EXTRACTION OF TAMANU OIL FROM *Calophyllum inophyllum* L. SEEDS BY ULTRASOUND-ASSISTED METHOD AND TESTING WOUND CARE TREATMENT

Lu Thi Mong Thy[1], Truong Le Khoi[2], Tran Do Dat[2], Phan Le Thao My[2], Vuong Hoai Thanh[2], Nguyen Duc Viet[2], Huynh Khanh Duy[2], Hoang Minh Nam[2], Mai Thanh Phong[2], Nguyen Huu Hieu[2,*]

[1]*Ho Chi Minh City University of Food Industry*

[2]*Ho Chi Minh City University of Technology - VNUHCM*

* Email: *nhhieubk@hcmut.edu.vn*

Received: 2 May 2020; Accepted: 24 July 2020

ABSTRACT

Tamanu oil was extracted from *Calophyllum inophyllum* Linn. by ultrasound-assisted method. Four solvents including *n*-hexane, ethanol, petroleum ether, and ethyl acetate were investigated. Response surface methodology was employed to investigate effects of time, temperature, and solvent-to-material ratio on the extraction yield. Tamanu oil extraction yield was achieved of 55.15% under conditions including time of 23 minutes, temperature of 42 °C, solvent-to-material ratio of 26 to 1 (v/w) mL/g. In addition, better physicochemical indices and similar fatty acid components compared to that of commercial oil were reported indicating the quality of the extracted oil. Wound healing abilities of the extracted tamanu oil was also tested on *Swiss albino* mice and a commercial product showing the potential agent for wound care treatment of tamanu oil based on the recorded better wound healing ability.

Keywords: Tamanu, ultrasound, response surface methodology, Box-Hunter, wound healing.

1. INTRODUCTION

The application of natural compounds for pharmaceutical and cosmetic uses from plants has been globally employed from over millennia. With a range of biological activities such as anticancer, antioxidant, antivirus, anti-inflammation, UV protection, and wound healing, *C. inophyllum* has been studied in many parts of the world [1, 2]. According to previous studies, it has been revealed that tamanu oil contains a great variety of components including free fatty acids, glycerides, sterols, triterpenoids, flavonoids, phenols, polyphenols, phospholipids, and unsaponifiable content [3, 4]. Because of the pharmacological properties, particularly for skin problems treatment including burns, dermatoses, skin allergies, acne, and open wounds, tamanu oil has been gained scientific interest from many researchers [5, 6]. In addition, the oil has been used externally for rheumatism, gout, joint pains, arthritis, and bruise [7]. As a result, several techniques have been utilized for tamanu oil extraction.

In general, solid-liquid extraction technology these days consists of traditional and modern methods. Conventional methods such as maceration and Soxhlet extraction have been known because of its simplification. However, these methods have several drawbacks such as the large quantity of solvent use and long extraction time [8]. It has been reported that applying mechanical pressing with a screw press for tamanu oil extraction could lead to several disadvantages including higher energy consumption, contamination between tamanu oil and

by-products as well as lower extraction yield [4, 9]. To overcome these issues of conventional methods, the development of modern approaches has been studied. Among numerous modern extraction techniques such as enzymatic extraction, microwave extraction, ultrasound-assisted extraction (UAE) has been developed as a green technology to enhance extraction yield when extracting oil from plants [10]. The method of using ultrasound has been cited with many advantages including less extraction time, low extraction temperature, and high extraction efficacy [11]. For elucidating ultrasound enhancement mechanism, cavitation bubbles has been mainly used as an important reason, which could contribute to the break of cell walls and increase interaction between solvents and target compounds leading to the acceleration of extraction yield [10].

This study aims to employ highly efficient ultrasound-assisted extraction of tamanu oil from seed kernel. Response surface methodology (RSM) with Box-Hunter design was used to investigate the simultaneous effects of multi-factors encompassing extraction time, temperature, and solvent-to-material ratio. The optimum combination of those factors was then determined to ensure optimum extraction efficiencies [12, 13]. The extracted oil was evaluated in terms of physicochemical indexes, composition, and wound healing activity.

