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Full Length Research Paper

Anticancer activity of persimmon (*Diospyros kaki* L.) calyx extracts on human cancer cells

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Natural products are the source of numerous therapeutic agents. Recent progress in the discovery of drugs from natural products has resulted in development of compounds for use in cancer treatment. Results from this study demonstrated that methanolic extracts of persimmon calyx (PCE) have cytotoxic effects in human cancer cells. Through MTT reduction assay, lactate dehydrogenase release assay, morphological assay, and colony formation assay, highly cytotoxic effects were demonstrated in human cancer cells treated with various concentrations of PCE. As expected, PCE inhibited growth of HT-29 cells in a dose-dependent manner. In particular, 500 µg/ml of PCE showed 31.1% inhibition against HT-29 cells. Interestingly, characteristics of apoptosis, including chromatin condensation and sharking, were shown to occur in HT-29 cells 24 h after treatment with 500 µg/ml of PCE. In addition, PCE also showed cytotoxic activity against HeLa and PANC-1 cells, as well as HT-29 cells. These results indicate that PCE contains bioactive materials and that it is a potential candidate for use as a chemotherapeutic agent against human cancer cells.

Key words: Natural products, persimmon calyx, cytotoxic effects, cancer cells, apoptosis.

INTRODUCTION

Despite advancements in options for early detection and treatment, in terms of morbidity and mortality, cancer continues to be a major health issue. In the current era cancer in its multifarious forms is one of the principal causes of human death. There are several primary types of cancer; these have been classified into three types: carcinomas, sarcomas, and lymphomas (Brown and Giaccia, 1998). In spite of advances in development of chemotherapeutic drugs, radiotherapy, and surgery, even in modern times, most advanced cancers remain incurable. All of these treatment options have significant side effects, and resistance to anti-cancer drugs is a serious problem (Lee et al., 2006; Panchal., 1998). Also, in normal tissue and cells, these agents have been associated with serious cytotoxicity (Jo et al., 2008; Manoj et al., 2001).

Therefore, for development of safer and better therapeutic drugs, the necessity for research has become critical. Overcoming limitations and improving the effectiveness of cancer chemotherapy requires isolation of new anticancer drugs that are specifically toxic to cancer cells and identification of new targets that destroy cancers when exposed to chemotherapy (Vaux and Korsmeyer, 1999). Many recent studies have reported on potential antitumor activity of natural products and compounds through induction of apoptosis (Park et al., 2002). Consequently, over the course of our screening program for evaluation of cytotoxic activity of natural products, we found that methanolic extracts of persimmon calyx (PCE) exerted cytotoxic effects against human cancer cells. Persimmon is an important fruit in Asian countries, including Japan, China, and Korea. Persimmon is also rich in antioxidant phenolic compounds other than tannins, and the potential of these compounds to reduce the risk of chronic disease through protection of tissues against free radical-mediated damage has been demonstrated (Ahn et al., 2002; Gorinstein et al., 1994). However, the anti-proliferative effects of PCE have not been elucidated.

In this study, we assessed the cytotoxic effects of PCE against human cancer cells, and subsequently, have

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proposed the mechanism responsible for its cell death activity.

MATERIALS AND METHODS

Materials and preparation of methanolic extracts from persimmon

Calyx, peel, and flesh of persimmon were kindly supplied by sweet persimmon Research Institute, Korea. Five gram of calyx, peel, and flesh from persimmon were extracted with 100 ml of methanol for 3 days at room temperature and filtered through Whatman No. 1 filter paper (Advantec, Tokyo, Japan). The methanol was then removed by evaporation *in vacuo*, and the dried methanolic extracts were obtained. These extracts were name persimmon calyx (PCE), persimmon peel (PPE), and persimmon flesh (PFE), respectively. These extracts were then dissolved in dimethyl sulfoxide (DMSO) with a concentration of 50 mg/ml for experiments.

