

Effects of the flaxseed lignans secoisolariciresinol diglucoside and its aglycone on serum and hepatic lipids in hyperlipidaemic rats

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The present study involved a comparative analysis of the effects of purified flaxseed lignans, secoisolariciresinol diglucoside (SDG) and its aglycone metabolite (SECO), in hyperlipidaemic rats. For hypercholesterolaemia, female Wistars (six rats per group) were fed a standard or 1% cholesterol diet and orally administered 0, 3 or 6 mg SDG/kg or 0, 1.6 or 3.2 mg SECO/kg body weight once daily for 4 weeks. Hypertriacylglycerolaemia was induced in male Sprague–Dawley rats (ten rats per group) by supplementing tap water with 10% fructose. These rats were orally administered 0, 3 or 6 mg SDG/kg body weight once daily for 2 weeks. Fasting blood samples (12 h) were collected predose and at the end of the dosing period for serum lipid analyses. Rats were killed and livers rapidly excised and sectioned for lipid, mRNA and histological analyses. Chronic administration of equimolar amounts of SDG and SECO caused similar dose-dependent reductions in rate of body-weight gain and in serum total and LDL-cholesterol levels and hepatic lipid accumulation. SDG and SECO failed to alter hepatic gene expression of commonly reported regulatory targets of lipid homeostasis. SDG had no effect on serum TAG, NEFA, phospholipids and rate of weight gain in 10% fructose-supplemented rats. In conclusion, our data suggest that the lignan component of flaxseed contributes to the hypocholesterolaemic effects of flaxseed consumption observed in humans. Future studies plan to identify the biochemical mechanism(s) through which flaxseed lignans exert their beneficial effects and the lignan form(s) responsible.

Purified flaxseed lignans: Hyperlipidaemia: Rats

Hypercholesterolaemia and hypertriacylglycerolaemia are independent risk factors of CVD and often occur in association with other CVD risk factors such as obesity, type 2 diabetes mellitus and elevated blood pressure⁽¹⁾. Therapeutic measures designed to reduce blood lipid parameters can decrease the risk for CVD⁽¹⁾. The need for very safe and effective lipid-lowering therapies directs significant attention toward flaxseed and its various products (for example, ground flaxseed, defatted flaxseed meal, flaxseed extracts) as sources of chemopreventive agents in CVD^(2,3). Recent human clinical trials and pathological animal model investigations with whole flaxseed or flaxseed products (for example, ground flax, flaxseed extracts, lignan-enriched flaxseed product) show clinically favourable changes in blood lipid profiles and other indices of lipid homeostasis with apparent safety^(4–8). Unfortunately, such studies cannot definitely ascribe the lipid-lowering effects of flaxseed consumption to any single flaxseed component. Whole flaxseed and its products may contain variable levels of fat (primarily α -linolenic acid), dietary fibre and soluble protein as well as lignans,

minerals, vitamins and carbohydrates⁽⁹⁾, which confound interpretations and probably explain the variable results reported with the use of such materials. Studies using enriched flaxseed lignan sources draw a clearer association between flaxseed lignans and the beneficial lipid-lowering effects of flaxseed^(3,10,11). However, optimisation of safety and efficacy of use of a particular chemopreventive therapy require a guarantee of product standardisation. Purified bioactives provide the greatest assurance of quality for determination of safety and efficacy. Yet no study has used purified flaxseed lignan sources for assessments in hypercholesterolaemic and hypertriacylglycerolaemic conditions.

Secoisolariciresinol diglucoside (SDG) is the principal lignan present in flaxseed. Following consumption, SDG is further converted to its aglycone secoisolariciresinol (SECO) and the mammalian lignans enterodiol and enterolactone within the intestine⁽¹²⁾. The literature provides little convincing evidence to attribute biological activity to either the plant or mammalian lignans or both. A comparative analysis of individual pure lignans in pathological lipid states is critical

Abbreviations: SDG, secoisolariciresinol diglucoside; SECO, secoisolariciresinol.

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to confirm which specific lignan(s) contributes to the lipid-lowering effects of flaxseed. However, availability of pure lignan is an important barrier to such assessments.

Assurance of lignan safety and efficacy also requires an understanding of their biological mechanism of action. The literature offers several explanations for lignan beneficial health effects, which include direct antioxidant activity⁽¹³⁾ and weak oestrogenic or anti-oestrogenic activity⁽¹⁴⁾. Very limited attention addresses potential presystemic mechanisms (i.e. inhibition of cholesterol absorption) or the potential of flaxseed lignans to influence transcriptional regulation of those genes involved in overall hepatic lipid homeostasis. Precedence exists for antihyperlipidaemic effects subsequent to transcriptional regulation of those factors controlling lipid homeostasis^(15–18). For example, fibrates⁽¹⁸⁾, statins⁽¹⁶⁾, soya isoflavones⁽¹⁷⁾ and SDG⁽³⁾ activate PPAR to mediate the hypolipidaemic effects associated with these compounds. Additionally, sesame lignans alter gene expression of enzymes involved in fatty acid oxidation and synthesis⁽¹⁵⁾. Such examples may identify a similar role for flaxseed lignans in the liver.

