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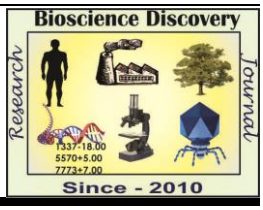

Acute Oral Toxicity Testing of Siamese Crocodile (*Crocodylus siamensis*) Oil in Wistar Rats

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Abstract

The Siamese Crocodile (*Crocodylus siamensis*) Oil are contain high level of essential fatty acids as omega 3, 6 and 9 up to 1,377.32, 21,748.72 and 41,062.98 mg/100g respectively. There are many claims of positive results. It includes fading of freckles, acne, pimple marks, dark lines, wrinkles and laugh lines, dark shadows, sun spots and other discolorations. It helps prevent the forming of discoloration and makes the skin softer, brighter and more attractive. But they did not show data about irritation and safety test. This study would like to rank classified of crocodile oil and acute oral toxicity study. The ranked and classified of crocodile oil by the Globally Harmonized System for the classification of chemical, which cause acute toxicity in Wistar 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 the 300 and 2,000 mg/kg body weight of crocodile oil did not produce any mortalities. No sign of toxicity were observed for 14 days. The results showed that crocodile oil was classified in GHS category 5 or unclassified, the LD50 cut off at 5,000- ∞ mg/kg body weight.

INTRODUCTION

Today, there are well over 2,500 farms in Cambodia, Vietnam and Thailand (Daltry *et al.*, 2016). Thailand has the largest crocodile farming industry in the world. It is a recognized source of the natural exotics skins. Department of Fisheries in Thailand reported that more than 520,000 crocodiles from nationwide farms were supplied to the industry every years. The main products of crocodile industry in Thailand are exotic crocodile skin and crocodile meat. In addition, crocodile bloods are also used as a dietary supplement (Chaeychomsri *et al.*, 2009). Although crocodile blood industry transformed into large industry in Thailand, it leaves many wastes from crocodile skin and meat productions such as internal organs and fat. Crocodile fat can be extracted to crocodile oil and develop products for health care, skin care and cosmeceutical.

Natural oil is extensively used in cosmetics and treatment for a growing number of conditions (Nielson, 2006). The natural products industry is a multibillion dollar industry and has grown enormously over the past few years.

Oil extracted from plant sources has a rich history of use by local people as a source of food, energy, medicine, and health products applications. It have been using in the personal care product as well as in the topical treatment of various conditions. The demand of seed oils as ingredients in cosmetics has greatly increased as the industry has been seeking for natural alternative (Vermaak *et al*., 2011). Natural oils are known for their medicinal properties and used in embalmment, preservation of food, antimicrobial, analgesic, sedative, anti-inflammatory, spasmolytic and local anesthetic remedies (Bakkali *et al*., 2007). It may be considers as potential natural antioxidants and could perhaps be formulated as a part of daily supplements or additives to prevent stress that contributes to many degenerative diseases including aging (Edris, 2007). Essential oils can be used in treatment of cancer, cardiovascular disease including atherosclerosis, thrombosis and diabetes (Stewart, 2005). Oils used in cosmetics and dietary supplements contain a range of fatty acids which contribute to several beneficial properties. Fatty acids are divided into saturated acids and unsaturated acids (Vermaak *et al.,* 2011). Fatty acids are very important as formulation agents and vehicles in health care products (Heinrich, 2004). The most common fatty acids include omega-3, omega-6 and omega-9 fatty acids. The omega-3 and omega-6 fatty acids are naturally occurring lipids, appearing in high concentration in certain fish, particularly in cold water, oily species and plants such as flax seed oil, tea tree oil, olive oil, grape seed oil, emu oil and crocodile oil (Stoll *et al*., 1999). The fat of Nile crocodile (*Crocodylus niloticus*) composition are contain high levels of palmitic (16:0), palmitoleic (16:1c9), stearic (18:0), oleic (18:1c9) and linoleic (18:2n6) acids (Magnino *et al*., 2009). Crocodiles are monogastric animals and therefore their diet strongly influences the fatty acid composition of the fat. Fish based diets result in greater amounts of longer fatty acids compared to chicken and beef diets (Osthoff *et al*., 2009). The commercial products are many claims of positive results. It includes fading of freckles, acne, pimple marks, dark lines, wrinkles and laugh lines(Venter, 2012). However, toxicity study of are not carried out before the initiation of human studies, further safety evaluation studies were required to be conducted in animals to provide data for toxicity assessment. Therefore in the study would like to report of fatty acid composition of Siamese crocodile (*Crocodylus siamensis*) Oil and the toxicity study on acute oral toxicity testing of Siamese crocodile (*Crocodylus siamensis*) Oil in Wistar Rats.

