1 **In vivo anti-inflammatory and antioxidant properties of ellagitannin**
2 **metabolite urolithin A**

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ABSTRACT

Urolithin A is a major metabolite produced by rats and humans after consumption of pomegranate juice or pure ellagitannin geraniin. In this study, we investigated the anti-inflammatory effect of urolithin A on carrageenan-induced paw edema in mice. The volume of paw edema was reduced at 1 h after oral administration of urolithin A. In addition, plasma in treated mice exhibited significant oxygen radical antioxidant capacity (ORAC) scores with high plasma levels of the unconjugated form at 1 h after oral administration of urolithin A. These results indicate strong associations among plasma urolithin A levels, the plasma ORAC scores, and anti-inflammatory effects and may help explain a mechanism by which ellagitannins confer protection against inflammatory diseases.

KEYWORDS: Ellagitannin; urolithin A; antioxidant activity; anti-inflammatory activity

41 Ellagitannins are natural antioxidants, which are found in many medicinal plants
42 and foods such as pomegranates, raspberries, blackberries, and walnuts.[1] Various
43 biological studies of ellagitannins have demonstrated antioxidant,[2] antiviral,[3]
44 antimutagenic,[4] antimicrobial,[5-7] anti-inflammatory,[8] and antitumor activities[9-10] and the
45 absorption and metabolism of ellagitannins have recently been reported in animal and
46 human studies. Consumption of ellagitannin-rich beverages, such as pomegranate juice,
47 results in the production of ellagitannin metabolites, ellagic acid and
48 3,8-dihydroxy-6*H*-dibenzo[*b,d*]pyran-6-one (urolithin A) (Fig. 1).[11-14] Furthermore, we
49 have isolated and characterized seven urinary and gut microbial metabolites in rats
50 including urolithin A after the ingestion of geraniin, which is a typical ellagitannin
51 found in *Geranium thunbergii*.[15] Urolithin A has been found to be the main metabolite
52 in plasma after the administration of geraniin in rats[16] and pomegranate juice in
53 humans[13] and it is the most potent antioxidant among major ellagitannin metabolites.[16]
54 Free radical-mediated peroxidation of membrane lipids and oxidative damage of
55 DNA are involved in a variety of pathological complications such as cancer,
56 atherosclerosis, and neurodegenerative diseases. Because of their antioxidant activity,
57 ellagitannins may play a vital role in protecting against these oxidative stress-mediated
58 pathological conditions. We previously reported that urolithin A exhibited more potent
59 antioxidant activity than intact ellagitannins, as indicated by oxygen radical absorbance
60 capacity (ORAC) measurements, suggesting that urolithin A may be a key mediator of
61 ellagitannin protection. In addition, because oxidative stress plays an important role in
62 the pathogenesis of inflammation, the ability of antioxidants to scavenge reactive
63 oxygen species (ROS) may also provide anti-inflammatory activity. Specifically, ellagic
64 acid, an ellagitannin metabolite, has been shown to inhibit activated biomarkers of

65 inflammation, such as tumor necrosis factor-α and interleukin (IL)-1β.[17] Recently,
66 urolithin A has been shown to inhibit prostaglandin E2 production induced by IL-1β[18]
67 and attenuate the effect of colonic inflammation in a colitis rat model.[19] In the present
68 study, we investigated *in vivo* anti-inflammatory and antioxidant properties of the
69 ellagitannin metabolite urolithin A in a carrageenan-induced paw edema model in
70 mice[20] and with an ORAC assay in order to clarify the possible role of ellagitannin
71 metabolites as biological antioxidants after consumption of ellagitannins.