A definitive identification of the bioactive lignan form and its underlying mechanism of action are critical to our understanding of flaxseed efficacy and safety in the management of CVD risk factors. The purpose of the present study was to separately administer SDG and its metabolite, SECO, to diet-induced dyslipidaemic rats and compare the extent to which each lignan decreased serum and hepatic lipid levels in hyperlipidaemic rats. In addition, we used quantitative gene expression analysis to provide a preliminary assessment as to whether SDG and/or SECO alter the expression of several regulators of lipid homeostasis to possibly explain their hypolipidaemic effects with chronic oral administration.

Methods

Animals and chemicals

Male and female Sprague–Dawley and Wistar rats were obtained from Charles River Canada (St Constant, Quebec) at age 6 or 8 weeks and male Wistar rats (*n* 2) were obtained from the Animal Resources Centre (University of Saskatchewan, Saskatoon, SK) at age 8 weeks. On the date of arrival, rats were randomly assigned to either control or dietary intervention and according to lignan dose. Rats were housed in groups of two or three or individually (depending on experiment) with controlled temperature ($22 \pm 2^\circ\text{C}$) and were maintained on a 12 h light–dark cycle (lights on from 07.00 to 19.00 hours). All rats were fed a standard rodent diet (Prolab[®] RMH 3000; Purina, Inc., Richmond, IN, USA) (22.5% protein; 4.5% fat, ether extract; 6.4% fat, acid hydrolysis; 4.0% crude fibre; 6.1% ash; 1.0% Ca; 0.75% P), with or without diet supplementation depending upon the dietary intervention, and provided water *ad libitum*. Rats were acclimatised to animal handling and oral dosage administration procedures for a period of 1 week before the start of the chronic dosing protocol. All procedures were conducted in accordance with the Canadian Council of Animal Care guidelines for the care and use of laboratory animals and were approved by the Animal Care and Supply Committee of the University of Saskatchewan.

SDG (> 99% pure) and SECO (> 95% pure) were kind gifts from Agriculture and AgriFood Canada (A. D. M.). RNeasy Midi kits were obtained from Qiagen Inc. (Mississauga, ON, USA) and RNAlater[™] solution was from Sigma–Aldrich (St Louis, MO, USA). For real-time RT-PCR we used a Quanti Tect SYBR Green RT-PCR Kit from Qiagen Inc. Commercial kits for total cholesterol, TAG, NEFA, phospholipids and HDL-cholesterol were from Stanbio Laboratory (Boerne, TX, USA) or Wako Chemicals USA, Inc. (Richmond, VA, USA). All other chemicals not otherwise specified were obtained from Sigma–Aldrich.

Dose-dependent effects with diet-induced hypercholesterolaemia study design

A pilot study was conducted to identify the appropriate rat strain and sex to compare lignan effects on hypercholesterolaemic rats. For the pilot study male and female Sprague–Dawley (*n* 3) and female Wistar rats (*n* 3) at age 6 weeks and male Wistar rats (*n* 2) at age 8 weeks were evaluated (as indicated for the main study below) and the appropriate sex and strain employed. Our pilot studies showed significant strain and sex differences in serum lipid parameters with SDG administration. After 4 weeks, only female Wistar and Sprague–Dawley control rats demonstrated serum total cholesterol beyond the normal range (250–700 mg/l), and with SDG administration only female Wistar rats demonstrated distinct reductions in serum total cholesterol (48%) and LDL-cholesterol (61%) (data not shown). Based on the results of the pilot study, female Wistar rats were selected for the subsequent study.

For the main study, upon arrival rats were randomised to either the standard rodent diet or a 1% cholesterol diet (1% cholesterol incorporated into Prolab[®] RMH 3000; Purina, Inc., Richmond, IN, USA). Following the 1-week acclimatisation period, rats on the same diet were randomised to one of six treatment groups (*n* 6/group) (0, 3 or 6 mg SDG/kg body weight or 0, 1.6 or 3.2 mg SECO/kg body weight). All rats were weighed daily and lignan (SDG or SECO) or vehicle (0.9% sodium chloride for SDG; 50:50 0.9% sodium chloride–polyethylene glycol 400 for SECO) (0.2 ml) was administered orally once daily. After a 12 h fast blood samples were collected under isoflurane anaesthesia at 0 (pre-dose baseline) and 2 weeks via saphenous venepuncture and at 4 weeks by cardiac puncture into sterilised centrifuge tubes containing no anticoagulant for serum lipid analyses. All blood samples were collected between 09.30 and 10.30 hours. At 4 weeks, rats were euthanised by an overdose of isoflurane anaesthesia and exsanguination. Livers were rapidly excised and sectioned for lipid, RNA and histological analyses. Samples for RNA analyses were stored in RNAlater[™] solution at -20°C . Segments of liver were snap-frozen in liquid N₂ and fixed in 10% formalin for routine histology.

We assessed food intake (standard diet) in individually housed female Wistars in vehicle control (*n* 6) and weight-matched SDG-treated (*n* 6) rats to determine whether SDG may alter food consumption. Each animal was administered a 0.2 ml volume of vehicle (0.9% sodium chloride) or SDG (6 mg SDG/kg body weight, dissolved in 0.9% sodium chloride) daily by oral administration for 4 weeks. Each animal was weighed daily and food intake was measured every

other day. SDG did not alter food intake (data not shown) at the highest dosing level.

