Inhibitory Activities of Antioxidant Flavonoids from *Tamarix gallica* on Amyloid Aggregation Related to Alzheimer’s and Type 2 Diabetes Diseases

Asma Ben Hmidene, Mizuho Hanaki, Kazuma Murakami, Kazuhiro Irie, Hiroko Isoda, and Hideyuki Shigemori*<sup>ad</sup>

<sup>a</sup> Graduate School of Life and Environmental Sciences, University of Tsukuba; Tsukuba, Ibaraki 305–8572, Japan;
<sup>b</sup> Graduate School of Biostudies, Kyoto University; Sakyo-ku, Kyoto 606–8502, Japan; <sup>c</sup> Alliance for Research on North Africa (ARENA), University of Tsukuba; Tsukuba, Ibaraki 305–8572, Japan; <sup>d</sup> Faculty of Life and Environmental Sciences, University of Tsukuba; Tsukuba, Ibaraki 305–8572, Japan.

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The prevention of amyloid aggregation is promising for the treatment of age-related diseases such as Alzheimer’s (AD) and type 2 diabetes (T2D). Ten antioxidant flavonoids isolated from the medicinal halophyte *Tamarix gallica* were tested for their amyloid aggregation inhibition potential. Glucuronosylated flavonoids show relatively strong inhibitory activity of Amyloid β (Aβ) and human islet amyloid polypeptide (hIAPP) aggregation compared to their aglycone analogs. Structure-activity relationship of the flavonoids suggests that the catechol moiety is important for amyloid aggregation inhibition, while the methylation of the carboxyl group in the glucuronide moiety and of the hydroxyl group in the aglycone flavonoids decreased it.

**Key words** amyloid aggregation; glucuronosylated flavonoid; *Tamarix gallica*; Alzheimer’s disease; type 2 diabetes

The incidence of both disturbances Alzheimer’s disease (AD) and type 2 diabetes (T2D) is increasing and has become a major public health concern in many industrialized countries. Despite intense research, best strategies to treat/prevent these costly diseases are still under investigation. However, it is now widely recognized that AD and T2D share many pathophysiological features including increased oxidative stress and amyloid aggregation.<sup>1–4</sup>

Amyloid β (Aβ) is the component of the amyloid deposits in the AD brain,<sup>5</sup> while the component of the amyloidogenic peptide deposit in the diabetic pancreatic islets of Langerhans is identified as islet amyloid polypeptide (IAPP), a 37-amino acid peptide.<sup>6,7</sup> Although the amino acid sequences of amyloidogenic proteins are diverse, they all adopt a similar structure in aggregates called cross-β-spine.<sup>5</sup> Extensive studies in the past years have found that, similar to Aβ<sub>1–42</sub>, IAPP forms early intermediate assemblies as spherical oligomers<sup>8,9</sup> that are recognized by soluble Aβ oligomers antibody,<sup>10</sup> implicating that these oligomers possess a common folding pattern or conformation.<sup>4</sup> The similarities in Aβ<sub>1–42</sub> and IAPP oligomers can be used to investigate the prevention of oxidative stress and amyloid aggregation inhibition related to AD and T2D, since potent therapeutic agents such as antioxidants with a catechol moiety, proved to inhibit Aβ aggregation,<sup>12–16</sup> may play a key role inhibiting the aggregation of human IAPP (hIAPP) in case of patients with diabetes.

The powerful antioxidant activity of the medicinal halophyte *Tamarix gallica* L. has been reported as exhibiting a remarkable spectrum of biochemical and pharmacological activities. Although traditionally used for the treatment of various liver disorders and marketed as a herbal medicine in many countries,<sup>17–19</sup> there have been no reports on the use of this plant for the treatment or prevention of AD or T2D. Therefore, the aim of this research is to investigate the protective effect of *T. gallica* towards both disturbances by isolation and identification of the Aβ and hIAPP aggregation inhibitors with antioxidant potential.

**MATERIALS AND METHODS**

**Materials** The aerial part of *T. gallica* was collected at Tunisia. The voucher specimen (UT-ARENA-01097) is maintained at Alliance for Research on North Africa (ARENA), University of Tsukuba. Forty two-Mer amyloid β-protein (Aβ<sub>1–42</sub>) was synthesized by the standard protocol.<sup>20</sup> hIAPP (Amylin 1–37, human, purity ≥95%) was purchased from KareBay Biochem Inc., U.S.A.

