JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Repeated Oral Administration of High Doses of the Pomegranate Ellagitannin Punicalagin to Rats for 37 Days Is Not Toxic

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The water-soluble ellagitanin punicalagin has been reported to be toxic to cattle. Taking into account that this antioxidant polyphenol is very abundant in pomegranate juice (≥2 g/L), the present study evaluated the possible toxic effect of punicalagin in Sprague−Dawley rats upon repeated oral administration of a 6% punicalagin-containing diet for 37 days. Punicalagin and related metabolites were identified by HPLC-DAD-MS-MS in plasma, liver, and kidney. Five punicalagin-related metabolites were detected in liver and kidney, that is, two ellagic acid derivatives, gallagic acid, 3,8-dihydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one glucuronide, and 3,8,10-trihydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one. Feedstuff intake, food utility index, and growth rate were lower in treated rats during the first 15 days without significant adverse effects, which could be due to the lower nutritional value of the punicalagin-enriched diet together with a decrease in its palatability (lower food intake). No significant differences were found in treated rats in any blood parameter analyzed (including the antioxidant enzymes gluthatione peroxidase and superoxide dismutase) with the exception of urea and triglycerides, which remained at low values throughout the experiment. Although the reason for the decrease is unclear, it could be due to the lower nutritional value of the punicalagin-enriched diet with respect to the standard rat food. Histopathological analysis of liver and kidney corroborated the absence of toxicity. In principle, the results reported here, together with the large safety margin considered, indicate the lack of toxic effect of punicalagin in rats during the 37 day period investigated. However, taking into account the high punicalagin content of pomegranate-derived foodstuffs, safety evaluation should be also carried out in humans with a lower dose and during a longer period of intake.

KEYWORDS: Antioxidant; ellagitannin; histopathology; phytochemical; polyphenol; pomegranate juice; punicalagin; rat; toxicity

INTRODUCTION

Pomegranate (*Punica granatum* L.) is mainly grown in the Near East, India, (southeastern) Spain, Israel, and the United States (California). Pomegranate is mainly consumed fresh or used to obtain juice. The health-beneficial effects of pomegranate fruit and derivatives such as juice have been claimed from ancient times (*1*). Recently, in vitro and ex vivo studies have demonstrated the antiatherosclerotic capacity (*2*−*4*) of pomegranate juice, probably mediated by its high antioxidant activity (*4*, *5*). Nowadays, more than 90 international patents claim the use of pomegranate juice and other pomegranate derivatives as sources of health-promoting effects due to their content of health-beneficial bioactive compounds (phytochemicals, phytoestrogens, etc.).

Pomegranate husk is very rich in ellagic acid derivatives such as the ellagitannins punicalagin [2,3-(*S*)-hexahydroxydiphenoyl-4,6-(*S*,*S*)-gallagyl-D-glucose] and punicalin [4,6-(*S*,*S*)-gallagyl-D-glucose] (**Figure 1**). In addition, some ellagic acid derivatives (ellagic acid hexoside, -pentoside, etc.) are also present, although in lesser amounts (*5*).

Commercial pomegranate juice is obtained by pressing the fruit, so the water-soluble compounds of the pomegranate husk, including punicalagin, are also found in significant amounts in the juice. This is the case of punicalagin with a content that can reach 2 g/L or even higher depending on the cultivar (*5*). This means that the regular consumption of pomegranate juice could involve a high intake of this water-soluble hydrolyzable ellagitannin.

The bioavailability and metabolism of punicalagin from pomegranate has been recently studied in rats (*6*). Punicalagin was detected in plasma with the subsequent relevant finding which indicated that punicalagin is the highest molecular weight

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10.1021/jf020842c CCC: $25.00
Published on Web 04/22/2003

