Phytochemical screening, proximate composition, and antihyperlipidemic activity of the barley-rich nutraceutical combination in triton X-100-induced hyperlipidemic Wistar rats

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ABSTRACT

Cardiovascular disorders are associated with hyperlipidemia, and statin therapy is the first line of treatment, with several side effects. To prevent hyperlipidemia and for cost-effectiveness with no side effects, the study aimed to develop a nutraceutical recipe. For 15 days, the Wistar rats were fed an oral diet supplemented with barley, foxtail millet, flaxseeds, moong, and fish oil. Each rat was treated with Triton X-100 intraperitoneally to induce hyperlipidemia. The serum lipid profiles were measured. Diet formulations rich in flaxseeds and barley significantly improved high-density lipoprotein levels in rats and reduced total cholesterol, triglycerides, and low- and very low-density lipoprotein levels. Fish oil, foxtail millet, and moong did not affect high-density lipoproteins. Our next step was to combine the nutraceuticals and test them similarly as above, along with analyzing the lipid profiles. It was found that the barley-rich combination reduced cholesterol most effectively among the four different combinations tested. Furthermore, alkaloids, carbohydrates, proteins, steroids, and cardiac glycosides were identified by phytochemical analysis. The proximate analysis revealed the presence of 30% moisture, 6% ash, 46% carbohydrate, 10% protein, 5% fat, and 1% fiber. The study concludes that the barley-rich nutraceutical combination is beneficial in preventing hyperlipidemia.

INTRODUCTION

Hyperlipidemia is characterized by an elevated triglyceride (TG) levels and free fatty acid content, a decrease in high-density lipoprotein (HDL-C) levels, and a normal or slightly elevated low-density lipoprotein cholesterol (LDL-C) level in circulation (Sheik *et al.*, 2022). Lipid metabolism is essential to maintain blood lipid levels and cardiovascular disease (CVD) is caused by dysfunction in lipid metabolism (Chen *et al.*, 2014). The cholesterol is absorbed in the intestine and transported in the form of chylomicrons to the liver as it is the predominant organ in maintaining the lipid homeostasis (Mansbach and Gorelick, 2007). The cholesterol absorption pathway has been recognized as an important pharmacological intervention for hyperlipidemia due to its close relationship with lipid metabolism. The intestinal absorption of cholesterol can, therefore, be reduced to prevent hyperlipidemia (El-Tantawy and Temraz, 2019)

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To treat hyperlipidemia, lifestyle modifications are required, including a low-fat diet, weight loss, exercise, and pharmacotherapy. Despite being first-line drugs, statins do not universally correct metabolic abnormalities and are associated with several side effects if taken for a long period. Hence, there is a need to develop natural hypolipidemic drugs for preventing and treating hyperlipidemia (Watts and Karpe, 2011). Nutraceuticals have been used long back by the people, but it is only recently that scientific and medical evidence began supporting their potential for effectiveness (Dillard and German, 2000). Hippocrates stated, "Let food be thy medicine and medicine be thy food," describing the significance of nutraceuticals as substances that can be considered foods or parts of foods, attributing to the therapeutic purpose including disease prevention and treatment (Elkhalifa *et al.*, 2021). The International Lipid Expert Panel has approved these therapies (Ji *et al.*, 2019). Studies on food consumption trends and ecological studies provide early evidence of the role of diet on CVD (McGill, 1979). Due to their pathophysiological role, nutraceuticals may block intestinal cholesterol absorption, inhibit liver cholesterol synthesis, and enhance LDL-C excretion. It is possible that these agents may be of considerable help to patients with low-to-moderate hyperlipidemia, as well as in those with statin-related side effects (Sanidas and Grassos, 2020). Some of the nutraceuticals which are inspected for hypolipidemic activity include *Tetracarpidium conophorum*, commonly known as African walnut, and *Buccholzia coriacea*, popular as wonderful kola (Oluchi Nwaichi *et al.*, 2017), the plant named *Capparis spinosa L*. (Mollica *et al.*, 2017), and lyophilized beetroot powder (Sarfaraz *et al.*, 2021).

