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Original Research Article

Synergistic anticancer activity of *Juniperus indica* Bertol extract plus 5-fluorouracil against oral squamous cell carcinoma *in vitro*

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Abstract

Purpose: *To investigate the potential effect and underlying mechanism of Juniperus indica Bertol extract (JIB) in combination with 5-fluorouracil (5-FU) for the treatment of oral squamous cell carcinoma (OSCC).*
Methods: *The OECM-1, and OSCC cells were exposed to JIB and/or 5-FU for 24–72 h, and subsequently, cell viability, cell cycle distribution, caspase activity, apoptotic cells, and protein expression were determined using the (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide; MTT) assay. Flow cytometry, fluorometric caspase activity assay kit, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay and western blotting were carried out.*
Results: *The combination of JIB and 5-FU significantly inhibited the growth of OECM-1 cells compared to JIB or 5-FU alone ($p < 0.05$). After treatment with JIB plus 5-FU, the cell cycle was significantly impeded by downregulating levels of CKD2/cyclin A and CDK4/cyclin D1 ($p < 0.05$). Furthermore, the combination treatment triggered apoptosis through activation of caspases and the regulation of Bax/caspase 9/caspase 3. In particular, JIB significantly suppressed the regrowth of 5-FU-treated cells and diminished the development of 5-FU resistance during OSCC treatment ($p < 0.05$)*
Conclusion: *Juniperus indica Bertol extract (JIB) in combination with 5-FU synergistically inhibits cell proliferation, alters cell cycle distribution, and triggers apoptosis compared to single drug. There is need to further explore the potential use of JIB-based combination therapies to improve the clinical application of 5-FU in OSCC.*

Keywords: *Oral squamous cell carcinoma, Juniperus indica Bertol, 5-Fluorouracil, Drug combination, Anticancer*

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is a highly metastatic neoplasm that constitutes 90 % of global oral cancers [1]. In clinical practice, primary therapeutic options for OSCC typically include surgery, radiation treatment, and drug-based chemotherapy. Owing to several factors,

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especially delay in diagnosis, most patients are diagnosed with cancer at an advanced stage, resulting in a 5-year survival rate of less than 30 % [2]. Furthermore, in cases of tumor progression or recurrence, chemotherapy may serve as a means of providing palliative care or as an adjunctive therapeutic approach to surgery or radiotherapy to enhance the possibility of a cure. 5-Fluorouracil (5-FU) is one of the most effective and frequently utilized drugs for the treatment of solid tumors [3]. However, the development of drug resistance due to continuous administration and side effects caused by non-selective cytotoxicity limits its clinical application. Consequently, it is necessary to develop innovative agents or strategies to enhance the efficacy of 5-FU in treating OSCC.

Juniperus genus, which belongs to the *Cupressaceae* family, consists of approximately 68 species and 36 varieties characterized by evergreen aromatic shrubs or trees that are predominantly found in cold and temperate regions of the Northern Hemisphere [4]. Scientific research has confirmed various pharmacological effects of *Juniperus species* which include diuretic, digestive, hypoglycemic, hypolipidemic, analgesic, and hepatoprotective effects [5].

Juniperus Indica Bertol, commonly known as Black Juniper, is distributed in several countries such as Bhutan, China, India, Nepal, and Pakistan [6]. It has been demonstrated that JIB exerts cytotoxic effects against human cervical cancer, lung carcinoma, and cutaneous carcinoma [7]. Furthermore, JIB has been shown to synergistically inhibit oral cancer and melanoma cells when used in combination with cisplatin. [8,9]. However, the mechanisms underlying the synergistic effects of JIB and 5-FU remain unclear. The current study investigated the effects of JIB combined with 5-FU on cell proliferation, cell cycle progression, and apoptosis in OSCC human oral squamous cell carcinoma cells.

EXPERIMENTAL

Cell culture

Human oral squamous cell carcinoma (OSCC) cell lines, OECM-1 developed from a Taiwanese male patient by Yang and Meng [10], and SCC-25, obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). SVEC (mouse vascular endothelial cells) was obtained from ATCC. OECM-1 and SVEC cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (FBS), 0.5 mM sodium pyruvate, 15 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HEPES), and 1 % penicillin/streptomycin. The SCC-25 cells were cultured in DMEM/F12 medium (Gibco, Grand Island, NY, USA) supplemented with 10 % FBS, 0.5 mM sodium pyruvate, 15 mM HEPES, 1 % penicillin/streptomycin, and 400 ng/mL hydrocortisone. All cells were cultured in a humidified incubator with 5 % CO_2 at 37 °C.

