

Sweet Elements of *Siraitia Grosvenori* Inhibit Oxidative Modification of Low-Density Lipoprotein

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This study examined the ability of sweet elements extracted from *Siraitia grosvenori* (SG) to inhibit the oxidation of LDL. We monitored the formation of conjugated diene during copper-mediated LDL oxidation in the presence or absence of sweet elements of whole extract of SG (SG extract) or cucurbitane glycosides (CGs) purified from SG extract as sweet elements. CGs consist of Mogroside IV (Mog.IV), Mogroside V (Mog.V), 11-Oxo-mogroside V (11-Oxo-mog.V), and Siamenoside I (Sia.I). In addition, the effect of these elements on human umbilical vein endothelial cell (HUVEC)-mediated LDL oxidation was tested by measuring production of lipid peroxides. SG extract inhibited copper-mediated LDL oxidation in a dose-dependent fashion, but neither glucose nor erythritol suppressed the oxidation. Among CGs, 11-Oxo-mog.V significantly inhibited LDL oxidation, and prolongation of the lag time during LDL oxidation by 11-Oxo-mog.V was dose-dependent. The lag time (119.7 ± 8.9 min) in the presence of $200 \mu\text{M}$ 11-Oxo-mog.V was significantly longer than that (76.8 ± 5.5 min) of control ($p < 0.01$). In addition, SG extract and 11-Oxo-mog.V inhibited HUVEC-mediated LDL oxidation in a dose-dependent manner. These results demonstrate that SG extract can inhibit LDL oxidation and that 11-Oxo-mog.V, a sweet element of SG extract, provides the anti-oxidative property of SG which might reduce the atherogenic potential of LDL. *J Atheroscler Thromb*, 2002; 9: 114-120.

Key words: Cucurbitane glycosides, Low-density lipoprotein, Oxidation, Antioxidant

Introduction

Considerable evidence supports the concept that oxidatively modified low-density lipoprotein (LDL) plays an important role in the initiation and progression of atheros-

clerosis (1-7). Several reports have demonstrated that naturally occurring polyphenols, such as flavonoids, reduce the mortality from coronary heart disease (CHD) through inhibition of both LDL oxidation and platelet aggregation (8-11). Indeed, the antioxidant effects of tea and wine on LDL have been well-established (11-14). Hence, biological effects of other food ingredients should be also investigated for preventing LDL oxidation.

Previous reports have shown that the LDL of diabetes mellitus (DM) patients is susceptible to oxidative modification and that DM patients have increased levels of plasma oxidized LDL and serum antibodies against oxidized LDL

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(15–18). Further, high concentrations of glucose promote LDL oxidation, possibly through a free radical pathway, and postprandial hyperglycemia contributes to oxidative stress in diabetic patients (19–22). Diet therapy is an essential element of therapy for patients with DM and impaired glucose tolerance. Low energy sweet elements are readily available as substitutes for glucose and saccharose.

The aim of the present study is to investigate whether cucurbitane glycosides (CGs), a sweet element of *Siraitia grosvenori* (SG), can inhibit oxidative modification of LDL.

Materials and Methods

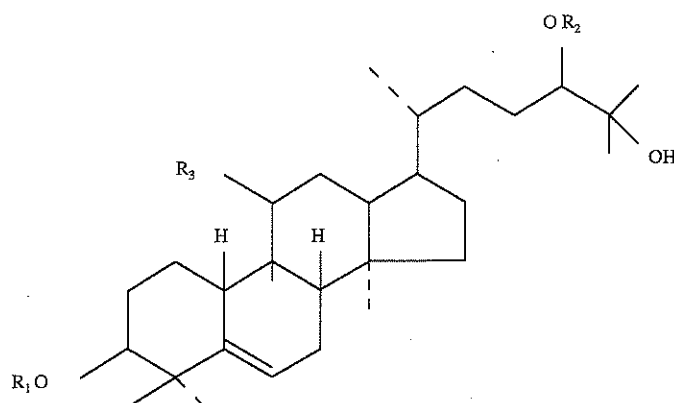
CGs are extracted from SG, a fruit plant, and separated by repeated column chromatography as reported previously (23, 24). Whole extract from SG prior to further purification is called SG extract, which contains less than 10 nmol vitamin E/g and no β -carotene or vitamin C. Fig. 1 shows the structure of CG, which is the representative sweet element of SG. Molecular weights (g/mol) of Mogroside IV (Mog.IV), Mogroside V (Mog.V), 11-Oxo-mogroside V (11-Oxo-mog.V), and Siamenoside I (Sia.I) are 1124, 1286, 1284, and 1124, respectively.

