

## DEVELOPMENT AND BIOFUNCTIONAL CHARACTERIZATION OF FLAXSEED BASED COOKIES WITH IN VIVO VALIDATION IN A HYPERLIPIDEMIC MICE MODEL

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### ABSTRACT

Flax is a plant that has been used as a food source for thousands of years and is high in  $\alpha$ -linolenic acid, lignans, and soluble fiber, which have shown potential cardiovascular-protective effects. However, its systemic addition to cereals in the form of functional food, with proven in vivo results, is scarce. This work aimed to develop and evaluate flaxseed-incorporated cookies for nutritional up gradation, antioxidative potential, as well as sensory acceptability and biological efficacy against a mouse-based hyperlipidemic model. Six formulas with 5–30% flaxseed flour mixed with chickpea, oat, and rice flours were produced and analyzed according to AOAC (2006) methods. Biological activities were measured using the Folin–Ciocalteu, aluminum chloride, DPPH, and FRAP methods. For in vivo assessment, Triton WR-1339–induced hyperlipidemic mice ( $n = 24$ ) were used, and their lipid profiles, hepatic markers, and histopathological changes were analyzed. The addition of flaxseed led to a significant ( $p < 0.001$ ) increase in protein ( $16.84 \pm 0.24\%$ ) and crude fiber ( $3.41 \pm 0.18\%$ ) content, while boosting the total phenolics content ( $188.7 \pm 4.3$  mg GAE/100 g) and antioxidant potential (FRAP  $312.4 \pm 10.7$   $\mu\text{mol Fe}^{2+}/\text{g}$ ). In vivo, animals fed flaxseed cookies exhibited lower serum total cholesterol levels ( $-38\%$ ), triglyceride levels ( $-33\%$ ), and LDL levels ( $-42\%$ ), as well as higher HDL levels by 29% ( $p < 0.01$ ). Liver enzyme levels recovered, and the hepatic architecture was restored, along with a reduction in aortic wall thickening as determined from histopathological measurements. These results suggest that flaxseed-enriched cookies may be a promising functional food with antioxidative and cardio protective efficacy and potentially could be used in the dietary treatment of dyslipidemias and risk profiles for cardiovascular diseases.

**Keywords:** Antioxidant activity, Cardio protection, Flaxseed cookies, Functional food, Hyperlipidemia, Lipid metabolism, Nutritional quality

### INTRODUCTION

Cardiovascular diseases (CVDs) persist as the number one cause of morbidity and mortality globally, accounting for more than 30% of deaths annually around the world (Thakur *et al.*, 2023). Diabetes mellitus, obesity and hyperlipidemia are major dietary risk factors known to aggravate the development of cardiovascular dysfunction via diet-induced mechanisms (Yao *et al.* 2020). Interestingly, the natural functional foods rich in bioactive compounds, dietary fibers, and essential fatty acids are being recognized as agents that may impact the lipid metabolism and oxidative stress and improve vascular health (Mondal *et al.* 2021). Naturally, vegetarian diets seem to play a sustainable and beneficial role in the nutrition of metabolic-related tasks.

Flaxseed (*Linum usitatissimum* L.) as a functional ingredient has received substantial scientific interest due to its astonishing chemical profile and bioactive properties (Mueed *et al.* 2022). Flaxseed contains  $\alpha$ -linolenic acid (ALA), a plant form of omega-3 fatty acid that reduces plasma cholesterol and triglycerides, and secoisolariciresinol

diglucoside (SDG), a strong antioxidant and estrogenic lignan (Abdrabou and Gouda 2025). In addition, its lipids contain a large proportion of soluble and insoluble dietary fiber, which influences gut microflora, intestine motility, and fat excretion. The well-regulated bioactive compounds in flaxseed, confer hypolipidemic, anti-inflammatory, and cardiomyopathic activity to the ingredient (Thakur *et al.* 2023), These properties have been adequately proven and even clinically validated in animal and human studies.

However, flaxseed inclusion in human consumables is minimal due to the availability of other flaxseed based healthy products in a dense and palatable form. Most people prefer to consume consolidated products, such as pastries (Cauvain and Clark 2019). Moreover, cookies categorically attract human attention and maintain stability, with distinct extraction profiles and physical attributes. Therefore, the formulating cookies with soluble and insoluble dietary fiber, along with flax seeds can promote the production of cookies with a viscera-breaking effect-produced cookies (Slade and Levine 1994). The potential lies to achieving optimal balance compromise between physicochemical, sensory, and nutritional features through the addition of high-fiber and lipid-rich ingredients, such as flaxseed.

In general, hyperlipidemic Triton WR-1339 induced murine models are suitable and reproducible models for understanding lipid metabolism and searching for hypolipidemic products (Zarzecki *et al.* 2014). The nonionic detergent, Triton WR-1339, causes acute and rapid hypercholesterolemia following an injection by inhibiting the lipoprotein lipo-barrier and plasma cholesterol and triglycerides accumulation (Hmidani *et al.* 2020). Therefore, one can screen diet by insulin type, which may affect synthesis, deposition, and degradation provoked by the hyperinsulinemic diet. Liver and vessel tissue histology analysis will provide mechanistic insight into tissue protective rooftops and underpin the global cellular action of cardiometabolic benefits of cookies with flax.

Given the aforementioned background, the present work was planned to provide an intermediate between food formulation and physiological validation. Flaxseed was added at graded levels (5–30%) to a composite flour blend of chickpea, oat and rice flours to establish a nutritionally fortified palatable cookie product. The proximate, phenolic, and flavonoid content, as well as the antioxidant activity (DPPH; FRAP) properties of all the formulations, were evaluated in detail, along with sensory acceptability (Zhao *et al.* 2022). The most acceptable formulation was further evaluated for its biological potential in hyperlipidemic mice, in terms of serum lipid profiles, hepatic enzyme markers, hematological indices, and tissue histopathology.

The findings of this study can be extrapolated beyond the health of individuals. Overall, the production and incorporation of flaxseed are aligned with global efforts to increase plant-food consumption and reduce red meat, develop sustainable food systems, and advocate for evidence-based nutrition with a public health approach to combat chronic diseases. Lastly, valorization precursor of flaxseed towards product innovation may push for agricultural diversification and rural entrepreneurship with enhanced added value in local food chain. Together, our study addresses a critical linkage by developing, characterizing and biologically validating flaxseed-enriched cookies for creating a functional food prototype in cardio-metabolic health. As such, our combined *in vitro* and *in vivo* implementation provides hard-won scientific evidence in developing a causal relationship of DFs formulation strategies that trigger quantifiable clinical benefits in lipid homeostasis and oxidative balance – a possible step in merging food technology with health care prevention.

