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Dietary Caffeic Acid, Ferulic Acid and Coumaric Acid Supplements on Cholesterol Metabolism and Antioxidant Activity in Rats

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ABSTRACT

This study was designed to test the lipid-lowering and antioxidative activities of three phenolic compounds, caffeic acid, ferulic acid and coumaric acid. Four groups of rats were given a semisynthetic diet containing 3% of cholesterol for 6 weeks. The control group only received a high cholesterol diet, whereas the other three groups received a diet including 0.2% of caffeic acid, 0.2% of ferulic acid and 0.2% of coumaric acid. The caffeic acid, ferulic acid and coumaric acid significantly lowered the plasma lipid and hepatic cholesterol levels compared to those in the control ($p < 0.05$). The hepatic HMG-CoA reductase activity was significantly lower in the caffeic acid group than in the ferulic acid and coumaric acid groups ($p < 0.05$), while the hepatic ACAT activity was significantly lower in the caffeic acid, ferulic acid and coumaric acid groups compared to the control group ($p < 0.05$). The overall potential of the antioxidant system was significantly enhanced by the caffeic acid, ferulic acid and coumaric acid supplements as the plasma and hepatic TBARS levels were lowered while the hepatic SOD activities and GSH concentration were elevated in the high-cholesterol-fed rats ($p < 0.05$). Furthermore, these results indicated that the supplementation of caffeic acid, ferulic acid and coumaric acid boosted the antioxidant activity in rats and promoted the excretion of neutral sterol and acidic sterol ($p < 0.05$), thereby leading to a decreased absorption of dietary cholesterol as well as lower plasma, hepatic cholesterol and promoted excretion of fecal sterols.

Key words: caffeic acid, ferulic acid, coumaric acid, HMG-CoA reductase, ACAT, fecal sterols, antioxidant enzymes

INTRODUCTION

Although flavonoids are non-energetic and not considered as indispensable vitamins, their supply, either individually or in a complex mix, may have a positive effect on health. A high consumption of flavonoids has been previously exert reported to contribute to a decreased risk of coronary heart diseases due to lowering the serum cholesterol and triglycerides in rats[(1)]. However, it is still unclear exactly which substances in fruits and vegetables are responsible for the observed inverse association with cardiovascular diseases, which could be attributed to an antioxidant or other constituents, such as fiber or phytochemicals[(2)]. Most flavononoids exhibit antioxidant activity in both aqueous and lipid-assay systems and their free radical scavenging activity can largely be predicted on the basis of their chemical structure. Flavonoids and phenolics are known to decrease the risk of tumors, inflammation and bacterial and viral infections[(3,4)], and they exhibit inhibitory effects on membrane lipid peroxidation[(5)].

Plasma cholesterol concentration can be regulated by the biosynthesis of cholesterol, removal of cholesterol from the circulation, absorption of dietary cholesterol and excretion of cholesterol via bile and feces. Cellular cholesterol homeostasis is very important for the prevention of cardiovascular diseases and numerous studies have already reported on the beneficial effects of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase and acyl-CoA; cholesterol acyltransferase (ACAT) inhibitors on hypercholesteolemia and atherosclerosis[(6,7)]. Previous reports also demonstrate that the flavonoids hesperidin and naringin plus their aglycones lower the plasma and hepatic cholesterol as well as HMG-CoA reductase activity[(8,9)].

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In association with cardiovascular diseases, there are relatively few reports on the physiological functions of caffeic acid, ferulic acid and coumaric acid. Polyphenol glycoside can relax smooth muscles and it was observed to lower hepatic and blood cholesterol levels[10]. Meanwhile, caffeic acid, ferulic acid and coumaric acid and other polyphenol compounds seemed to have direct antioxidant effects by scavenging free radicals as phenolic bioflavonoids[11]. However, the overall effects of bioflavonoids and phenolic compounds on the cholesterol metabolism and antioxidative status still require clarification. Accordingly, the present study was aimed to investigate caffeic acid, ferulic acid and coumaric acid supplements alter the cholesterol metabolism and activities of antioxidant enzymes.

MATERIALS AND METHODS

I. *Reagents*

Caffeic acid, ferulic acid and coumaric acid standards were purchase from Sigma (St. Louis, MO, USA).

II. *Animals*

Male weanling Wistar rats were purchased from the National Laboratory Animal Center. They were kept in an air-conditioned room (23 ± 1°C, 50-60% humidity) light for 12 hr/day (7 AM to 7 PM). Experimental protocol was approved by the Institutional Animal care and Use, Committee of Toko University. After acclimatizing for 2 week with a commercial non-purified diet (Rodent Laboratory Chow 5001, Purina Co., USA), 24 rats were divided six rats in each group into four groups. The composition of the basal and all treatment diets is listed in Table 1. In previous studies hypercholesterol diet induce increase cholesterol, triglyceride (TG) and oxidative stress[12-14]. The diets were synthesized as described previously by the American Institute of Nutrition[15] and included: basal diet (control diet), caffeic acid diet (0.2% caffeic acid in diet), ferulic acid diet (0.2% ferulic acid) and coumaric acid diet (0.2% coumaric acid).

