

Research Paper

Anti-inflammation and Anti-Cancer Activity of Ethanol Extract of Antarctic Freshwater Microalga, *Micractinium* sp.

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Abstract

Inflammation mediated by the innate immune system is an organism's protective mechanism against infectious environmental risk factors. It is also a driver of the pathogenesis of various human diseases, including cancer development and progression. Microalgae are increasingly being focused on as sources of bioactive molecules with therapeutic potential against various diseases. Furthermore, the antioxidant, anti-inflammatory, and anticancer potentials of microalgae and their secondary metabolites have been widely reported. However, the underlying mechanisms remain to be elucidated. Therefore, in this study, we investigated the molecular mechanisms underlying the anti-inflammatory and anticancer activities of the ethanol extract of the Antarctic freshwater microalga *Micractinium* sp. (ETMI) by several *in vitro* assays using RAW 264.7 macrophages and HCT116 human colon cancer cells. ETMI exerted its anti-inflammatory activity by modulating the main inflammatory indicators such as cyclooxygenase (COX)-2, interleukin (IL)-6, inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF)- α , and nitric oxide (NO) in a dose-dependent manner. In addition, ETMI exerted cytotoxic activity against HCT116 cells in a dose-dependent manner, leading to significantly reduced cancer cell proliferation. Further, it induced cell cycle arrest in the G1 phase through the regulation of hallmark genes of the G1/S phase transition, including *CDKN1A*, and cyclin-dependent kinase 4 and 6 (*CDK4* and *CDK6*, respectively). At the transcriptional level, the expression of *CDKN1A* gradually increased in response to ETMI treatment while that of *CDK4* and *CDK6* decreased. Taken together, our findings suggest that the anti-inflammatory and anticancer activities of the Antarctic freshwater microalga, *Micractinium* sp., and ETMI may provide a new clue for understanding the molecular link between inflammation and cancer and that ETMI may be a potential anticancer agent for targeted therapy of colorectal cancer.

Key words: Inflammation, cancer, *Micractinium* sp., proinflammatory cytokines, HCT116

Introduction

Inflammation is a defense response mediated by the innate immune system to reinstate cellular homeostasis against foreign pathogenic agents that disrupt the integrity of cellular homeostasis [1-3]. The biological mechanisms underlying inflammatory processes, which comprise three distinct steps,

initiation, regulation, and resolution, are tightly regulated to maintain physiological and cellular homeostasis inside the organism [4, 5]. If the tissue is damaged due to a pathogenic infection, the cells of our body activate a signal pathway including cytokines and chemokines to cause an immune

response. For example, macrophages, which are mainly located in the infected area, recognize the infection and secrete proinflammatory cytokines that attract other immune cells such as leukocytes and lymphocytes to the infected area, leading to inflammation. The recruited immune cells contribute to strengthening of the immune response against infection and the homeostatic state disrupted by infection is rapidly recovered into the normal state [6, 7]. If acute inflammation remains unresolved for any reason and progresses to chronic inflammation, which is the major driver of the pathogenesis of many diseases including cancer, the inflammatory cells are broken down and various inflammation inducers such as tumor necrosis factor (TNF)- α , interleukin-1 (IL)-1, and IL-6 are secreted, leading to DNA damage or mutations to form tumors [8-10]. In addition, inflammatory cells overexpress inflammatory cytokines or enzymes such as epidermal growth factor (EGF), matrix metalloproteinase (MMP), and monocyte chemo-attractant protein (MCP) [11]. These substances change the inflammatory microenvironment around the tumor, helping the tumor to proliferate and progress rapidly and thus making it easier for the tumor cells to break down nearby tissues and make the transition easier. To better understand the regulatory mechanisms underlying the progression of inflammation to cancer and development of inflammation-related cancers, it is necessary to determine the roles of the main regulatory molecules involved in the process, such as cytokines, nuclear factor (NF)- κ B, cyclooxygenase (COX)-2, and inducible nitric oxide synthase (iNOS) [12, 13]. Cytokines produced in immune/inflammatory cells, including ILs and TNF- α , play an important role in tumorigenesis through the modulation of transcription factors, such as NF- κ B, signal transducer and activator of transcription 3 (STAT3), and activator protein 1 (AP-1), to induce regulatory genes that promote cell proliferation and survival. Another key regulatory molecule in inflammation-mediated tumor development, NF- κ B, acts as a transcriptional factor to control the expression of many regulatory molecules in response to inflammation, such as IL-6, TNF- α , iNOS, COX-2, and chemokines [12-14]. In fact, NF- κ B and its downstream target genes, iNOS and COX-2, are constitutively activated in various cancer types and play crucial roles in tumorigenesis by regulating cellular proliferation, antiapoptotic activity, and angiogenesis, and enhancing metastasis [15, 16].