Human plasma was prepared from healthy men after a 12-h fast using blood in EDTA (1 mg/ml). LDL (density 1.019 to 1.063 g/ml) was isolated by a density gradient,

single spin ultracentrifugation (13, 25). LDL for oxidation experiments was dialyzed at 4°C for 24 h against phosphate-buffered saline (PBS, pH 7.4) to remove EDTA. LDL protein was determined according to the procedure of Lowry *et al.* (26) using bovine serum albumin (BSA) as a standard.

Oxidative susceptibility of LDL was examined following the method of Esterbauer *et al.* (13, 16, 27). Briefly, LDL (50 μ g/ml) was incubated with 2 μ M CuSO_4 as a prooxidant in the presence or absence of sweet elements such as SG extract, CGs, glucose, or erythritol at the indicated concentrations. Conjugated diene formation during copper-mediated LDL oxidation was monitored continuously for 4 h at 10 min intervals at 234 nm absorbance. LDL oxidation can roughly be divided into three consecutive phases, the lag phase, propagation phase, and decomposition phase. The lag phase is defined as the period where no oxidation occurs prior to propagation of the chain reaction to form lipid hydroperoxides. Lag time is determined by extrapolation of the linear part of the increase in absorption (propagation phase) to the time axis.

The free radical scavenging capacity of sweet elements was analyzed by the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay (28, 29). Each sweet element was diluted in ethanol at the indicated concentrations and mixed with 0.2 mM DPPH in 1 ml of ethanol. The time course of



Cucurbitane glycosides (CG)	R ₁	R ₂	R ₃
Siamenoside I (Sia.I)	-Glc	-Glc $\underline{2}$ Glc 6 Glc	-OH H
Mogroside IV (Mog.IV)	-Glc $\underline{6}$ Glc	-Glc $\underline{2}$ Glc	-OH H
11-Oxo-mogroside V (11-Oxo-mog.V)	-Glc $\underline{6}$ Glc	-Glc $\underline{2}$ Glc 6 Glc	= O
Mogroside V (Mog.V)	-Glc $\underline{2}$ Glc	-Glc $\underline{2}$ Glc 6 Glc	-OH H

Fig. 1. Structure of cucurbitane glycosides (CGs). Glc indicates β -D-glucopyranosyl.

change in optical density at 517 nm was monitored.

The effects of SG extract on cell-mediated LDL oxidation was tested using human umbilical vein endothelial cell (HUVECs). HUVECs were cultured with EBM-2 medium (Clonetics, Walkersville, MD) until 90% confluent, then switched to Ham's F-10 medium. LDL (100 $\mu\text{g}/\text{ml}$) was incubated for 24 h with HUVECs in Ham's F-10 medium (Life Technologies, GIBCO-BRL, Rockville, MD) in the presence or absence of sweet elements, glucose, or erythritol at the indicated concentrations. We measured the extent of LDL oxidation by agarose gel electrophoresis and lipid peroxide (LPO) assay using a commercially available kit (Determiner LPO, Kyowa, Tokyo, Japan) based on a calorimetric assay with the reaction of a leucomethylene blue derivative with lipid hydroperoxides in heme compounds (13, 14, 16).

Each experiment was performed three times, each time in triplicate. Results of the LPO assay are expressed as the mean \pm SD. Differences between groups of more than three were tested by ANOVA with the Fisher method, and differences between two groups were analyzed by the unpaired Student *t*-test. A value of $p < 0.05$ was accepted as statistically significant.

