JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antioxidant Isoflavones in Osage Orange, *Maclura pomifera* (Raf.) Schneid

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Recent findings that many human chronic diseases are associated with oxidative stresses have instigated the search for dietary antioxidants. Many phytochemicals, particularly phenolic compounds, have been found to possess strong antioxidant activity and reduce the risks of those diseases. Isoflavones, a special phenolic group found in soybean, have been found to act as antioxidants in some model systems. This study investigated the isoflavone content in a unique nonedible tree fruit, Osage orange [*Maclura pomifera* (Raf.) Schneid], and methods for the extraction, identification, and quantification of the two major isoflavones, osajin and pomiferin, were developed. The ethyl acetate extract contained 25.7% osajin and 36.2% pomiferin, and the two isoflavones were at 9.5 g kg^{-1} of fresh Osage orange. Two model systems, FRAP and β-CLAMS, were used to measure the antioxidant activity of these two isoflavones. Pomiferin was found to be a strong antioxidant in both systems, comparable to the antioxidant vitamins C and E and the synthetic antioxidant BHT. Osajin and the two soybean isoflavones (genistein and daidzein) showed no antioxidant activity. Although the Osage orange fruit is not a food source, it is considered to be safe and, therefore, a potentially good source of an antioxidant nutraceutical and functional food ingredient.

KEYWORDS: Osage orange; *Maclura pomifera*; hedge apple; osajin; pomiferin; isoflavones; antioxidant activity

INTRODUCTION

Phytochemicals from plants have been extensively studied for their antioxidant activity, a biological function that is important in keeping the oxidative stress level below the critical point in the body. Antioxidants are a special group of compounds that neutralize or quench free radicals and other reactive oxygen species (ROS) that are generated in the body. Free radicals and ROS are highly reactive and unstable chemical species. They are generated in the body during normal metabolism; however, many environmental factors such as infectious agents, pollution, UV light, and radiation can skew the balance to a point that harmful free radicals are not neutralized by the body's primary and secondary defense mechanisms. The imbalance will damage vital proteins, lipids, carbohydrates, and DNA and cause various chronic diseases.

The intake of antioxidant-rich diets has been associated with reduced incidence of chronic diseases such as cancer and cardiovascular diseases. Many polyphenolic compounds of plant origin, particularly the flavonoids, have been found to possess much stronger antioxidant activity than that of the antioxidant vitamins (*1*−*5*). Isoflavones (**Figure 1**), although categorized as polyphenolic, are often studied separately because these are a special group of flavonoids not commonly found in fruits and vegetables. Leguminous plants such as soybean and red clover, however, are known to be rich sources of isoflavones. These isoflavones can be aglycons or their glycosides. Some researchers suggest that isoflavones in the aglycon form are absorbed more efficiently than the glycosides (*6*); however, others found that this was not the case, although aglycons may be absorbed somewhat more quickly (*7*). Similar to many other phytochemicals, isoflavones are not essential nutrients; however, they play very important roles in preventing human diseases. Aside from the major role of isoflavones as phytoestrogens, the other major activity reported is their antioxidant activity. Isoflavones were found to significantly inhibit lipid peroxidation of rat liver microsomes (*8*). The antioxidant activity of soybean isoflavones was found in the following order: genistein > daidzein = genistin = biochanin A = daidzin > formononetin = ononin (*9*). However, using a similar method, Mitchell et al. (*10*) observed only weak antioxidant activity in genistein and daidzein, the two major isoflavones in soybean. In studies using other methods, isoflavones were found to inhibit the production of the $O_2^{\bullet -}$ produced by the xanthine/xanthine oxidase system (*11*) and the low-density lipoprotein (LDL) oxidation (*12*). Wei et al. (*13*) also found that genistein inhibited oxidative DNA damage induced by UV light and Fenton reaction.

The surge in the interest of the health benefits of isoflavones has prompted our effort in the search for isoflavones in other botanical sources. One such plant is the Osage orange [*Maclura*

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10.1021/jf0342369 CCC: $25.00 Published 2003 by the American Chemical Society
Published on Web 09/19/2003

