Moringa oleifera fruit induce apoptosis via reactive oxygen species-dependent activation of mitogen-activated protein kinases in human melanoma A2058 cells

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Abstract. The present study was performed to determine the effect of *Moringa oleifera* fruit extract on the apoptosis of human melanoma A2058 cells. A2058 cells were treated for 72 h with *Moringa oleifera* fruit extract at 50-100 μg/ml, and cell viability with apoptotic changes was examined. The involvement of reactive oxygen species (ROS) and mitogen-activated protein kinases (MAPKs) was examined. It was revealed that *Moringa oleifera* fruit extract significantly inhibited the cell viability and promoted apoptosis of A2058 cells in a concentration-dependent manner. *Moringa oleifera* fruit extract-treated A2058 cells exhibited increased activities of cleaved caspase-9 and caspase-3. It also caused an enhancement of MAPK phosphorylation and ROS production. The pro-apoptotic activity of *Moringa oleifera* fruit extract was significantly reversed by pretreatment with the c-Jun N-terminal kinase (JNK) inhibitor SP600125, extracellular-signal-regulated kinase (ERK) inhibitor PD98058 or ROS inhibitor N-acetyl-L-cysteine. Taken together, *Moringa oleifera* fruit extract is effective in inducing mitochondrial apoptosis of A2058 cells, which is mediated through induction of ROS formation, and JNK and ERK activation. *Moringa oleifera* fruit extract may thus have therapeutic benefits for human melanoma A2058 cells.

Introduction

Malignant melanoma is the most serious type of skin cancer and develops in melanocytes that produce melanin pigment (1). The most common site of malignant melanoma is the upper back, although it also occurs in the arms and legs (2). Previous studies have demonstrated that age, skin type and a family history of melanoma significantly affect the development of malignant melanoma. Surgery is the main treatment for early-stage malignant melanoma (3). However, there is no available treatment when melanoma passes early stage, or upon recurrence, making it an incurable disease with a high rate of metastasis (4,5). *Moringa oleifera* Lam is a tree of the Moringaceae family that can reach a height of between 5 and 10 m (6). *Moringa* is cultivated in Asia, Africa and Arabia, and is a good source of nutrition, since the plant is rich in proteins and vitamins (7). It has various pharmacological effects, including anti-hyperglycemic, anti-inflammatory and anticancer functions (8). *Moringa* leaves are the most nutritious part, as they are rich in β-carotene, proteins, vitamin C, calcium and antioxidants (9). There are two types of cell death process: Apoptosis and necrosis. Apoptosis is an important physiological mechanism in which apoptotic cells cause immune responses for removal of dead cells without destruction of surrounding cells, leading to characteristic cell changes, including cell shrinkage and membrane blebbing (10). This active process is mediated under the control of gene regulation (11). Reactive oxygen species (ROS) induce cancer and aging, formation of lipid peroxides, destruction of proteins and nucleic acids, and inhibition of various enzyme functions by attacking living cells. ROS are also mediators of intracellular signaling (12). However, excessive ROS production increases oxidative stress, resulting in cellular damage and inhibition of cellular functions and the cell cycle to cause apoptosis (13,14). Chemotherapeutic agents, including anticancer agents, exert relatively marked toxic effects, although certain cancer cells exhibit resistance. Once cancer cells acquire resistance to a particular anticancer agent, they have resistance to all anticancer agents operating via the same mechanism (15). Therefore, anticancer agents derived from natural compounds have been developed with decreased side effects and increased anticancer activity (16,17). It is important for patients to strengthen their immune systems upon occurrence of malignant melanoma. As synthetic anticancer agents may weaken the immune system, more studies on natural products for treatment of malignant melanoma are required. For the identification of cytoprotective agents from natural resources, the present study investigated the cytoprotective mechanisms underlying *Moringa oleifera* fruit, against mitochondrial apoptosis with respect to the induction of ROS formation, and c-Jun N-terminal kinase (JNK) and extracellular-signal-regulated kinase (ERK) activation in human melanoma A2058 cells.

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Materials and methods

Plants and sample extraction. *Moringa oleifera* fruit (MOF) were collected at Dar es Saalam, Tanzania, in September 2013. Botanical identification was made by Professor Henry Joseph Hjndangalasi, Department of Botany, Dar es Salaam University, Dar es Salaam, Tanzania. Dried MOF (13.0 g) were soaked in 70% ethanol and sonicated (40 kHz) for 3 h at room temperature. Extracts were evaporated in a dry oven at 60°C and stored at -20°C until used for the *in vitro* assay (yield, 0.2794 g).

