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석사학위 논문

Anti-cancer efficacy of *Dianthus japonicus* extract on papillary thyroid cancer

조선대학교 대학원

글로벌바이오융합학과

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이 논문을 이학 석사학위신청 논문으로 제출함

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ABBREVIATIONS

CDK	Cyclin-dependent kinase
CIN	Chromosomal instability
Cyt c	Cytochrome c
DAPI	4′,6-diamidino-2-phenylindole
DJEE	*Dianthus japonicus* ethanol extract
DMSO	Dimethyl sulfoxide
DTC	Differentiated thyroid cancer
FACS	Fluorescence activated cell sorter
FBS	Fetal Bovine Serum
GIN	Genomic instability
IC 50	Half maximal inhibitory concentration
MFI	Mean fluorescence intensity
MMP	Mitochondria Membrane Potential
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
OXPHOS	Oxidative Phosphorylation System
PARP	Poly ADP-ribose polymerase
PBS	Phosphate-buffered saline
PI	Propidium iodide
PTC	Papillary thyroid cancer
PVDF	Polyvinylidene difluoride
RPMI-1640	Roswell Park Memorial Institute Medium

SD	Standard deviation
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TMRM	Tetramethyl-rhodamine, methyl ester

ABSTRACT

Anti-cancer efficacy of *Dianthus japonicus* extract on papillary thyroid cancer

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Thyroid cancer is a malignant endocrine system tumor that develops in the thyroid gland on the lower side of the neck it shows a higher incidence in women than in men. The development of cancer diagnostic tools has made it possible to detect malignant tumors, enabling prompt treatment of thyroid cancer. Papillary thyroid cancer cells are observed in most thyroid cancer patients with a good prognosis and a 5-year survival rate. However, even patients with a good prognosis for papillary thyroid carcinoma must be treated. In addition, metastatic progression decreases the survival rate depending on the location and degree of progression. Papillary thyroid carcinoma variants are diverse, exhibit aggressive characteristics, are prone to metastasis, and exhibit a low response to radioactive iodine therapy. Therefore, therapeutic agents are needed to help reduce the size of or eliminate the tumor so that the cancer risk (attributed to multiple factors) is reduced.

The plant *Dianthus japonicus*, also known as Seashore pink, grows close to the seashore in the Republic of Korea and is distributed in Japan, China, and other locations. Recent studies have shown that this species exhibits anti-cancer effects against oral, liver, and other cancers. Nevertheless, the anti-cancer effect of *D. japonicus* against papillary thyroid cancer is unknown. I investigated the anti-cancer effect of *D. japonicus* ethanol extract (DJEE) on papillary thyroid cancer cells. An MTT assay was performed to confirm whether DJEE had cytotoxic effects or influenced the viability of papillary thyroid cancer cells. Cell viability was observed to decrease in a concentration-dependent manner. DAPI staining was conducted to detect nuclear DNA fragmentation and condensation as features of apoptosis. The results showed that the fluorescence intensity increased as the DJEE concentration increased. Therefore, I used western blotting to detect proteins involved in the intrinsic and extrinsic signaling pathways of apoptosis. The expression of the anti-apoptotic protein Bcl-2 was decreased, whereas that of the proapoptotic protein Bax was increased. The expression of pro-PARP-1—related to DNA fragmentation—and procaspase-3/-8/-9—which promote apoptosis—was decreased. Increased Bax expression and Bcl2 inhibition suggest that the decrease in the expression of procaspases activated apoptotic signaling pathways. The change in the mitochondrial membrane potential (MMP) in the apoptotic pathway was not significant. To confirm that changes in other factors had an impact, how increased expression of p53 impacted the cell cycle was evaluated. The results showed that the sub-G1 phase was increased. In summary, DJEE induces G0/G1 arrest by promoting caspase-mediated apoptosis and increasing p53 expression in papillary thyroid carcinoma cells. In addition, the butanolic and ethanolic fractions of DJEE reduce cell viability. Therefore, DJEE shows potential as a chemotherapeutic agent for papillary thyroid cancer.

국문 초록

갯패랭이꽃 추출물에 의한 갑상선암 항암효능 연구

손대현

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갑상선암은 체내의 항상성을 조절하는 내분비계인 갑상샘에서 발생한 악성 종양이다. 남성보다 여성에게서 발병률이 높고, 여러 종류의 갑상선암은 위험성과 특성이 각각 다르다. 대부분의 갑상선암 환자에게서 나타나는 유두상 갑상선암은 엽을 절제하는 수술과 잔여 암과 전이암을 제거하는 방사성 요오드 요법으로 치료하고, 치료 후 5년 생존율이 다른 갑상선암보다 예후가 좋다. 하지만 높은 위험성도 갖고 있다. 60세 이상인 환자는 다른 연령대의 환자보다 전이와 치료적 예후가 좋지 않아 생존율이 낮다. 그리고 유두상 갑상선암의 다양한 형태와 특징의 변이체는 방사성 요오드의 결합력이 낮아 치료의 어려움을 주어 예후가 좋지 않다. 주된 치료가 수술로 엽을 절제하는 방법을 사용하기 때문에 나타나는 여러 가지 기능적 문제와 1.5 ㎝ 이상으로 암이 커질 경우 전이가 될 수 있다. 그래서 예후가 좋은 암으로 알려졌지만 여러 위험 요소가 있는 악성종양이기 때문에

이러한 위험성을 줄이는 지속적인 치료제의 연구가 필요하다.

본 연구에서 사용한 갯패랭이꽃은 바닷가에 서식하는 식물이고, 이전에 진행된 항암 활성연구가 없다. 그래서 같은 속의 패랭이 추출물을 이용한 항암 효능의 연구 결과를 찾아보았고, 여러 암종에서 항암 효능을 갖는 것으로 밝혀져 있다. 따라서 갯패랭이꽃 추출물의 갑상선암에 대한 항암 효능을 확인하였다. 갯패랭이꽃 추출물이 SNU-790 유두상 갑상선 암세포의 생존능력을 감소시키는지 알아보기 위해서 MTT-assay를 하였다. 그 결과 세포독성으로 인한 생존능력이 농도 의존적으로 감소하였고, 세포사에 의한 것인지 확인이 필요하였다. 세포사의 과정 중 핵 내 DNA의 단편화와 응집이 유도되기 때문에 DAPI staining으로 확인하였다. 농도가 증가할수록 유두상 갑상선 암세포 핵 내의 형광의 세기가 강해지는 것으로 보아 응집이 유도되는 것으로 관찰되었다. 그래서 세포사의 신호 전달경로를 확인하기 위해 내인성, 외인성 세포사멸경로에 있는 단백질의 발현을 확인하였다. Pro-apoptotic 단백질인 Bax, p53이 증가하였고 반대로 Anti-apoptotic인 Bcl-2는 감소하였다. Caspase-3/-8/-9의 pro-form이 감소하고 최종적으로 caspase-3가 활성화되는 것으로 보아 세포사가 유도되는 것이 확인된다. 세포사의 내 인성 경로의 Bax, Bcl-2의 발현 변화가 확인되어서 미토콘드리아에 관여된 세포사인지 확인이 필요하였다. 미토콘드리아 막 전위에 영향을 주었는지 확인하기 위해 Tetramethyl-rhodamine, methyl ester (TMRM)을 이용하여 측정하였다. 180 min까지의 변화의 차이가 관찰되지 않았다. 다른 부분에서 영향을 확인하기 위해 세포주기를 분석하였고, 그 결과 농도가 증가함에 따라 Sub-G1기의 증가와 이수성 암세포의 구간이 감소하였다. 추가로 갯패랭이꽃 에탄올 추출물은 천연추출물로 여러 가지

화합물이 섞여져 있기 때문에 어떠한 유효성분이 존재하는지 확인이 필요하다. 그 결과 에탄올과 부탄올 층에서 분리된 화합물에서 기존의 DJEE보다 더 좋은 항암 활성을 갖는 것으로 보아 유효성분이 포함되었을 가능성을 시사한다. 그러므로 갯패랭이꽃 에탄올 추출물은 caspase에 관여된 세포사를 유도하고, 유두상 갑상선암에 사용할 수 있는 항암 화학요법제로서의 가능성을 확인하였다.

I. INTRODUCTION

A. Cancer

1. Anti-cancer therapy

Cancer is the most common cause of death worldwide and its incidence is increasing. Anti-tumor therapies have progressed from radiotherapy to chemotherapy, targeted therapy, and immunotherapy. Radiotherapy can control tumor cell growth and eradicate the disease by acting directly on DNA in tumor cells. Induction of DNA damage can reduce the risk of cancer recurrence and improve survival. Radiation therapy is used with other treatments to increase survival (Deloch et al. 2016).

