JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Dietary Administration of High Doses of Pterostilbene and Quercetin to Mice Is Not Toxic

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The aim of this study is to evaluate possible harmful effects of high doses of t-pterostilbene (t-PTER) and quercetin (QUER) in Swiss mice. Mice were fed during 28 days at doses of 0, 30, 300, and 3000 mg/kg body weight/day of t-PTER, QUER, or a mixture of both, t-PTER + QUER, which are equivalent to 5, 50, and 500 times, respectively, the estimated mean human intake of these polyphenols (25 mg/day). Daily oral administration of QUER, t-PTER, or a mixture of both of them did not cause mortality during the experimental period. There were no differences in food and water consumption on sex. No significant body weight gain in the male or female groups was observed. Red blood cell number and the hematocrit increased after polyphenols administration compared to control groups. Biochemical parameters were not affected. Histopathological examination revealed no alterations in clinical signs or organ weight at any dose.

KEYWORDS: Quercetin, t-pterostibene, 28-day feeding study, subchronic toxicity, mice

1. INTRODUCTION

Polyphenols are found ubiquitously in fruits, vegetables, nuts, seeds, and bark (*1*−*6*). These natural compounds are nonenergetic, but their dietary supply may have positive effects on human health. Different phenolic compounds, including resveratrol (*trans*-3,5,4′-trihydroxystilbene, RESV), show potent anti-inflammatory, antiallergic, and antioxidant effects and may have therapeutic applications in oxidative stress-related pathologies such as e.g. cancer or cardiovascular and coronary heart diseases (*3*, *7*−*12*). Cancer chemopreventive activity of RESV was previously reported (*9*). However, potential inhibition of cancer growth by RESV is strongly limited due to its low bioavailability in either sex (*13*).

Besides, polyphenols are among the most potent antioxidants because they show one or more of the following structural characteristics: an *o*-diphenolic group, a 2−3 double bond conjugated with the 4-oxo function, and OH groups in positions 3 and 5. Quercetin (3,3′,4′,5,6-pentahydroxyflavone, QUER) combines all these three properties and also exhibits antitumor properties, likely due to immune stimulation, free radical scavenging, alteration of the mitotic cycle in tumor cells, gene expression modification, antiangiogenesis activity, apoptosis induction, or a combination of these effects (*2*, *12*−*18*). QUER has attracted much attention from the standpoint of its possible role in the prevention of atherosclerosis (*5*, *15*, *19*).

t-Pterostilbene (3,5-dimethoxy-4′-hydroxystilobene, t-PTER), one of the most extensively studied secondary metabolites found in peanuts, berries, grapes, and wine, is an analogue of RESV but about 60−100 times stronger as an antifungal agent (*6*, *20*). t-PTER also has hypolipidemic and antioxidative activities. t-PTER is reported to scavenge free radicals and to inhibit the oxidation and lipid peroxidation in rat liver microsomes and in culture mammalian cell lines (*6*, *20*, *21*). t-PTER reduces cholesterol and glucose levels and increases plasma insulin levels significantly. It is used as an antidiabetic (*11*, *14*, *20*, *22*−*24*). Furthermore, t-PTER has been shown to be cancer-chemopreventive and antiinflammatory (*6*, *20*, *21*, *25*).

Depending on dietary habits, the human intake of flavones and flavonols (the most common flavonoids) is ∼3−70 mg/day, mainly QUER (60−75%) (major sources include tea, wine, berries, apples, and onions) (*1*, *2*). However, there are no reported estimations regarding t-PTER intake. However, PTER is presented, for example, in extracts of the heartwood of *Pterocarpus marsupium*, used in Ayurvedic medicine for the treatment of Diabetes mellitus. For diabetes treatment, Ayurvedic herbomineral formulations contain 20 mg of *P. marsupium* and other ingredients (*24*). t-PTER is also present in dark-skinned grapes; however, quantitative studies have shown that, for every

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10.1021/jf803579e CCC: $40.75
Published on Web 03/17/2009

10 parts of RESV, there is only 1−2 parts of t-PTER (*21*). In vivo studies reported in the literature for t-PTER showed dosages ranging from 10 mg/kg to 40 mg/kg body weight (*8, 11, 14, 22, 26, 27*). While, for QUER, higher doses (50−1000 mg/kg body weight/day) were assayed (*3, 16, 28−30*).

Recently, we have reported that after oral administration of 20 mg of t-PTER or QUER/kg (a dose that represents for an adult human being of 70 kg b.wt. ∼1000 times the maximum amount of t-PTER found in 1 kg of dark grapes and ∼20 times the maximum daily intake of QUER), plasma levels of t-PTER and QUER peaked at 60 and 10 min, respectively (*8*). However, the total levels of t-PTER and QUER (free unchanged polyphenols plus their metabolites and conjugated forms) were very different. The total t-PTER concentration was >10 μM between 30 and 240 min after administration, whereas the total QUER levels only remained >1 μM within the first 10 min. During those time periods, free t-PTER represented a small percent of the total (15−35%), whereas free QUER (excepting the first 5 min) was almost undetectable (<0.5%). The serum concentration vs time profile for t-PTER and QUER demonstrated a rapid decline in concentrations in the first hour. Bioavailable concentrations of t-PTER and QUER, measured in plasma after oral administration (20 mg/kg), failed to inhibit growth of the highly malignant B16 melanoma F10 in vitro (even when concentrations of these polyphenols were constant along the culture time), thus suggesting that oral administration is limiting systemic bioavailability and does not allow polyphenol to reach a tumor through blood circulation, at pharmacologically efficient concentrations. Nevertheless, in the case of primary tumors of the gastrointestinal tract, limitations caused by bioconjugation mechanisms are minimized, and oral administration can be useful to deliver biologically effective anticarcinogenic/antitumor concentrations of polyphenols. Moreover, Ferrer et al. (*8*) have proposed that the association of t-PTER and QUER could also be useful in other oxidative stress-related pathologies where doses required to obtain benefits could be similar or entirely different.