Numerous studies show synergistic and pleiotropic effects of highly bioactive components on CVD risk factors as outweighing their individual benefits (Ramprasath *et al.*, 2015), and hence there is a need for extensive research on supplements containing a mixture of nutraceuticals as the compounds present in the mixture may have the enhanced effect by interacting with each other (Santana-Gálvez *et al.*, 2019). Developing a nutraceutical diet as an adjunctive strategy to treat hyperlipidemia may reduce the adverse side effects of pharmacotherapy medications and also reduce healthcare expenses (Johnston *et al.*, 2017). Therefore, following identification and authentication by the nutritionist, the study selected five ingredients for preparation of the nutraceutical diet, including flaxseeds, foxtail millet, moong, barley, and fish oil, which were tested for the antihyperlipidemic activity both individually and in combination on the Triton X-100 hyperlipidemic rat model.

MATERIALS AND METHODS

Reagents and chemicals

The reagents and chemicals were purchased from Sigma-Aldrich, India, and they include 96% absolute ethanol (CN64-17-5), potassium mercuric iodide (CN7783-33-7), 20% alcoholic α-naphthol (CN90-15-3), concentrated sulfuric acid (CN77664-93-9), potassium sodium tartrate tetrahydrate (CN6381-59-5), copper sulfate (CN7758-98-7), sodium hydroxide (CN1310-73-2), ferric chloride (CN7705-08-0), lead acetate (CN 6080-56-4),

chloroform (CN67-66-3), sodium bicarbonate (CN 144-55-8), glacial acetic acid (CN64-19-7), and acetic anhydride (CN108-24-7).

Raw materials to prepare the nutraceutical diet

Raw materials such as barley (*Hordeum vulgare*), foxtail millet (*Setaria italic*), flaxseeds (*Linum usitatissimum*), and moong (*Vigna radiata*) were purchased at a local store in Manipal, India, and Seacod® cod-liver oil capsules were purchased at a local pharmacy in Manipal, India.

Preparation of the nutraceutical diet

The diet was prepared according to AIN-93 purified diets for laboratory rodents (Reeves, 1997), with slight modifications according to the Indian food composition tables (Longvah *et al.*, 2017) and Indian Council of Medical Research dietary guidelines for Indians (Manual, 2011). The raw ingredients used were identified and authenticated by the Department of Nutrition, Welcomgroup Graduate School of Hotel Administration in Manipal, Karnataka, India. The concentration of each nutraceutical being effective to reduce hyperlipidemia is selected based on the literature, and the composition of the diet is designed. An electronic balance was used to accurately weigh and mix feed ingredients in a clean bowl. The ingredients were washed, air-dried, fried on a smaller flame with a temperature of 100°C for 10 minutes, cooled, and ground into powder. The diets were made into square confectionaries (Table 1).

Experimental animals

Adult male Wistar rats (150–200 g, 12 weeks old) were obtained from the Central Animal Research Facility, Manipal, Karnataka, India. All rats were housed under identical conditions in an aseptic facility and given free access to water and food. The study was approved by the Institutional Animal Care and Use Committee of the Manipal Academy of Higher Education (approval no.: IAEC/KMC/66/2019).

Experimental design

Step 1: Grouping of animals
The Wistar rats were randomly allocated into 11 groups ($n = 6$):

1. Normal controls (NC) fed with basal diet.
2. Hyperlipidemic controls (HC) fed with basal diet.
3. Hyperlipidemic rats (HFS) fed with flaxseeds.
4. Hyperlipidemic rats (HFM) fed with foxtail millet.
5. Hyperlipidemic rats (HM) fed with moong.
6. Hyperlipidemic rats (HB) fed with barley.
7. Hyperlipidemic rats (HFO) fed with fish oil.

Step 2: The formulation is fed orally (15 g/day) to each rat in their respective cages for 15 days.

