A Thesis for the Degree of Master of Philosophy

Juniperus chinensis extract induces apoptosis via ROS generation in human pancreatic cancer cell lines

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August, 2018

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A thesis submitted in partial fulfillment of the requirement

For the degree of master of philosophy

June, 2018

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ABSTRACT

Pancreatic cancer is one of the dreadful tumors. Less symptoms or signs at early stage cause over a half of patients has distant metastases, which leads resistance to cancer therapies. In this study, we found *Juniperus chinensis* extract (*JCX*) inhibited the cell viability and migration activity of PANC-1 and SNU-213 in a dose-dependent manner. *JCX* increased capase-3 activation, and reactive oxygen species (ROS) generation. N-acetylcysteine (NAC) treatment blocked anti-viability activity of *JCX* and *JCX* -induced ROS generation. In addition, *JCX* downregulated the levels of p-FAK/FAK and p-ERK/ERK.

Altogether, these results indicated that *JCX* induced apoptosis in human pancreatic cancer cell lines via ROS generation and downregulation of FAK/ERK signaling and activation of caspase-3. We suggest the *JCX*-derived compounds could be an alternative medicine candidate for pancreatic cancer treatment.

1. Introduction

Pancreatic cancer is one of the most dreadful tumors. Its 5- year survival ratio is around 8%. [1] There are few symptoms or signs at early stage, which leads over 50 % of patients have distant metastases when their diagnosis [1]. Invasive pancreatic cancer has resistance against common cancer therapies as chemotherapy and radiation therapy [2]. It's necessary to improve the treatment strategies for pancreatic cancer.

Juniperus chinensis is native to East Asian countries such as Korea, China, Japan, and Mongolia. Extract of Juniperus genus exhibits various activities as antioxidants, antimicrobiotics, and antitumor agents. [3, 4, 5] The *Juniperus*–derived compounds have been reported inducing apoptosis in cancer cells, for example, nardosinen from *J. foetidissim*, deoxypodophyllotoxin from *J. communis*, and widdrol from *J. lucayana*. [6, 7, 8].

Apoptosis is a type of cell deaths. It takes place without inflammation, which is a distinct feature from other cell deaths. It occurs under control remarkably and is a important biological process such as ageing, embryogenesis, and diseases. Reactive oxygen species (ROS) is a byproduct of metabolisms, and acts in cell signaling and homeostasis. Oxidative stress arises between the production of ROS and the biological ROS removal system. Excessively high levels of ROS induce cell apoptosis or necrosis [9, 10]. ROS or oxidative stress involved in regulation of signal transductions such as FAK/ERK, which are related in tumorigenesis. Many therapeutic strategies are devised to elevate intracellular ROS levels with the goal to induce irreparable damages, subsequently resulting in tumor cell apoptosis [11] .

In this study, we report that *Juniperus chinensis* extract (*JCX*) inhibits cell-viability and -migration in human pancreatic cancer cells. *JCX* increased intracellular ROS levels, subsequently regulates FAK/ERK pathway and the apoptosis-relating proteins, revealing the

possibility of *JCX* as a therapeutic medicine in human pancreatic cancer.

2. Materials and Methods

2.1. Sample preparation and antibodies

The dried extract of *J. chinensis* leaf was obtained from Jeju Biodiversity Research Institute (JBRI), dissolved in 70% ethanol, and filtered with a 0.2 μm syringe filter. Antibodies against p-tyrosine, and GAPDH were purchased from Santa Cruz Bio Technology. Antibodies against p-FAK, FAK, p-ERK, ERK, caspase-3, cleaved caspase-3, and proliferating cell nuclear antigen (PCNA) were purchased from Cell Signaling Technology.

2.2. Cell culture

PANC-1, SNU-213, and 293T cells were obtained from Korean Cell Line Bank (Seoul, Korea). 293T and PANC-1 cells were maintained in Dulbbeco'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 ℃, and 5% CO_2.

2.3. Cell viability assay

PANC-1, SNU-213, and 293T 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, 15 μg/mL) of *JCX* for 72 h. Pretreatments of N-acetylcysteine (NAC) was implemented 1.5 h prior to treatments of *JCX* when performed. Cell viability was assessed by WST assay (EZ-cytox, DoGenbio). Each well was added WST solution 50μL, and incubated for 30 min at 37 ℃. The absorbance was measured by a microplate reader (ScanIt, BD) at 450 nm.

$$\text{Cell viability} = \frac{\text{OD (sample)}}{\text{OD (average of control)}}$$

2.4. Scratch wound healing assay

PANC-1 and SNU-213 cells were plated at a 5×10^5 cells/well in 6-well plates overnight, and medium were changed into 0.5% FBS medium (PANC-1) or 0.75% FBS medium (SNU-213) for 24 h. Confluent monolayer cells were manually wounded by scratchers (SPLScar^{TM}, SPL), washed with PBS, and incubated with fresh medium containing *JCX* (0, 10 μg/mL) for 24 h. At 0 and 24 h of incubation, the wounded area were photographed with a microscopy, and measured by imageJ.

$$\text{Wound healing rate} = 1 - \frac{\text{remained area (24 h)}}{\text{wounded area (0 h)}}$$

2.5. Western blot analysis

PANC-1, SNU-213, and 293T cells, were treated with *JCX* (0, 10 μg/mL) for 48 h. The total proteins were extracted with M-PER lysis buffer (Thermo science) containing protease inhibitors such as 1X complete protease inhibitor cocktail (Roche), 2 mM sodium vanadate, 30 mM sodium pyrophosphate, and 100 mM sodium fluoride . After heated at 95 ℃ for 5min, the same amounts of proteins were separated by 12% SDS-PAGE and transferred onto a nitrocellulose blotting membrane (GE healthcare life science). Membranes were blocked with 5% BSA in TBST, incubated with primary antibodies (1:1,000) overnight at 4 ℃. After washing with TBST, membranes were incubated with the corresponding secondary antibodies (1:4,000) room temperature for 3 h. The protein bands were detected by ECL kit (biosesang) on X-ray film (AGFA).

