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Nonenzymatic Antioxidative and Antiglycative Effects of Oleanolic Acid and Ursolic Acid

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Oleanolic acid and ursolic acid are two triterpenes presented in several herbs. This study analyzed the content of oleanolic acid and ursolic acid in eight locally available fresh herbs, also examined several nonenzymatic antioxidant activities of these two triterpenes, and used a liposome system to evaluate the influence of temperature and pH upon the antioxidant property of these two triterpenes. The impact of these two triterpenes on the production of nonenzymatic glycative products, pentosidine and carboxymethyllysine (CML), was also evaluated. α-Tocopherol was used for comparison. Results showed that the content of oleanolic acid and ursolic acid in glossy privet fruit and hawthorn fruit varied from season to season and was in the range of 200−650 μg/g of fresh weight. Both oleanolic acid and ursolic acid possessed greater antioxidant activity against 2,2′-azobis-(2-amidinopropane) dihydrochloride and less antioxidant activity against 2,2′-azobis(2,4-dimethylvaleronitrile) when compared with α-tocopherol at equal concentration (P <0.05). At 75 and 100 °C, oleanolic acid exhibited greater antioxidant activity than α-tocopherol and ursolic acid (P <0.05). At pH 2 and pH 4, oleanolic acid and ursolic acid showed greater antioxidant activity than α-tocopherol (P <0.05). These two triterpenes also exhibited a dose-dependent effect in superoxide anion scavenging activity, chelating effect, xanthine oxidase inhibition activity, and reducing power (P <0.05). Oleanolic acid significantly and dose-dependently inhibited pentosidine and CML formation (P <0.05). Ursolic acid also significantly suppressed CML formation (P <0.05). These data support that these two triterpenes possessed nonenzymatic antioxidative and antiglycative properties.

KEYWORDS: Oleanolic acid; ursolic acid; oxidation; glycation; glossy privet fruit; hawthorn fruit

INTRODUCTION

Glossy privet fruit (*Ligustrum lucidum* Ait.) and hawthorn fruit (*Crataegi pinnatifidae* Fructus) are two herbs commonly used in Chinese medicine. The antioxidative effect of these herbs has been observed *(1, 2*), and those authors indicated that the observed antioxidative activity of these herbs could be ascribed to their triterpene or glucoside components such as oleanolic acid and ursolic acid. Although several studies have reported that oleanolic acid and ursolic acid could exhibit antioxidant protection in experimental hypertension, leukemic cells, and rat liver microsomes (*3*−*5*), less attention was paid to their nonenzymatic antioxidative properties such as reducing power, metal ion chelating effect, and free-radical scavenging activity. On the other hand, the influence of temperature and pH on the antioxidant activity of these two compounds remains unknown. If these agents could exhibit antioxidative protection in a higher temperature or lower pH environment, they will be more useful in a wide variety of food systems.

Pentosidine and carboxymethyllysine (CML) are nonenzymatic advanced glycative end products formed from reactive carbonyl compounds with proteins, and they contribute to the progression of glycation-associated diseases such as diabetes and Alzheimer's disease (*6*−*8*). Several studies have indicated that the nonenzymatic antiglycative capability of an agent could be evaluated by measuring its in vitro inhibitory effect on the production of pentosidine and CML (*9, 10*). So far, it is unclear that oleanolic acid and ursolic acid possess antiglycative activity. If these two compounds could inhibit the formation of pentosidine, CML, or both, they might be used as a supplement for people with glycation-associated diseases to retard or alleviate glycative damage.

In this study, the content of oleanolic acid and ursolic acid in glossy privet fruit, hawthorn fruit, and other six locally available herbs was analyzed. A liposome system was used to study the influence of temperature and pH on the antioxidant activity of these compounds. Furthermore, the nonenzymatic antioxidant property of these two compounds was examined. The impact of these two triterpenes on the production of pentosidine and CML was also evaluated.

MATERIALS AND METHODS

Chemicals. Oleanolic acid (99%) and ursolic acid (98%) were purchased from Aldrich Chemical Co. (Milwaukee, WI). In this study, the antioxidant activities of these compounds were compared with α-tocopherol purchased from Sigma Chemical Co. (St. Louis, MO).

