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Anticancer Activities of Peanut (*Arachis hypogaea* L.) Testa Extract in Combination with Cisplatin and 5-Fluorouracil against Cholangiocarcinoma Cells In Vitro and in Mouse Xenograft Models

Jarckrit Jeeunngoi [1], Gulsiri Senawong [1], Sanun Jogloy [2], Jeerati Prompipak [1], Arunta Samankul [1], Suppawit Utaiwat [1], Khanutsanan Woranam [1], Banchob Sripa [3] and Thanaset Senawong [1,*]

[1] Department of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand; jarckrit@kkumail.com (J.J.), gulsiri@kku.ac.th (G.S.), jeerati.ppk@gmail.com (J.P.), s_arunta@kkumail.com (A.S.), suppawitu@gmail.com (S.U.), k.woranam@gmail.com (K.W.), sthanaset@kku.ac.th (T.S.)

[2] Department of Plant Science and Agricultural Resources, Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand; sanun@kku.ac.th (S.J.)

[3] WHO Collaborating Centre for Research and Control of Opisthorchiasis (Southeast Asian Liver Fluke Disease), Tropical Disease Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand; banchob@kku.ac.th (B.S.)

* Correspondence: sthanaset@kku.ac.th

Abstract: Cholangiocarcinoma is an aggressive cancer with limited treatment options. Current therapies, such as cisplatin and 5-fluorouracil (5-FU), often cause side effects and drug resistance. This study investigated a potential new approach by combining a peanut skin extract (KK4-PSE) with these drugs. The extract effectively inhibited the growth of cholangiocarcinoma cells in laboratory studies and showed even greater selectivity for cancer cells compared to healthy cells. When combined with cisplatin or 5-FU, KK4-PSE enhanced the drugs' effects, leading to cell cycle arrest and increased cell death. These combined treatments also reduced levels of proteins that promote cancer cell survival. In animal models, KK4-PSE combined with 5-FU showed superior tumor suppression compared to the combination with cisplatin. Overall, these findings suggest that KK4-PSE may be a promising synergistic agent to improve the effectiveness of chemotherapy for cholangiocarcinoma, warranting further clinical investigation.

Keywords: apoptosis; cholangiocarcinoma; cisplatin; 5-fluorouracil; mouse xenografts; peanut skin

1. Introduction

Cholangiocarcinoma (CCA) is a rare type of adenocarcinoma that results from the neoplastic transformation of bile duct epithelial cells in the intrahepatic or extrahepatic biliary ducts [1]. CCA is a complex disease characterized by complex interactions between the patient's genetic background and several risk factors. CCA is the second most common hepatobiliary malignancy, originating from bile duct epithelial cells, accounting for 1% of all cancer cases worldwide [2], with the elderly population bearing the majority of disease incidence. CCA incident rates vary by geography globally [3], reflecting various risk factors and genetic background in the area. The disease seems to have the highest rate in the north-eastern region of Thailand [4]. Parasitic infestation of the bile duct is an important endemic factor to CCA in Thailand, which is a certain risk factor for the disease caused by chronic liver fluke (*Opisthorchis viverrini*) [4,5]. In addition, chronic infection with the liver fluke is crucial for cholangiocarcinogenesis. Genetic modifications and epigenetic mechanisms are associated with the inflammatory process of liver fluke infection. Chronic inflammation could stimulate tumorigenesis by providing signals for pro-survival triggering genetic aberrations which can promote cholangiocarcinogenesis [6]. Surgery is currently the only CCA curative therapy, but the five-year relative survival rate is still below 5 percent [7].

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Chemotherapeutic resistance of CCA cells is a major cause of treatment failure [8]. The combination chemotherapy is therefore crucial in the treatment of CCA patients, resulting in better early treatment results but not in the later stage of a disease [9]. Chemotherapy, on the other hand, frequently has undesirable side effects. Patients may develop chemotherapeutic drug resistance, and surgical resection results are often insufficient due to tumor recurrence [10]. Consequently, new effective treatment strategies that contribute to the reduction of the side effects of chemotherapy drugs are also urgently needed. Indeed, plant-derived compounds such as tiliacorinine [11] and capsaicin [12] are gaining attention as potential CCA therapeutics. In addition, the medicinal herb extracts were also tested for their anticancer potential against CCA cell lines [13] and a significant positive result was reported. Our previous studies demonstrated that the Valencia KK4-type peanut (*Arachis hypogaea* L.) skin ethanolic extract (KK4-PSE) showed the highest phenolic content among the 15 peanut varieties of Valencia [14] and exhibited cytotoxicity against several human cancer cell lines, including HeLa, HCT-116, MCF-7, and Jurkat cells [15]. Moreover, KK4-PSE has been shown to possess histone deacetylase (HDAC) inhibitory activity, induce cell cycle arrest and cellular apoptosis through the up-regulation of caspases-8 and 3 proteins against CCA cells [16]. However, the anticancer activity of KK4-PSE in combination with current chemotherapeutic drugs (cisplatin and 5-fluorouracil (5-FU)) against CCA cells has not yet been investigated. In the present study, we investigated the effect of KK4-PSE combined with cisplatin and 5-FU against CCA cells both *in vitro* and in nude mouse xenograft models. In addition, the potential anticancer mechanism of KK4-PSE was also explored.

2. Materials and Methods

2.1. Materials and Reagents

RPMI-1640 medium was procured from Gibco-BRL (Gaithersburg, MD, USA), while fetal bovine serum (FBS) was acquired from Cytiva (Kremplstrasse, Pasching, Austria). Propidium iodide (PI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA), and Annexin V-FITC was sourced from Biolegend (San Diego, CA, USA). Cisplatin was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA), whereas 5-fluorouracil (5-FU) was acquired from PanReac Applichem (Castellar del Valles, Spain). Antibodies against p53 (2524), Bcl-2 (2870), Bax (2772), pERK1/2 (9107), ERK1/2 (4377), and Beta-Actin (4970) were all obtained from Cell Signaling Technology (Beverly, MA, USA).