Chemotherapy is the primary pharmacological method for treating cancer using natural compounds or chemicals. These compounds exhibit toxicity toward tumor cells by inducing apoptosis. However, other approaches are needed because of the toxic side effects of these drugs in normal tissues, in addition to the development of drug resistance following continuous use in tumors. Side effects are influenced by the patient's physical health status, quality of life, and various factors. Advances in molecular biology and genetics when other treatment modalities are needed to complement these chemotherapy side effects have allowed cancers to be identified as specific genes by their mutated DNA (DeVita et al. 2008).

Targeted therapy can be used to treat multiple tumor types through different mechanisms in a selective oncogene-targeting manner, causing minor damage to normal cells. The target molecules are specific receptors, and proteins involved in neoplastic processes. Antibodies selectively induce cell death by blocking molecular pathways or inhibiting proliferation through the activation of antibody-dependent cellular cytotoxicity pathways and the complement system. Advances in genomics, such as the

Genome Project, have enabled personalized treatments and contributed to improving the survival rates of patients with cancer (Tsimberidou 2015). However, the treatment is complex when cancer cells survive via other means unrelated to the targeted proteins or genetic mutations. Therefore, targeted therapy should be combined with chemotherapy or radiotherapy.

Cancer is recognized as non-self by the immune system. Immunotherapy targets cancers through its effects on the immune system. In immunotherapy, monoclonal antibodies for T cell surface protein receptors or tumor antigens are used to activate immunity. Antigen-presenting cells use the major histocompatibility complex to signal antigen information to the T-cell receptor on T-cells, which can be activated or inactivated T-cells through immune checkpoint signals. B7-1 and B7-2 of antigen-presenting cells can be co-stimulated to be activated via CD28 and inactivated by stimulation of CTLA-4 on T-cells. In addition, programmed death-ligand-1 presents on the tumor surface and binds to programmed cell death-1 on T-cells, which inactivates T-cells (Li et al. 2019). Monoclonal antibodies targeting immune checkpoint molecules increase the disease-free survival rate in patients and have been used to treat refractory tumors. However, this immunotherapy has side effects during treatment; additionally, some patients do not respond to immune checkpoint inhibitors and develop resistance to previous therapy. Other types of resistance include tumor heterogeneity, genetic factors, and immune evasion by the tumor microenvironment (Dobosz et al. 2022).

Numerous therapeutic agents for treating cancer have been developed. Individualized treatment methods, new therapeutic approaches, and therapies that utilize the body's systems can facilitate the development of more effective medicines (Falzone et al. 2018).

Numerous therapeutic agents for treating cancer have been developed.

Individualized treatment methods, new therapeutic approaches, and therapies that utilize the body's systems can facilitate the development of more effective medicines. Because various treatments can use to treat cancer, previous treatments should be subjected to continuous research. Demonstrating the anti-cancer efficacy of DJEE may expand the range of therapeutic agents for thyroid cancer, increase the cure rate, and lead to a better prognosis. The active compounds with anti-cancer effects in papillary thyroid cancer must also be identified. Therefore, I evaluated the anti-cancer agents in DJEE and confirmed their potential as adjuvants in other therapeutic agents.

2. Types of thyroid cancers

Thyroid cancer is a malignant tumor that originates in the endocrine thyroid gland in the neck. There were 586,000 cases of thyroid cancer worldwide in 2020, ranking ninth among all cancers. Worldwide incidence rates are three-fold higher in women than in men. Furthermore, the mortality rate is 0.5 in women and 0.3 in men per 100,000 people, for a combined total of 44,000 deaths (Sung et al. 2021). Recent advances in observation tools and blood tests have enabled the diagnosis of even small-diameter tumors, leading to increased survival rates. Ultrasonography patterns can be examined to identify suspected tumors. Fine needle aspiration (FNA) is recommended following ultrasound if the suspected tumor size is ≥1 cm. Additionally, follow-up ultrasonography for 3–6 months should be performed to observe growth. If the tumor size increases, reexamination using FNA is recommended; confirmed malignancies can be subsequently treated using surgery. However, although overdiagnosis increased incidence, mortality did not change significantly (Hoang et al. 2015; Feldkamp et al. 2016).

Thyroid cancer is categorized based on the cells from which it is derived, which can be from follicular epithelial cells involved in iodine metabolism in the thyroid gland or

parafollicular thyroid C cells that produce calcitonin. Follicular epithelial cells in thyroid carcinoma divide through differentiation, differentiated thyroid cancer generally includes carcinomas that progress slowly and have a good prognosis at the time of initial treatment. Papillary and follicular thyroid cancer account for 70–80% and 10–15% of thyroid cancer cases, respectively. Undifferentiated thyroid cancer includes anaplastic thyroid cancer, which is aggressive and characterized by a low survival rate. Cancers produced by parafollicular C-cells include medullary thyroid cancer and occur in 5–10% of thyroid cancer cases (Pstrag et al. 2018). Papillary thyroid cancer (PTC) cells are the most common type of thyroid malignant and have a good prognosis. However, the pathological sub-types of PTC are aggressive and show high recurrence and metastasis rates and low survival rates. Mutations in PTC increase the aggressiveness, recurrence rate, and metastasis rate of this cancer. The most aggressive variants include diffuse sclerosing, tall cell, columnar cell, solid, and hobnail variants. Patients treated with RAI have a low survival rate because radioactive iodine has no binding power. Here, the characteristics of the variant are briefly described. The diffuse sclerosing variant accounts for 6% of all PTC variants and indicates lung metastasis. The tall cell variant is characterized by an extended cell height and width and a columnar shape. Patients with this variant have an average age of 41–66 years, which is older than the average age of patients with PTC. The columnar cell variant has the lowest incidence, occurring in 0.15–0.2% of cases, and is characterized by rapid growth and a high recurrence rate, exhibiting hypercellular neoplasia and clear cytoplasm. Additionally, several variants with different characteristics have been identified. Most of these mutants show mutations in BRAF and can be detected using FNA but are most often diagnosed after surgery, as they do not represent the entire lesion (Coca-Pelaz et al. 2020). Therefore, the precise determination and molecular properties need further. In addition, the 5-year survival rate varies depending on the degree of metastasis,

mutation type, and age; the roles of these factors in PTC are not well understood (Kauffmann et al. 2018).

3. Thyroid cancer treatment

Thyroid cancer can be treated via thyroidectomy, which is the removal of the thyroid gland. If metastases are found in patients with high-risk features for T3–T4 tumors, lymph node dissection is performed. If there is a risk of recurrence or metastatic disease after surgery, radioactive iodine therapy or inhibition of thyroid-stimulating hormone can reduce the risk of recurrence and residual tumors (Haugen et al. 2016). However, it is ineffective in low-risk patients, and more effective in patients with intermediate- and high-risk tendencies (McDougall et al. 2001). These treatments are ineffective in low-risk patients but more effective in patients with intermediate- and high-risk patients. Various anticancer drugs are ineffective in thyroid cancer because thyroid cancer cells express high levels of Bcl-2 and Bcl-xL. Overexpression of the Bcl-2 family in cancer cells prevents access to mitochondrial secreted cytochromes during apoptosis. Therefore, anticancer therapies that can modulate the Bcl-2 family and induce apoptosis, such as Bax, are required (Stassi et al. 2003). Therapies targeting the MAPK pathway can be performed in addition to surgery. The MAPK pathway regulates cell proliferation, motility, and apoptosis. Treatment using methods that can target BRAF, RAS, PI3K-AKT, and mTOR exerts side effects (Schlumberger et al. 2015). In addition, immunotherapy effectively suppressed CTLA-4 and PD-L1 expression. In addition, immunotherapy can suppress CTLA-4 and PD-L1. Both these targeted therapies and immunotherapies are clinically effective but require improvement, which may be achieved by combining chemotherapy with treatment. I used *D. japonicus* extract to investigate compounds with anticancer effects and provide a basis for improving targeted therapeutic drugs (Naoum

et al. 2018; Laha et al. 2020).

4. Distinct apoptotic signaling pathways

Anticancer drugs induce apoptosis by targeting DNA or factors that promote DNA replication in cancer cells. Apoptosis is a form of regulated cell death that differs from necrosis (Fink and Cookson 2005). Apoptotic bodies are produced by intracellular caspase signaling, after which cell death proceeds through phagocytosis. Apoptotic pathways can be intrinsic or extrinsic (Elmore 2007). The extrinsic apoptosis pathway activates caspase-8 when death receptors present at the extracellular membrane are stimulated with anticancer drugs. Activated caspase-8 activates caspase-3 and proceeds to apoptosis. Interestingly, it can also share unique pathways in mitochondria by activating Bid containing the BH-3 domain. The extrinsic apoptosis pathway activates caspase-8 when death receptors on the outside of the cell membrane are stimulated with anticancer target drugs. Activated caspase-8 activates caspase-3 to induce apoptosis. It can also share unique pathways in mitochondria by activating Bid which contains the BH-3 domain. The intrinsic pathway induces intracellular stress, which leads to Bax expression in the outer membrane of mitochondria and inhibition of Bcl-2. The permeability of the outer membrane inside mitochondria is subsequently increased and cytochrome c is released. When cytochrome c is secreted, it attaches to Apaf1, forms the apoptosome, and activates caspase-9. Caspase-3 is then activated to induce apoptosis.