MATERIALS AND METHODS

Animals and Husbandry:

Healthy 6-8 weeks, 12 female, Wistar rats of body weight range 200 g ± 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±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(Council, 2010). The study was approved by National Laboratory Animal Center Animal Care and Use Committee (NLAC-ACUC), Mahidol University; Thailand.

Sample collection:

The Siamese crocodile (*Crocodylus siamensis*) oil was derived from crocodiles in Sri-Ayuthaya Gold Medal crocodile farm, Nong Khanak, Tha Ruea District, Phra Nakhon Si Ayutthaya province, Thailand. The study supported by Interdisciplinary Graduate Program in Bioscience, Faculty of Sciences, Kaestsart University, Thailand.

Crocodile Oil Extraction:

Several excellent methods are available for fat extraction (Christie, 2003). Oil was extracted by non-chemical methods which is stream rendering. Sample preparation was cut in small pieces (5-10 cm in diameter) and then stream at 90 °C for 45 minutes. The cooked fat was wrapped in fabric and pressed by pressing machine. Crude oil was separated to liquid fractions and the oil was obtained by centrifugation at 6000 g temperature 4 °C for 10 minutes and maintained in 4 °C until used.

Preparation of fatty acid methyl esters (FAMEs) and Gas Chromatography Analysis:

To analyze fat in foods for fatty acid information, AOAC Official method 996.06 is recommended (DeVries *et al.* 1999). The Procedure involves hydrolysis of oil samples using esters and acids, followd by non-chemical extraction of the released

fat, transesterification of extracted fat to fatty acid methyl esters (FAME) and determination of fatty acid profile by capillary gas chromatography (Christie, 2003) as GC is shown to be the best method for analyzing fatty acid profiles.

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 (Botham, 2004; Jonsson *et al.*, 2013). 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 that not exceed 1 ml per 100 g body weight. The crocodile oil 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 duringthe 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.

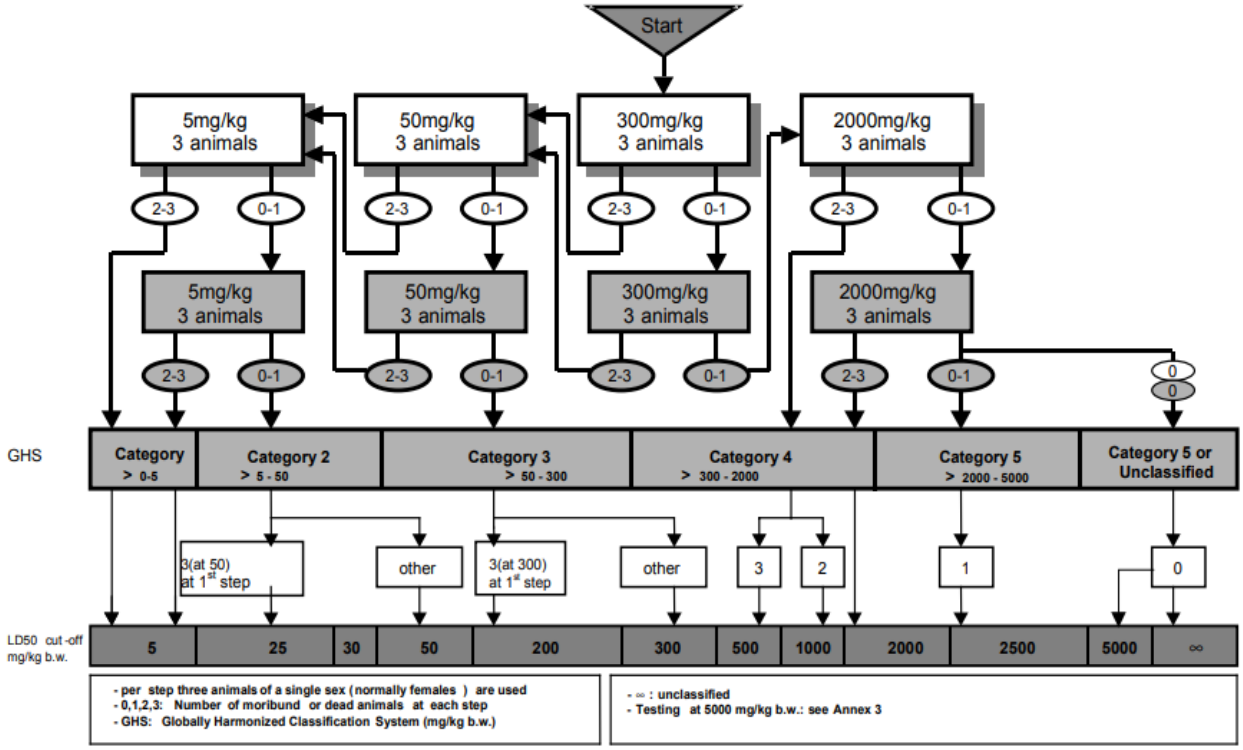

Figure 1. The OECD Guidelines for the testing of chemicals 423 (OECD 423 2001)

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 andrespiratory pattern), changes in gait, posture, response to handing, 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 CO_2 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.

