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Research article

Characterization of Red Palm Oil from *Elaeis guineensis* Produced by Multi-step Fractionation and Assessment of Anti-inflammatory Activity

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Abstract: An economical processing method for red palm oil production was developed for small and medium-sized businesses (SMEs). Crude palm oil was obtained from oil palm fruits (*Elaeis guineensis*). The optimal degumming conditions were determined. A multi-step fractionation at 34, 25, and 15 °C with the cooling rates of 60 °C /hr and 10 °C /hr were compared. The optimal degumming conditions were 20 min at a temperature of 90 °C, phosphoric acid to citric acid of 0.06:0.04%, and a cooling rate of 10 °C /hr. The red palm oil contained 43.54% oleic acid, 747.22 mg/kg carotenoids, 856.91 mg/kg vitamin E, and 55.70% yield at optimal conditions. The anti-inflammatory activity in RAW264.7 macrophages stimulated with lipopolysaccharide (LPS) showed that the oil could reduce the production of nitric oxide and prostaglandin E2 (PGE2) in LPS-stimulated RAW264.7 cells. The oil attenuated LPS-induced mRNA expression of inflammatory mediators and cytokines by inhibiting the NF-κB and MAPK signaling pathways in LPS-stimulated macrophages. The results suggest that the developed process could effectively produce red palm oil with promising anti-inflammatory properties.

Keywords: Red palm oil; Multi-step fractionation; Red palm fat; Anti-inflammation; Macrophages

1. Introduction

Red palm oil is generally produced using a mild process that does not involve hydrogenation, bleaching, or deodorizing processes to preserve the phytonutrients originally present in the crude palm oil [1]. Advanced techniques [2,3,4] have been used to produce red palm oil. However, advanced methods are complicated, expensive, and unsuitable for SMEs. Acid degumming is a more economical technique to produce red palm oil.

The oil palm (*Elaeis guineensis*) is Thailand's most widely cultivated variety [5, 6]. Degumming of crude palm oil derived from oil palm (*Elaeis guineensis*) using phosphoric and citric acids was previously reported by Chompoo et al. [7]. The acids dissociate metal–phospholipid complexes and remove impurities from crude palm oil. Optimizing temperature, time, and acid ratio is essential to maximize oil and carotenoid yields while preserving phytonutrients. Previous studies have identified optimal degumming conditions [7] but left gaps in understanding the effects of acid ratio and

broader temperature ranges. This study employed a factorial design to address these gaps and determine optimal conditions for pilot-scale production.

Primarily, the condition that provides high oil and carotenoid yields at a minimal heating temperature and operation time will be selected as the desired condition for red palm oil production. In the separation step, instead of using the centrifuge that has been used to a limited extent by the SMEs, the water washing process at 60 °C was developed to separate gum from the oil. The free fatty acids are eliminated by neutralization with 6–9% NaOH solution [8]. The suitable amount was reported at 7.0%, adequate to decrease the free fatty acid level to below the maximum of 0.6%, as stated by the product standards [9].

Pilot-scale equipment should be used to run the degumming and neutralization processes to reduce upscaling hazards. The optimal degumming and neutralization conditions attained from the lab-scale experiments were further used as guidance for scaling up the equipment at the capacity of 120 kg/batch, which is a suitable size for SMEs. The primary objectives for the development were to meet the SMEs' requirements without compromising the product quality. The design involved a variety of considerations, including cost, ease of handling by personnel, ease of maintenance, and degumming efficiency.

Following the neutralization step, the oil is moved to a fractionation step, a controlled cooling of the oil to form partially crystallized fat. The residual liquid, palm olein, is separated from the solid palm stearin fraction by filtration. The fractionation can be conducted in a single-step or multi-step operation. Multi-step fractionation is more suitable for bulk processing as the oil's viscosity restricts the crystallizing capability of a single-step procedure [10]. Moreover, the multi-step operation produces a variety of fats suitable for many applications. The fractionation of red palm oil using a single temperature of -15 °C has been reported [7]. However, based on a thorough review of earlier studies, little information about multi-step fractionation for Thailand's palm oil output is available. In this work, the fractionation was controlled in several stages at the designated intermediate temperatures to obtain red palm fats with different properties. The effect of the cooling rate was investigated, while the physicochemical properties of the red palm oil and red palm fats were analyzed to suggest their potential usage.

Finally, the anti-inflammatory properties of red palm oil produced through the multi-step fractionation process were assessed. Red palm oil possesses potential health benefits as it contains carotenoids, vitamin E, sterols, phospholipids, glycolipids, and squalene [11]. These phytonutrients have several beneficial effects, including enhancing human immunity and reducing the risk of developing diseases like cancer [12] and coronary heart disease [13]. Carotenoids have several therapeutic effects, including anti-cancer, immunomodulatory, anti-inflammatory, anti-bacterial, anti-diabetic, and neuroprotective [14,15]. Tocotrienols contribute inhibitory effects, including anti-tumor, anti-cancer [16,17], cardiovascular protection [18], and neuroprotection [19]. While studies focusing on individual phytonutrients in red palm oil have highlighted beneficial effects, current evidence concerning the relationship between red palm oil consumption and health effects remains inconclusive. Inflammation is a defensive response to harmful stimuli important for defensive immunity against external and internal infections [20]. Macrophages play a significant host role, including autoimmune diseases, inflammation, and infections [21]. Activated macrophages are created by inflammatory cytokines and associated inflammatory mediators such as nitric oxide and prostaglandin E2 (PGE2), which are produced by inducible nitric oxide synthase (*iNOS*) and cyclooxygenase-2 (*COX-2*), respectively [22,23]. Previous studies of anti-inflammatory capacity found that the essential oils from plants can reduce the production *in vitro* of nitric oxide, PGE2, *iNOS, COX-2*, tumor necrosis factor α (*TNF-*α), interleukin-1β (*IL-1*β), and *IL-6* by lipopolysaccharide (LPS)-activated RAW 264.7 macrophages [20,24,25]. Leaf extracts of palm oil reduce inflammation in aged Sprague Dawley rats [26]. The tocotrienol-rich fraction of palm oil suppresses the production of inflammatory mediators in human monocytic cells, demonstrating anti-inflammatory properties [27]. However, no studies have been published on the anti-inflammatory activity of red palm oil from *E. guineensis* on RAW264.7 cells, making this investigation a novel contribution to understanding its potential therapeutic effects."

2. Materials and Methods

2.1 Materials

The crude palm oil produced from oil palm (*Elaeis guineensis*) was obtained from Srisuk Palm Co., Ltd., Prachuap Khiri Khan, Thailand (11°59'08.7"N, 99°47'30.4"E). The chemicals used were either analytical or HPLC grade.

2.2 Methods

2.2.1 Physicochemical properties of crude palm oil

The crude palm oil samples were analyzed for free-fatty acid value [28], phosphorus [29], iron [29], copper [29], and melting point (DSC, Perkin Elmer, USA).

2.2.2 Degumming conditions

The degumming procedure was carried out by following the method of Chompoo et al. [7], with modification. Optimization of the degumming process was performed using 3 independent variables: (1) mixing ratio of acids, (2) temperatures, and (3) degumming times, while the responses include oil yield, carotenoids yield, and level of free fatty acid. In the first part of the experiment, 200 g of crude palm oil was degummed at 90 °C, 100 °C, and 110 °C with continuous stirring at 120 rpm (Heidolph, MR 3001 model, Germany) for 20, 25, and 30 min. The acids used in this study were a mixture of 85% phosphoric acid and 99.5% citric acid at percentage ratios of 0.06:0.04, 0.08:0.02, and 0.10:0.0 by oil weight. The acid mixtures were prepared as a 10% solution before mixing with the crude palm oil. The sedimented gum was separated from the oil by washing the acid oil with warm water at 60 °C. The washing was applied continuously until the pH of the washing water was back to neutral. Next, the sample was evaporated to remove water at 80 °C under vacuum conditions for 30 min using an evaporator (IKA, SV10 model, Germany). The degummed oil was analyzed for oil yield [30], carotenoid content [30], and free-fatty acid [28].

2.2.3 Neutralization process

After the oil was degummed, the free-fatty acid was removed through a neutralization process. A calculated amount of 4 N NaOH was added to the oil according to the molecular weight of oleic acid [31]. The neutralization was controlled at 80 °C for 30 min with constant stirring [32]. Separation of the resulting soap was carried out using a separate vessel. The oil samples were vacuum-dried at 50 °C and 150 mmHg until the moisture content was less than 0.1%. The oil was analyzed for oil yield [30], carotenoid content [30], free fatty acid [28], phosphorus content [29], iron [29], and copper [29].

2.2.4 Evaluation of the degumming and neutralization parameters by the pilot-scale equipment

The pilot-scale equipment was developed based on the optimal conditions obtained from the lab-scale experiments. The equipment included the degumming/neutralization tank and the evaporation tank, with a 120 kg/batch capacity. The 2-layer tank was made of 304 stainless steel and equipped with a 3,000 W heater and a 120 rpm stirring blade (Figures 1 and 2). Oil yield [30], carotenoid content [30], free-fatty acid [28], phosphorus [29], iron [29], and copper [29] of the oils produced by the lab-scale and the pilot-scale equipment were compared to evaluate the efficiency of the pilot-scale equipment.

2.2.5 Multi-step fractionation

The multi-step fractionation was performed after the neutralization process. Fractionations were compared at a fast cooling rate of 60 °C /hr and a slow cooling rate of 10 °C /hr. The fractionation was performed in a 3,000 ml glass reactor (Yuchem, JGR1L model, China) equipped with a cooling system (Drywell, SDC-6 model, China). The fractionation temperatures were predetermined by using the melting point of crude palm oil as guidance, where the first crystallization temperature was observed at 36 °C. The fractionation procedure started by adjusting the oil temperature to 70 °C for 15 min to dissolve fat crystals [33,34] and then reducing the oil temperature to predetermined levels. When the oil temperature reached each predetermined level, the temperature was maintained for 30 min. The sample was then vacuum filtered through the 50 μm filter cloth to separate the red palm fats from the oil fraction. The obtained red palm fats and the red palm oil were analyzed for fatty acids composition [29], yield [30], iodine value [28], free fatty acid [28], peroxide value [28], melting point (DSC, Perkin Elmer, USA), carotenoids content [30], vitamin E content

[35] and solid fat content (Pulse Nuclear Magnetic Resonance Spectrometers, Bruker, Germany). The optimal cooling rate was determined based on the yield and carotenoid content of the obtained red palm oil.