Chemicals and reagents. MTT and propidium iodide were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Primary mouse monoclonal antibodies against β-actin (catalog no. sc-47778; dilution, 1:1,000) and rabbit monoclonal antibodies against B-cell lymphoma-2 (Bcl-2; catalog no. sc-492; dilution, 1:1,000) and Bcl-2-associated X protein (Bax; catalog no. sc-493; dilution, 1:1,000) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Rabbit monoclonal antibodies against cleaved caspases-3 (dilution, 1:1,000; catalog no. 9661), -8 (dilution, 1:1,000; catalog no. 8592) and -9 (dilution, 1:1,000; catalog no. 7237) and poly (ADP-ribose) polymerase (PARP; dilution, 1:1,000; catalog no. 5625) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase (HRP) -conjugated goat anti-rabbit IgG (HRP; catalog no. sc-2004; dilution, 1:2,000) and HRP-conjugated goat anti-mouse IgG (catalog no. sc-2005; dilution, 1:2,000) secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. Primary antibodies against caspase-9 (catalog no. 9501; dilution, 1:1,000), caspase-3 (catalog no. 9664; dilution, 1:1,000), JNK (catalog no. 9258; dilution, 1:1,000), phosphorylated (p)-JNK (catalog no. 4668; dilution, 1:1,000), ERK (catalog no. 4695; dilution, 1:1,000), p-ERK (catalog no. 4370; dilution, 1:1,000), p38 (catalog no. 9212; dilution, 1:1,000) and p-p38 (catalog no. 4511; dilution, 1:1,000) antibodies were purchased from Cell Signaling Technology, Inc. All other chemicals and reagents were of the highest analytical grade.

Cell culture. The human melanoma A2058 and human keratinocyte HaCaT cell lines were purchased from the Korean Cell Line Bank (Seoul, Korea) and Amore Pacific (Yongin, Gyeonggi-do, Republic of Korea). respectively. Cells were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.). Cells were incubated at 37°C in an incubator with a humidified atmosphere containing 5% CO_2 and were subcultured every 2 to 3 days. Cell counts were performed using a hemocytometer from Hausser Scientific (Horsham, PA, USA).

Cell viability assay. The cytotoxic effects of MOF extract on the A2058 and HaCaT cell lines were estimated colorimetrically using the MTT method, which is based on the reduction of tetrazolium salt by mitochondrial dehydrogenase in viable cells (18). The cells were seeded on a 96-well plate (density,

2×10^6 cells/ml) and were then treated with MOF extract at final concentrations of 0, 50, 75 and 100 µg/ml. After 72 h of incubation at 37°C, 50 µl MTT solution (2 mg/ml) was added to each well at a final concentration of 0.4 mg/ml. After 2 h of incubation at 37°C, the supernatants were aspirated and replaced with 150 µl dimethyl sulfoxide to dissolve the formazan product. The absorbance at 540 nm was then read using a spectrophotometric plate reader (model 550; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The results were calculated as percentages relative to the unexposed control.

Nuclear staining with Hoechst 33258. The nuclear morphology of the cells was observed using the DNA-specific blue fluorescent dye Hoechst 33258. The viable cells were stained homogeneously, whereas the apoptotic cells that exhibited chromatin condensation or nuclear fragmentation were not stained (19). The A2058 cells were treated with MOF extract at the various concentrations (0, 50, 75, 100 µg/ml). Cells were then fixed for 30 min at 37°C in 100% methanol, washed with PBS and stained with 2 µg/ml Hoechst 33258 (Sigma-Aldrich; Merck KGaA). The cells were observed under a BX51 fluorescence microscope (magnification, x200) and images were captured with a DP70 camera (Olympus Corporation, Tokyo, Japan).

Determination of ROS levels. Intracellular ROS generation was assessed using the stable nonpolar dye 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Sigma-Aldrich; Merck KGaA), which readily diffuses into the cells (20). Following treatment with MOF extract (100 µg/ml) for 24 h at 37°C, the A2058 cells were incubated at 37°C with 25 µM H_2DCF-DA for 30 min, and then washed twice with ice-cold PBS. The ROS production was measured using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). To cells grown in 6-well plates (1×10^6 cells/ml) for 24 h, an antioxidant N-acetyl-L-cystein (NAC; 2 mM) was added for 1 h prior to exposing them to 100 µg/ml MOF extract for 72 h at 37°C. A total of 25 µM of H_2DCF-DA was then added and the cells incubated for 30 min at 37°C, and then washed twice with ice-cold PBS. The ROS production was evaluated using a FACSCalibur flow cytometer (BD Biosciences).