2.2. Cell Culture and Nude Mouse Xenograft Models

The KKU-M213B cell line was derived from a male patient with intrahepatic mass-forming CCA stage-4B [17]. The immortalized cholangiocyte cell line H69 was obtained from Prof. D. Jefferson (Tufts University, Boston, MA, USA). KKU-M213B cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco, New York, NY, USA). H69 cells were cultured in Dulbecco's minimal essential medium (DMEM; Gibco, Invitrogen Corporation) supplemented with 10% fetal bovine serum (FBS), 100 U/mL ampicillin, 100 U/mL streptomycin sulfate, adenine (25 µg/mL), epinephrine (1 µg/mL), insulin (5 µg/mL), EGF (10 ng/mL), T3T Triiodo-L-thyronine (13.60 ng/mL), hydrocortisone (0.62 µg/mL), and holo-transferrin (8.30 µg/mL). The cells were incubated at 37°C in a humidified atmosphere containing 5%CO_2. For all ensuing experiments, cells in the exponential growth phase were exclusively used. Female BALB/CAJcl-Nu/Nu mice (4–6 weeks of age, 25–30 g in weight) were obtained from Nomura Siam International (Bangkok, Thailand). The mice were housed in individual ventilated cages (IVCs) with environmental conditions maintained at 23 ± 2 °C, 30–60% humidity, and a 12-hour light/dark cycle (350–400 Lux). All procedures involving animals were carried out at the Northeast Laboratory Animal Center, Khon Kaen University.

2.3. Preparation of KK4-PSE

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The Field Crop Research Station of Khon Kaen University, Thailand, provided the KK4 Valencia-type peanut testae for the 2019-crop (October 2018 to February 2019). 1 g of powdered peanut testae was stirred in absolute ethanol (1:40, w/v) at room temperature for 48 hours. The samples were centrifuged at 900 ×g for 10 minutes and the supernatant was filtered through a filter paper. The filtrate was evaporated using a rotary evaporator until the solution was reduced to about 2 mL. The remaining filtrate was completely dried with a gentle stream of nitrogen gas. The KK4-PSE was stored at −20 ºC until use.

2.4. Cell Viability Assay

The MTT (3-(4,5-dimethylthiazol-2yl) 2,5-diphenyl tetrazolium bromide) assay was used to assess cell proliferation. KKU-M213B and H69 cells were seeded at a density of 8×10^3 cells/well in a 96-well plate. The cells were treated with various concentrations of single agent (Cis, 5-FU, and KK4-PSE) and a solvent control (0.5% ethanol + 0.5% dimethyl sulfoxide (DMSO)) for 24, 48, and 72 h. The combination treatments with KK4-PSE (various concentrations) and Cis or 5-FU (sub-toxic concentration; IC20) were performed at 24, 48, and 72 h exposures. After treatment, each well was added with 200 µL of 1.2 mM MTT, and the resulting formazan crystals were dissolved in DMSO. The absorbance of formazan was measured at 570 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) and the optical density of cellular debris measured at 655 nm was used to correct the absorbance of formazan. The following equation was used to calculate the cell viabilit. Where A and O.D. are the absorbance and optical density, respectively.

$$\%\ \text{Cell viability} = \frac{\text{A570 Sample} - \text{O. D. 655 Sample}}{\text{A570 Control} - \text{O. D. 655 Control}} \times 100 \qquad (1)$$

2.5. Drug Interaction Analysis

The combination index (CI) was calculated to determine the type of drug interactions (synergistic effect, additive effect, or antagonistic effect) according to the Chou–Talalay method [18]. The following equation was used to calculate the CI values for 50% growth inhibition:

$$\text{CI} = \left(\frac{\text{D1}}{\text{Dx1}}\right) + \left(\frac{\text{D2}}{\text{Dx2}}\right) + \alpha\frac{\text{D1D2}}{\text{Dx1Dx2}}\ ; \qquad (2)$$

where *D*1 is a dose of drug 1 (Cis or 5-FU) combined with drug 2 (KK4-PSE) to produce 50% cell viability; *Dx*1 is a dose of single drug 1 to produce 50% cell viability; *D*2 is a dose of drug 2 combined with drug 1 to produce 50% cell viability; *Dx*2 is a dose of single drug 2 to produce 50% cell viability; α = 1 for mutually non-exclusive modes of drug action. CI < 0.90 indicates a synergistic effect; CI = 0.90–1.10 indicates an additive effect, and CI > 1.10 indicates antagonism. The dose reduction index (DRI) was also calculated using the equation below to indicate the extent of dose reduction (fold) of the combined dose tested compared to the dose of a single agent:

$$\text{DRI} = \left(\frac{\text{Dx}}{\text{D}}\right)\ ; \qquad (3)$$

where *D* is a dose of a drug combined with the other drug to produce 50% cell viability; *Dx* is a dose of a single drug to produce 50% cell viability.

2.6. Cell Cycle Inhibition Analysis

KKU-M213B cells (1×10^6 cells/mL) were plated in a 5.5 cm culture dish and incubated for 24 h. The cells were then treated with various concentrations of KK4-PSE, either alone or in combination with the IC_{20} subtoxic dose of the chemotherapy drug (cisplatin or 5-FU). Propidium Iodide (PI) staining was performed as described previously [19]. In brief, cells were harvested and fixed in 70% ethanol and then incubated for 1 hour at 37 ºC in 250 µL of phosphate-buffered saline (PBS) containing 0.2 mg/mL RNase A. Finally, cells were stained for 30 minutes at room temperature with PBS containing PI (20 mg/mL). The PI-stained cells were analysed by the BD FACSCanto II flow

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cytometer (Becton Dickinson, San Jose, CA, USA). The service was provided by the Research Instrument Center, Khon Kaen University, Thailand.

