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Effects of citrus aglycone flavonoids, hesperetin and naringenin, on triacylglycerol metabolism in hamsters fed with a cholesterol diet

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Running title: Hypotriglyceridemic effect of hesperetin on hyperlipidemic hamster

SUMMARY

Effects of hesperetin and naringenin on the concentration of triacylglycerol in the serum and liver were studied in male golden hamster fed with the semipurified diet containing at 1% level of them for 3 weeks. The concentration of triacylglycerol in serum of the naringenin group decreased by 31%, whereas that in liver increased by 37% compared to the control group. The concentration of triacylglycerol in the serum and liver of the hesperetin group was slightly lower than the control group. The activity of microsomal phosphatidate phosphohydrolase in the liver, which is a key enzyme for biosynthesis of triacylglycerol, was significantly inhibited in the hesperetin group, whereas it was not affected in the naringenin group. The effect of hesperetin on phosphatidate phosphohydrolase was also measured *in vitro*. Hesperetin decreased the activity of phosphatidate phosphohydrolase with a dose-dependent manner. Both naringenin and hesperetin did not statistically affect the daily food consumption, body weight, liver weight, and total cholesterol in the serum. The observation accounts for the hypotriglyceridemic effect of hesperetin in the hyperlipidemic hamster.

Key words : Hesperetin; Naringenin; Triacylglycerol; Hamster; Phosphatidate Phosphohydrolase (PAP).

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INTRODUCTION

Hypertriglyceridemia is often found to be a risk factor for the premature development of atherosclerosis (Manninen *et al.*, 1992; Assamann and schulth 1992). The recently revised guidelines in Europe and the USA have focused attention on triacylglycerol (TG) and HDL-cholesterol as risk factors for coronary heart disease (Anonymous, 1992; 1993). Many drugs used for reduction of the plasma lipid level, some of the food ingredients, and some kinds of chemicals have more attention due to their possibility as hypolipidemic agents (Yanagita *et al.*, 1996; Yotsumoto *et al.*, 1997). Recent studies have shown that flavonoids in the diet were inversely associated with death from heart disease (Hertog, 1993). Relatively higher concentration of flavonoids are found in fruits, fruit juice, and vegetable (Kuhnan, 1976; Mouly *et al.*, 1994). It was reported that the amount of mixed flavonoids as a part of diet taken by adult American is about 1g/day from daily diet (Kuhnan, 1976). Flavanones, one of the most important groups of flavonoids, are the

most abundant components in the fruit of most citrus species which are composed mainly of hesperidin, hesperetin, naringin, and naringenin (Kuhnan, 1976; Mouly *et al.*, 1994). These flavanones have multiple pharmacological functions, such as capillary permeability and fragility-lowering effect (Kuhnan, 1976; Kimura *et al.*, 1981; Middeton and Kanandaswami, 1992), antioxidant (Chen *et al.*, 1990), fatty liver prevention (Cha *et al.*, 1999), autophagy and endocytosis protection (Gordon *et al.*, 1995), and lipase inhibition (Kawaguchi *et al.*, 1997). It has also been reported that these flavanones have hypotriglyceridemic and hypocholesterolemic effects in experimental animals (Choi *et al.*, 1991; Ikeda *et al.*, 1992; Hisatomi *et al.*, 1995). However, any other beneficial effect of those on lipid metabolism in hamsters with far-reaching analogies to man in lipid metabolism have not been well studied (Singhal *et al.*, 1982). In this study, two major flavanone-aglycone flavonoids, hesperetin and naringenin, were selected to evaluate their effect on the lipid metabolism in the harmster fed with a diet containing cholesterol. The effects of these flavanones on the phosphatidate phosphohydrolase (PAP) was examined *in vitro* to study on the biosynthesis of TG in the liver as a determinant of TG level in the plasma.

