COMPARATIVE ORAL PHARMACOKINETICS AND EFFICACY OF FLAXSEED LIGNAN-PURIFIED SDG AND SDG POLYMER

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ABSTRACT

Cardiovascular disease (CVD) is a major contributor to chronic disability and death globally while hypercholesterolemia is a major independent risk factor. Because of adverse effects of currently used CVD drugs (e.g. statins), safer alternatives including natural products might be considered as options for treatment of hypercholesterolemia. Studies have shown that treatment with flaxseed lignan, mainly as secoisolariciresinol diglucoside (SDG), can safely decrease blood cholesterol. However, purified SDG production is expensive; processes that concentrate the natural SDG polymer to 50% or more SDG from flax yield a more cost effective product. Unfortunately, the relative bioavailability of pure SDG and SDG polymer are unknown and the bioactivity of these two forms of SDG are also unknown.

This study compared the relative pharmacokinetics of purified SDG and SDG polymer in a single oral dose (40 mg/kg SDG dose) in rat. The concentration of SDG and SDG polymer including secoisolariciresinol (SECO), enterodiol (ED), and enterolactone (ENL) were determined in plasma after administration. The aglycone SECO could only be detected in early plasma samples at 0.25 to 4 h after administration. Unconjugated ED was detected and quantified after 8 (mean ± SD: 3.4 ± 3.3 ng/mL) and 12 h (6.2 ± 3.3 ng/mL) in most subjects, while total ED (unconjugated ED and ED conjugate) was detected in plasma from 2 h to 16 h in most rats. The time of the maximum plasma concentration (T_{max}) of total ED in rats fed SDG was 11.7 ± 1.1 h and 10.9 ± 1.9 h for rats fed the SDG polymer. The maximum concentration of total ED (C_{max}) for rats fed SDG was 262.2 ± 170.8 ng/mL while for those fed SDG polymer C_{max} was 207.2 ± 115.5 ng/mL. Total enterolactone (ENL) was determined for all plasma samples. T_{max} of total ENL in rats fed purified SDG was 12.6 ± 1.5 h while for those fed SDG polymer T_{max} was 12.7 ± 3 h. C_{max} of total ENL in the purified SDG and SDG polymer fed rats was 81.6 ± 23.8 ng/mL, and 65.9 ± 19.6 ng/mL, respectively. The relative bioavailability of total ED and ENL of SDG polymer is 111% and 89%, respectively, when compared to purified SDG. No significant difference in C_{max}, T_{max}, and AUC of total ED and ENL of purified SDG and SDG polymer was found.

In addition, we investigated the effects of chronic daily oral purified SDG (6 mg/kg) or an equivalent dosage of SDG polymer in female Wistar rats fed a 1% cholesterol diet for 1 week

before initiation of purified SDG or SDG polymer doses for 23 days. A significant reduction in normalized liver weight was observed in the group treated with purified SDG when compared to high cholesterol control. Both purified SDG and SDG polymer not significantly, but clinically induced a reduction in serum TAG (19% and 15%, respectively) and increase in HDL-C (15% and 24%, respectively). Furthermore, hypercholesterolemic rats given purified SDG or SDG polymer had clinically lower scores in steatosis and non-alcoholic fatty liver disease activity (NAS), when compared to controls.

In conclusion, no differences in the absorption kinetics and total exposure of bioactive metabolites was observed between pure SDG and SDG polymer. Moreover, apparent lipid lowering effects were observed following purified SDG and SDG polymer administration, without significant differences between the two groups. Thus, the pharmacokinetic characteristics and hypocholesterolemic effect of SDG polymer warrant its further investigation.

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DEDICATED TO

My parents Hong, Guiyue and Guo, Jintian, without whom endless support, care, love, optimism, generosity and patience I would not be the person I am,

My sister Guo, Yaqiu

My furry friend, Glue

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LIST OF ABBREVIATIONS

ATP binding cassette transporters	ABC
Acetonitrile	ACN
Archer Daniels Midland Company	ADM
α-Linolenic acid	ALA
Apolipoprotein B	APOB
Area under the curve	AUC
Coronary heart disease	CHD
Cardiovascular disease	CVD
p-Coumaric acid	CouAG
Drug Identification Number-Homeopathic Medicine	DIN-HM
Enterodiol	ED
Enterolactone	ENL
Ferulic acid	FeAG
Hydroxycinnamic acid glucosides	HCAG
Hematoxylin-eosin	H&E
Herbacetin diglucoside	HDG
High-density lipoprotein cholesterol	HDL-C
3-hydroxy-3-methyl-glutaryl	HMG
3- hydroxy-3-methyl glutaric acid	HMGA
3-hydroxy-3-methylglutaryl-CoA	HMG-CoA
High quality control	HQC
Insulin Induced Gene-1	INSIG-1
Internal standard	IS
Low-density lipoprotein cholesterol	LDL-C
LDL-C receptor	LDLR
Lower limit of quantification	LLOQ
Low quality control	LQC
Liver X receptor	LXR

New dietary ingredient	NDI
NAFLD activity score	NAS
Natural Health Products Directorate	NHPD
Natural Health Products Regulation	NHPR
Natural health products	NHPs
Niemann-Pick C1 like1	NPC1L1
Natural product number	NPN
Quality control	QC
Secoisolariciresinol diglucoside	SDG
Secoisolariciresinol	SECO
Sterol regulatory element binding protein-2	SREBP-2
Triglyceride	TAG
Total cholesterol	TC
Toronto Research Center	TRC
Very low-density lipoprotein cholesterol	VLDL-C

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1 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Cardiovascular disease (CVD) and non-alcoholic fatty liver disease result in considerable morbidity and mortality in human. Hypercholesterolemia and hypertriglyceridemia are major independent risk factors for CVD. To manage hypercholesterolemia, lifestyle changes and drug therapy, especially statins, are used. However, statins can lead to side effects. Preclinical and clinical studies suggest that oral consumption of the flaxseed lignan, secoisolariciresinol diglucoside (SDG), can reduce reduce serum total, and LDL-cholesterol, and lipid accumulation in the liver. Furthermore, both animal and human studies indicate that flaxseed and its lignan extract are safe. Hence, oral SDG supplementation might offer a safe alternative to pharmaceutical hypocholesterolemic agents in hypercholesterolemia management.

Flaxseed SDG is stored as a component of an ester-linked copolymer. Reported processes for hydrolyzing, extracting, and purifying SDG from the flaxseed copolymer to produce a purified SDG can be a costly procedure. Consequently, unless the cost of SDG isolation is reduced it is unlikely to be marketed as a nutraceutical. In 2007, Archer Daniels Midland Company (ADM) attained regulatory approval of a flaxseed lignan enriched complex called BeneFlax™ from both the U.S. Food and Drug Administration Agency and Health Canada. Hydrolysis of BeneFlax™ released 34%-38% SDG by weight and long-term supplementation with the complex was well tolerated and safe. However, ADM ceased commercial production of BeneFlax™ to focus on soy isoflavanoids. Recently, Prairie Tide Diversified Inc. began production of a more concentrated flax lignan copolymer that releases 50% or more SDG with hydrolysis. Biological activity of this SDG copolymer has never been studied.

Following oral consumption of the SDG copolymer, SDG is released in the stomach and intestine. Subsequent deglycosylation, releases the aglycone of SDG, secoisolariciresinol (SECO) which is further metabolized to mammalian lignans, enterodiol (ED) and enterolactone (ENL), by colonic microflora. Deglycosylation of SDG and further biotransformation to mammalian lignans proceed following administration of either purified SDG or SDG polymer. However, the extent to which SDG is released from the copolymer and subsequent bioavailability of the SDG metabolites is unknown.

Therefore, I conducted a relative bioavailability study between purified SDG and the SDG copolymer to understand the pharmacokinetics of the two SDG forms. As well, I evaluated the efficacy of the SDG copolymer in diet-induced hypercholesterolemic rats to determine if a diet including this product induced similar effects as purified SDG. These studies helped to determine if the value of using an SDG copolymer, a more economical product than purified SDG, is a candidate for management of hypercholesterolemia.

1.2 Cholesterol as a risk factor for cardiovascular disease and current therapies

Cardiovascular diseases (CVD) are diseases that involve heart or blood vessels[1]. Collectively these diseases remain the major contributor to chronic disability and death globally[2]. In 2015, there were about 17.92 million deaths due to CVD, increasing from 12.59 million deaths in 1990[2]. Different types of CVD include ischemic heart disease, cerebrovascular disease (stroke), peripheral vascular disease, heart failure, rheumatic heart disease and congenital heart disease[1,2]. Hypercholesterolemia and hypertriglyceridemia are major independent risk factors for CVD[3]. Elevated serum total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high serum triglyceride (TG) levels, and low serum high-density lipoprotein cholesterol (HDL-C) are associated with increased CVD[4].

1.2.1 Etiology of hypercholesterolemia

Hypercholesterolemia can be divided into familial hypercholesterolemia and polygenic hypercholesterolemia. Polygenic hypercholesterolemia, the most common form, is caused by the combination of unidentified genetic factors and unhealthy lifestyle factors, such as high-fat diets and lack of practical exercise[5]. High-fat diets include high levels of cholesterol, saturated fatty acids, trans fatty acids, and total caloric intake[6]. In most cases, familial hypercholesterolemia results from mutation of the LDL receptor gene, leading to elevated levels of plasma LDL-C[5, 7]. Other genetic causes of familial hypercholesterolemia are mutations in apolipoprotein B (APOB) and proprotein convertase subtilisin/kexin type 9 gene (PCSK9)[5, 7].

1.2.2 Impact of elevated cholesterol

The biggest threat associated with hypercholesterolemia is unfavorable cardiac events, with complications like heart disease, stroke, and peripheral vascular disease[5]. Increasing LDL-C can accumulate as arterial deposits or plaques and, if untreated, lead to atherosclerosis. There is an association between elevated serum TC/HDL-C and the presence of vulnerable plaques[8]. Furthermore, low HDL-C and high total cholesterol levels increased the incidence of coronary

heart disease (CHD)[9]. In a community study involving 8132 people aged 40 to 69 years, the incidence rate of CHD was 2.49x for individuals with 140-159 mg/dL non-HDL cholesterol and 3.13x for individuals with ≥ 180 mg/dL, compared to individuals with non-HDL cholesterol levels of < 100 mg/dL[10]. Nowadays, cholesterol reduction is considered as a practical strategy for the prevention of heart disease[9].

1.2.3 Treatment of hypercholesterolemia

Lifestyle changes and drug therapy are the mainstays of hypercholesterolemia treatment. Lifestyle modification might include improvement in diet, increase of physical exercise, maintenance of an optimal body weight, moderation of alcohol consumption, and cessation of smoking[11]. The usual targets for drug therapy include reduction of LDL-C level, enhancement of HDL-C level, and reduction in triglyceride concentration[12]. There are several biochemical mechanisms to reduce LDL-C levels including: 1) HMG-CoA reductase inhibition to inhibit synthesis of hepatic cholesterol (e.g. statins)[13]; 2) Inhibition of cholesterol absorption (e.g. ezetimibe and phytosterols)[12,14]; 3) bile acid sequestrants increasing clearance of circulating LDL-C (e.g. bile acid resins, colesevelam)[12]; and 4) Modulation of transcription of genes encoding for proteins that control lipoprotein metabolism. (e.g. fibrates) [15].

Inhibition of HMG-CoA reductase, which is the first and rate-limiting step in cholesterol synthesis by statins is the most common approach for lipid reduction[16]. The intensity of statin therapy is determined by the predicted percentage reduction in LDL-C levels, from < 30% to >50%. Only 38% of patients achieved their LDL-cholesterol goal in statin therapy[13]. Although trials indicated that statins lower LDL-C levels leading to reduction of major vascular events by 22%, CHD death by 20% and all-cause mortality by 10%, the outcomes of statin therapy have varied in particular patient groups[17]. For example, no clear benefit of statin therapy in patients with heart failure with reduced ejection fraction, and for patients aged >75 years, statin had less benefit in reduction of major vascular events when compared to younger patients[17]. Moreover, there are some side effects of statins including gastrointestinal disturbance, extremely high liver transaminases, rhabdomyolysis, myositis, and neuropathies[16].

The efficacy of other hypocholesterolemic drugs is also limited in patient groups with elevated serum cholesterol. For instance, Ezetimibe reduces LDL-C blood levels by about 17% – 22% and has modestly beneficial effects on TGs and HDL-C levels as well. No significant adverse effects of ezetimibe were found compared to placebo[12]. Fibrates are PPAR-α agonists, which predominantly reduce VLDL level[15]. Fibrates are used for treatment of primary hypertriglyceridemia, but in some hypercholesterolemic patients, no response or even a

paradoxical increase in LDL has been observed[15]. Potential adverse effects of fibrates are gastrointestinal complaints, increase in liver transaminases, and increased cancer has been observed in rodents[12, 15]. While current lipid-altering agents are commercially available, however, their varied effects in different patient groups and possible side effects has led to a need for more effective drugs with no adverse events.

1.3 Cholesterol

Cholesterol is a water insoluble sterol alcohol with a single hydroxy group, a double bond, and an eight carbon side chain[18]. Cholesterol is an important component of all mammalian cells and a precursor of steroid hormones, vitamin D, and bile acids[19]. It exists in its free form in cell membranes and in esterified forms in plasma. Total body cholesterol homeostasis is maintained by balancing dietary cholesterol intake, *de novo* synthesis, biliary excretion, and fecal excretion[18, 19]. Cholesterol biosynthesis regulation requires the balance of endogenous cholesterol synthesis and absorption of dietary and biliary cholesterol.

1.3.1 Intestinal cholesterol absorption and regulation

Intraluminal cholesterol absorption involves the digestion and hydrolysis of dietary lipids and micellar solubilization. Biliary cholesterol absorption is higher than dietary cholesterol absorption because biliary cholesterol enters the intestine in micellar solution, while dietary cholesterol is mostly dissolved in dietary triglycerides and must be transformed into micellar solution before adsorption is possible.

Cholesterol is released from micelles at the enterocyte brush border membrane and then absorbed into cells by a mechanism largely involving a protein called Niemann-Pick C1 like 1 protein (NPC1L1)[20]. However, a portion of the cholesterol entering the enterocyte is removed from the cells to the intestinal lumen by the action of ATP-binding cassette (ABCA1) transporters, ABCG5 and ABCG8 [20]. ABCA1 also plays a role in reverse cholesterol transport. Studies have showed that the ABCA1 transporter, mostly present in the liver and peripheral macrophages, assists the efflux of cholesterol and production of nascent HDL [21]. Overexpression of ABCA1 in mice increased plasma HDL cholesterol levels[21].

1.3.2 Cholesterol biosynthesis

Cholesterol biosynthesis begins with two acetyl-CoA molecules forming acetoacetyl-CoA, followed by reaction of HMG-CoA synthase with one more acetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). HMG-CoA is then converted to mevalonate by

HMG-CoA reductase[18]. Mevalonate undergoes conversion to isopentenyl-pyrophosphate and subsequently six isopentenyl-pyrophosphate are condensed in sequence to yield squalene. The precursor squalene is then converted to cholesterol[22]. The major site for cholesterol biosynthesis is liver, followed by the gastrointestinal tract[18].

No human enzyme can degrade the sterol ring structure of cholesterol. The sterol ring is acted on by enzymes to become more water soluble by the action of liver enzymes. The modified product is excreted in the bile and urine[18].

1.3.3 Regulation of cholesterol metabolism

Three mechanisms exist to maintain cholesterol homeostasis including transcriptional (liver X receptor (LXR) dependent and independent pathways), post-transcriptional, and post-translational (SREBP-2 pathway) regulatory mechanisms. LXR-dependent pathways regulate bile acid synthesis and sterol efflux from hepatocytes and peripheral cell via transcriptional upregulation of CYP7A1, ABCA1, ABCG5, and ABCG8[23]. LXR-independent pathways regulate sterol regulatory element binding protein-2 (SREBP-2), which is also a post-translation factor. SREBP-2 levels decline when cellular sterol and oxysterol levels are high, resulting cholesterol biosynthesis genes inhibition, including HMGR, HMG-CoA synthase, squalene synthase, and the LDL-C receptor (LDLR)[24]. High cellular oxysterol levels lead to increasing cellular mRNA levels of LXR, and protein content and enzyme activity of CYP7A1, ABCG5, and ABCG8[23].

1.3.4 Cholesterol transport

Cholesterol transport from peripheral tissues to the liver is required for oxidation and excretion. Free cholesterol is transported from peripheral cells by nascent HDL. This complex is secreted by the liver and intestine and consists primarily of phospholipids and apoA-1 but has very little cholesterol. In peripheral tissues nascent HDL matures by binding cholesterol esters for transport to form mature HDL-C. Cholesterol esters of HDL-C can be selectively absorbed by the liver and other steroidogenic tissues, or transferred to apoB-containing lipoproteins, including VLDL-C and LDL-C. Levels of LDL-C increase with increasing cholesterol supply[25].

1.3.5 Cholesterol regulation by lignan

According to a previous study by Alcorn et al. treatment of HepaRG cells with ENL and ENL-Gluc inhibits HMG-CoA reductase, a rate limiting activator of the mevalonate synthesis

pathway, and LDL-R transcription and translation. These findings suggest that ENL and ENL-Gluc might play an inhibitory effect on both cholesterol synthesis and uptake[26]. In other work by Alcorn et al. treatment of Caco-2 intestinal epithelium cell line with ENL and ENL-Gluc reduced enterocyte cholesterol accumulation[27]. ENL-Gluc significantly increased Insulin Induced Gene-1 (INSIG-1) regulation, and significantly downregulated SREBP-1, while ENL, distinctly but not significantly, upregulated INSIG-1[27]. INSIG-1 expression restricts adipocyte lipogenesis[28] and serves a dual function to regulate cholesterol homeostasis by binding to both SREBP cleavage-activating protein and HMG-CoA reductase[29], while SREBP-1 downregulation is correlated with reduction in cholesterol synthesis in mice[30]. Therefore, the glucuronidation form of ENL is perceived to have a potential role in cholesterol homeostasis.