## MATERIAL AND METHODS

### Experimental Design

The objective of the investigation was to formulate and characterize flaxseed-enriched cookies for their nutritional, instrumental, sensory properties and cardioprotective impacts. The study was designed in two parts, first, a formulation and analytical procedure of the flaxseed incorporated cookies formulations to characterize structural and lipid properties and second, an *in vivo* analysis of the simplest formulation with a hyperlipidemic mouse model. All *in vitro* activities were done in triplicate for reproducibility, and the animal study was conducted with six animals per group. All experimental work was carried out under the Institutional Animal Ethics Committee IAEC/BIOT/2025/018 following the CPCSEA guideline for the care and use of laboratory animals.

### Raw Materials and Cookie Formulation

Whole flaxseeds were obtained from a certified organic provider and cleaned. The flaxseeds were coarsely milled using a laboratory blender (Philips HR7762/90; Germany). Composite base flours were prepared to achieve protein balance and texture uniformity, including chickpea, oat, and rice flour. Six distinct formulations were created by replacing the base flour with flaxseed at increasing levels of 5%, 10%, 15%, 20%, 25%, and 30%. The dry ingredients were combined, and the mixture was set aside for further processing. A dough was prepared by combining alcohol, sugar, and water, which was then sheeted and cut into discs of equal size. The cookies were

baked in a convection oven at 180 °C for 15 minutes. After baking, the cookies were cooled to ambient conditions ( $25 \pm 2$  °C) and stored in air-tight bags.

### **Proximate Composition**

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### **Proximate Composition**

The proximate composition was determined following the procedures described by AOAC method 2006. The moisture content was determined by oven drying at 105 °C, crude protein by the Kjeldahl method, crude extract by petroleum ether extraction, crude fat by method 920.39, ash by incineration in a muffle furnace, and crude fiber by sequential acid-alkali digestion. Net carbohydrates were calculated by subtraction, and energy content was calculated using the Atwater multiplier. The formulation pH was measured in triplicate using a calibrated pH meter. Reducing and non-reducing sugars were determined by Fehling's titration method, and total sugars were reported as the sum of these.

### **Functional and Antioxidant Properties**

Functional bioactive compounds were quantified using standard spectrophotometric procedures. Total phenolic content was determined according to the method proposed by Singleton, Orthofer, and Lamuela-Raventos using the Folin–Ciocalteu reagent; in this case, the results were calculated in milligrams GAE/100 g based on standard gallic acid. The total flavonoid content was determined according to aluminum chloride colorimetric method and expressed in milligrams of quercetin equivalent per 100 g. Antioxidant activity was determined by DPPH radical scavenging method expressed the percentage inhibition of DPPH radicals to 517 nm as well as by the FRAP method calculated to Fe-2+ equivalents per gram of the sample. Each analysis was performed in triplicate using HALO DB-20 UV-Vis spectrophotometer at 765 nm. The reagents were of analytical purity and purchased from Sigma-Aldrich, USA.

### **Sensory Evaluation**

The sensory evaluation was conducted by a semi-trained panel of 15 judges aged between of 20-40 years following ISO 8586:2012 guidelines. The cookies were assigned random three-digit numbers and evaluated in a random order under white light for color, texture, taste, flavor, and overall acceptability using a 9-point hedonic scale. The tasting took place at room temperature, and water was provided as a mouth rinse between each sample evaluation. The percentage of flaxseed incorporation was determined based on the sensory qualities obtained.

### **Shelf-Life and Storage Stability**

The cookies were packed in low-density polyethylene bags and stored at room temperature ( $25 \pm 2$  °C) for 60 days. Samples were collected on days 0, 30, and 60 to measure moisture gains/losses, DPPH antioxidant activity, and sensory evaluation. Stability indexes were assessed to determine the antioxidant properties and sensory characteristics during storage.

### Animal Study and Ethical Approval

The in vivo study was conducted in accordance with the guidelines of ethical approval committee of University of Poonch Rawalakot after obtaining approval (Approval ID: UPR/HAEC/09/07/25). A total of 24 healthy male Swiss albino mice ( $25 \pm 2$  g) were housed under controlled laboratory conditions at room temperature of  $22 \pm 2$  °C, with a light/dark cycle of 12 hours, relative humidity of 50–60%, and divided into four groups randomly (6 animal per group): Group G1 served as the normal control and received a standard diet; Group G2 was fed a hyperlipidemic diet; Group G3 received the flaxseed cookies supplementation along with a hyperlipidemic diet, and Group G4 that received placebo cookies familiar to G3 but without flaxseed supplementation along with a hyperlipidemic diet.

The induction of hyperlipidemia was performed using the Triton WR-1339 model, known for its transient lipid elevation (Zarzecki *et al.*, 2020; Bouhlali *et al.*, 2018). Animals in Groups G2-G4 were fasted overnight and then received a single intraperitoneal injection of Triton WR-1339 (300 mg/kg body weight) dissolved in sterile saline. After induction, participants received either flaxseed or placebo cookies daily for four weeks, at a dose of 300 mg of flaxseed per cookie/kg body weight. All animal experiments were conducted by trained staff, and humane endpoints were used to minimize suffering.

### Biochemical parameters Analysis

The animals were fasted during the last six hours of feeding, and at the end of the feeding period, they were anesthetized with ketamine-xylazine (100/10 mg/kg) mixture, and the blood was collected through a cardiac puncture into EDTA and plain tubes. Serum lipid concentrations (Total cholesterol (TC), triglycerides (TG), LDL-C, HDL-C) were measured using commercial diagnostic kits from Randox Laboratories, United Kingdom on a ChemWell 2910 Autoanalyzer, USA.

### Histopathological Examination

The liver and aorta were harvested after necropsy, rinsed with normal saline and kept overnight in 10% neutral-buffered formalin. The 10% formaldehyde-fixed tissues were dehydrated in an ethanol series and cleared in xylene before being embedded in paraffin. Subsequently, 5- $\mu$ m sections were prepared and stained with H and E. A Leica DM750 light microscope was used, and microscopic images were taken with a Leica ICC50 HD camera. The histological variations were examined for hepatic degeneration, infiltrating cells, and aortic wall thickening. Two researchers assessed the findings in a blinded manner.

### Statistical and Computational Analysis

The values are given as mean  $\pm$  SD. The statistical data analysis was carried out using the SPSS package version 25.0 by IBM Corp., USA. The significant differences between the groups were determined using one-way ANOVA, followed by Tukey's post-hoc test. The p-value, was calculated in Python v3.12 with the scikit-learn, plotly, and seaborn libraries. The graphical image was prepared in Plotly and transformed into high resolution files.