To assess general lignan safety, blood was collected for haematology and blood chemistry for evaluation of SDG's effect (daily administration of 6 mg SDG/kg body weight for 4 weeks) on renal, hepatic and immune systems. Tissues were rapidly excised following euthanasia, weighed and assessed by gross morphological examination. Complete blood chemistry and haematology were performed in the clinical chemistry laboratory at the Royal University Hospital (Saskatoon, Saskatchewan, Canada).

Dose-dependent effects with diet-induced hypertriacylglycerolaemia study design

For consistency of the model system, initial studies were conducted with female Wistar rats (eight rats per group) obtained at age 6 weeks. We failed to induce hypertriacylglycerolaemia in female Wistars following 4 weeks of 10% fructose in water. Hence, we used the well-known male Sprague–Dawley rat model of hypertriacylglycerolaemia. Male Sprague–Dawley rats were housed individually and, after the acclimatisation period, 10% fructose in water replaced the regular tap water in those groups requiring fructose feeding. Animals were randomly assigned to four treatment groups (ten rats per group): (1) tap water; (2) 10% fructose in tap water; (3) 10% fructose in tap water with single daily dose of 3 mg SDG/kg body weight; (4) 10% fructose in tap water with a single daily dose of 6 mg SDG/kg body weight. Rats were weighed daily and SDG or vehicle (0.9% sodium chloride) was orally administered once daily within a 2 h period at the same time each day. Blood samples were collected following a 12 h fast under isoflurane anaesthesia at 0 (pre-dose baseline) weeks via saphenous venepuncture, and at 2 weeks via cardiac puncture into sterilised centrifuge tubes containing no anticoagulant for serum lipid analyses. All blood samples were collected within a 2 h period beginning at 06.00 hours. At 2 weeks, rats were euthanised by an overdose of isoflurane anaesthesia and exsanguination. Liver, heart and retroperitoneal adipose tissue were rapidly excised and weighed. Liver samples for RNA analysis were stored in RNAlater™ solution at –20°C. Portions of liver were flash-frozen in liquid N₂ and stored in –80°C and fixed in 10% formalin solution for routine histology.

Analyses of lipids

Serum total cholesterol, TAG, NEFA, phospholipids and HDL-cholesterol were determined by colorimetric analysis with commercial kits from Stanbio (Boerne, TX, USA) or Wako Chemicals USA, Inc. (Richmond, VA, USA) immediately following serum collection according to the manufacturer directions. LDL-cholesterol levels were determined using the Friedewald method⁽¹⁹⁾.

Histological analyses

Formalin fixed liver samples were embedded in paraffin, sectioned at 5 µm, stained with haematoxylin–eosin and examined by light microscopy. Determination of the extent of hepatic lipodosis was performed on oil red O-stained frozen

formalin-fixed tissue sections using image analysis software (Northern Eclipse v. 6.0; Empix Imaging, Mississauga, ON, Canada). From each liver sample, four digital images (each representing 3.69 mm² of hepatic section) were captured by camera (DVC-1310C; Digital Video Camera Company, Austin, TX, USA) using a light microscope (Olympus BH-2; Olympus Optical Co. Ltd, Tokyo, Japan) with a 6.3 × objective lens (Plan 6.3/0.16; Carl Zeiss, Jena, Germany). The area of empty spaces representing empty sinusoids and vessels as well as artifactual tissue separation was selected, determined and subtracted from the total area of the digital image to obtain the total area of hepatic parenchyma (hepatocytes and supportive stroma). The total area of oil red O staining was selected and determined to calculate the percentage of hepatic parenchyma accumulating oil red O-stained fat. The average percentage of hepatic parenchyma affected by hepatic lipodosis was derived from four digital images for each rat. All hepatic tissue sections were coded and analysed blindly by an American College of Veterinary Pathologists (ACVP) board certified pathologist without knowledge of treatment groups and diets.

RNA analyses

RNeasy Midi RNA isolation kits were used to isolate total cellular RNA from liver following the manufacturer's protocol. Quantitative real-time RT-PCR analyses were carried out using a SmartCycler® (Cepheid, Sunnyvale, CA, USA) and a Quanti Tect SYBR Green RT-PCR Kit (Qiagen). Primer sequences (Table 1) were designed using Primer3 software (www.broad.mit.edu/cgi-bin/primer/primer3). Sequences, product sizes and optimal annealing temperatures are listed in Table 1. The general real-time RT-PCR protocol for the SmartCycler for analysis of molecular targets is given in the footnotes of Table 1. Gene expression was quantified using the standard curve method for relative quantification.

Statistical analyses

All statistical analyses were conducted using GraphPad Prism 3.0 (GraphPad, San Diego, CA, USA). All results are expressed as mean values with their standard errors, except liver histological data, which are expressed as medians with their standard errors. Within a lignan treatment, comparisons between treatment groups for each diet were made using one-way ANOVA with a Tukey's post-test. A Kruskal–Wallis test (non-parametric one-way ANOVA) with a Dunn's post-test were used for the analysis of histological data from SDG-treated rats. Non-parametric analysis was employed due to a bimodal distribution in rats treated with 6 mg SDG/kg body weight. Comparisons between the lignan administered and treatment group after 4 weeks of lignan administration were made using two-way ANOVA with a Bonferroni post-test. For the food intake study, differences between mean daily food intake and rate of weight gain were tested using an unpaired two-tailed Student's *t* test. Effects were considered statistically significant when the *P* value was less than 0.05.

