Fishery Technology 62 (2025) : 337 - 343

In Vivo Assessment of the Nutritional Efficacy of Encapsulated Squalene in Male Wistar Rats

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Abstract

Squalene, a triterpenoid compound, possesses potent bioactivities by virtue of its high degree of unsaturation. In the present study, stable squalene microparticles prepared via spray drying-mediated encapsulation were subjected to a detailed nutritional assessment in male Wistar albino rats. Experimental animals were divided into four groups– I, II, III and IV, and were fed encapsulated squalene at the rate of 0, 150, 300 and 450 mg/kg body weight, respectively. The treatment groups fed with encapsulated squalene showed improved weight gain percentage and specific growth rate. Squalene supplementation could also lower cholesterol, low density lipoprotein and very low-density lipoprotein levels, suggesting its cardioprotective property. The findings of the study indicate that encapsulated squalene powder can be effectively used as a dietary supplement for improved growth performance along with lipid-lowering attributes.

Keywords: Squalene, whey protein, maltodextrin, lipid profiling

Introduction

Squalene, chemically 2, 6, 10, 15, 19, 23-hexamethyl-2, 6, 10, 14, 18, 22- tetracosahexane, is a polyunsaturated triterpenoid compound that is widely distributed in nature. It serves as a precursor for cholesterol and many other steroid compounds. Moreover, it plays an important role in the synthesis of fat-soluble vitamins, hormones, etc. (Gao, Ma, Zhou, Li, & Xiang, 2022). The presence of six double bonds makes the molecule highly unsaturated and, at the same time, highly reactive. By virtue of this high degree of unsaturation, squalene exhibits diverse biological activities such as antioxidant, anti-inflammatory, anti-cancerous, anti-bacterial, detoxificatifying, immunity enhancing effects, etc. highlighting its significance as a dietary supplement (Gohil, Bhattacharjee, Khambhati, Braddick, & Singh, 2019).

Reports state that in the human body, squalene constitutes 10-14% of sebum lipids and approximately 10% of surface lipids, making it an important biological constituent (Gohil et al., 2019). It has been reported that the human body can synthesize about 125-475 mg of squalene daily. However, the synthesis of squalene is reported to decrease with age (Cheng, Ji, Zhang, & Fang, 2024). Hence, it would be ideal if squalene could be supplemented through diet. The major sources of squalene in nature are shark liver oil, olive oil, amaranth oil, wheat bran oil, camellia oil etc. (Lekshmi et al., 2023). Extraction of squalene can be achieved through various methods such as supercritical fluid extraction, molecular distillation, ultrasound-assisted extraction, enzyme-assisted extraction, etc. (Cheng et al., 2024). However, even after extraction, the direct use of squalene in any food formulations/supplements is restricted owing to its oxidative instability imparted by the high degree of unsaturation. Stabilizing squalene through encapsulation can be a feasible strategy to address this oxidative instability. The success of squalene stabilization depends on the ideal selection of encapsulation methods, type of wall materials, etc. Wall materials such as sodium caseinate, chitosan, whey protein, gelatin, gum Arabic, chitosan-whey protein, etc. have been employed by various researchers for stabilization of squalene (Lekshmi et al., 2017; Lekshmi et al., 2020).

Received 7 January 2025; Revised 2 July 2025; Accepted 27 July 2025

Handling Editor: Dr. Remya S.

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Following encapsulation, physicochemical characterization coupled with *in vitro* and *in vivo* studies needs to be carried out to establish the safety, toxicity, bioavailability, *in vitro* release pattern, etc. In a previous study, we reported the preparation of stable squalene microparticles followed by their detailed characterization and *in vitro* release studies (Lekshmi et al., 2021). However, to get a complete insight into its dietary implications, an *in vivo* assessment of the squalene microparticles also needs to be carried out. The present study was hence carried out with an objective to elucidate the nutraceutical implications of squalene microparticles based on a Wistar rat model study.

Materials and Methods

Stable squalene microparticles were prepared as per the procedure detailed by Lekshmi et al. (2021). Subsequently, an *in vivo* nutritional assessment was performed as detailed below:

Wistar albino rats (male), weighing between 133–158 g, were selected for the study. The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and approved by the Institutional Animal Ethics Committee of the Central Institute of Fisheries Technology, Cochin, over a period of 45 days. During the acclimatization period, rats were provided a standard diet (M/s Sai Foods, Bangalore, India) and water *ad libitum*. The diet composition was as follows: carbohydrate 56.2%, crude protein 22%, ash 7.5%, total fat 4.2%, crude fibre 3%, glucose 2.5%, vitamin 1.8%, sand silica 1.4%, calcium 0.8%, phosphorus 0.6%, providing a metabolizable energy of 3600 kcal.