2.6. Flow cytometry analysis

For apoptosis analysis, PANC-1, SNU-213, and 293T cells (1×10^5 cells/well) were seeded in 6-well plates overnight, and treated *JCX* (0, 10 μg/mL) for 72 h. Cells were collected through tripsinization, and washed with PBS. Then, the cells were incubated with PI and Annexin V-FITC (FITC Annexin V apoptosis detection kit, BD pharmigen) in the dark for 15 min. The apoptotic cells were analyzed by flow cytometry (LSRFortessa, BD)

For intercellular ROS measurement, PANC-1 and SNU-213 cells (1.5×10^5 cells/well) were plated in 12-well plates overnight, pretreated NAC (0, 10 mM) prior 1.5 h to treat *JCX*, and treated *JCX* (0, 10 μg/mL) for 6 h. Cells were harvested, and stained with 10 μM H_2DCF-DA (ROS detection reagent, Invitrogen.) in the dark for 15 min. The intracellular ROS measurement was conducted by flow cytometry.

2.7. GC-MS MS analysis

The sample was eluted in methanol. The analysis was performed using a gas chromatograph (GC2010, Shimadzu Corporation). The GC was equipped with an AOC-20i auto-injector and a triple quadruple tandem mass spectrometer (TQ8040, Shimadzu Corporation). 1 μL volume of the sample was injected in splitless mode on Rtx-5MS (30 m × 0.25 mm, 0.25-μm film thickness) column (restek). The carrier gas and the collision gas were helium and argon, respectively. Oven temperature started from 80 ℃ (3-min hold time) to 310 ℃ (10-min hold time). The interface and ion source temperature were 280 °C and 250 °C, respectively. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. The data acquisition was started at 3.5 min.

2.8. Statistical analysis

Data were presented as average ± standard deviation (SD). The differences between multiple groups were analyzed using tukey's *post hoc* method. $P < 0.5$ was regarded statistically significant.

3. Results

3.1. *JCX* inhibits cell viability in human pancreatic cancer cell lines.

The picture of *J. chinensis* leaf is shown in Fig. 1. The WST assay was conducted to examine whether the *JCX* mediates the inhibition effects on the cell viability of PANC-1, SNU-213, and 293T. Cells were treated with different concentrations (0, 2.5, 5, 7.5, 10, 15 μg/mL) of *JCX* for 72 h. The results demonstrated the *JCX* inhibited the cell viability in a dose-dependent manner. (Fig. 2) We found *JCX* has higher inhibition in PANC-1 and SNU-213 human pancreatic cancer cells than 293T, derived from human embryonic kidney cells. Compared with the control, the cell viabilities were inhibited approximately 44% in PANC-1, 36% in SNU-213, and 13% in 293T at treatment of 10 μg/mL *JCX*.

Fig. 1 The picture of *Juniperus chinensis* leaf.

