Lipid-lowering Activity of Natural and Semi-Synthetic Sterols and Stanols

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ABSTRACT - Consumption of plant sterols/ stanols has long been demonstrated to reduce plasma cholesterol levels. The objective of this review is to demonstrate the lipid-lowering activity and anti-atherogenic effects of natural and semi-synthetic plant sterols/ stanols based on evidence from cell-culture studies, animal studies and clinical trials. Additionally, this review highlights certain molecular mechanisms by which plant sterols/ stanols lower plasma cholesterol levels with a special emphasis on factors that affect the cholesterol-lowering activity of plant sterols/stanols. The crystalline nature and the poor oil solubility of these natural products could be important factors that limit their cholesterol-lowering efficiency. Several attempts have been made to improve the cholesterol-lowering activity by enhancing the bioavailability of crystalline sterols and stanols. Approaches involved reduction of the crystal size and/or esterification with fatty acids from vegetable or fish oils. However, the most promising approach in this context is the chemical modification of plant sterols/ stanols into water soluble disodium ascorbyl phytostanyl phosphates analogue by esterification with ascorbic acid. This novel semi-synthetic stanol derivative has improved efficacy over natural plant sterols/ stanols and can provide additional benefits by combining the cholesterol-lowering properties of plant stanols with the antioxidant potential of ascorbic acid.

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INTRODUCTION

Phytosterols are integral components of plant cell membranes. They are structurally similar to cholesterol with some differences in the D-ring side chain (1-3). The term phytosterols is widely used to describe both plant sterols, which have a double bond at position 5 of the sterol ring, and plant stanols in which the double bond is saturated (4, 5) (Figure 1). Phytosterols are extracted from vegetable oils such as corn oil, rapeseed oil, soybean oil, sunflower oil, olive oil and tall oil either in free forms or as fatty acid esters (6). They are also present to a lower extent in nuts, seeds, fruits and vegetables (7). The most abundant plant sterols are sitosterol, campesterol and stigmasterol. They constitute approximately 65, 30, and 3% of dietary sterol intakes, respectively (3, 8). Stanols, on the other hand, are less abundant in nature (8, 9) and can be produced commercially by hydrogenation of plant sterols (10). Sitostanol and campestanol are the most common plant stanols. The dietary intake of plant sterols ranges from 150 to 450 mg/day and can be as high as 600 mg/day in vegetarians (11). In contrast, the dietary intake of plant stanols is usually only about 50 mg/day (9, 12). Plant sterols are poorly absorbed from gastrointestinal tract (0.4-5%) and the absorption of plant stanols is even lower (0.02-0.3%) (13, 14).

Due to the chemical similarity with cholesterol, plant sterols block cholesterol absorption. Therefore, these compounds are of interest as therapeutic agents to reduce plasma cholesterol. However, the use of plant sterols/stanols as supplements or ingredients for functional foods is restricted by their crystalline nature, low solubility in fats, insolubility in water, high melting point of 140–150 °C, in addition to poor palatability (15-18). Several attempts have been made to enhance the bioavailability of crystalline sterols by reducing the crystal size or by the use of microcrystalline suspensions (18, 19). Other approaches involve modification of phytosterols by esterification with fatty acids from vegetable (20, 21) or fish oils (22, 23), which leads to synthesis of semisynthetic sterols. Such modification can provide an easy way of introducing the daily amount of phytosterols required for optimal reduction of cholesterol (17).
Esterification not only changes the physical properties, but also can improve the efficacy of plant sterols since it allows greater solubilisation within food products and optimal dispersion within mixed micelles during intestinal absorption of lipids (17, 24). The first commercial application of fatty acid esterification of plant stanols was in the form of margarine (Benecol®) in 1995 in Finland by Raisio Benecol Ltd (12, 17). These compounds have been approved as food additives in the US and Canada since then, and are now found in products as diverse as juice, spreads, dressings, margarines and dietary supplements in capsule or tablet form (Table 1).

The objective of this review is to demonstrate the lipid-lowering activity and anti-atherogenic effects of natural and semi-synthetic plant sterols/stanols based on evidence from cell-culture studies, animal studies and clinical trials. Additionally, this review highlights molecular mechanisms by which plant sterols/stanols lower plasma cholesterol levels with a special emphasis on factors that affect the cholesterol-lowering activity of plant sterols/stanols.

**Cholesterol-lowering effect of phytosterols**

The cholesterol-lowering effects of phytosterols was demonstrated for the first time in chicks in the early 1950s by Peterson and co-workers (25-27). This initial observation was soon confirmed in humans by Pollak (28) who reported a significant reduction in plasma total cholesterol levels of about 28% in 26 healthy subjects fed plant sterols at a dose ranging from 5.7-10 g/day for 2 weeks. Thereafter, a series of experiments have been conducted on a number of animals including rabbits (28), mice (29), rats (30, 31) and hamsters (32). Results from these studies have shown that plant sterols and stanols significantly reduce total plasma cholesterol, LDL-cholesterol (LDL-C) and liver cholesterol concentrations. In most of the early studies, plant sterols were used in crystalline, un-esterified (or free) form, which is not well absorbed following oral administration, and therefore doses in excess of 5 g/day were necessary to achieve satisfactory cholesterol-lowering effects (18). However, more recently, the use of phytosterol-enriched foods or supplements allowed dose reduction as a result of enhanced bioavailability. Several randomized placebo-controlled trials have been carried out to evaluate the effect of various phytosterol containing products on serum lipid and lipoprotein concentrations (Table 2). Recent clinical trials have suggested that the LDL-C lowering effects of phytosterols can be achieved with 2-3 g/day, while higher intakes produce little additional benefit (Figure 2) (9, 33, 34).

