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Modulatory effects of ZYM-201 sodium succinate on dietary-induced hyperlipidemic conditions

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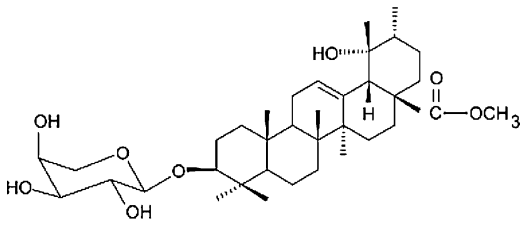
Sanguisorba officinalis, a well known and valuable medicinal plant in Korea, China and Japan has been used traditionally for the treatment of inflammatory and metabolic diseases such as diarrhea, chronic intestinal infections, duodenal ulcers, and bleeding. We studied the anti-hyperlipidemic effects of a chemically modified triterpenoid glycoside (ZYM-201 sodium succinate) isolated from *Sanguisorba officinalis* in rats in which hyperlipidemia had been induced by dietary administration of cholesterol and cholic acid. Oral administration of ZYM-201 sodium succinate (1 to 10 mg/kg) dose-dependently attenuated the diet-induced increases in body and liver weights. At 10 mg/kg, this compound also reversed the enhancement of serum levels of triglycerides (TG) and total cholesterol back to normal levels. In addition, imbalances in both serum and hepatic values of high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL) were prevented. Finally, this compound both blocked the generation of lipid peroxide and hydroxyl radicals and enhanced the activity of superoxide dismutase (SOD) in liver. Therefore, our data strongly suggest that ZYM-201 sodium succinate could play a role in modulating hyperlipidemic conditions, which could be used as a valuable remedy for the treatment of relevant disorders such as atherosclerosis and vascular diseases.

1. Introduction

Hyperlipidemia is a pathology that causes several serious diseases such as atherosclerosis, ischemic cerebrovascular disease, and peripheral vascular disease (Hvidtfeldt et al. 2008). Hyperlipidemic conditions can be easily induced by a high fat diet, and can become a major threat to human health (Connor and Connor 1990). Although it is not understood exactly how a high fat diet can lead to hyperlipidemia, there are numerous lines of evidence indicating that oxidative stress derived from anion production and from other radicals is one of serious causes underlying the induction of hyperlipidemia by a high fat diet. Therefore, direct suppression of the excess oxidative stress generated by a high fat diet can be considered as a major therapeutic approach to the management of hyperlipidemic diseases. In addition, abnormal metabolic conditions on lipid or cholesterol biosynthesis are also considered as another cause of hyperlipidemic symptoms (Kaysen 1992; Matsuo et al. 1995). Such cases can be approached to design or screen some chemical inhibitors or modulators with a structural similarity to lipidic molecules or cholesterols for restoring metabolic alteration (Leitersdorf 2002; Matsuo et al. 1995).

Sanguisorba officinalis L. (Rosaceae), is a medicinal plant with hemostatic, analgesic, and astringent properties (East 1955). In Far Eastern countries such as Korea, Japan and China, this plant has been used for the treatment of various inflammatory and metabolic symptoms such as diarrhea, chronic intestinal infections, duodenal ulcers, and bleeding (East 1955; Yu et al. 2011). Recently the ability of this plant to suppress infection, inflammation, cancer, allergy, and wrinkle formation, and its neuroprotective and anxiolytic activities have been explored through studies on its cellular and molecular mechanisms (Kim et al. 2001, 2008; Nguyen et al. 2008; Park et al. 2004; Yu et al. 2011). To date, saponin components such as triterpenes and their glycosides (eg. ziyuglycoside I and II), gallic acid, and disaccharide (5-*O*-alpha-D-(3-C-hydroxymethyl)lyxofuranosyl-beta-D-(2-C-hydroxymethyl) arabino furanose) have been reported as major active principles that possess the above-noted suppressive effects (Ban et al. 2008; Cho et al. 2006; Kim Y. H. et al. 2008; Park et al. 2004). Although numerous activities of this plant have



Ziyu-glycoside II methyl ester
(ZYM-201)

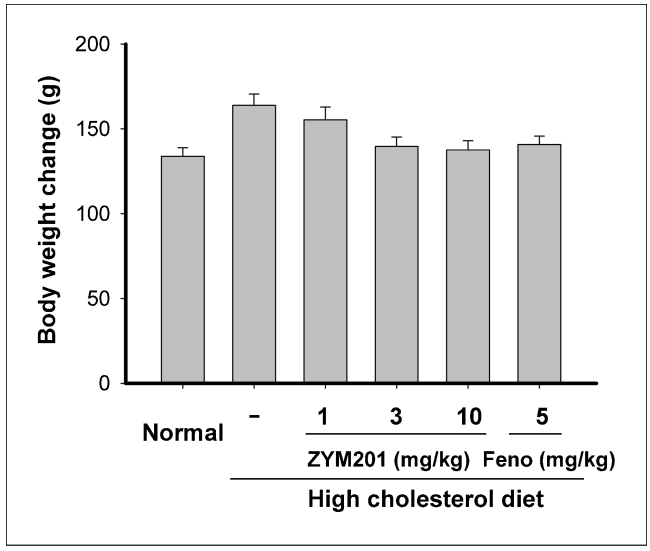
Fig. 1: Chemical structure of ZYM-201.