Step 3: The hyperlipidemia was induced in overnight fasted Wistar rats by administration of Triton X-100 ((100 mg/kg BW) dissolved in physiological saline (Parwin *et al.*, 2019), followed by blood withdrawal within 24 hours by puncturing the retro-orbital sinus. The serum was used to analyze the lipid profile.

Table 1. Composition of feed formulations (in g).

Ingredients	Basal diet	Flaxseeds	foxtail millet	Moong	Barley	Fish oil	Nutraceutical combinations			
							1	2	3	4
Wheat flour	22.5	24.0	24.0	29.0	24.0	22.5	-	-	-	-
Roasted Bengal gram flour	60.0	23.0	23.0	28.0	23.0	40.0	-	-	-	-
Flaxseeds	-	35.0	-	-	-	-	7.0	10.0	10.0	26.0
Foxtail millet	-	-	35.0	-	-	-	35.0	35.0	10.0	10.0
Moong	-	-	-	25.0	-	-	25.0	7.0	7.0	10.0
Barley	-	-	-	-	35.0	-	7.0	10.0	35.0	26.0
Fish oil	-	-	-	-	-	20.0	8.0	20.0	20.0	10.0
Skim-milk powder	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Casein	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Refined groundnut oil	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Salt mixture	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Vitamin and mineral mixture	0.5	1.0	1.0	1.0	1.0	0.5	1.0	1.0	1.0	1.0

Step 4: The nutraceutical combinations are formulated based on the results obtained after the individual nutraceutical effect on hyperlipidemia in Wistar rats.

Step 5: The nutraceutical combination is fed for 15 days to the below grouped rats (15 g/day, orally):

8. Hyperlipidemic rats (HC1) fed with nutraceutical combination 1.
9. Hyperlipidemic rats (HC2) fed with nutraceutical combination 2.
10. Hyperlipidemic rats (HC3) fed with nutraceutical combination 3.
11. Hyperlipidemic rats (HC4) fed with nutraceutical combination 4.

Step 6: Inducing hyperlipidemia similar to that of step 3 in the above groups. The serum was again used to analyze the lipid profile.

Biochemical analysis

Total cholesterol (TC), TG, and HDL-C were analyzed using the commercial kits available from Coral Clinical Systems, India, and the levels were determined using the semiautomatic biochemistry analyzer (Medsource Ozone Biomedicals Pvt. Ltd.). LDL-C and very low-density lipoprotein cholesterol (VLDL-C) concentrations were calculated using the Friedewald formula.

Extraction of the nutraceutical diet for phytochemical analysis

An extraction was obtained by using the hot Soxhlet method for 72 hours with 200 g of dried nutraceutical powdered diet soaked in 500 ml 96% absolute ethanol. A portion of the filtrate was collected inside an amber bottle for phytochemical analysis based on the amount of filtrate obtained.

***In vitro* phytochemical analysis**

The analysis was carried out to test for alkaloids (Mayer's test), steroids (Libermann–Burchard test and Salkowski's test),

phenols and tannins (ferric chloride test, lead acetate test, and alkaline reagent test), saponins (foam test and sodium bicarbonate test), flavonoids (Shinoda's test and sodium hydroxide test), carbohydrates (Benedicts test), proteins (Biuret test), and cardiac glycosides (Killer Kilani's test) (Usunomena and Ngozi, 2016).

***In vitro* proximate composition analysis**

The standard AOAC procedure was used to estimate the moisture, protein, fat, and ash content. Moisture was estimated gravimetrically using a hot air oven by heating at 105°C. Protein was estimated using total nitrogen by the Kjeldahl digestion and distillation. Fat was estimated using the Soxhlet apparatus using petroleum ether and ash by combustion using Muffle furnace at 550°C. The following formulae was used to calculate the total energy: Energy (kcal) = 4× (g protein+ g carbohydrate) + 9× (g fat) (Sharif *et al*., 2017).

