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A Glycoside Flavonoid in Kudzu (*Pueraria lobata*)

Identification, Quantification, and Determination of Antioxidant Activity

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Abstract

Kudzu (*Pueraria lobata*) foliage has been touted as a possible energy crop. High-performance liquid chromatography and mass spectrometry analysis of the methanolic kudzu foliage extracts confirmed the presence of robinin (kaempferol-3-*O*-robinoside-7-*O*-rhamnoside). Robinin accounted for 0.65 ± 0.16% (dry basis) of kudzu biomass. Fast performance liquid chromatography (FPLC) was employed to fractionate robinin from the crude extract. The antioxidant capacity of robinin was evaluated by an oxygen radical absorbance capacity (ORAC) assay. The ORAC values of pure standard were compared with those of the extract fractions. One milligram of the FPLC-fractionated robinin generated an ORAC value of 5.15 ± 2.00 μmol/mg of Trolox, whereas 1 mg of pure robinin generated an ORAC value of 12.34 ± 0.45 μmol/mg of Trolox. Because of its antioxidant properties, robinin may be a flavonoid worth extracting prior to energy production.

Index Entries: *Pueraria lobata*; high-performance liquid chromatography; oxygen radical absorbance capacity; antioxidant; robinin; flavonoids.

Introduction

Flavonoids, primarily categorized into flavonols, flavanols, flavones, flavanones, and anthocyanidins, are widely distributed in nature and are present in most fruits, vegetables, and certain beverages. Flavonoids may play a preventive role in the development of cancer and heart disease owing

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to their antioxidant activities *(1)*. Potential sources of antioxidant compounds have been found in several plant materials such as fruits, vegetables, leaves, oilseeds, barks, roots, spices, and herbs. Energy crops cultured or wildcrafted in the United States can be sources of antioxidants. Such crops include mimosa (*Albizia julibrissin*) foliage and seed, sericea lespedeza (*Lespedeza cuneata*), kudzu (*Pueraria lobata*), giant reed (*Arundo donax* L.), velvet bean (*Mucuna pruriens* L.), switchgrass (*Panicum virgatum* L.), and castor (*Ricinus communis* L.). With the oxygen radical absorbance capacity (ORAC) assay, the antioxidant potential of crude methanol extracts from nine different energy crops was reported *(2)*. The results showed that the crude extract of kudzu (*P. lobata*) had high radical-scavenging activity when compared to spinach (*Spinacia oleracea*). These results, coupled with the relative abundance of kudzu in the southeastern part of the United States *(3)*, prompted further study of this plant.

Kudzu is native to eastern Asia *(4)*. In 1876, it was imported from Japan into the United States as an ornamental cultivar and for its edible tuberous roots *(5)*. As a vine, kudzu grows outward in every direction and can reach 15 m in length in a single season. The deep root system, which makes the plant drought resistant, coupled with its ability to resist attacks by insects and disease, resulted in the selection of kudzu for erosion control *(6)*. In China, the roots and flowers of kudzu have been used to treat alcohol abuse effectively and safely for more than a thousand years *(7,8)*. The extract of kudzu root, which contains isoflavones such as puerarin, daidzin, genistin, daidzein, and genistein, is a source of phytoestrogens with high levels of estrogenic activity *(9)*. Phytoestrogens have been shown to prevent cancer, act as antioxidants, scavenge free radicals, lower serum cholesterol, and have antiestrogenic and antiproliferative effects. In addition, daidzin and daidzein, two isoflavones present in kudzu roots, have been shown to contribute to antidipsotropic (alcohol abuse) activity in rats *(8)*. In a dose-dependent fashion, genistein has been reported to inhibit growth of in vitro stomach cancer cells through activation of a signal transduction pathway for apoptosis *(10)*. In other studies, daidzein was determined to have antigiardial *(11)*, antioxidant *(12)*, and antidiabetic activities *(13)*. Most of the kudzu studies have focused on the root as a source of compounds beneficial to health. However, few reports have examined kudzu foliage for health benefits, along with its use as a biomass source for energy purposes. Hence, the purpose of the present study was to identify and then quantify the flavonoids isolated from kudzu foliage that are responsible for the displayed *(2)* antioxidant potential.

