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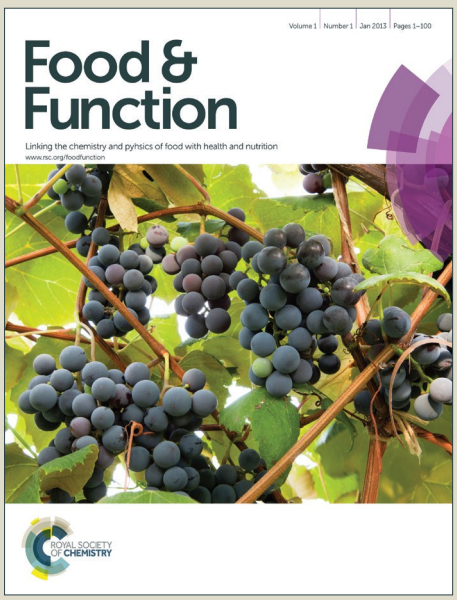


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# Safety Evaluation and Antihyperlipidemia Effect of Aqueous Extract from Fermented Puerh Tea

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**Abstract:**

Fermented puerh tea, undergone a long-time of secondary oxidization and fermentation, has been more and more popular in recent years. In the present paper, the safety evaluation of aqueous extract from fermented puerh tea (EFPT) was investigated, including oral acute toxicity study in rats and mice, mutation tests, mouse micronucleus test, mouse sperm abnormality test and 30 days feeding study in rats. Meanwhile, the antihyperlipidemia effect of EFPT was investigated as well. It was found that the oral maximum tolerated dose of EFPT was more than 10.0 g/kg body weight both in rats and mice. And it had no mutagenicity judged by negative experimental results of mutation test. No abnormal symptoms and clinical signs or deaths had been found in rats in each group throughout the experiments. In addition, EFPT in this study showed certain effects on hyperlipidemia.

**Keywords:** Aqueous extract, Safety, Toxicology, Fermented puerh tea, Antihyperlipidemia effect

## 1. Introduction

Puerh tea, a kind of special and prevalent Chinese tea, originally produced in Yunnan province of China about 1700 years ago. This tea is made from large leaves of *C. sinensis O. kuntze var. assamica Kitamura*.[1] Generally, on the basis of fermentation procedures, puerh tea can be divided into two types: non-fermented puerh tea (puerh green tea) and fermented puerh tea (puerh black tea). Fermented puerh tea undergoes a long-time of secondary oxidization and fermentation with microorganism such *as Aspergillus niger* (post-fermented), resulting in a unique type of tea.[2] Catechins are oxidized into quinone by polyphenol oxidase and then condensed to form theaflavin, theabrownin, and other high molecular components during the fermentation process.[3] These are regarded as the important biological components of fermented puerh tea, which provide multiple positive benefits for animal and human health, such as antihypoglycemic effect,[4-6] anti-oxidative activity,[7,8] anti-obesity effect,[9-11] anti-constipation effect,[12] and anti-cancer effect.[13-15]

Though the health benefits of puerh tea are focused worldwide, issues of safety should not be neglected when administered at a high dose as concentrated extracts or products.[16] Previously, Wang[17] reported acute and sub-chronic oral toxicities of puerh black tea in Sprague Dawley rats, and then evaluation of reproductive and developmental toxicities of puerh black tea extract in Sprague Dawley rats was investigated in another study.[18] Such results were helpful to evaluate the safety of puerh tea; however, mutagenic potential and teratogenic effect of puerh tea was neglected. Besides, puerh tea varied due to its special processing technologies, storage

periods, that affected its chemicals and flavor, and affected its safety and bioactivities further.[19] Thus, supplementary evidences are needed to illustrate the safety and bioactivities of puerh tea deeply.

In the present work, aqueous extract from fermented puerh tea (EFPT) was prepared, and its safety, including oral acute toxicity study in rats and mice, mutation tests made up of Ames test, mouse micronucleus test and mouse sperm abnormality test and 30 days feeding study in rats, was systematically evaluated, meanwhile its effect on antihyperlipidemia was investigated in rats with an objective of establishing a basis for its potential application in food industry and in the treatment of hyperlipidemia.

## 2. Materials and methods

### *2.1. Materials and chemicals*

The fermented puerh tea (product number 7572), produced in 2010, was obtained from Menghai Tea Industry Co. (Yunnan, China). Folin-Ciocalteu's phenol reagent, gallic acid, bovine serum albumin, theanine, glucose, and caffeine were purchased from Sigma Chemical Co. (Missouri, USA). Methanol and acetonitrile of HPLC grade were purchased from Tianjin Shield Co. (Tianjin, China). All other chemicals were analytical grade and purchased from Sinopharm Chemical Reagent Co. (Shanghai, China).

### *2.2. Preparation and characterization of EFPT*

EFPT was prepared from fermented puerh tea. Dry meshed puerh tea powders (100 g) were mixed with 1 L of distilled water at 100 $^{o}$C for 10 min and the extraction

was repeated twice. The extracts were filtered through filter paper and concentrated with a vacuum evaporator at 65 $^{o}$C. Finally the extract of puerh tea (EFPT) was dried with spray drier. Total phenolics were determined using the Folin-Ciocalteu method using gallic acid as standard.[20] Protein was analyzed by the method of Bradford[21] using bovine serum albumin as the standard. Amino acid content was determined by the ninhydrin assay method[22] using theanineas as standard. Total sugars were evaluated via the phonel-sulfate method,[23] using glucose as standard. Caffeine was determined according to the HPLC method,[24] described using a Shimadzu SCL-10A HPLC system (Shimadzu Corporation, Tokyo, Japan).

The results showed that EFPT was a grayish brown powder which was composed of 35.6% total phenolics, 26.3% total sugars, 11.2% caffeine, 6.8% protein, 4.7% amino acid and 5.5% moisture.

