste water effluents prior to dumping into the oceans or rivers. The concentrations of Phosphorus, Nitrogen, COD, and TSS are reduced by about 98.35 %, 97.38 %, 90.96 % and 85.32 % respectively.

C. Total phenolic content

The total phenolic content of different solvent extracts (methanol, acetone, hexane and water) of microalgae *Chlorella pyrenoidosa* was determined and the results are presented in Fig.2. The phenolic content at 500µg/ml concentration was found to be higher in methanol and hexane extract of 1.21 and 0.67 mg/g gallic acid equivalent were observed in *C. pyrenoidosa*. In recent times it was reported that *T. conoides, G. foliifera* and *H. tuna* exerted a similar or higher phenolic content obtained from southcoast of India [18].

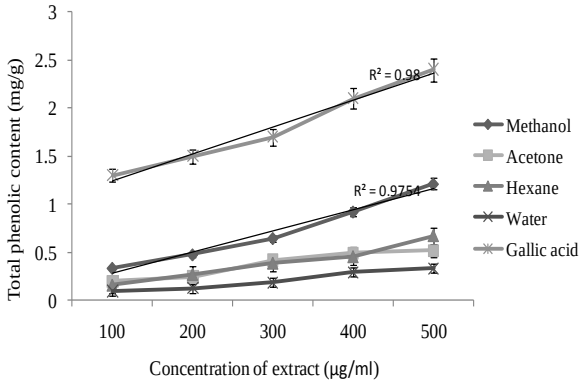

Fig.2 Total phenolic content assay

D. Hplc analysis

Ten major phenolic acids (*p*-hydroxybenzoic, caffeic, syringic, *o*-coumaric, *m*-coumaric, *p*-coumaric, gentisic, ferulic, sinapic, salicylic), were resolved under the chromatographic conditions described (Fig 3A). Chromatographic analysis of the extracted sample of *C. pyrenoidosa* revealed that phenolic acids found in appreciable concentrations include: *p*-hydroxybenzoic, caffeic, ferulic, sinapic. There are other unidentified compounds, which might stem from flavonoids with complex structure. Concentration and composition of the phenolic compounds in microalgae biomass could be affected by both species and growth conditions.

E. Total antioxidant and Ferric reducing antioxidant activity

Total antioxidant activity of algal extract was showed in Fig.4. The highest activity of 1.2 mg/g ascorbic acid equivalent was observed in 500µg concentration of *C. pyrenoidosa*. The ferric reducing activity of the microalgae extract was determined by reducing power assay varied as seen in Fig.5. The reducing power was found to be higher in methanolic extract 500µg concentration of *C. pyrenoidosa* (0.96 mg/g ascorbic acid equivalent). Lately, the ethanolic extract of *Enhalus acoroides* exerted the potential antioxidant and ferric reducing activity [19] which is in and around similar to our findings

