Antitumor activity of *Coptis chinensis* rhizome extract against PANC-1 cells

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Abstract: The rhizome of *Coptis chinensis* is known for its antibacterial, anti-inflammatory, and anticancer activities. This study aimed to investigate the anti-pancreatic cancer activity of *C. chinensis* rhizome and elucidate the molecular mechanism involved in such activity. The *C. chinensis* rhizome extract (CRE) significantly inhibited the cell viability of PANC-1 pancreatic cancer cells and arrested the cell cycle at the G0/G1 phase. Western-blot analysis revealed that CRE downregulated the protein level of Forkhead box M1 (FoxM1), an oncogenic transcription factor, and its downstream target proteins such as cyclin D1, c-Myc, and survivin. Berberine was identified as the main component in CRE, effectively reducing cell viability and FoxM1 expression. These findings indicate that CRE and berberine can exert anticancer activity by downregulating FoxM1 expression in pancreatic cancer cells and that berberine may partially contribute to the anti-pancreatic cancer properties of CRE. This study highlights that *C. chinensis* rhizome extract and its main ingredient, berberine, might have therapeutic potential against pancreatic cancer.

Keywords: anticancer, berberine, *Coptis chinensis*, FoxM1, pancreatic cancer

INTRODUCTION

Pancreatic cancer, the sixth leading cause of death among neoplastic diseases, has a poor prognosis [1]. It often remains asymptomatic in its early stages, leading to late diagnosis, and poor prognosis with a high lethality rate [2]. Pancreatic cancer treatment depends on surgical resection and chemotherapy for resectable tumors at an early stage of disease development. For most cases at a locally advanced stage, resection is difficult due to aggressive metastasis, and therefore, chemotherapy and radiotherapy are performed. However, chemotherapy and radiotherapy have limitations such as toxicity, drug resistance, and the potential for recurrence [3]. Therefore, effective, nontoxic, and preventive agents are urgently needed for pancreatic cancer.

Forkhead box M1 (FoxM1), a transcription factor in the Forkhead superfamily, can regulate cell differentiation, cell proliferation, and cell cycle progression [4]. Its dysregulation is highly associated with tumor initiation, promotion, and progression [4]. Elevated FoxM1 expression has been found in various cancers, including pancreatic cancer [5,6]. Extensive studies have revealed that aberrant FoxM1 expression is associated with poor prognosis and metastasis in pancreatic cancer [7,8]. Therefore, targeting FoxM1 expression could be a valuable strategy in developing therapeutics for treating pancreatic cancer.

The rhizome of *Coptis chinensis* (Ranunculaceae family) has been used as a traditional herbal medicine to treat diarrhea, vomiting, jaundice, diabetes, and eczema for centuries [9]. Recent studies have shown that *C. chinensis* has antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, and antitumor effects [10-15]. Reports support its anticancer potential against various types of malignancies. *C. chinensis* extract has been shown to inhibit cell proliferation and induce apoptosis in squamous carcinoma cells by modulating key signaling pathways such as cell cycle regulation, cell adhesion, and mitogen-activated protein kinase (MAPK) signaling [16]. Kim et al. [17] reported that *C. chinensis* extract effectively induced apoptosis in

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gefitinib-resistant lung cancer cells through downregulation of Mcl-1 and Bcl-2. Similarly, Coptidis Rhizoma extract has demonstrated antitumor activity in hepatocellular carcinoma Hep3B cells and xenograft models through the induction of reactive oxygen species (ROS)-mediated apoptosis and autophagy [18]. Among the bioactive constituents of *C. chinensis*, berberine, a major alkaloid, has been extensively studied for its anticancer properties [19,20]. Berberine has been reported to inhibit gastric cancer progression by regulating the JAK2/STAT3 pathway and downregulating interleukin-6 (IL-6) expression [21]. In pancreatic intraepithelial neoplasia, berberine suppresses tumor development by inhibiting glycolysis through the activation of the adenosine monophosphate-activated protein kinase (AMPK) pathway [22]. Another component of *C. chinensis*, coptisine, was reported to suppress the growth and metastasis of pancreatic cancer PANC-1 cells by inducing G1 phase arrest and inhibiting ERK signaling [23]. These findings suggest that *C. chinensis* and its derivatives may serve as potential therapeutic agents for various cancer types. The effect of *C. chinensis* or berberine on dysregulated FoxM1 expression in pancreatic cancer has not yet been reported. Thus, this study aimed to investigate the impact of *C. chinensis* rhizome extract and its major component, berberine, on PANC-1 pancreatic cancer cells, focusing on the regulation of FoxM1 expression to uncover the molecular mechanism.

MATERIALS AND METHODS

Ethics statement

This study was conducted entirely *in vitro* using a cell line. No humans or animals were involved.

