ANTI-AUSTERITY ACTIVITIES OF JAPANESE KAMPO MEDICINES AND ACTIVE CONSTITUENTS OF *ANDROGRAPHIS PANICULATA*

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DISSERTATION

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Abbreviations

AO	Acridine orange
$CaCl_2$	Calcium chloride
^{13}C NMR	Carbon nuclear magnetic resonance
$CHCl_3$	Chloroform
CH_3CN	Acetonitrile
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
EB	Ethidium bromide
EIMS	Electron ionized mass spectrometry
EtOAc	Ethyl acetate
EtOH	Ethyl alcohol
FBS	Fetal bovine serum
$Fe(NO_3)$	Iron(III) nitrate
HEPES	2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
^1H NMR	Proton nuclear magnetic resonance
KCl	Potassium chloride
$MgSO_4$	Magnesium sulfate
MPLC	Medium-pressure liquid chromatography
NDM	Nutrient deprived medium
MEM	Minimum essential medium
MeOH	Methyl alcohol
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
NaH_2PO_4	Sodium phosphate monobasic
NP	Normal phase
PBS	Phosphate buffered saline
PANC-1	Human pancreatic cancer cell line PANC-1
PC_{50}	50% Preferentially concentration
PSN-1	Human pancreatic cancer cell line PSN-1
RP	Reverse phase
TLC	Thin-layer chromatography
TMS	Tetramethylsilane
WST-8	Water-soluble tetrazolium salt-8

Abstract

Cancer is a major public health problem throughout the world. Among the different forms of cancer, pancreatic cancer is the most aggressive and has an exceptionally high global mortality rate which ranks 7th as the most frequent cause of cancer death. Pancreatic cancer rapidly metastases and leads to the death of patients in a short period after diagnosis. Indeed, the 5-year survival rate of patients with pancreatic cancer is the lowest among several cancers. Though surgery is the only treatment that offers any prospect of potential cure, chemotherapy with 5-fluorouracil and gemcitabine is also used for palliative therapy of advanced pancreatic cancer. However, pancreatic cancer is largely resistant to most known chemotherapeutic agents, including 5-fluorouracil and gemcitabine. Therefore, effective chemotherapeutic agents for pancreatic cancer are urgently needed.

Tumor cells, in general, proliferate very fast, and demand high essential nutrients and oxygen. The immediate environment of tumor cells rapidly proliferating increasing in size, however, often becomes heterogeneous and some regions of large cancers often possess micro environmental niches, which exhibit a significant gradient of critical metabolites, including oxygen, glucose, amino acids, and growth factors etc. Thus, many cancer cells obtain their critical metabolites by randomly recruiting new blood vessels, a phenomenon commonly known as angiogenesis, to survive under such severe conditions. However, human pancreatic cancer cells survival are known to with an extremely poor blood supply and becomes more malignant. The method by which pancreatic cancer survives is by obtaining a remarkable tolerance to extreme nutrient starvation. Therefore, to the eliminating the tolerance of cancer cells to nutrition starvation has been known as “anti-austerity” strategy for cancer therapy.

In this study, on the discovery of anticancer agents from natural products, I first examined preferential cytotoxicity of crude drugs available in Japan against human pancreatic cancer cell lines PANC-1 and PSN-1 based on anti-austerity strategy. Among the tested crude drugs, the extract of Andrographis Herba (No.17, aerial part of *Andrographis paniculata*) showed the most potent preferential cytotoxicity against PANC-1 and PSN-1cells. Hence, I investigated active constituents of *A. paniculata* and identified them for the preferential cytotoxicity against PANC-1 and PSN-1 cells. Furthermore, I examined detailed mechanism of the preferential cytotoxicity of the active constituent.

1. Preferential cytotoxicity of crude drugs available in Japan against human pancreatic cancer cell lines

The 70% EtOH extracts of 24 crude drugs available in Japan were screened for their preferential cytotoxicity against PANC-1 and PSN-1 cells. The preferential cytotoxicity was determined by comparing the cytotoxicities in normal medium (DMEM) and nutrient deprived medium (NDM). Of the 24 extracts examined, nine extracts of Isodonis Herba (extract No.1, aerial part of *Plectranthus japonicus*), Phellodendri Cortex (No.2, bark of *Phellodendron amurense*), Lacca Sinica Exsiccata (No.3, resin of *Rhus verniciflua*), Arctii Fructus (No.5, fruits of *Arctium lappa*), Lycopodium (No.15, spore of *Lycopodium clavatum*), Agrimoniae Herba (No.16, aerial part of *Agrimonia pilosa*), Andrographis Herba (No.17, aerial part of *Andrographis paniculata*), Panacis Japonici Rhizoma (No.18, rhizome of *Panax japonicus*), and Chorei (No.19, sclerotium of *Polyporus umbellatus*) induced cell death to both cell lines in NDM, but not in normal medium (DMEM). Among them, Andrographis Herba (No.17) exhibited the most potent preferential cytotoxicity against PANC-1 and PSN-1 cells with the PC_{50}

values of 9.72 μg/mL and 9.41 μg/mL, respectively. Isodonis Herba (No.1), Phellodendri Cortex (No.2), Lycopodium (No.15), and Panacis Japonici Rhizoma (No.18) showed also mild activity. The ethidium bromide (EB)/acridine orange (AO) double-staining on PANC-1 and PSN-1 cells treated by these extracts predicted that Isodonis Herba Herba (No.1), Lycopodium (No.15), and Panacis Japonici Rhizoma (No.18) induced the apoptosis-like cell death under nutrient starvation whereas Phellodendri Cortex (No.2) and Andrographis Herba (No.17) induced necrosis-like cell death.

2. Preferentially cytotoxic constituents of *Androgarphis paniculata*

To further investigate active constituents against PANC-1 and PSN-1 cells, the most potent crude drug, Andrographis Herba (No.17) was selected, and bioassay-guided isolation of constituents was carried out. The 70% EtOH extract was subjected to a series of chromatographic separation which led to the isolation of 20 compounds: stearic acid (**1**), pinostrobin (**2**), β-sitosterol (**3**), 5-hydroxy-6,7-dimethoxyflavone (**4**), pinocembrin (**5**), ermanin (**6**), ergosterol peroxide (**7**), oleanolic acid (**8**), 5,3′,4′-trihydroxy-7-methoxyflavone (**9**), andrograpanin (**10**), skullcapflavone I (**11**), loliolide (**12**), 5-hydroxy-7,8,2′,5′-tetramethoxyflavone (**13**), 3-oxo-*ent*-cleroda-8(17),11,13-trien-16,15-olide (**14**), 5,7,8-trimethoxyflavanone (**15**), 14-deoxy-11,12-didehydro-andrographolide (**16**), isoandrographolide (**17**), andrographolide (**18**), apigenin (**19**), and 14-deoxyandrographolide (**20**). Among these compounds, **2** and **5** have been isolated for the first time from the plants of *Andrographis* genus. Of the 20 compounds examined, **16** exhibited the most potent activity against PANC-1 and PSN-1 cells with the PC_{50} values of 10.0 μM and 9.27 μM, respectively. The six compounds, **7**, **8**, **10**, **12**, **13**, and **18** showed also the preferential cytotoxicity with mild potency (PC_{50}: 34–85 μM).

3. Preferential cytotoxicity of 14-deoxy-11,12-didehydroandrographolide (16)

In a continuing study, the nutrient-dependency of the preferential cytotoxicity of 14-deoxy-11,12-didehydroandrographolide (**16**), which showed the most potent activity, was examined. As a result, **16** inhibited survival of PANC-1 cells under deprivation of amino acids or serum, whereas **16** caused cell death of PSN-1 under deprivation of serum. On the other hand, the mechanism of cell death induced by **16** was examined using microscopical observation, EB/AO double staining, and flow cytometry with propidium iodide/annexin V double staining. Interestingly, microscopic observation of the PANC-1 and PSN-1 cells treated by **16** displayed the membrane bleb, nuclear fragmentation, and chromatin condensation, which are typical apoptosis-like morphological changes. Further EB/AO double-staining experiment in the cells treated with **16** under nutrient starvation indicated that condensed and/or fragmented chromatin is stained in orange, which allowed us to predict that **16** induces apoptosis-like cell death. Finally, flow cytometry with propidium iodide/annexin V double staining of **16** against PANC-1 and PSN-1 cells in NDM indicated that it also triggered apoptosis-like cell death in a concentration- and time-dependent manner. These results suggested that **16** induced apoptosis-like cell death to PANC-1 and PSN-1 cells under nutrient starvation.

Conclusions

In a course of search for anticancer agent based on a novel anti-austerity strategy, I found that 70% EtOH extracts of the crude drug extracts of Isodonis Herba (No.1), Phellodendri Cortex (No.2), Lycopodium (No.15), Andrographis Herba (No.17), and Panacis Japonici Rhizoma (No.18) showed the preferential cytotoxicity against PANC-1 and PSN-1 cells. Among them, Andrographis Herba (No.17) exhibited the most potent

preferential cytotoxicity against PANC-1 and PSN-1 cells. Phytochemical investigation of this active extract led to the isolation of 20 compounds consisting of six labdane-type diterpenes (**10**, **14**, **16**–**18**, **20**), six flavones (**4**, **6**, **9**, **11**, **13**, **19**), three flavanones (**2**, **5**, **15**), two sterols (**3**, **7**), a fatty acid (**1**), a triterpene (**8**), and a monoterpene (**12**). Among these, 14-deoxy-11,12-didehydroandrographolide (**16**) displayed the most potent preferential cytotoxicity against PANC-1 and PSN-1 cells with PC_{50} value of 10.0 μM and 9.27 μM, respectively. Microscopical observation, EB/AO double staining, and flow cytometry with propidium iodide/annexin V double staining predicted that **16** triggered apoptosis-like cell death in NDM with an amino acids and/or serum-sensitive mode. These results suggest that the Andrographis Herba (aerial part of *A. paniculata*) and its active constituent, 14-deoxy-11,12-didehydroandrographolide (**16**) may be key leads in the development of new drugs based on the anti-austerity strategy.

Chapter 1

Introduction

Cancer is a very serious disease throughout the world. About 10 million people are diagnosed with cancer on the world, and more than 50% of patients died in the world year in and year out. A correct cancer diagnosis is essential for adequate and effective treatment because every cancer type requires a specific treatment regimen which encompasses one or more modalities such as surgery, and/or radiotherapy, and/or chemotherapy. However, anti-cancer agents including chemotherapy and radiation therapy could cause a number of side effects.[1] Therefore, anti-cancer agents that affect selectively to cancer cells are required.

Natural products have been a prodigious source of medicinal drugs for centuries, originating from herbal remedies, and many of them are still being used for clinical today. Major advances in modern medicines and the pharmaceutical industry were driven by natural products, and more than 50% of therapeutics currently used are either natural products or their derivatives.[2] Plants are a significant sources for anticancer drug discovery. For instance, vincristine, irinotecan, etoposide, and paclitaxel derived from plants are employed for cancer treatment.[3]

Among the different forms of cancer, pancreatic cancer is one of the most aggressive disease with an exceptionally high global mortality rate, as well as with an estimated 330,372 deaths worldwide in 2012, which ranks 7th as the most frequent cause of cancer death. Moreover, it has been estimated that the number of deaths from pancreatic cancer will reach 540,000 by 2030.[4] Pancreatic cancer rapidly metastases and leads to the death of patients in a short period after diagnosis. Thus, the 5-year survival rate of patients with pancreatic cancer is the lowest among several cancers (Figure 1.1). Though surgery is the only treatment that offers any prospect of potential cure, chemotherapy with 5-fluorouracil and gemcitabine is also used for palliative therapy of advanced pancreatic cancer. However, pancreatic cancer is largely resistant to most

known chemotherapeutic agents including 5-fluorouracil and gemcitabine (Figure 1.1).[5] Therefore, the effective chemotherapeutic agents that target pancreatic cancer are urgently required.

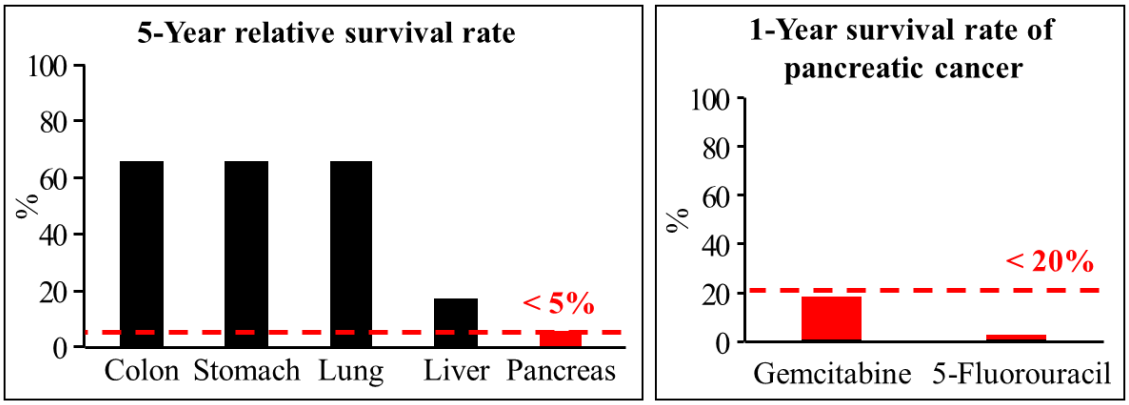


Figure 1.1 Survival rate in population-based cancer registry.

Cancer cells proliferate very fast because of high requirement for essential nutrients and oxygen. The immediate environment of cancers is increasing in size, however, often becomes heterogeneous and some regions of large cancers often possess micro environmental niches, which exhibit a significant gradient of critical metabolites, including oxygen, glucose, other nutrients, and growth factors.[6] Thus, many cancer cells obtain their critical metabolites by randomly recruiting new blood vessels, a phenomenon commonly known as angiogenesis, to survive under such severe conditions. However, human pancreatic cancer survives with an extremely poor blood supply and becomes more malignant.[7] The method by which pancreatic cancer cells, such as PANC-1, AsPC-1, BxPC-1, KP-3, PSN-1, and MiaPaCa-2, survives by obtaining a remarkable tolerance to extreme nutrient starvation (Figure 1.2).[8] Therefore, it has been hypothesized that eliminating the tolerance of cancer cells to nutrition starvation may provide a novel biochemical approach known as "anti-austerity" for cancer therapy.[9,10]

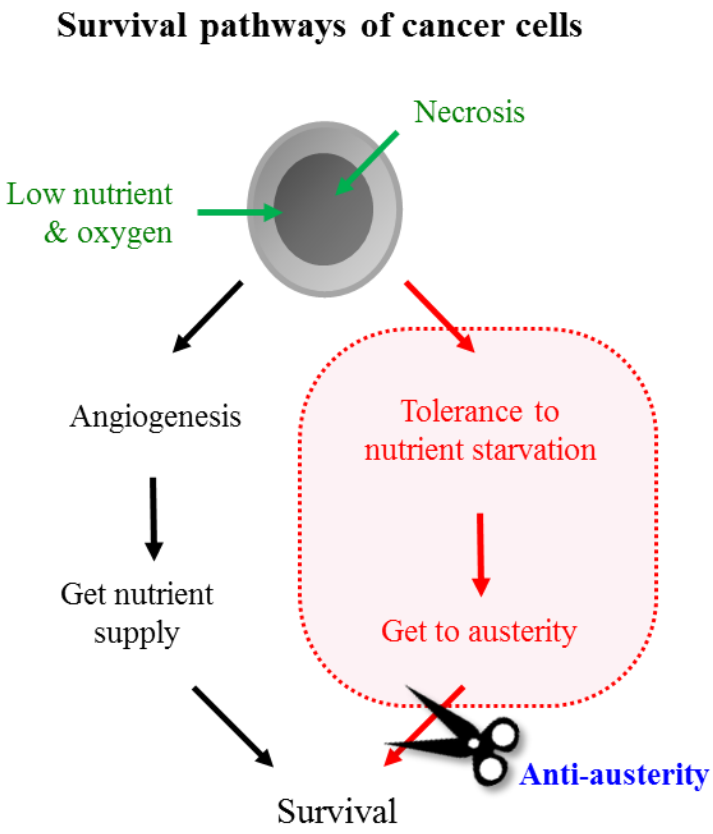


Figure 1.2 Tumor cell survival pathways.

Based on this hypothesis, a novel anti-austerity strategy has been developed to search candidates termed as anti-austerity agent, that preferentially show toxicity against cancer cells in nutrient-deprived medium (NDM), but not any toxicity in DMEM (Figure 1.3).[11]

In order to furnish anti-austerity agents, Esumi *et al*. have developed an anti-austerity activity assay system and that have found a novel compound kigamicin D[10] from culture of actinomycetes. An anthelminthic, pyrvinium pamoate[9] was also identified as an anti-austerity agent which has no antitumor activity against xenografts of human pancreatic cancers in nude mice.

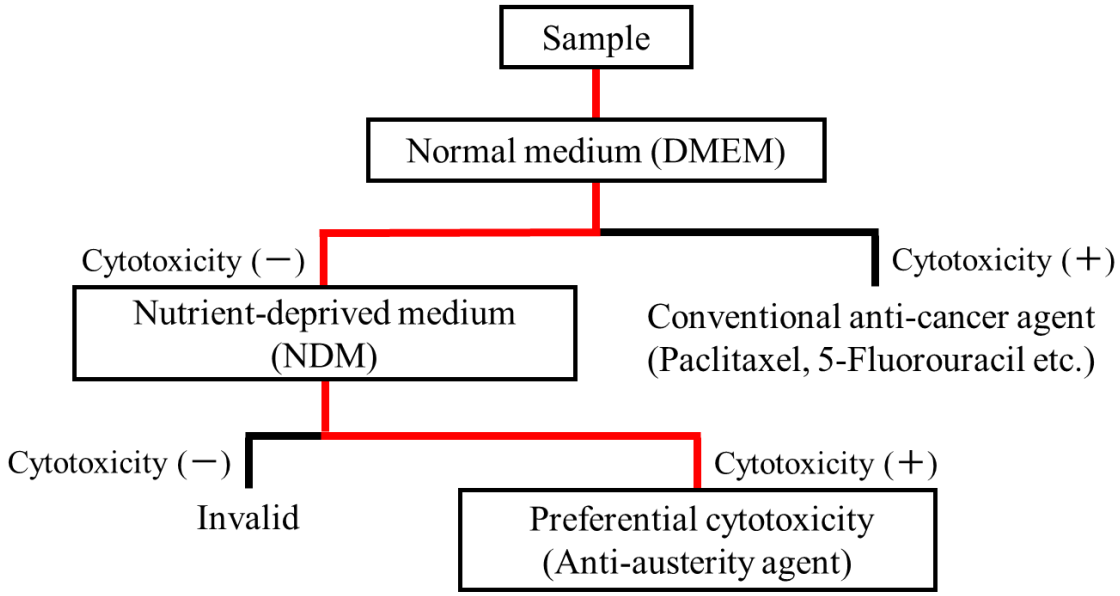


Figure 1.3 Anti-austerity strategy in anticancer drug discovery.
DMEM: Dulbecco's Modified Eagle Medium, NDM: Nutrient-Deprived Medium (absence of glucose, amino acids and serum)

According to these approaches, 500 crude drugs used in Japan and the active constituents of Arctii Fructus (fruits of *Arctium lappa*),[11] Angelicae Pubescentis Radix (root of *Angelica pubescens*),[12] Pini Resina (resin of *Pinus massoniana*),[13] and Notopterygii Rhizoma (rhizome and root of *Notopterygium incisum* or *N. forbesii*)[14] were screened by this assay system. Moreover, anti-austeric constituents of medicinal plants and propolis collected in Myanmar and Democratic Republic of Congo have been investigated to identify the anti-austerity agent in the laboratory.[15-19] Working on this strategy, numerous compounds possessing preferential *in vitro* cytotoxicity against PANC-l cells under NDM have been discovered as anti-austerity agents, such as arctigenin,[11] angelmarin,[12] nicolaioidesin B, panduratin A,[20] alkylated coumarins (kayeassamin A, B, C, D, E, and G),[21,22] matairesinol,[23] and damnacanthal[17] (Figure 1.4).

