RESEARCH ARTICLE

Editorial Process: Submission:05/29/2025 Acceptance:01/15/2026 Published:01/21/2026

Anti-Metastatic Effects of Crocodile Blood Powder through *E-cadherin* Activation and Matrix Metalloproteinase Inhibition in Hepatocellular Carcinoma Cells

Sasitorn Rungarunlert[1,2], Rassameepen Phonarknguen[3], Thanakorn Rawangchue[4], Kanjana Assawasuparerk[1]*

Abstract

Background: Crocodile blood is a rich and valuable source of bioactive compounds derived from natural products. Crocodile blood powder (CP) has garnered significant attention for its potential applications in human health treatment. **Objective:** This study aimed to investigate the effect of CP on the invasion and metastasis of hepatocellular carcinoma (HepG2) cells. **Methods:** We analyzed the protein content of CP using MS/MS techniques. The effects of CP on cell proliferation, apoptosis, metastasis, and invasion were assessed using immunofluorescence, a wound healing assay, a transwell invasion assay, and Western blot analysis, respectively. **Result:** The findings indicated that CP could inhibit the proliferation of HepG2 cell lines. Additionally, CP increased *caspase-3* expression, inducing apoptosis in HepG2 cells. CP treatment also reduced metastasis and invasion of HepG2 cells. Immunofluorescence and Western blot analyses revealed that CP upregulated *E-cadherin* expression, while downregulating *MMP-2* and *MMP-9* expression. **Conclusion:** Overall, this study demonstrated that CP inhibits HepG2 cell proliferation and promotes apoptosis. Furthermore, CP suppresses metastasis and invasion by increasing *E-cadherin* expression and downregulating *MMP-2* and *MMP-9*. Thus, CP may serve as a promising candidate for hepatocellular carcinoma therapy.

Keywords: Crocodile blood powder- Hepatocellular carcinoma- *E-cadherin- MMP-2- MMP-9*

Asian Pac J Cancer Prev, **27 (1)**, 183-192

Introduction

Hepatocellular carcinoma (HCC) is a primary liver cancer caused by mutations in liver cells [1]. It is the fifth most common cancer and the third leading cause of cancer-related deaths worldwide. HCC is among the most metastatic cancers, contributing significantly to its high mortality rate. Metastasis is a multistep process involving the spread of cancer cells from the primary tumor to distant organs. During this process, various proteolytic enzymes degrade the extracellular matrix (ECM) and basement membrane [2], creating space for cancer cells to migrate. To detach from the primary tumor. Next, cancer cells lose cell–cell adhesion often through the secretion of protease enzymes. *E-cadherin* is a key molecule involved in cell–cell adhesion in epithelial tissues. As a member of the cadherin family– calcium-dependent intercellular adhesion glycoproteins–*E-cadherin* is widely expressed and facilitates homotypic cell binding. Classical cadherins, including *E-cadherin* and N-cadherin, also play a crucial role in linking to the cytoskeleton [3]. The loss of *E-cadherin* is strongly associated with cancer progression, particularly in altering cell morphology during tumor metastasis. In addition, decreased expression of *E-cadherin* at the cell membrane is associated with increased cancer cell invasion and metastasis, whereas upregulation of *E-cadherin* expression has been shown to inhibit these invasive and metastatic behaviors [3, 4]. Therefore, *E-cadherin* is considered a tumor suppressor, and its loss is closely linked to cancer progression [5]. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases involved in the degradation of the basement membrane and ECM, playing a critical role in cancer cell invasion, migration, and metastasis. In particular, *MMP-2* and *MMP-9* contribute significantly to cancer progression through their ability to degrade type IV collagen, a major structural component of the basement membrane [6-8]. Elevated expression of *MMP-2* and *MMP-9* has been reported in nearly all human tumors and is associated with advanced tumor stages and poor

*[1]Department of Preclinic and Applied Animal Science, Faculty of Veterinary Science, Mahidol University, Nakhon Pathom, Thailand. [2]Laboratory of Cellular Biomedicine and Veterinary Medicine, Faculty of Veterinary Science, Mahidol University, Nakhon Pathom, Thailand. [3]The Monitoring and Surveillance Center of Zoonotic Disease in Wildlife and Exotic Animals, Faculty of Veterinary Science, Mahidol University, Nakhon Pathom, Thailand. [4]Prasu Arthorn Veterinary Teaching Hospital, Faculty of Veterinary Science, Mahidol University, Nakhon Pathom, Thailand. *For Correspondence: kanjana.ass@mahidol.ac.th*

prognosis [9, 10]. The Siamese crocodile (Crocodylus siamensis) is recognized as a valuable medicinal animal. In regions such as China, Hong Kong, and Taiwan, crocodile blood and meat have traditionally been believed to enhance the immune response. Crocodile blood is considered a particularly valuable source of biologically active proteins, amino acids, and short peptides [11]. Previous studies have reported that proteins and peptides in crocodile blood constitute a rich and promising source of bioactive compounds derived from natural products. It has received growing attention for its potential application in promoting human health [12]. Crocodile blood contains several bioactive components, including serum, plasma, white blood cells, and hemoglobin, which exhibit diverse biological activities such as antibacterial, anticancer, anti-inflammatory, antioxidant, and wound healing effects [13-15]. Research shown the components of crocodile blood can inhibit the proliferation of various cancer cells types, including human cervical cancer cells (HeLa), human lung cancer [15, 16], human colon cancer cells (Caco-2) [17, 18], human prostate cancer cells (LNCaP and PC-3), human breast cancer cells (MCF-7) [18], and hepatocellular carcinoma cells (HepG2) [19]. However, the underlying mechanism by which crocodile blood inhibits HepG2 cells remains unclear. In this study, we investigated the effect of crocodile blood powder (CP) on HepG2 cell invasion and metastasis. This is the first report demonstrating that CP exhibits anti-metastatic activity in HepG2 cells. Mechanistically, the bioactivity of CP may be linked to increased *E-cadherin* expression, which in turn suppresses *MMP-2* and *MMP-9* expression, leading to the inhibition of metastasis in HCC.

Materials and Methods

Chemicals and reagents

CP was obtained from private farms in Kanchanaburi province, Thailand. Reagents for cell culture were purchased from Invitrogen-Gibco (Grand Island, NY, USA). Transwell chambers were acquired from Corning Costar Corporation. Primary antibodies against *caspase-3*, *E-cadherin*, *MMP-2*, *MMP-9*, and *Ki67* were sourced from Santa Cruz Biotechnology, CA, USA, while β-actin was purchased from Cell Signaling Technology. Secondary antibodies were obtained from Abcam and ThermoFisher Scientific. Lysis buffer, dithiothreitol (DTT), and iodoacetamide (IAA) were supplied by Sigma Chemical, Inc.