Cell culture and treatments

HT-29 human colon cancer cells, HeLa human cervix adenocarcinoma cells and, PANC-1 human pancreatic cancer cells were obtained from the Korean Cell Line Bank (KCLB). HT-29 cells were maintained in RPMI1640 medium (Invtrogen, NY, USA) and HeLa, PANC-1 cells were maintained in DMEM medium (Invtrogen, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FSB), penicillin (100 U/ml), streptomycin (100 mg/ml), and 2 mg/ml $NaHCO_3$ in a 37℃ incubator with 5% CO_2. PCE, PPE, and PFE were added to culture medium so that the final concentration of DMSO was less than 1%.

Morphological analysis

HT-29 cells seeded in 6-well plate (2.0 x 10^5 cells/well) and incubated in RPMI 1640 at 37℃ under a 5% CO_2 for 24 h. The cells were pretreated with 100, 250, and 500 µg/ml of PCE. After incubation for 24 h, the cellular morphology was observed using a phase-contrast microscope (Nikon, Japan). The photographs were taken at x100 magnification.

MTT reduction assay

Cell viability was measured with blue formazan that was methabolized from MTT by mitochondrial dehydrogenase, which is active only in live cells. 3-(4,5-dimehtyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was provided by Sigma-Aldrich (St Louis, MO, USA). Human cancer cells were preincubated in a 96-well at a density of 1.0 x 10^5 cells/well for 24 h; cells were pretreated with various concentrations of PCE, PPE, and PFE. After incubation for 24 h, the MTT reagent (5 mg/ml) was added to each well, and the plate was incubated for an additional 1 h at 37℃. At the end of incubation the media were removed, and the intracellular formazan product was dissolved in 100 µl of DMSO. The absorbency of each well was measured at 540 nm using an ELISA reader (BioRad, Model 680, USA), and the percentage viability was calculated.

Lactate dehydrogenase release assay

Cytotoxicity was determined by measuring the release of lactate dehydrogenase (LDH). An LDH release assay kit was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). HT-29 cells were pretreated with various concentrations of PCE for 24 h and the supernatant was used to assay of LDH activity. The reaction was initiated by mixing 50 µl of cell-free supernatant with potassium phosphate buffer containing nicotinamide adenine dinucleotide (NADH) and sodium pyruvate, in a final volume of 100 µl, and added to the 96-well plate. A colorimetric assay was applied, according to which the amount of formazan salt is proportional to the LDH activity of the sample. The intensity of the red color formed in the assay, measured at 540 nm, was proportional to the LDH activity and number of damaged cells. The data were normalized to the activity of LDH released from vehicle-treated cells, and expressed as a percentage of the control.

Colony formation assay

HT-29 cells were seeded at 5.0 x 10^4 cells/well in 24-well plates, incubated overnight, and treated with 100, 250 and 500 µg/ml of PCE for 24 h. The cells were then diluted in new medium, replated at 1.0 x 10^3 cells/well in 6-well plates, and cultured for 7 to 8 days to form colonies. The colonies were then fixed with 10% formaldehyde, stained with 0.01% crystal violet, and counted. Cell survival was calculated by normalizing the survival of the control cells as 100%.

Cell staining

HT-29 cells from exponentially growing cultures were seeded in 6-well plates (2.0 x 10^5 cells/well). The cells were treated with or without PCE for 24 h, washed twice with phosphate-buffer saline (PBS) and then fixed in PBS containing 10% formaldehyde for 2 h at room temperature. The fixed cells were washed with PBS, and stained with Hoechst 33342 (Sigma) for 1 h at room temperature. Then the cells were washed twice more with PBS, and the Hoechst-stained nuclei were excitation and emission wave lengths set at 358 and 461 nm, respectively. Cells were examined using a fluorescence microscope (Nikon, Japan). The photographs were taken at a magnification of x400.

