치의과학석사 학위논문

Effects of *Lactobacillus plantarum* L14 extract on viability and migration of human malignant melanoma cells

Lactobacillus plantarum L14 추출물이

인간 악성 흑색종 세포의 생존 및 이동에 미치는 영향

2020 년 8 월

서울대학교 대학원

치의과학과 종양및발달생물학 전공

박 재 현

치의과학석사 학위논문

Effects of *Lactobacillus plantarum* L14 extract on viability and migration of human malignant melanoma cells

Lactobacillus plantarum L14 추출물이

인간 악성 흑색종 세포의 생존 및 이동에 미치는 영향

2020 년 5 월

서울대학교 대학원

치의과학과 종양및발달생물학 전공

박 재 현

ABSTRACT

Effects of *Lactobacillus plantarum* L14 extract on viability and migration of human malignant melanoma cells

Jaehyun Park

Department of Cancer and Developmental Biology

The Graduate School

Seoul National University

(Directed by Prof, Sangho Roh, D.V.M., Ph.D.)

Human malignant melanoma is the most aggressive type of skin cancers with high metastatic ability. Despite several traditional therapies, the mortality rate remains still high. *Lactobacillus plantarum* (*L. plantarum*), a species of the lactic acid bacteria, is being studied for cancer treatment. However, there are few researches about the relationship between *L. plantarum* and human malignant melanoma.

To investigate the effects of *L. plantarum* extract on melanoma, A375 human melanoma cells were used and treated with *L. plantarum* strain L14 extract. After the

treatment, the viability, the migration ability, the molecular changes of migration- and apoptosis-related genes, and the cellular location of cytochrome c was confirmed. As a result, the L14 extract inhibited viability, migration of A375 cells as well as reduced the expression of epithelial-mesenchymal transition-related genes. Also, it was confirmed that the L14 extract promoted the intrinsic apoptosis of A375 cells. Collectively, the present study demonstrated that the L14 extract exerted anti-growth and -migration effects and induced apoptosis on A375 cells. Therefore, these data suggest that the L14 extract could be developed as an anti-cancer agent for melanoma.

Keyword : *Lactobacillus plantarum*, anti-cancer, cell migration, human malignant melanoma, intrinsic apoptosis

Student Number : 2018-22152

CONTENTS

INTRODUCTION

Cancer is the second leading cause of death globally, which humans have not yet fully conquered [1]. Skin cancer is one of the most common cancers in the world and its incidence rate has increased by 50-times in 70 years [2]. the World Health Organization attributes the cause of the elevated incidence to increased exposure to ultraviolet (UV) due to the decrease in ozone level, or the use of UV-emitting tanning devices for cosmetic purposes. If a skin cancer is detected at an early stage and metastasis has not occurred, most of them could be treated via surgery with a high survival rate [3]. However, when metastasis has progressed, chemotherapy and radiotherapy as well as surgery should be used either alone or in combination, and the survival rate decreases dramatically [4]. About 80% of skin cancers are basal cell carcinoma, 16% are squamous cell carcinoma, and 4% are malignant melanoma [5]. While malignant melanoma has the smallest portion among skin cancers, it is responsible for most of the deaths related to skin cancer (more than 75%) because of its high metastatic and invasive abilities [6]. Although the therapies for malignant melanoma exist, the traditional therapies have harmful side effects such as the generation of reactive oxygen species or the toxicity to adjacent organs and tissues [7, 8]. Therefore, the development of treatments which can inhibit metastasis of melanoma and have fewer side effects is required.

Lactic acid bacteria (LAB), the bacteria derived from fermented foods, are the most commonly used probiotics [9]. LAB are administered to humans for various purposes because they and their metabolites have beneficial effects [10]. They are accepted as safe

in general when they are applied with adequate doses [11]. The previous studies have shown that LAB and their metabolites modulated the pathogen-induced immune response and enhanced the innate immunity [12, 13]. Also, Pochard et al. confirmed that LAB could mitigate allergic symptoms by reducing the cytokines production [14]. *Lactobacillus plantarum* (*L. plantarum*) is a major species of LAB, and the researches on the effects of *L. plantarum* on various cancers are being actively conducted [15, 16]. However, little is known about the effects of *L. plantarum* on human malignant melanoma.

Therefore, the present study investigated whether *L. plantarum* extract has any effects on human malignant melanoma cells. After treating human malignant melanoma cells, A375P (low metastatic) and A375SM (high metastatic), with *L. plantarum* extract, the morphological and molecular changes of the melanoma cells were analyzed.

MATERIALS AND METHODS

General materials

Fetal bovine serum (FBS) and penicillin/streptomycin (P/S) was purchased from HyClone (Logan, UT, USA) and Gibco (Grand Island, NY, USA), respectively. Antibodies were purchased from the following companies: N-cadherin, Slug, Bax, caspase-3 and cleaved caspase-3 from Cell Signaling Technology (Danvers, MA, USA), alpha-smooth muscle actin (α-SMA) and Vimentin from Abcam (Cambridge, UK), caspase-9 and cleaved caspase-9 from Cusabio (Wuhan, China), poly (ADP-ribose) polymerase (PARP) and cleaved PARP from Millipore (Burlington, MA, USA), Bcl-2 from Novus Biologicals (Centennial, CO, USA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from BioLegend (San Diego, CA, USA).

Preparation of *L. plantarum* cell-free extract

L. plantarum L14 strain used in this study was provided from NeoRegen Biotech (Suwon, Gyeonggi-do, Korea), and cultured in sterilized de Man, Rogosa and Sharpe (MRS; Hardy Diagnostics, Santa Maria, CA, USA) broth at 37℃ for 18 h. The cultured L14 was centrifuged at 10,000 × *g* for 15 min at 4℃ for harvest. The L14 pellet was washed with distilled water (DW) three times to remove the MRS broth medium. After washing, the L14 pellet was resuspended with DW and sonicated for 30 min on ice. The sonicated L14 was centrifuged at 12,000 × *g* for 15 min at 4 ℃. The supernatant was

filtered using a 0.45 μm filter (Sartorius, Göttingen, Germany) and freeze-dried for 72 h. The extract was dissolved at 50 mg/ml in phosphate-buffered saline (PBS) and filtered using a 0.45 μm filter for the following experiments.

Animals and xenograft model

All animal experiments were performed under the guidelines of the Institutional Animal Care and Use Committee of Seoul National University (approval number: SNU-161024-3). To induce tumors in mice, 5×10^6 cells of A375SM were injected subcutaneously into the right femoral region of immunodeficient BALB/c mice (n = 14). After 5 days for tumor growth, the tumor-induced mice were intraperitoneally treated with PBS (n = 6) and the L14 extract (500 mg/kg, n = 8) every 2 days. Following treating for 3 weeks, the mice were sacrificed. The tumors were dissected and analyzed. The tumor volume was calculated by the formula (length × width^2)/2.

Cell culture

Human dermal fibroblast (HDF) was provided from the American Type Culture Collection (Manassas, VA, USA). A375P and A375SM were purchased from the Korean Cell Line Bank (Seoul, Korea). HDF and both A375 cell lines were cultured in Dulbecco's Modified Eagle Medium (WELGENE, Daegu, Korea) with 10% FBS and 1% P/S at 37℃ with 5% CO_2, and the culture medium was replaced every 2 days.

Cell viability assay

HDF, A375P and A375SM were seeded at a density of 2.0×10^3 cells per well in 96-

well plates and allowed to adhere for 24 h. The medium was changed at various concentrations of the L14 extract and maintained. After 72 h, the effects of the extract on the cell growth were determined by Quanti-Max™ WST-8 cell viability kit (BIOMAX, Seoul, Korea).

