Effectiveness in the Treatment of Iron Deficiency Anemia in Sprague-Dawley Rats Using Freeze-Dried Crocodile Blood

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***Abstract*—The effectiveness of the treatment of Iron Deficiency Anemia (IDA) in rats using Freeze-dried Crocodile Blood (FCB) was performed by using 10 groups of 5, male, Sprague-Dawley rats. After 4 weeks of IDA induction (groups G2-G10), the rat's tail-vein blood was collected for hematological and serum protein investigations. The induced groups hemoglobin (5.59±0.25 g/dl) and hematocrit (26.46±6.45%) were significantly different (p<0.05) from the control group (G1). Rats (G2-G8) were treated with FCB in various doses as design for 24 weeks. Group G6 fed FCB 500 mg and vitamin C 50 mg was as effective as groups G4-G5, showed hemoglobin (12.20±0.82 gm/dl) and hematocrit (33.06±2.09%) significantly different (p<0.05) from groups G2-G3. These revealed that the FCB and low dose of vitamin C was efficient when used to increase hemoglobin and hematocrit values. After treatments, the FCB groups had no detrimental effect on internal organs - with the exception of group G10 fed with AIN93G^{-Fe} diet and 60 mg ferrous sulfate per day. The serum protein analysis demonstrated an alpha-1 proteinase inhibitor (alpha-1-inhibitor III) was released in high volume; depend on FCB treatment dose. Furthermore, haptoglobin and preprohapto-globin from the IDA rats were released in higher volumes than G1 or FCB groups.**

***Index Terms*—Iron deficiency anemia, Crocodile blood, Alpha-1 protease inhibitor, Haptoglobin, Preprohaptoglobin.**

I. Introduction

The practice of consuming crocodile blood for improving human health is found in the traditions of many Asian cultures. Recently, freeze-dried crocodile blood, FCB, in capsule form was developed as a supplemented food product [1]-[3]. It has been widely consumed not only for its nutritious composition, but also for its claimed medicinal value. Previous study [4] revealed that the FCB in concentration 1000 mg/1kg body weight of rat/day is safe and efficient for promoting hemoglobin and hematocrit values and may be used as a food supplement in anemia patients. FCB is high in bioavailability heme iron, an essential component of crocodile hemoglobin [5].

IDA is the most common micronutrient deficiency affecting over 3.5 billion men, women and children [6]. IDA during childhood has been associated with impaired work performance, behavioral development, cognition, psychomotor skills, stunted growth, decreased appetite and poor performance of the immune system. These indicators, collectively or independently, have a negative impact on social and economic development. It can cause fatigue (tiredness), shortness of breath, chest pain, and other symptoms. Severe IDA can lead to heart problems, infections, growth and development problems in children, and other complications [7]-[9]. FCB was considered as an iron supplement for promoting hematological values and use as a food supplement in anemia patients. The purpose of this study was to determine the efficacy of various doses of FCB intake on IDA Sprague-Dawley rats and determined its effect on the rat serum protein.

II. Materials and Methodology

A. Preparation of Freeze-Dried Crocodile Blood (FCB)

Fresh crocodile blood was taken weekly, using sterile techniques and kept in sterile containers. The blood was taken from live crocodiles in accordance with Thai petty patent No. 7468 [10]. The FCB was then prepared using a freeze dryer in accordance with Thai petty patent No. 5074 [11]. The prepared FCB was then stored at 4^{o}C before use.

B. Treatment of the Animals

The Animal Ethics Committee of Kasetsart University, Thailand approved the use of the laboratory animals for this study. Fifty male Sprague-Dawley rats were purchased from the National Laboratory Animal Center, Mahidol University, Salaya, Thailand. They were aged between 3 and 4 weeks with weight ranging from 60 to

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80g. They were housed in the animal facility at the Research and Development Unit, Institute of Food Research and Product Development, Kasetsart University. The animals were allowed free access to food and clean water under standard conditions of 12/12 hours dark-light periods, 30-70% relative humidity and temperatures between 25-29 ^{o}C.

