Effects of solvent polarity and acidity on the extraction efficiency and fatty acid compositions of oil from Nile crocodile

Yi Huang, Shengzhao Dong, Yun Liu

Abstract— **Background: Crocodile oil extracted from the fatty tissues of crocodiles is rich in monounsaturated and polyunsaturated fats, which have high economic and medical values. The present study describes and compares the effects of polarity and acid of extraction solvents on the extraction efficiency and fatty acid compositions of crocodile oil extracted by six solvents extraction systems, which are the combinations of three polarity levels (75% CH_2Cl_2, 50% CH_2Cl_2 and 25% CH_2Cl_2) and two acidity levels (non-acidified and acidified).**

Results: The oil extract was saponification with 0.5M KOH before re-suspected by n-hexane, and the fatty acid compositions of lipids was analyzed by gas chromatography-Mass Spectrometer (GC-MS). Analysis of variance (ANOVA) shows that solvent polarity not acidity of six extraction solvent systems has a significant effect on the oil yield. Different solvent systems could extract an obvious different type and amount of fatty acids compositions, which consisted of long carbon chains fatty acids. Among the six solvent systems examined, 50% CH_2Cl_2 + 46.7% methanol without acid was the best for extraction of oils from crocodile fat. DPPH antioxidation test indicated that crocodile oil extracted by solvent systems of 50% CH_2Cl_2 + 46.7% methanol without acid showed higher antioxidation activity (77.5%) than oil (56.0%) extracted by petroleum ether.

Conclusions: The findings in this work are very helpful to screen the extraction solvent systems to extract the oil from crocodile organism.

Index Terms— **Crocodile oil; Solvent extraction; Polarity; Acidified; GC-MS**

I. BACKGROUND

Crocodiles are large aquatic reptiles that live throughout the tropics in Africa, Asia, Americas and Australia, which have high economic and medical values [1]. Crocodile oil extracted from the fatty tissues of crocodiles is rich in monounsaturated and polyunsaturated fats. It has been reported that the fatty acids compositions of oil from crocodiles are consisting of long carbon chains of palmitic (16:0), palmitoleic (16:1 c9), stearic (18:0), oleic (18:1 c9) and linoleic (18:2n-6) acids [2]. Different fatty acid compositions were observed in different type of crocodiles, for instance captive and wild crocodiles, healthy and disease crocodiles [3]. Investigators showed that crocodile oil and meat have many functional activities of antibacterial [4], antifungal [5], anti-inflammatory [6], and wound healing [7]. Therefore, suitable fatty acid components are important for human health since they are the precursors for the biosynthesis of eicosanoids which is considered as an important bio-regulator for many cellular metabolic processes [8]. Furthermore, researchers reported that crocodile oil would be a potential source for biodiesel production, and the fuel product from crocodile oil was found to meet the ASTM specifications of biodiesel concerning kinematic viscosity, sulfur, free and total glycerin, flash point, cloud point, and acid number [9].

Currently, a variety of extraction methods of planty oil or animal fats have been available [10], including steam extraction or hydro-distillation, solvent extraction, and supercritical fluid extraction (SFE). In this work, dichloromethane/methanol mixture solvents extraction is employed to replace n-hexane or petroleum ether as a solven used in the extraction of oi from Nile crocodile. The mixture solvents consist of six solvents systems: system I, acidified 75% dichloromethane (22.5 mL of dichloromethane + 6.5 mL methanol + 1 mL of 2.5N HCl); B, system Π 75% dichloromethane (22.5 mL of dichloromethane + 6.5 mL methanol + 1 mL de-ionic H_2O); system ΙΙΙ, acidified 50% dichloromethane (14.5 mL of dichloromethane + 14.5 mL methanol + 1 mL of 2.5N HCl); system Ⅳ, 50% dichloromethane (14.5 mL of dichloromethane + 14.5 mL methanol + 1 mL of 2.5N H_2O); system Ⅴ, acidified 25% dichloromethane (7.5 mL of dichloromethane + 21.5 mL methanol + 1 mL of 2.5N HCl); system Ⅳ, 25% dichloromethane (7.5 mL of dichloromethane + 21.5 mL methanol + 1 mL of 2.5N H_2O).

