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Research article

Myricetin induces apoptosis and autophagy in human gastric cancer cells through inhibition of the PI3K/Akt/mTOR pathway

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ABSTRACT

Myricetin, a natural flavonoid present in berries, nuts, and green tea, is well-known for its anticancer properties. Even though several previous studies have reported the anticancer effects induced by myricetin, these effects have not yet been confirmed in the adenocarcinoma gastric cell line (AGS). Moreover, the exact mechanisms of myricetin-induced apoptosis and autophagy have not been clearly identified either. Therefore, in this study, we aimed to examine the role of myricetin in inducing apoptosis and autophagy in AGS gastric cancer cells. First, the survival rate of AGS gastric cancer cells was assessed using the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) cell viability assay. Thereafter, the rate of apoptosis was analyzed using4′,6-diamidino-2-phenylindole (DAPI) staining as well as annexin V and propidium iodide (PI) staining, and the expression of the proteins associated with apoptosis, PI3K/Akt/mTOR pathway, and autophagy was examined by western blotting. We observed that myricetin reduced the survival rate of AGS gastric cancer cells by inhibiting the PI3K/Akt/mTOR pathway, thereby inducing apoptosis and autophagy. Similar results were also obtained *in vivo*, and tumor growth was inhibited. Therefore, in the AGS gastric cancer cells, myricetin seems to inhibit the PI3K/Akt/mTOR pathway, which in turn leads to apoptosis *in vitro*and *in vivo*, cell-protective autophagy, as well as inhibition of cancer cell proliferation. These results indicate the potential of myricetin as a natural anticancer agent.

1. Introduction

Gastric cancer affects more than 1 million people around the world every year, and statistically, its incidence rate in humans (from birth to 74 years of age) is higher in men (1.87%) than in women (0.79%). Gastric cancer originates in the inner mucous membrane of the stomach, from where the cancer cells penetrate the stomach wall and metastasize to other organs, thereby showing rapid growth (Rawla and Barsouk, 2019). Since existing anticancer drugs have adverse effects on normal cells, there is ongoing research for anticancer drugs that can be made from natural products with proven safety (Lichota and Gwozdzinski, 2018).

Myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone) is a flavonoid that is commonly found in foods, such as berries, spinach, nuts, black tea, wine, and green tea. Recent pharmacological studies have revealed that myricetin has antioxidant effects, and that it can reduce hyperglycemia and improve insulin function (Ong and Khoo, 1997; Ross and Kasum, 2002; Weng and Yen, 2012; Sun et al., 2012). Moreover, its ability to function as an anticancer agent by inducing apoptosis of cancer cells has been identified in case of pancreatic, liver and colon cancers (Phillips et al., 2011; Li et al., 2019a, b; Cho and Choi, 2015). Nevertheless, the effect of myricetin on the adenocarcinoma gastric cell line (AGS) has not yet been identified.

Apoptosis is a type of programmed cell death characterized by cell contraction, condensation of the nucleus, and formation of bubbles in the cell membrane. Inhibition of apoptotic pathways may lead to the transformation of mutated cells into cancerous cells, thereby making apoptosis a representative anticancer mechanism (Elmore, 2007). Protein kinase B (Akt) is a serine/threonine kinase, which is activated by the signaling

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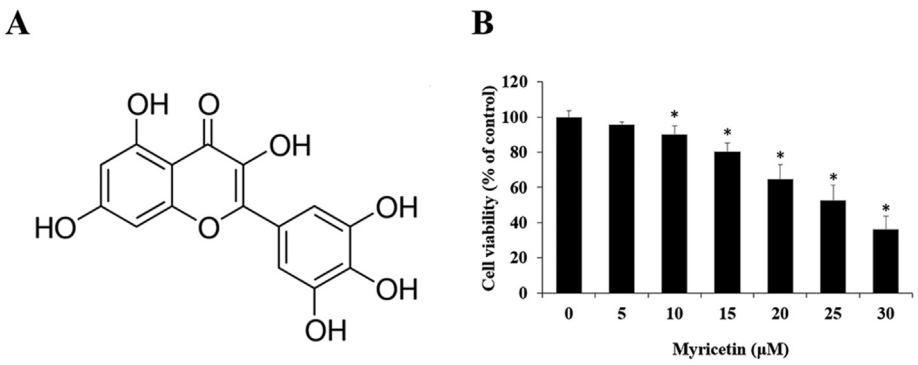

Figure 1. (A) The chemical structure of myricetin. (B)Myricetin reduces the viability of the AGSgastric cancer cell. The AGSgastric cancer cell viability was measured by MTT assay after treatment with different concentrations of myricetin (0, 5, 10, 15, 20, 25, and 30 μM) for 24 h. The results are representative of three independent experiments. $*p < 0.05$ vs. untreated control group.

pathway triggered by phosphoinositide 3-kinase (PI3K), an upstream kinase, and along with PI3K, it plays a key role in cell survival and proliferation (Chan et al., 2014; Markman et al., 2013). Akt can phosphorylate and activate the mammalian target of rapamycin (mTOR) protein (LoPiccolo et al., 2008), which, in turn, can promote cell growth by integrating signals from growth factors when there is a sufficient supply of nutrients and energy (Zou et al., 2020). An abnormal increase in the activity of the PI3K/Akt/mTOR pathway is associated with various malignancies, including gastric cancer. Therefore, modulation of the PI3K/Akt/mTOR signaling pathway may be a key to cancer treatment (Singh et al., 2015; Almhanna et al., 2011; Osaki et al., 2004).

Autophagy occurs prior to apoptosis under stressed conditions, and it generally blocks the induction of apoptosis by inhibiting caspase activity. However, excessive degradation of the cytoplasm by autophagy may lead to cell death along with apoptosis (Ouyang et al., 2012). When autophagy is induced within a cell, autophagosome are formed, and their fusion with lysosomes to form autolysosomes (Li et al., 2019a, b). These autolysosomes play an important role in cell survival by decomposing or recycling old proteins or cytoplasmic materials. The formation of the autophagosomes is affected by beclin 1 and the conversion of the microtubule-associated protein 1A/1B-light chain 3 (LC3)-I to LC3-II after binding to phosphatidylethanolamine (PE) (Runwal et al., 2019).

This study investigates whether the proliferation of AGS gastric cancer cells is inhibited by myricetin-mediated apoptosis both in vitro and in vivo. Furthermore, this study attempts to identify the role of the PI3K/Akt/mTOR pathway in the induction of apoptosis as well as determine the relationship between the induced apoptosis and autophagy.

2. Materials and methods

2.1. Materials

Myricetin (Figure 1A) used in this experiment was purchased from Sigma-Aldrich (St. Louis, MO, USA). The human gastric cancer cells (AGS) were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). The Roswell Park Memorial Institute (RPMI) -1640 medium was purchased from Welgene (Gyeonsan, Korea), and fetal bovine serum (FBS) and penicillin were purchased from Gibco (Grand Island, NY, USA). The 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT), 4′,6-diamidino-2-phenylindole (DAPI), and acridine orange were purchased from Sigma-Aldrich, and fluorescein isothiocyanate (FITC)-annexin-V detection kit was purchased from BD Pharmingen™ (San Diego, CA, USA). Primary and secondary antibodies including anti-B cell lymphoma 2 (Bcl-2), anti-Bcl-2 associated X (Bax), anti-polyadenosine diphosphate-ribose polymerase (PARP), anti-p-Akt, anti-p-mTOR, anti-light chain 3 (LC3),anti-beclin 1andLY294002 were purchased from Cell Signaling (Beverly, MA, USA). Anti-β-actin was purchased from Santa Cruz Biotechnology Inc. (Finnell St, Dallas, USA), and anti-p-PI3K was purchased from Abcam (Cambridge, UK).3-MAwaspurchasedformMedChemExpress (Monmouthjuncion,NJ,USA).

