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ORIGINAL CONTRIBUTION

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# Evaluation of the bioavailability and metabolism in the rat of punicalagin, an antioxidant polyphenol from pomegranate juice

**Summary** *Background & Aims* Punicalagin is an antioxidant ellagitannin of pomegranate juice. This compound is responsible for the high antioxidant activity of this juice. Nothing is known about the bioavailability and metabolism of punicalagin or other food ellagitannins. The present work aims to evaluate the bioavailability and metabolism of punicalagin in the rat as an animal model. *Design* Two groups of rats were studied. One fed with standard rat diet (n = 5) and another with the same diet plus 6 % punicalagin (n = 5). Samples of urine and faeces were taken during 37 days and plasma every week. The different metabolites were analysed by HPLC-MS-MS. *Results* The daily intake of punicalagin ranged from 0.6 to 1.2 g. Values around 3–6 % the ingested punicalagin were excreted as identified metabolites in faeces and urine. In faeces, punicalagin is transformed to hydrolysis products and partly metabolites by the rat microflora to 6H-dibenzo-[b,d]pyran-6-one derivatives. In plasma, punicalagin was detected at concentrations around 30 μg/mL, and glucuronides of methyl ether derivatives of ellagic acid were also detected. 6H-Dibenzo[b,d]pyran-6-one derivatives were also detected especially during the last few weeks of the experiment. In urine, the main metabolites observed were the 6H-dibenzo[b,d]pyran-6-one derivatives, as aglycones or glucuronides. *Conclusion* As only 3–6 % of the ingested punicalagin was detected as such or as metabolites in urine and faeces, the majority of this ellagitannin has to be converted to undetectable metabolites (i. e. $CO_2$) or accumulated in non-analysed tissues, however with only traces of punicalagin metabolites being detected in liver or kidney. This is the first report on the absorption of an ellagitannin and its presence in plasma. In addition, the transformation of ellagic acid derivatives to 6H-dibenzo-[b,d]pyran-6-one derivatives in the rat is also confirmed.

**Key words** ellagitannin – punicalagin – pomegranate juice – bioavailability – metabolism – rat – plasma – urine – faeces – HPLC-MS-MS

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## Introduction

Epidemiological studies have shown that diets rich in plant-derived food products lower the mortality by cardiovascular diseases [1, 2]. This positive effect has been associated with the content in natural antioxidant phytochemicals, as it has been demonstrated in in vitro and ex vivo experiments [3]. These results have been the driving force of many recent studies regarding the antioxidant activity of different food products and the evaluation of the constituents responsible for this activity.

In a recent study the high antioxidant activity of pomegranate juices has been reported, and this activity was associated with the high content (2 g/L) of phenolic compounds that were identified as punicalagin isomers, ellagic acid derivatives and anthocyanins (delphinidin, cyanidin and pelargonidin 3-glucosides and 3,5-diglucosides) [4]. Punicalagin is an ellagitannin in which gallagic and ellagic acids are linked to a glucose molecule. The

punicalagin isomers have been reported to be mainly responsible for the high antioxidant capacity of pomegranate juice. These compounds impart the characteristic yellow colour of pomegranate husk, and are extracted with the juice during processing [4]. The health-beneficial properties of pomegranate juice have recently been demonstrated in *in vivo* experiments with humans [5]. Punicalagin, however, has been reported as responsible for kidney and liver toxicity in cattle [6] although other studies have reported the lack of such toxic effects [7].

Very little is known about the bioavailability of food ellagic acid derivatives and ellagitannins [8]. Some studies on rats have reported the metabolic transformation of ellagic acid into 3,8-dihydroxy-6H-dibenzo[b,d]-pyran-6-one and it was excreted as such in urine and faeces [9], and this transformation was reported to be produced by the rat microflora. However, Smart et al. [10] and Teel and Martin [11] reported that in mice, these microflora metabolites were not detected and ellagic acid derivatives were excreted in urine instead. Nothing is known about the fate of ellagitannins in animals or humans [8]. As these compounds had a very high antioxidant activity in vitro, it would be essential to know their fate in animals and in humans.

Due to the possible beneficial and non-beneficial activities of punicalagin isomers, it is essential to evaluate the absorption and metabolism of these compounds in animal models after oral administration and follow their fate in the faeces, plasma and urine using the rat as an animal model. In order to facilitate and control the ellagitannin intake by rats, a rich source of these compounds (pomegranate husk) was included in the diet.

## Material and methods

### Animals and diets

Ten female Sprague-Dawley rats weighing 156–168 g were provided by the Animal Center of the University of Murcia (Spain). Rats were quarantined for 1 week. Rats were 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 a standard rat diet (Panlab, Barcelona, Spain). Both solid diet and water were consumed *ad libitum*. Handling and killing were in full accordance with the actual law for the care and management of laboratory animals. Rats were randomly divided into two groups, i. e. a control group (CG, n = 5) that was only fed the commercial diet, and a treated group (pomegranate group, PG, n = 5) which was fed the commercial diet containing 6 % punicalagin provided by mixing the commercial feeding-stuff with pomegranate husk extract. At the end of the experiment the rats were anaesthetised with ether and killed by exsanguination.

### Preparation of a punicalagin standard

One kg of pomegranate husk (var. Mollar de Albatera) was incubated in 2 L of distilled water at room temperature for 2 hours. The solution was then decanted and aliquots of 50 mL further filtered through a solid-phase extraction cartridge (a reverse phase C-18 cartridge; Waters Millipore, USA) which retained phenolic compounds and removed other highly hydrophilic compounds. The cartridges were previously activated with 10 mL methanol and 10 mL water. Phenolic compounds retained in each cartridge were then removed by passing air through it. Each cartridge was washed with 5 mL water. These fractions eluted with water were discarded. The remaining volume in each cartridge was eluted with 5 mL of MeOH. The methanol fractions of each cartridge were collected and filtered through a 0.45 µm filter. The pooled filtered solution was injected in a semi-preparative HPLC system equipped with a L-6000 pump, L-4000 UV detector, D-2500 Chromato-Integrator and 2 mL sample loop. Chromatographic separation was carried out on a reverse phase ODS-2 column (25x0.7 cm, 5 µm particle size) (Teknokroma, Barcelona, Spain) using an isocratic mixture of methanol/acetic acid/water (2:0.5:97.5) at a flow rate of 1.5 mL/min. Fractions were detected at 360 nm. The different fractions were analysed by HPLC-DAD-MS-MS to identify and quantify the punicalagin isomers. The purified fraction of punicalagin (50 mg) was freeze-dried and used as a pure 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 the diet and in the biological fluids analysed.

### HPLC-MS-MS analysis

Chromatographic separations of pomegranate extracts, as well as plasma, urine, kidney, liver and faeces from control and treated rats were carried out on a reverse phase $C_{18}$ LiChroCART column (25 x 0.4 cm, particle size 5 µm, Merck, Darmstadt, Germany) 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), 5 min 1 % (B), 20 min 20 % (B), 30 min 40 % (B), 35 min 95 % (B), 39 min 95 % (B), 41 min 1 % (B), 50 min 1 % (B). UV chromatograms were recorded at 255 and 360 nm.

