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Antioxidant Activity of Mixture Herbal Oil from *Siamese Crocodile* Oil (*Crocodylus siamensis*) Turmerica(*Curcuma longa*), Black Galingale (*Kaempferia parviflora*) and Plai (*Zingiber cassumunar* Roxb)

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Abstract

The 3 formulas of crocodile oil mixture with Thai medicinal plants *Zingiber cassumunar* Roxb (Phlai), *Curcuma longa* (Turmeric) and *Kaempferia parviflora* (Black galingale) were developed as an alternative to skincare products. The study characterized by gas chromatography, Crocodile oil contained high concentration of palmitic acid, stearic acid, oleic acid and linoleic acid, the mixture oil (3 formulas) contain same high sabinene, sabinene hydrate, and (E)-1-(3,4-dimethoxyphenyl) butadiene (DMPBD). The study shed light on 4 methods consists of phenolic content test, flavonoid content test, antioxidant efficiency test, and inhibition lipid peroxidation test of the mixture oil (3 formulas). The result indicated that crocodile oil mixed with Phlai has highest phenolic content 35.250 ± 0.902 mg GAE/ml, the crocodile oil mixed with turmeric has phenolic content 35.188 ± 1.659 mg GAE/ml, and the crocodile oil mixed with black galingale has phenolic content 16.406 ± 0.797 mg GAE/ml. The result of flavonoid contents test indicated that crocodile oil mixed with Turmeric has highest flavonoid contents 5.676±0.644 mg QE/ml, crocodile oil mixed with Black galingale has flavonoid contents 3.281±0.505 mg QE/ml and crocodile oil mixed with Phlai has flavonoid contents 3.275±1.132 mg QE/ml. The result of antioxidant efficiency by DPPH assay indicated that crocodile oil mixed with Phlai was highest 119.735 ± 1.058 mg TEAC/ml, crocodile oil mixed with turmeric has 64.797 ± 1.481 mg TEAC/ml, and crocodile oil mixed with Black galingale has 62.199 ± 0.581 mg TEAC/ml. The result of inhibition lipid peroxidation indicated that crocodile oil mixed with Turmeric has highest 40%, crocodile oil mixed with Phlai has 22%, and crocodile oil mixed with Black galingale 8%. The study showed that the mixed crocodile oils and 3 herbal plants have useful fatty acids for skincare and high anti-oxidant which can be developed to be massage oil and various relevant products in the future.

Keywords: Crocodile oil, Phlai, Turmeric, Black Galingale and Phenolic

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ฤทธิ์ต้านอนุมูลอิสระของน้ำมันสมุนไพรสูตรน้ำมันจระเข้พันธุ์ไทย (*Crocodylus siamensis*) ขมิ้นชัน (*Curcuma longa*) กระชายดำ (*Kaempferia parviflora*) และไพล (*Zingiber cassumunar* Roxb)