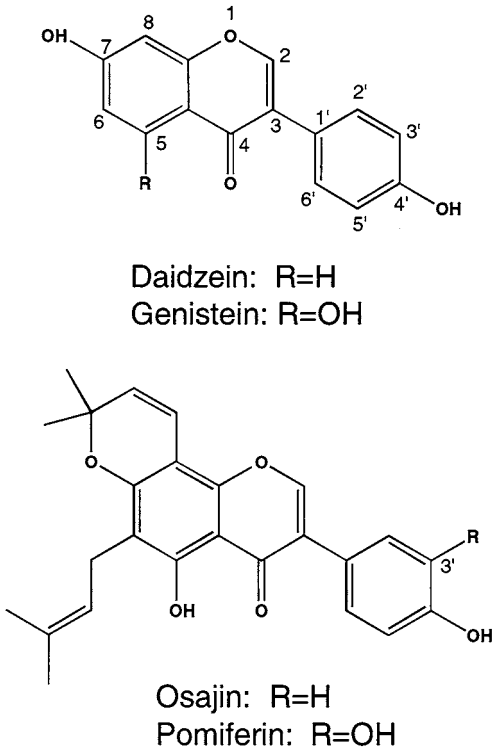

Figure 1. Structures of isoflavones.

pomifera (Raf.) Schneid], a tree of the Moraceae or mulberry family. It is a tree that is native to a small region in the United States that was also known as the home of the Osage Indians, hence the common name of Osage orange. The tree was widely planted as hedge trees throughout the Midwest of the United States and Ontario, Canada, therefore, also the name of hedge apple, and it played an important role in converting the prairies into productive agricultural land communities (*14*−*16*). Other than its uses as hedge trees and hardwood, the use of the fruit as an insect repellent is perhaps the most attractive folklore. Even today, the use of Osage oranges is an enduring pest management home remedy in the Midwest United States.

Phytochemical profiling of the Osage orange was done mostly in the first half of the 20th century. Scientists have been trying to find the magic compounds in the Osage orange that repel insects or kill fungi. Many secondary metabolites have since been isolated and identified (*15*, *17*), and some did show certain bioactivity in the laboratory; however, few of the compounds showed practical usage as medicines or pest control agents (*14*, *15*, *18*, *19*). Despite the many secondary metabolites found in the Osage orange, for some reason, there has been a lack of research interest in the biological activities of these compounds. Many of the bioactivities found in the Osage orange were measured using old technologies. Thus, it is advantageous to re-examine some of those with more advanced technologies.

Among the phytochemicals in the fruit of the Osage orange, isoflavones are the predominant group and are perhaps the most studied. Osajin and pomiferin were discovered and their structures identified more than half a century ago (*20*), and they are the two predominating isoflavones in the fruit of Osage orange (*19*, *21*). Osajin and pomiferin were found to be antimicrobial (*18*); however, of greater interest was the antioxidant activity of the isoflavones in the Osage orange. Clopton, in 1953 (*22*), first found that the dried and ground Osage orange fruit or crude solvent extracts delayed the onset of oxidative rancidity in lard, and pomiferin seemed to be more efficient an antioxidant than osajin. Schall and Quackenbush (*23*) found similar results but indicated that a synergistic effect might exist among antioxidants in the fruit of the Osage orange. Budincevic and Vrbaski reconfirmed the pioneer work by the previous two studies (*24*).

In this paper, we report the isolation, quantification, and antioxidant activity of the two major Osage orange isoflavones, osajin and pomiferin. The antioxidant activity was compared with that of the soybean isoflavones and antioxidant vitamins, and the potential use of Osage orange isoflavones as nutraceutical antioxidants is discussed.

MATERIALS AND METHODS

Chemicals and Solvents. Ascorbic acid, β-carotene, daidzein, genistein, α-tocopherol, 2,4,6-tripyridyl-*s*-triazine (TPTZ), and Tween 40 were purchased from Sigma Chemical Co. (St. Louis, MO); ferric chloride ($FeCl_3$), ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), linoleic acid, sodium acetate, Trolox, and 2,6-di-*tert*-butyl-4-methylphenol (BHT) were from Aldrich Chemical Co. (Milwaukee, WI); and acetic acid, acetonitrile (HPLC grade), chloroform ($CHCl_3$), dichloromethane, ethyl acetate (HPLC grade), hexanes, hydrochloric acid, anhydrous sodium sulfate, and methanol were from Caledon Laboratory Chemicals (Georgetown, ON).