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from A375P and A375SM after the 24 h treatment of the L14 extract at a concentration of 50 and 100 μg/ml using MiniBEST Universal RNA Extraction Kit (TaKaRa Bio Inc., Shiga, Japan). The isolated RNA was reverse transcribed to cDNA using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). Each of the genes was amplified and analyzed with TB Green™ Premix Ex Taq™ II (TaKaRa) and StepOnePlus (Applied Biosystems, Foster City, CA, USA). The primer sequences for qRT-PCR were listed in Table 1. The reagents were used according to the manufacturer's recommendations. GAPDH was used as an endogenous control and for normalization of the differences in individual samples.

Protein isolation and western blot

Both A375 cells were seeded at a density of 5×10^4 cells per well in 6-well plates and stabilized for 24 h. Then, the cells were treated with various concentrations of the L14 extract for 72 h. Total proteins were extracted by Passive lysis buffer (Promega) containing protease inhibitor and centrifuged at 12,000 × *g* for 15 min at 4℃ to remove any insoluble debris. An equal amount (10-20 μg) of proteins in the supernatants was loaded, isolated by performing sodium dodecyl sulfate-polyacrylamide gel

electrophoresis and transferred to polyvinylidene difluoride membranes. After blocking for 1 h at room temperature (RT) to prevent non-specific binding, the proteins were probed with primary antibodies diluted with blocking buffer overnight at 4℃ followed by washing with 0.1% Tween 20 (Sigma-Aldrich, Saint Louis, MO, USA) in tris-buffered saline. The primary antibodies were detected horseradish-peroxidase-conjugated secondary antibodies for 1 h at RT, which were visualized by ECL Western Blotting Substrate (Daeil Lab Service, Seoul, Korea).

Immunofluorescence for cytochrome c

A375SM was seeded at a density of 3×10^3 cells in a circular coverslip pre-coated with gelatin in 24-well plates. After 24 h of stabilization, the cells were treated with various concentrations of the L14 extract. Following 24 h, 48 h of the treatment, the culture medium was replaced with the fresh medium containing 100 nM MitoTracker (Invitrogen, Carlsbad, CA, USA). The cells were incubated for 30 min at 37℃, washed twice with PBS and fixed with 4% paraformaldehyde for 10 min at 37℃. After washing the cells three times with PBS, the cells were permeabilized with 0.15% Triton X-100 (Sigma) in PBS for 5 min at RT and washed three times with PBS. The cells were blocked with 3% bovine serum albumin (BSA; Bovogen, East Keilor, Australia) in PBS for 30 min at 37℃. After blocking, the blocking solution was replaced with anti-cytochrome c antibody (Novus Biologicals) diluted with 3% BSA in PBS and incubated overnight at 4℃. The cells were washed three times using 0.05% Tween-20 in PBS (PBST). The primary antibody was visualized by Alexa Fluor 647-conjugated secondary (Abcam) diluted with 3% BSA in PBS, and incubated for 30 min at RT followed by washing three times with

PBST. The coverslip was mounted with Hoechst 33342 (Invitrogen). All of the samples were scanned via LSM 800 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) at 630X magnification.

Cell migration assay

Both A375 cells were seeded in 12-well plates and cultured until they reached about 80% confluence. Then, both A375 cell lines were treated with mitomycin C (MMC; Cayman, Ann Arbor, MI, USA) for 3 h before the cell migration assay to exclude the effect by proliferation. A scratch was drawn in the center of the well using a pipette tip. Each well was washed by PBS three times to remove MMC and detached cells. A375 cells were treated with various concentrations of the L14 extract. The scratched monolayer of cells was photographed via EVOS XL Core microscope (Life Technologies, Carlsbad, CA, USA) at 24, 48, and 72 h of the treatment at 40X magnification

Statistical analysis

All values in the present study were obtained from three independent experiments in triplicate and displayed as the mean ± standard deviation. Results of $p < 0.05$ was considered statistically significant.

Table 1. Primer sequence used for real-time PCR

Gene		Sequence (5'-3')	Product size (bp)
Ki-67	Forward	AGT TTG CGT GGC CTG TAC TAA	202
	Reverse	AGA AGA AGT GGT GCT TCG GAA	
N-cadherin	Forward	ACA GTG GCC ACC TAC AAA GG	201
	Reverse	CCG AGA TGG GGT TGA TAA TG	
Vimentin	Forward	ATC CAA GTT TGC TGA CCT CTC TGA	99
	Reverse	GAC TGC ACC TGT CTC CGG TAC TC	
α-SMA	Forward	GAC GTA CAA CTG GTA TTG TG	144
	Reverse	TCA GGA TCT TCA TGA GGT AG	
Slug	Forward	TTG TGG CCT TCT TTG AGT TCG GTG	146
	Reverse	GGT GCC TCA GGT ACT CAG TCA	
Bcl-2	Forward	TTG TGG CCT TCT TTG AGT TCG GTG	111
	Reverse	GGT GCC TCA GGT ACT CAG TCA	
Bax	Forward	CCT GTG CAC CAA GGT GCC GGA ACT	99
	Reverse	CCA CCC TGG TCT TGG ATC CAG CCC	
GAPDH	Forward	CGC TGA GTA CGT CGT GGA GTC	172
	Reverse	GCT GAT GAT CTT GAG GCT GTT GTC	

RESULTS

The L14 extract affected growth and viability of A375P and A375SM

To investigate the effect of the L14 extract on human melanoma, the L14 extract was intraperitoneally injected into the mice with a A375SM-induced tumor. In the L14 extract-treated group, the tumors were evidently smaller than those of the control group (Figure 1A). The weight and the diameter of the tumors were measured and statistically analyzed, which showed significant decreases in the two factors (Figure 1B). To explore how the L14 extract reduced tumor mass, human skin-related cells, HDF, A375P and A375SM were treated with 50, 100 and 150 μg/ml L14 extract. After 72 h of treatment, the growth and the appearance of the cells in the well were observed using crystal violet solution. The growth of HDF was decreased in the 150 μg/ml-treated group (Figure 2A), whereas the growth of the melanoma cells was inhibited in all treated groups in a dose-dependent manner (Figure 2B and C). The morphological change of cells was not observed in all three cell lines after the treatment. For quantitative analysis, the viability of three cell lines was measured after 72 h of the treatment. The viability of HDF was not changed by the L14 extract under the 150 μg/ml (Figure 3A) while the viability was significantly decreased from over 100 μg/ml and 50 μg/ml L14 extract in a dose-dependent manner in A375P and A375SM, respectively (Figure 3B and C). The change of mRNA expression of Ki-67, a proliferation marker, confirmed the growth-inhibitory effect of the L14 extract. The mRNA expression level of Ki-67 in HDF was not affected by the L14 extract (Figure

4A). However, those of A375 cells were reduced in the 100 μg/ml-treated groups (Figure 4B and C). Thus, in the following *in vitro* experiments, the treatment concentrations of the L14 extract were determined to be 50 μg/ml, the minimum concentration at which the growth of A375SM began to be inhibited, and 100 μg/ml, the maximum concentration at which the growth of HDF was not inhibited.

The expression of mRNA and protein associated with apoptosis also was changed

To confirm whether the L14 extract induced apoptosis in A375 cells, the apoptosis-related genes were analyzed after the treatment of the L14 extract. The mRNA expression of Bcl-2, an anti-apoptotic marker, was significantly decreased by the L14 extract in both A375 cells while that of Bax, an apoptosis regulator, was increased only in A375SM (Figure 5). However, the protein expression showed the same tendency in both A375 cells. It was observed that the decrease in Bcl-2 protein, the increase in Bax protein and the activation of both caspase-9 and caspase-3. The L14 extract also induced the cleavage of PARP, a protein involved in DNA repair (Figure 6).