2.7. Apoptosis Induction Analysis

The apoptosis analysis was carried out using a Vybrant Apoptosis Assay Kit #2 (Molecular Probes, Invitrogen Corporation, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The cells were then treated with various concentrations of KK4-PSE, either alone or in combination with the IC_{20} subtoxic dose of the chemotherapy drug (cisplatin or 5-FU). After treatments, the cells were harvested and washed twice with ice-cold PBS. The cells were then resuspended in 500 µL of Annexin-binding buffer and stained with Annexin V-FITC and PI as directed by the manufacturer. Beckman Coulter CytomicsFC500 MPL flow cytometry (Beckman Coulter, Miami, FL, U.S.A.) was used to analyze the stained cells.

2.8. Western Blot Analysis

KKU-M213B cells were plated at a density of 1×10^6 cells/dish in a 5.5 cm culture dish. Synergistic concentrations of KK4-PSE and chemotherapy drugs were used to treat KKU-M213B cells for 24 h. RIPA lysis buffer (Amresco, Solon, OH, USA) supplemented with protease inhibitor cocktail was used to extract total proteins (60 µg). The Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) was employed to determine protein concentration. Proteins were separated by 12.5% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes. After 1 hour incubation at room temperature in TBST with 5% skim milk, membranes were incubated with primary antibodies overnight at 4 °C. Western blotting analysis utilized the following primary antibodies: anti-p53 (1:1,000), anti-Bcl-2 (1:1000), anti-Bax (1:1000), anti-pERK1/2 (1:2,000), anti-ERK1/2 (1:1,000), and anti-beta-actin (1:1000). Following TBST washing, membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA). Chemiluminescence reagent (Bio-Rad, CA, USA) was used to develop blots, which were then exposed to X-ray film.

2.9. Antitumor Activity of KK4-PSE in Nude Mouse Xenograft Models

Female nude mice (BALB/cAJcl-Nu/Nu, 6-8 weeks old, weighing 25-30 g) were obtained from Nomura Siam International (Bangkok, Thailand) and maintained at Khon Kaen University's Northeast Laboratory Animal Center. The animal experiments were approved by Khon Kaen University's Institutional Animal Care and Use Committee (approval No. KKUAE 21/2562; date of registration 21 March 2019) and performed in accordance with guidelines established by the National Research Council of Thailand's Ethical Principles and Guidelines for the Use of Animal in Scientific Purposes (License No. U1-00998-2558). The study was carried out in compliance with the ARRIVE guidelines. The animals were acclimatized for one week in an ABSL-2 standard cage, with each group consisting of five nude mice. The mice were fed a standard chow pellet. The standard conditions were 20-24 ºC, 30%-60% relative humidity, and light at 350-400 Lux, for a 12/12-hour light/dark cycle. Forty-five of BALB/cAJcl-Nu/Nu mice were randomly divided into nine groups (n = 5), which consisted of (1) control group (treated with normal saline solution), (2) 5-FU 10 mg/kg, (3) cisplatin 3 mg/kg, (4) KK4-PSE 100 mg/kg, (5) KK4-PSE 200 mg/kg, (6) 5-FU 10 mg/kg + KK4-PSE 100 mg/kg, (7) 5-FU 10 mg/kg + KK4-PSE 200 mg/kg, (8) cisplatin 3 mg/kg + KK4-PSE 100 mg/kg, and (9) cisplatin 3 mg/kg + KK4-PSE 200 mg/kg. Mice were injected subcutaneously at the right anterior lateral thoracic wall with 100 µL of KKU-M213B cells (2×10^6 cells) [20]. After inoculation, mice were acclimatized for 14 days to observe tumor development. The drugs and KK4-PSE dissolved in a normal saline solution (NSS) were administered to the mice by intraperitoneal injection. The administration was given once every two days for 14 days and the tumor diameter and body weights of a mice were also measured every two days. The following formula was used to calculate the tumor volumes from measurements taken using a digital vernier caliper. A digital vernier caliper was used to measure the tumor volume which was calculated every two days as the following formula:

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$$\text{Tumor volume} = \frac{\text{length} \times \text{width}^2}{2} \quad (4)$$

Animals were euthanized using inhalation anesthesia in a CO_2 chamber. The tumors, kidneys, liver, and spleen were removed and stored at -80 °C for further examination. The tumor growth inhibition (TGI) rate was calculated using the following formula:

$$\%\,TGI = \left(1 - \frac{RTV\ in\ experimental\ group}{RTV\ in\ control\ group}\right) \times 100 \quad (5)$$

The relative tumor volume (RTV) was determined using following formula:

$$\text{RTV} = \frac{\text{tumor volume on the measured day}}{tumor\ volume\ on\ day\ 0} \quad (6)$$

After 14 days, the mice were sacrificed, and the tumors and visceral organs were weighed before being fixed in 10% formalin solution for the further experiments.

2.10. Histological Examination

Tumors and other organs were preserved in 10% formalin. After at least 24 hours of fixation, the tissues were dehydrated in a serial dilution of alcohol using an automated tissue processor (Leica TP1020, Leica Microsystems, Wetzlar, Germany). Tissues which had been processed were paraffin embedded. Rotary Microtome is used to cut five micrometer thick sections from the paraffin block (Leica RM2255 Fully Automated Rotary Microtome, Leica Microsystems, Wetzlar, Germany). Hematoxylin and eosin were used to stain the tissue section (H&E). Light microscopy was used to examine each slide.

2.11. Terminal Deoxyuridine Nick-End Labeling (TUNEL) Staining

The terminal deoxyuridine nick-end labeling (TUNEL) assay was used to determine the level of apoptosis in the tissue sections. Deparaffinized tissue sections were washed with phosphate buffered saline before being used in the TUNEL study. The tissue sections were rehydrated using a series of alcohol dilutions. Thereafter, tissues were incubated with primary antibodies using an in-situ cell death detection kit, POD (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions. The tissue was then developed using 3,3-Diaminobenzidine (MERCK; Sigma-Aldrich) (St. Louis, MO, USA). The tissue slides were examined under a light microscope (Olympus CX31, Tokyo, Japan) and then sorted based on the technique described previously [21].

