Note

Anti-inflammatory Effects of 6,6′-bieckol and 6,8′-bieckol from Eisenia arborea on Mouse Ear Swelling

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The anti-inflammatory effects of two phlorotannins identified in the brown alga Eisenia arborea, i.e., 6,6′-bieckol and 6,8′-bieckol, were previously determined in vitro. Here we investigated the in vivo effects of these phlorotannins in mice. In ICR mice, ear swelling was induced by three sensitizers: arachidonic acid, 12-O-tetradecanoylphorbol-13-acetate (TPA) and oxazolone (OXA). Swelling was suppressed after the two phlorotannins were applied to the mouse ears. In particular, compared with epigallocatechin gallate, a typical natural inhibitor, the phlorotannins had a significantly greater effect on OXA-induced ear swelling (6,6′-bieckol, \( p < 0.05 \); 6,8′-bieckol, \( p < 0.01 \)). This is the first report to confirm the anti-inflammatory effects of 6,6′-bieckol and 6,8′-bieckol in vivo.

Keywords: anti-allergy, anti-inflammation, Eisenia arborea, phlorotannin

Introduction

Seaweed polyphenols (phlorotannins), such as eckol, dieckol, 6,6′-bieckol, 8,8′-bieckol and phlorofucofuroeckol (PFF)-A, are well-known components of brown algae (Ragan and Glombitza, 1986). Phlorotannins are useful physiologically active compounds that can be used to prevent lifestyle-related diseases, such as cancer and diabetes (Kim and Himaya, 2011). In our previous study, we isolated six phlorotannins (eckol, 6,6′-bieckol, 6,8′-bieckol, 8,8′-bieckol, PFF-A and PFF-B) with anti-allergic and anti-inflammatory activities from the brown alga Eisenia arborea (Sugiura et al., 2007). We have previously reported the anti-allergic and anti-inflammatory effects of phlorotannins in vitro (Sugiura et al., 2009). Four of the six identified phlorotannins, i.e., eckol, 8,8′-bieckol, PFF-A and PFF-B, were previously shown to suppress in vivo mouse ear swelling induced by sensitizers, such as arachidonic acid (AA), 12-O-tetradecanoylphorbol-13-acetate (TPA) and oxazolone (OXA) (Sugiura et al., 2013). However, in vivo experiments demonstrating the anti-inflammatory effects of the remaining two phlorotannins, 6,6′-bieckol and 6,8′-bieckol (Fig. 1), have not yet been conducted because of the limited availability of these phlorotannins. Therefore, in this study, we examined the suppressive effects of 6,6′-bieckol and 6,8′-bieckol on in vivo mouse ear swelling at appropriate, physiologically relevant doses. We investigated the effects of these two phlorotannins against inflammation induced by AA, TPA and OXA.

Materials and Methods

Materials E. arborea samples were collected from the coast of Mugizaki in Mie Prefecture, Japan. Harvested algae were dried and ground into powder according to the procedure outlined by Sugiura et al. (2008a). Previously, two phlorotannins, 6,6′-bieckol

Abbreviations: AA, arachidonic acid; EGCG, epigallocatechin gallate; OXA, oxazolone; TPA, 12-O-tetradecanoylphorbol-13-acetate

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and 6,8'-bieckol, were isolated from algal powder via methanol/chloroform (M/C) extraction and high performance liquid chromatography (HPLC) purification (Sugiura et al., 2009). The samples were stored at −80°C in methanol. Because of the long storage period, we conducted an HPLC analysis and a 1,1-diphenyl-2-picrylhydrazyl radical scavenging assay (Sugiura et al., 2008b) to evaluate phlorotannin decomposition. No decomposition was observed (data not shown). Epigallocatechin gallate (EGCG; Sigma-Aldrich, St. Louis, MO, USA), which exhibits well-known anti-allergic and anti-inflammatory properties, was used as a typical natural inhibitor (Tachibana, 2011).

Animals  ICR mice were used to generate a model of ear swelling. Six-week-old male mice were purchased from KBT Oriental Co., Ltd. (Tosu, Saga, Japan) and housed in individual cages maintained at 23°C–26°C under a 12-h light/dark cycle until testing. The mice had free access to MF diet (KBT Oriental) and tap water. All animal experiments were performed after obtaining permission from the “Committee for Use and Care of Laboratory Animals” at the National Fisheries University. These experiments were conducted in compliance with the “Guideline for Animal Experiments in Research Institutes under the Jurisdiction of the Ministry of Agriculture, Forestry and Fisheries” (Approval number 15–11; March 31, 2015).

