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# Biological Activities of Polyphenol Compounds Derived from Unripe Apple

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Biological activities of apple polyphenol derived from unripe apple were examined in this study. Peroxidation of low density lipoprotein was strongly inhibited by unripe apple polyphenol in in vitro assay and its potency was equal to that of tea catechin such as epigalocatechin gallate. Dietary unripe apple polyphenol lowered the levels of liver phospholipids hydroperoxides in rats in the dose-dependent manner. Dietary unripe apple polyphenol also decreased the activities of hepatic HMG-CoA reductase and Δ6 desaturase in rats. On the other hand, the activity of hepatic cholesterol 7α-hydroxylase was increased by dietary unripe apple polyphenol. Reflecting these modulations of enzyme activities, liver cholesterol level and Δ6 desaturation indices of tissue lipids were lowered and fecal excretion level of acidic steroids was increased in rats fed unripe apple polyphenol. The ratio of HDL-cholesterol/total cholesterol was increased by consumption of unripe apple polyphenol. The dimer of unripe apple polyphenol oligomer were identified in serum in rats fed unripe apple polyphenol. Moreover, many unidentified compounds derived from unripe apple condensed tannin were found in serum in rats fed unripe apple polyphenol, although these compounds were not identified in serum in rats fed polyphenol-free diet. Therefore, the absorbed and decomposed compounds derived from apple condensed tannin may exhibit various biological functions such as antioxidative action and regulation of lipid metabolism.
Key words: apple polyphenol, antioxidant lipid metabolism, rat

## 1. INTRODUCTION

Unripe apples, compared to ripe apple, contain a large amount of polyphenol. Existence of the condensed tannin in apple is confirmed [1]. Condensed tannin is made up of polymerized catechins (Fig.1) [2]. Biological functions of condensed tannin such as procyanidin B compounds from grapes or cacao have well known [3,4], however, bioactivities of unripe apple polyphenol have not been examined. Therefore, unripe apple polyphenol has not been used as useful material until now. We examined biological activities including antioxidative actions or lipid metabolism regulation of unripe apple polyphenol in this study.

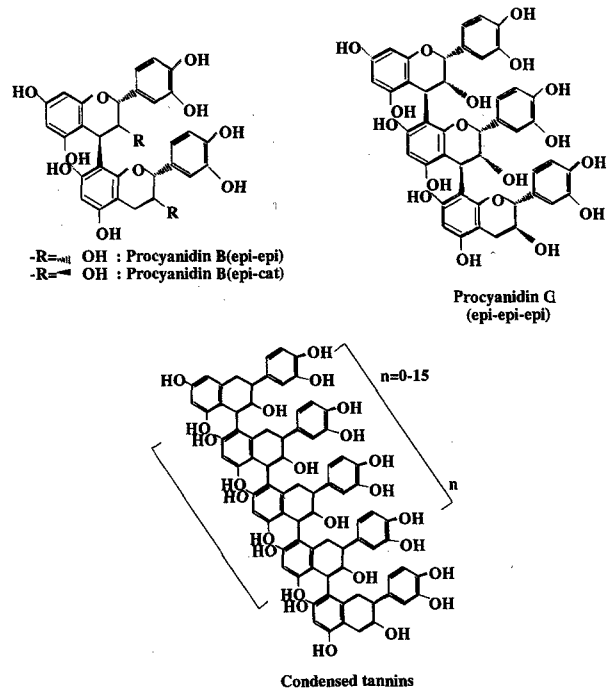


**Fig.1 Structure of Apple Condensed Tannin**

## 2. MATERIALS AND METHODS

### 2.1. Materials

Unripe apples were given from Hirosaki university, teaching and research center for bio-coexistence. Purified unripe apple polyphenol and condensed tannin were given from Nikka Whisky Co. Ltd.

### 2.2. Analysis of polyphenol

The level of polyphenol compounds from juice of 14 species of unripe apple was determined by the method of Folin-Ciocalteu [5].