After a seven-day acclimatization period, experimental animals were divided into four groups of six rats each. Group I (normal control) received the standard diet, Group II, III and IV received the standard diet and were orally administered with encapsulated squalene powder at the rate of 150, 300 and 450 mg/kg body weight, respectively, for a period of 45 days. The body weight of each rat was recorded at every seven days interval till the completion of the feeding trials. Weight gain (%) and specific growth rate (SGR) was calculated to monitor the growth performance using the following formulas:

$$\text{Weight gain} = \frac{\text{(Final weight-Initial weight)}}{\text{Initial weight}} \times 100 \quad (1)$$

$$\text{Specific growth rate (SGR)} = 100\ \frac{\text{(Log average final weight - log average initial weight)}}{\text{Number of days}}$$

At the end of 45 days of experiment, the animals were anesthetized with chloroform and subsequently sacrificed. Blood collected was transferred immediately to a clean test tube without an anticoagulant and kept in a slanting position for 3 h to allow serum separation. Serum was separated from blood by centrifuging at 6,000 rpm for 20 min at 4ºC and stored at -20°C until further analysis. Heart, brain, liver and kidneys were dissected out immediately, washed in physiological saline, and weighed. A 5% tissue homogenate was prepared using chilled sucrose solution (0.25 M) using a high-speed homogenizer, followed by centrifugation at 6000 g for 20 min at 4°C. The supernatants were collected and stored at -20°C till analysis. Serum cholesterol, triglycerides (TG), low density lipo-protein (LDL), very low density lipo-protein (VLDL) and high density lipo-protein (HDL) were determined using an automated lipid analyzer (Abacus-250). Antioxidant enzymes such as superoxide dismutase and catalase activities of the tissue homogenates were measured by employing the method of Takahara et al. (1960) and Misra and Fridovich (1972), respectively. The activity of metabolic marker enzymes such as Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) were assayed in the different tissue homogenates as described by Wooten (1964). The data obtained were subjected to one-way analysis of variance (ANOVA) using SPSS version 16.0 (Chicago IL. USA) at a significant level of $p \leq 0.05$.

Results and Discussion

Dietary supplementation with microencapsulated squalene was found to improve the weight gain and specific growth rate when compared to the control group (Table 1). There was a clear-cut, pronounced dose-dependent effect on the weight gain % and SGR with the highest weight gain % and SGR observed in rats fed with the highest dose of encapsulated squalene (450 mg/kg wt). Improvement in weight gain can be attributed to the core material, squalene and the wall materials, maltodextrin and whey protein isolate, which might

Table 1. Effect of encapsulated squalene dietary supplementation on weight gain and SGR

Treatments	Weight gain (%)	SGR
T1	43.3 ± 3.77[a]	0.8 ± 0.06[a]
T2	44.93 ± 6.31[a]	0.83 ± 0.10[a]
T3	50.02 ± 8.57[a]	0.90 ± 0.93[a]
T4	50.68 ± 9.82[a]	0.91 ± 0.15[a]

All data are presented as mean ± SD (n = 6). [a]Values with the same superscript indicate no significant differences ($p > 0.05$) between treatments.

have some potential health promoting properties. Chen, Gu, Zhao, Zhang, and Zhou (2020) reported that the dietary supplementation of liquid squalene at the rates of 250, 500, 1000, and 2000 mg/kg increased the average daily weight gain in broilers. However, the feed-to-gain ratio was found to decrease during the feeding period. Similarly, Gao et al. (2022) reported that the daily dietary supplementation of squalene (purity, 92%) at the rate of 250 mg/kg considerably improved the average daily weight gain, final body weight and average feed intake in experiments conducted on early-weaned piglets. Chen, Gu, Zhao, and Zhou (2021) also reported the positive effect of squalene supplementation at the rate of 1 g/kg in improving the growth performance, such as weight gain, average feed intake, etc., in the case of diquat-challenged broiler chicks.

