2,3,22,23-Tetrahydroxyl-2,6,10,15,19,23-hexamethyl-6,10,14,18-tetracosatetraene, an Acyclic Triterpenoid Isolated from the Seeds of *Alpinia katsumadai*, Inhibits Acyl-CoA : Cholesterol Acyltransferase Activity

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In order to isolate a cholesterol-lowering compound from *Alpinia katsumadai*, an inhibitor for acyl-CoA : cholesterol acyltransferase (ACAT), an enzyme responsible for the cholesterol ester formation in liver, was purified, its chemical structure was determined, and *in vivo* and *in vitro* inhibition activities were performed. In a high fat diet mouse model, we discovered that the ethanol extract of *Alpinia katsumadai* reduced plasma cholesterol, triglyceride, and low density lipoprotein (LDL) levels. An acyclic triterpenoid showing ACAT inhibitory activity was isolated from the extract of seeds of *A. katsumadai*. By NMR spectroscopic analysis of its ^{1}H-NMR, ^{13}C-NMR, ^{1}H–^{1}H correlation spectroscopy, heteronuclear multiple bond connectivity (HMBC), hetero multiquantum coherence (HMQC) and nuclear Overhauser effect, chemical structure of 2,3,22,23-tetrahydroxyl-2,6,10,15,19,23-hexamethyl-6,10,14,18-tetracosatetraene (1), were elucidated. The acyclic triterpenoid was found to be responsible for the ACAT inhibition activities of rat liver microsomes with IC_{50} values of 47.9 μM. It also decreased cholesteryl ester formation with IC_{50} values of 26 μM in human hepatocyte HepG2 cell. The experimental study revealed that the ethanol extract of *A. katsumadai* has a hypolipemic effect in high fat diet mice, and the isolated acyclic triterpenoid has ACAT inhibition activity, showing a potential novel therapeutic approach for the treatment of hyperlipidemia and atherosclerosis.

Key words atherosclerosis; triterpenoid; acyl CoA : cholesterol acyltransferase; inhibitor; *Alpinia katsumadai*

In humans, cholesterol is a chemical necessary for cellular sterol metabolism. When it is in excess, they are converted and stored as cholesterol esters. Cholesterol ester formation appears to be important in intestinal cholesterol absorption, hepatic lipoprotein production, and macrophage foam cell formation in atherosclerotic lesions.[1,2] The esterification of cholesterol is catalyzed by acyl-CoA : cholesterol acyltransferase (ACAT; EC 2.3.1.26).[3] In the human liver, ACAT is involved in the assembly and secretion of apolipoprotein-containing lipoprotein and is responsible for the main cholesteryl esters contained in very low-density lipoprotein (VLDL).[4]

Epidemiological studies have indicated that the majority of ischemic heart diseases are due to atherosclerosis involving atheroma in the coronary artery, and that elevation in serum cholesterol levels is a crucial factor in the development of the disease. In order to lower serum cholesterol levels, methods to inhibit cholesterol absorption in the small intestine, to inhibit biosynthesis of cholesterol in the liver, and to promote bile secretion have been suggested.[5] According to Sliskovic *et al.*, ACAT inhibitors have been effective in the prevention and treatment of hyperlipemia,[6] and have garnered interest as a therapeutic agent with a novel mechanism of action which is directly associated with the pathogenesis of atherosclerosis. During the last 20 years, numerous ACAT inhibitors have been synthesized and developed.[7] ACAT inhibitors, including Avasimibe (CI-1011),[8] F-1394,[9] and K604,[10] have been reported and their pharmacological activities have been evaluated in cells, animals, and humans. In addition, several natural ACAT inhibitors, such as pyripyropene,[11] pipercide in pepper,[12] and gabrol in licorice,[13] have been purified and exhibited. Recent studies have also shown that ACAT inhibitors, such as Avasimibe (CI-1011) and CP-113,818, substantially reduced the amyloid β secretion and protected from amyloid pathology in neuron cells, supporting ACAT inhibition as a strategy to treat Alzheimer's disease.[14,15]

Alpinia katsumadai L. (Zingiberaceae) is one of the commonly used traditional medicinal plants in many Asian countries. The dried ripe seed has been used to warm the stomach, arrest vomiting, and remove damp-cold in the spleen. A number of pharmacological activities of this plant extract has been reported, including antiviral activity,[16] antioxidant activity,[17] and anti-emetic effects.[18] However, there is no evidence of anti-atherosclerosis or hypolipemic effects of *A. katsumadai* to date.