### 2.3. Lipoprotein oxidation

Low density lipoprotein (LDL) was separated from fresh whole blood obtained from normolipidemic healthy male volunteers and concentrated by the method of Haverl et al [6]. LDL oxidation was performed for 48hr by dialysis in physiological saline containing 1μM $CuSO_4$ with or without antioxidative compounds. The formation of lipoperoxide in copper catalyzed lipoproteins was determined by LPO measurement kit (Determiner LPO, KYOWA Medix Co.).

### 2.4. Animal experiment

Male Sprague-Dawley rats (4 weeks old, CLEA Japan,

Tokyo, Japan) were divided into three groups of 7 or 8 each; one group was fed polyphenol-free diet (Control), second group was fed 0.25% apple polyphenol monomer and third group was fed 0.25% apple condensed tannin. Experimental diets were prepared according to the recommendation of the American Institute of Nutrition [7] and contained (weight %): casein, 20; α-cornstarch, 13.2; sucrose, 10; safflower oil, 10; cellulose, 5; mineral mixture (AIN-93G), 3.5; vitamin mixture (AIN-93), 1.0; L-cystine, 0.3; choline bitartrate, 0.25; tert-butylhydroquinon, 0.0014; and cornstarch to 100. After 26 days, rats were killed by withdrawing blood from the abdominal aorta under light diethyl ether anesthesia at night (01.00) for analysis of hepatic cholesterol metabolism. Feces were collected for 2 days, before killing, to analyze levels of neutral and acidic steroids in feces then lyophilized. The Hirosaki University Animal Policy approved this animal study, and rats were maintained according to the guidelines for the care and use of laboratory animals of Hirosaki University.

2.5. Enzyme activity

The activities of hepatic 3-hydroxy-3- methylglutaryl (HMG)-CoA reductase, cholesterol 7α-hydroxylase and Δ6 desaturase were measured by the methods of Shapiro et al. [8], Van Contfort et al. [9] and Svensson [10] respectively.

2.6.Lipids analyses

Liver and serum lipids were extracted and the concentrations of liver and serum phospholipid, liver and serum total cholesterol and liver triglyceride were measured as described previously [11]. The levels of serum triglyceride and HDL-cholesterol were measured using commercially available kits. Each lipid classes of liver were separated using preparative TLC, and their fatty acid composition was analyzed by gas-liquid chromatography [12]. The levels of fecal neutral and acidic steroids were analyzed by GLC [13].

Level of thiobarbituric acid reactive substances (TBARS) of an indicator of lipid peroxidation was measured according to the method of Yagi [14], although TBARS value does not necessarily exact lipoperoxide value. TBARS are expressed as malondialdehyde equivalents using 1,1,3,3-tetramethoxypropane as a standard. The levels of serum and liver α-tocopherol were measured by the method of Ueda and Igarash. [15]

2.7 Statistical analysis

Data were analyzed by Duncan's new multiple range test [16] to determine the exact nature of the difference ($P < 0.05$) among the groups.

## 3. RESULTS AND DISCUSSION

High level (900-2,200mg/ml) of polyphenol was contained in juice from 14 species of unripe apples. Especially, the juice from Kogyoku had polyphenol at the level of 2,200mg/ml. Moreover, unripe apples have polyphenol more than ten times that of ripe apple. Approximately 40-60% of polyphenol from unripe apple was the condensed tannin.

Peroxidation of low density lipoprotein (LDL) was inhibited by unripe apple polyphenol (Fig.2). This antioxidative activity was comparable to that of α-tocopherol or tea catechin.

Apple polyphenol from unripe apple polyphenol inhibited the generation of hydroxyl radical and superoxide anion. The scavenging activity of superoxide anion by unripe apple polyphenol was comparable to that of epigalocatechin gallate (Fig.3). Thus, unripe apple polyphenol exereted potent antioxidative activity as well as tea catechin [17].

