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IN VITRO CYTOTOXIC AND ANTIBACTERIAL ACTIVITY OF ETHANOLIC EXTRACT OF *Euglena viridis*

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ABSTRACT

Euglena viridis extract was evaluated for its *in vitro* cytotoxic activity against two prostate cancer cell line, PC3, Du145 and colon cancer cell line, HCT-116. The ethanolic extract of *Euglena* was fractionated with hexane and ethyl acetate solvent system. The active partial purified fraction was tested for cytotoxic activity by methyl thiazolyl tetrazolium (MTT) assay. The results showed that 50 µg & 100 µg of algal extract were the most effective concentrations against PC3, Du145, HCT-116 cells. Preliminary phytochemical analysis of the crude extracts was revealed the presence of alkaloid, flavonoid and reducing sugar in ethanolic extracts. Antibacterial activity of the fraction was done by disc diffusion method against nine bacterial pathogens (*Pseudomonas putida, P. aeruginosa, P. fluorescens, Aeromonas hydrophila, Vibrio anguillarum, V. alginolyticus, V. fluvialis, V. parahaemolyticus* and *Escherichia coli*). The extract showed highest antibacterial activity against *V. anguillarum* (20.08±0.561mm) and *E.coli* (21.33±0.457mm) with lowest minimum inhibitory concentration (MIC) values (40µg/ml & 60µg/ml). From the performed assay the results supports the use of *Euglena* extract as antimicrobial agents as well as anticancer agents and novel pharmaceutical leads. The alga needs the further investigation for the isolation of its active constituents.

KEY WORDS: ***Aeromonas hydrophila*, Antibacterial, *Euglena viridis*, cytotoxic, MTT assay**

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INTRODUCTION

In recent years the popularity of complementary medicine has increased. Over 50% of all modern clinical drugs are of natural product origin[1]and natural products play an important role in drug development programs of the pharmaceutical industry[2]. Cancer is one of the most serious threats to human health in the world and chemotherapy is still the standard treatment method. Most of the anticancer drugs currently used in chemotherapy are cytotoxic to normal cells and cause immunotoxicity which affects not only tumor development, but also aggravates patient's recovery. The discovery and identification of new antitumor drug with low side effects on immune system has become an essential goal in many studies of immunopharmacology[3]. With this aim, many attentions have been paid to natural compounds in plants, marine organism and microorganisms. Regarding the low side effects of plants and other natural compounds, scientists are interested in working on them to find new medications. Finding anticancer agents from plant sources started in the earliest 1950s with the discovery and development of vinca alkaloids, vinblastine and vincristine and the isolation of the cytotoxic podophyllotoxins[4]. Regarding to cancer cells resistance to antitumor drugs, finding new effective anticancer compounds with fewer side effects has been a field of interest for many scientists. There has been many attention to natural compounds obtained from plants or seaweeds to investigate about their medicinal properties. The antitumor activity was one of the most important activities in marine drugs, and lots of algae and their metabolites have been showed potent cytotoxicity. These metabolites have played an important role in leading to new pharmaceutical compounds from algae for antitumor drugs. In one study a research group has screened 39 algae from seacoast of China for their possible antitumor activities and they showed that four species of *Rhodophyta* algae and three species of *Phaeophyta* exhibited cytotoxic effects against KB and HT-29 cancer cell lines. More than 30 compounds including bromophenols, carotene and steroids were isolated and purified from them and their effects on cancer cell lines have been evaluated separately[5]. The glycoprotein derived from *Chlorella vulgaris* showed immunoactive antitumor activity[6].

