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Evaluating phenolic compounds and antioxidant activity of the pericarps and seed coats extracts of *Macadamia integrifolia* and preparing their nanoemulsions

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Abstract

The extraction efficiency of the pericarps of *Macadamia integrifolia* is ten times higher than that of the seed coats. The pericarps of *M. integrifolia* contained much more phenolic compounds than seed coats. The optimal extraction condition is for reflux extraction in the mixture of water and acetone (1:1) with the result for DPPH free radical scavenging activity IC_{50} = 34.4 µg mL^{-1}. Total phenolic contents of 11.88 mg and total flavanone and flavanonol contents of 9.00 mg can be extracted from one gram of dried macadamia skin. The efficiency of surfactants (lecithin, tween 20, tween 80) was evaluated by pseudo-ternary phase diagrams. The nanoemulsification efficiency was indicated by the nanoemulsion area. Tween 80 showed the largest nanoemulsion fields. For preparing nanoemulsions, the ratio of oil and surfactants is 1/3, which is suitable for both devices (a high shear mixer, and the ultrasonic technology). Additionally, nanoemulsions formed by a high shear mixer resulted in a particle size of 246.6 nm, lower than the ultrasonic technology of 127.4 nm. These particle size results are all in the nano-sized region and have a high potential for further research.

Keywords: DPPH, *Macadamia integrifolia*, Total flavanone and flavanonol contents, Total phenolic contents.

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1. Introduction

Nanoemulsions were defined as an emulsion system with a droplet size from 20 to 500 nm, where surfactants were common stabilizers for the oil-water dispersion formation [1]. The application of nanoemulsions was commonly various, such as oral, topical, ophthalmic products, or even drug delivery systems [2]. Nanoemulsions were divided into three main types, including the oil in water nanoemulsion (O/W – the dispersion of oil in the continuous aqueous phase), water in oil one (W/O – the dispersion of water in the continuous oil phase), and bi-continuous one (the inter-dispersion of water and oil in the emulsion). It was used to form a precise drug carrier due to its penetration and stability. Specifically, thanks to super small droplet sizes and high surface-to-volume ratio, drugs or functional compounds easily cross the skin barrier or are absorbed from the gastrointestinal tract, which enhances their bioavailability [2]. Nanoemulsions were not formed spontaneously since they are not equilibrium systems; therefore, they require energy input for droplet formation [1]. There are four methods for producing nanoemulsions: micro fluidization, high-pressure homogenization, sonication, and emulsion inversion point (EIP) method [2]. High-energy emulsification is a common technique for generating nanoemulsions due to fast and low equipment costs. The high-speed stirring method using a rotor-stator high-speed stirring system produced nanoemulsions with a narrow size distribution and particle sizes of about 135 nm [3]. A rotor-stator mixer consists of a stator and a rotor placed inside the stator with two or more blades. When the rotation of the rotor occurred, low pressure was formed to put the solution in and out of the system, which led to circulation and produced nanoemulsions [4]. The droplet sizes in this progress depend on processing time, rotation speed, diameter, and the slit size of the rotor [3,4]. Besides, the ultrasonicator was highly evaluated for the generation of emulsions. A sonicator probe was used to provide energy in ultrasonic nanoemulsions. When this probe contacts the solution, mechanical vibration, and cavitation are created to destroy liquid cavities and generate particles with super small sizes [5]. Yuan Long et al. have reported that garlic oil nanoemulsion made by ultrasonic technique had a particle size of 52.27 nm. Moreover, this nanoemulsion enhanced the bioavailability of antifungal activity against *Penicillium italicum* by reducing the minimum inhibitory concentration (MIC) from 3.7% to 0.01265% [6].