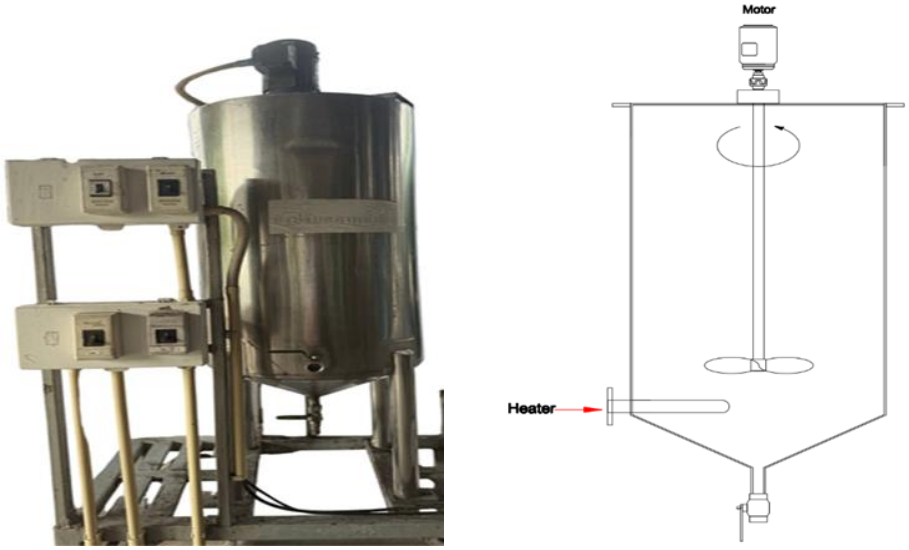

Figure 1. Degumming and neutralization tank.

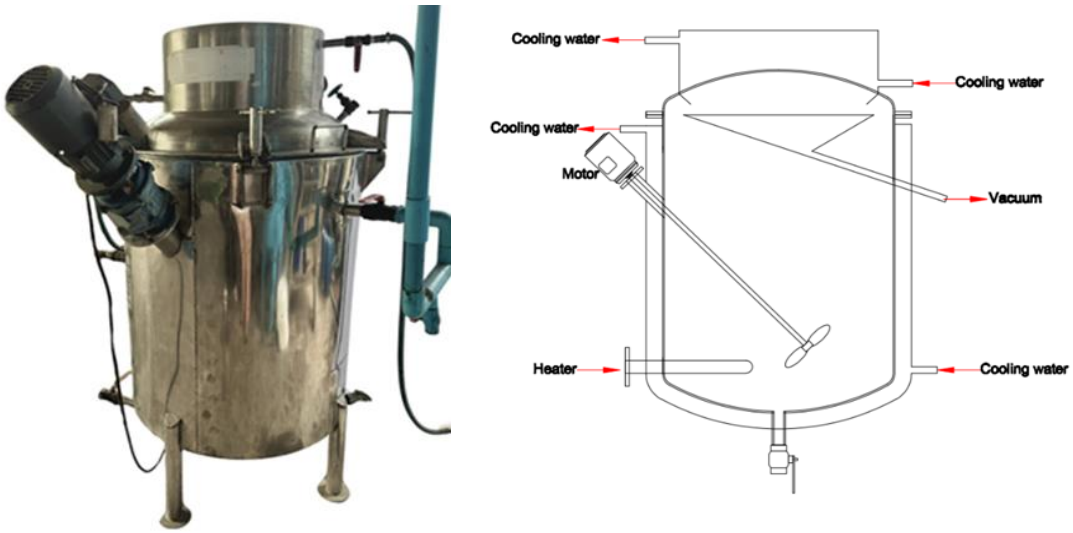

Figure 2. Evaporation tank.

2.2.6 Assessment of anti-inflammatory activity of red palm oil

2.2.6.1 Assay for macrophage proliferation

The proliferation of RAW264.7 cells, a murine macrophage cell line obtained from ATCC, was assessed using the WST-1 colorimetric assay. The suspensions of RAW264.7 cells (100 µL) were plated in a 96-well plate (1×10^5 cells/well) for 24 hr. The cells were exposed to different concentrations of fatty acids (5, 10, 20, and 40 µg/mL) for 24 hr. The cell cultures were kept in a humidified atmosphere containing 5% CO_2 at 37 °C. Following a 24-hour incubation period, cell proliferation was assessed using the WST-1 assay. Optical density was recorded at 450 nm using a microplate reader (EL-800; BioTek Instruments, Winooski, VT, USA). The absorbance (A) was converted into a macrophage proliferation (%) = $As/Am \times 100$, where *As* and *Am* represent the sample's and medium's absorbances, respectively.

2.2.6.2 Measurement of nitric oxide production

The concentration of nitric oxide was determined using the Griess method to measure its quantity [36]. This method detects the level of accumulated nitrite in the cell-free supernatants within the culture

medium of RAW264.7 cells. Briefly, cells were seeded (1 × 10^5 cells/well) in a 96-well plate for 24 hr. The nitric oxide levels were assessed after stimulation with various concentrations of red palm oil (5, 10, 20, and 40 μg/mL) and stimulation with LPS (1 μg/mL). Specifically, 100 μL of culture supernatant was combined with 100 μL of the Griess reagent (Sigma-Aldrich, USA). As detailed in previous reports, the nitrite concentration in the culture supernatant was quantified by measuring absorbance at 540 nm using a microplate reader [37].

2.2.6.3 Measurement of PGE2 production

The level of PGE2 in the culture medium's supernatant was measured using ELISA kits (Enzo Life Sciences, Farmingdale, NY, USA) following the manufacturer's guidelines.

Real-time PCR analysis

The RAW264.7 cells were seeded onto a 24-well plate at 2×106 cells/well density and incubated with red palm oil and LPS. After 24 hr incubation, cells were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The concentration of RNA was measured with a spectrophotometer before constructing cDNA with an oligo-(dT) 20 primer and Superscript III RT (Invitrogen, Carlsbad, CA, USA). Real-time quantitative PCR was conducted using a Light Cycler instrument (CFX Connect Real-Time PCR system, Bio-Rad) with the Fast Start DNA Master SYBR Green I kit. The results were expressed as the ratio of optimal density to *β-actin*, serving as an internal reference. The primer nucleotide sequences utilized were as previously documented [23]. The primer nucleotide sequences employed are listed as shown in Table 1.

Table 1. Primers sequences.

Genes	Primer	Sequence (5' to 3')
IL-1β	Forward	ATGGCAACTATTCCTGAACTCAACT
	Reverse	CAGGACAGGTATAGATTCTTTCCTTT
IL-6	Forward	TTCCTCTCTGCAAGAGACT
	Reverse	TGTATCTCTCTGAAGGACT
IL-10	Forward	TACCTGGTAGAAGTGATGCC
	Reverse	CATCATGTATGCTTCTATGC
TNF-α	Forward	ATGAGCACAGAAAGCATGATC
	Reverse	TACAGGCTTGTCACTCGAATT
iNOS	Forward	CCCTTCCGAAGTTTCTGGCAGCAGC
	Reverse	GGCTGTCAGAGCCTCGTGGCTTTGG
COX-2	Forward	AGAAGGAAATGGCTGCAGAA
	Reverse	GCTCGGCTTCCAGTATTGAG
β-actin	Forward	TGGAATCCTGTGGCATCCATGAAAC
	Reverse	TAAAACGCAGCTCAGTAACAGTCCG

2.2.6.4 Western blot analysis

The RAW264.7 cells (1 × 10^5 cells/well) were seeded in a 6-well plate and incubated with red palm oil, followed by stimulation with 1 μg/mL of LPS for 24 hr. After the removal of the supernatants, the cells were harvested. The total cellular proteins were extracted by incubating cells in a lysis buffer (Cell Signaling Technology, USA) on ice, and their concentration was measured using the BCA kit (Beyotime Biotech Co. Ltd., Shanghai, China). The protein (30 μg) was separated into 10% SDS-polyacrylamide gels. The separated proteins were electrophoretically transferred to nitrocellulose membranes and blocked with a 5% skimmed milk solution. After that, the membranes were incubated with primary antibody: anti-NF-κB-p65 (BD Biosciences), anti-phospho-JNK (pT183, pY185) rabbit polyclonal, anti-phospho-pERK1/2 (pT202, pY204) rabbit polyclonal, anti-phospho-p38 (pT180, pY182) rabbit polyclonal, and α-tubulin (Cell Signaling Technology, USA) at 4 °C overnight. Then, the membrane was incubated with 1:2000 goat anti-rabbit IgG-HRP (Proteintech® Co., Manchester, UK) for 2 hr at 25 °C. The protein signal was detected using an enhanced chemiluminescence (ECL) kit following the manufacturer's instructions. The bands were obtained using Image Lab software under the ChemicDoc TM imaging system.

2.2.7 Statistical analysis

All experiments were performed in triplicate (n = 3). The data are presented as the mean values with the standard deviation. Statistical differences were tested using a one-way analysis of variance (ANOVA, except the degumming study was tested using the 3x3x3 factorial in a completely randomized design. Duncan's multiple-range test determined the significance of differences between means. Statistical analyses were performed using the Statistical Analysis System (SAS Institute, Cary, NC, USA).

3. Results and Discussion

3.1 Physicochemical properties of crude palm oil

The physicochemical properties of crude palm oil were analyzed to determine their applicability as raw material for red palm oil production. The crude palm oil samples contained 639.90 ± 5.30 mg/kg carotenoids and 763.25 ± 21.75 mg/kg total vitamin E, indicating a rich source of valuable phytonutrients. However, when compared to the standard of cooking oil [9], the crude palm oil contained a free-fatty acid value of 3.10 ± 0.14% (max 0.6%), phosphorus content of 111 mg/kg (max 10 mg/kg), copper content of 55.56 mg/kg (max 10 mg/kg), and iron content of 20.27 mg/kg (max 0.10 mg/kg). High free-fatty acid level decreases storage stability. High amounts of phosphorus and trace metals contribute to a cloudy appearance, less sensory acceptance, and a short oil shelf-life [7]. Thus, the degumming and neutralization steps are required to produce red palm oil. Nevertheless, it is crucial that the operations were performed under the optimal process conditions as phytonutrients in the crude oil might be significantly decomposed by the severe conditions.

The DSC analysis showed that the melting point of the crude palm oil was 33.75 ± 0.63 °C. The result suggested that the first fractionation temperature of the multi-step fractionation process would be approximately 32-36 °C, and fractionation should begin at about 36 °C. This finding agreed with the previous study when observing an initial crystallization temperature of crude palm oil [31].

3.2 The optimal degumming condition

Optimization of degumming was conducted to determine the interaction between the ratio of phosphoric acid and citric acid, temperature, and degumming time. The desirable condition was optimized for oil yield, carotenoid yield, and free-fatty acid content. Oil yield and carotenoid yield are prioritized criteria for red palm oil production. Free fatty acid levels affect the neutralizing cost, and carotenoids are destroyed less during neutralization [11]. The acid has been used to remove the non-hydratable phosphatides by changing them to the hydratable form. Phosphoric acid forms a precipitate with Ca and Mg. Citric acid forms a complex with Ca and Mg. Both acids are commonly used because they are food-grade and sufficiently substantial. Phosphoric acid is a stronger acid compared to citric acid. Thus, phosphoric acid is known for its ability to remove gum. However, excessive use of phosphoric acid can increase the phosphorus content in the oil [38]. Therefore, it is important to carefully consider the mixing ratio of phosphoric acid and citric acid. This study examined different percentage ratios of phosphoric acid and citric acid (0.06:0.04, 0.08:0.02, and 0.10:0.00). Temperature and time also influence the stability of phytonutrients and production costs, necessitating a thorough assessment of optimal conditions.

Effects of acid ratio, temperature, and time on oil yield, carotenoids yield, and free-fatty acid are presented in Table 2 and Figure 3. The 3x3x3 factorial in a completely randomized design was used to optimize the interaction effects between variables for the degumming process. The results showed that the interaction between acid ratio and temperature on oil yield was statistically significant ($p \leq 0.05$). In contrast, the difference between acid ratio, temperature, and time on carotenoid yield and free fatty acid was insignificant ($p > 0.05$).

