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Safety Evaluation of an Oak-Flavored Milk Powder Containing Ellagitannins upon Oral Administration in the Rat

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Ellagitannins are phenolic compounds that occur in a number of dietary sources such as walnuts, raspberries, pomegranate, strawberries, and oak-aged wines. Ellagitannins have been acknowledged to have antioxidant and cancer chemopreventive activities. However, ellagitannins have also been considered to be "antinutrients" and have been reported to provoke adverse effects in animals. In this context, risk assessment studies are demanded to ensure that chemicals present in food do not compromise food safety. Oak-flavored milk can be obtained by thermal treatment of milk in the presence of oak shavings. The oak-flavored milk can be used to impart wood-like flavor to different recipes. The present study evaluated, under a subchronic approach, the safety of an oak-flavored milk powder containing ellagitannins in rats upon repeated oral administration for 96 days. The effect of 30% oak-flavored milk powder in the diet was evaluated. This amount involved a margin of safety equivalent to the daily intake of 134 yogurts by a 70 kg person for 96 days. Growth rate, food utility index, 9 hematological, and 13 serobiochemical parameters as well as the metabolism and tissue distribution of ellagitannins and derived metabolites were studied. Overall, no significant differences were found in oak-milk-fed rats in any blood parameter analyzed. No effect was found on growth rate and food utility index. Urolithin A was identified in feces of rats fed oak-flavored milk and the glucuronide derivative in urine. No metabolites were found in the liver, kidney, or uterus. Histopathological analysis of liver and kidney corroborated the absence of toxicity. Therefore, these results suggest that the intake of the oak-flavored milk powder assayed in this study is safe and does not involve potential hazard to human health.

KEYWORDS: Ellagitannin; oak; food safety; xenobiotic; ellagic; toxicity; urolithin; tannin; bioavailability; metabolism; tissue disposition

INTRODUCTION

Ellagitannins are esters of hexahydroxydiphenic acid and sugars (most often β-D-glucose or quinic acid) that can be hydrolyzed ("hydrolyzable tannins") to yield ellagic acid. These phenolics can reach high molecular weights (up to 4000). The occurrence of ellagitannins has been reported in walnut, persimmon, pomegranate (fruit and juice), oak-aged wines and other spirits (whiskey, brandy) aged in oak barrels, strawberry, raspberry, blackberry, peach, plum, muscadine grape and wine, etc. (*1, 2*). Ellagitannins and ellagic acid have been reported to show in vitro and in vivo antitumorigenic and antipromoting activities (*3, 4*). The dietary administration of ellagitannin-containing foodstuffs such as strawberries and raspberries to rats has been reported to inhibit events associated with both the initiation and promotion/progression of chemically induced esophageal cancer (*5, 6*). In addition, ellagitannins have been proposed as pro-phytoestrogens because the bioavailable ellagitannins colonic microflora derived metabolites, urolithins (*7*), show estrogenic and antiestrogenic activities in vitro in the range of those described for isoflavones (*8*).

However, despite the acknowledged health benefits attributed to ellagitannins, the perception is still widespread that hydrolyzable tannins, including ellagitannins, can be harmful or even toxic (*9–11*). Ellagitannins have been reported to be antinutrients

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in animals due to their ability to combine with dietary proteins, cellulose, pectin, minerals, etc., thus hampering and retarding their digestion (*12*). In addition, hydrolyzable tannins can also impair the digestive process by complexing with secreted enzymes and endogenous protein (*13*). General detrimental effects in ruminants and nonruminants are associated with lower feed intake, protein and dry matter digestibility, liveweight gains, milk yield, wool production, etc. (*12, 14*). However, most studies on tannin intoxications failed to state the type of tannin responsible, and only a few studies have investigated the toxicity of specific tannins. For example, administration of the ellagitannins castalin and vescalin from chestnut to lambs did not provoke toxic effects (*15*). Punicalagin, a pomegranate ellagitannins, has been reported to cause liver necrosis and nephrotoxicity in cattle (*16, 17*). In addition, Lin et al. (*18*) reported some antioxidant and hepatoprotective effects against acetaminophen-induced liver damage, but some detrimental effects were also detected at high doses of punicalagin. However, Cerdá et al. (*19*) did not observe any toxic effect in rats upon consumption of a high punicalagin amount for 5 weeks (approximately equivalent to 350 g/day of punicalagin for a 70 kg person). Oak ellagitannins have been reported to show different effects depending on the *Quercus* sp. For example, *Q. rotundifolia* acorns, which are very rich in ellagitannins, have proved to be excellent for growing and finishing Iberian pigs and ruminants (*20, 21*). On the contrary, toxic effects have been reported upon the administration of oak extracts from *Q. calliprinos*, *Q. incana*, and *Q. coccifera* to cattle, rabbits, and goats (*22, 23*). Oak wood-derived ellagitannins are present in our diet mainly through the intake of oak-aged wines and spirits such as whiskey and brandy, also aged in oak barrels. This maturation procedure normally occurs in *Q. alba* (American oak) or *Q. robur* (European oak). Oak staves are thermally treated ("toasted") to facilitate stave assembly in barrel production. In addition, the toasting improves the sensory quality of the beverage (*24*). Despite the common presence of oak-derived ellagitannins in wine drinkers, oak (*Q. robur*) poisoning in cattle has been also described (*25*). Therefore, the balance risk/benefit in the intake of ellagitannin-containing foodstuffs depends not only on the ellagitannin dose consumed but also on the source, chemical structure, and food matrix, which has given rise to a conundrum regarding tannins in animal nutrition and health as recently reviewed by Mueller-Harvey (*26*). With the above in mind, it seems that the safety evaluation of ellagitannin-containing foods should be studied for each specific case.