Visual observation, such as the appearance of the dispersion and the stability or pH of emulsions, is a fast and easy way to evaluate emulsions. Dynamic light scattering (DLS) or photon correlation spectroscopy (PCS) is the tool for analyzing the droplet size of nanoemulsions, detecting aggregation attendance, or evaluating the stability of components in emulsions. The polydispersity index (PDI) represents the width of the size distribution. Low PDI value was shown in homogenous nanoparticles. Mode implies the peak of the size distribution. The Z-average (mean) relates to the intensity of the mean hydrodynamic size of particles in nanoemulsion [7]. *Macadamia integrifolia*, belonging to Proteaceae family, is widely distributed in subtropical areas. Macadamia oil is good for human health since its main components are monounsaturated fatty acids such as palmitoleic and asclepic acids [8]. The presence of palmitoleic acid helped stroke prevention. It is also essential in cosmetic products due to smooth hair and skin properties. Macadamia nut has been widely known in the field of food for having high contents of unsaturated fatty acids, fiber, minerals, and no cholesterol, which is good for providing nutrition, improving blood circulation, and balancing cholesterol levels in the human body [9]. On the other hand, the pericarps of *M. integrifolia*, considered by-products, contain a large number of flavonoids, proanthocyanidins, and phenolic compounds, as well as antioxidants [10-13].

Therefore, this research was performed to determine phenolic contents and antioxidant capacity of the pericarps and seed coats extract of *M. integrifolia* and generate nanoemulsions loaded with this extract by rotor-stator high-speed stirring and ultrasonicator. This research aims to find the best solvent and extraction method for obtaining extract with high bioactivity and compare the effectiveness of each nanotechnology. From that, many waste products of *M. integrifolia* were solved.

2. Materials and methods

2.1. Plant materials and chemicals

The pericarps and seed coats of *Macadamia integrifolia* were supplied by Damaca Nguyen Phuong Company, Dak Lak Province, Vietnam, in October 2020. Other reagents and chemicals were of the highest analytical grade.

2.2. Conventional solid-liquid and ultrasound-assisted extraction

The powder-dried pericarps and seed coats of *M. integrifolia* (5 g) were refluxed in 1 h with 100 mL different solvents such as H_2O (100%), EtOH: H_2O (v/v, 50:50), Me_2CO: H_2O (v/v, 50:50). The ultrasound extraction was conducted by using PS-30A Ultrasonic cleaners (China) (200 W) in 1 h at 45 °C. The sample-to-solvent ratio and solvents used for ultrasonic-assisted extraction (UAE) were the same as those of the reflux method. A rotary evaporator evaporated the filtered solution to yield 12 extracts for both methods.

2.3. Determination of total phenolic content

The total phenolic content of each extract was determined based on using the Folin-Ciocalteau assay [14]. Briefly, diluted samples were added with 1 ml 10% Folin-Ciocalteau reagent. After 5 minutes, 1.25 mL Na_2CO_3 10% and H_2O were added to obtain the mixture (5 mL). It was shaken and incubated in the dark at room temperature for 30 minutes. Absorbance measurement was performed by Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) at 737 nm. The calculation of total phenolic content was based on the standard curve of gallic acid and expressed as gallic acid equivalent (mg GAE/1 g dry materials).

2.4. Determination of total flavonoid content

The total flavanone and flavanonol content of each extract was determined based on using a 2,4-dinitrophenylhydrazine assay [15]. 0.5 ml 2,4-dinitrophenylhydrazine was added to diluted samples, then diluted with methanol to 2.5 ml. It was shaken and incubated in the dark at 50 °C for 30 minutes. After cooling at room temperature, it was diluted with NaOH 1% to 5 ml. The mixture was centrifuged to separate the precipitate. Finally, 0.5 ml mixture was diluted with methanol to 5 ml. Absorbance measurement was performed by Shimadzu UV-1800 spectrophotometer at 485 nm. The total flavanone and flavanonol content calculation was based on the standard curve of 2*S*-pinostrobin and expressed as 2*S*-pinostrobin equivalent (mg 2*S*-PSE/1 g dry materials).

2.5 DPPH radical scavenging assay

The antioxidant capacity of each extract was performed by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay [16]. An aliquot (1.5 mL, 0.1 mM) of DPPH• in 90% ethanol was mixed with 1.5 mL of each sample at 100, 50, 25, and 10 μg/mL concentrations in 90% ethanol. The absorbance was measured at 517 nm by Shimadzu UV-1800 spectrophotometer after 30 minutes of incubation in the dark at room temperature. The results were expressed as the percentage inhibition I% = $(1 - A_{sample}/A_{control}) \times 100\%$. All experiments were performed in triplicate. The IC_{50} value (μg mL^{-1}) was defined as the test sample concentration required to scavenge 50% DPPH free radicals. Trolox was used as a positive control.

