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

Asma Ben Hmidene,a Mizuho Hanaki,b Kazuma Murakami,b Kazuhiro Irie,b Hiroko Isoda,c,d and Hideyuki Shigemori*a

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

Received October 12, 2016; accepted November 11, 2016

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.1–4)

Amyloid β (Aβ) is the component of the amyloid deposits in the AD brain,5) 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.6,7) Although the amino acid sequences of amyloidogenic proteins are diverse, they all adopt a similar structure in aggregates called cross-β-spine.8,9) Extensive studies in the past years have found that, similar to Aβ1–42, IAPP forms early intermediate assemblies as spherical oligomers10) that are recognized by soluble Aβ oligomers antibody,11) implicating that these oligomers possess a common folding pattern or conformation.4) The similarities in Aβ1–42 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,12–16) 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,17–19) 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 (Aβ1–42) was synthesized by the standard protocol.20) 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 CHCl3 (200 mL×2), EtOAc (200 mL×3), BuOH (200 mL×3), and H2O (200 mL). The CHCl3 layer was collected at 1.0 mL/min; MeCN/H2O–0.1% TFA (5:95) was purified by octadecyl-silane (ODS) HPLC [TSKgel ODS-80Ts (ϕ 250 mm, Tosoh Corporation, Japan), flow rate 1.0 mL/min; MeCN/H2O–0.1% trifluoroacetic acid (TFA) (5:95→30:70→40:60→40:60→55:55→100:0); detection UV (210, 254, 280 nm)] to yield naringenin (1, 3.4 mg, tR 41 min), quercetin21) (2, 1.2 mg, tR 43 min), rhamnetin21) (3, 2.9 mg, tR 46 min), and rhannazin22) (4, 1.6 mg, tR 72 min). Tamarixetin21) (5, 2.0 mg, tR 37 min) was purified from TGC-6 (100.1 mg) using ODS HPLC [TSKgel ODS-80Ts (ϕ4.6×250 mm), flow rate 1.0 mL/min; MeCN/H2O–0.1% TFA (5:95→30:70→40:60→40:60→55:55→100:0); detection UV (210, 254, 280 nm)].
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 (10:90–100:0), 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; MeOH/H2O–0.1% TFA (5:95→55:54→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, tk 37.5 min). TGE-6-3 (24.7 mg, tk 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, tk 9.3 min), and quercetin 3-O-β-D-glucuronide methyl ester (QGlcA-Me)23) (8, 9.0 mg, tk 17.6 min). Additionally, TGE-6-5 (20 mg, tk 32.3 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, tk 11.5 min), and kaempferol 3-O-β-D-glucuronide methyl ester (KGlca-Me)23) (10, 5.0 mg, tk 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 aggregating ability of the amyloid was evaluated at 37°C by the Th-T using Ab1–42 and hIAPP. Briefly, Ab1–42 was dissolved in 0.1% NH4OH at 250 mM and hIAPP in 1,1,3,3,4,4-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 2.5 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 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. Ab1–42 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 Ab1–42 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); 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,12,13,15,16 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.25 The SOD activity of flavonoids 1–10 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.

Acknowledgments We are grateful to Prof. Abderrazak Smaoui and Prof. Chedly Abdelly (Center of Biotechnology of Börj Cedria, Tunisia) for collecting the plant materials. This work was partially supported by the JST-JICA’s Science and Technology Research Partnership for Sustainable Development (SATREPS) and Grants-in-Aid for Scientific Research (C) (Grant No. 24580156) of Japan Society for the Promotion of Science (JSPS).

Conflict of Interest The authors declare no conflict of interest.

