Original Research

Phytochemical Profiling of Microalgae *Euglena tuba* and Its Anticancer Activity in Dalton's Lymphoma Cells

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Academic Editor: Josef Jampílek
Submitted: 7 January 2022   Revised: 22 February 2022   Accepted: 25 February 2022   Published: 1 April 2022

Abstract

Introduction: Natural phytochemicals are considered safe to use as therapeutic agents. There is a growing trend toward exploring anticancer effects of crude algal extracts or their active ingredients. *Euglena tuba*, a microalga, contains excellent antioxidant potential. However, the anticancer property of *E. tuba* has not been explored. This study investigates the chemical profiling as well as antitumor property of methanolic extract of *E. tuba* (ETME) against Dalton's lymphoma (DL) cells. **Materials and Methods**: *E. tuba*, procured from northern part of India, was extracted in 70% methanol, dried at room temperature, and stored at –20 °C for future use. A freshly prepared aqueous solution of ETME of different concentrations was employed into each experiment. The ETME mediated anti-tumor response in Dalton's lymphoma was evaluated in the inbred populations of BALB/c (H2d) strain of mice of either sex at 8–12 weeks of age. The cytotoxicity of ETME in cancer cells, effects on morphology of cell and nucleus, alteration in the mitochondrial membrane potential, and level of expression of proapoptotic proteins (Bcl-2, cyt C, Bax and p53) were done using known procedures. **Results**: The ETME contained high content of total alkaloids (96.02 ± 3.30 mg/100 mg), flavonoids (15.77 ± 2.38 mg/100 mg), carbohydrate (12.71 ± 0.59 mg/100 mg), ascorbic acid (12.48 ± 2.59 mg/100 mg), and phenolics (0.94 ± 0.05 mg/100 mg). Gas chromatography-mass spectrometry (GC-MS) analysis indicated the presence of 23 phytochemicals with known anticancer properties. DL cells treated with ETME exhibited significant and concentration dependent cytotoxicity. Florescent microscopy and flow cytometry of ETME treated DL cells indicated significant repair in cellular morphology and decreased mitochondrial potential, respectively. Western blot analysis displayed up-regulation of proapoptotic proteins (Bax, Cyt-c, p53) and down regulation of anti-apoptotic protein (Bcl$_2$) in DL cells treated with ETME. **Conclusions**: The findings of this study clearly indicated that the anticancer property of ETME was mediated via reduction in mitochondrial potential and induction of apoptotic mechanism. Further studies are warranted to explore the anticancer activities of active ingredients present in this microalga of pharmaceutical importance.

Keywords: *Euglena tuba*; Dalton's lymphoma cells; anticancer activity; cytotoxicity; antiproliferative activity; GC-MS; phytochemicals

1. Introduction

Cancer is a general term to define a number of diseases that are characterized by the uncontrolled proliferation of cells resulting from the altered signaling pathways, which regulate cellular functions [1,2]. In 2020, the International Agency for Research on Cancer (IARC), published a report showing total number on new cancer cases to be about 19.3 million with a global cancer death of 10 million people (2020). This data is expected to be double by end of 2040 due to increasing practices of smoking, unhealthy diet, physical inactivity, and fewer childbirths countries [3]. According to an estimate, about 1.81 million new cancer cases and 0.96 million cancer deaths were reported in India during 2020. The birth/death ratio has been reported to be 2.4. Despite rapid advancement in the health care sector, cancer is still a major health issue. At present, cancer is treated using chemotherapy, radiotherapy and surgery [4].

Though chemotherapy is an effective method for cancer treatment, there is still urgent requirement to develop safe, cost effective and novel anticancer agents with higher efficiency [5]. The synthetic/conventional anticancer compounds used in the treatment have been, however, reported to exert severe toxicity to the patients mostly via alterations in the cellular redox balance and functions of mitochondria. The efficacy and cytotoxicity of many anticancer compounds have been tested under varying conditions of cell culture with different cancer cell lines [6].

Nonconventional therapeutic approaches to treat cancer involve the phytochemicals isolated from different traditional medicinal plants. The plant based chemical ingredients have been shown to possess numerous medicinal properties. They could be used as alternative medicines to cure cancer, diabetes, inflammation and microbial infections [7,8]. The phytochemicals regulate various molecular

pathways and can be employed to inhibit proliferation of cancer cells, to inactivate the carcinogens, to induce apoptosis via arresting cell cycle and to increase the immune and redox systems [9]. The phytochemicals and their derivatives have been identified as suitable candidates for anticancer drug development due to their pleiotropic actions on targets and specific actions on tumor cells without affecting normal cells [10]. The biological targets of phytochemicals were found to be involved in antiproliferative, antiangiogenic, antimetastatic, and proapoptotic effects in mammalian cells or the ability to reduce oxidative stress [11,12]. The antioxidants and other phytochemicals contained in lower plants such as algae have been displayed to arrest carcinogenesis [13].

Euglena tuba (Carter) (Family-*Euglenaceae*) is a unicellular euglenozoa distributed in most aquatic bodies all over India throughout the year. The red coloured euglenozoa blooms from India were recognized as *Euglena tuba*, *E. haematodes* and *E. orientalis* [14]. Different *Euglena* species exhibit redox properties as it contains plenty of antioxidant compounds like vitamins A, B, C and E, β-carotene and phenolic compounds, which serve as the promising supplement of diet [15]. *Euglena* species are rich sources of dietary fiber, minerals, lipids, proteins, omega-3 fatty acids, essential amino acids, and polysaccharides. The compounds isolated from natural sources for the treatment of cancer are expected to exert either no or only minimum side effects. The scientists worldwide are engaged in designing the targeted therapeutics that can specifically exterminate cancer cells without harming the normal and healthy cells. The natural products isolated from algae [16], angiosperms [17–19] and lichens [20,21] have been utilized as viable sources of medicines to treat many diseases including cancer and injuries. Though, some studies have shown anti-oxidative properties of *Euglena* sp. [22–25], the anticancer properties of *E. tuba* extracts have not been elucidated properly. Several workers have reported the analysis of phytochemicals present in algal extracts using GCMS [26–31].

Due to the presence of the relatively nontoxic compounds in the algal extracts, their applications in human medicines against HIV-1, pathogenic microbes and genomic mutations, and tumor are quite popular. These compounds have also been shown to act as strong immunostimulants and immune-potentiator [32]. The algal bioactive compounds have potential to neutralize reactive oxygen species (ROS). ROS overproduction may result in genomic instability and cellular damage, as well as carcinogenesis. The present chemotherapeutic anticancer drugs are known to exert severe side effects and hence limit their long-term applications. Recently, more attention is being paid towards the discovery of plant based novel anticancer agents from the marine algae [33].

The compounds isolated from natural sources for the treatment of cancer are expected to exert either no or only minimum side effects. The scientists worldwide are engaged in designing the targeted therapeutics that can specifically exterminate cancer cells without harming the normal and healthy cells.

In this research paper, the chemical and biological characterization of the ETME has been done and its effect on various cellular properties such as cell viability, antiproliferation activity, nuclear morphology, mitochondrial membrane potential (Ψm), and the apoptotic processes of Dalton's lymphoma (DL) cells. We have used DL cells as they serve as viable experimental tools for studying progression of tumor cancer. DL cells have been reported to respond well against their treatment with different factors in laboratory. The findings of this study suggested that the phytochemicals present in the ETME have anticancer potential. ETME might be further explored for the development of novel drugs for the treatment of cancer.