2. EXPERIMENTAL

2.1. Materials

Tamanu seeds were purchased from Tan Phat Herb Company, Ho Chi Minh City, Vietnam. Seeds were washed, peeled off, sliced, and sun-dried in 2-3 days prior to grinding process. Petroleum ether, ethyl acetate, and *n*-hexane were purchased from Xilong Chemical, China. Ethanol was obtained from Vina Chemsol, Vietnam. All chemicals were used as received without further purification.

2.2. Selection of the appropriate solvent for extraction procedure

5.0 g of ground seeds was dispersed in 100 mL of solvent. Effects of four types of solvent including 99.5% ethanol, ≥ 95% *n*-hexane, 30-60% petroleum ether, and ≥ 99.5% ethyl acetate were investigated. The mixture was sonicated at 40 ^{o}C in 20 minutes and filtered to obtain extract. Solvent was removed using a vacuum rotary evaporator.

2.3. Ultrasound-assisted extraction

5.0 g of ground seeds was dispersed in solvent and sonicated in the ultrasonic bath with the minimum and maximum power at 120 W, and 1200 W, respectively. The mixture was centrifuged at 2000 rpm in 10 minutes and filtered to obtain extract. Solvent was removed using a vacuum rotary evaporator.

2.4. Effects of factors on extraction yield

Effects of three factors including time, temperature, and solvent-to-material ratio on extraction yield were investigated using (RSM) with Box-Hunter design. Variables including Z1 (time, min), Z2 (temperature, °C), and Z3 (solvent-to-material ratio, mL/g) were encoded at -1 level as 15 for Z1, 35 for Z2, and 14 to 1 for Z3; 0 level as 20 for Z1, 40 for Z2, and 20 to 1 for Z3; and 1 level as 25 for Z1, 45 for Z2, and 26 to 1 for Z3. Experiments were conducted with 20 runs as shown in Table 1. Analysis of variance was used to evaluate the significant

difference with $p < 0.05$. Design-Expert 11 was used to generate the Box-Hunter design and build up regression model.

2.5. Oil extraction yield

Oil extraction yield was calculated by the following equation (1).

$$Y = \frac{M_1}{M_o} 100\% \quad (1)$$

where Y is the oil extraction yield (%), M_o, M_1 are the weight of oil in seed kernel and oil extracted, respectively.

2.6. Physicochemical analysis

Acid value (W_A) was identified via equation (2) based on the standard ISO 660:2020 [14].

$$W_A = \frac{56.1CV}{m} \quad (2)$$

where C (mol/L) is the concentration of KOH (mol/L), V (mL) is the volume of KOH solution, and m (g) is the weight of sample.

Peroxide value (W_P) was identified by equation (3) based on the standard ISO 3960: 2017 [15].

$$W_P = \frac{0.0002538(V_1 - V_2)}{m} 100 \quad (3)$$

where V_1 and V_2 are the volume of $Na_2S_2O_3$ solutions, 0.002 N, titrating the sample and negative control, respectively, m (g) is the weight of sample.

Iodine value was identified by equation (4) based on the standard ISO 3961:2013 [16].

$$W_t = \frac{12.69(V_1 - V_2)c}{m} \quad (4)$$

where V_1 and V_2 (mL) are the volume of $Na_2S_2O_3$ solutions used for negative control and sample, respectively, c (mol/mL) is the concentration of $Na_2S_2O_3$ solution, and m (g) is the weight of sample.

Saponification value was identified by equation (5) based on the following equation ISO 3657:2013 [17].

$$l_s = \frac{(V_o - V_1)56.1c}{m} \quad (5)$$

where V_o and V_1 (mL) are the volume of HCl solutions used for negative control and sample, respectively, c (mol/L) is the concentration of HCl solution, and m (g) is the weight of sample.