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2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH), 2,2′-azobis-(2,4-dimethylvaleronitrile (AMVN), trichloroacetic acid (TCA), thiobarbituric acid (TBA), bovine serum albumin (BSA), and chemicals used for liposome preparation were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). All chemicals used in these measurements were of the highest purity commercially available.

Determination of Oleanolic Acid and Ursolic Acid Content. Eight locally available fresh herbs were used to analyze the content of oleanolic acid and ursolic acid. These herbs included glossy privet fruit (*Ligustrum lucidum* Fructus), hawthorn fruit (*Crataegi pinnatifidae* Fructus), *Perilla ocymoides* L., mint (*Mentha piperita*), *Glycyrrhiza globora*, *Paeonia lactiflora* Pall, *Hibiscus sabdariffa* L., and *Mours alba* L. These herbs, harvested in spring and fall 2005 were purchased from five botanical gardens in Taichung County, Taiwan. The content of oleanolic acid and ursolic acid in these herbs was analyzed by HPLC methods described in Liu et al. (*11*) and Cui et al. (*12*).

Liposome Preparation. Phosphatidylcholine (PC) with oleic acid (at the *sn*-1 position) and linoleic acid (at the *sn*-2 position) were purchased from Wako Pure Chemical Industries, Ltd. Liposomes (multilamellar vesicles) were prepared from PC, cholesterol, and dicetyl phosphate at 4 °C as described by Yin et al. (*13*). The buffer for a liposomes suspension was sodium citrate buffer (0.05 M, pH 2 and 4) or sodium phosphate buffer (PBS; 0.05 M, pH 6, 7, 8, and 10). After preparation, all samples were incubated at 4, 25, 37, 50, 75, and 100 °C for oxidation measurements.

Antioxidant Treatments. On the basis of lipid solubility, α-tocopherol, oleanolic acid, and ursolic acid were incorporated into multilamellar vesicles with phosphatidylcholine for liposome preparation. α-Tocopherol, oleanolic acid, and ursolic acid, at 5 or 10 μM, were used to measure the individual effectiveness of antioxidant action. The influence of temperature and pH on the antioxidant activity of these three compounds at 10 μM was measured.

Lipid Oxidation Measurements. For the individual antioxidant effectiveness of test compounds, 2 mM AAPH or 0.5 mM AMVN was added to liposomes to induce lipid oxidation. AMVN was first dissolved in 95% ethanol. The residue of ethanol in liposomes did not significantly affect the oxidation level (data not shown). For the effect of temperature and pH on the antioxidant activity of these test compounds, 10 μM $FeSO_4$ was added to liposomes to induce lipid oxidation. Lipid oxidation was measured by the TBA assay as described by Yin et al. (*13*). Briefly, 1 mL of sample was mixed with 0.5 mL of 30% TCA, and the mixture was centrifuged at 1400*g* for 5 min at 4 °C, and then 1 mL of supernatant was mixed with 1 mL of 0.02 M TBA and this mixture was stored in the dark for 20 h at 25 °C. The absorbance of the final solution was measured by a UV−vis spectrophotometer at 532 nm and recorded as the TBA number, which was directly used to express the lipid oxidation level. The lipid stability of purchased PC was examined, and the PC with TBA no. ϵ 0.01 was used for liposome preparation.

Superoxide Anion Production Assay. The production of superoxide anion was assayed by monitoring the reduction of cytochrome *c* method (*14*). Three test compounds at 5 or 10 μM were prepared in 50 mM PBS (pH 7). Control groups contained no test agent. Then, 1 mL of sample was mixed with 1 mL of solution containing 0.07 U/mL xanthine oxidase, 100 μM xanthine, and 50 μM cytochrome *c*. After incubating at room temperature for 3 min, the absorbance at 550 nm was determined by a spectrophotometer. Lower absorbance of the reaction mixture indicated greater superoxide anion scavenging activity. The inhibition percentage of superoxide anion generation was calculated according to the following formula: % inhibition = $(A_{control} - A_{sample})/A_{control} \times 100$.