2. Materials and Methods

2.1 Chemicals

Roswell Park Memorial Institute Medium (RPMI-1640) and the culture medium were obtained from HiMedia, Mumbai, India. Fetal bovine serum (FBS) was procured from Invitrogen, CA, USA. Rhodamine (RH-123) and 4,6-diamidino-2-phenylindole (DAPI) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). MTT was procured from Sigma Aldrich, India. Anti-Bcl$_2$, anti-Bax, anti-β-actin, anti-cytochrome c, anti-p53 antibodies, and HRP conjugated goat anti-mouse secondary antibodies were purchased from Cells Signaling Technology, Denver, MA, USA. All other chemicals were purchased from local firm with highest purity. FC reagent (Folin-Ciocalteu), Na$_2$CO$_3$, gallic acid, NaNO$_2$, AlCl$_3$, NaOH, quercetin, HCl, anthrone reagent, glucose, 2,4-dinitrophenylhydrazine reagent, 85% H$_2$SO$_4$, ascorbic acid, FeCl$_3$, 1,10 phenanthroline, reserpine, colchicines were procured from SRL (India). All other chemicals used were analytical grade.

2.2 Microalgae Collection and Extract Preparation

Algal sample were collected in the Baroh area of village Dhalwara, pond name Masran-ka-Talab, in the month of October 2016, in district Kangra of the state of Himachal Pradesh [34]. For identification of sample, *E. tuba* were preserved in formalin 4% and observed under the light microscope. The authenticity of *E. tuba* was verified by Dr. Rakesh Kumar (Algologist) and the voucher specimen was retained in the departmental herbarium for future reference with Accession No. WRS/GCD/DBH-002. It has been identified as *E. tuba* Carter belonging to family Euglenaceae, in Department of Botany, Wazir Ram Singh Govt. College Dehri, District Kangra (H.P.).

Algal samples were cleaned thoroughly three times in sterile distilled water currently and removed inappropriate materials adhered to it. Finally, it was centrifuged at 1000$\times$

g using a macro rotor to eliminate the bacterial contamination [35]. For extraction purpose, the collected pellet (biomass) of *E. tuba* was dried for 7 days at ambient temperature. The powder (10 g) was mixed with 100 mL solvent (methanol:water, 7:3) for 15 h using a magnetic stirrer and then centrifuged at 2850× g to obtain the supernatant. The process was repeated by mixing the precipitated pellet with 100 mL fresh 70% methanol. Both the supernatants were mixed and concentrated under reduced pressure in rotary evaporator, followed by lyophilization. The lyophilized 70% methanolic extract of *E. tuba*, designated as ETME, was kept at –20 °C for future use. A freshly prepared aqueous solution of ETME of different concentrations was employed into each experiment [35]. In this aqueous preparation of ETME, the final concentration of methanol was almost negligible.

2.3 Phytochemical Analysis

The ETME were evaluated for the presence of the phytochemical analysis by using the following standard qualitative methods as described [36]. The components analysed for phytochemicals were phenolics, flavonoids, carbohydrates, alkaloids and ascorbic acid.

2.3.1 Total Phenolic Content

The phenolic content was estimated by using 1 mL extract (ETME). It was mixed with 1.5 mL FC reagent (previously diluted to 1000 fold with distilled water) followed by addition of 0.06% Na_2CO_3 (1.5 mL) solution. After incubation at 22 °C for 90 min, the absorbance was taken at 725 nm. The phenolic content was evaluated from a gallic acid standard curve. Total phenolic content was done by method as described with some modifications [36].

2.3.2 Total Flavonoid Content

Total flavonoid content was determined using 0.5 mL extract. It was added to 5% $NaNO_2$ (0.15 mL). After incubation for 5 min at 25 °C, $AlCl_3$ (0.15 mL, 10%) was added, followed by 1 mM NaOH (1 mL). Finally, the reaction mixture was diluted with water and the absorbance was measured at 510 nm. The flavonoid content was calculated using quercetin as a standard. The flavonoid content was determined by the methods as described [36].

2.3.3 Quantification of Carbohydrate Content

Carbohydrate content of the extract was quantified using 10 mg of ETME. It was hydrolysed with 5 mL of 2.5N HCl. The mixture was diluted to 10 mL with distilled water, centrifuged and supernatant was used and mixed with 4 mL anthrone reagent. The mixture was incubated at water bath 95 °C for 8 min. After incubation, absorbance of the resultant dark green coloured solution was measured at 630 nm using glucose as a standard [36].

2.3.4 Quantification of Ascorbic Acid Content

Ascorbic acid content quantification was accomplished according to an elucidated technique [36]. In brief 1 mL aliquots of ETME (1 mg/mL) in water were mixed with 1 mL of 2,4-dinitro-phenylhydrazine reagent and incubated at 95 °C for 15 min. After incubation, 5 mL of 85% H_2SO_4 was added drop wise to the reaction mixture in ice cold condition. After 30 min, the absorbance was measured at 520 nm. All tests were performed six times. The ascorbic acid content was expressed as L-ascorbic acid equivalent.

2.3.5 Quantification of Alkaloid Content

Alkaloid content was quantified using 3 mL ETME extract. It was mixed with 0.3 mL of $FeCl_3$ (2.5 mM $FeCl_3$ in 0.5 MHCl) followed by addition of (0.3 mL, 0.05 M 1,10 phenanthroline). After incubation for 30 min at 70 °C at water bath, the absorbance was measured at 510 nm. The alkaloid content was quantified from the reserpine standard graph. The procedure was carried out with some modification using the methods of Chaudhuri *et al.* [36].

2.4 GCMS Analysis of ETME

The ETME was analysed using a Thermo Scientific Gas Chromatography-Traceultraver: 1100 and Mass Spectrometry- TSQ Duo. The oven temperature was maintained at 220 °C at a rate of 6 °C/min and flow rate of carrier gas was adjusted at 1 mL/min. The column of the GC was TG-5MS. Different parameters of the column were as such- the length of the column: 30 m, the diameter: 0.25 nm and the thickness of the film: 0.25 μm. The GCMS programming were done as follows: Injector temperature 215 °C, Transfer line temperature 218 °C, oven temperature program 80–280 °C with ramping 5 °C per min, carrier gas: Helium at 1.5 mL /min, individual components were identified by NIST MS 2.0 structural library. The split sampling technique was used to inject the sample in the ratio of 1:10. The time elapsed between elution and injection was recorded as the "retention time" (RT). RT represents the time from when the injection of filtered ETME was performed (Initial time) to when elution occurred.

The retention index of the compounds was identified by comparing the retention times and identification of each component was confirmed by the comparison of its retention index with data in the NIST library. Interpretation of Mass-Spectrum was carried out by using the database of the National Institute Standard and Technology (NIST) having more than 62,000 patterns. Spectrum of the known compound which are stored in NIST library was used to compare the spectrum of unknown component. The molecular weight, name, chemical structure and molecular formula of the components of the test materials were ascertained.

2.5 Animal Care and the Development of Tumor Model in Mice

For the evaluation of the ETME mediated anti-tumor response in Dalton's lymphoma, the inbred populations of BALB/c (H2d) strain of mice of either sex at 8–12 weeks of age were used. In conventional cages (2 animals in each cage) BALB/c (H2d) strain of mice received sterilized food and water *ad libitum*. All animals were kept and maintained with utmost care under the guidelines of the Institutional Animal Ethics Committee with CPCSEA Registration No. 839/GO/Re/04/CPCSEA, dated 30/08/2017, University of Allahabad, Allahabad, India. There is no any need to use euthanasia of animals in our experiment because we collected the DL cells from DL bearing mice and injected into the healthy mice of either sex at 8–12 weeks of age intraperitoneally (1.0×10^6 non-Hodgkin type of Dalton's lymphoma (DL cells) in 0.5 mL sterile PBS) using 20 gauge needle. The mice were not sacrificed. The DL cells for transplantation were obtained from ascitic fluid of DL-bearing mice, where the yield of the cells is higher and maintained in an ascitic form *in vivo* by serial transplantation.

