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Toxicity screening to human peripheral blood mononuclear cells and Wistar rats by *Crocodylus siamensis* liver extracts

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KEYWORDS
Crocodylus siamensis; Liver; Immunomodulatory; Byproducts.

ABSTRACT
Crocodile organs have been used as ingredients in traditional medicine in Asia for prevention and treatment of diseases. This study, *Crocodylus siamensis* liver was extracted to evaluate cytotoxicity and immune responses in Wistar rats. The soluble proteins part of C. siamensis' liver was prepared. In vitro cytotoxicity was assayed in human peripheral blood mononuclear cells (hPBMCs). In vivo chronic toxicity test was performed on Wistar rats with single dose. Body weights, serum biochemical parameters, serum cytokines and the histopathology were observed. The 50% cytotoxic concentration (CC50) of Crude Liver crocodile Extract (CLE) is 28.41 mg/ml. Oral administration showed no toxicity and no adverse effects for chronic period. Serum cytokines did not differ significantly between Th1 and Th2. We found the CC50 of the CLE in hPBMCs. No toxicity was observed in vivo at the study concentration, with the pro-inflammatory cytokines skewed. Since the using of traditional medicine as an alternative therapy is becoming more widely available, this study is an important beginning step to determine the toxicity of bioactive substances which found non-toxic to cells and animal model. For further study, the effectiveness of a biomolecule is compulsorily to identify. To research on their property and develop to supplement products.

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Introduction

Animal parts, including those from snakes, lizards, turtles and crocodiles, are used in traditional medicine. They can gain greater value in combination with modern and herbal drugs[1]. Crocodiles have been used in Chinese traditional medicine regimens for more than 5,000 years. *Crocodylus niloticus* blood help stabilize osmotic pressure in pregnant women[2]. Crocodile oil has been reported to heal burning skin and reduce scar formation in rats[3], which may be from its anti-inflammatory and antibacterial activities[4]. Crocodile egg protein extract combined with ginseng (*Ganoderma lucidum)* boosts the immune response[5]. The total soluble protein extracted from crocodile livers may be available to use as a supplement for the treatment of ailments.

Crocodile farms have been a growing business for meat and skin production in Thailand. Other crocodile parts, such as the bones and internal organs, including the liver, are byproducts. The liver plays a role in the synthesis of most proteins in the body and in the synthesis of detoxification enzymes. The complex functions of the liver are well described in both Western and Chinese resources[6]. However, like others natural compound, the toxicity concentration and their properties are important to be observed. In the present study, the *in vitro* and *in vivo* cellular toxicity and immune responses by Thai crocodile (*C. siamensis*) liver extract (CLE) were investigated, which may provide information concerning further applications in medicine and raise the economic value of the crocodile liver byproduct.

Materials and methods

Chemicals

Dimethyl sulfoxide (DMSO) (PubChem CID:679) was purchased from Merck KGaA, Darmstadt, Germany. Penicillin (PubChem CID:5904), streptomycin (PubChem CID:19649), bovine serum albumin (BSA), fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, sterile phosphate-buffered saline (PBS) pH 7.4, and trypan blue (PubChem CID:101417452) were purchased from Gibco (Grand Island, NY, USA). Ficoll-Paque™ PLUS was obtained from GE Healthcare (Uppsala, Sweden). Sterile normal saline solution was obtained from Klean & Kare™ Thailand. 3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) (PubChem CID:64965) was provided by Sigma Aldrich (USA).

Crocodile liver collection and extraction

Crocodile livers were obtained from Sriracha Moda Farm, Chonburi, Thailand. Two-year-old C. siamensis were harvested under a standard process. Their livers were immediately kept with standard precautions at -80 ºC until extraction.

The extraction procedure was modified from Pegg et al. (1982)[7]. Briefly, 1 kg of crocodile liver was chopped and homogenized with cold 0.9% sodium chloride at a ratio of 1:1 (w/v) using an aseptic technique. A separation step was completed by centrifugation at 12,000 × g for 40 min at 4 °C. The supernatant was collected, and the centrifugation was repeated. The clear supernatant was then removed and passed through a 0.2 µM syringe filter (PALL Life Sciences Acrodisc®, New York). This CLE was used for further experiments.

The Biuret method, modified from Nigam et al. (2007), was set up for measuring the protein present in the CLE[8]. Bovine serum albumin (BSA) was used for the standard curve construction. The CLE was mixed with 0.75 mM cupric sulfate and 94 mM sodium hydroxide in Folin-Ciocalteu's reagent. The copper (II) ions then formed a complex with the peptides, which became a violet color in the alkaline solution, and the absorbance at 540 nm was recorded and analyzed.