The HPLC system equipped with a DAD detector and mass detector in series consisted of a HPLC binary pump (G1312A), an autosampler (G1313A), a degasser (G1322A) and a photo-diode array detector (G1315B) controlled by software (v. A08.03) from Agilent Technologies (Waldbronn, Germany). The mass detector was an ion-trap mass spectrometer (G2445A, Agilent Technologies, Waldbronn, Germany) equipped with an elec-

trospray ionisation (ESI) system and controlled by a software (v. 4.0.25). The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. Mass scan (MS) and MS-MS spectra were measured from *m/z* 100 up 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 ionisation mode.

### Sampling procedure

#### Plasma

Blood was extracted with a cardiac punctation and collected in heparinised tubes every week. Blood was immediately separated by centrifugation at 14000 g for 15 minutes at 4 °C. Plasma was homogenised with MeOH:200 mM HCl (1:1, v/v). The mixture was vortexed for 30 s and centrifuged at 14000 g for 2 minutes at 4 °C. The supernatant was filtered through a 0.45 µm filter, and directly analysed by HPLC-DAD-MS-MS.

#### Urine and faeces

Samples were collected every two days and were immediately deep frozen at –70 °C and freeze-dried. Urine samples were diluted with 3 mL of $H_2O$/MeOH/HCOOH (80:19.9:0.1, 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 directly analysed by HPLC-DAD-MS-MS. Faeces samples (1 g) were homogenised with 10 mL of $H_2O$/MeOH/HCOOH (80:19.9:0.1, v:v). The mixture was homogenised with an Ultraturrax T25, and filtered through sterilised cheesecloth. The filtrate was centrifuged for 10 min at 4 °C at 14000 g. The supernatant was filtered through a 0.45 µm filter, and the filtrate analysed by HPLC-DAD-MS-MS.

#### Phenolic compounds identification and quantification

The different metabolites were identified by their UV spectra and the MS and MS-MS analyses after ion isolation using ion trap. Quantification of ellagic acid derivatives and punicalagin were carried out by chromatographic comparisons with pure standards of ellagic acid (Sigma) and the punicalagin standard prepared as reported above. All ellagic acid derivatives were quantified as ellagic acid at 360 nm. Punicalagin isomers, punicalin and gallagic acid were quantified as punicalagin at 360 nm. The ellagic acid metabolites (6H-dibenzopyran-6-one derivatives) were quantified by taking into account their molar absorptivity values at 360 nm [12].

## Results and discussion

### Pomegranate ellagitannins

An HPLC analysis of a water extract of pomegranate husk (results not shown) that was given to the rats contained three main peaks with a characteristic UV spectrum as punicalagin (Fig. 1A) and three additional peaks with the UV spectrum of an ellagic acid derivative (Fig. 1B) [4]. After HPLC-MS-MS analyses the three peaks with punicalagin-like UV spectrum were identified as three isomers with the same mass (M-H at 1083 m/z) consistent with a glucose substituted with a gallagyl and a hexahydroxydiphenoyl residue (Fig. 2). These compounds were previously identified as different isomers of punicalagin [13]. The MS-MS analysis revealed the presence of the three punicalagin isomers as well as the intermediate punicalin (gallagyl-glucose), gallagic acid and ellagic acid. In addition, the three ellagic acid derivatives coincided with an ellagic acid hexoside (M-H, m/z 463, and 301, more likely glucoside), a pentoside (M-H, m/z 433 and 301) and a deoxyhexoside (M-H, m/z 447, and 301, more likely rhamnoside) (results not shown).

Punicalagin was isolated from pomegranate husk in order to prepare an authentic standard that could be used for quantification purposes, and in order to evaluate the intake of these compounds during this experiment, and the occurrence of these compounds and their derivatives (gallagic and ellagic acids) in plasma and other biological fluids. Details on the isolation of this compound are provided above.

### Analysis of the different punicalagin and ellagic acid metabolites in the rat gastrointestinal tract and in biological fluids

#### Faeces

Clear differences were found between the methanol extracts obtained from freeze-dried faeces of control rats, fed the plain diet (Fig. 3A), and those of rats fed the diet with added pomegranate phenolics (Fig. 3B). Chromatograms of control faeces only showed a characteristic peak identified by its UV spectrum and MS analysis as ferulic acid (peak *a*), and could originate from the cereals present in the standard rat food. Faeces from rats fed with the punicalagin diet showed the occurrence of the three punicalagin derivatives (2, 3, 4) indicating that part of the ingested punicalagin derivatives were excreted as such with faeces. Part of the ingested punicalagin was hydrolysed during the gastrointestinal digestion to release punicalin (gallagyl glucose) (1) and the aglycones gallagic acid (5) and ellagic acid (6) (Fig. 2). The release of ellagic acid from strawberry ellagitannins un-

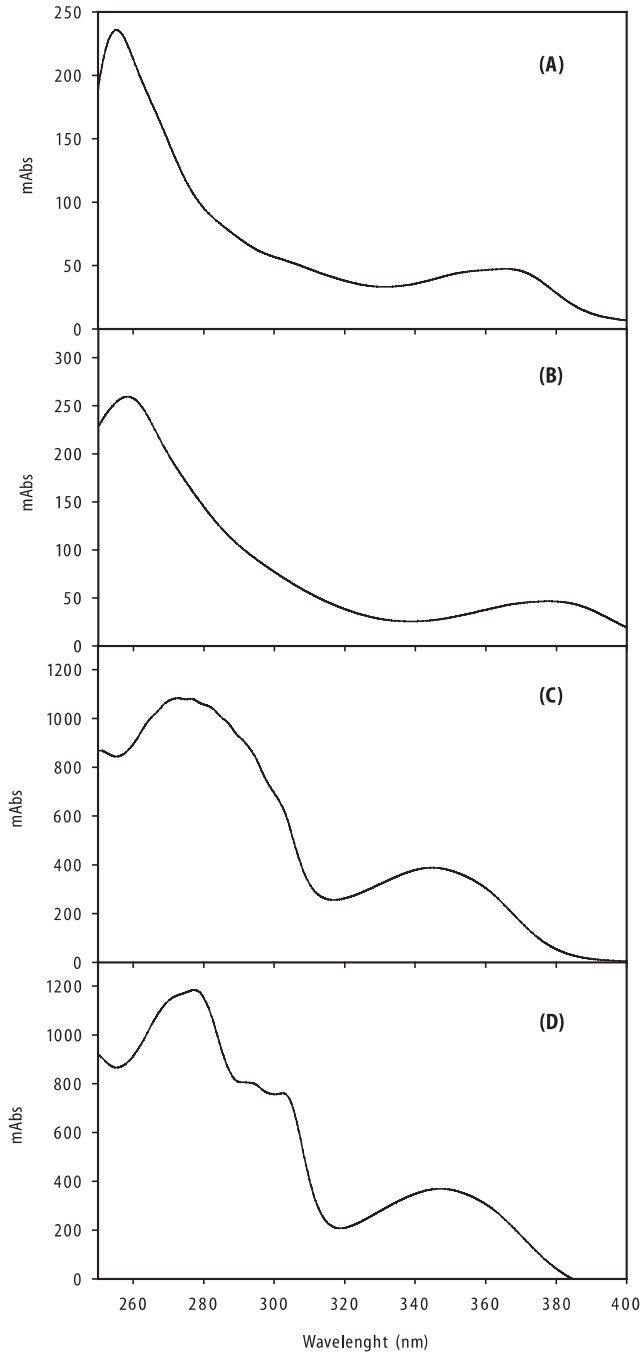


**Fig. 1** Characteristic UV spectra of different pomegranate ellagic derivatives and their microflora metabolites. **A** Ellagic acid derivatives; **B** gallagic acid derivatives; **C** 3,8,10-trihydroxy-dibenzo[b,d]pyran-6-one derivatives; **D** 3,8-dihydroxy-dibenzo[b,d]pyran-6-one derivatives