2.2. Cell culture

The AGS gastric cancer cells were initially grown in the RPMI-1640 medium supplemented with 5% FBS and 1% streptomycin/penicillin. The cell culture was maintained in an incubator at 37°C and 5% CO_2. The culture medium was changed every two to three days. When the cells occupied more than 70% of the 75 cm^2 flask, they were washed twice with phosphate buffered saline (PBS, pH 7.4), and then sub-cultured after treating with trypsin-ethylenediamine tetraacetic acid (EDTA).

2.3. MTT assay

The AGS gastric cancer cells were dispensed at 2×10^4 cells/mL in a 96-well plate for cell culture and incubated for 24 h, such that the cells could attach to the culture plate. Thereafter, the cells were treated with varying concentrations of myricetin (0, 5, 10, 15, 20, 25, and 30 μM) for 24 h. Subsequently, the culture medium was removed, and 40 μL of MTT solution was added. The MTT solution was then removed after a reaction time of 1 h and 30 min. Finally, the cells were treated with 100 μL of dimethylsulfoxide (DMSO), and the absorbance was measured at 595 nm with an enzyme linked immunosorbent assay (ELISA) reader (Bio-RadLaboratories Inc., Hercules, CA, USA).

2.4. DAPI staining

The AGS gastric cancer cells were dispensed into a 60 mm dish at 1×10^5 cells/mL, incubated for 24 h, and then treated with myricetin at concentrations of 0, 15, and 25 μM for 24 h. Thereafter, the medium to which myricetin was added was removed, the cells were washed three times with PBS, and fixed with 4% paraformaldehyde solution at room temperature for 15 min. Subsequently, the cells were washed three times with PBSagain, 2 mL of DAPI reagent was added, and the cells were allowed to react with the stain in the dark for 1 min. Finally, the changes in the shape of the nuclei were observed under a fluorescence microscope (Zeiss Fluorescence Microscope, Thornwood, NY, USA), and quantitation was expressed as a percentage by counting the DAPI-positive cells among the total number of cells on a screen of the same size.

2.5. Acridine orange staining

The AGS gastric cancer cells were dispensed into a 60 mm dish at 1×10^5 cells/mL, incubated for 24 h, and then treated with myricetin at

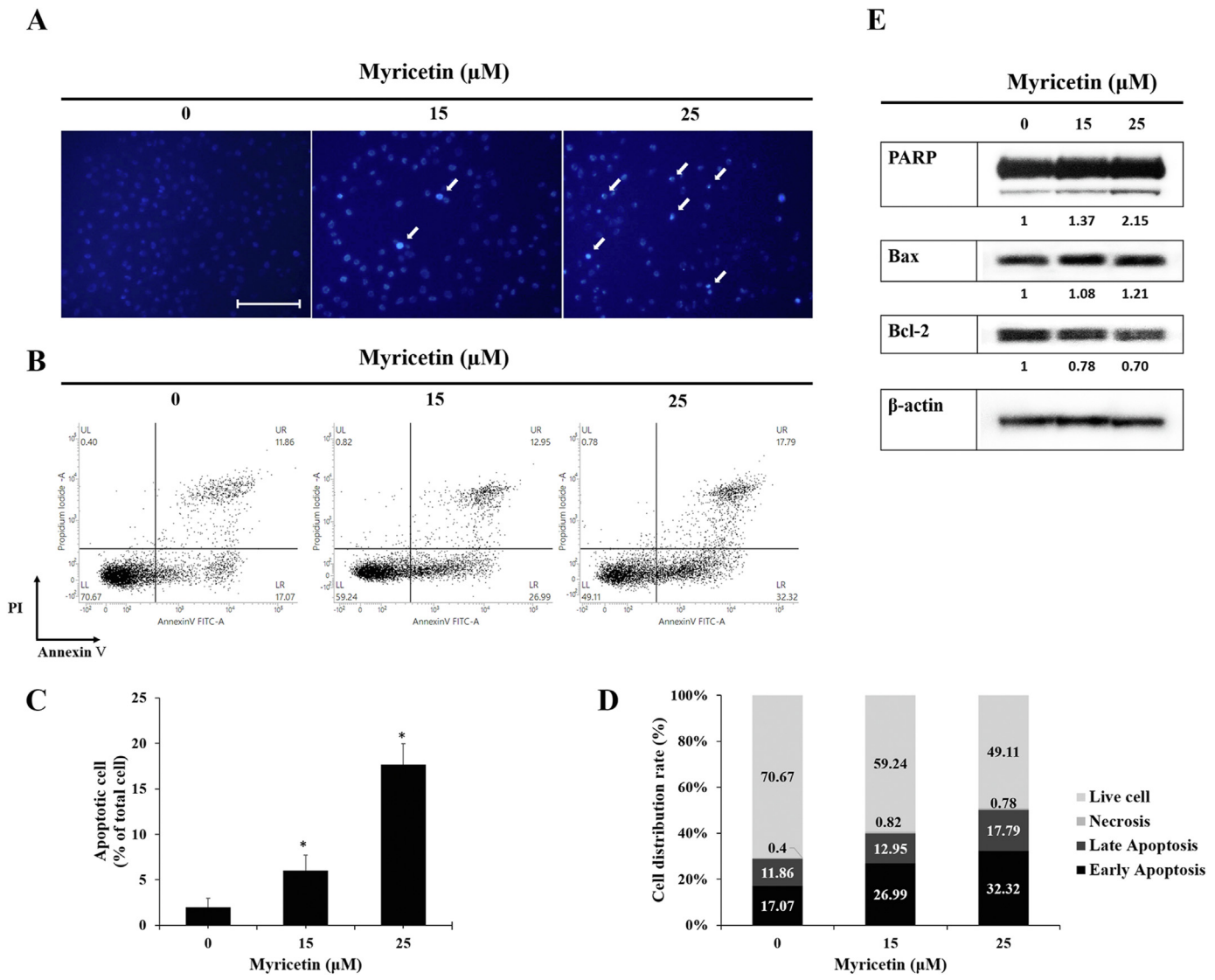

Figure 2. Myricetin induces apoptosis in the AGSgastric cancer cell. (A) The AGSgastric cancer cells were treated with myricetin (0, 15, and 25 μM) for 24 h, and the cells were subsequently stained with DAPI. The arrows indicate chromatin condensation in AGSgastric cancer cells. Scale bar, 10 μm. (B) The AGSgastric cancer cells were treated with 0, 15, and 25 μM doses of myricetin for 24 h, and the rate of cellular apoptosis was assayed by flow cytometry.(C)The bar graph represents the apoptotic bodies (% of total cells).(D)The bar graph represents the cell distribution rate.(E) The AGSgastric cancer cell was treated with myricetin (0, 15, and 25 μM) for 24 h, and the cells were harvested to measure the levels of PARP, Bax and Bcl-2proteins by western blotting. The results are representative of three independent experiments. $^*p < 0.05$ vs. untreated control group.

concentrations of 0, 15, and 25 μM for 24 h. After discarding the medium, the cells were washed three times with PBS, and fixed with 4% paraformaldehyde solution for 15 min. Thereafter, they were washed twice with PBS, stained with acridine orange, and observed under a fluorescence microscope (Zeiss Fluorescence Microscope) to identify the acidic vesicular organelles (AVOs).

