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Original Article

Experimental Study of *Ginkgo-Chunghyul-dan* on Anti-oxidant, Anti-platelet Aggregation, and Anti-hyperlipidemic Activity

Sang-Pil Yun[1], Hyung-Sup Bae[2], Seong-Uk Park[2], Woo-Sang Jung[3], Sang-Kwan Moon[3], Jung-Mi Park[2], Chang-Nam Ko[2], Ki-Ho Cho[3], Young-Suk Kim[3]

[1]Saint Paul's Oriental Medical Center, Seoul, Korea
[2]Stroke and Neurological Disorders Center, Kyung-Hee East-West Neo Medical Center, Seoul, Korea
[3]Department of Cardiovascular and Neurologic(Stroke Center), College of Oriental Medicine, Kyung-Hee University, Seoul, Korea

Objectives : *Ginkgo-Chunghyul-dan* (GCHD) is newly developed herbal medicine to prevent and treat stroke. In this study, we investigated whether the GCHD had antioxidant activity and anti-platelet aggregation effect *in vitro* and hypolipidemic activities *in vivo*.
Methods : Anti-oxidant activity of GCHD was measured using the Blois method, anti-platelet effect of GCHD was assessed by the Born method, and hypolipidemic activities of GCHD were evaluated in corn oil- or Triton WR-1339-induced and cholesterol-fed rats.
Results : GCHD showed anti-oxidant activity in the study inhibiting the formation of 1-diphenyl-2-picrylhydrazyl radicals and xanthine oxidase activity. GCHD had anti-platelet aggregation activity. GCHD significantly lowered total cholesterol (TC), triglyceride (TG), and low density lipoprotein cholesterol (LDL-C) in high cholesterol diet and Triton WR-1339 induced model TG in corn oil-induced model. GCHD had no acute toxicity at a single dosage.
Conclusion : These results suggest that GCHD has the potential to treat hyperlipidemia and stroke.

Key Words : *Ginkgo-Chunghyul-dan* anti-oxidant activity; anti-platelet effect; hypolipidemic activities.

Introduction

The mortality of stroke and cardiovascular disease is increasing in Korea. Stroke and cardiovascular disease are the second and the third leading causes of mortality above 50 years old after cancer.[1)] Hyperlipidemia, hypertension, diabetes mellitus, and smoking are common risk factors for stroke and cardiovascular disease.[2,3)] Lowering cholesterol levels can reduce the mortality of cardiovascular disease to 20-30% and decrease the incidence of stroke up to 16-30%.[4-7)]

The incidence of hyperlipidemia is 11% in Korea.[8)] As our life expectancy grows and our diet and life-style change to higher calorie foods and sedentary lifestyles, it is likely to increase over time. Thus, it is quite necessary to treat hyperlipidemia.

HMG-CoA (3-hydroxy-3-methyl glutaryl-coenyme) reductase inhibitor (statin) has a therapeutic effect on lowering cholesterol levels, but many adverse effects have been reported like gastric disturbance, liver function change, myopathy and rhabdomyolysis. Furthermore, cessation of administrating statins causes elevation of cholesterol levels.[9)]

Chunghyul-dan (CHD) is made of extract of *Scutellariae Radix, Coptidis Rhizoma, Phellodendri*

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· Correspondence to : Sang-Pil Yun
Saint Paul's Oriental Medical Center, Jeonnong-dong, Dongdaemun-gu, Seoul, Korea.
Tel : +82-2-958-2429, Fax : +82-2-969-9244, E-mail : yunpaul@yahoo.co.kr

Cortex, Gardeniae Fructus, and *Rhei Rhizoma*. Previous studies revealed that it inhibited cholesterol biosynthesis, HMG-CoA reductase, pancreatic lipase, nitrite oxide synthesis, and scavenged free radicals.[10-12] In clinical studies, CHD had [minor] adverse effects like renal or hepatic toxicity, lowered serum cholesterol levels, and elevated blood pressure.[13-15]

GCHD is a newly developed prescription adding *Ginkgo Folium* to CHD. It has been known that *Ginkgo Folium* has effects for treating hyperlipidemia, improving blood circulation, and protecting brain cells.[16-19]

In this study, we performed experimental study *in vitro* and *in vivo* with rats to evaluate the anti-oxidant activity, anti-platelet aggregation activity, and anti-hyperlipidemic effect of GCHD in comparison with CHD. We also assessed the acute toxicity of GCHD at a single dosage with mice.

Materials & Methods

1. Materials

1) Chemicals and instruments

Xanthine, xanthine oxidase, bovine serum albumin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitro blue tetrazolium (NBT), 2,2'-azobis-(2-amidinopropane) dihydro chloride (AAPH), and Triton WR-1339 were purchased from Sigma Chemical (U.S.A.). Cholesterol was purchased from Yakuri Pure Chemical (Japan). Cholic acid was purchased from Fluka Biochemica (Switzerland). Total cholesterol, triglyceride, and HDL-cholesterol assay kits were from Asan Pharm. (Korea). LDL-cholesterol assay kit was from BioMerieux Chemical (France). We used lovastatin (20mg/tab) from ChoongWae Pharma (Korea) and fenofibrate (100mg/tab) from Kuhnil Pharm. (Korea). We used high performance liquid chromatography (HLPC) (Waters Co., U.S.A.), spectrophotometer (Shimadzu Co., Model UV-160A, Japan),prime automatic clinical chemistry analyzer (BPC Biosed, Italy), aggregometer (Chrono-log Corporation, U.S.A.), rotary vacuum evaporator (EYELA Co., Model NE-1, Japan), and freeze dryer (EYELA Co., Model FD-1, Japan).

2) Preparation of GCHD

Scutellariae Radix, *Coptidis Rhizoma*, *Phellodendri Cortex*, Gardeniae Fructus, Rhei Rhizoma, and *Gingko Folium* were purchased from Kyunghee Oriental Medical Hospital (Seoul, Korea) and identified by Dr. Nam-Jae Kim, East-West Medical Research Institute, Kyunghee Medical Center (Seoul, Korea). Voucher specimens were deposited at East-West Medical Research Institute, Kyunghee Medical Center (Seoul, Korea).

CHD is composed of *Scutellariae Radix*, *Coptidis Rhizoma*, *Phellodendri Cortex*, Gardeniae Fructus, and Rhei Rhizoma (4:4:4:1). CHD (510 g) was extracted twice with water or 80% ethanol (170 g) in boiling water for 2 hours.These extracts were filtered and evaporated in a rotary vacuum evaporator. Finally CHD (137.7 g, dry weight corresponding to 27%) was made after being lyophilized with a freezing dryer. To standardize the quality of CHD, berberine in *Coptidis Rhizoma* and *Phellodendri Cortex*, baicalin in *Scutellariae Radix*, and geniposide in Gardeniae Fructus were quantitatively assayed according to the previous methods.[20] The contents were 0.51% for berberine, 6.93% for baicalin, and 2.28% for geniposide.