C. Effective Dose of Freeze-Dried Crocodile Blood Study

The Fifty Sprague-Dawley rats were divided into 10 groups of 5. The control group (G1) was fed on a high fat corn starch base AIN93G diet with iron (AIN93G^{+Fe}). Rats in G2 - G10 groups were induced to be IDA by receiving AIN93G diet without Fe (AIN93G^{-Fe}) for 4 weeks. After 4-12 weeks, G2 rats were fed only with AIN93G^{-Fe}, G3 – G5 rats were supplemented with 500, 1500, and 2500 mg of FCB/1kg body weight of rat/day respectively (AIN93G^{-Fe}+FCB$_{500}$, AIN93G^{-Fe}+FCB$_{1500}$ and AIN93G^{-Fe}+FCB$_{2500}$), G6 – G8 anemic rats were supplemented with 500 mg of FCB/1kg body weight of rat/day and vitamin C adding of 50, 100 and 150 mg/day, respectively (AIN93G^{-Fe}+FCB$_{500}$+Vc$_{50}$, AIN93G^{-Fe}+FCB$_{500}$+Vc$_{100}$ and AIN93G^{-Fe} +FCB$_{500}$+Vc$_{150}$). G9 rats were supplemented with 500 mg of FCB/1kg body weight of rat and 60 mg of ferrous sulphate, FS, per day (AIN93G^{-Fe}+FCB$_{500}$+FS$_{60}$) and G10 rats supplemented only 60 mg of FS per day (AIN93G^{-Fe}+FS$_{60}$).

Body weights and food intake were measured weekly and animals were monitored for signs of abnormalities throughout the study. After food supplementing, rat blood was sampled at 4, 8 and 12 weeks for hematological studies and serum protein determinations. A complete blood count with a white blood cell (WBC) differential was performed on the blood samples. Hematocrit (Hct) and hemoglobin (Hb) concentration were determined, the WBC was counted manually. Rat blood without anticoagulant was prepared for serum and then used in protein analysis. After 24 weeks, selected rat organs including intestine, kidney and liver were collected, weighed to determine relative organ weights and fixed with Bouin's fixative. The tissue slides of each organ were prepared and stained with hematoxylin and eosin. The slides were examined by a pathologist.

D. Serum Protein Analysis

Total serum protein was measured by Bradford assay [12]. Serum protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% gel and stained with Coomassie blue according to Siruntawineti *et al*. (2006) [13]. Samples subjected to SDS-PAGE were solubilized in sample buffer, containing 0.0625 M Tris/HCl, pH 6.8, 10% (v/v) glycerol, 10% (w/v) SDS, and 0.01% bromophenol blue. 2-Mercaptoethanol 0.1% (v/v) was conditionally added in the sample buffer. Then, the samples were applied in quantities of 20 µg protein/lane. Molecular weight marker proteins in broad range (Bio-Rad Laboratories, USA) were used as standard markers.

Electrophoresis was carried out using a vertical slab gel electrophoresis apparatus (AE-6530 mPAGE, Atto Corporation, Tokyo, Japan). Constant current (20 mA/gel) was applied to the electrophoresis gel. After electrophoresis, gels were stained with 0.25% Coomassie brilliant blue R-250 in 90 ml of methanol: acetic acid: water (5.7: 1: 7.5) with 10 ml glacial acetic acid and then destained in methanol: acetic acid: water (5.7: 45: 21). After the background of the gel was cleared, it was washed in water. The protein pattern and its band were determined. Images of gels were captured by using a gel document system (Gel Doc XR System, Bio-Rad, USA).

E. Sample Preparation and Mass Spectrometry (MS)

After 7.5% SDS- PAGE stained with Coomassie brilliant blue R-250, protein bands in the gel were excised and cut into 1×1 mm pieces. They were then subjected to a gel tryptic digestion preparation by washing with dH_2O, destained (50 mM ammonium bicarbonate, 50% v/v acetonitrile), dehydrating with 70% (v/v) acetonitrile and acetonitrile removing. Sample digestion was processed with 10 µl of 40 mg/ml trypsin solution (in 25 mM ammonium bicarbonate, 10% v/v acetonitrile) for 5 h at 37^{o}C. Sample (1 µl) was thoroughly mixed with 1 µl of a saturated matrix solution (recrystallised α-cyano-4-hydroxycinnamic acid in 50% v/v acetonitrile, 0.5% v/v TFA). Mixed sample (0.3 µl) was applied to the sample plate and left to dry at room temperature. Mass spectra were acquired using a Matrix-assisted laser desorption/ ionization-time-of-flight mass spectrometry (MALDI-TOF MS), Ettan MALDI-TOF/Pro instrument (Amersham Biosciences, Bucks, UK).

F. High-Resolution Method for the Analysis of Complex Protein in Serum

Differential protein bands between rat groups were selected by using a preparative electrophoresis system for the next step of MALDI-TOF MS analysis. The mass spectra obtained were used for protein identification by comparison of a peptide sequence tag with a sequence in rodent public domain database to identify protein markers.

G. Protein Identification

The data from MALDI-TOF MS was interpreted to MASCOT generic–format (mgf) peak lists and sent to MASCOT MS/MS ion search (Matrix Science, London, UK (Perkin *et al*., 1999)) [14]. Using the National Center for Biotechnology Information (NCBI) database, animal rats to taxonomy, trypsin was chosen as the digest enzyme. MASCOT database search, protein score is -10 × Log (P), where P is the probability that the observed match is a random event. Protein score greater than 82 was significant (p<0.05).