The Bcl-2 protein family is involved in the progression of apoptosis by altering mitochondrial membrane permeability. Bcl-2 is divided into three distinct subfamilies; 1) anti-apoptotic proteins such as Bcl-2; 2) pro-apoptosis proteins including Bax and Bak; and 3) Bad, Bim, Noxa, Puma, and Bmf which contain a BH-3 domain. It is a key molecule in mitochondria, where it undergoes structural changes and interacts directly

with the Bcl-2 protein. Therefore, it is considered an upstream molecule for cell suicide following the release of cytochrome c. Bcl-2 provides a basis for developing therapeutic agents with anti-cancer effects, as its primary function is to block cytochrome c release to prevent apoptosis. Cytochrome c functions as a redox intermediate in complexes III and IV in the mitochondrial electron transport chain. It is secreted during apoptosis progression, binds Apaf-1, and interacts with caspase signaling. Another factor released from mitochondria, an apoptosis-inducing factor, interacts with DNA to cause chromatin condensation and DNA fragmentation of 50 kb. (Wang and Youle 2009). Activation and regulation of these proteins result in apoptosis.

Therefore, to develop new anticancer drugs, studies are needed to understand the active pathways involving these molecules.

5. Cell cycle and aneuploid cancer

Cell proliferation and death must be controlled to maintain tissue homeostasis. Cyclin-dependent kinases that receive these growth factor signals monitor and regulate the cell cycle. Some checkpoints are controlled within the cell cycle when proliferating by cyclin-dependent kinases (Matthews et al. 2022). Checkpoints regulate proliferation at the G1/S phase as the restriction checkpoint, the G2/M phase as the DNA replication checkpoint, and metaphase/anaphase as the spindle apparatus checkpoint. Cell proliferation can pass these checkpoints after it is confirmed that there are no errors in the DNA, spindles, growth factors, etc.; otherwise, apoptosis is induced. Normal cells proceed through the G1, S, G2, and M phases. G1, S, and G2 phases are part of interphase, during which DNA replication occurs and organelles and the cell increase in size. The S phase is critical in the cell cycle, as DNA is copied during this step. After interphase, the mitotic (M) phase occurs, during which DNA condenses into chromosomes and is split in half by

the spindle to form two daughter cells after cytokinesis. The cells can then enter either the G0 or G1 phase again in a sustained mitotic state (Wenzel and Singh 2018).

Tumor protein p53 regulates cell cycle arrest, apoptosis, and DNA repair but is mutated in most cancers. p53 regulates p21 expression and affects cell proliferation by acting on the G1 and G2/M checkpoints. It can also increase the expression of Bax, inhibits Bcl-2, or directly promotes the mitochondrial outer membrane transmembrane to induce apoptosis. Thus, p53 is a critical factor because of its role in regulating the cell cycle and its association with apoptosis (Pucci et al. 2000; Marei et al. 2021).

Mis-segregation of mitotic spindles during the cell cycle results in the gain or loss of whole chromosomes. Aneuploidy in cancer cells is associated with poor prognosis, and cell cycle defects render cancer cells less susceptible to damage caused by chemotherapy. Aberrations in the original gene copy number in aneuploid cancer cells cause various intracellular stresses that delay G1 in the cell cycle and slow cell proliferation. In addition, cells with aneuploidy form a new cellular metabolic environment and lead to genomic instability, thus inducing resistance to chemotherapy and leading to poor prognosis (Replogle et al. 2020). Therefore, methods should be developed that target cell cycle-related factors and avoid resistance.

6. Mitochondria membrane potentials

Mitochondria are critical ATP-producing organelles present in multiple cell types and are dysfunctional in neurodegenerative, cardiovascular, and metabolic diseases as well as in cancer. Cells generate pyruvate through glycolysis in the cytoplasm; pyruvate then undergoes aerobic or anaerobic respiration to produce ATP. Mitochondria generate 36 ATP and CO_2 via oxidative phosphorylation and the tricarboxylic acid cycle under aerobic conditions. In contrast, in the anaerobic state, lactate and 2 ATP are produced.

Therefore, ATP production under aerobic conditions can maintain energy levels in cells. Membrane potential is generated by oxidative phosphorylation during ATP production via the proton pump and is important in glucose metabolism (Zorova et al. 2018). The proton concentration gradient gives the inner membrane a negative charge, whereas the outer membrane is comparatively rich in protons (Papa et al. 2012). Mitochondrial membrane potentials (MMPs) are directly linked to the production of ATP, which is the main energy source in cells. However, when MMPs are not maintained, increased outer membrane permeability leads to Bax upregulation, inhibition of Bcl-2 family expression, and release of cytochrome c, inducing apoptosis.

Cancer cells consume large amounts of energy because of their altered mitochondrial metabolism and sustained division, and thus are attracted glucose under aerobic conditions but produce lactic acid and 2 ATP under anaerobic conditions. This cancer metabolic reprogramming is known as the Warburg effect. Glucose that is not fully oxidized can be degraded into lactate and biomass to generate ATP and support cell growth (Vander Heiden et al. 2009; Kalyanaraman 2017). In addition, the metabolism of cancer cells, which absorbs a lot of glucose, inhibits the immune response.

The high glucose consumption by cancer cell metabolism inhibits the immune response and promotes regulated cell death. In addition, it reprograms by activation of anabolism by the Warburg effect, promotion of reductive metabolism, reduction of reactive oxygen species production, suppression of immune response, genomic acetylation, and apoptosis control. Therefore, mitochondria are promising targets for the development of new therapeutic agents. Preexisting chemotherapy, targeted therapy, radiation therapy, and immunotherapy approaches can kill or inactivate cancer cells (Porporato et al. 2018).

B. *Dianthus* herb

1. *Dianthus japonicus*.

Natural products extracted from plants and natural chemical structure-based drugs have been used to treat many diseases. *Dianthus japonicus* grows on the Gyeongsangnam-do and Jeju Island seashores and is distributed in Japan and China. In this study, I examined the anticancer effects of *D. japonicus* extract, which have not been evaluated previously. I searched for *Dianthus chinensis*, which belongs to the family Caryophyllaceae, and whether it was used for pharmacological applications. *Dianthus chinensis* is sold as a medicinal herb named as Gu-Maig and has been used pharmacologically since early times. Recent studies showed that *D. chinensis* is effective against oral and liver cancer. Orthodox medicine documents from the Joseon Dynasty period describe that *D. chinensis* was used pharmacologically, as indicated in Dongui-Bogam and Byeolcho-dan-bang documents. Therefore, the anticancer effects of *D. japonicus* were examined to identify its specific pharmacological functions. Natural products such as *D. japonicus* are widely available and can be applied in numerous fields such as medicine, food, and beauty, and can be competitive. Therefore, I examined the anti-cancer activity of *D. japonicus* and investigated its potential as a pharmacological agent.

II. MATERIALS AND METHODS

1. Reagents

Dianthus japonicus ethanol extract *(DJEE)* powder was extracted with 70% Ethanol and powdered by freeze-drying purchased from Gyeonggi-do Business & Science Accelerator (Suwon, Gyeonggi-do, Korea). 100× Penicillin-streptomycin Solution, 10× Trypsin-EDTA Solution, and RPMI-1640 Medium were purchased welgene (Gyeongsangbuk-do, Korea). Fetal Bovine serum (FBS) was purchased from Gibco (MA, United States). The reagent of 33-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St Louis, MO, USA). Prolong Gold anti-fade reagent with 4, 6-diamidino-2-phenylindole (DAPI) and tetramethyl-rhodamine, methyl ester (TMRM) bought from Invitrogen (Carlsbad, CA, USA). The Antibodies α-tubulin, Bax, Bcl-2, AIF, Cytochrome C, Caspase-3, and PARP were purchased by Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA) and Caspase-9, Caspase-8 were purchased Cell Signaling Technology (Danvers, Massachusetts, USA). The secondary Antibody Anti-goat Anti-mouse or rabbit IgG F(ab')2, polyclonal antibody (HRP conjugate) purchased from Enzo Biochem, Inc (Farmingdale, NY, USA).