2.5.1 MTT Assay

Evaluation of cytotoxicity of the extracts on DL cells was determined by the MTT assay. This test is based on MTT (3-(4,5- dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide), which is reduced by the living cells to a purple-blue precipitate of insoluble formazan. DL cells were harvested in RPMI 1640 medium (10% FBS and antibiotics solution) and seeded in 96 well culture plate at cellular density of 2×10^4 cells/well with varying ETME concentrations, i.e., 25, 50, 100, 200, and 250 μg/mL. The culture plates were incubated at 37 °C for 24 h and 48 h in 5% CO_2 incubator. After the incubation period, each well was added with MTT (5 mg/ mL) followed by the incubation of the plates in CO_2 incubator for 4 h. In the wells formed formazan crystal were dissolved in 100 μL of DMSO and took OD at 570 nm by ELISA plate reader (Bio-red, CA, USA) [37].

2.5.2 Nuclear Morphology Assay by DAPI (4, 6-diamidino-2 Phenylindole) Staining

From the mice bearing DL, the peritoneal DL cells were harvested. The nonadherent cells were collected and washed three times with chilled PBS. The DL cells (1×10^6/mL) were cultured in RPMI1640 medium supplemented with 10% FBS (v/v) and antibiotic solution in a humidified incubator at 37 °C with an experimental condition of 5% CO_2 and 95% air. The DL cells were treated with ETME (200 μg/mL) in a 6 well-plate for 24 h and 48 h. The cells were washed with PBS (pH 7.4), fixed with 4% PFA for 15 min. After that, in addition to 0.2% Triton-X100 (v/v) for 10 min and finally re-suspended in DAPI 10 μL (1 μg/mL) for 15 min at 37 °C followed by washing of the cells

with PBS. Then the cells were observed under fluorescence microscopy (Nikkon E 800, Japan) at ×20 magnification [38].

2.5.3 Effect of ETME on DL Cell Morphology Using Acridine Orange and Ethidium Bromide (AO/EB) Assay

The morphological evidence of apoptosis was detected by the treatment of DL cells (1×10^6/mL) in a 6 well-plate with acridine orange/ethidium bromide. The DL cells were treated with ETME (200 μg/ mL) for 24 and 48 h followed by washing of the cells with PBS (pH 7.4), 2 μL of acridine orange/ethidium bromide solution (100 μg/mL) and incubated for 5 min. After that the cells were washed with PBS and examined under fluorescence microscopy [38].

2.5.4 Flow Cytometric Determination of Mitochondrial Membrane Potential ($\Delta\Psi$m) by Rh-123

The changes in the mitochondrial membrane potential due to apoptosis induced condition were studied by flow cytometry using rhodamine-123 as described earlier [39]. The non-adherent cells were collected and washed twice with chilled PBS. The DL cells count (1×10^6 cells/mL) in a 6-well plate was adjusted and cultured in RPMI1640 medium supplemented with 10% FBS and antibiotics solution in a humidified incubator with an atmosphere of 95% air and 5% CO_2 at 37 °C treated with ETME (200 μg/mL) for 24 h and 48 h. The cells were washed and resuspended in PBS. Cells were stained at 37 °C with 2 μg/mL Rhodamine 123 for 30 min and observed under fluorescence microscope. The fluorescence intensity was analyzed in FL-1 channel on a BD FACS Calibur (Bec-ton Dickinson, USA). The decrease in fluorescence intensity due to the loss of mitochondrial membrane potential was analyzed in FL-1 channel [39].

2.5.5 Western Blot Analysis

The DL cells (1×10^6 cells/mL) in a 6 well plate were treated with ETME (200 μg/mL) for 24 h and 48 h. After treatment, the cells were washed with PBS three times and lysed with triple detergent cell lysis buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% Sodium azide, 0.1% Sodium dodecyl sulphate, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mg/mL aprotinin, 100 mg/mL phenyl methylsulfonyl fluoride) and the lysates were then centrifuged at 10000 $\times$ g for 30 min at 4 °C. The protein concentration was measured by Bradford method. The proteins (30 μg) in the cell lysates were resolved on 10–12% SDS-PAGE and transferred to the PVDF membrane using transfer buffer (190 mM Glycine, 25 mM Tris base, 20% Methanol, pH 8.3). The membranes were then blocked with 5% non-fat dry milk in TBS followed by incubation with corresponding primary antibodies separately overnight. Membrane was washed and incubated with goat antimouse HRP conjugated antibody (1:2000) for 1–2 h at

room temperature. The protein bands of β-actin, Bcl-2, cyt C, Bax and p53 on membrane were detected by enhanced chemiluminescence method (ECL). The protein expression pattern was obtained by normalizing the density to that of β-actin, respectively [40]. The measurements were performed using the Image J software (1.52A, NIH, MD, USA).

2.6 Statistical Analysis

The data was statistically analyzed by using one way analysis of variance (ANOVA) with Tuckey post hoc test by Graph Pad Prism software (9, GraphPad Software, CA, USA). p value < 0.05 were considered statistically significant. All the results were expressed as mean $\pm$ SD. All the above experiments were carried out minimum three times and more.

3. Results

3.1 Phytochemicals from E. tuba and Their Properties

In the present study, the ETME revealed that the presence of various bioactive phytoconstituents could be responsible for the therapeutic ability of ETME. The results obtained from the colorimetric analysis of the ETME demonstrated the presence of chemical components such as alkaloids, carbohydrates, flavonoids, phenols and ascorbic acid. The ETME analysis showed presence of phenolic contents to a significant level, i.e., 0.94 ± 0.05 mg/100 mg, equivalent to the corresponding gallic acid. The flavonoid contents were found to be 15.77 ± 2.38 mg/100 mg equivalent to quercetin. The alkaloid contents were 96.02 ± 3.30 mg/100 mg equivalent to corresponding value of colchicine. The total carbohydrate content was present as 12.71 ± 0.59 mg/100 mg equivalent to glucose. The ascorbic acid content was 12.48 ± 2.59 mg/100 mg equivalent to corresponding amount of L-ascorbic acid (data not shown).

It was found that the ETME contained highest amount of alkaloid, with considerable amounts of flavonoid, ascorbic acid, carbohydrates and very low amount of phenolic content. In this study, preliminary phytochemicals analysis was not sufficient to evaluate the content of secondary metabolites present in ETME. Therefore, the quantitative phytochemical analysis was carried out using a methanolic solvent system of the ETME.

In the present study, we have reported the presence of some of the important compounds in ETME as resolved by GC-MS analysis and also searched their biological activities from previous studies. The GC–MS chromatogram of ETME recorded a total of 631 peaks corresponding to different bioactive compounds that were recognized by relating their peak retention time, peak area (%), height (%) and mass spectral fragmentation patterns to that of the known compounds described by the National Institute of Standards and Technology (NIST) library. Out of 631 peaks, the major peaks based on the percentage area of peaks >0.1 represented the presence of a total of 23 compounds.