Risk assessments are conducted to ensure that chemicals present in food do not compromise food safety. Different institutions such as the U.S. Food and Drug Administration (FDA), the European Food Safety Authority (EFSA), and the International Life Science Institute (ILSI) supervise and provide science-based advice to avoid any potential hazard caused by foodstuffs, including novel foods and/or botanical-enriched preparations (www.fda.gov; www.efsa.europe.eu; www.ilsi.org).

In light of the above and to ensure food safety for potential consumers, the present study was designed to explore potential toxic effects of an oak-flavored milk powder containing ellagitannins upon subchronic oral administration in the rat. This powder could be tentatively used to impart a wood-like flavor to some foodstuffs.

MATERIALS AND METHODS

Reagents. Ellagic acid was purchased from Sigma (St. Louis, MO). Urolithins A and B were chemically synthesized by Kylolab (Murcia, Spain). All other reagents (formic acid, hydrochloric acid, methanol, etc.) were of analytical grade and supplied by Merck (Darmstadt, Germany). Milli-Q system (Millipore Corp., Bedford, MA) ultrapure water was used throughout this research.

Table 1. Compositions of the Diets Used in the Study

	control	control milk (30%)	oak-flavored milk (30%)
proteins (%)	14.5	20.9	19.4
carbohydrates (%)	63.9	60.0	62.1
fats (%)	4.0	3.2	3.1
energy (kcal/g)	3.2	3.3	3.3

Animals and Diets. Sprague–Dawley rats ($n = 24$) with weights ranging from 197 to 245 g were provided by the Animal Centre of the University of Murcia (Spain). Handling and killing of rats were in full accordance with national and international law and policies (*27*) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (*28*). These guidelines recommend the use of the minimum possible number of animals for experiments. Rats were quarantined for 1 week, housed in cages (four rats per cage), kept at 22 ± 2 °C with 55 ± 10% relative humidity and controlled lighting (12 h light/dark cycle) throughout the present experiment. Rats were fed standard feedstuff for rats (Panlab, Barcelona, Spain). The standard feedstuff contained 14.5% proteins, 63.9% carbohydrates, and 4% fat (3.2 kcal/g). Both solid diet and water were consumed ad libitum. Rats were distributed in three groups composed by two cages of animals: control (fed with standard feedstuff), control milk (fed with standard milk powder), and oak-flavored milk (fed oak-flavored milk powder). Both control milk and oak-flavored milk powders were provided by Laiteries Triallat (group Rians, France). Control (standard) milk powder contained 36% proteins, 51% carbohydrates, and <1.25% fats (3.6 kcal/g). The oak-flavored milk powder contained 31% proteins, 58% carbohydrates, and <1% fats (3.66 kcal/g) (data provided by the manufacturer). The final diets consumed by the rats are shown in **Table 1**. The procedure to obtain the oak milk powder is confidential but, briefly, consists of incubating oak shavings with boiling milk for some minutes. Afterward, the milk is filtered and spray-dried to obtain the final oak-flavored milk powder. The feedstuff (which is usually administered in the "pellet" form) was ground to ensure a full homogenization with the milk powders.

A preliminary study was conducted with eight rats to evaluate the acceptance of different milk powder contents in the diet. Rats were randomly divided into four groups ($n = 2$) that were fed increasing oak-flavored milk powder amounts, that is, 10, 20, 25, and 30% during 7 days for each amount. The final intervention was carried out with 24 female rats randomly distributed in the three groups described above and fed the highest oak-flavored milk powder amount (30%).

Skin, hair, eyes, nervous system conditions, and behavior were examined daily. Body weight and food and water consumption were recorded daily. Growth rate was calculated as the difference between the final and initial weights divided by the 96 day intervention period. Food utility index was calculated as the weekly body weight gain divided by the food consumption (*19*).

Ellagitannins and Ellagic Acid Extraction from Milk Powders. Milk powders (3 g of either control or oak-flavored milk) were dissolved in 50 mL of water at 40 °C and vigorously stirred. Afterward, 100 μL of phosphoric acid and 100 mL of methanol/HCl (99.9:0.1) were added to the milk solution. The mixture was stirred overnight. The solution was centrifuged at 3000*g* for 10 min at room temperature in a Centromix centrifuge (Selecta, Barcelona, Spain). The supernatants were concentrated under reduced pressure at 40 °C to remove the methanolic phase. The aqueous (10 mL) phase was filtered through a Sep-Pak cartridge (a reverse phase C-18 cartridge), which retained phenolic compounds and removed other highly hydrophilic compounds. The cartridges were previously activated with 10 mL of methanol and 10 mL of water. The remaining volume of each cartridge was removed by passing air through it. Each cartridge was washed with 10 mL of water. Those fractions eluted with water were discarded. The remaining volume in each cartridge was eluted with 2 mL of MeOH. The methanolic fractions of each cartridge were collected and filtered through a 0.45 μm filter and injected in the HPLC-DAD-MS-MS equipment. The extraction protocol was repeated four times.

Hydrolysis of Samples for Quantifying Ellagitannins. One gram of milk powder (either control or oak-flavored milk) was dissolved in 5 mL of 2 N HCL. The solution was bubbled with N_2 to remove the aqueous oxygen content. The solution was placed in an oven at 85 °C for 15 h. Afterward, 10 mL of diethyl ether was added to the solution and stirred, and the organic phase was recovered. The extraction with ether was repeated twice. The pooled organic phases were evaporated to dryness under reduced pressure. One milliliter of methanol was added to dissolve the dry matter. The solution was filtered through a 0.45 μm filter and injected in the HPLC-DAD-MS-MS equipment. The hydrolysis protocol was repeated five times.