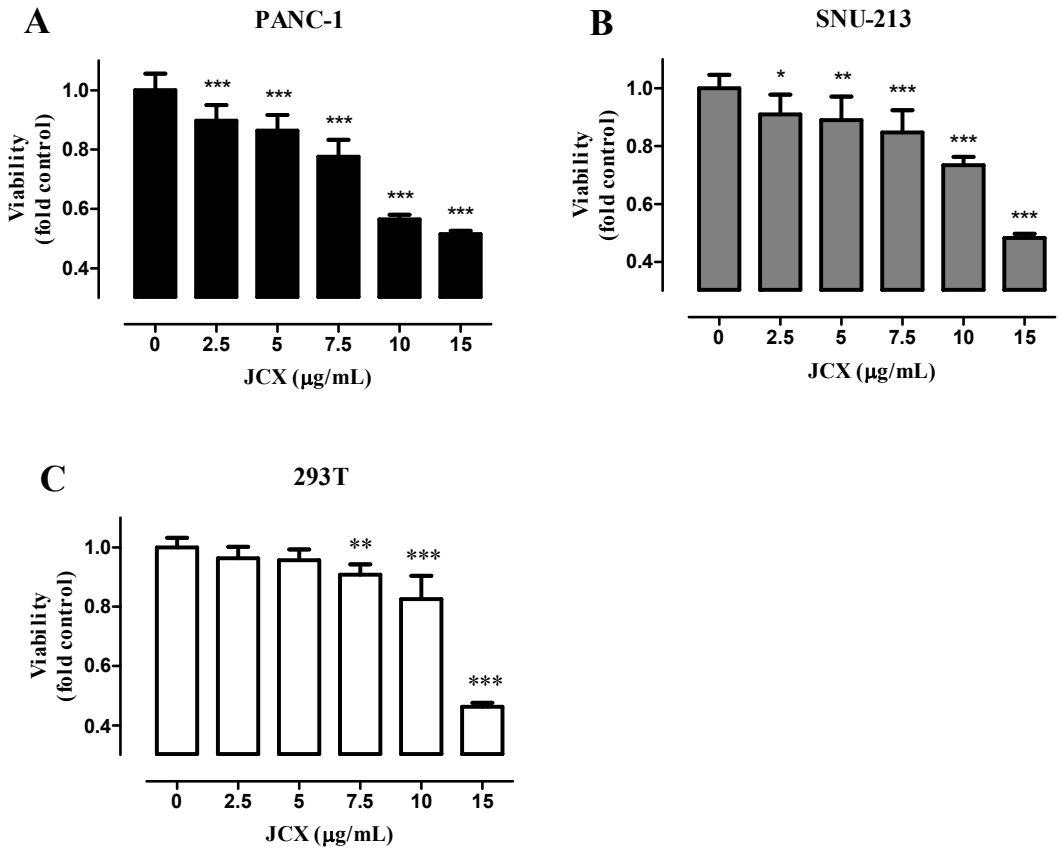

Fig. 2 *JCX* inhibited cell viability PANC-1 (A), SNU-213 (B), and 293T (C) cells were treated with various concentrations (0, 2.5, 5, 7.5, 10, 15 μg/mL) of *JCX* for 72 h. Cell viabilities were measured using WST assay. Each point is the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001

3.2. *JCX* inhibits cell migration in human pancreatic cancer cells lines.

The scratch wound healing assay was performed to assess the *JCX*-mediated inhibition on the cell migration of PANC-1, and SNU-213. Cells were treated with *JCX* (0,10 μg/mL) for 24 h, after scratchiness. The quantification was carried out by photoshoot and measuring the wounded area after 0 and 24 h treatment. As shown in Fig. 3, the wounded areas were reduced approximately 27% (PANC-1) and 23% (SNU-213), respectively.

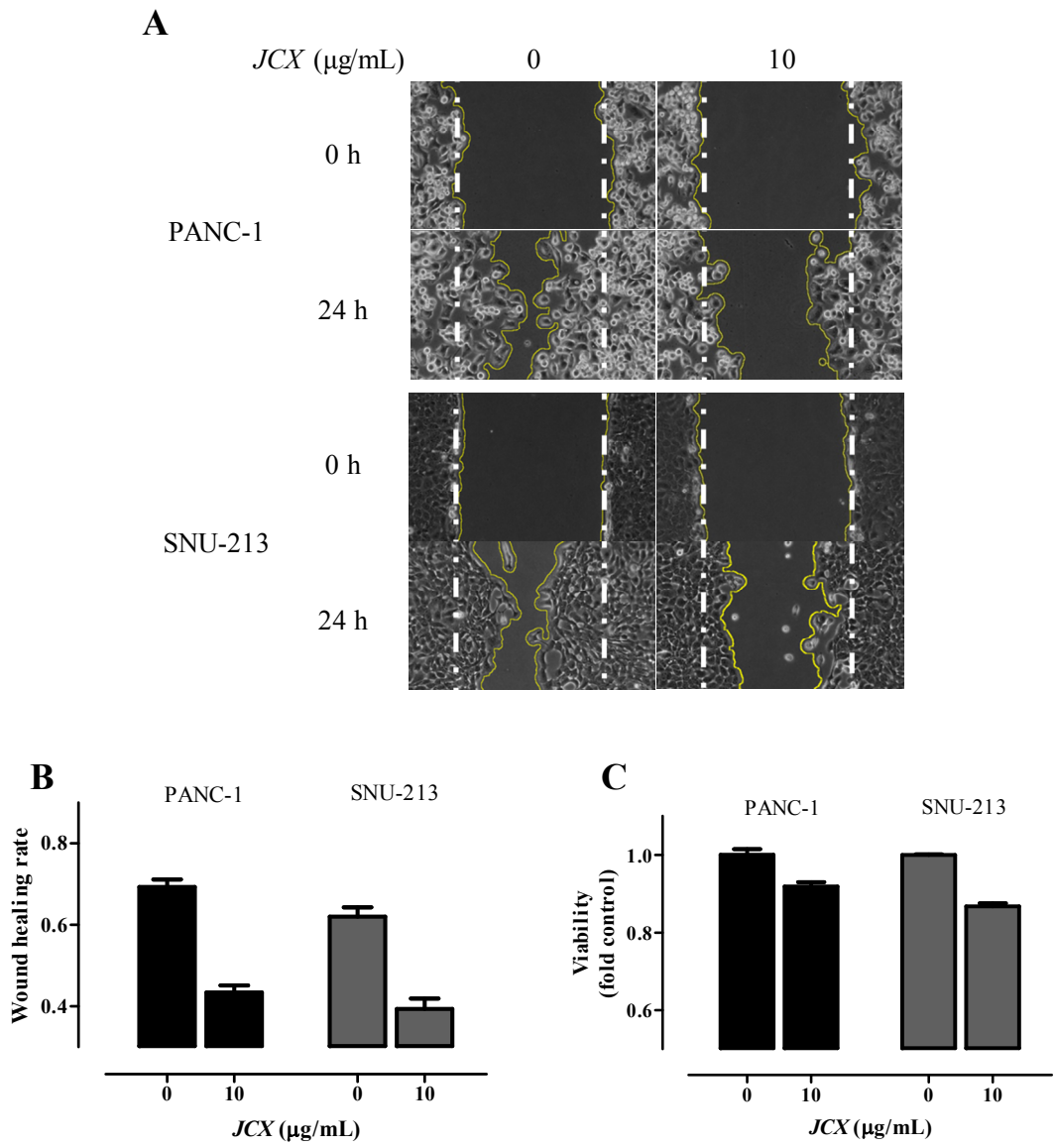

Fig. 3 *JCX* inhibited cell migration Pancreatic cancer cells were treated with *JCX* (0, 10 μg/mL) for 24 h. The wounded area pictures at 0, 24 h (A), the numeric results (B), and the viabilities of the PANC-1 and SNU-213 (C).