Preparation of *Coptis chinensis* rhizome extract

Dried rhizomes of *Coptis chinensis* Franch were obtained from an herbal medicine store (Seoul, Korea) and sourced from China. The *C. chinensis* rhizomes (103.3 g) were soaked with 1 L of methanol at 40°C for 1 h in an ultrasonic bath. This process was repeated three times. *C. chinensis* rhizome extract (CRE, 17.01 g) was then obtained by removing the solvent with a vacuum rotary evaporator. The extract was stored at 4°C until use. For cell treatment, it was prepared with dimethyl sulfoxide (DMSO). The concentrations of CRE for cell treatment were determined according to a prior report [24].

Cell culture

The PANC-1 human pancreatic cancer cell line was purchased from ATCC (Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), streptomycin (0.1 mg/mL), and penicillin (100 units/mL). Cells were maintained at 37°C in a 5% CO_2 incubator.

Cell viability assay

Cells were seeded into a 96-well plate at a density of 3×10^3 cells per well and incubated overnight. The cells were then treated with 5, 10, 25, 50, and 100 μg/mL of CRE or berberine (Chemfaces, Hubei, China) for 24 h, 48 h, or 72 h. After 5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well, the cells were incubated for 4 h. Formazan crystals produced by living cells were solubilized with DMSO. The absorbance of formazan dye solution per well was measured at 540 nm using a microplate reader.

Cell cycle analysis

Cells were plated onto 60-mm cell culture dishes at a density of 4×10^5 cells per dish and treated with 2 mM thymidine for 24 h to synchronize the cell cycle. Cells were then treated with CRE at different concentrations (25, 50, and 100 μg/mL). After 24 h, the cells were suspended in 70% cold-ethanol and fixed overnight at 4°C. The following day, the ethanol was removed, and RNase A solution was added to the fixed cells. After incubation for 15 min in a 37°C incubator, the cells were exposed to propidium iodide (PI) for 20 min at 4°C. Finally, 10,000 cells were transferred to a BD Biosciences flow cytometer (San Jose, CA, USA). Cell cycle distribution was analyzed using BD CellQues™ Pro software.

Western blot analysis

CRE or berberine was used to treat cells for 20 h. Cells were then lysed with a radioimmunoprecipitation assay (RIPA) lysis buffer. Protein lysates (20 µg) were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred onto polyvinylidene fluoride membranes. After blocking with skimmed milk, membranes were incubated overnight with primary antibodies against cyclin D1, c-Myc, and survivin (Cell Signaling Technology, Inc., Danvers, MA, USA), and FoxM1 (Bethyl Laboratories, Inc., Montgomery, TX, USA) at 4°C. The next day, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody solution at room temperature for 2 h. These membranes were then exposed to an enhanced chemiluminescence reagent. Protein bands were captured using an imaging system (VersaDoc 3000, Bio-Rad, Hercules, CA, USA). Densitometric quantification of the protein bands was performed with ImageJ (NIH, Bethesda, MD, USA).

Reverse transcription polymerase chain reaction (RT-PCR)

After 8 h of treatment with CRE, TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA) was added to the cells for total RNA extraction according to the manufacturer's instructions. Extracted RNAs were reverse transcribed using a complementary DNA (cDNA) synthesis kit (Cosmogenetech, Seoul, Korea). Prepared cDNAs were amplified using a PCR thermal cycler (Applied Biosystems, Foster City, CA, USA). β-Actin was used as an internal control to normalize gene expression levels. Amplified DNAs were detected by separating them on 2% agarose gels and staining them with ethidium bromide. Primers used for PCR were as follows: FoxM1 (forward) ATGGCAAATTTTTCGCTCC, (reverse) ATGTCACCAGAAATTCCCAGTT; β-Actin (forward) AAGGGACTTCCTGTAACAACG, (reverse) AGGATGC AGAAGGAGATCACT

High-performance liquid chromatography (HPLC) analysis

Stock solutions for berberine and CRE were prepared with methanol (HPLC grade) and passed through a 0.22-µm syringe filter before analysis. HPLC analysis was performed with an Agilent HPLC 1260 system (Agilent, Santa Clara, CA, USA). An ACE Excel 5 SuperC18 column (250 × 4.6 mm i.d., Avantor Inc., Radnor, PA, USA) was used. The injection volume was 10 µL. The mobile phase included water with 0.1% trifluoroacetic acid (A) and acetonitrile (B). The column was eluted with a gradient solvent system: 0-30 min, 25%-40% B; 30-35 min, 40%-90% B. The flow rate of the mobile phase was 1.0 mL/min and the detection wavelength was set at 350 nm.

Statistical analysis

All experiments were performed in triplicate. Data are presented as the mean±standard deviation. Statistical difference between groups was determined by a two-tailed Student's t-test. Statistical significance was considered when the probability (P) value was less than 0.05.