Liu, Hosokawa, and Miyashita (2018) also reported a significant weight gain when squalene was fed at the rate of 2%. In contrast to our results, Gabás-Rivera et al. (2014) reported no significant weight gain with squalene supplementation, suggesting that long term supplementation may be well tolerated within the bodily changes. Liu et al. (2009) reported weight gain in the control groups, whereas squalene-fed groups exhibited weight loss. Motawi, Sadik, and Refaat (2010) reported similar results, indicating an increased weight gain with squalene supplementation. Based on these findings, it is suggested that encapsulated squalene can be very well utilized as a health promoting functional food ingredient.

The tissue weights of the kidney and brain have shown a decreasing trend with the increasing squalene dosage; however, this decrease was not statistically significant ($p \geq 0.05$) (Table 2a). A non-significant ($p \geq 0.05$) increase in liver weight was also noticed. Similar to our results, Liu et al. (2018) reported that there was no significant influence of squalene supplementation on the tissue weight except in the case of the liver. Kumar, Narayan, Sawada, Hosokawa, and Miyashita (2016) also reported similar results, stating a significant increase in liver weight following squalene supplementation. A significant difference in protein content of tissues was also observed, with liver having the highest protein content, followed by the kidney. The brain and heart had the lowest protein levels (Table 2b).

The effect of squalene supplementation on serum lipid profile was analysed among control and treatment groups (Table 3). With the increase in dosage, serum cholesterol was found to decrease from 94 mg/dL to 58 mg/dL. Squalene, an isoprenoid compound, is a biochemical precursor for cholesterol and many other steroid compounds. Hence, it is expected that oral administration of squalene may increase the serum levels of cholesterol. However, in the present study, it was noticed that there was an initial increase in serum cholesterol level (94 mg/dL) in case of rats fed with the lower squalene dose (T1) compared to control (74 mg/dL) and had decreased with the increase in squalene dosage (58 mg/dL). This can be attributed

Table 2a. Effect of squalene supplementation on the weight of different tissues

Treatments	Liver (g)	Kidney (g)	Brain (g)	Heart (g)
T1	5.0 ± 0.38[a]	1.45 ± 0.23[a]	1.65 ± 0.05[ab]	0.54 ± 0.02[a]
T2	5.22 ± 0.16[a]	1.26 ± 0.02[ab]	1.78 ± 0.10[b]	0.58 ± 0.01[a]
T3	5.11 ± 0.70[a]	1.13 ± 0.17[a]	1.59 ± 0.04[ab]	0.55 ± 0.01[a]
T4	5.11 ± 0.17[a]	1.22 ± 0.03[ab]	1.56 ± 0.17[a]	0.64 ± 0.05[b]

All data are expressed as mean ± SD (n = 6). [a–b]Different superscript letters indicate significant differences between treatments ($p \leq 0.05$).

Table 2b. Effect of squalene supplementation on protein content (mg protein/g tissue) of various tissues

Treatments	Liver	Kidney	Brain	Heart
T1	129.16 ± 5.19^{c}	100.91 ± 1.31^{a}	65.18 ± 0.79^{a}	59.35 ± 1.03^{a}
T2	113.80 ± 2.07^{a}	102.18 ± 5.48^{a}	70.55 ± 2.39^{c}	68.41 ± 1.21^{b}
T3	122.07 ± 3.01^{b}	114.09 ± 5.48^{c}	67.77 ± 0.28^{b}	75.30 ± 1.33^{c}
T4	113.83 ± 0.87^{b}	105.31 ± 6.16^{ab}	70.89 ± 0.13^{c}	70.49 ± 0.76^{b}

Data are presented as mean ± SD (n = 6). $^{a–c}$Different superscript letters indicate significant differences between treatments ($p \leq 0.05$).

Table 3. Effect of squalene supplementation of lipid profile

Treatment	Cholesterol (mg/dL)	Triglycerides (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)	HDL (mg/dL)
T1	74	52	61.9	10.4	1.7
T2	94	84	73	16.8	4.1
T3	88	72	67.6	14	6
T4	58	42	41	8	9

to the effect of 3-hydroxy-3-methylglutaryl coenzyme A reductase, which is a key enzyme involved in the biosynthesis of cholesterol. It has already been studied and established about the feedback inhibition of the enzyme in the presence of exogenous squalene, thereby downregulating the conversion from acetyl CoA to cholesterol (Sawada et al., 2001). Kumar et al. (2016) reported that the supplementation of squalene has marked an increase in serum cholesterol and HDL. Similarly, Chan, Tomlinson, Lee, and Lee (1996) have reported that a daily squalene supplementation at the rate of 0.86 g reduced the serum cholesterol levels. Gabás-Rivera et al. (2014) have reported that modulation of cholesterol levels with dietary supplementation is dosage dependent, stating that low squalene dosage promotes cholesterol biosynthesis whereas higher doses reduce the cholesterol level. In contrast to our results, Strandberg, Tilvis, and Miettinen (1989) reported that dietary supplementation of squalene (900 mg/day) did not result in any significant increase in serum triglyceride or cholesterol levels. This can be possibly due to the fact that the cholesterol synthesized from squalene might have been excreted in the form of cholesterol itself or as bile acids.