3.3. *JCX* downregulates the tyrosine-phosphorylated proteins in human pancreatic cancer cell lines.

Western blot analysis was performed to determine *JCX* mediated molecular mechanisms of *JCX* such *as* the phosphorylation of tyrosine, FAK and ERK in PANC-1, SNU-213, and 293T. Cells were treated with *JCX* (0, 10 μg/mL) for 48 h, and then lysates were prepared. As shown in Fig. 4, *JCX* treatment downregulated the levels of tyrosine-phosphorylated proteins, which are sized between approximately 25 and 135 kDa in PANC-1 and SNU-213, but not in 293T. Furthermore, the results indicated the treatment of *JCX* decreased the levels of p-FAK and p-EKR in PANC-1 and SNU-213, whereas the expression of ERK and FAK were not decreased. (Fig. 5)

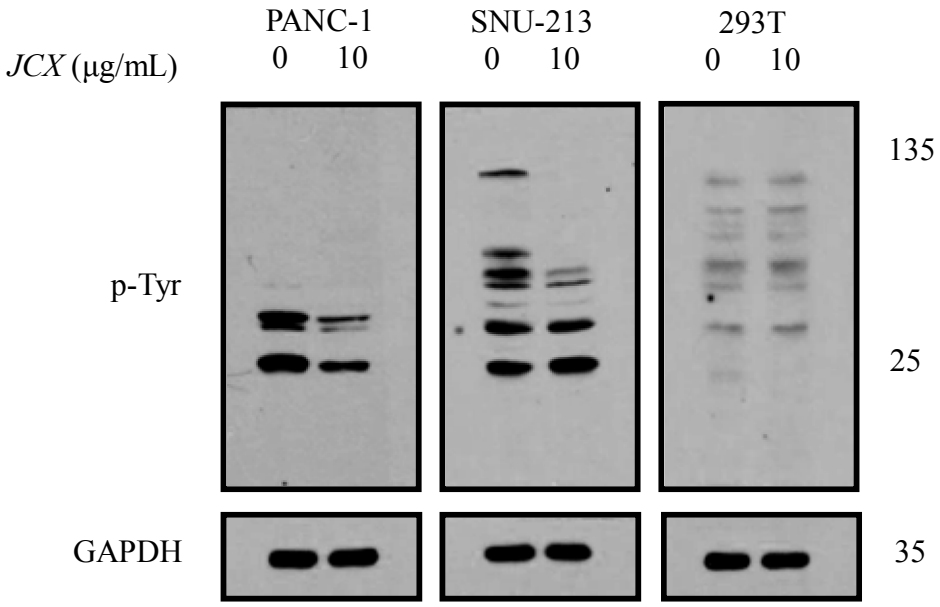

Fig. 4 *JCX* downregulated the tyrosine-phosphorylated proteins PANC-1, SNU-213, and 293T cells were treated with *JCX* for 48 h. The expression of tyrosine-phosphorylated proteins.

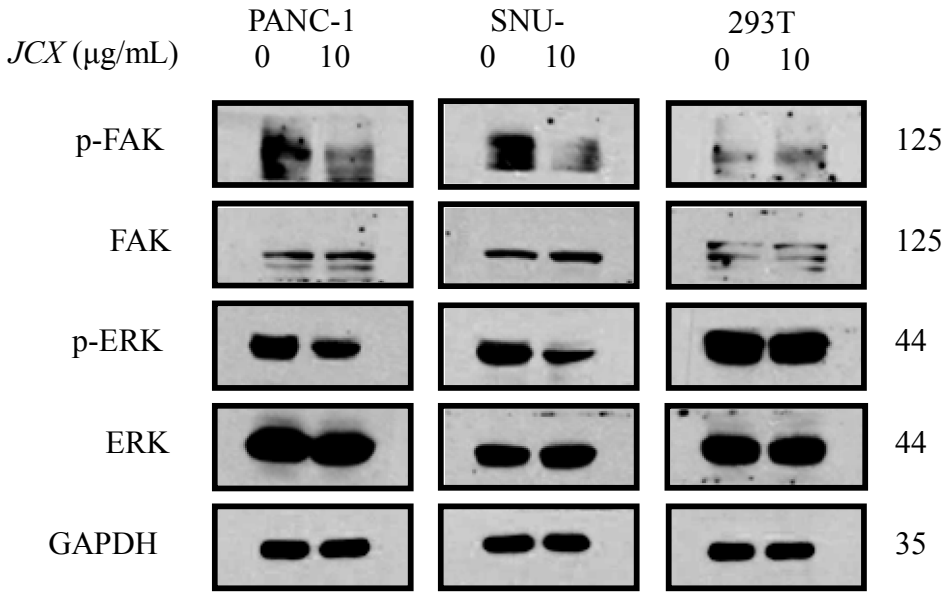

Fig. 5 *JCX* downregulated the p-FAK and p-ERK PANC-1, SNU-213, and 293T cells were treated with *JCX* for 48 h. The expression of p-FAK, FAK, p-ERK, ERK, and GAPDH.

3.4. *JCX* induces apoptosis through activation of caspase-3 in human pancreatic cancer cells lines.

To investigate whether *JCX* induces anti-viability due to apoptosis, PI and Annexin V-FITC staining was performed after treatment of *JCX* (0, 10 μg/mL) in PANC-1, SNU-213, and 293T for 72 h. Compared to untreated counterparts, the percentage of the apoptotic cells were increased 21.1% (PANC-1), 6.8% (SNU-213), and 0.6% (293T), respectively (Fig. 6).

