ORIGINAL ARTICLE

Apoptosis of AGS human gastric adenocarcinoma cells by methanolic extract of *Dictamnus*

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ABSTRACT

Background: The root bark of *Dictamnus dasycarpus* Turcz has traditionally been used in East Asia to treat skin diseases such as eczema, atopic dermatitis, and psoriasis. However, it has also been reported to exhibit an anti-proliferative effect on cancer cells. **Objective:** To investigate the anti-cancer effects of a methanol extract of *Dictamnus dasycarpus* root bark (MEDD) on AGS cells (a human gastric adenocarcinoma cell-line). **Materials and Methods:** An 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium assay, a caspase activity assay, cell cycle analysis, mitochondrial membrane potential (MMP) measurements, and western blotting were used to investigate the anti-cancer effects of MEDD on AGS cells. **Results:** Treatment with MEDD significantly and concentration-dependently inhibited AGS cell growth. MEDD treatment in AGS cells led to increased accumulation of apoptotic sub-G1 phase cells in a concentration-dependent manner. Also, MEDD reduced the expressions of pro-caspase-3, -8 and -9, and increased the active form of caspase-3. Furthermore, subsequent Western blotting revealed elevated levels of poly (ADP-ribose) polymerase protein. MEDD treatment reduced levels of MMP and anti-apoptotic Bcl-2 and Bcl-xL proteins. Pretreatment with SB203580 (a specific inhibitor of p38 mitogen-activated protein kinases), SP600125 (a potent inhibitor of C-Jun N-terminal kinases), or PD98059 (a potent inhibitor of extracellular signal-regulated kinases) did not modify the effects of MEDD treatment. However, pretreatment with LY294002 (a specific inhibitor of Akt) significantly enhanced MEDD-induced cell death. **Conclusion:** These results suggest that MEDD-mediated cell death is associated with the intrinsic apoptotic pathway and that inhibition of Akt signaling contributes to apoptosis induction by MEDD.

Key words: A human gastric adenocarcinoma cell-line, Akt, apoptosis, *Dictamnus dasycarpus*, gastric cancer, LY294002

INTRODUCTION

Gastric cancer originates from the glandular epithelium of gastric mucosa, and according to global cancer statistics, gastric cancer is the fourth most frequently diagnosed cancer in men and the third most common cause of cancer-related death.[1,2] Although cancer treatments such as surgery, radiation therapy, and chemotherapy have advanced, in gastric cancer these therapies are limited response and survival rates are poor.[3] Therefore, it is important that more effective strategies be developed to improve the survival rates of gastric cancer patients.

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Recently, traditional medicines such as Korean and Chinese traditional medicines and Ayurveda (a type of Hindu traditional medicine used in the Indian Subcontinent) have attracted considerable research attention as sources of novel anti-cancer agents.[4] *Dictamnus dasycarpus* Turcz is widespread throughout Asia and Europe, and its root bark is traditionally used in Korea and China to treat eczema, rubella, scabies, acute rheumatoid arthritis, jaundice, colds, and headaches.[5-7] Furthermore, the water extract of its root bark has been reported to inhibit the growths of several types of human pathogenic fungi *in vitro*.[8] According to recent studies, *D. dasycarpus* also has pharmacological properties such as anti-inflammatory,[9] anti-fungal,[10,11] and neuroprotective effects.[12] In addition, Obacunone from *D. dasycarpus* potentiates the cytotoxicities of anti-microtubule agents such as vincristine, vinblastine, and paclitaxel in cancer cells.[13] The known constituents of *D. dasycarpus* root bark include limonoids,[7,10,14-17] furoquinoline alkaloids,[17] flavonoids,[15,16] coumarins,[18]

sesquiterpenes,[19] sesquiterpene glycosides, and phenolic glycosides.[19-21]

Apoptosis (type 1 programmed cell death) is a highly conserved form of cell suicide and plays a central role in the differentiation of multicellular organisms and in the elimination of damaged and infected cells. Apoptosis is characterized by cytoplasmic shrinkage, chromatin condensation, deoxyribonucleic acid fragmentation, and extensive plasma membrane blebbing.[22] In general, two major pathways lead to apoptosis that is the death receptor pathway (extrinsic) and the mitochondria-dependent pathway (intrinsic). Interactions between apoptosis-inducing ligands and death receptors initiate the extrinsic pathway at cell membranes and subsequently activate caspase-8 by forming death-induced signaling complex (DISC). In addition, activated caspase-8 directly activates effector caspases such as caspase-3, or cleaves Bid to truncated Bid, both of which lead to intrinsic pathway activation via mitochondrial dysfunction. Resultantly, cytochrome *c* is released from mitochondria, causing the activations of caspase-9 and effector caspases, and eventually apoptotic cell death.[23-25]

In this study, we investigated the anti-cancer effects of the methanolic extract of *D. dasycarpus* Turcz root bark (MEDD) on AGS human gastric adenocarcinoma.