The purpose of this work is to qualify the effects of solvent polarity and acidity on the extraction efficiency and fatty acid compositions of oil from Nile crocodile fat. More comparisons of DPPH radical scavenging activities of crocodile oil extracted by dichloromethane/methanol mixture solvents and petroleum ether solvent have also been studied in this study.

II. METHODS

Gas Chromatography- Mass Spectrometer (GC-MS) analysis of crocodile oil

Fatty acids were trans-esterified to form methyl esters (FAME) using 0.5 N KOH in methanol and 14% boron trifluoride-ether solution in methanol. The FAME was quantified using a GC-MS QP 2010 flame ionization GC equipped with a mass spectrometer and a fused silica capillary column, HP-5 (100 m length × 0.25 mm ID × 0.2 μm film thickness). The column temperature was 120–280 °C with the temperature rate of 10 ºC/min. The FAME in hexane (1 μL)

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was manually injected into the column with a split ratio of 100:1. The injection port and detector were both maintained at 250 °C. Hydrogen was used as the carrier gas at 45 psi and nitrogen was the makeup gas. Chromatograms were recorded using N2000 Chromatography Software (Zhejiang, China). The percentage composition was obtained from electronic integration measurements using flame ionization detection [11].

DPPH radical scavenging activity of crocodile oil

1,1-diphenyl-2-picryl-hydrazil (DPPH) radical scavenging activity of extracted crocodile oil was modified according to the method by Dong et al [12]. DPPH with a concentration of 0.04 mM in ethanol was prepared. Then, 1.5 mL of this solution was added to 2 mL of extracted crocodile oil. The mixture was shaken and allowed to keep at 25 °C for 30 min. The absorbance was measured at 517 nm in the UV756CRT UV spectroscope. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The DPPH scavenging rate was calculated with Eq (2):

$$\text{Scavenging rate (\%)} = \frac{A_0 - (A_1 - A_2)}{A_0} \times 100\%$$

Eq. (2)

Where: A_0 was the absorbance of the blank control, A_1 was the absorbance in the presence of the samples and DPPH, A_2 was the absorbance of the samples alone without DPPH.

III. Statistical Analysis

Crocodile oil extracted by six solvent systems was carried out with a 3×2 factorial randomized complete block design (RCBD). Each block was used as a batch containing one replication of each of the six solvent systems. In each batch, the trials were completely randomized, and all of the conditions were maintained stable for each sample, to minimize the within block variance. Two factors were: polarity of the extraction solvents with three different polarity levels (75% dichloromethane in methanol, 50% dichloromethane in methanol, and 25% dichloromethane in methanol) and acidification with two acidification levels (non-acidified and acidified). The six solvent systems were the combination of the two factors and showed in section 2.2. Regression analysis was performed to evaluate the effect of polarity and acidity of extraction solvents on oil yields. Statistical analysis of Variance (ANOVA) and regression analyses were conducted by using IBM SPSS statistics V22 software (SPSS Inc., USA).

IV. Results and Discussion

Effects of polarity and acidification on oil extraction rate

The six solvent systems were the combinations of two factors: polarity of the extraction solvents with three different polarity levels (75% dichloromethane in methanol, 50% dichloromethane in methanol, and 25% dichloromethane in methanol) and acidification with two acidification levels (non-acidified and acidified). The oil extraction rate by six solvents systems was shown in Fig. 1.

Solvent system Π (22.5 mL of dichloromethane + 6.5 mL methanol + 1 mL de-ionic H_2O) was the two most efficient solvent system for crocodile oil extraction with the oil extraction rate of 87±5%. Six solvent systems show different efficiency of oil extraction rate from Nile crocodile, and their order is: system Π > system I > system Ⅳ > system Ⅲ > system Ⅴ > system Ⅵ.

ANOVA analysis in table 1 showed that the oil extraction rate by the six solvent systems resulted in significant differences ($P < 0.0001$). It was also noted that there was remarkable differences ($P < 0.0001$) among solvent systems with different polarities in extracting oil from Nile crocodile fat. However, solvent systems with acidity did not significantly affect the oil extraction efficiency ($P = 0.624 > 0.05$). Namely, there were significantly differ between solvent systems I, Π and solvent systems Ⅲ, Ⅳ and solvent systems Ⅴ, Ⅵ, but there were not significantly differ between solvent systems I and Π or solvent systems Ⅲ and Ⅳ or solvent systems Ⅴ and Ⅵ.

