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Original Article

# *Ginkgo biloba Leaf* Extract Regulates Cell Proliferation and Gastric Cancer Cell Death

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*Ginkgo biloba Leaf* Extract (GBE) is an extract from leaves of the Ginkgo biloba tree, widely used as a health supplement. GBE can inhibit the proliferation of several types of tumor cell. Although it is known to have anti-cancer effects in breast cancer and skin cancer, research related to gastric cancer is still insufficient. Based on results showing anti-cancer effects on solid cancer, we aimed to determine whether GBE has similar effects on gastric cancer. In this study, the anti-cancer effect of GBE in gastric adenocarcinoma was investigated by confirming the cell proliferation inhibitory effect of AGS cells. We also evaluated whether GBE regulates expression of the tumor suppressor protein p53 and Rb. GBE has apoptotic effects on AGS cells that were confirmed by changes in anti-apoptosis protein Bcl-2, Bcl-xl and pro-apoptosis protein Bax levels. Wound healing and cell migration were also decreased by treatment with GBE. Furthermore, we verified the effects of GBE on mitogenic signaling by investigating AKT target gene expression levels and revealed downregulated Sod2 and Bcl6 expression. We also confirmed that expression of inflammation-related genes decreased in a time-dependent manner. These results indicate that GBE has an anti-cancer effect on human gastric cancer cell lines. Further research on the mechanism of the anti-cancer effect will serve as basic data for possible anti-cancer drug development.

**Key Words:** AGS, Cell proliferation, Gastric cancer, *Ginkgo biloba Leaf* Extract (GBE)

## INTRODUCTION

Gastric cancer is a malignant tumor of the stomach and has been the most common cancer in Korea for many years (Sasako, 2019). It is caused by repeated gastric mucosal damage due to stress, carcinogens or *Helicobacter pylori* infection (Rabanal, 2018; Youn et al., 2019; Waddingham et al., 2021). The incidence of gastric cancer is increasing every year due to changes in eating habits worldwide, and there are a number of active studies following this trend (Rawla and Barsouk, 2019; Wang et al., 2020). Anti-cancer drugs and chemotherapy are current treatment options for stomach cancer, but there are side effects associated with these (Filho et al., 2021). Recovery from surgical treatment is difficult and the risk of recurrence is high (Tu et al., 2019). Therefore, new targeted drugs with reduced risk of side effects are required to minimize the burden on patients. Natural compounds can be novel targets in cancer treatment. *Ginkgo biloba Leaf* Extract (GBE) has anti-oxidant and anti-cancer effects by impacting the survival rate of melanoma cells (Barth et al., 2021; Liu et al., 2021). In recent studies,

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GBE was reported to have anti-tumor, anti-inflammatory and anti-aging effects and clinical research also indicates a role in regulating memory disorders (Tian et al., 2019b; Oliveira et al., 2020). This extract, derived from the Chinese Ginkgo biloba tree, is a popular supplement around the world. It is easily soluble in water, which makes it available to process into various nutritional supplements. It has also been used as a therapeutic agent for cerebrovascular diseases and dementia (Yihao et al., 2021; Yu et al., 2021). Recently, various studies have identified an anti-tumor effect of GBE on melanoma and breast cancer (Liu et al., 2021). Here, we investigated the anti-cancer effect of GBE on gastric cancer cell line. We observed reduced cell proliferation after GBE treatment. GBE also modulated the expression of tumor suppressor and inflammation-related genes. We further assessed the therapeutic mechanism of GBE by monitoring regulation of AKT target genes. These results suggest GBE can be a potent therapeutic target for gastric cancer.

## MATERIALS AND METHODS

### Cell culture and *Ginkgo biloba Leaf* Extract preparation

We purchased cancer cell lines, AGS, TC-1, B16F10 and Ovcar3, from American Type culture collection (ATCC, Rockville, MD, USA).

Cancer cells were cultured in RPMI1640 (Welgene, Korea), Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Corning Cellgro, USA), 1% antibiotics (Invitrogen) and Trypsin EDTA (Sigma) and maintained at 37℃ in a humidified incubator with a 5% $CO_2$ atmosphere. Cancer cells were seeded in 6-well plates and treated with GBE (0~100 μg/mL). *Ginkgo biloba Leaf* Extract was purchased from Korea plant Extract bank (Cheongju, Korea).

