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Comparison of Efficacy of Crocodile Blood Extract against Inhibition of Cell Viability in Hepatocellular Carcinoma and Human Cholangiocarcinoma Cell Lines

Rassameepen Phonarknguen[1] **Kanjana Assawasuparerk**[2] **and Thanakorn Rawangchue**[3]

[1]*The Monitoring and Surveillance Center for Zoonotic Disease in Wildlife and Exotic Animals,*

[2]*Department of Pre-Clinical and Applied Animal Science,*

[3]*The Center for Veterinary Diagnosis, Faculty of Veterinary Science, Mahidol University, Phutthamonthon, Nakhon Pathom 73170, Thailand*

ABSTRACT Crocodile *(Croccodylus siamensis)* blood extracts (CE) consisting of natural active peptides, which are used in cancer therapy. This study investigated the activity of CE and their effects on cell viability of hepatocellular carcinoma (HepG2) and human cholangiocarcinoma (HuCCA) cell lines by MTT assay. A range from 100 to 1,600 μg/mL of CE decreased the viability of HepG2 and HuCCA cells as compared with control group, while CE statistically reduced cell viability of HepG2 more than HuCCA cells ($p<0.001$). The 50% inhibitory concentration (IC_{50}) of CE on HepG2 and HuCCA cells dose suggested to approximately 120.36 μg/mL and 846.28 μg/mL, respectively. The treatment of CE could induce nuclear morphological changes apoptotic cells, nuclear chromatin condensation and fragmentation by Hoechst 33342 staining. In conclusion, CE could inhibit cell viability and induce apoptosis in HepG2 and HuCCA cells and it could reduce cell viability of HepG2 cells more than HuCCA cells. This study indicated that CE had more promising treatment of hepatocellular carcinoma than cholangiocarcinoma *in vitro* study.

Keywords: Crocodile blood extract, Cell viability, Apoptosis, Hepatocellular carcinoma, Cholangiocarcinoma

Corresponding author E-mail: thanakhorn.raw@mahidol.ac.th

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Introduction

Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) are two main histological types of liver cancer.[1] Liver cancer originated from either the liver or structure within liver, such as blood vessels and bile duct. HCC and CCA are malignant cancer. These cancers are crucial public health problem worldwide. The incidence of the cancer can find throughout the world. It is relatively common in developing countries.[2, 3] In particular, the incidence of cancer is very high in Thailand. The highest incidence is in the northeast of Thailand, where chronic infection with hepatitis B (HBV) and the liver fluke, *Opisthorchis viverini* is the major risk factor for the progression of liver cancer.[3] Both HCC and CCA are highly incurable disease.[1] Curative option in the cancer presently remains rather unclear. Moreover, resection of cancer, liver transplantation, radiotherapy and chemotherapy are still a matter strong debate with many reasons for the best treatment. One of the treatments for HCC and CCA is chemotherapy by 5-fluorouracil (5-FU), which however is rather ineffective.[4] The combined treatment of chemotherapy with radiotherapy in HCC and CCA causes severe side effects without significantly improving the prognosis of a patient.[5] Therefore, alternative therapies without side effects have been widely sought after. Natural products have been a source of medication efficacious drugs.[6] Crocodile blood from Siamese Crocodile *(Croccodylus siamensis)* has bioactive compounds such as hemoglobin, plasma, serum and white blood cell. These compounds have been suggested to contain numerous types of antimicrobial agents.[7, 8, 9] Antimicrobial agents have been reported to be a propitious anticancer remedy. Previous research suggested that some antimicrobial substances show anticancer properties which these substances can reduce cancer cell viability.[10] Therefore, antimicrobial substances are being vastly studied to define whether they can function as anticancer drugs.[11] White blood cells extract from Siamese crocodile are considered a prime source of natural active peptides, which are used in the treatment of cancer. Previous studies found that the crocodile leukocyte peptide could inhibit human cervical cancer cells (HeLa), human lung cancer,[12, 13, 14] human colon cancer cells (Caco-2)[13, 14] human prostate cancer cells (LNCaP and PC-3) and human breast cancer cells (MCF-7).[14] However, effect of crocodile blood extract on hepatocellular carcinoma and human cholangiocarcinoma cells has not formerly been determined. In this research, we evaluated the effect of crocodile blood extract on hepatocellular carcinoma (HepG2) and human chrolangiocarcinoma (HuCCA) cell viability *in vitro.* To our knowledge, this is the first report of crocodile blood extract to inhibit of HepG2 and HuCCA cells. In addition, this is comparison of the efficacy of crocodile blood extract against hepatocellular carcinoma and human cholangiocarcinoma cell inhibition.

