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Antitumor and Apoptosis-inducing Effects of Piperine on Human Melanoma Cells

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Abstract. *Background/Aim: Piperine is a major pungent alkaloid present in black pepper (Piper nigrum L). This study investigated the potential anticancer effects of piperine on human melanoma cells and explored the potential pharmacological mechanisms in vitro and in vivo. Materials and Methods: Studies were performed using the MTT assay, 4',6-diamidino-2-phenylindole (DAPI) staining, western blotting, a xenograft model, the terminal deoxynucleotidyl transferase dUTP nick end labeling assay, and immunohistochemistry. Results: Piperine inhibited the growth of melanoma cells. Several apoptotic events were observed following treatment, as revealed by DAPI staining. Piperine increased the expression of BCL2-associated X, apoptosis regulator (BAX), cleaved poly(ADP-ribose)polymerase, cleaved caspase-9, phospho-c-Jun N-terminal kinase and phospho-p38, and reduced that of B-cell lymphoma 2 (BCL2), X-chromosome-linked inhibitor of apoptosis, and phospho-extracellular signal-regulated protein kinase (ERK1/2) in a concentration-dependent manner. Treatment of mice for 4 weeks with piperine inhibited tumor growth without apparent toxicity. Piperine increased the expression of apoptotic cells and cleaved-caspase-3 protein and reduced the expression of phospho-ERK1/2 protein in melanoma tumors. Conclusion: Piperine suppressed the growth of human melanoma cells by the induction of apoptosis via the inhibition of tumor growth of human melanoma cells and tumor xenograft models.*

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Key Words: Piperine, melanoma, A375SM, A375P, xenograft model, apoptosis.

The mortality rate of cancer is increasing every year worldwide. Cancer is also the leading cause of death in South Korea (1, 2). The occurrence of skin cancer has consistently increased with concurrent increases in ultraviolet light exposure during outdoor activities. Comprising only 4% of all types of skin cancers, but with a mortality as high as 80%, melanoma has the highest rate of skin cancer malignancies and is caused by the malignant alteration of melanin cells (3, 4). Malignant human melanoma cells rapidly spread to internal organs such as the bones, liver and lungs through lymphatic ducts and blood vessels. Due to its high resistance to chemotherapy and radiotherapy, surgical excision after early detection is considered the only treatment for this disease. Human melanoma is an intractable disease, and no specific treatment is available in cases of recurrence (5, 6). A specific group of food materials, medications, and health supplements developed from various natural resources have also been used to treat melanoma (7).

Piper nigrum L., a member of the family Piperaceae, is used as spice in Asia and India, and has beneficial effects on dyspepsia and pain relief due to the presence of various phytochemicals with different biological activities (8). Piperine (Figure 1) is a major alkaloid-amide that is responsible for the distinct taste and scent of pepper (9). Piperine also reportedly has various biological activities including antioxidant (10), anti-inflammatory (11), anti-arthritic (12), antibacterial (13), and anticancer (14).

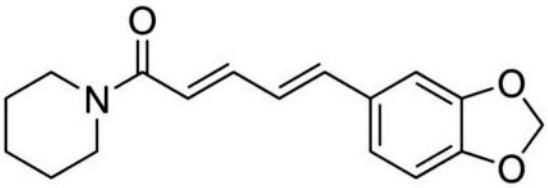

Figure 1. *Chemical structure of piperine.*

Cell death is classified into apoptosis, an intercellular activity also known as programmed cell death, and necrosis, a form of cell injury that induces an inflammatory response in tissues by externally leaking intracellular materials. Apoptosis is induced by the expression of various proteins, including those of the B-cell lymphoma 2 (BCL2) family, inhibitors of apoptosis (IAP) family, and mitogen-activated protein kinase (MAPK) pathway, when affected by DNA through physical or chemical stimulation (15). The BCL2 family proteins exist either in the mitochondrial membrane or move to the membrane due to a cell death-inducing signal, and then play an important role in regulating cell death (16). When expression of BCL2-associated X, apoptosis regulator (*BAX*; a pro-apoptotic protein) is increased, cytochrome c is released through the transformation of the mitochondrial membrane potential, generating apoptosome-associated complex (including cytochrome c/apoptotic peptidase activating factor 1/caspase-9) that induce apoptosis by activating caspase-3. As a common pathway for apoptosis signaling, caspase-3 is activated by an initiator caspase to cleave poly(ADP-ribose)polymerase (PARP), a protein that restores damaged DNA, to induce apoptosis (17). BCL2 (anti-apoptotic protein) inhibits apoptosis by inhibiting BAX movement to the mitochondria (18). In addition, IAP proteins combine with caspase-3 and caspase-9 to neutralize their activities, inhibiting apoptosis. Of the eight IAP families, X-chromosome-linked IAP (XIAP) is highly expressed in cancer cells (19). When XIAP expression is reduced by external stimuli, proliferation of prostate cancer and human melanoma cells is inhibited, due to the induction of apoptosis (20, 21). Mitogen-activated protein kinases (MAPK) are classified into extracellular signal-regulated protein kinase (ERK1/2), p38 MAPK, and c-Jun *N*-terminal kinase/stress-activated protein kinase (JNK/SAPK), and regulate biological activities such as cell signaling transmission (22).

This study used A375SM and A375P human malignant melanoma cells to examine the inductive effect of piperine on apoptosis *in vitro*, and examined the MAPK signaling transmission mechanism. In addition, the effect of piperine on *in vivo* tumor growth was also examined.