H. Statistical Analysis

The data was analyzed by one-way ANOVA. The significant differences between the experimental groups, at p<0.05, were compared by Duncan multiple range test to distinguish significant differences between groups at p<0.05. Each value represents Mean ± SE.

III. Results and Discussion

A. Effective Dose of Freeze-Dried Crocodile Blood

Throughout the experiment the rats appeared healthy, inquisitive and active. No illnesses or deaths occurred. During the study the body weight of the male rats that received FCB in groups G3-G8 were not significant different from the control group (G1) (data not shown). The Hb and Hct (7.49±0.95 g/dl and 20.91±1.56%) values of the induced anemic groups were significantly different (p<0.05) from group G1, 15.98±0.67 g/dl and 42.50±2.39%), respectively (Table I).

TABLE I. HEMATOLOGICAL VALUE (MEAN ± SE) OF THE RAT IN G1 AND G2-G10 GROUPS AFTER 4 WEEK OF ANEMIA INDUCING

Hematological Value	Rat group	
	G1 (n=5)	G2-G10 (n=45)
RBC ($\times 10^6$ cells/dl)	7.66±0.37	5.50±0.46*
Hb (g/dl)	15.98±0.67	7.49±0.95*
Hct (%)	42.50±2.39	20.91±1.56*
MCV (fl)	55.42±0.52	38.06±0.86*
MCH (pg)	20.84±0.18	13.65±1.40*
MCHC (g/dl)	37.58±0.60	35.85±3.64*
Plt (10^3 /ml)	951.60±150.33	1642.00±389.53*
RDW (%)	16.42±0.80	46.61±10.28*

* Significantly different between groups at the same time (p<0.05)
RBC (Red blood cell), Hb (Hemoglobin), Hct (Hematocrit), MCV (Mean corpuscular volume), MCH (Mean corpuscular hemoglobin), MCHC (Mean corpuscular hemoglobin concentration), Plt (Platelet) and RDW (Red cell distribution width)

After 4 weeks of treatment (Table II), the IDA groups (G4-G5) were effective which showed Hb (14.48±0.48, 15.20±0.32 g/dl) and Hct (37.70±1.03, 39.52±1.23%) respectively and significantly different (p<0.05) from groups G2-G3 which presented Hb (7.56±0.46, 9.96±0.23 g/dl) and Hct (22.12±1.28, 27.90±0.58%) respectively. Rats in IDA groups (G6-G8) were treated with FCB 500 mg/kg body weight of rat and supplemented with vitamin C in 50, 100 and 150 mg/kg body weight daily after 4 weeks were effective as G1-, G4- and G5-groups which demonstrated Hb (11.66±0.24, 11.18±0.40 and 10.90±0.60 g/dl) and Hct (31.72±0.99, 30.16±0.79 and 30.06±1.25 %) respectively and significantly different (p<0.05) from rats in G2- and G3-groups. After 8 weeks of treatment (Table III), the control and all groups that received FCB (G3-G10) showed Hb and Hct significantly different (p<0.05) from that of group G2. Interestingly, the results showed that vitamin C has an effect on red blood cell production especially in concentration of 50 mg. This was better than the results for 100 and 150 mg which had the same effect as in Groups G4 and G5 after 4 weeks of treatment. Rats in the IDA groups that received the FCB as a dietary supplement (G3-G8) had the same growth rate as the rats fed the normal diet, AIN93G$^{\pm Fe}$. It showed no detrimental effect on intestine, liver or pancreas after 24 weeks of treatment (Fig. 1). Data revealed that FCB has an effect on hematological values, especially in Hb and Hct values, on IDA rats and had efficiency to promote normal red blood cell production in at least 4 weeks of treatment and may very useful as a food supplement in anemia patients.

The data captured relating to the rats fed with FCB showed that large quantity of crocodile blood did not cause any toxicity to the body. On the contrary, it actually caused an improvement of their overall heath. Furthermore, tests showed that smaller quantities of FCB were also effective in health enhancement of IDA rats, but results would take longer to achieve. Vitamin C helps the body absorb iron [15]. Hence, consumption of FCB, in conjunction with vitamin C, resulted in better improvement of blood cells. FCB contains heme iron which has a higher bioavailability than nonheme iron, and other dietary components have less effect on the bioavailability of heme than nonheme iron [16]. It is recommended that FCB be ingested on an empty stomach, this would increase the body's ability to absorb essential nutrients. With regard to the enhancement of the blood system and immunity, improvements in red blood cell, white blood cell and platelet generation. Consumers are more able to fight against infectious diseases, became less exhausted because their red blood cells could efficiently carry oxygen to all parts of the body [3].

