Neuroprotective Effects of Bioavailable Polyphenol-Derived Metabolites against Oxidative Stress-Induced Cytotoxicity in Human Neuroblastoma SH-SY5Y Cells

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ABSTRACT: Oxidative stress is involved in cell death in neurodegenerative diseases. Dietary polyphenols can exert health benefits, but their direct effects on neuronal cells are debatable because most phenolics are metabolized and do not reach the brain as they occur in the dietary sources. Herein, we evaluate the effects of a panel of bioavailable polyphenols and derived metabolites at physiologically relevant conditions against H2O2-induced apoptosis in human neuroblastoma SH-SY5Y cells. Among the 19 metabolites tested, 3,4-dihydroxyphenylpropionic acid, 3,4-dihydroxyphenylacetic acid, gallic acid, ellagic acid, and urolithins prevented neuronal apoptosis via attenuation of ROS levels, increased REDOX activity, and decreased oxidative stress-induced apoptosis by preventing the caspase-3 activation via the mitochondrial apoptotic pathway in SH-SY5Y cells. This suggests that dietary sources containing the polyphenol precursors of these molecules such as cocoa, berries, walnuts, and tea could be potential functional foods to reduce oxidative stress associated with the onset and progress of neurodegenerative diseases.

KEYWORDS: gallic acid, ellagic acid, neuroprotective effects, phenolic acids, polyphenol, urolithins

INTRODUCTION

Oxidative stress is involved in neuronal cell death, which is one of the leading causes of neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, or cerebral ischemia reperfusion after stroke.1 Oxidative stress is characterized by the imbalance between antioxidants and the accumulation of reactive oxygen species (ROS) and reactive nitrogen species that can be spontaneously generated, such as superoxide anion and hydrogen peroxide, or made due to exogenous factors, such as drug exposure or radiation.2 ROS generally are found in the course of cell death, mainly by apoptosis caused by intracellular microenvironmental changes, and in the brain, leading to neurodegeneration and cognitive decline.3

There is much evidence showing that naturally occurring antioxidants such as vitamins, minerals, and phenolic compounds present in high amounts in fruits, vegetables, and natural products scavenge the free radicals, and their intake acts as an upstream preventive measure to neurodegeneration.4,5 In fact, dietary polyphenols have been reported to display potential neuroprotective effects as antioxidants preventing oxidation of proteins, lipid peroxidation, and generation of ROS as well as anti-inflammatory and anti-apotptic properties in several in vitro models of toxicity and animal models of neurological disorders.6,7 In this regard, previous studies have indicated the neuroprotective effect of red wine-, grape-, pomegranate-, cocoa-, and tea-derived polyphenols as sources of flavan-3-ols, stilbenes such as resveratrol (RSV), and ellagitannins (ETs) using animal models of neurodegenerative diseases.8−11 However, polyphenols are poorly bioavailable and cannot reach systemic tissues as they occur in the dietary sources. On the contrary, they are extensively metabolized and converted by colonic microbiota into other metabolic forms.6,12 Therefore, the actual metabolites responsible for the in vivo neuroprotective effects of polyphenols are not entirely known.

To date, only low concentrations of a few polyphenol-derived metabolites have been detected in the brain of animals, that is, gallic acid (GA), ellagic acid (EA) and its gut microbial derived metabolites urolithins,13−15 resveratrol and its microbial metabolites,16 and also anthocyanins.17,18 Nevertheless, many derived metabolites have been detected in the human bloodstream, so that they should be taken into account as potential candidates to exert the neuroprotective effects attributed to the polyphenolic dietary precursors.19,20

Whereas dietary polyphenols such as ETs, procyanidins, and stilbenes are poorly bioavailable, we hypothesize here that their derived metabolites, which can target the brain, could protect neuronal cells from oxidative stress-induced cell death. To check this hypothesis, we aimed at determining the anti-apoptotic and antioxidant effects of a panel of 19 polyphenol-derived metabolites (Figure 1), at physiologically relevant concentrations, on H2O2-induced neurotoxicity in human neuroblastoma SH-SY5Y cells.