Proteomic analysis

CP was lysed in buffer (8 M urea, 50 mM Tris-HCl, 150 mM NaCl, and protease inhibitors), centrifuged, and the supernatant collected. Protein concentration was determined using the BCA Protein Assay Kit (ThermoFisher Scientific). Proteins were reduced with 10 mM DTT at 37°C for 1 h, alkylated with 20 mM IAA at room temperature for 30 minutes, and digested overnight at 37°C with trypsin/P (1:50, w/w). Peptides and desalted using C18 StageTips. Peptides were analyzed using an Orbitrap-based mass spectrometer coupled with UPLC. Separation was carried out on a C18 column using a 90-minute gradient. The MS operated in data-dependent acquisition mode with a resolution of 120,000 for MS1 and 30,000 for MS2. Fragmentation was performed using CID at 35% energy. Raw MS/MS data were processed with Mascot (Matrix Science) against a crocodile protein database using the following parameters: enzyme, Trypsin/P, fixed modification, carbamidomethyl (C), variable modification, oxidation (M), Mass values: monoisotopic protein mass; peptide mass tolerance, ±1.6 Da; fragment mass tolerance, ±0.8 Da; maximum missed cleavages, 2; Instrument type default. A total of 18,225 queries were submitted, and proteins were identified with Mascot Ion Scores ($p < 0.05$). Peptide spectrum matches were manually validated. Data analysis was performed using Scaffold and Perseus for functional annotation.

Cell culture

Human hepatocellular carcinoma cell lines (HepG2) were obtained from the Monitoring and Surveillance Center for Zoonotic Diseases in Wildlife and Exotic Animals, Faculty of Veterinary Science, Mahidol University, Thailand. HepG2 cells were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 µg/mL). The cells were maintained in a humidified incubator at 37°C with 5% CO_2

Migration assay

The migratory ability of HepG2 cells was assessed using a wound-healing assay. Briefly, cells were seeded in 6-well plates at a density of 5×10^5 cells/well and incubated overnight at 37°C. The culture medium was aspirated, and a scratch was made using a 200 µL pipette tip. Floating cells and debris were removed by washing with PBS. The adherent cells were then treated with various concentrations of CP (100–1,600 µg/mL). Wound closure was photographed at 0, 24, and 48 h using a phase-contrast microscope at 40x magnification (Nikon Instruments Inc.). The wound area percentage was also evaluated. Migration data are expressed as mean ± SD, and independent triplicate measurements were carried out for all samples (n = 3).

Cell invasion assay

Cell invasion was assessed using a transwell invasion assay with modified two chambers inserts containing 8-µm pore-size membranes. The upper surfaces of the insert membranes were coated with Matrigel (Corning, NY, USA) diluted in serum-free medium. Then, 5 × 104 HepG2 cells were seeded into the upper chambers in serum-free medium, and CP at concentrations of 100–1,600 µg/mL was added and mixed gently. Complete medium containing serum was added to the lower chambers to serve as a chemoattractant. The plates were incubated in a humidified 5% CO_2 atmosphere at 37°C for 48 h. After incubation, the chambers were washed three times with PBS, fixed in ice-cold methanol for 15 min, and stained with crystal violet for 15 min. Non invading cells remaining on the upper surface of the membrane were gently removed with a cotton swab. Cells that had migrated to the lower surface through the Matrigel-coated membrane were

photographed and counted under a light microscope at 200x magnification (Nikon Instrument Inc.). The number of invading cells was quantified by counting cells in five random fields per insert. The invasion data are expressed as mean ± SD from five independent experiments (n = 5).

Immunofluorescence analysis

HepG2 cells (1 × 10^5 cells/well) were seeded onto coverslips placed in 6-well plates and treated with various concentrations of CP (100–1,600 µg/mL). After treatment, the medium was removed, and the cells were washed with PBS. Cells were then fixed with 4% paraformaldehyde in cold PBS at 37ºC for 15 min. Permeabilization was performed using 0.25% Triton-X 100 for 10 min, followed by blocking with 2% bovine serum albumin for 1 h. The cells were incubated overnight at 4°C with specific primary antibodies, followed by incubation with secondary antibodies. Details of the primary and secondary antibodies are provided in Table 1. The coverslips were mounted on glass slides using antifade mounting medium with DAPI (Vectashield, Vector Laboratories, Burlingame, CA, USA) and visualized under a fluorescence microscope at 400x magnification (Nikon, NIS Elements). The percentage of positive cells was measured in comparison with the control group. Data are presented as mean ± SD, and every sample was independently analyzed in triplicate. (n=3).

Western blot analysis

For Western blot analysis, HepG2 cells were seeded in 6-well plates at a density of 2 × 10^6 cells/well and treated with different concentrations of CP (100–1,600 µg/mL). After treatment, whole-cell lysates were prepared using ice-cold RIPA lysis buffer (Sigma-Aldrich, USA) according to the manufacturer's instructions. Protein concentrations were quantified using the BCA assay kit (Thermo Fisher Scientific). Western blotting was performed using the JessTM Simple Western System, an automated capillary-based size separation technique (ProteinSimple, San Jose, CA, USA) [20]. Target proteins were separated and quantified using 12–230 kDa Jess separation modules (SM-W004 ProteinSample), following the manufacturer's instructions. Primary and secondary antibodies used are listed in Table 1. Chemiluminescent detection was carried out using peroxide/luminol-S (ProteinSample). The chemiluminescent signal of the separated proteins in the capillary was acquired and analyzed using Compass Simple Western software version 5.0.1 (Build 0911, Protein Sample), which automatically calculated chemiluminescence intensity. The relative densitometric analysis following CP treatment. Data for positive cells and protein density are expressed as mean ± SD, and all measurements were performed independently in triplicate for each sample (n = 3).

Statistical analysis

All data are presented as mean ± SD from three independent experiments. Statistical analyses were performed using the GraphPad Prism program version 8.0.1 (244). One-way analysis of variance followed by Sidak's post hoc test was used to compare differences between treatment and control groups. A p-value < 0.05 was considered statistically significant.

Results

Protein analysis of CP

The analysis of proteins could be classified into seven major functional categories (Table 2).

(1) Transport and carrier proteins (2) Structural and cytoskeletal proteins

(3) Enzymes and catalytic proteins (4) Histones and DNA-associated proteins

(5) Cell signaling and regulatory proteins (6) Immune and stress response proteins (7) Other functional proteins.

