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Juniperus chinensis extract induces apoptosis via reaction oxygen species (ROS) generation in human pancreatic cancer cell lines

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Abstract Pancreatic cancer is among the most difficult-to-treat tumors. More than half of patients with this cancer have very few symptoms at the early stages, allowing the development of distant metastases and resistance to cancer treatment. In this study, we found that *Juniperus chinensis* extract (JCX) decreased the cell viability and migration activity of PANC-1 and SNU-213 pancreatic cancer cells in a dose-dependent manner. JCX increased caspase-3 activation and generation of reactive oxygen species (ROS). N-acetylcysteine treatment blocked JCX-induced ROS generation and the negative effects on pancreatic cancer cell viability. In addition, JCX down-regulated the levels of phospho-focal adhesion kinase (p-FAK) and phospho-extracellular signal-regulated kinase (p-ERK). Together, these results indicate that JCX induces apoptosis in human pancreatic cancer cell lines through ROS production, downregulating FAK/ERK signaling and activating caspase-3. We propose that JCX-derived compounds represent candidates for the development of alternative medicines for the treatment of pancreatic cancer.

Keywords Apoptosis · Cell viability · *Juniperus chinensis* · Migration · Pancreatic cancer · Rreactive oxygen species

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Introduction

Pancreatic cancer is one of the most dangerous cancers: the 5-year survival rate is less than about 8%, very low relative to other cancers. It has few early symptoms, and more than 50% of patients have developed distant metastases at the time of diagnosis [1]. Invasive pancreatic cancers are resistant to common cancer therapies such as chemotherapy and radiation therapy [2]. Accordingly, new strategies for the treatment of pancreatic cancer are in high demand.

Genus Juniperus is widely distributed in East Asian countries such as Korea, China, Japan, and Mongolia. Juniperus extracts have diverse physiological components, including flavones, lignans, and terpene [3,4]. These physiological components have anti-microbial, anti-bacterial, anti-insect, anti-viral, and anti-tumor activities [5-8]. For example, the leaf extract of *Juniperus chinensis* has a cytotoxic effect on two tumor cell lines, HeLa and HGC-27 [9].

Apoptosis differs from other types of cell death in that it progresses without inflammation. The process is under strict control and is strongly associated with biological processes such as aging, embryogenesis, and diseases. Reactive oxygen species (ROS) are metabolic byproducts that act on cell signaling and homeostasis, but a mismatch between the production of ROS and biological ROS removal results in oxidative stress. Excessively high levels of ROS cause apoptosis or necrosis [10,11]. ROS and oxidative stresses are involved in the regulation of signal transduction pathways associated with tumorigenesis. Some therapeutic strategies increase the level of intracellular ROS to induce damage to tumor cells and ultimately cause their death [12].

Here, we report that *Juniperus chinensis* extract (JCX) inhibits cell viability and migration in human pancreatic cancer cells. JCX increases intracellular ROS levels and regulates the FAK/ERK pathway and apoptosis-related proteins, suggesting that it could be used to develop substances for the treatment of pancreatic cancer.

Material and Methods

Sample preparation and antibodies

Crude extract of *Juniperus chinensis* leaves was obtained from Biodiversity Research Institute, dissolved in 70% ethanol, and passed through a 0.2 μm syringe filter. Antibodies against phospho-tyrosine and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phospho-FAK, FAK, phospho-ERK, ERK, caspase-3, cleaved caspase-3, and proliferating cell nuclear antigen (PCNA) were purchased from Cell Signaling Technology.

Cell culture

293T, PANC-1, and SNU-213 cells were obtained from the Korean Cell Line Bank (Seoul, Korea). 293T and PANC-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. SNU-213 cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum, 25 mM HEPES, 100 U/mL penicillin, and 100 μg/mL streptomycin. All cells were cultured in a humidified incubator at 37 °C and 5% CO_2.

Cell viability assay

293T, PANC-1, and SNU-213 cells were seeded at a density of 1×10^4 cells/well in 24-well plates overnight and then treated with different concentrations (0, 2.5, 5, 7.5, 10, and 15 μg/mL) of JCX for 72 h. Pretreatment with N-acetylcysteine (NAC) was performed 1.5 h prior to treatment with JCX. Cell viability was assessed by WST assay (EZ-Cytox, DoGenBio, Seoul, Korea). Fifty microliters of WST solution was added to each well, and the samples were incubated for 30 min at 37 °C. Absorbance was measured on a microplate reader (ScanIt, BD Biosciences, San Jose, CA, USA) at 450 nm.