MATERIALS AND METHODS

Materials and Reagents. Trypan blue, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), hydrogen peroxide solution, gallic acid (GA), ellagic acid (EA), 3,4-dihydroxyphenylpropionic acid (DHPPA), 3,4-dihydroxyphenylacetic acid, and hydrogen tetrazolium salt (CST). Materials and Reagents. Trypan blue, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), hydrogen peroxide solution, gallic acid (GA), ellagic acid (EA), 3,4-dihydroxyphenylpropionic acid (DHPPA), 3,4-dihydroxyphenylacetic

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acid (DHPPA), and trans-resveratrol (RSV) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The microbial EA-derived urolithins were chemically synthesized and purified by Villapharma Research S.L. (Parque Tecnológico de Fuente Alamo, Murcia, Spain). Urolithin D (Uro-D) and urolithin C (Uro-C) were purchased from Dalton Pharma Services (Toronto, Canada). RES-sulfate, RES-glucuronide, and the microbial RES-derived metabolites dihydror-esveratrol (DHR) and DHR-glucuronide were obtained as described elsewhere.

Metabolites against H2O2-induced oxidative stress, cells were pretreated with each test molecule (1, 5, or 10 μM) for 6 h and then exposed to 100 μM H2O2 for 24 h (i.e., pretreatment) or cells were not pretreated with the molecules but incubated jointly with H2O2 for 24 h (1, 5, or 10 μM test molecule + 100 μM H2O2) (i.e., cotreatment).

After each assay, cell viability was measured using the MTT assay as described elsewhere. The cytotoxic effect was calculated as the percentage of cell proliferation values with respect to the control cells (0.5% DMSO; 100%). Data are presented as the mean ± SD of at least five separate experiments.

Measurement of Reactive Oxygen Species. The measurement of ROS was performed using the ROS-sensitive 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) staining method (Sigma, St. Louis, MO, USA). Following treatments, the medium was removed and incubated with 25 μM H2DCFDA for 2 h at 37 °C in the dark. H2DCFDA, a cell permeable nonfluorescent, is de-esterified intracellularly and turns to the highly fluorescent permeant molecule 2′,7′-dichlorofluorescein (DCF) in the presence of intracellular ROS upon oxidation. Fluorescence intensity was measured at an excitation wavelength of 495 nm and an emission wavelength of 520 nm using a multimode microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany).

Measurement of Mitochondrial Oxidation–Reduction (REDOX) Activity. The measurement of REDOX activity was performed using the fluorogenic oxidation–reduction indicator resazurin (Life Technologies Inc., Rockville, MD, USA). After treatments, resazurin dissolved in water at a final concentration of 5 μM was added to the wells, and the fluorescence intensity was examined at an excitation of 530 nm and an emission of 590 nm. The plate was incubated for 2 h, and then fluorescence was measured using a microplate reader described above.

Analysis of Apoptosis by Annexin V/PI Assay. The analyses of apoptosis induction were performed in triplicate (n = 2 plates per experiment) for each treatment and were carried out using the Annexin V/propidium iodide (PI) detection kit (Molecular Probes, Life Technologies Inc.) as specified by the manufacturer. Briefly, floating and adherent cells (0.5 × 10⁶) were resuspended in 400 μL of binding buffer and incubated in the dark with both Annexin V and PI for 15 min. Then, a minimum of 25,000 cells per sample was analyzed by flow cytometry (Coulter, EPICS XL-MCL, Miami, FL, USA). Staurosporine (Sigma) 5 μM was assayed as a standard inducer of apoptosis. The percentage of living (negative in both Annexin V-FITC and PI), early apoptotic (positive in Annexin V-FITC and negative in PI), late apoptotic (positive in both Annexin V-FITC and PI), and necrotic cells (positive only in PI) was calculated.

Evaluation of Caspase Activation by Flow Cytometry. The activation of caspase-3, -8, and -9 was evaluated by flow cytometry using the caspase-specific FITC-labeled (FAM) FLICA apoptosis detection kits (Immunochemistry Technologies LLC, Bloomington, MN, USA). Experiments were carried out three times for each treatment (n = 2 plates per experiment) in FL1-A channel (Coulter, EPICS XL-MCL). A minimum of 2 × 10⁵ cells was analyzed for each sample.