Xanthine Oxidase Inhibition Assay. Xanthine oxidase activity was determined by measuring the formation of uric acid from xanthine. Three test compounds at 5 or 10 μM were prepared in 50 mM PBS (pH 7). Control groups contained no test agent. Then, 1 mL of sample was mixed with 1 mL of solution containing 0.4 U/mL xanthine oxidase and 100 μM xanthine. After incubating at room temperature for 3 min, uric acid production was determined by measuring the absorbance at 295 nm. Lower absorbance of the reaction mixture indicated greater xanthine oxidase inhibitory activity. The inhibition percentage of xanthine oxidase activity was calculated according to the following formula: % inhibition = $(A_{control} - A_{sample})/A_{control} \times 100$.

Chelating Effect on Ferrous Ions. The method of Shimada et al. (*15*) was used to determine the chelating effect of α-tocopherol, oleanolic acid, and ursolic acid on ferrous ions. Each agent in methanol (2 mg/mL) was mixed thoroughly with 200 μL of 1 mM tetramethyl murexide and 2 mL of a solution consisting of 30 mM hexamine, 30 mM potassium chloride, and 9 mM ferrous sulfate. Control groups contained no test agent. Absorbance at 485 nm was measured after 3-min incubation at 25 °C. Lower absorbance indicated higher iron chelating effect. In this study, the iron-chelating ability of the test agent was compared with that of EDTA and was expressed in percentage.

Reducing Power. The method of Oyaizu (*16*) was used to determine the reducing power of α-tocopherol, oleanolic acid, and ursolic acid. Each agent was dissolved in methanol (2 mg/mL) and then was mixed with a solution containing 2.5 mL of PBS (pH 6.6, 200 mM) and 2.5 mL of 1% potassium ferricyanide. After the mixture was incubated at 50 °C for 20 min, 2.5 mL of 10% TCA was added. Then, the resulting suspension was centrifuged at 650*g* for 10 min. The supernatant was mixed thoroughly with 5 mL of deionized water and 1 mL of 0.1% ferric chloride. Absorbance at 700 nm was measured and directly used to express reducing power. Higher absorbance indicated greater reducing power.

Glycative Product Formation and Determination. The method of Miyata et al. (*17*) was used for glycative product formation and measurement. Ten milligrams of fatty acid-free BSA was incubated with 100 mM glucose, 10 mM ascorbate, and 1 mM ribose in 5 mL of 0.1 M PBS (pH 7.2) for 4 weeks at 37 °C. Incubation was performed in the absence or presence of oleanolic acid, ursolic acid, or α-tocopherol. For pentosidine determination, 50 mL of sample was mixed with an equal volume of 10% TCA, and the resultant mixture was centrifuged at 5000*g* for 5 min. The pellet was washed with 300 mL of 5% TCA, dried under a vacuum, and hydrolyzed with 100 mL of 6 N HCl for 16 h at 110 °C under nitrogen. After being neutralized with 100 mL of 5 N NaOH and 200 mL of 0.5 M PBS (pH 7.2), the sample was filtered through a 0.5-mm-pore filter and finally diluted with PBS. Pentosidine level was analyzed by a HPLC equipped with a C18 reversed-phase column. Detection limit was 0.2 nmol of pentosidine/g of albumin. CML was determined by an ELISA technique using the CML-specific monoclonal antibody 4G9, and 6-(*N*-carboxymethylamino)caproic acid was used for calibration. Results are expressed in microgram per gram of albumin.

Statistical Analysis. The effect of each treatment was analyzed from 10 different preparations ($n = 10$). Data were reported as means ± standard deviation (SD) and subjected to analysis of variance (ANOVA). Differences among means were determined by the least significance difference test with significance defined at $P < 0.05$.

RESULTS

Both oleanolic acid and ursolic acid were detectable in glossy privet fruit and hawthorn fruit. The content of these two compounds in six other herbs was too low to be detected. As shown in **Figure 1**, the content of oleanolic acid and ursolic acid in glossy privet fruit and hawthorn fruit was in the range of 200−650 μg/g of fresh weight. Both herbs harvested in Fall had more oleanolic acid.