Besides, recent findings show that long-term administration of a RESV-enriched diet (equivalent to a daily intake of 20 mg of RESV/kg day) produces changes associated with longer life span, including increased insulin sensitivity, reduced insulin-like growth factor-1 (IGF-I) levels, increased AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) activity, increased mitochondrial number, and improved motor function (*31*), suggesting that to improve general health in mammals by means of using small molecules is an attainable goal and, moreover, pointing to new approaches for treating obesity-related disorders and diseases of aging.

Considering the potential beneficial effects of QUER on health, it is commercially available as a component of many herbal products and dietary supplements, together with other antioxidants, for example, t-PTER. Information in the literature regarding its toxicity is partial and contradictory. Regarding QUER, its LD_{50} in mouse is 160 mg/kg b.wt. after p.o. administration (*32*), it is mutagenic to *Salmonella typhimurium* based on the Ames test (*3, 17, 32, 33*), but it is also recognized as genoprotective against different mutagenic agents (*3, 18*). Thus, toxicological evaluation and safety assessment of these compounds needs to be carefully conducted by means of in vivo animal experiments receiving chronic ingestion of these natural polyphenols. The aim of this study was to evaluate the harmful effects on health of dietary oral administration of high doses of QUER and t-PTER. The present study examined the subchronic toxicity of t-PTER and QUER in Swiss mice to determine possible adverse effects. The effects on body weights, hematology, clinical chemistry, urinary parameters, and gross or microscopic changes in organs and tissues after daily oral intake of large doses of t-PTER and/or QUER during a period of 28 days were also studied.

Table 1. Dose of Quercetin and Pterostilbene Administered to Male and Female Swiss Mice during the Treatment Period of 28 days

sex	treatment	group number
male		
	commercial pelleted diet for rodents	control
	30 mg quercetin/kg b.wt./day	Q1
	300 mg quercetin/kg b.wt./day	Q2
	3000 mg quercetin/kg b.wt./day	Q3
	30 mg quercetin + 30 mg pterostilbene/kg b.wt./day	QP1
	300 mg quercetin + 300 mg pterostilbene/kg b.wt./day	QP2
	3000 mg quercetin + 3000 mg pterostilbene/kg b.wt./day	QP3
	30 mg pterostilbene/kg b.wt./day	P1
	300 mg pterostilbene/kg b.wt./day	P2
	3000 mg pterostilbene/kg b.wt./day	P3
female		
	commercial pelleted diet for rodents	control
	30 mg quercetin/kg b.wt./day	Q1
	300 mg quercetin/kg b.wt./day	Q2
	3000 mg quercetin/kg b.wt./day	Q3
	30 mg quercetin + 30 mg pterostilbene/kg b.wt./day	QP1
	300 mg quercetin + 300 mg pterostilbene/kg b.wt./day	QP2
	3000 mg quercetin + 3000 mg pterostilbene/kg b.wt./day	QP3
	30 mg pterostilbene/kg b.wt./day	P1
	300 mg pterostilbene/kg b.wt./day	P2
	3000 mg pterostilbene/kg b.wt./day	P3

2. MATERIALS AND METHODS

2.1. Chemicals. QUER dihydrate (CAS Number: 6151-25-3) (98% purity) was provided by Sigma Aldrich (Madrid, Spain), whereas t-PTER was synthesized in our laboratory following standard Witting and Heck reactions (*34*). Both compounds were stored at 2−8 °C and protected from light.

2.2. Animals and Experimental Conditions. Procedures involving animals were in conformity with the Ethics Committee of the Faculty of Pharmacy of the University of Valencia, which is in compliance with the European Council directive (86/609/EEC) for the use and care of laboratory animals (*35*). Male and female Swiss mice (B&K Universal G.L., Barcelona, Spain) weighing ∼22−25 g were used in the present study. Animals were housed by sex in groups of five per plastic cage (47 × 34 × 18 cm^3) on soft chip bedding, at room temperature (24 ± 1 °C) and 40−70% relative humidity, and maintained on a 12 h light/12 h dark cycle. Mice were allowed free access to food and water. All animals were acclimated for 1 week before initiation of subchronic dosing, and they were randomly assigned to a control or treatment group.

2.3. Preparation of Diets. The quantity of QUER and t-PTER that was additional to the commercial pellets rodent diet was calculated from the normal diet consumed ad libitum by a mouse during 1 week.

Animals were divided into 20 groups per sex (5 mice per group). The groups were dosed during the treatment period of 28 days as stated in **Table 1**.

