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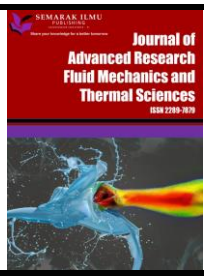

Extraction of Coumarin Mixture from Tamanu Oil using Food-Grade

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ABSTRACT

Indonesia's flora and fauna are divided into four significant ecosystems: freshwater (rivers and lakes), marine, semi-terrestrial (mangrove and riparian), and terrestrial. Tamanu (*Calophyllum inophyllum*) is a member of the mangrove-associated plant species belonging to the mangosteen family. It is also known as Alexandrian laurel, *bintangur* (Malaysia), Borneo-mahogany, *polanga* (India), and *nyamplung* (Indonesia). The tamanu seed can produce oil with a 60-75% yield. It is applicable to treat various skin problems, including eczema, psoriasis, herpes, acne, haemorrhoids, and certain allergies. It is also beneficial as an antimicrobial, anticancer, anti-HIV, anti-inflammation, and anticoagulant agent. This is because tamanu oil contains bioactive compounds named coumarin. Previous works isolated coumarins with toxic solvents and using methods that are hard to scale up. Therefore, this study aims to know the best concentration of food-grade ethanol, the number of stages, and the solvent system to extract crude coumarin mixtures from tamanu oil by batchwise solvent extraction. The free fatty acid and triglyceride removal was also studied. The initial contents of free fatty acid, coumarin mixture, and triglyceride in tamanu oil were 25.87%, 11.96%, and 51.16%, respectively. Tamanu oil was extracted simultaneously with binary solvents (n-hexane and 70%/80%/90% aqueous food-grade ethanol) and a single solvent (96% food-grade ethanol). Each fraction was analysed by Thin Layer Chromatography (TLC) and High Temperature-Gas Chromatography (HT-GC). The best result was obtained using 80% aqueous food-grade ethanol, eight extraction stages, and a binary solvent system, which produced 48.64±6.57% purity and 88.68±1.38% recovery of coumarin mixture. Moreover, it was found that the method removed 64.15% of free fatty acid and 99.96% of triglyceride from the crude tamanu oil after eight stages of batchwise extraction.

1. Introduction

Indonesia, alongside Brazil, Peru, Venezuela, Mexico, Ecuador, Colombia, United States, South Africa, Dominican Republic of Congo, Madagascar, India, China, Philippines, Malaysia, Australia, and Papua New Guinea, is recognized as one of the world's most biodiverse nations. These 17 megadiverse countries boast approximately 70% of the earth's biodiversity. Indonesia is situated on

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the equator, resulting in a relatively stable, warm, and humid climate. This condition increases the habitat's productivity, which supports the country in possessing an enormous species diversity. Indonesia's flora and fauna are scattered into four major ecosystems categorised: freshwater (rivers and lakes), marine, semi-terrestrial (mangrove and riparian), and terrestrial [1].

Mangrove is considered one of the distinctive and crucial ecosystems. The Indonesian mangrove ecosystem is home to 243 mangrove species, consisting of 99 flora and 144 fauna species. Mangroves' flora species are classified as true mangroves and associated mangroves. True mangroves are species found in mangrove habitats only. While associated mangroves are typical vegetation on the border of mangrove areas, they are usually never swamped by seawater [2]. At least 35 mangrove-associated plant species were found in the mangrove ecosystem [1].

Tamanu (*Calophyllum inophyllum*) is a member of the mangrove-associated plant species belonging to the mangosteen family. It is also known as Alexandrian laurel, *bintangur* (Malaysia), Borneo-mahogany, *polanga* (India), and *nyamplung* (Indonesia). The tamanu seed can produce oil with a yield of 60-75% [3,4]. Tamanu seed oil consists of triglycerides (78.3%), diglycerides (5.35%), monoglycerides (2.75%), free fatty acids (8.51%), and others (5.09%) [5]. It is applicable to treat various skin problems, including eczema, psoriasis, herpes, acne, haemorrhoids, and specific allergies [6]. It is also beneficial as an antimicrobial, anticancer, anti-HIV, anti-inflammation, and anticoagulant agent [7-11]. This is because tamanu seed oil contains bioactive compounds named coumarin.

Coumarin (e.g., inophyllum C, inophyllum E, calophyllolide, inophyllum P, inophyllum B) in tamanu seed oil was isolated (>90% purity) through column chromatography [7,12]. Gunawan *et al.,* [13] separated one of the bioactive compounds from tamanu seed oil (calophyllolide) using silica gel adsorption, but the purity was low (12.92%). From a chemical engineer's perspective, these methods are challenging to scale. Aparamarta *et al.,* [5] proposed a technique for triglyceride purification (98% purity) by eight stages-batchwise solvent extraction, which was easily to scale up. However, they did not investigate its bioactive compounds. Moreover, the solvents used in the previous works were toxic ones, such as methanol, and dichloromethane [5,7,13]. Therefore, this study aims to know the best concentration of food-grade ethanol, the number of stages, and the solvent system to extract coumarin mixture from tamanu seed oil by batchwise solvent extraction.

2. Methodology

2.1 Materials

Crude tamanu seed oil was ordered from Jarak Lestari company in Cilacap, Central Java, Indonesia. Aluminium plates (20 cm × 20 cm × 250 µm) for thin-layer chromatography (TLC) and acetic acid were purchased from Merck (Darmstadt, Germany). Food-grade ethanol, n-hexane, and ethyl acetate were supplied from CV. Chemical Indonesia Multi Sentosa (Surabaya, Indonesia).