Euglena viridis is unicellular flagellate algal protests which are both freshwater and marine forms and the term '*Euglena*' is coined by Ehrenberg[7]. The genus *Euglena* is the largest in the class Euglenophyceae with 154 or more species[8] and it is the most interesting genus which is a representative of animal as well as plant character. It is usually free swimming fusiform, elongate, lanceolate, spindle-shaped, flexible unicellular mobile form with usually one or rarely two flexible flagella issuing out of an anterior notch at the base of which is an oval aperture and distinctive red pigment spot known as eye-spot. *Euglena* forms red blooms in all type of water bodies when density is very high characterized by formation of haematochrome during a bright sunny days. The coloration is green in cloudy days[9]. *E. viridis* is characterized by a single stellate group of band shaped chloroplast and finely striated delicate periplast, varying from 40-150µ in length. Different *Euglena* sp. have a broad range of medicinal properties, such as antimicrobial[10], anti-mutagenic[11], anti-HIV[12], immunopotentiating[13] and antitumor[14] activity. It has been reported that the unicellular flagellate *Euglena gracilis* is a rich source of b-1,3, glucan and has applications in human and veterinary medicine as an immunostimulant and immunopotentiator[15]. The microalgae have a significant attraction as natural source of bioactive molecules, because they have the potential to produce

bioactive compounds in culture, which are difficult to produce by chemical synthesis[16]. Despite this potential, attention has been centered on marine algae, with very little on fresh water algae. Recently antibacterial activity of some freshwater algae has been studied[17-20].

The present research was performed with an algal extract consisting of several substances; the derivative was initially analyzed for the phytochemical characteristics of its constitution through the phytochemical screening and chromatographic profile and aimed at exploring their cytotoxicity activity and biomolecules of potential therapeutic interest.

MATERIALS AND METHODS

(i)*Algal material & extracts preparation[18]*: Samples of freshwater alga, *E. viridis* were collected from ponds of Central Institute of Freshwater Aquaculture, Bhubaneswar, India in the month of October 2010. All samples were brought to the laboratory in plastic bags containing pond water and then washed three times with distilled water to separate potential contaminants. The alga was identified as belonging to family Euglenophyceae following Records of Botanical Survey of India[9]. Harvested samples were dried at room temperature and ground in an electric grinder. Resulting powder was submitted to lipid soluble polar solvents (Hexane, Ethyl acetate, Ethanol and Methanol) for extraction, using a soxhlet extractor at 55- 60 ^{0}C. All samples were refluxed until saturation (24 h) and the respective extracts were dried in rotary evaporator (Heidolph, Laborota 4000 efficient) at low pressure. Subsequently the residual extracts were suspended in the respective solvents to a final concentration of 10 µg µl^{-1}.

(ii) *Preliminary phytochemical screening[21,22]*: The phytochemical screening of the crude extracts of *E. viridis* was performed to verify the presence of natural chemical constituents such as: alkaloids, flavonoids, tannin, steroids, reducing sugar and saponins by using standard methods of Sofowora[21] and Trease and Evans[22].

(iii) *Fractionation by Column chromatography:* The active crude ethanolic extract of *Euglena* (2.0g) was fractionated using silica gel (SRL, 100–200 mesh size) column chromatography[23]. The solvent system was fixed by a preliminary thin layer chromatographic (TLC) study[24]. The elution was carried out successively with hexane, different ratio of ethylacetate/ hexane (1:20, 1:5, 1:1, 4:5) and 1:20 chloroform/ methanol and 50-100 ml of each fraction were collected. Then the fractions were reduced to 5ml by distilling. After distillation collected fractions were mixed according to their TLC behavior and finally 8 fractions were obtained. Out of eight fractions the fraction obtained from 30%+50% ethylacetate/ hexane chromatographic elution (i.e EuF) was taken for antibacterial and cytotoxicity activity.

(iv) *Test organisms:* Antibacterial sensitivity was tested against the pathogenic Gram-negative strains of *Pseudomonas putida* (ATCC 49818), *P. aeruginosa* (PA1), *P. fluorescens* (PF1), *Aeromonas hydrophila* (MTCC 646), *Vibrio anguillarum* (VAN), *V. alginolyticus* (VAL), *V. fluvialis* (VFL), *V. parahaemolyticus* (VP) and *Escherichia coli* (O115). These pathogens maintained in the Fish Health Management Division (FHMD), CIFA, Bhubaneswar, were taken for the antibacterial sensitivity study. Pure cultures of different bacterial strains inoculated in brain heart infusion (BHI) broth (Hi-media, Mumbai, India) except *V. parahaemolyticus* which was maintained in BHI broth supplemented with 2.5% NaCl and incubated at 37 °C for 18 h and subsequently used for antibacterial assay.