1.4 Natural health products (NHPs)

Natural health products (NHPs) include herbal remedies, traditional medicines such as traditional Chinese and Ayurvedic medicines, probiotics, homeopathic medicines, vitamins and minerals, and other products like amino acids and essential fatty acids[31]. NHPs should be safe, effective, and of high quality; however, 12% of Canadians who use NHPs report that they have experienced unwanted side effects. Canadian regulations allow access to NHPs freely without a prescription. Generally, NHPs are consumed to maintain health, and treat or prevent minor, serious, or chronic disease[32]. Health Canada claimed that 71% of Canadians have used natural health products[33]. According to a survey involving 326 individuals, 82% of participants stated they preferred to take a NHP for a minor condition and 60% of them preferred the use of NHPs for chronic medical illnesses, as they perceive that NHPs are safe and have good quality[34].

1.4.1 Natural Health Products Regulation (NHPR)

The Natural Health Products Directorate (NHPD) of Health Canada was empowered to enforce Canadian NHP regulations on January 1, 2004. Prior to that time NHPs sold in Canada were regulated by the Food and Drugs Act (1985). The categorization of NHPs as foods or drugs is dependent upon any medicinal claims associated with the product. Generally, NHP regulations are stricter than food product regulations but less strict than drug regulations. All NHP marketers must provide science-based evidence of safety, efficacy, and quality to Health Canada[31]. Health Canada has published a "Pathway for Licensing Natural Health Products Making Modern Health Claims", a document that indicates evidence requirements for sale of NHPs[35]. Depending on product ingredients, proposed health claim(s) and overall risks, the level

of evidence required differs. For NHPs with low risk, data to support health claims can range from epidemiological studies and strong Phase II clinical trials. For NHPs with high risk and those used for treatment, cure, and prevention of serious diseases at least two Phase III clinical trials are required [35]. Sellers must submit a product license application to the NHPD to receive authorization for sale of their product in Canada. Once approved by NHPD, an eight-digit natural product number (NPN) or a Drug Identification Number-Homeopathic Medicine (DIN-HM) will be issued by the NHPD to the producer[31].

1.4.2 Flaxseed lignans as NHP candidates

Flaxseed lignans may be marketed as NHPs if they are both safe and the seller claims health benefits. Preclinical and clinical studies have shown that flaxseed lignans have potential health benefits that would be useful in treatment of chronic diseases such as breast cancer, prostate cancer, CVD, diabetes, and inflammation[36-47]. Safety studies in rabbit (40 mg/kg lignan complex once daily for 2 months)[48], rat (10% flax chow)[49], and human (200-600 mg/day SDG for 6 months)[50-52] indicate that flaxseed lignans are safe for most people to consume with the possibleexception of women during pregnancy and lactation[53, 54]. Safety will be discussed in section 1.5.4.

In 2008, NHPD accepted Archer Daniel's Midland (ADM)'s Natural Health Products Master File for a flax lignan concentrate (a 34%-38% SDG-enriched complex) called BeneFlax™ and issued a specific reference number for the ingredient[55]. The USFDA also accepted Archer Daniel Midland's new dietary ingredient (NDI) filing for BeneFlax™[56]. Therefore, the new SDG-enriched polymer is an approved NHP.

1.5 Flaxseed

Flax (*Linum usitatissimum*) is an oilseed crop, which is usually grown in cooler environments, primarily in the mid-west of United States and Canada[57]. Flaxseed varies in color, from golden yellow to reddish brown[58]. Although the nutritional composition of flaxseed varies with environment and cultivar, it typically has about 40% lipid, 30% dietary fiber, 20% protein, as well as lignans, minerals, and vitamins[59]. Flaxseed was grown to produce industrial oils, but recently it has received more attention as a functional food because of its three principle components: α-linolenic acid (ALA), soluble fiber, and lignan[58]. ALA is an omega-3 polyunsaturated essential fatty acid that comprises at least 55% of the total flax oil triglyceride of Canadian cultivars but lower levels occur in flaxseed grown elsewhere. The saturated fatty acid content of flaxseed oil is typically less

than 9%; therefore, flaxseed oil is considered a low saturated fat food[58]. Flaxseed soluble fiber, known as mucilage or gum, is composed of high molecular weight polysaccharides. This gum comprises approximately one-third of the total dietary fiber in flaxseed[60]. Flaxseed hull is one of the richest sources of plant lignans which occur mainly as a copolymer that contains SDG. The lignan content of flaxseed is 1-26 mg/g of whole flaxseed[61]. Based on a submission put forward by the Flax Council of Canada the NHPD allows a health claim for the consumption of products that contain whole flaxseed. The daily recommended consumption of flaxseed according to the health claim is 40 g (5 tablespoons) of ground whole flaxseed.

1.5.1 Flaxseed lignans

Lignans are a class of diphenolic compounds distributed widely in the plant kingdom, including flaxseed, sesame, rye bran, whole grain and vegetables[62]. Flaxseed lignans are concentrated in the seed coat mainly as SDG, as well as a small amount of the lignans, matairesinol, pinoresinol and isolariciresinol[63]. The lignan content of flaxseed varies with genotype, and environmental effects including location, harvest year, and seeding date[46]. Flaxseed lignan is present in a linear ester-linked copolymer where SDG bonded with 3-hydroxy-3-methyl-glutaryl units (HMG) is the repeated unit (Figure 1.1)[64, 65]. Ford et al. used ^{1}H and ^{13}C NMR and HRMS analysis, to deduce that SDG-containing polymer consists of mixtures of dimers, trimers, tetramers, and so forth[65]. Additionally, the glucosides of p-coumaric acid (CouAG) and ferulic acid (FeAG)[65-68] and herbacetin diglucoside (HDG)[69] may also be found as the terminal residue of the copolymer. However, in the study of Kamal-Eldin et al.(2001), ^{1}H NMR analysis showed peaks of p-coumaric acid and ferulic acid but no visible peaks of these phenolic compounds were observed when using ^{13}C NMR, as the latter is a much less sensitive method and the amount of these compounds in the copolymer is relatively small[70]. In their study, the oligomeric structure was composed of five SDG residues interconnected by four 3-hydroxy-3-methyl glutaric acid (HMGA) residues[70].

Figure 1.1 Schematic representation of the SDG polymer. The circle represents the backbone SDG, the rhombus represents the linker molecule HMGA, and the square represents terminal

units, which can be CouAG, FeAG (HCAG = CouAG or FeAG), or HMGA. Adapted from Struijs et al. [71].

1.5.2 Overall health benefits of flaxseed lignan

The breadth of biological effects of flaxseed lignan as an anti-oxidative, anti-oestrogenic, anti-inflammatory, and anti-carcinogenic compound have been reviewed[36, 37]. The reviews include potential health benefits of lignan as a treatment or prophylactic for chronic disease including cancer, CVD, diabetes, and inflammation. In two studies, one of 383 women with palpable cysts[38] while the other included 194 women diagnosed with breast cancer and a 208 community-based control[39], an inverse correlation was observed between serum EL concentration and breast cancer risk. SDG and its metabolites can inhibit lipid peroxidation by hydroxyl radical (·OH) scavenging indicating that the antioxidative ability is greater than Vitamin E [72, 73]. In addition, studies in rat and human showed that SDG mitigated the effects of type I and type II diabetes[40-43].

1.5.3 Hypocholesterolemic effect of flaxseed lignans

Studies have shown reductions in LDL-C and serum cholesterol in response to flaxseed meal or flaxseed lignan supplementation for both animal models [74-78] and human[44-47]. Several human studies indicate that flaxseed lignan only has hypolipidemic effects in hypercholesterolemic patients[46, 47] but not in normocholesterolemic subjects even with a dose of 543 mg/day SDG in BeneFlax™ Flax Lignan Concentrate [44, 45]. The ability to lower LDL-C in hypercholesteremic patients but have no effect in normocholesterolemic subjects is seen as an indicator that flaxseed lignan-enriched products are potentially safe for consumption by most people.

1.5.3.1 Animal studies

Various studies have been performed in different animal models to evaluate the hypocholesterolemic effect of flaxseed or purified SDG. In SHR/N-cp lean and obese rats receiving 20% of energy from flaxseed meal for 6 months, a significant reduction was found in LDL-C, HDL-C, plasma triglyceride, and liver fat deposition. However, the components responsible for the hypolipidemic effect are unknown[74]. Two studies by Penumathsa et al. (2008) indicated cardioprotective effects of SDG in rat models. Rats were fed a high cholesterol diet (2%) for 8 weeks followed by oral administration of SDG (20 mg/kg) for 2 weeks and blood lipids were compared to rats only fed the high cholesterol diet[79]. A significant reduction in total cholesterol, LDL-cholesterol,

triglycerides and an increase in HDL-cholesterol levels were observed in rats receiving SDG. Felmlee et al (2009) found that for hypercholesterolemic rats fed a 1% cholesterol diet and also administered 3 or 6 mg SDG/kg body weight exhibited a dose-dependent reduction in serum TC, LDL-C levels, hepatic lipid accumulation, and body-weight gain. In this study SDG did not alter hepatic gene expression of commonly reported regulatory targets of lipid homeostasis[80].

In rabbits fed a high cholesterol diet, flaxseed reduced development of aortic atherosclerosis without significantly lowering serum cholesterol. In normocholesterolemic rabbits, flaxseed increased serum total cholesterol without significantly affecting serum triglycerides[75]. Prasad et al.[76] also investigated diets that included solin flaxseed, which has similar oil and lignan content as regular flaxseed but has very little α-linolenic acid (2–3% of the total oil), and observed a similar reduction of serum TC and LDL-C in hypercholesterolemic rabbits fed flaxseed with normal α-linolenic acid content. This finding indicates that the antiatherogenic activity of flaxseed is not due to α-linolenic acid[76]. In 6 to 8 week old New Zealand White rabbit, treatment with 15 mg/kg body weight pure SDG (wrapped in lettuce) reduced hypercholesterolemic atherosclerosis with a decrease in serum cholesterol, LDL-C, and lipid peroxidation products as well as an increase in HDL-C and antioxidant reserve[77]. Similarly, in another rabbit study, a diet containing 0.04% SDG reduced the progression of atherosclerosis to a greater extent than controls, and reduced atherosclerosis[78]. They also conducted three studies of flax lignan complex in diet-induced hypercholesterolemic rabbits. They found that 40 mg/kg lignan complex treatment for two months reduced the development of atherosclerosis by 34% in rabbits fed a diet with 0.5% cholesterol which was associated with a reduction in serum TC by 20%, LDL-C by 14% and an increase in HDL-C by 30%[81]. In addition, their later study verified that flax lignan complex was effective in slowing progression of hypercholesterolemic atherosclerosis by 31% in rabbits fed a 0.25% cholesterol diet (2 months) followed by a 0.25% cholesterol diet plus 40 mg/kg body weight flax lignan complex orally daily (2 months), when compared to control rabbits fed a 0.25% cholesterol diet for 4 months[82]. However, the same group reported that flax lignan complex did not induce regression of atherosclerosis but prevented its acceleration[83]. The authors did not report the SDG content of the flax lignan complex in these three studies, thus it is difficult to determine the effects SDG on atherosclerosis. In 2016, a study of the therapeutic effect of a 15% flax-based diet on non-alcoholic fatty liver of laying hens indicated that hens

supplemented with whole flaxseed or defatted flaxseed meal had lower body weight and serum aspartate aminotransferase concentration[57].

Although most animal model studies suggest that flaxseed lignan has positive effects on CVD, the study of *Sano et al.* showed that SDG did not affect either atherogenesis or thrombosis in apolipoprotein E and low-density lipoprotein receptor deficient mice fed a diet with 0.05% cholesterol and SDG (0.06% w/w; 100 mg/kg body weight per day)[84].

1.5.3.2 Human studies

Several studies have revealed that flaxseed or flaxseed lignan lowers cholesterol in hypercholesterolemic subjects, while lignan supplementation had no effect on normocholesterolemic subjects. Moderately hypercholesterolemic men who received 100 mg of SDG for 12 weeks exhibited a significant reduction in the ratio of LDL-C/ HDL-C[47]. In a study conducted in fifty-five hypercholesterolemic patients, 600 mg SDG supplementation for 8 weeks significantly decreased serum TC, LDL-C and glucose concentrations. The observed serum cholesterol correlated negatively with concentrations of plasma SECO and ED[46]. However, a study among healthy postmenopausal women who consumed a low-fat muffin with a lignan complex (500 mg/day SDG) daily for 6 weeks showed that lignan complex had no effect on plasma lipid concentrations, serum lipoprotein oxidation resistance, or plasma antioxidant capacity[45]. Similarly, flaxseed lignan supplementation had no hypoglycemic or hypotensive effect in healthy older human participants receiving 543 mg/day SDG in the form of a 35% SDG-enriched complex[44].

1.5.3.3 Hypocholesterolemic effect of HMG

Several studies in rats and rabbits have showed that HMG had hypocholesterolemic effects. The study of Beg et al (1968) showed that 20 mg HMG kg/day i.p. injections treatment caused a significant decline in the levels of different serum lipids of hyperlipidemic rats[85]. In another study, rats receiving 25 mg HMG/kg had significantly lower serum triglyceride and phospholipid levels, but the serum cholesterol, and liver and aorta lipid content did not show any significant change[86]. Conversely, all serum, liver, and aorta lipids except aortic phospholipids were significantly decreased by administration of 50 mg HMG/kg[86] suggesting a dose-dependent lipid reduction effect of HMG. Cholesterol-fed rabbits receiving HMG had significantly lower levels of serum cholesterol, phospholipids, triglycerides, free fatty acids, and total lipids. However, liver cholesterol was slightly increased[87]. As SDG polymer include HMG, it is necessary to consider the effect of both components when evaluating hypocholesterolemic efficacy.

1.5.4 Safety and toxicity of flaxseed lignan

Sustained consumption of lignan complex (40 mg/kg body weight orally daily for 2 months) had no adverse effects on the hemopoietic system in normo- and hypercholesterolemic rabbits[48]. A study of male and female Fischer 344 rats indicated that consumption of 10% flax chow from the 18th day of gestation to the 86th day after giving birth did not have long-term effects on growth, development, and behavior[49]. Moreover, no signs of toxicity were observed in the subjects[49].

A 34%-38% SDG-enriched complex provided to subjects in a 600 mg/day SDG dose for six months (BeneFlax™, Archer Daniel's Midland) was safe and well tolerated by human subjects[50, 51]. Similarly, no evidence of hypotension, hypoglycemia, or other adverse events was found in older adults supplemented with 300 mg/ day SDG for six months[52].

A study showed that feeding rats flaxseed during pregnancy and lactation might reduced the risk of mammary cancer[88]. Flaxseed consumption was associated with lowered birth weight, irregular estrous cycles, and altered reproductive development of offspring. It was postulated that lignans transferred to the offspring via rat dam's milk induced the observed physiological changes[53,54]. The authors suggest that such effects would be of concern for subjects consuming flaxseed during pregnancy and lactation.

1.6 Pharmacokinetics of flaxseed lignan

1.6.1 Absorption and Gastrointestinal metabolism

Following flaxseed consumption, SDG and various combinations of SDG and HMGA are released from the lignan macromolecule in the stomach and small intestine[89]. A study using artificial human stomach and small intestine suggested that lignan polymer digestion begins in the stomach and small intestine. Pancreatic enzymes and bile salts[90] contribute considerably to digestion of the lignan complex. SDG can be deglycosylated to its aglycone form, SECO, by β-glucosidase and β-glucuronidase enzymes in the small intestine[91] and colon microflora[92]. Clavel et al. (2006) found that *Clostridium* sp. SDGMt85-3Db had the highest deglycosylation rate, but this strain was only found in two of twenty human fecal samples[93]. In their study, *Bacteroides distasonis* and *B. fragilis,* which were found in all human feces with proportion of 0.5% and 3.3% of total fecal bacteria, respectively, completely converted SDG into SECO within 20 h[93]. Similarly, in SDG-supplemented culture inoculating with human feces, SDG underwent deglycosylation to SECO within 12 h[94]. SECO is then partially absorbed into the systematic circulation or biotransformed to the

mammalian lignan, enterodiol (ED), by gut microflora via demethylation and dehydroxylation[95]. In SDG-supplemented microbial culture inoculated with human feces, SECO was successively dehydroxylated and demethylated to yield enterodiol (4–18% conversion) and enterolactone (0.2–6%) after 24 h[94]. In another *in vitro* experiment, both *Peptostreptococcus productus SECO-Mt75m3* and *Eggerthella lenta SECO-Mt75m2* transformed SECO to ED. Conversion of SECO was detected under anoxic conditions, but not observed during aerobic incubation[96]. ED is subsequently dehydrogenated to form enterolactone (ENL), and only small amounts of ED and ENL are absorbed into the systemic circulation[50, 80]. The pathway for conversion from SDG to ENL is displayed in Figure 1.2. A subdominant bacteria of the human intestine, *Lactonifactor longoviformis*, contributes to ENL formation from ED[97]. Other plant lignans in flaxseed such as matairesinol, pinoresinol and lariciresinol, can also be converted into the mammalian lignans, ED and ENL[50, 80].

Neither SDG nor its conjugates have been detected in plasma and urine[98]. In a permeability study of SDG and its metabolites using caco-2 human intestinal cells, SDG had very poor permeation characteristics, and passive diffusion is the principal mechanism of intestinal permeation for SECO, ED, and ENL [99]. Therefore, only SECO, ED, and ENL can be absorbed by the gastrointestinal tract. Kuijsten, Anneleen et al. found that crushing and milling of flaxseed significantly improved enterolignan bioavailability[100]. They found that relative bioavailability of enterolignans from whole flaxseed was 28% , while that of crushed ground flaxseed expeller cake was 43%[100].