Materials and Methods

Chemicals, drugs, and antibodies. Minimum essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM) and penicillin-streptomycin were purchased from Welgene (Gyeonsan, Republic of Korea). Fetal bovine serum was purchased from Thermo Fisher (Waltham, MA, USA). Piperine (Figure 1), 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Cell lysis buffer and 4',6-diamidino-2-phenylindole (DAPI) were obtained from Invitrogen (Carlsbad, CA, USA). Antibodies to β-actin, BAX, BCL2, caspase-9, caspase-3, PARP, XIAP, phospho(p)-p38 MAPK, p38 MAPK, p-JNK, JNK, p-ERK1/2, ERK1/2, and goat anti-rabbit IgG were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell lines and culturing. The A375SM (highly metastatic) and A375P (moderately metastatic) human melanoma cell lines were obtained from the Korean Cell Line Bank (Seoul, Republic of Korea). Melanoma cells were maintained in MEM or DMEM supplemented with 5% FBS and penicillin-EDTA at 37˚C with 5% CO_2. The culture media were renewed every 2–3 days. For piperine treatment, melanoma cells at 80-90% confluence were seeded in a 175 cm^2 flask (Nunc; Fisher Scientific, Loughborough, UK).

Cell viability assay. The cytotoxicity effects of piperine were assessed using the MTT assay. A375SM, and A375P cells were seeded in 96-well plates at a density of 2×10^4 cells/ml, and incubated for 24 h. The cells were then treated with 50, 100, 150 and 200 μM piperine for 24 h in triplicate. Following treatment, the media were discarded, followed by the addition of 40 μl of a 5 mg/ml MTT solution, and incubated for an additional 2 h. The MTT solution was then aspirated and a formazan was solubilized with the addition of 100 μl of DMSO. The absorbance was determined at an absorbance of 595 nm using a microplate reader (BioRad, Hercules, CA, USA). The percentage of viable cells was estimated and compared with that of untreated control cells.

DAPI staining. Apoptotic cell death was determined morphologically using the fluorescent nuclear dye, DAPI, which showed apoptotic cells with chromatin condensation and nuclear fragmentation. A375SM and A375P cells were incubated with phosphate-buffered saline (PBS) or different concentrations of piperine (0, 100 and 150 μM) for 24 h, harvested by trypsinization, then fixed in 70% ethanol overnight at 4˚C. The cells were then stained with DAPI, deposited onto slides, and viewed to detect apoptotic characteristics with a fluorescence microscope. Quantitative assay counted apoptotic cells among 100 randomly selected cells and was performed three times.

Western blot analysis. Cells were treated with 0, 100 and 150 μM of piperine for 24 h, and then protein concentrations were determined using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). Proteins in each cell lysate were separated, using different concentrations (6-14%) of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and were electrotransferred onto nitrocellulose membranes. The membranes were incubated with 5% (w/v) skim milk for 1 h at room temperature and were further incubated overnight at 4˚C with specific antibodies listed above diluted in blocking solution. After washing, the membranes were

incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. After washing, the protein bands were detected using enhanced chemiluminescence reagents (Pierce Biotechnology, Rockford, IL, USA). The density of each band was quantitatively measured using the ImageJ Launcher imaging program (provided by NCBI).

Animals and the in vivo xenograft tumor model. Five-week-old male BALB/c nude mice (nu/nu) were purchased from Orient Bio (Gyeonggi-do, Republic of Korea). Experiments on animals were performed in accordance with the Guidelines for the Care and Use of Animals of the Kongju National University Animal Care Committee (Chungcheongnam-do, Republic of Korea. Approval number: KNU_2018-5). Mice were maintained under a 12-h light/dark cycle, and housed under controlled temperature (23±3˚C) and humidity (40±10%) conditions. Mice were allowed access to pelleted food and water *ad libitum*. A375SM and A375P cells were maintained in MEM or DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37˚C in an incubator with 5% CO_2. The cells were harvested, using 0.25% trypsin and trypsinization was stopped with a solution containing 10% FBS. Cells were then washed twice and resuspended in MEM or DMEM. A total of 2×10^7 cells in 0.2 ml of medium were administered subcutaneously into the right flank of mice. One week after the subcutaneous injection, A375SM and A375P cells growing under the skin of nude mice established tumors. When the tumors were palpable, the mice were assigned randomly into three groups with five mice in each group: vehicle-treated controls, and groups treated with piperine. Piperine was administered orally five times per week at a dose of 50 or 100 mg/kg body weight, while the control group mice were administered vehicle (distilled water) only. Tumor volume and weight were measured twice each week for 28 days. Tumor volume was measured as: volume (mm^3)=$0.5\times a\times b^2$, where a was the long axis, and b the short axis of the tumor.

At the completion of the experiment, the mice were sacrificed and the tumors were excised to measure tumor weight. A portion of the tumor was embedded in paraffin and used for terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assays and immunohistochemistry.

Histological examination. In order to assess organ toxicity due to piperine administration, livers and kidneys from mice were excised, fixed in 10% neutral-buffered formalin and embedded in paraffin. Paraffin blocks were cut into 5 μm thick sections, followed hematoxylin and eosin (H&E) staining. The sections were qualitatively examined under an optical microscope (200×).

TUNEL assay. Paraffin-embedded tumor tissues were used for TUNEL staining, which was performed using the Dead End Colorimetric TUNEL system (Promega, Madison, WI, USA). Paraffin-embedded sections (5-μm-thick) were processed according to the manufacturer's instructions. The stained tissues were qualitatively observed using an optical microscope.

