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Anti-cancer effects of *Kochia scoparia* fruit in human breast cancer cells

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ABSTRACT

Background: The fruit of *Kochia scoparia* Scharder is widely used as a medicinal ingredient for the treatment of dysuria and skin diseases in China, Japan and Korea. Especially, *K. scoparia* had been used for breast masses and chest and flank pain. **Objective:** To investigate the anti-cancer effect of *K. scoparia* on breast cancer. **Materials and Methods:** We investigated the anti-cancer effects of *K. scoparia*, methanol extract (MEKS) *in vitro*. We examined the effects of MEKS on the proliferation rate, cell cycle arrest, reactive oxygen species (ROS) generation and activation of apoptosis-associated proteins in MDA-MB-231, human breast cancer cells. **Results:** MTT assay results demonstrated that MEKS decreased the proliferation rates of MDA-MB-231 cells in a dose-dependent manner with an IC_{50} value of 36.2 μg/ml. MEKS at 25 μg/ml significantly increased the sub-G1 DNA contents of MDA-MB-231 cells to 44.7%, versus untreated cells. In addition, MEKS induced apoptosis by increasing the levels of apoptosis-associated proteins such as cleaved caspase 3, cleaved caspase 8, cleaved caspase 9 and cleaved Poly (ADP-ribose) polymerase (PARP). **Conclusion:** These results suggest that MEKS inhibits cell proliferation and induces apoptosis in breast cancer cells and that MEKS may have potential chemotherapeutic value for the treatment of human breast cancer.

Key words: Apoptosis, breast cancer, cancer therapy, *Kochia scoparia*, reactive oxygen species

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INTRODUCTION

Apoptosis plays critical roles in anti-cancer treatments and it is characterized by the cross-linking of cell membrane proteins, nuclear fragmentation and disorganized arrangements of cytoplasmic organelles.[1,2] Two main pathways trigger apoptosis that are, the mitochondria-mediated intrinsic pathway and the death receptor-mediated extrinsic pathway.[3] Multiple caspases play essential roles in both of these pathways via self-cleavage. These caspase cascades are highly complex and exhibit a high degree of cross-talk.[3,4]

Several systemic chemotherapeutic agents are used to treat breast cancer, such as trastuzumab, bevacizumab, 5-fluorouracil, paclitaxel, doxorubicin, cyclophosphamide and tamoxifen.[5] However, despite their effectiveness, these drugs also have significant side effects including cardiac toxicity, bone marrow dysfunction, thrombocytopenia and mucositis.[6] Accordingly, novel, more effective drugs are required that can inhibit the development and progression of breast cancer with fewer side effects. In the context of side effects and toxicity, natural substances are usually relatively safe. For this reason, we screened and examined several agents in an effort to identify and develop novel and effective agents, by focusing on extracts of Korean herbs.

The mature fruit of *Kochia scoparia* (L.) Schrad. (family Chenopodiaceae) is harvested throughout China in the autumn and sun dried. In the Far East, including China, Korea and Japan, this fruit is widely used to treat dysuria and skin diseases. Especially, *K. scoparia* had been used for breast masses and chest and flank pain.[7,8]

Several types of compounds, such as, triterpenoid glycosides,[9] saponins,[10] and alkaloids have been isolated from *K. scoparia*. Furthermore, *K. scoparia* and components of extracts have been shown to have anti-inflammatory[11]

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and anti-allergic activities.[12] Recently, it was reported that *K. scoparia* has possible tumoricidal effects.[13] In addition, momordin Ic, a natural triterpenoid saponin component of several traditional medicines and of *K. scoparia*, has been reported to have anti-cancer effects.[14] However, no report has been issued on the anti-cancer mechanism of *K. scoparia*.

Accordingly, we evaluated the anti-cancer activity of the methanol extract of the dried fruit of *K. scoparia* (MEKS) by measuring proliferation rates and apoptosis in MDA-MB-231 human breast cancer cells. In addition, we sought to identify the molecular mechanisms responsible.