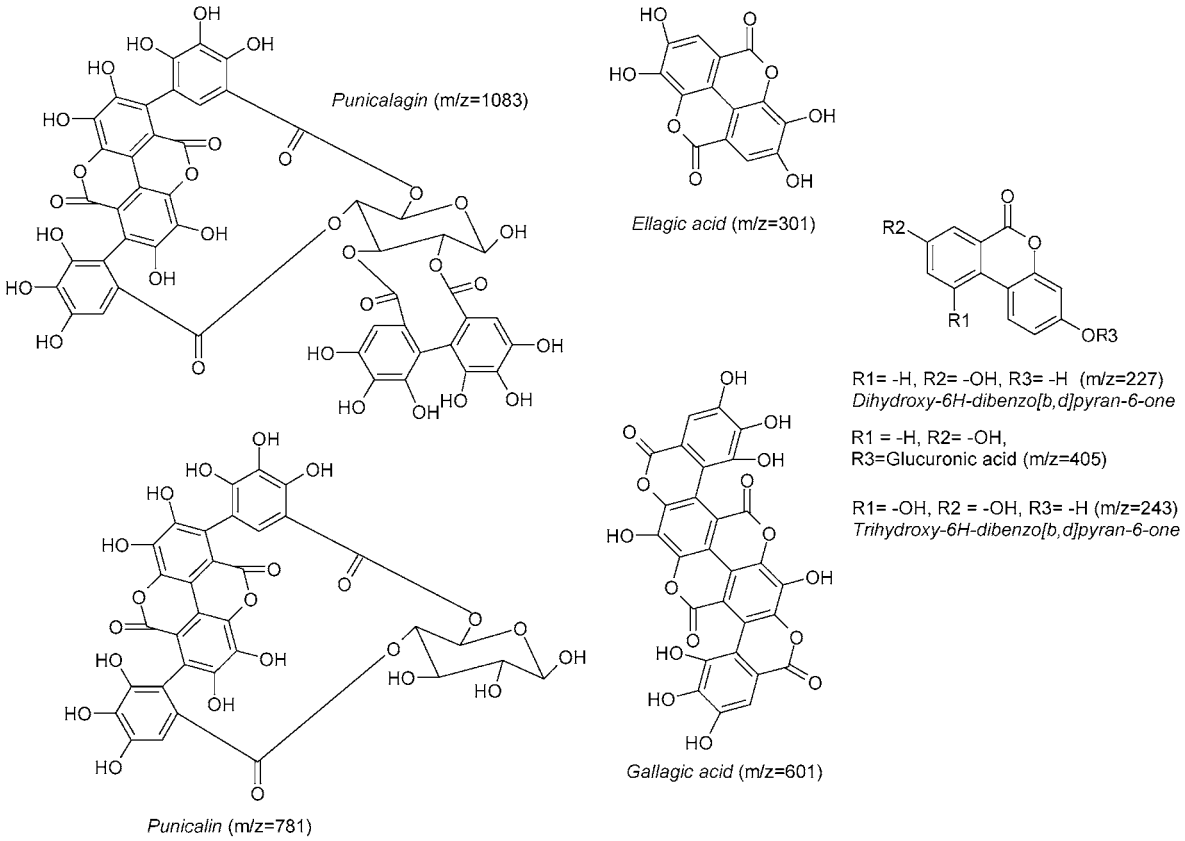

Figure 1. Structures of punicalagin and related metabolites.

compound absorbed throught the gut barrier so far (*7*). In addition, glucuronides of methyl ether derivatives of ellagic acid were also detected, as was in lesser amount the metabolite 6*H*-dibenzo[*b*,*d*]pyran-6-one. In urine, the main metabolites observed were 6*H*-dibenzo[*b*,*d*]pyran-6-one derivatives, as aglycons or glucuronides (*6*).

Punicalagin has been reported to provoke both liver necrosis and nephrotoxicity in cattle (*8*−*10*). However, antioxidant and hepatoprotective effects of punicalagin on acetaminophen-induced liver damage in rats have been recently reported, although harmful effects were detected with high doses (*11*).

Therefore, a high consumption of pomegranate juice favored by its increasingly claimed health-beneficial properties could involve a potential risk because of its high punicalagin content. Taking into account the apparent controversy concerning the toxicity of punicalagin together with the significant amount detected in pomegranate juice, it was the aim of the present study to assess the effect of repeated oral administration of punicalagin-containing pomegranate extracts to rats for 37 days. For this purpose, we evaluated the effect of daily high doses of punicalagin on growth, antioxidant enzymes, and hematological and clinical chemistry parameters of Sprague−Dawley rats as well as the effect on possible internal target organs (liver and kidney).

MATERIALS AND METHODS

Animals and Diets. Sprague−Dawley rats weighing 179−214 g were provided by the Animal Centre of the University of Murcia (Spain). Rats were quarantined for 1 week, housed in individual metabolic cages, 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 with standard feedstuff for rats (Panlab, Barcelona, Spain). Both solid diet and water were consumed ad libitum. Handling and killing of rats were in full accordance with national and international law and policies (*12*) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (*13*). These guidelines recommend the use of the minimum possible number of animals for experiments. This, together with the recent bibliography concerning experiments on rats prompted us to use 10 animals for the final investigation. A previous study was conducted with 12 rats to evaluate both the acceptance of different punicalagin contents in the diet and a preliminary effect on blood parameters. Rats were randomly divided into six groups ($n = 2$), which were fed with increasing pomegranate husk extract concentrations, that is, 0.5, 2, 5, 10, 20, and 40% during 7 days for each extract concentration. The final, more detailed study, was performed with 10 female rats randomly divided in two groups, that is, a control group ($n = 5$) that was fed only with the commercial diet and a treated group (pomegranate group, $n = 5$) that was fed with the commercial diet containing 20% pomegranate husk extract to reach an average of 6% punicalagin in the final mixture. The husk extract was kept at −20 °C. The mixture feedstuff−pomegranate extract was freshly made up every day to avoid possible instability of punicalagin. Body weight as well as food consumption was recorded every day for 37 days. At the end of the experiment the rats were anesthetized with ether and killed by exsanguination.

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 37 days. Feed efficiency or food utility index was calculated as the weekly body weight gain divided by the food consumption.

Reagents. Ellagic acid was purchased from Sigma (St. Louis, MO). 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.

Pomegranate Extract. Water-soluble compounds from pomegranate husk were extracted in order to mimic the pomegranate juice preparation. Ten kilograms of pomegranate husk (var. Mollar de Albatera) were freeze-dried. One kilogram of freeze-dried husk was squeezed in 2 L of distilled water (w/v), stirred vigorously, and left for 2 h at room

temperature. The corresponding extracted solution was pooled and freeze-dried. The protocol was repeated several times. The pooled freeze-dried powder was mixed with the standard rat feedstuff to get the ratio 20% pomegranate extract/80% standard diet, with a final 6% of punicalagin content in the mixture. The other water-soluble compounds present in the extracts (mainly soluble fiber such as mucilages and salts) are also found in pomegranate juice. In addition, and very importantly, pomegranate husk extracts did not contain alkaloids, which are found in both root and stem barks but not in fruit husk (*14*). The isolated effect of pure punicalagin was disregarded due to the huge amount of punicalagin needed for this purpose.

Purification of Punicalagin. One kilogram of pomegranate husk (var. Mollar de Albatera) was squeezed and incubated in 2 L of distilled water at room temperature for 2 h. Afterward, the solution was filtered through a Sep-Pak cartridge (a reverse phase C-18 cartridge; Waters Millipore), 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. A sample of 1 L was filtered by using 10 reused cartridges. Each cartridge was washed with 5 mL of water. These fractions eluted with water were discarded. The remaining volume in each cartridge was eluted with 5 mL of MeOH. The methanolic fractions of each cartridge were collected and filtered through a 0.45 μm filter. The pooled filtered solution was injected in a semipreparative HPLC system equipped with an L-6000 pump, an L-4000 UV detector, a D-2500 Chromato-Integrator, and a 2 mL sample loop. Chromatographic separation was carried out on a reverse phase ODS-2 column (25 $\times$ 0.7 cm, 5 μm particle size) (Teknokroma, Barcelona, Spain) using an isocratic flow of MeOH−H+/H_2O (2:98) (the acid was acetic acid 0.5%) at a flow rate of 1.5 mL/min. The UV chromatogram was recorded at 360 nm. The different eluted fractions were analyzed by HPLC-DAD-MS-MS to identify and quantify the punicalagin isomers. The purified fraction of punicalagin was freeze-dried and used as pure (50 mg) punicalagin standard. A calibration curve was performed using both UV and MS signals (r^2 = 0.995 and 0.997, respectively) to further quantify punicalagin in rat plasma. Quantification by using both methods, UV and MS, yielded coefficients of variation (CV) always <10%.

