Extracts of *Opuntia humifusa* Fruits Inhibit the Growth of AGS Human Gastric Adenocarcinoma Cells.

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ABSTRACT: *Opuntia humifusa* (OHF) has been used as a nutraceutical source for the prevention of chronic diseases. In the present study, the inhibitory effects of ethyl acetate extracts of OHF on the proliferation of AGS human gastric cancer cells and the mode of action were investigated. To elucidate the antiproliferative mechanisms of OHF in cancer cells, the expression of genes related to apoptosis and cell cycle arrest were determined with real-time PCR and western blot. The cytotoxic effect of OHF on AGS cells was observed in a dose-dependent manner. Exposure to OHF (100 µg/mL) significantly induced (P<0.05) the G1 phase cell cycle arrest. Additionally, the apoptotic cell population was greater (P<0.05) in OHF (200 µg/mL) treated AGS cells when compared to the control. The expression of genes associated with cell cycle progression (Cdk4, Cdk2, and cyclin E) was significantly downregulated (P<0.05) by the OHF treatment. Moreover, the expression of Bax and caspase-3 in OHF treated cells was higher (P<0.05) than in the control. These findings suggest that OHF induces the G1 phase cell cycle arrest and activation of mitochondria-mediated apoptosis pathway in AGS human gastric cancer cells.

Keywords: anticancer activities, gastric cancer cell, *Opuntia humifusa*

INTRODUCTION

The genus *Opuntia* is a member of the Cactaceae family and is widely distributed throughout the world (1). *Opuntia humifusa* (OHF) is one of the cold-hardy cactus species and can be grown during the winter in areas where temperatures reach −20°C (2,3). The therapeutic properties of the OHF cactus parts have been researched for the anti-diabetic effect of the stem (4), the anti-inflammatory activity of the fruit (5), and the prevention of osteoporosis of the seeds (6). Previously, we found that OHF extracts have potent antiproliferative effects on HeLa cervical carcinoma cells (7) and U87MG glioblastoma cells (8). These studies suggested that extracts of OHF might serve as a cell cycle arrest inducer to inhibit the growth of cancer cells. Additionally, it was reported that taxifolin and dihydrokaempferol were the main flavonoids in the extracts of OHF fruits (7). These phytochemicals have been considered as important contributors for the prevention of chronic diseases (9,10).

Gastric cancer is the second most common cause of cancer-related death in the world (11). The case-fatality ratio of gastric cancer is higher than those of colon and breast cancers (12). Despite widely accepted treatment options for cancer, including radiotherapy and chemotherapy, they have limited efficacy. Thus, the discovery of extracts or compounds from natural products is still necessary as an alternative for cancer treatment. Moreover, elucidating the critical events related to carcinogenesis provides an opportunity for inhibiting cancer development via dietary intervention, particularly with functional foods (13).

In spite of several studies related to the nutraceutical effects of OHF, the anticancer effects of OHF on human gastric cancer cells have not been evaluated. Therefore, the aim of the present study was to investigate the antiproliferative activities of OHF and the molecular mechanisms in the AGS human gastric adenocarcinoma cells.
MATERIALS AND METHODS

Extraction of the OHF fruit

OHF fruits were collected from a plantation located in the city of Goyang, Korea. Each fruit (seed-free) was freeze-dried using a freeze drier (EYELA, Tokyo, Japan) at −70°C and grounded using a cyclone mill (UDY Co., Fort Collins, CO, USA). The powder from OHF fruits was extracted using methanol 3 times at room temperature. Methanol extracts were subsequently filtered through Whatman No. 2 filter paper. Methanol-extracted samples were concentrated using a rotary evaporator (Buchi, Flawil, Switzerland) at 40°C. Methanol extracts were suspended in water and then partitioned in hexane to produce a hexane-soluble layer. The aqueous layer was then partitioned using ethyl acetate to yield ethyl acetate-soluble (OHF) and water soluble residues. All extracts were stored at −20°C.