### *2.3. Oral acute toxicity studies*

#### *2.3.1. Oral acute toxicity study in rats*

Four-week-old male and female SD rats (Grade II, certificate No. SCXK 2008-0033) were obtained from Zhejiang Experimental Animal Center (Hangzhou, China), with a body weight of 180 g to 220 g. The rats were housed in cages in a temperature-controlled animal room (20-24 °C) with a relative humidity of 50-70%. They were fasted overnight but allowed free access to water. 25 g of EFPT was dissolved in 100 ml distilled water and administered at a rate of 10 g/kg body weight. The rats were divided into two groups, with each group having ten males and ten females. EFPT was administered twice in 24 h by oral gavages at a dose of 20 ml/kg

body weight. The observations of general status, toxic symptom and mortality in rats were continued for 14 days after treatment. Eventually, the maximum tolerated dose and acute toxic classification were confirmed.[25] All experiments were performed in compliance with the relevant laws and institutional guidelines, and the Ethical Committee of ZheJiang University approved the experiments.

*2.3.2. Oral acute toxicity study in mice*

Six-week-old ICR male and female mice (Grade II, certificate No. SCXK 2008-0106) were also obtained from Zhejiang Experimental Animal Center (Hangzhou, China), with a body weight of 18 g to 22 g. The method was similar as above (as shown in section 2.3.1).

***2.4. Mutation tests***

*2.4.1. Ames test*

EFPT was examined for its mutagenic potency in four histidine-requiring *S. typhimurium* mutant strains $TA_{97}$, $TA_{98}$, $TA_{100}$ and $TA_{102}$, which were obtained from Shanghai disease control and prevention center, using the treat and plate method.[26] Tester bacteria were exposed to five concentrations of EFPT, 0, 8, 40, 200, 1000 μg/plate, with and without $S_9$ mixture (the liver of rats), respectively. Positive and negative controls were run simultaneously with the test. All the tests were performed twice.

*2.4.2. Mouse micronucleus test*

30 ICR male and female mice (Grade II, certificate No. SCXK 2008-0106), six weeks of age, from Zhejiang Experimental Animal Center (Hangzhou, China) were

used in this test. They were divided into three groups and each group was 10 mice. The micronucleus test was carried out according to the method of Lee.[27] EFPT was dissolved in distilled water and administered orally, twice in every 24 h, at doses of 1.26, 2.5, 5 g/kg body weight to five males and five females each at random. The distilled water and cyclophosphamide (CPA, 0.06 g/kg) was given to another groups of mice as a negative and positive control. 6 h after the final gavage, the mice were sacrificed and their sternum marrow cells were collected and analyzed after methanol fixing and Giemsa staining. The frequency of micronuclei (MN) was enumerated based on an examination of 1000 polychromatic erythrocytes (PCE) per mouse.

*2.4.3. Mouse sperm abnormality test*

The methods[28] (Jaqta *et al.*, 2014) involved 35 ICR male mice (Grade II, certificate No. SCXK 2008-0106) with a body weight range from 25 to 30 g, obtained from Zhejiang Experimental Animal Center (Hangzhou, China), which were assigned randomly to five groups. EFPT was dissolved in distilled water and administered orally, daily for five days, at doses of 1.26, 2.5, 5 g/kg body weight to 7 males. The distilled water and 2.0 mg/kg mitomycin C (MMC) were given to the same number of mice as a negative and positive control, respectively. Mice were sacrificed by cervical dislocation on the 35th day after the first dose. The bilateral epididymides of five mice selected at random were placed in physiological saline and minced with ophthalmic scissors. Smears were prepared on clean slides, fixed with methanol and stained with 1% eosin. Morphological evaluation of mice sperms was conducted with a microscope of high magnification. The number of abnormal sperm out of 1000 sperms

was recorded and abnormality was calculated.

### *2.5. 30 days feeding study in rats*

80 SD male and female rats (Grade II, certificate No. SCXK 2007-0005), weighing range from 60 to 80 g, were obtained from Shanghai Slac Experimental Animal Co. (Shanghai, China). EFPT was added to a basal feed at concentration of 0 (control group), 0.75 (low-dose group), 1.5 (middle-dose group) and 3.0 g/kg body weight (high-dose group) daily and it continued for 30 days.[29] The highest dose in the test was settled to 3.0 g/kg body weight because the dose was 100 times of estimated daily ingestion of EFPT by human (about 1.8 g/60 kg body weight per day). Rats were housed in their respective cages and were allowed free access to water and food. The body weight of rats and food consumption were recorded every week during the study. The food availability was calculated and peripheral blood was collected by piercing the ventral tail vein. Moreover, hematology values were detected by MEK-6318K automatic haemacytometer (Nihon Koden Co., Japan). Clinical chemical values were evaluated by Accute TBA-40FR automatic biochemical analysis meter (Toshiba Co., Japan) after the rats were sacrificed by decapitation. Each rat body was observed and the organs such as livers, kidneys, spleens and testes/ovaries were weighted, with organ/body weight ratios calculated. These organs were taken for further histopathologic examinations including paraffin section, H-E staining and photoscope detection.

### *2.6. Effect on hyperlipidemia in rats*

A total of 40 SD rats (Grade II, certificate No. SCXK 2008-0016), weighing

185±15 g, were obtained from Shanghai Sippr-Bk laboratory animals Co. Ltd. (Shanghai, China). The rats were fed with a basal feed at room temperature of 22±2□ and the humidity of 55±15 %. After seven days, the peripheral blood of rats was collected by piercing the ventral tail vein. The levels of total cholesterols (TC), triglycerides (TG), and high-density lipoprotein-cholesterol (HDL-C) of the serum were determined. According to HDL-C level of rats, 40 rats were divided randomly into four groups of ten each and were all fed with a high-fat diet which contained basal feed (78.8%, w/w), cholesterol (1%, w/w), yolk powders (10%, w/w), lord (10%, w/w), and ox bile extract (0.2%, w/w). EFPT was dissolved in distilled water and administered orally, daily for 30 days, at doses of 0.15, 0.30, 0.90 g/kg body weight. The same amount of distilled water was given as a control. The highest dose (0.90 g/kg body weight) in this test was 30 times of estimated daily ingestion of EFPT by human (about 1.8 g/60 kg body weight per day). During the experiment, the body weights of the rats were recorded. The rats were fasted for 16 h and the peripheral blood was collected by piercing the ventral tail vein on the 30th day after the first dose. The levels of serum TC, TG and HDL-C were determined again.