Sampling Procedure (Plasma, Liver, and Kidney). Blood was extracted every week by cardiac puncture and collected in heparinized tubes. Blood was immediately separated in plasma by centrifugation at 14000*g* for 15 min at 4 °C. Plasma was homogenized with MeOH/0.2 M HCl (1:1, v/v). The mixture was vortexed for 30 s and centrifuged at 14000*g* for 2 min at 4 °C. The supernatant was filtered with a 0.45 μm filter, and the filtered solution was analyzed by HPLC-DAD-MS-MS.

After sacrifice, liver and kidney were quickly excised and weighed. Two grams of liver and 0.8 g of kidney were homogenized with 4 and 2.4 mL, respectively, of MeOH/0.2 M HCl (1:1, v/v), vortexed, and centrifuged at 14000*g* for 5 min at 4 °C. The supernatant was filtered with a 0.45 μm filter, and the filtered solution was analyzed by HPLC-DAD-MS-MS.

HPLC-MS-MS Analysis. Chromatographic separations of pomegranate extracts, as well as plasma, kidney, and liver from control and treated rats were carried out on a reverse phase C_{18} LiChroCART column (25 $\times$ 0.4 cm, particle size 5 μm, Merck) using water/formic acid (95:5, v/v) (A) and methanol (B) as the mobile phases at a flow rate of 1 mL/min. The linear gradient started with 1% B, remained for 5 min at 1% B in A, to reach 20% B in A at 20 min, 40% B in A at 30 min, and 95% B in A at 35 min, and remained at 95% B in A until 39 min. UV chromatograms were recorded at 255 and 360 nm. The HPLC system equipped with a DAD detector and mass detector in series consisted of an HPLC binary pump, an autosampler, a degasser, and a photodiode array detector controlled by software (v. A08.03) from Agilent Technologies (Waldbronn, Germany). The mass detector was an ion-trap mass spectrometer from Agilent Technologies equipped with an electrospray ionization (ESI) system and controlled by software (v. 4.0.25). The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. Mass scan (MS) and daughter (MS-MS) spectra were measured from *m*/*z* 100 to *m*/*z* 1500. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, and the collision energy was set at 50%. Mass spectrometry data were acquired in the negative ionization mode. All HPLC analyses were performed in triplicate.

Hematology and Clinical Chemistry. Hematological parameters were determined every week in heparinized blood from both control (n = 5) and treated rats (n = 5) using an automated hematological analyzer (ABC Vet, ABX Hematologie), with specific software for rat blood samples. The parameters analyzed were packed cell volume (PCV); red blood cell number (RBC); hemoglobin concentration; erythrocytic indices, that is, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC); white blood cell count (WBC); and platelet number. PCV determined by the microhematocrit method and smears evaluation were used as quality control of the automated hematological analysis.

Diagnostic kits from Spin React (Gerona, Spain) were used for spectrophotometric analysis of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatine kinase (CK), glucose, urea, creatinine, cholesterol, triglycerides (TGs), bilirubin, calcium, and phophorous. All these biochemical measurements were made in heparinized plasma using a Cobas Mira Plus analyzer (ABX Diagnostic).

Histopathology. Livers (n = 5) and kidneys (n = 5) from control and treated rats were weighed and fixed in 10% buffered neutral formalin. Paraffin sections were prepared and stained with hematoxylin−eosin staining for histological examination.

Antioxidant Enzyme Activities. Glutathione peroxidase (GPx) and superoxide dismutase (SOD) were determined every week in heparanized blood with the use of commercial kits (Randox Laboratories). Temperature of the assay was 37 °C. Briefly, SOD activity was determined by using the xanthine/xanthine oxidase method to generate superoxide radicals, which reacted with 2,4-iodiphenyl-3,4-nitrophenol-5-phenyltetrazolium chloride to form a red formazan dye, which is determined at 500 nm. SOD activity was then measured by the degree of inhibition of this reaction. GPx activity was based on the method by which GPx catalyzes the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized glutathione was immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$, and the corresponding decrease in absorbance at 340 nm was measured.

Statistical Analysis. Results of **Tables 1** and **2** are expressed as the mean of both control (n = 5) and pomegrante-treated (n = 5) rats and normal values range (NVR) at the end of the experiment. This range (NVR) was calculated as the control mean $\pm$ 2 $\times$ SD. NVR is useful because values out of this interval are considered to be pathological. Serobiochemical and hematological parameters were analyzed with an analysis of variance (ANOVA) between the control group and the treated group. Differences were considered to be statistically significant at P > 0.05. Graphs of the experimental data and their statistical analysis were carried out by using the Sigma Plot 6.0 program for Windows.

RESULTS AND DISCUSSION

Punicalagin Content in the Rat Diet. A previous study was conducted both to evaluate the acceptance of different pomegranate husk concentrations in the diet by the rats and to ensure a rational high level of the compound for a toxicological study. For this purpose, 0.5, 2, 5, 10, 20, and 40% of extract concentration were administered to rats. The 20% pomegranate husk concentration in the diet was the highest proportion accepted by the rats (the 40% level significantly decreased feedstuff palatability) and also involved a high punicalagin concentration intake. The choice of this concentration was also supported by the fact that lower concentrations did not show any preliminary effect on blood parameters, so the 20% extract concentration (the maximal concentration accepted by rats) was chosen for a longer and more systematic study.