Materials and Methods

Plant Material

Samples of dried and ground (0.3 mm) kudzu foliage were provided by Dr. David Bransby of the Department of Agronomy and Soils at Auburn

University (Auburn, AL). Kudzu was collected from the wild and within a few hours placed in a forced-air oven at 60°C until a constant mass was reached. The process took approx 48 h. A voucher specimen has been deposited at the Department of Chemical Engineering, University of Arkansas (Fayetteville, AR).

Chemicals

High-performance liquid chromatography (HPLC)–grade methanol (extraction solvent), acetonitrile, and formic acid (HPLC solvents) were obtained from VWR (West Chester, PA), and 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) (antioxidant analysis) was purchased from Wako (Richmond, VA). The AAPH solution was prepared by dissolving 0.86 g of AAPH in 10 mL of phosphate buffers and holding them at 4°C. Kaempferol, luteolin (compound identification), fluorescein (3′, 6′-dihydroxyspiro [isobenzofuran-1[3H], 9′[9H]-xanthen]-3-one) (FL) and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (antioxidant analysis) were acquired from Sigma-Aldrich (St. Louis, MO). Robinin (compound identification) was purchased from Extrasynthese (Genay Cedex, France). Potassium phosphate dibasic (antioxidant analysis) was purchased from J. T. Baker (Mallinckrodt Baker, Philipsburg, NJ). The phosphate buffer was prepared by dissolving 130 g of potassium phosphate in 1 L of deionized water to make a 0.75 *M* solution (pH 7.0). A 1000 μ*M* concentration of Trolox stock solution was acquired by dissolving 25 mg of Trolox in 100 mL of phosphate buffer. Serial dilutions of the Trolox stock solution were performed with phosphate buffer to obtain Trolox standard concentrations of 6.25, 12.50, 25.00, and 50.00 μ*M*.

Extraction

Two grams of dried kudzu was extracted with 60 mL of methanol at 50°C by blending the mixture in a household blender for 10 min. The resulting mixture of solvent and solids was filtered through a 0.45-μm syringe filter (VWR). The filtered crude extracts were collected and stored at 4°C for subsequent analysis.

Identification of Compound

HPLC analysis was conducted on a Waters Instrument (Waters, Milford, MA), equipped with a 2996 photodiode array detector, with a 2795 separations module controlled with Mass Lynx software. A 50-μL sample was injected on a Symmetry® C_{18} (50 × 2.1 mm) column (Waters Milford, MA). Mobile phases used for the gradient consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The initial condition began at 90:10 solvent A:solvent B and was maintained for 6 min. The gradient was linearly increased to 80:20 solvent A:solvent B over 30 min. The gradient was increased again to 20:80 solvent A:solvent B over

2 min and was held for another 10 min before it was decreased to 90:10 solvent A:solvent B in 2 min. This process was followed by a reequilibration of the column at the final condition for 5 min. The flow rate was set at 0.15 mL/min, and the column was maintained at room temperature. Initially, the diode array detector acquired spectra between the wavelengths of 210 and 600 nm, but it was observed that optimum wavelength was obtained at 360 nm.

Mass Spectrometry Analysis

A Hewlett Packard (Palo Alto, CA) 1100 Series HPLC with a photodiode array detector set at 280 nm was coupled to a Bruker Esquire (Billerica, MA) mass spectrometer. The column used was a Symmetry (Waters) C_{18} column (250 × 4.6 mm). A 25-µL sample was injected via the autosampler. HPLC analysis was conducted using a modification of the Cho et al. *(14)* method, which has been used for the analysis of flavonoids in blackberry (*Rubus ursinus*), blueberry (*Vaccinium* spp.), and red grape (*Vitis vinifera*). The flow rate was set at 0.7 mL/min. The ultraviolet (UV) response during HPLC-mass spectrometry (MS) was monitored at 360 nm. The HPLC-MS was operated in both the positive and negative ionization mode from the electrospray ionization source. The temperature of the drying gas (N_2) was 300°C and flowed at 10 mL/min. The nebulizing pressure (N_2) was maintained at 2.1×10^5 Pa (30 psi). The HPLC system was directly connected to the mass spectrometer without stream splitting.