Reagents, chemicals and antibodies

Fruits of *Juniperus Indica* Bertol was obtained from Nepal and subjected to extraction using steam distillation [9]. The JIB and 5-FU (Sigma-Aldrich, MO, USA) were prepared as 20 mg/mL stock solution in dimethylsulphoxide (DMSO) and stored at 4 °C before use. The stock solution was diluted with the medium, resulting in a final DMSO concentration of < 0.5 % for cell treatment. Antibodies against CDK4, cyclin D1, CDK2, cyclin A, Rb, p-RB, caspase 9, and caspase 3 were obtained from Santa Cruz Biotechnology (CA, USA), as well as those against Bax, PARP, and β-actin were obtained from iReal Biotechnology (Hsinchu, Taiwan).

Cytotoxicity assay

The MTT colorimetric assay was performed to estimate the inhibitory effects of JIB and 5-FU. A total of 5×10^3 cells/well were seeded in 96-well culture plates and exposed to serial concentrations of JIB (0–200 μg/mL) and 5-FU (0–20 μg/mL) for 24–72 h. Subsequently, the MTT solution (Sigma, MO, USA) was added into each well, and cells were incubated for 6–8 h. After dissolving the formazan crystals, absorbance at 550 nm was measured using a SpectraMax M5 microplate reader (Molecular Devices, San Jose, CA, USA). Cell viability was determined as optical density percentage compared to control (100 %).

Drug combination analysis

Combined effect of JIB and 5-FU was evaluated based on Chou-Talalay method and quantified using the combination index (CI) with values less than 1.0 indicating the presence of a synergistic effect between the two drugs. The IC_{50} values were obtained from MTT assay, in which cells were treated with JIB (0 – 80 μg/mL) plus 4 μg/mL 5-FU (treatment I) or 5-FU (0 – 20 μg/mL) plus 30 μg/mL JIB (treatment II) for 24 – 72 h. Finally, a corresponding CI was calculated utilizing CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA).

Cell cycle analysis

The cells were seeded into 10 cm dishes at a density of 2 × 10^6 cells, and the cells were treated with JIB (30 μg/mL), 5-FU (4 μg/mL), and JIB (30 μg/mL) plus 5-FU (4 μg/mL) for 0 – 48 h. After washing with phosphate-buffered saline (PBS), the cells were harvested with 0.05 % trypsin and stained with propidium iodide (PI) solution (Sigma, MO, USA) containing RNase (Sigma, MO, USA) and then incubated at 4 °C overnight. After filtering cells through 35 μm nylon mesh (Becton Dickinson, Franklin Lakes, NJ, USA), the cell cycle distribution was analyzed using a flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and FlowJo software (Tree Star, San Carlos, USA).

Western blotting

An equal amount of protein (20 μg) was electrophoresed and transferred to polyvinylidene fluoride (PVDF) membranes (0.2 μm, Pall Corporation, Washington, NY, USA). After blocking with 10 % skim milk at room temperature, the membranes were probed with specific primary antibodies. After washing, the blots were incubated with secondary antibody (1:1000 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 2 h. The blots were reacted with horseradish peroxidase (HRP), and immunoreactive bands were determined using a chemiluminescence detection kit (T-Pro Biotechnology, Taipei, Taiwan) and a chemiluminescence imaging analyzer (GE LAS-4000, NJ, USA).

Caspases activity

Cells were exposed to JIB (30 μg/mL), 5-FU (4 μg/mL), and JIB (30 μg/mL) plus 5-FU (4 μg/mL) for 24 and 48 h. After harvest, the cells were incubated with caspase activity detection kit (AAT Bioquest, Sunnyvale, CA, USA) for 4 h, followed by centrifugation at 1200 rpm. Subsequently, the cells were re-suspended in assay buffer and analyzed using a flow cytometer (Becton Dickinson, NJ, USA) and FlowJo software (Tree Star, San Carlos, USA).

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay

The treated cells were harvested, fixed with 10 % formalin, smeared on slides, reacted with 3 % H_2O_2 in methanol, and incubated with a penetrating solution containing 0.1 % Triton-X 100 in 0.1 % sodium citrate. Subsequently, the cells were reacted with the TUNEL kit (Roche, Basel, Switzerland) for 2 h at 37 °C, followed by counterstaining with PI. Images were captured and counted using a fluorescence microscope (ZEISS Axio Imager A2, Bremen, Germany) at ×400 magnification.

