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# Siamese Crocodile White Blood Cell Extract Inhibits Cell Proliferation and Promotes Autophagy in Multiple Cancer Cell Lines

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Cancer represents one of the most significant threats to human health on a global scale. Hence, the development of effective cancer prevention strategies, as well as the discovery of novel therapeutic agents against cancer, is urgently required. In light of this challenge, this research aimed to evaluate the effects of several potent bioactive peptides and proteins contained in crocodile white blood cell extract (cWBC) against LU-1, LNCaP, PC-3, MCF-7, and CaCo-2 cancer cell lines. The results demonstrate that 25, 50, 100, and 200 µg/ml cWBC exhibits a strong cytotoxic effect against all investigated cell lines (IC$_{50}$ 70.34–101.0 µg/ml), while showing no signs of cytotoxicity towards noncancerous Vero and HaCaT cells. Specifically, cWBC treatment caused a significant reduction in the cancerous cells' colony forming ability. A remarkable suppression of cancerous cell migration was observed after treatment with cWBC, indicating potent antimetastatic properties. The mechanism involved in the cancer cell cytotoxicity of cWBC may be related to apoptosis induction, as evidenced by typical apoptotic morphology features. Moreover, certain cWBC concentrations induced significant overproduction of ROS and significantly inhibited the S-G$_2$/M transition in the cancer cell. The molecular mechanisms of cWBC in apoptosis induction were to decrease Bcl-2 and XIAP expression levels and increase the expression levels of caspase-3, caspase-8, and p53. These led to a decrease in the expression level of the cell cycle-associated gene cyclin-B1 and the arrest of cell population growth. Consequently, these findings demonstrate the prospect of the use of cWBC for cancer therapy.

**Keywords:** Antitumor, breast cancer, colorectal cancer, *Crocodylus siamensis*, lung cancer, prostate cancer

## Introduction

Population ageing poses a variety of problems that challenge public health worldwide. Extensive research has demonstrated that the number of adults aged 60 years and older will increase more rapidly in developing countries than in developed countries [1]. Ageing has been recognized as the first and foremost risk factor for noncommunicable diseases (NCDs), including tumors, heart diseases, hypertension, and diabetes [2]. The World Health Organization has concluded that deaths of over-60s caused by NCDs total over twice the number of those below 60 years of age. Cancer is regarded as a typical NCD, and represents one of the most serious health conditions on a global scale,

accounting for 8.2 million cancer-related deaths and an estimated 14.1 million new cancer patients in the year 2012 alone, according to the GLOBOCAN 2012 report [3]. Worldwide, the most commonly diagnosed cancer types include 1.8 million cases of lung, 1.7 million cases of breast, 1.4 million cases of colorectal, and 1.1 million cases of prostate cancers [3]. Among these, prostate cancer is associated with the highest incidence rate of all cancer types in men, and with an estimated 307,000 deaths per year, it constitutes the principal cause of death, representing 6.6% of male cancer mortality in total [3]. Its incidence rate has increased by more than 25-fold worldwide, not least owing to the advent of readily available high-throughput cancer screening techniques. Although the cases of prostate cancer in East Asian countries is much lower than in Western countries, the incidence and mortality rates of prostate cancer have recently grown rapidly in some Asian countries [4].

A cancer's development is characterized by a multistep progression that eventually enables cancer cells to spread to other areas far distant from a given primary tumor mass, which often leads to metastasis. As a result, death in more than 90% of cancer patients is not caused by the primary tumor itself, but occurs as a result of the development of metastases affecting the vital organs of the patient [5]. With regard to the type and stage of tumor, some combination of surgery, radiotherapy, chemotherapy, or immunotherapy treatments can eradicate the tumor or slow its proliferation. Furthermore, such therapy techniques have been found to display a relatively short period of clinical life before being rendered ineffective, while other very potent medicines often exhibit severe side effects on healthy or normal cells and tissues [6, 7].

Consequently, the increasing cancer mortality rate has led many researchers to search for novel, potentially active compounds derived from natural products with potent anticancer activity. Within this wealth of structurally and functionally diverse compounds, bioactive molecules associated with innate immune systems especially may open up new strategies for anticancer therapy [8, 9]. Antimicrobial peptides (AMPs) and proteins constitute an integral part of the innate immune mechanism for defending the host from pathogen infection [10] and are therefore interesting candidates for anticancer agents. The ability of certain AMPs called anticancer peptides (ACPs) to kill cancer cells is believed to derive from the positively charged AMPs playing a main role in the strong binding and selective disturbance of membranes [11]. The ACPs have two basic mechanisms, triggering apoptosis or necrosis in cancerous cells [12]. The killing, based on apoptosis through the mitochondrial lytic system and necrosis via cell membrane lysis, appears to be dependent on the presence of anionic lipids [12]. It is supported by several reports that cationic AMPs, which have effects on bacterial cells but no significant toxicity to healthy mammalian cells, exhibit a broad spectrum of cytotoxic properties against cancerous cells [11].

Crocodilians form an ancient group among the class of reptiles, and typically thrive in aquatic environments containing a high burden of infectious microbes [13]. Although violent fighting with other crocodiles or species occurs frequently, the resulting wounds and lesions appear to heal quickly and there are almost no symptoms from pathogen infections, in spite of the harsh natural conditions [14]. Furthermore, crocodiles rarely seem to show any evidence of cancer symptoms within their average span of life [15]. In the recent years, several studies have demonstrated a surprisingly broad range of biological activity for the blood of the Siamese crocodile (*Crocodylus siamensis*), including antibacterial [14, 16–18], antioxidant [19, 20], anti-inflammatory [19, 21], and wound healing activities [22]. Leukocytes (white blood cells; WBC), one of the major constituents of crocodile blood, belong to the family of effector cells in the innate immune mechanism, which act against pathogens by employing a variety of peptides that exhibit antimicrobial functions [16]. Although eosinophils, basophils, and natural killer cells in particular represent a source of antimicrobial molecules, a variety of other bioactive substances capable of destroying or inhibiting microbial pathogen growth are also expressed in leukocytes [23]. In our previous work, Siamese crocodile Leucrocin I–IV antibacterial peptides were isolated from leukocyte extracts [16]. Leucrocin I–IV showed strong antibacterial properties against *Staphylococcus epidermidis*, *Salmonella typhi*, and *Vibrio cholerae*. The targets for these four antimicrobial peptides were both outer and inner membranes of bacteria. This characteristic of AMPs is important in leading to the hypothesis that AMPs and ACPs share similar molecular principles for inhibiting the viability of cancer cells via cell membranes by electrostatic interactions, disrupting their function and resulting in the death of the cancer cells [10]. Hence, white blood cells derived from Siamese crocodile blood (cWBC) are considered an excellent source of naturally occurring active peptides, which are highly promising to serve as templates for the development of effective remedies for cancer. To determine the property of cWBC against multiple cancer cell lines, the experiment in the recent work was focused on functional analysis of the effects on the proliferation of human lung cancer cells (LU-1), human

prostate cancer cells (LNCaP and PC-3), human breast cancer cells (MCF-7), and human colon cancer cells (CaCo-2). Then, the colony formation, migration, autophagy, ROS signal, and cell cycle progression were evaluated to explain the mechanism of the cWBC on cancer cell inhibition.

## Materials and Methods

### Reagents

Sulforhodamine B (SRB), trichloroacetic acid (TCA), Tris (tris[hydroxymethyl] amino methane), and propidium iodide (PI) were obtained from Sigma-Aldrich (USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and penicillin/streptomycin were obtained from Invitrogen (USA). Trypsin-EDTA was obtained from Mediatech (USA), 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) was obtained from Biolegend (USA), and RNase A was purchased from Merck (Germany).

