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EMULSIFYING PROPERTIES AND ANTIOXIDANT ACTIVITY OF PHOSPHOLIPIDS FRACTIONS FROM PALM PRESSED FIBRE

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ABSTRACT

Palm pressed fibre (PPF) is a by-product of crude palm oil (CPO) extraction and has great potential as a source of phospholipids (PL). PPF was extracted from chloroform:methanol to extract total lipid, and polar lipid was separated using methanol. PL from PPF was fractionated by ethanol, and subsequently, the ethanol-soluble fraction was fractionated by acetone. Four fractions were obtained: ethanol-soluble, ethanol-insoluble, ethanol-acetone soluble, ethanol soluble-acetone insoluble. All fractions had different PL molecular species compositions. Different compositions of PL in the fractions resulted in different hydrophilic-lipophilic balance (HLB) values, emulsifying properties, and antioxidant activity. HLB value of crude PL from PPF was 6.07 that was suitable for water in oil (w/o) emulsion indicated by low emulsifying activity index (EAI) and emulsion stability index (ESI) in oil-water (o/w) emulsion systems. Ethanol-soluble and ethanol-acetone soluble fractions exhibited better emulsifying properties and higher HLB values of around 10. Crude PL and its fractions showed a potential antioxidant activity to prevent bulk soybean oil in a single antioxidant system. Synergism with α-tocopherol appeared in a binary antioxidant system indicated by low peroxide value compared to the single one. PL composition affected the ability of PL fractions as antioxidants.

Keywords: binary antioxidant system, emulsifying properties, fractionation, HLB value, palm pressed fibre.

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INTRODUCTION

Phospholipids (PL) are a mixture of phosphate-containing lipids mainly used in the food industry as emulsifiers. Commercially, PLs are well known as lecithin, a term that is commonly used for a mixture of amphiphilic compounds that mainly comprised of PL and other minor constituents of glycolipids, carbohydrates, triglycerides, and a trace amount of water, free fatty acids (FFA), and sterols (Arnold *et al*., 2014). PL are also used as wetting agents, lubricants, texture modifiers, and dispersants to improve the characteristics of food products such as solubility of instant powder, less stickiness of candy, good rheology of chocolate and spreads, and others. PLs are the cell membrane constituents, and the human body recognises these compounds well, thus, PL is a safe emulsifier.

The primary source of PL is of vegetable origins, mainly oil-bearing seeds (Ahmad and Xu, 2015) such as soybean, sunflower (Cabezas *et al.*, 2012), canola (Xie and Dunford, 2019), rapeseed (Arnold *et al.*, 2014), camelina seed (Belayneh *et al.*, 2018) that are obtained during degumming in oil refining. Vegetable lecithin is a mixture of naturally occurring lipids containing more than 50% PL

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(Robert *et al.*, 2020). The most widely used lecithin in the food industry is soybean lecithin. PL of animal origins are the egg yolk (Bernardo *et al.*, 2019), bovine milk (Nagai, 2012), and fish (Malaplate *et al.*, 2019). Different sources of PL have different characteristics and emulsifying properties.

Some residual PL is also found in the industrial food wastes such as rice bran (Garba *et al.*, 2020; Sun *et al.*, 2020), fish discard (Topuz *et al.*, 2021) and palm pressed fibre (PPF) (Choo *et al.*, 2004). PPF is a by-product of the extraction process of CPO. The fibrous structure of PPF entraps 5%-7% of the residual oil (Ooi *et al.*, 2019) with good oxidative stability that is contributed by PL (Lau *et al.*, 2006). PPF residual oil contains 46 800 ppm of PL. The quantity of PPF PL is considerably high due to palm oil's huge world production (Choo *et al.*, 2004). Thus, the PPF PL utilisation as the source of PL is important as an alternative to conventional PL sources.

PL are amphiphilic in nature because they have a hydrophobic tail consisting of two fatty acid chains at the sn-1 and sn-2 positions of a glycerol moiety and a polar phosphate head group at sn-3 (Robert *et al.*, 2020) that are attached to other hydrophilic molecules such as ethanolamine (PE), choline (PC), inositol (PI, phosphatidylinositol), serine (PS, phosphatidylserine), and glycerol (PG, phosphatidylglycerol). The most common bioactive PL in biological vegetable cells are PC, PE, PI and PS (Robert *et al.*, 2020).

Lecithin is usually modified or fractionated to have tailor-made lecithin that is suitable for the processing and characteristics of food products. The chemical modification of PL is aimed to improve emulsifying properties by means of acid or base-catalysed hydrolysis, acetylation, hydroxylation, and hydrogenation (Li *et al.*, 2019). Modification by enzyme is used to liberate fatty acid from sn-1 or sn-2 of the glycerol backbone of PL, resulting in better emulsifying properties for o/w emulsion (Estiasih *et al.*, 2013). Fractionation is one way to improve the emulsifying properties of PL without modification of their chemical structures. The fractionation of PL is usually conducted by solvent to have different polarity of PL. PL are the main components of native lecithin and have different solubility in acetone or ethanol (Cabezas *et al.*, 2012; Xie and Dunford, 2019). Solvent fractionation is used to obtain PL fractions with different compositions compared to the origin (Cabezas *et al.*, 2012). The PPF PL is still limitedly explored, and its fractionation is important to evaluate as the alternative of commonly modified lecithin.

Lecithin fractionation aims to obtain fractions with desirable functionalities. Ethanol is commonly used as the solvent for fractionation (Xie and Dunford, 2019). PC has better solubility in alcohol than the other PL. The ethanol-soluble fraction containing a high PC/PE ratio is found in the ethanol-soluble fraction, but the insoluble fraction has a low PC/PE ratio. PI, lysophosphatidylinositol (LPI), and phosphatidic acid (PA) are less soluble in alcohol and enriched in the ethanol-insoluble fraction (Cabezas *et al.*, 2012). PL fractionation using ethanol and acetone as solvents is compatible with the regulations required for subsequent food and pharmaceutical applications (Bernardo *et al.*, 2019).