The phytoconstituents in the ETME

extract were found to be Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15hexadecamethyl; Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl; Propyl-1-pentanol; 1-Hexanol,2-ethyl; Cyclohexasiloxane, dodecamethyl; Dimethyl phthalate; Dodecanoic acid, methyl ester; 2,4-Di-tert-butylphenol; Cyclotridecane; 1-Hexadecanol; Methyl tetradecanoate; 1-Hexadecanol; Methyl 13-methyltetradecanoate; Phthalic acid, butyl undecyl ester; Methyl 4,7,10,13-hexadecatetraenoate; Hexadecanoic acid, methyl ester; Methyl 2-ethylhexyl phthalate; 10-Octadecenoic acid, methylester; 9,12,15-Octadecatrienoic acid, methyl ester; Methyl stearate; 5,8,11,14-Eicosatetraenoic acid, methyl ester; cis-5,8,11,14,17-Eicosapentaenoic acid; Eicosane; Phthalic acid, di(2-propylpentyl) ester. The GC-MS chromatogram of ETME based on their retention time (RT) displays presence of 10 peaks as demonstrated in Fig. 1. The structure and biological functions of the some bioactive compounds present in the ETME are displayed in Table 1 (Ref. [41–63]).
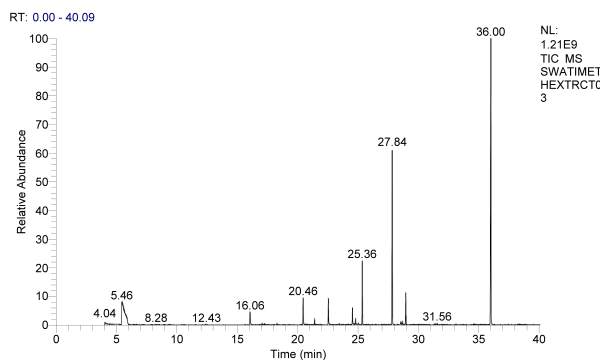

Fig. 1. GC-MS chromatogram of ETME. RT signifies retention time of the peaks. The relative abundance of peaks is shown; maximum being at RT value of 36.00.

The phytochemical and bioactive molecules present in ETME were Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl; Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl; Cyclohexasiloxane, dodecamethyl; Dodecanoic acid, methyl ester; 2,4-Di-tert-butylphenol; Phthalic acid, butyl undecyl ester; 10-Octadecenoic acid, methyl ester; Eicosane. The bioactive molecules such as 1Hexadecanol; Methyl 13-methyltetradecanoate; Methyl 4,7,10,13-hexadecatetraenoate; Hexadecanoic acid, methyl ester; Methyl 2-ethylhexyl phthalate; Methyl stearate; Phthalic acid, di(2-propylpentyl) ester is reported as anticancer, anti-inflammatory and antimicrobial, antioxidant activities. Another compound 9,12,15-Octadecatrienoic acid, methyl ester has been shown to exhibit the anti-inflammatory and anti-arthritic property and Methyl tetradecanoate compound show the plant metabolite, as a flavouring agent and a fragrance. The library search report and GC-MS

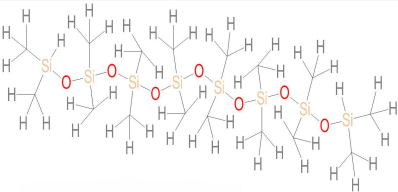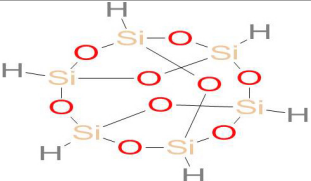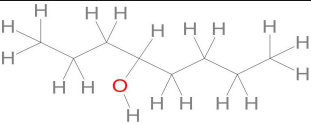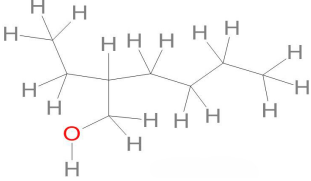**Table 1. The structure and function of bioactive compounds present in ETME.**
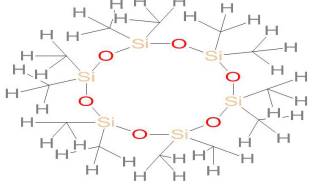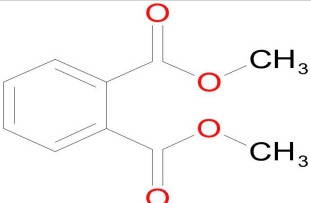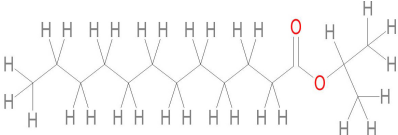
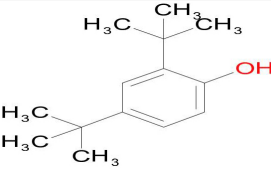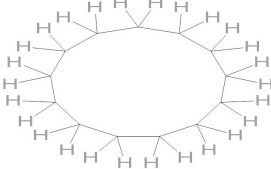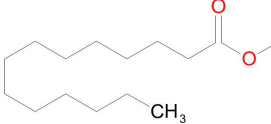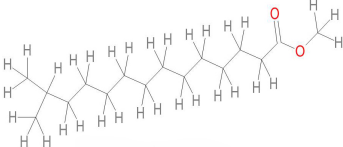| S.N.	Compound name	Molecular weight	Molecular formula	% Area	Retention time	Biological activity	Reference
1	OCTASILOXANE, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-HEXADECAMETHYL-	578	$C_{16}H_{50}O_7Si_8$	0.55	4.05	Antimicrobial	[41]
2	HEXASILOXANE, 1,1,3,3,5,5,7,7,9,9,11,11-DODECAMETHYL	430	$C_{12}H_{38}O_5Si_6$	0.12	5.18	Anti-microbial, antibacterial, anti-septic, hair conditioning agent, emollient	[42–44]
3	PROPYL-1-PENTANOL	130	$C_8H_{18}O$	6.94	5.45	Not reported	–
4	1-HEXANOL, 2-ETHYL-	130	$C_8H_{18}O$	0.86	5.88	Not reported	–
5	CYCLOHEXASILOXANE, DODECAMETHYL-	444	$C_{12}H_{36}O_6Si_6$	0.1	12.43	Antifungal properties	[45]
6	DIMETHYL PHTHALATE	194	$C_{10}H_{10}O_4$	1.96	16.06	Not reported	–
7	DODECANOIC ACID, METHYL ESTER	214	$C_{13}H_{26}O_2$	0.22	17.08	Antibacterial, antiviral, antifungal	[46]

Table 1. Continued.
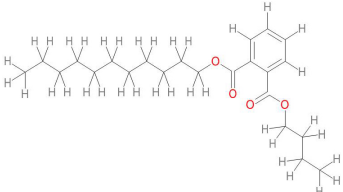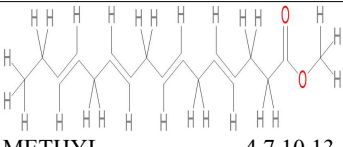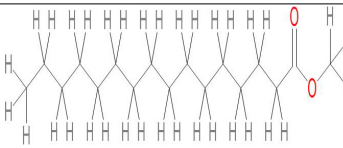
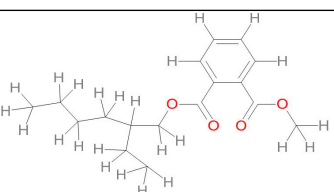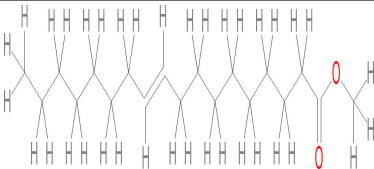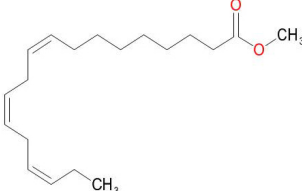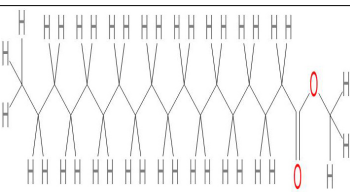| S.N.	Compound name	Molecular weight	Molecular formula	% Area	Retention time	Biological activity	Reference
8	2,4-DI-TERT-BUTYLPHENOL	206	$C_{14}H_{22}O$	0.24	17.26	Antibacterial, antiviral, antifungal activities	[47–49]
9	CYCLOTRIDECANE	182	$C_{13}H_{26}$	0.22	18.29	Not reported	–
10	1-HEXADECANOL	242	$C_{16}H_{34}O$	3.18	20.46	anticancer, anti-inflammatory and antimicrobial, antioxidant activities	[50]
11	METHYL TETRADECANOATE	242	$C_{15}H_{30}O_2$	0.63	21.41	plant metabolite, a flavouring agent and a fragrance	[51]
12	METHYL 13-METHYLTETRADECANOATE	256	$C_{16}H_{32}O_2$	0.17	23.44	Antioxidant, cancer-preventive, hypercholesterolemi, lubricant, nematicide	[52]
13	PHTHALIC ACID, BUTYL UN-DECYL ESTER	376	$C_{23}H_{36}O_4$	0.08	24.69	Antimicrobial, antibacterial, anti-inflamatory activity	[53]
14	METHYL 4,7,10,13-HEXADECATETRAENOATE	262	$C_{17}H_{26}O_2$	0.73	24.8	Antioxidant, antibacterial activity	[54]
15	HEXADECANOIC ACID, METHYL ESTER	270	$C_{17}H_{34}O_2$	6.98	25.36	Antioxidant, antimicrobial, hemolytic, hemolytic, 5-alpha reductase inhibitor cancer enzyme inhibitors in pharmaceutical, cosmetics, and food industries	[50]