The average punicalagin content in pomegranate juice (2 g/L or higher; *5*) involves an oral administration of 29 μg/g of body

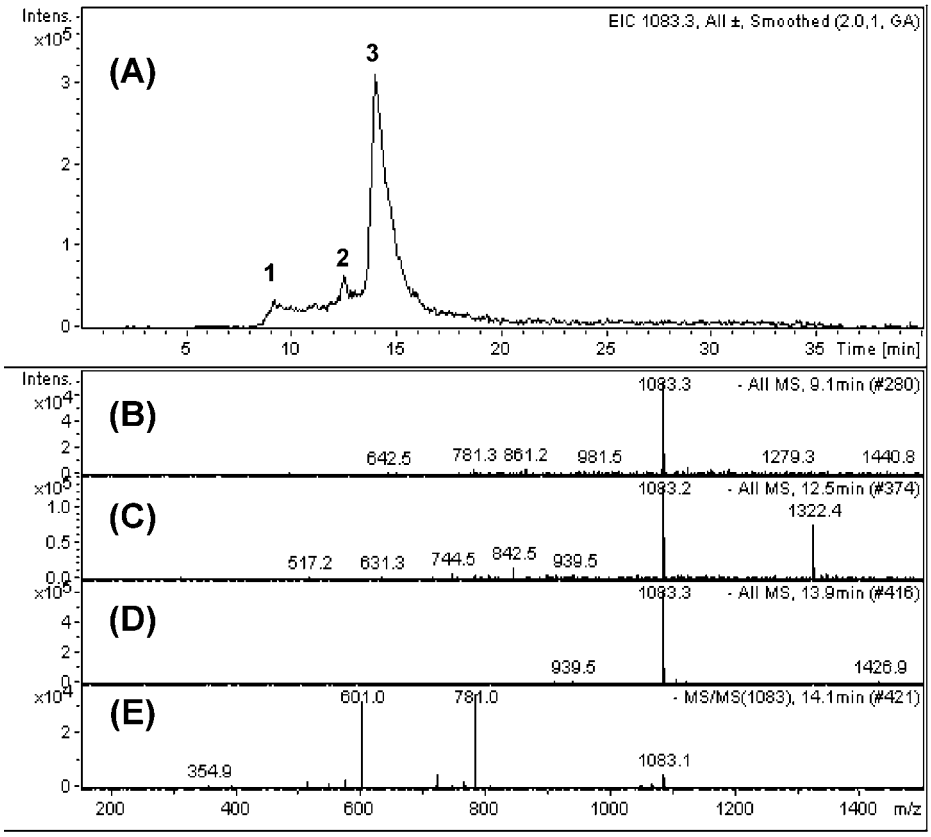

Figure 2. Punicalagin isomers in rat plasma: (A) extracted ion chromatogram (EIC) (*m*/*z* 1083) showing the three punicalagin isomers at 9, 12.5, and 13.9 min of retention time; (B, C, D) all MS detected at 9, 12.5, and 13.9 min of retention time, respectively; (E) representative MS-MS analysis of *m*/*z* 1083 showing the main fragments at *m*/*z* 601 (gallagic acid) and *m*/*z* 781 (punicalin).

weight for a 70-kg person. In the present study (including 20% pomegranate husk with the feedstuff), the rats consumed an increasing amount of punicalagin per day, that is, 0.39, 0.54, 1.12, 1.3, and 1.18 g/day, during the first, second, third, fourth, and fifth weeks, respectively. The mean oral consumption of 0.9 g/day (≈4.8 g/kg of body weight/day) punicalagin per rat was equivalent to the consumption of ∼194 L/day of pomegranate juice by a 70-kg person, which is, in principle, a large safety margin. This large margin was taken into account due to the increasing number of commercial pomegranate-containing nutraceuticals that claim health-beneficial effects. In fact, a detailed study about the absorption and metabolism of punicalagin has been recently carried out (*6*), but its possible adverse effects upon repeated and prolonged oral intake of high doses have not been evaluated so far.

The above safety margin should be also taken with caution. Although the punicalagin concentration ingested by the rats is equivalent to a very high pomegranate juice intake, this does not necessarily rule out the possible adverse effects of lower doses of punicalagin in humans, especially during longer periods.

Accumulation of Punicalagin in Rat Plasma. The HPLC-MS-MS analyses with the ion trap and subsequent fragmentation of the isolated ions allowed the clear identification of punicalagin in rat plasma. The single-ion HPLC-MS analyses allowed the identification of three peaks in the plasma of rats fed with pomegranate coincident with a molecular ion at *m*/*z* 1083 corresponding to three punicalagin isomers (**Figure 2A**−**D**).

Daughter (MS-MS) mass spectra of the isolated *m*/*z* 1083 ions mainly showed fragments for punicalin (*m*/*z* 781) and gallagic acid (*m*/*z* 601) (**Figure 2E**), which unequivocally confirmed that the detected molecule in plasma was punicalagin. This is a relevant finding because this is the molecule with highest molecular weight detected in plasma so far (*6*, *7*). Intact punicalagin was accumulated by following saturation kinetics to reach a steady-state (saturated) level from day 7 in which the mean concentration of 29 μg of punicalagin/mL plasma was reached (*6*). However, as no determination was carried out before day 7, faster saturation kinetics cannot be excluded. Punicalagin intake increased from 0.39 g/day during the first week to 1.18 g/day during the fifth week although there was a maximum intake in the middle of the experiment, during the fourth week (1.3 g/day) (**Figure 3A**). Therefore, a higher intake of punicalagin was not correlated with a higher accumulation in the plasma, which indicated a possible saturation of the absorption mechanism for this molecule with M − H at *m*/*z* 1083 (results not shown; *6*).

Body Weight and Food Consumption. Previous studies stated that the body weight of rats can be affected depending on the polyphenol administered because a 0.4% flavonoid-containing extract from grapefruit and 2% of proanthocyanidin-rich extract from grape seeds did not affect body weight (*15*, *16*), whereas significant worsening of the weight gain was observed in diets containing 1% polymeric grape seed tannins (*17*) or 1.9% catechins (*18*). In fact, according to previous reports, the compounds classified as tannins (including ellagitannins) strongly interact with proteins. The main consequence is that tannin-consuming animals are usually smaller than their counterparts. Dietary tannins mainly inhibit the digestion of endogenous proteins instead of dietary proteins (*19*).