To determine whether *J. chinesis* extract regulates the levels of caspase-3, cleaved caspase-3, and PCNA, which are related to apoptosis, in PANC-1, SNU-213, and 293T cells by Western blot analysis. As shown in Fig. 7, treatment of *JCX* increased the expression of cleaved csaspase-3, and decreased the expression of PCNA in PANC-1 and SNU-213. However, treatments of *JCX* in 293T seemed not causing the activation and expression of cleaved Caspase-3 and PCNA significantly as PANC-1 and SNU-213.

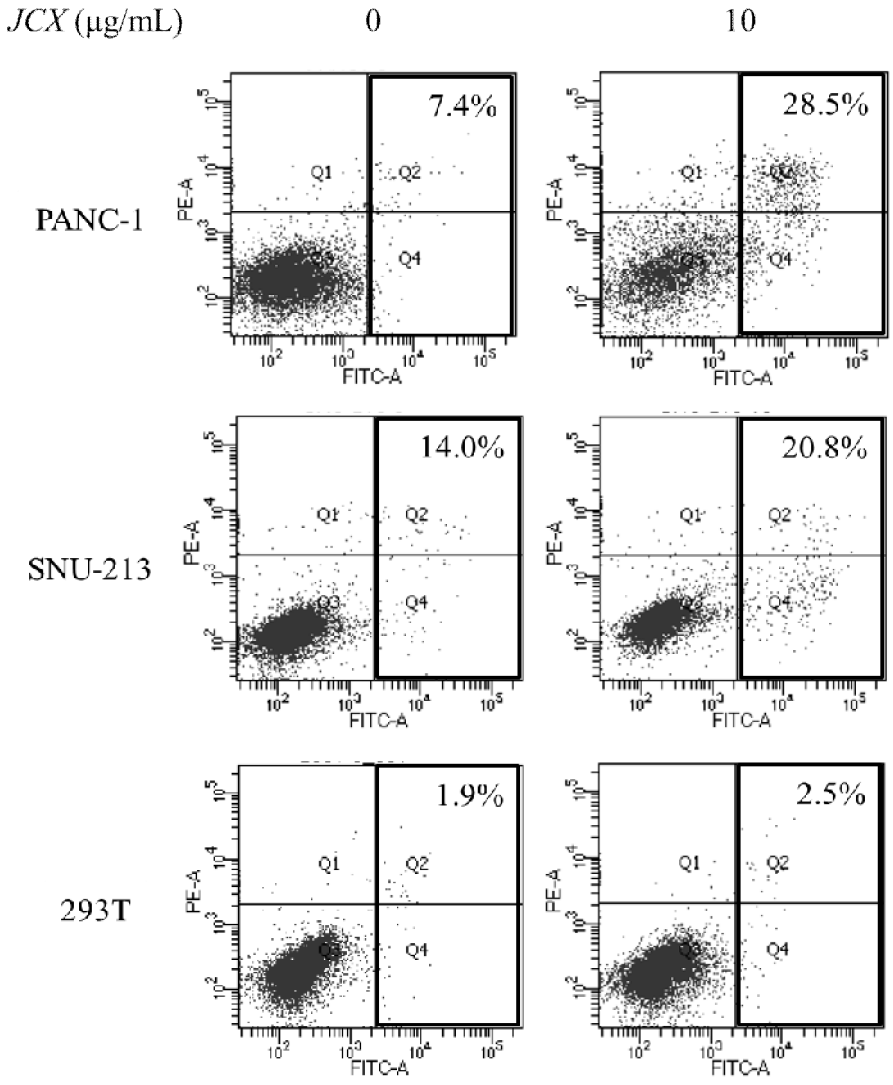

Fig. 6 *JCX* induced apoptosis PANC-1, SNU-213, and 293T cells were treated with *JCX* for 72 h. PI and Annexin V-FITC double staining, and then flow cytometry analysis

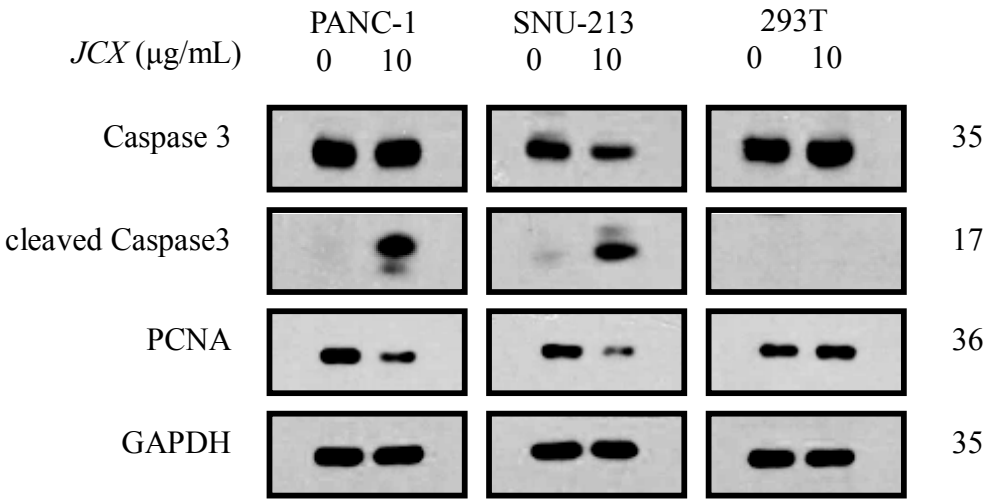

Fig. 7 *JCX* downregulated the apoptosis-related proteins PANC-1, SNU-213, and 293T cells were treated with *JCX* for 48 h. The expression of caspase3, cleaved caspase-3, PCNA, and GAPDH. DCF-DA staining, and then flow cytometry analysis

3.5. *JCX* induces ROS generation in PANC-1 and SNU-213

To determine whether *JCX* -induced anti-viability and apoptosis is coupled with oxidative stress level, H_2DCF-DA staining was performed after co-treatment of NAC and *JCX* in PANC-1 and SNU-213 for 6 h. As shown as Fig. 8, treatments of *JCX* increased intracellular ROS levels in PANC-1 (17.1%) and SNU-213 (14.2%), respectively, and 10 mM NAC pretreated cells showed less intracellular ROS levels.