Anti-inflammatory effects on mouse ear swelling  Arachidonic acid (AA)  A model of AA-induced ear swelling was established according to the method described by Young et al. (1984), with some modifications. Ten microliters of AA (Wako Pure Chemical Co. Ltd., Tokyo, Japan; 12.5 mg/mL in acetone, stored at −20°C until use) and 5 μL of methanol-dissolved phlorotannin (75 nmol/5 μL) were mixed and spread onto the mouse ear. As a control, AA solution and 5 μL of the methanol without phlorotannin were mixed and applied to the mouse ear. One hour later, ear swelling was determined using a thickness gauge (547 series; Mitsutoyo Corporation, Kawasaki, Kanagawa, Japan). The percentage of ear swelling suppression was calculated according to the following formula:

\[
\text{Percent Suppression (\%)} = \left[1 - \frac{(T - T_0)}{(C - C_0)}\right] \times 100 \quad \cdots \text{Eq. 1}
\]

where \(C_0\) is the ear thickness without phlorotannin treatment or AA exposure, \(C\) is the ear thickness after AA exposure without phlorotannin treatment, \(T_0\) is the ear thickness with phlorotannin treatment but without AA exposure, and \(T\) is the ear thickness after phlorotannin treatment and AA exposure.

12-O-tetradecanoylphorbol-13-acetate (TPA)  A model of TPA-induced ear swelling was established according to the method described by Young et al. (1984), with some modifications. Immediately before use, a stock solution of TPA (Wako; 800 μg/mL in acetone, stored at −20°C until use) was diluted to 80 μg/mL with acetone. Ten microliters of TPA and 5 μL of phlorotannin (75 nmol/5 μL) were mixed and spread onto the mouse ear. As a control, TPA solution and 5 μL of the methanol without phlorotannin were mixed and applied to the mouse ear. Four hours later, ear swelling was determined using a thickness gauge. The percentage of ear swelling suppression was calculated as described above.

Oxazolone (OXA)  A model of OXA-induced ear swelling was established according to the method described by Yoshino et al. (2010), with some modifications. The OXA (Sigma-Aldrich) was stored at 4°C until use. Fifty microliters of 1% OXA in ethanol were spread onto the abdominal region of a mouse whose hair had been locally carefully shaved with an animal shaver (Natsume Seisakusho Co, Ltd., Tokyo, Japan) while under diethyl ether anesthesia. Five days later, 10 μL of 0.5% OXA in acetone and 5 μL of phlorotannin (75 nmol/5 μL) were mixed and spread onto the mouse ear. For a control, the OXA solution and 5 μL of methanol without phlorotannin were mixed and applied to the ear. Twenty-four hours later, ear swelling was determined using a thickness gauge. The percentage of ear swelling suppression was calculated as described above.

Investigation of interactions between phlorotannins and sensitizer  When mixing a sensitizer with phlorotannin, we had to account for the possibility that sensitizer-mediated inflammation
Table 1. Suppressive effects of phlorotannins on AA-induced mouse ear swelling.

<table>
<thead>
<tr>
<th>Test groups</th>
<th>Control</th>
<th>6,6′-bieckol</th>
<th>6,8′-bieckol</th>
<th>EGCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ear swelling (mm)</td>
<td>0.180 ± 0.026</td>
<td>0.069* ± 0.062</td>
<td>0.080* ± 0.058</td>
<td>0.103 ± 0.027</td>
</tr>
<tr>
<td>Suppression (%)</td>
<td>—</td>
<td>61.8 ± 34.4</td>
<td>55.7 ± 32.1</td>
<td>42.7 ± 15.0</td>
</tr>
</tbody>
</table>

Suppressive effects at a dose of 75 nmol/mouse were calculated from the results of quadruplicate experiments (n = 4). Controls were sensitized by AA without phlorotannins. Values are shown as means ± standard deviations. The suppressive effects of phlorotannins did not differ significantly (p > 0.05) among the test groups. # indicates 0.05 < p < 0.1 in comparison with the control. Abbreviations: AA, arachidonic acid; EGCG, epigallocatechin gallate.

Table 2. Suppressive effects of phlorotannins on TPA-induced mouse ear swelling.