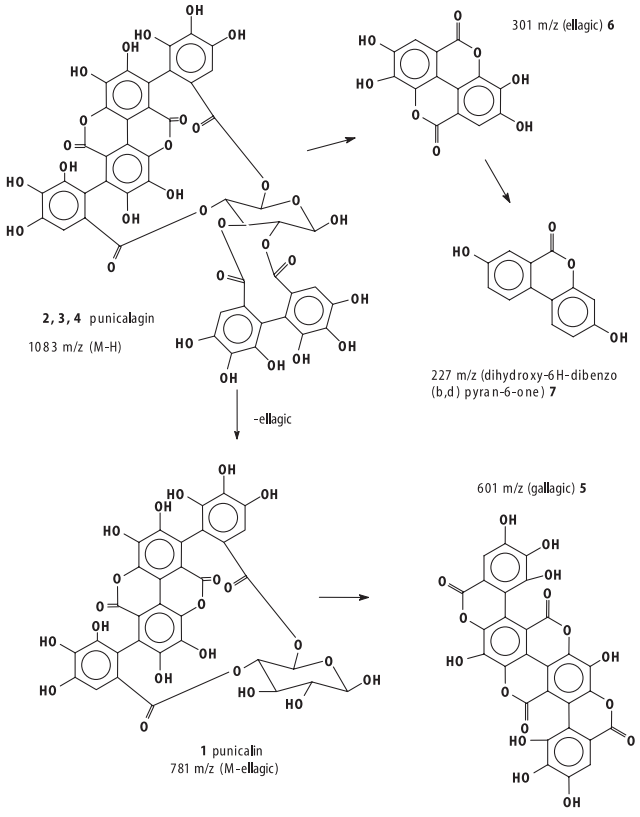


**Fig. 2** Gastrointestinal tract transformations of punicalagin

der the physiological conditions of the human gastrointestinal tract has already been reported [14]. In addition a new metabolite was observed in faeces of treated rats. This compound showed characteristic UV (Fig. 1D) and mass spectra (M-H at 227 m/z) as 3,8-dihydroxy-6H-dibenzo[b,d]pyran-6-one (7) (Figs. 2 and 3), a metabolite previously reported to be produced by rat microflora from ellagic acid [9]. This compound has also been reported as a hyaluronidase inhibitor in the faeces of Pteropi (*Trogopterus xanthipes*) [12], and in the beaver hair excretion [15], two animals that ingest large amounts of ellagitannins in their diets.

The amount of punicalagin and their hydrolysis products (punicalin, ellagic acid and gallagic acid) in faeces showed an increase up to day 18 starting then a slight decrease in the amounts of punicalagin and punicalin (Fig. 4A) while ellagic and gallagic acids remained quite constant (Fig. 4B). This decrease coincided with an increase in the microflora metabolite 3,8-dihydroxy-6H-dibenzo[b,d]pyran-6-one (Fig. 4C).

## Plasma

The HPLC analyses of metabolites present in plasma of control rats (Fig. 5A) and those fed with pomegranate phenolics (Fig. 5B) also showed clear differences, both qualitative and quantitative. The analysis with the diode array detector showed that some of the metabolites detected in the plasma from pomegranate-fed rats had UV spectra similar to that of the ellagic acid derivatives (peaks *5*, *12*, *6* and *13*) (Fig. 1) (Table 1). Compounds *8*,

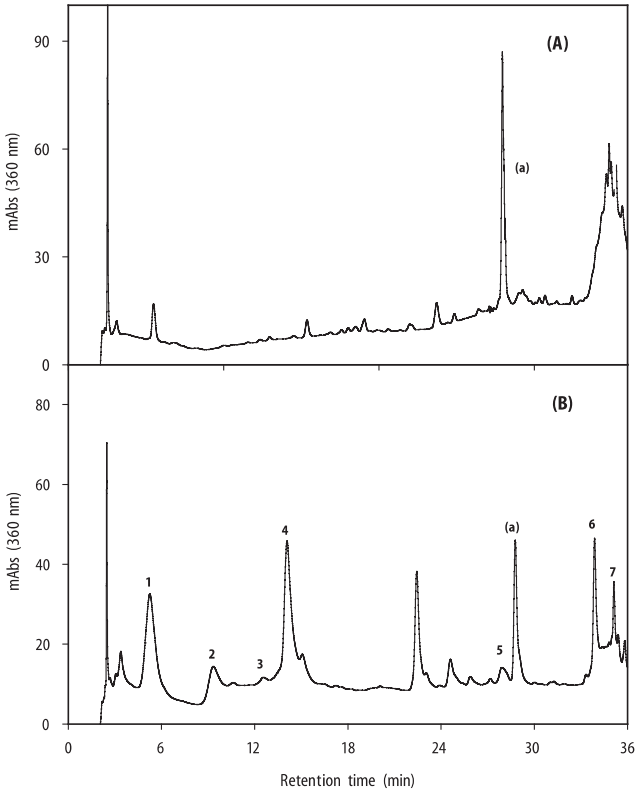


**Fig. 3** HPLC analysis of faeces from control (**A**) and pomegranate polyphenols fed (**B**) rats. (**1**) punicalin; (**2, 3, 4**) punicalagin isomers; (**5**) gallagic acid; (**6**) ellagic acid; (**7**) 3,8-dihydroxy-dibenzo[b,d]pyran-6-one; (**a**) ferulic acid