RESULTS

Cytotoxicity of CRE against PANC-1 cells

It has been reported that CRE can suppress cell proliferation in various types of human cancer cells [25,26]. The current study examined whether CRE could affect pancreatic cancer cell growth. To assess the effect of CRE on cell growth, PANC-1 human pancreatic cancer cells were treated with increasing concentrations (5, 10, 25, 50, and 100 µg/mL) of CRE for 24 h, 48 h, or 72 h. Cell viability was measured using the MTT assay. The result revealed that CRE potently suppressed the viability of PANC-1 cells in a concentration-dependent manner at all durations of exposure. In addition, a significant interaction between time and concentration of CRE on cancer cell viability was found. There was an obvious decline in cell viability following treatment with CRE, suggesting a negative correlation between concentrations of CRE and PANC-1 cell viability. These findings indicate that CRE can inhibit pancreatic cancer cell growth (Fig. 1).

Cell cycle arrest after treatment with CRE

Cell cycle progression is essential for cell proliferation and its disruption can lead to cell growth inhibition

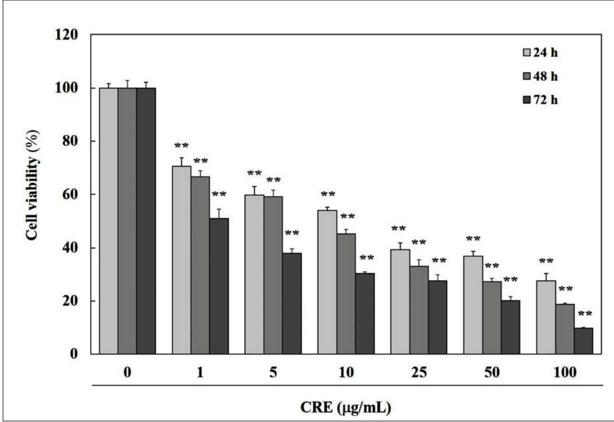

Fig. 1. Effects of CRE on cell viability in PANC-1 human pancreatic cancer cells. PANC-1 cells were treated with CRE at various concentrations for 24 h, 48 h, or 72 h. Cell viability was determined by the MTT assay. *P<0.05; **P<0.01 between CRE treatment and control.

[27]. Thus, the effect of CRE on cell-cycle progression was investigated using PANC-1 cells. Flow cytometry demonstrated that the cell population of the control group at the G0/G1 phase was 41.0%, increasing to 44.5%, 50.9%, and 59.3% after treatment with CRE at 25 μg/mL, 50 μg/mL, and 100 μg/mL, respectively. These observations suggest that CRE can arrest the cell cycle at the G0/G1 phase, thereby inhibiting PANC-1 cell proliferation (Fig. 2).

FoxM1 expression and its downstream target protein levels after treatment with CRE

FoxM1 has been verified as a key regulator in cell cycle progression [6], playing an important role in cell proliferation [6]. Overexpression of FoxM1 is associated with poor prognosis and high mortality in patients with pancreatic cancer [7,8]. Since CRE inhibited cell growth and caused cell cycle arrest in PANC-1 cells, the effect of CRE on FoxM1 expression was examined. Western blot analysis revealed that CRE reduced FoxM1 protein levels, whereas the control group had high levels of FoxM1 in PANC-1 cells. The RT-PCR results showed that CRE slightly suppressed the mRNA levels of FoxM1 compared to the control. The effects of CRE on the levels of FoxM1 downstream target proteins such as cyclin D1, c-Myc, and survivin were then evaluated. CRE concentration-dependently lowered the levels of cyclin D1, c-Myc, and survivin proteins involved in cell cycle progression. These findings suggest that CRE can downregulate FoxM1 expression in pancreatic cancer cells, subsequently leading to reduced protein levels of its downstream targets, including cyclin D1, c-Myc, and survivin (Fig. 3). These findings suggest that CRE can downregulate FoxM1 and its downstream effectors involved in cell cycle progression, leading to growth inhibition and cell cycle arrest in PANC-1 cells.