<table>
<thead>
<tr>
<th>Test groups</th>
<th>Control</th>
<th>6,6′-bieckol</th>
<th>6,8′-bieckol</th>
<th>EGCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ear swelling (mm)</td>
<td>0.280* ± 0.050</td>
<td>0.180** ± 0.040</td>
<td>0.095** ± 0.028</td>
<td>0.163** ± 0.038</td>
</tr>
<tr>
<td>Suppression (%)</td>
<td>—</td>
<td>35.7 ± 14.3</td>
<td>66.2** ± 10.1</td>
<td>41.8 ± 13.7</td>
</tr>
</tbody>
</table>

Suppressive effects at a dose of 75 nmol/mouse were calculated from the results of quadruplicate experiments (n = 4). Controls were sensitized by TPA without phlorotannins. Values are shown as means ± standard deviations. Differences between groups indicated by different letters are statistically significant (p < 0.05). An asterisk indicates that the suppression percentage of 6,8′-bieckol was significantly higher than that of EGCG (p < 0.05). # indicates 0.05 < p < 0.1 in comparison with the control. Abbreviations: TPA, 12-O-tetradecanoylphorbol-13-acetate; EGCG, epigallocatechin gallate.

Results and Discussion

As shown in Table 1, 6,6′-bieckol and 6,8′-bieckol tended to suppress AA-induced ear swelling at a dose of 75 nmol/mouse (6,6′-bieckol, p < 0.06; 6,8′-bieckol, p < 0.09). The suppression percentages associated with 6,6′-bieckol and 6,8′-bieckol were 61.8% and 55.7%, respectively. Although the means of the suppression percentages were both higher than that of EGCG (42.7%), this difference was not significant. The suppressive effect of EGCG was comparable to that observed in our previous study (Sugiura et al., 2013), in which EGCG at a dose of 75 nmol/mouse yielded a suppression percentage of 41.1% against AA-induced ear swelling. Therefore, phlorotannins might tend to more effectively suppress ear swelling, compared to EGCG.

As shown in Table 2, 6,8′-bieckol (p < 0.01) and EGCG (p < 0.05) significantly suppressed TPA-induced ear swelling and 6,6′-bieckol tended to suppress ear swelling compared with the control (p < 0.06). Regarding suppression percentages, treatment with 75 nmol/mouse of 6,6′-bieckol and 6,8′-bieckol suppressed TPA-induced ear swelling by 35.7% and 66.2%, respectively. The suppressive effect of 6,8′-bieckol (66.2%) was significantly greater than that of EGCG (41.8%). However, the suppressive effect of EGCG at the dose of 75 nmol/mouse was slightly stronger in this study than that observed in our previous study (33.5%) (Sugiura et al., 2013). Therefore, the phlorotannins from *E. arborea* seem to suppress AA-induced and TPA-induced mouse ear swelling at rates similar to those mediated by EGCG, a compound with well-known anti-inflammatory effects. The phlorotannins tested herein may therefore act against immediate inflammatory responses.

The suppressive effects of 6,6′-bieckol and 6,8′-bieckol on AA-induced mouse ear swelling were comparable to those of EGCG (Table 1). LOX-related activity has been associated with AA-
induced ear swelling (Young et al., 1984). The suppressive effects of the two investigated phlorotannins on TPA-induced mouse ear swelling were comparable to, or greater than those of EGCG (Table 2). The gene encoding COX-2 is rapidly expressed in the context of TPA-induced mouse ear swelling (Kujubu et al., 1991). In our previous studies, phlorotannins inhibited LOX activity. However, the IC50 values of 6,6′-bieckol and 6,8′-bieckol were higher than those of eckol, 8,8′-bieckol, PFF-A and PFF-B, and COX-2 activity was not affected (Sugiura et al., 2009). The estimated percentages of eckol, 8,8′-bieckol, PFF-A and PFF-B at a dose of 75 nmol/mouse, according to Sugiura et al. (2013), on AA-induced ear swelling were 37.3%, 59.8%, 53.0% and 59.6%, respectively; the corresponding values for TPA-induced ear swelling were 23.9%, 35.4%, 26.4% and 36.0%, respectively. Therefore, the inhibitory effects of 6,6′-bieckol and 6,8′-bieckol on the enzymatic activities underlying these inflammatory reactions were weaker in comparison to those of other phlorotannins, although their suppressive effects were similar or even greater. There are many possible explanations for this observation. First, the anti-degranulation effects of 6,6′-bieckol and 6,8′-bieckol in RBL-2H3 cells, a model line of cultured mast cells model, were stronger than those of eckol, 8,8′-bieckol and PFF-B (Sugiura et al., 2007). Second, the IC50 value of 6,6′-bieckol on phospholipase A2 activity, which plays a role in inflammatory reactions, was lower than those of eckol and 8,8′-bieckol (Sugiura et al., 2009). Third, the inhibitory effects of 6,8′-bieckol on hyaluronidase activity, which plays a role in topical inflammatory reactions, was greater than those of eckol, 8,8′-bieckol and PFF-B (Sugiura et al., 2008b). Moreover, 6,6′-bieckol suppressed COX-2 mRNA and protein expression in RAW 264.7 cells, a line of cultured mouse macrophages (Yang et al., 2012); accordingly, the suppressive effects of this agent on mouse ear swelling might be due to the suppression of COX-2 expression. Although the anti-oxidative effects of 6,8′-bieckol have been reported (Kwon et al., 2013), studies of its suppressive effects on COX-2 expression are required.