(v)*Antibacterial activity*[25, 26] : Antibacterial sensitive test of ethanolic fraction of *E. viridis* was done using single disc diffusion method as described by Chabbert. All bacteria were grown in BHI broth incubated at 37°C for 24 h and plated using a sterile swab, on to petridishes containing Antibiotic Assay Medium (Hi-media) adjusting the bacterial count to 10^7 CFU mL^{-1}. Each extract of 100 µg 10 $µl^{-1}$ concentration was applied to sterile filter paper discs (6 mm in diameter, Hi-Media). After solvent evaporation the discs were put on to inoculated plates and incubated at 37 °C. Discs with solvent (10 µl) used for dissolution were taken as control after evaporation of the solvent. Activity of the microalgae extracts against bacterial pathogens was determined after 24 h at 37°C by measuring the diameter of the halo around the discs (average of three experiments).

(vi) *Determination of minimal inhibitory concentration (MIC)*[27] : To determine the MIC, the extract of ethanolic fraction of Euglena was serially diluted in nutrient broth. Equal amount (2 mL) of bacterial suspension corresponding to 10^7 CFU mL^{-1} of the test organism was added to each of the test tube. The mixture was allowed to overnight incubation and the turbidity in each tube was visualized. The highest dilution of the algal extract in which there was no growth of the organism on the nutrient broth was observed to assess the susceptibility of the growth of the pathogen and to explain the lethality of the toxins present in the algal extracts.

(vii) *Cytotoxic Activity*[28,29]*:*
MTT Assay
The cancer cell lines (PC3, DU145 & HCT-116) were cultured in T-25 cm^2 tissue culture flask. Cells were harvested and resuspended at $1X10^6$ per ml. 100µl of the dilutions were plated out in triplicate into wells of a microtiter plate and incubated in 5% CO_2 incubator at 37^0 for 12 h or overnight to recover from handling. Three control wells of medium alone to provide the blanks for absorbance readings were included. After incubation ethanolic fraction of *Euglena*, EuF was dissolved in 1% DMSO (Dimethyl Sulfoxide) (Fisher Scientific, India) and added to the cell cultures at two fold serial dilution from (100µg to 6.25 µg). The cells were then incubated for 48h in 5% CO_2 incubator at 37^0C. 10ml of MTT reagent (Sigma Aldrich, US) was added to each well, including controls then return plate to cell culture incubator for 2h to 4h. When the purple precipitate is clearly visible 100µl of stop solution was added to all wells, including controls. The plate with cover was left in dark for 2h at room temperature and the absorbance of each well, including the blank was measured at 570 nm in a microtiter plate reader.

(viii) *Statistical Analysis*[30]*:* The results were analyzed using one way analysis of variance (ANOVA) and significant difference of treatments mean were compared to control by using Duncan's multiple range test (DMRT).

RESULTS

(i)*Phytochemical analysis:* The crude ethanol extract of *E. viridis* was qualitatively tested for the presence of alkaloids, tannins, flavonoids, steroids, saponins and reducing sugar and the results were given in Table. 1. The qualitative studies indicated the presence of alkaloids, flavonoids and reducing sugars.

Table 1
Preliminary phytochemical analysis of crude etanolic extract of Euglena (E:EtOH)

Phytochemical Tests		Crude Extract (E:EtOH)
Alkaloid	Dragendorff's Reagent	+
	Wagner's Reagent	+
	Mayer's Reagent	+
Tanin		
Flavonoid		+
Steroid		-
Saponin		-
Reducing sugar		+

'+'= present: '-'= absent

(ii) ***Antibacterial activity and MIC values:*** Table. 2 showed the results of antibacterial test and MIC values. The antibacterial potential of the extract was assessed against nine bacterial strains at the dose of 100µg /disc. The ethanolic fraction *Euglena*, EuF exhibited significant zone of inhibition against all the gram negative bacteria. Among three *Pseudomonas* spp. *P. putida* (ATCC 49818) was more sensitive towards the fraction (18.0±0.41mm) with MIC value 100 µg. *Vibrio anguillarum* (VAN) and *V. alginolyticus* (VAL) showed maximum zone of inhibition (20.08±0.56 & 16.91±0.46 mm) to the ethanolic fraction (EuF) of *Euglena* with lowest MIC values i.e. 40 µg and 50µg respectively. It was also noticed that the fraction, EuF is more effective (21.33±0.46 mm) against *E. coli* (O115) with MIC value 60 µg.