![Figure 1. Chemical structures of plant sterols/ stanols.](image-url)
| Product's brand name | Manufacturer | Products | Type of phytosterols | Amount of phytosterol per serving |
|----------------------|--------------|----------|----------------------|---------------------------------|
| Benecol® Raisio plc. | Yogurt drinks Dairy Free Fruit & Soya Drinks Spreads | Tall oil and vegetable oil stanol esters | 0.8g/ pot (120 g) 2 g/ bottle (67.5 g) 2 g/ bottle (65.5 g) 0.8 g/ tablespoon |
| Flora Pro.Activ® Unilever | Skimmed milk drink Spreads Mini-drink | Vegetable oil sterol esters | 0.75 g/ 250 mL 1.25 g/ 2 teaspoons (10 g) 2 g/ bottle (100 g) |
| Promise Activ™ (Formerly Take Control®) Unilever | Mini-drink Spreads | Unprocessed sterols, primarily sitosterol from soybean oil | 2 g/ 3ounce serving 1.7 g/ tablespoon |
| Lifetime® Lifeline Food Co. | Cheese | Vegetable sterol esters | 0.65 g/ portion (28g) |
| CocoaVia® Mars Inc. | Chocolates | Canola oil sterol esters | 1.1 g/ bar |
| Yoplait Healthy Heart Yogurt™ General mills | Yogurt | Vegetable oil sterol esters | 0.4 g/ 6 ounce, single-serving carton. |
| Premium Heart Wise® Minute Maid | Orange Juice | Cargill CoroWise® plant sterols | 1 g/ serving (240 mL) |
| Smart Balance® HeartRight Smart Balance Inc. | Fat free milk | Cargill CoroWise® plant sterols | 0.4 g/ cup (240 mL) |
| Reducol™ Forbes Medi-Tech Inc. | Phytosterol powder for incorporated into various food products including margarine, cheese, milk, yoghurt drinks and cooking oil | Plant sterols and stanols | NA |
| Listrin™ Plant Sterols Innovia Nutraceuticals | Tablets | β-sitosterol β-sitostanol | 225 mg of β-sitosterol and 79 mg of β-sitostanol/ tablet |
| Ultra™ Plant Sterols Vitabiotics | Tablets | β-sitosterols | 450 mg/ tablet |
| Heart health plant sterols Boots pharmaceuticals | Capsules | Plant sterol esters | 650 mg/ capsule |

NA: not applicable.
Several meta-analyses have been performed to evaluate the cholesterol-lowering effects of plant sterols and stanols (50). A meta-analysis of 41 clinical trials have shown that daily intake of 2 g/d of plant sterols/stanols reduced the LDL-C by 10% with little additional reduction at higher doses (9). Another meta-analysis of 84 clinical trials has reported an 8.8% reduction in serum LDL-C with a daily phytosterol intake of 2.15 g with no statistically significant differences were reported between plant sterols, stanols, or their esterified derivatives (34). Most recent meta-analysis have shown an average LDL-C lowering effect of 12% with a daily intake of 3 g (33). No alterations in serum HDL-cholesterol (HDL-C) concentrations have been reported in most animal and human studies (24, 51-53). However, in subjects with familial hypercholesterolemia (54) and type 2 diabetic patients (55), plant sterol consumption was associated with an increase in serum HDL-C levels.

Mechanisms of cholesterol-lowering effect of plant sterols and stanols
The precise mechanisms of action by which plant sterols/stanols decrease plasma cholesterol levels remain obscure. Figure 3 summarizes the different mechanisms of action proposed for the cholesterol-lowering effects of plant sterols and stanols.

**Competition with intestinal cholesterol for incorporation into mixed micelles**
In vitro studies have demonstrated that plant sterols/stanols reduce cholesterol solubility in bile salt micelles in the presence or absence of oleic acid and monoolein (12, 57-62). Phytosterols are more hydrophobic than cholesterol and have higher affinity to mixed micelles than cholesterol (1, 13). Ikeda and colleagues have shown that the presence of sitosterol at equimolar amounts with cholesterol in a binary mixture reduces the micellar solubility of cholesterol (63). Another study by Slota and co-workers demonstrated that the addition of β-sitosteryl to the mixed micelles mixture consisting of 10 mM taurocholate and 6 mM olate reduced cholesterol solubility to a greater extent than would be expected from an equimolar replacement of cholesterol by β-sitosterol (Figure 4) (59).
Evidence from in vivo studies in rats have indicated that intragastric administration of equimolar amounts of cholesterol and sitosterol as a single emulsified lipid meal significantly inhibits lymphatic absorption of cholesterol by 57% in 24 hr (Figure 5). However, when equimolar amounts of cholesterol and sitosterol were incorporated together in phospholipid bile-salt micelles, no inhibition of lymphatic cholesterol absorption was reported following administration. The simultaneous presence of both cholesterol and sitosterols in the mixed micelles before administration could abolish the ability of sitosterol to compete with cholesterol for incorporation into micelles (63).
**Figure 4.** Solubility of cholesterol and \( \beta \)-sitosterol in mixed micellar solutions containing 10 mM taurocholate and 6 mM oleate. \( \beta \)-sitosterol reduces the solubility of cholesterol below concentration that would be expected by an equimolar replacement of cholesterol by \( \beta \)-sitosterol. Adapted from Slota et al. (59) with the permission of BMJ Publishing Group Ltd.

**Figure 5.** Inhibitory effect of sitosterol consumption on exogenous cholesterol absorption in lymph fistula rats. Animals were administered intragastric emulsion containing 25 mg [\( ^3\)H] cholesterol alone (C) or plus 25 mg of sitosterol (C+S). Cholesterol radioactivity was measured following the collection of lymph at different time points over 24 hr. Data were expressed as mean ± SE for 6 replicates. * Significant difference of C vs. C+S, p<0.05. Adapted from Ikeda et al. (63) with the permission of the American Society for Biochemistry and Molecular Biology.
Effects of plant sterols and stanols on cholesterol trafficking and metabolism

Several in vitro and in vivo studies have suggested that plant sterols/stanols may exert their lipid-lowering effects by mechanisms other than inhibition of cholesterol incorporation into mixed micelles (64-73). Some researchers have demonstrated a significant reduction in plasma cholesterol levels in chicks (71) and hamsters (70) following subcutaneous administration of low doses of soya sterols. Therefore, one can assume that the reduction of plasma cholesterol levels might be not only due to the inhibition of intestinal cholesterol absorption, but also to other factors affecting cholesterol metabolism.