Results

SG extract suppressed copper-mediated LDL oxidation dose-dependently, and the lag phase was prolonged with increasing SG extract (Fig. 2). In addition, an effect of SG extract on HUVEC-mediated LDL oxidation was observed. SG extract inhibited HUVEC-mediated LDL oxidation in a dose-dependent manner (Fig. 3), and there was a similar trend in increased electrophoretic mobility of LDL on

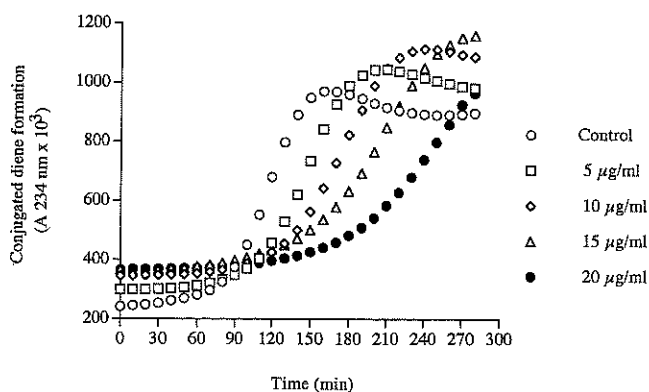
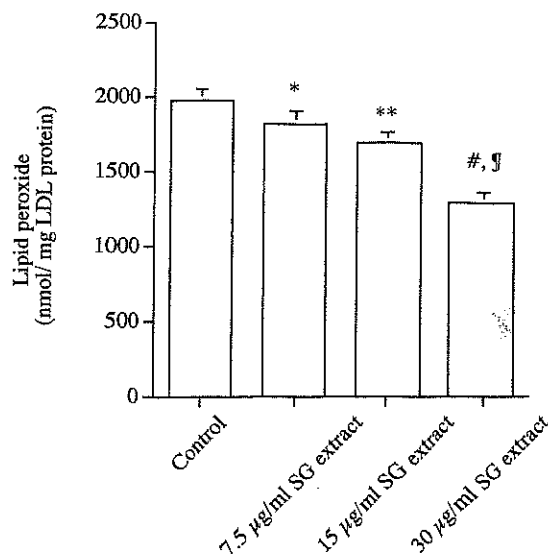


Fig. 2. Effect of SG extract on copper-mediated LDL oxidation. LDL (50 $\mu\text{g}/\text{ml}$ in PBS) was oxidized with 2 μM CuSO_4 at 25°C in the presence or absence of SG extract at the indicated concentrations, and conjugated diene formation during LDL oxidation was monitored by the change in absorbance at 234 nm as described in the Methods. Open circle shows the no-addition control. Open square, open diamond, open triangle, and closed circle indicate 5 $\mu\text{g}/\text{ml}$, 10 $\mu\text{g}/\text{ml}$, 15 $\mu\text{g}/\text{ml}$, and 20 $\mu\text{g}/\text{ml}$ of SG extract, respectively.



Additions to LDL during 24th incubation

Fig. 3. Effect of SG extract on HUVEC-mediated LDL oxidation. LDL (100 $\mu\text{g}/\text{ml}$ in Ham F-10 medium) was oxidized with HUVECs for 24 h in the presence or absence of SG extract at the indicated concentrations. The lipid peroxidation products were assessed by LPO assay as described in the Methods. All values are the mean \pm SD ($n=3$). ***, # significantly different from control: * $p < 0.05$, ** $p < 0.01$, # $p < 0.001$. ¶ $p < 0.001$, as compared with 15 $\mu\text{g}/\text{ml}$ of SG extract.

