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The effect of kalangkala seed (*Litsea angulata*) biscuits containing flavonoids and unsaturated fatty acids on low-density lipoprotein cholesterol levels (LDL-C) in hypercholesterolemic wistar rats

Pengaruh biskuit biji kalangkala (Litsea angulata) mengandung flavonoid dan asam lemak tidak jenuh terhadap kadar kolesterol low-density lipoprotein (LDL-C) pada tikus wistar hiperkolesterolemia

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Abstract

Cardiovascular disease remains a leading cause of death, and elevated low-density lipoprotein cholesterol (LDL-C) is a major modifiable risk factor. Kalangkala (*Litsea angulata*), a native Kalimantan plant, contains flavonoids and unsaturated fatty acids with potential hypolipidemic activity. This study aimed to evaluate the effects of kalangkala seed biscuits on serum LDL-C levels in hypercholesterolemic Wistar rats. A randomized pretest-posttest control group experiment was conducted from January to April 2021 at the Faculty of Pharmacy Laboratory, Lambung Mangkurat University. Eighteen male Wistar rats were allocated to three groups (n = 6 each): control, kalangkala seed biscuit (BBK), and kalangkala seed biscuit with duck egg yolk (BBKKT). LDL-C levels were measured before and after a 10-day intervention and analyzed using ANOVA after assumption testing, and Tukey's test was utilized to examine differences between groups for ΔLDL-C. Lowering of LDL-C levels was observed in both kalangkala seed biscuit groups, with a greater decrease in the biscuit treatment group than in the group undergoing continuous hypercholesterolemic induction. In contrast, LDL-C levels increased in the control group. This implies that LDL-C changes were significantly different between the groups. Kalangkala seed biscuits could lower LDL-C levels however the underlying pathways are unclear.

Keywords: LDL-C, hypercholesterolemia, functional food

Abstrak

Penyakit kardiovaskular masih menjadi penyebab utama kematian, dan peningkatan kadar LDL-C merupakan salah satu faktor risiko yang dapat dimodifikasi. Biji kalangkala (*Litsea angulata*), tanaman asli Kalimantan, mengandung flavonoid dan asam lemak tidak jenuh yang berpotensi memberikan efek hipolipidemik. Penelitian ini bertujuan menganalisis pengaruh biskuit biji kalangkala terhadap kadar LDL-C serum pada tikus Wistar hiperkolesterolemia. Eksperimen dengan rancangan randomized pretest-posttest control group dilaksanakan pada bulan Januari - April 2021 di Laboratorium Fakultas Farmasi Universitas Lambung Mangkurat. Sebanyak 18 tikus Wistar jantan dibagi menjadi tiga kelompok (n = 6): kontrol, biskuit biji kalangkala (BBK), serta biskuit biji kalangkala dan kuning telur bebek (BBKKT). Kadar LDL-C diukur sebelum dan setelah intervensi 10 hari, lalu dianalisis menggunakan uji ANOVA, dan uji Tukey digunakan untuk memeriksa perbedaan antar-kelompok terkait ΔLDL-C. Penurunan kadar LDL-C diamati pada kedua kelompok yang mengonsumsi biskuit biji kalangkala, dengan penurunan yang lebih besar terjadi pada kelompok perlakuan biskuit dibandingkan dengan kelompok yang menerima induksi hiperkolesterolemia berkelanjutan. Sebaliknya, kadar LDL-C meningkat pada kelompok kontrol. Hal ini menunjukkan adanya perbedaan yang signifikan dalam perubahan kadar LDL-C antar-kelompok. Biskuit biji kalangkala terbukti dapat menurunkan kadar LDL-C, namun mekanisme yang mendasarinya belum diketahui secara pasti.

Kata Kunci: *LDL-C,* Hiperkolesterolemia, Pangan Fungsional

Introduction

Noncommunicable diseases (NCDs) are the leading cause of mortality worldwide and represent a major global health challenge. According to the World Health Organization (WHO), cardiovascular diseases account for approximately 19.8 million deaths annually, representing more than 32% of all global deaths (World Health Organization, 2025). Similarly, the World Heart Federation has reported a growing burden of cardiovascular disease, particularly in developing countries, driven by urbanization, unhealthy lifestyles, and dietary patterns characterized by high saturated fat and low dietary fiber intakes (Cesare et al., 2023; Kesehatan, 2024). Hypercholesterolemia, characterized by elevated circulating low-density lipoprotein (LDL), is a major modifiable risk factor contributing to the development of cardiovascular disease (Kronenberg et al., 2022).

A similar trend was observed in Indonesia. The 2023 Indonesian Health Survey (SKI) reported an increasing prevalence of cardiovascular disease risk factors associated with unhealthy dietary habits, while cardiovascular diseases remain among the leading causes of mortality in Indonesia (Kesehatan, 2024). These findings highlight the need for preventive strategies beyond pharmacological treatment, including the development of functional foods that can improve lipid metabolism (Bjune et al., 2024; Zhang et al., 2025).

Bioactive compounds, including flavonoids, polyphenols, phytosterols, and unsaturated fatty acids, have demonstrated beneficial effects on lipid metabolism by inhibiting cholesterol synthesis, promoting bile acid excretion, modulating the gut microbiota, and reducing oxidative stress (Islam et al., 2021; Sun et al., 2021; Vignesh et al., 2024; Zhang et al., 2025). Flavonoids specifically reduce plasma LDL levels by inhibiting HMG-CoA reductase and upregulating hepatic LDL receptors, whereas plant-derived phytochemicals have consistently shown cardioprotective properties (Islam et al., 2021; Mohammadi, Farrell, et al., 2024; Sun et al., 2021; Xu et al., 2024).

Kalangkala (*Litsea angulata*), an endemic plant of Kalimantan belonging to the Lauraceae family, represents a promising local resource for functional food development. Previous phytochemical studies on the *Litsea* genus have identified flavonoids, alkaloids, tannins, phenols, and unsaturated fatty acids with antioxidant and antihyperlipidemic activities (Amit & Zinyin, 2021; Arifuzzaman et al., 2025; Dai et al., 2024; Rijai et al., 2023; Rikomah et al., 2024). Furthermore, LC-HRMS analysis has demonstrated that kalangkala seeds contain several antioxidant bioactive compounds (Mahfudh et al., 2024).

