Inflammation, Vol. 40, No. 1, February 2017 (© 2016)
DOI: 10.1007/s10753-016-0471-7

ORIGINAL ARTICLE

Protective Effect of Crocodile Hemoglobin and Whole Blood Against Hydrogen Peroxide-Induced Oxidative Damage in Human Lung Fibroblasts (MRC-5) and Inflammation in Mice

Santi Phosri,[1,2] Nisachon Jangpromma,[1,3] Rina Patramanon,[1,2] Bunkerd Kongyingyoes,[4] Pramote Mahakunakorn,[5] and Sompong Klaynongsruang[1,2,6]

Abstract—A putative protective effect of cHb and cWb against H_2O_2-induced oxidative damage was evaluated in detail using MRC-5 cells. In addition, the carrageenan (Carr)-induced mouse paw edema model and the cotton pellet-induced granuloma model were employed to examine the *in vivo* anti-inflammatory activity of cHb and cWb in mice. It was demonstrated that both cHb and cWb treatments significantly increased cell viability and inhibited morphology alterations in MRC-5 cells exposed to H_2O_2. Orally administered cHb and cWb significantly reduced Carr-induced paw edema volume and cotton pellet-induced granuloma formation. Moreover, cHb and cWb decreased the expression levels of important pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α), while only cWb was found to increase the expression of the anti-inflammatory cytokine IL-10 significantly. Finally, the activity of antioxidant enzymes (SOD, CAT, and GPx) in the liver improved after cHb and cWb treatment under acute and chronic inflammation. Taken collectively, the results of this study suggest that both cHb and cWb protect against hydrogen peroxide-induced damage in fibroblast cells. Moreover, cHb and cWb were found to exhibit anti-inflammatory activity in both the acute and chronic stages of inflammation and appear to enhance antioxidant enzyme activity and decrease lipid peroxidation in the livers of mice. Therefore, this study indicates that cHb and cWb have great potential to be used in the development of dietary supplements for the prevention of oxidative stress related to inflammatory disorders.

KEY WORDS: crocodile; antioxidant; anti-inflammatory; cytokines; animal models.

INTRODUCTION

Inflammation is the result of a complex network of biological immune responses to tissue injury or infection, resulting in the secretion of a variety of pro-inflammatory cytokines, such as interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α). These cytokines play an important role in acute and chronic inflammation [1–3] and therefore have received considerable attention in the target-driven development of anti-inflammatory drugs. Although naturally occurring bioactive compounds with anti-inflammatory properties represent a valuable and sustainable source for novel therapeutic

Santi Phosri and Nisachon Jangpromma contributed equally to this work.

[1] Protein and Proteomics Research Center for Commercial and Industrial Purposes (ProCCI), Faculty of Science, Khon Kaen University, Khon Kaen, 40002, Thailand
[2] Department of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen, 40002, Thailand
[3] Office of the Dean, Faculty of Science, Khon Kaen University, Khon Kaen, 40002, Thailand
[4] Department of Pharmacology, Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand
[5] Department of Pharmacognosy and Toxicology, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, 40002, Thailand
[6] To whom correspondence should be addressed at Department of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen, 40002, Thailand. E-mail: somkly@kku.ac.th

agents against diseases related to inflammation, their discovery requires reliable and generalizable models to assess *in vivo* anti-inflammatory activity. In this context, both Carr-induced mouse paw edema and cotton pellet-induced granuloma formation in mice have been recognized as suitable models for the *in vivo* evaluation of potential anti-inflammatory drugs. While the Carr model can be used to assay acute inflammatory states, the cotton pellet model induces chronic inflammation, including the infiltration of phagocytes, the formation of free radicals, and the release of inflammatory mediators [4].

A variety of reactive oxygen species (ROS), such as the superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) and reactive nitrogen species (RNS), including nitric oxide ($NO^{\cdot}$) and peroxynitrite ($ONOO^-$), are produced during normal physiological processes and assume specific roles in the regulation of cellular function. However, the overproduction of ROS and RNS due to exogenous stimuli and alterations in endogenous metabolism can cause a disturbance in redox homeostasis, leading to severe oxidative damage to lipids, proteins, and DNA [5]. During inflammation, high levels of free radicals and radical precursors, such as superoxide, hydrogen peroxide, and nitric oxide, are generated in cells, necessitating efficient cellular defense mechanisms to lower the level of free radicals and to minimize oxidative damage to the cells. Important constituents of this antioxidant defense are the superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) enzymes, which act as highly efficient scavengers of superoxide ions and hydrogen peroxide. Moreover, these enzymes prevent the formation of hydroxyl radicals, thus increasing the protection of cellular components from oxidative damage [2, 6]. Recently, several studies have focused on the role of natural products in the inhibition of inflammation-induced oxidative stress [2, 7, 8].

The Siamese crocodile (*Crocodylus siamensis*) is a critically endangered freshwater crocodile species that was originally distributed widely throughout parts of South-East Asia, including Thailand. Like other crocodilians populating aquatic habitats, *C. siamensis* is expected to be highly prone to opportunistic bacterial infection. However, severe adverse effects resulting from pathogenic infection are rather uncommon, suggesting that this species developed a remarkably effective innate immune system as part of its evolutionary adaptation. Consequently, several studies have focused on investigating the specific biological properties of *C. siamensis* blood related to immune function, revealing its pronounced antibacterial [9–14] and antioxidant activity [12, 15–17]. Recently, Phosri *et al*. [17] reported that *C. siamensis* blood components (crude leukocyte extract, hemoglobin, and plasma) exert potent anti-inflammatory activity by suppressing NO and IL-6 in a RAW 264.7 cell model. However, the mechanistic details of the observed anti-inflammatory effects *in vivo* remain speculative due to the lack of experimental evidence. Therefore, the present study aimed to utilize established animal models of both acute and chronic inflammation, in addition to detecting malondialdehyde (MDA) levels in the liver and TNF-α, IL-6, IL-1β, and IL-10 levels in the serum of mice subjected to either paw edema or granuloma to elucidate the impact of *C. siamensis* blood components on different aspects of the overall inflammatory process. In addition, the SOD, CAT, and GPx activities in the livers of mice were determined to gain further insight into the relationship between the anti-inflammatory activity of crocodile hemoglobin (cHb) and whole blood (cWb) and antioxidant enzyme activity.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), L-glutamine, fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Lonza (Walkersville, MD, USA); trypsin-EDTA was purchased from Mediatech (Manassas, VA, USA), and H_2O_2, λ-carrageenan (Carr), indomethacin (Indo), prednisolone (Pred); and other chemical reagents were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). *C. siamensis* blood was obtained from Sriracha Moda Farm, Chon Buri, Thailand.

Crocodile Blood Samples

Crocodile blood was collected from the supravertebral branch of the internal jugular vein using a sterile syringe and kept in 15-mL conical screw cap tubes containing 0.08 g EDTA. The blood was kept on ice until the erythrocytes settled to the bottom of the tubes, while the plasma formed the top layer and the leukocytes were enriched in the interphase layer. The red blood cell (RBC) suspension was collected from the bottom layer for the extraction of hemoglobin [12]. Whole blood samples (cWb) containing all blood components, including RBCs, white blood cells, and plasma, were lyophilized before storing at −70 °C until use [9–17].

Cell Culture

Human lung fibroblast cells (MRC-5) were cultured in DMEM containing 10 % heat-inactivated FBS, 1 % penicillin-streptomycin solution, and 5 % L-glutamine. Cells were maintained in an incubator at 37 °C, 5 % CO_2 with 90 % relative humidity. Cells were subcultured every 3 days using trypsin (0.05 %) to detach and separate the cells to form a single-cell suspension.

Cell Viability Assay

The viability of MRC-5 cells was evaluated using the modified sulforhodamine B (SRB) assay method of Vichai and Kirtikara [18]. Briefly, the suspended cells were seeded in 96-well plates at a density of 5×10^3 cells/well and incubated for 24 h prior to the experiment. The cells in the first group were incubated with defined concentrations of cHb and cWb (6.25, 12.5, 25, and 50 μg/mL) for 24 h. In the second group, cells were initially treated with predetermined concentrations of cHb and cWb for 1 h. Afterward, the cells were exposed to 500 μM H_2O_2 for 24 h. The cells in both groups were fixed with 100 μL cold 20 % trichloroacetic acid (TCA) for 30 min at 4 °C, after which the plate was washed with running tap water four times and dried at room temperature. Then, the cells were stained with 100 μL of 0.4 % SRB in 1 % acetic acid for 30 min. Stained cells were washed with 1 % acetic acid four times, and the plates were air-dried until no moisture was visible. Protein-bound dye was solubilized with 200 μL/well of 10 mM Tris base (pH 10) and shaken for 20 min. The optical density of the solubilized dye solution was measured at 531 nm with a Perkin Elmer 2030 multilabel reader.

Antioxidant Activity Assessment Using Microscopy

MRC-5 cells (1×10^5 cells/well) were seeded in a 6-well plate for 24 h, followed by pretreatment with defined concentrations of hemoglobin and whole blood (6.25, 12.5, 25, and 50 μg/mL) for 1 h. Then, the cells were exposed to 500 μM H_2O_2 for 24 h. After incubation, the medium was removed, and the cells were rinsed with PBS. Cells were fixed with methanol/acetic acid (3:1) for 15 min before staining with 0.5 % crystal violet for 25 min. The plate was washed with distilled water and left to dry at room temperature. The images were recorded using a phase contrast inverted microscope (Zeiss Axiovert 25, Germany).

Animals

Male ICR mice (6–8 weeks old) were obtained from the National Laboratory Animal Center of Mahidol University, Salaya, Nakorn Pathom, Thailand. The animals were housed in an environmentally controlled room (temperature 22 ± 1 °C; relative humidity 55 ± 5 %; 12-h dark–light cycle) and given food and water ad libitum. All procedures in this study complied with the Guide for the Care and Use of Laboratory Animals and were approved by the ethics committee of the Institutional Animal Care and Use Committee (IACUC) of Khon Kaen University, Khon Kaen, Thailand (AEKKU 14/2556).

Carrageenan-Induced Mouse Paw Edema

The Carr-induced hind paw edema model was used for the determination of anti-inflammatory activity [19]. After a 2-week adaptation period, male ICR mice (18–25 g) were randomly assigned to eight groups ($n = 6$), including control, indomethacin (positive control), three cHb-treated groups, and three cWb-treated groups. The animals received deionized water orally, indomethacin at a dose of 10 mg/kg, 62.5, 125, or 250 mg/kg cHb or 62.5, 125, or 250 mg/kg cWb. One hour after treatment, 20 μL of 1 % Carr solution was injected subplantarly into the left hind paw of each mouse to induce edema. The paw volume was measured at 1-, 2-, and 3-h intervals after Carr injection using a plethysmometer (model 7159, Ugo Basile, Varese, Italy). The inhibitory activity was calculated using the following formula.