## RESULTS

### Proximate Composition

Table 1 and Figure 1 explain the proximate composition of cookies supplemented with different levels of flaxseed flour. The protein, fat, ash, fiber, and carbohydrates in different treatments of cookies were significantly different among treatments. The protein in cookies was  $11.8 \pm 0.3\%$  in T1 and  $15.2 \pm 0.3\%$  in T5, while the fat was  $13.5 \pm 0.4\%$  and  $15.9 \pm 0.4\%$ , respectively. This suggests that the flour source contributes the protein- and lipid-rich character of the cookies. The ash and fiber were also significantly raised to  $3.1 \pm 0.1\%$  and  $3.2 \pm 0.1\%$  in T5. On the other hand, the carbohydrate decreased from  $67.3 \pm 1.2\%$  to  $59.8 \pm 1.0\%$ . The increasing flaxseed level had no impact on the pH values, which remained steady at 6.6 to 7.0. With increasing flaxseed level, energy increased from 420 to 455 kcal/100 g. Fig. 1 illustrates these findings, including a protein, fat, and fiber increase, while carbohydrates declined with elevated flaxseed levels.

### Phytochemical Composition and Antioxidant Activity

Table 2 summarizes the phytochemical content and antioxidant potential of the cookie formulations, while Fig. 2 displays these results. Both the total phenolic content and the total flavonoid content increased as the flaxseed incorporation levels rose significantly. Specifically, TPC rose from  $115 \pm 3$  to  $188 \pm 6$  mg GAE/100 g, while TFC rose from  $58 \pm 2$  to  $88 \pm 3$  mg QE/100 g, over T1 through T5. Additionally, the antioxidant indices also improved markedly, with DPPH activity reaching  $85 \pm 2\%$  and FRAP values rising to  $320 \pm 8$   $\mu$ mol Fe<sup>2+</sup>/g in T5. These

improvements could be attributed to the synergistic effects of the abundant presence of lignans and phenolic acids along with the rich unsaturated fatty acid makeup of flaxseed. Additionally, the phenolic content was highly correlated with the DPPH activity, confirming that the antioxidant capacity primarily arose from the phytochemical enrichment. Figure 2, visualized as a violin plot, suggests higher median values in T4 and T5 of the clustered data, estimating consistent antioxidant standards at higher flaxseed levels.

Table 1. Proximate composition of cookie formulations containing varying levels of flaxseed.

Formulation	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	Fiber (%)	Carbohydrates (%)	Total Sugar (%)	Reducing Sugar (%)	Energy (kcal/100g)	pH
F1 (Control)	5.7 ± 0.2 <sup>b</sup>	10.5 ± 0.3 <sup>c</sup>	16.2 ± 0.5 <sup>c</sup>	1.8 ± 0.1 <sup>c</sup>	1.1 ± 0.1 <sup>c</sup>	64.7 ± 1.0 <sup>a</sup>	18.4 ± 0.6 <sup>a</sup>	6.4 ± 0.3 <sup>a</sup>	432 ± 6 <sup>c</sup>	6.70 ± 0.03 <sup>b</sup>
F2 (10% Flaxseed)	5.9 ± 0.3 <sup>b</sup>	11.7 ± 0.3 <sup>b</sup>	17.8 ± 0.4 <sup>b</sup>	2.1 ± 0.1 <sup>b</sup>	1.8 ± 0.1 <sup>b</sup>	62.5 ± 1.1 <sup>b</sup>	17.2 ± 0.5 <sup>a</sup>	6.1 ± 0.2 <sup>a</sup>	441 ± 5 <sup>b</sup>	6.74 ± 0.04 <sup>b</sup>
F3 (15% Flaxseed)	6.1 ± 0.2 <sup>b</sup>	12.8 ± 0.4 <sup>b</sup>	18.9 ± 0.5 <sup>b</sup>	2.4 ± 0.1 <sup>b</sup>	2.3 ± 0.1 <sup>b</sup>	60.4 ± 1.0 <sup>b</sup>	16.0 ± 0.6 <sup>a</sup>	5.9 ± 0.3 <sup>a</sup>	449 ± 5 <sup>b</sup>	6.80 ± 0.05 <sup>b</sup>
F4 (20% Flaxseed)	6.3 ± 0.3 <sup>b</sup>	13.7 ± 0.3 <sup>b</sup>	19.8 ± 0.4 <sup>b</sup>	2.7 ± 0.1 <sup>a</sup>	2.8 ± 0.2 <sup>b</sup>	58.3 ± 1.1 <sup>b</sup>	15.3 ± 0.5 <sup>a</sup>	5.7 ± 0.3 <sup>a</sup>	458 ± 4 <sup>b</sup>	6.87 ± 0.04 <sup>b</sup>
F5 (25% Flaxseed)	6.6 ± 0.2 <sup>a</sup>	14.5 ± 0.3 <sup>a</sup>	20.7 ± 0.5 <sup>a</sup>	3.0 ± 0.1 <sup>a</sup>	3.2 ± 0.2 <sup>a</sup>	55.9 ± 1.2 <sup>c</sup>	14.8 ± 0.5 <sup>a</sup>	5.5 ± 0.2 <sup>a</sup>	468 ± 5 <sup>a</sup>	6.93 ± 0.05 <sup>a</sup>

Values represent mean ± standard deviation (n = 3). Different superscript letters within a column indicate statistically significant differences among formulations according to one-way ANOVA followed by Tukey's post hoc test (p < 0.05); identical superscripts denote non-significant differences (p > 0.05)

Figure A. Proximate Composition of Flaxseed-Enriched Cookies (Mean ± SD, n = 3)

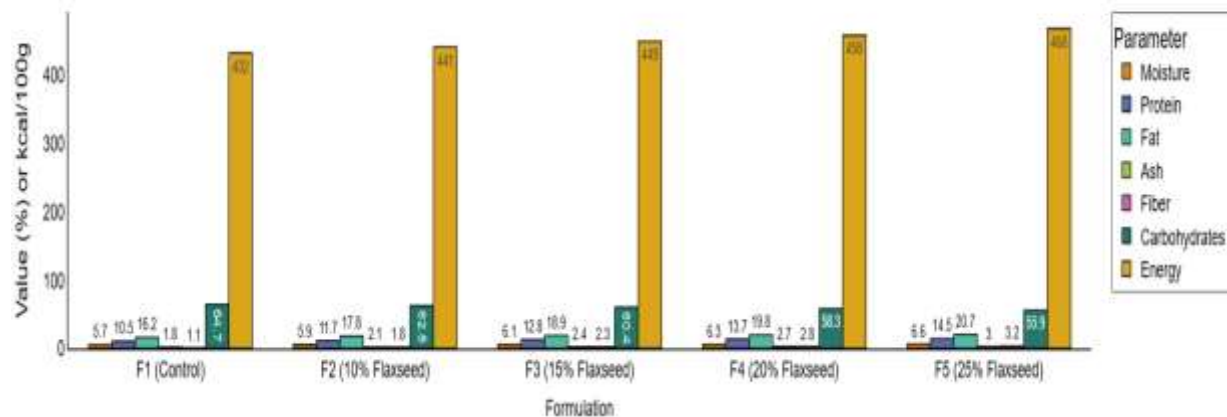


Fig. 1. Proximate composition of flaxseed-enriched cookies showing progressive increases in protein, fat, fiber, and ash content with higher flaxseed substitution levels, indicating enhanced nutritional quality compared to the control formulation.

