Plant Sterol-Enriched Low Fat Flavoured Milk Enhances The Attainment of Ldl-Cholesterol Goal In Hypercholesterolemic Subjects

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Abstract

The study was conducted to investigate the effect of phytosterols (PS) enriched flavoured milk on total and LDL (Low density lipoprotein) - cholesterol levels in subjects with hypercholesterolemia. Phytosterol is added at 3 different levels of concentration to enrich the low fat flavoured milk samples. The prepared samples were T1 (2 % PS), T2 (2.5 % PS), T3 (3 % PS) and control samples were also prepared and standardized. The developed products were analyzed at regular intervals for their physico chemical, nutritional, organoleptic properties and microbial count for the period of 1 month. The results were shown that the flavoured milk sample T1 (combination of toned milk, cocoa, sugar, stabilizer (500:4: 35:0.2) enriched with 2 % PS was more acceptable in terms of its sensory attributes. To evaluate the efficacy of phytosterol enriched flavoured milk (T1), twelve hypercholesterolemic human subjects were selected with cholesterol range of 192.0 - 221.8 mg/dl in the age group of 25-45 years. The selected subjects were received one 100 ml serving of either plain (control) low-fat or phytosterol enriched (2.0 g of free sterol equivalents) drinkable flavoured milk per day along with the main meal for 30 days. Enrichment of phytosterol in low fat flavoured milk to the experimental group, brought a significant ($p < 0.05$) reduction of total cholesterol(2.53 %) and LDL – cholesterol levels(2.62 %).Triglycerides and HDL – cholesterol reduced by 1.10 per cent and 1.22 per cent respectively but were not significant at 5 per cent level ($p > 0.05$). In the control group an insignificant increase in total cholesterol, LDL – cholesterol and decrease in HDL – cholesterol and decrease in triglycerides level were observed. The use of phytosterols as supplement can lower the total and LDL cholesterol levels, thereby reducing the risk of cardiovascular disease (CVD).

Keywords: phytosterols, flavoured milk, low density lipoprotein (ldl) - cholesterol

1. Introduction

Elevated LDL cholesterol is a major risk factor for cardiovascular disease. The serum LDL cholesterol level can be lowered through the reduction of cholesterol absorption or through synthesis. Plant sterols, which resemble cholesterol chemically, have been shown to inhibit the absorption of dietary and biliary cholesterol in the gut [6]. Plant sterols are minor constituents of vegetable oils (0.1–0.9 g/100 g) that have a chemical structure similar to that of cholesterol [17]. Normal dietary intake of plant sterols is ~160–360 mg/d, with a typical composition of 65% as β-sitosterol, 30% as campesterol and 5% as stigmasterol [13]. When consumed at levels 5–10 times higher than the normal intake, plant sterols have been shown to lower blood LDL cholesterol (LDL-C) levels. Phytosterols decrease cholesterol absorption rate, by displacing cholesterol from intestinal micelles and thus preventing cholesterol absorption. Phytosterols can enter the enterocyte, but are immediately released to the intestinal lumen through the ABCG5/G8 molecular system [18].The western diet contains about 150–400 mg of phytosterols and stanols per day [9]. By increasing this amount by threefold to fivefold, a clinically significant cholesterol-lowering effect can be observed. At least 1 g of phytosterols per day is necessary to obtain a significant 5–8% LDL- cholesterol reduction [15]. This effect is dosage-dependent until reaching a plateau of around 2 g per day [9]. Dietary phytosterol supplements are usually prescribed to moderately hypercholesterolemic patients; therefore it may be better to incorporate them in low-fat aliments along with low-fat, cholesterol poor and diets [8].

[14] observed that PSteE and non-esterified, free stanols, of which two-thirds were incorporated into low-fat foods including breakfast cereal and bread, lowered LDL-cholesterol by 13.6%. A recent study showed that consumption of a low-fat yoghurt (0.7 % total fat) providing 3 g/d of plant stanols in the form of plant stanol esters (PStaE) lowered LDL-cholesterol by 13.7% compared to the placebo group [12]. In addition, plant sterols incorporated into a low-fat yoghurt-based drink have also been shown to be effective in lowering cholesterol. A daily intake of 1 g of plant sterols resulted in a decrease in TC and LDL-cholesterol of 7% and 11% respectively; however when taking the reduction seen in the placebo group into account, the net effect was 4.4% for TC and 6.2 % for LDL-cholesterol [6]. As plant sterols or stanols, due to their lipophilic nature, have mostly been added to margarine and spreads, fewer studies have examined the

efficacy of plant sterols in low-fat or even non-fat, aqueous-type food carriers such as bread, cereals, yoghurt or milk [12]. As dietary guidelines recommend limiting total fat intake to around 30% of total energy intake, fat-based products may not always be the preferred choice for health-conscious consumers. In fact, consumer surveys have shown that the amount of PS-enriched spreads consumed even by frequent users is well below the required intake to achieve the desirable dose of 2 g/d of plant sterols for maximal LDL-cholesterol reduction [16].