72 Carrageenan-induced inflammation is a useful model to evaluate the effect of
73 potential anti-inflammatory agents after oral administration.[21] Paw edema was induced
74 in the right hind paw of ICR mice by the subcutaneous injection of 1% λ-carrageenan in
75 physiological saline (50 μL). The inflammation level was quantified by the volume of
76 paw edema. Urolithin A prepared by chemical synthesis[15] in 0.5%
77 carboxymethylcellulose suspension was orally administered to the mice at 1 or 6 h
78 before carrageenan injection. The anti-inflammatory effects of urolithin A on
79 carrageenan-induced edema in mice are summarized in Fig. 2. The volume of paw
80 edema of mice treated with urolithin A at 1 h before carrageenan injection decreased to
81 35%, 26%, and 34% relative to the control group after 3, 6, and 24 h of inflammatory
82 induction, respectively (Fig. 2A). The differences in mean values of the control group
83 were statistically significant at $p < 0.05$; however, treatment with urolithin A at 6 h
84 before inflammatory induction by carrageenan showed no effect (Fig. 2B). The edema
85 induced by carrageenan injection is believed to be biphasic in nature. The initial phase,
86 beginning 1 h after carrageenan administration, is due to the release of histamine and
87 serotonin. The second phase, occurring 2 to 5 h after carrageenan ingestion, is induced
88 by the release of bradykinin, proteases, prostaglandin, and lysozyme.[22] Our data suggest

89 that treatment with urolithin A at 1 h before inflammatory induction is effective on both
90 phases of inflammation induced by carrageenan.

91 Peripheral inflammatory responses have been mechanistically linked to enhanced
92 production of ROS, such as superoxide anion, peroxynitrite anion, hydroxyl radical, and
93 hydrogen peroxide radical, at the inflamed site.[23] Systematic comparison studies on the
94 antioxidant and anti-inflammatory effects of phytochemicals have recently been
95 performed.[24-26] Natural antioxidants such as polyphenols may protect against
96 oxidant-mediated inflammation and tissue damage by their ability to scavenge free
97 radicals. The antioxidant capacity of urolithin A proved more potent than that of the
98 intact ellagitannins, such as geraniin and corilagin, as measured by the ORAC assay,[16]
99 so that urolithin A is predicted to directly contribute to suppression of
100 carrageenan-induced inflammation after oral administration. The ORAC method is
101 based on the inhibition of peroxyl radical-induced oxidation and has the advantage of
102 utilizing a biologically relevant radical source.[27-28]

103 We investigated the association between the plasma ORAC scores and plasma
104 levels after oral administration of urolithin A in mice. Mouse plasma samples collected
105 at 1 h and 6 h after administration were employed for the ORAC assay[29] and estimation
106 of plasma urolithin A levels.[30] The ORAC scores were increased to 142% in plasma of
107 mice at 1 h after administration compared to those of control plasma samples obtained
108 before administration (Fig. 3). The scores were reduced to 118% of the control scores at
109 6 h.

110 Plasma levels of urolithin A analyzed by the HPLC-ESI-MS/MS method are
111 shown in Table 1. Total urolithin A levels reached 3.9 μM at 1 h after ingestion and
112 decreased to 1.3 μM at 6 h. On the other hand, the related metabolite,

113 3-hydroxy-6*H*-dibenzo[*b,d*]pyran-6-one (urolithin B) (Fig. 1), which may be a gut
114 microbial metabolite derived from urolithin A in mammals,[31] could not be detected in
115 any plasma samples. We recently demonstrated that urolithin A plasma levels in rats
116 reached a maximum of 0.45 μM at 6 h after ingestion of 5 mg/head of ellagitannin
117 geraniin.[16] Furthermore, Seeram *et al.* reported that plasma levels of urolithin A in
118 humans reached 0.04 μM and 0.11 μM at 0.5 h and 6 h, respectively, after consumption
119 of pomegranate juice (180 mL containing ellagitannin punicalagin 318 mg).[13] Both
120 studies revealed that plasma levels of the main metabolite, urolithin A, reached
121 maximum values 6 h after consumption of pure ellagitannin or pomegranate juice. In
122 this study, we were the first to demonstrate that urolithin A was rapidly absorbed and
123 had good bioavailability after oral administration.