2.7. Fatty acid composition

Fatty acid composition of tamanu oil was analysed by gas chromatography (Thermo, USA) with column DB-FFAP (30m×0.25mm×0.25µm), DBPX 70, split 1/25. The flow rate of helium was 1.2 mL/min, pressure of 12-14 psi. Temperature program was set up as column temperature 100 °C, increase speed of 7 °C/min to 230 °C/min and keep in 15 min. Temperature of injector and FID was 250 °C.

2.8. Wound healing test

Experiments were conducted at Department of Pharmacology, Faculty of Pharmacy, HCMC Medicine and Pharmacy University. Male *Swiss albino* mice aged 7-8 week old, weight of 25 ± 3 g was provided by Nha Trang Institute of Vaccines and Biological Medical. Mice were adapted to experimental environment in 5 days. Cages with size of 25×35×15 cm were used to keep mice (6 mice/cage). Food and water were provided during experimental period. Mice were divided into 5 groups with 6 mice/group as follows: normal group received no wound created, negative control group was treated with normal saline, positive control group was treated with povidone iodine 10%, tamanu oil group was treated with extracted tamanu oil, and commercial oil group was treated with commercial tamanu oil. The process was described elsewhere [6, 18]. Briefly, wound with 2.5 cm length and 1 mm depth was created on the back of mouse. Mice were treated with 20 µL of tested solution once a day. Weight of mice, wound status, wound size, and spleen index were recorded. The number of white blood cells in heart blood was determined in day 8 using Neubauer chamber. Statistics were expressed in mean ± standard error of mean. All experiments were approved by Department of Pharmacology of the Ho Chi Minh City University of Medicine and Pharmacy.

3. RESULTS AND DISCUSSION

3.1. Selection of the appropriate solvent for extraction procedure

Effect of various solvents on oil yield is shown in Figure 1. Oil extraction using *n*-hexane gave the highest yield of 56.8%. This result could be explained due to the fact that tamanu oil primarily includes non-polarized compounds as courmarin, xanthone, and fatty acids [19]. Thus, non-polarized solvent as *n*-hexane could well dissolve these substances [20-22]. Therefore, in following experiments, *n*-hexane was chosen to extract tamanu oil.

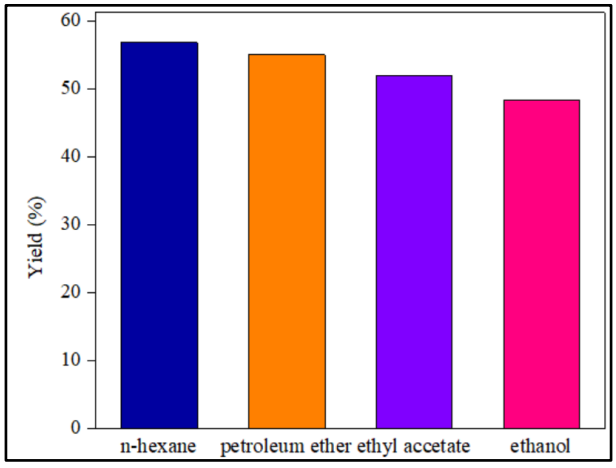

Figure 1. Effect of different solvents on oil yield

3.2. Condition of extraction

The yield of extraction according to experimental design based on three factors: time (Z1), temperature (Z2), solvent to material ratio (Z3) was shown in Table 1.

Table 1. Box-Hunter design and the response for extraction yield of tamanu oil

Run	Z1 (min)	Z2 (°C)	Z3 (mL/g)	Yield (%)
1	25	45	26	56.04
2	15	45	26	53.50
3	25	35	26	52.91
4	15	35	26	50.00
5	25	45	14	54.93
6	15	45	14	52.02
7	25	35	14	52.96
8	15	35	14	50.03
9	28.41	40	20	56.50
10	11.59	40	20	52.95
11	20	48.41	20	52.91
12	20	31.59	20	48.00
13	20	40	30.09	54.93
14	20	40	9.91	52.97
15	20	40	20	56.49
16	20	40	20	56.50
17	20	40	20	56.44
18	20	40	20	56.00
19	20	40	20	56.74
20	20	40	20	55.50

In Table 1, the result showed that extraction yield depended on affect factors and valued at 48.00-56.74%. Based on regression analysis, the fit of model was assessed by ANOVA analysis as shown in Table 2.