### *2.7. Statistical analysis*

The results were expressed as means ± SD and evaluated by analysis of variance (ANOVA) followed by Turkey's test carried out on the SAS system for Windows V9 (SAS Inst., Cary, N.C., USA), and $p < 0.05$ was regarded as statistically significant.

## 3. Results

### *3.1. Oral acute toxicity study in rats and mice*

The results of oral acute toxicity study in rats and mice were similar, which showed no deaths occurred in either the group or EFPT treated groups. The general status of mice and rats were all fine. The oral maximum tolerant dose was more than 10.0 g/kg body weight both in rats and mice. Thus, the oral acute toxicity both in rats and mice was no toxicity according to the criteria of cute toxic classifications.[30]

### *3.2. Mutation Test*

#### *3.2.1. Ames test*

As shown in Table 1, the results suggested that no significant increase ($p > 0.05$) in the number of revertant colonies occurred in the four test strains ($TA_{97}$, $TA_{98}$, $TA_{100}$, $TA_{102}$) at any concentrations of EFPT, either in the presence or absence of $S_9$ mixture. There was no obvious dose-response relationship among the group. Therefore, the results of the Ames test were negative according to the results.

#### *3.2.2. Mouse micronucleus test*

The occurrence of chromosome abnormality is a phenomenon of chromosome abnormality, and the high rate of micronucleus meant that chromosome aberration might happen. The results in Table 2 showed that the frequencies of micronucleus in EFPT treated groups were not significantly ($p > 0.05$) different from that of the negative control. However, the positive control (Cyclophosphamide, 60 mg/kg) showed a significantly ($p < 0.05$) higher frequencies of micronucleus than that of negative control. Thus the results of mouse micronucleus test for EFPT were negative.

#### *3.2.3. Mouse sperm abnormality test*

Sperm abnormality test was to determine the effect of the mutation in the process of the formation of sperm in mice by observing the morphological changes of sperm head or tail. The results (Table 3) suggested the frequencies of sperm abnormality of three different dose groups had no significantly differences ($p > 0.05$) with the negative control, while the positive control (Mitomycin C, 2.0 mg/kg) gave a statistically significant ($p < 0.01$) elevation of abnormal sperm heads compared to the negative control, which meant the frequencies of sperm abnormalities were not significantly affected by EFPT.

### *3.3. 30 days feeding study in rats*

#### *3.3.1. General observations*

In order to estimate the safety of EFPT, 30 days feeding study was preferentially chosen for estimating the toxicity of EFPT further on the basis of acute toxicity study according to the Technical Standards for Testing and Assessment of Health Food in China.[30] Based on the results, the rats in each group had no abnormal symptoms or clinical signs and no death was found during the experiment.

#### *3.3.2. Effects on body weight, food consumption and food availability of rats*

As shown in Fig. 1, the body weights of all groups went up during the experiments. However, no significant difference among EFPT treated group and the control group was observed during the experiment on body weight of rats. The mean body weight, food consumption and food availability of rats given EFPT had no significant difference ($p > 0.05$) with that of the control (Table 4).

#### *3.3.3. Effects of hematology of rats*

Hematology values such as hemoglobin, red blood cell count, white blood cell count, lymphocyte, monicyte and granulocyte (Table 5) were found to be no significant difference ($p > 0.05$) among three different EFPT treated groups including males and females based on the data.

*3.3.4. Effects on clinical chemistry of rats*

10 rats were observed and tested for each group during the experiment. No difference among groups on clinical chemistry values (Table 6) was observed. Clinical chemistry values like glutamic-pyruvic transaminase (GPT), aspartate transaminase (AST), urea nitrogen (UN), creatinine (CRE), total cholesterol (TC) and triglycerides (TG), blood glucose (BG), total protein (TP), albumin (ALB) and Globulin (GLO) were not significantly affected by EFPT based on the data ($p > 0.05$).

*3.3.5. Effects on organ weight, organ/body weight ratios of rats*

The organ weights including liver, spleen, kidney and genitals (testicle/ovary) weights were measured and the corresponding organ/body weight ratios of male and female rats were also calculated. As shown in Fig.2, the organ weights of males were heavier than those of females. No significant difference ($p > 0.05$) among groups on organ weights and organ/body weight ratios has been observed. Therefore, organ weight and organ/body weight ratios of rats were not significantly ($p > 0.05$) affected by EFPT.

*3.3.6. Histopathological examination in rats*

No obvious treatment-related changes in the organs of SD rats were observed during necropsy. Some microscopic changes such as epithelial cells of renal tubular

248 slight swelling were observed in some rats. However, there were no differences noted
249 between the control group and high-dose group considering the minor incidence. No
250 treatment-related changes in histopathological examination were observed.

251 ***3.4. Effect on hyperlipidemia***

252 *3.4.1. Effect on body weight of rats*

253 To evaluate whether EFPT had any beneficial effect on hyperlipidemia, we
254 measured the body weight of rats fed with a high-fat diet every day during the 30 days
255 feeding period (Fig.3). It was shown that the body weights of low concentration EFPT
256 level group (LL group, 0.15 g/kg) and middle concentration EFPT level group (ML
257 group, 0.30 g/kg) were higher than that of HF group (the high fat control) with no
258 significant difference ($p$ >0.05), while the body weight of high concentration EFPT
259 level group (HL group, 0.90 g/kg) was lower than that of HF group but still with no
260 significant difference ($p$ >0.05) on the 30th day. The results indicated that the body
261 weights of rats in this experiment were not significantly ($p$ > 0.05) affected by
262 different concentrations of EFPT (0.15 g/kg, 0.30 g/kg, and 0.90 g/kg) based on the
263 data.

