Research Article

Hypolipidemic activity of phytosterol derived from Indian rice bran oil deodorizer distillate in free and esterified form with alpha-linolenic acid.

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ABSTRACT

Introduction and Aim: The hypolipidemic activity, antioxidant activity and tissue fatty acid composition of high purity phytosterols (PS) obtained from rice bran oil deodorizer distillate (RBO DOD) was evaluated in free and esterified form. The phytosterol esters of ALA (PSE) were prepared by esterifying PS with alpha linolenic acid (ALA) obtained from linseed oil.

Materials and Methods: Male wistar rats weighing 40-50g was used for the study. The study had six groups and each group had six animals (n=6). The serum lipid profile such as total cholesterol (TC), triacylglycerol (TAG), low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) was analyzed for control and treated groups. The anti-oxidant activity such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and tissue fatty acid compositions (liver, adipose, serum, brain, eye and heart) were analyzed for control and treated groups.

Results: The TC, TAG and LDL-C contents in the treated groups GNO+PS, GNO+ALA, GNO+PSE-LD (lower dose), and GNO+PSE-HD (higher dose) were decreased. The HDL-C content was increased and the antioxidant enzymes (SOD and CAT) showed increased activity in the treated groups GNO+PS, GNO+ALA, GNO+PSE-LD and GNO+PSE-HD. The omega 6 to omega 3 fatty acid ratio were decreased in the treated groups such as GNO+ALA, GNO+PSE-LD and GNO+PSE-HD.

Conclusions: This is probably the first study which shows hypolipidemic, antioxidant and tissue fatty acid composition of PS (isolated from RBO DOD) and ALA in individual form and also in esterified form in dose dependent study.

Keywords: Phytosterol from Indian RBO DOD, Phytosterol esters of ALA, Hypolipidemic activity, Antioxidant enzymes and Fatty acid composition.

INTRODUCTION

The process of deodorization is the final step in the refining of edible vegetable oils. The end product of the step yields refined edible oil and deodorizer distillate (DOD) which is the waste byproduct. DOD is a complex mixture of free fatty acids, monoacylglycerols, diacylglycerols, triacylglycerols, phytosterols, steryl esters, tocopherols, hydrocarbons, pesticides and break down products of fatty acids (aldehydes and ketones) and oxidized triglycerides (1). DOD is rich in nutraceuticals like phytosterols, tocopherols, tocotrienols and squalene. Phytosterols and omega-3 fatty acids reduce blood LDL-C and TAG levels respectively, however fish oil (FO) PUFA appears to elevate the LDL-C level slightly (2). Traditionally phytosterols have been esterified to different kinds of fatty acids to improve its solubility, functionality which in turn helps in incorporation into different food products (2). The hypocholesterolemic and hypotriglyceridemic action of fish oil esters of phytosterols was studied in male golden Syrian hamster for 5 weeks. The fish oil esters decreased

plasma TC and non-HDL-C by 20% and 29% respectively (3). The hypolipidemic effect of phytosterol esters of EPA+DHA and phytosterol esters of ALA was studied in male wistar rats. The treated groups showed decrease in TG and non-HDL-C level, whereas the HDL-C was increased in the treated group (4).

The brain antioxidant enzyme and brain lipid composition of the rats fed phytosterol esters of EPA+DHA and phytosterol esters of ALA was studied. The treated groups ameliorate brain antioxidant enzyme levels and decreased brain malondialdehyde (MDA) levels (5). The cardiac lipid profile of rats fed phytosterol esters of EPA+DHA and phytosterol esters of ALA was studied. The TC and TG levels were decreased in the omega-3 fatty acid ester treated groups, however antioxidant enzyme activity was increased in the treated groups (6). The anti-atherogenic effects and plasma lipid profile of phytosterol esters of DHA was studied in apo-E deficient mice. The atherosclerotic lesions in the treated group showed three times smaller lesion than the control group (7). In all the above studies commercially available phytosterols were used. In the current study, high purity phytosterols prepared from RBO DOD were analyzed for its hypolipidemic activity in free and in esterified form. PSE was evaluated for its hypolipidemic activity, antioxidant enzymes and tissue fatty acid composition in male wistar rats.

MATERIALS AND METHODS

Materials

Groundnut oil (GNO), casein and starch were obtained from a local market in Mysuru city. Mixed fatty acid methyl ester (FAME) standards were obtained from Supelco (USA). Boron trifluoride (BF_3) and cholesterol were procured from Sigma-Aldrich Chemicals (St Louis, USA). Mineral mix, choline chloride, DL-methionine and bile salts were purchased from Sisco Research Laboratories (SRL), Mumbai. From Agappe Diagnostics Limited, Kochi, Kerala standard kits for estimation of serum lipid profile was obtained. Solvents, chemicals, and reagents used were of analytical grade.

Methods

Physico-chemical characteristics of groundnut oil

The free fatty acid (FFA) content and peroxide value (PV) of GNO was estimated by the official method of American Oil Chemists Society (AOCS) (8). The tocopherol and tocotrienol content of GNO was expressed as total tocopherol and determined by IUPAC method (9). The GNO was converted into fatty acid methyl esters (FAME) and fatty acid composition was analyzed by gas chromatography (GC) (10).

Preparation of phytosterol and ALA enriched fraction

The rice bran oil DOD was saponified to obtain unsaponifiable matter. The unsaponifiable matter was mixed with hexane and kept at -20°C for 72hrs. The whole contents were filtered under vacuum to obtain crude phytosterol extract. The crude mixture was further purified to obtain high purity phytosterol mixture (11). Linseed oil (LSO) was hydrolyzed using alkali treatment to obtain linseed oil fatty acids (LSOFA). The obtained fatty acids were subjected to urea fractionation to obtain ALA enriched fraction (12). The fatty acid composition of the ALA enriched fraction was analyzed by GC (10).

Preparation of PSE

Phytosterols and ALA enriched fraction was taken in 1:1 ratio followed by *Rhizomucor miehei* lipase (10%) was added. Ethyl acetate was added to bring fluidity to the reaction mixture and the whole contents were kept in shaking water bath (100rpm) at 50°C. The samples were incubated for 24hrs, followed by the completion of enzymatic reaction the presence of excess FFA was removed by alkali treatment. Silica gel column chromatography method was used to separate the esterified sterols and free phytosterols (12).