The main function of PL for food, pharmaceutical, and cosmetic industries is as an emulsifier since PL naturally have amphiphilic properties. Lecithin has been widely applied in both o/w and w/o emulsions (Zembyla *et al.*, 2020) by facilitating small droplet formation (Wu *et al.*, 2019), positioning themselves at the interface to stabilise emulsions (Zembyla *et al.*, 2020), and also reducing the interfacial tension (Wu *et al.*, 2019). The advantage of lecithin over other emulsifiers is the ability to provide a strongly negative surface charge over a wide pH range (Warren *et al.*, 2019). Different PL fractions exhibit different emulsifying properties. The PC-enriched fraction has improved o/w emulsifying properties; meanwhile, the fraction enriched with PI is suitable for w/o emulsions (Xie and Dunford, 2019). It is important to elucidate the suitability of PPF PL and its fractions as an emulsifier for the o/w or w/o system.

PL has been reported to inhibit lipid oxidation in bulk oil and emulsions, but the stabilisation mechanism still remains controversial (Cardenia *et al.*, 2011). PL are sometimes used as antioxidants in foods. Several mechanisms have been proposed for the antioxidant activity of PL. PL are chelators that could bind prooxidative metals, participating in Maillard reaction during lipid oxidation to produce antioxidative compounds, altering the other antioxidants to o/w emulsions' interface, or in a bulk oil regenerating primary antioxidants (Cui and Decker, 2016). One mechanism of the phospholipid-tocopherol synergism is the donation of a hydrogen atom by PL to the tocopheryl radical (Doert *et al.*, 2012). The ternary mixture of PL, tocopherol and ascorbic acid are powerful antioxidants for stabilising highly unsaturated oils (Doert *et al.*, 2017). Antioxidant activity of PPF PL and its fractions and synergism with other antioxidants have not been evaluated yet.

The emulsifying properties and antioxidant activity of PL from PPF and its fractions are interesting to examine since PPF might be an alternative source of PL. Ethanol and acetone fractionation of PL from PPF are supposed to produce different PL molecular species compositions with different emulsifying properties and antioxidant activity. The emulsifying properties of crude PL from PPF and its fractions were evaluated in the o/w emulsion system as a food major emulsion

system. The antioxidant activities of crude PL and PL fractions in a single and synergistic binary system of bulk oil were also evaluated.

MATERIALS AND METHODS

Materials

PPF was kindly obtained from a palm oil milling, Surya Dumai Inc., Riau Province, Indonesia. The variety of oil palm was *tenera*. The PPF was stored at room temperature before being used. The chemicals for PL extraction from PPF, emulsifying properties, and antioxidant activity analysis were analytical grade from Merck (Germany). Commercial soybean oil was used for emulsifying properties and antioxidant activity analysis. The purity of acetone and ethanol for fractionation was 99.7% and 98.0%, respectively. Standards for PL analysis comprised of PE (purity ≥98.0% by TLC), PC (purity ≥99.0% by TLC), PI (purity ≥98.0% by TLC), PA (purity ≥98.0%), PG (purity ≥99.0% by TLC), DPG (purity ≥97.0% by TLC), and TLC Silica Gel G60 plates were obtained from Sigma Aldrich (Singapore).

Extraction of Total Lipid from PPF

Yunoki *et al.* (2008) method was used to extract total lipids from PPF (*Figure 1*). A total of 100 g of fine and dry PPF was extracted twice with 100 mL chloroform:methanol (2:1 v/v), and once with 100 mL chloroform:methanol (1:2 v/v), subsequently. Each extraction was conducted for 1 hr at room temperature, and the solvent and the cake were separated by filtering through coarse filter paper. The solvent of each extract was removed with a rotary evaporator, and all lipid extracts were mixed.

Separation of Crude PL from Extracted Lipid

The Palacios and Wang (2005) method, with a slight modification, was used to separate crude PL from total lipid (*Figure 2*). Total lipid, 10 g, was dissolved in 40 mL chloroform for 10 min. The mixture was then centrifuged for 10 min at 1542 x g to separate the chloroform-soluble (supernatant) and insoluble fractions (residue or sub-natant). Chloroform from the supernatant was removed by rotary evaporation. The residue after evaporation was then extracted again with 30 mL of chloroform. After centrifugation at 1542 x g for 10 min, the chloroform from the supernatant of this second extraction was removed.

The chloroform-insoluble fraction from the first extraction was mixed with the chloroform-insoluble fraction from the second extraction. After removing the residual chloroform by rotary evaporation, the chloroform-insoluble fractions were extracted by methanol, 20 mL, to obtain methanol-soluble fraction. Methanol was removed from this fraction, and crude PL was obtained.

