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Research Article



The Evaluation of Acute Oral Toxicity Testing of Siamese Crocodile (*Crocodylus siamensis*) Bile in Sprague Dawley Rats in Compliance with OECD Guideline 423

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Abstract

Crocodiles have been used for medical purposes. By product of the crocodile industry contains a large amount of gallbladder. Crocodile bile is composition of crocodile gallbladder. Siamese Crocodile (*Crocodylus siamensis*) bile was exported for a long time. In many countries such as China, Hong Kong and Taiwan *etc.*, people used crocodile bile for an ingredient of traditional medicine. The purpose of the present study is ranked and classified of Siamese crocodile bile by the Globally Harmonised System for the classification of chemical (GHS) which cause acute toxicity in Sprague Dawley rats. The study was conducted in a stepwise procedure used starting dose 300 mg/kg body weight in compliance with OECD/OCDE, OECD Guidelines for the testing of chemicals 423, Acute Oral Toxicity – Acute Toxic Class Method (2001). The oral administration of 2,000 mg/kg body weight Siamese crocodile bile was produce moribund and mortalities in animals. After administrated with 2,000 mg/kg body weight Siamese crocodile bile, all animals were showed moribund within 24 hours, 2 animals were died within 24 and 48 hours respectively. The results suggested that Siamese crocodile bile was classified in GHS category 4, the LD₅₀ cut off at 500 mg/kg body weight.

INTRODUCTION

Siamese crocodile is an economic animal. Crocodile farm and industry are valuable additions to country. The crocodile product such as internal organ, blood, skin and hides can be sold at higher price than other animals that are allowed for commercial aquaculture farm (Praduptong *et al.*, 2018). Crocodile bile is by-product of the crocodile

industry. In many countries such as China, Hong Kong and Taiwan *etc.*, people used crocodile bile for an ingredient of traditional medicine. Siamese crocodile bile was exported for a long time. In traditional clinical practice can be used crocodile bile for drugs solvent and treatment of sepsis, hemorrhage and trauma) Picheinsutthorn and Jeerawong, 2003(.

Other animals are widely used as traditional medicine such as bear and snake. Bear bile contains high levels of ursodeoxycholic acid (UDCA) to be useful for treating liver and gall bladder.

Crocodile bile is traditional medicine, consisted of 0.8% of Cholestanol, 68% of Coprocholic acid, 9% of Coprochenodeoxycholic acid and derivative includes; 10% of 3-oxo-7 α , 12 α -dihydroxy-5 β -cholestanoic acid, 8% of 3 α , 7 α , 12 α -trihydroxy-5 β cholestanic acid, 7-oxo-3 α , 12 α -dihydroxy-5 β -cholestanoic acid, 3-oxo-7 α , 12 α -dihydroxy-5 α -cholestanoic acid, chenodeoxycholic acid, 5 α -cholestan-3 α , 7 α , 12 α , 26-tertrol, 5 β -cholestan-3 α , 7 α , 12 α , 25-tertrol, Ursodeoxycholic acid and 5 α -cholic acid (Chinese Pharmacopoeia Commission, 2010). Bile acids have been the subject of clinical research for many years. Coprocholic acid is bile acid, its main composition in crocodile bile. Bile acids are also steroidal amphipathic molecules derived from the catabolism of cholesterol, found predominantly in bile of mammals. Bile acids are physiological detergents that facilitate excretion, absorption and transportation of fats and sterols in the intestine and liver. And it has been implicated in the regulation of all the key enzymes involved in cholesterol homeostasis (Wishard DS *et al.*, 2007).

However, toxicology study of Siamese crocodile bile have not been carried out. Therefore, the purpose of the present study is evaluating of acute oral toxicity of Siamese crocodile bile in Sprague Dawley rats in compliance with OECD guideline 423.

