Print & Online, Open Access, Research Journal Available on http://jbsd.in
ISSN: 2229-3469 (Print); ISSN: 2231-024X (Online)
Research Article

Primary Skin Irritation Test of Siamese Crocodile (*Crocodylus siamensis*) Oil in New Zealand White Rabbit

Amon Praduptong[1,5] Passaraporn Srimangkornkaew[1,4], Warin Intana[6] Jindawan Siruntawineti[1,2], Sudawan Chaeychomsri[3], Natthawut Thitipramote[7], Win Chaeychomsri[1,2]*

1 Interdisciplinary Graduate Program in Bioscience, Faculty of Sciences, Kasetsart University, Thailand
2 Department of Zoology, Faculty of Sciences, Kaestsart University, Thailand
3 Central Laboratory and Greenhouse Complex, Faculty of Agriculture at Kamphaeng Saen, Kaestsart University, Thailand
4 National Laboratory Animal Center, Mahidol University, Thailand
5 Thailand Center of Excellence for Life Sciences (Public Organization), Thailand
6 School of Agriculture Technology, Walailak University, Thailand
7 School of Cosmetic Science, Mae Fah Luang University, Thailand
*For Correspondence, fscijwcc@ku.ac.th

Article Info

Received: 19-05-2018,
Revised: 04-09-2018,
Accepted: 19-09-2018

Keywords:
Delayed-type hypersensitization, crocodile oil, fatty acids, skin irritation

Abstract

Crocodile oil has many kinds of fatty acids including stearic acid, myristic acid, palmitic acid, palmitoleic acid, oleic acid, linoleic acid and alphalinoleic acid. Composition of the crocodile oil is very similar to human skin oil with only ingredients composition differ. To the best of our knowledge, the irritation effects from the crocodile oil has not been reported. Therefore, the study aimed to understand the effects of crocodile oils in New Zealand White Rabbit. This study followed guideline of ISO 10993-10 the Biological evaluation of medical device part 10: Tests for Irritation and Delayed-Type Hypersensitization. The data showed all animals appeared active and healthy during the study. Apart from the dermal irritation noted below, there were no other signs of toxicity, adverse pharmacologic effects or abnormal behavior. No edema was observed at any treated dose site. Under the conditions of this study the crocodile oil was classified as negligible irritating to the skin.

INTRODUCTION

The Siamese crocodile (*Crocodylus siamensis*) oil contains 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 (Praduptong *et al.*, 2018). There are many claims of positive results, including fading of freckles, acne, pimple marks, dark lines, wrinkles, 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 (Venter, 2012). In spite of these, irritation and safety tests have yet to be reported. The previous study showed the rank classified of crocodile oil and acute oral toxicity study by the Globally Harmonized System for the classification of chemical, which cause no 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 (OECD 423, 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 was 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 (Praduptong *et al.*, 2018).

The results are supported with crocodile oil profiling for food supplement products. In addition, the data support of crocodile oil for food. There are many applications of crocodile oil to support good skin by skin care products. There are claims described abound that it recovers skin problems such as burns, wounds, sunburns, and even eczema. They are used for dull, dry, itchy, irritated, cracked rough skin, wound healing agent and relieve swelling from insect bites by many kind of skin care products. However, the fact that no confirmation could be found in literature that any scientific studies have been done before. It is desirable to confirm irritation effect on skin. Therefore, the aim of the study was to investiage the skin irritation of Siamese crocodile (*Crocodylus siamensis*) oil in New Zealand White rabbit (NZW) skin in compliance with ISO 10993-10:the Biological evaluation of medical device part 10: Tests for Irritation and Delayed-Type Hypersensitization. The implication of this study may support the positive claims of skin care product from crocodile oil in the market and value adding of wasted fat from crocodile skin industry in the future.

MATERIALS AND METHODS

Sample Collection:

Crocodile fat was obtained from Siamese crocodile (*C. siamensis*) carcass that was slaughtered at Sri-Ayutthaya Gold Medal Crocodile Farm, Nong Khanak, Tha Ruea District, Phra Nakhon Si Ayutthaya Province, 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. The sample preparation was cut in small pieces (5-10 cm in diameter) and then stream at 90 ℃ for 45 minutes. The cooked fat was wrapped in fabric and pressed by pressing machine. The crude oil was separated to liquid fractions. After that, it was obtained by centrifugation at 6000 g, temperature 4 ℃ for 10 minutes and maintained in 4 ℃ until used (Praduptong *et al.,* 2018).