Statistical analysis

All data are the means of ± S.D. of three determinations and data was analyzed using the SPSS package for Windows (Version 11.5). The data were evaluated by one-way analysis of variance (ANOVA) followed by Scheffe's test. The differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Cytotoxic activity of PCE in human cancer cells

For determination of cytotoxicity in HT-29 human colon cancer cells, extracts from the calyx (PCE), peel (PPE), and flesh (PFE) of persimmon were measured by MTT reduction assay. HT-29 cells were originally derived from a human colon cancer, and were representative of a vascularized solid tumor. *In vivo* these cells exist in stressful microenvironments, including glucose deprivation (Duan et al., 2003; Matsuo and Ito, 1978). HT-29 cells were exposed for 24 h to various concentrations of three extracts (50 to 500 µg/ml). As shown in Figure 1, among the three extracts, PCE showed the highest cytotoxicity in HT-29 cells. Compared to the control, PCE

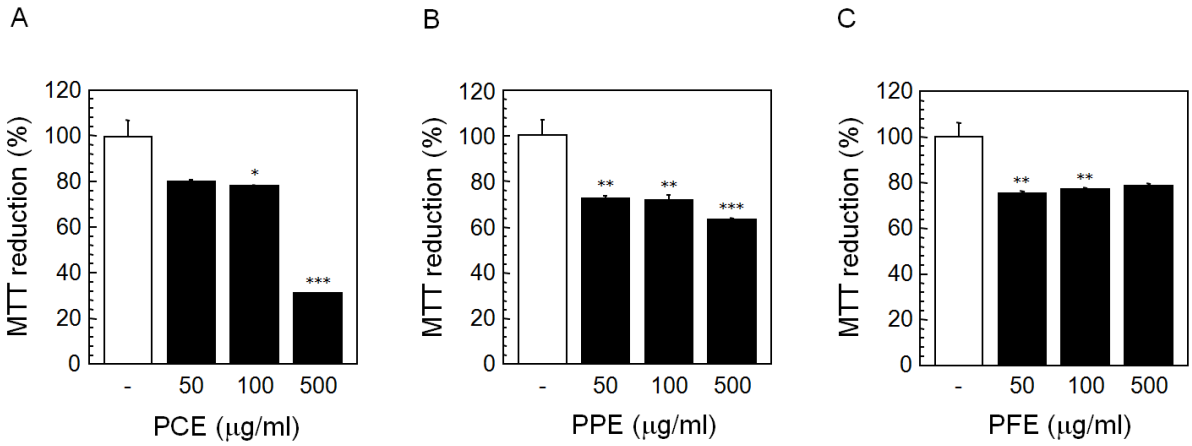

Figure 1. The extracts of persimmon induced cell death in HT-29 human colon cancer cells. The cells were exposed to the indicated various concentrations of extracts from persimmon for 24 h (A; PCE, B; PPE and C; PFE). After MTT reduction assay, the MTT reduction rate was calculated by setting each of the control survivals equal to 100%. Significant vs. control untreated cells (*p<0.05; **p<0.01; ***p<0.001). Source: Jo et al. (2008).