Cell viability = (OD (sample)) / (OD (average of control))

Scratch wound healing assay

PANC-1 and SNU-213 cells were incubated overnight by plating 5×10^5 cells/well in 6-well plates, and then the medium was replaced with 0.5% FBS medium (PANC-1) or 0.75% FBS medium (SNU-213 cells) for an additional 24 h incubation. Confluent monolayer cells were wounded manually with scratchers (SPL Life Science, Pochen, Korea), washed twice with PBS, and then incubated with fresh medium containing JCX (0, 10 μg/mL) for 24 h. At 0 and 24 h, the wounded area was photographed under a microscope and measured using ImageJ.

Wound healing rate = 1 – (remained area (24 h))/(wounded area (0 h))

Western blot assay

293T, PANC-1, and SNU-213 cells were treated with JCX (0, 10 μg/mL) for 48 h. Total proteins were extracted with M-PER lysis buffer (Thermo Science) containing protease inhibitors: 1× complete protease inhibitor cocktail (Roche), 2 mM sodium vanadate, 30 mM sodium pyrophosphate, and 100 mM sodium fluoride. After heating at 95 °C for 5 min, equal quantities of proteins were separated by 12% SDS-PAGE and transferred onto a nitrocellulose blotting membrane (GE Healthcare Life Science, Solingen, Germany). Membranes were blocked with 5% BSA in TBST and incubated with primary antibodies (1:1,000) overnight at 4 °C. After washing with TBST, membranes were incubated with the corresponding secondary antibodies (1:4,000) at room temperature for 3 h. Protein bands were detected using an ECL kit (Biosesang, Seoul, Korea) and recorded on X-ray film (AGFA).

Flow cytometry analysis

For analysis of apoptosis, 293T, PANC-1, and SNU-213 cells (1×10^5 cells/well) were seeded in 6-well plates overnight and treated with JCX (0 and 10 μg/mL) for 72 h. Cells were collected through trypsinization and washed with PBS. Next, the cells were incubated in the dark for 15 min with PI and Annexin V-FITC (FITC-Annexin V apoptosis detection kit, BD PharMingen, San Diego, CA, USA). Apoptotic cells were analyzed by flow cytometry (LSRFortessa, BD Biosciences). For measurement of intercellular ROS, PANC-1 and SNU-213 cells (1.5×10^5 cells/well) were plated in 12-well plates overnight, pretreated with NAC (0 and 10 mM) for 1.5 h, and then treated with JCX (0 and 10 μg/mL) for 6 h. Cells were harvested and stained with 10 μM H2DCF-DA (ROS detection reagent, Invitrogen, CA, Carlsbad, USA) in the dark for 15 min. Intracellular ROS measurement was conducted by flow cytometry.

Statistical analysis

Data were presented as averages ± standard deviation (SD). Differences among multiple groups were analyzed using Tukey's post hoc method. $p<0.05$ was regarded as statistically significant.

Results

Juniperus chinensis extract (JCX) decreases cell viability in human pancreatic cancer cell lines

We examined the effect of JCX on the viability of 293T, Panc-1, and SNU-213 pancreatic cancer cells using the WST assay (Fig. 1). All three cell lines were treated with various concentrations (0, 2.5, 5, 7.5, 10, and 15 μg/mL) of JCX for 72 h. JCX treatment decreased cell viability in a dose-dependent manner. JCX had a stronger effect in PANC-1 and SNU-213 human pancreatic cancer cells than in control 293T cells, which are derived from human embryonic kidney. Treatment with 10 μg/mL JCX decreased cell viability by 12 0±6.97, 44.0±5.30, and 33.65±7.06% in 293T, Panc-1, and SNU-213 cells, respectively.