Extraction, Purification, and Structure Identification of Osajin and Pomiferin. The Osage orange fruits were collected locally (Guelph, ON) in November 2002 and stored at −20 °C for 30 days before being processed. Five fruits (594 g total, whole fruits, not peeled) were semithawed, cut into 1.5−2.5 cm cubes, and soaked overnight in 700 mL of ethyl acetate. The mixture was filtered through Whatman no.1 paper (Whatman International Ltd., Maidstone, U.K.,), and the remaining fruit was rinsed three times, each with 300 mL of ethyl acetate, and filtered. The combined filtrate was concentrated to ∼300 mL with a rotary evaporator. The concentrated extract was partitioned with 3 equal volumes of ethyl acetate. The combined ethyl acetate was dried with anhydrous sodium sulfate, filtered through Whatman no.1 filter paper, and evaporated to dryness. A total of 9.12 g of crude extract was obtained, giving the final yield of 1.53% on a wet weight basis. The extract (9.12 g) was dissolved in 200 mL of methanol, diluted 500 times with methanol (1-mL aliquot to 500 mL), filtered with a 0.45 μm syringe filter (Gelman Laboratory, Ann Arbor, MI), and quantified by HPLC with UV detection for osajin and pomiferin.

Isolation and Identification of Osajin and Pomiferin. Osajin and pomiferin standards were prepared similarly as described by Peterson et al. (*19*). Briefly, crude extract was separated by performing thin-layer chromatography using Whatman preparative silica plates (Whatman International Ltd. Division, Clifton, NJ) and a solvent system of 2:1 hexane/ethyl acetate. The two major bands under the UV light (254 nm) were osajin and pomiferin ($R_f = 0.52$ and 0.35, respectively), and they were separately scraped off the plate, extracted with dichloromethane, and dried using a rotary evaporator. The identities of osajin and pomiferin were confirmed by using HPLC coupled to a photodiode array UV detector (Finnigan MAT Spectra System UV6000LP, San Jose, CA) and a Finnigan LCQ Deca electrospray ionization mass spectrometer (HPLC-ESI-MS) operated in the negative ion mode.

Quantification of Osajin and Pomiferin. An HPLC system (1100 series, Agilent Technologies, Palo Alto, CA) equipped with a quaternary pump, a degasser, a thermostatic autosampler, and a DAD was used for sample analysis and quantification. The analytical data were evaluated using the 3D Chemstation software. Separation of phenolic compounds was carried out in a Phenomenex (Torrance, CA) 5 μm ODS-2 C18 RP (150 × 4.6 mm i.d.) column with a C18 guard column. The mobile phase was composed of 2% acetic acid in water (solvent A) and acetonitrile (solvent B), pumped at a flow rate of 1 mL/min. The linear gradient elution conditions were as follows: 50% B to 100% B in 15 min, 100% B back to 50% B in 2 min. The injection volume for all samples was 10 μL. The analytes were monitored at 274 nm. UV spectra were recorded from 200 to 600 nm.

Ferric Reducing/Antioxidant Power (FRAP) Assay. The FRAP assay was first introduced by Benzie and Strain (*25*) for measuring the total antioxidant activity. The assay is based on the reducing power of a compound (antioxidant). A potential antioxidant will reduce the ferric