2.6 Construction of pseudo-ternary phase diagrams

The pseudo-ternary phase diagrams, including water, oil, and surfactant, were performed using the water titration method [17]. Oil (O) and surfactant (S) (lecithin, Tween 80, Tween 20) were mixed with different mass ratios of (1:0.1), (1:0.3), (1:0.5), (1:0.8), (1:1), (1:1.5), (1:2), (1:2.5), (1:3), (1:3.5), (1:4), (1:5), (1:6). Water (W) was titrated slowly into these mixtures. The pseudo-ternary phase diagrams were constructed with three axes representing oil, water, and surfactant. From those diagrams, the effective surfactant and nanoemulsion regions are determined.

2.7 Nanoemulsion formation

The pericarp extract of *Macadamia integrifolia* was dissolved in 5 ml water. 1% citric acid, oil, surfactant, and water poured into solution, respectively. Finally, the mixtures were stirred for 30 minutes using a rotor-stator system or ultrasonicator probe sonicator (Table 1). Nanoemulsions were evaluated based on *Z*-average, mode, and PDI values.

Table 1: Data of nanoemulsion produced by rotor-stator high-speed stirring (**1**–**7**) and ultrasonicator probe sonicator (**8**–**11**)

Sample	1	2	3	4	5	6	7	8	9	10	11
m_{oil} (g)	1.1546	1.2648	1.1496	1.1023	1.017	1.0595	1.2014	1.0156	1.1659	1.2622	1.1563
$m_{surfactant}$ (g)	1.1494	2.1263	3.1257	5.2015	3.106	5.2353	3.2594	1.1359	2.2633	3.0798	5.1562
$m_{extract}$ (g)	0.2564	0.2515	0.2601	0.2496	0.5194	0.5202	0.2602	0.2496	0.2496	0.2485	0.2602
$m_{citric\ acid}$ (g)	0.0226	0.0233	0.0212	0.0226	0.0235	0.0256	0.0239	0.0232	0.0194	0.0221	0.0226
V_{water} (mL)	15	15	15	20	15	20	15	15	15	15	20
Power (W)	–	–	–	–	–	–	–	7–8	7–8	7–8	7–8
Time (min)	–	–	–	–	–	–	–	30	30	30	30

3. Results and Discussions

3.1. The results of extraction yield, total phenol and flavonoid content, DPPH radical scavenging assay.

The extraction yield of UAE of the pericarps of *Macadamia integrifolia* ranged from 10.89% to 11.80%, which was lower than that of reflux extraction (from 14.05% to 17.96%). The extraction yield of UAE can be influenced by power, frequency, time, temperature, type of solvents, and pH environment [18]. With the conditions used in this study, the extraction efficiency by UAE is not as good as that of the reflux method. The solvent efficiency, as indicated by the extraction yield, was demonstrated in the following order: aqueous ethanol (50% EtOH) < aqueous acetone (50% Me_2CO) < 100% water. This research indicated that water is the best solvent for the extraction (17.96% in the reflux method), which can be explained by the fact that the presence of water in solvent extraction increased the dielectric constant and the extraction efficiency [19]. The solubility of carbohydrates and protein in water was higher than in organic solvents such as ethanol and acetone [20].