CP inhibits HepG2 cell proliferation and induces apoptosis

Cell proliferation and apoptosis were assessed using *Ki67* and *caspase-3* as markers, respectively. Immunofluorescence staining for *Ki67* revealed stronger nuclear localization in the control group compared to the CP-treated groups (Figure 1A). Treatment with CP at concentrations of 100–1,600 µg/mL significantly reduced the percentage of *Ki67*-positive cells relative to the control group (Figure 1B), a dose-dependent manner. Similarly, immunofluorescence staining for *caspase-3* demonstrated increased cytoplasmic localization in CP-treated cells compared to untreated controls (Figure 2A). CP treatment at 100–1,600 µg/mL significantly elevated the percentage of *caspase-3*-positive cells in a dose-dependent manner (Figure 2B). These findings suggest that CP induces apoptosis in HepG2 cells, with the apoptotic rate increasing proportionally with CP concentration.

Table 1. List of Antibodies Used for Immunofluorescence and Western Blotting

Antibodies	Dilution	Companies	Catalog number
Ki67	1/100	Santa cruz	sc-15402
Caspase-3	1/100	Santa cruz	sc-7148
E-cadherin	1/100	Santa cruz	sc-8426
MMP-2	1/100	Santa cruz	sc-13594
MMP-9	1/100	Santa cruz	sc-13520
Donkey anti rabbit IgG	1/1,000	Thermo Fisher scientific	A-21206
Donkey anti mouse IgG	1/1,000	Abcam	Ab150105
β-actin	1/500	Cell signaling	4970s

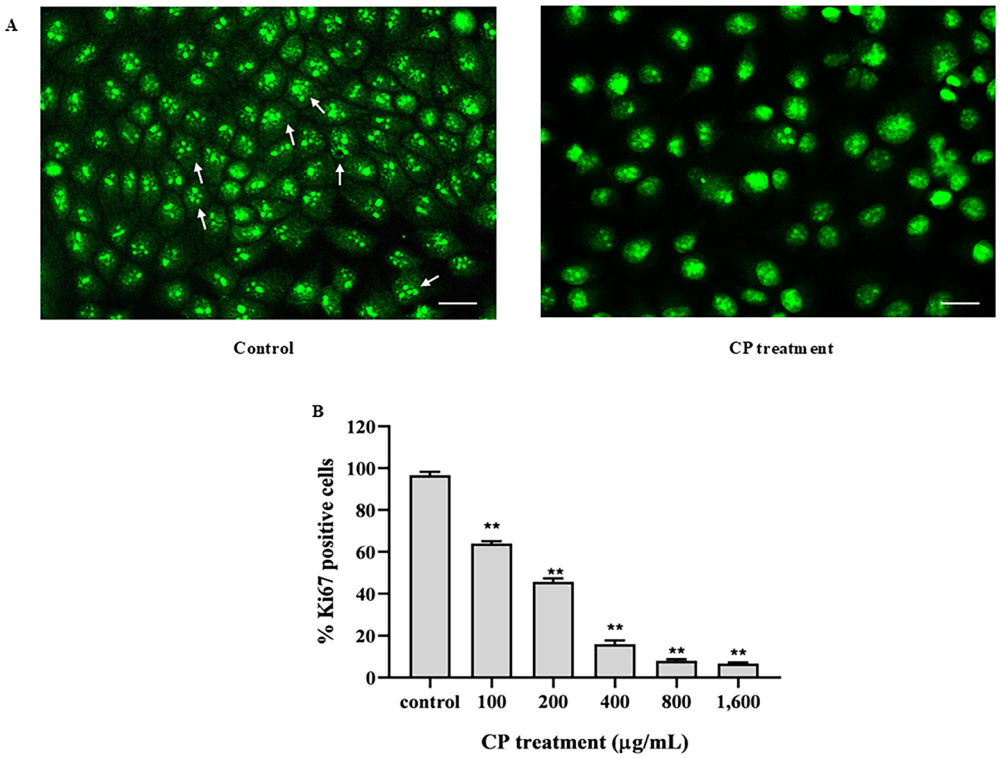

Figure 1. Immunofluorescence of *Ki67*. (A) *Ki67* staining indicates cellular proliferation; which showed nuclear localization staining (white arrow) was observed more prominently in the control group compared to the CP treatment group. (B) The percentage of *Ki67*-positive cells was measured in comparison with the control group. Data are presented as mean ± SD, and every sample was independently analyzed in triplicate. (n=3). ** indicates statistical significance at $p < 0.01$ compared to the control group.

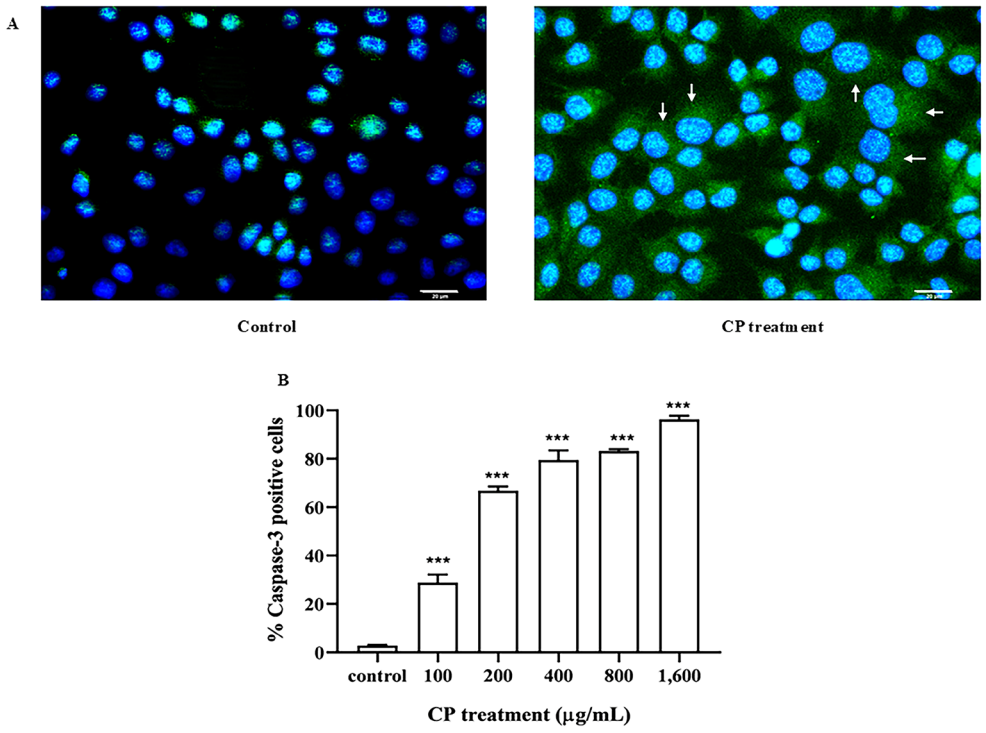

Figure 2. Immunofluorescence of *Caspase-3* and *DAPI*. *Caspase-3* (green color) for the detection of cytoplasmic localization and DAPI (blue color) for the detection of nuclei. (A) *Caspase-3* staining reflects apoptosis; cytoplasmic localization of *Caspase-3* (white arrow) was greater in the CP treatment group than in the control group. (B) The percentage of *Caspase-3* positive cells was measured in comparison with the control group. Data are expressed as mean ± SD, and every sample was independently analyzed in triplicate. (n=3). *** indicates statistical significance at $p < 0.001$ compared to the control group.