MATERIALS AND METHODS

Reagents and antibodies

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT), propidium iodide (PI), and JC-1 (5,5', 6,6'-tetrac hloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide) were purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) and caspase activity assay kits were purchased from GIBCO-BRL (Gaithersburg, MD) and R and D Systems (Minneapolis, MN), respectively. PD98059 (an extracellular signal-regulated kinases [ERK]-specific inhibitor), SP600125 (C-Jun N-terminal kinases [JNK]-specific inhibitor), SB203580 (a p38 mitogen-activated protein kinases (MAPK)-specific inhibitor), and LY294002 (an Akt-specific inhibitor) were purchased from Calbiochem (San Diego, CA, USA). Enhanced chemiluminescence (ECL) kits were purchased from Amersham (Arlington Heights, IL, USA). All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Preparation of methanolic extract of *Dictamnus dasycarpus* Turcz

The root bark of *D. dasycarpus* was purchased from Kwangmyungdang Medicinal Herbs (Ulsan, Korea). To produce the methanolic extract, 50 g of root bark was immersed in 1000 ml of methanol, sonicated for 30 min, and lowed to stand for 24 h. The mixture was filtered through Whatman (number 20) filter paper, and the filtrate was evaporated under reduced pressure using a vacuum evaporator (Eyela, Japan). The condensed extract so obtained was lyophilized using a freeze dryer (Labconco, Kansas City, MO, USA), and 2.8 g of lyophilized powder (MEDD) was obtained (yield, 5.6%). A sample to MEDD (voucher number. MH2010–010) was deposited at the Division of Pharmacology, School of Korean Medicine, Pusan National University.[5,6] MEDD was dissolved in dimethyl sulfoxide (DMSO) to produce a stock solution of concentration 100 mg/ml and stored at 4°C.

Cell culture

AGS cells (a human gastric adenocarcinoma cell-line) were obtained from the American Type Culture Collection (Rockville, MD, USA), and maintained at 37°C in a humidified 95% air/5% CO_2 atmosphere in RPMI1640 supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium assay

To investigate viabilities, cells were seeded in 6-well plates at a density of 2×10^5 cells per well and allowed to stabilize for 24 h. The cells were then treated with various doses of MEDD for predetermined times. MTT working solution (0.5 mg/ml) was then added to the culture plates and incubated at 37°C for 2 h. Culture supernatants were completely removed from the wells, and DMSO was added to completely dissolve the formazan crystals. Well, absorbances were measured at a wavelength of 540 nm using a microplate reader (Molecular Devices, Palo Alto, CA). The effect of MEDD on cell growth was assessed using cell viability percentages versus vehicle-treated controls.

Measurement of cell cycle

Following treatment with MEDD, AGS cells were trypsinized, washed with PBS, and fixed in 75% ethanol at 4°C for overnight. Prior to analysis, cells were again washed with PBS, suspended in cold (PI, Sigma) solution, and incubated at room temperature in the dark for 30 min. Flow cytometry was performed using a FACScan flow cytometry system (Becton-Dickinson, San Jose, CA, USA).

Protein extraction and Western blotting

Cells were harvested, washed twice in PBS at 4°C, and lysed in lysis buffer. Supernatants were collected, and protein concentrations were measured using protein assay reagents (Bio-Rad Laboratories, Hercules, CA, USA). For western blotting, equal amounts of proteins were denatured by boiling at 95°C for 3 min in sample

buffer (0.5 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.1% bromophenol blue, 10% β-mercaptoethanol) at a ratio of 1:1, subjected to 10–13% SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes (Schleicher and Schuell, Keene, NH) by electroblotting. Membranes were blocked with 5% nonfat dry milk in PBS containing Tween 20 buffer (PBS-T) (20 mM Tris, 100 mM NaCl, and 0.1% Tween 20; pH 7.5) for 50 min at room temperature. Membranes were then incubated overnight at 4°C with primary antibodies, probed with enzyme-linked secondary antibodies, and visualized using an ECL kit (Amersham Bioscience, Piscataway, NJ, USA).