Diets were prepared by the pulverization of a commercial pelleted rodent diet (Harlan Teklad, Indianapolis, IN) into a Bapitaurus food chopper (Taurus, Berlin, Germany), followed by the addition of the required amount of test compounds of each dose and 150 mL of 5% gum arabic solution (Sigma Aldrich). The mixture was carefully homogenized, and pellets were reconstituted using a stainless steel welded wire mesh and allowed to dry for 24 h. Concentration of polyphenols in reconstituted pellet was determined by high performance liquid chromatography (HPLC) analyses. Results of HPLC analyses demonstrated that the concentration of each polyphenol in each type

of pellet was close to that intended. The difference between the measured concentration and the intended concentration was less than 10%. Control groups were fed with commercial pelleted rodent diet added with 5% gum Arabic solution. Finally the food was stored at 4 °C and protected from light until used.

2.4. Experimental Design. The duration of the exposure was 28 days, according to the Organization for Economic Cooperation and Development guidelines, 1995 (*36*). The study was composed of 10 treatment groups, resulting in 20 groups (5 mice/sex/group) receiving diets containing 0 (controls), Q1, Q2, Q3, QP1, QP2, QP3, P1, P2, and P3 (**Table 1**). Food and water were available ad libitum. Diets were provided in stainless steel cans, and water was supplied in glass bottles.

Daily dietary intake of QUER was estimated by an adult individual in the United States and is around 25 mg (*32*). The doses used in this study (**Table 1**) were approximately 5, 50, and 500 times this estimated daily intake, which were estimated to provide a sufficiently large safety margin (*23, 32*). The translation of the doses from animals to humans was calculated by the human equivalent dose (HED) approach (*37*).

The general condition and behavior of all animals were examined daily. The individual body weights, food consumption per cage, and water intake per cage were recorded daily. The amounts of food and water were calculated before they were supplied to each cage, and the next day, the remaining water and food in each cage were measured, to calculate the differences, which were regarded as daily food and water consumption (g/mice/day). The efficiency of food utilization was calculated in weekly intervals and expressed as gram of body weight gain per gram of food consumed.

Freshly voided urine samples were collected from 1 mice/sex/group once a week, on days 4, 11, 18, and 25 of the 28 days study. Mice were placed in individual plastic metabolic cages (Tecniplast, Barcelona, Spain), which permitted separate collection of urine and feces for determining urine parameters. The samples were collected in the morning and were analyzed for specific gravity, pH, leukocytes, nitrite, protein, glucose, ketones, urobilinogen, bilirubin, and blood (erythrocytes and hemoglobin) using Combur 10 Tests (Roche, Barcelona, Spain).

At the end of day 28, all animals were weighed and anesthetized with isofluoran, and then, 2 mL of blood was taken by heart puncture for hematology and blood chemistry. Animals were then killed by exsanguinations from the heart.

2.5. Hematology and Blood Chemistry. For the evaluation of selected hematological and biochemical parameters, blood samples were collected into tubes containing EDTA 3K (TAPVAL, Madrid, Spain) and centrifuged at 1500*g* for 15 min in Eppendorf 5804-R (Hamburg, Germany) tubes to isolate the plasma.

Hematological parameters investigated included hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), red blood cell count (RBC), white blood cell count (WBC), neutrophils, eosinophils, basophiles, lymphocytes, monocytes, and platelets count (PLT). Serum biochemical parameters evaluated were alkaline phosphatase activity (AP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, glucose, bilirubin (Bil), cholesterol (Cho), total protein (TP), albumin (Alb), globulin (Glb), bilirubin creatinine (Bil.CRN), triglycerides (TG), high-density lipoproteins (HDL), and low-density lipoproteins (LDL). Serum electrolytes, including sodium (Na), potassium (K), calcium (Ca), chlorine (Cl), and phosphorus (P), were also measured.

2.6. Organ Weights and Histopathology. The relative (organ-to-body weight ratios) weights of following organs were measured for all survivors when they were sacrificed: liver, spleen, kidneys, heart, brain, lungs, and pancreas. Macroscopic examinations were conducted on tissues collected at necropsy. Histopathological evaluation was conduced for both sexes. Organs/tissues were fixed in 10% (v/v) neutral formalin, embedded in paraffin, and sectioned in 5-μm-thick slices. The sections were stained with hematoxylin and eosin stain for microscopic examination.

2.7. Statistics. The statistical analysis of the results was performed by one-way analysis of variance (ANOVA) test using the SPSS version 13 statistical program. A comparison of means with the Tukey test was applied to determine differences between control and dosed groups. Data were expressed as means ± SD, and group mean differences with an associated probability of less than 0.05 were considered to be statistically significant.

3. RESULTS

After daily oral administration of either of the doses tested for the 28 days period, there were no deaths. No evident treatment-related effect was observed on behavior. Clinical chemistry and hematological and histopathological analyses revealed that oral intake of high doses of t-PTER and QUER is safe, since this is not associated with significant local or systemic toxicity.

3.1. Animal Growth and Food and Water Consumption. Daily oral administration of QUER, t-PTER, or a mixture of both of them did not cause mortality during the experiment. **Figure 1** shows the real growth curves for Swiss mice treated with QUER, t-PTER, or a mixture of both of them during the 28 days of the experiment. **Figure 2** shows the mean food intake of QUER, t-PTER, or a mixture of both in treated Swiss mice every day for 28 days. There was no significant alteration in the mean body weight of the male or female mice during the experimental period. The groups did not differ in food or water consumption.

3.2. Biochemical and Hematological Observations. Biochemical and hematological determinations are presented in **Tables 2** and **3**. Although there were a few significant changes in several hematological parameters, they were not considered to be related to the substances under study.