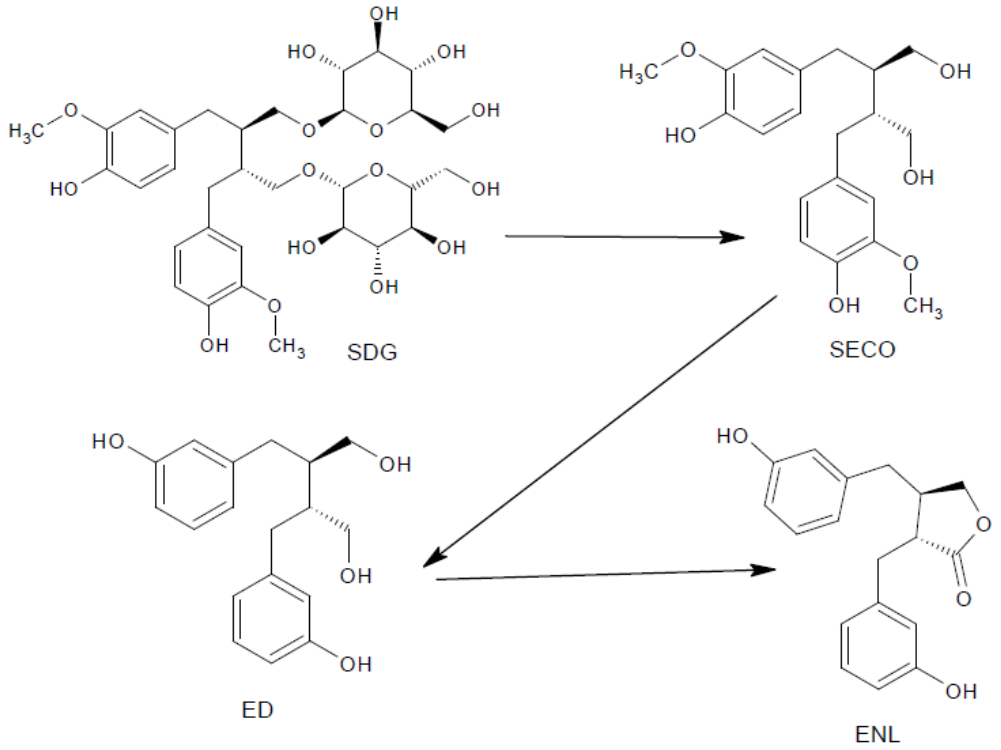


Figure 1.2 Pathways for the conversion of the plant lignan secoisolariciresinol diglucoside (SDG) to the mammalian lignan enterolactone (ENL). The intestinal β-glycosidase enzyme cleaves glucose groups from SDG and yields its aglycone form, secoisolariciresinol (SECO). The unabsorbed SECO undergoes dehydroxylation and demethylation to produce enterodiol (ED). ED is subsequently dehydrogenated to form ENL.

1.6.2 Metabolism

After absorption from the gastrointestinal tract, SECO, ED, and ENL undergo extensive first pass metabolism. Mainly, the compounds undergo conjugation reactions with glucuronic acid and sulfate by enterocyte and liver phase II enzymes[101]. In a polarized Human Caco-2 cell system used for permeability assessments due to its morphological and physiological similarity with the small intestine, SECO, ED, and ENL underwent passive permeation and extensive conjugative metabolism by intestinal Caco-2 cells[99]. An *in vitro* enzyme kinetic study of ENL glucuronidation in liver and intestinal microsomes from both human and rat indicated that the extent of hepatic microsomal glucuronidation surpassed intestinal glucuronidation in both human and rat[102]. The phase II metabolites may undergo enterohepatic recirculation, a process that could slow the clearance of lignans from the body[103].

As well, SECO, ED, and ENL can undergo metabolism by hepatic cytochrome P450, typically hydroxylation reactions at aliphatic and aromatic positions, to a minor extent[104, 105]. In a study of the oxidative metabolism of SECO in rat and human microsomes, Niemeyer et al. detected aliphatic and aromatic hydroxylation metabolites of SECO, but demethylation

products were not observed[106]. In female Wistar rat aromatic and aliphatic monohydroxylation products of ED and ENL were present in bile of rat that were administered interduodenal ED and EL (10 mg/kg) [107].

1.6.3 Distribution

Flaxseed lignans metabolites are widely distributed to the whole body in their conjugated forms. A study involving a single gavage study of ^{3}H-SDG (3.7 kBq/g body weight) in female Sprague-Dawley rats showed that tissue radioactivity was highest in the cecum[108]. Other tissues with measurable lignan concentrations included the small intestine, colon, stomach, liver, kidney, and uterus. Throughout the experimental period radioactivity in the blood was always <1% of the recovered dose. The highest radioactivity levels in the blood and blood components were noted at 12 h[108]. In male Sprague-Dawley rats ED and ENL accumulated in liver, testes, prostate, and lung tissue in a dose-dependent manner. Among these four tissues, prostate had the highest concentration of ED (1.899 pmol/mg) at the 60 mg/kg dose, suggesting that prostate accumulates lignan[109]. Saarinen and Thompson assessed tissue lignan distribution in rats after 1 d and 7 d administration of ^{3}H-SDG (3·7 kBq/g body weight)[110] and found that liver contained the majority of tissue lignans (48–56%) in both male and female rat after both exposure regimens[110]. When serum lignan concentrations reached a plateau, concentrations in skin and kidneys increased, which indicated tissue accumulation[110]. Therefore, serum concentrations alone do not fully reflect individual tissue concentrations.

1.6.4 Excretion

Urine and faeces are the major routes of lignan metabolite excretion. In a study in female rats with acute or chronic SDG treatment over 48 h, more than 80% of the lignan dose of lignan was excreted in both groups (feces > 50%, urine = 28–32%) by 48 hours[108]. Similarly, another study of lignan excretion in male and female Sprague–Dawley rats after 1 d and 7 d administration of ^{3}H-SDG (3.7 kBq/g body weight) showed that the majority of radioactivity was excreted in faeces (40–83%) and urine (1.2–5.2 %)[110]. A study of adult male chimpanzees consuming regular food showed that they excreted large amounts of ENL (14.1 + 3.5 nmol/mg cr.) and small amounts of ED, (0.4 +/- 0.2 nmol/mg cr.) in urine[111]. In male rats administered 47.2 mg/kg SDG or 25 mg/kg SECO orally ENL was a major metabolite identified in urine after SDG administration, with small amounts of SECO and ED detected as well. However, for rats had been given SECO, the majority of them excreted higher quantities of ED than ENL into urine[112].

Both urinary and fecal lignan increased with consumption of lignan containing foods. A study on the effect of flaxseed and wheat bran in 16 premenopausal women indicated that urinary lignan excretion significantly increased with flaxseed consumption, but intake of wheat bran did not significantly change excretion[113]. Thirteen premenopausal women consumed their usual diets supplemented with 10 g/day ground flaxseed[114]. Feces were collected on days 7-11 where ED and ENL in feces increased significantly with flax consumption, from 80.0 ± 80.0 (SD) to 2560 ± 3100, and 640 ± 480 to 10,300 ± 7580, respectively[114]. Similarly, young vegetarian women excreted significantly greater amounts of ENL than omnivores, while old vegetarians excreted similar amounts as the omnivore group[115].

In urine, lignans mainly exist as conjugates. In urine samples from two vegetarian or semivegetarian women and in two urine samples from men consuming an ordinary Finnish diet[116], ED, ENL and matairesinol were largely present as monoglucuronides (73-94%), and to a smaller extent as monosulfates (2-10%). Unconjugated lignans represented 0.3-1% of the total lignans[116]. Interestingly, in some studies, before flaxseed supplementation, EL glucuronide conjugates and sulfates occurred in all urine samples[94].

1.6.5 Pharmacokinetic Parameters

In a PK study involving male Wistar rats, the oral bioavailability of SDG, SECO, and ED were 0, 25%, and 1%, respectively[117]. SDG had the lowest apparent volume of distribution (0·76 litres/kg), systemic clearance (1.11 litres/h per kg) and half-life (0.52 h). SECO had a large apparent volume of distribution (44.1 litres/kg), systemic clearance (7.82 litres/h per kg), and half-life (4 h). Compared with SECO, ED had a larger systemic clearance (23.1 litres/h per kg), and a shorter half-life (1.8 h)[117]. In a second PK study involving male Wistar rats, SECO had smaller volume of distribution (17.7 litres/kg), and a smaller systemic clearance (3.1 litres/h per kg), while its oral bioavailability was about 25%.

A single oral dose of purified SDG (1.31 μmol/kg body wt) PK study in healthy women and men showed that enterolignans appeared in plasma 8–10 h after ingestion of the purified SDG[118]. In 14.8 ± 5.1 h and 19.7 ± 6.2 h, ED and ENL reached their maximum plasma concentration, respectively. The half-life of ED (4.4 h ± 1.3 h) was shorter than that of ENL (12.6 h ± 5.6 h)[118]. Another PK study in healthy postmenopausal women administering SDG orally reported that SECO reached its peak plasma concentrations after 5–7 h and disappeared with a plasma elimination half-life of 4.8 h[119]. Highest concentrations of ED and ENL were detected after 12–24 h and 24–36 h, respectively, and their half-lives were 9.4 h and 13.2 h[119].

2 RATIONALE

Cardiovascular disease (CVD) remains the major contributor to chronic disability and death globally. Hypercholesterolemia is one of the major independent risk factors of CVD. Statins are the most commonly used class of drugs for treatment of hypercholesterolemia; however, adverse effects of statins attract interest of scientists and investigators to find safer natural product alternatives.

Oral SDG supplementation might offer a safe alternative in the management of hypercholesterolemia. Studies have shown that SDG supplementation reduced LDL-C and serum cholesterol; however, purified SDG production is costly. Recently, Prairie Tide Diversified Inc. produced a more affordable SDG polymer that contains 50% or more SDG. Although SDG polymer undergoes deglycosylation in the gastrointestinal tract following consumption to yield SDG, the extent to which SDG is released from the polymer and subsequent bioavailability of the SDG metabolites is unknown. Our pharmacokinetic knowledge regarding flaxseed bioactives suggests that the mammalian lignans, enterodiol (ED) and enterolactone (ENL), and ENL-glucuronide play important role in high cholesterol treatment. Therefore, to understand the relative bioactivity of purified SDG and SDG polymer it is necessary to first understand the pharmacokinetics of the two SDG forms. This will be one of the aims of this thesis. In addition, the biological activity of this SDG polymer has never been studied. Hence, the effects of SDG polymer administration in treatment of hypercholesterolemia will be another aim of this work.

3 PURPOSE OF PROJECT

3.1 Hypothesis

3.1.1 Hypothesis 1

There will be no difference in the pharmacokinetics of SDG and its metabolites in female Wistar rats administered equivalent doses of SDG polymer or purified SDG.

3.1.2 Hypothesis 2

Chronic administration of SDG polymer will induce equivalent reductions in serum lipid levels and steatohepatitis as purified SDG in diet-induced hypercholesterolemic female Wistar rats, when compared to hypercholesterolemic controls.

3.2 Objectives

3.2.1 Objective 1

To compare the relative bioavailability of purified SDG and SDG polymer in rat by measuring SDG metabolites after administration of a single oral bolus.

3.2.2 Objective 2

To investigate the effects of oral purified SDG and SDG polymer administration in diet-induced hypercholesterolemic rats.

a. Assess the effects of purified SDG and SDG polymer on body weight, liver weight, and serum lipid parameters.
b. Analyze the effects of purified SDG and SDG polymer on steatohepatitis.

4 MATERIALS AND METHODS

4.1 Comparison of oral pharmacokinetics of purified SDG and SDG polymer in rat

4.1.1 Chemicals and reagents

Purified SDG (96.1%) and SDG polymer were kind gifts from Prairie Tide Diversified Inc. (Saskatoon, Canada). Chemicals including secoisolariciresinol (SECO), enterolactone (ENL), enterodiol (ED), PEG 400, and sodium acetate buffer were purchased from Sigma-Aldrich (Oakville, ON, Canada). Racemic enterolactone-^{13}C3, enterodiol-^{13}C3, and racemic secoisolariciresinol-D6 was from Toronto Research Chemicals (TRC) (Toronto, ON, Canada). MP Biomedicals™ Beta-Glucuronidase solution from *Helix pomatia* were purchased from Fisher Scientific (Ottawa, ON, Canada). Diethyl ether, 0.9% sodium chloride, LC/MS grade acetonitrile (ACN), LC/MS grade water, LC/MS grade methanol were acquired from ThermoFisher Scientific (Toronto, ON, Canada). Whatman Mini-UniPrep Syringeless Filter vials were procured from GE Healthcare Life Sciences (Mississauga, ON, Canada). Rat blank plasma was obtained directly from rats in UACC Animal & Tissue Share program. Double deionized water was provided from a MilliQ Synthesis Water Purification. Dow Corning silastic tubing and Intramedic polyethylene tubing were purchased from VWR (Mississauga, ON, Canada). All other chemicals used were analytical grade or better.

4.1.2 Animals

Wistar female rats (about 300 g) were obtained from Charles River Canada (St. Constant, PQ, Canada). Animals were housed under controlled temperature and maintained on a 12 hour dark-light cycle. The rats received a standard laboratory rodent chow and water *ad libitum* throughout a one-week acclimatization period. During this time, rats were acclimated to handling and restraint such that rats were familiar with the handler. This work was approved by the University of Saskatchewan's Animal Research Ethics Board (20180044) and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

4.1.3 Dosing vehicle

Considering SDG polymer's poor water solubility, SDG polymer was first dissolved in 70%

ethanol. Subsequently, PEG 400 and saline were added to the lignan solution to form the suspension. The final proportion of ethanol, PEG 400 and saline were 1:3:6. Purified SDG used the same dosing vehicle as SDG polymer.

4.1.4 Single dose oral pharmacokinetics study

Wistar female rats were randomized into two groups (n=12): one group received purified SDG (40 mg SDG/kg body weight) while the other group received SDG polymer (amount of SDG is equal to purified SDG). Rats had a jugular vein cannula surgically implanted one day prior to the PK study. After fasting overnight, rats were administered purified SDG or SDG polymer by single dose oral gavage. Rats were singly housed during the blood collection period. Due to the limited blood volume, rats of each treatment groups (n=12) were divided into two subgroups. Blood samples (300 µL per time) of first subgroup (n=6) were collected via jugular vein cannulation at 0 (pre-dose), 15, 30, 60, 90 min, and 2, 3, 4, 8 and 12 hours after the dosing. For second subgroup (n=6), jugular blood samples (300 µL per time) were collected at 0 (pre-dose), 12, 16, 20, 24, 32, 40 and 48 hours after the dosing. Food was provided to the rats two hours after dosing.

4.1.5 Plasma Preparation

Blood samples were collected in Lithium Heparin tubes and then centrifuged at 3000 rpm for 5 min. Separated plasma was transferred into 2 mL plastic micro-centrifuge tubes and stored at -80°C until analysis.

4.1.6 Quantitation of lignans

4.1.6.1 LC-MS/MS conditions

This assay was adopted from a published protocol of Jane Alcorn (2017)[120]. The chromatographic system used for analysis was an Agilent series 1200 binary pump (G1311A) with a degasser (G1322A) and auto sampler (G1329A) (Agilent Technologies, Mississauga, ON, Canada), and the analytes were detected with an AB Sciex API 4000 Q-Trap mass spectrometer (AB Sciex, Concord, ON, Canada) with a Turbo V electrospray ionization (ESI) source in the negative mode. A 2.1 x 50 mm, 2.7 µm, Poroshell 120 EC-C18 column with 2.1 x 5 mm, 2.7 µm, guard column (Agilent Technologies) was used and the column temperature was 20°C. Analytes were separated under gradient mobile phase conditions with a mobile phase that consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile, at a flow rate of 250 µL/min and injection volume of 5 µL. Lignans were

separated by a gradient starting with 85% A and 15% B for 0.5 minutes which was subsequently decreased to 5% A over 6 minutes. After each chromatogram the column was returned to starting conditions (85% A over 0.5 minutes) and held for 5 minutes under that condition before the next injection. The total chromatogram time per sample and regeneration is 12 min. In the AB Sciex API 4000 Q-Trap mass spectrometer, Curtain gas pressure was adjusted at 30 psi; GS1 and GS2 were set at 60 psi. The Ion spray voltage was maintained at -4500 V and ESI source interface temperature was set at 700°C. The flaxseed lignan metabolite fragment ionization conditions are listed in Table 4.1.

Table 4.1 ABSciex QTRAP 4000 mass spectrometry conditions for multiple reaction monitoring of analytes.

Analyte	Fragment	Declustering potential	Collision energy	Collision cell exit potential
SECO	361.019 > 164.800	-90	-36	-11
SECO-D6 (IS)	367.126 > 168.000	-95	-36	-13
ED	301.000 > 106.000	-85	-46	-5
ED-^{13}C3 (IS)	304.000 > 273.000	-95	-32	-13
EL	297.000 > 189.000	-90	-30	-7
EL-^{13}C3 (IS)	299.942 > 255.100	-75	-30	-13

4.1.6.2 Preparation of standard curve solutions and quality control

Stock solutions (1 mg/mL) of SECO, ED, EL and their internal standard (IS) SECO-D6, ED-^{13}C3, EL-^{13}C3, were dissolved in methanol and stored at -20°C. Mixed working solutions of SECO, ED and EL were prepared by serial dilution of stock solutions with water-acetonitrile (90:10) mixture to produce concentrations for the calibration curve (SECO: 100 – 1000 ng/mL; ED: 15 – 1000 ng/mL; EL: 50 – 1000 ng/mL) and the mixture of internal standards were diluted with water-acetonitrile (90:10) mixture to concentrations of SECO-D6, ED-13C3: 500 ng/mL; EL-13C3: 250 ng/mL. Quality control (QC) presenting the entire range of the standard curve: lower limit of quantification (LLOQ), low quality control sample (LQC) (3-fold the lower limit of quantification), middle quality control sample (MQC), and high quality control (HQC) (80% of the upper limit of quantification), were prepared using water-acetonitrile (90:10) mixture.

To prepare the standard curve, 10 µL of working solutions was added to 40 µL of blank rat serum to achieve calibration standard curve range of 20 – 200 ng/mL for SECO, 3 – 200 ng/mL for ED, and 10 – 200 ng/mL for EL. QC samples were prepared by spiking 40 µL of blank rat serum with 10 µL of QC working solutions.