Analysis of PSE

The phytosterols and ALA enriched fraction used in the study had mixture of different kinds of phytosterols and fatty acids. The PSE was saponified by alkali treatment to obtain phytosterol mixture. The obtained mixture was mixed with hexane and kept at 5°C for 2-5 hours.

The whole contents were filtered under vacuum to obtain high purity phytosterol mixture (12). The phytosterol composition of the obtained mixture was analyzed by HPLC using C18 column with UV detector (set at 206nm) (13). Followed by extraction of phytosterol, the soap was neutralized with 2N Hydrochloric acid (HCL) to release the fatty acids. Hexane was added to extract the fatty acids and later desolventized to obtain the fatty acid mixture (12). The obtained fatty acids were converted into FAME by treatment with boron triflouride(BF_3)-methanol and fatty acid composition was analyzed by GC (10).

Diet composition

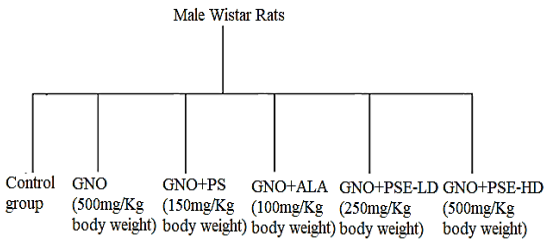
The ingredients in the control group diet follow as (g/kg), starch-650.0g, casein-200.0g, vitamin mix-10.0g, mineral mix-35.0g, choline chloride-2.0g, DL-methionine-3.0g, and GNO-100.0g. The ingredients in the treated groups diet follows as (g/kg), starch-630.0g, casein-200.0g, vitamin mix-10.0g, mineral mix-35.0g, choline chloride-2.0g, DL-methionine-3.0g, GNO-100.0g, cholesterol-10.0g, and bile salts-10.0g. All the diets were prepared freshly and stored in a cold room (4°C).

Experimental animals

The animal experiments were conducted according to the guidelines of Central Food Technological Research Institute (CFTRI) animal ethical committee. The institutional animal ethical committee (IEAC) number was IEAC# 292/2014 for the animal experiments. The male wistar rats weighing (40-50g) were obtained and housed in CFTRI animal house for 42 days. The rats were given free access to fresh diet and water every day. The left over diet was weighed and discarded.

Hypolipidemic effect of PSE of ALA in experimental rats

The schematic representation of different group involved in the study is given in Fig. 1. Male wistar rats (40-50g) were divided into following groups, and each group had six animals (n=6). Control group, groundnut oil (rats received GNO 500mg/Kg body weight/day), treated groups GNO+PS (Groundnut oil+phytosterols, rats received phytosterols of RBO DOD 150mg/Kg body weight/day), GNO+ALA (Groundnut oil+ALA, rats received ALA from LSO 100mg/Kg body weight/day), GNO+PSE-LD (Groundnut oil+PSE of ALA-LD, rats received PSE 250mg/Kg body weight/day) and GNO+PSE-HD (Groundnut oil+PSE-HD, rats received PSE 500mg/Kg body weight/day). Throughout the study, GNO+PSE-LD refers to a lower dose of PSE (250mg/kg) and GNO+PSE-HD refers to higher dose of PSE (500mg/kg). The control group contained fat (10%) in the diet, whereas the remaining groups GNO, GNO+PS, GNO+ALA, GNO+PSE-LD and GNO+PSE-HD fed with cholesterol (1%) in the diet and additional fat (GNO:20%) was given through oral intubation as a vehicle. The oral intubation of GNO, GNO+PS, GNO+ALA, GNO+PSE-LD and GNO+PSE-HD groups were administered based on the individual animal body weight measured daily. To analyze the hypolipidemic effect of PS and ALA separately, GNO+PS and GNO+ALA groups were included. The dosage of PS and ALA in GNO+PS and GNO+ALA groups were based on the lower dose of GNO+PSE–LD, which contained phytosterols (150mg) and ALA (100mg). So GNO+PS, GNO+ALA and GNO+PSE-LD show the effect of compounds individually and also in esterified form. The dose-dependent effect of PSE was studied at two different concentrations GNO+PSE-LD and GNO+PSE-HD.



Control group diet had 10% fat and zero cholesterol, Groups from GNO to GNO+PSE-HD diet had 10% fat, 1% cholesterol and the remaining 20% fat was given through oral gavage containing PS, ALA and PSE separetely.

Fig.1: Schematic representation of groups involved in animal experiments. For abbreviations see Table 1

Determination of food efficiency ratio

The diet consumption of the rats was measured daily and weights of the animals were measured weekly. The food efficiency ratio (FER) was calculated using the formula:

Food efficiency ratio = Average weekly diet intake (g) / Weekly gain in body weight (g).

Determination of serum lipid profile

After 42 days of treatment, the animals were fasted for overnight (12hrs) and sacrificed under ether anesthesia. Blood was collected by cardiac puncture and centrifuged at 4°C for 10min at 3000rpm. The supernatant serum was collected for the analysis of lipid profile and other parameters. The serum TC, HDL-C, LDL-C and TAG content was analyzed according to standard procedures as mentioned in the commercially available kits.

Analysis of antioxidant enzyme and lipid peroxidation in liver

The liver was homogenized with 0.74% potassium chloride (KCl) solution and the homogenate was centrifuged to get the supernatant. To the obtained supernatant, the superoxide dismutase (SOD) activity was analyzed for control and treated groups (14). The Catalase (CAT) and glutathione peroxidase (GPx) activity of the liver homogenate was analyzed for control and treated groups (15, 16). Lipid peroxidation of the liver homogenate was analyzed for control and treated groups (17). The lipid peroxidation of the liver homogenate was expressed as nmol MDA/mg protein.

Extraction of lipids and analysis of fatty acid composition in different tissues

Lipids from different tissue such as liver, adipose tissue, brain, eye and heart was extracted for control and treated groups (18). The extracted lipid was treated with BF_3-methanol and the prepared FAME was analyzed GC (10).

Statistical analysis

The obtained results were expressed as a mean ± standard deviation. Data were analyzed using statistical program Graph Pad In Stat Demo [DATASET1.ISD] and Tukeys Multiple Comparison Tests was used. A difference of *P* value ≤ 0.05 was considered to be significant.