Table 2. Results of ANOVA analysis

Factors	Coefficient	Standard error coefficient	F value	p-value Prob > F
Z_0	0.5628	0.0017	75.74	< 0.0001
Z_1	0.0126	0.0011	123.96	< 0.0001
Z_2	0.0138	0.0011	147.81	< 0.0001
Z_3	0.0043	0.0011	14.03	0.0038
Z_1Z_2	-0.0005	0.0015	0.1080	0.7492
Z_1Z_3	-0.0005	0.0015	0.1080	0.7492
Z_2Z_3	0.0033	0.0015	5.06	0.0482
${Z_1}^2$	-0.0056	0.0011	25.62	0.0005
${Z_2}^2$	-0.0207	0.0011	350.57	< 0.0001
${Z_3}^2$	-0.0083	0.0011	56.88	< 0.0001

The p-value Prob related to the F-test (Fisher test) in Table 2 was less than 0.05 ($p < 0.0001$) showed that the compatibility of regression equation with experiment which has statistical level of confidence. Table 2 pointed out that two interaction coefficients namely Z_1Z_2 and Z_1Z_3 were un-confidence. The linear regression equation with the coding variable presented in the equation (6):

$$Y=0.5628+0.0126Z_1+0.0138Z_2+0.0043Z_3-0.0033Z_2Z_3-0.0056Z_1^2-0.0207Z_2^2-0.0083Z_3^2 \quad (6)$$

Equation (6) was transformed into the linear regression equation with the real variable as in equation (7):

$$Y=-1.05174+0.012583Z_1+0.067135Z_2+0.005844Z_3-0.000111Z_2Z_3-0.000224Z_1^2-0.000828Z_2^2-0.000231Z_3^2 \quad (7)$$

The results of the analysis about the fit and significance of the model compare to empirical data were shown in Table 3.

Table 3. Statistics of tamanu oil model

Parameters	Value	Parameters	Value
Standard deviation	0.0042	R^2	0.9855
Mean (SD)	0.5392	R^2 adjusted	0.9725
Coefficient of variation (%)	0.7781	R^2 predicted	0.9414

The results analysis in Table 3 showed that the value of $R^2 = 0.9855$ was 1.45% of the variable sum not explained in the model. The correlation coefficient R^2 predicted = 0.9725 demonstrated the yield of tamanu oil approximately the predicted value of the model.

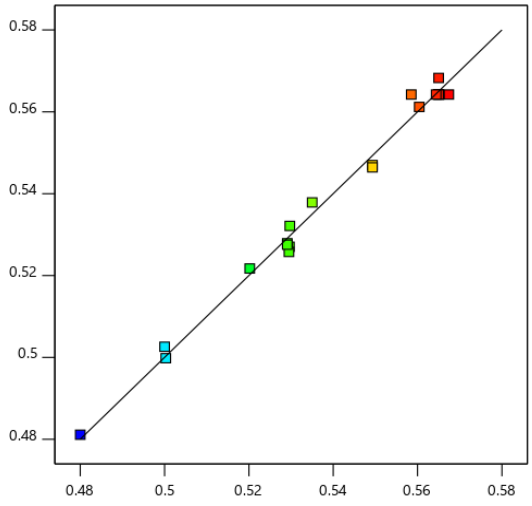

Figure 2. The yield of the extraction by experiment and simulation

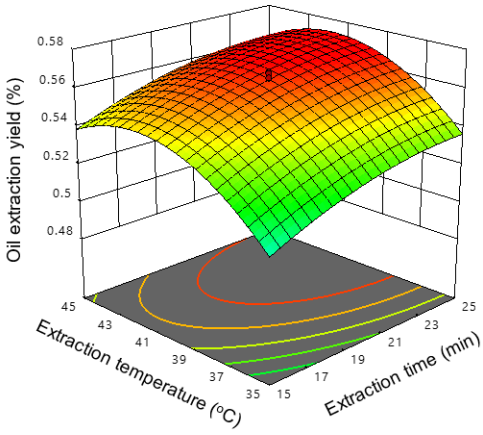

Figure 3. The effect of extraction temperature and extraction time on tamanu oil extraction yield