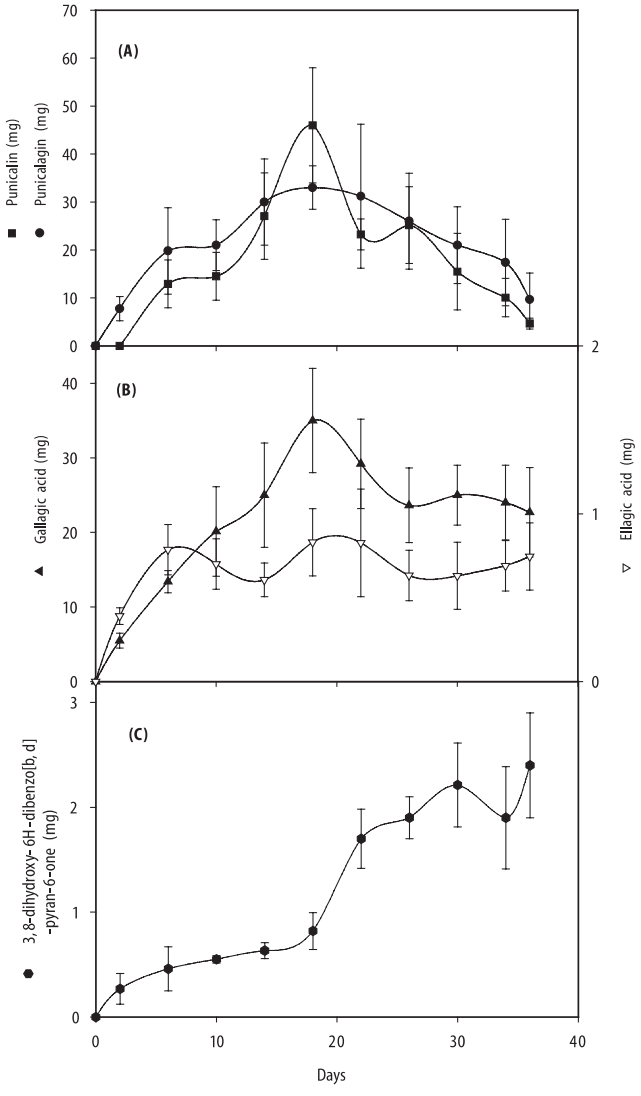


**Fig. 4** Evolution of punicalagin metabolites in faeces during the 37 days of experiment. Values are total amount of the individual metabolites excreted with faeces per period of time (in mg)

*9*, *10* and *11* showed characteristic UV spectra of 6H-dibenzo[b,d]pyran-6-one derivatives with a clear maximum around 360 nm (Fig. 1C, D). None of these metabolites was detected in the control plasma (Fig. 5A). The HPLC-MS-MS analyses with the ion trap and subsequent fragmentation of the isolated ions allowed the clear identification of some of these metabolites. The single ion HPLC-MS analyses allowed the identification of three peaks in the plasma of rats fed with pomegranate phenolics coincident with a molecular ion at 1083 m/z corresponding to the punicalagin isomers *2*, *3* and *4* (Fig. 5B). MS-MS analyses of the isolated 1083 ions clearly showed fragments for punicalin (781 m/z), gallagic acid (601 m/z) and ellagic acid (301 m/z) (Figs. 2 and 5B) confirming the presence of native punicalagin in plasma. The MS-MS analyses of compounds *5*, *12*, *6* and *13* (all of them with UV spectra similar to that of ellagic or gallagic acid derivatives) (Fig. 1), showed that *5* was gallagic acid, *12* was dimethyl-ellagic acid glucuronide, *6* free ellagic acid and *13* dimethyl ellagic acid glucuronide methyl ester (Table 1) (Fig. 6). As ellagic acid has two ortho-dihydroxyl groups, it can be expected that, in the liver via the activity of COMT (catechol *o*-methyl transferase), one methyl ether is introduced per dihydroxyl group (Fig. 6). These dimethyl ellagic acid metabolites would have a UV spectrum nearly identical to that of free ellagic acid, and an aglycone mass at m/z 330 as is the case of compounds *12* and *13*. In addition, both metabolites were further metabolised by combination with glucuronic acid and in the case of *13*, an additional methyl ester was introduced in the carboxylic residue leading to a rather lipophilic compound (Fig. 6). Compounds *8* and *9* showed characteristic UV spectra as dibenzo-pyran-6-one derivatives (Fig. 1C, D) that were reported to be microfora metabolites of ellagic acid in rats [9]. Compound *8* was identified as a trihydroxy-6H-dibenzo[b,d]pyran-6-one diglucuronide (Fig. 6) as shown by an M-H at 595 and fragments after MS-MS at m/z 420 (the monoglucuronide) and m/z 243 for the aglycone. The UV spectrum of this compound (Fig. 1C)

