

Pomegranate's Neuroprotective Effects against Alzheimer's Disease Are Mediated by Urolithins, Its Ellagitannin-Gut Microbial Derived Metabolites

Tao Yuan,[†] Hang Ma,[†] Weixi Liu,[†] Daniel B. Niesen,[†] Nishan Shah,[†] Rebecca Crews,[‡] Kenneth N. Rose,[†] Dhiraj A. Vattam,[‡] and Navindra P. Seeram^{*,†}

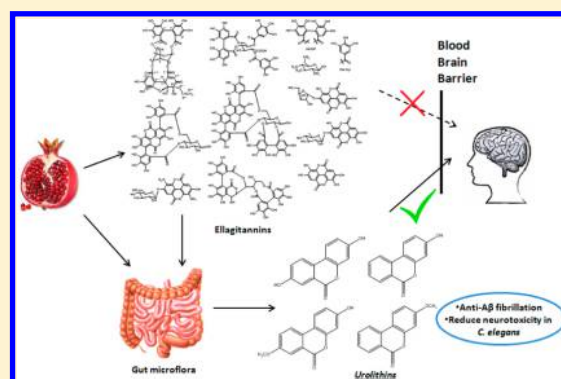
[†]Bioactive Botanical Research Laboratory, Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, Rhode Island 02881, United States

[‡]Nutrition Biomedicine and Biotechnology, Texas State University, San Marcos, Texas 78666, United States

S Supporting Information

ABSTRACT: Pomegranate shows neuroprotective effects against Alzheimer's disease (AD) in several reported animal studies. However, whether its constituent ellagitannins and/or their physiologically relevant gut microbiota-derived metabolites, namely, urolithins (6H-dibenzo[*b,d*]pyran-6-one derivatives), are the responsible bioactive constituents is unknown. Therefore, from a pomegranate extract (PE), previously reported by our group to have anti-AD effects in vivo, 21 constituents, which were primarily ellagitannins, were isolated and identified (by HPLC, NMR, and HRESIMS). In silico computational studies, used to predict blood-brain barrier permeability, revealed that none of the PE constituents, but the urolithins, fulfilled criteria required for penetration. Urolithins prevented β -amyloid fibrillation in vitro and methyl-urolithin B (3-methoxy-6H-dibenzo[*b,d*]pyran-6-one), but not PE or its predominant ellagitannins, had a protective effect in *Caenorhabditis elegans* post induction of amyloid β_{1-42} induced neurotoxicity and paralysis. Therefore, urolithins are the possible brain absorbable compounds which contribute to pomegranate's anti-AD effects warranting further in vivo studies on these compounds.

KEYWORDS: Pomegranate, Alzheimer's disease, microbial metabolites, ellagitannins, urolithins, blood-brain barrier



Alzheimer's disease (AD) is a degenerative brain disease that is projected to affect over 115 million people worldwide by 2050. AD is a leading cause of disability and morbidity among patients and is among the most costly chronic diseases known to society. Apart from its public health burden, the economic cost of AD exceeded 200 billion dollars for 2014 alone which is estimated to increase 5-fold by 2050.¹ If the incidence of AD continues on its current trajectory, it will cripple the health care systems of several countries including the United States where it is the sixth leading cause of death.²

Unfortunately, despite several decades of research, many approved drugs for AD have little effect on slowing disease progression. In fact, it is estimated that the brain changes for AD may begin more than 20 years before symptoms of the disease appear and it is often too late to reverse AD pathology by the time of diagnosis. Therefore, it is imperative that other approaches, such as the utilization of natural products as dietary intervention strategies, be explored as preventive and/or disease-modifying measures to slow or stop AD progression.

Among natural compounds, plant polyphenols have emerged as an important nonpharmacologic approach for AD prevention and treatment.^{3,4} However, the majority of polyphenols,

including the subclass known as ellagitannins, are poorly absorbed in the small intestine and do not achieve physiologically relevant concentrations in circulation.^{5,6} Instead, they reach the colon where they are extensively metabolized by gut microbiota to colonic-derived metabolites, which are implicated with a vast array of biological effects.^{7,8} Given considerable interindividual variability in microflora, and different phenotypes being observed with "metabolite-producers and nonproducers" after consumption of many polyphenol subclasses, including ellagitannins,⁹ further investigations into understanding the biological effects of these colonic metabolites are necessary.

The pomegranate (*Punica granatum* L.) fruit is a rich source of ellagitannins, primarily punicalagin (PA) and its hydrolysis product, ellagic acid (EA).¹⁰ Pomegranate juice and extracts have been reported to show neuroprotective effects against AD pathogenesis in several transgenic animal models but the bioactive compound/s responsible have not been character-