2.12. Statistical Analysis

Statistical analyses were performed using SPSS 22.0 (IBM, Manassas, VA, USA). Data are represented as mean ± standard deviation (SD) for individual experiments and mean ± standard error of the mean (SEM) for independent experiments. GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA) was utilized for graphical representations. To assess statistically significant differences between control and experimental groups, one-way analysis of variance (ANOVA) was employed, followed by Duncan's post hoc test for pairwise comparisons. P values < 0.05 were deemed statistically significant. All experiments were replicated three times.

3. Results

3.1. Antiproliferative Activities of KK4-PSE, Cisplatin, and 5-Fluorouracil against CCA (KKU-M213B) cells

The MTT assay was employed to assess the antiproliferative effects of KK4-PSE and current chemotherapeutic agents (cisplatin and 5-FU) against cervical cancer cells. Figure 1 demonstrates that KK4-PSE, cisplatin, and 5-fluorouracil suppressed KKU-M213B cell proliferation in both a dose- and time-dependent fashion. To determine cell viability, KKU-M213B cells were exposed to various

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concentrations of KK4-PSE, cisplatin, and 5-fluorouracil for periods of 24, 48, and 72 hours, followed by MTT assay analysis. MTT results showed that KK4-PSE and current chemotherapy drugs significantly inhibited proliferation of KKU-M213B cells in a dose- and time-dependent manner (Figure 1A–C). KK4-PSE inhibited proliferation of CCA cell line (IC_{50} = 37.22 ± 4.31, 26.27 ± 0.78, and 17.72 ± 0.50 μg/ml at 24 h-, 48 h-, and 72 h-exposures, respectively) more effective than that of the noncancer H69 cholangiocyte cell line (IC_{50} = 193.35 ± 6.55, 75.35 ± 1.00, and 57.41 ± 0.96 μg/ml at 24 h-, 48 h-, and 72 h-exposures, respectively) (Figure 1D), indicating that KKU-M213B cell line was more sensitive to KK4-PSE than the noncancer H69 cell line. In contrast, the noncancer H69 cells were more sensitive to both current chemotherapy drugs (cisplatin and 5-FU) than KKU-M213B cells (Figure 1E,F). Cisplatin inhibited the growth of KKU-M213B cells with IC_{50} values of 31.18 ± 0.60, 21.41 ± 1.19, and 6.62 ± 0.22 μM at 24 h-, 48 h- and 72 h-exposures, respectively, while 5-FU suppressed the growth of KKU-M213B cells with IC_{50} values of 672.37 ± 26.69, 63.91 ± 5.91, and 2.13 ± 0.03 μM at exposure times of 24, 48 and 72 h, respectively.

Figure 1. Antiproliferative effects of the single agent treatments of KK4-type peanut skin extract (KK4-PSE), cisplatin and 5-FU on KKU-M213B CCA (A, B, C), and non-cancer H69 (D, E, F) cells. Cells were treated with various concentrations of KK4-PSE, cisplatin and 5-FU for 24, 48 and 72 h. The percentages of cell viability were calculated relative to the solvent control (0.50% ethanol + 0.50% DMSO). Data are presented as mean ± SEM for three independent experiments. .

3.2. Antiproliferative Activities of KK4-PSE in Combination Treatments with Current Anticancer Drugs (Cisplatin and 5-FU) against CCA Cells

To elucidate the potential of KK4-PSE for enhancing the antiproliferative activities of current chemotherapeutic drugs (cisplatin and 5-FU) against KKU-M213B cells, we performed combination treatments of KK4-PSE with a subtoxic dose (IC_{20}) of cisplatin and 5-FU. The IC_{50} values of KK4-PSE in combinations with a subtoxic dose of cisplatin and 5-FU were shown in Table 1. The combination index (CI) values were calculated to determine the type of drug interaction based on the median-effect principle of the Chou & Talalay method [18]. The CI values for combination treatments of KK4-PSE and cisplatin against KKU-M213B cells at 24 h, 48 h, and 72 h exposures were 0.77, 1.01, and 1.23, indicating synergistic, additive, and moderate antagonistic effects, respectively (Table 1). The synergistic effect at 24 h exposure resulted in a dose reduction of 4.27-fold for cisplatin. The CI values for combination treatments of KK4-PSE and 5-FU against KKU-M213B cells at 24 h, 48 h, and 72 h exposures were 0.48, 0.66, and 1.13, indicating strong synergistic, synergistic, and moderate antagonistic effects, respectively (Table 1). The synergistic effects at 24 h and 48 h exposures in KKU-M213B cells resulted in the dose reductions of 25.50-fold and 16.53-fold for 5-FU. Taken together, these results suggest that KK4-PSE enhance anticancer activity of 5-FU against CCA cells more effective than cisplatin at both 24 h and 48 h exposure times.

Table 1. CI and DRI of the combination treatments between KK4-PSE, Cisplatin and 5-FU against KKU-M213B cells.

Exposure times	IC_{50} of KK4-PSE (μg/mL)		IC_{20} of Cis (μM)	IC_{20} of 5-FU (μM)	CI	DRI		
	Alone	Combination				Cis	5-FU	KK4-PSE
24 h	37.22 ± 4.31	19.71 ± 0.56	7.38 ± 0.87	-	0.77 ± 0.05	4.27	-	1.89
48 h	26.27 ± 0.78	18.45 ± 0.27	6.53 ± 0.87	-	1.01 ± 0.05	3.31	-	1.43
72 h	17.72 ± 0.50	14.63 ± 0.43	2.68 ± 0.26	-	1.23 ± 0.06	2.48	-	1.21
24 h	37.22 ± 4.31	16.11 ± 0.40	-	27.17 ± 5.87	0.48 ± 0.06	-	25.50	2.31
48 h	26.27 ± 0.78	15.64 ± 0.47	-	3.97 ± 0.80	0.66 ± 0.03	-	16.53	1.68
72 h	17.72 ± 0.50	15.08 ± 1.29	-	0.58 ± 0.02	1.13 ± 0.08	-	3.64	1.18

KK4-PSE: Valencia KK4-type peanut skin ethanolic extract; Cis: Cisplatin; 5-FU: 5-fluorouracil. Antagonism: CI > 1.3; Moderate antagonism: CI = 1.1-1.3; Additive effect: CI = 0.9-1.1; Slight synergism: CI = 0.8-0.9; Moderate synergism: CI = 0.6-0.8; Synergism: CI = 0.4-0.6; Strong synergism: CI = 0.2-0.4.