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บทคัดย่อ

สูตรน้ำมันจระเข้และสมุนไพรไทยสามชนิดคือไพล ขมิ้นชัน และกระชายดำ เป็นสามสูตรใหม่ที่พัฒนาขึ้นเพื่อเป็นทางเลือกของผลิตภัณฑ์ดูแลผิวพรรณ การศึกษาใช้แก๊สโครมาโตกราฟีในการตรวจหาสาระสำคัญในน้ำมันจระเข้ และในน้ำมันจระเข้ผสมสมุนไพร พบว่าในน้ำมันจระเข้มีกรดไขมันกรดปาล์มิติก (palmitic acid) กรดสเตียริก (steric acid) กรดโอเลอิก (oleic acid) และกรดลิโนลิอิก (linoleic acid) ในปริมาณสูงและพบสารซาบินีน (Sabinene) สารซาบินีน ไฮเดรต (sabinene hydrate) และสารไดเมททอกซีฟีนิล บูทาดีอีน (DMPBD)ในปริมาณสูงของทั้งสามสูตร นอกจากนี้ทำการตรวจหาปริมาณสารประกอบฟีนอลิก ฟลาโวนอยด์ การต้านอนุมูลอิสระของสารที่ได้ด้วยวิธี DPPHและการยับยั้งปฏิกิริยาออกซิเดชันของน้ำมันนี้ ผลการศึกษาพบว่าน้ำมันจระเข้ผสมไพลมีปริมาณสารประกอบฟีนอลิกสูงสุด 35.250±0.902 mg GAE/ml รองลงมาเป็นน้ำมันจระเข้ผสมขมิ้นชัน 35.188±1.659 mg GAE/ml และน้ำมันจระเข้กับกระชายดำ 16.406±0.797 mg GAE/ml ส่วนปริมาณสารประกอบฟีนอลิกพบในน้ำมันจระเข้ผสมไพลสูงสุด 35.250±0.902 mg GAE/ml รองลงมาเป็นน้ำมันจระเข้ผสมขมิ้นชัน 35.188±1.659 mg GAE/ml และน้ำมันจระเข้ผสมกระชายดำ 16.406±0.797 mg สำหรับประสิทธิภาพในการต้านอนุมูลอิสระที่วัดด้วยวิธี DPPH พบว่าน้ำมันจระเข้ผสมไพลให้ผลการต้านอนุมูลอิสระดีที่สุด 119.735±1.058 mg TEAC/ml รองลงมาเป็นน้ำมันจระเข้ผสมขมิ้นชัน 64.797±1.481 mg TEAC/ml และน้ำมันจระเข้ผสมกระชายดำ 62.199±0.581 mg TEAC/ml ส่วนการยับยั้งปฏิกิริยาออกซิเดชันของน้ำมันพบว่าในน้ำมันจระเข้ผสมขมิ้นชันร้อยละ 40 น้ำมันจระเข้ผสมไพลมีร้อยละ 22 และน้ำมันจระเข้ผสมกระชายดำร้อยละ 8 ซึ่งผลการทดสอบแสดงให้เห็นว่าสูตรน้ำมันสมุนไพรนี้มีกรดไขมันที่ดีต่อผิวและยังมีสารต้านอนุมูลอิสระสูงด้วย อาจพัฒนาเป็นน้ำมันนวดผลิตภัณฑ์ดูแลผิวพรรณและผลิตภัณฑ์อย่างอื่นได้ในอนาคต

คำสำคัญ: น้ำมันจระเข้ ไพล ขมิ้นชัน กระชายดำ และ สารประกอบฟีนอลิก

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Introduction

There are more than 925 crocodile farms in Thailand because of the market of crocodile skin has a high demand. More than 6,000 crocodiles are sent to slaughterhouses for month, and for each crocodile, the leftover fat is 5-10 kg (>30,000 kg / month). It is desirable to utilize the waste into oil for the development of value-added product. Siamese crocodile oil (*Crocodylus siamensis*) contain both saturated and Unsaturated fatty acids including Palmitic acid, *cis*-9-Oleic acid, Palmitoeic acid, *cis*-9-12-Linoleic acid, and others, which can be used as moisturizer for human skins (Praduptong *et al.,* 2018). In addition, crocodile oil contains high omega 6 especially linoleic acid and omega 9 fatty acid as well as oleic acid. Omega 6 fatty acids (essential fatty acids) retain moisture in skin and reduce skin dryness. Queen Cleopatra of Egypt used crocodile oil as a beauty product for her skin (Janos, 2004). Oil is an intermediate property and a good carrier of substance into the skin. The carrier oils are derived from nature, containing several good fatty acids. Moreover, carrier oils are used as the carrier to deliver substance when they are applied to the skin in massage and aromatherapy. (Rao *et al.,*2013).

