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Contributed Paper

Comparisons of Anti-inflammatory Activity of Crocodile (*Crocodylus siamensis*) Blood Extract

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ABSTRACT

Crocodile blood extracts; serum, plasma, hemoglobin, and crude leukocyte extract were prepared from whole blood of crocodiles (*Crocodylus siamensis*) by differential sedimentation. In this study, crocodile blood extracts possessed considerable cytotoxic activity. It was evident that, the serum, plasma, hemoglobin, and crude leukocyte extract have not significant cytotoxic potentials by the yellow tetrazolium bromide assay (MTT assay) using a macrophage-like cell, RAW 264.7. The anti-inflammatory effect of crocodile blood extracts, which were evaluated by examining their inhibitory effects on pro-inflammatory mediators in lipopolysaccharide (LPS)-stimulated murine macrophage RAW 264.7 cells. Both plasma and crude leukocyte extract significantly inhibited the anti-inflammation NO production and exhibited cytotoxicity at the whole tested concentration. In addition, crude leukocyte extract reduced the NO production in a dose-dependent manner, also similar pattern was observed in plasma. On the other hand, serum and hemoglobin cannot inhibit the anti-inflammation NO production. Taken together, these findings indicate that the anti-inflammatory properties of plasma and crude leukocyte extract may be due to the inhibition of NO production. Thus crocodile blood extracts, plasma and crude leukocyte extract may provide a potential therapeutic approach for inflammation related diseases.

Keywords: anti-inflammatory, blood extract, cytotoxicity, Siamese crocodile, nitric oxide

1. INTRODUCTION

The Siamese crocodile (*Crocodylus siamensis*) is a critically endangered species of freshwater crocodiles, originally distributed in most parts of southeast Asia. Crocodilians live with opportunistic bacterial infection but normally suffer no adverse effects. During fights, the limbs of crocodiles are sometimes torn and they are left with gaping wounds or even limbless. However, despite the harsh environment that they live in, they appear to heal rapidly and almost always infection-free [1-3].

Research revealed how powerful the crocodile's immune system is, as opposed to the human immune system [4-7]. It is able to effectively destroy resistant bacteria, as well as viruses including the human immune deficiency virus (HIV) [1, 3, 5, 8]. In China, the blood, oil, bile and gall bladder of crocodiles are used for conditions such as bronchitis, coughing, allergy, skin problems, high blood pressure and cancer [9]. Merchant *et al*. [10] reported potent antimicrobial activity of the serum from the American alligator (*Alligator mississippiensis*) against enveloped viruses in cell-based assays.

In our previous work, we reported the antibacterial activity of serum and plasma from the Siamese crocodile (*Crocodylus siamensis*) also showed potential of anti-bacterial activity against a variety of both Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus*, *Klebsiella pneumonia*, *Streptococcus epidermidis*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Vibrio cholera* [6-7].

Inflammation is typified by the activation of immunocytes such as monocytes and macrophages, and the secretion of inflammatory mediators such as nitric oxide (NO), prostaglandin E_2 (PGE_2), and tumor necrosis factor-α (TNF-α). Although NO plays a significant role in host immune defense, vascular regulation, neurotrans-mission, and other systems under normal conditions, aberrant NO expression is thought to cause severe inflammatory disease [11-12]. Overproduction of inducible NO synthase (iNOS) is especially related to various human diseases such as inflammatory and neuronal disorders because of the up regulation of NO [13-14]. Lipopolyssacharide (LPS)-activated macrophages have usually been used for evaluating the anti-inflammatory effects of various materials. LPS is a principle component of the outer membrane of Gram-negative bacteria, is an endotoxin that induces septic shock syndrome and stimulates the production of inflammatory mediators such as NO, TNF-α, interleukins, prostanoids and leukotrienes [15-17]. Therefore, the inhibition of these inflammatory mediators would be an effective therapeutic approach for regulating LPS-induced septic shock.

From the previous research, crocodile (*Crocodylus siamensis*) blood showed potential of antibacterial activity. Therefore, in the present study we aim to investigate the anti-inflammatory activity of crocodile (*Crocodylus siamensis*) blood extract by suppression of nitric oxide production.

2. MATERIALS AND METHODS

2.1 Crocodile Blood Samples

Crocodiles (*Crocodylus siamensis*) were captured and housed at the local Sriracha Moda Farm, Chon Buri, Thailand. The crocodiles (age ranging from 1-3 years) were housed in a single tank, and treated with electric shock. Blood samples were collected using 0.8 × 38 mm needles and heparinized 5 ml syringe. After blood began to settle, leukocytes appearing in the interphase layer between liquid layer (plasma layer) and red blood cells layer (hemoglobin). They were collected into a centrifuge tube using a transfer pipette and were kept at -70°C until use.