Based on the chart in Figure 2, the experimental values with three factors encompassing extraction time, temperature and material to solvent ratio were different from the simulations,

although the data points on the chart were close to the line with high correlation coefficients, indicating compatibility. The Box-Hunter model indicated the appropriate extraction yield was predicted 56.86% at time of 23 minutes, temperature of 42 °C, and solvent-to-material ratio of 26 to 1 (v/w) mL/g. After conducting experimental tests based on the above conditions, the yield obtained 55.15%, showing the difference was less than 5%. This indicated that the model was accurate and reasonable.

In addition to the impact of each factor, the extraction yield was also affected by pairs of factors. The effect of each pair of factors on the extraction yield was shown in Figure 3, Figure 4, and Figure 5.

3.2.1 Extraction temperature and extraction time

Figure 3 gives information of the effect of extraction temperature ranging from 35 to 45 ^{o}C and extraction time investigating from 15 to 25 minutes on tamanu oil extraction yield. It is clear that the optimal conditions for obtaining tamanu oil were at 41 ^{o}C during 21 minutes in ultrasonic bath. In accordance with other studies, it has been posited that high temperature could increase cavitation due to a decrease in the surface tension decrease and viscosity of solvent, resulting in the improvement of mass transfer kinetics and the interactions between solvent and target components [23, 24]. Besides, prolonged extraction time could raise tamanu oil content because it provides sufficient time for acoustic cavitation effect on the surface of materials that strongly contributes to the better penetration of solvents into materials to easily dissolve interest constituents [25]. However, temperature acceleration could lead to tamanu oil content reduction due to the relationship between temperature and vapor pressure, which was the primary cause of the target compounds damage and extraction efficacy decrease [11]. Therefore, the reduction of tamanu oil could be observed.

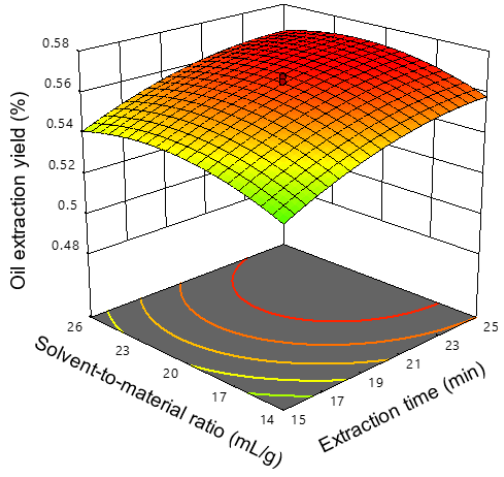

Figure 4. The effect of solvent-to-material ratio and extraction time on tamanu oil extraction yield

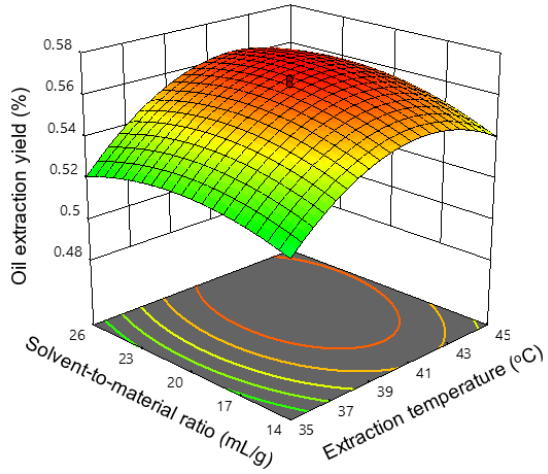

Figure 5. The effect of solvent-to-material ratio and extraction temperature on tamanu oil extraction yield