264 *3.4.2. Effect on lipid level*

265 Obesity often shows an increase in the risk of cardiovascular disease and exhibits
266 an abnormally higher TC and TG levels in serum. As shown in Fig.4A, the serum TC
267 levels of LL and ML group showed an obvious decline ($p$ < 0.05) compared to that of
268 HF group in the end of experiment. As shown in Fig.4B, the serum TG levels of ML
269 and HL group were significantly ($p$ < 0.05) lower than that of HF group on the 30th

day. The level of HDL-C more or less reflects the ability of lipid metabolism; therefore the higher HDL-C contributes to keep away from the obesity. According to Fig.4C, the serum HDL-C levels of EFPT group had no significant difference ($p > 0.05$) with that of HF group. The results suggested that EFPT had some beneficial effect on hyperlipidemia of rats, especially on serum TC and TG levels of rats.

## 4. Discussions

Puerh tea, known as a kind of slimming tea, is becoming more and more popular worldwide. Puerh tea was reported to lead great health benefits mainly associated with its bioactive constituents such as tea polyphenols, protein, amino acids, caffeine, tea polysaccharide, theabrownin etc.[31-32,7] The effect of puerh tea on body fat and lipid profiles was normally made by tea polyphenols,[33] tea polysaccharide,[34] theabrownin and statins.[35] Nowadays, the focus on the safety of natural products is increasing especially on some natural active components extracted from plants.[36-38]

As known, food safety is attracting more and more attention currently. The safety of extracts from puerh tea should be concerned seriously despite it is taken as a daily beverage from thousand years ago. Previously, Wang[17] reported acute and sub-chronic oral toxicities of puerh black tea in Sprague Dawley rats, and stated that the acute oral $LD_{50}$ value in SD rats was more than 10 g/kg, and a dose of 5 g/kg/day was identified as the no-observed-adverse-effect-level in sub-chronic toxicity study in rats. Similar results were obtained from our work. It was found that no significant toxicological effects of EFPT both in rats and mice because the oral dosage with EFPT at more than 10,000 mg/kg body weight which could be considered as no toxicity on animals.

There were also no adverse effects at the highest concentration administered in acute toxicity study. Meanwhile, according to the mutation tests (Ames test, mouse micronucleus test and mouse sperm abnormality test), negative results were obtained which meant the EFPT didn't exhibit any genotoxic or teratogenic potential in the experiments. No obvious toxicity was found in 30 days feeding study in rats and nearly no statistically significant differences were found in the essential organ weight and organ/body weight ratio. No dose dependency and nearly no adverse effects were observed histopathologically, which were not considered to be treatment-related effects. Thus, according to the results above, the maximum tolerated dose of EFPT in acute toxicity test was more than 10 g/kg body weight both in mice and rats.

Based on the results of EFPT's effect on hyperlipidemia, EFPT had no significant ($p > 0.05$) effect on the body weight and HDL-C level of rats. But the serum TC and TG levels were significant ($p < 0.05$) decreased by different concentrations of EFPT, which meant EFPT played a certain role on hyperlipidemia. Previously, several studies have reported that puerh tea extract had outstanding antihyperlipidemic effects.[39,40] Rats fed with fructose/pu-erh tea showed the greatest reduction in serum TG, cholesterol, insulin and leptin levels among three different kinds of tea.[41] However, by accessing the body weights and the lipid levels such as TC, TG, and HDL-C levels of rats in our study, the results showed that the antihyperlipidmic effects of puerh tea extract were not as excellent as those in previous reports.

The effect of EFPT on hyperlipidemia was probably due to tea polyphenols, tea

polysaccharide, theabrownin, statins etc. In fact, teas are divided depending on the different degree of fermentation which directly causes alteration in catechins through oxidative reaction.[42] Theabrownin is regarded as an important bioactive ingredient in puerh tea that lowers serum lipid levels because theabrownin can enhance the activity of hepatic lipase and hormone-sensitive triglyceride lipase (HSL) and increase the HSL mRNA expression in liver tissue and epididymis tissue. However, some studies have demonstrated that theabrownin is not a kind of homogeneous or heterogeneous polyphenol-derived polymers, but a kind of complex substance consisting of polyphenols, polysaccharides and protein.[33]

## 5. Conclusions

Base on the results obtained, it can be concluded that EFPT had low toxicity and positive effect on the serum TC and TG levels in rats, which indicated that EFPT could be applied as safe additives in food industry. However, as a concentrated agent of puerh tea, whether EFPT has more potential healthy benefits requires further study.

## Conflict of Interest

The authors declare that there are no conflicts of interest.

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409

410 **Figure Captions**

411

412 **Fig.1 Body weight changes of rats fed diets containing EFPT for 30 days.**

413 FC, female control group; FLL, female low level group (0.15 g/kg); FML, female
414 middle level group (0.30 g/kg); FHL, female high level group (0.90 g/kg); MC, male
415 control group; MLL, male low level group (0.15 g/kg); MML, male middle level
416 group (0.30 g/kg); MHL, male high level group (0.90 g/kg).

417

418 **Fig.2 Organs weight (A) and organ/body weight ratio (B) of rats fed EFPT for 30**
419 **days.**

420 LW, liver weight; SW, spleen weight; KW, kidney weight; GW, genitals weight; BW
421 body weight; FC, female control group; FLL, female low level group (0.15 g/kg);
422 FML, female middle level group (0.30 g/kg); FHL, female high level group (0.90
423 g/kg); MC, male control group; MLL, male low level group (0.15 g/kg); MML, male
424 middle level group (0.30 g/kg); MHL, male high level group (0.90 g/kg).

425

426 **Fig.3. The body weight of rats fed a high-fat diet every other day during the 30**
427 **days feeding period.**

428 HF, rats fed a high-fat diet; LL, rats fed a high-fat diet with 0.15 g/kg extracts; ML,
429 rats fed a high-fat diet with 0.30 g/kg extracts; HL, rats fed a high-fat diet with 0.90
430 g/kg extracts.

431

**Fig.4. Effect of extracts on serum TC (A), TG (B) and HDL-C (C) levels of rats.**

HF, rats fed a high-fat diet; LL, rats fed a high-fat diet with 0.15 g/kg extracts; ML, rats fed a high-fat diet with 0.30 g/kg extracts; HL, rats fed a high-fat diet with 0.90 g/kg extracts.