MATERIALS AND METHODS

Chemicals

Naringenin and hesperetin were provided by Nihon Shinyaku Ind. (Kyoto, Japan) and their purity was more than 97% through HPLC analysis. L-a-Phosphatidic acid and phosphatidylcholine sodium salts (98%, from Egg Yolk Lecithin) purchased from Sigma Chemical Co. (USA). Vitamin (AIN 93) and mineral mixtures (AIN 93) were purchased from Nihon Nosan Koygo (Tokyo, Japan). All other chemicals and reagents were of the best commercial grade possible.

Diet and animal experiment

Male golden hamsters were purchased from Kuydo Experimental Animal Co. (Tosu, Japan) and housed individually in suspended wire-mesh stainless steel cages in a temperature-controlled room (21-24℃) with a 12 h light/dark cycle (07:00-19:00) and maintained commercial chow diet for a week before the experiment started. The experimental diets were prepared by supplementation of the basal control diet with 1.0% level of naringenin and hesperetin. Each flavonoid was added to the diet at the expense of sucrose. The basal diet used in these studies contained 20% casein, 15% corn starch, 10% fat, 3.5% mineral mixture (AIN 93), 1% vitamin mixture (AIN 93), 0.2% choline bitartrate, 0.5% cholesterol, and 0.125% sodium cholate. Food and water were provided ad libitum for 3 weeks. Body weight was recorded every other

Lipid analysis

At the end of the treatment period, the animals were killed by decapitation between 8:00 and 9:00 am. Livers were removed, weighed, and kept frozen at -80℃ until analysis. Lipids were extracted from liver and purified using the method of Folch *et al.*(1957). Total cholesterol and TG in the lipid extract was measured by the method of Sperry and Webb and Fletcher, respectively. The serum was separated from the blood by centrifugation at 3,000 rpm for 15 min. Total cholesterol and TG in the serum were measured enzymatically by using commercial kits (Cholesterol E-Test and Triglyceride E-Test, respectively, from Wako Pure Chemical Ind., Osaka, Japan)

Preparation of liver microsomal fractions.

The fresh livers were excised and transferred to an ice-cold buffer solution containing 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 1 mM EDTA, and 0.2 mM dithiothreitol

(buffer-A solution). The livers were homogenized with 4 volumes of buffer-A and centrifuged at 20,000×g for 20 min at 4℃. The supernatant was filtered through nylon gauze, and then centrifuged at 105,000×g for 45 min at 4℃. The supernatant was collected as the cytosolic fraction. The pellet was gently homogenized with a small volume of buffer-A solution and used as the microsomal fraction. The fraction was used immediately or frozen at −80℃ for further assay. Protein concentration was assayed by the method of Lowry *et al.* (1951) using bovine serum albumin as.

Assay of enzyme activity

The activity of phosphatidic acid phosphohydrolase (EC 3.1.3.4) was assayed by a modified method of Walton and Possmayer. Reaction mixture contained 0.05 M Tris-HCl (pH 7.0), 1 mM phosphatidic acid, 1 mM phosphatidylcholine (prepared by sonication for 10 min in 0.9% NaCl with a model Branson Sonifier 250 (Branson, U. S. A.), 1.25 mM Na2-EDTA, and 50 to 100 ㎎ crude liver enzyme in a final assay volume of 0.2 ㎖. The mixtures were incubated for 15 min at 37℃ and the reaction was terminated by the addition of 0.8 ㎖ of a solution containing 0.13% sodium dodecyl sulfate, 1.25% ascorbic acid, 0.32% ammonium molybdate-4 H_2O, and 0.75 N H_2SO_4. The phosphomolybdate color was developed at 45℃ for 20 min (Chen *et al.*, 1956) and the absorbance was determined at 820 nm. The release of non enzymatic phosphate was determined with inactivated enzymes by boiling for 1 min. This assay method gave the good correlation between the activity and incubation time, or protein concentration. PAP assay *in vitro* was measured in various concentrations of hesperetin dissolved in dimethyl sulfoxide.

Statistical analyses

All values are presented as means ± SEM. Data were analyzed by one way ANOVA, followed by inspection of all differences by Duncan's new multiple-range test (Duncan, 1957). Differences were considered significant at $p<0.05$.