Human peripheral blood mononuclear cell (hPBMC) separation

The hPBMCs, separated from a healthy donor (blood donation and transfusion unit, Srinagarind Hospital, Khon Kaen, Thailand), were used as the normal primary immune cells. The study design of donor blood was approved under the office of the Khon Kaen University Ethics Committee for Human Research (HE611553). The separation procedure was modified from Heo et al. (2009)[9]. The buffy coat from 5 healthy donors was diluted with sterile 1X PBS at pH 7.4 at

a ratio of 1:3. The diluted blood was overlaid on Ficoll-Paque at a density of 1.077 g/ml, followed by centrifugation at 400× g for 40 min at 22 °C. The hPBMCs, which appeared as a cloudy layer in the middle of a centrifuge tube, were then removed and kept at -80 ºC until the next experiment.

In vitro cytotoxicity test of the CLE

The cytotoxicity of the CLE used a tetrazolium colorimetric assay modified from Twentyman and Luscombe (1987)[10]. The hPBMCs (2×10^5 cells per well), which represented immune cells, were cultured in RPMI-1640 medium containing 1% penicillin and streptomycin and 10% FBS and maintained at 37°C and 5% CO_2 under humidified conditions. The various protein concentrations of CLE, prepared by a ten-fold serial dilution with culture media, were directly added to the cell culture plates and incubated for 24 hours. The medium was then removed, and 200 µl of 0.5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) was added followed by a 3-hour incubation under the same conditions. Then, the formazan crystals, which represent viable cells, were dissolved in DMSO and the absorbance was measured by a spectrophotometer (Tecan, Tecan Trading AG, Switzerland) at 550 nm.

In vivo chronic toxicity test of CLE in Wistar rats

Twenty-six to twenty-eight weeks old male and female Wistar rats were provided by the National Laboratory Animal Centre, Mahidol University, Thailand. The rats were maintained at Northeast Laboratory Animal Centre, Khon Kaen University, Khon Kaen, Thailand. The animal experimental design was investigated in accordance with OECD No. 478 guidelines and was approved by the Khon Kaen University Ethics Committee (NELAC 21/2557). All rats were acclimated for one week under standard conditions (12 h light/dark cycle at 20-25°C and 50%–60% humidity with a commercial pellet diet and water with added chlorine at a concentration of 3-4 ppm). Next, the rats were randomly separated into four groups (5 per group) as follows: group 1) male rats receiving 3.0 mg of CLE/kg body weight; group 2) male rats receiving 1.0 ml of sterile normal saline/kg body weight; group 3) female rats receiving 3.0 mg of CLE/kg body weight; and group 4) female rats receiving 1.0 ml of sterile normal saline/kg body weight. All groups were orally given CLE or saline every other day by gavage for a chronic period (24 weeks). An animal pellet basal diet and drinking water were freely accessible. Rats were observed daily, and their body weights were measured every week. Blood was collected from the lateral tail vein every month for determination of the hematological parameters by using a Sysmex automated analyzer (Model xs-800i, Germany). Plasma biochemical parameters were determined by a Beckman Coulter automated analyzer (Model Synchron LX20pro, USA.), including a kidney function test (urea nitrogen, creatinine), liver enzymes (aspartate aminotransferase, AST; alanine aminotransferase, ALT; and alkaline phosphatase, ALP), and lipid profile (triglycerides; cholesterol; high density lipoprotein, HDL; and low density lipoprotein, LDL). After 24 weeks of the experiment, the rats were sacrificed with an anesthetic, and blood was drawn from the aorta. The internal organs, including the lungs, stomachs, intestines, kidneys and livers, were removed and fixed with 10% formaldehyde solution for evaluation of pathologic appearance by hematoxylin and eosin (H&E) staining.

Plasma cytokines assay

At the end of the experiment, the rats were sacrificed by euthanasia, and their whole blood was quickly drawn by aortic puncture and kept in a tri-potassium-ethylenediaminetetraacetic acid (3K-EDTA) tube. The plasma was separated by centrifugation at 3,500 rpm for 10 min and kept at -80°C. Plasma cytokine levels (IFN-γ, IL-2, IL-4, IL-10) and the Th1:Th2 ratio after 24 weeks of oral CLE administration were then analyzed by commercial ELISA kits (Bio-Plex assay®, Bio-Rad, USA).

Tissue processing and histological scoring

Rat kidney and liver tissues were fixed with 10% buffered formalin, followed by removal of the fixative in distilled water. Dehydration was performed by a tissue processor with serial dilutions of alcohol (70%, 90%, and 100%), clearing

of the samples with xylene and impregnating the tissues with molten paraffin wax. The tissues were embedded, sectioned (4 μm thickness) and stained with hematoxylin and eosin (H&E)[11].