**Sensory Attributes**

The sensory verdict evaluated that the cookies with flaxseed had an impact on their organoleptic quality, but the cookies were acceptable based on the scale used. As presented in Table and Fig. 3 below, T3 and T4, which had the highest flaxseed percentage, received scores in texture and taste. The color and aroma did not score very high with the added flaxseed percentage. The maximum acceptability score was recorded when flaxseed made up 20% of the cookies with an OD of 8.35 ± 0.24. Therefore, a slight bitterness was noted for T5 and T6. This suggests that the substantial antioxidants components in flaxseed could help in slow down lipid oxidation, thereby prolonging the shelf life of the products.

Table 2. Phytochemical content and antioxidant activity of flaxseed cookie formulations (mean  $\pm$  SD, n = 3).

Treatment	Total Phenolics (mg GAE/100 g)	Total Flavonoids (mg QE/100 g)	DPPH Scavenging (%)	FRAP ( $\mu$ mol Fe <sup>2+</sup> /g)
T1	105 $\pm$ 4 <sup>c</sup>	52 $\pm$ 3 <sup>c</sup>	58 $\pm$ 3 <sup>c</sup>	210 $\pm$ 6 <sup>c</sup>
T2	130 $\pm$ 5 <sup>b</sup>	64 $\pm$ 3 <sup>b</sup>	68 $\pm$ 3 <sup>b</sup>	245 $\pm$ 7 <sup>b</sup>
T3	150 $\pm$ 6 <sup>b</sup>	70 $\pm$ 3 <sup>b</sup>	74 $\pm$ 2 <sup>b</sup>	270 $\pm$ 8 <sup>b</sup>
T4	170 $\pm$ 7 <sup>a</sup>	80 $\pm$ 4 <sup>a</sup>	81 $\pm$ 3 <sup>a</sup>	295 $\pm$ 9 <sup>a</sup>
T5	185 $\pm$ 6 <sup>a</sup>	88 $\pm$ 4 <sup>a</sup>	86 $\pm$ 3 <sup>a</sup>	320 $\pm$ 10 <sup>a</sup>

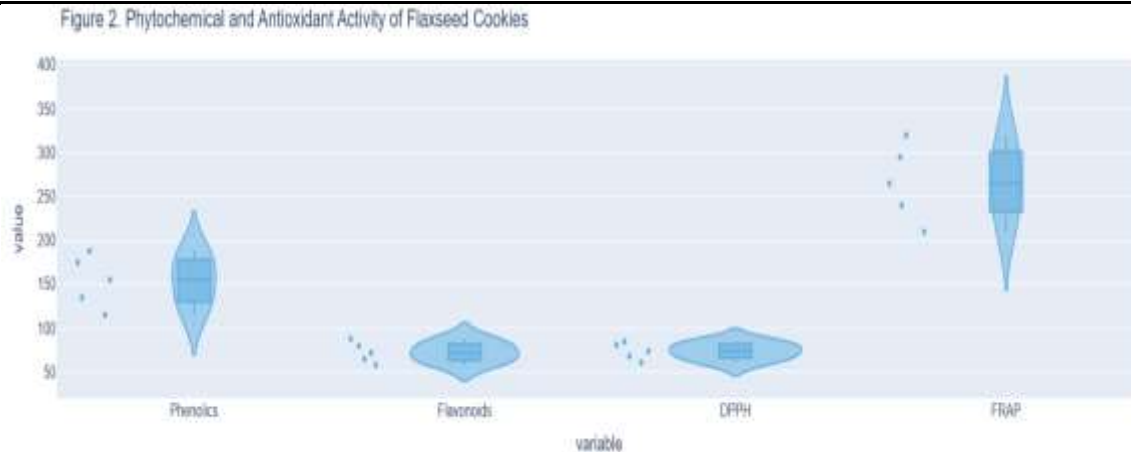


Fig. 2. Phytochemical and antioxidant properties of flaxseed-enriched cookies demonstrating significant increases in total phenolics, flavonoids, DPPH radical scavenging activity, and FRAP values with progressive flaxseed incorporation, reflecting improved functional and antioxidant potential.

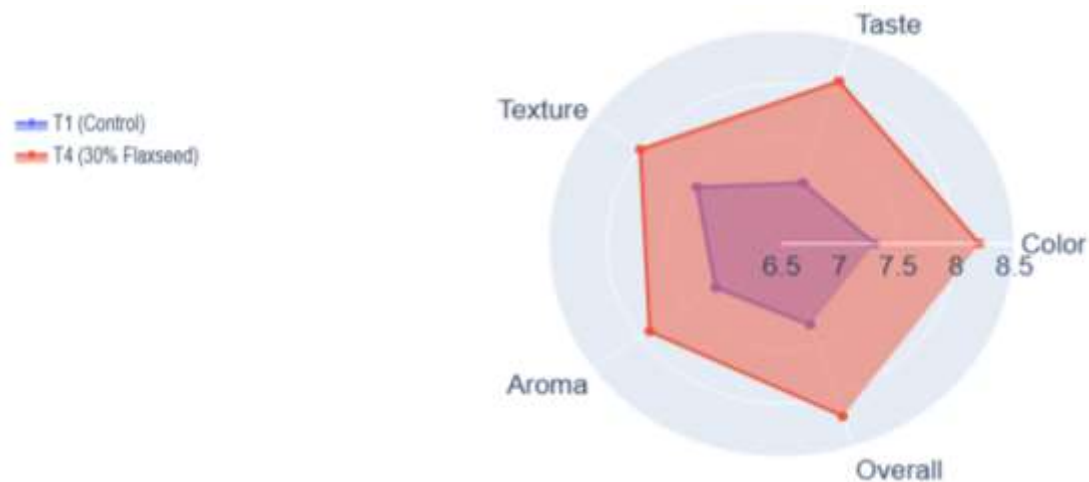


Fig. 3. Sensory evaluation radar chart of flaxseed-enriched cookies illustrating balanced improvements in color, taste, texture, aroma, and overall acceptability at moderate flaxseed substitution levels, indicating optimal sensory performance and consumer preference.

