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Contributed Paper

In vitro Anti-aging Activities of Crocodile (*Crocodylus siamensis*) Blood Extracts

Jiradej Manosroi [a, b, c, d], Charinya Chankhampan [a, c, d], Worapaka Manosroi [e] and Aranya Manosroi* [a, b, c, d]

[a] Manose Health and Beauty Research Center, Muang, Chiang Mai, 50200, Thailand.
[b] Science and Technology Research Institute (STRI), Chiang Mai University, Chiang Mai 50200, Thailand.
[c] Research Administration Center, Chiang Mai University, Chiang Mai 50200, Thailand.
[d] Faculty of Science and Technology, North-Chiang Mai University, Chiang Mai 50230, Thailand.
[e] Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand.
*Author for correspondence; e-mail: a.manosroi@gmail.com

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ABSTRACT

The objective of this study was to determine anti-aging activities of crocodile (*Crocodylus siamensis*) blood extracts. The extracts were prepared from the whole blood and the precipitated blood (in which the serum was separated). Each was extracted by six different processes including cold water (WC), hot water (WH), cold ethyl acetate (EC), hot ethyl acetate (EH), cold methanol (MC) and hot methanol (MH). The ethyl acetate and methanol extracts were dried by a hot air oven, while the water extracts and the seperated serum were by lyophilization. The total of 15 extracts was investigated for *in vitro* anti-aging activities including antioxidant, tyrosinase inhibition and gelatinolytic activity of MMP-2 inhibition on human skin fibroblasts as well as cytotoxicity. The dried blood serum (SR) gave the highest DPPH radical scavenging (SC_{50} value = 1.83±0.60 mg/ml) and lipid peroxidation inhibition (IPC_{50} value = 0.91±0.13 mg/ml). The precipitated blood extracted by cold ethyl acetate (POEC) showed the highest metal chelating (MC_{50} = 0.0085±0.007 mg/ml). The tyrosinase inhibition activity of POEC (IC_{50} = 0.016±0.003 mg/ml) was higher than kojic acid of 2.25 times. All extracts gave no cytotoxicity at all concentrations except the blood extracted by cold water (CBWC) at 1 mg/ml. The extract which indicated the highest cell viability at 1 mg/ml was the precipitated blood extracted by hot methanol (POMH). The blood extracted by cold ethyl acetate (CBEC) exhibited the highest MMP-2 inhibition activity on human skin fibroblasts with the percentages of pro MMP-2 inhibition at 47.30±10.51%, but lower activity than ascorbic acid of 1.93 times. The result from this study has demonstrated the commercial potential of crocodile blood to be developed as raw materials for an anti-aging product.

Keywords: crocodile blood, *Crocodylus siamensis*, antioxidant, tyrosinase inhibition, cytotoxicity, MMP-2 inhibition

1. INTRODUCTION

Crocodylus siamensis or Siamese crocodile is a critically endangered species of freshwater crocodile that was originally distributed throughout South East Asia. In Thailand, commercial crocodile farms produce hides and meat as major products, while the blood has been a waste. In fact, its blood composes of white blood cells, hemoglobin, plasma and serum. Siamese crocodile is a medicinal animal since its blood has been reported to have antimicrobial and antiviral activities. In many Asian cultures, crocodile blood has been traditionally consumed for improving human health. Recently, several pharmaceutical activities of crocodile blood have been scientifically investigated. Antimicrobial activity is the most important activity found in crocodile blood. The fresh serum and freeze dried serum from Siamese crocodile exhibited potential antibacterial (*Enterobacter aerogenes* ATCC 13048, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 27736, *Salmonella typhimurium* ATCC 13311 and *Pseudomonas aeruginosa* ATCC 27853) and antifungal (*Cryptococcus neoformans* 250309 and *Aspergillus niger*) activities [1]. Crocosin and leucrosin, the antimicrobial peptides, were recently identified from the Siamese crocodile plasma [2-3]. Crocodile serum has enhanced the macrophage phagocytic activity through the complement activity [4]. The serum and plasma of *C. siamensis* which inhibited the proliferation of HepG2, BGC823, HeLa and SKOV3 cell have been reported. The blood of *C. siamensis* gave the tumor inhibitory rate of up to 57.55% on S180 tumor [5]. Jandaruang et al. have reported that hemoglobin from *C. siamensis* exhibits anti-oxidant activity by the DPPH radical scavenging assay which depends on its structure and the pH [6]. Additionally, it has been found that the native α-globin and β-globin subunits separated from the hemoglobin molecule can provide the antioxidant activity [7]. The peptide powder from *C. siamensis* which was prepared using the enzymatic catalysis of trypsin gave strong antioxidant activity, by inhibiting the biphenolase activity of the mushroom tyrosinase [8]. However, no work has been performed on anti-aging activity of the crocodile blood extracts. The aim of this study was to investigate the anti-aging potential of the crocodile blood extracts by determining the *in vitro* biological activities including antioxidant activities, tyrosinase inhibition activity and gelatinolytic activity of MMP-2 inhibition on human skin fibroblasts as well as the cytotoxicity.