Flavonoids lower LDL levels through multiple mechanisms, including the inhibition of HMG-CoA reductase, upregulation of hepatic LDL receptors, prevention of LDL oxidation, and improvement of endothelial function (El-Rayyes et al., 2023; Jomova et al., 2025; Li & Zhang, 2023; Xu et al., 2024). Polyunsaturated fatty acids (PUFAs) synergistically contribute to these effects by promoting hepatic cholesterol metabolism and inhibiting its production. One major route of cholesterol removal is the increased conversion of cholesterol into bile acids; however, this pathway has been established mainly in research exploring specific bioactive chemicals rather than PUFAs (H. Duan et al., 2023; Luo et al., 2022). Moreover, flavonoids and PUFAs may exert synergistic lipid-lowering effects, suggesting that foods enriched with both compounds could provide an effective nutritional strategy for the prevention of hypercholesterolemia (Freitas et al., 2017; Wang et al., 2022).

In the present study, kalangkala seeds were developed into biscuits as a functional food vehicle because biscuits are shelf-stable, widely accepted by consumers, easy to distribute, and suitable for regular consumption. This formulation facilitates the daily intake of bioactive compounds and supports the practical application of functional foods in disease prevention (Mouafo et al., 2024; Vignesh et al., 2024).

Flavonoids, polyphenols, and polyunsaturated fatty acids (PUFAs) have been linked to improved lipid metabolism; however, earlier research has mostly focused on single chemicals, plant extracts, or nutraceutical formulations (Islam et al., 2021; P. Sun et al., 2021). Most existing studies on Litsea angulata have focused on its phytochemical composition and biological activities, particularly the antioxidant activity of its seeds (Ilmia, 2024; Mahfudh et al., 2024). However, evidence supporting the potential of L. angulata seeds as processed functional foods for improving lipid profiles remains inadequate. Specifically, the effect of kalangkala seed biscuits on LDL levels in hypercholesterolemic Wistar rats has not been well characterized.

Kalangkala seed biscuits are rich in flavonoids, alkaloids, tannins and polyunsaturated fatty acids (PUFAs) that may be involved in lipid metabolism. Therefore, the present study was conducted to analyze the alterations in LDL-C levels after the administration of kalangkala seed (Litsea angulata) biscuits in hypercholesterolemic Wistar rats. We anticipated that the administration of kalangkala seed biscuits would be associated with a reduction in blood LDL levels in hypercholesterolemic Wistar rats.

Methods

This study used a randomized pretest-posttest control group experimental design. A quantitative experimental investigation was conducted using a pretest-posttest randomized control group design. Eighteen healthy male Wistar rats were randomly divided into three groups. Hypercholesterolemia was induced using duck egg yolk. In the short term, a diet high in saturated fat can increase blood cholesterol levels (Hussain et al., 2022). Baseline (pretest) serum LDL levels were evaluated after the induction of anesthesia. The treatment groups were given kalangkala seed (Litsea angulata) biscuits with the optimal formulation of 50% kalangkala seed flour at a dose of 1.35 g/day. The control group received the control treatment. Serum LDL levels were retested (post-test) after the intervention period. Next, we examined group differences in the change in LDL levels from pretest to posttest.

The research process included two major phases: the production of kalangkala seed biscuits and preclinical tests with laboratory animals. The study was conducted from January to April 2021. Preparation of kalangkala seed flour and kalangkala seed biscuits was done in Food Technology Laboratory, Department of Nutrition, Poltekkes Kemenkes Banjarmasin and preclinical study was done in Laboratory of Faculty of Pharmacy, Lambung Mangkurat University. Kalangkala seed biscuits were analyzed for proximate composition, including moisture content (AOAC 925.10/934.01), ash content (AOAC 923.03), protein content (AOAC 2001.11 or 984.13; Kjeldahl method), fat content (AOAC 920.39/2003.05/2003.06), and carbohydrate content by difference, using standard methods of AOAC (George W Latimer, 2023). In addition, the biscuits were assayed for unsaturated fatty acid profile, total flavonoid and tannin levels, and antioxidant activity using spectrophotometric and DPPH assays.

The optimal formulation was 50% kalangkala seed flour (P3), based on the De Garmo efficacy index. The sensory evaluation results were used for selection. The sensory qualities were assigned based on their relative importance and effectiveness for each formulation. For the preclinical trials, the formulation with the highest index of effectiveness was selected. Hence, the P3 designation as the optimum formulation was not the highest concentration of individual bioactive chemicals but the overall sensory effectiveness. This difference is relevant because P3 showed the lowest flavonoid and tannin content but the highest antioxidant activity and total unsaturated fatty acid content in the formulations. The biscuit dose for preclinical intervention was calculated based on the recommended intake of 75 g/day for snacks in humans. The dose conversion factor for humans to rats based on body surface area scaling was 0.018. The equivalent dose was 1.35 g/day for a 200-g rat (75 g × 0.018). Since the experimental animals had varying body weights, the fixed dose of 1.35 g/day corresponded to an exposure range of approximately 6.75–9.25 g/kg body weight/day based on the observed body-weight range of 146–200 g. This dose was administered throughout the intervention period.

A preclinical experimental study was conducted on healthy male Wistar rats (Rattus norvegicus) aged 8–12 weeks, with a body weight of 146–200 g. Animals were sourced from the Laboratory of the Faculty of Pharmacy, Lambung Mangkurat University. The inclusion criteria were clinically healthy animals, active behavior, absence of apparent anatomical abnormalities, and no previous use in experimental experiments. Animals that exhibited signs of disease or injury, lost >10% of their body weight during the acclimatization period, or died before intervention were eliminated from the study. Upon arrival, the animals were acclimated over the course of three days to typical laboratory settings (22–25°C, 50–60% relative humidity, 12-h light/12-h dark cycle). The animals were housed in polypropylene cages (three animals per cage) and had free access to normal laboratory feed and water.