### Siamese Crocodile Blood Sample Collection

Blood of crocodiles was purchased from Sriracha Moda Farm, Chonburi, Thailand. The whole blood was transferred into a bottle containing EDTA (8 mg/ml) and stored overnight at 4°C. WBCs were collected after the cells in the whole blood sample were allowed to settle in the middle layer (buffy coat). The WBCs obtained were further extracted as described by Pata *et al.* [16]. Briefly, acetic acid (10% (v/v)) was added and mixed with the WBCs, followed by homogenization of the mixture using an ultrasonicator. The cWBC supernatant was obtained after centrifugation for 20 min at 12,000 ×*g* and subsequently kept at −70°C until used in further experiments.

### Noncancerous and Human Cancerous Cell Line Culture

African green monkey kidney cells (Vero), human keratinocytes (HaCaT), lung cancer cells (LU-1), prostate cancer cells (LNCaP and PC-3), breast cancer cells (MCF-7), and colorectal cancer cells (CaCo-2) were obtained from the American Type Culture Collection (ATCC, USA). Each cell line was grown on polystyrene tissue culture dishes and supplemented with DMEM containing 10% (v/v) heat-inactivated FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin and maintained at 37°C in an incubator with a 5% $CO_2$ humidified atmosphere. The cell lines were monitored daily and media were changed every 3−4 days. After attaining 90% confluence, the cancer cells were trypsinized from the dishes and resuspended in growth medium for further studies.

### Cell Viability by MTT Assay

Assessment of the cytotoxicity of cWBC in the noncancerous (Vero and HaCaT) cell lines was performed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [9]. In brief, cells were seeded in 96-well plates at a final density of $1 \times 10^4$ cells/well and cultured overnight at 37°C in a 5% $CO_2$ incubator. Each cell line was then treated with 15.62−500 μg/ml

cWBC, followed by the addition of 150 μl of 0.5 mg/ml MTT solution to each well and incubation for 4 h at 37°C. Afterwards, the medium was removed and the resulting MTT formazan product was solubilized in 100 μl of DMSO. The absorbance values at 550 nm were measured using a microplate reader and the cell viability was evaluated by comparing the measured absorbance with the absorbance of untreated cells. All experiments were done at least in three replicates.

### Cytotoxicity Assessment by SRB Assay

The in vitro cytotoxicity of cWBC was assessed using a procedure based on cell staining with SRB and measurement of the cellular protein content of adherent and suspended cultures in 96-well plates. The SRB assay employed was according to the experiment of Mehta and Pezzuto [24] with minor modifications. First, each cancer cell type was grown in 96-well plates ($5 \times 10^4$ cells/well) either in the absence or presence of 12.5−200 μg/ml cWBC and incubated for 72 h. Each cell line was fixed by adding 100 μl/well of ice cold 20% (w/v) TCA at 4°C for 30 min. After removing the fixative, the plate was washed with tap water 4 times and dried under air. Then, each cell was stained with 100 μl/well of 0.4% (w/v) SRB in 1% acetic acid for 30 min before rinsing off the unbound dye with 1% (v/v) acetic acid 4 times and drying under air. The bound dye was solubilized with 10 mM Tris base (pH 10; 200 μl/well), for 20 min and the optical density was recorded at 531 nm using a Perkin Elmer 2030 multilabel reader. The cytotoxic effect of the compound was calculated using the following formula:

Percentage of inhibition = $100 - (Abs_{test} - Abs_{background})/(Abs_{control} - Abs_{background}) \times 100$

The 50% inhibitory concentration ($IC_{50}$) was determined from dose-dependent curves.

### Morphological Observation of Apoptotic Cells by Phase-Contrast Inverted Microscopy

The morphological study of apoptotic cells was performed according to the method of Chen *et al.* [25] with a few modifications. In brief, cells ($5 \times 10^4$ cells/well) were incubated for 48 h in the absence or presence of 25, 50, 100, and 200 μg/ml cWBC in a 24-well plate. After that, the medium was discarded and each cell line was washed once with PBS, and then fixed with methanol and stained with Giemsa. The morphology of the apoptotic cells was monitored using a phase-contrast inverted microscope at 200× magnification (Zeiss Axiovert 25; Zeiss, Germany).

### Colony Forming Assay

A colony forming assay was constructed to investigate the long-term anticancer activity of cWBC. Therefore, each cancer cell type was seeded in a 24-well plate at about 400 cells/well for 24 h, and subsequently treated with 25, 50, 100, and 200 μg/ml cWBC for 48 h. Then, the medium was removed and the cells were continuously incubated in fresh medium at 37°C in 5% humidified $CO_2$ for a

duration of 10 days, during which the medium was changed once every 3 days. The cells were then rinsed with PBS and fixed for 15 min using methanol:acetic acid (3:1). Afterwards, each cell line was stained with 0.5% crystal violet for 20 min and then washed with PBS, and the number of colonies was counted manually using a stereo microscope, with a colony defined as consisting of at least 50 cells.

### Scratch Migration Assay

A scratch migration assay was conducted to estimate the "wound-healing" propensity, following the protocol of Liang *et al.* [26]. Therefore, each cell type was grown in a 6-well plate to 80% confluence and a scratch was created using a 200 μl pipette tip, followed by removal of floating cells and debris by washing with PBS. The cells were then photographed under a phase-contrast inverted microscope (time = 0) and subsequently treated with 25, 50, 100, and 200 μg/ml cWBC or incubated in the absence of cWBC for 24 h. Afterwards, the cells were gently rinsed with PBS, and the scratch wound area was photographed at the end of the incubation period of 24 h to count the number of cells that had migrated across the scratched lines. The percentage of control was expressed by assigning a value of 100% to the untreated wells. Since cWBC was found to exhibit the highest $IC_{50}$ values against LNCaP cells, this cell line was selected for further experiments.

### Measurement of Intracellular Reactive Oxygen Species (ROS) Levels

ROS levels were measured using $H_2DCFDA$ following the protocol of Liu *et al.* [27]. In brief, LNCaP cells ($2 \times 10^5$ cells/well) were treated with cWBC (25, 50, 100, and 200 μg/ml) for 24 h. After that, the cells were collected and washed with PBS. The cell suspension in PBS was then incubated with 10 μM $H_2DCFDA$ in the dark for 30 min at 37°C before measuring the ROS levels with a flow cytometer (BD FACSCanto II, USA). The ROS level was presented as the fluorescence of the treated cells relative to the fluorescence of the untreated (control) cells.

### Determination of Cell Cycle

To analyze the distribution of LNCaP cells in different phases of the cell cycle, the DNA content of the cells was quantified by PI staining and flow cytometry. Briefly, LNCaP cells ($8 \times 10^5$ cells/well) were grown in a 6-well plate and incubated with 25, 50, 100, and 200 μg/ml cWBC for 24 h. After incubation, the cells were harvested, suspended in fresh medium, and centrifuged at 1,500 ×*g* for 5 min. The cell pellets were washed with PBS and fixed by adding 500 μl of 70% ice-cold ethanol overnight at 4°C. Then, the cells were washed with PBS and incubated with 600 μl of PBS (containing 100 μg of PI and 50 μg of RNase A) in the dark for 30 min at 37°C. Afterwards, flow cytometric analysis was carried out using a BD FACSCanto II flow cytometer. The percentage of cells in different phases of the cell cycle was evaluated by MultiCycle DNA Content and Cell Cycle Analysis software.