3.2.2. Solvent-to-material ration and extraction time

Figure 4 presents the relationship between solvent-to-material ratio and extraction time on tamanu oil extraction. The range of solvent-to-material ratio from 14 to 1 (v/w) to 26 to 1 (v/w) mL/g

and extraction time from 15 to 25 minutes was conducted. The highest tamanu oil content could be obtained when the solvent-to-material ratio was of 20 to 1 (v/w) mL/g and time of 21 minutes. It has been cited that an adequate ratio between solvent and material could support the occurrence of diffusion scenario that significantly increases tamanu oil content during extraction process [26]. However, with higher increase in the solvent-to-material ratio, tamanu oil content would not be accelerated since the distance of diffusion from solvent to interior matrix could be prolonged that needs long extraction time to attain the optimal content [27]. Thus, the result was in agreement with previous studies [28].

3.2.3. Solvent-to-material ratio and extraction temperature

Figure 5 illustrates the effect of solvent-to-material with its range from 14 to 1 (v/w) to 26 to 1 (v/w) mL/g and extraction temperature varied between 35 and 45 ^{o}C on tamanu oil extraction. The optimal condition from the Figure 5 was solvent-to-material ratio of 20 to 1 (v/w) mL/g and extraction temperature of 41 ^{o}C. This phenomenon could be explained by a decrease in viscosity and an increase in solubility of solvents when raising temperature and using appropriate solvent-to-material ratio [28]. In contrast, high temperatures could be the main reason of molecular decomposition leading to tamanu oil content reduction [29]. Therefore, the observation could be persistent with preliminary research [30].

3.3. Physicochemical properties

Results of qualitative analysis of tamanu oil obtained under optimal conditions are presented in Table 4. The acid value of tamanu oil is significantly lower than that of commercial oil implying tamanu oil is more difficult to be oxidized and easier to be stored. This result could be explained that commercial oil is produced by physical pressing, which creates great friction and causes oxidation to happen, leading to higher acid value [31]. Tamanu oil has higher peroxide and iodine value, indicating the higher number of unsaturated fatty acid. This may result from the increasing temperature during sonication process oxidized tamanu oil [32, 33]. Saponification value describes the average molecular weight of fatty acids in oil [34, 35]. These values for commercial and tamanu oil are quite similar.

Table 4. Physicochemical indexes of commercial and tamanu oil

Samples	Acid value	Peroxide value	Iodine value	Saponification value
	mg KOH/g	m_{eq} O_2/kg	g I_2/100g	mg KOH/g
Commercial oil	74.40	3.87	113.60	202.00
Tamanu oil	8.42	10.40	9.90	193.50

3.4. Fatty acid composition

The composition and content of fatty acids in tamanu and commercial oil are presented in Table 5. Tamanu oil has 13 fatty acids including 6 saturated fatty acids and 7 unsaturated fatty acids with similar contents to commercial oil.

Table 5. Fatty acid composition of extracted tamanu and commercial oil

Components			Content (%)	
			Tamanu oil	Commercial oil
Saturated fatty acids	Myristic acid	C14:0	0.02	0.02
	Palmitic acid	C16:0	13.23	12.89
	Stearic acid	C18:0	14.77	14.03
	Arachidic acid	C20:0	0.79	0.8
	Behenic acid	C21:0	0.24	0.27
	Lignoceric acid	C24:0	0.06	0.07
Unsaturated fatty acids	Oleic acid	C18:1	38.43	38.86
	Linoleic acid	C18:2	31.68	31.82
	Linolenic acid	C18:3	0.24	0.27
	Eicosenic acid	C20:1	0.18	0.21
Omega	Omega 3		0.23	0.37
	Omega 6		31.56	31.85
	Omega 9		38.63	39.16
Total			99.54	99.46

3.5. Wound healing activity

Wound healing activities of tamanu oil and commercial oil are shown in Table 6. After 5 days, most of wounds closed. In 7th day, complete closure of wounds was recorded in some mice as shown in Figure 6. In day 8, the percentage of mice having complete closed wound was from 33.3 to 66.7%, as a result, experiments were ended in 8th day.