Sampling Procedure (Plasma, Urine, Feces, and Organs). Rats were anesthetized via intramuscular injection with a mixture of ketamine (Imalgéne 1000) (Merial Laboratorios, Barcelona, Spain) and xylazine (1:1) (Xilagesic 2%) (Laboratorios Calier, Barcelona, Spain) with a dose of 1 mL/kg of body weight. Blood was extracted every 2 weeks with a cardiac punction and collected in heparinized tubes. Heparinized blood was used to obtain the hematological profile. For the determination of serobiochemical parameters, blood was immediately separated in plasma by centrifugation at 14000*g* for 15 min at 4 °C in a Sigma 1-13 microcentrifuge (Braun Biotech. International, Germany). The plasma was immediately frozen at −80 °C for further analyses.At the end of the intervention study (96 days), animals, previously anesthetized as described above, were sacrificed by exsanguination. Blood, urine, feces, and main organs were collected.

Urine samples were obtained at the end of the experiment and stored with 1 g/L ascorbic acid at −80 °C until use. Samples (2.5 mL) were acidified with 2.5 μL of 6 N HCl and filtered through reversed-phase C_{18} Sep-Pak cartridges, washed with distilled water (10 mL), and further eluted with methanol (1 mL). A sample of 100 μL was analyzed by HPLC-DAD-MS-MS.

Plasma samples (180 μL) were mixed vigorously with 500 μL of 0.2 N MeOH/HCl. The mixture was centrifuged at 14000*g* for 5 min at 4 °C. The supernatant was filtered through a 0.45 μm filter and injected (100 μL) in the LC-MS-MS equipment.

Feces were collected at the end of the experiment and immediately frozen at −80 °C until use. Samples (1 g) were ground, vigorously mixed with 10 mL of MeOH/water/HCl (80:19.5:0.5), and exposed to ultrasound for 10 min. The mixture was centrifuged at 14000*g* for 10 min at 4 °C. The methanolic phase of the supernatant was removed with N_2. The remaining aqueous phase was filtered through a Sep-Pak cartridge as described above, eluted with 2 mL of MeOH and injected (100 μL) in the LC-MS-MS.

Organs were quickly excised and weighed. Brain, heart, liver, kidneys, spleen, thyroid gland, uterus, and gastrointestinal tract were submitted to macroscopic pathology examination (weight, visual appearance, etc.). Livers, kidneys, and uteri were collected to evaluate the presence of ellagitannin-derived metabolites. Livers and kidneys were submitted to histopathological examination.

Six grams of liver, 1.6 g of kidney, and 0.4 g of uterus were homogenized with 15, 8, and 6 mL of cold MeOH/water (80:20), respectively, using an IKA T10 Ultra-Turrax (Janke and Kunkel, Ika-Labortechnick, Germany) at 24000 rpm for 1 min at 4 °C. The mixture was centrifuged at 14000*g* for 5 min at 4 °C, and the supernatant obtained was kept at 4 °C. Each pellet was further extracted with 5 mL of the same methanolic solution. The mixture was also centrifuged as described above. Both supernatants from each organ were pooled and evaporated with N_2 at 40 °C. The dry matter was dissolved with 150 μL of MeOH, filtered through a 0.45 μm filter and injected (100 μL) in the LC-DAD-MS-MS equipment.

For the histopathological study, the organs were immediately cut and fixed in neutral buffered formalin (10%). Paraffin sections were prepared and stained with hematoxylin-eosin staining for histological examination. Lesions were scored from 0, for intact liver or renal parenchyma, to 4 (maximum), for severe degeneration areas in the tissue.

Hematology and Clinical Chemistry. Hematological parameters were measured with an automatic device (Abacus Junior Vet, CVM S.L., Navarra, Spain) with specific software to analyze blood rat parameters. The following parameters were analyzed: red blood cell number (RBC), hemoglobin concentration (Hb), hematocrit (HCT), erythrocytic indexes, that is, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cells count (WBC), and platelet number (PLT).

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatine kinase (CK), γ-glutamyl aminotransferase (GGT), amylase, glucose, creatinine, cholesterol, bilirubin, triglycerides, phosphorus, total protein, albumin, globulins, and urea were measured in heparinized plasma using a Cobas Mira Plus analyzer (HORIBA ABX Diagnostic, Montpellier, France) (*19*).

Reference values were obtained to establish the normal values range (NVR) for each parameter in our laboratory. NVR was calculated, at the beginning of the experiment, as the mean of reference values $\pm$ 2 $\times$ SD. Bilirubin, GGT, and CK were not parametrically distributed and were expressed as mean and percentiles.

LC-MS/MS Analyses. The HPLC system equipped with both a photodiode array detector and a mass detector in series consisted of a HPLC binary pump, autosampler, and degasser controlled by software from Agilent Technologies (Waldbronn, Germany). The mass detector was an ion-trap mass spectrometer (Agilent) equipped with an electrospray ionization (ESI) system (capillary voltage, 5 kV; drying temperature, 350 °C). Mass scan (MS) and MS/MS daughter spectra were measured from *m/z* 150–2000. Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, and the collision energy was set at 50%. Mass spectrometry data were acquired in the negative ionization mode.

Milk Powders. Chromatographic separations of milk powder samples were carried out on a 250 $\times$ 4 mm i.d., 5 μm, C_{18} Mediterranean sea column (Teknokroma, Barcelona, Spain) using water/formic acid (99:1, v/v) (A) and acetonitrile (B) as the mobile phases at a flow rate of 1 mL/min. The gradient started with 10% B in A to reach 25% B at 25 min, 30% B at 30 min, 60% B at 31 min, 90% B at 40 min for 6 min and returned at 47 min to the initial conditions (10% B). UV chromatograms were recorded at 280 and 360 nm. Oak ellagitannins were identified according to their MS spectra and MS/MS fragments. Ellagic acid was identified and quantified at 360 nm using the corresponding commercially available standard.