In the present study, the feedstuff intake was clearly lower for punicalagin-treated rats than for control rats (**Figure 3A**) during the first 15 days, which could be due to a decrease in the palatability of the standard diet in agreement with previous

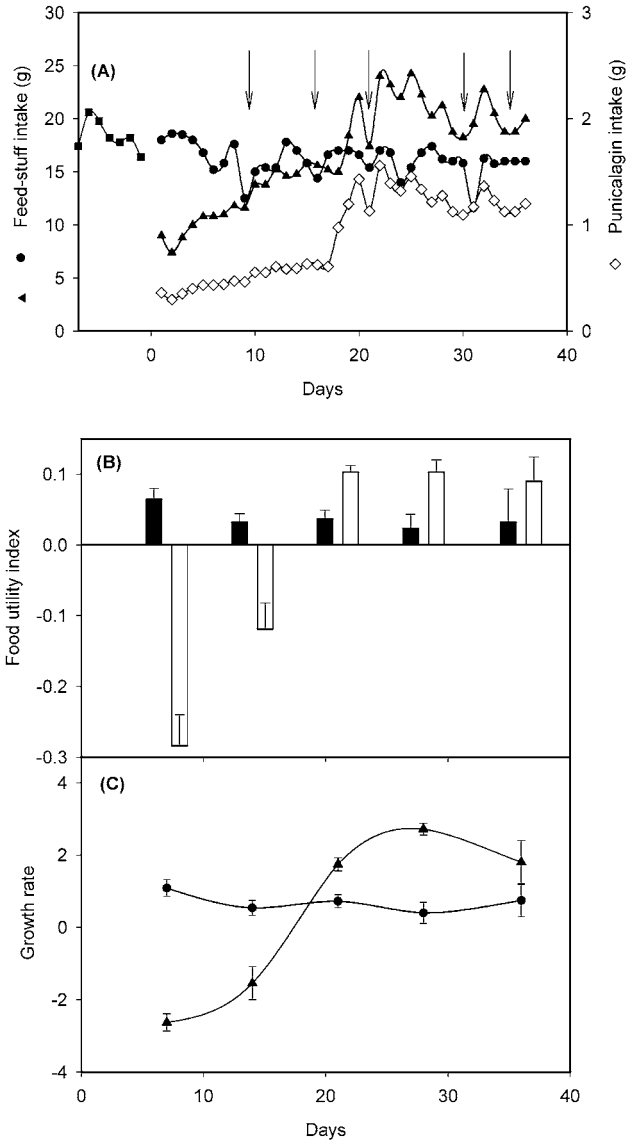

Figure 3. (A) Feedstuff and punicalagin intake of Sprague–Dawley rats: (■) quarantine period; (●) control rats ($n = 5$); (▲) pomegranate-treated rats ($n = 5$); (◇) punicalagin intake. (B) Food utility index: (■) control rats; (□) pomegranate-treated rats. (C) Growth rate: (●) control rats; (▲) pomegranate-treated rats. Arrows designate the cardiac puncture for obtaining blood sample. Coefficient of variation was always <10% for each determination.

studies (*19*). However, there was a drastic change from the third week in which food intake was higher for treated rats than for control rats (**Figure 3A**). The food utility index (**Figure 3B**, weekly body weight gain divided by the food consumption) and weekly growth rate (**Figure 3C**, the difference between the final and initial weight divided by 7 days) positively correlated the pattern of feedstuff intake (**Figure 3A**). The effect of the inclusion of punicalagin in the diet was clearly negative during the first 15 days, whereas during the last three weeks the rats were fully recovered with even higher growth rates in the pomegranate-treated rats (**Figure 3C**). The possible factors involved in this effect, apart from the above suggested decrease in the palatability, could be a decrease in the nutritive value of the diet because 20% of pomegranate extract (final 6% punicalagin) was included in the diet with the subsequent replacement of the standard feedstuff. Another possible reason could be the antinutritional effect of punicalagin by precipitating

Table 1. Hematological Parameters in Control and Punicalagin-Treated Rats[a]

parameter	control group	pomegranate group	NVR
PCV (%)	34.3 ± 1.4	29.9 ± 1.2	31.4–37.2
WBC ($10^3/\mu$L)	7.1 ± 1.1	6.9 ± 1.0	4.8–9.4
RBC ($10^6/\mu$L)	6.2 ± 0.2	6.1 ± 0.2	5.7–6.7
Hb (g/dL)	13.2 ± 0.5	12.4 ± 0.4	12.2–14.2
PLT ($10^3/\mu$L)	610.6 ± 188.4	567.8 ± 174.7	233.7–987.5
MCV (μm^3)	55.6 ± 1.3	54.0 ± 1.2	52.9–58.3
MHC (pg)	21.4 ± 0.5	21.3 ± 0.5	20.3–22.5
MCHC (g/dL)	38.6 ± 0.6	37.6 ± 0.5	37.3–39.9

[a] Results are expressed as mean ± SD. Control group ($n = 5$); pomegranate group ($n = 5$). Coefficient of variation (CV) of each measurement ($n = 3$) for each rat was always <10%. Normal values range (NVR) was calculated as the mean of control ± 2 × SD. Parameters were determined every week. Results shown correspond to the end of the experiment (day 37); no significant differences were observed throughout the experiment.

proteins in the alimentary tract, which could imply the formation of complexes and inhibition of digestive enzymes (*20*) as well as a decreased efficiency in converting the absorbed nutrients to new body substances (*21*) as described in tannin-supplemented diets. However, in the present study, after two weeks, feedstuff intake was higher in the pomegranate-treated rats with the concomitant improvement of the above indices (**Figure 3**). The reason for this change is not clear, but it could be postulated that the rats were used to the palatability of the diet after two weeks and thus could increase feedstuff intake to compensate for the lower nutritive value of the diet. This hypothesis (corroborated by the increase of growth rate in treated rats) could rule out the reason of the antinutritional effect of punicalagin

Hematology and Clinical Chemistry. The mean of the hematological parameters throughout the present study is shown in **Table 1**. Slight and nonsignificant decreases in PCV and hemoglobin values were observed in the group fed with punicalagin-containing extract.