### Cell proliferation analysis

Cancer cells (AGS, TC-1, B16F10, and Ovcar3) were seeded in 96-well culture plates at a density of $3\times10^3$ cells/well. After 24 h, cancer cells were treated with GBE for 24 h. WST plus-8 cell proliferation assay reagent (GenDEPOT, TX, USA) was added to each well. Cell viability was determined using ELISA reader at 450 nm.

**Table 1.** Primer lists and sequence for RT-PCR

| Primer | Sequence (5' to 3') |
|---|---|
| β-actin | Forward: CCAGTTGGTAACAATGCCATGT |
| | Reverse : GGCTGTATTCCCCTCCATCG |
| TGFβ | Forward: CGTCAGCCGATTTGCTATCT |
| | Reverse : CGGACTCCGCAAAGTCTAAG |
| TNFα | Forward: ATGAAAGTCTCTGCCGCCCTCA |
| | Reverse : TCCTTGGCAAAACTGCACCT |
| p53 | Forward: GGCCCACTTCACCGTACTAA |
| | Reverse : GTGGTTTCAAGGCCAGATGT |
| Rb | Forward: TGTATCGGCTAGCCTATCTC |
| | Reverse : AATTAACAAGGTGTGGTGG |
| Bcl-2 | Forward: CATGTGTGTGGAGAGCGTCAAC |
| | Reverse : CAGATAGGCACCCAGGGTGAT |
| Bcl-xl | Forward: CGGTACCGGCGGGCATTCAG |
| | Reverse : CGGCTCTCGGCTGCTGCATT |
| Bax | Forward: TTTGCTTCAGGGTTTCATCCA |
| | Reverse : CTCCATGTTACTGTCCAGTTCGT |
| Bcl6 | Forward: CTGCAGATGGAGCATGTTGT |
| | Reverse : TCTTCACGAGGAGGCTTGAT |
| Sod2 | Forward: CTGAGGAGAGCAGCGGTCGT |
| | Reverse : CTTGGCCAGCGCCTCGTGGT |
| GADD45 | Forward: CGTTTTGCTGCGAGAACGAC |
| | Reverse : GAACCCATTGATCCATGTAG |
| CAT | Forward: GCGAATGGAGAGGCAGTGTAC |
| | Reverse : GAGTGAGTTGTCTTCATTAGCACTG |

*Abbreviations: Beta-actin (β-actin), Transforming Growth Factor-β (TGF-β), Tumor Necrosis Factor (TNF-α), Tumor protein p53 (p53) Retinoblastoma protein (Rb), B-cell lymphoma 2 (Bcl-2), B-cell lymphoma-extra large (Bcl-xl), Bcl-2-associated X protein (Bax), B-cell lymphoma 6 (Bcl6), Superoxide Dismutase 2 (SOD2), Growth Arrest and DNA Damage-inducible 45 (GADD45), Catalase (CAT)

### Total RNA isolation, reverse transcription polymerase chain reaction

RNA was isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using a reverse transcription system (TOYOBO, Tokyo, Japan) (primers listed in Table 1). PCR was performed using instructions in Ex-Taq (TaKaRa, Kyoto, Japan) manual. Real-time PCR was performed using SYBR Premix Ex Taq (Clontech Laboratories, Mountain View, CA, USA) with ABI instruments (Applied Biosystems Inc, Foster City, CA, USA). All results were normalized by

β-actin.

### Western blot analysis

Cell lysate extractions were isolated with RIPA buffer (1% Triton X-100; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate; 150 mM NaCl; 50 mM Tris-HCl, pH 7.5; and 2 mM EDTA, pH 8.0). Cell lysates were incubated for 20 min on ice and centrifuged at 4℃ for 25 min at 13,200 rpm. Supernatant concentration was measured with protein assay reagent (Thermo Scientific, MA, USA). Protein samples were loaded into wells of the SDS-PAGE gel and transferred to PVDF or nitrocellulose membranes (Merck Millipore, MA, USA). The membranes were blocked with 5% skim milk or BSA for 1 h at room temperature. After blocking, membranes were incubated with primary antibodies overnight at 4℃. The membranes were washed 3 times for 10 min with PBST and incubated with HRP-conjugated secondary antibodies (Bethyl Laboratories, TX, USA) for 1 h at room temperature. The membranes were washed 3 times for 10 min with PBST. The FUSION SOLO S (Vilber, Eberhardzell, Germany) was used for image detection according to manufacturer's instructions. Antibodies used were anti-β-actin, anti-p53, anti-Rb, anti-$Bcl_2$, anti-$Bclx_L$, and anti-Bax (Santa Cruz, TX, USA).