The histological features of the livers and kidneys were evaluated for histotoxicity with broad categories. The histological scoring definitions are shown in Table 1, which was modified from[12,13] and (Table S1), which was modified from[14]. The tissues were scored under a light microscope in 5 random fields per sample (N = 3) by two independent pathologists.

Table 1 Histological scoring definitions of the liver

Item	Definition (% of tissue affected)	Grade
Steatosis	< 5%	0
	5-33%	1
	33-66%	2
	66-100%	3
Hepatocellular injury	None	0
Ballooning cells	Few ballooning cells	1
	Many ballooning cells/prominent ballooning	2
Acidophil bodies	None to rare	0
	Many	1
Fibrosis	Mild, perisinusoidal/periportal/zone 3	1
	Moderate, perisinusoidal/periportal/zone 3	2
	Bridging fibrosis	3
	Cirrhosis	4
Miscellaneous features	None to rare	0
	Many	1

Statistical analysis

The cytotoxicity data were compared by the nonparametric t-test. Animal experiments were analyzed by the nonparametric one-way, Mann-Whitney *U* Test (Graph Pad Prism software version 5, GraphPad Software, CA, USA) for comparing groups and cell types. Statistically significant differences were judged at a *p*-value < 0.05.

Results

Cytotoxicity of CLE on human peripheral blood mononuclear cells (hPBMCs)

To examine the toxicity of CLE against hPBMCs, the cells were extracted from normal healthy donors. The hPBMCs represent normal immune cells and were treated with CLE at various concentrations: 0, 0.006, 0.01, 0.02, 0.1, 0.5, 1.0, 1.5, 3.0, and 6.0 mg/ml for 24 h. The concentration of 0 mg/ml was the control condition (medium alone), which represents 100% cell viability. The percent cell viability was measured with the MTT assay. CLE at a concentration of 28.41 mg/ml showed 50% toxicity to hPBMCs (Figure 1).

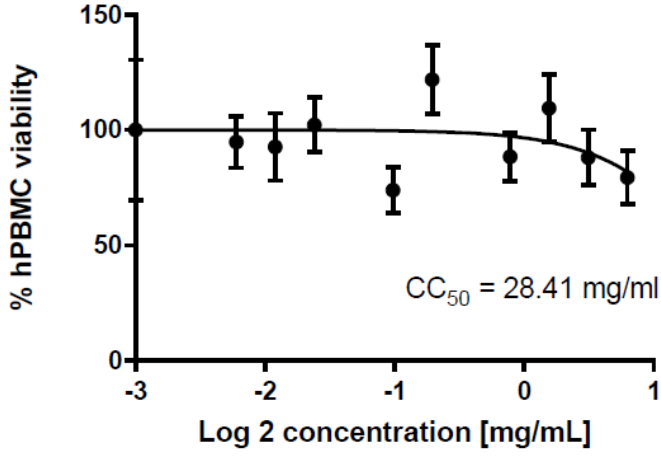

Figure 1 CC_{50} of the CLE test on hPBMCs. Data from triplicate experiments were repeated 5 times. Each bar shows the mean ± SEM.

Chronic toxicity of CLE to Wistar rats

CLE, at a concentration of 3.0 mg/kg rat body weight, was fed to rats for 24 weeks. All rats survived until the end of the experiment with no morbidity and no behavioral changes. Only one female rat, which was treated with CLE, showed illness and lethargy during the last week of the experiment. The body weight data were recorded every week (Figure 2A, B). The mean body weight of the CLE-treated group showed no significant difference compared with the control group (p-value > 0.05). This result implied that CLE had no effect on the growth of rats.

