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Screening of new antileukemic agents from essential oils of algae extracts and computational modeling of their interactions with intracellular signaling nodes

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ABSTRACT

Microalgae are very rich in bioactive compounds, minerals, polysaccharides, poly-unsaturated fatty acids and vitamins, and these rich constituents make microalgae an important resource for the discovery of new bioactive compounds with applications in biotechnology. In this study, we studied the antileukemic activity of several chosen microalgae species at the molecular level and assessed their potential for drug development. Here we identified *Stichococcus bacillaris*, *Phaeodactylum tricornutum*, *Microcystis aeruginosa* and *Nannochloropsis oculata* microalgae extracts with possible antileukemic agent potentials. Specifically we studied the effects of these extracts on intracellular signal nodes and apoptotic pathways. We characterized the composition of essential oils of these fifteen different algae extracts using gas chromatography–mass spectrometry (GC–MS). Finally, to identify potential molecular targets causing the phenotypic changes in leukemic cell lines, we docked a selected group of these essential oils to several key intracellular proteins. According to results of rank score algorithm, five of these essential oils analyzed might be considered as in silico plausible candidates to be used as antileukemic agents.
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1. Introduction

Leukemia is defined by an abnormal and uncontrolled proliferation of one or more type of hematopoietic cell (Wang et al., 2014). Deterioration of the coordination between differentiation and proliferation of leukemic cells can be caused by a variety of factors such as chromosomal translocations, activation of oncogenes and the accumulation of inhibitory mutations in proliferation-related genes (Ma et al., 2013). Among the genes that commonly are mutated in leukemia are those that encode growth factors, intracellular signal transducers and tumor suppressors. Overexpression or inhibition of various genes can block differentiation and an increase in uncontrolled proliferation (Daigle et al., 2011).

Microalgae are very rich in bioactive compounds, minerals, polysaccharides, poly-unsaturated fatty acids and vitamins, and these rich constituents make microalgae an important resource for the discovery of new bioactive compounds with applications in biotechnology (Atasever Arslan et al., 2015; Ngo and Kim, 2013; Shariati and Hadi, 2011; Zemke et al., 2013). Notably, the Chlorophyceae family is intensively studied for the purposes of alternative energy and nutrition (Azmuddin et al., 2014). For this purpose, the *Dunaliella*, *Spirullina*, *Chlorella*, and *Scenedesmus* species have already been optimized for open and closed full-scaled production. In the current study, we studied the antileukemic activity of several chosen microalgae species at the molecular level and assessed their potential for drug development.

In this study, we focused on 10 different species of Chlorophyceae, 3 different species of Cyanophyceae, and one species from each Prymnesiophyceae, Eustigmatophyceae, and Bacillariophyceae. We cultured these different algae species, extracted cellular components and assessed their activity on human leukemia cell lines. Specifically we studied the effects of these extracts on intracellular signal nodes and apoptotic pathways. We characterized the composition of essential oils of these fifteen different algae extracts using gas chromatography–mass spectrometry (GC–MS). Finally, to identify potential molecular targets causing the phenotypic changes in leukemic cell lines, we docked a selected group of these essential oils to several key intracellular proteins.

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2. Materials and methods

2.1. Algae culture and lysate extraction

Microalgae species that are used in this study are *Haematococcus pluvialis* (UTEX Collection Culture No #2505), *Scenedesmus obliquus* (UTEX Collection Culture No #393), *Chlorella vulgaris* (UTEX Collection Culture No #265), *Spirulina platensis* (UTEX Collection Culture No #LB2340), *Closterium acerosum* (UTEX Collection Culture No #LB1075), *Neochloris oleoabundans* (UTEX Collection Culture No #1185), *Microcystis aeruginosa* (UTEX Collection Culture No #LB2061), *Nannochloropsis oculata* (UTEX Collection Culture No #2164), *Stichococcus bacillaris* (UTEX Collection Culture No #2542), *Klebsormidium flaccidum* (UTEX Collection Culture No #323), *Botryococcus braunii* (UTEX Collection Culture No #572), *Ulothrix acuminata* (UTEX Collection Culture No #1178), *Phaeodactylum tricornutum* (UTEX Collection Culture No #640), and *Synechococcus elongatus* (UTEX Collection Culture No #LB563). These cultures were obtained from UTEX and cultured according to established protocols in sterile conditions in the laboratories at SU and UU (http://www.sbs.utexas.edu/utex/mediaDetail.aspx?mediaID=166). Lipid enriched extracts of microalgae were extracted using absolute methanol. The soluble fraction was concentrated at room temperature in a rotavapor. These extracts were again dissolved in methanol using an ultrasonic bath, and filtered using a 25 μm filter just before conducting cytotoxicity assays.

2.2. Mammalian cell culture

Cytotoxic potential of the microalgae extracts were assessed on HL60 (human promyelocytic leukemia cell line) and K562 (human chronic myeloid leukemia cell line). ECV304 (human umbilical vein endothelial cell line) was used as non-cancerous cellular control because ECV304 cell line presents many features of endothelial cells (Suda et al., 2001). The cell lines were purchased from ATCC and cultured under BSL2 conditions at UU.

RPMI medium for suspension cells (Panbiotech P04-16100) and DMEM medium for adhesive cells (HyClone, 16777-133) were supplemented with 10% fetal bovine serum (HyClone, SH3007003HI), 1% penicillin–streptomycin, 1% L-Glutamine and 0.1%, and MEM non-essential amino acids. Cell lines were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C. 75 ml polystyrene cell culture flasks and 96-well plates (Greiner) were used for cell culture and viability assays, respectively. Adhesive ECV304 cells, were detached by 0.5% Trypsin–EDTA solution (Sigma, T3924), washed once with 10% FBS containing medium and twice with PBS and resuspended in DMEM at density of 1×10^5 cells/ml.