Table 1. Primer sequences, amplicon sizes and optimisation parameters (annealing temperature and primer efficiency) for real-time RT-PCR analysis of targets important in cholesterol and TAG pathways*

Gene	Accession number	Sense primer	Antisense primer	Product size (bp)	Temperature (°C)
ACAT2	AB075946	caggacaccagcatcagg	aggatggacagcagcagagc	162	58
ApoE	NM138828	cgcggtgtgagtctatcc	acctcctctaggaggctcgc	179	57
β -Actin	NM031144	agcgtggctacagcttcacc	tgccacaggattccataccc	237	58
Cyp7a1	NM012942	gggcatctcaagcaaacacc	gcactggaaagcctcagagc	226	55
HMGCR	X55286	ggatgcagcacagaatgtgg	acgcccctgaacacctagc	182	57
LDLR	X13722	cccactcaccgaagtccacc	agctgaggatcctccttgc	176	56
PPAR- γ	AB011365	tggggatgtctcacaatgcca	ttcctgtcaagatgcacctcg	200	58
SREBP-2	XM216989	cgcaaccagctttcaagtcc	agcgactgtctgactgtgg	175	57
PPAR- α	M88592	aaggctatcccaggcttgc	gatgtcgcagaatggctcc	189	57
SREBP-1c	L16995	gtgctcagcttggcagctgg	atgcctcggtatgtgaagg	236	57

ACAT2, acetyl-CoA acetyltransferase 2; Cyp7a1, cytochrome P450 7a1; HMGCR, HMG-CoA reductase; LDLR, LDL receptor; SREBP, sterol response element binding protein.

* General real-time RT-PCR protocol for the analysis of mRNA levels: reverse transcription, 30 min at 50°C; PCR, initial activation step at 95°C for 15 min, denaturation at 94°C for 15 s, annealing at temperatures indicated above for 30 s, extension at 72°C for 30 s.

Results

Comparison of secoisolariciresinol diglucoside and secoisolariciresinol in hypercholesterolaemia

SDG (3.0 and 6.0 mg/kg body weight) and SECO (1.6 mg/kg body weight) decreased rate of weight gain in standard diet-fed female Wistar rats (Table 2). Both low- and high-dose SDG and SECO decreased rate of weight gain in 1% cholesterol-fed female Wistar rats (Table 3). High-dose SECO also decreased absolute liver weight (Table 2). The differences in weight parameters corresponded to a greater degree of mesenteric fat accumulation and diffuse yellow discoloration of the liver (suggesting hepatic lipidosis) in 1% cholesterol-fed control rats relative to lignan-treated rats (data not shown). No changes in body weight and mesenteric fat accumulation were observed in rats fed a standard diet treated with either SDG or SECO (data not shown).

Serum lipid parameters after 4 weeks of SDG or SECO treatment remained unchanged in female Wistar rats fed a standard diet (data not shown). In rats fed the 1% cholesterol diet, 3 and 6 mg SDG/kg decreased total cholesterol by 8 and 27%, respectively ($P > 0.05$), and LDL-cholesterol by 11 and

39%, respectively ($P > 0.05$) (Fig. 1). Similarly, 1.6 and 3.2 mg SECO/kg decreased total cholesterol by 16 and 15%, respectively ($P > 0.05$), and LDL-cholesterol by 24 and 34%, respectively ($P > 0.05$) (Fig. 1). Equimolar administrations of SDG and SECO resulted in a similar magnitude of reduction in serum total cholesterol and LDL-cholesterol in 1% cholesterol-fed female Wistar rats.

In rats fed a diet supplemented with 1% cholesterol, livers exhibited a distinct yellow discoloration on gross examination. This was not observed in rats fed a standard diet with or without SDG or SECO administration. In rats fed the 1% cholesterol diet, 3 and 6 mg SDG/kg decreased hepatic fat accumulation by 8 and 24%, respectively. However, hepatic fat accumulation increased by 7% with 1.6 mg/kg SECO, but decreased by 24% following 3.2 mg/kg SECO (Fig. 2). Figure 3 illustrates hepatic accumulation of lipids (as visualised by oil red O staining) in hypercholesterolaemic control rats but a decreased amount of hepatic lipids in rats treated with lignan. The variable degree of hepatic lipidosis had positive and significant correlations ($r > 0.44$; $P < 0.05$) with serum lipid parameters of the same rats (data not shown). No other histopathological changes were observed in the livers of these rats.

Table 2. Body and liver weight indices for female Wistar rats (n 6 per treatment group) fed standard rat chow (5 weeks) and treated with oral doses of 0.0, 3.0 or 6.0 mg secoisolariciresinol diglucoside (SDG)/kg body weight (BW) or 0.0, 1.6 or 3.2 mg secoisolariciresinol (SECO)/kg BW once daily (same time of day) for 4 weeks§||

(Mean values with their standard errors)

Weight parameter	SDG (mg/kg BW)						SECO (mg/kg BW)					
	0.0		3.0		6.0		0.0		1.6		3.2	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Final BW (g)	250	5.0	247	7.9	228*	1.8	257	10	263	5.9	251	6.8
Absolute weight gain (g)	47.5	4.4	47.5	4.0	36.0	3.8	87.3	5.1	88.2	5.2	97.8	6.1
Rate of weight gain (g/d)	1.76	0.035	1.43*	0.006	1.28*†	0.029	3.25	0.07	3.04*	0.046	3.29*‡	0.056
Liver weight (g)	7.38	0.43	7.17	0.42	6.47	0.34	8.43	0.61	8.60	0.49	8.45	0.23
Liver weight/BW (g/kg)	29.4	1.4	29.0	1.2	28.3	1.5	32.7	1.3	32.6	1.5	33.8	1.4

* Mean value was significantly different from that of the control group ($P < 0.05$).