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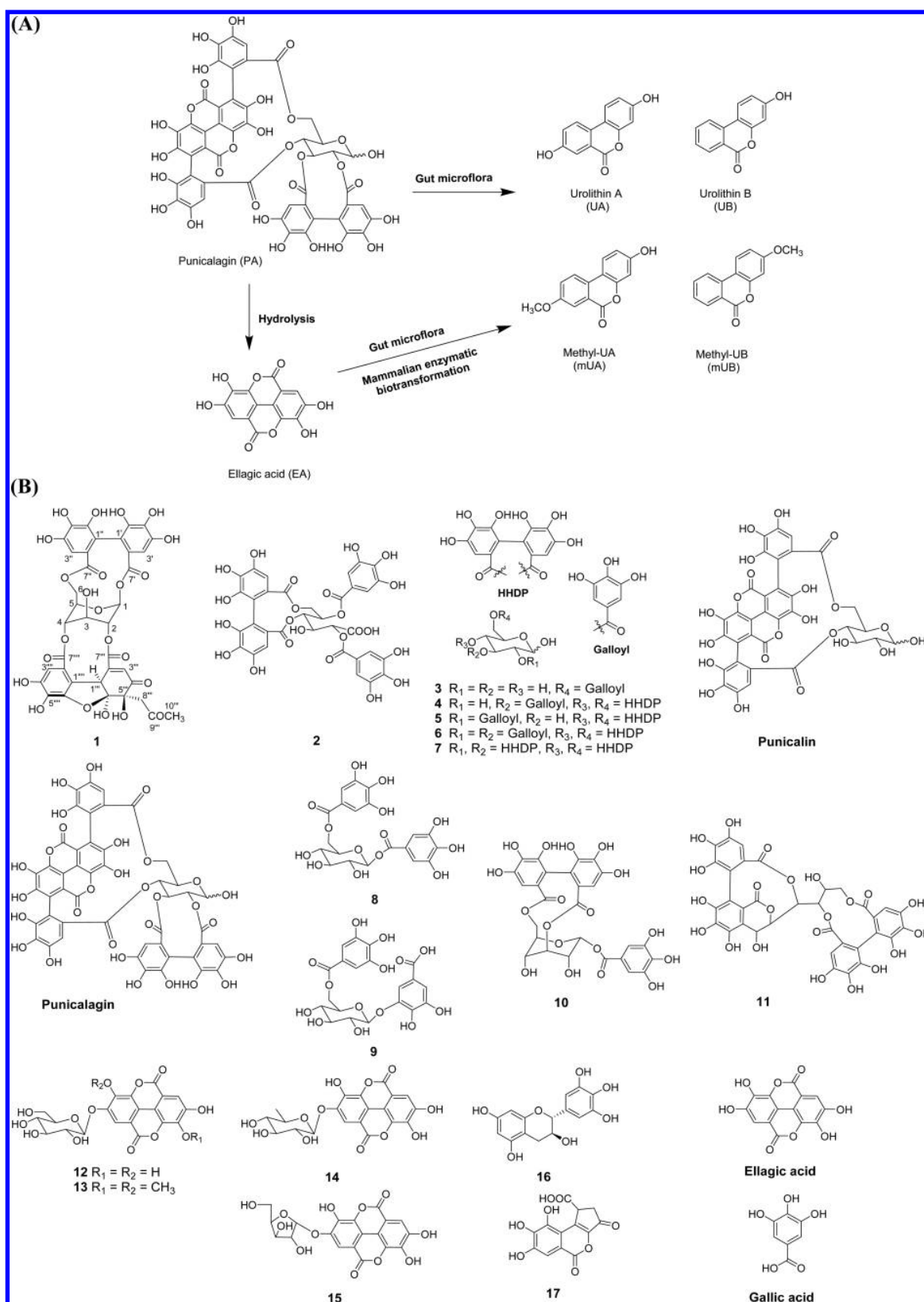


Figure 1. (A) Chemical structures of punicalagin (PA) and ellagic acid (EA) and their gut microbial metabolites, urolithins. (B) Chemical structures of compounds identified in the pomegranate extract (PE).

ized.^{11–14} Furthermore, the identity of the brain absorbable compounds, whether they are the natural ellagitannin constituents present in pomegranate, and/or their *in vivo* colonic-derived metabolites, is not known.

The bioavailability and metabolism of ellagitannins in human subjects, after the consumption of pomegranate juice and pomegranate extracts, are well established.^{5,6} The major pomegranate ellagitannins, PA and others, are not found intact

Table 1. BBB Penetrability of Compounds Present in the PE and Urolithins (UA, UB, mUA, and mUB) Using Computational Methods and Software Developed by ACD/Labs (Toronto, Ontario, Canada)

compd	log P	pK _a	fraction unbound in plasma	penetration rate (log PS)	penetration extent (log BB)	sufficient BBB penetration
1	-0.88	6.10	0.64	-8.10	-2.00	no
2	0.76	1.90	0.27	-8.20	-2.00	no
3	-1.26	7.80	0.76	-5.00	-2.00	no
4	0.56	6.10	0.52	-6.90	-2.00	no
5	0.67	6.10	0.41	-6.80	-2.00	no
6	1.82	6.10	0.36	-7.10	-2.00	no
7	1.64	5.80	0.34	-7.40	-2.00	no
8	-0.33	7.50	0.71	-5.50	-2.00	no
9	-0.07	4.30	0.22	-6.60	-2.00	no
10	0.80	6.10	0.53	-6.70	-2.00	no
11	0.60	5.80	0.46	-8.10	-2.00	no
12	-0.08	7.00	0.49	-4.70	-0.27	no
13	-0.58	6.60	0.38	-5.80	-2.00	no
14	-0.10	6.60	0.30	-5.20	-2.00	no
15	0.80	6.60	0.57	-5.10	-2.00	no
16	0.27	8.70	0.30	-3.60	-0.42	no
17	0.14	3.50	0.40	-4.90	-0.41	no
punicagalin	3.20	5.70	0.07	-8.30	-2.00	no
punicalin	0.69	5.20	0.26	-8.00	-2.00	no
ellagic acid	1.91	6.30	0.35	-3.70	-0.16	no
gallic acid	0.65	4.30	0.17	-4.20	-0.71	no
UA	2.87	9.00	0.09	-1.40	0.01	yes
UB	2.98	9.10	0.08	-1.20	0.03	yes
mUA	3.54	9.10	0.09	-1.60	0.38	yes
mUB	3.81	None	0.02	-1.10	-0.03	yes