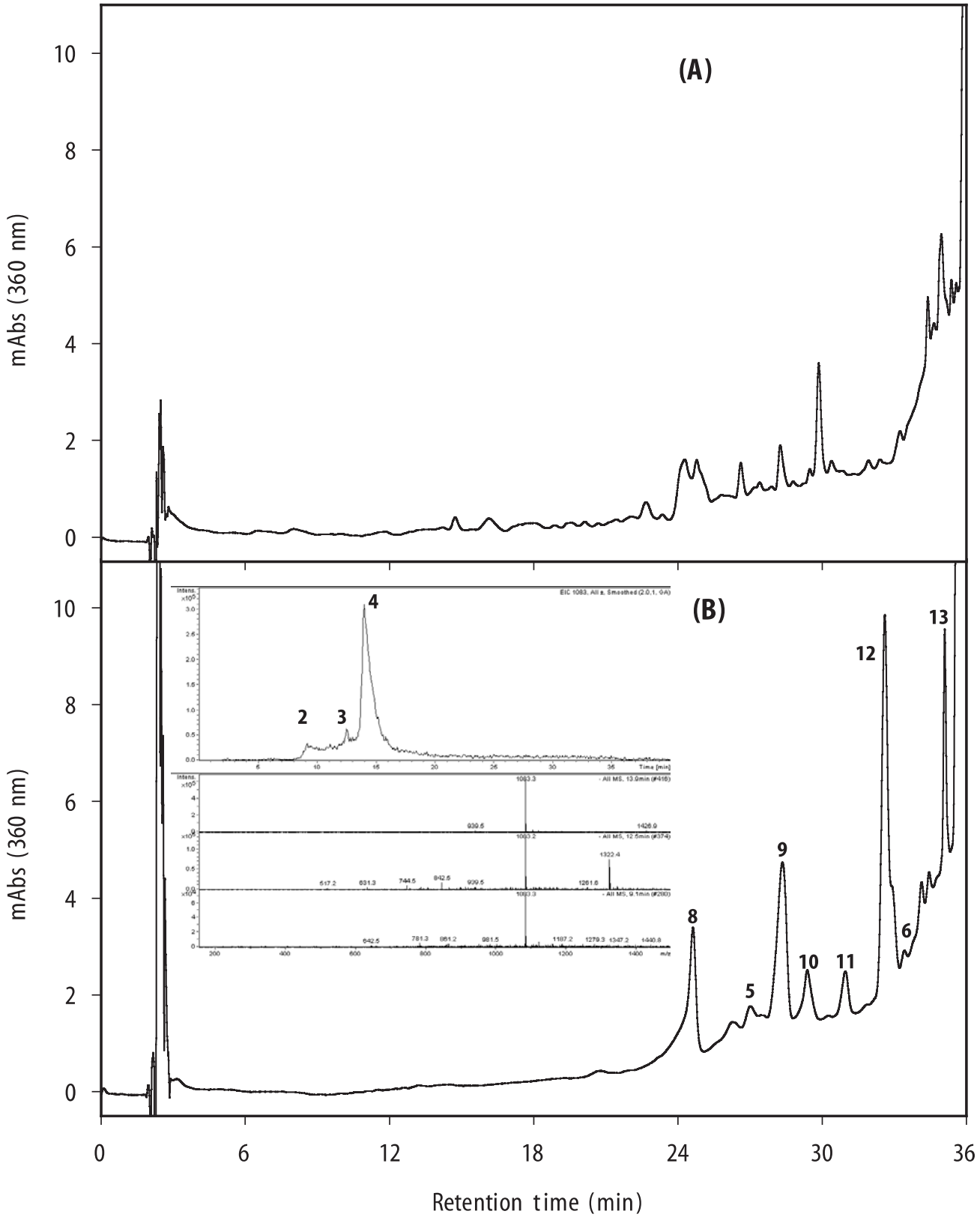


**Fig. 5** HPLC-MS-MS analysis of plasma from control (**A**) and pomegranate- polyphenols-fed (**B**) rats. Window inside **B** is the extracted ion chromatogram analysis of ion 1083 m/z. For compound identification see Table 1

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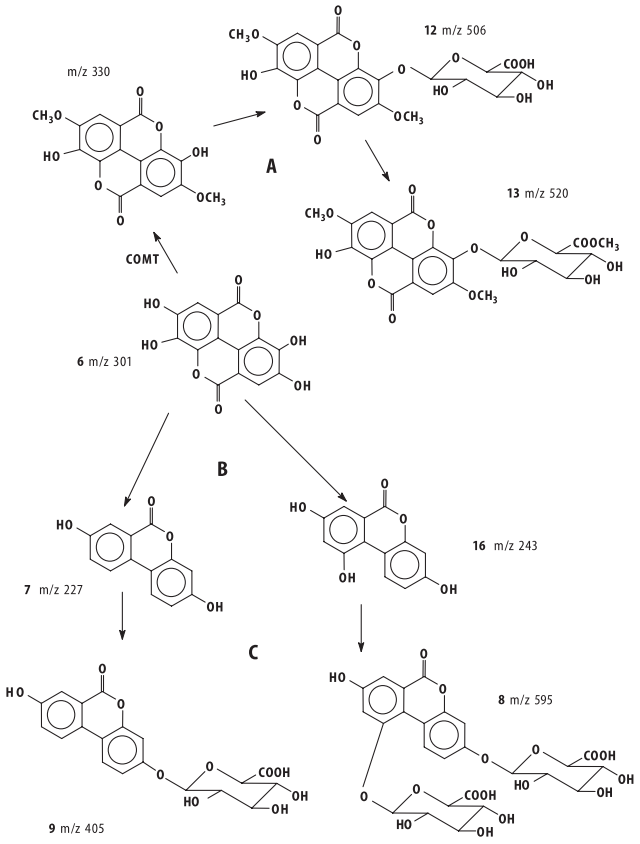


**Fig. 6** Ellagic acid metabolites. **A** Ellagic acid conjugation in liver. **B** Rat microflora metabolites. **C** Microflora metabolites conjugation in rat tissues

and the mass spectrum of the aglycone (243 m/z) coincided with those reported for 3,8,10-trihydroxy-6H-dibenzo[b,d]pyran-6-one, a metabolite recently reported from *Trogopterus xanthipes* faeces [12]. Compound *9* was a 3,8-dihydroxy-6H-dibenzo[b,d]pyran-6-one glucuronide, as supported its M-H at 405 m/z and a fragment after MS-MS of the isolated ion at 227 m/z and a characteristic UV spectrum (Fig. 1D). Hydrolysis with β-glucuronidase released the aglycone from compound *9*, confirming its conjugation with glucuronic acid. Other trihydroxy-6H-dibenzo[b,d]pyran-6-one derivatives were also detected (compounds *10* and *11*) as they showed characteristic UV spectra and the aglycone at m/z 243 after MS-MS (Table 1).

The concentration of the different punicalagin metabolites in plasma was followed during 5 weeks (Fig. 7). Punicalagin isomers (*2*, *3* and *4*) were the main metabolites detected showing a rather constant concentration around 30 µg/mL. A similar pattern was observed for free ellagic acid (*6*), which was always present in much smaller concentrations (around 3–5 µg/mL). The ellagic acid conjugation metabolites showed different kinetics of accumulation in plasma. Ellagic acid dimethyl ether glucuronide (*12*) showed a concentration curve that increased during the first three weeks, to reach a maximum concentration of 20 µg/mL, then decreasing to stabilise at around 12 µg/mL. The methyl ester of the previous metabolite (*13*) started to increase mainly during the last two weeks to a concentration around 5–7 µg/mL, and this increase could explain, at least partly, the decrease observed during the last two weeks in the other ellagic acid methyl ether glucuronide (Fig. 7). Gallagic acid (*5*) was only detected in plasma during the last three weeks of the experiment. The microflora metabolites [trihydroxy- (*16*) and dihydroxy-6H-dibenzo[b,d]pyran-6-one (*7*)] were detected in

