Identification of *Ophiocordyceps gracilioides* as an antitumor natural drug resource

抗腫瘍性天然薬物資源としての*Ophiocordyceps gracilioides* の同定

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Contents

Abbreviations

BSA	Bovine serum albumin
^{13}C NMR	Carbon Nuclear Magnetic Resonance
CCK-8	Cell counting kit-8
J	Coupling constant
DAD	Diode array detection
$CDCl_3$	Deuterated chloroform
DMSO	Dimethyl sulfoxide
ESI	Electrospray ionization source
ELISA	Enzyme-linked immunosorbent assay
HPLC-MS	High performance liquid chromatography-mass spectrometry
IL-6	Interleukin-6
δ	ppm
^{1}H NMR	Proton Nuclear Magnetic Resonance
STAT3	Signal transducer and activator of transcription 3
TLC	Thin Layer Chromatography
NF-κB	Nuclear factor-kappaB
NMR	Nuclear Magnetic Resonance
MS	Mass
m/z	Mass-to-charge ratio
MHz	Megahertz
*μ*M	Micro molar
*μ*g/mL	Microgram per milliliter

Preface

This thesis is submitted for the degree of Doctor of Philosophy (Ph.D.) to the Affiliation of Graduate School of Medicine and Pharmaceutical Sciences for Education, University of Toyama, Japan. This work was carried out in the Laboratory of Cancer Biology and Immunology, Section of Host Defences, Institute of Natural Medicine. The results within this thesis were published in two papers as follow:

1. Shin, M.-K., Sasaki, F., Ki, D.-W., Win, N.N., Morita, H., Hayakawa, Y. (2021). Identification of *Ophiocordyceps gracilioides* by its anti-tumor effects through targeting the NFκB-STAT3-IL-6 inflammatory pathway. *Biol. Pharm. Bull.*, 44 (5), 686-690. doi: 10.1248/bpb.b20-01032

2. Shin, M.-K., Sasaki, F., Ki, D.-W., Win, N.N., Morita, H., Hayakawa, Y. (2021). Anti-metastatic effects of ergosterol peroxide from the entomopathogenic fungus *Ophiocordyceps grailioides* on 4T1 breast cancer cells. *J. Nat. Med.*, 1-9, Online published (Apr 27th). doi: 10.1007/s11418-021-01520-2

Chapter 1

General introduction

1.1 Background

1.1.1 Entomopathogenic fungus

An entomopathogenic fungus has a number of referential terms such as *Cordyceps sensu lato* (*s.l.*), caterpillar fungus. *Cordyceps s.l.* is a type of medicinal mushroom, it is called *To Chu Ka So* in Japanese (a worm in winter transform into herb in summer) [1]. The origin of *Cordyceps* name was derived from the Greek word “kordyle”, and the Latin stem “-ceps” meaning club and head, respectively [2]. Most fungus of *Cordyceps s.l.* parasite on the insect (host) in winter and multiplies until it turs into a fungal hyphae, and then it usually erupts out of host head part to keep the fruiting body in summer (Figure 1) [3]. The main differences for occurrence of *Cordyceps s.l.* are the host insect what they infect and the locality where they grow [2]. The *Cordyceps s.l.* fungus invade to various species of insects at different life cycles (egg, larva, pupa, and adult stage) and then reproduces fruiting body (Figure 2) [4]. Those are distributed all over the world especially Japan, Korea, China, Nepal, Thailand, and Bhutan, and are most abundant in highlands, equivalent to ecological environment factors such as biogeography, temperature, humidity, and light [5].

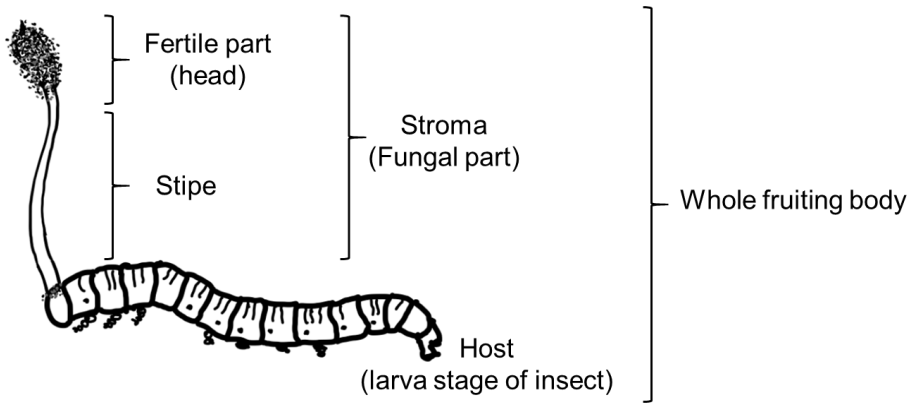


Figure 1. Morphological features of *Ophiocordyceps sinensis*, best known as a *Cordyceps s.l.*.

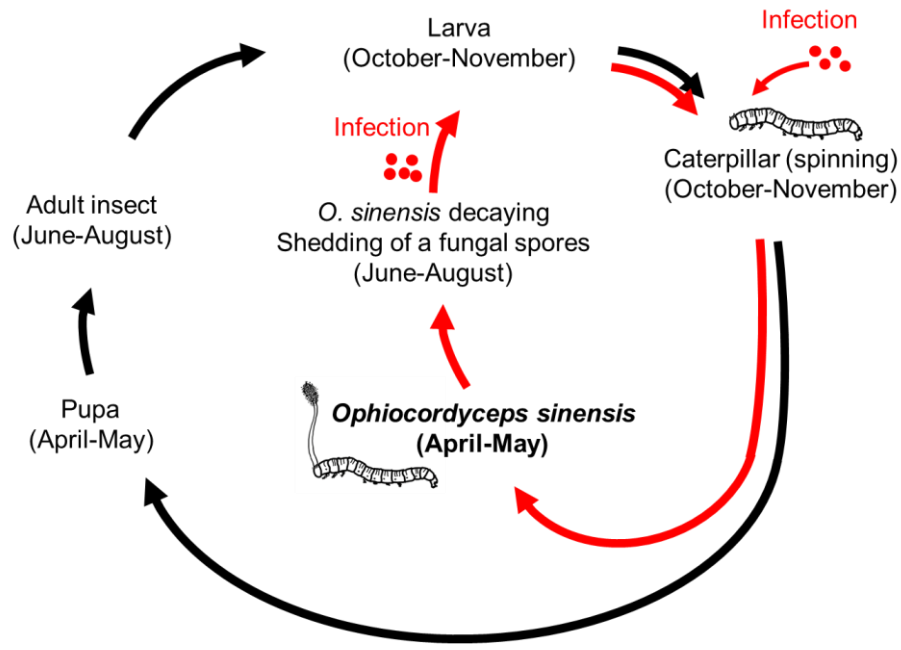


Figure 2. The life cycle of the insect (black color arrow) and the infection cycle of *Ophiocordyceps sinensis* (red color arrow).

The usage of a fungus so-called "*Cordyceps*" that parasitic to insect was documented first in 1757 in old medical Chinese book [6]. It is valuable as a tonic to strengthen the lung and kidney on human health for thousands of years [7]. In recent decades, *Cordyceps s.l.* is increasing popular, and has been collected from worldwide of approximately 600 species [8]. Meanwhile, the genus *Cordyceps* is classified in family Clavicipitaceae, order Hypocreales, division Ascomycota, kingdom Fungi by Fries (1818) to accommodate various species which have different features of producing specific shape of the stromata [9]. Besides, many *Cordyceps* species re-classified to new genera based on molecular phylogenetic analyses; however, many more *Cordyceps* species still not categorized and another problem is that a single fungus has multiple names, because of lack of molecular phylogenetic studies or inconclusive morphological and ecological assessment [10]. Innumerable name authorized to one fungus was renamed and applied to a single generic name by the ICN (International Code

of Nomenclature for algae, fungi, and plants; known as Melbourne Code) in 2011 [11]. Complying with ICN code (one name of one fungus), the phylogenetic arrangement of *Cordyceps s.l.* (a large genus *Cordyceps*) discussed and divided to family Clavicipitaceae, Ophiocordycipitaceae, and Cordycipitaceae [12]. The explanation of classification by ICN illustrated in detail in Figure 3.

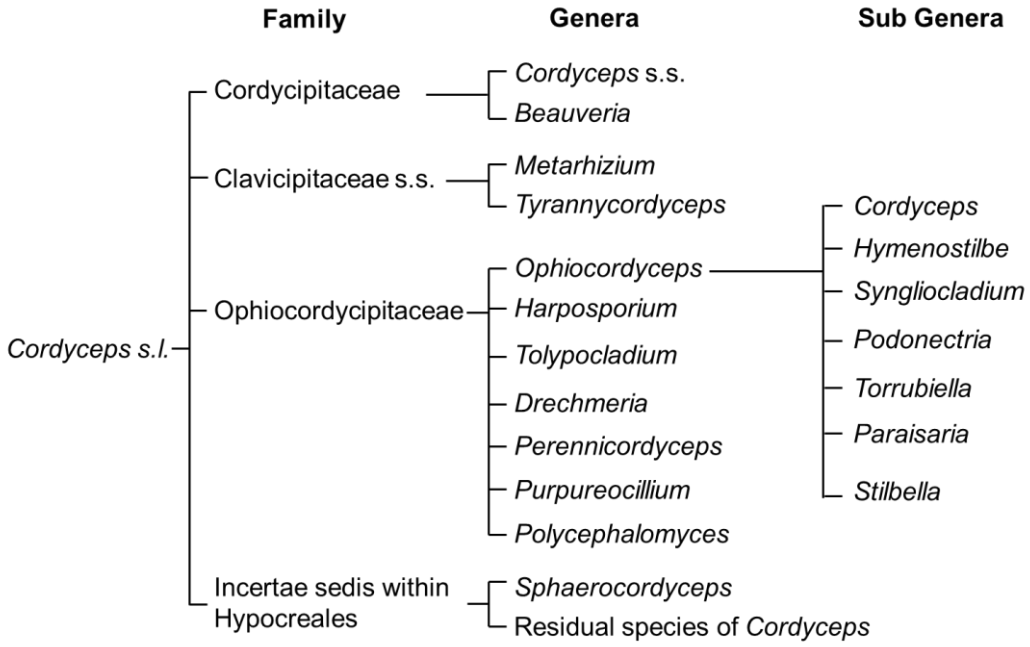
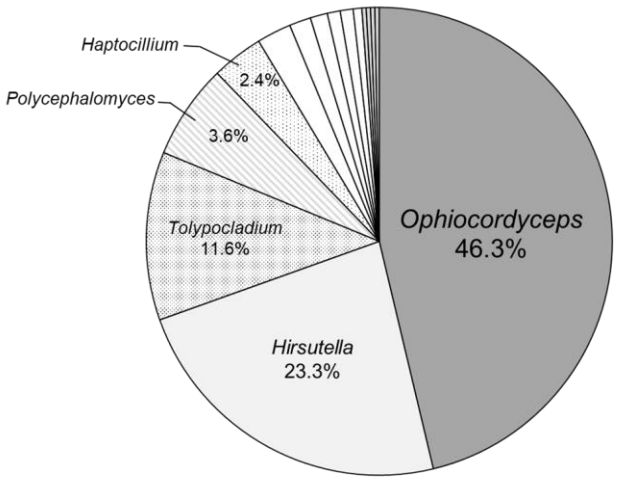


Figure 3. Modern taxonomy of *Cordyceps s.l.* (Reproduced from *Mycology*, 2017; 8 (4), 293-302) [12].

Out of several species in *Cordyceps s.l.*, genus *Ophiocordyceps* is the largest genus composed to approximately 46.3% amongst classification of family Ophiocordycipitaceae (Figure 4) [13]. Among them, *Ophiocordyceps sinensis* (previously named *Cordyceps sinensis*) is the most famous fungal species in the genus *Ophiocordyceps* [14].



Ophiocordycipitaceae	
Genus (*n* =15)	Species (*n*)
Ophiocordyceps	155
Hirsutella	78
Tolypocladium	39
Hymenostilbe	22
Polycephalomyces	12
Haptocillium	8
Syngliocladium	5
Perennicordyceps	4
Didymobotryopsis	3
Purpureocillium	3
Paraisaria	2
Blistum	1
Elaphocordyceps	1
Synnematium	1
Trichosterigma	1

Figure 4. Latest classification of Ophiocordycipitaceae family in *Cordyceps s.l.* (Reproduced from *Int J Med Mushrooms*, 2018; 20 (12), 1149-1162) [13].

O. sinensis is rare and expensive to collect owing to small amount in nature and over-collection [15]. To overcome these problems, therefore, *O. sinensis* has been artificially cultivated in the laboratory and most of studies have evaluated the potent biological activity using artificially cultured one, and these researches have yielded results suggesting that artificially cultured one have the same bioactivity as wild-grown species [16]. Till now, *Cordyceps s.l.* is attracting attention due to it has been reported to have a variety of biological activities, especially anti-tumor activity (Figure 5) [2]. Regarding its potential applications on anti-tumor effect, studies have shown that *O. sinensis* prevent to growth various cancer cells through suppressing NF-κB pathway [17], PI3K/Akt pathway [18], or mTOR signaling [19]. *O. sinensis* has also reported to have anti-metastatic activity against 4T1 breast cancer cells [20]. In addition, *Cordyceps militaris* in *Cordyceps s.l.*, which has been exhibited to have similar components and pharmacological properties with *O. sinensis*, inhibits proliferation of cancer cells by suppressing NF-κB activation [17]. Due to NF-κB pathway, which plays an essential role in inflammation and increases cancer initiation and progression, *O. sinensis* is also demonstrated to down-regulate an inflammatory factors [21].

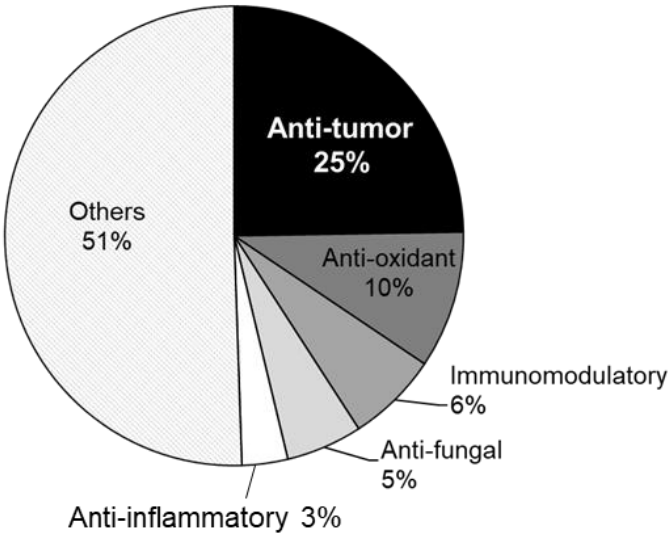


Figure 5. Pharmacological activities of *Cordyceps s.l.* species (Reproduced from *J Tradit Complement Med*, 2013; 3 (1), 16-32) [22]**.**

Although the pharmacologically active components such as nucleosides/nucleotide derivatives, polysaccharides, sterols, fatty acids, and proteins have been extracted from *O. sinensis*, generally, cordycepin (3'-deoxyadenosine; Figure 6) belonging to nucleosides proposed as important active constituents of *O. sinensis* for anti-tumor efficacy [2].

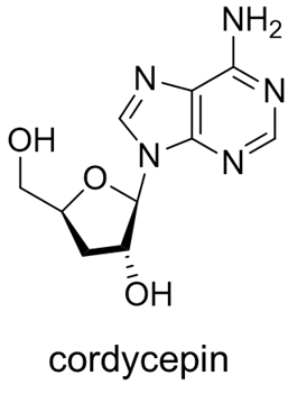


Figure 6. Chemical structure of cordycepin, one of the active component of *O. sinensis*.