### Wound healing assay

AGS cells were seeded in 6-well plates, allowed to incubate for 24 h and treated with 50 μg/mL GBE. A straight wound was created by scratching a confluent monolayer with a pipette tip. After scratching, AGS cells were washed by 1x PBS and maintained in serum-free RPMI1640 medium.

### Statistical analysis

Statistical evaluations of the data were expressed as the mean ± SEM. Statistically significant differences between mean values for the treatment groups were analyzed by Student's *t*-tests and one-way analysis of variance (ANOVA) using GraphPad Prism software (Version 6; GraphPad Software Inc., La Jolla, CA). Data were considered statistically significant as follows: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

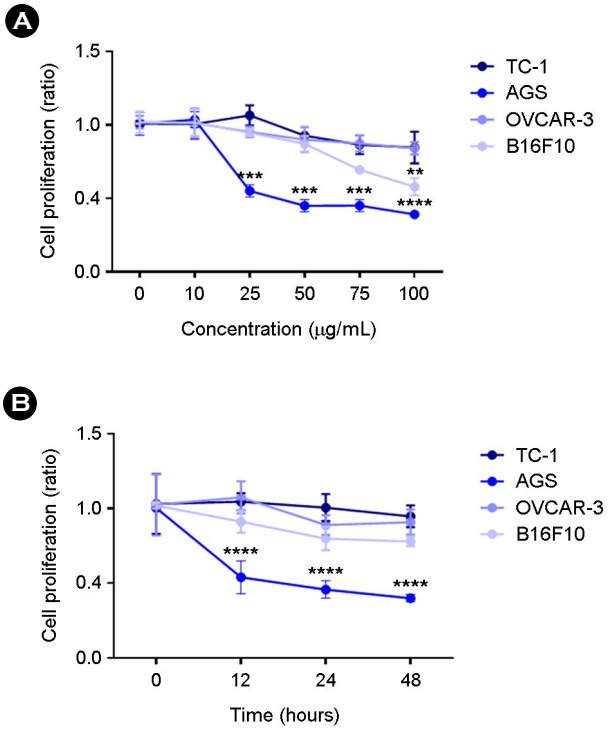


**Fig. 1.** *Ginkgo biloba Leaf* Extract inhibits cell proliferation in cancer cells. (A) WST assay exhibits that GBE regulates cell proliferation of TC-1, AGS, OVCAR-3 and B16F10 in dose dependent manner. TC-1, AGS, OVCAR-3 and B16F10 cells were treated with 0, 10, 25, 50, 75 and 100 μg/mL of GBE for 24 hr. (B) The concentration of the GBE was treated at 50 μg/mL, at 0, 12, 24, and 48 hr, we confirmed that the cell proliferation in TC-1, AGS, OVCAR-3 and B16F10 cells.

## RESULTS

### *Ginkgo biloba Leaf* Extract inhibits cancer cell proliferation

We investigated whether GBE regulates cell proliferation by treating various cancer cells (TC-1, AGS, OVCAR-3 and B16F10) with GBE in a dose-dependent manner (0~100 μg/mL). The degree of cell differentiation in AGS decreased following GBE 25 μg/mL treatment. B16F10 also showed a reducing tendency with increasing concentration, most significant (~50%) with 50 μg/mL treatment. As the concentration of GBE increased, AGS cell proliferation rate decreased (Fig. 1A). Cancer cell proliferation was observed by treatment with 50 μg/mL GBE in a time-dependent manner. Cell proliferation rate of AGS decreased in time-dependent manner. A reduction in cell differentiation was