The release of cytochrome c from mitochondria was observed following the treatment of the L14 extract

The process of the apoptosis pathway includes the release of cytochrome c from mitochondrial intermembrane space to the cytosol. Therefore, the location of intracellular cytochrome c was probed by immunofluorescence. The non-treated A375SM had the intact nuclear morphology and the same location of mitochondria and cytochrome c

(Figure 7A). In the 50 μg/ml-treated group at 24 h of the treatment, the morphological change of nucleus and the location of cytochrome c were not different to control group while the fragmented nucleus and the release of cytochrome c were observed at 48 h (Figure 7B). Both characteristics were detected from 24 h in the 100 μg/ml-treated group (Figure 7C).

The L14 extract inhibited the migration of A375P and A375SM

To evaluate the anti-migratory effect of the L14 extract on A375P and A375SM, their migration distance was measured after the treatment of the L14 extract. The L14 extract-treated groups showed decreased migration compared to the control groups in a dose-dependent manner in both A375 (Figure 5A and C). The concentration of the L14 extract was adjusted lower (25, 50 and 75 μg/ml) than other experiments because the anti-growth effect of the L14 extract was promoted by the inhibition of the cell division due to MMC. In the case of A375SM, the migration distance of the group with the treatment of 75 μg/ml L14 extract rather retreated because the anti-migratory effect was added to the reduction of viability by the L14 extract. The anti-migratory effect was much greater in A375SM than in A375P. The wounding area of cells was quantified (Figure 5B and D), which showed that the migration distance was reduced in a dose-dependent manner in both A375 cell lines.

The L14 extract changed the expression of mRNA and protein associated with metastasis of cancers

The molecular changes of mesenchymal gene expression after the treatment were detected by qRT-PCR and western blot. The mRNA expression of mesenchymal cell

markers including N-cadherin, Vimentin, α-SMA and Slug was decreased in A375SM while the mRNA level of Vimentin was not decreased in A375P (Figure 9A and B). Consistent with the result of the qRT-PCR, the protein expression also was down-regulated (Figure 10).

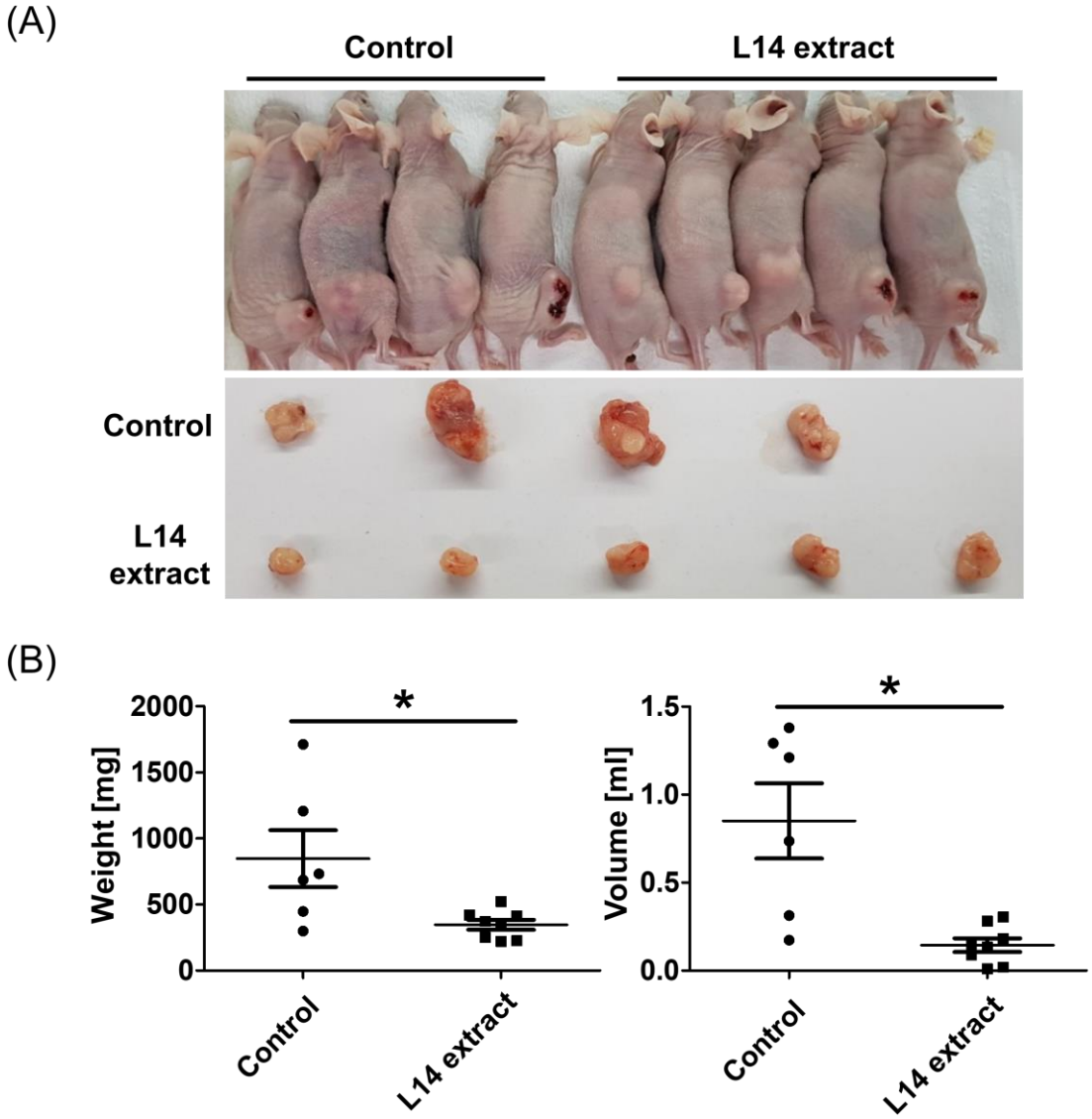

Figure 1. The tumor suppression effect of the L14 extract *in vivo*. 5×10^6 cells of A375SM were injected subcutaneously into the right femoral region of immunodeficient BALB/c mice. After tumor growth, PBS (control group) and the L14 extract was treated intraperitoneally every 2 days. (A) The mice with tumor and dissected tumors. (B) The quantification of tumor weight and volume. * $p < 0.05$.

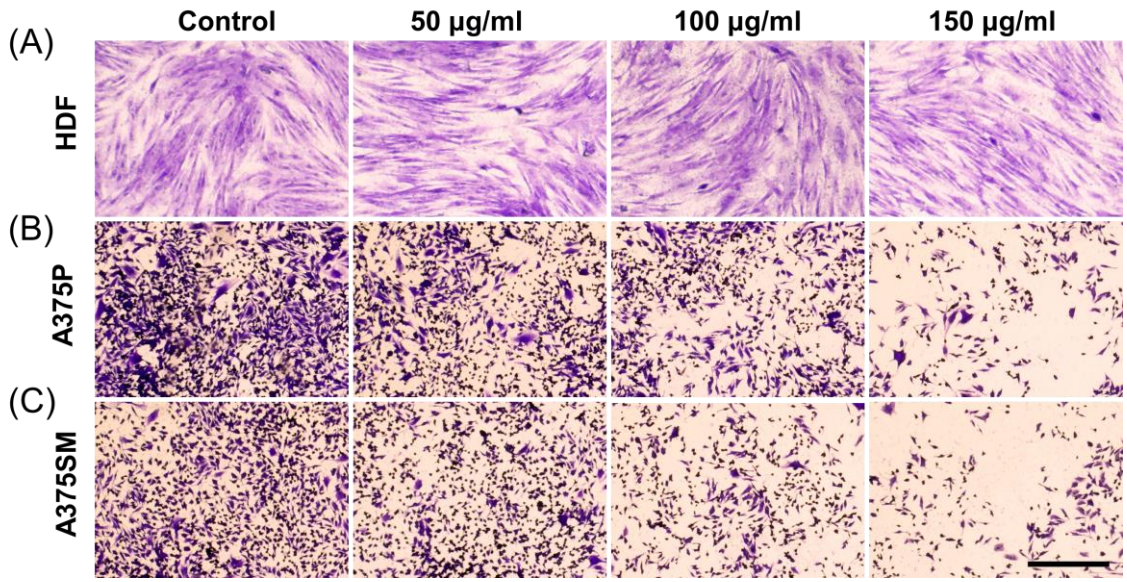

Figure 2. The effect of the L14 extract on the morphology and the growth of skin-related cell lines. The morphology and the growth were investigated after the 72 h treatment of the L14 extract using crystal violet solution. (A) Human dermal fibroblast (HDF; non-neoplastic cells). (B-C) A375P and A375SM (Human malignant melanoma cells). Scale bar = 200 µm.

