

The Effect of Unsaponifiable Fraction from Palm Fatty Acid Distillate on Lipid Profile of Hypercholesterolaemia Rats

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Abstract Physical refining of palm oil by deodorization produces palm fatty acid distillate (PFAD) that contains some bioactive compounds such as vitamin E (tocopherol and tocotrienols), phytosterol, and squalene. These bioactive compounds were accumulated in unsaponifiable fraction. This study aimed to use USF as a source of bioactive compounds for lowering cholesterol that tested in vivo by using hypercholesterolaemia rats. The rats were divided into 4 groups that administered by USF of 0, 200, 500, 1000 mg/kg bw/day. As comparison, one group of rats was fed by commercial squalene supplement of 90 mg/kg bw/day. One group of normal rats was used as a control. The results showed that USF of PFAD contained vitamin E 1.96%, phytosterols 0.55%, and squalene 32.30%. Vitamin E of USF comprised of 83% tocotrienols and the remaining is tocopherol. Phytosterols of USF contained 0.42% beta sitosterol and 0.13% campesterol. The result showed that USF doses significantly affected blood serum total cholesterol reduction. Higher dose of USF caused greater reduction of serum blood cholesterol level. The highest decline of blood serum total cholesterol was found at USF dose of 1000 mg/kg bw/day. At dose of 1000 mg/kg bw/day, blood serum cholesterol level reached normal after USF administration for 4 weeks. The blood serum triglyceride level decreased in line with increasing USF doses. The sharp decline of blood serum triglyceride level was found at USF dose of 1000 mg/kg bw/day and at the end of experiment (week 4), the blood serum triglyceride level reached normal. Higher dose of USF resulted on greater decrease of LDL cholesterol level. The highest decline of LDL cholesterol level was found on USF 1000 mg/kg bw/day. Increasing USF dose resulted in increasing blood serum HDL cholesterol level. The higher rise of blood serum HDL cholesterol level was found at USF dose of 1000 mg/kg bw/day. Commercial squalene at dose of 90 mg/kg bw/day was less effective in reducing total cholesterol, LDL cholesterol, and triglyceride, as well as in rising HDL cholesterol level than USF 200 mg/kg bw/day (equivalent to squalene 64.60 mg/kg bw/day). This finding proved that multi components of bioactive compounds in USF improved blood lipid profile synergistically.

Keywords: hypercholesterolaemia, lipid profile, palm fatty acid distillate, phytosterol, squalene, tocopherol, tocotrienol, unsaponifiable fraction

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1. Introduction

Palm frying oil is produced through several refining steps, consisted of degumming, bleaching, and physical refining to remove odorous compounds and free fatty acids. Physical refining process by deodorization produces palm fatty acid distillate (PFAD) as a by-product. Gapoor et al (2002) reported that PFAD contained mainly free fatty acids, and other minor constituent comprised of tocopherols, tocotrienols, phytosterols, and squalene [1].

For every 100 ton of CPO, PFAD is produced about 3.66 ton [2], because its production is 4% based on CPO [3]. Unfortunately, PFAD is used as fatty acid source for non food industries [4] such as soap, feed, and oleo chemical industries [5] by neglecting the existence of bioactive compounds. PFAD could be used integratively as a source of bioactive compounds and free fatty acids for oleo chemical industries [6].

As much as 5-57% of tocotrienols and tocopherols lost from CPO during deodorization and mostly are accumulated in PFAD with concentration of 0.7-1.0% [7].

Squalene was found in PFAD that reaches 1.03% [8]. Other minor bioactive compound that found in PFAD is phytosterols with concentration of 0.37% [1]. All of these bioactive compounds are not saponified during saponification, therefore they are accumulated in unsaponifiable fraction (USF) of PFAD [9]. Our previous study showed that USF could be separated from saponifiable fraction by simple saponification. The bioactive compounds of USF are vitamin E of 0.80%, phytosterols of 9.16%, squalene of 1.14% [6]. The difference of PFAD as vitamin E containing material from other vitamin E sources is rich in tocotrienols [10,11]. Lewis (2001) indicated that tocotrienols have hypocholesterolaemic property and have the ability to reduce LDL cholesterol level [12].

Phytosterol lowers blood cholesterol level by inhibiting cholesterol absorption [13], so it has hypocholesterolaemic effect [14,15,16]. The mechanism of cholesterol lowering properties of phytosterol is still unclear, but Bonsdorff-Nikander (2005) proposed that phytosterols compete with cholesterol in micelle formation, phytosterols cocrystallize with cholesterol, and phytosterols inhibit ACAT activity in cholesterol synthesis [17].