### Real-Time PCR

LNCaP cells were grown in a 6-well plate to a final density of $5 \times 10^5$ cell/well and left for 24 h in the absence or presence of cWBC at different concentrations (25, 50, 100, and 200 μg/ml). Then, the cancer cells were trypsinized from the plate for further RNA extraction. Total RNA was extracted by a TRIzol reagent (Invitrogen, USA). The oligo(dT18) primer was used to initiate cDNA synthesis from 1 μg of total derived RNA, following the RevertAid First-Strand cDNA synthesis kit manual (Fermentas, USA). The real-time PCR was carried out with the LightCycler 480 real-time PCR system (Roche, Switzerland) using SYBR Green PCR master mix (Roche, Switzerland). The primer sequences and annealing temperature of each gene are shown in Table 1. The expression levels of Bcl-2, XIAP, caspase-3, and caspase-8 were used as markers to identify the induction of apoptosis in cells,

**Table 1.** Primers sequences for real-time PCR.

| Gene | Sequence (5′ → 3′) | Annealing (°C) | Product size (bp) | Reference sequence |
|---|---|---|---|---|
| Bcl-2 | F_TGTGGCCTTCTTTGAGTTCGGTG | 63 | 114 | NM_000633.2 |
| | R_GTGCCGGTTCAGGTACTCAGTCA | | | |
| XIAP | F_GGGGTTCAGTTTCAAGGAC | 58 | 91 | LT727091.1S |
| | R_TGCAACCAGAACCTCAAGTG | | | |
| Caspase-3 | F_GTGGAATTGATGCGTGATGT | 55 | 113 | NM_001354777.1 |
| | R_ACAGGTCCATTTGTTCCAAAA | | | |
| Caspase-8 | F_AGAGTCTGTGCCCAAATCAAC | 55 | 78 | XM_005246894.3 |
| | R_GCTGCTTCTCTCTTTGCTGAA | | | |
| Cyclin-B1 | F_TTGATACTGCCTCTCCAAGCC | 55 | 122 | NM_001354844.1 |
| | R_AGCTCCATCTTCTGCATCCAC | | | |
| P53 | F_AGATAGCGATGGTCTGGC | 55 | 381 | LT739496.1 |
| | R_TTGGGCAGTGCTCGCTTAGT | | | |
| β-Actin | F_GCATGGGTCAGAAGGATTCCT | 55 | 106 | NM_001101.4 |
| | R_TCGTCCCAGTTGGTGACGAT | | | |

whereas the expression levels of p53 and cyclin-B1 were used as markers to investigate cell cycle progression. The housekeeping gene β-actin was used as the normalized control to find out whether there was any change in the level of expression. The relative change in gene expression from the real-time quantitative PCR data was presented by the comparative $2^{-\Delta\Delta CT}$ method [28].

### Statistical Analysis

All data are expressed as the mean ± SD. A one-way analysis of variance followed by Dunnett's test and Duncan's multiple range tests were used to analyze the experimental data. Values of $p < 0.05$ were statistically significant. All experiments were carried out at least in triplicate.

## Results

### Effect of cWBC on the Viability of Noncancerous Cells

Prior to the assessment of the putative anticancer ability of cWBC, the cytotoxicity of cWBC was evaluated towards noncancerous (Vero and HaCaT) cell lines, for which the results are illustrated in Figs. 1A and 1B. The cWBC had no
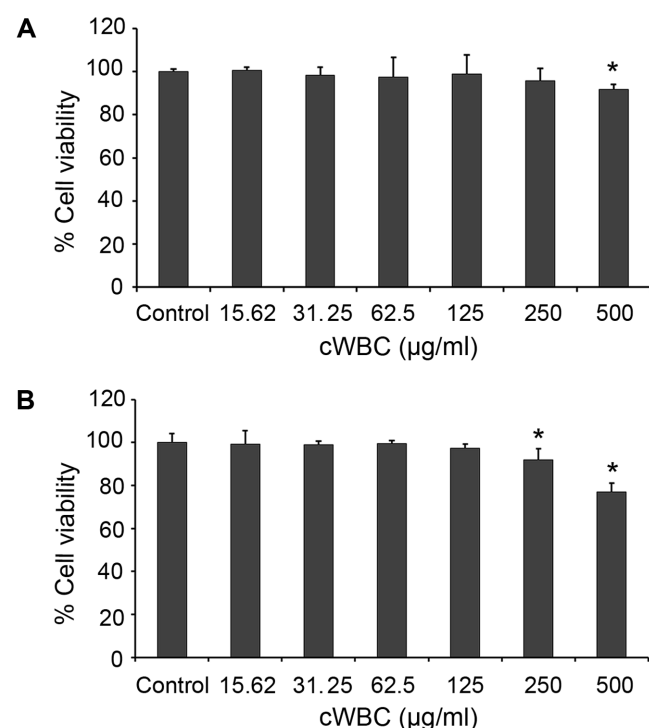
effect on Vero cell viability within a range of 15.62−250 µg/ml cWBC. When the concentration was increased to around 500 µg/ml, a slight decrease in the percentage of cell viability was observed (Fig. 1A). In the same way, cWBC did not show any significant cytotoxic effect on the viability of HaCaT cells in a concentration range of 15.62−125 µg/ml, whereas higher concentrations (250 µg/ml) of cWBC led to a slightly decreased percentage of viability (Fig. 1B).

### Effect of cWBC on the Viability of Cancerous Cells

The anticancer efficiency of cWBC on different cancer cell lines was determined using the SRB method, measuring cellular protein content. A significant cytotoxic effect induced by cWBC was observed after 72 h of treatment, resulting in a concentration-dependent decrease of cell viability in all tested cancerous cells (Fig. 2). The $IC_{50}$ values for LU-1, LNCaP, PC-3, MCF-7, and CaCo-2 were 101.0, 51.4, 101.05, 95.93, and 70.34 µg/ml, respectively. At 100 µg/ml of cWBC, the proliferation of LU-1, LNCaP, PC-3, MCF-7, and CaCo-2 cells was significantly inhibited by 49.5%, 90.8%, 49.5%, 52.1%, and 69.0%, respectively (Fig. 2).

### Effect of cWBC on Cancer Cell Colony Formation

The colony forming assay was carried out to evaluate the long-term effects of cWBC on the proliferation of LU-1, LNCaP, PC-3, MCF-7, and CaCo-2 cells. The colony count indicated that cWBC induced a dose-dependent decrease in the colony formation ability in all tested cell lines (Fig. 3A). Dramatic effects of cWBC on the colony formation ability were observed at a concentration of 100 µg/ml (in all tested cells), and the formation of colonies was found to be completely inhibited at 200 µg/ml. Notably, 50 µg/ml cWBC resulted in almost complete inhibition of colony