2.6. Annexin V and propidium iodide (PI) staining

After culturing AGS gastric cancer cells in a 75 cm^2 flask, they were treated with myricetin at concentrations of 0, 15, and 25 μM for 24 h. Subsequently, the cells were suspended in trypsin-EDTAand then centrifuged at 1,200 rpm for 5 min at 4 °C to collect pellets. The suspension was adjusted to 1 × 10^6 cells/mL by using the 1×binding buffer; thereafter, FITC-conjugated annexin V and phycoerythrin-conjugated PI were added, the suspension was incubated for 15 min, and finally the number of apoptotic cells were analyzed with a flow cytometer.

2.7. Western blot analysis

After culturing the AGS gastric cancer cells in a 75 cm^2 flask, they were treated with myricetin at concentrations of 0, 15, and 25 μM for 24 h. Thereafter, the cells were suspended in trypsin-EDTA and then centrifuged at 1,200 rpm for 5 min at 4 °C to collect pellets. Subsequently, the proteins were isolated by centrifugation in a cell lysis buffer (Invitrogen Corp., Carlsbad, CA, USA); the extracted proteins were quantified by the Bradford protein assay (Bio-Rad Laboratories Inc.). The proteins were separated according to size by using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane (Bio-Rad Laboratories Inc.), and blocked using 5% skim milk. Thereafter, the primary antibody was added and incubated overnight. Next, the membrane was washed and the secondary antibody was added and allowed to react at room temperature for 2 h. Finally, the results of the Western blot analysis were confirmed using enhanced chemiluminiscence (ECL) detection reagents (Pierce, Rockford, IL, USA). The AGS cells were harvested to measure the levels of

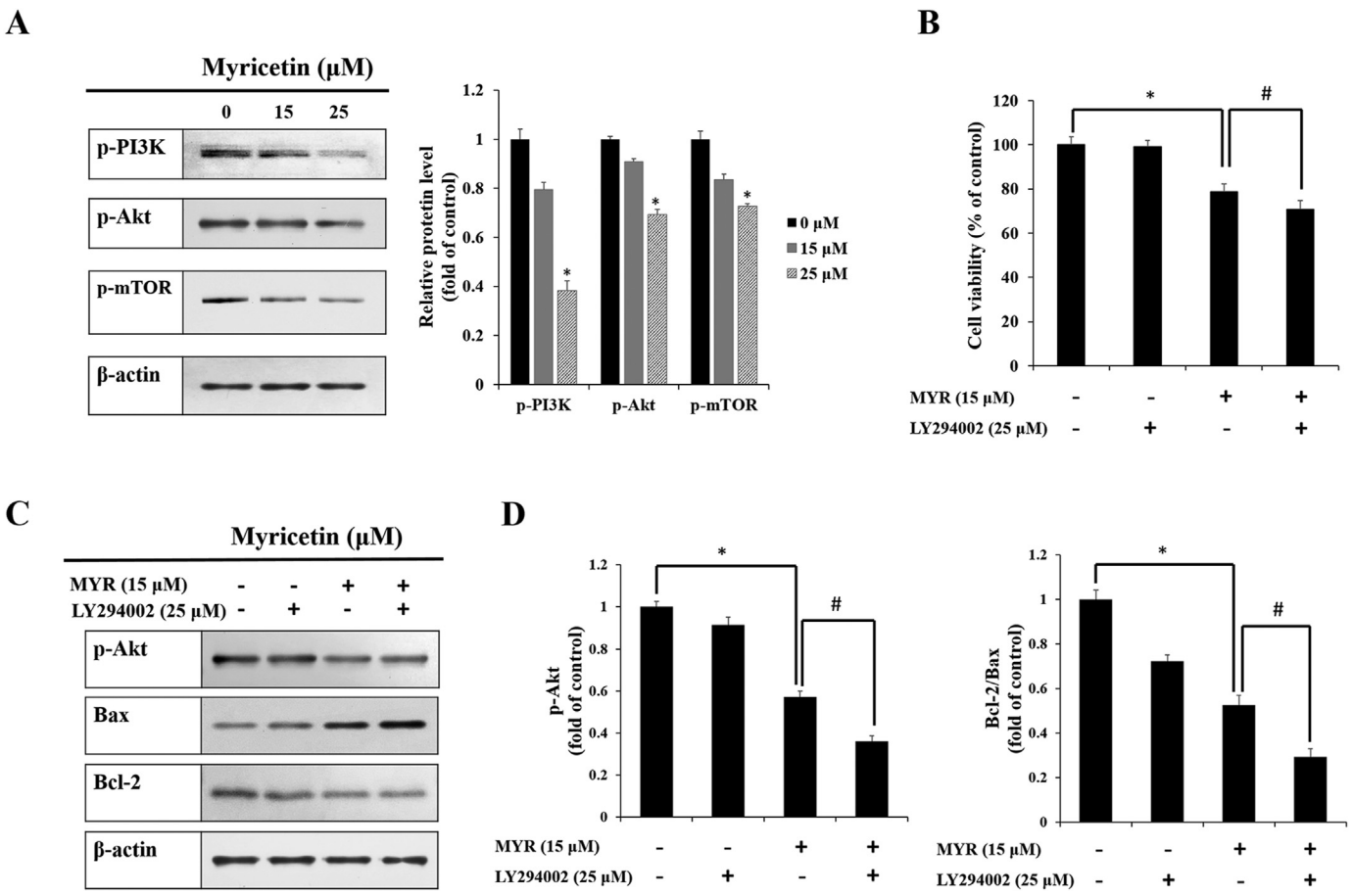

Figure 3. Myricetin induces apoptosis via inhibition of the PI3K/Akt/mTOR pathway in the AGSgastric cancer cell.(A) The AGSgastric cancer cells were treated with myricetin (0, 15, and 25 μM) for 24 h, and the cellular lysates were subjected to western blotting with p-PI3K, p-Akt and p-mTOR. (B) The AGSgastric cancer cell viability was measured by the MTT assay. The AGSgastric cancer cells were pretreated with PI3K inhibitor LY294002 (25 μM) for 2 h, followed by myricetin treatment (0 and 15 μM) for 24 h. (C) and (D) The AGS cells were harvested to measure the levels of p-Akt, Bax and Bcl-2proteins by western blotting. The blots were also probed with β-actin antibody to confirm equal sample loading. The results are representative of three independent experiments. $^{*}p < 0.05$ vs. untreated control group, $^{\#}p < 0.05$ vs. treated myricetin group.

proteins by western blotting (Supplementary Figure). Protein density was measured by Image J Launcher (provided by NCBI).