Squalene as one of bioactive compounds in PFAD, also has cholesterol lowering property [18] by increasing fecal excretion of bile acid [19,20,21]. In contrary, other study showed that squalene increase cholesterol level [22], due to the increasing extent of cholesterol synthesis [23].

It is possible that bioactive compounds of USF synergistically have beneficial effects on lipid profile in hypercholesterolaemia condition. At this study, the ability of USF from PFAD as cholesterol lowering compounds was investigated including the effective dose to improve lipid profile.

2. Materials and Methods

2.1. Materials

PFAD was kindly obtained from a palm oil refinery, PT Salim Ivomas Pratama at Surabaya, Indonesia. Chemical reagents used were vitamin E standard (α tocopherol, α tocotrienol, β tocotrienol, δ tocotrienol), squalene, phytosterols (campesterol, stigmasterol and β sitosterol), MSTFA (methyl N-trimethylsilyl-trifluoroacetamide) (Santa Cruz Biotech USA), HPLC grade solvents (Merck) and other chemical reagents for analysis (Merck), Wistar male rats with body weight of 150-200 g and age of 8-12 weeks, AIN-93M standard diet with slight modification, atherogenic diets, commercial squalene supplement, and serum blood lipid profile analysis kit (Diasys).

2.2. USF Preparation from PFAD

The preparation of USF was conducted by saponification method [6]. Samples of PFAD and USF were analyzed for vitamin E, phytosterols, and squalene.

2.2.1. Phytosterols Analysis

Phytosterols analysis was followed method of Khatoun et al (2010). About 1 mg of unsaponifiable fraction of PFAD was added by ethanol 1 mL and subsequently filtered. 20 μ L of sample solution was injected into high

performance liquid chromatography (Shimadzu LC20AT) with C18 PPODS column 250 \times 4.6 mm and uv vis detector (Shimadzu SPD20A) at wavelength of 206 nm. Mobile phase was methanol: water (99:1 v: v) with flow rate of 1 mL/min. Identification and quantification was conducted by using phytosterols (stigmasterol, β sitosterol, campesterol) standard (Santa Cruz Biotech, USA) that injected into HPLC separately [9].

2.2.2. Vitamin E analysis

This procedure was according to Ball (1988). About 1 mg of unsaponifiable fraction of PFAD was added by ethanol 1 mL and then filtered. 20 μ L of sample solution was injected into high performance liquid chromatography (Shimadzu LC20AT) with C18 PPODS column 250 \times 4.6 mm and uv vis detector (Shimadzu SPD20A) at wavelength of 295 nm. Mobile phase was methanol: water (95:5 v: v) with flow rate of 1 mL/min. Identification and quantification was conducted by using α tocopherol, α tocotrienol, β tocotrienol, δ tocotrienol and γ tocotrienol standards (Santa Cruz Biotech, USA) that injected into HPLC separately [24].

2.2.3. Squalene Analysis

This analysis was using GC MS with derivatization method according to Mendez et al (2003) by using MSTFA. About 10 mg of unsaponifiable fraction of PFAD was put into reaction tube and added by MSTFA 10 μ L. The mixture was heated at 60°C for 15 min. External standard of 1-docosanol was used for quantification that prepared by diluting 1 mg of this standard with MSTFA 100 μ L and the mixture was heated at 60°C for 15 min. 1 μ L of derivatized sample was injected into GC MS (GCMS-QP2010S Shimadzu) (with column (AGILENT DB-1) 30 m, i.d. 0.25 mm, helium as carrier gas and ionization EI 70 Ev. GC operational condition was column oven temperature of 80°C, injector temperature of 310°C, pressure of 16.5 kPa, total flow rate of 80.0 mL/min, column flow rate of 0.50 mL/min, linear velocity of 26.1 cm/s, supporting gas rate of 3.0 ml/min and split ratio of 153.0. Initial temperature was 80°C and held for 5 min. Temperature was increased with rate of 10°C/min to the temperature of 305°C and held for 25 min. MS analysis was conducted at ion source temperature of 250°C, interface temperature of 310°C, solvent cut time of 4.80 min, scan speed of 1250, start m/z 28.0 and end m/z 600.0. Squalene standard (Santa Cruz Biotech, USA) was used as external standard [25].