Anti-oxidants are potential therapeutic agents to prevent free radical in human body. Usually the sources of anti-oxidants are extracted from herbal plants such as *Curcuma longa*, *Zingiber cassumunar* Roxb and *Kaempferia parviflora.* *Curcuma longa* has biological active compound of exhibit anti-inflammatory, anti-bacteria, antioxidant effects (Araujo and Leon, 2001). *Kaempferia parviflora* has flavonoids content that can exhibited antifungal (Yenjai *et al.,* 2009). And active compound in *Zingiber cassumunar* Roxb against cytokine-induced in cartilage degradation in Rheumatoid arthritis (Chaiwongsa *et al.,* 2013).

Crocodile oil has high fatty acid and all of 3 Thai medicinal plants has high anti-oxidants substance and it could be combined to make more benefit of the new formulation oil to support skincare products.

This study aims to increase economic value of discarded *Siamese crocodile* fat in Thailand and combine with Thai herbal plant to have opportunity to substitute other massage oil in the market and other skincare products.

Materials and Method

Sampling and extraction

Siamese crocodile oil (*Crocodylus siamensis*) was collected from Sri Ayutthaya Rianthong Company Limited which has a Thai crocodile farm located at Nong Khanak, Tha Ruea District, Phra Nakhon Si Ayutthaya Province, Thailand. These fats can be considered as waste from the slaughterhouses. Collected fat are cleaned, packaged and freezed at -20°C (Praduptong *et al*, 2018). They are cut into small pieces (5-10 cm) and oil are extracted in steam pressure machine at 100°C for 45 minutes. Crude oil was obtained using compression machine. The mixture was released to liquid and centrifuged at 6,000 g, 4°C for 10 minutes. Oils were collected in brown bottle glass and stored at 4°C (Praduptong *et al.*, 2018). Dried *Curcuma longa*, *Zingiber cassumunar* Roxb, and *Kaempferia parviflora*, Clove flowers, and Camphor were bought from Samunprai thaprachan co.,ltd, pak klong paseechalern subdistrict, Paseechalern subdistrict, Bangkok.

Massage oil preparations

The method was applied extraction process from herbal oil ancient formula

Curcuma longa 500 g (*Zingiber cassumunar* Roxb, and *Kaempferia parviflora* were used same weight)

Clove flowers	100	g
Camphor	100	g
Crocodile oil	1,000	ml

Gas chromatography analysis

The composition of all essential oils were analyzed using Gas chromatography (Shimizu -2010, Japan) with flame ionization detector (FID). DB-5MS capillary column (J&W Scientific, Folsom, CA) was used to separate two microliter samples. Sample were dissolved in absolute ethanol (1: 100 v / v) and injected into the automatic column. Initially, the column temperature was set to 50°C, then the temperature was programmed to increase from 50 to 220°C at a rate of 4°C/min. The inlet temperature was maintained at 230°C. And injection the sample 0.5 µl at inject port. The carrier gas used helium, flow rate at 1.2 mL/min. The fatty acids composition of crocodile oil compared retention time to Supelco TM 37 component FAME, the chemical composition of mixed oil samples (3 formulas) were compared retention time to terpinene. The composition was reported as a percentage from the area calculation under curve (Manochai *et al*., 2007).

DPPH radical scavenging activity

Chemical and machine preparation used

- DPPH (2,2-diphenyl-1-picryl-hydrazyl, (Sigma, Japan))
- BHT (3,5-di-tert-butyl-4-hydroxytol synonym Butylated hydroxyl toluene (Supelco, USA))
- absolute ethanol
- Spectrophotometer (Shimizu, UV-1601PC)

Control preparation used

- Prepared DPPH at concentration $6x10^{-5}$ M in absolute ethanol.
- Prepared BHT at concentration 100, 75, 50, 25, 12.5, 6.5, 3.125, and 1.562 µg/mL in absolute ethanol.

Control used DPPH solution and BHT solution at 1:1 v/v, shake vigorously and leaved to stand for 30 minutes at room temperature.

Sample preparation used

- Sample prepared at concentration 100, 75, 50, 25, 12.5, 6.5, 3.125, and 1.562 µg/ mL in absolute ethanol.