To investigate whether *JCX*-induced anti-viability and apoptosis is related in ROS generation, the cells were co-treated with *JCX* and NAC. The pretreatment of NAC leaded to a inhibition of *JCX*-mediated anti-viability effect in a dose-dependent manner (Fig. 9). Compared to control, the viabilities of 10 mM NAC pretreatments and 10 μg/mL *JCX* treatment were 111 % (PANC-1), 122 % (SNU-213), and 107 % (293T), respectively. These results demonstrate that pretreatment of 10 mM NAC offset the anti-viability effect of *JCX* in PANC-1, SNU-213, and 293T.

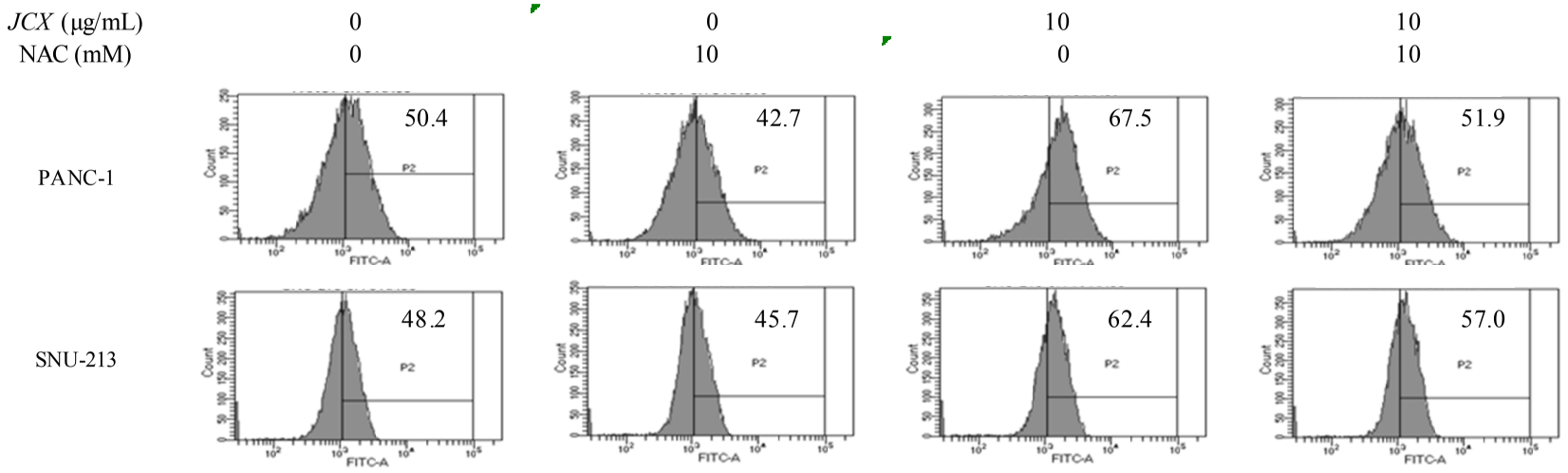

Fig. 8 *JCX* induced ROS generation Pretreatment of NAC or vehicle prior to treatment of *J. chinensis* extract. After 6 hour-treatment of *J. chinensis* extract. H_2DCF-DA staining, and then flow cytometry analysis.