been reported and published, not many reports have explained the mechanism for the anti-metabolic activity of *Sanguisorba officinalis*. Accordingly, the present study was designed to investigate the anti-hyperlipidemic effects of orally administered *Sanguisorba officinalis* derived-triterpenoid glycoside (ziyuglycoside II methylester [ZYM201] sodium succinate) (Fig. 1) in rats in which hyperlipidemia was induced by a high fat diet containing cholesterol (2.0%) and sodium cholate (0.5%).

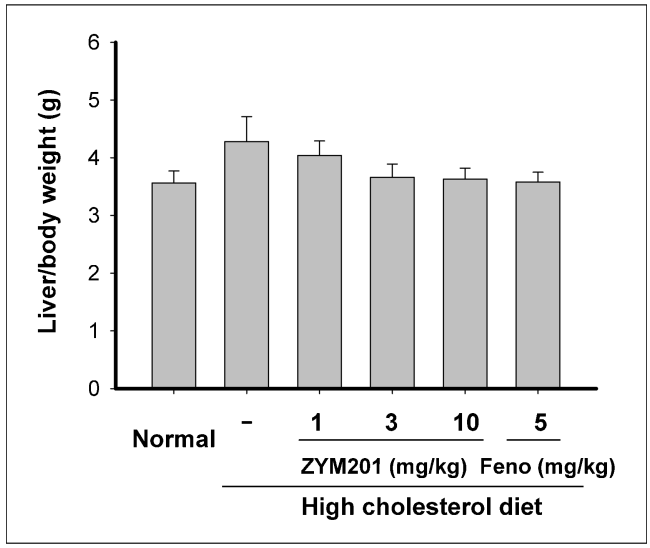
2. Investigations, results and discussion

Sanguisorba officinalis L. (Rosaceae), a well defined medicinal plant in Korea, China, and Japan, is traditionally used for the treatment of inflammatory and metabolic diseases such as diarrhea, chronic intestinal infections, duodenal ulcers, and bleeding. Recent studies have determined that several natural products such as fungal polysaccharides (eg, lentinan) composed of the basic structure of a β-1,3-glucan with β-1,6-glucopyranosidic branches and red *Ginseng*-derived acid polysaccharides have anti-hyperlipidemic activity (Franz 1989; Lee et al. 2008; Soeda et al. 1994). In addition, triterpenoids such as *Ginseng* saponins have also been reported to ameliorate various metabolic diseases such as diabetes and hyperlipidemic symptoms. To date, however, no reports have demonstrated whether triterpenoid glycosides from *Sanguisorba officinalis* and their structural derivatives (e.g., ZYM-201 and its succinate form ZYM-201 sodium succinate) are capable of modulating metabolic diseases. As part of our continuing evaluation of ZYM-201 sodium succinate pharmacology, we therefore investigated its anti-hyperlipidemic effects using a diet-induced hyperlipdemic rat model.

Our data indicate that ZYM-201 sodium succinate can regulate hyperlipidemic conditions as effectively as do other anti-hyperlipidemic materials (Soeda et al. 1994). For example, orally administered ZYM-201 sodium succinate (1 to 10 mg/kg) significantly reversed the increases in body weight (Fig. 2A) and liver weight (Fig. 2B) back down to normal levels in a dose dependent manner. The enhanced levels of triglycerides and total cholesterol in serum were also significantly suppressed by this compound, although there was not a large alteration in lipase activity (Table 1). Furthermore, ZYM-201 sodium succinate treatment restored normal levels of HDL, LDL, and VLDL in hyperlipidemic rats as in the case of fenofibrate-treated group (Table 2). In agreement with these results, ZYM-201 sodium succinate (10 mg/kg) reversed the up-regulation of the atherosclerotic index (AI) back down to normal levels (Fig. 3). A similar pattern was observed for hepatic lipid levels. Thus, ZYM-201 sodium succinate dose-dependently decreased the enhanced hepatic levels of total lipids, total cholesterol, and triglycerides (Table 3). However, under our conditions, there was no change in the activity of liver HMG-CoA reductase between control and drug treated groups (Fig. 4). On the contrary, ZYM-201 sodium succinate strongly suppressed the generation of lipid



(A)



(B)

Fig. 2: Effect of ZYM-201 sodium succinate on body weight and liver weight changes in hyperlipidemic rats. Rats fed with a high cholesterol diet for 4 weeks were orally administered ZYM-201 sodium succinate or fenofibrate (Feno) for 1 week. Body weight changes (A) and liver/body weight ratios (B) were examined.

peroxides and hydroxyl radicals (Table 4), while it up-regulated the SOD activity that had been decreased by hyperlipidemic conditions (Fig. 5).

