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Food and Chemical Toxicology 42 (2004) 959–967

Food and Chemical Toxicology

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The toxicology and safety of apple polyphenol extract

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Received 4 August 2003; accepted 10 February 2004

Abstract

Apple polyphenol extract has strong antioxidant activity and various physiological functions, and is used in Japan as a food additive and nutritional supplements. Here, we tested the consumption safety of Applephenon®, which is a polyphenol extract produced from unripe apples. The Ames test without S9 mixture revearled that Applephenon®, had slight mutagenicity at a high concentration of 2500 μg/plate; however, both chromosomal aberration test and the micronucleus test found no significant mutagenicity. Furthermore, an acute oral-toxicity test, and a 90-day subchronic-toxicity test showed no significant hematological, clinical, chemical, histopathological, or urinary effects at a dose of 2000 mg/kg. These results confirme that Applephenon® is safe and no toxic at average dietary level.

Keywords: Applephenon®; Unripe apple; Polyphenol extracts; Acute oral-toxicity test; 90-day subchronic oral-toxicity test; Mutagenicity

1. Introduction

Polyphenols are widely common secondary metabolites of plants, the content of which varies greatly between different species, and cultivars, and with maturity, season, region and yield. Polyphenols are classified according to their structure as phenolic acids derivatives, flavonoids, stilbenes or lignans (Harborne, 1988). They are further sub-divided on the basis of the hydroxylation of phenolic rings, glycosylation, acylation with phenolic acids and the existence of stereoisomers. They are present in many beverages (e.g., red wine and green tea) and foods (e.g., chocolate, grapes, and apples).

Several recent studies have reported physiological functionalities of polyphenols (Frankel et al., 1993; Hertog et al., 1993; Koga et al., 1999; Eberhardt et al., 2000; Richelle et al., 2000). For example, epidemiological studies have indicated that the consumption of red wine might prevent coronary heart disease, because it contains polyphenols that protect against the oxidation of LDL-cholesterol. These several findings have led to extensive research on the polyphenol content of human foods and beverages.

Apples (*Rosaceae Malus* sp.) have been one of the human diet since ancient times and are one of the most commonly consumed fruits in worldwide. They are eaten both raw and in processed products such as juice, cider, brandy, jam and vinegar. Apples contain many types of phenolic acid derivatives and flavonoids (flavan-3-ols, flavonols, procyanidins, chalcones, and anthocyanins) (Spanos et al., 1990; Lister et al., 1994; Ohnishi-Kameyama et al., 1997; Suárez et al., 1998; Mangas et al., 1999; Shoji et al., 2003).

Apple polyphenols have been reported to have various physiological functions including in vivo and clinical anti-allergic activity (Kanda et al., 1998; Akiyama et al., 2000; Kojima et al., 2000), in vivo anti-caries activity (Yanagida et al., 2000), and in vitro and in vivo inhibitory activity against some enzymes and receptors (Shoji et al., 2000; Saito et al., 2002).

Polyphenols have been shown to be safe in toxicological studies of green tea (Jain and Sethi, 1991; Yamane et al., 1996) and grape seed extract (Yu and Swaminathan, 1987; Bentivegna and Whitney, 2002; Yamakoshi et al., 2002; Wren et al., 2002; Erexson, 2003). However, the

Abbreviations: APTT, activation part thromboplastin time; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CPK, creatine phosphate kinase; Hgb, hemoglobin; IP, inorganic phosphorous; LDH, lactate dehydrogenase; LUC, large unstained cells; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; PCE, polychromatic erythrocyte; PT, prothrombin time; TC, total cholesterol.

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doi:10.1016/j.fct.2004.02.008

polyphenol content of apples differs from that of both green tea and grape seed. The main polyphenols in green tea are flavan-3-ols such as epigallocatechin gallate and catechin (Shahidi and Naczk, 1995), and those present in grape seed extract are mostly proanthocyanidins (Fuleki et al., 1997; Krueger et al., 2000; Waterhouse et al., 2000). By contrast, apple polyphenols contain mainly phenolic acid derivatives and other flavonoids, with the exception of procyanidins.

Therefore, to investigate the safety of apple polyphenol extract (AP) we carried out a range of toxicological tests on Applephenon® (Asahi Breweries Ltd., Japan), which is a polyphenol rich extract that is produced from unripe apples.

2. Materials and methods

2.1. Manufacturing process of apple polyphenol extract

Applephenon® was prepared from unripe apples, which contain higher concentrations of polyphenols than ripe apples (Lister et al., 1994; Mayr et al., 1995), using the method described by Tanabe et al. (1994). Briefly, unripe apples weighting ~5–25 g per fruit were crushed and pressed whilst 10% $Na_2S_2O_5$ solution was added. Pectolytic enzyme was used to clarify the juice obtained and the mixture was centrifuged and/or filtered with diatomaceous earth. The clarified juice was passed through a column with aromatic synthetic adsorbents. Subsequently, the column was washed with distilled water in order to remove sugars and organic acids. Thereafter, AP was eluted with approximately 50% ethanol and concentrated using an evaporator. Finally, the concentrated fraction was dried using a spray drier to obtain the AP as a brown powder.