Analyzing Fatty Acid Profiles:

Normally, fat in foods is analyzed for fatty acid information related to AOAC Official method 996.06 recommendations (DeVries *et al.,* 1999). The procedure involved hydrolysis of oil samples using esters and acids, followed 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).

The Quantitative Methods for Macroscopic Finding:

The primary skin irritation study followed the Biological evaluation of medical device part 10: Tests for Irritation and Delayed-Type Hypersensitization. The study used 3 female Mlac:NZW rabbits those were necropsied after skin irritation test with Siamese crocodile (*C. siamensis*) oil. In both of first and last days, rabbits' back was prepared by clipping and shaving. Then, clinical sign of skin was red and irritation, some area was also ulceration.

Animals Husbandry and Experimental Design:

Healthy young female NZW rabbits, body weight range 2000 – 3000 g, were obtaining from Office of Laboratory Animal Production, National Laboratory Animal Center (NLAC), Mahidol University, Thailand. The animals were kept under standard conditions 12:12 (day:night cycles) at 22±3 ℃ and 30–70% relative humidity. The animals were housed individually in stainless cages, Animal Room 1 Mini Clean Room 2, Research Building with food (086, Perfect Companions, Thailand) and 5-6 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 1996; NIH publication number #85-23, revised 1996) 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. Then, applied 5 ml of crocodile oil directly to each test skin site as shown in Figure 1 and covered the application site with a 2.5 cm x 2.5 cm on for 4 h. At the end of the contact time, removed residual test material by appropriate means, such as washing with lukewarm water or other suitable non irritating solvent and careful drying.

Key 1. Cranial end 2. Test site 3. Control site 4. Clipped dorsal region 5. Caudal end

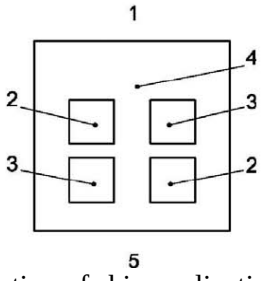

Figure 1. Location of skin application site

Single exposure test:

For single exposure test, recorded the appearance of each application site at (1+0.1) h, (24+2) h, (48+2) h and (72+2) h following removal of the patches. Extended observation can be necessary if there are persistent lesions, in order to evaluate the reversibility or irreversibility of the lesions over a period of time not exceeding 14 days (OECD 423, 2001).

Repeated exposure test:

Repeated exposure shall only be carried out after completion of an acute single exposure test (after at least 72+2) h of observation. For repeated exposure tests, recorded the appearances of the application site at (1+0.1) h after removal of the patches and immediately prior to the next application. After the last exposure, noted the appearance of each application site at (1+0.1) h, (24+2) h, (48+2) h and (72+2) h following removal of the patches (Jonsson, 2013). Extended observation can be necessary if there are persistent lesions, in order to evaluate the reversibility or irreversibility of the lesions over a period of time not exceeding 14 days.

Evaluation of results:

For single exposure test, determined the primary irritation index (PII) as follows: Used only (1+0.1) h, (24+2) h, (48+2) h and (72+2) h observation for calculations. Observation made prior to dosing or after 72h to monitor recovery was not used in the determination. After the 72h grading, all erythema grades plus edema grades (1+0.1) h, (24+2) h, (48+2) h and (72+2) h were totaled separately for each test sample and blank for each animal. The primary irritation score for an animal was calculated by dividing the sum of all the score by 6 (two test/observation sites, 4 time points). To obtain the primary irritation index for the test sample added all the primary irritation scores of the individual animals and divided by the number of animals (generally three). When blank or negative control was used, calculated the primary irritation score for the controls and subtracted that score from the score using the test material to obtain the primary irritation score. For repeated exposure assays the primary irritation index for each animal was be calculated according to the principle mention above, taking into consideration all evaluation points. For repeated exposure, the cumulative irritation index as follow was determined. Then, the irritation scores of all animals were added together and divided by the total number of animals. This value was the cumulative irritation index.

The cumulative irritation index was compared with the categories of irritation response given in Table 1 and the appropriate response category was recorded for the report. For any response, record the maximum primary irritation score given in Table 1. For each animal, the time of onset of the response and the time to maximum response were noted. The primary or cumulative irritation index was characterized by number (score) and description (response category) given in Table 2. In case different extracts had been tested, the one giving the highest PII determined the response category.