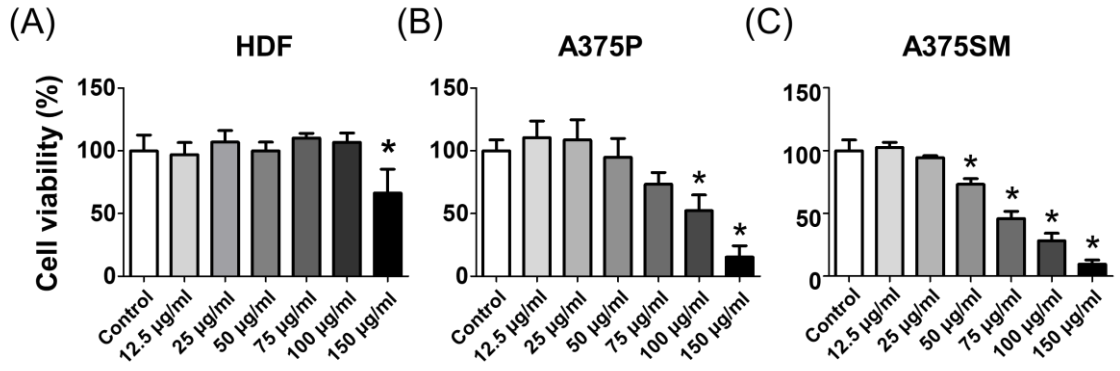

Figure 3. The cell viability after the treatment of the L14 extract. The cell viability was investigated after the 72 h treatment of various concentrations L14 extract by WST-8 viability assay. The viability of (A) HDF, (B) A375P and (C) A375SM. * $p < 0.05$ versus the control group.

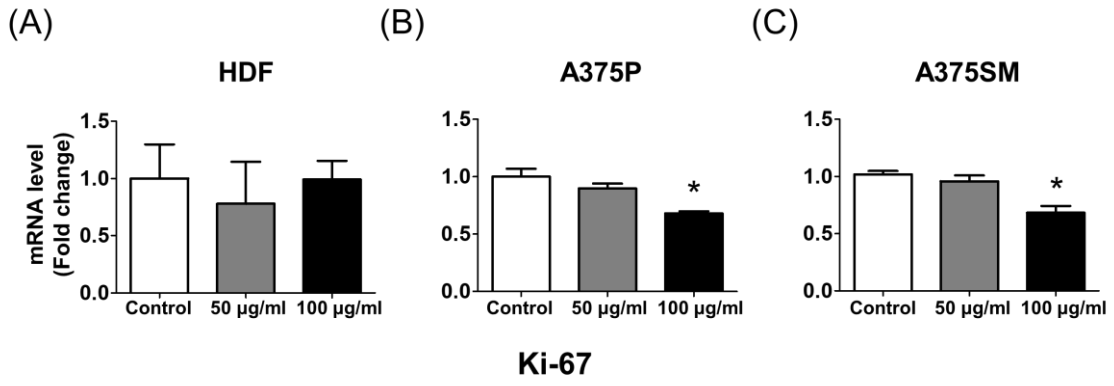

Figure 4. mRNA expression of the proliferation gene after the treatment of the L14 extract. The mRNA expression of Ki-67 was measured by qRT-PCR in (A) HDF, (B) A375P and (C) A375SM. Values were normalized against GAPDH and depicted as fold change values. * $p < 0.05$ versus the control group.

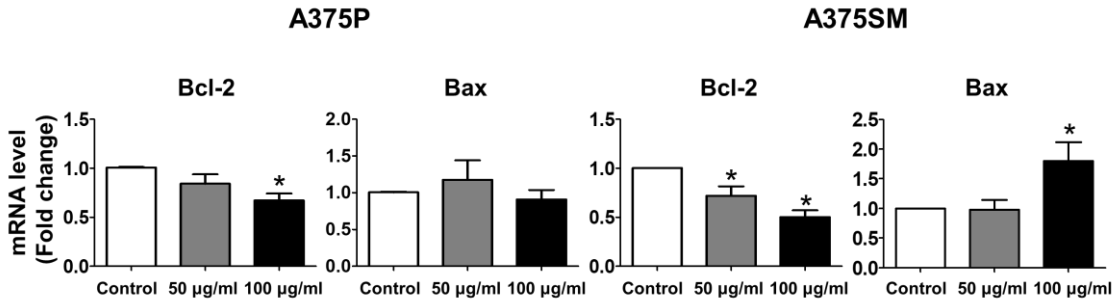

Figure 5. mRNA expression of the apoptosis genes after the treatment of the L14 extract. The mRNA expression of Bcl-2 and Bax was measured by qRT-PCR in A375P and A375SM. Values were normalized against GAPDH and depicted as fold change values. * $p < 0.05$ versus the control group.