Low-fat dairy products such as milk and yoghurt are healthy and acceptable foods for the consumer [16] Thus, incorporating plant sterols into low-fat dairy products would offer additional dietary options to maintain a healthy cholesterol concentration or to maximize the effectiveness of a cholesterol-lowering treatment. Hence with the view of promising health benefits of phytosterols leads to the development of functional foods, including components with cholesterol-lowering capacity, has increased the tools available to control cholesterol levels. Among them, the use of phytosterol-enriched aliments is widely accepted [18].

Therefore, the objective of this study is to assess the effects of phytosterol-enriched low-fat flavored milk supplementation to the hypercholesterolemia subjects for the attainment of LDL-cholesterol goal by assessing their blood samples using a method of CHOD-PAP, GPO-PAP, and PEG-CHOD-PAP methods [7].

2. Materials and methods

Materials and chemicals

For the present investigation, toned milk was purchased from Heritage Foods India Ltd (Hyderabad, India), Cocoa powder obtained from Morde Foods Private Ltd (Mumbai, India), stabilizer (CEKOL® cellulose gum 30000A) procured from Cpkelco A Huber Company(USA) and analysis kits for Triglycerides, cholesterol, and fluoride treated tubes were procured from Medsourse ozone Biomedicals Pvt.Ltd (Delhi, India). All other chemicals were purchased from Qualigens Fine Chemicals (Mumbai, India) or Molychem India Pvt. Ltd. (Mumbai, India). Microbiology media were obtained from Hi-Media Laboratories (Mumbai, India). Phytosterol powder was procured from Reducol™ original Powder (Forbes medi – Tech lnc) USA based company. Unless otherwise mentioned all chemicals used were of analytical grade.

Physico-chemical and nutritional characteristics

Total fat, SNF (Solid Not Fat), Specific gravity, Total solids and pH of developed low fat PS enriched flavoured milk samples were determined as per the AOAC methods [1]. Acidity was calculated by titrating against 0.1 N NaOH and expressed as percentage of lactic acid. Protein, Carbohydrates, total sugars, calcium and phosphorus were determined using approved AOAC methods [1].

Microbial analysis

Ten gram analytical unit of each food sample [low fat PS enriched flavoured milk] was homogenized with 90 mL of sterile Ringer's solution for 2 min and then 10 fold serial dilutions were prepared in sterile Ringer's solution [2].Briefly, individual serial decimal dilutions, starting with the prepared sample and each of the subsequent dilutions were prepared in 9 mL volume of sterile Ringer's solution up to 1×10^{-6} dilution, of the original food sample. Triplicate 1 mL inoculums of appropriate dilutions were pour plated, on the following media; for enumeration of total plate counts (TPC) on plate count agar and for enumeration of yeast and moulds on potato dextrose agar. The inoculated petri plates were incubated at 37°C for 48 h for TPC and at 25°C for 48 h for yeast and moulds, respectively. Colonies were counted and expressed as colony forming units (cfu) per gram. Standard enumeration procedures were followed [19].

Sensory analysis

The sensory assessments were conducted at the Post Graduate & Research Centre, Acharya N.G. Ranga Agricultural University, Hyderabad. A panel of 12 members consisting of staff and students of university evaluated the products. To ensure that there was no bias towards the products, it was ensured that the panelists chosen were naive to project objectives. The control was compared with the prepared low fat PS enriched flavoured milk samples were T1 (2 % PS), T2 (2.5 % PS), T3 (3 % PS). Prior to sensory evaluation the samples were chilled to 10ºC. Samples were coded using random three-digit numbers and served chilled. 25 ml of each sample was served, with the order of presentation counter balanced. Panelists were provided with a glass of water and, instructed to rinse their palate with water and drink water between samples. They were given written instructions and asked to rate the coded samples on color, sourness, flavor, sweetness and overall acceptability, using a nine-point hedonic scale [1=like extremely to 9 =dislike extremely] [3].