Chromatographic Separation

The fractionation of crude kudzu was carried out with an AKTA (Amersham Biosciences, Piscataway, NJ) fast performance liquid chromatography (FPLC). The 20-mL column and sample were preconditioned with 0.1% formic acid in 10% aqueous acetonitrile solution for 80 min before a 10-mL sample was injected. To wash out unbound compounds, the column was eluted with 10 mL of 0.1% formic acid in 10% aqueous acetonitrile. Mobile phases were identical to those described for the HPLC analysis. The gradient began with 90:10 solvent A:solvent B and was linearly changed to 40:60 solvent A:solvent B over 150 min. The gradient was increased to 0:100 solvent A:solvent B in 1 min, and the final gradient was held for 50 min followed by a reequilibration of column with 90:10 solvent A:solvent B for 30 min. The flow rate was set at 1 mL/min. The FPLC was equipped with a fixed-wavelength (280 nm) detector. Although not optimum, monitoring was conducted at 280 nm.

Antioxidant Analysis

An ORAC assay was conducted using the method of Prior et al. *(15)* with FL as a fluorescent probe. A microplate reader (FLUORstar Optima; BMG, Durham, NC) used for the assay was preassembled with two internal

pipettors and 48 sample wells. The assay was run at 37°C with a total of 28 reading cycles, each consisting of a 197-s duration with zero pausing time between cycles. The duration of the reading cycle and the number of reading cycles could vary depending on the number of wells assayed. For the assay, 40 µL of each sample was diluted with phosphate buffer, and a blank consisted of only the phosphate buffer. Trolox (40 µL of 6.25, 12.5, 25, and 50 µ*M*) solutions were used as standards. A stock solution of 1.2 m*M* FL was diluted to 94 n*M* with phosphate buffer. The FL solution (400 µL) was added to each well first via a multichannel pipet on cycle 2 followed by a 150-µL injection of the prepared AAPH solution (0.137 g of AAPH/16 mL of phosphate buffer) on cycle 4 to begin the oxidation reaction. Excitation and emission filters of 490 and 520 nm, respectively, were used. Each sample was analyzed in triplicate, and the average value was recorded. Data were expressed in micromolar Trolox equivalent per gram of sample analyzed.

Results and Discussion

Amid several smaller peaks, the HPLC chromatogram of kudzu crude extract showed the presence of one main peak, with a retention time (RT) of 35 min (see Fig. 1). The MS positive ion mode analysis of the main peak (RT = 35 min) showed the presence of four major significant ions at *m/z* 287, 433, 595, and 763 (see Fig. 2), which were referred to as the first ion, second ion, third ion, and fourth ion, respectively. The difference in mass between the first and second ions was 146, which corresponded to the mass of deoxyglycoside. Similarly, the difference in mass between the second and third ions was 162 and could be attributed to a glycoside compound. However, the difference in mass between the third and fourth ions was 168 and could not be accounted for by the mass of a glycoside compound. The MS negative ion mode analysis of the dominant compound presented in Fig. 1 was conducted and showed the presence of an ion with an *m/z* of 739, which corresponds to the *m/z* 763 in the positive ion mode (see Fig. 3). A difference of 24 from the positive and negative ion modes was inferred as the presence of sodium ion. This assumption was verified by the observation that sodium ion occurred only in the positive ion mode. Moreover, the mass of compounds in the negative ion mode was found to be 2 Daltons less than the corresponding mass in the positive ion mode, indicating that the neutral compound mass would be the average of the two values. Therefore, the main peak was concluded to be formed by an aglycone with a mass of 286, plus 2 mol of deoxymonosaccharide and 1 mol of monosaccharide.