Percentage inhibition

$$= (\mathrm{Vt–Vo})_{\mathrm{control}} – (\mathrm{Vt–Vo})_{\mathrm{treated}} / (\mathrm{Vt–Vo})_{\mathrm{control}} \times 100$$

Vt volume of paw after Carr injection
Vo volume of paw before Carr injection

After complete measurement of the swollen paw volumes, the animals were anesthetized with carbon dioxide and oxygen. Blood samples were withdrawn from the abdominal aorta and centrifuged at ×1000*g* for 5 min. Serum samples were removed and stored at −70 °C until the determination of IL-6, IL-1β, TNF-α, and IL-10 levels. Likewise, the liver was rinsed in ice-cold normal saline, immediately placed in three volumes of cold normal saline and stored at −70 °C for the MDA and antioxidant enzyme (CAT, SOD, and GPx) activity assays.

Cotton Pellet-Induced Granuloma in Mice

This assay was performed according to the method of Winter and Porter [20]. The mice were divided into five groups of six animals each. Each animal was anesthetized, and its fur was shaved. Then, a sterile pre-weighed cotton pellet (10 ± 1 mg) was inserted into the axillary region of each mouse through a single needle incision. cWb (62.5, 125, and 250 mg/kg) and the standard prednisolone (5 mg/kg) were administered orally to the respective groups of animals for seven consecutive days from the day of cotton pellet implantation. On the 8th day, the animals were anesthetized with carbon dioxide and oxygen; the cotton pellets were carefully removed and cleaned of extraneous tissue. The pellets were dried at 60 °C, weighed, and compared with those from the control group.

On the eighth day, blood samples were collected from the abdominal aorta and centrifuged at ×1000*g* for 5 min. The serum samples were removed and stored at −70 °C for the determination of IL-6, IL-1β, TNF-α, and IL-10 levels. Likewise, the liver was isolated and stored at −70 °C for the determination of MDA and CAT, SOD, and GPx activity.

Measurement of IL-6, IL-1β, TNF-α, and IL-10 in Serum

The serum levels of IL-6, IL-1β, TNF-α, and IL-10 were determined using a commercially available ELISA kit (R&D, Minneapolis, MN, USA) according to the manufacturer's instructions. IL-6, IL-1β, TNF-α, and IL-10 concentrations were determined from a standard curve.

Liver Protein Extraction

The livers were homogenized in cold 0.01 M PBS (0.5 g sample per 2 mL 0.01 M PBS) with a hand homogenizer in an ice bath. The protein concentration in the homogenized samples was determined according to the Bradford method [21] and compared with bovine serum albumin (BSA) as the standard.

Measurement of Catalase (CAT)

Catalase activity was assessed following the colorimetric method of Goth [22]. Samples were diluted 20 times in 0.01 M PBS, and 15 μL of diluted sample solution was pipetted into a 96-well plate. Fifty microliters of 130 μM hydrogen peroxide (H_2O_2) substrate and 35 μL of 60 mM potassium phosphate buffer, pH 7.4, were added, and the reaction plate was incubated at 37 °C for 1 min. Then, 65 μL of 32.4 mM ammonium molybdate was added to stop the reaction. The yellow complex between H_2O_2 and ammonium molybdate was measured at 405 nm. Catalase activity was calculated with the following equation:

$$\text{Catalase activity}\left(\text{kU}/\text{L}\right) = \left[\left(A_{\text{sample}} - A_{\text{blank sample}}\right)/\left(A_{\text{control}} - A_{\text{blank control}}\right)\right] \times 271$$

A_{sample}	contained sample, H_2O_2 substrate, and ammonium molybdate.
$A_{\text{blank sample}}$	contained sample and ammonium molybdate.
A_{control}	contained H_2O_2 substrate, ammonium molybdate, and phosphate buffer.
$A_{\text{blank control}}$	contained ammonium molybdate and phosphate buffer.

Measurement of Superoxide Dismutase (SOD)

Superoxide dismutase activity was investigated according to previously described methods [23, 24]. One hundred microliters of liver homogenate was added to 60 μL chloroform and 100 μL ethanol and vortexed for 1 min. The mixture was centrifuged at ×13,000*g* and 4 °C for 30 min, and 12.5 μL supernatant was pipetted into a 96-well plate. Then, reaction mixture (107.5 μL) containing 3 mM xanthine, 0.6 mM ethylenediaminetetraacetic acid (EDTA), 0.75 mM nitroblue tetrazolium (NBT), 400 mM sodium carbonate (Na_2CO_3), and 1 g/L BSA was added to each well, followed by the addition of 20 μL of 8 U/mg protein xanthine oxidase solution (17.5:2.5, *v*/*v*) and incubation of the plate at 25 °C for 20 min. The reaction was stopped by adding 20 μL of 0.8 mM copper chloride ($CuCl_2$). The absorbance was measured at 550 nm, and the percentage of inhibition was calculated as follows.

$$\%\ \text{Inhibition} = \left[\left(A_{\text{control}} - A_{\text{sample}}\right)/A_{\text{control}}\right] \times 100\%$$

A_{control}	contained deionized water.
A_{sample}	contained samples.

Measurement of Glutathione Peroxidase (GPx)

The glutathione peroxidase activity in the liver was determined according to the method of Pinto and Bartley [25], with some modifications. Briefly, the reaction mixture containing 60 μL of liver homogenate, 12 μL of 50 mM phosphate buffer at pH 7.4, 12 μL of 0.4 mM EDTA, and 2 μL of 0.1 mM sodium azide (NaN_3) was incubated at room temperature for 10 min. Then, 10 μL of

50 mM reduced glutathione (GSH) and 2 μL of 150 mM H_2O_2 were added. To stop the reaction, 160 μL of 5 % sulfosalicylic acid was then added, and the mixture was incubated at 4 °C for 10 min. The mixture was centrifuged at ×1900*g* at room temperature for 30 min, the OD of the supernatant was measured at 405 nm, and the GPx activity was calculated as U/mg protein. One unit of GPx activity was calculated from 1 μmol GSSG (oxidized glutathione) formed per minute in the reaction at 30 °C and pH 7.4.

Measurement of Malondialdehyde (MDA)

The malondialdehyde level was determined using the method of Ohkawa *et al.* [26], with some modifications. Briefly, 100 μL of liver homogenate was mixed with 200 μL sodium dodecyl sulfate (SDS) (8.1 %, *w*/*v*), 1.5 mL acetic acid (20 %, *w*/*v*), 1.5 mL thiobarbituric acid (TBA) (0.8 %, *w*/*v*), and 600 μL deionized water, followed by incubation of the reaction mixture in a heated water bath (95 °C) for 1 h. After the mixture was cooled to room temperature, n-butanol and pyridine (15:1 *v*/*v*) were added, and the mixture was vortexed. The mixture was centrifuged at ×1000*g* for 10 min, and the upper layer was removed for colorimetric measurement at 532 nm. The amount of MDA was calculated from a standard curve of 1, 1, 3, 3-tetramethoxypropane (TMP).

Statistical Analysis

The data are presented in as the mean ± SD. Differences between groups were analyzed with one-way analysis of variance (ANOVA followed by Dunnett's test). Statistical significance was accepted at $P < 0.05$.

RESULTS

Cell Viability

MRC-5 cells were treated with defined concentrations of cHb and cWb (6.25, 12.5, 25, and 50 μg/mL) for 24 h to evaluate potential toxic effects on the cells. However, cHb and cWb did not display any cytotoxicity toward MRC-5 cells (Fig. 1a, b), while 500 μM H_2O_2 reduced the cell viability to 69.37 %. Pretreatment of the cells with cHb and cWb before H_2O_2 exposure significantly improved the cell viability in a concentration-dependent manner. The results show an increase in cell viability from 69.37 to 91.33 %, 91.54, 94.28, and 104.88 % at 6.25, 12.5, 25, and 50 μg/mL cHb, respectively. For 6.25, 12.5, 25, and 50 μg/mL cWb, the cell viability increased to 83.08, 84.74, 85.00, and 90.03 %, respectively (Fig. 1c, d).

Antioxidant Activity Assessment Using Microscopy

To assess the protective effects of cHb and cWb against H_2O_2-induced oxidative stress, H_2O_2-induced changes in the morphology of MRC-5 cells were visualized with microscopy. A visible difference in the control group upon addition of H_2O_2 was the loss of typical fibroblast appearance, as well as an overall change in cell morphology. In contrast, pretreating cells with defined concentrations of cHb and cWb before exposure to H_2O_2 effectively prevented cell morphology alterations. At each concentration tested, significant attenuation of H_2O_2-induced cytotoxicity was observed. These results indicate that cHb and cWb treatment can prevent or at least alleviate H_2O_2-induced cytotoxicity in MRC-5 cells (Figs. 2 and 3).

Carrageenan-Induced Mouse Paw Edema

The anti-inflammatory activity of cHb and cWb in Carr-induced edema in the left hind paws of mice is summarized in Table 1. A gradual increase in paw edema volume in control mice (Carr-treated group) was observed over a 3-h period. The control indomethacin (10 mg/kg) could clearly inhibit the induction of hind paw edema within 1, 2, and 3 h after injection of Carr (40.91, 53.85, and 48.72 %, respectively). Upon treatment with cHb and cWb, a significant decrease in paw edema volume was observed in a dose-dependent manner. In the cHb group, a marked reduction of hind paw edema was visible at a dose of 125 mg/kg 1–3 h after treatment. Treatment with the highest dose of cHb (250 mg/kg) led to a reduction in edema volume by 31.82, 38.46, and 35.90 % after 1, 2, and 3 h, respectively. The group treated with cWb showed a significant decrease in paw edema volume at all doses (62.5, 125, 250 mg/kg) and all times (1, 2, 3 h). Maximal decreases of 40.91, 42.31, and 41.03 % (at 1, 2, and 3 h, respectively) relative to the Carr group were found in the cWb group at a dose of 250 mg/kg.