436 **Table 1** Ames test results for EFPT in four strains of *Salmonella typhimurium.*

| Strain | Dose (μg/plate) | Revertant colonies (means ± SD) | | | |
|---|---|---|---|---|---|
| | | $-S_9$ | | $+S_9$ | |
| | | Test 1 | Test 2 | Test 1 | Test 2 |
| $TA_{97}$ | 0 | 121±2.6 | 128±6.6 | 138±2.6 | 138±8.1 |
| | 8 | 114±3.1 | 118±5.5 | 132±4.0 | 132±2.6 |
| | 40 | 122±3.6 | 125±4.0 | 133±4.6 | 138±2.0 |
| | 200 | 126±4.7 | 130±7.0 | 146±5.7 | 149±4.9 |
| | 1000 | 130±6.1 | 134±1.5 | 151±4.4 | 155±5.3 |
| | ICR-191 [a] | 3012±99.0 | 2880±166.2 | / | / |
| | 2-AF [a] | / | / | 1923±164.2 | 1857±194.7 |
| $TA_{98}$ | 0 | 34±2.1 | 35±1.5 | 39±1.0 | 38±1.5 |
| | 8 | 32±0.6 | 33±1.0 | 34±1.2 | 34±1.0 |
| | 40 | 33±0.6 | 34±1.0 | 36±1.5 | 36±0.6 |
| | 200 | 35±1.0 | 35±1.0 | 38±1.0 | 38±1.5 |
| | 1000 | 37±1.5 | 38±1.0 | 42±1.2 | 42±1.2 |
| | Daunomycin [a] | 2196±162.1 | 1951±128.5 | / | / |
| | 2-AF [a] | / | / | 2351±173.9 | 1940±72.3 |
| $TA_{100}$ | 0 | 128±3.0 | 131±9.7 | 144±4.5 | 143±2.5 |
| | 8 | 125±5.5 | 125±4.2 | 135±3.5 | 132±4.0 |

| | | | | | |
|---|---|---|---|---|---|
| | 40 | 126±1.5 | 126±3.8 | 141±6.7 | 138±3.2 |
| | 200 | 128±3.8 | 129±3.1 | 151±2.6 | 143±3.1 |
| | 1000 | 131±4.4 | 133±2.1 | 157±4.6 | 157±4.4 |
| | $NaN_3$ [a] | 2115±119.2 | 1965±217.8 | / | / |
| | 2-AF [a] | / | / | 1822±125.2 | 1912±187.1 |
| | 0 | 266±9.7 | 269±8.1 | 274±5.0 | 278±7.0 |
| | 8 | 261±7.0 | 256±8.0 | 262±9.7 | 260±6.1 |
| | 40 | 263±8.0 | 264±5.7 | 272±6.0 | 268±7.5 |
| $TA_{102}$ | 200 | 268±8.5 | 273±5.0 | 276±6.7 | 288±8.0 |
| | 1000 | 276±8.0 | 277±9.2 | 286±9.2 | 294±5.5 |
| | Mitomycin C [a] | 1848±108.6 | 1819±97.7 | / | / |
| | 1,8- DAA [a] | / | / | 1849±79.1 | 1819±151.0 |

[a] 1.0 μg/plate of 2-methoxy-6-chloro-9-(3-(2-chloroethyl) aminopropylamino) acridine (ICR-191), 10 μg/plate of 2-Aminofluorene (2-AF), 6.0 μg/plate of Daunomycin, 1.5 μg/plate of Sodium azide ($NaN_3$), 0.5 μg/plate of Mitomycin C and 50 μg/plate of 1,8-dihydroxyanthraquinoe (1,8- DAA) were used as positive controls. All the tests were performed twice (Test 1 and Test 2).

443 **Table 2** Results of micronucleus test for EFPT using mice.

| Group | Treatment (g/kg) | PCE[b] No. | MNPCE[b] No. | MN[b] (%) (mean ± SD) | P value | PCE/RBC |
|---|---|---|---|---|---|---|
| | 0 | 5000 | 6 | 1.2±0.8 | / | 1.30±0.28 |
| | 1.25 | 5000 | 6 | 1.2±0.8 | 0.554 | 1.21±0.22 |
| Females | 2.5 | 5000 | 7 | 1.4±0.6 | 0.394 | 1.22±0.25 |
| | 5.0 | 5000 | 8 | 1.6±0.9 | 0.256 | 1.38±0.18 |
| | Cyclophosphamide[a] | 5000 | 92 | 18.4±3.58** | <0.001 | 0.99±0.27 |
| | 0 | 5000 | 7 | 1.4±1.1 | / | 1.35±0.33 |
| | 1.25 | 5000 | 8 | 1.6±0.6 | 0.401 | 1.34±0.34 |
| Males | 2.5 | 5000 | 8 | 1.6±0.6 | 0.401 | 1.29±0.30 |
| | 5.0 | 5000 | 6 | 1.2±0.8 | 0.699 | 1.41±0.36 |
| | Cyclophosphamide[a] | 5000 | 101 | 20.2±6.02** | <0.001 | 1.14±0.10 |

444 [a] Cyclophosphamide was treated as the positive control at dose of 60 mg/kg.

445 [b] PCE meant polychromatic erythrocytes, and MN meant frequencies of

446 micronucleus.

447 ** meant a statistically significant elevation of micronucleus compared to the negative

448 control ($p < 0.01$).

449 **Table 3** Result of sperm abnormality test for EFPT using mice.

| Treatment (g/kg) | Mice No. | Sperm No. | Abnormality No. | Frequencies of sperm abnormalities (%) | P value |
|---|---|---|---|---|---|
| 0 | 5 | 5000 | 116 | 2.32±0.23 | / |
| 1.25 | 5 | 5000 | 112 | 2.24±0.11 | >0.05 |
| 2.5 | 5 | 5000 | 113 | 2.26±0.38 | >0.05 |
| 5 | 5 | 5000 | 123 | 2.46±0.47 | >0.05 |
| Mitomycin C[a] | 5 | 5000 | 272 | 5.44±1.14** | <0.01 |

450 [a] Mitomycin C was treated as the positive control at dose of 2.0 mg/kg.

451 ** meant a statistically significant elevation of frequencies of sperm abnormalities

452 compared to the negative control ($p < 0.01$).