A few studies have suggested that plant sterols / stanols may competitively inhibit cholesterol uptake by brush boarder membrane of intestinal mucosal cells (63). It has been demonstrated that both cholesterol and sitosterol can bind to rat brush border membranes in vivo (74), rat brush border membranes of jejunal loops in situ (75), isolated rat brush border membranes (63, 74) or isolated jejunal villus cells (76). Although the affinity of sitosterol to brush border membranes in each case was lower than that of cholesterol, high doses of sitosterol have been suggested to inhibit cholesterol binding (63). Recently, it has been shown that the uptake of cholesterol from intestinal lumen into the enterocytes is mediated by specific interactions with the luminal N-terminal domain (NTD) of the NPC1L1 protein (77). While the luminal NTD specifically binds cholesterol and mediates their endocytosis, plant sterols have not been shown to interact with this terminal domain and therefore competition with dietary cholesterol for intestinal absorption is an unlikely mechanism of plant sterols/ stanols in lowering plasma cholesterol levels (63, 77, 78). In line with this, some studies have demonstrated that plant sterols reduce plasma cholesterol without affecting intestinal Niemann-Pick C1-like 1 protein gene expression (79-82).

Other suggested mechanisms involve local activation of nuclear liver-X receptor (LXR) resulting in induction of expression of a number of target genes (73, 83). Among those genes, the ATP binding cassette (ABCA1 and ABCG5/ABCG8) efflux transporter genes have been shown to be involved in intestinal cholesterol excretion (64, 73, 84). Increased expression of ABCA1 have been reported in vitro in Caco-2 cells following incubation with mixed micelles enriched with sitostanol or cholesterol plus sitostanol (64). Furthermore, feeding mice plant sterols has been shown to reduce fractional cholesterol absorption and to stimulate fecal cholesterol excretion via a non-biliary route. These effects were partially mediated by the Abcg5/Abcg8 heterodimer (84). However, some studies have suggested that plant sterols reduce intestinal cholesterol absorption and increase fecal neutral sterol excretion without inducing intestinal LXR target gene expressions (79, 81). These findings suggest that modulation of LXR gene expression cannot be considered as the main mechanism by which plant sterols reduce intestinal cholesterol absorption.

There is also some evidence that plant sterols/ stanols interfere with the cholesterol esterification process inside the enterocytes. This process is necessary for the incorporation of cholesterol into chylomicrons and is mediated by the activity of the enzyme acyl coenzyme A: cholesterol acyltransferase (ACAT). A significant drop in the activity of this enzyme has been reported in the jejunum and ileum of rabbits fed with β-sitosterol (65). In addition, down-regulation of ACAT has been demonstrated in hamsters that were fed high-fat diet enriched with β-sitosterol and stigmasterol (69). However, in an in vitro study, incubation of Caco-2 cells with micelles containing cholesterol and β-sitosterol has not been shown to alter the activity of ACAT enzyme (66). Instead, there was a decrease in the uptake of micellar cholesterol resulting in an attenuation of the influx of plasma membrane cholesterol and an inhibition of secretion of cholesteryl esters derived from the plasma membrane. Furthermore, β-sitosterol decreased cholesterol synthesis and HMG-CoA reductase activity despite the reduction in intracellular cholesterol concentration (66). In line with this, Δ^{22}-unsaturated phytosterols (stigmasterol, brassicasterol and ergosterol) have inhibited cholesterol synthesis by competitive inhibition of sterol Δ^{24}-reductase (an enzyme which catalyses the saturation of the double bond at C-24 during cholesterol biosynthesis) in mammalian cells (67). Another study have shown that stigmasterol (0.5% w/w) feeding of Wistar and WKY rats over 6 weeks resulted in significant reduction in HMG-CoA reductase activity by 44% and 77%, respectively (85).

Plant sterols/ stanols could also affect the expression of microsomal triacylglycerol transfer protein (MTP) which is involved in the assembly of very low density lipoprotein and chylomicrons (13). A study by Liang et al. (69) have shown that the cholesterol-lowering activity of sitosterol was associated with a decrease in the mRNA level of MTP in male golden Syrian hamsters.
Factors affecting the cholesterol-lowering activity of plant sterols and stanols:
Several factors have been shown to influence the hypcholesterolemic effects of plant sterols and stanols. In general, the LDL-C lowering effects of plant sterols are greater among subjects with higher baseline cholesterol levels compared to those with normal or borderline levels (50, 51, 86). Moreover, a strong correlation was reported between the cholesterol-lowering effects of plant sterols and age. More efficient reduction of LDL-C levels was observed in older than in younger subjects. However, when the LDL-C lowering effect of plant sterols were corrected for the baseline LDL-C levels, the differences between various age groups were non-significant (9, 10).

The time of intake of plant sterols and stanols is also another important factor affecting cholesterol-lowering activity. It has been suggested that plant sterols should be present simultaneously with cholesterol in the intestinal lumen for the maximum effectiveness (87). In general, plant sterols/ stanols are to be administered with or immediately after the main meal for the optimal cholesterol-lowering activity (78). However, when taken before breakfast, no significant reduction in serum LDL-C concentrations was reported (86). In line with this, Doornbos and co-workers observed a significantly higher reduction in LDL-C concentrations in subjects taking sterol-enriched yoghurt drink with or immediately after lunch, compared to those taking the drink before breakfast (88).