Statistical analysis

Statistical analysis was carried out using Jamovi 2.0 and data are expressed as mean ± standard deviation (SD). One-way analysis of variances (Welch's method) with subsequent *post-hoc* comparison with the help of the Games Howell test was used. P-value < 0.05 was considered statistically significant.

RESULTS

The effect of individual nutraceuticals on the lipid profile levels in hyperlipidemic rats

TC, TG, LDL-C, and VLDL-C levels were significantly increased ($p < 0.001$) in the HC rats except the levels of HDL-C when compared to the NC rats. The treatment with flaxseeds reduced TC, TG, LDL-C, and VLDL-C significantly ($p < 0.001$) and increased the HDL-C ($p < 0.05$) when compared to the HC rats. Similarly, treatment with barley reduced TC ($p < 0.05$), TG ($p < 0.001$), LDL-C ($p < 0.05$), and VLDL-C ($p < 0.001$), and improved the HDL-C ($p < 0.05$) when compared to the HC rats.

Treatment with Foxtail millet reduced TC ($p \le 0.05$), TG ($p < 0.05$), LDL-C ($p < 0.05$), and VLDL-C ($p < 0.05$) when compared to the HC rats. The rats receiving moong reduced TC ($p < 0.001$), TG ($p < 0.001$), LDL-C ($p < 0.01$), and VLDL-C ($p < 0.001$). Similarly, the rats receiving fish oil significantly reduced TC ($p \le 0.05$), TG ($p < 0.001$), VLDL-C ($p < 0.01$), and LDL-C ($p < 0.001$) when compared to the HC rats. There was no significance observed in the levels of HDL-C in rats receiving foxtail millet, moong, and fish oil. Flaxseeds and barley appeared to have an effect on each of the five components of the lipid profile, as indicated by the results shown in Figure 1).

The effect of nutraceutical combinations on the lipid profile levels in hyperlipidemic rats

Table 2 presents the levels of the serum lipid profiles in hyperlipidemic rats given the nutraceutical combination. Nutraceutical combinations 1, 2, and 4 were not effective in either improving the HDL-C or reducing the other lipid profile when compared to the HC rats. Rather, it was observed that nutraceutical combination 4 significantly increased TC ($p < 0.05$) and LDL-C ($p \le 0.05$). Nutraceutical combination 3, rich in barley, effectively reduced TC ($p < 0.01$), TG ($p < 0.001$), LDL-C ($p < 0.001$), and VLDL-C ($p < 0.001$), and improved the HDL-C ($p < 0.05$) when compared to the HC rats.

***In vitro* phytochemical analysis**

Table 3 presents the qualitative phytochemical analysis of the nutraceutical combination rich in barley. Phytoconstituents such as alkaloids, carbohydrates, proteins, steroids, and cardiac glycosides were present. However, tannins, saponins, phenols, and flavonoids were absent.

***In vitro* proximate composition analysis**

The proximate analysis revealed that the barley-rich nutraceutical combination is a rich source of carbohydrate (46.34 g), protein (10.87 g), ash (6.07 g), and fat (5.2 g). It contained a substantial amount of fiber (1.18 g), and the total energy value was calculated to be 275.64 kcal/100 g of dry matter (Table 4).

DISCUSSION

Hyperlipidemia is the key contributing factor to the development of CVD (Tietge, 2014) and it results from the abnormality of cholesterol homeostasis. Through 3-hydroxy-3-methylglutaryl-CoA reductase, the liver plays a vital role in cholesterol homeostasis (Li, 2014). CVD can be prevented by reducing serum lipids. The consumption of a healthy diet has improved serum lipid profiles, decreased bad cholesterol (LDL-C) levels, and increased good (HDL-C) cholesterol levels (Chen *et al.*, 2014). Hence, the present study investigated to develop a nutraceutical combination that can act as an adjuvant in pharmacotherapy to prevent hyperlipidemia.