**Isolation and Identification of Flavonoids** Dried and crushed aerial parts (200 g) of *T. gallica* were extracted with 70% ethanol and kept at room temperature for 24 h. After filtration, the solvent of the extract was evaporated, and partitioned into CHCl<sub>3</sub> (200 mL×2), EtOAc (200 mL×3), BuOH (200 mL×3), and H<sub>2</sub>O (200 mL). The CHCl<sub>3</sub> layer (TGC, 1.1 g) was chromatographed on a silica gel column (ϕ3.0×35 cm, Nacalai Tesque, Inc., Japan) with acetone–hexane (0:100→100:0), which yielded eight fractions, TGC-1–8. TGC-5 (299.4 mg) eluted with acetone–hexane (40:60) was purified by octadecyl-silane (ODS) HPLC [TSKgel ODS-80Ts (ϕ4.6×250 mm, Tosoh Corporation, Japan), flow rate 1.0 mL/min; MeCN/H<sub>2</sub>O–0.1% trifluoroacetic acid (TFA) (5 : 95→30:70→40:60→55:54→100:0); detection UV (210, 254, 280 nm)→238 Vol. 40, No. 2

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The EtOAc-soluble portion (TGE, 987 mg) was eluted on an ODS column (Cosmosil 75 C18-PREP, φ3.0×35 cm, Nacalai Tesque, Inc.) with MeOH−H₂O (10:9→100:0), yielding six fractions, TGE-1–6. TGE-6 (228.3 mg) eluted with MeOH−H₂O (100:0) was fractionated by ODS HPLC [TSKgel ODS-80Ts (φ4.6×250 mm), flow rate 1.0 mL/min; MeCN−H₂O−0.1% TFA (5:95→55:45→85:15→100:0); detection UV (280, 320 nm)] to yield eight sub-fractions, TGE-1–8. TGE-6-8 was identified as kaempferol23) (6, 3.3 mg, tr 37.5 min). TGE-6-3 (24.7 mg, tr 28.1 min) was purified by ODS HPLC [TSKgel ODS-80Ts (φ4.6×250 mm), flow rate 1.0 mL/min; MeCN−H₂O (5:95→25:75); detection UV (210, 254, 280 nm)] to yield quercetin 3-O-β-D-glucuronide (QGlca)23,24) (7, 6.3 mg, tr 9.3 min), and quercetin 3-O-β-D-glucuronide methyl ester (QGlca-Me)23) (8, 9.0 mg, tr 17.6 min). Additionally, TGE-6-5 (20.2 mg, tr 12.3 min) was also purified by ODS HPLC [TSKgel ODS-80Ts (φ4.6×250 mm), flow rate 1.0 mL/min; MeCN−H₂O (5:95→30:70); detection UV (210 nm)] to yield kaempferol 3-O-β-D-glucuronide (KGlca)23) (9, 2.0 mg, tr 11.5 min), and kaempferol 3-O-β-D-glucuronide methyl ester (KGlca-Me)23) (10, 5.0 mg, tr 12.3 min). The structure of these substances were confirmed by NMR (Avance 500, Bruker, Germany) spectral analyses (supplementary data) and compared with the literature.21–24

Thioflavin-T (Th-T) Fluorescence Assay The aggregability of the amyloid was evaluated at 37°C by the Th-T using Aβ₁–42 and hIAPP. Briefly, Aβ₁–42 was dissolved in 0.1% NH₄OH at 250 mM and hIAPP in 1,1,3,3,3-hexafluoro-2-propanol. The amyloid solution was diluted 10-fold with 50 mM phosphate buffered saline (PBS) (pH 7.4), and the solution was incubated with or without samples. A 5 µL volume of a peptide solution was added to 250 µL of 1 mM Th-T in 50 mM Gly-NaOH (pH 8.5). The fluorescence intensity was measured at an excitation wavelength of 420 nm and an emission wavelength of 485 nm by a Multi-detection Microplate Reader and IC₅₀ was calculated from the inhibitory rate (%) of each wavelength of 485 nm by a Multidetection Microplate Reader and IC₅₀ was calculated from the inhibitory rate (%) of each compound (100, 10, 1 µM) on amyloid aggregation after 24 h of incubation. Aβ₁–42 was incubated for 6, 12 and 24 h, with and without samples and hIAPP was incubated for 4, 8 and 24 h, with and without samples. Quercetin (2) was selected as the reference substance for this experiment.

Transmission Electronic Microscopy (TEM) The effect of the flavonoids on Aβ₁–42 and hIAPP fibrillogenesis was investigated by using TEM. The incubating solution was the same as that used for preparing the samples for Th-T assay. A 5 µL volume of each sample was spotted on to a glow-discharged, Formvar-carbon-coated grid and was incubated for 2 min, then washed twice with 5 µL of pure water. The grid was negatively stained twice for 1 min each with 5 µL of 0.4% silicotungstic acid, and the solution was removed. After air drying for 5 min, each sample was examined with a JEOL JEM-1400 electron microscope.