Reis and co-workers [13] compared five different extraction solvents systems (dichloromethane+methanol, chloroform+methanol, acidfied chloroform+methanol, tert-butyl methyl ether+methanol, and hexane+isopropanol）on oil lipid extraction yield. They found that extraction solvents systems of dichloromethane+methanol showed the most effective for the extraction of oil lipid and acidification of solvents had no significance effect on oil extraction. Pérez-Palacios and co-workers [14] also reported that mixture solvents systems of dichloromethane+methanol possessed high extraction efficiency of oil lipids from meat and meat products. Howeever, Lin and Giusti [15] found solvent polarity and acidity had significantly effect on targeting extracted production when they used the combinations solvents of three polarity levels (83% acetonitrile, 80% methanol, and 58% acetonitrile) and two acidity levels (nonacidified and acidified) to extract isoflavones from soybeans. In our work, we demonstrated that solvent polarity showed significant effect on oil extraction yield but acidification had no significant effect. This findings agreed well with that reported by Murphy et al. [16], who found no differernce of isoflavones extraction yield by mixture solvents systeme with or without HCl acidification.

Based on the oil extraction rate by six solvent systems, a polynomial Eq. (3) was obtained from the RCBD design.

$$Y = 1.187 - 1.183X \qquad \text{Eq. (3)}$$

Where: Y is the dependent variable of oil extraction rate, X is the polarity of solvent system.

The *p*-value of model was less than 0.0001 and the coefficient of determination (R2) of Eq. (3) was 91.4%. These values suggested that Eq. (3) was highly significant, and fitted the regression analysis of the data. The predicted values agreed well with the observed or experimental values (Fig. 2).

Fatty acid compositions of extracted oils by GC-MS

Crocodile oil extracted by six solvent systems was analyzed by GC-MS, resulting in the identification of 9 compounds which represented 99% of the oil. The composition of fatty acids from the crocodile oil is shown in Table 2.

From Table 2. It is evident that 12 factty acids were detected and 8 were unsaturated fatty acid. Crocodile oil extracted by six solvent systems was rich in monounsaturated fatty acid and polyunsaturated fatty acids, and among them the main constitutes were C16:1, C18:1, C18:2, C20:1, C20:3 and C20:4. Saturated fatty acids with low concentration

account for the remainder of the oil. Remarkably, some content of heptadecanoic acid (6.6%) and 10-heptadecen-8-ynoic acid (0.61%) were firstly obtained among the crocodile oil extracted by solvent system Ⅲ. oxiraneundecanoic acid of 0.45% percentage was also observed in the crocodile oil by solvent system Ⅳ. Buthelezi and co-workers [17] detected sixteen fatty acids in crocodile oil with oleic, palmitic and linoleic acid being the major component of the oil. And they demonstrated that crocodile oil showed antimicrobialandanti-inflammatoryactivities. Hoffman and co-workers [18] reported that the fatty acid compositions of Nile crocodile consisted of 37.7% saturated, 51.1% monounsaturated and 10.7% polyunsaturated. Oleic acid was predominant (43.1%), whilst palmitic acid (25.4%), stearic acid (9.9%) and linoleic acid (9.1%) were also present in high concentrations.

Antioxidant activity of extracted oils

Crocodile oil samples was extracted by petroleum ether and solvent system Ⅳ, respectively. DPPH radical scavenging activity of the two oil samples was evaluated and the results are shown in Fig. 3.

