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Original Article

Ethanol Extract of *Croton kongensis* Promotes Cell Cycle Arrest and Consequently Inhibits Anchorage-Independent Growth of Cervical Cancer Hela Cells

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Abstract: Extracts from *Croton kongensis (C. kongensis)* exhibit anticancer activities on various cancers. However, there is no research conducted to investigate the effects of *C. kongensis* extracts on cervical cancer as well as on zebrafish. In this study, we demonstrated that *C. kongensis* ethanol extract expressed high toxicity to cervical cancer Hela cells with an IC50 dose of 20.4 µg/mL and to zebrafish embryos with malformations, lethality and hatching inhibition at 72-hpf at effective dose of 125 µg/mL. Interestingly, treatment with *C. kongensis* ethanol extract caused cell cycle arrest at the G2 phase. Particularly, percentages of *C. kongensis* ethanol extract-treated cells in G1, S, G2/M were 70%, 6% and 23%, while percentages of control cells in G1, S, G2/M were 65%, 15% and 18%, respectively. Consistent with cell cycle arrest, the expressions of *CDKN1A*, *CDNK2A* and *p53* in *C. kongensis* ethanol extract-treated cells were up-regulated 2.0-, 1.65- and 1.8-fold, respectively. Significantly, treatment with *C. kongensis* ethanol extract inhibited anchorage-independent growth of Hela cells; the number of colonies formed in soft-agar of *C. kongensis* ethanol extract-treated cells was only one-fourth of that of control cells. In conclusion, we suggest that *C. kongensis* ethanol extract could be used as a traditional medicine for treatment of cervical cancer.

Keywords: Croton kongensis, Hela cells, Zebrafish, Cell cycle arrest, Anchorage-independent growth.

1. Introduction

Many endemic plants of Vietnam have been used as traditional medicines for thousands of years [1-4].

Recently, cervical cancer accounts for about 7.5% of all female cancer deaths world-wide; of those deaths, approximately 90% occurred in the developing countries with weakness of public health [5, 6]. In Vietnam, there are more than 5000 new cases and about 3000 deaths each year [5]. *C. kongensis* is one of the endemic medicinal plants displaying different biological activities including antioxidant, antibacterial, anti-inflammatory, and anticancer

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[7-11]. Previous studies demonstrated that the products originating from *C. kongensis* could induce the death of liver cancer cells [9, 11], colorectal cancer cell [10], and other cancer cells [7] by activating the apoptosis pathway. However, the molecular mechanisms underlying these actions have not been thoroughly investigated. In addition, so far, no study addressed the anticancer activity of *C. kongensis* on cervical cancer cells or its putative toxicity on zebrafish embryos, an effective and popular *invivo* experimental model for drug screening [12, 13]. Therefore, in this study, we examined the biological and pharmaceutical properties, focusing on cell-targeted toxicity, organism-targeted toxicity (zebrafish) and anticancer activity on cervical cancer *invitro*, of the ethanol extract of the medicinal plant *C. kongensis* (CK-EE), an endemic plant of Vietnam and to be used as traditional medicine for a long period.

2. Materials and Methods

2.1. Preparation of Plant Extracts

Medicinal plant was collected from the North-Western mountainous areas of Vietnam in the Dry season in 2018-2019. They were identified, coded and placed at the Museum of Biology, Faculty of Biology, VNU University of Science, Vietnam National University, Hanoi, Vietnam. The whole plant was used for extraction. The extraction protocol was described in previous study [14]. The obtained extract was dissolved in DMSO for further analysis and exposure on cells and zebrafish embryos.

2.2. Cell Culture

Cervical cancer Hela (ATCC: CCL-2) cell line was provided by Health Science Research Resources Bank, Japan and cultured in DMEM, supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin at 37 ^{o}C in 5% CO_2.

2.3. Zebrafish Embryos Toxicity Test

Adult zebrafish wild type strain AB (*Danio rerio*) [13] were cultured in glass rectangular pools measuring 40x50x30 (cm^3). Fish were reared in a Techniplast recirculating system under 14:10-h light/dark photocycle. Healthy embryos that showed normal cleavage were distributed into 6-well plates at 25 embryos/well for the embryotoxicity tests. Fish embryo acute toxicity was determined according guidelines of OECD (OECD 2002) [12].