Table2
Antibacterial activity and MIC values of EuF (30+50% EA:Hex) fraction of ethanolic extract of Euglena against pathogenic bacteria

Sl.No	Pathogenic bacteria	Code	Zone of inhibition (mm)	MIC values (µg)
1	*Pseudomonas putida*	ATCC 49818	18.0±0.41	100
2	*P. aeruginosa*	PA1	12.91±0.46	100
3	*P. fluorescens*	PF1	15.08±0.46	50
4	*Aeromonas hydrophila*	MTCC 646	13.91±0.46	100
5	*Vibrio anguillarum*	VAN	20.08±0.56	40
6	*V. alginolyticus*	VAL	16.91±0.46	60
7	*V. fluvialis*	VFL	11.66±0.50	75
8	*V. parahaemolyticus*	VP	10.41±0.41	100
9	*Escherichia coli*	O115	21.33±0.46	60

Values represent mean±S.D

(iii) ***Cytotoxic activity:*** The results of cytotoxicity activity of fractionated ethanolic extract of *E. viridis* against two prostate cancer cells (PC3, DU145) and one colon cancer cell (HCT-116) were shown in Figures 1, 2 and 3 respectively. Increase in extracts concentration of up to 100 µg, could reduce the cell viabilities significantly ($P \leq 0.05$) in a dose-dependent manner in both cell lines. Cytotoxicity of the fraction

against prostate cancer cell lines, PC3 and DU145 are shown in Fig. 1 and Fig. 2. This fraction with concentration 100 µg appeared to be significantly ($P \leq 0.05$) more cytotoxic (cell viability of 5.83%) to prostate cancer cell line, DU145. It was noticed that, 100 µg of ethanolic fraction decreased the PC3 and DU145 cell viability to 16.89% & 5.83% while the change in colon cancer cell, HCT-116 viability was 11.26%.

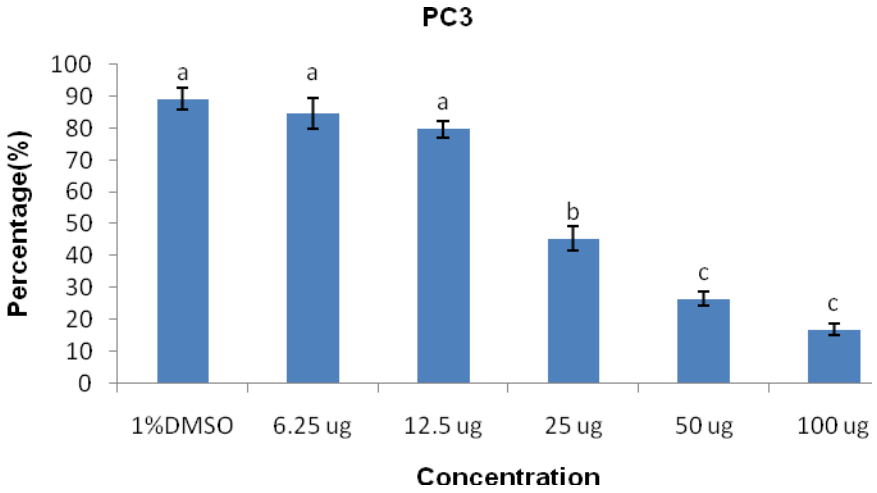

Figure 1

Effect of fractionated ethanolic extract of E.viridis on PC3 cell viability. All values represent mean ± standard deviation, P ≤ 0.001 indicate significant difference compared to the control