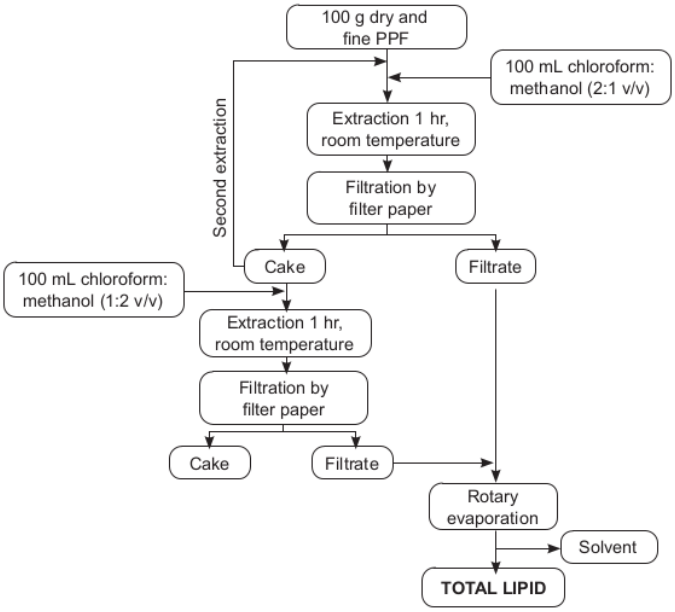

Figure 1. Total lipid extraction from PPF.

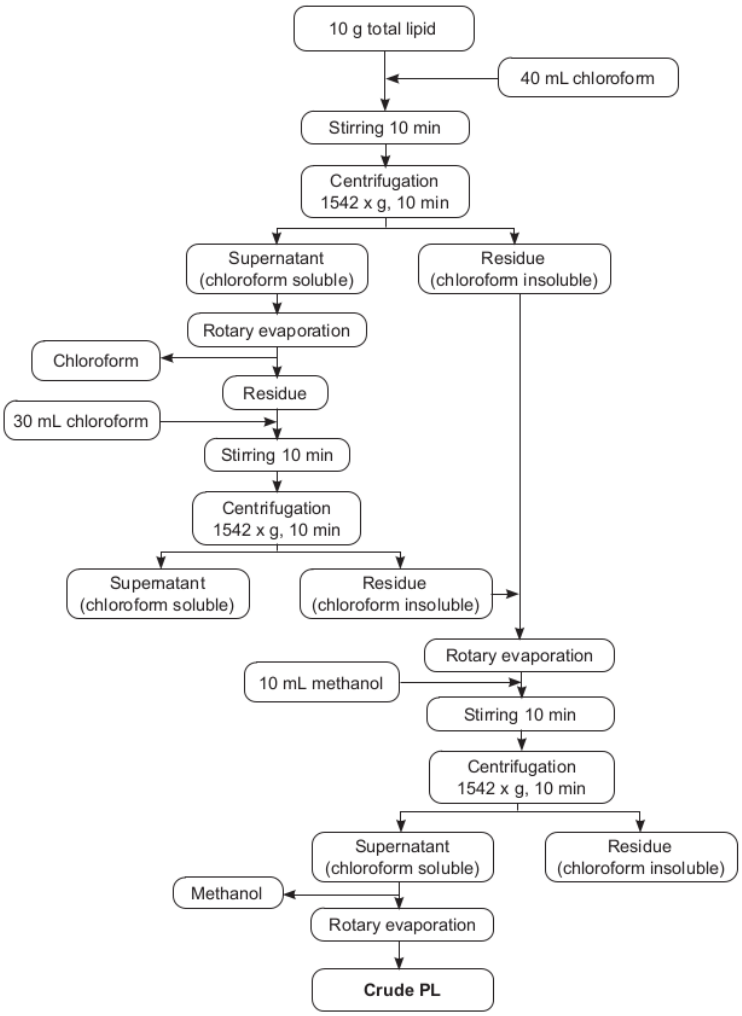

Figure 2. Separation of crude PL from the total lipid of PPF.

PL Fractionation

The fractionation of PL was conducted by using acetone and ethanol at room temperature (*Figure 3*). Extraction by ethanol was used as the first fractionation. Crude PL, 5 g, was dissolved in 20 mL ethanol and agitated for 60 min by using a magnetic stirrer. The mixture was centrifuged for 10 min at 15 000 x g. The residue was an ethanol-insoluble fraction, and the supernatant contained an ethanol-soluble fraction. A rotary evaporator removed ethanol from the supernatant and residual ethanol in an ethanol-insoluble fraction to obtain ethanol-soluble and ethanol-insoluble fractions.

Some of the ethanol-soluble fraction was taken and further fractionated by using acetone through dissolving in acetone at a ratio of the ethanol-soluble fraction to acetone 1:4 (w:v). The mixture was agitated for 60 min by a magnetic stirrer. Acetone soluble fraction and acetone-insoluble fraction were separated by centrifugation at 15 000 x g for 10 min. A rotary evaporator removed the residual acetone from each fraction. By solvent fractionation, four fractions were obtained: Ethanol-soluble fraction, ethanol-insoluble fraction, ethanol-acetone soluble fraction, and ethanol soluble-acetone insoluble fraction; and crude PL without fractionation as a control.

Analysis of PL Profile

The analysis of the PL profile was according to the method of Nzai and Proctor (1998). This analysis used silica gel G60 plates with a