MATERIALS AND METHODS

Reagents and antibodies

Paclitaxel, Hoechst 33342, MTT (3, 4, 5-dimethyl N-methylthiazol-2-yl-2, 5-d-phenyl tetrazolium bromide) and propidium iodide (PI) solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2, 7-Dichloro-fluorescein diacetate (DCFH-DA) was obtained from Eastman Kodak (Rochester, NY, USA). The ANNEXIN V-FITC apoptosis detection kit, anti-rabbit IgG antibody and anti-mouse IgG antibody were purchased from Enzo Life Sciences (Farmingdale, NY, USA). The antibodies targeting cleaved caspase 3, cleaved caspase 8, cleaved caspase 9 and cleaved Poly (ADP-ribose) polymerase (PARP) were purchased from Cell Signaling Technology (Beverly, MA, USA), while anti-beta actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Preparation of MEKS

The dried fruit of *K. scoparia* was purchased from Hwalim Medicinal Herbs (Pusan, Korea). Extraction was performed using a standard extraction process, as previously described.[15] Briefly, 100 g of the dried fruit of *K. scoparia* were immersed in 1 L of methanol, sonicated for 30 min and then allowed to stand for 48 h. The obtained extract was filtered through No. 20 Whatman filter paper, evaporated under reduced pressure using a vacuum evaporator (Eyela, Japan) and lyophilized using a freeze dryer (Labconco, Kansas City, MO, USA). Finally, 4.46 g of lyophilized powder was obtained (yield, 4.46%). A sample of the lyophilized powder (MEKS, Voucher No. MH2013-006) was deposited at the Division of Pharmacology, School of Korean Medicine, Pusan National University.

Cell culture

MDA-MB-231 cell (a human breast cancer cell line) was purchased from the Korean cell line bank (Seoul, Korea) and cultured in DMEM (Hyclone Laboratories, Logan, UT, USA) supplemented with 10% FBS (Hyclone Laboratories) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA).

MTT assay

Proliferation rates of MDA-MB-231 cells were measured using a MTT proliferation assay. Briefly, cells were seeded in 24-well plates (5×10^4 per well) and cultured overnight to allow attachment. They were then treated with MEKS at the concentration of 0, 3, 6, 12, 25 and 50 μg/ml for 4 h, respectively, and then in fresh MEKS-free media for 20 h. Control cells were treated with vehicle (dimethyl sulfoxide; DMSO) for 4 h. MTT activities were measured in triplicate for control and experimental groups. MTT solution was added to each well, and then the cells were incubated at 37°C for 4 h in a 5% CO_2 atmosphere. Media were then removed and the formazan crystals produced were dissolved in 100 μl DMSO. Absorbances were read at 570 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Cell morphology

A phase contrast microscope (Olympus, Tokyo, Japan) was used to determine the cell morphologies of untreated and MEKS-treated MDA-MB-231 cells.

ANNEXIN V and 7-AAD double staining

Cells were seeded in 6-well plates (3×10^5 per well), incubated overnight and treated with MEKS in indicated concentrations for 4 h. MEKS was then removed and cells were cultured in fresh media for a further 20 h. Control cells were treated with vehicle (DMSO) for 4 h. Thirty nM of paclitaxel was used as positive control. After incubation, cells were trypsinized, harvested, washed with phosphate-buffered saline (PBS), resuspended in 500 μl of Binding Buffer (ANNEXIN V-FITC apoptosis detection kit, Enzo Life Sciences) and stained using the ANNEXIN V-FITC apoptosis detection kit (Enzo Life Sciences) at room temperature for 5 min in the dark, according to the manufacturer's instructions. Stained cells were analyzed using a Flow cytometer (BD Biosciences, Heidelberg, Germany) and the data obtained was analyzed using the FACS (Fluorescence Activated Cell Sorting)-Canto II software.

Hoechst 33342 staining

Untreated and MEKS-treated MDA-MB-231 cells were stained with Hoechst 33342 to determine whether nuclei were condensed or fragmented. Cells were seeded on cover slips in 24-well plates (5×10^4 per well) and cultured overnight. After treatment with MEKS at the concentration of 0, 3, 6, 12, 25 or 50 μg/ml for 4 h, cells were fixed with 4% paraformaldehyde solution for 15 min and washed with PBS twice. They were then stained with 10 μM Hoechst

33342 solution for 10 min and washed with PBS. Nuclear morphologies were determined using a fluorescence microscope (Olympus, Tokyo, Japan).