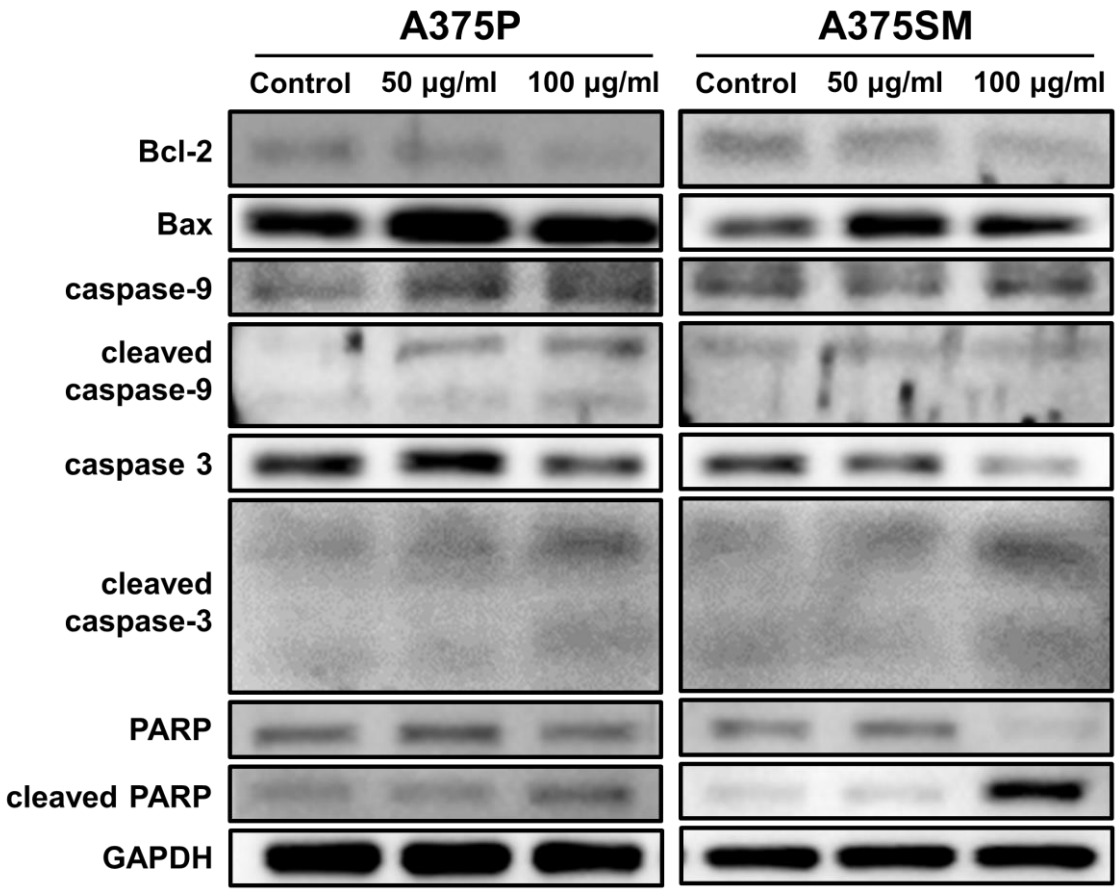

Figure 6. Protein expression of the apoptosis genes after the treatment of the L14 extract. The relative protein level of Bcl-2, Bax, caspase-9, cleaved caspase-9, caspase-3, cleaved cacspase-3, PARP, and cleaved PARP was measured by western blot analysis in A375P and A375SM. The housekeeping protein GAPDH was used to control for loading.

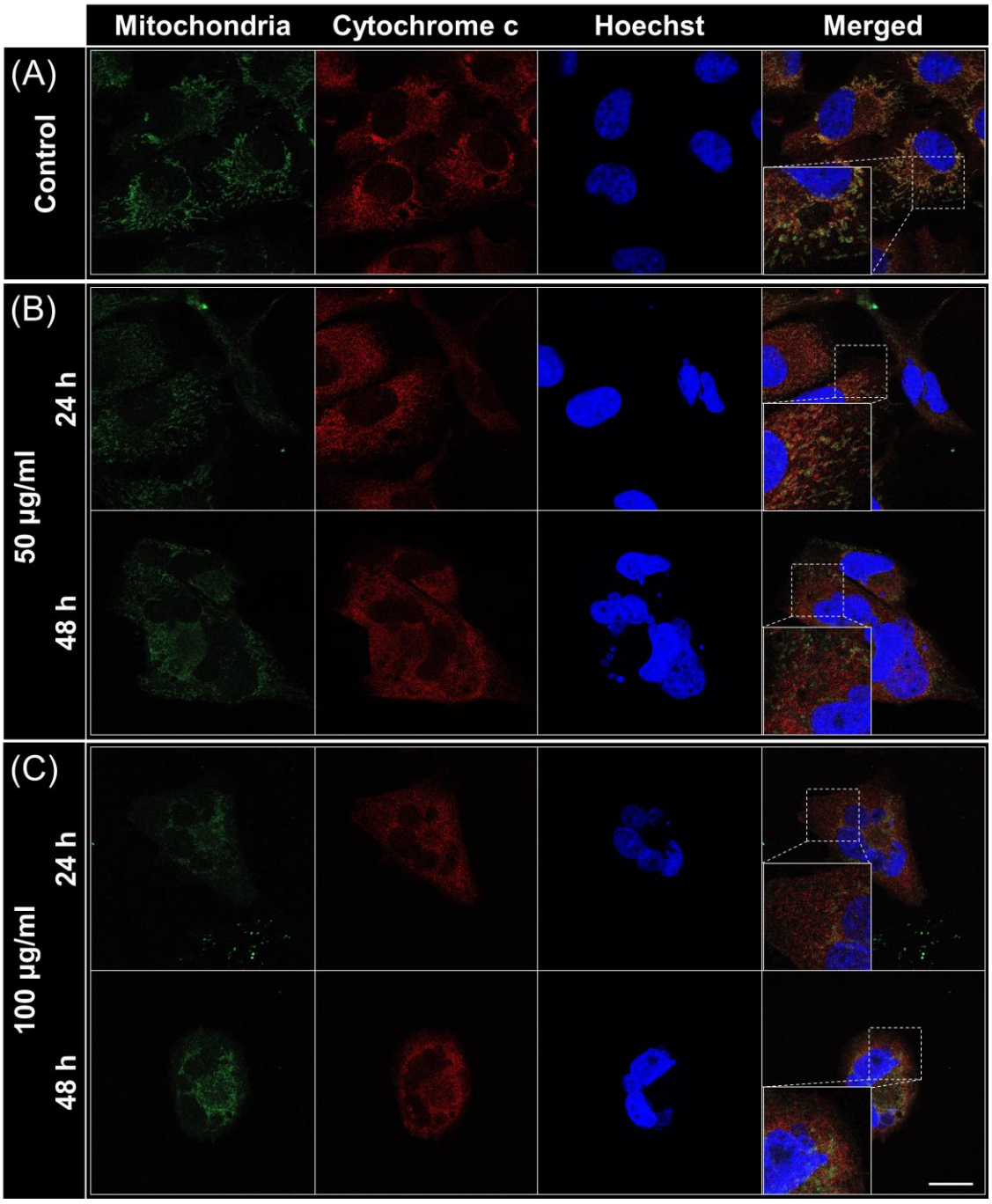
Mitochondria
Cytochrome c
Hoechst
Merged
(A)
Control
(B)
50 µg/ml
24 h
48 h
(C)
100 µg/ml
24 h
48 h

Figure 7. Immunofluorescence for the location of cytochrome c in A375SM after the treatment of the L14 extract. The locational change of cytochrome c was detected by staining mitochondria (green), cytochrome c (red). Hoechst indicates nuclei. (A) The non-treated A375SM, (B) the 50 μg/ml-treated group and (C) the 100 μg/ml-treated group. In the L14 extract treated groups, the upper panels were at 24 h of the treatment and the lower panels were at 48 h of the treatment. Scale bar = 20 μm.