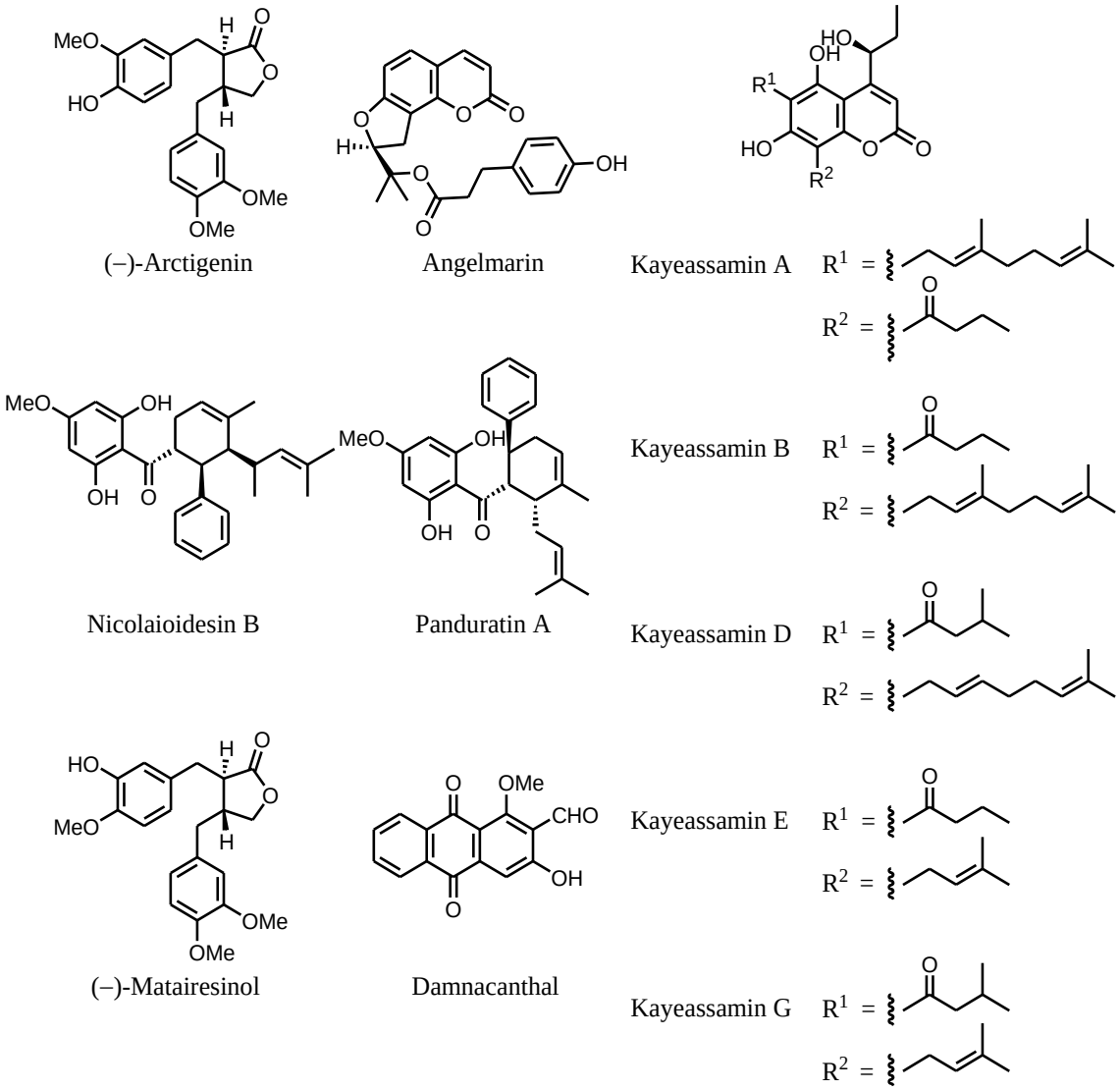


Figure 1.4 Structures of anti-austerity agents discovered from natural sources.

Among reported anti-austeric agents, it was reported that pyrvinium pamoate, kigamicin D, arctigenin induced necrotic cell death when glucose was depleted.[9–11] Troglitazone inhibited the survival of PANC-1 cells and caused necrosis under glucose and serum deprivation,[8] whereas (+)-grandifloracin induced apoptosis under glucose or serum deprivation.[24] 1,6,8-Trihydroxy-2,3,4,5-tetramethoxyxanthone and 1,6-dihydroxy-2,3,4,5,8-pentamethoxy-xanthone caused cytotoxicity under glucose or amino acids deprivation in the presence of serum.[19] LY294002 led to inhibition under

amino acid deprivation and caused apoptosis, although the effect was more prominent in the presence of serum.[8] Damnacanthal induced the cell death under serum deprivation.[17]

Among them, arctigenin has been discovered as the most potent anti-austerity agents. It also strongly suppressed tumor growth in a xenograft model using pancreatic cancer cells. However, arctigenin induced necrotic cell death when nutrients were depleted. While necrotic agent stimulates a host inflammatory response, apoptotic and autophagic agents don't provoke inflammation. Therefore, effective and different mechanism from other anti-austeric agents are urgently needed.

This study aims to search for active crude drugs and their active constituents based on anti-austerity strategy. In this study, the preferential cytotoxicity of crude drugs available in Japan is examined against human pancreatic cancer cell lines PANC-1 and PSN-1. Furthermore, active constituents of the most active crude drug were examined and the detailed mechanism of the preferential cytotoxicity of the isolated active compound are investigated.

Chapter 2

Preferential cytotoxicity of crude drugs available in Japan against human pancreatic cancer cell lines

2.1 Introduction

When normal human fibroblasts were subjected to extreme nutrient starvation by culturing in a medium without serum, glucose, and amino acids, cells died within 24 h. In contrast, human pancreatic cancer cell lines, PANC-1, AsPC-1, BxPC-1, KP-3, PSN-1, and MiaPaCa-2, survived for remarkably longer periods. In particular, PANC-1 cells are the most potent remarkable tolerance to extreme nutrient starvation. In contrast, PSN-1 cells are relatively sensitive to extreme nutrient starvation in comparison with other pancreas cancer cell lines.[8]

The laboratory of division of natural products chemistry has conducted a preliminary screening against human pancreatic cancer cell line PANC-1 of 500 crude drugs available in Japan and identified active constituents of Arctii Fructus (fruits of *Arctium lappa*),[11] Angelicae Pubescentis Root (root of *Angelica pubescens*),[12] Pini Resina (resin of *Pinus massoniana*),[13] and Notopterygii Rhizoma (rhizome and root of *Notopterygium incisum* or *N. forbesii*).[14] Moreover, anti-austeric constituents of medicinal plants used in Myanmar and Democratic Republic of Congo and of propolis.[15-22]

However, the preliminary screening of 500 crude drugs had not also been identified in terms of the activities of each crude drug with 50% preferential cytotoxicity (PC_{50}) value level in this study. Therefore, 24 crude drugs (Table 2.1) were selected based on the activity of crude drugs in the preliminary screening, and their PC_{50} against PANC-1 cells were evaluated. To investigate whether the preferential cytotoxicity was limited to PANC-1 cells or was observed against other pancreatic cancer cell lines, the effect on survival of PSN-1 cells was further examined.

Table 2.1 List of selected crude drugs available in Japan.

Extract No.	Crude drug name	Japanese name	Origin	Part used	Kracie No.
1	Isodonis Herba	Enmeiso (延命草)	*Plectranthus japonicus* Koidz. (Lamiaceae)	Aerial part	WA-1
2	Phellodendri Cortex	Obaku (黄柏)	*Phellodendron amurense* Rupr. (Rutaceae)	Bark	N-39
3	Lacca Sinica Exsiccata	Kanshitsu (乾漆)	*Rhus verniciflua* Stokes (Anacardiaceae)	Resin	WA-18
4	Coriandri Fructus	Kozuishi (胡荽子)	*Coriandrum sativum* L. (Apiaceae)	Fruit	WA-4
5	Arctii Fructus	Goboshi (牛蒡子)	*Arctium lappa* L. (Asteraceae)	Fruit	WA-2
6	Sesami Semen	Gomashi (胡麻子)	*Sesamum indicum* L. (Pedaliaceae)	Seed	WA-19
7	Leonuri Herba	Yakumoso (益母草)	*Leonurus sibiricus* L. (Lamiaceae)	Aerial part	WA-20
8	Asteris Radix	Shion (紫苑)	*Aster tataricus* L.f. (Asteraceae)	Root and Radix	WA-6
9	Hominis Placenta	Shikasha (紫河車)	*Homo sapiens* L. (Hominidae)	Placenta	WA-7
10	Lycii Radicis Cortex	Jikoppi (地骨皮)	*Lycium chinense* Mill. (Solanaceae)	Root bark	WA-21
11	Potentilla Hebiichigo Herba	Jabai (蛇苺)	*Potentilla hebiichigo* Yonek. & H.Ohashi (Rosaceae)	Aerial part	WA-8
12	Pini Resina	Shoko (松香)	*Pinus massoniana* Lamb. (Pinaceae)	Resin	WA-9
13	Polygoni Orientalis Fructus	Suikokashi (水紅花子)	*Carthamus tinctorius* L. (Asteraceae)	Fruit	WA-10
14	Hirudo	Suitetsu (水蛭)	*Whitmania pigra* Whitman (Hirudidae)	Whole body	WA-11
15	Lycopodium	Sekishoshi (石松子)	*Lycopodium clavatum* L. (Lycopodiaceae)	Spore	WA-3
16	Agrimoniae Herba	Senkakuso (仙鶴草)	*Agrimonia pilosa* Ledeb. (Rosaceae)	Aerial part	WA-12
17	Andrographis Herba	Senshinren (穿心蓮)	*Andrographis paniculata* Nees (Acanthaceae)	Aerial part	WA-5
18	Panacis Japonici Rhizoma	Chikusetsuninjin (竹節人参)	*Panax japonicus* C.A.Mey. (Araliaceae)	Rhizome	K-20
19	Polyporus	Chorei (猪苓)	*Polyporus umbellatus* Fries (Polyporaceae)	Sclerotium	W-C-1
20	Angelicae Pubescentis Root	Dokkatsu (独活)	*Angelica pubescens* Maxim. (Umbeliferae)	Root	W-9-11
21	Scutellariae Herba	Hanshiren (半枝蓮)	*Scutellaria barbata* D.Don. (Lamiaceae)	Aerial part	WA-13
22	Atractylodis Rhizoma	Byakujutsu (白朮)	*Atractylodes ovata* DC. (Asteraceae)	Rhizome	Z-B-1
23	Eriobotryae Folium	Biwayo (枇杷葉)	*Eriobotrya japonica* Lindl. (Rosaceae)	Leaf	WA-15
24	Akebiae Caulis	Mokutsu (木通)	*Akebia quinata* Dence. (Lardizabalaceae)	Climbing stem	WA-17

2.2 Preferential cytotoxicity against PANC-1 and PSN-1 cells

The 70% EtOH extracts of the 24 crude drugs available in Japan were screened for their preferential cytotoxicity against human pancreatic cancer cell lines PANC-1 and PSN-1 (Table 2.1). The preferential cytotoxicity was determined by comparing the cytotoxicities in DMEM and NDM. Of the 24 extracts examined, nine extracts of Isodonis Herba (extract No.1, aerial part of *Plectranthus japonicus*), Phellodendri Cortex (No.2, bark of *Phellodendron amurense*), Lacca Sinica Exsiccata (No.3, resin of *Rhus verniciflua*), Arctii Fructus (No.5, fruits of *Arctium lappa*), Lycopodium (No.15, spore of *Lycopodium clavatum*), Agrimoniae Herba (No.16, aerial part of *Agrimonia pilosa*), Andrographis Herba (No.17, aerial part of *Andrographis paniculata*), Panacis Japonici Rhizoma (No.18, rhizome of *Panax japonicus*), and Chorei (No.19, sclerotium of *Polyporus umbellatus*) induced cell death to both cell lines in NDM, but not in DMEM (Figures 2.1 and 2.2).

Among them, Andrographis Herba (No.17) exhibited the most potent preferential cytotoxicity against PANC-1 and PSN-1 cells with the PC_{50} values of 9.72 μg/mL and 9.41 μg/mL, respectively. Isodonis Herba (No.1), Phellodendri Cortex (No.2), Lycopodium (No.15), and Panacis Japonici Rhizoma (No.18) showed also mild activity (Table 2.2). In contrast, Arctii Fructus (No.5), from which the potent anti-austeric agent, arctigenin, had been isolated,[11] showed the preferential cytotoxicity against PANC-1 and PSN-1 cells with the PC_{50} values of 71.7 μg/mL and 78.1 μg/mL, respectively.

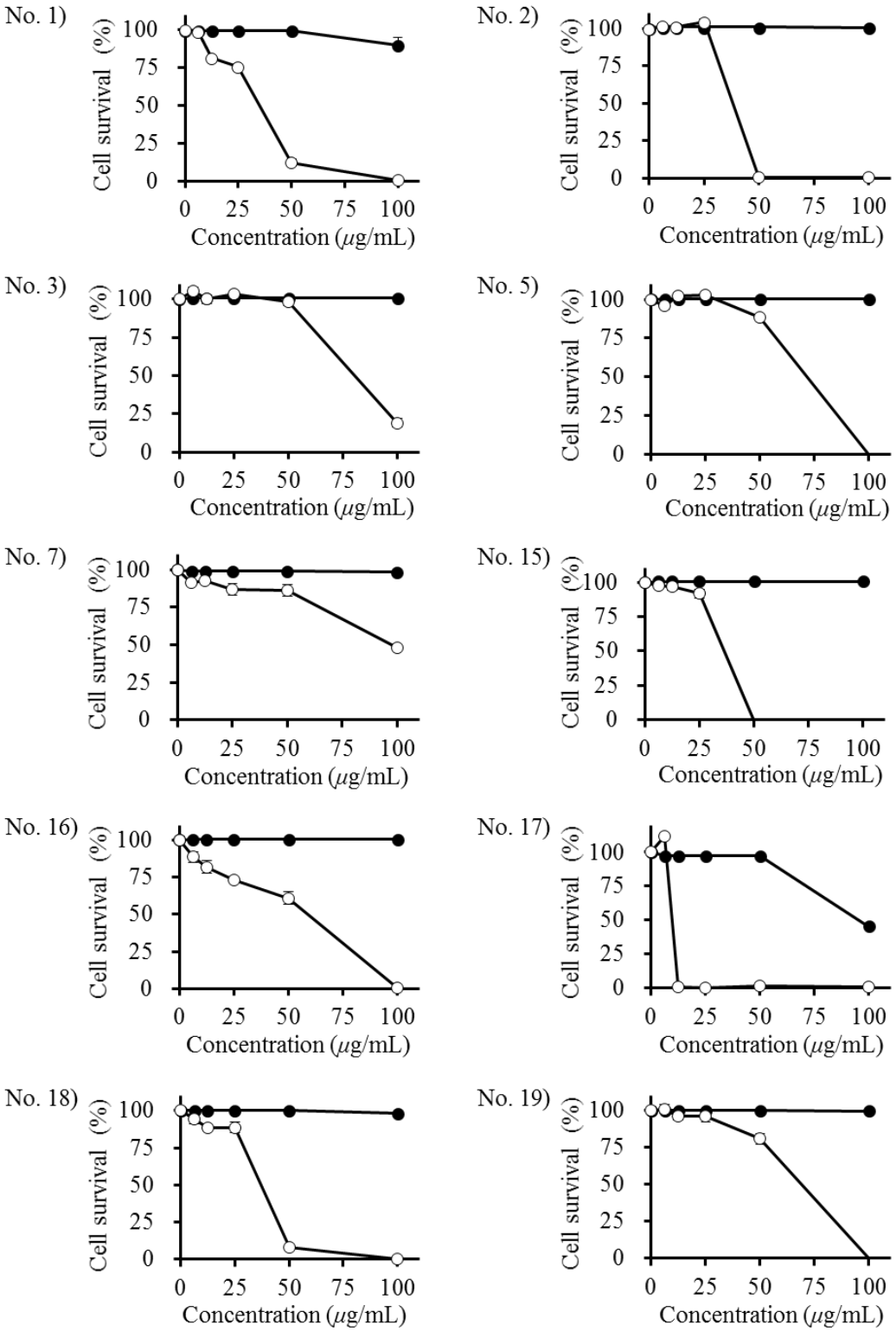


Figure 2.1 Preferential cytotoxicity of crude drug extracts on cell survival in the pancreatic cancer cell line PANC-1 under NDM (○) or DMEM (●) condition. Points are mean from triplicate experiments. The cell number at the start of the starvation was considered to be 100%.

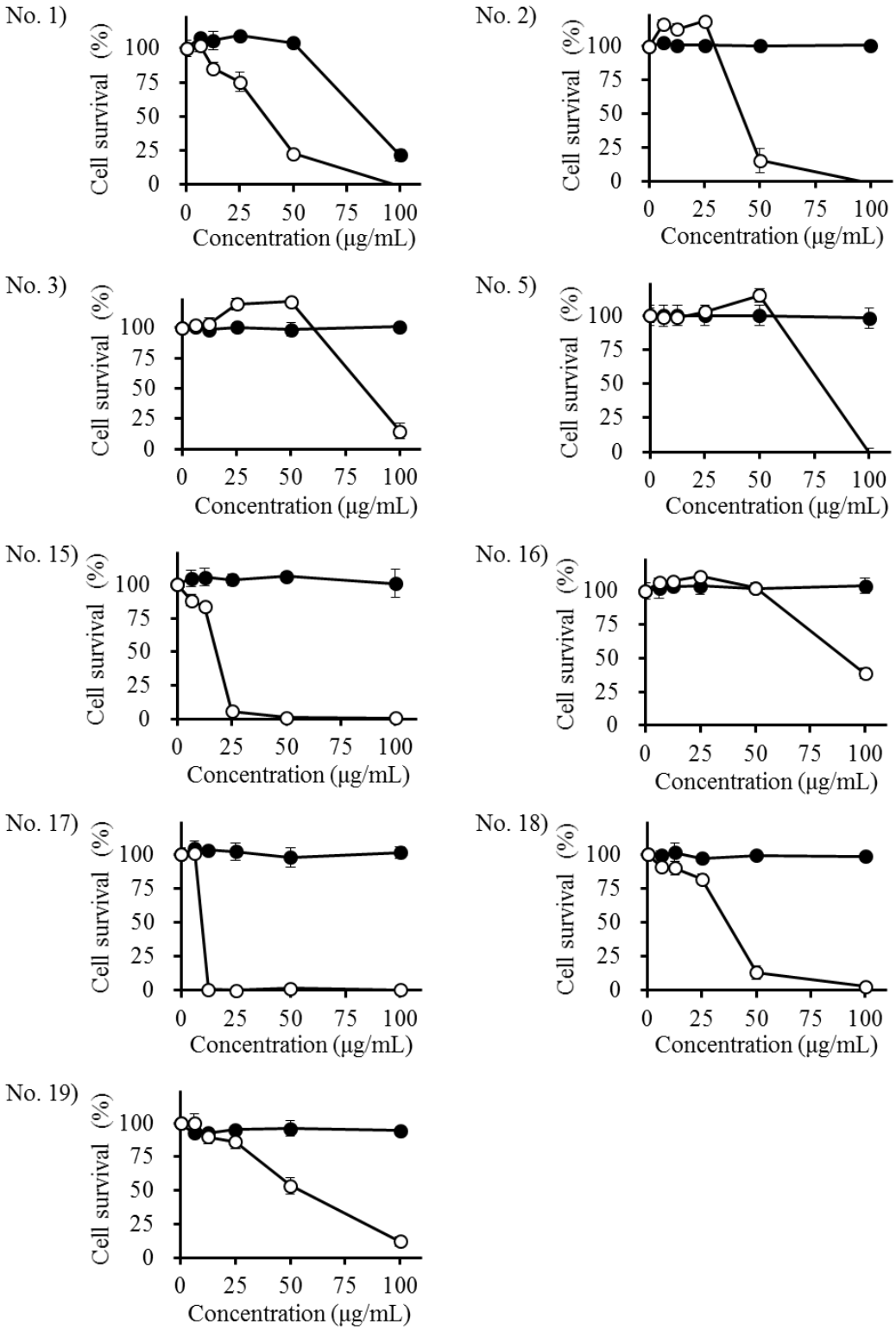


Figure 2.2 Preferential cytotoxicity of crude drug extracts on cell survival in the pancreatic cancer cell line PSN-1 under NDM (○) or DMEM (●) condition. Points are mean from triplicate experiments. The cell number at the start of the starvation was considered to be 100%.

Table 2.2 Preferential cytotoxicity of crude drugs available in Japan against human pancreatic cancer cell lines PANC-1 and PSN-1.

Extract No.	PC_{50} (μg/mL)[a]	
	PANC-1	PSN-1
1	35.1	37.1
2	38.1	41.6
3	80.5	83.6
5	71.7	78.1
7	97.1	> 100
15	36.3	17.9
16	59.2	91.0
17	9.72	9.41
18	37.0	36.6
19	69.0	54.2
Others	> 100	> 100
Arctigenin[b]	0.29	0.47
Paclitaxel[c]	> 100	> 100

[a] Preferentially 50% growth inhibitory concentration in NDM.

[b,c] Positive and negative control, respectively.

In addition, time- and concentration-dependent preferential cytotoxicity of five active crude drug extracts against PANC-1 and PSN-1 cells in NDM were evaluated, in order to investigate possibility as candidates that selectively target tumor cells in susceptible phases of the cell cycle. All of them exhibited the preferential cytotoxicity in a time- and concentration-dependent manner (Figure 2.3).

