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***Artemisia scoparia* extract attenuates non-alcoholic fatty liver disease in diet-induced obesity mice by enhancing hepatic insulin and AMPK signaling independently of FGF21 pathway**

Zhong Q. Wang^a, Xian H. Zhang^a, Yongmei Yu^a, Russell C. Tipton^a, Ilya Raskin^b, David Ribnicky^b, William Johnson^c, and William T. Cefalu^{a,*}

^aNutrition and Diabetes Research Laboratory, Pennington Biomedical Research Center, LSU System, Baton Rouge, LA 70808, USA

^bDepartment of Plant Biology and Pathology, Rutgers University, New Brunswick, NJ 08901, USA

^cBiostatistics, Pennington Biomedical Research Center, LSU System. Baton Rouge, LA 70808, USA

Abstract

Objective—Nonalcoholic fatty liver disease (NAFLD) is a common liver disease which has no standard treatment. In this regard, we sought to evaluate the effects of extracts of *Artemisia santolinaefolia* (SANT) and *Artemisia scoparia* (SCO) on hepatic lipid deposition and cellular signaling in a diet-induced obesity (DIO) animal model.

Materials/Methods—DIO C57/B6J mice were randomly divided into three groups, i.e. HFD, SANT and SCO. Both extracts were incorporated into HFD at a concentration of 0.5% (w/w). Fasting plasma glucose, insulin, adiponectin, and FGF21 concentrations were measured.

Results—At the end of the 4-week intervention, liver tissues were collected for analysis of insulin, AMPK, and FGF21 signaling. SANT and SCO supplementation significantly increased plasma adiponectin levels when compared with the HFD mice ($P < 0.001$). Fasting insulin levels were significantly lower in the SCO than HFD mice, but not in SANT group. Hepatic H&E staining showed fewer lipid droplets in the SCO group than in the other two groups. Cellular signaling data demonstrated that SCO significantly increased liver IRS-2 content, phosphorylation of IRS-1, IR , Akt1 and Akt2, AMPK 1 and AMPK activity and significantly reduced PTP 1B abundance when compared with the HFD group. SCO also significantly decreased fatty acid synthase (FAS), HMG-CoA Reductase (HMGR), and Sterol regulatory element-binding protein 1c (SREBP1c), but not Carnitine palmitoyltransferase I (CPT-1) when compared with HFD group. Neither SANT nor SCO significantly altered plasma FGF21 concentrations and liver FGF21 signaling.

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*Corresponding author. Nutrition and Diabetes Research Laboratory, Pennington Biomedical Research Center, LSU system, 6400 Perkins Road, Baton Rouge, LA 70808, USA. Tel.: +1 225 763 2654, fax: +1 225 763 0391. cefaluwt@pbrc.edu (W.T. Cefalu).

Conflicts of interest

Authors have no conflicts of interest.

Author's contributions

W.T. Cefalu designed the study, reviewed the data, and reviewed and edited the manuscript. X.H. Zhang, Y.M. Yu, and R.T. researched data. D. Ribnicky and I. Raskin reviewed and edited manuscript, provided sources of material for study, W.J. provided statistical support. Z.Q.W. conducted the study, wrote the manuscript, and analyzed the data. Dr. Z.Q. Wang had full access to all the data, and takes full responsibility for the integrity of data and the accuracy of data analysis. All authors read and approved the final manuscript.

Conclusion—This study suggests that SCO may attenuate liver lipid accumulation in DIO mice. Contributing mechanisms were postulated to include promotion of adiponectin expression, inhibition of hepatic lipogenesis, and/or enhanced insulin and AMPK signaling independent of FGF21 pathway.

Keywords

Obesity; Insulin resistance NAFLD; FGF21; AMPK

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) refers to hepatic steatosis, or the accumulation of fat in the liver, in the absence of excess alcohol consumption. The prevalence of NAFLD is reported to be as high as 30% in developed countries and nearly 10% in developing nations, making NAFLD one of the most common liver conditions in the world [1]. This fatty liver disease is epidemiologically strongly associated with obesity and insulin resistance, leading to a speculation of a reciprocal cause–effect relationship and a vicious cycle of pathology [2]. Thus, it creates a need for the development of more efficacious and safer drugs for treatment options. In this regard, an evaluation of botanical preparations and their possible benefits represents an alternative focus in this field of study.