2. MATERIALS AND METHODS

2.1 Materials

2, 2-diphenyl-1-picrylhydrazyl radical (DPPH), ethylenediaminetetraacetic acid (EDTA), sulphorodamine B (SRB), vitamin C (l-(+)-ascorbic acid, 99.5%), dimethyl sulfoxide (DMSO), kojic acid (99.0%), ammonium thiocyanate (NH_4SCN), acrylamide (minimum 99%) and glycerol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mushroom tyrosinase (4276 U/mg), l-tyrosine, linoleic acid (99.0%), N, N'-methylenebisacrylamide and TEMED (N, N, N', N'-tetramethyl ethylenediamine) from Fluka (Switzerland) and ammonium sulfate and glycine from BDH Limited Poole, England were used. Tris (hydroxymethyl) methylamine was purchased from Fisher Scientific UK Limited, UK. Sodium dodecyl sulfate and coomassie brilliant blue R-250 from Bio-Rad Laboratories, UK, and bromophenol blue dye and ammonium persulfate from Amersco Inc., USA were used. Dulbecco's modified Eagle's culture medium (DMEM), antibiotics (penicillin and streptomycin), fetal bovine serum (FBS) and

trypsin were purchased from HyClone (Logan, UT, USA). All other chemicals and reagents were analytical grade.

2.2 Preparation of the Extracts

The dry whole blood powder was provided from Warodom Clinic, Nakhon Pathom in Thailand. The 50 g of the whole blood were dispersed with 1,250 ml of water and mixed for 1 h. After filtration, the filtrate was concentrated under vacuum by a rotary evaporator (R-124 Buchi, Switzerland) and lyophilized. The precipitated blood was collected and dried by a hot air oven (Memmert GmbH & Co. KG, Germany) at 45±2°C. The extracts were prepared from the whole blood and the precipitated blood by the hot and cold processes using 3 different solvents (water, methanol and ethyl acetate). For the hot process, the dried powder of the whole blood or the precipitated blood was boiled with the solvent at its boiling point [water (98±2°C), methanol (65±2°C) and ethyl acetate (75±2°C)] for 1 h. For the cold process, the dried powder was mixed with the solvents and sonicated in a sonicator bath (Chest ultrasonics corp., NJ, USA) at ambient temperature (27±2°C) for 1 h. The extracts from the hot and cold methods were centrifuged, filtered and concentrated by a rotary evaporator (Rotavapor R210, Buchi, Flawil, Switzerland) under vacuum at 50±2°C and dried by lyophilization (water extracts) or a hot air oven at 45±2°C (methanol and ethyl acetate extracts). The percentage yield was calculated on a dried weight basis. The obtained extracts were kept at 4±2°C in the air tight container with cap until use.

2.3 Antioxidant Activities

2.3.1 DPPH radical scavenging assay

Free radical scavenging activity of the extracts was determined by a modified DPPH assay [9]. Briefly, 50 µl of the extracts dissolved in distilled water or 20%DMSO in 70%ethanol at the concentrations of 0.001, 0.01, 0.1, 1 and 10 mg/ml, and 50 µl of DPPH in ethanol solution were put in each well of a 96-well microplate (Nalge Nunc International, NY). The reaction mixtures were allowed to stand for 30 min at room temperature (27±2°C), and the absorbance was measured at 515 nm by a well reader (Model 680 microplate reader, Bio-Rad Laboratories Ltd., Corston, UK) against the negative control (distilled water or 20%DMSO in 70%ethanol). Ascorbic acid (0.001-10 mg/ml) was used as a positive control. The experiments were done in triplicate. The percentages of free radical scavenging activity were calculated as the following: Scavenging (%) = $[(A-B)/A]\times100$, where A was the absorbance of the negative control and B was the absorbance of the sample. The sample concentration providing 50% of scavenging (SC_{50}) activity was calculated from the graph plotted between the percentages of the scavenging activity and the sample concentrations.

