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Biological Activity of Chemical Constituents Isolated from Strain *Chlamydomonas* sp. KSF108 (Chlamydomonadaceae)

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Abstract – This study focused on investigation of the immunosuppressive inhibitory effect through determination of IL-2 production of nine compounds (**1** – **9**) isolated from *Chlamydomonas* sp. KSF108. Among them, compounds **1**, **5**, and **6** displayed moderately inhibitory effects on IL-2 production at a concentration of 100 μM. In addition, the related ones including cytotoxic, anti-inflammatory, and anti-oxidant activities were also elucidated. **6** further displayed cytotoxic activity against the MCF-7 cell line, with an IC_{50} value of 17.2 μM and **4**, **6** − **7**, and **9** possessed significant DPPH radical scavenging activity, with IC_{50} values ranging from 3.1 to 4.4 μM. To the best of our knowledge, this is the first report on the bioactivity of isolated chemical constituents from the genus *Chlamydomonas.* Compounds **1** and **5** investigated for the first time in the activity of immunosuppressivity and **6** may come to serve as the most important marker in broad-spectrum activities of the secondary metabolites identified from *C.* sp. KSF108.
Keywords – Chlamydomonadaceae, *Chlamydomonas* sp. KSF108, Immunosuppressive, Anti-inflammatory, Cytotoxic, Anti-oxidant

Introduction

The *Chlamydomonas* genus comprises unicellular chlorophyte algae with two anterior flagella, a basal chloroplast surrounding one or more pyrenoids, and a distinct cell wall. Species within the genus have been distinguished by differences in overall size, body shape, positions of the chloroplast and pyrenoids, flagellar length, number and position of contractile vacuoles, and more subtle structural features that are visible at the light microscope level.[1] Previous chemical investigations of the algae have reported the presence of carotenoids, fatty acids, sterols, mycosporine-like amino acids, phycobilins, polyketides, pectins, halogenated compounds, and toxins. In addition, algal extracts and secondary metabolites have shown anti-inflammatory and anti-cancer activities, and their pharmacological and therapeutic potentials have increasingly been noted.[2] In our recent studies, we have focused on strains of fungi, green yeast samples, collected from the unexplored environment of the King Sejong Antarctic Station. These objects were maintained in a suitable life environment and tested for the priority of IL-2 production. The results indicated that the methanolic extract of *C.* sp. KSF108 exhibited selectivity activity (data not shown), and this strain was therefore chosen for further follow-up research. In this study, nine compounds (**1** − **9**), including one norisoprenoid (**1**), one polyol-glycoside (**2**), three sterols (**3** − **5**), three phenols (**6**, **8**, and **9**), and one unsaturated fatty acid compound (**7**), from *C.* sp. KSF108 are elucidated for their immunosuppressive effect by determining the IL-2 production, as well as related activities such as the cytotoxic effect on three human tumour cell lines, anti-inflammatory activity in the inhibition of NO production, and anti-oxidant activity with the determination of DPPH radical scavenging properties.

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Experimental

Microalgae – This strain collected from freshwater of King Sejong Antarctic Station (62° 13' S, 58° 47' W) in 2014 and maintained in Bold's basal medium (BBM) consist of 10 mL $NaNO_3$ 2.94 mM, 10 mL $CaCl_2 \cdot 2H_2O$ 0.17 mM, 10 mL $MgSO_4 \cdot 7H_2O$ 0.3 mM, 10 mL K_2HPO_4 0.43 mM, 10 mL KH_2PO_4 1.29 mM, 10 mL NaCl 0.43 mM, 1 mL mixture of alkaline EDTA solution 17.10 mM and EDTA (Titriples III) KOH 55.30 mM, 1 mL $FeSO_4 \cdot 7H_2O$ 0.18 mM, 1 mL H_3BO_3 18.50 mM, and 1 mL mixture of $ZnSO_4 \cdot 7H_2O$ 8.82 mM, $MnCl_2 \cdot 4H_2O$ 1.44 mM, MoO_3 0.71 mM, $CuSO_4 \cdot 5H_2O$ 1.57 mM, and $Co(NO_3)_2 \cdot 6H_2O$ 0.49 mM and 1000 mL of distilled water in a Erlenmeyer flask keeping at pH value of 6.6 and incubated at 2 – 3 °C under continuous lighting from light-emitting diodes at an intensity of 35 μmol photons $m^{-2}s^{-1}$. Gene sequence of strain was compared with those deposited in public databases. The morphological, gene sequence, and phylogenetic data indicated that this strain was representative member of the genus *Chlamydomonas* and referred as strain of *Chlamydomonas* sp. KSF108. Green yeast identification was performed by Professor Sanghee Kim and a voucher specimen (KPRI-KSF108) was deposited at the Division of Polar Life Sciences, Korea Polar Research Institute, Korea. *C.* sp. KSF108 was continuously refermented on a large scale in the same medium previously used to prepare the fractionation.