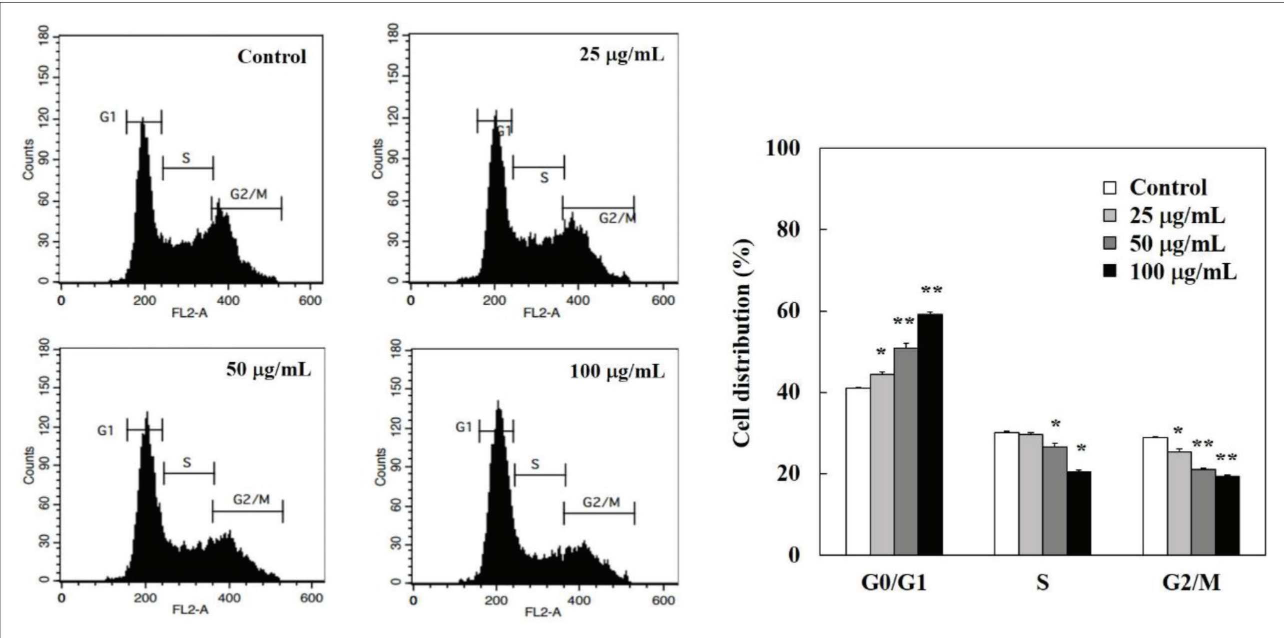

Fig. 2. Effects of CRE on cell cycle distribution in PANC-1 human pancreatic cancer cells. PANC-1 cells were treated with CRE for 24 h. Cell cycle progression was evaluated by flow cytometry. *P<0.05; **P<0.01 between CRE treatment and control at each stage.