Artemisia species are a rich source of herbal remedies with antioxidant and anti-inflammatory properties. For example, it was reported that *Artemisia herba-alba* Asso (Asteraceae) had beneficial antioxidant effects [3]. A recent study observed that *Artemisia scoparia* hydromethanolic extract possesses anti-nociceptive, anti-inflammatory and antipyretic activities [4]. In addition, two variants of *Artemisia princeps* Pampanini, [*Sajabalssuk* (SB) and *Sajuarissuk* (SS)], were shown to partly improve lipid dysregulation and fatty liver in db/db mice by suppressing hepatic lipogenic enzyme activities [5]. *Artemisia campestris* aqueous extract was found to be effective for correcting hyperglycemia and preventing diabetic complications [6] and *Artemisia herba-alba* Asso (AHA) had antihyperglycaemic and antihyperlipidemic effects in HFD-induced diabetic mice [7]. Moreover, *Artemisia princeps* (APE) inhibited hepatic fatty acid synthase (FAS) and suppressed the elevation of plasma leptin, but had no effect on adiponectin levels in the high-fat diet mice [8]. Our previous studies showed that ethanolic extracts of *Artemisia dracunculus* L (termed PMI-5011) reduced protein tyrosine phosphatase 1B (PTP1B) content in cultured muscle cells and increased insulin sensitivity and enhanced insulin receptor signaling in an insulin resistant animal model [9,10]. Recently, we observed that PMI 5011 attenuated the insulin signaling induced by ceramide accumulation in cultured muscle cells [11].

Artemisia santolinaefolia (SANT) and *Artemisia scoparia* (SCO) are closely related species (Supplemental material 1) in the same genus within the family Asteraceae and have reported biological activity [12,13]. Essential oil obtained from the aerial parts of *Artemisia scoparia* showed strong radical scavenging capacity and antioxidant activity against hydroxyl radical and hydrogen peroxide [14]. More specifically, scoparone (6,7-dimethoxycoumarin), a coumarin isolated from *Artemisia scoparia* used as a Chinese herb, was found to have anti-atherogenic activity in treated hyperlipidaemic diabetic rabbits and was shown to attenuate advanced atherosclerosis and lower plasma cholesterol [15]. In addition, an aqueous extract of *SCO* significantly reduced blood sugar levels in Streptozotocin-induced diabetic rats at doses of 125 and 250 mg/kg body weight for 3 weeks [16]. However, the effects of SANT and SCO on glucose and lipid metabolism in mice fed high fat diets and the effect on hepatic lipid accumulation are largely unknown.

It is well documented that adiponectin is a protein hormone secreted from adipose tissue that modulates a number of metabolic processes, including glucose regulation, insulin sensitivity and fatty acid catabolism [17–20]. Adiponectin enhances insulin sensitivity and glucose tolerance and activates AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor alpha (PPAR α) signaling in the liver and skeletal muscle [21]. Recently, a novel protein, fibroblast growth factor 21 (FGF21), has been demonstrated to be involved in glucose and lipid metabolism. Treatment of animals with FGF21 results in increased energy expenditure, fat utilization and lipid excretion [22]. Thus, FGF21 represents a novel and potential therapeutic agent for Type 2 diabetes mellitus, obesity, dyslipidemia, cardiovascular and fatty liver diseases [22,23]. It is not clear, however, if the beneficial effects of botanical extracts, and in particular, SANTA and SCO, are secondary to modulation of these proteins. Thus, we sought to determine if botanical extracts, i.e. SANT and SCO, attenuate lipid accumulation in the liver by altering cellular signaling pathways and lipid synthesis related enzymes. To test our hypothesis, the effects of SANT and SCO on plasma glucose, adiponectin, liver lipid content, hepatic insulin, AMPK and FGF21 signaling were comprehensively investigated in a diet induced obesity (DIO) murine model.

2. Materials and methods

2.1. Animals

All animal experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee of Pennington Biomedical Research Center. Fifteen 5-week-old male C57BL/6 J mice were ordered from Charles River Laboratories (Wilmington, MA) and housed under conditions that were maintained at constant temperature and humidity (21 ± 2 °C with humidity 65%–75%) with a 12:12-h light–dark cycle. Given that each group contained 5 mice, mice were housed either as one/cage or two/cage, and allowed access to water and high-fat diet [58% of energy from fat (D-12331), Research Diets (New Brunswick, NJ)] ad libitum for 10 weeks to induce obesity and insulin resistance.