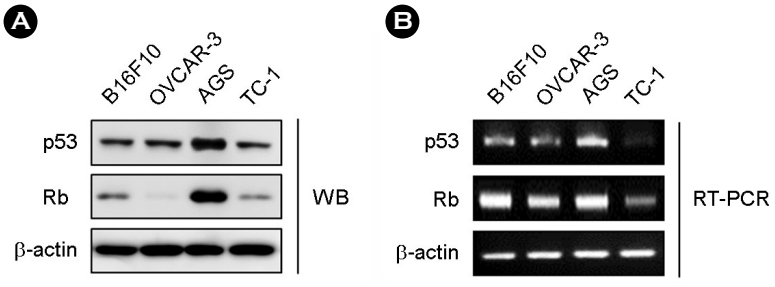


**Fig. 2.** *Ginkgo biloba Leaf* Extract regulates cancer suppressor genes in gastric cancer cell line. (A) Protein levels of p53, Rb in TC-1, AGS, OVCAR-3 and B16F10 cells after treated with 50 μg/mL at 24 hr detected by western blotting. (B) RNA levels were measured with RT-PCR in p53, Rb in TC-1, AGS, OVCAR-3 and B16F10 cells after treated with 50 μg/mL at 24 hr. β-actin used as control.

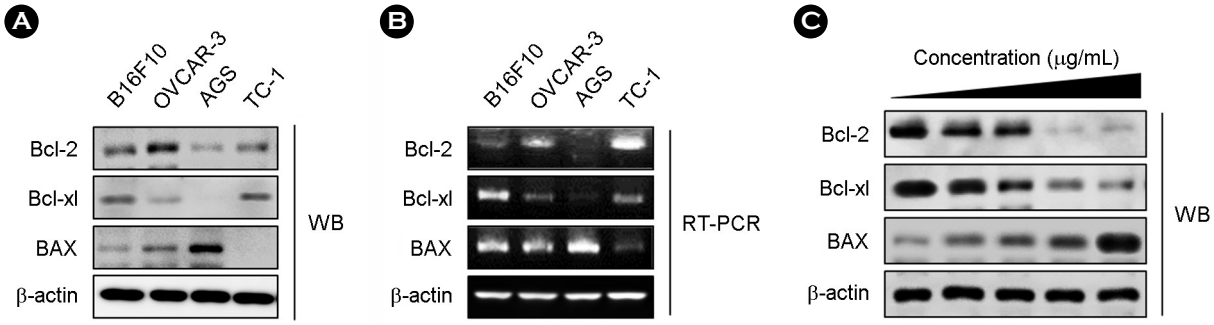


**Fig. 3.** *Ginkgo biloba Leaf* Extract adjust apoptosis related proteins and genes. (A) Western blotting based detection of Bcl-2, Bcl-xl, Bax in TC-1, AGS, OVCAR-3 and B16F10 cells treated with 50 μg/mL. (B) RNA levels of Bcl-2, Bcl-xl, Bax in TC-1, AGS, OVCAR-3 and B16F10 cell treated with 50 μg/mL were measured by RT-PCR. (C) protein levels of Bcl-2, Bcl-xl, Bax in AGS with 0, 25, 50, 75 and 100 μg/mL. β-actin is loading control.

observed after 12 h of treatment; this phenomenon was not apparent in other cancer cells (Fig. 1B). These results indicate that GBE regulates the growth and differentiation of cancer cells. Based on the effects in AGS, a gastric cancer cell, it is evident that as GBE concentration increases, degree of cell differentiation and cell apoptosis decrease in a time-dependent manner.

### *Ginkgo biloba Leaf* Extract regulates cancer suppressor genes in gastric cancer cell line

Since it was confirmed that GBE regulates the proliferation of cancer cells, the following experiment was conducted to determine the level of protein and RNA associated with cancer. Western blotting was used to confirm the levels of tumor suppressor markers p53 and Rb. Various cancer cells were treated with 50 μg/mL GBE, and after 24 h, cells were collected to identify proteins. p53 protein was expressed in all cancer cells, most common in AGS. Rb protein was weakly expressed in B16F10 and TC-1 and strongly in AGS (Fig. 2A), similar to RNA level (Fig. 2B). These results indicate that GBE affects cancer cell growth by regulating tumor suppressor protein and RNA levels.