Similar to cholesterol, triglycerides and low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) also showed a similar decreasing trend with increasing dosage of squalene supplementation. The triglyceride level increased from 52 mg/dL in control to 84 mg/dL when squalene was fed at lower doses (150 mg/kg). However, with the increase in squalene dosage, triglyceride levels decreased accordingly. Similarly, LDL levels declined from 73 mg/dL to 41 mg/dL, and VLDL levels from 10.4 mg/dL to 8 mg/dL, with increasing squalene supplementation. These findings are in line with Kumar et al. (2016) who reported decreased LDL levels with squalene supplementation. Liu et al. (2009) also reported that squalene supplementation reduced the serum cholesterol and triglyceride levels. Gabás-Rivera et al. (2014) reported similar results, noting that squalene supplementation decreased the triglyceride levels.

Conversely, high density lipoprotein (HDL) exhibited an increasing trend with increasing squalene dosage. The HDL level increased from 1.7 mg/dL in the control group to 9 mg/dL in squalene-fed groups. Previous studies have suggested an inverse correlation between HDL cholesterol levels and coronary heart disease (Sharrett et al., 2001). Farrer (2018) reported that even 1 mg/dL increase in HDL level can reduce the risk of cardiovascular diseases, LDL cholesterol, and triglyceride levels by 2-3%. These observations suggests that squalene supple-

Table 4. Effect of squalene supplementation on Catalase, SOD, AST and ALT in different tissues

Catalase (EC 1.11.1.6)				
Treatments	Liver	Kidney	Brain	Heart
T1	19.94 ± 2.04^{a}	39.15 ± 1.08^{a}	9.09 ± 1.61^{b}	14.51 ± 1.85^{a}
T2	37.52 ± 1.80^{b}	42.08 ± 2.43^{a}	9.45 ± 0.14^{a}	9.27 ± 0.75^{a}
T3	39.84 ± 0.77^{b}	38.78 ± 1.22^{a}	3.94 ± 1.50^{ab}	12.54 ± 3.17^{a}
T4	30.17 ± 0.18^{b}	41.39 ± 0.63^{a}	2.70 ± 4.91^{ab}	7.43 ± 0.23^{a}
SOD (EC 1.15.1.1)				
Treatment	Liver	Kidney	Brain	Heart
T1	258.25 ± 1.39^{b}	201.36 ± 5.11^{b}	63.19 ± 5.43^{a}	58.63 ± 2.70^{a}
T2	219.5 ± 5.6^{a}	170.87 ± 1.046^{a}	87.44 ± 2.21^{a}	71.99 ± 2.20^{b}
T3	247.06 ± 1.08^{b}	224.91 ± 1.19^{c}	84.86 ± 3.48^{a}	85.98 ± 4.70^{c}
T4	220.07 ± 1.25^{a}	193.31 ± 1.73^{ab}	81.22 ± 6.65^{a}	85.24 ± 4.72^{c}
AST (EC 2.6.1.1)				
Treatments	Liver	Kidney	Brain	Heart
T1	4.14 ± 0.20^{b}	7.82 ± 0.21^{c}	10.98 ± 0.10^{c}	13.98 ± 0.86^{c}
T2	5.58 ± 0.49^{d}	6.98 ± 0.55^{b}	8.90 ± 0.51^{a}	10.60 ± 1.22^{b}
T3	4.66 ± 0.10^{c}	6.36 ± 0.35^{ab}	9.63 ± 0.08^{b}	6.93 ± 0.12^{a}
T4	3.53 ± 0.07^{a}	5.60 ± 0.45^{a}	9.46 ± 0.03^{b}	8.17 ± 0.29^{a}
ALT (EC 2.6.1.2)				
T1	5.19 ± 0.15^{a}	1.22 ± 0.11^{b}	6.27 ± 0.08^{a}	3.77 ± 0.05^{a}
T2	5.19 ± 0.33^{a}	0.89 ± 0.02^{a}	3.33 ± 1.24^{a}	4.26 ± 0.12^{a}
T3	5.79 ± 0.19^{b}	2.07 ± 0.29^{c}	3.82 ± 0.63^{a}	4.67 ± 1.04^{a}
T4	5.48 ± 0.32^{ab}	1.37 ± 0.13^{b}	4.10 ± 0.39^{a}	4.14 ± 0.18^{a}