While investigating cholesterol-lowering compounds from natural resources, we have observed that the ethanol seed extract of *Alpinia katsumadai* (eAKA) reduced plasma cholesterol and low-density lipoprotein (LDL) levels in a mouse model. In this paper, we report the effects of eAKA on mice fed on a high fat diet, as well as isolation, structure, and ACAT inhibitory activities of an acyclic triterpenoid from *A. katsumadai*.

MATERIALS AND METHODS

General UV spectrum was obtained using a Hewlett Packard model 8453 spectrophotometer. ^{1}H-NMR (300 MHz), ^{13}C-NMR (75 MHz), distorsionless enhancement by polarization transfer (DEPT), heteronuclear multiple quantum cohrence (HMQC), and heteronuclear multiple bond

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connectivity (HMBC) spectra were obtained with a Varian Unity 300 spectrometer using CD_3OD as a solvent. Electrospray ionization (ESI)-MS was measured on a Finning TM navigator spectrometer. The semi-preparative HPLC system consisted of a Shimaduz Model LC-6AD pump, SPD-10A detector, and a C-R8A recorder.

Reagents and Chemicals Minimal essential medium (MEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), penicillin G, and streptomycin were purchased from GIBCO BRL. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bovine serum albumin (BSA), and cholesterol were purchased from Sigma Chemical Co. [1-^{14}C]-Oleoyl CoA was obtained from Amersham. All solvents from Burdick & Jackson were analytical grades.

Animals All the animal experiments were performed at Pharmacology and Toxicology Lab., Dong Wha Pharm, Ind., Co., Ltd., Korea. Female ICR mice were obtained from SLC, Japan, and tested at 8 weeks of age. Animals were kept in a temperature (20–25°C)- and humidity (40–45%)-controlled room on a 12 h light/dark cycle. The animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals by the Institute of Laboratory Animal Resources, National Research Council, U.S.A., in 1996.

Animal Treatment Prior to the experiment, the mice were randomly divided into four treatment groups with five mice in each group. These groups were used throughout all tests. Mice were fed a normal diet containing 5% corn oil, 1% vitamin mixture, 3.5% salt mixture, 20% casein, and 20% sucrose. For high fat diets (HFD), normal diet plus 40% tallow AIN-76A purified rodent high fat diet (Dieyts, U.S.A.) were supplied to ICR mice from 1 week before initiation of sample dosing throughout the 12 weeks of sample-dosing periods. In the control group, normal pellet diets were supplied free to access instead of HFD. Oral administration of samples began 1 week after the initiation of HFD, once a day for 12 weeks at 200 mg/kg (of body weight) in a volume of 10 mL of 5% ethanol. Total cholesterol, LDL cholesterol, HDL cholesterol and triglyceride levels in serum were determined using an automated blood analyzer (AU400, Olympus, Japan).

Extraction and Isolation The dried seeds of *Alpinia katsumadai* were purchased from an herbal store in Daejeon, Korea. A voucher specimen (KRIB-0009862) was deposited in the Korea Plant Extract Bank, Korea Research Institute of Bioscience and Biotechnology. The seed of *Alpinia katsumadai* (1 kg) were extracted with ethanol (1 L) for 7 d at room temperature. The ethanol extracts (200 g) were evaporated and suspended in distilled water and divided into two fractions with chloroform as non-aqueous phase. The chloroform layer was concentrated *in vacuo* and its crude extracts (73.5 g) were chromatographed on a silica gel (230–400 mesh, 1 kg, Merck) using a step gradient of $CHCl_3$–MeOH solvent system (100 : 0, 90 : 1, 70 : 1, 50 : 1, 30 : 1, 15 : 1, 5 : 1, 1 : 1; each 2 L) to create 8 fractions. Each fraction was tested for ACAT inhibitory activity. The active fractions ($CHCl_3$–MeOH=70 : 1 fractions, 650 mg) were further fractionated using reverse-phase column chromatography (100 g, YMC-Gel RP-18, 70-230 mesh) with the step gradient elution of MeOH–H_2O (70 : 1, 80 : 1, 90 : 1, 100 : 0; each 2 L) to create 4 sub-fractions. After being tested for ACAT inhibitory activity of each fraction, the active fractions (MeOH–H_2O=90 : 1 fractions, 555 mg) were subjected to HPLC. Final purification was achieved by HPLC (column, YMC-J'sphere ODS H-80, 20×250 mm; solvent, 90% aqueous MeOH; flow rate, 6 mL/min; UV, 210 nm). Compounds **1** and **2** were eluted with retention times of 25 and 40 min, respectively. Oily compounds of **1** and **2** were obtained in quantities of 508 mg and 31 mg, respectively.