Table 1. Continued.

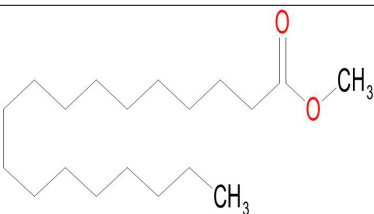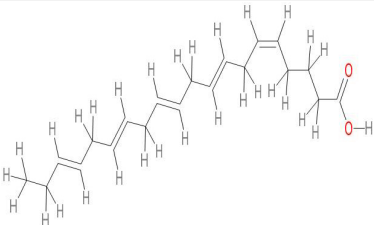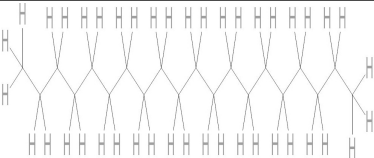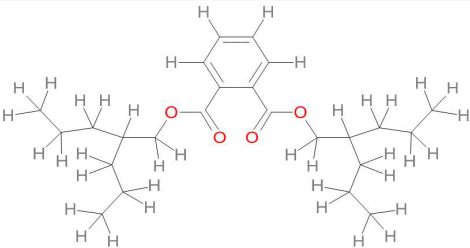| S.N.	Compound name	Molecular weight	Molecular formula	% Area	Retention time	Biological activity	Reference
16	METHYL 2-ETHYLHEXYL PHTHALATE	92	$C_{17}H_{24}O_4$	21.95	27.83	Antimicrobial and Cytotoxic Activity	[55]
17	10-OCTADECENOIC ACID, METHYL ESTER	296	$C_{19}H_{36}O_2$	0.55	28.57	Antimicrobial potential	[56]
18	9,12,15-OCTADECATRIENOIC ACID, METHYL ESTER	292	$C_{19}H_{32}O_2$	0.38	28.69	Anti-inflammatory and anti-arthritic	[57,58]
19	METHYL STEARATE	298	$C_{19}H_{38}O_2$	3.65	28.96	Antidiarrheal, cytotoxic and antiproliferative activities	[59,60]
20	5,8,11,14-EICOSATETRAENOIC ACID, METHYL ESTER	318	$C_{21}H_{34}O_2$	0.17	31.39	Antifungal, antibacterial, antitumor cytotoxic effects	[61]
21	CIS-5,8,11,14,17-EICOSAPENTAENOIC ACID	302	$C_{20}H_{30}O_2$	0.18	31.56	Not reported	–
22	EICOSANE	282	$C_{20}H_{42}$	0.11	33.12	Antifungal compound	[62,63]

Table 1. Continued.

S.N.	Compound name	Molecular weight	Molecular formula	% Area	Retention time	Biological activity	Reference
23	PHTHALIC ACID, DI(2-PROPYLPENTYL) ESTER	390	$C_{24}H_{38}O_4$	0.08	35.28	Anticancer activity	[55]

chromatogram of ETME are displayed in Fig. 1.

3.2 Evaluation of Effect of ETME on DL Cells

In the *in vitro* condition, the treatment with ETME may induce the death of DL cells. In order to assess the cytotoxic activity of ETME on DL cells, MTT assay was performed as described in Materials and Methods. The data presented in Fig. 1 indicated that ETME exhibited anticancer potential to markedly inhibit the growth of DL cells. After the incubation periods of 24 h (Fig. 2A) and 48 h (Fig. 2B), the percentage cell death was calculated. The results indicated that ETME significantly inhibited the proliferation of DL cells in the concentration dependent manner (**$p < 0.01$, ***$p < 0.001$) (Fig. 2). However, the cytotoxicity of DL cells increased after increasing the treatment duration from 24 h to 48 h. It was observed that ETME at different concentrations (50, 100, 200, 250) for 24 h treatment duration reduced cell viability to 78.9, 64.2, 54.2, 40.5%, while for 48 h it reduced the cell viability to 62.6, 53.2, 40.7, 31.5% of DL cells.

3.3 Effect of ETME on the Nuclear Morphology of DL Cells as Observed by DAPI Staining

We studied the mode of death of DL cells upon treatment with ETME by observing the nuclear morphology using DAPI staining method as described above. The results reflected that the nuclear abrogation of DL cells in the ETME (200 μg/mL) treated group was more as compared to control. The ETME was capable of inducing apoptosis in DL cells by inducing apoptotic feature. The results presented in Fig. 2 demonstrated that the DL cells exhibited significant morphological changes, which were associated with apoptosis such as nuclear fragmentation, migration of nucleus and chromatin condensation upon treatment with ETME. The ETME mediated cytotoxicity of DL cells was time dependent, i.e., more number of DL cells died after longer treatment duration (48 h) than at smaller duration (24 h) (Fig. 3).
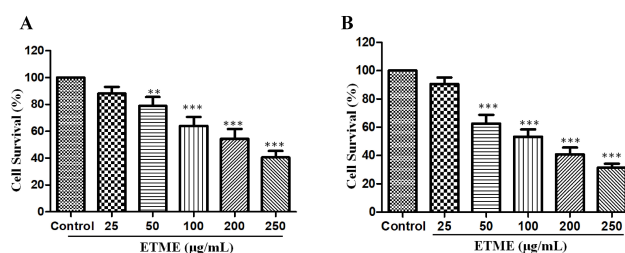

Fig. 2. Effect of ETME on viability and growth of DL cells. MTT assay of DL cells treated with different concentrations of ETME (25, 50, 100, 200, 250 μg/mL) was performed as described. The cells were incubated for 24 h (A) and 48 h (B). The results were expressed as cell viability (% of control). The values are demonstrated as mean $\pm$ SD (n = 3). Significant at **$p < 0.01$, ***$p < 0.001$.

3.4 Analysis of Effect of ETME on the Morphology of DL Cells Using Acridine Orange/Ethidium Bromide (AO/EB) Staining

The cellular morphological changes in DL cells upon treatment with ETME were also monitored by acridine orange/ethidium bromide (AO/Eb) staining as described in Materials and Methods. The treatment of DL cells with ETME revealed changes associated with apoptosis as indicated by the arrows. In both the groups viz. control and the treated DL cells, appearance of green fluorescence with intact green nucleus represents live cells. Morphological changes such as membrane blebbing, cell shrinkage and a number of red/orange cells (apoptotic cells) were observed in DL cells on treatment with ETME (200 μg/mL) (Fig. 4). These results validated that the treatment with ETME conferred apoptotic induction and death in the DL cells.