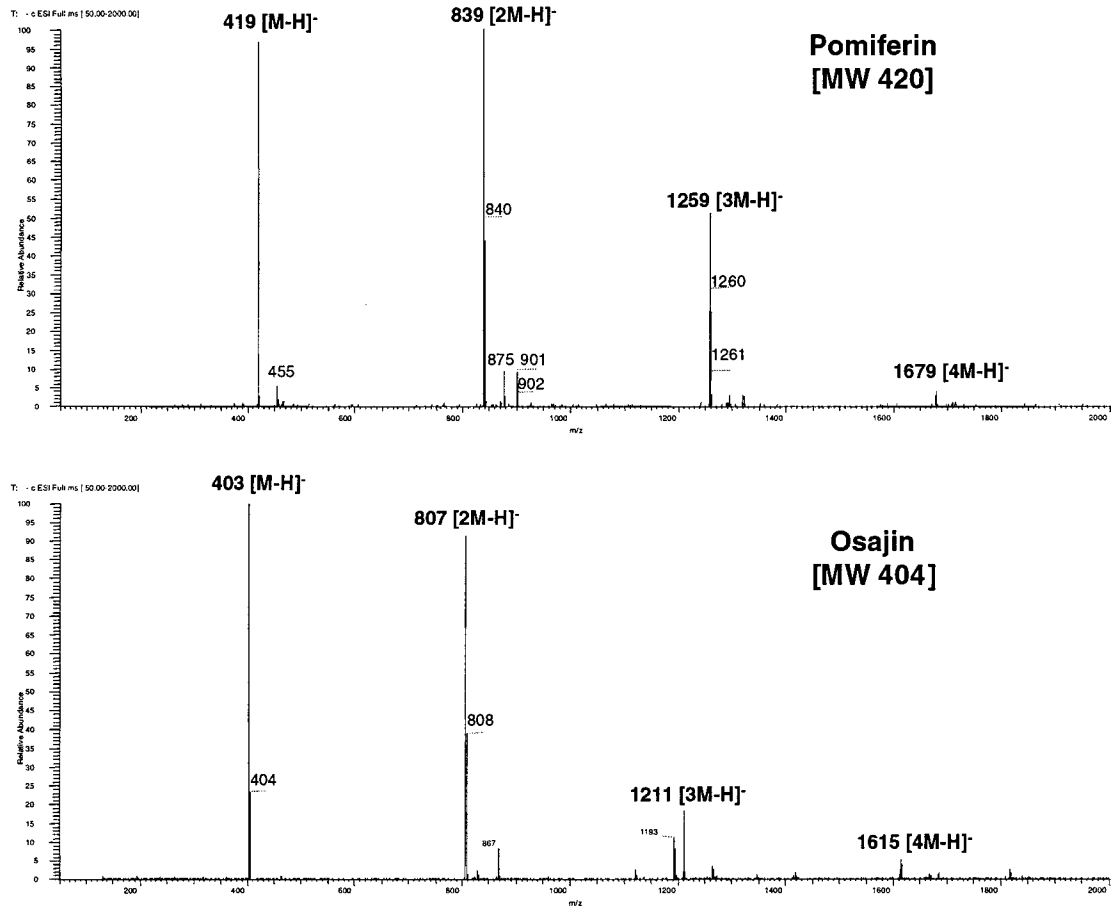

Figure 2. Mass spectra of osajin and pomiferin obtained from the HPLC-ESI-MS experiment.

ion (Fe^{3+}) to the ferrous ion (Fe^{2+}); the latter forms a blue complex (Fe^{2+}/TPTZ), which increases the absorption at 593 nm. In this study, the above method was modified for the 96 well microplate reader. Briefly, the FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3$ at 10:1:1 (v/v/v). All standards and samples were prepared at 500 μM in water or methanol. The 300 μL reagent and the 10 μL standard ($FeSO_4 \cdot 7H_2O$) or sample solutions were added to the well and mixed well. The absorbance readings were taken at 593 nm immediately after and 4 min after using a visible−UV microplate kinetics reader (EL 340, Bio-Tek Instruments, Inc., Winooski, VT). The plate was incubated at 37 °C for the duration of the reaction. All treatments were run in triplicate. The FRAP value of the samples was calculated on the basis of 500 μM Fe^{2+} ($FeSO_4 \cdot 7H_2O$) as follows:

$$\text{FRAP value } (\mu\text{M}) = (0-4 \text{ min } \Delta A_{593\text{nm}} \text{ test sample})/(0-4 \text{ min } \Delta A_{593\text{nm}} \text{ standard}) \times 500\ (\mu\text{M})$$

***β*-Carotene−Linoleic Acid Model System (*β*-CLAMS).** The *β*-CLAMS (*26*, *27*) method is based on the decoloration of *β*-carotene by the peroxides generated during the oxidation of linoleic acid (a free radical chain reaction) at elevated temperature. In this study the *β*-CLAMS was modified for the 96 well microplate reader. In brief, *β*-carotene (0.5 mg) was dissolved in ~2 mL of $CHCl_3$ in a 200 mL round-bottom flask, to which 25 μL of linoleic acid and 200 mg of Tween 40 were added. $CHCl_3$ was removed using a rotary evaporator. Oxygenated HPLC grade water (100 mL) was added, and the flask was shaken vigorously until all material dissolved. The oxygenated water was obtained by bubbling the water with compressed oxygen gas for at least 2 h at room temperature. This test mixture was prepared fresh and used immediately. To each well was added 250 μL of the test mixture and 35 μL of sample solution or water (blank). The plate was incubated at 45 °C. Readings were taken at 490 nm immediately after and every 15 min for 300 min using the same microplate kinetics reader as stated above. All antioxidant standards and samples were prepared at 200 ppm, and all treatments were run in triplicate.

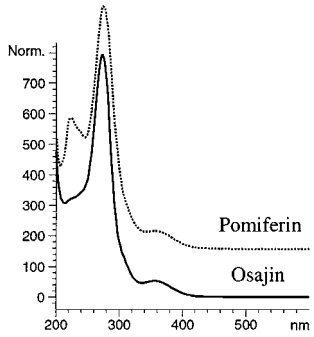

Figure 3. UV spectra of osajin and pomiferin.