Plasma, Urine, and Organs. Chromatographic separations of samples were carried out on a 25 mm $\times$ 4 mm i.d., 5 μm, C_{18} LiChroCART column (Merck, Darmstadt, Germany) using water/formic acid (99:1, v/v) (A) and acetonitrile (B) as the mobile phases at a flow rate of 1 mL/min. The gradient started with 1% B in A to reach 25% B at 20 min, 55% B at 30 min, and 90% B at 31 min. UV chromatograms of extracts were recorded at 280, 305, and 360 nm. Ellagic acid, urolithins A and B, and the derived metabolites were identified according to their UV and MS spectra as well as MS/MS fragments as described elsewhere (*7, 29, 30*).

Statistics. Hematological and serobiochemical data were analyzed using Levene's test to check homocedasticity (homogeneity of variances). Those data with homogeneous variances were analyzed using ANOVA followed by Tukey's test. Data with no homocedasticity were treated using the nonparametric Mann–Whitney *U* test. Data were deemed to be significant at $p < 0.05$ (*).

RESULTS AND DISCUSSION

Oak-Flavored Milk Powder and Ellagitannin Content in the Rat Diet. The analyses of oak-flavored milk powder revealed the presence of $[M - H]^-$ ions at *m/z* 631 with MS/MS fragments at 301, tentatively identified as the isomers vescalin or castalin; free ellagic acid (m/z^- 301; MS/MS 257, 229); pedunculagin (m/z^- 783, MS/MS 481, 301, 169); a dimer of ellagic acid with sodium adduct (m/z^- 625, MS/MS 301), and an unknown ellagitannin with ion at m/z^- 707 and MS/MS fragments at 479, 301, and 227 because it shows an MS/MS similar pattern to that of other ellagitannins with ellagic acid as fragment ion (m/z^- 301) (*31*). The chromatographic profile of oak-flavored milk powder extracts at different wavelengths showed ellagic acid as the only UV-identifiable phenolic (**Figure 1**).

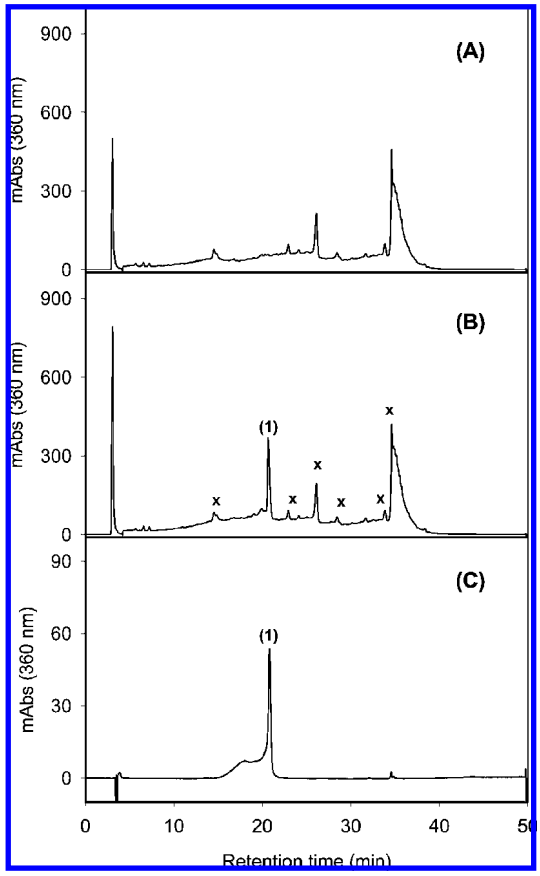

Figure 1. HPLC chromatograms of hydrolyzed control and oak-flavored milk powders: (**A**) control milk; (**B**) oak-flavored milk powder; (**C**) ellagic acid standard. (**1**) Ellagic acid. Crosses (×) designate compounds present in normal milk.

The main oak wood ellagitannins are castalagin, vescalagin, grandinin, pedunculagin, and roburins A−E (*37*) (**Figure 2**). However, these macromolecular ellagitannins are degraded upon thermal treatments such as the "toasting" for producing oak barrels, yielding castalin, vescalin, and ellagic acid in accordance with the results presented here (*24, 31*). Ellagitannins are most reliably estimated by hydrolysis into ellagic acid followed by estimation of ellagic acid by HPLC (*32*). The analyses of the nonhydrolyzed oak-flavored milk powder yielded 1 mg of ellagic acid. In the case of the hydrolyzed oak-flavored milk powder a total amount of 2.1 ± 0.15 mg of ellagic acid/100 g was found. This indicated that the difference between both values (1.1 mg) occurred in the form of ellagitannins.

A preliminary study was carried out to evaluate the acceptance of different oak-flavored milk powder concentrations in the diet by the rats as well as to ensure a large enough safety margin, especially because the ellagitannin content in the oak-flavored milk powder was rather low. To this purpose, the standard feedstuff was supplemented with 10, 20, 25, and 30% of oak-flavored milk powder during 7 days for each amount. The highest concentration (30%) was fully accepted by the rats, and it was finally chosen to conduct the study described here. A higher milk powder amount was not assayed to avoid an excessive displacement of other nutrients in the diet (*33*). The final diets consumed by the rats are shown in **Table 1**. Overall, the main difference among diets was the higher content of proteins in the diets containing both control milk and oak-flavored milk powders (approximately 5% more proteins).