Ginkgo Folium(1 kg) was extracted twice with 95% ethanol (5 L) in boiling water for 2 hours. These extracts were then filtered and evaporated by using the rotary vacuum evaporator, and then ethanol extract (205 g) (GE, dry weight corresponding to 20.5%) was made after being lyophilized. The ethanol extract was dissolved in distilled water, and that was extracted by 3 parts of saturated water n-BuOH, and then each part of n-BuOH layer was combined, and this n-BuOH layer was filtered and evaporated by using the rotary vacuum evaporator, and then n-BuOH fraction (50 g) (GEB, dry weight corresponding to 5%) was made by lyophilizing that n-BuOH. Subsequently, this n-BuOH fraction was dissolved in the distilled water, and that was fractionated by 3 parts of fraction with n-hexane, and then water layer without n-hexane was evaporated. Finally *Ginkgo Folium* fraction (42 g) (GEBW,

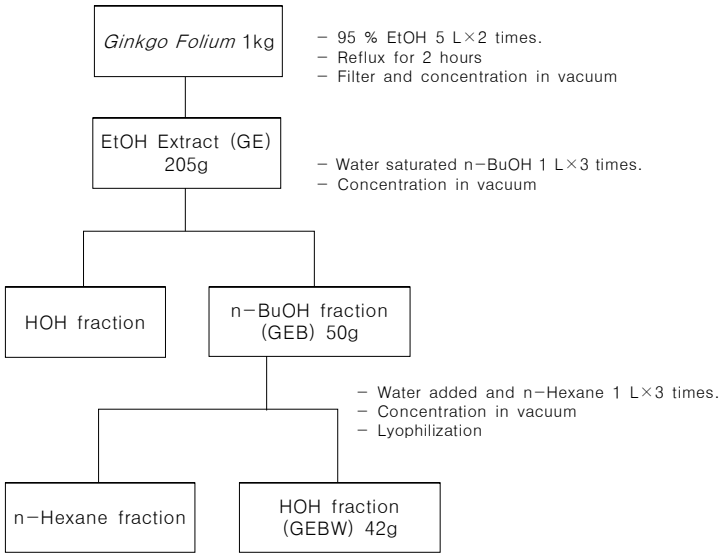

Fig. 1. Fractionation of Ginkgo Folium

dry weight corresponding to 4.3%) was made after being lyophilized (Fig.1). Water extract (320 g) (GW, dry weight corresponding to 32%) was used as a sample extracted from *Ginkgo Folium* which was added to the distilled water and filtered and evaporated by using the rotary vacuum evaporator. It was then lyophilized. GCHD is composed of CHD and GEBW (10:1).

3) Animals

Male and female ICR mice (about 30 g weight) and male Sprague-Dawley rats (about 250 g weight) were bought from Samtaco (Korea) and fed a commercial diet (Samyang, Korea). These animals were kept for at least 7 days before the experiments

2. Methods

1) Assay of anti-oxidant activity

Free radical scavenging activity was measured by DPPH using Blois' method.[21] A sample (4 ml) was added to the 1×10^{-4}M DPPH radical in methanol (1 ml) and the mixture was shaken and held for 30 minutes at room temperature. Absorbance was measured at 520 nm with a spectrophotometer.

The scavenging potential for superoxide anion was measured using the xanthine oxidase method.[22,23] A sample solution was added to the mixture (0.02 ml), consisting of 3 mM xanthine, 3 mM ethylenediaminetetraacetic acid (EDTA), 0.15% bovine serum albumin (BSA) and 0.24 mM NBT in 0.05 M sodium carbonate buffer (0.48 ml). The mixture (0.02 ml) was diluted with distilled water and held for 10 minutes at room temperature. Xanthine oxidase (6 mU) and $CuCl_2$ (0.02 ml) were added, followed by incubation in a shaking water bath at 25℃ for 20 minutes, the absorbance was monitored spectrophotometrically at 560 nm.

The inhibition of hemolysis caused by AAPH induced lipid oxidation in erythrocyte membranes was measured using Miki's method.[24] Blood was obtained from aortic vein of rat and collected into a heparinized tube. Erythrocytes were separated from the plasma and the buffy coat and then washed three times with 10 volumes of phosphate-buffered saline (PBS). During every wash, the erythrocytes were centrifuged at 1000 revolutions perminute (rpm) for 10 minutes to obtain a packed cell preparation. After the last wash, the packed erythrocytes were

suspended in 10 volumes of PBS. Erythrocyte oxidative hemolysis was induced by AAPH, a peroxyl radical indicator. Addition of AAPH to the suspension of washed erythrocytes induces the oxidation of membrane lipids and proteins, resulting in hemolysis. The erythrocyte suspension (0.2 ml) after pre-incubation for 5 minutes at 37℃ was mixed with 0.2 ml PBS containing varying amounts of samples. 0.2 ml of 100 mM AAPH in PBS was then added to the mixture. The reaction mixture was incubated for 3 hours at 37℃ with gentle shaking. After incubation, the reaction mixture was diluted with 20 volumes of PBS and centrifuged at 1000 rpm for 10 minutes. The antioxidant activity was measured at 540 nm using a spectrophotometer.

Hemolysis rate (%) =
(A_{540nm} of 0.15 M sodium chloride supernatant / B_{540nm} of distilled water supernatant)×100

The percent inhibition was calculated as follows:
% Inhibition = (H_{AAPH}-H_{sample}) / H_{AAPH}×100
Where: H_{sample} s the absorbance of the sample extracts and HAAPHis the absorbance of the control group.

In the present study, 2 steps were taken. First, *Ginkgo Folium* fractions (GF) were compared. Then, CHD and GCHD (the GF which proved to be the most effective to anti-oxidant activity was added to CHD) were compared.

2) Assay of anti-platelet aggregation activity

The platelet aggregation induced by adenosine diphosphatase (ADP) and collagen was assessed by Born's method.[25] Specimens of blood samples were collected using 2.2% sodium citrate at the ratio 1:9 with the blood in syringe. Samples of blood and anticoagulants were gently inverted up and down, avoiding shaking. Platelet rich plasma (PRP) was prepared by centrifuging at 1000 rpm for 10 minutes. Platelet poor plasma (PPP) was prepared by centrifuging at 3000 rpm for 30 minutes. Platelet aggregation studies in PRP were performed at 37°C with constant rate of stirring at 1200 rpm in the aggregometer using PPP as reference, and samples and control (distilled water) 10 μl were added to PRP. During the experiments the optical density was continuously recorded. After a stable baseline was observed for 3 minutes, 10 μl of an aggregation-inducing agent was added to 390 μl PRP. The following concentrations of aggregation inducing agents were used: 10 μg/ml collagen and 20 μM ADP.