RESULTS AND DISCUSSION

Extraction, Identification, and Quantification of Osajin and Pomiferin. Although alcohols were used by the others in extracting osajin and pomiferin from dried or fresh fruit (*18*, *20*), due to the nature of the higher lipophilicity of the Osage orange isoflavones, we used ethyl acetate to directly extract the targeted compounds. Osajin and pomiferin were purified using thin-layer chromatography as described by Peterson et al. (*19*), and their identities were confirmed by mass spectrometry and UV spectrophotometry (**Figures 2** and **3**). The calculated molecular mass of osajin is 404, which appeared at 403 as an $[M-1]^-$ peak in the mass spectrum obtained with electrospray ionization (ESI). In the ESI experiment, molecular ions may aggregate to form characteristic peaks of dimer, trimer, and tetramer ions, which help determine which of the many ions

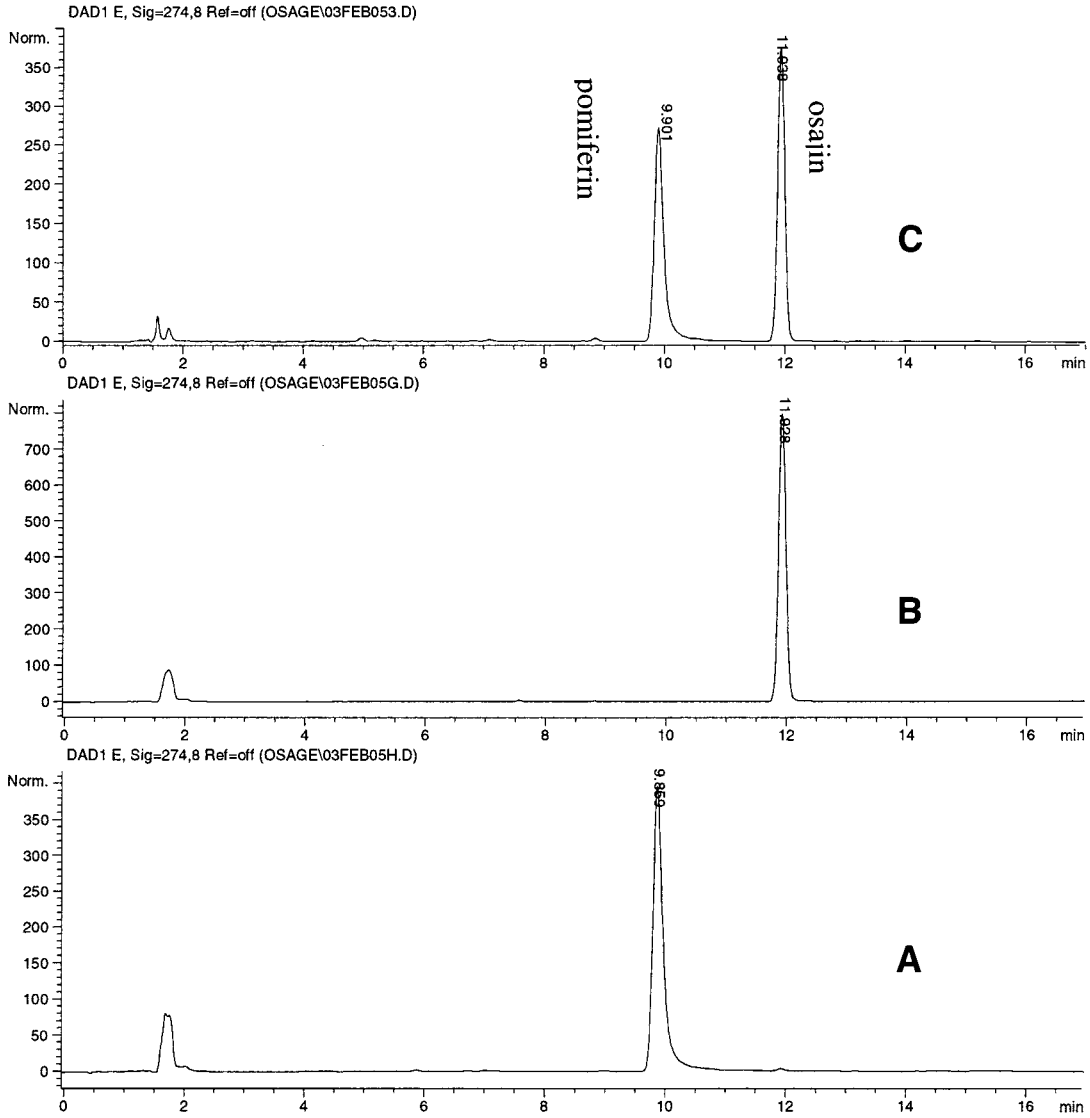

Figure 4. HPLC chromatograms of pomiferin (**A**), osajin (**B**), and the ethyl acetate extract of the Osage orange fruit (**C**).