2.8. Xenograft

Four-week-old female BALB/c nude mice were purchased from Nara Biotech (Seoul, Korea). All the animal experiments were approved by the Animal Care Committee of Gongju University (IACUC, KNU_2021-01), and all experimental guidelines were followed. Mice (2 groups, n: 4) were reared in 12-hour cycles of light and darkness at a controlled temperature of 23 ± 3°C and a humidity of 40 ± 10%. The AGSgastric cancer cells that were cultured in a CO_2 incubator were administered subcutaneously into the shoulder of nude miceat 5×10^6 cells/mL in PBS containing 10% FBS. When a tumor developed in the shoulder of the nude mice, the solid tumors were excised, and 2 mm cube-shaped tumor sections were implanted subcutaneously into the experimental group consisting of 8 mice. Thereafter, the mice were randomly divided into a control group and a treatment group with four mice in each group, and the latter received a daily dose of myricetin (25 mg/kg) intraperitoneally for 28 days (Wang et al., 2014). The tumor size and body weight of all the mice were measured once every 3 days, and the tumor volume was calculated using the following equation: Tumor volume (mm^3) = 0.5 × length of tumor × (width of tumor)^2.

2.9. Immunohistochemistry (IHC)

The tumors excised from the control as well as treatment groups of mice were fixed in 10% formaldehyde, embedded in paraffin, sliced to a thickness of 5 μm, and then attached to a slide; thereafter, they were sequentially deparaffinized and hydrated. Subsequently, the tissue sections were blocked with skim milk for 1 h at 37 °C, incubated with the primary p-Akt antibody at 4 °C, and the secondary antibody at room temperature for 2 h. Finally, the sections were treated with 3,3′-diaminobenzidine (DAB) solution and H_2O_2 and observed under an optical microscope (olympus microscope bx41, Tokyo, Japan).

2.10. Histological examination

Sectioned liver and kidney tissues isolated from the control as well as treatment groups of mice were deparaffinized using xylene and hydrated with alcohol. Subsequently, the tissues were stained with hematoxylin and

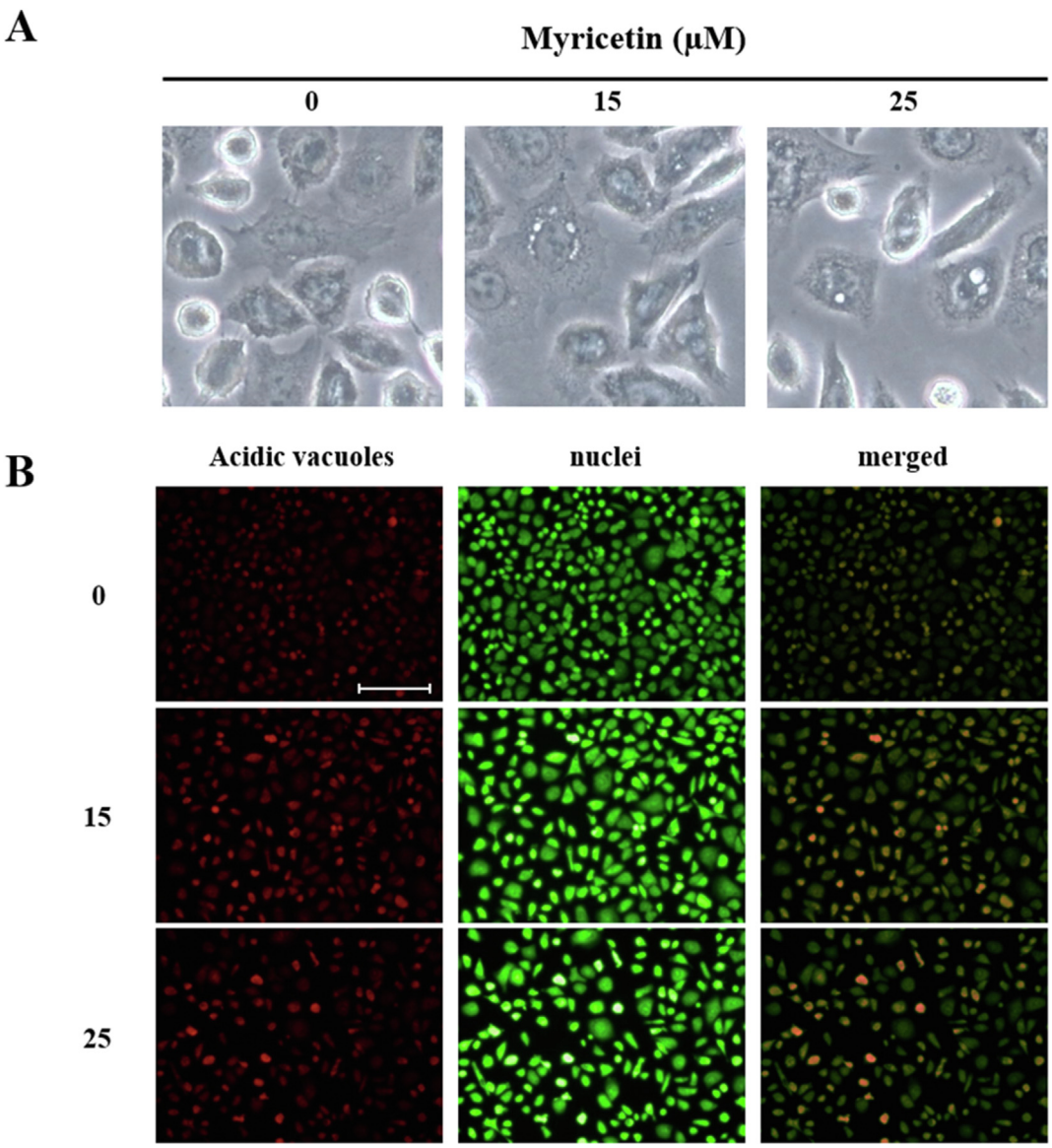

Figure 4. Myricetin effects on the morphological change in the AGSgastric cancer cell. The AGS gastric cancer cells were treated with myricetin (0, 15, and 25 μM) for 24 h. (A) Morphological changes, particularly autophagy vacuoles, were observed under a fluorescence microscope. (B) The AGSgastric cancer cells were stained with acridine orange to detect acidic vesicular organelles (AVOs) and analyzed using a fluorescence microscope. The cytoplasm and the nucleus stained fluorescent green, and the AVOs stained fluorescent red (scale bar, 10 μm).

eosin (H&E) at room temperature and observed under an optical microscope.

2.11. Statistical analysis

Statistical analysis of the results was expressed as mean ± standard deviation (SD). After the homogeneity of variance test, a one-way analysis of variance (ANOVA) was performed, and for the items for which the F-value was found to be significant, a Dunnett's *t*-test was carried out between the control group and the treatment group. The difference in the mean values for each group was determined to be significant if $*p < 0.05$.