Experimental design and diets

Twelve modestly hypercholesterolemic men and women were recruited from the University health center were screened on the basis of the following selection criteria: aged between 25–45 years with fasting total cholesterol between 192.0 and 221.8 mg/dl. Subjects consuming plant sterol enriched flavoured milk samples were permissible provided they had at least a 3-week washout prior to study commencement. All

subjects gave their informed written consent before the start of the study. Participants were asked to maintain their usual dietary habits throughout the study. The selected subjects were received one 100mL serving of either plain (control) low-fat or phytosterol enriched (2.0 g of free sterol equivalents) drinkable flavoured milk per day along with the main meal for 30 days. Fasting blood samples were drawn from the subjects on the 0 day and after 30 days in the morning between 8am and 9am using disposable syringes with the help of a trained laboratory technician at the university (ANGRAU) health centre. 5mL of venous blood sample was collected into fluoride treated tubes. Serum was separated immediately by centrifuging at 2000 rpm for 10-15 min and transferred to plastic storage vial. The vials were immediately covered with aluminum foil. Lipid profile (Total cholesterol, TG, HDL and LDL) was determined in samples collected before and at the end of the experimental period in both the groups using CHOD-PAP, GPO-PAP, and PEG-CHOD-PAP methods [7].

Statistical analysis

Statistical analysis was carried out at the end of the study. The data was subjected to students paired‘t’ test, two-way analysis of variance (ANOVA), karl Pearson’s correlation coefficient and means were tested for significance by critical difference [5].

Development of low fat PS enriched flavoured milk

The milk (toned milk) was received and preheated to 60°C/1 minute and homogenized at 2500 psi at 55-60ºC/1 min and then clarified. To the warm milk, the desired amount of cocoa-mix, sugar and stabilizer were slowly added and stirred so as to dissolve them properly. The cocoa powder was added in the form of syrup, and the stabilizer in the form of solution. The mixture was then pasteurized at 71ºC/30 minutes, cooled rapidly to 5°C. Phytosterol powder was added (2g , 2.5g, 3g/100mL), homogenized at 2000 psi at room temperature per 2 minutes, then pasteurized at 71ºC/30 minutes and bottled by using glass bottles and kept under refrigeration (5ºC) until used. The received milk was homogenized to prevent or delay the rising of cream. It could be homogenized after addition of cocoa and sugar, but this increases sedimentation. Stabilizer was added to delay or prevent settling cocoa particles and also preventing cream rising.

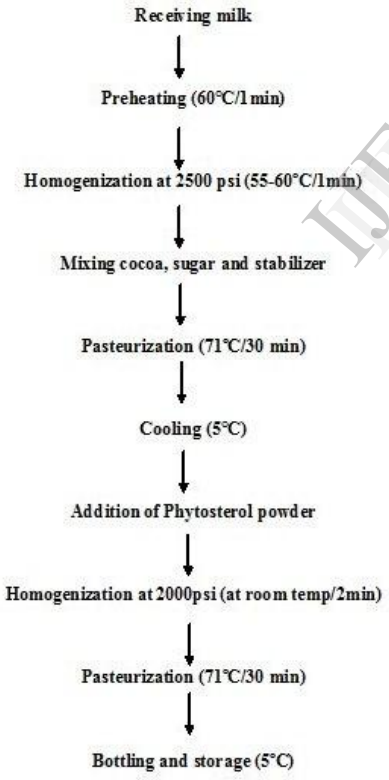

Figure 1. Methodology for the preparation of PS enriched low fat flavored milk

3. Results and discussion

Standardization of low fat phytosterol enriched flavored milk

Initial trials were conducted to optimize the formulations of low fat flavoured milk, after that phytosterols were added at different concentrations to enrich the flavored milk. The PS enriched low fat flavored milk was prepared by blending the ingredients at different level (Tab 1).