453

454 **Table 4** Body weight increase, food consumption and food availability of rats fed
455 EFPT for 30 days.

| Parameter | EFPT in the diet (g/kg) | | | |
|---|---|---|---|---|
| | 0 | 0.75 | 1.5 | 3 |
| Females | | | | |
| Body weight increase (g) | 144.4±13.3 | 137.8±11.5 | 133.2±9.9 | 130.8±11.4 |
| Food consumption (g) | 624.9±28.1 | 632.2±37.5 | 619.5±41.6 | 608.2±17.7 |
| Food availability (%) | 23.1±1.9 | 21.8±1.9 | 21.5±1.4 | 21.6±2.3 |
| Males | | | | |
| Body weight increase (g) | 254.9±17.9 | 241.1±12.8 | 247.3±25.0 | 236.1±24.6 |
| Food consumption (g) | 766.9±39.9 | 755.5±35.3 | 766.0±21.1 | 748.6±38.9 |
| Food availability (%) | 33.3±1.9 | 32.0±1.6 | 32.2±2.7 | 31.5±2.2 |

456

457 **Table 5** Hematological values of rats fed EFPT for 30 days.

| Parameter | Control group | | EFPT group | |
|---|---|---|---|---|
| | Females | Males | Females | Males |
| Hemoglobin (g/L) | 158.4±13.2 | 149.3±11.2 | 145.9-161.4 | 153.6-157.9 |
| RBC[a] ($\times 10^{12}$/L) | 8.0±0.7 | 7.6±0.5 | 7.7-8.2 | 7.6-7.9 |
| WBC[b] ($\times 10^{9}$/L) | 18.4±3.9 | 20.5±3.9 | 15.7-17.9 | 18.3-21.2 |
| Lymphocyte (%) | 68.7±6.8 | 69.2±6.2 | 69.4-73.3 | 70.2-71.8 |
| Monocytes (%) | 1.3±0.2 | 1.4±0.2 | 1.3-1.3 | 1.3-1.3 |
| Granulocyte (%) | 30.0±6.8 | 29.4±6.2 | 27.4-29.3 | 26.9-28.6 |

458 [a] RBC represented for red blood cell count.

459 [b] WBC represented for white blood cell count.

460 Mean ± SD values in EFPT groups were not shown in the table.

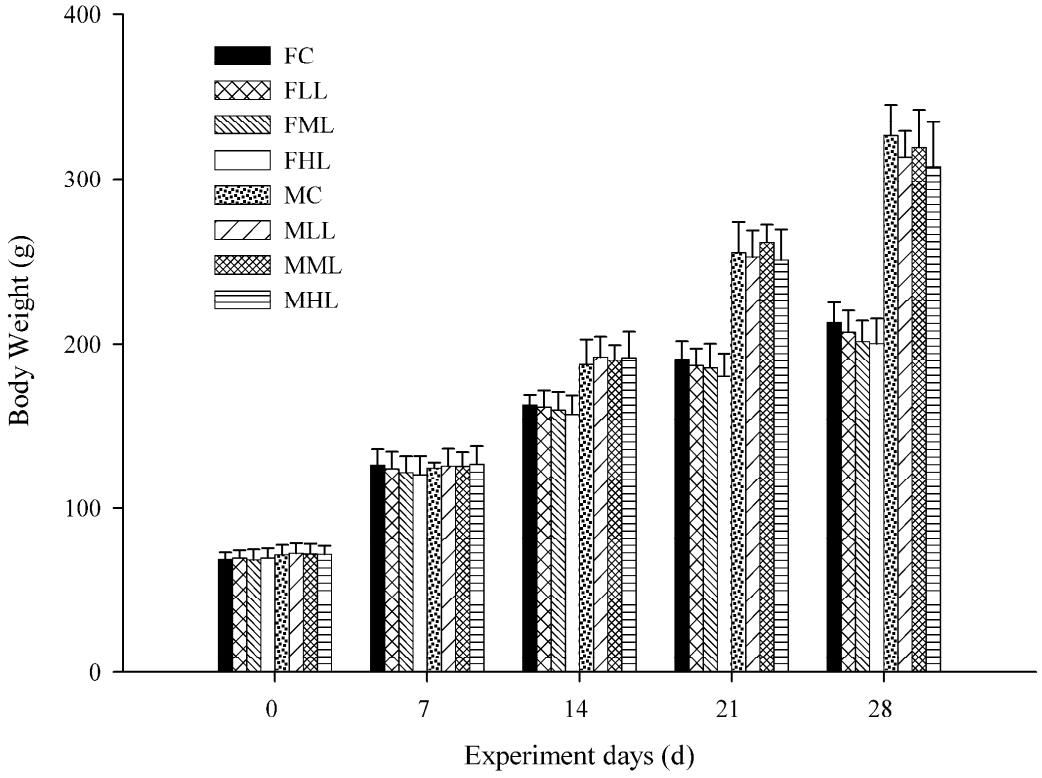
461

462 **Table 6** Clinical chemistry values of rats fed EFPT for 30 days.

| Parameter [a] | EFPT in the diet (g/kg) | | | |
|---|---|---|---|---|
| | 0 | 0.75 | 1.5 | 3 |
| Females | | | | |
| GPT | 44.5±3.7 | 43.3±7.3 | 40.5±7.7 | 45.7±8.1 |
| AST | 191.0±32.5 | 185.5±34.6 | 183.9±37.9 | 224.5±44.6 |
| UN | 5.80±1.36 | 6.98±0.83 | 6.99±0.82 | 6.66±1.20 |
| CRE | 65.3±2.4 | 65.4±2.4 | 65.6±3.2 | 66.4±3.1 |
| TC | 1.86±0.40 | 1.95±0.35 | 1.92±0.61 | 1.50±0.33 |
| TG | 0.78±0.25 | 1.02±0.40 | 0.98±0.22 | 1.04±0.25 |
| BG | 7.0±10.8 | 7.1±0.5 | 7.5±1.2 | 8.0±0.7 |
| TP | 66.6±1.9 | 69.1±2.0 | 68.7±2.3 | 69.2±2.9 |
| ALB | 34.4±1.2 | 35.8±1.2 | 35.6±1.0 | 35.7±1.6 |
| GLO | 32.2±1.1 | 33.3±1.5 | 33.1±1.7 | 33.5±1.6 |
| Males | | | | |
| GPT | 45.5±7.3 | 48.8±9.4 | 44.5±5.1 | 53.1±11.3 |
| AST | 194.9±27.2 | 183.4±52.6 | 167.9±42.7 | 205.2±83.2 |
| UN | 5.60±0.50 | 5.61±1.40 | 5.78±0.72 | 6.16±0.89 |
| CRE | 63.2±3.6 | 60.1±4.5 | 60.2±3.3 | 63.6±2.5 |
| TC | 1.85±0.24 | 1.54±0.25 | 1.82±0.56 | 1.56±0.31 |
| TG | 0.81±0.17 | 0.71±0.21 | 0.91±0.25 | 0.71±0.22 |
| BG | 7.1±0.8 | 7.7±2.3 | 7.0±0.8 | 7.1±0.5 |