Latest literature reported that *O. sinensis* has biological activity through various mechanisms and its active ingredients [22]. Nevertheless, various species fungus of *Cordyceps s.l.* are used traditionally (folk use), but scientific evidence is still unidentified. This could serve the way to lead the development of new drugs through validation of biological activities and establishment of the principle of activity. Previous reviews have focused on *O. sinensis*, however, herein I propose to find scientific evidence of existing species that are still being discovered. The 10 species of genus *Ophiocordyceps* used in this thesis are described in detail in chapter 2 (Table 1). Further studies are required to identify active compounds on biological capacities *in vitro* and *in vivo* experiments.

1.1.2 NF-κB/STAT3 inflammatory pathway in breast cancer

Nuclear factor-kappaB (NF-κB) and signal transducer and activator of transcription 3 (STAT3) collaboratively induce inflammatory signals and its persistence are major factor inducing cancer progression [23-25]. Lasting NF-κB activation upregulates inflammatory factors, such as interleukin-6 (IL-6) cytokine, and then induces DNA damage to promote cell proliferation in a variety types of tumor, such as breast cancer [26], lung cancer [27], liver cancer [28], etc. The STAT protein, a potential cytoplasmic transcription factor for nuclear activation, were described to regulate cell growth, survival, and differentiation in cells. Among STAT proteins, STAT3 plays an important factor in promotion of oncogenesis with chronic inflammation, and is known to be activated after IL-6 stimulation.

Both NF-κB and STAT3, which are activated in malignant tumors, mutually regulate transcriptional activity, as well coordinate by binding at a subset of gene promoter in the nucleus [29]. Moreover, IL-6 cytokine induced by NF-κB/STAT3 positively stimulated to induce NF-κB/STAT3 activation (Figure 7) [30]. As such, NF-κB/STAT3 signaling has been proposed to promote tumorigenesis. In several literatures, natural compounds reported targeting NF-κB or STAT3 in cancer cells, and suggested as a substitute for anti-tumor [29].

Based on these evidences, targeting the cancer-related NF-κB/STAT3 inflammatory pathway in cancer to select new chemical agents is appropriate as a chemotherapeutic approach on anti-tumor effects. Therefore, in this thesis, it was selected as a method to find a substance that inhibits the NF-κB and STAT3 activation in 4T1 breast cancer cells.

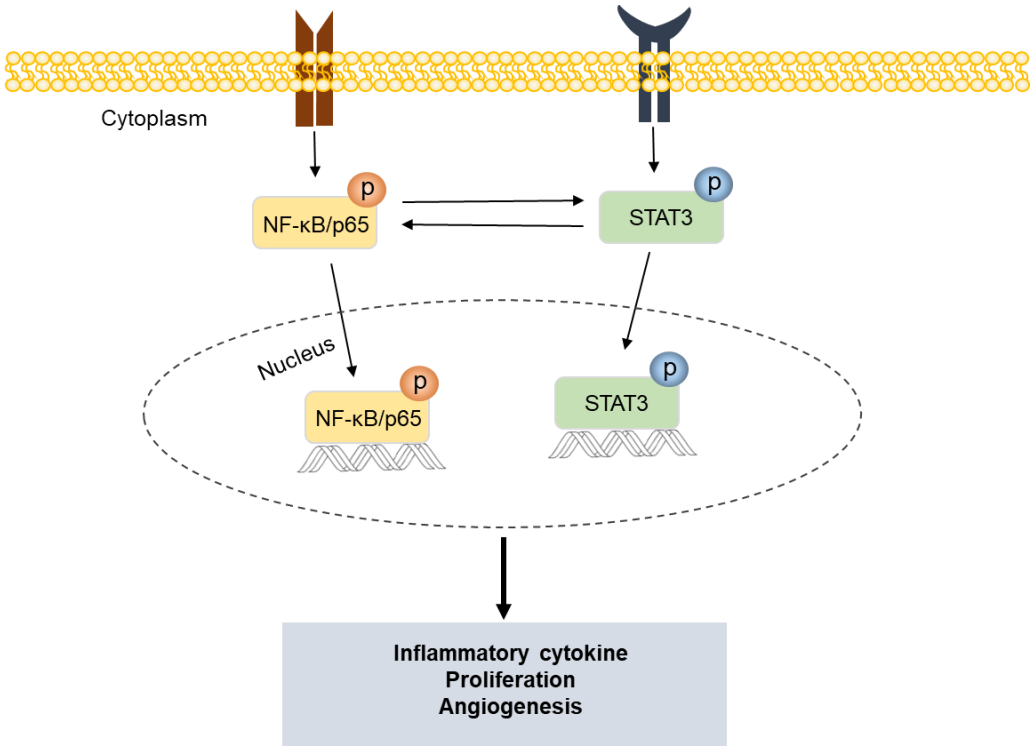


Figure 7. Scheme of NF-κB and STAT3 inflammatory signaling in breast cancer.

1.2 Aims and objectives

A hundreds of *Cordyceps s.l.* have been used widely as a traditional medicine. *Cordyceps s.l.* including *Ophiocordyceps sinensis*, has been elucidated a variety of activities on the anti-tumor, anti-inflammation, and anti-aging, but most of those have not been investigated in their potential for medicinal use. Recently, NF-κB and STAT3 regulate a number of genes involved in cell cycle progression and survival pathways, in addition to regulating a cytokines in breast cancer.

In this thesis, out of ten species in genus *Ophiocordyceps* collected in Japan, *Ophiocordyceps gracilioides* is the best inhibitory activity in both NF-κB and STAT3. *O. gracilioides* was expected that its active ingredient on anti-tumor activity would be a cordycepin, major candidate of *O. sinensis*, but it was confirmed that it was not contained. Thereby, I highlight that inhibition effect by targeting NF-κB/STAT3 activities on *O. gracilioides* treated 4T1 breast cancer cells and purification its active compounds.

In the chapter 2, I determined best one that out of 10 species in genus *Ophiocordyceps*, which is an unexplored species in *Cordyceps s.l.*, suppressed both NF-κB and STAT3 activation, which was regulated by IL-6 expression in 4T1 breast cancer cells. *O. gracilioides* out of 10 *Ophiocordyceps* species suppressed NF-κB and STAT3 activation, and IL-6 production. Regarding the potential anti-cancer effects on *O. gracilioides,* it showed to have different HPLC profiles with cordycepin. Although it was confirmed that *O. gracilioides* did not contain cordycepin as a pharmacologically active ingredient, I expected it had unique active components to have anti-tumor efficacy.

Additionally, a bio-active components were purified from *O. gracilioides* and anti-tumor effect was examined targeting NF-κB/STAT3 activities in the murine 4T1 breast cancer cells. Regarding NF-κB/STAT3 inflammatory pathway, leading to the development of invasive breast cancer, ergosterol and ergosterol peroxide purified from *O. gracilioides* were exhibited anti-metastatic effect both *in vitro* and *in vivo* experiments.

Collectively, these studies present the potential use of *O. gracilioides*, which is an unexplored on bioactive effects, by targeting NF-κB/STAT3 activities on anti-tumor and anti-metastasis capacities in 4T1 breast cancer cells.

Chapter 2

Identification of anti-tumor activity on *Ophiocordyceps gracilioides* and its active compounds by targeting NF-κB/STAT3 inflammatory pathway in 4T1 breast cancer cells

2.1 Introduction

Cordyceps s.l. is an attractive materials used as a traditional medicinal material that has proven safety *in vivo* experiments with various pharmacological activities [31, 32]. A number of *Cordyceps s.l.* collected in nature is limited, various studies have reported aiming to preserving strain (mycelium of *Cordyceps s.l.*) and mass production through artificial cultivation [10, 33, 34]. In nature, *Cordyceps s.l.* causes the hyphae to invade the inside of the host (insect) and becomes depleted of nutrients, it releases the stromata to the outside of the insect, resulting in the fruiting body (Figure 2). In other words, artificial cultivation is carried out in a medium suitable (e.g. Sabouraud media) for growth by separating the mycelium from the insect body [34, 35].

Ophiocordyceps is the largest genus in family Ophiocordycipitaceae on *Cordyceps s.l.*. Unfortunately, however, *Cordyceps s.l.* has been poorly investigated and this affects identification of species and medical applications and uses [5]. As such, several studies are actively reporting the biological activity of more than 600 species of *Cordyceps s.l.* discovered so far [36]. Two representative species of *Cordyceps s.l.*, *Ophiocordyceps sinensis* and *Cordyceps militaris*, have been reported to have anti-tumor activity targeting the NF-κB pathway. In addition, it has been reported that the NF-κB pathway causes inflammation with STAT3 activation in breast cancer, and continuous stimulation affects pre-cancerous properties by induction of the IL-6 cytokine [21].

Therefore, screening was performed through the NF-κB/STAT3 inflammatory pathway in 4T1 breast cancer cells to select the *Ophiocordyceps* species that has the most effective activity. The samples used were 10 species belonging to genus *Ophiocordyceps* collected in Japan, whose reports for activity were insufficient and unknown. The information of the samples is listed in Table 1. I also identified the active components from *Ophiocordyceps* samples and its biological activity was confirmed *in vitro* and *in vivo* experiments.

Table 1. List of *Ophiocordyceps* species used in this study

Sample number	*Ophiocordyceps* species		Region of collection in Japan	Host (life cycle /substratum)	References
	Scientific name	Japanese name			
1	*Ophiocordyceps entomorrhiza*	Osamushitanpotake	Tomakomai, Hokkaido	Larva (Coleoptera)	[10]
2	*Ophiocordyceps* cf. *elongatistromata*	Tsukinukihachitake	Toyama, Toyama	Adult stage *Vespula spp* (Hymenoptera)	[37]
3	*Ophiocordyceps gracilioides*	Usuirotanpotake	Toyama, Toyama	Larva Coleopteran (Elateridae)	[38-40]
4	*Ophiocordyceps heteropoda*	Oosemitake	Toyama, Toyama	Nymph Homoptera (Cicadidae)	[41, 42]
5	*Ophiocordyceps neovolkiana*	Koganemushitanpotake	Nantan, Kyoto	Larva (Coleoptera)	[38]
6	*Ophiocordyceps nikkoensis*	Teppoumushitake	Tomakomai, Hokkaido	Larva (Erebidae)	[38]
7	*Ophiocordyceps nutans*	Kamemushitake	Toyama, Toyama	Adult stage stink bug (Hemiptera)	[43]
8	*Ophiocordyceps sobolifela*	Semitake	Kanazawa, Ishikawa	Nymph Cicada (Hemiptera)	[10]
9	*Ophiocordyceps sphecocephala*	Hachitake	Toyama, Toyama	Adult stage *Vespula spp* (Hymenoptera)	[44, 45]
10	*Ophiocordyceps tricentri*	Awafukimushitake	Toyama, Toyama	Adult stage spittle bug (Hemiptera)	[10]

2.2 Materials and methods

2.2.1 Reagent

Cordycepin was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), dissolved in phosphate-buffered saline (PBS) at 100 mM, and stored at -20°C. D-luciferin was obtained from Promega (Madison, WI, USA), and dissolved in PBS at a concentration of 10 mg/mL, and stored at -20°C. The primary antibodies for western blotting analysis against STAT3, p-STAT3, p65, and p-p65 were purchased from Cell Signaling Technology (Beverly, MA, USA), and the antibody against *β*-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All the extract, fractions, and sub-fractions were dissolved in dimethyl sulfoxide (DMSO; FUJIFILM Wako Pure Chemical Corporation) at a concentration of 10 mg/mL. These stock solutions were stored at -20°C, and diluted in the relevant assay medium and 0.01 % DMSO served as vehicle controls.

2.2.2 General experimental procedures for isolation and identification

^{1}H and ^{13}C NMR spectra were measured in $CDCl_3$ on a JEOL ECA500II spectrometer (Tokyo, Japan). The chemical shifts are expressed in δ (ppm) and calibrated to the residual proton and carbon resonances of chloroform (δ_H 7.26 ppm, δ_C 77.2 ppm). HPLC-MS analysis was performed using an Agilent Technologies 1260 Infinity II LC system, which consisted of a G7112B binary pump and G7117B diode array detector. MS spectra were scanned using Agilent Technologies 6420 triple quad LC-MS (ESI voltage: + 3.0 kV) by an ESI interface in the positive or negative mode.

Column chromatography was performed with normal phase silica gel (silica gel 60N, spherical, neutral, 40-50 *μ*m) (Kanto Chemical, Tokyo, Japan). Thin layer chromatography (TLC) was performed on silica gel GF_{254} precoated plates (Merck, Kenilworth, New Jersey, USA), and the spots were visualized with a UV lamp (254 and 365 nm) by spraying a *p*-

anisaldehyde stain solution (Nacalai Tesque, Kyoto, Japan) before heating at 120°C for 5-10 min in a drying cabinet.

2.2.3 Mycelium materials

The fresh specimens of 10 *Ophiocordyceps* species were collected in Japan and identified by *Dr. Fumito Sasaki*, Forestry Research Institute, Toyama Prefectural Agricultural, Forestry and Fisheries Research Center in Japan according to the method in the previous paper [43, 46], and listed to Table 1.

2.2.4 Mycelium sample preparation

To prepare the samples, a mycelia of fertile part from fruit body collected was sterilely inoculated on sabouraud liquid medium at 23°C in a dark room for 3 months until getting enough sample. Thereafter, formed mycelium was filtered and then freeze-dried to obtain dried powder (Figure 8). The dried powder was dissolved in PBS at a concentration of 10 mg/mL. These stock solutions were stored at -20°C.

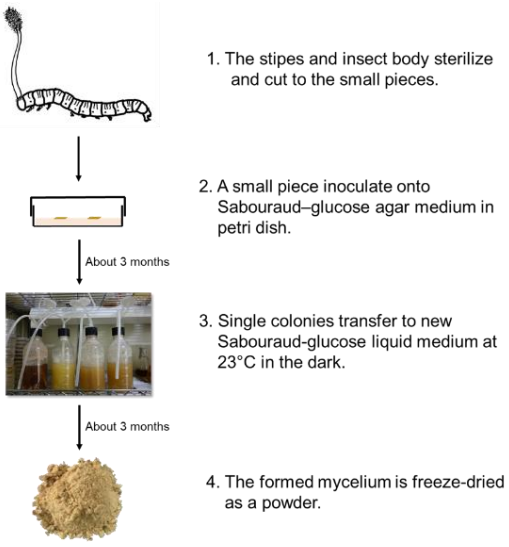


Figure 8. Flow chart for sample preparation of *Ophiocordyceps* species (mycelium).

2.2.5 Extraction and isolation

Based on luciferase reporter assay for anti-tumor activity in 4T1 breast cancer cells, active layers were selected and partitioned to isolate the active components by separatory funnel using solvents with increasing polarities. The *O. gracilioides* (in 10 g dry weight) was macerated with methanol (100 mL × 3) for 1 h with sonication at room temperature. The crude extract (1.5 g) was concentrated under vacuum, suspended in water (100 mL), and sequentially partitioned with *n*-hexane (503.5 mg), chloroform (48.8 mg), ethyl acetate (187.3 mg), and *n*-butanol (428.6 mg containing sodium chloride), respectively. All partitioning were repeated three times in each steps. The detailed scheme is provided in Figure 9.

The *n*-hexane layer was subjected to normal phase silica gel column chromatography, and eluted with *n*-hexane/ethyl acetate/methanol (100:1:0, 50:1:0, 20:1:0, 10:1:0, 5:1:0, 2:1:0, 1:1:0, 0:4:1, 0:1:1, 0:0:1) with increasing polarities. In total, 99 fractions of 20 mL each were collected and pooled into 9 combined fractions after TLC profiling. Ergosterol peroxide (1.2 mg) was acquired from the combined fraction 7 which obtained from *n*-hexane-soluble portion using silica gel column chromatography eluted with *n*-hexane/ethyl acetate (2:1, 1:1).