Rats were fasted for 10 hr before the experiment, blood was obtained by tail vein puncture and the rats were weighed and euthanized (with diethyl ether) on the 6th week. The plasma of blood samples were collected by centrifugation (1000 × g for 15 min) of the whole blood and examined for aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) activites. The cholesterol, HDL-cholesterol and TG, levels in the plasma and hepatic lipids were determined by enzymatic assay using commercially enzymatic kit with Selectra Analyser (Merck Co. Ltd, Germany). Livers and kidneys of the rats were quickly excised and weighed. Both relative ratios of liver and kidney weight

Table 1. Composition of the experimental diets for animal diet of caffeic acid, ferulic acid and coumaric acid

Ingredient	Diets			
	Base diet (%)	Caffeic acid diet (%)	Ferulic acid diet (%)	Coumaric acid diet (%)
Casein	20	20	20	20
Methionine	0.3	0.3	0.3	0.3
Cellulose	5	5	5	5
Corn oil	2	2	2	2
Cholesterol	3	3	3	3
Caffeic acid	0	0.2	0	0
Ferulic acid	0	0	0.2	0
Coumaric acid	0	0	0	0.2
Choline	0.2	0.2	0.2	0.2
Mineral mix[(a)]	3.5	3.5	3.5	3.5
Vitamin mix[(b)]	1	1	1	1
Corn starch	30	29.8	29.8	29.8
Sucrose	35	35	35	35

(a) Minerals per 100 g diet: NaCl 7.4 g, $K_2C_6H_5O_7$ H_2O 22 g, K_2SO_4 5.2 g, $CaHPO_4$ 50 g, MgO 2.4 g, $FeC_6H_5O_7$ $5H_2O$ 0.6 g, $MnCO_3$ 0.35 g, $CuCO_3$ 30 mg, $CrK(SO_4)_2$ $12H_2O$ 55mg, $CoCl_2$ $6H_2O$ 10 mg, KI 1 mg, $ZnCO_3$ 160 mg.

(b) Vitamin per 100 g diet: thiamine 100 mg, riboflavin 150 mg, pyridoxine HCl 100 mg, nicotinamide 1000 mg, D-panthenate 500 mg, folic acid 50 mg, vitamine B_{12} 0.1 mg, vitamin A 2.5×10^5 IU, vitamin E 100 mg, calciferol 2×10^4 IU, vitamin C 3.7×10^3 mg.

to body weight were obtained. The liver was stored at -40°C for glutathione (GSH) and thiobarbituric acid-reactive substances (TBARS) determinations.

III. *HMG-CoA Reductase*

Microsomal HMG-CoA reductase activity was measured using [^{14}C] HMG-CoA and an NADPH regenerating system as previously described[16]. Each reaction mixture contained 0.1 mg of protein, as determined by a biuret protein assay, and was incubated for 10 min. The product [^{14}C] mevalonate was converted to the lactone and isolated by thin layer chromatography[16]. Enzyme activity was expressed as nmol/min/mg of microsomal protein.

IV. *ACAT Activities*

The intestine was opened longitudinal, mucosal cells were scraped off using a microscope slide and suspended in 0.154 M potassium phosphate (pH 7.4) containing 0.25 M sucrose. The liver or intestinal mucosal cell suspension was homogenized in 0.25 M sucrose solution and centrifuged to obtain a microsome fraction[17,18]. The activity of ACAT was determined by the formation of cholesteryl [^{14}C] oleate from [^{14}C] oleoyl-CoA and endogenous cholesterol as previously described[18]. The radioactivity was expressed as PSL (photo stimulated luminescense). Cholesterol in liver microsome was extracted with chloroform methanol (2:1 v/v) and measured enzymatically.

