Abstract. Background/Aim: (–)-Epicatechin is a major constituent of Bulnesia sarmienti, which is known to possess anticancer properties. Here we report that (–)-epicatechin isolated from B. Sarmienti inhibited growth and induced apoptosis of SW480 human colon cancer cells. Materials and Methods: Cells were treated with different concentrations (0, 25, 50, and 100 μmol/ml) of (–)-epicatechin. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, 40,6-diamidine-2-phenylindole dihydrochloride (DAPI) staining, colony-forming assay, DNA fragmentation analysis, reverse transcription-polymerase chain reaction (RT-PCR), annexin V- fluorescein isothiocyanate (FITC) staining, and immunoblot analyses were then carried out. Results: (–)-Epicatechin was found to have cytotoxic activity, and cells treated with this compound had fragmented nuclei, fragmented DNA, and underwent apoptosis. mRNA and protein expression levels of BCL2-associated X protein (BAX) and p53 were up-regulated and those of B-cell lymphoma-2 (BCL2) were down-regulated, while p21 mRNA levels were significantly increased in cells treated with (–)-epicatechin in a concentration-dependent manner. Conclusion: (–)-Epicatechin from B. Sarmienti inhibited colon cancer cell growth and induced apoptosis.

Colon cancer is a significant public health problem in the Western world and the third leading cause of cancer-related death in human (1, 2). Disease survival is related to tumor stage. For example, patients with stage I colorectal cancer, in which the carcinoma remains localized in the submucosa of the colonic epithelium, have an overall 5-year survival rate of over 90%. In contrast, the 5-year survival rate for individuals with metastatic disease (stage IV) drops to less than 10% (1). Since metastasis accounts for over 90% of colon cancer deaths, therapies that target this process and block disease progression are of major concern. Although early-stage colorectal cancer can be cured by surgical removal, most often surgery is combined with adjuvant radiotherapy and chemotherapy. Radiotherapy is often co-administered with one or more chemotherapeutic agents. While advances in developing effective strategies for treating colorectal cancer, continue to be made, chemotherapy is still often limited by severe side-effects and dose-limiting toxicities. Therefore, there is a need to develop compounds that can effectively treat this form of cancer.

Some epidemiological and intervention studies suggest that regular consumption of fruits, vegetables, and green tea is associated with reduced risk of chronic and degenerative disorders, including cancer, Parkinson’s disease, and cardiovascular disease, in both humans and animals (3, 4). Polyphenols are naturally-occurring compounds that are widely found in vegetables, fruits, and beverages. These compounds have different beneficial biological activities such as antioxidant, anti-inflammatory, antiviral, and anti-carcinogenic properties (3, 4). Bulnesia sarmienti (B. sarmienti) is a tree that inhabits in a part of the Gran Chaco area in South America. The essence of this tree is known to have skin-healing properties and the bark is used to treat gastrointestinal problems (5). B. sarmienti has attracted great attention because of its beneficial characteristics, particularly regarding the polyphenolic compounds present in products of this tree (6). The bark extracts of B. sarmienti have been analyzed by high performance liquid chromatography (HPLC) which showed that the main active ingredients are various types of catechins, such as (–)-epigallocatechin (EGC), (–)-epicatechin (EC), (–)-epicatechin gallate (ECG), and (+)-catechin gallate (CG) (7). EC is a common dietary polyphenol found in green tea and cocoa (8). This compound is an important part of the...
human diet and is extensively metabolized during absorption in the intestine. Many studies have shown that tea polyphenols are potent chemopreventive agents that protect against many types of cancer, and these effects are mediated through the inhibition of cell growth or cell-cycle arrest and the induction of apoptosis (9, 10). Granado-Serrano et al. reported that EC and chlorogenic acid regulate apoptotic and survival/proliferation pathways in a human hepatoma cell line (11). Previous studies of our group also demonstrated the growth-inhibitory and apoptotic effects of B. sarmienti extracts against human lung cancer H460 and A549 cells, as well as against sarcoma 180 cells, both in vitro and in vivo (7, 12). However, the mechanisms underlying the effects of EC on SW480 cells have not yet been elucidated. The aim of the present study was to evaluate the effects of EC on the growth and proliferation of SW480 human colon cancer cells. On this regard, we evaluated changes in survival/proliferation pathways, as well as the regulation of apoptosis-related genes and proteins.