Recent studies have demonstrated that oxidative stress plays a crucial role in induction of hyperlipidemia, although an abnormal cholesterol biosynthesis pathway is regarded as another critical cause of this pathology. In particular, oxidative stress in the arterial wall is linked to the initiation and progression of the cardiovascular dysfunction associated with hyperlipidemia (Inagi et al. 2006). The toxic radicals trigger lipid peroxidation which results in the formation of aldehyde by-products such as malondialdehyde (MDA) and damages the liver (Jenkins et al. 2008). It is well known that SOD and glutathione peroxidase (GSH-PX) are among the main defensive antioxidant agents that scavenge oxygen free radicals (Miller et al. 1998). Indeed, a high fat diet enhances the production of lipid peroxides and hydroxyl radicals under our conditions (Table 4); in parallel, SOD activity was strongly decreased under hyperlipidemic conditions (Fig. 5), whereas there was no significant enhancement of HMG-CoA reductase activity (Fig. 4). ZYM-201 sodium

Table 1: Effect of ZYM-201 sodium succinate on serum levels of triglyceride (TG), total cholesterol (TC), and lipase

Treatment		Dose (mg/kg)	Day	TG (mg/dl)	TC (mg/dl)	Lipase (mg/dl)
Normal				65.1 ± 3.94	66.3 ± 5.70	0.93 ± 0.08
Hyper-lipidemic diet	ZYM-201 sodium succinate	0		$92.6 \pm 6.06^{\#}$	$104.9 \pm 7.35^{\#\#}$	0.98 ± 0.14
		1	7	88.7 ± 5.52	105.6 ± 7.45	1.02 ± 0.15
		3	7	$67.3 \pm 4.16^{**}$	$73.1 \pm 6.38^{**}$	0.99 ± 0.06
		10	7	$65.9 \pm 3.70^{**}$	$68.9 \pm 6.20^{**}$	1.05 ± 0.19
	Fenofibrate	5	7	$64.9 \pm 3.62^{**}$	$69.9 \pm 6.29^{**}$	$1.95 \pm 0.24^{*}$

Rats fed a high cholesterol diet for 4 weeks were orally administered ZYM-201 sodium succinate or fenofibrate (Feno) for 1 week. Serum levels of TG, TC and lipase were examined. Data represent mean ± SEM of three independent observations performed with 10 rats. #: $p < 0.05$ and ##: $p < 0.01$ compared to normal group, and *: $p < 0.05$ and **: $p < 0.01$ compared to control group (high cholesterol diet alone).

Table 2: Effect of ZYM-201 sodium succinate on serum levels of lipoproteins

Treatment		Dose (mg/kg)	Day	Lipoprotein (mg/dl)		
				HDL	LDL	VLDL
Normal				28.6 ± 3.12	22.0 ± 3.66	14.9 ± 0.94
Hyper-lipidemic diet	ZYM-201 sodium succinate	0		20.2 ± 1.93	$66.1 \pm 5.73^{\#\#}$	$20.5 \pm 1.64^{\#}$
		1	7	21.6 ± 2.52	63.1 ± 4.82	19.8 ± 1.06
		3	7	24.8 ± 2.76	$36.2 \pm 4.19^{**}$	$15.8 \pm 1.14^{**}$
		10	7	25.9 ± 2.85	$32.8 \pm 3.93^{**}$	$15.3 \pm 0.98^{**}$
	Fenofibrate	5	7	26.2 ± 3.07	$31.7 \pm 3.75^{**}$	$15.5 \pm 1.03^{**}$

Rats fed a high cholesterol diet for 4 weeks were orally administered ZYM-201 sodium succinate or fenofibrate (Feno) for 1 week. Levels of HDL, LDL, and VLDL were examined in serum. Data represent mean ± SEM of three independent observations performed with 10 rats. #: $p < 0.05$ and ##: $p < 0.01$ compared to normal group, and **: $p < 0.01$ compared to control group (high cholesterol diet alone).