124 Most of the polyphenolic compounds present in the blood circulatory system exist
125 in conjugated forms, such as glucuronide and sulfate, so that biological activity of some
126 conjugates are believed to be reduced compared to free form. In a tandem mass
127 spectrometry, the bonds of glucuronides and sulfates are easily cleaved in the collision
128 cell to generate product ions of $[M\text{-}H\text{-}176]^-$ and $[M\text{-}H\text{-}80]^-$ respectively, which
129 correspond to the fragments resulting from the deprotonated molecule. The neutral loss
130 scan is a powerful tool for identifying the existence of conjugated forms in biofluids.
131 For detection of urolithin A conjugates in mouse plasma, neutral loss scans were
132 performed for glucuronide and sulfates (Fig. 4). The peak due to glucuronide was
133 observed at 2.5 min in the neutral loss of 176 dalton scan data (Fig. 4A) and the mass
134 spectrum of the peak at 2.5 min showed the ion peak at *m/z* 403, corresponding to
135 urolithin A monoglucuronide (Fig. 4B). The peak corresponding to sulfates could not be
136 detected in any plasma samples.

137 Plasma levels of free urolithin A were estimated by treatment with and without
138 β-glucuronidase, and it was determined that urolithin A was present as free form in
139 77.2% and 65.7% of the plasma samples at 1 h and 6 h after administration, respectively
140 (Table 1). Lysosomal enzymes, including β-glucuronidase, are released from
141 inflammatory cells such as neutrophils and macrophages at the inflammatory site. Some
142 flavonoid glycosides have been reported to be deconjugated into aglycone by
143 β-glucuronidase released from neutrophils after the induction of inflammation.[32-33]
144 Urolithin A glucuronide in plasma may also serve to reduce the inflammation after
145 deconjugation at the inflamed site. Our findings indicate a strong association among
146 plasma urolithin A levels, the plasma ORAC scores, and anti-inflammatory effects in
147 the carrageenan-induced paw edema mice model. Thus, the potent antioxidant capacity
148 of urolithin A in mouse plasma may contribute to the anti-inflammatory response at the
149 affected sites after oral administration.
150 In this study, we investigated anti-inflammatory activity in the
151 carrageenan-induced paw edema mice model and *in vivo* antioxidant activity of
152 urolithin A. Our data indicate that urolithin A has profound anti-inflammatory effects,
153 which are associated with the significant ORAC scores and high plasma levels of free
154 urolithin A at 1 h after oral administration. These findings suggest that urolithin A as an
155 antioxidative metabolite of ellagitannins may contribute to the prevention of
156 inflammatory diseases after oral administration and could help explain the protective
157 effects of ellagitannin consumed from natural sources.
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159 **ACKNOWLEDGMENTS**

160

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166 **References and notes**

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200 20. Six-to-eight-old female ICR mice, weighing 20–32 g, were obtained from Japan
201 SLC (Shizuoka, Japan). The mice were kept at a controlled temperature of 24 °C
202 under a 12 h light/dark cycle. Each mouse was placed in a cage (Natsume
203 Seisakusho, Tokyo, Japan) with MF standard diet (Oriental Yeast, Tokyo, Japan)
204 and water *ad libitum*, but fasted for 24 h before the experiment. Urolithin A (300
205 mg/kg) was orally administered to mice in the form of suspension in 0.5%
206 carboxymethylcellulose (CMC). The mice in the control group were administered
207 with 0.5% CMC. Urolithin A solution was administered at 1 or 6 h before
208 injection with 50 μL 1% λ-carrageenan dissolved in physiological saline to the
209 right hind paw. After carrageenan injection, the hind paw volume was measured
210 at 3, 6, and 24 h. Volume of the edema was immediately measured after 3, 6, and
211 24 h of carrageenan injection with a plethysmometer (TK-101; Unicom, Tokyo,
212 Japan). The percentage protection was calculated in comparison to the control
213 group. Data are reported as means ± SEM. The experimental protocol was

approved by the animal research control committee of Okayama University.