All data are given as mean ± SD, n=6; $^{a-c}$Different letters with mean value indicate differences ($p \leq 0.05$) between treatments, Units: milllimols H_2O_2 decomposed min^{-1} mg^{-1} protein at 37°C (Catalase); 50% inhibition of epinephrine auto oxidation mg^{-1} protein min^{-1}) (SOD); nano moles oxalo acetate released/mg protein/min at 37°C (AST); nano moles of sodium pyruvate formed/mg protein/min at 37°C (ALT).

mentation may have significant cardioprotective effects through its mode of action. Farvin et al. (2004) already reported that daily squalene supplementation had a significant cardioprotective effect. Overall, the results indicated that the cholesterol-LDL and VLDL lowering effect is highly dose-dependent. It can be concluded that squalene can also positively modify the HDL metabolism.

Catalase activity of liver and brain exhibited a significant change, whereas no significant change in the kidney and heart activity was observed (Table 4). Among all the tissues, kidney exhibited the highest catalase activity, followed by the liver, whereas the lowest activity was noted in the brain. The catalase activity was found to decrease with the increase in squalene supplementation. Similar to catalase, SOD activity also showed a significant difference in the activity of the liver, kidney, and heart. The highest SOD activity was recorded in liver tissues, followed by the kidney and the lowest activity in the heart and brain. Similar to catalase, SOD activity was also found to decrease with an increase in squalene supplementation.

Catalase, a common antioxidant enzyme, is highly efficient and can break down millions of hydrogen peroxide molecules. Superoxide dismutase is an-

other important endogenous antioxidant enzyme that acts against reactive oxygen species (ROS). As the first line of antioxidant defence enzymes, both are expressed mostly in stressful conditions and helps in protecting the organism from cellular damage. A lower expression of catalase and SOD enzymes in squalene-fed groups suggested that squalene alone was sufficient to act against ROS. The scavenging activity of squalene may be attributed to its isoprenoid structure. Moreover, the free radical scavenging activity of squalene might have helped in maintaining the cell viability and keeping the enzyme activity at its lowest level. In contradiction to our results, Kumar et al. (2016) reported an increased level of antioxidant enzymes such as catalase and SOD upon squalene supplementation. Nonetheless, it can be concluded that dietary supplementation of squalene itself is enough to take care of radical scavenging effects and thereby restore the activities of antioxidant enzymes.

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities are mostly employed as biological markers for hepatic injury. In the present study, there was a significant ($p \leq 0.05$) change in the AST activity of different tissues with squalene supplementation (Table 4). With increase in squalene dosage, AST activity was found to decrease significantly, with the highest value recorded in the brain. However, the ALT activity was found to increase with an increase in squalene dose except in the brain tissue. The higher ALT activity was noted in the liver tissue and comparatively lower levels in the kidney. Generally, amino acids are deaminated, and the intermediates produced in the TCA cycle are used as substrates for gluconeogenesis. Both AST and ALT helps to reuse the amino nitrogen for the synthesis of new amino acids. Farvin et al. (2004) observed elevated levels of AST and ALT when the effect of squalene supplementation on isoproterenol-induced myocardial infarction was analysed. This indicates that both AST and ALT enzymes are highly expressed in case of cellular damage or injuries. Hence, the lowered expression of AST is justified as the treatment groups have not been exposed to any stressful condition.

The present study tried to analyse the influence of dietary supplementation of encapsulated squalene in male Wistar rats. Nutritional evaluation of the encapsulated squalene conducted on Wistar male rats indicated that it had cholesterol, triglycerides, LDL and VLDL level lowering effects and the effects were observed to be entirely dosage dependent. Additionally, squalene supplementation also helped in restoring the level of antioxidant enzymes emphasizing their antioxidant potential. The supplementation of encapsulated squalene was also found to improve the growth performance. The study points out that squalene encapsulated with maltodextrin-whey – whey protein can very well be promoted as a dietary supplement.

Acknowledgements

The authors sincerely express their gratitude to the Director, ICAR-CIFT, for providing all the support needed for the study.

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