In vitro resistance assay

Cells were inoculated into 96 wells at 8 × 10^2 cells/well and continuously exposed to JIB (30 μg/mL), 5-FU (4 μg/mL), and JIB (30 μg/mL) plus 5-FU (4 μg/mL) for 5, 9, and 16 days. Treated cells were fixed with -20 °C methanol and stained with 0.1 % crystal violet. The images were photographed using AlphaImager HP (Alpha Innotech, San Leandro, CA) under white light. After adding 10 % acetic acid, the absorbance at 590 nm was measured using a SpectraMax M5 microplate reader (Molecular Devices, San Jose, CA, USA).

Statistical analysis

Data were expressed in mean ± standard deviation (SD; n = 3). Unpaired Student's t-test or one-way analysis of variance (ANOVA) was employed to identify differences among groups. $P < 0.05$ was considered statistically significant.

RESULTS

Juniperus indica Bertol extract (JIB) plus 5-FU exhibited synergistic effects on growth inhibition in OECM-1 cells

Results revealed that 50 μg/mL of JIB continuously inhibited cell viabilities of OSCC cells from 24 to 72 h and the inhibition rates exceeded 70 % (Figure 1). In normal cells, JIB (50 μg/mL) caused approximately 15 – 20 % inhibition rates from 24 to 72 h in SVEC cells. These results indicate that JIB was more cytotoxic to OSCC cells compared to normal cells. The IC_{50} values of JIB at 72 h in OSCC cells were approximately 2 folds lower than those in normal cells (Table 1). However, the IC_{50} values of 5-FU in OSCC cells (2.11 ± 0.05 to 2.80 ± 0.20 μg/mL) were higher than normal cells (0.65 ± 0.03 μg/mL) at 72 h (Table 1).

Cell viability

The OECM-1 cells were treated with JIB alone, 5-FU alone, JIB plus 5-FU, and 5-FU plus JIB for 24, 48, and 72 h, and cell viability was evaluated using MTT assay. The results revealed that cell viability was significantly decreased in combination groups compared to when the drugs were used alone.

Table 1: The IC_{50} values of JIB and 5-FU-treated OSCC and normal cells

Cell lines	JIB			5-FU		
	24 h	48 h	72 h	24 h	48 h	72 h
OECM-1	41.21±1.24	41.69±1.59	39.41±1.44	16.33±0.19	8.40±0.36	2.80±0.20
SCC-25	41.76±1.06	39.56±0.47	39.08±1.05	21.47±0.98	12.89±2.21	2.11±0.05
SVEC	72.58±1.45	73.32±1.48	81.86±0.80	17.16±6.23	1.00±0.04	0.65±0.03

Note: IC_{50} was the concentration required for 50 % growth inhibition. All values were presented as means ± SD (µg/mL; n = 3)