After that, used the UV-Vis spectrophotometer to measure the adsorption value of control and each sample at 517 nm. Every sample measured 3 times and calculating the percentage of inhibition (%) of free radical DPPH as follows:

% radical scavenging activity = $[(A_0–A_1/A_0]x100$

Where A_0 represents the absorbance sample, while A_1 represents the absorbance of the controlled (Bua-in and Paisooksantivatana, 2009).Then convert % of DPPH radical inhibition to Trolox equivalent, expresses the relation between the concentrations of standard Trolox solutions and % of DPPH inhibition expressed as mg TEAC/ml.

Phenolic contents analysis

The study applied the Folin Ciocalteu method for phenolic content analysis (Velioglu *et al.,* 1998). Prepared sample 0.1 ml and added ethanol until the volume up to 10 ml. Then, pipette this solution 0.5 ml, added ethanol until the volume up to 10 ml. After that added Folin–Ciocalteu 0.5 ml and mixed, incubate for 5 minutes and added 2 mL sodium carbonate concentration (Na_2CO_3) 10% w/v and incubate for more 10 minutes. The absorbance of the mixture oil (3 formulations) was measured by the UV-Vis spectrophotometer at 725 nm. The result compared with standard gallic acid expressed as mg GAE/mL.

Flavonoid contents analysis

The study followed aluminum chloride colorimetric method (Chang and Kim, 2018). Total flavonoid contents used quercetin 5.0 mg

in 10 mL methanol, then prepare standard solution by serial dilution used methanol (5-200 µg/mL). Quercetin standard solution and oil sample separated mixed with 2% aluminum chloride 0.6 mL, mixed it and room temperature incubation for 60 minutes. Blank, standard, and sample measured at 420 nm wavelength with spectrophotometer (Shimizu, UV-1601PC) expressed as mg quercetin equivalent (QE)/ml.

Inhibition Lipid Peroxidation

The study applied the thio-barbitiric acid-reactive species (TBARS) assay (Chang and Kim, 2018). The mixed oil (3 formulations) mixed with 10% Gum arabic and homogenized 16 minutes (AM-8 Nissei, Japan), incubated at 30°C or 70°C, then water bath (JISIO Tech, Korea) and collected 10 µL at 0,7,10 day, diluted the sample to 5, 0.25, 0 mg/mL by (1,1,3,3-tetramethoxypropane) in tube on ice then added 40 µL of 20 nM phosphate buffer (pH 7.0) after that added

- 50 µL of 3% sodium dodecyl sulfate
- 200 µL of 0.1 N HCL
- 30 µL of 10% phosphotungstic acid
- 100 µL of 0.7% of 2-thiobarbituric acid

were added in each tube, the tube boiled at 100°C for 30 minutes in water bath and mixed 400 µL n-butanol and centrifuge at 3,000 rpm for 10 minutes. The supernatants were collected and loaded to 96-well plate. fluorescence intensity measurement used a microplate reader (VICTOR, PerkinElmer, Korea) was read at the excitation wavelengths of 515 nm and sample oil wavelengths of 555 nm.

Results

DPPH radical scavenging activity

The result of the antioxidant effect crocodile oil mixed with Phlai, Turmeric, and Black galingale were showed crocodile oil mixed with Phlai 119.735±1.058, crocodile oil mixed with Turmeric 64.797±1.481 and crocodile oil mixed with Black galingale 62.199±0.581 mg TEAC / ml, recently. The electron donation ability of this essential oil extract was determined using DPPH purple-colored solution bleaching assay. Aqueous the oil extract significantly increases the degree of color change in a dose-dependent manner, indicating significant free radical scavenging activity as shown in table 1.