FCB is a product that contains a good source of natural iron in heme-form of red blood cell [5]. In general, people who suffer with anemia are given a supplement of ferrous sulfate (synthetic iron pills). The long term use of these iron pills has resulted in the enlargement of liver and spleen in anemic patients. Thus, a comparative study on the consumption of FS 60 mg/day (G10, AIN93G^{-Fe}+FS_{60}) and G5 which received FCB 2500 mg/kg body weight per day as a food supplement for 24 weeks was conducted. At the end of experiments, when the rat carcasses were examined, it was found that rats in group G10 showed an enlargement of liver and spleen. Whereas the Group G5 rats which did not show any enlargement of these organs (Fig. 2).

B. Serum Protein Analysis

Separation of serum proteins in IDA rats that received FCB consumption was studied by using SDS-PAGE and identified proteins at 4, 8 and 12 weeks after treatment operated by MALDI-TOF MS. The results showed that alterations in expression pattern from 4-12 weeks.

The protein expression profiles (Fig. 3) can be divided into two groups: the control group, G1, received AIN93G^{+Fe} and anemic groups (G2-G10) received AIN93G-^{Fe} diet supplemented with various doses of FCB and ferrous sulfate for 8 weeks. The results demonstrated that these expressed proteins were distinguished into 20 protein bands, named as band A-T as shown in Fig. 3. They were removed and analyzed by MALDI-TOF-MS (Table IV). The proteins corresponded to bands: A, B and I, despite their relative high intensity, they were not identified.

TABLE II. HEMATOLOGICAL VALUE (MEAN ± SE) OF G1-G10 RAT GROUPS AFTER 4 WEEKS OF TREATMENT WITH VARIOUS DOSES OF FCB FERROUS SULPHATE.

Hema.Value	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10
RBC (10^6/ml)	8.65±0.10	7.31±0.57	9.00±0.26	10.48±0.24	10.14±0.22	9.99±0.26	9.53±0.25	9.53±0.44	8.39±0.30	7.92±0.04
Hb (g/dl)	16.86±0.32	7.56±0.46*	9.96±0.23*	14.48±0.48	15.20±0.14	11.66±0.24	11.18±0.40	10.90±0.60	17.60±0.45*	16.54±0.10*
Hct (%)	43.66±0.52	22.12±1.28*	27.90±0.58*	37.70±1.03	39.52±0.55	31.72±0.99	30.16±0.79	30.06±1.25	45.20±1.47	41.62±0.63
MCV (fl)	50.48±0.12*	30.50±0.83*	31.04±0.36*	35.90±1.01	39.10±0.88	31.76±0.70	31.62±0.23	31.58±0.49	53.88±0.65*	52.48±0.75*
MCH (pg)	19.44±0.18*	10.42±0.22*	11.08±0.09*	13.84±0.43	15.02±0.30	11.70±0.15	11.68±0.12	11.44±0.20	21.02±0.35*	20.84±0.11*
MCHC (g/dl)	38.58±0.32	34.12±0.32*	35.66±0.26*	38.50±0.34*	38.46±0.19*	36.86±0.74	36.98±0.37	36.18±0.53	39.02±0.55*	39.76±0.60*
WBC (10^3/ml)	8.18±1.03	15.36±0.83*	10.70±0.66	13.18±0.93	10.04±0.55	11.04±1.23	10.58±0.42	9.82±1.56	8.30±0.96	8.58±0.94
RDW (%)	17.16±0.44*	35.78±1.14	32.66±1.04	34.60±1.29	35.08±1.35	31.72±1.10	29.62±0.14	31.96±1.14	19.14±0.61*	18.70±0.50*

*Significantly different between groups at the same time (p<0.05). RBC (Red blood cell), Hb (Hemoglobin), Hct (Hematocrit), MCV (Mean corpuscular volume), MCH (Mean corpuscular hemoglobin), MCHC (Mean corpuscular hemoglobin concentration), WBC (White blood cell) and RDW (Red cell distribution width).

TABLE III. HEMATOLOGICAL VALUE (MEAN ± SE) OF G1-G10 RAT GROUPS AFTER 8 WEEKS OF TREATMENT WITH VARIOUS DOSES OF FCB FERROUS SULPHATE.