2.3.2 Lipid peroxidation inhibition assay

The antioxidant activity of the extracts was assayed by the modified ferric-thiocyanate method [9]. An amount of 50 µl of five serial concentrations of the extracts (0.001-10 mg/ml) dissolved in distilled water or 20%DMSO in 70%ethanol was added to 50 µl of linoleic acid in 50% (v/v) DMSO. The reaction was initiated by the addition of 50 µl of NH_4SCN (5mM) and 50 µl of $FeCl_2$ (2mM). The mixture was incubated at 37±2°C in a 96-well microplate for 1 h and measured at 490 nm. The solution without the sample was used as a negative control. Ascorbic acid (0.001-10 mg/ml) was used as a positive control. All determinations were performed in

triplicate. The inhibition percentages of lipid peroxidation of linoleic acid were calculated by the following equation: *Inhibition of lipid peroxidation (%) = [(A-B)/A]×100*, where *A* was the absorbance of the control and *B* was the absorbance of the sample. The sample concentration providing 50% inhibition of lipid peroxidation (IPC_{50}) was calculated from the graph plotted between the percentages of lipid peroxidation inhibition and the sample concentrations.

2.3.3 Metal ion chelating assay

The metal ion chelating activity of the extracts was assayed by the modified ferrous ion chelating method [9]. Briefly, 100 µl of five serial concentrations of the samples (0.001-10 mg/ml) dissolved in distilled water or 20%DMSO in 70%ethanol were added to the solution of 2 mM $FeCl_2$ (50 µl) in distilled water. The reaction was initiated by the addition of 5 mM ferrozine (50 µl) and the total volume was adjusted to 300 µl by distilled water. Then, the mixture was left at room temperature (27±2°C) for 15 min. Absorbance of the resulting solution was then measured at 570 nm by a microplate reader. EDTA (0.001-10 mg/ml) was used as a positive control. The negative control contained $FeCl_2$ and ferrozine, was the complex formation molecules. All experiments were performed in triplicate. The inhibition percentages of ferrozine-Fe^{2+} complex formation were calculated by the following equation: *Metal chelating activity (%) = [(A-B)/A]×100*, where *A* was the absorbance of the negative control and *B* was the absorbance of the sample. The sample concentration providing 50% metal chelating activity (MC_{50}) was calculated from the graph plotted between the percentages of metal chelating activity and the sample concentrations.

2.4 Tyrosinase Inhibition Assay

The tyrosinase inhibition activity of the extracts was assayed by the modified dopachrome method using tyrosine as a substrate [10]. Briefly, 50 µl of five serial concentrations of the samples (0.001-10 mg/ml) dissolved in distilled water or 20%DMSO in 70%ethanol, and 50 µl of 100 units mushroom tyrosinase solution in 0.1 M phosphate buffer, 50 µl of 1 mg/ml tyrosine solution in 0.1 M phosphate buffer and 50 µl of 0.1 M phosphate buffer were added into each well of a 96-well plate. The mixture was incubated at 37±2°C for 60 min and the absorbance at 450 nm was measured. Kojic acid (0.001-10 mg/ml) was used as a positive control. The solution without the sample was used as a negative control. All experiments were performed in triplicate. The percentages of tyrosinase inhibition were calculated according to the following equation: *Tyrosinase inhibition activity (%) = [(A-B)-(C-D)/(A-B)]×100*, where *A* was the absorbance of the blank after incubation, *B* was the absorbance of the blank before incubation, *C* was the absorbance of the sample after incubation, and *D* was the absorbance of the sample before incubation. The sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted between the percentages of tyrosinase inhibition activity and the sample concentrations.

2.5 Cytotoxicity on Human Skin Fibroblasts

2.5.1 Cell culture

The normal human skin fibroblasts were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured under the standard conditions in the complete culture medium containing Dulbecco's Modified Eagle's Medium

(DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 mg/ml). Cells were incubated in a temperature-controlled and humidified incubator (Shel Lab, model 2123TC, Cornelius, USA) with 5% CO_2 at 37°C.

2.5.2 The SRB assay

The extracts were tested for cytotoxicity on the normal human skin fibroblasts (6th passage) by the SRB assay as previous described [11]. The cells were plated at the density of 1×10^5 cells/well in 96-well plates and left overnight for cell attachment on the plate in 5% CO_2 at 37°C. Cells were then exposed to five serial concentrations of the samples (0.0001-1 mg/ml) for 24 h. After incubation, the adherent cells were fixed *in situ*, washed and dyed with SRB. The bound dye was solubilized and the absorbance was measured at 540 nm by a well reader. Ascorbic acid (0.0001-1 mg/ml) was used as a positive control. The experiments were done in triplicate. The percentages of cell viability were calculated according to the following equation: $cell\ viability\ (\%) = (A/B)\times100$, where *A* was the absorbance of the cells treated with the samples and *B* was the absorbance of the control cells.