is the parent ion. In this case, the identity of osajin was further confirmed by the presence of peaks at 807 $[2M - H]^-$, 1211 $[3M - H]^-$, and 1615 $[4M - H]^-$, that is, the dimer, trimer, and tetramer ions of the molecular ion of osajin, respectively (**Figure 2**). Similarly, the molecular ion for pomiferin was at 419 $[M - H]^-$, and the corresponding polymers were at 839 $[2M - H]^-$, 1259 $[3M - H]^-$, and 1679 $[4M - H]^-$ (**Figure 2**). The UV spectra of osajin and pomiferin showed a distinctive maximum absorption at 274 nm and a shoulder at 356 nm for both compounds (**Figure 3**). The two were not scandenone and auriculasin, the respective linear isomers of osajin and pomiferin, because the maximum UV absorptions for the former two were at 286−290 nm and had no absorption at near 356 (*21*).

The HPLC profile of the ethyl acetate extract of the Osage orange showed that it contained nearly exclusively osajin and pomiferin as their aglycons at 274 nm (**Figure 4**), similar to what was found by Peterson et al. (*19*). The HPLC method developed in this study well separated the two isoflavones, contrary to what was reported by Delle Monache et al. (*21*). Osajin and pomiferin were quantified using UV calibration curves generated from serial dilutions of purified standards (1−100 ppm, $R^2 = 0.9999$). The concentrations of osajin and pomiferin were 0.39 and 0.55%, respectively, in the fruit (wet mass), and 25.7 and 36.2%, respectively, in the crude ethyl acetate extract (**Table 1**). The total of these two compounds took up 61.9% of the crude extract, or 9.5 g kg^{-1} of the Osage orange on a wet weight basis. This would be considerably higher than the total isoflavones in the soybean (1−4 mg kg^{-1}, on a dry weight basis) (*28*) or any other major or minor crops containing isoflavones (*29*). There is no clear boundary between the cuticle and flesh of the Osage orange fruit; therefore, the isoflavone contents reported here are considered to occur in both tissues.

Table 1. Concentrations of Osajin and Pomiferin in Osage Orange Fruit and Its Ethyl Acetate Extract

	% of fruit (wet mass)	concn (% of crude extract)
osajin	0.39	25.7
pomiferin	0.55	36.2

Antioxidant Activity of Osajin and Pomiferin. Although different in vitro methods have been used to evaluate the antioxidant capacity of different phytochemicals, not all methods have been found to correlate well with each other. There is no perfect system available to help us know about the "true" antioxidant power or capacity of a complex medium (*30*, *31*). The FRAP and β-CLAMS assays, despite their disadvantages,

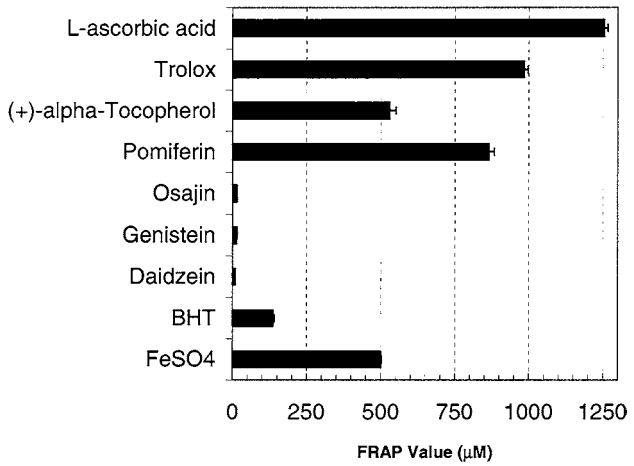

Figure 5. Antioxidant activities of isoflavones and vitamins measured by the FRAP assay. Values are the average of three replicates.

are still frequently used by many researchers for rapid evaluation of antioxidants with nutraceutical potential (*4*, *25*, *32*−*34*). Although these two methods are based on two different mechanisms, they were found to correlate well in our previous study (*5*).