It was obviously seen from Fig. 3 that the highest DPPH radical scavenging value of crocodile oil by solvent system Ⅱ was 68% and the adding EC_{50} value of oil was 0.17 mL, while the highest DPPH radical scavenging value of crocodile oil by petroleum ether was 56% and the adding EC_{50} value of oil was 0.19 mL. It indicated that crocodile oil extracted by different solvents would show different antioxidant activity. Santos and co-workers [19] demonstrated that the fatty acid profile from gabiroba seeds extracted by different solvens (hexane, chloroform, ethyl acetate, and alcohol) showed different antioxidant activity, and the ethanol extract showed the highest antioxidant potential. Tavakoli and co-workers [20] revealed the DPPH radicals antioxidant activity of the extracts from *Ficaria kochii* by different polarity solvents systems, and H_2O proved to be the most efficient solvent for the extraction of antioxidants, as the H_2O extract contained the highest amount of phenolic compounds (2.78±0.23 GAE/g dry matter) and also exhibited the strongest antioxidant capacity in all the assays used, then the order of scavenging effect was H_2O > MeOH > EtOH > acetone. All these researches indicated that the extracts by different solvents systems showed different antioxidant activities.

V. Experimental

Chemicals

Tissue samples of Nile crocodiles were collected from the Wuhan crocodile farm, Wuhan, Hubei province, China. Chloroform, dichloromethane, methanol, sodium chloride of analytical reagents were all purchased from Beijing Chemical Company, Beijing, China. Boron trifluoride-ether solution, potassium hydroxide, 1,1-Diphenyl-2-picrylhydrazyl radical 2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) were bought from China Pharmaceutical Group, Shanghai, China.

Solvents systems

Six solvent systems were examined in this work. They were as follows: system Ⅰ, acidified 75% dichloromethane (22.5 mL of dichloromethane + 6.5 mL methanol + 1 mL of 2.5N HCl); B, system Ⅱ 75% dichloromethane (22.5 mL of dichloromethane + 6.5 mL methanol + 1 mL de-ionic H_2O); system Ⅲ, acidified 50% dichloromethane (14.5 mL of dichloromethane + 14.5 mL methanol + 1 mL of 2.5N HCl); system Ⅳ, 50% dichloromethane (14.5 mL of dichloromethane + 14.5 mL methanol + 1 mL of 2.5N H_2O); system Ⅴ, acidified 25% dichloromethane (7.5 mL of dichloromethane + 21.5 mL methanol + 1 mL of 2.5N HCl); system Ⅳ, 25% dichloromethane (7.5 mL of dichloromethane + 21.5 mL methanol + 1 mL of 2.5N H_2O).

Crocodile oil extraction

The tissue of Nile crocodile fat was ground for 1.5 min at intervals of 15 s using a LT-BL01P Electric Kitchen Blender (local market, Beijing, China). Two grams of the grounded crocodile fat was mixed with 40 mL of one of the six solvents for 2 h at room temperature (25 ºC). The mixture was then vacuum filtered through Whatman no. 41 filter paper using a Buchner funnel. The filtrate containing lipids was evaporated under vacuum in a rotary evaporator, and the oil extract was obtained. Extractions using each of the six solvents were carried out in triplicate. The oil extraction rate was calculated with the following Eq. (1):

$$\text{Oil extraction rate (\%)} = \frac{W_{oil}}{W_m} \times 100\% \quad \text{Eq (1)}$$

Where: W_{oil} was the mass weight of extracted oil, W_m was the mass weight of crocodile fat material.

VI. Conclusions

The polarity of the solvent systems was desirable for oil extraction efficiency from Nile crocodile, while acidification of the extraction solvent did not favor oil extraction. Among the six solvent systems examined in this study, 50% dichloromethane (14.5 mL of dichloromethane + 14.5 mL methanol + 1 mL of 2.5N H_2O) without acidification was the best solvent for oil extraction from Nile crocodile, since it yielded the widest overview of fatty acid components among crocodile oil. With regard to DPPH antioxidant activity, the crocodile oil extracted by solvent system Ⅳ was better against the oil sample extracted by petroleum ether. In summary, solvent polarity not acidification of extraction systems have signification effect on oil yield, and the extracts by different extraction solvents showed different antioxidant activities.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

Yun Liu made substantial contributions to conception and design, acquisition of data and revised the manuscript; Yi Huang was involved in drafting the manuscript; Shengzhao Dong finished the expeirments, analyzed and interpreted data.