Immunohistochemistry. The embedded paraffin sections were rehydrated and deparaffinized by sequential immersion in xylene and alcohol solutions. The sections were then incubated at 4˚C with antibodies specific for cleaved-caspase-3 and p-ERK1/2 overnight, and then incubated for 1 h at room temperature with a peroxidase-conjugated goat anti-rabbit antibody. The tumor sections were visualized using 3,3'-diaminobenzidine solution, treated with mounting agent, and qualitatively observed under an optical microscope (200× magnification).

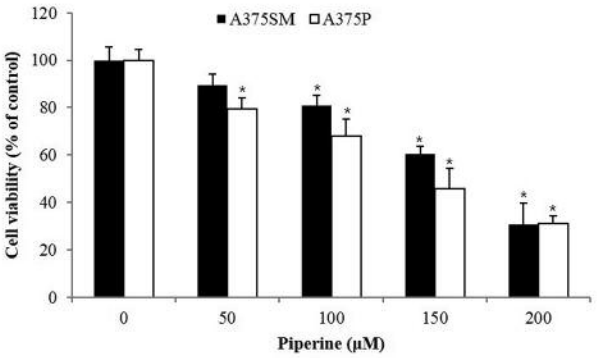

Figure 2. *Effects of piperine on the cell viability of melanoma cells. Cell viability was measured by MTT assay. The results are presented as the mean±SD from three independent experiments performed in triplicate. Significance was determined by Dunnett's t-test. *Statistically significant at p<0.05 when compared with nontreated controls.*

Statistical analysis. The results are presented as the mean±standard deviation (SD). Differences between mean values for piperine-treated and control groups were assessed by one-way analysis of variance with Dunnett's *t*-tests. Differences with a value of $p<0.05$ was considered to indicate statistical significance.

Results

Effect of piperine on the viability of melanoma cells. To evaluate the effects of piperine (0, 50, 100, 150, and 200 μM) on A375SM and A375P melanoma cells, an MTT assay was performed to determine survival rates after 24 h. Piperine significantly reduced survival of A375P cells at all concentrations used and of A375SM cells at 100 μM and higher (Figure 2). These findings suggest that piperine has significant effects on the survival of A375SM and A375P human melanoma cells.

Effects of piperine on morphological changes in A375SM and A375P cells. To confirm whether the effect of piperine on melanoma cells observed in the MTT assay was caused by induction of apoptosis, the morphological changes of the nucleus and the phenomenon of chromatin condensation were observed through staining using DAPI, which specifically reacts with DNA. When A375SM and A375P melanoma cells were treated with 0, 100, and 150 μM, multiple apoptotic-positive cells were observed (Figure 3A). One hundred cells from five random sections taken at ×200 through a fluorescent microscope were quantified to analyze the amount of apoptosis induced. The number of apoptotic cells, such as those with apoptotic bodies and chromatic

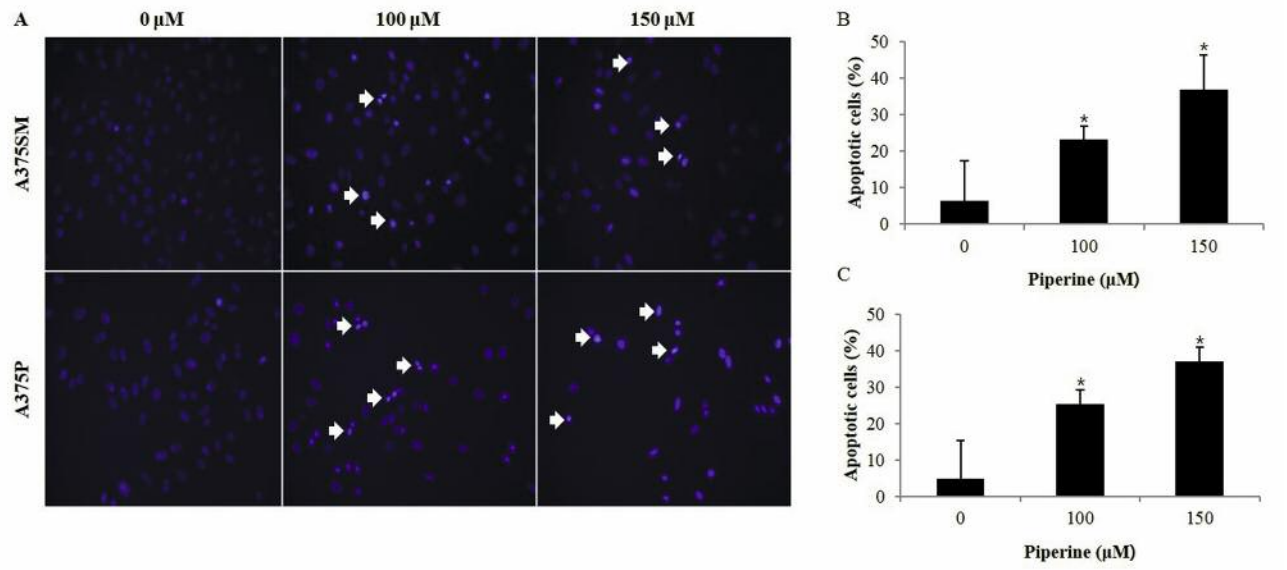

Figure 3. *Effects of piperine on A375SM and A375P cells. A: A375SM and A375P cells were treated with/without piperine (0, 100 and 150 μM) for 24 h, and apoptotic bodies were stained with 4',6-diamidino-2-phenylindole (DAPI). Chromatin condensation was then examined using a fluorescence microscope (200×). The arrows indicate chromatin condensation in A375SM and A375P cells. Quantification of DNA fragmentation and nuclear condensation determined by DAPI in A375SM (B) and A375P (C) cells treated with piperine (0, 100 and 150 μM) for 24 h. Each bar represents the mean±SD calculated from five independent experiments. Significance was determined by Dunnett's t-test. *Statistically significant at p<0.05 when compared with nontreated controls.*

condensation, was significantly increased in the piperine-treated groups compared with the control group. A375SM cells showed an increase in apoptotic cells of more than 3-fold (Figure 3B) and A375P cells of more than 5-fold (Figure 3C) at piperine concentrations of 100 and 150 μM. These findings show that apoptosis induction occurs through DNA fragmentation by DNA cleavage of nucleosome (23), and suggest that piperine induces apoptosis of A375SM and A375P cells.

