

## Urolithins, Intestinal Microbial Metabolites of Pomegranate Ellagitannins, Exhibit Potent Antioxidant Activity in a Cell-Based Assay

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Many health benefits of pomegranate products have been attributed to the potent antioxidant action of their tannin components, mainly punicalagins and ellagic acid. While moving through the intestines, ellagitannins are metabolized by gut bacteria into urolithins that readily enter systemic circulation. In this study, the antioxidant properties of seven urolithin derivatives were evaluated in a cell-based assay. This method is biologically more relevant because it reflects bioavailability of the test compound to the cells, and the antioxidant action is determined in the cellular environment. Our results showed that the antioxidant activity of urolithins was correlated with the number of hydroxy groups as well as the lipophilicity of the molecule. The most potent antioxidants are urolithins C and D with IC<sub>50</sub> values of 0.16 and 0.33 μM, respectively, when compared to IC<sub>50</sub> values of 1.1 and 1.4 μM of the parent ellagic acid and punicalagins, respectively. The dihydroxylated urolithin A showed weaker antioxidant activity, with an IC<sub>50</sub> value 13.6 μM, however, the potency was within the range of urolithin A plasma concentrations. Therefore, products of the intestinal microbial transformation of pomegranate ellagitannins may account for systemic antioxidant effects.

**KEYWORDS:** *Punica granatum* L.; punicalagins; ellagic acid; urolithins; antioxidant activity; gut bacterial metabolites of polyphenols

### INTRODUCTION

Pomegranate products, including juice, tea, wine, and extracts, are widely consumed and recognized for their health benefits, including prevention of cancer and vascular diseases (1). In recent years, various health advantages of pomegranate have been attributed to the potent antioxidant activity of ellagitannins, mainly punicalagins, punicalins (we prefer to use the plurals punicalins and punicalagins, because these compounds exist in solution as the α- and β-anomers as well as the acyclic hydroxylaldehyde analog), and ellagic acid (Figure 1) (2, 3). In fact, increased antioxidant properties of plasma were found in human volunteers after consumption of pomegranate products (4). Although the punicalagins showed high antioxidant activity in in vitro assays (5), they were not detected in human systemic circulation after consumption of large amounts of pomegranate products (4, 6), and only trace amounts of ellagic acid have been found in human blood (7). Punicalagins undergo partial hydrolysis at the pH levels of the small intestine and under physiological conditions of human cell lines (8). The remaining ellagitannins and ellagic acid are retained unabsorbed in the gut lumen where they can interact with complex intestinal bacteria.

The contribution of human gut microbiota toward health improvement and genesis of various diseases has been widely recognized (9, 10). One important function of intestinal bacteria is the fermentation of undigested food components leading to the production of metabolites of different physiological significance. The products of microbial modifications can be potentially harmful, like in the case of metabolites produced by *Bacteroides* and *Clostridia* sp. (11). Other metabolites formed by gut bacteria may exhibit significant health beneficial actions, for example, the short chain fatty acids released by probiotic lactic bacteria (9). Human intestinal bacteria are able to metabolize dietary polyphenolic flavonoids by cleavage of the C-ring, hydroxylation, dehydroxylation, reduction of carbon–carbon double bonds, and lengthening or shortening of aliphatic chains (12). Therefore, by the formation of different phenolic acids and other aromatic derivatives, gut microbiota can modify the bioactivity of the original compounds. For instance, the isoflavone daidzein is transformed into equol, a bioavailable metabolite with potent estrogenic activity (13). In vitro fermentation of punicalagins and ellagic acid by human gut bacteria resulted in the formation of a dibenzopyranone urolithin A (Figure 2) (14). Urolithin A and related analogs (Figure 2) were also confirmed as intestinal microbial metabolites of dietary ellagitannins in animal studies (15, 16). Common bacterial metabolites of various ellagitannins indicate that ellagic acid, the product