Structure Elucidation of the Compound Compound **1** was a yellow-colored oil and exhibited a molecular ion peak at *m/z* 501 $[M+Na]^+$ in the ESI-MS with the molecular formula of $C_{30}H_{54}O_4$. ^{1}H-NMR (300 MHz), ^{13}C-NMR (75 MHz), DEPT, HMQC, and HMBC spectra were obtained with a Varian Unity 300 spectrometer using CD_3OD as a solvent. The ^{13}C-NMR spectrum has fifteen peaks, which is half the carbon numbers expected from the ESI-MS spectrum data, suggesting that this compound has a symmetrical structure with 30 carbons. In the ^{1}H-NMR spectrum, four olefinic protons (δ 5.13, 5.18), four methyl protons (δ 1.59, 1.61), and six methylene protons δ 2.08 (H_2-8, 17), δ 2.01 (H_2-9, 12, 13, 16) were attached to the olefinic backbone. In addition, four methylene protons were observed at δ 1.40 (Ha-4, 21), 1.58 (Hb-4, 21), 2.06 (Ha-5, 20), and 2.23 (Hb-5, 20), as well as two methine protons attached to the hydroxyl at δ 3.36 (dd, J=2.0, 10.5 Hz), and four terminal methyl protons at δ 1.15 and 1.19.

Cell Culture The human liver cell line HepG2 was purchased from the American Type culture Collection (ATCC). The cells were cultured in MEM medium supplemented with penicillin G (100 U/mL), streptomycin (100 U/mL), and 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 humidified atmosphere and routinely sub-cultured using 2.5 mg/mL trypsin–ethylenediaminetetraacetic acid solution.

Cell Free ACAT Activity Assay ACAT activity was assayed according to a previously reported method,[19] with several modifications. The reaction mixture contained 4 μL of rat liver microsomes (10 mg/mL protein), 15 μL of bovine serum albumin (fatty acid free, 40 mg/mL), 2.0 μL of cholesterol in acetone (20 mg/mL), 51 μL of water, and 10 μL of sample in a total volume of 92 μL was pre-incubated for 30 min at 37°C. The reaction was initiated by the addition of 8 μL of [1-^{14}C]-oleoyl CoA solution (0.05 μCi: final concentration 10 μM). After 30 min, 1.0 mL of isopropanol–heptane (4 : 1) solution was added to stop the reaction. A mixture of heptane and 0.1 M assay buffer was then added to separate the cholesterol oleate in the upper (heptane) phase. The radioactivity in the upper phase was measured using a liquid scintillation counter (Beckman LS 6000, U.S.A.).

Assay for Cholesteryl Ester Formation in HepG2 Cell HepG2 cells were seeded in a 6-well plate at the density of 1×10^6 cells/mL/well and cultured in Dulbecco's modified Eagle's medium supplemented 10% FBS for 2 d. The medium was removed and cells were incubated with 2.5 μL of sample or 0.1% dimethyl sulfoxide as a vehicle, and [1-^{14}C]oleic acid (0.5 μCi) for 6 h. The medium was then removed, and cells were washed three times with PBS. The cells were lysed by adding 0.5 mL of PBS containing 0.1% sodium dodecyl sulfate, and intracellular lipids were extracted with hexane–isopropanol (3 : 2 v/v). After the organic phase had evaporated under nitrogen, the total lipid was separated on a silica gel TLC plate in petroleum ether–diethyl ether–acetic acid (90 : 10 : 1), followed by assessing the amount of radioactivity with a bio-imaging analyzer (BAS 1500, Fuji, Japan).