Caspase activity assay

The activities of caspases were determined using colorimetric assay kits that utilized synthetic tetrapeptides (Asp-Glu-Val-Asp (DEAD) for caspase-3; Leu-Glu-His-Asp (LEHD) for caspase-9, respectively) labeled with p-nitroaniline (pNA). Briefly, MEDD treated or untreated cells were lysed in the supplied lysis buffer, supernatants were collected, and incubated with the supplied reaction buffer containing dithiothreitol and DEAD-pNA or LEHD-pNA as substrates at 37°C. Activities were determined by measuring changes in absorbance at 405 nm using a microplate reader.

Measurement of mitochondrial membrane potentials

Mitochondrial membrane potentials (MMPs) were assessed using JC-1 (a dual-emission potential-sensitive probe). Briefly, cells were collected, incubated with 10 μM JC-1 for 20 min at 37°C in the dark, washed once with PBS, and analyzed by flow cytometry, as previously described.[26]

Statistical analyses

Unless indicated, results are expressed as means ± standard deviations of results obtained in triplicate. Statistical analysis was performed using the paired Student's *t*-test. Statistical significance was accepted for *P* < 0.05.

RESULTS

Methanolic extract of *Dictamnus dasycarpus* Turcz inhibited cell growth and induced apoptosis

To determine the effect of MEDD on the growth of AGS cells, cells were treated with various concentrations of MEDD for 24 h, and cell viabilities were then assessed using the MTT assay. As shown in Figure 1a, cell viabilities were significantly and concentration-dependently decreased by MEDD. The flow-cytometric analysis was used to detect apoptotic cells to determine whether MEDD-induced cell death resulted from apoptosis. As shown in Figure 1b,

MEDD concentration-dependently increased numbers of apoptotic sub-G1 phase cells.

Methanolic extract of *Dictamnus dasycarpus* Turcz activated caspases in AGS cells

Caspases are important mediators of apoptosis in both intrinsic and extrinsic pathways. This phase induces the activations of the cytoplasmic endonuclease, which degrades nuclear material and activates proteases. These proteases in turn, degrade cytoskeletal and nuclear proteins and cleave various substrates, including poly (ADP-ribose) polymerase (PARP), and the cleavage product of PARP serves as a marker of apoptosis.[22] As shown in Figure 2a, MEDD concentration-dependently reduced the expressions of pro-caspase-3,-8 and-9, and increased the active form of caspase-3. Furthermore, subsequent Western blotting revealed elevated levels of PARP protein. We also attempted to quantify the proteolytic activation of caspases by MEDD, and as shown in Figure 2b, MEDD was found to increase the activities of caspase-3 and -9. These results indicate that MEDD induced apoptosis by activating caspases in AGS cells.

Methanolic extract of *Dictamnus dasycarpus* Turcz induced loss of mitochondrial membrane potential by modulating Bcl-2 and Bcl-xL protein levels

The intermembrane space of mitochondria contains many pro-apoptotic proteins, including cytochrome *c*, and disruption of the outer mitochondrial membrane induced by various events such as diminished levels of the anti-apoptotic proteins Bcl-2 and Bcl-xL results in the release of cytochrome *c*, which leads to the activations of caspase-9 and effector caspases that eventually cause apoptotic cell death.[27-30] We examined the effects of MEDD on MMP levels by flow cytometer using JC-1 (a mitochondrial-specific probe). As shown in Figure 3a, MEDD treatment concentration-dependently reduced MMP levels, indicating that MEDD depolarized mitochondrial membranes. We next investigated changes in the levels of anti-apoptotic proteins. As shown in Figure 3b, MEDD reduced levels of anti-apoptotic Bcl-2 and Bcl-xL.