**Fig. 1.** Effect of Siamese crocodile white blood cell extract (cWBC) on the viability of noncancerous cells, measured by MTT assay.
(**A**) Vero and (**B**) HaCaT cells were incubated with cWBC at different concentrations (15.62, 31.25, 62.5, 125, 250, and 500 µg/ml) for 24 h. Each bar represents the mean ± SD ($n = 8$). *Significant at $p < 0.05$.
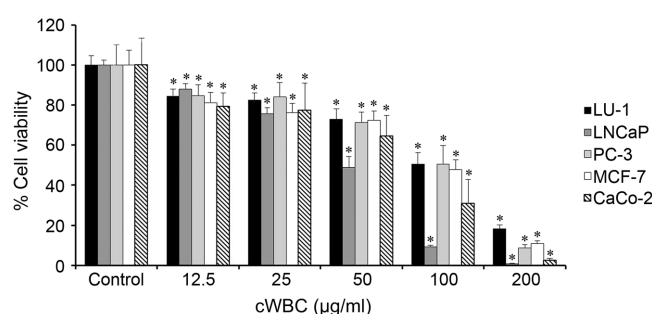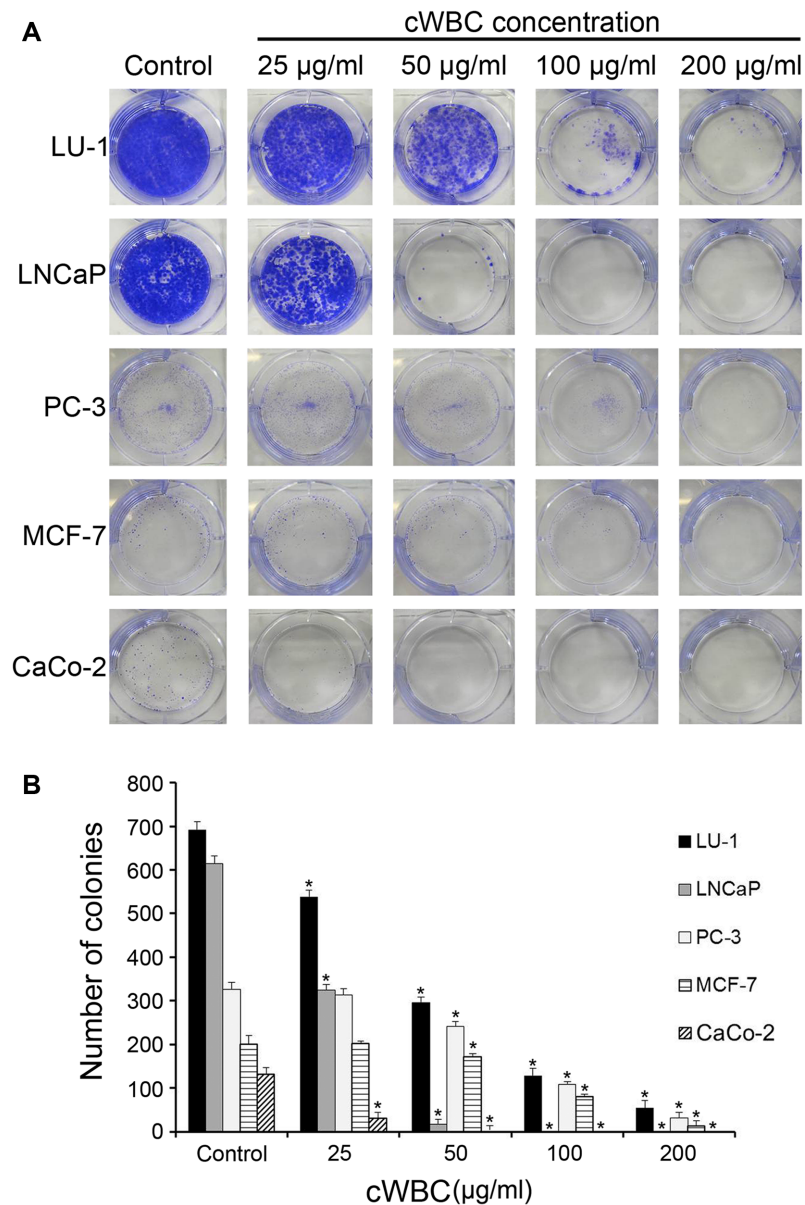


**Fig. 2.** Effects of cWBC on the viability of LU-1, LNCaP, PC-3, MCF-7, and CaCo-2 cells, determined by the SRB assay.
Cells were incubated with 12.5, 25, 50, 100, and 200 µg/ml of cWBC for 72 h. Results are expressed as the percentage of control. Each bar represents the mean ± SD ($n = 6$). *$p < 0.05$; significant difference from control (one-way ANOVA followed by Dunnett's test).

**Fig. 3.** Effect of cWBC on the colony formation ability of LU-1, LNCaP, PC-3, MCF-7, and CaCo-2 cells.
Cells were treated with cWBC at different concentrations (25, 50, 100, and 200 μg/ml) for 48 h. After 48 h, the inhibitor was removed and the cells were grown in their respective culture media alone for 10 days. (**A**) Representative images of colonies in crystal violet. (**B**) Number of colonies counted. The data are presented as the mean ± SD ($n = 3$). *$p < 0.05$; significant difference compared with the control group.

formation in the cases of LNCaP and CaCo-2 cells. The total numbers of colonies for LU-1, LNCaP, PC-3, MCF-7, and CaCo-2 cells were found to be 127, 1, 108, 81, and 0 after challenge with cWBC at a concentration of 100 μg/ml, respectively (Fig. 3B).

**Anticancer Cell Migration**
The scratch migration assay was conducted to assess the

effect of cWBC on the cell migration ability (Fig. 4). Figs. 4A and 4F display the migration and the corresponding percentages of migrated LU-1 cells after exposure to cWBC. After scratching, the remaining cells were exposed to cWBC at different concentrations and inspected microscopically over time, as the cells migrated to fill the damaged area. Migration of untreated LU-1, LNCaP, and PC-3 cells (Figs. 4A−4C) to heal the scratch occurred after 24 h,
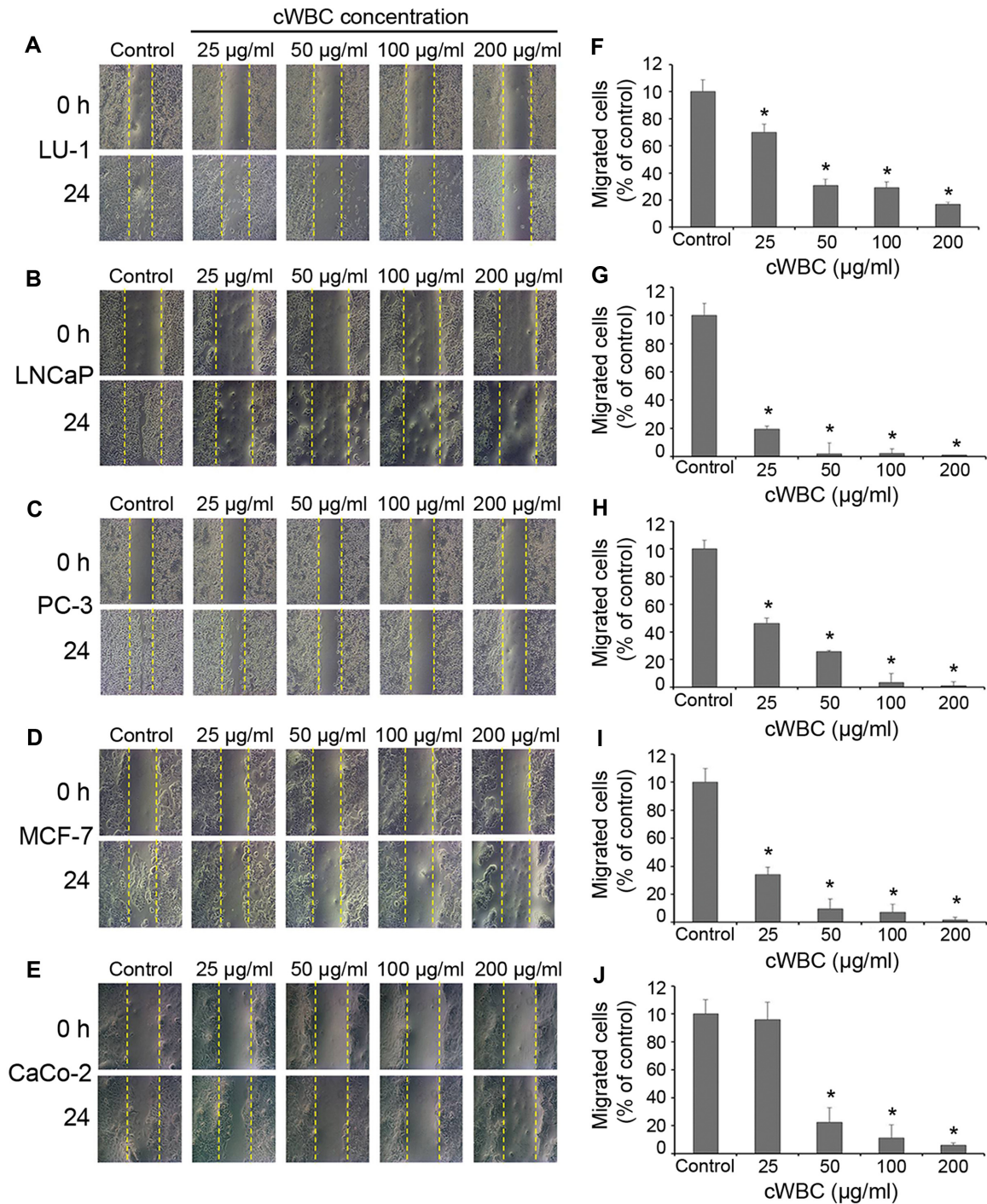
**Fig. 4.** Effect of cWBC on the migration of LU-1, LNCaP, PC-3, MCF-7, and CaCo-2 cells.
(**A–E**) Images showing inhibition of cWBC on LU-1, LNCaP, PC-3, MCF-7, and CaCo-2 cells, respectively. (**F–J**) Graphical representation of the percentage of migrated cells. The data are presented as the mean ± SD ($n = 3$). *$p < 0.05$; significant difference compared with the control group.