Cell cycle analysis. Cell cycle analysis was performed to determine the proportion of apoptotic sub-G1 hypodiploid cells (21). The A2058 cells were plated on 6-well plates (1×10^6 cells/ml) and incubated for 24 h at 37°C. The cells were treated with MOF extract (0, 50, 75, 100 µg/ml) and incubated for 72 h at 37°C, following which they were trypsinized, harvested and washed with PBS. The pellet was fixed using ice-cold 70% ethanol at 4°C for 30 min. The cells were then washed once with PBS and resuspended in 50 µg/ml ice-cold propidium iodide (PI) containing 50 µg/ml RNase A in PBS (pH 7.4) for 30 min in the dark. Fluorescence emitted from the PI-DNA complex was quantified using a FACSCalibur flow cytometer.

Apoptosis analysis. An Annexin V-PI double staining assay was performed to differentiate early and late apoptosis stages. The assay was determined using an ApoScan™ Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BioBud Co. Ltd., Seoul, Korea) in the MOF

extract-treated A2058 cells. Cells were trypsinized, harvested and washed with PBS, and were subsequently were resuspended in 500 μl 1X binding buffer (50 mM HEPES, 700 mM NaCl, 12.5 mM CaCl$_2$, PH 7.4) and incubated with 1.25 μl Annexin V-FITC (200 μg/ml) at room temperature for 15 min. The supernatant was then removed following centrifugation at 400 x g for 10 min at 4˚C. The cells were then resuspended in 500 μl 1X binding buffer, and cell suspensions were stained with 10 μl PI (30 μg/ml) at 4˚C in the dark. Fluorescence was quantified using a FACSCalibur flow cytometer. The number of cells in early and late apoptosis was determined as the percentage of Annexin V$^+$/PI$^-$ or Annexin V$^+$/PI$^+$ cells, respectively.

Western blot analysis. Western blot analyses were performed as previously described (22). The cells were cultured, harvested and lysed on ice for 30 min in lysis buffer (120 mM NaCl, 40 mM, pH 8.0, and 0.1% nonyl phenoxypolyethoxyle-thanol) and were then centrifuged at 13,000 x g for 15 min at 4˚C. Lysates from each sample were mixed with 5X sample buffer (0.375 M Tris-HCl, 5% SDS, 5% 2-mercaptoethanol, 50% glycerol and 0.05% bromophenol blue, pH 6.8) and were then heated to 95˚C for 5 min. Equal amounts of protein (25 μg) were separated by SDS-PAGE (12% gel) and were transferred onto nitrocellulose membranes. The membranes were then washed with TBS (10 mM Tris-HCl and 150 mM NaCl) containing 0.05% Tween-20 (TBST), and were then blocked in TBST containing 5% nonfat dried milk. The membranes were then incubated with the aforementioned specific primary antibodies overnight at 4˚C. Subsequent to 3 washes in TBST, the membranes were incubated with the appropriate HRP-conjugated secondary antibodies for 1 h at room temperature. The membranes were then washed 3 times in TBST with 15 min between each step, and protein detection was performed using an enhanced chemiluminescence western blotting detection ECL kit (Bio-Rad, Hercules, CA, USA). The intensity of each band was analyzed using Image J software (version k 1.45; National Institute of Health, Bethesda, MD, USA). The expression level of individual proteins in the MOF-treated cells was evaluated and expressed relative to that of the control, of which the expression level was designated as 1.0.

Statistical analysis. SPSS software (version 22.0; IBM SPSS, Armonk, NY, USA) was used to analyze the data. The results were subjected to analysis of variance, followed by Tukey's range test in order to analyze the differences between conditions. In each case, P<0.05 was considered to indicate a statistically significant difference. All measurements were made in triplicate, and all values are presented as the mean ± standard deviation.

Results

Cytotoxic effects of MOF extract in A2058 and HaCaT cell lines. To examine the proliferation inhibitory effects of MOF extract, human melanoma A2058 cells were exposed to MOF extract at concentrations of 50, 75 and 100 μg/ml for 72 h, prior to the cell viability being determined using an MTT assay. As presented in Fig. 1A, MOF extract decreased the viability
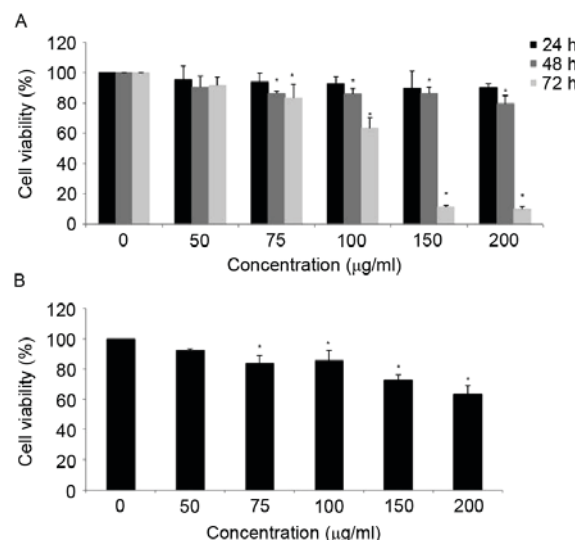