Table 3: Effect of ZYM-201 sodium succinate on hepatic levels of lipoproteins [total lipid (TL), total cholesterol (TC), and triglyceride TG)]

Treatment		Dose (mg/kg)	Day	Lipid level (mg/g tissue)		
				TL	TC	TG
Normal				14.7 ± 1.11	2.27 ± 0.20	9.67 ± 0.69
Hyper-lipidemic diet	ZYM-201 sodium succinate	0		$33.6 \pm 2.83^{\#\#}$	$4.10 \pm 0.29^{\#}$	$28.1 \pm 3.99^{\#\#}$
		1	7	33.4 ± 3.19	4.07 ± 0.27	27.6 ± 3.75
		3	7	$16.3 \pm 1.07^{**}$	$2.47 \pm 0.18^{**}$	$11.8 \pm 0.92^{**}$
		10	7	$16.0 \pm 1.12^{**}$	$2.40 \pm 0.17^{**}$	$11.3 \pm 0.98^{**}$
	Fenofibrate	5	7	$15.7 \pm 1.15^{**}$	$2.37 \pm 0.15^{**}$	$11.1 \pm 0.96^{**}$

Rats fed a high cholesterol diet for 4 weeks were orally administered ZYM-201 sodium succinate or fenofibrate (Feno) for 1 week. Levels of TL, TC, and TG were examined for liver. Data represent mean ± SEM of three independent observations performed with 10 rats. #: $p < 0.05$ and ##: $p < 0.01$ compared to normal group, and **: $p < 0.01$ compared to control group (high cholesterol diet alone).

succinate strongly suppressed radical generation (Table 4) and enhanced the activity of the protective enzyme SOD (Fig. 5), suggesting that the anti-hyperlipidemic mechanism of ZYM-201 sodium succinate could be due to its anti-oxidant properties. This compound did not strongly block HMG-CoA reductase activity (Fig. 4), indicating less involvement of this enzyme.

Because oxidative stress induces vascular endothelial injury leading to apoptosis, a critical pathogenic factor in the development of cardiovascular diseases, we next examined whether ZYM201 sodium succinate was able to scavenge reactivity of SNP-releasing radicals (nitric oxide). In fact, SNP increased chemically reactive NO release, as determined with Griess reagent, in PBS solution in a dose-dependent manner (Fig. 6A). However, ZYM201 sodium succinate did not block the NO release from SNP up to 400 μM (Fig. 6B). A Similar effect was observed in experiments with RAW264.7 cells, a murine macrophage cell line. Thus, toxic radicals derived from SNP strongly and dose-dependently enhanced cell death of RAW264.7 cells up to 95% at 40 mM (Fig. 6C). The apoptotic condition of RAW264.7 cells induced by reac-

Table 4: Effect of ZYM-201 sodium succinate on serum levels of lipid peroxides (LP) and hydroxyl radicals (HR)

Treatment		Dose (mg/kg)	Day	LP	HR
Normal				22.3 ± 1.31	2.50 ± 0.05
Hyper-lipidemic diet	ZYM-201 sodium succinate	0		$34.7 \pm 3.02^{\#}$	$4.91 \pm 0.28^{\#\#}$
		1	7	32.8 ± 2.68	4.63 ± 0.25
		3	7	$23.6 \pm 1.86^{**}$	$2.88 \pm 0.18^{**}$
		10	7	$23.1 \pm 1.70^{**}$	$2.77 \pm 0.12^{**}$
	Fenofibrate	5	7	$23.3 \pm 1.57^{**}$	$2.69 \pm 0.10^{**}$

Rats fed a high cholesterol diet for 4 weeks were orally administered ZYM-201 sodium succinate or fenofibrate (Feno) for 1 week. Levels of LP and HR were examined in serum. Data represent mean ± SEM of three independent observations performed with 10 rats. #: $p < 0.05$ and ##: $p < 0.01$ compared to normal group, and **: $p < 0.01$ compared to control group (high cholesterol diet alone).