Analysis of the hematological profile revealed an increase in RBC count, hematocrit, and neutrophils, whereas monocytes decreased (**Tables 2** and **3**). In t-PTER- and t-PTER + QUER-treated mice, RBC counts were significantly ($p < 0.001$) higher compared to controls in both sexes. Consequently, the hematocrit was also higher in treated than in control groups (**Tables 2** and **3**), although in both cases these increases were not dose-related. Significant differences ($p < 0.05$) were observed in neutrophils levels that increase in Q1, Q3, and P2 male groups and QP3 female groups, whereas monocytes significantly decreased in Q1 and Q3 male groups (**Tables 2** and **3**).

As shown in **Table 2**, in the Q3 male mice group, blood glucose was significantly ($p < 0.05$) lower at the end of the 28-day period compared to controls. Cho-LDL levels decreased in all treated male mice groups, with the exceptions of the Q3, QP1, and QP3 groups (**Table 2**). Only in the 30 mg QUER/kg b.wt./day (Q1) female group was a decrease detected as compared to the control group. In addition, there was a slight, but not significant, increase in these parameters in the other female groups. Albumin was significantly decreased ($p < 0.05$) in the QP1 and QP2 male groups (**Table 2**), and globulin significantly increased ($p < 0.05$) in the Q2 and Q3 male groups.

Calcium in circulating blood was only significantly ($p < 0.05$) elevated in the PQ-1 male group (**Table 2**), whereas platelets decrease in the QP-3 female group (**Table 3**). Various liver and kidney function-related parameters were investigated. Total bilirubin significantly increased ($p < 0.05$) in the Q2 male group. Phosphatase activity significantly ($p < 0.05$) increased in the QP3 group (**Table 2**). AST significantly ($p < 0.05$) increased in the P3 female treated group and ALT in the QP2 and QP3 female treated groups (**Table 3**). These statistical differences were isolated; most of them occurred in male groups and were not dose-dependent. Thus, they were considered within the range of normal variability, apparently unrelated to the polyphenol administration and without toxicological significance.

3.3. Urinalysis Observations. Table 4 presents urinary analyses obtained on days 4, 11, 18, and 25 of the 28-day study. As can be noted, no effect related to the oral administration of polyphenols was observed.

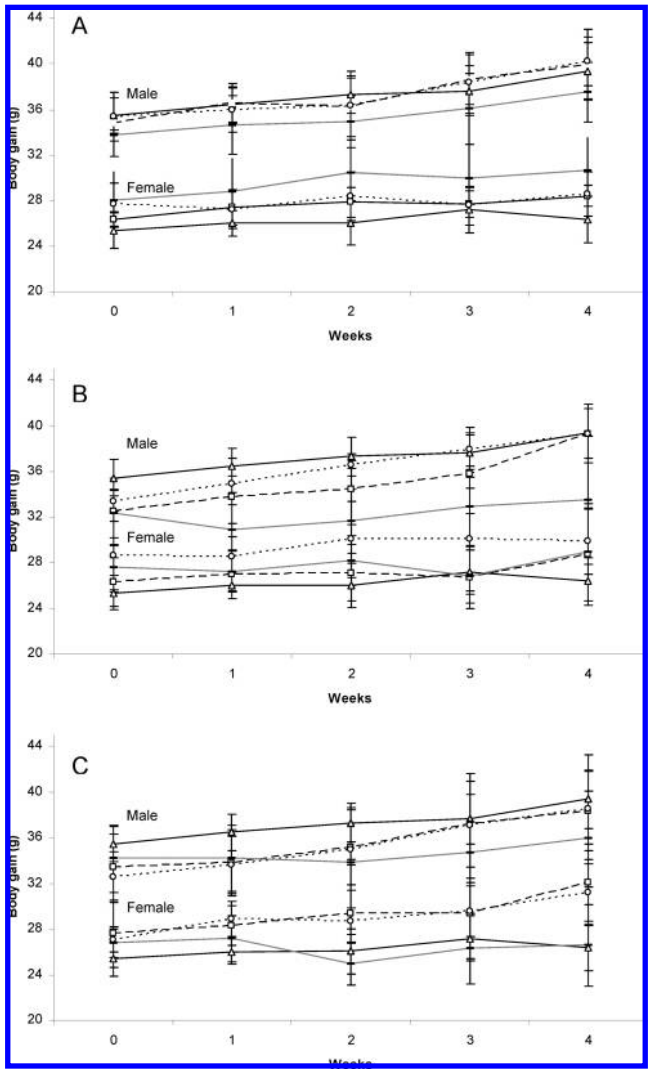

Figure 1. Growth curves for Swiss mice treated with quercetin (Q), pterostilbene (t-PTER), or a mixture of both of them (QP) for 4 weeks (28 days): (**A**) quercetin treatment (control, △; Q1, □; Q2, ○; Q3, −); (**B**) t-pterostilbene treatment (control, △; P1, □; P2, ○; P3, −); (**C**) quercetin plus t-pterostilbene treatment (control, △; QP1, □; QP2, ○; QP3, −).

3.4. Organ Weights and Histopathological Analysis. The relative organ weights (as a percent of body weight ± SD) are shown in **Table 5**. The histological examination showed that no toxicity-related alterations were found in either of the organs examined in all groups. Postmortem examination of internal organs did not show macroscopic differences in size, color, or texture between control and treated groups. Neither pathological signs nor gross lesions were observed in the vital organs examined by microscopy.