Materials and Methods

Reagents and HPLC analysis. EC was isolated from a hot water extract of B. sarmienti by chromatographic separation with DIAION HP 20 resin (Mitsubishi Chemical Industries Limited, Tokyo, Japan), followed by passage through an ODS column (Capcell Pak C18 UG120, 4.6×250 mm; Shiseido, Tokyo, Japan). EC was eluted with a mixture of methanol and water (15:85 v/v, containing 0.1% phosphoric acid) at a flow rate of 1.0 ml/min at 40˚C. We confirmed the presence of a separate EC peak by HPLC (Figure 1). An HPLC-grade EC standard was purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade. The analyses of the sample were performed by HPLC (Waters Co. Milford, MA, USA), consisting of a pump (Waters 2795) and a UV detector (Waters 2487). The chromatographic column used was a SunFireTM C18 (4.6×150 mm, 5 μm, Waters Co.). The mobile phase consisted of water (eluent A) and acetonitrile (eluent B). The gradient was as follows: 0-20 min, linear gradient from 8% to 12% B; 20-40 min, linear gradient from 12% to 20% B. Elution was performed at a solvent flow rate of 1.0 ml/min. Detection was accomplished with a UV detector and chromatograms were recorded at 280 nm. The voucher specimen of B. sarmienti has been deposited in Kyungpook National University, Laboratory of Veterinary Toxicology.

Cell lines and culture. SW480 human colon cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, and 1% penicillin/streptomycin. The cells were incubated at 37˚C in 5% CO₂ and 95% air.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells seeded in 96-well plates at a density of 5×10⁴ cells per well were incubated with EC for 48 h. The medium was removed and cells were then incubated with 100 μl of a 3-(4,5-
dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) solution (5 mg/ml) for 2-4 h. After discarding the medium, 100 μl DMSO were added and gently mixed for 10 min. Finally, the absorbance at 570 nm was measured by an enzyme-linked immunosorbent assay (ELISA) reader (VERSAmax; Molecular Devices, Sunnyvale, CA, USA). Three replicates per condition were assayed, and the average values from three to five separate experiments are presented. Data are expressed as a percentage of the control.

DAPI staining. 4,6-Diamidino-2-phenylindole dihydrochloride (DAPI) staining was conducted according to the method described by Lock et al. (13). Briefly, cells treated with EC (0, 25, 50, and 100 μmol/ml) for 48 h were harvested and washed twice with cold phosphate buffered saline (PBS), fixed with 4% ethanol for 30 min at room temperature, and then washed twice again with PBS. They were then incubated with a 2 μg/ml DAPI solution for 30 min, and cell morphology was evaluated by BX50 fluorescence microscopy with BX-FLA (Olympus, Tokyo, Japan). Cells showing chromatin condensation, nuclear fragmentation, and nuclear condensation were recognized as apoptosis.

Colony- forming assay. The antiproliferative effect of EC on SW480 cells was assayed by a colony-formation assay, as described by Roy et al. (14). Briefly, 0-500 cells were plated into each well of 6-well plates in triplicate and incubated for 24 h. Thereafter, the cells were treated with EC (0, 25, 50, and 100 μmol/ml) and incubated at 37˚C for 13 days. It was expected that each individual surviving cell would proliferate and form a colony during this period. On day 14, the colonies were washed with PBS and stained with 0.5% crystal violet. The colonies that had more than 50 cells per colony were counted using a dissecting microscope.

DNA fragmentation assay. Fragmented DNA was isolated from cultivated cells. In brief, cells (2×10^5) were treated with the EC for 48 h and then collected by centrifugation (2,000 ×g, 10 min). The cells were then resuspended in a 0.5 ml DNA lysis buffer [2% sodium dodecyl sulfate (SDS), 10 mM EDTA, and 10 mM Tris-HCl, pH 8.5]. The lysis was immediately incubated with 0.1 mg/ml proteinase K for 3 h at 37˚C. After adding isopropanol to the lysate, DNA was precipitated with 70% ethanol. The suspension was then centrifuged, and the isolated DNA was treated with 100 μl of 10 mM Tris-HCl and 0.5 mg/ml RNase A (Biosesang, Inc, Seongnam, Korea) at 37˚C for 24 h. Samples of the DNA were then loaded onto a 2% agarose gel containing ethidium bromide and underwent electrophoresis. The DNA bands were visualized under ultraviolet illumination.

Reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from cells that had been treated with EC (0, 25, 50, and 100 μmol/ml) for 48 h and was subjected to RT-PCR. The primer sequences were as follows: BCL2-associated X protein (BAX), forward: 5'-TCTGACGGCAACTTCAACTG-3' and reverse: 5'-TCACTTGTGGCTCAGATAGGC-3'; p53, forward: 5'-GGAGGATTGTGGCCTTCTTT-3' and reverse: 5'-GCACCTCAAAG

Fig. 2. The antiproliferative effect of (−)-epicatechin (EC) isolated from Bulnesia sarmienti on SW480 human colon cancer cells. Cells were treated with different concentrations (0, 25, 50, and 100 μmol/ml) of EC for 48 h. Cell viability was then measured by an 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Growth inhibition was calculated as a percentage of growth compared to that of the untreated controls. Each data point represents the mean±SD of four wells from three independent experiments (n=4, *p<0.05, compared to the control).
Figure 3. The effect of (−)-epicatechin (EC) on morphological changes in the nucleus. A: Control cells without EC treatment; B: cells treated with 25 μmol/ml EC; C: cells treated with 50 μmol/ml EC, and D: cells treated with 100 μmol/ml EC for 48 h. Nuclei morphology was then evaluated by 40,6-Diamidine-20-phenylindole dihydrochloride (DAPI) staining. Arrows show condensed and fragmented nuclei, indicative of apoptosis. Magnification, ×100.

Figure 4. The antiproliferative effect of (−)-epicatechin (EC) on SW480 cells assessed by a colony-formation assay. Cells (0-500) were plated into each well of 6-well plates in triplicate and incubated for 24 h. The cells were then treated with EC (0, 25, 50, and 100 μmol/ml) and incubated at 37°C for 13 days. On day 14, the colonies were washed with phosphate buffered saline (PBS) and stained with 0.5% crystal violet.
pellets were resuspended in 100 μl of 1× annexin-binding buffer and 1 μl of an annexin V-Fluorescein isothiocyanate (FITC) working solution was added to each 100 μl of the cell suspensions. The suspension volume was brought to 250 μl with 1× binding buffer. The stained cells were placed on a glass slide and covered with a glass coverslip. The cells were observed under a fluorescence microscope using a filter set for FITC detection. Alternatively, the stained cells were immediately analyzed by FACScan flow cytometry (FACS Calibur, Becton Dickinson, San Jose, CA, USA). For each measurement, at least 20,000 cells were counted.

Statistical analysis. The values are expressed as the mean±SD Differences between the control and treatment groups were determined by the Student’s t-test. p-Values less than 0.05 were considered to be statistically significant.

Results

Cell viability. In order to determine the potential effects of EC on the viability of SW480 human colon cancer cells, the cells were exposed to different concentrations (25, 50, and 100 μmol/ml) of EC for 48 h. MTT assay results showed that SW480 cell proliferation was inhibited by EC treatment in a concentration-dependent manner (Figure 2). The 50% inhibitory concentration (IC50) was less than 60 μmol/ml of EC. Treatment with 100 μmol/ml of EC resulted in a maximum decrease (69.86%) in cell viability after 48 h of incubation.

DAPI staining. Figure 3 shows the apoptotic morphological changes induced by EC treatment using DAPI staining. Untreated SW480 cells with normal morphology served as the control group. SW480 cells treated with EC (25, 50, and 100 μmol/ml) for 48 h exhibited condensed and fragmented nuclei, indicative of apoptosis. These results suggest that EC is capable of inducing marked apoptotic morphological changes in SW480 cells.

Figure 5. (−)-Epicatechin (EC) induces apoptosis of SW480 cells. The cells were treated with 0, 25, 50, and 100 μmol/ml of EC for 48 h. The fragmented DNA was isolated and subjected to 2% agarose gel electrophoresis. The bands were then visualized and photographed.

Figure 6. The effects of (−)-epicatechin (EC) on the expression of apoptosis-related proteins in SW480 cells. A: Total RNA obtained from SW480 cells treated with 0, 25, 50, and 100 μmol/ml of EC for 48 h. The expression of p53, p21, and BCL2 family proteins was measured by reverse transcription-polymerase chain reaction (RT-PCR). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as the internal control. B: Total cell protein (30 μg) isolated from EC-treated SW480 cells was subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with antibodies against BAX, p53, and BCL2. β-Actin was used as a loading control. Immunoblot data presented are representative of the results obtained from three separate experiments.
EC inhibits SW480 cellular clonogenic survival. The effect of EC on the clonogenic survival of SW480 cells was examined. There was a decrease in the ability of the SW480 cells to form colonies with increasing concentration of EC (Figure 4). Concentrations of 50 and 100 μmol/ml clearly inhibited cell proliferation, although some colonies were formed by 14 days. These results indicate that EC has antiproliferative and antimetastatic effects on SW480 cells.