In the FRAP assay, all antioxidants were prepared and tested at the same concentration of 500 μM. On the basis of the standard (Fe^{2+}), the FRAP value of pomiferin was 866 μM. Although not as high as that of vitamin C (ascorbic acid), it was comparable to that of Trolox, a water soluble vitamin E analogue (**Figure 5**). It was a better antioxidant than α-tocopherol (vitamin E) (FRAP = 530 μM) and the synthetic antioxidant BHT (FRAP = 136 μM) (**Figure 5**). Whereas pomiferin showed strong antioxidant activity, it was interesting to note that osajin, the Osage orange isoflavone with one hydroxyl group less than pomiferin (**Figure 1**), did not have any antioxidant activity (FRAP = 15 μM) (**Figure 5**). Two of the major soybean isoflavones, genistein and daidzein, had no antioxidant activity in the FRAP assay (**Figure 5**), consistent with the findings of Mitchell et al. (*10*), although these compounds were reported as antioxidants in other systems (*9*−*11*).

Table 2. Antioxidant Activity of Isoflavones and Other Known Standards Calculated Using Different Endpoints in the β-CLAMS Assay

	AOA[a] (mean ± SD)	av absorbance[b] (mean ± SD)	initial slope[c]
L-ascorbic acid	−71.10 ± 8.43	0.20 ± 0.01	–[d]
BHT	82.57 ± 4.65	0.71 ± 0.07	−0.0017
daidzein	27.08 ± 7.55	0.39 ± 0.04	−0.0059
genestein	27.51 ± 10.61	0.39 ± 0.02	−0.0058
osajin	8.32 ± 7.11	0.35 ± 0.03	−0.0068
pomiferin	80.35 ± 0.42	0.74 ± 0.01	−0.0020
(+)-α-tocopherol	88.96 ± 1.09	0.82 ± 0.01	−0.0011
Trolox	85.98 ± 0.25	0.80 ± 0.02	−0.0015
blank	–[e]	0.34 ± 0.01	−0.0073

[a] Antioxidant activity (AOA) calculated using the 90 min data points according to the method of Emmons et al. (*36*). [b] Antioxidant activity calculated by averaging the absorbance unit (AU) values taken from 0 to 300 min at 15 min intervals (*33*). [c] Values are means of slope coefficients (% initial absorbance min^{-1}) calculated by linear regression of the initial linear portion (before 90 min) of the absorbance values according to the method of Fukumoto and Mazza (*35*). [d] Nonlinear. [e] Data for blank control were used for calculating the antioxidant activity of others.

In the β-CLAMS assay, pomiferin showed once again a very strong antioxidant activity (**Table 2**). Different endpoints have been used for evaluating the antioxidant activity in β-CLAMS. An average absorbance value over the assay period was used by Birch et al. (*33*); however, the slope for the initial linear portion of the plot was used by Fukumoto and Mazza (*35*). Degradation rate was also used by Emmons et al. (*36*). We used the initial 90 min absorbance values of the tested compounds and calculated the antioxidant activity according to all three methods. As **Table 2** shows, all three calculations generally agreed with one another. Pomiferin consistently showed a good antioxidant activity that was comparable to those of BHT, α-tocopherol, and Trolox by all three endpoints. At the same concentration of 200 ppm, the half-life of β-carotene was >300 min when pomiferin was present, greater than that of BHT and close to that of α-tocopherol and Trolox (**Table 2**; **Figure 6**). Genistein and daidzein showed only slight antioxidant activity in the β-CLAMS assay; using the initial 90 min linear decaying region (R^2 = 0.9980 and 0.9988, respectively), the half-lives of β-carotene in the presence of these two soybean isoflavones