2.3. Bioassay of Cholesterol Lowering Effect of USF

The protocol of bioassay had been approved for ethical clearance No. 190-KEP-UB from Animal Care and Use Committee, Brawijaya University. As many as 24 Wistar male rats were used in this study. Each rat was caged individually and adapted to laboratory environment for 7 days. During adaptation, rats were fed by standard diet of AIN-93 M with slight modification (Table 1) [26]. Rats were divided into 6 groups and each group comprised of 4 rats. One group was normal rats without USF treatment. Other four groups were hypercholesterolaemia rats treated by USF 0, 200, 500, 100 mg/kg bw/day and one group was hypercholesterolaemia rats treated by commercial

squalene at dose of 90 mg/kg bw/day. All groups of hypercholesterolaemia rats were fed by atherogenic diet (Table 1).

Table 1. Composition of modified AIN-93M and atherogenic diet

Component	Composition (g/kg diet)	
	Modified AIN-93M [1]	Atherogenic Diet
Maize starch	620, 692	620, 692
Casein	140	116,528
Sucrose	100	100
Soybean oil	40	40
CMC	50	50
AIN mineral mix	35	35
AIN vitamin mix	10	10
L-cystin	1,8	1,8
Choline bitartrate	2,5	2,5
TBHQ	-	-
Cholic acid	-	2
Cholesterol crystal	-	20
Tallow	-	185

Rats were treated by USF and squalene administration for 4 weeks, and body weight of each rat was measured daily as well as the quantity of feed consumption. Treatment by USF administration was conducted after total cholesterol level of rats reached >130 mg/dL. Lipid profile was analyzed at week 0, 1, 2, 3, and 4 to measure LDL cholesterol, triglyceride, and total cholesterol of serum blood. Blood was taken by *retro orbital plexus* method and centrifuged at 4000 rpm for 10 min to separate blood serum. Blood serum total cholesterol, triglyceride, and HDL cholesterol levels were measured by method of CHOD-PAP, GPO-PAP, and triglyceride kit, respectively. Meanwhile, blood serum LDL level was calculated based on the data of total cholesterol, triglyceride, and HDL cholesterol.

3. Results

3.1. Bioactive Compounds of PFAD and USF

Bioactive compounds found in PFAD are vitamin E, phytosterols, and squalene. Generally, phytosterols were the most prominent bioactive compound found in PFAD (Table 2). USF and PFAD had different concentration and composition of bioactive compounds. USF had higher vitamin E and squalene concentration than PFAD (Table 2), meanwhile phytosterols of USF was lower than that in PFAD.

Table 2. The concentration of bioactive compounds of PFAD and USF

Bioactive Compound	PFAD		USF	
	ppm	%	ppm	%
Vitamin E	195,60	0,1956	19.600	1,96
Phytosterols	7.476,56	0,7476	5.500	0,55
Squalene	2.373,27	0,2373	323.000	32,30

The increase of vitamin E and squalene was related to elimination of free fatty acid as the major component of PFAD during saponification. But, the decrease of phytosterols was still unknown. Our previous study showed that all of the bioactive compounds increased in USF after PFAD saponification, including phytosterols with the concentration in USF was 9.18 and 7.77% [6].

The concentration of bioactive compounds in USF was high. The phytosterols content of USF was 0.55% or 5.5 mg/g, vitamin E 1.96% or 19.6 mg/g, and squalene 32.30% or 323 mg/g. Vitamin E of USF comprised of 83% tocotrienols and the remaining is tocopherol.

Phytosterols was the lowest concentration of bioactive compounds of PFAD. The concentration of phytosterols in USF was 0.55% that meant 1 g of USF contained 5.5 mg of phytosterols. Phytosterols of USF contained 0.42% beta sitosterol and 0.13% campesterol. Meanwhile, squalene was the highest bioactive compound in USF with concentration of 32.30% or in 1 g of USF would provide 323 mg squalene.

3.2. The Effect of USF on Blood Serum Total Cholesterol Level

USF administration to hypercholesterolaemia rats decreased blood serum total cholesterol level. The degree of total cholesterol level decline depended on USF doses (Figure 1). Group of hypercholesterolaemia rats without USF administration showed high blood serum total cholesterol level and total cholesterol level slightly decreased during 4 weeks experiment. USF administration at dose of 1000 mg/kg bw/day revealed blood serum total cholesterol level that reached normal (blood serum total cholesterol level \pm 100 mg/dL) after 4 weeks administration.