In the current study, 2 steps were taken. First, each GF was compared. Then, CHD and GCHD (the GF which proved to be the most effective to anti-platelet aggregation activity was added to CHD) were compared.

3) Assay of anti-hyperlipidemic activity

To evaluate the anti-hyperlipidemic effect, three kinds of hyperlipidemic animal models were prepared. First, hyperlipidemic rat model by corn oil was prepared according to the method of Duhault *et al*.[26] Six rats were used per group. Corn oil (3g/kg) was orally administered 2 hours after each sample (CHD 400 mg/kg, GCHD 200 and 400 mg/kg, and fenofibrate 800 mg/kg) was orally administered. Each sample was orally administered once per experiment. At 2 hours after the administration of corn oil, blood sample of rats was drawn by cardiac puncture under ether anesthesia.

Second, hyperlipidemic rat model by Triton WR-1339 was prepared according to the method of Kusama *et al*.[27] Triton WR-1339 was injected at theend of the regular 16-hour fasting period as a 10% solution in saline at a dose of 200 mg/kg into the tail veins of rats under light ether anesthesia. Six rats were used per group. These rats were anesthetized with ether 18 hours after Triton WR-1339 injection and 1-1.5 ml blood was withdrawn by cardiac puncture. Tested samples (CHD 400 mg/kg, GCHD 200 and 400 mg/kg, and lovastatin 50 mg/kg) were administered orally once a day for 3 days. The final administrations of these samples were performed 1 hour before Triton WR-1339 injection.

Third, hyperlipidemic rat model by high cholesterol diets was prepared according to the method of Niiho *et al*.[28] Rats were divided into 5 groups (normal, control group, GCHD 200 and 400 mg/kg, and lovastatin 50 mg/kg administered group). Each group contained 6 rats.normal groupreceived a solid normal diet (rodent chow: 32% protein, 5% fat, 2% fiber, and 60% nitrogen extract) alone. The control group was fed on normal diet supplemented with 1% cholesterol, 0.25% cholic acid, and 2.5% olive oil (CCO) for 21 days. The GCHD 200 and 400 mg/kg, and lovastatin 50 mg/kg administered groups were fed on normal diet supplemented with CCO and orally administered GCHD 200 and 400 mg/kg, and lovastatin 50 mg/kg. After 24 hour fasting period from final administration of sample, blood samples of rats were drawn by cardiac puncture under ether anesthesia.

4) Determination of serum lipid levels

Serum was obtained from a portion of the blood sample by centrifugation at 3000 rpm for 30 minutes. All serum samples were stored at room temperature before analysis.

Serum total cholesterol (TC) and triglyceride (TG) were measured by the enzyme method designed by Allain *et al*. and Vanhadel and Zilversmit.[29,30] Serumdensity lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) were measured by the method designed by Mainard and Madec and Lopes *et al*.[31,32]

5) Acute toxicity

Toxicity study was carried out using female and male ICR mice. Mice were divided into 6 groups (5 GCHD administered groups and control group) with 5 males and 5 females in each group. GCHD was orally administered by gavage at a dosage of 312.5, 625, 1250, 2500, and 5000 mg/kg. The general behavior of the mice was observed continuously for 6 hours immediately after administration and then intermittently observed for a period of 14 days. The mice were further observed once a day for up to 14 days after treatment. General condition, any signs of toxicity, and death were also recorded. All mice weighed 4 times; before treatment and 3, 10, and 14 days after treatment. At the end of the experiment, mice were anesthetized under ether and sacrificed by cervical dislocation. The outward appearance and abnormality of the organs were carefully examined with the unaided eye. The weight of the heart, liver, spleen, and kidney were measured and recorded.[33]

6) Statistical analysis

All data were expressed as mean ± standard error and statistical significance was determined using Student's t-test. P-values less than 0.05 were considered as significant.

Results

1. Anti-oxidant activity of GF

The anti-oxidant activity of the sample was expr-

Table 1. Anti-oxidant activity of GF

Samples	IC_{50} (mg/mL)		
	DPPH*	Xanthine oxidase†	Hemolysis‡
GW	0.076	0.026	5.13
GE	0.087	0.024	5.42
GEB	0.050	0.011	2.22
GEBW	0.038	0.005	1.54

GW: water extract GE: EtOH extract GEB: n-BuOH fraction GEBW: HOH fraction
*: Absorbance of DPPH radical at 520 nm
†: Absorbance of NBT at 560 nm
‡: Absorbance of RBC at 540 nm

essed as IC_{50}. The IC_{50} value was defined as the concentration of extracts that scavenged the DPPH radicals, xanthine oxidase activity, and red blood cell (RBC) membrane hemolysis by 50%.

As shown in Table 1, IC_{50} of GEBW was 0.038, 0.005, and 1.54 mg/mL for the DPPH scavenging activity, the inhibition of xanthine oxidase activity, and that of RBC membrane hemolysis respectively. GEBW proved to be the most effective to anti-oxidant activity among GF.

2. Anti-oxidant activity of CHD and GCHD

As shown in Table 2, IC_{50} of CHD and GCHD were 0.048 and 0.021 mg/mL, respectively, for the inhibition of xanthine oxidase activity. GCHD proved to be more effective at inhibiting xanthine oxidase than CHD.

3. Anti-platelet aggregation of GF

The anti-platelet aggregation activity of the extract was expressed as IC_{50}. The IC_{50} value was defined as the concentration of extracts that inhibits platelet aggregation induced by ADP and collagen by 50%.

As shown in Table 3, IC_{50} of GEBW was 2.2 and 6.2 mg/mL for the inhibition of ADP and collagen induced platelet aggregation, respectively. GEBW proved to be the most effective at inhibiting the platelet aggregation induced by ADP and second most at inhibition of platelet aggregation induced by collagen among GF.