Decreases in PCV and hemoglobin have been previously found with propyl gallate (*22*) and gallic acid (*23*) diet administration in rats. Further studies about iron content and digestibility of the diet used should be developed to fully characterize this slight decrease in red blood cell parameters.

Serum biochemical results showed only significant differences between control and pomegranate-treated groups in both urea and TGs (**Table 2**). The decrease of serum urea, which appears from the first day of sampling and persists during the entire trial, could be due to liver failure as reported previosuly (*24*). However, no liver failure was detected in the present study; liver parameters (ALT, AST, ALP, and bilirubin) were normal. Another possible reason could be either a protein deficiency in the diet or a deficient protein digestibility of the diet, which was not enough to meet normal rat requirements, especially significant during the first 15 days (**Figure 3**). In fact, decreases of the nutritional value of the diet with reduction in protein digestibility and protein efficiency ratio after supplementation with large amounts of vegetable extracts have been recently described (*25*). However, in our study, the food utility index and growth rate after 15 days were even better in the pomegranate-treated group than in the control rats (**Figure 3B**). Therefore, the antinutritional effect cannot be fully accepted as the reason for such behavior. Although the main unanswered question is why urea was not restored concomitant with the increase of food utility index, the decrease in the palatability of the pomegranate-enriched diet appeared to be the main reason for triggering the decrease in urea.

Table 2. Serobiochemical Constituents and Antioxidant Enzymes of Control and Punicalagin-Treated Rats

parameter	control group	pomegranate group	NVR
AST (units/mL)	125.2 ± 40.2	140.6 ± 45	44.8–205.6
ALT (units/mL)	61.1 ± 20.1	70.7 ± 22.6	20.7–101.4
ALP (units/L)	135.6 ± 31.1	117.0 ± 27	73.5–197.7
TP (g/dL)	5.1 ± 0.4	4.6 ± 0.4	4.4–5.8
Alb (g/L)	2.7 ± 0.1	2.7 ± 0.1	2.5–2.9
Bil (mg/dL)	0.3 ± 0.05	0.3 ± 0.04	0.2–0.4
Glc (mg/dL)	135.0 ± 15.1	108.7 ± 12.2	104.8–165.2
Chol (mg/dL)	70.7 ± 11.8	67.2 ± 11.3	47.1–94.3
TG (mg/dL)	60.9 ± 18.0	30.6 ± 8.9	24.8–96.9*
Urea (mg/dL)	31.6 ± 4.9	12.1 ± 1.9	21.7–41.6*
Crea (mg/dL)	0.5 ± 0.1	0.4 ± 0.1	0.29–0.69
CK (units/L)	446.1 ± 168.1	353.2 ± 160.1	110.1–782.3
P (mg/dL)	5.1 ± 0.9	6.2 ± 1.1	3.3–6.9
Ca (mg/dL)	10.0 ± 0.6	9.5 ± 0.6	8.8–11.2
antioxidant enzymes			
GPx (units/mL)	132.1 ± 33.5	93.3 ± 23.6	65–199
SOD (units/mL)	72.9 ± 12.5	70.8 ± 12.1	47.2–98.7

[a] Results are expressed as mean ± SD. CG, control group ($n = 5$); PG, pomegranate group ($n = 5$). Coefficient of variation (CV) of each measurement ($n = 3$) for each rat was always <10%. Normal values range (NVR) was calculated as mean of control ± 2 × SD). Parameters were determined every week. Results shown correspond to the end of the experiment (day 37); no significant differences were observed throughout the experiment. The differences observed in both urea and TGs were detected the first week and remained at the same values until the end of the experiment (results shown in the table). *Significant differences.

The decrease of serum TGs has been previously described upon feeding rats with flavone-enriched diets from skullcap, anthocyanins from chokeberry, as well as with diets containing condensed tannin extracts from faba bean (*25*). As in the present study, the rats also presented normal cholesterol levels (**Table 2**). Although further studies would be required to establish the possible pathophysiological mechanism for this situation, a relationship between decreased TGs and a lower HDL cholesterol level has been recently reported (*25*). In the present study, the content of TGs was significantly lower in the pomegranate-treated group than in the control group. However, TGs content remained in the lowest limit within the normal values range (NVR; **Table 2**).

Regarding the relationship between plant extracts and liver toxicity, a previous study reported oak (*Quercus incana*) leaf poisoning in cattle due to hydrolyzable tannins (*26*) with significant reductions of blood hemoglobin and mean corpuscular hemoglobin as well as important elevations in serum bilirubin, AST, and ALP activities after feeding of oak leaves (98 mg of tannic acid equivalents/g of dry leaves) to cattle for only 2 days. However, identification of the responsible tannin was not carried out. Lin et al. (*27*) reported hepatoprotective activity of punicalagin and punicalin on carbon tetrachloride-induced liver damage in rats after subcutaneous injection of 25 and 12.5 μg/g of body weight of punicalagin and punicalin, respectively. The hepatoprotective effect was correlated with the decrease in both AST and ALT values. However, harmful effects were observed with 25 μg/g of body weight of punicalin. Contrarily to flavonoids and other plant extracts (*15*, *25*), the presence of punicalagin did not cause increases of ALT in our study. Furthermore, all biochemical parameters used to assess hepatocyte integrity (AST, ALT), bile duct alterations (ALP), and liver function (bilirubin, albumin) were normal. These results were in accordance with those obtained in previous studies in which AST, ALP, and γ-GT were not affected in rats fed 2.5% green tea leaf extracts (*27*).

According to our results and despite the apparent controversy existing in the literature, in principle, it could be concluded from the biochemical results obtained that the consumption of punicalagin has no negative effect on rat liver hepatocyte integrity or function.

The above controversy regarding punicalagin toxicity can be extended in the safety evaluation of other phenolic compounds such as gallic acid because a no-adverse-effect-level (NOAEL) value similar to that reported in the present study (5 g/kg of body weight) has been reported for mice (*28*), whereas the same value was toxic in F344 rats (*23*).

Punicalagin Accumulation in Possible Target Organs. Both liver and kidney have been reported to be target organs of punicalagin in cattle (*8*−*10*), which prompted us to try to identify punicalagin and/or derived metabolites in these organs.