Data Analysis All data was calculated as mean value± S.D. Multiple comparison tests for different dose groups were

Table 1. Changes in the Plasma Cholesterol and Triglyceride Levels among Mice after HFD and Test Article Administration

Groups	Serum levels (mg/dL)	
	Total cholesterol	Triglyceride
Controls		
Normal	78.40±16.47	73.60±11.93
HFD	163.00±28.13*	150.60±32.95*
eAKA	117.40±16.04	111.20±18.36*
Simvastatin	119.20±23.76**	84.80±7.05$^{\#}$

Simvastatin was used as a positive control to compare with eAKA. Values are mean±S.D. (n=5, each group); *p<0.01 and **p<0.05 compared to normal control; $^{\#}p$<0.05 compared to HFD control.

Table 2. Changes in the Plasma LDL and HDL Levels among Mice after HFD and Test Article Administration

Groups	Serum levels (mg/dL)	
	Low density lipoprotein	High density lipoprotein
Controls		
Normal	11.80±3.27	93.76±18.76
HFD	24.20±5.12*	60.60±14.33
eAKA	11.40±2.88$^{\#}$	80.86±9.60
Simvastatin	14.80±3.27$^{\#\#}$	77.76±11.81

n=5; (mean±S.D.); *p<0.01 compared to that of normal control; $^{\#}p$<0.01 and $^{\#\#}p$<0.05 compared to HFD control.

conducted. Variance homogeneity was examined using the Levene test. If the Levene test indicated no significant deviations from variance homogeneity, the data were analyzed by a one-way analysis of variance (ANOVA) test, followed by Tukey HSD test to determine which pairs of group comparison was significantly different.

RESULTS AND DISCUSSION

Synthetic,[20,21] fungal[22] ACAT inhibitors, and ACAT2 antisense oligonucleotide[23] were reportedly shown to lower plasma cholesterol, triglyceride (TG), or VLDL levels. Therefore, we began to screen plant materials that could decrease plasma cholesterol and TG, or increase HDL contents in mice fed on a high fat diet. Among various candidates, the ethanol extract of dried seeds of *Alpinia katsumadai* (eAKA) was efficacious in lowering the serum lipid levels of hyperlipidemic mice (Table 1). Significant (p<0.01) increases of serum total cholesterol and TG levels were detected in HDF control compared to normal control. However, dramatic decreases on the plasma cholesterol and TG levels were detected in both the eAKA and positive control simvastatin-dosing groups compared to HFD control. In HFD control, the serum total cholesterol levels showed a 107.9% change compared to the intact control, and they were 27.9 and 26.9% decreases in the eAKA and simvastatin-dosing groups, respectively, compared to HFD control. The triglyceride level changes compared to HFD control were −26.2 and −43.7% in the eAKA and simvastatin-dosing groups, respectively. These results suggested that the eAKA might effectively inhibit ACAT activity in either the liver or intestine. Furthermore, lipoprotein analyses indicated that the reduction of plasma cholesterol levels was attributed to decreased cholesterol levels in the hyperlipidemic LDL. The administration of the eAKA significantly (p<0.01) reduced serum levels of LDL by 52.9% and non-significantly increased HDL levels by 33.4%, compared to the HFD control group (Table 2).

Based on the *in vivo* efficacy of eAKA, it is therefore likely that the administration of eAKA decreased plasma lipid levels by inhibiting the liver ACAT activity. Therefore, in order to identify the active compound capable of inhibiting ACAT enzyme activity, the ethanol extract of the seeds of *Alpinia katsumadai* was fractionated with silica gel chromatography and reverse phase column chromatography. We also discovered that chloroform extract of the seeds of *Alpinia katsumadai* has an inhibitory activity against the ACAT enzyme of rat liver microsomes (71.6% inhibition at 100 μg/mL, 42.3% inhibition at 30 μg/mL). By HPLC, the active compounds **1** and **2** were eluted with retention time of 25 and 40 min, respectively. Of the two ACAT inhibitory compounds, compound **1** which exhibited greater inhibitory activity was further purified with preparative HPLC. The characteristics of the NMR data and other physicochemical data of the isolated compound were in concurrence with the chemical structure from previous reports.[24,25] Thus, compound **1** was identified as 2,3,22,23-tetrahydroxyl-2,6,10,15,19,23-hexamethyl-6,10,14,18-tetracosatetraene, an acyclic triterpenoid (Fig. 1) previously reported in the stem bark of *Ekebergia capensis*.