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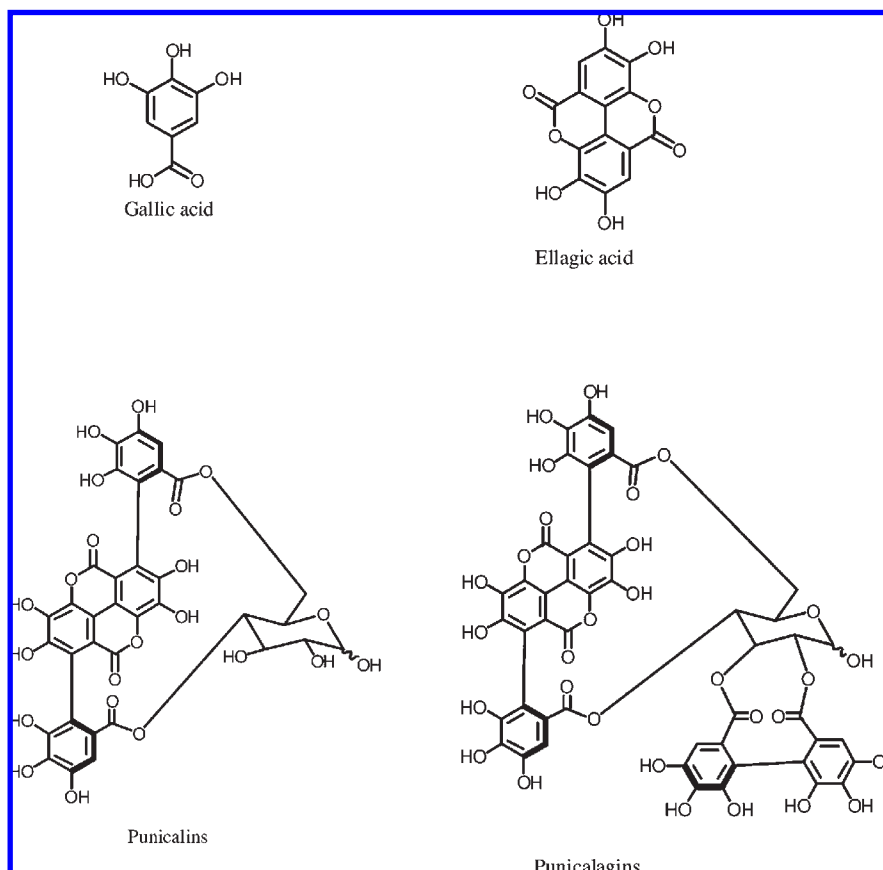


Figure 1. Chemical structures of pomegranate polyphenols.

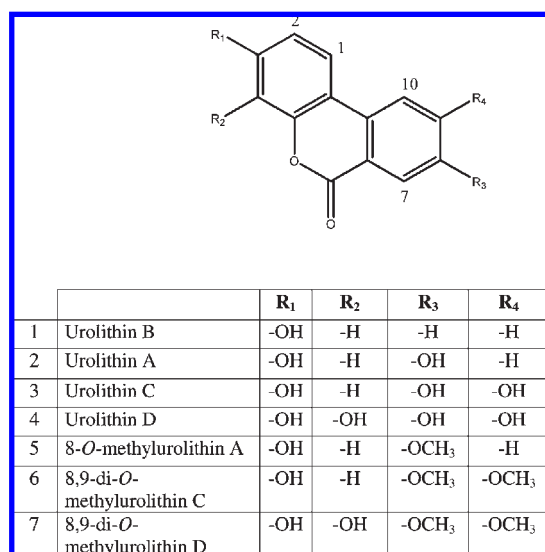


Figure 2. Chemical structure of urolithins.

of hydrolysis of ellagitannins, may serve as substrate in the formation of urolithins. A comprehensive investigation of the production and bioavailability of urolithins was performed using the Iberian Pig model (17).

Urolithins appear in human systemic circulation within a few hours of consumption of pomegranate products, reaching maximum concentrations between 24 and 48 h. They are present in the plasma and urine for up to 72 h, in free and conjugated forms (1, 4, 14). Therefore, microbial metabolites could account for the increased antioxidant properties of plasma in human

volunteers after consumption of pomegranate products (4). However, previous investigations reported urolithins as insignificant antioxidants compared to the original ellagitannins (15, 16).