Figure 1. Cytotoxic effects of MOF extract on (A) A2058 and (B) HaCaT cells. Cell viability at the indicated concentrations of MOF extract from the A2058 and HaCaT cell lines was assessed for 72 h, using MTT assays. *P<0.05 vs. control cells. MOF, *Moringa oleifera* fruit.

of A2058 cells in a dose-dependent manner. The viability of MOF extract-treated A2058 cells was decreased to 91.6, 83.1 and 63.5% at concentrations of 50, 75 and 100 μg/ml for 72 h, respectively. At concentrations of 150 and 200 μg/ml, the MOF extract markedly decreased the cell proliferation to 11.3 and 10.1%, respectively. To compare the cytotoxic effects of MOF extract in human keratinocyte HaCaT cells, the HaCaT cells were treated with concentrations of MOF extract of 50, 75, 100, 150 and 200 μg/ml for 72 h. The results indicated that HaCaT cells were more resistant to MOF extract-induced cytotoxicity compared with human melanoma A2058 cells (Fig. 1B). Therefore, MOF extract concentrations of 50, 75 and 100 μg/ml were selected for subsequent experiments.

Induction of apoptosis in A2058 cells. The apoptogenic property of MOF extract was investigated through morphological changes in A2058 cells. Nuclear Hoechst 33258 staining was performed in order to determine whether the anti-proliferative effect of MOF extract was due to apoptosis. As presented in Fig. 2, A2058 cells treated with MOF extract exhibited a number of morphological changes, including membrane blebbing, nuclear fragmentation, chromatin condensation and an increased density of apoptotic bodies compared with the untreated control cells. The number of apoptotic cells increased in a dose-dependent manner, which is consistent with the results of the MTT assay.

Effects on generation of ROS. To investigate the possible underlying mechanisms of MOF extract-induced apoptosis, ROS production was examined using the specific fluorescent probe H$_2$DCF-DA. Using flow cytometry, MOF extract caused a rightward shift for H$_2$DCF-DA, indicating increased production of hydroxyl radicals and hydrogen peroxide, compared with the control, followed by cell death (Fig. 3A). However, co-treatment with MOF extract and NAC slightly attenuated ROS production, compared with MOF extract alone, indicating that NAC may act as a scavenger of ROS (Fig. 3B). The results
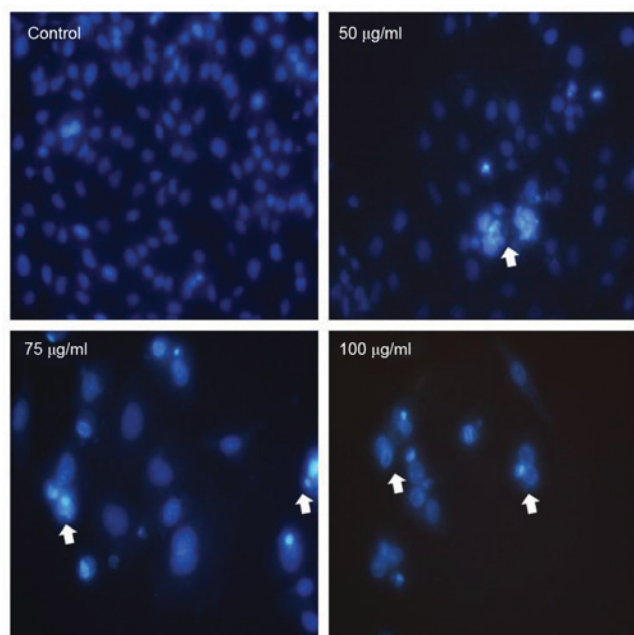
Figure 2. Induction of apoptosis by *Moringa oleifera* fruit on A2058 cells at the concentrations indicated. The formation of apoptotic bodies (arrows) in Hoechst 33258-stained cells was observed using fluorescence microscopy (magnification, x400).
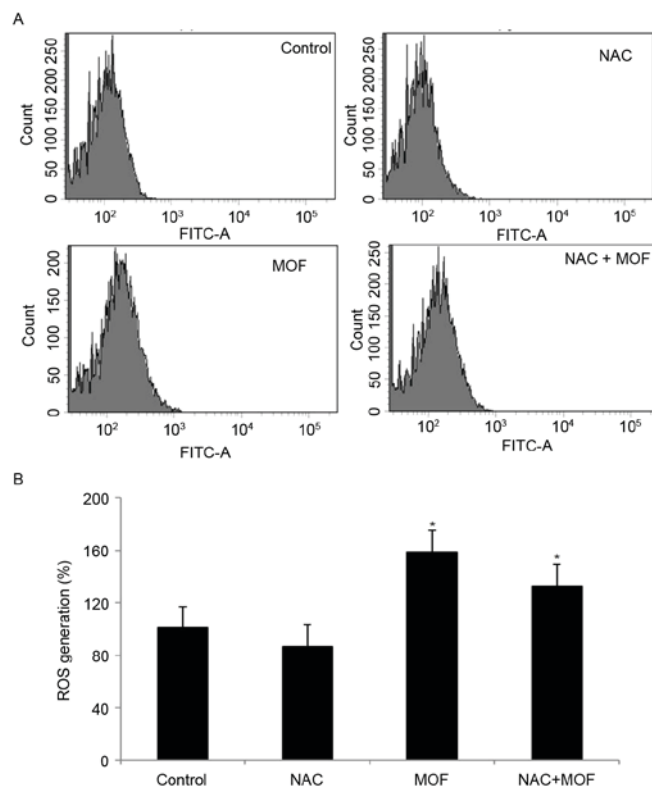