Cell cycle analysis

After treatment with MEKS for 4 h, MDA-MB-231 cells were trypsinized, harvested and washed with PBS twice. After homogenisation, cells were fixed with 75% ethanol and washed with PBS. They were then re-suspended in PBS containing RNase (40 μg/ml) for 30 min and treated with PI solution (10 μg/ml). Cells were then transferred to FACS tubes and DNA contents were analysed using a FACS Scan flow cytometer. Data were analysed using FACS-Canto II software.

Intracellular reactive oxygen species detection

The levels of intracellular (ROS) were measured using DCFH-DA. Briefly, MDA-MB-231 cells (2.5×10^4) were cultured overnight in a 96-well plate. Then, 50 μM of DCFH-DA was added and the cells were cultured for a further 45 min. Cells were then treated at the concentration of 0, 6, 12, 25, or 50 μg/ml of MEKS and fluorescence intensities were measured using a TECAN Infinite M200 fluorometric plate reader (excitation at 485 nm, emission at 530 nm, Männedorf, Switzerland).

Western blot analysis

MDA-MB-231 cells were treated with MEKS at the concentration of 25 or 50 μg/ml for 4 h. After treatment, the cells were collected and lysed with RIPA buffer (Cell Signaling Technology) for 30 min. Then, the supernatant was taken by 20 min centrifugation at 12,000 rpm, diluted in sodium dodecyl sulfate (SDS) buffer, and boiled for 5 min. After quantification, 50 μg of protein extract were subjected to electrophoresis using 10% SDS-polyacrylamide gel, then transferred to polyvinylidene fluoride membrane for 2 h. Blocking was performed using TNE buffer [50 mm Tris–HCl (pH 7.4), 100 mm NaCl, 0.1 mm EDTA] containing 5% skim milk and 0.1% tween-20. Antibodies against cleaved PARP, cleaved caspase 3, cleaved caspase 8 and cleaved caspase 9 were incubated overnight for 4 °C. Then, it was applied with horseradish-conjugated secondary antibody and detected using SuperSignal West-Femto reagent (Pierce, Rockford, IL, USA).

Statistical analysis

The Student's-t test in Window PASW (Predictive Analytics SoftWare) version 18.0 (SPSS Inc, Chicago, IL, USA) was used to analyse the significance of differences between the control and MEKS-treated groups. Results are presented as means ± standard errors and statistical significance was accepted for $P < 0.05$, $P < 0.01$ or $P < 0.001$, as indicated.

RESULTS

MEKS treatment inhibited cell proliferation and modified the morphology of breast cancer cells

MEKS inhibited cell proliferation in a dose-dependent manner [Figure 1A], with an IC_{50} value (50% growth inhibition) of 36.2 μg/ml. After treatment with MEKS, cells became smaller and rounder and lost their cellular processes [Figure 1B]. Taken together, these results demonstrated that MEKS inhibited cell proliferation and induced cell death.

Meks induced early and late apoptosis in MDA-MB-231 cells

7-AAD and ANNEXIN V FITC double staining demonstrated that MEKS increased the number of apoptotic cells in a dose-dependent manner. In particular, MDA-MB-231 cells treated with 25 μg/ml of MEKS showed early and late apoptosis proportions of 19.7% and 10.8%, respectively. In addition, treatment with of