MATERIALS AND METHODS

Sample Collection:

One hundred of captive Siamese crocodile (*C. siamensis*) gall bladders were collected from Sriyuthaya Gold Medal co., Ltd., Phra Nakhon Si Ayutthaya Province, Thailand. They were 3 – 5 years of age and 160 – 250 cm in length.

Freeze Dried Crocodile Bile Preparation:

Fresh crocodile gall bladders were collected by using sterile techniques and kept in sterile container. Freeze dried crocodile bile were performed accordance with Thai patent application No. 0901001231, Kasetsart University (Chaeychomsri and Siruntawineti, 2009) as following. The gall bladders without fat tissue outside were surface sterile with 70% ethyl alcohol and the crocodile bile were collected in tray and freeze-drying using freeze dryer (Lyomaster, USA).

The freeze-dried crocodile bile was powdered by graining at sterile condition. The crocodile bile powder was kept at 4°C until use.

Preparation of the Dose Concentrations:

Freeze-dried crocodile bile were calculated at 300 and 2,000 mg/kg body weight and freshly mixed with vehicle prior to gavage and weighed using balance 4 digits.

Animals Husbandry:

Healthy 6-8 weeks, 12 female, Sprague Dawley rats of body weight range 200 g \pm 20% were obtained from Office of Laboratory Animal Production, National Laboratory Animal Center, Mahidol University, Thailand. The animals were kept under standard conditions 12 hours light, 12 hours dark at 22 \pm 3°C and 30-70% relative humidity. The animals were housed in stainless steel cages with food (082, Perfect Companions, Thailand) and 5-7 ppm chlorinated water *ad libitum*. All the animals were acclimatized for at least 5 days prior to the study. Guidelines of “Guide for the care and use of laboratory animals” (Institute of laboratory animal resources, National academic press 2011; 8th Edition NIH publication number #85-23, revised 2011) were strictly followed throughout the study. The animal ethics was approved by National Laboratory Animal Center Animal Care and Use Committee (NLAC-ACUC), Mahidol University; Thailand.

Experimental Design:

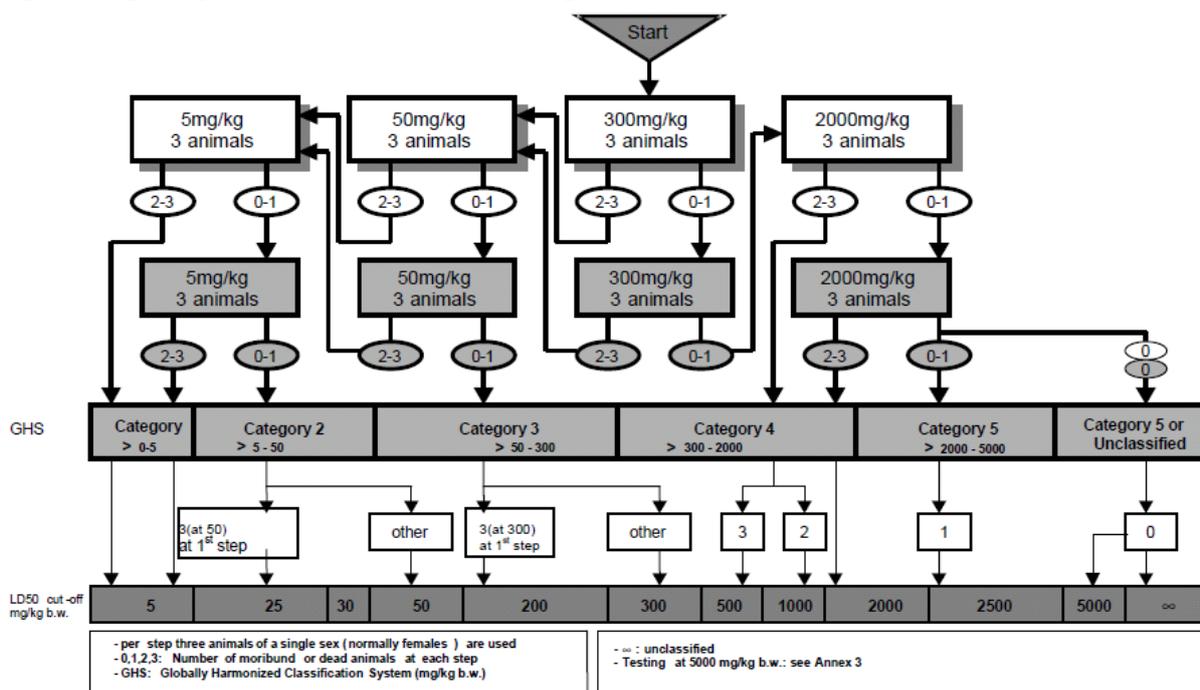
The acute oral toxicity test was in compliance with the OECD/OCDE, OECD Guidelines for the testing of chemicals 423, Acute Oral Toxicity – Acute Toxic Class Method (2001). This study was stepwise procedure with the use of 3 female Sprague Dawley rats per step, depending on the mortality and/or moribund status of animals. The starting dose was 300 mg/kg body weight. All animals were kept for overnight (15-18 hours) fasting (feed but not water) prior to administration. Each animal was administrated in a single dose by gavage. The dosage of administration to each animal was calculated based on the body weight of animal prior to administrate at a constant volume not exceed 1 ml per 100 g body weight. The crocodile bile was administered orally to 3 animals at 300 mg/kg body weight, if no animals or 1 animal were shown moribund state or mortalities, new 3 animals were repeated 300 mg/kg body weight. After that, 3 animals were administrated with the next dose level (2,000 mg/kg body weight) if no animal or 1 animal were shown signs of toxic