whereas MCF-7 and CaCo-2 cells (Figs. 4D and 4E) did not completely cover the scratched area within the same timeframe. In the treated group, all tested cancerous cells demonstrated significant differences in the percentage of migrated cells compared with the respective controls. Specifically, treatment of LU-1, LNCaP, PC-3, and MCF-7
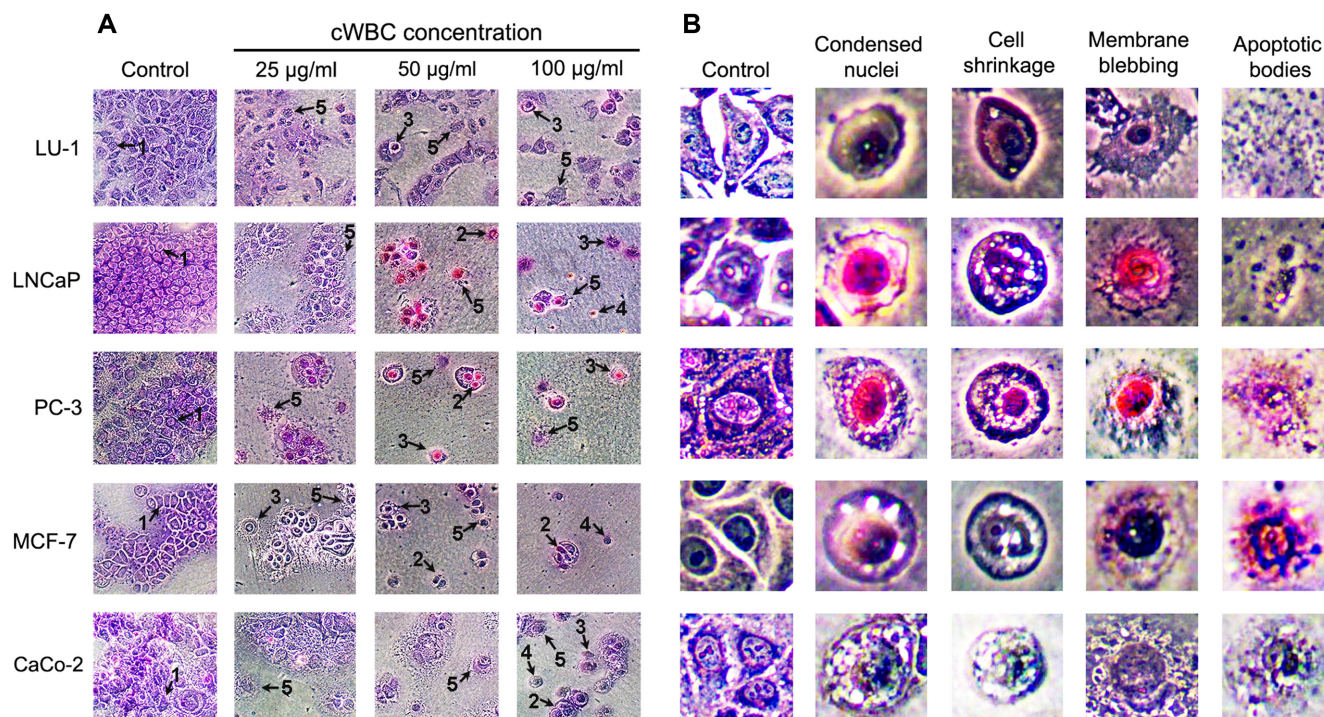
**Fig. 5.** Morphological appearance of LU-1, LNCaP, PC-3, MCF-7, and CaCo-2 cells treated with cWBC, under a phase-contrast inverted microscope.

Giemsa staining; 1: untreated control; 2: condensed nuclei; 3: cell shrinkage; 4: apoptotic bodies; and 5: membrane blebbing as evidence for apoptosis (**A**). The enlargement of each morphological hallmark of apoptosis in all cancer cells is shown in (**B**). Figures shown were obtained from at least three independent experiments with similar parameters.

cells with 25 μg/ml cWBC led to a significantly diminished quantity of migrated cells at 29.9%, 80.7%, 53.8%, and 69.5% of the control, respectively. At a cWBC concentration of 50 μg/ml, CaCo-2 cells also showed a significant decrease in migrated cells at 77.6% of the control. Because they exhibited the greatest sensitivity to cWBC treatment, the LNCaP cells were selected for all subsequent studies.

### Effect of cWBC on the Morphological Changes of Cancer Cells

Morphological changes of cells can be applied as an early indicator of responses to apoptotic stimuli [29]. Hence, after incubation with 25, 50, and 100 μg/ml of cWBC, morphological alterations in LU-1, LNCaP, PC-3, MCF-7, and CaCo-2 cells were monitored in comparison with untreated cells used as controls (Fig. 5). The results indicate that challenge of cancer cells with cWBC for 48 h induces the formation of condensed nuclei (arrow No. 2), cell shrinkage (arrow No. 3), apoptotic body formation (arrow No. 4), and membrane blebbing (arrow No. 5), as compared with control cells (arrow No. 1; normal cell) (Fig. 5A). The

enlargement of each morphological hallmark of apoptosis in all the cancer cells shown in Fig. 5B confirms that cWBC induced marked morphological changes in all the cancer cell lines investigated.

### Effect of cWBC on Reactive Oxygen Species Generation in Cells

The induced ROS level in LNCaP cells was determined through interaction with a specific fluorescent dye, $H_2DCFDA$, which results in enhanced fluorescence intensity in response to reactive metabolite generation within the cells [30]. Fig. 6A demonstrates that the extent of ROS expression was significantly elevated, compared with the untreated cells ($p < 0.05$). Incubation with cWBC (25, 50, 100, and 200 μg/ml) resulted in significantly elevated levels of ROS in a concentration-dependent manner, totaling up to 2.00, 2.64, 3.12, and 4.29 times that of the control, respectively (Fig. 6B).

### Effect of cWBC on Cell Cycle Arrest

To determine the effect of cWBC on LNCaP cell cycle phases, the intracellular DNA content was measured by