The ethyl acetate layer was applied to normal phase silica gel column chromatography and eluted with chloroform/methanol/water solvent systems (30:1:0, 20:1:0, 10:1:0, 5:1:0, 2:1:0, 1:1:0, 0:1:0, 6:4:1) with increasing polarities. In total, 123 fractions of 13 mL each were collected and pooled into 11 fractions after TLC profiling. The combined fraction 3 obtained from ethyl acetate-soluble portion was further subjected to normal phase HPLC [column: COSMOSIL 5SL-II packed column (10 mm I.D. × 250 mm) (Nacalai Tesque, Inc., Kyoto, Japan); ethyl acetate:*n*-hexane (1:4), flow rate: 1 mL/min] and monitored at wavelength of 254 nm and 280 nm, to yield ergosterol (2.5 mg, t_R 24 min).

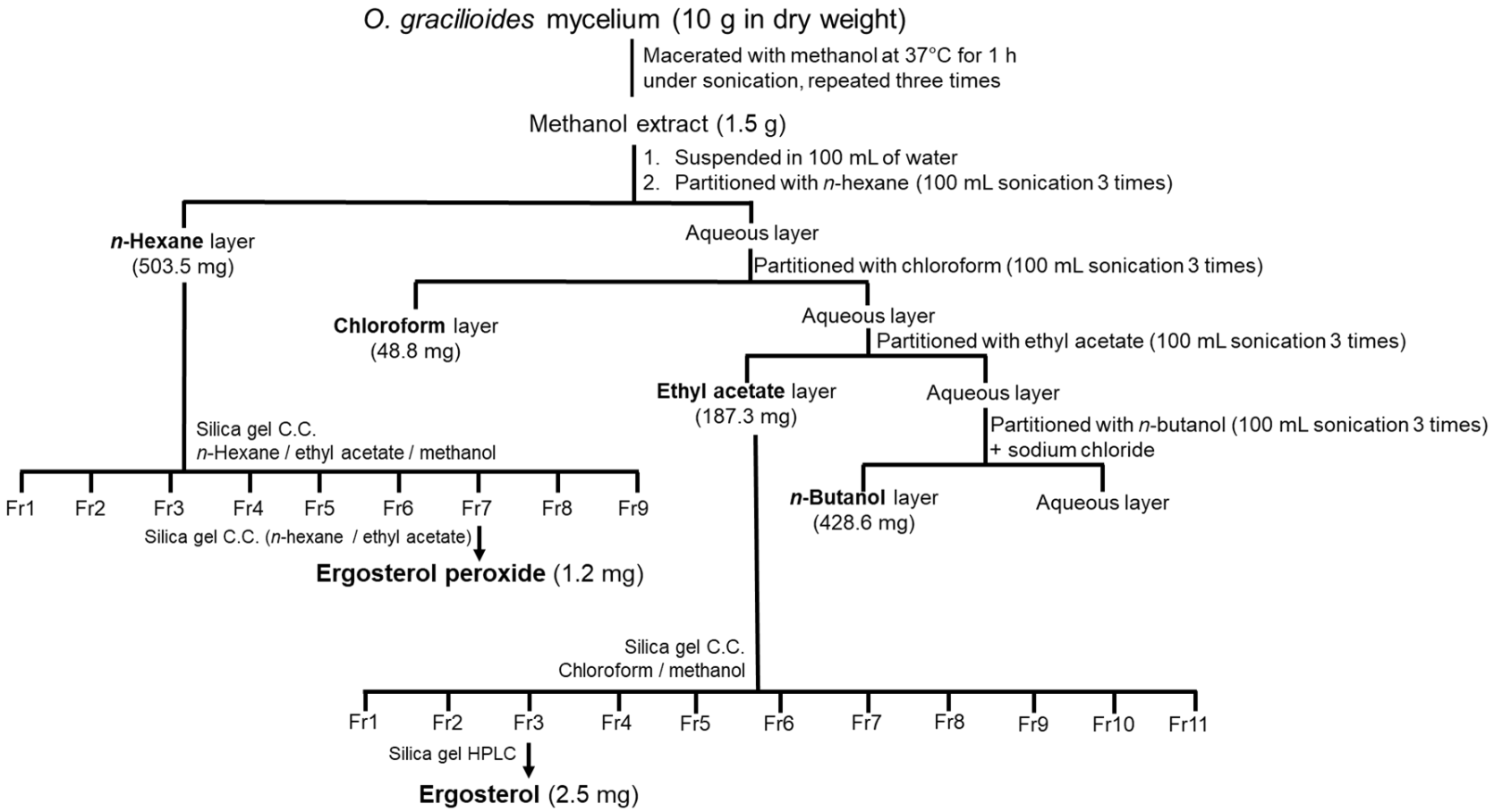


Figure 9. Scheme of bio-guided fractionation and isolation of compounds from *O. gracilioides* methanolic extract.

2.2.6 Cell culture

All cells were maintained in RPMI1640 medium containing 10% fetal bovine serum (FBS; Nichirei Biosciences, Tokyo, Japan), 0.2% (w/v) sodium bicarbonate ($NaHCO_3$; Wako Pure Chemical Co., Osaka, Japan), 1mM L-glutamine (Life Technologies, Gaithersburg, MD, USA), and 1% antibiotics (100 units/mL penicillin and 100 mg/mL streptomycin; Meiji Seika Pharma Co., Ltd, Tokyo, Japan) at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

The parental mouse 4T1 breast cancer cells were obtained from American Type Culture Collection (ATCC; VA, USA) and the 4T1 luciferase reporter-expressing cell lines (4T1-luc2, 4T1-NF-κB-luc2, and 4T1-STAT3-luc2) were established as described previously [47-49]. In briefly, to establish NF-κB-mediated luciferase gene expressing 4T1 cells (4T1-NF-κB-luc2) and STAT3-mediated luciferase gene expressing 4T1 cells (4T1-STAT3-luc2), 4T1 cells were seeded in 6-well plate at a density 5×10^5/well. Then, pGL4.32 vector or pGL4.26 vector (Promega) was transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The cells were selected with Hygromycin B (100 μg/mL) and cloned by limiting dilution. To evaluate the response of NF-κB or STAT3 transfectants or 4T1 control cells were culture in 96-well plate and treated with TNF-α (10 ng/mL). After the 6 h incubation, luciferase activity was measured with multi plate reader (2030 ARVO X, Perkin Elmer Life Science, Boston, MA).

2.2.7 Luciferase reporter assay

For the detection of luciferase activity, cells (4T1-NF-κB-luc2 or 4T1-STAT3-luc2 cells) were placed at a final concentration of 1×10^4 cells per well in 96-well black plate (Ref 655090; Greiner Bio-One, Kremsmünster, Austria) in screening experiments. After day, cells were treated with extracts, layers, fractions, isolated compounds, or an equal concentration of the vehicle for 24 h. After incubation, the luminescence of the firefly luciferase was measured using the imaging system (IVIS Lumina II; Caliper Life Sciences, Hopkinton, MA, USA) which was previously added a final concentration of 150 μg/mL D-luciferin (Promega) for 30 min.

2.2.8 Cell viability assay

Viability of cells was assessed using a Cell counting kit-8 (CCK-8) assay kit (Dojindo Co., Kumamoto, Japan) according to the manufacturers' instruction. The experimental conditions for cell viability were similar to the previous luciferase reporter assay. Twenty-four hours after treatment with extracts, layers, fractions, isolated compounds, or an equal concentration of the vehicle, 10 μL CCK-8 reagent was added in 100 μL culture media and incubated for another 2 h (37°C, 5% CO_2). The absorbance at 450 nm/620 nm was measured using a microplate reader (Sunrise; Tecan, Grödig, Austria).

2.2.9 Western blotting analysis

4T1 cells (1×10^6 cells/well in 6-well plate) were treated with test samples. The treated cells were rinsed one time in cold PBS, scraped, and lysed in whole-cell lysis buffer (25 mmol/L pH 7.7 *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), 300 mmol/L sodium chloride, 1.5 mmol/L magnesium chloride, 0.2 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.1% Triton X-100, 20 mM/L β-glycerophosphate, 1 mM/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonylfluoride, 1 mM/L dithiothreitol, 10 mg/mL aprotinin, and 10 mg/mL leupeptin). Cell lysates were subjected to electrophoresis in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred to an Immobilon-P polyvinylidene fluoride (PVDF) membrane (Merck Milipore, Darmstadt, Germany). The membranes were treated with Block Ace (Dainippon Pharmaceutical, Co., Ltd., Osaka, Japan) for at least 4 h, and probed with the indicated primary antibodies overnight, followed by horseradish peroxidase-conjugated secondary antibodies (DAKO, Glostrup, Denmark). Bands were visualized using Pierce™ ECL Western Blotting Substrate (Thermo Scientific, Waltham, MA, USA). Primary antibodies used (at a dilution of 1:1,000) were specific to STAT3 (79D7, #4904), p-STAT3 (Tyr705) (Y705, #9131), p-STAT3 (Ser727) (S727, #9134),

p65 (L8F6, #6956), p-p65, (93H1, #3033), and *β*-actin (C4, sc-47778).

2.2.10 Enzyme-linked immunosorbent assay (ELISA)

4T1 cells (2 × 10^5 cells/well in 24-well plate) were treated with *Ophiocordyceps gracilioides* or isolated compounds varying concentrations and the supernatant was collected. The amounts of cytokines in collected supernatants for IL-6 were diluted to 1:2 in media, and standard of IL-6 was prepared with 1% bovine serum albumin (BSA) in PBS. The samples were measured using ELISA MAX™ Standard Set Mouse IL-6 (BioLegend, San Diego, CA, USA) according to the manufacturer's instructions.

2.2.11 High performance liquid chromatography-mass spectrometry (HPLC-MS)

Extracts (10 samples of *Ophiocordyceps* species) and cordycepin were dissolved in methanol into 5 mg/mL and 1 mg/mL, respectively. The high performance liquid chromatography (HPLC) analysis was performed with Agilent Technologies 1260 Infinity II Series (Agilent, Waldbronn, Germany) consisting of a binary pump (G7112B), a vial sampler (G7129A), a 1290 photodiode array detector (G7117B) and TSK-gel ODS-80Ts (4.6 mm × 150 mm, 5 *μ*m) column (Tosoh Co., Tokyo, Japan). The following the solvent system was used: mobile phase: water (solvent system A) and methanol (solvent system B) in a gradient mode (B from 0% to 90% in 60 min), with a flow rate of 0.5 mL/min. Mass spectra were acquired on an Agilent Technologies 6420 Triple Quadrupole LC-MS spectrometer (Agilent, Waldbronn, Germany) equipped with an electrospray ionization source (ESI). The following mass (MS) settings were applied: High-purity nitrogen was used as dry gas at a flow rate of 5 L/min, gas temperature of 300°C, and fragment voltage of 135V. Nitrogen was used as nebulizer pressure at 15 psi and capillary voltage of + 3000V and - 2000V.

2.2.12 *In vitro* wound healing assay

4T1-luc2 cells were seeded in a 24-well plate at 2 × 10^5 cells/well and allowed to form a confluent monolayer. The cell monolayer was scratched using a sterile pipette tip (200-μL) on 90% confluence, replaced with the serum-free fresh media in the absence or presence of compounds (ergosterol or ergosterol peroxide), and incubated for 12 h. Scratched area was photographed under Biozero BZ-8000 microscope (Keyence, Osaka, Japan) at 0 h and then after 12 h (200× magnification). The ratio of wound closure was measured within the captured images using Image *J* software, and the percentage of inhibition of migrated cells was expressed using 100% as the value of the control group.

2.2.13 Transwell matrigel invasion assay

The transwell chamber (Costar No. 3422; Cambridge, MA) with 8 μm pore size of polycarbonate filter (Whatman, Clifton, NJ, USA) was pre-coated with 10 μg fibronectin (Fujifilm Wako Pure Chemical Corporation, Tokyo, Japan) on the lower surface and with 1 μg Matrigel (Becton Dickinson, Bedford, MA) on the upper surface of the filter. A total of 1 × 10^6 cells/well (4T1-luc2 cells) were pre-treated with test compounds for 12 h. After trypsinization, cells (3 × 10^4 cells/100 μL/chamber) were plated into the upper compartment of transwell in serum free media, and placed in 24-well plate (lower compartment of chamber) with media contained 0.1% (v/v) BSA, and incubated at 37°C for 6 h. The invaded cells on lower surface of the membrane were fixed in methanolic and stained with hematoxylin and eosin. Non-invaded cells in the upper chamber was discarded using a cotton swab. Invaded cells in five randomly selected fields were counted and photographed under an inverted microscope using Biozero BZ-8000 microscope (Keyence, Osaka, Japan) at a 400× magnification. The percentage of inhibition of invaded cells was expressed using 100% as the value of the control group.

2.2.14 Experimental lung metastasis *in vivo* model

Female BALB/c mice (six-week old) were purchased from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan). The mice were kept in the temperature of 24 ± 2°C for one week. All experiments for lung metastasis model were approved and performed according to the guidelines of the Care and Use of Laboratory Animals of University of Toyama. 4T1-luc2 cells were pre-treated with or without test compounds for 12 h, and then inoculated intravenously (2 x 10^5 cells/200-μL PBS/mouse) into mice. D-Luciferin (2 mg, *In Vivo* Glo™ Luciferin, Promega) was injected intraperitoneally into mice at day seventh after the tumor inoculation. After 10 min, the lungs were dissected for bioluminescence assay using an *in vivo* imaging system (IVIS Lumina II, Caliper Life Sciences, Hopkinton, MA, USA).

2.2.15 Statistical analysis

Statistical significance was calculated using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA). Data are presented as the mean ± standard deviation of three independent experiments. Statistical differences between groups were composed using one-way analysis of variance (ANOVA) with Dunnett's test. A *p*-value less than 0.05 was considered to indicate a statistically significant difference.

2.3 Results

2.3.1 Evaluation of anti-tumor effects of supplied ten *Ophiocordyceps* species by inhibiting NF-κB and STAT3 activation in 4T1 breast cancer cells

To identify novel anti-tumor agents in *Cordyceps s.l.*, 10 *Ophiocordyceps* species (Table 1; at 500 *μ*g/mL) were screened for their effects on NF-κB or STAT3 activities in 4T1 cells by confirming both luciferase reporter assay and cell viability assay. As shown in Figure 10, ten *Ophiocordyceps* species inhibited the STAT3 transcriptional activation compared to control, while only nine *Ophiocordyceps* species (*Ophiocordyceps* cf. *elongatistromata*, *Ophiocordyceps entomorrhiza, Ophiocordyceps gracilioides*, *Ophiocordyceps heteropoda*, *Ophiocordyceps neovolkiana*, *Ophiocordyceps nikkoensis*, *Ophiocordyceps nutans*, *Ophiocordyceps sobolifela*, *Ophiocordyceps sphecocephala*) except no.10 (*Ophiocordyceps tricentri*) suppressed the NF-κB activation compared to control in 4T1 luciferase reporter gene. For instance, STAT3 activation of ten *Ophiocordyceps* species were decreased from 1.00 (control group) to 0.40 (no.1), 0.78 (no.2), 0.51 (no.3), 0.65 (no.4), 0.62 (no.5), 0.45 (no.6), 0.81 (no.7), 0.50 (no.8), 0.66 (no.9), and 0.77 (no.10), respectively. Additionally, the NF-κB activation were suppressed from 1.00 (control group) to 0.95 (no.1), 0.95 (no.2), 0.67 (no.3), 0.71 (no.4), 0.86 (no.5), 0.87 (no.6), 0.91 (no.7), 0.79 (no.8), and 0.84 (no.9) in 4T1 cells after 24 h treatment of ten *Ophiocordyceps* species, respectively. Among those samples, no.3 (*O. gracilioides*) most strongly suppressed NF-κB and STAT3 activation in 4T1 cells than other tested nine *Ophiocordyceps* species (Figure 10). So, I selected *O. gracilioides* as the best inhibitor on NF-κB and STAT3 among the samples presented in Table 1 for next experiments.