Cotton Pellet-Induced Granuloma in Mice

The anti-inflammatory activity of cHb and cWb in the cotton pellet-induced granuloma model of chronic inflammation model in mice is shown in Table 2. cHb and cWb treatment caused significant ($P < 0.05$) dose-dependent decreases in the weight of wet and dry cotton pellets relative to the control group (cotton pellet-induced inflammation only). The granuloma in the control group of animals

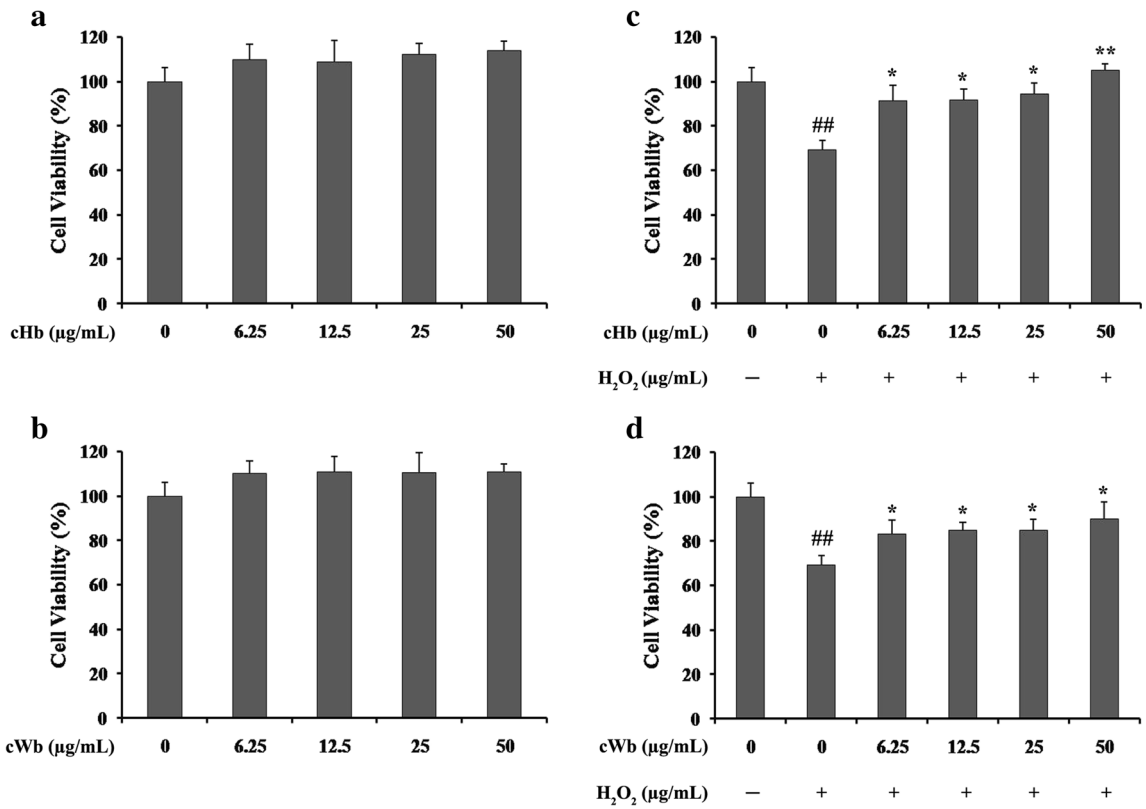

Fig. 1. Effects of cHb and cWb on the viability of MRC-5 cells. Cells were pretreated with defined concentrations of cHb and cWb and/or H_2O_2 (500 μM), and cell viability was assessed with the SRB assay. The data are expressed as the mean ± SD. $^{\#\#}P < 0.01$; significant difference compared to the untreated group. $^{**}P < 0.01$, $^{*}P < 0.05$; significant difference when compared to the H_2O_2-only group (one-way ANOVA followed by Dunnett's test).

exhibited a 116.23 ± 7.64 mg wet weight and a 25.80 ± 1.36 mg dry weight. A considerable reduction in granuloma weight to 32.48 % (wet weight) and 27.91 % (dry weight) was obtained upon treatment with the highest cHb dose (250 mg/kg). Similarly, the highest dose of cWb (250 mg/kg) led to a reduction in granuloma weight to 33.62 % (wet weight) and 30.62 % (dry weight). Prednisolone (5 mg/kg), used as a reference, reduced granuloma formation by 46.27 % (wet weight) and 32.17 % (dry weight).

Effects of cHb and cWb on Pro-Inflammatory Cytokines

Upon induction of Carr-induced acute inflammation, a significant decrease in IL-6 production was observed for all preparations compared to the media controls (Fig. 4a, b). The activity of cHb ranged from 24.09 to 51.10 % (936–603 pg/mL) (Fig. 4a), and the cWb activity was between 21.90 and 70.51 % (963–364 pg/mL) (Fig. 4b) for the doses of 62.5–250 mg/kg. Indomethacin (10 mg/kg) was also found to inhibit IL-6 production by 49.72 % (620 pg/mL). On the other hand, cHb and cWb did not decrease IL-6 production in the cotton pellet-induced chronic inflammation model (Fig. 4c, d).

Figure 5a shows that cHb significantly ($P < 0.05$) inhibited the IL-1β level. Likewise, cWb could reduce the IL-1β level in a dose-dependent manner, with the highest observed inhibition of 27.96 % (3800 pg/mL) relative to the Carr-only group occurring at a dose of 250 mg/kg (Fig. 5b). Regarding cotton pellet-induced chronic inflammation, the inhibitory effect of cHb on IL-1β production was 27.23 % (2,165 pg/mL) at the highest concentration (Fig. 5c). cWb also significantly reduced the IL-1β level (Fig. 5d).

As illustrated in Fig. 6a, b, cHb and cWb at the highest concentration decreased the production of TNF-α to 49.19 % (51.23 pg/mL) and 43.50 % (56.97 pg/mL), respectively. In the positive control group, indomethacin also inhibited the production of TNF-α to 50.00 % (50.41 pg/mL) of that observed in the Carr model. When

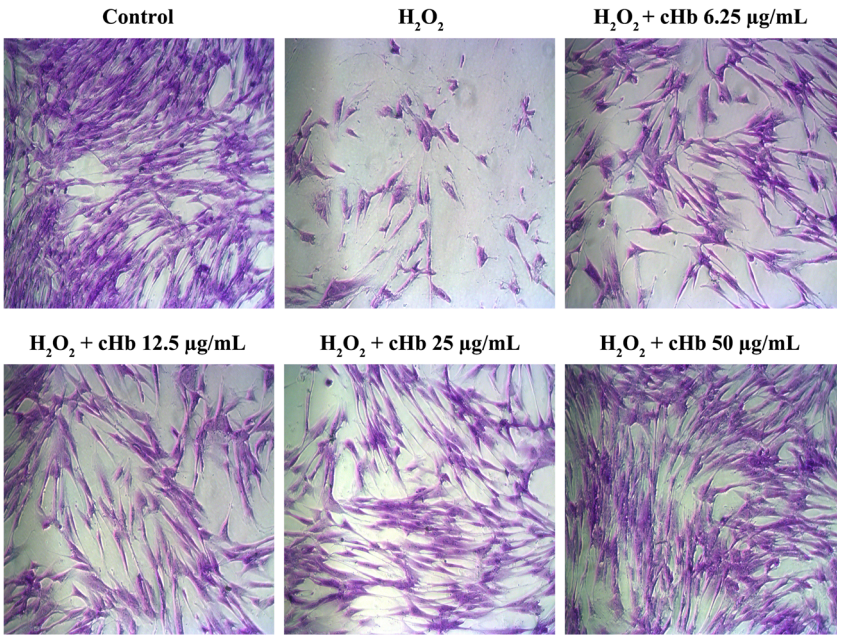

Fig. 2. Inverted microscope images of MRC-5 fibroblast cells (×200) pretreated with defined concentrations of cHb. Cells (except control) were exposed to 500 µM H_2O_2 for 24 h.

cHb and cWb were administered in the granuloma formation experiments, cHb at doses of 62.5, 125, and 250 mg/kg significantly inhibited TNF-α levels by 22.09 % (67.38 pg/mL), 28.17 % (62.1 pg/mL), and 42.18 % (50.00 pg/mL) (Fig. 6c), respectively, while cHb at doses of 62.5, 125, and 250 mg/kg significantly inhibited TNF-α levels by 38.70 % (53.01 pg/mL), 39.34 % (52.46 pg/mL), and 40.05 % (51.84 pg/mL) (Fig. 6d), respectively.

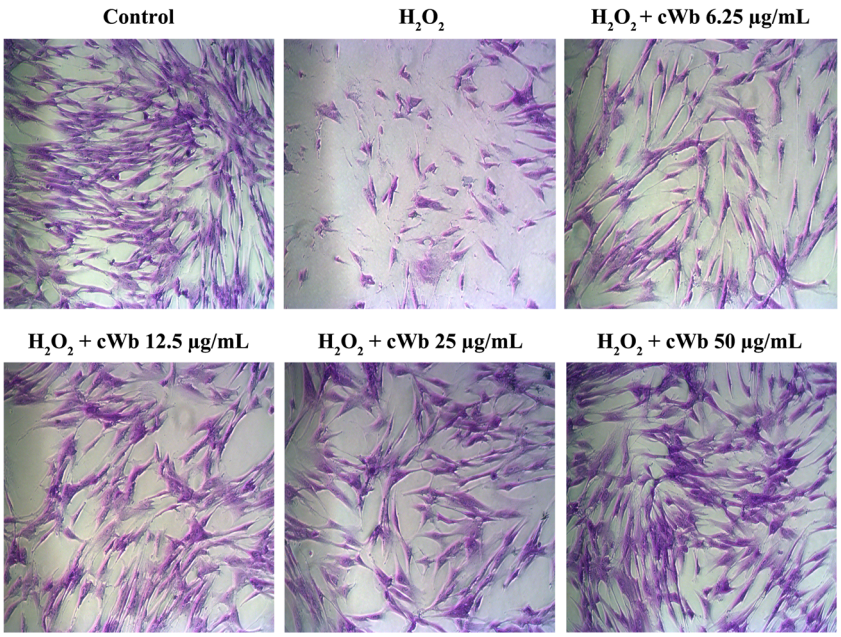

Fig. 3. Inverted microscope images of MRC-5 fibroblast cells (×200) pretreated with defined concentrations of cWb. Cells (except control) were exposed to 500 µM H_2O_2 for 24 h.