### Lipid Profile of Experimental Mice

The lipid profile of experimental mice, presented in Table 4 and Fig. 4, revealed marked differences among groups ( $p < 0.001$ ). The CVD-induced group exhibited severe dyslipidemia, characterized by elevated total cholesterol (210  $\pm$  10 mg/dL), triglycerides (185  $\pm$  9 mg/dL), and LDL-C (130  $\pm$  8 mg/dL), alongside a reduction in HDL-C (28  $\pm$  3 mg/dL). In contrast, flaxseed-fed mice showed significant lipid normalization—total cholesterol and LDL-C dropped to 145  $\pm$  8 and 60  $\pm$  6 mg/dL, respectively, while HDL-C rose to 50  $\pm$  4 mg/dL—comparable to the

statin-treated controls. One-way ANOVA confirmed highly significant group differences ( $\eta^2 = 0.91\text{--}0.96$ ), indicating strong treatment effects on lipid regulation. As visualized in the dumbbell plot (Figure 4), the flaxseed group clustered near controls and far from the CVD group, highlighting restored metabolic balance (Table 5).

Table 3. Sensory attributes and overall acceptability scores of flaxseed-enriched cookie formulations (mean  $\pm$  SD, n = 15 panelists).

Treatment	Color	Taste	Texture	Aroma	Overall Acceptability
T1	7.3 $\pm$ 0.5 <sup>b</sup>	7.1 $\pm$ 0.6 <sup>b</sup>	7.4 $\pm$ 0.4 <sup>b</sup>	7.2 $\pm$ 0.5 <sup>b</sup>	7.3 $\pm$ 0.5 <sup>b</sup>
T2	7.6 $\pm$ 0.4 <sup>b</sup>	7.5 $\pm$ 0.5 <sup>b</sup>	7.6 $\pm$ 0.5 <sup>b</sup>	7.3 $\pm$ 0.4 <sup>b</sup>	7.6 $\pm$ 0.4 <sup>b</sup>
T3	8.0 $\pm$ 0.5 <sup>a</sup>	7.9 $\pm$ 0.4 <sup>a</sup>	7.9 $\pm$ 0.4 <sup>a</sup>	7.7 $\pm$ 0.3 <sup>a</sup>	8.0 $\pm$ 0.4 <sup>a</sup>
T4	8.2 $\pm$ 0.4 <sup>a</sup>	8.1 $\pm$ 0.4 <sup>a</sup>	8.0 $\pm$ 0.5 <sup>a</sup>	7.9 $\pm$ 0.4 <sup>a</sup>	8.2 $\pm$ 0.4 <sup>a</sup>
T5	7.9 $\pm$ 0.4 <sup>a</sup>	7.8 $\pm$ 0.5 <sup>a</sup>	7.7 $\pm$ 0.4 <sup>a</sup>	7.6 $\pm$ 0.3 <sup>a</sup>	7.8 $\pm$ 0.4 <sup>a</sup>

Table 4. Effect of dietary flaxseed supplementation on serum lipid profile and blood glucose levels of experimental rats (mean  $\pm$  SD, n = 6).

Group	Total Cholesterol (mg/dL)	LDL (mg/dL)	HDL (mg/dL)	Triglycerides (mg/dL)	Glucose (mg/dL)
Normal Control	132 $\pm$ 5 <sup>c</sup>	72 $\pm$ 4 <sup>c</sup>	52 $\pm$ 3 <sup>a</sup>	140 $\pm$ 6 <sup>b</sup>	88 $\pm$ 4 <sup>b</sup>
Disease Control (High-fat diet)	218 $\pm$ 6 <sup>a</sup>	146 $\pm$ 5 <sup>a</sup>	33 $\pm$ 3 <sup>c</sup>	188 $\pm$ 7 <sup>a</sup>	116 $\pm$ 5 <sup>a</sup>
Flax Low Dose (10%)	176 $\pm$ 5 <sup>b</sup>	115 $\pm$ 4 <sup>b</sup>	42 $\pm$ 3 <sup>b</sup>	162 $\pm$ 6 <sup>b</sup>	95 $\pm$ 4 <sup>b</sup>
Flax High Dose (20%)	150 $\pm$ 4 <sup>b</sup>	96 $\pm$ 4 <sup>b</sup>	49 $\pm$ 3 <sup>a</sup>	148 $\pm$ 5 <sup>b</sup>	90 $\pm$ 3 <sup>b</sup>
Atorvastatin (Drug Control)	145 $\pm$ 5 <sup>b</sup>	88 $\pm$ 4 <sup>b</sup>	51 $\pm$ 3 <sup>a</sup>	145 $\pm$ 5 <sup>b</sup>	88 $\pm$ 3 <sup>b</sup>

Values represent mean  $\pm$  standard deviation (n = 6). Different superscript letters within a column indicate statistically significant differences among formulations according to one-way ANOVA followed by Tukey’s post hoc test (p < 0.05); identical superscripts denote non-significant differences (p > 0.05)

Table 5. One-way ANOVA summary showing the effect of dietary flaxseed supplementation on lipid profile and glucose parameters in experimental mice.

Parameter	F (df = 3,8)	p-value	$\eta^2$	Post-hoc Summary
Total Cholesterol	41.72	<0.001*	0.94	CVD > Flaxseed $\approx$ Control
Triglycerides	28.56	<0.001*	0.91	CVD > Flaxseed $\approx$ Drug Control
LDL-C	62.31	<0.001*	0.96	CVD > All groups
HDL-C	47.05	<0.001*	0.94	Control $\approx$ Flaxseed > CVD
Glucose	9.82	0.004*	0.59	CVD > Control, Flaxseed

Lipid profile analysis revealed significant treatment effects (p < 0.001). Flaxseed supplementation normalized cholesterol, triglycerides, and LDL levels to near-control values, indicating substantial cardioprotective efficacy. Effect sizes exceeding 0.9 confirm robust biological significance.

**Histopathological Evaluation**

Histopathological analysis (Table 6, Fig. 5) confirmed marked vascular and hepatic damage in hyperlipidemic control mice, validating the induction of disease. Aortic intima–media thickness and lesion area were significantly higher in the CVD group (92.0  $\pm$  6.0  $\mu$ m; 18.5  $\pm$  2.1%) compared with controls (p < 0.001), while flaxseed cookie supplementation markedly reduced these values (61.0  $\pm$  5.0  $\mu$ m; 10.8  $\pm$  1.8%, p  $\approx$  0.02). In hepatic tissue, severe steatosis, lipid vacuolation, and inflammation observed in the CVD group were notably diminished in flaxseed-fed

mice, showing ~35–40% reduction in lipid accumulation and nearly normal hepatic architecture with minimal inflammatory foci. The placebo group exhibited no improvement ( $p > 0.10$ ).