4.1.6.3 Extraction procedure

Internal standard (10 µL) was added to all samples and vortexed 15s to mix, after which 3 mL of diethyl ether was added. The mixture was shaken for 10 min to extract the lignans and internal standard, followed by centrifugation for 5 min at 1000 x g and then placed at -80°C for 5-10 minutes to freeze the lower plasma layer. The upper layer was transferred into disposable glass tubes (VWR® Culture Tubes, Disposable, Borosilicate Glass and dried to evaporation under a stream of air (about 20 minutes). Samples were reconstituted in 150 µL of water-acetonitrile (90:10) mixture, vortexed for 15 s, transferred into Whatman Mini-UniPrep Syringeless Filter vials, PTFE membrane, 0.2 µm pore size (Fisher Scientific Canada, Ottawa, ON) for analysis.

4.1.6.4 Glucuronidase/Sulfatase Sample Pretreatment

In order to measure total concentrations of SECO, ED, and EL in rat plasma after purified SDG or SDG polymer administration, 50 µL plasma was incubated with a 9 µL β-glucuronidase sulfatase solution (5000 U/mL) and 50 µL 0.1 mol/L sodium acetate buffer (pH5) prior to LC-MS/MS analysis. The mixture was then incubated in a shaking incubator at 37°C for 4 hours followed by the extraction with diethyl ether as described in 4.6.1.3.

4.1.6.5 Method validation

Since the assay was previously validated in the laboratory, a partial validation process was performed according to USFDA guidelines. A standard curve was constructed each time prior to analysis. QC samples were analyzed with each analysis run as acceptance criteria for each run. The lowest limit of quantification (LLOQ) was determined at the lowest concentration that signal-to-noise ratio was 5. The intra- and inter-day precision and accuracy of the assay was determined by analyzing six replicates at each of LLOQ, LQC, MQC, and HQC on three consecutive days. Precision (%) was expressed as relative standard deviation (RSD). Accuracy (%) was expressed as (observed amount/nominal concentration×100). The ratio of peak areas of lignans and their internal standard were plotted against the nominal concentrations of the calibration curve samples. A linear least squares regression analysis, using 1/X as weighting factor, was used to determine slope, intercept, and coefficient of determination (r^2) to indicate linearity of the method. Matrix effect was measured at LQC and HQC samples in triplicates. Post-extracted LQC and HQC samples were first prepared using water-acetonitrile (90:10) mixture, and then spiked into extracted blank rat plasma matrices using the same extraction method mentioned above. The absolute peak areas of the analytes were compared to those of analogues QC samples prepared in neat solvents.

4.1.7 Pharmacokinetic parameter estimation and statistical analysis

Pharmacokinetic parameters were estimated from concentration vs. time data using GraphPad Prism 7.0 (GraphPad software, San Diego, CA, USA). PK parameters were expressed as mean ± SD. Area under the curve (AUC) was estimated from concentration-time data using the linear trapezoidal rule-extrapolation method. Relative SDG polymer bioavailability was calculated using Equation 4.1. Linear regression using the terminal slope of natural logarithmic plasma lignan concentration versus time response supported the calculation of a log-linear terminal rate constant, k. The half-life ($t_{1/2}$) was estimated as 0.693/k. C_{max} and AUC of purified SDG and SDG polymer were compared using t test. T_{max} of purified SDG and SDG polymer were compared using Mann-Whitney U test (non-parametric). P value of 0·05 was considered as the threshold for significance testing.

$$Frel = \frac{AUC_{\text{SDG Polymer}}}{AUC_{\text{purified SDG}}} \times 100\% \qquad \text{Equation 4.1}$$

4.2 Hypocholesterolemic efficacy study of purified SDG and SDG polymer

4.2.1 Chemicals and reagents

Purified SDG (96.1%) and SDG polymer were kind gifts from Prairie Tide Diversified Inc. (Saskatoon, Canada). PEG 400 was purchased from Sigma-Aldrich (Oakville, ON, Canada). Ensure® Nutritional Drinks (strawberry flavor) was bought from Real Canadian Superstore (Saskatoon, SK, Canada). Rat blank plasma was obtained directly from rats in UACC Animal & Tissue Share program. Double deionized water was provided from a MilliQ Synthesis Water Purification. Stanbio Cholesterol Kit and Triglycerides kit were bought form Fisher Scientific (Ottawa, Ontario). HDL and LDL/VLDL Quantitation Kit was purchased from Sigma-Aldrich. RNAprotect Tissue Reagent was purchased from Qiagen (Hilden, Germany). All other chemicals used were analytical grade.

4.2.2 Animals

Female Wistar rats (N=35) were obtained from Charles River Canada (St. Constant, Quebec) at 10 weeks of age. The rats were housed in groups of two or three under controlled temperature (22 +/- 2°C) and maintained on a 12-hour light:dark cycle.

4.2.3 Dosing vehicle

Considering SDG polymer's poor water solubility, SDG polymer was first dissolved in 70%

ethanol. Subsequently, PEG 400 and saline were added to the lignan solution to form the suspension. To increase the taste, strawberry flavored Ensure was added to the suspension. The final proportion of ethanol, PEG 400, saline, and Ensure were 1:3:6:15. Purified SDG used the same dosing vehicle as SDG polymer.

4.2.4 Study design

On the date of arrival, female Wistar rats (10-week old, 200 - 250g) were randomized and divided into four groups. First group (n=5) were fed the standard rodent diet (LabDiet® Prolab® RMH 3000, 5P00), the other three groups (n=10) were fed a 1% cholesterol diet (Modified LabDiet® Prolab® RMH 3000, 5P00, with 1% total cholesterol). Animals consumed food and water *ad libitum*. After a one-week period on the diet and for acclimatization, rats in the 1% cholesterol diet groups, were administered vehicle, 6 mg/kg purified SDG or SDG Polymer at a dose equivalent to purified SDG using a modified oral gavage technique (with syringe)once daily for 23 days. This dose is equivalent to human studies which use 200 – 600 mg SDG daily. All rats were weighed daily. Fasted blood samples were collected under isoflurane anaesthesia at 2 weeks via saphenous venipuncture and at 23 days by cardiac puncture for serum lipids analysis. Then rats were euthanized by exsanguination following isoflurane anaesthesia. Livers were rapidly excised, weighed. Parts of liver were fixed in 10% formalin solution for routine histological analysis. Portions of liver were stored in RNAprotect Tissue Reagent for mRNA and protein analysis.

4.2.5 Serum lipids analysis

Blood samples were allowed to clot for 30 min at a room temperature and then centrifuged in an Eppendorf centrifuge at 3000 rpm for 10 min. Separated serum was transferred into 1.5 mL plastic micro-centrifuge tubes and was stored at -80°C until analysis.

Serum total cholesterol (TC) and triglycerides (TG) were determined by Stanbio Cholesterol LiquiColor colorimetric kits and Stanbio LiquiColor Triglycerides kits. In the manufacturer's brochure, TC and TAG are measured with cuvets and spectrophotometer. However, due to the large sample size, after validation we used 96-well plates and microplate reader instead. Sample volume was adjusted proportionally. High density lipoprotein cholesterol (HDL-C) were determined by Sigma HDL and LDL/VLDL Quantitation kit. LDL-cholesterol levels were determined using the Friedewald method[121], which subtracts HDL-C and VLDL-C from TC. VLDL calculated as one-fifth the level of TG.

For total cholesterol, cholesterol standard (200 mg/dL) was serially diluted with water to produce concentrations for the standard curve (0-200 mg/dL). In 96 well plate, 2 µL unknown serum or standard solutions were added to 200 µL of cholesterol reagent (Stanbio No. 1010) and pipette up and down to mix thoroughly. Following a 10-minute incubation at room temperature, the absorbance was measured at 500 nm using a Microplate Reader (Synergy HT, Biotek Instruments, Inc., Oakville, ON, Canada).

For triglyceride determination, Triglyceride Activator was added into Triglyceride Reagent. The following steps were the same as that of total cholesterol analysis except a triglyceride standard replaced the cholesterol standard. Serum TC and TAG concentration can be determined from the standard curve.

To separate HDL-C, 100 µL serum was mixed with 100 µL of the Precipitation Buffer in a microcentrifuge tube, incubated for 10 min at room temperature to allow LDL/VLDL precipitation and then centrifuged at 2000 x g for 10 min. The supernatant fraction (HDL) was transferred to a new tube. For HDL-C analysis, Reaction Mix were made by mixing Assay Buffer, Probe, Enzyme mix and Esterase together according to the manufacturer direction. 0, 4, 8, 12, 16, 20 µL of the 25 ng/ µL Cholesterol standard solution and 15 µL of each serum samples were added into a 96 well plate. Each well plate was brought to a final volume of 100 µL with cholesterol assay buffer and 50 µL Reaction Mix. After mixing well and incubated at 37 °C for 1h, the absorbance was measured at 570nm using a Microplate Reader.

The amount of HDL-C present in the samples can be determined from the standard curve. Concentration of HDL was calculated with Equation 4.2

$$C_{HDL\text{-}C} = S_a / S_v \times Df \qquad \text{Equation 4.2}$$

Where: S_a = Amount of cholesterol in unknow sample

S_v = Serum sample volume added into the wells

Df = The dilution factor (due to the 1:1 dilution with the Precipitation buffer, Df=2)

4.2.6 Histology and pathology analysis

Liver samples of rats were fixed in neutral buffered formalin for 48 hours. After fixation, tissue processing was done in an automated tissue processor. In the processor, the water from the livers were removed by dehydration with a series of alcohols, from 70% to 95% to 100%. The next step was called "clearing" and consisted of removal of the dehydrant with xylene that

was miscible with the embedding medium (paraffin). Finally, the tissue was infiltrated with the embedding agent paraffin.

Following processing, tissues were embedded in paraffin, and sectioned at 5 mm with Leitz microtome. Finally, the slides were stained with hematoxylin-eosin (H&E) for steatohepatitis grading. Morphological analysis was performed using a Nikon Eclipse E200 microscope with a *Motic*® Moticam S2 Microscope Tablet Camera.

A pathologist who was blinded to treatment groups assessed the liver sections for the degree of steatohepatitis, using grading criteria according to the NASH Clinical Research Network Scoring System[122]. The scoring system definition is detailed in Table 4.2. NAFLD activity score (NAS) was calculated as the sum of steatosis, lobular inflammation and ballooning.

Table 4.2 NASH Clinical Research Network Scoring System Definition and Scores

Item	definition	score
Steatosis	<5%	0
(Low- to medium-power	5%-33%	1
evaluation of parenchymal	>33%-66%	2
involvement by steatosis)	>66%	3
Lobular inflammation	No foci	0
(Overall assessment of all	<foci per 200×field	1
inflammatory foci)	2-4 foci per 200×field	2
	>4 foci per 200×field	3
Ballooning	None	0
	Few balloon cells	1
	Many cells/prominent ballooning	2

4.2.7 Statistical analysis

All data were analyzed using SPSSv26 (IBM SPSS Statistics 26, NY, US). All results were presented as means and standard deviation, except for serum lipids levels and histological NAFLD activity score, which expressed as mean and standard error. Because my purpose is to determine the effect of SDG and SDG polymer in diet-induced hypercholesterolemic rats, I performed a statistical test in three groups with 1% cholesterol diet. Five rats for the negative

control (standard diet) were used to confirm diet-induced hypercholesterolemia. I performed statistical test between standard diet and 1% cholesterol diet without lignan intervention. Comparisons of body weight, liver weight, and serum lipid levels between 1% cholesterol diet control group and standard diet group used t-test, while comparisons of NAFLD scores use non-parametric Mann – Whitney U test. Comparisons of body weight, liver weight, and serum lipid levels among 1% cholesterol diet control, purified SDG, and SDG polymer treatment groups used one-way ANOVA and Tukey post-hoc test, while comparisons of NAFLD scores used non parametric Kruskal-Wallis H test. Differences were considered significant at p-value < 0.05.

5 RESULTS

5.1 Comparison of oral pharmacokinetics of purified SDG and SDG polymer in rat

5.1.1 LC-MS/MS method validation

Figure 5.1 presents representative LC-MS/MS spectrums of lignans and their respective internal standards spiked into rat plasma. The spectra demonstrated that the method is specific with the absence of endogenous peaks that co-elute with the lignans and internal standards.

Before conducting the PK study in rat, a partial validation process of a previously validated LC-MS/MS assay for lignan and lignan metabolites in human plasma was performed. Therefore, the lignans quantitation assay was suitable for a rat PK study. SDG was not included in the analysis as previous studies in the lab, including rat *in vivo*[117, 118] PK and *in vitro* Caco-2[99] transwell permeation assays, failed to detect SDG.

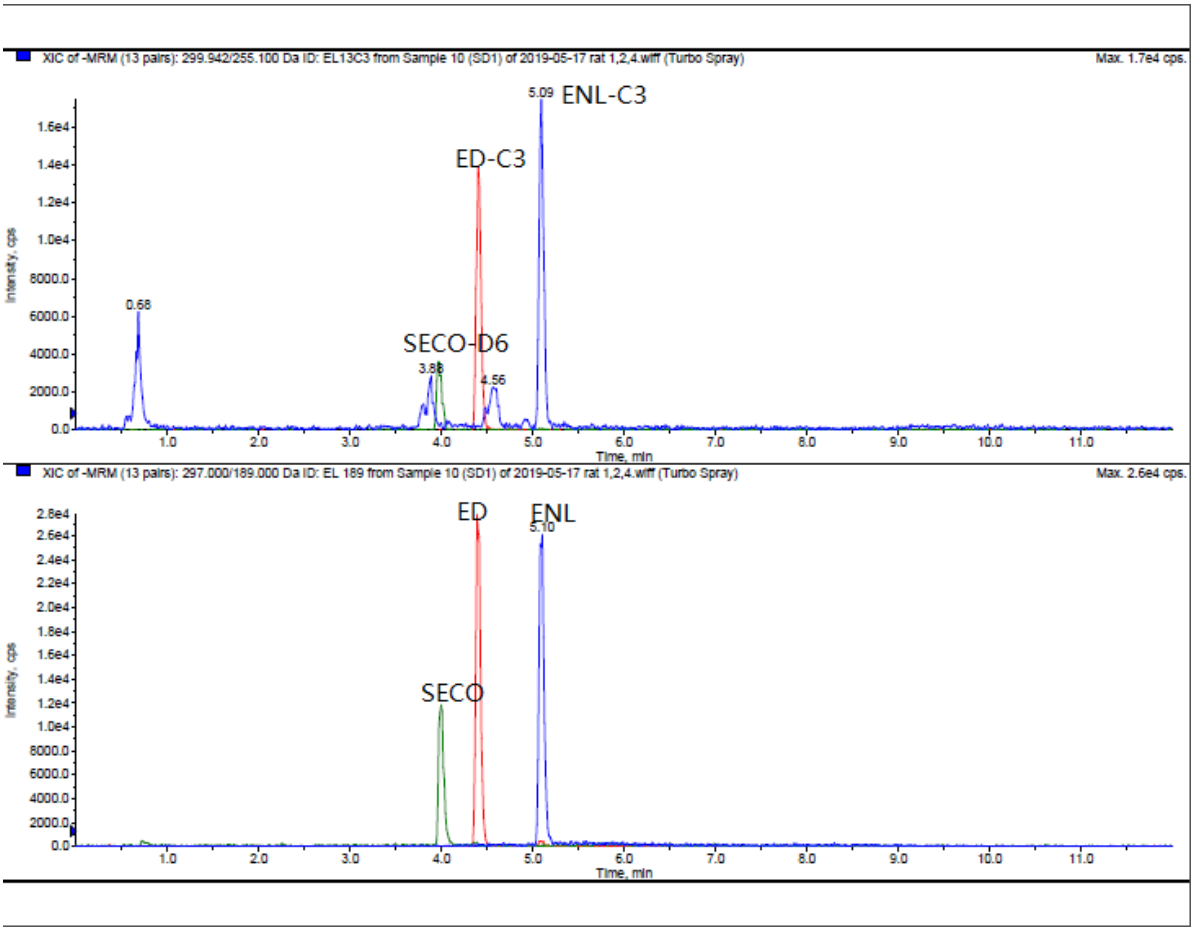


Figure 5.1 Representative LC-MS/MS peaks of rat blank plasma for SECO (200 ng/mL), ED (200 ng/mL) and ENL (200 ng/mL). The analyte peaks from left to right in the lower spectrum were SECO [m/z 164], END [m/z 106] and ENL [m/z 189]. The internal standards used for these analytes were SECO-D6, END-^{13}C3, and ENL-^{13}C3, which were in the upper spectrum at the same retention time as the analysts in the lower spectrum.

The limit of detection (LOD) for SECO, ED, and ENL was 10, 1.5, and 5 ng/mL respectively, and the lowest limit of quantification (LLOQ) was 20, 3, and 10 ng/mL, respectively. The method was linear over a concentration range of 20 – 200 ng/mL for SECO, 3 – 200 ng/mL for ED, and 10 – 200 ng/mL for ENL with coefficient of determination values greater than 0.995 for all calibration curves. The matrix factor of SECO, ED, and ENL was 104% ± 10.2%, 121% ± 6.8%, 133% ± 23%, which means the majority of analytes suffered from low ion enhancement effect. Intra-day and inter-day accuracy and precision values of SECO, ED and ENL are listed in Table 5.1, 5.2, 5.3.

Table 5.1 Intra-day (n=6) and inter-day (three consecutive days, n=18) accuracy and precision

values of secoisolariciresinol (SECO) determination by LC-MS/MS in rat plasma.

SECO nominal concentration (ng/mL)	Intra-day accuracy and precision			Inter-day accuracy and precision		
	Observed concentration (mean ± SD, ng/mL)	Accuracy (%)	Precision (CV%)	Observed concentration (mean ± SD, ng/mL)	Accuracy (%)	Precision (CV%)
LLOQ : 20	20.3±2.25	102	11	19.8±0.50	99	3
LQC: 60	60.9±5.17	101	9	61.9±0.98	103	2
MQC: 80	74.7±6.40	93	9	81.6±6.09	102	7
HQC: 160	165.0±10.90	97	7	172.2±7.12	108	4

Table 5.2 Intra-day (n=6) and inter-day (three consecutive days, n = 18) accuracy and precision values of enterodiol (ED) determination by LC-MS/MS in rat plasma.