2.6 Gelatinolytic Activity on MMP-2 Inhibition Zymography

The extracts were tested for gelatinolytic activity of MMP-2 inhibition in comparing to ascorbic acid [10]. A monolayer of 5×10^5 cells of the normal human skin fibroblasts (6th passage) was maintained in the culture medium without FBS for 24 h, treated with the samples and ascorbic acid at 1 mg/ml dissolved in 5% (v/v) DMSO and incubated for 48 h. From our preliminary study, the area and intensity of bands on the zymograms from the cell culture supernatant after 24 h incubation were unclear and also could not be detected and evaluated by gel documentation. So, an increase incubation time was performed. In fact, Oum'hamed et al. have demonstrated that the MMP-2 gelanolytic activity increased with times (from 1 to 48 h) and reached the maximal intensity at 48-72 h [12]. The culture supernatants were collected to assess the gelatinolytic activities of MMP-2. The SDS-PAGE zymography using gelatin as a substrate was performed. Briefly, 20 µl of the cell culture supernatant were suspended in the loading buffer [0.125 M Tris (pH 6.8), 4% (w/w) SDS and 0.04% bromophenol blue] and run on 10% SDS polyacrylamide gel containing 0.1% (w/v) gelatin. After electrophoresis, gels were washed to remove SDS and incubated for 20 min in the renaturing buffer [50 mM Tris, 5 mM $CaCl_2$, 0.02% NaN_3 and 2.5% Triton X-100]. The gels were then incubated for 24 h at 37°C in the developing buffer [50 mM Tris (pH 7.5), 5 mM $CaCl_2$, 0.02% NaN_3 and 1% Triton X-100]. Gels were subsequently stained with 0.5% Coomassie brilliant blue G-250 and de-stained in 30% methanol and 10% acetic acid to detect the gelatinolytic activity. The gel was documented by a gel documentation system (Bio-Rad Laboratories, UK) and analyzed by the Quantity 1-D analysis software. The area multiplied by intensity (mm^2) of the bands on the gel was determined as the relative MMP-2 content. The percentages of MMP-2 inhibition in comparing to the control (the untreated systems) were calculated by the following equation: $MMP\text{-}2\ inhibition\ (\%) = 100\text{-}[(A/B)\times100]$, where *A* was MMP-2 content of sample and *B* was MMP-2 content of control. The assays were done in three independent separately experiments. The MMP-2 inhibition potency of the samples was compared with the positive control (ascorbic acid).

2.7 Statistical Analysis

Data were expressed as mean ± SD. Statistical analysis was carried out using the ANOVA using the software SPSS 13.0 for Windows and p at less than 0.05 was considered as statistical significance.

3. RESULTS AND DISCUSSION

3.1 Physical Characteristics and Percentage Yields of the Extracts

Most whole blood and precipitated blood extracts were semi-solid in yellow-brown color, except the whole blood extract prepared by water extraction and the blood serum were dried powder in red-brown color. Table 1 demonstrated the percentage yields of the crocodile blood extracts. The blood serum dried by lyophilization (SL) gave the highest percentage yield (18.24%). The high yield of the blood serum may be due to the water solubility of proteins, minerals and enzymes which were the constituents found in the blood serum [13]. The blood serum is composed of protein, carbohydrate and lipid of about 69.0, 8.2 and 6.9 g/100g, respectively [14]. The precipitated blood extracted by cold ethyl acetate (POEC) gave the lowest percentage yield (0.34%). The mean percentage yields of the extracts were decreased in the order of the following solvents: methanol > water > ethyl acetate. The different solubility of the solute in different polarity solvent is expected. The highest percentage yield of the methanolic extracts may be due to various polar and non-polar compounds containing in the extracts which can be dissolved by methanol.

Table 1. Percentage yields, antioxidative and tyrosinase inhibition activities of the crocodile blood extracts.

Extracts			Code	%yield	Antioxidative activities			Tyrosinase inhibition activity
					SC_{50} (mg/ml)	IPC_{50} (mg/ml)	MC_{50} (mg/ml)	IC_{50} (mg/ml)
Crocodile whole blood	Whole		CB	ND	>1000	-	-	-
	Water	Hot	CBWH	5.06	2.64±1.14	-	-	-
		Cold	CBWC	10.93	>1000	-	-	-
	Methanol	Hot	CBMH	9.58	53.83±12.83	-	-	-
		Cold	CBMC	10.70	>1000	-	0.36±0.10	1.17±0.47
	Ethyl acetate	Hot	CBEH	0.96	>1000	-	0.24±0.09	0.54±0.39
		Cold	CBEC	0.74	>1000	-	0.32±0.07	0.99±0.28
Crocodile precipitated blood	Oven		PO	17.75	>1000	-	-	-
	Water	Hot	POWH	3.36	3.28±0.08	-	-	1.08±0.87
		Cold	POWC	2.70	23.29±2.70	-	-	3.86±2.50
	Methanol	Hot	POMH	5.63	>1000	-	0.013±0.01	-
		Cold	POMC	4.71	>1000	-	0.022±0.01	-
	Ethyl acetate	Hot	POEH	0.59	-	-	0.037±0.04	0.083±0.035
		Cold	POEC	0.34	-	-	0.0085±0.007	0.016±0.003
Crocodile blood serum	Lyophilize		SL	18.24	4.05±0.24	3.60±0.91	>1000	-
	Evaporate		SR	17.54	1.83±0.60	0.91±0.13	-	-
Standard					0.14±0.02 (ascorbic acid)	0.15±0.04 (ascorbic acid)	0.058±0.01 (EDTA)	0.036±0.007 (kojic acid)