Table 2. Summary of Protein Expression Analysis in Crocodile Blood Powder (CP)

Protein Family	Proteins Identified	Biological Function
Transport and Carrier Proteins	Albumin domain-containing protein, Apolipoprotein A1, A-IV, C-III, Hemopexin, Transferrin (Fragment), Ovotransferrin, Ceruloplasmin, Solute carrier family 4 member 11	Transport and storage of molecules
Structural and Cytoskeletal Proteins	Actin family (Actin beta, Actin gamma 1, Actin alpha 2, smooth muscle, Actin aortic smooth muscle-like, etc.), Vimentin, Integrin subunit alpha V, Collagen type X alpha 1 chain, Tight junction protein 1, Xin actin binding repeat containing 2, Gelsolin, Microtubule associated protein 1B	Cell structure and mobility
Enzymes and Catalytic Proteins	Carbonic anhydrase, Lysyl oxidase homolog, Malic enzyme, Pantetheinase-like, Rho GTPase-activating protein 7	Metabolic and catalytic processes
Histones and DNA-a ssociated Proteins	Histone H2A, H2B, H3, H3.7 histone (putative), H2A/H2B/H3 domain-containing protein, Condensin-2 complex subunit H2, Nucleoporin 153, Short stature homeobox 2	DNA packaging and gene regulation
Cell Signaling and Regulatory Proteins	RING-type E3 ubiquitin transferase, PHD and ring finger domains 1, Zinc finger MYND-type containing 11, Protein cereblon, Piezo-type mechanosensitive ion channel component, SRP receptor subunit alpha	Cell signaling and protein regulation
Immune and Stress Response Proteins	10 kDa heat shock protein, mitochondrial, 60 kDa heat shock protein, mitochondrial, Myeloid protein 1-like, Putative complement component C3 (Fragment), von Willebrand factor A domain containing 3A	Cellular stress response and immunity
Other Functional Proteins	26S proteasome non-ATPase regulatory subunit 6, STIL centriolar assembly protein, Early endosome antigen 1, Shroom family member 1, Leucine rich repeat containing 2, Cochlin,Cell division cycle 23, Beta-microseminoprotein-like, Uncharacterized protein	Various cellular functions

Effect of CP on HepG2 cell migration and invasion

HepG2 cells were treated with various concentrations of CP (100–1,600 µg/mL) for 24 and 48 h. The inhibition of cell migration is illustrated in Figure 3A. In the untreated control group, cells along the edges of the scratch migrated into the wound area and nearly closed the gap within 48 h. In contrast, CP-treated cells showed a reduced migration rate, and the scratch area remained incompletely filled. Quantitative analysis revealed that CP at concentrations of 200–1,600 µg/mL significantly

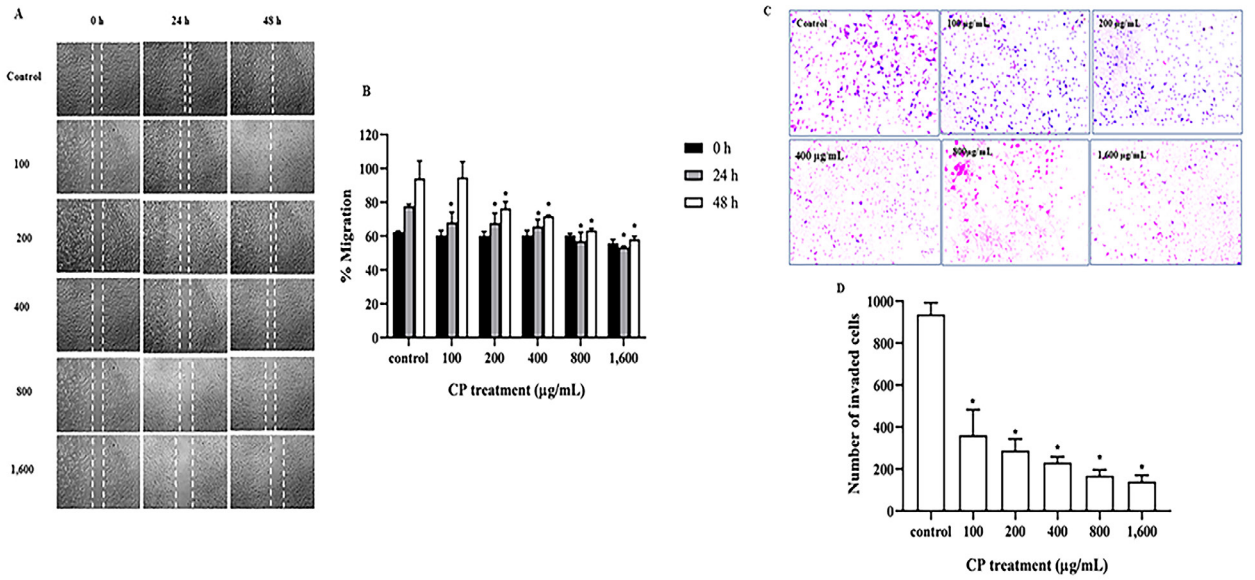

Figure 3. The Effect of CP on the Migration and Invasion of HepG2 Cells was assessed Using Wound- Healing and Transwell Invasion Assays. (A) Cell migration was observed under phase contrast microscopy. (B) The percentage of cell migration quantified relative to untreated control cells. (C) Cell invasion was visualized using crystal violet staining. (D) The number of invaded HepG2 cells per field was evaluated. Migration data are expressed as mean ± SD, and independent triplicate measurements were carried out for all samples (n = 3), and invasion data are expressed as mean ± SD from five independent experiments (n = 5). * indicates statistical significance at $p < 0.05$ compared to the control group.