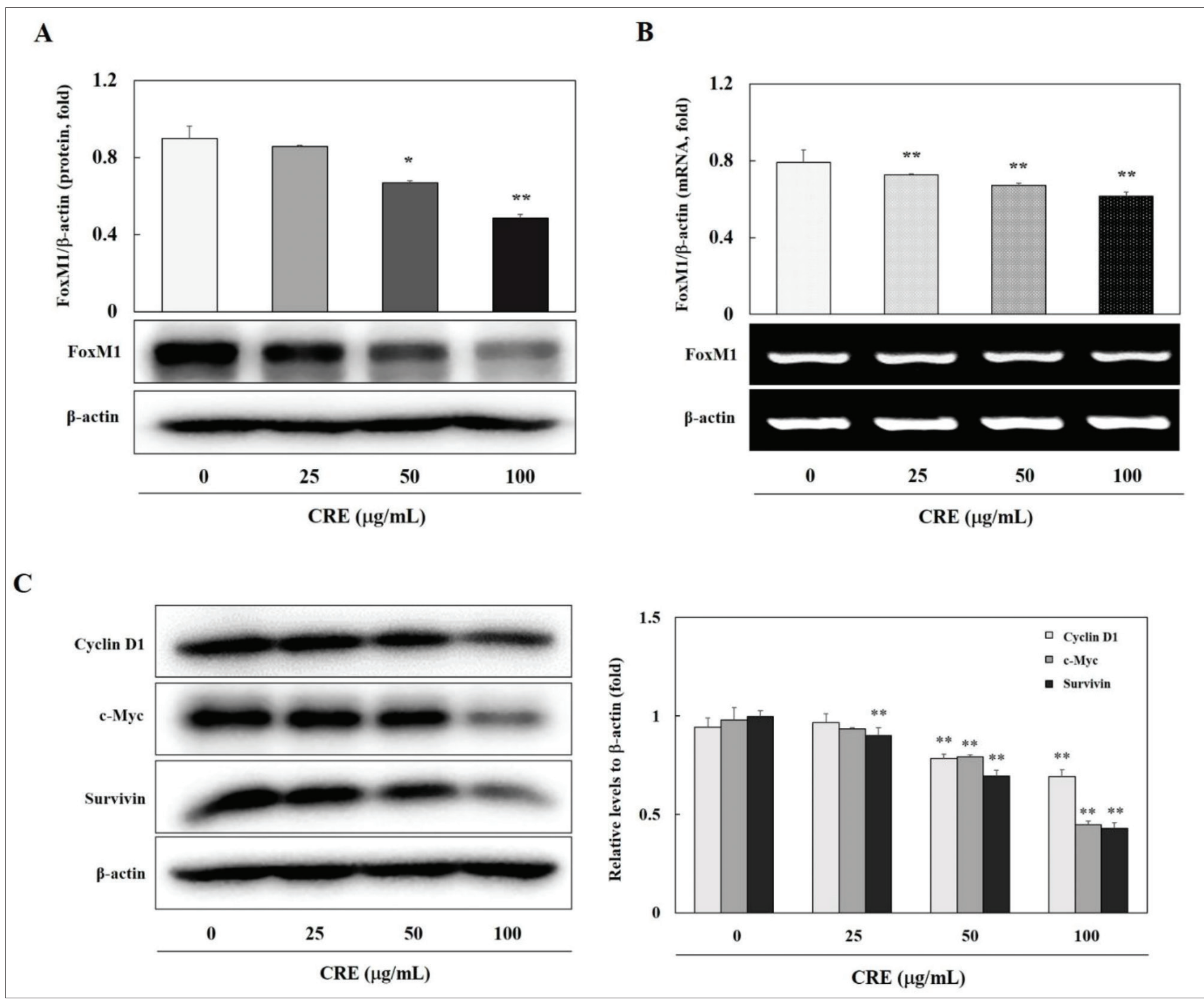

Fig. 3. Effects of CRE on FoxM1 expression and its target protein levels. **A** – PANC-1 cells were treated with CRE for 20 h. The protein level of FoxM1 was detected by Western blotting. **B** – mRNA levels of FoxM1 in CRE-treated PANC-1 cells were measured by RT-PCR. **C** – Levels of FoxM1-target proteins such as cyclin D1, c-Myc, and survivin were evaluated by Western blotting. Protein and mRNA levels were quantified using ImageJ. *P<0.05; **P<0.01 indicate significant differences compared to control.

Table 1. Linear regression data, limit of detection (LOD), limit of quantification (LOQ), and the amount of berberine

Compound	Linear regression data			LOD (μg/mL)	LOQ (μg/mL)	Amount (mg/g of extract)
	Test range (μg/mL)	Calibration curve	R^2			
berberine	**0.5 ~ 500**	$y = 32.378x + 47.706$	**0.9999**	**0.046**	**0.152**	**289.264±3.810**

y – peak area; x – concentration; R^2 – correlation coefficient

Downregulation of FoxM1 by berberine

HPLC was performed to identify and quantify berberine, a major component of CRE. Chromatographic analysis showed that berberine has a major peak in CRE (Supplementary Fig. S1). The linearity and sensitivity of the method were validated for the quantification of berberine. The linear regression range for berberine was 0.5~500 μg/mL with a regression coefficient of 0.9999. The limits of detection (LOD) and quantification (LOQ) of berberine were 0.046 μg/mL, and 0.152 μg/mL, respectively (Table 1). The berberine content