RESULTS

The effects of hesperetin and naringenin on the daily food intake, body weight, and liver weight in hamsters are shown in Table 1. No significant differences of daily food intake, body weight, and liver weight were observed in hamsters fed with both flavonoids and control diet for 3 weeks. The concentration of TG in the serum of the hesperetin and naringenin groups significantly decreased by 48% and 31%, respectively (Fig. 1).

Table 1. Effects of hesperetin and naringenin on body weight, food intake, and liver weight in hamsters.

	Control	Hesperetin	Naringenin
Initial body weight (g)	76.73±2.22	76.62±1.95	76.87±2.12
Final body weight (g)	93.15±9.25	90.18±8.48	89.30±6.03
Food intake (g/day)	7.11±1.49	7.58±0.49	7.20±0.71
Liver weight			
Total weight (g)	5.27±0.37	5.20±0.64	4.59±0.05
g/100g body weight	5.67±0.17	5.72±0.18	5.17±0.29

Values are means ± SE of six hamsters.

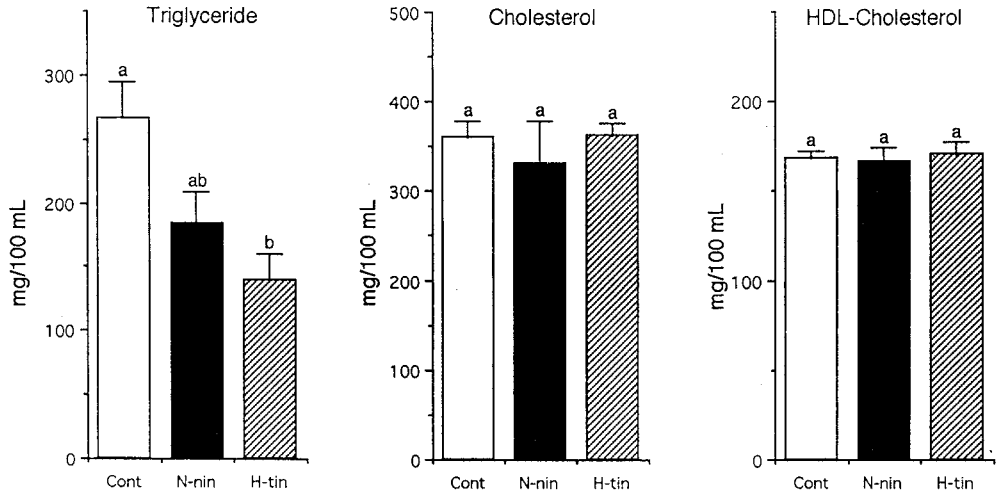

Fig. 1. Effect of hesperetin and naringenin on the concentrations of serum lipids. Hamsters were fed the semipurified diets containing either naringenin or hesperetin at the 1% level for 3 weeks. Values are means ± SEM of 6 hamsters. Values with different letters are significantly different at $p < 0.05$.
N-nin : naringenin, H-tin : hesperetin.

The concentrations of total cholesterol and HDL-cholesterol in the serum did not show difference among the groups. The concentration of TG in the liver was increased in the naringenin group, whereas tended to decrease in the hesperetin group (Table 2).

Table 2. Effects of hesperetin and naringenin on the concentrations of liver lipids in hamsters.

	Control	Hesperetin (mg/g liver)	Naringenin
Triacylglycerol	13.71±2.87[a]	11.50±2.34[a]	18.73±2.65[b]
Cholesterol	19.69±0.54[a]	25.46±1.98[b]	22.19±3.91[a]
Phospholipid	25.4±1.02	25.1±0.66	25.1±1.10

Values are means ± SE of six hamsters. Values with different letters are significantly different at $p < 0.05$.

The activity of microsomal PAP in the liver of the hesperetin group was significantly inhibited, whereas that in the naringenin group did not change (Table 3).

Table 3. Effects of hesperetin and naringenin on the activity of phosphatidate phosphohydrolase in hamsters.