3. Results

3.1. Myricetin reduced the viability of AGS gastric cancer cells

The MTT assay was performed to investigate the effect of myricetin on the cell viability of AGS gastric cancer cells. The results were analyzed after 24 h of treating with 0, 5, 10, 15, 20, 25, and 30 μM myricetin. The cell viability of AGS gastric cancer cells was 95.8% with 5 μM myricetin, 90.3% with 10 μM myricetin, 80.6% with 15 μM myricetin, 64.6% with 20 μM myricetin, 52.7% with 25 μM myricetin, and 36.3% with 30 μM myricetin, thereby indicating that as the concentration of myricetin increased, the viability of AGS gastric cancer cells gradually decreased as compared to that of the control group (Figure 1B).

3.2. Myricetin induced apoptosis in AGS gastric cancer cells

The nuclei of the AGS gastric cancer cells were observed post DAPI staining to determine whether the decrease in cell viability, as observed in the MTT assay, was due to apoptosis. The DNA fragments, which are one of the main characteristic features of apoptosis, stain positively with the DAPI stain and appear to glow; these are called the apoptotic bodies. We observed apoptotic bodies in the AGSgastric cancer cells treated with myricetin at concentrations of 0, 15, and 25 μM for 24 h. There was a significant increase in the number of apoptotic bodies with an increase in

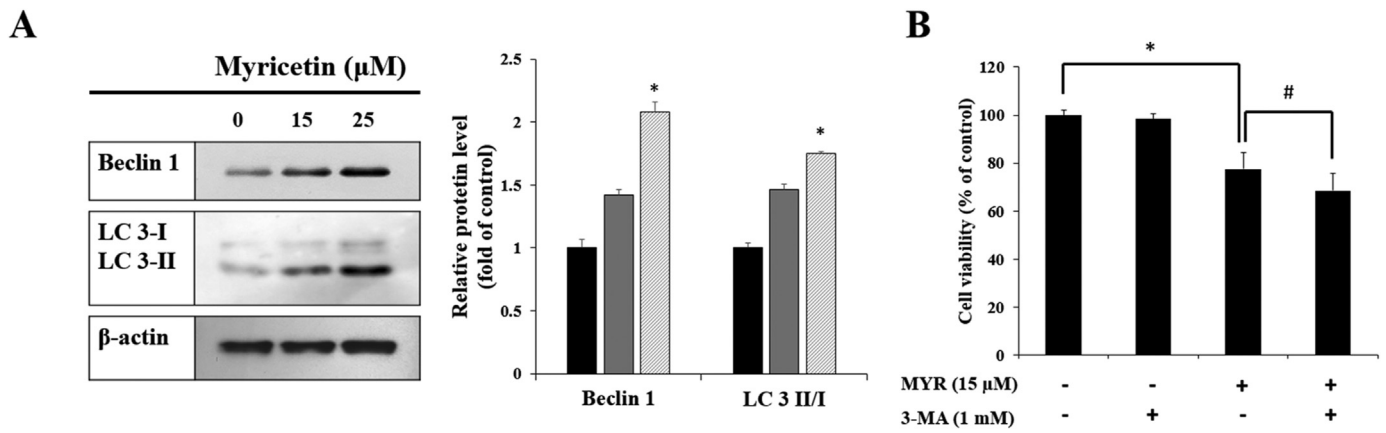

Figure 5. Myricetin induces autophagy in the AGSgastric cancer cell. (A) The AGSgastric cancer cells were treated with myricetin (0, 15, and 25 μM) for 24 h, and the cells were harvested to measure the levels of beclin 1 and microtubule-associated protein LC3 proteins by western blotting. (B) The AGSgastric cancer cells were pretreated with 3-MA, an autophagy inhibitor (1 mM), for 1 h, followed by treatment with myricetin (0 and 15 μM) for 24 h. The AGSgastric cancer cell viability was measured by MTT assay. Data are presented as mean ± standard deviation (SD); $^{*}p < 0.05$ vs. untreated control group, $^{\#}p < 0.05$ vs. treated myricetin group.

the concentration of myricetin, particularly by 2.0% in the control group, by 6.0% in the 15 μM myricetin treatment group, and by 17.7% in the 25 μM myricetin treatment group (Figure 2A and C).

For quantitative analysis of the apoptosis, which was identified by DAPI staining, the cells were stained with annexin V and PI after being treated with myricetin at concentrations of 0, 15, and 25 μM for 24 h. The number of apoptotic cells was counted by flow cytometry. The proportion of apoptotic cells increased significantly from 28.93% in the control group (0 μM myricetin) to 39.94% in the 15 μM treatment group and 50.11% in the 25 μM treatment group (Figure 2B and D). Western blot