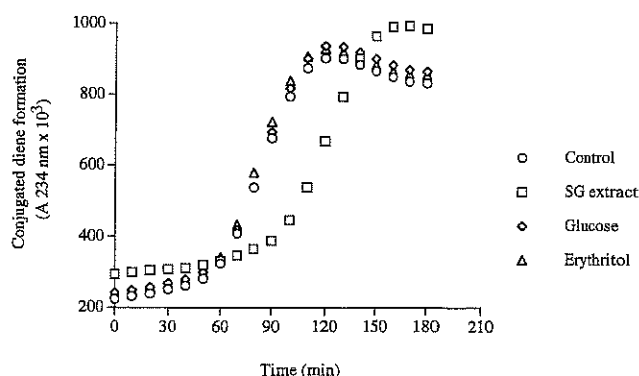


Fig. 4. Effect of sweet elements on copper-mediated LDL oxidation. LDL (50 $\mu\text{g}/\text{ml}$ in PBS) was oxidized with 2 μM CuSO_4 at 25°C in the presence or absence of 10 $\mu\text{g}/\text{ml}$ of SG extract, 10 μM glucose and 10 μM erythritol, and conjugated diene formation during LDL oxidation was monitored by the change in absorbance at 234 nm as described in the Methods. Open circle, open square, open diamond, and open triangle indicate no addition, 10 $\mu\text{g}/\text{ml}$ of SG extract, 10 μM glucose and 10 μM erythritol, respectively.

agarose gel to the LPO results (data not shown). As 10 $\mu\text{g}/\text{ml}$ of SG extract is equivalent to 10 μM CG, we used 10 μM as the experimental concentration of sweet elements in subsequent experiments. Fig. 4 shows that

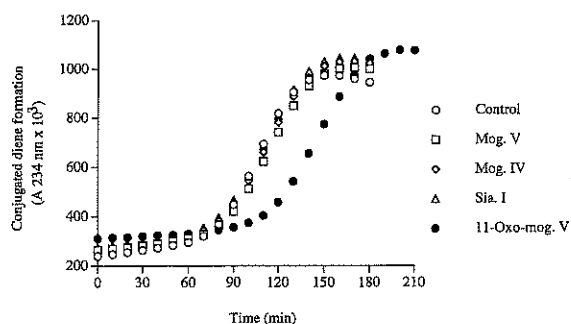


Fig. 5. Effect of CGs on copper-mediated LDL oxidation. LDL (50 $\mu\text{g/ml}$ in PBS) was oxidized with 2 μM CuSO_4 at 25°C in the presence or absence of CGs and conjugated diene formation during LDL oxidation was monitored by the change in absorbance at 234 nm as described in the Methods. Open circle, open square, open diamond, open triangle, and closed circle indicate no addition, 100 μM Mog.V, 100 μM Mog.IV, 100 μM Sia.I, and 100 μM 11-Oxo-mog.V, respectively.

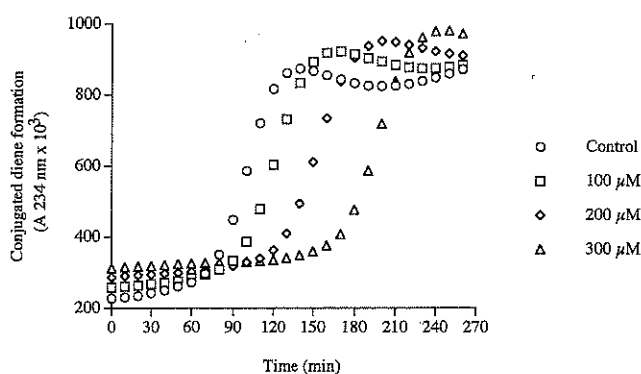
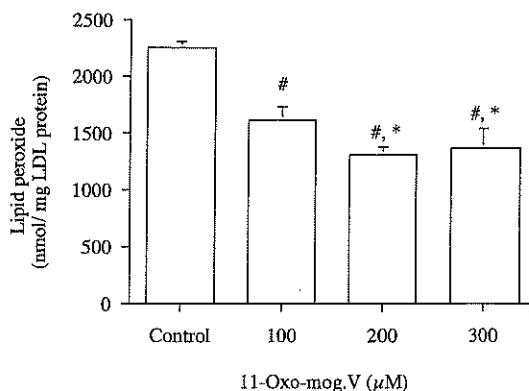