2.2 Extraction of Coumarin Mixture using Binary Solvents

The coumarin mixture was extracted by multistage batchwise solvent extraction as described in Aparamarta *et al.,* [5] with solvent modification. First, tamanu seed oil (50 g) was poured into the beaker glass. Then, the solvents were introduced to it. The ratio of tamanu seed oil to the solvents was 1:5 (g/g). The solvents consisted of n-hexane and aqueous food-grade ethanol with a 3:1 (g/g) ratio. The concentrations of food-grade ethanol were 90%, 80%, and 70% in water. After that, the mixture was stirred with a magnetic stirrer (300 rpm) for 5 min. Next, the mixture was put in a separatory funnel to form the upper layer (n-hexane phase) and lower layer (aqueous food-grade ethanol phase). Then, the aqueous food-grade ethanol phase in the lower layer was separated from

the upper layer and designated as Polar Lipid Fraction stage 1 (PLF 1) after solvent removal. Next, fresh aqueous food-grade ethanol with the same concentration and mass as in stage 1 was mixed with the previous n-hexane phase in a beaker glass. The mixture was stirred at 300 rpm for 5 min. Then, the mixture was conveyed into a separatory funnel for the two layers to separate. The lower layer was separated and it was designated as PLF 2. This process was repeated until PLF 8 was obtained. Each PLF was analysed by TLC and HT-GC.

2.3 Extraction of Coumarin Mixture using a Single Solvent

Tamanu seed oil (50 g) was mixed with a polar solvent, 96% food-grade ethanol, in a beaker glass. The mass of food-grade ethanol added was equal to food-grade addition in the process of coumarin extraction using binary solvents (62.5 g). The mixture was stirred with a magnetic stirrer (300 rpm) for 5 min. Then, the mixture was put into a separatory funnel to form the upper layer (food-grade ethanol phase) and lower layer (oil-rich phase). The food-grade ethanol phase in the upper layer was separated from the oil-rich phase. Food-grade ethanol phase was designated as Polar Lipid Fraction stage 1 (PLF 1) after solvent removal. Next, fresh food-grade ethanol with the same concentration and volume as in stage 1 was mixed with the previous oil-rich phase in a beaker glass. The mixture was stirred at 300 rpm for 5 min. Then, the mixture was transferred into a separatory funnel and let rest to form the upper and lower layers. After that, the food-grade ethanol phase was separated from the oil-rich phase, and designated as PLF 2. This process was repeated until PLF 8 was obtained. The composition of PLF was analysed with TLC and HT-GC.

2.4 Thin-Layer Chromatography (TLC)

The TLC was used for qualitative analysis, as described by Aparamarta *et al.,* [5]. The mobile phase was prepared by combining n-hexane, ethyl acetate, and acetic acid with a volume ratio of 80:20:1. Each sample was stained in the aluminium TLC plate using a capillary column. After that, the plate was submerged in the mobile phase within a sealed container. The plate was removed from the container after the mobile phase reached the end line. Then, the plate was air-dried at ambient temperature and put under UV light (λ_{254} nm) for observation.

2.5 High Temperature-Gas Chromatography (HT-GC)

The composition of extracts was analysed quantitatively using Shimadzu GC-2010 (Kyoto, Japan) equipped with a Flame Ionized Detector (FID). A nonpolar column, Agilent DB-5HT ((5%-phenyl)-methylpolysiloxane (15 m × 0.32 mm i.d.; Agilent Technologies, Palo Alto, CA), was used as a static phase, and nitrogen was utilized as a carrier gas. The sample was split with a ratio of 1:50, and hydrogen was used to ignite the flame. Firstly, both the injector and detector were set at 370 °C. Meanwhile, the column was set at 80 °C, then with a rate of 15 °C/min, it was increased to 365 °C, and the temperature was held at 365 °C for 8 min. Twenty milligrams of the sample were dissolved in ethyl acetate (1 mL) and introduced into the HT-GC instrument.

2.6 Statistic Analysis

A trial version of Minitab 18 was utilized for conducting statistical analysis. The experiment was designed using a general full factorial approach and executed in duplicate. In the meantime, we

employed analysis of variance (ANOVA) to assess the significance of variables. A variable was deemed significant if its *p*-value fell below 0.05.

3. Results

Solid-liquid (maceration) or liquid-liquid extraction has been widely employed and investigated for bioactive compound extraction. These methods are easy because they require unsophisticated equipment and recyclable solvents. Commonly employed solvents for extracting bioactive compounds from medicinal herbs are acetone, ethanol, ethyl acetate, methanol, and water [14-16]. Solvent selection is crucial because it affects the selectivity and recovery of the desired compound [17]. Mostly, bioactive compounds are semipolar and hardly dissolve in water. For this reason, ethanol, methanol, ethyl acetate, and acetone are primarily used to extract higher yields of natural compounds. However, people using herbal distillate products for a long time might be at risk for toxicity if high concentrations of those solvents are present [18]. The toxicity of methanol, ethyl acetate, and ethanol in terms of LD50 in rats are 5600 mg/kg, 5620 mg/kg, and 7060 mg/kg, respectively [19]. LD50 is a metric used to gauge the toxicity of a substance. It signifies the quantity or dosage of a substance that, when given to a test group (typically laboratory animals), leads to the mortality of 50% of those subjects within a defined period. Therefore, ethanol is the least toxic solvent among them.

According to Alzeer and Hadeed [20], three types of ethanol are absolute, denatured, and food-grade ethanol. Food-grade ethanol is produced by fermentation of sugars. Meanwhile, absolute ethanol contains additives, such as 2-butanone, toluene, and heptane. Denatured ethanol contains isopropanol and methanol, which are harmful to human consumption. Only the quality of food-grade ethanol can be used in personal care, pharmaceuticals, food, and beverage products. Therefore, food-grade ethanol was employed in this study to minimize the product's toxicity. In addition, the toxicity of n-hexane is considered low. According to the United States (U.S.) Food and Drug Administration [21], the allowed concentration of hexane residue in the final product is not to exceed 25 parts per million. Tamanu seed oil used for this study consisted of triglyceride (51.16%), diglyceride (7.89%), monoglyceride (0.53%), free fatty acid (25.87%), coumarin mixture (11.96%), and others (2.96%).