in circulation, but rather are hydrolyzed to release EA and then subsequently biotransformed by gut microbiota to yield urolithins (6*H*-dibenzo[*b,d*]pyran-6-one derivatives) (see Figure 1A). These urolithins and their phase-2 enzyme conjugates [methylated (i.e., conversion of hydroxyl to methoxyl or methyl ether), sulfated, and glucuronidated forms]¹⁵ achieve physiologically relevant concentrations through enterohepatic recirculation and persist in vivo following the regular consumption of pomegranate food stuffs.^{5–8} Therefore, the poor bioavailability and extensive metabolism of pomegranate ellagitannins to urolithins suggest that these latter metabolites may be relevant bioactive compounds in vivo.⁵ Moreover, urolithins have been reported to show anti-inflammatory,⁵ and antiglycative and neuroprotective effects in vitro.¹⁶ However, it is also possible that there are unidentified compounds, yet to be isolated from pomegranate, which are responsible for its neuroprotective effects.

Our group has recently reported on the biological effects of an ellagitannin-enriched pomegranate extract (PE) in an aged AD transgenic animal model.¹⁷ Therefore, from this PE, herein we sought to (1) isolate and identify its chemical constituents; (2) conduct in silico computational studies to evaluate whether the PE constituents and several urolithin analogues [urolithin A (UA) and urolithin B (UB) and their methyl derivatives, mUA, and mUB, respectively], can cross the blood-brain barrier (BBB); (3) evaluate the in vitro effects of PE, its constituents [PA, EA, and gallic acid (GA)], and the urolithins, on A β _{1–42} fibrillation and; (4) evaluate the in vivo ability of PE and the aforementioned pure compounds (constituents and urolithins) to abrogate A β _{1–42} induced neurotoxicity and paralysis in *Caenorhabditis elegans*. This is the first study to investigate a PE, its constituents, and the urolithins, for in silico BBB penetrability and in vitro and in vivo anti-AD potential.

Given that the PE was previously reported to show anti-AD effects in an animal model,¹⁷ we first sought to isolate and identify all of its constituents. Twenty-one compounds (see Figure 1B), predominantly ellagitannins, were identified from the PE (by HPLC, NMR, and mass spectral data; described in the Supporting Information). The isolates included PA, EA, and gallic acid (GA) and other compounds common to pomegranate.^{10,18} In addition, a new ellagitannin was isolated and assigned the common name of pomellatannin (1). This compound is a dehydroellagitannin acetone condensate, and its structure is in accordance to similar ellagitannin-acetone derivatives previously reported.¹⁹ The remaining isolates 2–17 were identified based on ¹H NMR and/or ¹³C NMR data and by comparison of these data to published literature reports as follows: punigluconin (2),²⁰ 6-*O*-galloyl-D-glucose (3),²¹ gemin D (4),²² hippomanin A (5),²² praecoxin B (6),²³ pedunculagin (7),²⁴ 1,6-di-*O*-galloyl- β -D-glucose (8),²¹ gallic acid-3-*O*- β -D-(6'-*O*-galloyl)-glucopyranoside (9),²¹ isocorilagin (10),²⁵ casuariin (11),²⁶ ellagic acid-4-*O*- β -D-glucopyranoside (12),²⁷ 3,3'-di-*O*-methyl-ellagic acid-4-*O*- β -D-glucopyranoside (13),²⁸ 4-*O*- α -L-rhamnopyranosyl-ellagic acid (14),²⁹ 4-*O*- α -L-arabinofuranosyl-ellagic acid (15),³⁰ galocatechin (16),³¹ and brevifolincarboxylic acid (17).³²

Having obtained the structures of the natural constituents present in the PE, which were primarily ellagitannins, we next sought to investigate whether the isolates and their colonic derived microbial metabolites, namely urolithins, could potentially cross the BBB. Therefore, using in silico computational methods as previously reported,³³ the 21 constituents identified in PE, as well as UA, UB, mUA, and mUB, were evaluated for BBB penetrability. Interestingly, none of the isolates, but all of the urolithins, fulfilled criteria required for BBB penetration (Table 1).

