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Original article

Effect of iridoid (containing plants) on AGE formation and degradation

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Abstract

***Objectives*:** Glycation is a non-enzymatic reaction between reducing sugars and the amino groups of proteins and lipids. This reaction produces advanced glycation end products (AGEs). AGEs are involved in diabetic complications, and plants containing iridoids are reported to prevent diabetes complications. However, it is unknown if these increase AGE degradation. In this study, the abilities of extracts from iridoid containing plants (*Morinda citrifolia*, *Cornus officinalis* and *Olea europaea*) to prevent AGE formation were tested with three model proteins (Human serum albumin, collagen and keratin). Further, the AGE degrading activity of these extracts was determined by examining their impact on the α-diketone structure of 1-phenyl-1, 2-propanedione (PPD).

***Method*:** To measure the inhibition of AGE formation, each model protein was incubated with glucose and samples at 60°C. Changes in detectable fluorescent AGEs (excitation wavelength 370 nm, detection wavelength 440 nm) were used to determine the percent inhibition of AGE formation of each sample. Determination of AGE degradation activity involved reacting PPD solution with samples at 37°C, for 8 h. Changes in measured benzoic acid content (by HPLC), produced from cleavage of the α-diketone structure, was used to calculate AGE degrading activity.

***Result*:** *Morinda citrifolia* seed extract was the most active sample in preventing AGE formation in all model proteins. The other extracts were also active. But inhibition intensity differed by protein type. At 1 mg/mL, *Cornus officinalis* fruit juice extract was the strongest AGE breaker (degradation rate; 29.8%), followed by Olea europaea leaf extract (26.5%) and *Morinda citrifolia* seed extract (18.2%).

***Conclusion*:** The results demonstrate that iridoid containing plants may prevent AGE formation, as well as degrade existing AGEs.

***KEY WORDS*:** *Morinda citrifolia*, *Cornus officinalis*, *Olea europaea*, advanced glycation end products (AGEs), anti-glycation, cross-link break, iridoids

Introduction

Glycation is a non-enzymatic reaction between reducing sugars and the amino groups of proteins, lipids, and nucleic acids. Advanced glycation end products (AGEs), which are irreversible glycation products, trigger "glycative stress" and play an important role in the pathogenesis of diabetic complications [1,2], atherosclerosis [1,2] and osteoporosis [3], and in aging [4,5]. There has been a great deal of interest in glycative stress inhibitors. Researchers have developed some approaches such as: (1) managing blood sugar levels, (2) inhibiting AGE formation, and (3) breaking preexisting AGE derived cross-links. Inhibition of AGE formation has been investigated widely. One well-known antiglycating agent is aminoguanidine (AG), which prevents the development of diabetic complication in vivo by inhibiting the formation of AGEs [6]. On the one hand, It has been reported that *N*-phenacylthiazolium bromide (PTB) may have therapeutic value in metabolizing AGEs due to its ability to cleave C-C bonds of alpha-diketone structures [7]. But studied less intensively are mechanisms of involving the breaking of preexisting AGE derived cross-links. There is limited evidence for the clinical effectiveness and safety of AG [8]. But plant-based AGE inhibitors, whose safety profiles are supported by long consumption histories, are also expected to reduce glycative stress.

Noni (*Morinda citrifolia*), cornelian cherry (*Cornus officinalis*) and olive (*Olea europaea*) are traditional food sources and have been also been used as traditional medicines. French Polynesians and other indigenous groups throughout the tropics have used noni fruits, leaves, seeds, and flowers for general health problems [9]. Cornelian cherry is a major herb in Traditional Chinese Medicine, with dried fruit

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powders being used for nutritive and analeptic purposes [10]. Olive leaf was used medicinally in Mediterranean countries, and multiple researchers report various health benefits [11].

These herbs contain a common phytonutrient group, iridoids. These include deacetylasperulosidic acid (DAA) in noni, loganin in cornelian cherry and oleuropein in olive [12-14]. Iridoids in noni, cornelian cherry, and olive are reported to mitigate diabetes induced liver and kidney damage [15-18]. Further, cornelian cherry inhibits AGE formation and improves diabetic conditions [18]. The object of this study was to evaluate the ability of iridoid containing plants to inhibition AGE formation and reduce preexisting AGE cross-links.