The animals were randomly distributed into a control cohort and a hypercholesterolemic induction group after an acclimatization period. The control cohort was fed a regular diet in the laboratory and did not undergo hypercholesterolemic induction. The induction cohort was hypercholesterolemic, induced by oral ingestion of 2 mL duck egg yolk daily for 10 consecutive days. At the end of the induction period, blood LDL levels were evaluated as the baseline state after hypercholesterolemic induction. The animals in the induction cohort were randomly assigned to two groups using a computer-generated random number sequence: the BBK and BBKKT groups. The BBK group received a regular laboratory diet with kalangkala seed (*Litsea angulata*) biscuits, while the BBKKT group received a standard laboratory diet, continued duck egg yolk administration, and kalangkala seed biscuits. The control group (K) was fed a conventional laboratory diet without hypercholesterolemic induction or kalangkala seed biscuit delivery. Therefore, the final experimental design comprised three groups: K, BBK, and BBKKT. Serum LDL levels were evaluated at baseline and at the end of the 10-day intervention period. The primary outcome was the difference in LDL levels between the pre- and post-intervention measures. Outcome assessment was not blinded to group allocation. Initially, the sample size was determined based on the Federer formula, $(t - 1)(n - 1) > 10$. For the three experimental groups ($t = 3$), the equation implies that $2(n - 1) > 10$, that is, $n > 6$. Thus, to achieve the above condition, at least seven animals in each group would be needed, which is a minimum of 21 animals. The number of animals that completed the protocol was less than the minimum calculated, as several animals died throughout the experimental period. Animals that died were not replaced, and the final number of animals analyzed was reported using the actual number of animals that completed the intervention.

In the preclinical trial stage, all rats were acclimated through the administration of standard feed and water ad libitum for three days. After acclimation, the rats were divided into two groups of uneven size: control and hypercholesterolemic induction groups. Hypercholesterolemia was induced by oral gavage of 2 mL duck egg yolk daily for 10 successive days. Baseline blood samples were collected from all animals to measure low-density lipoprotein (LDL) levels before the intervention. The hypercholesterolemic rats were subsequently divided into two treatment groups. The control group (K) was provided with a standard Diet, the BBK group was given a standard diet supplemented with kalangkala seed biscuits, and the BBKKT group was given a standard diet, duck egg yolk, and kalangkala seed biscuits. Treatment was administered for 10 days, after which blood samples were collected to check the level of LDL (post-test).

Rats were fasted for 14 h with free access to water before blood collection to minimize postprandial fluctuations in lipid values. Blood samples were collected at baseline (pretest) before the intervention and at the conclusion of the intervention period (posttest) between 08:00 and 10:00 a.m. to minimize circadian variability. Animals were sedated before sampling with ketamine–xylazine (80–100 mg/kg and 5–10 mg/kg). Blood was collected from the retro-orbital venous sinus using a sterile heparinized microcapillary tube into sterile 1.5 mL serum separator tubes (Eppendorf®, Hamburg, Germany) at a volume of (0.5-1.0 mL). The blood samples were allowed to coagulate for 15 min at room temperature and centrifuged at 4,000 rpm for 10 min. Serum LDL-C values were determined using a direct homogeneous enzymatic colorimetric assay (CHOD-PAP principle) at Prodia Clinical Laboratory, Banjarmasin, Indonesia, according to the laboratory standard operating procedures.

The main outcome was the change in serum LDL-C concentration (ΔLDL), which was computed as the posttest result minus the pretest result for each rat. If the change scores were normally distributed, a paired-samples t-test was used to examine within-group pre- and post-test differences; otherwise, the Wilcoxon signed-rank test was employed. The main between-group analysis involved comparing ΔLDL among the three groups using one-way ANOVA after testing for normality using the Shapiro–Wilk test and homogeneity of variance using Levene's test. If the assumptions of ANOVA were met, pairwise comparisons were performed using Tukey's HSD test. When the assumption of homogeneity was violated, heteroscedastic analysis and the appropriate post hoc technique were utilized. P-values with effect sizes and 95% confidence intervals are presented. The statistical unit was the individual rat, and one ΔLDL per animal was included in the study. Statistical significance was set at $\alpha = 0.05$, and analyses were conducted using IBM SPSS Statistics.

The study protocol was evaluated and approved by the Health Research Ethics Committee of the Health Polytechnic of Malang, Ministry of Health, Indonesia (Reg. No. 832/KEPK-POLKESMA/2020, authorized on May 4, 2020). This committee granted ethical permission because, at the time the study was conducted, the Health Polytechnic of Banjarmasin, Ministry of Health, had not yet established an institutional ethics committee. All animal experiments were conducted according to the internationally accepted principles of laboratory animal welfare and biomedical research ethics, including the principles of replacement, reduction, and refinement (3Rs), and the institutional guidelines for the care and use of laboratory animals. Animals were examined daily for pain, distress, decreased food intake, considerable weight loss, limited mobility, or serious sickness; animals reaching these humane endpoints were removed from the trial and terminated promptly. Before invasive procedures, rats were sedated with ketamine (80–100 mg/kg body weight) and xylazine (5–10 mg/kg body weight) via intraperitoneal injection to decrease discomfort and distress. Animals were humanely euthanized by an overdose of anesthetic drugs at the end of the experiment in accordance with standard laboratory animal care practices. All biological specimens and animal carcasses were handled and discarded as biohazardous waste in accordance with the institutional biosafety and laboratory waste management protocols. The confidentiality of the research data was preserved, and the data were used only for academic and scientific objectives.

Result and Discussion

Proximate Composition of Kalangkala Seed Biscuits

Proximate properties are among the essential factors in determining the quality of functional foods because of their direct association with the nutrient content, stability of the food, bioactive content, and possible physiological effects. For a functional food to be sufficiently efficient, it should possess a well-balanced combination of macronutrients and bioactive content, such that its physiological effect will be optimal. Considering this fact, a proximate study of kalangkala seed cookies is necessary to ensure that the product not only has sufficient bioactive content but also has good nutritional qualities to serve as a healthy food.