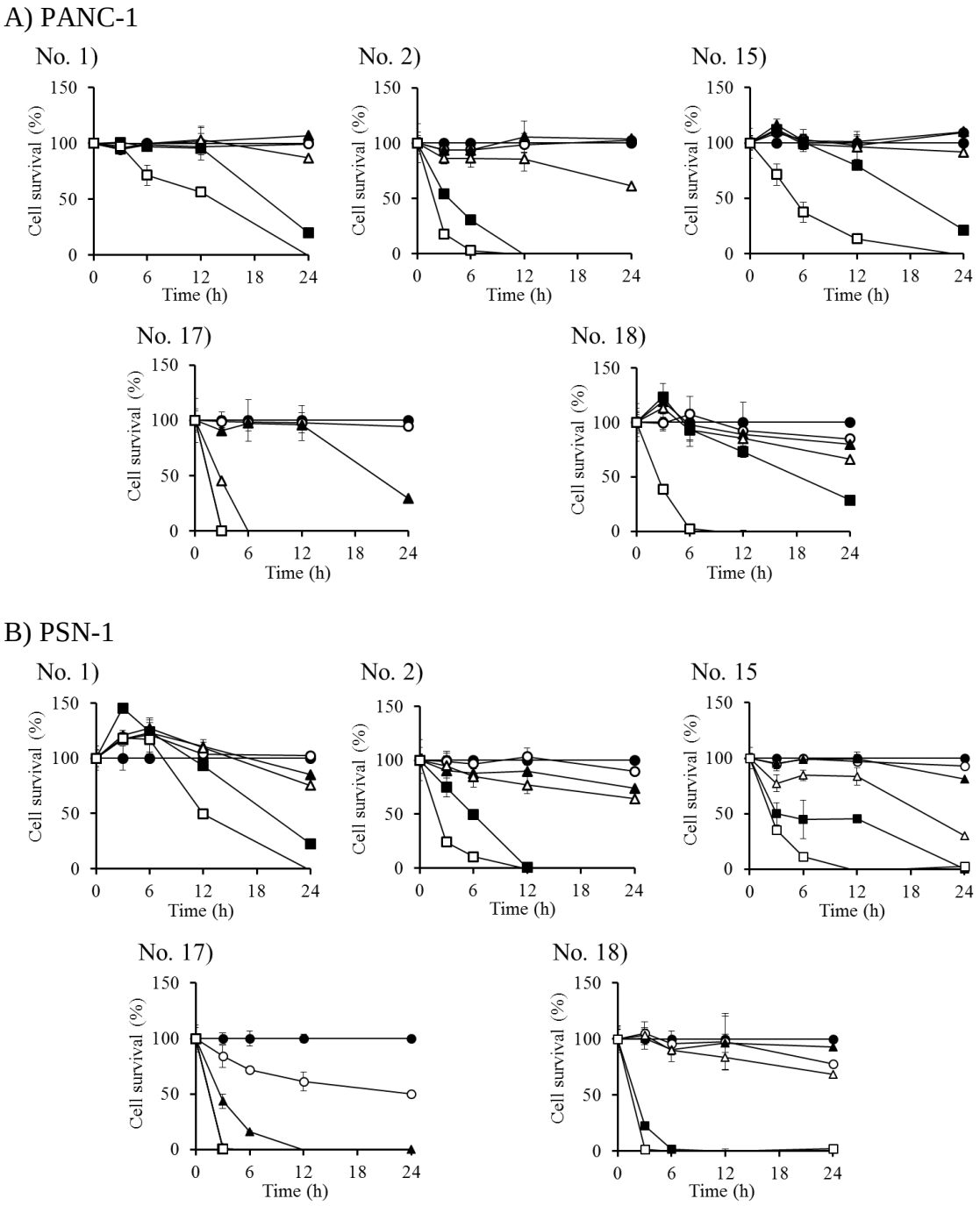


Figure 2.3 Time-dependent preferential cytotoxicity of extracts of Isodonis Herba (No.1, aerial part of *P. japonicus*), Phellodendri Cortex (No.2, bark of *P. amurense*), Lycopodium (No.15, spore of *L. clavatum*), Andrographis Herba (No.17, aerial part of *A. paniculata*), and Panacis Japonici Rhizoma (No.18, rhizome of *P. japonicus*) against PANC-1 (A) and PSN-1 (B) cells in NDM. Final concentration of each extract was 0 μg/mL (●), 6.25 μg/mL (○), 12.5 μg/mL (▲), 25 μg/mL (△), 50 μg/mL (■), and 100 μg/mL (□). Points are mean from triplicate experiments. The cell number at the start of the starvation was considered to be 100%.

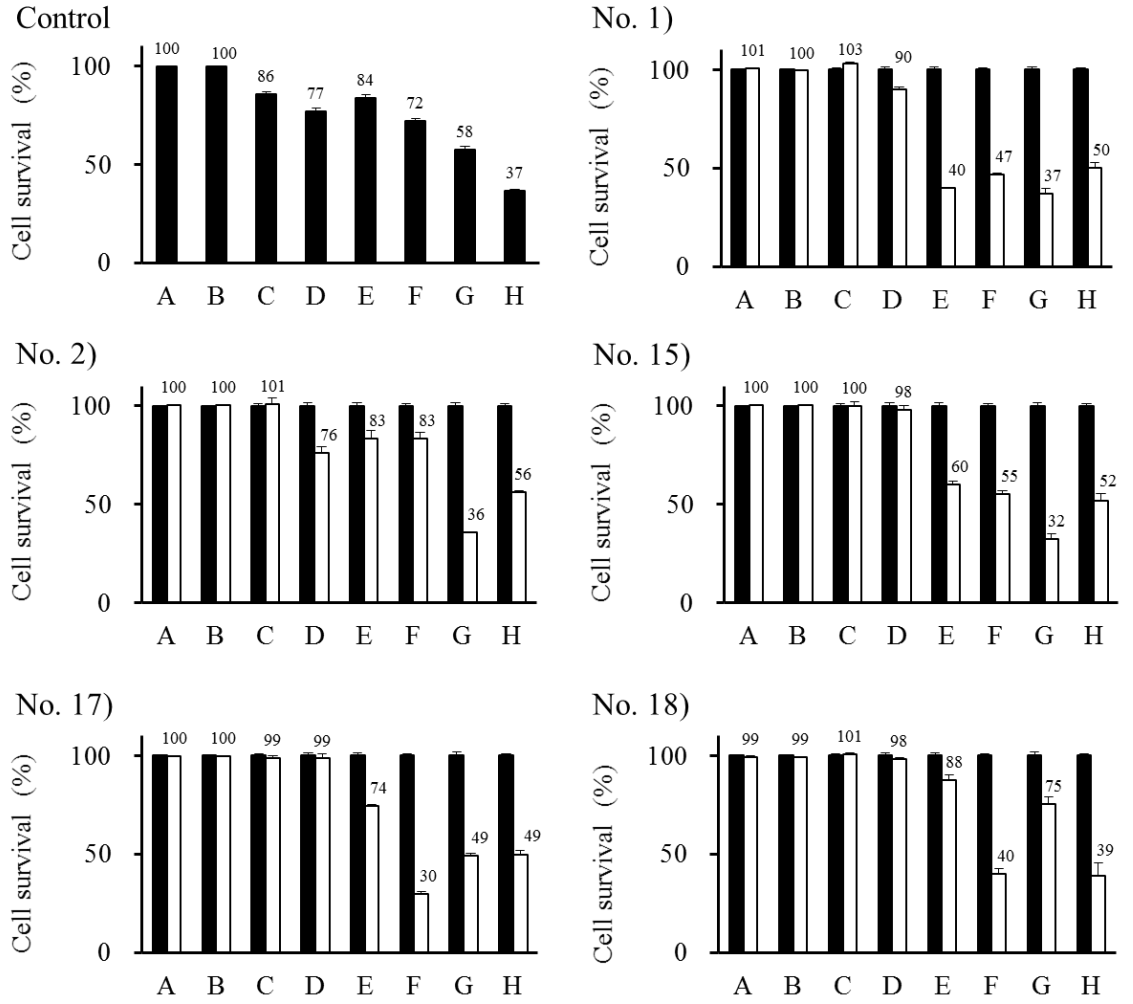
2.3 Nutrient-dependent preferential cytotoxicity against PANC-1 and PSN-1 cells

Depletion of nutrients such as glucose, amino acids, and serum from culture medium is sensitive to the cytotoxicity and is important in the anti-austerity assay. In order to confirm the nutrient selectivity of the drugs, the preferential cytotoxicity of the five active crude drug extracts was examined by varying the components of nutrients.

Interestingly, the five extracts inhibited survival of PANC-1 cells under deprivation of serum. The result demonstrated that serum is the key component that determined the sensitivity of PANC-1 cells of the extracts (Figure 4.2A).

On the other hand, glucose is key factors to the extract of Phellodendri Cortex (No.2, bark of *P. amurense*), while serum is found to be important to the preferential cytotoxicity of extract of Andrographis Herba (No.17, aerial part of *A. paniculata*) against PSN-1 cells (Figure 2.4B).

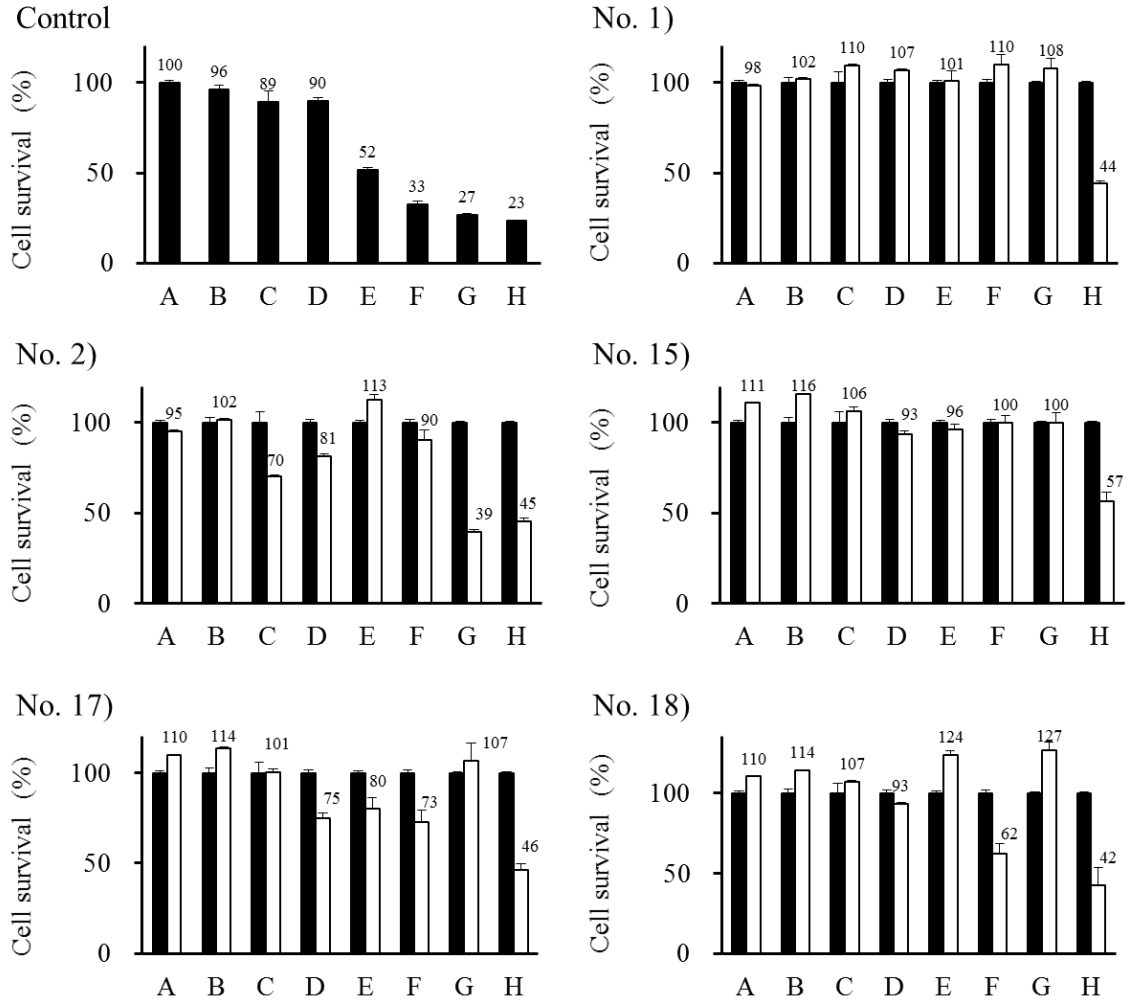
A) PANC-1



Nutrients	A	B	C	D	E	F	G	H
Glucose	+	+	−	−	+	+	−	−
Amino acids	+	−	+	−	+	−	+	−
Serum	+	+	+	+	−	−	−	−

Figure 2.4A Nutrient-dependent preferential cytotoxicity of extracts of Isodonis Herba (No.1, aerial part of *P. japonicus*), Phellodendri Cortex (No.2, bark of *P. amurense*), Lycopodium (No.15, spore of *L. clavatum*), Andrographis Herba (No.17, aerial part of *A. paniculata*), and Panacis Japonici Rhizoma (No.18, rhizome of *P. japonicus*) against PANC-1 cells. The white and black columns mean the data with and without the extract, respectively. The final concentration was set to the PC_{50} values of each extract and the cell survival rate was examined at 24 h after nutrient starvation. On the control panel, the cell survival rate under DMEM (condition A) was arbitrary setted to 100%, while on the panel of each extract cell survival rate of the control under corresponding condition was arbitrary set to 100%. Data are expressed as the mean ± s. d. (n = 3).

B) PSN-1



Nutrients	A	B	C	D	E	F	G	H
Glucose	+	+	−	−	+	+	−	−
Amino acids	+	−	+	−	+	−	+	−
Serum	+	+	+	+	−	−	−	−

Figure 2.4B Nutrient-dependent preferential cytotoxicity of extracts of Isodonis Herba (No.1, aerial part of *P. japonicus*), Phellodendri Cortex (No.2, bark of *P. amurense*), Lycopodium (No.15, spore of *L. clavatum*), Andrographis Herba (No.17, aerial part of *A. paniculata*), and Panacis Japonici Rhizoma (No.18, rhizome of *P. japonicus*) against PSN-1 cells. The white and black columns mean the data with and without the extract, respectively. The final concentration was set to the PC_{50} values of each extract and the cell survival rate was examined at 24 h after nutrient starvation. On the control panel, the cell survival rate under DMEM (condition A) was arbitrary setted to 100%, while on the panel of each extract cell survival rate of the control under corresponding condition was arbitrary set to 100%. Data are expressed as the mean ± s. d. (n = 3).

2.4 EB/AO double staining

EB/AO double-staining (ethidium bromide/acridine orange) can identify the viable, apoptotic, and necrotic cells based on color and appearance. In this method, live cells appear green with intact nuclei; early-apoptotic cells appear green with condensed and/or fragmented chromatin; late-apoptotic cells appear orange with condensed and/or fragmented chromatin; necrotic cells appear reddish orange without structural abnormality. Thus, the mechanism of cell death induced by the five extracts in PANC-1 and PSN-1 cells was investigated using the EB/AO double-staining method.[25]

PANC-1 cells in control were alive and stained in green, whereas PANC-1 cells treated with the extracts of Isodonis Herba (No.1, aerial part of *P. japonicus*), Lycopodium (No.15, spore of *L. clavatum*), and Panacis Japonici Rhizoma (No.18, rhizome of *P. japonicus*) were stained in orange. On the other hand, PANC-1 cells treated with extracts of Phellodendri Cortex (No.2, bark of *P. amurense*) and Andrographis Herba (No.17, aerial part of *A. paniculata*) were stained in red (Figure 2.5A). These results predicted that the extracts of Isodonis Herba (No.1), Lycopodium (No.15), and Panacis Japonici Rhizoma (No.18) induced the apoptosis-like cell death against PANC-1 cells under nutrient starvation, while the extracts of Phellodendri Cortex (No.2) and Andrographis Herba (No.17) induced necrosis-like cell death.

As in the case for PANC-1 cells, PSN-1 cells treated with the extracts of Isodonis Herba (No.1), Lycopodium (No.15), and Panacis Japonici Rhizoma (No.18) were stained in orange, while, PSN-1 cells treated with extracts of Phellodendri Cortex (No.2) and Andrographis Herba (No.17) were stained in red (Figure 2.5B). These results suggested that the extracts of Isodonis Herba (No.1), Lycopodium (No.15), and Panacis Japonici Rhizoma (No.18) induced also the apoptosis-like cell death against PSN-1 cells under nutrient starvation and the extracts of Phellodendri Cortex (No.2) and Andrographis Herba (No.17) induced also the necrosis-like cell death.

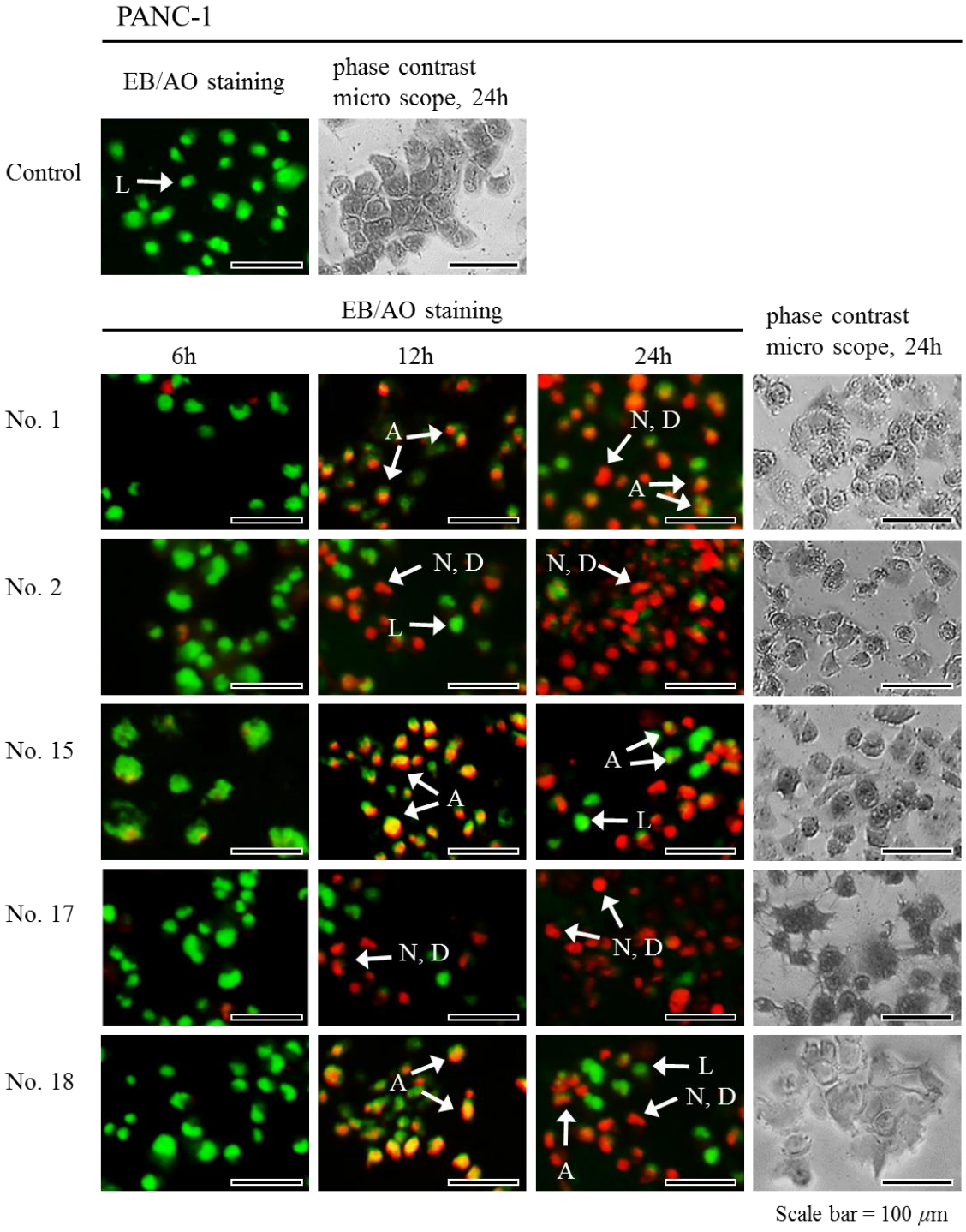


Figure 2.5A EB/AO double-staining of PANC-1 cells treated with extracts at PC_{50} concentration of Isodonis Herba (No.1, aerial part of *P. japonicus*), Phellodendri Cortex (No.2, bark of *P. amurense*), Lycopodium (No.15, spore of *L. clavatum*), Andrographis Herba (No.17, aerial part of *A. paniculata*), and Panacis Japonici Rhizoma (No.18, rhizome of *P. japonicus*). The control data were obtained at 24 h of the treatment. L: live cell, A: apoptotic-like cell, N: necrotic-like cell, D: dead cell.