2.4. Cellular Toxicity MTT Assay

MTT (Sigma Chemical Co., St. Louis, MO) assays were carried out as previously described in [15]. A purple formazan product produced by viable cells on the 96-well plate was read using an ELISA reader at 570 nm for absorbance density values and the percentage of viable cells was calculated.

2.5. Cell Cycle Analysis

Cells were stained with propidium iodide (PI) (BD Biosciences) to measure the DNA content by FACSCanto system [16].

2.6. Real-time PCR Analysis

Total RNA was isolated from cells using the RNA Isolation Kit (Thermo Scientific, USA), according to the protocol of the Kit. Then cDNA was synthesized from total RNA by reverse transcription, according to the protocol of the cDNA Synthesis Kit (Thermo Scientific, USA). Real-time quantitative RT-PCR with SYBR green was performed using power SYBR1 Green PCR master mix in a LightCycler@96 Instrument.

2.7. Colony Formation Assay

A colony formation assay was performed to assess the development of tumor in vitro, according to the previous report [17]. Colonies on the soft agar dish exceeding 50 µm in

diameter were counted and presented as an activity of anchorage-independent growth.

2.8. Statistical Analysis

Statistical analysis in this study was performed as previously described [14]. Results from three independent experiments in each group were statistically analyzed by a Student's t-test. The SPSS (version 18) software package (SPSS JapanInc.) was used for statistical analyses, and the significance level was set to $p < 0.05$.

3. Results

3.1. CK-EE Displayed the Highest Cytotoxicity on Cervical Cancer Hela Cells

The ethanol extract of *C. kongensis* (CK-EE) displayed the quite high toxic activity on Hela cells, with a concentration for fifty percent inhibiton (IC50) of 20.4 µg/mL; While paclitaxel (taxol), a drug approved by FDA for cancer treatment and used as a positive control, had its value at 13.5 ng/mL on Hela cells (Data not shown). In general, an agent with IC50 value in the range of 0-50 µg/mL is listed as a toxicant; thus the CK-EE was used for further experiments.

3.2. Toxicity of CK-EE on Zebrafish Embryos

The effects of CK-EE on the development of zebrafish embryos at different stages were investigated. CK-EE at different concentrations of 0, 7.8, 15.6, 31.3, 62.5, 125, 250, 500, 1000 and 2000 (mg/L) were applied. At each time point (24, 48, 72, and 96 hours post-fertilization (hpf)), the presence of developmental malformations, lethality and specific endpoint of hatching of the zebrafish embryos (larvae) were assessed (Figure 1A, B, C). At concentrations up to 62.5 mg/L, CK-EE had almost no effect on the development of defects or death of zebrafish larvae with malformation of 5%, lethality of 2%, and hatching efficiency of 90% (Figure 1A, B, C). However, at 125 mg/L a big increase in developmental malformations with tail truncation defect, and heart/yolk sac oedema on 60% larvae at 72 hpf and 96 hpf (Figure 1 E, F); while normal development was observed in the figure 1D. Similarly, at dose of 125 mg/L, an increase in embryo and larva death was observed with lethality percentages of 10%, 35% and 44% at 48 hpf, 72 hpf and 96 hpf, respectively (Figure 1B). At high concentration of 250 mg/L, CK-EE expressed its very toxicity to zebrafish embryos because of the developmental defects of 65% at 72 hpf and 98% at 96 hpf, (Figure 1A, E, F) and death rates of 85% at 72 hpf and 98% at 96 hpf (figure 1B, G). In addition, we monitored the effect of CK-EE at high concentrations on the hatching percentage of the embryos. Normally, the zebrafish hatching takes place between 48 and 72hpf, as illustrated in the control fish. Specifically, at 250 mg/L CK-EE strongly decreased the hatching percentage of zebrafish embryos to around 10% at both 72 hpf and 96 hpf (Figure 1C).