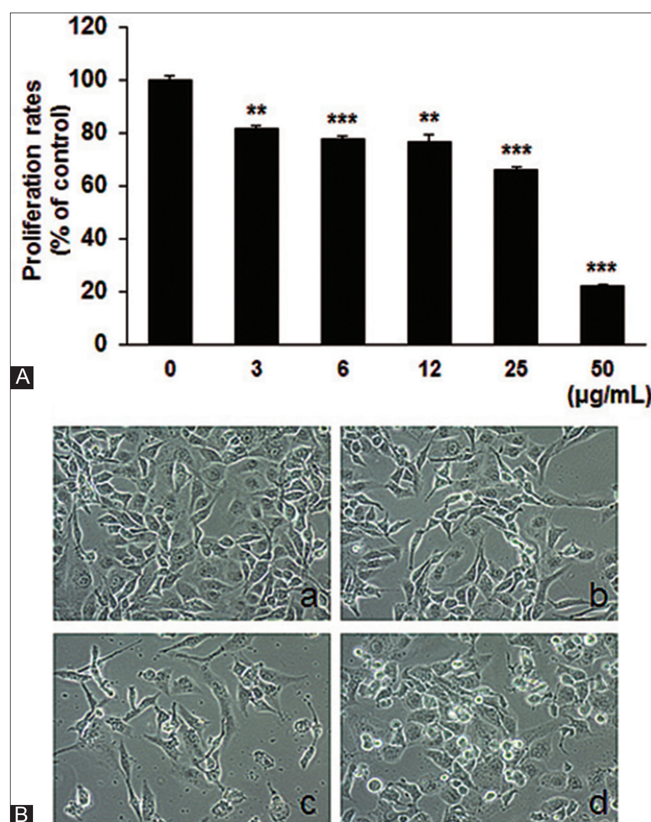

Figure 1: Effects of MEKS on the proliferation rates of human breast cancer cells. (A) MDA-MB-231 cells were treated with 0, 3, 6, 12, 25, or 50 μg/ml of MEKS for 4 h and proliferation rates were measured by MTT assay. Values are presented as means ± standard errors., $^{**}P < 0.01$; $^{***}P < 0.001$. (B) Cells were treated with 0 μg/ml (a), 25 μg/ml (b) or 50 μg/ml (c) of MEKS for 4 h, or 30 nM of paclitaxel (d) for 24 h. Cell morphologies were observed under a phase-contrast microscope (Original Magnification, X200)

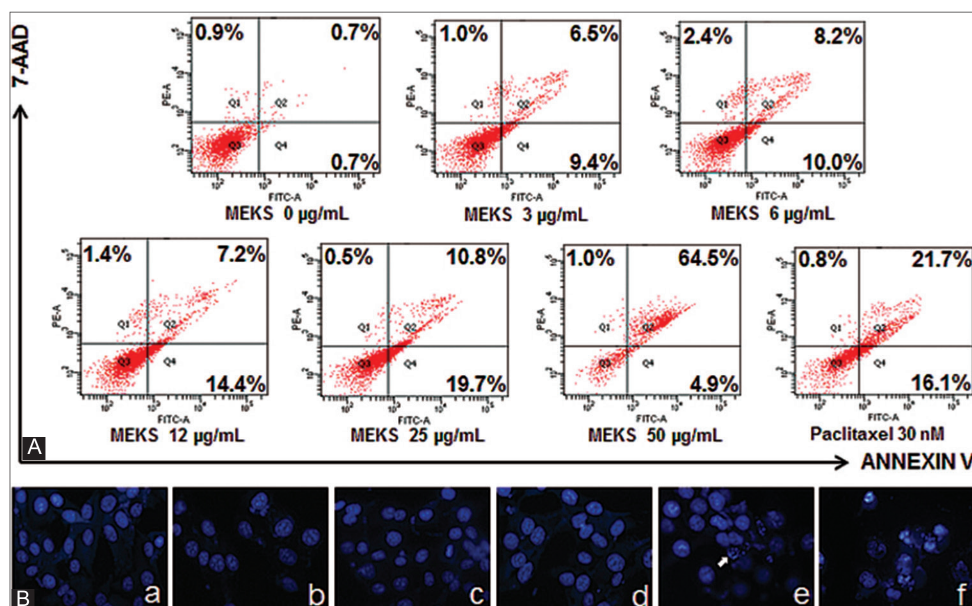

Figure 2: Effects of MEKS on the induction of apoptosis. (A) MDA-MB-231 cells were treated with various concentrations of MEKS for 4 h or with 30 nM paclitaxel for 24 h (positive control). Cells were stained with Annexin-V and 7-AAD and analysed by flow cytometry. Early apoptotic cells were stained by Annexin-V but not by 7-AAD (lower right quadrant, Q4), whereas late apoptotic cells are stained by both upper right quadrant, Q2.(B) Cells were treated with 0 μg/ml (a), 3 μg/ml (b), 6 μg/ml (c), 12 μg/ml (d), or 25 μg/ml (e) of MEKS, or 30 nM of paclitaxel for 24 h (f). Apoptosis was identified by Hoechst 33342 staining (Original Magnification, X400). The filled arrow indicates the presence of the characteristic morphological changes of apoptosis, including chromatin condensation and nuclear fragmentation