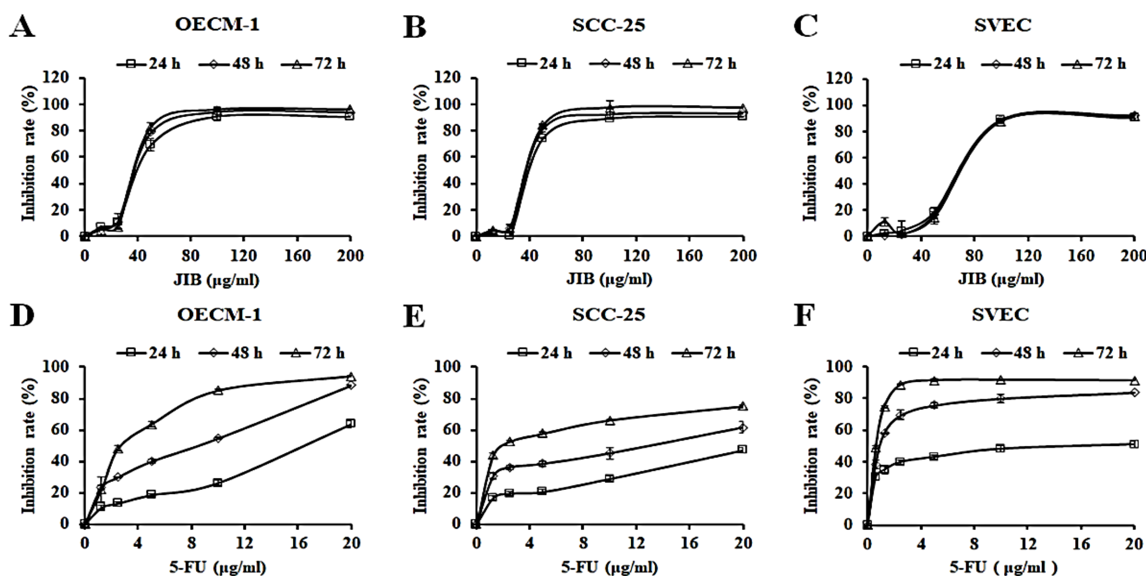

Figure 1: Effect of JIB and 5-FU on cell viability in OSCC and normal cells. The cell viabilities of OECM-1 (A), SCC-25 (B), and SVEC (C) cells exposed to JIB (0–200 µg/mL), as well as OECM-1 (D), SCC-25 (E), and SVEC (F) cells exposed to 5-FU (0–20 µg/mL) for 24–72 h, were determined using MTT assay

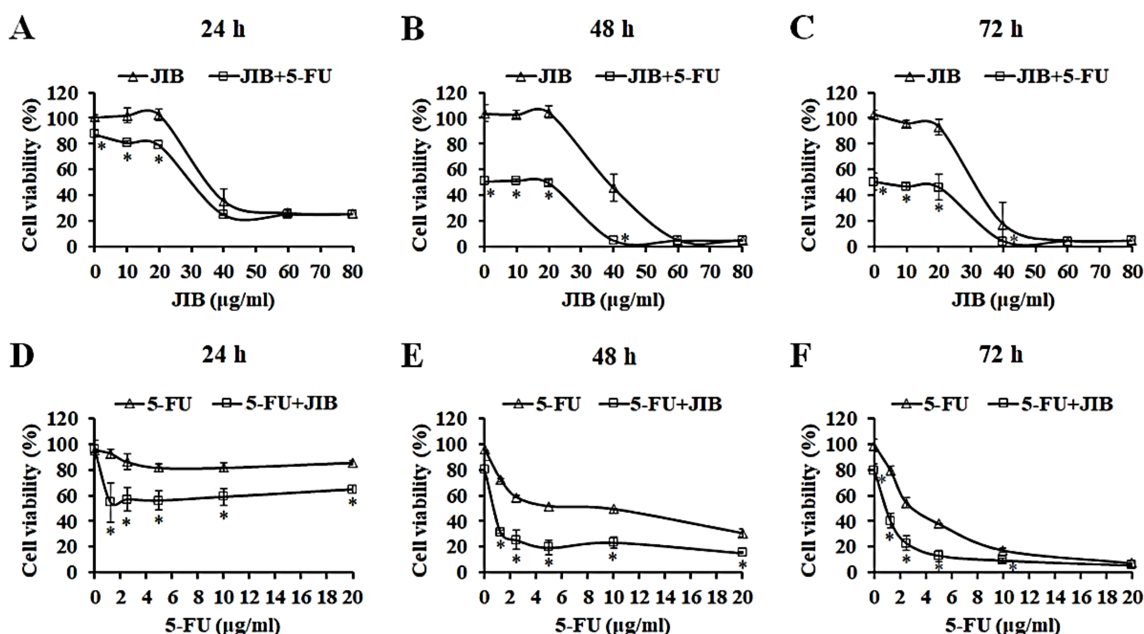

Figure 2: JIB combined with 5-FU synergistically inhibited the growth of OECM-1 cells. Cells were treated with (A–C) JIB (0–80 µg/mL) plus 5-FU (4 µg/mL), (D–F) 5-FU (0–20 µg/mL) plus JIB (30 µg/mL) for 24–72 h, and cell viability was determined using an MTT assay

To evaluate the combination effects of JIB and 5-FU, the combination index (CI) was estimated, with an index < 1 indicating a synergistic combination. The combination index was validated as 1.00, 0.53, and 0.31 at 24, 48, and 72 h, respectively. These results showed that JIB combined with 5-FU effectively repressed cell growth in a time-dependent manner and exerted synergistic effects on growth inhibition in OECM-1 cells (Figure 2).