As shown in Table 3, the 6,8′-bieckol-mediated reduction in OXA-induced ear swelling differed significantly from the reductions associated with control (p < 0.01) and EGCG treatment (p < 0.05). Ear swelling in response to 6,6′-bieckol was significantly (p < 0.01) reduced, compared to the control. Regarding suppression percentages, 6,6′-bieckol and 6,8′-bieckol reduced OXA-induced ear swelling by 58.6% and 77.6%, respectively. The suppressive effects of these phlorotannins were significantly stronger than that of EGCG (28.0%). Therefore, 6,8′-bieckol had a significant effect on OXA-induced ear swelling, similar to its ability to suppress TPA-induced ear swelling. The estimated suppression percentages of eckol, 8,8′-bieckol, PFF-A and PFF-B at a dose of 75 nmol/mouse, according to Sugiura et al. (2013), on OXA-induced ear swelling were 49.0%, 55.3%, 52.9% and 49.9%, respectively. Therefore, our results indicate that 6,6′-bieckol and 6,8′-bieckol may be more effective than the four previously investigated phlorotannins against OXA-induced inflammation, which underlies delayed-type (type IV) allergic reactions (Xu et al., 1996). These data demonstrating the suppressive effects of 6,6′-bieckol and 6,8′-bieckol on OXA-induced type IV allergic reactions provide unique and valuable information about the use of marine-based food products to improve human health.

Table 4 summarizes the suppressive effects of both the M/C extract and EGCG on mouse ear swelling. Even if they were applied separately (i.e., not mixed with AA, TPA or OXA), they significantly suppressed AA-, TPA- and OXA-induced ear swelling compared with the controls. Therefore, the suppressive effects of 6,6′-bieckol and 6,8′-bieckol (Table 1-3) can be attributed to the true effects of these phlorotannins, rather than to inactivation of the sensitizer by the phlorotannins upon mixing. When the M/C extract or EGCG was mixed with any of the sensitizers, the suppressive effects of the former (0.1 mg/mouse) were 57.1% (AA), 38.7% (TPA) and 62.5% (OXA), whereas those of the latter (0.1 mg/mouse) were 59.1% (AA), 54.4% (TPA) and 75.7% (OXA) (Sugiura et al., 2013). Because the M/C extract and EGCG exerted higher percentages of suppression against AA or TPA-induced immediate inflammation (Table 4) than the values reported previously, administration of the sample alone, rather than mixed with sensitizer, might yield a more effective immediate inflammation response. In other words, the suppressive ability of phlorotannins on AA or TPA-induced ear swelling might be decreased by mixing with sensitizers. Regarding OXA-induced delayed type inflammation, we did not observe definitive

### Table 3. Suppressive effects of phlorotannins on OXA-induced mouse ear swelling.

<table>
<thead>
<tr>
<th>Test groups</th>
<th>Control</th>
<th>6.6′-bieckol</th>
<th>6.8′-bieckol</th>
<th>EGCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ear swelling (mm)</td>
<td>0.253 ± 0.042</td>
<td>0.105 ± 0.051</td>
<td>0.057 ± 0.023</td>
<td>0.182 ± 0.019</td>
</tr>
<tr>
<td>Suppression (%)</td>
<td>—</td>
<td>58.6 ± 20.2</td>
<td>77.6 ± 9.2</td>
<td>28.0 ± 7.3</td>
</tr>
</tbody>
</table>