| | | | | |
|---|---|---|---|---|
| TP | 66.5±1.7 | 65.9±2.5 | 65.5±2.3 | 67.4±1.9 |
| ALB | 33.7±0.7 | 33.0±1.1 | 33.0±0.9 | 33.8±0.7 |
| GLO | 32.7±1.3 | 32.9±1.8 | 32.5±2.0 | 33.6±1.3 |

463 [a] Parameters biochemistry: glutamic-pyruvic transaminase (GPT), aspartate
464 transaminase (AST), urea nitrogen (UN), creatinine (CRE), total cholesterol (TC) and
465 triglycerides (TG), blood glucose (BG), total protein (TP), albumin (ALB) and
466 Globulin (GLO).




467

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469 FC, female control group; FLL, female low level group (0.15 g/kg); FML, female

470 middle level group (0.30 g/kg); FHL, female high level group (0.90 g/kg); MC, male

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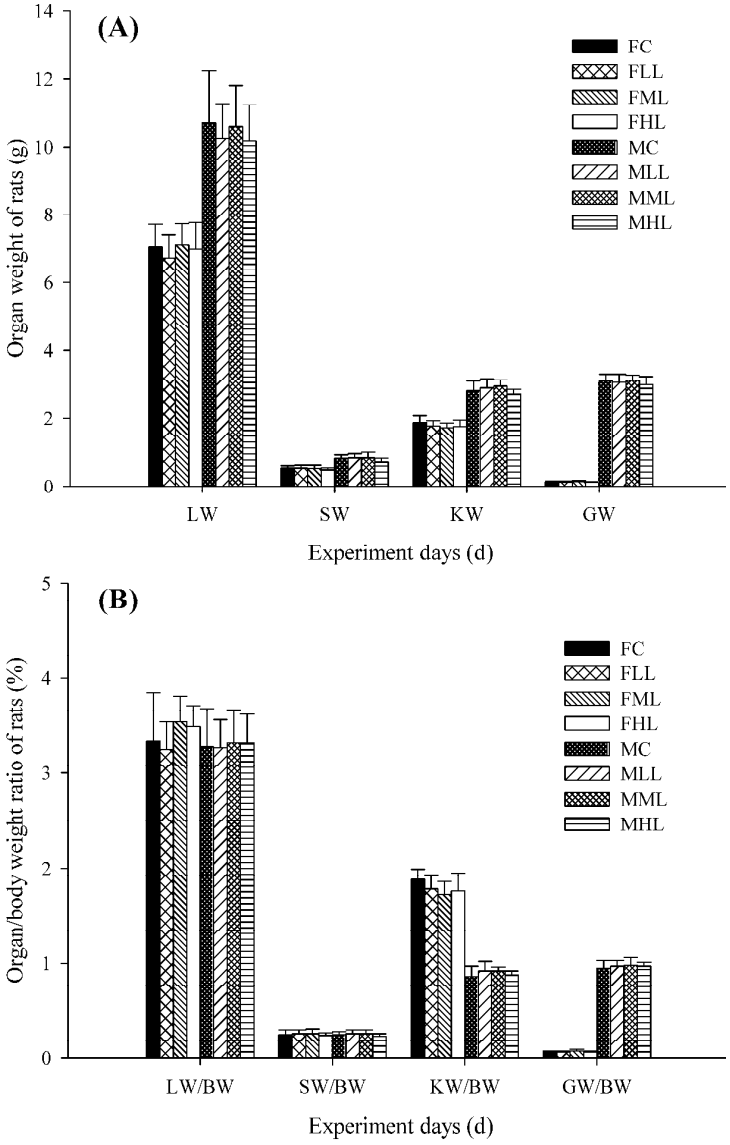


473

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475 days.

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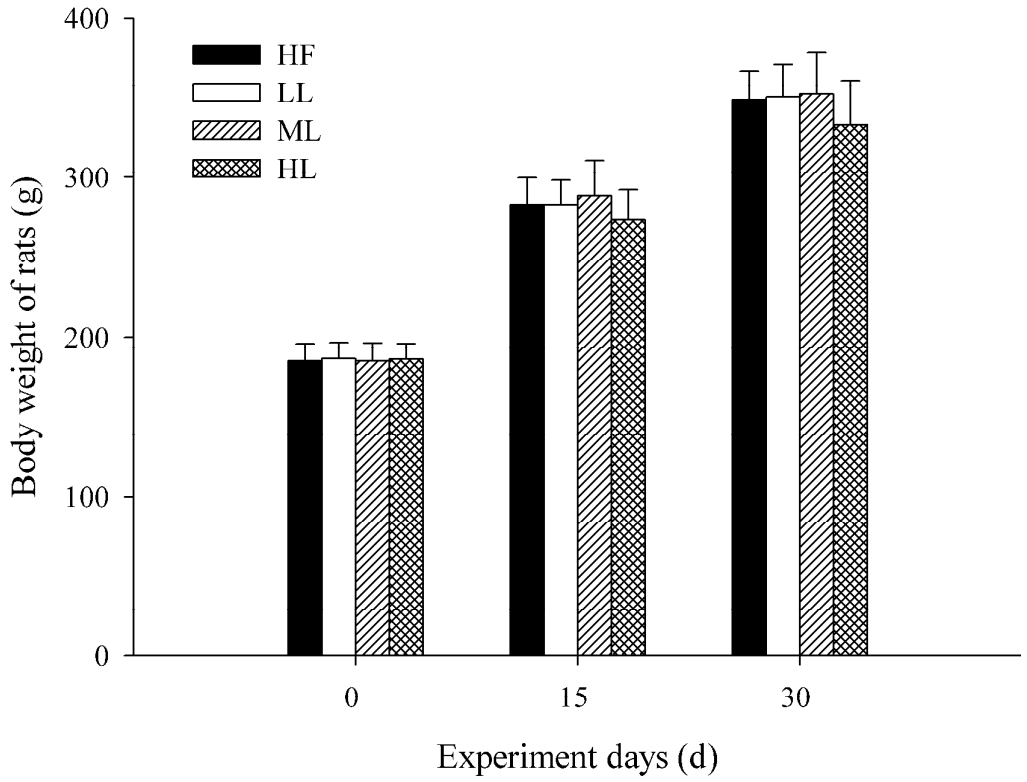
477 body weight; FC, female control group; FLL, female low level group (0.15 g/kg);