The relative weights of lungs in the female QP1 group and male QP2 and QP3 groups were reduced with respect to control groups, and the effect was most pronounced in females than in males. This decline in weights was statistically significant ($p < 0.05$) in both sexes (**Table 5**). In contrast, the weight of lungs in the female Q2 group increased significantly ($p < 0.05$) when compared to controls.

The relative liver weight also was significantly increased in the P3 ($p < 0.05$) and QP3 ($p < 0.001$) female groups and in the P2 ($p < 0.1$) male group at the end of the 28-day period, as compared to controls; although there was no effect on liver weight in male mice receiving t-PTER, QUER, or t-PTER + QUER (**Table 5**). The relatively highly statistically significant enlargement of the liver in these groups could not be considered conclusive because it was not observed for all the groups treated with the same dose of pterostilbene or for all the groups of the same sex. A reasonable hypothesis to justify this finding could be that pterostilbene is metabolized in the liver. In the P3 male mice group, kidney weight was significantly ($p < 0.05$) decreased at the end of the 28-day treatment period.

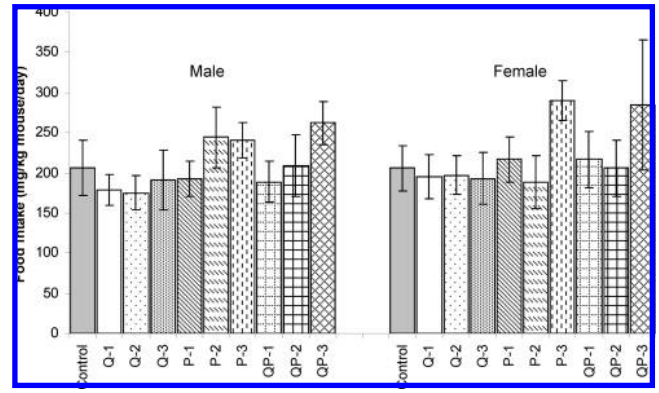

Figure 2. Mean food intake of quercetin (Q), t-pterostilbene (P), and a mixture of both of them (QP) in treated Swiss mice during the experimental period.

A significant increase ($p < 0.05$) in brain weight was observed in males in the P1, P2, and QP3 groups compared to controls (**Table 5**). In the female Q2, Q3, P3, and QP1 groups, brain weight was statistically ($p < 0.1$) higher than that in the control female group.

Only in the QP3 female mice group did heart and pancreas weight increase ($p < 0.1$) compared to controls, whereas pancreas weight in male Q2 and QP2 was statistically ($p < 0.1$) higher than that in the control group. The spleen weight increased significantly ($p < 0.1$) in the Q1, P1, P2, P3, and QP3 female groups with respect to controls.

Male mice treated with t-PTER, QUER, or t-PTER + QUER did not show significant changes of heart and spleen weights in comparison to controls. Besides, female treated mice did not show differences in the weights of their kidneys, compared to controls (**Table 5**).

The gross of microscopic pathological changes observed in mice sacrificed at the end of the study were considered incidental and typical of spontaneously occurring natural lesions commonly observed in mice of that age and strain.

4. DISCUSSION

4.1. Body Weight Gain. Oral administration of P1, P2, and P3 during 28 days did not affect the final body weight or the mean growth rate, in agreement with data from other authors (*38*) who assayed t-RESV at a dose of 20 mg/(kg day) during a similar treatment period. Our results indicate that, in terms of growth, t-PTER is well tolerated by animals at the doses and routes of administration tested (**Figure 1**). Besides, total female or male body weight after QUER administration was found to be similar to that found for controls (**Figure 1**), in accordance with results from other authors, who administered 400 mg/kg of QUER over 410 days to rats (*28*) or 25 mg PTER/kg to hamsters (*11*).

The National Toxicology Program (NTP) investigated the toxic effects of quercetin by feeding F344/N rats diets consisting of up to 1900 mg/kg quercetin for 728 days. Reduced body weight gain in male and female rats was observed by week 15, and the final mean body weights were 87% of controls at week

Table 2. Hematological and Biochemical Findings in Blood of Male Swiss Mice Treated with Quercetin, Pterostilbene, or a Mixture of Both of Them for 28 Days[a]