Effect of piperine on apoptosis-related proteins of melanoma cells. Western blotting was performed to examine changes in the expression of apoptosis-associated proteins in A375SM and A375P cells treated with 0, 100, and 150 μM piperine. The results showed that the expression of BAX, cleaved PARP, and cleaved caspase-9 (pro-apoptotic proteins) increased in A375SM and A375P cells after treatment with 100, and 150 μM piperine, while BCL2 (anti-apoptotic protein) expression decreased. Expression of XIAP, which inhibits caspase-9, did not significantly change in A375SM cells, while it was reduced in A375P cells in a concentration-dependent manner after piperine treatment (Figure 4). These findings suggest that piperine regulates expression of apoptosis proteins in A375SM and A375P cells to induce apoptosis.

Effect of piperine on MAPK pathway proteins in melanoma cells. Various kinases in the MAPK pathway inhibit different activities depending on intracellular and extracellular environmental changes. A375SM and A375P cells were treated with different concentrations of piperine to examine its effects on proteins in the MAPK pathway. Piperine treatment (100, and 150 μM for 24 h) resulted in increased expression of p-JNK and p-p38 in a concentration-dependent manner, while the expression of p-ERK 1/2 decreased in both A375SM and A375P cells (Figure 5). These findings suggest that piperine regulates the MAPK signaling pathway in A375SM and A375P melanoma cells to induce apoptosis.

Effect of piperine on tumor growth in an in vivo animal model. A375SM and A375P melanoma cells were implanted into the hypodermis of nude mice. Piperine was then administered orally at 50 mg/kg or 100 mg/kg five times a week for 4 weeks to examine its effects on tumor growth. Tumor size was measured twice a week. Compared with the control group, significant inhibition of tumor growth was observed beginning at day 18 in the A375P group and at day 21 for the A375SM group (Figure 6A). The tumor weight significantly decreased in the group treated with piperine (Figure 6B). When tumor size was compared between the control and the piperine-treated groups, the A375SM group showed 53% reduction in tumor growth on treatment with a low piperine concentration and 43% reduction on treatment with a high concentration, while the A375P groups showed corresponding reductions of 57% and 35% (Table I). These

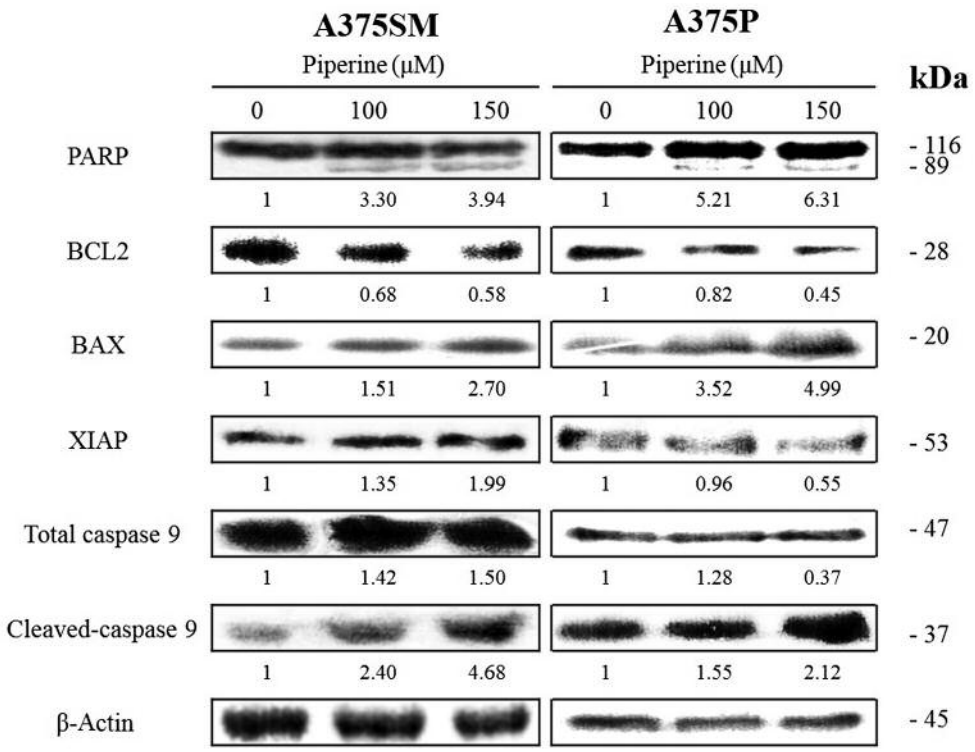

Figure 4. *A375SM and A375P cells were treated with piperine for 24 h, and the cells were harvested to measured protein levels of B-cell lymphoma 2 (BCL2), BCL2-associated X, apoptosis regulator (BAX), cleaved poly(ADP-ribose)polymerase (PARP), X-chromosome-linked inhibitor of apoptosis (XIAP), and caspase-9 by western blotting. The blots were probed with an antibody specific to β-actin to confirm equal sample loading.*

findings suggest that piperine has an inhibitory effect on A375SM and A375P melanoma cell growth.