Figure 3 (A) shows the interaction between acid ratio and temperature on oil yield ($p \leq 0.05$). Figure 4 demonstrates the interaction between acid ratio and temperature on oil yield at each heating time. Results showed that oil yield increased with the increase in the ratio of phosphoric acid when using a high degumming temperature (110 °C). In contrast, the oil yield decreased with the increase in the ratio of phosphoric acid when degumming at medium and low temperatures (100 °C and 90 °C). The reason might be due to the outcome of degumming efficiency, which is favored by strong acid and elevated temperature. When using a high ratio of phosphoric acid and high heat treatment, gum or phosphatide was better changed into the polar state. This form allows the separation of gum more easily. Therefore, the oil yield was higher.

Degumming temperature, heating time, and ratio of acids did not show significant differences in terms of a reduction in the carotenoids yield ($p>0.05$) (Table 2). Still, a decrease tended to be noticeable when using higher temperatures and longer heating times, particularly when using 0.06% phosphoric acid and 0.04% citric acid per oil weight (Figure 3). Yields of carotenoids decreased in response to temperature and heating time because heat enhances the decomposition of carotenoid molecules and increases the conversion of *trans* to *cis* isomers [39]. Phosphoric acid is more potent than citric acid. Thus, a higher phosphoric acid concentration caused a lower carotenoid yield, particularly when degumming at elevated temperatures for a long time.

For free fatty acids, Table 2 showed that the acid ratio or temperature did not significantly influence the level of free fatty acids but was significantly increased with longer degumming times ($p \leq 0.05$). The lowest free fatty acid content was observed at 20 min. The free fatty acid level is important for the subsequent neutralization step. A higher amount of remaining free fatty acid requires more NaOH. When alkalin increases, it affects production cost and oil quality.

Overall, the results of this study indicated that the optimal degumming condition was 20 min at 90 °C and that phosphoric acid to citric acid was 0.06:0.04%. Chompoo et al. found the optimal degumming conditions of crude palm oil were at a temperature of 90 °C and a heating time of 25 min [7]. The time discrepancy may be due to the preparation of the degumming acids. The acid mixture used by Chompoo et al. was not diluted before mixing with crude palm oil, while the one used in this study was diluted to a 10% acid solution. Using a dilute acid solution may increase the hydration of the hydratable phosphatides, leading to reduced degumming duration. Using a longer time in the previous study led to lower oil yield and carotenoid content, as reported [7], compared to this study. These results confirmed that exposure of crude palm oil to a longer degumming time resulted in lower oil recovery and reduced stability of carotenoids.

Table 2. Effects of percentage ratio of phosphoric acid and citric (RA), temperature (TEMP), and time (TIME) in degumming process on oil yield, carotenoids yield, and free fatty acid.

Treatment		Oil yield	Carotenoids yield	Free fatty acid
RA	0.06:0.04	87.78 ± 3.86	80.67 ± 5.53	4.37 ± 0.63
	0.08:0.02	86.25 ± 6.24	79.08 ± 5.65	4.42 ± 0.65
	0.10:0.00	87.54 ± 5.02	78.10 ± 5.92	4.08 ± 0.71
TEMP	90	84.29 ± 4.16	78.78 ± 6.45	4.20 ± 0.73
	100	86.58 ± 5.12	78.52 ± 4.72	4.20 ± 0.68
	110	90.70 ± 3.89	80.55 ± 5.88	4.46 ± 0.59
TIME	20	86.48 ± 5.36	79.90 ± 5.46	3.99 ± 0.65[a]
	25	87.52 ± 5.01	78.87 ± 5.39	4.29 ± 0.62[ab]
	30	87.56 ± 5.09	79.09 ± 6.44	4.58 ± 0.63[b]
P-VALUE				
	RA	0.3937	0.2559	0.1008
	TEMP	<0.0001	0.3692	0.2028
	TIME	0.5950	0.7844	0.0037
	RA*TEMP	0.0354	0.0760	0.0521
	RA*TIME	0.5769	0.4858	0.4709
	TEMP*TIME	0.7898	0.6519	0.8661
	RA*TEMP*TIME	0.6627	0.5823	0.8642

[a, b] Means with similar letters in each sub-RED class within a column did not differ from another at $p<0.01$.

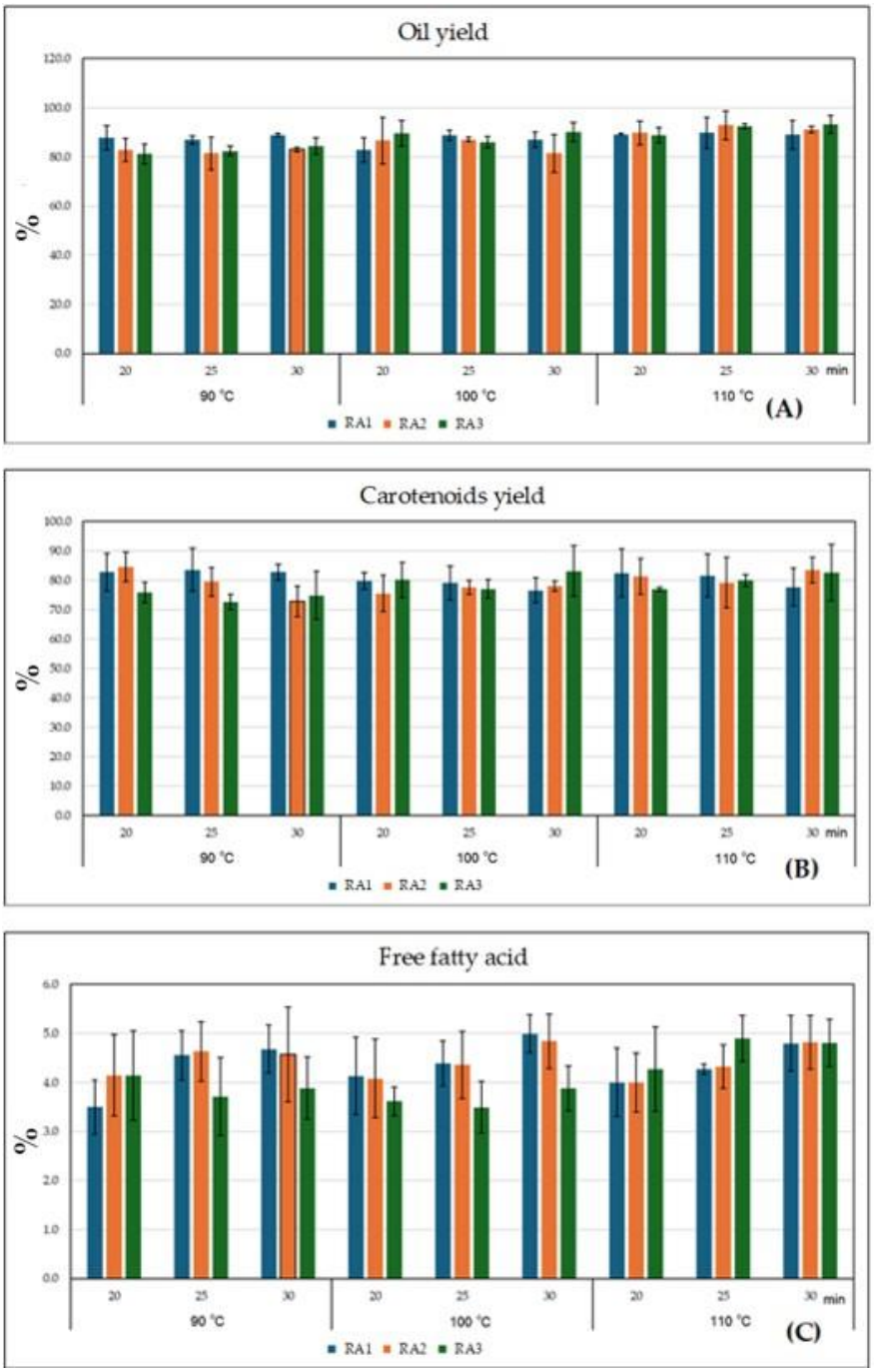

Figure 3. (A) oil yield, (B) carotenoids yield, and (C) free fatty acid of the degummed oil influenced by percentage ratio of phosphoric acid and citric acid (RA1 = 0.06:0.04, RA2 = 0.08:0.02 and RA3 = 0.10:0; temperature (90, 100 and 110 °C) and time (20, 25, 30 min).

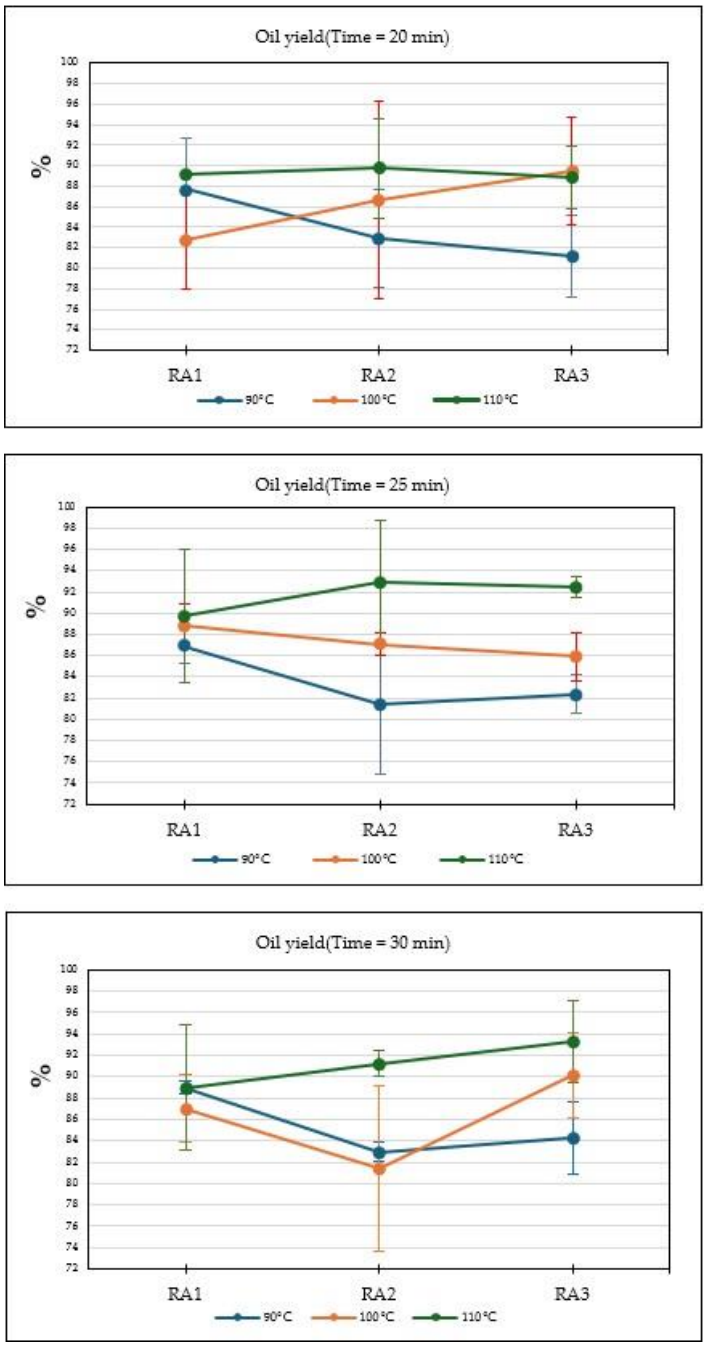

Figure 4. Oil yields are influenced by the percentage ratio of phosphoric acid and citric acid (RA1 = 0.06:0.04, RA2 = 0.08:0.02 and RA3 = 0.10:0; temperature (90, 100, and 110 °C) and time (20, 25, 30 min).