Material and Methods

Chemical and reagents

Crocodile blood was obtained from a farm in Kanchanaburi province. Dulbecco's Modified Eagle Medium (DMEM) was purchased from Invitrogen-Gibco (Grand Island, NY, USA). Fetal

bovine serum (FBS) was purchased from Hyclone USA. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5-fluorouracil (5-FU), Hoechst 33342 and Dimethylsulfoxide (DMSO) were purchased from Sigma Inc., USA. Other common chemicals and reagents were received from the Monitoring and Surveillance Center for Zoonotic Diseases in Wildlife and Exotic Animals, Faculty of Veterinary Science, Mahidol University Thailand.

Cell culture and cell lines

Human hepatocellular carcinoma cell line (HepG2) was received from the Monitoring and Surveillance Center for Zoonotic Diseases in Wildlife and Exotic Animals, Faculty of Veterinary Science, Mahidol University. Human cholangiocarcinoma cell line (HuCCA) was provided by Associate Professor Dr. Adisak Wongkajornslip, the Department of Pharmacology, Faculty of Medicine Siriraj Hospital, Mahidol University. HepG2 and HuCCA cells were cultured in DMEM added 10% FBS, Penicillin (100IU/mL) and streptomycin (100 µg/mL). Both cells were kept at 37 °C in an incubator with a humidified atmosphere of 95% and 5% (v/v) CO_2.

Crocodile blood extract (CE) preparation

Crocodile blood extracts were prepared as formerly explained.[9, 15] Concisely, the crocodile blood extracts were sonicated with a High Intensity Ultrasonic Processor (Sonic, VC 500) (pulse on 5s, pulse off 10s, 40% amplitude, at 4 °C for 20 min) and centrifuged with refrigerator centrifuge HERMLE Z383K at 4 °C 12,000 rpm for 20 min. The supernatant was dried using vacuum chamber and freeze drier. Next, the extracts were dissolved in 1x PBS (pH 7.4). The protein concentrations were measured by BCA protein Assay Kit (Thermo Fisher Scientific).

The research protocol was conducted by National Research Council (NRC), Thailand and was approved by a committee of Faculty of Veterinary Science, Mahidol University, Thailand, animal use license no: U1-01313-2558.

Treatment of cell lines with CE and cytotoxicity analysis

HepG2 and HuCCA cells were seed into 96 well plates and incubated under 5% CO_2 at 37 °C for 24 h. After that both cells were treated with different concentration of ether CE or 5-FU (100, 200, 400, 800, 1,600 µg/mL), control (0 µg/mL) for 48 h. Later moving the media, the 100 µL of MTT solution was added in each well and incubated for 4 h at 37°C.[16] After 4 h, the media was forsaken and added serum-free media. Then, DMSO 100 µl was added to each well to solubilize the purple formazan yield and incubated for 5 min in the dark at room temperature. Absorbance was measured at 490 nm using a microplate reader (Bio-Rad, Hercules, CA). The results were used to assess 50% inhibition concentration (IC_{50}) or concentration that cause 50% loss of cell viability. The IC_{50} was calculated by GraphPad Prism program version 8.0.1 (244) Software. The cytotoxicity was measured by The MTT assay. The experiments were done three independent trials.