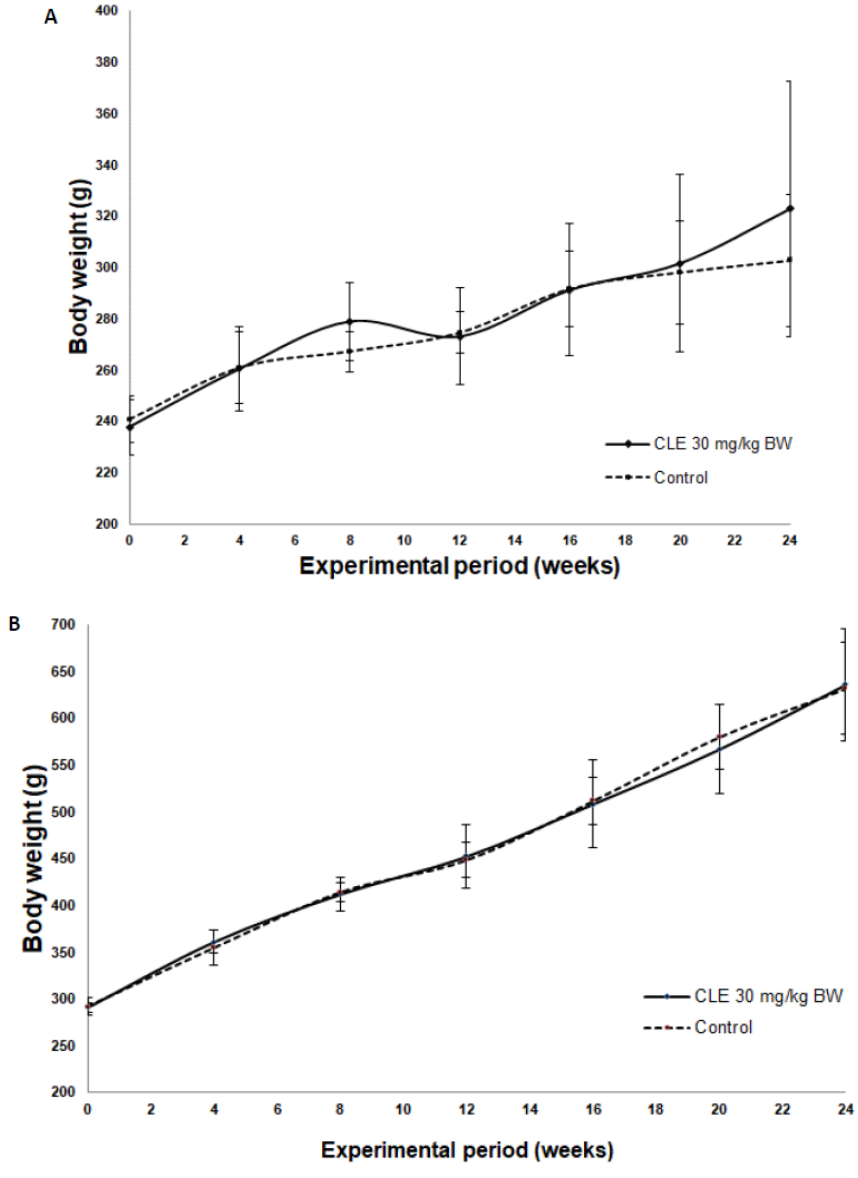

Figure 2 Effect of the oral treatment of CLE to (A) female and (B) male rat body weights (N = 5 per group). Each dot represents the result of body weight measurements at weeks 0, 4, 8, 12, 16, 20, and 24.

Clinical chemical and hematological parameters

The effects of CLE on the clinical chemical and hematological parameters in normal male and female rats were assessed after 24 weeks of the experiment. Compared with the control group, there was no statistically significant change in any of the blood hematological or clinical chemistry parameters (Tables S2, S3) of both the male and female groups treated with 3.0 mg/ml CLE. The rat intestines showed no pathological appearance (data not shown).

Plasma cytokines and Th1: Th2 cytokine balance

Th1 (IFN-γ, IL-2) and Th2 (IL-4, IL-10) plasma cytokines were measured in both female and male rats to evaluate the effect of CLE or their

metabolites on cytokine patterns, as shown in Table S4. The levels of IFN-γ, IL-2, IL-4 and IL-10 independent analysis was not significantly different between the control and CLE group in both male and female. The Th1:Th2 ratio was calculated to observe the type of immune response. Figure 3A shows that the Th1:Th2 ratio in female rats treated with CLE showed significant differences: IFN-γ/IL-4 (*p*-value = 0.008) and IFN-γ/IL-10 (*p*-value = 0.019) compared to the control group. The result of male group including IFN-γ/IL-4 (*p*-value = 0.0008), IFN-γ/IL-10 (*p*-value = 0.016), IL-2/IL-4 (*p*-value = 0.004), and IL-2/IL-10 (*p*-value = 0.001), are shown in Figure 3B.

