Journal of Molecular Structure 1156 (2018) 390–396

Contents lists available at ScienceDirect

Journal of Molecular Structure

journal homepage: http://www.elsevier.com/locate/molstruc

Isolation, characterization, antioxidant activity, and protein-precipitating capacity of the hydrolyzable tannin punicalagin from pomegranate yellow peel (*Punica granatum*)

Boualem Oudane [a], Djalila Boudemagh [a, b, *], Mahmoud Bounekhel [a], Widad Sobhi [c], Michel Vidal [d, e], Sylvain Broussy [d, **]

[a] Laboratoire des Matériaux Polymériques Multiphasiques (LMPMP), Département de Génie des Procédés, Faculté de Technologie, Université Ferhat Abbas, Sétif 19000, Algeria
[b] Unité de Recherche Sciences des Matériaux et Applications, Université Constantine 1, Constantine 25000, Algeria
[c] Laboratoire de Biochimie Appliquée, Faculté des Sciences de la Nature et de la Vie, Université Ferhat Abbas Sétif 1, Sétif 19000, Algeria
[d] UMR 8638 CNRS Université Paris Descartes, Faculté de Pharmacie de Paris, Sorbonne Paris Cité, 4 Avenue de l'Observatoire, Paris 75006 France
[e] UF Pharmacocinétique et Pharmacochimie, Hôpital Cochin, AP-HP, 27 Rue du Faubourg Saint Jacques, Paris 75014, France

ARTICLE INFO

Article history:
Received 6 August 2017
Received in revised form
29 November 2017
Accepted 29 November 2017
Available online 30 November 2017

Keywords:
Punicalagin
Pomegranate yellow peel
HPLC
Antioxidant activity
Protein precipitating capacity

ABSTRACT

This work describes the isolation and characterization of the hydrolysable tannin punicalagin, obtained from the yellow peel of pomegranate (*Punica granatum*, belonging to the family *Lythraceae*). The natural product was present as a mixture of α and β anomers, rapidly interconverting under acidic pH conditions. A fast and efficient purification method was established using semi-preparative high performance liquid chromatography (HPLC). The chemical structure of the molecule was confirmed as punicalagin based on IR spectroscopy, ^{1}H and ^{13}C NMR, and MALDI-TOF mass spectrometry studies. The lowest energy conformations of the α and β anomers were calculated by quantum chemical methods, and their ratio compared to the experimental value (experimental: α/β = 2.2 to 2.5; calculated α/β = 2.4, in aqueous solution at acidic pH). The antioxidant activity of pure punicalagin was determined with a DPPH radical scavenging assay (IC_{50} = 1.9 ± 0.2 μg/mL) and was comparable to that of tannic acid (IC_{50} = 1.3 ± 0.2 μg/mL). Finally, we demonstrated that punicalagin, when used above a threshold concentration, was able to precipitate bovine serum albumin (BSA), although less efficiently than tannic acid.

1. Introduction

The peel of pomegranate is a rich source of ellagitannins including punicalagin [2, 3-(*S*) hexahydroxydiphenoyl-4, 6-(*S*, *S*)-gallagyl-D-glucose], as well as lower amounts of punicalin [4, 6-(*S*, *S*)-gallagyl-D-glucose]), gallic acid (GA), ellagic acid (EA) and EA-glycosides (hexoside, pentoside, rhamnoside). Punicalagin is the first most abundant component in pomegranate peel with high molecular weight, and it was considered as a characteristic compound of this part of the fruit [1–3]. Punicalagin is a water-soluble ellagitannins, found naturally under two anomeric structures α and β (Fig. 1). It is listed into the chemical class of hydrolyzable tannins [4,5]. Various potential therapeutic applications of punicalagin have been proposed, resulting mostly from its high antioxidant capacity, owing to the presence of sixteen phenolic hydroxyl groups in its structure [1,2,4–9]. In particular, anti-proliferative [10], anti-inflammatory [11,12], hepatoprotective [13], and antigenotoxic [14] activities have been reported. These biological activities could also be explained in part by the astringent properties of tannins, which interact with proteins and alter their functions [8,15–19]. Various chromatographic methodologies have been described for the isolation of punicalagin from the crude extract of pomegranate [2,9,10,20–22]. Most of them needed pre-purification steps and column chromatography with different stationary phases and solvent systems (C18, polyamides, cellulose, Sephadex LH-20 lipophilic, Diaion HP20 resin, silica gel, Amberlite XAD-16). Some of them, like preparative HPLC [21] and high speed counter current

* Corresponding author. Laboratoire des Matériaux Polymériques multiphasiques (LMPMP), Département de Génie des Procédés, Faculté de Technologie, Université Ferhat Abbas, Sétif 19000, Algeria.
** Corresponding author.
E-mail addresses: djalila.boudemagh@gmail.com (D. Boudemagh), sylvain.broussy@parisdescartes.fr (S. Broussy).