3.5 Impact of ETME on the Mitochondrial Membrane Potential ($\Delta\Psi m$)

After treatment with ETME, the status of mitochondrial membrane potential ($\Delta\Psi m$) in DL cells was studied by using Rh-123 and the fluorescence was measured by flow cytometer. The results presented in Fig. 5 demonstrated a high level of Rh-123 fluorescence in untreated control cells
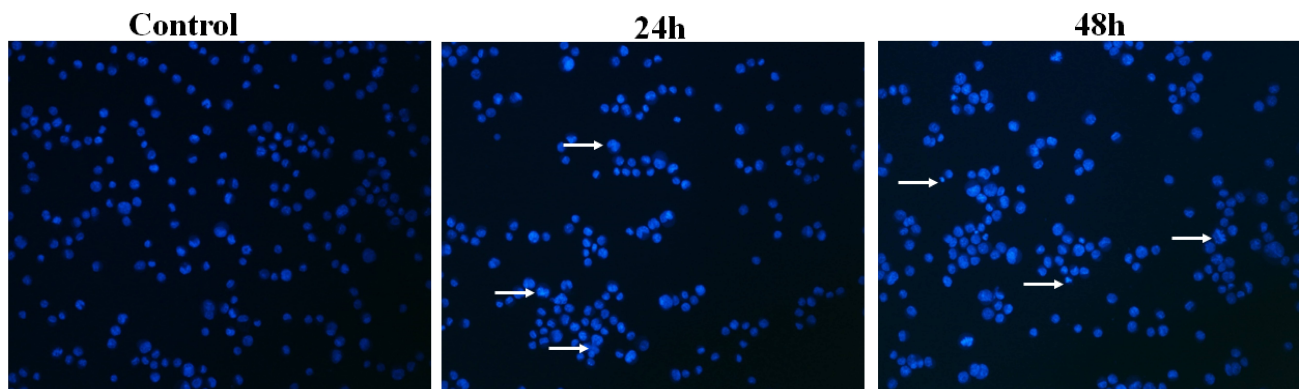
Fig. 3. Effect of ETME on nuclear morphology of DL cells as observed by DAPI staining. DL cells were treated with ETME (200 μg/mL) for 24 h and 48 h as described. The cells were harvested, fixed and stained with DAPI. The changes in nuclear morphology were observed by fluorescent microscopy at 200 ×. The arrows indicate chromatin condensation and nuclear fragmentation in treatment conditions.
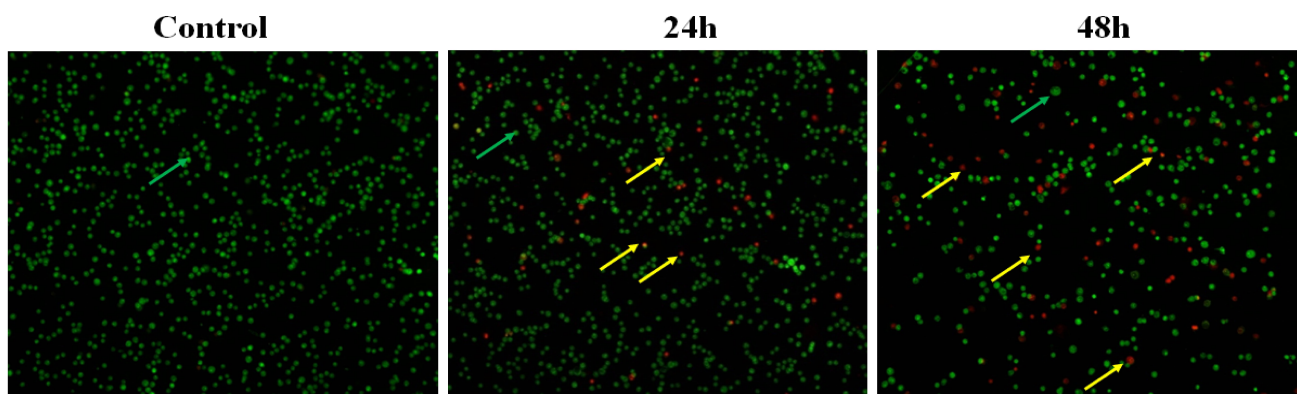
Fig. 4. Composite images of acridine orange/ethidium bromide (AO/EtBr) stained DL cells after treatment with ETME. The yellow and green arrows represent apoptotic and live cells, respectively. DL cells were treated with ETME (200 μg/mL) for 24 h and 48 h. Cells were stained with AO/EtBr and then were observed by fluorescence microscopy (×100 magnification). The red cells indicate the dead DL cells.
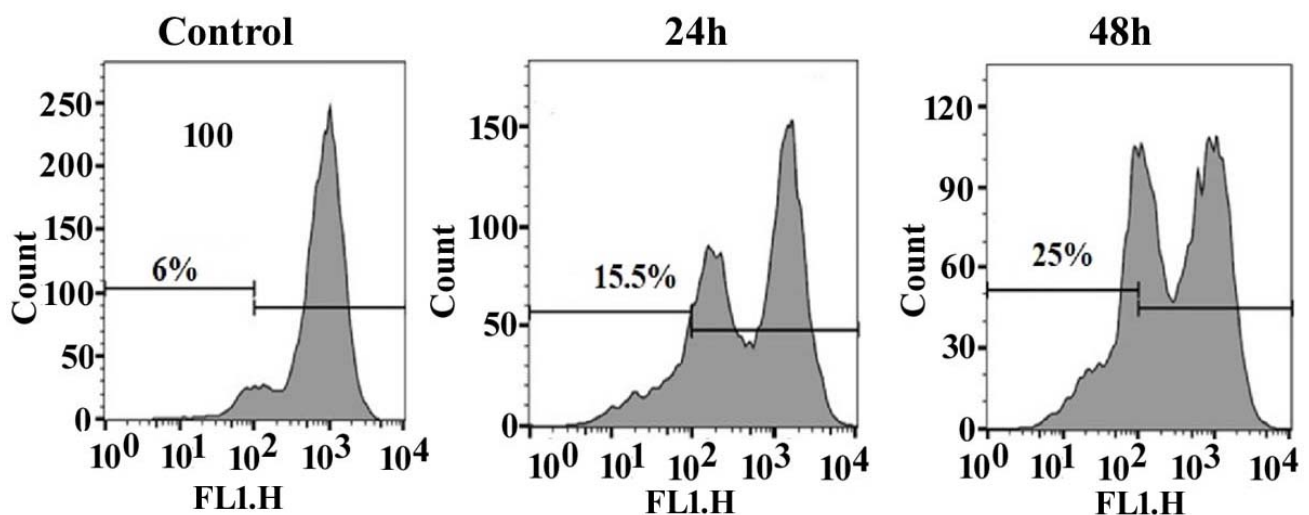
Fig. 5. Effect of ETME on the mitochondrial membrane potential (ΔΨm). The DL cells were treated with ETME (200 μg/mL) for 24 h and 48 h as mentioned above. The harvested cells were then stained with Rh-123 and flow cytometry was performed to analyze mitochondrial membrane potential. The inserted histograms show the left shifting due to loss of Rh-123 intensity in treated cells.

due to functionally active mitochondria. Exposure of cells with ETME for 24 h and 48 h resulted in mitochondrial damage which caused significant loss in $\Delta\Psi$m, as reflected by marked decrease in Rh-123 fluorescence. These results indicated that ETME was able to induce DL cells apoptosis via mitochondrial dependent pathway (Fig. 5). The results indicated that the apoptosis induced by ETME was through intrinsic apoptotic pathway. It was confirmed by loss in $\Delta\Psi$m as a result of mitochondrial damage. The leakage of proapoptotic proteins from mitochondria to cytosol due to the widening of mitochondrial permeability transition pores (PTP) may also attribute to the loss of mitochondrial membrane potential.