	Control	Hesperetin	Naringenin
	(nmol/min/mg protein)		
Microsome	10.01±0.82[a]	5.71±0.37[c]	12.83±0.92[b]
Cytosol	11.39±1.15[a]	10.14±0.82[a]	14.27±0.53[b]

Values are means ± SE of six hamsters. Values with different letters are significantly different at $p < 0.05$.

It seem that hesperetin directly react with PAP on the basis of our result that the hesperetin significantly inhibited the PAP in the liver microsome *in vivo*. The fraction of hepatic microsome isolated from hamsters fed with a basal control diet was used as an enzyme source for studying on the effect of hesperetin on the activity of PAP *in vitro*. The activity of PAP with various concentrations of hesperetin was measured. The inhibition of PAP by the hesperetin was observed with a dose-dependent manner (Fig. 2).

DISCUSSION

This study showed that citrus aglycone flavonoids such as hesperetin and naringenin could affect the concentration of lipid in the liver and serum and the activity of PAP involved in biosynthesis of hepatic TG. There is a great deal of evidence that dietary flavonoids influence cholesterol levels in plasma and atherogenesis in hyperlipidemic animals (Gordon *et al.*, 1995; Hisatomi *et al.*, 1995; Igarashi and Ohmura, 1995; Cha *et al.*, 1999). The effect of dietary flavonoids on the TG level and its metabolism has not been fully studied. The concentration of TG in the serum of the hesperetin group was significantly lower than that of the control group in this study (Fig. 1). The concentration of TG in the liver of the same group also decreased (Table 2). Decreased concentration of TG in the liver and serum was accompanied with the reduced activity of microsomal membrane-bounded PAP, which is the key enzyme for biosynthesis of TG (Table 3). The change in the concentra- tion of TG in the liver was correlated with that in the activity of PAP in microsome ($r = 0.613$) as previously reported (Fremont and Gozzelino, 1996; Cha *et al.*, 1998). According to previous our result, the citrus flavonoid, hesperetin decreased the activity of PAP in the fatty liver induced by orotic acid (Cha *et al.*, 1999). The dietary flavonoids such as isorhamnetin, rhamnetin, and baicalein, were reported to decrease the concentration of TG in liver (Kimura *et al.*, 1981; Igarashi and Ohmura, 1995). The recent study also showed that the concentration of TG in plasma of rats fed with a diet containing 10% hesperidine, a glycoside flavonoid of hesperetin, was significantly lower than that of a diet without hesperidin (Kwaguchi *et al.*, 1997). However, the mechanism by which flavonoids decreases the TG level of the serum and liver in the hamster is not clear. Inhibition of biosynthesis of TG may be due to change in the activity of PAP or diacylglycerol acyltransferase and decrease in the activity of lipogenic enzymes involved in fatty acid synthesis (Ide and Murada, 1993; Ikeda *et al.*, 1998). The most recent result have showed that PAP is considered an important enzyme in the regulation of serum TG level (Fremont and Gozzelino, 1996; Cha and Cho, 1997; Cha *et al.*, 1998).