The antioxidant activity of α-tocopherol, oleanolic acid, and ursolic acid against AAPH or AMVN is shown in **Table 1**. Both oleanolic acid and ursolic acid exhibited a dose-dependent antioxidant effect against AAPH or AMVN-induced TBA formation ($P < 0.05$). When compared with α-tocopherol at equal concentration, oleanolic acid and ursolic acid showed greater antioxidant effect against AAPH and less antioxidant effect against AMVN ($P < 0.05$). The influence of temperature and pH on the antioxidant activity of α-tocopherol, oleanolic acid, and ursolic acid is shown in **Tables 2** and **3**. At 75 and 100 °C, oleanolic acid exhibited greater antioxidant activity than α-tocopherol and ursolic acid ($P < 0.05$). At lower pH environments such as pH 2 and pH 4, oleanolic acid and ursolic acid showed greater antioxidant activity than α-tocopherol ($P < 0.05$).

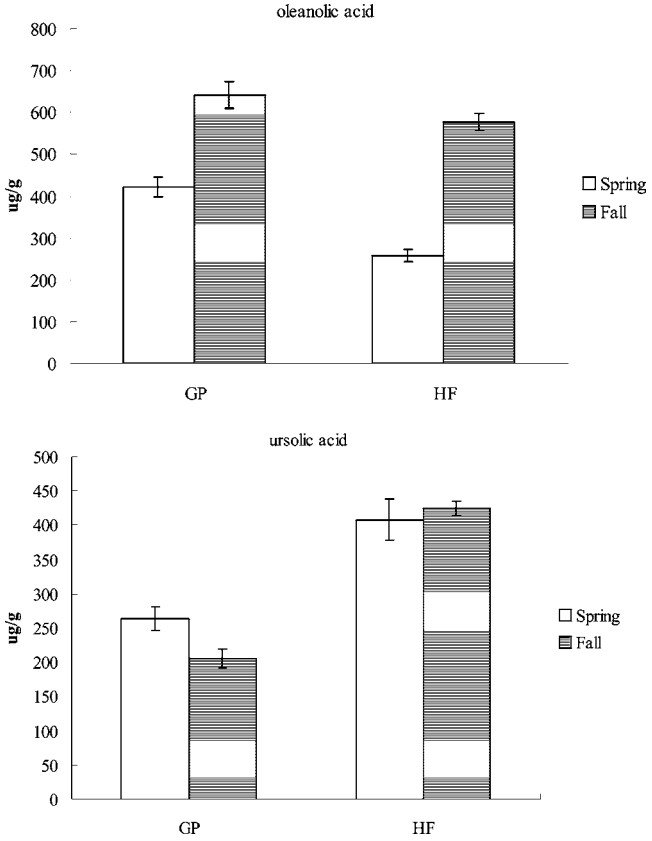

Figure 1. Content (μg/g of fresh weight) of oleanolic acid and ursolic acid in glossy privet fruit (GP) and hawthorn fruit (HF) harvested in spring and fall 2005. Data are mean ± SD ($n = 5$).

Table 1. Individual Antioxidant Activity of α-Tocopherol (α-Toc), Oleanolic Acid, and Ursolic Acid against AAPH- or AMVN-Induced TBA Formation (TBA No.) after a 72-h Incubation at 37 °C, pH 7.0 (Data Are Mean ± SD ($n = 10$))

		TBA no.	
treatment	conc (μM)	AAPH	AMVN
control[a]		0.583 ± 0.029 e	0.565 ± 0.034 f
α-Toc	5	0.386 ± 0.021 d	0.278 ± 0.028 d
	10	0.335 ± 0.016 c	0.168 ± 0.014 b
oleanolic acid	5	0.321 ± 0.025 c	0.350 ± 0.026 e
	10	0.238 ± 0.019 b	0.261 ± 0.020 c
ursolic acid	5	0.342 ± 0.024 c	0.375 ± 0.031 e
	10	0.260 ± 0.018 b	0.293 ± 0.026 d

[a] Controls contained no antioxidant agent. b–f: Least-squares means with a common letter within a column are not different at the 5% level.