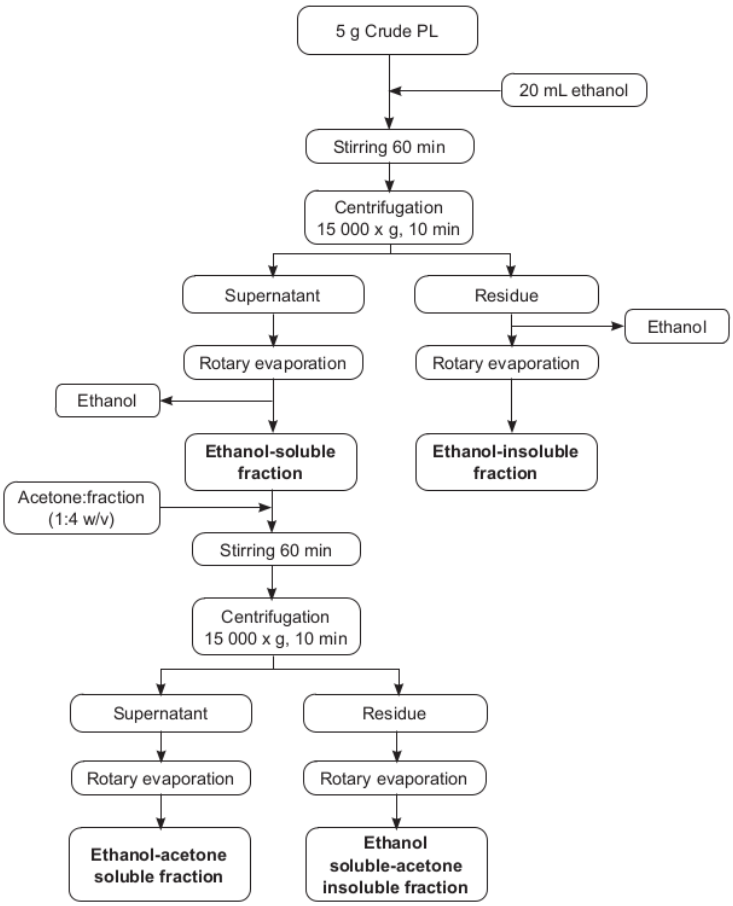

Figure 3. Fractionation of crude PL from PPF.

chloroform:methanol:water (75:25:3 v/v/v) system as a developing solution. Each standard PL, crude PL, and the fractions were dissolved separately in a chloroform:methanol solution (95:5 v/v) at 1 mg mL^{-1} concentration. After activating the plates by drying for 20 min in an oven at 100°C, the PL standard, crude PL, and the fraction solution in a volume of 10 µL were spotted separately. The plate development was run for 40 min, and then the plates were dried at room temperature for 10 min, and heated in the oven for 10 min at 90°C. TLC scanner (CAMAG TLC Scanner 3) was used for the quantification of the molecular species of PL. PL concentration in the samples was calculated on the basis of area curves of each PL from the densitogram of the TLC scanner.

Analysis of Emulsifying Properties

Hydrophilic lipophilic balance (HLB). The suitability of PL from PPF and its fractions to stabilise emulsions are measured by HLB value. HLB plays an essential role in balancing the interfacial tension between oil and water in an emulsion. The HLB value permits the ability of an emulsifier to stabilise w/o or o/w emulsions (Alam *et al.*, 2020). HLB value ranges from 0 to 20 and was measured based on saponification number (*S*) (AOAC, 1997) and acid value (*Av*) (AOAC, 1980) with the Equation (1) as follows:

$$\text{HLB} = 20\ (1 - \frac{S}{Av}) \quad (1)$$

Emulsifying activity index (EAI) and emulsifying stability index (ESI). In this study, the capability of crude PL and its fraction in stabilising o/w emulsions was evaluated by EAI and ESI. The EAI represents the emulsifiers' ability to be absorbed in the interface during the emulsion formation, and a higher EAI represents a stronger emulsification capability (Chen *et al.*, 2020). The emulsifying

activity index represents the surface area covered by the emulsifier. Thus, the higher EAI value indicates the emulsifier's greater ability to produce smaller droplets of the dispersed phase. The turbidity method was used to measure EAI and ESI of crude PL from PPF and its fractions (Pearce and Kinsella, 1978). The emulsion was prepared by mixing 0.5 g of soybean oil with 9.5 mL of distilled water to have an oil fraction of 0.5%. As much as 0.05 g crude PL or its fractions was added to the mixture of oil and water. The mixture was homogenised by agitating with a homogeniser (Ultra Turrax) at a speed of 12 500 rpm for 1 min. EAI and ESI were measured by mixing 0.12 mL of emulsion with phosphate buffer (0.005 M, pH 7) containing sodium dodecyl sulphate (SDS) 0.3% to a volume of 10 mL, to have an oil volume fraction of 6×10^{-5}. When emulsion and phosphate buffer were mixed, the time was t_0 and the absorbance at 500 nm was A_0. The mixture was allowed to stand for 10 min (t_{10}), and the absorbance at t_{10} was expressed as A_t. The following Equation (2) was used to calculate EAI ($m^2\ g^{-1}$) and ESI (min):

$$EAI = \frac{(2.303)(2)(A_0 \times \text{dilution factor})}{C \times \Phi \times 10\,000} \quad (2)$$

A_0 = absorbance at min 0
C = emulsifier concentration (g/mL)
Φ = oil volume fraction
A_t = absorbance at min 10
Δt = 10 min

$$ESI = A_0 \times \frac{\Delta t}{A_0 - A_t}$$

Antioxidant Activity

The antioxidant activity measurement was based on the ability to inhibit lipid peroxidation of bulk soybean oil. The ability of crude PL and its fraction as a single antioxidant was measured in 5 mL of soybean oil that was added by crude PL or its fractions at concentration of 0.5% or 5000 ppm (w/v). The mixture was incubated at 37°C for five days, and every day a sample was removed to measure the peroxidation value by the ferric thiocyanate method (Hills and Thiel, 1946). For binary antioxidant systems, crude PL and its fraction were added separately to soybean oil. α-tocopherol of 1000 ppm was added to the system and homogenised by agitation with a homogeniser (Ultra Turrax) at the speed of 1500 rpm. The mixture was incubated for five days, and every day, the peroxide value was measured. Commercial antioxidant BHT at a concentration of 100 ppm and α-tocopherol at a 1000 ppm concentration were used as controls.