Fig. 6. Dose-dependent effect of 11-Oxo-mog.V on copper-mediated LDL oxidation. LDL (50 $\mu\text{g/ml}$ in PBS) was oxidized with 2 μM CuSO_4 at 25°C in the presence or absence of 11-Oxo-mog.V, and conjugated diene formation during LDL oxidation was monitored by the change in absorbance at 234 nm as described in the Methods. Open circle shows the no-addition control. Open square, open diamond, and open triangle indicate 100 μM , 200 μM , and 300 μM 11-Oxo-mog.V, respectively.

copper-mediated LDL oxidation was inhibited by 10 $\mu\text{g/ml}$ of SG extract but not by 10 μM glucose or 10 μM erythritol. Subsequently, we observed that neither 10 mM glucose nor 10 mM erythritol inhibited copper-mediated LDL oxidation (data not shown).

The effect of CGs on copper-mediated LDL oxidation was tested to investigate which sweet elements of SG extract inhibited the oxidation. CGs had no major effect on LDL oxidation at concentrations of less than 50 μM (data not shown). Fig. 5 demonstrates that only 11-Oxo-mog.V among CGs suppressed LDL oxidation at 200 μM . The addition of 11-Oxo-mog.V made LDL less susceptible to copper-mediated oxidation in a dose-dependent manner (Fig. 6). Moreover, 11-Oxo-mog.V significantly in-



Additions to LDL during 24th incubation

Fig. 7. Effect of 11-Oxo-mog.V on HUVEC-mediated LDL oxidation. LDL (100 $\mu\text{g/ml}$ in Ham F-10 medium) was oxidized with HUVECs for 24 h in the presence or absence of 11-Oxo-mog.V at the indicated concentrations. The lipid peroxidation products were assessed by LPO assay as described in the Methods. All values are the mean \pm SD ($n=3$). * $p < 0.05$ (vs. 100 μM 11-Oxo-mog.V), # $p < 0.001$ (vs. control).

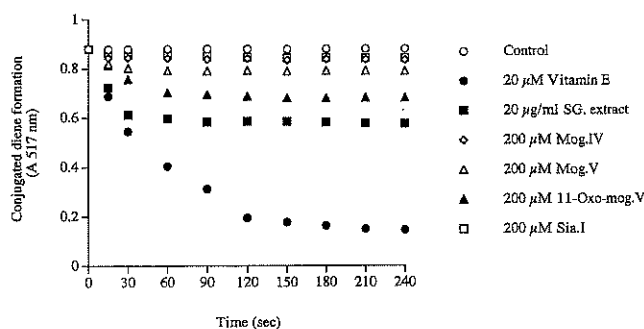


Fig. 8. Free radical scavenging capacity of SG extract and CGs. SG extract and CGs at the indicated concentrations were incubated with 0.2 mM DPPH, and kinetic determination of the absorbance at 517 nm was performed. One representative experiment out of 3 with a similar pattern is shown. Vitamin E (20 μM) was used as a positive control for the free radical scavenger.

creased the lag time of LDL oxidation from 76.8 \pm 5.5 min (mean \pm SD) for control to 119.7 \pm 8.9 min at 200 μM ($p < 0.01$). Subsequently, the experiment to test the effect of 11-Oxo-mog.V on HUVEC-mediated LDL oxidation revealed that 11-Oxo-mog.V inhibited the oxidation in a dose-dependent manner (Fig. 7). The extent of inhibition of HUVEC-mediated LDL oxidation by 11-Oxo-mog.V is likely to be consistent with that by SG extract (Figs. 3, 7).

The inhibitory effect of SG extract and 11-Oxo-mog.V on LDL oxidation may be related to the free radical scavenging capacity. In the DPPH assay, a time-dependent reduction in absorbance at 517 nm was observed with SG extract and 200 μM 11-Oxo-mog.V, but not with the other CGs (Fig. 8). After 4 min of incubation, SG

extract and 11-Oxo-mog.V reduced the absorbance at 517 nm by 35% and 23%, respectively. For comparison, an 85% reduction in absorbance was obtained with 20 μ M vitamin E, a positive free radical scavenger antioxidant.