3.3 The neutralization procedure

The oil degummed under the optimal conditions contained 3.5 ± 0.55% free fatty acid, 625.92 ± 6.59 mg/kg carotenoids content, 82.7 ± 6.49% carotenoids yield, 68.49 mg/kg phosphorus, 19.25 mg/kg iron, and 0.84 mg/kg copper. Compared to the amounts in the crude palm oil, the most significant decreases occurred in phosphorus and copper. However, compared with the standard of edible oil, the free-fatty acid and phosphorus levels were still high. After neutralization, the oil meets the quality standard of edible oil, with 74.48 ± 9.39% oil yield, 637.60 ± 8.64 mg/kg carotenoids, 74.21 ± 9.35% carotenoids yield, 0.15 ± 0.0 5 mg/kg iron, and < 0.10 mg/kg copper. The free fatty acid level decreased from 3.5 ± 0.55% to 0.46 ± 0.05%, which is within the standard of not more than 0.6%. Meanwhile, the phosphorus content reduced from 68.49 mg/kg to an undetectable level.

Washing the soap using warm water reduced the phosphorus content because phosphorus was trapped in the soap [31,40]. In this study, the volumes of washing water and oil were 1.5:1.0, which was higher than that reported by Mayamol et al. [34], where the volumes of water and oil were 1:1. The reason for using more amount of washing water in this study was because a lower concentration of NaOH was employed in neutralization. As a result, oil and soap developed a more substantial emulsion power, so additional water was required to remove the soap.

3.4 Evaluation of the degumming and neutralization parameters at the pilot-scale equipment

The pilot-scale equipment was employed to run the degumming and neutralization processes to reduce the risks of upscaling. The optimal conditions attained from the lab-scale experiments were further used to guide the equipment scaling up. The important properties of the oils were examined to compare the degumming and neutralization efficiencies between the lab-scale and pilot-scale equipment (Table 3). Results indicated higher efficiency of the pilot-scale prototype, particularly oil and carotenoid yields. The increase in oil yield could be due to the better design outcome. The laboratory experiment was performed using a beaker, whereas the pilot scale employed a conical bottom tank with a drainage port inserted at the lowest point of the cone. This design is better for decanting processes, including oil separation from the sediment gum. Similarly, the pilot scale yielded the oil with lower contents of P and Cu ($p \leq 0.05$). The reason is that the pilot-scale mixing was conducted using the stirring blade, which is more effective mixing equipment than the laboratory magnetic stirrer.

Table 3. Properties of the oils after degumming and neutralization by lab and pilot scale equipment.

Oil samples	Properties	Lab-scale equipment	Pilot-scale equipment
After degumming	Oil yield (%)	84.35 ± 10.69^{b}	95.46 ± 1.90^{a}
	Free fatty acid (%)	3.24 ± 0.55^{a}	3.21 ± 0.31^{a}
	Carotenoids (mg/kg)	625.92 ± 6.59^{a}	630.92 ± 5.54^{a}
	Carotenoids yield (%)	82.70 ± 6.49^{b}	94.23 ± 2.50^{a}
	P (mg/kg)	68.49 ± 0.29^{a}	59.34 ± 0.34^{b}
	Fe (mg/kg)	19.25 ± 0.10^{a}	15.34 ± 0.09^{a}
	Cu (mg/kg)	0.84 ± 0.03^{a}	0.51 ± 0.02^{b}
After neutralization	Oil yield (%)	74.48 ± 5.39^{b}	80.74 ± 3.97^{a}
	Free fatty acid (%)	0.46 ± 0.05^{a}	0.39 ± 0.04^{a}
	Carotenoids (mg/kg)	637.60 ± 8.64^{a}	643.90 ± 6.53^{a}
	Carotenoids yield (%)	74.21 ± 6.35^{b}	81.37 ± 4.67^{a}
	P (mg/kg)	ND	ND
	Fe (mg/kg)	0.14 ± 0.04^{a}	0.11 ± 0.02^{a}
	Cu (mg/kg)	< 0.10	< 0.10

The values presented were the means and the standard deviations of triplicate analysis. The mean values within the same row followed by different superscript letters were significantly ($p \leq 0.05$) different. ND = not detected

3.5 The multi-step fractionation process

Crude palm oil contains a high amount of saturated fat that gives a cloudy texture at room temperature. Appropriate control of the fractionation conditions is important to yield desirable-quality products [31]. According to the melting point of crude palm oil, the fractionation temperature was suggested to begin at 36 °C. The exact fractionation temperatures varied according to the fatty acid composition retained in the sample. The shorter chains and less saturated fatty acids have lower crystallization temperatures. The influence of minor components was also reported [41]. Then, predetermination in the fractionation temperatures before measuring the exact temperatures was essential for selecting the appropriate fractionation points. This procedure was performed by cooling the sample until a visible solid fraction developed. At that point, the temperatures were recorded. The oil samples were found to have fractionation temperatures at 34, 25, and 15 °C. The red palm fats are separated at first, second, and third fractionation temperatures, which are

called first, second, and third red palm fat, respectively. The red palm oil was attained after the final separation of the red palm fats (Figure 5).

Figure 5. Red palm fats were obtained from the multi-step fractionations at 34 °C, 25 °C, and 15 °C and red palm oil was obtained at 15 °C (from left to right).

The effect of cooling rate on the oil products' crystallization temperatures, yield, and carotenoid content was investigated. Table 4 showed that cooling rates did not affect the crystallization temperatures and slightly affected the yield and carotenoid content of the red palm fats and oil. A slow cooling rate of 10 °C/hr required a longer operating time, but it produced red palm oil with higher yield and carotenoid content. When a slight decrease in temperature occurred, the triglyceride molecules that had a slight difference in crystallization temperatures were crystallized at different periods. This allowed the separation of fat fractions within the oil. Conversely, when fast cooling was applied, triglyceride molecules with a wide range of crystallization temperatures were crystallized almost simultaneously. These crystals accumulated to one another to form a larger granular. Subsequently, the oil fraction was partly trapped inside the granule, leading to a lower oil yield. For red palm oil production, the slow cooling was a better condition for the multi-step fractionation process.

Table 4. Yields and carotenoids content of red palm fats and red palm oil obtained from multi-step fractionation under different cooling rates.

Samples	Fractionation temperatures (°C)	Yield (%)		Carotenoids (mg/kg)	
		Slow cooling (10 °C /hr)	Fast cooling (60 °C /hr)	Slow cooling (10 °C /hr)	Fast cooling (60 °C /hr)
1st Red palm fat	34	19.30 ± 3.32^{bc}	15.13 ± 4.83^{c}	451.4 ± 47.18^{c}	445.03 ± 21.45^{c}
2nd Red palm fat	25	16.25 ± 6.31^{bc}	22.80 ± 5.58^{b}	551.75 ± 35.66^{b}	480.47 ± 16.05^{c}
3rd Red palm fat	15	5.75 ± 0.86^{d}	4.38 ± 1.14^{d}	571.90 ± 32.43^{b}	491.30 ± 18.77^{c}
Red palm oil	15	53.45 ± 2.85^{a}	52.58 ± 1.74^{a}	747.22 ± 22.49^{a}	742.45 ± 23.35^{a}

The values were the means and the standard deviations of triplicate analysis. The mean values within the same row followed by different superscript letters were significantly ($p \leq 0.05$) different.

Red palm oil produced by the slow cooling rate not only contained higher carotenoids but also higher unsaturated fat, mainly monounsaturated fat, compared to those produced by the fast cooling process (Table 5, 6). The unsaturated fatty acid in red palm oil was mainly oleic acid (C18:1), comprising 43.54 and 41.70 % for slow and fast cooling fractionations, respectively. The high amount of oleic acid was associated with oxidative stability [42]. The main saturated fatty acid was palmitic acid (C16:0), while others were identified in small amounts. Red palm oil from slow cooling contained more unsaturated fatty acids than from fast cooling. This was because the slow cooling rate allowed better separation of fat fractions within the oil. As previously described, the fast cooling rate caused incomplete separation as unsaturated fatty acids were partially trapped inside the granule.

The trend of increased saturated fat over the first, second and third red palm fats was expected. At the first fractionation temperature, the amount of saturated fat was maximized. This was because palmitic acid, which has a high melting point and was the dominant fatty acid in the oil sample, turned into solid fat. Similarly, the remaining fatty acids in the samples influenced the amounts of saturated fats obtained at the second and third fractionation temperatures. The first red palm fat could be used to manufacture cocoa butter substitutes or animal feed, particularly a cocoa butter substitute for chocolate consumed in warm climates [43,44].

Based on the cooling rate study, the slow cooling rate was selected to prepare oil products. The properties of red palm oil and fats were further analyzed, as shown in Table 7. When comparing properties to the specification [9], the red palm oil and red palm fats complied with the requirements for free fatty acids and peroxide value. The iodine value directly indicates the unsaturation of fats and oils and is a significant property related to the texture of the samples. The iodine value of red palm oil (60.11 ± 2.31) was within the range values of super olein or double-fractionated palm olein established by the Codex Alimentarius Commission, indicating a minimum iodine value of 60 [44]. The 55% unsaturated fat in red palm oil was found to be 3% higher than the level found in palm olein [45]. Therefore, the red palm oil obtained from this study has better stability and clarity and is less likely to turn cloudy than normal olein [31].

Table 5. Fatty acid composition of red palm fats and red palm oil obtained from multi-step fractionation with a slow cooling rate of 10 °C /hr.