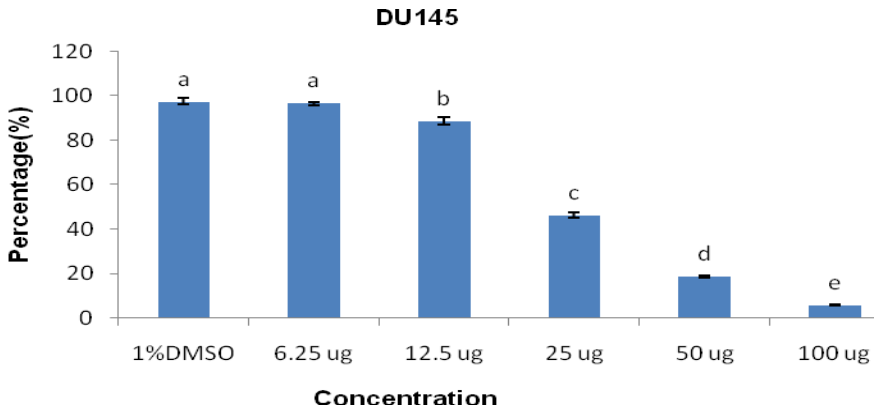

Figure 2

Effect of fractionated ethanolic extract of E.viridis on DU145 cell viability. All values represent mean ± standard deviation, P ≤ 0.001 indicate significant difference compared to the control

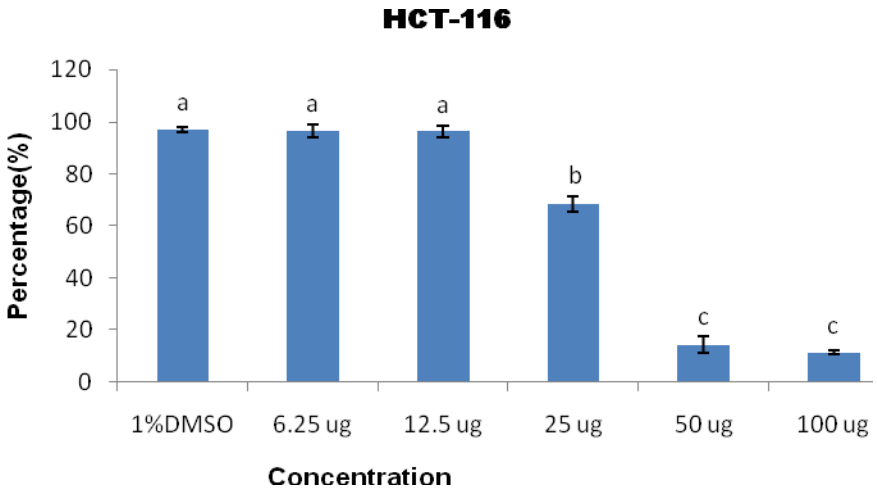

Figure 3

Effect of fractionated ethanolic extract of E.viridis on HCT-116 cell viability. All values represent mean ± standard deviation, P ≤ 0.001 indicate significant difference compared to the control

DISCUSSION

Based on the preliminary screening results, selected ethanolic fraction was chosen to test the efficacy of ethanolic extracts of *E. viridis* against nine bacterial pathogens. We had found that the antibacterial effect of crude extract of *E. viridis* was already demonstrated by our previous works[10, 18], but no work was realized on its partially purified fractionated product against the tested bacterial pathogens. The result showed that the ethanolic fraction, EuF could effectively inhibit the growth of three *Pseudomonas* spp. two *Vibrio* species and *E. coli* with lowest MIC values. It has been indicated that the antibacterial activity is due to different chemical agents present in the extract, including flavonoids and triterpenoids and other compounds of phenolic nature or free hydroxyl group, classified as active antimicrobial compounds[31]. Similarly our phytochemical screening indicated the presence of alkaloids, flavonoids and reducing sugar, which are mainly responsible for the remarkable antimicrobial effect of this alga. Similar type of observations was found by many workers[32,33] that the ethanolic extract of plants (*Plectranthus glandulosis*) shows the presence of tannins, alkaloids, glycosides, steroids and flavonoids. Polyphenols, such as tannins and flavonoids, are important antibacterial substances[34]. The presence of alkaloids has shown as antimicrobial[35,36] and antioxidant activity[37]. Ethanol is considered as a safe solvent and ethanol turned out to be the most suitable solvent in extracting antioxidant components from *Spirulina* since ethanol extracts showed a high antioxidant activity together with a high extraction yield. Higher amount of chlorophyll a together with a lower content of carotenoids was present in ethanol extract opposite to petroleum ether and hexane extracts[38].