Table 1. Proximate composition of kalangkala seed biscuits

Proximate	Treatment		
	P1	P2	P3
Moisture Content (%)	2.00 ± 0.10	2.00 ± 0.08	1.40 ± 0.10
Ash Content (%)	0.03 ± 0.02	1.21 ± 0.01	1.64 ± 0.02
Protein (%)	6.33 ± 1.42	6.07 ± 0.13	6.56 ± 0.22
Fat (%)	40.45 ± 0.08	45.31 ± 0.10	44.21 ± 0.22
Carbohydrates (%)	18.87 ± 0.22	38.67 ± 0.08	29.93 ± 2.37

Note: P1 biscuits with kalangkala seed flour 100%; P2 biscuits with kalangkala seed flour 75%; P3 biscuits with kalangkala seed flour 50%

The proximate composition of the kalangkala seed biscuits is shown in Table 1. All analyses were performed in six separate replicates (n = 6), and the results are expressed as mean ± standard deviation (SD). The moisture content ranged from 1.40 ± 0.10% to 2.00 ±

0.10%. The lowest value was reported for the P3 formulation. The ash concentration varied considerably among the formulations, from 0.03 ± 0.02% in P1 to 1.64 ± 0.02% in P3. The protein level ranged from 6.07 ± 0.13% to 6.56 ± 0.22%, fat content ranged from 40.45 ± 0.08% to 45.31 ± 0.10%, and carbohydrate content ranged from 18.87 ± 0.22% to 38.67 ± 0.08%.

One-way analysis of variance (ANOVA) indicated significant differences between the formulations in terms of moisture content ($F = 67.50$, $p<0.001$), ash content ($F = 17,372.50$, $p<0.001$), fat content ($F = 1,538.18$, $p<0.001$), and carbohydrate content ($F = 260.63$, $p<0.001$). In contrast, no significant difference was observed in the protein content among the three formulations ($F = 0.43$, $p=0.656$). The results showed that the proximate features of the biscuits were considerably ($p<0.05$) affected by the differences in the amount of kalangkala seed flour, except for the protein content, which was generally stable across the formulations. This indicates that variations in the composition of kalangkala seed flour in different formulations affect the chemical properties of the cookies.

The low moisture content in all the formulas suggests that the biscuits may have improved shelf stability. According to the standards for biscuit production and research on dry foods, low moisture content prevents microbial growth and delays food degradation during storage (Haider et al., 2022). The findings of this study are consistent with those reported by Ling et al. regarding the low moisture content of products based on seeds from the Litsea genus owing to drying and baking processes (Ling et al., 2022). Moreover, the low moisture content is an indicator that the processes involved in manufacturing were successful in reducing the moisture content. This is a benefit in terms of product development because it increases the shelf life and makes it easy for distribution on an industrial level.

The highest ash content in the P3 formulation indicates an increase in its mineral content. In general, ash content represents the amount of inorganic residue remaining after the combustion of food ingredients and is therefore often used as an indicator of the total mineral content (Haider et al., 2022). Thus, the results show that changes in the ingredient composition of the formulations contribute to variations in the mineral content of the biscuits. These findings are consistent with those of Amit and Zinyin, who reported that various species within the genus Litsea are sources of essential minerals that can support various metabolic processes in the body (Amit & Zinyin, 2021).

The use of kalangkala seed flour as a substitute did not change the protein content. This is evident from the results of the uniform protein across all formulations. Biscuits are a means of introducing protein into the diet (Mouafo et al., 2024; Suresh & Hemalatha, 2025). This study compared the protein content of biscuits with that of other foods formulated as functional foods. Other local foods have been intentionally designed as functional foods for various reasons, including their protein content and other nutritional constituents (Vignesh et al., 2024; Zhang et al., 2025). The results showed that the addition of kalangkala seed flour did not change the protein content, which is advantageous for developing functional foods. This is important for developing functional foods, as one can maintain the protein content of the food while increasing other constituents.

One of the major findings of this study was the elevated fat levels in all sample formulations, especially in formulation P2. This finding coincides with the reports of Mahfudh et al. and Ling et al., who described the Litsea genus as having a higher-than-normal lipid count with the presence of unsaturated lipid fractions (Ling et al., 2022; Mahfudh et al., 2024). The relatively high fat content of the biscuits should not be considered nutritionally unfavorable, as the lipid fraction was characterized by a predominance of unsaturated fatty acids, which have been associated with more favorable cardiometabolic outcomes than saturated fatty acids (Ho et al., 2020; Mirmiran et al., 2020). A predominance of polyunsaturated fatty acid (PUFA)-based fats can aid in the support and proper function of cardiovascular health and lipid metabolism, in contrast to saturated fats (Mirmiran et al., 2020; Yang et al., 2025). Similarly, the elevated fat levels in the kalangkala seed cookies should be viewed along with the PUFAs indicated in the fatty acid profiling; thus, these cookies may aid in lipid management and support as a functional food beyond being a product with higher caloric value.

The higher carbohydrate content of formulation P2 was likely attributable to the differences in the proportions of the ingredients among the formulations. Carbohydrates are major dietary energy sources that contribute to flavor and aroma.

Carbohydrates are also important contributors to the sensory characteristics of biscuits, especially texture and crispness (Mamat & Hill, 2018; Mouafo et al., 2024; Suresh & Hemalatha, 2025). The differences in carbohydrate content among the formulations were likely attributable to differences in the proportions and composition of the raw materials used. These observations correlate well with those of Vignesh et al., who mentioned that functional formulations of food employ different proportions of carbohydrates owing to variations in raw material characteristics and processing protocols (Vignesh et al., 2024).