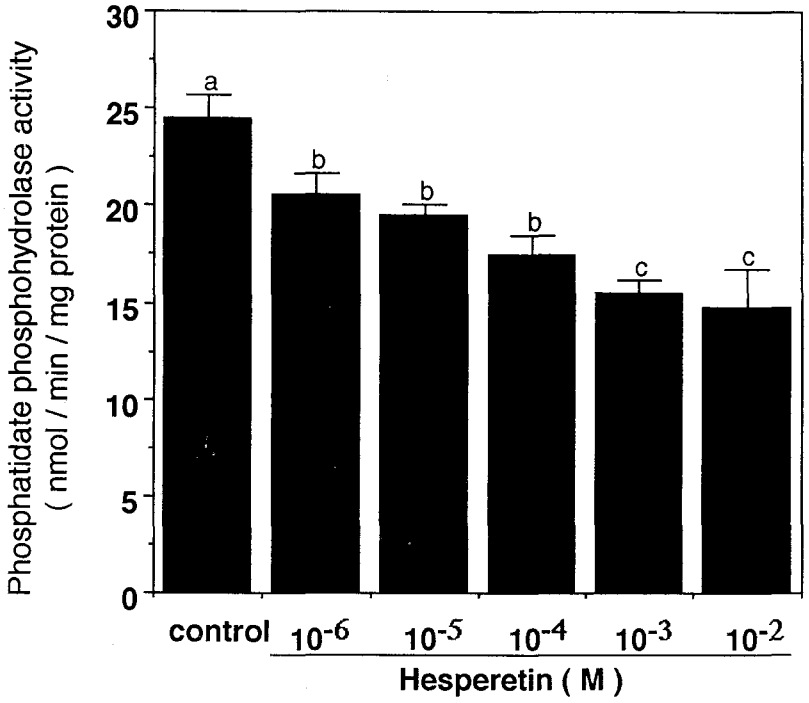

Fig. 2. Effect of hesperetin on the activity of phosphatidate phosphohy-drolase in the hepatic microsomal fraction of hamster in vitro. Phosphatidate phosphohydrolase activity was measured in the 10^{-6}-10^{-2}M concentration of hesperetin. Values are means ± SEM of 5 samples. Values with different letters are significantly different at $p < 0.05$.

As shown in table 3, the activity of PAP in hamster fed with the hesperetin diet decreased whereas that with naringenin did not change. On the basis of the result that hesperetin inhibited the activity of PAP in the hepatic microsome *in vivo*, the effect of hesperetin was examined to confirm if it directly react with PAP activity *in vitro* or not. Hesperetin was found to be the more effective compound to decrease the activity of PAP than any other flavonoids tested (Cha and Cho, 1997). The inhibitory effect of hesperetin was observed in a dose-dependent manner from the concentration of 10-6 M in this study (Fig. 2). This result indicates that decreased concentration of TG in the serum may be due to the reduced activity of PAP *in vivo*. The other possible mechanism of flavonoid action may be decreased absorption of TG in the intestine. Ikeda *et al.* reported that catechin mixtures significantly decreased absorption of fatty acid in rats until 3 h after administration of the test emulsion. Pancreatic lipase is the key enzyme for absorption of dietary fat so some inhibitors acted this lipase could alter absorption rate of them. Hesperidin, a glycoside flavonoid of hesperetin, decreased the activity of lipase whereas narigin, a aglycone flavonoid of naringenin, did not affected it (Kawaguchi *et al.*, 1997).

We have previously observed that naringenin and hesperetin showed hypocholesterolemic effect in rats fed with the cholesterol-enriched diet (Hisatomi *et al.*, 1995). However, the result from this work showed that the concentration of cholesterol in the serum and liver of hamsters fed with the hesperetin and naringenin diets did not change. The administration of hesperetin 5-O-glucoside (20 ㎎/㎏) in rats fed with the high fat diet showed no effect on the total cholesterol in the serum in previous study (Choi *et al.*, 1991). The fact that (+)-catechin did not induce hypocholesterolemic effect in a normal rats was confirmed. There was no influence observed in the mice for 15 days when dietary quercetin was added into the diet at the level of 0.5% (Kato *et al.*, 1983). These differences in the results may have been due to dietary conditions and the animals used. Our date suggested that the effect of hesperetin in the hypercholesterole-mic state in the hamster can be attributed to the lowering concentration of TG without any alteration in total cholesterol.

The disposition of citrus flavonoids in human was investigated (Ameer *et al.*, 1995). The intact naringing and hesperidin were not found in plasma, urine, and blood cell in human body after orally ingestion because they were completely converted to their aglycone types of naringenin and hesperetin. This result indicated that both flavonoids absorbed from the intestine may affect the lipid metabolism in the serum and liver. Thus, both hesperetin and naringenin may have been shown to possess a variety of biochemical and pharmacological activities including hypotriglycerolemic effect in the hamster.