Suppressive effects at a dose of 75 nmol/mouse were calculated from the results of quadruplicate experiments (n = 4). Controls were sensitized by OXA without phlorotannins. Values are shown as means ± standard deviations. Differences between groups indicated by different letters are statistically significant (p < 0.05). Asterisks indicate that the suppression percentages of phlorotannins were significantly higher than those of EGCG (*p < 0.05 and **p < 0.01). Abbreviations: OXA, oxazolone; EGCG, epigallocatechin gallate.
differences in the suppressive effects in the present study or in the previous report (Sugiuara et al., 2013). Because the duration for determining anti-inflammatory effects is longer for OXA (24 h) than for AA (1 h) and TPA (4 h), the effects of these samples on delayed-type inflammation might differ from the effects on immediate inflammation responses.

As shown in Fig. 1, 6,6′-bieckol and 6,8′-bieckol are isomers. However, the suppression percentages of 6,8′-bieckol on both TPA and OXA-induced ear swelling differed significantly from those of EGCG (TPA, \( p < 0.05 \); OXA, \( p < 0.01 \)), whereas 6,6′-bieckol caused significant suppression of OXA-induced swelling (\( p < 0.05 \)) only. Among the terrestrial plant polyphenols, flavonoids and tea catechins possessing anti-allergic activities, the locations of hydroxyl groups are crucial mediators of strong anti-allergic and inflammatory effects (Matsuo et al., 1997; Takano-Ishikawa et al., 2006); therefore, the observed differences in suppressive effects between the two phlorotannins might be attributable to the locations of hydroxyl groups in the molecular structure. Accordingly, the hydroxyl group bound to C-4′ might be critical to the anti-inflammatory effect. The proximity of the hydroxyl group in 6,6′-bieckol to O-5′ or another hydroxyl group bound to C-7 might cause an electro-organic interaction. On the other hand, the hydroxyl group bound to C-4′ is located external to the molecular surface of 6,8′-bieckol and is therefore not influenced by interactions with nearby moieties. Accordingly, the hydroxyl group may be able to play important anti-inflammatory roles. Additional studies that include other phlorotannins are needed to determine correlations between structure and activity.

Thus, phlorotannins suppress the inflammatory reactions associated with mouse ear swelling. This is the first report to demonstrate that 6,6′-bieckol and 6,8′-bieckol exert anti-inflammatory effects in vivo. However, orally administered EGCG, flavonoids and isoflavones have also been shown to suppress mouse ear swelling (Ueda et al., 2004; Yoshino et al., 2010; Nagano et al., 2016); therefore, the anti-inflammatory effects of orally administered phlorotannins on mouse ear swelling need to be examined in the context of food-based treatment.

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References

<table>
<thead>
<tr>
<th>Test groups</th>
<th>Control</th>
<th>M/C extract</th>
<th>EGCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Ear swelling (mm)</td>
<td>0.170±0.039</td>
<td>0.033b±0.017</td>
</tr>
<tr>
<td></td>
<td>Suppression (%)</td>
<td>—</td>
<td>80.9±10.0</td>
</tr>
<tr>
<td>TPA</td>
<td>Ear swelling (mm)</td>
<td>0.205±0.030</td>
<td>0.020b±0.008</td>
</tr>
<tr>
<td></td>
<td>Suppression (%)</td>
<td>—</td>
<td>90.2±4.0</td>
</tr>
<tr>
<td>OXA</td>
<td>Ear swelling (mm)</td>
<td>0.280±0.042</td>
<td>0.120b±0.012</td>
</tr>
<tr>
<td></td>
<td>Suppression (%)</td>
<td>—</td>
<td>57.1±4.1</td>
</tr>
</tbody>
</table>

The suppressive effects at a dose of 0.1 mg/mouse were calculated from the results of quadruplicate experiments (\( n = 4 \)). Reproducibility was confirmed by repeated runs. Controls were sensitized only (no M/C extract). Values are shown as means ± standard deviations. Differences between groups indicated by different letters are statistically significant (\( p < 0.01 \)). Abbreviations: M/C, methanol/chloroform; AA, arachidonic acid; TPA, 12-O-tetradecanoylphorbol-13-acetate; OXA, oxazolone; EGCG, epigallocatechin gallate.