Table 1. Primary or cumulative irritation index categories in a rabbit

Mean score	Response category
0 to 0.4	Negligible
0.5 to 1.9	Slight
2.0 to 4.9	Moderate
5.0 to 8.0	Severe

RESULTS

Fatty acid profile of the extracted Siamese crocodile oil:

The fatty acid content of C. siamensis oil was recorded and presented in Figure 2 and Table 3. The crocodile oil has fatty acid composition include stearic acid, myristic acid, palmitic acid, palmitoleic acid, oleic acid, linoleic acid and alphalinoleic acid, that show the percentage are 5.11, 0.74, 23.71, 5.13, 40.91, 20.98 and 1.13 respectively. The presented profile as like composition in human skin oil that it shows 11.2, 2.1, 20.2, 3.8, 30.8, 15.1 and 0.3, respectively (Venter, 2012). It is only different in the percentages of the ingredients composition with human skin oil.

Skin irritation evaluation:

One day prior to the experiment, hair on the backside area of three New Zealand white rabbits were removed by hair clippers (area of 10 cm × 15 cm) and washed with normal saline. Crocodile oil was applied on the skin surface with the dosage of 5 ml.

Table 2. Scoring system for skin reaction in the tested rabbit.

Reaction	Irritation score
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well defined erythema	2
Moderate erythema	3
Severe erythema	4
Edema formation	
No edema	0
Very slight edema (barely perceptible)	1
Well defined edema	2
Moderate edema	3
Severe edema	4
Maximum possible score for irritation	8
Other adverse changes at the skin sites shall be recorded and reported	

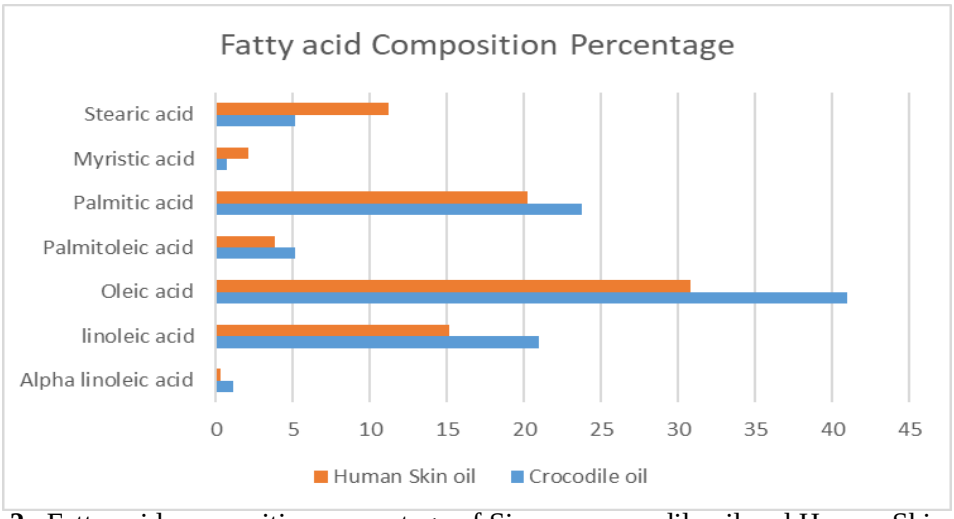

Figure 2. Fatty acid composition percentage of Siamese crocodile oil and Human Skin oil

All animals appeared active and healthy during the study. A primary skin irritation rabbit test was conducted to determine the potential for original hydrogels to produce irritation after a single topical application. Individual skin irritation scores and a summary thereof were used for calculation of Primary Dermal Irritation Index. In Single exposure test**,** erythema and edema were checked on test areas applied with Crocodile oil and control of all three rabbits after 1, 24, 48 and 72 h. An erythema and edema with the score of 0 was found at the skin sites of all rabbits. In repeated exposure, erythema and edema were checked on test areas applied with Crocodile oil and control of all three rabbits after 1, 24, 48 and 72 h. No erythema and edema were found. The response of the Crocodile oil applied on the rabbit skin surface compared with control sites applied the skin surface, were evaluated and primary irritation index (PII) was calculated and matched with primary or cumulative irritation index categories (Table 2). The Primary Dermal Irritation Index for the Crocodile oil is 0.0