The present study demonstrates that the hypotriglyceridemic effect of dietary hesperetin in the hamster may have been due in part to the reduced activity of PAP in *in vivo* and *in vitro*.

REFERENCES

Ameer B, Weintraub R, Johnson J. (1995) Metabolism of naringin and hesperidin. *Clin. Pharmacol. Ther.* **57**. 186.

Anonymous (1992) Prevention of coronary heart disease: Scientific background and new clinical guidelines. *Nutr. Metab. Cardiovasc. Dis.* **2**, 113-156.

Anonymous (1993) NIH Consensus Development Panel on triglyceride, HDL andcoronary heart disease. *JAMA* **269**, 505-510.

Assmann G, Schulth H (1992) Relation of HDL cholesterol and triglycerides to incidence of atherosclerotic coronary artery disease (The PROCAM Experimence). *Am. J. Cardiol.* **70**, 733-737.

Cha JY, Cho YS. (1997) Effects of hesperidin, naringin and their aglycones on the in vitro assay phosphatidate phosphohydrolase, and on the proliferation in cultured human hepatocytes HepG2 cells. *Agri. Chem. Biotechnol.* **40**, 577-582.

Cha JY, Cho YS, Kim I, Yanagita T. (1999) Effect of hesperetin, a citrus flavonoid, on the liver triacylglycerol content and phosphatidate phosphohydrolase activity in orotic acid-fed rats. (in submitted).

Cha JY, Mameda Y, Oogami K, Yamamoto K, Yanagita T. (1998) Association between hepatic triacylglycerol accumulation induced by administering orotic acid and enhanced phosphatidate phosphohydrolase activity in rats. *Biosci. Biotechnol. Biochem.* **62**, 508-513.

Chen PS, Toribara TY, Warner H. (1956) Microdetermination of phosphorus. *Anal Chem.* **28**, 1756-1758.

Chen YT, Zheng RL, Jia ZJ, Ju Y. (1990) Flavonoids as superoxide scavengers and antioxidants. *Free Radical Biol. Med.* **9**, 19-21.

Choi JS, Yokozawa T, Oura H. (1991)

Antihyperlipidemic effects of lavonoids from Prunus Davidiana. *J. Nat. Prod.* **54**, 218-224.

Duncan DB. (1957) Mutiple range test for correlated and heteroscedastic means. *Biometrics* **13**, 164-176.

Fletcher MJ. (1968) A colorimetric method for estimating serum triglyceride. *Clin. Chim. Acta* **22**, 393-397.

Folch J, Lees M, Sloane-Starley GH. (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**, 494-509.

Fremont L, Gozzelino MT. (1996) Dietary sunflower oil reduces plasma and liver triacylglycerols in fasting rats and is associated with decreased liver microsomal phosphatidate phosphohydrolase activity. *Lipids* **31**,871-878.

Gordon PB, Holen I, Seglen PO. (1995) Protection by naringin and some flavonoids of hepatocytic autophagy and endocytosis against inhibition by okadaic acid. *J. Biol. Chem.* **270**, 5830-5838.

Hertog MGL, Fesken EJM, Hollman PCH, Katan MB, Kromhout D. (1993) Dietary antioxidant flavonoids and risk of coronary heart disease the Zutphen Elderly Study. *Lancet*. **342**, 1007-1011.

Hisatomi T, Hara E, Furukawa J, Cha JY, Anno N, Yanagita T. Effect of dietary hesperidin and its aglycone on the lipid metabolism in rats. Annual Regional Meeting of Agricultural Chemical Society of Japan at Hiroshima, Oct 1995, Proceeding Abstract p.121.

Ide T, Murada M. (1993) The acyl-acceptor specificity of microsomal diacylglcerol acyltransferase as a possible determinant in regulating hepatic triacylglycrol synthesis in rats fed a polyunsaturated fat diet. *J. Nut. Biochem.* **4**, 229-235.