Incorporation of plant sterols into different food matrices or pharmaceutical formulations has also been suggested to alter the cholesterol-lowering capacity of plant sterols and stanols (48, 89). The physical state of the administered plant sterols is also a crucial factor affecting the partitioning of plant sterols and cholesterol over different phases in the intestinal contents (78, 87). A study by Clifton et al. (89) has demonstrated that plant sterol added to low-fat milk is almost three times more effective in lowering cholesterol levels than sterol-enriched bread and cereal products. Similarly, sitostanol administration in lecithin micelles at a dose of 700 mg has been shown to reduce cholesterol absorption more efficiently than 1 g sitostanol in crystalline powder form (90). On the other hand, phytosterol esters intake as capsules have been shown to be ineffective in reducing plasma LDL-C levels in subjects with mild to moderate hypercholesterolemia (48). In another study, however, the relative LDL-C lowering efficacy of plant sterols/ stanols provided as supplements (tablets/capsules) have not been shown to differ from that of phytosterols-enriched dietary products (16). Moreover, some clinical trials have demonstrated that the cholesterol-lowering efficacy of esterified sterols and stanols is independent of the food matrix (18, 39).

Other factors include whether the plant sterols/ stanols are taken as their free forms or as fatty acid esters. In this context, an early study by Mattson and co-workers has suggested that the esterified form of plant sterols, although has the advantage of being readily incorporated into dietary fat, is less effective in reducing intestinal cholesterol absorption compared with the free form (87). Another study in a thoracic duct cannulated rat model has shown that intra-gastric administration of un-esterified plant sterols was associated with significant reduction in lymphatic recovery of radio labeled cholesterol compared with administration of sterol ester (Figure 6) (91).

An in vitro study has shown that intact plant sterol esters and their simulated hydrolysis products differentially affect the solubility of cholesterol in mixed bile-salt/phosphatidylcholine micelles. While free sterols and fatty acids reduce cholesterol solubility in a dose dependent manner, intact plant sterol esters do not incorporate into mixed micelles nor do they alter cholesterol solubility (57). The free plant sterols can displace cholesterol from the micellar phase, while the major part of the esterified forms accumulate in the oil phase (92, 93). Therefore, plant sterol esters need to be hydrolysed first to non-esterified forms in the intestinal lumen before they can be incorporated into mixed micelles. The hydrolysis is catalysed by the enzyme pancreatic cholesterol esterase and is thought to be a rate limiting step for cholesterol absorption inhibition (91).

Contrary to these findings, a number of studies have reported a comparable cholesterol-lowering efficacy of esterified and un-esterified plant sterols in humans (9, 94) and hamsters (95). Such discrepancies could be explained by the rapid rate of hydrolysis of plant sterol esters in human intestinal lumen (92). Furthermore, un-hydrolysed plant sterol esters may affect cholesterol absorption by creating a lipid phase in which cholesterol becomes trapped (96). On the other hand, Nissinen and colleagues has shown that the free plant sterols could become partially esterified during the duodenal transit resulting in partial loss of their capacity to inhibit intestinal cholesterol absorption. Although, the authors were not able to provide clear explanation to the esterification of free plant sterols, reversed action.
of pancreatic cholesterol esterase has been suspected (92).

The fatty acid moiety of phytosterols has been suggested to influence their cholesterol-lowering efficacies (96, 97). A study in hamsters has shown that consumption of phytosterol esters enriched with stearic acid, compared to linoleic acid, significantly reduced cholesterol absorption and total plasma and hepatic cholesterol concentrations (98). The enhanced efficacy of phytosterol stearate ester has been attributed to the ability of stearic acid itself to reduce intestinal cholesterol absorption and increase fecal neutral sterol excretion secondary to alteration of bile acid composition, resulting in lower plasma and liver cholesterol concentrations (99, 100). Another study in hyperlipidemic men and women has shown that combined supplementation of phytosterols with long-chain polyunsaturated fatty acids synergistically lowered plasma LDL-C and triglyceride concentrations with an improvement in HDL-C levels (101). Similarly, fish oil esters of plant sterols have demonstrated marked reduction in fasting and postprandial plasma triacylglycerol concentrations, in hyperlipidemic subjects, compared to sunflower oil esters of plant sterols (23).

Several researchers have claimed that plant stanols are more effective in reducing circulating cholesterol levels than plant sterols (102-109). This claim was supported by the finding that stanols reduce the in vivo micellar solubility of cholesterol more efficiently than sterols (62). The superiority of plant stanols over sterols in reducing cholesterol absorption and serum LDL-C levels has been reported in a number of animal studies (102, 109, 110) and in humans (105, 111, 112). In one study in humans, intestinal infusion of sitostanol was found to be more effective in reducing cholesterol absorption by 85% compared with 50% reduction in the case of sitosterol (105). In another study, sitostanol supplementation in children with severe familial hypercholesterolemia at a dose four-fold lower than that of sitosterol has been shown to be more effective in reducing elevated levels of LDL-C than sitosterol. The higher LDL-C lowering effects of sitostanol were associated with higher fecal neutral sterol excretion than sitosterol (111).

On the other hand, comparable cholesterol-lowering activity and anti-atherogenic effects of plant sterols and stanols were reported by Pritchard et al. (113) in Apolipoprotein E (Apo E)-deficient mice over a 14 week experimental course.