Table 1 DPPH radical scavenging activity of sample oil

Sample	DPPH (mg TEAC/ml)
Crocodile oil mixed with Phlai	119.735±1.058
Crocodile oil mixed with Turmeric	64.797±1.481
Crocodile oil mixed with Black galingale.	62.199±0.581

Note: TEAC= Trolox equivalent anti-oxidation capacity.

The total phenolic contents

The total phenolic contents of crocodile oil mixed with Phlai, crocodile oil mixed with Turmeric and crocodile oil mixed with Black galingale extract were 35.250±0.902 mg GAE/L, 35.188±1.659 mg GAE/L and 16.406±0.797 mg GAE/L, recently (Table 2).

Table 2 Phenolic contents of sample oil

Sample	Phenolic (mg GAE/ml)
Crocodile oil mixed with Phlai	35.250±0.902
Crocodile oil mixed with Turmeric	35.188±1.659
Crocodile oil mixed with Black galingale.	16.406±0.797

Note: GAE= Gallic acid equivalent.

The total flavonoid contents

The total flavonoid contents of crocodile oil mixed with Turmeric 5.676±0.644 mg QE/mL, crocodile oil mixed with Black galingale 3.281±0.505 mg QE/mL and crocodile oil mixed with Phlai 3.275±1.132mg QE/mL (Table 3).

Table 3 Flavonoid contents of sample oil

Sample	Flavonoid (mg QE/ml)
Crocodile oil with Phlai	3.275±1.132
Crocodile oil with Turmeric	5.676±0.644
Crocodile oil with Black galingale.	3.281±0.505

Note: QE= Quercetin equivalent.

The Inhibition Lipid Peroxidation

The Inhibition Lipid Peroxidation of crocodile oil mixed with Phlai 22%, crocodile oil mixed with Turmeric 40% and crocodile oil mixed with Black galingale 8% (Table 4).

Table 4 Inhibition Lipid Peroxidation of the mixed oil (3 formulations)

Sample	Lipid Peroxidation (% Inhibition)
Crocodile oil mixed with Phlai	22
Crocodile oil mixed with Turmeric	40
Crocodile oil mixed with Black galingale	8

Chemical composition of the mixed oil (3 formulations)

The oil of crocodile oil mixed with Phlai, Turmeric, and Black galingale were analyzed by gas Chromatography. The result showed that the major compositions were containing high level of sabinene, sabinene hydrate, (E)-1-(3,4-dimethoxyphenyl) butadiene as shown in Table 5.

Chemical composition of crocodile oil

Crocodile oil has the main saturated fatty acid composition was Palmitic acid 26.19%, Stearic acid 9.96 %, Myristic acid 9.89%. The main unsaturated fatty acid was Palmitoeic acid 5.13%, *cis*-9-Oleic acid 40.91%, *cis*-9-12-linoleic acid, gamma-Linolenic, alpha- Linolenic, Arachidonic acid, *cis*-5,8,11,14,17-Eicosapentaenoic acid (EPA), and 4,7,10,13,16,19-Decosahexaenoic acid (DHA), in crocodile oil had 20.04, 0.23, 1.13, 0.6, 0.07, 0.18% respectively as shown in Table 6.

Table 5 Percentage of chemical compositions crocodile oil mixed with *Curcuma longa*, *Kaempferia parviflora*, and *Zingiber cassumunar* Roxb

Peak	Chemical compositions	Retention time Std. (min.)	Percentage chemical composition Crocodile oil mixed with		
			C. longa	*K. parviflora*	*Z. cassumunar* Roxb
1	α-thujene	7.70	0.70	0.88	0.53
2	α-pinene	7.98	1.10	1.18	1.13
3	sabinene	9.27	38.50	42.15	33.90
4	β-mycrene	9.45	2.25	2.45	2.20
5	α-terpinene	9.75	1.53	1.70	1.47
6	p-cymene	10.78	2.49	2.88	2.59
7	β-phellanddrene	11.06	1.10	1.15	1.36
8	Y-terpinene	11.31	1.48	1.10	1.64
9	Sabinene hydrate	12.28	5.22	5.81	5.55
10	terpinolene	13.30	1.10	1.24	1.22
11	terpinen	16.94	19.98	17.76	24.36
12	Terpinyl acetate	29.05	2.50	1.72	1.74
13	β-sesquiphellanddrene	31.09	1.28	1.30	1.26
14	DMPBD	32.29	18.30	21.72	20.40
15	unknown	37.55	1.12	1.19	2.18