Effects of JIB plus 5-FU on cell cycle progression in OECM-1 cells

To further study the mechanism of growth suppression by JIB plus 5-FU, cells were treated with JIB, 5-FU, and JIB plus 5-FU and harvested cells were used in flow cytometry analysis. Results revealed that JIB alone induced the accumulation of cell population in the G0/G1 phase at 48 h while reducing the cell populations in the S and G_2/M phases (Figure 3). Also, 5-FU alone mainly blocks the cell cycle at the S phase accompanied by reduction of G0/G1 and G_2/M phase. Treatment with JIB plus 5-FU group significantly impeded S phase at 6–24 h and obstructed at G0/G1 phase at 48 h ($p < 0.05$). In addition, results of western blotting showed that JIB or 5-FU alone decreased CDK4/cyclin D1, CDK2/cyclin A, or p-Rb expression, inducing G0/G1 or S cell cycle arrest. Furthermore, JIB plus 5-FU significantly decreased expression of these proteins ($p < 0.05$) to trigger the G0/G1 or S phase arresting at different time points, suggesting that JIB and 5-FU exert their pharmacological effects individually to block cell cycle progression, leading to the inhibition of cell proliferation.

JIB plus 5-FU triggered apoptosis via activation of the intrinsic pathway

The percentage of cells in the subG1 phase was identified during the cell cycle analysis. The drug alone and the combination caused < 20 % of the subG1 phase within 24 h, and JIB plus 5-FU significantly triggered the highest percentage of subG1 phase at 48 h ($p < 0.05$; Figure 4 A).

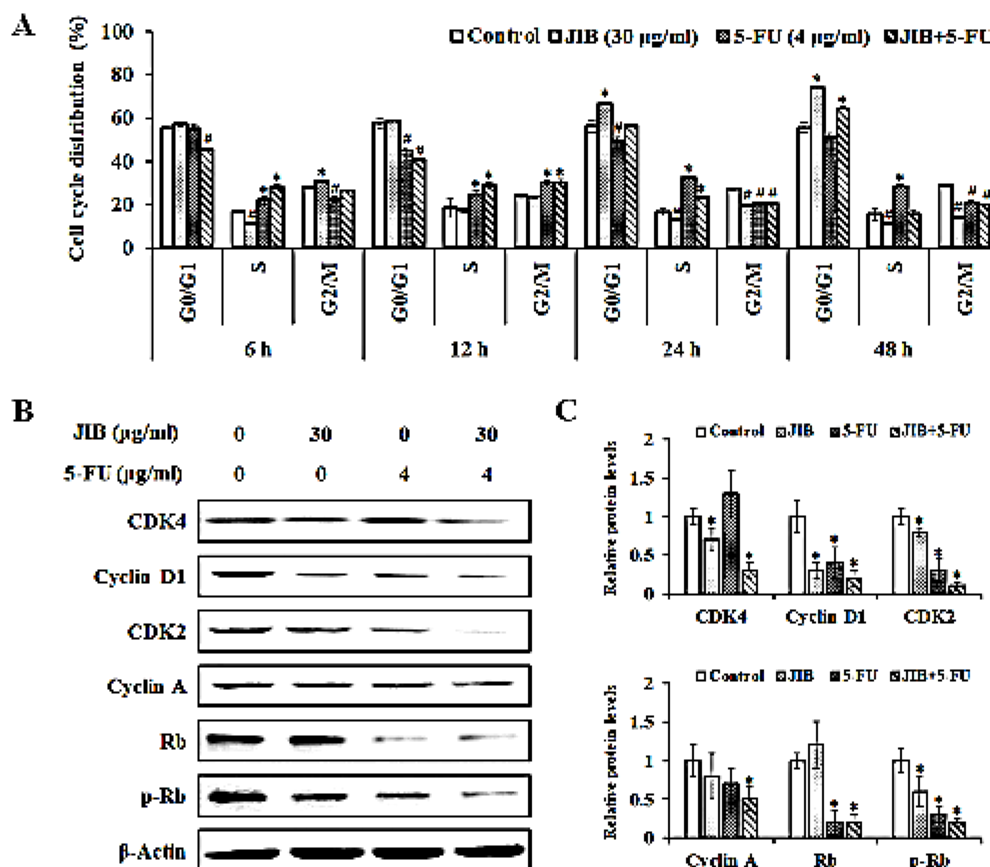

Figure 3: Effect of JIB plus 5-FU on cell cycle progression in OECM-1 cells. (A) Cell proportion in different cell cycle phases in OECM-1 cells exposed to JIB (30 μg/mL), 5-FU (4 μg/mL) or JIB plus 5-FU, determined by flow cytometry. (B) Western blotting of CDK4, cyclin D1, CDK2, cyclin A, Rb, p-Rb, and β-actin protein expression of OECM-1 cells. (C) Quantitative analysis of protein expression adjusted with β-actin. *$P < 0.05$ vs control with a significant increase. $^{\#}P < 0.05$ vs control with a significant decrease