In the present study, a cellular assay was applied for the first time to comprehensively evaluate the antioxidant potency of seven urolithins. This method reflects bioavailability of the test compounds to the cell and employs cellular enzymes to reveal the antioxidant action, hence better characterizing biological systems in action. Six of the urolithins were earlier described as bacterial metabolites of dietary ellagitannins (4, 6, 7, 16, 17). We also included an *O*-methylated model derivative, 8,9-di-*O*-methylurolithin D for a comprehensive evaluation of the role of phenolic hydroxy groups in the antioxidant activity.

## MATERIALS AND METHODS

**Chemicals.** Resorcinol, 2-bromobenzoic acid, 2-bromo-4,5-dimethoxybenzoic acid, and chlorobenzene were purchased from Sigma Aldrich, St. Louis, MO, 2-bromo-5-methoxybenzoic acid, from Alfa Aesar, Ward Hill, MA, pyrogallol from Acros Organics, and copper sulfate, sodium hydroxide, and aluminum chloride from Fisher Scientific, Pittsburgh, PA. HL-60 cells were from ATCC Manassas, VA. DCFH-DA was from Molecular Probes, Eugene, OR.

**Purification of Compounds.** The HPLC system consisted of a Waters Delta 600, Waters 600 controller, Waters 996 Photodiode Array Detector, and 10 × 250 mm column (Phenomenex, Prodigy ODS 10 μm C18). The purification was performed in the gradient system **A**, 2.5% HOAc, **B**, 2.5% HOAc in MeOH, starting from 100% **A** for 5 min, 0–60% **B** for 15 min, and 60–100% **B** for 15 min. The flow rate was 1 mL/min and the pressure 600–800 mmHg. The elution of metabolites was monitored at 254 nm.

**Identification of Compounds.** The molecular mass of compounds was confirmed using an LC-MS system consisting of a Waters Micromass ZQ mass spectrometer, Waters 2695 Separation Module, and Waters 996 Photodiode Array Detector. Mass spectra were recorded in the

negative mode. The capillary voltage was 4000/3500 V, gas temperature 300 °C, and a 3.0 × 150 mm analytical column (Phenomenex, Luna 5 μ C18 100 Å) was used. The analyses were performed in the gradient system A, 1% formic acid, B, 1% formic acid in MeOH, starting from 100% A for 5 min, 0–60% B for 15 min, and 60–100% B for 15 min. The flow rate was 0.3 mL/min and the pressure 900–1500 mmHg. The elution of metabolites was monitored at 254 nm.

**Synthesis of Urolithins.** The urolithins were synthesized by the condensation of either resorcinol or pyrogallol with the appropriately substituted benzoic acids, via modification of literature protocols (16, 18). The identity of urolithins was confirmed based on their molecular mass and <sup>1</sup>H NMR spectra.

**Urolithin B (3-Hydroxy-6H-dibenzo[b,d]pyran-6-one).** 2-Bromobenzoic acid (1 g), resorcinol (1.1 g), and NaOH (0.4 g) in water (5 mL) were heated under reflux for 30 min. After the addition of aqueous CuSO<sub>4</sub> (5%, 2 mL) the mixture was refluxed again for 10 min, during which time urolithin B (0.62 g) precipitated as a white powder. The precipitate was filtered and subjected to HPLC for purification.

**8-O-Methylurolithin A (3-Hydroxy-8-methoxy-6H-dibenzo[b,d]pyran-6-one).** 2-Bromo-5-methoxybenzoic acid (1 g), resorcinol (1 g), and NaOH (0.375 g) in water (4.5 mL) were heated under reflux for 30 min. After the addition of aqueous CuSO<sub>4</sub> (5%, 1.8 mL) the mixture was refluxed for a further 10 min, during which time 8-O-methylurolithin A (0.72 g) precipitated as a white powder. The precipitate was filtered and subjected to HPLC for purification.

**Urolithin A (3,8-Dihydroxy-6H-dibenzo[b,d]pyran-6-one).** A solution of 2-bromo-5-methoxybenzoic acid (2.5 g) and AlCl<sub>3</sub> (7.5 g) in chlorobenzene (75 mL) was refluxed for 2.5 h. After cooling, the mixture was added to ice and extracted with diethyl ether (3 × 125 mL). The ether was evaporated to yield 2-bromo-5-hydroxybenzoic acid (2 g). The 2-bromo-5-hydroxybenzoic acid (1.53 g), resorcinol (1.6 g), and NaOH (0.58 g) in water (8 mL) were heated under reflux for 30 min. After the addition of aqueous CuSO<sub>4</sub> (5%, 3 mL) the mixture was refluxed for a further 10 min, during which time urolithin A (1 g) precipitated as a white powder. The precipitate was filtered and subjected to HPLC for purification.