3.3. The CK-EE Induced Hela Cell Cycle Arrest at the G2 Phase

Hela cells were treated with CK-EE or paclitaxel (positive control) at the IC50 doses for 24 hr. The cells were stained with propidium iodide (PI). DNA in the nucleus of cells was bound with PI. PI-bound DNA contents were measured by flow cytometer FACs CANTO. The proportion of cells in G1, S, and G2/M phases were determined and presented as the DNA content histograms in the Figure 3. The percentages of control Hela cells distributed in the G1, S, and G2/M phases were around 64%, 15% and 18%, respectively (Figure 2A), while the percentages of CK-EE-treated Hela cells in the G1, S, and G2/M phases were 70%, 6% and 23%, respectively. These results indicate that CK-EE significantly induced cell cycle arrest in the G2/M phase (Figure 2B). In similar manner, paclitaxel arrested the cell cycle at the G2/M phase, as indicated by the percentages of cells distributed in the G1, S, and G2/M phases of 36%, 21% and 26%, respectively (Figure 2C); however, paclitaxel expressed its higher ability in inducing cell apoptosis with 17% cells entering apoptotic phase, compared with CK-EE.

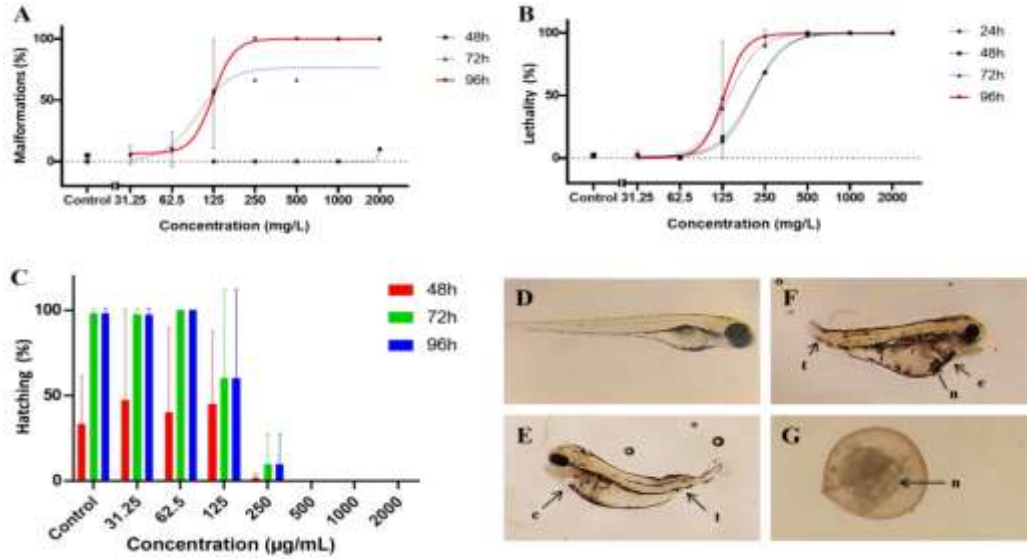

Figure 1. Effect of CK-EE on development and survival of zebrafish embryos at different time points. The effects of CK-EE at various doses (0, 7.8, 15.6, 31.3, 62.5, 125, 250, 500, 1000 and 2000 (mg/L)) on developmental defects (A), lethality (B), and hatching (C) of zebrafish embryos at 48, 72 and 96 hpf, respectively are presented as dose-response curves. Representative images of 96hpf zebrafish larvae presenting various developmental defects upon CK-EE exposure at different doses; (D) 0 mg/L (negative control), (E) 62.5 mg/L, (F) 125 mg/L, (G) 250 mg/L. Illustrated defects on larvae include yolk-sac oedema (e), truncate defect (t), and necrosis (n) as indicated with arrows.