† Mean value was significantly different from that of the group that received SDG at 3.0 mg/kg BW ($P < 0.05$).

‡ Mean value was significantly different from that of the group that received SECO at 1.6 mg/kg BW ($P < 0.05$).

§ Data were analysed with one-way ANOVA with Tukey's post-test.

|| On a molar basis the doses are equivalent to 0.0, 4.4 and 8.8 μ mol/kg BW.

Table 3. Body and liver weight indices for female Wistar rats (*n* 6 per treatment group) fed a 1% cholesterol diet (5 weeks) and treated with oral doses of 0.0, 3.0 or 6.0 mg secoisolariciresinol diglucoside (SDG)/kg body weight (BW) or 0.0, 1.6 or 3.2 mg secoisolariciresinol (SECO)/kg BW once daily (same time of day) for 4 weeks†§

(Mean values with their standard errors)

Weight parameter	SDG (mg/kg BW)						SECO (mg/kg BW)					
	0.0		3.0		6.0		0.0		1.6		3.2	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Final BW (g)	269	4.9	260	4.4	257	3.7	277	11	271	6.9	261	4.1
Absolute weight gain (g)	59.0	6.7	55.5	5.1	57.2	0.60	110	5.8	103	3.2	102	2.9
Rate of weight gain (g/d)	2.06	0.080	1.96*	0.03	1.95*	0.040	4.01	0.08	3.54*	0.060	3.63*†	0.040
Liver weight (g)	9.25	0.35	8.37	0.16	8.15	0.44	11.0	0.36	9.52	0.57	9.33*	0.22
Liver weight/BW (g/kg)	34.2	1.6	32.3	0.88	31.6	1.4	39.7	0.86	35.2	2.3	35.7	0.30

* Mean value was significantly different from that of the control group ($P < 0.05$).

† Mean value was significantly different from that of the group that received SECO at 1.6 mg/kg BW ($P < 0.05$).

‡ Data were analysed with one-way ANOVA with Tukey's post-test.

§ On a molar basis the doses are equivalent to 0.0, 4.4 and 8.8 $\mu\text{mol/kg}$ BW.

Hepatic enzymes and proteins involved in cholesterol metabolic and uptake pathways were selected for analysis based on significant ability to regulate serum lipid levels as identified in the literature. For hypercholesterolaemia the targets included transcription factors (sterol response element binding protein (SREBP)-2 and PPAR- γ), the cholesterol metabolic enzymes (HMG-CoA reductase, cytochrome P450 7a1 (Cyp7a1) and acetyl-CoA acetyltransferase 2 (ACAT2)) and proteins involved in cholesterol hepatic uptake and transport (LDL

receptor and apoE). SDG administration to rats fed a diet supplemented with 1% cholesterol resulted in a 54 and 66% increase ($P < 0.05$) in ACAT2 expression at 3 and 6 mg/kg, respectively (Table 4). No statistically significant changes in gene expression were observed for the other targets. Following the 3.2 mg SECO/kg dosing administration, Cyp7a1 expression decreased by 72% ($P < 0.05$). No other significant changes in expression levels were observed for the other targets (Table 4).