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29. The blood samples were collected at 1 h and 6 h after oral administration of urolithin A to mice at 300 mg/kg by abdominal aorta puncture in a heparin-coated syringe under diethyl ether anesthesia. Each blood sample was centrifuged at 7830 × g for 10 min at 4 °C, to obtain plasma samples for plasma ORAC assay and HPLC-ECI-MS-MS analysis. The collected plasma sample (70 μL) was deproteinized with acetone/water/acetic acid (140 μL; 70:29.5:0.5, v/v) and was subsequently centrifuged at 10,000 × g for 10 min at 4 °C. Fluorescein and trolox were dissolved in phosphate buffer (75 mM). The plasma sample, blank (phosphate buffer), or trolox solution (20 μL; 125, 250, 500, and 1000 μM) were added to the wells of a 96-well plate. After adding 200 mL of fluorescein solution (94.4 nM) to each well, the plate was preincubated for 10 sec at 37 °C.

238 2,2′-Azobis(2-amidinopropane)dihydrochloride (AAPH) (75 μL; 307 mM) in
239 phosphate buffer solution at 37 °C was added. Fluorescence was recorded every 2
240 min for 90 min at excitation and emission wavelengths of 485 and 528 nm,
241 respectively, using Powerscan HT (DS Pharma Biomedical, Osaka, Japan). Data
242 are reported as means ± SEM.

243 30. The collected plasma (200 μL) was incubated with or without β-glucuronidase
244 (40 μL, 2000 units, G7896, Sigma, CA, USA) for 4 h at 37 °C. The plasma
245 samples were deproteinized with methanol/hydrochloric acid (600 μL; 95:5, v/v).
246 The mixture was centrifuged at 10,000 × g for 10 min at 4 °C, and the resulting
247 supernatant was evaporated to dryness. The residue was dissolved in
248 acetonitrile/water/formic acid (200 μL; 50:50:0.1, v/v) and filtered (PTFE
249 membrare, 0.45 μM; Milipore, Bedford, MA, USA), followed by injection (10
250 μL) into the HPLC-ESI-MS-MS system. HPLC-ESI-MS-MS analysis was
251 performed on a Shimadzu LC system (LC-20AD delivery pump, SIL-20AC
252 autosampler, CTO-20AC column oven and CBM-20A system controller;
253 Shimadzu, Kyoto, Japan) coupled to a triple quadrupole mass spectrometer
254 (API-4000; Applied Biosystems, Creemore, ON, Canada). The chromatographic
255 column was a Hydrosphere C18 column (50 × 2 mm i.d., particle size 3 μm;
256 YMC, Kyoto, Japan) maintained at 40 °C, and the mobile phase consisted of
257 acetonitrile/water/formic acid (95:5:0.1, v/v) (solvent A) and
258 acetonitrile/water/formic acid (20:80:0.1, v/v) (solvent B). A gradient was applied
259 as follows: the proportion of solvent B in the eluent increased from 0 % to 25 %
260 (t = 1 min), remained at 25 % (t = 3 min), increased from 25 % to 100 % (t = 10
261 min), and decreased back to 0 % (10.1 min) until the next injection (t = 15 min).

262 The gradient with a flow rate of 0.5 ml/min was directed into the mass
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Figure Legends

Figure 1. Chemical structures of urolithins A and B.

Figure 2. Anti-inflammatory effects of urolithin A on paw edema induced by carrageenan in mice at 1 (A) and 6 (B) h after oral administration. Data are expressed as means of the difference between the final and initial volumes ± SEM (n = 10). Mean value was significantly different from control: $^{*}p < 0.05$.

Figure 3. Plasma Oxygen Radical Absorbance Capacity (ORAC) scores after urolithin A intake by mice. Data are expressed as means ± SEM (n = 7–10). Mean value was significantly different from the value at 0 h: $^{**}p < 0.01$.