2.2 Cell Line

The mouse macrophage cell line, RAW 264.7 was obtained from the European Collection of Cell Cultures (ECACC) and cultured in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin 100 units/ml, streptomycin 100 μg/ml, and amphotericin B 25 μg/ml were maintained at 37°C in a 5% CO_2 humidified atmosphere.

2.3 Cytotoxicity Assay

The murine macrophage cell line RAW 264.7 were plated onto 96-well plates and treated with different concentrations (62.5, 125, 250, 500 and 1000 μg/ml) of crocodile blood (serum, plasma, hemoglobin and crude leukocyte extract) for 24 h at 37°C in a 5% CO_2 humidified atmosphere. The cell viability of RAW 264.7 was measured after 24 h exposure to the test crocodile blood extracts by colorimetric assay, based on the ability of mitochondria in viable cells to reduce MTT [3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]. An aliquot of MTT solution 0.5 mg/ml was added to each well, and after 30 min incubation at 37°C, the medium was discarded, and the formazan blue formed in the cells was dissolved in dimethyl sulfoxide (DMSO). Absorbance at 570 nm was determined with a microplate reader. The absorbance of the formazan formed in non-treated cells was taken as 100% viability. The cell viability was defined as the % of untreated control cells [i.e. viability (% control) = 100 × {(absorbance of blood extract-treated sample)/(absorbance of control)}].

2.4 Nitric Oxide (NO) Assay

Nitric oxide (NO) production was assayed by measuring the accumulation of the stable oxidative metabolite, nitrite (NO_2^-), in culture supernatants [18]. Briefly, RAW 264.7 cells (2×10^5 cells/ml) were plated onto 96-well plates and treated with LPS (*E. coli* 0111:B4) (1 μg/ml) and different concentrations (62.5, 125, 250, 500 and 1000 μg/ml) of crocodile blood (serum, plasma, hemoglobin and crude leukocyte extract) for 24 h at 37°C in a 5% CO_2 humidified atmosphere. Briefly, the sample supernatants were mixed with equal volume of Griess reagent (2% sulfanilamide in 4% phosphoric acid and 0.2% naphthylethylenediamine dihydrochloride) and then incubated at room temperature for 10 min. The absorbance was measured at 540 nm on a microplate reader. LPS (*E. coli* 0111:B4) (1 μg/ml) and L-NANE (10 μM) were used as a negative and positive control, respectively.

2.5 Statistical Analysis

All data were derived from at least three independent experiments. Statistical analyses were conducted using SigmaPlot software (version 6.0). Values were presented as mean ± SD. Significant differences between the groups were determined using the unpaired Student's *t*-test. Statistical significance was set at $p < 0.05$.

3. RESULTS AND DISCUSSION

The present study was undertaken to elucidate the anti-inflammatory potential of different crocodile blood extracts; serum, plasma, hemoglobin, and crude leukocyte extract. Therefore, we prepared crocodile blood extracts from Siamese crocodile to test their biological effects on RAW 264.7 macrophage-like cell viability and on the production of inflammatory mediators in macrophages RAW 264.7 upon stimulation with LPS.

To assess the cytotoxicity of the crocodile blood extracts; serum, plasma, hemoglobin, and crude leukocyte extract were used to administer to the cells, macrophages RAW 264.7. Although the MTT assay is an indirect measurement of cell density or the number of living cells attached to the culture plate by formation of colored formazan crystals, the crocodile blood extracts exhibited almost similar viability of more than 96% to that of non-treated cells, which was taken as 100% viability (Figure 1). The results obtained indicated that the crocodile blood extracts was not significant cytotoxic to the cells.

These result similar with previous reported by Kommanee *et al*. [7], cells were treated with the indicated concentrations of crocodile plasma in the presence or absence of LPS for 24 h and analyzed cell viability by MTT assay, to assess the effects of crocodile plasma on the viability of RAW 264.7 cells found that crocodile plasma is not toxic to RAW 264.7 cells.