### *Ginkgo biloba Leaf* Extract affects apoptosis-related proteins and genes

We treated several cancer cells with 50 μg/mL GBE to identify the expression of apoptosis-related genes and identified proteins and RNA after 24 h. Anti-apoptosis-related protein was expressed in various cancer cells. Protein expression of Bcl-2 and Bcl-xl was reduced in AGS in B16F10, OVCAR3, AGS, and TC-1. On the contrary, expression of Bax, a pro-apoptosis related protein, was increased in AGS cells (Fig. 3A). This was also the case at the RNA level (Fig. 3B). Based on these results, an experiment was conducted to confirm the change in expression of apoptosis-related proteins according to GBE concentration. Cells were treated with GBE (0~100 μg/mL) and western blotting was performed after 24 h. Expression of Bcl-2 and Bcl-xl decreased as GBE concentration increased, and Bax expression also increased (Fig. 3C). These results suggest that GBE affects

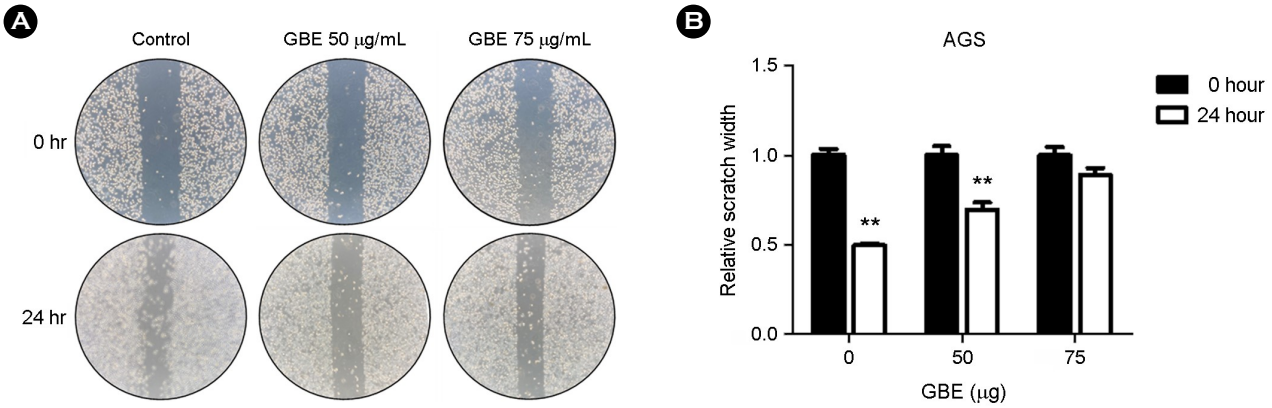


**Fig. 4.** *Ginkgo biloba Leaf* Extract regulates cell migration of gastric cancer cell line. (A-B) measurement of scratch wound healing assay in AGS treated with 50 and 75 μg/mL at 0, 24 hr.