2.2. Characterization of the AP

The total procyanidin content of the AP was determined using the method of Porter et al. (1986) with a procyanidin B2 standard (Funakoshi Co., Ltd., Japan). Reversed phase HPLC was used to detect the main apple polyphenols: chlorogenic acid, (+)-catechin, (−)-epicatechin, phloridzin, procyanidin B1, procyanidin B2 and procyanidin C1. This was carried out using an HPLC equipped with an L-6200 intelligent pump (Hitachi Ltd., Japan), an AS-2000 autosampler (Hitachi), an L-4200 UV-VIS detector (Hitachi) at 280 nm and an Inertsil ODS-3 (GL Sciences Inc., Japan) reversed phase column (250×4.6 mm i.d.) at 40 °C. Briefly, a mixture of 10 mM KH_2PO_4 solution (pH 2.0) and methanol was used as the mobile phase with a flow rate of 1.0 ml/min. An eluent of 10% methanol was used for the first 10 min, followed by a linear gradient from 10 to 50% methanol for 40 min. The methanol concentration was held at 50% for 15 min, then returned to the initial conditions to re-equilibrate for 10 min.

Procyanidins were analyzed according to the degree of polymerization by normal phase HPLC using the apparatus described above and an Inertsil SIL column (250×4.6 mm i.d.; GL Science Inc., Tokyo, Japan) at 25 °C. A mixture of hexane, methanol and ethyl acetate was used as the mobile phase, with a flow rate of 1.0 ml/min. The initial eluent was a 7:3:1 mixture of hexane, methanol and ethyl acetate, followed by a linear gradient to 2:3:1 for 50 min. Detection was carried out at 280 nm. The injection volume of 3 mg/ml Applephenon® solution was 10 μl. The procyanidin standards were prepared according to the methods of Yanagida et al. (2000b).

The polyphenol profiles of AP analyzed by reversed and normal phase HPLC are shown in Fig. 1. Applephenon® was a complex mixture, mostly made up of polyphenols. It contained 63.8% procyanidins, which comprised 11.1% dimers, 12.3% trimers, 8.7% tetramers, 5.9% pentamers, 4.9% hexamers and 20.9% other polymers. It also contained 12.4% flavan-3-ols (monomers), 6.5% other flavonoids and 10.8% non-flavonoids.

The structure formulae of the main components of the AP are shown in Fig. 2. In addition to polyphenols, it also contained 1.8% moisture, 2.1% protein and 0.4% ash. The extract specifications are summarized in Table 1.

2.3. Toxicological studies

Mutagenicity tests, including a reverse mutation test in bacteria and a chromosomal aberration test in cultured mammalian cells, were carried out by Fuji Biomedix Co., Ltd. (Kitakoma, Yamanashi, Japan). A rat micronucleus test and an acute oral toxicity test were performed at the Mitsubishi Chemical Safety Institute Ltd. (Minato, Tokyo, Japan). A 90-day subchronic toxicity test was carried out by Panapharm Laboratories Co., Ltd. (Uto, Kumamoto, Japan). All of the animals involved were maintained on a standard diet.

2.4. Mutagenicity test

2.4.1. Ames test

The *Salmonella typhimurium* strains TA 100 (Japan Bioassay Research Center) and TA98, TA1535 and TA1537 (Institute of Environmental Toxicology) and the *Escherichia coli* strain WP2*uvr*A (Japan Bioassay Research Center) were used in the reverse mutation test, both with and without S9 mixture. Testing was carried out according to the methods of Ames et al. (1975) using AP concentrations of 156, 313, 625, 1250, 2500 and 5000 μg/plate.

2.4.2. In vitro chromosomal aberration test with CHL cells

A chromosomal aberration test was performed using CHL/IU cells (Dainippon Pharmaceutical Co., Ltd) in