Statistical Analysis

Correlations between PL molecular species from PPF PL fractions and EAI, ESI, and the mean of peroxide values during five days incubation in single and antioxidant systems were conducted by multivariate Pearson Correlation using IBM SPSS Statistic 24. All the treatments were replicated three times. The data were analysed by one-way analysis of variance using SPSS software, and Duncan Multiple Range Test further analysed the differences among fractions at a significance level of 5%.

RESULTS AND DISCUSSION

PL Composition of Crude PL from PPF and its Fractions

Crude PL and its fractions had a different PL profile (*Table 1*). Predominant PL in the crude PL from PPF were PC, followed by PG, PE, DPG, PI and PA in decreasing order of concentration. Three species of PL and their concentrations from PPF extracted by hexane were PC>PE>PG, and four species of PL were found in the ethanol extract (PC>PE>PG>PA) (Choo *et al.*, 2004). Meanwhile, Choo *et al.* (2004) reported four species of PL in PPF, which were PG>PC>PE>LPC (lysophosphatidylcholine). The concentration of PL in the ethanol extract was much higher than in hexane extract. This study found more molecular species of PL. Gradual extraction using chloroform:methanol to extract total lipids and separation of polar lipids using methanol from chloroform-insoluble fraction resulted in more PL species. The purity of PL extract from PPF was 61.67%. Choo *et al.* (2004) reported that the concentration of PL in the ethanol extract was 4.68% and in the hexane extract was 0.14%. Chua *et al.* (2009) extracted the PL from PPF by chloroform:methanol 2:1 (v/v), and the concentration of PL in the extract ranged from 1.01% to 5.33%, depending on the ratio of dry PPF to solvent (w/v).

The FFA found in the extract were impurities. FFA were more polar than triglycerides that were soluble in the methanol during PL separation. Less polar PL, such as PG, DPG, PI and PA, were found in the extract. The extraction of total lipid from PPF used the mixture of chloroform and methanol. Polar and nonpolar lipids were extracted in this step. Separation of PL from total lipids was conducted by chloroform that PL was insoluble and more polar residue than chloroform soluble matters. Further extraction by methanol extracted more polar lipid such as PC. However, less polar PL including PE, PG, DPG, PI and PA were also found due to the suitable polarity with methanol.

Fractionation by ethanol resulted in an increased purity of the PL (*Table 1*). PC increased dramatically in the ethanol extract compared to the original crude PL. Lower PI, PE, PG and DPG were found meanwhile, PA concentration was similar to the origin. The concentration of PC increased in the ethanol-soluble fraction. On the other hand, the ethanol-insoluble fraction was enriched with PI and PE (Cabezas *et al.*, 2009). In this study, the purity of the ethanol-insoluble fraction was higher than that of the soluble one. A sharp increase of PE and PI was found in the ethanol-insoluble fraction. This finding is in accordance with the results of the study by Xie and Dunford (2019) that PE, PI and LPI increased in ethanol-insoluble fraction. This finding was in accordance with the Palacios and Wang (2005) report that PC increased after ethanol extraction. In this study, the PL quantification was based on the percentage of each PL peak area curve. The decrease in one species of PL would increase other species of PL. PE, PG and DPG concentration increased in ethanol insoluble fraction, indicating that the ethanol-insoluble fraction contained higher, less polar PL.

Further fractionation of ethanol-soluble fraction by acetone resulted in an acetone soluble fraction that was enriched with PC. Meanwhile, the ethanol soluble-acetone insoluble fraction was rich in PI, PE, PG and DPG. The concentration of more polar PL was found in the ethanol-acetone soluble. In contrast, the concentration of less polar PL was found in the ethanol soluble-acetone insoluble fraction. Acetone and ethanol have different polarities in that ethanol is more polar than acetone. Acetone is usually used to purify PL so that the acetone insoluble fraction matters indicate the PL's purity. Acetone is used to separate nonpolar lipids from the PL. Therefore, the less polar PL species were found in the acetone-insoluble fraction.

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Emulsifying Properties of Crude PL from PPL and its Fractions

An emulsion contains two immiscible liquids that are thermodynamically unstable. One way to stabilise emulsions is to reduce interfacial tension in the boundary between the immiscible liquids by using emulsifiers. An emulsifier's effectiveness to stabilise emulsions might be affected by several factors, such as HLB value, concentration, and emulsifier flexibility to reposition at the interface (Feng *et al.*, 2018). The HLB values of crude PL from PPF and its fraction are shown in *Table 2*.

The crude PL from PPF had a low HLB value of 6.07 that might be contributed by PL molecular species composition (*Table 1*). The predominant PL in crude PL from PPF were less polar PL, such as PA, PG, DPG, PI and PE, which caused hydrophobic moieties to dominate, resulting in low HLB value. Some lecithin ingredients are not particularly good at stabilising o/w emulsions when used in isolation because of low or intermediate HLB numbers of 2 to 8 (Mc Clements and Gumus, 2016). Thus, the crude PL from PPF might be suitable for w/o emulsion.