2.2. Experimental design and diet

Diet-induced obese mice were randomly divided into three groups: high-fat diet control (HFD), SANT treated and SCO treated groups ($n = 5/\text{group}$). Extracts of SANT and SCO dried herb were prepared at Rutgers University using 80% ethanol (1:20 w/v) which was removed by evaporation after 12 h of extraction. These extracts were incorporated into the high-fat diet at a concentration of 0.5% (W/W) which equaled ~250 mg/kg body weight. This dose was chosen based on our *in vitro* experiments and a previously reported *in vivo* study using a scoparia extract [4]. Food intake and body weight were recorded weekly. Fasting plasma glucose and insulin concentrations were measured at weeks 0, 1, 2 and 4 of the study respectively. Intraperitoneal insulin tolerance testing (IPITT) was measured at week 3. At the end of the study (week 4), the mice were euthanized. The liver and other tissues were dissected immediately, placed in liquid nitrogen, and stored at -80 °C for future measurements.

2.3. Blood chemistry and hormone analysis

Blood samples were collected after 4 h of fasting by tail stick. Plasma glucose levels were measured by a colorimetric hexokinase glucose assay (Sigma Diagnostics, St Louis, MO). Plasma insulin levels were determined by a rat/mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (Millipore Billerica, MA). Plasma cholesterol and triglyceride concentrations were measured by a Triglyceride assay kit (Eagle Diagnostics, DeSoto, TX) and a cholesterol quantitation kit (BioVision, Milpitas, CA), respectively. Plasma FGF21 was measured using Mouse FGF-21 ELISA Kit according to the

manufacturer's instructions (R & D Systems, Minneapolis, MN). Intra-assay and inter-assay CVs of FGF21 were 4.51% and 6.14%, respectively. FGF21 quality control result was 278 pg/ml (range 191–319 pg/ml). Fasting plasma leptin concentrations in mice were determined using mouse leptin ELISA kit by following the manufacturer's instructions (Millipore Billerica, MA). The intra-assay and inter-assay CVs of leptin were 2.53% and 4.1 %, respectively.

2.4. Intraperitoneal insulin tolerance test (IPITT)

After 4-h fasting, IPITT was conducted by IP injection of insulin at 0.75 U/kg body weight. Blood glucose concentrations were measured from the tail vein at time 0 (baseline), 15, 30, 60 and 120 min after insulin injection using the Freestyle blood glucose monitoring system (Thera Sense, Phoenix, AZ).

2.5. Liver FGF21 content assessment

Liver tissues (~25 mg) were placed in Eppendorf microcentrifuge tubes and added to ten volumes (w/v) of homogenization buffer (1% Triton X-100, 100 mmol/L Tris [pH7.4], 100 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 10 mmol/L EDTA, 10 mmol/L sodium vanadate, 2 mmol/L phenylmethylsulfonyl fluoride, and 0.1 mg/mL aprotinin), minced with scissors, and homogenized by micro-homogenizer. Tubes were centrifuged at $14,000 \times g$ for 15 min. FGF21 content in the liver supernatant (50 μ g protein) was assayed using a FGF21 ELISA kit as described above.

2.6. Hepatic PTP 1B activity assay

Liver PTP 1B activity was measured using a PTP 1B activity assay kit (Millipore, Temecula, CA) and performed according to the manufacturer's instruction.

2.7. Liver lipid extract for TG and cholesterol measurement

Liver lipid extracts were prepared by the Folch's procedure [24]. Briefly, liver tissues (about 25 mg) were added to five volumes of PBS (w/v), minced with scissors in Eppendorf microcentrifuge tube, and homogenized by sonication. The liver lysates were added to ten volumes of an extract solvent containing chloroform and methanol in the ratio of 2:1 (v/v). After vortexing, the tubes were centrifuged at $5000 \times g$ for 10 min. Aliquots of 100 μ l were removed from the bottom of the tube, transferred to a new tube and dried under nitrogen gas. After adding 100 μ l of PBS to the tube, 10 μ l of mixture was taken to measure triglyceride and cholesterol content using a Triglyceride assay kit (Eagle Diagnostics, DeSoto, TX) and a cholesterol quantitation kit (BioVision, Milpitas, CA), respectively. The results were normalized by protein concentration.