Cell culture

Human gastric cancer cell line AGS was purchased from the Korean Cell Line Bank. Cells were maintained in RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (Gemini, West Sacramento, CA, USA), 1% penicillin/streptomycin (Caisson Labs, North Logan, UT, USA). Cells were grown at 37°C and 5% CO₂ in humidified air.

Assay for cytotoxicity

Cell cytotoxicity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,3 diphenyltetrazolium bromide (MTT) assay (Roche, Mannheim, Germany). This assay is used to indirectly determine cytotoxicity by measuring the activity of mitochondrial enzymes. MTT, a yellow tetrazolium salt, is metabolized by mitochondrial dehydrogenases to form blue formazan (methyltriazole). Briefly, cells were plated in 96-well plates at an initial density of 4×10⁴ cells per well. After incubation for 24 h at 37°C, cells were treated with OHF (100 μg/mL or 200 μg/mL) and incubated for 24 h. Upon completion of treatment with OHF, the cells were collected by trypsinization and combined with floating cells collected from the media. Cells were double stained with 50 μg PI (Sigma-Aldrich Co.) and FITC Annexin V (BD Pharmingen, San Jose, CA, USA) at a dilution of 1:100, and cell death was determined by flow cytometry. The percentage of cells undergoing apoptosis was determined using a FACSCalibur (Becton Dickinson) equipped with CellQuestPro software (Becton Dickinson).

RNA isolation and quantitative real-time PCR

The total RNA from AGS human gastric cancer cells was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Isolated RNA was further purified by using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) with RNase-free DNase (Qiagen) and quantified by the use of a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Synthesis of cDNA was performed using the iScript cDNA synthesis kit (BioRad) and adjusted for GAPDH Ct values for each sample to determine ΔΔCt and relative expression of the target mRNA. Data were presented as 2−ΔΔCt (14). The primers used in this study included the following: p53 (NM_001126115.1), forward: 5′-GGC CAT CTA CAA GCA GTC ACA GCA C-3′ and reverse: 5′-CTC ATT CAG CTC TCG GAA CAT CTC G-3′; p21 (NM_001291549.1), forward: 5′-CCC GTG AGC GAT GGA ACT-3′ and reverse: 5′-CGA GGC ACA AGG GTA CAA GA-3′; Cdk4 (NM_000075.3), forward: 5′-GAG GGG GCC TCT CTA GCT T-3′ and reverse: 5′-CAC GGT TGT AAG TGC CAT CT-3′; Cyclin D1 (NM_053056.2), forward: 5′-ACG GAG TAC AGG GGA GTT TTG-3′ and reverse: 5′-GAA ATA GTG CGG GGT CAT TG-3′; Cdk2 (NM_052827.3), forward: 5′-CAC TGA GAC TGA GGG TGT GC-3′ and reverse: 5′-GGA CTC CAA AAG TCT GGG C-3′; Cyclin E1 (NM_001238.2), forward: 5′-CAT GAT GCC GAG GGA GGC-3′ and reverse: 5′-TTT GCC CAG CTC AGT ACA GG-3′; Bax (NM_001291428.1), forward: 5′-ACC TTT TGC TTC AGG GTT TCA T-3′ and reverse: 5′-ACA GGG ACA GTA GTC GCT GC-3′; Bcl-2 (EU_287875.1), forward: 5′-CTC GTC GCT ACC GTC GTG ACT TGG-3′ and reverse: 5′-CAG ATG CCG GTT CAG GTA CTC AGT C-3′; Caspase-3 (NM_004346.3), forward: 5′-CAA