https://doi.org/10.1016/j.molstruc.2017.11.129
0022-2860/

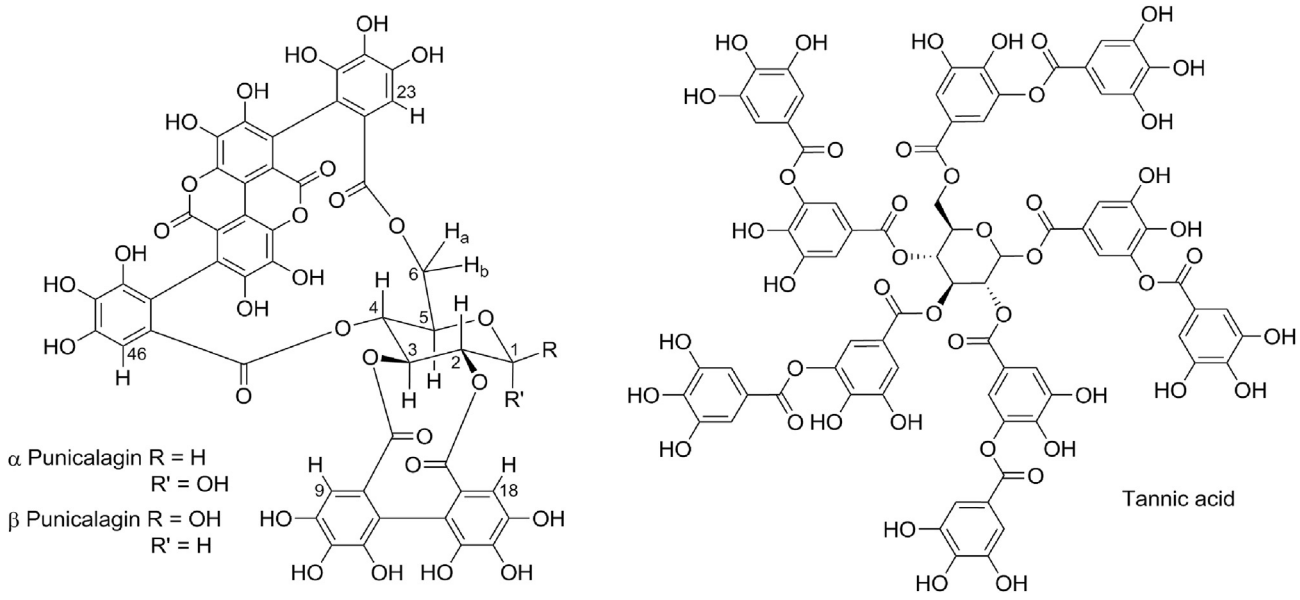

Fig. 1. Structures of punicalagin and tannic acid.

chromatography (HSCCC) [22], exhibited higher handling capacity for large scale production. However, purities greater than 99% have rarely been achieved with the above-mentioned methods.

The aims of our work were (i) to develop an efficient method for the isolation of punicalagin from the yellow peel of pomegranate in multi-milligram quantities and very high purity, (ii) to confirm its structure by spectroscopic methods and to study its conformations and (iii) to compare its antioxidant activity and protein-precipitating capacity with a commercially available hydrolyzable tannin (tannic acid, Fig. 1), and to explain the observed differences based on structural and conformational information. Therefore, the present article describes the purification of punicalagin in one step from a crude extract of the yellow peel of pomegranate, using semi-preparative HPLC. Different techniques, including MALDI-mass spectrometry, ^{1}H and ^{13}C NMR, and IR spectroscopy, were employed to confirm the chemical structure of isolated punicalagin, as an equilibrating mixture of α and β anomers in aqueous solution. Quantum chemical calculations were carried out to determine the lowest energy conformations and to calculate the ratio of these two anomers in aqueous solution. Moreover, the antioxidant activity of isolated punicalagin was evaluated in an assay based on DPPH radical scavenging [23,24]. Another assay was used to quantify the amount of punicalagin precipitated by a standard protein, bovine serum albumin (BSA) [25]. Both the antioxidant activity and the protein precipitating-capacity of isolated punicalagin were compared to those of tannic acid under the same assay conditions.

2. Experimental

2.1. Plant materials

The pomegranates (*Punica granatum*) were collected from the east of Setif, Algeria, during the months of October and November 2014. They were identified and authenticated. The yellow peels were separated manually from the fruit and air-dried at room temperature for 60 days, in the dark to prevent photo-oxidation. All samples were then grounded using a coffee grinder.

2.2. General experimental procedures

Tannic acid, sodium dodecyl sulfate, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), α-cyano-4-hydroxycinnamic acid, and tri-ethanolamine were purchased from Sigma-Aldrich Ltd. Bovine serum albumin Fraction V (BSA) was purchased from Eurobio (France). Analytical grade methanol, ethanol, trifluoro-acetic acid (TFA), and HPLC grade acetonitrile were supplied from Carlo Erba (France).

For MALDI mass spectrometry (MS) analysis, a mixture of 2 μL matrix solution (α-cyano-4-hydroxycinnamic acid, in 50% acetonitrile aqueous solution) and 2 μL compound (approximately 50 μM in acetonitrile aqueous solution) was loaded on a 96-well plate. After evaporation of solvents, the sample was analyzed on a Voyager-DE™ PRO Biospectrometry™ Workstation, PerSeptive Biosystems. The spectrum was recorded over a mass range from 660 to 1500 Da, in negative ion mode. ^{1}H and ^{13}C NMR spectra were recorded in D_2O on Bruker Avance NMR spectrometers operating at 400 and 600 MHz (^{1}H frequencies). A sample of 10 mg punicalagin was dissolved in 500 μL of D_2O. All spectra were referenced relative to residual solvent peak. The IR spectrum of solid punicalagin was recorded on a Perkin-Elmer Spectrum 65 FT-IR spectrometer. The software Spectrum was used for the acquisition and analysis of data. The semi-preparative HPLC was performed with a Shimadzu Prominence HPLC system (SPD-20AV detector) equipped with a reversed-phase C18 column (250 mm × 10 mm, 5 μm, Grace-Vydac) and the analytical HPLC was performed with a Shimadzu Prominence HPLC system (SPD-20A detector) equipped with an analytical reversed-phase C18 column (250 mm × 4.6 mm, 5 μm, Phenomenex).