TABLE 1. PL PROFILE (%) CRUDE PL FROM PPF AND ITS FRACTIONS

PL	Crude PL (%)	Fractions (%)			
		Ethanol soluble	Ethanol insoluble	Ethanol-acetone soluble	Ethanol soluble-acetone insoluble
Free fatty acid (FFA)	18.61 ± 0.87c	21.28 ± 0.98b	10.53 ± 0.46d	24.21 ± 1.04a	11.19 ± 0.54d
Phosphatydic acid (PA)	7.02 ± 0.51c	7.44 ± 0.24b	7.36 ± 0.36bc	7.65 ± 0.34ab	8.34 ± 0.56a
Phosphatidylglicerol (PG)	21.24 ± 1.65b	15.79 ± 0.98c	24.67 ± 1.23a	14.46 ± 0.88c	21.72 ± 1.02b
Diphosphatidylglicerol (DPG)	8.88 ± 0.26c	7.92 ± 0.56d	17.38 ± 0.32b	7.72 ± 0.44d	18.18 ± 0.56a
Phosphatidylethanolamine (PE)	13.21 ± 1.01b	3.31 ± 0.23d	13.13 ± 0.89b	7.47 ± 0.78c	16.31 ± 1.24a
Phohatidylinositol (PI)	8.87 ± 0.47c	3.23 ± 0.28e	21.28 ± 0.89a	3.91 ± 0.13d	16.72 ± 1.12b
Phosphatidylcholine (PC)	22.17 ± 1.09c	41.03 ± 2.34a	3.65 ± 0.23e	34.58 ± 1.78b	7.54 ± 0.65d
Purity (%)	61.67 ± 1.05	65.78 ± 0.78	71.44 ± 0.67	67.06 ± 0.59	68.49 ± 0.98

Note: The data with different notification means different significantly at α<0.05 for the same row.

TABLE 2. EMULSIFYING PROPERTIES AND ANTIOXIDANT ACTIVITY OF CRUDE PL FROM PPF AND ITS FRACTIONS

PL and fraction	HLB value	EAI ($m^2 g^{-1}$)	ESI (min)	Average PV (meq kg^{-1}) during 5-day storage	
				Single antioxidant system	Binary antioxidant system
Crude PL	6.07 ± 0.24c	197.80 ± 7.89c	62.51 ± 1.29c	5.20 ± 0.23c	4.18 ± 0.11cd
Ethanol soluble	10.85 ± 0.58a	282.36 ± 2.98b	78.43 ± 2.21b	6.70 ± 0.37a	5.12 ± 0.23b
Ethanol insoluble	5.68 ± 0.16b	196.88 ±3 .45c	52.70 ± 1.87d	5.35 ± 0.52bc	4.33 ± 0.16c
Ethanol-acetone soluble	10.11 ± 0.78a	309.81 ± 9.88a	94.07 ± 3.43a	5.80 ± 0.26b	4.27 ± 0.11c
Ethanol soluble-acetone insoluble	5.54 ± 0.22b	179.49 ± 4.56d	43.89 ± 2.01e	5.87 ± 0.32b	4.05 ± 0.11d
α-tocopherol				6.30 ± 0.66ab	6.55 ± 0.22a
Butylated hydroxytoluene				3.63 ± 0.40d	3.83 ± 0.21e

Note: The data with different notification means different significantly at α<0.05 for the same column.

The fractionation by ethanol increased the HLB value of the ethanol-soluble fraction to 10.85, which is suitable for the o/w emulsion (Alam *et al.*, 2020). Increasing HLB value was related to the dramatic increase in PC, meanwhile less polar PL decreased. Analysis of Pearson correlation showed that PC positively correlated with HLB value. The ethanol-insoluble fraction had a significantly lower HLB value of 5.68 (α<0.05) than the HLB value of the original crude PL from PPF. A sharp increase in concentration was found in PI and DPG.

Meanwhile, other less polar PL increased to some extent. Increasing PI and DPG might lower HLB values, as indicated by a negative Pearson correlation between both with HLB value. The HLB value of the ethanol soluble-acetone insoluble fraction was not significantly different from that of the ethanol-insoluble fraction. The composition of PL in both fractions was similar, which was lacking in PC but rich in the less polar PL. Meanwhile, the ethanol-acetone soluble fraction had an increased HLB value compared to the crude PL from PPF. This HLB value was not significantly different (α<0.05) from that of the ethanol-soluble fraction. Both fractions were rich in PC, and the composition of PL in the fractions was also almost similar. Based on the HLB value, fractionation of crude PL from PPF by ethanol and acetone produced fractions with different HLB values. However, the ethanol-soluble and ethanol-acetone soluble HLB values were not significantly different, and the same characteristics were found for ethanol insoluble and ethanol soluble-acetone insoluble fractions. Therefore, further acetone fractionation after ethanol fractionation was not required because no significant HLB values were produced.

Different fractions of PL from PPF showed different PL compositions that might result in different emulsifying properties. Data in *Table 2* show that PL fractions which were able to create the highest specific surface area were found in the ethanol-acetone soluble fraction. EAI of this fraction was significantly higher than that of the ethanol-soluble fraction, although the HLB values of both fractions were similar. Although the ethanol-acetone soluble fraction had a lower PC concentration (*Table 1*), it had a higher purity and lower concentrations of less polar PL such as DPG and PG, thus, revealing better emulsifying properties than the ethanol-soluble fraction. PL with a high PC level was reported to produce smaller oil droplets (Cabezas *et al.*, 2012). Analysis of Pearson correlation showed that PC attributed positively with EAI. Other PL had a negative correlation. Meanwhile, the presence of a high amount of PI corresponded to w/o emulsions (Belayneh *et al.*, 2018), which means the lower concentration of PI in ethanol-acetone soluble fraction resulted in better emulsifying properties for o/w emulsion.

The EAI of the ethanol-insoluble fraction was not significantly different from that of crude PL from PPF, although both PL compositions were also different. Crude PL had higher PC than the ethanol-insoluble fraction, but the levels of PI and PG, as less polar PL, were much lower. Thus, the EAI values of both were similar. PI, PG, DPG and PA are less polar PL suitable for w/o emulsions; therefore, the capability to create smaller droplets in o/w emulsion was lower. The ethanol soluble-acetone insoluble fraction showed the lowest EAI and also had the lowest HLB value. This fraction was suitable for w/o emulsion because PG and other less polar PL dominated the PL composition.