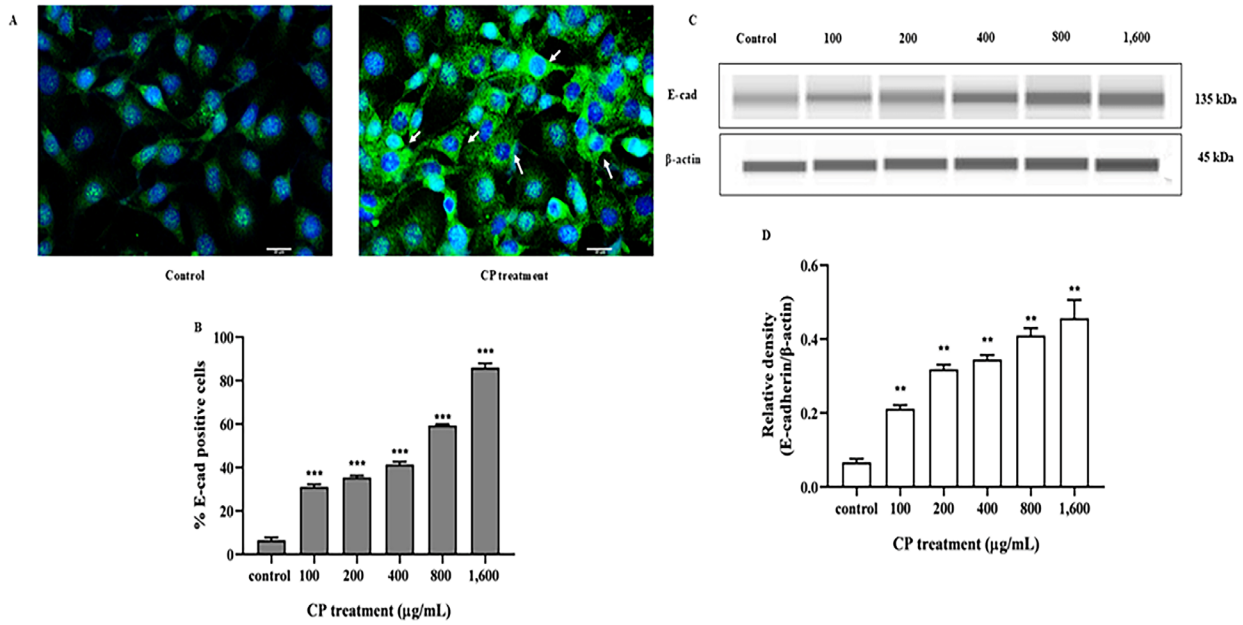

Figure 4. Effect of CP on *E-cadherin* Protein Expression in HepG2 Cells. (A) Immunofluorescence staining of E-cadherin and DAPI. E-cadherin (green color) for the detection of membrane localization and DAPI (blue color) for the detection of nuclei. Immunofluorescence staining for E-cadherin showed increased membrane localization (white arrow) in the treatment group compared to the control. (B) The percentage of E-cadherin-positive cells was quantified relative to untreated control cells. (C) Western blot analysis of E-cadherin expression. (D) Relative densitometric analysis of E-cadherin following CP treatment. Data for E-cadherin-positive cells and protein density are expressed as mean ± SD, and all measurements were performed independently in triplicate for each sample (n = 3). ** and *** indicates statistical significance at $p < 0.01$ and $p < 0.001$, respectively, compared to the control group.

inhibited the migration of HepG2 cells at 48 h compared to both untreated cells and lower CP concentrations. These findings suggest that CP suppresses HepG2 cell migration in a dose-dependent manner (Figure 3B). Regarding cell invasion treatment with CP at concentrations of 100–1,600 µg/mL significantly reduced the number of invading

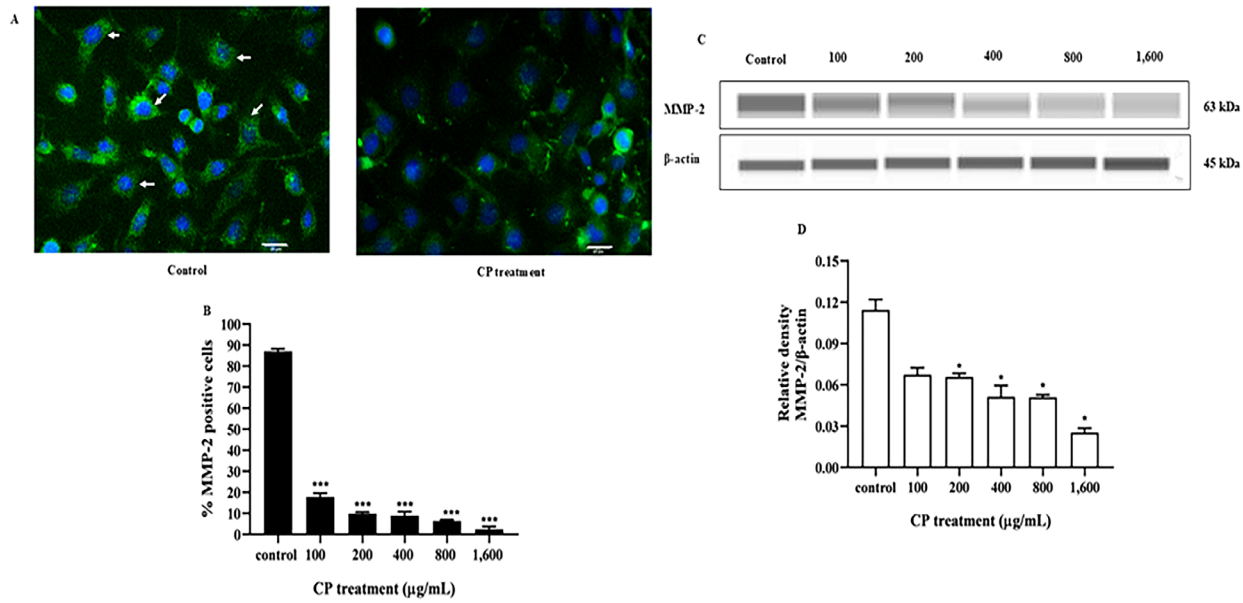

Figure 5. Effect of CP on MMP-2 protein Expression in HepG2 Cells. (A) Immunofluorescence staining of MMP-2 and DAPI. MMP-2 (green color) for the detection of cytoplasmic localization and DAPI (blue color) for the detection of nuclei. Immunofluorescence staining for MMP-2 revealed decreased cytoplasmic localization (white arrow) in the treatment group compared to the control. (B) The percentage of MMP-2 positive cells was quantified relative to untreated control cells. (C) Western blot analysis of MMP-2 expression. (D) Relative densitometric analysis of MMP-2 following CP treatment. Data for MMP-2-positive cells and MMP-2 density are expressed as mean ± SD, and every sample was independently analyzed in triplicate (n = 3). * and *** indicate statistical significance at $p < 0.05$ and $p < 0.001$, respectively, compared to the control group.