4. Anti-platelet aggregation of CHD and GCHD

As shown in Table 4, IC_{50} of CHD and GCHD were 25.5 and 22.4 mg/mL, respectively, for the inhibition of ADP induced platelet aggregation and

Table 2. Anti-oxidant activity of CHD and GCHD

Samples	IC_{50} (mg/mL)		
	DPPH*	Xanthine oxidase†	Hemolysis‡
CHD	0.037	0.048	1.31
GCHD	0.032	0.021	1.48

CHD: Chunghyul-dan GCHD: Ginkgo-Chunghyul-dan
*: Absorbance of DPPH radical at 520 nm
†: Absorbance of NBT at 560 nm
‡: Absorbance of RBC at 540 nm

Table 3. Anti-platelet aggregation activity of GF

Samples	IC_{50} (mg/mL)	
	ADP	Collagen
GW	7.2	5.8
GE	4.3	6.7
GEB	4.1	12.6
GEBW	2.2	6.2

GW: water extract GE: EtOH extract GEB: n-BuOH fraction GEBW: HOH fraction

Table 4. Anti-platelet aggregation activity of CHD and GCHD

Samples	IC_{50} (mg/mL)	
	ADP	Collagen
CHD	25.5	44.7
GCHD	22.4	34.9

CHD: Chunghyul-dan GCHD: Ginkgo-Chunghyul-dan

44.7 and 34.9 mg/mL, respectively, for that of collagen-induced platelet aggregation. GCHD proved to be more effective at the inhibition of platelet aggregation than CHD.

5. Anti-hyperlipidemic effects of CHD and GCHD in corn oil-induced hyperlipidemic rats

As shown in Table 5, there was no significant effect on serum level of TC 2 hours after treatment with corn oil (3g/kg). However, TG level increased by 230 mg/dL compared with normal. Conversely, when rats were administered CHD 400mg/kg and GCHD 400mg/kg, serum level of TG decreased significantly by 102.8 and 123.3 mg/dL, respectively, compared with the group with corn oil alone. There was no significant difference between the CHD 400 mg/kg and the GCHD 400 mg/kg groups.

6. Anti-hyperlipidemic effects of CHD and GCHD in Triton WR-1339-induced hyperlipidemic rats

As shown in Table 6, when rats were treated with Triton WR-1339 (200 mg/kg) for 18 hours, the amount of TG and TC increased by 741.3 and 172.9 mg/dL, respectively, compared with the normal group. In contrast, when rats were administered CHD 400 mg/kg and GCHD 400 mg/kg, serum levels of the CHD and the GCHD group markedly reduced by 249 and 267.1 mg/dL, respectively, for TG and 42.2 and 56.2 mg/dL,respectively for TC, compared with the group treated with Triton WR-1339 alone. There was no significant difference between the CHD and the GCHD groups, however GCHD had a tendency to lower serum TG and TC levels more than CHD.

7. Anti-hyperlipidemic effects of GCHD in cholesterol diet induced rats

As shown in Table 7, when rats were treated with a high cholesterol diet for 3 weeks, the amount of TC, TG, and LDL-C increased by 198.4, 24, and 111.3 mg/dL, respectively, compared with the normal group. In contrast, when rats were administered with GCHD 200, 400 mg/kg, and lovastatin 50 mg /kg, serum levels of lipoproteins in the GCHD 200

Table 5. Anti-hyperlipidemic effects of CHD and GCHD in 30% corn oil-induced hyperlipidemic rats

Groups	Dose (mg/kg, p.o.)	TG (mg/dL)	TC (mg/dL)
Normal	-	114.3±8.92	77.8±4.43
Control	-	344.3±46.2*	83.0±6.93
CHD	400	241.5±17.7†	75.2±6.71
GCHD	400	221.0±14.7†	81.7±5.31

CHD: Chunghyul-dan GCHD: Ginkgo-Chunghyul-dan
Values are mean ± standard error of 6 rats.
: Significantly different from the normal value (: $p < 0.001$)
†: Significantly different from the control value (†: $p<0.05$)

Table 6. Anti-hyperlipidemic effects of CHD and GCHD in Triton WR-1339-induced hyperlipidemic rats

Groups	Dose (mg/kg, p.o.)	TG (mg/dL)	TC (mg/dL)
Normal	-	163.0±12.3	80.3±10.1
Control	-	904.3±79.4 *	253.2±13.4*
CHD	400	655.3±71.1†	211.0±10.1†
GCHD	400	637.2±54.0†	197.0±19.3†

CHD: Chunghyul-dan GCHD: Ginkgo-Chunghyul-dan
Values are mean ± standard error of 6 rats.
: Significantly different from the normal value (: $p < 0.001$)
†: Significantly different from the control value (†: $p<0.05$)

Table 7. Anti-hyperlipidemic effects of GCHD in high cholesterol diet-induced hyperlipidemic rats

Groups	Dose (mg/kg, p.o.)	TC (mg/dL)	TG (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)
Normal	-	76.3±4.18	78.0±7.86	36.5±1.41	21.2±2.29
Control	-	174.7±10.0*	102.0±5.28†	20.0±2.02*	132.5±14.1*
GCHD	200	144.0±13.4	82.0±7.13‡	22.5±1.05	105.5±12.2
	400	139.0±9.31‡	74.7±8.01‡	26.0±1.62‡	94.2±8.08‡
Lovastatin	50	140.7±6.55‡	84.0±4.49‡	21.2±1.64	96.3±7.39‡

GCHD: Ginkgo-Chunghyul-dan
Values are mean ± standard error of 6 rats.
* and †: Significantly different from the normal value (*: $p<0.001$ and †: $p<0.05$)
‡: Significantly different from the control value (‡: $p<0.05$)

Table 8. Antihyperlipidemic effects of GCHD in 30% corn oil-induced hyperlipidemic rats

Groups	Dose (mg/kg, p.o.)	TC (mg/dL)	TG (mg/dL)
Normal	-	77.8±5.86	125.7±18.0
Control	-	91.7±4.03	335.7±26.6*
GCHD	200	84.5±5.94	256.3±24.8†
	400	85.5±5.77	246.8±25.3†
Fenofibrate	800	68.3±2.93‡	142.8±14.5‡

GCHD: Ginkgo-Chunghyul-dan
Values are mean ± standard error of 6 rats
*: Significantly different from the normal value (*: $p<0.001$)
†: Significantly different from the control value (†: $p<0.05$)
‡: Significantly different from the control value (‡: $p<0.001$)

mg/kg group reduced by 30.7, 20, and 27 mg/dL for TC, TG, and LDL-C, respectively, compared with the group treated with a high cholesterol diet. The GCHD 400 mg/kg and lovastatin 50 mg/kg groups significantly reduced by 35.7 and 34mg/dL, respectively, for TC, 27.3 and 18 mg/dL, respectively, for TG, and 38.3 and 36.2 mg/dL, respectively, for LDL-C, compared with the group treated with a high cholesterol diet. HDL-C level decreased by 16.5 mg/dL compared with the normal group. However, after treatment with GCHD 200, 400 mg/kg and lovastatin 50 mg/kg, the serum concentrations of HDL-C increased by 2.5, 6.0, and 1.2 mg/dL, respectively, compared with the group with a high cholesterol diet alone.