3.6 Evaluation of ETME Mediated Regulation of the Expression of bcl-2 Family Proteins

After observing sharp decrease in the mitochondrial membrane potential into DL cells due to ETME treatment, the expression of some key proteins involved in the regulation of apoptosis of DL cells by Western blot analysis were further examined. The results presented in Fig. 6 indicated an increase in pro-apoptotic protein, Bax, which may contribute to the release of cytochrome C (Cyt-c) from mitochondria and activation of intrinsic apoptotic pathway. The results shown in Fig. 6 indicated that ETME was able to down-regulate the expression of antiapoptotic protein Bcl_2. The ETME exposure also caused increase in the expression of Cyt-c. Further, the level of p53 was observed to be significantly increased in ETME treated conditions as compared to control (Fig. 6).
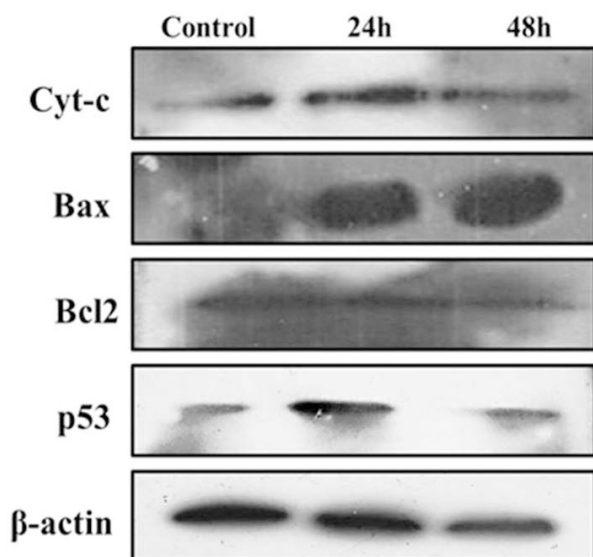

Fig. 6. Effect of ETME on the levels of expression of apoptosis related protein. Western blotting was carried to decipher the effect of ETME on the expression of apoptotic proteins viz. p53, Bcl_2, Bax, and Cyt-c after 24 h and 48 h.

4. Discussion

The analysis and extraction of plant material plays a vital role in the growth, development, upgrading, and quality control of herbal formulations [64]. The study of medicinal plants helps to explore their therapeutic potential to cure the humans and animals from various diseases. The current investigations on ETME revealed the presence of various phytoconstituents. These bioactive phytoconstituents could be responsible for the therapeutic ability of various extracts of *E. tuba*. The microalgae possess numerous metabolites [65–68] responsible for their varied physiological activities with biomedical significance. Flavonoids and phenolic compounds are known to contain antioxidant, anti-inflammatory, anti-cancer, anti-diabetic, and anti-pypertic activities [68–73].

Alkaloids extracted from plants show antimicrobial [74], antitumor and antiviral activities [75,76]. Similar to our results, recently some phytochemicals in ETME such as steroids, alkaloids, phenols, flavonoids, saponins, tannins, anthro-quinone have been reported to contain potential antibacterial, anticancer properties [77]. The results from the study conducted by others workers have also shown the presence of flavonoids, carbohydrates, tannins, phenols, amino acids, alkaloids, steroids, proteins, terpenoids, phytosterols, saponins and diterpenes in *E. tuba*, which could be responsible for its therapeutic ability [78].

The GC–MS analysis of ETME revealed the presence of 23 phytochemical compounds, which could contribute to the medicinal properties of this plant species [79–81]. The analysis also reported that the compound Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl has the antimicrobial activity [41]. The compound Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl possesses the anti-microbial, antibacterial, anti-septic, hair conditioning agent, emollient [42–44]. The Cyclohexasiloxane, dodecamethyl is known to exhibit antifungal properties [45]. The Dodecanoic acid, methyl ester was found to have antibacterial, antiviral, antifungal activity [46]. The 2,4-Di-tert-butylphenol or 2,4-bis (1,1-dimethylethyl)-phenol (2,4-DTBP), contained the antibacterial, antiviral, antifungal activities [47–49], while 1-Hexadecanol present in ETME exhibiting anticancer, anti-inflammatory, antimicrobial and antioxidant activities [50]. The compound, methyl tetradecanoate, acts as a flavouring and fragrance agent [51]. The compound Methyl 13-methyltetradecanoate had antioxidant, cancer-preventive, hypercholesterolemia, lubricant, nematicide properties [52]. Other bioactive compounds such as phthalic acid, butyl undecyl ester have been documented to contain a broad spectrum of antimicrobial, antibacterial, anti-inflammatory activities [53]. However, Methyl 4,7,10,13-hexadecatetraenoate was found to possess characteristic of antioxidant and antibacterial activity [54].

Ouyang *et al*. [50] have reported the hexadecanoic acid, and its methyl ester with the antioxidant, antimicro-

bial, and hemolytic functions. These molecules also acted as 5-alpha reductase inhibitor (cancer enzyme). The methyl 2-ethylhexyl phthalate, has been reported to be capable of exhibiting high antimicrobial and cytotoxic properties [55]. The compound,10-octadecenoic acid, and its methyl ester are reported to act as antimicrobial agents [56]. Another compound 9,12,15-Octadecatrienoic acid, methyl ester has been shown to exhibit the anti-inflammatory and anti-arthritic property [57,58]. The Methyl stearate compound has been suggested to contain potential bioactivities, such as antidiarrheal, cytotoxic and antiproliferative activities [59,60]. The compound 5,8,11,14-Eicosatetraenoic acid, methyl ester has antifungal, antibacterial, antitumor cytotoxic effects [61]. Some workers have shown that the compound Eicosane possessed the antifungal property [62,63]. Phthalic acid, di (2-propylpentyl) ester from ETME was found to be capable of producing high multi therapeutic properties such as anticancer activity [55]. Out of 23 compounds present in ETME only 6 compounds such as 1-Hexadecanol; Methyl 13-methyltetradecanoate; Methyl 2-ethylhexyl phthalate; Methyl stearate; 5,8,11,14-Eicosatetraenoic acid; Phthalic acid, di(2-) ester were reported to contain anticancer property. There was no evidence found for the presence of any biological activity of the compounds such as Propyl-1-pentanol; 1-Hexanol, 2-ethyl; Dimethyl phthalate; Cyclotridecane and cis-5,8,11,14,17-Eicosapentaenoic acid.

Cancer being a very serious health problem represents the second most threatening disease in humans after cardiac complications [82]. The cancer related research has always been paid a great attention, particularly in the direction of design and development of effective and safe drugs for its chemotherapy, specially derived from plants in journal and microalgae in particular [83]. Different Genus such as Chlorella, Cladophoropsis, Codium, Dunaliella, Enteromorpha, Helimenda, Udocea, and Ulva were previously reported to be able to produce high value compounds (HVC) with potential bioactive property against cancer [82,84–88]. These findings have suggested the need of further investigation of algal species inhabiting in both the marine and sweet water to explore active natural molecules to complement the existing anticancer medicines with very small and no toxicity. In order to address the complexity of cancer, some novel bioactive compounds with multipronged efficacy are urgently required to be investigated [89].