Statistical Analysis. Data are presented as mean values ± SD. Graphs of the experimental data were performed using Sigma Plot 13.0 (Systat Software, San Jose, CA, USA). Two-tailed unpaired Student’s t test was used for statistical analysis of the data. A p value <0.05 was considered significant.

Results

Bioavailable Phenolics and Derived Metabolites Protect SH-SY5Y Cells from H2O2-Induced Cytotoxicity. The compounds did not exert cytotoxic effects when incubated for 6–48 h using the highest concentration (10 μM) as cell growth or viability (always >90%) was not significantly affected (data not shown). H2O2 alone decreased SH-SY5Y cell viability around 40–50% compared with the DMSO control (Figure 2).
Both the pretreatment and cotreatment experiments with GA, EA, DHPPA, and DHPAA (10 μM) significantly attenuated the H₂O₂-induced cytotoxicity (p < 0.05) (Figure 2A) compared with the H₂O₂ treatment alone. The pretreatment with urolithins (10 μM) showed slight neuroprotective effects, although significant (p < 0.05), except for Uro-B glucuronide, whereas no effects were observed with the cotreatment for any urolithin (Figure 2B). No protective effects were observed for any compound at 1 or 5 μM (data not shown), except for DHPPA, DHPAA, GA (cotreatment and pretreatment), and EA (only pretreatment) (p < 0.05) at 5 μM (Figure 2C).

We next investigated the potential mechanisms involved in the neuroprotective effects, so that only the most effective compounds (DHPPA, DHPAA, GA, EA, and urolithin A (Uro-A), a representative urolithin) were used for subsequent experiments.

Effects on H₂O₂-Induced Intracellular ROS Accumulation and REDOX Activity. The effects of the selected metabolites (DHPPA, DHPAA, GA, EA, and Uro-A) on intracellular ROS levels and REDOX activity are shown in Tables 1 and 2, respectively. As cell viability was reduced by

### Table 1. Effect of Metabolite Pretreatment and Cotreatment on H₂O₂ Generation in SH-SY5Y Cells

<table>
<thead>
<tr>
<th>Treatment (10 μM)</th>
<th>control</th>
<th>H₂O₂</th>
<th>Uro-A</th>
<th>DHPPA</th>
<th>DHPAA</th>
<th>GA</th>
<th>EA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h, no oxidative stress</td>
<td>100.0 ± 3.5</td>
<td>100.0 ± 7.8</td>
<td>100.0 ± 11.3</td>
<td>100.0 ± 7.8</td>
<td>100.0 ± 11.3</td>
<td>100.0 ± 7.8</td>
<td>100.0 ± 11.3</td>
</tr>
</tbody>
</table>

Values (%) are expressed as the mean ± SD (n = 5). (a) Significant difference (p < 0.05) compared to control cells. (b) Significant difference (p < 0.05) compared to H₂O₂ treatment alone.

### Table 2. Effect of Metabolite Pretreatment and Cotreatment on REDOX Activity in SH-SY5Y Cells

<table>
<thead>
<tr>
<th>Treatment (10 μM)</th>
<th>control</th>
<th>H₂O₂</th>
<th>Uro-A</th>
<th>DHPPA</th>
<th>DHPAA</th>
<th>GA</th>
<th>EA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h, no oxidative stress</td>
<td>100.0 ± 6.1</td>
<td>100.0 ± 7.8</td>
<td>100.0 ± 9.1</td>
<td>100.0 ± 7.8</td>
<td>100.0 ± 9.1</td>
<td>100.0 ± 7.8</td>
<td>100.0 ± 9.1</td>
</tr>
</tbody>
</table>

Values (%) are expressed as the mean ± SD (n = 3). (a) Significant difference (p < 0.05) compared to control cells. (b) Significant difference (p < 0.05) compared to H₂O₂ treatment alone.