Methanolic extract of *Dictamnus dasycarpus* Turcz -induced cell death was enhanced by LY294002 pretreatment

The PI3K/Akt signaling pathway functions as a critical regulator of cell survival and proliferation,[31] and the MAPKs such as p38 MAPK, ERK, and JNK play fundamental roles in survival, proliferation, and apoptosis.[32] To determine whether these signaling pathways play a role in MEDD-induced apoptotic response, we pretreated AGS cells with specific inhibitors of PI3K/Akt or MAPK and
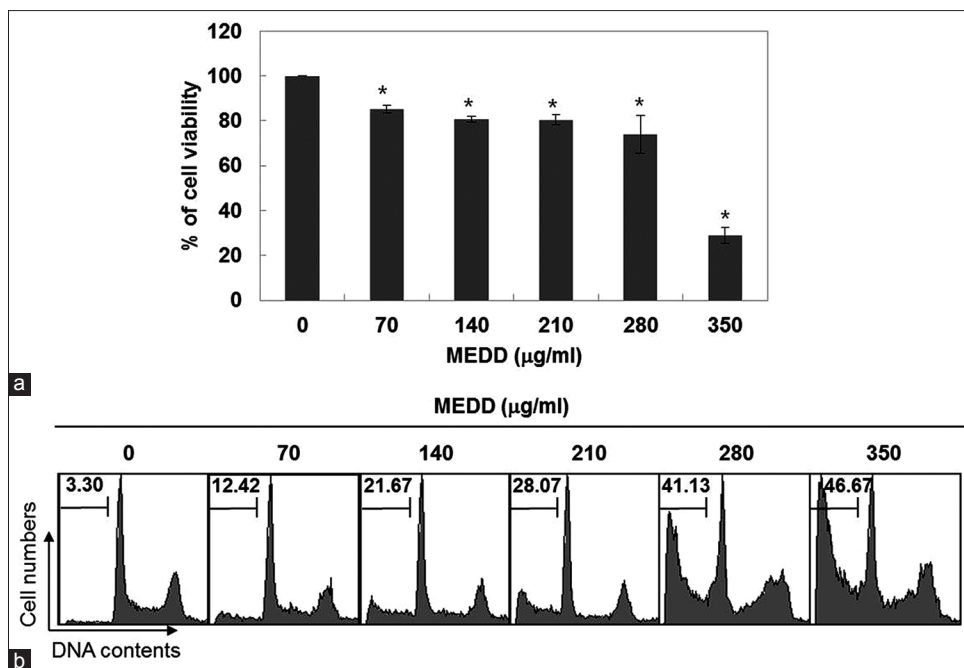
Figure 1: Induction of apoptosis by methanolic extract of *Dictamnus dasycarpus* (MEDD) Turcz in AGS cells. (a) Cells were treated with the indicated concentrations of MEDD for 24 h. Cell viabilities were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium assay. The significances of difference were determined using the Student's *t*-test (*$P < 0.05$ vs. untreated cells) (b) to quantify the degree of apoptosis induced by MEDD, cells grown under the same conditions as (a) were evaluated by flow cytometry for sub-G1 DNA contents (a surrogate of apoptotic DNA degradation). Results are the mean ± standard deviations of two different experiments
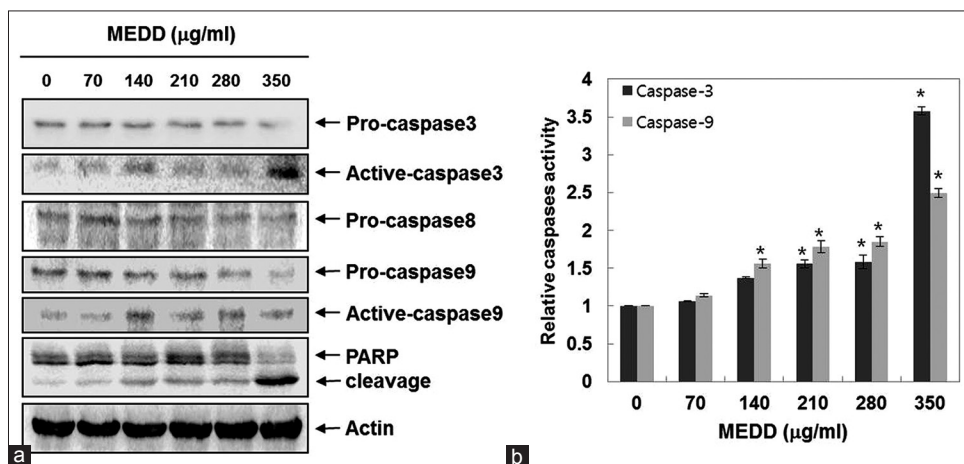