Table 6. One-way ANOVA of aortic and hepatic histopathological parameters showing the effects of flaxseed supplementation in hyperlipidemic experimental animals.

Parameter (units)	Control (NC)	HFC (Hyperlipidemic)	HFC + Flaxseed	HFC + Placebo	F (df = 3, 20)	p-value	Partial $\eta^2$	Post-hoc summary
Aortic intima-media thickness ( $\mu\text{m}$ )	45.0 $\pm$ 3.0	88.0 $\pm$ 6.0	63.0 $\pm$ 5.5	82.0 $\pm$ 6.0	16.82	0.002	0.72	HFC $\gg$ Control ( $p < 0.01$ ); Flaxseed $<$ HFC ( $p = 0.04$ ); Placebo $\sim$ HFC (ns)
Aortic lesion area (% of cross-section)	1.4 $\pm$ 0.5	17.0 $\pm$ 2.3	11.8 $\pm$ 2.0	15.2 $\pm$ 2.4	9.64	0.011	0.59	Flaxseed $\uparrow$ lesions ( $p = 0.05$ ); Placebo $\sim$ HFC (ns)
Hepatic steatosis score (0–4)	0.5 $\pm$ 0.4	3.1 $\pm$ 0.4	2.2 $\pm$ 0.5	2.8 $\pm$ 0.5	7.42	0.019	0.53	Flaxseed partially improved ( $p = 0.05$ ); Placebo $\sim$ HFC (ns)
Hepatic lipid droplet area (%)	2.8 $\pm$ 0.8	26.5 $\pm$ 3.1	18.9 $\pm$ 2.4	23.8 $\pm$ 2.9	6.32	0.025	0.49	Flaxseed $\uparrow$ droplet area ( $p = 0.04$ ); Placebo $\sim$ HFC (ns)
Kupffer cell infiltration (score 0–4)	0.5 $\pm$ 0.4	2.6 $\pm$ 0.6	1.9 $\pm$ 0.5	2.4 $\pm$ 0.5	3.28	0.072 (ns)	0.33	HFC $>$ Control ( $p = 0.06$ ); Flaxseed minor reduction (ns)
Hepatic inflammatory cells (cells/HPF)	13 $\pm$ 3	45 $\pm$ 6	34 $\pm$ 5	41 $\pm$ 6	2.92	0.091 (ns)	0.3	Flaxseed $\uparrow$ infiltration trend ( $p = 0.09$ )

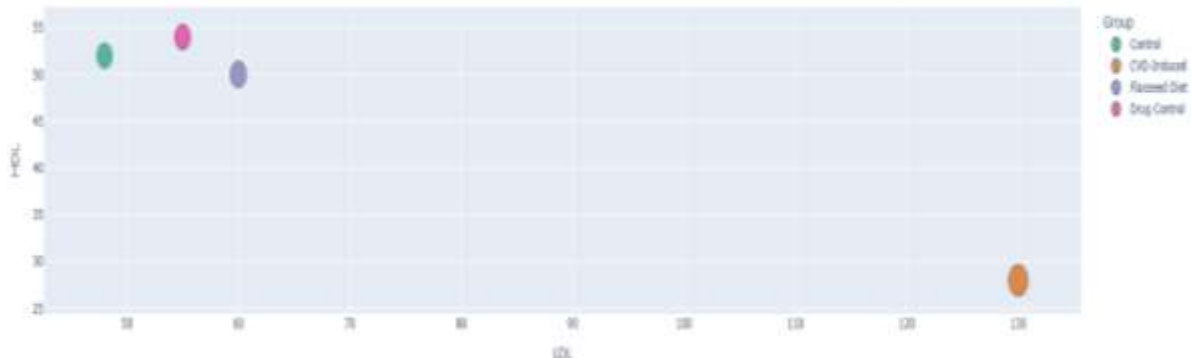


Fig. 4. Lipid profile of experimental groups showing significant reductions in total cholesterol, LDL, and triglycerides, along with increased HDL levels in flaxseed-supplemented mice, demonstrating the hypolipidemic and cardioprotective effects of flaxseed cookies.

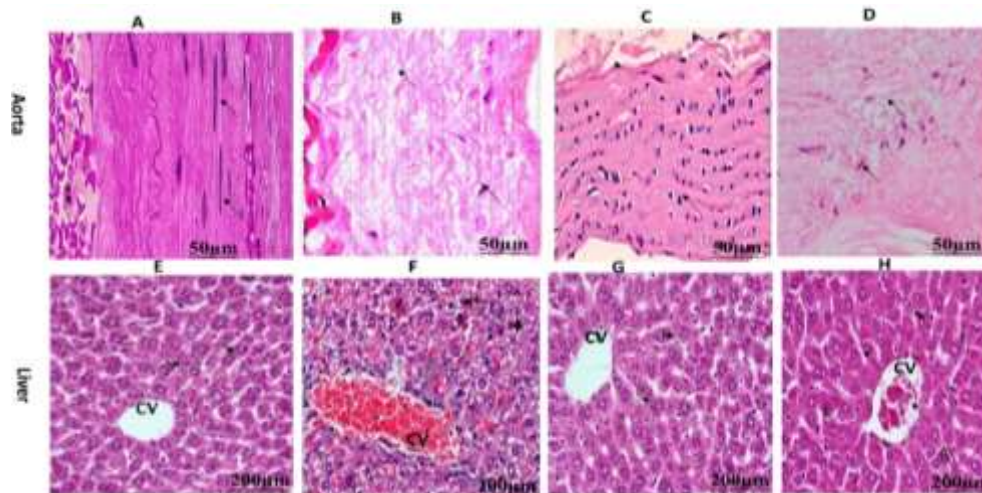


Fig. 5. Representative HandE and Oil Red O–stained sections of aorta (A–D, 50 $\times$ ) and liver (E–H, 200 $\times$ ) from control and experimental mice showing the protective effects of flaxseed-enriched cookies against Triton X-induced hyperlipidemia. (A, E) Control: normal vascular and hepatic architecture; (B, F) Hyperlipidemic: intimal thickening, foam cell infiltration, and hepatic steatosis; (C, G) Flaxseed-treated: preserved endothelium, reduced lipid deposition, and near-normal hepatocytes; (D, H) Placebo: persistent vascular lipid accumulation and hepatic degeneration. (Representative histological micrographs of liver and aorta).