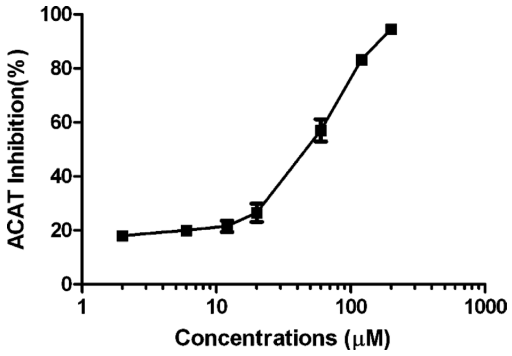

Fig. 2. Inhibition of Rat Liver Microsomal ACAT Activity by Compound **1**

The enzyme reaction was performed at 37°C for 30 min.

The inhibitory effect of compound **1** on microsomal ACAT enzyme activity was examined using the extract of rat liver microsomes. The compound **1** exhibited inhibitory activity against ACAT enzyme in a dose-dependent manner with an

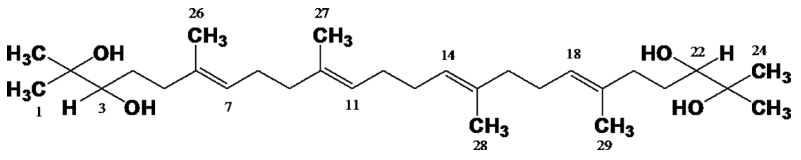

Fig. 1. Chemical Structure of Compound **1**

Table 3. Effects of Compound **1** on Cholesteryl Ester Formation in HepG2 Cells

Sample (μM)		Cholesteryl [1-^{14}C]oleate (nM/protein))
Compound **1**	12.5	688.77±11.32
	25	528.16±10.08
	50	342.47±7.64
	100	154.66±4.24
Pyripyropene A	2.6	333.63±7.32
(+)		1022.34±10.01

Cells were incubated with [1-^{14}C]oleic acid (33.4 μM) at varying concentrations of compound **1**. Pyripyropene was used as a positive control. (+): HepG2 cells+ [1-^{14}C]oleic acid, protein concentration: 1.85±0.082 mg/mL.

IC_{50} value of 47.9 μM (Fig. 2). This activity was compared with pyripyropene A as a positive control, which inhibited ACAT activity with an IC_{50} value of 70 nM.[26)]

Several studies have reported that blocking hepatic and intestinal cholesterol ester formation would lower the plasma levels of the atherogenic apolipoprotein B-containing lipoprotein.[27)] In order to identify the effect of compound **1** on cellular cholesterol esterification, the incorporation of [1-^{14}C]-oleic acid into cellular cholesterol was measured in an intact cell assay with the human hepatocarcinoma cell line HepG2, a suitable model to study liver cholesterol metabolism in human hepatocytes *in vitro*.[28)] The compound **1** inhibited cholesteryl [1-^{14}C]oleate synthesis in a dose-dependent manner with an IC_{50} value of 26 μM (Table. 3). This compound in HepG2 cell was almost twice as likely to inhibit the ACAT activity as in rat liver microsomes. The compound **1** did not cause any cytotoxic effects on HepG2 cells at the concentrations employed in this study (data not shown). These data indicate that the hypolipidemic activity of *A. katsumadai* among high fat diet mice may be related to these ACAT inhibitory effects, suggesting that the ACAT inhibition in the liver can be effective against hyperlipidemia or atherosclerosis. Although compound 1 has previously shown various pharmacological activities, including antiviral, antioxidant activity, and anti-emetic effect, this is the first report to reveal its ACAT inhibition activity.

In conclusion, after identifying the hypolipemic effect of eAKA on high fat diet mice, 2,3,22,23-tetrahydroxyl-2,6,10,15,19,23-hexamethyl-6,10,14,18-tetracosatetraene was purified from *A. kasumadai*, and was identified with inhibitory activity against ACAT in rat microsome and human HepG2 cell. This is the first report to demonstrate that an acyclic triterpenoid isolated from *A. katsumadai* has ACAT inhibition activity, and thus, providing a potential novel therapeutic approach for the treatment of hyperlipidemia and atherosclerosis. To date, we do not have direct evidence that eAKA inhibits ACAT activity *in vivo*. It seems likely that some of the hypolipemic effects of eAKA on high fat diet mice may be related to inhibition of ACAT. There has been a number of reports which indicate that the favorable hypolipemic activities were exhibited by ACAT inhibitors in animal models.[6,29)] Further experiments should be focused on determining biochemical mechanisms and *in vivo* significance of ACAT inhibition by eAKA.

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