Unsaturated Fatty Acids

The fatty acids in kalangkala seed biscuits were identified by gas chromatography (GC) analysis after FAME derivatization, and the results revealed the presence of six unsaturated fatty acids: myristoleic, palmitoleic, linoleic, linolelaidic, α-linolenic, and arachidonic acids. Data are expressed as mean ± SD of six separate determinations (n=6). The most abundant monounsaturated fatty acid among the identified substances was palmitoleic acid, which increased considerably in the formulations, from 10.00 ± 0.49% in P1 to 18.00 ± 0.99% in P3 ($p < 0.001$). Moreover, linoleic acid, linolelaidic acid, and α-linolenic acid were significantly different among the treatments ($P < 0.05$), whereas myristoleic acid was unaffected ($P = 0.099$). Importantly, only arachidonic acid (15.64 ± 0.92%) was detected in P1.

The total unsaturated fatty acid content differed considerably among the formulations ($p < 0.001$), with the greatest value being observed in P3 (54.45 ± 2.65%), followed by P1 (51.34 ± 1.69%) and P2 (46.80 ± 2.47%). The enrichment of palmitoleic, linoleic, and α-linolenic acids was found in P3, indicating that the integration of kalangkala seeds has the potential to improve the nutritional quality of biscuit formulations. Considering the documented importance of unsaturated fatty acids in lipid homeostasis and cardiovascular health, these compositional features may contribute, at least in part, to the LDL-lowering benefits observed in the hypercholesterolemic rat model. The results are shown in Table 2.

Dietary polyunsaturated fatty acids diet are involved in regulate cholesterol levels and inflammation. The lipid composition of functional foods also influences liver lipid metabolism and blood lipoproteins via various metabolic pathways (Bjune et al., 2024; H. Duan et al., 2023). The presence of α-linolenic acid in all formulations is an important finding, even though it is a well-known essential omega-3 fatty acid, and the human body cannot produce it. α-Linolenic acid has been demonstrated to improve hepatic cholesterol metabolism, induce LDL clearance, and inhibit inflammatory signals related to atherosclerosis (Bjune et al., 2024; H. Duan et al., 2023; Sherratt et al., 2023). The consumption of PUFAs, especially omega-3 fatty acids, has often been linked to better lipid profiles and a lower likelihood of cardiovascular disease (Bernasconi et al., 2021). These findings are consistent with those of previous studies, in which omega-3 metabolites blocked the assembly of inflammatory mediators while simultaneously supporting improved endothelial function in blood vessels (J. Duan et al., 2021; Sherratt et al., 2023). Thus, the α-linolenic acid content of kalangkala seed biscuits may be associated with the observed reduction in LDL-C levels.

Table 2. Unsaturated fatty acid content

Unsaturated Fatty Acid (%)	Treatment		
	P1	P2	P3
Myristoleic Acid (C14:1)	0.73 ± 0.14	0.95 ± 0.20	0.95 ± 0.22
Palmitoleic Acid (C16:1)	10.00 ± 0.49	14.46 ± 1.44	18.00 ± 0.99
Linoleic Acid (C18:2n6c)	1.26 ± 0.12	1.96 ± 0.62	2.37 ± 0.66
Linolelaidic Acid (C18:2n6t)	16.88 ± 0.77	19.45 ± 1.78	23.07 ± 0.86
α-Linolenic Acid (C18:3n3)	6.84 ± 0.88	9.99 ± 1.01	10.06 ± 1.08
Arachidonic Acid (C20:4n6)	15.64 ± 0.92	ND	ND
Total unsaturated fatty acids (%)	51.34 ± 1.69	46.80 ± 2.47	54.45 ± 2.65

Note: ND (not detected)

These findings are consistent with those of Ling et al., who reported that Litsea garciae seeds contain a range of unsaturated fatty acids, including long-chain fatty acids that may contribute to favorable health effects (Ling et al., 2022). This overall similarity indicates that members of the genus Litsea may have lipid profiles relevant to the development of functional foods. However, the fatty acid profile, especially the proportions, measured here did not fully correspond to what earlier work reported. In other words, there are some differences. These shifts are probably caused by plant species variations, environmental growing conditions, the level of seed maturity, the lipid extraction process, and the processing steps used when turning things into flour and later cookies. These factors are known to influence the stability and composition of fatty acids in food ingredients (Amit & Zinyin, 2021).

The finding that the P3 formulation had the highest total content of unsaturated fatty acids indicates that differences in formulation composition also influence the lipid quality of the final product. A higher ratio of unsaturated fatty acids may improve the functional value of a food product, as unsaturated fatty acids are more strongly associated with beneficial regulation of lipid metabolism and cardiometabolic health than saturated fatty acids (Liu et al., 2025; Mirmiran et al., 2020). However, a high level of unsaturated fatty acids has stability implications during storage. These unsaturated fatty acids are more susceptible to oxidation, which can lower both the sensory appeal and nutritional quality of the food (Wang et al., 2023). Therefore, even if the P3 formulation shows a more favorable lipid profile, oxidative stability must remain a primary concern when developing the product on an industrial scale.

The fatty acid profile should be read by discriminating between cis-unsaturated and trans fatty acids. Cis-unsaturated fatty acids are usually considered beneficial components of dietary fat, especially when they displace saturated or trans fatty acids in the diet. However, linolelaidic acid, a trans isomer of linoleic acid, was identified in all biscuit formulations, with higher levels in P3, the formulation employed in the animal intervention. Higher circulating linolelaidic acid levels have been linked to negative cardiometabolic outcomes, including elevated blood pressure and hypertension (Luan et al., 2024). The presence of linolelaidic acid may be related to thermal processing. Experimental data have shown cis–trans isomerization of unsaturated fatty acids and generation of linolelaidic acid under high-temperature conditions (He et al., 2023). However, the precise process of linolelaidic acid synthesis in the biscuits was not directly studied. Therefore, the total unsaturated fat content should not be consistently viewed as cardioprotective because it contains both cis- and trans-unsaturated fatty acids. The health effects of linolelaidic acid also depend on its concentration and actual consumption. Its presence alone does not allow any conclusions to be drawn regarding its potential safety. Comprehensive characterization of individual cis- and trans-fatty acids, their dose-dependent effects, and their safety should be explored in further research.

Phytochemical

The phytochemical study revealed considerable differences in the tannin and flavonoid concentrations in the kalangkala seed biscuit formulations (Table 3). Six independent replicates ($n=6$) were examined for each treatment, and the findings are expressed as the mean ± standard deviation (SD).