8. Anti-hyperlipidemic effects of GCHD in corn oil-induced rats

As shown in Table 8, there was no significant increase in serum level of TC 2 hours after treatment with corn oil (3 g/kg). However, TG level increased by 210 mg/dL, compared with normal. When rats were administered GCHD 200 or 400 mg/kg, or fenofibrate 800 mg/kg, TC level significantly decreased by 23.4 mg/dL only in the fenofibrate 800 mg/kg group, compared withthe group with corn oil alone. Serum levels of TG decreased significantly by 79.4, 88.9, and 192.9 mg/dL, respectively, compared with the group with corn oil alone. Fenofibrate 800 mg/kg was most effective at lowering serum TG levels.

9. Anti-hyperlipidemic effects of GCHD in Triton WR-1339-induced rats

As shown in Table 9, when rats were treated with Triton WR-1339 (200 mg/kg) for 18 hours, the amount of TC, TG, and LDL-C increased by 227.9, 767.3, and 81.6 mg/dL, respectively, compared with

Table 9. Anti-hyperlipidemic effects of GCHD in Triton WR-1339-induced hyperlipidemic rats

Groups	Dose (mg/kg, p.o.)	TC (mg/dL)	TG (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)
Normal	-	71.8±10.4	57.0±5.87	49.0±2.99	19.7±1.25
Control	-	299.7±16.8*	824.3±30.9*	39.8±0.72*	101.3±6.22*
GCHD	200	268.5±16.8	746.0±64.7	41.5±3.56	89.0±3.54
	400	227.3±22.2†	643.0±44.3‡	44.2±1.56†	80.0±6.72†
Lovastatin	50	232.3±18.4†	704.3±33.9†	46.5±2.91†	87.3±1.99†

GCHD: Ginkgo-Chunghyul-dan
Values are mean ± standard error of 6 rats
*: Significantly different from the normal value (*: $p < 0.001$)
†: Significantly different from the control value (†: $p<0.05$)
‡: Significantly different from the control value (‡: $p<0.01$)

the normal group. In contrast, when rats were administered with GCHD 200, 400 mg/kg, and lovastatin 50 mg/kg, serum levels of lipoproteins in the GCHD 200 mg/kg group reduced by 31.2, 78.3, and 12.3 mg/dL for TC, TG, and LDL-C, respectively, compared with the group treated with Triton WR-1339 alone. The GCHD 400 mg/kg and the lovastatin 50 mg/kg group significantly reduced by 72.4 and 67.4 mg/dL, respectively, for TC, 181.3 and 120 mg/dL, respectively, for TG, and 21.3 and 14 mg/dL, respectively, for LDL-C, compared with the group treated with Triton WR-1339 alone. HDL-C level was decreased by 9.2 mg/dL, compared with normal group. However, after treatment with GCHD 200, 400 mg/kg and lovastatin 50 mg/kg, the serum concentrations of HDL-C increased by 1.7, 4.4and 6.7 mg/dL, respectively, compared with the group treated with Triton WR-1339 alone.

10. Acute toxicity of GCHD

Oral administration of GCHD in a dose of 312.5 to 5000 mg/kg did not produce behavioral changes

Table 10. Survival time and body weight changes in mice orally administered with single dosage of GCHD (5 male and 5 female mice per group)

Sex	Dose (mg/kg)	Days after administration					
		Number of deaths		Body weight (g)			
		7	14	0	3	10	14
Male	-	0	0	31.8±0.82	32.8±0.96	35.2±0.82	36.2±1.08
	312.5	0	0	30.4±0.57	31.8±0.74	34.0±0.71	35.8±1.08
	625	0	0	30.6±0.27	32.4±0.57	34.6±0.57	35.4±0.45
	1250	0	0	30.6±0.84	31.2±0.74	34.2±1.08	35.0±1.17
	2500	0	0	31.2±0.42	31.8±0.74	34.8±0.89	36.6±0.97
	5000	0	0	31.4±0.57	32.6±0.67	35.2±0.74	37.2±0.96
Female	-	0	0	25.2±0.74	26.4±0.84	27.6±0.97	28.4±0.76
	312.5	0	0	26.2±0.42	26.8±0.65	28.2±0.74	29.0±0.50
	625	0	0	25.2±0.89	25.8±1.24	26.6±1.30	26.4±1.40
	1250	0	0	25.0±0.61	26.2±0.65	26.6±0.76	27.2±0.96
	2500	0	0	23.6±0.82	23.8±0.72	26.1±0.61	29.4±0.71
	5000	0	0	25.6±1.04	26.8±0.42	27.6±0.76	28.0±0.79

Values are mean ± standard error

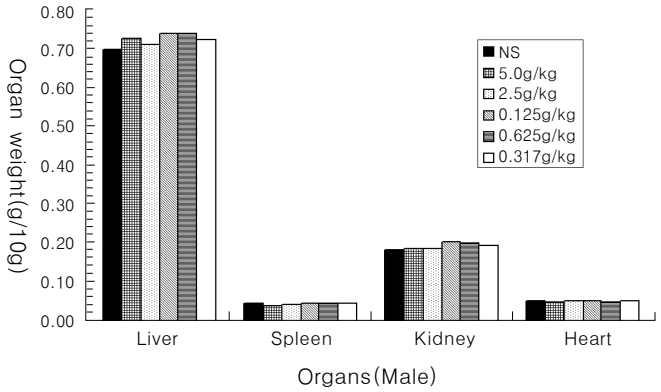

Fig. 2. Organ weight change in male mice orally administered with single dose of GCHD.

nor did death of mice occur (Table 10). Autopsy study with naked eye showed no visible change. There was no significant difference in body weight and organ weight after the oral administration of GCHD 312.5 to 5000 mg/kg (Table 10, Fig. 2, 3). These effects were observed during the experimental period. These results showed that the medium lethal dose (LD50) was higher than 5000 mg/kg for male and female mice

Discussion

The purpose of this study was to develop new herbal medicine having a therapeutic effect on prevention of stroke through the mechanisms of scavenging free radicals, inhibiting platelet aggregation, and lowering cholesterol levels. Thus, we investigated anti-oxidant activity, anti-platelet aggregation activity, and anti-hyperlipidemic activity of GCHD in comparison with CHD.

First, we performed comparatively studies with GW, GE, GEB, and GEBW to find which *Ginkgo Folium* fraction was the most effective to anti-oxidant and anti-platelet aggregation activity. GEBW proved to be the most effective, so we made GCHD by adding GEBW to CHD.