Hema.Value	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10
RBC (10^6/ml)	9.07±0.14	7.07±0.49	9.88±0.27	9.70±0.28	9.17±0.13	9.87±0.29	9.98±0.034	10.13±0.25	8.98±0.10	8.92±0.21
Hb (g/dl)	17.52±0.34	7.50±0.36*	11.14±0.27	16.02±.90	16.76±0.26	12.20±0.36	12.02±0.66	12.70±0.43	17.22±0.30	16.48±0.61
Hct (%)	45.84±0.83	21.76±0.98*	29.94±0.55	40.90±2.08	42.72±0.51	33.06±0.93	31.60±1.53	32.90±1.08	45.16±0.46	43.20±1.28
MCV (fl)	50.52±0.15	31.04±1.17*	30.30±0.30*	42.00±1.19	46.60±1.02	33.54±0.32	31.66±0.48	32.50±0.68	50.28±0.56	48.38±0.44
MCH (pg)	19.32±0.14	10.69±0.37*	11.32±0.09*	16.44±0.52	18.26±0.49	12.36±0.10	12.04±0.26	12.52±0.24	19.18±0.25	18.44±0.37
MCHC (g/dl)	38.26±0.23	34.48±0.33*	37.30±0.30*	39.10±0.46	39.22=0.26	36.90±0.47	38.08±0.29	38.52±0.23	38.12±0.36	38.08±0.43
WBC (10^3/ml)	8.37±0.75	15.38±2.29	10.33±0.69	8.17±1.02	9.96±0.34	8.53±0.70	11.04±0.59	10.85±1.57	7.01±0.42	7.01±0.88
RDW (%)	18.12±0.22*	32.34±1.33	29.86±0.96	31.54±2.42	22.62±2.30	30.72±0.77	30.56±1.11	32.12±1.13	18.46±0.36*	19.62±1.06*

*Significantly different between groups at the same time (p<0.05). RBC (Red blood cell), Hb (Hemoglobin), Hct (Hematocrit), MCV (Mean corpuscular volume), MCH (Mean corpuscular hemoglobin), MCHC (Mean corpuscular hemoglobin concentration), WBC (White blood cell) and RDW (Red cell distribution width).

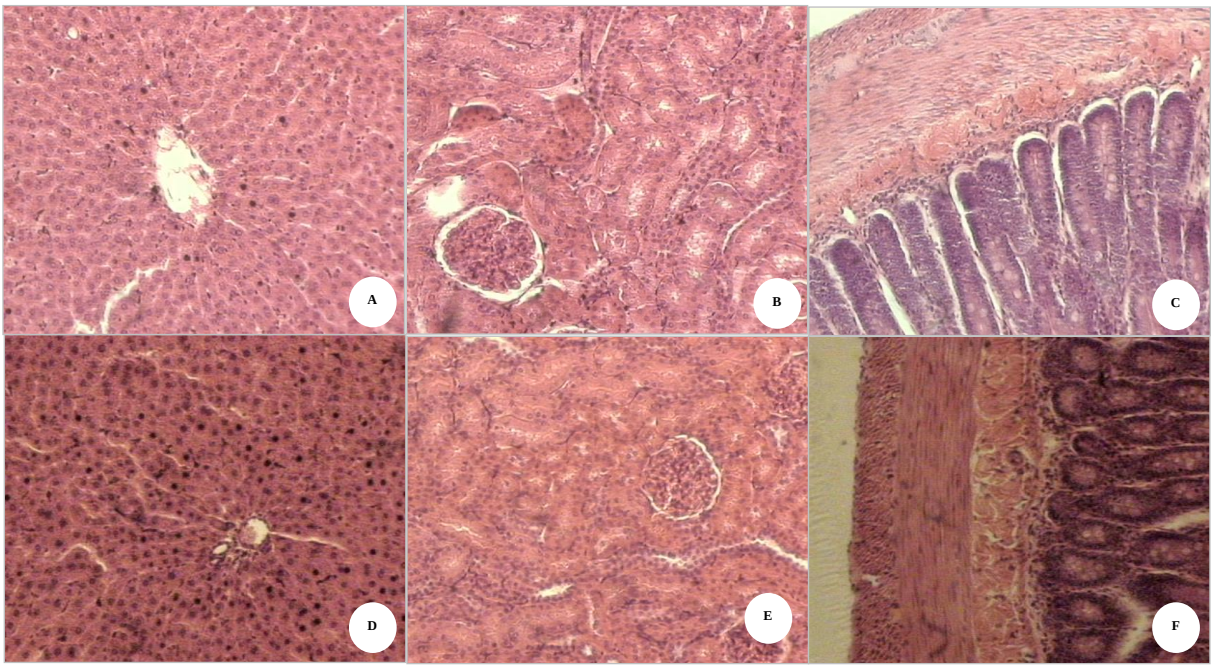


Figure 1. Characteristic of cells from liver, kidney and intestine of control (G1) and ferrous sulphate group (G10) after 24 weeks of treatment. Control group; A: liver, B: kidney and C: intestine. Ferrous sulphate group; D: liver, E: kidney and F: intestine.

TABLE IV. MASCOT DATABASE DETAIL OF MS/MS SCORE INDENTIFIED WITH >95% CONFIDENCE PROTEIN WITHIN INDIVIDUAL PROTEIN BANDS IN SDS-PAGE ANALYSIS OF RAT SER UM AT 3 MONTHS ON 7.5% BIS–TRIS GEL, STAINED WITH COOMASSIE BRILLIANT BLUE.