The tannin content, expressed as percentage (w/w) tannic acid equivalents (TAE) per 100 g of sample, ranged from 0.446 ± 0.103% to 1.399 ± 0.119%. The maximum tannin content was recorded in formulation P1 (1.399 ± 0.119% TAE/100 g), followed by P2 (0.829 ± 0.153% TAE/100 g) and P3 (0.446 ± 0.103% TAE/100 g). One-way ANOVA showed a significant effect of formulation on the tannin levels ($F = 71.72$, $p<0.001$). Subsequent Tukey's HSD analysis showed that all treatments were significantly different from each other (P1 > P2 > P3; $p < 0.05$).

Likewise, the overall flavonoid content (as a percentage (w/w) of quercetin equivalents (QE) per 100 g of sample) was significantly different across the treatments. P1 had the highest flavonoid concentration (0.070 ± 0.014% QE/100 g), followed by P2 (0.020 ± 0.011% QE/100 g) and P3 (0.017 ± 0.008% QE/100 g). One-way ANOVA revealed significant differences in flavonoid concentrations among the formulations ($F = 36.39$, $p<0.001$). Tukey's post hoc test revealed that P1 had considerably greater flavonoid levels than P2 and P3 ($p<0.05$), but there was no significant difference between P2 and P3 ($p = 0.866$).

The results showed that higher levels of kalangkala seed flour in the biscuit formulation significantly increased the levels of bioactive substances, including tannins and flavonoids. Higher amounts of these phytochemicals might be linked to the functional features of kalangkala seed biscuits, such as potential antioxidant and hypolipidemic actions. The phytochemical composition of the kalangkala seed cookies is presented in Table 3.

Table 3. Phytochemical composition of kalangkala seed biscuits

Phytochemical	Treatment		
	P1	P2	P3
Tannins (%)	1.399 ± 0.119	0.829 ± 0.153	0.446 ± 0.103
Flavonoids (%)	0.070 ± 0.014	0.020 ± 0.011	0.017 ± 0.008

The higher flavonoid content in the P1 formulation further supports the hypothesis that increasing the use of kalangkala seed flour enhances the biological activity of the product. This is because flavonoids are generally known to act as free radical scavengers, inhibit lipid oxidation, and regulate cholesterol metabolism by inhibiting HMG-CoA reductase activity, an enzyme that participates in cholesterol biosynthesis (Bjune et al., 2024; Xu et al., 2024). According to Bjune et al., different classes of flavonoids can exert antihyperlipidemic activity by upregulating the expression of LDL receptors while simultaneously downregulating cholesterol synthesis in the liver (Bjune et al., 2024). This result is in line with that of Xu et al., who showed that flavonoids can suppress oxidative stress and improve endothelial function through a mechanism that inhibits LDL oxidation (Xu et al., 2024). Thus, the high flavonoid content in the P1 formulation provides a biological basis for the results of this study, especially concerning the LDL level reduction in the treatment group that consumed kalangkala seed biscuits.

In addition to flavonoids, the relatively high tannin content of the P1 formulation also contributes to the health benefits of the product. Tannins are polyphenolic compounds that can bind to proteins and lipids in the digestive system, thereby obstructing the absorption of cholesterol and fats (Islam et al., 2021; P. Sun et al., 2021). According to Islam et al., tannins and other polyphenolic compounds help lower cholesterol levels by increasing bile acid excretion and reducing lipid absorption in the intestine (Islam et al., 2021). This leads to a reduced availability of cholesterol as a precursor for the formation of atherogenic lipoproteins, including LDL. Thus, the higher tannin content in formulation P1 may have contributed to the cholesterol-lowering effect of the kalangkala seed cookies.

The current data are consistent with previous reports showing that Litsea species are a valuable source of potentially bioactive phenolic compounds. The LC-HRMS profile of Litsea angulata seeds revealed many phenolic constituents and antioxidant compounds that may be related to its biological activities (Mahfudh et al., 2024). Similar to previous studies, L. angulata also has diverse secondary metabolites, such as flavonoids and tannins, suggesting that this plant could be investigated for the development of drug candidates from natural resources (Rikomah et al., 2024). The information presented in this review supports the potential of kalangkala seeds as a natural source of bioactive phytochemicals; however, the variability in the amount and type of phytochemicals produced is a function of the specific extraction techniques and processing conditions employed to produce the product matrix or formulation.

Antioxidant Activity

The antioxidant activities of the kalangkala seed biscuit formulations were determined using the DPPH radical scavenging assay. The results showed a substantial formulation influence on the antioxidant activity (one-way ANOVA, $F(2,15) = 12.37$, $p = 0.001$). The maximum antioxidant activity was observed in formulation P3 (74.60 ± 1.20%), followed by P2 (72.63 ± 0.95%) and P1 (71.24 ± 1.05%) (mean ± SD, n = 6). Post hoc analysis (Tukey's HSD test) revealed that P3 had considerably higher antioxidant activity than P1 ($p = 0.001$) and P2 ($p = 0.028$); however, no significant difference was observed between P1 and P2 ($p = 0.134$). The results showed that the formulation composition significantly affected the free radical-scavenging capacity of the kalangkala seed biscuits. The greater antioxidant activity of P3 could be due to the higher availability or synergistic effect of bioactive substances, such as phenolic compounds and flavonoids. Antioxidant activity was measured using the DPPH radical scavenging assay, and the results are presented in Figure 1.