Superoxide anion scavenging activity, chelating effect, xanthine oxidase inhibition activity, and reducing power of α-tocopherol, oleanolic acid, and ursolic acid are presented in **Table 4**. Both oleanolic acid and ursolic acid showed a dose-dependent effect in superoxide anion scavenging activity, chelating effect, xanthine oxidase inhibition activity, and reducing power ($P < 0.05$). When compared with α-tocopherol at equal concentration, oleanolic acid has higher superoxide anion scavenging activity and xanthine oxidase inhibition activity ($P < 0.05$).

The effect of oleanolic acid and ursolic acid on the production of pentosidine and CML is shown in **Figure 2**. Oleanolic acid exhibited significantly dose-dependent inhibition against pentosidine and CML formation ($P < 0.05$). Ursolic acid also significantly suppressed CML formation ($P < 0.05$), but its inhibition on pentosidine was not significantly ($P > 0.05$).

DISCUSSION

The presence of oleanolic acid and ursolic acid in hawthorn fruit and glossy privet fruit has been reported (*12*, *18*). Our present study further indicated that the content of oleanolic acid and ursolic acid in these herbs was in the range of 200−650 μg/g of fresh weight, and varied from season to season. Because more oleanolic acid was presented in both herbs harvested in Fall; thus, it is highly possible that seasonal temperature affects the synthesis of this component in these herbs.

The antioxidant activity of oleanolic acid and ursolic acid has been observed (*3*−*5*), and those authors reported these two compounds could scavenge free radicals. Our present study further found that these two compounds could protect liposomes against AAPH- and AMVN-induced oxidation; thus, these results agreed that these compounds possessed antioxidant activity. Furthermore, we found that these two compounds exhibited several nonenzymatic antioxidant activities such as superoxide anion scavenging activity, metal ion chelating effect, xanthine oxidase inhibitory effect, and reducing power. These findings partially explained their antioxidative action modes and once again supported that these two agents could provide antioxidant protection at least via these nonenzymatic actions. Our results also found that oleanolic acid and ursolic acid could decrease lipid oxidation at 37 °C, and the antioxidant activity from oleanolic acid was marked at 50 °C or higher temperatures; these findings suggested that these two agents might provide antioxidative protection under human physical status, and oleanolic acid could be used for foods before or after a heating process. On the other hand, we found that these two agents could exhibit antioxidant activity in lower or higher pH environments. This advantage suggested that these two agents were more useful in acidic or basic food systems for antioxidant protection.

Ursolic acid and oleanolic acid have similar chemical structures but differ in the position of one methyl group on the E ring. Ovesna et al. (*4*) indicated that these two compounds had antioxidant activity; however, the different position of one methyl group in their chemical structures caused moderately different biological activities on three leukemic cell lines. Our present study also found that these two compounds exhibited different effects in scavenging superoxide anion and reducing power. Furthermore, it was noted that ursolic acid exhibited

Table 2. Influence of Temperature on the Antioxidant Activity of 10 μM α-Tocopherol (α-Toc), Oleanolic Acid, and Ursolic Acid against Fe^{2+}-Induced Lipid Oxidation in Liposomes after a 72-h Incubation at pH 7.0 (Data Are Mean ± SD ($n = 10$))

	TBA no.					
agent	4 °C	25 °C	37 °C	50 °C	75 °C	100 °C
control[a]	0.098 ± 0.010 c	0.374 ± 0.025 c	0.532 ± 0.031 c	1.052 ± 0.104 c	1.452 ± 0.132 e	1.916 ± 0.115 e
α-Toc	0.034 ± 0.008 b	0.113 ± 0.017 b	0.218 ± 0.025 b	0.674 ± 0.036 b	0.771 ± 0.032 c	1.063 ± 0.084 c
oleanolic acid	0.046 ± 0.014 b	0.135 ± 0.020 b	0.243 ± 0.019 b	0.637 ± 0.027 b	0.706 ± 0.024 b	0.954 ± 0.047 b
ursolic acid	0.03*b* ± 0.011 a	0.140 ± 0.013 b	0.225 ± 0.021 b	0.680 ± 0.041 b	0.860 ± 0.038 d	1.235 ± 0.121 d

[a] Controls contained no antioxidant agent. b–d: Least-squares means with a common letter within a column are not different at the 5% level.