Based on the observed mass and photodiode array spectra, the common *m/z* 286 was assigned to be either kaempferol or luteolin. Both compounds were selected based on their proximity in mass to the compound shown in Fig. 1. The MS/MS product ion spectrum obtained during HPLC separation and the reference spectra from reagent grade kaempferol and

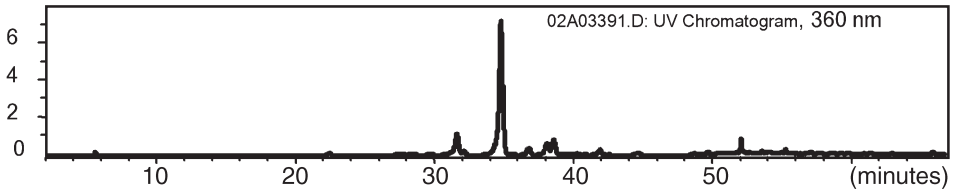

Fig. 1. HPLC chromatogram of kudzu crude extract at wavelength of 360 nm.

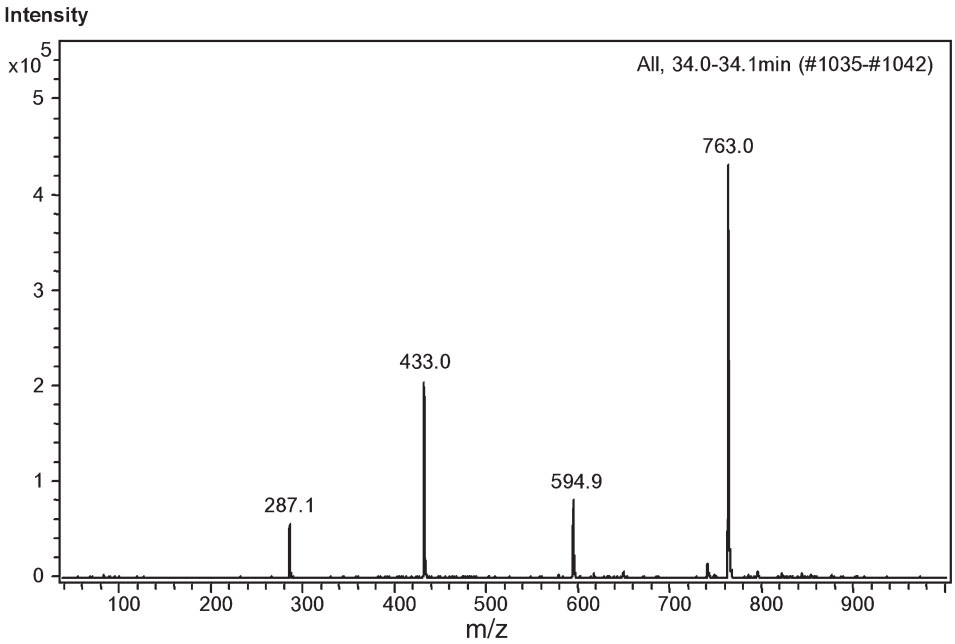

Fig. 2. MS spectrum of *m/z* $[M + H]^+$ chromatograms of peak (RT = 35 min) from kudzu extract.

luteolin were compared as shown in Fig. 4A–C. The MS/MS product ion spectrum of luteolin (see Fig. 4C) did not bear a resemblance to the main peak (RT = 35 min) of the kudzu extract. By comparison of the mass spectrum, the common mass of 286 was confirmed as kaempferol (see Fig. 4B). Kaempferol, a common flavonol found in leaves and petals, contributes the yellow colors found in plants *(16)*.