RESULTS

Physico-chemical characterization of GNO

The FFA content and PV of the GNO were 0.09 ± 0.01% and 1.98 ± 0.03 Meq O_2/kg. The total tocopherol (mg/100g) content of the GNO was 55.2 ± 2.1. The fatty acid composition (%) of the GNO (used as vehicle) was palmitic acid (C16:0):13.60 ± 1.0, stearic acid (C18:0):4.20 ± 0.4, oleic acid (C18:1):44.50 ± 1.2, linoleic acid (C18:2):32.90 ± 1.0 and arachidic acid (C20:0):4.50 ± 0.4.

Analysis of phytosterol and ALA

The phytosterol purified from RBO DOD had the following sterol composition (%) β-sitosterol:38.2 ± 1.07, stigmasterol:34.9 ± 1.05, campesterol:9.5 ± 0.1 and minor sterols:17.4 ± 1.2. The fatty acid composition (%) of the ALA enriched fraction was palmitic acid (C16:0):0.5 ± 0.2, stearic acid (C18:0):0.2 ± 0.01, oleic acid (C18:1):7.4 ± 0.07, linoleic acid (C18:2):14.2 ± 0.12 and linolenic acid (C18:3):74.2 ± 0.9. The esterification yield was 80.6% under the optimized reaction conditions such as substrate ratio (1:1), enzyme concentration (10%), incubation period (24h) and temperature (50°C).

Analysis of PSE

The PSE had the following phytosterol composition (%) β-sitosterol:50.47 ± 0.66, stigmasterol:34.7 ± 0.35, campesterol:8.41 ± 1.48 and minor sterols:6.0 ± 0.5. The PSE had the following fatty acid composition (%) oleic acid (C18:1):3.28 ± 0.2, linoleic acid (C18:2):17.1 ± 0.2 and alpha linolenic acid (C18:3):78.5 ± 0.34. Beta sitosterol esters of fish oil and linseed oil were prepared and the prepared sterol esters were further hydrolyzed to analyze phytosterol content and fatty acid composition of the esters (19). Similarly, even in the current study the sterol esters were hydrolyzed to analyze the phytosterol content and fatty acid composition of the esters.

Food intake, body weight gain and FER

The daily diet intake of different groups did not show a significant difference (Table 1). The initial body weights of all the groups (Control, GNO, GNO+PS, GNO+ALA, GNO+PSE-LD, and GNO+PSE-HD) were normalized that ranged from 77.30 to 77.56g, whereas the final body weight was in the range of 220.93-254.83g. The gained body weights of all the groups (Control, GNO, GNO+PS, GNO+ALA, GNO+PSE-LD and

GNO+PSE-HD) were in the range of 143.63-177.27g at the end of the study. Feeding with normal diet had gained less body weight (~220.93g), whereas in the group with additional intubation of GNO alone as vehicle had increased body weights (~248.83g). Treatment with PSE in GNO reduced the body weight when compared with other groups, however there was no significant difference between the treated groups when compared with GNO treatment alone. The FER ratio for the control group was 0.46, but the ratio of the GNO group was decreased (17.39%) and remained same with treated groups of with or without treatment of esters or non-ester forms of ALA and PS.

Organ weights

The difference in organ weights such as liver, adipose tissue, kidney, brain and heart are given (Table 1).

Table 1: Weight gain, food efficiency ratio & organ weights of rats fed control & treated groups.

Groups	Control	GNO	GNO + PS	GNO + ALA	GNO + PSE-LD	GNO + PSE-HD
Food intake (g/d/rat)	14.80 ± 1.8[a]	14.56 ± 1.7[a]	15.61 ± 1.6[a]	14.69 ± 1.7[a]	15.07 ± 1.4[a]	14.93 ± 1.8[a]
Final body weight (g)	220.93 ± 10.7[a]	248.83 ± 20.4[b]	254.83 ± 17.6[b]	253.16 ± 24.6[b]	242.20 ± 23.8[b]	244.80 ± 17.2[b]
Food efficiency ratio	0.46 ± 0.10[a]	0.38 ± 0.07[b]	0.39 ± 0.07[b]	0.37 ± 0.07[b]	0.38 ± 0.07[b]	0.39 ± 0.08[b]
Organ Weights (g)						
Liver	7.96 ± 0.60[a]	13.01 ± 1.53[b]	13.1 ± 1.66[b]	12.81 ± 2.57[b]	11.10 ± 0.96[b]	12.40 ± 1.18[b]
Adipose	2.43 ± 0.58[a]	3.23 ± 0.86[a]	3.56 ± 0.31[a]	3.10 ± 0.99[a]	2.70 ± 0.41[a]	3.56 ± 0.81[a]
Kidney	1.55 ± 0.18[a]	1.66 ± 0.08[a]	1.70 ± 0.21[a]	1.68 ± 0.23[a]	1.80 ± 0.15[a]	1.58 ± 0.04[a]
Brain	1.68 ± 0.18[a]	1.81 ± 0.09[a]	1.83 ± 0.10[a]	1.83 ± 0.08[a]	1.94 ± 0.05[a]	1.88 ± 0.04[a]
Heart	0.76 ± 0.08[a]	0.85 ± 0.05[a]	0.82 ± 0.08[a]	0.73 ± 0.08[a]	0.74 ± 0.05[a]	0.80 ± 0.10[a]

Values in the table are means ± SD (n=6 rats). Values given in column followed by different alphabetical superscript are significantly different at $P \leq 0.05$. GNO-Groundnut oil, GNO + PS-Groundnut oil + Phytosterol, GNO+ALA-Groundnut oil + Alpha linolneic acid, GNO+PSE-LD-Groundnut oil + Phytosterol esters of ALA-lower dose, GNO+PSE-HD-Groundnut oil + Phytosterol esters of ALA-higher dose.

The weight of liver was 7.96g in the control group, while in the group intubated with GNO alone it was increased (13.01g) by 1.63 times, whereas the weights were in the range of 11.10-13.1g for the treated groups (GNO+PS, GNO+ALA, GNO+PSE-LD and GNO+PSE-HD).

Similarly, the weight of adipose tissue was 2.43g for the control group and intubation with GNO alone increased weight by 1.32 times, whereas the weight of adipose tissue for the following treated groups such as GNO+PS, GNO+ALA, GNO+PSE-LD and GNO+PSE-HD were in the range of 2.70-3.56g. The weight of kidney, brain and heart for all the groups such as control, GNO, GNO+PS, GNO+ALA, GNO+PSE-LD and GNO+PSE-HD ranged from 1.55-1.80g, 1.68-1.94g and 0.73-0.85g respectively. The intubation of GNO alone increased the weights of kidney, brain, and heart by 7.1%, 7.73% and 11.84% respectively. Treatment with esters of ALA reversed the effect of GNO at various levels with different doses.