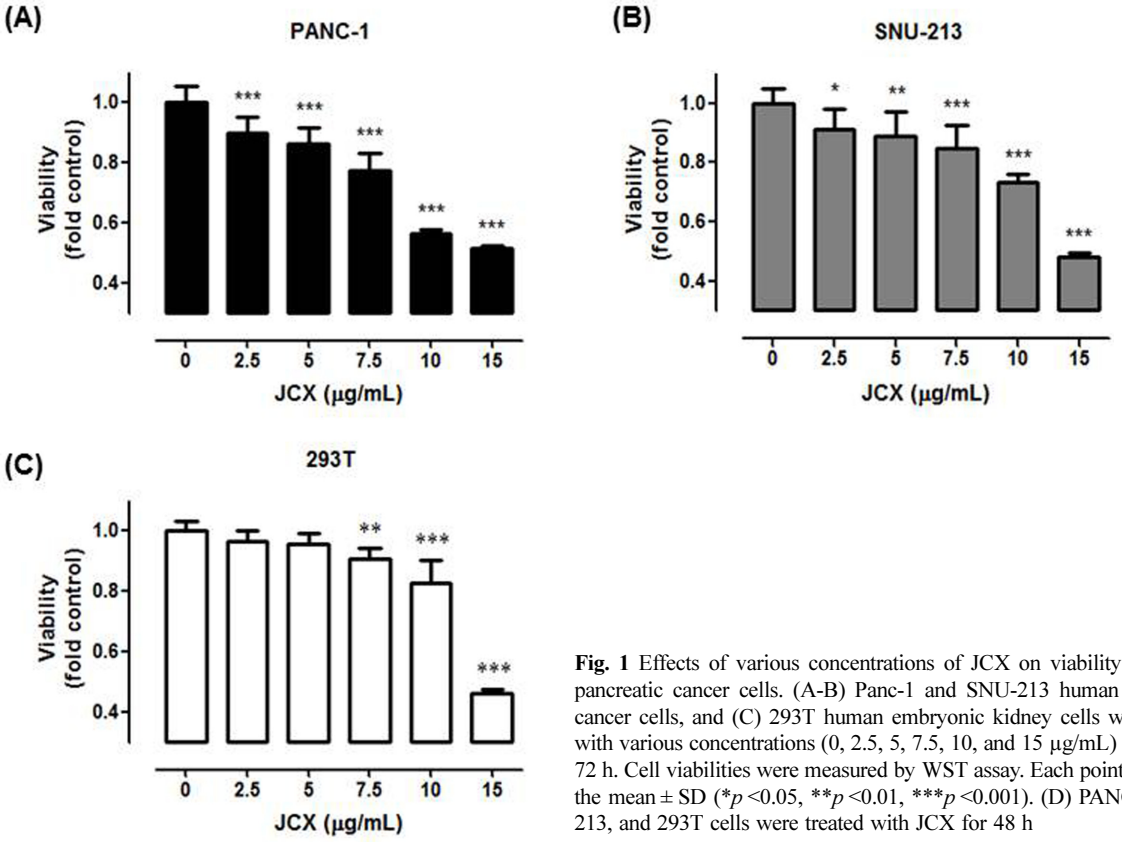

Fig. 1 Effects of various concentrations of JCX on viability of human pancreatic cancer cells. (A-B) Panc-1 and SNU-213 human pancreatic cancer cells, and (C) 293T human embryonic kidney cells were treated with various concentrations (0, 2.5, 5, 7.5, 10, and 15 μg/mL) of JCX for 72 h. Cell viabilities were measured by WST assay. Each point represents the mean ± SD (*p <0.05, **p <0.01, ***p <0.001). (D) PANC-1, SNU-213, and 293T cells were treated with JCX for 48 h

JCX inhibits cell migration in human pancreatic cancer cells lines

Next, we investigated the anti-migratory effect of JCX in Panc-1 and SNU-213 cells by using a scratch wound healing assay (Fig. 2). After scratching, cells were treated with 10 μg/mL JCX for 24 h, which inhibited the migration potential of Panc-1 and SNU-213 cells by 25.90±6.25 and 22.7±8.87%, respectively. There was no significant difference in viability of cells under this condition (Fig. 2C). To identify the migration signaling pathways inhibited by JCX treatment, we examined the phosphorylation levels of FAK and ERK in cells. As shown in Fig. 3A, JCX treatment decreased the levels of p-FAK and p-ERK in PANC-1 and SNU-213 cells, but it did not change the overall expression of ERK and FAK.