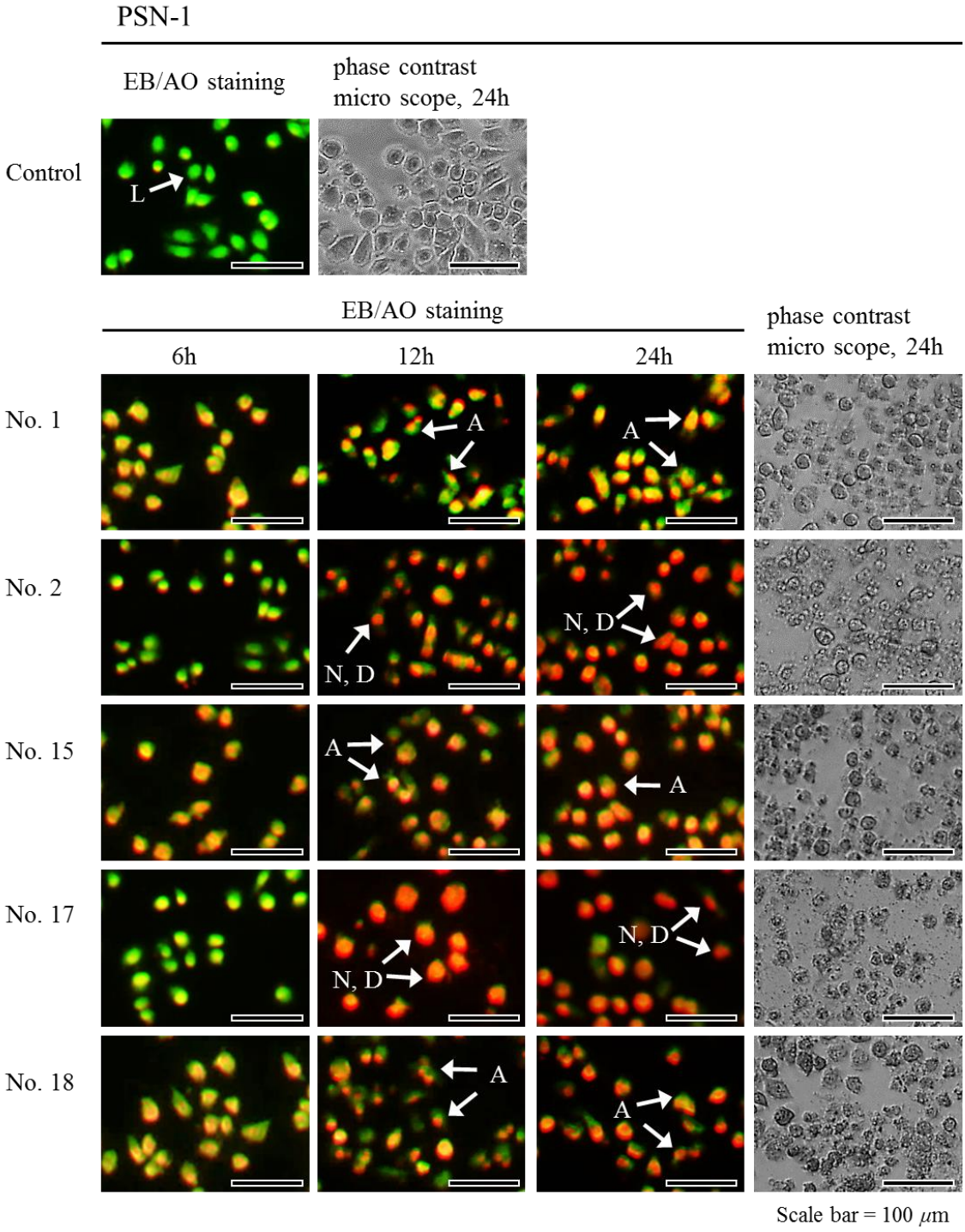


Figure 2.5B EB/AO double-staining of PSN-1 cells treated with extracts at PC_{50} concentration of Isodonis Herba (No.1, aerial part of *P. japonicus*), Phellodendri Cortex (No.2, bark of *P. amurense*), Lycopodium (No.15, spore of *L. clavatum*), Andrographis Herba (No.17, aerial part of *A. paniculata*), and Panacis Japonici Rhizoma (No.18, rhizome of *P. japonicus*). The control data were obtained at 24 h of the treatment. L: live cell, A: apoptotic-like cell, N: necrotic-like cell, D: dead cell.

2.5 Summary of chapter 2

The following remarks can be summarized from chapter 2.

Our results demonstrated that the crude drug extracts of Isodonis Herba (extract No.1, aerial part of *Plectranthus japonicus*), Phellodendri Cortex (No.2, bark of *Phellodendron amurense*), Lacca Sinica Exsiccata (No.3, resin of *Rhus verniciflua*), Arctii Fructus (No.5, fruits of *Arctium lappa*), Lycopodium (No.15, spore of *Lycopodium clavatum*), Agrimoniae Herba (No.16, aerial part of *Agrimonia pilosa*), Andrographis Herba (No.17, aerial part of *Andrographis paniculata*), Panacis Japonici Rhizoma (No.18, rhizome of *Panax japonicus*), and Chorei (No.19, sclerotium of *Polyporus umbellatus*) induced cell death to both human pancreatic cancer cell lines PANC-1 and PSN-1 in NDM, but not in DMEM.

Among them, Andrographis Herba (No.17) exhibited the most potent preferential cytotoxicity against PANC-1 and PSN-1 cells with the PC_{50} values of 9.72 μg/mL and 9.41 μg/mL, respectively. Isodonis Herba (No.1), Phellodendri Cortex (No.2), Lycopodium (No.15), and Panacis Japonici Rhizoma (No.18) showed also mild activity with the PC_{50} values less than 50 μg/mL. The EB/AO double staining on cells treated by these extracts suggested that Isodonis Herba (No.1), Lycopodium (No.15), and Panacis Japonici Rhizoma (No.18) induced the apoptosis-like cell death under nutrient deprivation whereas Phellodendri Cortex (No.2) and Andrographis Herba (No.17) induced the necrosis-like cell death.

Chapter 3

Preferentially cytotoxic constituents of *Andrographis paniculata*

3.1 Introduction

In the previous chapter, Andrographis Herba (No.17, aerial part of *Androgarphis paniculata*) exhibited the most potent preferential cytotoxicity against human pancreatic cancer cell lines PANC-1 and PSN-1 with the PC_{50} values of 9.72 μg/mL and 9.41 μg/mL, respectively. In a continuing study, the most potent crude drug, Andrographis Herba (No.17) was selected, and bioassay-guided isolation of constituents in the drug was carried out, to investigate active constituents against PANC-1 and PSN-1 cells.

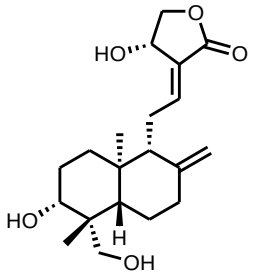
Andrographis Herba consists of the dried aerial parts of *A. paniculata* (Burm. f.) Nees (Acanthaceae),[26-28] which was and widely found and was cultivated in tropical and subtropical Asia, south-east Asia and India.[29,30]

Figure 3.1 *Andrographis paniculata*

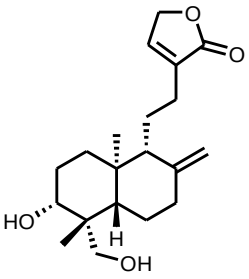
Studies on chemical constituents the ethanol or methanol extracts of the whole plant, leaf, and stem of *A. paniculata* have reported the presence of diterpenoids and flavonoids.[31-35] Andrographolide is the major diterpenoid in *A. paniculata*, making up about 4%, 0.8~1.2%, and 0.5~6% in dried whole plant, stem, and leaf extracts

respectively.[31,33,36] The other main diterpenoids are deoxyandrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographide, and isoandrographolide.[31,37] 5-Hydroxy-7,8-dimethoxyflavone, 5-hydroxy-7,8,2',5'-tetramethoxyflavone, 5-hydroxy-7,8,2',3'-tetramethoxyflavone, 5-hydroxy-7,8,2'-trimethoxyflavone, and 7-*O*-methylwogonin were isolated as the main flavonoids from EtOAc-soluble fraction of the ethanol or methanol extracts (Figure 3.2).[37-40]

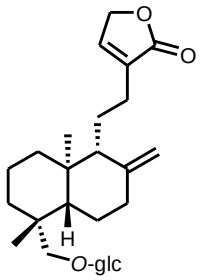
Andrographolide exhibits multiple pharmacological properties and is a potential chemotherapeutic agent. Andrographolide contains an α-alkylidene γ-butyrolactone moiety and three hydroxyl groups at C-3, C-14, and C-19 responsible for the cytotoxic activities of andrographolide against many cancer cell lines.[41] Among the andrographolide analogues, 14-deoxy-11,12-didehydroandrographolide is immunostimulatory, anti-infective and anti-atherosclerotic; neoandrographolide is anti-inflammatory, anti-infective, and anti-hepatotoxic; 14-deoxyandrographolide is immunomodulatory and anti-atherosclerotic. Among the less abundant compounds from *A. paniculata*, andrograpanin is both anti-inflammatory and anti-infective; 14-deoxy-14,15-dehydroandrographolide is antiinflammatory; isoandrographolide, 3,19-isopropylideneandrographolide and 14-acetylandrographolide are tumor suppressive; arabinogalactan proteins are anti-hepatotoxic. The four flavonoids from *A. paniculata*, namely 7-*O*-methylwogonin, apigenin, onysilin, and 3,4-dicaffeoylquinic acid are anti-atherosclerotic.[42]



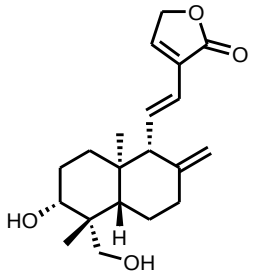
Andrographolide



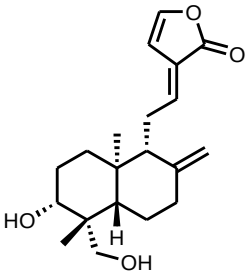
14-Deoxyandrographolide



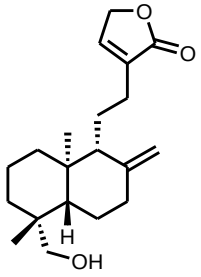
Neoandrographolide



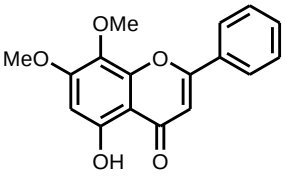
14-Deoxy-11,12-didehydro-andrographolide



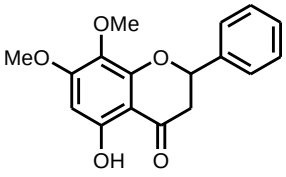
14-Deoxy-14,15-didehydro-andrographolide



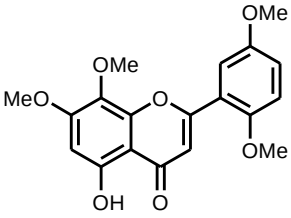
Andrograpanin



5-Hydroxy-7,8-dimethoxyflavone



5-Hydroxy-7,8-dimethoxyflavanone



5-Hydroxy-7,8,2',5'-tetramethoxyflavone

Figure 3.2 Chemical structures of reported diterpenoids and flavonoids from *A. paniculata*.

3.2 Extraction and isolation of active constituents

The aerial part of *A. paniculata* (850 g) were extracted with 70% EtOH under sonication to yield extract (61.0 g). This extract (30.0 g) was suspended in distilled water and partitioned with $CHCl_3$ to give the $CHCl_3$-soluble extract (3.5 g). The $CHCl_3$-soluble extract exhibited the potent preferential cytotoxicity with the PC_{50} value of 19.0 μg/mL (Figure 3.3).

Figure 3.3 Extraction procedures of aerial part of *A. paniculata*.
PC_{50} is preferentially 50% growth inhibitory concentration in NDM

Accordingly, the $CHCl_3$-soluble extract (3.0 g) was chromatographed on a silica gel column to yield 16 fractions. The 16 fractions exhibited the preferential cytotoxicity against PANC-1 cells. Among them, fr. 9 (PC_{50}: 73.7 μg/mL), fr. 10 (PC_{50}: 99.2 μg/mL), fr. 11 (PC_{50}: 34.5 μg/mL), fr. 12 (PC_{50}: 34.6 μg/mL), fr. 13 (PC_{50}: 74.9 μg/mL), fr. 14 (PC_{50}: 11.1 μg/mL), fr. 15 (PC_{50}: 10.1 μg/mL), and fr. 16 (PC_{50}: 24.3 μg/mL) displayed the preferential cytotoxicity against human pancreatic cancer cell line PANC-1.

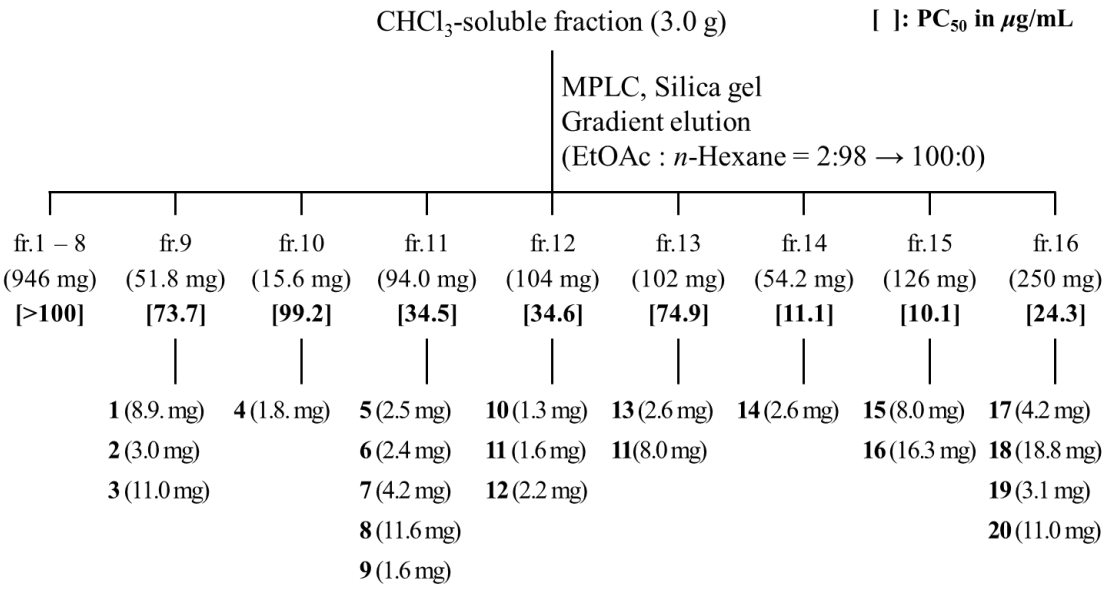
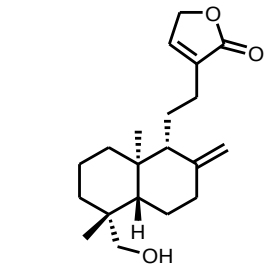


Figure 3.4 Bioassay guided isolation from the $CHCl_3$-soluble extract of *A. paniculata*. PC_{50} is preferentially 50% growth inhibitory concentration in NDM

The preferentially cytotoxic fractions 9-16 from the $CHCl_3$-soluble extract of *A. paniculata* were thus subjected to a series of chromatographic separation which led to the isolation of 20 compounds (Figure 3.4). Their structures were identified by analyses of spectroscopic data and comparison with the literature data to be: stearic acid (**1**, 8.9 mg),[43] pinostrobin (**2**, 3.0 mg),[44] *β*-sitosterol (**3**, 11.0 mg),[45] 5-hydroxy-6,7-dimethoxyflavone (**4**, 1.8 mg),[46] pinocembrin (**5**, 2.5 mg),[47] ermanin (**6**, 2.4 mg),[48] ergosterol peroxide (**7**, 4.2 mg),[49] oleanolic acid (**8**, 11.6 mg),[50] 5,3′,4′-trihydroxy-7-methoxyflavone (**9**, 1.6 mg),[51] andrograpanin (**10**, 1.3 mg),[52] skullcapflavone I (**11**, 9.6 mg),[53] loliolide (**12**, 2.2 mg),[54] 5-hydroxy-7,8,2′,5′-tetramethoxyflavone (**13**, 2.6 mg),[37] 3-oxo-*ent*-cleroda-8(17),11,13-trien-16,15-olide (**14**, 2.6 mg),[55] 5,7,8-trimethoxy-flavanone (**15**, 8.0 mg),[56] 14-deoxy-11,12-didehydroandrographolide (**16**, 16.3 mg),[57] isoandrographolide (**17**, 4.2 mg),[58] andrographolide (**18**, 18.8 mg),[59] apigenin (**19**, 3.1 mg),[60] and 14-deoxyandrographolide (**20**, 11.0 mg)[61] (Figure 3.5). Among these compounds, compounds **2** and **5** have been isolated for the first time from the plants of *Andrographis* genus.



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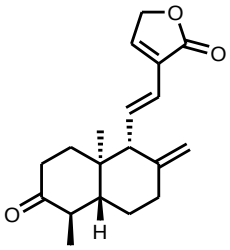

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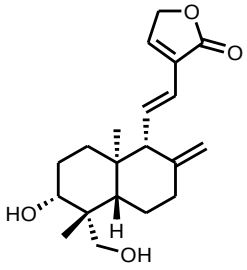

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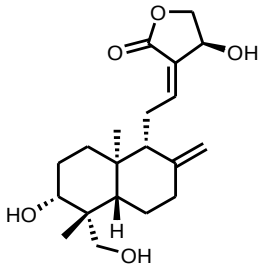

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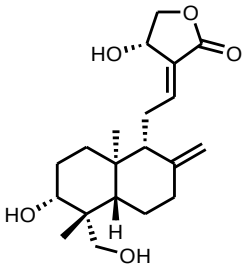

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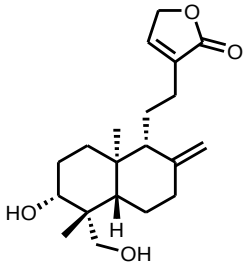

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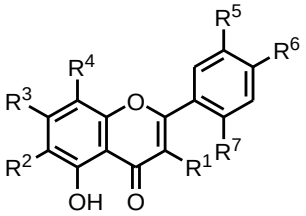

4 $R^1 = R^4 = R^5 = R^6 = R^7 = H$, $R^2 = R^3 = OMe$
6 $R^1 = R^6 = OMe$, $R^2 = R^4 = R^5 = R^7 = H$, $R^3 = OH$
9 $R^1 = R^2 = R^4 = R^7 = H$, $R^3 = OMe$, $R^5 = R^6 = OH$
11 $R^1 = R^2 = R^5 = R^6 = H$, $R^3 = R^4 = OMe$, $R^7 = OH$
13 $R^1 = R^2 = R^6 = H$, $R^3 = R^4 = R^5 = R^7 = OMe$
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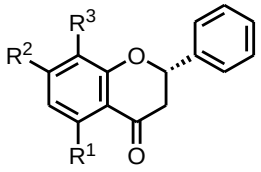
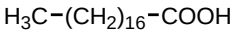


2 $R^1 = OH$, $R^2 = OMe$, $R^3 = H$
5 $R^1 = R^2 = OH$, $R^3 = H$
15 $R^1 = R^2 = R^3 = OMe$



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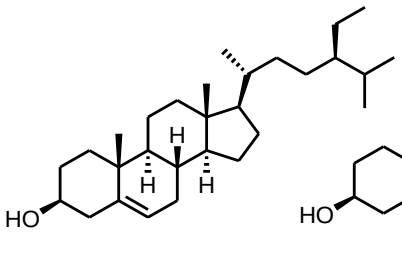

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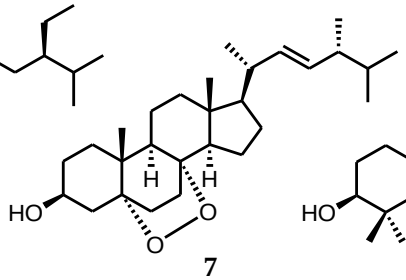

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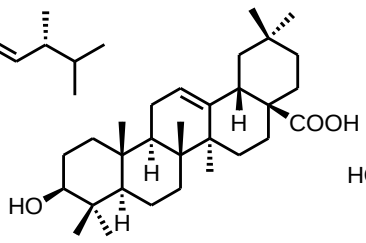

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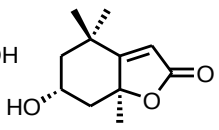

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Figure 3.5 Structures of compounds isolated from the $CHCl_3$-soluble extract of *A. paniculata*

3.3 Preferential cytotoxicity of isolated compounds against PANC-1 and PSN-1 cells

Compounds **1**–**20** were evaluated for their preferential cytotoxicity against human pancreatic cancer cell lines PANC-1 and PSN-1. Compounds **7**, **8**, **10**, **12**, **13**, **16**, and **18** induced cell death against both PANC-1 and PSN-1 cells in NDM in a concentration-dependent manner, but not in DMEM (Figure 3.6). Among these, only 14-deoxy-11,12-didehydroandrographolide (**16**) had the most potent activity with PC_{50} values of 10.0 μM and 9.27 μM against PANC-1 and PSN-1 cells, respectively. The six compounds, **7**, **8**, **10**, **12**, **13**, and **18** showed also the preferential cytotoxicity with mild potency (PC_{50}: 34–85 μM) (Table 3.1).