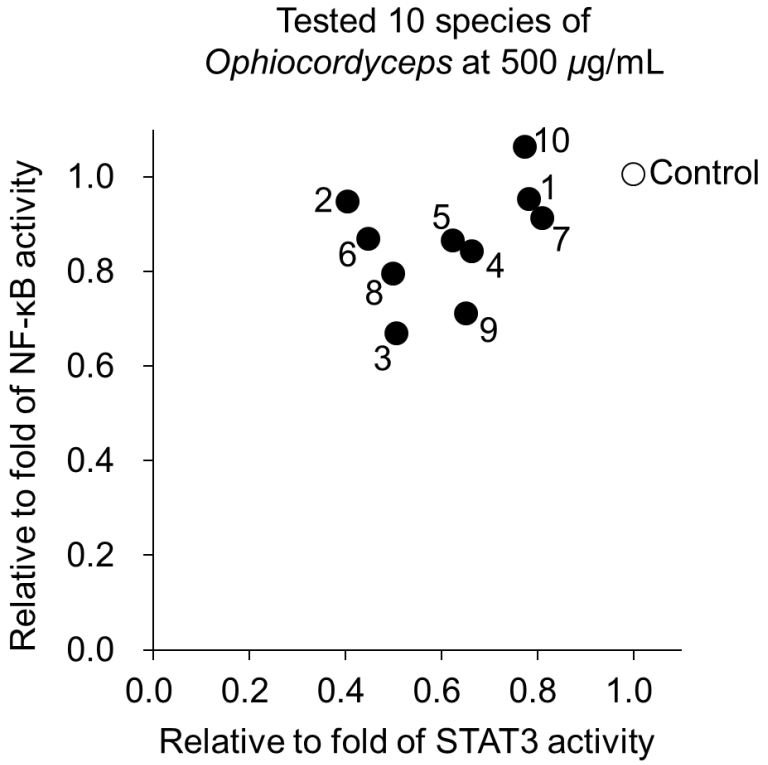


Figure 10. Screening for antitumor activity of ten *Ophiocordyceps* species on NF-κB and STAT3 activities in murine 4T1 breast cancer cells.

4T1 cells with stably expression of NF-κB or STAT3-mediated luciferase gene (4T1-NF-κB-luc2 and 4T1-STAT3-luc2) were seeded onto 96-well plate and treated with 10 *Ophiocordyceps* sample (at 500 μg/mL, Table 1) for 24 h. D-Luciferin (150 μg/mL) was added and incubated for another 30 min, and the luminescence was measured by IVIS imaging system. Then, the cell viability of the culture was determined using CCK-8 reagent. The relative activities of NF-κB and STAT3 transcription were calculated by dividing the luminescence with the cell viability in each group, and compared to the control (0 μg/mL).

2.3.2 Evaluation of anti-tumor properties on *O. gracilioides*-treated 4T1 cells

In order to evaluate whether *O. gracilioides* (Figure 11) at low doses inhibits the NF-κB and STAT3 transcriptional activities in 4T1 cells, it was conducted using luciferase reporter assay and western blotting. In the luciferase reporter assay, the NF-κB and STAT3 activities were significantly suppressed in a concentration-dependent manner in 4T1 cells (Figure 12A), and cytotoxicity was not observed (cell viability ≥ 80%) at low concentrations (doses of *O. gracilioides* ≤ 125 *μ*g/mL) (Figure 12B). The expression of phosphorylation of both the p65 subunit of NF-κB and STAT3, but not that of total protein, were down-regulated in 4T1 cells exposed to *O. gracilioides* (62.5, 125, 250, and 500 *μ*g/mL) for 24 h (Figure 13). Consequently, *O. gracilioides* inhibits the transcriptional activities of NF-κB and STAT3, as well as suppress its phosphorylation.

To further assess the pro-inflammatory cytokine IL-6, which is known to be regulated by NF-κB and STAT3, IL-6 production was examined in *O. gracilioides*-treated 4T1 cells. As shown in Figure 14, *O. gracilioides* significantly suppressed the IL-6 production in a concentration-dependent manner, especially at a concentration of 125 *μ*g/mL, which is not cytotoxic, decreased by 36% compared to control. In sum, these results indicated that the anti-tumor effect of *O. gracilioides* might result from inhibition of NF-κB/STAT3 inflammatory pathway in 4T1 breast cancer cells.

Figure 11. Natural wild specimen of *Ophiocordyceps gracilioides* collected in Japan.

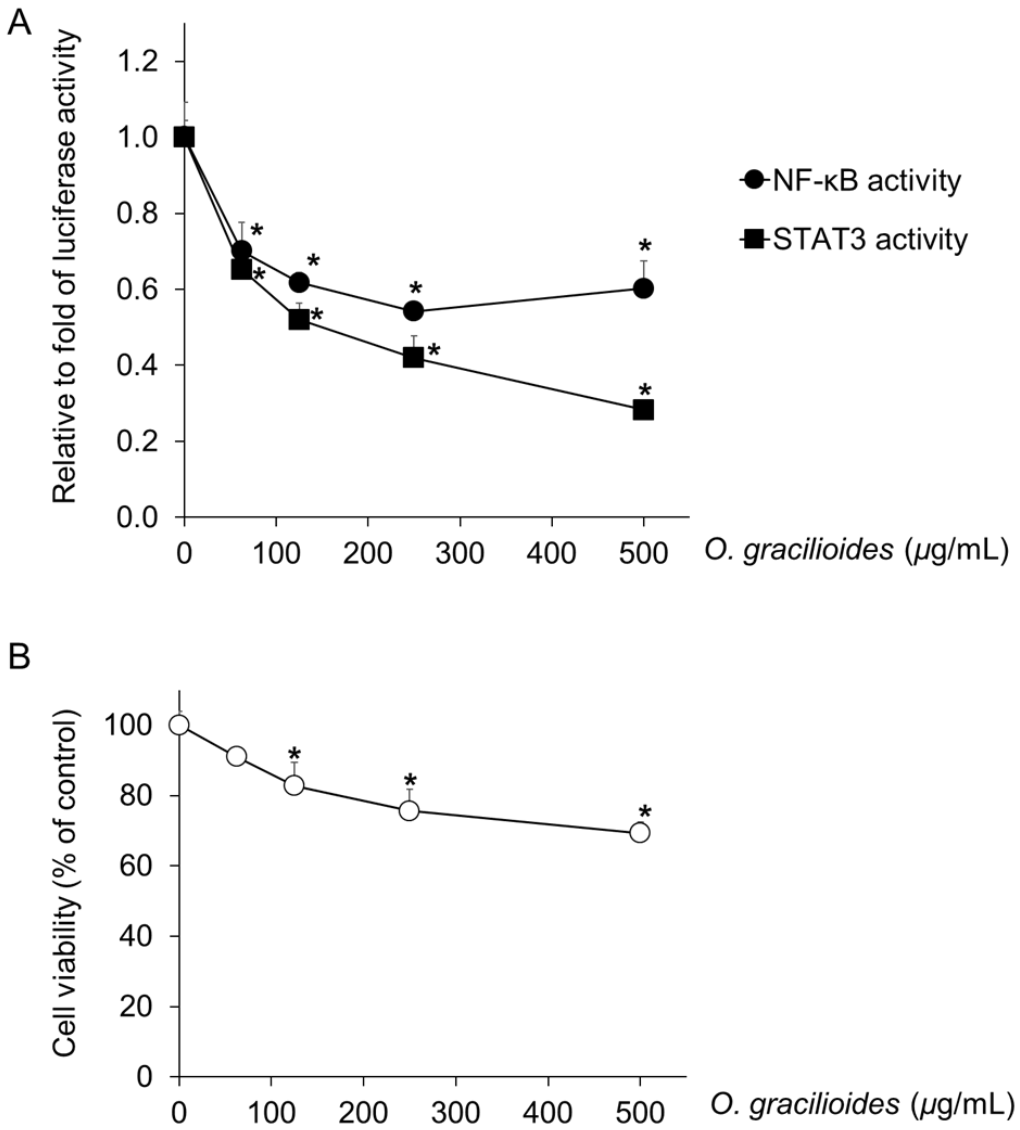


Figure 12. Inhibitory effects of *O. gracilioides* on the NF-κB and STAT3 activities in 4T1 cancer cells.

(A) 4T1-NF-κB-luc2 or 4T1-STAT3-luc2 cells were treated with *O. gracilioides* for 24 h. The activation on NF-κB or STAT3 relative to controls were determined. (B) Then, the cell viability was conducted in the same plate after luciferase reporter assay. Cell viability was determined using a CCK-8 assay and shown as a percentage of the control. Significance was assessed by one-way ANOVA with Dunnett's test. *p <0.05 versus control (0 μg/mL).

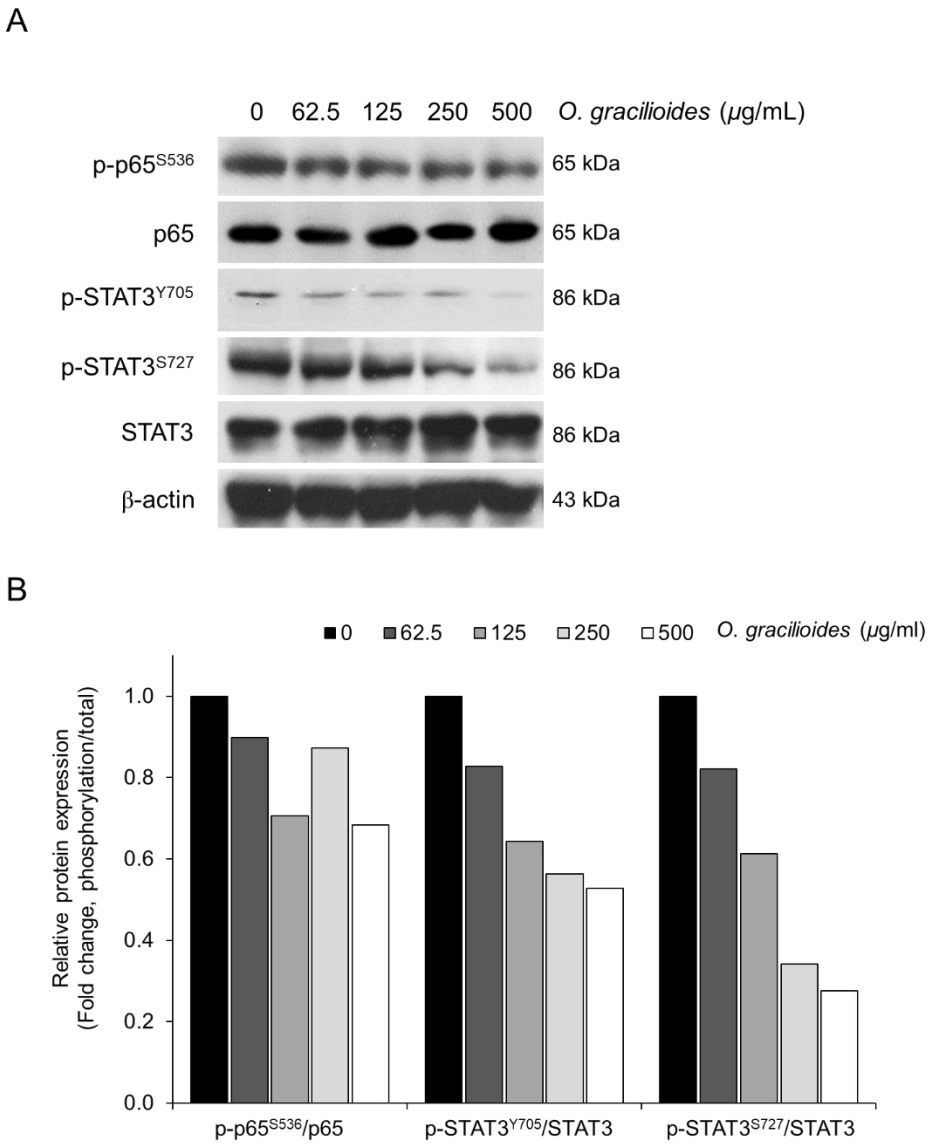


Figure 13. *O. gracilioides* inhibits the NF-κB and STAT3 phosphorylation in 4T1 breast cancer cells.

4T1 cells (10^6 cells/well) were placed in 6-well plates and treated with the indicated concentrations of *O. gracilioides* for 24 h, and equal amounts of protein in cell lysates were analyzed by western blotting (A). The β-actin protein level was used to confirm that equal amounts of protein were subjected to electrophoresis. The band intensities of phosphorylated proteins were assessed relative to non-phosphorylated protein using ImageJ software (B).

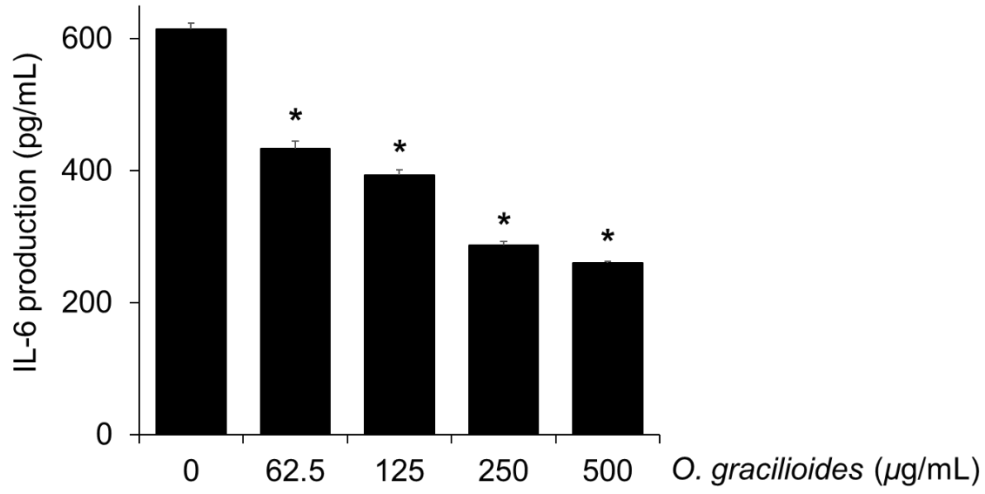


Figure 14. *O. gracilioides* suppresses the IL-6 production in 4T1 breast cancer cells.

4T1 cells (2×10^5 cells/well) were seeded in 24-well plates and treated with the indicated dose of *O. gracilioides*. After 24 h, culture supernatants were collected and cytokine IL-6 was quantified using the ELISA kit according to the manufacturer's instructions. The data are presented as the mean ± SD. Significance was assessed by one-way ANOVA with Dunnett's test. **p* <0.05 versus control (0 *μ*g/mL).

2.3.3 Comparison of constituents profile on *O. gracilioides* with cordycepin

To comparison of constituent profile of 10 *Ophiocordyceps* species, the samples were subjected to total ion chromatography (TIC) on HPLC. All samples showed similar peak patterns from 0 to 12 min of retention time, but an unique peak in *O. gracilioides* samples, which was confirmed to be the best inhibitor on NF-κB and STAT3 activities in 4T1 cells, was detected at 39.5 min (Figure 15). So, it was examined whether *O. gracilioides* contained cordycepin, one of the representative major active compound in *Cordyceps s.l.*, using HPLC-MS in positive ion mode. As shown in Figure 16, a peak of cordycepin appeared at 14.8 min in the chromatogram and the peak exhibited a molecular ion at *m/z* 252 (Figure 16A). The HPLC chromatogram of *O. gracilioides* had a peak at the retention time of 14 min, while the mass (MS) spectrum was not consistent with cordycepin (Figure 16B). In turn, regarding the potential contribution of cordycepin in the anti-tumor activity of *O. gracilioides*, I did not see any peak of cordycepin at 14.8 min. I therefore concluded *O. gracilioides* might have unique constituents other than conventional active ingredients of other *Cordyceps s.l.*.