The ESI indicates the emulsifier capability to remain at the (o/w) interface in emulsions after a period of storage (Chen *et al.*, 2020). Emulsion stability is related to PL's molecular species composition (McClements and Gumus, 2016). The molecular composition of the PL affects emulsion stability (Guiotto *et al.*, 2013). Data in *Table 2* show that the ESI of crude PL and its fraction was in accordance with the EAI. The highest EAI of PL fraction also

revealed the highest ESI. The smaller droplets produced during emulsification that was stabilised by PL fractions resulted in higher emulsion stability. The highest ESI was found in the ethanol-acetone soluble fraction, and the order pattern of ESI of each PL fraction was similar to the EAI. The PL molecular species composition similarly affected ESI and EAI. PL fractions containing a high level of PC produced o/w emulsions with high stability, and PL fractions containing less polar PL resulted in lower o/w emulsion stability. Analysis of Pearson correlation showed that PC had a positive contribution to ESI. Meanwhile, other PL showed a negative correlation.

Antioxidant Activity

Some studies revealed that PL has the ability to prevent oxidation in o/w emulsion (Cardenia *et al.*, 2011) and bulk oil (Cui *et al.*, 2014; Charanyaa *et al.*, 2019). In this study, crude PL capability from PPF and its fractions to inhibit oxidation was evaluated in bulk soybean oil. The primary oxidation products (peroxide value, pv) were monitored during 5 day-storage at 37°C. A commercial synthetic antioxidant butylated hydroxytoluene (BHT) and a natural antioxidant α-tocopherol were used as controls.

In a single antioxidant system, crude PL and its fractions revealed antioxidant activity (*Figure 4*). Their activity at a concentration of 5000 ppm was comparable to BHT's antioxidant activity at 100 ppm. α-tocopherol at a 1000 ppm concentration failed to retard oxidation indicated by a sharp increase of peroxide value after 4-day storage. The ethanol-insoluble fraction showed the highest antioxidant activity among PL fractions and always had the lowest daily peroxide value. Except for the ethanol-insoluble fraction, crude PL from PPF and its fractions did not show significantly different antioxidant activity. Fractionation by ethanol and acetone did not produce fractions with better inhibition of peroxidation.

The groups of PE and PA in PL structures significantly contributed to the antioxidant activity, while inositol showed little effect (Doert *et al.*, 2017). The PL fraction with the highest PI (ethanol-insoluble fraction) showed the lowest antioxidant activity in this study. Cardenia *et al.* (2011) reported that PL's antioxidant activity was affected by the type of phosphate head group. PC with oleic or palmitic acids are the most effective antioxidant to inhibit hydroperoxide formation in an emulsion. The PL antioxidant activity is related to their ability to form a layer at the emulsion droplets interface or to chelate metals. A physical barrier to oxygen contributes to PC's antioxidant activity in the w/o emulsion (Choe and Choe, 2016). The different structures of emulsions and bulk oil might result in different mechanisms of PL inhibition to oxidation. In this study, PC and PA showed a positive correlation with average daily peroxide values; other PL were negatively correlated.

Some mechanisms have been proposed for the antioxidant activity of PL. Some PL effectively inhibits lipid oxidation in emulsion due to their capacity to scavenge free radicals (McClements and Gumus, 2016) by hydrogen donation (Choe and Choe, 2016). PL could chelate prooxidative metals, alter other antioxidant locations, regenerate primary antioxidants, and produce antioxidative compounds through Maillard reactions during lipid oxidation (Cui and Decker, 2016). In this study, the proposed mechanism of antioxidant activity of crude PL from PPF and its fractions was scavenging activity because no prooxidant metal and primary antioxidants were added to the bulk oil, and there was no protein or amine as precursors for Maillard reaction in the system.

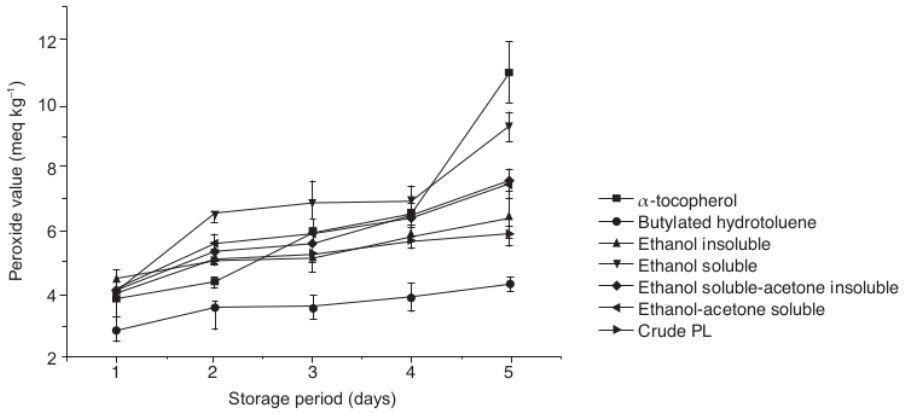

Figure 4. The effect of crude PL from PPF and its fractions in inhibiting peroxidation of bulk soybean oil during 5-day storage in a single antioxidant system. The concentration of crude PL and its fractions was 5000 ppm, BHT was 100 ppm, and α-tocopherol was 1000 ppm.

Crude PL and its fractions were added to soybean oil to evaluate their ability to regenerate the primary antioxidant α-tocopherol. *Figure 5* shows that α-tocopherol in the bulk oil-PL system produced lipid oxidation of 4.30% to 35.27% lower than lipid oxidation of a single antioxidant system, depending on the PL fractions. All of the PL fractions and crude PL exhibited synergism with α-tocopherol, which was indicated by lower daily peroxide values of soybean oil added by the binary antioxidant system. The reductions were 4.30% for insoluble ethanol fraction, 35.27% for ethanol-soluble fraction, 26.32% for ethanol-acetone soluble fraction, 30.96% for ethanol soluble-acetone insoluble fraction, and 19.52% for crude PL. The lowest antioxidant activity was found in the binary system stabilised by the ethanol-soluble fraction, and the highest one was produced by the ethanol soluble-acetone insoluble fraction (*Table 2*). Other PL fractions did not show a significant difference in antioxidant activity.