RESULTS AND DISCUSSION

Fatty acid composition of Siamese Crocodile (*Crocodylus siamensis*) Oil:

Crocodile oil and other natural oils mainly contain saturated and unsaturated fatty acids. The unsaturated fatty acids namely omega-3,-6,-7 and -9 are responsible for the positive effects on human skin (Nielsen 2006). Fatty acids have an even number of carbon atoms, in the range of 12-24. Fatty acids with only single bonds between adjacent carbon atoms are referred to as saturated, where's those with at least one C=C double bond are called unsaturated. The polyunsaturated fatty acids have two or more double bonds and are named according to the position of these bonds and the total chain length. For example, docosahexzenoic acid (DHA; 22:6) is an omega-3 (n-3) fatty acid with 22 carbon atoms and 6 double bonds. The term 'n-3' indicates that, counting from the methyl (CH_3) end of the molecule, the first double bonds are located between the third and fourth carbons. As the degree of unsaturation in fatty acids increases, the melting point decreases which confers the attribute of fluidity on n-3 polyunsaturated fatty acids (Venter, 2012). Figure 2 shows the comparison of Siamese crocodile oil and fish oil fatty acids composition. The crocodile oil has 1,377.32 mg/100g, 21,784.72 mg/100g, 5,130.00 mg/100g and 41,062.98 mg/100g of Omega-3, Omega-6, Omega-7 and Omega-9, respectively. In contrast, the fish oil has, 26,324.95 mg/100g, 5,520.52 mg/100g, 9,860.00 mg/100g and 11,102.09 mg/100g, respectively. The amount of Omega-6 and Omega-9 fatty acids from Siamese crocodile oil are 4 time higher than those measured in fish oil

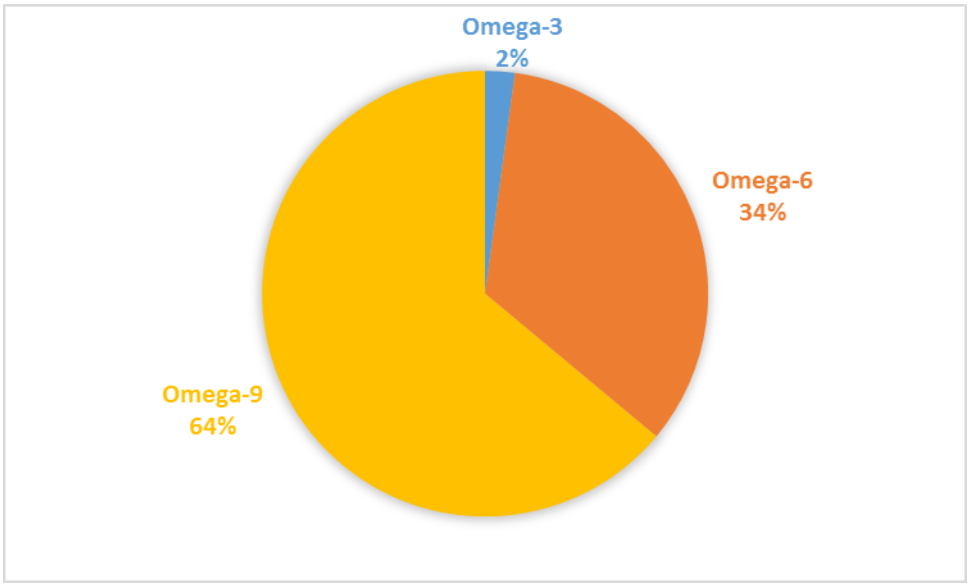

Figure 1. Fatty acid composition of Siamese crocodile oil mg/ in 100g
Alpha linoleic acid (Omega-3), Linoleic acid (Omega-6), Palmitoleic acid (Omega-7 and Oleic acid (Omega-9) are shown in green, orange, yellow, sky blue respectively.