Figure 6. Effect of un-esterified plant sterols and plant sterol oleates on lymphatic recovery of radiolabeled cholesterol in thoracic duct cannulated rat model. Data are expressed as mean ± SE of 6-7 rats. Filled circle = 10 mg radiolabeled cholesterol, filled square = 10 mg cholesterol +10 mg un-esterified plant sterol, filled triangle= 10 mg radiolabeled cholesterol + 16.5 mg plant sterol oleate (equivalent to 10 mg plant sterol). a= significant difference of un-esterified sterols vs. sterol oleate at p<0.05; b= significant difference of un-esterified sterol vs. control and sterol oleate at p<0.05; c= significant difference between un-esterified sterols, sterol oleate and control at p<0.05. Adapted from Kobayashi et al. (91) with permission from J-STAGE.
Results from meta-analyses comparing the LDL-C lowering efficacy of plant stanols and sterols are conflicting (9, 114, 115). In an early meta-analysis, the pooled LDL-C reduction for plant stanols (10.1%; mean dose, 2.5 g/day) was not significantly different from that for plant sterols (9.7%; mean dose, 2.3 g/day) (9). In another meta-analysis, the efficacy of plant stanols and plant sterols was compared head-to-head at doses ranging from 0.6 to 2.5 g/day in healthy subjects and patients with hypercholesterolemia. Again, no statistically or clinically significant difference were reported between plant sterols and plant stanols in their ability to modify total plasma cholesterol, LDL-C, HDL-C or triglyceride levels (115). However, more recent meta-analysis has shown that the maximal LDL-C reduction achievable with plant stanols is approximately two-fold greater than that achieved with plant sterols (114).

Triglyceride-lowering effects of plant sterols and stanols
Numerous clinical and animal studies have shown that consumption of plant sterols and stanols was associated with significant reduction of plasma triglyceride levels (50, 116-118). Pooled analysis of twelve randomised controlled trials including 935 hypercholesterolemic subjects has shown that a daily consumption of 1.6 - 2.5 g of plant sterols/stanols exerts a modest triglyceride-lowering effect of about 6% (0.12 mmol/L). The triglyceride-lowering effects of plant sterols are more pronounced in people with elevated baseline triglyceride levels (117). In metabolic syndrome patients with moderate hypertriglyceridemia, consumption of a plant stanol ester-enriched yogurt drink (2 g/d) for 8 weeks lowered serum triglyceride concentrations by 27%. In that study, the reduction in triglyceride levels was accompanied by decrease in hepatic production of both large (> 60 nm) and medium size (35-60 nm) VLDL particles (119). In another study, plant sterols supplementation (4 g/d) in patients with metabolic syndrome for 2 months period decreased triglyceride levels by 19.1% (36). The proposed mechanism behind the triglyceride-lowering effect of plant sterols is a reduction in triglyceride-rich VLDL particles production by the liver (118, 120). There is also similar evidence from experimental mouse models suggesting that consumption of plant sterols/stanols was associated with significant reduction of plasma triglyceride levels with or without reduction of hepatic triglyceride levels (52, 116, 118). In these animal studies, the triglyceride-lowering effects of plant sterols were accompanied by inhibition of hepatic VLDL secretion (118) and an increase in fecal excretion of the saturated fatty acids, palmitate and stearate, as a result of interference with intestinal fatty acid absorption (116).

Direct effects of plant sterols/stanols on atherosclerotic lesions and endothelial function
The role of plant sterols/stanols in prevention and treatment of cardiovascular and atherosclerotic diseases is mediated not only by their cholesterol-lowering properties but also by possessing anti-atherogenic activities (15).

Evidence from cell culture-based studies
The anti-atherogenic effects of phytosterols have been investigated in a number of in vitro studies (121-123). Results from these studies have shown that plant sterols suppress aortic endothelial cells and vascular smooth muscle cell proliferation (121-123), inhibit platelet aggregation (122) and stimulate prostacyclin (PGI2) production (a potent inhibitor of platelet aggregation with vasodilator and anti-inflammatory actions) (121). The anti-inflammatory effects of phytosterols have been demonstrated in vitro in human aortic endothelial cells. Phytosterols reduced the expression of vascular and intracellular adhesion molecules (VCAM)-1 and (ICAM)-1 in TNF-α stimulated human aortic endothelial cells, attenuated the phosphorylation of nuclear factor-κB and inhibited the binding of monocytes to TNF-α stimulated human aortic endothelial cells (124). The concentrations of plant sterols used in these studies were distributed over wide ranges. In some studies the concentrations of plant sterols were relevant to the physiologically achieved plasma levels in humans (121-123). However, in other studies higher concentrations were employed (124).

Evidence from animal studies
Anti-atherogenic activity of plant sterols have been reported for the first time in chickens by Peterson and co-workers, who observed a significant reduction in the severity and extent of atherosclerotic lesions upon inclusion of soybean sterols in cholesterol rich diet (26). Soon thereafter, similar effects were reported in rabbits by Pollak (28). The beneficial effects of dietary sterol supplementations on endothelial and vascular smooth muscle cell functions have also been reported in atherosclerotic rat and mice models (22, 29, 30, 52, 125-127). Administration of plant sterols/stanols to Apo E-deficient mice has been shown to decrease the size and severity
Nevertheless, reduction in circulating concentrations of oxidized LDL have been reported in few studies (143).

**Short and long term safety and toxicity of sterols and stanols**

Evidence from clinical trials and experimental models have indicated a good safety profile of plant sterols/stanols (144). However, long-term use has raised concerns of a potential increase in the risk of cardiovascular events, probably due to elevation of plasma and tissue plant-sterol concentrations (145, 146). High plasma plant-sterol levels could be detrimental in sitosterolemia (a rare genetic disorder caused by genetic mutation of ABCG5/ABCG8 transporter genes and characterised by high plasma levels of sitosterol and campesterol, premature atherosclerosis and increased risk of coronary heart and aortic valve disease) (1).

A number of studies have suggested that phytosterols consumption could be a risk factor of cardiovascular disease (147-150). Despite that, currently available data could neither confirm an increased cardiovascular risk with plant sterols nor rule it out (144, 151). It has been suggested that plant sterols may induce inflammation and reduce cholesterol efflux from macrophages, conditions that are directly implicated in the development of atherosclerosis (145). A study by Weingärtner et al. (152) have shown that food supplementation with plant sterol impairs endothelial function, exacerbates atherogenesis and ischemic brain injury in mice and increases tissue sterol concentrations in humans. Vergès and Fumeron (151) recently reviewed studies examining the relationship between plasma phytosterol levels and cardiovascular disease events. They reported conflicting results of a potential connection between elevated plasma plant-sterol levels and cardiovascular risk. Some studies suggest a positive association while other show no or even inverse correlation. Although the review included a total of 225,437 subjects from different case control, prospective and genome-wide association studies, it was difficult to draw a firm conclusions even though the large prospective trails and genome wide association studies have suggested a positive correlation which cannot be ruled out.