As shown in Table 1, H₂O₂ remarkably boosted the intracellular ROS levels in SH-SY5Y cells, yielding DCF-fluorescence intensity around 4-fold higher (p < 0.05) than in control cells. Both pretreatment and cotreatments with DHPPA, DHPAA, GA, and EA, and Uro-A led to a significant decrease in ROS levels compared to H₂O₂ treatment alone (p < 0.05), except for the cotreatment with Uro-A (Table 1). With regard to REDOX activity, H₂O₂ treatment led to a significant decrease of REDOX activity (p < 0.05) compared to control cells (Table 2), which was attenuated by both pretreatment and cotreatment with DHPPA, DHPAA, GA, and EA (p < 0.05), but not with Uro-A (Table 2). No differences were observed in REDOX activity between control cells and pretreatment with each metabolite alone for 6 h, except in the case of Uro-A, in which a slight decrease (p < 0.05) was observed.

Bioavailable Phenolics and Derived Metabolites Decrease H₂O₂-Induced Apoptosis. H₂O₂ treatment significantly increased the percentage of late apoptotic cells (~17%, p < 0.05) when compared to the control (Figure 3). Consistent with the neuroprotective effects observed in the

50% in H₂O₂-treated cells, with the subsequent loss of fluorescence, we evaluated the intracellular ROS generation and REDOX activity at shorter incubation time (2 h) to confirm the protective effect of the metabolites on H₂O₂-induced oxidative stress. We also evaluated the intracellular ROS generation and REDOX activity of each metabolite alone, without H₂O₂ treatment, after 6 h of incubation.

Figure 2. Effect of polyphenol-derived metabolites (10 μM) (6 h pretreatment and 24 h cotreatment with H₂O₂, 100 μM) on cell viability (%) in SH-SY5Y cells (A, B). Effect of DHPPA, DHPAA, GA, and EA (5 and 1 μM) (pretreatment and cotreatment with H₂O₂) on cell viability (%) in SH-SY5Y cells (C). Values (%) are expressed as the mean ± SD (n = 5). (a) Significant difference (p < 0.05) compared to control cells. (b) Significant difference (p < 0.05) compared to H₂O₂ treatment alone.
MTT assay, pretreatment with DHPPA, DHPAA, GA, EA, or Uro-A significantly decreased the percentage of late apoptosis (8−12%, p < 0.05) compared to H2O2 treatment, with no significant differences between each metabolite (Figure 3A). A similar trend was observed after cotreatment with DHPPA, DHPAA, GA, or EA, except in the case of Uro-A, for which the cotreatment did not protect SH-SY5Y cells from apoptosis (Figure 3B).

Prevention of H2O2-Induced Caspase Activation by Bioavailable Phenolics and Derived Metabolites. Figure 4 shows the activation of caspase-3 and -9 (10−14%, p < 0.05), but not caspase-8, after H2O2 treatment. Pretreatment with each metabolite significantly attenuated H2O2-induced activation of both caspases-3 and -9 (p < 0.05) (Figure 4A). The cotreatment with each metabolite exerted a similar attenuation in the activation of both caspases (p < 0.05), except for Uro-A (Figure 4B). Notably, although no differences were found between these metabolites, higher attenuations were observed by DHPPA and DHPAA.

DISCUSSION
Epidemiological studies link the adherence to healthy diets such as the Japanese and Mediterranean diets to a lower risk of neurodegenerative diseases. Given the involvement of ROS in neurodegenerative diseases, research has focused on the potential beneficial effects of natural antioxidant compounds due to their ability to inhibit ROS generation and protect neuronal cells from oxidative damage or death. On this point, a number of studies have highlighted the potential role of dietary polyphenols in neurodegenerative disease prevention and treatment, in which the attenuation of ROS generation, apoptotic signal, or inflammatory response has been proposed as a mechanism of action. However, clinical evidence supporting the specific role of dietary polyphenols in the prevention of neurodegenerative diseases is still very limited. In this regard, better knowledge about the bioavailability, tissue distribution, and metabolism of dietary polyphenols will facilitate the identification of the putative actors in the
prevention of neurodegenerative diseases following polyphenol-rich diets.20 However, to date, most studies have been performed following exposure of cultured neuronal cells to food extracts, complex polyphenols, or single compounds in molecular forms and concentrations that do not occur in vivo. This means that the in vivo potential neuroprotective effect of dietary polyphenols remains unclear. Indeed, the ability of dietary phenolics or their derived metabolites produced in vivo to prevent oxidative stress in the central nervous system will depend on their bioavailability as well as their capacity to cross the blood–brain barrier (BBB).19,24 Therefore, there is a need to design improved in vitro studies that better reflect, at least partially, the in vivo environment. For the above reasons, and taking into account previous bioavailability human studies, we assayed here some bioavailable phenolic compounds and their phase II conjugates as well as microbial-derived metabolites, which can reach the bloodstream and even pass through the BBB.14