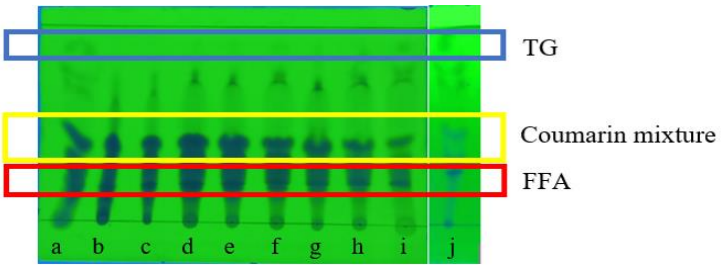
3.1 Extraction of Coumarin Mixture using Binary Solvents

This study used n-hexane and aqueous food-grade ethanol to extract coumarin mixture from the crude tamanu seed oil. Ethanol is considered a semipolar solvent with a polarity index of 5.2, because its molecule contains hydroxyl group bonded to the ethyl group [22]. The addition of water to the mixture promotes the polarity index of ethanol, thereby increasing selectivity against coumarin. This study used 70%, 80%, and 90% of ethanol in aqueous food-grade ethanol. The polarity index of aqueous food-grade ethanol can be calculated by adding the multiplication of volume fraction and polarity index. Therefore, 70%, 80%, and 90% of aqueous food-grade ethanol polarity indexes are 6.34, 5.96, and 5.58, respectively.

On the other hand, n-hexane is a hydrocarbon made up of carbon and hydrogen atoms linked together in a straight chain. There are solely carbon-hydrogen (C-H) bonds in an n-hexane molecule and no electronegative atoms such as oxygen or nitrogen. Carbon and hydrogen have similar electronegativity, which means they evenly distribute electrons in their covalent bonds. As a result, the electron distribution in the molecule is rather uniform, and no large dipoles or partial charges are

present. Because the electron cloud surrounding the carbon and hydrogen atoms is symmetrical, n-hexane is a nonpolar molecule with zero polarity.

After the mixture of tamanu seed oil, n-hexane, and aqueous food-grade ethanol was stirred at room temperature for 5 min, it was placed in the separatory funnel for some time. The compounds of tamanu seed oil were distributed between the two layers based on their polarity. This refers to the principle of "like dissolve like". The upper layer consisted of n-hexane and nonpolar compounds of tamanu oil since pure n-hexane has a lower density (0.65 g/ml) than aqueous food-grade ethanol. Meanwhile, the lower layer consisted of aqueous food-grade ethanol and polar compounds. The extraction was terminated at the eighth stage because of the insignificant contents of the FFA and coumarin mixture in the seventh and eighth stages. PLFs from stage 1 to stage 8 were analysed by TLC and HT-GC. Subsequently, these PLFs were mixed to enhance the retrieval of the coumarin mixture from the crude tamanu seed oil. A typical thin-layer chromatogram of crude tamanu seed oil, PLFs, and NPLF is demonstrated in Figure 1. It shows that the spot of TG is detected in the crude tamanu seed oil and NPLF 8. It means that the method successfully eliminated the main component of tamanu seed oil, TG, from the PLF. However, the concentration and recovery of each component were further analysed by HT-GC.



Description:

a. Crude tamanu seed oil
b. PLF 1
c. PLF 2
d. PLF 3
e. PLF 4
f. PLF 5
g. PLF 6
h. PLF 7
h. PLF 7
j. NPLF 8

Fig. 1. Typical thin-layer chromatogram of crude tamanu seed oil, PLF, and NPLF extracted from binary solvent

The typical GC chromatogram can be seen in Figure 2. The results indicate the same outcome as those shown by the TLC analysis. The peaks of TG were not detected in PLFs, instead of NPLF. In addition, the peaks of DG were absent in the PLFs, indicating that DG was dissolved in n-hexane. The typical GC chromatogram shows that the response of coumarin mixture purity in each stage gradually decreased, in contrast to the response of FFA purity. The HT-GC analysis results of 70%, 80%, and 90% aqueous food-grade ethanol are presented in Table 1, Table 2, and Table 3, consecutively.

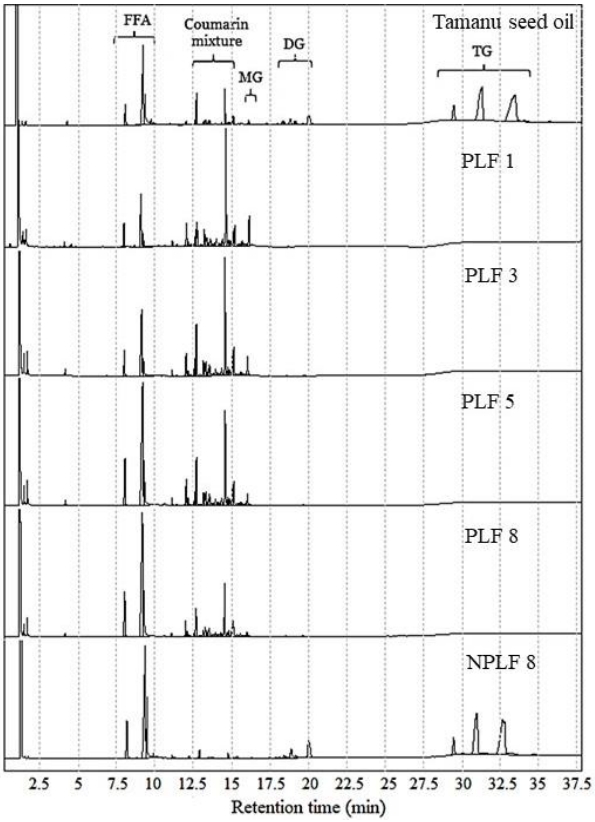


Fig. 2. A typical GC chromatogram of crude tamanu seed oil, PLFs, and NPLF extracted from the binary solvent system

Based on the results of extraction using 70% aqueous food-grade ethanol and n-hexane in Table 1, FFA was increased from 17.36±9.95% (stage 1) to 25.62±10.92% (stage 8). Meanwhile, the purity of the coumarin mixture decreased from 64.99±7.01% (stage 1) to 61.02±7.67% (stage 8). The coumarin mixture purity decreased because FFA, MG, and other compounds were extracted to PLF instead of NPLF. Based on its nature, each compound tends to dissolve in the lipophilic or hydrophilic solvent. The term expressing the partition of coefficient of a molecule between two immiscible solvents commonly considered as octanol (lipophilic) and water (aqueous) is designated as LogP. This is the most frequently used method to convey the compound's lipophilicity [23]. It is determined by dissolving the compound in a biphasic system consisting of lipids and water, which do not mix, allowing us to ascertain the proportion of the solute dissolved in each of the two phases. It is possible to compute it using computer applications in cases where the experimental value is unavailable, such as with software like MarvinSketch 5.4.0.1 or EPI Suite [24]. A higher value of LogP means that the compound has lipophilic nature and vice versa. LogP of each compound in crude tamanu seed oil was compiled in Table 2. Calophyllolide was used to represent the coumarin mixture since it was found in several previous works [7,8,12].

