

Chemical constituent of *Isochrysis galbana* microalgae extract and its cytotoxic activities on leukemic cell lines

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ABSTRACT

Background and Aims: This study was aimed to investigate the anti-cancer effect of *Isochrysis galbana* microalgae extract.

Methods: In the study, the chemical composition of *Isochrysis galbana* microalgae extract was analyzed, and its cytotoxic effect against K562, HL60, U937, MOLT-4, and Raji cancer cells was investigated. ECV304 endothelial cells were used as a healthy cell line for the understanding of its selective cytotoxicity. To determine the effects of *Isochrysis galbana* extract, an MTT cytotoxicity assay was performed.

Results: According to the results of the experiments, the highest cytotoxic effect of *Isochrysis galbana* microalgae extract was shown at about 24.07±6.48% cytotoxicity against Raji cells. There were a large number of bioactive molecules in the extract, and these molecules showed a specific response to Raji cells when considering the synergistic and antagonistic effects of these molecules on each other.

Conclusion: According to the results of GC-MS analysis of *Isochrysis galbana* microalgae extract, the most intense molecules in the content were Dodecanoic acid, 3-hydroxy- (CAS) Beta-Hydroxy Dodecanoic Acid, and Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-isonide. The investigation of the effect of these molecules specifically against Raji cells is important to determine the possible anti-leukemic molecules and their combinations that show cytotoxicity against this cell line.

Keywords: Microalgae, Cancer, Cytotoxicity, GC-MS

INTRODUCTION

Algae, which can be unicellular or multicellular, are classified as micro or macroalgae according to their size. There are more than 50,000 species of microalgae, one of the oldest life forms in the world, that can live in fresh and salt water, moist soils, and trees (Kuşoğlu Gültekin, 2020; Richmond, 2003). Different species of microalgae are known to be rich in carbohydrates, proteins, lipids, and molecules with therapeutic properties (Becker, 2003). Due to their special cellular components, microalgae have been used by humans for various purposes for many years.

Microalgae are becoming the focus of attention in drug discovery day by day due to their high biodiversity, ease of production, and the diversity of bioactive molecules they have. It is known that many bioactive molecules obtained from macro and micro-

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algae have cytotoxic effects on cancer cells. However, the fact that a substance has shown cytotoxicity to cancer cells in anti-cancer drug research is not an adequate result. For a substance to have drug potential, it is important that it exhibits selective cytotoxicity.

Isochrysis galbana, a marine flagellated microalgae, belongs to the phylum Haptophyta, class Coccolithophyceae, subclass Prymnesiophycidae, order Isochrysidales, family Isochrysidaceae. Due to its small size (4–7 µm), it can be easily taken into cells by marine animals, and these animals can be added to the food chain by being consumed by other living things (Sadovskaya et al., 2014). *I. galbana* cells have high levels of soluble and insoluble polysaccharides and proteins as well as high amounts of polyunsaturated fatty acids (Sánchez, Martínez, & Espinola, 2000). It has been determined that the carbohydrate components, which make up approximately 13% of the dry weight of *I. galbana*, are a complex mixture of biopolymers containing various proportions of glucose, galactose, mannose, xylose, arabinose, fucose, and rhamnose (Batista, Gouveia, Bandarra, Franco, & Raymundo, 2013). According to the results of the *I. galbana* GC-MS analysis conducted by Hafsa et al. in 2017, *I. galbana* aqueous extract has been reported to contain 56.88% glucose, 38.8% mannitol, and 20.32% inositol (Hafsa et al., 2017).

An abnormality resulting from uncontrollable proliferation in one or more hematopoietic cell lines is called leukemia. There is a balance between differentiation and proliferation in the cell. Disruption of the coordination between differentiation and proliferation, chromosome translocations, mutations of proliferation suppressor genes and accumulation of oncogenes cause the development of leukemia. These mutated genes may be genes that synthesize proteins or growth factors that are active in the cell cycle or that are functional in the intracellular signal transduction pathway. Excessive increase or suppression of the expression of these genes causes the cell to be unbalanced, blocking differentiation and increasing proliferation (Barata et al., 2004).

Acute leukemias occur as a result of cancerization and clonal spread of hematopoietic progenitor cells during their differentiation stages. Some features of leukemia cells are the same as normal cells, but they cannot respond to cytokines that transmit differentiation information. This suppresses differentiation in leukemia cells and causes an increase in proliferation (Karawajew et al., 2000).

The aim of this study was to investigate the anti-cancer effect of *I. galbana* microalgae extract. For this reason, the chemical composition of *I. galbana* microalgae extract was analyzed with a gas chromatography-mass spectrometry, and its cytotoxic effect against chronic myelogenous leukemia cell line (K562), Caucasian promyelocytic leukemia (HL60), Human Caucasian histiocytic lymphoma (U937), Human acute T lymphoblastic leukaemia (MOLT-4), and Human Burkitt's lymphoma (Raji) cancer cells was investigated with an MTT cytotoxicity assay. ECV304 endothelial cells were used as healthy cell line to understand the selective cytotoxicity of the extract.