V. *Fecal Sterols*

Analysis of fecal neutral sterols were carried out in a Hewlett Packard Gas Chromatographer (GC) Model 873 equipped with a hydrogen flame ionization detector and a dual pen recorder. A 6 ft long (3 mm i.d.) u-shaped column was silanized and packed with 3% SE-30 on 100-120 mesh Supelcoport. Helium was used as a carrier gas at a flow rate of 40-50 mL/min and an inlet pressure of 40 psi. Operating temperatures for the column, inlet and detector were 260, 280 and 280°C, respectively. Daily neutral sterol excretion was calculated based on the amount of cholesterol, coprostanol and coprostanone in each sample. The fecal acid sterols were determined by using a Hitachi Liquid Chromatographer (Hitachi, Ltd, Tokyo) consisting of a Model L-6200 pump, a Rheodyne Model 7125 syringe loading sample injector, a Model L-4000 UV-Vis detector set at 210 nm and a Model D-2500 Chromato-integrator. A Lichrospher 100 RP-18 reverse-phase column (5 μm, 25 × 0.3 cm^2 i.d., E. Merck) was used for separation. The mixed solvent of 0.3% ammonium carbonate solution-acetonitrile was used as the mobile phase. The system of mobile phase was as follows: the ratio of both solution from 73:27 for 10 min, 68:32 for 10 min and then to 50:50 for 10 min. The flow rate was 0.8 mL/min[19]. The daily acid sterol excretion was calculated based on the amount of cholic acid, deoxycholic acid, lithocholic acid and glycocholic acid in each sample.

VI. *Antioxidant Activities*

Appropriate liver tissues were dissected, weighed, immersed in liquid N_2 generally within 60 s of death, and kept frozen at -70°C. Prior to enzyme determinations, thawed tissue samples were homogenized in 20 volumes of ice cold 50 mM phosphate buffer (pH 7.4), centrifuged at 3200 × 3 g for 20 min at 5°C. The supernatant fraction was used for the determination of antioxidant enzymes.

(I) *Catalase Activity*

Catalase activity of 1% Triton X-100-treated supernatant was determined at 20°C by the disappearance of H_2O_2 at 240 nm[20,21]. One unit of catalase represents the decrease of 1 μmol of H_2O_2 per minute. The Se-dependent isoenzyme of glutathione peroxidase (Se-GPx) was assayed at 25°C[21,22], where NADPH oxidation was followed by the absorbance at 340 nm in the presence of reduced glutathione (GSH) and H_2O_2[22]. To correct for spontaneous reactions in the absence of enzyme, blanks were run without sample and then subtracted from the assay values[21]. One unit of GPx is defined as the amount of enzyme that oxidizes 1 μmol of NADPH per minute. Total superoxide dismutase (Total-SOD) activity was assayed by the inhibition, at 25°C, of pyrogallol autoxidation by SOD (with and without sample) and was followed kinetically at 420 nm[23]. One unit of SOD is defined as the amount of enzyme that causes 50% inhibition of pyrogallol autoxidation. Changes in absorbance were read on a SPECTRAmax Plus microplate spectrophotometer (Molecular Devices Corp. Sunnyvale, CA, USA) and analyzed using SOFTmax Pro software (Molecular Devices Corp.). All enzyme activities are expressed per mg protein. Protein content for separate tissue samples was determined by the method of Lowry *et al*.[24].

(II) *SOD Activity Assays*

SOD activity was assayed using a SOD assay kit based on the cytochrome c-xanthine oxidase method[25,26]. One unit of activity was defined as the amount of enzyme which inhibits the rate of cytochrome c reduction by 50%. Different concentrations of $MnCl_2$ were added to the assay system in order to address the effects of $MnCl_2$ on the activities.

(III) *Levels of GSH Measurement*

GSH reacts non-enzymatically with DTNB to yield GSSG and TNB. GSSG is then reduced enzymatically by NADPH and GR to regenerate GSH. Concentrations of DTNB, NADPH and GR are chosen such that the rate of the overall reaction is linearly proportional to the concentration of total GSH. The rate of formation of TNB is

followed spectrophotometrically, and assay is calibrated using standards. If the sample is reacted with 2-vinylpyridine and GSH is derivatized, and only GSSG is detected during subsequent assay[27].

(IV) *TBARS Concentration*

Lipid peroxidation activities in the liver and plasma were assayed by measurement of MDA, an end-product of peroxidized fatty acids, and TBA reaction product. The sample of 20% liver homogenate was mixed with 1.0 mL 0.4% TBA in 0.2 N HCl and 0.15 mL 0.2% BHT in 95% ethanol. The samples were incubated in a 90°C water-bath for 45 min. After incubation, the TBA-MDA adduct was extracted with isobutanol. The isobutanol extract was mixed with methanol (2:1) prior to injection into the HPLC system. The supernatant was examined by using the HPLC system at an excitation 515 nm and an emission 550 nm on a Hitachi Fluorescence Detector (Japan)[28].

VII. *Atherogenic Index*

The atherogenic index (AI) was calculated using the following formula: AI = (total cholesterol-HDL-cholesterol)/HDL-cholesterol.

VIII. *Statistical Analysis*

Statistical analysis for differences among experimental groups was performed by the one-way analysis of variance procedure and Duncan's new multiple range tests[29]. A p value < 0.05 was considered statistically significant.

Table 2. Effect of caffeic acid, ferulic acid and coumaric acid supplementation on the body weight, liver and kidney weight to body weight in high cholesterol-fed rats.