Materials and methods

Plant materials

Morinda citrifolia fruits (MCF), leaves (MCL) and seeds (MCS) were collected in French Polynesia. The fruits were separated into flesh and seeds by hand. The flesh was then freeze-dried. The leaves and seeds were air-dried. Aqueous ethanol extracts of noni fruit flesh, leaves and seeds were prepared at Kinki University, as described previously [19]. *Cornus officinalis* fruit (COF), grown in China, was extracted with aqueous ethanol. *Olea europaea* leaves (OEL), grown in Morocco, were extracted with ethanol.

Reagents

Deacetylasperulosidic acid (DAA) was purchased from Chengdu Biopurify Phytochemicals Ltd. (Sichuan, China). Loganin was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Oleuropein was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Human serum albumin (HSA) was purchased from Sigma-Aldrich Japan (Tokyo, Japan). Collagen type I (bovine skin, Pepsin-solubilized) was purchased from Nippi, Incorporated (Tokyo, Japan). Keratin was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Aminoguanidine hydrochloride and quinine sulfate dihydrate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 1-phenyl-1, 2-propanedione (PPD) was obtained from Sigma-Aldrich Japan (Tokyo, Japan). *N*-phenacylthiazolium bromide (PTB) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

HSA, collagen and keratin glycation tests

Glycation tests were performed according to the methods described by Hori *et al.* [20], with minor modifications. Glucose in phosphate-buffer (PB; pH 7.4) was combined with HSA, collagen and keratin, as described in ***Table 1***. The test samples and aminoguanidine (the positive control) were dissolved in

Table 1. Preparation of glycation test reaction solution

A. HSA-glucose model

	Final conc.	Glucose (+)	Glucose (-)
PB (100 mM)	50 mM	500 µL	500 µL
Glucose solution (2M)	0.2 M	100 µL	
Water	–	100 µL	200 µL
HSA solution (40 mg/mL)	8 mg/mL	200 µL	200 µL
Sample (50% DMSO)	5% DMSO	100 µL	100 µL

B. Collagen-glucose model

	Final conc.	Glucose (+)	Glucose (-)
PB (100 mM)	50 mM	500 µL	500 µL
Glucose solution (2M)	0.4 M	200 µL	
Water	–		200 µL
Collagen solution (3 mg/mL)	0.6 mg/mL	200 µL	200 µL
Sample (50% DMSO)	5% DMSO	100 µL	100 µL

C. Keratin-glucose model

	Final conc.	Glucose (+)	Glucose (-)
PBS (100 mM)	50 mM	500 µL	500 µL
Glucose solution (2M)	0.4 M	200 µL	
Water	–		200 µL
Keratin solution (3 mg/mL)	0.6 mg/mL	200 µL	200 µL
Sample (50% DMSO)	5% DMSO	100 µL	100 µL

dimethyl sulfoxide (DMSO) and diluted with water to a DMSO concentration of 50% v/v. The control consisted of only 50% DMSO in water. After incubation at 60°C, the fluorescence of reaction products was measured in a microplate reader (SpectraMax Paradigm Malti-Mode Microplate Reader; Molecular Devise, Sunnyvale, CA, USA) with excitation at 370 nm and emission at 440 nm. Each fluorescence intensity was calculated as a relative value, comparing emission values (reported by the microplate reader) of samples and controls to that of 5 μg/mL quinine sulfate in 0.1 N sulfuric acid aqueous solution (this was set at 1000). Inhibition of fluorescent AGE formation was calculated by the following formula:

Inhibition (%) = (Control $_{FI*}$ - Sample $_{FI*}$) / Control $_{FI*}$ × 100.

*FI: fluorescence intensity = (emission value of Glucose (+) – emission value of Glucose (-)) / quinine sulfate × 1000.