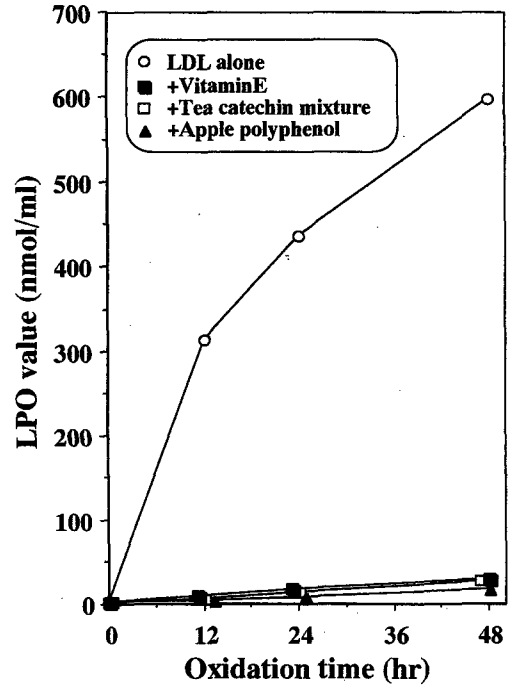


**Fig.2. Effect of unripe apple polyphenol on LDL oxidation. LDL (1mg/mL) alone or with antioxidative compounds (20μg) was dialyzed in 0.9% NaCl solution containing 2μM $CuSO_4$ at 4°C.**

Tissue TBARS values were lower in rats given unripe apple polyphenol than in those given control diet, particularly in rats fed apple condensed tannin. Dietary unripe apple polyphenol also lowered liver pospholipid peroxide level in the dose-dependent manner (data not shown).

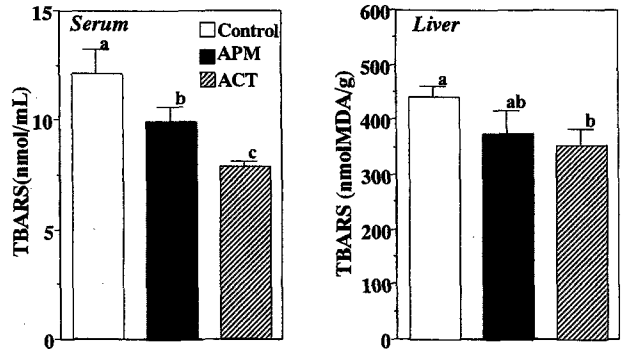


**Fig.3. Effect of dietary unripe apple polyphenol on tissue lipoperoxide levels. Data are presented as mean±SE for 7 rats in each group. [a]Values without a common superscript letter are significant at P<0.05. MDA, malondialdehyde; TBARS, thiobarubituric acid reactive substance. Control, polyphenol-free diet; APM, apple polyphenol monomer diet; ACT, apple condensed tannin diet.**

Dietary unripe apple polyphenol also lowered the consumption of α-tocopherol in tissue and this observation was significant in rats fed apple condensed tannin. These observations are shown in animal fed tea catechin [18] or wine polyphenol diet [19]. The metabolites of polyphenol may be scavenger of reactive oxygen species. Therefore the consumption of α-tocopherol seems to be regulated by scavenging action of reactive oxygen species by the metabolites of polyphenol. Thus, the positive intake of unripe apple polyphenol may strengthen biological antioxidative system.

Hepatic key enzymes of cholesterol (HMG-CoA reductase and cholesterol 7α-hydroxylase) and fatty acid (Δ6 desaturase) metabolism were modulated by dietary apple polyphenol (Fig.4). These modulations were significant in rats fed unripe apple condensed tannin.