With kaempferol identified as the aglycone of the main peak in Fig. 1, analysis of the sugar moiety was undertaken. Robinin (kaempferol-3-*O*-robinoside-7-*O*-rhamnoside, Fig. 5) was tested based on the similarity between the mass of robinin and the main antioxidant compound in kudzu. Direct comparison with robinin reference compound was performed. The chromatograms of kudzu crude extract, robinin standard, and kudzu extract spiked with the robinin standard are shown in Fig. 6A–C. Through the use of co-chromatography, standard addition, and tandem

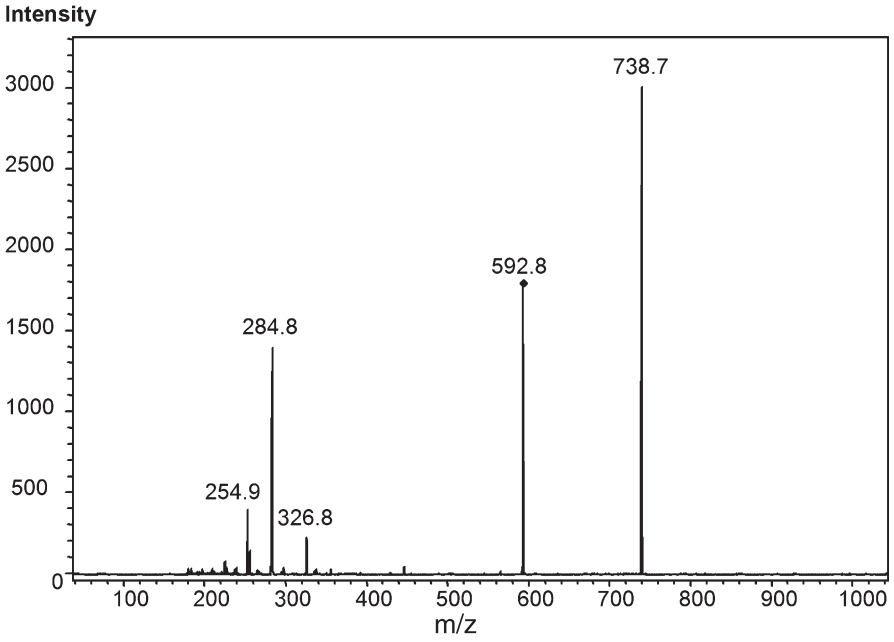

Fig. 3. MS spectrum of *m/z* $[M + H]^-$ chromatograms of peak (RT = 35 min) from kudzu extract.

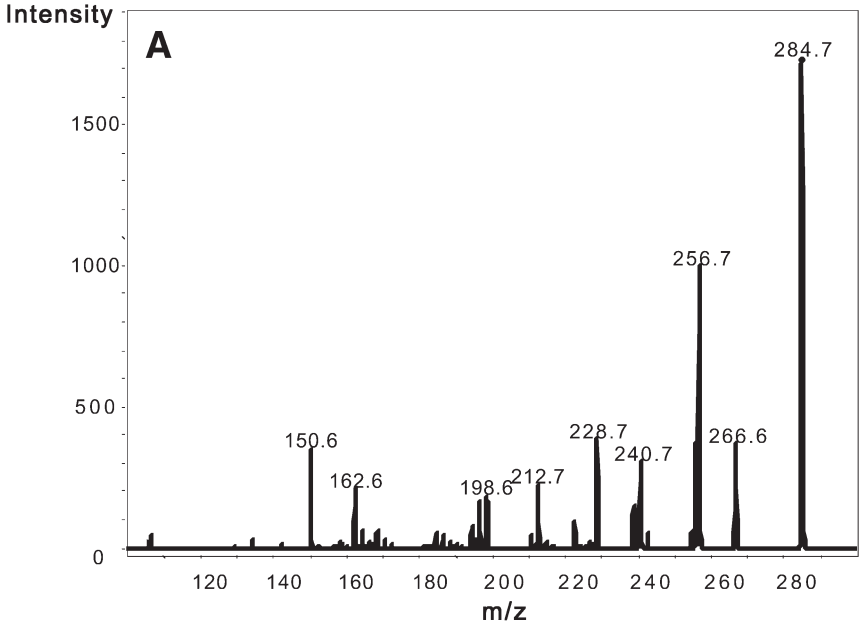

Fig. 4. MS/MS spectra of *m/z* $[M + H]^-$ chromatograms of **(A)** peak (RT = 35 min) kudzu extract, **(B)** authentic kaempferol, and **(C)** authentic luteolin.