Note: ND = not determined, - = no activity, SC_{50} value (mg/ml) was the concentration of the sample that scavenged 50% of the DPPH radicals. IPC_{50} value (mg/ml) was the concentration of the sample that inhibited 50% of the lipid peroxidation. CC_{50} value (mg/ml) was the concentration of the sample that chelated 50% of the metal ion. IC_{50} value (mg/ml) was the concentration of the sample that inhibited 50% of the tyrosinase enzyme.

3.2 Antioxidative Activities

Antioxidative activities including free radical scavenging, lipid peroxidation inhibition and metal chelating of the crocodile blood extracts were shown in Table 1.

3.2.1 DPPH radical scavenging activity

The SR exhibited the highest free radical scavenging activity (SC_{50} values of 1.83±0.60 mg/ml), but lower than ascorbic acid (0.14±0.02 mg/ml) of 13.07 times. Protein containing in the crocodile blood serum are albumin and globulin [15]. Albumin is the major extracellular source of the reduced sulphydryl groups which are present on a single exposed cysteine residue at position 31 in the molecule. These sulphydryl groups, termed thiols, are avid scavengers of reactive oxygen and nitrogen species, especially the superoxide hydroxyl and peroxynitrite radicals. Albumin can also limit the production of these reactive species by binding free copper (Cu^{2+}), an ion known to be particularly important in accelerating the production of free radicals [16]. Moreover, free radical scavenging activity may be from the minerals containing in the blood serum such as selenium and zinc which are well known for their ability to scavenge reactive oxygen species (ROS). The selenium and zinc contents in plasma of male American alligators have been reported in the range of about 0.17-0.2 and 0.42-0.44 µg/ml, respectively [13]. Free radical scavenging activity of the crocodile blood cell has also been reported. The white blood cell extracts from *C. siamensis* blood at 11, 1.375 and 0.37 mg/ml exhibited DPPH free radical scavenging activity (percentage of antioxidant index; %AI) at 88.05%, 81.70% and 54.67 %, respectively [17]. Theansungnoen et al. have demonstrated that leukocyte extract of *C. siamensis* containing peptides give strong antioxidant activity in reducing free radicals on the DPPH molecules [18].

3.2.2 Lipid peroxidation inhibition activity

Only two extracts including SR and SL gave lipid peroxidation inhibition activity at the IPC_{50} values of 0.91±0.13 and 3.60±0.91 mg/ml, but lower than ascorbic acid (0.15±0.04 mg/ml) of 6.07 and 24 times, respectively. This antioxidant capacity might be due to albumin and globulin found in the blood serum. Lipid peroxidation inhibitory activity of the peptide fractions of albumin and globulin has been reported. These peptide fractions interfered the propagation cycle of lipid peroxidation and thereby slowing radical-mediated linoleic acid oxidation [19]. Bovine serum albumin has been previously demonstrated to inhibit lipid peroxidation by preventing the interaction of iron chelate with membranes, followed by a decrease of its availability for the initiation of membrane lipid peroxidation [20]. The peptide which contains hydrophobic amino acids such as Phe and Gly in its sequence may contribute to the peroxidation inhibition by increasing the solubility of peptide in lipid and thereby facilitating better interaction with radical species. Moreover, the activity of histidine in the peptides has also been reported to act against lipid peroxidation. An imidazole ring in histidine structure may involve in hydrogen donation and lipid radical trapping ability [21].