Figure 4. Neutral loss scan data of (A) 176 dalton for glucuronide form of urolithin A obtained using HPLC-ESI-MS/MS method and (B) mass spectrum of the peak at 2.5 min containing urolithin A monoglucuronide at *m/z* 403.

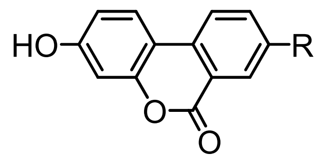
Table 1

Plasma levels of total or free urolithin A treated with or without β-glucuronidase[a]

Time (h)	Total urolithin A (μM)	Free urolithin A (μM)	Percentage of free urolithin A (%)[b]
1	3.88 ± 0.25	2.85 ± 0.32	77.2 ± 10.9
6	1.27 ± 0.06	0.83 ± 0.05	65.7 ± 4.4

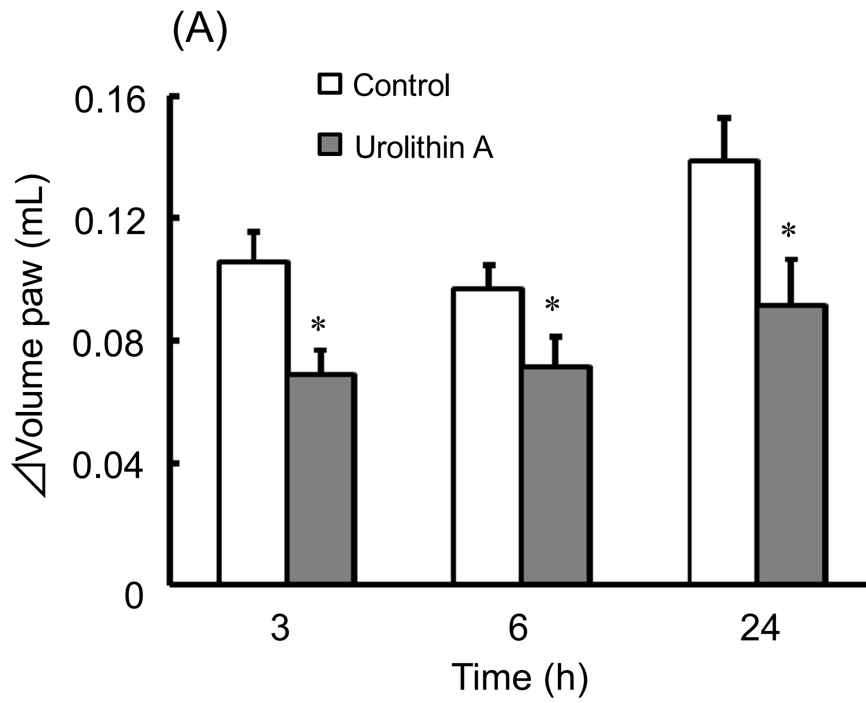
[a] Data are expressed as means ± SEM (n = 5–10)