To confirm the effect of JIB plus 5-FU on cancer cell apoptosis, caspase activity and TUNEL assays were performed. The results showed that JIB, 5-FU, and combination induced about 260 %, 230 %, and 310 % caspase activity at 48 h (Figure 4 B), respectively. Furthermore, JIB and 5-FU alone induced approximately 40 % and 30 % TUNEL-positive cells, and JIB plus 5-FU enhanced apoptosis, which reached 50 % TUNEL-positive cells (Figure 4 C and D). These results suggest that JIB and 5-FU significantly and synergistically induced apoptosis in OSCC cells, resulting in cell death. Also, JIB or 5-FU alone decreased levels of pro-caspase 9 and 3 while increasing the levels of Bax, cleaved-caspase 9, 3, and cleaved PARP (Figure 4 E and F). Furthermore, JIB plus 5-FU significantly increased the expression of Bax and activation of caspases and PARP. These results confirmed that JIB plus 5-FU potentiated OECM-1 cell death by activating intrinsic apoptotic pathway.

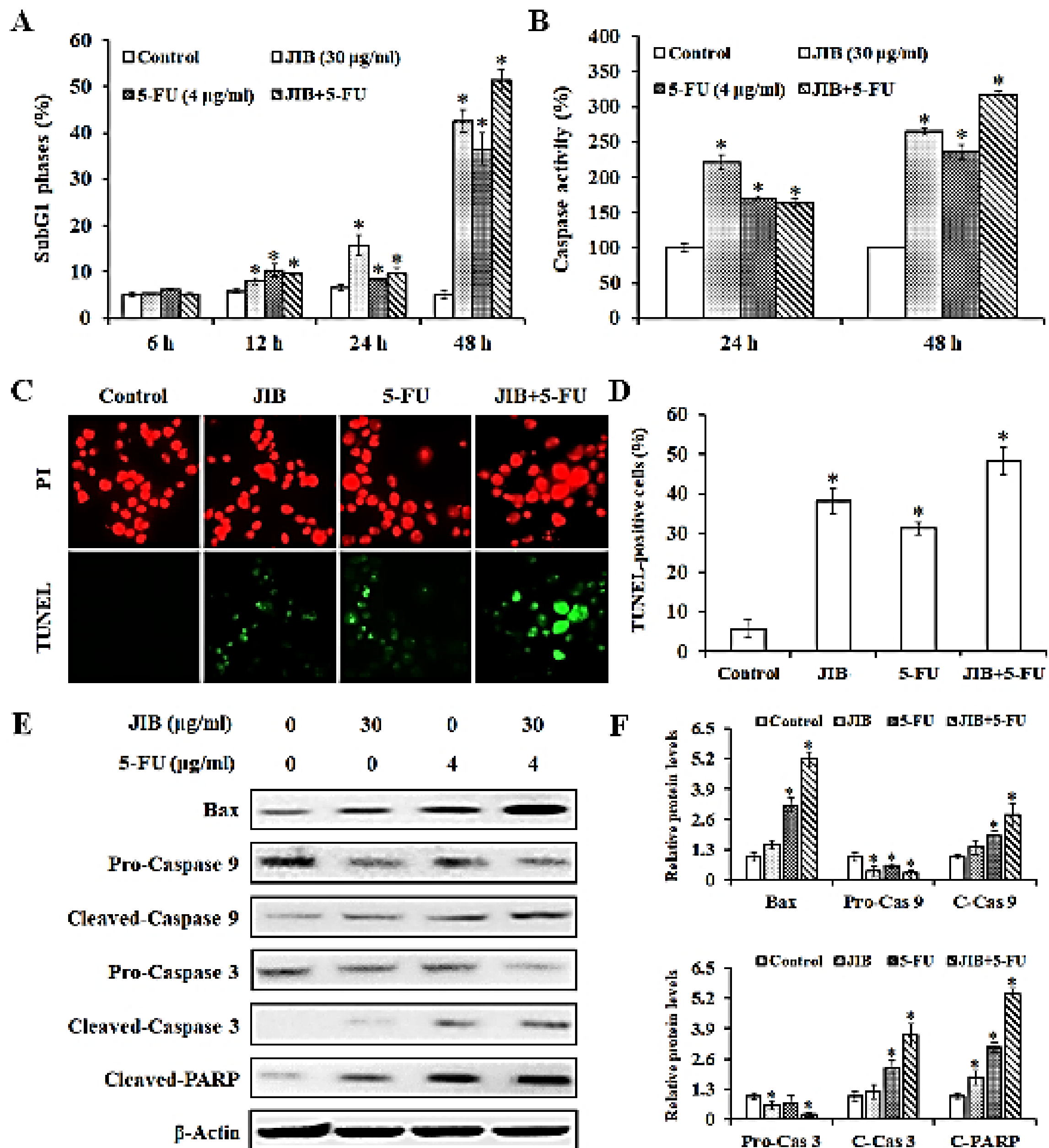

Figure 4: Combination of JIB and 5-FU enhanced apoptosis of OECM-1 cells. Percentages of subG1 phase (A) and caspases activity (B) in cells exposed to JIB (30 µg/mL), 5-FU (4 µg/mL) or JIB plus 5-FU by flow cytometry analysis. (C) Apoptosis was determined by the TUNEL assay and observed by fluorescence microscopy. (D) Quantitative analysis of TUNEL-positive cells. (E) Levels of proteins in cells were measured by western blotting. (F) Density of a specific band was quantified and normalized. *P < 0.05 vs control

JIB attenuated the development of resistance to 5-FU in OECM-1 cells

To determine the effect of JIB on the development of resistance during 5-FU treatment, cells were incubated in a medium containing 5-FU with or without JIB for 5, 9, and 16 days. The cells were inhibited by 5-FU treatment on day 5 and showed a continuous tendency to regrow from day 5 to 16, indicating that OECM-1 cells might become resistant to 5-FU (Figure 5). However, the groups treated with 5-FU and JIB showed reduced cell regrowth. These results demonstrated that JIB attenuated the development of resistance to 5-FU during OSCC treatment.

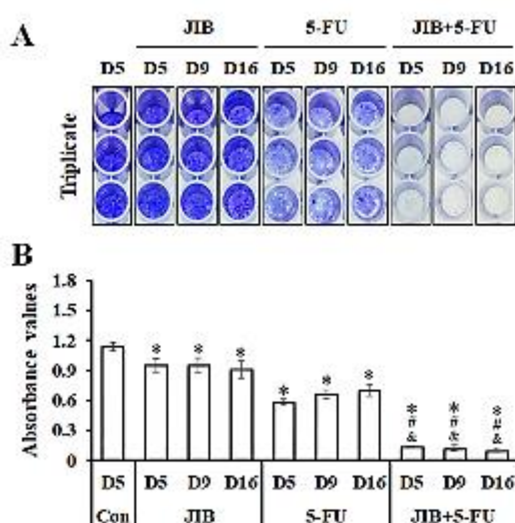