Recently, microalgae have been increasingly highlighted as a source of bioactive molecules with pharmacological and therapeutic potential against inflammation and cancer. For example, the

cytotoxicity of microalgal extracts and their secondary metabolites such as ramalin and lobaric acid has been demonstrated against different types of human cancer [17, 18]. In addition, many studies have shown the antioxidant, anti-inflammatory, and anticancer potentials of microalgae species and their secondary metabolites, including carotenoids, fatty acids, glycolipids, and polysaccharides [19]. However, the physiological and molecular mechanisms underlying biological activities of the microalga-derived metabolites remain to be clearly elucidated.

The main purpose of this study was to assess the anti-inflammatory and cytotoxic effects of the ethanol extract from the polar microalga *Micractinium* sp. (ETMI) on the human colon cancer cell line, HCT116. In the present study, the anti-inflammatory activity of ETMI was determined by evaluating the expression patterns of inflammatory regulatory molecules, including IL-6, TNF-, COX-2, and iNOS, by using the well-characterized LPS-induced inflammation model in RAW 264.7 cells. The antiproliferative and cytotoxic effects of ETMI on the cancer cells were determined using two different assays, the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT) and cell cycle assays, which showed a significant suppression of cellular proliferation and induction of cell cycle arrest in response to ETMI. These data suggest that ETMI may be a promising candidate as an anticancer agent for targeted therapy of colorectal cancer.

Materials and Methods

Preparation of ETMI

The microalga, *Micractinium* sp., was obtained from near King Sejong Station (62° 13' S, 58° 47' W). To identify the microalga, its 18S rDNA sequences were analyzed by Basic Local Alignment Search Tool (BLAST) for sequence similarities by using the NCBI GenBank database. A microalga sample was deposited at Korea Polar Research Institute, Republic of Korea (# KSF105) (Fig. 1). ETMI was prepared as described previously [20].

Cell culture

RAW 264.7 macrophages and HCT116 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin at 37°C in a humidified CO₂ incubator. RAW 264.7 macrophages were incubated with or without different concentrations of ETMI for 1 h prior to LPS (0.5 μ g/mL) stimulation. The HCT116 cells were seeded at a density of 5×10^3 cells/well in a 96-well plate and incubated in 5% CO₂ at 37°C.

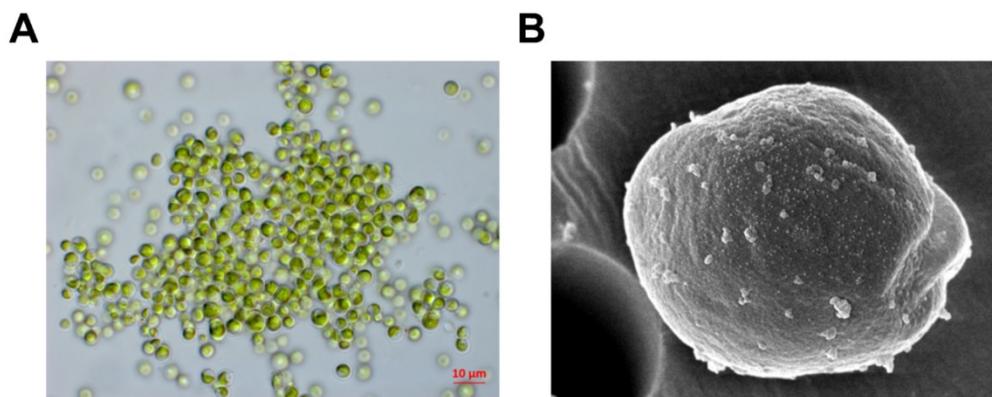


Figure 1. Morphological observation using light (A) and scanning electron microscopy (B)

Measurement of NO production

NO concentration in the RAW 264.7 cell culture supernatant was measured using Griess reagent. Briefly, 100 µL of the supernatant was mixed with an equal amount of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine). The mixture was incubated for 10 min at room temperature, and then the absorbance of each well was determined at a wavelength of 540 nm by using a microplate reader. Nitrite concentration was determined using a sodium nitrite calibration curve (0-100 µM).