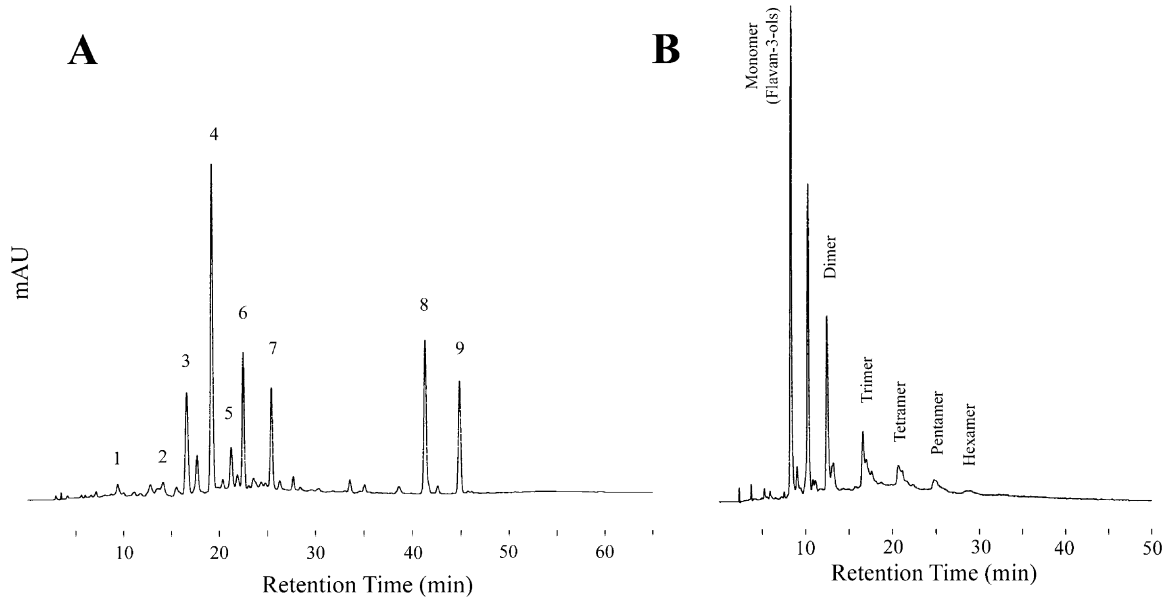

Fig. 1. Profiles of apple polyphenol extract, Applephenon® by reversed phase (A) and normal phase (B) HPLC. **1**, procyanidin B1; **2**, (+)-catechin; **3**, procyanidin B2; **4**, chlorogenic acid; **5**, procyanidin C1; **6**, (−)-epicatechin; **7**, *p*-coumaroyl quinic acid; **8**, phloretin-xyloglucoside; **9**, phloridzin.

both a non-activated and an activated system, with and without S9 mixture, for 6 h, and also in a non-activated system without S9 mixture for 25 h, at doses of 0.039, 0.078, 0.156, and 0.313 mg/ml.

2.4.3. Micronucleus test

A micronucleus test was carried out using 7-week old male Sprague–Dawley (Crj: CD (SD) IGS) rats. Each group contained five males. The AP was tested at doses of 500, 1000 and 2000 mg/kg by double administration (Hayashi et al., 1983). Each sample was dissolved in distilled water and 10 ml/kg was administered orally. Genotoxicity was evaluated by measuring the frequency of polychromatic erythrocyte (PCE) cells in bone marrow. Cyclophosphamide (Sigma, USA) was used as a positive control.

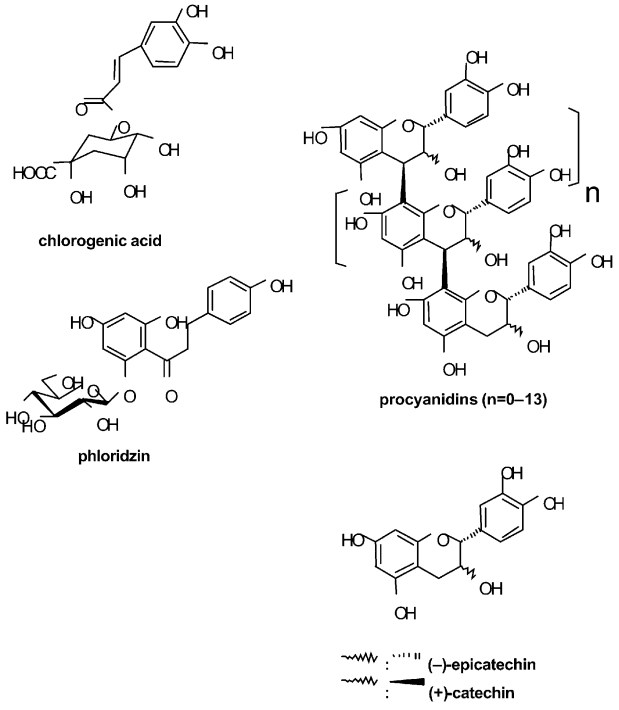

Fig. 2. Structural formulae of the main components of Applephenon®.

Table 1
Specifications of apple polyphenol extract, Applephenon®

Parameters	Specification	Methods
Total procyanidins	63.8%	Porter et al. (1986)
Dimer fr.	11.1%	Normal phase HPLC
Trimer fr.	12.3%	Normal phase HPLC
Tetramer fr.	8.7%	Normal phase HPLC
Pentamer fr.	5.9%	Normal phase HPLC
Hexamer fr.	4.9%	Normal phase HPLC
Over Polymer fr.	20.9%	Normal phase HPLC
Other flavonoids	18.9%	Reversed phase HPLC
Non-flavonoids	10.8%	Reversed phase HPLC
Moisture	1.8%	Air oven
Ash	0.4%	Ignition at 550 °C
Protein	2.1%	Kjeldahl
Metals		
Total heavy metals as lead	<20 mg/kg	Sodium sulfide colorimetric method
Arsenic	<2 mg/kg	Atomic absorption spectroscopy
Microbiological analysis		
Total plate count	<300 CFU/g	Standard agar
Yeast and mold	<30 CFU/g	Potato dextrose agar
Coliform	N.D.	BGLB broth
Fungal toxin		
Patulin	<0.05 μg/g	HPLC

N.D.: not detected; CFU : Colony forming units.