2.3. Preparation of the extract and isolation of punicalagin

Crude extract of *Punica granatum* was prepared from the ground sample according to a previously described method [26] with slight modifications. Approximately 20 g of the ground sample were extracted with 200 mL of methanol using a homogenizer at room

temperature for 24 h. During extraction, the sample container was protected from light in order to prevent any photo-oxidation of the extract. The supernatant was collected by filtration through a filter paper (particle retention 5–13 μm), and the methanol was evaporated under reduced pressure on a rotary evaporator at temperatures below 30 °C. The resulting extract was then dissolved in 300 mL of water. After centrifugation (5 min, 2000 rpm 670 g) to remove the pellet, the extract was filtered through a 0.45 μm syringe filter. The final extract was then lyophilized, yielding 9.2 g of a fluffy brown powder. The punicalagin was purified by semi-preparative HPLC: 100 mg of the crude extract were dissolved in 1 mL of water and injected into the column equilibrated with initial chromatographic conditions. The mobile phase was a linear gradient of eluent A (0.1% TFA aqueous) and eluent B (0.09% TFA in 70% aqueous acetonitrile). The gradient elution procedure was as follows: 0–40 min, 0–50% B; 40–42 min, 50–100% B; 42–47 min, 100% B; 47–50 min, 100-0% B. The flow rate was 2.0 mL/min and the effluent was monitored by UV detection at 254 nm. All peak fractions of punicalagin were manually collected according to the chromatogram and were analyzed by analytical HPLC with the following gradient elution procedure: 0–30 min, 0–50% B; 30–35 min, 50–100% B; 35–40 min, 100-0% B; followed by a 10 min re-equilibration before the next injection. The flow rate was 1.0 mL/min, and 15 μL samples were injected. The effluent was monitored by UV detection at 254 nm. The fractions which showed the presence of pure punicalagin were collected and lyophilized to give 23 mg (23% yield) of faint yellow to dark yellow powder of punicalagin.

2.4. DPPH radical scavenging activity

The DPPH radical scavenging activity of isolated punicalagin and tannic acid were assayed following a previously described method with recommendations given in Ref. [24]. The DPPH radical stock solution (100 μg/mL, 254 μM) was prepared in ethanol and the natural products stock solutions were prepared in water (1 mg/mL). The reactions were carried out in the dark at 25 °C in 1 mL final volume (900 μL ethanol + 100 μL water), containing 20 μg DPPH radical (51 μM) and 0–100 μg natural product. The absorbance was read at 517 nm after 30 min incubation. The absorbance of control experiments without natural product was used to calculate the % inhibition.

2.5. Evaluation of protein precipitating capacity

The BSA solution (1 mg/mL) was prepared in 0.2 M acetate buffer containing 0.17 M NaCl, and the pH was adjusted to 4.9 with NaOH. Solutions of tannic acid and punicalagin (ranging from 0.10 to 1.0 mg) were prepared in distilled water. The capacities of tannic acid and punicalagin to form an insoluble complex with BSA were assayed according to the protein precipitation method of Hagerman and Butler [25]. Briefly, after precipitation and isolation of the tannin-BSA complex, the precipitate was dissolved in a SDS/triethanolamine solution and then reacted with $FeCl_3$. The complex formed between tannin and Fe^{3+} was measured by UV absorption at $\lambda = 510$ nm.

3. Computational details

A conformational search on α and β anomers of punicalagin was carried out with the algorithm BEST in the program Catalyst [27] implemented in Discovery Studio [28]. The lowest energy conformer was selected for further *ab initio* geometry optimization (in water with the PCM solvation model) at the indicated level of theory in GAMESS [29]. Default parameters were chosen, except for OPTTOL = 5.10^{-4} (for all levels) and QMTTOL = 10^{-4} (for the HF/6-31 + G(d) level only). IR frequencies were calculated at the HF/6-31G level to confirm the identification of an energy minimum (no imaginary frequencies). The thermal Gibbs free energies corrections were also computed at 298.15 K at the HF/6-31G level. They were found very close for the two anomers (relative energies of 0.112 kcal/mol) and were neglected for the calculations of anomer populations. Frequency calculations and thermochemical analysis were not achieved for higher levels of theory because of the high computational cost. The anomer populations were calculated from the total free energy in water (sum of electronic energy and solvation energy) using the Boltzmann equation. Graphical representations were done with Discovery Studio.

4. Results and discussion

4.1. Isolation of punicalagin

The pomegranate peel polyphenols are mainly extracted by methanol or combinations of methanol with other organic solvents by conventional extraction techniques [26]. In our procedure, the dry powder was first extracted with methanol. The yield of the extract was high (50%), probably due to the recovery of both low and high molecular weight compounds. Then, it was solubilized in water and centrifuged, in order to isolate the more polar compounds (hydrolyzable tannins) and to remove the insoluble, less polar compounds. After lyophilization, the crude extract of pomegranate yellow peel was analyzed by HPLC. It contained several compounds (Fig. 2, top). The punicalagin content of the crude extract was 59% based on the ratio of peak areas at 254 nm. The retention times of α and β punicalagin were 20.82 and 19.33 min, respectively.