Measurement of proinflammatory cytokine production

RAW 264.7 macrophages were seeded at a density of 5×10^5 cells/well in 24-well plates and treated with various concentrations of ETMI (0, 5, 10, 20, and 40 µg/mL) for 1 h and then stimulated with 0.5 µg/mL LPS for 24 h. The cell culture media were collected and the levels of proinflammatory cytokines, TNF- α and IL-6, were analyzed using enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Cytotoxicity assay

RAW 264.7 macrophages and HCT116 cells were seeded at a density of 1×10^5 cells/well in a 96-well plate. The macrophages were incubated with various concentrations of ETMI (12.5, 25, and 50 µg/mL) for 1 h and then stimulated with LPS (1 µg/mL) for 24 h. Thereafter, cellular proliferation was analyzed by CellTiter 96AQ_{ucous} One Solution Cell Proliferation Assay (Promega).

Colony-forming assay

HCT116 cells were seeded at a density of $\sim 1 \times 10^3$ in 6-well culture plates. To ensure cell adherence, the cells were incubated at 37°C for 24 h and then treated

with various concentrations of ETMI (12.5, 25, 50, and 100 µg/mL) for 12 h. ETMI containing the culture medium was then removed; the cells were washed in phosphate-buffered saline (PBS) and incubated in regular medium until viable colonies were observed. The cells were then fixed with methanol-acetic acid (3:1), stained with the staining solution, and counted as described previously [20].

RNA extraction and qRT-PCR

To measure the mRNA expression of COX-2, IL-6, iNOS, and TNF- α , total RNA was isolated from ETMI-treated cells according to the manufacturer's instruction for TRIzol Reagent (Invitrogen). The first-strand copy DNA (cDNA) was synthesized using the Super Script first-strand cDNA synthesis kit (Invitrogen). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using SYBR green real-time PCR master mixes (ThermoFisher Inc., USA) with gene expression primers. Relative mRNA levels were normalized to those of the housekeeping gene β -actin. The primers used were as follows: iNOS: (F) 5'-GGAGCCTTTAGACCTCAACAGA-3', (R) 5'-TGA ACGAGGAGGGTGGTG-3'; COX-2: (F) 5'-GAAGTCT TTGGTCTGGTGCCTG-3', (R) 5'-GTCTGCTGGTTTGAATAGTTGC-3'; IL-6: (F) 5'-GAGGATAACACTC CCAACAGACC-3', (R) 5'-AAGTGCATCGTTGTTCATACA-3'; TNF- α : (F) 5'-CATCTTCTCAA AATTCGAG TGACAA-3', (R) 5'-TGGGAGTAGACAAGGTACAA CCC-3'; β -actin: (F) 5'-TGTTTGAGACCTTCAACA CC-3', (R) 5'-CGCTCATTGCCGATAGTGAT-3'.

Western blotting

RAW 264.7 cells were seeded at a density of 5×10^5 cells/mL in a 6-well plate. After overnight incubation, they were pretreated with different concentrations of ETMI for 1 h before the addition of LPS (0.5 µg/mL). After incubation for 24 h, the cells were collected and lysed in radioimmunoprecipitation assay (RIPA) buffer. Western blotting was performed as previously described [19]. Anti-iNOS

antibody was purchased from Sigma-Aldrich (Cat# N7782), and anti-COX-2 and anti- β -actin antibodies were obtained from Cell Signaling (Cat# 4842 and Cat# 3700, respectively). Immunoblot signals were compared with those for β -actin and the relative protein expression was determined.