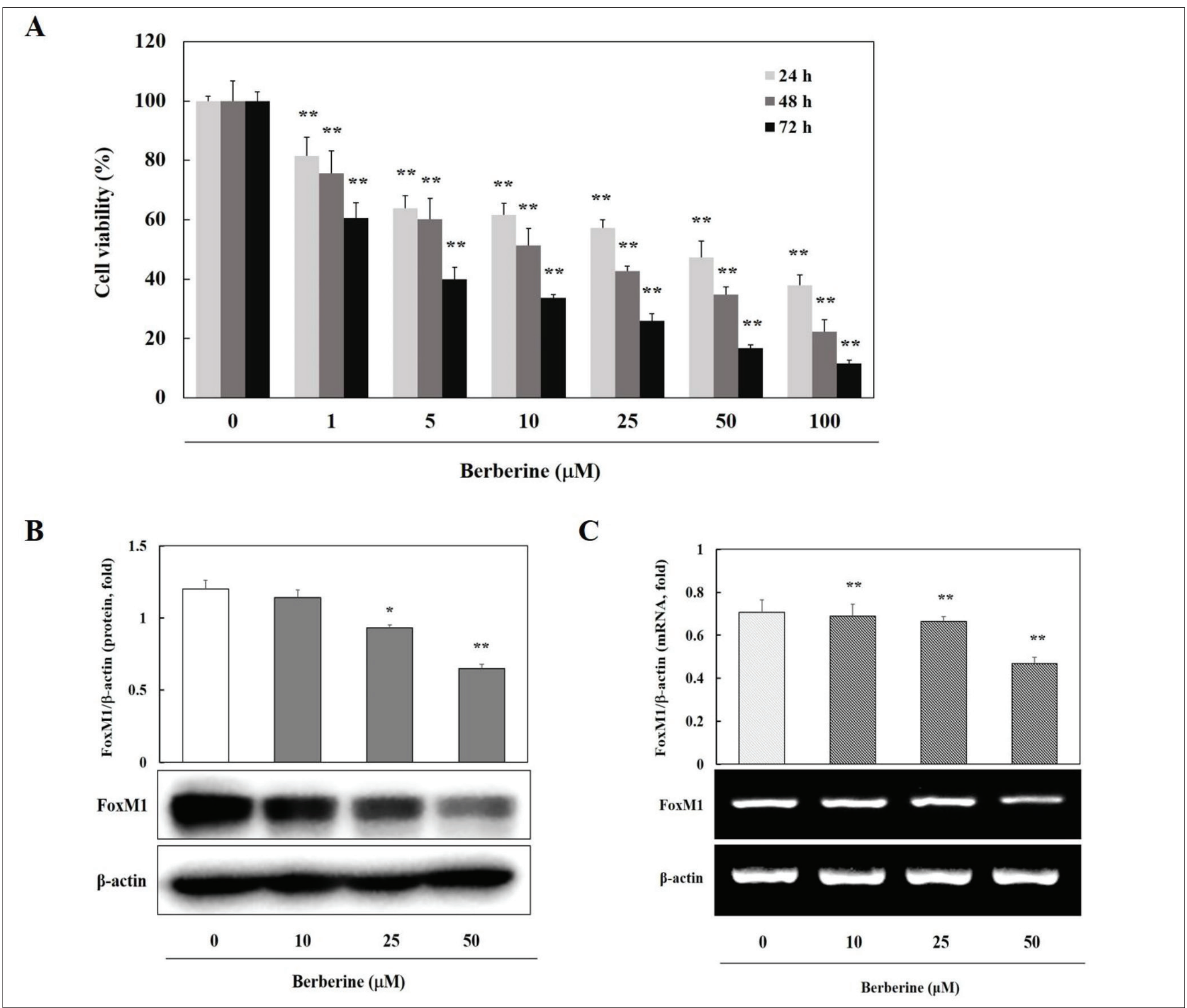

Fig. 4. Effects of berberine on cell viability and FoxM1 expression in pancreatic cancer cells. **A** – Cell viability in berberine-treated PANC-1 cells was measured by the MTT assay. **B, C** – FoxM1 expression levels in berberine-treated PANC-1 cells were measured by Western blotting and RT-PCR. Protein and mRNA levels were quantified using ImageJ. *P<0.05; **P<0.01 point to a significant difference between berberine treatment and control.

in CRE was 289.264 mg/g of extract (Supplementary Fig. S2). To explore whether berberine could affect the growth of pancreatic cancer cells, an MTT assay was performed. Although 100 µM slightly exceeded the estimated maximum content of berberine in CRE, it was included in the MTT assay to evaluate potential dose-dependent effects of berberine beyond the levels present in CRE. Berberine reduced the viability of PANC-1 cancer cells in a concentration-dependent manner. To examine the effect of berberine on FoxM1 regulation, protein and mRNA levels of FoxM1 in PANC-1 cells were detected by Western blotting and RT-PCR, respectively. Berberine suppressed both FoxM1 protein and mRNA levels (Fig. 4). For these molecular analyses, berberine was applied at concentrations up to 50 µM to maintain consistency with the biologically relevant range observed in CRE-treated cells. These results of berberine were consistent with those of CRE. Therefore, berberine, a main component of *Coptis chinensis*, could powerfully suppress the growth of pancreatic cancer cells by downregulating FoxM1 expression. This suggests that berberine contributes to the anti-pancreatic cancer activity of CRE, at least in part.

DISCUSSION

The present study focused on the potential anticancer effects of *C. chinensis* rhizome extract (CRE) against pancreatic cancer. Although significant progress has been made in cancer research, pancreatic cancer continues to be associated with poor outcomes and high mortality rates [28,29]. While many etiological factors, such as *Kras* oncogene mutation, inactivation of tumor suppressor genes, and gene amplification, are known to contribute to pancreatic cancer, a reverse link between this cancer and the consumption of fruits, vegetables, and fiber has been observed [30]. Therefore, developing effective therapeutics for pancreatic cancer derived from herbal sources and elucidating their molecular mechanisms is valuable and warranted.

Coptis chinensis is a perennial herb characterized by its yellow and branched rhizomes. The rhizome of *C. chinensis* (CR) has been used in traditional herbal medicine in Asian countries, including China, Korea, and Japan. Modern research has indicated that CR and berberine, a key alkaloid found in CR, possess various biological and pharmacological activities, including antitumor effects [9]. Studies have demonstrated the inhibitory effects of CR and berberine against multiple types of cancers [31-34]. CR and berberine exhibit significant antiproliferative activities against pancreatic cancer cell lines [35]. Park et al. [36] reported that berberine can inhibit the growth of pancreatic cancer cells by inducing G1 phase arrest. In this study, CRE reduced cancer cell viability and arrested the cell cycle at the G0/G1 phase. These results align with previous reports suggesting that CRE is an important inhibitor of cancer proliferation. CR and berberine have demonstrated inhibitory effects against various cancers through multiple mechanisms, including upregulation of mitogen-activated protein kinase (MAPK) p38, modulation of the phosphoinositide 3-kinase (PI3K)/Akt pathway, downregulation of the Rho/Rho-associated kinase (ROCK) signaling pathway, and suppression of signal transducer and activator of transcription 3 (STAT3) phosphorylation [37-40]. However, the effects of CR and berberine on FoxM1 expression in pancreatic cancer cells have not been previously reported.