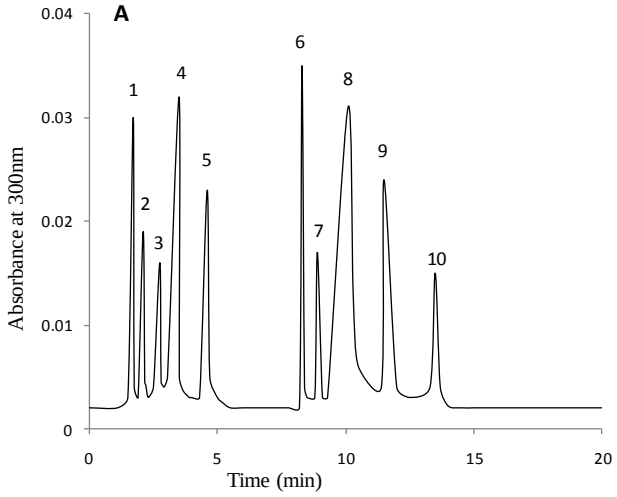

Fig 3A. HPLC chromatogram of phenolic acids standards

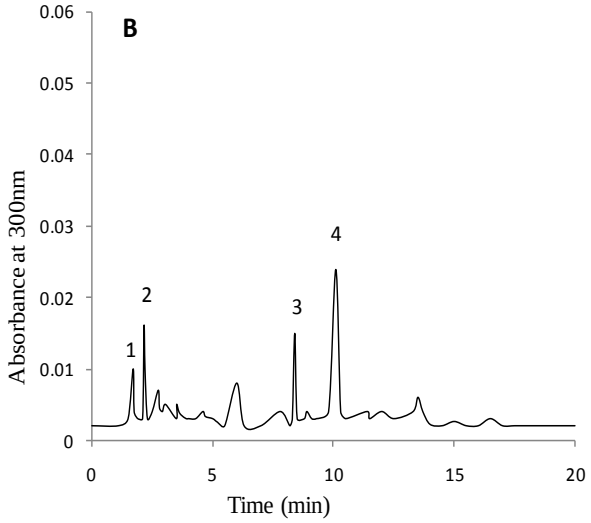

Fig 3B. HPLC chromatograms of phenolic acids in extract

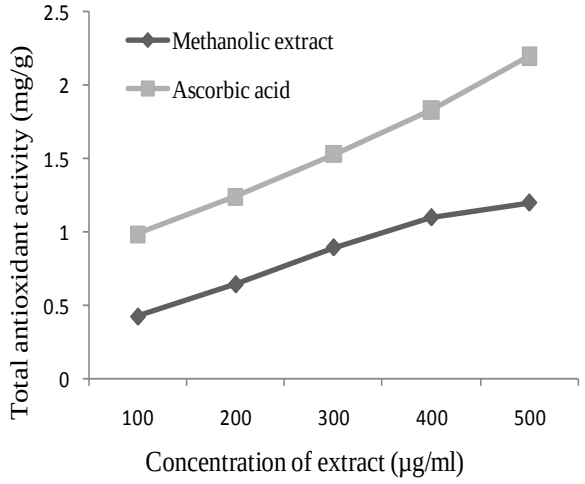

Fig.4 Total antioxidant activity assay

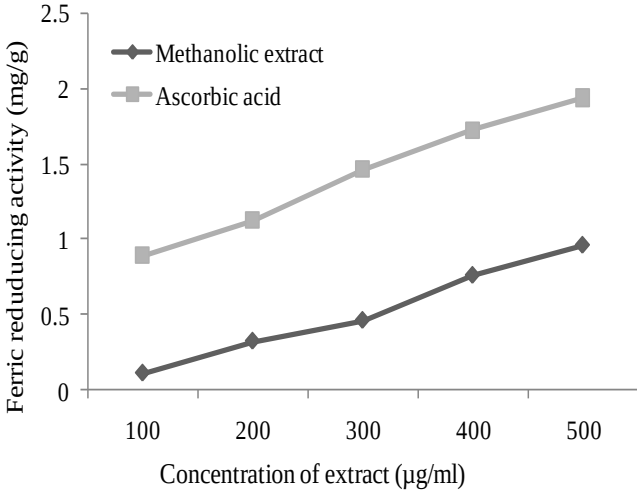

Fig.5 Ferric reducing activity assay

F. DPPH radical scavenging and β-carotene degradation assay

DPPH radical scavenging and β-carotene degradation activity of microalgae is presented in Fig.6. The extract haunted the ability to scavenging DPPH at various degrees; with the 500µg concentration of *C. pyrenoidosa* (47.15%) was bring into being the most potent scavenger. Degradation of β-carotene was found to be higher at 500µg concentration which exerted 58.62% of antioxidant activity than other concentrations. Here, β-carotene degradation activity was significantly compared with BHA (positive control). Recently, the microalgae's *Pterocladiella capillacea* and *Osmundaria obtusiloba* also exerted more or less similar β-carotene degradation activity [20].

G. Cell proliferative assay

Using the MTT assay, we investigated the potential of algal extract to inhibit growth of HT-29 cells. As shown in Fig. 7, exposure of cells to increasing concentrations (100-500µg/ml) of algal extract for 24 h and 48 hours inhibited the viability of HT-29 cell line. Results indicate that the anti-cancer effect strengthens with increase in the concentration of algal extract. Therefore Chlorella pyrenoidosa extract can able to activate the mitochondrial dehydrogenase enzyme of living cells to cleave the tetrazolium rings of the yellow MTT drug. The cytotoxicity

of algae is based on the presence of bioactive cytotoxic compounds like antitumor metabolites. In recent times it was reported that *Ulva lactuca* and *Enteromorpha intestinalis* have shown cytotoxicity at a very low IC50 doses against human lung carcinoma A549 cells, human colon carcinoma LS174 cells [21].