Cell cycle analysis

HCT116 cells were treated with various concentrations (0, 25, 50, and 100 $\mu\text{g}/\text{mL}$) of ETMI and then incubated for 24 h. The cells were then harvested and washed with PBS and fixed in 70% ice-cold ethanol for at least 4 h. Next, the cells were stained with a solution of PBS containing 50 g/mL propidium iodide and 50 g/mL RNase A. Cell cycle distribution was determined by flow cytometry (Becton Dickinson).

Statistical analysis

Values are presented as mean \pm SEM of three independent biological experiments. Statistically significant differences between each treated group and the control group were determined using

one-way analysis of variance followed by Student's *t*-test; *p* values < 0.05 were considered to indicate a statistically significant difference.

Results

Effect of ETMI on RAW 264.7 cell viability

To determine the cytotoxicity of ETMI in RAW 264.7 cells, the cells were exposed to various concentrations of ETMI, from 5 $\mu\text{g}/\text{mL}$ to 320 $\mu\text{g}/\text{mL}$, and then treated with 1 $\mu\text{g}/\text{mL}$ LPS for 24 h. Thereafter, the cell growth rate was determined using the MTT assay. As shown in Fig. 2A, the proliferation of RAW 264.7 cells was not significantly affected by treatment with ETMI at concentrations up to 40 $\mu\text{g}/\text{mL}$ compared to that of the cells without LPS or ETMI treatment.

Effects of ETMI on LPS-induced NO production and iNOS/COX-2 expression

To determine the inhibitory effect of ETMI on the LPS-induced proinflammatory reaction, the expression levels of the main proinflammatory mediators, iNOS and COX-2 genes, in ETMI-treated RAW 264.7 cells were determined. The cells exposed to ETMI at concentrations of 5, 10, 30, and 40 $\mu\text{g}/\text{mL}$ for 1 h did not show a cytotoxic effect; thereafter, the cells were treated with 1 $\mu\text{g}/\text{mL}$ LPS for 24 h. As shown in Fig. 2B and 3, LPS-stimulated expression of both iNOS and COX-2 significantly reduced in a dose-dependent manner at both the transcriptional (Fig. 2B) and translational levels (Fig. 3) compared to that in the control cells, which received only LPS stimulation and not ETMI pretreatment. Next, the level of NO, which is one of the principal mediators of inflammation, was determined using Griess reagent under the same condition. We found that ETMI markedly decreased LPS-stimulated NO production in a dose-dependent manner (Fig. 2C). Taken together, these findings suggest that ETMI has high anti-inflammatory activity even at low concentrations through the modulation of the main regulators of inflammation at both the transcriptional and translational levels.

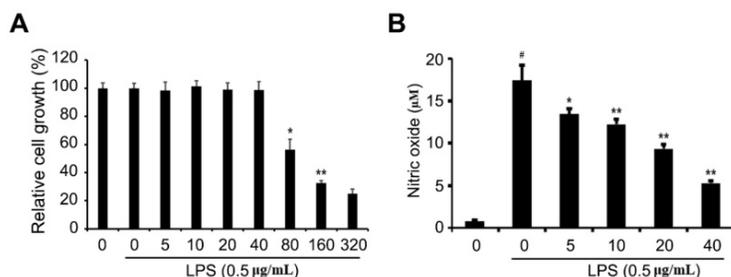


Figure 2. Effects of ETMI on cell viability and NO production in LPS-induced RAW 264.7 macrophages. After 24 h of treatment, cell viability was evaluated using an MTT assay (A) and NO production was determined by Griess reaction (B). The results are shown as mean \pm SD values of three experiments. **p* < 0.05 , ***p* < 0.01 , and #*p* < 0.001 . ETMI: ethanol extract of *Micractinium* sp.; LPS: lipopolysaccharide; MTT: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; NO: nitric oxide