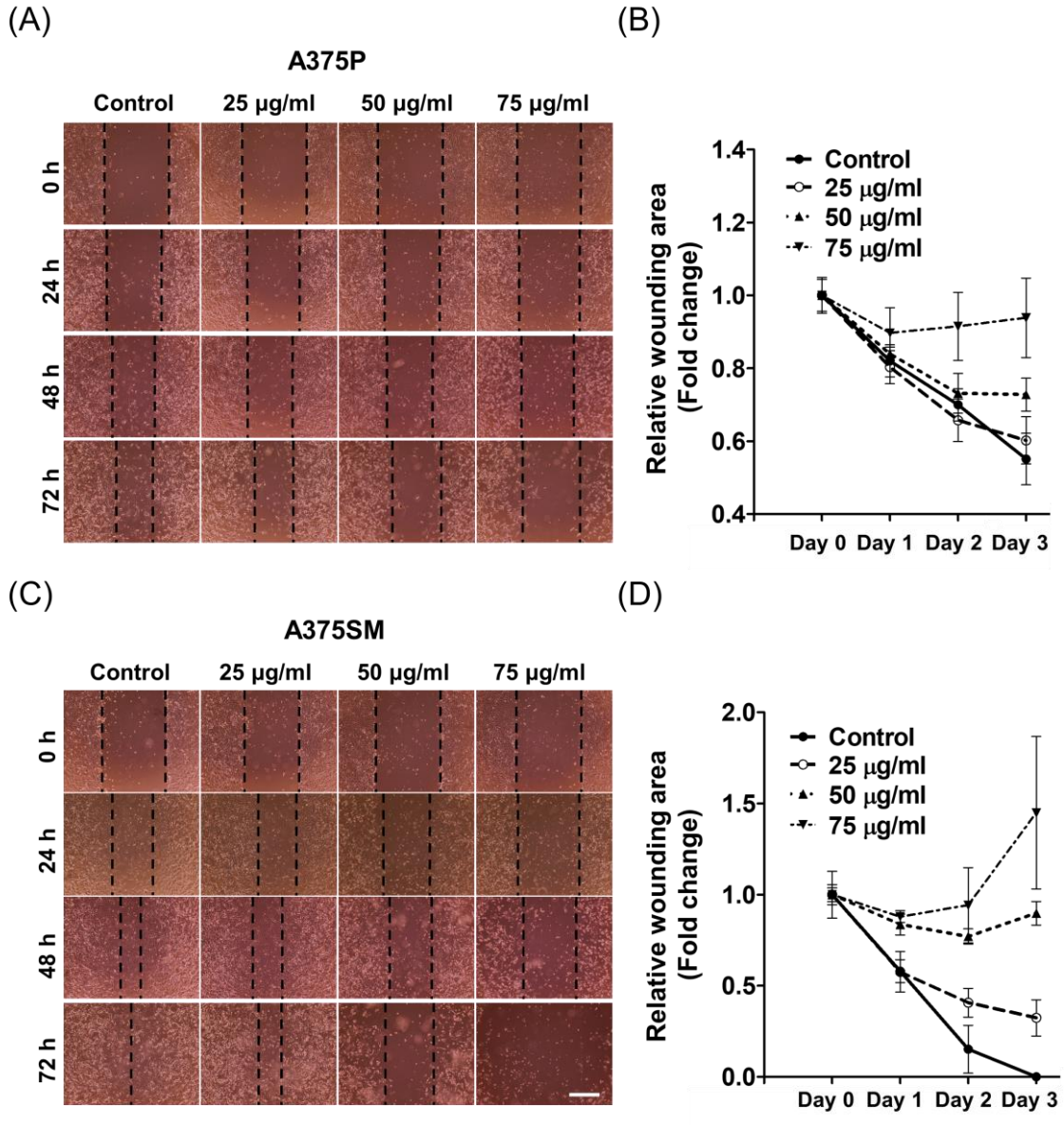

Figure 8. Analysis of cell migrative ability after the treatment of the L14 extract. The cells were cultured with the L14 extract after scratching using a pipette tip. (A) The A375P cells were observed every 24 h and (B) the wounding area was quantified. (C and D) The A375SM cells were analyzed in the same manner. Scale bar = 200 μm.

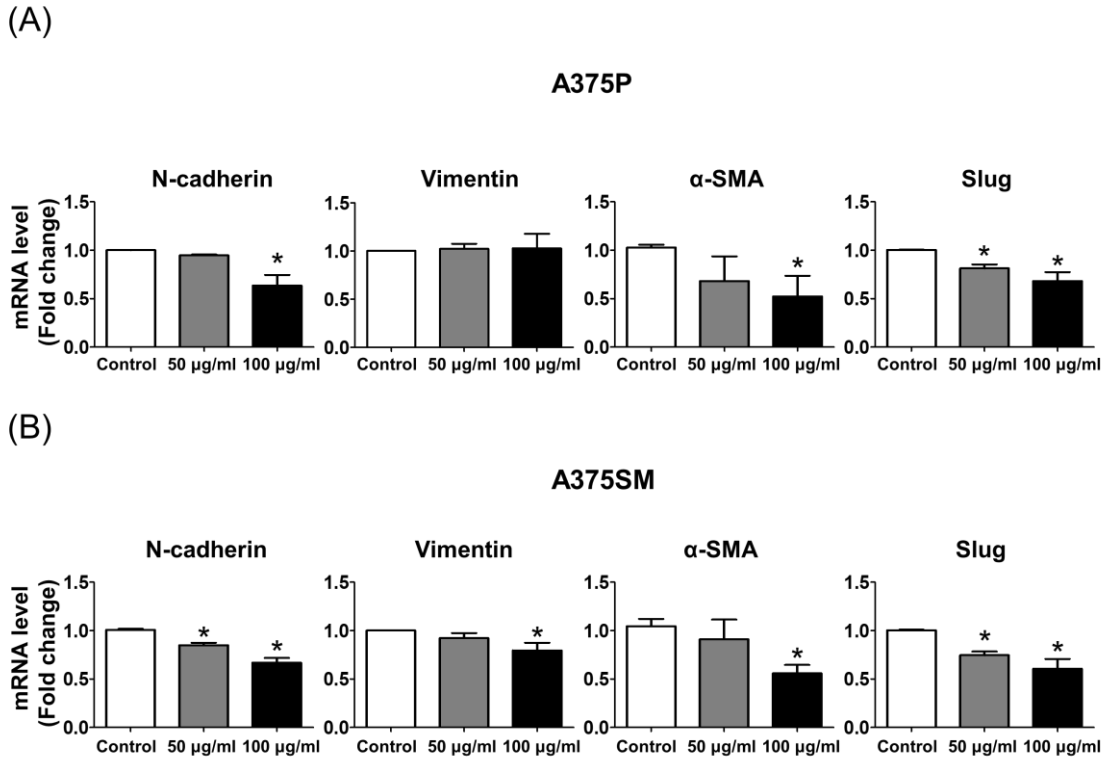

Figure 9. mRNA expression of the migration-related genes after the treatment of the L14 extract. The mRNA expression of N-cadherin, Vimentin, α-SMA and Slug was measured by qRT-PCR in (A) A375P and (B) A375SM. Values were normalized against GAPDH and depicted as fold change values. * $p < 0.05$ versus the control group

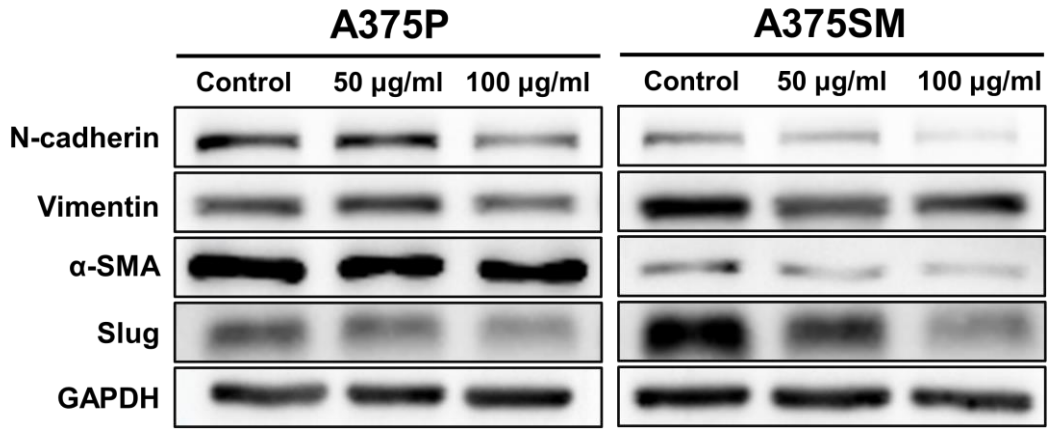

Figure 10. Protein expression of the migration-related genes after the treatment of the L14 extract. The relative protein level of N-cadherin, Vimentin, α-SMA and Slug was measured by western blot analysis in A375P and A375SM. The housekeeping protein GAPDH was used to control for loading.

DISCUSSION

Malignant melanoma is one of the most aggressive cancers with a high metastasis ability. Despite the attempts to develop therapies for melanoma, the mortality rate remains high. For example, even dacarbazine, the only food and drug administration-approved chemotherapy applied to patients with stage IV, does not increase the overall survival rate [17]. Therefore, the development of new drugs for melanoma is constantly required.

LAB are widely used for human health. Recently, there have been attempts to cure cancers using LAB. However, the effects of *L. plantarum* extract on human malignant melanoma have not been investigated. Accordingly, this study was conducted to demonstrate the potential of *L. plantarum* strain L14 extract as a treatment for human malignant melanoma using A375P and A375SM.