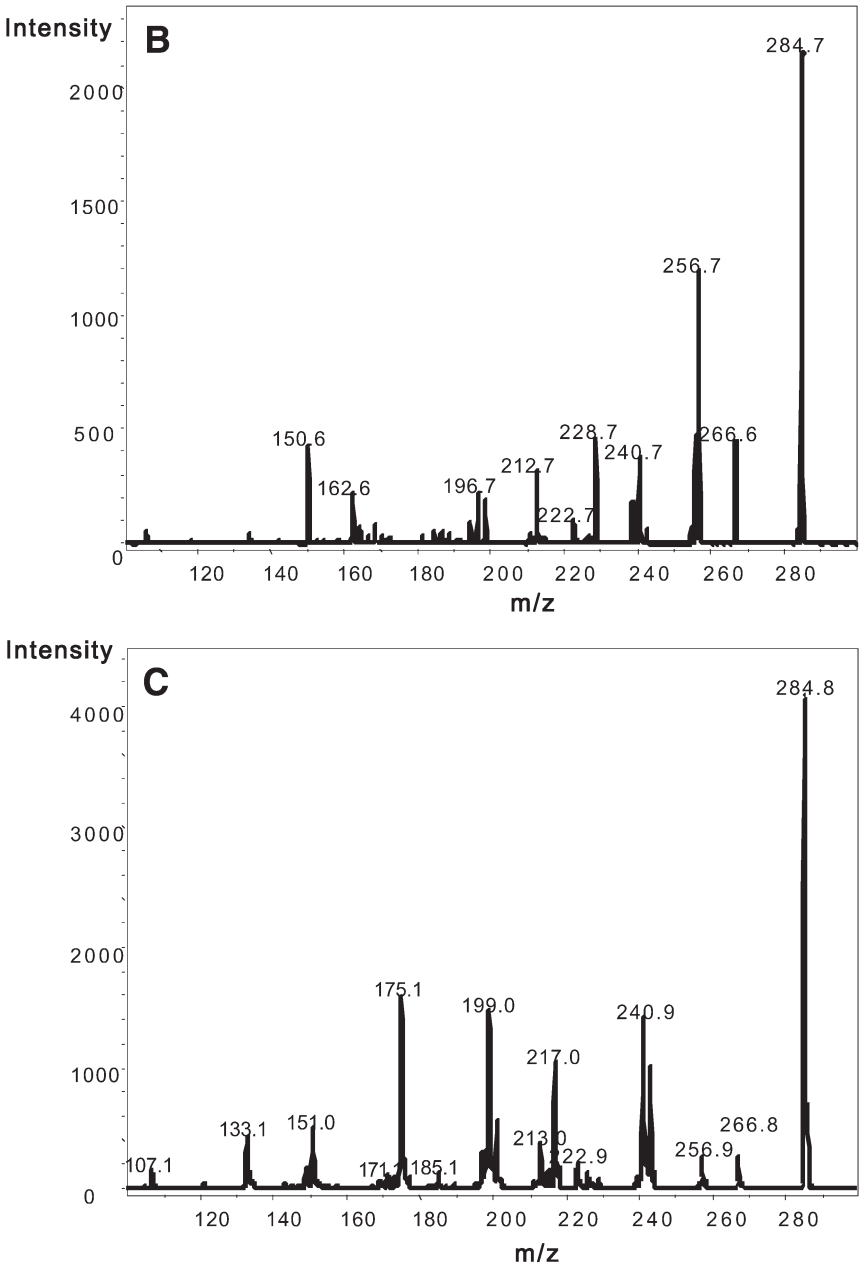

Fig. 4. *Continued.*

MS procedures, the major peak in kudzu crude extract was confirmed as robinin, which accounted for 0.65 ± 0.16 % (dry basis) of kudzu biomass.