Groups	Control*	Caffeic acid	Ferulic acid	Coumaric acid
Body weight (g)	385 ± 23	386 ± 26	392 ± 25	393 ± 28
Liver weight/body weight (%)	2.23 ± 0.15	2.26 ± 0.16	2.23 ± 0.15	2.27 ± 0.16
Kidney weight/body weight (%)	2.56 ± 0.15	2.58 ± 0.13	2.6 ± 0.16	2.63 ± 0.18

*Data represent mean ± SD.

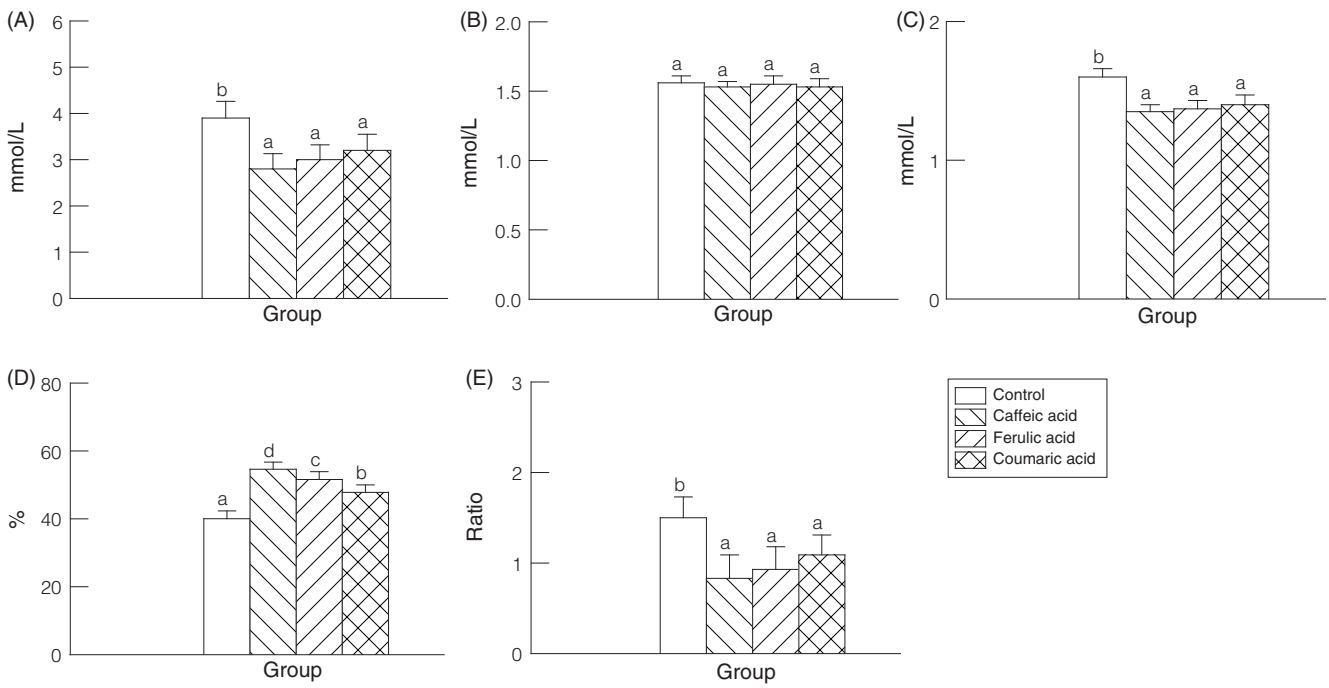

Figure 1. Effect of caffeic acid, ferulic acid and coumaric acid supplementation on plasma lipids in high cholesterol-fed rats. A-E: values in the same week with different superscript are significantly different (p < 0.05). (A): Plasma (total cholesterol); (B): Plasma (HDL-cholesterol); (C): Plasma (TG); (D): Plasma (HDL-C/total-C); (E): Plasma (atherogenic index, AI).

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RESULTS

I. *Body Weight, Liver and Kidney Weight to Body Weight*

There was no significant difference in the body weight, liver and kidney weight to body weight among various groups (Table 2). Accordingly, these areas were not seemingly affected by the three flavonoid supplements.

II. *Plasma and Hepatic Lipids*

The supplementation of caffeic acid, ferulic acid and coumaric acid significantly lowered both plasma cholesterol and triglyceride concentrations compared to the control group (Figure 1). The HDL-cholesterol concentration did not differ between the groups, however, the supplementation of caffeic acid significantly increased the HDL-C/total-C ratios compared to the other groups. The AI was significantly lower in the caffeic acid, ferulic acid and coumaric acid groups than in the control group. In addition, the AI and total cholesterol level in plasma were both significantly lower in the caffeic acid, ferulic acid and coumaric acid group than in the control group.