Determination of the apoptotic cell population

AGS human gastric cancer cells (3×10⁵ cells/well) were plated in 6-well plates. Cells were treated with OHF (100 μg/mL or 200 μg/mL) and incubated for 24 h. Upon completion of treatment with OHF, the cells were collected by trypsinization and combined with floating cells collected from the media. Cells were double stained with 50 μg PI (Sigma-Aldrich Co.) and FITC Annexin V (BD Pharmingen, San Jose, CA, USA) at a dilution of 1:100, and cell death was determined by flow cytometry. The percentage of cells undergoing apoptosis was determined using a FACSCalibur (Becton Dickinson) equipped with CellQuestPro software (Becton Dickinson).
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**Western blot analysis**

Whole cells were lysed in a lysis buffer [20 mM Tris-HCl (pH 8.0), 1% nonyl phenoxypolyethoxylethanol-40, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 50 mM NaF, and 1 mM Na$_3$VO$_4$] supplemented with a protease inhibitors cocktail (Roche Applied Science, Indianapolis, IN, USA). After incubation on ice, lysed tissues were centrifuged, and equal amounts of protein were dissolved in 4× sodium dodecyl sulfate (SDS) sample buffer. Samples were separated in 10% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA) and blocked with 5% nonfat dried milk. The membrane was incubated with the primary antibody (1:1,000 dilution) at 4°C overnight. Antibodies against p53, p21, Cdk4, Cyclin D1, Cdk2, Cyclin E1, Bax, Bcl-2, Caspase-3, and β-actin were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The membrane was washed with Tris-buffered saline with Tween 20, followed by incubation with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc.). Proteins were detected using the chemiluminescence (Western ECL substrate, BioRad). Membrane images were recorded using a ChemiDoc XRS system (BioRad). Protein bands were quantitated by densitometry using the UN-SCAN-IT gel software (Silk Scientific, Orem, UT, USA).

**Statistical analysis**

Data are presented as the mean±SE. Differences between the mean values for individual groups were assessed with a one-way analysis of variance with Duncan’s multiple-range test (SAS version 9.3, SAS Institute Inc., Cary, NC, USA). Differences were considered significant when $P<0.05$.

**RESULTS AND DISCUSSION**

**OHF inhibit cell proliferation on AGS human gastric cancer cells**

The inhibitory effects of OHF on AGS human gastric cancer cells proliferation were evaluated using the MTT
assay. The AGS cell proliferation was significantly suppressed ($P<0.05$) by the OHF treatment in a dose-dependent manner (Fig. 1). Treatment with OHF100 and OHF200 decreased the proliferation of AGS cells by 34.7% and 55.6%, respectively. These results indicated that OHF had cytotoxic effects against AGS cells. Similar to this finding, the inhibitory effects of the extracts of OHF fruits were observed in U87MG glioblastoma cells and HeLa cervical cancer cells (7,8). In addition, OHF fruits contained the flavonoids taxifolin and dihydrokaempferol (7). These compounds have been shown to possess antiproliferative effects on colon cancer cells (15, 16). Therefore, flavonoids in OHF may induce the inhibitory action on AGS cells growth.

Based on the results of the MTT assay, we investigated the effects of OHF on cell cycle progression. It is known that inhibition of the cell cycle is a target for the management of cancer development (17). As shown in Fig. 2, OHF100 significantly increased ($P<0.05$) the number of cells in the G1 phase. This result indicated that OHF have the potential for induction of G1 phase cell cycle arrest in AGS cells. Additionally, an increment of sub-G1 cells was observed ($P<0.05$) in cells treated with OHF 200 (Fig. 2). To further characterize the cell death induced by OHF in AGS cells, cells were analyzed by PI/FITC Annexin V staining with flow cytometry. Exposure to OHF resulted in significant apoptosis (Fig. 3). The treatment of OHF100 and OHF200 increased ($P<0.05$) the early apoptotic cell populations significantly by 14.2% and 31.1%, respectively, compared with the control value of 5.6%. Moreover, significant increases of late apoptotic cells were observed ($P<0.05$) in OHF treated cells. Cancer has been associated with dysregulated apoptotic processes, leading to the inhibition of cell death (13). Thus, apoptosis induction of OHF in AGS cells is also an important event for inhibition of cancer cell proliferation.