We then used semi-preparative HPLC to purify the crude extract. Punicalagin was isolated in very high purity (>99% based on HPLC analysis at 254 nm, Fig. 2, bottom) by a single column chromatography. Moreover, the yield of the isolated punicalagin was 23% from the methanol extract, on a 100 mg scale. Therefore, our method is convenient to obtain rapidly multi-milligram quantities of pure compound.

4.2. Identification of purified punicalagin, structural confirmation and conformational study by quantum chemical calculations

The structure of the isolated punicalagin was determined by various spectroscopic techniques. IR data exhibited stretching vibrations at 3380 cm^{-1} (OH) and 1717 cm^{-1} (CO) absorption bands, besides other stretching at 1319 and 1057 cm^{-1}, among other signals. These values were in good agreement with previously published data [30]. The MALDI-MS spectrum of the compound confirmed the presence of punicalagin, with an *m/z* peak at 1084 (Fig. 3). Another *m/z* peak at 800 indicated the subsequent fragment ion of punicalin. The presence of punicalin in the spectrum was due to punicalagin degradation under the high energy conditions used in the analysis. This is consistent with the published literature [20], which showed that MS analysis of punicalagin resulted in partial conversion into punicalin (M-H^- at *m/z* 799). Therefore, the MS analysis of the isolated punicalagin confirmed its high purity.

In addition, ^1H NMR and ^{13}C NMR analyses of the isolated punicalagin matched the previously reported spectral data acquired in D_2O solutions at acidic pH [31]. ^1H and NOESY spectra obtained in our study are shown in the Supporting information. The NOESY spectrum was similar to the ROESY spectrum, which was also recorded.

We carried out computational studies on punicalagin α and β

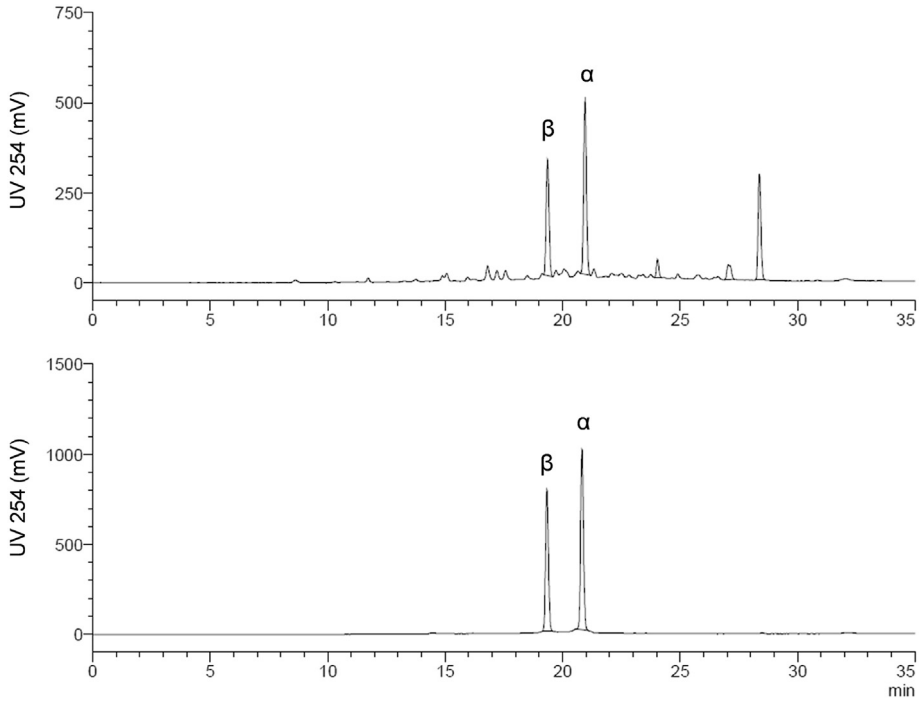

Fig. 2. Analytical HPLC chromatogram of the crude extract of pomegranate yellow peel (top) and of pure punicalagin anomers (bottom) isolated from the crude extract, after purification by semi-preparative HPLC. Peaks of the α and β anomers of punicalagin are indicated, and were identified by comparison of their retention times with a commercial standard of punicalagin anomers.