Analysis of apoptosis by nuclear staining

The nuclear condensation and fragmentation indicative of apoptosis was evaluated by Hoechst 33342 (Sigma, USA) nuclear staining. After incubation with CE for 48 h, the culture media were removed, the cells were washed two times with cold phosphate-buffered saline (PBS) and fixed with cold methanol and acetic acid (3/1 v/v) at 4 °C overnight. Afterward, the cells were stained with Hoechst 33342 for 30 min the dark at room temperature. Next, cells were washed with PBS two times and assessed under a fluorescent microscope (Nikon, NIS-Elements).

Statistical analysis

Data of cell viability were presented as mean ± SD from three independent experiments. Statistical analyses were performed using GraphPad Prism program version 8.0.1 (244) Software. Two-way analysis of variance (ANOVA) with Bonferroni post-test were used to compare the differences between treatments and control group. All data were considered statistically significant at p-values < 0.05.

Results

Cytotoxicity of CE on HepG2 and HuCCA cells

After treatment with CE for 48 h, the viabilities of HepG2 and HuCCA cells were decreased in a concentration-dependent manner from 100-1,600 μg/mL as compared with control group. CE could decrease cell viability of HepG2 cells more than HuCCA cells (Figure 1). In addition, the effect of CE on HepG2 and HuCCA cell viability were compared with treatment 5-FU for 48 h. The results showed that CE could reduce HepG2 cell viability similar effect to the 5-FU treatment. At the CE dose 1,600 μg/mL, the inhibitory effect on cell viability was 1.99 times more than 5-FU at the same dose (Figure 2A). Whereas, HuCCA cells treated with CE found that CE could decrease HuCCA cell viability lower than 5-FU treatment (Figure 2B). The average 50% inhibitory concentrations (IC_{50}) of CE on HepG2 and HuCCA cells were 120.36 μg/mL and 846.28 μg/mL, respectively. Meanwhile, IC_{50} of 5-FU on HepG2 and HuCCA cells were 77 μg/mL and 103 μg/mL (Table 1).

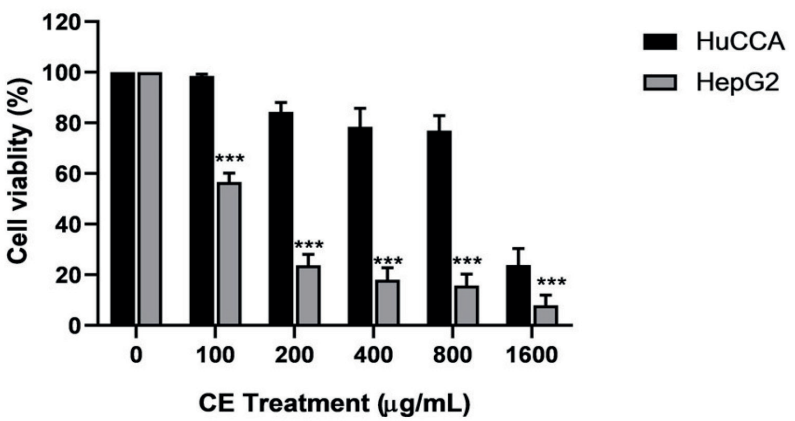

Figure 1 Comparison the cell viability of HepG2 and HuCCA cells after treatment with CE at various concentration for 48 h. All values are presented in mean ± SD, ***p<0.001.