Table 1. Effects of cHb and cWb on Carr-Induced Mouse Paw Edema

Treatment group	Dose (mg/kg)	Edema volume (mL)			% inhibition		
		1 h	2 h	3 h	1 h	2 h	3 h
Control	–	0.022 ± 0.0017	0.026 ± 0.0021	0.039 ± 0.0029	–	–	–
Indo	10	0.013 ± 0.0012***	0.012 ± 0.0013***	0.020 ± 0.0011***	40.91	53.85	48.72
cHb	62.5	0.020 ± 0.0028	0.023 ± 0.0013*	0.032 ± 0.0015*	9.09	11.54	17.95
cHb	125	0.018 ± 0.0011*	0.018 ± 0.0011**	0.030 ± 0.0022*	18.18	30.77	23.08
cHb	250	0.015 ± 0.0019**	0.016 ± 0.0010***	0.025 ± 0.0017**	31.82	38.46	35.90
cWb	62.5	0.018 ± 0.0017*	0.020 ± 0.0019**	0.033 ± 0.0006*	18.18	23.08	15.38
cWb	125	0.015 ± 0.0013*	0.017 ± 0.0006**	0.026 ± 0.0015**	31.82	34.62	33.33
cWb	250	0.013 ± 0.0010***	0.015 ± 0.0010***	0.023 ± 0.0015***	40.91	42.31	41.03

Values are expressed as the mean ± SD ($n = 6$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$; significant difference compared to the control (Carr-only) group

Prednisolone (5 mg/kg) also significantly decreased the TNF-α level by 38.70 % (53.01 pg/mL).

With regard to the level of the anti-inflammatory factor IL-10, cHb did not cause a significant increase in production, while indomethacin increased the IL-10 level by 44.97 % (6,480 pg/mL) relative to the Carr-only group (Fig. 7a). Interestingly, cWb was found to increase the serum levels of IL-10, with a maximum activity of 89.53 % (8,472 pg/mL) at a dose of 250 mg/kg, which is higher than that of the indomethacin group in the Carr-induced inflammatory process (Fig. 7b). cHb did not increase the level of the anti-inflammatory factor IL-10 in the granuloma model (Fig. 7c), whereas cWb significantly increased the IL-10 level by 51.88 % (7260 pg/mL) at a dose of 250 mg/kg, which was comparable to that observed in the prednisolone group (57.74 %, 7540 pg/mL) (Fig. 7d).

Measurement of Catalase (CAT)

The activity of CAT in each test sample was determined to examine the effect of cHb and cWb on the antioxidant system. In the Carr model, treatment with cHb resulted in a significant increase in CAT activity at a dose of 250 mg/kg (6.25 ± 1.55 U/mg protein). cWb concentrations ranging from 125 to 250 mg/kg increased CAT activity to 6.10 ± 0.27–7.77 ± 1.37 U/mg protein (Table 3). Similar results were obtained in the cotton pellet model, in which cHb doses of 62.5–250 mg/kg could improve CAT activity by 16.64 ± 1.04, 20.99 ± 2.67, and 24.72 ± 3.81 U/mg protein. Similarly, cWb (62.5–250 mg/kg) significantly increased CAT activity to 20.34 ± 1.43 to 27.97 ± 1.09 U/mg protein in comparison to the cotton group (Table 4).

Measurement of Superoxide Dismutase (SOD)

SOD activity in mouse liver homogenates was measured to determine the effect of cHb and cWb on the antioxidant system. cHb increased the activity of SOD by 67.88 ± 4.63, 69.73 ± 5.06, and 71.49 ± 9.33 U/mg protein in the Carr group and 72.10 ± 5.11, 77.32 ± 2.00, and 83.64 ± 6.35 U/mg protein in the cotton pellet model at a concentration range of 62.5–250 mg/kg (Table 3). However, cWb did not

Table 2. Effects of cHb and cWb on Cotton Pellet-Induced Granuloma in Mice

Treatment group	Dose (mg/kg)	Wet weight of granuloma (mg)	% inhibition	Dry weight of granuloma (mg)	% inhibition
Control	–	116.23 ± 7.64	–	25.80 ± 1.36	–
Pred	5	62.45 ± 9.83***	46.27	17.50 ± 0.30**	32.17
cHb	62.5	89.13 ± 9.62*	23.32	21.68 ± 2.67*	15.99
cHb	125	87.43 ± 4.20*	24.78	21.18 ± 1.00*	17.93
cHb	250	78.48 ± 9.35**	32.48	18.60 ± 5.37*	27.91
cWb	62.5	95.65 ± 4.60*	17.70	21.03 ± 2.00*	18.48
cWb	125	80.53 ± 5.43**	30.71	20.37 ± 2.43*	21.10
cWb	250	77.15 ± 3.18**	33.62	17.90 ± 2.40**	30.62

Values are expressed as the mean ± SD ($n = 6$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$; significant difference compared to the control (cotton-only) group

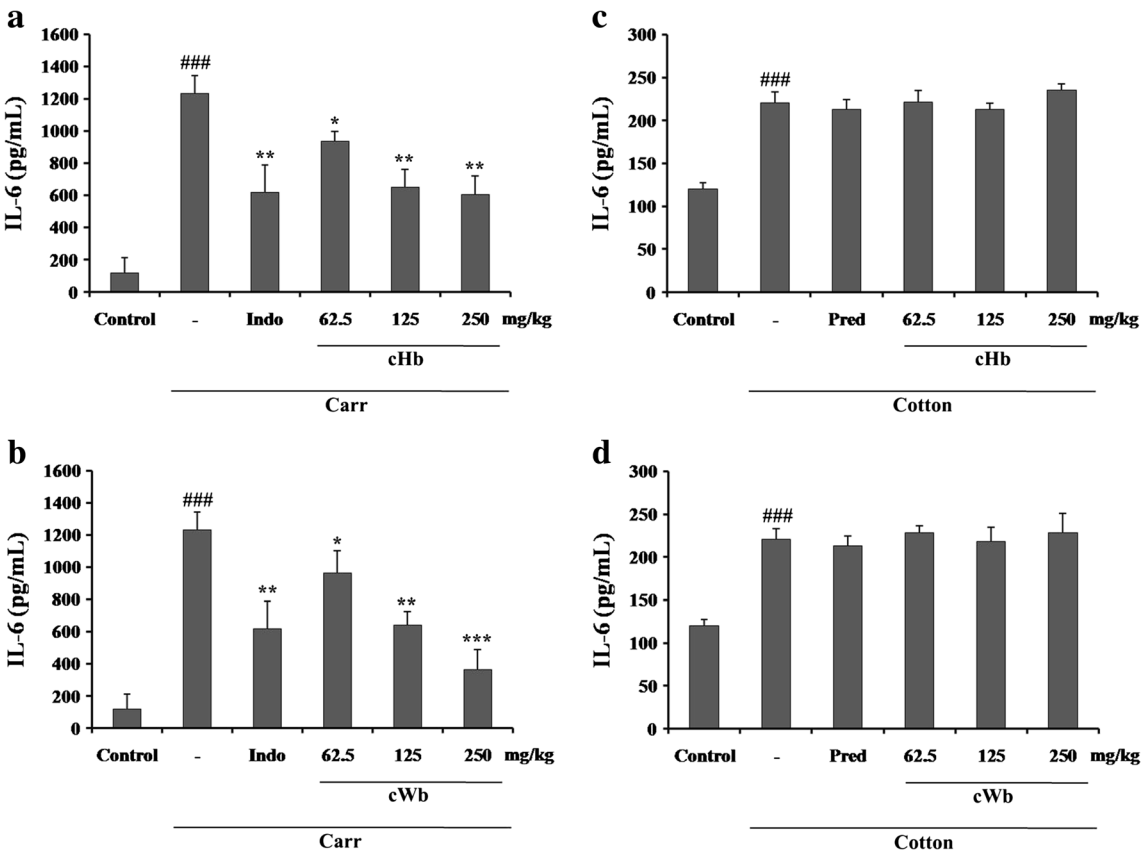

Fig. 4. Effects of cHb and cWb on IL-6 levels in Carr-induced mouse paw edema (**a**, **b**) and cotton pellet-induced granuloma in mice (**c**, **d**). Each experiment was conducted in triplicate. The data are expressed as the mean ± SD. $^{###}P < 0.001$; significant difference compared to the normal control group. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$; significant difference when compared to the Carr-only or cotton-only group (one-way ANOVA followed by Dunnett's test).

induce comparable SOD activity enhancement in the Carr model. In the cotton pellet model, cWb (62.5, 125, and 250 mg/kg) significantly increased SOD activity by 79.76 ± 3.67, 82.16 ± 3.01, and 88.61 ± 2.04 U/mg protein (Table 4).

Measurement of Glutathione Peroxidase (GPx)

The GPx activity in the liver homogenate of Carr-induced mice after treatment with cHb and cWb is shown in Table 3. cHb significantly increased GPx activity in a dose-independent manner. In a similar fashion, cWb at concentrations of 62.5, 125, 250 mg/kg could improve the activity of GPx by 5.61 ± 0.50, 5.77 ± 0.16, and 6.15 ± 0.15 U/mg protein, respectively. In the cotton pellet model, cHb over a concentration range of 62.5–250 mg/kg increased GPx activity from 5.06 ± 0.46 to 6.71 ± 0.74 U/mg protein (Table 4). cWb showed a dose-dependent effect on GPx activity (6.51 ± 1.72–8.11 ± 0.45 U/mg protein), albeit to a lesser extent than prednisolone (8.78 ± 1.41 U/mg protein) (Table 4).

Measurement of Malondialdehyde (MDA)

The MDA level was elevated in the Carr control group with respect to the normal control group. The groups treated with cHb at concentrations of 62.5, 125, and 250 mg/kg exhibited significantly decreased levels of MDA at 0.70 ± 0.09–0.61 ± 0.08 μM/mg protein. The cWb-treated group also featured a significantly decreased MDA level of 0.69 ± 0.07–0.58 ± 0.14 μM/mg protein (Table 3). In the cotton pellet model, the MDA level dropped significantly upon cHb treatment (62.5–250 mg/kg) to 0.80 ± 0.10–0.74 ± 0.19 μM/mg protein. Likewise, cWb treatment resulted in an analogous reduction in the MDA level to 0.68 ± 0.10–0.63 ± 0.15 μM/mg protein. The standard prednisolone, on the other hand, was not found to influence the MDA level significantly (Table 4).