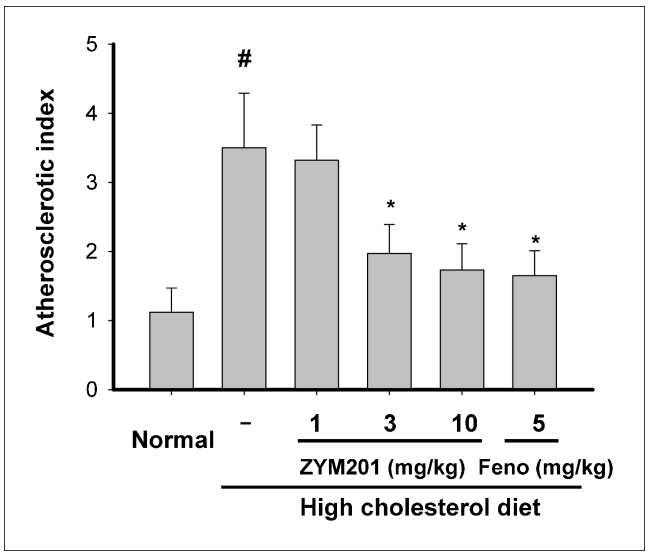


Fig. 3: Effect of ZYM-201 sodium succinate on the atherosclerosis index (AI) in hyperlipidemic rats. Rats fed a high cholesterol diet for 4 weeks were orally administered ZYM-201 sodium succinate or fenofibrate (Feno) for 1 week. AI values were calculated from an equation [AI = (total cholesterol-HDL-cholesterol)/HDL-cholesterol]. Data represent the mean ± SEM of three independent observations performed with 10 rats. #: $p < 0.05$ compared to the normal group, and *: $p < 0.05$ compared to the control group (high cholesterol diet alone).

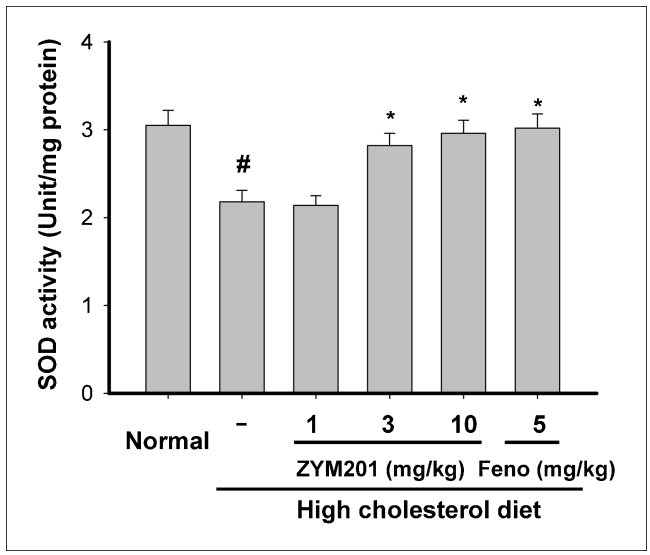


Fig. 5: Effect of ZYM-201 sodium succinate on SOD activity in hyperlipidemic rats. Rats fed a high cholesterol diet for 4 weeks were orally administered ZYM-201 sodium succinate or fenofibrate (Feno) for 1 week. SOD activity was examined in serum. Data represent mean ± SEM of three independent observations performed with 10 rats. #: $p < 0.05$ compared to normal group, and *: $p < 0.05$ compared to control group (high cholesterol diet alone).

tive radicals from SNP was not abrogated by the treatment of ZYM201 sodium succinate (50 and 100 μM) (Fig. 6D), indicating that there is no cytoprotective activity by this compound. However, we could not test more than 100 μM of ZYM201 sodium succinate, due to its cytotoxicity (Fig. 6E). Therefore, these results could suggest both that ZYM201 sodium succinate does not act as an anti-oxidative or radical scavenging compound and that anti-hyperlipidemic activity of ZYM-201 sodium succinate could be due not to anti-oxidative activity but to any other pharmacological property (eg. a metabolic modulation).

What chemical feature of ZYM-201 sodium succinate leads to its anti-hyperlipidemic effect is not understood. Several glycosidic triterpenoids have been reported to exhibit anti-hyperlipidemic activity. Recently, we reported that Korean red *Ginseng*-derived acid polysaccharides can negatively modulate the pathophysiological parameters seen in hyperlipidemic conditions induced by corn oil and Triton WR1339 (Kwak et al. 2010). Furthermore, β-glucans have been found to display anti-diabetic and anti-hyperlipidemic activities (Keogh et al. 2003). Glucose analogs (eg. pynitol and D-chiroinositol) are also known as anti-diabetic and anti-hyperlipidemic biomaterials (Geethan and Prince 2008, Ortmeyer et al. 1995). Considering these reports, structural characteristics of glycosides or their analogs could be an important feature of the anti-hyperlipidemic mechanism of ZYM-201 sodium succinate. How such a chemical feature can be directly linked to its anti-hyperlipidemic property is not known. Therefore, future experiments will elucidate its anti-hyperlipidemic mechanism in terms of its chemical structure, contributing to inhibition of certain metabolic enzymes, by preparing several synthetic derivatives such as the aglycone form of ZYM-201 sodium succinate or analogs of ZYM-201 sodium succinate with additional glycoside groups.