In contrast to the plasma lipids, hepatic triglyceride level was not different among groups (Figure 2), yet the supplementation of caffeic acid, ferulic acid and coumaric acid did significantly lower the hepatic cholesterol content compared to the control group.

III. *Hepatic HMG-CoA Reductase and ACAT Activities*

The hepatic HMG-CoA reductase activity was significantly lowered by the caffeic acid supplement compared to other groups (Figure 3). However, the hepatic ACAT activity was lower in the caffeic acid, ferulic acid and coumaric acid groups than in the control group.

IV. *Fecal Sterols*

The daily excretion of fecal sterols is shown in Figure 4. The effect of the caffeic acid, ferulic acid and coumaric acid supplementation did result in certain changes in the fecal sterol under the cholesterol-fed conditions. As such, when compared with the control group, the caffeic acid supplement induced a significant increase in the excretion of neutral, and total fecal sterols, while the coumaric acid supplement induced an increase in the excretion of acidic and total fecal sterols.

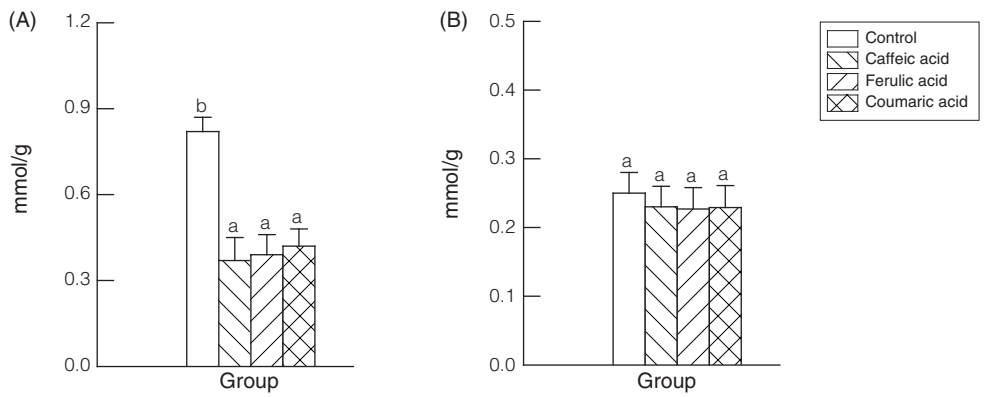

Figure 2. Effect of caffeic acid, ferulic acid and coumaric acid supplementation on hepatic lipids in high cholesterol-fed rats. A-B: values in the same week with different superscript are significantly different ($p < 0.05$). (A): Liver (cholesterol); (B): Liver (TG).

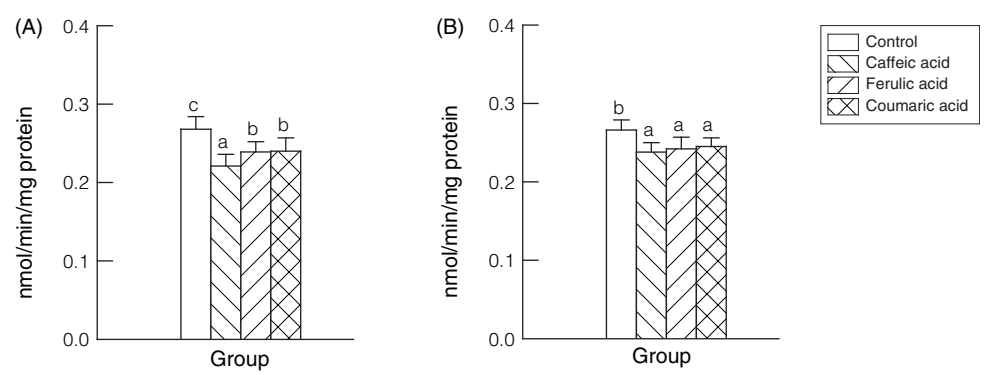

Figure 3. Effect of caffeic acid, ferulic acid and coumaric acid supplementation on hepatic ACAT activity and HMG-CoA reductase activity in highcholesterol fed rats. A-B: values in the same week with different superscript are significantly different ($p < 0.05$). (A): HMG-CoA reductase; (B): ACAT activity.