3.2.3 Metal ion chelating activity

Most extracts from the precipitated blood showed high metal ion chelating activity. The precipitated blood extracted by cold ethyl acetate (POEC) exhibited the highest metal ion chelating activity (MC_{50} values of 0.0085±0.007 mg/ml), higher than EDTA (0.058±0.01 mg/ml) of 6.82 times. Several studies have found that the main bioactive compound of the crocodile blood is the peptide. As known, acidic and/or basic

amino acids play an important role in the chelation of metal ions by carboxyl and amino groups in their side chains. The presence of the histidine residues which are frequently observed in sequences of peptide ion chelators was presumed to be this observed activity. More specifically, the presence of histidine at the N-terminal of the peptide sequence was effective in metal ion chelation [22]. Porphyrin, the central component of hemoglobin in red blood cell is also a chelating agent. It can coordinate to a metal using the four nitrogen atoms as electron-pair donors, and hence is a polydentate ligand.

3.3 Tyrosinase Inhibition Activity

Aging is often associated with alterations in skin pigmentation, with one of the most noticeable alterations being age spots or melasma. Tyrosinase is involved in melanin biosynthesis and the abnormal accumulation of melanin pigments leading to hyperpigmentation [23]. Thus, any compound which can inhibit tyrosinase will have anti-aging activity. Tyrosinase inhibition activity of the crocodile blood extracts were shown in Table 1. Most extracts from ethyl acetate extraction, especially the precipitated blood gave high tyrosinase inhibition activity. The precipitated blood extracted by cold ethyl acetate (POEC) exhibited the highest tyrosinase inhibition activity (IC_{50} values of 0.016±0.003 mg/ml), higher than kojic acid (0.036±0.007 mg/ml) of 2.25 times. Porphyrin, the component of the red blood cell may play an important role in chelating the copper ion at the active site of the tyrosinase enzyme. In fact, the porphyrin ring is an excellent chelating ligand, with the four nitrogen atoms binding strongly to a co-ordinated metal atom in a square planar arrangement. Tyrosinase activity depends on the function of copper ions. So, the agents with affinity to bind to these ions can inhibit the activity of tyrosinase [24]. Interesting, most extracts showing high tyrosinase inhibition were from the ethyl acetate extraction, since ethyl acetate can extract several lipids. Several lipids purified from natural sources exhibited tyrosinase inhibitory activity. A triacylglycerol, trilinolein, which is a byproduct lipid isolated from sake lees has been proved to be as potent as kojic acid for the inhibition of diphenolase activity of mushroom tyrosinase [25]. The fatty acids such as octanoic acid, hexanoic acid and butyric acid can potently inhibit both monophenolase and diphenolase activity of tyrosinase [26]. The membrane of red blood cell is composed of proteins and lipids such as oleic acid, linoleic acid, α-linolenic acid, palmitic acid, stearic acid and docosahexaenoic acid [27]. As known, unsaturated fatty acids, such as oleic acid, linoleic acid and α-linolenic acid can decrease melanin synthesis and tyrosinase activity. Linoleic acid decreased the DOPA reaction of tyrosinase and tyrosinase protein [28]. Docosahexaenoic acid has been reported to decrease α-MSH-induced melanin synthesis through increasing tyrosinase degradation [29].

3.4 Cytotoxicity on Human Skin Fibroblasts

The viability percentages of human skin fibroblast treated with the crocodile blood extracts were presented in Table 2. All samples at the concentration range of 0.0001 to 1 mg/ml gave cell viability of more than 75% with no significant difference at $p<0.05$, except the blood extracted by cold water (CBWC) at 1 mg/ml which showed 66.64±7.01 % cell viability. CBWC was from the whole blood which contained the complex mixture of biological compounds that might be toxic to human skin fibroblasts. This may be due to the cationic peptide

contents of the crocodile blood. It has been found that these peptides can bind to the lipopolysaccharide (LPS) of the bacterial cells through ionic interactions (ion binding), and after that they form a structure that disrupts and permeabilizes the bacterial cell membrane [1]. Thus, the positively charges at the antimicrobial peptides are related to cytotoxicity. The positively charges of the antimicrobial peptides are related to cytotoxicity of the normal cell membrane. Nogueira et al. have investigated the toxicity of the cationic nanovesicles in skin cell lines including murine Swiss albino fibroblasts (3T3) and spontaneously immortalized human keratinocyte (HaCaT) [30]. The cationic charge of the vesicles is responsible for the initial binding to the negatively charged cell membrane by the ionic interaction thereby causing cell death. When the concentrations of the extracts increased, cell viability was increased. This indicated the stimulation effects of the extracts on collagen synthesis of the human skin fibroblasts, which may be beneficial for the anti-aging property. The extract which indicated the highest cell viability at 1 mg/ml of 207.69±17.80% was the precipitated blood extracted by hot methanol (POMH). Previous study has reported that the Siamese crocodile plasma was not cytotoxic to the macrophage-like cell, RAW 264.7 [26]. The crocodile plasma at 62.5, 125, 250, 500 and 1,000 µg/ml exhibited almost similar viability of more than 96% to that of the non-treated cells, which was taken as 100% viability. However, there was no report on the cytotoxicity of human skin fibroblast cells treated with the crocodile blood extracts. This present study is the first report.