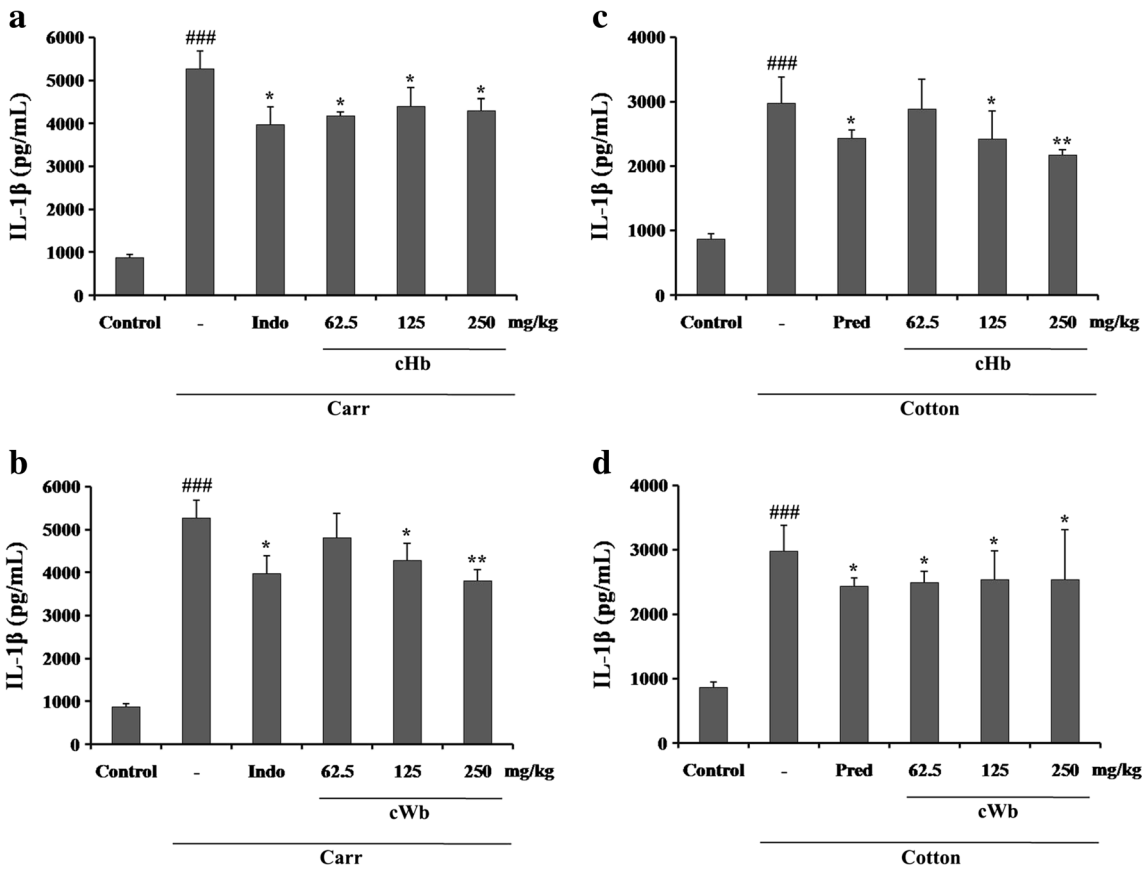

Fig. 5. Effects of cHb and cWb on IL-1β levels in Carr-induced mouse paw edema (**a**, **b**) and cotton pellet-induced granuloma in mice (**c**, **d**). Each experiment was conducted in triplicate. Each value is presented as the mean ± SD. $^{###}P < 0.001$; significant difference compared to the normal control group. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$; significant difference when compared to the Carr-only or cotton-only group (one-way ANOVA followed by Dunnett's test).

DISCUSSION

In a previous study, we demonstrated that crocodile blood (hemoglobin, crude leukocyte extract, and plasma) displays significant free radical scavenging activity and anti-inflammatory effects via inhibiting the release of inflammatory mediators and cytokines in RAW 264.7 macrophage cells. Based on these results, the focus of the present study was set on exploring the *in vivo* anti-inflammatory effects of hemoglobin and whole blood from *C. siamensis*.

H_2O_2-induced oxidative damage to MRC-5 cells was used to determine the antioxidant activity of cHb and cWb. Hydrogen peroxide is a comparatively stable ROS and can freely diffuse through cell membranes, where it readily damages various cellular components. Furthermore, H_2O_2 is a known precursor of other, more reactive ROS, such as the hydroxyl radical, via Fenton's reaction. It can also induce the formation of RNS, including nitric oxide ($NO^{\cdot}$) and peroxynitrite ($ONOO^{-}$) ions [5]. Free radicals originating from H_2O_2 and radical chain reactions during lipid peroxidation are most likely involved in the oxidative damage and death of MRC-5 cells. In normal cell metabolism, free radicals and other reactive oxygen species are quenched by antioxidants in the cell. However, when the cellular redox balance is disrupted under oxidative stress, excess radicals can readily modify amino acid side chains and cause proteins to denature and aggregate. Along with the oxidation of the cell membrane, these processes lead to the loss of cell integrity, toxin build-up, and ultimately, the death of the cell [27, 28]. In this study, direct evidence for a protective effect of cHb and cWb against H_2O_2-induced toxicity in MRC-5 cells was found with the observed improvement in cell viability. Similarly, intact human red blood cells were shown to be efficient scavengers of H_2O_2 [29]. In addition to improved cell viability, cHb and cWb

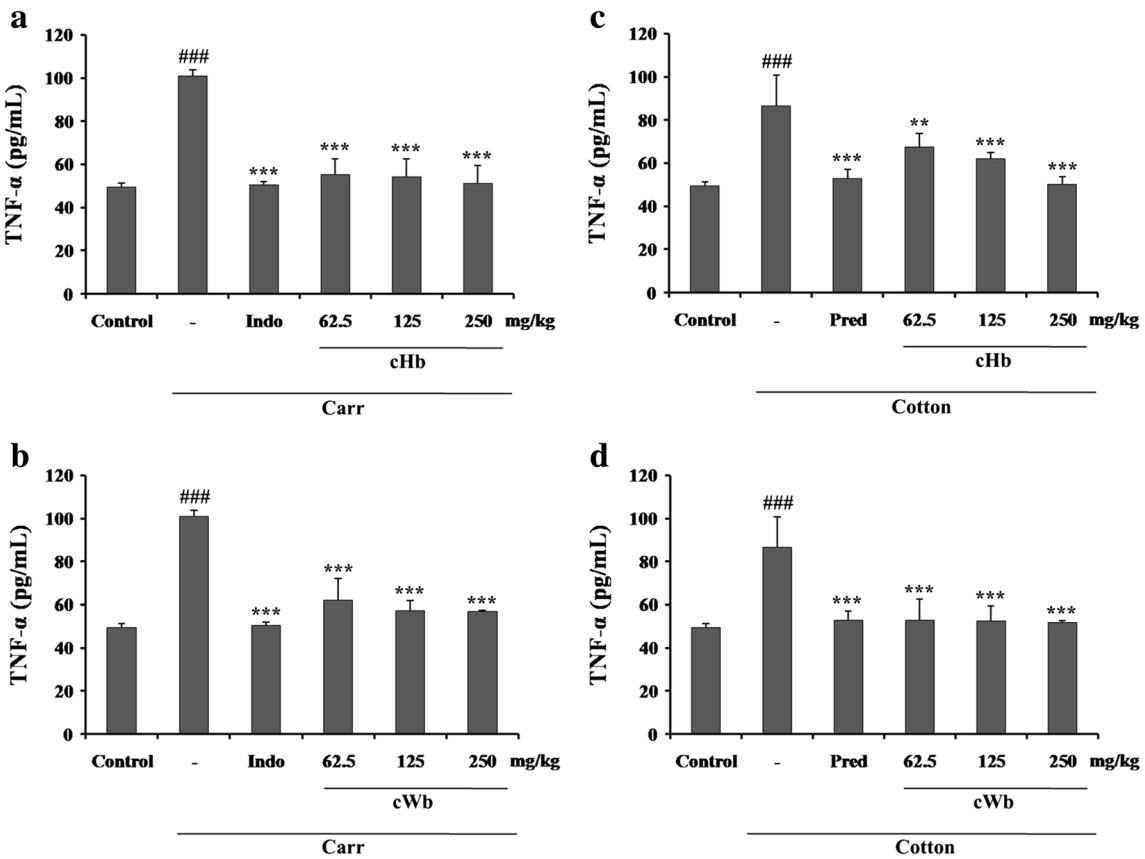

Fig. 6. Effects of cHb and blood on TNF-α level in Carr-induced mouse paw edema (**a**, **b**) and cotton pellet-induced granuloma in mice (**c**, **d**). The experiment was conducted in triplicate. The data are presented as the mean ± SD. $^{###}P < 0.001$; significant difference compared to the normal control group. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$; significant difference when compared to the Carr-only or cotton-only group (one-way ANOVA followed by Dunnett's test).

were also found to preserve the morphology of MRC-5 cells against changes induced by exposure to H_2O_2. This effect is presumed to be attributed to the neutralization of free radicals by cHb and cWb.

The *in vivo* anti-inflammatory activity of *C. siamensis* blood in the acute and chronic phases was evaluated using two representative animal models, i.e., Carr-induced mouse paw edema and cotton pellet-induced granuloma in mice. The Carr-induced paw edema model is a well-known and widely used method to assess the anti-inflammatory activity of test agents in the acute stage of inflammation [8]. The progressive formation of hind paw edema induced by Carr injection can be divided into two phases: The first phase is characterized by the release of histamine, serotonin, and bradykin in during the first hour. Later, the second phase (2–3 h) is linked to neutrophil infiltration and the production of neutrophil-derived free radicals (hydrogen peroxide, superoxide, and hydroxyl radicals) [2], as well as the creation of pro-inflammatory cytokines such as IL-1β and TNF-α. cHb and cWb showed a significant inhibitory effect on paw edema volume during the first and second phases of inflammation.

The cotton pellet-induced granuloma model is widely used for the screening of anti-inflammatory substances in chronic inflammation associated with the transudative, exudative, and proliferative components of the chronic stage. During cotton pellet implantation, the surrounding fluid absorbed by the pellet significantly influences the wet and dry weight of the pellet, which relates to the formation of granulomatous tissue [30, 31]. The chronic inflammatory process leads to the release of numerous pro-inflammatory cytokines, such as IL-6, IL-1β, and TNF-α [32]. cHb and cWb at 250 mg/kg were found to significantly inhibit the formation of cotton pellet-induced granuloma, similar to the potent anti-inflammatory agent prednisolone. These data provide strong evidence for the anti-inflammatory activity of cHb and cWb in the chronic phase of inflammation.