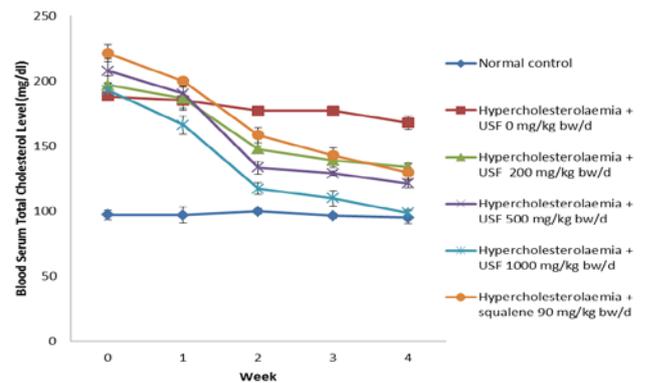


Figure 1. Blood serum total cholesterol level after USF administration at various doses

USF doses significantly affected blood serum total cholesterol reduction. Higher dose of USF caused greater reduction of serum blood cholesterol level. Thus, the highest decline of blood serum total cholesterol was found at USF dose of 1000 mg/kg bw/day. At the lowest dose (200 mg/kg bw/day), the phytosterol was 1.1 mg/kg bw/day and squalene was 64.60 mg/kg bw/day. The USF at dose of 200 mg/kg bw/day was more effective in reducing blood serum total cholesterol than pure squalene at dose of 90 mg/kg bw/day, although the dose of squalene at USF was lower.

Administration of USF at dose of 200 and 500 mg/kg bw/day showed similar pattern, but the decline of blood serum cholesterol level was sharp at dose of 1000 mg/kg bw/day. At dose of 1000 mg/kg bw/day, blood serum cholesterol level reached normal after USF administration for 4 weeks.

3.3. The Effect of USF on Blood Serum Triglyceride Level

Serum triglyceride level is one of indicator of blood lipid profile. The changes of blood serum triglyceride level of each group of rats were shown at Figure 2. The blood serum triglyceride level decreased in line with

increasing USF doses. The sharp decline of blood serum triglyceride level was found at USF dose of 1000 mg/kg bw/day and at the end of experiment (week 4), the blood serum triglyceride level reached normal.

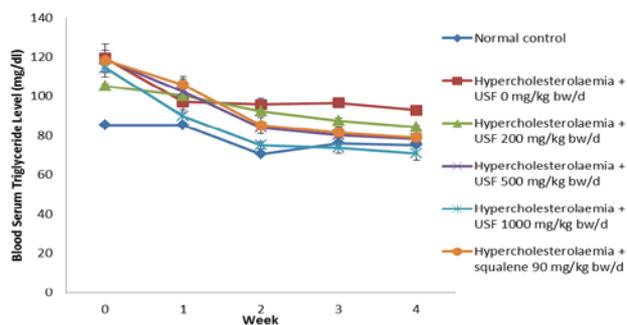


Figure 2. Blood serum triglyceride level after USF administration at various doses

As blood serum total cholesterol level, the decrease of blood serum triglyceride level of USF at dose 200 mg/kg bw/day almost similar to squalene supplement administration at dose of 90 mg/kg bw/day, although at USF dose of 200 mg/kg bw/day the squalene dose was 64.60 mg/kg bw/day. It meant that USF was more effective in lowering blood serum triglyceride level. USF administration at dose of 1000 mg/kg bw/day for 4 weeks resulted in normal triglyceride level.

3.4. The Effect of USF on Blood Serum LDL Cholesterol Level

The effect of USF doses on blood serum LDL cholesterol level was shown in Figure 3. Blood serum LDL cholesterol level decreased in all USF doses, meanwhile control groups still showed no reduction in LDL cholesterol level. The decrease in blood serum LDL cholesterol level had occurred since week 1 of the treatments. Group of rat administered by USF 1000 mg/kg bw/day had approximate normal LDL cholesterol level at week 4.

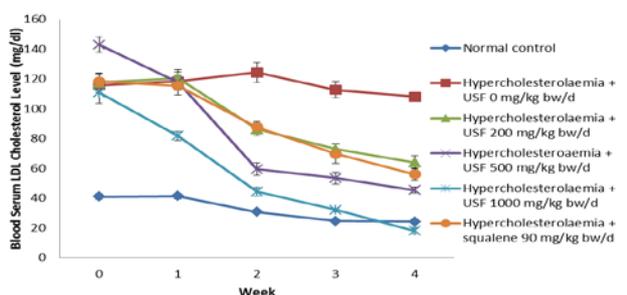


Figure 3. Blood serum LDL cholesterol level after USF administration at various doses

Blood serum LDL cholesterol level reduction due to USF treatment showed that USF was effective to lower LDL cholesterol level. This reduction was caused by the presence of phytosterols, squalene, and vitamin E in USF. Higher dose of USF resulted on greater decrease of LDL cholesterol level. The highest decline of LDL cholesterol level was found on USF 1000 mg/kg bw/day. In reducing LDL cholesterol level, squalene supplement at dose of 90 mg/kg bw/day showed almost as effective as USF dose of 200 mg/kg bw/day that contained 64.60 mg squalene.