Figure 3. Alterations in ROS levels on MOF extract-treated A2058 cells. (A) 2',7'-dichlorodihydrofluorescein diacetate staining was conducted to measure alterations in the ROS level. Following incubation with NAC and MOF extract for 72 h, ROS levels were measured by flow cytometry. (B) Statistical analysis of apoptosis. *P<0.05 vs. control cells. FITC, fluorescein isothiocyanate; ROS, reactive oxygen species; MOF, *Moringa oleifera* fruit; NAC, N-acetyl-L-cysteine.

demonstrated that ROS is involved in MOF extract-mediated apoptotic processes.

Effects on cell cycle progression in A2058 cells. To investigate the effect of MOF extract on cell cycle progression, flow cytometry was performed with 50, 75 and 100 μg/ml MOF extract. As presented in Fig. 4, 13.9% of control cells were in sub-G_1 phase, whereas 29.9, 31.1 and 57.5% of MOF extract-treated cells were in sub-G_1 phase, at concentrations of 50, 75 and 100 μg/ml, respectively. This was accompanied by a significant decrease in the numbers of A2058 cells in S phase of 28.5, 20.5 and 16.3%, and G2/M phase of 23.1, 18.7 and 6.9 at 50, 75 and 100 μg/ml, respectively.

Effects on apoptosis in A2058 cells. In order to quantify the percentage of apoptotic cells, flow cytometry analysis was performed using double staining with Annexin V and PI. The Annexin V$^-$/PI$^-$ population was considered to account for unaffected cells, the Annexin V$^+$/PI$^-$ population represented early apoptosis, the Annexin V$^+$/PI$^+$ population represented late apoptosis and the Annexin V$^-$/PI$^+$ population represented necrosis. The results demonstrated that the treatment of the cells with MOF extract significantly increased the percentage of apoptotic cells compared with untreated control cells (Fig. 5). MOF extract-treated A2058 cells exhibited increased proportions of early apoptotic cells to 6.5, 27.3 and 31.9% at 50, 75 and 100 μg/ml, respectively, compared with 3.2% for the control. The proportions of late apoptotic cells also increased to 11.2, 21.6 and 26.6% at 50, 75 and 100 μg/ml, respectively, compared with 9.0% for the control. The total number of apoptotic cells increased from 12.2% in control cells to 17.7, 48.9 and 58.5% at 50, 75 and 100 μg/ml, respectively. The proportion of early apoptotic cells was increased at 50 μg/ml compared with that of late

apoptotic cells. However, the population of late apoptotic cells was increased compared with that of early apoptotic cells. From these results, it is hypothesized that anti-proliferative effects of MOF extract in A2058 melanoma cells may be mediated by the induction of cell apoptosis in a dose-dependent manner.