Isolation of tested compounds – Isolation of compounds (**1** – **9**) from *C.* sp. KSF108 described in our previous study.[2] Their structures are presented in Fig. 1.

Immunosuppressive activity – Jurkat T cells viability after 24 h of continuous exposure to the compounds were measured with a colorimetric assay based on the ability of mitochondria in viable cells to reduce MTT. Total RNA was isolated from Jurkat T using TRIZOL reagent (JBI, Korea). cDNA was synthesized by performing reverse transcription PCR using RT Pre-Mix (Enzynomics, Korea). The mRNA level of the IL-2 and GAPDH gene were measured by PCR with primers. The primers and PCR conditions for each gene were as follows: human IL-2, 5'-CACGTCTTGCACTTGTCAC-3' and 5'-CCTTCTTGG GCATGTAAAACT-3'; human GAPDH, 5'-CGGAGTCA ACGGATTTGGTCGTAT-3' and 5'-AGCCTTCTCCATG GTGGTGAAGAC-3'. The amplification profile consisted of denaturation at 94 °C for 30s, annealing at 60 °C for 20s, and extension at 72 °C for 40s. The 30 cycles were preceded by denaturation at 72 °C for 7 min.[3]

Cytotoxic activity – The MCF-7, HL-60, and Hela cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 with 15 mM HEPES buffer, L-glutamine, and pyridoxine hydrochloride supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a 96-well plate at a density of 6×10^4 cells/mL. After reaching confluence (2×10^5 cells/mL), the cells were treated with the compounds. The compounds were dissolved in dimethylsulfoxide (DMSO) and the final concentration of DMSO was less than 0.1% (*v*/v). Various concentrations of tested compounds were prepared with serial dilutions. The experiment allowed to proceed for 48h at 37 °C in a humidified 5% CO_2 atmosphere. At the end of this period, supernatants were discarded. To minimize the interference of supernatant residue, the adherent cells were washed twice with Dulbecco's Phosphate-Buffered Saline (DPBS), and then 20 μL of MTT stock solution (5 mg/mL) was added to each well and the plates were further incubated for 3h at 37 °C. DMSO (100 μL) was added to each well to solubilize the water-insoluble purple formazan crystals. After 1h, the absorbance was measured at 570 nm with a microplate reader. Adriamycin, a commercial standard anticancer agent, was used as a positive control. The 50% reduction in cell number

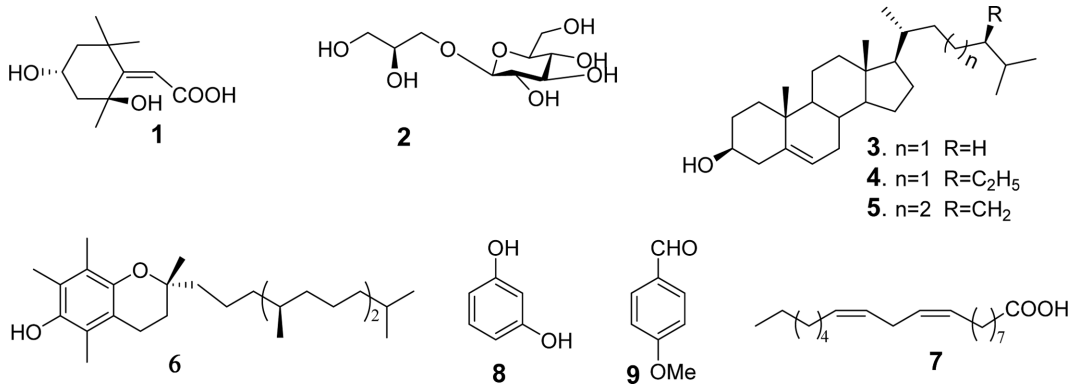

Fig. 1. The structure of compounds (**1** – **9**) isolated from *C.* sp. KSF108.

relative to the control (IC_{50}) was estimated visually. The results are presented as mean ± standard error of mean (SEM).[4]