3.5. The Effect of USF on Blood Serum HDL Cholesterol Level

USF intake for 4 weeks increased blood serum HDL level (Figure 4). HDL cholesterol increase related to USF administration that reduce total cholesterol and LDL cholesterol levels simultaneously. Increasing USF dose resulted in increasing blood serum HDL cholesterol level.

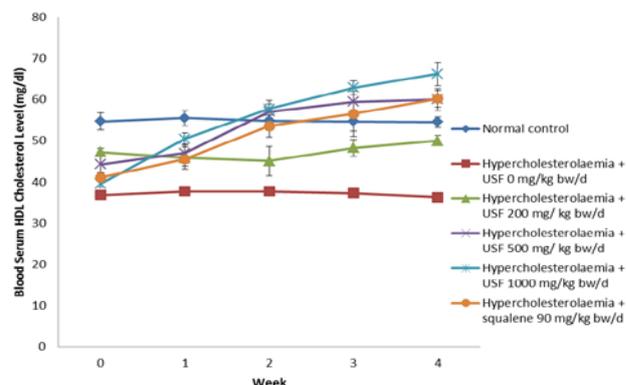


Figure 4. Blood serum HDL cholesterol level after USF administration at various doses

The higher rise of blood serum HDL cholesterol level was found at USF dose of 1000 mg/kg bw/day. Commercial squalene at dose of 90 mg/kg bw/day was less effective in rising HDL cholesterol level than USF 200 mg/kg bw/day (equivalent to squalene 64.60 mg/kg bw/day). This finding proved that multi components of bioactive compounds in USF improved blood lipid profile synergistically.

4. Discussions

4.1. Bioactive Compounds of PFAD

Bioactive compounds found in PFAD are vitamin E, phytosterols, and squalene. Palm oil contained vitamin E 600-1,000 ppm, phytosterols 300-620 ppm, carotenoid 500-700 ppm, squalene 250-540 ppm, phospholipids 20-100 ppm, co-enzyme Q10 10-80 ppm, and polyphenol 40-70 ppm [18]. Meanwhile, our previous study [27] showed that PFAD from several palm oil refineries contained vitamin E 65-281 ppm, phytosterols 407-6012 ppm, and squalene 206-1274 ppm. Vitamin E of PFAD comprised of α -tocopherol 9-135 ppm, α -tocotrienol 12-52 ppm, δ -tocotrienol 5-64 ppm, γ -tocotrienol 32-118 ppm. Meanwhile phytosterols of PFAD consisted of β -sitosterol 382-3,576 ppm, stigmasterol 11-1548 ppm, and campesterol 19-1,720 ppm.

USF and PFAD had different concentration and composition of bioactive compounds. Generally, USF had higher bioactive compounds than PFAD (Table 2) because they were accumulated in unsaponifiable fraction [9]. Vitamin E increased 245% in USF compared to PFAD, phytosterols decreased 73%, and squalene increased 13611%. The increase of vitamin E and squalene was related to elimination of free fatty acid as the major component of PFAD during saponification. But, the decrease of phytosterols was still unknown. Our previous study [6] showed that all of the bioactive compounds increased in USF after PFAD saponification, including

phytosterols with the concentration in USF was 9.18 and 7.77%. The phytosterols content of USF was 0.55% or 5.5 mg/g, vitamin E 1.96% or 19.6 mg/g, and squalene 32.30% or 323 mg/g.

The concentration of bioactive compounds in USF was high. Consumption of 1 g USF per day will provide 19.6 mg that meets the daily requirement of vitamin E or 140% of daily reference value. Other sources of vitamin E are rice bran (90.9 mg/100 g), wheat sprout (153.7 mg/100 g), coconut (1.1 mg/100 g), soybean (7.8 mg/100 g), and olive (11.9 mg/100 g) [28]. Meanwhile, USF contained vitamin E of 1960 mg/100 g.