**8,9-Di-O-methylurolithin C (3-Hydroxy-7,8-dimethoxy-6H-dibenzo[b,d]pyran-6-one).** 2-Bromo-4,5-dimethoxybenzoic acid (1.3 g), resorcinol (1.1 g), and NaOH (0.4 g) in water (5 mL) were heated under reflux for 30 min. After the addition of aqueous CuSO<sub>4</sub> (5%, 2 mL) the mixture was heated again for 10 min, during which time 8,9-di-O-methylurolithin C (0.82 g) precipitated as a white powder. The precipitate was filtered and subjected to HPLC for purification.

**Urolithin C (3,7,8-Trihydroxy-6H-dibenzo[b,d]pyran-6-one).** A solution of 8,9-Di-O-methylurolithin C (0.563 g) and AlCl<sub>3</sub> (1.69 g) in chlorobenzene (16.9 mL) was refluxed for 2.5 h. After cooling, the mixture was added to ice and extracted with diethyl ether (3 × 25 mL). The ether was evaporated and subjected to chromatography over a 43 × 1.1 cm Sephadex LH-20 column developed with EtOH → EtOH/MeOH (1:1) → MeOH, to yield urolithin C (0.211 g) from eluates of EtOH and EtOH/MeOH.

**8,9-Di-O-methylurolithin D (2,3-Dihydroxy-7,8-dimethoxy-6H-dibenzo[b,d]pyran-6-one).** 2-Bromo-4,5-dimethoxybenzoic acid (1.3 g), pyrogallol (1.26 g), and NaOH (0.4 g) in water (5 mL) were heated under reflux for 30 min. After the addition of aqueous CuSO<sub>4</sub> (5%, 2 mL), the mixture was heated again for 10 min, during which time 8,9-di-O-methylurolithin D (0.9 g) precipitated as a yellow powder (0.656 g). The precipitate was filtered and subjected to HPLC for purification.

**Urolithin D (2,3,7,8-Tetrahydroxy-6H-dibenzo[b,d]pyran-6-one).** 8,9-Di-O-methylurolithin D (0.5 g) in a mixture of a glacial HOAc (30 mL) and aqueous HBr (15 mL) was heated under reflux for 11 h. The starting material dissolved in 2 h and upon cooling the product precipitated as dark brown solid (0.295 g) that was filtered and subjected to HPLC for purification.

**Antioxidant Assay.** Antioxidant activity was determined by the DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) method as described earlier (5, 19). Myelomonocytic HL-60 cells (1 × 10<sup>6</sup> cells/mL, ATCC; Manassas, VA) were suspended in RPMI-1640 medium containing 10% fetal bovine serum, penicillin (50 units/mL), and streptomycin (50 units/mL). The cell suspension (125 μL) was added to wells of a 96-well plate. After treatment with different concentrations (0.01–31.25 μg/mL) of the test compounds for 30 min, cells were stimulated with phorbol