		QUER mg/kg b.wt./day			PTER mg/kg b.wt./day			QUER + PTER mg/kg b.wt./day		
parameters	control	30	300	3000	30	300	3000	30	300	3000
glucose, mg/dL	195 ± 9	198 ± 19	188 ± 20	182 ± 8*	201 ± 5	185 ± 19	191 ± 19	201 ± 10	197 ± 12	200 ± 17
Cho-LDL, mg/dL	44 ± 8	28 ± 4*	28 ± 7*	37 ± 16	33 ± 4*	30 ± 9*	25 ± 3*	33 ± 7	30 ± 9*	36 ± 8
Alb, g/L	24 ± 0.5	24 ± 2	23 ± 2	25 ± 3	23 ± 2	23 ± 1	24 ± 2	22 ± 1*	22 ± 1*	23 ± 1
Glb	32 ± 3	33 ± 3	36 ± 2*	39 ± 4*	34 ± 5	33 ± 2	35 ± 4	33 ± 3	32 ± 2	31 ± 2
total Bil, mg/dL	0.4 ± 0.1	0.5 ± 0.3	0.7 ± 0.2*	0.5 ± 0.2	0.5 ± 0.2	0.4 ± 0.1	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.1	0.5 ± 0.2
Ca, mg/dL	9.6 ± 1.1	9 ± 0.7	9 ± 1	9 ± 0.5	9 ± 2	9 ± 1	9.4 ± 1	11 ± 0.4*	10 ± 0.3	10 ± 0.5
phosphatase, U/L	120 ± 23	108 ± 43	112 ± 20	178 ± 197	132 ± 22	130 ± 23	118 ± 54	116 ± 25	92 ± 19	185 ± 50*
RBC/mm^3	7.8 ± 0.4	9 ± 0.3*	8 ± 0.4	8 ± 0.2	10 ± 0.2**	10 ± 0.4**	10 ± 0.3**	10 ± 0.3**	9 ± 0.2**	9 ± 0.4*
ht, %	39.4 ± 0.2	43 ± 0.1**	42 ± 0.3**	41 ± 0.2**	49 ± 0.3**	47 ± 0.3**	47 ± 0**	46 ± 0.1**	45 ± 1**	43 ± 0.1**
neutrophiles (%)	22 ± 4	32 ± 3*	24 ± 4	30 ± 3*	24 ± 3	28 ± 2*	27 ± 6	26 ± 8	26 ± 3	27 ± 7
monocytes (%)	74 ± 4	64 ± 3*	72 ± 5	66 ± 3*	73 ± 4	70 ± 4	69 ± 6	72 ± 9	71 ± 4	70 ± 7

[a] Data are expressed as mean ± SD ($n = 5$). * and ** indicate a significant difference from the controls at the levels of $p < 0.05$ and $p < 0.001$, respectively. Hematocrit (Ht), red blood cell count (RBC), bilirubin (Bil), cholesterol (Cho), albumin (Alb), globulin (Glb), calcium (Ca). Normal reference range for each parameter taken from Zúñiga et al. (*40*).

Table 3. Hematological and Biochemical Findings in Blood of Female Swiss Mice Treated with Quercetin, Pterostilbene, or a Mixture of Both of Them for 28 Days[a]

		QUER mg/kg b.wt./day			PTER mg/kg b.wt./day			QUER + PTER mg/kg b.wt./day		
parameter	control	30	300	3000	30	300	3000	30	300	3000
Cho, mg/dL	107 ± 26	72 ± 4*	79 ± 8	87 ± 18	124 ± 25	85 ± 24	114 ± 28	99 ± 33	88 ± 21	104 ± 31
AST, U/L	176 ± 41	321 ± 350	234 ± 152	242 ± 139	235 ± 123	322 ± 154	296 ± 98*	265 ± 79	288 ± 113	356 ± 213
ALT, U/L	45 ± 16	83 ± 74	64 ± 30	71 ± 36	57 ± 23	108 ± 99	79 ± 30	66 ± 18	87 ± 34*	91 ± 35*
RBC/mm^3	8 ± 0.4	9 ± 0.3*	8 ± 0.3	8 ± 0.2	10 ± 0.5**	10 ± 0.3**	9 ± 0.1*	10 ± 0.3**	9 ± 0.4*	9 ± 0.2*
PLT 10^5/mm^3	98 ± 10	91 ± 6	97 ± 9	90 ± 6	102 ± 7	96 ± 8	93 ± 9	97 ± 6	87 ± 5	85 ± 3*
ht, %	39 ± 0.1	42 ± 0.1**	41 ± 0.1**	40 ± 0.3*	48 ± 0.4**	47 ± 0.2**	46 ± 0.1**	45 ± 0.1**	44 ± 0.2**	43 ± 0.1**
neutrophils (%)	25 ± 8	27 ± 4	21 ± 4	21 ± 6	24 ± 3	24 ± 4	31 ± 5	26 ± 5	28 ± 5	35 ± 4*

[a] Data are expressed as mean ± SD ($n = 5$). * and ** indicate a significant difference from the controls at the levels of $p < 0.05$ and $p < 0.001$, respectively. Hematocrit (Ht), red blood cell (RBC) count, cholesterol (Cho), aspartate amino transferase (AST), alanine amino transferase (ALT), platelets count (PLT). Normal reference range for each parameter taken from Zúñiga et al. (*40*).

Table 4. Urinary Balance in Male and Female Swiss Mice Treated Orally with the Highest Dose Administrated of Quercetin, Pterostilbene, or a Mixture of Both of Them for 28 days (Results Were Obtained by the Combur^{10}-Test from Roche)

	male				female			
	control	Q3	P3	QP3	control	Q3	P3	QP3
specific weight (×10^3)	1.007 ± 2.8	1.010 ± 4	1.011 ± 2.5	1.015 ± 4	1.01 ± 2.5	1.00 ± 2.5	1.01 ± 2.5	1.01 ± 5
pH	8.2 ± 0.5	8.0 ± 0	6.5 ± 0.6	7.0 ± 0	8.2 ± 0.9	8.5 ± 0.6	6.5 ± 0.6	7 ± 0.8
leukocites μL^{-1}	negative	negative	negative	negative	negative	negative	negative	negative
nitrite	negative	negative	negative	negative	negative	negative	negative	negative
protein (g L^{-1})	0.2	0.15	0.3	0.5	0.15	0.15	0.15	0.3
glucose	normal	normal	normal	normal	normal	normal	normal	normal
ketones	negative	negative	negative	positive	negative	negative	negative	positive
urobilinogen	normal	normal	normal	normal	normal	normal	normal	normal
bilirubin	negative	negative	negative	negative	negative	negative	negative	negative
blood/hemoglobin (erythrocytes μL^{-1})	ca. 11	negative	ca. 25	ca. 11	ca. 10	negative	ca. 23	ca. 11

104 (*32*). The finding observed in this study led us to understand that 28-days are not enough days to observe an alteration in the body weight gain of mice from our study.