Fatty acids (g/100g)	**Red palm fats**			**Red palm oil**
	First fractionation (34 °C)	**Second fractionation (25 °C)**	**Third fractionation (15 °C)**	
C8:0	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
C10:0	0.06 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.05 ± 0.01
C12:0	0.12 ± 0.02	0.13 ± 0.01	0.14 ± 0.01	0.15 ± 0.01
C14:0	1.03 ± 0.07	1.08 ± 0.13	1.04 ± 0.01	0.91 ± 0.02
C15:0	0.06 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.01
C16:0	50.97 ± 0.44	45.11 ± 0.23	44.32 ± 0.47	38.27 ± 0.45
C16:1	0.11 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.16 ± 0.01
C17:0	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.10 ± 0.02
C18:0	5.25 ± 0.38	4.84 ± 0.49	4.97 ± 0.11	4.49 ± 0.06
C18:1	32.82 ± 0.75	37.96 ± 0.55	38.62 ± 0.37	43.54 ± 0.68
C18:1*trans*	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.01
C18:2	8.45 ± 0.23	9.54 ± 0.12	9.53 ± 0.21	11.18 ± 0.17
C18:3	0.24 ± 0.05	0.24 ± 0.03	0.25 ± 0.01	0.32 ± 0.02
C20:0	0.39 ± 0.02	0.38 ± 0.04	0.38 ± 0.01	0.37 ± 0.01
C20:1	0.12 ± 0.02	0.14 ± 0.01	0.13 ± 0.01	0.16 ± 0.01
C20:2	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
C22:0	0.07± 0.01	0.06 ± 0.01	0.07 ± 0.00	0.06 ± 0.01
C23:0	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.00
C24:0	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.00	0.07 ± 0.01
Unsaturated fatty acids	41.84 ± 0.87	48.11 ± 0.44	48.78 ± 0.53	55.46 ± 0.56
Saturated fatty acids	58.16 ± 0.82	51.90 ± 0.40	51.23 ± 0.58	44.54 ± 0.51

Concentrations of carotenoids and vitamin E in the red palm oil obtained from the multi-step fractionation increased by about 17% and 12%, respectively, compared to the contents in the crude oils. This trend is similar to the carotenoid results reported by Kellens et al. [44]. The main form of vitamin E is γ-tocotrienol, which has potent antioxidant, anti-inflammatory, and neuroprotective activities [12]. The carotenoids and vitamin E contents in red palm oil were higher than those in red palm fats. The increase of vitamin E and carotenoid concentrations positively correlated with the increase of iodine values, indicating better solubility in the oil fraction. In addition, the solubility of carotenoids and vitamin E increased with the degree of unsaturation of the liquid fraction (red palm oil >first red palm fat >second red palm fat >third red palm fat) (Table 7).

Red palm fats have differences in iodine value and melting point (Table 7). The first red palm fat with a low iodine value and high melting point might be a confectionery ingredient. The second red palm fats with medium iodine value and medium melting point can be used to produce margarine. Finally, the third red palm fat with a high iodine value and low melting point can produce cacao butter substitutes [24]. In addition, all red palm fats have fair contents of carotenoids and vitamin E, which is crucial for consumers looking for health benefits from their food. The present study showed that multi-step fractionation is an effective tool for producing several fractions that differ markedly in their properties.

Table 6. Fatty acid composition of red palm fats and red palm oil obtained from multi-step fractionation with a fast cooling rate of 60 °C /hr.

Fatty acids (g/100g)	**Red palm fats**			**Red palm oil**
	First fractionation (34 °C)	**Second fractionation (25 °C)**	**Third fractionation (15 °C)**	
C8:0	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
C10:0	0.04 ± 0.00	0.03 ± 0.01	0.02 ± 0.00	0.03 ± 0.00
C12:0	0.09 ± 0.01	0.10 ± 0.00	0.10 ± 0.00	0.13 ± 0.02
C14:0	1.15 ± 0.05	1.15 ± 0.03	1.15 ± 0.04	0.99 ± 0.05
C15:0	0.08 ± 0.01	0.06 ± 0.01	0.06 ± 0.00	0.05 ± 0.00
C16:0	55.1 ± 0.56	47.00 ± 0.61	45.8 ±0.85	39.3 ± 0.71
C16:1	0.13 ± 0.02	0.15 ± 0.03	0.15 ± 0.02	0.17 ± 0.02
C17:0	0.14 ± 0.03	0.11 ± 0.02	0.11 ± 0.01	0.10 ± 0.01
C18:0	5.39 ± 0.41	4.88 ± 0.13	5.19 ± 0.14	4.65 ± 0.12
C18:1	29.1 ± 0.85	35.5 ± 0.56	36.7 ± 0.86	41.70 ± 0.47
C18:1*trans*	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
C18:2	7.93 ± 0.12	10.00 ± 0.05	9.70 ± 0.10	11.8 ± 0.15
C18:3	0.22 ± 0.03	0.02 ± 0.00	0.26 ± 0.03	0.31 ± 0.03
C20:0	0.36 ± 0.04	0.36 ± 0.04	0.39 ± 0.06	0.37 ± 0.05
C20:1	0.11 ± 0.01	0.12 ±0.02	0.12 ± 0.01	0.14 ± 0.01
C20:2	0.00 ± 0.00	0.00 ± 0.00	0.06 ± 0.00	0.05 ± 0.00
C22:0	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.00
C23:0	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.00
C24:0	0.06 ± 0.00	0.07 ± 0.02	0.07 ± 0.01	0.07 ± 0.00
Unsaturated fatty acids	37.49 ± 0.78	46.10 ± 0.56	46.90 ± 0.51	54.20 ± 0.83
Saturated fatty acids	62.51 ± 0.54	53.90 ± 0.93	53.10 ± 0.68	45.80 ± 0.75

Table 7. Physicochemical properties of various fractions obtained from the multi-step fractionation at slow cooling rate of 10 °C /hr.

Properties	First red palm fat	Second red palm fat	Third red palm fat	Red palm oil
Yield (%)	20.3 ± 3.32^{b}	17.25 ± 6.31^{b}	6.75 ± 0.86^{c}	55.70 ± 2.85^{a}
Iodine value (g I_2/100g)	34.21 ± 3.03^{d}	43.28 ± 2.27^{c}	49.68 ± 0.05^{b}	60.11 ± 2.31^{a}
Free fatty acid (%)	0.24 ± 0.06^{a}	0.27 ± 0.05^{a}	0.26 ± 0.01^{a}	0.26 ± 0.07^{a}
Peroxide value (meq. O_2/kg)	7.45 ± 0.17^{a}	7.60 ± 0.10^{a}	7.65 ± 0.15^{a}	7.76 ± 0.23^{a}
Melting point (°C)	53.20 ± 0.53^{a}	40.93 ± 0.12^{b}	34.23 ± 6.79^{b}	NA
Carotenoids (mg/kg)	451.4 ± 4 7.18^{c}	551.75 ± 35.66^{b}	571.9 ± 32.43^{b}	747.22 ± 22.49^{a}
α-TP (mg/kg)	205.38 ± 11.16^{b}	220.14 ± 12.13^{b}	235.03 ± 18.07^{b}	288.22 ± 14.91^{a}
δ-TP (mg/kg)	ND	ND	ND	ND
γ-TP (mg/kg)	ND	ND	ND	ND
Total TP (mg/kg)	205.38 ± 11.16^{b}	220.14 ± 12.13^{b}	235.03 ± 18.07^{b}	288.22 ± 14.91^{a}
α-TT (mg/kg)	92.18 ± 11.37^{c}	98.73 ± 12.55^{bc}	110.38 ± 7.70^{b}	180.18 ± 10.59^{a}
δ-TT (mg/kg)	31.52 ± 2.54^{c}	36.81 ± 2.93^{c}	47.53 ± 2.87^{b}	67.39 ± 6.92^{a}
γ-TT (mg/kg)	88.12 ± 10.29^{d}	113.15 ± 15.17^{c}	131.59 ± 7.12^{b}	321.12 ± 19.30^{a}
Total TT (mg/kg)	211.80 ± 20.69^{c}	248.69 ± 26.06^{c}	289.49 ± 14.40^{b}	569.69 ± 23.13^{a}
Total Vitamin E (mg/kg)	417.20 ± 30.60^{d}	468.83 ± 38.18^{c}	524.52 ± 31.71^{b}	856.91 ± 30.36^{a}

Mean values within each row followed by different superscript letters were significantly different ($p \leq 0.05$)., NA = not analyzed, ND = undetected, TP = tocopherol, TT = tocotrienol.

3.6 Anti-inflammatory activity of red palm oil

3.6.1 Effect of red palm oil cytotoxicity on macrophages

The fatty acid composition and natural antioxidants in oils directly relate to their nutritional value and biological functions in humans. Oils high in oleic acid, for example, exhibit anti-apoptotic and anti-inflammatory properties, which are important for reducing inflammation and preventing related diseases [46]. Additionally, oils rich in antioxidants, such as carotenoids and vitamin E, offer further health benefits, including disease prevention. Therefore, evaluating the anti-inflammatory activity of red palm oil is relevant to human health, as it could help manage chronic inflammation, a factor in various diseases. Before evaluating its anti-inflammatory effects, assessing cytotoxicity is crucial to ensure that the oil does not harm cells, particularly macrophages, which play a central role in the immune response. This study aimed to assess the anti-inflammatory activity of red palm oil produced through multi-step fractionation at a slow cooling rate, as it contains high amounts of oleic acid, carotenoids, and vitamin E. The first step involved determining the potential cytotoxicity of the prepared red palm oil on RAW264.7 cells by culturing them with varying concentrations of the oil (5, 10, 20, and 40 μg/mL) using the WST-1 assay.

As shown in Figure 5, red palm oil did not exhibit any toxic effects on the cells at any concentration (5, 10, 20, or 40 μg/mL). Furthermore, treatment with LPS (1 μg/mL) alone did not cause any changes in RAW264.7 cell viability compared to untreated cells (RPMI). The lack of cell death or reduced viability further suggests that red palm oil does not harm macrophage cells, which are essential for immune function. Since the oil showed no toxicity, it is safe to proceed with evaluating its anti-inflammatory effects.

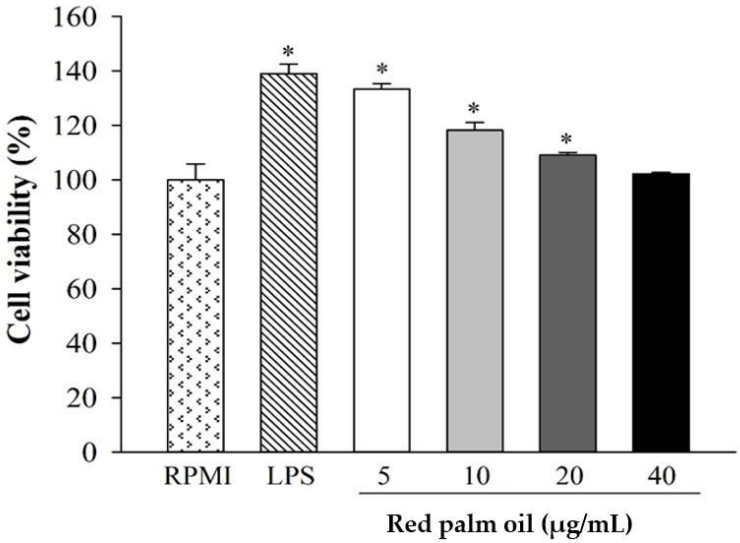

Figure 5. Effect of cell viability of RAW264.7 cells treated with different concentrations of red palm oil. Significant differences were p≤0.05 compared with RPMI (*).

3.6.2 Effect of red palm oil on nitric oxide production and iNOS expression on macrophages

Nitric oxide is a significant inflammatory mediator generated by activated RAW264.7 cells [47,48]. To determine the potential anti-inflammatory activities of red palm oil on LPS-stimulated macrophages, RAW264.7 cells were treated with red palm oil at 5, 10, 20, and 40 μg/mL before stimulating inflammation with LPS. Figure 6A shows red palm oil cannot suppress LPS-induced nitric oxide production following the oil concentrations. The highest concentration of red palm oil, at 40 μg/mL, demonstrated the most pronounced inhibitory effect on nitric oxide production, with values very close to those of the control group (RPMI). In addition, the expression of *iNOS*, a key enzyme that generates nitric oxide, was reduced dose-dependent after treatment with the oil concentration (Figure 6B). Likewise, similar results have been reported for various essential oils derived from citrus peels and red calyces [49,50]. These results demonstrated that red palm oil could reduce LPS-induced nitric oxide generation by reducing iNOS expression at the transcription level.