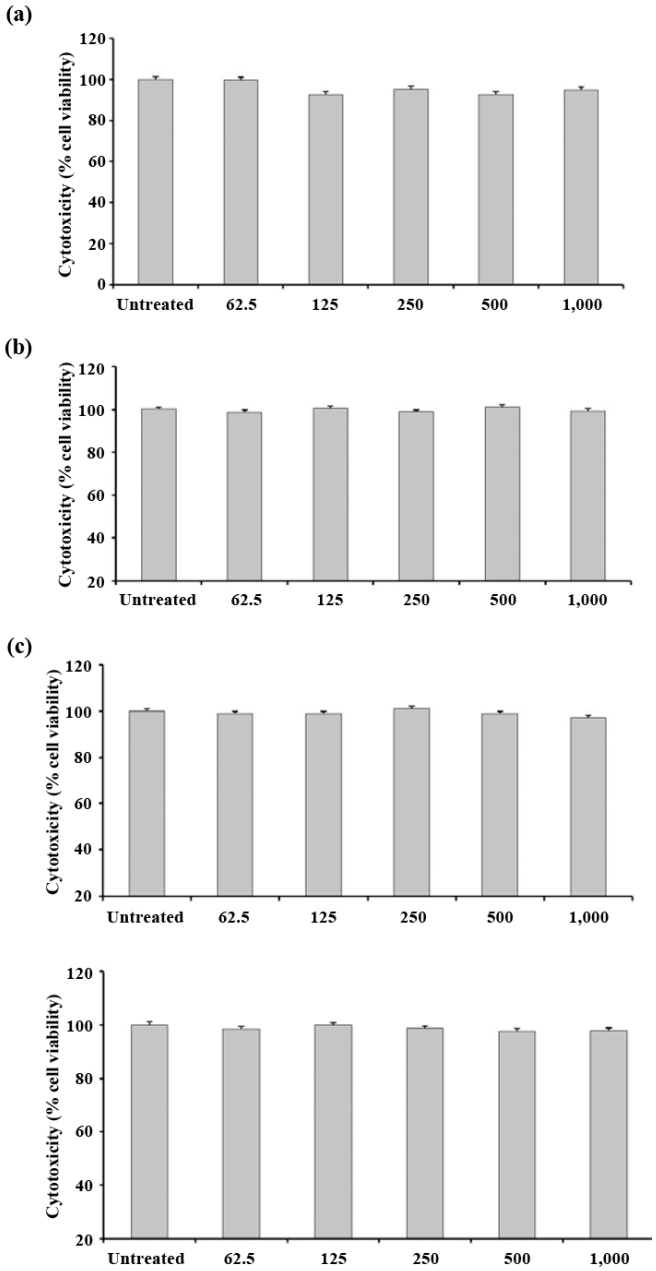

Figure 1. The effect of the Siamese crocodile blood extract; (a) plasma, (b) crude leukocyte extract (WBC), (c) serum, (d) hemoglobin on RAW 264.7 macrophage-like cell viability. Viability of cells treated with LPS alone has been taken as reference (100%). Bars represent the mean and standard deviations from three different experiments performed in triplicate.

Macrophages play an important role in both, host-defence mechanisms and inflammation. Activated macrophages secrete a number of different inflammatory mediators, including NO, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6). The over production of these mediators has been implicated in several inflammatory diseases and cancer [19]. Thus, inhibition of activation of these cells appears to be an important target for the treatment of inflammatory diseases. NO is one ubiquitous cellular mediator of physiological and pathological processes, being largely released at inflammatory sites [20]. It is well known that NO is the main macrophage-derived inflammatory mediators [21-22]. Aberrant production of NO induces an inflammatory response that causes damage to neighboring cells and tissues of the host. Therefore, it has been thought a good strategy to reduce LPS induced inflammation through the suppression of inflammatory mediators (NO). Among the overproduced inflammatory mediators, NO has been strongly implicated in the pathogenesis of several disease processes such as septic shock, rheumatoid arthritis, and autoimmune diabetes [23].