Second, we compared GCHD with CHD for effects on inhibiting cholesterol biosynthesis, HMG-

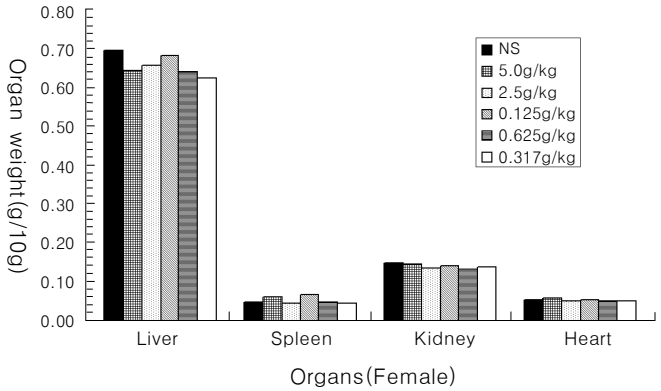

Fig. 3. Organ weight change in female mice orally administered with single dose of GCHD.

CoA reductase, pancreatic lipase, nitrite oxide synthesis, and scavenging free radicals to evaluate anti-oxidant activities.[10-12] GCHD showed a more potent xanthine oxidase inhibiting activity than CHD. This result suggests the possibility that GCHD could be used for the treatment and prevention for stroke because oxygen-derived free radicals seem to be involved in the pathophysiology of cerebral ischaemia as well as in reperfusion[34], and agents with a capacity to scavenge their action proved to be effective to the recovery of stroke.[35,36]2

We also conducted platelet aggregation study with GCHD and CHD. Platelet activation plays a multifactorial role in the production of cerebral ischemia. Platelet activation contributes to the formation of atherosclerosis of the carotid artery.[37] During the acute stage of ischemic cerebrovascular disease, a significant increase in platelet aggregation occurs.[38,39] Ischemic damage may be propagated following an acute ischemic event as platelet aggregates may contribute to ongoing ischemic damage.[40] Determination of platelet aggregation by aggregometers involves adding an aggregating agent to plasma-containing platelets. The aggregating agents used in this study included ADP and collagen. ADP acts on platelets via ADP receptor and collagen acts via the combined glycoprotein Ia/IIa receptor.[41] When high concentrations of ADP and collagen are present, platelet aggregability is increased. In this study, GCHD had a more anti-platelet aggregation effect than CHD. This result suggests the possibility that GCHD could be used for stroke prevention because many anti-platelet agents have been used for treatment and prevention of stroke.[42-44]

We also investigated which one was more effective in lowering serum cholesterol levels between GCHD and CHD. Though there was no significant difference in lowering serum cholesterol levels between CHD and GCHD in corn oil and Triton WR-1339-induced hyperlipidemic models, GCHD had a tendency to reduce the serum cholesterol levels more than CHD. Thus, GCHD could be used as an agent for treating hyperlipidemia and preventing stroke because CHD is used for lowering serum cholesterol levels and prevention of stroke. We further investigated whether GCHD had anti-hyperlipidemic potential at the level of serum lipids in high cholesterol diet-, corn-oil-, and Triton-WR 1339-induced rats, too. Triton WR-1339 is one of the many well-known non-ionic detergents that induce increases in plasma cholesterol and TG by increasing hepatic cholesterol biosynthesis. The mechanisms underlying this phenomenon have been suggested to be the consequence of the trapping of cholesterol and TG in the blood compartment and the subsequent reduction of the influx of plasma in the liver.[45,46] Corn oil contains abundant oleic acid and linoleic acid, which are known to influence plasma TG levels in mammals.[47]

In the present study, the results showed that GCHD 400 mg/kg significantly reduced TG, TC, and LDL-C levels in high cholesterol diet-, corn oil-, and Triton WR-1339-induced hyperlipidemic rats, indicating that GCHD was a potent anti-hyperlipidemic agent.

With respect to the hypolipidemic activity of GCHD, there are two possible explanations. One is a synergistic effect of the six components. *Scutellariae Radix* has anti-oxidant activity by DPPH radical scavenging activity[48,49] and anti-hyperlipidemic activity by inhibiting HMG-CoA reductase and pancreatic lipase.[10] *Coptidis Rhizoma* has anti-hyperlipidemic activity by reducing lipid peroxidation[50] and inhibiting HMG-CoA reductase and pancreatic lipase.[10] *Phellodendri Cortex* has anti-thrombic activity.[51] Gardeniae Fructus has anti-hyperlipidemic activity by inhibiting pancreatic lipase.[52] Rhei Rhizoma has anti-oxidant activity by direct scavenging of nitric oxide[53] and anti-hyperlipidemic activity by inhibiting HMG-CoA reductase and pancreatic lipase.[10] *Ginkgo Folium* has anti-oxidant activity by scavenging free radicals, anti-platelet aggregation activity,and anti-hyperlipidemic effect by reducing oxidized LDL-C and circulating free cholesterol level.[54-57] The other explanation is that the hypolipidemic activity of GCHD is involved

with the inhibition of cholesterol synthesis and interference with lipoprotein distribution through anti-oxidant activity of GCHD.

In the current study, LDL-C level was dose-dependently decreased in the GCHD administered groups in high cholesterol diet hyperlipidemic rats, and GCHD 400 mg/kg was better to lower LDL-C level than lovastatin 50 mg/kg. This result suggested the possibility that GCHD 400 mg/kg could be used for treating hyperlipidemia and preventing stroke. This possibility can be explained by the fact that LDL-C level is a target therapy point for hyperlipidemia, elevated LDL-C is associated with increasing the incidence of stroke,58-61 lowering LDL-C level can prevent the attack and recurrence of stroke[62] and statin appears to be associated with significant reduction in stroke risk.[4,6,63]

There was no acute toxicity at a single dosage in the present study; GCHD proved to be a safe drug.

In conclusion, GCHD had anti-oxidant, anti-platelet aggregation and anti-hyperlipidemic activities. GCHD was a safe and effective herbal medicine used for treating hyperlipidemia and preventing stroke. But, in this study, there was some limitation, as this was *in vitro* and *in vivo* experimental study, so further research will be needed to be undertaken to elucidate the biochemical mechanism of anti-oxidant, anti-platelet aggregation, and hypolipidemic activity of GCHD and clinical study will be needed. Further study will also be needed to determine GF dose.

Conclusions

1. GCHD proved to have anti-oxidant activity in the study inhibiting the formation of DPPH radicals, xanthine oxidaseactivity, and RBC membrane hemolysis.
2. GCHD proved to have anti-platelet aggregation activity in ADP and collagen-induced platelet aggregation study.
3. GCHD 400 mg/kg significantly lowered TC, TG, and LDL-C levels in high cholesterol diet-induced model.
4. GCHD 400 mg/kg lowered TG level in corn oil-induced model.
5. GCHD 400 mg/kg significantly lowered TC, TG, and LDL-C levels in Triton WR-1339-induced model.
6. GCHD had no acute toxicity at a single dosage.