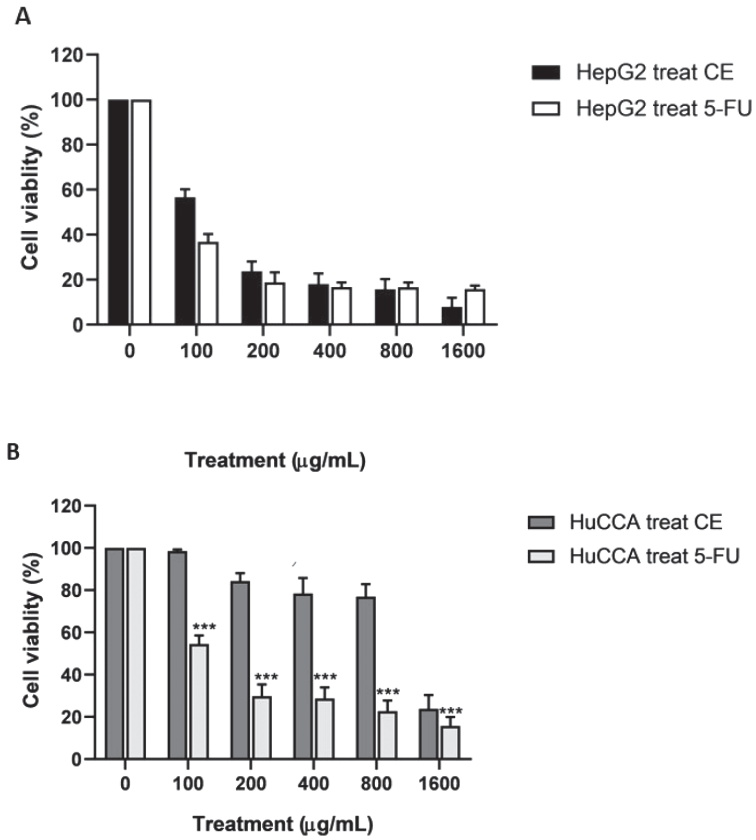

Figure 2 Cell viability of HepG2 and HuCCA cells after treatment with CE at various concentration for 48 h compared with 5-FU. (A) Cell viability of HepG2 cells. (B) Cell viability of HuCCA cells. All values presented in mean ± SD. ***p<0.001.

Table 1 The average 50% inhibitory concentration (IC_{50}) of CE and 5-FU on HepG2 and HuCCA cells.

Cell	Treatment	IC50 µg/mL
HepG2	CE	120.36
	5-FU	77
HuCCA	CE	846.28
	5-FU	103

CE induced HepG2 and HuCCA cells apoptosis with characteristic morphological changes

The nuclear morphology changes of treated cells were determined by Hoechst 33342 staining. The results showed chromatin condensation and fragmentation which refer to nuclear damage were presented in treated cells (Figure 3B and 3D) whereas round nuclei were observed in the control cells (Figure 3A and 3C). This result suggested that CE could lead HepG2 and HuCCA cells to under apoptosis.