2.8. Histological studies in the liver

Sections of liver tissues from the center of the largest liver lobes were fixed in 10% buffered formaldehyde, and then embedded in paraffin. A 5 μ m-thick section cut from a paraffin-embedded block was stained with hematoxylin and eosin (HE staining). All specimens were observed and photomicrographed using an Olympus 1X71 inverted microscope and Olympus PP72 camera, Olympus America (Melville, NY).

2.9. Western blotting analysis

Plasma adiponectin levels (denatured) and high molecular weight of adiponectin levels (undenatured) in mice at week 4 were measured using an antibody of adiponectin (Millipore, Billerica, MA). Liver lysates were prepared by homogenization in buffer A (25 mmol/L HEPES, pH 7.4, 1% Nonidet P-40 (NP-40), 137 mmol/L NaCl, 1 mmol/L PMSF, 10 μ g/ml aprotinin, 1 μ g/ml pepstatin, 5 μ g/ml leupeptin) using a PRO 200 homogenizer (PRO

scientific, Oxford, CT). The samples were centrifuged at $14,000 \times g$ for 20 min at 4°C and protein concentrations of the supernatants were determined by Bio-Rad protein assay kit (Bio-Rad laboratories, Hercules, CA). Supernatants (50 μg) were resolved by 8% or 12% SDS-PAGE and subjected to immunoblotting. The protein abundance was detected with antibodies against IRS-1 p^(Tyr612), IRS-1, IRS-2, IR p^(Tyr1150-1151), IR , PI 3 K, Akt1p^(Ser473), Akt1, Akt2 p^(Ser474), Akt2, PTP 1B, FGF21, FGFR1, AMPK p^(Thr172), AMPK 1, PPAR , ACC p^(Ser79), ACC, HMGR and PGC-1 (Millipore, Temecula, CA), fatty acid synthase (FAS) (Abcam, Cambridge, MA), FGFR3 (Bioworld Technology, Louis Park, MN), AMPK 2, CPT-1, SREBP 1c, klotho (Santa Cruz, CA), and -actin (Affinity Bioreagents, Golden, CO) using Chemiluminescence Reagent Plus from PerkinElmer Life Science (Boston, MA), and quantified via densitometer. All the proteins were normalized to -actin.

2.10. AMPK activity assay

Briefly, AMPK 1 and 2 were immunoprecipitated from 200 μg of liver lysate using anti-AMPK 1 (Millipore, Billerica, MA) or AMPK 2 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies in 500 μl of buffer A with protein A beads or protein A & G beads (50 mmol/L Tris _HCl, pH 7.4, 150 mmol/L NaCl, 50 mmol/L NaF, 5 mmol/L sodium pyrophosphate, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.1 mmol/L benzamidine, 1 mmol/L phenylmethylsulfonyl fluoride, 5 $\mu\text{g}/\text{ml}$ aprotin) at 4°C for 2 h. Immunocomplexes were washed with buffer A three times, buffer B containing 0.5 M NaCl and 62.5 mmol/L NaF one time, and then the reaction buffer (50 mmol/L HEPES, pH 7.4, 1 mmol/L DTT) three times. AMPK activity of immunocomplexes was determined by phosphorylation of SAMS peptide in the reaction buffer containing 0.25 mmol/L SAMS, 5 mmol/L MgCl_2 , and 10 μCi of [-32P]ATP for 10 min at 30°C with or without 200 μM AMP stimulation. The reaction was terminated by spotting reaction mixtures onto P81 filter paper and rinsing in 1% (vol/vol) phosphoric acid with gentle stirring to remove free ATP. The phosphorylated substrate was measured by scintillation counting.

2.11. Statistical analysis

All analyses were performed employing SAS® Software (Version 9.3; SAS Institute, Cary NC). Proc univariate with the normal option and the QQ plot statement was used to test normality of the data distributions. On-study changes in outcomes (e.g., fasting blood glucose levels and fasting plasma insulin levels) were assessed as differences by subtracting baseline (week 0) values from values recorded at each observation time (weeks 1, 2, 4). Mean differences for the three treatment groups (HFD, SANT and SCO) were compared using *proc mixed* to analyze mixed effects linear models with repeated measures across observation times. Mean differences were adjusted for baseline values when appropriate. Results were summarized as mean $\pm$ SEM. P values < 0.05 were considered statistically significant.