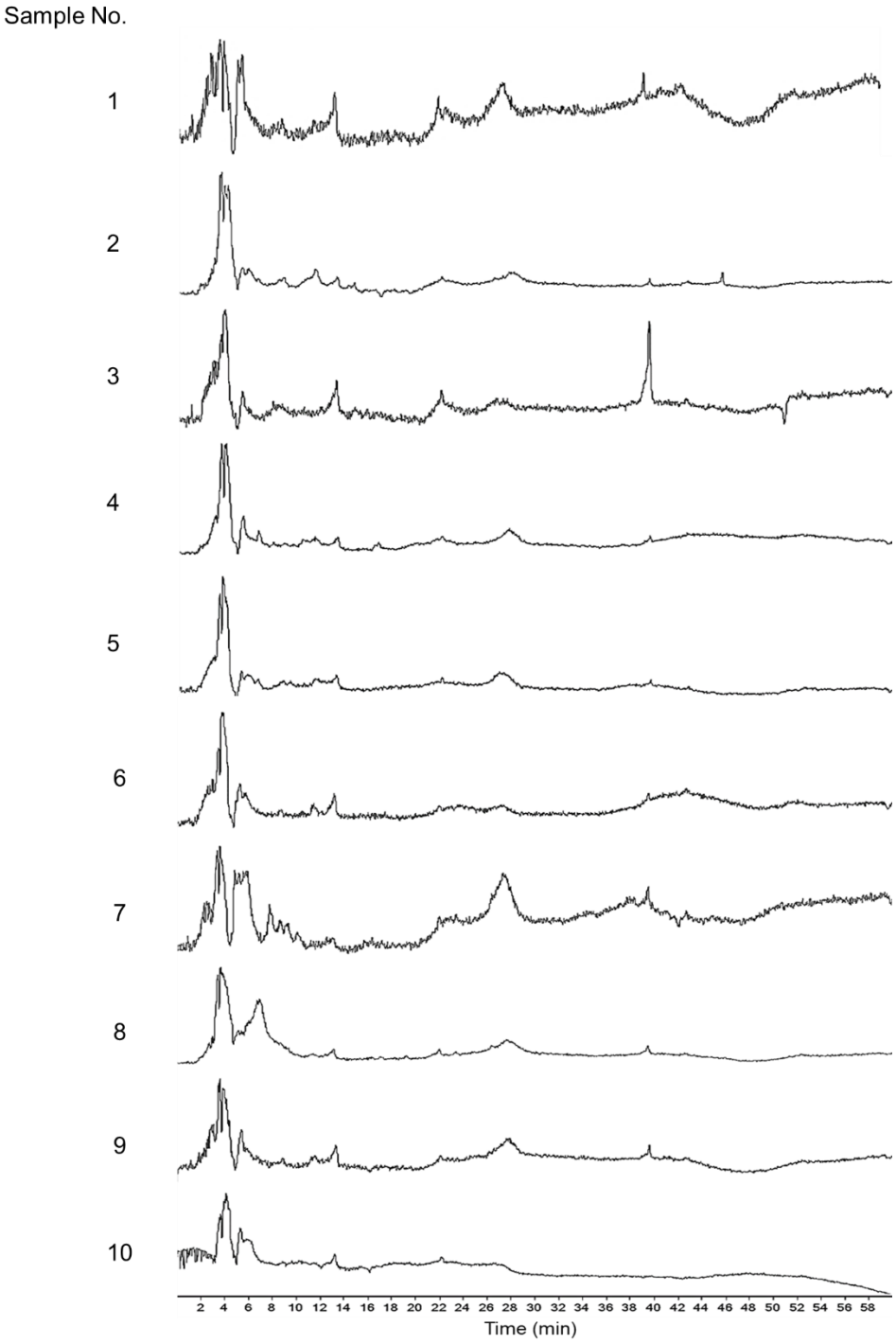


Figure 15. Total ion chromatography (TIC) of ten *Ophiocordyceps* species

All samples (ten *Ophiocordyceps* species) were dissolved in methanol to 5 mg/mL, and different TIC patterns of *Ophiocordyceps* species were identified using HPLC.

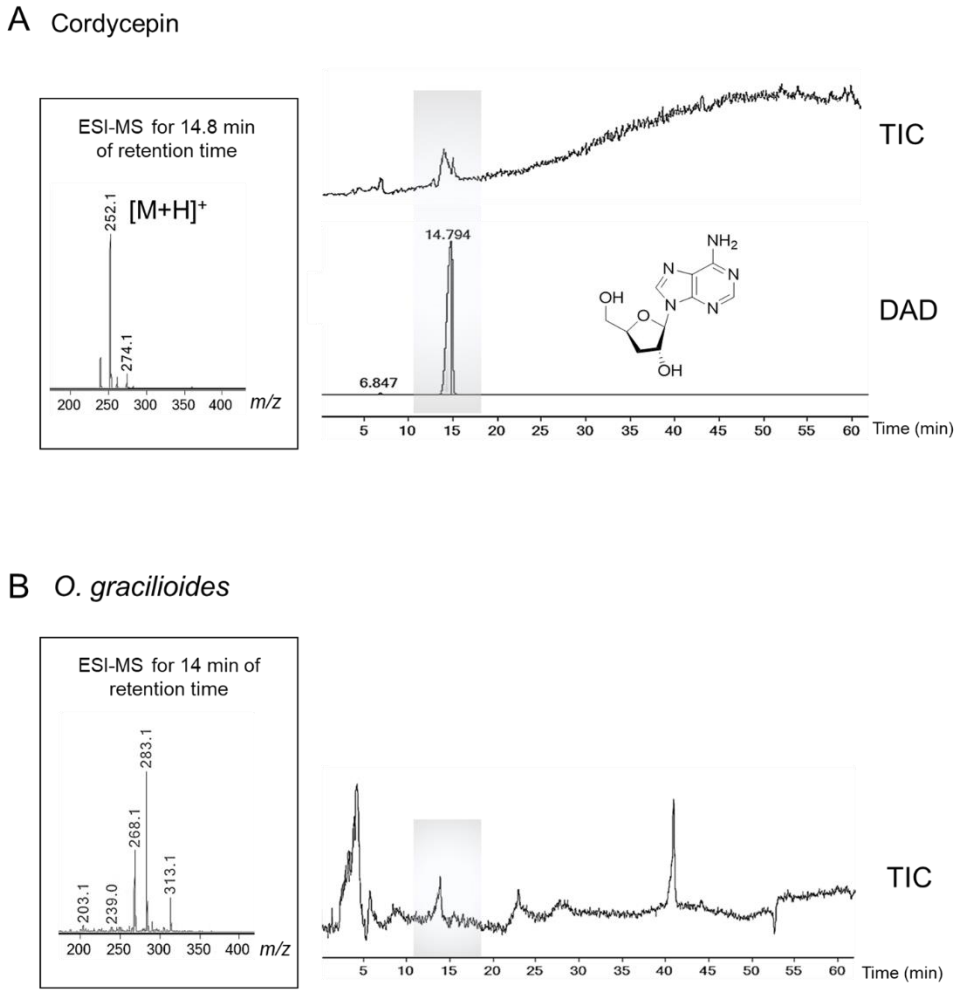


Figure 16. A chromatographic profiles at 254 nm and mass spectrum of cordycepin (A) and *O. gracilioides* (B).

Cordycepin and *O. gracilioides* were dissolved in methanol to 1 mg/mL and 5 mg/mL, respectively, and chromatograms of *O. gracilioides* and cordycepin were estimated using HPLC-MS. A chromatography was carried out on a TSK-gel ODS-80Ts (4.6 mm × 150 mm, 5 *μ*m) column. The following gradient was used: mobile phase: H_2O (solvent system A) and methanol (solvent system B) in a gradient mode (B from 0% to 90% in 60 min) with a flow rate of 0.5 ml/min. The MS spectrometer was coupled with an electrospray ionization (ESI) interface. The chromatography of cordycepin using diode array detection (DAD) was conducted at 254 nm in wavelength.

2.3.4 Identification of solvent fractioning on *Ophiocordyceps gracilioides* methanolic extract to suppress NF-κB and STAT3 activities in 4T1 breast cancer cells and its structure elucidation

The detailed steps for isolation of the active components are provided in Figure 9. In order to select the active layers from *O. gracilioides* methanolic extract, the respective layers (*n*-hexane, chloroform, ethyl acetate, and *n*-butanol) were confirmed on the inhibition of NF-κB and STAT3 activities in 4T1 breast cancer cells using luciferase reporter assay. The inhibitory activities on NF-κB and STAT3 of *n*-hexane and ethyl acetate layers were showed to be superior to that of other layers (Figure 17). Then, I next tried to identify the active component from *n*-hexane and ethyl acetate layer of *O. gracilioides* methanolic extract.

The *n*-hexane layer (480 mg) was eluted with *n*-hexane/ethyl acetate/methanol gradient in silicagel column chromatography. Amongst yield fractions, the active fraction 7 on both NF-κB and STAT3 inhibition (Figure 18A) was further eluted with *n*-hexane/ethyl acetate gradient on silicagel column chromatography to obatin ergosterol peroxide, which was identified using mass spectrometry and ^{1}H-NMR (Figure 19) and HPLC-MS analyses (Figure 20) by comparisons of its experimental data with literature [50].

The ethyl acetate layer (156 mg) was eluted with chloroform/methanol gradient on silicagel c.c., and fraction 3, which showed NF-κB and STAT3 inhibition (Figure 18B), was purified by normal phase HPLC with 20% ethyl acetate in *n*-hexane solvent system, and as a result ergosterol was obtained. Ergosterol was identified using ^{1}H-NMR (Figure 21) and ^{13}C-NMR analyses compared with literature (Figure 22) [51]. In another fraction from ethyl acetate layer, I tried to obtain active compounds against NF-κB and STAT3 activities, but the isolated compounds were fatty acids or extremely small amount of compounds that could not be identified by NMR and HPLC-MS (data not shown). The structures of the components identified are presented in Table 2, and their spectral details are shown below. Chemical structures of two sterols extracted from *O. gracilioides* are shown in Figure 23.

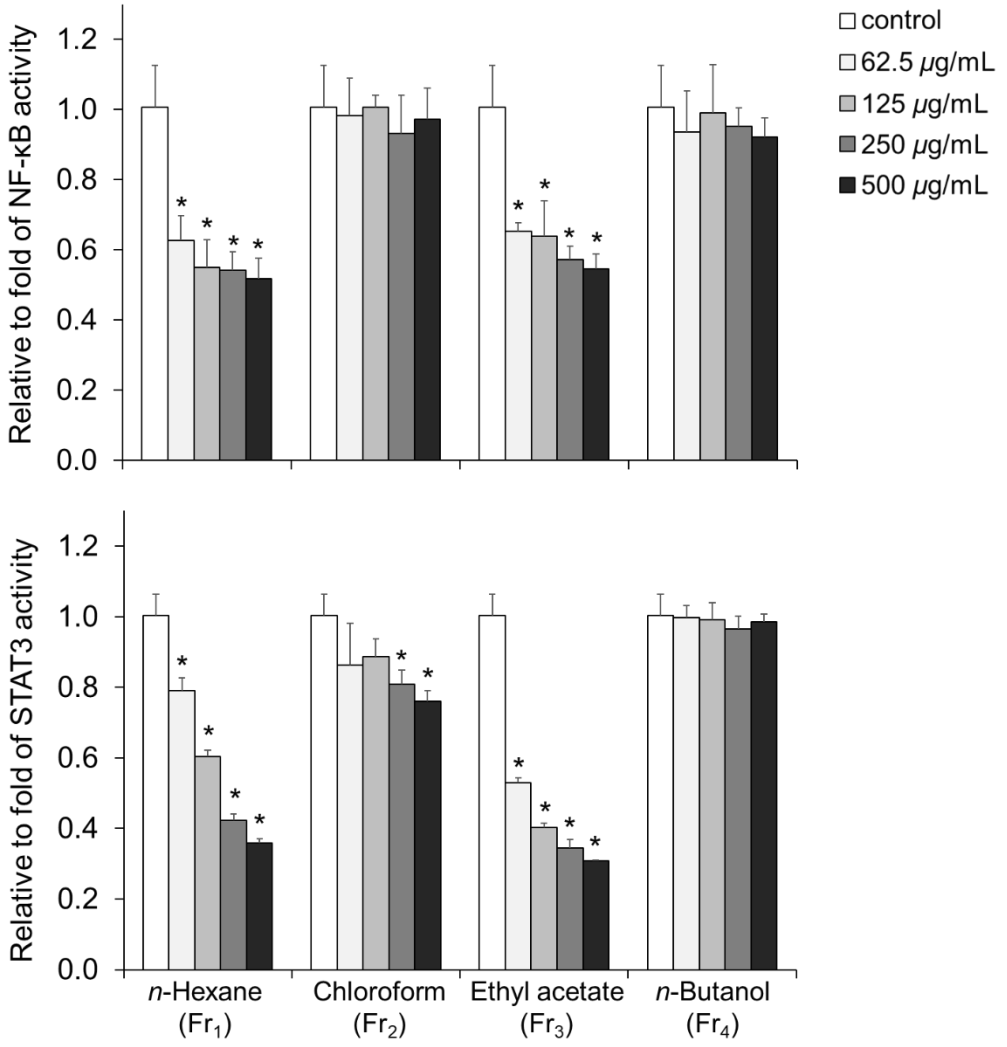


Figure 17. Identification of the active layers of *O. gracilioides* methanolic extract that inhibit NF-κB (upper graph) and STAT3 (lower graph) activation.

4T1-NF-κB-luc2 cells or 4T1-STAT3-luc2 were treated with the four layers (*n*-hexane, chloroform, ethyl acetate, and *n*-butanol) at range of 62.5 - 500 μg/mL for 24 h. Inhibition of NF-κB or STAT3 activation were determined relative to the control. Data are presented as the mean ± SD. Significance was assessed by one-way ANOVA with Dunnett's test. **p* <0.05 versus control (0 μg/mL).

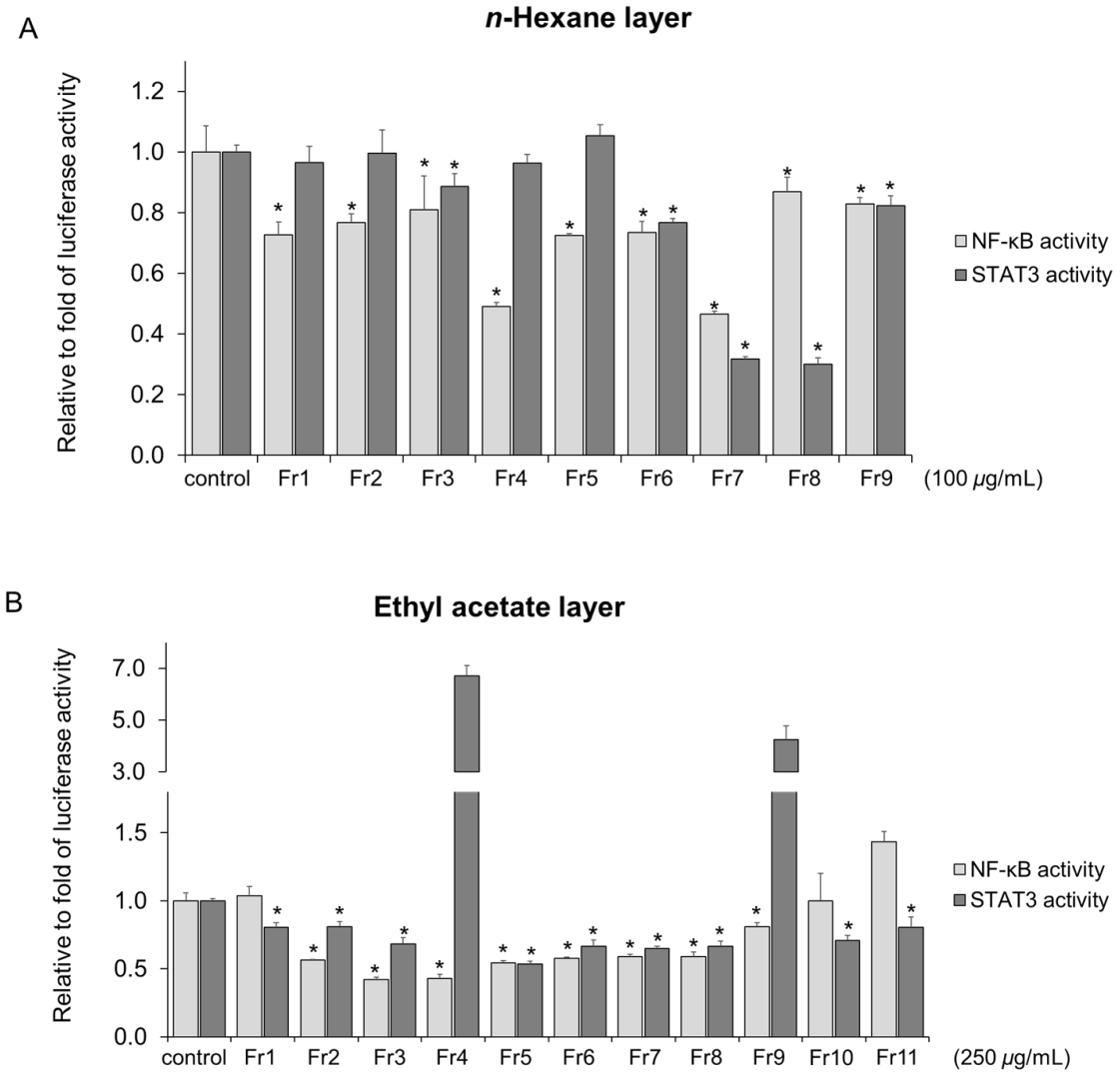


Figure 18. Identification of the active fractions of *n*-hexane (A) and ethyl acetate (B) layers from *O. gracilioides* methanolic extract that inhibit NF-κB and STAT3 activation.