Table 3.1 Preferential cytotoxicity of compounds **1**–**20** on human pancreatic cancer cells lines PANC-1 and PSN-1 in NDM.

Compound	PC_{50} (μM)[a]		Compound	PC_{50} (μM) [a]	
	PANC-1	PSN-1		PANC-1	PSN-1
7	60.5	79.2	**16**	10.0	9.27
8	40.4	58.6	**18**	34.3	48.3
10	46.0	43.9	Others	>100	>100
12	85.0	>100	Arctigenin[b]	0.48	0.68
13	74.9	74.1	Paclitaxel[c]	>100	>100

[a] PC_{50}: Preferentially 50% growth inhibitory concentration in NDM

[b,c] Positive and negative control, respectively.

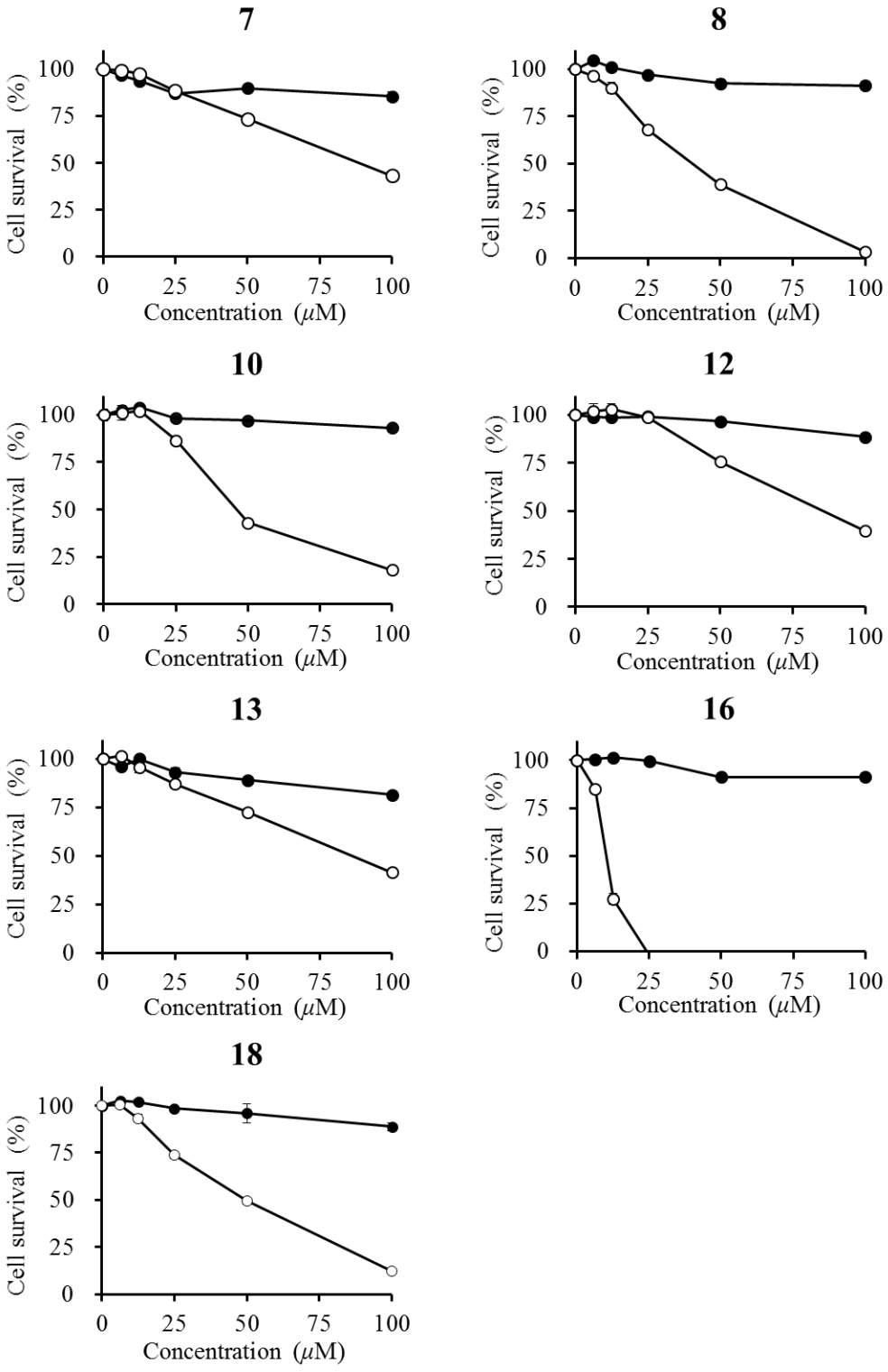


Figure 3.6A Effects of active compounds **7**, **8**, **10**, **12**, **13**, **16**, and **18** on cell survival in the PANC-1 cells under NDM (○) or DMEM (●) condition. Points are mean from triplicate experiments. The cell number at the start of the starvation was considered to be 100%.

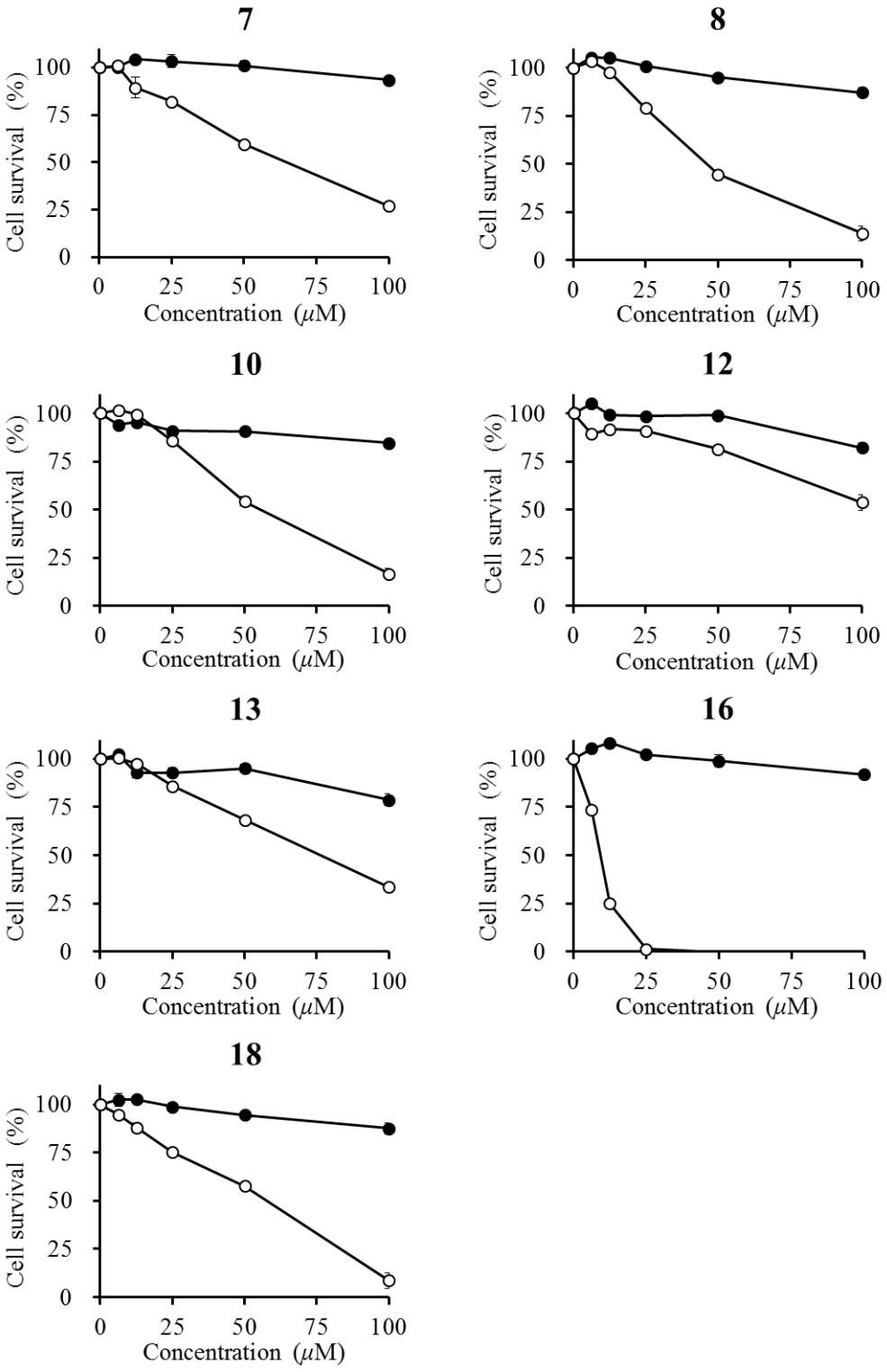


Figure 3.6B Effects of active compounds **7**, **8**, **10**, **12**, **13**, **16**, and **18** on cell survival in the PSN-1 cells under NDM (○) or DMEM (●) condition. Points are mean from triplicate experiments. The cell number at the start of the starvation was considered to be 100%.

In particular, 14-deoxy-11,12-dihydroandrographholide (**16**), labdane-type diterpene, showed the most potent activity, whereas andrograpanin (**10**) and andrographolide (**18**) displayed the mild activity. The other labdane-type diterpenes (**14**, **17**, **20**) did not show any cytotoxicity. These results would suggest the importance of the γ-butyrolactone moiety as reported previously.[62,63] It was reported that the double bond at C-12 and C-13 is responsible for the cytotoxic activity. However, our results demonstrated the importance of that the double bond at C-11 and C-12, not at C-13 and C-14, suggesting that the mechanism of the cytotoxicity of **16** and **17** are different.

Furthermore, the presence of the hydroxyl substituent at C-3 or hydroxyl methyl substituent at C-4 would potentiate the preferential cytotoxicity, since the activity of **16** is more potent than **14** (PC_{50} > 100 μM). In addition, the presence of the double bond at C-11 and C-12 would potentiates the preferential cytotoxicity against PANC-1 and PSN-1 cells, since the activity of **16** is more potent than **20** (PC_{50} > 100 μM).

Andrographolide (**18**) exhibited activity against PANC-1 and PSN-1 cells with PC_{50} values of 34.3 and 48.3 μM, respectively. It has been reported that andrographolide (**18**) an α-alkylidene γ-butyro-lactone moiety and three hydroxyls at C-3, C-19, and C-14, which are responsible for the cytotoxic activity against other cancer cells.[24] In addition to the previous findings, we propose that the hydroxyl at C-14 or the double bond at C-12 and C-13 of andrographolide (**18**) may play a certain role in the preferential cytotoxicity of **18**, since the activity of **18** is more potent than **20** (PC_{50} > 100 μM).

Finally, among the active labdane diterpenes, andrograpanin (**10**) exhibited activity against PANC-1 and PSN-1 cells with PC_{50} values of 46.0 and 43.9 μM, respectively. We suspected that a hydroxyl deficiency at C-3 of andrograpanin (**10**) may play a crucial role in the preferential cytotoxicity of **10**, since the activity of **10** is more potent than **20** (PC_{50} > 100 μM).

On the other hand, 5-hydroxy-7,8,2′,5′-tetramethoxyflavone (**13**) exhibited mild

activity against PANC-1 and PSN-1 cells with PC_{50} values of 74.9 and 74.1 μM, respectively. The presence of two methoxyl at B-ring would potentiate the preferential cytotoxicity against PANC-1 and PSN-1 cells, since the activity of **13** is more potent than **11** (PC_{50} > 100 μM).

It has been reported that flavanons are not responsible for the cytotoxic activity against PANC-1 cells.[16] Likewise, in this study, three flavanons (**2**, **5**, **16**) did not display preferential cytotoxicity against PANC-1 and PSN-1 cells with PC_{50} values less than 100 μM.

3.4 Summary of chapter 3

The following remarks can be summarized from chapter 3.

Bioassay guided isolation on the 70% EtOH extract of *Andrographis paniculata*, which showed preferential cytotoxicity against human pancreatic cancer cell lines PANC-1 and PSN-1 in NDM, led to the isolation of 20 compounds including six labdane-type diterpenes (**10**, **14**, **16**–**18**, **20**), six flavones (**4**, **6**, **9**, **11**, **13**, **19**), and three flavanones (**2**, **5**, **15**). Among these compounds, **2** and **5** have been isolated for the first time from the plants of *Andrographis* genus.

Of the 20 compounds examined, 14-deoxy-11,12-didehydroandrographolide (**16**) exhibited the most potent activity with the PC_{50} values of 10.0 μM and 9.27 μM against PANC-1 and PSN-1 cells, respectively. The six compounds, ergosterol peroxide (**7**), oleanolic acid (**8**), andrograpanin (**10**), loliolide (**12**), 5-hydroxy-7,8,2',5'-tetramethoxyflavone (**13**), and andrographolide (**18**) showed also the preferential cytotoxicity with mild potency (PC_{50}: 34–85 μM).

Chapter 4

Preferential cytotoxicity of 14-deoxy-11,12-didehydro-andrographolide

4.1 Introduction

The 14-deoxy-11,12-dihydroandrographholide (**16**) isolated from the $CHCl_3$-soluble extract of *Andrographis paniculata* exhibited the most potent preferential cytotoxicity against human pancreatic cancer cell lines PANC-1 and PSN-1 with the PC_{50} values of 10.0 μM and 9.27 μM, respectively. In the continuing study, time- and nutrient-dependency of the preferential cytotoxicity of **16** was investigated against PANC-1 and PSN-1 cells, as in the case of crude drugs mentioned in chapter 2. Furthermore, the mechanism of cell death induced by **16** was examined using microscopical observation, EB/AO double staining, and flow cytometry with propidium iodide/annexin V double staining.

4.2 Time- and concentration-dependent effect of compound 16

In order to investigate possibility as a candidate that selectively target tumor cells in susceptible phases of the cell cycle, time- and concentration-dependent preferential cytotoxicity of 14-deoxy-11,12-dihydroandrographhholide (**16**) against PANC-1 and PSN-1 cells in NDM was evaluated.

Compound **16** exhibited cytotoxicity against PANC-1 cells (Figure 4.1), depending on the incubation time and its concentration. In particular, compound **16** inhibited the growth of PANC-1 cell from 4 h to 24 h over 12.5 μM. Compound **16** also showed the cytotoxicity against PSN-1 cells, depending on the time and concentration, as in case of those against PANC-1 cells. However, its time- and concentration-dependency were different from the case of those against PANC-1 cells. Thus, **16** inhibited significantly PSN-1 cell survival from 8 h to 20 h over 12.5 μM. Furthermore, **16** significantly inhibited the PSN-1 cell survival at 50 μM within 12 h.

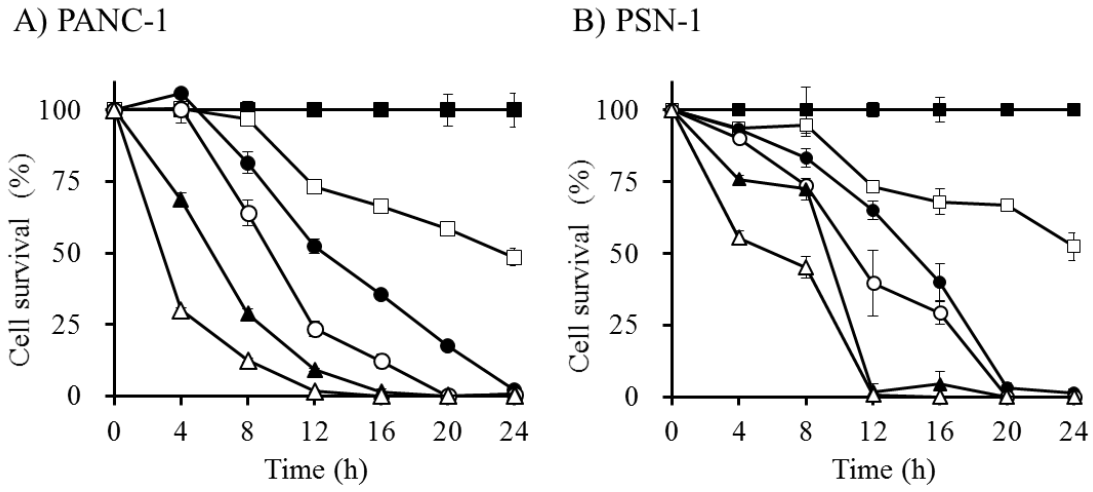


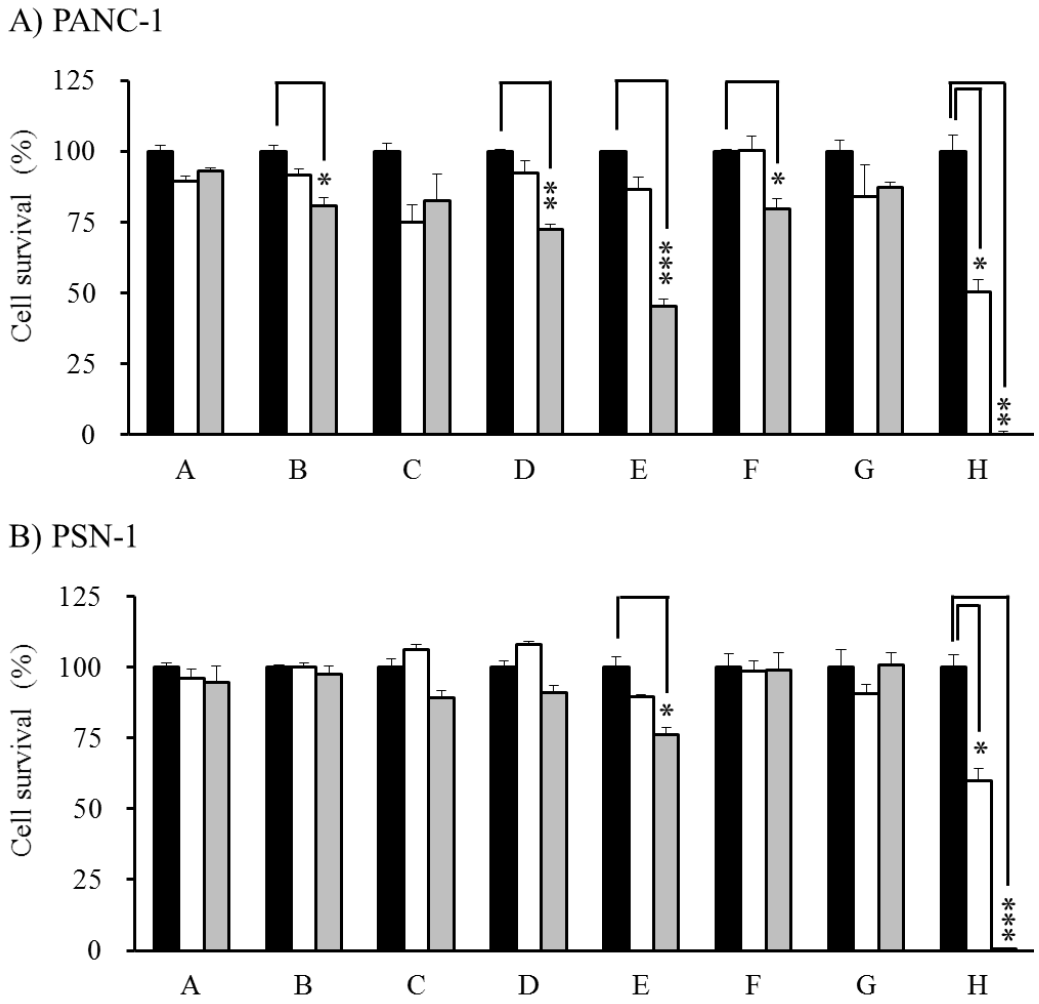
Figure 4.1 Time- and concentration-dependent effect of 14-deoxy-11,12-dihydroandrographhholide (**16**) on PANC-1 (A) and PSN-1 (B) cells. Final concentration of each extract was 0 μM (■), 6.25 μM (□), 12.5 μM (●), 25 μM (○), 50 μM (▲), and 100 μM (△). Points are mean from triplicate experiments. The cell number at the start of the starvation was considered to be 100%.

4.3 Nutrient-dependent preferential cytotoxicity of compound 16

It was reported that pyrvinium pamoate, kigamicin D, arctigenin induced necrotic cell death when glucose was depleted.[9-11] Troglitazone inhibited the survival of PANC-1 cells and caused necrosis under glucose and serum deprivation,[8] whereas (+)-grandifloracin induced apoptosis under glucose or serum deprivation.[24] 1,6,8-Trihydroxy-2,3,4,5-tetramethoxyxanthone and 1,6-dihydroxy-2,3,4,5,8-pentamethoxy-xanthone caused cytotoxicity under glucose or amino acids deprivation in the presence of serum.[18] LY294002 led to inhibition under amino acid deprivation and caused apoptosis, although the effect was more prominent in the presence of serum.[8] Damnacanthal induced the cell death under serum deprivation.[17]

To compare with the reported anti-austeric agents, the nutrient-dependency of the preferential cytotoxicity of 14-deoxy-11,12-dihydroandrographholide (**16**) was investigated. Interestingly, **16** inhibited survival of PANC-1 cells under deprivation of amino acids or serum (Figure 4.2A). The result demonstrated that amino acids or serum is the key component that determines the sensitivity of PANC-1 cells to the cytotoxicity of **16**. Interestingly, when serum was supplemented with amino acids, the effect of cell death is facilitated. Thus, the serum seemed to counteract the protective effect of amino acids.