**A**

Control

cWBC 25 µg/ml    cWBC 50 µg/ml

cWBC 100 µg/ml    cWBC 200 µg/ml

**B**
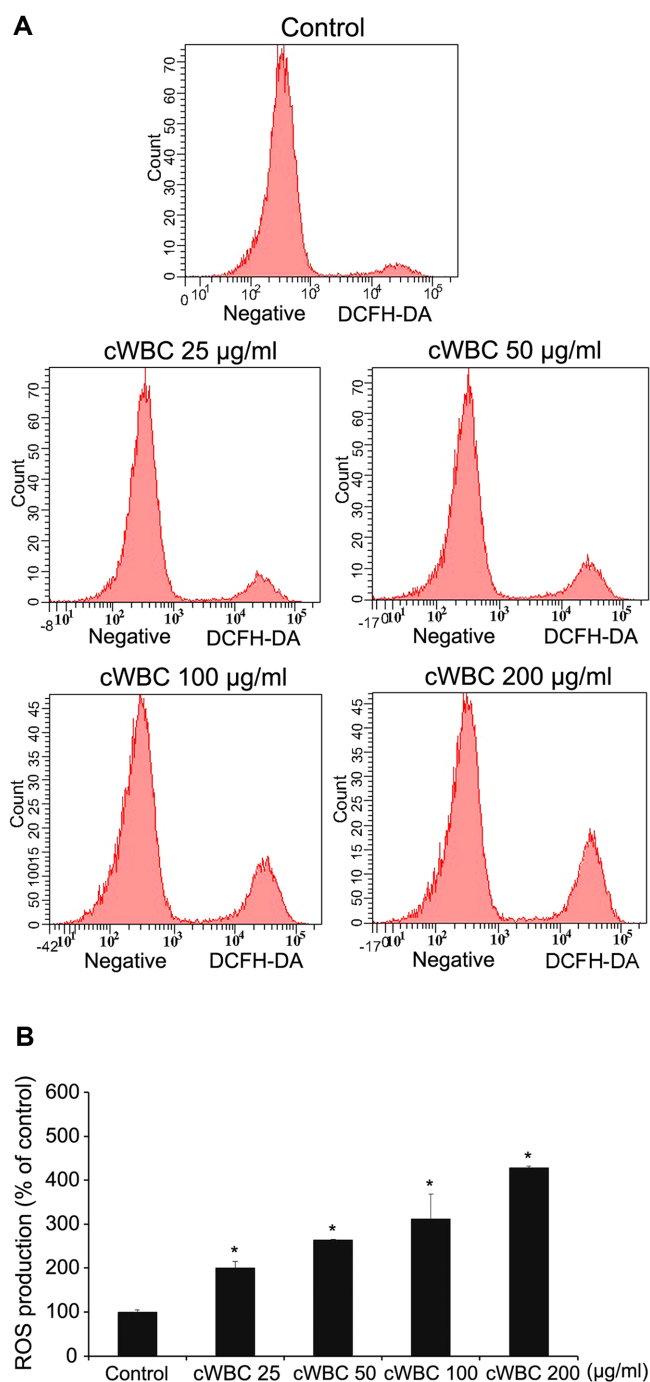
**Fig. 6.** Intracellular ROS generation in LNCaP cells induced by cWBC.
After treatment with cWBC for 24 h, cells were incubated with 10 µM $H_2DCFDA$ for 30 min and then immediately subjected to flow cytometric analysis. (**A**) Chromatogram of flow cytometry. ROS generation was expressed as the ratio of relative fluorescence intensity compared with the control group (**B**). The data are presented as the mean ± SD ($n = 3$). *$p < 0.05$; significant difference compared with the untreated cells (one-way ANOVA followed by Dunnett's test).

flow cytometry (Fig. 7). Fractions of cells in the S and $G_2/M$ phases were found to be significantly increased, together with significant reduction in the $G_0/G_1$ phases after 24 h of cWBC treatment (Fig. 7A). Interestingly, incubation with cWBC (25, 50, 100, and 200 µg/ml) enhanced S phase arrest by 8.1 ± 0.3%, 10.4 ± 0.2%, 13.1 ± 0.5%, and 14.9 ± 0.7%, respectively (Fig. 7B). In addition, cWBC treatment at 25–200 µg/ml for 24 h induced an increase in the number of cells in the $G_2/M$ phase, while the number of cells in the $G_0/G_1$ phase of the cell cycle declined when compared with the untreated control (Fig. 7B). These data demonstrate that the cWBC treatment inhibited S-$G_2/M$ transition in the cells, leading to cell population growth arrest in LNCaP cells.

**Effect of cWBC on Expression Levels of Apoptotic and Cell Cycle Genes**

The expression levels of Bcl-2, XIAP, caspase-3, and caspase-8 in LNCaP cells treated with various concentrations of cWBC were investigated to confirm the induction of the apoptosis process. As shown in Figs. 8A–8D, cWBC at all concentrations (25–200 µg/ml) strongly and significantly altered the apoptosis inhibitors, namely Bcl-2 and XIAP, in LNCaP cells by decreasing their expression level. Conversely, the treatment of cWBC inhibited the growth of LNCaP cells and led to cell death by dose-dependently increasing the expression level of caspase-3, caspase-8, and p53 genes. In addition, modifications of cell cycle genes by an increase in p53 and a decrease in cyclin-B1 expression were observed when treating cancer cells with various concentrations of cWBC (Figs. 8E and 8F).

## Discussion

According to estimations of the World Health Organization, the number of new cancer diagnoses and cancer-related deaths increased to around 14.1 million and 8.2 million, respectively, in 2012, as compared with 12.7 million new cases and 7.6 million deaths in 2008 [3, 31]. Among these, breast, lung, prostate, and colon cancers have been associated with the highest prevalence worldwide, together accounting for about 50% of the global incidence burden [3]. Current cancer treatment is often accompanied with a number of detrimental side effects; for instance, cardiotoxicity and congestive heart failure in the case of doxorubicin [32]. In addition, the success rate of chemotherapy may be further limited by the development of drug resistance, rendering the development of alternative therapeutics an urgent task for pharmaceutical research.

Owing to the broad range of biological properties of
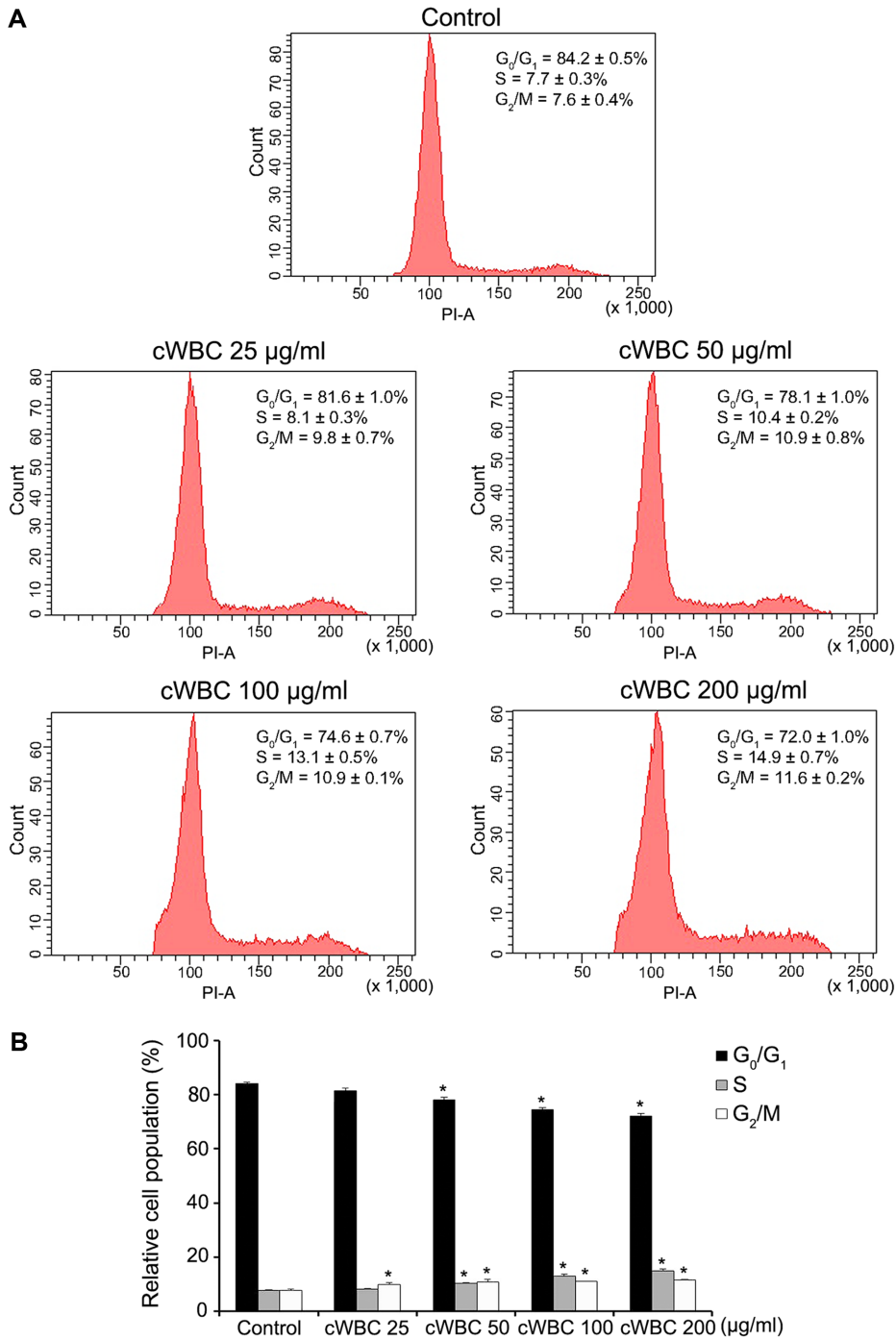
**Fig. 7.** Alterations of the cell cycle measured in LNCaP cells after treatment with cWBC.
(**A**) Panel showing the representative profiles of cell cycle status for cancer cells. (**B**) Graphical representation for $G_0/G_1$, S, and $G_2/M$ peak patterns of cancer cells treated with cWBC at different concentrations. Percentages of cell count of each cell cycle phase in each treatment group are shown as the mean ± SD ($n = 3$). *$p < 0.05$; significant difference versus untreated cells (one-way ANOVA followed by Dunnett's test).