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Figure captions

Figure 1 The oil extraction rate by six solvents systems
Figure 2 Regression analysis of the data
Fig. 3 DPPH radical scavenging activity of the two extracted oil samples

Table 1 ANOVA analysis for six solvents systems extracting crocodile oil

Source	Sum squares	Df	Mean square	F-values	*P*-value
Model	M	5	0.244	75.496	0.0001
Intercept	6.389	1	6.389	1980.132	0.0001
Acidification	0.001	1	0.001	0.254	0.624
Polarity	1.206	2	0.603	186.93	0.0001
Interaction	0.011	2	0.005	1.684	0.227
Error	0.039	12	0.003		
Total	7.646	18			
Corrected Total	1.257	17			

1. R^2 = .969 (Adjusted R^2 = .956); 2. d.f.: degree of freedom

Table 2 Fatty acid compositions of Crocodile oil extracted by different solvent systems with different polarity and acidification

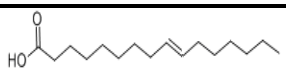
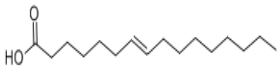
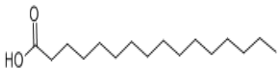
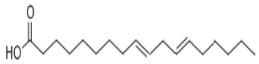
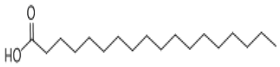
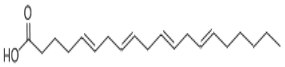
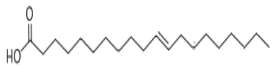
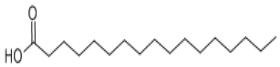
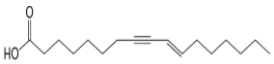
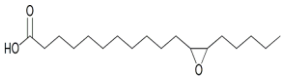
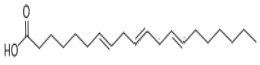
Fatty acid compositions	Molecular formula	Structure	Six solvent systems					
			I	II	III	IV	V	VI
7-Hexadecanoic acid	$C_{16}H_{30}O_2$		-	-	-	0.30	-	0.19
9-Hexadecanoic acid	$C_{16}H_{30}O_2$		5.55	5.36	5.59	5.72	5.58	5.78
Hexadecanoic acid	$C_{16}H_{32}O_2$		24.86	25.21	25.73	27.46	25.2	24.8
9,12-Octadecadi enoic acid	$C_{18}H_{32}O_2$		14.02	14.73	14.58	13.25	13.92	13.65
9-octadecenoic acid	$C_{18}H_{34}O_2$		48.84	48.19	46.89	46.52	49.07	49.26
octadecanoic acid	$C_{18}H_{36}O_2$		5.65	6.5	-	6.11	5.78	5.45
5,8,11,14-Eicosa tetraenoic acid	$C_{20}H_{32}O_2$		0.50	-	-	0.18	-	0.87
11-Eicosenoic acid	$C_{20}H_{38}O_2$		0.58	-	-	-	-	-
heptadecanoic acid	$C_{17}H_{34}O_2$		-	-	6.60	-	-	-
10-heptadecen-8-ynoic acid	$C_{17}H_{28}O_2$		-	-	0.61	-	-	-
oxiraneundecano ic acid	$C_{18}H_{34}O_3$		-	-	-	0.45	-	-
7,10,13-eicosatri enoic	$C_{20}H_{34}O_2$		-	-	-	-	0.45	-