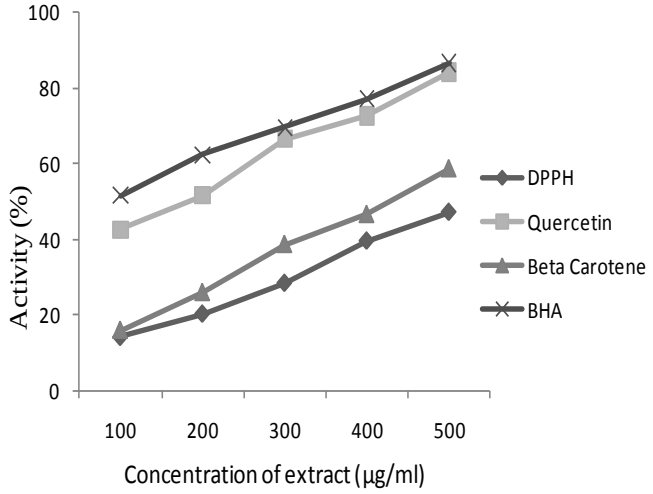

Fig.6 DPPH and β-carotene activity

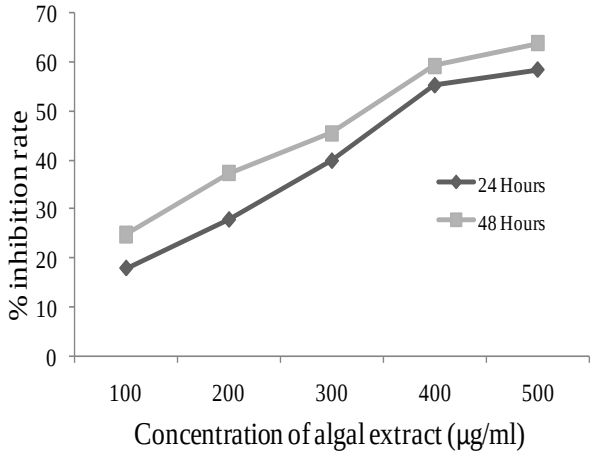

Fig.7 Cell proliferation rate for different concentration of algal extracts (100-500µg/ml) at 24 and 48 hours treatment

H. Fluorescence microscopy analysis:

Apoptosis was detected by the Hoechst 3324 staining method after 48 h of continuous exposure to different concentrations of algal extract followed by fluorescence microscopy. Algal extract induced apoptosis in a concentration and time dependent manner (Fig.8), which suggests that when exposed to algal extract, HT-29 cells underwent the typical morphologic changes of apoptosis. Bright white colored cells represent apoptotic cells exhibiting chromatin condensation and nuclear fragmentation. This suggests an apoptotic pathway for the mechanism of cytotoxicity of the extracts and stress further evaluation for illumination of the potential apoptotic pathway. It was also reported previously that curcumin induces apoptosis through the mitochondria-mediated apoptotic pathway in HT-29 cancer cell lines [22].

VI. CONCLUSION

Microalgae civilized in wastewater under air stripping gained a relevant removal process for micro contaminants. In this study, the algal extract of *C. pyrenoidosa* showed sensible antitumor activity against HT-29 human adenocarcinoma cell lines. The antioxidant activity and radical scavenging activity of *C. pyrenoidosa* also considerably in and around comparable to other microalgae previously reported. Also, in regards to the significant results of this study, evaluation of *in vivo* anticancer activity of *C. pyrenoidosa* extract is recommended and may lead to the finding of a new effective natural antitumor compound(s).