4T1-NF-κB-luc2 cells or 4T1-STAT3-luc2 were treated with each fractions at 100 or 250 μg/mL for 24 h. Inhibition of NF-κB or STAT3 activities were determined relative to the control. Data are presented as the mean ± SD. Significance was assessed by one-way ANOVA with Dunnett's test. *p <0.05 versus control (0 μg/mL).

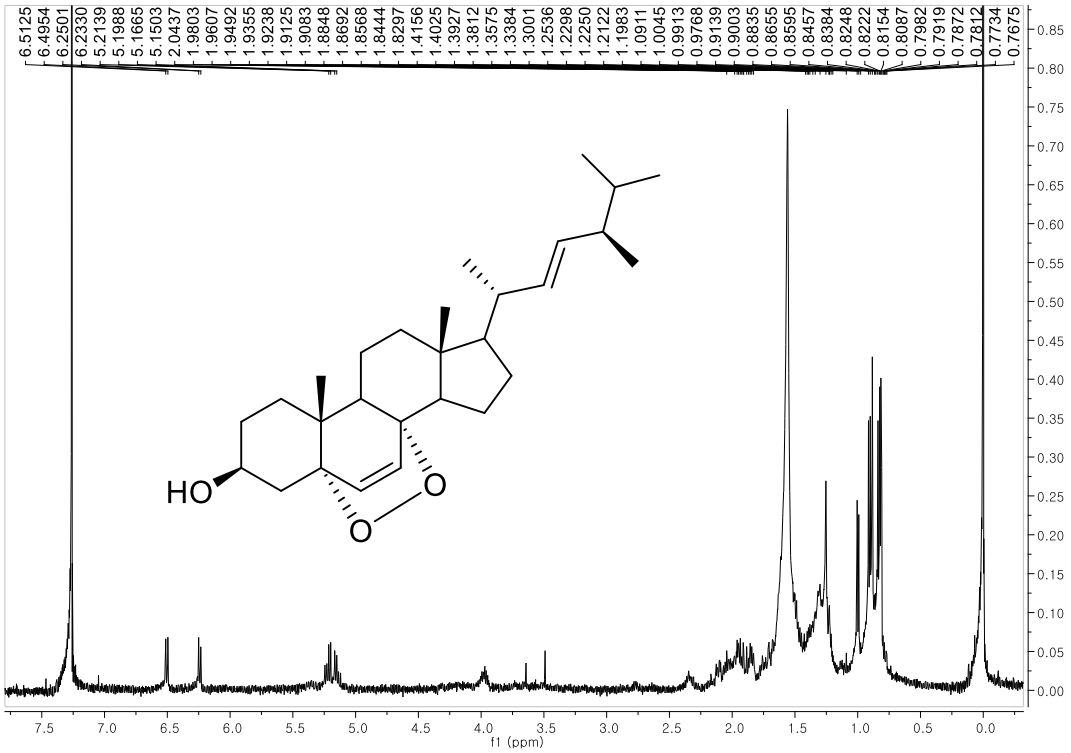


Figure 19. ^{1}H NMR spectrum (500 MHz) of ergosterol peroxide in $CDCl_3$.

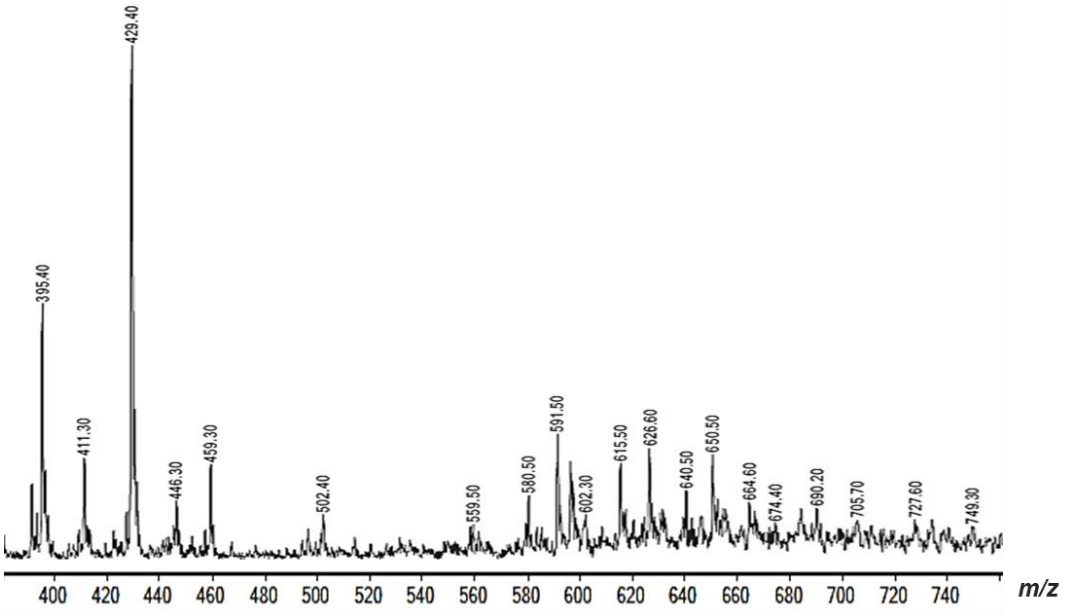


Figure 20. ESI-MS spectrum of ergosterol peroxide.

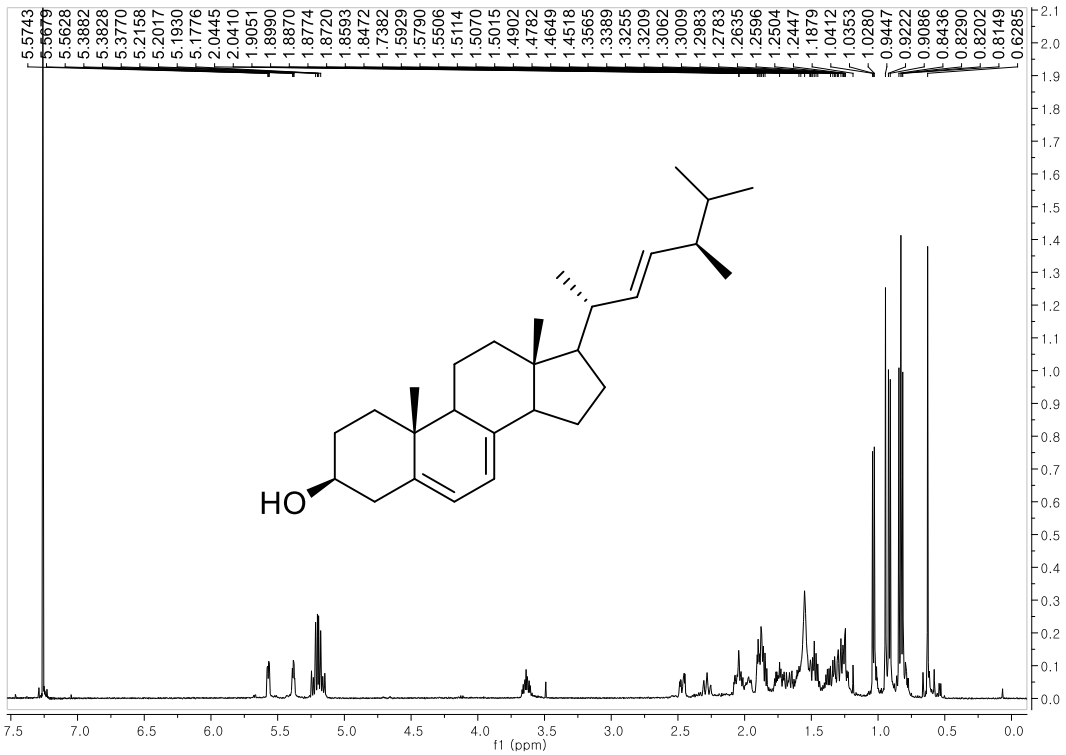


Figure 21. ^{1}H NMR spectrum (400 MHz) of ergosterol in $CDCl_3$.

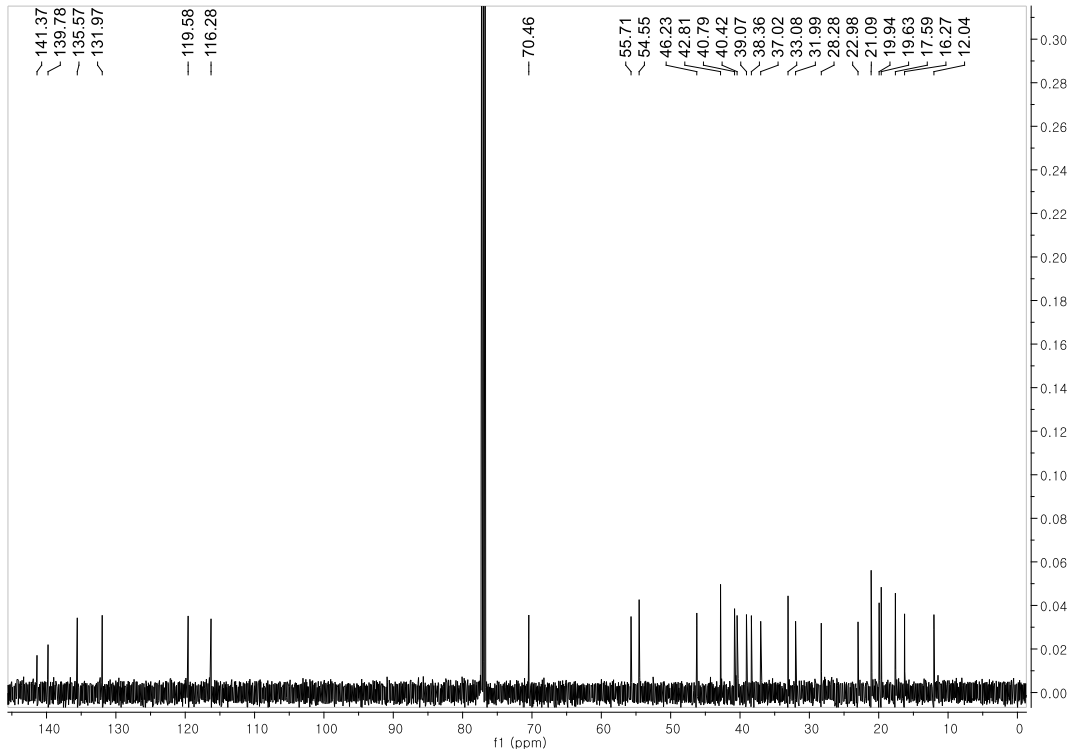


Figure 22. ^{13}C NMR spectrum (125 MHz) of ergosterol in $CDCl_3$.

Table 2. ^{1}H- and ^{13}C-NMR data for two isolated compounds (ergosterol peroxide and ergosterol) from *O. gracilioides* in $CDCl_3$ (δ in ppm and *J* values in (*Hz*) in parentheses)

Position	Ergosterol peroxide[a]	Ergosterol[a]	
	δ_H	δ_H	δ_C
1			38.4
2			32.0
3	3.96 (m)	3.63 (m)	70.5
4			40.8
5			139.8
6	6.22 (d, 8.6)	5.57 (dd, 5.7, 2.5)	119.6
7	6.50 (d, 8.6)	5.38 (dd, 5.7, 2.5)	116.3
8			141.4
9			46.2
10			37.0
11			21.1*
12			39.1
13			42.8*
14			54.6
15			23.0
16			28.3
17			55.7
18	0.81 (s)	0.62 (s)	12.1
19	0.88 (s)	0.93 (s)	16.3
20			40.4
21	1.00 (d, 6.6)	1.03 (d, 6.6)	21.1*
22	5.19 (dd, 15.5, 7.5)	5.20 (dd, 15.3, 7.7)	135.6
23	5.17 (dd, 15.5, 7.5)	5.19 (dd, 15.3, 7.7)	132.0
24			42.8*
25			33.1
26	0.82 (d, 6.8)	0.82 (d, 7.0)	20.0
27	0.83 (d, 6.8)	0.83 (d, 7.0)	19.6
28	0.91 (d, 6.6)	0.91 (d, 6.8)	17.6

[a] ^{1}H NMR and ^{13}C NMR at 500 MHz measured in $CDCl_3$.

* Overlapping resonances within the same column.

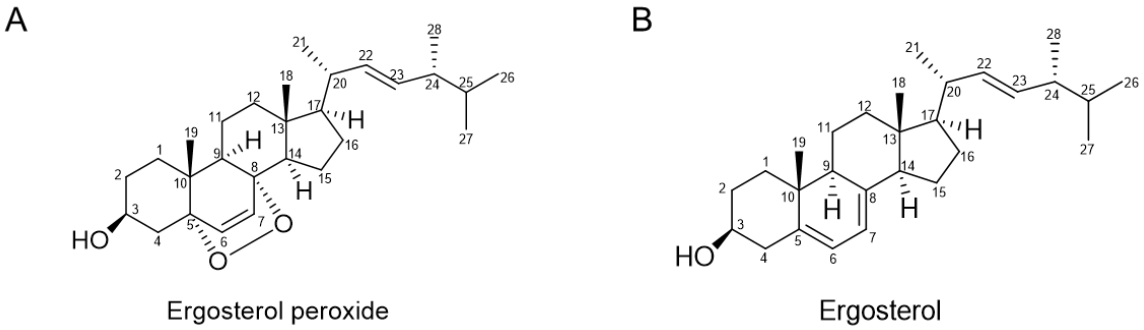


Figure 23. Structures of isolated ergosterol peroxide (A) and ergosterol (B) from *O. gracilioides*.