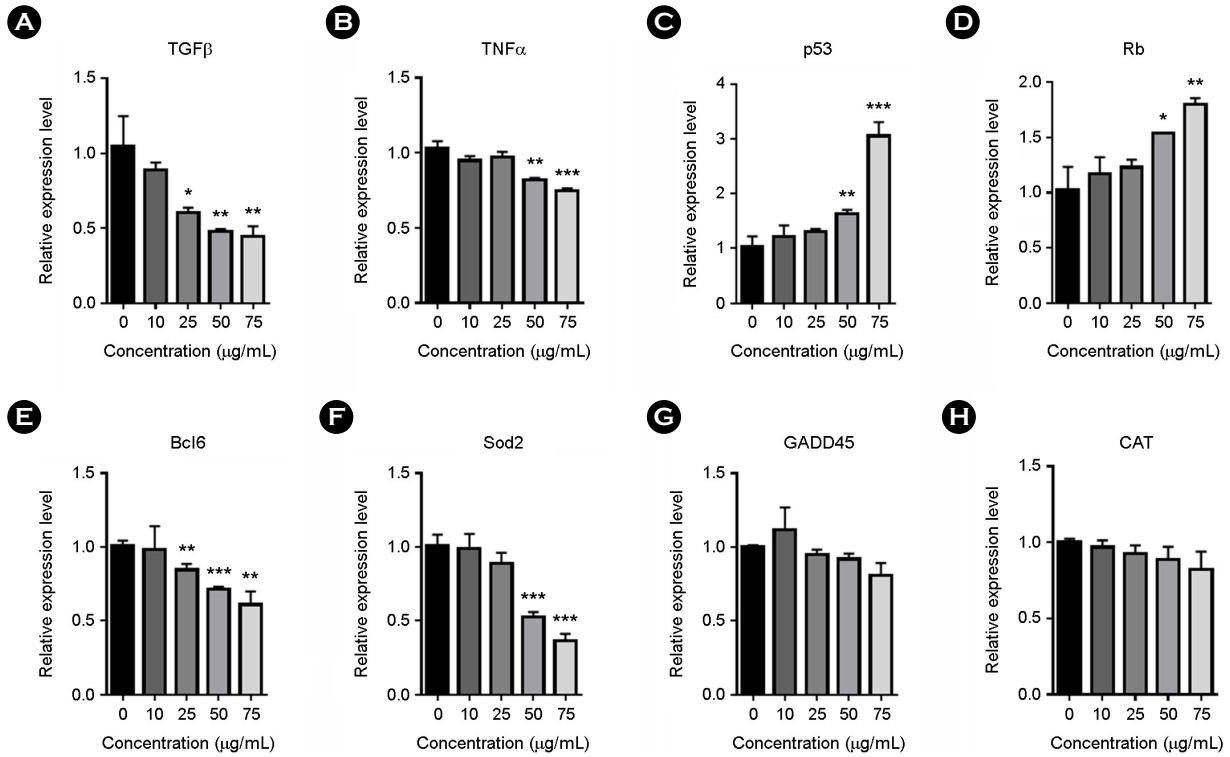


**Fig. 5.** *Ginkgo biloba Leaf* Extract regulates mitogenic related genes and inflammation related genes in AGS cells. (A-B). RNA expression of TGFβ and TNFα in AGS cells treated for 24 hr with GBE 0, 10, 25, 50 and 75 μg/mL. (C-D). qRT-PCR indicates the dose dependent effects of GBE on p53 and Rb after 24 hr. (E-H). Bcl6, Sod2, GADD45, CAT known as AKT signaling target markers measured using qRT-PCR at 24 hr in dose dependent manner. β-actin used as loading control. Significant differences are indicated by an asterisk (**P*<0.05, ***P*<0.01), and *P* values were calculated using the Student's *t* test.

cell growth and apoptosis by regulating apoptosis-related signaling within cells.

### *Ginkgo biloba Leaf* Extract regulates cell motility of gastric cancer cell line

Since we confirmed that GBE influenced the growth and

differentiation of cancer cells by regulating tumor suppressor proteins, an experiment was conducted to determine whether cell motility was also affected. AGS was seeded in a 6-well plate and treated with 50 and 75 μg/mL GBE after 24 h, and a wound healing assay was performed. After 24 h, untreated cells had a narrowing clearance in half response. In addition, the scratch gap of GBE-treated cells was wider than that of untreated AGS cells. The same was true at 50 and 75 μg/mL (Fig. 4A, 4B). After 48 h, GBE-treated cells induced cell death. These findings confirmed that GBE inhibits cancer cell metastasis by reducing cell migration in gastric cancer cells.

**Inflammation-related genes and mitogenic signaling target genes are downregulated by *Ginkgo biloba Leaf* Extract in AGS**

Since the anti-cancer effect of GBE was specifically exhibited in AGS cells, expression change of inflammation-related genes was examined. After GBE treatment (0~75 μg /mL), cells were collected and experiments were conducted by identifying RNA. When changes in expression of TGFβ and TNFα, well-known inflammatory genes, were observed, gene expression was confirmed to decrease as GBE concentration increased (Fig. 5A, 5B). It was verified that GBE had an anti-inflammatory effect, as expression of the GBE inflammatory gene decreased according to the concentration. In addition, to validate the anti-cancer effect, changes in p53 and Rb expression were investigated. As the concentration increased, the expression of p53 and Rb genes increased together, suggesting an anti-cancer effect of GBE on AGS cells (Fig. 5C, 5D). Therefore, experiments were conducted to determine the molecular mechanisms by which GBE had anti-cancer and anti-inflammatory effects on AGS through regulation. We demonstrated the change in expression of target genes of AKT signaling among several cell signaling pathways, known to have a negative effect on the growth and differentiation of cancer cells. Changes in expression of Bcl6, sod2, GADD45 and CAT were observed. Bcl6 and sod2 expression were found to decrease with increasing GBE concentration, and there was no change in expression of GADD45 and CAT (Fig. 5E, 5F, 5G, 5H). These results showed that GBE inhibits cancer cell growth and differentiation by regulating AKT signaling and has anti-cancer effects.