478 FML, female middle level group (0.30 g/kg); FHL, female high level group (0.90

479 g/kg); MC, male control group; MLL, male low level group (0.15 g/kg); MML, male

480 middle level group (0.30 g/kg); MHL, male high level group (0.90 g/kg).




481

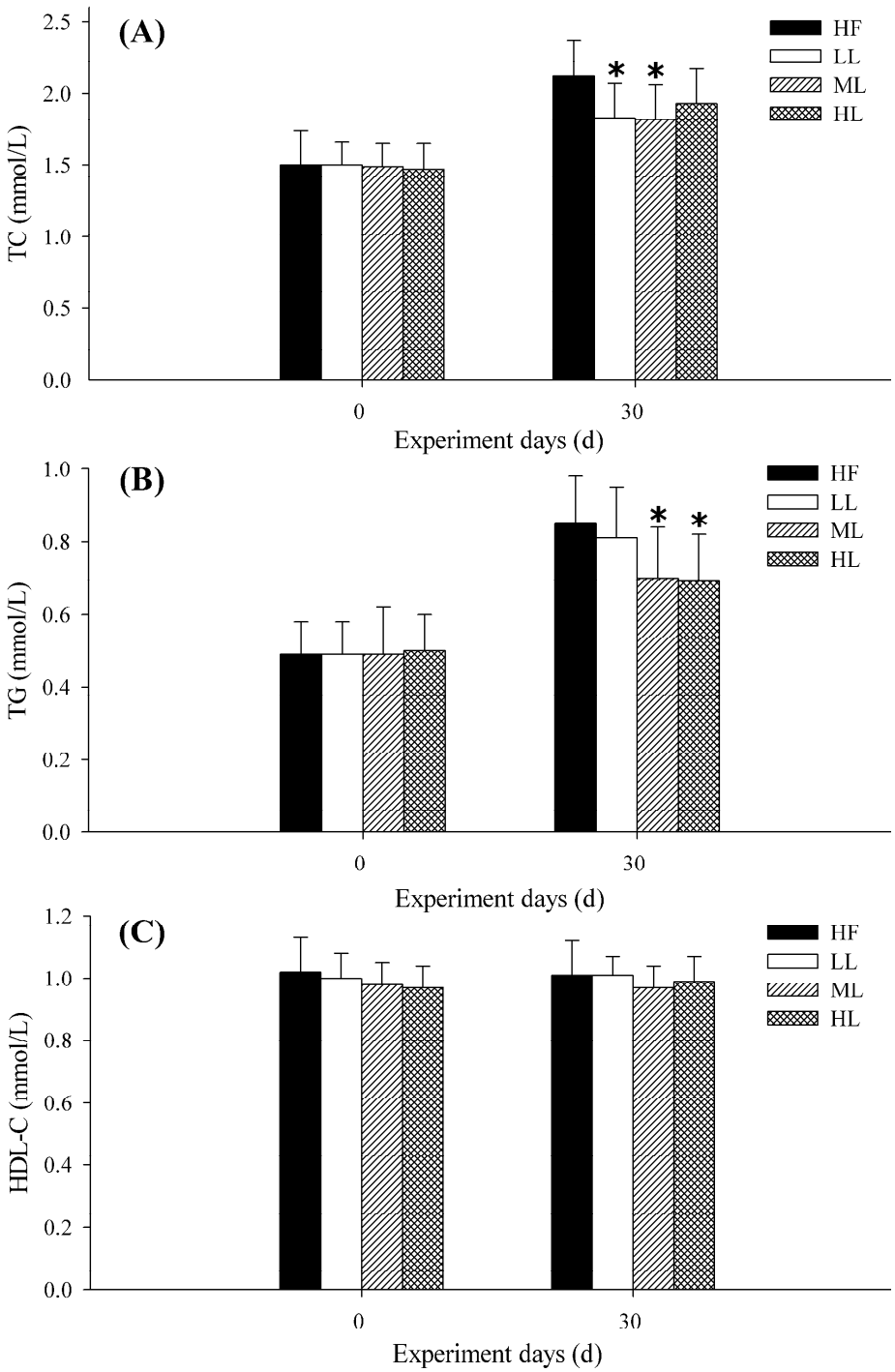
482 **Fig.3** The body weight of rats fed a high-fat diet every other day during the 30 days

483 feeding period.

484 HF, rats fed a high-fat diet; LL, rats fed a high-fat diet with 0.15 g/kg extracts; ML,

485 rats fed a high-fat diet with 0.30 g/kg extracts; HL, rats fed a high-fat diet with 0.90

486 g/kg extracts.




487

488 **Fig.4** Effect of extracts on serum TC (A), TG (B) and HDL-C (C) levels of rats.

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490 rats fed a high-fat diet with 0.30 g/kg extracts; HL, rats fed a high-fat diet with 0.90

491 g/kg extracts.