blood, the individual components of blood are considered an excellent source of naturally occurring, active peptides. Previously, the cationic peptides Leucrocin I−VI [16, 18, 33], cathelicidin [34], scolopendrasin VII [8], and hepcidin [35], have been isolated and were shown to exhibit potent antimicrobial activity. These small and generally amphipathic
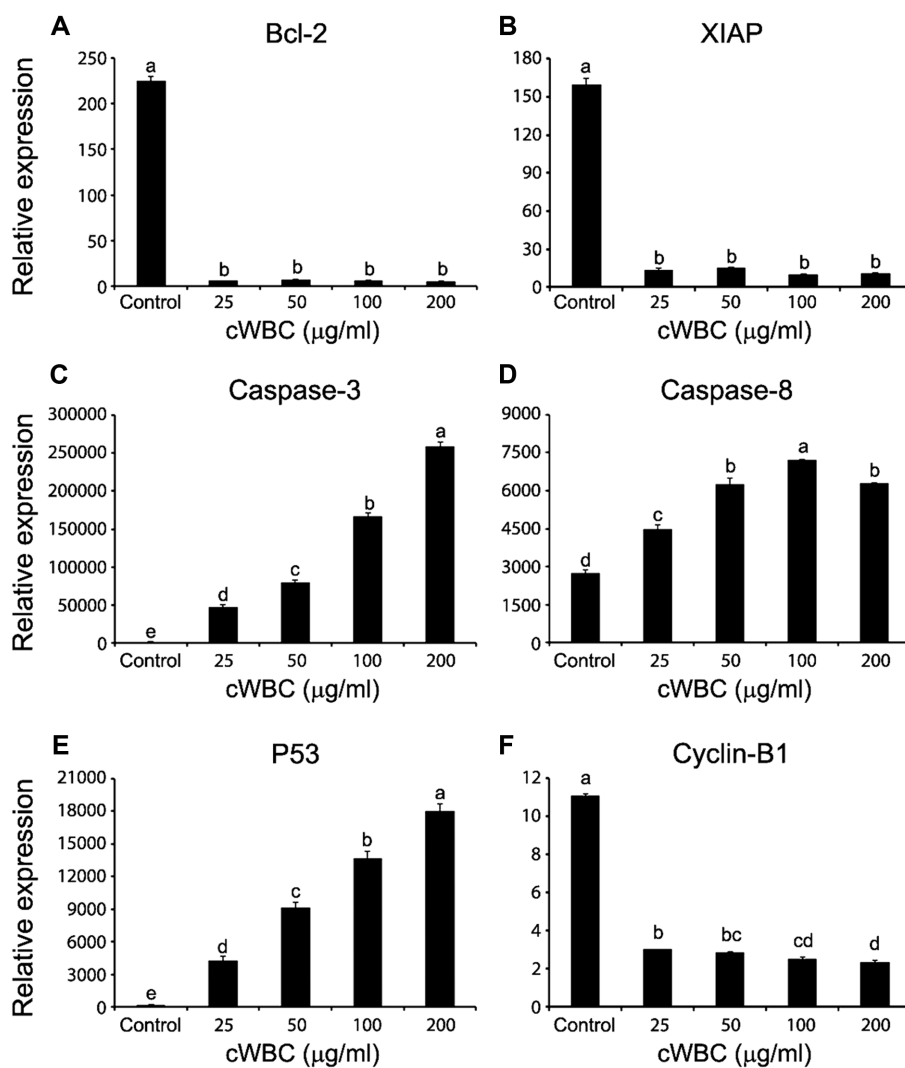
**Fig. 8.** Effect of cWBC on Bcl-2 (**A**), XIAP (**B**), caspase-3 (**C**), caspase-8 (**D**), p53 (**E**), and cyclin-B1 (**F**) mRNA expression in LNCaP cancer cells as determined by real-time PCR.

Each bar represents the mean and standard deviations in at least biological triplicates from the different experiments. Different letters (a−d) on the top of each bar indicate statistically significant differences ($p < 0.05$).

molecules constitute a pivotal mechanism of the immune defense with low antigenicity [36] and contain cationic and hydrophobic residues that facilitate the interaction with microbial membranes [37] through nonspecific interactions with membrane lipids [38]. Notably, the negative charge of the bacterial cell membrane surface is a property also shared by cancer cells [11], leading to the hypothesis that AMPs and ACPs might share similar molecular principles for activity and selectivity [10]. Recently, Patathananone *et al.* [9, 39] have reported WBC extract from *C. siamensis* exhibiting anticancer activity by inducing apoptosis, and inhibiting the migration and invasion process in HeLa cells. In light of

the global threat of cancer towards human health, these findings motivated us to explore the anticancer activity of cWBC against human lung cancer (LU-1), prostate cancer (LNCaP, PC-3), breast cancer (MCF-7), and colorectal cancer (CaCo-2) cell lines in more detail.

The fundamental abnormality resulting in the development of cancer has been ascribed to the continual unregulated proliferation of cells [40]. Hence, the first part of this research was designed to elucidate the effect of cWBC treatment on the proliferation propensity of LU-1, LNCaP, PC-3, MCF-7, and CaCo-2 cancer cells using the SRB assay. The results demonstrate that cWBC exhibited dose-dependent

anticancer property against several human cancerous cells with a degree of cytotoxicity in the order of LNCaP > CaCo-2 > MCF-7 > PC-3 > LU-1. Moreover, cWBC displayed potential long-term cytotoxic effects, as confirmed via colony formation assays in which treatment of cells with cWBC resulted in a largely similar inhibitory effect on the colony formation ability. Notably, cWBC displayed superior inhibitory ability on the colony formation of LNCaP cells in comparison with the other cancer cell lines. Therefore, the collected data indicate that cWBC shows a broad spectrum of antiproliferative activity against various types of cancerous cells, and further suggests cWBC to be useful for providing long-term inhibitory effects on cancer cell growth, which is in excellent agreement with the observed antiproliferative activities of cWBC against HeLa cancer cells reported by Patathananone *et al.* [39].

The ability to migrate from the primary tumor to a distant secondary site constitutes another characteristic property of cancer cells, and is generally regarded as one of the most problematic and harmful aspects of the disease [32]. Thus, a scratch cell migration assay was carried out to elucidate the possible influence of cWBC on cancer cell migration, revealing that cWBC was able to impede the migration of all investigated cancer cell lines (LU-1, LNCaP, PC-3, MCF-7, and CaCo-2) in a dose-dependent manner. It is speculated that this peculiar effect of cWBC may be associated with the reduction of the activity and protein expression levels of specific gelatinases (MMP-2, MMP-9) [9]. In this context, the disruption of vascular endothelial growth factors and integrin-signaling cascades by bioactive agents would also result in diminished MMP-2 and MMP-9 activity [9].