Protein bands	Protein ID	Accession number	Peptide sequence matches	Protein score	MW
A	alpha-1-macroglobulin	gi /81872093	13	288	168388
B	alpha-1-inhibitor III [*Rattus norvegicus*]	gi/83816939	31	183	165038
C	protein mixtures on alpha-1-inhibitor III [*R. norvegicus*]+ complement component 3 [*R. norvegicus*]	gi/83816939 + gi/158138561	57	251	-
D	alpha-1-inhibitor III [*R norvegicus*]	gi/83816939	42	257	165038
E	ceruloplasmin, isoform CRA_a [*R. norvegicus*]	gi/149048530	27	135	121387
F	inter-alpha-inhibitor H4 heavy chain [*R. norvegicus*]	gi/126722991	32	186	103862
G	protein mixtures on complement component 3 [*R. norvegicus*] + Inter-alpha-inhibitor H4 heavy chain [*R. norvegicus*]	gi/158138561 + gi/2292988	57	305	-
H	protein mixtures on complement factor B, isoform CRA_d [*R. norvegicus*] + unnamed protein product [*R. norvegicus*]	gi/149028000 + gi/55628	24	144	-
I	protein mixtures on LRRGT00127 [*R. norvegicus*] + similar to centromere protein F (350/400kD) [*R. norvegicus*]	gi/45478094 + gi/109499266	38	131	-
J	Transferrin [*R. norvegicus*]	gi/61556986	19	128	78512
K	protein mixtures on hemopexin [*R. norvegicus*] + transferrin [*R. norvegicus*]	gi/16758014 + gi/61556986	32	268	-
L	serum albumin	gi/124028612	15	104	70682
M	protein mixtures on vitamin D-binding protein precursor + unnamed protein product [*R. norvegicus*]	gi/203941 + gi/55628	16	136	-
N	apolipoprotein A-IV [*R. norvegicus*]	gi/114008	17	158	44438
O	protein mixtures on alpha-1-macroglobulin + unnamed protein product [*R. norvegicus*]	gi/81872093 + gi/55628	28	150	-
P	haptoglobin or preprohaptoglobin	gi/60097941 / gi/204657	15	95	39052 or 30428
Q	Chain B, Human Zinc-Alpha-2-Glycoprotein / Chain A, Crystal Structure Of The Complex Formed Between Mhc-Like Zinc Alpha2-Glycoprotein And Prolactin Inducible Protein At 3 A Resolution	gi/4699583 / gi/145579640		164	31854/32336
R	ORF2	gi/202959	16	147	38359
S	unnamed protein product [*R. norvegicus*]/ serum albumin	gi/55628 / gi/124028612	12	100	770670 /0682
T	apolipoprotein A-I [*R. norvegicus*]	gi/6978515	21	198	30100

Figure 2. Carcasses of anemia Sprague-Dawley rats, groups G5 and G10 after 24 weeks of treatment with FCB 2500 mg/kg bodyweight /day (A) and ferrous sulphate 60 mg/day (B).

Bands C, G, H, K, M, O and Q were protein mixture components. Proteins in bands D and P of FCB treatment rats exhibit significant changes at the position of the protein pattern. Both bands may be showed as protein marker of polypeptide components in the serum fraction. Band P in anemic rat group G2 was identified by comparison with mobility of standards. It appeared as the dominant band on anemic rat, no appearance in the control group (G1) because it was healthy rats; anemic rat groups (G3-G8) received AIN93G^{-Fe} with FCB 500, 1500 and 2500 mg/kg body weight, respectively, at FCB and Vitamin C treatment groups by received FCB 500 mg/kg body weight with vitamin C 50, 100 and 150 mg/kg body weight, respectively and at ferrous sulphate treatment

groups (G9-G10). This protein band was identified, by peptide mass sequence analysis, to be haptoglobin (39.5 kDa) or preprohaptoglobin (30.4 kDa) as same score. The presence of a possible same score in band could suggest that the protein is not pure, or the sample has been subject to some degradation. Both preprohaptoglobin and haptogobin are involved in the protection of tissue damage from hemoglobin-induced oxidative stress [17].

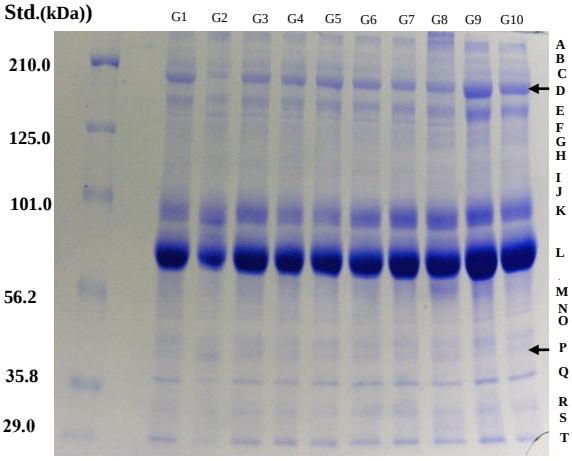


Figure 3. Protein profile of rat sera of the control group, G1, and anemic groups (G2-G10) after 8 weeks of treatment on% 7.5 SDS-PAGE gel stained with Coomassie brilliant blue. Std.: standard protein markers; kDa: kilodalton.