JCX induces ROS generation in PANC-1 and SNU-213 cells

To characterize the effects of JCX on cell viability, we examined the levels of caspase-3, cleaved caspase-3, and PCNA in Panc-1 and SNU-213 cells. As shown in Fig. 3B, treatment of JCX increased the level of cleaved caspase-3 and decreased the level of PCNA in both cell types. By contrast, in 293T cells, JCX treatment did not trigger activation of caspase-3 nor decrease expression of PCNA. To determine whether JCX-induced apoptosis and loss of viability are coupled to oxidative stress levels, we performed H2DCF-DA staining after co-treatment with NAC and JCX for 6 h. As shown in Fig. 4A, treatment with JCX increased intracellular ROS levels in PANC-1 (17.1%) and SNU-213 cells (14.2%), but not in 293T cells. NAC treatment lowered the ROS levels of cells. Pretreatment with NAC inhibited the JCX-mediated loss of viability in a dose-dependent manner (Fig. 4B).

Discussion

Cancer cells acquire properties distinct from those of normal cells, making them resistant to apoptosis, which is an important consideration for anticancer therapy. Cancer cells avoid apoptosis by inducing anti-apoptotic mechanisms and/or by downregulating the pro-apoptotic program [13]. To date, however, the induction of apoptosis by JCX in pancreatic cancer cell lines has not been investigated.

In this study, we found that JCX decreased viability in a dose-dependent manner in human pancreatic cancer cells. Treatment with 10 μg/mL JCX slightly reduced cell viability by approcimately 12% in control 293T cells, but significantly reduced cell viability by approximately 44 and 34% in Panc-1 and SNU-213 cells. The up-regulation of cleaved (active) caspase-3 and the down-regulation of PCNA demonstrated that JCX induced apoptosis. Caspase-3 plays a role in controlling the execution of apoptosis, and PCNA is essential for DNA replication. Focal adhesion kinase (FAK) is a non-receptor kinase localized to focal adhesions [14]. Extracellular

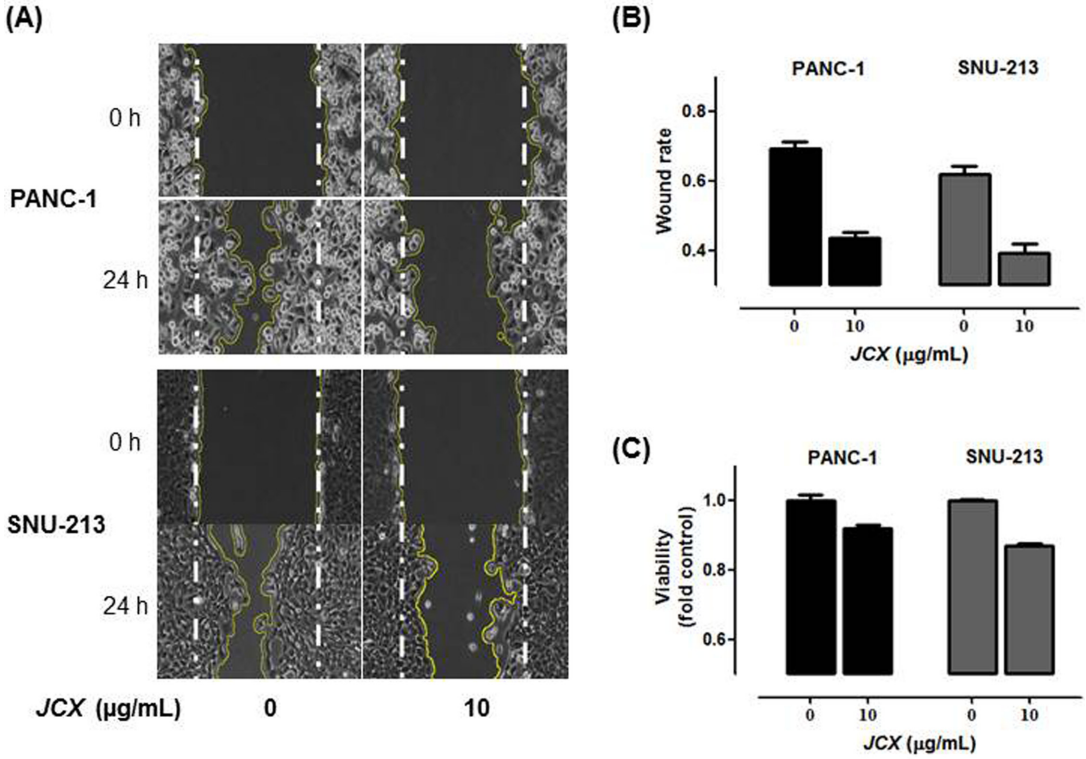

Fig. 2 Effects of various concentrations of JCX on migration of human pancreatic cancer cells. (A) Migration of Panc-1 and SNU-213 cells was monitored by wound healing assay. (B) Wound area was calculated from a representative of at least three independent experiments. (C) Cell viabilities were measured by WST assay