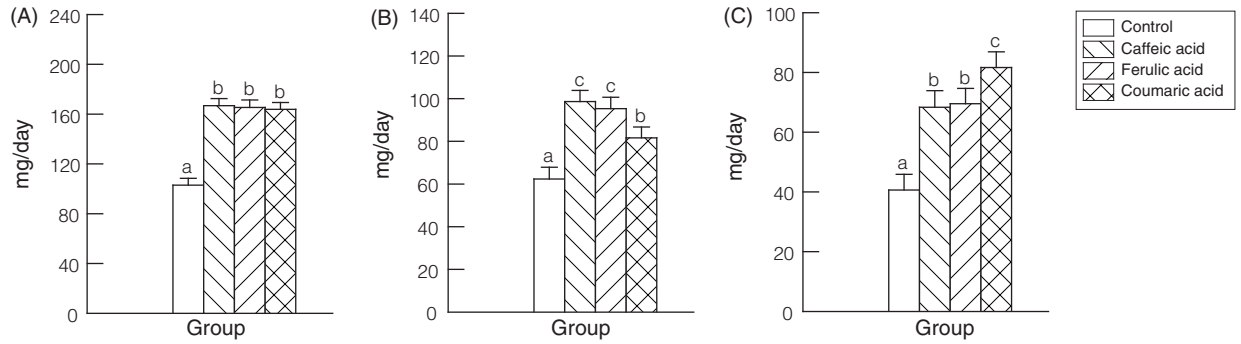

Figure 4. Effect of caffeic acid, ferulic acid and coumaric acid supplementation on fecal sterol concentration content in high cholesterol-fed rats. A-C: values in the same week with different superscript are significantly different ($p < 0.05$). (A): Total fecal sterol; (B): Neutral sterol; (C): Acidic sterol.

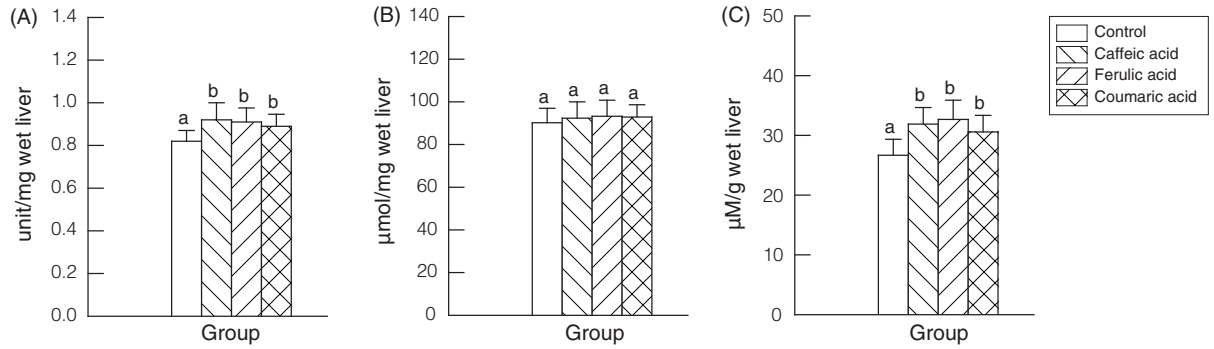

Figure 5. Effect of caffeic acid, ferulic acid and coumaric acid supplementation on SOD, CAT and GSH activities in high cholesterol-fed rats. A-C: values in the same week with different superscript are significantly different ($p < 0.05$). (A): SOD; (B): CAT; (C): GSH.

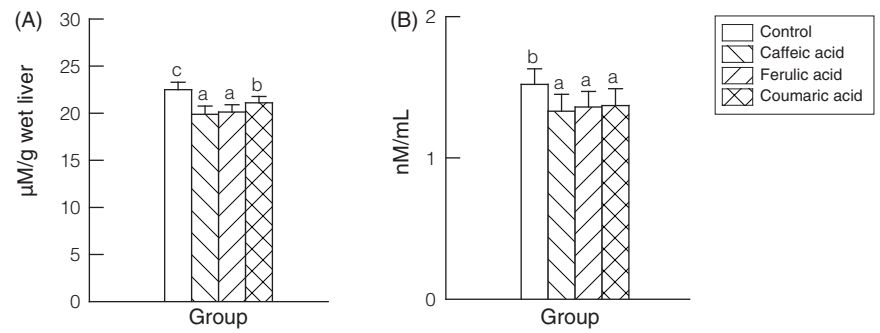

Figure 6. Effect of caffeic acid, ferulic acid and coumaric acid supplementation on plasma and hepatic TBARS concentrations in high cholesterol-fed rats. A-B: values in the same week with different superscript are significantly different ($p < 0.05$). (A): Liver (TBARS); (B): Plasma (TBARS).

V. *Hepatic Antioxidant Enzyme Activities and Plasma and Hepatic TBARS Levels*

No marked differences in the CAT activities were observed among groups (Figure 5). However, the activities of SOD and GSH were significantly higher in the caffeic acid, ferulic acid and coumaric acid groups than in the control group.

The levels of plasma and hepatic TBARS were significantly lower in the caffeic acid, ferulic acid and coumaric acid groups than in the control group (Figure 6). Among all three compounds, the caffeic acid and ferulic acid supplement was more effective in lowering the hepatic TBARS level.