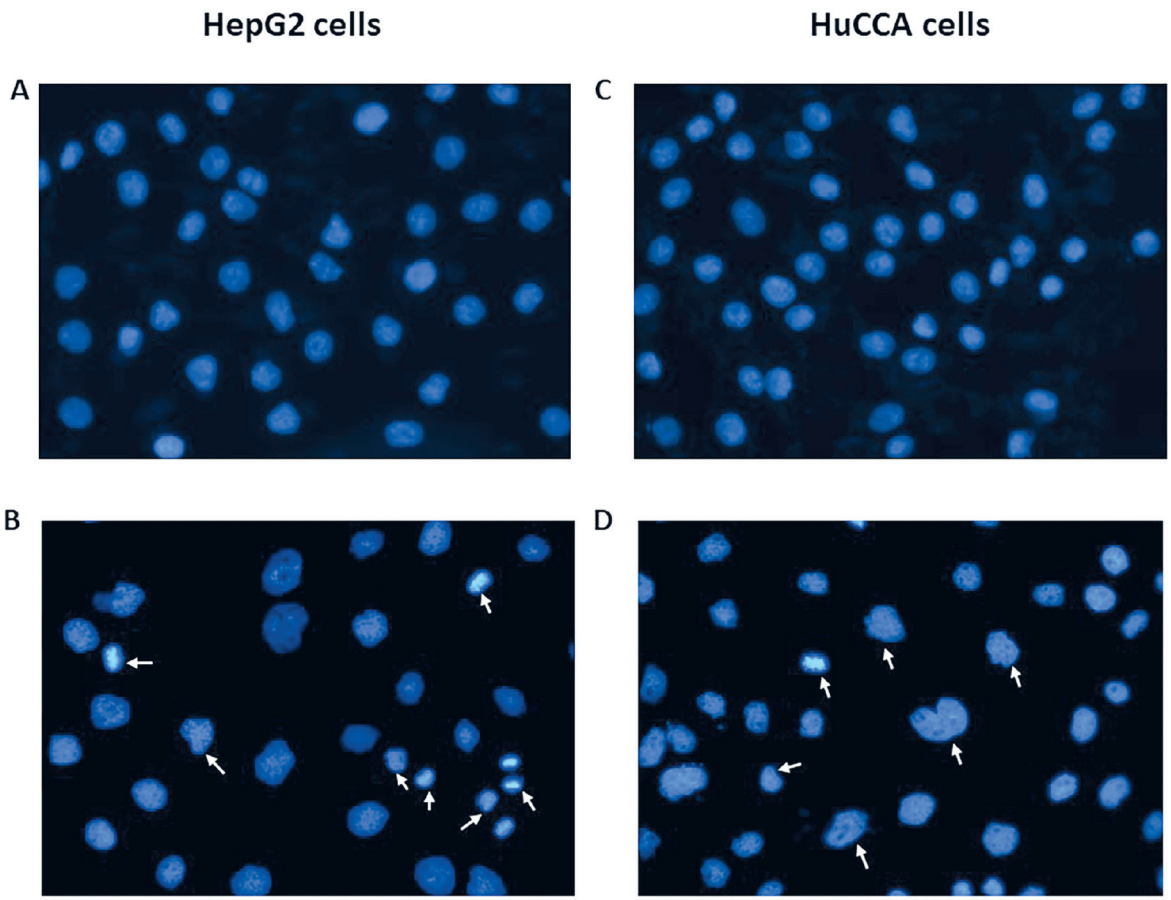

Figure 3 Detection of nuclear morphological changes in HepG2 (A–B) and HuCCA cells (C–D) following CE treatment for 48 h by Hoechst 33342 staining, as observed by fluorescence microscopy at 40x magnification. Control group (A, C) and CE treatment group (B, D). Arrow indicate chromatin condensation and fragmentation.

Discussion

Hepatocellular carcinoma and human cholangiocarcinoma are an important public health problem worldwide. These cancers are relatively common in developing countries. In Thailand, high incidence of hepatocellular carcinoma and human cholangiocarcinoma are especially found in the northeast region. These cancers are highly mortal disease because the cancer is characterized by a poor prognosis and poor response to contemporary therapeutics.[3, 17] Recently, it has been reported that treatment of hepatocellular carcinoma and human cholangiocarcinoma with chemotherapy and radiotherapy cause severe side effects without improving the prognosis of the patients.[5] The discovery and development of an effective treatment control of the cancer are desired. The use of natural products for cancer treatment has received a great deal of concentration toward their various health benefits and notable lack of toxicity and side effects. Crocodile blood has a broad range of biological properties. The crocodile blood peptides have antimicrobial potential.[9, 18, 19, 20] Additionally, extracts from crocodile *(Croccodylus siamensis)* leukocytes have been reported

anticancer activity[11, 14] against human lung cancer (LU-1), prostate cancer (LNCaP, PC-3), breast cancer, colorectal cancer (CaCo-2) and HeLa cells[11, 14] While, showed non toxicity to normal cells such as Vero cells and HaCaT cells.[14] The effect of CE on HepG2 and HuCCA cells have not formerly reported and this is the first study that we examined its effects on HepG2 and HuCCA cells. The results indicated that treatment with CE could decrease cell viability of HepG2 and HuCCA cells. Notably, CE showed decrease cell viability of HepG2 cells by 2.08 times higher than HuCCA cells. Thus, the data demonstrated that CE shows a broad spectrum of antiproliferation against HepG2 and HuCCA cells. Moreover, the data showed that CE and 5-FU displays the similar potentiality on the decrease in HepG2 cell viability at the same dosage. However, CE treatment reduced the viability of HuCCA cells by 1.84 times less than 5-FU treatment. This study indicated that apoptosis was induced following treatment with CE. HepG2 and HuCCA cells were likely adjusted through apoptosis by which nuclear morphological changes consist of chromatin condensation and fragmentation. Hence, the CE treatment of HepG2 and HuCCA cells could reduce cell viability due to increase apoptotic cells. Mechanism of apoptosis in HepG2 and HuCCA cells by CE also awaits further study.