MATERIAL AND METHODS

Isochrysis galbana culture conditions

Isochrysis galbana was obtained from the University of Texas (USA) Algae Culture Collection. The culture medium recommended by the collection was used for the microalgae strain *I. galbana* (UTEX Collection Culture No #LB987, Prymnesiophyceae, Erdschreiber's Medium). The culture was done in temperature and humidity controlled Erlenmeyer flasks.

Preparation of *Isochrysis galbana* microalgae extract and gas chromatography-mass spectrometry (GC-MS) samples

The *Isochrysis galbana* microalgae culture was incubated in Erdschreiber's Medium for 10 days and their development was followed by daily spectrophotometric measurements. At the end of the 10-day incubation period, the liquid microalgae culture was centrifuged at 8000 rpm for 10 minutes. The resulting microalgae pellet was dried at 60°C overnight at room temperature. 100 mg of dried microalgae samples were weighed, homogenized in 10 mL of methanol with the help of sonication, and then incubated for 1 day at 4°C in the dark with stirring. At the end of the incubation, the sample was centrifuged at 1200 rpm at 4°C for 10 minutes, the supernatant was collected and kept in the dark at -80°C until use. The concentration to be used in the study was determined according to the volume/volume (µg/mL) calculation since the amounts of bioactive components were not known. *I. galbana* GC-MS samples prepared in this way were measured by injecting 2 µL into the sample chamber in the GC-MS device.

Characterization of *Isochrysis galbana* microalgae extract using gas chromatography-mass spectrometry (GC-MS) method

The content of the *I. galbana* microalgae extract prepared using methanol was determined by a Shimadzu brand gas chromatography device (QP5050, NY, USA) carrying an Rtx®-5MS column (30 mx 0.25 mm ID, 0.10 µm film thickness).

Nitrogen gas was used as a carrier gas in the analysis; the average gas flow rate was set as 1 mL/min. The oven temperature program was set from 110 °C (2 min) to 200 °C, increasing by 10 °C per minute, then increasing to 300 °C by 5 °C per minute, and finally, incubation at 300 °C for 9 minutes after analysis. Injector and detector (FID) temperatures were determined as 250 °C and 280 °C, respectively. The MS electron impact mode was set to 70 eV, and the ion source temperature was set to 200 °C. Mass spectrum results were obtained in the scan mode, in the range of m/z 45-450 (Paul, De Nys, & Steinberg, 2006).

Analyzing GC-MS results

The National Institute Standard and Technology (NIST) database (<http://www.nist.gov/srd/nist1a.cfm>), which contains more than 62,000 patterns, was used for mass spectrum analysis. For *I. galbana* microalgae, 3 GC-MS samples were prepared, and the potential peaks (considering the area under the peak) observed during the GC-MS analysis of all 3 samples were automatically compared and characterized with the patterns in the NIST database. The molecular weights, structural properties, and names of the components were verified with informa-

tion from the NIST database. The percentages of the identified molecules in the cell relative to each other were calculated.

Cell lines and culture conditions

In this study, leukemic cell lines K562, HL60, U937, MOLT-4, Raji obtained from the American Type Culture Collection (ATCC), and ECV304 endothelial cell line as healthy cells were used. Leukemia cells were grown in an RPMI medium containing penicillin/streptomycin, L-glutamine, and 10% Fetal Bovine Serum (FBS) while ECV304 endothelial cells were grown in a DMEM medium containing penicillin/streptomycin, L-glutamine, and 10% Fetal Bovine Serum (FBS). Cells were stored frozen in liquid nitrogen at -196°C in an ultra-cold environment.

Cytotoxicity test

An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) cytotoxicity assay was performed using K562, HL60, U937, MOLT-4, Raji leukemia cells, and ECV304 cells to determine the drug potentials of *I. galbana* microalgae.

K562, HL60, U937, MOLT-4, Raji leukemia cells, and ECV304 cells were counted and prepared for the cytotoxicity experiment at 100,000 cells per mL. Five different concentrations, selected according to preparatory studies, of *I. galbana* microalgae species (500, 50, 10, 5, 1 µg/mL) were placed on microplates, and 10 µL of the medium was added as a control. 90 µL of cells prepared as 100,000 cells per mL were added to these wells. In addition, the culture plates were incubated for 48 h in a humid environment in a 37 °C incubator containing 5% CO₂, and then 10 µL of MTT (5 mg/mL) was added to each well and incubated for 4 hours in the incubator. After incubation, 100 µL of