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3.3. Effect of the Combination Treatments of KK4-PSE and 5-FU or Cisplatin on Cell Cycle Arrest and Apoptosis Induction

Based on the synergistic effect of KK4-PSE in combination with cisplatin and 5-FU against KKU-M213B cells, we further investigated whether the inductions of cell cycle arrest and apoptosis were related to the potency of KK4-PSE to induce cytotoxicity in combination treatments. The combination treatment of KK4-PSE and cisplatin caused a non-significant increase of G2/M phase arrest (37.30 ± 1.88%) compared with cisplatin (36.80 ± 1.58%) single drug treatment, but significant increase compared with KK4-PSE (23.30 ± 2.32%) single drug treatment (Figure 2A,B). In contrast, the combination treatment of KK4-PSE and 5-FU caused a significant increase of Sub G1 (9.10 ± 1.35%) compared with the 5-FU (5.70 ± 0.82%) and KK4-PSE (5.60 ± 0.68%) single drug treatments (Figure 2A,B).

Figure 2. Effects of KK4-PSE, cisplatin, and 5-FU in single and combination treatments on cell cycle distribution and apoptosis induction in KKU-M213B cells. (A) Representative DNA histograms represent the cell cycle distribution of KKU-M213B cells after treatments. The percentages shown are the mean ± SEM from three independent experiments. (B) The percentages of cells at each cell cycle phase are shown as bar graphs of the mean ± SEM from three independent experiments. (C) The representative dot plots display the flow cytometric analysis of apoptosis induction in KKU-M213B cells. (D) The bar graphs show the mean ± SEM of the percentages of apoptotic cells from three independent experiments. KKU-M213B cells were treated with solvent control (0.25% ethanol + 0.25% DMSO), 5-FU (27.17 μM), Cisplatin (7.38 μM), KK4-PSE (21.35 μg/mL) for 24 h exposure. Asterisks "*" and "**" indicate a significant increase ($p < 0.05$) when compared with the solvent control and single agent treatments, respectively.

KK4-PSE (21.35 ◎g/mL) alone treatment did not cause increased apoptosis in KKU-M213B cells compared with the solvent control treatment, however, KK4-PSE in combinations with cisplatin and 5-FU significantly caused increased apoptosis compared with the single agent treatments (Figure 2C,D). This finding demonstrated that synergistic apoptosis induction may underpin the potency of KK4-PSE to enhance anticancer activity of cisplatin and 5-FU against KKU-M213B cells.

3.4. KK4-PSE Enhances Anticancer Activities of Cisplatin and 5-FU by Inhibiting the ERK/MAPK Signalling Pathway and Regulating the Bax/Bcl-2 Expression at the Synergistic Conditions

To gain insight and understanding of the mechanisms involved in the anticancer effect of KK4-PSE in combination with chemotherapy drugs at the synergistic conditions, western blot analysis was performed to investigate the expression of proteins involved in cell proliferation and cell death in KKU-M213B cells. The results revealed that the levels of p-ERK, Bcl2 and p53 were significantly reduced, in the combination treatments (Figure 3A,B). The combination treatments of KK4-PSE with cisplatin and 5-FU caused increased Bax and decreased Bcl2 levels compared with the solvent control treatment (Figure 3A,B). The relative ratios of Bcl2/Bax were significantly decreased in the combination treatments of KK4-PSE with both cisplatin and 5-FU (Figure 3B). These findings suggest that KK4-PSE enhances anticancer activities of cisplatin and 5-FU via downregulations of p-ERK and Bcl2, and upregulation of Bax.

Figure 3. Effects of combination treatments of KK4-PSE with cisplatin and 5-FU on apoptosis-related proteins and ERK signaling pathway. KKU-M213B cells were treated with the solvent control (0.25% ethanol + 0.25% DMSO), KK4-PSE (21.35 μg/mL), and cisplatin (7.38 μM) or 5-FU (27.17 μM) for single and combination treatments under the synergistic conditions at 24 h exposure. (a) Representative western blots are shown. Total ERK1/2 and beta-actin were used as loading controls. (b) The relative fold of protein expression was shown as a bar graph from the intensity of the protein band compared with a loading control. Bar graph represents mean ± SEM from three independent experiments. Asterisks "*" and "**" denote a statistically significant difference ($p < 0.05$) compared with the solvent control and single agent treatments, respectively.

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3.5. Antitumor Effect of KK4-PSE in Combination with 5-FU or Cisplatin on CCA Xenograft Mice.