Conclusion

In conclusion, our study indicated that CE could inhibit the viability of HepG2 and HuCCA cells by the induction of apoptotic cells. Furthermore, CE could decrease cell viability of HepG2 cells more than HuCCA cells. Therefore, CE has more promising remedy of hepatocellular carcinoma than cholangiocarcinoma *in vitro*, which can be used for information further study *in vivo*.

Acknowledgements

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การเปรียบเทียบประสิทธิภาพของสารสกัดเลือดจระเข้ในการยับยั้งเซลล์มะเร็งตับและเซลล์มะเร็งท่อน้ำดีมนุษย์

รัศมีเพ็ญ โพธิ์นาคเงิน[1] กาญจนา อัศวศุภฤกษ์[2] และธนกร ระวังชื่อ[3]

[1]ศูนย์เฝ้าระวังและติดตามโรคจากสัตว์ป่า สัตว์ต่างถิ่น และสัตว์อพยพ

[2]ภาควิชาปรีคลินิกและสัตวศาสตร์ประยุกต์

[3]ศูนย์ตรวจวินิจฉัยทางการสัตวแพทย์ คณะสัตวแพทยศาสตร์ มหาวิทยาลัยมหิดล พุทธมณฑล นครปฐม 73170

บทคัดย่อ สารสกัดเลือดจระเข้ (Crocodile blood extract) ประกอบด้วยเปปไทด์ธรรมชาติซึ่งถูกใช้ในการรักษามะเร็ง การศึกษาครั้งนี้มีวัตถุประสงค์ในการประเมินประสิทธิภาพของสารสกัดเลือดจระเข้ต่อการมีชีวิตอยู่ของเซลล์มะเร็งตับและเซลล์มะเร็งท่อน้ำดีมนุษย์ด้วยวิธี MTT assay ผลการศึกษาพบว่าสารสกัดเลือดจระเข้ขนาด 100 ถึง 1,600 ไมโครกรัม/มิลลิลิตร ลดการมีชีวิตอยู่ของเซลล์มะเร็งตับได้มากกว่าเซลล์มะเร็งท่อน้ำดีเมื่อเปรียบเทียบกับกลุ่มควบคุม ค่าความเข้มข้นของสารสกัดเลือดจระเข้ที่ออกฤทธิ์ยับยั้งได้ 50% (IC_{50}) ต่อเซลล์มะเร็งตับและเซลล์มะเร็งท่อน้ำดีมนุษย์ คือ 120.36 และ 846.28 ไมโครกรัม/มิลลิลิตร นอกจากนี้ยังพบว่าสารสกัดเลือดจระเข้จะเหนี่ยวนำทำให้เกิดการตายแบบอะพอพโทซิสโดยทำให้มีการเปลี่ยนแปลงรูปร่างของนิวเคลียส เช่น มีการหดตัวและการแตกของสายโครมาติน ด้วยการย้อม Hoechst 33342 สรุปผลการศึกษาได้ว่าสารสกัดเลือดจระเข้สามารถยับยั้งการมีชีวิตของเซลล์มะเร็งตับและเซลล์มะเร็งท่อน้ำดีมนุษย์ได้โดยสารสกัดเลือดจระเข้จะยับยั้งการมีชีวิตของเซลล์มะเร็งตับได้มากกว่าเซลล์มะเร็งท่อน้ำดีมนุษย์ การศึกษาครั้งนี้บ่งชี้ให้เห็นว่าสารสกัดเลือดจระเข้มีแนวโน้มในการรักษามะเร็งตับมากกว่ามะเร็งท่อน้ำดีที่ได้จากการทดลองในหลอดทดลอง

คำสำคัญ: สารสกัดเลือดจระเข้, การมีชีวิตอยู่ของเซลล์, อะพอพโทซิส, มะเร็งตับ, มะเร็งท่อน้ำดี