In summary, we found that oral administration of ZYM-201 sodium succinate was able to modulate various pathophysiological symptoms of hyperlipidemia such as increased body weight, enhanced total lipids, triglycerides, and total cholesterol, and up-regulation of lipid peroxides and hydroxyl radicals in serum and liver. Considering that vascular diseases have become the most serious causes of human death, preventing the development of pathological changes caused by a high fat diet is absolutely necessary to avoid vascular diseases. Furthermore, according to acute toxicity test performed with rats and beagle dogs by single dose of 5 or 2.5 g/kg, this compound was shown to be non-toxic and did not alter any change of body weights (Cho et al., in preparation). Therefore, our data strongly suggest that ZYM-201 sodium succinate can play a role in modulating hyperlipidemic conditions, which can be used as a valuable remedy for the treatment of hyperlipidemia without displaying any adverse effects. Whether ZYM-201 sodium succinate can be developed as either a functional food or a new drug with anti-hyperlipidemic properties will be further examined in the next pre-clinical trial.

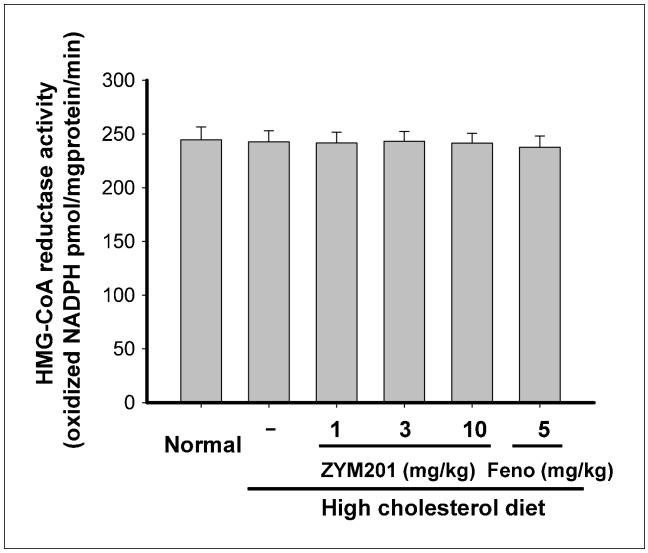
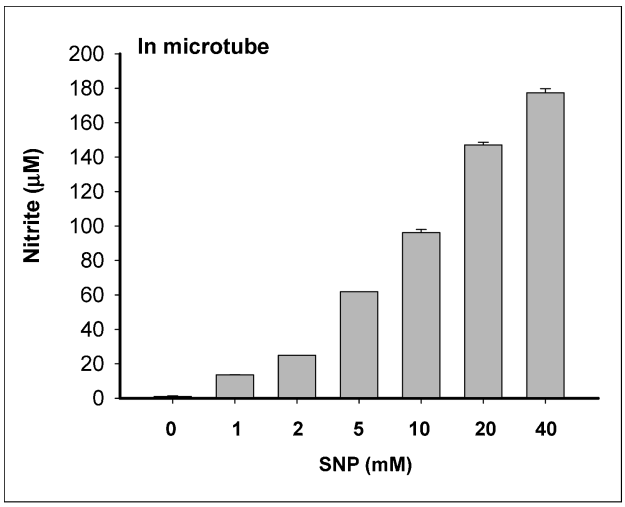


Fig. 4: Effect of ZYM-201 sodium succinate on hepatic HMG-CoA reductase activity in dietary hyperlipidemic rats. Rats fed a high cholesterol diet for 4 weeks were orally administered ZYM-201 sodium succinate or fenofibrate (Feno) for 1 week. Hepatic HMG-CoA reductase activity was examined using liver microsomal fractions. Data represent mean ± SEM of three independent observations performed with 10 rats.



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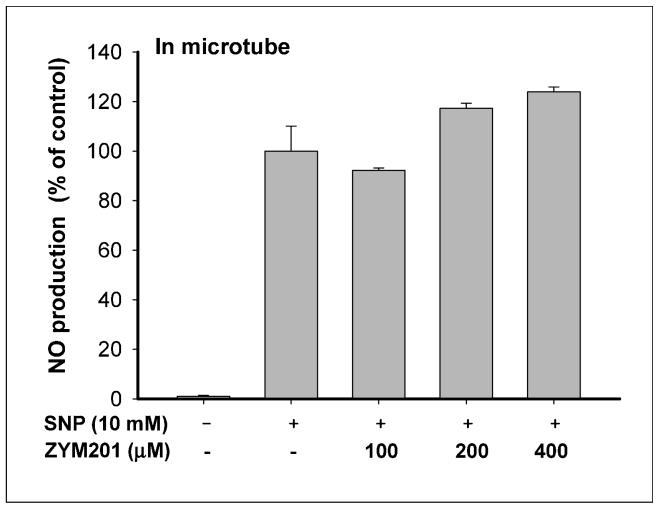