Figure 2: Activations of caspases and degradation of poly (ADP-ribose) polymerase protein by methanolic extract of *Dictamnus dasycarpus* (MEDD) in AGS cells. (a) AGS cells were treated with the indicated concentrations of MEDD for 24 h. Membranes were probed with the indicated antibodies. Actin was used as the internal control. (b) After 24 h incubation with the indicated concentrations of MEDD, cells were lysed, and aliquots were assayed for *in vitro* caspase-3 and -9 activities using DEVD-p-nitroaniline (pNA) and LEHD-pNA as substrates, respectively. Results are the mean ± standard deviations of three different experiments. *$P < 0.05$ versus untreated control

then measured cell viabilities using the above-mentioned MTT assay. As shown in Figure 4a, pretreatment with SB203580 (a specific inhibitor of p38 MAPK), SP600125 (a potent inhibitor of JNK), or PD98059 (a potent inhibitor of ERK) did not modify the effects of MEDD treatment. However, pretreatment with LY294002 (a specific inhibitor of Akt) significantly enhanced MEDD-induced cell death [Figure 4b].

Pretreatment with LY294002 increased apoptosis by methanolic extract of *Dictamnus dasycarpus* Turcz

AGS cells in sub-G1 phase was increased by MEDD, indicating that the number of apoptotic cells was significantly increased by LY294002 treatment prior to MEDD as compared with treatment with MEDD [Figure 5a]. In addition, pretreatment with LY294002 reduced the pro-form of PARP and increased its cleaved
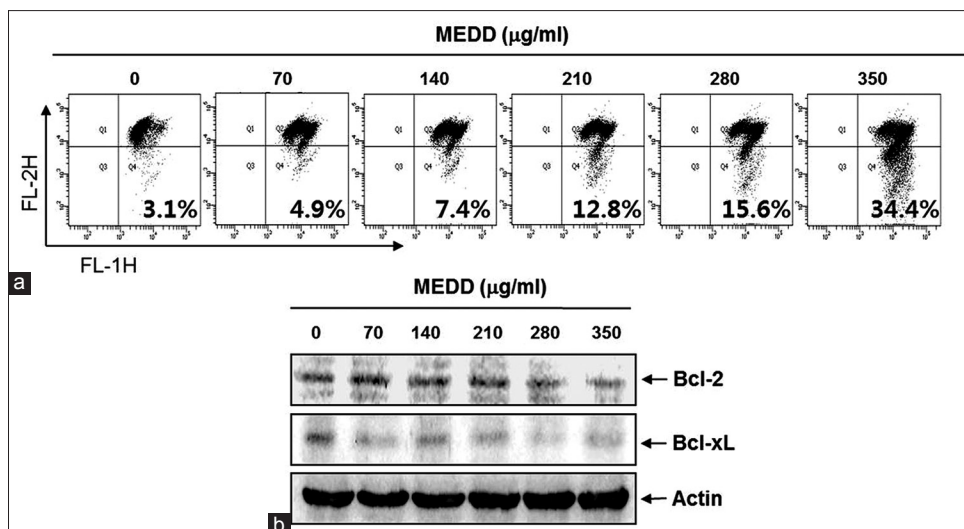
Figure 3: Effects of methanolic extract of *Dictamnus dasycarpus* (MEDD) on the protein levels of Bcl-2 and Bcl-xL and on mitochondrial membrane potential levels in AGS cells. (a) Cells were treated with the indicated concentrations of MEDD for 24 h, collected, and incubated with JC-1 (10 μM) for 20 min at 37°C in the dark. They were then washed once with PBS and subjected to deoxyribonucleic acid flow cytometry. (b) Cell lysates obtained from cells grown under the same conditions as (a) They were separated in SDS-polyacrylamide gels and transferred to nitrocellulose membranes, which were probed with the indicated antibodies. Actin was used as an internal control
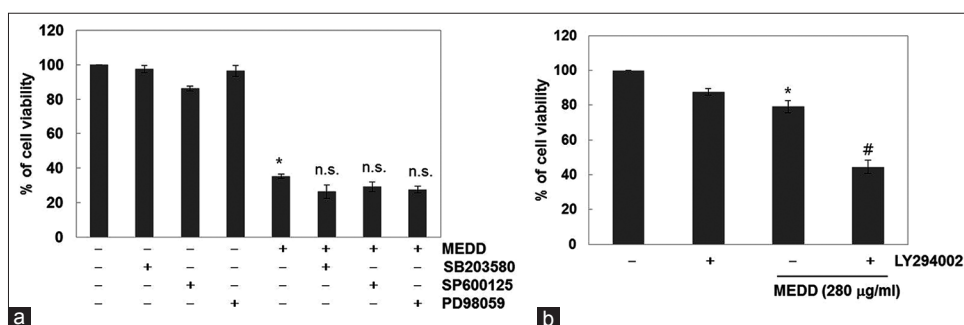