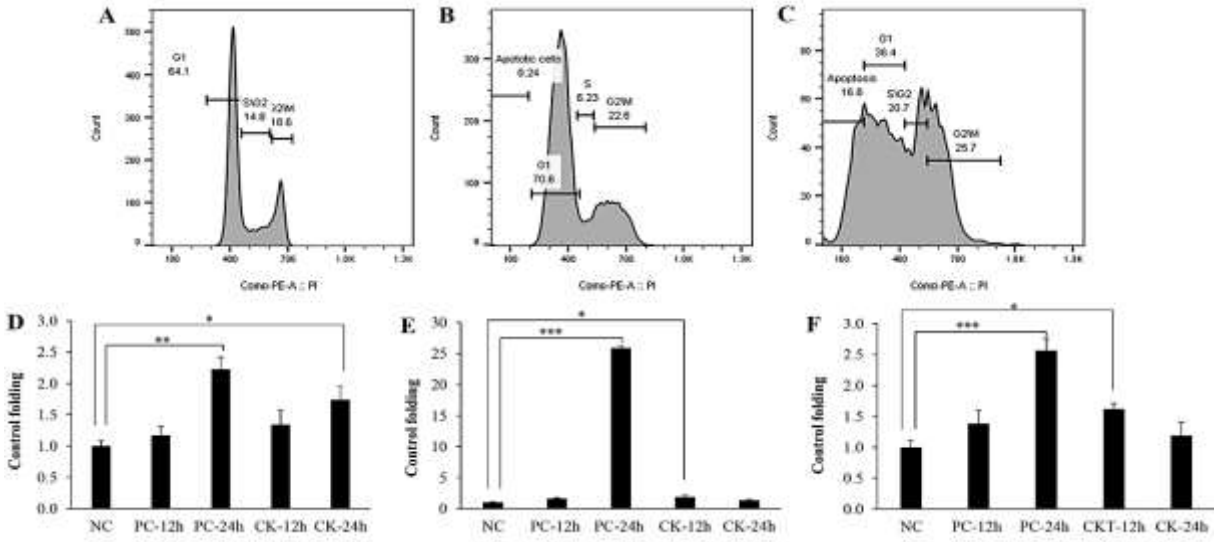

Figure 2. Hela cells were treated CK-EE at IC50 dose of 20.4 mg/L or paclitaxel (positive control) at IC50 dose of 13.5 µg/L for 24 hr before collecting, fixing, staining with propidium iodide (PI) and subjecting to flow cytometry system to measure of cellular DNA content. Representative data sets of cell cycle pattern of control Hela cells (A), CK-EE treatment (B), and paclitaxel treatment (C) were presented. Transcript levels of p53 (D), CDKN1A (E), and CDKN2A (F) in Hela cells treated with CK-EE and paclitaxel for 12 h and 24 h. *, ** and *** significant differences with P values < 0.05, < 0.01 and < 0.001, respectively. TBP was used as internal control gene. NC: negative control, PC: positive control.

Additionally, to study molecular mechanisms underlying the cell cycle arrest, the transcript levels of p53, *CDKN1A (encode for* $p21^{CIP1}$*), and CDKN2A (encode for* $p16^{INK4A}$ and $p14^{ARF}$*)* genes that encode for corresponding p53, p21 and p16 proteins, which in turn would regulate the cell cycle were investigated. Importantly, treatment with either CK-EE or paclitaxel significantly induced the transcript levels of *p53*, *CDKN1A* and *CDKN2A* in Hela cells (Figure 2D, E, F). Interestingly, although CK-EE and paclitaxel promoted the transcript levels of *CDKN1A* and *CDKN2A*, CK-EE expressed its effectiveness at earlier time of 12 h, while paclitaxel presented its effectiveness at later time of 24 h. In consistent with G2-phase retardation, the transcript levels of *p53*, *CDKN1A*, and *CDKN2A* in the CK-EE-treated cells were up-regulated 2.0-, 1.65-fold at 24 h and 1.80-fold, compared with those in the control cells, respectively (Figure 2D, E, F). In the other hand, paclitaxel strongly increased the expression levels of both *CDKN1A* and *CDKN2A* genes at the time of 24 h of 2.2-, 26- and 2.57-fold, respectively (Figure 2D, E, F).

3.4. CKbilit-EE Inhibited Anchorage-independent Growth A y of Hela Cells

Anchorage-independent growth ability of Hela cells was assessed by performing a colony formation assay. The results showed that exposure to CK-EE at IC50 dose of 20.4 mg/mL caused a reduction of colony numbers as well as decreased the size of colonies of cells in soft agar (Figure 3C, D) compared with control cells (Figure 3A, B). Particularly, number of colonies in case of control cells was 4.2-fold higher than that in CK-EE-treated cells (Figure 3E). Meanwhile, treatment with paclitaxel at the IC50 dose of 13.5 ng/mL totally inhibited colony formation of cells (Figure 3E).