The ability to produce antimicrobial substances may be significant not only as a

defensive instrument for the algal strains but also as a good source of the new bioactive compounds from a pharmaceutical point of view. Rania and Hala[39] reported that the ethanol, acetone, diethyl ether and methanol extracts of *S. platensis* revealed antibacterial activity on *E. coli*, *S. aureus* and *P.aeruginosa*. Screening efforts aimed to identify antimicrobial agents in microalgae have revealed several promising lead compounds. Numerous macroalgae have shown potent cytotoxic activities and certain authors have suggested the consumption of algae as a chemo-preventive agent against several cancers. Subsequent experiments were also done for determining the cytotoxic activities of tested ethanolic fraction of *Euglena* against two prostate cancer cell line and one colon cancer cell line. The ethanolic fraction showed cytotoxicity effects at various concentrations. Two concentrations of the fraction as 50 and 100 µg were examined for 48h incubation period. The highest cytotoxicity activity was shown by the fraction in 100µg concentration with 5.83% cell viability against DU145 cell line. However, in three concentrations (25 µg, 50 µg & 100µg) of the fraction, exceed 50% against both the prostate carcinoma cell line.

Similar type of result was observed by *Spyridia filamentosa* extract in 100 g ml^{-1} concentration, which exhibits both antimicrobial as well as strong cytotoxic activity with less than 10% cell viability against the DU145 cell line and about 20% cell viability against the MCF-7 cell line after treatment[40]. Ktari and Guyot[41] reported that the dichloromethane extract of *P. pavonica* showed high cytotoxic activity against the human buccal epidermal carcinoma (KB) cells in 10 g ml^{-1} concentration, while dichloromethane/methanol extract showed moderate inhibition. Many side chain-oxygenated sterols have been isolated from a number of brown algae, showed cytotoxic activity[42]. Zubia *et al*[43] investigated the cytotoxic activities of crude extracts from *Sargassaceae* and those from *Desmarestia ligulata* and *Dictyota dichotoma* showed strong cytotoxicities against the human cancer cell lines. Various diterpenes have been identified by Zubia *et al*[43] as the bioactive compounds in several species of the genus Cystoseira. In this study, the results of cytotoxic activities of the fraction were found more efficient in 100 µg concentrations and up to 12.5 µg showed low toxicities against all the cell lines. Crude or partially purified polysaccharides from various brown algae showed antitumor activities against the experimental tumor. In the report of Noda *et al*[44] the brown algae *S. lomentaria* (69.8% inhibition) and *Sargassum ringgoldianum* Harvey showed antitumor activity against implanted Ehrlich carcinoma. Xu *et al*[5] investigated 39 species of macroalgae from China coasts for their antitumoral activities and found that the ethanol extract of *S. lomentarius* had strong selective cytotoxic effect against KB cells. The ethanol extract of *Phyllanthus acidus* L. bark showed significant zone of inhibition against gram negative bacteria and also revealed the cytotoxic activity. Chloroform fraction of the red alga *Polysiphonia lanosa* possesses cytotoxic activities against HCT-116 cells[45]. Most anticancer drugs have been discovered through random screening of plant materials. Nowadays, isolation and elucidation of novel compounds have become an important part of cancer research for development of potential anticancer agents[46]. It should be borne in mind that the present study was based on partial purified ethanolic fraction of Euglena and detail investigation should be carried out to isolate the bioactive compounds, in particular from the alga, which is responsible for the cytotoxic effect on PC3, DU145, HCT-116 cells. A variety of compounds are present in the algal extracts (phenolic, flavonoid and alkaloids) and in this condition, the effects can be compounded.

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