2.3.5 Evaluation of anti-tumor properties in ergosterol or ergosterol peroxide treated 4T1 breast cancer cells

In order to examine the anti-tumor effects of two compounds (ergosterol or ergosterol peroxide) isolated from *O. gracilioides* methanolic extract, I tested the effect on NF-κB and STAT3 activities by luciferase reporter assay using 4T1 luciferase reporter expressing cells (4T1-NF-κB-luc2 and 4T1-STAT3-luc2 cells). During treatment for 24 h, ergosterol showed weak inhibitory activities against NF-κB (Figure 24C) and STAT3 (Figure 24D) without any cytotoxic (Figure 24A), however, it was confirmed that ergosterol peroxide suppressed the activities of NF-κB (Figure 24C) and STAT3 (Figure 24D) more prominent inhibition than ergosterol. When cells treated for 24 h with ergosterol and ergosterol peroxide at high dose (400 μM), NF-κB activities were 78.3 and 68.4%, and STAT3 activities were 68.2 and 8.8%, respectively (Figure 24C and 24D). Since ergosterol peroxide has cytotoxicity against 4T1 cells at concentrations above 200 μM, it is appropriate to select concentration at 100 μM for next experiment (Figure 24B). At 100 μM ergosterol and ergosterol peroxide inhibited NF-κB activity in 4T1 cells starting from 3 h after treatment (Figure 24E). Ergosterol peroxide suppressed STAT3 activity in 4T1 cells after 6 h treatment (Figure 24F). Moreover, as shown in Figure 25, the expression of phosphorylated p65 and STAT3 were inhibited by ergosterol peroxide. Of note, at a 100 μM of ergosterol peroxide inhibited the phosphorylation of STAT3 in 4T1 cells, especially at the S727 phosphorylation site, starting from 3 h after treatment (Figure 25).

To further assess the pro-inflammatory cytokine such as IL-6, the cell culture supernatant with or without two isolated compounds were treated for 12 h in 4T1 cells. As shown in Figure 26, the IL-6 production were inhibited in both ergosterol and ergosterol peroxide.

To evaluate the biological significance of two compounds as a potential anti-tumor agent, I confirmed its proliferation at 24, 48 and 72 h on 4T1 cells. The percentage of proliferation

versus control on ergosterol treated 4T1 cells at 24, 48 and 72 h were 94.9, 89.6 and 87.6%, whereas proliferation percentage after treatment with ergosterol peroxide at 24, 48 and 72 h were 92.0, 79.6 and 48.3%, respectively. As a results, ergosterol had no cytotoxic even long term incubation while ergosterol peroxide showed cell cytotoxic effect at 48 h or 72 h incubation (Figure 27A). Also, ergosterol peroxide (100 μM) showed cytotoxic effect in MDA-MB-231 cells at 48 h and 72 h (Figure 27B).

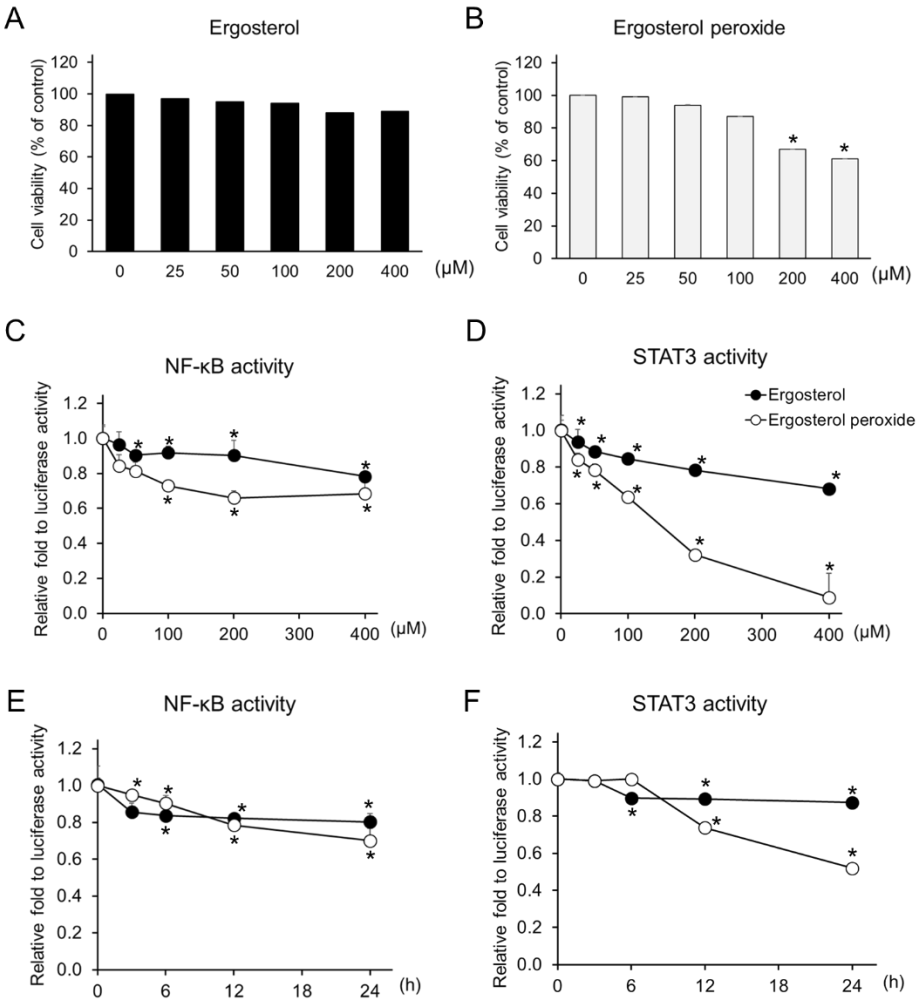


Figure 24. Effects of ergosterol and ergosterol peroxide on NF-κB and STAT3 transcriptional activities in 4T1 cells.

4T1 cells were treated dose- and time-dependently with ergosterol or ergosterol peroxide. (A and B) cell viability assay. 4T1 cells were treated with ergosterol and ergosterol peroxide in a different concentrations (25, 50, 100, 200, and 400 μM) for 24 h. 4T1-NF-κB-luc2 (10^4) and 4T1-STAT3-luc2 (10^4) cells were incubated with different concentrations (25, 50, 100, 200, and 400 μM) of ergosterol (C) or ergosterol peroxide (D) for 24 h, and subjected to IVIS. Cells were treated with 100 μM of ergosterol (E) or ergosterol peroxide (F) for 3, 6, 12, and 24 h, and subjected to IVIS. Results are expressed as the mean ± SD. Significance was assessed by one-way ANOVA with Dunnett's test. *p <0.05 versus control (0 μM or 0 h).

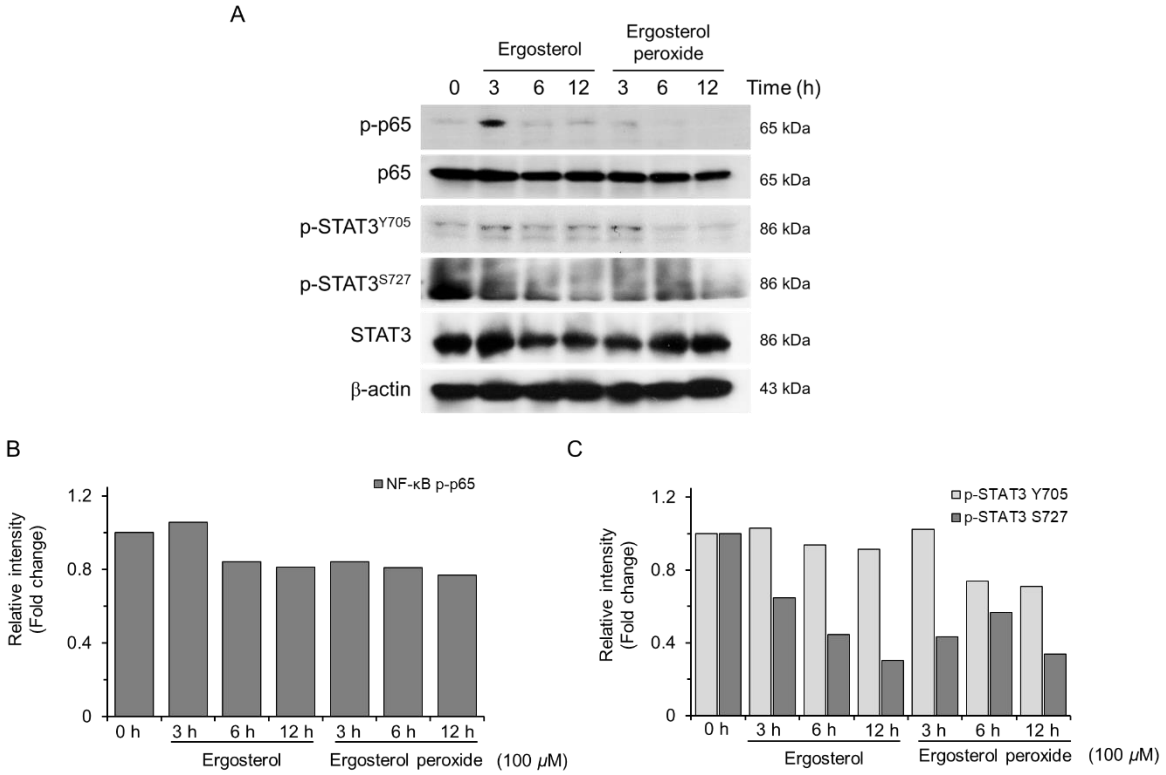


Figure 25. Effects of ergosterol and ergosterol peroxide on phosphorylation of p65 and STAT3 in 4T1 cells.

4T1 cells (10^6 cells per well in 6-cm dish) were treated with 100 μM of ergosterol and ergosterol peroxide for 3, 6, and 12 h. After treatment, cells were harvested, and equal amounts of protein in cell lysates were analyzed by western blotting analysis (A). The β-actin protein levels were used to confirm that equal amounts of protein were subjected to electrophoresis. (B and C) Quantitative analyses of western blotting analysis are presented by densitometry using ImageJ software.

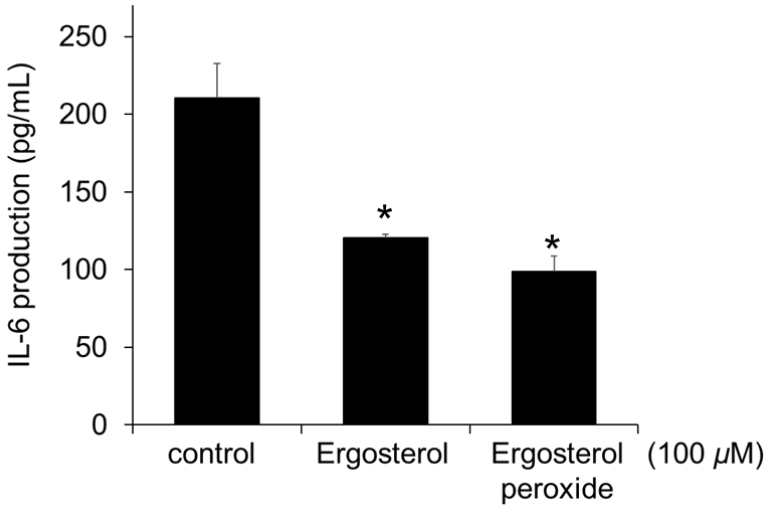


Figure 26. Effects of ergosterol and ergosterol peroxide on IL-6 production by 4T1 cells.

4T1 cells were treated with 100 μM of ergosterol and ergosterol peroxide for 12 h. After treatment incubation, culture supernatants were collected and IL-6 cytokine was quantified using the ELISA kit according to the manufacturer's instructions. The data are presented as the mean ± SD. Significance was assessed by one-way ANOVA with Dunnett's test. *p <0.05 versus control.

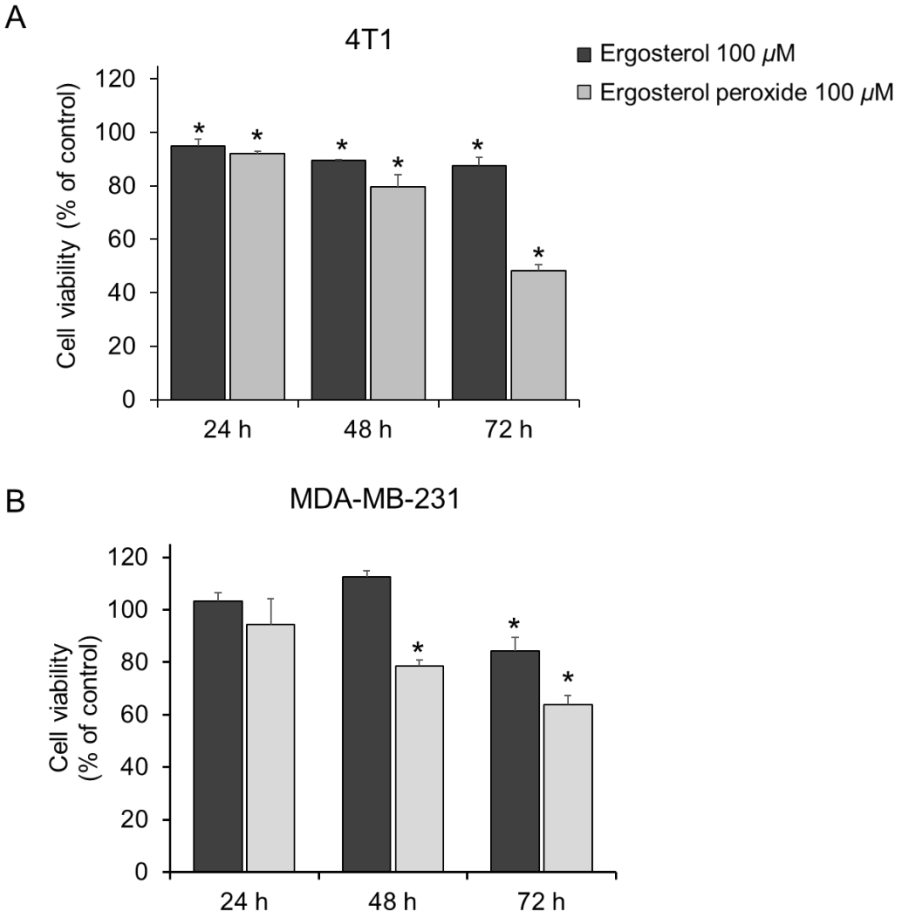


Figure 27. Effect of ergosterol and ergosterol peroxide on the growth of breast cancer cells.

4T1 cells (1×10^4 cells/well) and MDA-MB-231 (2×10^4 cells/well) were treated with 100 μM of ergosterol or ergosterol peroxide for 24, 48 and 72 h, and cell viability was evaluated as the relative cell viability to untreated control. The data are presented as the mean ± SD. Significance was assessed by one-way ANOVA with Dunnett's test. *p <0.05 versus control (0 h).

2.3.6 Ergosterol peroxide reduces metastatic potential of 4T1 cells

To examine the biological significance of ergosterol and ergosterol peroxide as a potential anti-metastasis agent, I evaluated migratory and invasive efficacies in 4T1 cells by wound healing assay and transwell matrigel invasion assay. Given the relevance of NF-κB and STAT3 activities to the distant metastasis of cancer cells have been recognized, I tested the effects of ergosterol and ergosterol peroxide on cell migration and invasion of 4T1 cells. As a results, two compounds (ergosterol and ergosterol peroxide) showed inhibition of the cell migration of 4T1 cells, as determined by the wound healing assay (Figure 28A). In addition, pre-treatment with two compounds significantly suppressed the invasion of breast cancer cells (Figure 28B). Collectively, these results clearly indicate that ergosterol and ergosterol peroxide reduce that the metastatic potential of 4T1 cells *in vitro* by regulating cellular migration or invasion, especially ergosterol peroxide in accordance with the inhibitory activities of NF-κB or STAT3 activation. I then tested the therapeutic potential of ergosterol and ergosterol peroxide in breast cancer using an *in vivo* animal model. In an experimental lung metastasis model of 4T1 breast cancer cells, I found that pre-treatment with ergosterol peroxide significanlty inhibited the metastatic lung colonization of 4T1 cells (Figure 29).