## DISCUSSION

Natural plant extracts have traditionally been used as medicinal ingredients for various diseases (Guerra et al., 2018). *Ginkgo biloba Leaf* Extract (GBE) is a natural drug with few chemical side effects known to be effective in improving brain function and expanding blood vessels (Tian et al., 2019a; Barth et al., 2021). GBE contains a substance called flavonoid, which acts as an inhibitor of platelet-activating factor (PAF) and has various medicinal effects as an infectious phospholipid. It appears to reduce cancer cell proliferation and metastasis by inhibiting PAF and most likely regulates various cell death-associated proteins. Studies have shown that GBE exhibits antioxidant and anti-inflammatory effects, and controls the expression of genes responsible for regulating cell growth and division (Oliveira et al., 2020; Yalcin et al., 2020). Recent research has revealed that cancer cells have the ability to inhibit cell growth while being involved in regulating cell signals that affect cell differentiation (Guo et al., 2015; Kim et al., 2021).

Gastric cancer is caused by genetic and environmental factors and DNA damage. Chemotherapy or surgical treatment is used to induce cell death, but mortality is still rising (Chue et al., 2020; Lerner and Llor, 2020). Cancer cells are malignant cells characterized by infinite and invasive proliferation which infiltrates other organs. According to these characteristics, anti-cancer drugs are used to reduce proliferation rate and metastasis by inhibiting the cell cycle or inducing cell death (Sasako, 2019). Existing chemical-based anti-cancer drugs induce cancer cell death, but at the same time induce the death of normal and immune cells that perform defense functions (Lyons et al., 2017). Therefore, the effects of natural drugs that can improve treatment outcomes while weakening cell damage have recently been in the spotlight (Choi et al., 2018). Research is being actively conducted on plant extracts with anti-inflammatory and anti-cancer effects (Lafferty, 2018; Kim et al., 2020). Several studies have indicated that GBE, a natural plant extract, is effective in solid cancer; however, research on the specific

mechanisms for regulating gastric cancer cell differentiation inhibition are insufficient.

This study was conducted to determine whether GBE is involved in differentiation and growth of cancer cells, to reduce side effects and induce effective inhibition of cancer cell proliferation. Among the various cancer cell types, AGS specifically exhibited the effect of GBE. It can be inferred that this effect is caused by gastric cancer cells with high specificity to the GBE component. We confirmed that GBE downregulates apoptosis of AGS, a gastric cancer cell line. Thus, we verified the expression of p53 and Rb tumor suppressor genes, and observed that their expression increased. In addition, the protein and gene expression of Bax, apoptosis marker, was elevated after GBE treatment. Conversely, the expression of anti-apoptosis markers Bcl-2 and Bcl-xl decreased. Therefore, GBE was confirmed to induce apoptosis in AGS. In addition, a wound healing assay was conducted to check whether the extract affected cancer cell migration. It was found that the scratch gap did not decrease compared to the control, which indicated that cell migration decreased after GBE treatment. In addition, we observed the mitogenic signaling pathway to control the anti-cancer effect of GBE. AKT signaling pathway is known to promote cell growth and differentiation as a phosphorylase (Roszak et al., 2017; Li et al., 2018). Excessive AKT activity induces infinite cell proliferation and division, leading to cancer; many studies have validated AKT as a cancer-related gene (Han et al., 2018). Expression of AKT target genes Bcl6, Sod2, CAT, and GADD45 were found to decrease. A molecular mechanism of the anti-cancer effects of GBE was thereby observed. However, there is a limitation in that the expression change of downstream AKT proteins was not confirmed. As a result, AKT was found to be the most controlled by GBE compared to other signaling pathways. In addition to AKT target genes, Mapk-related genes were discovered. Further experiments regarding Mapk-related protein expression levels should be conducted in future. Experiments on the downstream signaling mechanism of AKT related to GBE should also be progressed. Moreover, protein levels of cell signaling substances should be identified and additional apoptosis monitoring conducted through FACS analysis. Therefore, we would like to perform an *in vitro* mechanism study of GBE and investigate relevant cell signaling pathways.

In conclusion, GBE downregulates cell proliferation and migration of AGS cells. We demonstrated that GBE inhibits cell growth associated with apoptosis-related genes and the AKT cell signaling pathway. Therefore, GBE can play an important role in regulating the canister progression of gastric cancer and we suggest GBE as a potential future therapeutic target.

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**CONFLICT OF INTEREST**

No potential conflict of interest relevant to this article was reported.

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