As can be seen in Table 2, TG becomes the most lipophilic compound in tamanu seed oil because it is composed mainly of lengthy hydrocarbon chains linked to glycerol through ester bonds. Most triglyceride molecules comprise three fatty acid chains, each consisting of extended hydrocarbon structures composed of carbon and hydrogen atoms. These hydrocarbon chains exhibit nonpolar characteristics because the carbon-hydrogen (C-H) bonds within them are relatively nonpolar, owing to the similar electronegativities of carbon and hydrogen. Consequently, there are no notable partial charges or dipoles along these chains. Moreover, TG exists when three fatty acids react with glycerol, leading to the formation of ester bonds. Although the ester bond itself is polar due to the difference

in electronegativity between the oxygen and carbon atoms involved, the overall molecule retains its nonpolar nature. This occurs because the polar features of the ester bonds are surrounded by the nonpolar hydrocarbon chains, which cancel of any overall polar characteristics.

Table 1
The purity and recovery of each component in the PLF using 70% aqueous food-grade ethanol and n-hexane

Stage	FFA (%)	Coumarin mixture (%)	MG (%)	DG (%)	TG (%)	Others (%)
1	17.36 ± 9.95[a]	64.99±7.01	6.05±2.25	0.15±0.22	0.35±0.50	11.10±1.40
	(1.31 ± 0.60)[b]	(11.07±2.60)	(23.64±11.56)	(0.04±0.05)	(0.01±0.02)	(8.74±2.21)
1-2	18.58 ± 10.14	65.40±7.15	5.44±2.13	0.25±0.36	0.16±0.23	10.17±1.44
	(2.83 ±1.67)	(21.22±1.22)	(39.50±13.57)	(0.13+0.18)	(0.01±0.02)	(15.21±1.38)
1-3	20.04±9.94	64.94±6.76	4.93±2.12	0.25±0.35	0.18±0.26	9.67±1.66
	(4.46 ±2.68)	(30.18±0.54)	(50.61±16.04)	(0.19±0.27)	(0.02±0.03)	(20.65±1.05)
1-4	21.71±11.65	63.57±8.48	4.70±2.27	0.25±0.30	0.27±0.38	9.50±1.58
	(6.18 ±4.33)	(36.61±2.59)	(58.91±17.29)	(0.25±0.31)	(0.04±0.06)	(25.19±0.95)
1-5	22.59±11.36	63.17±8.26	4.43±2.22	0.29±0.32	0.24±0.35	9.28±1.55
	(7.71 ±5.23)	(43.68±3.57)	(66.37±20.27)	(0.34±0.40)	(0.05±0.07)	(29.50±1.33)
1-6	23.65±10.92	62.50±7.88	4.19±2.15	0.28±0.32	0.22±0.30	9.15±1.52
	(9.25 ±5.95)	(49.71±4.38)	(72.04±22.86)	(0.39±0.47)	(0.05±0.07)	(33.48±1.61)
1-7	24.63±10.79	61.78±7.66	4.00±2.12	0.27±0.32	0.19±0.27	9.13±1.60
	(10.73 ±6.69)	(54.86±5.03)	(76.58±25.53)	(0.42±0.51)	(0.05±0.07)	(37.22±1.48)
1-8	25.62±10.92	61.02±7.67	3.84±2.04	0.30±0.30	0.21±0.29	9.02±1.79
	(12.23 ±7.47)	(59.45±5.24)	(80.71±27.29)	(0.49±0.54)	(0.05±0.08)	(40.26±0.57)

[a]Purity; [b]Recovery (in parentheses)

Table 2
LogP of compounds and solvent

No	Compounds	LogP
1	Triglyceride	
	Tristearin	23.93
	Triolein	23.29
	Tripalmitin	20.99
2	Diglyceride	
	1,3-distearin	15.07
	1,3-diolein	14.64
	1,3-dipalmitin	13.11
3	Free fatty acid	
	Stearic acid	7.94
	Oleic acid	7.73
	Palmitic acid	6.96
4	Monoglyceride	
	Monostearin	6.62
	Monoolein	6.40
	Monopalmitin	5.63
5	Coumarin	
	Calophyllolide	5.21
6	Ethanol	-0.14
7	Water	-1.38

Source: EPI Suite v.4.11

DG is often classified as semi-polar or amphipathic molecules rather than purely nonpolar because its structure combines polar and nonpolar elements. In a DG molecule, two fatty acid chains are attached to a glycerol molecule through ester linkages, similar to triglycerides. The fatty acid chains within diglycerides comprise extended hydrocarbon chains of carbon and hydrogen atoms. These hydrocarbon chains are nonpolar because the carbon-hydrogen (C-H) bonds within them are relatively nonpolar due to the similar electronegativities of carbon and hydrogen. This nonpolar component of the diglyceride contributes to its overall nonpolar nature. The glycerol portion of diglycerides contains polar hydroxyl (-OH) groups. These hydroxyl groups are electronegative and create a polar region within the molecule. Although the polar portion is smaller than the nonpolar hydrocarbon chains, it still imparts some polar characteristics to the DG.

Coumarin mixture, FFA, and MG molecules have at least a hydroxyl group, which can make hydrogen bonds with water or ethanol molecules. MG comprises of a glycerol molecule with a fatty acid chain attached to it through an ester bond. The glycerol part contains polar hydroxyl (-OH) groups, which are electronegative and create a polar and water-attracting (hydrophilic) region within the molecule. The fatty acid chain connected to the glycerol acts as the nonpolar and water-repelling (hydrophobic) segment of the molecule. This chain is made up of a long hydrocarbon structure containing carbon and hydrogen atoms. The carbon-hydrogen (C-H) bonds in this hydrocarbon chain are relatively nonpolar due to the similar electronegativities of carbon and hydrogen.