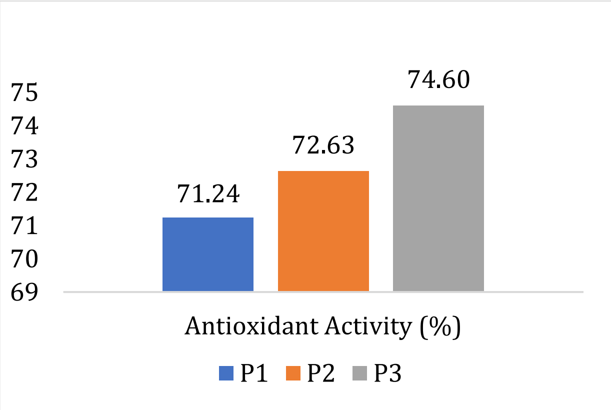

Figure 1. Antioxidant activity of kalangkala seed biscuits

Functional foods containing bioactive chemicals from plants may protect against oxidative stress via antioxidant mechanisms that help avoid metabolic diseases such as hypercholesterolemia (Arifuzzaman et al., 2025). The antioxidant activity observed in the kalangkala seed biscuit formulations supports their potential as functional foods, although in vitro DPPH activity should not be interpreted as direct evidence of in vivo protection against oxidative stress.

The observed antioxidant activity is believed to have resulted from the multitude of bioactive molecules present in the kalangkala seeds, including flavonoids, tannins, and unsaturated fatty acids. Flavonoids act as electron or hydrogen atom donors, and their supply causes the quenching of free radicals and termination of oxidative chain reactions (Xu et al., 2024). Tannins bind pro-oxidant metal ions, inhibiting the formation of reactive oxygen species (ROS) (Sun et al., 2021). Recent studies have shown that phenolic compounds enhance the oxidative stability of food matrices rich in unsaturated fatty acids by suppressing lipid oxidation, thereby maintaining their nutritional and functional value (Arslan et al., 2025; Joshi et al., 2024). In summary, the observed antioxidant activity of the kalangkala seed biscuits may be ascribed to the complementary roles of multiple bioactive constituents found within its food matrix.

Interestingly, formulation P3 exhibited the best antioxidant activity, while formulation P1 had the highest overall flavonoid and tannin concentrations. The formulation-level mean values of the three biscuit formulations ($n = 3$) were subjected to an exploratory descriptive evaluation of the relationship between the chosen bioactive components and antioxidant activity. Since the study was based on only three formulation-level observations, the resulting correlation coefficients were regarded as descriptive and hypothesis-generating rather than inferential, and no causal interpretation was provided. No significant association was identified between total flavonoid content and antioxidant activity ($r = -0.846$, $p = 0.358$) or between total tannin content and antioxidant activity ($r = -0.978$, $p = 0.135$). The results of this study indicate that the antioxidant activity of kalangkala seed biscuits cannot be explained by the total quantities of flavonoids or tannins alone.

The correlation study was performed on only three formulations ($n = 3$), which greatly reduced the statistical power and did not allow firm conclusions to be drawn regarding causality. Thus, the apparent contradiction between phytochemical content and antioxidant activity should be regarded as a preliminary observation and not as evidence of an adverse relationship between the two.

However, similar occurrences can be explained by the complex interactions of food elements that influence the bioactivity of specific molecules post-processing (Wang et al., 2023). As previously proven, the antioxidant activity is not necessarily linearly related to the total flavonoid or tannin content of the sample. Previous research has shown that the structural features of phenolic compounds, food component interactions, and transformations during processing can affect antioxidant activity (Dias et al., 2021; Mohammadi et al., 2024; Toydemir et al., 2022; Vargas-ramella et al., 2026). However, these pathways were not tested in the present study. Further investigations, including a larger number of formulations and extensive phenolic profiling, are needed to elucidate the molecular rationale for the better antioxidant activity observed in formulation P3, despite its low flavonoid and tannin concentrations. The enhanced antioxidant activity detected in formulation P3 could therefore be related to the more beneficial mixture of bioactive components or the greater availability of specific molecules with antioxidant activity generated during processing.

The present findings are in agreement with other investigations that have shown the presence of a variety of phenolic compounds and other secondary metabolites with high antioxidant activity in the seeds of Litsea angulata, as evidenced by LC-HRMS analysis (Mahfudh et al., 2024). Other studies have also revealed that L. angulata has great pharmacological potential as a natural source of antioxidants due to its flavonoids, alkaloids, and tannins, which all contribute to its biological activity (Rikomah et al., 2024). The present results are consistent with those of earlier studies, further supporting the idea that antioxidant activity is a major biological feature of L. angulata, which can be significantly preserved following the transformation of the seeds into food products.

Low-Density Lipoprotein

Figure 2. Changes in LDL cholesterol (ΔLDL = post-pre) following treatment. Bars represent mean ± SD (n = 6/group). One-way ANOVA and Tukey's post hoc tests were used to assess differences among groups. The overall group impact was significant (F (2,15) = 82.71, p<0.001, η2=0.917). Tukey's test revealed significant differences between the BBK and BBKKT (p=0.001), BBK and K (p<0.001), and BBKKT and K (p<0.001) groups.

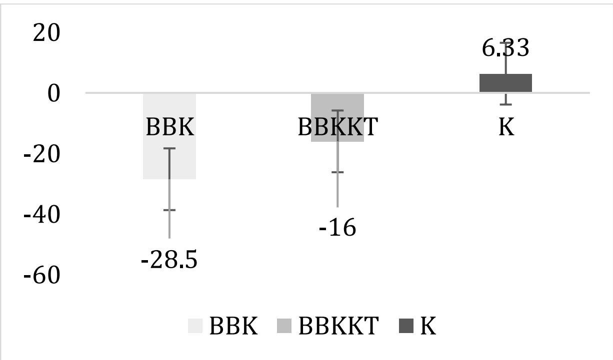

Figure 2. Mean change in serum LDL-C (postintervention minus preintervention) across the three study groups

Functional foods contain bioactive chemicals that can improve lipid metabolism via various mechanisms, including the suppression of hepatic HMG-CoA reductase activity, upregulation of LDL receptor expression, and modulation of genes involved in cholesterol transport and lipid homeostasis (Bjune et al., 2024; El-Rayyes et al., 2023; Palmado et al., 2024). In the present study, kalangkala seed biscuits contained flavonoids, tannins, and unsaturated fatty acids, which may modulate cholesterol metabolism and contribute to the decrease in blood LDL levels.