ED nominal concentration(ng/mL)	Intra-day accuracy and precision			Inter-day accuracy and precision		
	Observed concentration (mean ± SD, ng/mL)	Accuracy (%)	Precision (CV%)	Observed concentration (mean ± SD, ng/mL)	Accuracy (%)	Precision (CV%)
LLOQ : 3	2.7±0.23	91	9	2.9±0.17	96	6
LQC: 9	8.7±0.23	96	3	8.8±0.40	97	5
MQC: 80	79.2±2.23	99	3	80.8±1.43	101	2
HQC: 160	169.3±6.98	100	4	173.6±4.03	109	2

Table 5.3 Intra-day (n=6) and inter-day (three consecutive days; n = 18) accuracy and precision values of enterolactone (ENL) determination by LC-MS/MS in rat plasma.

ENL nominal concentration(ng/mL)	Intra-day accuracy and precision			Inter-day accuracy and precision		
	Observed concentration (mean ± SD, ng/mL)	Accuracy (%)	Precision (CV%)	Observed concentration (mean ± SD, ng/mL)	Accuracy (%)	Precision (CV%)
LLOQ : 10	10.4±1.52	104	15	10.4±0.34	104	3
LQC: 30	31.7±1.57	106	5	31.8±0.47	106	1
MQC: 80	81.0±3.10	101	4	83.5±3.31	104	4
HQC: 160	166.7±2.88	98	2	179.1±11.60	112	6

Collectively the data suggest that this LC-MS assay is sensitive, accurate, and precise for

the quantification of SECO, ED, and ENL. Therefore, it is suitable for the following pharmacokinetic study which requires an accurate, precise, and sensitive (to detect and quantify small changes in drug concentration over time) analytical technique.

5.1.2 48-hour pharmacokinetic study of purified SDG and SDG polymer

A 48-hour study was conducted to assess SECO, ED, and EL concentration response, based on the results of previous study in our lab indicating that two metabolites with bioactivity may not be detected after 24 hours, while another metabolite is detected in 15-36 hours. Tables 5.4 and 5.5 summarize concentrations of total ED of the 48-hour study. Tables 5.6 and 5.7 summarize concentration of total ENL of the 48-hour study. Unconjugated SECO, total SECO, unconjugated ED, and unconjugated ENL could only be detected in a few samples at different time point collections.

For rats receiving purified SDG, total SECO was detected in 0.25, 0.5, 1.5 and 3 hour samples of rat 6. It was also detected in 2 and quantitated in 3 hour (25.9 ng/mL) samples of rat 3, and detected in 3 and 4 hour sample of rat 1 and 2 (quantified in 4 hour sample of rat 2: 24.3 ng/mL). Total SECO was detected at its highest level in 3 hour sample in rat 7 (58.7 ng/mL). Unconjugated SECO was only detected at one time point (8h) of rat 3 following administration of Purified SDG.

In both purified SDG and SDG polymer group, unconjugated ED was detected in 8 (3.4 ± 3.3 ng/mL) and 12 h (6.2 ± 3.3 ng/mL) time points of most rats. Unconjugated ED was also detected at 3 and 4 h time samples in rat 4 (Purified SDG dose) and in 16 h sample in rat 13 and 24 (SDG polymer dose).

Unconjugated ENL was not detected in rats administered either purified SDG group or SDG Polymer.

Table 5.4. Serum concentration versus time response of total enterodiol (ED) after single oral dose of purified secoisolariciresinol diglucoside (SDG) (40 mg/kg, n=7) or SDG polymer (equivalent amount of SDG as purified SDG, n=5) by gavage in female Wistar rats from 0 h to 12 h.

Time (h)		0	0.25	0.5	1	1.5	2	3	4	8	12
Purified SDG (ng/mL)	Rat 1	0	0	0	0	0	D	4.11	4.2	14.2	200
	Rat 2	0	0	0	0	0	0	D	D	31.5	300
	Rat 3	0	0	0	0	D	3.1	4.71	6.2	54.3	238
	Rat 4	0	0	0	0	0	3.6	23.5	47.6	588	227
	Rat 5	0	0	0	0	0	0	D	D	44.4	477
	Rat 6	0	0	0	0	0	0	D	4.1	98	362
	Rat 7	0	0	0	0	0	D	D	3.6	54.6	427
	mean	0	0	0	0	D	1.7	6.2	9.9	126.4	318.7

SDG polymer (ng/mL)	Rat 8	0	0	0	0	0	0	6.7	11.4	90.4	248
	Rat 9	0	0	0	D	D	6.2	12.6	18.5	251	286
	Rat 10	0	0	0	0	D	D	7.5	9.1	172	93.5
	Rat 11	0	D	0	0	D	3.9	9.0	11.8	389	126
	Rat 12	0	0	0	0	0	D	7.5	14.5	229	15.5
	mean	0	D	0	D	1.4	3.0	8.7	13.1	226.3	153.8

D – detected (below the LOQ of the assay: 3 ng/mL)

Table 5.5 Serum concentration versus time response of total enterodiol (ED) after single oral dose of purified secoisolariciresinol diglucoside (SDG) (40 mg/kg, n=6) or SDG polymer (equivalent amount of SDG as purified SDG, n=6) by gavage in female Wistar rats from 12 h to 48 h.

Time (h)		0	12	16	20	24	32	40	48
Purified SDG (ng/mL)	Rat 13	0	256	33.3	D	D	0	0	D
	Rat 14	0	131	6.9	9.1	10.4	0	0	0
	Rat 15	0	26.5	3.2	0	0	0	0	0
	Rat 16	0	279	14.4	0	0	0	0	0
	Rat 17	0	33.2	D	0	0	0	0	0
	Rat 18	0	90.6	4.1	0	0	0	0	0
	mean	0	136.1	10.7	1.8	2.1	0	0	0
SDG polymer (ng/mL)	Rat 19	0	9.8	D	0	0	0	0	0
	Rat 20	0	102	6.4	0	0	0	0	0
	Rat 21	0	130	29.8	0	0	0	0	0
	Rat 22	0	234	20.4	0	0	0	0	0
	Rat 23	0	113	11.1	4.2	D	0	0	0
	Rat 24	0	366	88.3	20.1	D	0	0	0
	mean	0	159.1	31.2	4.1	D	0	0	0

D – detected (below the LOQ of the assay: 3 ng/mL)

Table 5.6 Serum concentration versus time response of total enterolactone (ENL) after single oral dose of purified secoisolariciresinol diglucoside (SDG) (40 mg/kg, n=7) or SDG polymer (equivalent amount of SDG as purified SDG, n=5) by gavage in female Wistar rats from 0 h to 12 h.

Time (h)		0	0.25	0.5	1	1.5	2	3	4	8	12
Purified SDG (ng/mL)	Rat 1	15.5	20.8	23.3	20.1	14.4	13.8	22.9	21.5	27.9	54.1
	Rat 2	20.9	16.7	25.4	18.0	10.0	11.3	17.6	20.7	37.7	60.2
	Rat 3	19.5	13.6	14.4	8.4	10.9	10.9	12.7	8.8	20.7	41.8
	Rat 4	11.5	10.0	9.2	8.0	5.7	5.3	8.0	18.8	54.6	108.0
	Rat 5	13.5	20.5	12.3	8.9	11.2	14.1	16.2	11.1	39.3	61.9
	Rat 6	25.5	29.6	20.0	8.9	13.2	11.2	28.5	13.0	43.9	122.0
	Rat 7	21.7	19.3	18.3	25.8	28.0	29.5	8.3	26.7	45.8	84.5
	mean	18.3	18.6	17.6	14.0	13.3	13.7	16.3	17.2	38.6	76.1
SDG polymer (ng/mL)	Rat 8	15.2	11.9	12.0	11.9	11.0	11.6	16.8	23.6	49.4	66.4
	Rat 9	5.6	3.8	3.8	7.5	7.5	8.4	10.4	12.4	25.8	75.4
	Rat 10	41.7	24.2	26.4	20.5	21.8	25.9	30.2	24.7	60.1	58.6
	Rat 11	19.4	23.4	23.5	21.6	15.7	25.8	13.4	15.2	37.0	36.8
	Rat 12	27.0	13.5	10.8	15.5	16.4	19.8	16.1	20.7	60.9	85.0
	mean	21.8	15.4	15.3	15.4	14.5	18.3	17.4	19.3	46.6	64.4

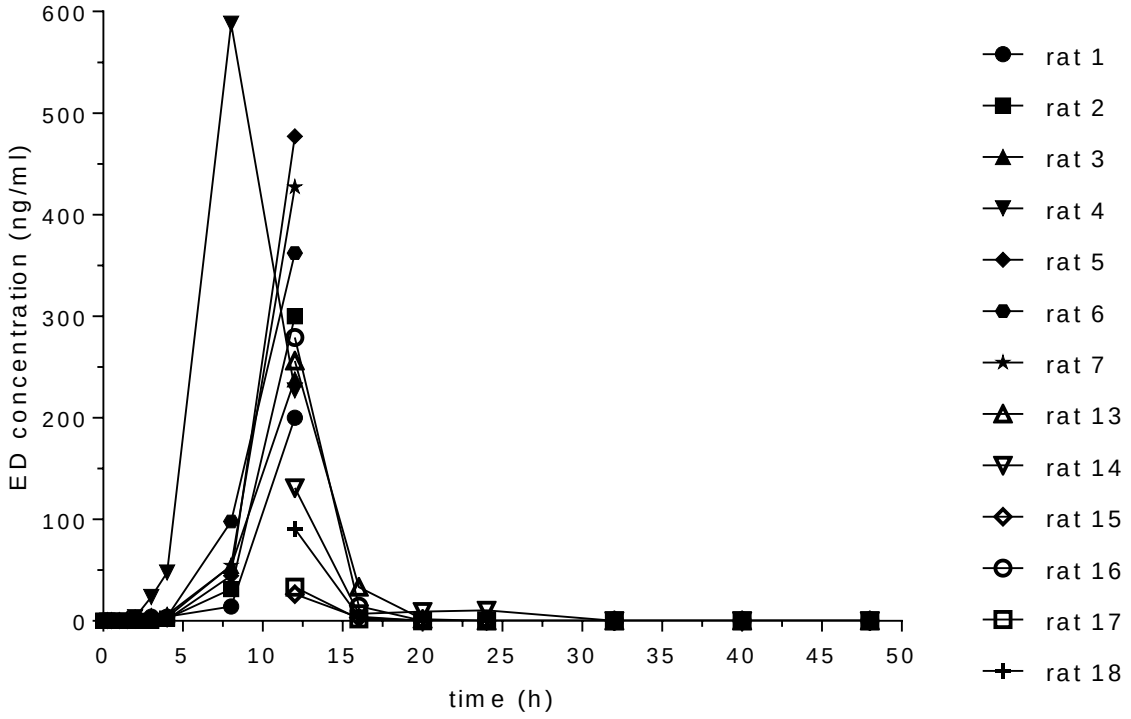
Table 5.7 Serum concentration versus time response of total enterolactone (ENL) after single oral dose of purified secoisolariciresinol diglucoside (SDG) (40 mg/kg, n=6) or SDG polymer (equivalent amount of SDG as purified SDG, n=6) by gavage in female Wistar rats from 12 h

to 48 h.

Time (h)		0	12	16	20	24	32	40	48
Purified SDG (ng/mL)	Rat 13	27.5	60.9	68.9	61.2	33.1	24.5	21.8	28.9
	Rat 14	0.0	104.0	81.7	80.4	65.6	14.4	13.1	20.2
	Rat 15	36.4	100.0	93.6	72.5	51.2	37.3	17.4	19.3
	Rat 16	3.6	58.9	80.6	61.0	44.0	29.2	20.2	25.9
	Rat 17	25.8	98.1	64.6	48.2	36.1	26.0	22.9	16.6
	Rat 18	12.1	76.7	75.0	61.4	51.3	32.5	28.2	23.5
	mean	17.6	83.1	77.4	64.1	46.9	27.3	20.6	22.4
SDG polymer (ng/mL)	Rat 19	14.3	98.7	101.0	73.6	57.6	31.4	48.7	22.7
	Rat 20	10.4	75.3	81.9	59.9	45.3	22.0	27.2	43.6
	Rat 21	14.3	62.2	72.4	44.5	34.5	14.0	12.8	13.9
	Rat 22	9.8	37.4	56.3	38.4	32.6	16.5	19.0	22.2
	Rat 23	12.3	44.3	34.4	43.8	25.3	15.3	18.0	13.8
	Rat 24	13.6	45.6	37.8	46.4	40.7	17.9	20.7	13.4
	mean	12.5	60.6	64.0	51.1	39.3	19.5	24.4	21.6

Total ED concentration versus time in individual rats of Purified SDG treatment group and SDG polymer treatment group are depicted in Figure 5.2. Mean value of total ED concentration versus time of purified SDG treated or SDG polymer treated rats is presented in Figure 5.3

(a)



(b)

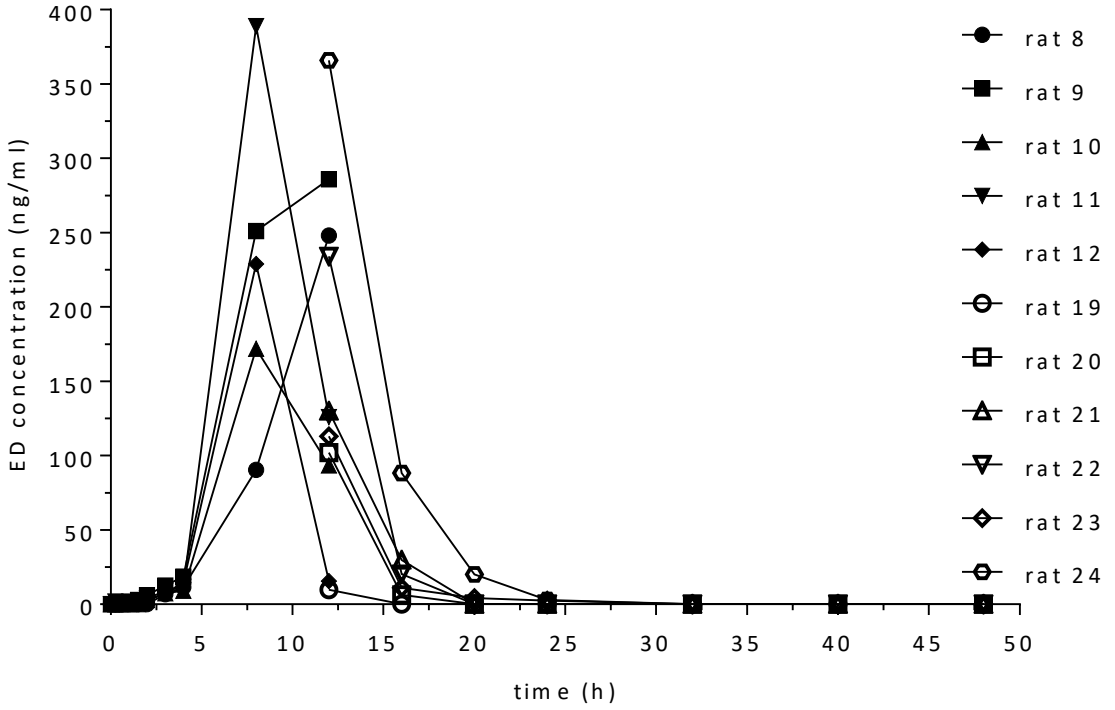


Figure 5.2 Total enterodiol (ED) serum concentration versus time response after a single oral dose of (a) purified secoisolariciresinol diglucoside (SDG) (40 mg/kg bwt); (b) SDG polymer (40 mg/kg bwt SDG) in female Wistar rats.