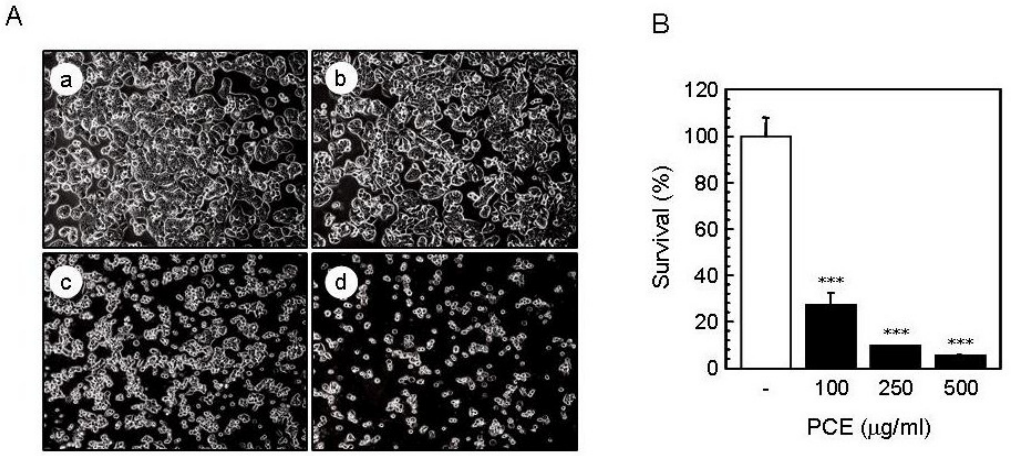

Figure 2. PCE induced cell death in HT-29 cells. The cells were exposed to various concentrations of PCE for 24 h. Cell viability were measured using a morphological analysis (A), and colony formation assay (B). The cells were exposed to various concentrations of PCE, and morphological changes were monitored for 24 h (a; control, b; 100 μg/ml, c; 250 μg/ml, and d; 500 μg/ml). After colony formation, the survival rate was calculated by setting each of the control survival rates. After 7 to 8 days, the formed colonies were fixed with 10% formaldehyde, stained with 0.01% crystal violet, and counted. ***significant vs. control untreated cells (p<0.001). Source: Jo et al. (2008).

effected an 80 to 30% reduction in cell viability, which occurred in a dose-dependent manner. Next, for assessment of cytotoxicity in PCE-treated HT-29 cells, the cells were incubated with PCE, and morphological alterations were confirmed through phase-contrast microscopy. As shown in Figure 2A, after 24 h of incubation with 100, 250 and 500 μg/ml of PCE, many of the cells showed cytoplasmic shrinkage, and became detached from each other or floated in the medium. Furthermore, the cytotoxic effects of PCE on cultured HT-29 cells were determined by measurement of extent of LDH leakage into the medium. Loss of intracellular LDH and its release into the culture medium are indicators of irreversible cell death due to cell membrane damage. As shown in Table 1, PCE caused cell death in a dose dependent manner by an 18 to 43% increase in LDH-release. For further clarification of these results, we examined the effects of PCE on cell viability using a colony formation assay. The highest concentration of PCE (500 μg/ml) induced an extreme reduction in cell viability, as demonstrated by 95% inhibition of colony formation (Figure 2B). In addition, PCE showed cytotoxic

Table 1. PCE induced cell death in HR-29 cells. Cytotoxicity was measured using a LDH release assay.

Treatment	Concentrations (µg/ml)	LDH release (%)
Control	-	28.9 ± 0.137
PCE	250	21.4 ± 1.467
	500	43.1 ± 0.699

For the results of the LDH release assay, the data were normalized to the activity of LDH released from vehicle-treated cells, and expressed as a percentage of the control.

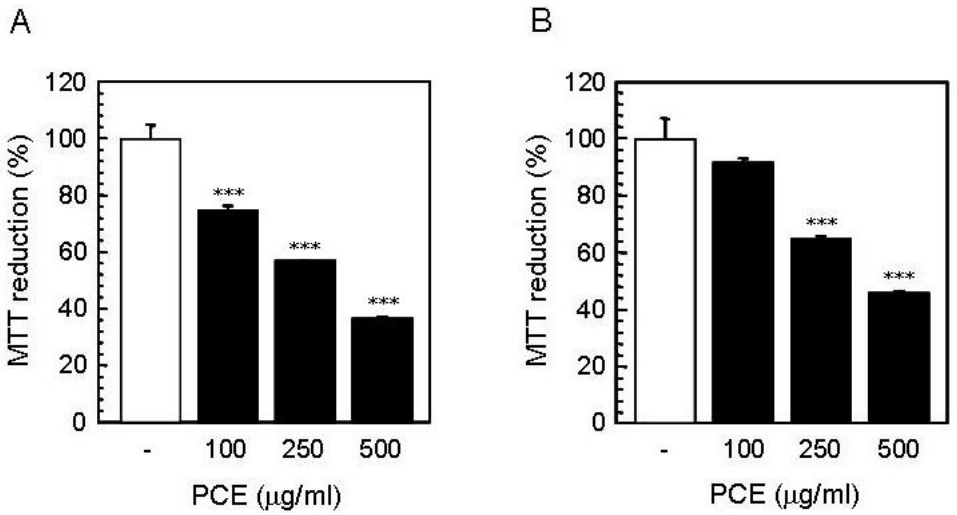

Figure 3. PCE induced cell viability in human cancer cells. The cells were exposed to the indicated various concentrations of PCE for 24 h (A**;** HeLa cells, B; PANC-1 cells). After an MTT assay, the MTT reduction rate was calculated by setting each of the control survivals equal to 100%. ***significant vs. control untreated cells ($p<0.001$). Source: Jo et al. (2008).

activity against HeLa and PANC-1 cells, as well as HT-29 cells, in a dose-dependent manner (Figure 3). These results clearly indicate that PCE inhibited cell growth and induced cell death in human cancer cells.