The methyl derivatives of UA and UB (i.e., mUA and mUB) may have in vivo relevance since these compounds could be formed from the metabolism of UA and UB by phenol-O-methyltransferase, a mammalian enzyme which is highly localized in the liver, and can transfer the methyl group of S-adenosylmethionine to phenols.³⁴ Indeed, mUA has previously been detected in tissues of mice after oral delivery of UA.³⁵ However, apart from these methyl derivatives, we also evaluated other potential mammalian enzyme-biotransformation products, namely, sulfated and glucuronidated derivatives of UA and UB for BBB penetrability. Interestingly, while none of these latter metabolites fulfilled criteria required for BBB penetration, the dimethyl derivative of UA (3,8-dimethoxy-6H-dibenzo-[b,d]pyran-6-one) did fulfill the criteria required for BBB penetration (data not shown). Therefore, it is possible that the increased lipophilicity caused by methylation of the hydroxyl group/s on UA and UB (to yield the methyl ether/methoxyl derivatives) increases BBB penetrability unlike increased hydrophilicity which is imparted by sulfation or glucuronidation. Methylated conjugates of other polyphenols, including monomeric flavanols (catechins), have been reported to remain intact and persist in vivo since they are not susceptible to enzymatic deconjugation unlike sulfated and glucuronidated metabolites which can be deconjugated in vivo by sulfatases and β -glucuronidases, respectively.³⁶

Apart from the dietary relevance of the urolithins and their conjugates due to their formation from ellagitannins by colonic microbiota, their subsequent biotransformation by mammalian enzymes, and their persistence in vivo through enterohepatic circulation,^{5–8} they could also be explored for pharmaceutical potential given that structural analogues, and accompanying SAR (structure–activity related) studies, can yield compounds with enhanced activity and BBB penetrability.

It is necessary, though, that the in silico data reported herein be substantiated by future animal brain tissue disposition studies. Interestingly, a recent report (using mass spectroscopic methods) has detected UB in brain tissues of rats after intravenous delivery.³⁷ However, animal studies to evaluate brain deposition after repeated oral exposure of PE, as well as the individual urolithin analogs, is warranted. These studies are necessary since it has been reported that the brain bioavailability of certain polyphenols, such as monomeric flavanols (catechins), can increase after repeated oral dosing as has been observed with a polyphenol-rich grape seed extract.³⁸

We next sought to use biophysical methods to evaluate the effects of PE, its constituents (PA, EA, and GA), and the urolithins on $A\beta$ fibrillation. This is because elevated levels of $A\beta$ fibrillation and oligomerization in brain are associated with neurotoxicity in AD and are characteristic hallmarks which play significant roles in both early and late stages of AD.^{39,40} Therefore, agents which target the formation of $A\beta$ fibrils and oligomers could serve as therapeutic approaches for AD prevention and/or treatment. $A\beta_{1-42}$ fibrillation was confirmed by the ThT assay which showed a significant increase in fluorescence which was then correlated to binding levels of ThT to $A\beta$ fibril content, β -sheet formation⁴⁰ and peptide oligomerization.³⁹ The PE treated samples reduced $A\beta$ fibrillation by 35.9% and 76.4%, at 10 and 100 $\mu\text{g}/\text{mL}$, respectively. The purified PE constituents and urolithins reduced $A\beta$ fibrillation at levels ranging 6.5–65.4% (at 10 μM) and 20.2–76.3% (at 100 μM) (Figure 2). These inhibitory levels were similar to those of resveratrol (37.6% at 10 μM and 74.4% at 100 μM), the well-known grape/red wine polyphenol,

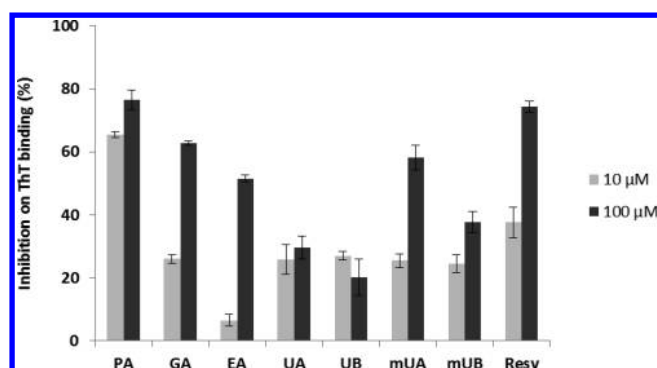


Figure 2. Inhibition on $A\beta_{1-42}$ fibrillation measured by the ThT binding assay. Treatments include 10 and 100 μM of PE constituents (PA, EA, and GA), urolithins (UA, UB, mUA, and mUB), and the positive control, resveratrol (Resv). The inhibition level on ThT binding of each treated solution was expressed as a percent inhibition value (% inhibition) relative to the negative control. % inhibition was calculated based on arbitrary fluorescence (FU) using the following equation: % inhibition = [(FU of negative control – FU of treated solution)/FU of negative control] \times 100%. Data was obtained from triplicate experiments ($n = 3$).

which has also been previously reported to reduce $A\beta$ fibrillation in vitro.⁴¹ Therefore, the preventive abilities of the urolithins on the assembly of neurotoxic $A\beta$ fibril structures may contribute to the overall neuroprotective effect reported for pomegranate but further studies would be required to confirm this.