Reflecting enzyme activity, level of liver cholesterol was lowered in rats fed unripe apple polyphenol, particularly in those fed apple condensed tannin (Fig.5). Same tendency was observed in triglyceride level. Level of serum cholesterol also lowered in rats fed apple polyphenol, particularly in those fed apple condensed tannin. Contrary to these observations, HDL-cholesterol levels in rats fed apple polyphenol were significantly higher than that of control group.

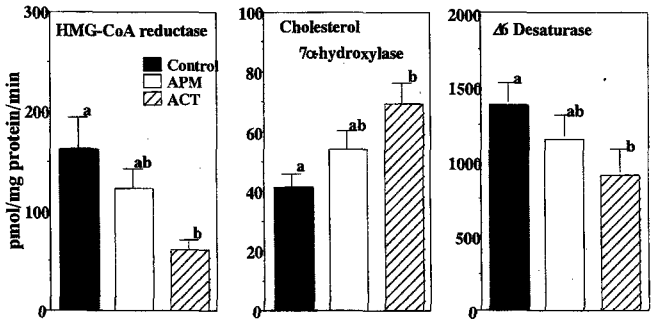


**Fig.4. Effect of dietary unripe apple polyphenol on hepatic key enzyme activities of cholesterol metabolism and linoleic acid desaturation in rats. Data are presented as mean±SE for 7 rats in each group. [ab]Values without a common superscript letter are significant at P<0.05. Abbreviations are the same as in Fig.3.**

Serum triglyceride level was significantly lower in rats fed polyphenol than those fed control diet. These obseravations are shown in animal fed tea catechin [20]. Δ6 Desaturation indices (dihomo-γ-linolenic acid+arachidonic acid/linoleic acid) of liver and serum lipids were lower in rats fed apple polyphenol than in those fed control group. This observation was significant in serum lipids. Thus, Δ6 desaturation of tissue lipids was modulated by intake of apple polyphenol. Thus, dietary unripe apple polyphenol, particularly consdensed tannin, may strongly modulate activities of fatty acid desaturase. We speculated that the mRNA level of hepatic Δ6 deaturase also may be modulated by dietary unripe apple polyphenol, although we do not have data. This action may affect eicosanoids production because level of arachidonic acid, which is substrate of eicosanoids, was lowered by intake of unripe apple polyphenol. In fact, Raso et al. found that various naturally occurring flavonoids inhibited $PGE_2$ production and cyclooxygenase-2 enzyme expression in vitro [21]. The latest research also indicated that flavonoids derived from cocoa inhibit 5-lipoxygenase, and contribute to a putative anti-inflammatory effect of cocoa products [22]. Therefore, these actions of apple polyphenol, particularly condensed tannin, may lead to the possibility of inhibition on inflammatory response.

The fecal excretion of acidic steroids was significantly higher in rats fed apple condensed tannin than those fed control diet. This action may lead to hypocholesterolemic response. The excretion levels of neutral steroids into feces also tended to be higher in rats fed polyphenol than in those fed control diet. However, level of coprostanol was significantly lowered in rats fed condensed tannin compared to other two groups. Therefore, dietary apple condensed tannin may affect the conversion of sterol by bacteria in intestine.

We observed various bioactivities of unripe apple polyphenol as described above, however, the bioavailability of apple polyphenol has not been elucidated until now. HPLC-ECD analysis of metabolized polyphenol in serum was performed after extraction of polyphenol compounds from serum and hydrolysis of polyphenol conjugates using β-glucronidase. Polyphenol dimers such as procyanidin $B_1$ or procyanidin $B_2$ were identified in serum of rats fed apple condensed tannin (Fig.6). Moreover, many unknown peaks were identified in serum of rat fed apple condensed tannin in HPLC/ESI/MS analysis, although these peaks were not found in serum of rats fed control diet. Therefore, a very small amount of condensed tannin may be absorbed from intestine and transfered into blood as intact form. Moreover, a part of condensed tannin may be decomposed compounds through some metabolic process and absorbed as small molecule. Further studies concerning the bioavailability of condensed tannin will need to elucidate exact mechanism of bioactivity of unripe apple polyphenol.