Primary Initiation score (PIS) at test site 1)Crocodile oil(

PIS at test site 1on Rabbit No.1 = $\frac{0+0+0+0+0+0+0+0}{8} = \frac{0}{8} = 0$

PIS at test site 1on Rabbit No.2 = $\frac{0+0+0+0+0+0+0+0}{8} = \frac{0}{8} = 0$

PIS at test site 1on Rabbit No.3 = $\frac{0+0+0+0+0+0+0+0}{8} = \frac{0}{8} = 0$

Primary Initiation score (PIS) at test site 2)Control(

PIS at test site 2 on Rabbit No.1 = $\frac{0+0+0+0+0+0+0+0}{8} = \frac{0}{8} = 0$

PIS at test site 2 on Rabbit No.2 = $\frac{0+0+0+0+0+0+0+0}{8} = \frac{0}{8} = 0$

PIS at test site 2 on Rabbit No.3 = $\frac{0+0+0+0+0+0+0+0}{8} = \frac{0}{8} = 0$

Primary Initiation score (PIS) at test site 3)Crocodile oil(

PIS at test site 3 on Rabbit No.1 = $\frac{0+0+0+0+0+0+0+0}{8} = \frac{0}{8} = 0$

PIS at test site 3 on Rabbit No.2 = $\frac{0+0+0+0+0+0+0+0}{8} = \frac{0}{8} = 0$

PIS at test site 3 on Rabbit No.3 = $\frac{0+0+0+0+0+0+0+0}{8} = \frac{0}{8} = 0$

Primary Initiation score (PIS) at test site 4)Control(

PIS at test site 4 on Rabbit No.1 = $\frac{0+0+0+0+0+0+0+0}{8} = \frac{0}{8} = 0$

PIS at test site 4 on Rabbit No.2 = $\frac{0+0+0+0+0+0+0+0}{8} = \frac{0}{8} = 0$

PIS at test site 4 on Rabbit No.3 = $\frac{0+0+0+0+0+0+0+0}{8} = \frac{0}{8} = 0$

Primary irritation index, PII

Primary irritation index (PII) of Test site (20% Crocodile oil) = $\frac{0+0+0}{3} = 0.0$

Primary irritation index (PII) of Control = $\frac{0+0+0}{3} = 0.0$

DISCUSSION

Crocodile oils are rich of essential fatty acids which is very important for the skin rejuvenation. It has been utilized to treat several skin disease including eczema and psoriasis as well as burn (Venter, 2012). To understand the origin of these properties, crocodile oils were characterized and found to have several fatty acids, including linoleic acid (treatment used in several skin condition), omega 3, 6, 9 (healing properties from burn and cut) and oleic acid (rejuvenates cells). The results are consistent with the report of characterization and clinical testing of *Crocodylus niloticus* (Osthoff, 2009*).*

CONCLUSION

All animals appeared active and healthy during the study. Apart from the dermal irritation noted below, there were no other signs of gross toxicity, adverse pharmacologic effects, or abnormal behavior. No edema was observed at any treated dose site. In Single exposure test**,** erythema and edema were checked on test areas applied with Crocodile oil of all three rabbits after 1, 24, 48 and 72 h. An erythema and edema with the score of 0 was found at the skin sites of all rabbits. In repeated exposure test which disappeared after 1, 24, 48 and 72 h. No erythema and edema were found. The results of Crocodile oil skin irritation evaluation showed had no skin irritation. Under the conditions of this study the Crocodile oil was classified as negligible to the skin which indicated its safety and acceptability for topical administration.

ACKNOWLEDGEMENT

Special thanks to C S G Products (Thailand) Co., Ltd.; Sri-Ayutthaya Gold Medal Crocodile Farm; Department of Zoology, Faculty of Science & Central Laboratory and Greenhouse Complex, Kasetsart University; National Laboratory Animal Center, Mahidol University, School of Cosmetic Science, Mae Fah

Luang University; Center of Excellence for Life Sciences (Public Organization). This study was supported by National Research Council of Thailand. We appreciate those whose names are not mentioned here but have greatly inspired and encouraged us until this independent study comes to a perfect end.