Figure 4: Effects of Akt inhibition on methanolic extract of *Dictamnus dasycarpus* (MEDD)-induced AGS cell death. (a) Cells were pretreated with the indicated mitogen-activated protein kinases inhibitors (SB203580 (20 μM), SP600125 (20 μM), or PD98059 (50 μM)) for 1 h and then treated with MEDD (350 μg/ml) for 24 h. (b) cells were pretreated with the Akt inhibitor (LY294002 (20 μM)) for 1 h and then treated with MEDD (280 μg/ml) for 24 h. Cell viabilities were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium assay. Results are the means ± standard deviations of two different experiments. *$P < 0.05$ versus untreated cells; #$P < 0.05$ versus MEDD-treated cells; n.s.: Not significant versus MEDD-treated cells

form [Figure 5b]. Taken together, these results suggest that MEDD-induced apoptosis in AGS cells is potentiated by the inhibition of Akt.

DISCUSSION

Although recent studies have revealed that the root bark of *D. dasycarpus* has various pharmacological effects such as anti-inflammatory, anti-allergic, anti-fungal, and neuroprotective effects,[8-10,12] its anti-cancer effect lacked experimental confirmation. Therefore, in the present study, we sought to investigate the anti-cancer effect of the root bark of *D. dasycarpus* and the mechanism involved. We used the methanolic extract of its root bark (MEDD) and investigated whether this extract could induce the apoptosis of AGS cells (a human gastric adenocarcinoma cell-line).

Our results indicate that MEDD concentration-dependently induced the apoptosis of AGS cells, and this was confirmed by increases in the sub-G1 population and cleaved PARP protein levels [Figures 1 and 2a].

The extrinsic apoptotic pathway can be triggered by interactions between transmembrane death receptors and their cognate ligands, and these interactions result in the recruitment of the associated death domain (FADD) and caspase-8 to DISC, which leads to the activation of caspase-8.[22,33] The intrinsic apoptotic pathway involves nonreceptor-mediated stimuli that produce mitochondrial mediated signals, which result in the opening of mitochondrial permeability transition pore, loss of MMP, and release of pro-apoptotic proteins like cytochrome c. Bcl-2 family proteins, which include
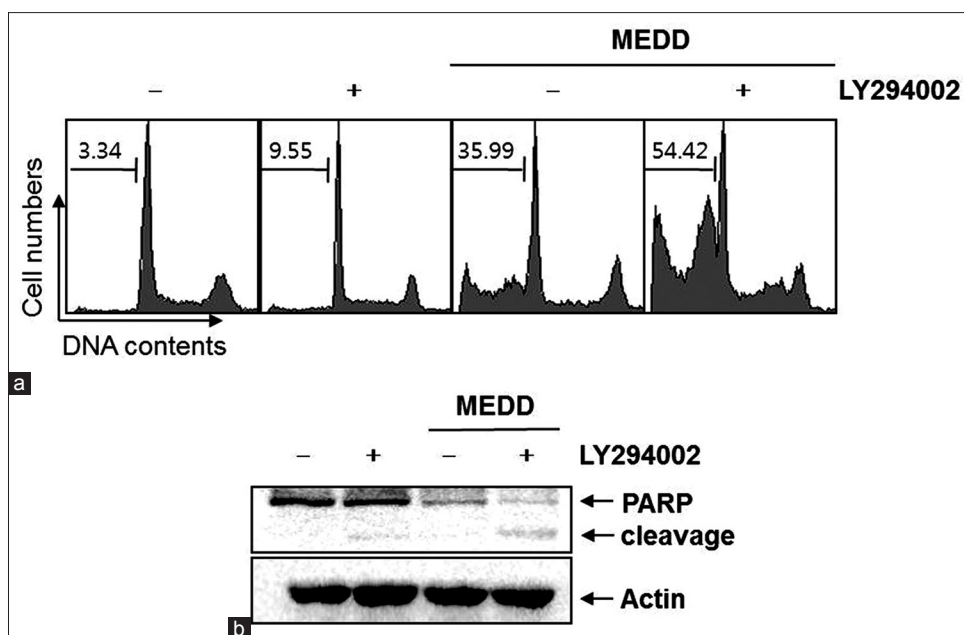
Figure 5: Effects of Akt inhibition on methanolic extract of *Dictamnus dasycarpus* (MEDD)-induced apoptosis in AGS cells. Cells were pretreated with Akt inhibitor (LY294002 (20 μM)) for 1 h and then treated with MEDD (280 μg/ml) for 24 h. (a) Cells were collected and deoxyribonucleic acid contents were analyzed by flow cytometry. (b) Equal amounts of cell lysates were extracted, separated in SDS-polyacrylamide gels and transferred to nitrocellulose membranes, which were probed with the indicated antibodies. Proteins were visualized using an enhanced chemiluminescence detection system. Actin was used as an internal control