Serum lipid profile

The TC content in control and GNO treated group was 50.87 and 106.80mg/dL respectively. In GNO+PS and GNO+ALA treated groups the TC content came down to 74.49 and 79.51mg/dL respectively. Whereas GNO+PSE-LD and GNO+PSE-HD treated groups, the TC content was further reduced to 70.88 and 65.06mg/dL. The TC content decreased by 30.25%, 25.55%, 33.36% and 39.08% in the following groups GNO+PS, GNO+ALA, GNO+PSE-LD and GNO+PSE-HD (Table 2). The HDL-C content in control & GNO treated group was 18.90 & 17.43mg/dL. The HDL level was further increased to 26.56, 26.93, 33.0 & 32.56mg/dL in GNO+PS, GNO+ALA, GNO+PSE-LD and GNO+PSE-HD treated groups respectively. The percentage increase in HDL-C content was 52.38%, 54.50%, 89.32% and 86.80% in GNO+PS, GNO+ALA, GNO+PSE-LD and GNO+PSE-HD treated groups respectively (Table 2).

The LDL-C content was highest in GNO treated group (33.36 mg/dL) (Table 2). Later the LDL level decreased by 21.23, 21.66, 12.82 and 13.52mg/dL in the GNO+PS, GNO+ALA, GNO+PSE-LD and GNO+PSE-HD treated groups. The percentage decrease in LDL-C levels content for GNO+PS, GNO+ALA, GNO+PSE-LD and GNO+PSE-HD treated groups were

36.36%, 35.07%, 61.57% and 59.47% respectively. The results indicated that PS, ALA and esters of ALA have the ability to ameliorate various types of lipoproteins in serum. The TAG content was 206.59 and 242.01mg/dL in control and GNO alone treated groups (Table 2). Treatment with GNO+PS and GNO+ALA reduced the contents to 215.9 and 102.7mg/dL. The TAG content further decreased to 118.9 and 103.12mg/dL in GNO+PSE-LD and GNO+PSE-HD treated groups. The percentage decrease in TAG levels in various treated groups such as GNO+PS, GNO+ALA, GNO+PSE-LD and GNO+PSE-HD were 10.78%, 57.56%, 50.86%, and 57.39% respectively. Rats treated with ALA alone had the ability to reduce TAG levels. However, the esters of ALA had dose-dependent effect in declining the levels of TAG.

Hepatic antioxidant enzymes activities

The SOD activity in GNO alone treated group was 0.59U/mL, whereas GNO+PS, GNO+ALA, GNO+PSE-LD and GNO+PSE-HD treated groups had a positive role in increasing the activity of SOD. In GNO+PS and GNO+ALA treated groups the activity was increased by 2.98 and 1.59 times respectively. The dose dependent study on GNO+PSE-LD and GNO+PSE-HD showed an increase in SOD activity by 2.11 and 3.22 times when compared with GNO alone treated group (Table 2).

The CAT activity was decreased in GNO alone treated groups when compared with control (Table 2). In GNO+PS and GNO+ALA treated group, the CAT activity was increased by 27.89% and 15.89%, whereas the activity was increased by 24.36% and 21.18% in GNO+PSE-LD and GNO+PSE-HD treated groups respectively. The GNO+PS treated group showed highest CAT activity among all the groups. However, the CAT activity was recovered in GNO+PSE-LD and GNO+PSE-HD groups significantly when compared with GNO alone treated group.

The GPx activity was 0.66U/mL and 0.58U/mL for control and GNO alone treated group. In GNO+PS treated group, the enzyme activity was increased by 1.1 times, whereas GNO+ALA and GNO alone treated groups did not show a significant difference in GPx activity. The dose dependent study of PSE of ALA revealed that the activity of the enzyme increased at a higher dose. The GNO+PSE-LD and GNO+PSE-HD groups had 1.12 and 1.22 times increased the activity of GPx when compared with GNO alone treated group (Table 2).

Malondialdehyde levels

The control group had 0.17nm/mg protein of malondialdehyde (MDA) levels in liver, but GNO treated group showed increased MDA levels (0.31nm/mg protein), and it was almost double in GNO alone treated group. The MDA levels were decreased by 25.80%, 29.03%, 38.70% and 29.03% in the treated groups (GNO+PS, GNO+ALA, GNO+PSE-LD, and GNO+PSE-HD) (Table 2). The treated groups showed decreased MDA level significantly when compared to GNO alone treated group.

Table 2: Antioxidant activity, lipid peroxidation & serum lipid profile of rats fed control & treated groups.

Parameters	Control	GNO	GNO + PS	GNO + ALA	GNO + PSE-LD	GNO + PSE-HD
Serum Lipid Profile (mg/dL)						
TC	50.87 ± 7.0^{a}	106.82 ± 4.04^{b}	74.49 ± 5.11^{c}	79.51 ± 4.54^{c}	70.88 ± 6.57^{c}	65.06 ± 6.0^{c,d}
HDL-C	18.9 ± 2.64^{a}	17.43 ± 3.68^{a}	26.56 ± 4.16^{b}	26.93 ± 3.11^{b}	33.0 ± 3.44^{c}	32.56 ± 4.90^{c}
LDL-C	6.78 ± 0.90^{a}	33.36 ± 0.91^{b}	21.23 ± 2.38^{c}	21.66 ± 2.07^{c}	12.82± 2.30^{d}	13.52 ± 2.37^{d}
TAG	206.59 ± 15^{a}	242.01 ± 18.0^{b}	215.9 ± 26.5^{a}	102.7 ± 5.89^{c}	118.9 ± 9.75^{c}	103.12 ± 6.14^{c}
Antioxidant Activity (U/mL)						
SOD	1.61 ± 0.04^{a}	0.59 ± 0.07^{b}	1.76 ± 0.02^{c}	0.94 ± 0.04^{d}	1.25 ± 0.04^{e}	1.90 ± 0.04^{f}
CAT	69.57 ± 2.83^{a}	54.09 ± 1.57^{b}	69.18 ± 3.33^{a}	62.69 ± 2.31^{a}	67.27 ± 2.85^{a}	65.55 ± 3.33^{a}
GPx	0.66 ± 0.04^{a}	0.58 ± 0.03^{a}	0.64 ± 0.03^{a}	0.59 ± 0.06^{a}	0.65 ± 0.03^{a}	0.71 ± 0.03^{b}
Lipid Peroxidation Values (nmoles/mg of protein)						
MDA	0.17 ± 0.09^{a}	0.31 ± 0.05^{b}	0.23 ± 0.011^{c}	0.22 ± 0.018^{c}	0.19 ± 0.024^{c}	0.22 ± 0.024^{c}