2. Cell culture and DJEE treatment

SNU-790 PTC cells were obtained from the Korea Cell Line Bank (Seoul, South Korea) with a cell line derived from a 72-year-old human male. SNU-790 PTC cells were incubated with RPMI-1640 supplemented with heat-inactivated Fetal bovine serum and 1% Penicillin-streptomycin and incubation at 37℃ in a humidified atmosphere containing 5% CO_2 incubator (Thermo Scientific). The population doubling time of PTC was 33 h,

and the sub-cultivation ratio was 1:3 to 1:4 when sub-cultured once 2–3 days. Trypsin-EDTA was added to 2 ml, and 2–3 min later, the pipettes were transferred to a 15 ml tube and neutralized using the RPMI-1640 media. The centrifugation at 1,500 RPM for 5 min at RT, leaving a pellet and sucking the supernatant. After suspension with new RPMI-1640 media, the cells were cultured in a new 100 mm culture plate. DJEE was dissolved to 200 mg/ml using a Dimethyl sulfoxide (DMSO). Then, it was diluted with RPMI-1640 media and used to make 400 μg/ml. Media was added according to the concentration of the group to be treated and treated with DJEE. The vehicle control group was treated with the same concentration of 0.2% (v/v) DMSO and DMSO was diluted in media when treated.

3. Cell viability assay

Cell viability of SNU-790 PTC cells treated with DJEE is measured by MTT assay to confirm the appropriate concentration. SNU-790 PTC cells (8×10^3 cells/well) were seeded into 96-well cell plates and treated with DJEE at a concentration of 25, 50, 100, 200, 300, and 400 μg/ml. After 24 h, discard the treated medium and add 0.5 mg/ml MTT reagent diluted in PBS. Then, the MTT reagent was incubated at 37°C for 4 hours. Formazan was dissolved using equal volumes of DMSO and Ethanol. Since the color that appears after dissolving the formazan is purple, it is measured using a plate reader at 570 nm. The measured values were calculated and graphed, and Standard deviation (SD) values were added.

4. DAPI staining

SNU-790 PTC cells (8×10^4 cells/well) were seeded in a 12-well culture plate with a round coverslip and incubation at 37℃. Each cell was treated with different concentrations (0, 25, 50, 100, 200, 300, and 400 μg/ml) and cultured for 24 h. After washing with PBS, cells were fixed by adding 100% methanol. The cell plate was wrapped in foil and incubated at -20°C. A round coverslip was removed from the plate for staining and the methanol was eliminated. 4′,6-diamidino-2-phenylindole (DAPI) was heated in a 45°C water bath for 10 – 15 min before staining. Permanent DAPI dye was placed on slide glass in the dark and cover a round coverslip. After staining, it was hardened at room temperature for 24 h. Then, using a fluorescence microscope (Eclipse Ts2-FL Diascopic and Epi-fluorescence illumination, Nikon) in a dark, the fluorescence intensity of the DAPI-stained cells at each concentration was observed. Photographs were analyzed.

5. Cell Cycle analysis

SNU-790 PTC cells (3×10^6cells/ dish) were seeded on a 100 mm plate and treated with different concentrations (0, 25, 50, 100, 200, 300, and 400 μg/ml). After 24 h, it was detached using Trypsin-EDTA and washed with cold PBS. After 24 h, the plate was detached with trypsin-EDTA and then centrifuged at 1,500 RPM for 5 min at 4℃ The supernatant was suctioned, and the pellet was suspended in PBS. After adding 9 ml of PBS, centrifugation was performed at 800xg, 5 min, and 4°C. The cells were fixed overnight using cold 70% ethanol. After removing the ethanol, it was washed with cold PBS. The supernatant was removed, and 300 μl of 50 μg/ml Propidium iodide (PI) diluted in PBS was added for staining. The stained cells were transferred into Micro Test Tubes (Bio-RAD) and incubated at 4°C for 15 min. Then, 20,000 cells were analyzed using a

Fluorescence-activated cell sorter (FACS) (Beckman, FC500). Analyzed data were identified and graphed for % Total values for each cell cycle through Kaluza Analysis Software.

6. Western blot analysis

SNU-790 PTC cells (2×10^6 cells/ dish) were seeded in RPMI-1640 media and treated with DJEE. Then, cells at each concentration were collected**.** After centrifugation, discard the supernatant and wash the cell pellet with PBS. And then the cell was down using a centrifuge, and the supernatant was removed and lysed using RIPA buffer (component: 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 50mM Tris, protease inhibitor cocktail, phosphatase inhibitor cocktail, pH8.0). Suspended cell pellets by lysis buffer were sonicated and centrifuged to obtain a supernatant. The concentrations of each protein were determined using the Bicinchoninic acid assay kit (Pierce, USA). Proteins for each DJEE concentration were quantified, and 20μg of protein was loaded on 12% SDS PAGE gel and separated by protein size. Then, the proteins separated by size from the SDS PAGE were transferred to PVDF. And it was blocked with 5% skim milk at room temperature for 1h. PVDF membranes were TBS-T 3 times, 10 min each. And the appropriately diluted primary antibody was incubated overnight at 4°C. Primary antibodies Bax, Bcl2, AIF, cytochrome C, p53, Caspase-3, 8, 9 were used (Table 1.) An anti-rabbit or mouse secondary antibody suitable for the primary antibody was used and incubated at 1h 30 min at RT. And developed a membrane using chemiluminescence (ECL solution, Abbkine). And by time, the blotted membrane was exposed using an imaging system.

Table 1. Information on primary antibodies used in Western blot

Primary antibody (Company, Cat. No)	Secondary antibody	Dilution ratio
Bax (Santa Cruz, sc-526)	Rabbit	1 : 2000
Bcl-2 (Santa Cruz, sc-7382)	Mouse	1 : 2000
AIF (Santa Cruz, sc-5586)	Rabbit	1 : 2000
Cytochrome C (Santa Cruz, sc-13560)	Mouse	1 : 2000
Caspase-3 (Santa Cruz, sc-7148)	Rabbit	1 : 2000
Caspase-8 (Cell signaling, #4790)	Rabbit	1 : 2000
Caspase-9 (Cell Signaling, #9508)	Mouse	1 : 2000
p53 (Santa Cruz, sc-6243)	Rabbit	1 : 2000

7. Mitochondria membrane potential (MMP, ΔΨm) assay

SNU-790 PTC cells (3×10^6 cells/dish) were seeded in 100 mm plates. DJEE was diluted with medium and treated with 300 μg/ml for each time (0, 15, 30, 60, 120, and 180 minutes). Cells were detached by treatment with Trypsin-EDTA and neutralized by treatment with RPMI-1640 media in the same volume. Centrifuged at 1,500 RPM for 5 min at 4°C and removed the supernatant. Cells were washed with cold PBS and centrifuged at 1,500 RPM for 5 min at 4°C. Then, the cell is fixed by cold 70% ethanol overnight at 20°C To remove ethanol, it was washed with PBS and centrifuged at 1,500 RPM for 5 min at 4°C. Tetramethyl-rhodamine, methyl ester (TMRM) dye was diluted with PBS to make 50 nM, and 300 μl was added to each cell. Stained cells were transferred to Micro Test Tubes (Bio-RAD) and incubated at 4°C for 15 min. The membrane potential of 10,000 stained cells was quantified as an average value using a Fluorescence-activated cell sorter (Beckman FC500). Results were analyzed for membrane potential values using Kaluza software. The analyzed data were graphed after significance evaluation.

8. Statistical analysis

The data are shown as mean ± SD. Data were analyzed using SPSS27 (IBM, USA). Data were evaluated by the Bonferroni method and a One-way ANOVA was used. Statistical significance was accepted at P Value (*$p<0.05$, **$p<0.005$, ***$p<0.001$).

RESULTS

1. *Dianthus japonicus* ethanol extract (DJEE) reduces cell viability of SNU-790 PTC cells

Experiments were performed to verify the anticancer effect of DJEE on PTC cells. I first examined whether DJEE affected the proliferation of PTC cells using an MTT assay, which is a commonly used method for studying cellular metabolic activity, cell viability, and therapeutic toxicity. MTT is taken up by cells, converted to formazan upon metabolic activation, and exported outside the cell. Formazan is dissolved, after which its absorbance is measured. DJEE was used to treat PTC cells at concentrations of 0, 25, 50, 100, 200, 300, and 400 μg/ml during incubation for 24 h. DJEE decreased the cell survival of PTC cells in a dose-dependent manner (Fig. 1). The antibiotic effects of DJEE are shown in Table 2; the 50% inhibitory concentration (IC 50) values were 300–400 μg/ml. Because this extract is a natural product containing various components, the precise molarity could not be determined. DJEE reduced the survival of PTC cells and showed toxic effects. I next examined the type of cell death that occurred, focusing on apoptosis.