[b] Free urolithin A / total urolithin A × 100



Urolithin A: R = OH
Urolithin B: R = H

Figure 1



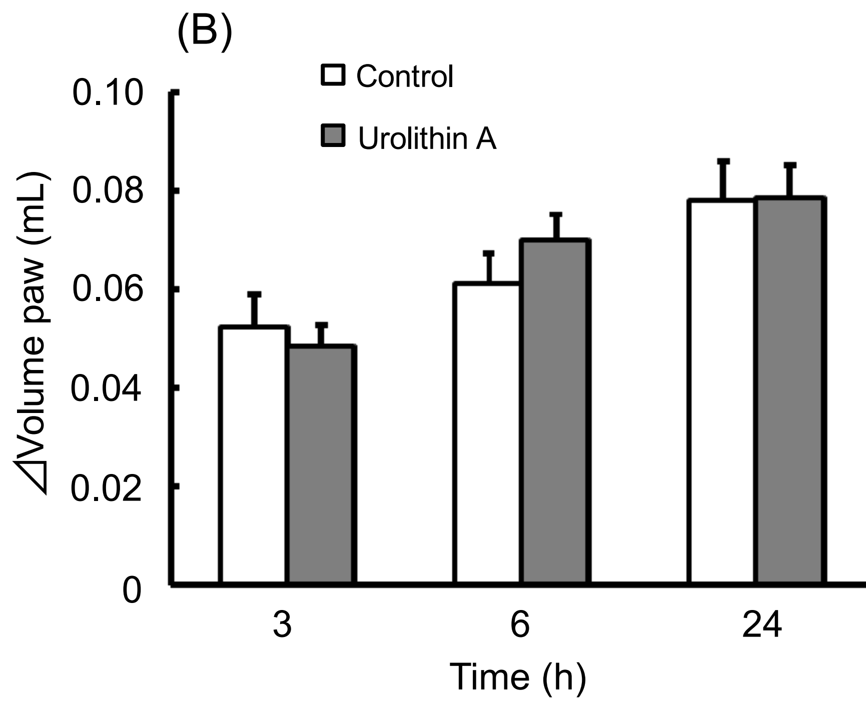


Figure 2

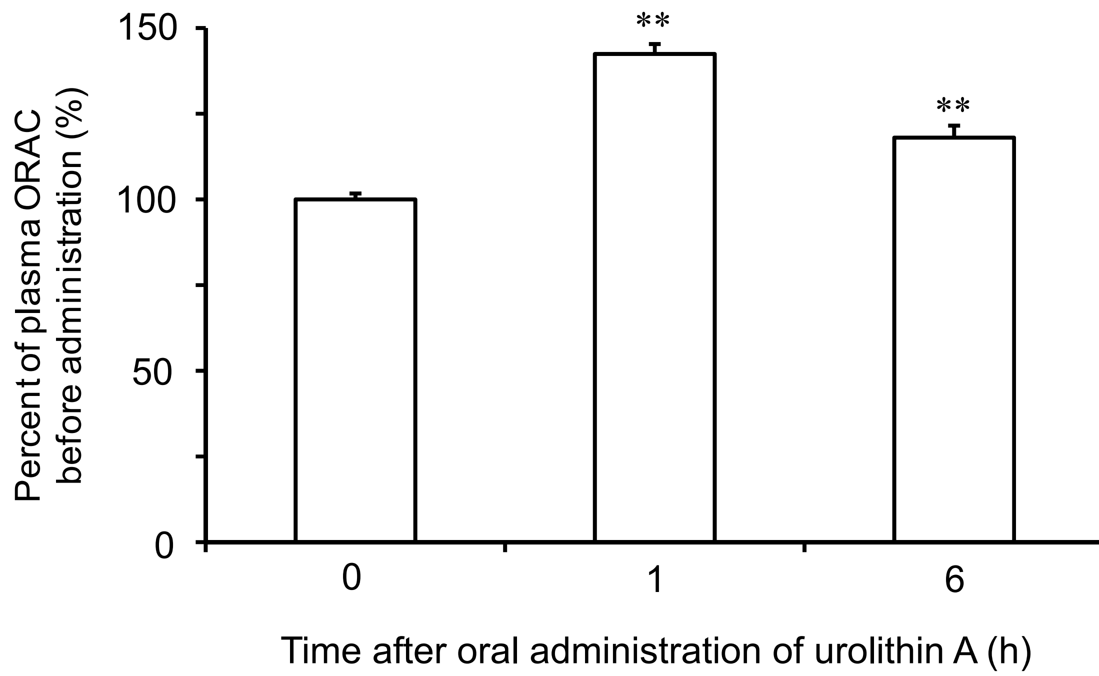


Figure 3

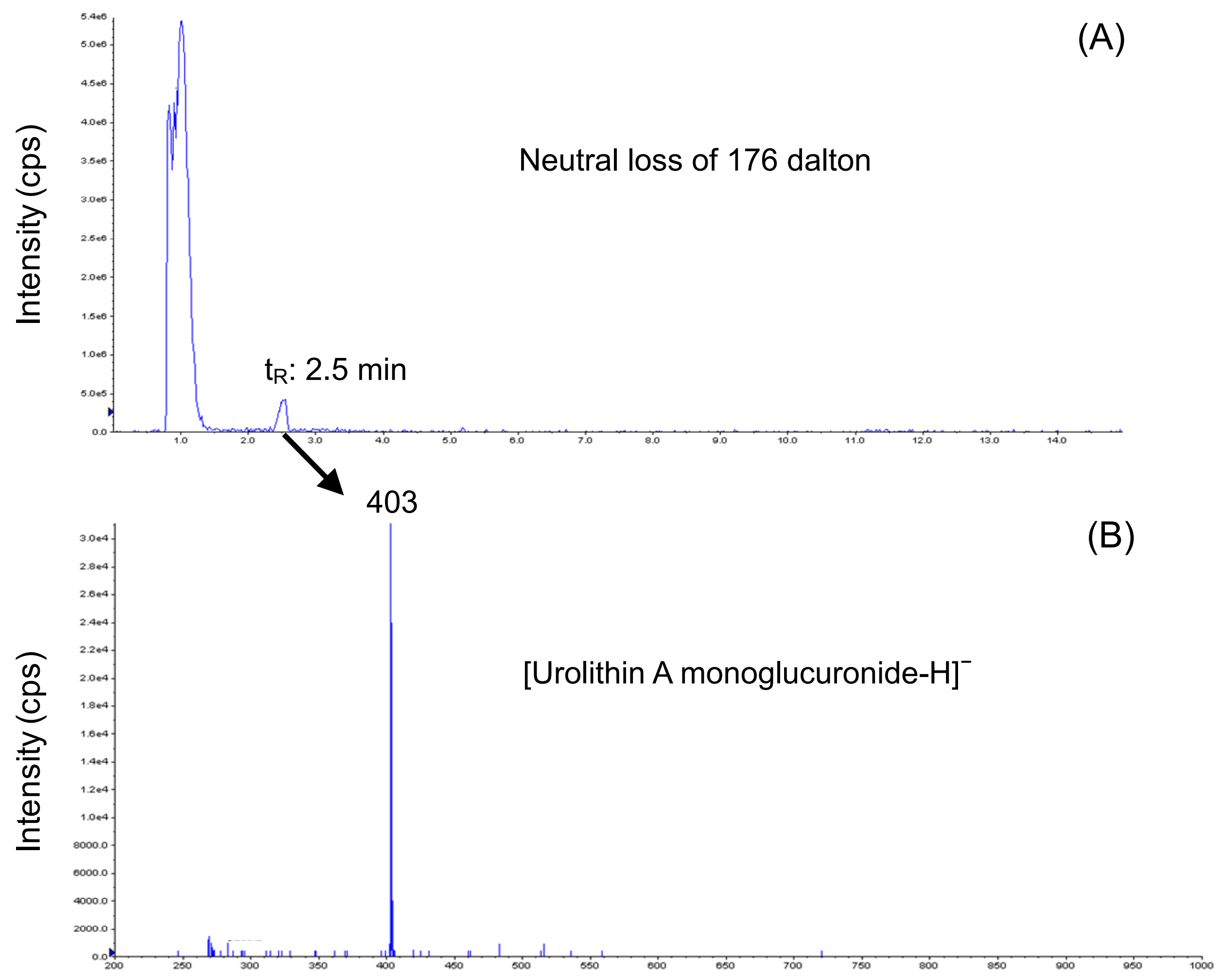


Figure 4

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In vivo anti-inflammatory and antioxidant properties of ellagitannin metabolite urolithin A

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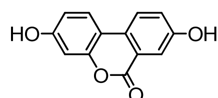

We investigated anti-inflammatory activity of a major ellagitannin metabolite urolithin A on carrageenan-induced paw edema in mice and antioxidant activity of urolithin A in mouse plasma after the oral administration by the ORAC assay.