PL molecular species composition affected the antioxidant activity in the binary system. The ethanol soluble-acetone insoluble fraction had the highest level of PE. PE is a PL species with an amine group that has the ability to donate H^+ to a tocopheryl radical. PL and phenolic antioxidants such as α-tocopherol synergistically inhibit lipid oxidation (Doert *et al.*, 2012). PE and PS can regenerate α-tocopheryl quinone radical to α-tocopherol. As a non-amine PL, PC does not show a synergistic effect on lipid oxidation inhibition (Doert *et al.*, 2017). The ethanol soluble-acetone insoluble fraction had the highest level of PE and showed the highest antioxidant activity. Meanwhile, the ethanol-soluble fraction had the highest PC level and exhibited the lowest antioxidant activity in the binary system compared to other fractions.

All of the PL fractions had similar levels of PA, and presumably, this PL had an equal contribution to the antioxidant activity of each fraction. Phosphoric moiety in PA contributes to the antioxidant activity of PL (Doert *et al.*, 2017). PA acts as an antioxidant synergist and acidity regulator (Silva and Lidon, 2016). PA from PL fractions might also regenerate α-tocopherol, thus having a role in lowering the peroxidation of soybean oil with a binary antioxidant system. Other PL species, PG and DPG, had not been reported for their role as antioxidants. Both had glycerol moiety, which might have no role in regenerating primary antioxidants, such as inositol in PI. PI has been reported to have no capability in regenerating primary antioxidants (Doert *et al.*, 2012).

Process for Scaling Up

Before scaling up, it is very important to consider the feasibility of PL production from PPF and its fractionation. Production of PL from PPL requires some equipment in the pilot plant scale, including a mixing tank to extract the total lipid, separation of PL from total lipid, and fractionation. A pilot-scale centrifuge is required to separate supernatant and sub-natant (residue) in total lipid extraction, PL separation, and fractionation. Evaporation solvent or residual solvent from total lipid, crude PL, and its fractions after centrifugation required a pilot-scale rotary evaporator. Scale-up is very important for commercial production of such products, including PL and its fractions from PPF. Some parameters should be established in scaling up, such as time for extraction, separation, centrifugation, and the speed for stirring and centrifuging. Therefore, further study is required to produce PL from PPF and its fractions on a pilot plant and commercial scale.

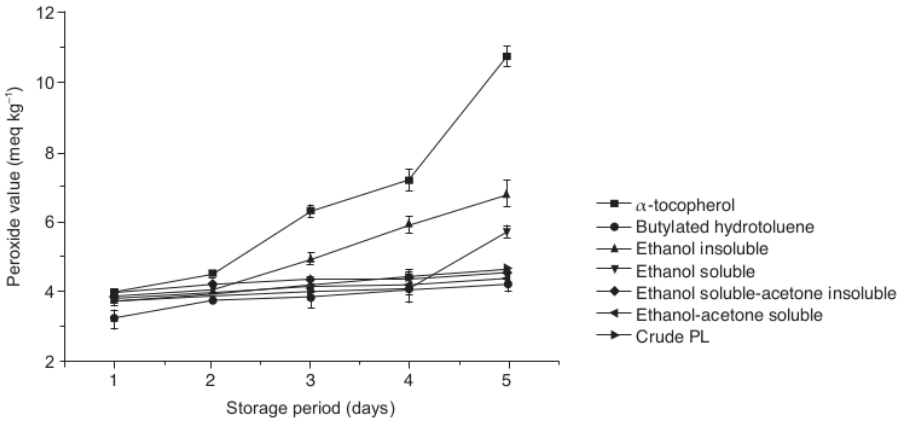

Figure 5. The effect of crude PL from PPF and its fractions (each was 5000 ppm) in inhibiting peroxidation of bulk soybean oil during 5-day storage in binary antioxidant systems with α-tocopherol of 1000 ppm. BHT of 100 ppm was used as control.

CONCLUSION

PPF could be used as the source of PL. Crude PL from PPF and its fractions had different PL profiles, and the predominant PL in crude was PG, followed by PC, PE, DPG, PI and PA. Fractionation by ethanol resulted in the increasing purity of the PL with PC as predominant PL in the ethanol-soluble fraction, and less polar PL was dominant in the ethanol-insoluble fraction. The crude PL from PPF had a low HLB, and fractionation by ethanol produced a soluble fraction with a higher HLB value, but a lower value was found in the insoluble fraction. Further fractionation by acetone did not produce significantly different HLB values. It was found that crude PL from PPF was not suitable to stabilise o/w emulsions. Ethanol and acetone fractionation resulted in better emulsifying properties and emulsion stability. Ethanol soluble fraction from PPF PL was a good candidate as an emulsifier for o/w food emulsion systems.

Meanwhile, crude PPF PL and ethanol insoluble fraction were better for the w/o system. Fractionation by ethanol was sufficient to stabilise o/w emulsion system. Crude PL from PPF and its fractions showed antioxidant activity in the bulk oil system and exhibited synergism with α-tocopherol. Antioxidant activity was affected by PL composition in that the high level of PC had a significant contribution at preventing lipid oxidation in a single antioxidant system. PPF PL was better to use as an antioxidant in binary rather than single systems. Fractionation by ethanol followed by acetone was required to have better antioxidant activity.

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