Phytosterols was the lowest concentration of bioactive compounds of PFAD. The concentration of phytosterols in USF was 0.55% that meant 1 g of USF contained 5.5 mg of phytosterols. This concentration was low compared to other sources such as crude corn fiber oil (8.79%), crude corn kernel oil (7.94%), refined corn kernel oil (1.11%), and commercial corn oil (0.74%) [29]. In palm oil, phytosterol is a minor component that comprises of campesterol, stigmasterol, and β -sitosterol [11]. Squalene was the highest bioactive compound in USF with concentration of 32.30% or in 1 g of USF would provide 323 mg squalene.

4.2. The Effect of USF on Lipid Profile Improvement

USF administration to hypercholesterolaemia rats decreased blood serum total cholesterol level. The degree of total cholesterol level decline depended on USF doses (Figure 1). Group of hypercholesterolaemia rats without USF administration showed high blood serum total cholesterol level and total cholesterol level slightly decreased during 4 weeks experiment. USF administration at dose of 1000 mg/kg bw/day revealed blood serum total cholesterol level that reached normal (blood serum total cholesterol level \pm 100 mg/dL) after 4 weeks administration.

The mechanism of USF in reducing blood serum total cholesterol level was related to the action of phytosterols. Phytosterols inhibits cholesterol absorption in the digestive system. The higher the dose of USF made higher cholesterol absorption inhibition. According to Mel'nikov et al (2003), phytosterols are the compounds that have very similar structure to cholesterol [30]. Phytosterols/stanols, which are structurally related to cholesterol, are thought to compete with cholesterol for solubilisation into mixed micelles. This leads to a reduced absorption of cholesterol and/or to an enhanced efflux of cholesterol back into the intestinal lumen due to a higher expression of the ABC transporter. Both mechanisms ultimately result in an increased faecal output of cholesterol [31]

Equal contents of cholesterol and either campesterol, stigmasterol, sitosterol, or stigmastanol (sitostanol) decreased cholesterol solubility in micelles by approximately 50% compared to no phytosterol present, with stigmasterol performing slightly better than sitosterol. Phytosterols competed with cholesterol in a dose-dependent manner. Unsaturated fatty acids increased the micelle solubility of sterols as compared with saturated or no fatty acids. No differences were detected in the size of the model micelles. Together, these data indicate that stigmasterol combined with saturated fatty acids may be

more effective at lowering cholesterol micelle solubility *in vivo*. Four different phytosterols decreased cholesterol solubility to a very similar extent. Stigmasterol was, by a small margin, the most potent of the four, decreasing the concentration of micellar cholesterol and total solubilized sterols [32]. In this study, stigmasterol was not found in USF of PFAD. It was supposed that β sitosterol and campesterol in USF had a role in decreasing cholesterol level.

Addition of phytosterol ester decreased the incorporation of cholesterol into the duodenum aqueous phase including micelles. Phytosterol ester either dispersed in fat during a meal or micro-dispersed in a liquid form after a meal resulted in a markedly reduced occurrence of meal-derived cholesterol in the circulation at a comparable extent [33]. Previously, the study of Chen et al (2010) showed that phytosterols intake of 2 g/kg feed for 5 weeks reduced 40% cholesterol level in the liver, 31% in aorta, and 19% in renal [34].

According to Kamal-Eldin and Moazzami (2009), the role of phytosterols is not only by inhibiting cholesterol absorption but also inhibiting cholesterol synthesis through HMG-CoA reductase inhibition [35]. In phytosterolemia, a condition that indicated by elevated sitosterol level, the activity of HMG CoA reductase is low [36].

Bartnikowska (2009) concluded that the extent of the hypocholesterolemic effect of phytosterols depends on various factors of which the most important are: (i) phytosterols origin determining their profile, (ii) the dose and manner of dosage (as one dose or divided into portions), (iii) duration of treatment, and (iv) age of the patient as well as the type and intensity of lipid metabolism disorders [37].

USF contained squalene of 32.30%. Some studies showed that squalene from animal and vegetable have different effect on cholesterol metabolisms. Animal squalene such as shark squalene has hypercholesterolaemic property, meanwhile vegetable squalene such as amaranth squalene shows otherwise [19,20,21,22]. This study showed that palm squalene in USF had hypocholesterolaemic effects, as well as squalene supplement also lowered total cholesterol, although the effect was not less effective compared to squalene in USF. The level of squalene intake from USF at various doses was 0 mg (USF 0 mg/kg bw/day), 64.60 mg (USF 200 mg/kg bw), 161.5 mg (USF 500 mg/kg bw/day), and 323 mg (USF 1000 mg/kg bw/day). Study of Miettinen *et al.* (1994) showed that administration of squalene 0.5 g/day decreased the serum lipid concentration.