Table 6. Size of un-healing wound and wound healing rate

Samples		Negative control	Positive control	Extracted tamanu oil	Commercial oil
Size of non-healing wound (mm)	Day 1	26.7 ± 0.4	25.0 ± 0.5	26.0 ± 0.6	25.0 ± 1.0
	Day 5	25.3 ± 0.4	21.5 ± 1.8*	13.5 ± 3.3**	22.7 ± 0.8*
	Day 7	21.2 ± 1.0	10.0 ± 2.4**	5.2 ± 2.1**	12.8 ± 2.3*
	Day 8	13.3 ± 1.2	6.5 ± 2.2*	2.2 ± 1.4**	5.3 ± 3.1
Healing wound rate (%)	Day 5	5.28 ± 1.15	13.42 ± 7.94	48.41 ± 12.87**	9.09 ± 2.25
	Day 7	20.42 ± 4.28	60.54 ± 9.71*	80.16 ± 8.44**	48.47 ± 9.12
	Day 8	50.32 ± 4.19	74.40 ± 8.94*	91.68 ± 5.27**	78.43 ± 12.15

(*$p < 0.05$, **$p < 0.01$ significant difference compared with negative control)

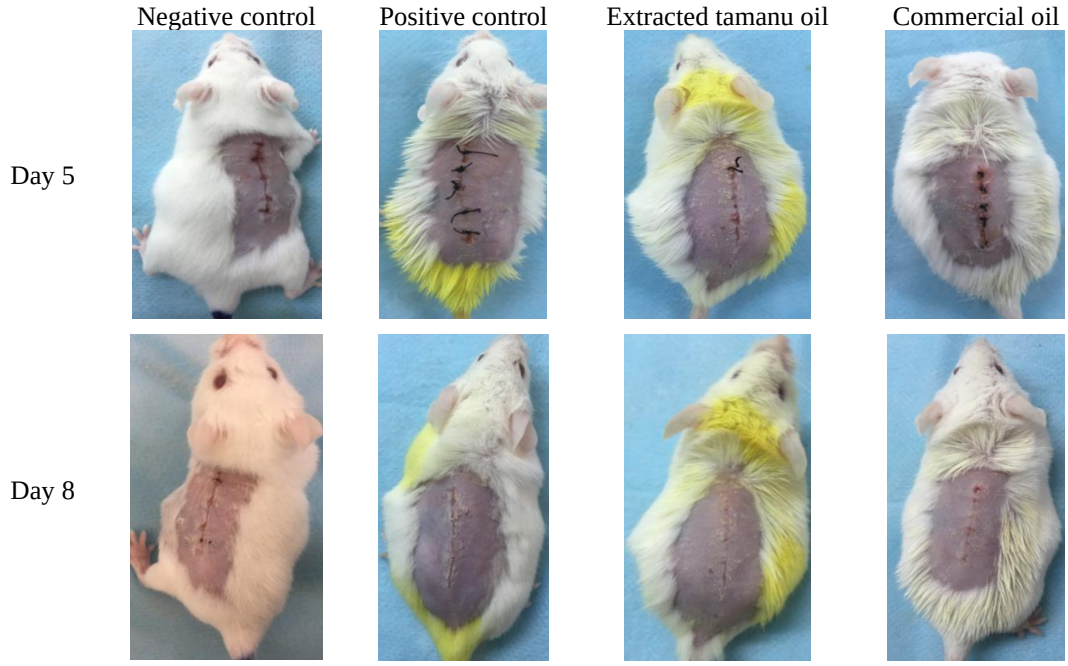

Figure 6. Wound observation in macro-level

Table 7 shows the body weight and spleen index of mice group in day 8. The spleen index indicates the inflammatory level of wound since spleen creates immune cells in the white pulp. When pathogens are detected, the white pulp increases in size, thus increasing spleen weight [16, 17]. As can be seen, the body weight of negative group was lower, but the spleen index was higher than other groups. These results showed the effectiveness of treating wound with positive control, extracted tamanu oil, and commercial oil.