To investigate the antitumor effects of KK4-PSE both single and combination drug treatments, the xenograft tumor models were established by implanting KKU-M213B CCA cancer cells into BALB/cAJcl-Nu/Nu nude mice. After implanting KKU-M213B cells in female BALB/cAJcl-Nu/Nu mice until the tumor volume reached ~100 mm^3 (14 days), the KKU-M213B xenograft mice were intraperitoneally (i.p.) injected every other day with normal saline solution as a vehicle control, KK4-PSE, cisplatin, and 5-FU alone and in combination for 14 days as depicted in Figure 4A. The length and width of the tumors were measured using a digital Vernier caliper at each time point to calculate the tumor volume (Figure 4B). Following the end of the treatments, the tumors were dissected and photographed (Figure 4C) after the mice were euthanized in a CO_2 chamber. The single drug treatments with KK4-PSE (100 and 200 mg/kg), cisplatin (3 mg/kg), and 5-FU (10 mg/kg), caused a reduction in tumor volumes when compared with the vehicle control treatment (Figure 4(b)). Furthermore, the combination treatments of KK4-PSE (100, 200 mg/kg) with both cisplatin and 5-FU showed a greater decrease in tumor volume when compared with vehicle control and single drug treatments (Figure 4B). Mice receiving the highest dose of KK4-PSE (200 mg/kg) combined with both cisplatin and 5-FU showed significantly greater tumor volume and tumor weight reductions compared with the control group (Figure 4B,D). Combination treatment of the lower dose of KK4-PSE (100 mg/kg) with cisplatin exhibited a nonsignificant increase of tumor growth inhibition (TGI), while the higher dose of KK4-PSE (200 mg/kg) combined with cisplatin showed a significant increase of TGI, compared with the cisplatin single drug treatment (Figure 4E). Whereas the mice treated with the combinations of 5-FU (10 mg/kg) with both doses of KK4-PSE (100 and 200 mg/kg) exhibited significant increases of %TGI values, compared with the 5-FU single drug treatment (Figure 4E).

Figure 4. Effects of KK4-PSE (100, 200 mg/kg), cisplatin (3 mg/kg), and 5-FU (10 mg/kg) alone or in combination on KKU-M213B xenograft mice. (a) Experimental design of the administration of KK4-PSE, cisplatin, and 5-FU alone or in combination is shown. (b) Tumor volumes after treatments with KK4-PSE, cisplatin, and 5-FU alone or in combination are shown. (c) Representative photographs of mouse tumors after treatments are shown. (d) Tumor weights after surgical excision are shown. (e) The percentages of tumor growth inhibition (%TGI) after treatments with KK4-PSE, cisplatin, and 5-FU alone or in combination are shown. Asterisk "*" indicates a significant difference between the single and combined drug treatments ($p < 0.05$).

3.6. Toxicological Evaluation in Nude Mouse Xenograft Models

Tumor sections from mice treated with the combined therapy exhibited features of apoptotic cells with nuclear condensation (Figure 5A). TUNEL analysis was also used to look at the apoptotic DNA fragmentation area in the treated tumors. The TUNEL results showed that increased brownish cells signifying cellular apoptosis were predominantly observed in the treatment groups compared with the vehicle control group (Figure 5B,C). Notably, combination treatments of KK4-PSE (both 100 and 200 mg/kg) with both cisplatin and 5-FU caused significant increases of TUNEL-positive cells compared with the single drug treatments (Figure 5B,C). The toxicities of the drugs on xenograft mice were evaluated by monitoring body weight changes (BWC), organ weight, and histopathology of the organs (liver, kidneys, and spleen). The initial and final body weights are shown in Table 2. The body weights of mice in vehicle control, 5-FU, KK4-PSE (100 mg/kg), KK4-PSE (200 mg/kg), 5-FU + KK4-PSE (100 mg/kg), and 5-FU + KK4-PSE (200 mg/kg) groups were increased by 5.04%, 22.06%, 7.66%, 2.03%, 5.04% and 3.82%, respectively. In contrast, the body weights of mice in the groups treated with cisplatin, 5-FU + KK4-PSE (100 mg/kg), and 5-FU + KK4-PSE (200 mg/kg) groups were decreased by 0.92%, 28.56% and 24.65%, respectively. In addition, the organ index was calculated as a ratio between organ weight and body weight. Liver weight was significantly reduced in mice treated with cisplatin alone. In contrast, liver weights of mice treated with both doses of KK4-PSE in single drug treatments were significantly increased. The kidney weights of all treated mice were not significantly changed compared with that of the vehicle control group. Single drug treatments with 5-FU and KK4-PSE caused a significant increase in the spleen weight, however, combination treatments of KK4-PSE and 5-FU exhibited no significant effect on the spleen weight.

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Figure 5. Effects of KK4-PSE, cisplatin, and 5-FU in single and combination treatments on apoptosis induction in KKU-M213B xenograft. (a) Hematoxylin and eosin staining (H&E) was used to analyze the histopathology of mouse tumor slices under a light microscope. (b) In situ apoptosis of the tumor sections were detected by TUNEL staining. (c) Bar graph shows the mean ± SD of TUNEL-positive cells as representing the level of apoptosis. Asterisks "*" and "**" denote a statistically significant difference ($p < 0.05$) compared with the solvent control treatment and single agent treatments, respectively. Scale bar = 100 μm.

Table 2. Body weights and relative organ weights of nude mice in the vehicle control and treated groups. *Results are expressed as mean ± S.D. from five mice. 5-FU: 5-fluorouracil; KK4-PSE: KK4 peanut skin ethanolic extract. a $p < 0.05$ versus vehicle control.

Groups	Initial body weight (g)	Final body weight (g)	%BWC	Organ Index (g/100 g Body weight)		
				Liver	Kidney	Spleen
Vehicle Control	21.49±0.39	22.63±0.28	5.04	7.1±0.86	0.95±0.06	0.96±0.30
Cisplatin 3 mg/kg	19.79±1.07[a]	19.61±2.24[a]	-0.92 [a]	5.86±0.19[a]	0.82±0.09	0.63±0.52
5-FU 10 mg/kg	20.45±0.71	26.23±2.22[a]	22.06 [a]	7.16±0.38	0.93±0.10	1.97±0.32[a]
KK4-PSE 100 mg/kg	20.61±1.39	23.32±1.88	7.66	8.32±0.55[a]	0.98±0.06	2.03±0.69[a]
KK4-PSE 200 mg/kg	21.12±1.11	21.55±1.12	2.03	8.41±0.66[a]	1.04±0.09	2.60±1.00[a]
KK4-PSE 100 mg/kg + Cis	21.52±0.97	16.74±2.93[a]	-28.56 [a]	7.44±1.59	0.89±0.20	0.59±0.33
KK4-PSE 200 mg/kg + Cis	22.14±1.29	17.76±1.73[a]	-24.65 [a]	7.50±1.60	0.87±0.21	1.22±0.56
KK4-PSE 100 mg/kg + 5-FU	21.49±0.39	22.63±0.28	5.04	7.5 ± 0.35	1.07±0.10	1.30±0.48
KK4-PSE 200 mg/kg + 5-FU	21.98±0.65	22.85±0.99	3.82	7.32±0.28	1.04±0.08	1.51±0.50