The total phenol and flavonoid contents of the pericarps of *M. integrifolia* ranged from 4.67 ± 0.24 to 11.88 ± 0.67 mg GAE/1 g dry materials and from 4.09 ± 0.15 to 7.92 ± 0.42 mg 2*S*-PSE/1 g dry materials while those of the seed coats were from 0.40 ± 0.07 to 2.36 ± 0.10 mg GAE/1 g dry materials, and from 1.96 ± 0.29 to 8.92 ± 0.49 mg 2*S*-PSE/1 g dry materials (Table 2). The results showed that the pericarps of *M. integrifolia* contained much more phenolic compounds than seed coats (p-value < 0.05) (Figure 1). For both reflux and ultrasound extraction, ethanol (50%) and acetone (50%) gave a higher recovery of phenolic compounds than that of water (p-value < 0.05). Aqueous acetone mixtures were considered to be a suitable solvent for the extraction of phenols. A similar trend was observed in *Ananas comosus* Merr., *Musa paradasiaca*, *Psidium guajava* L. [21], *Triticum aestivum* L. [22]. Similar to total phenolic content, the pericarps of *M. integrifolia* possessed a higher number of flavonoids than its seed coats, with no significant difference between them (p-value > 0.05). The acetone–water mixture is the efficient solvent for the recovery of flavonoids, similar to the extract of *Ananas comosus* Merr., *Musa paradasiaca*, *Psidium guajava* L. [21]. All extracts were tested for the DPPH radical scavenging assay. The result showed that 11/12 extracts exhibited IC_{50} values < 100 µg mL^{-1} (Figure 2). The IC_{50} value in the DPPH radical scavenging activity assay of the extraction by various solvents increased by 50% aqueous acetone < 50% aqueous ethanol < 100% water. There are no significant differences between the pericarps and seed coats of *Macadamia integrifolia* (p-value > 0.05) and between reflux extraction and ultrasound one (p-value > 0.05). However, the water-ethanol heat reflux extract of seed coats had the lowest phenolic content, and its antioxidant capacity showed the most potent activity. The mechanism of action of antioxidants may explain this. In the first mechanism, a free radical removes the antioxidant (ArOH) from a hydrogen atom. The second one is the one-electron donation of the antioxidant [23].

The mobile phase performance index (MPI_i) was calculated based on four factors: extraction yield, DPPH radical scavenging assay, phenol, and flavonoid contents (Figure 3) [24]. MPI was used to choose the best solvent for the reflux extraction of the pericarp and seed coats of *M. integrifolia*. The mixture of water and acetone showed the highest results, with MPI values of 0.76 and 0.99, respectively. From the above analysis, conventional solid-liquid extraction or reflux is effective for the extraction of the pericarps and seed coats of *M. integrifolia*. Additionally, the mixture of acetone and water is a potential solvent for the extraction of polyphenols and antioxidant compounds, as shown by MPI values in Figure 3. Phenolic compounds are responsible for the antioxidant properties of plants, and most of these compounds are considered hydrophilic antioxidants. The extraction of phenolic compounds and flavonoids was based on interactions (hydrogen bonds) between the polar sites of the molecules and the solvents [25]. Additionally, the presence of acetone in the solvent systems, which is the less polar solvent, is suitable for the extraction of lipophilic polyphenols [21].

3.2. Pseudo-ternary phase diagram

The result showed that with the 5% surfactant, while the percentage of oil in the emulsion using lecithin is about 40%, it is 50% in that of using Tween 20 and Tween 80 (Table 3). The nanoemulsion area of Tween 80 is the largest compared with that of lecithin and Tween 20 (Figure 4). Four ratios of oil and surfactant, including 1/1, 1/2, 1/3, and 1/4, were further researched based on the nanoemulsion area. Hydrophilic-lipophilic balance (HLB) is the balance of the size and strength of hydrophilic and lipophilic parts of surfactants. The required HLB value of surfactants for forming oil in water (O/W) nanoemulsion is commonly from 8 to 18 [26; 215]. Lecithin, Tween 20, and Tween 80 are chosen for surfactants with HLB values of 7.0, 16.7, and 15.0, respectively.

Table 2. Total phenol content, total flavonoid contents, DPPH free radical scavenging assay of the pericarps and seed coats of *Macadamia integrifolia*