About 25 yr ago, Saiiad et al. *(17)*, in a work published in a Russian language journal, also detected a component that they attributed to robinin in kudzu leaves. The English translation of their abstract stated that this was based on "spectral analysis and the analysis of the products

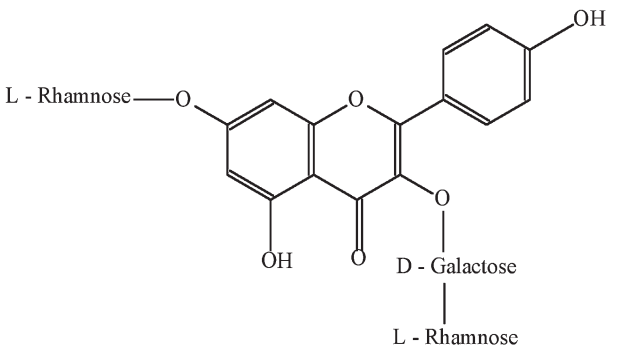

Fig. 5. Molecular structure of robinin.

of fermentative and acid hydrolysis." Our more recent results are based on modern reverse-phase HPLC and tandem MS analysis using authentic reference compounds. The current work confirms Saiiad et al.'s (*17*) assignment, which was tentative at best, being based on chromatographic data and spectrophotometry analysis. The content of robinin in their raw material was determined to be 1.87%. The smaller amount of robinin reported in the current study is attributed, at least in part, to improvements in chromatography in the 25 yr between the two studies and also the higher specificity of tandem MS compared to spectrophotometry. For example, the large number of theoretical plates available in modern HPLC columns allowed us to separate compounds that likely would have been very difficult to separate previously. Our separations included some "just resolved" components that necessitated the use of standard addition techniques to confirm coelution of the analyte. Had robinin not been resolved from these other components, the calculated concentration would have been higher and closer to their value. In a review, Morris *(18)* also noted the presence of compounds related to robinin in kudzu biomass. However, this review gave little or no detail on either amounts or methods. Morris (*18*) cited work that reported the presence of genistein, daidzin, daidzein, and starch in kudzu, but without quantification. Moreover, robinin was not specifically mentioned. Robinin has been detected in several herbaceous plants such as Amaranthaceae (*Alternanthera brasiliana*), the seed of sword bean (*Canavalia gladiata*), and flowers of *Robinia pseudoacacia* L. Robinin has also been detected in *Vinca erecta (19)*, *Robinia viscosa (20),* and the aerial part of *Astragalus shikokianus (21).*

Robinin, being a flavonoid, should display antioxidant activity in tests such as ORAC. To assess the ORAC activity of robinin, crude kudzu foliage extract was fractionated by FPLC. One gram of kudzu crude extract generated 6.45 ± 1.55 (dry basis) of robinin. The purity of the fraction was confirmed by HPLC with detection at 360 nm. One milligram of the FPLC-fractionated robinin generated ORAC values of 5.15 ± 2.00 μmol/mg of Trolox. On the other hand, 1 mg of pure robinin generated ORAC values