Values in the table are means ± SD (n=6 rats). For abbreviations of different treatment groups please refer Table 1. SOD-Superoxide dismutase, CAT-Catalase, GPx-Glutathione peroxidase, MDA-Malondialdehyde, TC-Total cholesterol, HDL-C-High density lipoprotein cholesterol, LDL-C-Low density lipoprotein cholesterol, TAG-Triacylglycerol.

Tissue fatty acid composition

The fatty acid composition of different organs such as liver, adipose, serum, brain, eye and heart was analyzed to ascertain whether or not ALA given in the form of PSE will increase the tissue omega-3 fatty acid contents. Control, GNO, and

GNO+PS treated groups did not receive ALA, whereas GNO+ALA, GNO+PSE-LD and GNO+PSE-HD treated groups received ALA in the form of LSO or PSE. The same is the reason to pool individual organs from all the animals in the group (n=6) for fat extraction. The extracted fat was used for the analysis of fatty acid composition and values are given (Tables 3-8). Increases in tissue omega-3 fatty acid content were analyzed for GNO+ALA, GNO+PSE-LD and GNO+PSE-HD treated groups.

Table 3: Liver fatty acid profile (%) of rats fed control and treated groups.

Fatty Acids Profile	Control	GNO	GNO + PS	GNO + ALA	GNO + PSE-LD	GNO + PSE-HD
Myristic acid (C 14:0)	0.73	1.07	0.83	0.77	0.69	0.83
Palmitic acid (C 16:0)	22.3	18.24	17.28	18.19	17.68	18.91
Palmitoleic acid (C 16:1)	1.7	4.18	2.66	3.21	1.66	2.34
Stearic acid (C 18:0)	9.36	3.27	3.64	3.89	5.65	3.4
Oleic acid (C 18:1)	24.82	37.91	39.98	38.19	32.89	36.35
Linoleic acid (C 18:2, ω-6)	20.25	28.47	28.84	27.24	29.37	30.52
Alpha linolenic acid (C 18:3, ω-3)	ND	ND	ND	0.47	0.67	1.16
Arachidonic acid (C 20:4, ω-6)	20.81	6.83	6.75	7.38	10.03	5.14
Eicosapentaenoic acid (C 20:5, ω-3)	ND	ND	ND	ND	0.11	0.2
Docosapentaenoic acid (C 22:5, ω-3)	ND	ND	ND	0.12	0.23	0.34
Docosahexaenoic acid (C 22:6, ω-3)	ND	ND	ND	0.49	0.94	0.77
Total ω-6	41.06	35.3	35.59	34.62	39.4	35.66
Total ω-3	NA	NA	NA	1.08	1.95	2.47
Ratio: ω-6/ω-3	NA	NA	NA	32.05	20.2	14.43

ND-Not detected, NA-Not applicable, For abbreviations of different treatment groups please refer Table 1.

Fatty acid composition of liver

In liver, the ALA content was increased by 0.47, 0.67 and 1.16 times in the treated groups GNO+ALA, GNO+PSE-LD and GNO+PSE-HD (Table 3). The DHA content in GNO+ALA, GNO+PSE-LD and GNO+PSE-HD treated groups was increased by 0.49-0.94 times. Similarly, the increase in eicosapentaenoic acid (EPA) and docosapentaenoic acid (DPA) content was in the range of 0.11-0.20 and 0.12-0.34 times in the treated groups GNO+ALA, GNO+PSE (LD & HD). The ratio of omega-6 to omega-3 fatty acids was 35.30 for GNO alone treated groups. On the other hand, the ratio came down to 14.43-32.05 in the treated groups. The GNO alone treated group did not show the presence of omega-3 fatty acids such as ALA, EPA, DPA and DHA.

Table 4: Adipose tissue fatty acid profile (%) of rats fed control and treated groups.

Fatty Acid Profile	Control	GNO	GNO + PS	GNO + ALA	GNO + PSE-LD	GNO + PSE-HD
Myristic acid (C 14:0)	3.39	3.14	3.66	2.23	2.3	3.16
Palmitic acid (C 16:0)	26.87	25.31	29.45	25.97	23.77	28.23
Palmitoleic acid (C 16:1)	5.01	5.13	5.77	5.48	3.77	5.22
Stearic acid (C 18:0)	2.39	2.27	2.04	2.45	2.48	2.21
Oleic acid (C 18:1)	37.33	37.83	34.85	38.93	38.83	35.16
Linoleic acid (C 18:2, ω-6)	24.78	26.03	23.94	24.07	27.59	24.35
Alpha linolenic acid (C 18:3, ω-3)	ND	ND	ND	0.58	0.93	1.44
Total ω-6	24.78	26.03	23.94	24.07	27.59	24.35
Total ω-3	NA	NA	NA	0.58	0.93	1.44
Ratio: ω-6/ω-3	NA	NA	NA	41.5	29.66	16.9

ND-Not detected, NA-Not applicable, For abbreviations of different treatment groups please refer Table 1.

Fatty acid composition of adipose tissue

Adipose tissue is the organ where the lipid is stored. Among the omega-3 fatty acids, ALA alone was present in adipose tissue, and it was increased by 0.58-1.44 times in the following groups GNO+ALA, GNO+PSE-LD and GNO+PSE-HD (Table 4). The ratio of omega-6 to

omega-3 fatty acids was 26.03 for GNO alone treated group, whereas the ratios were in the range of 16.90–41.50 for GNO+ALA, GNO+PSE-LD and GNO+PSE-HD treated groups.