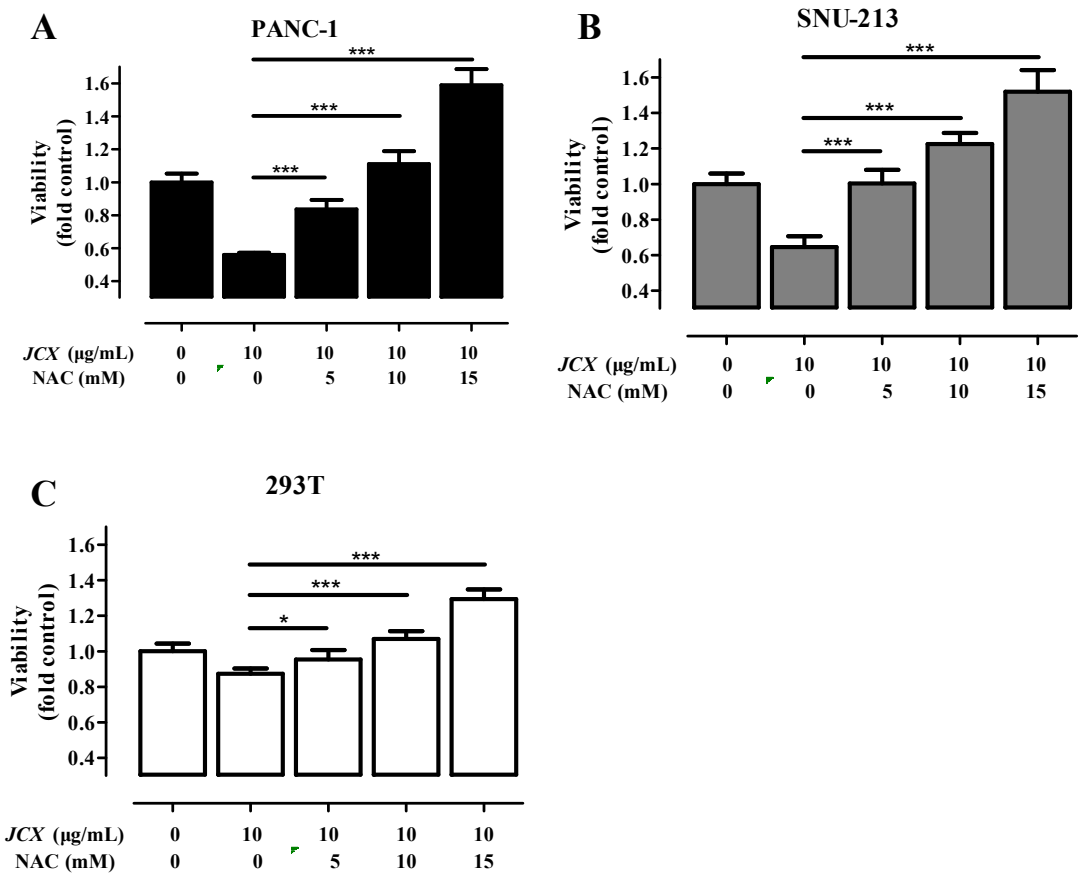

Fig. 9 NAC offset the anti-viability effect of *JCX* Pretreatment of NAC or vehicle prior to treatment of *JCX*. PANC-1, SNU-213, and 293T cells were incubated with *JCX* for 72 h. WST assay. Each point is the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001

3.6. GC-MS MS analysis

We performed GC-MS MS analysis to choose candidates among the compounds in *JCX*. The identified chemicals were shown in Fig. 10 and Table. 1.

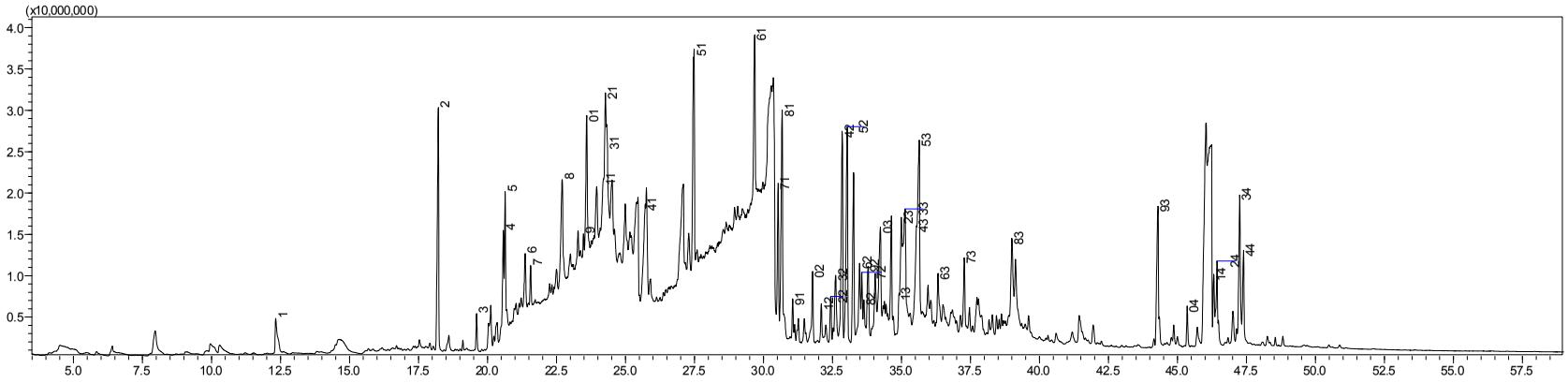

Fig. 10 Gas chromatography tandem mass spectrometer analysis The dried *J. chinensis* leaf extract was diluted in methanol. 1 μL of sample was injected. The carrier gas and the collision gas were helium and argon, respectively. Oven temperature started from 80 ℃ (3-min hold time) to 310 ℃ (10-min hold time).