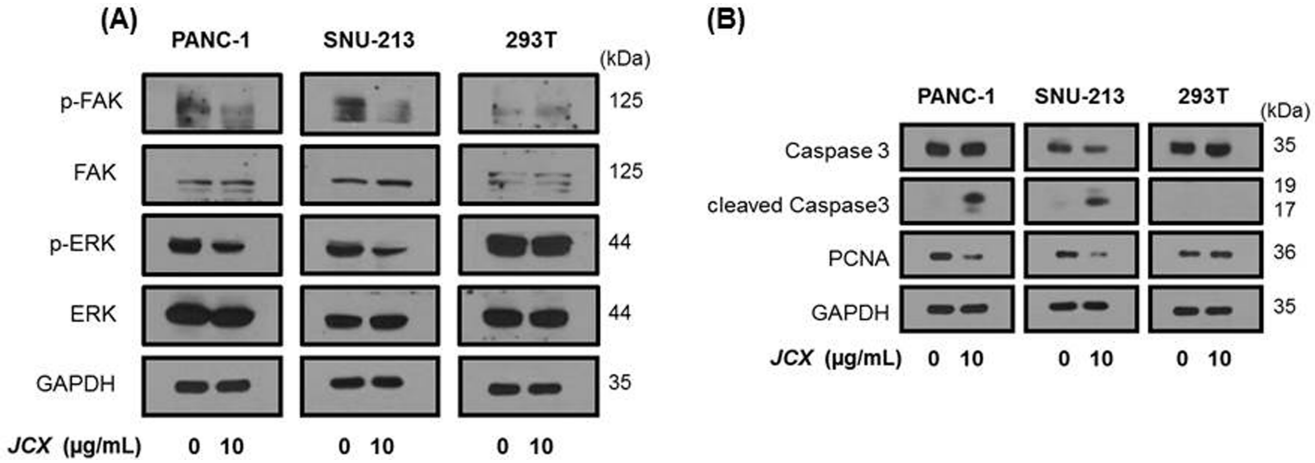

Fig. 3 Intracellular signaling by JCX in human pancreatic cancer cells. (A) Panc-1, SNU-213, and 293T cells were treated with JCX for 48 h, and the lysates were subjected to western blot analysis using antibodies for p-FAK, FAK, p-ERK, ERK, and GAPDH. (B) Expression of caspase-3, cleaved caspase-3, PCNA, and GAPDH

signal-regulated protein kinases 1/2 (ERK1/2) are members of the superfamily of mitogen-activated protein kinases. The regulation of FAK/EKR modulates cell proliferation, migration, adhesion, apoptosis, and differentiation [15-17].

The anticancer effect of JCX was revealed in a previous study, which showed that it inhibits TPA-induced phospholipid formation in HeLa cells [9]. Widdrol from JCX induces apoptosis by promoting proteolytic cleavage of PARP and activation of AMP-activated protein kinase (AMPK) in HT29 colon adenocarcinoma cells [18,19]. Overexpression of FAK blocks caspase-3-mediated apoptosis, whereas inhibition of FAK induces AMPK activation [20,21]. We found that JCX inhibits cell viability and migration