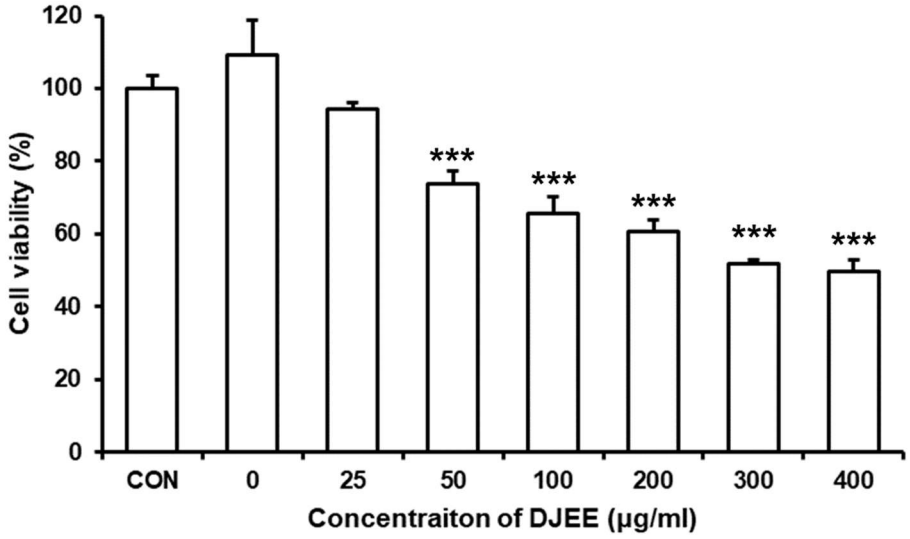

Fig. 1. DJEE decreases the cell viability of SNU-790 PTC cells in a dose-dependent manner. SNU-790 PTC cells were seeded in a 96-well plate and mixed with DJEE and RPMI-1640 media at concentrations of 25, 50, 100, 200, 300, and 400 μg/ml. The experiment is represented in the "Materials and Methods" part. After 24 h of incubation, an MTT reagent was added, and cell viability was measured. In the data, CON was only treated with RPMI-1640 media and only 0.2% DMSO at 0 μg/ml. The cell viability of each concentration draws on a graph as a percentage based on the CON value. The mean (± SD) of the independent three wells is shown and the experiment was performed in triplicate. Statistically significant results are indicated. (*$p<0.05$, **$p<0.005$, ***$P<0.001$).

Table 2. Statistical analysis of cell viability (mean ± SD)

Concentration (μg/ml)	Cell viability (%)
25	**94.23 ± 1.98**
50	**73.77 ± 3.41*** **
100	**65.73 ± 4.58*** **
200	**60.66 ± 3.02*** **
300	**51.74 ± 1.09*** **
400	**49.82 ± 3.19*** **

*, **, and *** suggest significant differences from the control group by the Bonferroni test (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$).

2. DJEE induces DNA condensation in SNU-790 PTC cells

DJEE reduced the viability of PTC cells. Therefore, I evaluated whether there was evidence of apoptosis in these cells (fig. 1). Apoptotic cells exhibit nuclear shrinkage and chromatin condensation. Upon activation of apoptosis, chromosomal DNA is fragmented into oligo-nucleosomes. DAPI staining of apoptotic cells results in high fluorescence intensity, as cancer cells have aneuploid characteristics and apoptosis is more condensed than mitosis. Therefore, I used DAPI to examine changes in the fluorescence caused by DNA condensation that occurs during apoptosis as described in the Materials & Methods. Cells treated at all DJEE concentrations were evaluated under the same conditions using a fluorescence microscope. In Fig. 2, 10× and 20× photographs of cells treated with 25 and 50 μg/ml DJEE did not show strong fluorescence intensity because of DNA condensation. Cells treated with DJEE concentrations of 100, 200, 300, and 400 μg/ml showed fluorescence. In addition, at DJEE concentrations of 200–400 μg/ml, cell viability was reduced compared to that in the control group according to the MTT assay, so the total cell amount appeared to decrease. These results show that in SNU-790 PTC cells, the fluorescence intensity of DAPI bound to fragmented and condensed DNA in the nucleus increased with increasing DJEE concentrations. As SNU-790 PTC cells treated with DJEE exhibited characteristics of apoptosis, I examined whether this process occurred through the intrinsic or extrinsic pathway.

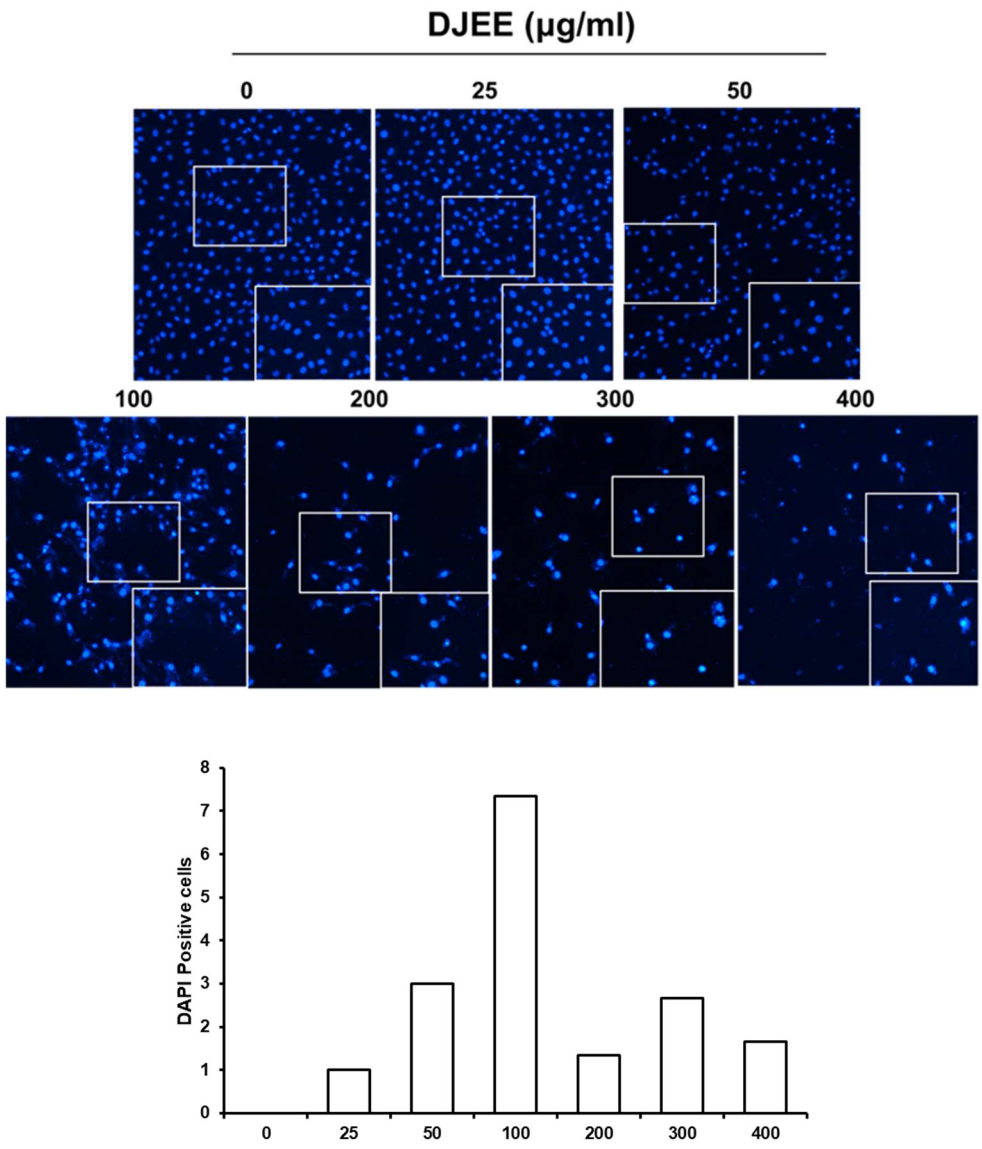

Fig. 2. DJEE affects nuclear DNA condensation in SNU-790 PTC cells. SNU-790 PTC cells were seeded in 12-well plates and treated at different concentrations. It was stained with DAPI in the same manner as in the Materials and Methods section after 24 h incubation. Data show representative pictures out of three independent experiments.