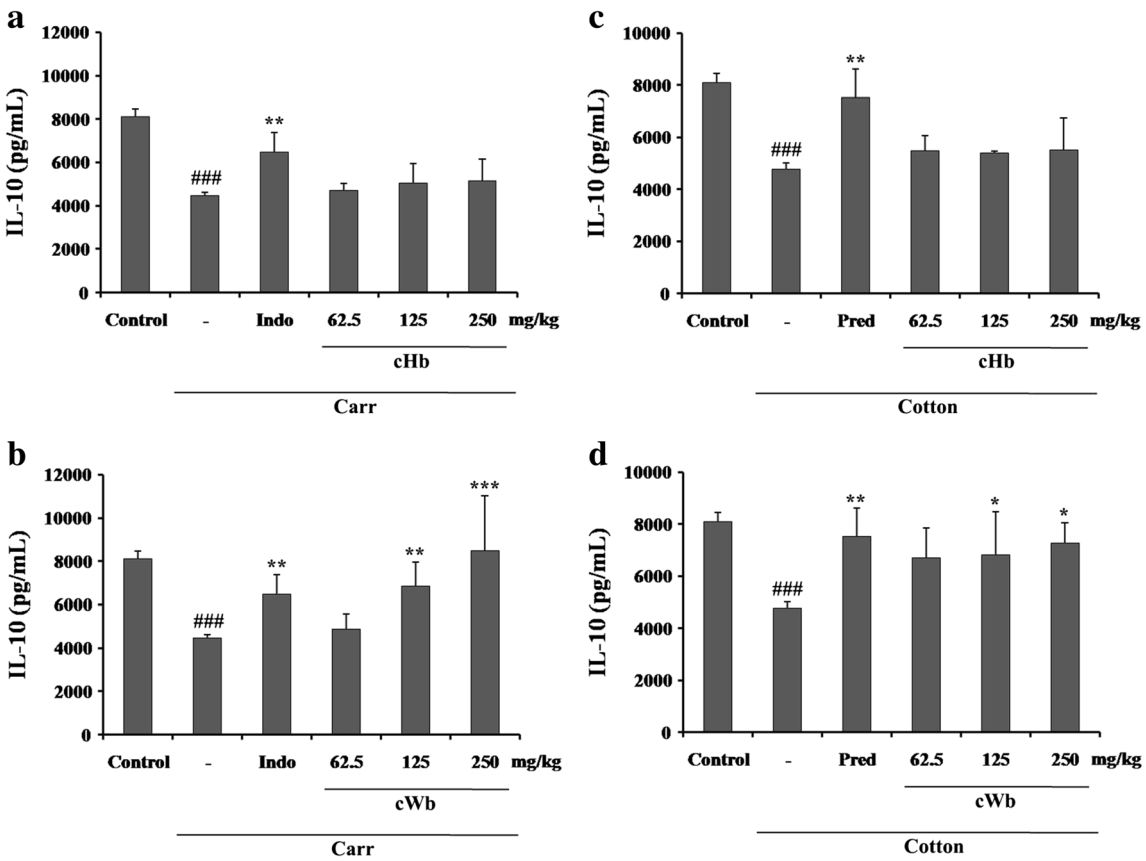

Fig. 7. Effects of cHb and blood on IL-10 level in Carr-induced mouse paw edema (**a**, **b**) and cotton pellet-induced granuloma in mice (**c**, **d**). Each experiment was conducted in triplicate. The data are expressed as the mean ± SD. $^{###}P < 0.001$; significant difference compared to the normal control group. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$; significant difference when compared to the Carr-only or cotton-only group (one-way ANOVA followed by Dunnett's test).

During the acute and chronic inflammatory processes, a variety of pro-inflammatory mediators, especially nitric oxide (NO), prostaglandin E_2 (PGE_2), and several pro-inflammatory cytokines (IL-6, IL-1β, TNF-α), are produced by macrophages [2, 33]. As shown in our results, cHb and cWb inhibit the release of pro-inflammatory cytokines, in particular, IL-6, IL-1β, TNF-α, whereas only cWb was found to increase the

Table 3. Effects of cHb and cHb on Liver Antioxidant Enzyme Levels and Lipid Peroxidation in the Carr-Induced Mouse Paw Edema Model

Treatment group	Dose (mg/kg)	CAT (U/mg protein)	SOD (U/mg protein)	GPx (U/mg protein)	MDA (μM/mg protein)
Control	–	9.88 ± 1.00	74.04 ± 6.92	5.80 ± 1.67	0.67 ± 0.15
Carr	–	$5.13 \pm 0.40^{##}$	$58.14 \pm 4.35^{##}$	$4.01 \pm 0.18^{##}$	$1.02 \pm 0.07^{##}$
Indo	10	$10.12 \pm 0.82^{**}$	$73.29 \pm 6.02^{**}$	$5.68 \pm 0.86^{**}$	$0.66 \pm 0.09^{**}$
cHb	62.5	5.04 ± 2.48	$67.88 \pm 4.63^{*}$	$5.19 \pm 0.54^{*}$	$0.70 \pm 0.09^{*}$
cHb	125	4.52 ± 1.52	$69.73 \pm 5.06^{*}$	$6.70 \pm 0.40^{***}$	$0.65 \pm 0.23^{**}$
cHb	250	$6.25 \pm 1.55^{*}$	$71.49 \pm 9.33^{**}$	$5.80 \pm 0.27^{**}$	$0.61 \pm 0.08^{***}$
cWb	62.5	4.07 ± 1.80	57.20 ± 2.64	$5.61 \pm 0.50^{**}$	$0.69 \pm 0.07^{**}$
cWb	125	$6.10 \pm 0.27^{*}$	61.81 ± 7.01	$5.77 \pm 0.16^{**}$	$0.59 \pm 0.06^{***}$
cWb	250	$7.77 \pm 1.37^{*}$	63.83 ± 1.61	$6.15 \pm 0.15^{***}$	$0.58 \pm 0.14^{***}$

Each value is presented as the mean ± SD ($n = 6$). $^{##}P < 0.01$; significant difference compared with the normal control group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; significant difference compared with the Carr-only group

Table 4. Effects of cHb and cHb on Liver Antioxidant Enzyme Levels and Lipid Peroxidation in the Cotton Pellet-Induced Granuloma Model in Mice

Treatment group	Dose (mg/kg)	CAT (U/mg protein)	SOD (U/mg protein)	GPx (U/mg protein)	MDA (μM/mg protein)
Control	–	25.15 ± 1.75	89.03 ± 0.50	6.81 ± 3.09	0.71 ± 0.15
Cotton	–	$10.37 \pm 0.85^{\#\#}$	$68.19 \pm 5.10^{\#\#}$	$4.44 \pm 1.02^{\#\#}$	$1.24 \pm 0.14^{\#\#}$
Pred	5	$25.69 \pm 4.45^{**}$	$85.47 \pm 6.94^{**}$	$8.78 \pm 1.41^{***}$	1.29 ± 0.20
cHb	62.5	$16.64 \pm 1.04^{*}$	72.10 ± 5.11	$5.06 \pm 0.46^{*}$	$0.80 \pm 0.10^{*}$
cHb	125	$20.99 \pm 2.67^{*}$	$77.32 \pm 2.00^{*}$	$5.10 \pm 1.04^{*}$	$0.75 \pm 0.08^{**}$
cHb	250	$24.72 \pm 3.81^{**}$	$83.64 \pm 6.35^{**}$	$6.71 \pm 0.74^{**}$	$0.74 \pm 0.19^{**}$
cWb	62.5	$20.34 \pm 1.43^{*}$	$79.76 \pm 3.67^{*}$	$6.51 \pm 1.72^{*}$	1.14 ± 0.28
cWb	125	$20.37 \pm 2.67^{*}$	$82.16 \pm 3.01^{**}$	$7.11 \pm 3.12^{**}$	$0.68 \pm 0.10^{**}$
cWb	250	$27.97 \pm 1.09^{**}$	$88.61 \pm 2.04^{**}$	$8.11 \pm 0.45^{***}$	$0.63 \pm 0.15^{***}$

Each value is presented as the mean ± SD ($n = 6$). $^{\#\#}P < 0.01$; significant difference compared with the normal control group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; significant difference compared with the cotton-only group

level of the anti-inflammatory cytokine IL-10 [34]. cHb and cWb were also found to exhibit anti-inflammatory effects in the cotton pellet model by reducing IL-1β and TNF-α levels, while only cWb enhanced the expression of the anti-inflammatory cytokine IL-10. cHb and cWb, however, did not cause a significant decrease in the IL-6 level in the chronic stage. Based on these observations, the *in vivo* anti-inflammatory properties of cHb and cWb are likely to be attributed to a complex molecular mechanism that effectively suppresses the production of the pro-inflammatory cytokines IL-6, IL-1β, and TNF-α and simultaneously elevates the level of the anti-inflammatory cytokine IL-10. The pronounced anti-inflammatory effects of cHb, which has also been observed for hemoglobins of other species, including humans [35], rats [36], and sheep [37], are presumably caused by strong inhibition of the production of NO [36]. In the case of cWb, IL-10-mediated heme oxygenase-1 (HO-1) [38], an antioxidant enzyme found in various cells, especially in leukocytes [39, 40] is assumed to play a crucial role in the observed anti-inflammatory effect. HO-1 mediates the transformation of heme into carbon monoxide (CO) and biliverdin, which is subsequently converted to bilirubin, a known potent antioxidant. Both CO and bilirubin have potent anti-inflammatory activity and can decrease the release of inflammatory mediators, such as NO and IL-6, and the LPS-mediated stimulation of the nuclear transcription factor NF-κB [41, 42]. In crocodile genera such as the American alligator (*Alligator mississippiensis*), ubiquitination in leukocytes has been reported to serve several functions in cellular processes, such as the control of apoptosis, the immune response, antigen processing, and especially, inflammation [43]. Moreover, the anti-inflammatory mechanism of plasma involves the inhibition of NO, which is released during inflammatory processes. This function is related to the anti-inflammatory effect of activated protein C (APC), with Esmon [44] revealing the ability of this protein to inhibit the synthesis of NF-κB components and to suppress inflammatory cytokine production through NF-κB.