References

1. Annual report on the cause of death statistics in 2005. Korean National Statistical Office, 2006.
2. D'Agostino RB, Russell MW, Huse DM, Ellison RC, Silbershatz H, Wilson PWF *et al*. Primary and subsequent coronary risk appraisal: new results from the Framingham study. Am Heart J. 2000;139:272-81.
3. Sacco RL. Pathogenesis, classification and epidemiology of cerebrovascular disease. In Rowland (ed): Merritt's Neurology 10th ed, Philadelphia: Lippincott Williams & Wilkins; 2000, p.217-29.
4. Lewis SJ, Moye LA, Sacks FM, Johnstone DE, Timmis G, Mitchell J *et al*. Effect of pravastatin on cardiovascular events in older patients with myocardial infarction and cholesterol levels in the average range. Results of the Cholesterol and Recurrent Events (CARE) trial. Ann Intern Med. 1998;129:681-9.
5. Downs JR, Clearfield M, Weis S, Whitney E, Shapiro DR, Beere PA *et al*. Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study. JAMA. 1998;279:1615-22.
6. Scandinavian Simvastatin Survival Study Group. Randomized trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). Lancet. 1994;344:1383-9.

7. The Stroke Prevention by Aggressive Reduction in Cholesterol Levels (SPARCL) Investigators, High-dose atorvastatin after stroke or transient ischemic attack. N Engl J Med. 2006;355:549-59.
8. Kim JG, Song JH, Cho HI, Kim SI. Prevalence of hyperlipidemia and other risk factors associated with coronary artery disease in Koreans. Korean Clinical Pathology. 1991;11:341-7.
9. Insull W, Kafonek S, Goldner D, Zieve F. Comparison of efficacy and safety of atorvastatin (10mg) with simvastatin (10mg) at six weeks. ASSET Investigators. Am J Cardiol. 2001;87 :554-9.
10. Kim YS, Jung EA, Shin JE, Chung JC, Yang HK, Kim NJ *et al*. Daio-Orengedokuto inhibits HMG-CoA reductase and pancreatic lipase. Biol. Pharm. Bull. 2002;25:1442-5.
11. Yang HK, Kim YS, Bae HS, Cho KH, Shin JE, Kim NJ *et al*., Rhei Rhizoma and chunghyuldaninhibit pancreatic lipase. Natural Product Sciences. 2003;9:38-43.
12. Cho KH, Kim YS, Bae HS, Moon SK, Jung WS, Park EK *et al*., Inhibitory effect of chunghyuldan on prostaglandin E2 and nitric oxide biosynthesis of lipopolysaccharide-induced RAW 264.7 cells. Biol. Pharm. Bull. 2004;27:1810-3.
13. Cho KH, Jung WS, Park SU, Moon SK, Kim YS, Bae HS. Clinical assessment on the safety of chunghyul-dan. J Korean Oriental Med. 2003; 24:45-50.
14. Cho KH, Kang HS, Jung WS, Park SU, Moon SK. Efficacy and safety of chunghyul-dan (qingwie-dan) in patients with hypercholesterolemia. Am J Chin Med. 2005;33:241-8.
15. Yun SP, Jung WS, Park SU, Moon SK, Ko CN, Cho KH *et al*. Anti-hypertensive effect of chunghyul-dan (qingxue-dan) on stroke patients with essential hypertension. Am J Chin Med. 2005; 33:357-64.
16. Agnoli A, Rapin Jr, Scapagnini V. Effects of Ginkgo biloba extract on organic cerebral impairment. Proceedings of the International Symposium. London: John Libbey & Company Ltd; 1985, p. 1-106.
17. Spinnewyn B, Blavet N, Clostre F. Effects of Gingko biloba extract on a cerebral ischemia model in gerbils. Presse Med. 1986;15:1511-5.
18. Kleijnen J and Knipschild P. Ginkgo biloba. Lancet. 1992;340:1136-9.
19. Francis V. Defeudis. Bilobalide and neuroprotection. Pharmacol. Res. 2002;46:565-8.
20. Hayakawa J, Noda N, Yamada S, Mikami E, Uno K, and Yakugadu Z. Studies on physical chemical quality evaluation of crude drugs preparations. III. Analysis of gardenia fruits and its preparations. Yakugaku Zasshi. 1986;105:996-1000.
21. Blois MS. Antioxidant determination by the use of a stable free radical. Nature. 1958;181:1199-1200.
22. Okamura, H., Mimura, A., Yakou, Y., Niwano, M., Takahashi, Y.activity of tannins and flavonoids in Eucalyptus Phytochemistry. 1993;33: 557-61.
23. Amakura Y, Umino Y, Tsuji S, Ito H, Hatano T, Yoshida T *et al*. Constituents and their antioxidative effects in eucalyptus leaf extract used as a natural food additive. Food Chemistry. 2002;77:47-56.
24. Miki, M, Tamai H, Mino M, Yamamoto Y, Niki E. Free-radical chain oxidation of rat red blood cells by molecularand its inhibition by α-tocopherol. Arch. Biochem. Biophys. 1987;258:373-80.
25. Born GV and Cross MJ. The aggregation of blood platelets. J. Physiol. 1963;168:178-95.
26. Duhault J, Boulanger M, Beregi L, Sicot N, Bouvier F. 780 SE: A new type of hyperlipidemic agent. Comparative assay in rats. Atherosclerosis. 1976;23:63-72.
27. Kusama H, Nishiyama M, Ikeda S. Pharmacological investigation of bezafibrate, a hypolipidemic

agent (I). Effect of bezafibrate on normal and experimental hyperlipidemia in rats. Folia Pharmacol. Japon. 1988;92:175-80.