Our results revealed that among 19 different metabolites examined, the pretreatment and cotreatment of SH-SYSY cells with DHPPA, DHPPA, GA, or EA as well as the pretreatment with urolithins exerted a potent protection against H2O2-induced oxidative stress. It is well-known that H2O2-induced oxidative stress markedly increases the intracellular ROS levels and may induce apoptosis or necrosis by intracellular microenvironmental changes.25,26 In our study we performed the exogenous H2O2 treatment that has been widely used to induce oxidative stress27 in the human neuroblastoma SH-SYSY cell line. Our data confirmed both the increase of ROS levels and apoptosis induction through the intrinsic pathway because caspase-9 is activated, which finally leads to caspase-3 activation, without affecting caspase-8 activation in H2O2-treated SH-SYSY human neuroblastoma cells. In general, these metabolites reduced intracellular ROS levels, increased REDOX activity, and decreased oxidative stress-induced apoptosis by preventing caspase-3 activation via the mitochondrial apoptotic pathway in SH-SYSY cells. On the contrary, other studies have reported the activation of both extrinsic and intrinsic apoptotic pathways28,29 as well as the activation of caspase-3 only via caspase-8 after higher treatments with H2O2 on SH-SYSY cells.30

To the best of our knowledge, this study represents the first attempt to identify the molecule(s) responsible for the neuroprotective effects of dietary phenolic compounds in a more realistic approximation to the in vivo scenario.

Resveratrol (RSV) is a plant-derived polyphenol with promising activity against neurodegenerative diseases.31,32 However, most studies aiming at identifying mechanisms related to the potential neuroprotective activity of RSV did not take into account its limited bioavailability, microbial biotransformation into DHR, and subsequent phase-II metabolism to yield different conjugated metabolites, which are more likely to interact with systemic tissues such as the central nervous system.32 In fact, RSV and different metabolites, both microbial and phase II conjugates, have been detected in the cerebrospinal fluid (CSF) and brain of pigs after oral RSV administration.16 In our study, unlike previous papers that showed protection against H2O2-induced oxidative injury after treatment with high RSV glycone concentrations,15,34 we did not observe significant protection against oxidative stress-induced damage using physiologically relevant RSV metabolites and low concentrations. Remarkably, Turner et al.35 recently described the occurrence of RSV and derived metabolites in the CSF of patients with mild–moderate Alzheimer’s disease. Despite the high RSV amount administered (from 0.5 to 2 g daily for 1 year), no clinical effects were observed in these patients.35 Therefore, our in vitro results support that physiologically relevant concentrations of RSV and its derived metabolites do not seem to be useful in the prevention of oxidative-triggered neurodegenerative processes.

With regard to EA and GA, both phenolics are well-known antioxidant compounds with reported neuroprotective activity in different models of neurodegeneration, neurotoxicity, and oxidative stress, although more studies are needed to verify their real implication in the neuroprotective effect.36,37 To the best of our knowledge, the present study shows for the first time that EA exerted neuroprotective activity by preventing H2O2-induced oxidative stress. A recent pharmacokinetic human study has reported that EA bioavailability is not as low as previously described and can reach low micromolar levels in the bloodstream.38 EA has also been detected in the rat brain after the consumption of EA.15 Therefore, EA could be a putative actor in the neuroprotection associated with the intake of certain plant foods.39 With regard to GA, it has also been detected in the brain of a mouse model of Alzheimer’s disease following grape seed extract administration13 and also in rats after intravenous administration.14 Our data support previous in vitro studies conducted in different neuronal models that have reported the neuroprotective effect of GA by inhibiting neuroinflammation in microglia and Neuro-2A cells40 and by the attenuation of ROS generation in human astrocytoma U373 MG cells.41