3. Results

The effects of SANT and SCO extracts on body weight, food intake, plasma glucose, insulin, and leptin concentrations were studied in the DIO mice. The results showed that differences among outcome means for HFD, SANT and SCO were not statistically significant at baseline (Week 0). After 4 weeks of intervention, there was no difference in body weight or food intake among the groups (supplemental material 2A and B). Plasma glucose concentrations in the SANT and SCO groups were slightly lower than in the HFD group, but there were no significant differences among the three groups (Fig. 1A). The reduction in plasma insulin concentrations was significantly greater in SCO mice than in HFD mice at weeks 1, 2 and 4, with $P = 0.007$, $P = 0.001$ and $P = 0.031$, respectively (Fig.

1B). The reduction in insulin was greater in santa mice than HFD mice across weeks 1, 2 and 4 but the difference was significant only at week 2 ($P = 0.037$) and marginally significant at week 1 ($P = 0.051$). Reduction in insulin tended to be greater in SCO mice than Santa mice at weeks 1, 2 and 4, but none of the differences were statistically significant. Likewise, both extracts increased the effect of insulin on glucose disposal relative to HFD mice, but only the SCO effect was significant ($P < 0.05$, Fig. 1C). Plasma cholesterol levels were modestly lower, but triglyceride concentrations were significantly lower in the SCO than in other two groups (Fig. 1D and E, $P < 0.05$). There were no differences in cholesterol and triglyceride levels between HFD and Santa groups. Plasma leptin concentrations did not differ between groups (Fig. 2A). Plasma adiponectin levels were measured in these mice using western blotting. It was observed that SANT and SCO significantly increased plasma adiponectin content when compared with the HFD animals, and adiponectin levels were significantly higher in the SCO group than in the SANT group ($P < 0.001$ and $P < 0.05$, Fig. 2B).

Effects of the extracts on the liver of the mice were determined based on histological assessments and lipid content. Hepatic H&E staining showed fewer lipid droplets in the SCO group than in the other two groups (Fig. 3A). Hepatic triglyceride and cholesterol concentrations were also significantly lower in the SCO group than in the HFD and SANT groups ($P < 0.05$, Fig. 3B & C). Moreover, there was a significant correlation between triglyceride and cholesterol levels in the liver of the all animals ($r = 0.9036$, Fig. 3D).

SCO significantly enhanced hepatic insulin signaling in mice. The content of insulin signaling proteins in the liver was measured using western blotting and showed that SCO treatment significantly increased IRS-2 content as well as the phosphorylation of IRS-1, IR, Akt1 and Akt2. SCO significantly reduced PTP 1B protein abundance when compared with the HFD group (Fig. 4). Hepatic PTP 1B activity in the SCO group was significantly lower than in the HFD group as well, but there was no significant difference between SCO and SANT groups (PTP 1B activities are 71.74 ± 4.56 pmol/mg/min, 58.67 ± 3.65 pmol/mg/min and 52.87 ± 2.14 pmol/mg/min in HFD, SANT and SCO group, respectively). SCO did not alter PI 3K abundance in the liver. SANT, however, significantly increased insulin stimulated phosphorylation of Akt1 but significantly decreased basal Akt2 phosphorylation in the liver relative to the HFD mice.

The effects of SANT and SCO on AMPK signaling and lipogenesis in the liver were also assessed. SCO supplementation significantly increased AMPK α 1 protein abundance (Fig. 5A). AMPK α 1 and AMPK α 2 activity assay in the liver showed that SCO but not SANT significantly increased basal and AMP stimulated AMPK α 1 activity when compared with HFD animals ($P < 0.05$, Fig. 5B). Both SCO and SANT slightly reduced AMP stimulated AMPK α 2 activity but this was not felt to be statistically significant (Fig. 5C). It was observed that SCO treatment significantly reduced FAS, SERBP 1c, and HMGCR content, and increased ratio of ACC p/ACC, but did not affect CPT-1 abundance in the liver in comparison with HFD group. However, SANT only significantly decreased hepatic FAS after normalized by β -actin ($P < 0.05$) (Fig. 6).

SANT and SCO did not affect hepatic FGF21 signaling in mice. Plasma FGF21 concentrations and hepatic FGF21 content were measured using the FGF21 ELISA assay and the results showed that neither SANT nor SCO altered plasma or liver FGF21 levels relative to the HFD mice. Additionally, it did not appear that these two extracts altered protein abundance of FGFR1, FGFR3, PGC-1 α , or PPAR α abundances in comparison to the HFD animals (data not shown).