FoxM1, an oncogenic transcription factor, is known to be involved in the overgrowth ability of cancer cells [41]. CRE was found to suppress FoxM1 expression at both the protein and mRNA levels, with a more pronounced reduction observed at the protein level. This might be due to increased instability at the translational step in addition to suppressed mRNA expression by CRE, which could be investigated in future studies. Since CRE downregulated FoxM1 expression, it was necessary to determine whether its downstream target proteins were also affected.

Cyclin D1 is known to be transactivated by FoxM1 [42]. Cyclin D1, a sensor for the transition of G1 to S phase, has been reported to be dysregulated in tumor cells. It is recognized as an oncogene that can promote tumor cells and tissues [43]. In the present study, cyclin D1 levels were decreased in association with cell cycle arrest at the G0/G1 phase. Other key regulators of cell cycle progression, such as c-Myc and survivin, are also downstream targets of FoxM1 [44,45]. c-Myc is known to promote cell cycle progression. Deregulation of c-Myc can lead to uncontrolled cell proliferation in cancers and other hyperproliferative diseases [46]. Overexpression of c-Myc allows cancer cells to move into the cell cycle and hasten cell cycle progression [47]. Survivin is known as an inhibitory protein of apoptosis and a regulator of cell division and cell cycle progression. Consistent with its role, survivin is upregulated in a variety of human cancers [48]. In this study, CRE decreased the expression of c-Myc, survivin, and cyclin D1, meaning that protein levels of cyclin D1, c-Myc, and survivin were declined through downregulation of FoxM1 by CRE. These findings demonstrate that CRE can downregulate FoxM1 expression and its downstream target protein levels, leading to cell cycle arrest and cell growth inhibition in pancreatic cancer. These results indicate that CRE inhibits cell growth and induces cell cycle arrest in PANC-1 human pancreatic cancer cells by modulating FoxM1 expression, highlighting its potential as a therapeutic target for pancreatic cancer.

Berberine, an isoquinoline alkaloid, is a significant component of CR [9]. Numerous studies have reported that berberine exhibits various biological activities, including anticancer effects. These activities of berberine align with those of CR [19,20]. Kang et al. [31] have demonstrated that CR contains berberine as a primary ingredient and that berberine is the active compound responsible for the anticancer effect of CR. In the present study, the concentration of berberine in CRE quantified by HPLC analysis was 289.264 mg/g of extract, indicating that 100 μg/mL of CRE contained

approximately 86 μM of berberine. CRE and berberine were assessed under comparable experimental conditions using the same pancreatic cancer cell line (PANC-1). Therefore, the anticancer effects of CRE, including the inhibition of cell growth and the downregulation of FoxM1 expression, are likely attributable, at least in part, to the presence of berberine. Multiple concentrations of berberine (1-50 μM) displayed inhibitory effects on the growth of PANC-1 pancreatic cancer cells. In line with these effects of CRE, berberine also reduced the protein and mRNA levels of FoxM1 expression in pancreatic cancer cells. This indicates that berberine downregulates FoxM1 expression, leading to the inhibition of cancer cell growth. These findings closely align with the antipancreatic activity of CRE. Therefore, it can be speculated that berberine may contribute, at least partially, to the anti-pancreatic cancer activity of CRE. However, the potential contributions of other constituents in CRE, possibly through synergistic or additive actions, cannot be excluded.

In conclusion, CRE repressed the growth of PANC-1 pancreatic cancer cells by inducing cell cycle arrest at the G0/G1 phase. Moreover, CRE downregulated FoxM1 at both the protein and mRNA levels, and its downstream targets, including cyclin D1, c-Myc, and survivin. Berberine, a major isoquinoline alkaloid of CRE, inhibited the growth and FoxM1 expression in pancreatic cancer cells. Taken together, these results demonstrate that CRE exerts anti-pancreatic cancer effects by downregulating FoxM1, with its major component, berberine, contributing at least in part to these activities. These results suggest that CRE and its principal isoquinoline alkaloid, berberine, might have valuable therapeutic potential against human pancreatic cancer.