	Extraction yield (%)	Total phenol content (mg GAE/1 g dry materials)	Total flavanone and flavanonol content (mg 2S-PSE/1 g dry materials)	DPPH (IC_{50}, μg mL^{-1})
Pericarp, reflux (H_2O 100%)	17.96	8.43 ± 0.51	6.35 ± 0.56	46.0
Pericarp, reflux (H_2O: EtOH 50:50)	14.05	10.12 ± 0.77	7.92 ± 0.42	34.9
Pericarp, reflux (H_2O: Me_2CO 50:50)	14.16	11.88 ± 0.67	9.00 ± 0.18	34.4
Pericarp, UAE (H_2O 100%)	11.80	4.67 ± 0.24	4.09 ± 0.15	96.6
Pericarp, UAE (H_2O: EtOH 50:50)	10.89	5.17 ± 0.74	5.14 ± 0.38	64.4
Pericarp, UAE (H_2O: Me_2CO 50:50)	11.52	8.91 ± 0.84	5.81 ± 0.58	53.9
Seed coat, reflux (H_2O 100%)	1.61	2.14 ± 0.11	4.78 ± 0.45	67.1
Seed coat, reflux (H_2O: EtOH 50:50)	1.24	1.49 ± 0.04	4.19 ± 0.23	25.9
Seed coat, reflux (H_2O: Me_2CO 50:50)	1.62	2.36 ± 0.10	8.92 ± 0.49	27.7
Seed coat, UAE (H_2O 100%)	1.02	0.40 ± 0.07	1.96 ± 0.29	>100
Seed coat, UAE (H_2O: EtOH 50:50)	1.03	1.18 ± 0.03	3.52 ± 0.61	70.3
Seed coat, UAE (H_2O: Me_2CO 50:50)	1.10	1.47 ± 0.08	5.33 ± 0.81	27.2
Trolox[a]				10.9

[a] Positive control

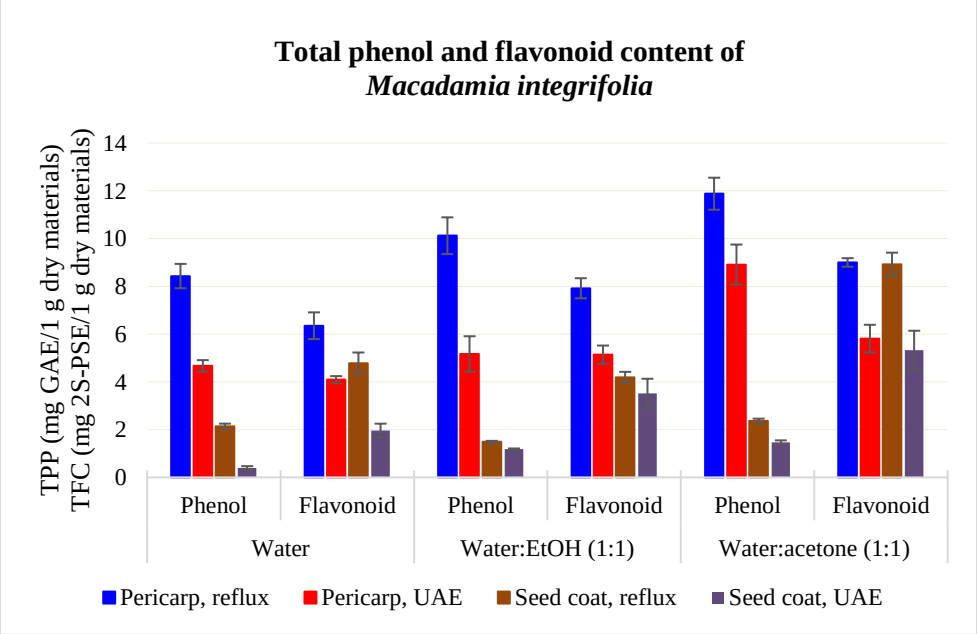

Figure 1. Total phenol and flavonoid contents of the pericarps and seed coats of *M. Integrifolia*