Effects on Bcl-2, Bax and caspase expression in A2058 cells. In order to investigate the effects of MOF extract on apoptosis in A2058 cells, the expression levels of apoptotic regulatory proteins, including Bcl-2, Bax and caspases, were examined. As presented in Fig. 6, MOF extract increased Bax protein expression, but decreased the expression of Bcl-2, in a dose-dependent manner. The disruption of the mitochondrial plasma membrane by MOF extract was followed by the activation of the cleaved caspase-3 and 9 and target protein, PARP, respectively. These results, together with the Bax/Bcl-2 ratio, indicated that the MOF extract may induce apoptosis through the regulation of apoptosis-associated protein expression in human melanoma A2058 cells.

Effects on mitogen-activated protein kinase (MAPK) expression in A2058 cells. The MAPKs, including JNK, ERK and p38 kinase, are present in all eukaryotes and have been demonstrated to perform central roles in regulating cell proliferation, differentiation and apoptosis (23). In order to investigate the effects of MOF extract in A2058 cells, the
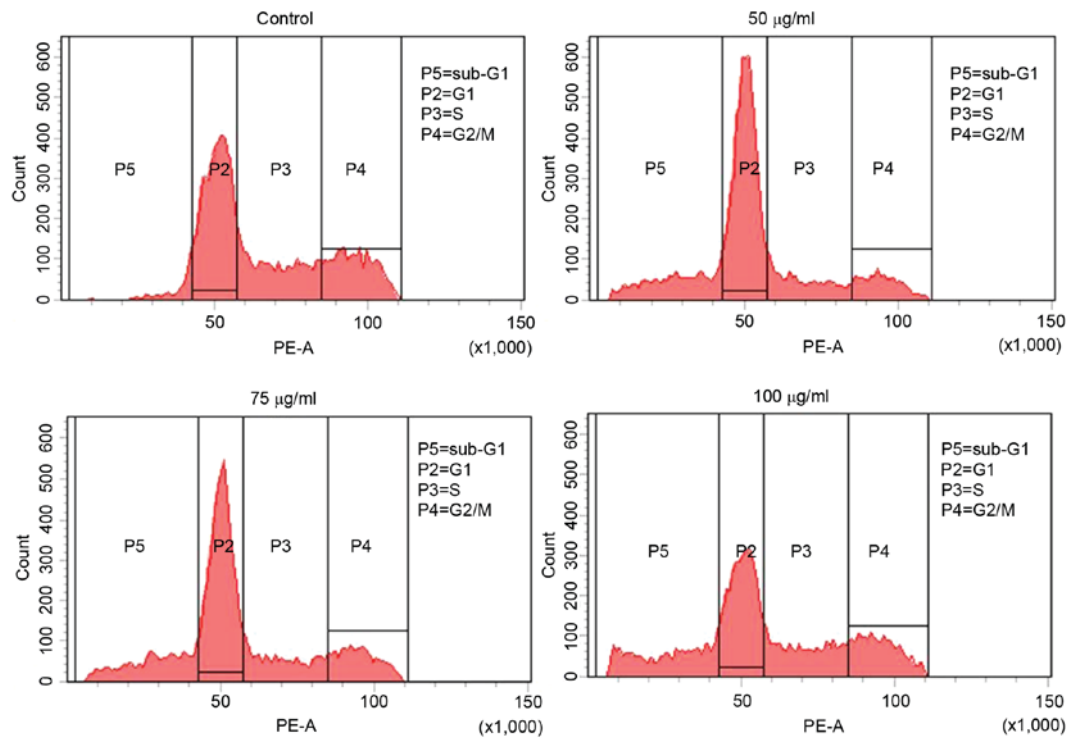
Figure 4. Effects of various concentrations of *Moringa oleifera* fruit on the cell cycle distribution in A2058 cells. The sub-G1 cells were detected by flow cytometry following propidium iodide staining.
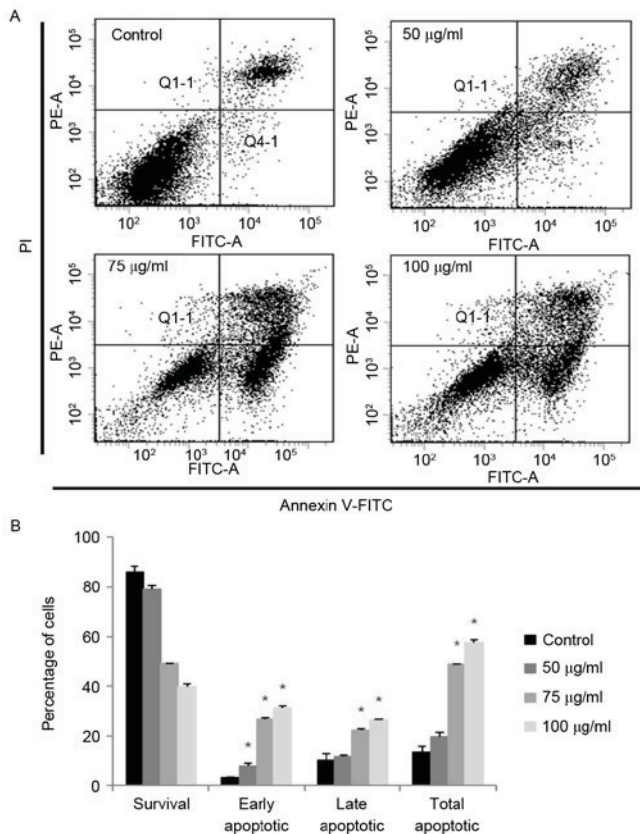