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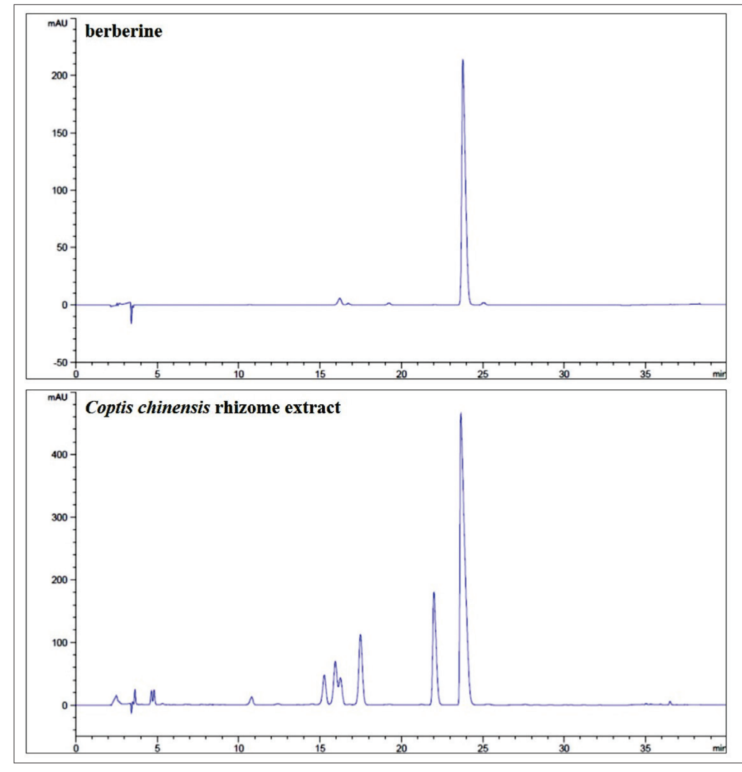
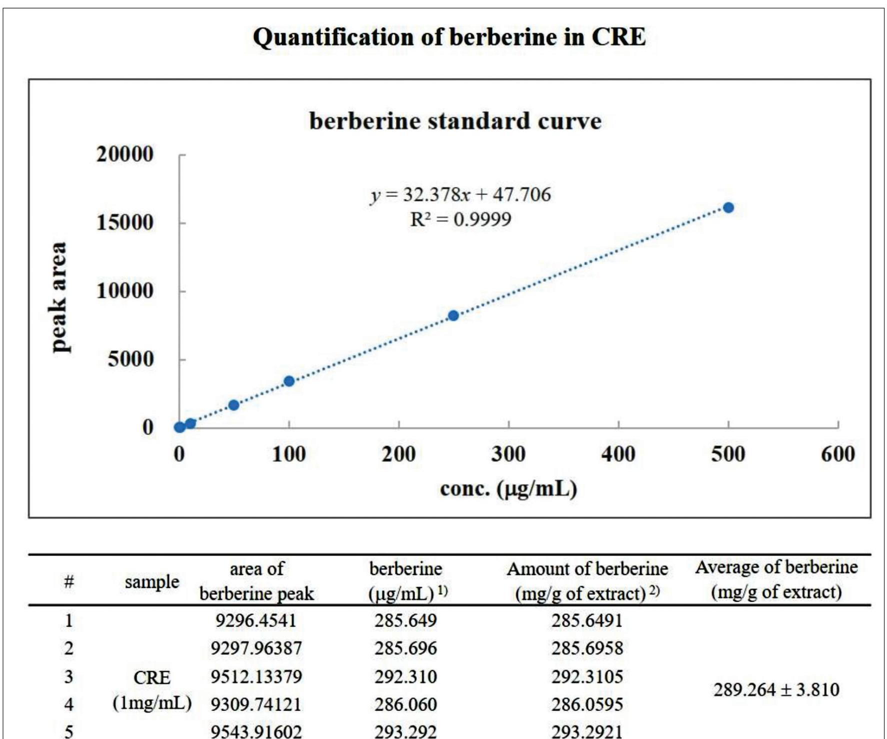
SUPPLEMENTARY MATERIAL



Supplementary Fig. S1. Chromatograms of berberine (standard) (upper) and *Coptis chinensis* rhizome extract (CRE) (lower).



#	sample	area of berberine peak	berberine (μg/mL) 1)	Amount of berberine (mg/g of extract) 2)	Average of berberine (mg/g of extract)
1	CRE (1mg/mL)	9296.4541	285.649	285.6491	289.264 ± 3.810
2		9297.96387	285.696	285.6958	
3		9512.13379	292.310	292.3105	
4		9309.74121	286.060	286.0595	
5		9543.91602	293.292	293.2921	
6		9520.86133	292.580	292.5800	

1) calculated using by equation $y = 32.378x + 47.706$ (y: peak area, x: conc.)

2) converted as follows; berberine conc. (μg/mL) in CRE (1mg/mL)

= berberine amount (mg) in CRE 1g

Supplementary Fig. S2. Quantification of berberine in CRE

ONLINE SUPPLEMENTARY RESEARCH DATASET

The raw data underlying this article is available as an online supplementary research dataset: https://www.serbiosoc.org.rs/NewUploads/Uploads/Jeong%20and%20Lee_Research%20Datset.pdf