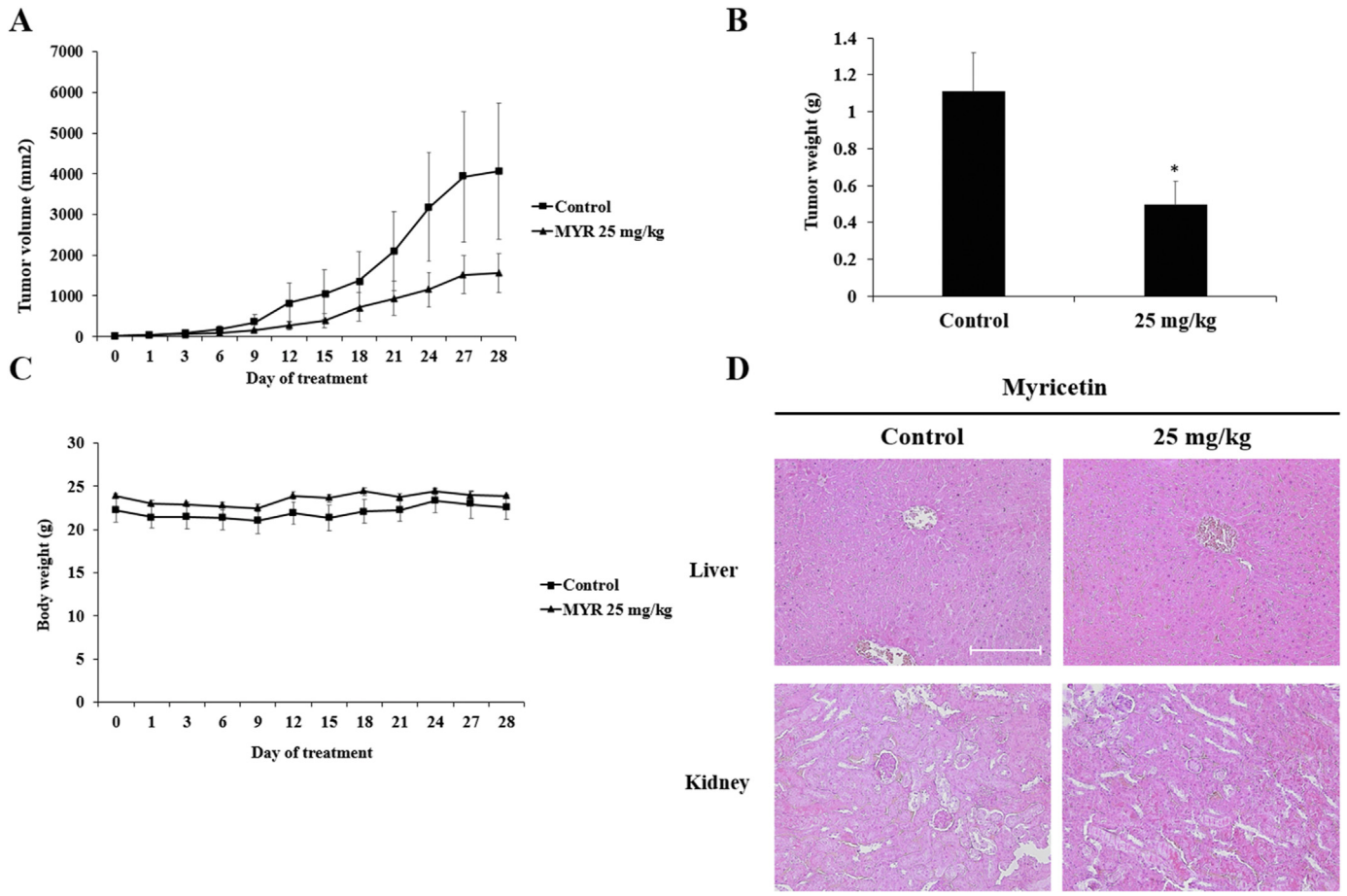

Figure 6. Myricetin inhibits tumor growth in the *in vivo*conditions. NudeBALB/c mice bearing the adenocarcinoma gastric cell line (AGS) as a xenograft were treated with myricetin (25 mg/kg) for 28 days, following which, the (A) tumor volume, (B) tumor weight, and (C) body weight were measured. The body weight of mice was measured every 3 days. Data are presented as the mean ± standard error of the mean. (D) Histological observations of myricetin-treated nude mice. The microscopic analysis of liver and kidney specimens do not show any evidence of adverse systemic toxicity following myricetin treatment. Slides were observed under a microscope (scale bar, 10 μm). Data are presented as mean ± standard deviation (SD); $^{*}p < 0.05$ vs. control group.

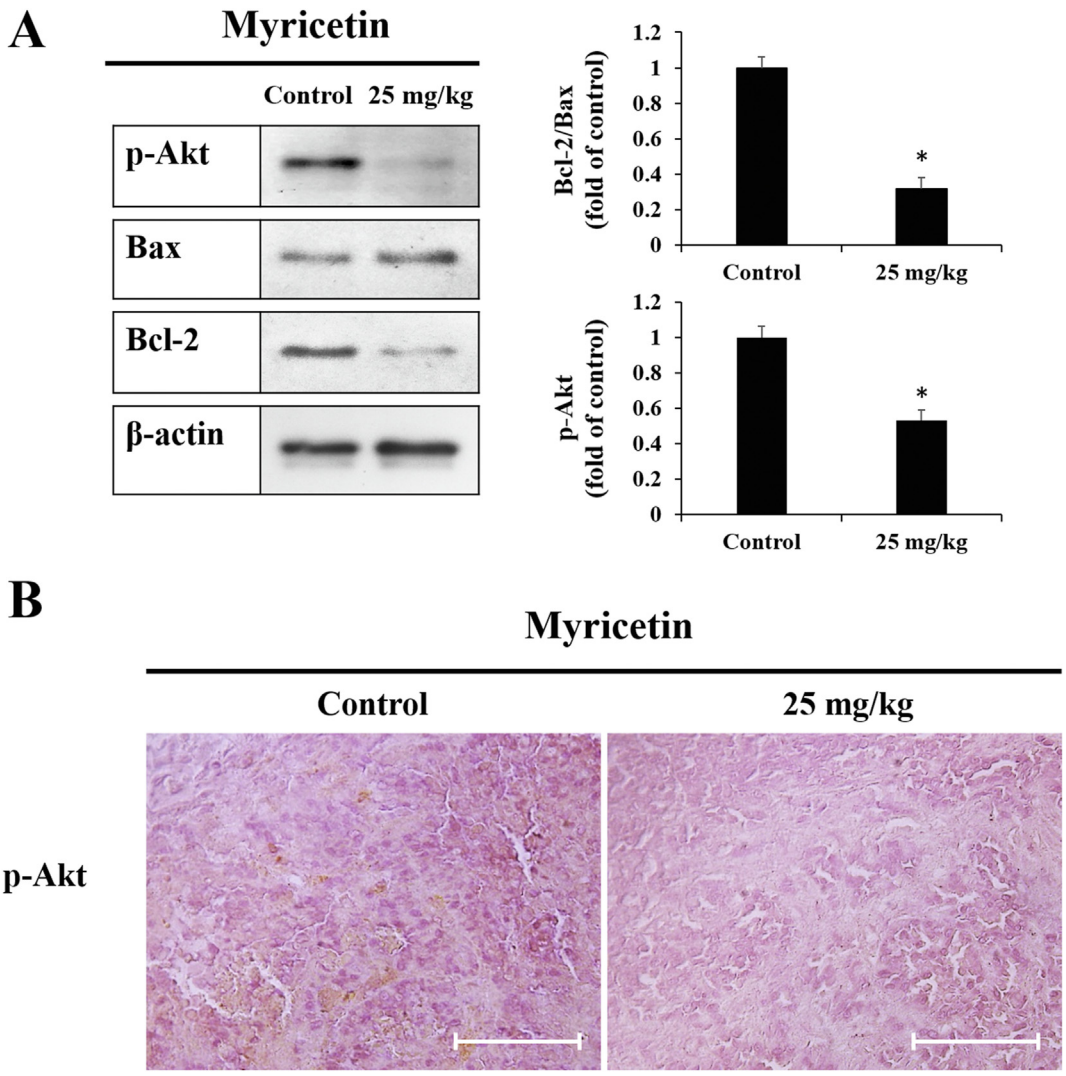

Figure 7. Myricetin induces apoptosis via Akt pathway in a xenograft model. (A) Protein expressions forp-Akt, Bax and Bcl-2 were observed by western blotting. The blots were also probed with β-actin antibody to confirm equal sample loading. (B) The p-Akt positive cells were observed under a light microscope (scale bar, 10 μm). Data are presented as mean ± standard deviation (SD); $*p < 0.05$ vs. control group.

analysis was performed to confirm the expression of apoptosis-related proteins, such as PARP, Bax, and Bcl-2, in the AGS gastric cancer cells treated with myricetin at concentrations of 0, 15, and 25 μM for 24 h. Interestingly, the expression of pro-apoptosis proteins, Bax and cleaved-PARP, had increased, while that of anti-apoptosis protein, Bcl-2, had decreased in the myricetin (15 and 25 μM) treatment groups as compared to that in the control group (Figure 2E).

3.3. Myricetin induced apoptosis by inhibiting the PI3K/Akt/mTOR pathway

To identify the apoptotic pathway activated by myricetin in the AGS gastric cancer cells, we examined the expression of proteins related to the PI3K/Akt/mTOR pathway. The expression of p-PI3K, p-Akt and p-mTOR decreased in a concentration-dependent manner in the myricetin (15 and 25 μM) treatment groups as compared to that in the control group (Figure 3A). Moreover, the AGS gastric cancer cells were pretreated with the PI3K inhibitor LY294002 25μM for 2 h to inhibit the PI3K/Akt/mTOR pathway and induce an inactivation state; subsequently, these cells were treated with 0 and 15 μM myricetin, and the cell viability and protein expression were analyzed using MTT assay and western blotting, respectively. We observed that the cell viability of the 15 μM myricetin treatment group pretreated with the PI3K inhibitor was lower than that of the cell group treated with myricetin alone (Figure 3B). Additionally, the cells in the myricetin treatment group pretreated with PI3K inhibitor exhibited a significant decrease in the Bcl-2/Bax ratio as well as the expression of p-Akt as compared to that in the group treated with myricetin alone (Figure 3C and D).