Induction of apoptosis by PCE

Next, we evaluated the question of whether or not the cytotoxic activity of PCE in HT-29 cells was a result of apoptosis. Apoptosis, or programmed cell death, is an intrinsic program of cell death involved in regulation of various physiological and pathological processes. Apoptosis is an evolutionary conserved, intrinsic program of cell death that occurs in various physiological and pathological situations. Apoptosis is critical for tissue homeostasis, which depends on balance between proliferation and cell death (Johnstone et al., 2002; Lowe and Lin, 2000). Apoptosis is characterized by distinct morphologic changes that include cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation, and formation of apoptotic bodies (Herr and Debatin, 2001).

In order to determine whether or not the cytotoxic activity of PCE was a result of apoptosis, HT-29 cells were treated for 24 h with various concentrations of PCE. Cells stained with Hoechst 33342 (10 µM) revealed marked chromatin condensation and apoptotic body formation, and were then examined through fluorescence microscopy (Figure 4). We confirmed that PCE induced apoptosis in HT-29 cells. In conclusion, PCE is highly cytotoxic against human cancer cells and induces cell death through apoptosis. Therefore, our results suggest that PCE may have potential for development as a novel cancer chemotherapeutic agent originating from a natural product.

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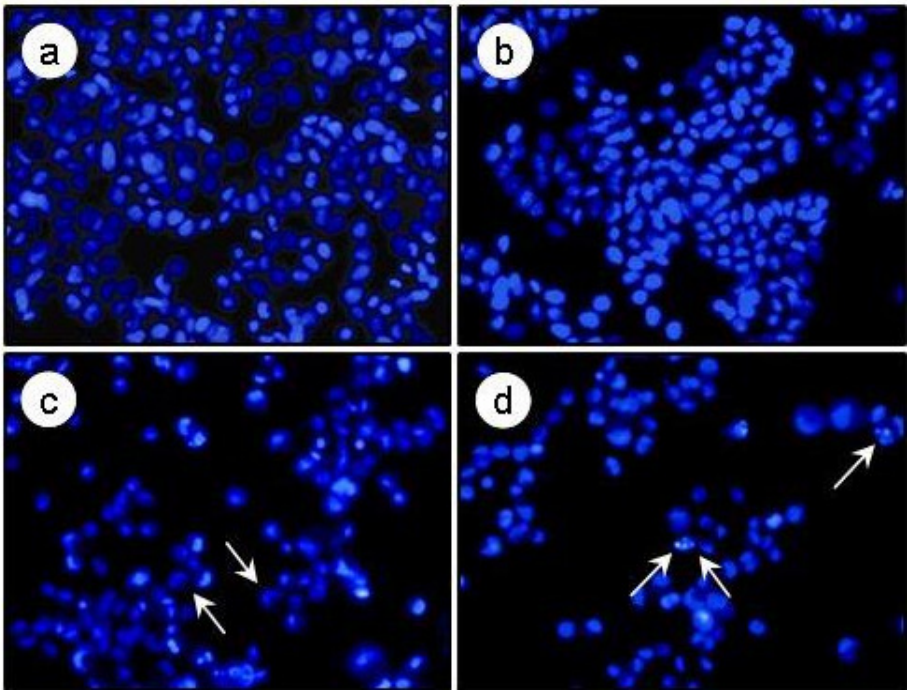

Figure 4. PCE induced apoptosis in HT-29 cells. The cells were treated with various concentrations of PCE for 24 h. Fixed cells were stained with Hoechst 33342 (10 µM) and examined by fluorescence microcopy (a; control, b; 100 µg/ml, c; 250 µg/ml, and d; 500 µg/ml). The arrows indicate apoptotic cells. ***significant vs. control untreated cells ($p<0.001$). Source: Jo et al. (2008).

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