Cross-link breakage test

Following the method described by Yagi *et al.*[21], with a modification, 1-phenyl-1, 2-propanedione (PPD), dissolved in 50% acetonenitrile, was used as a reactive substrate in the AGE cross-link model. In this test, 100 μL of sample solution as added to 100 μL PPD (10 mM) and 400 μL PB (200 mM) and mixed. The test samples and PTB (as a positive control) were dissolved into 50% DMSO (final concentration 5% DMSO v/v). The mixture was incubated at 37°C for 8h. After incubation, the reaction was stopped by adding 200 μL hydrochloric acid (2M), followed by centrifugation at 10000 rpm for 2 min. The supernatant (50 μL) was analyzed by high performance liquid chromatography (HPLC: LC-10A; Shimadzu, Kyoto, Japan) for benzoic acid. HPLC conditions were: Cadenza CD-C18 column (75 × 4.6 mm ID, Imtakt, Kyoto, Japan); 40°C column temperature; mobile phase of 0.2% acetic acid in 2 mM ethyenediamine-*N, N, N', N'*-tetraacetic acid-disodium salt (EDTA-2Na)-dehydrate/acetonitrile (70/30, v/v); 1.0 mL/min flow rate; and detection at 270 nm and 3.3 min retention time. Benzoic acid is released as the alpha-diketone structure of PPD is cleaved [7]. Cross-link breaking activity was determined by dividing benzoic acid concentration by the original PPD concentration of the reaction solution. Preexisting benzoic acid concentrations of the samples were subtracted from the values measured in the final reaction solutions.

Statistical analysis

Means and standard deviations (SD) were determined for each of experiments described in the figures or tables. Intergroup differences were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test with Statcel Ver. 3 (OMS Publishing Inc., Saitama, Japan, 2011).

Results

Inhibition of HSA, collagen and keratin glycation

All herbal extracts (MCF, MCL, MCS, COF, and OEL) suppressed *in vitro* formation of fluorescent AGEs in all three protein models. All activities were in a concentration-dependent manner and statistically significant, compared to the control (***Table 2-A, B, C***). The HSA model (***Table 2-A*** HSA) revealed that all herbal extracts, except MCF, were very effective inhibitors of AGE formation with IC_{50}s lower than AG. In the collagen model, most herbal extracts suppressed AGE formation more effectively than AG (***Table 2-B***). In the keratin model, the IC_{50} of noni leaf (MCL) could not be determined due to the weak inhibition while other herbal samples showed strong activity (***Table 2-C***). These results supported our hypothesis that iridoid-containing plants (noni, cornelian cherry, and olive) had anti-glycation properties. However, their efficacies varied by the type of protein involved. For example, the IC_{50} values of AG in the HSA, collagen, and keratin tests were, respectively, 196, 150, and 516 μg/mL. Protein-dependent results were also observed for MCL. The IC_{50} values were 168 and 134 μg/mL with HSA and collagen. But no inhibition was observed when keratin was the glycation target. MCF and OEL were most effective at inhibiting AGE formation in the HSA model. The COF extract displayed the strongest inhibition activity in the collagen model. In all protein models, MCS extract exhibited the greatest anti-AGE activity. Taken together, the data suggest that anti-AGE efficacy of the iridoid sources varies by the type of protein involved.

Cross-link breaking activity of MCF, MCL, MCS, COF, OL and PTB

Glycative stress can be alleviated if accumulated AGEs were selectively degraded. Reportedly, AGEs that form intermolecular cross-links, such as pentosidine, are degraded and reduced by breaking the cross-links [7]. The PPD molecule is an AGE model compound because of its unique alpha-diketone structure. Because of this, we evaluated the ability of iridoid-containing herb extracts to degrade PPD. Analysis of benzoic acid released during the cleavage of the alpha-diketone structure of PPD revealed that cross-link breaking activities of COF, OEL, and MCS (1mg/mL) were 29.8%, 26.5%, and 18.2%, respectively (***Fig. 1***).

Cross-link breaking activity of DAA, loganin, oleuropein and PTB

Results from the cross-link breakage tests suggest that iridoids in the plant extracts cleave AGE cross-links. To confirm this, we investigated the breaking activity of individual iridoids, such as loganin in COF, oleuropein in OEL, and DAA in MCS. DAA and loganin demonstrated weak cross-link breaking activity, ***Fig. 2***. However, oleuropein from OEL caused benzoic acid release in a dose-dependent manner. This seems to indicate that OEL's cross-link breaking activity is due in part to oleuropein.

Discussion

Many researchers report that the accumulation of AGEs in tissues cause the progression of age related diseases. In particular, hyperglycemia induced AGEs are related to diabetic complications and may be partly responsible for the onset of diabetes. AGE inhibitors have been actively developed to prevent or alleviate these conditions. Because noni, cornelian cherry, and olive are reported to improve liver or kidney malfunctions caused by diabetes, we hypothesized that they possess anti-AGE properties through inhibition of AGE formation and by cross-link breaking. Previous studies have revealed that these herbs possess anti-glycation activities.