Anti-inflammatory activity – The RAW264.7 cell viability after 24 h of continuous exposure to the compounds was measured with a colorimetric assay based on the ability of mitochondria in viable cells to reduce MTT. The level of NO production was determined by measuring the amount of nitric oxide from the cell culture supernatants as described previously. Briefly, the RAW264.7 cells (1×10^5 cells/well) were stimulated with 1 μg/mL of LPS for 24 h in the presence or absence of the test compounds (1 – 100 μM). The cell culture supernatant (100 μL) was then reacted with 100 μL of Griess reagent. Celastrol was used as a positive control.[5]

Anti-oxidant activity – The DPPH assay was carried out in 96-well plates (Greiner F bottom) and each compound was tested at five concentrations of 500, 125, 31.25, 15.62, and 7.81 μM in DMSO. In the well of 100 μL, 5 μL of each solution was added, and filled with 95 μL of DPPH solution (158 mM in EtOH 50%). A blank control was prepared with a mixture of 5 μL DMSO in 95 μL of EtOH 50% and the positive controls were prepared by using the mixture of 5 μL of L-ascorbic acid (at 125.0, 31.25, 15.62, 7.81, and 3.90 μM) and 95 μL of DPPH solution. The control solution was a mixture of 5 μL DMSO and 95 μL DPPH. The assay was performed at 37 °C in the dark during 60 min and the decrease in absorbance was measured at 570 nm with a microplate reader FLUO star Omega.[6]

Results and Discussion

From MeOH soluble fraction, nine compounds (**1** – **9**), consisting of (*Z*)-2-(2,4-dihydroxy-2,6,6-trimethylcyclohexylidene)acetic acid (**1**),[7] lilioside D (**2**),[8] cholesterol (**3**),[9] *β*-sitosterol (**4**),[10] hurgadacin (**5**),[11] *α*-tocopherol (**6**),[12] 9*Z*,12*Z*-nonadecadienoic acid (**7**),[13] *m*-hydroxyphenol (**8**),[14] and *p*-methoxybenzaldehyde (**9**),[15] were isolated and identified by comparison of spectroscopic data with those of compounds reported in the literature (Fig. 1).

To determine the immunosuppressive effects of the nine isolated compounds, we assessed the inhibitory effect of IL-2 production on T cell activation and tested the compounds by evaluating IL-2 mRNA expression. Among the compounds, **1**, **5**, and **6** exhibited the moderate inhibition of IL-2 expression in activated Jurkat T cells. The Jurkat T cells were pre-incubated with **1**, **5**, and **6** at a concentration of 100 μM, which led to a moderate inhibition of IL-2 expression (Fig. 2). It could be concluded that **1**, **5**, and **6** potentially exert inhibitory effects on IL-2 expression. In this study, for the first time, immunosuppressive investigations of **1**, a norisoprenoid, and **5**, a sterol, were conducted to obtain the inhibitory effect of IL-2 production, whereas **6** was confirmed to show this expression at a concentration of 100 μM, and the mechanism of these effects was shown through the modulation of PPAR-*γ*, I*κ*B*α*, and apoptotic pathway in activated splenocytes.[16] This result confirmed the long-established notion that the expression of IL-2 production plays an important role in cancer immunotherapy, and that it should clearly be approved for monotherapy or combined therapies of metastatic renal cell carcinoma and melanoma.[17] Among the isolated compounds, **3**, **4**, and **5** were found to have similar structures, but they showed different function units in the side chain, with *exo*-methylene instead of protons or an ethyl group in **5**, which led to the inhibition effect of IL-2 production in the order of **5** > **3** and **4**.

To understand whether the effects of the nine isolated compounds were influenced by cytotoxicity as well as the relationship between immunosuppressivity and cytotoxicity, we investigated cytotoxic activity by determining the growth inhibitory regulations of MCF-7, HL-60, and Hela cancer cell lines. To this end, these cell lines were treated with various concentrations of the tested compounds and incubated for 48 h. Their cytotoxic effects on MCF-7, HL-