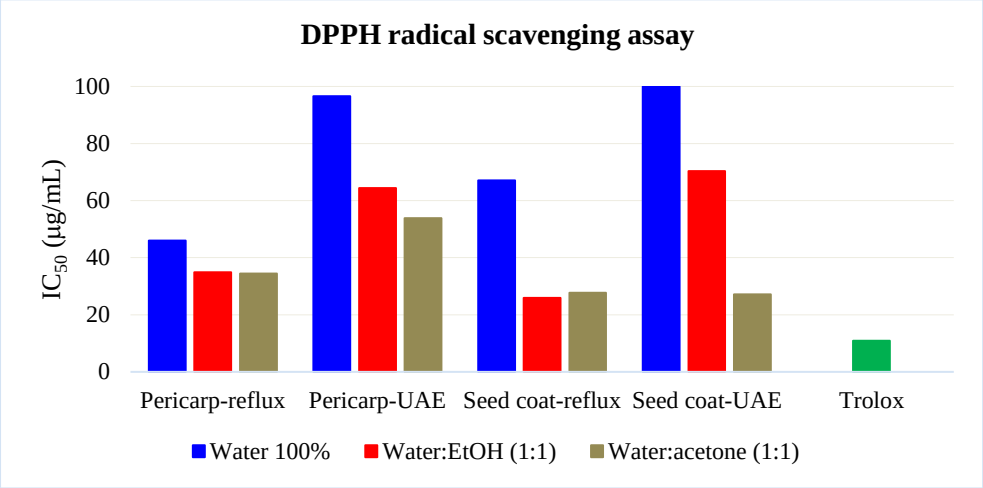

Figure 2. DPPH radical scavenging assay of the pericarps and seed coats of *M. integrifolia*

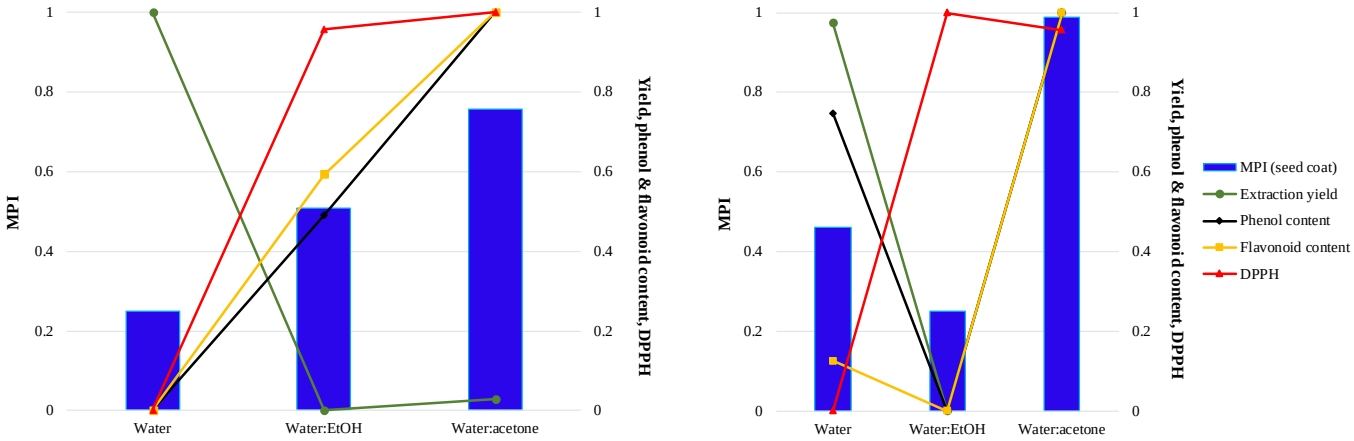

Figure 3. MPI plot of selected solvents for the pericarps and seed coats of *M. integrifolia* (reflux)

Table 3. Stable nanoemulsion formulations with different surfactants

O/S ratio	Lecithin			Tween 80			Tween 20		
	%O	%S	%W	%O	%S	%W	%O	%S	%W
1:0.1	34.5	3.4	62.1	52.6	5.3	42.1	50	5.0	45.0
1:0.3	30.3	9.1	60.6	41.7	12.5	45.8	29.4	8.8	61.8
1:0.5	20.0	10.0	70.0	33.3	16.7	50.0	30.3	15.2	54.6
1:0.65	12.9	8.4	78.7	–	–	–	–	–	–
1:0.8	8.7	6.6	84.7	29.4	23.5	47.1	22.2	17.8	60.0
1:1	6.7	6.7	86.6	17.5	17.5	65.0	18.2	18.2	63.6
1:1.5	3.6	5.4	91.1	13.3	20.0	66.7	14.1	21.1	64.8
1:2	2.5	4.9	92.6	11.5	23.0	65.5	11.1	22.2	66.7
1:2.5	2.2	5.1	92.7	10.4	26.1	63.5	9.5	23.8	66.7
1:3	–	–	–	8.8	26.6	64.6	8.3	24.8	66.9
1:3.5	–	–	–	8.3	29.2	62.5	7.7	26.9	65.4
1:4	–	–	–	5.8	23.4	70.8	6.2	24.7	69.1
1:5	–	–	–	4.6	23.4	72.1	5.8	29.1	65.1
1:6	–	–	–	4.8	28.7	66.5	5.0	29.8	65.2