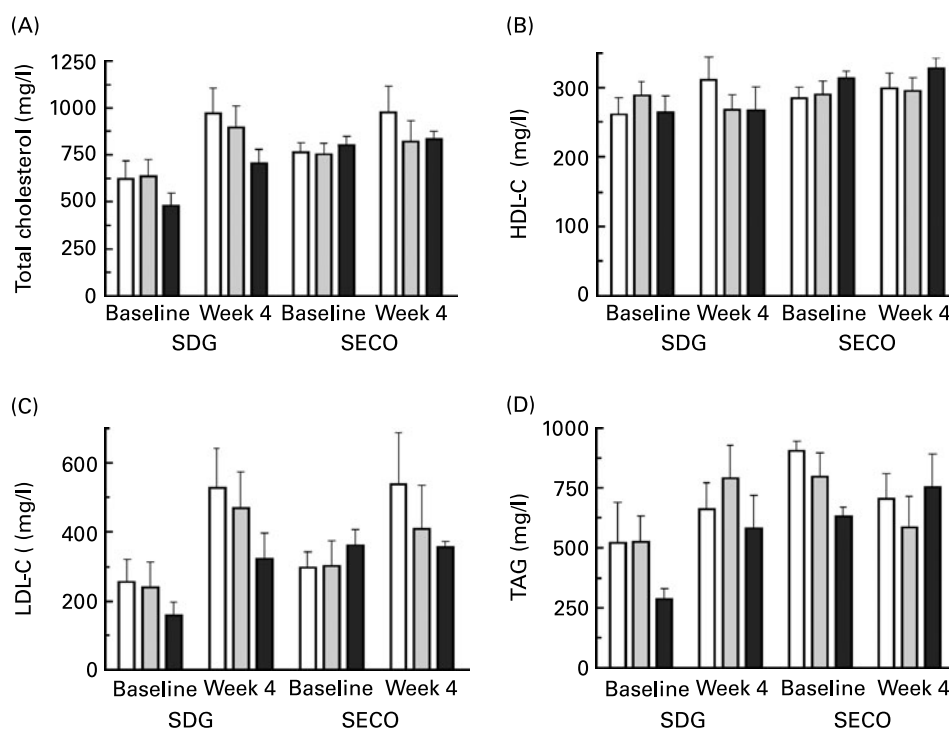


Fig. 1. Serum lipid parameters (total cholesterol (A), HDL-cholesterol (HDL-C) (B), LDL-cholesterol (LDL-C) (C) and TAG (D)) following chronic daily oral secoisolariciresinol diglucoside (SDG) or secoisolariciresinol (SECO) administration in female Wistar rats fed a 1% cholesterol diet for 1 week before initiation of SDG or SECO dosing and during the lignan dosing interval (4 weeks). Rats (*n* 6) were randomly assigned to 0.0 (□), 3.0 (▨) or 6.0 (■) mg SDG/kg body weight or 0.0 (□), 1.6 (▨) or 3.2 (■) mg SECO/kg body weight. (Note that on a molar basis the doses are equivalent to 0, 4.4 and 8.8 $\mu\text{mol/kg}$ body weight.). SDG or SECO was administered orally once daily (same time of day). Blood was collected from the same animal following an overnight fast via saphenous venepuncture under isoflurane anaesthesia just before initiation of dosing (baseline) and then again at 4 weeks. Values are means, with their standard errors represented by vertical bars.

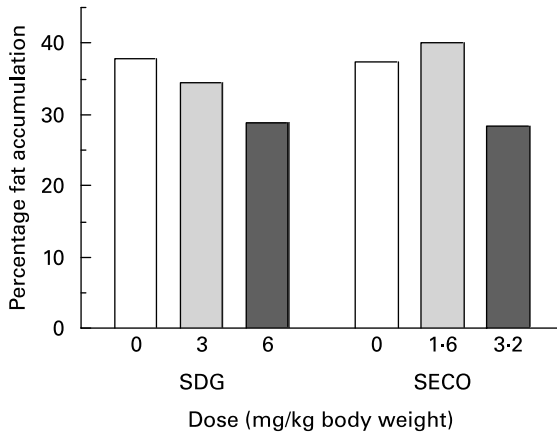


Fig. 2. Median percentage fat accumulation (*n* 6) in hepatic parenchyma of female Wistar rats fed a 1% cholesterol diet for 5 weeks. Rats were randomly assigned to 0.0, 3.0 or 6.0 mg secoisolariciresinol diglucoside (SDG)/kg body weight or 0.0, 1.6 or 3.2 mg secoisolariciresinol (SECO)/kg body weight administered orally once daily for 4 weeks. (Note that on a molar basis the doses are equivalent to 0, 4.4 and 8.8 μ mol/kg body weight.) Histological evaluation of oil red O-stained liver slices involved a semi-quantitative blinded analysis and fat accumulation was measured by digital analysis of stained liver sections.

Effects of secoisolariciresinol diglucoside in hypertriacylglycerolaemia

SDG had no effect on the weight parameters of male Sprague–Dawley rats supplemented with 10% fructose in tap water relative to controls (Table 5). Analysis of serum TAG, NEFA and phospholipids showed that 2 weeks of supplementation with 10% fructose in water induced hypertriacylglycerolaemia in male Sprague–Dawley rats (data not shown). However, no statistically significant changes in serum TAG, phospholipids or NEFA were observed with SDG administration relative to non-supplemented rats (data not shown). In rats supplemented with 10% fructose in tap water, livers

exhibited a distinct yellow discolouration on gross examination, but SDG had no effect on hepatic lipids (data not shown). The 10% fructose in water supplementation resulted in an expected down-regulation of PPAR- α ⁽²⁰⁾ and up-regulation of SREBP-1c⁽²¹⁾, but SDG administration had no impact on expression levels relative to 10% fructose in water-supplemented controls (Table 6).

Safety of orally administered secoisolariciresinol diglucoside

Daily oral administration of SDG (3 mg/kg body weight) for 4 weeks caused no adverse health effects or changes in body or organ weights in female Wistar juvenile rats (data not shown). Haematology and blood chemistry analysis also showed no changes, except in the erythrocyte distribution width (data not shown). However, the values for erythrocyte distribution width in both groups were within the normal range.

Discussion

The present study represents the first comparative analysis of the effects of purified flaxseed plant lignans in hyperlipidaemic rat models. Many studies have investigated the lipid-lowering effects of whole flaxseed or flaxseed products^(4–7,11,22), and one study reported the effects of purified SDG on serum lipid parameters in high-fat diet-induced obese mice⁽³⁾. In the present study, we evaluated several indices of lipid homeostasis in diet-induced hypercholesterolaemic and hypertriacylglycerolaemic rat models, including serum lipid parameters, body and liver weight, steatosis, and transcriptional changes in commonly reported targets of hepatic lipid homeostasis. We assessed these indices to compare the activity of purified SDG *v.* its aglycone metabolite, SECO, on lipid homeostasis and to glean some preliminary information on possible mechanisms of action. Our data suggest that chronic