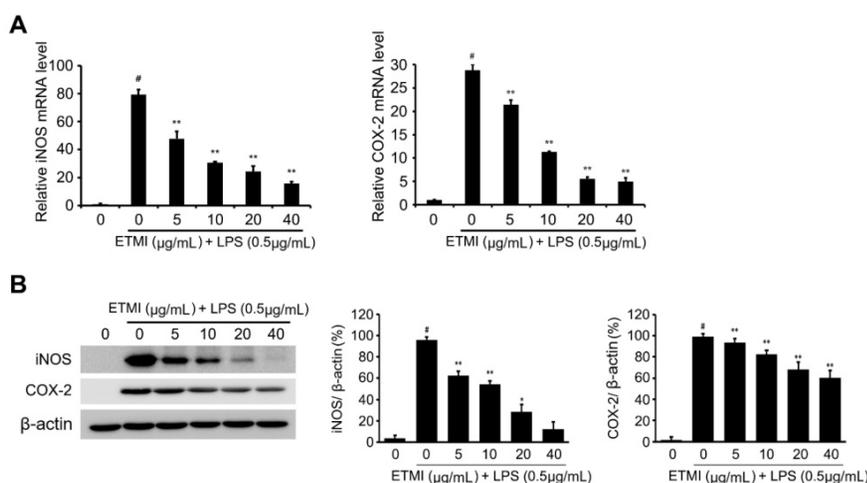


Figure 3. Effects of ETMI on the expression levels of proinflammatory mediators. The Transcriptional (A) and translational (B) levels of iNOS and COX-2 genes in response to ETMI treatment. Results are shown as mean \pm SD values of three experiments; **p* < 0.05 , ***p* < 0.01 , and #*p* < 0.001 . ETMI: ethanol extract of *Micractinium* sp.; iNOS: inducible nitric oxide synthase; COX: cyclooxygenase

Effects of ETMI on LPS-stimulated proinflammatory cytokine production

Many proinflammatory cytokines including TNF- α and IL-6 can be important regulators of inflammatory processes and diseases [15, 16]. To evaluate the effect of ETMI on the expression patterns of proinflammatory cytokine genes, RAW 264.7 macrophages were treated with different concentrations of ETMI from 5 to 40 $\mu\text{g}/\text{mL}$ for 1 h and then stimulated with 1 mg/mL LPS for 24 h. We found that the LPS-induced expression of TNF- α and IL-6 significantly decreased in the ETMI-treated cells in a concentration-dependent manner compared to that in the untreated cells with or without LPS stimulation (Fig. 4). Next, we investigated the effect of ETMI on the production of cytokines in RAW 264.7 macrophages by detecting TNF- α and IL-6 using ELISA. As shown in Fig. 4, ETMI, at a concentration of 40 $\mu\text{g}/\text{mL}$, significantly decreased the LPS-mediated increase in the level of IL-6 in RAW 264.7 cells by approximately 5-fold over that in the controls, while the LPS-stimulated TNF- α production slightly decreased in a dose-dependent manner despite a significant decrease in its mRNA level. These data suggest that ETMI may be a promising regulator of inflammatory processes through the regulation of proinflammatory cytokine production.

Cytotoxic effect of ETMI on HCT116 cells

Chronic inflammation has been reported to cause cancer through the modulation of oncogenic signaling pathways [21]. We wondered whether ETMI can inhibit tumorigenesis by exerting anti-inflamm-

atory activity. To evaluate the growth-inhibitory effect of ETMI on HCT116 cells, a colorimetric MTS assay was performed. HCT116 cells were treated with various concentrations of ETMI (0, 12.5, 25, 50, and 100 $\mu\text{g}/\text{mL}$) for 24 h. As shown in Fig. 5A, at concentrations of ≥ 25 $\mu\text{g}/\text{mL}$, ETMI suppressed the proliferation of the HCT116 cells in a concentration-dependent manner, suggesting that ETMI exhibits concentration-dependent inhibitory activity against HCT116 cells. Furthermore, the conclusion was supported by the colony formation of ETMI-treated cancer cells: the number of ETMI-treated cell colonies decreased compared to those of the control cells (Fig. 5B).