On the basis of the oak-flavored milk powder analyses and feedstuff intake, the rats consumed daily 0.094 mg of ellagitannins (quantified as ellagic acid), with a total intake in the whole experiment of 9.024 mg. This would involve the daily intake of 29.4 mg when extrapolated to a 70 kg person (2.8 g in the whole study). This value is very low when compared to the ellagic acid content of a single serving of some dietary products such as strawberries (190 mg of ellagic acid), raspberries (422 mg of ellagic acid), walnuts (190 mg of ellagic acid), and oak-aged wine (5.4 mg of ellagic acid) (*7*). Although the ellagitannin content in the oak-flavored milk powder did not seem to suggest any potential toxic effect upon repeated oral administration, nonpolyphenolic constituents including combustion products (melanoidin-type compounds, volatile molecules, etc.) could exert potential adverse effects. This prompted us to assess the subchronic systemic toxicity of the oak-flavored milk powder (30% in the diet) for 96 days in the rat by determining the effect on food intake and growth rate as well as macroscopic, histopathological, hematological, and serobiochemical examinations (*33*).

The mean feedstuff intake of the rat during the study was 15 g, and the mean rat body weight was 225 g. According to the information provided by the oak-flavored milk powder manufacturer regarding the required amount of oak-flavored milk powder to prepare a yogurt serving (125 g), the presence of 30% oak-flavored milk powder in the rat diet would be equivalent to the daily intake of 134 yogurts by a 70 kg adult person, which is, in principle, a large safety margin.

Effect on Growth Rate and Food Utility Index. Ellagitannins have been considered to be antinutrients by binding to dietary proteins and altering their metabolism (*34*). To this purpose, the growth rate and food utility index (weekly body weight gain divided by the food consumption) were determined. No differences were observed in the growth curves for the three rat groups (control, control milk, and oak-flavored milk) over the 96 day study (**Figure 3A**). The mean growth rates (weight gain divided by time) were 0.66 ± 0.11 g/day for the control group, 0.75 ± 0.12 g/day for the control milk group, and 0.63 ± 0.15 g/day for the oak-flavored milk group. The food utility index logically decreased after the first month due to the lower weight gain during the second and third months. No differences were observed among the three groups (**Figure 3B**). These results indicated that no antinutritional effect was observed upon repeated oral administration of 30% oak-flavored milk powder in the rat diet.

Hematological and Serobiochemical Parameters. Hematology results are shown in **Table 2**. Normal erythroid parameters recorded over the study indicated the absence of anemia. These results allowed us to think that hemorrhage, hemolysis, or diseases causing nonregenerative anemia such as renal insufficiency, chronic, or medullary disease were absent during the study. Similarly, no differences were observed in the leukogram and platelet profile. Therefore, inflammatory conditions or primary hemostatic disorders could be also discarded (*35*).

A high variability in normal values of rat biochemical analytes has been reported in the literature. These variations are due to the use of different reagents, assay methods, equipment, or even blood extraction systems, contributing to the high interlaboratory variability (*35*). For this reason, the calculation of reference values (results not shown) for the establishment of a normal values range (NVR) for each parameter by each laboratory is highly recommended.

Serum biochemistry results are shown in **Table 3**. In general, the results presented here are in accordance with other values of serobiochemical parameters previously reported in the rat (*19, 36–38*).