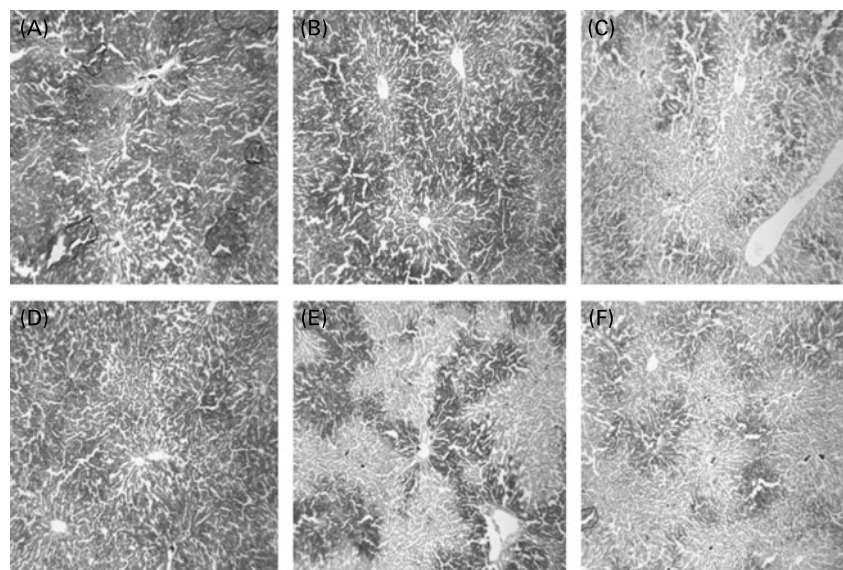


Fig. 3. Oil red O-stained frozen hepatic tissue sections to identify the extent of hepatic lipidosis. Female Wistar rats (*n* 6) were fed a 1% cholesterol diet and randomly assigned to 0.0, 3.0 or 6.0 mg secoisolariciresinol diglucoside (SDG)/kg body weight (A, B or C, respectively) or 0.0, 1.6 or 3.2 mg secoisolariciresinol (SECO)/kg body weight (D, E or F, respectively).

Table 4. Percentage changes in hepatic mRNA expression levels of key genes involved in cholesterol metabolism in female Wistar rats (*n* 6) fed a 1% cholesterol diet and treated with daily oral administration of secoisolariciresinol diglucoside (SDG) or secoisolariciresinol (SECO)†

Gene	SDG (mg/kg BW)		SECO (mg/kg BW)	
	3.0	6.0	1.6	3.2
ACAT2	↑ 54%*	↑ 66%*	0%	↓ 7%
ApoE	↓ 35%	↓ 21%	↑ 35%	↓ 0.6%
Cyp7a1	↓ 7%	↑ 10%	↓ 36%	↓ 71%*
HMGCR	↓ 27.5%	↑ 35%	↑ 8%	↓ 6%
LDLR	↓ 32%	↑ 6%	↓ 6%	↓ 24%
PPAR-γ	↓ 14%	↑ 2%	↑ 17%	↑ 17%
SREBP-2	↓ 27%	↓ 19%	↓ 15%	↓ 22%

BW, body weight; ACAT2, acetyl-CoA acetyltransferase 2; Cyp7a1, cytochrome P450 7a1; HMGCR, HMG-CoA reductase; LDLR, LDL receptor; SREBP-2, sterol response element binding protein-2.

* Change was significantly different from that of the control group ($P < 0.05$).

† Rats were randomly assigned to 0.0, 3.0 or 6.0 mg SDG/kg BW or 0.0, 1.6 or 3.2 mg SECO/kg BW dose groups and mRNA expression was assessed after 4 weeks using real-time reverse transcription-PCR.

administration of equimolar levels of SDG and SECO cause similar dose-dependent reductions in serum and hepatic cholesterol levels, but neither SDG nor SECO caused significant changes in hepatic gene expression of lipid homeostasis targets. Furthermore, SDG had no effect on serum TAG in our model of hypertriacylglycerolaemia.

We observed a dose-related decrease ($P > 0.05$) in total and LDL-cholesterol in rats supplemented with 1% cholesterol in their diet and dosed daily with SDG and SECO. SDG and SECO also decreased rate of weight gain and hepatic parenchymal fat accumulation in rats fed the high-cholesterol diet. These data provide supportive evidence that flaxseed lignans contribute to the serum and hepatic cholesterol-lowering effects of flaxseed observed in humans and in animal studies. Interestingly, similar reductions in serum total cholesterol and LDL-cholesterol (31 and 32%, respectively) in a diet-induced hypercholesterolaemic rabbit model⁽²³⁾ required 2.5 times higher SDG doses than the dose used in the present study. This may suggest that: (1) SDG exerts a greater cholesterol-lowering effect in rats; (2) significant interspecies differences

exist in the pharmacokinetics of flaxseed lignans; and/or (3) the effects of SDG may plateau with increasing dose. Although comparisons between the hypocholesterolaemic effects of purified lignans or lignan-enriched flaxseed products with other flaxseed products are difficult, the enriched lignan products generally produce greater reductions in serum lipid parameters than reported in the literature. Recently, significant reductions in serum cholesterol levels in hypercholesterolaemic patients administered a lignan-enriched (35% lignan) flaxseed polymer was reported⁽¹¹⁾, which exceeded reductions reported with other flaxseed products. One would expect to observe smaller reductions in serum lipid levels with lignan-enriched flaxseed products if the hypocholesterolaemic effects involved a synergism between two or more components of flaxseed. Our data support a role for the lignan component in mediating the hypocholesterolaemic effects associated with flaxseed consumption⁽¹¹⁾.