Figure 5: Effect of JIB on development of resistance to 5-FU in OECM-1 cells. (A) After long-term culture with JIB (30 μg/mL) and/or 5-FU (4 μg/mL) for 5, 9, and 16 days. The treated cells were fixed, stained, and photographed. (B) The optical density measurement was conducted at 560 nm using a microplate reader. $^{*}P < 0.05$ vs control, $^{\#}p < 0.05$ vs JIB. $^{\&}P < 0.05$ vs 5-FU

DISCUSSION

Numerous studies have demonstrated that cell cycle disruption is associated with the development of various tumors, indicating that controlling cell cycle progression is an effective strategy for cancer treatment [8,11,12]. The main components of JIB (molecular weights < 500 Da) included δ-3-Carene (46.43 %), δ-cadinene (8.73 %), D-Limonene (8.31 %), α-Pinene (7.92 %), β-Cubebene (5.11 %) and others, which have been determined by gas chromatography-mass spectrometry (GC-MS) [9]. Cell cycle arrest serves as a protective mechanism that enables tumor cells to undergo DNA repair or trigger the apoptotic pathway, resulting in cell death [11].

It is widely recognized that the cell cycle is primarily categorized into G0/G1, S, G2, and M stages and is mediated by various cyclin-dependent kinases (CDKs) along with their functional cell cycle protein chaperones [12]. These OSCC cells exposed to JIB and 5-FU were blocked in the G0/G1 and S phases of the cell cycle respectively, within 48 h. The synergistic intervention resulted in cell cycle arrest in the S phase at 6–24 h, followed by arrest in the G0/G1 phase at 48 h, indicating the distinct temporal regulatory effects of JIB in combination with 5-FU on cell cycle. The CDKs/cyclins, a group of heterodimeric kinases, have significant functions in facilitating cell cycle progression through various phases such as G0/G1 (CDK4/6 and cyclin D), S (CDK2 and cyclin A), and G2/M phases (CDK1 and cyclin B) [12]. Phosphorylated retinoblastoma protein (pRb) functions as a crucial regulator of the cell cycle, facilitating the transition from G1 phase to S phase [12]. The findings indicated that the combined administration of JIB and 5-FU led to significant reduction in levels of CDK4/cyclin D1, CDK2/cyclin A, and p-Rb, thereby inducing cell cycle arrest in G0/G1 or S phase at varying time intervals.