2.3. Cytotoxicity assay

The cytotoxic effects of *H. pluvialis*, *S. obliquus*, *C. vulgaris*, *C. acerosum*, *N. oleoabundans*, *M. aeruginosa*, *N. oculata*, *S. bacillaris*, *K. flaccidum*, *B. braunii*, *U. acuminata*, *P. tricornutum and S. elongatus* extracts on HL60, K562 and ECV304 cells were measured with MTT (Sigma, M-5655) assay as previously described (Pırıldar et al., 2010; Svobodova et al., 2012). K562 and HL60 cells were resuspended at 1×10^6 cells/ml in RPMI medium, ECV304 cells were resuspended at 1×10^6 cells/ml in DMEM medium at 37 °C in a humidified atmosphere containing 5% CO_2.

Stock solutions of the microalgae extracts were prepared in methanol at a concentration of 10 mg/ml. Serial dilutions of the stock solution (5000, 500, 100, 50, 10 μg/ml) were prepared in absolute methanol. 90 μl of cell cultures was dispensed into 96-well round-bottom plates containing 10 μl of the algae extract dilutions. Thus, the final concentrations of the extracts were 500, 50, 10, 5, and 1 μg/ml. As a negative control, only 10 μl of medium containing appropriately diluted methanol was used instead of algal extracts. After 48 h of incubation, 10 μl freshly prepared MTT solution (5 mg/ml) in phosphate buffer saline (PBS) was added to each well and the plates were incubated 3 h at 37 °C. Supernatants were removed from all wells and 100 μl of sodium dodecyl sulfate (SDS, pH: 5.5) containing isopropyl alcohol was added to the wells and the microplates were stored at room temperature in the dark, in order to dissolve the formazan crystals formed by reduction of MTT in living cells. Optical density of each well was measured at 570 nm test wavelength and at 655 nm reference wavelength on an Bio-Rad Benchmark Microplate Reader. Cytotoxicity index was calculated with the formula: (cytotoxicity index = 1 − [OD (treated wells) / OD (control wells)] × 100). The cytotoxicity assay was repeated six times for each concentration of the extracts. The microalgae extracts that exhibited cytotoxicity more than 50% for K562 and HL60 cells were further investigated in this study.

2.4. Flow cytometry analysis

To determine the apoptotic and necrotic effects of the microalgae extracts, cancer cells (K562 and HL60) were treated with the selected extracts, which showed more than 50% cytotoxicity in the MTT assays, stained with Annexin V and Propodium iodide (PI) (Biolegend Inc.) and analyzed by flow cytometry. Treated K562 and HL60 cells, were trypsinized from plates and harvested by centrifugation to remove the media and then washed with 1× FACS buffer (1× HBSS, 0.5% sodium azide and 0.5% BSA). Annexin V was diluted 1:100 with Annexin V binding buffer (0.1 M HEPES/NaOH (pH 7.4), 1.4 M NaCl, 0.05 M $CaCl_2$) and cells were resuspended in 500 μl of this mixture. Samples were incubated for 20 min at room temperature in the dark, stored on ice and PI (50 μg/ml) was added immediately before analysis. Flow cytometric measurement of the samples was performed on a FACS Canto flow cytometer (BD Biosciences). Data were analyzed with FlowJo 9.4.3 software (Treestar).

2.5. DNA fragmentation assay

After K562 and HL60 cells were incubated with the extracts for 24 h, cells were collected by centrifugation at 13,200 rpm for 20 s at room temperature. The supernatant was discarded and the pellet was transferred to a fresh microcentrifuge tube containing 600 μl of ice-cold cell lysis buffer (10 mM Tris–Cl (pH 8), 1 mM EDTA (pH 8), 0.1% (w/v) SDS). Three microliters of proteinase K solution (20 mg/ml) was added and incubated for 3 h at 55 °C followed by 1.5 μl of RNAse (4 mg/ml) and an additional incubation for 15 min at 37 °C. Then, samples were cooled down to room temperature and 200 μl potassium acetate solution was added and mixed. The samples were centrifuged (13,200 rpm) for 3 min at 4 °C. Supernatants were transferred to fresh microcentrifuge tubes followed by the addition of 600 μl isopropanol. The solution was mixed well and centrifuged at 13,200 rpm for 1 min at room temperature. The supernatant was removed and 600 μl of 70% ethanol was added to the DNA pellet. The tube was inverted several times and centrifuged at 13,200 rpm for 1 min at room temperature. The supernatant was removed and the DNA pellet air dried for 15 min before being dissolved in 100 μl TE buffer (Paul et al., 2006). Isolated genomic DNA was resolved by 2% agarose gel electrophoresis for 40 min at 100 V, stained in an Ethidium bromide solution (0.625 mg/ml) for x minutes and analyzed on a gel imaging system (Vilber Lourmat-02).

2.6. Spectrophotometric analysis of intracellular signal nodes and apoptotic signal pathways

CST PathScan® Signaling Nodes and Apoptosis Multi-Target Sandwich ELISA kits (Cell Signaling Technologies, USA) were used to determine the effect of microalgae extracts which showed more than 50% cytotoxicity in the MTT assays, on signaling nodes and intracellular apoptotic signal pathways. Algae extract-treated K562 and HL60 cells were processed according to the manufacturer's instructions. Briefly, 1 million cells were lysed in lysis buffer, lysates aliquoted onto 96 well

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