The larger decrease in LDL values in the BBK group may be due to the phytochemical composition of the kalangkala seed biscuits. The intervention material was chemically analyzed before the animal trial and showed measurable amounts of flavonoids (0.017% ± 0.008), tannins (0.446% ± 0.103), unsaturated fatty acids, and antioxidant activity. In addition, the dose of biscuits given to the rats was based on human-to-rat dose conversion, providing a standard amount of exposure during the intervention. Flavonoids have been reported to possess antihyperlipidemic properties in previous studies through various mechanisms, including the inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in hepatic cholesterol biosynthesis, and the upregulation of hepatic low-density lipoprotein receptor (LDLR) expression, which leads to the enhancement of the clearance of circulating LDL particles (El-Rayyes et al., 2023). Flavonoids have also been observed to upregulate the expression of LDL receptors in hepatocytes, promoting the hepatic clearance of circulating LDL particles (Bjune et al., 2024). El-Rayyes et al. also showed that the expression of HMG Co-A reductase was downregulated and the expression of LDLR was increased in hepatic cells by the flavonoid derivative, isorhamnetin (El-Rayyes et al., 2023).

The lowered LDL-C levels found may be considered in relation to the earlier suggested effects of flavonoids and other bioactive substances on cholesterol metabolism. However, these pathways were not directly investigated in the present study. Liver HMG-CoA reductase, LDLR expression, PCSK9 and oxidative-stress indicators were not evaluated. Thus, the results suggest that the delivery of chemically defined kalangkala seed biscuits is associated with alterations in LDL levels but do not indicate that the reaction is mediated by a specific bioactive molecule or molecular pathway. The role of these pathways is still hypothetical and needs to be explored in future mechanistic investigations employing suitable molecular and biochemical indicators.

In addition to flavonoids, the tannin content of kalangkala seed biscuits may also play a role in the decrease in LDL levels. Tannins have been shown to combine with dietary proteins and digestive enzymes, inhibiting the intestinal absorption of lipids and cholesterol and lowering cholesterol bioavailability for systemic absorption (Islam et al., 2021; Sun et al., 2021). Islam et al. also showed that polyphenols and tannins in plant diets have hypocholesterolemic effects by inhibiting the absorption of cholesterol and increasing the excretion of fecal sterols (Islam et al., 2021). Thus, the presence of flavonoids and tannins in kalangkala seeds may have complementary mechanisms that synergize to improve LDL-C-lowering activity.

The LDL-C-lowering effect in the present study may also be partly contributed by unsaturated fatty acids contained in kalangkala seed cookies. Fatty acid composition analysis showed the presence of α-linolenic, linoleic, and palmitoleic acids in all biscuit formulations. PUFAs have been widely shown to improve lipid metabolism by upregulating hepatic LDL receptor activation, downregulating very low-density lipoprotein (VLDL) production, and boosting hepatic cholesterol catabolism (Ling et al., 2022). Furthermore, Toydemir et al. showed that the synergistic effect of PUFAs and polyphenolic substances exerted benefits on lipid metabolism and cardiovascular health through antioxidant and anti-inflammatory pathways (Toydemir et al., 2022). The decrease in LDL-C concentrations in the treatment groups may be partly associated with the PUFA content of the kalangkala seed biscuit.

The BBKKT group showed a smaller reduction in LDL-C than that of the BBK group. The continued consumption of egg yolk throughout the intervention period could explain this finding by increasing the intake of exogenous cholesterol and possibly diminishing the hypocholesterolemic effect of the kalangkala seed biscuits. Similar tendencies have been described in prior functional food intervention studies, where changes in lipid profiles were identified but did not approach statistical significance in a consistent manner, especially in pilot studies with very short intervention periods and small sample sizes, corresponding with the present results (Brito et al., 2025).

In line with earlier investigations, the present findings are compatible with the bulk of evidence that flavonoids and other polyphenolic substances possess antihyperlipidemic properties. Many experimental investigations have indicated that flavonoids can lower serum total cholesterol, triglyceride, and LDL-C levels and improve lipid metabolism in vitro and in vivo models of hyperlipidemia (Long et al., 2021; Sun et al., 2021). Similarly, Xu et al. demonstrated that quercetin and other flavonoids enhance endothelial function and lower the risk of atherosclerosis by preventing low-density lipoprotein cholesterol (LDL-C) oxidation (Xu et al., 2024). However, the present study differs from these earlier studies as the intervention was administered in a processed functional food product in the form of biscuits, rather than with purified extracts or isolated bioactive chemicals. These results indicate that processing kalangkala seeds into functional food products does not eliminate their ability of kalangkala seeds to improve lipid profiles.

The observed reduction in LDL-C was associated with the administration of the full kalangkala seed biscuit intervention. Because the entire biscuit matrix was analyzed, the individual impacts of flavonoids, tannins, PUFAs, or other individual bioactive compounds and possible interactions between these compounds could not be experimentally isolated. Thus, LDL-C response cannot be explained by any single component or synergistic interactions. Further mechanistic investigations using isolated compounds or modified formulations are required to elucidate the roles of specific bioactive components.

There are several limitations to consider when interpreting these findings. The intervention was relatively short, only one biscuit recipe (P3) was examined in vivo, and the sample size was small. Cholesterol metabolism and oxidative stress-related molecular indicators were not evaluated. There was no examination of a full lipid profile or histological evaluation. Furthermore, the exploratory correlation analysis based on the mean values of the three formulation-level variables (n=3) had only limited inferential value and was thus read descriptively rather than as evidence for a reliable association or causal relationship.

Conclusion

Administration of kalangkala seed biscuits was associated with a reduction in blood LDL-C levels in hypercholesterolemic Wistar rats. Significant variations were also observed in LDL-C levels across the groups. Post-hoc comparisons supported the differences between the biscuit intervention and control groups. However, the whole-biscuit intervention was examined without the experimental isolation of individual components; therefore, the LDL-C response cannot be attributed to specific flavonoids, tannins, unsaturated fatty acids, or their possible synergistic interactions. Further investigations with a larger sample size and longer intervention duration should compare P1–P3 formulations in vivo and include a full lipid profile, oxidative stress indicators, histopathology, and mechanistic biomarkers such as hepatic HMG-CoA reductase and LDLR.

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