2.5. Acute oral toxicity test

The AP was administered to male ($n=5$) and female ($n=5$) 5-week old Sprague–Dawley (Crj: CD) rats (Charles River Japan Inc., Japan) at a dose of 2000 mg /kg. The AP was dissolved in 0.5% CMC-Na solution (Iwai Chemicals Co., Ltd., Tokyo, Japan) and 10 ml/kg of the sample was injected intragastrically by direct stomach intubation. Male rats weighed 119–133 g and female rats weighed 100–106 g. The rats were not fed for 3 h following administration.

General condition and body weight were monitored for 14 days after administration. On day 14, all animals were sacrificed and subjected to necropsies. The following organs were examined: heart, spleen, trachea, lungs, stomach, duodenum, ileum, jejunum, cecum, colon, rectum, liver, kidneys, urinary bladder, testis, pituitary, thyroids, parathyroid, adrenals gland, brain, submaxillary glands, thymus, seminal vesicle, prostate, ovary, and uterus.

2.6. 90-day subchronic oral toxicity test

The AP was administered to groups of 6-week old male ($n=10$) and female ($n=10$) Sprague–Dawley (Crj: CD) rats (Charles River Japan Inc., Japan) at the concentrations of 0, 500, 1000, and 2000 mg/kg by intragastric injection of 10 ml/kg of the sample using direct stomach intubation. Each group consisted of ten rats. Mean body weight of male rats on the first day of the study was 196.8 ± 5.7 g and that of female rats was 148.2 ± 7.7 g. Body weight was subsequently recorded once per week, and immediately before necropsy.

The general physical condition of each animal was observed thoroughout the test period, and food consumption was measured once per week. On day 90, a blood sample was collected from the abdominal aorta of each animal, under sodium pentobarbital induced anesthesis. Analysis of the following hematological parameters was carried out: leukocytes, erythrocytes, hemoglobin (Hgb), hematocrit, platelets, mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), differential leukocyte count, reticulocytes, prothrombin time (PT), activated partial thromboplastin time (APTT), eosinophils, neutrophils, lymphocytes, basophils, monocytes and large unstained cells (LUC).

In addition, a portion of each blood sample was centrifuged to obtain plasma ($1870g\times10$ min at 4 °C) for analysis of the following clinical chemistry parameters: total protein, α1-globulin, α2-globulin, β-globulin, γ-globulin, albumin, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-GTP, alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine phosphate kinase (CPK), total cholesterol (TC), triglycerides, phospholipids, glucose, blood urea nitrogen (BUN), creatinine, inorganic phosphorous (IP), Ca^{++}, Mg^{+}, Na^{+}, K^{+} and Cl^{-}.

The urine produced by each animal was collected over a 24 h period at day 90, in order to analyze: urinary volume, osmotic pressure, specific gravity, Na^{+}, K^{+} and Cl^{-} levels.

Autopsies were performed on all animals at the end of the study and the weights of the following organs were measured: brain, pituitary, submaxillary glands, thyroids, heart, lungs, thymus, liver, spleen, kidneys, adrenal glands, seminal vesicle, prostate, testes, ovary and uterus.

In the control and the high-dose groups, hsistopathological examinations were made of the following organs and tissues: oral cavity, tongue, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, submaxillary gland, sublingual gland, parotid gland, liver, pancreas, nasal cavity, trachea, lung, thymus, submaxillary lymph node, mesenteric lymph node, pancreatic lymph node, Peyer's patch, spleen, bone marrow (from the sternum and femur), heart, aorta, urinary bladder, testis, epididymis, prostate, seminal vesicle, ovary, oviduct, uterus, vagina, mammary gland, pituitary, thyroid, parathyroid, adrenal gland, brain, spinal cord, optic nerve, sciatic nerve, eye, harderian glands, Zymbal's gland, skeletal muscle, sternum, femur and integument. All samples were fixed in 10% neutral buffer formalin and stained with hematoxylin-eosin. The eye, optic nerve, and Harderian gland were pre-fixed in 2.5% glutaraldehyde solution, and the nasal cavity, testis, and epididymis were pre-fixed in Bouin's solution.

2.7. Statistical analysis

The data obtained from the mutagenicity tests were statistically analyzed using the Student's *t*-test. Data from the micronucleus test were analyzed using the method of Kastenbaum and Bowman to determine the frequency of micronucleated PCE cells, and the Student's *t*-test was used to analyze the number of PCE cells among the erythrocytes.

One-way parametric ANOVA with Dunnett's test was used to examine the organ weight, body weight, food consumption, hematological and blood chemistry data produced by the oral-toxicity test. Steel's test was used to analyze the urinary data, Fisher's exact test was used to evaluate the autopsy data and the Mann–Whitney U-test was used to analyze the histopathological data.

3. Results

3.1. Mutagenicity test

In the reverse mutation test without S9 mixture, *S. typhimurium* TA98 showed a slight increase in the

number of revertants at a dose of 2500 μg/plate. However, none of the other bacterial strains tested (TA 100, TA1535, WP2*uvrA* and TA1537) showed an increase in revertants with or without S9 mixture, at a dose of 5000 μg/plate (Table 2).