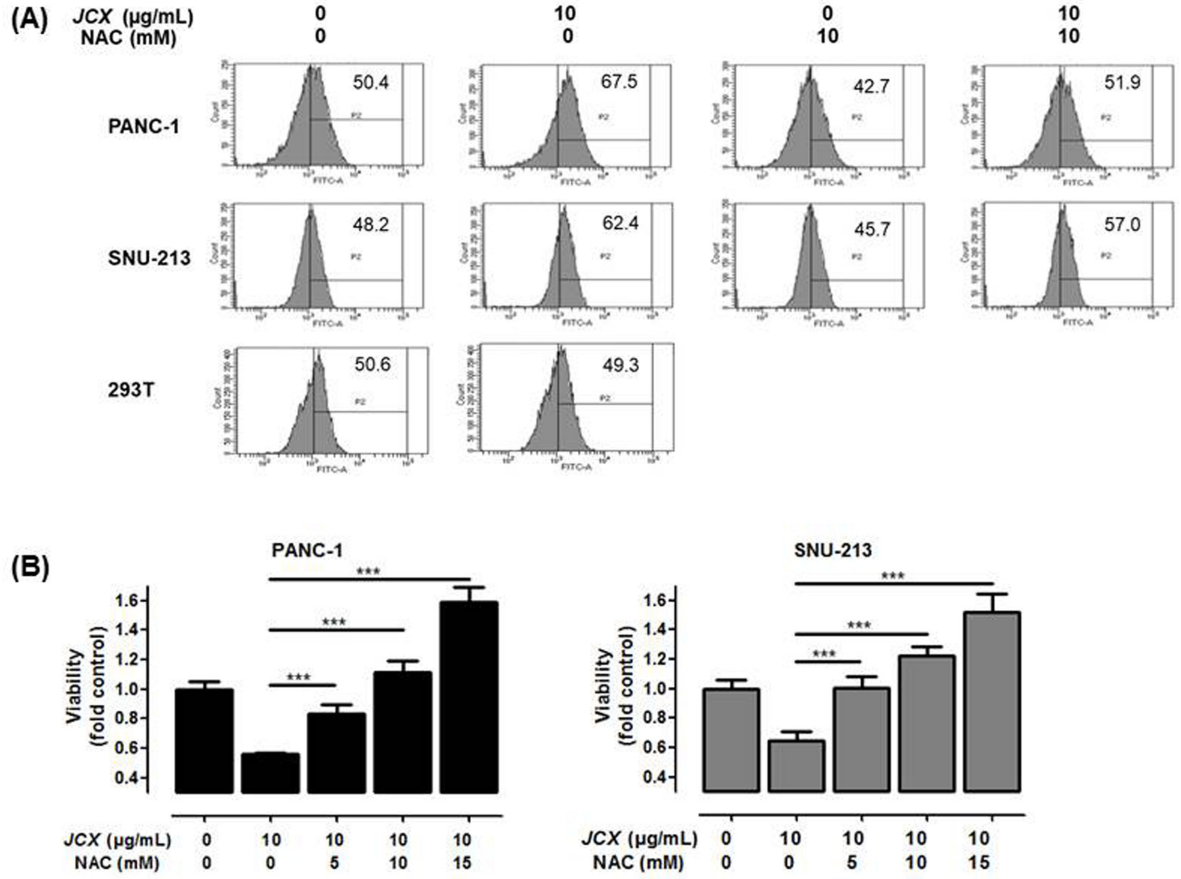

Fig. 4 Generation of reactive oxygen species (ROS) in human pancreatic cancer cells treated with JCX. (A) Flow cytometry assay of ROS generation under various concentrations of JCX. (B) NAC offsets the effect of JCX on viability. Cells were pretreated with NAC or vehicle prior to treatment with JCX. PANC-1 and SNU-213 cells were incubated with JCX for 72 h. Cell viabilities were measured by WST assay. Each point represents the mean ± SD. *p <0.05, **p <0.01, ***p <0.001

via downregulating p-FAK in pancreatic cancer cells. Our results with previous research suggest that the point of action of JCX (including widdrol) lies upstream of AMPK, or alternatively that other functional substances are present in JCX. Through GC/MS analysis, we found that 2-methoxy-4-vinylphenol is contained in JCX (data not shown). 2-Methoxy-4-vinylphenol is known to inhibit pancreatic cancer metastasis [25].

ROS is a mediator in the regulation of cell signaling. However, excessive ROS levels cause extreme oxidative stress in pancreatic cancer cells, eventually leading to apoptosis [15]. Substances such as epigallocate-3-gallate from tea, capsaicin from pepper, and benzyl isothiocynanate from papaya seeds share a similar mechanism of inducing apoptosis by increasing intracellular ROS levels [22-24]. Our study also revealed that the JCX-mediated effects on viability and migration generation in human pancreatic cancer cells are associated with ROS, and that pretreatment with NAC, a ROS scavenger, offsets the inhibitory effect of JCX. These findings are expected not only to enable the development of alternative anticancer agents based on JCX, but also to accelerate the study of the functional substances contained in JCX.

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