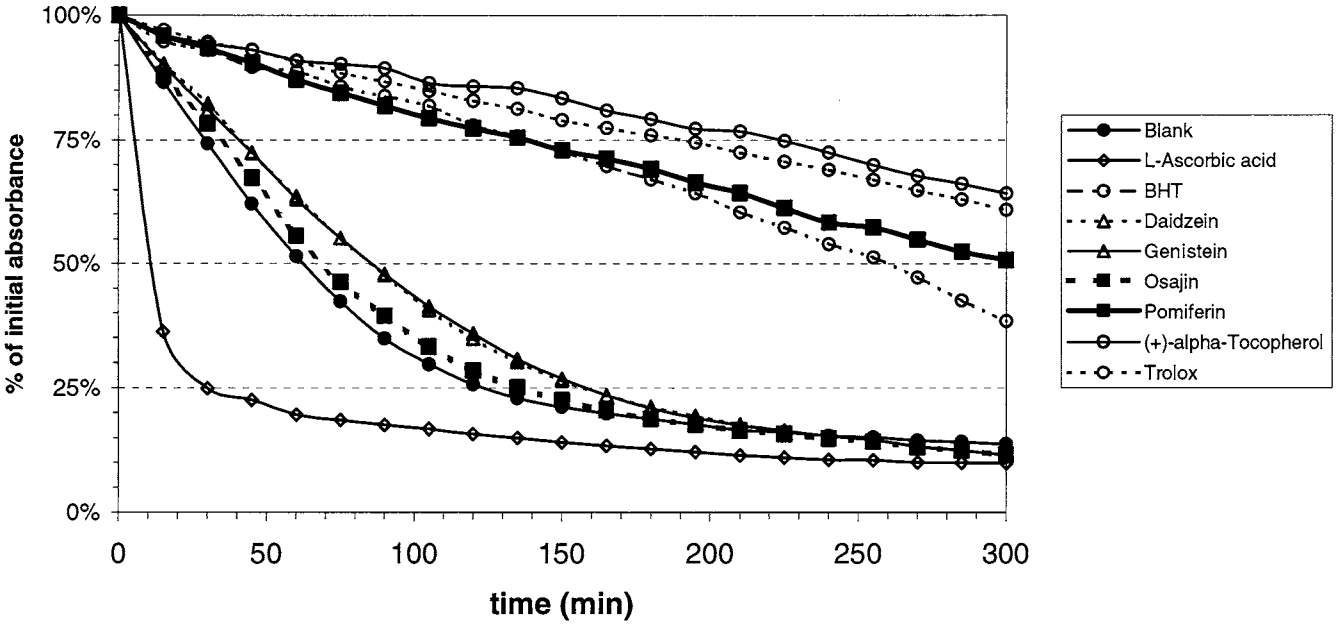

Figure 6. Antioxidant activities of isoflavones and vitamins measured by the β-CLAMS assay. Values are the average of three replicates.

were both 84 min. The decaying curve for osajin (half-life = 72 min, $R^2 = 0.9957$) was similar to that of the blank (half-life = 65 min, $R^2 = 0.9917$), suggesting that osajin has nearly no antioxidant activity in the β-CLAMS assay, a result similar to what was found in the FRAP assay. Contrary to the fact that vitamin C is a good antioxidant in the FRAP assay, it had no antioxidant activity in the β-CLAMS assay. Furthermore, the negative antioxidant activity (AOA) value of L-ascorbic acid indicated that it might be a pro-antioxidant at the concentration used in this study (**Table 2**; **Figure 6**). AOA was defined by Emmons et al. (*36*). A similar result was found in our other study (*5*). The antioxidant activity of pomiferin was persistent, as were those of known antioxidants over an extended assay period (300 min) (**Figure 6**).

The antioxidant activity measured in the β-CLAMS assay correlated well to those found in the FRAP assay. The correlation coefficient (*R*) between the AOA and FRAP (without L-ascorbic acid) was 0.76, and the *R* was 0.81 between average absorbance and FRAP, an indication that the antioxidant activities found in the two model systems supported each other.

The antioxidant activity of the Osage orange fruit or crude solvent extracts was first reported during the initial exploitation period of the fruit in the1950s (*22*, *23*). The objective of those studies was to find antioxidants that prevent fat or fat-rich food from going rancid. The only relatively recent publication on the antioxidant activity of crude extract was by Budincevic and Vrbaski (*24*); however, the paper did not offer new information other than the use of a β-carotene−linoleic acid system. The role of antioxidants in human disease prevention and health promotion is only recently recognized. Plant-derived polyphenols, including isoflavones, have particularly been attractive for their high antioxidant power. Although the antioxidant activity found in an in vitro experiment is only indicative of the potential health benefit, these methods remain important as the first step in screening phytochemical antioxidants. Other in vitro methods have been used in the determination of antioxidant capacity of isoflavones, and such studies did show that genistein and daidzein were good antioxidants in those model systems (*9*, *11*). However, as found in this study, these two soybean isoflavones are not good antioxidants in the β-CLAMS and FRAP assays. The completely different antioxidant activities between osajin and pomiferin indicated that the structure, particularly whether a hydroxy group is attached on the 3′-position, may play an important role in the structure−activity relationship of the Osage orange isoflavones (**Figure 1**). The hydroxy group on the 5-position of the soybean isoflavones did not seem to affect the antioxidant activity (**Figure 1**).

Although Osage orange is nonedible, the fruit has been deemed safe (*37*, *38*). Its high total isoflavone content lends itself for further exploitation as a potential source of nutraceuticals and functional food ingredients.

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Received for review March 10, 2003. Revised manuscript received August 5, 2003. Accepted August 13, 2003. This is the Scientific Publication S134 of the Food Research Program, Agriculture and Agri-Food Canada, Guelph.

JF0342369