**Table 1.** Antioxidant Activity of Pomegranate Tannins and Microbial Metabolites<sup>a</sup>

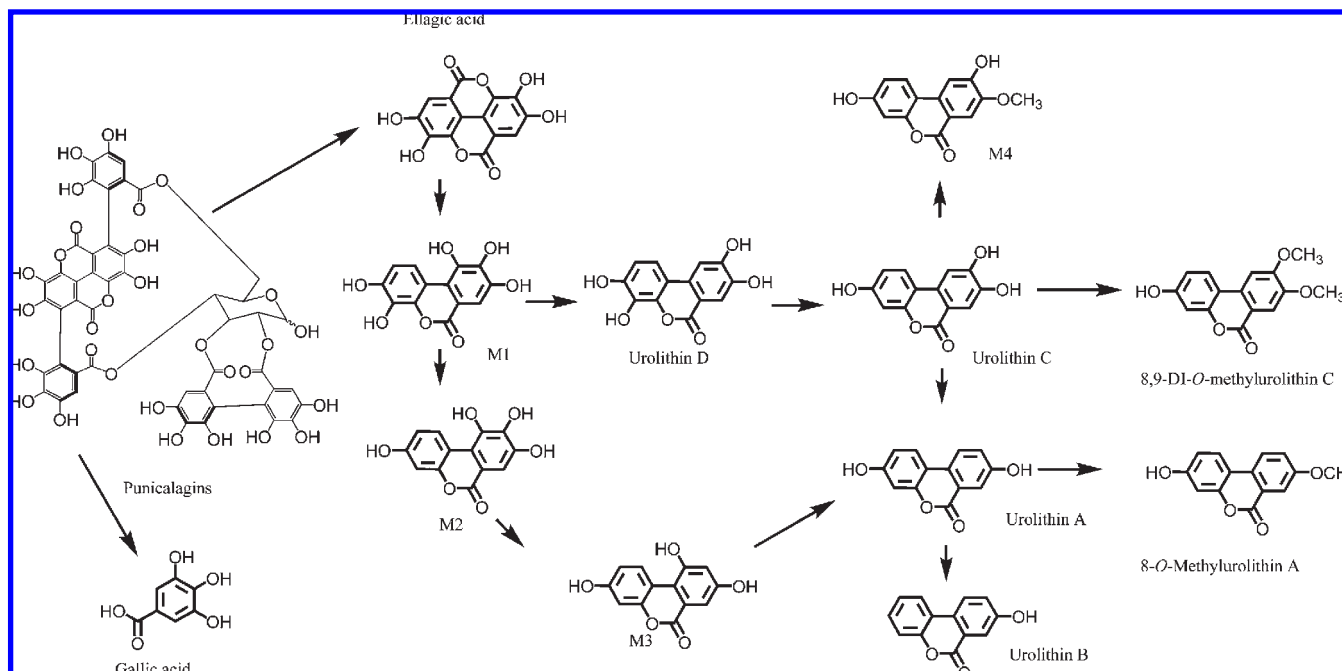
compound	antioxidant activity (IC <sub>50</sub> μg/mL)	cytotoxicity HL-60 (IC <sub>50</sub> μg/mL)
urolithin A	3.1 (13.6 μM)	>31.25
urolithin B	NA	31.25
urolithin C	0.04 (0.16 μM)	NC
urolithin D	0.085 (0.33 μM)	NC
8-O-methylurolithin A	NA	>31.25
8,9-di-O-methylurolithin C	NA	NA
8,9-di-O-methylurolithin D	NA	NC
punicalagins	1.6 (1.4 μM)	NC
punicalins	1.8 (2.3 μM)	NC
ellagic acid	0.33 (1.1 μM)	NC
gallic acid	2.1 (3.2 μM)	NC
vitamin C	0.35 (1.9 μM)	NT
doxorubicin	NT	0.25

<sup>a</sup> Inhibition of intracellular generation of ROS was determined in PMA-induced cells. IC<sub>50</sub> values were obtained from the dose response curves of % inhibition versus test concentrations. NA: not active up to the highest test concentration of 31.25 μg/mL; NC: not cytotoxic up to the highest test concentration of 31.25 μg/mL; NT: not tested; IC<sub>50</sub>: the concentration of compound that causes a 50% inhibition of intracellular ROS generation.

12-myristate-13-acetate (PMA, 100 ng/mL) for 30 min. DCFA-DA (5 μg/mL) was added and cells were further incubated for 15 min. Vitamin C (0.05 μg/mL–12.5 mg/mL) was used as positive control. Levels of fluorescent DCF (produced by reactive oxygen species (ROS) catalyzed oxidation of DCFH) were measured on a PolarStar with excitation wavelength of 485 nm and emission of 530 nm. DCFH-DA is a nonfluorescent probe that diffuses into the cells, where cytoplasmic esterases hydrolyze it to the nonfluorescent 2',7'-dichlorodihydrofluorescein (DCFH). ROS generated within HL-60 cells oxidize DCFH to the fluorescent dye 2',7'-dichlorofluorescein (DCF). The ability of the test compounds to inhibit ROS mediated oxidation of DCFH in PMA-treated HL-60 cells is measured in comparison to the vehicle control. The cytotoxicity to HL-60 cells was also determined after incubation of cells (2 × 10<sup>6</sup> cells/well in 225 μL) with test samples for 48 h by the XTT method as described earlier (5, 19).