In the 9-wk study 85% of squalene was absorbed but cholesterol synthesis measured by fecal analysis was insignificantly increased. Subsequent conversion of squalene to cholesterol had markedly inhibited presqualene cholesterol synthesis and HMG-CoA reductase activity and that these inhibitions may be enhanced by long-term squalene administration. The effect of squalene on blood LDL cholesterol is dose dependent. Small squalene doses obviously inhibit endogenous presqualene cholesterol synthesis with no effects on LDL receptors or serum cholesterol. Large amounts of squalene-rich olive oil with long-term use might have harmful effects on the serum cholesterol concentration [38].

Beside squalene and phytosterols, USF also contained tocotrienols 1.96%. It was reported that tocotrienols, mainly gamma and delta tocotrienols, down regulate cholesterol synthesis by affecting gene that responsible to HMG reductase synthesis [39]. Administration of USF at doses of 200, 500, and 1000 mg/kg bw/day provided tocotrienols 2.92 mg, 9.8, and 19.6 mg/kg bw/day, respectively.

USF doses significantly affected blood serum total cholesterol reduction. Higher dose of USF caused greater reduction of serum blood cholesterol level. Thus, the highest decline of blood serum total cholesterol was found at USF dose of 1000 mg/kg bw/day. At the lowest dose (200 mg/kg bw/day), the phytosterol was 1.1 mg/kg bw/day and squalene 64.60 mg/kg bw/day. The USF at dose of 200 mg/kg bw/day was more effective in reducing blood serum total cholesterol than pure squalene at dose of 90 mg/kg bw/day, although the dose of squalene at USF was lower. This was due to the synergistic effect of other bioactive compounds in USF such as phytosterols and vitamin E.

Administration of USF at doses of 200 and 500 mg/kg bw/day showed similar pattern, but the decline of blood serum cholesterol level was sharp at dose of 1000 mg/kg bw/day. At dose of 1000 mg/kg bw/day, blood serum cholesterol level reached normal after USF administration for 4 weeks.

4.3. The Effect of USF on Blood Serum Triglyceride Level

Serum triglyceride level is one of indicator of blood lipid profile. The changes of blood serum triglyceride level of each group of rat were shown at Figure 2. The blood serum triglyceride level decreased in line with increasing USF doses. The sharp decline of blood serum triglyceride level was found at USF dose of 1000 mg/kg bw/day and at the end of experiment (week 4), the blood serum triglyceride level reached normal.

As blood serum total cholesterol level, the decrease of blood serum triglyceride level of USF at dose 200 mg/kg bw/day almost similar to squalene supplement administration at dose of 90 mg/kg bw/day, although at USF dose of 200 mg/kg bw/day the squalene dose was 64.60 mg/kg bw/day. It meant that USF was more effective in lowering blood serum triglyceride level.

The phytosterols in USF was also supposed to decrease blood serum triglyceride level. Bile acid secretion increased by phytosterols administration from USF and affected blood serum triglyceride level. Bile acid influenced fat absorption, and fat absorption was interfered by high bile acid secretion that caused in the decrease of blood serum triglyceride level. USF administration at dose of 1000 mg/kg bw/day for 4 weeks resulted in normal triglyceride level. Low level of triglyceride leads to reduction of cholesterol level. Triglyceride in our body is catabolized into acetyl Co A, a precursor in cholesterol synthesis [23]. Thus, low triglyceride level affects the cholesterol synthesis that imply to low blood cholesterol level.

4.4. The Effect of USF on Blood Serum LDL Cholesterol Level

Elevated concentrations of plasma LDL-cholesterol are recognised as a major risk factor for the development of premature CVD [40]. Among lipoproteins, the property of LDL cholesterol is contrary to the HDL, that high LDL cholesterol leads to atherosclerosis meanwhile HDL cholesterol has the preventive effect. In LDL, lipid is incorporated into lipoprotein in higher amount than protein. USF administration increased HDL cholesterol in concomitant with LDL cholesterol level reduction. The effect of USF doses on blood serum LDL cholesterol level was shown in Figure 3.