4. Discussion

NAFLD is a hepatic manifestation of metabolic syndrome and is currently considered as one of the most common liver diseases [25]. Histologically, NAFLD occurs across a spectrum from mild hepatic steatosis, to nonalcoholic steatohepatitis (NASH), characterized by hepatocellular injury and inflammation, and eventually to cirrhosis. It has been reported that insulin resistance and excess adiposity are associated with increased lipid influx into the liver and increased de novo hepatic lipogenesis, promoting hepatic triglyceride accumulation [1]. Defects in lipid utilization such as mitochondrial lipid oxidation and lipid export may also contribute to hepatic lipid build-up [2]. The DIO mice used in this study appeared to have characteristics suggestive of NAFLD. As such, our data revealed that a high-fat diet supplemented with SCO significantly reduced fasting plasma insulin concentrations and attenuated hepatic lipid accumulation when compared with HFD animals even though food intake and body weight remained constant. SANT showed some similar overall trends to those in the SCO animals, but its effects on these parameters were much lower than the SCO.

A novel finding of this study was both SCO and SANT interventions significantly increased plasma adiponectin concentrations including high molecular weight adiponectin (Supplemental material 3) when compared to HFD animals. It has been noted that adiponectin levels are reduced in NAFLD patients and genetic variants of adiponectin have been frequently associated with type 2 diabetes and insulin resistance [26]. Low adiponectin levels and high leptin levels have a strong independent association with presumed early-stage NASH [27]. In contrast to the effects of *Artemisia princeps* (APE) on suppressing the elevation of plasma leptin without altering adiponectin levels in the high-fat diet mice [7], we observed that SANT and SCO significantly increased plasma adiponectin levels but did not affect plasma leptin concentrations when compared with HFD mice (Fig. 2A). Serum adiponectin levels have been reported to be related to insulin resistance, and inversely associated with NAFLD, independent of potential cofounders, suggesting that hypoadiponectinaemia may contribute to the development of NAFLD [28,29]. Thus, adiponectin has been postulated to prevent liver injury by reversing hepatic stellate cell (HSC) activation and maintaining HSC quiescence [30]. The significantly elevated plasma adiponectin in the SCO treated animals was associated with a reduction of fat accumulation in the liver of HFD mice.

Given the results of the study that the SCO extract reduced hepatic lipids, the possible mechanisms of action for this effect by botanicals can be suggested. First, skeletal muscle insulin resistance is considered to be the initiating or primary defect responsible for the altered plasma adiponectin levels in patients with metabolic syndrome and type 2 diabetes [30]. The high-fat diet induced obese animal models have similar characteristics to human subjects with metabolic syndrome. Our data revealed that SCO supplementation may significantly improve insulin sensitivity by restoring plasma adiponectin levels. Second, SCO and SANT may be argued to possess a unique potential effect on promoting adiponectin secretion and possibly adiponectin gene expression, which was also suggested in 3T3L1 adipocyte culture (Supplemental material 4A & B). In addition, depressed adiponectin levels are associated with subclinical inflammation [31]. Thus, it is possible that the beneficial effects of SCO on elevated plasma adiponectin concentrations provide strong evidence that SCO extract has the features of anti-inflammatory and antioxidant activity [4]. Plasma concentrations of adiponectin in humans correlate with insulin sensitivity [32]. SCO supplementation significantly improved insulin sensitivity as measured by IPITT and enhanced hepatic insulin signaling by increasing IRS-2 content, phosphorylation of IRS-1, IR , Akt1 and Akt2, and reduced PTP 1B activity when compared with the HFD group. It is known that Akt1 is mainly involved in cell growth and survival, and Akt2 is implicated in