Testing acceptability of product by sensory evaluation

Organoleptic scores for different parameters of sensory evaluation are presented in (Tab 2). It is seen that as per the mean score obtained the experimental product T1 was rated higher than experimental products T2 and T3. The colour, taste, flavour and overall acceptability of the experimental product T1 did not differ much from the control. Both control and experimental product T1were rated better for texture, (4.0 $\pm$ 0.7). The experimental products T2 and T3 scored equal (3.7 $\pm$ 0.4) for overall acceptability**.** It was observed that for control product, the mean score for colour was 4.3 $\pm$ 0.8 whereas for experimental product (T1) it was 4.4 $\pm$ 0.8 and experimental products T2 and T3 scored similarly (3.6 $\pm$ 0.6) for colour. Score of 4.0 $\pm$ 0.6 was obtained for flavour for control and 4.2 $\pm$ 0.7 for experimental product (T1). The mean score of 4.1 $\pm$ 0.8 was obtained for control and 4.2 $\pm$ 0.7 for experimental (T1). Score of 4.4 $\pm$ 0.6 was obtained for overall acceptability for control and 4.5 $\pm$ 0.6 for experimental (T1) product. When compared to T1 (2g/100mL), phytosterol powder per cent was high in experimental products T2 (2.5g/100mL) and T2 (3g/100mL). As a result all the variables scored lower than the control and T1. The overall acceptability scores was observed in which T1 (4.5) scored higher followed by control (4.4) and lowest score was observed for T2 and T3 (3.7%). Organoleptic evaluation showed that of experimental product T1 was better than other samples. After product development and acceptability studies, it was observed that the product T1 (combination of toned milk, cocoa, sugar, stabilizer (500:4: 35:0.2) and phytosterol powder (2g/100mL)) was found to be superior in all aspects. Hence T1 product was selected for further study.

Physico-chemical and nutritional analysis of the products

Physico- chemical and nutritional composition of the phytosterol enriched flavoured milk (T1) and control samples are presented in (Tab 3).

(Tab 3) indicates that the fat per cent of experimental product was 3.0 and that of control was found to be 3.1. SNF (solid not fat) per cent of the experimental product was found to be 12.0 % and control was 12.4%. Specific gravity of the experimental product was 1.043% and control was 1.044%.Total solids of the experimental product was 14.52% and control was 15.50%. Acidity of the experimental product was found to be 0.1865% of lactic acid and control was 0.1896% of lactic acid. pH of the experimental product was found to be 6.6 and control was 6.5. There was a slight variation between the experimental and control values of Fat, SNF, Total solids, Specific gravity, Acidity and pH. Protein, calcium, Phosphorus, carbohydrate and sugars content of control and experimental samples are almost similar**.** Physico-chemical and nutritional parameters of the experimental product did not differ much from the control.

Table 1**.** Composition of different flavoured milk prepared**.**

Ingredients used	Flavoured milk			
	T1	T2	T3	Control
Toned milk	500mL	500mL	500mL	500mL
Cocoa powder	4g	5g	6g	4g
Sugar	35g	45g	55g	35g
Stabilizer	0.2g	0.3g	0.2g	0.2g
Phytosterol powder	2g/100mL	2.5g/100mL	3g/100mL	-

Table 2. Mean scores obtained for control and experimental products.

S.NO.	Variables	Products			
		Control	T1	T2	T3
1	Colour/appearance	4.3±0.8	4.4±0.8	3.6±0.6	3.6±0.6
2	Flavour	4.0±0.6	4.2±0.7	4.0±0.5	3.9±0.7
3	Taste	4.1±0.8	4.2±0.7	4.0±0.5	3.8±0.7
4	Texture/consistency	4.0±0.7	4.0±0.7	3.5±0.5	3.4±0.5
5	Overall acceptability	4.4±0.6	4.5±0.6	3.7±0.4	3.7±0.4

Note: Values are expressed as mean ± SD. **Control:** Flavoured milk with out phytosterols, **T1:** Phytosterols (2g) enriched flavoured milk, **T2:** Phytosterols (2.5g) enriched flavoured milk, **T3:** Phytosterols (2g) enriched flavoured milk.

Table 3**.** Physico-chemical and nutritional parameters of experimental and control products (per 100g sample).