Data are expressed as mean ± S.D. (n = 5). Cis: Cisplatin; 5-FU: 5-fluorouracil; KK4-PSE: KK4 peanut skin ethanolic extract. [a]$p < 0.05$ versus vehicle control, [b]$p < 0.05$ versus Cisplatin, [c]$p < 0.05$ versus 5-FU, [d]$p < 0.05$ versus KK4-PSE.

Histological analysis of liver and kidney tissues revealed no significant differences among mice treated with individual therapies or their combinations when compared to the vehicle control group (Figure 6). However, the liver of mice treated with a combination of cisplatin and KK4-PSE (200 mg/kg) exhibited signs of hepatocyte injury, characterized by vacuolar or hydropic degeneration (Figure 6A, blue arrows). Other treatment groups showed no evidence of hepatocellular damage (Figure 6A). Furthermore, examination of kidney and spleen tissues revealed no notable histological differences between the treated groups and the vehicle control group (Figure 6B,C).

Figure 6. Histopathology of mouse organs after treatments. The tissues, (a) liver, (b) kidney and (c) spleen, were stained by hematoxylin and eosin (H&E) and examined under a light microscope. The blue arrows indicate hepatocyte degeneration, also known as vacuolar or hydropic degeneration. The Scale bar = 100 μm.

4. Discussion

CCA is the second most common type of liver cancer and has aggressive features that make it resistant to chemotherapy, resulting in a poor prognosis [22]. Currently, the combination of gemcitabine and cisplatin is an acceptable recommended treatment for advanced cholangiocarcinoma [23]. Previous research has shown that CCA cells are resistant to a variety of chemotherapeutic agents [24]. To overcome this limitation, effective therapeutic strategies, and drugs to combat this lethal tumor must be developed [25]. A combination strategy involving multiple mechanisms of action could be a potential chemotherapy against tumor development. Indeed, several studies on the efficacy of polyphenol compounds such as resveratrol, tannic acid, escin, capsaicin, and green-tea polyphenols as effective alternative therapies to conventional chemotherapy strategies for CCA have been published in recent years [20,26,27]. Previous research ddemonstrated that phenolic-rich extracts of two Valencia peanut (*Arachis hypogaea* L.) skins, KK4 and ICG15042,

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inhibited the growth of various cancer cell types in vitro [15,28]. In the present study, our results indicate that co-treatments of KK4-PSE with cisplatin and 5-FU exhibit anticancer potential in CCA cells. Combinations of KK4-PSE with cisplatin and 5-fluorouracil resulted in cancer cell cycle arrest at the G2/M and S phases, respectively (Figure 2A,B). Moreover, inductions of cell cycle arrest and apoptosis were also observed, together with decreases in pERK1/2 and Bcl2/Bax signals. KK4-PSE combination with 5-fluorouracil exerted greater anti-proliferative efficacy than the combination with cisplatin against the KKU-M213B CCA cells both in vitro (Table 1) and in vivo (Figure 4E).

Apoptosis pathways are critical for suppressing cancer cell growth which is one of the predominant anticancer mechanisms of antineoplastic drugs, and it is used to assess the efficacy of anticancer drugs. Many studies have shown that Valencia peanut testa extracts (KK4 and ICG15042) induce apoptosis by modulating the expression of many apoptosis-related proteins in a series of apoptosis cascades, inhibiting cell cycle progression, and inhibiting HDAC activity against CCA cell lines [16,28]. The current study revealed that the treatments with combined KK4-PSE and current chemotherapeutic drugs caused more increased apoptotic cells than the treatments with individual chemotherapeutic drugs. The proapoptotic protein Bax and the antiapoptotic protein Bcl2 are critical regulators of apoptosis [29]. In this study, the treatments with combined KK4-PSE and current chemotherapeutic drugs caused a decrease in the Bcl2/Bax ratio (Figure 3A,B).

ERK1/2 activation leads to the phosphorylation of numerous intracellular proteins that regulate a variety of cellular functions ranging from cell proliferation to apoptosis [30]. Previous study reported that some polyphenol compounds induced apoptosis through ERK1/2 activation [31]. Furthermore, ERK activation is also involved in the induction of apoptosis and cell cycle arrest as a response mechanism to DNA damage or oxidative stress [32]. However, previous study demonstrated that ERK inactivation (decreased pERK1/2 level) was related to oxidative stressed-mediated proteolytic degradation of Bcl2 [33]. This proteolytic degradation of Bcl2 was triggered by tumor necrosis factor-alpha (TNF-α) activation. Furthermore, inhibition of the ERK1/2 has been shown to sensitize pancreatic cancer cells to the apoptotic effect of chemotherapy in vitro and in vivo [34]. In this study, treatments with KK4-PSE combinations with current chemotherapeutic drugs caused downregulation of pERK, suggesting that ERK inactivation is linked to cell death caused by downregulation of Bcl2 but not linked to p53 activation as downregulation of p53 is observed instead (Figure 3).