MEKS at 50 μg/ml caused the apoptosis of 69.4% of cells and treatment with paclitaxel at 30 nM caused the apoptosis of 37.8% of cells [Figure 2A]. Cells were also stained with Hoechst 33342 to determine nuclear morphologies. Control (DMSO-treated) cells had large round nuclei with no apoptotic features, whereas the ratio of early and late apoptotic cells increased in a dose-dependent manner and cells exposed to 25 μg/ml of MEKS exhibited the typical characteristics of apoptosis, including cell shrinkage and nuclear fragmentation [Figure 2B].

MEKS induced sub-G1 arrest and increased the number of apoptotic cells

Only 1.0% of control cells were in the sub-G1 phase. However, cells treated with 25 μg/ml MEKS showed a marked increase in the number of cells undergoing sub-G1 arrest. The proportions of sub-G1 arrested cells in the 25 μg/ml MEKS and 30 nM paclitaxel groups were 44.7% and 19.9%, respectively [Figure 3].

MEKS elevated intracellular ROS levels

Treatment with MEKS markedly increased intracellular ROS levels, as shown in Figure 4. ROS was found to accumulate in a time-dependent manner, with peak ROS production occurring following exposure to 25 μg/ml of MEKS. After 90 min of incubation with 25 μg/ml of MEKS, intracellular ROS levels were approximately 2.5 fold higher than in the untreated control group [Figure 4].

MEKS triggered the intrinsic and extrinsic apoptotic pathway

The caspase family plays critical roles in apoptosis. Treatment of MDA-MB-231 cells with MEKS markedly activated intrinsic pathway-related proteins such as caspase 8 and extrinsic pathway-related proteins such as caspase 9, caspase 3 and cleaved PARP. Furthermore, MEKS significantly increased the levels of cleaved caspase 8, cleaved caspase 9, cleaved caspase 3 and cleaved PARP in a dose-dependent manner [Figure 5].

DISCUSSION

Recently, the identification of anti-cancer drugs among substances extracted from traditional medicines has emerged as a novel strategy. In our studies, we have focused on the development of anti-cancer agents in substances derived from traditional Korean medicine.

K. scoparia is used to treat patients with skin diseases and dysuria in traditional Korean Medicine,[7] and has recently been reported to influence immune response and been used to treat inflammation and allergies.[11,12] In a recent study, Mazzio and Soliman[13] reported *K. scoparia* has an anti-proliferative effect on human cancer cells but did not identify the mechanism involved.