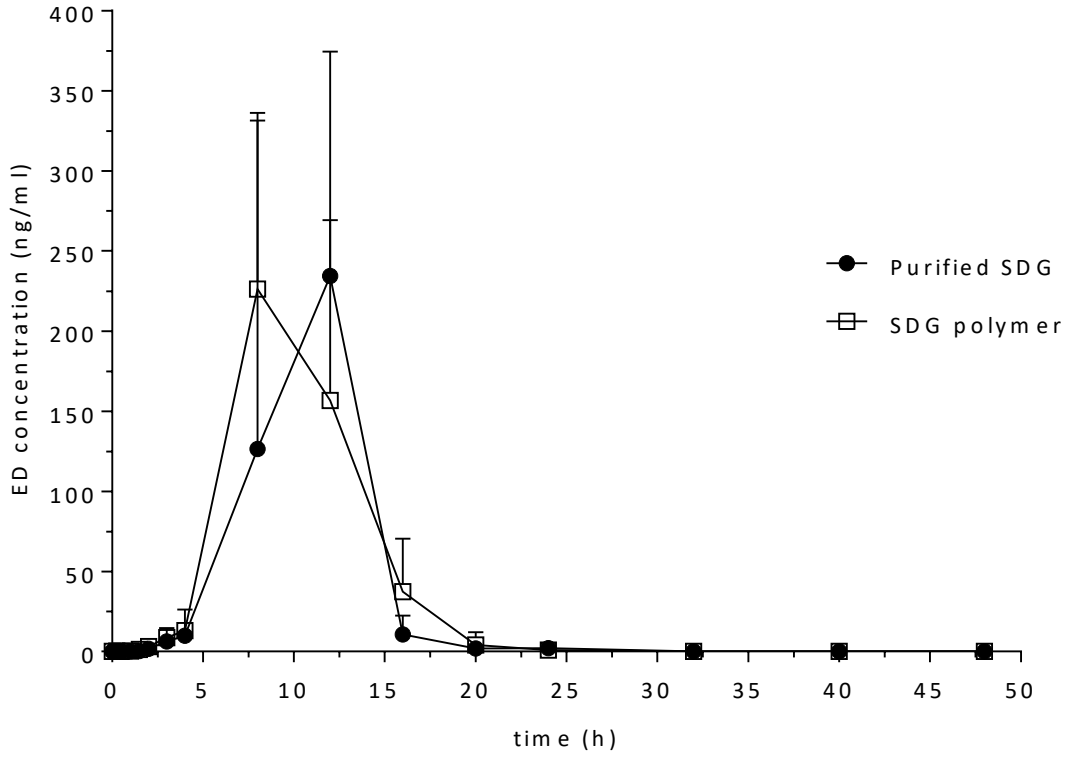
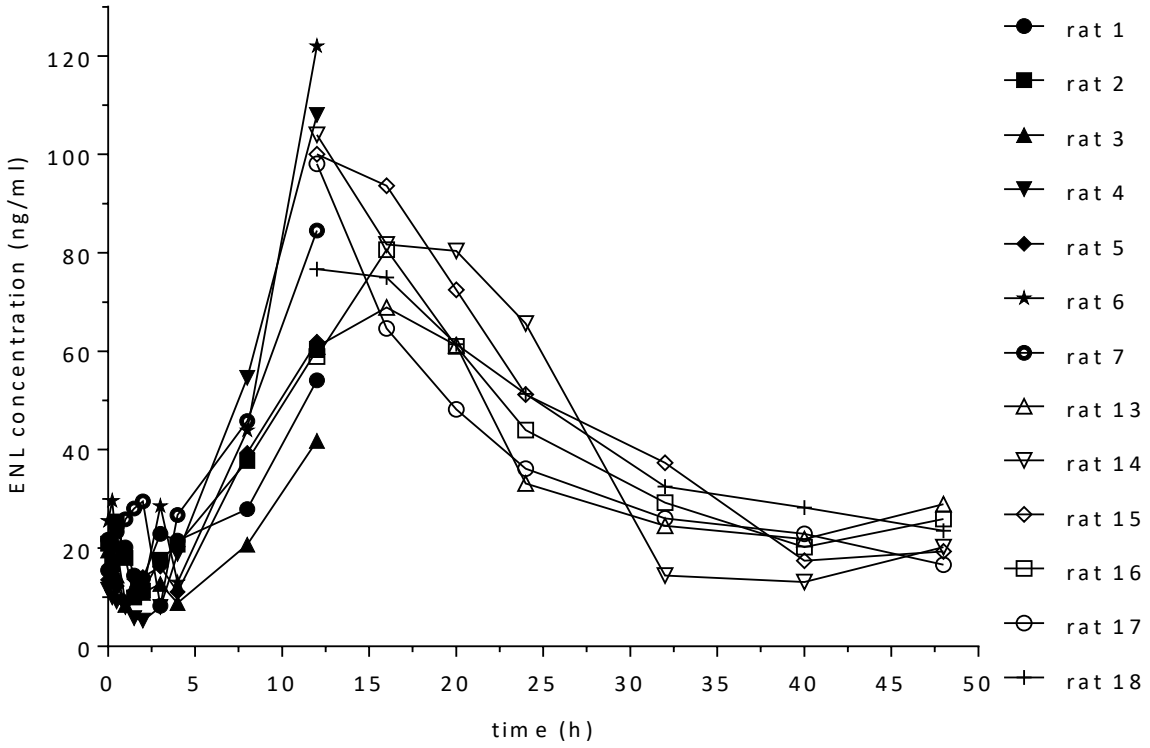


Figure 5.3 Total enterodiol (ED) serum mean concentration versus time plot after single oral dose of purified secoisolariciresinol diglucoside (SDG) (40 mg/kg bwt) or SDG Polymer (40 mg/kg bwt SDG) in female Wistar rats.

Total ENL concentration versus time in individual rats of Purified SDG treatment group and SDG polymer treatment group are also depicted in Figure 5.4 and Figure 5.5, respectively. Mean value of total ENL concentration versus time of Purified SDG treated or SDG Polymer treated rats are presented in Figure 5.6.

(a)



(b)

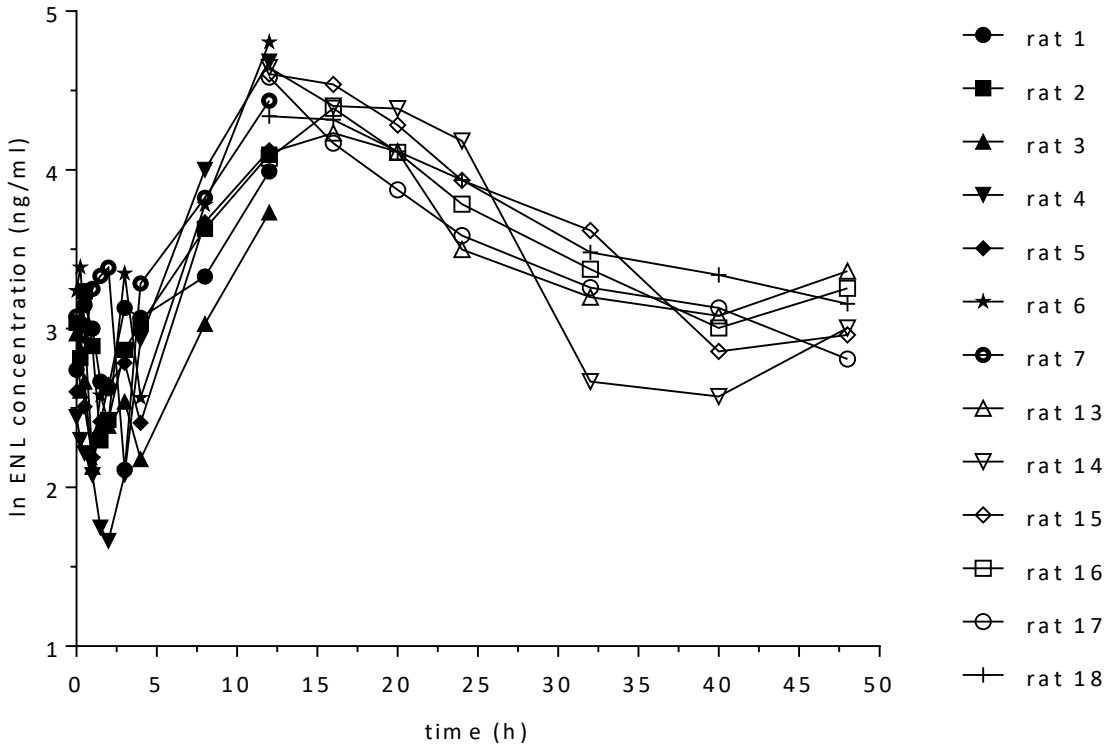
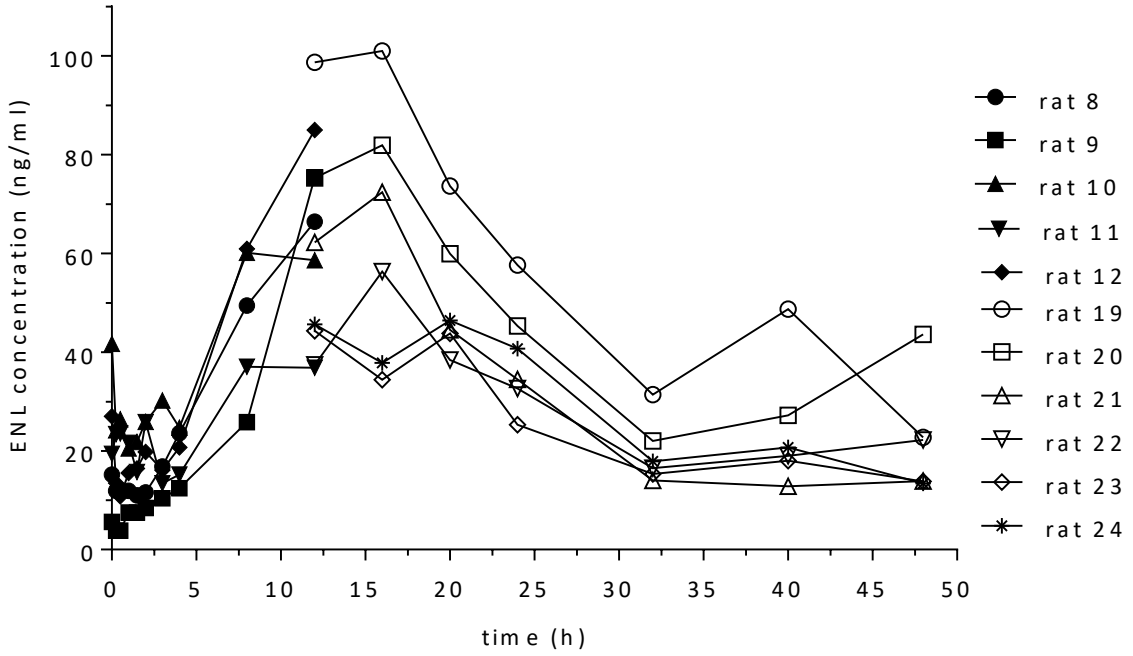


Figure 5.4 Total enterolactone (ENL) serum concentration versus time plot after a single oral

dose of purified secoisolariciresinol diglucoside (SDG) (40 mg/kg bwt) in female Wistar rats: (a) rectilinear plot; (b) semilogarithmic plot.

(a)



(b)

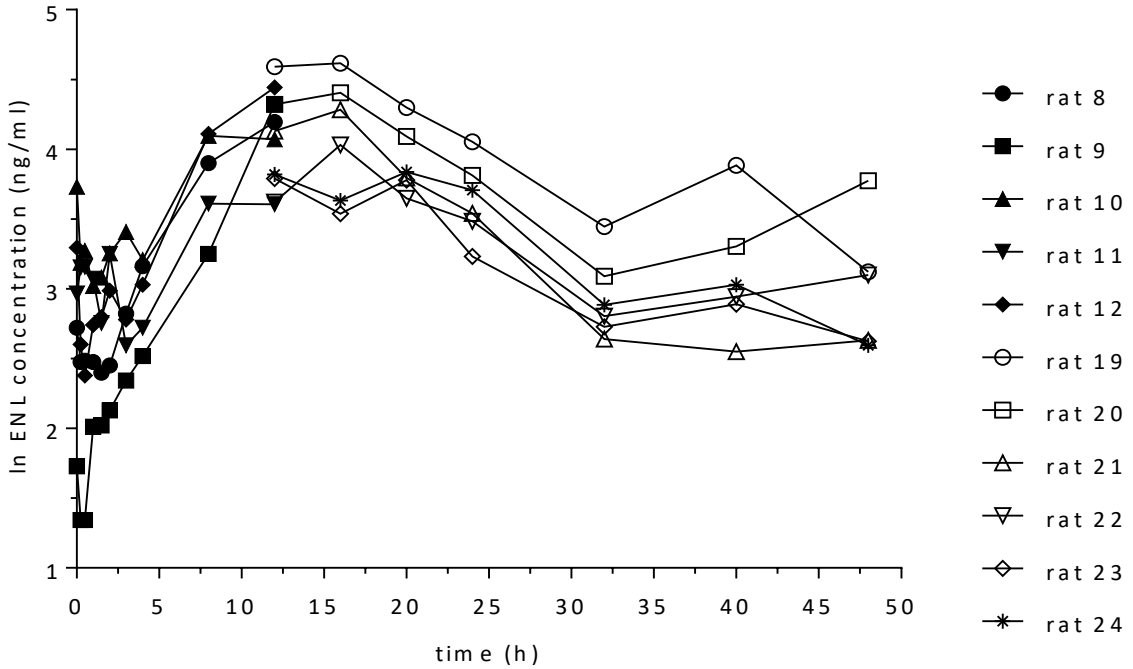


Figure 5.5 Total enterolactone (ENL) serum concentration versus time plot after single oral dose of secoisolariciresinol diglucoside (SDG) Polymer (40 mg/kg bwt SDG) in female Wistar rats: (a) rectilinear plot; (b) semilogarithmic plot.

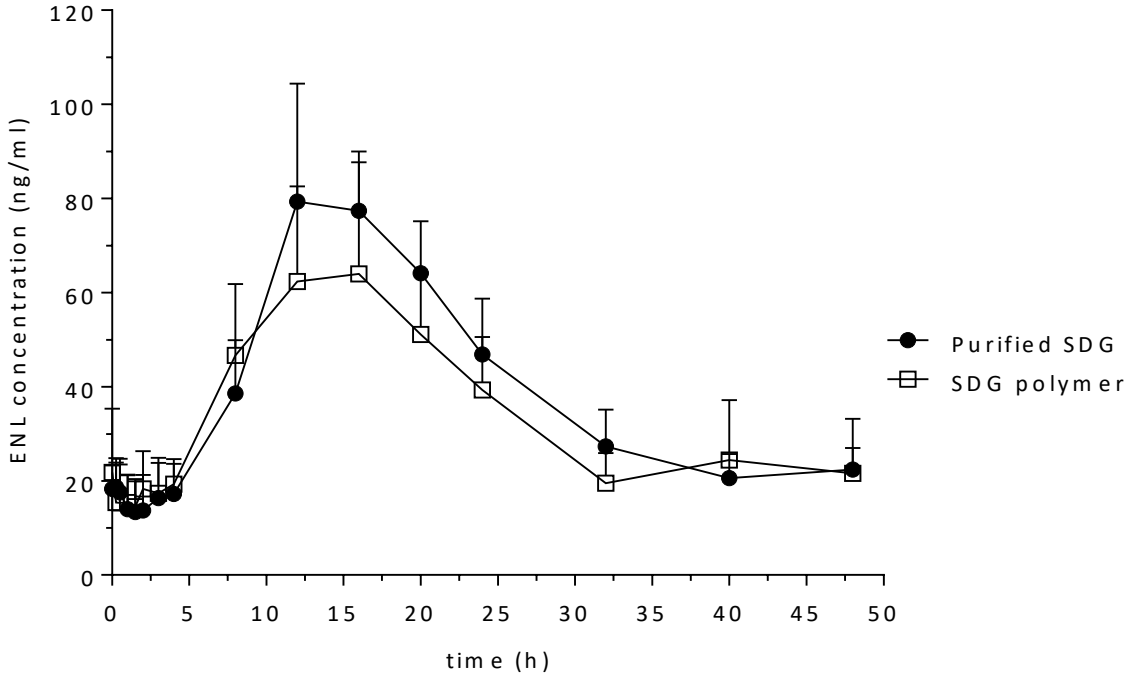


Figure 5.6 Total enterolactone (ENL) serum mean concentration versus time plot after single oral dose of purified secoisolariciresinol diglucoside (SDG) (40mg/kg bwt) or SDG Polymer (40 mg/kg bwt SDG) in female Wistar rats.

5.1.3 Pharmacokinetic Parameter Estimation

Non-compartmental method was used for estimation of ED and ENL PK parameters of Purified SDG treatment group and SDG Polymer group. Data collected during the terminal phase of natural logarithmic plasma ED concentration versus time study was not sufficient to provide an estimate of the elimination rate constant (k) and half-life, following a linear regression. The PK parameters are summarised in Table 5.8.

Table 5.8 Pharmacokinetic parameter estimates calculated by a non-compartmental pharmacokinetic analysis using GraphPad Prism 7 following a single oral dose administration of 40 mg/kg secoisolariciresinol diglucoside (SDG), or equivalent dosage of SDG polymer in female Wistar rats

Pharmacokinetic parameters	ED		ENL	
	Purified SDG	SDG polymer	Purified SDG	SDG polymer
C_{max} (ng/mL)	262.2 ± 170.8	207.2 ± 115.5	81.6 ± 23.8	65.9 ± 19.6

T_{max} (h)	11.7 ± 1.1	10.9 ± 1.9	12.6 ± 1.5	12.7 ± 3
k	—	—	0.058	0.075
$T_{1/2}$ (h)	—	—	12.0	9.2
AUC (ng×h/mL) (95% confidence interval)	1532 (472 to 2543)	1702 (715 to 2687)	1889 (1637 to 2143)	1677 (1111 to 2243)
F_{rel} (%, compared to purified SDG)	—	111	—	89

No significant difference was observed in T_{max}, C_{max} and AUC of total ED in purified SDG and SDG polymer. (P>0.05, Mann-Whitney U test for T_{max}, t test for C_{max} and AUC)
No significant difference was observed in T_{max}, C_{max} and AUC of total ENL in purified SDG and SDG polymer. (P>0.05, Mann-Whitney U test for Tmax, t test for Cmax and AUC)

The relative bioavailability (F_{rel}) of SDG polymer in total ED is 111%, while F_{rel} of SDG polymer in total ENL is 89%, as compared to that of purified SDG. There are no significant differences in AUC of total ED and total ENL between purified SDG and SDG polymer.

5.2 Hypocholesterolemic efficacy study of purified SDG and SDG polymer

5.2.1 Body weight and liver weight

The body and liver weight after 23 days of purified SDG or SDG Polymer administration with 30 days of 1% cholesterol diet feeding to hypercholesterolemic rats are listed in Table 5.9. When comparing 1% cholesterol diet treated rats (with no SDG) with those of standard diet, significant increases were observed in final body weight (P<0.05, 9%), liver weight (P<0.01, 23%), and normalized liver weight (P<0.0113%). Upon examination of gross liver morphology, differences in liver color were apparent between normo- and hypercholesterolemic rats. A diffuse yellowing of the liver was observed in rats of three groups consuming 1% cholesterol diet, suggesting hepatic fat accumulation.

Table 5.9. Body and liver weight indices (Mean ± SD) for female Wistar rats (10 weeks old) fed a standard diet or 1% cholesterol diet (30 days) and treated with oral doses of 0, 6.0 mg purified secoisolariciresinol diglucoside (SDG)/kg body weight (BW) or SDG polymer with equivalent SDG once daily for 23 days.