Another marker protein band (band D) detected in the serum fraction corresponded to crocodile blood feeding, FCB, treatments. This band was based lower on its relative band proportion in anemic rat (G2) than on the control (normal) group, G1, and FCB blood treatments (groups G3-G8). However, all protein brands in low intensities of FCB treatments had no dose dependent diminution. This band protein could be assigned to alpha-1-inhibitor III, an alpha-1-proteinase inhibitor, whose molecular weight value was estimated to be 165.0 kDa. Fig. 4 and Fig. 5- Fig. 6 show the peptide mass spectra for bands D and P, respectively, as revealed by MALDI-TOF. The mass spectra of band D and P present a high level of similarity (protein score $\geq$ 82) by matching the peptide masses generated following the trypsin digestion with those available in the database. It was confirmed that both band proteins (bands D and P) corresponded to the rat acute phase proteins: alpha-1-inhibitor III and haptoglobin (or preproheptoglobin), respectively.

Alpha-1 inhibitor III is a proteinase inhibitor and negative acute-phase protein as levels usually decrease in inflammatory responses [18]. In humans, it is known as alpha-1 proteinase inhibitor ($alpha_1$-PI) that belonging to the serpin superfamily. It is generally known as serum trypsin inhibitor isolated from alpha-1 globulin fraction in 1962 was named alpha-1-antitrypsin. Due to its capability to inhibit a number of serine proteases other than trypsin, then its name was changed to $alpha_1$-PI [19]. It protects tissues from enzymes of inflammatory cells, especially neutrophil elastase, and has a reference range in blood of 1.5-3.5 mg/dL or micromoles, but the concentration can rise manyfold upon acute inflammation. In the acute phase reaction, a further elevation is required to confine the damage caused by activated neutrophil granulocytes and their enzyme elastase, which breaks down the elastin component of connective tissue fiber [20].

Preprohaptoglobin is the primary translation product of haptoglobin (Hp). It is an abundant hemoglobin binding protein which functions as an antioxidant, anti-inflammatory, immune cell regulation and angiogenic promoter. Biological function of Hp is initially in destination of hemoglobin released from red blood cells after either intravascular or extravascular hemolysis. Hp represents many important roles in processes that are basic to cellular function. It is usually a marker of hemolytic anemia that has been related to the release of haemoglobin from red blood cells. It releases in low levels of haptoglobin with -IDA, severe malarial anemia [21] and blood transfusions [22]. When an Hp level is decreased, along with a higher reticulocyte count and a reduced RBC count, hemoglobin, and hematocrit, then it means some degree of hemolytic anemia with red blood cells destroyed in the circulation. These results are in agreement with the findings of other research because it due to iron-deficient anemia rat that can cause intravascular hemolysis which red blood cells break down in the blood stream, releasing iron that is then lost in the urine. IDA rats showed increased Hp which may be suggestive of an inflammatory component taking part in the course of iron deficiency anemia status.

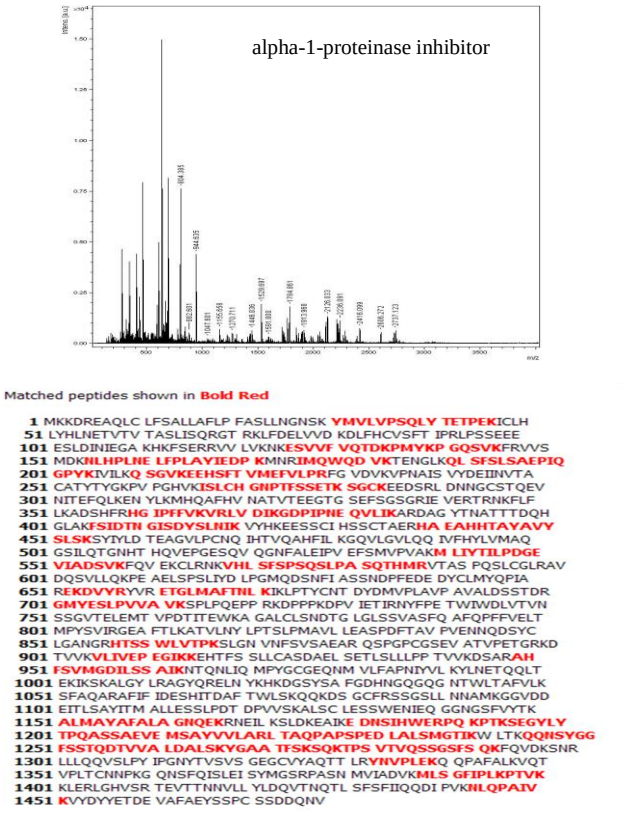
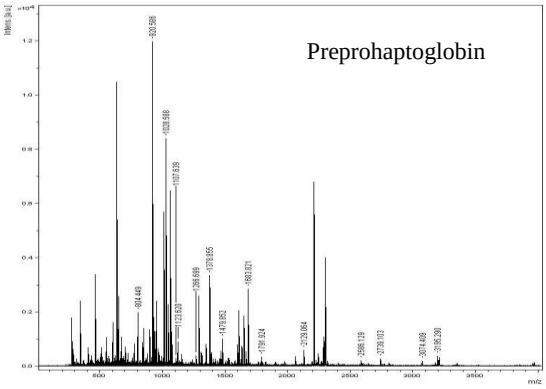