On the other hand, **16** caused cell death of PSN-1 under deprivation of serum (Figure 4.2B). This result demonstrated that only serum is the key component that determines the sensitivity of PSN-1 cells to the cytotoxicity of **16**.



Nutrients	A	B	C	D	E	F	G	H
Glucose	+	+	−	−	+	+	−	−
Amino acids	+	−	+	−	+	−	+	−
Serum	+	+	+	+	−	−	−	−

Figure 4.2 Nutrient-dependency of preferential cytotoxicity of 14-deoxy-11,12-dihydroandrographholide (**16**) against PANC-1 (A) and PSN-1 (B) cells. Compound **16** was added to the medium, and cell survival was examined at 24 h after the start of nutrient deprivation. Final concentration of compound was 0 μM (■), 12.5 μM (□), and 25 μM (▣). Data are expressed by the mean values of triplicate determinations ± SD. *** $P < 0.0001$, ** $P < 0.001$, * $P < 0.01$ compared with control.

4.4 Mechanism of preferential cytotoxicity of compound 16

In continuous study, the mechanism of cell death induced by 14-deoxy-11,12-dihydroandrographholide (**16**) in PANC-1 and PSN-1 cells was investigated using the microscopical observation and EB/AO double staining[25] as in the case of the crude drugs as mentioned in chapter 2, as well as flow cytometry with propidium iodide/annexin V double staining.[64,65]

Microscopic observation of the PANC-1 and PSN-1 cells treated by **16** at 6.25 or 12.5 μM displayed the membrane bleb, nuclear fragmentation, and chromatin condensation (Figure 4.3), which are typical apoptosis-like morphological changes.

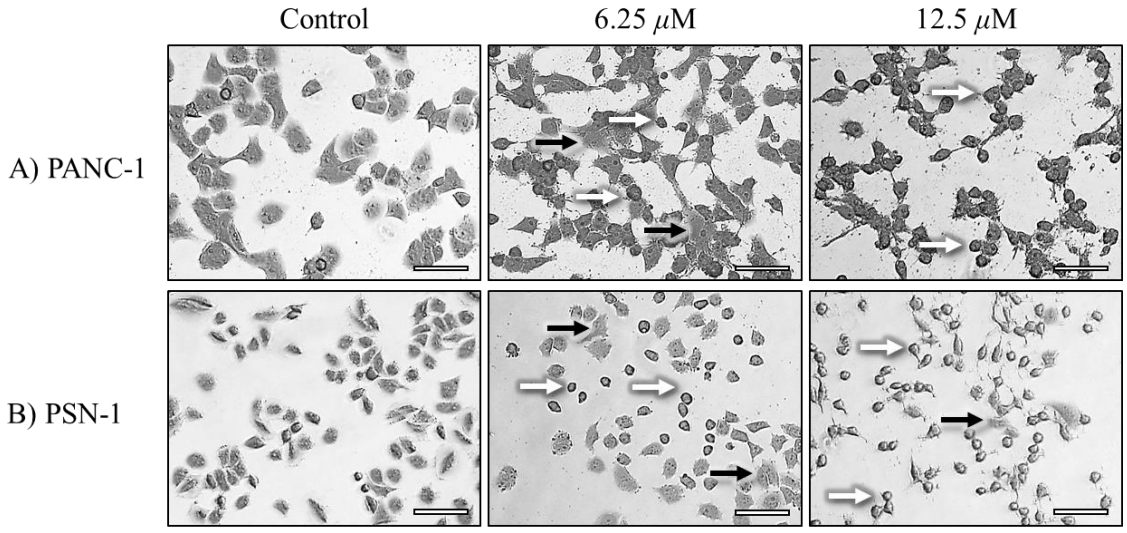


Figure 4.3 Morphological change of PANC-1 (A) and PSN-1(B) cells in NDM after 24 hours exposure with 6.25 and 12.5 μM of 14-deoxy-11,12-dihydroandrographholide (**16**). (White arrow: nucleus fragmentation and condensation; Black arrow: membrane bleb)

Furthermore, EB/AO double staining revealed that PANC-1 cells treated with 6.25 or 12.5 μM of **16** under nutrient starvation appear orange with condensed and/or fragmented chromatin (Figure 4.4A). These results suggested that **16** induced the apoptosis-like cell death against PANC-1 cells under NDM. PSN-1 cells treated with **16** under nutrient starvation appear orange (Figure 4.4B). These results suggested also that **16** induced the apoptosis-like cell death against PSN-1 cells under nutrient starvation.

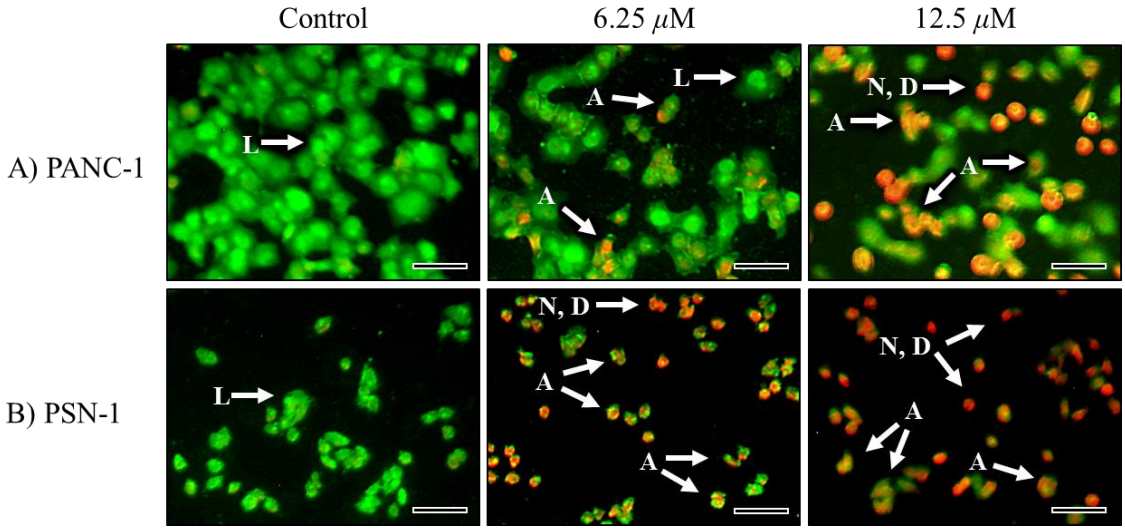


Figure 4.4 EB/AO staining of PANC-1 (A) and PSN-1 (B) cells treated with 14-deoxy-11,12-dihydroandrographholide (**16**) in NDM. Cells were treated with 6.25 and 12.5 μM of 12 hours and stained with EB/AO (L: live cell, A: apoptotic-like cell, N: necrotic-like cell, D: dead cell).

Flow cytometry with propidium iodide/annexin V double staining showed that PANC-1 cells treated in NDM with 6.25 or 12.5 μM of **16** for 8 h displayed propidium iodide-negative/annexin V-positive staining at levels of 34% and 55%, respectively. These ratios were raised to 47% and 55% by 16 h treatment (Figure 4.5A). PSN-1 cells treated in NDM with 6.25 or 12.5 μM of **16** for 8 h displayed propidium iodide-negative/annexin V-positive at levels of 54% and 73%, respectively (Figure 4.5B). These ratios were raised to 58% and 82% by 16 h treatment. These results suggest that **16** induced apoptosis-like cell death of PANC-1 and PSN-1 cells.

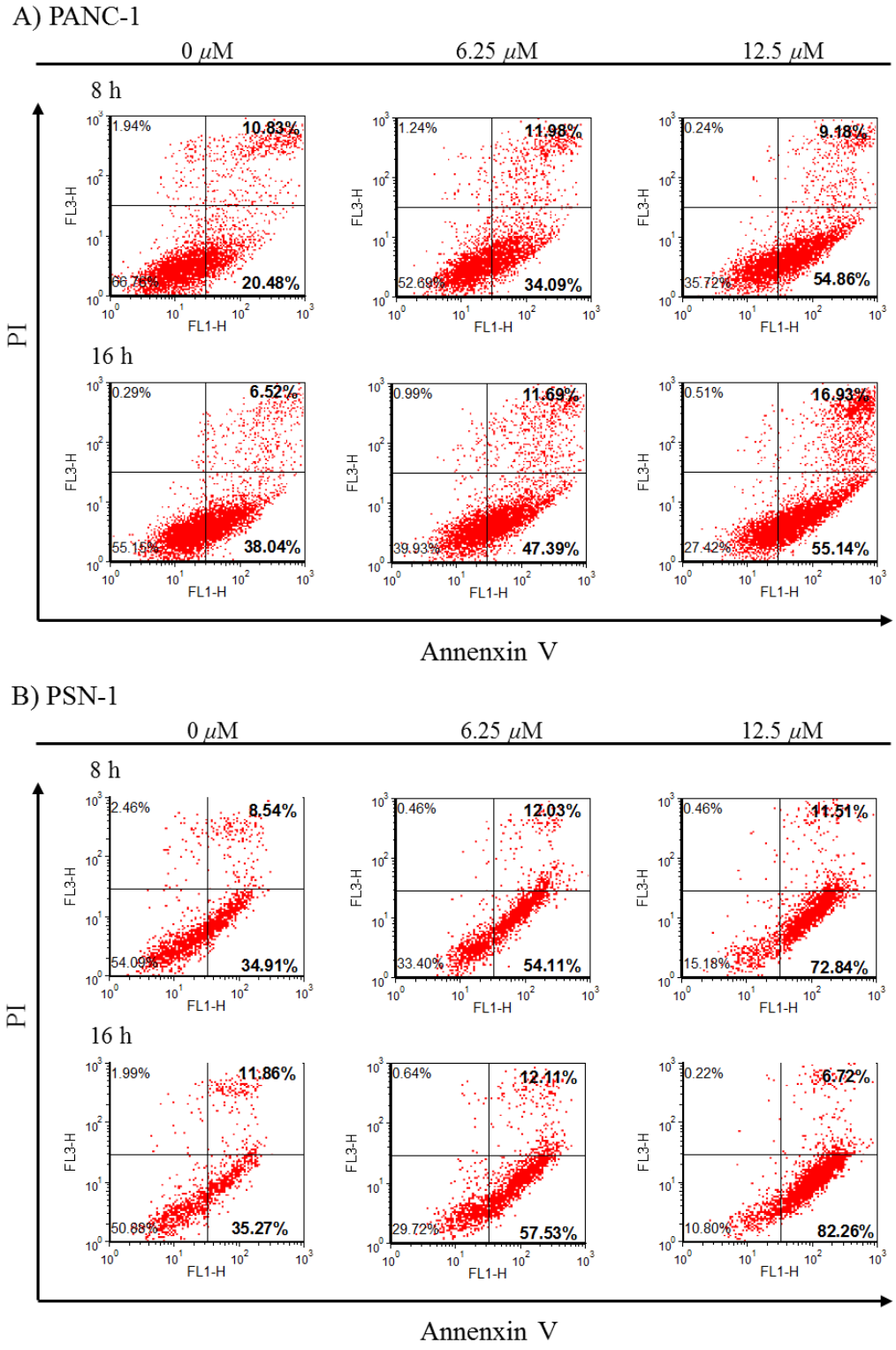


Figure 4.5 Flow cytometric analysis with propidium iodide/annexin V double staining of PANC-1 (A) and PSN-1 (B) cells treated with 14-deoxy-11,12-dihydroandrographholide (**16**) in NDM for 8 and 16 h.

4.5 Summary of chapter 4

The following remarks can be summarized from chapter 4.

14-Deoxy-11,12-didehydroandrographolide (**16**) inhibited survival of human pancreatic cancer cell line PANC-1 under deprivation of amino acids or serum, whereas **16** caused cell death of human pancreatic cancer cell line PSN-1 under deprivation of serum. On the other hand, the microscopic observation of the PANC-1 and PSN-1 cells treated by **16** displayed the membrane bleb, nuclear fragmentation, and chromatin condensation, which are typical apoptosis-like morphological changes. Further EB/AO double-staining experiment in the cells treated with **16** under nutrient starvation indicated that the condensed and/or fragmented chromatin is stained in orange, which allowed us to predict that **16** induces apoptosis-like cell death. Finally, flow cytometry with the propidium iodide/annexin V double staining of **16** against PANC-1 and PSN-1 cells in NDM predicted that it also triggered apoptosis-like cell death in a concentration- and time-dependent manner.

These results suggested that **16** induced apoptosis-like cell death to human pancreatic cancer cell lines PANC-1 and PSN-1 under nutrient starvation.

Conclusions

The 70% EtOH extracts of Isodonis Herba (extract No.1, aerial part of *Plectranthus japonicus*), Phellodendri Cortex (No.2, bark of *Phellodendron amurense*), Lacca Sinica Exsiccata (No.3, resin of *Rhus verniciflua*), Arctii Fructus (No.5, fruits of *Arctium lappa*), Lycopodium (No.15, spore of *Lycopodium clavatum*), Agrimoniae Herba (No.16, aerial part of *Agrimonia pilosa*), Andrographis Herba (No.17, aerial part of *Andrographis paniculata*), Panacis Japonici Rhizoma (No.18, rhizome of *Panax japonicus*), and Chorei (No.19, sclerotium of *Polyporus umbellatus*) exhibited preferential cytotoxicity against human pancreatic cancer cells lines PANC-1 and PSN-1 under nutrient-deprived medium (NDM). Among them, Andrographis Herba (No.17) exhibited the most potent preferential cytotoxicity against PANC-1 and PSN-1 cells with the PC_{50} values of 9.72 μg/mL and 9.41 μg/mL, respectively.

Isodonis Herba (No.1), Lycopodium (No.15), Andrographis Herba (No.17), and Panacis Japonici Rhizoma (No.18) showed the preferential cytotoxicity serum-dependently under NDM, while the extract of Phellodendri Cortex (No.2) showed the selective cytotoxicity glucose-dependently. PANC-1 and PSN cells treated with extracts of Isodonis Herba (No.1), Lycopodium (No.15), and Panacis Japonici Rhizoma (No.18) showed the apoptosis-like cell death under nutrient starvation, while the cells treated with extracts of Phellodendri Cortex (No.2) and Andrographis Herba (No.17) showed the necrosis-like cell death under nutrient starvation.

Bioassay guided isolation of the 70% EtOH extract of *A. paniculata*, which showed preferential cytotoxicity against PANC-1 and PSN-1 cells in NDM, led to the isolation of 20 compounds consisting of stearic acid (**1**), pinostrobin (**2**), β-sitosterol (**3**), 5-hydroxy-6,7-dimethoxyflavone (**4**), pinocembrin (**5**), ermanin (**6**), ergosterol peroxide (**7**), oleanolic acid (**8**), 5,3′,4′-trihydroxy-7-methoxyflavone (**9**), andrograpanin (**10**), skullcapflavone I (**11**), loliolide (**12**), 5-hydroxy-7,8,2′,5′-tetramethoxyflavone (**13**), 3-oxo-*ent*-cleroda-8(17),11,13-trien-16,15-olide (**14**), 5,7,8-trimethoxyflavanone (**15**), 14-deoxy-11,12-didehydroandrographolide (**16**), isoandrographolide (**17**), andrographolide

(**18**), apigenin (**19**), and 14-deoxyandrographolide (**20**). Among these compounds, compounds **2** and **5** have been isolated for the first time from the plants of *Andrographis* genus.

The compounds **7**, **8**, **10**, **12**, **13**, **16**, and **18** induced cell death against both PANC-1 and PSN-1 cells in NDM in a concentration-dependent manner, but not in DMEM. Among these, only 14-deoxy-11,12-didehydroandrographolide (**16**) had the most potent activity with the PC_{50} values of 10.0 μM and 9.27 μM against PANC-1 and PSN-1 cells, respectively. The six compounds, ergosterol peroxide (**7**), oleanolic acid (**8**), andrograpanin (**10**), loliolide (**12**), 5-hydroxy-7,8,2',5'-tetramethoxyflavone (**13**), and andrographolide (**14**) showed also the preferential cytotoxicity with mild potency (PC_{50}: 34–85 μM).

As previously mentioned, Andrographis Herba (No.17, aerial part of *A. paniculata*) exhibited the most potent preferential cytotoxicity against PANC-1 and PSN-1 cells with the PC_{50} values of 9.72 μg/mL and 9.41 μg/mL, respectively. In contrast, Arctii Fructus (No.5, fruits of *Artium lappa*), from which the potent anti-austeric agent, arctigenin had been isolated, showed the mild preferential cytotoxicity against PANC-1 and PSN-1 cells with the PC_{50} values of 71.7 μg/mL and 78.1 μg/mL, respectively. Therefore, the extracts of Andrographis Herba (No.17), was expected to contain more active constituent(s). However, the most potent 14-deoxy-11,12-didehydroandrographolide (**16**) obtained from *A. paniculata* showed only the PC_{50} values of 10.0 μM and 9.27 μM against PANC-1 and PSN-1 cells, respectively, which is significantly weaker than arctigenin (PC_{50} < 1.0 μM). 14-Deoxy-11,12-didehydroandrographolide reported the major diterpenoid in *A. paniculata*, making up about 0.5~6% in dried plant ethanol extract.[66] In the present study, 14-deoxy-11,12-didehydroandrographolide (**16**) was isolated about 0.5% from the dry weight of the extract. Therefore, the high content of 14-deoxy-11,12-didehydroandrographolide (**16**)

in *A. paniculata* would potentiate the preferential cytotoxicity against PANC-1 and PSN-1 cells.

14-Deoxy-11,12-didehydroandrographolide (**16**) inhibited survival of PANC-1 cells under deprivation of amino acids or serum, whereas it caused the cell death of PSN-1 under deprivation of serum. On the other hand, the microscopic observation of PANC-1 and PSN-1 cells treated by **16** displayed the membrane bleb, nuclear fragmentation, and chromatin condensation, which are typical apoptosis-like morphological changes. Further EB/AO double staining experiment in the cells treated with **16** under nutrient starvation indicated that condensed and/or fragmented chromatin is stained in orange, which allowed us to predict that **16** induces apoptosis-like cell death. Finally, flow cytometry with propidium iodide/annexin V double staining of **16** against PANC-1 and PSN-1 cells in NDM also indicated that it triggered apoptosis-like cell death in a concentration- and time-dependent manner. These results suggested that **16** induced apoptosis-like cell death to PANC-1 and PSN-1 human pancreatic cancer cells under nutrient starvation.

The serine/threonine kinase Akt/mTOR pathway is constitutively activated in a majority of human pancreatic cancer cell lines. Activation of this pathway has been attributed to the survival of cancer cells in the heterogeneous tumor microenvironment, which confers resistance to chemotherapy and radiotherapy.[8] Akt has been found to be overexpressed in pancreatic cancer cells during extreme nutrient deprivation. Increased Akt expression is one of the austerity markers that enables tumor cells to survive and proliferate in the hostile hypovascular tumor microenvironment.[8] Therefore, inhibition of the Akt pathway might have therapeutic value in cancer patients. A number of antiausterity agents such as arctigenin, kigamicin D, and pyrvinium pamoate have been found to strongly suppress Akt activation, which suggests that inhibition of Akt phosphorylation by these compounds is partially responsible for the preferential cytotoxicity observed under nutrient deprivation.[8-11] Accordingly, 14-deoxy-11,12-

didehydroandrographolide (**16**) may be an inhibitor of the principal survival factors, Akt and mTOR, in human pancreatic cancer cell lines under nutrient starvation.

LY294002, a phosphatidylinositol 3-kinase inhibitor, has reported to kill PANC-1 cells only under nutrient starvation conditions. LY294002 induced apoptosis by inhibiting PI3 kinase.[8] Therefore, it might be also possible that 14-deoxy-11,12-didehydroandrographolide (**16**) induced apoptosis by mechanism the inhibition of PI3 kinase.

Recently, 14-deoxy-11,12-didehydroandrographholide (**16**) has been reported to induce apoptotic cell death on human promonocytic leukemia THP-1 cells through reduction of endogenous reduced glutathione (GSH) content.[67] Therefore, it might be also possible that **16** induced apoptosis by the same mechanism.

14-Deoxy-11,12-didehydroandrographolide (**16**) induced apoptosis-like cell death to PANC-1 and PSN-1 cells under nutrient starvation. While necrotic agent stimulates a host inflammatory response, apoptotic and autophagic agents doesn't provoke inflammation. Therefore, **16** may be the high bioavailable anti-austeric agent.

In conclusion, this research demonstrated that the Andrographis Herba (aerial part of *Andrographis paniculata*) and its active constituent, 14-deoxy-11,12-didehydro-andrographolide (**16**) may be key leads in the development of new drugs based on the anti-austerity strategy.

Experimental

I. Experiment on chapter 2

Crude drugs

Crude drugs used in this study were supplied by Dr. Norimoto of Kracie Pharma, Ltd. (Takaoka, Japan) and were authenticated by a botanist of Kracie Pharma, Ltd. Voucher specimens are preserved in Kampo Research Laboratories, Kracie Pharma, Ltd., Takaoka, Japan.