Programmed cell death (apoptosis) plays an important role in cancer therapy and is triggered by many physiological and pathological stimuli, including chemotherapy and radiotherapy [13]. It is characterized by the accumulation of subG1 cells, anoikis, chromatin condensation, caspase activation, DNA fragmentation, and formation of apoptotic bodies [13]. Compared to the drug alone, co-treatment with JIB and 5-FU elevated the number of cells in subG1 phase and increased caspase activity in OECM-1 cells. Furthermore, the TUNEL results confirmed that the combination of JIB and 5-FU had a significantly better effect in inducing apoptosis in OECM-1 cells compared to single drug. The intrinsic apoptotic pathway is regulated by Bax/Bcl-xL proteins, and an increase in this ratio promotes the formation of pores in the outer mitochondrial membrane and the release of cytochrome C [14]. Cytochrome C forms an apoptosome with Apaf-1 and inactive caspase 9, which activates the caspase cascade and causes PARP cleavage, thus promoting apoptosis [14]. Compared with JIB or 5-FU alone, the combination treatment significantly elevated levels of Bax and enhanced activation of caspase 9, caspase 3, and PARP suggesting that JIB combined with 5-FU induces apoptosis by activating the mitochondria-mediated caspase family. 5-Fluorouracil (5-FU) is a drug that acts by blocking the function of thymidylate synthase to suppress DNA replication and has been

utilized alone or in combination with other chemotherapeutic agents for the treatment of a variety of malignancies [3]. Resistance to 5-FU-based chemotherapy is commonly observed in patients with recurrent or metastatic OSCC, leading to unfavorable prognosis [15]. Thus, 5-FU is frequently used in combination with other anticancer agents, including cisplatin and docetaxel, to enhance the overall survival rate of patients experiencing tumor recurrence or metastasis [16]. However, the use of a combined treatment approach has been associated with a significant increase in adverse effects such as oral mucositis, shallow immunity, fatigue, diarrhea, anemia, and nausea [3]. Therefore, overcoming drug resistance and minimizing adverse reactions are important for enhancing the prognosis of patients with OSCC.

Owing to the multi-targeting capabilities and low toxicity, natural compounds from herbs are widely regarded as promising candidates for the suppression of malignant tumors or as adjuvants combined with conventional drugs [17]. Using combination therapies instead of a single drug has shown many benefits, such as reduced drug concentration and toxicity, better efficacy, multiple targeting pathways, increased cellular sensitivity to treatment, and preventing the development of drug resistance [18]. The findings of this study indicated that the combination of JIB and 5-FU synergistically enhanced the growth inhibition of OSCC cells. Moreover, JIB significantly inhibited the regrowth of 5-FU-treated cells, suggesting that it attenuates the development of OSCC cells to 5-FU. This suggests that JIB has the potential to be used in the treatment of OSCC and sensitize resistant cells to 5-FU, justifying its broad clinical applications against cancers.

CONCLUSION

Juniperus indica Bertol extract (JIB) in combination with 5-fluorouracil (5-FU) inhibits cell viabilities, induces cell cycle arrest, and apoptosis of OSCC cells, and attenuates the development of resistance to 5-FU with reduced 5-FU dose. The findings suggest that researchers may explore the potential use of JIB-based combination therapies to improve the clinical application of 5-FU in OSCC.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Ju-Huei Chien and Nu-Man Tsai conceived the study and provided the funding; Ju-Huei Chien, Kai-Fu Chang and Hung-Hsiu Liao performed the experiments and analyzed data; Xiao-Fan Huang conducted statistical analysis; Ju-Huei Chien, Kai-Fu Chang and Xiao-Fan Huang wrote the manuscript. Nu-Man Tsai modified the manuscript. Nu-Man Tsai served as supervisor. Ju-Huei Chien and Kai-Fu Chang contributed equally to this work. All authors reviewed the manuscript and approved the submitted version.

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