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Inhibitory effects of *Chrysanthemum* species extracts on formation of advanced glycation end products

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ABSTRACT

The corolla of *Chrysanthemum* species (*C. morifolium* R. and *C. indicum* L.) has long been used to treat eye and inflammatory disease. However, little is known about the antiglycation properties of *Chrysanthemum* species. Our study sought to characterise their activity against the formation of advanced glycation end products (AGEs) in glycation model reactions. In BSA/glucose (fructose) systems, both *Chrysanthemum* species strongly inhibited the formation of AGEs and N^{ε}-(carboxymethyl)lysine (CML). *C. morifolium* R., not *C. indicum* L., also acted to inhibit the formation of fluorescent AGEs, including pentosidine. This difference correlated with the values of polyphenol and flavonoid components. We characterised the active components in these plants by liquid chromatography-diode array detector-atmospheric pressure chemical ionisation/mass spectrometry, which showed that *C. morifolium* R. contains large amounts of chlorogenic acid, flavonoid glucoside varieties, and apigenin, while *C. indicum* L. contains large amounts of caffeic acid, luteolin, and kaempferol. Our findings raise hopes for the successful treatment of pathogenesis in conditions associated with diabetic complications and aging.

1. Introduction

Glycation is a non-enzymatic browning reaction caused by amino-carbonyl reactions between reducing sugars and amino groups of proteins and lipids. Several reports mention the damage done by such reactions within the body, identifying them as the pathogenesis in conditions associated with diabetic complications and aging (Monnier & Cerami, 1981; Monnier, Kohn, & Cerami, 1984). The non-enzymatic reaction leads to cumulative chemical modifications of tissue proteins, called AGEs, resulting in functional disturbances in proteins such as collagen, low density lipoprotein, and lens crystallines (Garlick, Mazer, Chylack, Tung, & Bunn, 1984; Lyons, Bailie, Dyer, Dunn, & Baynes, 1991; Monnier et al., 1984; Press and Wilding, 1989). Glycation initially progresses to form the reversible Schiff base formation. Subsequently, the products of the Amadori rearrangement involving Maillard reactions give rise to a number of complex reactions (e.g., dehydration, oxidation, cyclisation, scission), all leading to the formation of AGEs, including fluorescent (e.g., pentosidine) and non-fluorescent (e.g., CML) adducts (Ahmed, Thorpe, & Baynes, 1986; Akagawa, Sasaki, Kurota, & Suyama, 2005; Sell and Monnier, 1989).

Amino acids, proteins, and sugars account for a large share of the functional constituents of living systems, these reactions play significant roles in our daily lives (Brownlee, Vlassara, Kooney, Ulrich, & Cerami, 1986; Uchida et al., 1997). Various therapeutic agents that inhibit or reverse the progress of glycation have been examined. A representative drug is aminoguanidine (AG), a hydrazine compound, which prevents AGE formation by trapping intermediates at the initial glycation stages (Brownlee et al., 1986). Recent attention has focused on the benefits of medicinal plants with both antiglycation and antioxidant properties (Booth, Khalifah, & Hudson, 1996; Kiho, Usui, Hirano, Aizawa, & Inakuma, 2004; Osawa and Kato, 2005). Cervantes-Laurean et al. (2006) report that rutin and its metabolites effectively inhibit the formation of CML but do not inhibit the formation of fluorescent adducts such as pentosidine, indicating that such inhibitory effects do not apply equally to all AGEs. Preventing the accumulation of AGE varieties in diabetic complications and in the aging process, will likely require the combination of several approaches.

The corolla of *Chrysanthemum morifolium* R. is a herb widely used in traditional medicine in China and Japan to treat eye and inflammatory disease and is used in formulas as an analgesic and antipyretic agent. The corolla of *C. indicum* L. is used as a folk medicine in China and as a traditional medicine in Japan. Several recent

Abbreviations: AGEs, advanced glycation end products; AG, aminoguanidine; BSA, bovine serum albumin; *C. indicum* L. and *morifolium* R., *Chrysanthemum indicum* L. and *morifolium* R.; CML, N^{ε}-(carboxymethyl)lysine; DPPH, 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate buffered-saline; Glu, glucoside; AGlu, acetyl glucoside; Neo, neohesperidoside; CQAs, caffeoylquinic acids.

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reports indicate that the *Chrysanthemum* species possess antimicrobial activity and vasodilating effects in coronary hemodynamics (Kato et al., 1986; Shunying, Yang, Huaidong, Yue, & Guolin, 2005). Moreover, the *Chrysanthemum* species have been demonstrated to inhibit aldose reductase (Matsuda, Morikawa, Toguchida, Harima, & Yoshikawa, 2002; Terashima, Shimizu, Horie, & Morita, 1991), suggesting that these plants have therapeutic benefits against diabetic disease. However, little is known about the antiglycation activity of these two *Chrysanthemum* species. In the present study, we verified the effects of the *Chrysanthemum* species on the formation of AGEs such as CML and pentosidine using glycation model systems. We show that these two *Chrysanthemum* species strongly inhibit the formation of various AGEs. The results reported herein should create new avenues for exploring pharmacological treatments to prevent glycation and related disease conditions.