Induction of cell cycle arrest in the ETMI-treated HCT116 cells

To further understand the potential mechanisms by which ETMI exhibits its inhibitory activity, the effect of the extract on cell cycle distribution in HCT116 cells was investigated using a flow cytometry assay. As shown in Fig. 6, the cell population in the G0/G1 phase significantly increased in a dose-dependent manner, while that in the S phase decreased. Of the control cells, 38.61% were in the G0/G1 phase, 36.93% were in the S phase, and 24.45% were in the G2/M phase. However, the percentage of extract-treated cells in the G1 phase (38.61%) gradually increased with an increase in the concentrations of the extract up to 100 $\mu\text{g}/\text{mL}$ (38.61% at 0 $\mu\text{g}/\text{mL}$, 43.83% at 25 $\mu\text{g}/\text{mL}$, 46.77% at 50 $\mu\text{g}/\text{mL}$, and 51.83% at 100 $\mu\text{g}/\text{mL}$). The cell population in the S phase decreased at the same concentrations (36.94% at 0 $\mu\text{g}/\text{mL}$, 37.32% at 25 $\mu\text{g}/\text{mL}$, 31.47% at 50 $\mu\text{g}/\text{mL}$ and 27.16 $\mu\text{g}/\text{mL}$) (Fig. 6A). These data provide strong evidence of cell cycle arrest at the G1 phase, and in turn, the inhibition of HCT116 cell division by ETMI. To further investigate the effect on cell cycle distribution, the transcriptional levels of marker proteins of G1 phase transition were analyzed by qRT-PCR. The results (Fig. 6B) indicated that ETMI treatment led to significantly increased levels of p21 mRNAs in a concentration-dependent manner, whereas the expression of CDK4 and CDK6 mRNAs decreased. These data suggest that ETMI suppressed proliferation, at least in part, by modulating the expression levels of regulatory genes in the G1/S transition phase.

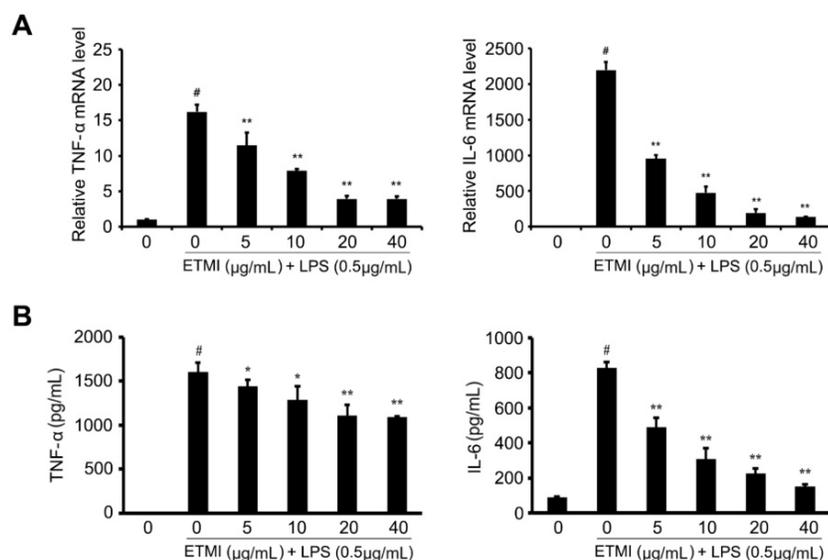


Figure 4. Effects of ETMI on the expression and production of proinflammatory cytokines. Transcriptional levels (A) and production (B) of cytokines, TNF- α and IL-6. Results are shown as mean \pm SD values of three experiments. * $p < 0.05$, ** $p < 0.01$, and # $p < 0.001$. ETMI: ethanol extract of *Micractinium* sp.; TNF: tumor necrosis factor; IL: interleukin