Lastly, we evaluated PE, its purified constituents, and the urolithins (all at 10 $\mu\text{g}/\text{mL}$), using an in vivo *C. elegans* model of AD. The mobility curves for the CL4176 *C. elegans* strain after the $A\beta_{1-42}$ induction of muscular paralysis at 25 $^{\circ}\text{C}$ are shown in Figure 3 and the mean, maximum, and median survival of the worms post heat shock are shown in Table 2. Compared to the control worms, treatment with PE (Figure 3B) did not have any significant effect on the mean, maximum, or median survival/mobility in *C. elegans* post induction of $A\beta_{1-42}$ induced neurotoxicity and paralysis. Treatment with EA (Figure 3D), UB (Figure 3F), mUA (Figure 3G), and GA (Figure 3I) did not have any effect on the mean or medium survival/mobility in *C. elegans* but significantly ($p < 0.0001$) increased the maximum survival/mobility by 10.6, 8.5, 12.6, and 16.4%, respectively (Table 2). Among all of the samples, only treatment with mUB (Figure 3H) significantly ($p < 0.0001$) increased mean, maximum, and median survival/mobility in *C. elegans* post induction of $A\beta_{1-42}$ induced neurotoxicity and paralysis by 5.6, 13.0, and 10.3% respectively (Table 2). Treatment with PA (Figure 3C) and UA (Figure 3E) did not have any significant effect on the maximum or median survival/mobility, but significantly ($p < 0.0001$) decreased the mean survival/mobility in *C. elegans* post induction of amyloid β_{1-42} induced neurotoxicity and paralysis by 5.6% (Table 2).

The uptake of urolithins, specifically UA and UB, has previously been reported in different cell lines^{42,43} but to date, similar studies have not been conducted with *C. elegans*. Therefore, using a protocol reported for *C. elegans* uptake studies with the polyphenol, quercetin,⁴⁴ wild type N2 nematodes were exposed to UA and subsequent liquid chromatography mass spectroscopy (LC-MS/MS) analyses revealed its uptake into the tissues of the worms (details provided in the Supporting Information). However, further