Meanwhile, FFA is commonly classified as a polar compound, although it contains a nonpolar hydrocarbon chain. The presence of a polar carboxyl (-COOH) group at one end of the molecule causes free fatty acids to be polar. The carboxyl group consists of a carbon atom (C) that forms a double bond with an oxygen atom (O) and a hydroxyl group (-OH) that is single-bonded to another oxygen atom (O). Due to the varying electronegativities of oxygen and carbon, this arrangement creates a strongly polar region within the molecule. The oxygen atom strongly attracts electrons, resulting in partial negative and positive charges on the oxygen and carbon atoms. The rest of the free fatty acid molecule is made up of a long hydrocarbon chain made up of carbon and hydrogen atoms. Because carbon and hydrogen have similar electronegativities, the carbon-hydrogen (C-H) bonds within this hydrocarbon chain are mainly nonpolar. Coumarin, represented by calophyllolide, has a relatively polar nature. This polarity is caused by polar functional groups in its structure, specifically carbonyl (C=O) and hydroxyl (-OH) groups. Electronegative atoms (oxygen) are bound to carbon in these functional groups, resulting in areas of partial negative and positive charges inside the molecule. Calophyllolide's polarity renders it less soluble in nonpolar liquids.

The recovery of the coumarin mixture in PLF 8 (59.45±5.24%) increased significantly compared to that in PLF 1 (11.07±2.60%) by adding extraction stage. Other compounds' recovery, such as FFA, MG, and DG, were also increased concurrently. However, the increase in the recovery of TG and DG is very small. These results indicate that components with a higher LogP value (>13.11) hardly dissolve in 70% aqueous food-grade ethanol. Also, 87.77% of FFA, 99.51% of DG, and 99.95% of TG in the crude tamanu seed oil were removed by using 70% aqueous food-grade ethanol.

Table 3 shows that adding extraction stages decreases the coumarin mixture's purity. Also, the purity of the coumarin mixture obtained from 80% aqueous food-grade ethanol was lower than that obtained from 70% aqueous food-grade ethanol. The purity of the coumarin mixture decreased from 63.86±0.53% (PLF 1) to 48.64±9.29% (PLF 8). Reducing water content in the aqueous food-grade ethanol reduced its hydrophilic nature. It caused a considerable amount of FFA soluble in the PLF and decreased the coumarin mixture purity, even though the recovery of the coumarin mixture increased. Less water concentration in aqueous ethanol enhanced the extraction of FFA because its polarity became more compatible with the polarity of FFA. However, 80% aqueous food-grade ethanol removed 64.15% of FFA, 97.73% of DG, and 99.96% of TG from the crude tamanu seed oil.

Table 3
The percentage of each component in every stage of PLF using 80% aqueous food-grade ethanol and n-hexane

Stage	FFA (%)	Coumarin mixture (%)	MG (%)	DG (%)	TG (%)	Others (%)
1	20.66±0.86[a] (2.63±0.15)[b]	63.86±0.53 (17.60±0.13)	4.70±0.15 (29.20±0.46)	0.19±0.26 (0.08±0.11	0.00±0.00 (0.00±0.00)	10.60±0.44 (13.49±0.35)
1-2	25.84±3.80 (7.34±2.04)	60.15±3.09 (36.46±3.00)	3.91±0.33 (53.34±2.61)	0.32±0.26 (0.31±0.28)	0.00±0.00 (0.00±0.00)	9.78±0.64 (27.34±1.86)
1-3	27.92±6.24 (11.99±4.60)	58.94±5.55 (53.35±3.93)	3.48±0.34 (70.98±4.89)	0.45±0.42 (0.68±.69)	0.00±0.00 (0.00±0.00)	9.21±0.77 (38.54±3.26)
1-4	31.20±8.64 (17.52±7.64)	56.49±7.73 (66.25±2.17)	3.13±0.43 (82.81±2.73)	0.51±0.43 (0.98±0.93)	0.00±0.00 (0.00±0.00)	8.66±0.91 (47.04±3.06)
1-5	34.44±9.13 (22.78±9.83)	53.88±8.06 (74.34±2.02)	2.85±0.44 (88.64±1.82)	0.60±0.48 (1.35±1.24)	0.04±0.01 (0.01±0.00)	8.19±1.11 (52.27±2.18)
1-6	36.53±10.06 (27.24±12.04)	52.28±8.82 (81.09±0.71)	2.66±0.47 (92.97±0.17)	0.66±0.50 (1.68±1.48)	0.06±0.00 (0.02±0.00)	7.82±1.27 (56.02±0.87)
1-7	39.05±10.43 (31.90±13.79)	50.17±9.20 (85.24±0.70)	2.48±0.46 (95.21±0.88)	0.73±0.56 (2.04±1.79)	0.09±0.02 (0.04±0.02)	7.49±1.35 (58.75±0.30)
1-8	40.90±10.49 (35.85±15.15)	48.64±9.29 (88.68±1.38)	2.35±0.46 (96.73±1.88)	0.76±0.57 (2.27±1.99)	0.09±0.04 0.04±0.02)	7.25±1.36 (61.04±0.75)

[a]Purity; [b]Recovery (in parentheses)

In Table 4, the purity of the coumarin mixture decreases from 53.26±0.06% (PLF 1) to 27.63±0.16% (PLF 8) by employing 90% aqueous food-grade ethanol. In contrast, the recovery of coumarin mixture was increased from 28.59±0.28% (PLF 1) to 87.32±1.59% (PLF 8). The purity of the coumarin mixture decreased because the recovery of FFA, DG, and others was significantly increased in the PLF. Less water in the aqueous ethanol reduces its polarity level and facilitates the FFA, DG, MG, and coumarin mixture to dissolve in 90% aqueous food-grade ethanol. However, 90% aqueous food-grade ethanol removed 10.72% of FFA, 83.73% of DG, and 99.55% of TG. In addition, components with a LogP value less than 15.08 are soluble in 90% of food-grade ethanol.