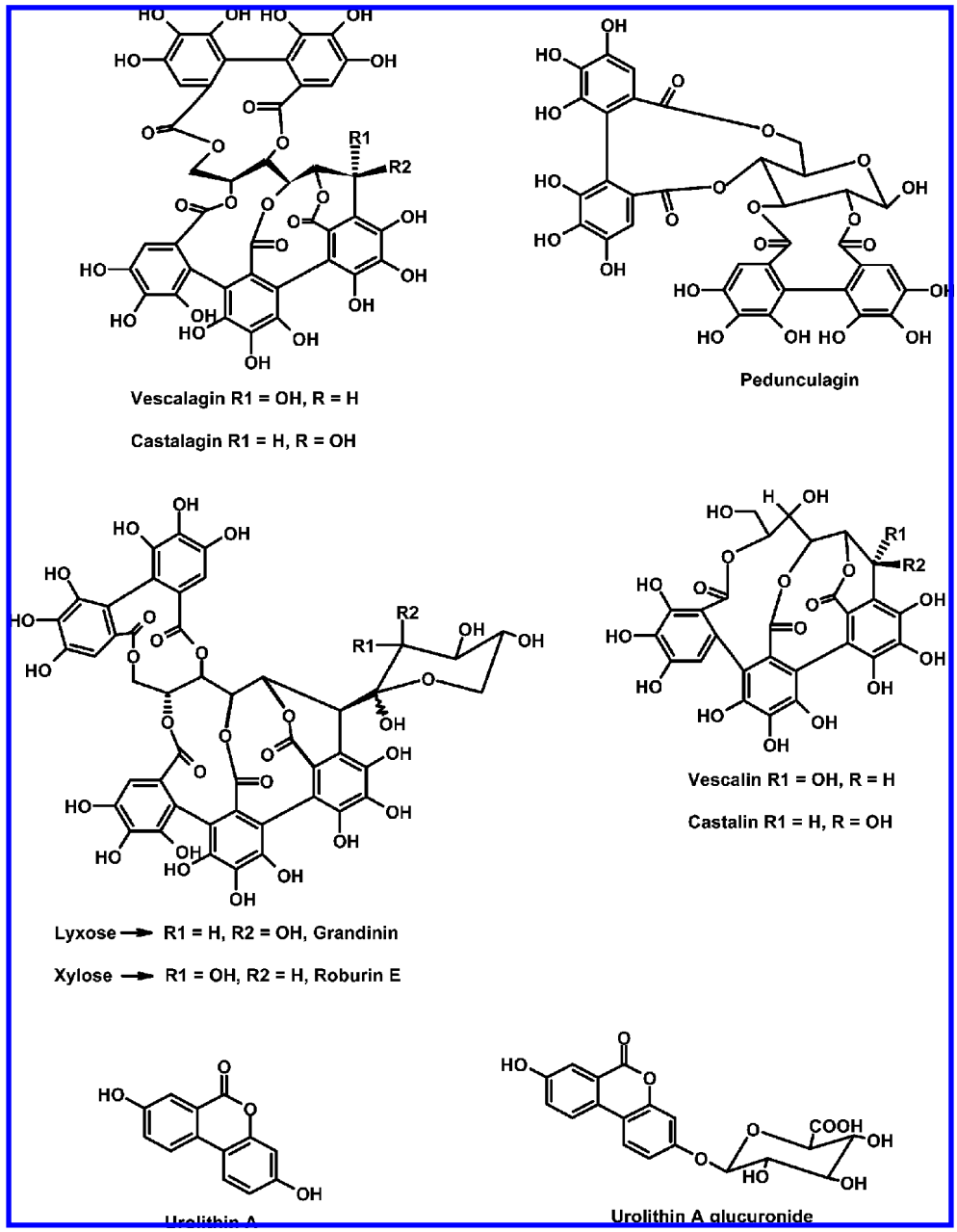

Figure 2. Some representative oak ellagitannins (vescalagin, castalagin, grandinin, roburin E, pedunculagin), combustion-derived products from vescalagin and castalagin (vescalin and castalin, respectively), and in vivo produced metabolites upon consumption of ellagitannins (urolithin A and urolithin A glucuronide).

Glucose did not show significant differences over the present study, although the values obtained were relatively high, which could be due to the stress produced in animals during handling and blood collection (*36*). The values for ALP and AST were also higher than in previous papers (*36–38*), which could be due to differences between laboratories. In addition, some other effects such as sex or lineage could be implicated in these differences. For example, higher values for glucose and ALP have been described in males than in females (*37*), and some variability has been also described between different lineages of rats in some parameters such as glucose, ALT, AST, and ALP (*38*).

Urea and creatinine concentrations are used to assess renal function. Creatinine is a better indicator of glomerular function because urea can be influenced by other factors such as protein content in diet or dehydration (*35*). Urea levels tended to increase in the three groups along the study with final values within the NVR (**Table 3**). The highest value was found in the control-milk group, although the increase was mild and lower than that reported by other authors to be clinically relevant as indicative of renal failure or decrease in glomerular filtration rate (*35*). In addition, no significant changes were observed in the values of creatinine (**Table 3**). Phosphorus, which is usually elevated in renal disease (*35*), showed values within the NVR (**Table 3**). Therefore, the mild increases in urea, especially in the control milk group, could be related to the protein in the diet or hydration status and would not reflect a decrease in glomerular filtration rate or renal failure.

Analytes for assessment of liver function (bilirubin), hepatocyte integrity (ALT and AST), and cholestasis (ALP and GGT) were included in this study because liver toxicity has been reported due to plant extracts in mammals with important elevations in serum bilirubin, AST, and ALP (*9*). Bilirubin increases due to hepatobiliary disease, bile duct disease, or in

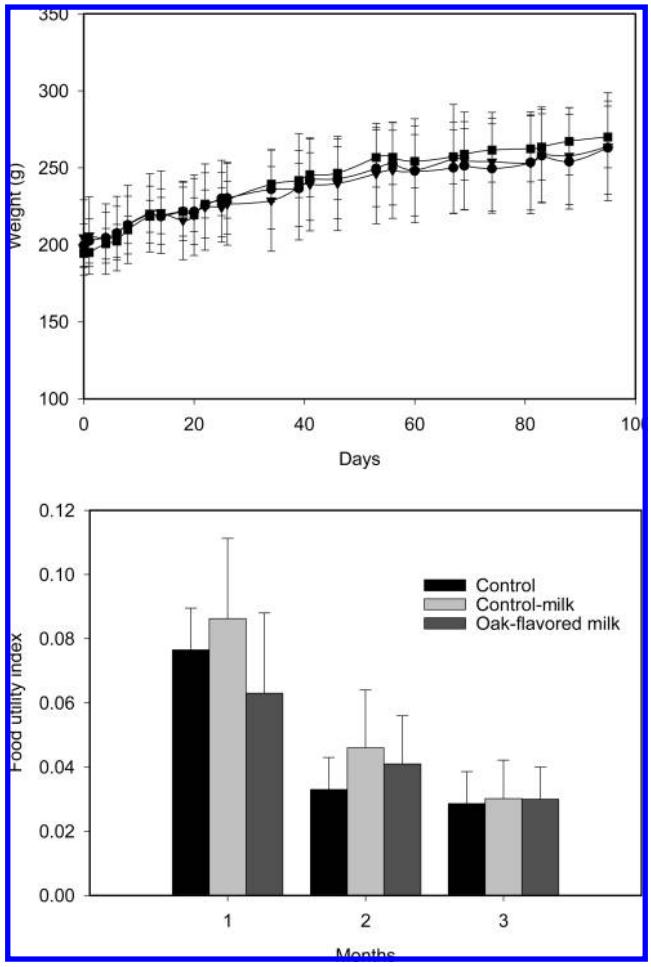

Figure 3. Growth curves and food utility index for rat groups: (**A**, top) growth curves [(▼) control group; (■) control milk group; (●) oak-flavored milk powder group]; (**B**, bottom) food utility index.