The chromosomal aberration test using CHL/IU mammalian cells did not reveal any abnormalities, with or without S9 mixture, associated with the AP at a dose of 0.313 mg/ml (data not shown).

No significant differences in body weight or other clinical data were found between the treated rats and controls in the micronucleus test. The frequency of micronucleated PCE cells observed in the bone marrow of rats that were treated with Applephenon® at a dose of 2000 mg/kg did not significantly differ from the control group (data not shown).

3.2. Acute oral toxicity test

All rats treated with the AP at a dose of 2000 mg/kg survived the 14-day observation period; the weight of all animals increased during this time. No significant changes were observed in any organs at the necropsy on day 14 (data not shown). Therefore, the acute oral minimum fatal dose of Applephenon® for Sprague–Dawley rats is >2000 mg/kg body weight.

3.3. 90-day subchronic oral toxicity test

Administration of the AP produced no clinical signs, adverse effects or deaths in the animals tested. Body weight gain was unaffected by the AP during the test period. Food consumption was slightly increased on day 64 in females receiving a dose of 1000 mg/kg, and on days 8, 15, 29, 36 and 43 in males receiving a dose of 2000 mg/kg. However, these findings were within the normal range and did not affect the overall changes in body weight. These differences were therefore attributed to normal biological variation.

Data from the hematological, clinical chemistry and urinary tests are shown in Tables 3–5, respectively. There were statistically significant differences between male and female rats in several parameters. However, these were within the normal range of physiological background data and were not correlated with the AP dosage.

Gross necropsy findings did not reveal adverse changes in any of the organs examined. Statistically significant differences were found in the lungs of males given a dose of 1000 mg/kg, however these changes were within the normal physiological range (Table 6). Histopathological examinations of the organ tissue did not reveal any changes that were associated with the AP.

4. Discussion

Previous studies have focused on the taste, color, stabilities and interaction of polyphenols with proteins and carbohydrates in foods and beverages. Recently, as polyphenols have been reported to possess variable physiological functions, polyphenol extracts obtained from various plants are used as a supplements and food ingredient.

Although catechins in green tea and proanthocyanidins in grape seeds extract have been reported to be safe for use as food supplements and ingredients, limited safety testing has been carried out on the phenolic acid derivatives and minor flavonoids that are present in apples.

The mutagenicity and genotoxicity of Applephenon® were evaluated using the Ames, chromosomal aberration and micronucleus tests. In the reverse mutation Ames test, only one strain of *S. typhimurium* (TA98) showed a slight increase in the number of revertants at the highest dose tested. Previously, catechins of green tea and proanthocyanidins of grape seeds extracts have shown no toxicity in mutagenicity tests (Yu and Swaminathan, 1987; Takahashi et al., 1999; Duarte-Silva et al., 2000; Yamakoshi et al., 2002). Furthermore, chlorogenic acid, which is the main phenolic acids derivatives in apple, dose not significantly increase mutagenic activity with

Table 2
Mutagenic activity (means) of Applephenon® in *S. typhimurium* TA 98, TA 100, TA1535, TA1537 and *E. coli* WP2*uvrA*

Concentration (μg/plate)	Without S9					With S9				
	TA100	TA1535	WP2*uvrA*	TA98	TA1537	TA100	TA1535	WP2*uvrA*	TA98	TA1537
0	147	10	23	24	7	152	7	19	32	8
156	138	8	24	22	10	148	8	23	36	13
313	141	8	24	24	7	139	9	27	30	7
625	140	11	27	34	10	142	5	30	29	10
1250	133	6	27	29	8	133	5	29	39	7
2500	132	9	31	54[a]	13	133	4	21	41	8
5000	168	7	28	71[a]	9	141	11	31	59	9

[a] 2 > counts in sample/counts in control.

Table 3
Hematological findings (means±S.D.) in rats after 90 days subchronic oral administration of Applephenon®