The representative characteristics of cancers are unlimited proliferation, metastasis and evasion of apoptosis [18]. The main objectives of various anti-cancer drugs are to hamper these characteristics of cancers and to return to the normal state. The present study confirmed that the L14 extract inhibited the growth of tumors induced by A375SM *in vivo*. In the subsequent *in vitro* experiments to determine the causes, it was confirmed that the L14 extract also inhibited the growth of human melanoma cells, and down-regulated the mRNA expression of Ki-67. It has been known that the expression of Ki-67 is strongly associated with the growth and proliferation of cancer, allowing that it can be used as a marker of tumor malignancy. The previous studies have suggested that Ki-67 can be used

as a tool for the diagnosis and prognosis of cancer, indicating the potential to be a target for anti-cancer drugs [19, 20].

The decrease in the viability by the L14 extract could be associated with the induction of apoptosis. Apoptosis is typically characterized by the nuclear fragmentation, the changes of apoptosis-related gene expression, the cleavage of apoptosis-related proteins, and the locational change of cytochrome c. Apoptosis is divided into two major pathways, intrinsic and extrinsic pathway. In the intrinsic or mitochondrial apoptosis, Bax increases the permeabilization of the mitochondrial membrane, causing the release of cytochrome c from the mitochondria [21]. The released cytochrome c converts pro-caspase-9 into caspase-9 [22]. Subsequently, the caspase-9 activates the executioner caspase-3, which thereafter induces the cleavage of PARP. I confirmed the features of apoptosis after the treatment of the L14 extract; nuclear fragmentation, up-regulation of Bax, down-regulation of Bcl-2 and the cleavage of caspase-9, caspase-3 and PARP while the mRNA expression of BAX was not changed in A375P. Also, it was detected that cytochrome c was released from mitochondria to cytosol, which has often been regarded as the point of no return in the intrinsic apoptosis [23]. This study indicated that the L14 extract induced the intrinsic pathway of apoptosis in human melanoma cells

In the following experiment, the anti-migratory effect by the L14 extract showed an additional possibility as a drug for melanoma. The inhibition of epithelial-mesenchymal transition (EMT), a process by which cells lose their epithelial junctions and gain the migratory and invasive ability, is important to the development of new therapy for cancer metastasis [24]. The previous studies suggested these mesenchymal genes are involved in the metastasis of cancer and can be therapeutic targets [25-28]. The treatment of the

L14 extract reduced both the mRNA and protein expression of mesenchymal markers, such as N-cadherin, Vimentin, α-SMA and Slug [29]. Consistent with the gene expression, it was also confirmed that the L14 extract inhibited migration of both A375 cells at the cellular level. It has been reported that the resistance to apoptosis was conferred by the induction of EMT [30, 31]. Therefore, the induction of apoptosis by the L14 extract could be closely related to the reduction of EMT.

In the development of anti-cancer drugs, the induction of apoptosis is the most effective non-surgical method [32]. Although chemotherapies to induce apoptosis have been actively studied, the traditional treatments have moderate-to-severe toxicities and side effects [33]. However, the L14 extract induced the inhibition of growth and apoptosis at a lower concentration in both A375 than in HDF, indicating that the L14 extract could have fewer side effects on normal cells.

CONCLUSION

Taken together, I demonstrated that the L14 extract decreased migratory ability via the reduction of mesenchymal cell-related gene expression and induced apoptosis and the activation of the intrinsic pathway by up-regulating of Bax, down-regulating Bcl-2, cleaving caspase-9, caspase-3 and PARP as well as releasing cytochrome c from mitochondria in human malignant cells, A375. While exerting these effects, the L14 extract acted on non-neoplastic cells, HDF, at a higher concentration than both A375 cells. Although further studies are required to find exactly what the effect molecule is via fractionation of the extract and why the efficacy of the L14 extract was different in A375P and A375SM , the results in the present study indicate that the L14 extract could be a worthwhile candidate for the treatment of melanoma.

REFERENCES

1. Van der Walt, N. B.; Zakeri, Z.; Cronje, M. J., The induction of apoptosis in A375 malignant melanoma cells by *Sutherlandia frutescens*. *Evid. Based. Complement. Alternat. Med.* **2016,** 2016, 4921067.
2. Guy, G. P.; Ekwueme, D. U., Years of potential life lost and indirect costs of melanoma and non-melanoma skin cancer: A systematic review of the literature. *Pharmacoeconomics* **2011,** 29, (10), 863-74.
3. Balch, C. M.; Gershenwald, J. E.; Soong, S. J.; Thompson, J. F.; Atkins, M. B.; Byrd, D. R.; Buzaid, A. C.; Cochran, A. J.; Coit, D. G.; Ding, S.; Eggermont, A. M.; Flaherty, K. T.; Gimotty, P. A.; Kirkwood, J. M.; McMasters, K. M.; Mihm, M. C., Jr.; Morton, D. L.; Ross, M. I.; Sober, A. J.; Sondak, V. K., Final version of 2009 AJCC melanoma staging and classification. *J. Clin. Oncol.* **2009,** 27, (36), 6199-206.
4. Song, X.; Zhao, Z.; Barber, B.; Farr, A. M.; Ivanov, B.; Novich, M., Overall survival in patients with metastatic melanoma. *Curr. Med. Res. Opin.* **2015,** 31, (5), 987-91.
5. Freeman, K.; Dinnes, J.; Chuchu, N.; Takwoingi, Y.; Bayliss, S. E.; Matin, R. N.; Jain, A.; Walter, F. M.; Williams, H. C.; Deeks, J. J., Algorithm based smartphone apps to assess risk of skin cancer in adults: Systematic review of diagnostic accuracy studies. *Br. Med. J.* **2020,** 368, m127.
6. Faiao-Flores, F.; Coelho, P. R.; Arruda-Neto, J.; Maria, D. A., Boron neutron

capture therapy induces cell cycle arrest and DNA fragmentation in murine melanoma cells. *Appl. Radiat. Isot.* **2011,** 69, (12), 1741-4.

7. Ozben, T., Antioxidant supplementation on cancer risk and during cancer therapy: An update. *Curr. Top. Med. Chem.* **2015,** 15, (2), 170-8.

8. Cosentino, D.; Piro, F., Hyaluronic acid for treatment of the radiation therapy side effects: A systematic review. *Eur. Rev. Med. Pharmacol. Sci.* **2018,** 22, (21), 7562-7572.

9. Zielinska, D.; Kolozyn-Krajewska, D., Food-origin lactic acid bacteria may exhibit probiotic properties: Review. *Biomed Res. Int.* **2018,** 2018, 5063185.

10. Ren, D.; Zhu, J.; Gong, S.; Liu, H.; Yu, H., Antimicrobial characteristics of lactic acid bacteria isolated from homemade fermented foods. *Biomed Res. Int.* **2018,** 2018, 5416725.

11. Bintsis, T., Lactic acid bacteria: Their applications in foods. *J. Bacteriol. Mycol.* **2018,** 6, (2), 89-94.

12. Perdigon, G.; Alvarez, S.; Rachid, M.; Aguero, G.; Gobbato, N., Immune system stimulation by probiotics. *J. Dairy. Sci.* **1995,** 78, (7), 1597-606.

13. Corr, S. C.; Gahan, C. G.; Hill, C., Impact of selected *Lactobacillus* and *Bifidobacterium* species on Listeria monocytogenes infection and the mucosal immune response. *FEMS Immunol. Med. Microbiol.* **2007,** 50, (3), 380-8.

14. Pochard, P.; Gosset, P.; Grangette, C.; Andre, C.; Tonnel, A. B.; Pestel, J.; Mercenier, A., Lactic acid bacteria inhibit TH2 cytokine production by mononuclear cells from allergic patients. *J. Allergy Clin. Immunol.* **2002,** 110, (4), 617-23.