Daily consumption of 3.24 g of phytosterols over a period of 3.5 weeks produced no significant alteration in serum levels of alkaline phosphatase, alanine transaminase, aspartate transaminase and gamma-glutamate transaminase (41). Few clinical trials have shown that the use of high doses of plant sterols/stanols was...
associated with a modest reduction in levels of carotenoids, fat-soluble vitamins and antioxidants (40, 94, 153-155). Similar changes were reported in rats treated with high doses of stanol ester for 13 weeks (156). However, when the levels of these vitamins were adjusted to the lowered LDL-C concentrations, the reduction was not significant for vitamin E, D and K (9, 41, 106), while plasma levels of α-, β -carotene and lycopene remained low (9, 41, 153, 157). The effects of plant sterols on fat soluble vitamins can be overcome by maintaining adequate daily intake of fruits and vegetables (1, 9, 158).

Although consumption of plant sterols have been associated with increased plant sterol levels in erythrocyte membranes, no detrimental effects have been reported in humans with normal sterol metabolism (157, 159-161). However, increased RBC fragility with episodes of haemolysis have been reported in patients with sitosterolemia (162, 163). The increased RBC fragility by plant sterols have also been shown to shorten the life span of stroke-prone spontaneously hypertensive (SHRSP) rats (164).

Some studies have suggested potential estrogenic effects of plant sterols/stanols (165-167). However, these assumptions have been disproved by extensive safety evaluations using series of in vitro and in vivo experiments (168, 169). Indeed, consumption of plant sterol in humans have not been shown to affect hormone levels in males (free and total testosterone) and females (luteinizing hormone, follicle stimulating hormone, β-estradiol and progesterone) (157) and plant sterols do not bind to oestrogen receptors (168).

A promising semisynthetic stanol: disodium ascorbyl phytostanyl phosphate (FM-VP4).
Commercially available plant sterols and stanols are semisynthetic, hydrophobic fatty acid esters and their use as food additives are limited to oil-based foods such as margarines (170). Many attempts have been made to improve the lipid-lowering activity of plant sterols by modification of their chemical structure to increase their micellar incorporation capacity. One of such well characterized phytostanol analogue is disodium ascorbyl phytostanyl phosphates, also known as FM-VP4 (21). Disodium ascorbyl phytostanyl phosphate is a novel hydrophilic stanol analogue that can be formulated into a wide range of delivery vehicles (171). FM-VP4 is composed of a mixture of two components; disodium ascorbyl sitostanyl phosphate and disodium ascorbyl campestanyl phosphate (Figure 7). FM-VP4 is produced by hydrogenation of sitosterol and campesterol to form sitostanol and campestanol followed by esterification with ascorbic acid by a phospodiester bond (172). FM-VP4 has an improved therapeutic efficacy over plant sterols/ stanols, most probably resulting from the combined cholesterol-lowering properties of the plant stanols and the antioxidant potential of ascorbic acid (173).

The lipid-lowering effects of FM-VP4 have been reported in a number of experimental animals and human studies. Evidence from previous studies has suggested that FM-VP4 is more effective in lowering plasma cholesterol levels than plant sterols/ stanols (159, 174, 175). In an early study, Apo E-deficient mice have been used to evaluate the lipid-lowering effects and anti-atherosclerotic properties of FM-VP4. The Apo E-deficient mouse is particularly popular model because of its propensity to spontaneously develop athersclerotic lesions similar to human counterparts even on a standard chow diet (176). In that study, the incorporation of FM-VP4 into animal’s diet at a dose of 0.5% (w/w) or drinking water at a dose of 0.5% (w/v) significantly reduced total plasma cholesterol levels and aortic athersclerotic lesion by 75% over the 12 weeks study period. Transient reduction in plasma triglyceride levels has also been reported in the first 8 weeks of FM-VP4 treatment.

**Figure 7.** Chemical structure of disodium ascorbyl phytostanyl phosphate; $R=\text{CH}_3$ (disodium ascorbyl campestanyl phosphate), $R=\text{C}_2\text{H}_5$ (disodium ascorbyl sitostanyl phosphate).

*Lipid-lowering effects and anti-atherosclerotic activity of FM-VP4*
On the other hand, administration of un-esterified plant stanol with or without ascorbic acid is associated with less significant reduction of total plasma cholesterol and no triglyceride-lowering effects (72).

In another study, 4 weeks' administration of FM-VP4 to gerbils at a dose of 1 to 2% (w/w) significantly reduced total plasma cholesterol and LDL-C levels without affecting plasma triglyceride or HDL-C levels (170). However, a longer period of administration (8 weeks) with a higher dose of 4% (w/v) in drinking water resulted in a significant reduction of total plasma triglyceride levels and a significant increase in HDL-C as well (177). Administration of 2% (w/w) FM-VP4 for 12 continuous weeks in mice fed either with a high fat or a low fat diet significantly reduced total plasma cholesterol levels by 32% and 45%, respectively, compared with animals that received no treatment (178). In hamsters, the cholesterol-lowering effect of FM-VP4 was found to be three-fold greater than that of free stanols. Five weeks of feeding hamsters with 0.71% or 1.43% (w/w) FM-VP4 (corresponding to 0.5 or 1% free plant stanol) reduced plasma cholesterol levels by 34% and 46%, respectively. On the other hand, free stanols reduced plasma cholesterol levels by only 14%. The non-HDL-C levels were reduced by 39 and 54% in animals supplemented with 0.71% or 1.43% (w/w) FM-VP4, respectively. Moreover, plasma triglyceride levels were reduced by 42% and 49%, respectively, compared to hamsters that were fed un-esterified stanols (159, 174). Interestingly, the cholesterol-lowering effects of FM-VP4 were associated with a significant reduction in abdominal fat and less body weight gain in diet-induced obese mice (178) and hamsters (174).