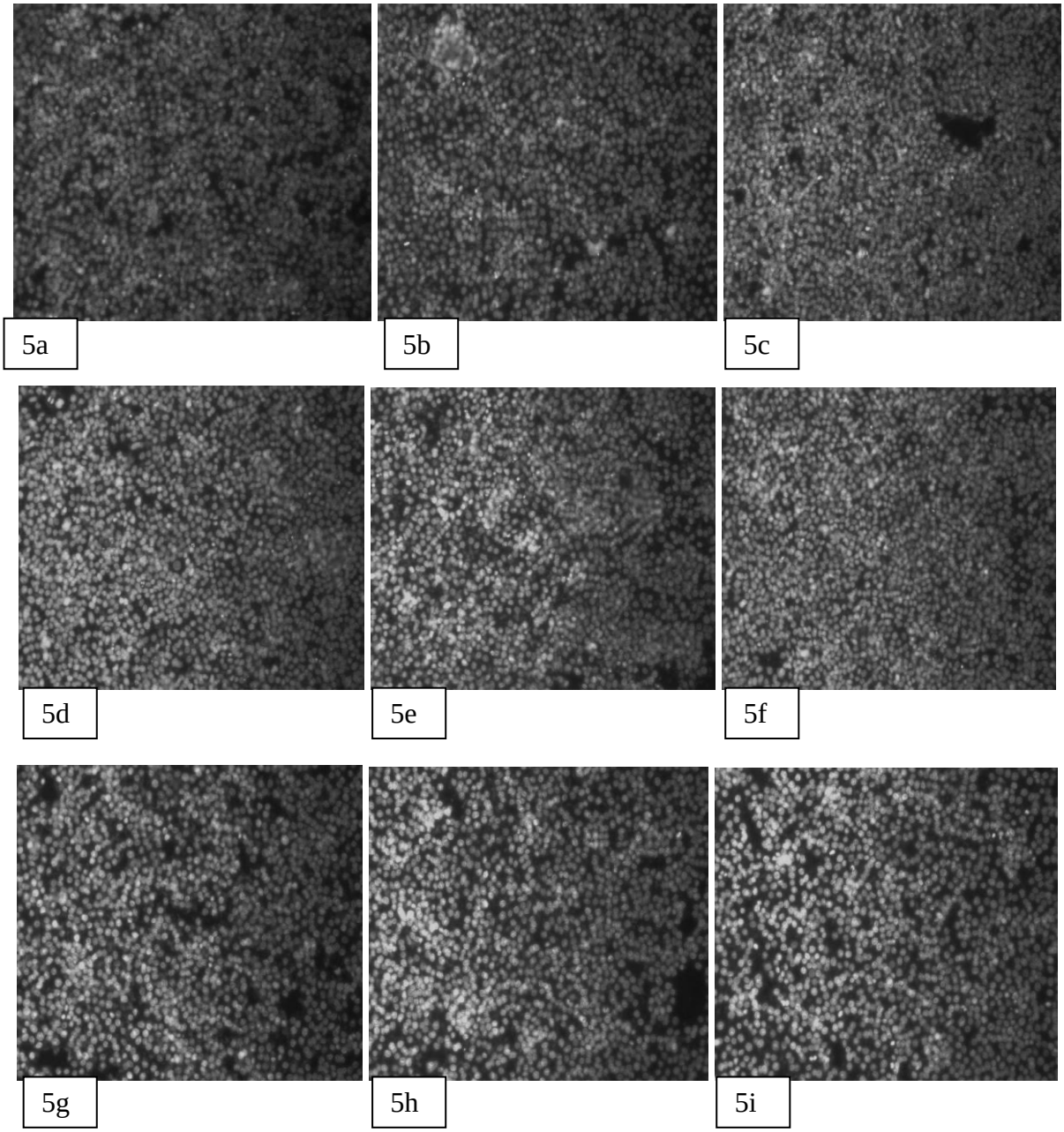

Fig.8 Hoechst 3324 staining analysis for the effects of algal extract on apoptosis. HT-29 cells were treated without algal extract (5a) and with algal extract 100µg/ml (5b), 200µg /ml (5c), 300µg /ml (5d) and 400µg /ml (5e) for 24 hours and HT-29 cells were treated with algal extract 100µg /ml (5f), 200µg /ml (5g), 300µg /ml (5h) and 400µg /ml (5i) for 48 hours.

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