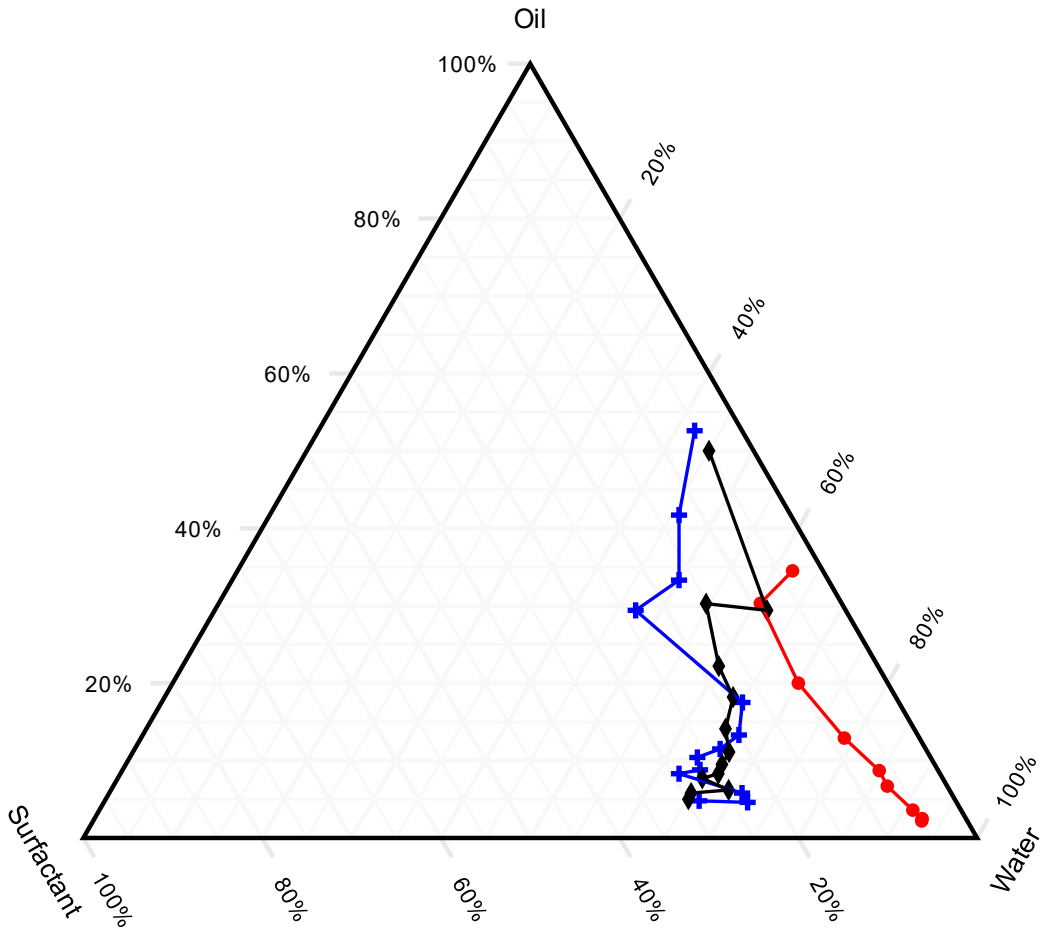

Figure 4. Pseudo-ternary phase diagram using lecithin (red solid line), Tween 80 (blue solid line), and Tween 20 (black solid line) as surfactants

Table 4. DLS data of nanoemulsion made by rotor-stator high-speed stirring (**1**–**7**) and ultrasonicator probe sonicator (**8**–**11**)

Sample	O/S ratio	Mode (nm)	*Z*-Average (nm)	PDI
1	1:1	–	–	–
2	1:2	–	–	–
3	1:3	262.1	246.6	0.417
4	1:5	426.8	431.3	1.991
5	1:3	261.7	343.1	1.109
6	1:5	855.8	–	–
7	1:3	616.1	524.2	0.556
8	1:1	–	–	–
9	1:2	181.0	127.4	0.588
10	1:3	181.2	186.8	0.412
11	1:5	333.5	240.0	0.709