Table 2. Inhibition of AGE formation by MCF, MCL, MCS, COF, OL and AG

A. Glucose-HSA model

Samples	Conc. (μg/mL)	FI [a)]	Inhibition (%)	IC_{50} value [b)] (μg/mL)
Control	–	255.4 ± 2.8	–	–
MCF extract	200	174.4 ± 3.0**	32	349
	400	123.0 ± 3.5**	52	
	800	48.6 ± 0.8**	81	
MCL extract	100	172.0 ± 0.6**	33	168
	200	111.4 ± 2.4**	56	
	400	57.1 ± 1.1**	78	
MCS extract	13	197.1 ± 7.0**	23	34
	25	149.9 ± 6.5**	41	
	50	102.1 ± 2.9**	60	
COF extract	25	209.3 ± 1.1**	18	66
	50	155.2 ± 4.8**	39	
	100	90.1 ± 5.6**	65	
OEL extract	100	167.8 ± 28.2**	34	181
	200	112.6 ± 20.1**	56	
	400	85.2 ± 23.6**	67	
AG	100	195.3 ± 19.8**	24	196
	200	131.0 ± 15.5**	49	
	400	50.4 ± 7.3**	80	

[a)] FI: fluorescence intensity. [b)] IC_{50} is the concentration of sample required to inhibit 50% of AGE formation.
Each value represents the mean ± SD of 3 experiments. **: $p<0.01$, significantly different from the control group.

B. Glucose-collagen model

Samples	Conc. (μg/mL)	FI [a)]	Inhibition (%)	IC_{50} value [b)] (μg/mL)
Control	–	210.2 ± 2.9**	–	–
MCF extract	200	100.1 ± 1.6**	52	175
	400	55.0 ± 2.5**	74	
	800	23.5 ± 1.2**	89	
MCL extract	200	91.8 ± 6.1**	56	134
	400	69.0 ± 0.8**	67	
	800	46.0 ± 4.7**	78	
MCS extract	6	158.7 ± 1.6**	25	15
	13	113.0 ± 0.1**	46	
	25	79.4 ± 1.7**	62	
COF extract	13	111.1 ± 1.7**	47	15
	25	85.1 ± 1.5**	60	
	50	59.1 ± 2.0**	72	
OEL extract	25	138.0 ± 2.9**	34	42
	50	95.2 ± 4.7**	55	
	100	48.2 ± 2.8**	77	
AG	100	129.6 ± 1.0**	38	150
	200	87.6 ± 0.6**	58	
	400	44.8 ± 1.7**	79	

[a)] FI: fluorescence intensity. [b)] IC_{50} is the concentration of sample required to inhibit 50% of AGE formation.
Each value represents the mean ± SD of 3 experiments. **: $p<0.01$, significantly different from the control group.

C. Glucose-keratin model

Samples	Conc. (μg/mL)	FI [a)]	Inhibition (%)	IC_{50} value [b)] (μg/mL)
Control	–	233.6 ± 2.5	–	–
MCF extract	50	181.8 ± 0.9**	22	146
	100	142.3 ± 1.3**	39	
	200	96.6 ± 0.5**	59	
MCL extract	50	145.2 ± 1.1**	38	–
	100	130.5 ± 0.8**	44	
	200	124.3 ± 1.2**	47	
MCS extract	6	190.7 ± 1.0**	18	20
	13	153.9 ± 3.1**	34	
	25	101.1 ± 6.2**	57	
COF extract	50	158.7 ± 1.1**	32	101
	100	121.2 ± 3.4**	48	
	200	72.2 ± 2.5**	69	
OEL extract	50	135.1 ± 2.7**	42	67
	100	89.6 ± 1.1**	62	
	200	64.5 ± 6.9**	72	
AG	200	182.0 ± 1.8**	22	516
	400	136.4 ± 4.4**	42	
	800	85.5 ± 6.0**	63	

[a)] FI: fluorescence intensity. [b)] IC_{50} is the concentration of sample required to inhibit 50% of AGE formation.
Each value represents the mean ± SD of 3 experiments. **: $p<0.01$, significantly different from the control group.