effects or mortalities, new 3 animals are repeated 2,000 mg/kg body weight. All animals were observed for toxic effects after administration at the first 30 minutes with special attention given during the first 4 hours, periodically during the first 24 hours. The time between treatment groups was determined until confident of previously dose animals, 24 hours for this study. Survived animals were general clinical observed once daily for a total of 14 days. In case of animals were showed moribund state, they were euthanized according to animal welfare. General Clinical observations were observed outside the home cage at least once a day, at the same times for 14 days, which include; feed and water consumption, skin and fur, mobility and behavior. Health examinations were performed weekly, which include changes in skin, fur, eyes and mucous membrane, occurrence of secretions and excretions and autonomic activity (lacrimation, piloerection, pupil size and respiratory pattern), changes in gait, posture, response to handling,

presence of clonic or tonic movement and stereotypes (excessive grooming and repetitive circling) and bizarre behavior (self-mutilation and walking backwards) and abnormality of lymph node. Animal body weights, feed consumption and water consumption were measured and recorded weekly. On day 15, the animals were euthanized using CO₂ inhalation. All animals were sacrificed after euthanized. The positions, shapes, sizes and colours of internal organs were evaluated. Histopathology examinations were performed on showing lesions organs. Organs for histopathology examinations were trimmed, embedded and section using paraffin section technique. The tissues slides were stained with Hematoxylin and Eosin.

Analysis of Result:

The analysis of results were ranked and classified follows the indicated arrow, that depending on the number of humanely killed or dead animals according to OECD guideline 423.



OECD/OCDE, OECD Guidelines for the testing of chemicals 423, Acute Oral Toxicity – Acute Toxic Class Method (2001)

RESULTS AND DISCUSSION

For evaluation of acute oral toxicity, after administrated with 300 mg/kg body weight of crocodile bile were not shown signs of toxic, moribund and mortality. All administrated animals were normally consumed feed and water and no changed in skin, fur, eyes and mucous membrane, occurrence of secretions and excretions and

autonomic activity (lacrimation, piloerection, pupil size and respiratory pattern), no changed in gait, posture, response to handling, presence of clonic or tonic movement and stereotypes (excessive grooming and repetitive circling) and not shown any bizarre behavior (self-mutilation and walking backwards) and not shown any clinical symptoms of lymph node.