a mixture of sodium dodecyl sulfate (SDS, pH:5.5) dissolved in 50% isopropyl alcohol was added to the microplate. The prepared SDS mixture broke up the formazan crystals formed by MTT. The resulting color was measured at 570 nm, according to the reference wavelength of 630 nm, in the ELISA spectrometer. Cells that were incubated with only the medium were used as the negative control. For negative control, cells were incubated with 10 mL medium. This experiment was repeated 6 times independently. Cytotoxicity values of microalgae were calculated by comparison with controls (Arslan, Isik, Gur, Ozen, & Catal, 2017; Kaya, Atasever-Arslan, Kalkan, Gür, & Ülküseven, 2016). Six independent repeats were carried out, and the results were evaluated using the GraphPad Prism® 8 program.

RESULTS

The cytotoxic effects of *I. galbana* microalgae extract against HL60, K562, U937, MOLT-4, and Raji cells were investigated using the MTT cytotoxicity test. Non-cancer ECV304 cells were used as controls. According to the experimental results, *I. galbana* microalgae extract showed cytotoxicity below 50% in all cell lines (Figure 1). Therefore, IC₅₀ values of *I. galbana* microalgae were more than 500 µg/mL for these cells. It showed that 500 µg/mL concentration had the highest cytotoxic effect against Raji cells at a rate of 24.07±6.48%. There are many bioactive molecules in the extract and considering the synergistic and antagonistic effects of these molecules with each other, it shows 25% cytotoxicity against Raji cells while the absence of this effect against other cells shows that it creates a specific response to Raji cells.

Table 1. Molecules found over 1% in *Isochrysis galbana* extract as a result of GC-MS analysis.

Molecules	rt*	<i>I. galbana</i> (%)
Dodecanoic acid, 3-hydroxy- (CAS) Beta-Hydroxy Dodecanoic Acid	2.58	24.61
Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-isonide	6.14	23.19
Isopropyl dodecanoate	6.57	4.02
Decyl sulfide	6.94	8.43
2,6-Diisopropyl-naphthalene	7.56	1.42
Iron, tricarbonyl [N-(phenyl-2-pyridinylmethylene) benzenamine-N,N']-	7.75	2.12
Decanoic acid, decyl ester	7.83	3.74
Benzoic acid, 2-ethylhexyl ester	7.99	2.81
Tetradecanoic acid, methyl ester (CAS) Methyl myristate	8.15	2.54
Octadecane	9.12	1.02
Tricosane (CAS) n-Tricosane	9.24	1.10
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	10.05	1.68
Hexadecanoic acid, methyl ester (CAS) Methyl palmitate	10.55	4.96
Pentadecanal-	11.68	1.21
Octadecanoic acid, methyl ester	12.77	5.13
1H-Purin-6-amine, [(2-fluorophenyl) methyl]-	15.53	3.09
Eicosanoic Acid, 2,3-Bis[(Trimethylsilyl)Oxy]Propyl Ester (Cas) Trimethylsilylether Derivative Of 1-Monoarachidin	16.37	1.39

*rt, retention time (min)

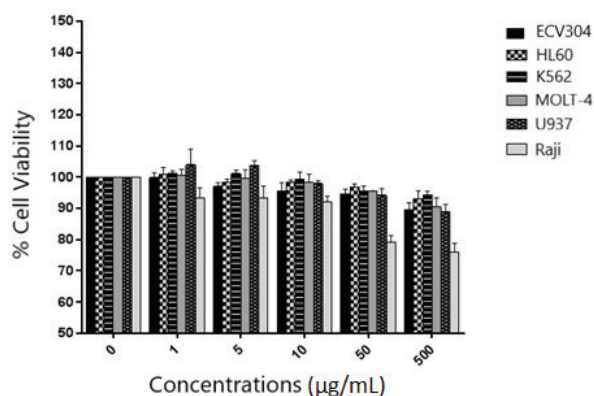


Figure 1. The figure shows the cytotoxic effects of *Isochrysis galbana* microalgae extract against ECV304, HL60, K562, MOLT-4, U937, and Raji cells. The control was accepted as 100% and considered as baseline, then the amount of decrease in cell viability was shown at 1, 5, 10, 50 and 500 µg/mL, respectively.

Therefore, investigation of the molecules that make up the chemical composition of *I. galbana* microalgae extract and their combinations in these cells may lead to the identification of agents with specific anti-leukemic effects against Raji cells.

The extract showed $10.37 \pm 4.79\%$, $7 \pm 5.99\%$, $11.1 \pm 5.48\%$, and $9.45 \pm 6.39\%$ cytotoxicity against ECV304 (control cells), HL60, U937, and MOLT-4 cells, respectively, and $5.44 \pm 2.44\%$ cytotoxicity against K562 cells. It is an important result that shows low cytotoxicity against control cells.