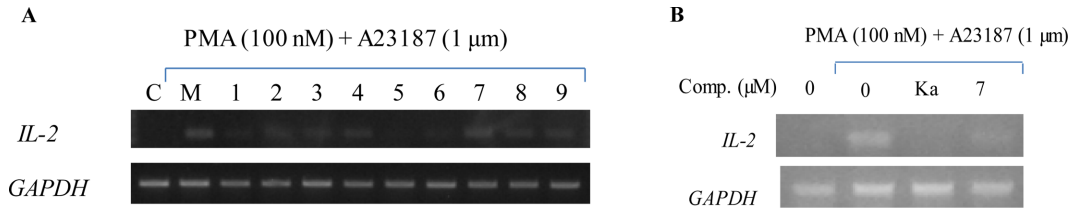

Fig. 2. The inhibition of isolated compounds on IL-2 production in activated T cells. Jurkat T cells (1×10^6/well) were pre-incubated for 30 min with isolated compounds at concentration of 100 μM, and then the cells were stimulated for 6 h with PMA (100 nM)/A23187 (1.0 μM). After 6h, IL-2 mRNA levels were detected by PCR. (A) The inhibitory effect of all isolated compounds (**1** – **9**). C: Blank; M: mRNA level of Jurket T cell without stimulation and compound. (B) The comparison of compound **7** and kaempferol (Ka, 100 μM) as positive control.

Table 1. Inhibitory effect of **1** − **9** on MCF-7, HL-60, Hela cells, and DPPH radical scavenging

Compounds	Anti-cancer			Anti-oxidant
	MCF-7	HL-60	Hela	DPPH
	IC_{50}, μM[a]			
1	-	>100	>100	-
2	-	>100	>100	-
3	-	>100	>100	-
4	-	>100	>100	3.1 ± 0.3
5	-	>100	>100	-
6	17.2 ± 0.7	>100	>100	4.3 ± 0.5
7	-	>100	>100	4.2 ± 0.2
8	>100	>100	>100	-
9	>100	>100	>100	4.4 ± 0.3
Adriamycin[b]	$(2.5 \pm 0.4) \times 10^{-2}$	$(2.4 \pm 0.6) \times 10^{-3}$	0.49 ± 0.05	
Ascorbic acid[b]				34.9 ± 1.2

[a]The results are presented as mean ± SEM (n = 3).
[b]Positive control

60, and Hela cancer cell lines are summarized in Table 1. The IC_{50} values indicated that most of compounds were less sensitive on the three tested cell lines, except for **6**, which showed cytotoxic activity against the MCF-7 cell line with an IC_{50} value of 17.2 μM. In previous papers, α-tocopherol was used as a natural product in the design of effective chemotherapy and showed enhanced cytotoxic, immunosuppressive, and anti-oxidant activities. Moreover, α-tocopherol was demonstrated to have anticancer activities on several cell lines including ORL-48 (oral squamous carcinoma), T47D (estrogen-receptor positive), MDA-MB-231 (estrogen-receptor negative), MCF-7 (breast cancer), and CRL-1740 (prostate carcinoma).[18-21] The cytotoxic activity of this compound on MCF-7 cell line was confirmed by the results of the present study. The evidence presented above indicates that a combination of anti-cancer immunotherapies can be considered as a potent treatment method for breast cancer in the future, but that further studies are required to investigate the mechanisms of these integrated therapy pathways.

To determine the anti-inflammatory effects of the compounds isolated from *C.* sp. KSF108 on RAW264.7 cells, the cells were treated in the presence or the absence of various concentrations of the nine isolated compounds ranging between 1 – 100 μM that were incubated with 1 μg/mL of LPS for 24h, then reacted with Griess reagent. The results indicated that none of compounds displayed the inhibition of NO production in activated RAW264.7 cells (data not shown).

The anti-oxidant activities of all of the compounds were determined by DPPH radical scavenging assay, and the results, including IC_{50} values, are summarized in Table 1. Among compounds, **4** displayed the strongest antiradical activity with an IC_{50} value of 3.1 μM, then **6**, **7**, and **9** also showed effects with IC_{50} values ranging from 4.2 to 4.4 μM. Compounds **3, 4**, and **5** have similar structures, but the ethyl terminal group linked to the side chain in **4** appears to influence the DPPH radical scavenging activity. For compound **6**, it was reported that the potent anti-oxidant activity is caused by its possession of a free phenol hydroxy group in functional moiety, which increases the role of the anti-oxidant activity in the inhibition and destruction of MCF-7 cancer cell development. The DPPH radical scavenging activity of **6** was confirmed in this study with an IC_{50} value of 4.3 μM.[18,19]

In conclusion, bioactive investigation of the isolated chemical constituents from the genus *Chlamydomonas* reported for the first time. In this study, we provide evidence showing that Antarctic microalgae source, represented flagellated single-celled strains, could distribute as potential candidates in finding of new agents for immunosuppressive and anti-cancer drugs but further studies are required to explore their molecular mechanisms in greater detail.

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