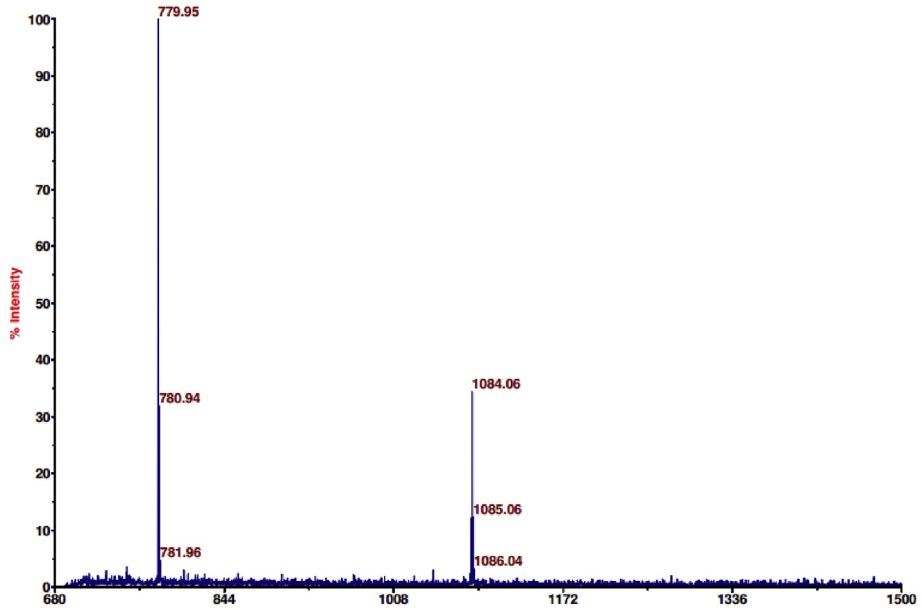

Fig. 3. MALDI mass spectrum, in the negative ionization mode, of purified punicalagin, at $m/z = 1084$ (molecular ion). The most intense peak at $m/z = 800$ corresponds to the subsequent fragment ion of punicalin.

anomers to identify their lowest energy conformers. The NMR data was used to validate each step. An initial set of conformations was generated with the algorithm BEST in the program Catalyst [27]. Unlike tannic acid, punicalagin is a very rigid molecule, with very few rotatable bonds, and it is therefore a good candidate for conformational studies. The lowest energy conformers obtained had the glucose in a chair conformation, in agreement with ^{1}H NMR coupling constants. Moreover, these conformers matched the NOE correlations (H_5/H_{6b} and H_4/H_{6a} for each anomer, see the Supporting information). Also, the large upfield shift of H_{6b} compared to H_{6a} could be explained by the proximity of the ellagic acid. The α and β punicalagin anomer conformations were subsequently optimized by *ab initio* quantum chemical calculations, starting at the HF/6-31G level of theory. The population of each anomer was calculated from their energy difference. A better agreement with experimental results was obtained by increasing

Table 1
Calculated total (E) and relative energies (ΔE) and corresponding proportions of α and β anomers of punicalagin in water (PCM solvation model) at the indicated level of theory.

Punicalagin anomer	E (Hartrees)	ΔE (kcal/mol)	Population %
HF/6-31G			
α	−4078.20995	0	97.2
β	−4078.20589	2.55	2.8
HF/6-31G(d)			
α	−4079.93381	0	88.2
β	−4079.93191	1.19	11.8
HF/6-31 + G(d)			
α	−4080.04553	0	71.0
β	−4080.04468	0.53	29.0

the basis set size to 6-31 + G(d), as summarized in Table 1. The ratio α/β reached 2.4, in the range of ratios observed at acidic pH: 2.2 to 2.5 (reference [31] and our results). This computational study concerned only one conformer of each anomer and was achieved at a relatively low level of theory. However, the good agreement with experimental results (in particular NOESY NMR and equilibrium constant) indicate that the optimized conformations displayed in Fig. 4 are likely among the lowest energy conformers on the potential energy surface.

4.3. DPPH radical scavenging activity and protein precipitating capacity of tannic acid and punicalagin

The interesting biological properties of punicalagin have been mainly explained by its antioxidant activity. Therefore, we assayed the antioxidant activity of our isolated punicalagin and compared it with previously published results. The DPPH radical is intensely colored and is sufficiently stable (at room temperature and in the dark) to carry out colorimetric radical scavenging experiments, and thereby to quantify the antioxidant activity of compounds [23,24]. Isolated punicalagin and commercial tannic acid were assayed in ethanol. Kinetic measurements (up to 60 min) were initially carried out, indicating that the reactions were essentially finished after 30 min, even for the lowest concentrations of natural products. Therefore, a 30 min incubation time was chosen. For 20 μg/mL (51 μM) solutions of DPPH radical, almost complete radical scavenging was achieved for both compounds above 5 μg/mL

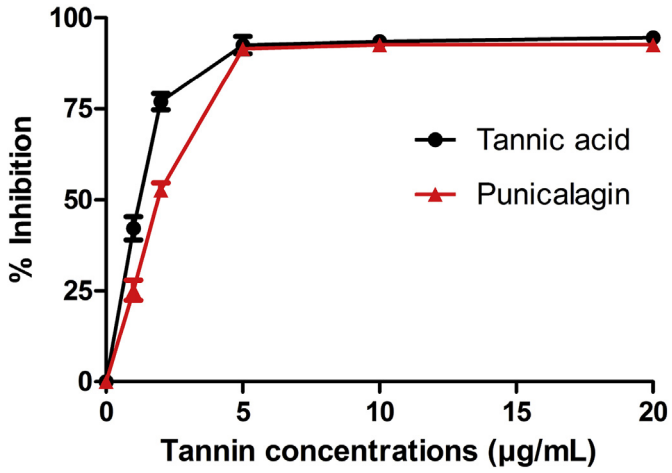

Fig. 5. DPPH radical scavenging activity of punicalagin and tannic acid. The error bars are standard deviations from triplicate experiments.