Blood serum LDL cholesterol level decreased in all USF doses, meanwhile control groups still showed no reduction in LDL cholesterol level. The decrease in blood serum LDL cholesterol level had occurred since week 1 of the treatments. Group of rat administered by USF 1000 mg/kg bw/day had approximate normal LDL cholesterol level at week 4.

USF was effective to lower LDL cholesterol level due to the presence of phytosterols, squalene, and vitamin E. Piironen *et al.* (2000) and Cohen (2008) indicated that phytosterols inhibit cholesterol absorption from foodstuffs and phytosterols also inhibit reabsorption of cholesterol from bile acid [15,41]. Phytosterols have similar structure to cholesterol. Therefore they can replace cholesterol from micelle. According to Kamal-Eldin and Moazzami (2009), daily consumption of phytosterols 1-2 g can reduce LDL cholesterol level 10–20% [35]. This research showed that administration of phytosterols 1.1 mg-5.5 mg/kg bw/day reduced LDL cholesterol level 7-140%.

Other bioactive compounds in USF that responsible to lower LDL cholesterol were squalene and vitamin E mainly tocotrienols. Some studies showed that there were inconsistent results on LDL cholesterol level after squalene intake [21,22]. Long-term study showed that the serum concentration of total cholesterol was increased by the large squalene intake (1 g/d) because of the augmented number of LDL particles with low surface lipids (free cholesterol and phospholipids), possibly a consequence of increased cholesterol synthesis from squalene [38]. Shin *et al.* (2004) supposed that the sources of squalene influenced the effect of squalene on LDL cholesterol level. Squalene from vegetable origin tended to decrease LDL cholesterol level, meanwhile squalene from animal has otherwise effect [20].

Response of plasma and lipoproteins to tocotrienols may be determined by the dose of tocotrienol supplementation. Tocotrienol isomers (α , γ , and δ tocotrienols) were present in the circulating plasma and lipoproteins (Triglyceride Rich Particle/TRP, LDL, and HDL) after tocotrienol supplementation [42]. This study showed tocotrienols from USF might have a role in reducing LDL cholesterol level.

Higher dose of USF resulted on greater decrease of LDL cholesterol level. The highest decline of LDL cholesterol level was found on USF 1000 mg/kg bw/day. In reducing LDL cholesterol level, squalene supplement at dose of 90 mg/kg bw/day showed almost as effective as USF dose of 200 mg/kg bw/day that contained 64.60 mg squalene/kg bw/day. This was due to not only one bioactive compound that responsible to LDL cholesterol reduction, but also multi bioactive components that have synergistic role.

4.5. The Effect of USF on Blood Serum HDL Cholesterol Level

HDL has a function to transport cholesterol from peripheral tissue to liver. Low blood HDL cholesterol level related to high risk of coronary heart disease [43]. USF intake for 4 weeks increased blood serum HDL level (Figure 4). HDL cholesterol increase related to USF administration that reduced total cholesterol and LDL cholesterol levels simultaneously. Increasing USF dose resulted in increasing blood serum HDL cholesterol level.

In blood plasma, cholesterol is not soluble so it requires HDL for transporting cholesterol into liver. Liver will eliminate the excess of cholesterol by bile acid synthesis and secretion. The cholesterol transport into liver is one mechanism to maintain cholesterol level and balance in blood plasma. The decline in LDL cholesterol also causes decreasing cholesterol transport into liver and concomitantly increases HDL cholesterol. HDL cholesterol increases cholesterol transport from blood into liver thus reduces blood total cholesterol. In liver, cholesterol is converted into bile acid that was secreted into feses [41].

The highest rise of blood serum HDL cholesterol level was found at USF dose of 1000 mg/kg bw/day. Commercial squalene at dose of 90 mg/kg bw/day was less effective in rising HDL cholesterol level than USF 200 mg/kg bw/day (equivalent to squalene 64.60 mg/kg bw/day). This finding proved that multi components of bioactive compounds in USF improved blood lipid profile synergistically.

5. Conclusions

USF from PFAD that was obtained by saponification contained vitamin E 1.96% (19.6 mg/g), phytosterols 0.55% (5.5 mg/g), and squalene 32% (320 mg/g). Multi component of bioactive compounds in USF synergistically improved blood lipid profile in hipercholesterolaemia condition. USF had a role to decrease blood serum total cholesterol, LDL cholesterol, and triglyceride levels, and concomitantly increased blood serum HDL cholesterol level. Increasing doses of USF resulted in better blood lipid profile improvement. USF was more effective in improving blood lipid profile than commercial squalene supplement.

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Conflict of Interest

The authors have no competing interest.

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