3. DJEE activates the caspase-mediated apoptosis signaling pathway in SNU-790 PTC cells

Next, western blot analysis was performed to evaluate the levels of proteins involved in the intrinsic and extrinsic apoptotic signaling pathways (Fig. 2). The intrinsic pathway increases the expression of Bax protein in mitochondria when intracellular stress is induced by chemotherapeutic reagents, whereas the expression of Bcl-2 is inhibited, and cytochrome c is released and binds with Apaf-1 to form the apoptosome. After caspase-9 is activated by the apoptosome, caspase-3 is activated to induce apoptosis. In extrinsic pathways, activation of death receptors (Fas and TNF) on the cell membrane either activates caspase-8 and proceeds to caspase-3, or caspase-8 activates Bid via the BH-3 domain and shares an intrinsic pathway. Additionally, when p53 is activated, the pro-apoptotic gene activates transcription, represses Bcl-2 and inhibitors of apoptosis transcription, and enhances apoptosis. As shown in Fig. 3, pro-caspase-3, -8, and -9 were found to be activated based on their decreased expression. Therefore, activation of FADD through the extrinsic receptor may activate caspase-8 and induce extrinsic cell death. Additionally, the expression of Bax is increased, and that of Bcl-2 is decreased. Further, pro-caspases-9 and -3 are activated in response to decreased levels of pro-caspases-9 and -3, thereby inducing the intrinsic pathway and causing apoptosis. As shown in Fig. 2, as the DNA condensation progressed, the fluorescence intensity increased with increasing DJEE concentrations. Therefore, I evaluated the expression of PARP-1, which is involved in nuclear DNA condensation and fragmentation. Pro-PARP-1 is reduced and activated and is thought to be involved in DNA fragmentation. I confirmed the expression of proteins involved in the extrinsic and extrinsic pathways and showed that caspase activation leads to apoptosis. Analysis of the expression of proteins involved in the apoptotic pathway showed that p53 expression increased in concentration. Therefore, the

increase in Bax expression and suppression of Bcl-2 expression is associated with increased p53 expression. DJEE was considered to have anticancer effects on SNU-790 PTC cells via apoptosis but not necrosis. As p53 showed increased expression, I examined the effects of DJEE on the cell cycle. In addition, alterations in mitochondrial membrane permeability and the MMP were evaluated.

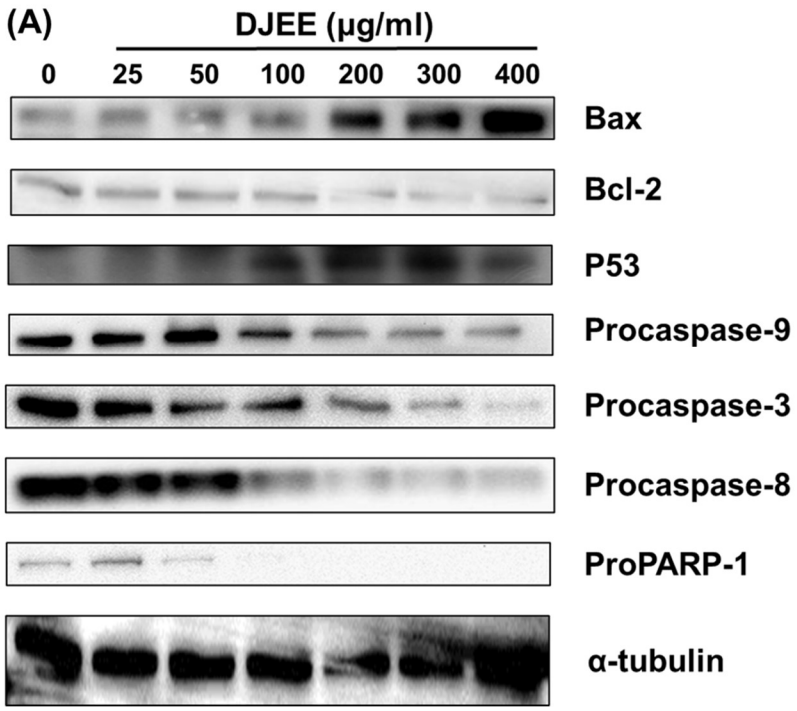

Fig. 3. (Continued)

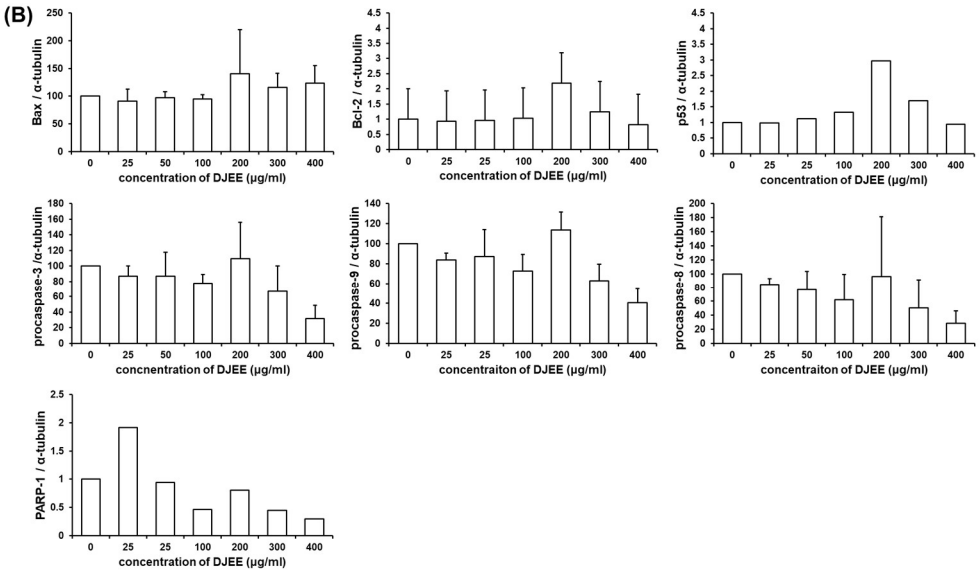

Fig. 3. DJEE activates pro-apoptotic protein and triggers the caspase-mediated apoptosis signaling pathway in SNU-790 PTC cells. SNU-790 PTC cells were incubated with DJEE (25–400 μg/ml) for 24 h, and proteins were isolated. After protein quantification, they were separated on a 12% SDS-PAGE gel and transferred to a PVDF membrane. Patterns were confirmed using factors (Bax, Bcl-2, procaspase-3/-8/-9, p53, and proPARP-1, α-tubulin). Representative results from many experiments are shown.

4. DJEE-induced apoptosis does not affect MMP (ΔΨm) in SNU-790 PTC cells

Mitochondria are important mediators of signal transduction and can reprogram energy metabolism and chemical resistance in cancer cells. Chemotherapeutic drugs targeting mitochondria in cancer cells can alter membrane resistance, release cytochrome c, and initiate apoptosis. In addition, the MMP can result in ATP production, as oxidative phosphorylation occurs when the concentration gradient generated by the proton pump is maintained. Therefore, I investigated whether DJEE induced MMP dysfunction in PTC cells. Dysfunction in mitochondria leading to intrinsic apoptosis may be a suitable drug target. Tetramethyl-rhodamine, methyl ester (TMRM) was used as a dye to measure the membrane potential. When oxidative phosphorylation provides a negative charge to the inner mitochondrial membrane, TMRM accumulates in the inner membrane and generates high fluorescence intensity. However, when apoptosis occurs, the MMP cannot be maintained, TMRM spreads throughout the cytoplasm, and fluorescence decreases. Fluorescence-activated cell sorting was performed after treating the cells with TMRM. The histogram shown in Fig. 4A revealed that increasing DJEE concentrations did not affect the MMP. No significance was found in the MFI values between the three independent experiments (Fig. 4B). Thus, DJEE did not affect the MMP in PTC cells and was not a major factor affecting apoptosis. Therefore, cell cycle analysis was performed to identify other factors influencing cell death.