concentrations. The IC_{50} values were 1.9 ± 0.2 μg/mL (approximately 2 μM) for punicalagin and 1.3 ± 0.2 μg/mL (approximately 1 μM) for tannic acid (Fig. 5). A previous report on tannic acid indicated a strong quenching activity at 15 μg/mL, but the IC_{50} was not measured and the assay conditions were different (for example the initial absorbance value at 517 nm, A_{517}, was higher than 2, whereas it was kept at 0.5 in our work) [32]. Previous studies on punicalagin reported IC_{50} values of 16.7 μg/mL [9], 17.1 μg/mL [10], 26 μg/mL [33] and 109.53 μg/mL [34]. The assay conditions were different, which may explain the differences in IC_{50} values (use of buffered methanol at pH 7.4 or high initial concentrations of DPPH radical for example). The last value of 109.53 μg/mL corresponded to an IC_{50} of approximately 100 μM, and seemed very high to scavenge 50% of a DPPH radical solution at 60 μM. Nevertheless, we demonstrated herein that both punicalagin and tannic acid are very efficient antioxidant compounds in ethanol solutions.

The tannin content of a sample is often determined by a precipitation assay, which is based on the capacity of tannins to interact with proteins in a manner that results in the formation of insoluble aggregates [25]. Few studies describe the interaction of

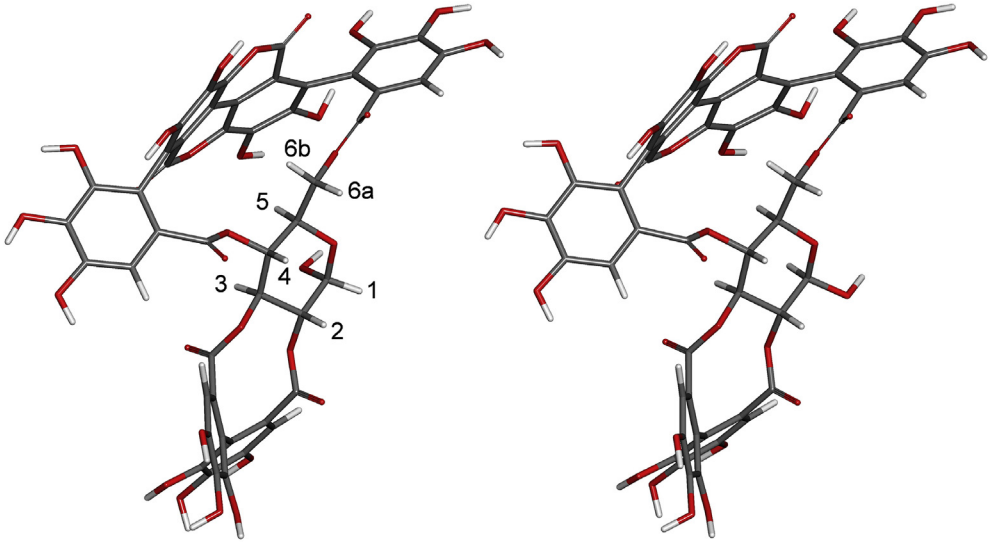

Fig. 4. Lowest energy conformations of α (left) and β (right) anomers of punicalagin. Geometries were optimized *ab initio* at the HF/6-31 + G(d) level of theory.

punicalagin with proteins. The binding interaction (without precipitation) of punicalagin with BSA [8] and with salivary proteins [16] was studied by fluorescence and STD-NMR. The ability of the natural product to actually precipitate BSA [33] and salivary proteins [19] was also studied recently. Interestingly, wine polysaccharides were able to disrupt the interaction between punicalagin and salivary proteins [19]. An ED_{50} of 0.31 μmol (0.34 mg) was measured for the interaction between punicalagin and BSA (this value is indicative, but will strongly depend on the assay conditions used) [33].

We studied the BSA-precipitating capacity of punicalagin and tannic acid following a previously described procedure based on UV absorbance of an iron-tannin complex [25]. Our results confirmed that punicalagin has the ability to precipitate BSA. We also found that the relationship between the quantity of precipitate (proportional to the absorbance values measured) and the tannin concentrations was nearly linear over the range from 0.1 to 1.0 mg tannin (correlation coefficients are given in Fig. 6). Moreover, compared to tannic acid, punicalagin has a lower BSA precipitation capacity (Fig. 6). This observation can be explained by the structural differences existing between punicalagin and tannic acid. Indeed, the size, conformational flexibility, and number of binding sites (hydrophobic and hydrogen bonding sites) of the tannin molecules have a large influence on their protein-precipitation capacities [35,36]. Tannic acid presents more phenolic hydroxyl groups and ester bonds than punicalagin, and therefore more potential binding sites to the protein (Fig. 1). It is also much more flexible than punicalagin, which could favor binding to multiple proteins and their subsequent precipitation. The standard curves for commercial tannic acid and punicalagin did not pass through the origin. The y intercept were - 0.14 and - 0.08 respectively, which suggests that there exist a threshold level required for BSA precipitation. This observation is consistent with previous reports on hydrolyzable tannins [25,37,38].

5. Conclusions

In this study, we have established a rapid method for the isolation of pure punicalagin (α and β anomers) from yellow peel of pomegranate fruit with high yields. The spectroscopic characterization was achieved by mass spectrometry, IR and NMR, and matched previously reported results, confirming the identity of the natural product. In addition, we carried out a conformational search followed by quantum chemical calculations to identify low energy conformers of each punicalagin anomer. These conformer structures were in good agreement with the NMR data.