insulin-mediated regulation of glucose homeostasis and in hepatic lipid accumulation [33]. The enhancing insulin sensitivity in SCO animals may partially result from elevated circulating adiponectin levels and reduced hepatic fat accumulation e.g. inhibition of fatty acid and cholesterol synthesis. It is well documented that FAS [34] and SREBP 1c are required for de novo lipogenesis [35]. HMGR (3-Hydroxy-3-Methylglutaryl-CoA Reductase) is a rate-limiting enzyme for cholesterol synthesis [36] and CPT 1 is a rate-limiting enzyme for fatty acid oxidation in muscle, fat and liver [37]. Consistent with Jung's findings that two variants of *Artemisia princeps* partly improve lipid dysregulation and fatty liver in db/db mice by suppressing hepatic lipogenic enzyme activities [5], we demonstrated that SCO treatment significantly reduced FAS, SREBP 1c and HMGR abundance, and increased the ratio of ACC p/ACC without affecting CPT-1 (Fig. 6), PPAR alpha and PGC-1alpha in comparison with HFD animals, suggesting that SCO reduces triglyceride and cholesterol accumulation in the liver by suppressing de novo lipogenesis instead of altering fatty acid oxidation. Moreover, we observed that SCO directly enhanced cellular signaling in cultured muscle cells in the absence of adiponectin via an increase of IRS-1, IRS-2 abundance, and phosphorylation of ACC in C2C12 myotubes (Supplemental material 5). AMPK p^(Thr172) antibody detects both α 1 and α 2 isoforms of the catalytic subunit, therefore, the phosphorylation level of AMPK reflects total phosphorylation of AMPK α 1 and AMPK α 2. SCO significantly increased AMPK α 1 which resulted in slightly increased AMPK p relative to HFD. The assessment of AMPK α 1 and AMPK α 2 activity verified that SCO significantly increased both basal and AMP-stimulated activation of AMPK α 1 instead of AMPK α 2 in the liver. Different effects of SCO on increasing AMPK α 1 and α 2 protein abundance in the liver and in muscle may contribute to our observations. AMPK α 2 expression is dominant in muscle and AMPK α 1 is dominant in the liver where AMPK α 1 isoform accounts for approximately 94% of the enzyme activity measured using the SAMS peptide substrate [38]. On the other hand, we observed that SCO treatment increased AMPK α 2 abundance in the cultured muscle cells without affecting AMPK α 1 (Supplemental material 5).

Adiponectin binding to its receptor activates several intracellular signaling pathways, primarily AMPK but also other pathways are affected [39]. AMPK phosphorylation promotes glucose utilization and fatty acid oxidation, increases glucose uptake in the muscle, and reduces gluconeogenesis in the liver. SCO treatment significantly increased hepatic AMPK α 1 protein abundance and its activity relative to the HFD and SANT groups, which may contribute to significantly elevated adiponectin levels. In contrast to SCO, SANT significantly increased plasma adiponectin concentrations, but did not affect insulin and AMPK signaling. Plasma adiponectin levels were significantly lower in the SANT group than in the SCO group, which may partially explain why SANT increased plasma adiponectin levels, but did not significantly enhance hepatic insulin and AMPK signaling in mice. It is most likely that the bioactives of the SANT are simply less effective or present at a lower concentration than those of the SCO extract. Recently, a novel protein, FGF-21, has been identified that plays an important role in liver and adipose tissue metabolism. Pharmacologic studies show that FGF21 possess broad metabolic actions in obese rodents and primates that include enhancing insulin sensitivity, decreasing triglyceride concentrations, and causing weight loss [40]. However, it is a surprising finding that FGF-21 was increased in obesity e.g. so called FGF21 resistance, which is frequently associated with metabolic syndrome and NAFLD [41] as well as in newly diagnosed type 2 diabetes with NAFLD [42]. In order to investigate the effects of SANT and SCO extracts on FGF21 signaling in mice, plasma and hepatic FGF21 levels as well as hepatic FGF21 signaling protein abundances were assessed. The results revealed that neither SANT nor SCO extracts altered FGF21 signaling in the liver. A recent finding showed that serum FGF21 levels were not correlated to serum adiponectin levels in subjects with T2DM and did not affect serum FGF21 levels [43]. Similarly our study demonstrated that SCO significantly increased

adiponectin levels but did not alter FGF21 signaling, suggesting adiponectin may be not involved in the regulation of FGF21 signaling in DIO mice.

5. Conclusion

This study suggests that SCO, but not SANT, attenuated/reversed liver lipid accumulation in DIO mice and the contributing mechanisms may include promotion of adiponectin secretion, suppressing hepatic lipogenesis, and/or enhancing the signaling of insulin and AMPK independently of FGF21. Future studies will need to be conducted to confirm the potential of SCO to serve as a viable therapeutic option for modulation of NAFLD and metabolic dysregulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

FGF21	Fibroblast growth factor 21
FGFR	Fibroblast growth factor receptor
NAFLD	nonalcoholic fatty liver disease
IPGTT	intraperitoneal glucose tolerance test
AMPK	AMP-activated protein kinase

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.metabol.2013.03.004>.