Fatty acid composition of serum

The ratio of omega-6 to omega-3 fatty acids was 39.87 in GNO alone treated groups while the ratio came down to 16.89-33.43 in GNO+ALA, GNO+PSE-LD and GNO+PSE-HD treated groups in serum (Table 5). The ALA content was increased by 0.36-0.86 times and DHA content was increased by 0.64-1.05 times in GNO+ALA, GNO+PSE-LD and GNO+PSE-HD treated group. The GNO+ALA, GNO+PSE-LD and GNO+PSE-HD treated groups showed EPA and DPA content increased by 0.10-0.25 and 0.15-0.35 times. The omega-3 fatty acids such as ALA, EPA, DPA and DHA were absent in the GNO alone treated groups.

Table 5: Serum fatty acid profile (%) of rats fed control and treated groups.

Fatty Acids Profile	Control	GNO	GNO + PS	GNO + ALA	GNO + PSE-LD	GNO + PSE-HD
Myristic acid (C 14:0)	0.82	0.67	0.52	0.62	0.61	0.88
Palmitic acid (C 16:0)	23.04	20.83	18.66	19.8	19.61	20.25
Palmitoleic acid (C 16:1)	2.1	2.73	2.01	2.24	1.36	1.84
Stearic acid (C 18:0)	5.83	6.92	7.15	6.5	6.98	6.57
Oleic acid (C 18:1)	23.56	28.94	29.35	27.75	25.95	25.49
Linoleic acid (C 18:2, ω-6)	23.28	25.1	26.05	25.52	27.6	28.67
Alpha linolenic acid (C 18:3, ω-3)	ND	ND	ND	0.36	0.51	0.86
Arachidonic acid (C 20:4, ω-6)	18.94	14.77	16.24	16.27	15.95	13.74
Eicosapentaenoic acid (C 20:5, ω-3)	ND	ND	ND	0.1	0.16	0.25
Docosapentaenoic acid (C 22:5, ω-3)	ND	ND	ND	0.15	0.24	0.35
Docosahexaenoic acid (C 22:6, ω-3)	ND	ND	ND	0.64	0.96	1.05
Total ω-6	42.22	39.87	42.29	41.79	43.55	42.41
Total ω-3	NA	NA	NA	1.25	1.87	2.51
Ratio: ω-6/ω-3	NA	NA	NA	33.43	23.28	16.89

ND-Not detected, For abbreviations of different treatment groups please refer Table 1.

Fatty acid composition of brain

The ratio of omega-6 to omega-3 fatty acids was 2.57 for GNO alone treated group in brain. However the ratio had come down to 1.26-1.63 for GNO+ALA, GNO+PSE-LD and GNO+PSE-HD treated group (Table 6). The GNO+ALA, GNO+PSE-LD and GNO+PSE-HD treated groups showed DHA content increased by 1.32-1.59 times, whereas the ALA content increased by 0.37-0.42 times. The GNO alone treated group did not show the presence of ALA. Rats fed ALA and PSE showed decrease in arachidonic acid (AA) content in the brain when compared with the GNO alone treated groups.

Table 6: Brain fatty acid profile (%) of rats fed control and treated groups.

Fatty Acids Profile	Control	GNO	GNO + PS	GNO + ALA	GNO + PSE-LD	GNO + PSE-HD
Myristic acid (C 14:0)	1.7	1.85	2.18	1.67	1.83	1.41
Palmitic acid (C 16:0)	32.23	33.03	29.75	30.16	30.52	28.08
Stearic acid (C 18:0)	18.56	18.38	17.83	18.78	18.87	18.88
Oleic acid (C 18:1)	27.88	24.7	28.77	26.74	25.74	28.44
Linoleic acid (C 18:2, ω-6)	1.73	2.06	1.85	1.84	1.84	1.89
Alpha linolenic acid (C 18:3, ω-3)	ND	ND	ND	0.4	0.37	0.42
Arachidonic acid (C 20:4, ω-6)	12.21	13.78	13.53	12.19	12.14	11.03
Docosahexaenoic acid (C 22:6, ω-3)	5.65	6.16	6.06	8.18	8.66	9.81
Total ω-6	13.94	15.84	15.38	14.03	13.98	12.92
Total ω-3	5.65	6.16	6.06	8.58	9.03	10.23
Ratio: ω-6/ω-3	2.46	2.57	2.53	1.63	1.54	1.26

ND-Not detected, NA-Not applicable, For abbreviations of different treatment groups please refer Table 1.

Fatty acid composition of eye

The treated groups GNO+ALA, GNO+PSE-LD and GNO+PSE-HD showed DHA and ALA contents were increased in the range of 1.52-2.19 and 0.39-1.10 times respectively in the eye (Table 7). The DPA content was increased by 0.15-0.38 times in GNO+ALA, GNO+PSE-LD and GNO+PSE-HD treated groups. The fatty acids ALA and DPA were absent in the GNO alone

treated groups. The ratio of omega-6 to omega-3 fatty acids was 3.61 for GNO alone treated group, whereas the ratio came down to 1.56-1.93 in the treated groups GNO+ALA, GNO+PSE-LD and GNO+PSE-HD. The eye fatty acid composition analysis revealed that AA content was slightly increased in the treated groups when compared with GNO alone treated group.

Table 7: Eye fatty acid profile (%) of rats fed control and treated groups.

Fatty Acids Profile	Control	GNO	GNO + PS	GNO + ALA	GNO + PSE-LD	GNO + PSE-HD
Myristic acid (C 14:0)	4.79	4.48	3.06	5.13	4.27	2.69
Palmitic acid (C 16:0)	32.87	32.81	30.78	32.86	29.15	30.17
Palmitoleic acid (C 16:1)	2.2	2.91	2.81	2.68	2.32	2.82
Stearic acid (C 18:0)	15.97	16.44	15.62	15.25	16.23	14.29
Oleic acid (C 18:1)	21.44	22.19	21.28	20.07	20.26	21.51
Linoleic acid (C 18:2, ω-6)	7.64	7.64	8.79	7.88	7.38	8.46
Alpha linolenic acid (C 18:3, ω-3)	ND	ND	ND	1.01	1.1	0.39
Arachidonic acid (C 20:4, ω-6)	9.03	8.91	10.47	7.93	9.55	9.19
Docosapentaenoic acid (C 22:5, ω-3)	ND	ND	ND	0.15	0.28	0.38
Docosahexaenoic acid (C 22:6, ω-3)	6.01	4.58	7.16	6.99	9.41	10.06
Total ω-6	16.67	16.55	19.26	15.81	16.93	17.65
Total ω-3	6.01	4.58	7.16	8.15	10.79	10.83
Ratio: ω-6/ω-3	2.77	3.61	2.68	1.93	1.56	1.62

ND-Not detected, For abbreviations of different treatment groups please refer Table 1.