Table. 1 Gas chromatography tandem mass spectrometer analysis

Peak No.	AREA %	NAME
1	1.36	2-Methoxy-4-vinylphenol
2	5.35	Elemol
3	0.53	2-Butanone, 4-(2,6,6-trimethyl-2-cyclohexen-1-ylidene)-
4	1.70	beta-Eudesmol
5	2.02	10-epi-.gamma.-eudesmol
6	0.64	AROMADENDRENEPOXIDE-(I)
7	0.63	.delta.-Cadinol
8	2.68	(-)-Caryophyllene oxide
9	0.28	Tetradecanoic acid, ethyl ester
10	3.01	Bicyclo[5.3.0]decane, 2-methylene-5-(1-methylvinyl)-8-methyl-
11	0.67	alpha-Eudesmol
12	2.65	B-Norcholestan-3-one, cyclic 1,2-ethanediyl acetal, (5.alpha.)-
13	1.80	Ethanol, 2-(9-octadecenyloxy)-, (Z)-
14	1.10	LONGIFOLENALDEHYDE
15	5.63	Hexadecanoic acid, ethyl ester
16	3.58	3,7,11,15-Tetramethyl-2-hexadecene
17	2.50	Ethyl linoleate
18	4.76	ETHYL LINOLEOLATE
19	0.53	Octadecanoic acid, ethyl ester
20	1.06	5-Hydroxymethyl-1,1,4a-trimethyl-6-methylenedecahydronaphthalen-2-ol
21	0.52	Cyclohexane, hexaethylidene-
22	0.82	1-Phenanthrenecarboxaldehyde, 1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-, [1S-(1.alpha.,4a.alpha.,10a.beta.
23	1.78	Kaur-16-en-19-ol
24	4.79	Totarol
25	5.26	Cycloisolongifolene, 9,10-dehydro-
26	1.40	Podocarpa-6,8,11,13-tetraen-12-ol, 13-isopropyl-, acetate
27	0.99	Ferruginol
28	0.58	(Albicanol) Decahydro-2-methylene-5,5,8a-trimethyl-1-naphthalenemethanol
29	1.78	Vitamin A alcohol
30	2.89	METHYL TRANS-COMMUNATE
31	0.62	Methane, phenyl(2,2,3-trimethylcyclopentylidene)-
32	2.83	1-Phenanthrenecarboxylic acid, 1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-, [1R-(1.alpha.,4a.beta.,10a.alpha
33	3.83	1-Phenanthrenecarboxylic acid, 1,2,3,4,4a,10a-hexahydro-1,4a-dimethyl-7-(1-methylethyl)-, methyl ester, [1R-(1.alpha.,4a.beta.,
34	2.12	Bicyclo[3.1.1]hept-2-ene, 2,2'-(1,2-ethanediyl)bis[6,6-dimethyl-
35	4.40	Methyl abietate

36	0.72	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
37	1.24	BORNYL ESTER OF 3-ISOPROPYLIDENE-CYCLOPENTANECARBOXYLIC ACID
38	2.25	9,12-Octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester
39	3.13	(-)HINOKININ
40	0.64	Sesamin
41	0.98	Bicyclo[2.2.1]hept-2-en-7-ol, 7-(4-methoxyphenyl)-, anti-
42	1.15	Racemic Savinin [2-[(3,4-Methylenedioxy)benzylidene]-3-[(3,4-methylenedioxy)benzyl]-.gamma.-butyrolactone isomer]
43	3.35	Stigmast-5-en-3-ol, (3.beta.)-
44	1.86	deoxypodorhizon

4. Discussion

Cancer cells are having acquired capabilities unlike normal cells. Resistance to apoptosis is an important index for cancer cells. Cancer cells are avoiding apoptosis with encouraging the anti-apoptotic mechanisms and/or downregulating the pro-apoptotic program. [12, 13] In this study, we found *JCX* induces anti-viability in a dose-dependent manner. The upregulation of cleaved (active) caspase-3 and downregulation of PCNA is proving *JCX*-mediated apoptosis in human pancreatic cancer cells. Caspase-3 plays a centrol role in the execution of apoptosis and PCNA is essential for DNA replication. Likewise, the *Juniperus*-derived compounds reported inducing caspase-dependent apoptosis in cancer cells, for examples, nardosinen from *J. foetidissim* in MCF-7 breast cancer cell, deoxypodophyllotoxin from *J. communis* in breast cancer cells, and widdrol from *J. lucayana* in HT-29 colon cancer cells. [6, 7, 8] In addition, the crude leaf extract from *Juniperus chinensis* was also suggested as a antitumor-promoting and antitumor candidate. [5]

Focal adhension kinase (FAK) is a non-receptor kinase localized to focal adhension. [14] Extracellular signal-regulated protein kinases 1/2 (ERK1/2) are members of the mitogen-activated protein kinase super family. The regulation of FAK/ERK modulates cell proliferation, migration, adhension, apoptosis, and differentication. [15, 16, 17] The increased FAK complex increases Extracellular signal Regulated Kinase 1/2 (ERK1/2) signal pathway, subsequently boosting the ability of cancer cell survival, and growth in a cell detached condition [18]. Overexpression of FAK has been shown to block the caspase-3-mediated apoptosis [19]. In this study, we found *JCX* inhibits cell viability and migration via downregulations of p-FAK and p-ERK in pancreatic cancer cells.

ROS is known as a mediator regulation of cell signaling. However, exorbitant ROS levels lead to extreme oxidative stress, and eventually apoptosis in pancreatic cancer cells. [20] For

example, for pancreatic cancer, to date only few treatment strategies have been discovered as effective for therapy. Like the combinations of gemcitabine with trichostatin A, epigallocate-3-gallatae (EGCG), capsaicin and benzyl isothiocyanate (BITC), these share the same mechanism to elevate intracellular ROS levels to trigger apoptosis [20, 21, 22, 23] 30,31,32,33]. In this study, *JCX* -mediated anti-viability and -migration is coupled with ROS generation. The pretreatment of NAC, a ROS scavenger, offset the inhibition effect of *JCX*.

We suggest *JCX* as an alternative anticancer medicine. *JCX* leads to a caspase-related apoptosis in human pancreatic cancer cells via ROS-mediated FAK/EKR signaling. Furthermore, to find the major compounds having anticancer activity in *JCX*, the next study is in progress.

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ACKNOWLEDGMENT

석사학위 과정을 뒤돌아보니 정말 많은 것을 경험하고 배운 시간이었습니다. 제가 석사연구생 생활을 하는 동안 많은 분들의 도움이 있었습니다. 이 짧은 지면으로나마 감사의 마음을 전합니다.

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다 적지는 못했지만 많은 분들의 도움으로 제가 석사 졸업을 할 수 있었습니다. 모두 건승하시고 행복하시길 바랍니다.