anti-apoptotic Bcl-2 and Bcl-xL, and pro-apoptotic proteins, such as, Bax, Bid, and Bad, regulate the release of cytochrome *c* from mitochondria by modulating mitochondrial membrane permeability. Furthermore, release of cytochrome *c* contributes to the activation of caspase-9 and the subsequent sequential activation of caspase-3 and cleavage of PARP.[23,24,34] Our results show MEDD-induced apoptosis was associated with the caspase cascade, the down-regulation of Bcl-2 and Bcl-xL, and loss of MMP [Figures 2 and 3], indicated that MEDD induced mitochondrial dysfunction via Bcl-2 family regulation.

Many researches are currently investigating the anti-cancer effects of herbal medicines on gastric cancer cells. *Sophorae radix* and *Orostachys japonicas* have been reported to inhibit the growth and survival of gastric adenocarcinoma cells via the involvements of transient receptor potential melastatin 7 (TRPM7) ion channels.[35,36] In a previous study, we suggested that human gastric adenocarcinoma cells express TRPM7 channel, which is essentially required for cell survival and a potential pharmacologic target for gastric cancer treatment.[37] Therefore, in the future, we intend to determine the involvements of TRPM7 channels in the anti-cancer effects of MEDD. Buxus Microphylla var. Koreana Nakai Extract (BMKNE) is as a folk remedy for malaria and venereal disease.[38] However, BMKNE has also been reported to inhibit the growth and survival of gastric cancer cells by blockading TRPM7 channel and MAPK signaling.[39] *Flos carthami* (FC) is used in traditional

Asian medicine to treat blood stagnation and its associated diseases,[40] and FC has anti-proliferative effects on human gastric cancer cells and is therefore considered a starting point for the development of agents against gastric cancer.[41]

Recent studies have shown that the PI3K/Akt pathway modulates cell survival, cell cycle progression and cellular growth, and hyperactivation of this pathway in various cancers increases proliferation and reduces apoptosis.[31,42] MAPKs, family of serine/threonine kinases, including p38 MAPK, JNK, and ERK also play important roles in apoptosis and cell proliferation in a variety of cancers,[32,43] and thus, are also considered possible therapeutic targets. Our results show pretreatment with LY294002 (a specific Akt inhibitor) significantly increased MEDD-induced apoptosis, but that pretreatment with MAPK inhibitors did not affect cell death in AGS cells [Figures 4 and 5].

CONCLUSIONS

Our study shows that the methanolic extract of D. *dasycarpus* root bark (MEDD) reduces AGS cell proliferation and induces apoptosis as confirmed by an accumulation of cells in the sub-G1 phase. Furthermore, MEDD-induced apoptosis was found to be associated with activations of caspases and mitochondrial dysfunction via reductions in the levels of Bcl-2 and Bcl-xL proteins. In addition, inhibition of the Akt pathway caused by pretreating

LY294002 enhanced MEDD-induced apoptosis in AGS cells. These findings suggest that MEDD be considered a potential agent for the treatment of human gastric adenocarcinoma.

ACKNOWLEDGMENTS

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