3.4. Myricetin induced cell protective autophagy in AGS gastric cancer cells

Vacuoles, which are one of the hallmarks of cells undergoing autophagy, were observed in the myricetin-treated AGS gastric cancer cells, and the number of vacuoles in the myricetin (15 and 25 μM) treatment groups was higher than that in the control group (Figure 4A). Moreover, autolysosomes were identified in the AGSgastric cancer cells using acridine orange stain, which binds to the AVOs. In the control group, very few cells stained positively with acridine orange, whereas the number of positively stained cells gradually increased with increase in the concentration of myricetin (Figure 4B).

Western blotting was performed to identify the expression of autophagy-related proteins, namely beclin 1 and LC3. The myricetin (15 and 25 μM) treatment groups showed a significant increase in the levels of beclin 1 protein as well as the LC3-II/LC3-I ratio, as compared to the control group (Figure 5A).

To investigate the correlation between myricetin-induced apoptosis and autophagy, the change in the cell viability of the AGS gastric cancer cells was measured by the MTT assay after treating the cells with 3-methyladenine (3-MA), which is an autophagy inhibitor acting at the initial stages of autophagy. We observed that the cell group treated with 1 mM 3-MA for 1 h prior to the 15 μM myricetin treatment had a lower cell viability as compared to that of the group treated with myricetin alone (Figure 5B).

3.5. Myricetin induced apoptosis of tumor cells via the Akt pathway

Xenograft was performed to determine whether the anticancer effects induced by myricetin in the *in vitro* conditions are effective *in vivo*. The experiment included a control group of mice, which were treated with 0 mg/kg of myricetin, and a treatment group of mice, which were administered 25 mg/kg of myricetin. Interestingly, in the myricetin treatment group, both tumor size and weight decreased as compared to that in the control group (Figure 6A and B). However, there was no significant difference in body weight of the mice in the control and treatment groups (Figure 6C), and H&E staining revealed that there were no histopathological differences in the liver and kidneys between the two groups (Figure 6D). Additionally, Western blot analysis was performed using the proteins extracted from the tumors. The Bcl-2/Bax ratio and the p-Akt level tended to decrease significantly in the myricetin treatment group (Figure 7A). Furthermore, IHC staining for the p-Akt protein revealed a decrease in the number of positively stained cells in the myricetin treatment group as compared to that in the control group (Figure 7B).

4. Discussion

Myricetin is a flavonoid found in berries, nuts, and green tea, and recent pharmacological studies have reported its anticancer properties, antioxidant effects, and its role in overcoming hyperglycemia as well as insulin resistance (Ong and Khoo, 1997; Ross and Kasum, 2002; Weng and Yen, 2012; Sun et al., 2012). Moreover, its anticancer effect through induction of apoptosis in cancer cells has been confirmed in case of pancreas, liver and colon cancers (Phillips et al., 2011; Li et al., 2019a, b; Cho and Choi, 2015). However, similar effects have not yet been elucidated in AGS gastric cancer cells. Therefore, an experiment was conducted to examine whether myricetin exhibits anticancer effects in AGS gastric cancer cells.

First, an MTT assay was performed to examine whether myricetin could reduce the cell viability of the AGSgastric cancer cells. We observed that the cell viability declined significantly as the myricetin concentration gradually increased from 10 to 30 μM, thereby suggesting that myricetin can decrease the cell viability of the AGS gastric cancer cells. Subsequently, the myricetin-treated AGSgastric cancer cells were subjected to DAPI staining to determine whether the previously identified decrease in the cell viability was caused by apoptosis. When apoptosis occurs, the cell membrane gets ruptured, thereby increasing the membrane permeability, and the DNA undergoes fragmentation (Degli Esposti and Dive, 2003). Therefore, in apoptotic cells, a greater amount of DAPI can penetrate the nucleus and bind to the fragmented DNA as compared to the cells in which apoptosis has not occurred. This process leads to the observation of brightly lit areas called apoptotic bodies in these cells (Carrington et al., 2017). In this experiment, we observed that the proportion of the brightly stained apoptotic bodies in the AGS gastric cancer cells increased with the increase in the concentration of myricetin (0, 15, and 25 μM). The differences in the nuclear permeability of annexin V and PI can determine whether cell death is caused by apoptosis or necrosis; where annexin V binds to the plasma membrane while PI stains the nucleus (Rieger et al., 2011). The combined results of the DAPI staining and the annexin V and PI staining suggest that the myricetin-induced reduced viability of AGS gastric cancer cells is due to apoptosis.

Subsequently, we subjected the myricetin-treated AGS gastric cancer cell lysates to western blotting and analyzed the expression of the PARP, Bax, and Bcl-2 proteins, all of which are representative proteins of apoptosis. Incidentally, PARP plays an important role in various cellular processes, such as regulation of transcription, replication, and recombination of chromatin structure, as well as DNA repair; the fragmentation of PARP occurs due to activated caspase, thereby resulting in the expression of cleaved PARP (Morales et al., 2014). Hence, the occurrence of cleaved PARP in cells is an important marker of apoptosis. Similarly, Bax is a pro-apoptosis protein that forms a permeable transition pore in mitochondria and induces apoptosis through the release of cytochrome C. On the contrary, Bcl-2 is an anti-apoptosis protein that inhibits the activity of Bax (Elmore, 2007). In this experiment, the expression of Bax and cleaved-PARP increased, while that of Bcl-2 decreased in the myricetin (15 and 25 μM) treatment groups as compared to those in the control group. These results suggest that the myricetin-induced apoptosis in AGS gastric cancer cells occurs due to modulations in the concentrations of Bax and Bcl-2.

A previous studies reported the anticancer efficacy of adenocarcinoma gastric cell lines HGC-27 and SGC7901 cells, but the PI3K/AKT/mTOR pathway in AGSgastric cancer cell was not known (Feng et al., 2015). The PI3K/Akt/mTOR pathway consists of three major proteins, namely PI3 kinase, Akt, and mTOR, and it is an important intracellular signaling pathway that regulates cell growth, survival, and migration during the progression and metastasis of cancer (Vo et al., 2013; Chang et al., 2013; Cantley, 2002). The PI3K/Akt/mTOR pathway is highly active in cancer cells, which may be because of mutations in the PI3K and Akt or the loss of PTEN activity (Engelman, 2009). Therefore, inhibition of the PI3K/Akt/mTOR pathway can play an important role in lowering the viability of cancer cells. In this experiment, the expression of p-Akt, p-PI3K, and p-mTOR in the AGSgastric cancer cells decreased in a concentration-dependent manner in the myricetin (15 and 25 μM) treatment groups as compared to that in the control group. Additionally, the group treated with the PI3K inhibitor LY294002 (25 μM) prior to the myricetin (15 μM) treatment had a decreased survival rate as compared to the survivability of the cell group treated with myricetin (15 μM) alone; moreover, the ratio of Bcl-2/Bax and the expression of p-Akt protein were significantly reduced in the former group. These observations suggest that myricetin induces apoptosis in AGS gastric cancer cells by interfering with their growth and survival via modulation of the PI3K/Akt/mTOR pathway.