Figure 5. Induction of apoptosis by MOF extract in A2058 cells. (A) Flow cytometric analysis of A2058 cells incubated with MOF extract for 72 h. The lower-right quadrant represents Annexin V-stained cells (early-phase apoptotic cells). The upper-right quadrant represents PI- and annexin V-stained cells (late-phase apoptotic cells). (B) Quantification of apoptosis. *P<0.05 vs. control cells. MOF, *Moringa oleifera* fruit; PI, propidium iodide; FITC, fluorescein isothiocyanate.
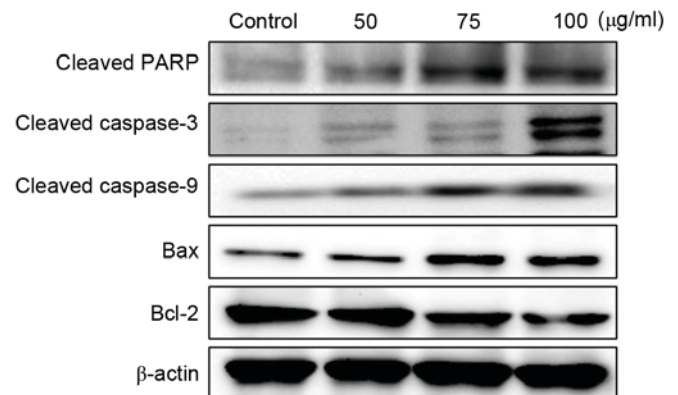

Figure 6. Expression of caspase-associated proteins by *Moringa oleifera* fruit by A2058 cells. Cell lysates were electrophoresed, and cleaved caspase-3, caspase-9 and PARP were detected using western blot analysis. PARP, poly (ADP-ribose) polymerase; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein.

phosphorylation of MAPKs was determined. As presented in Fig. 7A, expression levels of non-phosphorylated ERK, JNK and p38 were unchanged with MOF extract. By contrast, accumulation of p-ERK and p-JNK markedly increased in a time-dependent manner. These results indicated that MOF extract induces apoptosis through activation of ERK and JNK in A2058 cells. In order to determine the effects of ERK and JNK on MOF extract-induced A2058 cytotoxicity, the kinase-specific inhibitors SP600125 and PD98058 were used. As presented in Fig. 7B, co-treatment with MOF extract and inhibitors blocked MOF extract-mediated p-ERK and p-JNK accumulation. The cumulative results
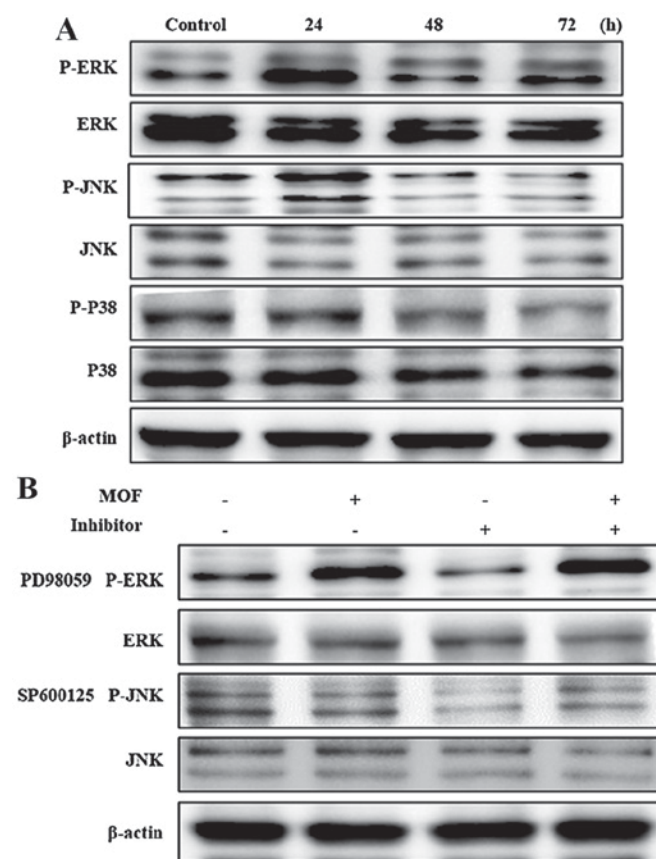
Figure 7. Regulation of mitogen-activated protein kinase signaling by MOF extract-induced A2058 cells. (A) Equal amounts of cell lysates were electrophoresed and apoptosis-associated protein expression was detected using western blot analysis. (B) The cells were stimulated with MOF extract following pretreatment with SP600125 and PD98059, respectively. MOF, *Moringa oleifera* fruit; P-, phosphorylated; ERK, extracellular-signal-regulated kinase; JNK, c-Jun N-terminal kinase.