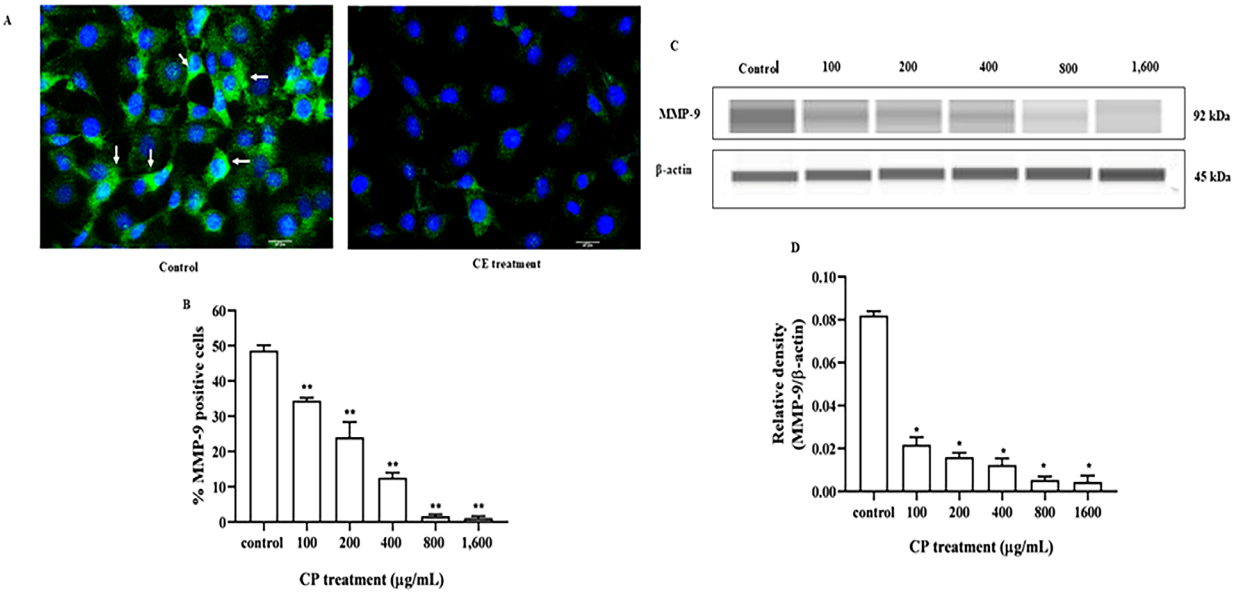

Figure 6. Effect of CEs on MMP-9 protein Expression in *HepG2* Cells. (A) Immunofluorescence staining of MMP-9 and DAPI. MMP-9 (green color) for the detection of cytoplasmic localization and DAPI (blue color) for the detection of nuclei. Immunofluorescence staining for MMP-9 showed reduced cytoplasmic localization (white arrow) in the treatment group compared to the control. (B) The percentage of MMP-9 positive cells was quantified relative to untreated control cells. (C) Western blot analysis of MMP-9 expression. (D) Relative densitometric analysis of MMP-9 following CP treatment. Data for MMP-9 positive cells and MMP-9 density are expressed as mean ± SD, and each sample was measured independently three times (n = 3). * and ** indicate statistical significance at $p < 0.05$ and $p < 0.01$, respectively, compared to the control group.

HepG2 cells at 48 h in a concentration-dependent manner compared to the control group (Figure 3C, 3D).

Effect of CP on protein expression levels

To evaluate the effect of CP on the expression of proteins involved in cancer metastasis, HepG2 cells were treated with various concentrations of CP (100–1,600 μg/mL) for 48 h. Protein expression levels were subsequently assessed using immunofluorescence and Western blot analyses, focusing on key proteins related to cell migration and invasion. Immunofluorescence staining for *E-cadherin* in the CP-treated group showed increased membrane localization compared to the control group (Figure 4A). The percentage of *E-cadherin*-positive cells increased significantly in response to CP treatment (100–1,600 μg/mL) compared to untreated cells (Figure 4B). Western blot analysis confirmed that treatment markedly upregulated *E-cadherin* expression (Figure 4C), and quantification showed a significant increase in protein levels across all CP concentrations compared to the control group (Figure 4D). In contrast, *MMP-2* staining in the control group revealed more intense cytoplasmic localization than in the CP treated group (Figure 5A). The percentage of *MMP-2*-positive cells significantly decreased in response to CP treatment (100–1,600 μg/mL) compared to untreated cells (Figure 5B). Western blot analysis showed a substantial downregulation of *MMP-2* expression with CP treatment (Figure 5C), and protein quantification confirmed a significant reduction in *MMP-2* levels across all doses (Figure 5D). Similarly, *MMP-9* immunofluorescence in the control group exhibited stronger cytoplasmic staining than in the CP treated group (Figure 6A). The percentage of *MMP-9*-positive cells significantly declined with CP doses of 100–1,600 μg/mL (Figure 6B). Western blot results demonstrated that CP treatment significantly downregulated *MMP-9* expression (Figure 6C), with corresponding reductions in protein level of *MMP-9* indicating CP treatment doses of 100–1,600 μg/mL significantly decreased compared to the control group (Figure 6D).

Discussion

HCC is a highly aggressive cancer characterized by aggressive invasion and metastasis, contributing to its poor prognosis. Cancer cell migration and invasion are key steps in the metastatic cascade for many cancer types [21]. Therefore, there is a pressing need to develop novel therapeutic agents that enhance treatment efficacy and target these metastatic mechanisms. The search for natural products with anticancer potential has gained increasing attention among researchers.

Crocodile blood is considered a valuable medicinal substance. In traditional Chinese medicine, it is believed that consumption of CP can boost the immune response. In addition, modern studies have shown that crocodile blood possesses anti-inflammatory, antioxidant, and anti-cancer properties [14, 15]. In the present study, we analyzed the protein composition of CP and identified several proteins involved in the regulation of cell growth and cancer metastasis, including apolipoprotein [22], cystatins [23, 24], gelsolin [25, 26], integrin [27], and

vimentin [28]. These findings support our experimental data, which demonstrated that treatment inhibits the growth and metastasis of HepG2 cells. Furthermore, our results showed a decrease in *Ki-67* expression following CP treatment, suggesting that CP suppresses HepG2 cell proliferation. This observation is consistent with findings by Phonarknguen and colleagues, who reported that crocodile blood extract reduces HepG2 cell viability [19].

Apoptosis plays a vital role in eliminating damaged cells to maintain homeostasis under normal physiological conditions. However, impairments in the apoptotic signaling pathway can contribute to carcinogenesis and promote cancer cell survival [29]. Therefore, inducing apoptosis in cancer cells is a key therapeutic strategy for cancer treatment. A previous study reported that crocodile blood extract can induce apoptosis in HepG2 cells by promoting nuclear morphological changes characteristic of apoptotic cells [19]. In the present study, we examined the effect of CP on the expression of the apoptosis-related protein *caspase-3* in HepG2 cells. *caspase-3*, a critical executioner in the intrinsic apoptotic pathway, is influenced by mitochondrial signals. Our finding demonstrated that CP treatment increased the expression of cleaved *caspase-3* in HepG2 cells, correlating with enhanced levels of apoptosis.