The absorption of flavonoids from food depends on the form in which QUER is presented (*4*). Quercetin glycosides are better absorbed than its aglycone (*23*). Hollman et al. (*23*) speculated that intestinal sugar carriers may play a role in flavonoid absorption, especially when they are present as β-D-glycosides. The quercetin group might thus be drawn into the enterocyte by its glucose moiety, which is transported by the glucose carrier. The aglycone (i.e., free quercetin) would then fail to be absorbed because it lacks a sugar (*23*). QUER would probably be greater because it was administered as powders in food. However, no significant difference in body weight gain between QUER-treated and control mice (**Figure 1**) was observed. Besides, the different response related to sex could be explained on the basis of previous studies made by Erlund et al. (*2*), who found that QUER-3-rutinoside was more bioavailable in women compared to men. This fact was likely due to gender-related differences in gastrointestinal microbiota, absorption, metabolism, and/or biotransformation mechanisms.

4.2. Organ Weight and Histopathology. Small increases in organ weight (**Table 5**) observed in mice were not accompanied by changes in clinical chemistry (**Tables 2** and **3**) or histopathology and were therefore not considered an adverse treatment-related effect. The NTP studies of quercetin pointed out that the principal lesions associated with the administration of quercetin occurred in the kidney of dosed male rats but not in dosed female rats. Parathyroid hyperplasia frequently accompanies nephropathy, because the progressive loss of renal function disrupts calcium

Table 5. Relative Organ Weights of Female and Male Swiss Mice Treated with Quercetin, Pterostilbene, or a Mixture of Both of Them for 28 days[a]

	lung	heart	liver	pancreas	spleen	kidney	brain
				Female			
control	0.76 ± 0.09	0.48 ± 0.08	5.19 ± 0.4	0.45 ± 0.06	0.30 ± 0.04	1.47 ± 0.14	1.62 ± 0.34
Q1	0.73 ± 0.06	0.56 ± 0.09	5.28 ± 0.28	0.51 ± 0.08	0.37 ± 0.07*	1.37 ± 0.13	1.47 ± 0.22*
Q2	0.88 ± 0.06**	0.49 ± 0.03	5.32 ± 0.65	0.53 ± 0.05	0.34 ± 0.05	1.42 ± 0.19	1.53 ± 0.1
Q3	0.69 ± 0.07	0.41 ± 0.08	5.61 ± 0.6	0.47 ± 0.06	0.33 ± 0.02	1.36 ± 0.32	1.44 ± 0.24*
P1	0.77 ± 0.09	0.48 ± 0.08	5.41 ± 0.52	0.51 ± 0.10	0.39 ± 0.10*	1.37 ± 0.46	1.63 ± 0.31
P2	0.72 ± 0.03	0.51 ± 0.07	5.79 ± 0.85	0.55 ± 0.12	0.35 ± 0.05*	1.30 ± 0.23	1.42 ± 0.19
P3	0.83 ± 0.12	0.45 ± 0.1	6.56 ± 0.7**	0.53 ± 0.09	0.37 ± 0.08*	1.37 ± 0.19	1.64 ± 0.11*
QP1	0.63 ± 0.06**	0.52 ± 0.09	5.63 ± 0.75	0.49 ± 0.04	0.29 ± 0.02	1.35 ± 0.11	1.37 ± 0.13*
QP2	0.67 ± 0.07	0.48 ± 0.08	5.40 ± 0.49	0.53 ± 0.08	0.33 ± 0.05	1.33 ± 0.16	1.40 ± 0.11
QP3	0.81 ± 0.12	0.49 ± 0.07*	7.48 ± 0.47***	0.52 ± 0.09*	0.26 ± 0.03*	1.44 ± 0.2	1.69 ± 0.14
				Male			
control	0.62 ± 0.03	0.49 ± 0.04	5.19 ± 0.45	0.58 ± 0.08	0.41 ± 0.06	1.62 ± 0.11	1.1 ± 0.1
Q1	0.64 ± 0.12	0.47 ± 0.03	4.83 ± 0.58	0.61 ± 0.06	0.38 ± 0.03	1.53 ± 0.17	1.21 ± 0.09
Q2	0.60 ± 0.09	0.48 ± 0.06	5.06 ± 0.55	0.55 ± 0.09*	0.42 ± 0.09	1.58 ± 0.19	1.1 ± 0.24
Q3	0.61 ± 0.07	0.52 ± 0.05	5.13 ± 0.44	0.59 ± 0.06	0.49 ± 0.09	1.66 ± 0.21	1.25 ± 0.13
P1	0.6 ± 0.06	0.5 ± 0.06	4.63 ± 0.91	0.56 ± 0.1	0.40 ± 0.07	1.57 ± 0.35	1.22 ± 0.05**
P2	0.61 ± 0.06	0.48 ± 0.04	5.03 ± 0.39*	0.55 ± 0.05	0.37 ± 0.07	1.6 ± 0.08	1.26 ± 0.07**
P3	0.66 ± 0.13	0.47 ± 0.1	6.57 ± 1.44	0.58 ± 0.09	0.39 ± 0.09	1.45 ± 0.13**	1.5 ± 0.34
QP1	0.62 ± 0.07	0.48 ± 0.04	4.89 ± 0.39	0.56 ± 0.07	0.51 ± 0.15	1.62 ± 0.1	1.28 ± 0.18
QP2	0.56 ± 0.02**	0.48 ± 0.02	4.98 ± 0.18	0.56 ± 0.05*	0.63 ± 0.07	1.58 ± 0.14	1.26 ± 0.25
QP3	0.55 ± 0.03**	0.53 ± 0.03	5.72 ± 1.12	0.65 ± 0.05	0.40 ± 0.14	1.6 ± 0.15	1.38 ± 0.17**