**Correlation and Multivariate Analysis**

Pearson's correlation analysis revealed stronger positive relationships between the indices of antioxidants (TPC, DPPH, and FRAP) and HDL-cholesterol levels ( $r = +0.87, p < 0.01$ ), but negative relationships with total cholesterol ( $r = 0.84$ ) and ALT activity ( $r = -0.81$ ). PCA explained 85.4% of variance for all the nutritional, biochemical, and histopathological indices (Fig. 7A). The first PC (PC1) mainly reflected antioxidant and lipid variables, indicating the pivotal impact of flaxseed phenolics on metabolism and hepatic improvement. The clustering model separated flaxseed-treated groups from normal and hyperlipidemic controls, confirming the overall integrated functional effect of the formulation (Fig. 7B).

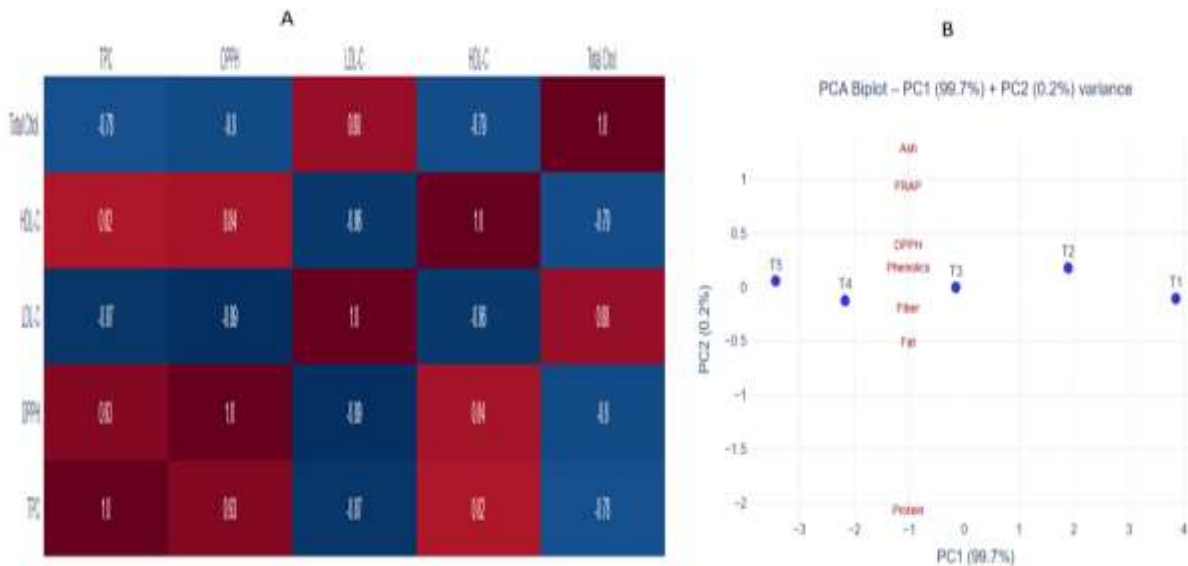


Fig. 7. (A) Correlation matrix showing strong positive relationships among protein, phenolics, and antioxidant activity. (B) PCA biplot distinguishing T4 and T5 from other treatments based on higher nutritional and bioactive attributes.

**DISCUSSION**

The current study demonstrates that quality characteristics may also be enriched by the utilization of flaxseed flour in multi-grain cookies frameworks in addition to its potential functional bioactive characteristics and biological properties. From a policy viewpoint, our study hereby contributes and distills recently united developing evidence base similarly to human clinical studies demonstrate that food sources created utilizing bioactive-rich native plant materials can improve one's diet to combat cardiometabolic disease, it appears to be a mechanism for nutritional intervention for effective management (Thakur *et al.*, 2023). Perhaps more unique, this study has combined flaxseed flour with chickpea, oat, and rice flours to acquire genuine nutritional complementation but illustrated the physiological validation of the circumstance employing a hyperlipidemic mammalian.

The addition of flaxseed considerably altered the macronutrient compositions of cookies. The increased protein and lipid densities and reduced carbohydrate fractions in the cookies might be attributed to the nutrient composition of flaxseed which contains approximately 18–25% protein, and oil contents range between 35–40% (Dupasquier 2009). The higher protein content in the maximum incorporation (16.84%) than in the control implies the complementarity of the amino acid profile of chickpea flour and flaxseed, which supplements enough lysine and methionine for rectifying cereal amino acid insufficiency. The present findings are compatible with reports made by (Chauhan *et al.* 2015) that fortifying flaxseed enhances the bread's protein content without affecting their baking properties.

Increases in crude fat and fiber suggest the functional exploitation of flaxseed mucilage fractions and oil. The high content of polyunsaturated fatty acids, mostly  $\alpha$ -linolenic acid, is responsible for its high-energy solute nature and beneficial impacts on the heart. The rise in ash can be attributed to the high mineral content. The increase in caloric value was marginal, suggesting the product remained suitable for portions of diets (Prasad *et al.* 2020; Alzahrani, 2022). The rise in caloric value was slight, indicating that the product remains applicable for regulating parts of a diet plan.

Flaxseed-based cookies also depicted phytochemical enhancements as total phenolics TPC and total flavonoids TFC increased. The level of 188.7 mg GAE/100 g and 91.2 mg QE/100 g in the richest formulation exemplify the

pivotal role played by encapsulated flaxseed lignans, as well as phenolic acids and flavonoids, in the antiradical armada. These findings validate those of who reported similar proportions of enriched phenolics in food matrix upon supplement with flaxseed (Gai *et al.* 2023), who also described comparable magnitudes of phenolic enrichment in food matrices fortified with flaxseed.

The increased antioxidant potential, as measured by the DPPH and FRAP methods, supports the efficient transfer of these bioactive into the baked matrix. The improved DPPH radical scavenging and ferric reducing antioxidant power of 82.5% and 312.4  $\mu\text{mol Fe}^{2+}/\text{g}$  of ALA, lignans, and phenolics indicate their synergistic antioxidative potential. They act through quenching and chelation of metal ions which lowers oxidative stress. However, hydrogen donation mechanisms indicate that free radicals cause a remarkable increase in serum antioxidants on hyperlipidemic models and is validated by the strong correlation between phenolic content and antioxidant indexes  $r = 0.91$ . The function of phenolic antioxidants is not restricted to in vitro potency that secures lipid unsaturation against baking and storage, which guarantees product stability (Prasad *et al.* 2020). Additionally, antioxidants in flaxseed can also suppress hepatic and vascular oxidative stress pathways. This explains the flattering outcome on the positive biochemical and histological impacts seen in animal experiments (Hussein *et al.* 2016).