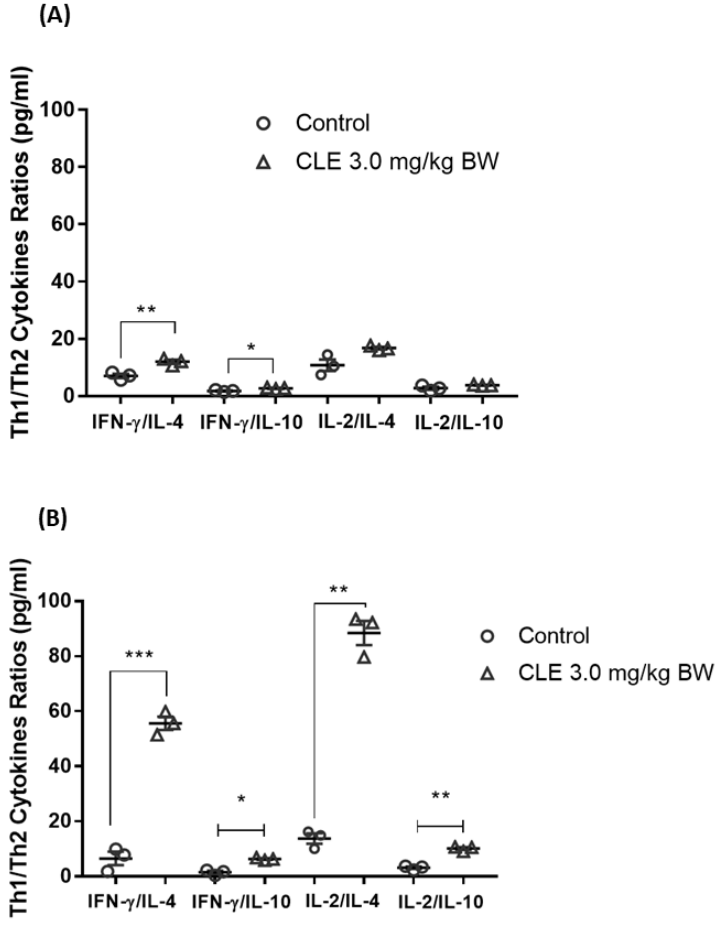

Figure 3 Th1: Th2 cytokine balance pattern of the rats after 24 weeks of CLE oral administration (3.0 mg/kg body weight). INF-γ/IL-4, INF-γ/IL-10, IL-2/IL-4, IL-2/IL-10 ratios were plot; (A) female (B) male. The mean ± SEM is displayed on each bar graph. $^{*}p$-value < 0.05; ** *p*-value < 0.01; *** *p*-value < 0.001.

Histological scores from CLE chronic oral administration to rats

After termination of 24 weeks of chronic oral administration, the internal organs such as the lungs, stomachs, intestines, kidneys, and livers were observed macroscopically for abnormalities of the prepared sections. There were no significant abnormalities found in the organ and tissue sections from the lungs, stomachs, intestines, and kidneys between the control group and the CLE 3.0 mg/kg BW treatment groups (data not shown), both male and female. However, the livers from both the male control and male CLE-treated groups and the female CLE-treated group showed

lipid droplets around the hepatic portal vein, as shown in figure 4. The histological scores of the kidneys were not affected (≤ 1) in all groups, and there was no significant difference between the groups in terms of gender control, as detailed in Table S5. The liver examinations of CLE-treated male rats group scored indicated that significantly higher the hepatocellular injury concerning the number of ballooning cells than the control male rats and showed mild steatosis (> 1) that was not significant, detail in Table S6.