Parameter	Samples	
	T1	Control
Fat (g)	3.0±0.04	3.1±0.02
SNF (g)	12.0±0.02	12.4±0.07
Specific gravity	1.043±0.08	1.044±0.09
Total solids (g)	14.52±0.03	15.50±0.05
Acidity (% of lactic acid)	0.18±0.04	0.18±0.07
pH	6.6±0.06	6.5±0.04
Protein (g)	2.98±0.01	2.96±0.06
Calcium (mg)	137.37±0.02	137.47±0.03
Phosphorus (mg)	123.54±0.09	123.34±0.02
Carbohydrates (g)	4.60±0.03	4.64±0.01
Sugars (g)	6.32±0.04	6.37±0.09

Effect of supplementation of phytosterols on lipid profile (Hypocholesterolemic Effect):

The effect of supplementation of phytosterols on hypercholesterolemia was assessed in human subjects for a period of thirty days and the mean total cholesterol values of the study are given in (Tab 4). Supplementation of phytosterol enriched flavoured milk to the experimental group, (Tab 4) brought a significant ($p < 0.05$) reduction of 2.53 % in total cholesterol and 2.62 % in LDL – cholesterol levels. Triglycerides and HDL-cholesterol were reduced by 1.10 per cent, 1.22 per cent respectively and were not significant at 5 per cent level ($p > 0.05$). The similar results were also reported by[16], wherein daily consumption of plant sterol-enriched milk (2.0 g/day) significantly decreased total, and LDL cholesterol compared with plain milk and was therefore an interesting and convenient aid in managing mild to moderate hypercholesterolemia. Significant changes were not observed in HDL-cholesterol and triglycerides.

Table 4.Effect on lipid profile of the control and experimental subjects (mg/dL).

Parameter	Control group		Experimental group	
	Initial	Final	Initial	Final
Cholesterol	214.59 ±13.77	215.8 ^{NS}±16.00	222.29±7.32	216.66* ±6.21
Triglycerides	187.89 ±28.00	186.46*±28.06	190.05±4.02	187.95^{NS} ±4.18
LDL-C	132.1 ±8.62	133.94 ^{NS}±8.68	136.82±5.09	133.23* ±6.14
HDL-C	44.90 ±8.37	44.44 ^{NS} ±7.20	47.33 ±6.03	46.75^{NS} ±8.45

* Significance at $P < 0.05$ NS – not significant at 5 per cent level.

In this study also, experimental group, Phytosterol did not affect upon the HDL-C and triglyceride levels. A meta-analysis by [10] showed that a dose of ≥2g/d of plant sterols or stanols lead to an average reduction in serum LDL-cholesterol (0.33 to 0.54 mmol/L or 9 to 14%) depending on the age of the participants studied. Another meta-analysis of 41 trails confirmed these findings and concluded that 2g/d of plant sterols or stanols lowered LDL- cholesterol by 10% [9]. In the control group an increase in total cholesterol, LDL – cholesterol and decrease in HDL – cholesterol was not significant at 5 percent level. However, decrease in triglycerides was significant at 5 per cent level.

Similar study reported that the consumption of plant sterols decreased plasma concentrations of cholesterol without adverse effects in human subjects, in a randomized blind study with healthy subjects and hypercholesterolemic patients, both groups being treated with phytosterol enriched milk (2g/d). In another study hyperchelesterolemic group was used as a control group. After 15days of supplementation, healthy individuals showed lowered total cholesterol and LDL-c levels, by 8.31% ($p = 0.05$) and 11% ($p < 0.05$), respectively. After 30 days of the trial, these values did not change significantly. Hypercholesterolemic patients treated with phytosterol enriched milk demonstrated significantly diminished levels of total cholesterol and LDL-C concentrations, by 9.62% ($p < 0.05$) and 12.20% ($p < 0.05$), respectively [4]**.**

The results obtained in this study indicate that the phytosterol enriched flavoured milk is a good vehicle for reducing plasma cholesterol in hypercholesterolemic subjects. Interparameter correlations showed a significant ($p< 0.05$) positive correlation between serum cholesterol, LDL-cholesterol, triglycerides and

HDL- cholesterol.It can be inferred that the inclusion of phytosterols powder in the diets of hyperlipidemic subjects could have therapeutic effect.

4. Conclusion

Plant sterols have gained a prominent position in strategies to lower CVD risk because of their serum LDL-Cholesterol lowering effects. Our results are fully consistent with these observations. Interestingly, we have found that plant sterols also lowered serum triglyceride concentrations and slightly decreased the HDL cholesterol levels in the experimental subjects of the study. It is evident from the foregoing discussion that a phytosterol- enriched flavoured milk can be used effectively to reduce plasma total and LDL cholesterol levels in hypercholesterolemic individuals. The data suggest that the incorporation of phytosterol enriched flavoured milk into a balanced diet represents a practical dietary strategy in the management of serum cholesterol levels.

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