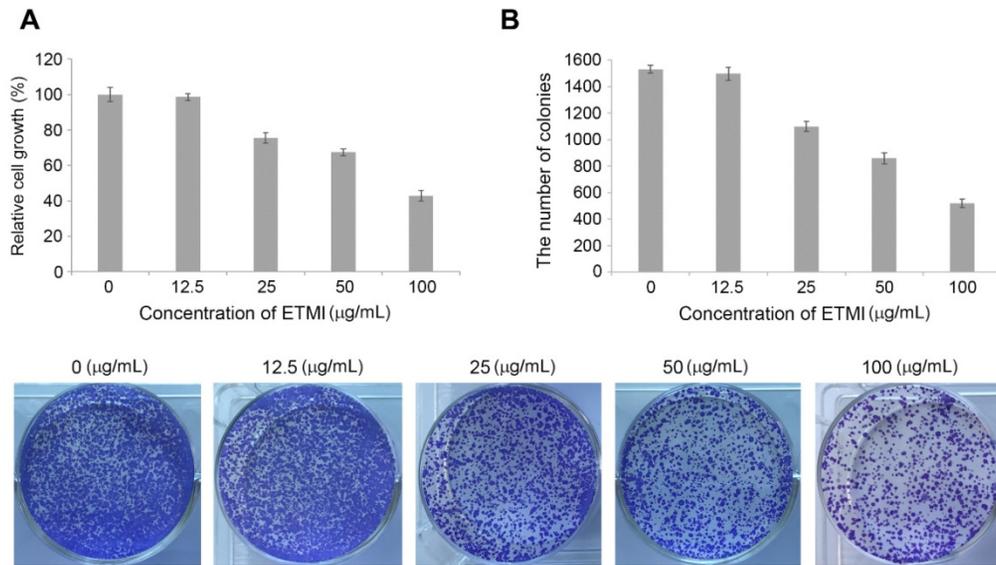


Figure 5. Effects of ETMI on the growth of human colon cancer cells, HCT116. Inhibition of cell proliferation (A) and colony formation (B) in response to ETMI treatment. ETMI: ethanol extract of *Micractinium* sp.

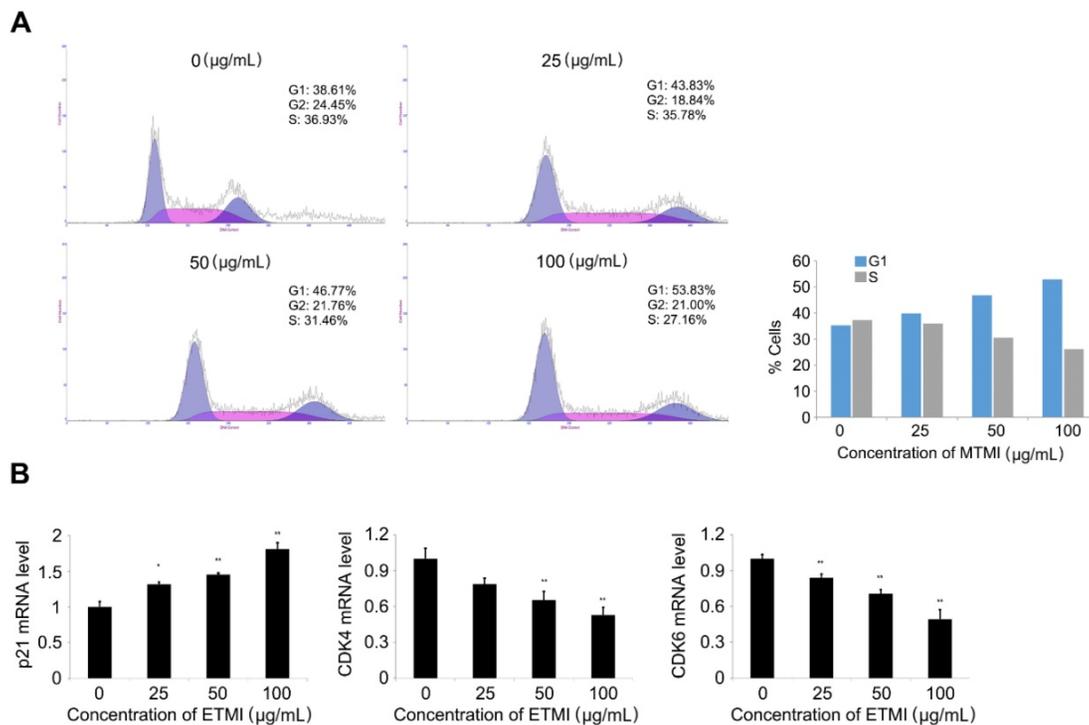


Figure 6. Induction of cell cycle arrest (A) and expression pattern of the cell cycle-regulated genes in response to ETMI treatment (B). Results are shown as mean \pm SD values of three experiments. * $p < 0.05$, ** $p < 0.01$. ETMI: ethanol extract of *Micractinium* sp.