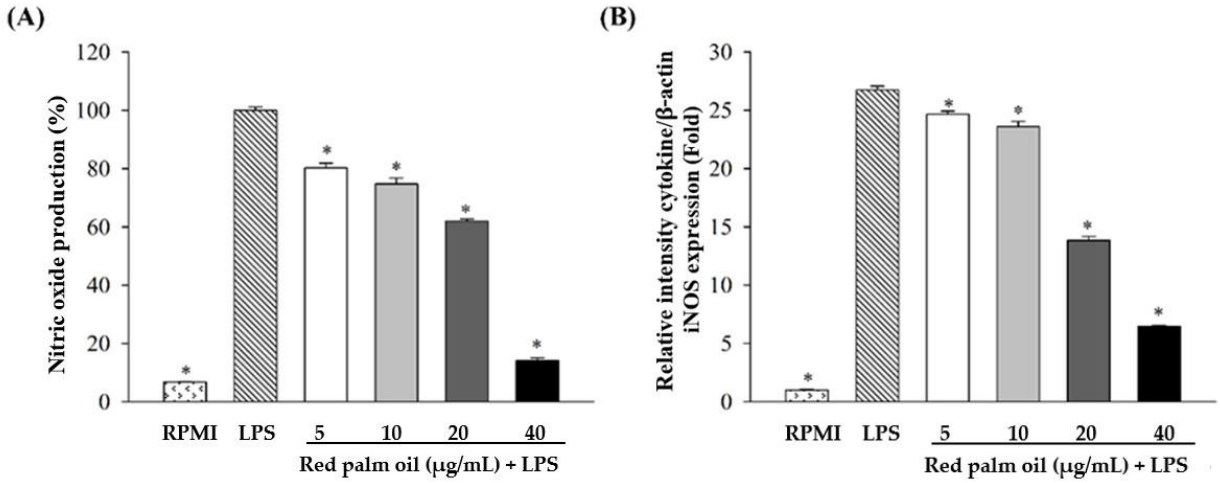

Figure 6. Effects of red palm oil on nitric oxide production and iNOS expression in LPS-stimulated RAW264.7 cells. (A) LPS-induced nitric oxide production; (B) LPS-induced iNOS expression. Significant differences were p≤0.05 compared with LPS (*).

3.6.3 Effect of red palm oil on PGE2 production

PGE2, a primary metabolite of the *COX-2* pathway, has been implicated in the pathogenesis of both acute and chronic inflammatory conditions [51]. The PGE2 levels were assessed following LPS-induced inflammation. As illustrated in Figure 7, the PGE2 levels were dose-dependent and decreased with higher oil concentrations in LPS-stimulated RAW 264.7 cells. PGE2 production was markedly decreased when red palm oil was applied compared to cells treated with LPS alone, exhibiting a strong anti-inflammatory effect in LPS-stimulated RAW264.7 cells.

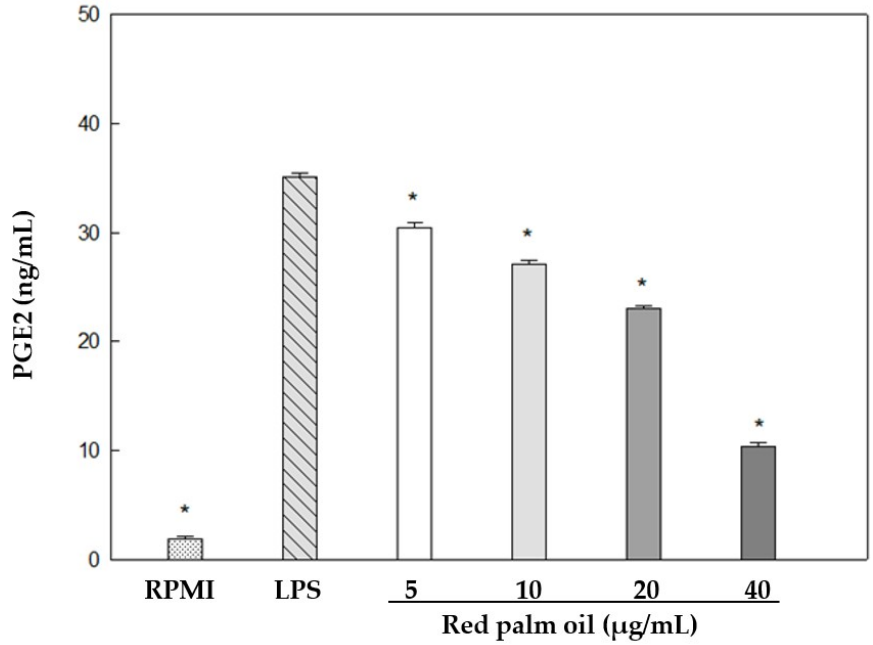

Figure 7. Effects of red palm oil on PGE2 production in LPS-stimulated RAW264.7 cells. Significant differences were $p \leq 0.05$ compared with LPS (*).

3.6.4 Effect of red palm oil on cytokine expressions

Studies have indicated that cytokines, such as *TNF-α*, *IL-6*, and *IL-1β*, exhibit pro-inflammatory properties both *in vitro* and *in vivo* [48,49]. In LPS-stimulated murine macrophage cells, the expression levels of most immune-associated genes were dose-dependently inhibited depending on the concentration of the oil (Figure 8, A-C). The mRNA expression levels of pro-inflammatory cytokines, including *IL-1β*, *IL-6*, and *TNF-α* genes, showed a decrease in the same pattern of expression levels. The expression of *IL-1β*, *IL-6*, and *TNF-α* was dose-dependently decreased after treatment with the red palm oil at 5, 10, 20, and 40 μg/mL concentrations (Figure 8, A-C). However, the oils also reduced the mRNA expression of anti- inflammatory IL-10 cytokine (Figure 8, D). Consistent with these results on red palm oil, it has recently been found that polysaccharides of Mytilus coruscus decrease the expression and production of IL-10 in macrophage cells [52]. These results indicate that red palm oil could substantially decrease the inflammatory response by elevating anti-inflammatory cytokines and reducing pro-inflammatory cytokines in LPS-stimulated RAW264.7 cells.

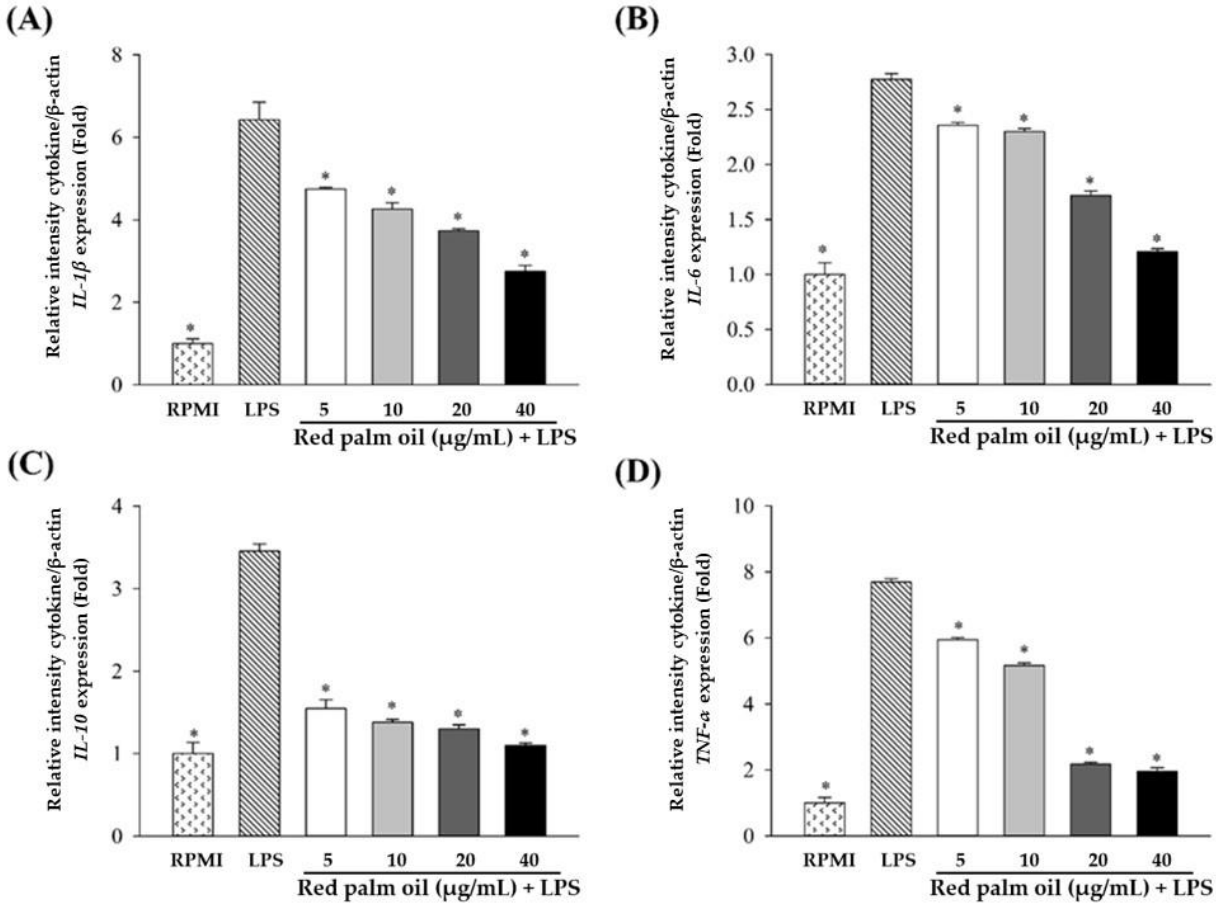

Figure 8. Quantification of red palm oil on inflammatory genes in relative expression (fold) in LPS-stimulated RAW264.7 cells. The mRNA levels of (A) *IL-1β*; (B) *IL-6*; (C) *IL-10*; and (D) *TNF-α*. Significant differences were $p \leq 0.05$ compared with LPS (*).

3.6.5 Effect of red palm oil on MAPK and NF-κB signaling pathways

Additional research was carried out to clarify how red palm oil triggered the synthesis of inflammatory mediators like nitric oxide and pro-inflammatory cytokines in RAW264.7 cells through the activation of nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK). RAW264.7 cells treated with LPS served as a positive control, and after LPS stimulation, the cells were treated with red palm oil before being assessed by western blotting. (Figure 9, A-D). The MAPK family of proteins consists of Ser/Thr kinases that convert extracellular stimuli into cellular responses, which are regulated by extracellular signal-related kinases (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 [53]. These proteins play a crucial role in regulating pro-inflammatory molecules, which are used as molecular targets for anti-inflammatory effects [22]. As shown in Figure 9, B-D, red palm oil also significantly inhibited the phosphorylation of p38, JNK, and ERK1/2 in a dose-dependent manner compared with the LPS control group, especially at a 40 μg/mL concentration of red palm oil. Moreover, NF-κB is recognized as a key transcription factor responsible for regulating the expression of proteins that facilitate the release of inflammatory response genes, including iNOS and cytokines associated with macrophage activity. It plays a crucial role in immunity and cellular proliferation. As shown in Figure 9, E, red palm oil (5–40 μg/mL) dose-dependently decreased the phosphorylation of NF-κB p-65 in the NF-κB signaling pathway, similar to *Artemisia fukudo* essential oil, which also showed an inhibitory effect of NF-κB activation via decreasing nuclear p50 and p65 protein levels as well as the phosphorylation of p38, ERK, and JNK [47]. The result suggested that the red palm oil inhibited inflammation through the MAPK and NF-κB signaling pathways.