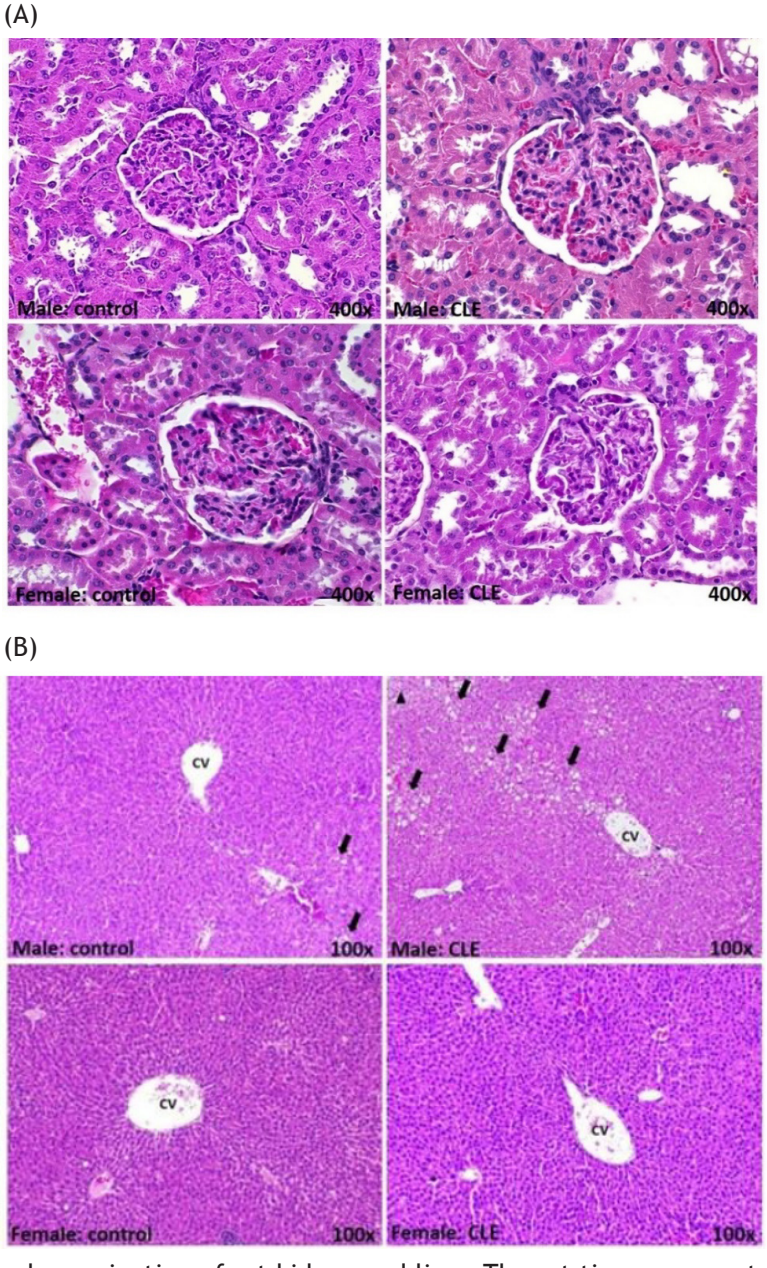

Figure 4 Histological examination of rat kidney and liver. The rat tissues were stained with H&E and observed under light-microscope (A). The kidney tissues represent with glomerulus and renal tubules each group with 400x magnification (B). The liver examinations represent hepatic structure including central vein (CV), steatosis (arrow) and ballooning cells (head arrow) with 100x magnification.

Discussion

Crocodile liver remains popular as a health-supportive bioactive ingredient in Asia and Africa traditional medicine[15]. Moreover, the farm industry of this species is seen as their internal organs to be a byproduct. The utilization of crocodile liver gives two important outcomes. The utilization of crocodile liver gives two important outcomes. First, to reduce environmental problems from the agricultural industry. Second, it is necessary to clarify the toxicity concentration of natural extracted resources for edibility safety. The overall parameters affected by CLE are determined in this study including body weight, serum biochemical, hematological, cytokines secretion, and histopathological appearance. The *in vitro* cytotoxicity test was used initially to examine the harmless concentration of CLE. The hPBMCs with which the primary cells were mixed is advantageous to use since they reflect lymphocyte, T cells, and monocyte responses. An example of peptide immunomodulatory activity was reviewed by Gauthier et al. (2006), which showed proliferation, cytokine expression, antibody production, and immune cell function[16]. In the present study, the 50 percent cytotoxicity concentration is 28.41 mg/ml of CLE. With little cyto-toxicological effect, 0.1 of CC_{50}, (3.0 mg/kg body weight) was selected as safely edible concentration for chronic period oral administration in Wistar rat. We tested the *in vivo* toxicity under OECD guidelines. A severe toxicological effect was not found, with no ruffled fur or mortality until the end of the experiment. Rat body weight gain did not decrease, indicating that the compound CLE is well-tolerated and does not produce mortality or any adverse effects in both male and female rats, which mean CLE does not risk to normal growth. According to the baseline normal range of blood parameters, the observed results are well defined in their hematological and clinical chemistry parameters[17]. No abnormal hematological parameters were found. Liver enzymes, including AST, ALT and ALP, and lipid profiles including cholesterol, triglycerides, and LDL, increased in the male CLE-treated group. In the female group, there was an increase in ALP and the lipid profile, including cholesterol, triglycerides, and LDL.

Histopathological observations focused on the kidneys and livers and reflected the toxicity of CLE with metabolite formation after long-term administration. The hepatic toxicity scoring system was modified following the studies of Zheng et al. (2005) and Kleiner et al. (2005)[13,14]. In the present study, fatty droplets around the portal vein were observed to be related to the increasing lipid profiles, while changes in other organs were not found. A fatty liver is the primary toxic effect that is mainly caused by liver metabolism or detoxification. The increase of serum lipids by more than 1 S.D. is associated with decreasing infectious diseases such as bacteremia, nervous system infection, and miscellaneous viral infections from a cohort study in both men and women. During inflammation, cytokines may alter lipid metabolism and induce lipoprotein-binding endotoxins, resulting in interruption of the cytotoxic effects[18]. Inflammatory activation and toll-like receptor activation can be induced by cholesterol accumulation by macrophages. Peripheral blood cholesterol increases resulting from decreases in macrophage cholesterol efflux probably followed the amplification of TLR signaling in macrophage cells via the ABCA1 receptor[19]. The long-term oral administration of CLE found that cholesterol, triglycerides, and LDL might be relevant to reverse the cholesterol transport pathway.