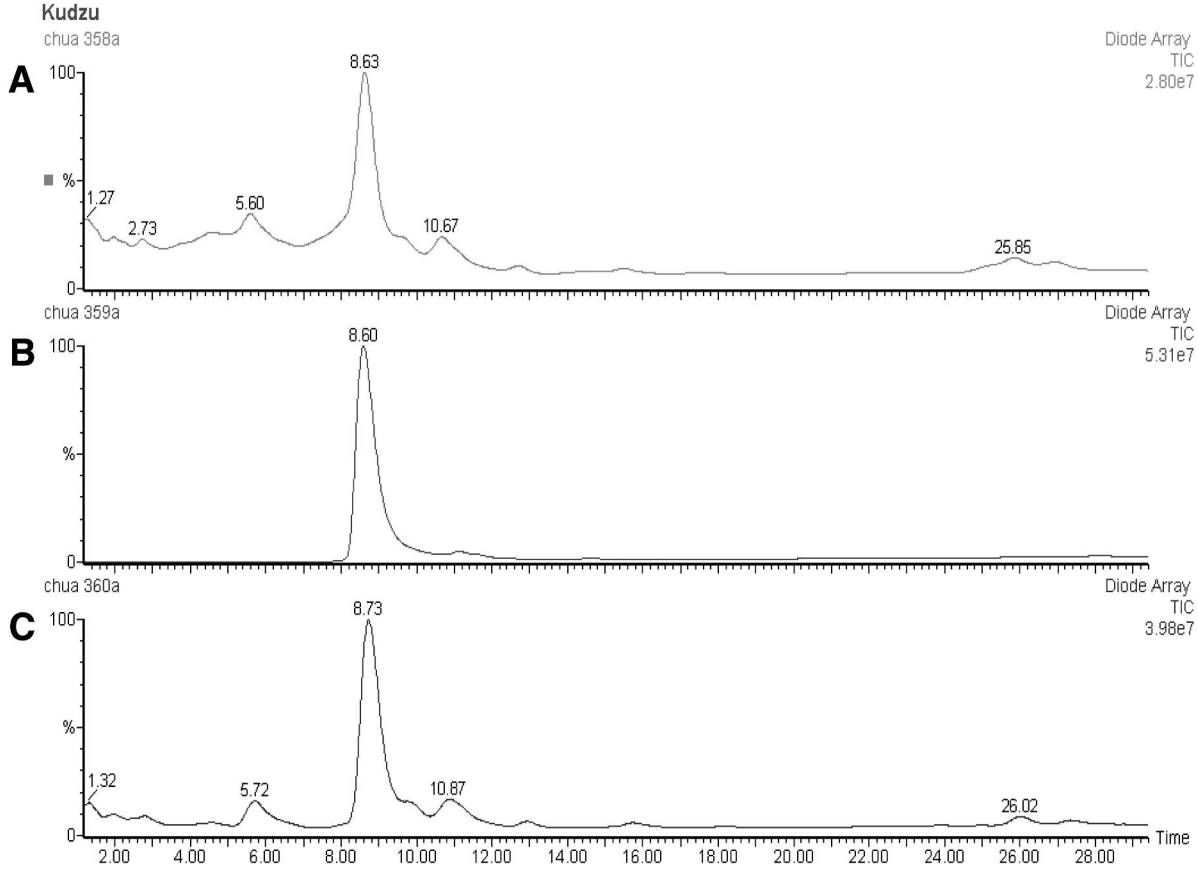

Fig. 6. HPLC-UV analyses of **(A)** kudzu foliage crude extract, **(B)** robinin, and **(C)** co-chromatography of kudzu crude extract with robinin.

of 12.34 ± 0.45 μmol/mg of Trolox. The difference in ORAC values could possibly be explained by the presence of moisture or salts in the fractionated robinin. Although all efforts were made to keep moisture away from the crude and purified material, water may have adsorbed on the samples throughout processing, resulting in an increase in mass. In addition, some residual salts may have contaminated the FPLC-fractionated sample. In sum, these impurities could contribute to the overall lower ORAC value of robinin obtained from FPLC fractionation. Flavonoids display diuretic, anabolic, and antiviral properties, of which the ORAC test can only serve as a preliminary indicator of biologic activity. The ORAC test does not shed light on the mechanism underlying the relationship between robinin on the immune, cardiovascular, gastrointestinal, and central nervous systems. Further in vitro and in vivo studies must be conducted to understand the relationship between robinin and the underlying mechanism of studied disease. Nonetheless, robinin may be a flavonoid worth extracting prior to energy production.

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