Effect of piperine on the apoptosis induction in melanoma tumor tissue. Xenograft experiments were performed on nude mice to examine the anticancer effect of piperine on human melanoma cells *in vivo*, and a TUNEL assay was performed on the tumor tissue to observe effects on apoptosis. Compared to the control group, the number of apoptosis-positive cells increased significantly in the A375SM and A375P groups administered 100 mg/kg piperine (Figure 6C). The results indicate that piperine induces DNA fragmentation in the nucleus, which in turn results in apoptosis of A375SM and A375P tumor cells *in vivo*.

Effect of piperine on caspase-3 and ERK1/2 expression in melanoma tumor tissue. To determine the effects of piperine on apoptosis-related proteins in xenografted human melanoma tumor tissue, the expressions of caspase-3 and p-ERK1/2 were measured by immunohistochemistry. Caspase-3 and ERK1/2 are important modulators of various biological activities that affect cell survival and proliferation. In this experiment, piperine was injected into nude mice with xenograft tissue, and immunohistochemistry was then performed to examine the expression of cleaved caspase-3 and p-ERK1/2 in the tumor tissues (Figure 7). Immunohistochemical analysis revealed that the level of cleaved caspase-3 increased, whereas that of the p-ERK1/2 decreased in the piperine-treated mice.

Table I. *Effect of piperine on tumor inhibition in nude mice bearing A375SM and A375P melanoma tumor administered piperine for 4 weeks.*

Tumor model	Piperine (mg/kg)	Tumor size (mm^3)		Inhibition rate (%)[a]
		Pre treatment	Post treatment	
A375SM	0 (Control)	121	3,819	
	50	123	1,783	53
	100	124	2,194	43
A375P	0 (Control)	103	1,815	
	50	109	784	57
	100	117	1,175	35

[a]Relative to the control. Inhibition rate=growth rate of treated group/growth rate of control×100%.

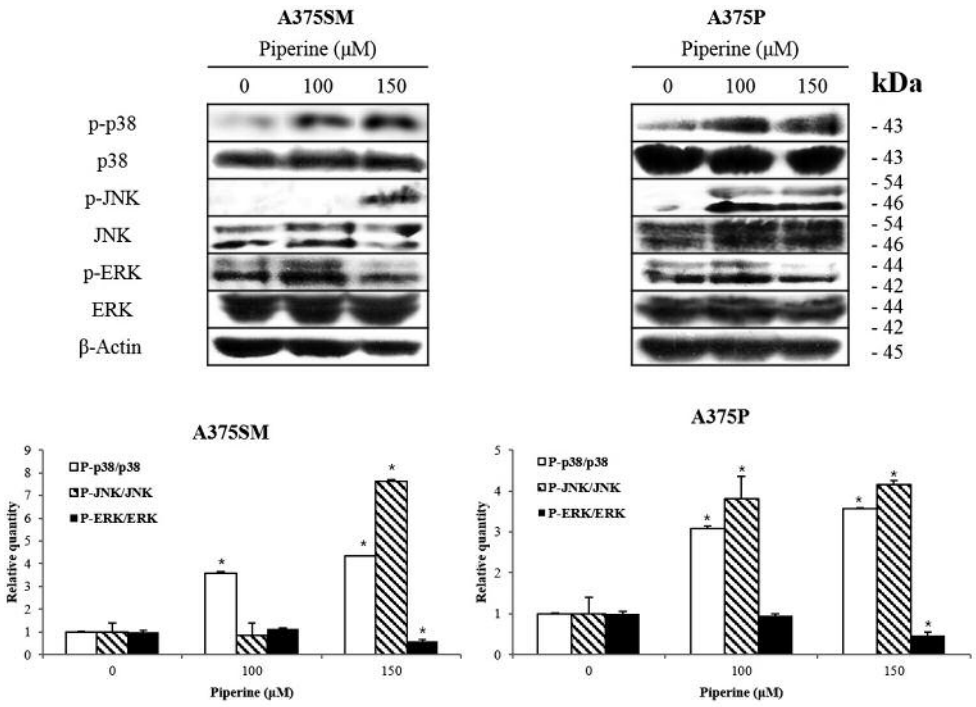

Figure 5. *Effect of piperine on the activation of mitogen-activated protein kinase (MAPK) pathway in A375SM and A375P cells, as measured by western blotting. Each bar represents the mean±SD calculated from independent experiments. Significance was determined by the Dunnett's t-test. (p)-JNK: (Phospho-)c-Jun N-terminal kinase; (p)-ERK1/2) (phospho-)extracellular signal-regulated protein kinase. *Statistically significant at p<0.05 when compared with nontreated controls.*

Histopathological changes in melanoma tumor tissues after piperine treatment. To assess organ toxicity due to piperine administration, liver and kidney tissues from tumor-xenografted mice were histologically examined by hematoxylin and eosin staining followed by light microscopy (Figure 8). No histopathological abnormality was detected, indicating that piperine did not cause any detectable toxic effect.