Weight parameter	Standard diet n=5	1% cholesterol diet, no SDG n=10	1% cholesterol diet with Purified SDG n=10	1% cholesterol diet with SDG Polymer n=10
Final body weight (g)	297±16.8	323±16.0[a]	323±12.8	330±15.0

Weight gain (%)	25.4±3.42	29.2±3.49	30.9±3.79	32.0±4.97
Liver weight (g)	8.8±0.52	10.8±0.89[b]	10.0±0.98	10.4±0.88
Liver weight/body weight (g/kg)	29.6±1.16	33.5±2.38[c]	31.0±2.16*	31.5±1.98

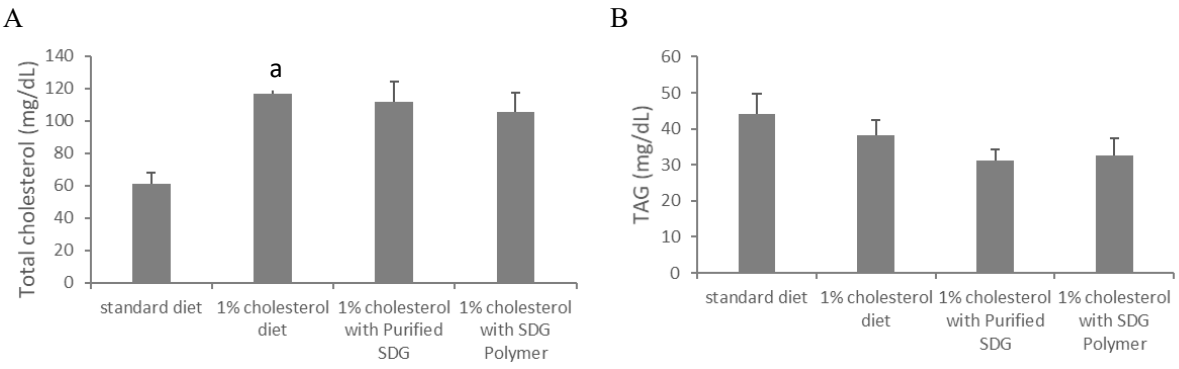
[a, b, c]Significantly different from standard diet control group (t-test, $P<0.05$)
*Mean value was significantly different from that of the 1% cholesterol diet without lignan group (One-way ANOVA, $P< 0{\cdot}05$).

In the three hypercholesterolemic treatment groups, no difference was observed in final body weight, weight gain, and liver weight. However, a significant reduction in normalized liver weight ($P<0.05$, 7%) was observed in Purified SDG treatment group when compared to control. Also, based on gross liver morphology, liver color was less yellow in both lignan treated groups in comparison to the hypercholesterolemic control group.

5.2.2 Serum lipid responses

Serum lipid parameters of rats fed a 1% cholesterol diet for 1 week before initiation of purified SDG or SDG polymer dosing for 23 days are presented in Figure 5.7. Rats in 1% cholesterol diet demonstrated a significant increase in TC (44%) and LDL-C (211%) when compared with rats in standard diet. They also had lower HDL-C level (51%) than those in standard diet.

Hypercholesterolemic rats given purified SDG demonstrated a distinctive, but not statistically significant reduction ($P>0.05$)in TAG (19%), and a slight reduction in TC (5%) and LDL-C (6%), when compared to hypercholesterolemic rats in control group. Similarly, hypercholesterolemic rats treated with SDG Polymer had a marked reduction ($P>0.05$)in TAG (15%), LDL-C (14%) and TC (10%). Both treatment groups showed an increase serum HDL-C level of 15% and 24%, respectively.



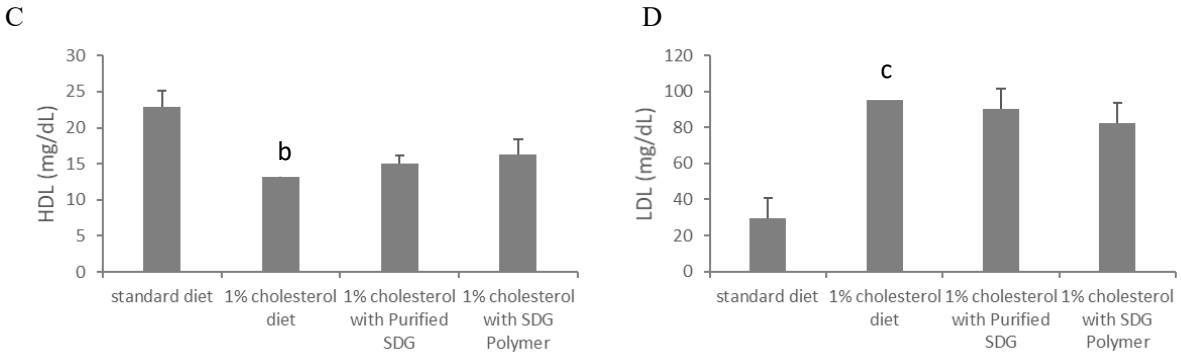
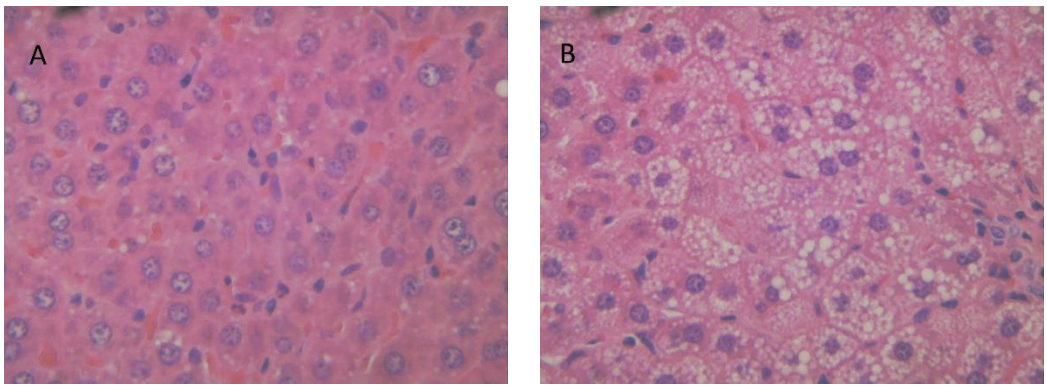


Figure 5.7 Serum lipid parameters (total cholesterol (A)), triglyceride (B) HDL-cholesterol (C) and LDL-cholesterol (D) following chronic daily oral purified secoisolariciresinol diglucoside (SDG) or SDG polymer administration in female Wistar rats fed a 1% cholesterol diet for 1 week before initiation of purified SDG or SDG polymer dosing for 23 days. Results are expressed as mean ± SEM. (Comparison between standard diet group and 1% cholesterol diet control group used t-test; comparison among three treatment groups with 1% cholesterol diet used one-way ANOVA, *P* set at 0.05).

[a, b, c] Significantly different from standard diet control group (t test, P<0.05).

5.2.3 Histology and pathology analysis

Histological assessment was conducted using H&E staining. Representative images of each groups are presented in Figure 5.8. A pathologist who was blinded to treatment groups assessed the liver sections for the degree of steatohepatitis, using grading criteria according to the NASH Clinical Research Network Scoring System. NAFLD scores of each group are listed in Table 5.10



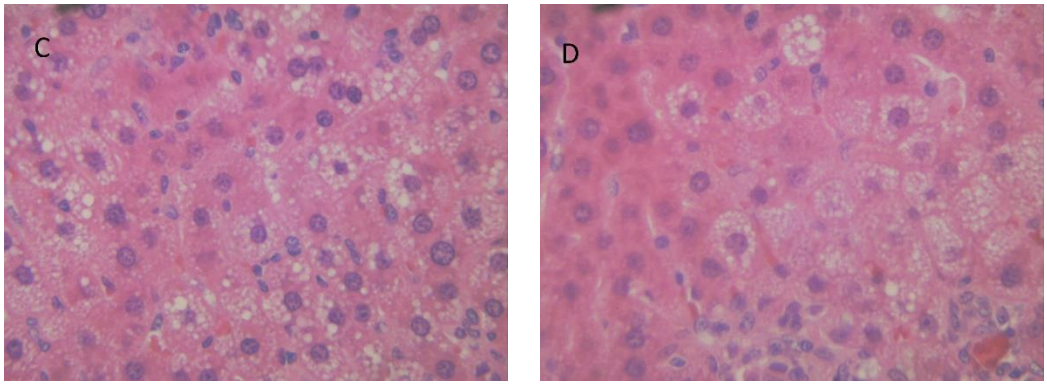


Figure 5.8 Liver histology by hematoxylin and eosin (H&E) staining of female Wistar rats (10 weeks old) fed a standard diet or 1% cholesterol diet (30 days) and treated with oral doses of 0, 6.0 mg purified SDG/kg body weight (BW) or SDG polymer with equivalent SDG once daily after 23 days. A: standard diet; B: 1% cholesterol diet; C: 1% cholesterol diet with purified SDG; D: 1% cholesterol diet with SDG Polymer. Representative images of H&E stained liver tissue are presented at 40×.

Table 5.10 NAFLD Scores (mean values and standard error) for female Wistar rats (10 weeks old) fed a standard diet or 1% cholesterol diet (30 days) and treated with oral doses of 0, 6.0 mg purified secoisolariciresinol diglucoside (SDG)/kg body weight (BW) or SDG polymer with equivalent SDG once daily after 23 days

Treatment	Standard diet	1% cholesterol diet	1% cholesterol with purified SDG	1% cholesterol with SDG Polymer
Steatosis	0	1.8 ±0.20[a]	1.3 ±0.26	1.4 ±0.31
Lobular inflammation	0.2 ±0.16	1[b]	0.7 ±0.15	1 ±0.15
NAS (NAFLD activity score)	0.2 ±0.16	2.8 ±0.20[c]	2 ±0.37	2.4 ±0.37

No balloon cell was found in any liver sections. Therefore, ballooning score was 0 in all groups.
[a, b, c] Significantly different from standard diet control group (Non parametric Mann – Whitney U test, $P<0.05$).
No significant differences were found among 1% cholesterol control, purified SDG, and SDG polymer in steatosis, lobular inflammation and NAS (Kruskal-Wallis H test, $P>0.05$).
Scoring for hepatic steatosis, lobular inflammation, and NAS was significantly lower with standard diet than those with 1% cholesterol diet.

Hypercholesterolemic rats given purified SDG demonstrated lower scores in steatosis (27.8%), lobular inflammation (30%) and NAS (28.5%), when compared to hypercholesterolemic rats in control group ($P>0.05$). Similarly, hypercholesterolemic rats treated with SDG Polymer had lower scores ($P>0.05$) in steatosis (22.2%) and NAS (14.3%). However, its mean score for lobular inflammation was the same as that of hypercholesterolemic control group. It should be noted that hepatocyte ballooning was not presented in all groups.

6 DISCUSSION

CVD remains the leading cause of morbidity and mortality worldwide. Hypercholesterolemia is one of the major independent risk factors for CVD. Lifestyle changes which combine a healthy diet and regular physical exercise, and drug therapy, specially statins, are the mainstays of hypercholesterolemia treatment. However, some side effects in organs are associated with statin treatment[16]. Several animal[74-78] and human[44-47] studies suggest that oral consumption of the SDG results in reduction in serum total and LDL-cholesterol and lipid accumulation in the liver. Therefore, oral SDG or SDG containing supplements are considered as a safe alternative to pharmaceutical hypocholesterolemic agents to manage hypercholesterolemia. Nevertheless, the process of hydrolyzing, extracting, and purifying SDG from the SDG polymer in flaxseed to produce a pure SDG is a costly procedure. In the market, 100 mg pure SDG (98%) is sold for $480. One g flaxseed lignan extract (SDG polymer: 40% SDG) is $5. If the recommended daily dose is 300 mg SDG, that means the cost is $1,469 when consuming pure SDG. Instead, the daily cost for consumption of SDG polymer is $3.75. SDG can also be formed in the GI tract following SDG polymer consumption[89], and undergoes subsequent bio-transformation to SECO, ED and ENL. The extent to which SDG is released from the polymer and subsequent bioavailability of the SDG metabolites was previously unknown. Furthermore, the biological activity of this SDG polymer has never been studied. We aimed to compare the pharmacokinetics and hypocholesterolemic effect of pure SDG and SDG polymer, to confirm the value of using an SDG polymer as a more economical product than purified SDG in the future application and management of hypercholesterolemia. To accomplish the aim of this study, two major objectives were addressed.

6.1 Comparison of oral pharmacokinetics of purified SDG and SDG polymer in rat

My first objective was to compare the relative bioavailability of SDG metabolites after single oral bolus administration of purified SDG and SDG polymer in rat. Rat is a common practical model for preclinical evaluation, particularly for pharmacokinetic evaluations. A complete plasma drug concentration versus time response can be recorded from a single rat allowing for pharmacokinetic parameter estimation. Rat strains also are nearly genetically identical which can lead to more consistent results, and their genetic, biological and behavior

characteristics closely resemble those of humans. However, an important limitation of the rat model is that there are considerable interspecies differences in first-pass metabolism in the gastrointestinal tract and liver[123]. Consequently, relative bioavailability determinations in rat may not translate to human relative bioavailability.

In the present study, the oral relative bioavailability (F_{rel}) was determined following comparison of AUC values of the mammalian lignans following oral administration of purified SDG and SDG polymer to parallel groups of rats. The F_{rel} of SDG polymer compared to that of purified SDG for total ED is 111%, while F_{rel} of total ENL is 89%. Statistical evaluation of AUC of total ED and total ENL between purified SDG and SDG polymer showed no differences. Various factors influence the availability of drugs prior to their entry into the systemic circulation, including but not limited to: physical properties of the drug (hydrophobicity, pKa, solubility), formulation, co-ingested factor, gastric emptying, intestinal motility, variability in microbiome, and first pass metabolism[124]. As for relative bioavailability of SDG polymer as compared to purified SDG, physicochemical properties, formulation, and inter-individual differences in intestinal microflora are the affecting factors. SDG polymer is poorly water soluble and more lipophilic. In the present study, we dissolved these two compounds in the same formulation to avoid differences of dissolution, which is considered as a prerequisite to the drug absorption process. Furthermore, SDG polymer undergoes hydrolysis to its monomer units, 3HMG and SDG, before further transformation to enterolignans [89]. Incomplete hydrolysis to SDG, then, may contribute to reductions in the relative bioavailability of SDG polymer. The lack of statistically different total mammalian lignan AUC values suggest hydrolysis of the SDG polymer to SDG is efficient within the rat gastrointestinal tract.

Comparison of total enterolignan AUC values is not the conventional approach towards determination of relative bioavailability. Conventionally, relative bioavailability is determined by comparing AUC of parent compound (SDG) after administration of two differentformulations of the same compound. However, lack of SDG bioavailability due to its poor permeation characteristics precluded assessment in this fashion. Instead, we compared AUC of mammalian lignans (total) because only the SDG metabolites SECO, ED, and ENL, can be absorbed into systemic circulation. Moreover, studies suggest that ED, ENL, and ENL-glucuronide play important role in high cholesterol treatment[26, 27].

Total enterolignan AUC values showed considerable interindividual variation amongst different rats. Important considerations in the variation in bioavailability include SDG's poor permeability characteristic, reliance on gastrointestinal flora for conversion to the mammalian

lignans, extensive first pass metabolism, and analytical sensitivity. Only the SDG metabolites SECO, ED, and ENL, are absorbed into systemic circulation. Interindividual differences in permeation of these lignan forms as well as differences in first-pass metabolism by the gastrointestinal tract and liver contribute to the interindividual variation in AUC values amongst the lignan forms. Previous research had found that SECO, ED, and ENL undergo extensive first pass metabolism by phase II enzymes in enterocytes and hepatocytes[99, 102]. This causes low bioavailability of lignans as a result of extensive first pass metabolism. In the current study, lignans in the systematic circulation are mostly present in their conjugated forms, which is consistent with the literature where it was reported the oral bioavailability in rat was 0, 25 and < 1% for SDG, SECO and ED, respectively[117]. Furthermore, variations in lignan-converting bacteria also contributes to the marked interindividual differences observed for enterolignan production[97]. Although rats are raised in the same environment and given the same diet, the rat gut microbiota can diversify due to the harvesting of more or less nutrients from this basic diet[125, 126]. Finally, robust AUC determination is hampered by limited analytical sensitivity. Total exposure is measured by AUC from time zero to infinity, calculating as AUC from time zero to the last measurable concentration (AUC_{0-t}) plus the last measurable drug concentration (C_{last}) divided by the terminal elimination rate constant (k). Therefore, incomplete concentration vs time response data also affects AUC estimation. Since we do not record continuous concentrations, sampling intervals also contributes to the variability.

6.1.1 Plasma SECO concentration versus time course in rat PK samples

SECO, the aglycone of SDG, is produced following hydrolysis of the glucose groups. SECO has known bioavailability (26%) in rat based on previous work[117]. However, it undergoes extensive phase II metabolism as well. Following administration of 40 mg/kg equivalent SDG oral doses, few rat plasma samples contained detectable levels of unconjugated SECO. Total SECO was detected from 15 min to 4 hours and quantifiable in three samples within 2 to 4 h, only following administration of pure SDG. Thus, we could not use SECO AUC for F determinations due to lack of concentration vs time response data for this metabolite.

Our findings are consistent with a previous PK study of SDG and its metabolites in Wistar rat from our lab, no unconjugated SECO was detected after administering SDG orally[127]. One possible explanation is that the systemic concentration of SECO was below our limit of detection. The systemic concentrations of SECO depend upon the rate and extent of conversion of SDG, extent of transformation to ED, as well as the rate and extent of its absorption, i.e. oral bioavailability. Possibly, the conversion rate from SDG to SECO was low, or most of SECO

were transformed into ED so that the systemic concentration of SECO was not large enough to be detected by our analytical method. However, in a single oral dose of 40 mg/kg SECO study in rat, unconjugated SECO was quantitated from 5 min to 18 h after administration (21.15 ng/ml - 1487.26 ng/ml) and reached bimodal peaks in 10 min and 1 h after dosing[117]. It was also reported that SECO was quantified from 2 h to 12 h after 86 mg SDG was given orally to human participants, and it reached peak plasma concentrations after 5–7 h and disappeared with a plasma elimination half-life of 4.8 h[119]. Another possible explanation is SECO appears in the systemic circulation and was distributed extensively and eliminated quickly, resulting in undetectable levels in the current PK study. Indeed a previous PK study involving IV administration of SECO in rat, SECO had short half-life (4.7±3.6 h), large volumes of distribution (44.1 ± 12.2 L/kg), and high systemic clearance (7.82 ± 1.11 L/h·kg)[117]. Also, SECO has a shorter half-life than the mammalian lignans in both rat and human[80, 119]. Considering the hydrolysis process from SDG to SECO and the short half-life of SECO, our sampling points may miss the time when SECO was present in systemic circulation.