To investigate whether crocodile blood regulates NO production, RAW 264.7 cells were pretreated with crocodile blood extract for 1 h before treatment with LPS (1 μg/ml) and L-NAME (10 μM) for 24 h, and NO production was measured by performing the Griess reagent assay. NO production was reflected in the accumulation of nitrite in the cell culture medium. Treatment with LPS resulted in significant up regulation of nitrite production, compared to the untreated control (Figure 2). However, RAW 264.7 cells treated with crocodile plasma and crude leukocyte extract at different concentrations (62.5-1000 μg/ml) displayed a marked significant decrease in the induction of nitrite after stimulation with LPS (Figure 2a, 2b). From figure 2a and 2b, it was found that crocodile plasma and crude leukocyte extract at different concentrations (62.5-1000 μg/ml) can reduce NO production which was in the range of 59-46% and 50-23%, respectively (Figure 2a, 2b). According to the results of the present study, crocodile plasma and crude leukocyte extract suppressed the production of NO in LPS stimulated RAW 264.7 cells. In addition, crude leukocyte extract reduced the NO production in a dose-dependent manner, also similar pattern was observed in plasma (Figure 2a, 2b). On the other hand, crocodile serum and hemoglobin cannot decrease the NO production (Figure 2c, 2d). Rezaie [24] reported that the protein C is a vitamin K-dependent anticoagulant serine protease zymogen in plasma, activated protein C (APC) binds to endothelial protein C receptor (EPCR) in lipid-rafts/caveolar compartments to activate protease-activated receptor 1 (PAR-1) thereby eliciting anti-inflammatory and cytoprotective signaling responses in endothelial cells. In the case of hemoglobin, it cannot decrease the NO production maybe cause from the polymerization of hemoglobin, probably related to NO scavenging, free radical induction [25]. From the result above, plasma and crude leukocyte extract have a potential anti-inflammatory capacity, suggesting that they could be regarded as a potential source of natural anti-inflammatory agents [26].

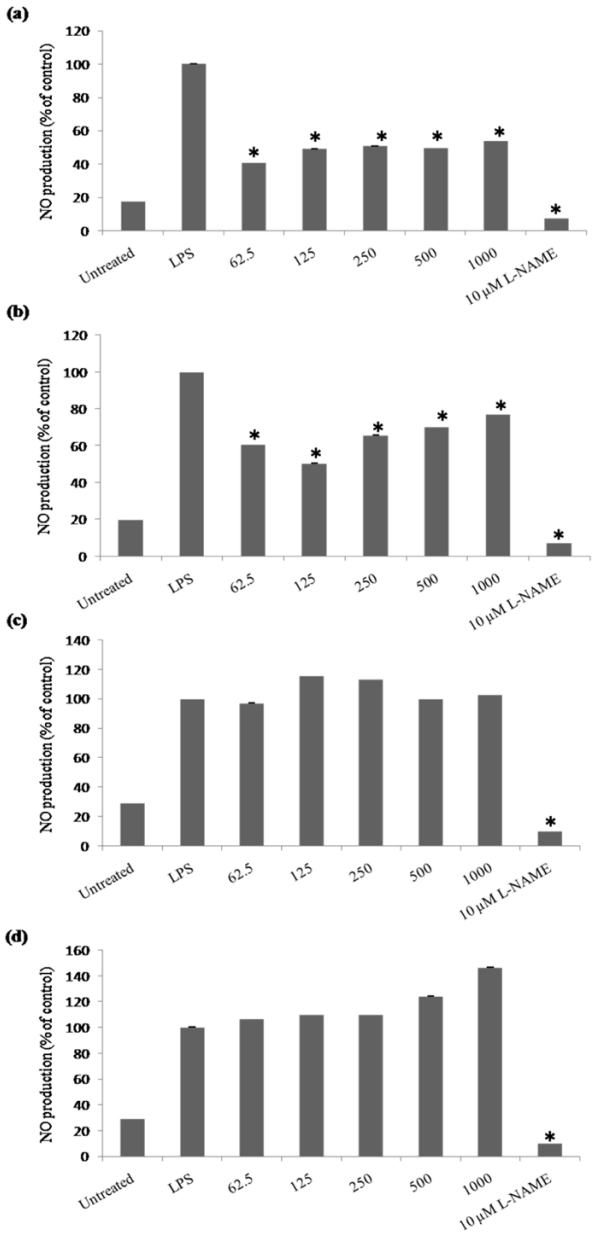

Figure 2. The effect of the Siamese crocodile blood extract; (a) plasma, (b) crude leukocyte extract (WBC), (c) serum, (d) hemoglobin on LPS-induced inflammatory mediators in RAW 264.7 macrophage-like cells. NO production is expressed as percentages of that of the group treated with LPS alone. Values show the means and standard deviations of three different experiments performed in triplicate. $p < 0.05$ significantly different from the LPS group.

4. CONCLUSION

Comparative analysis of the crocodile (*Crocodylus siamensis*) blood extract was studied. The results revealed that the crocodile plasma, crude leukocyte extract, serum, and hemoglobin were not cytotoxic to the cells. Crocodile plasma and crude leukocyte extract has anti-inflammatory effect by

suppressed the production of NO in RAW 264.7 cells. Crocodile crude leukocyte extract reduced the NO production in a dose-dependent manner, also similar pattern was observed in plasma. On the other hand, crocodile serum and hemoglobin cannot decrease the NO production. Therefore crocodile plasma and crude leukocyte extract may provide a potential therapeutic approach for inflammation related diseases.

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