Table 7. Spleen index

Group	Body weight (g)	Spleen weight (mg)	Spleen index (mg/g)
Normal	30.63 ± 2.80	131.5 ± 10.4	4.409 ± 0.384
Negative control	26.10 ± 2.78	132.0 ± 21.1	4.980 ± 0.640
Positive control	27.48 ± 1.16	126.5 ± 23.7	4.535 ± 0.797
Extracted tamanu oil	23.10 ± 1.57	108.9 ± 25.1	4.490 ± 0.756
Commercial oil	27.77 ± 1.93	131.6 ± 15.7	4.686 ± 0.296

Table 8 shows the number of white blood cells in blood flowing through heart in day 8. The number of white blood cells in negative group doubled that of normal group. This result was consistent with the observed inflammatory results. Other groups had similar white blood cell amount to normal group.

Table 8. Number of white blood cells

Groups	White blood cell (cell/mm^3)
Normal	6107.5 ± 463.0
Negative control	11716.3 ± 1236.6**
Positive control	6772.5 ± 1216.0*
Extracted tamanu oil	7227.5 ± 306.0*
Commercial oil	7638.8 ± 788.5*

(**$p < 0.01$ significant difference to normal group, *$p < 0.05$ significant difference to negative control)

4. CONCLUSIONS

Tamanu oil extraction with ultrasound-assisted extraction in *n*-hexane resulted in the highest yield compared to those obtained by ethanol, petroleum ether, and ethyl acetate extraction. The optimal yield could be achieved at 55.15% when extracted at 42 ^{o}C with time of 23 minutes, and solvent-to-material ratio of 26 to 1 (v/w) mL/g. The extracted oil had better physicochemical properties and similar fatty acid composition to commercial oil. The wound healing activity of extracted tamanu oil was higher than commercial oil. Thus, the tamanu oil could be regarded as a potential agent for wound care treatment.

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TÓM TẮT

TRÍCH LY DẦU MÙ U TỪ HẠT *Calophyllum inophyllum* L. VỚI SỰ HỖ TRỢ CỦA SIÊU ÂM VÀ THỬ NGHIỆM KHẢ NĂNG LÀM LÀNH VẾT THƯƠNG

Lữ Thị Mộng Thy[1], Trương Lê Khôi[2], Trần Đỗ Đạt[2], Phan Lê Thảo My[2],
Vương Hoài Thanh[2], Nguyễn Đức Việt[2], Huỳnh Khánh Duy[2],
Hoàng Minh Nam[2], Mai Thanh Phong[2], Nguyễn Hữu Hiếu[2,*]
[1]*Trường Đại học Công nghiệp Thực phẩm TP.HCM*
[2]*Trường Đại học Bách khoa - ĐHQGHCM*
* Email: *nhhieubk@hcmut.edu.vn*

Trong nghiên cứu này, dầu trích ly từ hạt mù u (*Calophyllum inophyllum* Linn.) với sự hỗ trợ của sóng siêu âm. Đánh giá khả năng trích của 4 loại dung môi bao gồm *n*-hexane, ethanol, ether dầu hỏa, và etyl acetat; và sử dụng phương pháp bề mặt đáp ứng để khảo sát ảnh hưởng đồng thời của các yếu tố gồm thời gian, nhiệt độ, tỷ lệ dung môi:nguyên liệu đến hiệu suất quá trình trích ly dầu mù u. Điều kiện tối ưu được xác định tại 23 phút, 42 °C, tỷ lệ dung môi:nguyên liệu 26:1 (v/w) mL/g với hiệu suất trích ly đạt là 55,15%. Bên cạnh đó, các chỉ tiêu hóa lý của dầu mù u thu được cao hơn và thành phần axit béo tương đồng với một sản phẩm thị trường. Ngoài ra, khả năng chữa lành vết thương của dầu mù u trích ly được thử nghiệm trên chuột *Swiss albino* tốt hơn sản phẩm thị trường. Vì vậy, dầu mù u trích ly trong nghiên cứu có tiềm năng sử dụng trong điều trị vết thương.

Từ khóa: Dầu mù u, siêu âm, phương pháp bề mặt đáp ứng, làm lành vết thương.