[a] Data are expressed as mean ± SD ($n = 5$). *, **, and *** indicate a significant difference from the controls at the levels of $p < 0.1$, $p < 0.05$, and $p < 0.001$, respectively.

homeostasis (*32*). Our results demonstrated normal appearance of vital organs, including kidney, in the macroscopic examinations after necropsy (not shown), suggesting that t-PTER and QUER are nontoxic even at very high doses. However, a significant decrease ($p < 0.05$) in P3 male kidney weight, with an increase in serum Ca concentration of QP1, was observed (**Table 5**). In agreement, Jang et al. (*9*) observed no signs of RESV-induced toxicity in mice treated topically, as judged by visual inspection of animals or gross morphological examination of major organ systems, relative to controls. Rimando et al. (*21*) administered t-PTER to animals during various physiologic stages, and their results demonstrated that this polyphenol is a free radical scavenger that effectively scavenges radicals and reduces peroxidation, thus suggesting that its antioxidant activity could be responsible for its capability to prevent organ lesions.

4.3. Urinalysis. Urinary volume and density were not significantly different from control values. Similarly, there were no treatment-related effects on urinalysis parameters (**Table 4**).

4.4. Hematological Parameters. Hematological parameters and serum chemistry measurements were also found to be within a normal range upon t-PTER and/or QUER treatment, except for the increase in RBC and hematocrit levels found in the treated mice. In relation to female mice, the slight increase observed in the RBC level can be correlated with the spleen weight gain, but this is not the case in male mice. Polycythemeia or an increase in RBC and hematocrit was observed in male and female treated mice, which can be associated directly with an increase of RBC in bone production or indirectly with dehydration.

Considering the hypolipidemic properties of flavonoids, it is no surprise to observe a decrease in serum cholesterol levels in QUER-treatment experimental animals (*29*). These effects are in agreement with those of Rimando et al. (*21*), who observed that hypercholesterolemic hamsters fed with 25 ppm of t-PTER showed a 29% decrease in plasma low density lipoprotein (LDL) cholesterol. In accordance with our results (**Table 2**), Satheesh and Pari (*26*) fed rats with a high dose of pterostilbene and observed lowering action on cholesterol in diabetic rats for six weeks. Similarly, Nakamura et al. (*3*) observed a significant decrease in serum HDL-cholesterol and triglyceride levels in rats orally administered quercetin at 0.5 g/kg and 1.0 g/kg, respectively. Indeed, several in vitro and in vivo studies have shown that t-PTER and its glycoside inhibited the accumulation of cholesterol and triglycerides (*20*, *26*, *27*). Results obtained by Rimando et al. (*11*) indicated that PTER acts as an inductor of endogenous peroxisome proliferator-activated receptor (PPARα) in H4IIEC3 cells. PPARα is involved in fatty acid and lipid catabolism, through the activation of genes involved in fatty acid oxidation in the liver, heart, kidney, and skeletal muscles; moreover, in liver, it also decreases triglyceride and very low density lipoprotein (VLDL) synthesis. They also demonstrated that PTER lowers lipid/lipoprotein levels in hypercholesterolemic hamsters through activation of PPARα. Yamamoto et al. (*15*) pointed out the same effect on PPARα from rat plasma previously administered intragastrically with 40 μmol of QUER. Ohmuma (*39*) reported that QUER lowers the serum total cholesterol in rats fed with a cholesterol-enriched diet and also reduces hepatic total cholesterol in rats fed with a cholesterol-free diet.

Nakamura et al. (*3*) administered orally quercetin to rats at various doses. No toxicological symptoms were observed in rats administered quercetin in a dose as high as 1.0 g/kg body weight/day for 22 days. Moreover, oral administration showed dose-dependent antioxidant properties. Similarly, human studies have not shown any adverse effects associated with oral administration of quercetin in a single dose of up to 4 g or after one month of 500 mg twice daily (*28*, *30*) In conclusion, the present 28-days subchronic study is apparently consistent with previous findings that orally administered t-PTER and QUER, even at the highest dose administered, were nontoxic.

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Received for review December 3, 2008. Revised manuscript received February 17, 2009. Accepted February 23, 2009. This research was supported by grant programs from the Universitat de Valencia (UVEG2004-ALIFUNC-03-03) and from the Ministerio de Educación y Ciencia (AGL2005-00831).

JF803579E