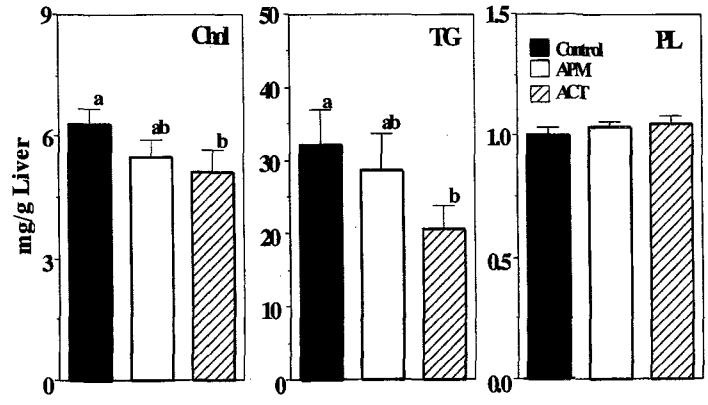


**Fig.5. Effect of dietary unripe apple polyphenol on liver lipids levels in rats. Data are presented as mean±SE for 7 rats in each group. [ab]Values without a common superscript letter are significant at P<0.05. Abbreviations are the same as in Fig.3.**

In conclusion, we found that unripe apple polyphenol, especially apple condensed tannin, has useful biological functions such as antioxidative action and regulative functions of lipid metabolism in this study. Therefore, the positive intake of unripe apple polyphenol may reduce the risk of some cardiovascular disease or inflammation.

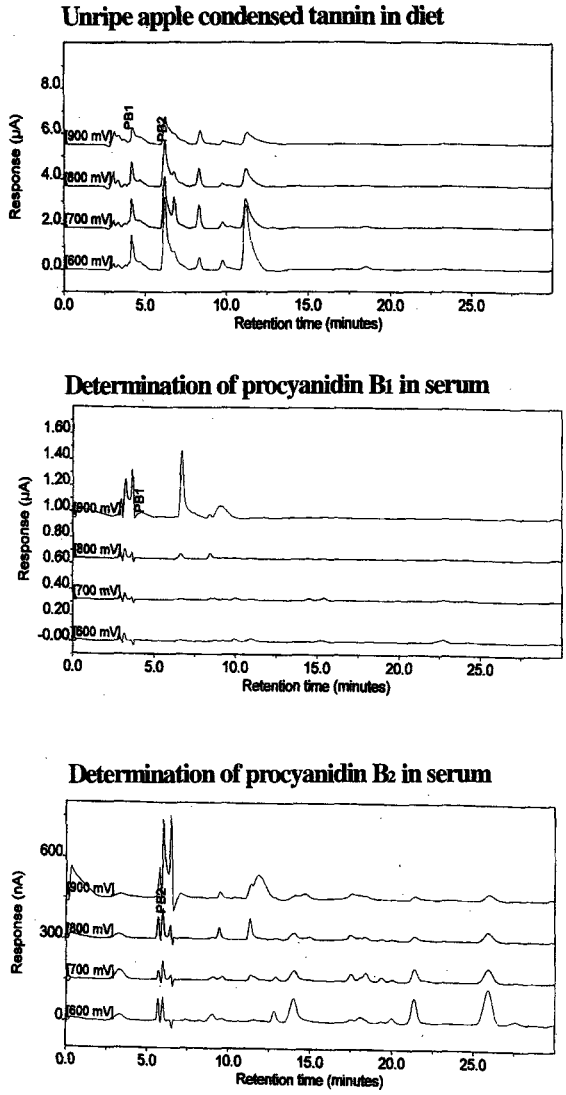


**Fig.6. HPLC chromatographic pattern of metabolized apple condensed tannin in rats.**

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