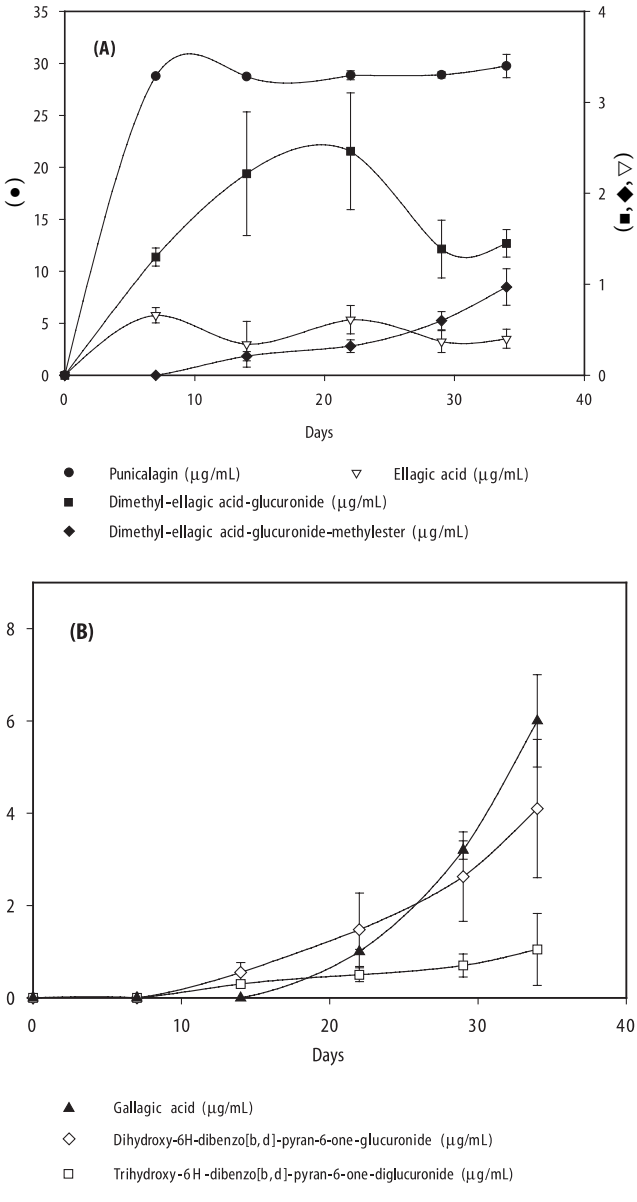


**Fig. 7** Evolution of punicalagin metabolite concentration in plasma during the 37 days of experiment

**Table 1** MS and MS/MS of punicalagin and its main metabolites in rat

| Peak number | Compound | MS | MS/MS | UV spectrum* |
|---|---|---|---|---|
| 1 | Punicalin | 781 | 601, 301 | B |
| 2 | Punicalagin isomer | 1083 | 781, 601, 301 | B |
| 3 | Punicalagin isomer | 1083 | 781, 601, 301 | B |
| 4 | Punicalagin isomer | 1083 | 781, 601, 301 | B |
| 5 | Gallagic acid | 601 | 301 | A |
| 6 | Ellagic acid | 301 | 301 | A |
| 7 | 3,8-dihydroxy-6H-dibenzo[b,d]-pyran-6-one | 227 | – | D |
| 8 | Trihydroxy-6H-dibenzo[b,d]-pyran-6-one-diglucuronide | 595 | 420, 243 | C |
| 9 | Dihydroxy-6H-dibenzo[b,d]-pyran-6-one-glucuronide | 405 | 228 | D |
| 10 | Unidentified trihydroxy-6H-dibenzo [b,d]-pyran-6-one derivative | – | 243 | C |
| 11 | Unidentified trihydroxy-6H-dibenzo [b,d]-pyran-6-one derivative | – | 243 | C |
| 12 | Dimethyl-ellagic acid-Glucuronide | 506 | 330 | A |
| 13 | Dimethyl-ellagic acid-Glucuronide-methylester | 520 | 330 | A |
| 14 | Unidentified ellagic acid derivative | – | 301 | A |
| 15 | Unidentified ellagic acid derivative | – | 315 | A |
| 16 | 3,8,10-trihydroxy-6H-dibenzo[b,d]-pyran-6-one | 243 | 227 | C |
| 17 | Unidentified dihydroxy-6H-dibenzo-[b,d]-pyran-6-one derivative | – | 227 | D |
| 18 | Unidentified dihydroxy-6H-dibenzo-[b,d]-pyran-6-one derivative | – | 227 | D |
| 19 | Unidentified ellagic acid derivative | – | 301 | A |
| 20 | Unidentified ellagic acid derivative | – | 301 | A |

* UV spectra from Fig. 2

plasma as glucuronide derivatives (*8* and *9*, respectively) (Fig. 6). They accumulated during the last few weeks of the experiment to reach final concentrations around 1 and 4 μg/mL, respectively (Fig. 7). This increase coincided with the increase in the non-conjugated metabolite (*7*) and a decrease in punicalagin and punicalin in faeces (Fig. 4).

## Urine

Clear differences were also found in the metabolites present in the urine obtained from the control rats (Fig. 8A) and those fed with pomegranate phenolics (Fig. 8B). The main compounds detected were those of the dihydroxy- and trihydroxy-6H-dibenzo[b,d]pyran-6-one type (compounds *7, 8*, *9*, *16*, *17*, *18*) although ellagic acid derivatives were also detected (*12*, *14*, *15*, *19*, *20*). Compounds *8* and *9* coincided with those detected in plasma as the glucuronide derivatives of trihydroxy-6H-dibenzo[b,d]pyran-6-one (*8*, aglycone fragment at m/z 243) and dihydroxy-6H-dibenzo[b,d]pyran-6-one (*9*, aglycone fragment at m/z 228) (Table 1) (Fig. 6). The other unidentified dihydroxy-6H-dibenzo[b,d]pyran-6-one derivatives were detected by their characteristic UV spectra (Fig. 1C, D) and by their aglycone fragments at m/z 227 or 243 after MS-MS. Some of these metabolites are the main compounds detected in urine of rats fed on pomegranate polyphenols (Fig. 8B). After performing a single ion chromatogram analysis for ion 1083 m/z (punicalagin), this compound was also detected in small amounts in urine, as well as the metabolite punicalin at m/z 781 (Fig. 8B). These two compounds started to appear in urine only after two weeks of the experiment (Fig. 9A) and in very small amounts (amounts generally below 0.25 mg of punicalagin and punicalin recovered in the urine produced during one day). This amount then remained quite constant until the end of the experiment (Fig. 9A). Ellagic acid derivatives were detected in urine in small amounts that prevented their full characterisation, although their characteristic UV spectra allowed their detection (Fig. 1A). The ellagic acid microflora metabolites trihydroxy- and dihydroxy-6H-dibenzo[b,d]-pyran-6-one derivatives followed an accumulation rate similar to those found in faeces and plasma, as these compounds, as glucuronic conjugates or aglycones, were present as the main urine metabolites during the last few weeks of the experiment (Fig. 9B) to reach values around 10 mg of compound in the urine