Discussion

Similar to the overall occurrence of cancer, the frequency of melanoma, the most aggressive type of skin cancer, is increasing worldwide (3, 4). Melanoma can be treated with surgery, radiotherapy, and chemotherapy, which can also result in a wide range of side-effects (5, 6). Consequently, there are increasing studies on non-toxic carcinostatic substances derived from natural agents that have fewer side-effects (7). As a major alkaloid-amide, piperine is responsible for the distinct scent and smell of black pepper (8). Piperine has been reported by recent studies to have anticancer effects in various types of cancers, but little is known about the effect of piperine on melanoma (14). This study examined the anticancer effects of piperine on A375SM and A375P human melanoma cells. MTT assay showed the survival of A375SM and A375P cells decreased depending on the piperine concentration used. Lin *et al*. also reported a concentration-dependent decrease in the survival rate of A549 lung cancer cells after piperine treatment (25). Yaffe *et al*. also observed significant concentration-and time-dependent inhibition of cancer cell growth when HRT-18 colorectal carcinoma cells were treated with different concentrations of piperine for varying amounts of time (26). These studies suggest that piperine has a concentration-dependent inhibitory effect on cell survival.

DAPI staining was performed to confirm whether the findings of survival inhibition of A375SM and A375P was the result of apoptosis. When A375SM and A375P were treated with piperine, cells with chromatin condensation and apoptotic bodies, as a characteristic of apoptosis, indeed increased. To quantify the degree of induced apoptosis, DAPI-positive cells were counted. In a comparison with the control group, DAPI-positive cells increased by more than 3-fold depending on the concentration of piperine used. Shin *et al*. reported the concentration-dependent increase in apoptotic bodies in AGS stomach cancer cells treated with piperine in (100 and 150 μM) (27), while Kim *et al*. observed apoptotic bodies in HT-29 colon cancer cells treated with 40 μM piperine (28). These findings suggest that the decreases in the survival rates of A375SM and A375P treated with piperine were caused by apoptosis induction.

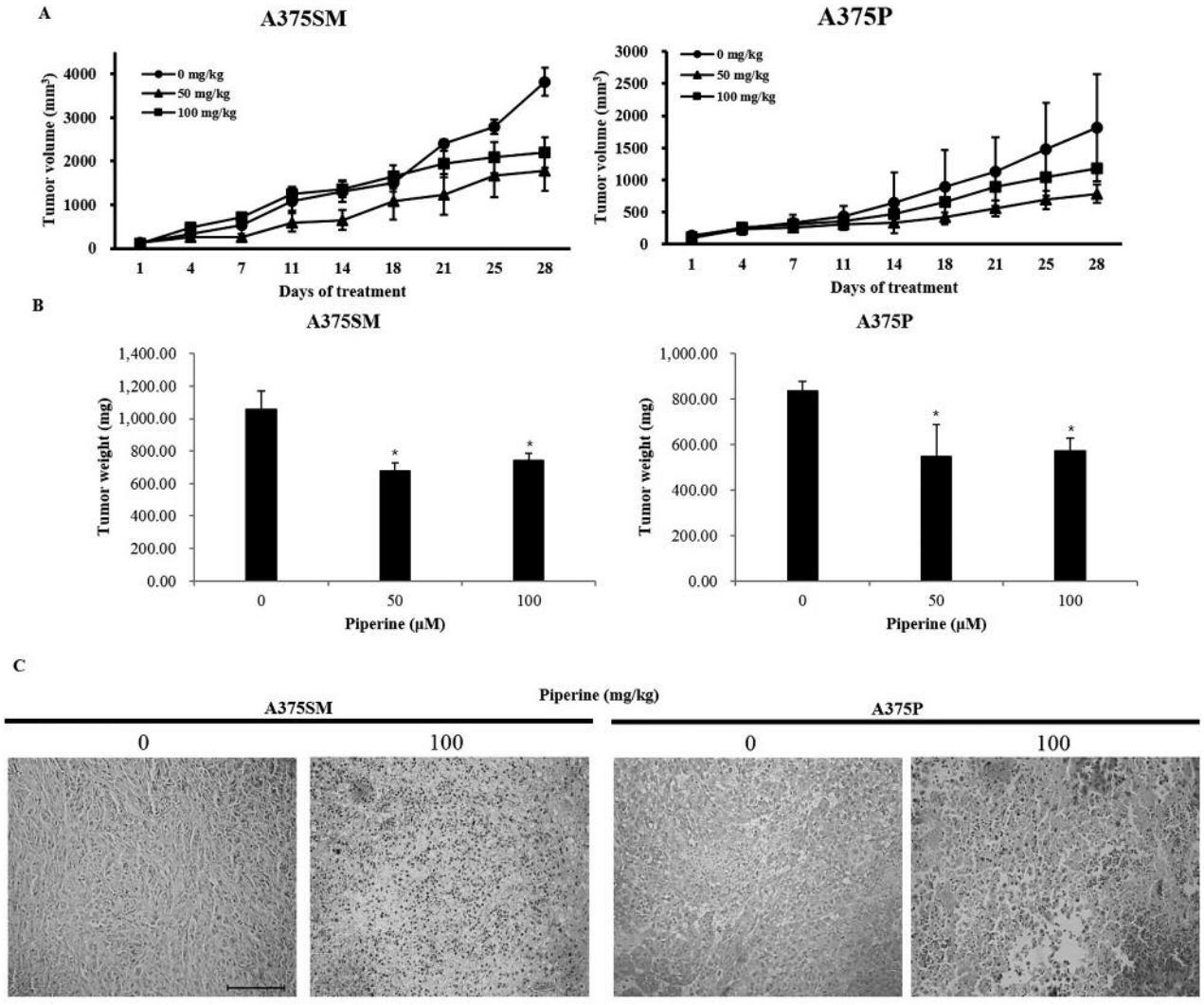

Figure 6. *Effects of piperine on melanoma tumor growth and apoptosis in tumor tissues. Nude mice bearing A375SM and A375P cells as xenograft models were treated with piperine for 28 days, and tumor volume (A) and weight (B) were determined. Each value represents the mean±SE. C: Apoptosis was measured in tumor tissues using terminal deoxynucleotidyl transferase dUTP nick-end labeling assay. Slides were observed under a microscope (200×). Scale bar: 10 μm. Significance was determined by Dunnett's t-test. *Statistically significant at p<0.05 when compared with nontreated controls.*