The CI value is used to assess the effect of combined drugs on cancer cells, which may be additive (CI = 0.90 – 1.10), synergistic (CI < 0.90), or antagonistic (CI > 1.10) [18]. In this study, the combinations of KK4-PSE and cisplatin indicated a synergism at 24 h-exposure (CI = 0.77 ± 0.05) and an additive at 48 h-exposure (CI = 1.01 ± 0.05), while the combinations of KK4-PSE and 5-FU indicated synergisms at 24- and 48 h-exposures (CI = 0.48 ± 0.06 and 0.66 ± 0.03, respectively) in KKU-M213B cells (Table 1). The synergistic and additive effects of combined KK4-PSE and cisplatin at 24- and 48-h exposures resulted in dose reductions for cisplatin of 4.27- and 3.31-folds, respectively, while the synergistic effects of combined KK4-PSE and 5-FU at 24- and 48-h exposures resulted in dose reductions for 5-FU of 4.27- and 3.31-folds, respectively (Table 1). These results suggest that the KK4-PSE exhibited a promising drug combination to enhance anticancer activity of cisplatin and 5-FU for the treatments of CCA.

The nude mouse KKU-M213B xenograft model was used to assess the empirical basis for the preclinical administration of cisplatin/5-FU in combination with KK4-PSE. In this study, KK4-PSE enhanced tumor suppression activity of 5-FU greater than that of cisplatin, even though single drug treatment of cisplatin showed a greater tumor suppression than that of 5-FU (Figure 4B–E). This is in accordance with the in vitro findings, in which KK4-PSE synergistically enhances the antiproliferative activity of 5-FU with CI values of 0.48 and 0.66 at 24 h- and 48 h-exposures, respectively, while the interactions of KK4-PSE and cisplatin are synergistic and additive with CI values of 0.77 and 1.01 at 24 h- and 48 h-exposures, respectively (Table 1). There were no significant differences in tumor volumes and tumor weights of mice treated with cisplatin combined with 100 mg/kg KK4-PSE and mice treated with cisplatin alone, while the tumor volumes and tumor weights of mice treated with cisplatin combined with 200 mg/kg KK4-PSE were significantly reduced when compared with a

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cisplatin single drug treatment (Figure 4B,D). In contrast, mice treated with both concentrations (100 and 200 mg/kg) of KK4-PSE combined with 5-FU exhibited significant reductions of tumor volumes and tumor weights when compared with 5-FU alone treated mice (Figure 4B,D). These results were correlated with the tumor growth inhibition ratios (Figure 4E). In addition, the percentages of TUNEL-positive cells representing apoptotic-positive cells in the mouse tumors were significantly increased in the combination treatments when compared with that of single drug treatments (Figure 5B,C). These results suggest that KK4-PSE effectively suppress KKU-M213B CCA cells in vivo especially in combination drug treatments.

Toxicological evaluation revealed that nude mice treated with 5-FU alone caused significantly increased BWC (%) and spleen weight, whereas 5-FU combination treatment with KK4-PSE show no significant changes on BWC (%) and spleen weight following the intervention sessions when compared with a vehicle control group (Table 2), suggesting that spleen toxicity in mice may be relieved by the 5-FU combination treatment with KK4-PSE to minimize 5-FU toxicity. The nude mice treated with cisplatin alone caused significantly increased BWC (%) and spleen weight, whereas 5-FU combination treatment with KK4-PSE show no significant changes on BWC (%) and spleen weight following the intervention sessions when compared with a vehicle control group (Table 2). Furthermore, our data showed that KK4-PSE alone treatments, both 100 and 200 mg/kg, significantly increased the mouse liver and spleen weights, while the KK4-PSE combination treatment with 5-FU showed no effect on mouse liver and spleen weights (Table 2), suggesting that the combination treatment could reduce toxicities of mouse liver and spleen caused by KK4-PSE treatment. Interestingly, the treatments with 5-FU alone and KK4-PSE alone significantly caused increased spleen weights, however, the combination treatment of 5-FU and KK4-PSE showed no effect on spleen weight, suggesting the combination treatment could reduce a spleen toxicity. The presence of tumors increases the population of tumor-associated neutrophils (TANs) and other leukocytes, which leads to spleen enlargement [35]. Splenomegaly has also been reported in patients with cholangiocarcinoma [36,37].

KK4-PSE may contain several bioactive compounds that promote cancer cell inhibition. The phytochemical composition of KK4-PSE contains gallic acid, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid, and sinapinic acid (Figure S1), which are the same phenolic acids identified in the previous lot of KK4-PSE [15]. In this study, KK4-PSE was not administered orally because the enzymatic digestion of polyphenol agents in the extract may cause a poor bioavailability, reducing their therapeutic potential [38,39]. In addition, pharmacokinetic properties have already limited the therapeutic outcome of natural compound products taken orally. It also includes the ability of these substances to be absorbed in the gastrointestinal tract [40]. The KK4-PSE has the potential to be used as a chemoprevention and chemosensitizer. Our results provide the evidence that combination treatment of current chemotherapeutic drug with plant extract could be a new strategy for CCA treatment. However, further study in clinical trial is required.

5. Conclusions

This study investigated the combined effects of Valencia KK4-type peanut skin ethanolic extract (KK4-PSE) with cisplatin or 5-fluorouracil (5-FU) on cholangiocarcinoma (CCA) cells in vitro and in vivo. KK4-PSE demonstrated superior antiproliferative activity against CCA cells compared to non-cancer cholangiocytes. Combinations of KK4-PSE with cisplatin or 5-FU enhanced apoptosis induction and cell cycle arrest in KKU-M213B cells. These combinations downregulated pERK1/2 and Bcl2, and decreased the Bcl2/Bax ratio, promoting apoptosis. In BALB/cAJcl-Nu/Nu xenograft models, KK4-PSE combined with 5-FU more effectively suppressed tumor growth compared to combinations with cisplatin. These findings suggest that KK4-PSE may serve as an effective synergistic agent with 5-FU and cisplatin in CCA chemotherapy. The enhanced anticancer effects observed in both in vitro and in vivo experiments highlight the potential of KK4-PSE as a complementary treatment for CCA. Further clinical investigations are warranted to validate these

promising results and explore the therapeutic potential of KK4-PSE in combination with conventional chemotherapeutic agents for CCA treatment.

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Data Availability Statement: The datasets generated and/or analysed during the study are available from the corresponding author on reasonable request.

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