Table 4
The percentage of each component in every stage of PLF using 90% aqueous food-grade ethanol and n-hexane

Stage	FFA (%)	Coumarin mixture (%)	MG (%)	DG (%)	TG (%)	Others (%)
1	33.07±0.32[a] (8.21±0.04)[b]	53.26±0.06 (28.59±0.28)	3.32±0.08 (40.21±0.17)	0.43±0.00 (0.35±0.05)	0.00±0.00 (0.00±0.00)	9.92±1.60 (24.59±0.40)
1-2	41.58±0.46 (20.84±0.60)	46.28±0.05 (50.16±0.86)	2.67±0.02 (65.20±0.68)	0.08±0.08 (1.29±0.02)	0.79±0.00 (0.00±0.00)	8.68±1.20 (43.45±1.16)
1-3	47.15±0.19 (34.37±0.63)	41.25±0.58 (65.22±0.68)	2.29±0.05 (81.63±0.98)	0.12±0.12 (3.10±0.78)	1.29±0.09 (0.02±0.03)	7.95±1.01 (58.09±3.97)
1-4	50.78±0.68 (46.51±1.86)	38.02±0.36 (75.29±0.15)	2.04±0.02 (91.30±1.03)	0.14±0.14 (5.00±0.46)	1.66±0.07 (0.07±0.03)	7.34±0.91 (67.19±5.47)
1-5	54.60±1.05 (59.32±3.77)	34.35±0.13 (80.61±0.09)	1.80±0.02 (95.27±0.37)	0.16±0.16 (7.59±1.09)	2.13±0.02 (0.15±0.02)	6.85±0.89 (74.38±7.14)
1-6	57.31±1.11 (71.12±5.66)	31.53±0.08 (84.49±1.46)	1.62±0.01 (97.80±1.33)	0.17±0.17 (10.45±1.02)	2.57±0.05 (0.26±0.01)	6.56±0.85 (81.37±8.24)
1-7	59.34±1.61 (80.72±8.12)	29.43±0.01 (86.31±1.41)	1.50±0.03 (99.01±0.80)	0.19±0.19 (13.34±0.88)	2.99±0.04 (0.34±0.00)	6.25±0.82 (84.97±9.40)
1-8	60.91±1.88 (89.28±9.24)	27.63±0.16 (87.32±1.59)	1.40±0.03 (99.47±0.72)	0.20±0.20 (16.27±0.34)	3.40±0.16 (0.45±0.08)	6.06±0.79 (88.69±9.36)

[a]Purity; [b]Recovery (in parentheses)

3.2 Extraction of Crude Coumarin Mixture Using a Single Solvent

Single solvent extraction of coumarin mixture from tamanu seed oil minimizes the solvent usage. In addition, 96% food-grade ethanol is miscible with n-hexane, resulting in no component separation [22]. Therefore, the extraction was conducted in the absence of n-hexane to achieve component distribution. The mixture of tamanu seed oil and 96% food-grade ethanol formed two layers, 96% food-grade ethanol phase in the upper layer and an oil-rich phase in the bottom layer. Figure 3 shows the typical GC chromatogram of crude tamanu seed oil, PLF, and NPLF extracted from a single solvent system. The purity and recovery of each component in the PLF are shown in Table 5.

Table 5
The percentage of each component in the PLF using 96% food-grade ethanol

Stage	FFA (%)	Coumarin mixture (%)	MG (%)	DG (%)	TG (%)	Others (%)
1	62.71±3.42[a] (61.27±1.50)[b]	14.14±0.69 (49.74±6.33)	0.81±0.10 (37.52±1.33)	6.25±0.58 (34.07±5.80)	11.78±2.89 (10.19±3.27)	4.31±0.64 (56.60±3.97)
1-2	61.84±2.20 (76.02±2.26)	14.51±0.78 (62.92±3.06)	0.83±0.14 (47.48±7.53)	6.48±0.57 (43.49±4.03)	11.02±3.27 (11.68±3.52)	4.33±0.72 (70.54±11.39)
1-3	62.87±2.12 (83.42±2.42)	14.12±1.02 (67.16±4.52)	0.81±0.13 (51.13±8.25)	6.77±0.54 (49.88±4.22)	11.21±3.44 (13.02±4.06)	4.22±0.71 (75.41±12.30)
1-4	62.36±2.22 (87.36±2.26)	13.78±1.07 (69.17±4.73)	0.80±0.13 (53.15±8.37)	7.04±0.41 (54.74±3.75)	11.89±3.66 (14.60±4.62)	4.14±0.65 (78.13±11.47)
1-5	61.70±2.68 (90.35±3.01)	13.46±1.15 (70.67±5.31)	0.79±0.14 (54.70±8.89)	7.29±0.25 (59.25±2.61)	12.69±4.34 (16.29±5.72)	4.07±0.63 (80.27±11.57)
1-6	61.10±2.64 (92.54±3.08)	13.22±1.10 (71.79±5.24)	0.78±0.13 (56.09±9.02)	7.52±0.14 (63.24±1.78)	13.34±4.31 (17.70±5.88)	4.04±0.57 (82.41±10.89)
1-7	60.31±3.00 (93.80±2.74)	12.98±1.19 (72.36±5.17)	0.77±0.14 (56.78±8.89)	7.65±0.14 (66.07±2.52)	14.29±4.76 (19.52±6.88)	4.00±0.58 (83.73±10.37)
1-8	60.91±1.88 (94.25±3.16)	12.78±1.03 (82.60±5.42)	0.76±0.13 (57.13±9.24)	7.70±0.06 (67.72±0.96)	15.31±4.01 (21.24±5.69)	3.95±0.54 (84.40±10.98)

[a]Purity; [b]Recovery (in parentheses)