The individual body weights of all animals were continued to gain throughout the study. Feed and water consumption were normal, the data are consecutive data, the results were transient change and no effect on animal health. The individual body weights of animal no. 1 - 6 were measured weekly and on the date of terminated. The individual body weights, feed and water consumption are presented in Table 1 and 2 respectively. The necropsy finding of animal no. 1, 2 and 3 were found slightly red intestinal mucosa and especially duodenum and animal No. 4, 5 and 6 were unremarkable lesions.

Table 1 Body weight (g) and body weight change (%)

No.	Body Weight (g)				Body Weight Change (%)
	Acclimatize	Week 1	Week 2	Terminate	
1	214	219	225	243	13.55
2	216	218	227	242	12.04
3	232	231	256	278	19.83
4	204	212	221	232	13.73
5	219	226	237	251	14.61
6	221	233	245	257	16.29

Table 2 Feed and water consumption (g)

No.	Feed Consumption (g)			Water Consumption (g)		
	Acclimatize	Week 1	Week 2	Acclimatize	Week 1	Week 2
1	14	18	11	26	27	24
2	11	17	15	25	28	26
3	16	13	19	29	36	30
4	9	13	10	18	20	19
5	12	16	17	28	30	31
6	12	11	15	26	11	34

For evaluation of acute oral toxicity, after administrated with 2,000 mg/kg body weight of crocodile bile were shown signs of toxic, moribund and mortality. The results shown, animal no. 7, 8 and 9 were shown depress, piloerection, lacrimation, inactivity, loss of mobility, unresponsiveness and coma and reached stage of predictable death, moreover animal no. 8 was died within 24 hours. And before 48 hours, animal no. 7 was died, then animal no. 9 was euthanized prior to schedule sacrifice. The individual body weights of animal no. 7 - 9 were measured weekly and on the date of terminated. The individual body weights are presented in Table 3. The necropsy finding of animal no. 7, 8 and 9 were found predominantly gastric lesions are severe diffuse red mucosal

stomach and mucosal erosion/hemorrhage. The stomach of all animals were found gas filled, patchy hemorrhage)severe(, redness and mucosal thinning, moreover the duodenum were found redness, thin and gas distension. Gastrointestinal lesions had a relative with sign of soft feces to diarrhea. For microscopic finding, typical lesion is moderate gastric erosion and acute proximal tubular necrosis. Gastric mucosa was founds loss of full mucus secreting cells and upper parietal cells, diffuse superficial to deep epithelial necrosis, numerous active neutrophil and mononuclear infiltration intra lamina propria with diffuse acute hemorrhage. Duodenum was found focal erosion and necrosis, increase cellularity in lamina propria. Jejunum shows focal erosion.

Table 3 Body weight (g) and body weight change (%)

No.	Body Weight (g)				Body Weight Change (%)
	Acclimatize	Week 1	Week 2	Terminate	
7	215	N/A	N/A	186 (Carcass)	-15.59
8	230	N/A	N/A	196 (Carcass)	-17.34
9	213	N/A	N/A	180	-18.33

Both sides of kidneys were found diffuse proximal tubular hydropic change that progress to proximal tubular necrosis without inflammatory infiltration. This lesion was limited in 30-70% of cortex.

Discussion:

For evaluation of acute oral toxicity of crocodile bile, at 300 mg/kg body weight, all animals were not shown signs of toxic, moribund and mortality. On the other hand, at 2,000 mg/kg body weight of crocodile bile, all animals were shown signs of toxic, moribund and mortality within 24 hours. Moreover the macroscopic and microscopic finding were shown gastrointestinal lesions, that lesions were related in all animals.

In summary, the crocodile bile administered orally is cause acute toxicity in animals. The result suggested that crocodile bile was classified in GHS category 4, the LD50 cut off at 500 mg/kg body weight. A sub chronic and chronic toxicities study should be further carried out to assess the long-term safety.

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