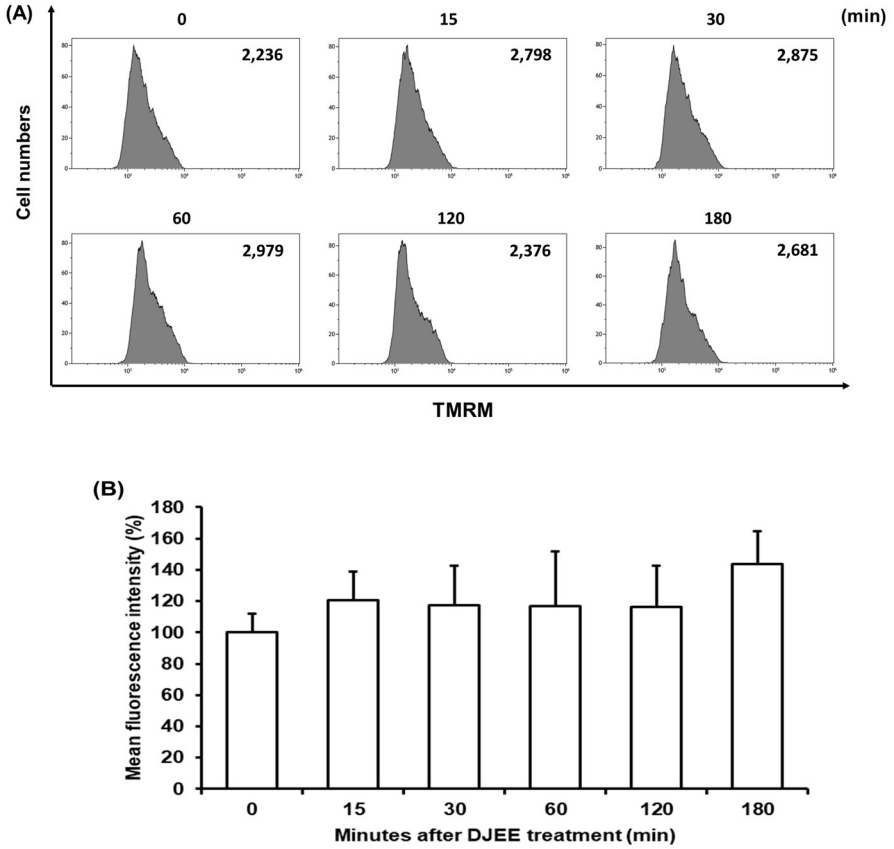

Fig. 4. DJEE does not damage MMPs during apoptosis of SNU-790 PTC cells. SNU-790 PTC cells were treated with 300 μg/ml, allowed to proceed for 15, 30, 60, 120, and 180 min, and stained with 50 nM TMRM. Fluorescence intensity was measured by flow cytometry. And (A) is shown as the mean fluorescence intensity (MFI) value of the histogram. In (B), the values up to 180 min were graphed based on 0 min in the experiment repeated three times, and significance was evaluated. Significance was confirmed by the Bonferroni method. (*p<0.05, **p<0.005, ***P<0.001).

5. DJEE arrests the cell cycle of SNU-790 PTC cells

Cancer cells divide in an uncontrolled manner. As shown in Fig. 3, p53 expression increased in SNU-790 PTC cells with increasing concentrations of DJEE. p53 plays important roles in cell division, maintenance of genomic stability, and regulation of apoptosis. However, p53 is mutated in most cancers and compromises the activity of cyclin-dependent kinases which regulate DNA damage and repair. As shown in Fig. 5, I analyzed aneuploidy in cancer cells. Cancer is caused by genetic mutations and aneuploidy; however, the effects of aneuploidy in cancer are not well-understood. Aneuploid carcinomas are characterized by genomic and chromosomal instability, anchorage-independent growth, transcriptional and metabolic reprogramming, and immune escape. Chromosomal instability can prevent the targeting of specific oncogenes, and alterations in the epigenome can indicate drug resistance. As mutations in the p53 and pRb cell cycle regulatory pathways induce aneuploidy in the genome and chromosomal instability, their effects on aneuploidy phases during the cell cycle and whether SNU-790 PTC cells (> 4N phase in the cell cycle) were affected by DJEE were analyzed. As shown in Fig. 5A and B, the sub-G1 phase was increased according to the fluorescence-activated cell sorting histograms and graphs. Propidium iodide binds to DNA within necrotic or late apoptotic cells, and its fluorescence can be analyzed using flow cytometry. Apoptotic cells have a decreased DNA content and exhibit fragmented DNA, resulting in a low fluorescence intensity owing to low levels of propidium iodide. DJEE increased the number of SNU-790 PTC cells in the sub-G1 phase to increase apoptosis. In addition, the number of aneuploid thyroid cancer cells with 4N or more chromosomes was decreased significantly in response to high DJEE concentrations. Table. 3 shows the statistical analysis of the data in Fig. 5B; the number of cells with sub-G1 and aneuploidy phases (> 4N) was significantly increased at DJEE treatment concentrations of 300–400 μg/ml.

Thus, the DJEE-induced arrest of the SNU-790 PTC cell cycle increased the sub-G1 phase and reduced the number of cells with aneuploidy that affect drug resistance, showing anticancer efficacy.

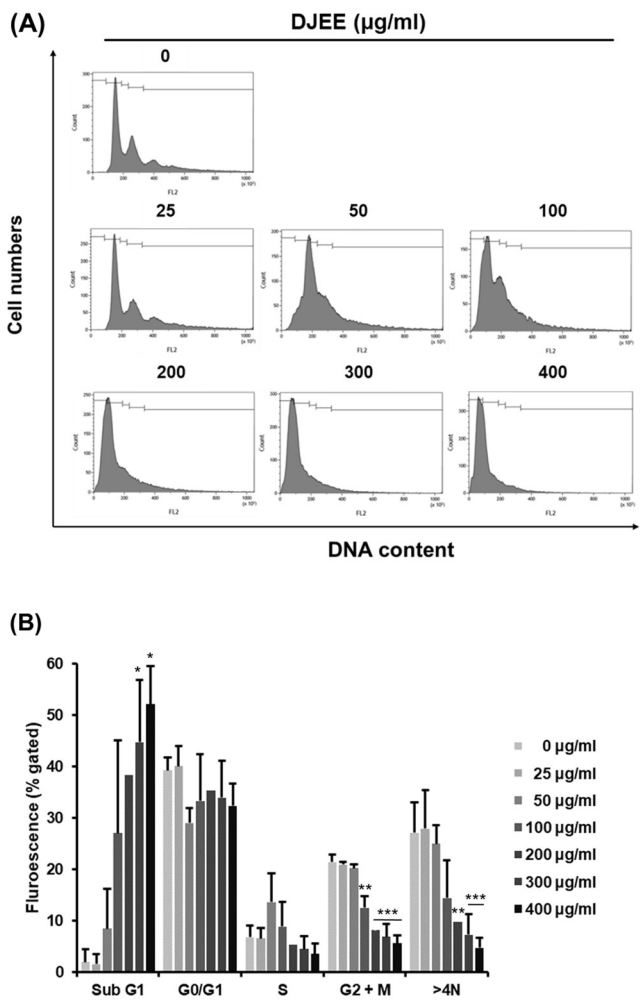

Fig. 5. Cell cycle analysis of SNU-790 PTC cells treated with DJEE. (A) Cell cycle histogram by the concentration of DJEE using flow cytometry. (B) Graphs represent the average values analyzed by flow cytometry. PTC cells were treated with different concentrations of DJEE for 24 h. It proceeded as in Materials and method. Data were indicative for three independent experiments. Differences represent standard deviation, and significance considered by the Bonferroni method. (*p<0.05, **p<0.005, ***P<0.001).

Table 3. Results of cell cycle analysis (± SD)

Group	Cell cycle distribution (%)				
	Sub-G1	**Go/G1**	**S**	**G2/M**	**>4N**
0 μg/ml	2 ± 2.39	39.15 ± 2.39	6.79 ± 2.19	21.39 ± 1.42	27.17 ± 5.88
25 μg/ml	1.56 ± 1.97	40.08 ± 3.88	6.55 ± 2.07	20.91 ± 0.48	27.89 ± 7.55
50 μg/ml	8.48 ± 7.77	29.03 ± 2.85	13.65 ± 5.59	20.24 ± 0.75	24.96 ± 3.71
100 μg/ml	27.04 ± 18.1	33.29 ± 9.12	8.86 ± 4.83	12.54 ± 2.31 **	14.40 ± 7.38
200 μg/ml	38.39 ± 17.7	35.37 ± 9.02	5.39 ± 3.06	8.15 ± 2.77 ***	9.8 ± 7.21 **
300 μg/ml	44.72 ± 12.09 *	33.98 ± 7.13	4.48 ± 2.49	6.89 ± 2.44 ***	7.26 ± 3.98 ***
400 μg/ml	52.11 ± 7.43 *	32.34 ± 4.36	3.58 ± 1.95	5.63 ± 1.61 ***	4.76 ± 1.97 ***

*, **, and *** suggest significant differences is using the Bonferroni test (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$).

6. The compound isolated from DJEE decrease the cell viability of SNU-790 PTC cells

Previous data confirmed that DJEE decreased the viability and apoptotic pathways in SNU-790 PTC cells. However, as DJEE is a natural extract, the active compounds must be identified. SNU-790 PTC cells were treated with 400 μg/ml of compounds separated using different solvents, and cell viability was evaluated in an MTT assay (Fig. 6A). SNU-790 PTC cells showed decreased cell viability in the ethanol and butanol fractions. Other compounds also affected viability but to lesser extents. The effects of concentrations of 100, 200, 300, and 400 μg/ml are shown in Fig. 6B. Thus, compounds isolated from DJEE using ethanol and butanol decrease the viability of SNU-790 PTC cells in a concentration-dependent manner. These compounds may exert more effective anticancer effects. I did not determine the structure of the compound, which should be further evaluated.