Carbohydrate-rich diets are known to stimulate lipogenesis in both the liver and adipose tissue to cause increased plasma and hepatic TAG levels⁽²⁴⁾. Supplementation with 10% fructose in tap water effectively induces hypertriacylglycerolaemia in male rats⁽²⁰⁾. SDG administration at either dosing level caused no significant changes in body and organ weights, serum TAG, phospholipids and NEFA, and hepatic steatosis relative to vehicle control fructose-fed rats. Given the similar dose-response effects of SDG and SECO in our high-cholesterol diet-fed rats and limited availability of purified SECO, we omitted SECO administration in this model of hypertriacylglycerolaemia. Interestingly, administration of flaxseed products in dyslipidaemic models reported in the literature has shown variable effects on serum TAG levels^(6,14,23,25,26). Human clinical trials also report variable effects of flaxseed consumption on serum TAG^(2,7,8,22,27,28). The present study contributes to this lack of consensus. Interestingly, a close structural analogue of SECO, masoprocol (nordihydroguaiaretic acid), significantly decreased serum TAG levels in a rat model of hypertriacylglycerolaemia⁽²⁹⁾. This offers intriguing evidence for a possible effect of SECO on TAG homeostasis and may depend upon the species, strain, age, sex and method of dietary manipulation used to induce hypertriacylglycerolaemic states.

Table 5. Weight indices for male Sprague-Dawley rats (*n* 10 per treatment group) supplemented with 0 or 10% fructose in tap water and treated with oral doses of 0.0, 3.0 or 6.0 mg secoisolariciresinol diglucoside (SDG)/kg body weight (BW) once daily (same time of day) for 2 weeks†‡

(Mean values with their standard errors)

Weight parameter	No fructose and no SDG		10% Fructose + SDG (mg/kg BW)					
			0.0		3.0		6.0	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Final BW (g)	281	6.3	306	7.6	285	5.9	296	7.9
Absolute weight gain (g)	91.0	13	100	38	83.0	32	106	20
Rate of weight gain (g/d)	6.90	0.080	8.40	0.10	6.90	0.10	7.90	0.10
Liver weight (g)	8.80	0.30	16.1*	0.83	15.2*	0.65	14.6*	0.92
Liver weight/BW (g/kg)	31.6	0.74	52.7*	2.4	53.4*	1.9	49.1*	2.1
Retroperitoneal adipose weight (g)	2.27	0.18	4.51*	0.61	3.63	0.36	3.68	0.35

* Mean value was significantly different from that of the no-fructose, no-SDG control group ($P < 0.05$).

† Data were analysed with one-way ANOVA with Tukey's post-test.

‡ On a molar basis the doses are equivalent to 0.0, 4.4 and 8.8 μmol/kg BW.

Table 6. Hepatic mRNA expression levels (expressed as percentage of non-fructose-supplemented control) of key transcription factors involved in hepatic TAG metabolism in male Sprague–Dawley rats (*n* 10) with or without supplementation of 10% fructose in tap water and treated with 0.0, 3.0 or 6.0 mg secoisolariciresinol diglucoside (SDG)/kg body weight (BW) after 2 weeks using real-time RT-PCR

Gene	No fructose and no SDG	10% Fructose in water + SDG (mg/kg BW)		
		0.0	3.0	6.0
PPAR- α	100	66.1	89.9	86.6
SREBP-1c	100	1640	1490	1010

SREBP-1c, sterol response element binding protein-1c.

We observed almost no statistically significant changes in hepatic gene expression following SDG or SECO administration. We evaluated targets that represented major regulatory control points in cholesterol or TAG homeostasis in the liver. The lack of lignan affect on the expression levels of these targets suggests that lignans act through other primary mechanism(s) not yet identified. Nevertheless, SDG and SECO seem to act with apparent safety at pharmacologically relevant doses, as daily SDG administration for 4 weeks resulted in no gross morphological changes or changes in body and organ weights, and no obvious toxicity to the liver, kidney and haematopoietic systems. These findings confirm the general safety of flaxseed lignans noted in the literature^(11,30).

In conclusion, our data provide supportive evidence that the lignan component of flaxseed contributes to the hypocholesterolaemic effects of flaxseed consumption observed in humans and animals. SDG and SECO caused similar dose-dependent reductions in serum and hepatic cholesterol profiles in high-cholesterol diet-fed rats, but had no effect on TAG levels in high-carbohydrate diet-fed rats. More studies are necessary to identify the exact biochemical mechanism(s) through which flaxseed lignans exert their beneficial effects and the exact identity of the active lignan form (i.e. plant or mammalian). Importantly, the pharmacokinetics of flaxseed lignans remain poorly understood, yet the biological activity and potential toxicity of lignans *in vivo* depend upon their pharmacokinetic characteristics. Identification of the mechanism through which flaxseed lignans improve lipid parameters, an understanding of lignan pharmacokinetics, and additional investigations into the comparative efficacy and safety of plant and mammalian lignans may lead to the emergence of flaxseed, or its purified lignan forms, as an invaluable therapeutic strategy in the management of hyperlipidaemic patients.

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