Apoptosis is induced by interactions between various proteins. The BCL2 family maintains the balance between pro-apoptotic and anti-apoptotic proteins. When this balance breaks, apoptosis is likely to be induced (16, 18). As well as these proteins, PARP, known for its major role in DNA repair, and XIAP interact with caspase-9 to block the active domain in an abnormal form (24). In light of this, western blotting was performed to examine the changes in protein expression caused by apoptosis induced in A375SM and A375P treated with piperine. As a result, the expression of BAX (pro-apoptotic protein) increased depending on the concentration, while the expression of BCL2 (anti-apoptotic protein) conversely decreased in the same manner. The expressions of cleaved caspase-9 and cleaved PARP increased, depending on the concentration. Lin *et al*. reported increases in the expression of BAX and cleaved caspase-9 and a decrease in BCL2 expression in A549 lung cancer cells treated with different concentrations of piperine (25), while Lai *et al*. reported the concentration-dependent regulation of the expression of BAX and BCL2 in piperine-treated 4T1 mouse breast cancer cells (29). The expression of XIAP showed no significant change in A375SM cells, but was down-regulated in piperine-treated A375P cells. Shin *et al*. also reported the concentration-dependent increase in the expression of BAX,

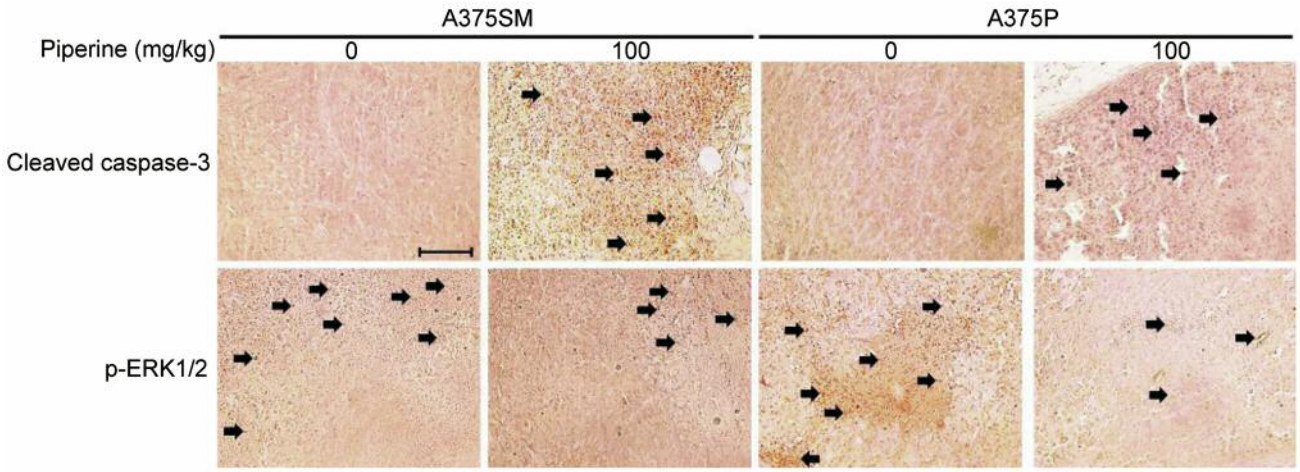

Figure 7. *Effects of piperine on cleaved-caspase-3 and phospho (p)-ERK1/2 expression in melanoma tumor tissues. Nude mice bearing A375SM and A375P cells were administered piperine (100 mg/kg) for 4 weeks and excised tumors were assayed by immunohistochemistry using antibodies specific for cleaved caspase-3 or p-ERK1/2. Slides were observed under a microscope (200×). The arrows indicate staining of cleaved caspase-3, and phospho-extracellular signal-regulated protein kinase (p-ERK1/2) in cells. Scale bar: 10 μm.*