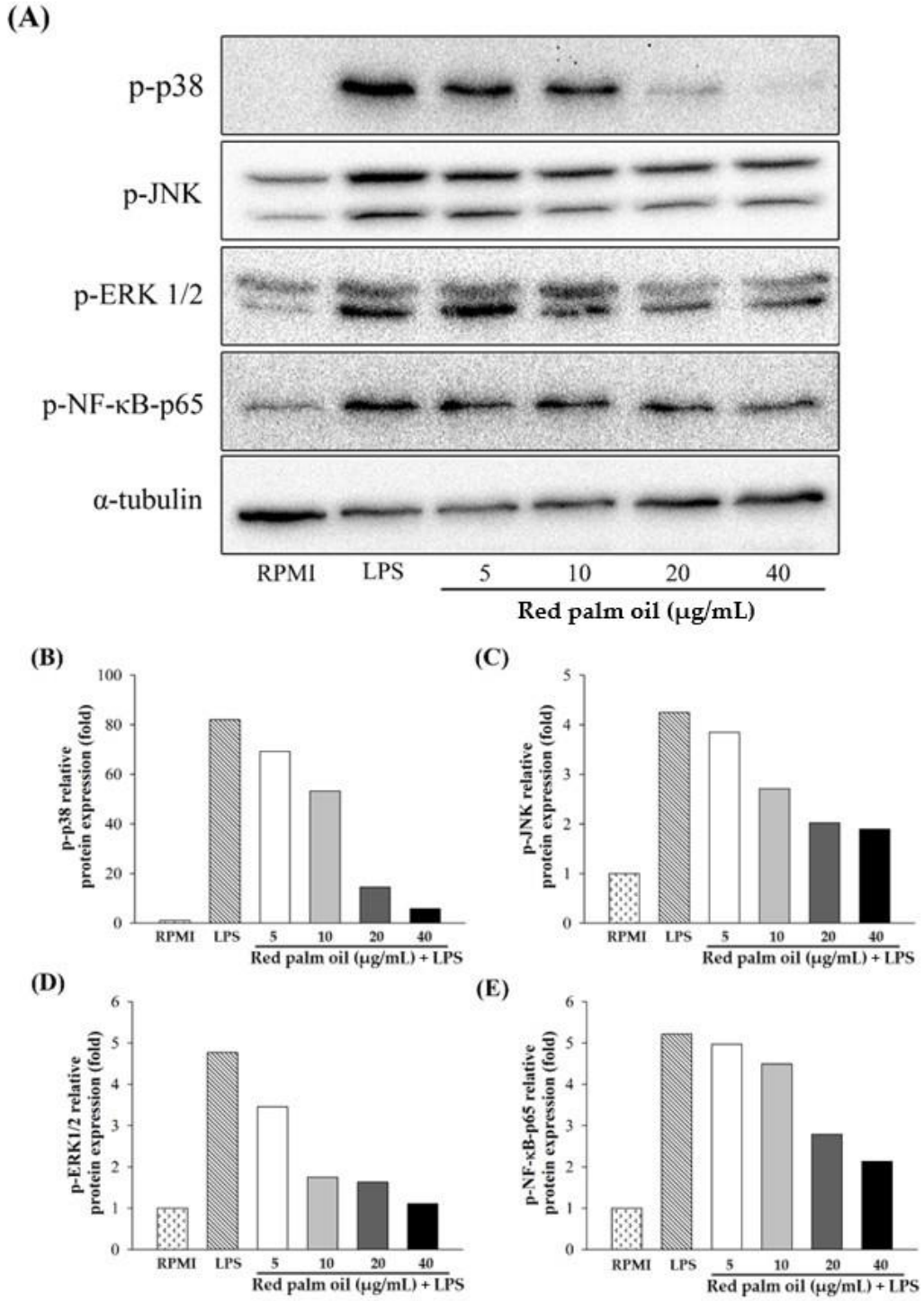

Figure 9. Effect of red palm oil on protein expression levels in the NF-κB and MAPK pathways in LPS-stimulated macrophages. (A) Western blotting of protein-treated with red palm oil and LPS in RAW264.7 cells; Relative band intensity of phosphorylated p38 (B), phosphorylated JNK (C), phosphorylated ERK1/2 (D), and phosphorylated NF-κB p-65 (E) proteins.

4. Conclusions

The acid degumming and multi-step fractionation effectively produce red palm oil and fats for SMEs. The optimal degumming conditions were 20 min at a temperature of 90 °C and phosphoric acid to citric acid of 0.06:0.04%. The optimal cooling rate of the multi-step fractionation was 10 °C /hr. The red palm oil produced

at the optimal conditions contained 43.54% oleic acid, 747.22 mg/kg carotenoids, 856.91 mg/kg vitamin E, and a 55.70% yield. The high presence of these phytonutrients in the oil obtained through these processes contributed to its anti-inflammatory activity. A study in RAW264.7 macrophages stimulated with lipopolysaccharide (LPS) showed that the oil could reduce the production of nitric oxide and prostaglandin E2 (PGE2) in LPS-stimulated RAW264.7 cells. The oil attenuated LPS-induced mRNA expression of inflammatory mediators and cytokines by inhibiting the NF-κB and MAPK signaling pathways in LPS-stimulated macrophages. Overall, the findings suggested that the developed red palm oil possessed anti-inflammatory properties and showed promise as an anti-inflammatory agent, thereby expanding its therapeutic potential. Nonetheless, further research is needed to confirm the anti-inflammatory effects of red palm oil in models of inflammatory diseases. These findings are of fundamental importance for producers and will be beneficial for promoting red palm oil to consumers worldwide.

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Conflicts of Interest: The authors declare no conflict of interest.

References

[1] Sumarna, D.; Sumarlan, S.H.; Wijaya, S.; Hidayat, N. Processing of olein fraction red palm oil with minimal refining method and optimization of deodorization process. *Advances in Biological Sciences Research* **2021**, *17*, 167-175. https://doi.org/10.2991/absr.k.220102.026

[2] Nagendran, B.U; nnithan, U.R.; Choo, Y.M.; Kalyana, S. Characteristics of red palm oil, a carotene-and vitamin E-rich refined oil for food uses. *Food and Nutrition Bulletin* **2000**, *21*(2), 189–194. https://doi.org/10.1177/156482650002100213

[3] Lee, W.J.; Tan, C.P.; Sulaiman, R.; Chong, G.H. Solubility of red palm oil in supercritical carbon dioxide: Measurement and modelling. *Chinese Journal of Chemical Engineering* **2018**, *26*(5), 964–969. https://doi.org/10.1016/j.cjche.2017.09.024

[4] Azmi, R.A.; Goh, P.S.; Ismail, A.F.; Lau, W.J.; Ng, B.C.; Othman, N.M.; Noor, A.M.; Yusoff, M.S.A. Deacidification of crude palm oil using PVA-crosslinked PVDF membrane. *Journal of Food Engineering* **2015**, *166*, 165-173. https://doi.org/10.1016/j.jfoodeng.2015.06.001

[5] Chooklin, S.; Kaewsichan, L.; Kaewsrichan, J. Potential Utilization of Sap from Oil Palm (Elaeis guineensis) for Lactic Acid Production by Lactobacillus casei. *Journal of Sustainable Energy & Environment* **2011**, *2*, 99-104.

[6] Silalertruksa, T.; Gheewala, S.H.; Pongpat, P.; Kaenchan, P.; Permpool, N.; Lecksiwilai, N.; Mungkung, R. Environmental sustainability of oil palm cultivation in different regions of Thailand: Greenhouse gases and water use impact. *Journal of Cleaner Production* **2017**, *167*, 1009-1019. https://doi.org/10.1016/j.jclepro.2016.11.069

[7] Chompoo, M.; Damrongwattanakool, D.; Raviyan, P. Properties of healthy oil formulated from red palm, rice bran and sesame oils. *Songklanakarin journal of science and technology* **2019**, *41*(2), 450-458. https://doi.org/10.14456/sjst-psu.2019.56

[8] O' Brien, R.D. *Fat and oil. Formulating and processing for applications*. 3rd ed.; CRC Press, New York, USA, **2009**, pp 1-680.

[9] Codex Alimentarius International Food Standards. *Standard for named vegetable oils*: CODEX STAN 210-1999. Geneva, Switzerland, **1999**.

[10] Shi-Cheng, T.; Teck-Kim, T.; Yee-Ying, L. A review on the fundamentals of palm oil fractionation: Processing conditions and seeding agents. *European Journal of Lipid Science and Technology* **2021**, *123*(12), Article 2100132. https://doi.org/10.1002/ejlt.202100132

[11] Tan, C.H.; Lee, C.J.; Tan, S.N.; Poon, D.T.S.; Chong, C.Y.E.; Pui, L.P. Red palm oil: A review on processing, health benefits and its application in food. *Journal of Oleo Science* **2021**, *70*(9) 1201-1210. https://doi.org/10.5650/jos.ess21108

[12] Loganathan, R.; Subramaniam, K.M.; Radhakrishnan, A.K.; Choo, Y.M.; Teng, K.T. Health-promoting effects of red palm oil: Evidence from animal and human studies. *Nutrition Reviews* **2017**, *75*(2), 98-113. https://doi.org/10.1093/nutrit/nuw054

[13] Khatoon, S.; Reddy, S.R.Y. Plastic fats with zero-trans fatty acids by interesterification of mango, mahua and palm oils. *European Journal of Lipid Science and Technology* **2005**, *107*(11), 786-791. https://doi.org/10.1002/ejlt.200501210

[14] Arain, M.A.; Mei, Z.; Hassan, F.U.; Saeed, M.; Alagawany, M.; Shar, A.H.; Rajput, I.R. Lycopene: A natural antioxidant for prevention of heat-induced oxidative stress in poultry. *World's Poultry Science Journal* **2018**, *74*(1), 89-100. https://doi.org/10.1017/S0043933917001040

[15] Bennedsen, M.; Wang, X.; Willén, R.; Wadström, T.; Andersen, L.P. Treatment of H. pylori infected mice with antioxidant astaxanthin reduces gastric inflammation, bacterial load and modulates cytokine release by splenocytes. *Immunology Letters* **2000**, *70*(3), 185-189. https://doi.org/10.1016/s0165-2478(99)00145-5

[16] Goh, S.H.; Hew, N.F.; Norhanom, A.W.; Yadav, M. Inhibition of tumour promotion by various palm-oil tocotrienols. *International Journal of Cancer* **1994**, *57*, 529-531. https://doi.org/10.1002/ijc.2910570415

[17] Takahashi, K.; Loo, G. Disruption of mitochondria during tocotrienol-induced apoptosis in MDA-MB-231 human breast cancer cells. *Biochemistry and Pharmacology* **2004**, *67*(2), 315-324. https://doi.org/10.1016/j.bcp.2003.07.015