Our results showed a gradual decrease in the number of

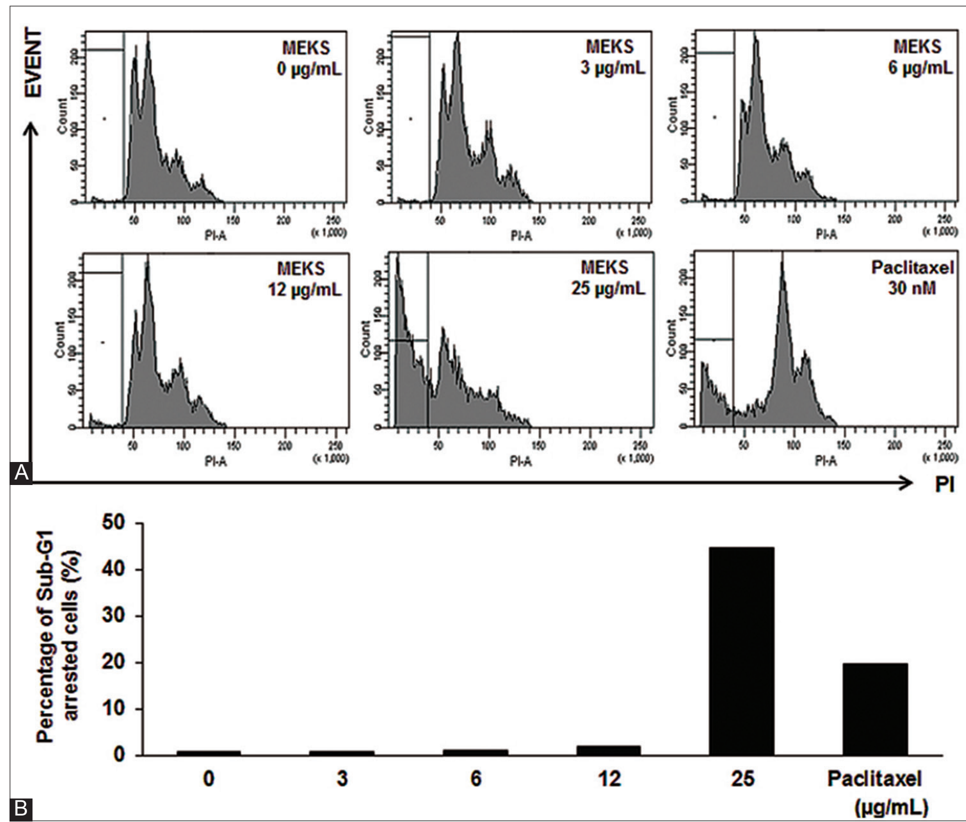

Figure 3: Effects of MEKS on the cell cycle arrest of MDA-MB-231 cells. (A) Cells were treated with various concentrations of MEKS for 4 h or with 30 nM of paclitaxel for 24 h as a positive control. Cells were stained with PI and analysed by flow cytometry. (B) Percentage of sub-G1 arrested cells by various concentrations of MEKS and 30nM of paclitaxel

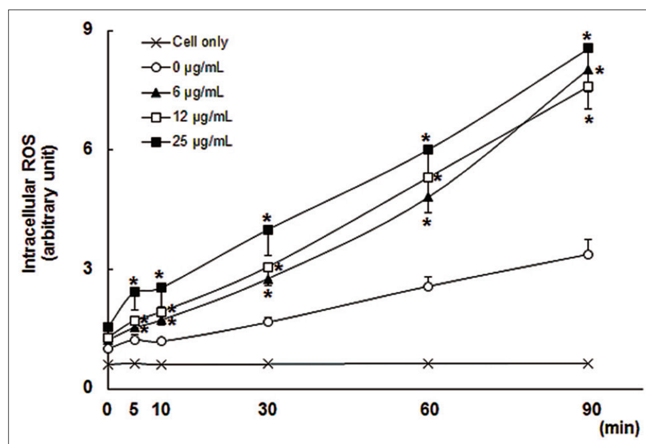

Figure 4: Effects of MEKS on intracellular ROS levels. MDA-MB-231 cells were treated with 0, 6, 12 or 25 µg/ml of MEKS for 90 min. Cells were then loaded with the ROS-specific probe, DCFH-DA and fluorescence was measured using a fluorometric plate reader. Values are means ± standard errors. *, P<0.05

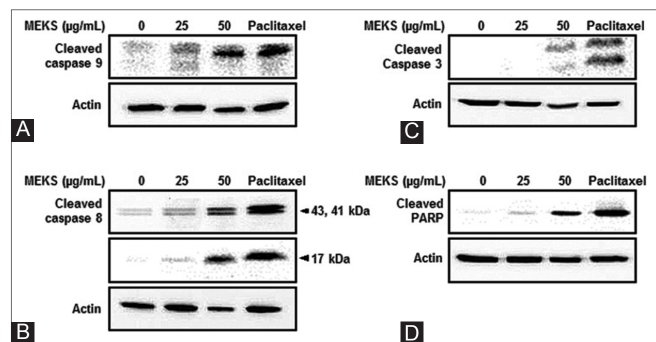

Figure 5: Effects of MEKS on the activations of apoptosis-related proteins. MDA-MB-231 cells were treated with 0, 25, or 50 µg/ml of MEKS for 4 h or 30 nM paclitaxel for 24 h (positive control). Protein expressions of cleaved caspase 3 (C), 8 (B) and 9 (A), cleaved PARP (D) and beta-actin (the loading control) were detected by Western blotting

viable MDA-MB-231 cells after treatment with increasing concentrations of MEKS. Furthermore, the cells lost their normal morphology and began to display a morphology characteristic of apoptosis. In addition, the percentage of sub-G1 arrested cells and of early and late apoptotic cells increased with increasing MEKS concentrations. Taken together, these findings show that the cell death induced by MEKS occurs via apoptotic mechanisms.