In the present study, the hyperlipidemia in rats is manifested by intraperitoneally inducing Triton X-100 which through the process of emulsification increases intestinal lipid absorption, hepatic cholesterol synthesis, inhibits lipoprotein lipase, and blocks the extrahepatic tissues from absorbing lipoproteins from circulation, leading to an increase in blood lipid concentrations (Abdelgadir *et al.*, 2020).Thus, the level of lipid profile is significantly increased ($p < 0.001$) with a significant decrease in HDL-C ($p < 0.001$) in the HC rats when compared to the NC. Treatment with flaxseeds and barley significantly decreased TC, TG, LDL-C, and VLDL-C ($p < 0.001$) and increased the HDL-C ($p < 0.05$); this can be attributed to the presence of bioactive cardioprotective component α-linolenic acid (ALA) (57%), the essential omega-3 fatty acid and linoleic acid(LA) (16%), the essential omega-6 fatty acid in flaxseeds (Anurag *et al.*, 2020), and β-glucan in barley (Bacchetti *et al.*, 2015). Similarly, individual nutraceuticals, such as foxtail millet, moong, and fish oil, significantly reduced the lipid profile ($p < 0.05$) but they did not improve the HDL-C levels. The lipid lowering effect of foxtail millet is accredited to the improvement in insulin sensitivity and improvement in cholesterol metabolism by increasing the adiponectin concentration (Choi *et al.*, 2005). Moong, during the process of digestion, forms propionate, short-chain fatty acid involved in regulating the metabolic pathway and lowering TC and TG (Ganesan and Xu, 2018). Fish oil has a cardioprotective bioactive component, n-3 polyunsaturated fatty acid (Owen *et al.*, 2004).

The bioactive components present in the nutraceuticals mentioned above may also be present in other foods. However, they do not take into account the possibility of other compounds interacting and even interfering with their activity. Furthermore, people usually eat a wide variety of foods in different combinations, which can alter the activity of compounds of interest by interacting with the compounds in other foods (Santana-Gálvez *et al.*, 2019). Hence, new research of combining the nutraceuticals to target the multiple factors associated to metabolic syndrome is emerging (Efferth and Koch, 2011). In support, the current study focused on developing effective nutraceutical combinations. Among the four different nutraceutical combinations studied, the one rich in barley significantly reduced the lipid profile ($p < 0.001$) and improved the HDL-C ($p < 0.05$). The desired effect can occur due to the interactions between the nutraceuticals, and the type of interaction can be assessed by calculating the combination index (CI). CI determines the type of interaction. There is an additive effect if CI = 1, a synergistic effect if CI < 1, and an antagonistic effect if CI > 1. The CI for the mixtures of more than two nutraceuticals can be calculated using the formula:

$$(CI)n = \sum_{j=1}^{n} \frac{(C)j}{(Cy)j}$$

where (CI)n represents the CI for an n-nutraceutical combination, (C)j is the individual concentration of each component that produces effect y, and (Cy)j is the individual concentration of each component that produces effect y in the mixture (Santana-Gálvez *et al.*, 2019). The CI of the effective
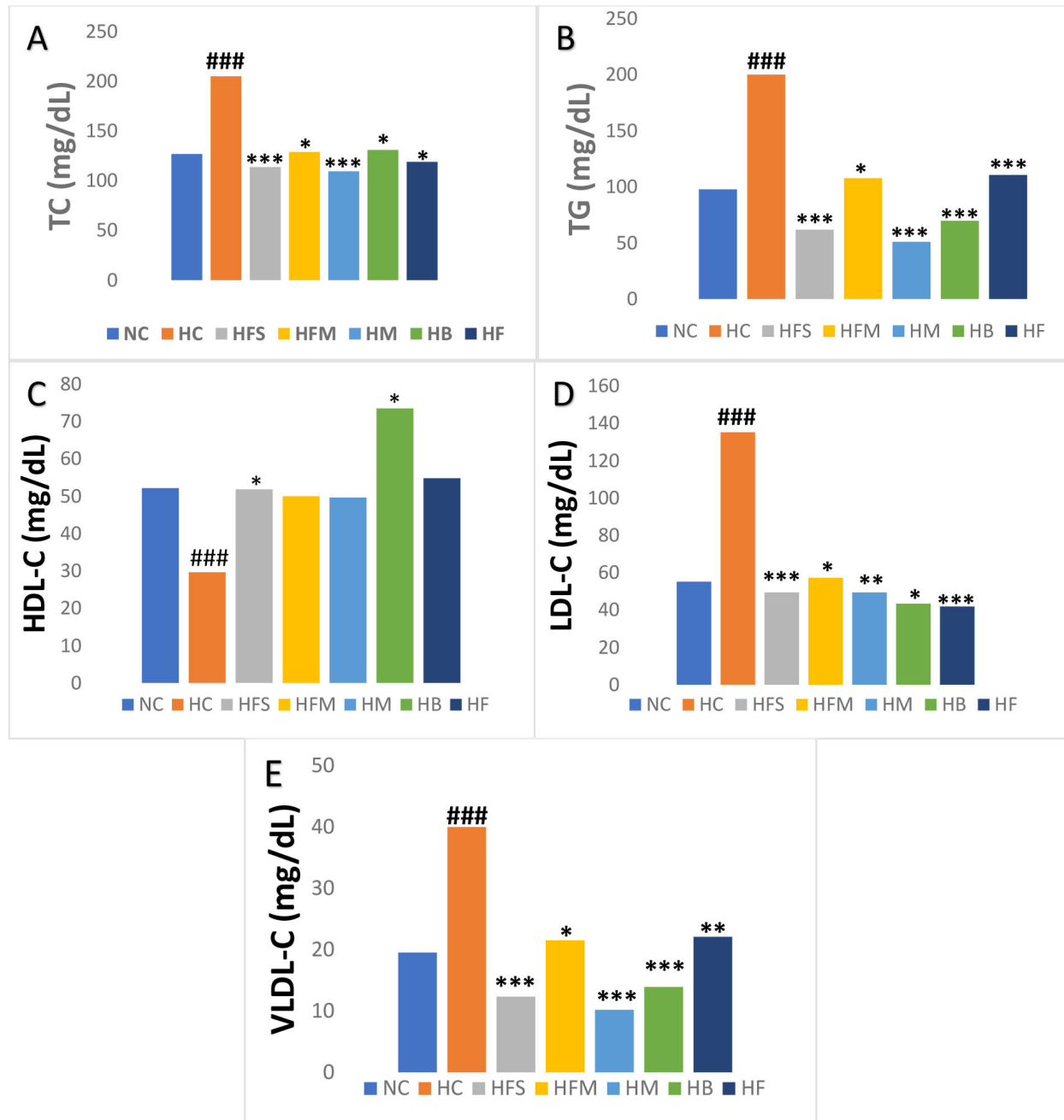
Figure 1. The effect of individual nutraceuticals on the lipid profile (mg/dl) levels in hyperlipidemic rats. The results are expressed as mean ± SD (*n* = 6). ###*p*-value < 0.001 versus NC; *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001 versus HC.Note: NC, Normal control given basal diet; HC, Hyperlipidemic controls given basal diet; HFS, Hyperlipidemic rats given flaxseeds; HFM, Hyperlipidemic rats given with foxtail millet; HM, Hyperlipidemic rats given moong; HB, Hyperlipidemic rats given barley; HFO Hyperlipidemic rats given fish oil.

barley-rich nutraceutical combination according to the above formulae is found to <1, which indicates the synergistic effect of the combination to prevent hyperlipidemia. Additionally, the barley-rich nutraceutical combination is found to be rich in β-glucan which may increase the viscosity of the gut content, increase excretion of bile acids and cholesterol in feces, and decrease the levels of TC, TG, and LDL-C with a moderate

increase in the levels of HDL-C (Dongowski *et al.*, 2002; Zeng *et al.*, 2020). Systemic HDL-C levels and CVD risk have an inverse relationship and the increase in HDL-C levels in the present study indicates the favorable effect of the nutraceutical combination in cholesterol efflux capacity with a strategy to counteract cholesterol accumulation (Adorni *et al.*, 2017; Nicholls and Nelson, 2019).