**Effect of OHF on cell cycle- and apoptosis-related gene expression**

To further analyze the cell cycle arrest associated molecular mechanisms of OHF, real-time PCR (Table 1) and

| Gene | Control | OHF100 | OHF200 |
|------|---------|--------|--------|
| p53  | 1.05±0.28<sup>b</sup> | 1.32±0.34<sup>b</sup> | 2.52±0.25<sup>a</sup> |
| p21  | 1.14±0.25<sup>b</sup> | 2.03±0.20<sup>b</sup> | 3.30±0.29<sup>a</sup> |
| Cdk4 | 1.04±0.13<sup>b</sup> | 0.57±0.26<sup>b</sup> | 0.36±0.24<sup>a</sup> |
| Cdk2 | 1.12±0.24<sup>b</sup> | 0.59±0.21<sup>b</sup> | 0.55±0.23<sup>b</sup> |
| Cyclin D1 | 1.23±0.32<sup>a</sup> | 1.09±0.19<sup>a</sup> | 0.29±0.28<sup>b</sup> |
| Cyclin E | 1.14±0.25<sup>a</sup> | 0.56±0.22<sup>b</sup> | 0.49±0.29<sup>b</sup> |
| Bax  | 1.08±0.13<sup>a</sup> | 4.49±0.19<sup>a</sup> | 5.57±0.20<sup>a</sup> |
| Bcl-2 | 1.44±0.18<sup>a</sup> | 1.21±0.25<sup>a</sup> | 0.32±0.22<sup>b</sup> |
| Caspase-3 | 1.20±0.10<sup>a</sup> | 1.69±0.17<sup>a</sup> | 1.84±0.14<sup>a</sup> |

Data are presented as the mean±SE. Values with different letters (a-c) in the same row are significantly different ($P<0.05$) according to Duncan’s multiple-range test. The cells were treated with OHF at 100 µg/mL (OHF100) or 200 µg/mL (OHF200) and incubated for 24 h.

![Fig. 3. Effects of *Opuntia humifusa* (OHF) on apoptotic cell numbers in AGS human gastric cancer cells. (A) Apoptotic cell death (right upper and lower windows) and non-apoptotic cell death (left upper window) were measured by double staining propidium iodide (PI)/FITC Annexin V and FACS-based quantification. OHF significantly induced apoptosis in AGS cells. Data shown are representative of triplicate experiments. (B) Significant increases of early (PI−/Annexin V+) and late (PI+/Annexin V+) apoptotic cells were observed in OHF treated cells. Data are presented with the mean±SE. Mean values with different letters (a-c) above the bars are significantly different ($P<0.05$) according to Duncan’s multiple-range test. The cells were treated with OHF at 100 µg/mL (OHF100) or 200 µg/mL (OHF200) and incubated for 24 h.]
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**Fig. 4.** Effects of *Opuntia humifusa* (OHF) on the apoptosis and cell cycle regulatory proteins in AGS human gastric cancer cells. (A) Representative blot showing protein expression following OHF treatment. (B) Band intensity was measured by densitometric analysis and expressed as fold change of the control. Data are presented with the mean±SE. Mean values with different letters (a-c) above the bars are significantly different (*P* < 0.05) according to Duncan’s multiple-range test. The cells were treated with OHF at 100 µg/mL (OHF100) or 200 µg/mL (OHF200) and incubated for 24 h.