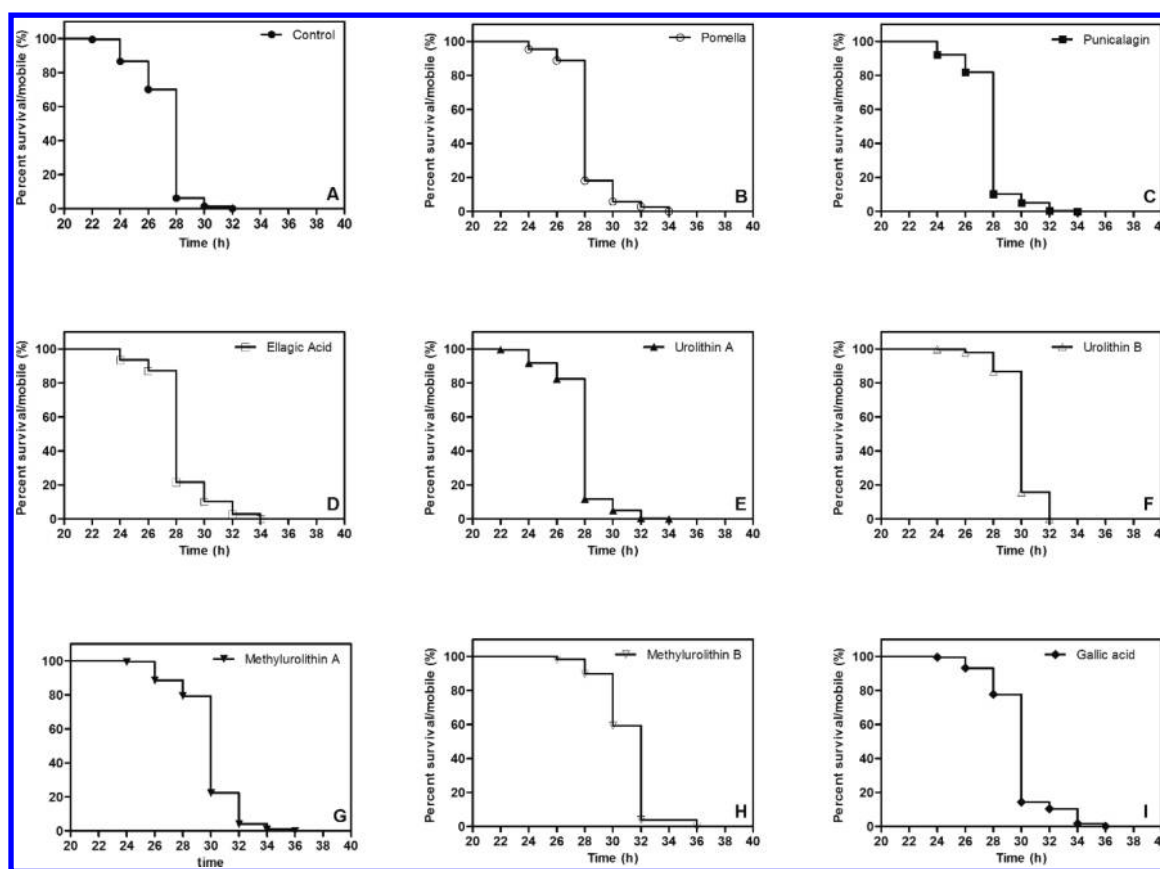


Figure 3. Mobility curves of transgenic (CL4176) *C. elegans* 20 h post $A\beta_{1-42}$ induction of muscular paralysis at 25 °C. Kaplan–Meier mobility plots of *C. elegans* worms fed on (A) control [NGM]; (B) PE [NGM + 10 $\mu\text{g}/\text{mL}$ PE; pomella extract]; (C) PA [NGM + 10 $\mu\text{g}/\text{mL}$ punicalagin]; (D) EA [NGM + 10 $\mu\text{g}/\text{mL}$ ellagic acid]; (E) UA [NGM + 10 $\mu\text{g}/\text{mL}$ urolithin A]; (F) UB [NGM + 10 $\mu\text{g}/\text{mL}$ urolithin B]; (G) mUA [NGM + 10 $\mu\text{g}/\text{mL}$ methyl-urolithin A]; (H) mUB [NGM + 10 $\mu\text{g}/\text{mL}$ methyl-urolithin B]; and (I) GA [NGM + 10 $\mu\text{g}/\text{mL}$ gallic acid].

Table 2. Survival (Mean, Median, and Maximum) of (CL4176) *C. elegans* Worms Treated with PE or Pure Compounds (10 $\mu\text{g}/\text{mL}$) 20 h Post $A\beta_{1-42}$ Induction of Muscular Paralysis at 25 °C^a

survival (h)	concn (10 $\mu\text{g}/\text{mL}$)								
	ctrl	PE	PA	EA	GA	UA	UB	mUA	mUB
mean	29.45	28.20	27.80*	28.30	29.94	27.80*	30.00	29.90	31.11*
maximum	29.47	31.70	31.1	32.60*	34.35*	29.5	32.00*	33.02*	33.33*
median	29.00	28.00	28.0	28.00	30.00	28.00	30.00	30.0	32.00*

^aNote: $p < 0.05$ log rank test (Mantel Cox).

quantitative and metabolism studies of urolithins in *C. elegans* are warranted.

In summary, we isolated and identified the naturally occurring constituents present in a PE previously reported to have anti-AD effects in an animal model.¹⁷ None of these compounds, but urolithins (gut microbial metabolites derived from ellagitannins), fulfilled in silico criteria required for BBB penetration. Moreover, the urolithins prevented β -amyloid fibrillation in vitro and methyl-urolithin B, but not PE or its constituents, protected *C. elegans* post induction of $A\beta_{1-42}$ induced neurotoxicity and paralysis. Therefore, further studies to evaluate the neuroprotective effects of urolithins and their structural analogs in animal models of AD are warranted.

METHODS

Pomegranate Extract (PE). The pomegranate extract (PE) used for the isolation studies is of the same lot number as the PE recently reported by our group to show anti-AD effects in a transgenic animal model.¹⁷ The PE is a whole pomegranate fruit extract (Pomella)

provided by Verdure Sciences (Noblesville, IN) standardized to PA (ca. 30%) and EA (2.3%).

Isolation and Identification of Compounds from the PE. Details of the isolation and identification of the compounds are provided in the [Supporting Information](#)

Urolithins. Urolithins (6*H*-dibenzo[*b,d*]pyran-6-one derivatives) including urolithin A (3,8-dihydroxy-6*H*-dibenzo[*b,d*]pyran-6-one; UA), methyl-urolithin A (3-hydroxy-8-methoxy-6*H*-dibenzo[*b,d*]pyran-6-one; mUA), urolithin B (3-hydroxy-6*H*-dibenzo[*b,d*]pyran-6-one; UB), and methyl-urolithin B (3-methoxy-6*H*-dibenzo[*b,d*]pyran-6-one; mUB) were synthesized in our laboratory according to previously reported methods.⁴⁵ Their structures were verified by NMR and mass spectral analyses and they are all >98% purity.

In Silico Computational Approach. Using previously reported methods,³³ BBB penetrability data including brain transfer descriptors (Table 1) were obtained using prediction software developed by ACD Laboratories (Toronto, Ontario, Canada).

$A\beta_{1-42}$ Thioflavin T (ThT) Binding Assay. The thioflavin T binding assay was used to measure human $A\beta_{1-42}$ fibril formation as reported previously.³⁹ Briefly, $A\beta_{1-42}$ peptide was dissolved in

ammonium hydroxide, lyophilized, and redissolved in PBS buffer to obtain a final concentration of 50 μM . Treatments included 10 or 100 $\mu\text{g}/\text{mL}$ of PE, and 10 or 100 μM of PE constituents (PA, GA, and EA), urolithins (UA, UB, mUA, and mUB), and the positive control, resveratrol. After 10 days incubation at 37 $^{\circ}\text{C}$, 20 μL of each sample was added to 100 μL of ThT solution (10 μM) and fluorescence was measured using a Spectra Max M2 spectrometer (Molecular Devices, Sunnyvale, CA) at excitation and emission wavelengths of 450 and 483 nm, respectively. To evaluate the inhibitory effects on ThT binding, the activity of each treatment was expressed as a percent inhibition value (% inhibition) relative to the negative control. Percent inhibition was calculated by [(FU of negative control – FU of treated solution)/FU of negative control] \times 100% based on arbitrary fluorescence (FU). Statistical significance was analyzed by one-way factorial ANOVA with Tukey–Kramer post hoc comparisons. A significance value of $p < 0.05$ were set to evaluate the group difference, $n = 3$.