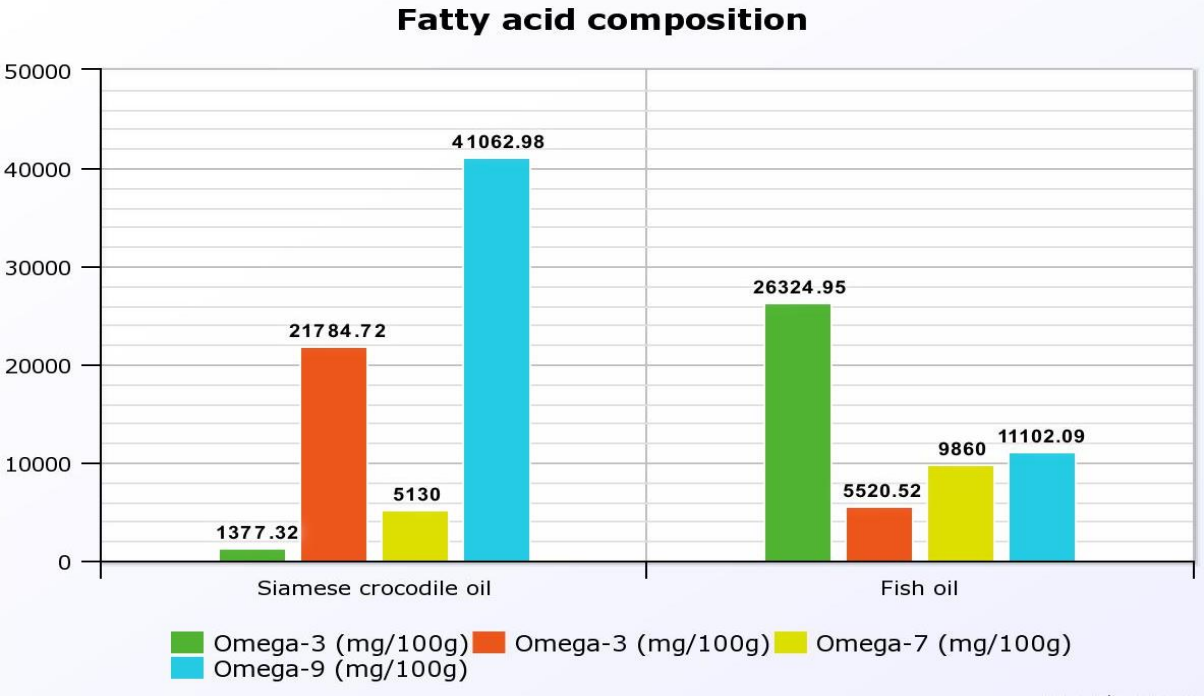

Figure 2. Fatty acid composition of Siamese crocodile oil mg/ in 100g
Alpha linoleic acid (Omega-3), Linoleic acid (Omega-6), Palmitoleic acid (Omega-7 and Oleic acid (Omega-9) are shown in green, orange, yellow, sky blue respectively.

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

No.	Body Weight (g)					Body Weight Change (%)
	Quarantine	Acclimatize	Week 1	Week 2	Terminate	
1	187	206	224	235	236.05	26.22
2	197	206	218	227	234.38	18.97
3	210	214	228	245	245.62	16.96
4	193	206	222	234	239.34	24.01
5	197	203	222	245	247.71	25.74
6	204	209	226	238	245.08	20.14
7	186	208	217	227	236.51	27.16
8	199	213	232	245	250.95	26.11
9	204	212	228	238	243.42	19.32
10	187	201	212	223	233.16	24.68
11	198	207	218	233	236.71	19.56
12	204	215	229	238	249.58	22.34

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	11	14	18	33	25	28
2	14	12	15	23	17	24
3	16	15	15	27	29	21
4	16	14	16	25	27	33
5	14	12	15	27	23	22
6	16	12	19	24	20	33
7	10	16	18	31	27	31
8	15	16	19	26	20	32
9	14	13	15	23	29	28
10	12	16	16	13	23	35
11	17	15	10	34	28	16
12	11	16	17	20	28	30

Acute oral toxicity test in Wistar Rats:

For evaluation of acute oral toxicity, after administrated with 300 and 2,000 mg/kg body weight of crocodile oil, all animals 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 handing, 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 necropsy findings of animals were not found any lesion.

The individual body weights of animals were measured weekly and on the date of terminated. The individual body weights, feed and water consumption are presented in Table 3 and 4 respectively.

Discussion:

For evaluation of acute oral toxicity, after administrated with 300 and 2,000 mg/kg body weight of crocodile oil, all animals were not shown signs of toxic effects, moribund and mortality. The health observations and examinations results of all 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 handing, 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. Moreover the macroscopic findings of all animals were no remarkable lesions in all organs. The body weights of all animals were continued to gain throughout the study. Feed and water consumption were normal. The feed and water consumption data of animals are consecutive data. The results were transient change and no effect on animal health.

The result suggested that crocodile oil was classified in GHS category 5 or unclassified, the LD50 cut off at 5,000- ∞ mg/kg body weight. In summary, the crocodile oil administered orally did not cause acute toxicity in Wistar rats. A sub chronic and chronic toxicities study should be further carried out to assess the long-term safety of the test item.

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