The ability to induce the programmed death of individual cells, a process also known as apoptosis, represents a significant mechanism for the eradication of damaged and mutated cells, whereas its dysregulation may lead to carcinogenesis [41]. Thus, apoptosis induction in cancer cells is regarded as an attractive strategy to combat inadvertent malignancy [41], and constitutes a key property of antitumor drug candidates [42]. Apoptosis itself is recognized as a general mode of cell death characterized by unique biochemical and morphological features [27]. Therefore, in order to assess the effect of cWBC treatment on the apoptotic process in cancer cells, the resulting alterations of cellular morphology were closely monitored by phase-contrast inverted light microscopy. Several morphological changes distinctive to apoptotic cells and commonly used for the identification of apoptosis [43, 44] were found to be induced by cWBC treatment of LU-1, LNCaP, PC-3, MCF-7, and CaCo-2 cells; specifically, membrane blebbing, cell

shrinkage, chromatin condensation, and the formation of apoptotic bodies. These findings closely resemble observations made by Patathananone *et al.* [39], who reported that cWBC can induce apoptosis in HeLa cells via both caspase-dependent and -independent pathways. Based on these findings, and in agreement with the antiproliferation (SRB assay) results, it is concluded that cWBC effectively induces cancer cell apoptosis.

Because prostate cancer (LNCaP) cells showed the highest susceptibility towards cWBC extract, LNCaP cells were selected as the representative cancer cell line for further elucidation of the mechanisms leading to cWBC treatment-induced cell death. In this study, the molecular mechanistic demonstration of the ability of cWBC to induce apoptotic processes was associated with a decrease in expression of Bcl-2 and XIAP and an increase in expression of caspase-3 and caspase-8. Bcl-2 is a member of the protein family that plays a critical role in regulating apoptosis via a mitochondria-dependent pathway [45]. XIAP (X-linked inhibitor of apoptosis protein) plays a critical role of anchoring the inhibitor onto the caspase, which stops apoptotic cell death [41]. The results of real-time PCR showed a decrease of the anti-apoptotic Bcl-2 and XIAP mRNA expression level in LNCaP cells treated with cWBC; hence the expression levels of caspase-3 and caspase-8 were significantly increased by cWBC at all treatment concentrations. Caspase-8 is involved in both extrinsic and intrinsic apoptotic signaling pathways, and functions via caspase-3 and Bid proteolytic cleavage [46]. The literature has demonstrated that in many human cancers, caspase-8 is inactivated, leading to progression and resistance of cancer cells to chemotherapeutics [46]. Taken together, our data show that cWBC decreases the Bcl-2 and XIAP expression levels and also increases caspase-3 and caspase-8 levels of gene expression, leading to the apoptotic cell morphological changes and dose-dependent inhibition of the proliferation of LNCaP.

Moreover, a pivotal role in the promotion of cellular apoptosis has been ascribed to excessive oxidative stress [47]. Although constituting vital signaling molecules involved in the regulation of diverse cellular functions, the profuse accumulation of ROS is known to induce oxidative stress, and has been associated with promoting a variety of different diseases, including the pathogenesis of inflammation-related cancers [48, 49]. On the other hand, increased ROS-derived stress in cancerous cells may also provide a biochemical basis for the development of novel therapeutic strategies exploiting these characteristics. Hence, by increasing ROS expression in the cell, suitable pharmacological agents

may be able to stimulate the natural apoptotic process to kill cancer cells [27, 50, 51]. In a recent study, a significant overproduction of ROS was observed in LNCaP cells after treatment with cWBC. This finding adds support to the assumption that sufficiently high ROS levels induce apoptosis by triggering pro-apoptotic signaling molecules within the tumor [51]. Furthermore, the results indicate that at high concentrations of cWBC, the apoptotic process in LNCaP cells is correlated with an increase of ROS concentration in a dose-dependent manner, and cWBC may therefore exert a pro-oxidant effect in LNCaP cancer cells.

The cycle of cells is governed by a complex series of signaling pathways by which cell growth, DNA replication and cell division are controlled. The cell cycle regulates genomic replication and cell division [52], as well as the two main checkpoints, $G_1/S$ and $G_2/M$ transitions, which permit the cells to control any modification in DNA content. Checkpoint loss leads to genomic instability and carcinogenesis induction, whereas cell cycle arrest causes a stopping point in the cell cycle, where it no longer participates in processes related to cell duplication and division. Thus, the triggering of cell cycle arrest in cancer cell lines constitutes a crucial strategy in the most prevalent methods to inhibit or limit the spread of cancerous cells [53].

The recent work indicates that treatment with cWBC caused S and $G_2/M$ phase arrest in LNCaP cells, resulting in the initiation of apoptosis. The cWBC-induced cell S phase arrest in the cell cycle may be associated with the reduction of DNA synthesis, by initiating DNA damage and causing replication fork arrest [54]. The cell cycle arrest induction at the $G_2/M$ phase by cWBC may involve an increase in p53 and a decrease in cyclin-B1 mRNA expression level (Figs. 8E and 8F). The literature reveals that the $G_2$ checkpoint is regulated by activation of multiple processes that act together to inhibit the activity of the cyclin-B1/Cdc2 kinase complex [45]. In addition, several researchers have indicated that p53 plays a crucial role in $G_2$ checkpoint regulation [45, 55]. Regarding the activity to inhibit DNA replication, cationic peptides derived from crocodile leukocyte extract have previously been demonstrated to exhibit specific DNA binding activity depending on the charge of the peptide [18, 33]. This evidence suggests that crocodile crude leukocyte extract contains a mixture of cationic peptides. After these crude cationic peptides bind to DNA, stopping or arresting at the S phase occurs. This binding inhibits RNA synthesis and DNA replication in cells and in cell-free systems, which might result in the increase of the $G_2/M$ phase [56].

Taken collectively, the data gathered in this study provide strong evidence for the potent anticancer activity of cWBC, which is thought to be related to several active peptides contained in the extract. The experiments conducted demonstrate that cWBC exerts anticancer property against five human cancerous cells, namely LU-1, LNCaP, PC-3, MCF-7, and CaCo-2, by reducing the viability and colony formation ability of the cancer cells, inducing apoptosis, inhibiting cancer cell migration, increasing intracellular ROS levels, and inducing cell cycle arrest. Moreover, no evidence for cytotoxic effects against normal cells could be found. Recent publications have revealed that *C. siamensis* cWBC, and the purified peptides derived from it, contain antibacterial peptides with remarkable activity against various pathogenic bacterial strains [16]. The fundamental mechanisms of the antibacterial activity of cWBC and the purified antibacterial peptides contained may be closely related to the mechanisms associated with the observed anticancer activity. Basic differences exist between the cell membranes of normal cells and cancer cells, enabling the ability of certain antibacterial peptides to kill cancer cells (ACPs), but to spare healthy cells. In this context, electrostatic interactions between cationic ACPs and anionic cell membranes are supposed to be a crucial factor in the selective killing of cancer cells by these peptides. The membranes of cancer cells normally provide a net negative charge that is higher than the normal abundance of anionic molecules on the surface, such as phosphatidylserine in cellular membranes and glycosylated mucins. Moreover, the negative membrane potential of cancer cells may also contribute to the selective cytotoxic activity of ACPs [11]. Since cWBC did not display any toxicity towards the noncancerous Vero and HaCaT cell lines, it might serve as a useful starting material in the development of novel pharmaceuticals for the treatment of various types of cancers.

## Acknowledgments

## Conflict of Interest

The authors have no financial conflicts of interest to declare.

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