Study day	Males				Females			
	Control	500 mg/kg	1000 mg/kg	2000 mg/kg	Control	500 mg/kg	1000 mg/kg	2000 mg/kg
Leukocytes (10^3/μl)	8.37±2.93	9.21±1.22	9.27±1.72	10.58±2.74	4.62±0.97	6.08±1.87	5.32±1.10	6.49±1.54
Erythrocyes (10^4/μl)	836±56	865±31	842±40	821±18	770±27	766±24	736±30a	740±31
Reticulocyte (10^4/μl)	20.4±7.1	15.1±2.0	14.7±2.4	14.7±2.1	14.7±2.7	13.2±2.1	13.0±2.0	10.7±2.7[b]
Platelets (10^4/μl)	108.8±12.5	108.0±5.7	104.7±12.4	112.4±18.2	107.7±7.0	110.6±8.2	111.9±10.2	115.0±14.1
Hgb (g/dL)	14.5±0.3	15.3±0.5[b]	15.1±0.5[a]	14.6±0.5	14.4±0.5	14.5±0.5	13.9±0.5	14.0±0.4
Hematocrit (%)	42.6±1.2	44.8±1.4[b]	43.7±1.5	42.5±1.2	41.1±1.4	41.4±1.4	39.6±1.1	39.6±1.4
MCV (fL)	51.2±3.2	51.7±1.2	52.0±1.2	51.7±1.3	53.3±1.5	54.0±1.0	53.9±1.8	53.5±1.3
MCH (pg)	17.4±1.0	17.7±0.4	17.9±0.5	17.8±0.6	18.7±0.5	19.0±0.4	18.9±0.7	18.9±0.5
MCHC (g/dL)	34.0±0.7	34.2±0.3	34.5±0.3	34.3±0.4	35.0±0.3	35.1±0.5	35.1±0.3	35.4±0.5
PT (sec)	14.6±1.9	15.0±1.7	14.9±1.2	16.7±3.2	11.7±0.4	12.2±0.5[a]	11.8±0.4	11.9±0.4
APTT (sec)	23.9±1.7	23.5±1.5	23.4±1.0	23.7±2.0	20.0±1.5	20.3±1.2	19.3±1.0	18.9±1.4
Eosinophis (10^2/μl)	1.4±0.5	1.6±0.4	1.8±0.6	1.9±0.7	0.8±0.4	0.9±0.4	1.0±0.4	1.1±0.3
Neutrophils (10^2/μl)	15.4±5.0	17.4±4.6	20.5±6.1	27.6±9.6[b]	7.9±2.6	11.6±6.7	11.4±4.7	16.5±7.4[b]
Lymphocytes (10^2/μl)	65.0±26.5	70.7±9.7	68.1±14.1	73.4±22.3	36.3±8.4	46.7±13.1	39.5±8.5	45.4±10.4
Basophils (10^2/μl)	0.1±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0±0	0.1±0.1[a]	0.1±0	0.1±0[a]
Monocytes (10^2/μl)	1.4±0.3	1.9±0.4[a]	1.8±0.5	2.2±0.8[a]	0.8±0.2	1.0±0.6	0.8±0.5	1.4±0.6
LUC (10^2/μl)	0.4±0.2	0.4±0.1	0.4±0.2	0.6±0.3	0.3±0.2	0.5±0.2	0.4±0.2	0.5±0.3

Hgb, hemoglobin; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; APTT, activated partial thromboplastin time; PT, prothrombin time; LUC, large unstained cells.

[a] $P<0.05$.

[b] $P<0.01$.

Table 4
Clinical chemistry findings (means±S.D.) in rats after 90 days subchronic oral administration of Applephenon®

Study day	Males				Females			
	Control	500 mg/kg	1000 mg/kg	2000 mg/kg	Control	500 mg/kg	1000 mg/kg	2000 mg/kg
Total protein (g/dl)	5.9±0.2	5.8±0.2	5.5±0.2[b]	5.3±0.3[b]	6.3±0.3	6.2±0.3	6.3±0.4	5.5±0.4[b]
α1-Globulin (g/dl)	1.11±0.13	1.08±0.11	0.94±0.09[b]	0.81±0.13[b]	0.98±0.09	0.94±0.11	0.92±0.05	0.75±0.08[b]
α2-Globulin (g/dl)	0.37±0.03	0.37±0.04	0.36±0.04	0.37±0.04	0.31±0.05	0.33±0.03	0.31±0.04	0.29±0.01
β-Globulin (g/dl)	0.94±0.04	0.91±0.09	0.87±0.06	0.80±0.08[b]	0.87±0.09	0.86±0.05	0.80±0.09	0.73±0.06[b]
γ-Globulin (g/dl)	0.34±0.08	0.33±0.06	0.33±0.03	0.29±0.06	0.35±0.08	0.39±0.06	0.34±0.07	0.32±0.09
Alubumin (g/dl)	3.09±0.08	3.07±0.11	3.02±0.18	3.02±0. 19	3.79±0.30	3.72±0.32	3.93±0.36	3.41±0. 27
A/G ratio	1.12±0.07	1.15±0.08	1.21±0.11	1.34±0.15[b]	1.52±0.15	1.49±0.16	1.66±0.15	1.64±0.08
$T_{οταλ}$-Bilirubin (mg/dl)	0±0	0±0	0±0	0±0	0.1±0.1	0±0.1	0±0.1	0±0[a]
AST (IU/L)	89±12	84±9	84±13	88±16	88±20	86±19	112±102	73±13
ALT (IU/L)	23±4	23±3	22±4	26±9	24±8	23±9	35±40	15±4[a]
γ-GTP (IU/L)	0.2±0.3	0.1±0.2	0.2±0.3	0.2±0.2	0.4±0.4	0.7±0.6	0.3±0.2	0.2±0.2
ALP (IU/L)	174±23	161±30	161±27	148±35	83±20	91±28	78±18	78±25
LDH (IU/L)	76±18	89±31	79±30	72±16	76±19	70±24	82±58	55±16[a]
CPK (IU/L)	78±16	87±18	103±99	84±31	55±12	67±40	54±7	59±8
TC (mg/dl)	69±7	66±17	64±4	72±14	84±7	77±9	84±12	68±13[b]
Triglycerides (mg/dl)	48±20	64±38	52±10	53±16	25±10	29±35	33±19	21±8
Phospholipids (mg/dl)	117±8	113±23	110±5	122±19	162±12	151±21	168±30	133±21[a]
Glucose (mg/dl)	130±12	131±8	134±15	125±14	132±10	124±11	132±13	114±11[b]
BUN (mg/dl)	16.3±1.9	15.5±2.1	14.1±1.5	12.7±2.2[b]	17.5±2.8	17.9±1.7	16.2±2.2	15.2±4.9
Creatinine (mg/dl)	0.5±0.1	0.4±0.1	0.4±0.1	0.4±0.1	0.5±0.1	0.5±0.1	0.5±0.1	0.5±0.1
IP (mg/dl)	6.8±0.7	6.8±0.7	6.6±0.5	6.8±0.5	5.2±0.8	5.8±0.8	5.5±0.7	5.5±0.7
Ca^{++} (mg/dl)	10.1±0.2	10.2±0.3	10.0±0.4	9.9±0.3	10.3±0.2	10.4±0.3	10.3±0.4	9.9±0.3[a]
Mg^{++} (mg/dl)	2.2±0.2	2.2±0.2	2.1±0.2	2.3±0.2	2.5±0.2	2.5±0.2	2.5±0.2	2.5±0.2
Na^+ (mEq/dl)	146.4±1.2	146.6±0.5	146.8±1.1	146.2±1.2	144.3±0.8	144.5±1.1	144.7±1.4	144.7±1.4
K^+ (mEq/dl)	4.35±0.24	4.33±0.12	4.24±0.16	4.35±0.21	4.17±0.33	4.16±0.37	4.18±0.16	4.08±0.21
Cl^- (mEq/dl)	107.1±1.2	107.1±1.0	107.1±1.4	108.1±1.0	108.2±2.0	107.9±2.7	108.0±1.7	108.4±1.8