DISCUSSION

Flavonoids are now widely accepted as physiologic antioxidants that have a significant potential to protect

against many degenerative diseases linked to free radical-related tissue damage. The health benefits of polyphenols would appear to arise from their antioxidant activity and capacity to protect critical macromolecules, such as chromosomal DNA, structural proteins and enzymes, LDL and membrane lipids, from damage from exposure to reactive oxygen species (ROS)[30,31]. Interest in polyphenols as antioxidants has been centered on a group referred to as flavonoids, which share a common molecular structure of diphenylpropane.

The present study investigated the effects of dietary caffeic acid, ferulic acid and coumaric acid in order to determine their possible roles in a high-cholesterol fed state. The results suggest that the plasma lipid-lowering and antioxidative effects of caffeic acid, ferulic acid and coumaric acid supplements were very potent in high cholesterol-fed rats.

Liver is the major site for the synthesis and net excretion of cholesterol, and hepatic HMG-CoA reductase activity can normally be suppressed under high-cholesterol feeding conditions by negative feedback control[32]. In the current study, the plasma cholesterol concentration was lowered by caffeic acid, ferulic acid and coumaric acid supplements, although caffeic acid exhibited a more potent biological effect on lowering the plasma cholesterol and hepatic HMG-CoA reductase activity than ferulic acid and coumaric acid. These results can also be supported by other previous findings. For instance, Santos *et al.* [33] reported on the hypolipidemic effect of phenolic acid by the intraperitoneal route with a dose of 5 mg/body weight. Furthermore, the intraperitoneal injection of caffeic acid at 30 mg/week for a period of 10 weeks was previously found to lower the plasma triglyceride, total-C, and LDL-C levels in spontaneously hypertensive rats, while increasing the plasma HDL-C level in normal Wistar rats[34]. Hypocholesterolemic effects have been observed in both genetically hypercholesterolemic (RICO) and normocholesterolemic (RAIF) rats when fed a high fat diet[35]. In the current study, the plasma triglyceride concentration was lower in the three flavonoid supplemented groups, although the hepatic triglyceride content did not differ significantly among groups. The reduced ACAT activity in these groups may have led to less cholesteryl ester available for VLDL packing, thereby resulting in a reduction of VLDL secretion from the liver, as suggested by Carr *et al.*[36].

The cholesterol-lowering activity of other flavonoids was also identified in previous studies[8,9]. As such, the present results are somewhat different from previous observations, where the supplement of a citrus bioflavonoid lowered both the plasma cholesterol concentration and the fecal neutral sterol in high cholesterol-fed rats[8,9]. Recently, Kurowska *et al.*[37] reported that citrus juices induce the reduction of LDL cholesterol as well as cholesterol excretion in rabbits fed a semi-purified cholesterol-free diet. Furthermore, same studies suggested that citrus bioflavonoids inhibit hepatic HMG-CoA reductase during their cholesterol-lowering action[8,9].

According to the results of the current study, although caffeic acid inhibited the hepatic HMG-CoA reductase activity, the mode of its cholesterol-lowering action was not the same as that of other citrus bioflavonoids in high cholesterol-fed rats. Meanwhile, Chan *et al.*[38] recently reported that epicatechins produce a hypolipidemic effect in a high fat diet without affecting the hepatic HMG-CoA reductase and ACAT activities. As such, it would appear that the decrease in the plasma cholesterol concentration resulting from the caffeic acid, ferulic acid and coumaric acid supplements may be resulted due to the increased fecal sterol, which in turn led to a decreased absorption of dietary cholesterol. The action of ferulic acid and coumaric acid in the cholesterol-lowering mechanism appeared to be similar to that of caffeic acid except for the HMG-CoA reductase activity, which is known to be down-regulated by the body cholesterol and can be inhibited by HMG-CoA reductase inhibitors. In this case, ferulic acid and coumaric acid did not exhibit any effective inhibition of the HMG-CoA reductase activity.

Dietary caffeic acid, ferulic acid and coumaric acid did have a significant affect on the two hepatic antioxidant enzyme activities, SOD and GSH, along with the plasma and hepatic TBARS levels. SOD converts superoxide radicals into hydrogen peroxide, which is then converted to water by both CAT and GSH. As a result, these three enzymes can prevent damage by detoxifying reactive oxygen species. The levels of plasma and hepatic TBARS in the caffeic acid, ferulic acid and coumaric acid treated animals showed a significant reduction, thereby indicating a decreased rate of lipid peroxidation. The overall potential of an antioxidant defense was greater in the caffeic acid supplemented rats than in the ferulic acid and coumaric acid group. A concomitant increase in the antiperoxidative enzymes, namely SOD and GSH, was observed in the caffeic acid group, and both enzymes play an important role in scavenging toxic intermediates of incomplete oxidation in the body. The significant elevation of SOD activity also suggests that the free-radical scavenging activity of this enzyme is only effective when it is accompanied by an increase in the activity of catalase and/or GSH. This is because SOD generates hydrogen peroxide as a metabolite, which is more toxic than oxygen radicals in cells and needs to be scavenged by catalase or GSH[39]. As such, a concomitant increase in catalase and/or GSH activity is essential if a beneficial effect from the high SOD activity is expected.