[18] Vasanthi, H.R.; Parameswari, R.P.; Das, D.K. Multifaceted role of tocotrienols in cardioprotection supports their structure: function relation. *Genes and Nutrition* **2012**, *7*(1), 19-28. https://doi.org/10.1007/s12263-011-0227-9

[19] Khanna, S.; Parinandi, N.L.; Kotha, S.R.; Roy, S.; Rink, C.; Bibus, D.; Sen, C.K. Nanomolar vitamin E alpha-tocotrienol inhibits glutamate-induced activation of phospholipase A2 and causes neuroprotection. *Journal of Neurochemistry* **2010**, *112*(50), 1249-1260. https://doi.org/10.1111/j.1471-4159.2009.06550.x

[20] Park, J.W.; Kwon, O.K.; Ryu, W.H.; Paik, J.H.; Paryanto, I.; Yuniato, P.; Choi, S.; Oh, S.R.; Ahn, K.S. Anti-inflammatory effects of *Passiflora foetida* L. in LPS-stimulated RAW264.7 macrophages. *International Journal of Molecular Medicine* **2018**, *41*(6), 3709-3716. https://doi.org/10.3892/ijmm.2018.3559

[21] Pierce, G.F. Macrophages: important physiologic and pathologic sources of polypeptide growth factors. *American Journal of Respiratory Cell and Molecular Biology* **1990**, *2*(3), 233-234. https://doi.org/10.1165/ajrcmb/2.3.233

[22] Du, L.; Li, J.; Zhang, X.; Wang, L.; Zhang, W.; Yang, M.; Hou, C. Pomegranate peel polyphenols inhibits inflammation in LPS-induced RAW264. 7 macrophages via the suppression of MAPKs activation. *Food and Nutrition Research* **2019**, *63*, 3392. https://doi.org/10.29219/fnr.v63.3392

[23] Lee, J.Y.; Li, C.; Surayot, U.; Yelithao, K.; Lee, S.M.; Park, W.; Tabarsa, M.; You, S.G. Molecular structures, chemical properties and biological activities of polysaccharide from *Smilax glabra* rhizome. *International Journal of Biological Macromolecules* **2018**, *120*(10), 1726-1733. https://doi.org/10.1016/j.ijbiomac.2018.09.138

[24] Wei, J.; Zhang, X.; Bi, Y.; Miao, R.; Zhang, Z.; Su, H. Anti-inflammatory effects of Cumin essential oil by blocking JNK, ERK, and NF-κB signaling pathways in LPS-stimulated RAW264.7 cells. *Evidence-Based Complementary and Alternative Medicine* **2015**, *2015*, 474509. https://doi.org/10.1155/2015/474509

[25] Chang, M.; Qiu, F.; Lan, N.; Zhang, T.; Guo, X.; Jin, Q.; Liu, R.; Wang, X. Analysis of phytochemical composition of *Camellia oleifera* oil and evaluation of its anti-inflammatory effect in lipopolysaccharide-stimulated RAW264.7 macrophages. *Lipids* **2020**, *55*(4), 353-363. https://doi.org/10.1002/lipd.12241

[26] Anyanji, V.U.; Mohamed, S.; Zokti, J.A.; Ado, M.A. Anti-inflammatory properties of oil palm leaf *(Elaeis guineensis Jacq*.) extract in aged rats. *International Journal of Pharmacy and Pharmaceutical Sciences* **2013**, *5*(4), 134-136.

[27] Wu, S.J.; Liu, P.L.; Ng, L.T. Tocotrienol-rich fraction of palm oil exhibits anti- inflammatory property by suppressing the expression of inflammatory mediators in human monocytic cells. *Molecular Nutrition and Food Research* **2008**, *52*(8), 921-929. https://doi.org/10.1002/mnfr.200700418

[28] AOAC. *Official Methods of Analysis*, 20th Ed.; The Association of Official Analytical Chemists, Maryland, USA. **2000**.

[29] AOAC. *Official Methods of Analysis*, 19th Ed.; The Association of Official Analytical Chemists, Washington DC, USA. **2016**.

[30] Ribeiro, H.S.; Chu, B.S.; Ichikawa, S.; Nakajima, M. Preparation of nanodispersions containing β-carotene by solvent displacement method. *Food Hydrocolloids* **2008**, *22*(1), 12-17. https://doi.org/10.1016/j.foodhyd.2007.04.009

[31] Lai, O-M.; Phuah, E-T.; Lee, Y-Y.; Basiron, Y. Palm Oil. In Shahidi, F. (Eds.), *Bailey's Industrial Oil and Fat Products*. John Wiley & Sons, Ltd., West Sussex, **2020**, pp. 385-486.

[32] Aung, L.L.; Pe, M.; Hla, P.K. Palm Oil Fractionation Process. *Journal of the Myanmar Academy of Arts and Science* **2009**, *7*(1), 435-446.

[33] Chen, C.W.; Lai, O.M.; Ghazali, H.M.; Chong, C.L. Isothermal crystallization kinetics of refined palm oil. *Journal of the American Oil Chemist' Society* **2002**, 79(4), 403-410. https://doi.org/10.1007/s11746-002-0496-4

[34] Mayamol, P.N.; Balachandran, C.; Samuel, T.; Sundaresan, A.; Arumughan, C. Process Technology for the production of micronutrient rich red palm olein. *Journal of the American Oil Chemists' Society* **2007**, *84*, 587–596. Retrieved from https://link.springer.com/article/10.1007/s11746-007-1078-9

[35] Shammugasamy, B.; Ramakrishnan, Y.; Manan, F.; Muhammad, K. Rapid reversed-phase chromatographic method for determination of eight vitamin E isomers and γ-oryzanols in rice bran and rice bran oil. *Food Analytical Methods* **2015**, *8*(3), 649–655. https://doi.org/10.1007/s12161-014-9929-0

[36] Green, L.C.; Wagner, D.A.; Glogowski, J.; Skipper, P.L.; Widhnok. J.S.; Tannenbaum, S.R. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Analytical Biochemistry* **1982**, *126*(1), 131-138. https://doi.org/10.1016/0003-2697(82)90118-x

[37] Surayot, U.; Yelitho, K.; Tabarsa, M.; Lee, D.H.; Palanisamy, S.; Prahu N.M.; Lee, J.; You, S.G. Structural characterization of a polysaccharide from *Certaria islandica* and assessment of immunostimulatory activity.*Process Biochemistry* **2019**, *83*(1), 214-221. https://doi.org/10.1016/j.procbio.2019.05.022

[38] Dijkstra, J. About water degumming and the hydration of non-hydratable phosphatides. *European Journal of Lipid Science and Technology* **2017**, *119*, 1600496. https://doi.org/10.1002/ejlt.201600496

[39] Bonnie, T.Y.P.; Choo, Y.M. Valuable minor constituents of commercial red palm olein: carotenoids, vitamin E, ubiquinones and sterols. *Journal of Oil Palm Research* **2000**, *12*(1), 97-108.

[40] De Greyt, W. Edible oil refining: Current and future technologies. In: Hamm,W.; Hamilton, R.J.; Calliauw, G. (Eds.), *Edible oil processing*, Wiley-Blackwell, West Sussex, **2013**, pp. 127-148.

[41] Yang, D.; Lee, Y.Y; Lu, Y.; Wang, Y; Zhang, Z. Internal Factors Affecting the Crystallization of the Lipid System: Triacylglycerol Structure, Composition, and Minor Components. *Molecules* 2024, 29(8), 1847. https://doi.org/10.3390/molecules29081847

[42] Zahran, H.A.; Tawfeuk, H.Z. Physicochemical properties of new peanut (*Arachis hypogaea* L.) varieties. *Oil seeds and Fats* **2019**, *26*(2). https://doi.org/10.1051/ocl/2019018

[43] Edem, D.O. Palm oil: Biochemical, physiological, nutritional, hematological and toxicological aspects: A review. *Plant Foods Human Nutrition* **2002**, *57*, 319-341. https://doi.org/10.1023/a:1021828132707

[44] Kellens, M.; Gibon, V.; Hendrix, M.; Grey, W.D. Palm oil fractionation. *European Journal of Lipid Science and Technology* **2007**, *109*(4), 336–349. https://doi.org/10.1002/ejlt.200600309

[45] Kritchevsky, D. Impact of red palm oil on human nutrition and health. *Food and Nutrition Bulletin* **2000**, *21*(2), 182-188. https://doi.org/10.1177/156482650002100212

[46] Schwingshackl, L.; Hoffmann, G. Adherence to Mediterranean diet and risk of cancer: A systematic review and meta-analysis of observational studies. *International Journal of Cancer* **2014**, *135*(18), 1884-1897. https://doi.org/10.1002/ijc.28824

[47] Yoon, W.J.; Moon, J.Y.; Song, G.; Lee, Y.K.; Han, M.S.; Lee, J.S.; Ihm, B.S.; Lee, W.J.; Lee, N.H.; Hyun, C.G. Artemisia fukudo essential oil attenuates LPS-induced inflammation by suppressing NF-κB and MAPK activation in RAW 264.7 macrophages. *Food and Chemical Toxicology* **2010**, *48*(5), 1222-1229. https://doi.org/10.1016/j.fct.2010.02.014

[48] Kim, K.N.; Ko, Y.J.; Yang, H.M.; Ham, Y,M.; Roh, S.W.; Jeon, Y.J.; Ahn, G.; Kang, M.C.; Yoon, W.J.; Kim,D.; Oda, T. Anti-inflammatory effect of essential oil and its constituents from fingered citron (Citrus

medica L. var. sarcodactylis) through blocking JNK, ERK and NF-κB signaling pathways in LPS-activated RAW 264.7 cells. *Food and Chemical Toxicology* **2013**, *57*, 126-131. https://doi.org/10.1016/j.fct.2013.03.017

[49] Shen, C.Y.; Zhan, T.; Zhang, W.L.; Jiang, J.G. Anti-inflammatory activities of essential oil isolated from the calyx of Hibiscus sabdariffa L. *Food and Function* **2016**, 7(10), 4451-4459. https://doi.org/10.1039/c6fo00795c

[50] Yang, J.; Lee, S.Y.; Jang, S.K.; Kim, K.J.; Park, M.J. Anti-Inflammatory Effects of Essential Oils from the Peels of Citrus Cultivars. *Pharmaceutics* **2023**, *15*(6), 1595. https://doi.org/10.3390/pharmaceutics15061595

[51] Ghosh, N.R.; Chaki, V.; Mandal, S.C. COX-2 as a target for cancer chemotherapy. *Pharmacological Reports* **2010**, *62*(2), 233-244. https://doi: 10.1016/s1734-1140(10)70262-0

[52] Xiang, X.W.; Wang, R.; Yao, L.W.; Zhou, Y.F.; Sun, P.L.; Zheng, B.; Chen, Y.F. Anti-inflammatory effects of Mytilus coruscus polysaccharide on RAW264.7 cells and DSS-induced colitis in mice. *Marine drugs* **2021**, *19*(8), 468. https://doi.org/10.3390/md19080468

[53] Kaminska, B.; Gozdz, A.; Zawadzka, M.; Aleksandra, E.M.; Lipko, M. MAPK signal transduction underlying brain inflammation and gliosis as therapeutic target. *The Anatomical Record* **2009**, *292*(12), 1902-1913. https://doi.org/10.1002/ar.21047