AST, aspartate aminotransferase; ALT, alanie aminotranseferase; ALP, mean cell hemoglobin; LDH, Lactate dehydrogenase; CPK, Creatine Phosphate Kinase; TC, total cholesterol; BUN, blood urea nitrogen; IP, inorganic phosphorous; Ca^{++}, calcium; Mg^{++}, magnesium; .Na^+, sodium; K^+, potassium; Cl^-, chloride.

[a] $P<0.05$.

[b] $P<0.01$.

Table 5
Urinary findings (means±S.D.) in rats after 90 days subchronic oral administration of Applephenon®

Study day	Males				Females			
	Control	500 mg/kg	1000 mg/kg	2000 mg/kg	Control	500 mg/kg	1000 mg/kg	2000 mg/kg
Urine volume (ml/24 h)	15.5±3.1	18.4±8.1	15.1±2.8	16.7±6.8	17.9±9.0	10.4±4.0	9.8±3.6[a]	5.4±1.9[a]
Osmotic pressure (Osm/kg)	1.490±0.261	1.441±0.316	1.520±0.181	1.364±0.436	1.023±0.264	1.390±0.340	1.458±0.506[a]	1.808±0.412[b]
Specific gravity	1.044±0.009	1.046±0.011	1.050±0.006	1.050±0.016	1.032±0.008	1.046±0.012	1.051±0.018[a]	1.069±0.016[b]
Na^+ (mEq/24 h)	1.212±0.458	1.199±0.379	1.246±0.295	0.952±0.336	1.180±0.317	0.854±0.354	0.809±0.344[a]	0.539±0.218[b]
K^+ (mEq/24 h)	2.693±0.452	3.216±0.517	3.164±0.415	2.943±0.659	2.362±0.575	1.905±0.544	1.870±0.683	1.314±0.480[b]
Cl^- (mEq/24 h)	1.696±0.425	1.860±0.404	2.064±0.461	1.919±0.520	1.621±0.457	1.209±0.415	1.202±0.541	0.836±0.362[b]

[a] $P<0.05$.
[b] $P<0.01$.

Table 6
Organ weights (means±S.D.) in rats after 90 days subchronic oral administration of Applephenon®