Igarashi K, Ohmuma M. (1995) Effects of isorhamnetin, rhamnetin and quercetin on the concentration of cholesterol and lipoperoxide in the serum and liver on the blood and liver antioxidative enzyme activities of rats. *Biosci. Biotechnol. Biochem.* **59**, 595-601.

Ikeda I, Cha JY, Yanagita T, Nakatani N, Oogami K, Imaizumi K, Yazawa K. (1998) Effects of dietary a-linolenic,eicosapentaenoic and docosahexaenoic acids on hepatic lipogenesis and b-oxidation in rats. *Biosci. Biotechnol. Biochem.* **62**, 675-680.

Ikeda I, Imasato Y, Sasaki E, Nakayama M, Nagano H, Takeo T, Yayabe F, Sugano M. (1992) Tea catechins decrease micellar solubility and intestinal absorption of cholesterol in rats. *Biochim. Biopys. Acta* **1127**, 141-146.

Kato N, Tosa N, Doudou T, Imamura T. (1983) Effects of dietary quercetin on serum lipids. *Agri. Biol. Chem.* **47**, 2119-2120.

Kawaguchi K, Mizuno T, Aida K, Uchino K. (1997) Hesperidin as an inhibitor of lipases from porcine pancreas and pseudomonas. *Biosci. Biotechnol. Biochem.* **61**, 102-104.

Kimura Y, Kubo M, Tani T, Arichi S, Okuda H. (1981) Studies on scutellariae radix.IV, Effects on lipid peroxidation in rat liver. *Chem. Pharm. Bull.* **29**, 2610-2617

Kuhnan J. (1976) The Flavonoids. A class of semi-essential food components: their role in human nutrition. *World Rev. Nutr. Diet* **24**, 117-191.

Lowry OH, Rosenbrough NJ, Farr AL, Ranll RJ. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.

Manninen V, Tenkanen L, Koskinen P, Huttunen JK, Manntari M, Heinonen OP, Frick MH. (1992) Triglycerides and LDL cholesterol and HDL cholesterol concentrations on coronary heart disease risk in the Helsinki Heart Stury. *Circulation* **85**, 37-45.

Middeton E, Kanadaswami C. (1992) Effects of flavonoids on immune and inflammatory

cell functions. *Biochem. Pharmacol.* **43**, 1167-1179.

Mouly PPM, Arzouyan CG, Gaydou EM, Estienne JM. (1994) Differentiation of citrus juices by factorial discriminate analysis using liquid chromatography of flavanone glycosides. *J. Agri. Food Chem.* **42**, 70-79.

Singhal AK, Finver-Sadowsky J, McSherry CK, Mosbach EH. (1982) Effect of cholesterol and bile acids on the regulation of cholesterol metabolism in hamster. *Biochim. Biophys. Acta* **752**, 214-222.

Sperry WM, Webb M. (1950) A revision of the shoenheimer-sperry method for cholesterol determination. *J. Biol. Chem.* **187**, 97-106.

Yanagita T, Hara E, Yotsumoto H, Rahman SM, Han SY, Cha JY, Yamamoto K. (1995) NK-104, a potent new 3-hydroxy-methylglutaryl coenzyme A reductase inhibitor, enhances posttranslation catabolism of polipoprotein B-100 and inhibits secretion of apolipoprotein B-100 and triacylglycerols from HepG2 cells. *Cur. Therapeutic. Res.* **60**, 423-434.

Yotsumoto H, Yanagita T, Yamamoto K, Ogawa Y, Cha JY, Mori Y. (1997) Inhibitory effects of Oren-Gedoku-to and its components on cholesteryl ester synthesis in cultured human hepatocyte HepG2 cells: Evidence from the cultured HepG2 cells and *in vitro* assay of ACAT. *Planta Med.* **63**, 141-145.

Walton PA, Possmayer F. (1985) Mg^{2+}-dependent phosphatidate phosphohydrolase of rat lung: Development of an assay employing a defined chemical substrate which reflects the phosphohydrolase activity measured using membrane-bound substrate. *Anal. Biochem.* **151**, 479-48