Cancer cell migration and invasion are critical steps in the metastatic process of many cancer types [21]. Therefore, we evaluated the inhibitory effects of CP on the migration and invasion of HepG2 cells. Our results indicated that CP significantly suppressed HepG2 cells migration in a dose-dependent manner. Similarly, CP treatment reduced the invasive ability of HepG2 cells in a dose-dependent fashion. These findings demonstrate that CP effectively inhibits both migration and invasion in HepG2 cells. To investigate the underlying mechanisms, we assessed the expression of proteins associated with cancer migration and invasion, including *E-cadherin*, *MMP-2*, and *MMP-9*. *E-cadherin* is a transmembrane protein and a key component of cell–cell adhesion complexes [30]. *E-cadherin* is intimately associated with the metastasis of cancer [31]. The development in various cancer cells displayed the reduced expression of *E-cadherin* [30]. The high expression of *E-cadherin* could induce conversion of cultured cancer cells from an invasive mesenchymal phenotype to a non-invasive epithelial phenotype [3]. The results of the present study showed that increased *E-cadherin* expression significantly reduced the migration and invasion of HepG2 cells treated with CP. These findings suggested that the inhibitory effect of CP on HepG2 migration and invasion is mediated by the upregulation of *E-cadherin*, leading to enhanced cell adhesion and tissue integrity. Previous studies have reported that *E-cadherin* can be cleaved by specific enzymes in various cancer cell types, resulting in diminished cell adhesion and tissue cohesion, thereby facilitating metastasis and invasion [32-34]. MMPs are key enzymes responsible for cleaving *E-cadherin* and generating extracellular fragments [35]. The MMP family, including *MMP-2* and *MMP-9*, plays a crucial role in ECM degradation [6, 36]. ECM and basement membrane degradation by MMPs are essential steps in cancer metastasis and invasion. *MMP-2* and *MMP-9*, in particular, have been strongly implicated in these processes [9]. Our data demonstrated that CP treatment downregulated *MMP-2* and *MMP-9* expression in HepG2 cells. Collectively, these results confirm that CP exerts an inhibitory effect on metastasis and invasion in HepG2 cells. Further animal studies will be conducted to validate the therapeutic potential of CP.

In this study, we analyzed the protein composition of CP and its role in inhibiting cancer cell growth and metastasis. We further investigated the effects of CP on proliferation, apoptosis, metastasis, and invasion in HepG2 cells. The results demonstrated that CP inhibited cell proliferation and induced apoptosis via the mitochondrial apoptotic pathway. Additionally, CP treatment suppressed metastasis and invasion by modulating key proteins, inducing *E-cadherin*, *MMP-2*, and *MMP-9*. Specifically, CP upregulated *E-cadherin* expression while downregulating *MMP-2* and *MMP-9* levels. To our knowledge, this is the first study to identify the protein components of CP and report its apoptotic, anti-invasive, and anti-metastatic mechanisms in HepG2 cells. These findings suggest that CP holds promise as a novel therapeutic approach for the treatment of HCC.

Author Contribution Statement

Research design, K.A., R.P., and S.R.; methodology, K.A. and R.P.; investigation, K.A., R.P., T.R., and S.R.; data analysis, K.A., R.P., and S.R.; data curation, K.A. and R.P.; conclusion, K.A., R.P., and S.R.; writing original draft preparation, K.A.; project administration, K.A.; All authors have read and agreed to the published version of the manuscript.

Acknowledgements

This research project has been supported by Mahidol University (Fundamental Fund: fiscal year 2025 by National Science Research and Innovation Fund (NSRF); Grant number FF-168/2568). The authors are thankful for the instrumentation support for the Jess automated Western blotting from Mahidol University Frontier Research Facility (MU-FRF), Mahidol University, for providing Jess automated Western blotting. Authors would also like to thank the Monitoring and Surveillance Center for Zoonotic Disease in Wildlife and Exotic Animals, Faculty of Veterinary Science, Mahidol University, for support regarding lab space for this research.

Ethical approval

The research protocol was approved by the National Research Council (NRC), Thailand, and approved by a committee of the Faculty of Veterinary Science, Mahidol University, Thailand (COA: NO. MUVS-2020-07-27). How the ethical issue was handled (name the ethical committee that approved the research)

Conflict of Interest

All authors declare that they have no conflict of interest.