In this study, ETME has been screened for its probable anticancer activity using cell culture system involving DL cells. It was observed that ETME was able to inhibit the growth of DL cells through the induction of apoptosis. However, the cytotoxic effects of an algae, *A. armata*, in cell lines have been reported by Zubia *et al*. [90] against the human cervical cancer cell line, i.e., HeLa. The methanolic extracts of two green algae, *E. intestinalis* and *R. riparium* exhibited antiproliferative activity [91]. The methanolic fraction of another microalgae, *S. triplicate*, was ob-

served to be highly active against the progression of human lung cancer cells (A549). Further, the extracts of other microalgae namely *H. cornea* and *G. longissima* were found to elicit the cytotoxic effects on RAW264.7 cells at low concentration as measured by the MTT assay; its anticancer potential being associated to the presence of high amount of many halogenated phenolic compounds [92]. These compounds have been reported for their cytotoxic potential against some cancer cell lines [93]. The aqueous extract of *Gracilaria corticate*, a microalgae, have been evaluated by some workers for their antitumor activities in the human leukemic cell lines such as Jurkat and molt-4 cells and their results indicated that these extracts were able to significantly reduce the tumor cells proliferation [94].

The anticancer agents are known to possess the characteristic to induce apoptosis in cancer cells, though various other mechanisms have also been reported by which different molecules cause attenuation of tumor angiogenesis [95] including induction of apoptosis or necrosis or immune- stimulation [96]. The mechanisms by which algae bioactive function mainly rely on to their distinct chemical constituents and biological properties [97] have demonstrated that fucoidan (a complex polysaccharide found in many species of brown seaweed functions against the proliferation of lung cancer through causing delay in the development of cancer, elimination of cancer cells, and display of the synergistic effect with the anticancer chemotherapeutics.

The characteristic feature of apoptosis includes altered cellular and nuclear morphology, as well as reduction in the mitochondrial potential. The bioactive compounds present in the ETME have been reported to possess antimicrobial, antiviral, and antioxidant properties [98]. The results from the present study have indicated that ETME (200 μg/mL) was effective in inducing apoptosis in DL cells. The nuclear fragmentation has been considered as an apoptotic mode of cell death [99,100]. In the present study, the cell death, nuclear and cellular morphological changes involved in apoptosis were monitored by the staining of DL cells with DAPI, a fluorescent DNA-binding agent, and AO/EtBr and the results reflected the anticancer potential of ETME. The DL cells treated with ETME reflected the fragmented apoptotic bodies, shrunken and marginated nuclei in contrast to the normal and large nucleus in the untreated cells, proving the apoptotic potential of the extract.

The decrease in the mitochondrial membrane potential ($\Delta\Psi$m) acts as an indicator of apoptosis and helps to distinguish the mode of cell death from necrosis [101]. The rhodamine-123 dye is reduced by healthy mitochondria into fluorescent probe and that fluorescence is measured by flow cytometer in FL-1 channel. In the present study, the ETME treated DL cells stained with rhodamine-123 were used to measure the mitochondrial membrane potential (MMP) loss in DL cells. These results suggested that ETME decreased the binding ability of mitochondria to Rhodamine 123, re-

sulting in a decrease of fluorescent dye entering cells and an increase of the percentage of cells with weak fluorescence, and a marked decrease in MMP. The mitochondrial functions can be assessed by monitoring changes in MMP and it could be used as a key indicator of cell apoptosis [102]. MMP reflects the functional status of the mitochondrion [103] and a decrease in MMP is linked to apoptosis [104]. Mitochondria and the mitochondrial ROS are known to play an important role in the regulation of apoptosis [105]. Apoptotic cell death is an important mechanism of antitumor therapy and most anticancer drugs exploit apoptotic signaling pathways to induce death of the cancer cells [106]. The apoptosis of DL cells induced by ETME treatment in the present study indicates its anti-tumor potential.

The expression levels of the proapoptotic Bax and antiapoptotic Bcl_2 proteins are considered as the important regulators of apoptosis; the balance between the Bcl_2 family protein being critical for cell survival and death [107]. The results of the effect of ETME expression levels of some Bcl_2 family proteins in DL cells (present study) demonstrated the increase in proapoptotic protein Bax and decrease in antiapoptotic protein Bcl_2, which indicated activation of intrinsic apoptotic pathway contributing in the release of cytochrome C from mitochondria as expressed by other workers. Some workers have demonstrated the anticancer activity of a microalga, *Botryidiopsidaceae* sp., using human cancer cell lines such as A537, HCT116, HeLa, and Hs678T [108,109]. They observed that the extract of this plant significantly inhibited the cancer cells progression and induced the apoptosis of cancer cells through the structural and functional modulation of apoptotic genes such as p53, Bcl-2, caspase-3 and p53 genes. The Bcl_2 is responsible for the regulation of cell death and proliferation of cells via inhibition of apoptosis Brentnall *et al.* [109].

It is known that the transmission of the apoptotic signals to the apoptotic regulatory system of a living cell takes place primarily through two pathways known as extrinsic and intrinsic. In addition, it also involves perforin/granzyme pathway [110]. The "extrinsic" pathway involves the death receptors which bind their specific ligands. The receptor-ligand binding undergoes conformational changes and causes activation of the caspase cascade [111]. This event may be responsible for inducing the release of mitochondrial cytochrome C into the cytoplasm, thereby leading to the activation of effector caspases. In contrast, "intrinsic" pathway is directly triggered by numerous apoptotic signals that facilitate cytochrome C release [111,112]. The release of Cyt-c in cytosol further activated caspase dependent apoptosis. In general, apoptotic pathway consists of sequential biochemical events including the activation of caspases as described [113], a family of cysteine proteases that plays crucial roles in apoptotic signaling.

The tumor suppressor protein p53 has been shown to play a key role in the regulation of apoptosis and cell cy-

cle [113]. In addition, p53, a tumor suppressor gene, functions as a negative regulator of Bcl_2 at the transcriptional level [114]. Several stresses causing DNA damage may lead to the activation of p53. Bcl_2 acts as a major regulator of survival or death of a cell and functions as an apoptosis inhibitor responsible for tumor progression [115]. However, p53 performs as a tumor suppressor through modulation of multiple biological events associated to the antiproliferative activities involving cell cycle arrest and apoptosis [115,116]. The results of the present study showed that the protein levels of p53 increased significantly with increasing exposure time to ETME. The extracts from many algal species have been shown to exhibit antitumor activities and cytotoxicity against different types of cancer cell lines [117–119]. The antitumor properties of these extracts may be exploited for the development of new pharmaceutical compounds against cancer [120].

5. Conclusions

The results obtained involving *in vitro* studies with DL cells demonstrated ETME mediated morphological changes in the cells such as nuclear fragmentation, migration of nucleus and chromatin condensation associated to apoptosis. The cytotoxicity of ETME in DL cells, however, exhibited a positive correlation with time, i.e., ETME exerts more anticancer effect at higher time of exposure duration. The ETME treatment also caused an increase in the expression of the proapoptotic protein Bax, which would have contributed to the release of cytochrome C (Cyt-c) from mitochondria into cytosol causing marked reduction in the mitochondrial membrane potential and activation of intrinsic apoptotic pathway. The ETME was able to cause downregulation of the expression of antiapoptotic protein Bcl_2, upregulation of the expression of Cyt-c as well as p53. The present investigation indicated that the ETME contained anticancer property. The cytotoxic effect of ETME on DL cells was mediated via induction of apoptosis and antiproliferative effects. The information generated from the present study may be exploited for developing cost effective, safe and potential antitumor agents from *E. tuba* in future. However, extensive research is still required to further characterize these molecules present in ETME for understanding their detailed structural, pharmaceutical and therapeutic potential.

Author Contributions

SPG collected *E. tuba* and prepared the methanolic extract, generated the DL induced ascites in the Balb/c mice, cultured the cells and conducted the required experiments and wrote the primary manuscript. RKS, PKV, SK planed the experiments, and statistically analysed the results. NJS, HAK, SHA and ASA organized the contents, removed the plags and edited the manuscript. AA and BS examined the display of results in tables and figures and prepared the final draft of the manuscript.

Ethics Approval and Consent to Participate

Acknowledgment

Funding

Conflict of Interest

The authors declare no conflict of interest.

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