Table 3. Influence of pH on the Antioxidant Activity of 10 μM α-Tocopherol (α-Toc), Oleanolic Acid, and Ursolic Acid against Fe^{2+}-Induced Lipid Oxidation in Liposomes after a 72-h Incubation at 37 °C (Data Are Mean ± SD (n = 10))

	TBA no.				
agent	pH 2	pH 4	pH 6	pH 8	pH 10
control[a]	0.976 ± 0.067 d	0.526 ± 0.029 d	0.355 ± 0.031 c	0.602 ± 0.035 c	0.897 ± 0.042 c
α-Toc	0.729 ± 0.045 c	0.305 ± 0.017 c	0.127 ± 0.020 b	0.259 ± 0.022 b	0.451 ± 0.031 b
oleanolic acid	0.349 ± 0.031 b	0.228 ± 0.016 b	0.155 ± 0.019 b	0.277 ± 0.018 b	0.475 ± 0.027 b
ursolic acid	0.303 ± 0.028 b	0.217 ± 0.025 b	0.143 ± 0.014 b	0.295 ± 0.023 b	0.483 ± 0.033 b

[a] Controls contained no antioxidant agent. b–d: Least-squares means with a common letter within a column are not different at the 5% level.

Table 4. Superoxide Anion (SA) Scavenging Activity, Chelating Effect, Xanthine Oxidase (XO) Inhibition Activity, and Reducing Power of α-Tocopherol (α-Toc), Oleanolic Acid and Ursolic Acid at 5 and 10 μM (Data Are Mean ± SD (n = 10))[a]

agent	conc (μM)	SA scavenge (%)	chelating effect (%)	XO inhibition (%)	reducing power
α-Toc	5	21.8 ± 1.6 a	23.4 ± 2.1 c	15.5 ± 1.4 a	0.38 ± 0.09 b
	10	38.3 ± 2.1 c	37.5 ± 2.5 d	29.6 ± 2.1 c	0.54 ± 0.11 c
oleanolic acid	5	27.2 ± 1.4 b	12.5 ± 0.9 a	31.8 ± 1.8 c	0.19 ± 0.07 a
	10	50.5 ± 1.8 d	21.3 ± 2.0 c	48.6 ± 1.5 e	0.33 ± 0.12 b
ursolic acid	5	18.7 ± 0.7 a	17.8 ± 1.5 b	21.5 ± 2.0 b	0.34 ± 0.14 b
	10	33.5 ± 1.7 c	34.2 ± 2.1 d	37.4 ± 2.3 d	0.63 ± 0.11 c

[a] a–e: Least-square means with a common letter within a column are not different at the 5% level.