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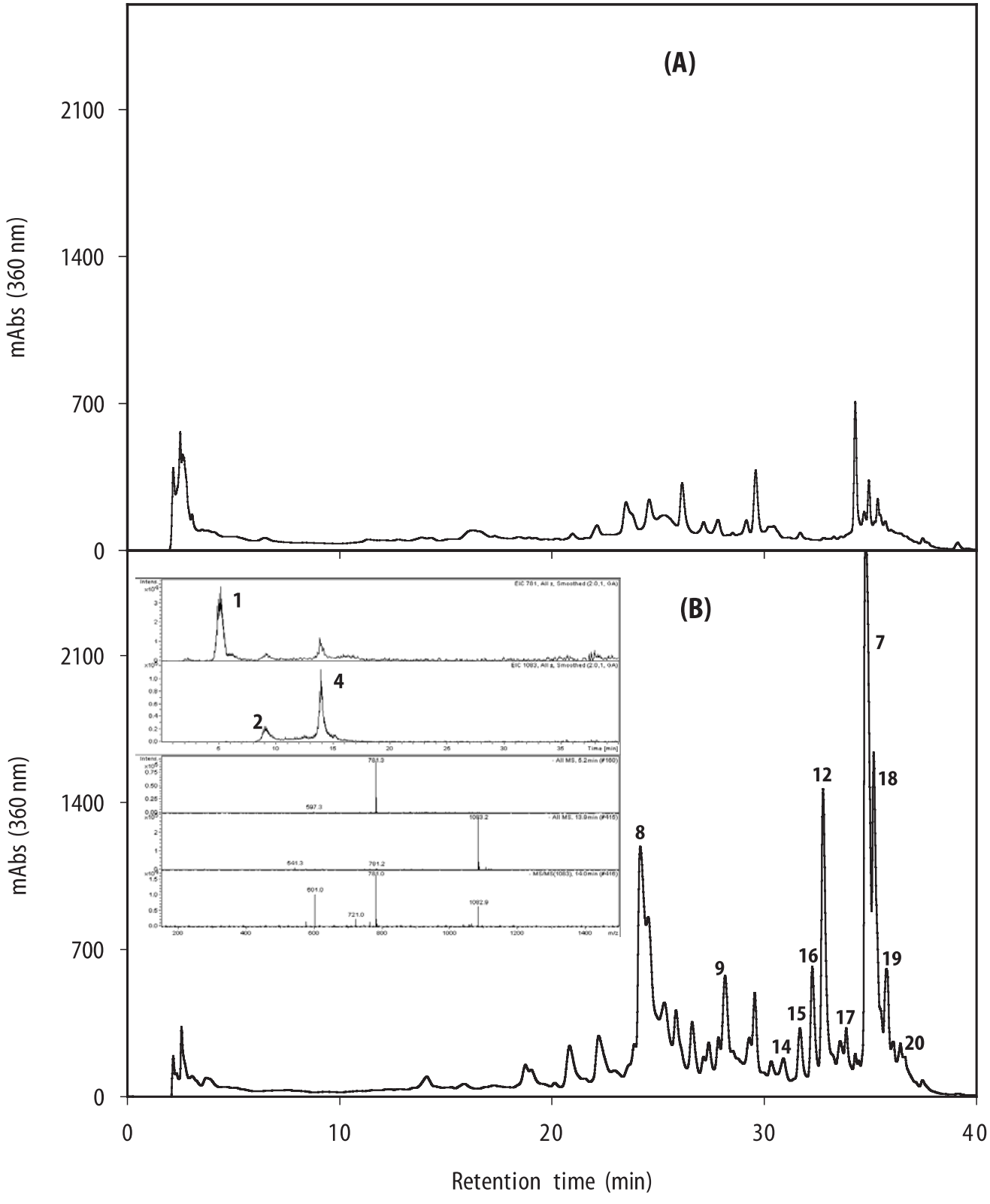


**Fig. 8** HPLC-MS-MS analysis of urine from control (**A**) and pomegranate-polyphenols-fed (**B**) rats. Window inside **B** is the extracted ion chromatogram analysis of ions 1083 and 781 m/z. For compound identification see Table 1

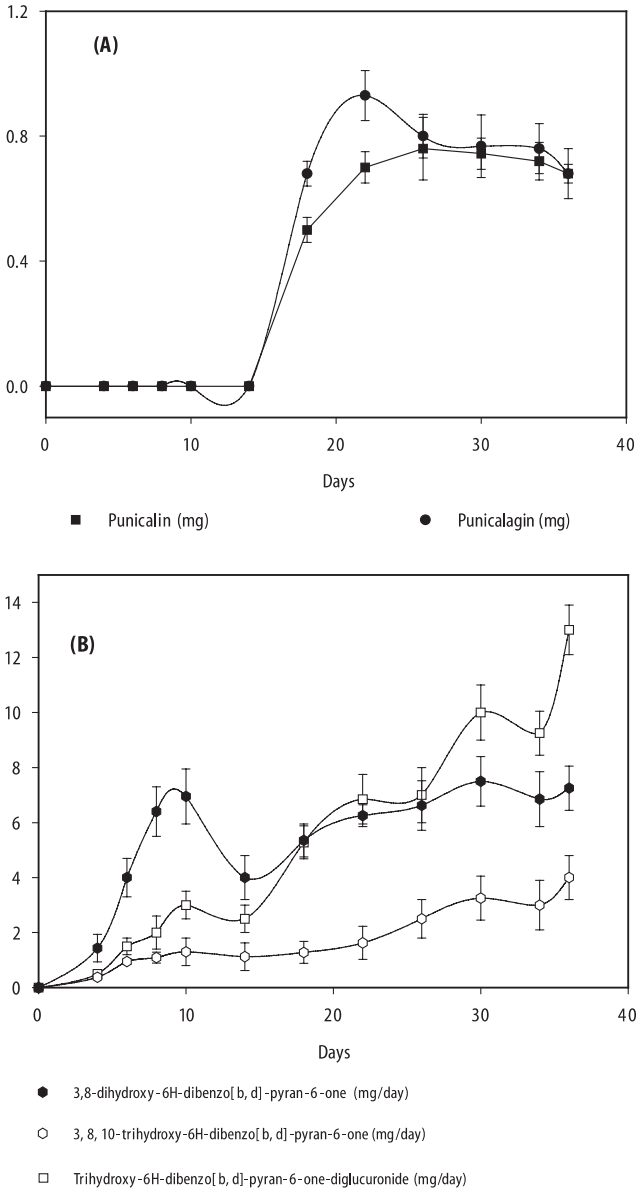


**Fig. 9** Evolution of punicalagin metabolites in urine during the 37 days of experiment. Values are average amount of the individual metabolites excreted with urine per period of time (in mg)

collected during one day. These values were 40 times higher than those found for punicalagin or punicalin.

## Fate of the ingested punicalagin in the rat

The total average (mg/day) punicalagin intake by rats is shown in Fig. 10A. Punicalagin intake values indicated that the average intake of punicalagin during the first week reached values around 400 mg/day to reach around 1200 mg/day at the end of the experiment (Fig. 10A). When looking at the values accumulated for punicalagin and its metabolites during one week in urine, it was clear that the higher values reached 37 mg/day, which is approximately 4 % of the ingested punicalagin, values that are slightly higher then those previously reported for other polyphenols [8]. When the total punicalagin-derived metabolites were quantified in the faeces and urine produced during the same time, the maximum excretion rate was observed during the second week with a mean excretion rate of 6 % mg/day (Fig. 10B) showing that the majority of the ingested punicalagin was metabolised and/or absorbed. This means that most of the ingested punicalagin has to be transformed to non-detected metabolites or accumulated in tissues. Analysis of extracts from liver and kidney, however, showed that