The single-ion HPLC-MS analyses showed the presence in both liver and kidney of rats fed with pomegranate molecular ions at *m*/*z* 301 corresponding to two ellagic acid derivatives (**Figures 1** and **4A**), *m*/*z* 601 corresponding to gallagic acid (**Figures 1** and **4B**), *m*/*z* 403 corresponding to 3,8-dihydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one glucuronide (**Figures 1** and **4C**), and *m*/*z* 243 corresponding to 3,8,10-trihydroxy-6*H*-dibenzo-[*b*,*d*]pyran-6-one (**Figures 1** and **4D**).

MS-MS analyses of the isolated ion *m*/*z* 403 yielded the fragment at *m*/*z* 227, which confirmed the presence of the above glucuronide derivative (results not shown). **Figure 4A$_1$**−**D$_1$** shows all MS detected at the retention times of the above extracted ion chromatograms (arrows designate the ion *m*/*z* of the different metabolites). The metabolites 3,8-dihydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one and 3,8,10-trihydroxy-6*H*-dibenzo[*b*,*d*]-pyran-6-one (**Figure 1**) have been previously reported to be produced by rat microflora from ellagic acid (*27*) and in the feces of *Trogopterus xanthippes* (*30*).

All of the above metabolites were found in very small amounts including traces of punicalagin, which *m*/*z* ion at 1083 was also found (results not shown). The unequivocal identification of these metabolites was supported by the analysis of their characteristic spectra (**Figure 5**) as well as by the purification of these metabolites and further NMR analysis (unpublished results). The scarce accumulation of these metabolites in both liver and kidney of rats after 37 days with a mean intake of 0.9 g of punicalagin/day also suggested the extensive metabolism of punicalagin.

Histopathological Analysis. To corroborate the apparent lack of toxicity in both kidney and liver as suggested above by the analysis of blood parameters, a histopathological study of these organs was also carried out. No difference was observed in the weight of organs belonging either to control or to treated rats. Histological examination of liver and kidney as target organs showed no marked differences between the control and treated groups, and no evident alterations were detected (results not shown). Niho et al. (*23*) observed dose-dependent centrilobular liver cell hypertrophy and deposition of a fine granular brown pigment in proximal tubular cells in rats treated with gallic acid. Previous studies reported severe liver necrosis in mice fed with punicalagin- and punicalin-containing extracts (1.3 mg of tannin/g of body weight) from *Terminalia oblongata* after 2 days (*9*). The same authors reported liver necrosis with an oral dose of 0.5 mg of tannin/g of body weight after 2 days. This concentration is 17-fold higher than the equivalent punicalagin intake of a 70-kg person after drinking 1 L of pomegranate juice. Biochemical results with no increase of liver (ALT, AST, ALP, bilirubin) and kidney function (urea, creatinine) parameters

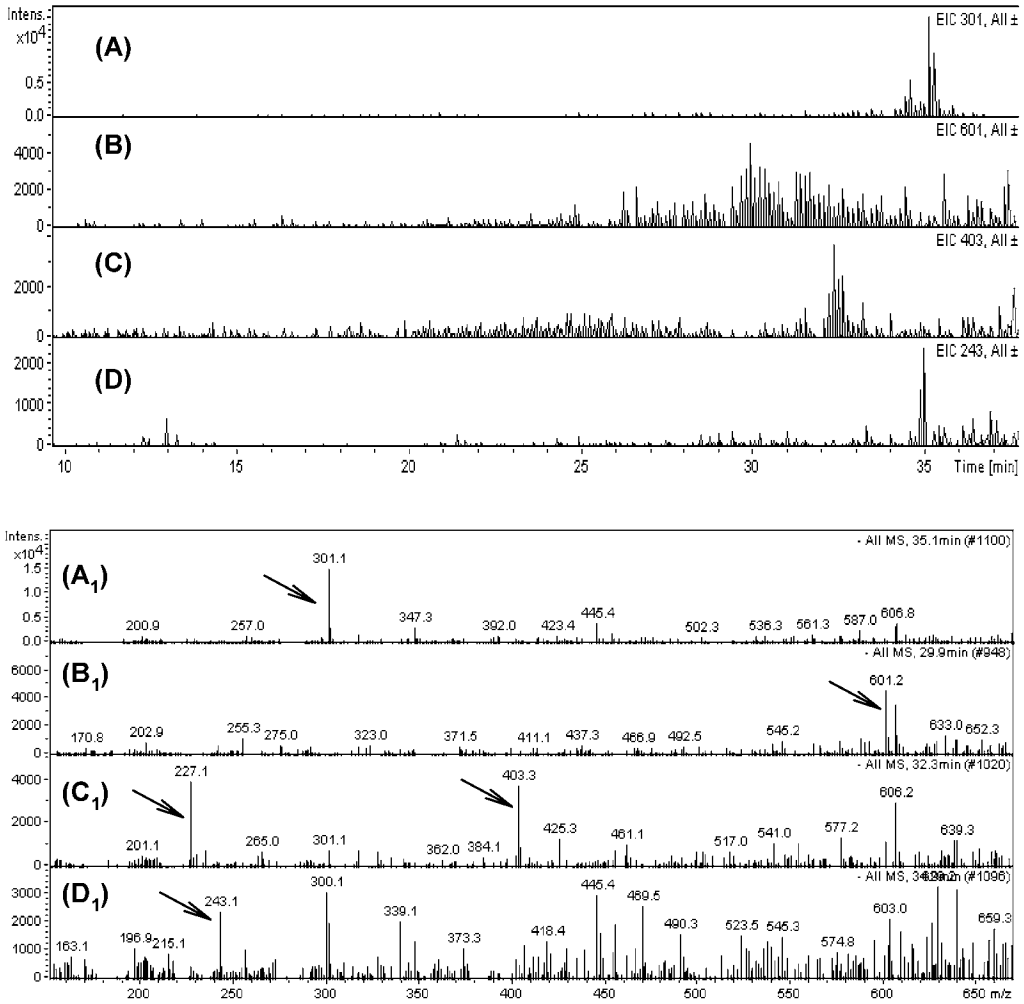

Figure 4. (A–D) Extracted ion chromatograms (EIC) (A, *m*/*z* 301; B, *m*/*z* 601; C, *m*/*z* 403; D, *m*/*z* 243) of punicalagin metabolites in liver extract of pomegranate-treated rats after 37 days. The same result was obtained in kidney extracts (result not shown). (A_1–D_1) All MS detected at the retention times of the above EIC. Arrows designate the ion *m*/*z* of the different metabolites. The fragment at *m*/*z* 227 in C_1 is the aglycon, 3,8-dihydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one, from its corresponding glucuronide (*m*/*z* 403) (see **Figure 1**).

would be in accordance with the histological patterns obtained in our study.