Table 2. Effect of the nutraceutical combinations on the lipid profile (mg/dl) levels in hyperlipidemic rats.

Groups	TC (mg/dl)	TG (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)	VLDL-C (mg/dl)
NC	126.83 ± 23.90	97.66 ± 15.27	52.16 ± 10.57	55.13 ± 19.44	19.53 ± 3.05
HC	204.83 ± 11.23[###]	200 ± 10.25[###]	29.66 ± 4.08[###]	135.16 ± 9.97[###]	40 ± 2.05[###]
HC1	183.33 ± 74.92	178.17 ± 68.86	65.67 ± 29.28	82.03 ± 75.82	35.63 ± 13.77
HC2	175.17 ± 63.57	130.83 ± 75.95	35.00 ± 16.30	114.00 ± 61.52	26.17 ± 15.19
HC3	119.83 ± 29.77**	90.50 ± 18.21***	58.33 ± 14.79*	43.40 ± 19.51***	18.10 ± 3.64***
HC4	300.00 ± 50.16*	211.00 ± 67.67	61.67 ± 23.49	196.13 ± 38.66*	42.20 ± 13.53

Note: NC, Normal control given basal diet; HC, Hyperlipidemic controls given basal diet; HC1, Hyperlipidemic rats given nutraceutical combination 1; HC2, Hyperlipidemic rats given nutraceutical combination 2; HC3, Hyperlipidemic rats given nutraceutical combination 3; and HC4, Hyperlipidemic rats given nutraceutical combination 4.
The results are expressed as mean ± SD ($n = 6$).
###p-value < 0.001 versus NC, *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001 versus HC.

Table 3. Qualitative analysis of the phytochemical constituents in the experimental diet.

Phytochemical constituents	Barley-richnutraceutical combination
Alkaloids	+
Carbohydrates	+
Proteins	+
Steroids	+
Cardiac glycosides	+
Tannins	−
Saponins	−
Phenols	−
Flavonoids	−

+ = Present, − = Absent.

Table 4. Proximate composition of experimental diet (unit: g/100 g).

Parameters	Value
Carbohydrate	46.34 ± 1.30
Protein	10.87 ± 0.2
Fat	5.20 ± 0.2
Fiber	1.18 ± 0.18
Moisture	30.05 ± 2.05
Ash	6.07 ± 0.8

The values are mean ± SD of triplicate experiments.

CONCLUSION

Nutraceuticals are naturally occurring compounds found in foods that can lower the incidence and mortality of CVDs by possessing both preventative and therapeutic properties. The study of nutraceutical blends is an emerging field since only a few nutraceuticals have been tested together of the thousands known. In the conclusion of this investigation, preliminary scientific data on the nutraceutical value (phytochemical and proximate composition) of the nutraceutical combination was presented, as well as its efficacy in reducing the lipid profile synergistically, with the exception that HDL-C levels increased, making it a possible therapeutic strategy to prevent primary hyperlipidemia. Furthermore, the effectiveness of the tested nutraceutical combination over a long term needs to be evaluated in future studies.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

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AUTHORS' CONTRIBUTIONS

Samreen M. Sheik and Revathi P. Shenoy significantly contributed to the concept and design; analysis and interpretation of data was done by Samreen M. Sheik, Mamatha B.S., Vani Lakshmi R., and Revathi P. Shenoy; draft was written by Samreen M. Sheik and critical revision of the manuscript was done by all the authors. Final approval was obtained for publication by all authors in the journal.

ETHICAL APPROVALS

The study was approved by the Institutional Animal Care and Use Committee of the Manipal Academy of Higher Education (approval no.: IAEC/KMC/66/2019).

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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