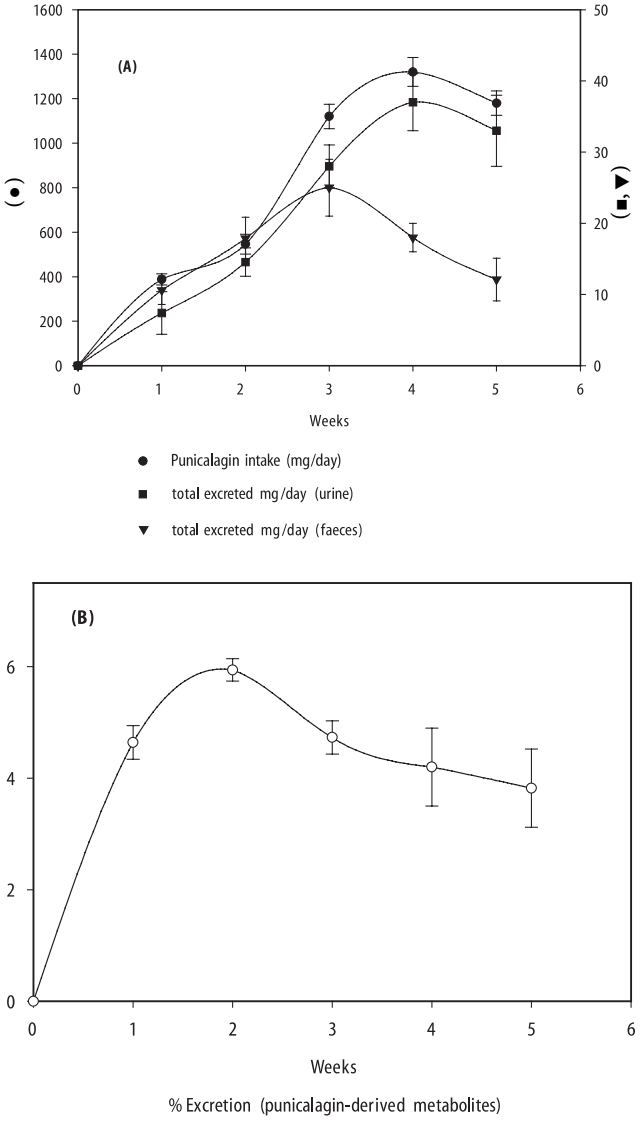


**Fig. 10** Punicalagin intake by rats and excretion of metabolites in faeces and urine. Values are mg of punicalagin ingested or excreted as such or as the corresponding metabolites per day (mean value n = 5)

these compounds were only present as traces (data not shown). In a recent study it was demonstrated that quercetin is mainly excreted in humans through the lungs as $CO_2$ [16]. This could explain, at least in part, the results found in the present experiment.

The results shown here indicate that there are two main stages in this experiment. The first stage during the first 20 days in which the main metabolites present in biological fluids are those derived from punicalagin by hydrolysis and further conjugation (methyl ethers or glucuronic acid derivatives) (Fig. 6). After this time there is a change and the microflora metabolites start to be apparent in faeces, and their conjugate metabolites (glucuronides) in plasma and urine where they become the main metabolites. These dramatic changes could be associated with a change in the rat microflora, which was then able to metabolise ellagic acid derivatives to produce the 6H-dibenzo[b,d]pyran-6-one metabolites. This would explain the quite different results found in studies on the disposition of ellagic acid in rats that showed that the main excreted compounds were 6H-dibenzopyran-6-one derivatives [9] while in mice these compounds were not detected and ellagic acid conjugation metabolites were detected instead [10–11]. In the present study, during the first two weeks of the experiment, the behaviour would be similar to that reported for mice, while after 3 weeks the microflora metabolites were the predominant metabolites as has been previously reported for rats.

This is the first time that an ellagitannin is reported to be absorbed as such in animals, as punicalagin has been clearly detected in plasma by HPLC-MS-MS analysis. In a recent review, Clifford and Scalbert [8] reported that the absorption of native ellagitannins in animals has never been definitively demonstrated, although it has been suggested that gallotannins occurring in commercial tannic acid do cross the intestinal barrier of sheep [17].

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## References

1. Arai Y, Watanabe S, Kimira M, Simio K, Mochizuki R, Kinae N (2001) Dietary intakes of flavonols, flavones and isoflavones by Japanese women and the inverse correlation between quercetin intake and plasma LDL cholesterol. J Nutr 130:2378–2383
2. Geleijnse JM, Launer LJ, van der Kuip DAM, Hofman A, Witteman JCM (2002) Inverse association of tea and flavonoid intakes with incident myocardial infarction: the Rotterdam study. Am J Clin Nutr 75:880–886
3. Pataki R, Bak I, Kovacs P, Bagchi D, Das DK, Tosaki A (2002) Grape seed proanthocyanidins improved cardiac recovery during reperfusion after ischemia in isolated rat hearts. Am J Clin Nutr 75:894–899
4. Gil MI, Tomás-Barberán FA, Hess-Pierce B, Holcroft DM, Kader AA (2000) Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. J Agric Food Chem 48:4581–4589
5. Aviram M, Dornfeld L, Rosenblat M, Volkova N, Kaplan M, Coleman, R, Hayek T, Presser D, Fuhrman B (2000) Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL, and platelet aggregation: studies in humans and atherosclerotic apolipoprotein E-deficient mice. Am J Clin Nutr 71:1062–1076
6. Filippich LJ, Zhu J, Alsalami MT Hepatotoxic and nephrotoxic principles in Terminalia oblongata (1991) Res Vet Sci 50:170–177
7. Castonguay A, Gali HU, Perchellet EM, Gao XM, Boukharta M, Jalbert G, Okuda T, Yoshida T, Hatano T, Perchellet JP (1997) Antitumorigenic and antipromoting activities of ellagic acid, ellagitannins and oligomeric anthocyanin and procyanidin. Int J Oncol 10:367–373
8. Clifford MN, Scalbert A (2000) Elagitannins–nature, occurrence and dietary burden. J Sci Food Agric 80:118–1125
9. Doyle B, Griffiths LA (1980) The metabolism of ellagic acid in the rat. Xenobiotica 10:247–256
10. Smart RC, Huant MT, Chang RL, Sayer JM, Jerina DM, Conney AH (1986) Disposition of the naturally occurring antimutagenic plant phenol, ellagic acid, and its synthetic derivatives 3-O-decylellagic acid and 3,3'-di-O-methylellagic acid in mice. Carcinogenesis 7:1663–1667
11. Teel RW, Martin RM (1988) Disposition of the plant phenol ellagic acid in the mouse following oral administration by gavage. Xenobiotica 18:397–405
12. Jeong SJ, Kim NY, Kim DH, Kang TH, Ahn NH, Miyamoto T, Higuchi R, Kim YC (2000) Hyaluronidase inhibitory active 6H-dibenzo[b,d]pyran-6-ones from the faeces of Trogopterus xanthippes. Planta Med 66:76–77
13. Doig AJ, Williams DH, Oelrichs PB, Baczynskyj L (1990) Isolation and structure elucidation of punicalagin, a toxic hydrolysable tannin from Terminalia oblongata. J Chem Soc Perkin Trans 1:2317–2322
14. Gil-Izquierdo A, Zafrilla P, Tomás-Barberán FA (2002) An in vitro method to simulate phenolic compound release from the food matrix in the gastrointestinal tract. Eur Food Res Technol 214:155–159
15. Alo BI, Kandil A, Patil PA, Sharp MJ, MA, Shinekus VJ (1991) Sequential directed ortho methylation-boronic acid cross-coupling reactions. A general regiospecific route to oxygenated dibenzo[b,d]pyran-6-ones related to ellagic acid. J Org Chem 56:3763–3768
16. Walle T, Walle UK, Halushka PV (2001) Carbon dioxide is the major metabolite of quercetin in humans. J Nutr 131: 2648–2652
17. Kessler FJ, Blumenberg FW (1961) Die Aufspaltung des Gallotannins in Darm. Arzneimittel Forsch 11:286–288