Fatty acid composition of heart

The ratio of omega-6 to omega-3 fatty acids was 81.81 in the GNO alone treated group. However, the ratio came down to 19.61-26.65 in GNO+ALA, GNO+PSE-LD and GNO+PSE-HD treated groups in heart (Table 8). The groups treated with GNO+ALA, GNO+PSE-LD and GNO+PSE-HD had DHA and DPA content increased by 1.81-2.65 and 0.29-0.45 times respectively. The ALA content was increased by 0.36-0.53 times in GNO+ALA, GNO+PSE-LD and GNO+PSE-HD treated groups. The ALA, DPA and DHA were not detected in the GNO alone treated group. The levels of AA acid were decreased in rats fed GNO+ALA and GNO+PSE-HD.

Table 8: Heart tissue fatty acid profile (%) of rats fed control and treated groups.

Fatty Acids Profile	Control	GNO	GNO + PS	GNO + ALA	GNO + PSE-LD	GNO + PSE-HD
Myristic acid (C 14:0)	1.56	1.36	1.69	1.49	1.13	1.35
Palmitic acid (C 16:0)	20.55	17.38	15.24	17.89	15.25	15.97
Palmitoleic acid (C 16:1)	1.53	0.81	0.87	0.9	0.36	0.62
Stearic acid (C 18:0)	15.21	14.64	17.13	17.68	21.07	18.76
Oleic acid (C 18:1)	22.07	20.23	18.18	16.37	12.94	13.97
Linoleic acid (C 18:2, ω-6)	23.25	27.73	27.44	28.42	29.12	32.08
Alpha linolenic acid (C 18:3, ω-3)	ND	ND	ND	0.36	0.36	0.53
Arachidonic acid (C 20:4, ω-6)	15.22	17.27	18.93	15.56	17.82	14.79
Docosapentaenoic acid (C 22:5, ω-3)	ND	ND	ND	0.29	0.45	0.45
Docosahexaenoic acid (C 22:6, ω-3)	0.58	0.55	0.56	1.0	1.46	1.41
Total ω-6	38.47	45	46.37	43.98	46.94	46.87
Total ω-3	0.58	0.55	0.56	1.65	2.27	2.39
Ratio: ω-6/ω-3	66.32	81.81	82.8	26.65	20.67	19.61

ND-Not detected, For abbreviations of different treatment groups please refer Table 1.

DISCUSSION

The incorporation of PS, ALA and PSE did not have any adverse effect on the daily food consumption, gain in body weights of the treated groups when compared with the control group. The weight of liver did not differ significantly between the treated groups, except the GNO alone treated group. However, the weights of organs such as kidney, brain and heart did not differ significantly among the treated groups when compared with the GNO alone treated group (3). Phytosterol esters of FO fatty acids reduced the plasma lipid profile, but it did not alter the fragility in red blood cells (3). The comparative study of plasma and liver lipid profile of rats fed phytosterol esters of FO and LSO fatty acids showed reduced serum non-HDL-C and TAG levels (4). In the current study PS from PSE is the

reason for decrease in serum LDL cholesterol level in the treated groups. The ALA from PSE is the reason for the decrease in triglyceride level in the treated groups. The effect of FO and LSO phytosterol esters on brain lipid profiles were analyzed in hypercholesterolemic rats. The results showed an increase in cerebroside, ceramide and brain phospholipid content (5). Reduction in TC and TAG content on cardiac and aorta were also observed in rats fed FO and LSO phytosterol esters. The phospholipid content was increased in the ester treated groups (6).

The plasma lipid lowering effect and atherosclerotic lesions by phytosterol esters of DHA was studied in apo-E deficient mice (7). Phytosterol esters of DHA provide a synergistic effect in reducing the risk factor for the cardiovascular disease (CVD). The TAG-lowering effect of the PSE is credited to the ALA, which is later converted into EPA and DHA by undergoing series of enzymatic conversion. ALA is metabolized to stearidonic acid (SDA C18:4 n-3), which later gets converted into DHA via a serious enzymatic reaction. Similarly, LA is converted into gamma linolenic acid (GLA, C18:3, n-6), which later gets metabolized to AA.

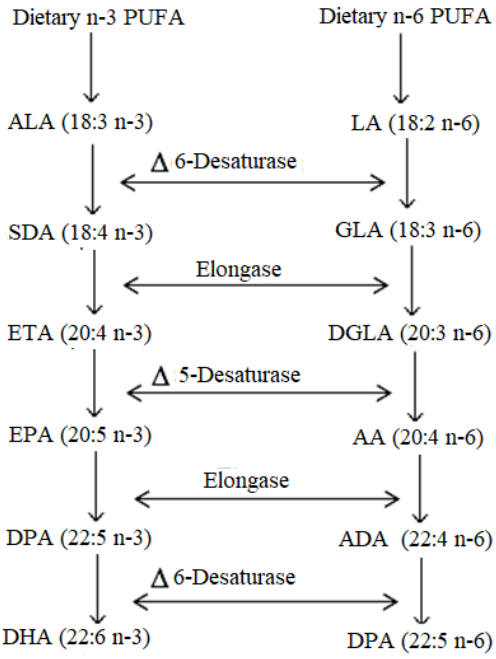


Fig.2: Schematic representation of different steps involved in metabolic pathway of omega 3 and omega 6 fatty acids in humans.

The metabolism of AA produces pro-inflammatory 2-series eicosanoids such as prostaglandins and thromboxanes via cyclooxygenase pathway (COX). Similarly, metabolism of AA via lipooxygenase pathway (LOX) generates leukotrienes 4-series. Metabolism of DHA and EPA generates anti-inflammatory 3-series prostaglandins and thromboxanes via COX pathway and 5-series leukotrienes via LOX pathway (20). The metabolic pathway in conversion of ALA (omega 3 fatty acid) and LA (omega 6 fatty acid) are given in Fig 2.

The TAG-lowering effect of omega 3 fatty acids are carried out by suppressing the hepatic expression of sterol regulatory element binding protein-1 (SREBP-1), which plays a major role in activating genes involved in fatty acid synthesis (20).