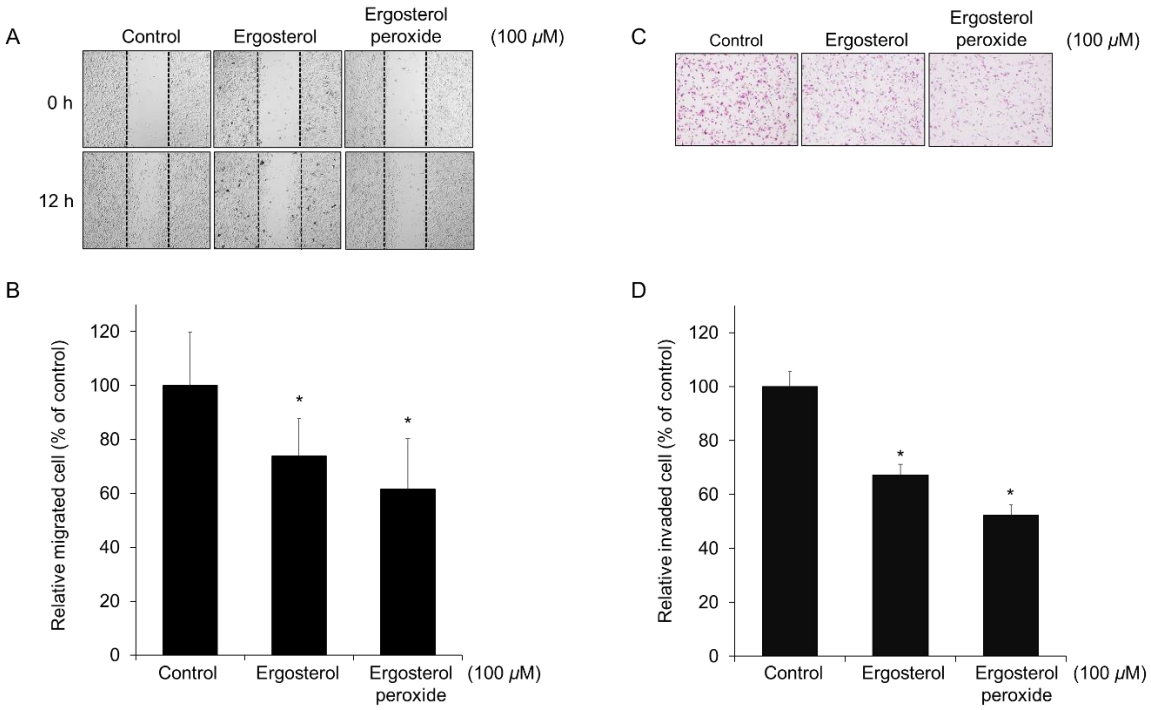


Figure 28. Anti-metastasis effects of ergosterol and ergosterol peroxide on the migratory and invasive activities of 4T1 cells.

(A) Wound healing assay. 4T1 cells (2 × 10^5 cells/well) were seeded in 24-well plates and incubated until confluency. A vertical scratch was made on the cell monolayer using a 200-μL pipette tip. After washing by the media, the cells were treated with 100 μM of ergosterol or ergosterol peroxide, and a snapshot was taken using an inverted microscope. After a 12 h incubation, snapshots were taken again. The black lines indicated the section occupied by the initial scraping. (C) Invasion assay. 4T1 cells (1 × 10^6 cells/well) were seeded in 6-well plate and treated with ergosterol or ergosterol peroxide. After 12 h, the cells (3 × 10^4 cells/chamber) were seeded in transwell chambers and incubated for 6 h. Snapshots were taken using an inverted microscope. The migratory (B) or invasive (D) activity were measured as the relative % of migrated or invaded cells to control cells. The data are presented as the mean ± SD. Significance was assessed by one-way ANOVA with Dunnett's test. *p <0.05 versus control.

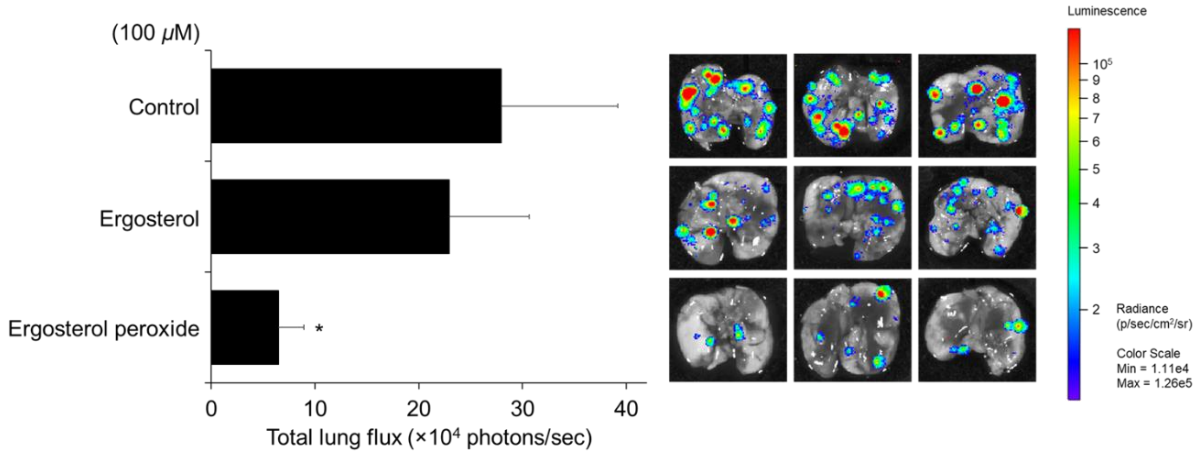


Figure 29. Anti-metastatic effect of ergosterol and ergosterol peroxide in experimental lung metastasis model on 4T1 cells.

4T1-luc2 cells were pretreated with 100 μM of ergosterol or ergosterol peroxide. After a 12 h incubation, cells were inoculated intravenously (2×10^5 cells/200 μL of PBS/mouse) into mice. Mice were sacrificed 7 days after the tumor inoculation and lungs were removed for bioluminescent assay using an *in vivo* imaging system. The data are presented as the mean luminescence ± SD (n = 6). * p<0.05 compared with the control.

2.4 Discussion

In this study, I investigated whether ten species of genus *Ophiocordyceps* belonging to the family Ophiocordycipitaceae of *Cordyceps s.l.* collected in Japan can deactivate the NF-κB and STAT3 in 4T1 breast cancer cells. *Cordyceps s.l.* has been used as a traditional herbal medicine for thousands of years. To date, several studies have been conducted to verify the medicinal properties for knowledge of traditional medicine, discover new pharmacological activities, and identify its bioactive components [2]. Nevertheless, most studies (about 60% of published articles on *Cordyceps s.l.*) mainly focus on two species of *Cordyceps s.l.*, *Ophiocordyceps sinensis* and *Cordyceps militaris*, while other species have not been comprehensively investigated for their biological activity and chemical composition.

It has been reported that both *O. sinensis* and *C. militaris* enhance anti-tumor effect by suppressing the growth and metastasis in 4T1 breast cancer cells [20, 52, 53] via the inhibition of NF-κB activation [17, 54]. Recently, NF-κB has been reported to cooperate with STAT3 to induce a variety of target gene transcription, in addition to regulating IL-6 inflammatory cytokine [55-57].

In the present study, therefore, the ten species of *Cordyceps s.l.* (Table 1) were screened for those NF-κB and STAT3 deactivation in 4T1 cells and one of the best species from them was explored for anti-tumor properties targeting NF-κB and STAT3 activities. When the 4T1 luciferase reporter-expressing cell lines were treated with nine samples (from no.1 to no.9; 500 *μ*g/mL) except no.10 (*Ophiocordyceps tricentri*) for 24 h, there were the inhibition on NF-κB and STAT3 activation. In particular, it was confirmed that no. 3 (*Ophiocordyceps gracilioides*) is the best inhibitor against the NF-κB and STAT3 activity in 4T1 cells. Besides, *O. gracilioides* downregulated phosphorylation STAT3 and NF-κB p65, but not total STAT3 and NF-κB p65 levels. Moreover, this report also examined to reduce the IL-6 production on *O. gracilioides* treatment in 4T1 cells.

In the nature, entomopathogenic fungus absorb the energy/nutrients needed to maintain primary metabolism from the host (insect, plant, soil, etc.) through the hyphae of the mycelium [58]. Under adverse conditions due to nutrient depletion, however, the fungus slows down in growth and switches from the growth pathway to the biochemical pathways; i.e. the primary metabolites that have produced in the fungus are converted into secondary metabolites [59-61]. Several studies of a number of secondary metabolites from *Cordyceps s.l.* have reported to isolate compounds with different biological properties, such as anti-tumor [62, 63], anti-inflammatory [64, 65], anti-oxidant [66, 67] and anti-bacteria activities [68]. For instance, *Ophiocordyceps sinensis*, a representative species belonging to the same family and same genus with *O. gracilioides*, mainly contains secondary metabolites such as cordycepin etc., which is proven to have anti-tumor activity [62]. However, cordycepin was not contained in *O. gracilioides* (Figure 16), so I expected that *O. gracilioides* would be contained special active ingredients other than cordycepin.

According to literature search, although, there is one report on the anticancer activity of no.8 (*Ophiocordyceps sobolifela*) in human breast cancer cells by regulating the cell cycle [69], while *O. gracilioides* was not reported other than a biological identification and classification. *O. gracilioides* belongs to the family Ophiocordycipitaceae, which is the same family with *O. sinensis*. It was first described by Kobayasi as *Cordyceps gracilioides* from Japan, infecting the larval stages of Elateridae (Coleoptera) [38]. It was then reported from Japan [70], Korea [10], China [39, 71], Colombia [40], Thailand [72], and Mexico [73, 74]. *C. gracilioides* was transferred to *Ophiocordyceps* by Sung *et al*. [10] by morphological and molecular analyses, hence, it was renamed *O. gracilioides* [10]. Asexual morph of *O. gracilioides* is classified to *Paraisaria gracilioides* in family Ophiocordycipitaceae [75]. A sexual morph of *O. gracilioides* is characterized by the production of dark red to brown to tan capitate stromata, and its asexual morph white colored stromata with immersed perithecia, cylindrical morph of stipe, and globose of fertile head [40, 75].

A further study, I confirmed the isolation and structural identification of two known compounds (ergosterol peroxide and ergosterol) from *O. gracilioides* methanolic extract. Moreover, the impact of active components isolated from *O. grcilioides* methanolic extract on 4T1 breast cancer cells was investigated by targeting NF-κB/STAT3 inflammatory pathway in terms of *in vitro* and *in vivo* experiment.

The structures of ergosterol and ergosterol peroxide are similar, and ergosterol can be converted into its peroxide by photo-oxidation, which is induced by a cell membrane damage. Regarding to biological activity, ergosterol peroxide exerted the anti-tumor effect in 4T1 breast cancer cells through the NF-κB and STAT3 inhibition and anti-metastasis effects via attenuating migration/invasion *in vitro* and suppressing the lung metastasis of a breast cancer cells *in vivo* experiment. In order to isolate the active ingredients against NF-κB/STAT3 activities in 4T1 cells, *O. gracilioides* investigated by a bio-guided method using luciferase reporter assay in 4T1 luciferase reporter-expressing cell lines. In consequence, ergosterol peroxide and ergosterol, which are contained in *n*-hexane and ethyl acetate layers respectively, were purified from methanolic extract of *O. gracilioides* mycelium.

In recent decades, many researchers began to report the ergosterol peroxide and ergosterol, bioactive secondary metabolites, from medicinal fungi and to examine their mechanisms on chronic diseases. Steroids such as ergosterol and ergosterol peroxide play a critical roles in maintaining the normal structure and function of cell membranes and act as precursors for the synthesis of metabolites, and have been proven to have various biological activities including anti-tumor [76-78], immune-modulating [79], and anti-oxidative [80]. A number of studies have demonstrated that ergosterol and its peroxides (such as ergosterol peroxide) might contribute to potential health benefits, such as reducing pain related to inflammation, reducing the incident of cardiovascular disease, and inhibiting the tumor development. Ergosterol is a part of cytoplasmic membrane in fungi and contained in medicinal fungi [81], yeast [82], and sponge [83]. It has the ability to maintain membrane

structure, fluidity, and permeability [84]. In addition, ergosterol purified from medicinal mushroom has been reported to have anti-tumor effect of MDA-MD-231 human breast cancer cells by inducing cell death and inhibiting cell cycle progression [85]. According to the results of present experiment, however, ergosterol showed a tendency to decrease compared to control in 4T1 murine breast cancer cells, but no significant difference.

Ergosterol peroxide, 5*α*,8*α*-endoperoxide sterol derivative of ergosterol, is known to metabolites of fungal species [86] or marine sponges [87-91], but it is rare [92]. In fungi, ergosterol peroxide is synthesized by the conversion of ergosterol to its epidioxide, may play a role in the detoxification reaction of reactive oxygen species [93]. It has been reported that ergosterol peroxide purified from medicinal mushroom inhibits cancer growth by-upregulating multiple tumor suppressors in human liver cancer cells [94] and human breast cancer cells [95]. Consistently, the result of this study demonstrated that ergosterol peroxide attenuated the proliferation of MDA-MB-231 human breast cancer cells and 4T1 murine breast cancer cells. Another researcher have presented that ergosterol peroxide exerts anti-tumor activity through STAT3 pathway, which is inducing the angiogenesis, and Wnt/β-catenin pathway in myeloma cells, colon cancer, and ovarian cancers [96-99]. Additionally, ergosterol peroxide exhibited to have inhibitory activities through IKK-β on NF-κB pathway [100, 101]. However, I demonstrated the anti-metastatic effect of ergosterol peroxide through the inhibition of both NF-κB and STAT3 activities in breast cancer cells. Moreover, I evaluated the activities of ergosterol and ergosterol peroxide on 4T1 breast cancer cells, and as a result, it is showed that ergosterol peroxide of the oxysterol type is superior to ergosterol on anti-tumor and anti-metastatic activities.

Herein, these results suggest for the first time that the nine *Ophiocordyceps* species (*Ophiocordyceps* cf. *elongatistromata*, *Ophiocordyceps entomorrhiza*, *O. gracilioides*, *Ophiocordyceps heteropoda*, *Ophiocordyceps neovolkiana*, *Ophiocordyceps nikkoensis*, *Ophiocordyceps nutans*, *O. sobolifela*, *Ophiocordyceps sphecocephala*) have anti-tumor

ability in 4T1 breast cancer cells with inhibitory activity against NF-κB/STAT3/IL-6 inflammatory pathway, of which *O. gracilioides* was the most effective. In sum, I identified ergosterol peroxide as one of the active constituent of *O. gracilioides* with anti-metastatic effects through the inhibition of NF-κB/STAT3 inflammatory pathway in 4T1 breast cancer cells.

Conclusion

In this study, it was investigated that *Ophiocordyceps gracilioides* has anti-tumor efficacy by inhibiting NF-κB or STAT3 activation against 4T1 breast cancer cells, and further isolated its active ingredients. A methanolic extract of *O. gracilioides* mycelium was confirmed that the *n*-hexane and ethyl acetate layers were most effective one on inhibition of NF-κB or STAT3 activity in 4T1 cells using bio-guided method, and two known compounds (ergosterol and ergosterol peroxide) were isolated from each fractions.

O. gracilioides collected and purified by *Dr.* Fumito Sasaki, a Forestry Research Institute, Toyama Prefectural Agricultural, Forestry and Fisheries Research Center in Japan shows promising anti-tumor effects on 4T1 breast cancer cells, which are mediated by both NF-κB and STAT3 activities. After being treated with the purified ergosterol peroxide and ergosterol, the NF-κB and STAT3 activation were inhibited in 4T1 breast cancer cells. In particular, ergosterol peroxide purified from *O. gracilioides* mycelium, the 4T1 breast cancer cells were suppressed more than ergosterol in terms of proliferation, migration and invasion *in vitro*, and metastasis *in vivo*.

This work is the first to focus on the pharmacological activity and isolation of active components from *O. gracilioides* mycelium, which is a species of *Cordyceps s.l.*, *O. gracilioides* contained ergosterol peroxide may has as a potential source on anti-tumor properties.

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