References

1. Aravalli RN, Steer CJ, Cressman EN. Molecular mechanisms of hepatocellular carcinoma. Hepatology. 2008;48(6):2047-63. https://doi.org/10.1002/hep.22580.
2. Harlozinska A. Progress in molecular mechanisms of tumor metastasis and angiogenesis. Anticancer Res. 2005;25(5):3327-33.
3. Vleminckx K, Vakaet L, Jr., Mareel M, Fiers W, van Roy F. Genetic manipulation of *E-cadherin* expression by epithelial tumor cells reveals an invasion suppressor role. Cell. 1991;66(1):107-19. https://doi.org/10.1016/0092-8674(91)90143-m.
4. Wijnhoven BP, Dinjens WN, Pignatelli M. *E-cadherin*-catenin cell-cell adhesion complex and human cancer. Br J Surg. 2000;87(8):992-1005. https://doi.org/10.1046/j.1365-2168.2000.01513.x.
5. Nawrocki-Raby B, Gilles C, Polette M, Martinella-Catusse C, Bonnet N, Puchelle E, et al. *E-cadherin* mediates mmp down-regulation in highly invasive bronchial tumor cells. Am J Pathol. 2003;163(2):653-61. https://doi.org/10.1016/s0002-9440(10)63692-9.
6. Stamenkovic I. Matrix metalloproteinases in tumor invasion and metastasis. Semin Cancer Biol. 2000;10(6):415-33. https://doi.org/10.1006/scbi.2000.0379.
7. Stetler-Stevenson WG. The role of matrix metalloproteinases in tumor invasion, metastasis, and angiogenesis. Surg Oncol Clin N Am. 2001;10(2):383-92. https://doi.org/https://doi.org/10.1016/S1055-3207(18)30071-1.
8. Jodele S, Blavier L, Yoon J, Yves A. Jodele s, blavier l, et al. Modifying the soil to affect the seed: Role of stromal-derived matrix metalloproteinases in cancer progression. Cancer Metastasis Rev. 2006;25:35-43. https://doi.org/10.1007/s10555-006-7887-8.
9. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer. 2002;2(3):161-74. https://doi.org/10.1038/nrc745.
10. Zucker SJ, Lysik RM, Zarrabi MH, Moll UM. Mr 92,000 type iv collagenase is increased in plasma of patients with colon cancer and breast cancer. Cancer Res. 1993;53:140-6.
11. Pakdeesuwan A, Araki T, Daduang S, Payoungkiattikun W, Jangpromma N, Klaynongsruang S. In vivo wound healing activity of crocodile (crocodylus siamensis) hemoglobin and evaluation of antibacterial and antioxidant properties of hemoglobin and hemoglobin hydrolysate. J Microbiol Biotechnol. 2017;27(1):26-35. https://doi.org/10.4014/jmb.1603.03046.
12. Pakdeesuwan A, Araki T, Payoungkittikun W, Neubert L, Jangpromma N, Klaynongsruang S. Development, characterization and determination of biological properties of crocodile hemoglobin (crocodylus siamensis) tablets. J Food Biochem. 2018;42. https://doi.org/10.1111/jfbc.12503.
13. Pata S, Yaraksa N, Daduang S, Temsiripong Y, Svasti J, Araki T, et al. Characterization of the novel antibacterial peptide leucrocin from crocodile (crocodylus siamensis) white blood cell extracts. Dev Comp Immunol. 2011;35(5):545-53. https://doi.org/10.1016/j.dci.2010.12.011.
14. Phosri S, Mahakunakorn P, Lueangsakulthai J, Jangpromma N, Swatsitang P, Daduang S, et al. An investigation of antioxidant and anti-inflammatory activities from blood components of crocodile (crocodylus siamensis). Protein J. 2014;33(5):484-92. https://doi.org/10.1007/s10930-014-9581-y.
15. Patathananone S, Thammasirirak S, Daduang J, Chung J, Temsiripong Y, Daduang S. Inhibition of hela cells metastasis by bioactive compounds in crocodile (crocodylus siamensis) white blood cells extract. Environmental toxicology. 2015;31. https://doi.org/10.1002/tox.22138.
16. Ou Y, Ho WS. Crocodile blood extract induces the apoptosis of lung cancer cells through pten activity. Oncol Rep. 2016;36(3):1457-66. https://doi.org/10.3892/or.2016.4914.
17. Maraming P, Klaynongsruang S, Boonsiri P, Maijaroen S, Daduang S, Chung JG, et al. Antitumor activity of rt2 peptide derived from crocodile leukocyte peptide on human colon cancer xenografts in nude mice. Environ Toxicol. 2018;33(9):972-7. https://doi.org/10.1002/tox.22584.
18. Phosri S, Jangpromma N, Chang LC, Tan GT, Wongwiwatthananukit S, Maijaroen S, et al. Siamese crocodile white blood cell extract inhibits cell proliferation and promotes autophagy in multiple cancer cell lines. J Microbiol Biotechnol. 2018;28(6):1007-21. https://doi.org/10.4014/jmb.1712.12002.
19. Phonarknguen r, assawasuparerk k, rawangchue t. Comparison of efficacy of crocodile blood extract against inhibition of cell viability in hepatocellular carcinoma and human cholangiocarcinoma cell lines. Bull dept med sci. 2021;63:618-27.
20. Edouard S, Jaafar R, Orain N, Parola P, Colson P, La Scola B, et al. Automated western immunoblotting detection of anti-sars-cov-2 serum antibodies. Eur J Clin Microbiol Infect Dis. 2021;40(6):1309-17. https://doi.org/10.1007/s10096-021-04203-8.
21. Zhang T, Li J, Dong Y, Zhai D, Lai L, Dai F, et al. Cucurbitacin e inhibits breast tumor metastasis by suppressing cell migration and invasion. Breast Cancer Res Treat. 2012;135(2):445-58. https://doi.org/10.1007/s10549-012-2175-5.
22. Ren L, Yi J, Li W, Zheng X, Liu J, Wang J, et al. Apolipoproteins and cancer. Cancer Med. 2019;8(16):7032-43. https://doi.org/10.1002/cam4.2587.
23. Cox JL. Cystatins and cancer. Front Biosci (Landmark Ed). 2009;14(2):463-74. https://doi.org/10.2741/3255.
24. Cox J. Cystatins as regulators of cancer. Medical Research Archives. 2017;5(7).
25. Yuan X, Wang W, Li J, Zheng P, Dong P, Chen L, et al. Gelsolin suppresses gastric cancer metastasis through inhibition of pkr-p38 signaling. Oncotarget. 2016;7(33):53459-70. https://doi.org/10.18632/oncotarget.10557.
26. Hsieh CH, Wang YC. Emerging roles of plasma gelsolin in tumorigenesis and modulating the tumor microenvironment. Kaohsiung J Med Sci. 2022;38(9):819-25. https://doi.org/10.1002/kjm2.12578.
27. Desgrosellier JS, Cheresh DA. Integrins in cancer: Biological implications and therapeutic opportunities. Nat Rev Cancer. 2010;10(1):9-22. https://doi.org/10.1038/nrc2748.
28. Berr AL, Wiese K, Dos Santos G, Koch CM, Anekalla KR, Kidd M, et al. Vimentin is required for tumor progression and metastasis in a mouse model of non-small cell lung cancer. Oncogene. 2023;42(25):2074-87. https://doi.org/10.1038/s41388-023-02703-9.
29. Zhu M, Li W, Dong X, Chen Y, Lu Y, Lin B, et al. Benzyl-isothiocyanate induces apoptosis and inhibits migration and invasion of hepatocellular carcinoma cells in vitro. J Cancer. 2017;8(2):240-8. https://doi.org/10.7150/jca.16402.
30. van Roy F, Berx G. The cell-cell adhesion molecule *E-cadherin*. Cell Mol Life Sci. 2008;65(23):3756-88. https://doi.org/10.1007/s00018-008-8281-1.
31. Chan AO. *E-cadherin* in gastric cancer. World J Gastroenterol. 2006;12(2):199-203. https://doi.org/10.3748/wjg.v12.i2.199.
32. Kuefer R, Hofer MD, Gschwend JE, Pienta KJ, Sanda MG, Chinnaiyan AM, et al. The role of an 80 kda fragment of *E-cadherin* in the metastatic progression of prostate cancer. Clin Cancer Res. 2003;9(17):6447-52.

33. Rios-Doria J, Day KC, Kuefer R, Rashid MG, Chinnaiyan AM, Rubin MA, et al. The role of calpain in the proteolytic cleavage of *E-cadherin* in prostate and mammary epithelial cells. J Biol Chem. 2003;278(2):1372-9. https://doi.org/10.1074/jbc.M208772200.
34. Chan AO, Chu KM, Lam SK, Wong BC, Kwok KF, Law S, et al. Soluble *E-cadherin* is an independent pretherapeutic factor for long-term survival in gastric cancer. J Clin Oncol. 2003;21(12):2288-93. https://doi.org/10.1200/jco.2003.08.078.
35. Symowicz J, Adley BP, Gleason KJ, Johnson JJ, Ghosh S, Fishman DA, et al. Engagement of collagen-binding integrins promotes matrix metalloproteinase-9-dependent *E-cadherin* ectodomain shedding in ovarian carcinoma cells. Cancer Res. 2007;67(5):2030-9. https://doi.org/10.1158/0008-5472.Can-06-2808.
36. Woessner JF, Jr. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. Faseb j. 1991;5(8):2145-54.