Construction of a pseudo-ternary phase diagram was carried out to find the effect of the ratio of composition of the constituents on emulsions for predicting the zones of formation of the efficient emulsion, which is transparent, has small particle size, and the highest amount of oil as well as the lowest number of surfactants in order to increase extract or drug solubility and their bioavailability [27, 28]. The nanoemulsion area was used for evaluating the efficiency of surfactants. The larger nanoemulsion fields show greater nanoemulsification efficiency [29]. Besides, the nanoemulsion areas of various surfactants increased in the following order: lecithin < tween 20 < tween 80. Therefore, tween 80 was chosen for further research. Tween 80 was helpful since it increased the permeability of numerous drugs or combined with nanoparticles to improve brain-specific delivery of several drugs [30].

3.3. Fabrication of nanoemulsions using rotor-stator high-speed stirring

With O/S ratios of 1:1 and 1:2, the oil film presented on samples 1 and 2 surfaces. Sample 3, with an O/S ratio of 1:3, showed the particle size distributions ranged from 100 to 500 nm (Table 4). With an O/S ratio of 1:5, samples 4 and 6 showed large and unspecified PDI values, respectively. Using rotor-stator systems, the particle sizes of samples with less surfactant (1:3) were better than others with more surfactant. This result could explain why increasing the surfactant amount may lead to increasing the surface viscosity, inhibition of surfactant diffusion to the aqueous phase, and the formation of nanoemulsions with big particle sizes [31].

Regarding the weight of the extracts, there was a similarity of mode values of samples 3 and 5 when increasing the extract of *M. integrifolia* pericarps, with mode values of 262.1 and 261.7 nm, respectively. There was a slight difference in *Z*-average and PDI values between them, with *Z*-average values of 246.6 and 343.1 nm and PDI values of 0.417 and 1.109, respectively. Regarding the rotor-stator types, the results showed that the particle size distribution of sample 3 (made by rotor/stator/slit sizes of 10/16/1 mm) was smaller than that of sample 7 (made by rotor/stator/slit sizes of 15/20/1.5 mm), with mode values of 262.1 and 616.1 nm, respectively. The droplet sizes of the sample made by a rotor with a slit size of 1.0 mm were smaller than others made by the device with a larger slit size. Reducing the rotor slit size increases the shear intensity. It decreases the volume shear of the emulsion, which leads to a higher energy input into the emulsion and a smaller droplet size [32; 14].

3.4. Fabrication of nanoemulsions using ultrasonicator probe sonicator.

There was still oil film in sample 8 at an O/S ratio of 1:1 due to the lack of surfactant. Overall, the droplet sizes of nanoemulsions processed by ultrasonicator probe sonicator were smaller than that by high shear mixer (< 300 nm). The particle size distributions of samples 9–11 were obtained in 100–500 nm, much better than when using a high-speed stirrer (Table 4). The samples with the larger O/S ratios showed smaller *Z*-average values of 127.4, 186.8, and 240.0 nm, respectively. In addition, sample 11 exhibited the largest mode and PDI values of 333.5 nm and 0.709, respectively. The difference between sample 11 and others was caused by increasing surfactant. From the results, 1/2 or 1/3 (the oil/the surfactant) was suitable for this device since the droplet sizes of all samples (< 200 nm) were nano size (100–500 nm). Compared to high-speed stirring, nanoemulsion produced by ultrasonic technology has better droplet sizes. Although using the ultrasonicator has benefits such as low equipment cost and ease of cleaning and maintenance, it should be used to produce small nanoemulsions in the laboratory since more energy is required and more heat was generated.

4. Conclusions

The average extraction yields of the pericarp and seed coat of *M. integrifolia* were 13.40% and 1.27%, respectively. The extraction yield of reflux method was more effective than that of maceration. Aqueous acetone was suitable solvent for the extraction of phenolic compounds. Besides, nanoemulsions preparing by the ultrasonic technology was better results than that formed by a high shear mixer. All particle size results belonged to the nano-sized region.

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Conflicts of Interest

The authors declare that there is no conflict of interests.

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