It has been reported that a single oral gavage of FM-VP4 with [3H] cholesterol co-administered in Intralipid® fat emulsion to rats significantly reduced intestinal [3H] cholesterol absorption and decreased plasma [3H] cholesterol level in a dose-dependent manner. The cholesterol-lowering effects of FM-VP4 have been attributed to displacement of cholesterol from bile salts micelles and inhibition of cholesterol uptake by enterocytes (Figure 8) (179, 180).

Figure 8. [3H]-Cholesterol plasma concentration-versus-time curve on a linear graph following a single oral dose of [3H]-cholesterol (227 ng/ml), unlabelled cholesterol (1 mg/ml) and FM-VP4 (0, 5, 10, 20, 50 and 100 mg/kg) co-administered in 10% Intralipid® to fasting Sprague Dawley male rats. Data are shown as mean ± SD. Adapted from Wasan et al. (180) with the permission from Canadian Society for Pharmaceutical Sciences.
Results from clinical trials in humans have shown that administration of FM-VP4 at a dose of 400 mg/day reduced LDL-C by 6.5% compared with baseline levels over four weeks’ treatment period. Similar reduction in LDL-C levels was reported with plant sterol and stanol esters but at doses 2-3 times higher than that of FM-VP4 (175).

Mechanism of action of disodium ascorbyl phytostanyl phosphate

The exact mechanisms of action of disodium ascorbyl phytostanyl phosphate have not been fully elucidated yet. FM-VP4 exerts its cholesterol-lowering effect by acting locally within the gastrointestinal tract. Following oral administration, only a small proportion of FM-VP4 is absorbed and becomes available in the systemic circulation and the vast majority is temporarily retained within the gastrointestinal tract and then removed via the feces (180).

It has been suggested that FM-VP4 could inhibit cholesterol incorporation into mixed micelles and thereby reduce the extent of cholesterol solubilisation and absorption into intestinal epithelial cells (175, 181). FM-VP4 actually has a significant advantage over other plant sterols/stanols in that it has greater solubility in GI mixed micelles. This advantage is achieved by incorporation of hydrophilic ascorbyl residue to the hydrophobic campestanol and sitostanol tail through a phosphodiester bond. The unique amphiphilic structure of FM-VP4 allows self-assembly into micelle structures in aqueous solutions in the absence of bile salt (175, 182).

In this context, a study using an in vitro lipolysis model has shown that disodium ascorbyl phytostanyl phosphate significantly reduced cholesterol distribution into the micellar phase, displacing it to the sediment phase and making it unavailable for absorption. This model mimics the digestion process that happens in the physiological environment of the upper gastrointestinal tract. In this model, the distribution of cholesterol into the oil, aqueous and sediment phases after ultracentrifugation provides insight into its probable fate of cholesterol in the intestine when consumed with FM-VP4. Cholesterol distributed into the aqueous phase is most readily available for absorption; cholesterol recovered in the oil phase is most likely to have a delayed absorption; while cholesterol found in sediment is most likely to be excreted. Because of its structural similarity to bile salts, FM-VP4 has been suggested to act as a surfactant and thus compete with bile salts for adsorption into lipid surface. However, the lack of hydroxyl groups prevents it from proper interaction with pancreatic lipase and/or co-lipase, thus hinders the lipid digestion process (181). Pancreatic lipase/colipase mediate partial hydrolysis of the triacylglycerol into monoacylglycerol and non-esterified fatty acids which is required for dietary cholesterol to be absorbed as emulsified substrate by intestinal cells (183). Therefore, the inhibitory effect of FM-VP4 on lipid digestion could be attributed to its competition with bile salts during the lipolysis process (181).

The other suggested mechanism involves competition between FM-VP4 and cholesterol for uptake by intestinal epithelial cells. This mechanism has been illustrated by an in vitro cholesterol uptake study using cells derived from the duodenal and jejunal segments of rat small intestine (184). In that study, rat intestinal epithelial crypt cells (IEC-6) were incubated with [3H] cholesterol micelles in the presence of increasing concentrations of FM-VP4 and the extent of cholesterol radioactivity associated with cell monolayers was determined at the end of incubation period. Results showed that 50 µM of FM-VP4 reduced [3H] cholesterol accumulation into IEC-6 cells by 60% within 150-30 min of exposure. Similarly, cholesterol has been shown to inhibit the accumulation of radiolabeled FM-VP4 by 62 % within 60 min of exposure suggesting that FM-VP4 and cholesterol may compete for the same binding site on IEC-6 cells (184). Comparable results were reported in another study using Caco-2 cells. Interestingly, the inhibition of cholesterol accumulation by FM-VP4 was independent of pancreatic lipase activity, P-glycoprotein-mediated cholesterol influx or cholesterol incorporation into micelles (171).

Some reports have suggested that FM-VP4 could affect the expression of regulatory genes that are involved in controlling cholesterol homeostasis (171, 185). Mendez-Gonzalez and co-workers have reported transcriptional changes in hepatic and intestinal genes that are involved in regulating cholesterol and bile acids metabolism in female mice fed with an FM-VP4-enriched diet. They showed that FM-VP4 up-regulates the expression of hepatic LXRα, ABCA1, ABCG5/ABCG8, SR-B1 and HMG-CoA reductase genes. In addition, the study showed that FM-VP4 down-regulates several farnesoid-X receptor (FXR) target genes and induces intestinal reabsorption of bile acids, resulting in an increase in the hepatic and intestinal bile acid contents and reduction of fecal bile acid output. Although these
Changes were associated with significant reductions in intestinal cholesterol absorption as well as reductions in plasma and liver cholesterol concentrations, it is not clear whether such changes were due to direct actions of FM-VP4 or promoted as compensatory mechanism to its cholesterol-lowering effects (185).