2. Materials and methods

2.1. Materials

Acacetin, apigenin, chlorogenic acid, kaempferol, luteolin, quinic acid, and quercetin were purchased from Sigma–Aldrich (St. Louis, MO). Aminoguanidine hydrochloride and bovine serum albumin (BSA) (fraction V; fatty acid free) were purchased from Wako Pure Chemicals Industries (Osaka, Japan). Antibodies against AGE (6D12) and CML (CMS-10) was purchased from Trans Genic Inc. (Hyogo, Japan). Goat anti-mouse horseradish peroxidase was purchased from BD Biosciences (San Jose, CA). Quantification of pentosidine in BSA/fructose system was determined by a pentosidine kit (Fushimi Pharmaceutical Co., Kagawa, Japan). All other chemicals were of the highest commercially available grade.

2.2. Extract preparation

Samples of *C. indicum* L. and *C. morifolium* R. were prepared from the corolla of each *Chrysanthemum* species purchased commercially and pulverised in liquid nitrogen. Extracts were obtained from each species (500 g) *via* extraction with 4 l of water at room temperature over the course of 3 days. The resulting solution was centrifuged and filtered and the supernatant removed, evaporated, and freeze-dried under a vacuum. The residue (100 mg) was then dissolved in 1 ml of water.

2.3. Formation of AGE in the BSA/glucose and BSA/fructose systems

AGE was formed in *in vitro* systems by the method previously described (Kiho et al., 2004). In brief, BSA (10 mg/ml) in phosphate buffered-saline (PBS, pH 7.4) containing 0.02% sodium azide was incubated with glucose (500 mM) at 37 °C for 0, 7, 14, 21, and 28 days in the absence (control) and presence of each of the extracts (2.5–5.0 mg/ml) or AG (1 mM). The protein, sugar, and prospective inhibitor were simultaneously introduced into the incubation mixture. BSA (5 mg/ml) was also incubated with fructose (100 mM) in PBS (pH 7.4) containing 0.02% sodium azide at 37 °C. AG (1 mM) was used as a positive inhibitor; control reactions in the absence of each of the extracts (2.5–5.0 mg/ml) as prospective inhibitors were also established. Each solution was kept in the dark in a capped vial, and incubation was allowed to proceed in triplicate vials. For time course experiments involving fluorescent AGE formation, we measured characteristic fluorescence (excitation wavelength of 370 nm and emission wavelength of 440 nm) with a 1420 ARVO series multilabel counter (Perkin–Elmer Japan Corp., Ltd., Kanagawa, Japan). Each sample taken was immediately frozen at −80 °C to await analysis by Western blot and ELISA.

2.4. Western blot

Each sample was fractionated on a polyacrylamide-SDS gel, after which the proteins were transferred to nitrocellulose membranes using a semidry blotter (BIO CRAFT Co., Ltd., Tokyo, Japan) and incubated in blocking solution (5% non-fat dry milk in PBS containing 0.1% Tween-20) for 1 h to reduce non-specific binding. Membranes were then exposed to primary antibodies (overnight at 4 °C). Thereafter, the blot was washed, exposed to HRP-conjugated secondary Abs for 1 h, and finally detected using an ECL Plus Western blotting detection system (GE Healthcare Biosciences, Piscataway, NJ).

2.5. ELISA

We performed an ELISA assay of pentosidine according to the manufacturer's instructions. In brief, each sample was subjected to pronase digestion at 55 °C for 1.5 h. The resulting reaction mixtures were boiled at 100 °C for 15 min to inactivate the enzyme, after which an EDTA solution (0.2 M) was added. The samples were dispensed into each well of a 96-well plate and incubated with a primary antibody at 37 °C for 1 h. After the plate was washed with TPBS, the wells were incubated with goat anti-rabbit horseradish peroxidase at room temperature for 1 h. After washing, 3,3′,5,5′-tetramethylbenzidine substrate solution was added and the samples incubated at room temperature until adequate colour developed. The enzyme reaction was stopped by adding a stop solution to each well. The absorbance at 450 nm was measured with a 1420 ARVO series multilabel counter, using 630 nm as a reference wavelength. Pentosidine was quantified from a calibration curve ranging from 5.0×10^{-5} to 5.0 (mg/ml).

2.6. Determination of total phenolic compounds

The concentration of total phenolic compounds in the water extracts was evaluated spectrophotometrically using Folin-Ciocalteu reagent, following a method slightly modified from that previously described (Julkunen-Tiitto, 1985). In brief, 100 μl of samples and the standard, previously dissolved in ethanol, was diluted with water to 0.45 ml, 0.5 ml of Folin-Ciocalteu phenol reagent was added, and the test tubes vigorously agitated. This was followed by the addition of 0.4 ml of 1 M sodium carbonate solution after which the mixtures were thoroughly agitated once again. The mixtures were allowed to stand at room temperature for 1 h, protected from light. The absorbance of the resulting reaction mixtures at 750 nm was measured with a GE Healthcare Biosciences Ultrospec 4300 pro UV/visible spectrophotometer. The concentration of total phenolic compounds for each extract was calculated on the basis of a standard curve obtained using gallic acid.

2.7. Determination of total flavonoids

The quantification of total flavonoids in each extract was performed by the method of Lamaison and Carnat modified for a microplate assay (Gálvez, Martín-Cordero, Houghton, & Ayuso, 2005). Each sample (100 μl) was added to a 96 well plate followed by 100 μl of a 2% $AlCl_3$ solution in methanol. After 10 min, absorbance at 415 nm was measured with a 1420 ARVO series multilabel counter. A standard curve was developed using quercetin.

2.8. HPLC-DAD

HPLC was performed with a JASCO Corp. gradient system equipped with dual Model PU-2089 plus pumps (10 ml pump heads), a Rheodyne (Cotati, CA) Model 7725i equipped with a 100 μl sample loop, and a JASCO Corp. Model MD-2010 plus mul-