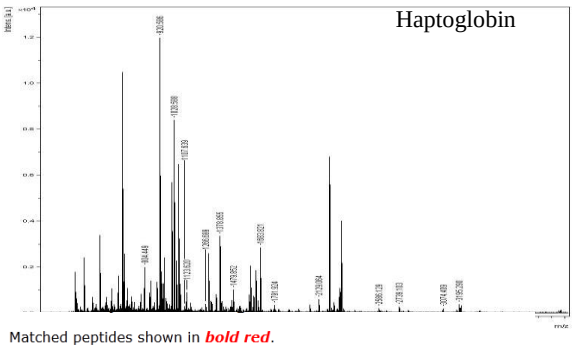
Figure 4. Mass spectrum and peptide mass fingerprinting of alpha-1 proteinase inhibitor which found in SDS–PAGE analysis of IDA rat serum after 8 weeks of treatment on % 7.5 Bis–Tris gel, stained with Coomassie brilliant blue by MALDI-TOF.



Matched peptides shown in ***bold red***.

```
  1 NPAAGDKLPK CEAVCGKPKH PVDQVQRIIG GSMDAKGSFP WQAKMISRHG
 51 LTTGATLISD QWLLTTAQNL FLNHSENATA KDIAPTLTLY VGKNQLVEIE
101 KVVLHPERSV VDIGLIKLKQ KVLVTEKVMP ICLPSKDYVA PGRMGYVSGW
151 GRNVNFRFTE RLKYVMLPVA DQEKCELHYE KSTVPEKKGA VTPVGVQPIL
201 NKHTFCAGLT KYEEDTCYGD AGSAFAVHDT EEDTWYAAGI LSFDKSCAVA
251 EYGVYVRATD LKDWVQETMA KN
```

Figure 5. Mass spectrum and peptide mass fingerprinting for band 16 (preprohaptoglobin) which found in 7.5% SDS-PAGE analysis stained with Coomassie brilliant blue by MALDI-TOF of rat serum at 3 months.



Matched peptides shown in ***bold red***.

```
  1 MRALGAVVTL LLWGQLFAVE LGNDATDIED DSCPKPPEIA NGYVEHLVRY
 51 RCRQFYKLQT EGDGIYTLNS EKQWVNPAAG DKLPKCEAVC GKPKHPVDQV
101 QRIIGGSMDA KGSFPWQAKM ISRHGLTTGA TLISDQWLLT TAQNLFLNHS
151 ENATAKDIAP TLTLYVGKNQ LVEIEKVVLH PERSVVDIGL IKLKQKVLVT
201 EKVMPICLPS KDYVAPGRMG YVSGWGRNVN FRFTERLKYV MLPVADQEKC
251 ELHYEKSTVP EKKGAVSPVG VQPILNKHTF CAGLTKYEED TCYGDAGSAF
301 AVHDTEEDTW YAAGILSFDK SCAVAEYGVY VRATDLKDWV QETMAKN
```

Figure 6. Mass spectrum and peptide mass fingerprinting for band 16 (haptoglobin) which found in 7.5% SDS-PAGE analysis stained with Coomassie brilliant blue by MALDI-TOF of rat serum at 3 months.

In conclusion, the good status in IDA Sprague-Dawley rats was enhanced by FCB, a natural rich iron product from Siamese crocodile material. The data revealed that the consumption of FCB in high doses is safe as a dietary supplement. Low dose feeding of FCB coupled with vitamin C demonstrated an effective health improvement in IDA rats. Consequently, the identification of differentially expressed serum proteins such as acute phase proteins (alpha-1 proteinase inhibitor and haptoglobin or preproheptoglobin) in normal and IDA rats might reveal some mechanism in homeostasis. This study provides further support for the use of FCB as an optimal nutritional food supplement for IDA treatment.

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