Table 3. Fatty acids composition of Siamese crocodile oil

Peak	Fatty acid composition (Carbon atom)	Retention time Standard. (min.)	Crocodile oil g/100g
Saturated Fatty acid			**30.16**
1	Lauric acid (C12:0)	13.633	0.29
2	Tridecanoic acid (13:0)	14.542	-
2	Myristic acid (C14:0)	15.637	0.74
3	Pentadecanoic acid (C15:0)	16.912	0.10
4	Palmitic acid (C16:0)	18.367	23.71
5	Heptadecanoic acid (C17:0)	20.004	0.14
6	Stearic acid (C18:0)	21.842	5.11
7	Arechidic acid (C20:0)	26.219	0.07
8	Heneicosanoic acid (C21:0)	28.781	-
9	Behenic acid (C22:0)	31.685	-
10	Tricosanoic acid (C23:0)	34.883	-
Unsaturated Fatty acid			**69.85**
Monounsaturated Fatty acid			**46.52**
11	Myristoeic acid (C 14:1)	16.746	0.14
12	Palmitoeic acid (C16:1n7)	19.518	5.13
13	*cis*-9-Oleic acid (C18:1n9c)	23.075	40.91
14	*cis*-11-Eicosenoic acid (C20:1n11)	27.654	0.18
15	Nervonic acid (C24:1n9)	39.826	0.16
Polyunsaturated Fatty acid			**23.33**
16	*cis*-9-12-linoleic acid (C18:2n6)	25.063	20.74
17	gramma-Linolenic(C18:3n6)	26.736	0.24
18	alpha- Linolenic(C18:3n3)	27.703	1.13
19	*cis*-11,14-Eicosadienoic acid (C20:2)	30.107	0.16
20	*cis*-8,11,14-Eicosatrienoic acid (C20:3n6)	32.059	0.21
21	*cis*-11,14,17-Eicosatrienoic acid (C20:3n3)	33.214	-
22	Arachidonic acid (C20:4n6)	33.648	0.60
23	*cis*-5,8,11,14,17-Eicosapentaenoic acid C20:5n3(EPA)	37.381	0.07
24	4,7,10,13,16,19-Decosahexaenoic acid C22:6n3(DHA)	44.307	0.18
Trans Fat			**-**
Omega-3 (mg/100g)			**1,377.32**
Omega-6(mg/100g)			**21,784.72**
Omega-9(mg/100g)			**41,062.98**

REFERENCES

Christie WW, 2003. *Analysis of conjugated linoleic acid: an overview*. AOCS Press, Illinois United States of America, Pp 1-12.

Council NR, 2010. *Guide for the care and use of laboratory animals*. National Academies Press, Washington United States of America, Pp 21-48.

DeVries JW, Kjos L, Groff L, Martin B, Cernohous K, Patel H, Payne M, Leichtweis H, Shay M and Newcomer L, 1999. Studies in improvement of Official Method 996.06. *Journal-Aoac International*, **82**(5):1146-1155.

Jonsson M, Jestoi M, Nathanail AV, Kokkonen U-M, Anttila M, Koivisto P, Karhunen P and Peltonen K, 2013. Application of OECD Guideline 423 in assessing the acute oral toxicity of moniliformin. *Food and chemical toxicolog*, **53**:27-32.

OECD 423, 2001. *OECD Guidelines for the Testing of Chemicals. Paris: Organisation for Economic Cooperation and Development*. OECD, Paris France, Pp 10.

Osthoff G, Hugo A, Bouwman H, Buss P, Govender D, Joubert CC and Swarts JC, 2009. Comparison of the lipid properties of captive, healthy wild, and pansteatitis-affected wild Nile crocodile (*C. niloticus*). *Comparitve Biochemistry and Physiology*, **155**:64-69.

Praduptong A, Srimangkornkaew p, Siruntawineti j, Chaeychomsri S and Chaeychomsri W, 2018. Acute Oral Toxicity Testing of Siamese Crocodile (*Crocodylus siamensis*) Oil in Wistar Rats. *Bioscience Discovery*, **9**(3):409-415.

Stewart D, 2005. *The chemistry of essential oils made simple: God's love manifest in molecules*. Care Publications, Missouri United States of America, Pp 848.

Venter T, 2012. *Characterisation, toxicology and clinical effects of crocodile oil in skin products*. North-West University, Potchefstroom South Africa, Pp 2.

How to cite this article

Amon Praduptong , Passaraporn Srimangkornkaew, Warin Intana Jindawan Siruntawineti, Sudawan Chaeychomsri, Natthawut Thitipramote, Win Chaeychomsri, 2018. Primary Skin Irritation Test of Siamese Crocodile (*Crocodylus siamensis*) Oil in New Zealand White Rabbit. *Bioscience Discovery*, **9**(4):478-484.