Plasma cytokines may reflect systemic activation by CLE and its metabolites of Wistar rats. We expect to see the balance between proinflammatory and anti-inflammatory cytokines in rats treated with CLE. Interestingly, we found Th1 skewed. Th1 is dominant in several conditions, including viral or intracellular pathogen infection, the fighting of cancer cells and stimulation delayed-type hypersensitivity skin reactions, whereas Th2 is dominant when the body is exposed to extracellular pathogens. Under normal conditions, the Th1/Th2 ratio balancing hypothesis is still argued[20]. Th1 dominant caused by IFN-γ and IL-2 increase, suggested that CLE might able an antigenicity to induce cellular. The ratio of IFN-γ/IL-4, IFN-γ/IL-10, IL-2/IL-4, and IL-2/IL-10 increased in male rats due to the secretion raise in Th1 cytokines, while there were higher levels of IFN-γ/IL-4 and IFN-γ/IL-10 ratio in female

rats treated with CLE. The Th1/Th2 ratio in both males and females treated with CLE exhibited Th1 dominance. This implied that CLE may affect rat cellular immunity. Taken together, CLE may induce immune cell proliferation, differentiation as Th1 was increased in the U-937 cell line (data not shown). We suggest further investigations of cytokine mediation and other immune functions with varies dose of CLE.

Conclusion

Our results are the first toxicity data from CLE. There were 40 grams of CLE powder from one kilogram of crocodile liver (wet weight) in the extraction. The *in vitro* and *in vivo* safety assessment results are related to the noncellular toxic effects in normal cells and the absence of adverse effects in rats. The cytokine pattern exhibits balancing and skewing toward cellular immunity. Detection of fat droplets in hepatocytes is a reversible phase of toxicity related to an increase in the lipid profile, and fat removal is an important the next step of the application. Furthermore, the *in vivo* plasma cytokine results relate to the *in vitro* cell base assay. The safety assessment of CLE is important to determine for future applications. In a further study, we attempted to test other different immune functions in both *in vitro* and *in vivo* pathological condition.

Clinical implications

- Cytotoxicity of crude liver extract as an edible supplement is compulsory to be evaluated.
- In vivo study act for the toxicity of the extract at chronic oral administration.
- The study model might be a fundamental experiment for supplement test.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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Supplementary

Table S1 Histological scoring definitions of the kidneys for glomerular injury, tubular cast formation, tubular necrosis, and interstitial inflammation

	Grading				
	0	1	2	3	4
Percent of tissue affected	No disease	1 - 25%	26 - 50%	51 - 75%	76 - 100%

Table S2 Effect of CLE oral administration (3.0 mg/kg body weight) for 24 weeks on the hematological parameters of rats[1]

Parameter	Unit	Male rats		Female rats	
		Control	CLE	Control	CLE
RBC	$\times 10^6$/µl	9.16 ± 0.34	10.08 ± 0.71	8.16 ± 1.50	9.75 ± 0.39
Hemoglobin	g/dl	16.65 ± 0.78	17.27 ± 0.55	14.65 ± 2.76	16.2 ± 0.35
Hematocrit	%	52.40 ± 0.71	50.73 ± 2.19	45.00 ± 10.75	47.60 ± 2.16
MCV	Fl	57.25 ± 1.34	48.60 ± 1.08	54.85 ± 3.04	48.84 ± 2.36
MCH	Pg	18.15 ± 0.21	16.53 ± 0.57	17.95 ± 0.07	16.62 ± 0.40
MCHC	g/dl	31.80 ± 0.99	34.07 ± 0.55	32.80 ± 1.70	34.08 ± 1.49
RDW	%	19.05 ± 0.21	22.93 ± 1.43	17.05 ± 2.19	21.44 ± 1.44
WBC	$\times 10^3$/µl	7.24 ± 2.09	7.55 ± 1.91	3.55 ± 0.65	7.29 ± 1.21
Neutrophils	%	14.75 ± 1.34	11.58 ± 1.60	17.05 ± 0.21	14.0 ± 6.06
Eosinophils	%	0.70 ± 0.00	1.64 ± 0.40	1.15 ± 0.78	2.32 ± 2.44
Lymphocytes	%	86.60 ± 7.78	83.78 ± 2.06	78.55 ± 0.78	78.5 ± 8.75
Monocytes	%	4.55 ± 0.21	2.86 ± 1.15	3.25 ± 1.77	3.72 ± 2.49
Basophils	%	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Platelets	$\times 10^3$/µl	828.00 ± 77.78	1007.67 ± 247.66	761.50 ± 3.54	712.80 ± 300.03
MPV	Fl	10.50 ± 1.41	8.50 ± 0.92	9.20 ± 0.28	8.36 ± 1.14

Note: [1] N = 5 per group; Control = rats received 1 ml of sterile normal saline/100 g of body weight; Fl, Femtolitre; Pg, Picogram; MCV, Mean corpuscular volume; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration; MPV, Mean platelet volume; RDW, Red blood cell distribution width; RBC, Red blood cell; WBC, White blood cell.