The potential biological activity of the EA microbial-derived metabolites urolithins is gaining increasing interest.42 However, the evidence regarding their role in neuroprotection is still very limited. To date, only an in vitro study has reported the potential effect of Uro-A and urolithin B (Uro-B) on the survival of human neuroblastoma SK-N-MC cells subjected to mild oxidative stress by incubation with 2,3-dimethoxy-1,4-naphthoquinone (DMNQ).43 In addition, a recent study has also shown that Uro-A, Uro-B, and their methylated conjugates prevented the fibrillation of β-amyloid proteins in vitro and exerted a protective effect post-induction of β-amyloid induced neurotoxicity and paralysis in the nematode Caenorhabditis elegans.44 Notably, urolithins are able to cross the BBB, suggesting their possible role in the neuroprotective activity following the intake of ET-rich foods.14 On this point, our present study describes for the first time the potential neuroprotective activity of urolithins (Uro-A, -B, -C, and -D) and their bloodstream-occurring glucuronide conjugates against H2O2-induced cell injury. Our results showed that neither the differential hydroxylation pattern of urolithins or the phase II metabolism was critical for its neuroprotective effect, showing no differences among all of the urolithins, aglycones, and glucuronide conjugates, except the lack of effect for Uro-B. Although our results suggest a lower neuroprotective activity of urolithins against oxidative stress-induced cell death than their parent compound EA, further studies are needed to identify the real bioactive components responsible for the neuroprotective effects observed in preclinical studies after ET-rich food intake.39

Finally, the strongest neuroprotective activity was observed with the phenolic acids DHPPA and DHPPA. In agreement with our results, the 30 min pretreatment of DHPPA acid and DHPPA at similar doses exhibited a slight, but significant, protective effect in human neuroblastoma SK-N-MC cells.
subjected to DMNQ-induced oxidative stress. These phenolics are the primary microbial-derived phenolic acids that can be found in the bloodstream (up to 10 μM) after the ingestion of plant foods containing proanthocyanidins and flavan-3-ols such as cocoa, tea, and wine. Therefore, our data suggest that these compounds could play an important role in the neuroprotective actions associated with flavanol-rich food intake in human studies.

In conclusion, our approach provides further evidence regarding the neuroprotective activity of physiologically relevant polyphenol-derived metabolites. Unlike previous impressive in vitro effects reported for polyphenols as they occur in the dietary source, the activity exerted by the circulating metabolites is much lower. Overall, our results suggest that a diet rich in foods containing polyphenol precursors of DHPPA, DHPAA, GA, and EA such as cocoa, tea, strawberry, walnut, and pomegranate could contribute to reduce oxidative stress in relation to the onset of neurodegenerative diseases. However, further studies are needed to investigate the detailed molecular mechanisms underlying the neuroprotective effects of these polyphenol-derived metabolites.

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**Notes**
The authors declare no competing financial interest.

**ABBREVIATIONS USED**
DCF, 2′,7′-dichlorofluorescein; DHPPA, 3,4-dihydroxyphenylacetic acid; DHPAA, 3,4-dihydroxyphenylpropionic acid; DHR, dihydroresveratrol; DMNQ, 2,3-dimethoxy-1,4-naphthoquinone; DMSO, dimethyl sulfoxide; EA, ellagic acid; ECACC, European Collection of Authenticated Cell Cultures; EMEM, Eagle’s minimal essential medium; ETs, eilagittinins; FAM, carboxyfluorescein; FBS, fetal bovine serum; GA, gallic acid; glur, glucuronide; H2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; IsoUro-A, isourilithin A; MTT, 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2-H-tetrazolium bromide; PBS, phosphate-buffered saline; PI, propidium iodide; ROS, reactive oxygen species; RSV, trans-resveratrol; SD, standard deviation; sulf, sulfite; Uro-A, urolithin A; Uro-B, urolithin B; Uro-C, urolithin C; Uro-D, urolithin D

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