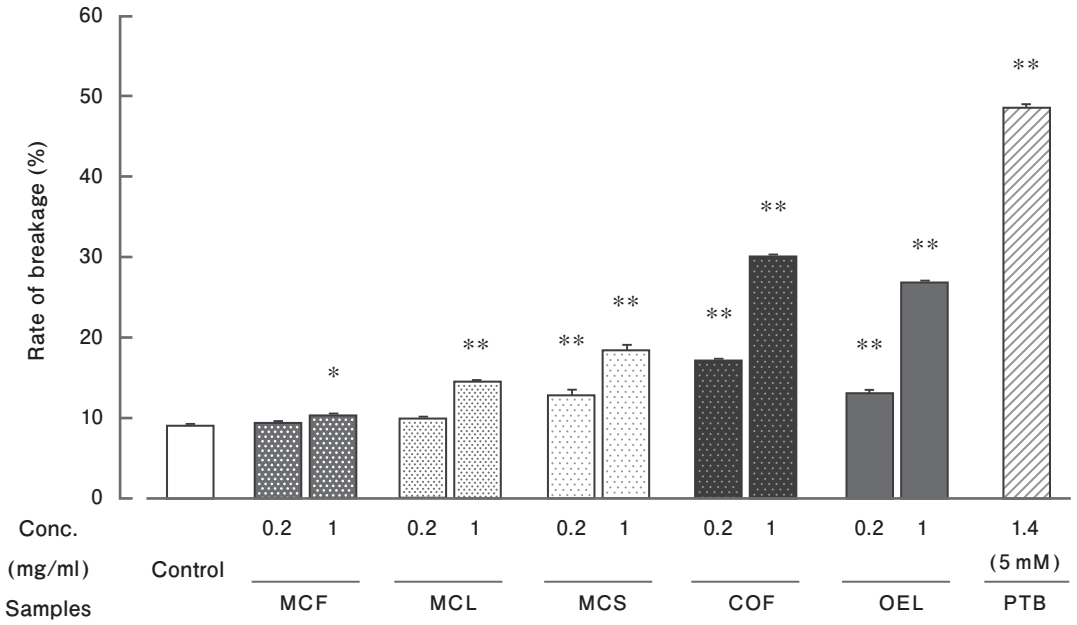

Fig. 1. **Cross-link breaking activity of MCF, MCL, MCS, COF, OL and PTB**

Each value represents the mean ± SD of 3 experiments. *: $p<0.05$, **: $p<0.01$, significantly different from the control group.

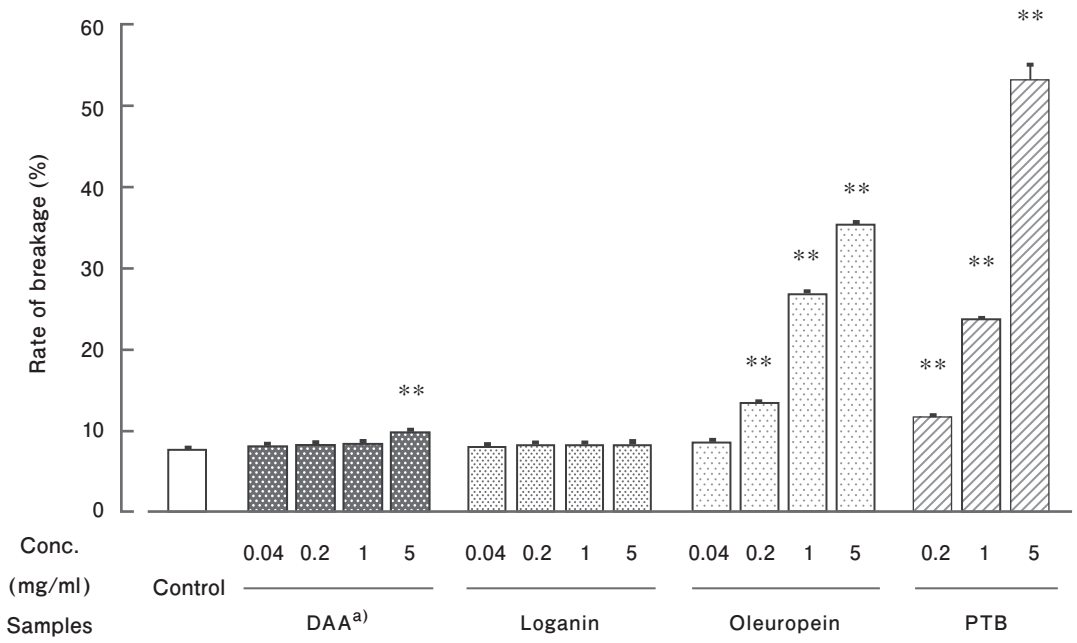

Fig. 2. **Cross-link breaking activity of MCF, MCL, MCS, COF, OL and PTB**

[a)] DAA; deacetylasperulosidic acid. Each value represents the mean ± SD of 3 experiments. **: $p<0.01$, significantly different from the control group.