Table S3 Effect of CLE oral administration (3.0 mg/kg body weight) for 24 weeks on the clinical chemistry parameters of rats[1]

Parameter	Unit	Male rats		Female rats	
		Control	CLE	Control	CLE
Urea nitrogen	mg/dl	25.00 ± 3.61	24.80 ± 1.48	23.00 ± 4.30	24.60 ± 2.51
Creatinine	mg/dl	0.58 ± 0.23	0.71 ± 0.09	0.60 ± 0.15	0.72 ± 0.13
AST	U/l	105.67 ± 60.08	171.40 ± 42.51	227.40 ± 280.40	163.40 ± 38.74
ALT	U/l	57.00 ± 11.79	129.60 ± 84.18	116.80 ± 162.90	123.00 ± 68.12
Alkaline phosphatase	U/l	36.67 ± 7.37	99.60 ± 41.14	53.00 ± 29.89	116.60 ± 28.60
Triglyceride	mg/dl	126.50 ± 2.12	240.40 ± 70.61	117.80 ± 40.36	169.00 ± 46.53
Cholesterol	mg/dl	54.50 ± 19.09	79.60 ± 8.44	48.40 ± 9.42	70.40 ± 13.01
HDL	mg/dl	39.50 ± 13.44	36.60 ± 5.86	31.20 ± 4.32	35.60 ± 5.77
LDL	mg/dl	41.96 ± 10.50	98.25 ± 16.58	44.07 ± 27.81	77.47 ± 37.82

Note: [1] N = 5 per group; Control = rats receiving 1 ml of sterile normal saline/100 g body weight; AST, Aspartate transaminase; ALT, Alanine transaminase; HDL, High-density lipoprotein; LDL, Low-density lipoprotein; U, unit; Fl, Femtolitre; Pg, Picogram; MCV, Mean corpuscular volume; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration; MPV, Mean platelet volume; RDW, Red blood cell distribution width; RBC, Red blood cell; WBC, White blood cell.

Table S4 Plasma cytokine concentrations of rats after 24 weeks of CLE oral administration (3.0 mg/kg body weight)

Cytokine (pg/ml)	Male rats		*p*-value	Female rats		*p*-value
	Control	CLE		Control	CLE	
IFN-γ	97.89 ± 25.11	80.39 ± 35.36	0.703	109.70 ± 11.57	104.20 ± 18.65	0.812
IL-2	187.10 ± 17.84	1147.50 ± 52.11	0.524	168.50 ± 31.41	170.40 ± 4.86	0.957
IL4	19.06 ± 2.55	15.84 ± 5.73	0.634	17.64 ± 4.08	15.87 ± 1.36	0.721
IL-10	76.91 ± 9.41	57.83 ± 18.39	0.407	64.75 ± 9.27	46.42 ± 5.95	0.157

Note: Control = rats receiving 1 ml of sterile normal saline/100 g body weight.

Table S5 Histological scores of the kidneys

Item	Male		Female	
	Vehicle control	CLE 3.0 mg/kg BW	Vehicle control	CLE 3.0 mg/kg BW
Glomerular injury	0.10 ± 0.05	0.03 ± 0.05	0.13 ± 0.09	0.13 ± 0.00
Tubular cast formation	0.07 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Tubular necrosis	0.30 ± 0.05	0.27 ± 0.09	0.27 ± 0.09	0.17 ± 0.05
Interstitial inflammation	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Table S6 Histological scores of the livers

Item	Male		Female	
	Vehicle control	CLE 3.0 mg/kg BW	Vehicle control	CLE 3.0 mg/kg BW
Steatosis	0.30 ± 0.05	1.44 ± 0.63	0.23 ± 0.14	0.00 ± 0.00
Hepatocellular injury				
• Ballooning cells	0.09 ± 0.03	1.16 ± 0.41*	0.18 ± 0.06	0.00 ± 0.00
• Acidophil bodies	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Fibrosis	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Miscellaneous features	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Note: * statistically significant at *p*-value < 0.05.