Table 2. Hematological Parameters in Rat Groups[a]

parameter	control	control milk	oak-flavored milk	NVR
WBC (1 mL)	7.0 ± 0.4	7.5 ± 2.1	7.8 ± 1.5	6.1–7.9
RBC (1 L)	7.5 ± 0.4	7.2 ± 0.4	7.7 ± 0.4	6.7–8.2
Hb (g/dL)	14.3 ± 0.8	14.0 ± 0.6	15.1 ± 1.1	12.7–15.9
HCT (%)	42.0 ± 2.0	40.5 ± 1.8	43.9 ± 2.3	37.9–46.0
MCV (μm^3)	56.3 ± 0.9	56.5 ± 1.0	56.8 ± 1.2	54.5–58.0
MCH (pg)	19.1 ± 0.7	19.5 ± 0.7	19.5 ± 0.6	17.8–20.5
MCHC (g/dL)	34.1 ± 1.0	34.4 ± 0.7	34.3 ± 0.9	32.1–36.0
PLT (1 mL)	383.9 ± 41.5	430.0 ± 73.9	428.4 ± 53.0	300.9–466.8

[a] Results are expressed as mean ± SD. Normal values range (NVR) was calculated as the mean of reference values ± 2 × SD. Results shown correspond to the end of the experiment (day 96). No significant differences were observed throughout the study.

vivo hemolysis (*35*). In rats, ALT is very specific to liver cells and a good indicator of hepatocellular damage (*35*). Hepatocellular injury is suspected with 10- or 20-fold increases of this enzyme. For example, a >20-fold increase in serum ALT activity has been reported in rats with acetaminophen-induced toxic hepatitis (*39*). On the other hand, 2−3-fold increases could be produced by hemolysis or slight muscle damage (*35*). Both bilirubin and ALT were slightly increased in some blood samples taken over the study (results not shown), but the final values were always within the NVR (**Table 3**). The very mild increases observed in our study in bilirubin and ALT could be produced by in vitro hemolysis of the samples. Therefore, our results suggest that rats had an adequate liver function during the study.

In contrast to ALT, AST is less specific for hepatic tissue because it could be present also in muscle (*35*). Hepatic injury has been described with elevation >10-fold in serum AST activity in rats with acetaminophen-induced toxic hepatitis (*39*). In the present study, the highest ALT values were observed in the oak-flavored milk powder, although within NVR, supporting the lack of liver injury (**Table 3**). CK is an enzyme that is present in muscle tissue. An increased CK serum concentration is an indicator of muscle damage or in vitro hemolysis (*35*). CK values were high in the three groups (although not clinically relevant), supporting the conclusion that both hemolysis and muscle damage induced by blood extraction (cardiac puncture) would be the most probable causes of raised AST and CK in our study (**Table 3**).

ALP and GGT activities are increased due to bile duct alteration or drug induction (*35*). In our work, ALP values tended to decrease in all groups during the experiment (results not shown), although the values at the end of the experiment were within the NVR (**Table 3**). GGT activity has been reported to be negligible in the rat, which was in accordance with our results (*40*). No significant differences were noted in any group (only very mild elevations in a few animals; results not shown). These results indicate that rats did not suffer any bile duct alteration through the study.

The protein profile included total protein content, albumin, and globulins. Total protein, albumin, and globulin concentrations showed some increases during the study (results not shown), although the final values were within our NVR (**Table 3**). The increases in total protein and globulins could be related with dehydration (*35*). Although total protein is rather elevated in the three groups, the absence of low albumin would rule out the presence of any inflammatory process in the animals.

Serum lipid concentration was also studied because some authors reported variations in lipids due to some plants in the diet of rats (*41*). Normal serum cholesterol values in rats are typically about one-third those of humans, and, in contrast to humans, 60% of the cholesterol is transported as high-density lipoprotein (HDL) cholesterol (*35*). In rats, cholesterol values are increased in bile duct obstruction and hepatic lipidosis (*35*). In our study, cholesterol concentrations were within the NVR. These results were not compatible with a possible lipid degeneration of the liver. Although values of triglycerides tended to increase in the three groups, no statistically significant increases were found (**Table 3**).

Amylase activity tended to decrease, especially in both control milk and oak-flavored milk groups. The clinical significance of this enzyme is not well understood due to the great variety of isoenzymes described (*36*), but an increase of amylase concentration could be related with pancreatitis in rats (*36*). Therefore, the decrease observed in our study does not have any clinical relevance.

Absorption and Disposition of Ellagitannin-Derived Metabolites. The bioavailability and metabolism of ellagitannins have been previously described in the rat (*19, 42, 43*), and our group described the metabolism of ellagitannins in humans and pigs for the first time (*7, 29, 30, 44*). The main in vivo derived ellagitannin metabolites are the colonic microflora urolithins (*45*). These metabolites have been proposed as urinary biomarkers of human exposure to dietary ellagitannins (*7*). In

Table 3. Serobiochemical Parameters in Rat Groups[a]