28. Niiho H, Yamazaki T, Nakajima Y, Itoh H, Takeshita T, Kinjo J *et al.*: Pharmacological studies on Puerariae Flos. II. The effects of Puerariae Flos on alcohol-induced unusual metabolism and experimental liver injury in mice. Yakugaku Zasshi. 1990;110:604-11. Clin. Chem. 1974;20: 470-5.
29. Allain CC, Poon LS, Chan CS, Richmond W, Fu PC. Enzymatic determination of total serum cholesterol.
30. Van Handel E and Zilversmit DB. Micromethod for the direct determination of serum triglycerides. J Lab Clin Med. 1957;50:152-7.
31. Mainard F and Madec Y. Cholesterol, phospholipid and apoB composition of LDL: Comparison of precipitation and ultracentrifugation methods. Ann. Biol. Clin. 1986;44:618-23.
32. Lopes-Virella MF, Stone P, Ellis S, Colwell JA. Cholesterol determination in high density lipoproteins separated by three different methods. Clin. Chem. 1977;23:822-4.
33. Twaij HA, Kery A, Al-Khazraji NK. Some pharmacological, toxicological and phytochemical investigations on Centaurea Phyllocephala. J. Ethnopharmacol. 1983;9:299-314.
34. SiesjöBK, Agardh C-D, Bengtsson F. Free radicals and brain damage. Cerebrovasc. Brain Metab. Rev. 1989;1:165-211.
35. Yamaguchi T, Sano K, Takakura K, Saito I, Shinohara Y, Asano T.Ebselen in acute ischemic stroke: A placebo-controlled double-blind clinical trial. Ebselen Study group. Stroke 1998;29:12-7.
36. Lees KR, Sharma AK, Barer D, Ford GA, Kostulas V, Cheng YF *et al.* Tolerability and pharmacokinetics of the nitrone NXY-059 in patients with acute stroke. Stroke. 2001;32:675 -80.
37. Fateh-Moghadam S, Li Z, Ersel S, Reuter T, Htun P, Plockinger U *et al.* Platelet degranulation is associated with progression of intima-media thickness of the common carotid artery in patients with diabetes mellitus type 2. Arterioscler. Thromb. Vasc. Biol.2005;25:1299-1303.
38. Weksler BB: Platelet function and antiplatelet therapy in ischemic cerebrovascular disease; in Barnett HJM (ed): Stroke: Pathophysiology, Diagnosis and Management. New York, Churchill Livingstone, 1992, pp 913-7.
39. Akyuz A, Bolayir E, Dener S, Topalkara K, Topaktas S. The effect of aspirin, ticlopidine and their low-dose combination on platelet aggregability in acute ischemic stroke: a short duration follow-up study. Eur J Neurol. 1999;6:57-61.
40. Danton GH, Prado R, Truettner J, Watson BD, Dietrich WD. Endothelial nitric oxide synthase pathophysiology after nonocclusive common carotid artery thrombosis in rats. J. Cereb. Blood Flow Metab. 2002;22:612-9.
41. Nieuwenhuis HK, Akkermen JWN, Houdijk WPM, Sixma JJ. Human blood platelets showing no response to collagen fail to express surface glucoprotein Ia. Nature. 1985;318:470-2.
42. Chinese Acute Stroke Trial (CAST) Collaborative Group. Randomised placebo-controlled trial of early aspirin use in 20,000 patients with acute ischaemic stroke. Lancet. 1997;349:1641-9.
43. Caro JJ and Migliaccio-Walle K. Generalizing the results of clinical trials to actual practice: The example of clopidogrel therapy for the prevention of vascular events. CAPRA (CAPRIE Actual Practice Rates Analysis). Clopidogrel versus Aspirin in patients at risk of ischemic events. Am J Med. 1999;107:568-72.
44. Antithrombotic Trialists'Collaboration. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. BMJ. 2002;324:71-86.
45. Friedman M and Byers SO. The mechanism resp-

onsible for the hypercholesteremia induced by Triton WR-1339. J. Exp. Med. 1953;97:117-30.
46. Hirsch RL and Kellner A. The pathogenesis of hyperlipidemia induced by means of surface-active agents. I. Increased total body cholesterol in mice given Triton WR-1339 parenterally. J. Exp. Med. 1956;104:1-13.
47. Toda T and Oku H. Effect of medium-chain fatty acids on cholesterolemia and atherosclerosis in Japanese quails. Nutr. Res. 1995;15:99-113.
48. Okawa M, Kinjo J, Nohara T, Ono M. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of flavonoids obtained from some medicinal plants. Biol. Pharm. Bull. 2001;24: 1202-5.
49. Huang WH, Lee AR, Yang CH. Antioxidative and anti-Inflammatory activities of polyhydroxyflavonoids of Scutellaria Baicalensis GEORGI. Biosci Biotechnol. Biochem. 2006 ;70:2371-80.
50. Yokozawa T, Ishide A, Cho EJ, Nakagawa T. The effects of *Coptidis Rhizoma* extract on a hypercholesterolemic animal model. Phytomedicine. 2003;10:17-22.
51. Chang GT, Min SY, Kim JH, Kim SH, Kim JK, Kim CH. Anti-thrombic activity of Korean herbal medicine, Dae-Jo-Whan and its herbs. Vascul. Pharmacol. 2005;43:283-8.
52. Lee IA, Lee JH, Baek NI, Kim DH Antihyperlipidemic effect of crocin isolated from the fructus of Gardenia Jasminoides and its metabolite Crocetin. Biol. Pharm. Bull. 2005;28:2106-10.
53. Yokozawa T, Chen CP, Tanaka T. Direct scavenging of nitric oxide by traditional crude drugs. Phytomedicine. 2000;6:453-63.
54. Hibatallah J, Carduner C, Poelman MC. In-vivo and in-vitro assessment of the free-radical-scavenger activity of Ginkgo flavone glycosides at high concentration. J. Pharm. Pharmacol. 1999; 51:1435-40.
55. Dutta-Roy AK, Gordon MJ, Kelly C, Hunter K, Crosbie L, Knight-Carpentar T *et al*. Inhibitory effect of Ginkgo biloba extract on human platelet aggregation. Platelets. 1999;10:298-305.
56. Yao ZX, Han Z, Drieu K, Papadopoulos V. Ginkgo biloba extract (Egb 761) inhibits beta-amyloid production by lowering free cholesterol levels. J. Nutr. Biochem. 2004;15:749-56.
57. Kudolo GB, Delaney D, Blodgett J. Short-term oral ingestion of Ginkgo biloba extract (EGb 761) reduces malondialdehyde levels in washed platelets of type 2 diabetic subjects. Diabetes Res. Clin. Pract. 2005;68:29-38.
58. Benfante R, Yano K, Hwang LJ, Curb JD, Kagan A, Ross W. Elevated serum cholesterol is a risk factor for both coronary heart disease and thromboembolic stroke in Hawaiian Japanese men. Implications of shared risk. Stroke. 1994;25:814 -20.
59. Byington RP, Davis BR, Plehn JF, White HD, Baker J, Cobbe SM *et al*. Reduction of stroke events with pravastatin: the Prospective Pravastatin Pooling (PPP) Project. Circulation. 2001; 103:387-92.
60. Iso H, Jacobs DR Jr, Wentworth D, Neaton JD, Cohen JD. Serum cholesterol levels and six-year mortality from stroke in 350,977 men screened for the multiple risk factor intervention trial. N Engl J Med.. 1989;320:904-10.
61. Plutzky J and Ridker PM. Statins for stroke: the second story? Circulation. 2001;103:348-50.
62. Amarenco P, Labreuche J,Lavallee P, Touboul PJ. Statins in stroke prevention and carotid atherosclerosis: systemic review and up-to-date meta-analysis. Stroke. 2004;35:2902-9.
63. The Long-term Intervention with Pravastatin in Ischemic Disease (LIPID) study Group. Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range or initial cholesterol levels. N. Engl. J. Med. 1998; 339:1349-57.