Preparation of test solutions

Each crude drug (30.0 g) was cut into small pieces and extracted with 70% EtOH under sonication (210 mL, 90 min, × 3). The 70% EtOH solution was filtered and evaporated under reduced pressure to produce 70% EtOH extracts. Each extract was dissolved initially in DMSO, followed by dilution with the corresponding medium to give a final DMSO concentration of 0.05–1%. The diluted solutions were examined to determine their preferential cytotoxicity against the human pancreatic cancer cell lines PANC-1 and PSN-1.

Cells and culture conditions

Human pancreatic cancer cell lines PANC-1 and PSN-1 were provided by Dr. H. Esumi of National Cancer Center Hospital East. Human pancreatic cancer cell lines PANC-1 and PSN-1 were maintained in DMEM medium. These media were supplemented with 10% fetal bovine serum (FBS), 0.1% sodium bicarbonate and 1% antibiotic–antimycotic solution (Sigma–Aldrich Inc., St. Louis, MO, USA).

NDM contained 265 mg/L $CaCl_2 \cdot 2H_2O$, 0.1 mg/L $Fe(NO_3) \cdot 9H_2O$, 400 mg/L KCl, 200 mg/L $MgSO_4 \cdot 7H_2O$, 6400 mg/L NaCl, 700 mg/L $NaHCO_3$, 125 mg/L NaH_2PO_4, 15 mg/L phenol red, 1 M HEPES buffer (pH 7.4, Wako Pure Chemical Industries, Ltd.), and 10 mL MEM vitamin solution (Gibco Life Technologies, Inc., Rockville, MD, USA). The final pH was adjusted to 7.4 with 10% $NaHCO_3$

Preferential cytotoxicity against PANC- 1 and PSN-1 cells

Preferential cytotoxicity was determined as previously described.[11] In brief, PANC-1 or PSN-1 cells (2×10^4 cells/well or 1.5×10^4 cells/well) were seeded in 96 well plates (Corning Inc., Corning, NY, USA) and incubated in fresh DMEM at 37 ℃ under 5% CO_2 and 95% air for 24 h. The cells were washed with Dulbecco's phosphate-buffered saline (PBS, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) before the medium was replaced with either DMEM or NDM containing serial dilutions of the test samples. After 3, 6, 12, and 24 h incubation, the cells were washed with PBS, and 100 μL of DMEM containing 10% WST-8 cell counting kit solution (Dojindo Laboratories, Kumamoto, Japan) was added to the wells. After 2 h of incubation, the absorbance was measured at 450 nm. Cell viability was calculated from the mean values for three wells using the following equation:

$$\text{Cell viability (\%)} = [(Abs_{(\text{test samples})} - Abs_{(\text{blank})})/(Abs_{(\text{control})} - Abs_{(\text{blank})})] \times 100$$

The preferential cytotoxicity was expressed as the concentration at which 50% of cells died preferentially in NDM (PC_{50}). (−)-Arctigenin (purity 97%; Enzo Life Sciences, NY, USA) and paclitaxel (purity 99.5%; LC Laboratories, MA, USA) were used as a positive and negative control, respectively. Data are expressed by the mean values of triplicate determinations ± SD.

Morphological assessment

PANC-1 (1×10^4 cells/well) or PSN-1 cells (0.75×10^4 cells/well) were seeded in 96 well plates and incubated in DMEM at 37 ℃ under 5% CO_2 and 95% air for 24 h. The medium was replaced with NDM (100 μL/well) with or without the test samples. After 24 h incubation, cells were observed using a phase contrast microscope.

EB/ AO double staining

EB/AO double staining was performed according to the procedure previously described.[25] In brief, PANC-1 (1×10^4 cells/well) or PSN-1 cells (0.75×10^4 cells/well) were seeded in 96-well plates and incubated in DMEM at 37 ℃ under 5% CO_2 and 95% air for 24 h. The medium was replaced with NDM (100 μL/well) with or without the test sample. After 6, 12, or 24 h of incubation, 10 μL of EB/AO solution [100 μg/mL EB (Sigma–Aldrich Inc.) and 100 μg/mL AO (Wako Pure Chemical Industries, Ltd.) in PBS] were added to each well, and the cells were observed using a fluorescent microscope (Biozero BZ-8000, Keyence Corp., Osaka, Japan). Cells stained by EB were detected with excitation and emission at 560 nm and 630 nm, respectively, while cells stained by AO were detected with excitation and emission at 480 nm and 510 nm, respectively.

II. Experiment on chapter 3

General methods

^{1}H and ^{13}C NMR spectra were recorded using a JEOL JNMLA400 or Bruker Avance DMX 500 spectrometer with TMS (tetramethylsilane) as an internal standard. EIMS measurements were carried out on JEOL JMS-GC mate II mass spectrometer. Medium-pressure liquid chromatography (MPLC) was performed using the Büchi Sepacore system (Büchi Labortechnik AG, Flawil, Switzerland). Silica gel 60 N (spherical, neutral, 40–50 μm, Kanto Chemical Co., Inc., Tokyo, Japan) was used for column chromatography. Analytical and preparative thin-layer chromatography (TLC) was performed using Merck precoated silica gel 60 F_{254} plates and RP-18F_{254} (0.25 or 0.5 mm thickness, Merck KGaA, Darmstadt, Germany).

Plant material

Andrographis Herba (aerial part of *Andrographis paniculata*) used in this study was supplied by Dr. Norimoto of Kracie Pharma, Ltd. (Takaoka, Japan) and was authenticated by a botanist of Kracie Pharma, Ltd. Voucher specimens (No. WA-5) are preserved in Kampo Research Laboratories, Kracie Pharma, Ltd., Takaoka, Japan.

Extraction and isolation

The aerial part of *Andrographis paniculata* (850 g) was extracted with 70% EtOH under sonication (2 L, 90 min, ×3) at room temperature and the solvent was evaporated under reduced pressure to give a 70% EtOH extract (61.0 g). The 70% EtOH extract

(30.0 g) was suspended on distilled water and extracted with $CHCl_3$ to give a $CHCl_3$-soluble extract (3.5 g). The $CHCl_3$-soluble extract (3.0 g) was chromatographed on silica gel with MPLC to yield 16 fractions, to which preferential cytotoxicity were determined against human pancreatic cancer cell line PANC-1 [fr. 1: *n*-hexane–EtOAc (98:2) eluate, 6.4 mg, PC_{50} > 100 μg/mL; fr. 2: *n*-hexane–EtOAc (97:3) eluate, 30.4 mg, PC_{50} > 100 μg/mL; fr. 3: *n*-hexane–EtOAc (95:5) eluate, 3.2 mg, PC_{50} > 100 μg/mL; fr. 4: *n*-hexane–EtOAc (90:10) eluate, 9.4 mg, PC_{50} > 100 μg/mL; fr. 5: *n*-hexane–EtOAc (85:15) eluate, 377 mg, PC_{50} > 100 μg/mL; fr. 6: *n*-hexane–EtOAc (85:15) eluate, 273 mg, PC_{50} > 100 μg/mL; fr. 7: *n*-hexane–EtOAc (85:15) eluate, 221 mg, PC_{50} > 100 μg/mL; fr. 8: *n*-hexane–EtOAc (80:20) eluate, 26.0 mg, PC_{50} > 100 μg/mL; fr. 9: *n*-hexane–EtOAc (75:25) eluate, 51.8 mg, PC_{50} 73.7 μg/mL; fr. 10: *n*-hexane–EtOAc (75:25) eluate, 15.6 mg, PC_{50} 99.2 μg/mL; fr. 11: *n*-hexane–EtOAc (70:30) eluate, 94.0 mg, PC_{50} 34.5 μg/mL; fr. 12: *n*-hexane–EtOAc (60:40) eluate, 105 mg, PC_{50} 34.6 μg/mL; fr. 13: *n*-hexane–EtOAc (40:60) eluate, 102 mg, PC_{50} 74.9 μg/mL; fr. 14: *n*-hexane–EtOAc (30:70) eluate, 54.2 mg, PC_{50} 11.1 μg/mL; fr. 15: *n*-hexane–EtOAc (20:80) eluate, 127 mg, PC_{50} 10.1 μg/mL; fr. 16: EtOAc eluate, 250 mg, PC_{50} 24.3 μg/mL].

Fraction 9 (51.8 mg) was subjected to reversed-phase preparative TLC with MeOH–CH_3CN–H_2O (5:10:1) to give stearic acid (**1**, 9.8 mg),[43] pinostrobin (**2**, 3.0 mg),[44] and β-sitosterol (**3**, 11.0 mg).[45]

Fraction 10 (15.6 mg) was subjected to reversed-phase preparative TLC with MeOH–CH_3CN–H_2O (5:8:1) to give 5-hydroxy-6,7-dimethoxyflavone (**4**, 1.8 mg).[46]

Fraction 11 (94.0 mg) was subjected to reversed-phase preparative TLC with MeOH–CH_3CN–H_2O (5:5:1) to give pinocembrin (**5**, 2.5 mg),[47] ermanin (**6**, 2.4 mg),[48]

ergosterol peroxide (**7**, 4.2 mg),[49] oleanolic acid (**8**, 11.6 mg),[50] and 5,3′,4′-trihydroxy-7-methoxyflavone (**9**, 1.6 mg).[51]

Fraction 12 (104 mg) was subjected to reversed-phase preparative TLC with MeOH–CH_3CN–H_2O (5:5:1) to give andrograpanin (**10**, 1.3 mg),[52] skullcapflavone I (**11**, 1.6 mg),[53] and loliolide (**12**, 2.2 mg).[54]

Fraction 13 (102 mg) was subjected to reversed-phase preparative TLC with MeOH–CH_3CN–H_2O (3:3:1) to give skullcapflavone I (**11**, 8.0 mg) and 5-hydroxy-7,8,2′,5′-tetramethoxyflavone (**13**, 2.6 mg).[37]

Fraction 14 (54.2 mg) was subjected to normal-phase preparative TLC with benzene–acetone (95:5) to give four subfractions. Subfraction 14-1 (13.6 mg) was purified by recrystallization to give 3-oxo-*ent*-cleroda-8(17),11,13-trien-16,15-olide (**14**, 2.3 mg).[55]

Fraction 15 (126 mg) was subjected to normal-phase preparative TLC with benzene–CH_3CN (95:5) to give six subfractions. Subfraction 15-1 (24.9 mg) was subjected to reversed-phase preparative TLC with MeOH–H_2O (4:1) to give 5,7,8-trimethoxyflavanone (**15**, 8.0 mg).[56] Subfraction 15-2 (54.2 mg) was separated by reversed-phase preparative TLC with MeOH–CH_3CN–H_2O (2:2:1) to give 14-deoxy-11,12-didehydroandrographolide (**16**, 16.3 mg).[57]

Fraction 16 (250 mg) was subjected to normal-phase preparative TLC with benzene–CH_3CN (95:5) to give three subfractions. Subfraction 16-1 (39.4 mg) was separated by reversed-phase preparative TLC with MeOH–H_2O (3:1) to give isoandrographolide (**17**, 4.2 mg)[58] and andrographolide (**18**, 18.8 mg).[59] Subfraction 16-2 (72.2 mg) was separated by reversed-phase preparative TLC with MeOH–CH_3CN–

H_2O (1:1:1) to give 5,7,4′-trihydroxyflavone (**19**, 3.1 mg)[60] and 14-deoxyandrographolide (**20**, 11.0 mg).[61]

Stearic acid (**1**): white powders with the molecular of $C_{18}H_{36}O_2$ (*m*/*z* 284 $[M]^+$); ^1H NMR (500 MHz, pyridine-d_5) δ 2.49 (2H, t, *J* = 7.0 Hz, α-CH_2, H-2,3), 1.77 (2H, m, β-CH_2, H-2,3), 1.26 (28H, m, 14 × CH_2, H-4–16), 0.83 (6H, t, *J* = 7.0 Hz, 2 × CH_3 H-17,18); ^{13}C NMR (125 MHz, pyridine-d_5) δ 175.9 (C-1), 34.7 (C-2), 31.9 (C-3), 29.7 (12 × CH_2), 25.4 (C-16), 22.7 (C-17), 14.1 (C-18). The above data are identical with those of stearic acid in the literature.[43]

Pinostrobin (**2**): white powders with the molecular of $C_{16}H_{14}O_4$ (*m*/*z* 270 $[M]^+$); ^1H NMR (500 MHz, $CDCl_3$) δ 5.44 (1H, dd, *J* = 14.0, 3.0 Hz, H-2), 3.10 (1H, dd, *J* = 17.0, 13.5 Hz, H-3), 2.83 (1H, dd, *J* = 17.0, 3.0 Hz, H-3), 6.08 (1H, s, H-6), 6.09 (1H, s, H-8), 7.41–7.46 (5H, m, H-2–6), 12.03 (1H, s, OH-5), 3.82 (3H, s, OMe-7); ^{13}C NMR (125 MHz, $CDCl_3$) δ 79.2 (C-2), 43.4 (C-3), 195.8 (C-4), 164.1 (C-5), 95.1 (C-6), 164.1 (C-7), 94.3 (C-8), 162.8 (C-9), 103.1 (C-10), 138.3 (C-1′), 126.1 (C-2′), 128.9 (C-3′), 128.9 (C-4′), 128.9 (C-5′), 126.1 (C-6′), 55.7 (7-OMe). The above data are identical with those of pinostrobin in the literature.[44]

β-Sitosterol (**3**): white powders with the molecular of $C_{29}H_{50}O$ (*m*/*z* 414 $[M]^+$); ^1H NMR (500 MHz, $CDCl_3$) δ 3.53 (1H, m, H-3), 5.36 (1H, br s, H-5), 0.92 (3H, d, *J* = 6.5 Hz, H-19), 0.85 (3H, t, *J* = 7.5 Hz, H-24), 0.84 (3H, d, *J* = 7.5 Hz, H-26), 0.82 (3H, d, *J* = 7.5 Hz, H-27), 0.68 (3H, s, H-28), 1.01 (3H, s, H-29); ^{13}C NMR (125 MHz, $CDCl_3$) δ 37.3 (C-1), 31.7 (C-2), 71.8 (C-3), 42.3 (C-4), 140.8 (C-5), 121.7 (C-6), 31.9 (C-7), 31.9 (C-8), 50.1 (C-9), 36.5 (C-10), 21.1 (C-11), 39.8 (C-12), 42.3 (C-13), 56.8 (C-14), 24.3 (C-15), 28.3 (C-16), 56.1 (C-17), 36.2 (C-18), 19.4 (C-19), 34.0 (C-20), 26.1 (C-21), 45.8 (C-22), 23.1 (C-23), 12.0 (C-24), 29.1 (C-25), 19.9 (C-26), 19.4 (C-27), 18.8 (C-

28), 11.9 (C-29). The above data are identical with those of *β*-sitosterol in the literature.[45]

5-Hydroxy-6,7-dimethoxyflavone (**4**): pale yellow powders with the molecular of $C_{17}H_{14}O_5$ (*m/z* 298 $[M]^+$); ^1H NMR (500 MHz, $CDCl_3$) *δ* 6.69 (1H, s, H-3), 6.45 (1H, s, H-8), 7.95 (1H, d, *J* = 7.0 Hz, H-2′), 7.56 (1H, m, H-3′), 7.54 (1H, m, H-4′), 7.56 (1H, m, H-5′), 7.95 (1H, d, *J* = 7.0 Hz, H-6′), 12.57 (1H, s, OH-5), 3.96 (3H, s, OMe-6), 3.95 (3H, s, 7-MeO); ^{13}C NMR (125 MHz, $CDCl_3$) *δ* 164.0 (C-2), 105.0 (C-3), 182.8 (C-4), 157.6 (C-5), 132.0 (C-6), 158.8 (C-7), 95.9 (C-8), 149.5 (C-9), 105.4 (C-10), 131.4 (C-1′), 126.4 (C-2′), 129.2 (C-3′), 126.4 (C-4′), 129.2 (C-5′), 126.4 (C-6′), 61.7 (6-OMe), 56.4 (7-OMe). The above data are identical with those of 5-hydroxy-6,7-dimethoxyflavone in the literature.[46]

Pinocembrin (**5**): yellow needles with the molecular of $C_{15}H_{12}O_4$ (*m/z* 256 $[M]^+$); ^1H NMR (500 MHz, acetone-d_6) *δ* 5.50 (1H, dd, *J* = 12.5, 3.0 Hz, H-2), 3.11 (1H, dd, *J* = 17.0,12.5 Hz, H-3), 2.76 (1H, dd, *J* = 17.0, 3.0 Hz, H-3), 5.97 (1H, d, *J* = 1.5 Hz, H-6), 5.97 (1H, d, *J* = 1.5 Hz, H-8), 7.52 (1H, d, *J* = 7.0 Hz, H-2′), 7.40 (1H, t, *J* = 7.0 Hz, H-3′), 7.36 (1H, br d, *J* = 7.0 Hz, H-4′), 7.40 (1H, t, *J* = 7.0 Hz, H-5′), 7.52 (1H, d, *J* = 7.0 Hz, H-6′), 12.14 (1H, s, OH-5); ^{13}C NMR (125 MHz, acetone-d_6) *δ* 79.1 (C-2), 42.7 (C-3), 196.4 (C-4), 164.4 (C-5), 96.1 (C-6), 166.6 (C-7), 95.1 (C-8), 163.1 (C-9), 102.3 (C-10), 139.2 (C-1′), 126.4 (C-2′), 128.6 (C-3′), 128.6 (C-4′), 128.6 (C-5′), 126.4 (C-6′). The above data are identical with those of pinocembrin in the literature.[47]

Ermanin (**6**): yellow powders with the molecular of $C_{17}H_{14}O_6$ (*m/z* 314 $[M]^+$); ^1H NMR (500 MHz, acetone-d_6) *δ* 6.32 (1H, d, *J* = 1.0 Hz, H-6), 6.66 (1H, d, *J* = 1.5 Hz, H-8), 8.05 (1H, d, *J* = 8.5 Hz, H-2′), 7.02 (1H, d, *J* = 8.5 Hz, H-3′), 7.02 (1H, d, *J* = 8.5 Hz, H-5′), 8.05 (1H, d, *J* = 8.5 Hz, H-6′), 3.92 (3H, s, OMe-3), 3.88 (3H, s, OMe-4′); ^{13}C NMR (125 MHz, DMSO-d_6) *δ* 155.0 (C-2), 139.1 (C-3), 177.8 (C-4), 161.1 (C-5),

98.3 (C-6), 164.1 (C-7), 93.5 (C-8), 156.3 (C-9), 104.3 (C-10), 113.8 (C-1′), 130.8 (C-2′), 122.1 (C-3′), 161.2 (C-4′), 122.1 (C-5′), 130.0 (C-6′), 59.5 (OMe-3), 55.3 (OMe-4′). The above data are identical with those of ermanin in the literature.[48]

Ergosterol peroxide (**7**): white solids with the molecular of $C_{28}H_{44}O_3$ (*m*/*z* 428 $[M]^+$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.71 (2H, m, H-1), 3.98 (2H, m, H-2), 6.51, (1H, d, *J* = 8.5 Hz, H-6), 6.24, (1H, d, *J* = 8.5 Hz, H-7), 1.23 (1H, m, H-11), 1.53 (1H, m, H-11), 1.25 (1H, m, H-12), 1.94 (1H, m, H-12), 1.58 (1H, m, H-14), 1.42 (1H, m, H-15), 1.67 (1H, m, H-15), 1.34 (1H, m, H-16), 1.84 (1H, m, H-16), 1.25 (1H, m, H-17), 0.88 (3H, s, H-18), 0.88 (3H, s, H-19), 2.02 (1H, m, H-20), 1.00 (3H, d, *J* = 6.5 Hz, H-21), 5.14 (1H, dd, *J* = 15.5, 7.5 Hz, H-22), 5.22 (1H, dd, *J* = 15.5, 7.5 Hz, H-23), 1.85 (1H, m, H-24), 1.50 (3H, m, H-25), 0.81 (1H, m, H-26), 0.83 (3H, d, *J* = 8.0 Hz, H-27), 0.91 (3H, d, *J* = 6.5 Hz, H-28); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 34.7 (C-1), 30.1 (C-2), 66.5 (C-3), 37.0 (C-4), 82.2 (C-5), 135.4 (C-6), 130.8 (C-7), 82.2 (C-8), 51.7 (C-9), 36.9 (C-10), 20.9 (C-11), 39.3 (C-12), 44.6 (C-13), 51.1 (C-14), 23.4 (C-15), 28.7 (C-16), 56.2 (C-17), 12.9 (C-18), 18.2 (C-19), 39.8 (C-20), 19.6 (C-21), 135.2 (C-22), 132.3 (C-23), 42.8 (C-24), 33.2 (C-25), 20.0 (C-26), 20.6 (C-27), 17.5 (C-28). The above data are identical with those of ergosterol peroxide in the literature.[49]