Discussion

It is known that chronic inflammation causes various diseases including cancer [22]. Therefore, if the inflammatory reaction is not controlled early, it can develop into a chronic infection, an autoimmune disease, or a metabolic disease, due to loss of function and disruption of homeostasis in the body. Recent studies that investigated whether chronic inflammation increases cancer incidence have shown that

inflammation and cancer have a common signaling pathway involving a vicious cycle linkage at the molecular level [23, 24]. Inflammatory substances secreted by inflammatory cells are closely related to the proliferation, survival, and metastasis of cancer cells. In the inflammatory tumor microenvironment, inflammation not only promotes the survival and proliferation of cancer cells but also induces angiogenesis and metastasis by destroying the

adaptive immune response and altering the response to hormones and drugs. For example, when acute inflammation is not resolved and progresses to chronic inflammation, inflammatory cells are broken down and TNF- α , IL-1, and IL-6 cause DNA damage or mutagenesis. Inflammatory cells overexpress inflammatory cytokines or enzymes such as EGF, MMP, and MCP [12-15]. In addition, in the inflammatory tumor microenvironment, hypoxia-induced factor produces vascular endothelial growth factor (VEGF) [25]. These substances can change the inflammatory microenvironment around the tumor and help the tumor to proliferate and induce metastasis by making it easier for the tumor cells to break down nearby tissues and spread to other organs. In the present study, we demonstrated that ETMI exhibited anti-inflammatory and anticancer activities, using several *in vitro* assays in RAW 264.7 macrophages and HCT116 cells. Based on our data, ETMI exerts anti-inflammatory effects by modulating the expression levels of inflammatory regulators such as COX-2, IL-6, iNOS, and TNF- α , and the production of inflammation-stimulated NO and proinflammatory cytokines such as IL-6 and TNF- α . In particular, anti-inflammatory activity was observed at a relatively low concentration (5 $\mu\text{g}/\text{mL}$) of ETMI, indicating a decrease in the expression levels of inflammatory mediators compared to those reported previously [26, 27]. ETMI may include bioactive molecules with strong anti-inflammatory activities, which are necessary for adaptation in harsh conditions such as those in the Antarctic region. In general, to adapt to extreme environments such as those in the Antarctic region, organisms including microalgae have developed specific self-defense mechanisms by synthesizing toxic secondary metabolites, which have been found to be pharmaceutically useful. In this aspect, bioactive compounds from Antarctic organisms may be promising pharmaceutical targets for developing new drugs. Based on our data on inflammatory molecular mechanisms, ETMI effectively regulated the transcription level of proinflammatory mediators. However, whereas both transcription levels and the production of proinflammatory cytokine IL-6 were significantly inhibited by ETMI treatment, TNF- α production slightly reduced in a dose-dependent manner compared to the marked reduction in its mRNA levels. This regulatory discrepancy between TNF- α mRNA and production may occur because the rapid turnover of TNF- α mRNA does not have a large effect on TNF- α biosynthesis in macrophages. In fact, it has been reported that TNF- α mRNA is short-lived even in conditions of maximal TNF- α production during tumor necrosis, suggesting that TNF- α mRNA

rapid turnover is not involved in the regulatory mechanism of TNF- α biosynthesis [28]. On the other hand, ETMI exerts anticancer activity to inhibit proliferation of human colon cancer cells by arresting the cell cycle. In particular, ETMI induced G1 phase arrest in a dose-dependent manner by modulating the expression level of G1/S regulators such as *CDKN1A*, *CDK4*, and *CDK6*. This result suggests that some bioactive molecules in ETMI play an important role in the tight regulation of G1/S phase transition.

In the present study, we demonstrated that ethanol extract from Antarctic freshwater microalga, *Micractinium* sp., has anti-inflammatory and anticancer activities against macrophages and colon cancer cells, respectively. Recently, to better understand the regulatory mechanism associated with the inflammation-to-cancer transition, investigation of the specific roles of the main regulatory molecules involved in this process has been emphasized. The identification of bioactive compounds that mediate the common signaling pathway between inflammation and cancer and the development of drugs that interfere with the function of these proteins may be an effective approach for treating cancer by eliminating the link between inflammation and cancer. Taken together, our results indicate that ETMI may be a promising candidate for the development of a pharmaceutically effective drug for cancer therapy.

Acknowledgement

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Competing Interests

The authors have declared that no competing interest exists.

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