Determination of Antioxidant Enzyme Activity Superoxide dismutase (SOD) activity was determined with the use of kits obtained from Dojindo Molecular Technologies, Inc., Japan. The absorbance was taken at 550 nm by a spectrophotometer. When the samples are containing SOD, the superoxide anion radicals could be distinctively inhibited and as a result, the nitrite was reduced. The SOD activity of samples was measured according to the formula: SOD activity (%)=|(blank 1–blank 3)−(sample–blank 2)|/(blank 1−blank 3);

Table 1. IC₅₀ of Aβ and hIAPP Aggregation Inhibitors

<table>
<thead>
<tr>
<th>Substance name</th>
<th>IC₅₀ (µM)</th>
<th>Aβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naringenin (1)</td>
<td>10.3</td>
<td>9.3</td>
</tr>
<tr>
<td>Quercetin (2)</td>
<td>1.8</td>
<td>15.7</td>
</tr>
<tr>
<td>Rhamnetin (3)</td>
<td>13.4</td>
<td>131.8</td>
</tr>
<tr>
<td>Rhamnadin (4)</td>
<td>&gt; 100</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>Tamarixetin (5)</td>
<td>10.6</td>
<td>90.7</td>
</tr>
<tr>
<td>Kaempferol (6)</td>
<td>25.4</td>
<td>51.6</td>
</tr>
<tr>
<td>QGlca (7)</td>
<td>1.7</td>
<td>3.8</td>
</tr>
<tr>
<td>QGlca-Me (8)</td>
<td>22.6</td>
<td>31.6</td>
</tr>
<tr>
<td>KGlca (9)</td>
<td>18.5</td>
<td>19.4</td>
</tr>
<tr>
<td>KGlca-Me (10)</td>
<td>28.2</td>
<td>22.4</td>
</tr>
</tbody>
</table>

with blank 1 being the coloring without inhibitor, blank 2 the sample blank and blank 3 the reagent blank. Epigallocatechin gallate (EGCG) was used as a positive control.

Statistical Analysis Statistical differences between groups were determined using a Student’s t-test, with p<0.05 set as the level of significance.

RESULTS AND DISCUSSION

Amyloid aggregation inhibition and oxidative stress potential were investigated using 10 flavonoids (Fig. 1) purified from the ethyl acetate and chloroform layer of a 70% ethanol T. gallica extract. Some of the compounds contained a catechol moiety, and others were non-catechol containing substances. The Th-T assay showed that both types of substances suppressed the aggregation of amyloid, but with different degrees. In fact, the flavonoids that possessed a catechol moiety had a lower IC₅₀ than the non-catechol type substances (Table 1), indicating that the position and number of hydroxyl group on the aromatic ring is important for the determination of aggregation inhibition potency. This result is consistent with
previous reports concerning Aβ but confirmed, in this research, for hIAPP aggregation inhibition.

From Table 1, it was deduced that among all the isolated flavonoids, QGlcA (7) showed the strongest effect with an IC₅₀ equal to 3.8 and 1.7 µM towards Aβ and hIAPP, respectively, leading to the conclusion that the glucuronide moiety considerably increases the inhibition potential of the aglycone form. Structure–activity relationship of those compounds (Table 1) suggests that, apart from the catechol moiety, a carboxyl moiety in the sugar and the presence or absence of a double bond between C2 and C3 in the C-ring are important functional groups for amyloid aggregation inhibition. Furthermore, O-methylation significantly reduces the inhibition potential of the aglycone form (2).

TEM images of Aβ and hIAPP fibrils, in the presence of QGlcA (7), the aglycone form quercetin (2), and the methylated analogue QGlcA-Me (8) show that fibril formation was relatively strongly inhibited by 7 (Figs. 2B, 3B) compared to 2 (Figs. 2C, 3C) and 8 (Figs. 2D, 3D).

Biological effects of reactive oxygen species (ROS) are controlled in vivo by a wide spectrum of enzymatic and non-enzymatic defense mechanisms, in particular SOD, that catalyze dismutation of superoxide anions to hydrogen peroxide and catalase, after which they convert H₂O₂ into molecular oxygen and water. The role of those enzymes as protective enzymes is well known and has been investigated extensively. The SOD activity of flavonoids 1–10 (50 µM) are represented in Fig. 4, and the result shows that rhamnazin (4), tamarixetin (5), QGlcA (7), QGlcA-Me (8), and KGlcA (9) have the highest inhibition percentage. The antioxidant potential of QGlcA (7) correlated with its amyloid aggregation inhibition potential, proving that it can be also used for the prevention of oxidative stress.

The present in vitro study reports, for the first time, that QGlcA (7) and KGlcA (9) function as hIAPP aggregation inhibitors and suggests that the flavonoids with catechol and glucuronide moieties, that show Aβ aggregation inhibition, can also be used for hIAPP aggregation prevention. Extending this study to other amyloidogenic diseases would also be of interest.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.
REFERENCES