Oleanolic acid (**8**): white powers with the molecular of $C_{30}H_{48}O_3$ (*m*/*z* 456 $[M]^+$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.22 (1H, dd, *J* = 10.5, 3.5 Hz, H-3), 5.28 (1H, br s, H-12), 2.82 (1H, dd, *J* = 14.0, 3.5 Hz, H-18), 0.99 (3H, s, H-23), 0.75 (3H, s, H-24), 0.93 (3H, s, H-25), 0.77 (3H, s, H-26), 1.13 (3H, s, H-27), 0.93 (3H, s, H-29), 0.90 (3H, s, H-30); ^{13}C NMR (125 MHz, $CDCl_3$) δ 38.5 (C-1), 27.2 (C-2), 79.2 (C-3), 38.8 (C-4), 55.3 (C-5), 18.3 (C-6), 32.7 (C-7), 39.2 (C-8), 47.7 (C-9), 37.2 (C-10), 23.5 (C-11), 122.6 (C-12), 143.3 (C-13), 41.7 (C-14), 27.8 (C-15), 23.1 (C-16), 46.6 (C-17), 41.1 (C-18), 45.9 (C-19), 30.8 (C-20), 33.8 (C-21), 32.5 (C-22), 28.2 (C-23), 15.6 (C-24), 15.4 (C-25),

17.1 (C-26), 26.0 (C-27), 182.7 (C-28), 33.2 (C-29), 23.8 (C-30). The above data are identical with those of oleanolic acid in the literature.[50]

5,3′,4′-Trihydroxy-7-methoxyflavon (**9**): yellow powers with the molecular of $C_{16}H_{12}O_6$ (*m*/*z* 300 $[M]^+$); ^{1}H NMR (500 MHz, acetone-d_6) δ 6.62 (1H, s, H-3), 6.32 (1H, d, *J* = 2.0 Hz, H-6), 6.69 (1H, d, *J* = 2.0 Hz, H-8), 7.51 (1H, br s, H-2′), 6.99 (1H, d, *J* = 8.0 Hz, H-5′), 7.49 (1H, d, *J* = 8.0 Hz, H-6′), 13.00 (1H, s, OH-5), 3.93 (3H, s, OMe-7); ^{13}C NMR (125 MHz, acetone-d_6) δ 164.1 (C-2), 103.0 (C-3), 181.8 (C-4), 157.3 (C-5), 97.5 (C-6), 165.2 (C-7), 92.1 (C-8), 161.5 (C-9), 104.6 (C-10), 121.3 (C-1′), 109.8 (C-2′), 148.1 (C-3′), 151.2 (C-4′), 115.6 (C-5′), 120.2 (C-6′), 55.5 (OMe-7). The above data are identical with those of 5,3′,4′-trihydroxy-7-methoxyflavone in the literature.[51]

Andrograpanin (**10**): colorless needles with the molecular of $C_{20}H_{30}O_3$ (*m*/*z* 318 $[M]^+$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.10 (1H, t, *J* = 2.0 Hz, H-14), 4.74 (2H, m, H-15), 4.58 (1H, s, H-17), 4.86 (1H, br s, H-17), 0.98 (3H, s, H-18), 3.75 (1H, d, *J* = 11.2 Hz, H-19), 3.46 (1H, d, *J* = 11.2 Hz, H-19), 0.66 (3H, s, H-20); ^{13}C NMR (100 MHz, $CDCl_3$) δ 39.0 (C-1), 18.9 (C-2), 35.3 (C-3), 39.5 (C-4), 56.3 (C-5), 24.4 (C-6), 38.5 (C-7), 147.5 (C-8), 56.4 (C-9), 38.9 (C-10), 21.7 (C-11), 24.5 (C-12), 134. 8 (C-13), 70.1 (C-14), 174. 3 (C-15), 174.3 (C-16), 106.9 (C-17), 27.0 (C-18), 65.0 (C-19), 15.3 (C-20). The above data are identical with those of andrograpanin in the literature.[52]

Skullcapflavone (**11**): pale yellow needles with the molecular of $C_{17}H_{14}O_6$ (*m*/*z* 314 $[M]^+$); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.13 (1H, s, H-3), 6.58 (1H, s, H-6), 7.04 (1H, d, *J* = 8.0 Hz, H-3′), 7.40 (1H, br t, *J* = 8.0 Hz, H-4′), 7.00 (1H, t, *J* = 8.0 Hz, H-5′), 7.86 (1H, d, *J* = 8.0 Hz, H-6′), 12.74 (1H, s, OH-5), 3.90 (3H, s, OMe-7), 3.80 (3H, s, OMe-8); ^{13}C NMR (125 MHz, DMSO-d_6) δ 161.7 (C-2), 108.6 (C-3), 182.1 (C-4), 156.6 (C-5), 95.8 (C-6), 158.4 (C-7), 128.4 (C-8), 149.4 (C-9), 103.6 (C-10), 117.2 (C-1′), 157.5

(C-2′), 117.4 (C-3′), 133.1 (C-4′), 119.2 (C-5′), 128.3 (C-6′), 56.8 (OMe-7), 61.2 (OMe-8). The above data are identical with those of skullcapflavone I in the literature.[53]

Loliolide (**12**): white powders with the molecular of $C_{11}H_{16}O_3$ (*m*/*z* 196 $[M]^+$); ^1H NMR (500 MHz, $CDCl_3$) δ 1.54 (1H, m, H-2α), 1.98 (1H, br d, *J* = 14.0 Hz, H-2β), 4.33 (1H, br s, H-3), 1.76 (1H, m, H-4α), 2.46 (1H, br d, *J* = 14.0 Hz, H-4β), 5.69 (1H, s, H-8), 1.47 (3H, s, H-9), 1.28 (3H, s, H-10), 1.78 (3H, s, H-11); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 36.0 (C-1), 47.3 (C-2), 66.8 (C-3), 45.6 (C-4), 86.8 (C-5), 182.5 (C-6), 112.9 (C-7), 170.0 (C-8), 26.5 (C-9), 30.7 (C-10), 27.0 (C-11). The above data are identical with those of loliolide in the literature.[54]

5-Hydroxy-7,8,2′,5′-tetramethoxyflavone (**13**): yellow solids with the molecular of $C_{19}H_{18}O_7$ (*m*/*z* 358 $[M]^+$); ^1H NMR (500 MHz, $CDCl_3$) δ 7.10 (1H, s, H-3), 6.43 (1H, s, H-6), 6.98 (1H, d, *J* = 9.0 Hz, H-3′), 7.05 (1H, dd, *J* = 8.5, 3.2 Hz, H-4′), 7.54 (1H, d, *J* = 2.5 Hz, H-5′), 3.95 (3H, s, OMe-7), 3.93 (3H, s, OMe-8), 3.91 (3H, s, OMe-2′), 3.86 (3H, s, OMe-5′); ^{13}C NMR (125 MHz, $CDCl_3$) δ 161.0 (C-2), 110.6 (C-3), 183.2 (C-4), 157.5 (C-5), 95.5 (C-6), 158.6 (C-7), 128.9 (C-8), 104.9 (C-9), 149.6 (C-10), 120.7 (C-1′), 152.6 (C-2′), 113.1 (C-3′), 118.3 (C-4′), 153.5 (C-5′), 114.0 (C-6′), 56.3 (OMe-7), 61.6 (OMe-8), 56.2 (OMe-2′), 55.8 (OMe-5′). The above data are identical with those of 5-hydroxy-7,8,2′,5′-tetramethoxyflavone in the literature.[37]

3-Oxo-*ent*-cleroda-8(17),11,13-trien-16,15-olide (**14**): white amorphous powders with the molecular of $C_{19}H_{24}O_3$ (*m*/*z* 300 $[M]^+$); ^1H NMR (400 MHz, $CDCl_3$) δ 6.95 (1H, dd, *J* = 16.0, 10.0 Hz, H-11), 6.15 (1H, d, *J* = 16.0 Hz, H-12), 7.20 (1H, br s, H-14), 4.83 (2H, d, *J* = 2.0 Hz, H-15), 4.62 (1H, d, *J* = 2.0 Hz, H-17), 4.87 (1H, d, *J* = 2.0 Hz, H-17), 1.02 (3H, br s, H-18), 1.10 (3H, s, H-19); ^{13}C NMR (100 MHz, $CDCl_3$) δ 40.0 (C-1), 37.9 (C-2), 212.5 (C-3), 45.2 (C-4), 52.7 (C-5), 27.2 (C-6), 35.5 (C-7), 147.7 (C-8), 59.6 (C-9), 38.5 (C-10), 135.9 (C-11), 121.4 (C-12), 129.1 (C-13), 143.1 (C-14),

69.5 (C-15), 172. 1 (C-16), 109.9 (C-17), 11.4 (C-18), 13.2 (C-19). The above data are identical with those of 3-oxo-*ent*-cleroda-8(17),11,13-trien-16,15-olide in the literature.[55]

5,7,8-Trimethoxyflavanone (**15**): pale yellow needles with the molecular of $C_{18}H_{18}O_5$ (*m*/*z* 314 $[M]^+$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.47 (1H, dd, *J* = 12.4, 3.2 Hz, H-2), 3.02 (1H, dd, *J* = 16.8, 12.3 Hz, H-3), 2.88 (1H, dd, *J* = 16.0, 3.6 Hz, H-4), 6.14 (1H, s, H-6), 7.43 (1H, m, H-2′), 7.36 (1H, m, H-3′), 7.33 (1H, m, H-4′), 7.36 (1H, m, H-5′), 7.43 (1H, m, H-6′), 3.81 (3H, s, OMe-5), 3.93 (3H, s, OMe-7), 3.95 (3H, s, OMe-8); ^{13}C NMR (100 MHz, $CDCl_3$) δ 79.1 (C-2), 45.6 (C-3), 189.5 (C-4), 157.9 (C-5), 89.4 (C-6), 89.4 (C-7), 131.0 (C-8), 156.3 (C-9), 106.3 (C-10), 138.9 (C-1′), 126.0 (C-2′), 128.7 (C-3′), 126.0 (C-4′), 128.7 (C-5′), 126.0 (C-6′), 56.1 (OMe-5), 56.2 (OMe-7), 61.2 (OMe-8). The above data are identical with those of 5,7,8-trimethoxyflavanone in the literature.[56]

14-Deoxy-11,12-didehydroandrographolide (**16**): colorless crystals with the molecular of $C_{20}H_{28}O_4$ (*m*/*z* 332 $[M]^+$); ^{1}H NMR (400 MHz, MeOD-d_4) δ 1.83 (1H, m, H-1), 1.40 (1H, m, H-1), 1.74 (2H, m, H-2), 3.34 (1H, m, H-3), 1.37 (1H, m, H-5), 1.81 (2H, m, H-6), 2.45 (1H, m, H-7), 2.08 (1H, m, H-7), 1.84 (1H, m, H-9), 6.87 (1H, dd, *J* = 15.6, 10.0 Hz, H-11), 6.17 (1H, d, *J* = 16.0 Hz, H-12), 7.45 (1H, br s, H-14), 4.50 (2H, d, *J* = 0.8 Hz, H-15), 4.76 (1H, d, *J* = 1.6 Hz, H-17), 4.83 (1H, br s, H-17), 1.23 (3H, s, H-18), 3.39 (1H, d, *J* = 11.2 Hz, H-19), 4.13 (1H, d, *J* = 11.2 Hz, H-19), 0.85 (3H, s, H-20); ^{13}C NMR (100 MHz, MeOD-d_4) δ 39.7 (C-1), 29.0 (C-2), 81.3 (C-3), 43.9 (C-4), 56.0 (C-5), 24.6 (C-6), 37.9 (C-7), 150.3 (C-8), 63.0 (C-9), 39.8 (C-10), 136.6 (C-11), 122.7 (C-12), 129.7 (C-13), 146.9 (C-14), 71.7 (C-15), 175.0 (C-16), 109.2 (C-17), 23.5 (C-18), 65.2 (C-19), 16.4 (C-20). The above data are identical with those of 14-deoxy-11,12-didehydroandrographolide in the literature.[57]

Isoandrographolide (**17**): colorless amorphous powders with the molecular of $C_{20}H_{30}O_5$ (*m*/*z* 350 $[M]^+$); ^{1}H NMR (400 MHz, MeOD-d_4) δ 1.85 (1H, m, H-1), 1.37 (1H, m, H-1), 1.77 (2H, m, H-2), 3.35 (1H, m, H-3), 1.33 (1H, m, H-5), 1.84 (2H, m, H-6), 2.38 (1H, m, H-7), 1.98 (1H, m, H-7), 1.94 (1H, m, H-9), 2.87 (2H, t, *J* = 6.8 Hz, H-11), 6.50 (1H, dt, *J* = 6.8, 1.2 Hz, H-12), 4.70 (1H, m, H-14), 4.40 (1H, dd, *J* = 10.0, 6.0 Hz, H-15), 4.04 (1H, dd, *J* = 10.0, 2.4 Hz, H-15), 4.81 (1H, d, *J* = 1.6 Hz, H-17), 4.50 (1H, d, *J* = 1.6 Hz, H-17), 1.20 (3H, s, H-18), 4.10 (1H, d, *J* = 10.8 Hz, H-19), 3.35 (1H, d, *J* = 10.8 Hz, H-19), 0.72 (3H, s, H-20); ^{13}C NMR (100 MHz, MeOD-d_4) δ 38.2 (C-1), 29.2 (C-2), 81.1 (C-3), 43.8 (C-4), 56.6 (C-5), 25.4 (C-6), 39.2 (C-7), 149.2 (C-8), 58.0 (C-9), 40.2 (C-10), 24.9 (C-11), 151.3 (C-12), 129.6 (C-13), 70.0 (C-14), 75.1 (C-15), 171.7 (C-16), 108.7 (C-17), 23.6 (C-18), 65.1 (C-19), 15.8 (C-20). The above data are identical with those of isoandrographolide in the literature.[58]

Andrographolide (**18**): colorless amorphous powders with the molecular of $C_{20}H_{30}O_5$ (*m*/*z* 350 $[M]^+$); ^{1}H NMR (500 MHz, pyridine-d_5) δ 1.90 (1H, m, H-1), 1.27 (1H, m, H-1), 1.68 (1H, m, H-2), 1.79 (1H, m, H-2), 3.67 (1H, m, H-3), 1.34 (1H, m, H-5), 1.95 (2H, m, H-6), 2.33 (1H, m, H-7), 2.00 (1H, m, H-7), 1.90 (1H, m, H-9), 2.71 (2H, br t, H-11), 7.18 (1H, dt, *J* = 6.8, 1.2 Hz, H-12), 5.39 (1H, br t, H-14), 4.62 (1H, dd, *J* = 12.0, 7.5 Hz, H-15), 4.53 (1H, dd, *J* = 12.0, 2.0 Hz, H-15), 4.87 (1H, br s, H-17), 4.89 (1H, br s, H-17), 1.52 (3H, s, H-18), 4.46 (1H, d, *J* = 12.0 Hz, H-19), 3.60 (1H, br s, H-19), 0.69 (3H, s, H-20); ^{13}C NMR (125 MHz, pyridine-d_5) δ 37.2 (C-1), 28.9 (C-2), 79.7 (C-3), 43.1 (C-4), 55.1 (C-5), 24.2 (C-6), 38.0 (C-7), 147.8 (C-8), 56.2 (C-9), 39.0 (C-10), 24.8 (C-11), 146.9 (C-12), 130.1 (C-13), 65.8 (C-14), 75.3 (C-15), 170.6 (C-16), 108.7 (C-17), 23.6 (C-18), 64.0 (C-19), 15.0 (C-20). The above data are identical with those of andrographolide in the literature.[59]

5,7,4′-Trihydroxyflavone (**19**): yellow crystals with the molecular of $C_{15}H_{10}O_5$ (*m*/*z* 270 $[M]^+$); ^{1}H NMR (500 MHz, MeOD-d_4) δ 6.57 (1H, s, H-3), 6.82 (1H, br s, H-6),

6.70 (1H, br s, H-8), 7.84 (2H, d, *J* = 8.5 Hz, H-2′), 6.92 (2H, d, *J* = 8.5 Hz, H-3′), 6.92 (2H, d, *J* = 8.5 Hz, H-5′), 7.84 (2H, d, *J* = 8.5 Hz, H-6′); ^{13}C NMR (125 MHz, MeOD-d_4) δ 164.4 (C-2), 106.3 (C-3), 180.5 (C-4), 164.9 (C-5), 104.8 (C-6), 160.2 (C-7), 99.3 (C-8), 160.7 (C-9), 109.4 (C-10), 123.2 (C-1′), 129.4 (C-2′), 117.0 (C-3′), 162.7 (C-4′), 117.0 (C-5′), 129.4 (C-6′). The above data are identical with those of 5,7,4′-trihydroxyflavone in the literature.[60]

14-Deoxyandrographolide (**20**): white powders with the molecular of $C_{20}H_{29}O_4$ (*m*/*z* 334 $[M]^+$); ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.66 (1H, m, H-1), 1.14 (1H, m, H-1), 1.60 (2H, m, H-2), 3.21 (1H, m, H-3), 1.21 (1H, m, H-5), 2.24 (1H, m, H-6), 2.00 (1H, m, H-6), 2.33 (1H, d, *J* = 12.5 Hz, H-7), 1.88 (1H, m, H-7), 1.56 (1H, m, H-9), 1.71 (1H, m, H-11), 1.56 (1H, m, H-11), 1.71 (1H, m, H-12), 1.32 (1H, m, H-12), 7.47 (1H, s, H-14), 4.81 (2H, br s, H-15), 4.83 (1H, br s, H-17) 4.59 (1H, br s, H-17), 1.07 (3H, s, H-18), 3.82 (1H, d, *J* = 11.7 Hz, H-19), 3.22 (1H, m, H-19), 0.60 (3H, s, H-20); ^{13}C NMR (125 MHz, DMSO-d_6) δ 36.5 (C-1), 28.1 (C-2), 78.6 (C-3), 42.3 (C-4), 54.7 (C-5), 24.1 (C-6), 37.9 (C-7), 147.8 (C-8), 55.6 (C-9), 38.8 (C-10), 21.7 (C-11), 24.3 (C-12), 132.2 (C-13), 147.0 (C-14), 70.5 (C-15), 174.2 (C-16), 106.8 (C-17), 106.8 (C-18), 62.8 (C-19), 14.9 (C-20). The above data are identical with those of 14-deoxyandrographolide in the literature.[61]

II. Experiment on Chapter 4

Morphological assessment

PANC-1 (1×10^4 cells/well) or PSN-1 cells (0.75×10^4 cells/well) were seeded in 96 well plates and incubated in DMEM at 37 ℃ under 5% CO_2 and 95% air for 24 h. The medium was replaced with NDM (100 μL/well) with or without 6.25 or 12.5 μM of **16**. After 24 h incubation, cells were observed using a phase contrast microscope.

EB/ AO double staining

EB/AO double staining was performed according to the procedure previously described.[25] In brief, PANC-1 (1×10^4 cells/well) or PSN-1 cells (0.75×10^4 cells/well) were seeded in 96-well plates and incubated in DMEM at 37 ℃ under 5% CO_2 and 95% air for 24 h. The medium was replaced with NDM (100 μL/well) with or without **16** (6.25 or 12.5 μM). After 12 h of incubation, 10 μL of EB/AO solution [100 μg/mL EB (Sigma–Aldrich Inc.) and 100 μg/mL AO (Wako Pure Chemical Industries, Ltd.) in PBS] was added to each well, and the cells were observed using a fluorescent microscope (Biozero BZ-8000, Keyence Corp., Osaka, Japan). Cells stained by EB were detected with excitation and emission at 560 nm and 630 nm, respectively, while cells stained by AO were detected with excitation and emission at 480 nm and 510 nm, respectively.

Flow cytometry

Flow cytometry was performed with propidium iodide/annexin-V double staining (Annexin-V-FLUOS staining kit, Roche Diagnostics, Indianapolis, IN, USA). In brief,

PANC-1 or PSN-1 cells (1×10^5 cells/well) were seeded in 6-well plates and incubated in DMEM containing 10% FBS at 37 ℃ under 5% CO_2 and 95% air for 24 h. The cells were washed twice using PBS and the medium was replaced with NDM containing **16** (6.25 or 12.5 μM). After 8 or 16 h incubation, floating and trypsinized adherent cells were collected, washed with PBS, and incubated with 2.5% annexin V in binding buffer for 10 min, followed by 2.5% propidium iodide in binding buffer. Stained cells (1×10^4 cells) were analyzed using a flow cytometer (BD Accuri™ C6, BD Biosciences, San Jose, CA, USA).

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List of publications

This doctoral thesis summarizes the full contents of the following publications.

1) Lee, S.; Dibwe, D. F.; Li, F.; Morita, H.; Tezuka, Y. Preferential cytotoxicity of crude drugs used in Japanese Kampo medicines against human pancreatic cancer PANC-1 and PSN-1 cells. *Traditional & Kampo Medicine* **2015**, DOI: 10.1002/tkm2.1016.

2) Lee, S.; Morita, H.; Tezuka, Y. Preferential cytotoxic constituents of *Andrographis paniculata* and their preferential cytotoxicity against human pancreatic cancer cell lines. *Nat. Prod. Commun.* in Press

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