Effect of EC on DNA fragmentation. Typical ladder patterns of internucleosomal DNA fragmentation are a known hallmark of apoptosis. To determine whether EC induces apoptosis, DNA was isolated from control and EC-treated cells, and then separated by agarose gel electrophoresis. A typical DNA ladder pattern was observed in cells treated with EC in a concentration-dependent manner. This finding further demonstrates that EC treatment induces apoptosis of SW480 cells (Figure 5).

Changes of mRNA expression levels of several genes. After treatment with EC (25, 50 and 100 μmol/ml), significant increase in the mRNA expression of BAX was detected (p<0.05). However, there was a significant decrease of anti-apoptotic BCL2 mRNA. p53 is a transcription factor which is encoded by the Tumor protein P53 (TP53) gene in humans. This factor is important in multicellular organisms, where it regulates the cell cycle and thus functions as a tumor suppressor that protects against cancer. The expression of p21 gene is tightly controlled by the tumor suppressor protein p53, through which this protein mediates the p53-dependent cell-cycle G1 phase arrest in response to a variety of stress stimuli. The p53 and p21 mRNA levels were significantly increased in a concentration-dependent manner in cells treated with EC (Figure 6A).

Changes in p53, BAX, and BCL2 protein expression following EC treatment. Immunoblot analyses of proteins associated with growth arrest and/or apoptosis were performed of SW480 cells treated with EC (25, 50, and 100 μmol/ml) for 48 h (Figure 6B). p53 protein was increased after treatment with EC in a concentration-dependent manner. Treatment with EC caused a marked and
concentration-dependent decrease in BCL2 expression in the cells, but increased the expression of BAX, thus leading to an increased ratio of BAX to Bcl2.

EC induces apoptosis of SW480 cells. After staining with annexin V-FITC, the cells were observed with a fluorescence microscope and analyzed by flow cytometry. The results show that EC treatment significantly increased apoptosis of SW480 cells in a concentration-dependent manner. Viable cells were negative for annexin V staining; truly apoptotic cells were positive for annexin V staining (Figure 7A). Apoptosis was also measured by flow cytometric analysis in cells treated with EC, and labeled with annexin V. We found that EC caused a concentration-dependent increase in SW480 cell apoptosis (Figure 7B). It was observed that treatment of SW480 cells with 25, 50, and 100 μmol/ml of EC for 48 h increased the percentage of early apoptotic cells from 9.93% to 19.99% in a concentration-dependent manner compared to the untreated control cells. These results suggested that apoptosis may be a major contributor to the biological efficacy of EC in SW480 cells.

Discussion

Cancer cells generally exhibit highly proliferative, migrative, and matrix-invasive characteristics by modulating expression of signaling molecules (15). Promising anticancer agents with strong inhibitory effects on survival-related proteins or the ability to activate apoptosis-associated proteins have been proposed, especially agents found in natural sources, since natural compounds are considered safe as they can be derived from commonly consumed food. We previously reported that catechins are the major compounds in extracts from B. sarmienti with HPLC analysis (12). EGCG, the most abundant catechin, has effects on various biological functions. Antitumor effects of EGCG have been reported to be mediated by apoptosis (16). EC might be an active ingredient of extracts of B. sarmienti. Recently, some researchers have reported that EC in green tea also affects apoptosis, cell growth inhibition, and membrane transporter function (17, 18). However, the EC in extracts of B. sarmienti have not been studied in detail.

In the present study, we determined whether EC isolated from B. sarmienti inhibits SW480 cell proliferation, prevents their metastasis, and promotes their apoptosis. The cytotoxic effects of EC in the cells were demonstrated by reduced cell viability.

Necrosis and apoptosis are involved in cell death. There are some key features for apoptosis that are shared between different cell death pathways. Schmid et al. reported that microscopic examination of stained cell nuclei is the most direct and reliable test for apoptosis (19). This technique is also the most direct and reliable test for nuclear and chromatin disintegration, and provides a way to distinguish viable cells from cells that are apoptotic and necrotic. In the present study, DAPI staining showed that the appearance of apoptotic morphological features, such as condensed and fragmented nuclei, is clearly evident after 48 h of EC treatment. Apoptosis is also characterized by cell shrinkage, loss of contact with neighboring cells, formation of cytoplasmic vacuoles, membrane blebbing, chromatin condensation, DNA fragmentation, and formation of apoptotic bodies (20).