According to the GC-MS analysis results of *I. galbana* microalgae extract, the most concentrated molecules found as Dodecanoic acid, 3-hydroxy- (CAS) Beta-Hydroxy Dodecanoic Acid and Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-isonide (Table 1). The investigation of the effects of these molecules against Raji cells is important in terms of determining the molecules and their combinations showing cytotoxicity against this cell line.

DISCUSSION

In recent years, the search for natural products that can be an alternative to chemicals used in the maintenance of health or the treatment of various diseases has accelerated. Algae are seen as potential natural therapeutic molecule producers for researchers due to their relatively cheap production cost, rapid proliferation under favorable conditions, and being rich in various bioactive molecules. Algae have a very rich content in terms of various bioactive compounds, minerals, polysaccharides, polyunsaturated fatty acids, and vitamins. Due to these properties, it is important to investigate its anti-cancer activity potential and to identify a new anti-cancer agent in cancer research.

Folmer et al. showed that some algal species have an inhibitory effect on the NF- κ B transcription factor (Folmer et al., 2009). Kwon and Nam showed that *Capsosiphon fulvescens* algae inhibit the anti-apoptotic molecule Bcl-2 in gastric cancer cells (Kwon & Nam, 2007). However, the effect of this species on healthy cells has not been demonstrated. In recent years, studies on algae species have shown that various algae species

have hepatic and renal protective activities, antioxidant, anti-tumor, anti-inflammatory, anti-coagulant, and anti-viral activities. However, the effects on healthy cells are still not fully clear (Samarakoon & Jeon, 2012; Gamal-Eldeen, Ahmed, & Abo-Zeid, 2009; Mayer & Gustafson, 2004; Samarakoon & Jeon, 2012). Harada and Kamei found that the algae species they used in their study showed cytotoxic effects against leukemia cell lines in the concentration range of 15-375 µg/mL (Harada & Kamei, 1997). Machana, Weerapreeyakul, Barusru, Thumanu, & Tanthanuch, found that *Polyalthiaevecta* algae species showed anti-cancer effects on HepG2 hepatoma cells (Machana, Weerapreeyakul, Barusru, Thumanu, & Tanthanuch, 2012). However, in this study, a very high concentration of 500 µg/mL was observed, and *Polyalthiaevecta* also showed cytotoxicity against Vero cells used as healthy cells (Ferdous, Norhana, & Yusof). It has been shown that fucoxanthin, one of the tetraterpenoids isolated from *Isochrysis galbana*, has antioxidant activity, and violaxanthin has antioxidant and anti-inflammatory activities (Ferdous, Norhana, & Yusof, 2021). In the study conducted by Matos et al. various biological properties of *I. galbana* such as anti-oxidant, cytotoxicity, and hypocholesterolemic were examined; it has been shown that ethanolic extraction of *I. galbana* had significant cytotoxicity against HeLa human cervical cancer cells, with IC_{50} values of 0.32 and 0.28 mg/mL⁻¹ (Matos et al., 2019). With the increase of *I. galbana* content, cell proliferation decreases. Cell proliferation reached 42.7% and 13.8% at *I. galbana* concentrations of 31.25 and 500 µg/mL, respectively. Cytotoxic activity was evaluated on human HeLa cervical cancer cells. HeLa cell proliferation has been shown to be completely inhibited after treatment with *I. galbana* (1 mg/mL) (Hafsa et al., 2017). In their study, Sadovskaya et al. showed that polysaccharide extracts from *I. galbana* inhibited the proliferation of U937 human leukemic monocyte lymphoma cells (30% at 100 µg/mL) and consequently had potential anti-tumor activity (Sadovskaya et al., 2014). Ho et al. observed a 41% cytotoxic effect in HeLa cells at a concentration of 200 µg/mL. In this study, low cytotoxicity values were found compared to the high concentration (Ho et al., 2007). Bechelli et al. found that the algae species they studied showed cytotoxic effects against both AML cell lines and normal hematopoietic cells at low concentration (Bechelli, Coppage, Rosell, & Liesveld, 2011). In various studies, they detected algae species showing cytotoxicity on leukemia cell lines (Zandi et al., 2010; Ishikawa et al., 2008; Kotake-Nara, Terasaki, & Nagao, 2005).

CONCLUSION

After the data obtained, it was found that there are a wide variety of bioactive molecules in the microalgae extract. According to GC-MS results, Dodecanoic acid, 3-hydroxy- (CAS) Beta-Hydroxy Dodecanoic Acid and Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-isonide are the most abundant molecules in the microalgae extract. Among the cell lines studied, it was determined that *Isochrysis galbana* extract had a high cytotoxic effect especially against Raji cells. For this reason, this study is important in terms of investigating the effects of the mentioned molecules against leukemic Raji cells and determining possible molecules and combinations that have cytotoxicity against this cell line.

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