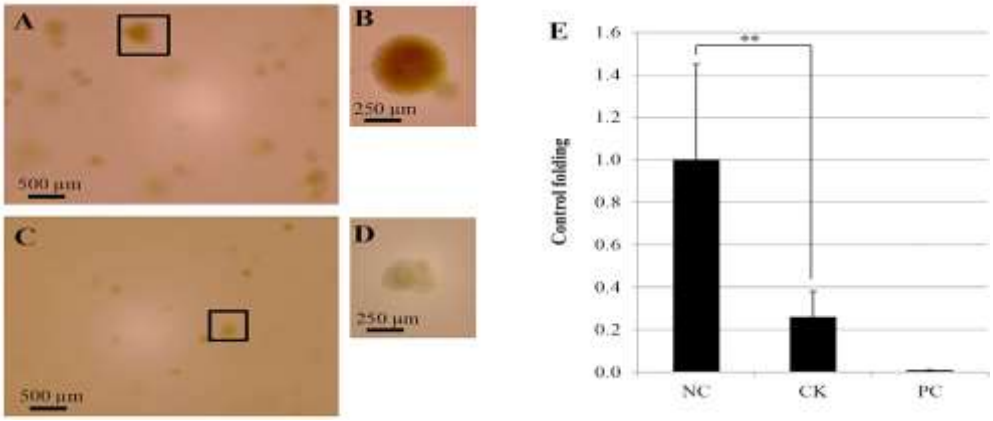

Figure 3. Anchorage-independent growth of Hela cells. Colonies formed on the soft agar in case of control cells (A and B) and CK-EE-treated cells (C and D) at magnifications of 2X (A and C) and 5X (B and D). The differences in colony formation ability on soft agar of Hela cells are presented in a graph (E). ** significant differences with P values < 0.01 by Student' ttest.

4. Discussion

Previous studies showed that diterpenoids extracted from *C. kongensis* had many biological activities *in vitro*, including antioxidant, anti-inflammatory, antibacterial, and anticancer [1-4]. Particularly, the anticancer activity of these diterpenoids had been presented on liver cancer cells and colorectal cancer cells by inducing apoptosis via activating related signaling pathways [9-11]. However, so far, the effects of CK-EE on cervical cancer cells as well as on zebrafish (a living organism) have not been examined yet.

We, for the first time, demonstrated that CK-EE promoted Hela cell cycle arrest of Hela cells at the G2/M phase. In consistent with this fact, the expression levels of genes encoding proteins involved in regulation of the cell cycle, including *p53*, *CDKN1A* ($p21^{CIP1}$), and *CDKN2A* ($p16^{INK}$) were examined. Our results showed transcript level of *p53*, *CDKN1A* ($p21^{CIP1}$), and *CDKN2A* ($p16^{INK}$) were significantly up-regulated in Hela cells treated with CK-EE. As well known, *p53* is generally involved in cell cycle arrest induced by DNA damage [5], and can also stimulate the transcription of *CDKN1A and CDKN2A* [6, 18], which responses for G1/S phase and G2/M phase of cell cycle [19, 20].

Anchorage-independent growth is a hallmark of cancer cells. It represents for the proliferative ability of cancer cells in the absence of adhesion to extracellular matrix proteins and correlates closely with tumorigenesis [21, 22]. Thus, we assessed the anchorage-independent growth ability of cancer cell by conducting colony formation. We found that CK-EE significantly inhibited both number and size of colonies of Hela cells formed in soft agar. The ability in inhibiting the colony formation of cancer cells is one of the most important effects of drug for cancer treatment, especially for cancers with solid tumors [21, 22]. And, in this study, we revealed the ability of CK-EE in decreasing the anchorage-independent growth of Hela cells.

Further, we demonstrated that CK-EE at the concentrations below 65 mg/L had almost no effect on zebrafish embryo development and survival. However, at higher concentrations starting at 125 mg/L, CK-EE strongly induced developmental defects and death of the larvae. These data suggest that further investigations about the effect of CK-EE *in vivo* are needed before applying on human beings.

5. Conclusions

Conclusively, we suggest that CK-EE can be used as a traditional medicine for treatment of cervical cancer; however, the epidemiological data and pharmacokinetic studies should be addressed. Moreover, because of the ability of CK-EE in causing hatching inhibition, the malformations and death of zebrafish larvae, it should be cautious with applying dose.

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