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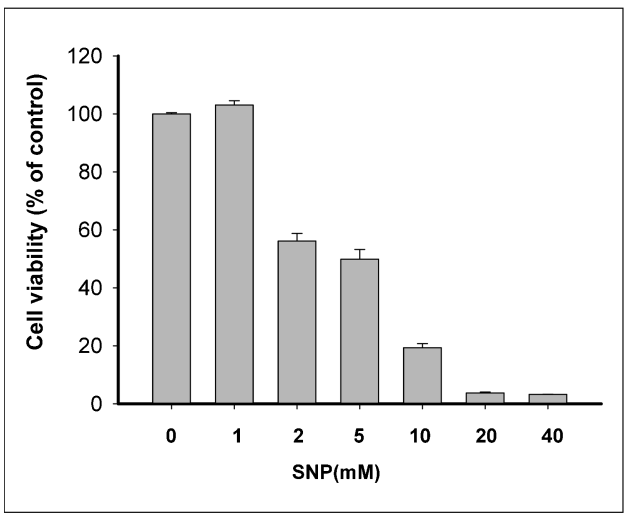

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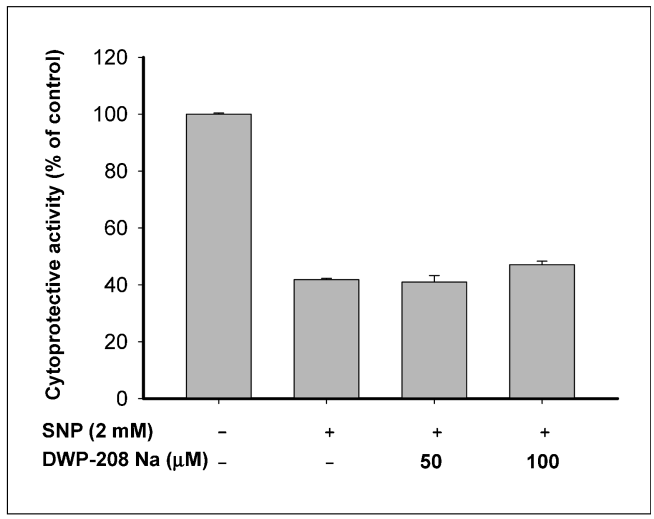
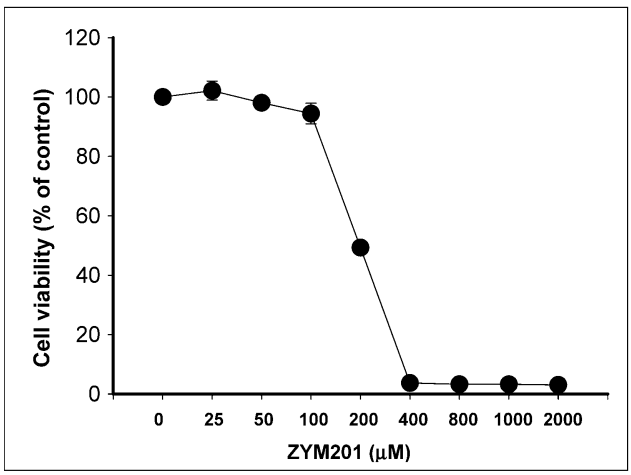


(D)



(E)

Fig. 6: Scavenging effect of ZYM-201 sodium succinate on the reactivity of SNP. (A) Chemical reactivity of SNP was determined by measuring its NO-releasing activity in microtube, assessed by Griess assay. (B) Neutralizing activity of ZYM-201 sodium succinate on the reactivity of NO released from SNP (10 mM) in microtube. (C and E) RAW264.7 cells (1×10^6 cells/ml) were treated with SNP or ZYM-201 sodium succinate for 24 h. Cell viability was determined by the MTT assay. (D) Cytoprotective effect of ZYM-201 sodium succinate on SNP-induced cytotoxicity of RAW264.7 cells (1×10^6 cells/ml) was examined by the MTT assay. *: $P < 0.05$ and **: $P < 0.01$ compared to control.

3. Experimentals

3.1. Materials

The sodium succinate form (ZYM-201 sodium succinate) of ziyuglycoside II methylester, ZYM-201 (Cho et al. 2006), was synthesized by adding succinic anhydride and sodium-2-ethylhexanoic acid. The yield of ZYM-201 sodium succinate was 82% and its purity was more than 95% according to HPLC analysis. Fenofibrate, cholesterol, (3–4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT), sodium cholic acid, succinic anhydride, sodium nitroprusside (SNP), and sodium-2-ethylhexanoic acid were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO USA). Absolute alcohol was purchased from

Table 5: Composition of basal and hyperlipidemic diets

Ingredient	Content (%)	
	Basal diet	Hyperlipidemic diet
Casein	20.0	20.0
Methionine	0.3	0.3
Corn starch	15.0	15.0
Sucrose	50.0	48.5
Fiber	5.0	5.0
Corn oil	5.0	5.0
AIN-mineral mixture	3.5	3.5
AIN-vitamin mixture	1.0	1.0
Choline bitartrate	0.2	0.2
Cholesterol	–	1.0
Sodium cholate	–	0.5

Fluka (Buchs, Switzerland). All other chemicals and reagents used were of Sigma grade. Foetal bovine serum and RPMI1640 were obtained from GIBCO (Grand Island, NY). RAW264.7 cells were purchased from ATCC (Rockville, MD).