Table 6 Percentage fatty acid compositions of crocodile oil

Peak	Fatty acid composition	Retention time Std. (min.)	Crocodile oil (%)
1	Lauric acid	13.633	0.29
2	Myristic acid	15.637	0.74
3	Pentadecanoic acid	16.912	0.10
4	Palmitic acid	18.367	23.71
5	Heptadecanoic acid	20.004	0.14
6	Stearic acid	21.842	5.11
7	Arechidic acid	26.219	0.07
8	Myristoeic acid	16.746	0.14
9	Palmitoeic acid	19.518	5.13
10	*cis*-9-Oleic acid	23.075	40.91
11	*cis*-11-Eicosenoic acid	27.654	0.18
12	Nervonic acid	39.826	0.16
13	*cis*-9-12-linoleic acid	25.063	20.74
14	gramma-Linolenic	26.736	0.24
15	alpha- Linolenic	27.703	1.13
16	*cis*-11,14-Eicosadienoic acid	30.107	0.16
17	*cis*-8,11,14-Eicosatrienoic acid	32.059	0.21
18	Arachidonic acid	33.648	0.60
19	cis-5,8,11,14,17-Eicosapentaenoic acid (EPA)	37.381	0.07
20	4,7,10,13,16,19-Decosahexaenoic acid (DHA)	44.307	0.18

Discussion

The crocodile oil, after extraction process, has to be added anti-rancidity substance. Because the fatty acid composition of crocodile oil has high unsaturated fatty acid can be cleaved by free-fatty acid reactions involving molecular oxygen. This reaction causes the release of rancidity. This study used tocopherols (vitamin E) added into the oil for anti-rancidity that it consistent to Hras, A.R. *et al.,*2000 on the topic of comparison of ant oxidative and synergistic effects of rosemary extract with α-tocopherol, ascorbyl palmitate and citric acid in sunflower oil.

The mixture of crocodile oil and 3 Thai herbal plants showed that it has a potential to be used as new healthy oil products. The mixture can be named as natural herb. It has high phenolic contents and high antioxidant activities especially for crocodile oil mixed with Phlai and crocodile oil mixed with Turmeric. However, the mixture has yellow color when applied to skin. On the other hand, crocodile oil mixed with Black galingale oil did not displayed purple color when applied to skin. Crocodile oil contained high concentration of palmitic acids, stearic acids, oleic acids and linoleic acids. These fatty acids are classified as Omega 6 and 9 fatty acids. Omega 6 fatty acids (especially linoleic acids) increase the tenacity of skin moisture and reduce skin dryness which agrees with the paper in Biological and Pharmaceutical Bulletin, Linoleic acid enhanced skin whitening effect (Shigeta *et al.,*2004).

The mixture oil (3 formulas) showed same high Sabinene and sabinene hydrate and DMPBD that it good substances to skin healthy which agrees with the paper in Journal of Essential Oil Research, it supported the treatment of skin diseases (Endris *et al.,* 2016.). In addition, the mixture oil (3 formulas) have high phenolic compounds and flavonoids compounds. phenolic compounds are among the most studied natural antioxidant and pharmaceuticals, flavonoids are a diverse group of polyphenols with antioxidant properties which agrees with the paper of applied to use phenolic and flavonoid to traditional Indian skin care formulation, published in Journal of ethno pharmacology (Biswas *et al.,*2016) .

Data form this study suggest that crocodile oil and Thai medicinal plants can be combined to develop the new formulation of skincare products such as a massage oil, cream, gel and other skin care products.

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