Safety of FM-VP4

A clinical study of 100 subjects demonstrated that administration of FM-VP4 at doses up to 800 mg/day for 4 weeks is safe and well tolerated. Mild adverse effects were reported including dizziness, headache and fatigue (175). These adverse effects were resolved spontaneously. Phase 1 clinical trial has shown that FM-VP4 had no effects on blood pressure or heart rate. However, a slight reduction of systolic blood pressure was reported in phase 2 trial after 4 weeks of treatment with no changes in diastolic blood pressure. Furthermore, the pre- and post-dose ECG findings were normal in both phases of the trial. Plasma levels of fat soluble vitamins (A and E) were unaffected by 4 weeks treatment of FM-VP4 at a dose up to 800 mg/day (175). No apparent hepatic or renal toxicities have been reported with FM-VP4 administration in both experimental animals (178) and humans (175). Assessment of liver enzyme activities (aspartate aminotransferase; AST and plasma alanine aminotransferase; ALT) and renal function (plasma creatinine) revealed no significant changes following 12 weeks of feeding mice with 2% (w/w) FM-VP4 (178) or eight weeks of feeding gerbils with 2% - 4% FM-VP4, either incorporated into the diets or drinking water (177). Five weeks of feeding hamsters with 0.7% - 1.4 % (w/w) of FM-VP4 has not been shown to affect erythrocyte fragility (159). No mutagenic activity has been reported with FM-VP4 (186).

Conclusions

Based on abundant evidence from scientific literature, plant sterols/ stanols and their semisynthetic analogues have promising roles in prevention and treatment of elevated plasma cholesterol levels and atherosclerotic cardiovascular disease. The enhanced safety profile of plant sterols/ stanols allows them to be used either as an adjuvant or alternative therapy especially for those patients who are intolerant to statin therapy. Several factors have been suggested to affect the cholesterol-lowering activity of plant sterols/stanols. Modifying these factors could be valuable in optimizing their efficacy. Synthesis of semisynthetic sterols/stanols by esterification with ascorbic acid improves the cholesterol-lowering efficacy of plant sterols and stanols and expands their capacity to lower elevated plasma triglyceride levels. FM-VP4 is a promising synthetic stanol that has shown substantially increased efficacy compared to other sterols and stanols.

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Conflict of Interest

The authors declare no conflicts of interest.

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Table 2. Randomized clinical trials on plant sterols/stanols showing the lipid lowering effects of different products.

| Study                          | Study design       | Supplementation format | Type of phytosterol                  | Dose* (g/day) | Duration (Weeks) | TC reduction (%) | LDL-C reduction (%) | TG reduction (%) |
|--------------------------------|--------------------|------------------------|-------------------------------------|---------------|------------------|------------------|---------------------|------------------|
| Weststrate and Meijer, (40)    | Balanced           | Margarine              | Esterified soya bean-oil sterols    | 3.3           | 3.5              | -8.3             | -13                 | NA               |
| Hendriks et al. (41)           | Balanced           | Spread                 | Esterified soya bean-oil sterols    | 0.83          | 3.5              | -4.9             | -6.7                | 8.4              |
|                                |                    |                        |                                     | 1.61          | 3.5              | -5.9             | -8.5                | -6.1             |
|                                |                    |                        |                                     | 3.24          | 3.5              | -6.8             | -9.9                | -4.8             |
| Sierksma et al. (37)           | Crossover          | Spread                 | Non-esterified soya bean oil sterols| 0.8           | 3                | -3.8             | -6                  | -5               |
| Maki et al. (38)               | Parallel           | Reduced-fat spread     | Esterified plant sterols            | 2.2           | 5                | -6.6             | -8.1                | -10.4            |
| Mensink et al. (39)            | Parallel           | Low-fat yoghurt        | Esterified plant stanols            | 3             | 4                | -8.7             | -13.7               | -2.7             |
| Plana et al. (35)              | Parallel           | Low-fat fermented milk | Esterified plant sterol/ stanols    | 1.6           | 6                | -7.99            | -10.49              | -6.58            |
| Sialvera et al. (36)           | Parallel           | Yogurt drink           | Non-esterified plant sterols        | 4             | 8.5              | -15.9            | -20.3               | -19.1            |
| McPherson et al. (42)          | Parallel           | Tablets                | Soy stanol-lecithin                 | 1.26          | 6                | -4.8             | -10.4               | 5.3              |
| Goldberg et al. (43)           | Parallel           | Tablets                | Soy stanol-lecithin                 | 1.8           | 6                | -5.7             | -9.1                | -0.3             |
| Woodgate et al. (44)           | Parallel           | Softgel capsules       | Esterified phytostanols             | 1.6           | 4                | -8               | -9                  | 0.6              |
| Carr et al. (45)               | Parallel           | Capsules               | Stearate-enriched phytosterol esters| 1.8           | 4                | NA               | -11                 | 2.8              |
| Maki et al. (46)               | Crossover          | Tablets                | Non-esterified plant sterol/ stanols| 1.8           | 6                | -2.8             | -4.9                | 4.2              |
| Maki et al. (47)               | Crossover          | Softgel capsules       | Esterified plant sterol/ stanols    | 1.8           | 6                | -7.4             | -9.2                | -9.1             |
| Ottestad et al. (48)           | Crossover          | Softgel capsules       | Esterified plant sterols            | 2             | 4                | -1.8             | -2.7                | -9.1             |
| McKenney et al. (49)           | Crossover          | Softgel capsules       | Esterified plant sterol/ stanols    | 1.8           | 6                | -3.5             | -4.3                | -3.2             |

*Phytosterol dose was expressed as free sterol/stanol equivalents; NA indicates that data are unavailable.