Western blot analysis (Fig. 4) for p21, cyclins, and Cdk's were performed. The mRNA and protein levels of p21 in AGS cells were upregulated (*P* < 0.05) by the OHF treatment, compared to the control, whereas the expressions of Cdk4 and Cdk2 were downregulated (*P* < 0.05). Additionally, OHF200 significantly decreased (*P* < 0.05) the
expression levels of cyclin D1 and cyclin E in AGS cells. Cyclin D1 related to the cell cycle progression through the G1 phase by interactions with Cdk2 and Cdk4 (18). When cyclin D1 is overexpressed at the G1 phase, cell proliferation is accelerated (19). The overexpression of cyclin D has been observed in various tumor cells (20, 21). Thus, inhibition of cell cycle progression is an important incident for anticancer activity. The cell cycle inhibitory proteins negatively regulate the cell cycle and discontinue the cell cycle process to the next phase (22). The induction of the G1 phase cell cycle arrest is correlated with the upregulation of Cdk inhibitor, p21 is associated with the inhibition of Cdk2 and Cdk4 (23). The effective approach to inhibit cyclin D1 activity is by reducing its associated kinases Cdk4 (24). Therefore, OHF arbitrated the G1 phase cell cycle arrest by disrupting the expression of cyclin D1 and Cdk4 via the upregulation of p21. It has also been reported that extracts of OHF fruits induced the increment of p21 protein expression in human cervical carcinoma cells (7).

To elucidate the mechanisms underlying the apoptosis by OHF, the expression of p53, Bax, Bcl-2, and caspase-3 was measured by real-time PCR and western blot. As shown in Table 1 and Fig. 4, the mRNA and protein expression levels of Bax were significantly increased (P<0.05) in OHF treated groups when compared to the control AGS cells. Moreover, OHF200 inhibited (P<0.05) Bcl-2 expression, which caused a dose-dependent increase of the Bax/Bcl-2 ratio. With OHF treatment, the expression of caspase-3 was elevated (P<0.05) when compared to the control. Furthermore, OHF200 treatment increased (P<0.05) the expression level of p53.

The anti-apoptotic protein Bcl-2 is expressed on the outer mitochondrial membrane surface and prevents the release of cytochrome c into the cytosol (25,26). In contrast, Bax permeabilizes the outer membrane of the mitochondria and admits the release of cytochrome c, which triggers the activation of caspases to induce apoptosis of the cell (27). The activation of caspase-3 is an important downstream event in the apoptotic process (28). The increase in the Bax/Bcl-2 ratio also leads to the activation of caspase-3 (29). Thus, the balance modulation between Bax and Bcl-2 is decisive for the induction of apoptosis. In the present study, apoptosis induction by the OHF treatment was related to upregulation of Bax and downregulation of Bcl-2, leading to an increase of caspase-3 expression in AGS cells. The expression level of p53 was also increased in OHF treated AGS cells. p53 also advances apoptosis through the mitochondrial pathways (30). The p53-dependent apoptosis leads to mitochondrial apoptotic changes via activation of the Bax and caspases cascade (31). Furthermore, p53 activates the p21 protein to instigate a the G1 phase cell cycle arrest (32). In the current study, the different p53 expression level was observed in the 2 doses of OHF treatment. Therefore, the distinct increased levels of p53 expression were considered to be supportive for the induction of cell cycle arrest and apoptosis in OHF treated AGS cells.

In conclusion, the present study demonstrated that OHF inhibits the growth of AGS cells by causing cell cycle arrest and the induction of apoptosis. OHF induced cell cycle arrest through the p21-mediated G1 phase arrest. Moreover, the activation of the mitochondria-mediated apoptosis pathway is the main events in the process of apoptosis incidence by OHF treatment. OHF was implicated the regulation of Bax and Bcl-2, followed by the significant increment of caspases-3 expression. This research provides a new insight into understanding the inhibition mechanisms of AGS cell proliferation by OHF. Further studies may be needed to identify the phytochemical constituents in OHF.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