In the inflammatory response, the overproduction of reactive oxygen species (ROS) is evidently linked with pathophysiological processes leading to cellular damage. ROS generated during inflammation are known to initiate lipid peroxidation and cause the inactivation of antioxidant enzymes by free radicals [45]. Lipid peroxides represent one of the major end-products of the attack of free radicals on cell membranes, which results in the release of oxidative damage biomarkers such as malondialdehyde (MDA) [46]. Our results demonstrate that cHb and cWb cause a significant decrease in MDA formation in both the Carr and the cotton pellet model, whereas the standard prednisolone did not alter the level of MDA. This is in excellent agreement with the results of Kirimi *et al.* [47], who reported that although prednisolone is suitable for treating the clinical and pathological effects of inflammation, it is either incapable of reducing oxidative stress effectively or induces oxidative stress itself with increasing doses [48]. In the present study, a significant decrease in MDA level upon cHb or cWb treatment was observed, presumably due to the antioxidant properties of plasma proteins in the blood reported elsewhere [49].

In addition to the mechanisms discussed above, free radicals can also cause cell damage by inactivating several crucial antioxidant enzymes, such as SOD,

CAT, and GPx, all of which play an essential role in protecting cells from the damage caused by specific free radicals. SOD is an important enzyme found in all living cells that can protect cells against the harmful effects of free superoxide ($O_2^{\cdot-}$) radicals. By alternately adding or removing an electron from the superoxide molecules it encounters, it converts $O_2^{\cdot-}$ into one of two less damaging species: either molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). CAT is an essential enzyme found abundantly in cells and catalyzes the decomposition of H_2O_2 to oxygen and water. In a similar fashion, GPx catalyzes the detoxification of peroxides and hydroperoxides, including H_2O_2, to water, comprising an integral part of the cellular antioxidant defense mechanism [50]. The overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in Carr-induced acute and cotton pellet-induced chronic inflammation results in an imbalance of antioxidant enzymes in cells [51, 52]. Our study shows that cHb greatly supports the recovery of CAT, SOD, and GPx activity in both the Carr and cotton pellet model. A significant contribution to the overall antioxidant capacity of cHb is expected to arise from thiol groups, which are easily oxidized to form disulfides or higher oxides under physiological conditions. This is underlined by the observation that β93Cys in human hemoglobin seems to play an essential role in the red blood cell antioxidant network [53]. More evidence for the importance of thiol groups in antioxidant defense was found in the occurrence of high plasma levels of organic disulfides, which are formed by oxidation and subsequent dimerization of thiol-containing proteins, during episodes of oxidative stress [54, 55]. Owing to their comparatively large cysteine content, the hemoglobins of several species in the crocodilian group have been reported to reduce ROS and RNS toxicity [56]. In light of these facts, it is plausible that the antioxidant activity of cHb observed in the present study originates from the abundance of thiol groups and other antioxidative amino acid residues in its primary structure [12]. Furthermore, a number of biologically active organic thiols and thioethers, including glutathione (GSH), N-acetylcysteine (NAC), captopril (CAP), homocysteine (HCYS), and cysteine (CYS), also show strong antioxidant activity, which may confer additional protective effects against oxidative damage [57]. Apart from the sulfur-containing Cys and Met residues, the antioxidant activity of cHb is presumed to involve certain other amino acids, i.e., His, Tyr, Trp, Phe, and Asp. The antioxidant properties of peptides containing His and Tyr may involve the ability to chelate and deactivate reactive metal species and bind lipid peroxide radicals via the imidazole ring in His, as well as effective hydrogen donation by the hydroxyl group in Tyr [12]. Similarly, Asp may contribute to the overall antioxidant activity by donating its acidic hydrogen atoms near neutral pH. Furthermore, the sulfhydryl group of Cys may act as a strong reducing agent; hence, a contribution to the reduction of ferric iron produced by oxidation appears plausible. In a similar way, Trp and Phe were shown to benefit H_2O_2-scavenging activity, which is attributed to the susceptibility of the aromatic side chain to oxidation [58]. Investigation of the effect of cWb on the enzymatic antioxidant system using the Carr model revealed an improvement in the catalytic activity of CAT and GPx only, whereas CAT, SOD, and GPx activity was enhanced in the cotton model. These effects may be traced back to specific blood components, including two specific peptides with the amino acid sequences TDVLGLPAK (912.5 Da) and DPNAALPAGPR (1,148.6 Da) in the crude leukocyte extract of *C. siamensis*, which display antioxidant activity in the DPPH assay [16]. Moreover, the antioxidant properties of plasma might be related to albumin, which constitutes the most abundant protein in plasma and may confer antioxidant activity through the presence of a cysteine thiol group (Cys34) [59].

CONCLUSION

In summary, the collected data suggest that the potent *in vivo* anti-inflammatory activity of cHb and cWb is attributed to an inhibitory effect on lipid peroxidation (MDA formation) and augmentation of the antioxidant activity of the protective enzymes SOD, CAT, and GPx. Treatment with cHb or cWb was directly associated with a significant reduction in paw edema volume and granuloma formation via decreases in pro-inflammatory cytokine levels (IL-6, IL-1β, and TNF-α). In addition, cHb and cWb were found to enhance the release of the anti-inflammatory cytokine IL-10 in both the Carr-induced paw edema and cotton pellet-induced granuloma formation models in mice. Taken collectively, the results of the present study thus underline the potential usefulness of cHb and cWb and may serve as a starting point in the development of crocodile blood-derived health supplements for the prevention and treatment of acute and chronic inflammation.

ACKNOWLEDGMENTS

This work was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, Khon Kaen University, Thailand, the Science Achievement Scholarships of Thailand (SAST), and the Protein and Proteomics Research Center for Commercial and Industrial Purposes (ProCCI), Faculty of Science, Khon Kaen University. The Department of Pharmacology, Faculty of Medicine, Khon Kaen University provided laboratory facilities to perform the Carr-induced mouse paw edema assay. The authors would like to thank Sriracha Moda Co., Ltd., Chon Buri, Thailand for providing the necessary facilities to collect crocodile blood samples.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare no conflict of interest.

REFERENCES

1. Cai, Y., Q. Luo, M. Sun, and H. Corke. 2004. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sciences* 74: 2157–2184.
2. Deng, J.S., C.S. Chi, S.S. Huang, P.H. Shie, T.H. Lin, and G.J. Huang. 2011. Antioxidant, analgesic, and anti-inflammatory activities of the ethanolic extracts of *Taxillus liquidambaricola. Journal of Ethnopharmacology* 137: 1161–1171.
3. Yu, T., H.M. Ahn, T. Shen, K. Yoon, H.J. Jang, Y.J. Lee, et al. 2011. Anti-inflammatory activity of ethanol extract derived from *Phaseolus angularis* beans. *Journal of Ethnopharmacology* 137: 1197–1206.
4. Salvemini, D., Z.Q. Wang, D.M. Bourdon, M.K. Stern, M.G. Currie, and P.T. Manning. 1966. Evidence of peroxynitrite involvement in the carrageenan-induced rat paw edema. *European Journal of Pharmacology* 303: 217–220.
5. Calloni, C., R.D. Agnol, L.S. Martinez, F.D.S. Marcon, S. Moura, and M. Salvador. 2015. Jaboticaba (*Plinia trunciflora* (O. Berg) Kausel) fruit reduces oxidative stress in human fibroblasts cells (MRC-5). *Food Research International* 70: 15–22.
6. Naaz, F., S. Javed, and M.Z. Abdin. 2007. Hepatoprotective effect of ethanolic extract of *Phyllanthus amarus* Schum. et Thonn. on aflatoxinB_1-induced liver damage in mice. *Journal of Ethnopharmacology* 113: 503–509.
7. Miguel, M.G. 2010. Antioxidant and anti-inflammatory activities of essential oils: a short review. *Molecules* 15: 9252–9287.
8. Huang, S.S., C.S. Chiu, T.H. Lin, M.M. Lee, C.Y. Lee, S.J. Chang, et al. 2013. Antioxidant and anti-inflammatory activities of aqueous extract of *Centipeda minima. Journal of Ethnopharmacology* 147: 395–405.
9. Preecharram, S., S. Daduang, W. Bunyatratchata, T. Araki, and S. Thammasirirak. 2008. Antibacterial activity from Siamese crocodile (*Crocodylus siamensis*) serum. *African Journal of Biotechnology* 7: 3121–3128.
10. Preecharram, S., P. Jearranaiprepame, S. Daduang, Y. Temsiripong, T. Somdee, T. Fukamizo, et al. 2010. Isolation and characterisation of crocosin, an antibacterial compound from crocodile (*Crocodylus siamensis*) plasma. *Animal Science Journal* 81: 393–401.
11. Pata, S., N. Yaraksa, S. Daduang, Y. Temsiripong, J. Svasti, T. Araki, et al. 2011. Characterization of the novel antibacterial peptide Leucrocin from crocodile (*Crocodylus siamensis*) white blood cell extracts. *Developmental & Comparative Immunology* 35: 545–553.
12. Srihongthong, S., A. Pakdeesuwan, S. Daduang, T. Araki, A. Dhiravisit, and S. Thammasirirak. 2012. Complete amino acid sequence of globin chains and biological activity of fragmented crocodile hemoglobin (*Crocodylus siamensis*). *The Protein Journal* 31: 466–476.
13. Kommanee, J., S. Preecharram, S. Daduang, T. Yosapong, A. Dhiravisit, Y. Yuzo, et al. 2012. Antibacterial activity of plasma from crocodile (*Crocodylus siamensis*) against pathogenic bacteria. *Annals Clinical Microbiology and Antimicrobial* 11: 22.
14. Yaraksa, N., T. Anunthawan, T. Theansungnoen, S. Daduang, T. Araki, A. Dhiravisit, et al. 2014. Design and synthesis of cationic antibacterial peptide based on Leucrocin I sequence, antibacterial peptide from crocodile (*Crocodylus siamensis*) white blood cell extracts. *The Journal of Antibiotics* 67: 205–212.
15. Jandaruang, J., J. Siritapetawee, K. Thumanu, C. Songsiriritthigul, C. Krittanai, S. Daduang, et al. 2012. The effects of temperature and pH on secondary structure and antioxidant activity of *Crocodylus siamensis* hemoglobin. *The Protein Journal* 31: 43–50.
16. Theansungnoen, T., N. Yaraksa, S. Daduang, A. Dhiravisit, and S. Thammasirirak. 2014. Purification and characterization of antioxidant peptides from leukocyte extract of *Crocodylus siamensis. The Protein Journal* 33: 24–31.
17. Phosri, S., P. Mahakunakorn, J. Lueangsakulthai, N. Jangpromma, P. Swatsitang, S. Daduang, et al. 2014. An investigation of antioxidant and anti-inflammatory activities from blood components of crocodile (*Crocodilus siamensis*). *The Protein Journal* 33: 484–492.
18. Vichai, V., and K. Kirtikara. 2006. Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nature Protocols* 1: 1112–1116.
19. Winter, C.A., E.A. Risley, and G.W. Nuss. 1962. Carrageenin-induced edema in hind paw of the rat as an assay for antiinflammatory drugs. *Proceedings of the Society for Experimental Biology and Medicine* 111: 544–547.
20. Winter, C.A., and C.C. Porter. 1957. Effect of alteration in side chains upon anti-inflammatory and liver glycogen activities in hydrocortisone esters. *Journal of the American Pharmacists Association* 46: 515–519.
21. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248–254.
22. Goth, L. 1991. A simple method for determination of serum catalase activity, and revision of reference range. *Clinica Chimica Acta* 196: 143–152.
23. Sun, Y., L.W. Oberley, and Y. Li. 1988. A simple method for clinical assay of superoxide dismutase. *Clinical Chemistry* 34: 497–500.
24. Chatuphonprasert, W., L. Udomsuk, O. Monthakantirat, Y. Churikhit, W. Putalun, and K. Jarukamjorn. 2013. Effects of *Pueraria mirifica* and miroestrol on the antioxidation-related enzymes in ovariectomized mice. *Journal of Pharmacy and Pharmacology* 65: 447–456.
25. Pinto, R.E., and W. Bartley. 1969. The effect of age and sex on glutathione reductase and glutathione peroxidase activities and on aerobic glutathione oxidation in rat liver homogenates. *Biochemical Journal* 112: 109–115.
26. Ohkawa, H., N. Ohishi, and K. Yagi. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry* 95: 351–358.