Fig. 1. The effects of SANT and SCO on plasma glucose, insulin and IPITT in mice. Plasma glucose (A) and insulin concentrations (B) were measured weekly for 4 weeks. IPITT was performed at week 3 of the study (C). Plasma cholesterol (D) and plasma triglyceride levels (E) were measured at end of study. SCO significantly reduced fasting plasma insulin and triglyceride concentrations as well as increased glucose disposal in comparison with HFD. Data are mean $\pm$ SEM (n = 5/group). * P < 0.05 and *** P < 0.001, SCO vs. HFD. # P < 0.05. SANT vs. HFD.

Fig. 2.

The effects of extracts on plasma leptin and adiponectin content in mice. Fasting plasma leptin concentrations were measured using mouse leptin ELISA kit. Plasma was diluted with 1× sample buffer at 1:20 (v/v) dilution. After denaturation at 100 °C for 5 min, samples were subjected 10% SDS-PAGE, adiponectin was detected with specific anti-adiponectin antibody. Bands were quantitated with densitometer; the fold change was measured against the mean value of adiponectin in the HFD. (A) Leptin levels in mice, and (B) plasma adiponectin results. SANT and SCO significantly increased plasma adiponectin levels without affecting leptin concentrations. Data were represented as mean ± SEM (n = 5/group). *** P < 0.001, SANT or SCO vs. HFD. # P < 0.05, SANT vs. SCO.

Fig. 3.

Liver H & E staining and lipid content in mice. At the end of the study, liver samples were collected and fixed in 10% buffered formaldehyde, and then embedded in paraffin. Slides of liver samples were stained with H&E method. (A) A large amount of lipids accumulated in the livers in the HFD and SANT treated mice, whereas SCO decreased lipids in the liver of the mice. (B and C) Hepatic triglyceride and cholesterol content, respectively. (D) The correlation between triglyceride and cholesterol in the liver tissues of all animals. Circle stands for HFD, delta is SANT treated animals, and diamond is the SCO treated mice. SCO significantly reduced lipid accumulation in the liver when compared with SANT and HFD groups. Data are mean $\pm$ SEM ($n = 5/\text{group}$). * $P < 0.05$, SCO vs. HFD, ** $P < 0.01$, SCO vs. SANT.

Fig. 4.

Insulin signaling pathway protein analysis in mouse livers. Liver lysates were subjected to SDS-PAGE, and then transferred to nitrocellulose membranes; insulin signaling proteins were detected with corresponding antibodies indicated in the legend. Results were normalized by β -actin, and Akt 1 p and Akt 2 p were normalized by Akt 1 and Akt 2, respectively. SCO significantly increased hepatic IRS-2 and decreased PTP 1B protein abundance. SCO increased phosphorylation of IR β , Akt 1 and Akt 2 in basal and insulin stimulated conditions in comparison with HFD mice. SCO also significantly increased IRS-1 phosphorylation while SNT only significantly increased Akt 1 phosphorylation under insulin stimulation. Data were represented as fold change of HFD, mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, SNT or SCO vs. HFD. # $P < 0.05$, SCO vs. SNT respectively.

Fig. 5. The effects of SANT and SCO on AMPK signaling proteins in mouse livers. Hepatic AMPK p, AMPK $\alpha 1$ and AMPK $\alpha 2$ were measured by western blotting. SCO significantly increased hepatic AMPK $\alpha 1$ content, AMPK $\alpha 1$ activity in basal and AMP stimulated conditions when compared with HFD mice. However, SANT did not affect AMPK signaling of liver. Data were represented as fold change of HFD, mean $\pm$ SEM. * $P < 0.05$, and *** $P < 0.001$, SCO vs. HFD. ### $P < 0.001$, SCO vs. SANT group.

Fig. 6. The effects of SANT and SCO supplementation on hepatic lipogenic enzyme abundance. Lipid metabolism related enzymes were determined using a western blotting as shown in legend. SCO significantly increased ratio of ACC p/ACC, reduced FAS, HMGR, and SREBP protein abundance in the liver without altering CPT 1 in compared with HFD mice, but SANT only significantly reduced FAS content. Data were normalized by β -actin and presented as mean $\pm$ SEM. * $P < 0.05$ and ** $P < 0.01$, and $P < 0.001$ SANT or SCO vs. HFD. # $P < 0.05$, ## $P < 0.01$, and $P < 0.001$. SANT vs. SCO, respectively.