REFERENCES

1. Acuña UM, Atha DE, Ma J, Nee MH, Kennelly EJ. 2002. Antioxidant capacities of ten edible North American plants. Phytother Res 16: 63-65.
2. Kim J, Jho KH, Choi YH, Nam SY. 2013. Chemopreventive effect of cactus (Opuntia humifusa) extracts: radical scavenging activity, pro-apoptosis, and anti-inflammatory effect in human colon (SW480) and breast cancer (MCF7) cells. Food Funct 4: 681-688.
3. Goldstein G, Nobel PS. 1994. Water relations and low-temperature acclimation for cactus species varying in freezing tolerance. Plant Physiol 104: 675-681.
4. Hahn SW, Park J, Son YS. 2011. Opuntia humifusa stems lowered blood glucose and cholesterol levels in streptozotocin-induced diabetic rats. Nutr Res 31: 479-487.
5. Lee JA, Jung BG, Kim TH, Lee SG, Park YS, Lee BJ. 2013. Dietary feeding of Opuntia humifusa inhibits UVB radiation-induced carcinogenesis by reducing inflammation and proliferation in hairless mouse model. Photochem Photobiol 89: 1208-1215.
6. Park J, Hahn SW, Son YS. 2011. Effects of cheononyuncho (Opuntia humifusa) seeds treatment on the mass, quality, and the turnover of bone in ovariectomized rats. Food Sci Biotechnol 20: 1517-1524.
7. Hahn SW, Park J, Oh SY, Lee CW, Park KY, Kim H, Son YS. 2015. Anticancer properties of extracts from Opuntia humifusa against human cervical carcinoma cells. J Med Food 18:
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31-44.

8. Hahm SW, Park J, Son YS. 2010. Opuntia humifusa partitioned extracts inhibit the growth of U87MG human glioblastoma cells. Plant Foods Hum Nutr 65: 247-252.

9. Nabel EG. 2003. Cardiovascular disease. N Engl J Med 349: 60-72.

10. Gebhardt R. 2003. Variable influence of kaempferol and myricetin on in vitro hepatocellular cholesterol biosynthesis. Planta Med 69: 1071-1074.

11. Heidari S, Akrami H, Gharaei R, Jalili A, Mahdiani H, Golezar E. 2014. Anti-tumor activity of Ferulago angulata Boiss. extract in gastric cancer cell line via induction of apoptosis. Iran J Pharm Res 13: 1335-1345.

12. Tan J, Qi H, Ni J. 2015. Extracts of endophytic fungus XKC-s03 from Prunella vulgaris L. spica inhibit gastric cancer in vitro and in vivo. Oncol Lett 9: 945-949.

13. Zhao X, Kim SY, Park KY. 2013. Bamboo salt has in vitro anti-cancer activity in HCT-116 cells and exerts anti-metastatic effects in vivo. J Med Food 16: 9-19.

14. Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative Ct method. Nat Protoc 3: 1101-1108.

15. Lee HS, Cho HJ, Yu R, Lee KW, Chun HS, Park JH. 2014. Mechanisms underlying apoptosis-inducing effects of kaempferol in HT-29 human colon cancer cells. Int J Mol Sci 15: 2722-2737.

16. Green DR, Kroemer G. 2009. Cytoplasmic functions of the tumour suppressor p53. Nature 458: 1127-1130.

17. Alonso-Castro AJ, Ortiz-Sánchez E, García-Regalado A, Ruiz G, Núñez-Martínez JM, González-Sánchez I, Quintanar-Jurado V, Morales-Sánchez E, Domínguez F, López-Toledo G, Cerbón MA, García-Carrancá A. 2013. Kaempferitrin induces apoptosis via intrinsic pathway in HeLa cells and exerts antitumor effects. J Ethnopharmacol 145: 476-489.

18. Graña X, Reddy EP. 1995. Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). Oncogene 11: 211-219.

19. Drobnjak M, Osman I, Scher HI, Fazzari M, Cordon-Cardo C. 2000. Overexpression of cyclin D1 is associated with metastatic prostate cancer to bone. Clin Cancer Res 6: 1891-1895.