parameter	control	control milk	oak-flavored milk	NVR
glucose (mg/dL)	296.8 ± 97.9	245.0 ± 86.3	283.9 ± 31.7	149.6–336.8
urea (mg/dL)	31.0 ± 4.3	43.2 ± 11.0	34.1 ± 4.1	21.4–37.2
cholesterol (mg/dL)	104.1 ± 12.2	82.1 ± 13.5	98.2 ± 14.8	71.9–121.5
triglycerides (mg/dL)	76.6 ± 35.5	64.3 ± 12.0	82.6 ± 19.8	28.0–86.5
creatinine (mg/dL)	0.45 ± 0.08	0.62 ± 0.11	0.57 ± 0.07	0.3–0.7
total protein (g/L)	7.21 ± 1.17	6.36 ± 0.39	6.90 ± 0.44	5.2–8.0
albumin (g/L)	3.61 ± 0.14	3.41 ± 0.16	3.62 ± 0.15	3.0–3.8
globulins (g/L)	3.60 ± 1.15	2.95 ± 0.28	3.28 ± 0.44	2.2–4.2
bilirubin (mg/dL)	0.25 (0.14–0.40)	0.14 (0.10–0.17)	0.21 (0.13–0.27)	0.17 (0.12–0.27)
ALT (units/L)	48.4 ± 4.6	42.7 ± 15.5	41.3 ± 7.1	20.2–70.2
AST (units/L)	100.9 ± 22.0	97.7 ± 30.7	122.1 ± 31.5	36.9–160.1
ALP (units/L)	196.1 ± 61.1	179.5 ± 77.1	163.9 ± 24.7	105.4–378.6
GGT (units/L)	0.1 (0.00–0.00)	0.4 (0.00–0.92)	0.1 (0.00–0.07)	ND
CK (units/L)	578.1 (389.00–756.00)	532.2 (339.25–701.50)	805.3 (315.7–1398.5)*	411.0 (261.0–591.2)
amylase (units/L)	682.5 ± 52.8	575.8 ± 119.4	589.0 ± 87.6	565.8–819.0
phosphorus (mg/dL)	4.78 ± 0.62	5.40 ± 1.47	5.02 ± 0.66	2.8–8.4

[a] Results are expressed as mean ± SD except in bilirubin, GGT, and CK in which mean and percentiles (25–75) are shown. Normal values range (NVR) was calculated as the mean of reference values ±2 × SD. Results shown correspond to the end of the experiment (day 96). ND, not detected. *, $p < 0.05$.

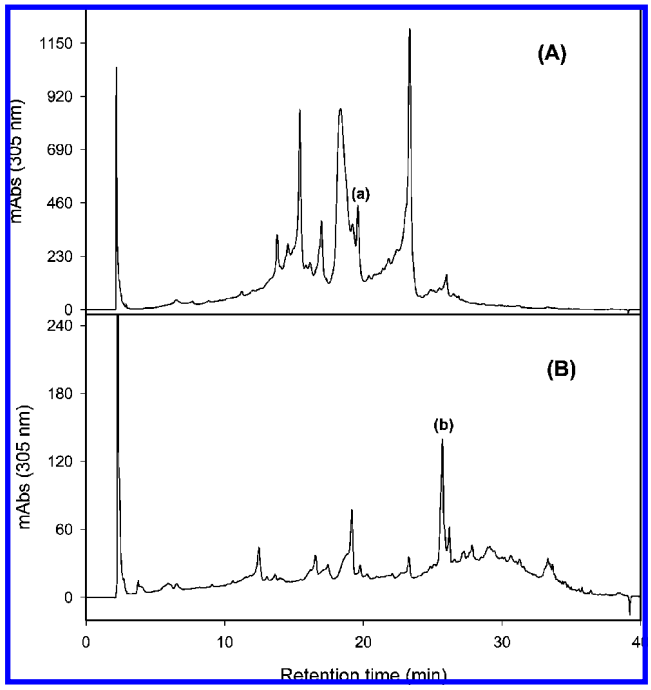

Figure 4. HPLC analyses of urine (**A**) and feces (**B**) of rats fed oak-flavored milk powder. Peaks: (a) urolithin A glucuronide; (b) urolithin A.

addition, our group described for the first time that these metabolites can display estrogenic and antiestrogenic activities (*8*).

Accumulation of ellagitannins such as punicalagin in both liver and kidney has been reported to be toxic in cattle (*17*). Punicalagin and derived metabolites were also detected in rats fed a high punicalagin amount (0.9 g/day for 5 weeks) (*19*). In the present study, neither ellagitannins nor derived metabolites were found in liver, kidney, and uterus (results not shown). This is not surprising because the amount of ellagitannins administered to rats was very low (0.094 mg/day) compared with the studies cited above. However, urolithin A glucuronide (m/z^- 403; MS-MS 227, 176) was detected in urine and urolithin A (m/z^- 227; MS-MS 209, 185, 178, 149) in the feces (**Figure 4**), which agree with previous studies that described the production of urolithins upon consumption of different ellagitannin-containing foodstuffs (*7, 19, 29, 30, 43, 44*). This is of particular interest because it confirms the production of microflora-derived urolithins independently of ellagitannin structure.

Histopathological Analyses. Although neither ellagitannin nor derived metabolite accumulation was detected, liver and kidneys were examined to investigate potential specific tissue damage. The histological examination of liver and kidney as potential target organs upon oral administration of plant extracts has been previously reported. Whereas some papers have described severe liver necrosis in mice fed punicalagin (*17*), other studies did not find any effect (*19*). In the present study, the histopathological examination of livers and kidneys corroborated the lack of toxicity suggested by serobiochemical parameters (ALT, AST, ALP, bilirubin, urea, and creatinine). No difference was observed in the weight of organs belonging to the three groups. Livers from the control group showed a typical deep-red color and scored 1, with small hydropic regions (cytoplasm with less coloration). Livers from rats fed the diet containing the milk powders (both control and oak-flavored milk) were slightly more yellowish than control livers. Livers from rats fed the milk powders were scored 2, showing some necrotic hepatocytes with mild hydropic areas located in centrilobular areas. This could be due to the higher protein content in the milk-containing diet compared to the control diet and independent of the presence of oak-derived compounds. Regarding the kidneys, no adverse sign was found in any sample.

In light of the above results, 30% oak-flavored milk in the rat diet for 96 days did not provoke significant serum biochemical, hematological, and tissue alterations. The mild effects observed in some parameters, although without clinical relevance, were due to the high protein content in the rat diet rather than to the presence of oak-derived compounds. Therefore, the results presented here suggest that the intake of the oak-flavored milk powder assayed in this study is safe and does not involve any potential hazard to human health.

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