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

1) Barbagallo M, Dominguez L. Type 2 diabetes mellitus and Alzheimer's disease. *World J. Diabetes*, 5, 889–893 (2014).
2) Blázquez E, Valdez E, Hurtado-Carneiro V, Ruiz-Albusac JM. Insulin in the brain: Its pathophysiological implications for states related with central insulin resistance. Type 2 diabetes and Alzheimer's disease. *Front. Endocrinol.*, 5, 161 (2014).
3) Takeda S, Sato N, Rakugi H, Morishita R. Molecular mechanisms linking diabetes mellitus and Alzheimer's disease: beta-amyloid peptide, insulin signaling, and neuronal function. *Mol. Biosyst.*, 7, 1822–1827 (2011).
4) Zhao WQ, Townsend M. Insulin resistance and amyloidogenesis as a common molecular foundation for type 2 diabetes and Alzheimer's disease. *Biochim. Biophys. Acta*, 1792, 482–496 (2009).
5) Haass C, Koo EH, Mellon A, Hung HY, Selkoe DJ. Targeting of cell-surface beta-amyloid precursor protein to lysosomes: alternative processing into amyloid-bearing fragments. *Nature*, 357, 500–503 (1992).
6) Cooper GJ, Willis AC, Clark A, Turner RC, Sim RB, Reid KB. Purification and characterization of a peptide from amyloid-rich pancreases of type 2 diabetic patients. *Proc. Natl. Acad. Sci. U.S.A.*, 84, 8628–8632 (1987).
7) Janson J, Laedtke T, Parisi JE, O'Brien P, Petersen RC, Butler PC. Increased risk of type 2 diabetes in Alzheimer's disease. *Diabetes*, 53, 474–481 (2004).
8) Toyama BH, Weissman JS. Amyloid structure: Conformational diversity and consequences. *Angew. Rev. Biochem.*, 80, 557 (2011).
9) Porat Y, Koulshchev S, Jelinek R, Gazit E. The human islet amyloid polypeptide forms transient membrane-active prefibrillar assemblies. *Biochemistry*, 42, 10971–10977 (2003).
10) Green JD, Goldsbury C, Kistler J, Cooper GJ, Aebi U. Human amyloid oligomer growth and fibril elongation define two distinct phases in amyloid formation. *J. Biol. Chem.*, 279, 12206–12212 (2004).
11) Kayed R, Head E, Thompson JL, McIntire TM, Milton SC, Cotman CW, Glabe CG. Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. *Science*, 300, 486–489 (2003).
12) Sato M, Murakami K, Uno M, Nakagawa Y, Katayama S, Akagi K, Masuda Y, Takegoshi K, Irie K. Site-specific inhibitory mechanism for amyloid β42 aggregation by catechol-type flavonoids targeting the Lys residues. *J. Biol. Chem.*, 288, 22322–22324 (2013).
13) Sato M, Murakami K, Uno M, Ikubo H, Nakagawa Y, Katayama S, Akagi K, Irie K. Structure–activity relationship for (+)-taxifolin isolated from silymarin as an inhibitor of amyloid β aggregation.
14) Miyamae Y, Han J, Sasaki K, Terakawa M, Isoda H, Shigemori H. 3,4,5-Tri-O-cafeoylquinic acid inhibits amyloid β-mediated cellular toxicity on SH-SY5Y cells through the upregulation of PGAM1 and G3PDH. *Cytotechnology*, 63, 191–200 (2011).
15) Miyamae Y, Kurisu M, Murakami K, Han J, Isoda H, Irie K, Shigemori H. Protective effects of cafeoylquinic acids on the aggregation and neurotoxicity of the 42-residue amyloid β-protein. *Bioorg. Med. Chem.*, 20, 5844–5849 (2012).
16) Kurisu M, Miyamae Y, Murakami K, Han J, Isoda H, Irie K, Shigemori H. Inhibition of amyloid β aggregation by acetoside, a phenyl-ethanoid glycoside. *Biosci. Biotechnol. Biochem.*, 77, 1329–1332 (2013).
17) Tabassum N, Chatturvedi S, Agrawal SS. Effect of *Tamarix gallica* leaves on experimental liver cell injury. *JK Practitioner*, 13, 43–44 (2016).
18) Ksouri R, Falleh H, Megdiche W, Trabelsi N, Hmadhi B, Chaieb K, Bakrouf A, Magne C, Abdellly A. Antioxidant and antimicrobial activities of the edible medicinal halophyte *Tamarix gallica* L. and related polyphenolic constituents. *Food Chem. Toxicol.*, 47, 2083–2091 (2009).
19) Mayuresh R, Andrzej P, Patrycja LK, Wojciech Z, Rafal F. Herbal medicine for treatment and prevention of liver diseases. *J. Preclinical Clin. Res.*, 8, 55–60 (2014).
20) Murakami K, Irie K, Ohigashi H, Hara H, Nagao M, Shimizu J, Shirasawa T. Formation and stabilization model of the 42-mer Abeta radical: implications for the long-lasting oxidative stress in Alzheimer’s disease. *J. Am. Chem. Soc.*, 127, 15168–15174 (2005).
21) Saewan N, Koysonbboom S, Chantrapromma K. Anti-tyrosinase and anti-cancer activities of flavonoids from *Blumea balsamifera* DC. *J. Med. Plants Res.*, 18, 1018–1025 (2011).
22) Paya M, Manez S, Villar A. Flavonoid constituents of *Rhamnus lycioides* leaves on experimental liver cell injury. *JK Practitioner*, 13, 202–208 (2016).
23) kayed R, Head E, Thompson JL, McIntire TM, Milton SC, Cotman CW, Glabe CG. Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. *Science*, 300, 486–489 (2003).
24) Maltese F, Erkelens C, van der Kooiy F, Choi YH, Verpoorte R. Identification of natural epimeric flavanone glycosides by NMR spectroscopy. *Food Chem.*, 116, 575–579 (2009).
25) Park J, Ryu J, Jin LH, Bahn JH, Kim JA, Yoon CS, Kim DW, Han KH, Eum WS, Kwon HY, Kang TC, Won MH, Kang JH, Cho SW, Choi SY. 9-Polylysine protein transduction domain: Enhanced penetration efficiency of superoxide dismutase into mammalian cells and skin. *Mol. Cells*, 13, 202–208 (2002).