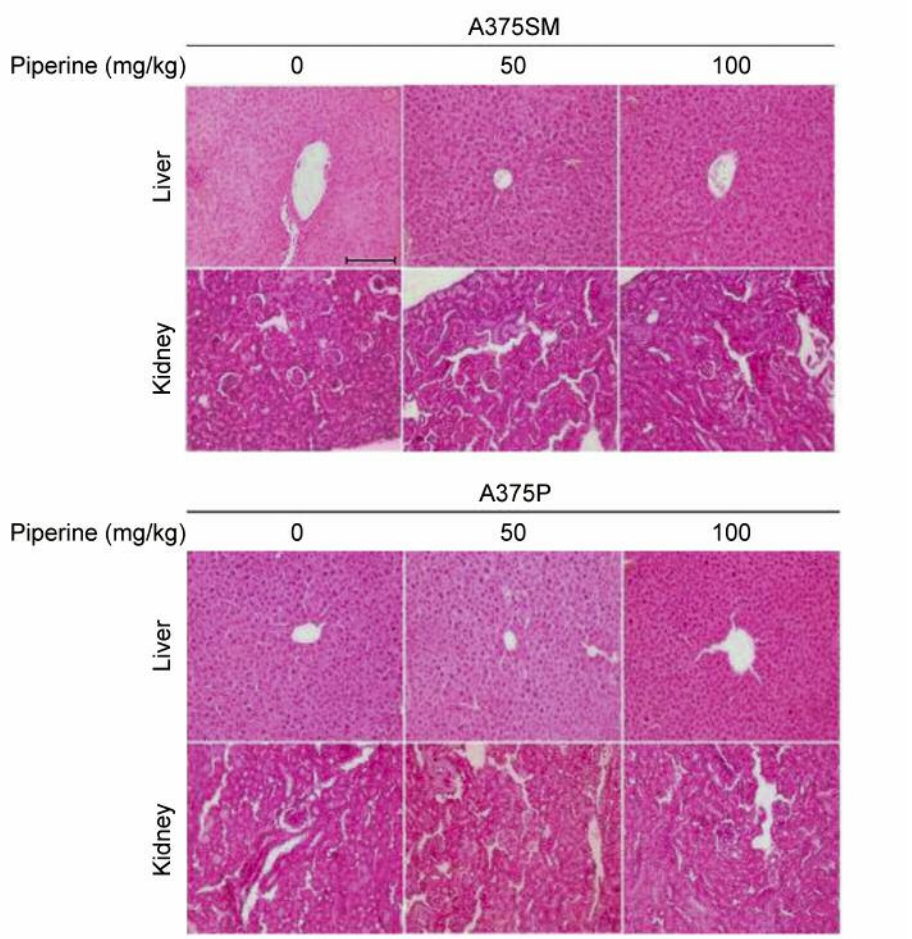

Figure 8. *Mice received an injection of A375SM or A375P cells, and piperine was orally administered five times per week for 4 weeks. The liver and kidney were then excised and evaluated by hematoxylin and eosin staining (200×). The dose of piperine had no detectable toxic effect on liver and kidney in nude mice. Bar: 10 μm.*

cleaved caspase-9, and cleaved PARP, and concentration-dependent decreases in the expression of BCL2 and XIAP in AGS cells treated with 100 and 150 μM piperine (27). Abdelhamed *et al*. reported the time-dependent decrease in the expression of XIAP in 4T1 cells treated with 200 μM piperine (30). These findings suggest that piperine regulates the expression of apoptosis proteins in A375SM and A375P cells, depending on the concentration, to induce apoptosis.

The MAPK signaling pathway regulates cell proliferation, death, migration, and differentiation. This study showed that the expression of p-ERK1/2 was reduced, while those of phospho-p38 and phospho-JNK were increased in piperine-treated cells. Yaffe *et al*. reported that there was a concentration-dependent increase in the expression of po-p38 and p-JNK in HT-29 cells treated with piperine (75 and 100 μM) (31). Zhang *et al*. also found that piperine (100, 150 and 200 μM) had a concentration-dependent effect on increasing p-p38 and p-JNK expression in HOS and U2OS osteosarcoma cells (32). Do *et al*. reported a concentration-dependent decrease in the expression of p-ERK1/2 in overexpressing breast cancer cells treated with piperine at different concentrations (33). These findings suggest that piperine induces apoptosis and inhibits growth of A375SM and A375P cells *in vitro*.

A375SM and A3775P cells were implanted in nude mice to examine the effects of piperine injection on tumor growth. Piperine was orally given at 50 and 100 mg/kg for 4 weeks. Compared to the control group, significant inhibition of tumor growth was observed beginning at day 18 in the A375P group and at day 21 for the A375SM group. Lai *et al*. reported similar tumor growth inhibition in 4T1 murine breast cancer cells in BALB/c mice treated with piperine for 30 days at different concentrations (29). Marklov *et al*. also reported a decrease in tumor growth as a result of injecting 50 mg/kg piperine into PC-3 prostate cancer cell xenografts in C.B17/Icr-SCID mice compared with the control group (34). Samykutty *et al*. observed a significant inhibitive effect on tumor growth in nude mice injected with DU-145 prostate cancer cells and treated with piperine compared to the control group (35). In our study, TUNEL assay showed that apoptosis-positive cells significantly increased in piperine-treated (100 μM) groups compared to the control. These findings suggest that piperine inhibits A375SM and A375P tumor growth.

Activated caspase-3 and p-ERK1/2 induce decomposition of multiple target proteins, ultimately inducing apoptosis and inhibiting cell growth and survival (21, 22). In this experiment, immunohistochemistry showed that compared to the control group, the expression of cleaved caspase-3 increased in the piperine-treated group. Piperine treatment significantly increased the level of cleaved casapse-3 and suppressed the phosphorylation of ERK1/2 in tumor tissue. These results indicate that piperine induced the apoptosis of A375P and A375SM cells and inhibited tumor growth.

To summarize these findings, the growth of A375SM and A375P melanoma cells was inhibited by piperine at different concentrations. Given that this inhibition was due to induction of apoptosis, our findings suggest that piperine induces apoptosis *in vitro* and *in vivo* to inhibit melanoma growth.

Conflicts of Interest

The Authors declare that there is no conflict of interest in regard to this research.

Authors' Contributions

Study concept and design: JY Jung, ES Yoo, GS Choo. Acquisition of data: ES Yoo, GS Choo, SH Kim, JS Woo, HJ Kim, YS Park, BS Kim, SK Kim, BK Park. Analysis and interpretation of data: ES Yoo, GS Choo, YS Park, BS Kim, SK Kim, BK Park, SD Cho, JS Nam, CS Choi, JH Che, JY Jung. Drafting of the manuscript: JY Jung, ES Yoo, GS Choo. Study supervision: JY Jung, JH Che.

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