Antioxidant Enzymes. Antioxidant enzymes such as glutathione peroxidase (GPx), catalase, and superoxide dismutase (SOD), among others, have been reported to be lower in transformed cells and/or tumors (*31*) so that the enhancement of the activity of these enzymes is accepted as a possible positive cancer chemopreventive action.

Increases in both GPx and SOD activities have been reported in tetrachloride-induced liver damage in rats further treated with both punicalagin and punicalin. In rats fed with 2.5% green tea extract an increase in SOD activity was also detected (*28*). It seems that depending on the phenolic compound the effect on antioxidant enzymes could be different. In fact, differential effects of dietary flavonoids on antioxidant enzymes (GPx and SOD among others) in rats have been previously reported (*32*).

In the present study, no significant differences were found between both groups (control and treated rats) of animals in GPx and SOD activities (**Table 2**). Although the mean values of the 37 day experiment period are shown in **Table 2**, GPx and SOD activities were monitored weekly, and no significant changes were observed (results not shown). This could indicate that the punicalagin-containing extract did not cause any variation in the enzymatic defense against reactive oxidative species. Recently, similar results were found with diets containing grapefruit flavonoid extracts (*25*).

It seems that there was a change in the metabolism of punicalagin by the rats, which could be critical to overcoming a possible toxicity. Although weekly analyses of biochemical and hematological parameters indicated the lack of toxicity, the HPLC-MS-MS and histopathological analyses of both liver and kidney were carried out only at the end of the experiment. Therefore, a more pronounced effect on liver and kidney tissues during the first 15 days cannot be completely ruled out, which could support the previous toxic effect on mice (*9*).

Therefore, the above results indicated that a 6% punicalagin content in the rat diet (4.8 g of punicalagin/kg of body rat weight/day) during 37 days did not provoke tissue alterations, and most of the serum biochemical and hematological parameters were normal. However, a decrease in both urea and TGs was observed. Although the final explanation for this decrease remains unknown, we postulate that the decrease in the nutritive value of the diet (replacement of the normal diet by the presence of 20% pomegranate extract) together with a low palatability (low food intake) could be mainly responsible for triggering the decrease in the above parameters rather than a toxic effect of punicalagin.

However, it should be noted that the present study was conducted in rats so that a further extrapolation to humans

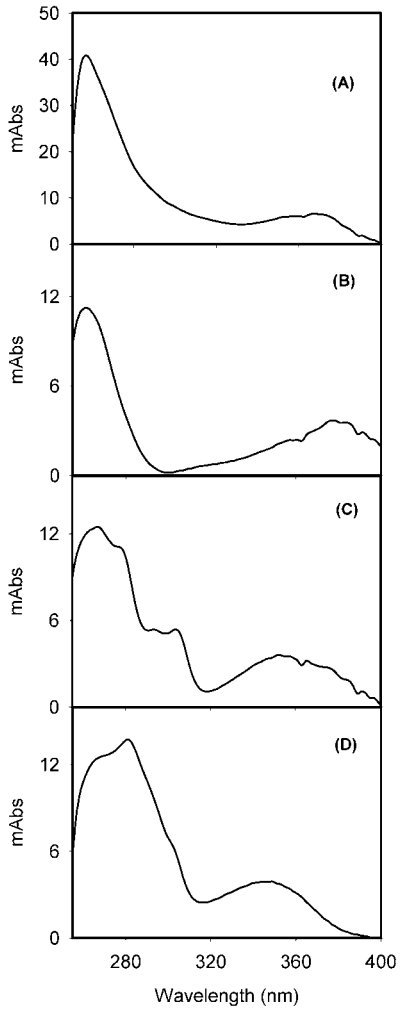

Figure 5. Spectra of punicalagin metabolites detected in liver and kidney of rat: (A) ellagic acid-like spectrum (*m*/*z* 301); (B) gallagic acid-like spectrum (*m*/*z* 601); (C) 3,8-dihydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one-like spectrum (MS/MS *m*/*z* 227); (D) 3,8,10-trihydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one-like spectrum (*m*/*z* 243).

should be made with the logical caution. In addition, the aim of this study was to evaluate the possible adverse effects upon the oral administration of very high doses of punicalagin during a rather short period (37 days). Therefore, the present study establishes the basis for a further needed study concerning the safety evaluation of punicalagin-containing foodstuffs such as pomegranate juice in humans with a longer safety margin of exposure and lower punicalagin concentration intake in order to mimic a more realistic context.

ABBREVIATIONS USED

Alb, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Bil, bilirubin; Ca, calcium; Chol, cholesterol; CK, creatine quinase; Crea, creatinine; Glc, glucose; GPx, gluthatione peroxidase; Hb, hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MHC, mean hemoglobin corpuscular; NVR, normal values ranges; P, phosphorus; PCV, packed cell volume; PLT, platelets; RBC, red blood cell number; SOD, superoxide dismutase; TP, total proteins; TGs, triglycerides; WBC, white blood cell count.

ACKNOWLEDGMENT

We are grateful to José Pastor Navarro from Agrudeco (Elche, Spain) for supplying pomegranates.

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Received for review July 31, 2002. Revised manuscript received March 10, 2003. Accepted March 14, 2003. This work has been supported by Spanish CICYT, AGL2000-2014 and by the "Fundación Séneca", Project 02068/c/02. B.C. has a fellowship from the Spanish Consejo Superior de Investigaciones Científicas (CSIC) and ESF (I3P Program).

JF020842C