## RESULTS AND DISCUSSION

The cellular injury caused by oxidative stress and excess of free radicals has been associated with aging and linked to over 200 clinical disorders, including cancer, heart disease, liver damage, and other degenerative diseases related to inflammation (20). A growing body of evidence shows that dietary antioxidants offer effective protection from peroxidative damage caused by ROS and other free radicals (21). Plant-derived polyphenols are known for their strong antioxidant potency, acting as ROS scavengers, peroxide decomposers, quenchers of singlet oxygen, electron donor, labile hydrogen donor, and inhibitors of lipoygenase (22). In the previous report, the cell-based assay revealed a potent antioxidant activity of pomegranate extracts and individual tannin components (punicalagins, punicalins, ellagic acid, and gallic acid) (5). The antioxidant potency of the compounds has been attributed to multiple phenolic hydroxy groups in the hexahydroxydiphenyl (HHDP) and gallagyl moieties with potential to form *o*- or *p*-quinones. In the present study, antioxidant properties of urolithins, the gut bacterial metabolites of pomegranate ellagitannins, were evaluated in terms of inhibition of intracellular generation of ROS. In contrast to earlier reports (15, 16), our results showed that urolithins exhibited a significant antioxidant action correlated with the number of hydroxy groups as well as lipophilicity of the molecules. The highest antioxidant activity was detected for urolithin C, carrying hydroxy groups at C-3, C-4, and C-8 (IC<sub>50</sub> 0.16 μM), and urolithin D, with one additional hydroxy group at C-9 (IC<sub>50</sub> 0.33 μM; Table 1). The



**Figure 3.** Proposed pathway of punicalagins transformation to urolithins by intestinal bacteria.

higher activity of the trihydroxydibenzopyranone, urolithin C, over the tetrahydroxydibenzopyranone, urolithin D, can be explained in terms of the superior lipophilicity, hence, increased bioavailability of urolithin C (17). Both urolithins C and D had higher antioxidant potency than ellagic acid and the punicalagins with  $IC_{50}$  values of 1.1 and 1.4  $\mu\text{M}$ , respectively (Table 1). They also showed higher activity than vitamin C (Table 1). The dihydroxydibenzopyranone, urolithin A, with hydroxy groups at C-3 and C-8, exhibited less significant antioxidant activity ( $IC_{50}$  13.6  $\mu\text{M}$ ). The monohydroxylated urolithin B, as well as methylated compounds, 8,9-di-*O*-methylurolithin C, 8,9-di-*O*-methylurolithin D, and 8-*O*-methylurolithin A, did not show antioxidant activity (Table 1). The general decrease in the antioxidant activity with the reduced number of phenolic hydroxy groups, as well as the lack of activity of *O*-methyl urolithins, reconfirms the importance of free hydroxy groups in antioxidant mechanisms.

Previously, urolithin A was reported as a less potent antioxidant compared to the punicalagins, with a 42- and 3500-fold lower activity in the DPPH (1,1'-diphenyl-2-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) assays (15). In our cell-based assay, urolithin A exhibited a 10-fold lower activity than the punicalagins (Table 1). In another study, the antioxidant properties of urolithins A and C were screened in assays for SOD (super oxide dismutase)-like activity generated by (1) the xanthine/XOD (xanthine oxidase system) and by (2) the PMS (phenazine methyl sulfate)/NADH system. A weak antioxidant activity was found for urolithin C, with an  $IC_{50}$  of 51  $\mu\text{M}$ , when compared to 1.3  $\mu\text{M}$  of the precursor ellagitannin geraniin. In addition, urolithin C showed a radical scavenging effect in the DPPH assay with an  $IC_{50}$  of 1.9  $\mu\text{M}$ , compared to 0.6  $\mu\text{M}$  of geraniin (16).