Table 2. The percentages of cell viability on human skin fibroblasts at the 6th passage by the SRB assay of the crocodile blood extracts.

	Extracts		Code	%cell viability				
				0.0001 mg/ml	0.001 mg/ml	0.01 mg/ml	0.1 mg/ml	1 mg/ml
Crocodile whole blood	Whole		CB	75.45±8.15	93.67±12.71	89.75±19.48	110.58±21.51	152.43±13.35
	Water	Hot	CBWH	103.84±20.91	120.03±19.77	137.86±15.18	154.80±13.33	165.14±18.20
		Cold	CBWC	139.45±17.52	131.34±10.58	128.59±12.46	98.67±12.62	66.64±7.01
	Methanol	Hot	CBMH	149.51±16.25	125.52±11.41	119.00±16.14	119.57±10.51	117.42±15.78
		Cold	CBMC	127.95±11.84	151.17±18.54	144.80±15.53	147.64±17.56	140.90±6.70
	Ethyl acetate	Hot	CBEH	128.05±22.43	122.50±16.83	148.32±4.69	115.20±13.33	94.78±13.84
		Cold	CBEC	125.63±13.03	117.28±26.57	108.36±19.98	104.88±17.42	94.78±6.93
Crocodile precipitated blood	Oven		PO	119.37±6.38	115.79±7.38	114.42±1.51	107.50±10.07	96.64±9.51
	Water	Hot	POWH	92.21±8.55	97.16±9.77	121.59±16.26	138.57±15.23	156.79±19.56
		Cold	POWC	119.29±15.81	105.55±19.20	103.76±21.66	101.87±16.05	97.82±14.18
	Methanol	Hot	POMH	103.49±8.99	130.94±18.63	163.24±12.15	168.30±17.35	207.69±17.80
		Cold	POMC	149.56±15.07	134.69±3.23	128.08±16.54	118.93±13.99	110.63±17.92
	Ethyl acetate	Hot	POEH	107.88±14.00	123.32±13.41	136.28±19.41	154.51±12.79	197.97±7.56
		Cold	POEC	140.15±17.50	155.02±14.98	97.08±16.58	87.93±14.44	82.08±12.34
Crocodile blood serum	Lyophilize		SL	117.31±18.17	102.84±14.32	111.48±5.23	133.04±13.57	157.75±18.56
	Evaporate		SR	146.34±24.34	163.88±15.97	170.08±16.31	174.95±13.82	189.52±16.28
Ascorbic acid				108.15±2.10	113.56±8.36	117.42±3.40	140.21±2.46	167.38±4.44

Note: Cell viability (%) = (absorbance$_{\text{treated cell}}$/ absorbance$_{\text{control cell}}$) ×100

3.5 Gelatinolytic Activity on MMP-2 Inhibition

The atrophy of collagen and elastin fibers in aged skin is predominantly from the increased expression of the degradative enzymes, collagenases (MMP-1), gelatinases (MMP-2 and -9), and elastases. Collagen fibers can be degraded by MMP-1 and MMP-2 while the elastin fibers by elastases, MMP-2, and MMP-9 [31]. Thus, any compound which can inhibit MMP-2 will have anti-aging activity. Figure 1 presented the zymograms of the gelatinolytic activity on MMP-2 inhibition of the crocodile blood extracts at 1 mg/ml. The zymograms which gave the reduction in the area or intensity of bands represented the MMP-2 inhibition activity, while those indicated the increasing in the area or intensity of bands represented the MMP-2 stimulation activity. Table 3 demonstrated the gelatinolytic activity of the crocodile blood extracts. The nine crocodile blood extracts including CBWC, CBMH, CBEH, CBEC, PO, POEH, POEC, SL and SR inhibited pro MMP-2. The blood extracted by cold ethyl acetate (CBEC) indicated the highest pro MMP-2 inhibition activity with the percentages of pro MMP-2 inhibition at 47.30±10.51%, but lower activity than ascorbic acid (%MMP-2 inhibition of 91.26±17.48%) of 1.93 times. The precipitated blood extracted by hot water (POWH) demonstrated 2 bands which indicated the two types of pro-form (upper band) and active-form (lower band) of *MMP-2*. POWH gave the active MMP-2 stimulation activity which was an undesirable activity for anti-aging. MMP-2 has been described to play an important role in the degradation of the fibrillar collagens leading to aging skin. However, POWH can be applied for the treatment of keloids and hypertrophic scars which have excessive accumulation of collagen. Most extracts from ethyl acetate extraction showed high MMP-2 inhibition activity. This may be due to the non-polar compounds containing in the extracts which can be dissolved by ethyl acetate. Fatty acids such as oleic acid have been proven to have an important inhibitory effect on gelatinase A (MMP-2) [32]. Gamma linoleic acid (GLA) has also been reported to be associated with a decrease in MMP-2 mRNA expression in the orthotopic C6 glioma model [33].