indicated that ERK and JNK are involved in the regulation of MOF extract-induced mitochondrial apoptosis in A2058 cells.

Discussion

The incidence rate of malignant melanoma has continued to increase by between 3 and 7% each year in Western countries (24). In addition, as the climate changes, exposure to harmful ultraviolet (UV) rays has become a concern due to increasing incidence of various types of skin cancer (25). As malignant melanoma exhibits resistance to chemotherapy or immunotherapy, complete treatment has become problematic (26,27).

Seeds, roots, leaves, flowers and bark of *Moringa oleifera* have been used as materials for cosmetics and medicine (28). As the anti-inflammatory, anticancer and antioxidant effects of MOF extract have been validated (29,30), the present study investigated the effects of MOF extract on apoptosis and ROS accumulation in malignant melanoma cells.

ROS are generated by active processes in normal cells and are associated with various biological processes, including cell differentiation and gene expression (31). Therefore, ROS homeostasis is important for cell viability and survival. Although the association between ROS and apoptosis has

been reported to be contradictory, ROS perform a role as an intermediate mediator that delivers signals for apoptosis (32).

MAPKs are protein kinases activated by a variety of stimuli, and regulate cell proliferation, differentiation, proliferation and death (33,34). MAPKs consist of JNK, p38 and ERK proteins (35). In general, ERK is activated by growth factors, cytokines and phorbol ester and is involved in cell proliferation or differentiation (36). By contrast, JNK and p38 are activated by proinflammatory cytokines, UV irradiation, heat, osmotic shock, hydrogen peroxide and DNA damage induced by stress and is involved in proliferation inhibition and apoptosis (37-40). However, previous studies have reported that ERK, JNK and p38 are involved in cell survival and death (41,42). The present study revealed that JNK and ERK are involved in the apoptotic effects of MOF extract.

Apoptosis, also termed programmed cell death, is required to control the number of normal cells and is induced by various types of damage (43). In addition, apoptosis serves as an important control mechanism of homeostasis, as it is involved in the removal of harmful cells, and is induced by internal and external signaling (44). Upon apoptosis, cells are morphologically characterized by membrane detachment, membrane blebbing, nuclear condensation, exposure of phosphatidylserine to the extracellular space and DNA fragmentation (45). Apoptosis may be initiated by one of two pathways: The intrinsic or the extrinsic pathway (46). In the intrinsic pathway, loss of mitochondrial transmembrane potential results in the release of cytochrome *c* into the cytosol, formation of apoptotic protease-activating factor 1 (Apaf-1) and activation of caspase-9. Caspase-9 then activates downstream caspases-3, -6 and -7 (47,48). In the extrinsic pathway, Fas-associated death domain protein and caspase-8 are activated through Fas and tumor necrosis factor receptors, which are death receptors (49). Bax and Bak proteins are widely known for their cell killing activity (50). Alterations in the expression of Bcl-2 family proteins inhibit dimerization of Bcl-2 family members on the outer mitochondrial membrane, which induces release of mitochondrial proteins, including cytochrome *c*, Apaf-1, second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI and apoptosis-inducing factor, and promotes apoptosis (51,52). Caspase-3 is generally inactive (pro-caspase-3) in cells and activated by death signaling, which catalyzes proteolytic cleavage of PARP as an apoptosis-specific marker (53,54). In the present study, the Bax/Bcl-2 ratio was identified to have increased and PARP was cleaved by caspase-3 via the caspase-9 signaling pathway.

The results of the present study revealed the apoptotic effects of MOF extract via ROS production in melanoma cells. Therefore, MOF extract inhibits the proliferation of human melanoma A2058 cells by generating ROS, which regulate expression of proteins involved in survival and apoptosis of cancer cells.

Acknowledgements

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