Study day	Males				Females			
	Control	500 mg/kg	1000 mg/kg	2000 mg/kg	Control	500 mg/kg	1000 mg/kg	2000 mg/kg
Final body weights (g)	528.6±38.7	534.7±50.5	531.8±57.1	502.1±47.1	285.8±21.7	280.0±24.4	277.1±28.3	264.4±17.2
Brain (g)	2.17±0.11	2.21±0.08	2.17±0.09	2.20±0.07	1.99±0.07	1. 97±0.08	1.96±0.06	1.94±0.06
Pituitary (mg)	13.5±2.0	14.2±1.4	13.6±1.6	13.4±1.4	15.9±1.5	15.4±1.3	16.7±1.7	15.3±1.2
Submaxillary glands (g)	0.71±0.05	0.72±0.10	0.69±0.07	0.71±0.05	0.45±0.05	0.44±0.03	0.40±0.06	0.44±0.06
Thyroids (mg)	23.8±3.9	24.4±5.1	24.6±2.5	22.6±3.1	16.4±3.7	17.4±2.3	17.0±1.8	16.3±3.6
Heart (g)	1.53±0.15	1.58±0.11	1.60±0.10	1.58±0.18	0.95±0.08	0.97±0.11	0.92±0.09	0.89±0.09
Lungs (g)	1.49±0.17	1.54±0.13	1.65±0.15[a]	1.56±0.09	1.11±0.07	1.11±0.08	1.08±0.07	1.15±0.11
Thymus (g)	0.30±0.09	0.27±0.06	0.28±0.06	0.25±0.06	0.26±0.05	0.26±0.07	0.27±0.04	0.26±0.06
Liver (g)	14.14±1.49	14.37±1.81	14.28±2.27	13.45±1.54	7.35±0.48	7.26±0.70	7.31±0.73	7.57±0.65
Spleen (g)	0.94±0.09	0.86±0.14	0.93±0.10	0.85±0.07	0.54±0.08	0.58±0.07	0.53±0.07	0.58±0.09
Kidneys (g)	3.36±0.33	3.43±0.34	3.54±0.57	3.37±0.35	1.82±0.16	1.81±0.09	1.86±0.12	1.81±0.14
Adrenals (mg)	59.2±8.1	63.2±8.4	61.5±7.2	61.4±8.3	64.7±7.4	73.1±11.2	65.5±10.7	69.1±11.9
Seminal (g)	2.04±0.26	2.17±0.32	2.01±0.24	2.08±0.32				
Prostate (g)	1.24±0.14	1.36±0.16	1.19±0.22	1.25±0.19				
Testes (g)	3.53±0.19	3.46±0.22	3.61±0.20	3.54±0.15				
Ovaries (mg)					75.7±9.6	73.7±10.4	71.1±10.9	74.7±12.7
Uterus (g)					0.75±0.09	0.66±0.06	0.74±0.07	0.74±0.24

[a] $P<0.05$.

or without S9 mixture (Hossain et al., 1976; Stich et al., 1981). Therefore, it is likely that other minor components in the AP caused the slight increase observed in revertants.

We also carried out a chromosomal aberration test using CHL/IU cells with or without the S9 mixture, and a micronucleus test in rats. The AP did not cause any abnormalities in both the non-activated and activated system. Furthermore, as Popp and Schimmer (1991) reported, the administration of AP did not significantly alter changes in body weight, clinical signs or the frequency of micronucleated PCE cells in rat bone marrow. These results strongly indicate that the AP did not induce mutagenicity.

Oral acute toxicity study revealed the lethal dose of Applephenon® to be >2000 mg/kg in both male and female rats. Acute oral lethal value of grape seed extract reported to be approximately 4000 mg/kg (Yamakoshi et al., 2002). These results were confirmed a 90-day subchronic oral toxicity study using Sprague–Dawley rats. No abnormal symptoms or effects on body weight, food consumption, clinical chemistry or histopathological parameters were associated with the AP at a dose of 2000 mg/kg. The acute oral lethal value of grape seed extracts is >4000 mg/kg and grape seed proanthocyanidins were shown to be non-toxic in a 90-day subchronic study (Wren et al., 2002; Yamakoshi et al., 2002).

In generally, polyphenols are poorly absorbed and are partially metabolized by the intestinal microflora from intestine. After absorption, they are transported to the liver, where they form glucuronide and/or sulfate or methyl conjugates. The absorbed polyphenols and their metabolites might reveal various functionalities in vivo (Santos-Buelga and Scalbert, 2000; Scalbert and Williamson, 2000), which are eliminated normally thorough the urinary and fecal systems.

Total intake of polyphenols can be calculated using data on the polyphenol concentration and consumption

of foods and beverages. Average polyphenols of consumption was recently estimated as 18–31 mg/day in the Spanish diet, with wine and apples being the main source (De Pascual-Teresa et al., 2000). Similarly, average polyphenol consumption was estimated to be 50 mg/day in the Dutch diet (Arts et al., 2001). Scalbert and Williamson (2000) reported total dietary intake of polyphenols to be approximately 1 g/day, regardless of the methods of polyphenol concentration used such as HPLC or the Folin–Ciocalteu method.

Apple has been reported to be 1217 and 212 mg of polyphenols equivalent as chlorogenic acid/l in *Jonathan* and *Golden Delicious*, respectively (Cilliers et al., 1990). Scalbert and Willamson (2000) reported apple contained 440 mg as catechin/200 g of apple. Our analysis of the total polyphenol content in apples cultivated in Japan (*Fuji*, *Jonagold*, *Jonathan*, *Ohrin*, *Starcking Delicious*, *Tugaru*) revealed 2365 ± 525 mg/l as chlorogenic acid.

Assuming an average polyphenol intake 1 g of per day, about 2.3 apples (200 g weighting per fruit) or about 420 ml of apple juice would need to be consumed. As our result, the 90-day subchronic study was equivalent to the human consumption of 272 apples per 60 kg body weight per day. This is an 120-fold higher dose than the estimated average human dietary intake. Therefore, the average total dietary intake of polyphenols for human is estimated to be considerably less than those levels that were shown to be safe in our analyses.

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