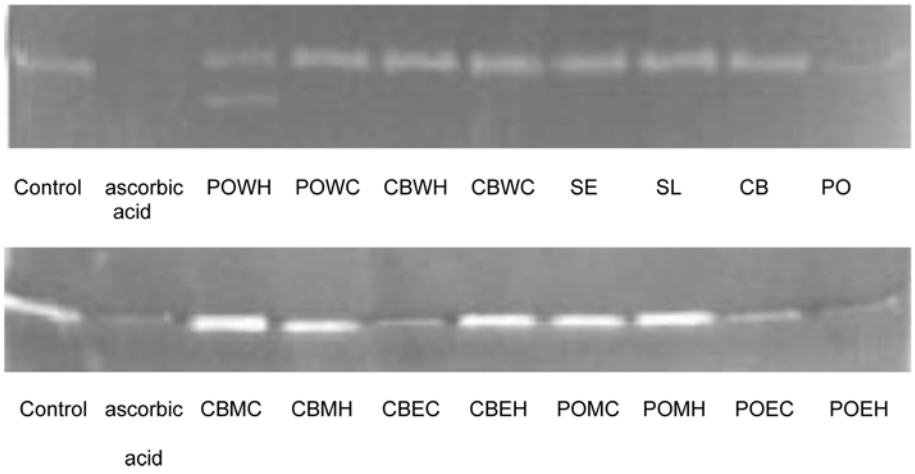

Figure 1. Zymograms of MMP-2 inhibition on human skin fibroblasts of the crocodile blood extracts at 1 mg/ml.

Table 3. Gelatinolytic activity of the crocodile blood extracts at 1 mg/ml.

Extracts			Code	%pro MMP-2 inhibition/stimulation
Crocodile whole blood	Whole		CB	-9.55±1.57
	Water	Hot	CBWH	+3.98±0.60
		Cold	CBWC	-17.89±8.44
	Methanol	Hot	CBMH	-2.33±0.91
		Cold	CBMC	+41.28±6.83
	Ethyl acetate	Hot	CBEH	-22.60±11.81
		Cold	CBEC	-47.30±10.51
Crocodile precipitated blood	Oven		PO	-31.18±5.49
	Water	Hot	POWH	+3.30±0.61
		Cold	POWC	+1.64±0.88
	Methanol	Hot	POMH	+39.18±6.80
		Cold	POMC	+36.34±6.51
	Ethyl acetate	Hot	POEH	-26.95±12.50
		Cold	POEC	-35.46±4.82
Crocodile blood serum	Lyophilize		SL	-21.51±2.60
	Evaporate		SR	-11.78±5.88
Ascorbic acid				-91.26±17.48

Note: MMP-2 inhibition (%) = 100-[(MMP-2 content of sample/ MMP-2 content of control)×100], - = MMP-2 inhibition activity and + = MMP-2 stimulation activity

It is still unknown for the mechanism of the crocodile blood extracts on the MMP-2 inhibition. One possible mechanism is the antioxidative activity. It has been reported that the exogenous hydrogen peroxide and endogenous reactive oxygen species (ROS) can induce the MMP expression in the endothelial cells, cardiac fibroblasts, macrophages and breast cancer cells [34]. ROS can affect the gene expression including the induction of MMPs through the signal transduction pathway [35]. MMPs which are upregulated by an increased formation of ROS, antioxidant approaches can thus decrease the MMP-2 upregulation [36]. Thus, antioxidant activities including DPPH radical scavenging, metal ion chelating and lipid peroxidation inhibition may be responsible for the MMP-2 expression inhibition.

4. CONCLUSIONS

This study has demonstrated the anti-aging activities of crocodile blood extracts. The blood serum which was evaporated before lyophilization (SR) showed the highest DPPH radical scavenging and lipid peroxidation inhibition activities, while the precipitated blood extracted by cold ethyl acetate (POEC) indicated the highest metal chelating and tyrosinase inhibition activities. All extracts gave no cytotoxicity at all concentrations except the blood extracted by cold water (CBWC) at 1 mg/ml. The blood extracted by cold ethyl acetate (CBEC) gave the highest MMP-2 inhibition determined by gelatin zymography. The results from this study have indicated the possible application of crocodile blood extracts to be developed as an anti-aging product.

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