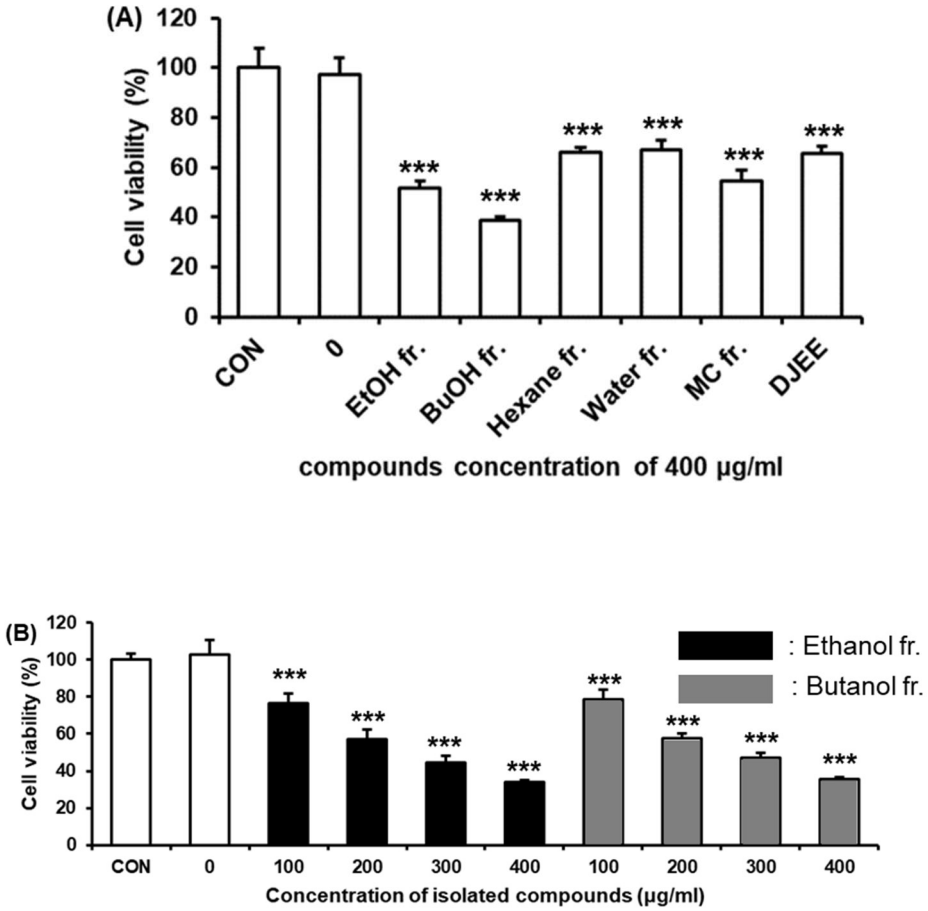

Fig. 6. Ethanol and butanol-isolated compounds from DJEE reduce the cell viability of SNU-790 PTC cells in a concentration-dependent manner. SNU-790 PTC cells were seeded in 96-well plates, treated with compounds isolated from DJEE for 24 h, and treated with MTT. (A) All fraction compounds were treated at 400 μg/ml. (B) Cells were treated with ethanol and butanol fraction concentrations of 100, 200, 300, and 400 μg/ml to confirm cytotoxicity. (*p<0.05, **p<0.005, ***P<0.001).

IV. DISCUSSION

Thyroid cancer is a malignant tumor that arises in the hormone-secreting endocrine system and is difficult to treat. Diagnosis using commonly advanced ultrasonography and fine-needle aspiration biopsy enables early detection and reduces recurrence and mortality rates. Because treatment is mostly surgical, you can face some of the problems that arise from thyroidectomy. The papillary thyroid cancer used in this paper is the most common type of thyroid cancer in patients. Most of the treatment methods for thyroid cancer with a good prognosis are advanced as surgery as described above. However, there is a need for a treatment method suitable for papillary thyroid cancer with various mutations (Raue and Frank-Raue 2016). Several molecular genetic foci that are predominantly mutated within thyroid cancer have not been identified. In previous studies, the most common mutations in thyroid cancer included RAS, PI3K, p53, and BRAF (Fagin et al. 1993; Xing et al. 2013). The expression of p53 increased as the concentration of DJEE increased, according to the experiments in this paper. As such, it can be an efficient therapeutic agent for thyroid cancers that exhibit low levels of mRNA expression. Cell cycle analysis also confirmed a reduction in Sub-G1 and aneuploid cancer cells, and p53 is a protein that can regulate the expression of Bax, Bcl-2, and thus could serve as a therapeutic agent (Chipuk et al. 2004). Recently, much has been done about BRAF-related research. 30-67% of papillary thyroid carcinomas are mutated and are closely associated with metastasis, progression, recurrence, and poor prognosis (Xing et al. 2005, Kim et al. 2012). Therefore, it can be a prognostic marker or a target for therapeutic agents.

I also confirmed the reduction of aneuploid cancer cells by DJEE. In general, 90% of solid tumors are aneuploid and have abnormal chromosomal phases. Chromosomal instability indicates altered RNA and protein expression. Previous studies have been

controversial as to whether it is a parental or anti-tumor property for aneuploid cancer cells (Simonetti et al. 2019). Aneuploid cancers with chromosomal instability have been shown to have a worse prognosis and be more resistant to chemotherapy than cancers with stable chromosomes (Lee et al. 2011; Replogle et al. 2020). Therefore, the reduction of the population of aneuploid cancer cells is induced by DJEE, which can better alter the therapeutic prognosis. In this paper, I confirmed the apoptotic pathway and mitochondrial membrane potential. I thought that caspase-9 activation was caused by changes in mitochondrial membrane potential. However, no change in membrane potential was observed, caspase-8 appears to be activated, and t-Bid induces Bax expression through the receptor. Therefore, it has been argued that cell death through the extrinsic pathway may be induced (Fig7).

Previously researched papers showed that the compound from *D. chinensis* and *Dianthus superbus*, which belongs to the genus *Dianthus*, contains many compounds, such as Saponins, flavonoids, anthraquinones, phenolic acids, amides, and phenyl-propyl, and induces apoptosis (Liu et al. 2022). Expected compounds include compounds of the saponin family. I need to confirm that the compound belongs to this series, and I need to look up the chemical structure. Finally, DJEE is likely to belong to extracts with high prospects. If the effect can be verified in more types of cancer and can be made and used as a drug, it will become a treatment that can be used as an adjuvant or a single treatment for multiple treatments.

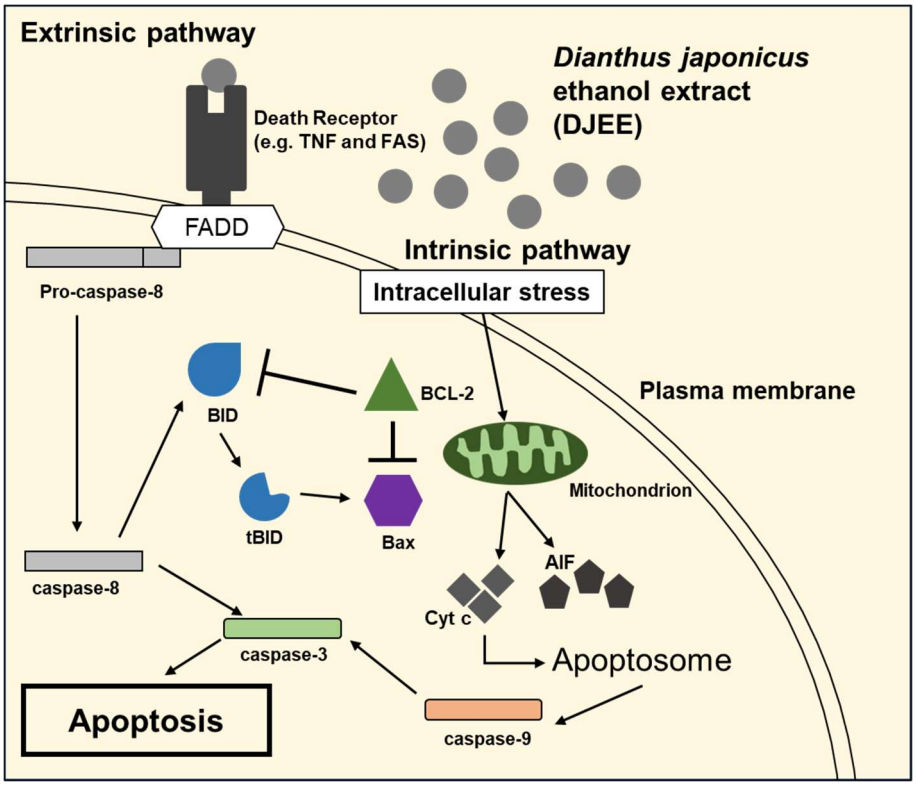

Fig. 7. DJEE induces apoptosis as activation of caspase signaling in SNU-790 PTC cells. Predicted cell death intrinsic or extrinsic signaling pathway by DJEE. It appears that apoptosis is induced by caspase activation.

V. REFERENCES

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