Kusirisin *et al*. reported noni (fruits and leaves) ethanol extracts had strong anti-glycation activity (IC_{50}; 0.01 μg/mL) [22]. Park *et al*. reported that an aqueous ethanol extract of cornelian cherry (25 μg/mL) inhibited AGE formation by 12.8% [22]. Kontogianni *et al*. demonstrated that an aqueous methanol extract of olive leaf reduced AGE formation significantly [24]. Previous studies have shown that AGE formation depends on the reaction condition and source of protein [20, 25]. In the present study, we also demonstrated that the degree of inhibition was affected by protein type. As such, a single method may not enough to evaluate overall anti-glycation activity, and the target protein has to be carefully selected to align with testing purposes. Further study is also important to evaluate non-fluorescent AGEs, such as N^{ε}-(carboxymethyl) lysine (CML).

Even though there are many reports of botanical extracts reducing AGE formation, there are very few which report both inhibition of formation and breaking of existing cross-links. Here we developed two approaches for testing anti-AGE capacity. The first involved a more typical anti-glycation assay. The second approach involved the evaluation of cross-link breaking activity as determined by the release of benzoic acid after incubation of PPD with COF, OEL, MCF, or MCS.

Our findings indicate that olueropein is an active component of OEL that is capable of breaking AGE cross-links. Yagi *et al*. investigated the breaking activity of ellagitannin compounds in *Punica granatum* and reported that trihydroxybenzenes and dihydroxybenzene exhibited the strongest effects [21]. Oleuropein, unlike other iridoids, has the dihydroxybenzene structure (***Fig. 3***). This chemical structure may contribute the observed cleaving activity. This study is an important early step towards understanding how iridoid sources may treat glycative stress through the breaking of AGE cross-links.

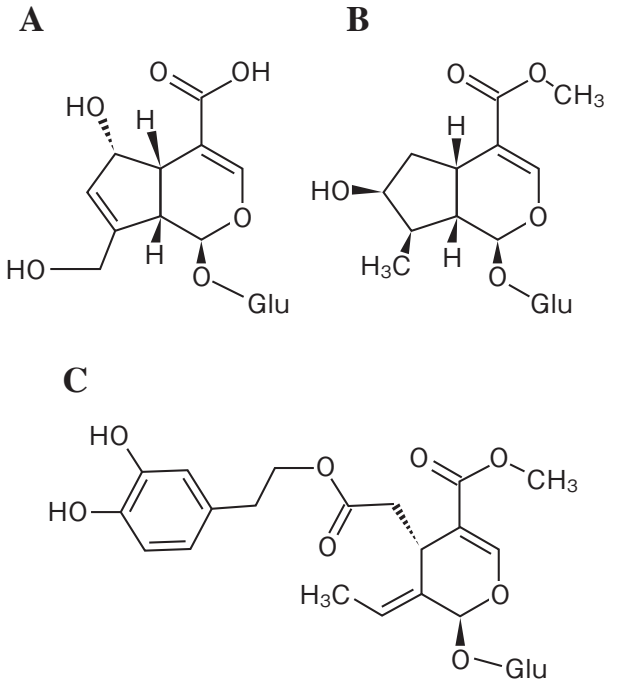

Fig. 3. **Chemical structure of deacetylasperulosidic acid (A), loganin (B), oleuropein (C)**

Conclusion

In all protein models, MCS suppressed AGE formation more significantly than the other extracts. MCF and MCS inhibited AGE formation in three protein models but did not exhibit substantial cross-link breaking properties. But MCS did have modest cross-link breaking activity. The anti-AGE properties of COF and OEL involved both inhibition of AGE formation as well as cross-link breaking activity. These results suggest that MCS, COF, and OEL are potential therapeutic materials which may help prevent and treat glycative stress.

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Conflict of interest

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