Fig. 1

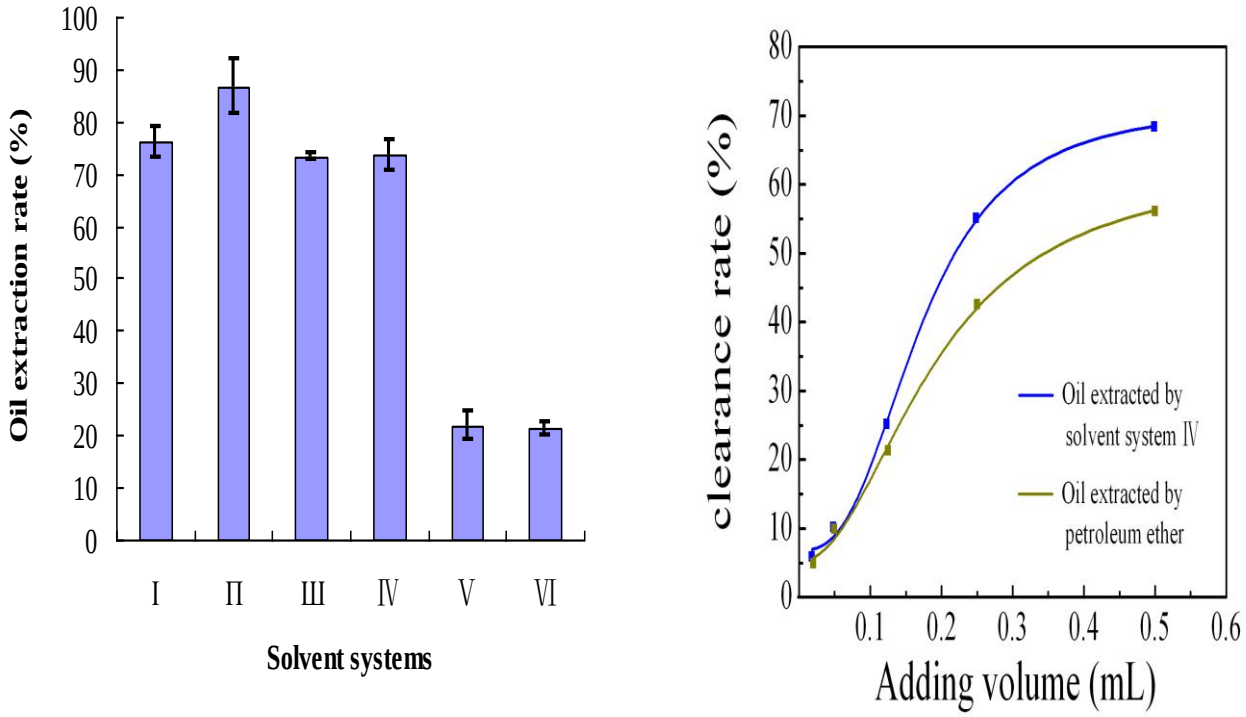

Oil extraction rate (%)
100
90
80
70
60
50
40
30
20
10
0
I
II
III
IV
V
VI
Solvent systems
clearance rate (%)
80
70
60
50
40
30
20
10
0
0.1
0.2
0.3
0.4
0.5
0.6
Adding volume (mL)
Oil extracted by solvent system IV
Oil extracted by petroleum ether

Fig. 2

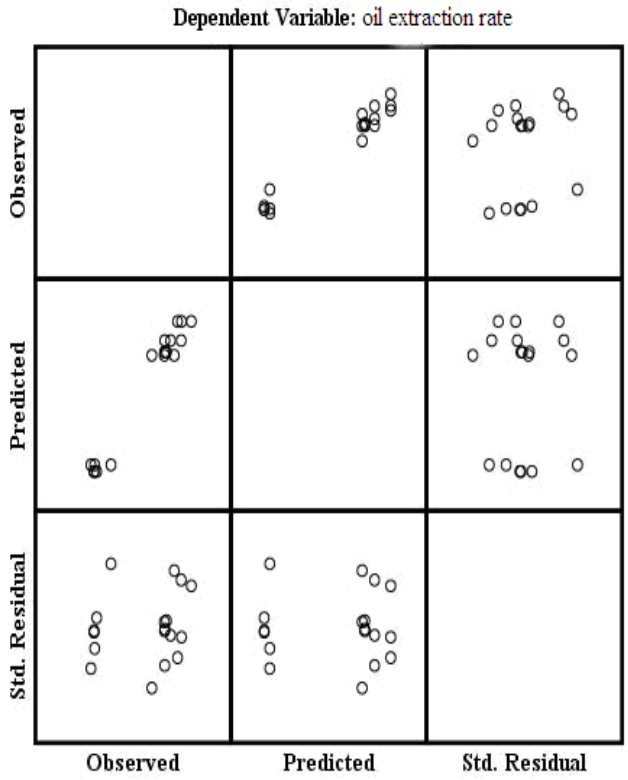

Dependent Variable: oil extraction rate
Observed
Predicted
Std. Residual
Observed
Predicted
Std. Residual

Fig. 3