C. elegans Strains, Maintenance, and Assays. Transgenic *C. elegans* strain CL4176, developed to express human amyloid β_{1-42} in the muscle tissue in response to heat shock,⁴⁶ were obtained from the *Caenorhabditis* Genetics Center (CGC) (University of Minnesota, Minneapolis, MN). Worms were grown and maintained at 16 $^{\circ}\text{C}$ on 60 mm culture plates with Nematode Growth Medium (NGM) (1.7% agar, 0.3% NaCl, 0.25% peptone, 1 mM CaCl₂, 1 mM MgSO₄, 5 mg/L cholesterol, 2.5 mM KPO₄ at 16–20 $^{\circ}\text{C}$). Media was poured aseptically into culture plates (10 mL for 60 mm) using a peristaltic pump and allowed to solidify for 36 h. NGM culture plates were then inoculated with 50 μL of *Escherichia coli* OP50 (CGC, University of Minnesota, Minneapolis, MN) overnight cultures and incubated for 8 h at 37 $^{\circ}\text{C}$. Strains of *C. elegans* were maintained by picking 2–3 young adult worms onto freshly inoculated NGM plates every 4–7 days.

Age Synchronization of *C. elegans*. Prior to the beginning of the experiment, *C. elegans* were age synchronized as previously described.^{47,48} Briefly, 10 worms at L4 stage (F0) were transferred to single NGM plates and incubated at 16 $^{\circ}\text{C}$ until they progressed to adulthood and laid eggs. Adults were immediately removed from the plates and the eggs were allowed to hatch (F1) and grow to L4 at 16 $^{\circ}\text{C}$. L4 worms of the F1 generation were again transferred to fresh NGM plates and allowed to mature into gravid adults and lay eggs at 16 $^{\circ}\text{C}$. Adults were quickly removed from the plates and returned to a 16 $^{\circ}\text{C}$ incubator to facilitate egg hatching. L1 worms (F2 generation) were collected from the plate by washing with S-basal buffer (0.59% NaCl, 5% 1 M KPO₄, 5 mg/mL cholesterol in ethanol) into a sterile 15 mL centrifuge tube. Worms from a minimum of five plates were pooled into a single centrifuge tube and centrifuged at 8000 rpm for 10 min at 10 $^{\circ}\text{C}$. The supernatant was carefully aspirated and the worms were washed again in S-basal buffer, centrifuged, and aspirated to leave approximately 1 mL of S-basal buffer in the centrifuge tube. The tube was gently agitated to disperse the worms and 20 μL was pipetted onto a slide and the number of worms was counted under a stereo microscope. The concentration of the worms was adjusted to 10–15 worms by diluting with S-complete liquid media (97.7% S-basal, 1% potassium citrate, 1% trace metals, 0.3% CaCl₂, 0.3% MgSO₄). A 100 mg/mL suspension of *E. coli* OP50 was prepared by centrifuging 100 mL of an overnight *E. coli* OP50 culture in LB broth at 3500 rpm. Spent LB broth was aspirated and pellet was washed several times by resuspension and centrifugation in sterile distilled water. The weight of the resultant pellet was determined and adjusted to 100 mg/mL using S-complete medium.

AD Assay and Treatments in Transgenic *C. elegans*. Prior to the beginning of the experiment, *C. elegans* were age synchronized at 16 $^{\circ}\text{C}$ and L1 worms from the F2 generation were transferred to control or treatment plates and allowed to mature gravid adult stage to lay eggs. For *C. elegans* treatment, stock solutions (1 mg/mL) of the samples were prepared as follows: PE and PA were dissolved in a 1:1 (v/v) mixture of S-basal buffer and methanol and diluted in S-basal buffer to a final concentration of 1 mg/mL. EA, UA, UB, mUA, and mUB were dissolved in DMSO and diluted in S-basal buffer to a final concentration of 1 mg/mL. GA was dissolved in methanol and diluted in S-basal buffer to a final concentration of 1 mg/mL. For preparing the treatment plates, stock solutions of PE, PA, EA, GA, UA, UB,

mUA, and mUB were added directly to the NGM media to obtain a final concentration of 10 $\mu\text{g}/\text{mL}$. A test concentration of 10 $\mu\text{g}/\text{mL}$ (i.e., 10 ppm) was selected for these assays since the test samples included an extract (i.e., PE) along with pure compounds. However, for the pure compounds, the 10 $\mu\text{g}/\text{mL}$ concentration is equivalent to the following μM concentrations: PA = 9.2 μM ; EA = 33.1 μM , GA = 58.8 μM , UA = 43.8 μM , mUA = 41.3 μM , UB = 47.2 μM , and mUB = 44.2 μM . Control and treatment NGM plates were then inoculated with 25 μL of *Escherichia coli* (*E. coli*) OP50 suspension (100 mg/mL) and incubated for 24 h at 23 $^{\circ}\text{C}$. The OP50 used for inoculation of treatment plates also contained the different treatments at a final concentration of 10 $\mu\text{g}/\text{mL}$. To standardize the food supply, the plates were then incubated under UV light in a Stratagene UV Stratalinker 2400 (La Jolla, CA) at maximum dose for 5 min to arrest growth of the *E. coli* OP50. Upon development of the eggs to the L3 stage, the incubation temperature of the plates was increased from 16 to 25 $^{\circ}\text{C}$, in order to induce the expression of amyloid β_{1-42} .⁴⁶ Mobility scoring was conducted beginning 20 h after temperature upshift and continued in 2 h increments until all of the worms were paralyzed. Three replicates per experiment were performed with a minimum of 200 worms. Failure to respond to touch (prodding with a worm pick) and absence of pharyngeal pumping were used to score paralyzed/dead worms. Survival curves were plotted to calculate the mean, median, and maximum survival of post heat shock treatment.