3.2. Cell culture

RAW264.7, a murine macrophage cell line, and U937 cells, a human promonocytic cell line, were maintained in RPMI1640 supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% foetal bovine serum. Cells were grown at 37 °C and 5% CO_2 in humidified air.

3.3. Animals

Male Sprague-Dawley (SD) rats weighing 120–130 g were purchased from Daehan Biolink (Daejeon, Korea) and were given access to a commercial diet (Samyang, Korea) and water *ad libitum*. They were housed in a temperature-controlled room at 20°C with lighting on from 7 a.m. to 7 p.m. Humidity was maintained between 50–70%. The animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institute of Health (NIH Publication, revised in 1985).

3.4. Induction of hyperlipidemic conditions

Diet-induced hyperlipidemia was induced in rats by having them ingest a dietary pellet (Table 5) containing cholesterol (1%) and sodium cholic acid (0.5%) for 4 weeks as reported previously (Hofbauer et al. 1996). After this period, ZYM-201 sodium succinate (0 to 10 mg/kg) or fenofibrate (5 mg/kg) were orally administered once a day for 1 week. Based on previous reports (Takahashi et al. 2003; Yuan et al. 2010), animals were anesthetized and blood was drawn by cardiac puncture 2 h after final drug administration and serum was obtained by centrifugation at 3,000 rpm for 15 min.

3.5. Biochemical analysis of serum

Serum was separated by centrifugation at 1000 × g for 15 min. The levels of total cholesterol (TC), total triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL) were determined using an automatic analyzer (Hitachii 7020, Japan) (Kim et al. 2010c). Determination of hydroxyl radicals was done by methods previously published (Sagone et al. 1980). Lipase activity in serum was also determined by a method previously published (Giada et al. 1988). Lipid peroxidation levels in liver and serum were measured using an assay for thiobarbituric acid reactive substances (TBARS) (Fraga et al. 1988; Lee et al. 2010a). Superoxide dismutase (SOD) activity was assayed in the blood and liver using a technique that involves inhibition of pyrogallol auto oxidation at pH 8.0 (Lee et al. 2010c; Wahlund et al. 1992).

3.6. Biochemical analysis of liver samples

Liver samples for enzymatic analyses were prepared according to a previous report (Ametaj et al. 2003). Each liver sample (1 g) was homogenized (10%, w/v) in 4 volumes of 0.1 M potassium phosphate buffer (pH 7.5), then centrifuged at 600 × g for 10 min, 10,000 × g for 20 min, and 105,000 × g for 60 min to prepare the microsomal fraction. The microsomal fraction was used for the assay of 3-hydroxy-3-methyl-glutaryl (HMG)-coenzyme (Co) A reductase. Total lipids (TL), TC, and TG in the liver homogenate prepared with 0.9% NaCl were enzymatically determined using assay kits (Asan Pharmacy, Seoul, Korea). Protein concentrations were determined by the Bradford method (Bradford 1976) using BSA as the standard.

3.7. Determination of NO reactivity generated from SNP

SNP dissolved in PBS were further incubated with ZYM201 sodium succinate for 3 h. The released level of NO from SNP was determined by Griess assay, as described previously (Lee et al. 2010b).

3.8. Cell viability test

After the preincubation of RAW264.7 cells (1×10^6 cells/ml) for 18 h, single or cotreatment of SNP or ZYM201 sodium succinate were added to the cells and incubated for 24 h. The cytotoxic effect of these drugs was then evaluated by a conventional MTT assay, as reported previously (Kim et al. 2010a; Yeon et al. 2010). At 3 h prior to culture termination, 10 μl MTT solution (10 mg/ml in a phosphate buffered-saline, pH 7.4) was added and the cells were continuously cultured until termination. The incubation was halted by the addition of 15% sodium dodecyl sulphate into each well, solubilising the formazan (Kim et al. 2010b). The absorbance at 570 nm ($OD_{570-630}$) was measured by a Spectramax 250 microplate reader.

3.9. Statistical analysis

A Student's *t*-test and a one–way ANOVA were used to determine the statistical significance of differences between values for the various experimental and control groups. Data are expressed as means ± standard errors (SEM) and the results are taken from at least three independent experiments performed in triplicate. P values of 0.05 or less were considered to be statistically significant.

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