Autophagy, an important recycling pathway for cells to remove old proteins and damaged organelles, proceeds sequentially through the processes of double membrane formation, maturation, fusion with lysosomes, and finally, elimination (Lamb et al., 2013; Livneh et al., 2016; Kroemer et al., 2010). The representatives of the autophagy-related proteins include LC3 and beclin 1. LC3-I binds to the PE protein present on the surface of nascent autophagosomes and gets converted to LC3-II. These autophagosomes engulf the non-functional proteins/organelles, fuse with the lysosome to form the autolysosomes, and finally degrade the engulfed particles. Incidentally, beclin 1 affects the interaction between autophagy and apoptosis (Phillips et al., 2011; Runwal et al., 2019; Mizushima and Komatsu., 2011). Therefore, LC3 and beclin 1 can be utilized as autophagy markers. In this experiment, we observed the presence of vacuoles, one of the characteristics of autophagy, as well as a high proportion of AVOs in the myricetin (15 and 25 μM) treatment group. Additionally, we analyzed the expression of autophagy-related proteins through western blotting, and found that the myricetin (15 and 25 μM) treated AGSgastric cancer cell group had a significantly higher expression of beclin 1 protein and LC3-II/LC3-I ratio as compared to the control group. Therefore, it may be suggested that the morphological changes and the increase in the levels of the autophagy marker proteins in the myricetin (15 and 25 μM) treated AGSgastric cancer cells occur in a concentration-dependent manner. When cancer cells are exposed to multiple stimuli, autophagy may occur either for cell protection or for cell death. Therefore, it is necessary to understand the

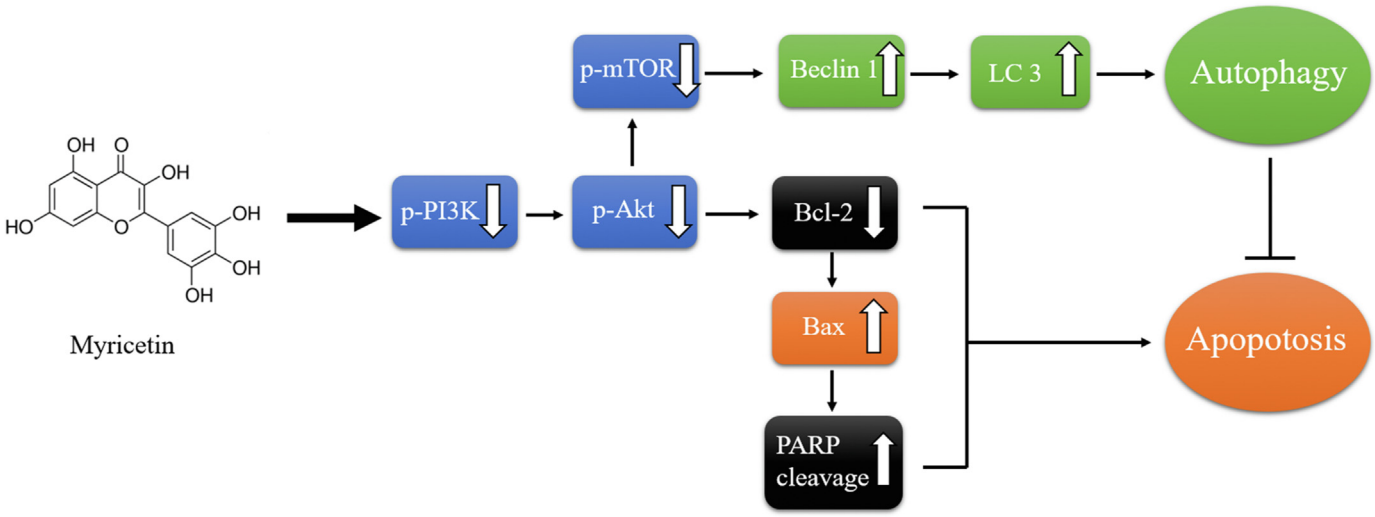

Figure 8. Proposed mechanisms of myricetin-induced apoptosis and autophagy in AGS cells.

relationship between apoptosis and autophagy by using inhibitors (Zhao et al., 2021). To confirm the relationship between autophagy and cell viability, the MTT assay was performed using 3-MA, an autophagy inhibitor, and it was observed that the cell group that was treated with 3-MA prior to the treatment with 15 μM of myricetin had reduced cell viability as compared to the group treated with myricetin (15 μM) alone. As a result, it can be inferred that autophagy is induced by myricetin in AGS gastric cancer cells, and the induced autophagy seems to play a role in protecting the cells.

With respect to the in vivo xenograft mouse models, both tumor size and weight decreased in the myricetin (25 mg/kg) treatment group as compared to those in the control group, and the decrease in the weight of the tumor was statistically significant. Since there were no significant differences between the control and treatment groups with respect to the body weights of the mice or the histopathology of their liver and kidneys, it can be suggested that in case of gastric adenocarcinoma, myricetin inhibits tumor growth in vivo without any biotoxicity. Additionally, the results of the Western blot analysis corroborated with those of the in vitro experiments, i.e., the Bcl-2/Bax ratio and p-Akt level were significantly lower in the myricetin (25 mg/kg) treatment group than in the control group. Moreover, a decrease in the number of positively stained cells with respect to the p-Akt-specific IHC staining in the myricetin treatment group as compared to that in the control group suggests that myricetin induces apoptosis through the regulation of p-Akt in the xenograft model for AGS. Furthermore, it would be necessary to conduct an experiment to determine the relationship between autophagy and an autophagy inhibitor.

5. Conclusion

Even though the anticancer properties of myricetin, a natural flavonoid, have been established in pancreatic, liver, and colorectal cancers, to the best of our knowledge, this was the first study to investigate its effects on AGS gastric cancer cells. Our study revealed that myricetin produced an anticancer effect in AGS gastric cancer cells by inhibiting signal transduction through modulation of the PI3K/Akt/mTOR pathway *in vitro* and *in vivo*; moreover, the increase in the LC3-II/LC3-I ratio and beclin 1 protein level can regulate the viability of gastric cancer cells by inducing autophagy (Figure 8). These results suggest that myricetin has a potential to be utilized as a natural anticancer agent for gastric cancer. However, there is a limitation in the lack of studies on the relationship between autophagy and apoptosis in vivo. Therefore, it is considered that such an in vivo study is necessary.

Declarations

Author contribution statement

So-Hee Han: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Jae-Han Lee: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Joong-Seok Woo: Performed the experiments.

Gi-Hwan Jung; Soo-Hyun Jung; Eun-Ji Han: Analyzed and interpreted the data.

Bumseok Kim; Jeong Seok Nam; Jeong Hwan Che; Ji-Youn Jung: Contributed reagents, materials, analysis tools or data.

Sung Dae Cho: Conceived and designed the experiments.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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