From Figure 3, it can be seen that all peaks of components are detected in the PLF 1 chromatogram. After eight stages of extraction, TG's peaks were higher than those of FFA, indicating a considerable amount of TG in the PLF. In Table 4, the purity of FFA in the first stage (62.71±3.42%) was relatively high, decreasing to 60.91±1.88% after eight extraction stages. At the same time, the purity of the coumarin mixture decreased from 14.14±0.69% (PLF 1) to 12.78±1.03 (PLF 8). By the end of extraction, 96% food-grade ethanol increased the recovery of FFA, coumarin mixture, DG, TG, and other compounds. This phenomenon occurred because ethanol had the most minor water (4%) on it, making it less polar. Therefore, the relatively nonpolar compounds, like DG and TG, could dissolve on 96% food-grade ethanol. The recovery of FFA in 96% food-grade ethanol was higher than using a binary solvent system because the FFA structure consists of a long chain of hydrocarbon, making this compound have a nonpolar nature. However, the purity of the coumarin mixture in PLF 8 was extremely low (12.78±1.03%) even though the recovery was relatively high (82.60±5.42%). The removal of FFA, DG, and TG from tamanu seed oil was 5.75%, 32.28%, and 78.76%, respectively. The method using a single solvent system of 96% food-grade ethanol shows its potential to obtain a high concentration of TG from tamanu seed oil as an alternative material for cooking oil. It is because FFA, coumarin mixture, DG, MG and other components are soluble in 96% ethanol fraction.

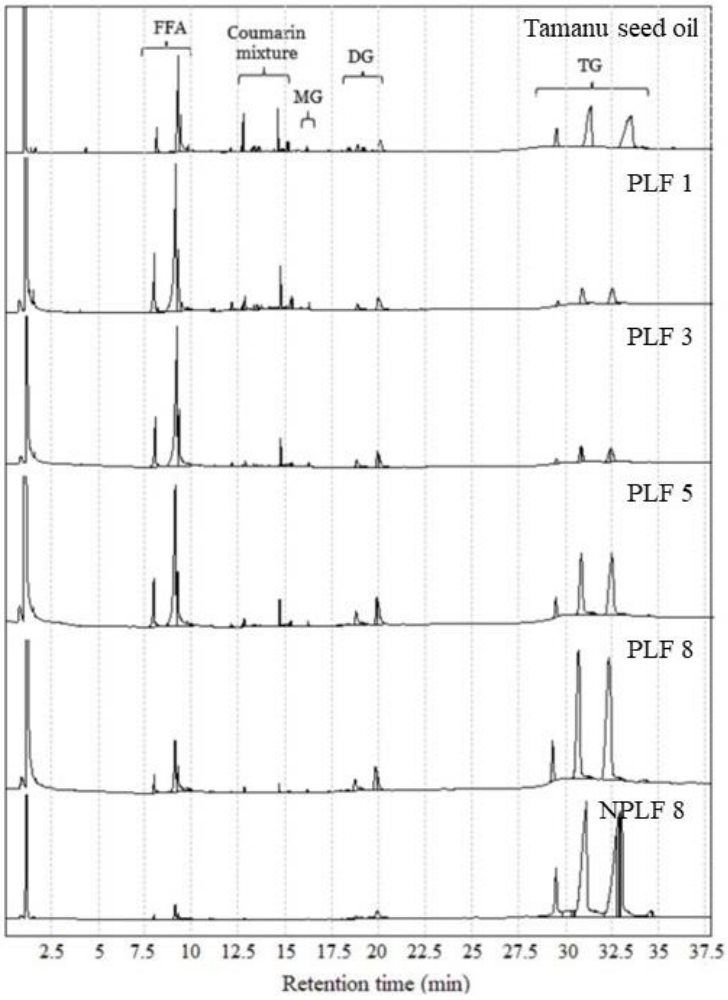
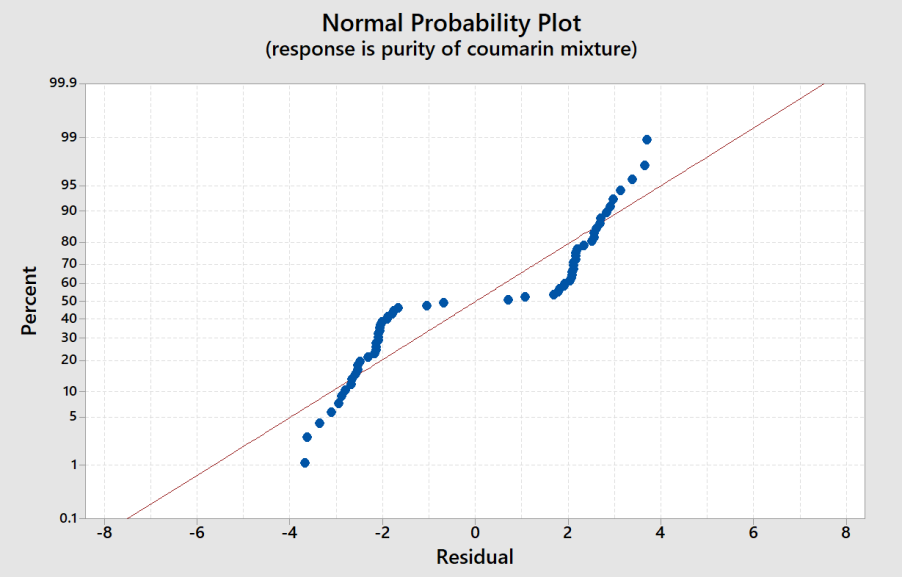