Punicalagin can be used in appropriate *in vitro* models for health related studies. Here, the antioxidant activity and BSA-precipitating capacity of isolated punicalagin was confirmed. Through comparison with commercial tannic acid under the same assay conditions, we showed that punicalagin had a similar antioxidant activity, but a lower ability to precipitate BSA. Our studies suggest that the difference between the chemical structures of two compounds was responsible for their different interaction with the protein.

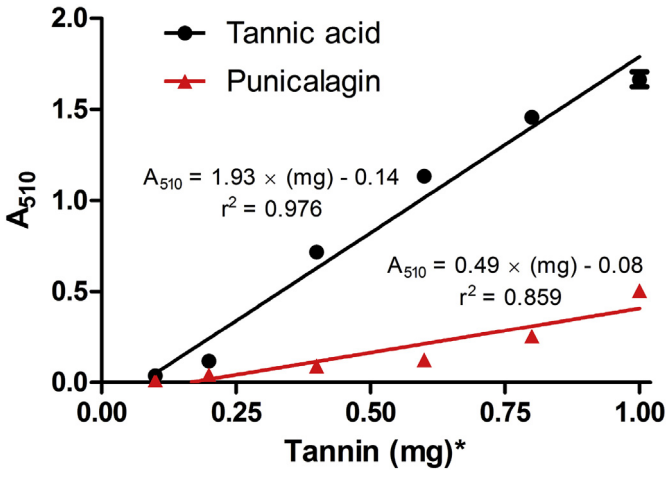

Fig. 6. BSA precipitation capacity of punicalagin and tannic acid. *The assay procedure was the same as described in method [25]. The error bars are standard deviations from triplicate experiments.

Acknowledgments

We thank the French CNRS and the Paris Descartes University. We thank Pr. O. Laprévote (Paris Descartes University) for the use of the MALDI mass spectrometer.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.molstruc.2017.11.129.

References

[1] M.I. Gil, F.A. Tomás-Barberán, B. Hess-Pierce, D.M. Holcroft, A.A. Kader, Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing, J. Agric. Food Chem. 48 (2000) 4581–4589.
[2] B. Cerdá, J.J. Cerón, F.A. Tomás-Barberán, J.C. Espín, Repeated oral administration of high doses of the pomegranate ellagitannin punicalagin to rats for 37 days is not toxic, J. Agric. Food Chem. 51 (2003) 3493–3501.
[3] B. Cerdá, R. Llorach, J.J. Cerón, J.C. Espín, F.A. Tomás-Barberán, Evaluation of the bioavailability and metabolism in the rat of punicalagin, an antioxidant polyphenol from pomegranate juice, Eur. J. Nutr. 42 (2003) 18–28.
[4] N.P. Seeram, Y. Zhang, J.D. Reed, C.G. Krueger, J. Vaya, Pomegranates: Ancient Roots to Modern Medicine, CRC Press, Taylor and Francis Group, Boca Raton, FL, 2006.
[5] E. Bakkalbaşi, Ö. Menteş, N. Artik, Food ellagitannins - occurrence, effects of processing and storage, Crit. Rev. Food Sci. Nutr. 49 (2009) 283–298.
[6] J.M. Landete, Ellagitannins, ellagic acid and their derived metabolites: a review about source, metabolism, functions and health, Food Res. Int. 44 (2011) 1150–1160.
[7] M.C. Nicoli, M. Anese, M. Parpinel, Influence of processing on the antioxidant properties of fruit and vegetables, Trends Food Sci. Technol. 10 (1999) 94–100.
[8] A.P. Kulkarni, H.S. Mahal, S. Kapoor, S.M. Aradhya, In vitro studies on the binding, antioxidant, and cytotoxic actions of punicalagin, J. Agric. Food Chem. 55 (2007) 1491–1500.
[9] A.P. Kulkarni, S.M. Aradhya, S. Divakar, Isolation and identification of a radical scavenging antioxidant – punicalagin from pith and carpellary membrane of pomegranate fruit, Food Chem. 87 (2004) 551–557.
[10] F. Aqil, R. Munagala, M.V. Vadhanam, H. Kausar, J. Jeyabalan, D.J. Schultz, R.C. Gupta, Anti-proliferative activity and protection against oxidative DNA damage by punicalagin isolated from pomegranate husk, Food Res. Int. 49 (2012) 345–353.
[11] L.S. Adams, N.P. Seeram, B.B. Aggarwal, Y. Takada, D. Sand, D. Heber, Pomegranate juice, total pomegranate ellagitannins, and punicalagin suppress inflammatory cell signaling in colon cancer cells, J. Agric. Food Chem. 54 (2006) 980–985.
[12] C.-C. Lin, Y.-F. Hsu, T.-C. Lin, Effects of punicalagin and punicalin on carrageenan-induced inflammation in rats, Am. J. Chin. Med. 27 (1999) 371–376.
[13] C.-C. Lin, Y.-F. Hsu, T.-C. Lin, H.-Y. Hsu, Antioxidant and hepatoprotective effects of punicalagin and punicalin on acetaminophen-induced liver damage in rats, Phytother. Res. 15 (2001) 206–212.
[14] P.-S. Chen, J.-H. Li, T.-Y. Liu, T.-C. Lin, Folk medicine *Terminalia catappa* and its major tannin component, punicalagin, are effective against bleomycin-induced genotoxicity in Chinese hamster ovary cells, Cancer Lett. 152 (2000) 115–122.
[15] H.I. Oh, J.E. Hoff, Effect of condensed grape tannins on the in vitro activity of digestive proteases and activation of their zymogens, J. Food Sci. 51 (1986) 577–580.
[16] M.S. Silva, I. García-Estevez, E. Brandão, N. Mateus, V. de Freitas, S. Soares, Molecular interaction between salivary proteins and food tannins, J. Agric. Food Chem. 65 (2017) 6415–6424.
[17] V. de Freitas, N. Mateus, Protein/polyphenol interactions: past and present contributions. Mechanisms of astringency perception, Curr. Org. Chem. 16