Liquid Chromatography Mass Spectroscopy (LC-MS/MS) Measurement of Urolithin Uptake by *C. elegans*. The uptake of urolithins in *C. elegans* (using UA as the model compound) were performed as previously reported for the polyphenol, quercetin.⁴⁴ Briefly, a confluent plate of wild type N2 nematodes (ca. 115 000 worms) grown in NGM/OP50 (1.7% agar, 0.3% NaCl, 0.25% peptone, 1 mM CaCl₂, 1 mM MgSO₄, 5 mg/L cholesterol, 2.5 mM KPO₄/100 μL of overnight OP50 *E. coli* culture) were washed from the plate with two \times 5 mL of S-basal buffer (0.59% NaCl, 5% KPO₄, 5 mg/mL cholesterol) and transferred to a 15 mL centrifuge tube. Treatment samples consisted of UA (100 μL of a 50 μM solution of UA dissolved in 0.05% DMSO in water) or the solvent control (100 μL of 0.05% DMSO in water) which were individually added to the nematode-buffer solution. The nematodes were incubated at 20 $^{\circ}\text{C}$ for 4 h then centrifuged at 1200 rpm for 5 min. The supernatant was decanted, and the nematodes were washed consecutively with 10 mL of PBS + 1% BSA, followed by 10 mL of PBS + 0.01% Tween20, and finally 10 mL of PBS. After the final wash, the supernatant was decanted and the worm pellet was stored at –80 $^{\circ}\text{C}$. After freezing, a 1 mL solution of MeOH/H₂O/HCl (50:49.9:0.1, v/v/v) was added to the frozen worm pellet which was then sonicated for 15 min in an ice water bath. Three rounds of freeze-sonication cycles were conducted to ensure rupturing of the worms which was confirmed by visualizing under a microscope (Nikon Eclipse E400 POL). Samples were centrifuged (Eppendorf Centrifuge 5804) at 5000 rpm for 5 min to pelletize the ruptured worm biomass and the resulting supernatant was dried in vacuo. The dried supernatant was reconstituted in deionized water (1 mL), sonicated for 10 min, and then loaded onto a C18 Solid Phase Extraction (SPE) cartridge (50 mg bed weight, 1 mL capacity; Alltech, Deerfield, IL). Before loading of the samples, the SPE cartridge was pre-equilibrated by eluting with methanol (5 mL) followed by deionized water (2 mL). The adsorbed sample was eluted with deionized water (1 mL) followed by 2 mL of tetrahydrofuran (THF). The organic layer was collected, dried in vacuo, reconstituted in 200 μL of THF, and then injected (10 μL) for LC-MS/MS analyses (further details and original spectra are provided in the [Supporting Information](#)).

Statistical Analyses. Results are expressed as mean \pm standard deviation. For the AD assay, the Kaplan–Meier method was used to compare the lifespan survival curves and the survival differences were tested for significance ($p < 0.05$) using the Log rank test (Mantel Cox). Both tests used GraphPad Prism software 6.0 (GraphPad Software, Inc., San Diego, CA).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchemneuro.5b00260.

General experimental procedures; isolation and identification of compounds from the pomegranate extract (PE); identification of compounds in the PE; structure elucidation of compound 1; fluorescence reading from ThT assay; liquid chromatography mass spectroscopy (LC-MS/MS) measurement of urolithin uptake by *C. elegans* including MS/MS transitions of the fragmentation of UA standard, and untreated and UA-treated worm samples (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Mailing address: 7 Greenhouse Road, Kingston, RI 02881, United States. Fax: 401-874-5787. Phone: 401-874-9367. E-mail: nseeram@uri.edu.

Author Contributions

T.Y. isolated and identified the compounds; W.L. and H.M. conducted the biophysical experiments; R.C. conducted the nematode neurotoxicity and paralysis experiments; N.S. conducted the in silico computational experiments; K.N.R. conducted the nematode uptake experiments; D.B.N. conducted the urolithin syntheses and mass spectral analyses for the nematode uptake experiments; H.M., D.A.V., and N.P.S. wrote the manuscript; H.M. and N.P.S. conceived and designed the overall project.

Notes

The authors declare no competing financial interest.

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■ NOTE ADDED AFTER ASAP PUBLICATION

This paper was published ASAP on November 17, 2015 with an incorrect abstract graphic and Figure 1A and incomplete text corrections. The corrected version was reposted on December 9, 2015.