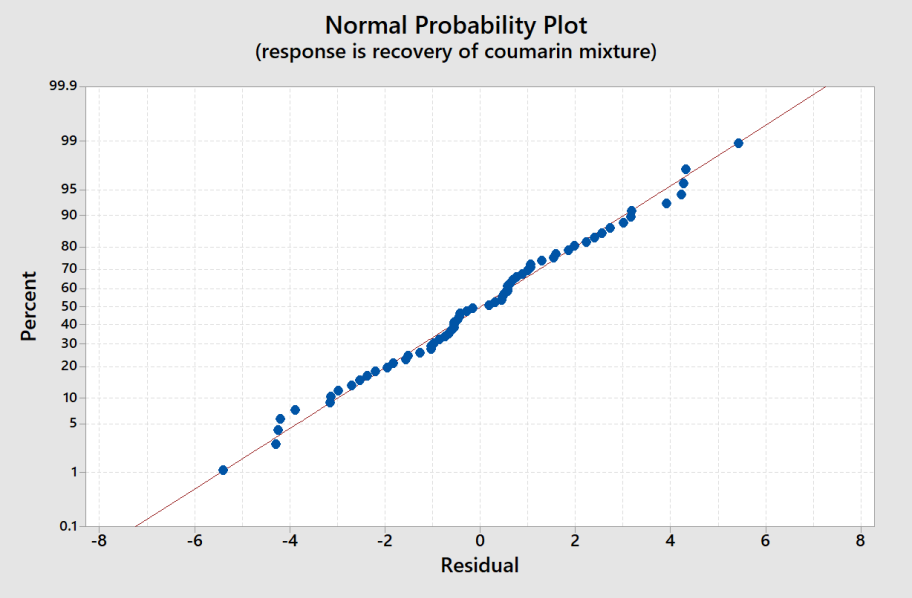
Fig. 3. A typical chromatogram of crude tamanu seed oil, PLF, and NPLF extracted from a single solvent system (96% food-grade ethanol)

3.3 Statistical Analysis

The concentration of aqueous food-grade ethanol and the number of stages were investigated in a general full factorial design to determine their impact on the purification and recovery of coumarin mixtures from tamanu seed oil. Aqueous food-grade ethanol has four concentration levels (70, 80, 90, and 96%) and eight extraction stages (1st to 8th). As shown in Figure 4, a normal probability plot was used to check the model fit for purity and recovery of the coumarin mixture. The data of coumarin mixture recovery was distributed normally. ANOVA analyses in Table 6 and Table 7 show that purity and recovery of coumarin mixture are significantly affected by concentration and the number of stages (p-value < 0.05). The interaction of the two factors is also statistically significant (p-value < 0.05). The best parameters for achieving the highest extraction efficiency, including purity and recovery, are utilizing an 80% concentration of food-grade ethanol and conducting eight extraction stages. In the design of experiment, composite desirability is often used to optimize the process with multiple variables. These optimal conditions are highlighted in Figure 5 and correspond to a composite desirability score of 0.7867. This indicates that the result has led to a combination of input factors or conditions that are advantageous for attaining the maximum values for all the responses at the same time [25].



(a)



(b)

Fig. 4. Normal probability plot of residuals for (a) purity and (b) recovery of coumarin mixture

Table 6
Analysis of variance of coumarin mixture purity

Source	DF	Adj SS	Adj MS	F-value	P-value
Model	32	25636.4	801.14	66.84	0.000
Linear	10	24463.4	2446.34	204.09	0.000
Concentration	3	23584.8	7861.61	655.87	0.000
Stage	7	878.5	125.50	10.47	0.000
2-Way Interactions	21	643.9	30.66	2.56	0.009
Concentration*Stage	21	643.9	30.66	2.56	0.009
Error	31	371.6	11.99		
Total	63	26008.0			

Table 7
Analysis of variance of coumarin mixture recovery

Source	DF	Adj SS	Adj MS	F-value	P-value
Model	32	30267.8	945.87	84.56	0.000
Linear	10	27294.1	2729.41	1.77	0.000
Concentration	3	9982.5	3327.49	244.02	0.000
Stage	7	17311.7	2473.09	297.49	0.000
2-Way Interactions	21	2953.9	140.66	12.58	0.000
Concentration*Stage	21	2953.9	140.66	12.58	0.000
Error	31	346.7	11.19		
Total	63	30614.6			

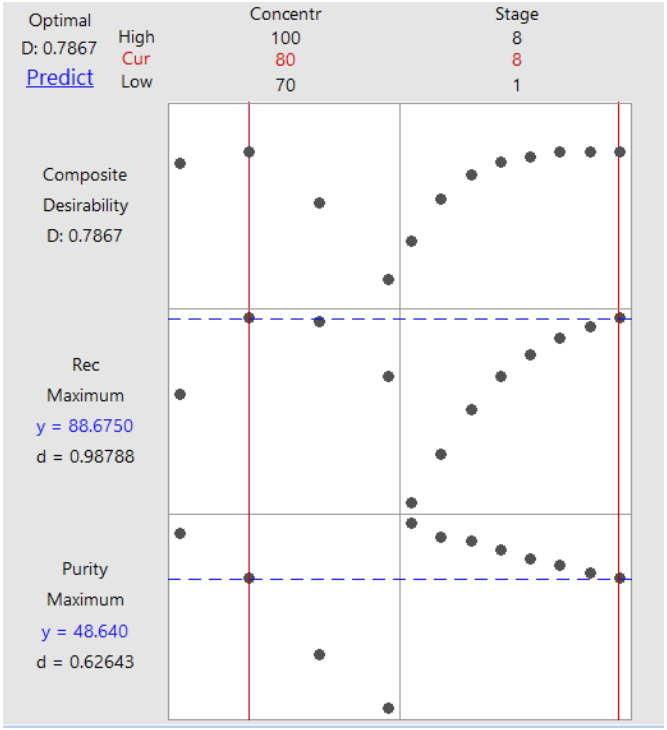


Fig. 5. Optimization response of coumarin mixture's purity and recovery

4. Conclusion

This study aims to know the best concentration of food-grade ethanol, the number of stages, and the solvent system to extract coumarin mixture from tamanu seed oil by batchwise solvent extraction. The results show that the coumarin mixture was best extracted using binary solvent system, 80% aqueous food-grade ethanol (48.64±6.57% purity, 88.68±1.38% recovery). The initial contents of free fatty acid and triglyceride in tamanu oil were 25.87% and 51.16%, respectively. After eight stages of batchwise extraction, it was found that the method could remove 64.15% of free fatty acid and 99.96% of triglyceride from the crude tamanu seed oil. A general full factorial design was used to determine the impact of food-grade ethanol concentration and the number of stages on the purification and recovery of coumarin mixtures. Purity and recovery of coumarin mixture were significantly affected by concentration and the number of stages (p-value < 0.05). The interaction of the two factors is also significant (p-value < 0.05).

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