27. Kong, B., X. Peng, Y.L. Xiong, and X. Zhao. 2012. Protection of lung fibroblast MRC-5 cells against hydrogen peroxide-induced oxidative damage by 0.1–2.8 kDa antioxidative peptides isolated from whey protein hydrolysate. *Food Chemistry* 135: 540–547.
28. Jia, N., T. Li, X. Diao, and B. Kong. 2014. Protective effects of black currant (*Ribesnigrum* L.) extract on hydrogen peroxide-induced damage in lung fibroblast MRC-5 cells in relation to the antioxidant activity. *Journal of Functional Foods* 11: 142–151.
29. Winterbourn, C.C., and A. Stern. 1987. Human red cells scavenge extracellular hydrogen peroxide and inhibit formation of hypochlorous acid and hydroxyl radical. *The Journal of Clinical Investigation* 80: 1486–1491.
30. Babu, N.P., P. Pandikumar, and S. Ignacimuthu. 2009. Anti-inflammatory activity of *Albizia lebbeck* Benth., an ethnomedicinal plant, in acute and chronic animal models of inflammation. *Journal of Ethnopharmacology* 125: 356–360.
31. Boonyarikpunchai, W., S. Sukrong, and P. Towiwat. 2014. Antinociceptive and anti-inflammatory effects of rosmarinic acid isolated from *Thunbergia laurifolia* Lindl. *Pharmacology, Biochemistry and Behavior* 124: 67–73.
32. Lefkowitz, D.L., M.P. Gelderman, S.R. Fuhrmann, S. Graham, J.D. Starnes, S.S. Lefkowitz, et al. 1999. Neutrophilic lysozyme-macrophage interactions perpetuate chronic inflammation associated with experimental arthritis. *Clinical Immunology* 91: 145–155.
33. Badole, S.L., A.A. Zanwar, A.E. Ghule, P. Ghosh, and S.L. Bodhankar. 2012. Analgesic and anti-inflammatory activity of alcoholic extract of stem bark of *Pongamia pinnata* (L.) Pierre. *Biomedicine & Aging Pathology* 2: 19–23.
34. Da Rosa, J.S., B.M. Facchin, J. Bastos, M.A. Siqueira, G.A. Micke, E.M. Dalmarco, et al. 2013. Systemic administration of *Rosmarinus officinalis* attenuates the inflammatory response induced by carrageenan in the mouse model of pleurisy. *Planta Medica* 79: 1605–1614.
35. Collins, P., J. Burman, H.I. Chung, and K. Fox. 1993. Hemoglobin inhibits endothelium-dependent relaxation to acetylcholine in human coronary arteries *in vivo. Circulation* 87: 80–85.
36. Dreier, J.P., K. Korner, N. Ebert, A. Gorner, I. Rubin, T. Back, et al. 1998. Nitric oxide scavenging by hemoglobin or nitric oxide synthase inhibition by N-nitro-L-arginine induce cortical spreading ischemia when K+ is increased in the subarachnoid space. *Journal of Cerebral Blood Flow & Metabolism* 18: 978–990.
37. Gibson, Q.H., and F.J.W. Roughton. 1957. The kinetics and equilibria of the reactions of nitric oxide with sheep haemoglobin. *The Journal of Physiology* 136: 507–526.
38. Niess, A., F. Passek, I. Lorenz, E. Schneider, H. Dickhuth, H. Northoff, et al. 1999. Expression of the antioxidant stress protein heme oxygenase-1 (HO-1) in human leukocytes. *Free Radical Biology and Medicine* 26: 184–192.
39. Lee, T.S., and L.Y. Chau. 2002. Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. *Nature Medicine* 8: 240–246.
40. Sathyapriya, K., and C.S. Parameswari. 2011. Effect of dialysis on heme oxygenase-1 (HO-1) expression in peripheral blood leukocytes of end staged renal diseased patients. *Asian Journal of Biochemistry* 6: 416–425.
41. Ryter, S.W., J. Alam, and A.M. Choi. 2006. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiological Reviews* 86: 583–650.
42. Habtezion, A., R. Kwan, A.L. Yang, M.E. Morgan, E. Akhtar, S.P. Wanaski, et al. 2011. Heme oxygenase-1 is induced in peripheral blood mononuclear cells of patients with acute pancreatitis: a potential therapeutic target. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 300: 12–20.
43. Darville, L.N.F., M.E. Merchant, A. Hasan, and K.K. Murray. 2010. Proteome analysis of the leukocytes from the American alligator (*Alligator mississippiensis*) using mass spectrometry. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics* 5: 308–316.
44. Esmon, C.T. 2012. Protein C anticoagulant system—anti-inflammatory effects. *Seminars in Immunopathology* 34: 127–132.
45. Zang, Y.X., L. Wang, E.L. Xiao, S.J. Li, J.J. Chen, B. Gao, et al. 2013. Ginsenoside-Rd exhibits anti-inflammatory activities through elevation of antioxidant enzyme activities and inhibition of JNK and ERK activation *in vivo. International Immunopharmacology* 17: 1094–1100.
46. Gao, Y., L. Fang, R. Cai, C. Zong, X. Chen, J. Lu, et al. 2013. Shuang-Huang-Lian exerts anti-inflammatory and anti-oxidative activities in lipopolysaccharide-stimulated murine alveolar macrophages. *Phytomedicine* 21: 461–469.
47. Kirimi, E., O. Tuncer, M. Kosem, E. Ceylan, A. Tas, I. Tasal, et al. 2003. The effects of prednisolone and serum malondialdehyde levels in puppies with experimentally induced meconium aspiration syndrome. *Journal of International Medical Research* 31: 113–122.
48. Pingsusaen, P., P. Kunanusorn, P. Khonsung, N. Chiranthanut, A. Panthong, and C. Rujjanawate. 2015. Investigation of anti-inflammatory, antinociceptive and antipyretic activities of *Stahlianthus involucratus* rhizome ethanol extract. *Journal of Ethnopharmacology* 162: 199–206.
49. Frei, B., R. Stocker, and B.N. Ames. 1988. Antioxidant defenses and lipid peroxidation in human blood plasma. *Proceedings of the National Academy of Sciences of the United States of America* 85: 9748–9752.
50. Yu, M., S.M. Li, X.Y. Li, B.J. Zhang, and J.J. Wang. 2008. Acute effects of 1-octyl-3-methylimidazolium bromide ionic liquid on the antioxidant enzyme system of mouse liver. *Ecotoxicology and Environmental Safety* 71: 903–908.
51. Jung, H.J., J.H. Nam, J. Choi, K.T. Lee, and H.J. Park. 2005. Anti-inflammatory effects of chiisanoside and chiisanogenin obtained from the leaves of *Acanthopanax chiisanensis* in the carrageenan and Freund's complete adjuvant-induced rats. *Journal of Ethnopharmacology* 97: 359–367.
52. Uzkeser, H., E. Cadirci, Z. Halici, F. Odabasoglu, B. Polat, T.N. Yuksel, et al. 2012. Anti-inflammatory and antinociceptive effects of salbutamolon acute and chronic models of inflammation in rats:involvement of an antioxidant mechanism. *Mediators of Inflammation* 2012: 438912.
53. Vitturi, D.A., C.W. Sun, V.M. Harper, B. Thrash-Williams, N. Cantu-Medellin, B.K. Chacko, et al. 2013. Antioxidant functions for the hemoglobin β93 cysteine residue in erythrocytes and in the vascular compartment *in vivo. Free Radical Biology and Medicine* 55: 119–129.
54. Prakash, M., M.S. Shetty, P. Tilak, and N. Anwar. 2009. Total thiols: biomedical importance and their alteration in various disorders. *Online Journal of Health and Allied Science* 8: 2.
55. Chakrabarty, J., M.S. Vidyasagar, D. Fernandes, V. Bhat, J.G. Nagalakshmi, and S.S. Mayya. 2013. Effectiveness of pranayama on the levels of serum protein thiols and glutathione in breast cancer patients undergoing radiation therapy: a randomized controlled trial. *Indian Journal of Physiology and Pharmacology* 57: 225–232.
56. Reischl, E., A.L. Dafre, J.L. Franco, and F.D. Wilhelm. 2007. Distribution, adaptation and physiological meaning of thiols from vertebrate hemoglobins. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 146: 22–53.
57. Demirkol, O., C. Adams, and N. Ercal. 2004. Biologically important thiols in various vegetables and fruits. *Journal of Agricultural and Food Chemistry* 52: 8151–8154.
58. Udenigwe, C.C., and R.E. Aluko. 2011. Chemometric analysis of the amino acid requirements of antioxidant food protein hydrolysates. *International Journal of Molecular Sciences* 12: 3148–3161.
59. Taverna, M., A.L. Marie, J.P. Mira, and B. Guidet. 2013. Specific antioxidant properties of human serum albumin. *Annals of Intensive Care* 3: 4.