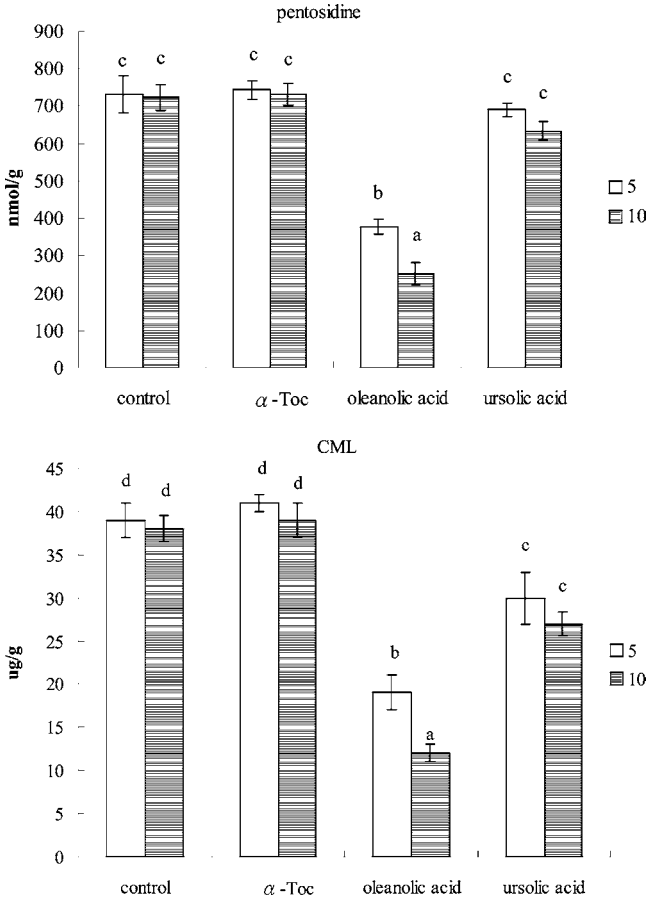

Figure 2. Effect of α-tocopherol (α-Toc), oleanolic acid, and ursolic acid at 5 and 10 μM on the production of pentosidine (nmol/g) and CML (μg/g). Data are mean ± SD (n = 10).

weaker antioxidant protection than oleanolic acid at 100 °C. These results agreed that these two compounds could not provide identical antioxidant capability. Regarding the test nonenzymatic antioxidant activities in our present study, oleanolic acid was greater than ursolic acid. It is highly possible that the position of this methyl group affects the stability of this molecular or affects the affinity of this molecular toward reactants.

Our present study is the first report regarding the antiglycative activity of oleanolic acid and ursolic acid. We found that the presence of these two compounds could effectively suppress in vitro formation of pentosidine, CML, or both, two glycative products. Furthermore, our results indicated that oleanolic acid exhibited a greater effect than ursolic acid in inhibiting the formation of pentosidine and CML. It is well-known that these glycative products contribute to the progression of glycation-associated diseases such as diabetes, Alzheimer's disease, atherosclerosis, osteoarthritis, inflammatory arthritis, and cataracts (*6*−*8*, *19*). Therefore, glycative product inhibitors or breakers like oleanolic acid or ursolic acid are potential therapeutic agents for these diseases. Since these two agents could provide marked antioxidative and antiglycative protection, the supplement of these agents for patients with these above diseases might be able to delay the deterioration of these diseases. Further in vivo study is necessary to verify the antioxidative and antiglycative activities of oleanolic acid and ursolic acid. Our present study also noted that oleanolic acid and ursolic acid exhibited different effects against the in vitro formation of glycative products although ursolic acid and oleanolic acid have similar chemical structures. Apparently, the different position of one methyl group in their chemical structures interfered with their affinity or reactive rate toward glucose or albumin used in this in vitro glycative system. These results suggested that the selection of ursolic acid or oleanolic acid for antioxidative or antiglyative protection should be carefully considered.

Several studies have indicated that the interaction of oxidation and glycation plays an important role in the pathogenesis of atherosclerosis and diabetic nephropathy (*20*−*22*). Thus, any agents like oleanolic acid and ursolic acid possessed of both antioxidative and antiglycative activities may provide greater preventive or alleviative effects because both oxidative and glycative stress could be simultaneously suppressed by these agents. On the other hand, it should be pointed out that glycative products are formed within Maillard reactions in many food systems (*23*−*25*). Thus, it may not be appropriate to use these compounds for certain food systems in which the Maillard reaction is preferred to produce special color or flavor.

In conclusion, the content of oleanolic acid and ursolic acid in glossy privet fruit and hawthorn fruit was in the range of 200−650 μg/g of fresh weight and varied from season to season. These two compounds exhibited dose-dependent effects in superoxide anion scavenging activity, chelating effect, xanthine oxidase inhibition activity, and reducing power. Oleanolic acid dose-dependently inhibited pentosidine and CML formation. Both oleanolic acid and ursolic acid possessed nonenzymatic antiglycative and antioxidative activities; thus, the application

of these two compounds may provide protection against oxidative and glycative damage.

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