6.1.2 Plasma ED concentration versus time course in rat PK samples

SDG is converted eventually to the mammalian lignans (ED and ENL) but this process largely occurs in the large intestine as it requires colonic bacteria. This explained the expected delay in the appearance of ED in plasma. In both purified SDG and SDG polymer group, unconjugated ED was detected and quantified in 8 h (3.4 ± 3.3 ng/mL) and 12 h (6.2 ± 3.3 ng/mL) time points of most rats, while total ED (unconjugated ED and ED conjugate) was quantified from 2 h to 16 h in most rats. T_{max} of total ED in purified SDG group and SDG polymer group was 11.7 ± 1.1 h, and 10.9 ± 1.9 h, respectively. C_{max} of total ED in purified SDG group and SDG polymer group was 262.2 ± 170.8 ng/mL, and 207.2 ± 115.5 ng/mL, respectively. Half-life of ED could not be determined due to lack of enough points in the terminal phase to calculate the elimination rate constant (k). That the concentrations of ED conjugates are much higher than its unconjugated form is supported by the finding that ED and EL were exclusively found as glucuronic acid and sulfate conjugates in urine, bile, and the portal vein of the rats following an oral lignan administration[128]. After oral administration, C_{max} and T_{max} are dependent on the extent and the rate of lignan absorption and disposition. No significant difference was observed in either T_{max} or C_{max} of total ED, suggesting similar SDG conversion to ED. There are no differences in processes that influence rate and extent of absorption in pure SDG and SDG polymer. In a PK study, maximum serum concentration of ED was attained after 12-24h in postmenopausal women after oral intake of 86 and 172 mg of

SDG[119]. Transit times in rodents are inevitably shorter than that in humans owing to their short gut length. Total colonic transit time is 36.2 ± 5.1 h in human, while the colonic transit time of rat is 15.6 h[129]. Since physiological factors, such as gastric emptying and small intestinal transit time, can affect lignan absorption, differences in intestinal transit time can explain differences in T_{max} in humans and rats.

Kuijsten's oral PK study in humans given 0.9 mg/kg SDG found that the elimination half-life (4.4 ± 1.3 h) and mean residence time (21 h) of ED were much shorter than that of ENL ($t_{1/2}$=12.6 ± 5.6 h, MRT=36 h), which may explain that ED was undetected after 24h[118]. In a previous PK study of our lab, unconjugated ED was detected from 8h to 48h (3 ng/mL - 10 ng/mL) in rats following a single oral 40 mg/kg SECO, while interfering endogenous peaks precluded quantification of the conjugated lignans[130]. Another PK study following IV administration indicated that ED had poor bioavailability (less than 1%), large volumes of distribution (54.2 ± 27.5 L/kg), and high systemic clearance (23.1 ± 4.51L/h·kg)[117].

6.1.3 Plasma ENL concentration versus time course in rat PK samples

The mammalian lignan ENL comes from ED and this largely occurs in the large intestine as gut microflora is needed for the conversion. Unconjugated ENL was not detected in rats administered either purified SDG group or SDG Polymer, because ENL appears to be more rapidly metabolized to its conjugates in human colon epithelial cells[131]. Total ENL can be quantified in all plasma samples including pre-dose sample, which was confirmed by other studies where ENL in pre-dose samples are not zero due to the abundance of lignans in foods[45, 46, 118]. Unlike ED, ENL responses showed a gradual increase from 4 hours, reaching the highest concentration in 12 or 16 hours, and then gradually declining to baseline in 32 hours. T_{max} of total ENL in the purified SDG group and SDG polymer group was 12.6 ± 1.5 h, and 12.7 ± 3 h, respectively. T_{max} of total ENL were shorter than T_{max} of total ED in both groups, demonstrating the transformation process of ED to ENL. C_{max} of total ENL in purified SDG group and SDG polymer group was 81.6 ± 23.8 ng/mL, and 65.9 ± 19.6 ng/mL, respectively. Half-life of ENL for pure SDG and SDG polymer were 12.0 h and 9.2 h, respectively. No significant difference was observed in either T_{max} or C_{max} of total ENL, suggesting similar SDG conversion to ENL. In a PK study involving postmenopausal women, peak serum concentration of ENL was reached after 24-36 h after oral intake of 86 and 172 mg of SDG[119]. Differences of T_{max} in humans and rats can be explained by species differences in intestinal transit time.

In a previous PK study of our lab in rat administered a single IV dose of 20 mg/kg SECO or a single oral dose of 40 mg/kg SECO, ENL was only detected in 15, 18, and 36 hour samples

of one rat (IV dose, less than 50 ng/mL). It was also detected in 21 and 36 hour samples of one rat (oral dose, less than 50 ng/mL)[130]. Further studies with more time points blood collections from 8 to 16 hours after administering SDG are necessary to unambiguously determine T_{max} and C_{max}.

In conclusion, there are no differences in the absorption kinetics between pure SDG and SDG polymer. The process of hydrolyzing, extracting, and purifying SDG from the SDG polymer in flaxseed to produce a purified SDG is a costly procedure. Since pure SDG and SDG polymer have similar absorption kinetics and total exposure of bioactive metabolites, SDG polymer is a more economical alternative of pure SDG for future application.

6.2 Hypocholesterolemic efficacy study of purified SDG and SDG polymer

Previous studies have determined the potential hypocholesterolemic effect of flaxseed and flaxseed lignan using rabbit[81], mouse[132], and rat[80] models of diet-induced hypercholesterolemia. The rat model may be the most suitable model for this study because of the relatively low animal cost, a requirement for lower amounts of purified SDG and SDG polymer relative to the rabbit, ease of lignan administration in this species, relatively short induction time for development of hypercholesterolemia and hepatic lipidosis, and ability to relate to lignan PK conducted in rat. The rat model allows for precise dose administration. Use of a modified oral gavage technique (syringe) provides opportunity to deliver an exact dose with minimal stress the rat rather than incorporation of the lignan into the diet as is often required in mouse studies. This avoids unfavorable inter-animal variation due to variable amounts of lignan consumption per day based on the dietary intake of individual animals. Since rat provided sufficient blood volume for the pharmacokinetic study, the rat model was utilized for the efficacy study for consistency. In the previous hypocholesterolemic efficacy study of SDG administration in diet-induced hypercholesterolemic rats in our lab, female Wistar rat demonstrated a clinically relevant response to 1% cholesterol diet and hypocholesterolemic response to oral SDG administration[80]. Because my objective is to compare the hypocholesterolemic efficacy of purified SDG and SDG polymer, in a previous study the female Wistar rat has a clear and predictable response to SDG[80], I also utilized female Wistar rat for efficacy study. In other animal chronic studies (rats and rabbits), 3 mg/kg, 6 mg/kg or 15 mg/kg SDG were used. In human studies, 100 mg to 600 mg SDG were used per day, which when normalized to body weight 70 kg, the dose was 1.4 mg/kg to 8.6 mg/kg daily. I chose 6 mg/kg SDG daily as the dose. An animal study (3 mg/kg and 6 mg/kg SDG) and a human study (300 mg and 600 mg SDG)[46] have shown that hypocholesterolemic effects of flaxseed lignan are dose-dependent.

The 1% cholesterol diet fed for 30 days increased final body weight, liver weight, and normalized liver weight, serum lipid levels, and hepatic steatosis and hepatocellular injury relative to rats fed a standard diet. This is consistent with the previous study where rats were fed 1% cholesterol diet for 37 days, their body weight, liver weight, and serum lipid levels, increased when compared to rats fed a standard diet[133]. The current study showed trends in reductions in normalized liver weight, liver steatosis and hepatocellular injury, as well as serum lipid levels in hypercholesterolemic rats (10 weeks) with pure SDG or SDG polymer treatment. However, no difference was observed in final body weight and liver weight. This is consistent with a previous study with a similar experiment design in our lab that clinically relevant reductions in serum and hepatic lipid levels of rats following 30 days 6 mg/kg SDG intervention was observed[80]. Although consistent, the current study did not show similar reductions in rate of weight gain and serum lipid parameters probably due to the shorter duration of the dietary and lignan intervention. Both the current study and the previous study fed the 1% cholesterol diet for one week before administration of the lignan, but the current study (30 days for 1% cholesterol diet and 23 days for lignan treatment) is one week shorter than the previous one (37 days for 1% cholesterol diet and 30 days for lignan treatment). Moreover, this inconsistency may be explained by the different ages and growth phase of rats in the reported studies. As for liver histological analysis, both purified SDG and SDG polymer treatment not statistically, but clinically demonstrated lower scores in steatosis and NAS, when compared to hypercholesterolemic rats in the control group. Purified SDG treatment demonstrated lower scores in steatosis (27.8%), lobular inflammation (30%) and NAS (28.5%) as compared to the hypercholesterolemic controls. Similarly, SDG Polymer treatment had lower scores in steatosis (22.2%) and NAS (14.3%). No significant difference in pathology parameters was found between pure SDG and SDG polymer. Microscopic visual artifacts can potentially cause misdiagnosis of samples. Related to lipidosis, results of oil red O-stained liver slices, a semi-quantitative analysis, in the previous study with a similar experiment design in our lab where 6 mg SDG/kg decreased hepatic fat accumulation by 24 %, we concluded that both purified SDG and SDG polymer can partially attenuate steatosis and hepatocellular injury caused by high cholesterol diet in a similar extent.

In the current study, hypercholesterolemic rats given SDG polymer demonstrated not statistically significant but clinical reduction in TC (10%), and an increase in HDL-C by 24%, which is similar in magnitude to the results of Jae B. Park's study (10% reduction in TC and 30% increase in HDL-C following a high fructose and high fat diet with 0.02% SDG enriched

complex, similar dosage to the present study)[134]. Besides, we only observed a distinctive, but not statistically significant reduction in TAG (15%) and a slight reduction in LDL-C (14%) after 23 days SDG polymer consumption. Within Jae B. Park's study, significant reductions were found in TAG (27%) and LDL-C (47%) [134]. These different results may due to the short duration of lignan administration in the present study. Other supportive evidence demonstrated that 23 days of lignan intervention may not be enough to induce significant differences in serum lipid parameters. Prasad et al found that TC and LDL-C in rabbits treated with 1% cholesterol and 15mg/kg SDG) at month 2 was significantly lower than those in 1% cholesterol control group. However, at month 1, SDG consumption only had slight reductions in TC and LDL-C[77]. Similar results were observed in their other similar study, in which TC and LDL-C values of rabbits were 20% and 14% lower in rabbits with 0.5% cholesterol and 40 mg/kg lignan complex (~15 mg/kg SDG)) as compared to those in 0.5% cholesterol control group at 2 months[81]. Also, HDL-C were significantly higher at month 1 (93%) and at month 2 (30%). Nevertheless, TC and LDL-C were even higher with lignan complex at month 1 when compared to those without lignan complex[81].

Although no statistical difference was found after 23 days intervention of either purified SDG or SDG polymer in hypercholesterolemic rats when compared to hypercholesterolemic controls, apparent lipid lowering effect was observed following purified SDG and SDG polymer administration. Therefore, from an economic perspective, SDG polymer can be considered as a more economical natural product in the management of mild hypercholesterolemia. This efficacy similarity was supported by the comparative PK study of pure SDG and SDG polymer where no differences were found in AUC of total ED and total ENL. When combined with the PK study where glucuronides of ED and ENL were the major lignan metabolites in plasma, the pharmacological effect of their conjugates should be considered and need more examination.

6.3 Challenges and limitations

This research encountered several challenges, ranging from technical to instrumental challenges. In the PK study, I used LC-MS/MS to quantify SDG metabolites levels in rat plasma. The assay was previously developed and validated in human plasma in our lab, with great sensitivity. However, the rat plasma volume used in analysis was much lower than human plasma resulting in lower sensitivity and a higher LLOQ. Lower sensitivity may explain why unconjugated and total SECO, unconjugated ED and unconjugated ENL could not be detected in most samples. Also, an incomplete plasma concentration versus time curve was collected due

to ethical constraints associated with limited volume blood collections in rodents. In addition, the LC-MS 4000 Q-Trap had some technical problems for a considerable period of time during my attempts to validate the assay for rat serum. Another challenge was that many sources of beta-glucuronidase contained an endogenous interfering substance that affected the quantification of SECO. This interfering peak eventually disappeared after using MP Biomedicals™ Beta-Glucuronidase solution. These analytical challenges took time to resolve.

For the efficacy study, we planned to feed rats 1% cholesterol diet for 7 days prior to initiation of the lignan intervention. Subsequently, we planned to continue the dietary intervention an additional 30 days coupled with once daily administration of purified SDG or SDG polymer during this 30 day time period. However, I encountered a shortage of the high cholesterol diet, so the study was terminated ahead of schedule (23 days). This early termination may explain the lack of significant difference in lignan treatment and control group. The previous study demonstrated greater increases in serum lipid levels and hepatic lipidosis as compared with the current study, suggesting induction of hypercholesterolemia was incomplete in the current study. Another technical problem was encountered with histopathology assessments. The liver blocks were overhydrated following tissue processing in an automated tissue processor. Thus, we soaked liver blocks in softening solutions for different periods of time to rehydrate. Nevertheless, slide preparation was less than ideal, which affected the ability to reliably assess for hepatocellular lipid levels and inflammatory changes. Given the incomplete induction of hypercholesterolemia, it is very likely that further investigation of liver samples for evaluation of the expression of molecular targets involved in cholesterol metabolism may not provide further supportive evidence to enhance our understanding of lignan effects on cholesterol regulation.

6.4 Conclusion

In the present study, we compared the relative bioavailability of SDG metabolites after single oral bolus administration of purified SDG and SDG polymer in rat. The unconjugated form of SECO, ED, and ENL was detected in only a few samples at different time point collections likely due to extensive first pass metabolism such that the lignans are present in the systematic circulation mostly in their conjugated forms. The relative bioavailability of total ED and ENL of SDG polymer was 111% and 89%, respectively, when compared to purified SDG. No significant differences in C_{max}, T_{max}, and AUC of total ED and ENL between purified SDG and SDG polymer were found. Since pure SDG and SDG polymer have similar absorption kinetics and total exposure of bioactive metabolites, combining the extra production process

and extra cost of pure SDG, SDG polymer is a more economical alternative of pure SDG for future application.

We observed an improved physiological condition in hypercholesterolemic rats treated with purified SDG or SDG polymer, including reductions in normalized liver weight, TAG, TC and LDL-C, and an increase in serum HDL-C. Both purified SDG and SDG polymer treatment demonstrated lower scores in liver steatosis and NAS, meaning that purified SDG and SDG polymer can partially attenuate steatosis and hepatocellular injury caused by high cholesterol diet. No significant difference was observed between purified SDG and SDG polymer treatment. Therefore, from an economic perspective, SDG polymer can be considered as a more economical natural production in the management of hypercholesterolemia.

6.5 Future work

Firstly, a single dose escalation pharmacokinetic study of purified SDG and SDG polymer can be conducted to understand whether lignans demonstrate dose-dependent pharmacokinetics. If the relationship between plasma AUC and dosage is not proportional, then lignans exhibit dose-dependent pharmacokinetics, which would complicate the design of dosage regimens and prediction of efficacy and toxicity. Many health and economic assessments, as well as regulatory decisions, often depend on the integrity of this relationship.

If lignans exhibit dose-dependent kinetics, the numbers of clinical trials would increase and it would cost a considerable amount of money. In addition, multiple dose pharmacokinetic studies determine the steady state pharmacokinetic parameters. Since multiple doses of purified SDG or SDG polymer will be administered if SDG is taken as a medication, steady state pharmacokinetics (C_{ss}, C_{max}, C_{min}) can affect efficacy and safety. Therefore, multiple dose pharmacokinetic studies of purified SDG and SDG polymer should be conducted to determine their steady state pharmacokinetic parameters. Purified SDG and SDG polymer can be administered following multiple doses once daily and blood samples (at $C_{SS,max}$ and $C_{SS,min}$) will be collected over the duration of the experiment to determine steady state pharmacokinetic parameters.

Studies have shown that the hypocholesterolemic efficacy of flaxseed lignans are dose dependent and it is critical to know appropriate dose and treatment duration as well as safety. Dose and duration range finding studies can be conducted in two phases. Firstly, in a fixed period of time, a single escalating dose of purified SDG and SDG polymer can be administered orally to diet - induced hypercholesterolemic rats. Also, a fixed dose of purified SDG and SDG polymer can be administered for different durations. The fixed dose for second phase will be

determined based on the first dose study and pharmacokinetics. Efficacy (body weight and serum lipids levels) and safety (liver enzymes and adverse events) should be assessed. At the end of the study, any abnormal physiological and histopathological changes will be investigated. No effect level dose (NEL), dose with no observed adverse effect level (NOAEL,) and maximum tolerated dose (MTD) will be determined. Applicable duration can also be determined.

Several studies have revealed that whole flaxseed or flaxseed lignan has hypocholesterolemic effects in hypercholesterolemic subjects. A comparative placebo controlled efficacy study of purified SDG and SDG polymer can also be conducted in hypercholesterolemic patients. The patient will receive purified SDG, equivalent dosage of SDG polymer, or a placebo for 12 weeks. The pilot study can start with 600 mg/day SDG and equivalent dosage of SDG polymer for 12 weeks because a 600 mg/day SDG dose for six months in human subjects are safe and well tolerated[50, 51]. Participants can mix the lignan form in a small amount of yoghurt once daily. Their plasma lignan levels, body weight, serum lipids level and other related physiological conditions will be monitored. Patients can record their body weight daily at home. Blood can be collected at Predose (baseline), one-month, two-month, and the end of intervention.

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