(2012) 724–746.
[18] R. Dias, R. Perez-Gregorio, N. Mateus, V. de Freitas, The interaction between tannins and gliadin derived peptides in a celiac disease perspective, RSC Adv. 5 (2015) 32151–32158.
[19] E. Brandão, M.S. Silva, I. García-Estévez, P. Williams, N. Mateus, T. Doco, V. de Freitas, S. Soares, The role of wine polysaccharides on salivary protein-tannin interaction: a molecular approach, Carbohydr. Polym. 177 (2017) 77–85.
[20] N. Seeram, R. Lee, M. Hardy, D. Heber, Rapid large scale purification of ellagitannins from pomegranate husk, a by-product of the commercial juice industry, Sep. Purif. Technol. 41 (2005) 49–55.
[21] J. Lu, K. Ding, Q. Yuan, One-step purification of punicalagin by preparative HPLC and stability study on punicalagin, Sep. Sci. Technol. 46 (2010) 147–154.
[22] J. Lu, Y. Wei, Q. Yuan, Preparative separation of punicalagin from pomegranate husk by high-speed countercurrent chromatography, J. Chromatogr. B 857 (2007) 175–179.
[23] M.S. Blois, Antioxidant determinations by the use of a stable free radical, Nature 181 (1958) 1199–1200.
[24] O.P. Sharma, T.K. Bhat, DPPH antioxidant assay revisited, Food Chem. 113 (2009) 1202–1205.
[25] A.E. Hagerman, L.G. Butler, Protein precipitation method for the quantitative determination of tannins, J. Agric. Food Chem. 26 (1978) 809–812.
[26] M. Çam, Y. Hişil, Pressurised water extraction of polyphenols from pomegranate peels, Food Chem. 123 (2010) 878–885.
[27] I.-J. Chen, N. Foloppe, Conformational sampling of druglike molecules with MOE and Catalyst: implications for pharmacophore modeling and virtual screening, J. Chem. Inf. Model. 48 (2008) 1773–1791.
[28] Discovery Studio BIOVIA, Discovery Studio Client V17, Dassault Systèmes, San Diego, 2017.
[29] M.W. Schmidt, K.K. Baldridge, J.A. Boatz, S.T. Elbert, M.S. Gordon, J.H. Jensen, S. Koseki, N. Matsunaga, K.A. Nguyen, S. Su, T.L. Windus, M. Dupuis, J.A. Montgomery, General atomic and molecular electronic structure system, J. Comput. Chem. 14 (1993) 1347–1363.
[30] A.J. Doig, D.H. Williams, P.B. Oelrichs, L. Baczynskyj, Isolation and structure elucidation of punicalagin, a toxic hydrolysable tannin, from Terminalia oblongata, J. Chem. Soc. Perkin Trans. 1 (1990) 2317–2321.
[31] M. Kraszni, A. Marosi, C.K. Larive, NMR assignments and the acid–base characterization of the pomegranate ellagitannin punicalagin in the acidic pH-range, Anal. Bioanal. Chem. 405 (2013) 5807–5816.
[32] İ. Gülçin, Z. Huyut, M. Elmastaş, H.Y. Aboul-Enein, Radical scavenging and antioxidant activity of tannic acid, Arab. J. Chem. 3 (2010) 43–53.
[33] Y. Wang, H. Zhang, H. Liang, Q. Yuan, Purification, antioxidant activity and protein-precipitating capacity of punicalin from pomegranate husk, Food Chem. 138 (2013) 437–443.
[34] P. Aguilar-Zárate, J.E. Wong-Paz, M. Michel, J. Buenrostro-Figueroa, H.R. Díaz, J.A. Ascacio, J.C. Contreras-Esquivel, G. Gutiérrez-Sánchez, C.N. Aguilar, Characterisation of pomegranate-husk polyphenols and semi-preparative fractionation of punicalagin, Phytochem. Anal. 28 (2017) 433–438.
[35] E.R. Deaville, R.J. Green, I. Mueller-Harvey, I. Willoughby, R.A. Frazier, Hydrolyzable tannin structures influence relative globular and random coil protein binding strengths, J. Agric. Food Chem. 55 (2007) 4554–4561.
[36] M. Naczk, S. Grant, R. Zadernowski, E. Barre, Protein precipitating capacity of phenolics of wild blueberry leaves and fruits, Food Chem. 96 (2006) 640–647.
[37] J.C. Schultz, I.T. Baldwin, P.J. Nothnagle, Hemoglobin as a binding substrate in the quantitative analysis of plant tannins, J. Agric. Food Chem. 29 (1981) 823–826.
[38] H.P.S. Makkar, R.K. Dawra, B. Singh, Protein precipitation assay for quantitation of tannins: determination of protein in tannin-protein complex, Anal. Biochem. 166 (1987) 435–439.