15. Kassayova, M.; Bobrov, N.; Strojny, L.; Orendas, P.; Demeckova, V.; Jendzelovsky, R.; Kubatka, P.; Kiskova, T.; Kruzliak, P.; Adamkov, M.; Bomba, A.; Fedorocko, P., Anticancer and immunomodulatory effects of *Lactobacillus plantarum* LS/07, inulin and melatonin in NMU-induced rat model of breast cancer. *Anticancer Res.* **2016,** 36, (6), 2719-28.

16. Chuah, L. O.; Foo, H. L.; Loh, T. C.; Mohammed Alitheen, N. B.; Yeap, S. K.; Abdul Mutalib, N. E.; Abdul Rahim, R.; Yusoff, K., Postbiotic metabolites produced by *Lactobacillus plantarum* strains exert selective cytotoxicity effects on cancer cells. *BMC Complement. Altern. Med.* **2019,** 19, (1), 114.

17. Yue, Y.; Liu, L.; Liu, P.; Li, Y.; Lu, H.; Li, Y.; Zhang, G.; Duan, X., Cardamonin as a potential treatment for melanoma induces human melanoma cell apoptosis. *Oncol. Lett.* **2020,** 19, (2), 1393-1399.

18. Fouad, Y. A.; Aanei, C., Revisiting the hallmarks of cancer. *Am. J. Cancer Res.* **2017,** 7, (5), 1016-1036.

19. Li, L. T.; Jiang, G.; Chen, Q.; Zheng, J. N., Ki67 is a promising molecular target in the diagnosis of cancer (review). *Mol. Med. Rep.* **2015,** 11, (3), 1566-72.

20. Brown, D. C.; Gatter, K. C., Ki67 protein: The immaculate deception? *Histopathology* **2002,** 40, (1), 2-11.

21. Liu, X.; Kim, C. N.; Yang, J.; Jemmerson, R.; Wang, X., Induction of apoptotic program in cell-free extracts: Requirement for dATP and cytochrome c. *Cell* **1996,** 86, (1), 147-57.

22. Jiang, X.; Wang, X., Cytochrome c promotes caspase-9 activation by inducing nucleotide binding to Apaf-1. *J. Biol. Chem.* **2000,** 275, (40), 31199-203.

23. Ferraro, E.; Pulicati, A.; Cencioni, M. T.; Cozzolino, M.; Navoni, F.; di Martino, S.; Nardacci, R.; Carri, M. T.; Cecconi, F., Apoptosome-deficient cells lose cytochrome c through proteasomal degradation but survive by autophagy-dependent glycolysis. *Mol. Biol. Cell* **2008,** 19, (8), 3576-88.

24. Boyer, B.; Thiery, J. P., Epithelium-mesenchyme interconversion as example of epithelial plasticity. *APMIS.* **1993,** 101, (4), 257-68.

25. Wels, C.; Joshi, S.; Koefinger, P.; Bergler, H.; Schaider, H., Transcriptional activation of ZEB1 by Slug leads to cooperative regulation of the epithelial-mesenchymal transition-like phenotype in melanoma. *J. Invest. Dermatol.* **2011,** 131, (9), 1877-85.

26. Lee, H. W.; Park, Y. M.; Lee, S. J.; Cho, H. J.; Kim, D. H.; Lee, J. I.; Kang, M. S.; Seol, H. J.; Shim, Y. M.; Nam, D. H.; Kim, H. H.; Joo, K. M., Alpha-smooth muscle actin (ACTA2) is required for metastatic potential of human lung adenocarcinoma. *Clin. Cancer Res.* **2013,** 19, (21), 5879-89.

27. Mrozik, K. M.; Blaschuk, O. W.; Cheong, C. M.; Zannettino, A. C. W.; Vandyke, K., N-cadherin in cancer metastasis, its emerging role in haematological malignancies and potential as a therapeutic target in cancer. *BMC Cancer* **2018,** 18, (1), 939.

28. Li, M.; Zhang, B.; Sun, B.; Wang, X.; Ban, X.; Sun, T.; Liu, Z.; Zhao, X., A novel function for vimentin: The potential biomarker for predicting melanoma hematogenous metastasis. *J. Exp. Clin. Cancer Res.* **2010,** 29, 109.

29. Zeisberg, M.; Neilson, E. G., Biomarkers for epithelial-mesenchymal transitions. *J. Clin. Invest.* **2009,** 119, (6), 1429-37.

30. Gotzmann, J.; Mikula, M.; Eger, A.; Schulte-Hermann, R.; Foisner, R.; Beug, H.; Mikulits, W., Molecular aspects of epithelial cell plasticity: Implications for local tumor invasion and metastasis. *Mutat. Res.* **2004,** 566, (1), 9-20.

31. Chang, J. C., Cancer stem cells: Role in tumor growth, recurrence, metastasis, and treatment resistance. *Medicine (Baltimore)* **2016,** 95, (1 Suppl 1), S20-5.

32. Pfeffer, C. M.; Singh, A. T. K., Apoptosis: A target for anticancer therapy. *Int. J. Mol. Sci.* **2018,** 19, (2).

33. Wu, S.; Singh, R. K., Resistance to chemotherapy and molecularly targeted therapies: Rationale for combination therapy in malignant melanoma. *Curr. Mol. Med.* **2011,** 11, (7), 553-63.

국문초록

Lactobacillus plantarum L14 추출물이 인간 악성 흑색종 세포의 생존 및 이동에 미치는 영향

박재현

종양및발달생물학 전공

치의과학과

서울대학교 대학원

(지도교수: 노상호, D.V.M., Ph.D.)

인간 악성 흑색종은 높은 전이능력을 가지고 있는 가장 공격적인 피부암 유형이다. 여러가지의 전통적인 치료 방법들이 있음에도 불구하고 사망률은 여전히 높다. *Lactobacillus plantarum* (*L. plantarum*)은 유산균의 하나로 암치료를 위해 연구가 되고 있다. 그러나 *L. plantarum* 추출물이 인간 악성 흑색종에서 어떤 영향을 끼치는 지에 대한 연구는 아직 보고된 바가 없다.

따라서 본 연구에서는 *L. plantarum* 이 인간 악성 흑색종 세포에 어떤 영향을 끼치는 지 확인하고자 하였다. 이를 위해 *L. plantarum* 균주 L14 추출물을 인간 악성 흑색종 세포인 A375 세포에 다양한 농도로 처리하여 나타나는 효과를 세포생존능력 분석, 유전자 분석, 단백질 분석, 면역형광법과 세포이동능력 평가를 통해 분석하였고, 이와 같은 결과는 통계적 처리법을 통해 유의적인 차이를 확인하였다. L14 추출물은 A375 세포에서 사람 피부 섬유아세포에서보다 낮은 농도에 민감하게 반응하여 생존능력을 떨어뜨렸고 A375 세포를 이용해 유도한 종양의 성장도 억제하였다. 이러한 결과는 증식관련 유전자와 세포사멸에 관련된 유전자 발현의 변화에 기인한 것임을 확인하였고 세포내 cytochrome c 의 위치를 확인하여 L14 에 의한 세포사멸이 내인성 세포사멸을 유도한다는 것을 확인하였다. 또한, L14 추출물이 A375 의 세포이동을 억제하고 이동과 관련된 유전자의 발현 또한 억제하는 것을 확인하였다. 따라서, 이러한 연구 결과는 L14 추출물이 인간 악성 흑색종에 대한 항암 물질로써 개발될 수 있음을 암시한다.

주요어 : 유산균, 항암, 세포이동, 인간 악성 흑색종, 내인성 세포사멸

학 번 : 2018-22152