The relatively weak antioxidant potency of the urolithins reported in the earlier studies could be explained by the fact that test methods utilized previously were based on physical and enzymatic processes. In contrast, in the present assay, the inhibition of intercellular generation of ROS was measured and the antioxidant potential of test compounds was determined in the cellular environment. Our results showed that the

urolithins display considerable activity cognizant of the increased plasma antioxidant properties demonstrated in human volunteers after consumption of pomegranate products (4). This also seems to be supported by the production and distribution of urolithins as described in the Iberian Pig experiment (17). Based on data reported in this model (17) and on the time course profile of in vitro ellagitannin transformations by rat intestinal microbials (16), we propose the pathway in Figure 3, to account for the formation of the urolithins in the intestinal tract. The ingestion of ellagitannins involves abiotic hydrolysis at the pH levels of the small intestines and spontaneous internal lactone formation to give ellagic acid, which probably constitutes the substrate for the formation of the urolithins in bacterial metabolism (15). Significant amounts of urolithins D and C and trace amounts of urolithin A were detected in the jejunum of Iberian pigs fed with an ellagitannin-rich diet (17). Therefore, urolithin formation starts as early as in the small intestines, and bacteria present in the small intestines are able to metabolize ellagitannins to a number of degradation metabolites (17). Because urolithin B was not detected in the small intestines, it suggests that the bacterial metabolism of ellagitannins continues in the colon and culminates with the formation of urolithin B as the final product (17). The distribution of urolithins in the digestive tract also points to urolithin D as the first product of microbial transformation of ellagic acid and subsequent modifications lead to intermediates with a decreasing number of phenolic hydroxy groups: urolithin C (3,7,8-trihydroxydibenzopyranone), urolithin A (3,8-dihydroxydibenzopyranone), and finally urolithin B (3-hydroxydibenzopyranone) (Figure 3). The alternative pathway, based on in vitro transformation of the ellagitannin, geraniin by rat colon bacteria, proposes a metabolite hydroxylated at positions C-3, C-8, C-9, and C-10, as alternative to urolithin D in urolithin formation. Subsequent deoxygenations at positions C-9 and C-10 lead to urolithin A (Figure 3). It should be noted that metabolites M1–M4 as well as 8,9-di-*O*-methylurolithin C were regarded as rat feasible intermediates but have not been detected in human systemic circulation nor in tissues of the Iberian pig (4, 6, 7, 16, 17).

Urolithins C and D, the first products of bacterial transformations, are present in significant concentrations in the intestines. The urolithins are present in plasma at trace concentrations because of enteropathic circulation (17). However, they can still provide health beneficial effects resulting from their extremely high antioxidant potency (Table 1). Urolithin A is the major metabolite of ellagitannins present in plasma, urine, tissues, and digestive tract (4, 6, 17). In the present study, urolithin A exhibited a relatively weak antioxidant activity compared to its precursors, that is, punicalagins and ellagic acid (Table 1). Despite this, the antioxidant IC<sub>50</sub> value of 13 μM is still in the range of the plasma concentrations of urolithin A, that is, 4–18 μM (23, 24). This compound remains in systemic circulation for up to 72 h (14), and although urolithin A is the major metabolite, urolithins C and D with their much superior antioxidant capacity will, no doubt, also contribute to the total antioxidant potency. Hence, the regular consumption of pomegranate products should maintain an efficient concentration of urolithins in the systemic circulation to provide protection against oxidative stress. Urolithin B, the final product of ellagitannin microbial transformation is present in significant amounts in plasma and urine (14), but it did not exhibit any antioxidant properties (Table 1). However, other health protective properties, including anticarcinogenic and anticancer activities, were demonstrated for this molecule (25). It is also important that the urolithins did not exhibit toxicity against mammalian cells at concentrations up to 31.25 μg/mL (Table 1).

In conclusion, urolithins, the bioavailable products of the intestinal microbial transformation of pomegranate ellagitannins may account for systemic antioxidant effects and protection against oxidative stress. In addition, the original ellagitannins as well as some of the urolithins retained in the gut may also provide potential local gastrointestinal tract beneficial effects.

#### ACKNOWLEDGMENT

We thank POM Wonderful, Los Angeles, for financial support. United States Department of Agriculture (USDA), agricultural research service specific cooperative agreement, No. 58-6408-2-0009, is also acknowledged for partial support of the work. We also thank Katherine Martin for her excellent technical support.

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**Received for review July 24, 2009. Revised manuscript received October 1, 2009. Accepted October 5, 2009.**