The regulation of apoptosis is an important developmental strategy in the search for anti-cancer treatments. Chen *et al*. reported that the treatment of cancer cells with the demethylated form of cantharidin (NCTD) induced the upregulation of p21, which caused the activation of

caspase 3 and subsequent apoptosis in HepG2 cells.[16] Our results demonstrate that MEKS inhibited cell cycle progression, resulting in sub-G1 arrest, which suggests that MEKS is a candidate treatment for breast cancer.

Intracellular ROS can activate the extrinsic pathway as well as the intrinsic apoptotic pathway.[17] Choi *et al*. demonstrated that apoptosis occurred via ROS generation, due to DNA damage caused by ROS generation.[18] Consistent with our results, Yaseen *et al*. reported that resveratrol mediated the up-regulation of death receptor by ROS and subsequently triggered the extrinsic apoptotic pathway, leading to the release of mitochondrial death proteins, such as cytochrome c.[19] Our results show that MEKS elevated intracellular ROS levels significantly. We consider that the elevation of intracellular ROS gives rise to the initiating both intrinsic and extrinsic apoptotic pathways as well as cell cycle arrest.

There are some debates on the anti-cancer effects of *K. scoparia* and its fractions. Mazzio and Soliman reported tumoricidal effects of *K. scoparia*.[13] In addition, Wang *et al*. reported that momordin Ic, a triterpenoid saponin enriched in various medicinal plants such as *K. scoparia*, had anti-cancer effects in the HepG2 cell.[14] On the contrary, Balsevich *et al*. reported that saponin-rich extract from *K. scoparia* had no anti-proliferative activity in the MDA-MB-231 cell.[20] In our results, MEKS showed both anti-proliferative and tumoricidal effects. In addition, momordin Ic was hardly detected in MEKS probably because its concentration in MEKS was too low (data not shown). These mean that the anti-cancer effect verified through the MEKS for the present study is due to other influential and novel components, besides momordin Ic. Therefore, we are carrying out an experiment on separating ingredients from various fractions, in order to accurately separate this ingredient having anti-cancer effects.

In the present study, MEKS inhibited MDA-MB-231 cell growth at lower concentrations (IC_{50} 36.2 μg/ml) than those reported for other natural products extracted from herbs.[21-23] For example, the IC_{50} values reported for *Gracilaria tenuistipitata* (methanol extract) and *Moricandia arvensis* (methanol extract) on the proliferation rate of cancer cells is 326 μg/ml and 875 μg/ml, respectively.[21-23]

MEKS showed high cytotoxicity even at low concentrations and when it was administered in combination with the anti-cancer agent paclitaxel, potent anti-cancer activity was evident at even lower concentrations (data not shown). We are now investigating the anti-cancer effects of MEKS and of its various fractions in combination with conventional anti-cancer drugs in an animal cancer model.

Taken together, this study describes a novel mechanism for the anti-cancer effect of MEKS. In particular, our results show that MEKS induces MDA-MB-231 cell apoptosis via the extrinsic and intrinsic apoptotic pathways, which suggests that MEKS is a novel potential chemotherapeutic agent for breast cancer.

CONCLUSIONS

Our study shows that the methanol extract of *K. scoparia* fruit (MEKS) inhibits the proliferation, induces sub-G1 arrest, ROS generation and triggers the apoptosis of breast cancer cells. Furthermore, the MEKS-induced apoptosis of breast cancer cells was found to be associated with the activation of the caspase cascade via ROS generation. These findings suggest MEKS possesses anti-cancer properties and that it should be regarded as a novel potential therapeutic agent.

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