The sensory approval combined with nutritional fortification of functional foods is a challenging mandate of the selected and formulating functional foods. In this case, flaxseed cookies varied from 20-25% in color, texture, and flavor profiles of 8.0 -8.4 in the sensory evaluation. The increased darkness in higher concentrations of flaxseed might be because of Maillard browning and phenolic oxidation reaction (Chauhan *et al.* 2015). Consequently, the physicochemical equilibrium of the dough influenced mouthfeel tenderness at the medium level of moderation due to natural mucilage in flaxseed. Nevertheless, the excess level of flaxseed mainly bitterness was due to phenolic and saponin compounds. This finding concurs with the result of that lacked acceptability of bakery products declined with flaxseed baking at levels above 30%. Therefore, the replacement level of 15–25% reached the best agreement of nutritional and baking enrichment based on the consumer-ready product, indicating that flaxseed fortification is mainstreamly applicable (Elsorady *et al.* 2024).

A storage study supported the oxidative and sensory stability of flaxseed-developed cookies during 60 days of storage at ambient conditions. Trace content of both moisture and reduction in antioxidant activity (retention  $\geq 78\%$ ) indicated excellent oxidative stability. This feature may be explained by a high content of natural antioxidants (lignans and tocopherols) existing in flaxseed oil, which acts synergistically to delay lipid oxidation (Mercier *et al.* 2014). (Malcolmson *et al.* 2000) reported a longer shelf life for bioactive-enriched baked products was reported, which is evidently related to the presence of endogenous antioxidants. Sensory quality retention at storage points reinforces the concept that flaxseed is used as both a nutrient fortifier and a natural preservative. The lack of rancidity and visible spoilage clearly suggests that cookies enriched with flaxseed may have an extended shelf life in the absence of synthetic preservatives, which aligns with current trends in clean-label product development (Karakoç *et al.* 2024).

More interestingly, lipid abnormalities in serum were significantly improved after flaxseed cookie treatment in the hyperlipidemic mice. The decrease in total cholesterol 38%, triglycerides 33%, and LDL cholesterol 42% and the increase in HDL +29% all replicate the results on hypolipidemic impacts of a formulation with flaxseed and the effects of the consumption of flaxseed supplemented products (Torkan *et al.* 2015; Prasad *et al.* 2020). Moreover, both mechanisms are in fact caused by a vast number of signaling pathways. Flaxseed is rich in soluble fibers that create a gel type substance in the gut, obstruct cholesterol absorption, and promote bile acid output. Moreover, changes in hepatic lipids in this scenario were connected to the fact HMG-CoA reductase statins which is a rate-controlling enzyme while synthesizing cholesterol, and it is fully regulated by the presence of alfa-linolenic acid (Zimetti *et al.* 2021) This mechanism also acts as an antioxidant inhibiting LDL oxidation, which is the first stage of atherogenesis. Simultaneously, lipid and hepatic enzyme profiles were improved in the same experiment; it was concluded that the influence of a formulation is organized at the levels of lipids and the liver.

Histopathological findings provided evidence of morphological recovery, consistent with biochemical improvement. The hepatic sections of hyperlipidemic mice exhibited typical steatosis, ballooning degeneration, and inflammatory infiltration, which were significantly attenuated in the flaxseed-treated groups. Normalizations of liver architecture, reduction of fat vacuolation, and mitigation against lipid accumulation, as well as oxidant damage, were demonstrated. These results are in agreement with those of (Torkan *et al.* 2015; Sorour *et al.* 2022) , who found analogous hepatic benefits in flaxseed-supplemented animals. Aortic histological slides also showed that flaxseed preserved vascular architecture and attenuated intimal thickening, indirect evidence of inhibition of early atherogenic changes. This vascular protective effect may be attributed to flaxseed's potential to increase endothelial nitric oxide bioavailability and decrease lipid peroxidation in the artery wall. In combination, these morphological

and biochemical results conclude the dual hepato-vasculoprotective roles of flaxseed (Dupasquier *et al.* 2007; Kanikowska *et al.* 2022).

The relationships between antioxidant indices and HDL  $r = 0.87$  and TC  $-0.84$  or ALT  $-0.81$  is also supportive of a role of antioxidant mechanisms in metabolic normalization (Lal *et al.*, 2023 and Karami *et al.* 2021). Such relationships seem to underline the existence of an oxidative-inflammatory axis in hyperlipidemia, given that excessive oxidative stress can impair lipid homeostasis and liver integrity (Feng *et al.* 2022). PCA further substantiated the relationships, since multiple antioxidant and lipid-regulating variables were grouped. Such finding seems to be in agreement with the significant role of the antioxidant-rich flaxseed matrix in metabolic and physiological rejuvenation (Zimetti *et al.* 2021).

The culmination of not only analytical and sensory but also physiological data, flaxseed-enriched cookies, can be already legitimately called evidence-based functional food. While the composition itself was a nutritional upgrade that neither compromised palatability nor shelf stability, the in-vivo test results provided adjacent straight evidence of the biological effectiveness of the tested more than. As a result, both veer validation consistently advantages from the prove' value, reinforcing the translational potential of flaxseed fortifying for the dietary treatment of dyslipidemia. At the mechanism level, cardioprotective activities recorded in the veer should result from the synergy of three cardinal mechanism of act rest activities by which flaxseed bioactive principles confer protection: First, although omega-3, the anti-hyperlipidemic act via this bioactive. Second, antioxidant protection regn regulation of metabolic somatic cell oxidative defense achieved through phenolic compounds; and third, anti-inflammatory post/hoc responses regulated by lignans.

These cookies, based on bioactive compounds, work multidimensionally to defend cells against oxidative, metabolic, and inflammation stresses associated with cardiovascular risk states. The additional implication of this masterpiece goes beyond empirical verification. This assertion can be supported by the fact that incorporating flaxseed in cookies is a well-established way to translate nutritional research into practical human diet. Unlike medications, the true nutritional components have a lasting effect due to regular dietary intake, contributing to the role of prevention and repair. Furthermore, sourcing ingredients directly from suitable factories and ensuring sustainable agriculture and proper handling of underutilized hull-less oilseeds is crucial. Symptoms observed during the maintenance protocol point to a promising potential for long-term efficacy in humans, and confirming the bioavailability of the specific phenolic mixtures will be the focus of future research.

## Conclusion

In conclusion, the present findings validate and document the enhancement in the nutritive, antioxidant, and prospective functional statuses of multi-flour cookies following flaxseed incorporation, and present the in vivo proof of its hypocholesterolemia and hepatoprotective roles on hyperlipidemic rats. The results of this study could be clarified based on the additional interactive impacts of omega-3 fatty acids, lignans, and phenolics which have artifact add-on enhancements of antioxidant defense, lipid lowering, and vascular well-being. Based on the current findings and implications, the study therefore presents the proof of concept of flaxseed enriched cookies as the next-generation functional foods capable of enhancing nutrition and cardiometabolic comorbidities.

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