Cholesterol diet may induce lipid peroxidation and reduce diet palatability in rats. TBARS is an end-product of lipid peroxidation. The level of TBARS in the liver was increased when the rats were fed with cholesterol. This means that the oxidative stress caused by feeding with cholesterol was due to the induction of the lipid peroxidation of liver cells. The level of TBARS in the liver of rats was significantly reduced when the rats were

fed with the supplement of caffeic acid, ferulic acid or coumaric acid. This result is the same as that reported previously[40-44]. Therefore, it is reasonable to assume that caffeic acid, ferulic acid and coumaric acid may act as a good scavenger in reducing the production of lipid peroxidation[45-50]. Meanwhile, the function of GSH to protect biological organisms from xenobiotic injuries is well known[51,52-54].

Lipid peroxidation is a chemical mechanism capable of disrupting the structure and function of the biological membranes that occurs as a result of free radical attacking on lipids. When reactive oxygen species (ROS) begin to accumulate, hepatic cells exhibit a defensive mechanism by various antioxidant enzymes. The main detoxifying systems for peroxides are catalase and GSH[55]. Catalase is an antioxidant enzyme, which destroys H_2O_2 that can form a highly reactive hydroxyl radical in presence of iron as a catalyst[56]. By participating in the glutathione redox cycle, GSH together with GSH-Px converts H_2O_2 and lipid peroxides to non-toxic products. Reduced activity of one or more antioxidant systems due to the direct toxic effect of cholesterol leads to increased lipid peroxidation, oxidative stress, and hepatotoxicity. In the current study, cholesterol depleted GSH reservoir and reduced catalase and GSH-Px activities. These results are in harmony with other investigations[57]. For example, cholesterol induced hepatotoxicity was exacerbated by GSH depletion. In the current study, the depletion of GSH reservoir can account for the inhibition of GSH-Px activity. In addition, high levels of peroxides may explain catalase activity inhibition[58].

Caffeic acid, ferulic acid and coumaric acid supplementation in our study significantly mitigated cholesterol induced oxidative stress. In addition, caffeic acid, ferulic acid and coumaric acid inhibited lipid peroxidation, diminished the decrease in catalase and GSH-Px activities, and abrogated GSH depletion induced by cholesterol.

Caffeic acid, ferulic acid and coumaric acid has been demonstrated to function as a direct antioxidant that scavenges or quenches oxygen free radicals, thus inhibiting lipid peroxidation, and as an indirect antioxidant that prevents the increase in membrane permeability resulted from oxidant injury in many tissues including liver and plasma[59]. Caffeic acid, ferulic acid and coumaric acid might stimulate *s*-nitrosylation of GSH producing *s*-nitrosoglutathione, which is approximately 100 times more potent than the classical GSH. In addition, *s*-nitrosylation of cysteine residues by nitrosoglutathione can inactivate caspase-3, thus preventing hepatic cell apoptosis[60]. Moreover, caffeic acid, ferulic acid and coumaric acid might lessen cholesterol induced oxidative injury and may be capable of lowering or slowing down oxidative-stress[61,62]. In summary, the data presented in this paper show that administration of caffeic acid, ferulic acid and coumaric acid is a safe and effective way of lowering cholesterol.

This study idemonstrated a decrease in the plasma lipid and hepatic cholesterol levels, increase in the fecal sterols and enhanced antioxidative capacity in rats supplemented with caffeic acid, ferulic acid and coumaric acid. Accordingly, these results indicate that caffeic acid, ferulic acid and coumaric acid supplements both decreased the cholesterol absorption in high cholesterol-fed rats. Since the cholesterol intake was about the same for all groups, the supplementation of these compounds appeared to promote an increase in the fecal sterol. It is also worth mentioning that caffeic acid exhibited a greater inhibitory effect on hepatic HMG-CoA reductase than ferulic acid and coumaric acid. However, more studies are needed, with various animal models, to explain the lipid-lowering action of these bioflavonoids.

In conclusion, we suggest that: (a) the imbalance between production of oxygen free radicals and the endogenous antioxidant defense system, as a result of the effect of cholesterol, is the main mechanism responsible for peroxide accumulation; and (b) caffeic acid, ferulic acid and coumaric acid reduces the oxidative stress through the inhibition of lipid peroxidation (a widely known mechanism).

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