Discussion

DM is associated with a markedly increased risk of atherosclerotic cardiovascular disease (30-32). The accelerated atherosclerosis of DM is thought to be caused by the modification of LDL by oxidation, glycosylation, or both, resulting in subsequent endothelial injury and increased cholesterol accumulation by monocyte-macrophages, leading to foam cell formation in the arterial intima (31-34). Dietary treatment and exercise comprise the basic recommendations of health care professionals for patients with DM. However, several reports have demonstrated that free-radical production and lipid peroxidation increase during acute moderate aerobic exercise, resulting in oxidative stress (35, 36). If low calorie sweet elements can inhibit the oxidation of LDL, they may help to prevent cardiovascular disease, a complication of DM.

The present study demonstrated that SG extract significantly inhibits copper-mediated and cell-mediated LDL oxidation in a dose-dependent fashion (Figs. 2-4), and 11-Oxo-mog.V is the candidate for the active component of the extract (Figs. 5-7). Previous reports have shown that glucose promotes LDL oxidation. DM patients have oxidative stress and increased susceptibility of LDL to oxidative modification (15-21). In the present study, glucose did not promote copper-mediated LDL oxidation at concentrations up to 10 mM, but it did not inhibit LDL oxidation, either. SG extract is likely to be beneficial in DM patients with increased oxidative stress. SG extract contains tiny amounts of vitamin E, but the content of vitamin E in 10 μ g/ml of SG extract is less than 0.1 nM. Thus, vitamin E in SG extract is unlikely to affect LDL oxidation because concentrations of more than 1 μ M are required to significantly inhibit copper-mediated LDL oxidation (37, 38).

The DPPH assay (Fig. 8) was undertaken to examine if the inhibitory effect of SG extract and CGs on LDL oxidation might be related to free radical scavenging capacity. Our results suggest that SG extract and 11-Oxo-mog.V both scavenge free radicals, but less effectively than vitamin E. The criteria for effective radical scavenging include not only the number of hydroxy groups, but also their positioning and arrangement on phenolic or aromatic rings in the case of flavonoids (39, 40). For flavonoids, the 4-oxo function of the C ring partly enhances antioxidant activity by providing electron delocalization, resulting in stabilization of radicals by the resonance effect of the aromatic nucleus. The oxo-function at the R₃ position in the structure of 11-Oxo-mog.V, as shown in Fig. 1, may be relevant to its anti-oxidative activity. In addition, copper-induced LDL oxidation is

inhibited not only by radical scavenging but also by chelating metal ion. Moreover, the physiological or pharmacological concentration of SG extract in plasma has not been defined. Hence, the mechanisms by which SG extract and 11-Oxo-mog.V inhibit LDL oxidation remain to be elucidated.

Limitations of the present study should be discussed. First, it remains to be defined whether purified CGs from SG can be substantially absorbed from intestine in humans and how much can exist in plasma after the intake of purified CGs. Second, whether the susceptibility of LDL to *ex vivo* oxidation bears a close correlation to susceptibility to atherosclerosis is not clear, because there is insufficient evidence to allow a confident prediction of the anti-atherosclerotic effectiveness of a compound from its antioxidant effectiveness *ex vivo* (5, 6). Therefore, the antioxidative effect *in vitro* is not necessarily likely to reflect the anti-atherosclerotic effect.

The four purified CGs from SG could in part explain the inhibitory effect of SG extract. Since 11-Oxo-mog.V inhibited HUVEC-mediated LDL oxidation as well as did SG extract, 11-Oxo-mog.V is likely to play a major role in the anti-oxidative action of SG. However, other unknown sweet elements of SG may also play a role in the inhibition of LDL oxidation by SG. Further study is needed to resolve the mechanisms of SG-related anti-oxidative effects and discover any new sweet elements of SG. Since SG extract and CGs are naturally occurring substances, they are less likely to have toxic effects. Whether SG extract or 11-Oxo-mog.V can reduce atherogenic potential in DM patients or dyslipidemias awaits further investigation.

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