ALA-Alpha linolenic acid, LA-Linoleic acid, SDA-Stearidonic acid, GLA-Gamma linolenic acid, ETA-Eicosatrienoic acid, DGLA-Dihomo gamma linolenic acid, EPA-Eicopentaenoic acid, AA-Arachidonic acid, DPA-Docosapentaenoic acid, AdA-Adrenic acid, DHA-Docosahexaenoic acid.

The hydrolysis of phytosterol esters is necessary for the release of free sterols which play an important role in reducing the blood cholesterol levels (21). The Nieman-Pick C1 like 1 protein (NPC1L1) is required for the absorption of phytosterol in the enterocyte and the exact mechanism of absorption is still not known (2). The ATB binding cassette (ABC) proteins (ABCG5 and ABCG8) were involved in transporting the phytosterols back to the intestinal lumen. Therefore, it may be concluded that by regulating the gene expression of intestinal NPC1L1, ABCG5 and ABCG8 phytosterols may decrease the cholesterol absorption (2). The ALA-rich phytosterol esters did not have any role in the mRNA expression of NPC1L1, ABCG5 and ABCG8 genes in the intestine. Phytosterols esters of ALA decreased the cholesterol level independently of the genes expression involved in the absorption, influx and efflux transporters (22). However, the phytosterol esters may have a possible role in protein expression or may have a role in post-transcriptional activity on the sterol transporters or by inhibiting the 3-hydroxy-3-

methylglutaryl-CoA reductase (HMGR) which has a role in endogenous cholesterol synthesis (21).

The increase in hepatic antioxidant enzyme activity such as SOD, CAT, GSH and GPx, was observed in rats fed FO and LSO sitosterol esters (4). Even in the current study the increase in hepatic antioxidant enzyme activity such as SOD, CAT and GPx was attributed to the presence of ALA and PSE in liver. The brain and cardiac antioxidant enzyme activity such as SOD, CAT, GSH and GPx showed amelioration of antioxidant enzyme activities after treatment with phytosterols esters of FO and LSO (5-6). Omega 3 fatty acids have specific effects on gene expression by regulating the activity of transcription factor. The increase in antioxidant activity was carried out by regulating the transcription factors such as peroxisome proliferator activator receptors (PPARs), liver X receptor (LXRs), hepatic nuclear factor-4 (HNF-4), and sterol element binding proteins (SREBPs) (6). The decrease in MDA levels in the treated groups are attributed to the presence of ALA and PSE. The MDA levels in brain and cardiac tissue are decreased due to the presence of beta sitosterol esters of FO and LSO (5-6), which is agreeing with the current study.

In the current study, omega 3 fatty acid contents were increased in different tissues such as serum, liver, brain, eye and heart due to the presence of ALA and PSE in the treated groups. Feeding phytosterol esters of LSO and FO increased the overall PUFA content in brain (5). Adipose tissue can accumulate ALA, however the conversion of ALA into long chain PUFA such as EPA and DHA is limited (23, 24). Even in the current study ALA content was increased in the adipose tissue, however its elongation to EPA and DHA was limited. The EPA and DHA content was increased in serum fatty acid compositions of rats fed FO and LSO in different forms (23, 24). In the current study, feeding PSE increased the serum EPA and DHA content. The brain fatty acid composition of rats fed blended, transeseterified and structures lipid of native and blended linseed oil was studied. The brain DHA content was increased in the treated groups (23). In the present study, DHA content in the brain was increased even though ALA was given in the form of PSE.

The fatty acid composition of eye was analyzed for rats fed native, blended oil and structured lipid prepared using RBO and LSO showed DHA content was increased in the treated groups (24). Even in the current study, though ALA was given in the form of esters the DHA content was increased in eye in the treated group. The heart fatty acid composition of rats fed FO and LSO in different forms showed an increase in DHA content which is agreeing with the current study (23, 24). The incorporation of different omega-3 fatty acids such as ALA and DHA in different tissues may be attributed to the difference in uptake of these fatty acids from blood lipids that are released from adipose tissue via lipolysis. The D6 desaturase enzyme acts on both omega-3 and omega-6 fatty acids and both the fatty acids compete for the same enzyme. The linoleic acid (LA) is converted into AA by the action of D6 desaturation and D5 elongation, whereas the ALA is converted into EPA and DHA by the same D6 desaturation and D5 elongation enzymes. The hypolipidemic effect of phytosterol esters of FO and LSO fatty acids are well documented in various studies (3-7, 21). Whereas the tissue fatty acid composition of rats fed PSE of LSO were not documented. This study also throws light on the fatty acid composition of various tissues such as liver, adipose, serum, brain, eye and heart of rats fed GNO+ALA, GNO+PSE-LD, and GNO+PSE-HD. This is probable the first study shows the hypolipidemic activity of phytosterols obtained from Indian RBO DOD in free and in esterified form.

CONCLUSION

The enzymatically synthesized PSE was evaluated for its hypolipidemic effect in male wistar rats. Feeding PS, ALA and PSE did not have any adverse effect on diet consumption, body weight gain and feed efficiency ratio. Feeding PS, ALA and PSE did not have any adverse effect on organ weights. Groups fed PS, ALA and PSE decreased the TC, TAG and LDL-C content. The HDL-C content was increased in the groups fed PS, ALA and PSE. The antioxidant and lipid peroxidation activity of the groups fed PS, ALA and PSE

showed a positive effect. The omega-3 fatty acids such as ALA, EPA, DPA and DHA were increased in organs such as liver, serum, adipose, heart, eye and brain even though ALA was given in the form of PSE. The ratio of omega-6 to omega-3 fatty acid were high in the GNO alone treated group, whereas the ratios had decreased in GNO+ALA, GNO+PSE-LD and GNO+PSE-HD treated groups because of the presence of ALA in LSO and PSE.

The study did not focus on exploring the underlying synergistic mechanism of PSE at genetic level. Whether PSE may have role in gene expression or post transcriptional modification or modifying the protein expression still needs to be understood. This may be considered as one of the limitation of the study. This study can be extended to other DOD obtained from soybean or sunflower oil to evaluate its hypolipidemic activity. This is probably the first study which shows hypolipidemic, antioxidant activity and tissue fatty acid composition of PS (isolated from RBO DOD) and ALA in individual form and also in esterified form in dose dependent study.

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CONFLICT OF INTEREST

The authors claim no conflict of interest.

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