The ability of SW480 cancer cells to form colonies in culture plates was confirmed in the present study. The treatment of cells with EC inhibited colony formation. EC induces apoptosis through the induction of BAX mRNA expression. When BAX expression exceeds that of BCL2, programmed cell death is accelerated and the cell death-suppressive effects of BCL2 are oppressed. In BAX-deficient mice, it was demonstrated that pro-apoptotic BAX can function as a tumor suppressor (21). Apoptosis can be induced by p53 through several pathways, one of which involves BCL2 family members. BAX, as a p53 target, promotes the release of cytochrome c from the mitochondria. p21 is a target gene of the tumor suppressor p53 and prevents cancer cell growth due to its ability to transiently or permanently stop proliferation, it is thus an important component of tumor suppressor mechanisms (22). Anticancer drugs may exert antiproliferative effects by altering the expression of target genes such as p53 and p21, with consequent cell-cycle arrest and apoptosis.

In the present study, we found that BAX, p53, and p21 gene expression increased and BCL2 gene expression decreased, in a concentration-dependent manner in SW480 cells, following EC treatment. Addressing the activation of apoptotic factors in response to EC will offer a better understanding of how EC promotes apoptosis. Under normal conditions, p53 plays a crucial role in inducing apoptosis of human and murine cells following DNA damage (23). Furthermore, p53 is the most commonly mutated tumor suppressor gene in several types of cancers and the sensitivity of cancer cells to chemotherapeutic agents is greatly influenced when the function of p53 is abrogated (24).

The role of BCL2 family proteins in apoptosis, particularly of BAX and BCL2, has received much attention. Upon apoptosis induction, BAX in the cytosol translocates to the mitochondria and executes its pro-apoptotic function (23). BCL2 forms heterodimers with BAX to exert its anti-apoptotic effect (25). In fact, alterations in the proportions of BAX and BCL2 play paramount roles in determining whether cells will undergo apoptosis. In the present study, we found that EC-mediated the up-regulation of BAX and down-regulation of BCL2. These findings provide strong evidence that increased BAX/BCL2 ratios are responsible for cytochrome c release from the mitochondria that leads to programmed cell death.
p21 is an important cellular checkpoint protein for G1 and G2 arrest and can induce permanent cell-cycle arrest in cancer cells (26). Thus, up-regulation of p21 in cancer cells could be an effective antitumor therapeutic strategy (27). Our results support the notion that EC-induced p21 might mediate apoptosis of SW480 cells.

Apoptosis is an important homeostatic mechanism that balances cell division and cell death, and helps maintain the appropriate numbers of cells in the body. Disturbances of the apoptotic process in cancer cells have been studied in detail, and induction of apoptosis in cancer cells is one of the strategies for anticancer drug development (28). There are several crucial cellular and molecular biological features associated with apoptosis, including cell shrinkage, chromatin disorganization, externalization of phosphatidylserine (PS), and caspase activation (29). Annexin V is a dye that preferentially binds PS, which is exposed during the early stages of apoptosis due to cell-surface phospholipid asymmetry. In the current study, by using annexin V we showed that EC inhibited the proliferation of SW480 cells by inducing apoptosis. The ratios of apoptotic cells detected by fluorescent microscopy after annexin V-FITC/PI staining were increased significantly following treatment with EC, suggesting that apoptosis plays an important role in the antiproliferative effects of EC on SW480 cells.

In conclusion, EC was identified here as a possible anticancer agent. The present study demonstrated that EC isolated from B. sarmienti exerts a variety of effects on SW480 human colon cancer cells, including inhibition of cell growth and nuclei fragmentation. The mRNA and protein levels of the pro-apoptotic factor BAX were up-regulated following treatment with EC. In contrast, the mRNA and protein expression of the antiapoptotic factor BCL2 was inhibited. p53 and p21 mRNA and p53 protein levels were increased. These results provide further evidence that EC is a potential therapeutic agent for colon cancer treatment. However, further studies are needed to elucidate the upstream signaling pathways associated with EC action.

Conflicts of Interest

There are no conflicts of interest for any of the Authors.

Acknowledgements

This study was supported by a grant of the Korea Health technology R&D Project, Ministry of Health & Welfare, Republic of Korea (#A111345).

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Received August 1, 2012
Revised October 11, 2012
Accepted October 12, 2012