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Effect of *Albizia julibrissin* Water Extracts on Low-Density Lipoprotein Oxidization

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High-value phytochemicals could be extracted from biomass prior to the current cellulosic pretreatment technologies (i.e., lime, ammonia, dilute acid, or pressurized hot water treatments) provided that the extraction is performed with a solvent that is compatible with the pretreatment. This work reports on the extraction of flavonoids from *Albizia julibrissin* biomass. While extracting *A. julibrissin* foliage with 50 °C water, 2.227 mg/g of hyperoside and 8.134 mg/g quercitrin were obtained, which is in the realm of what was obtained with 60% methanol. *A. julibrissin* foliage, flower, and whole plant extracts were tested in terms of their potential to inhibit low-density lipoprotein (LDL) oxidization. The highest inhibition was obtained with foliage water extracts, which were standardized at 2.5 μM of flavonoids. Also, the 2.5 μM foliage water extract resulted in a reduction from 43% to only 1% of the observed monocyte adherence. To have commercial application, *A. julibrissin* water extracts should be devoid of toxicity. The *A. julibrissin* foliage, flower, and whole plant water extracts were not toxic to Vero 76 cells. In summary, *A. julibrissin* biomass can be extracted with 50 °C water to yield an antioxidant stream, showing that it may be possible to couple extraction of valuable phytochemicals to the cellulosic pretreatment step.

KEYWORDS: Biomass; low-density lipoprotein; antioxidants; *Albizzia julibrissin*; value-added products

INTRODUCTION

With the increasing energy appetite of the world and skyrocketing crude oil prices, biofuels are becoming economically viable. In the United States, yearly corn-to-ethanol conversion is already in the realm of 4.5−5 billion gallons. However, this still falls short of the necessary 180 billion gallons of gasoline and diesel needed yearly by the United States. In addition to producing ethanol from corn, this liquid fuel can also be produced from cellulosic material. Depending on the conversion technology employed, 10−25 million tons of dry cellulosic material are required to produce one billion gallons of ethanol. In most cases, the dry cellulosic feedstock (agricultural wastes, forestry residues, energy crops) must be purchased. At $40/ton, feedstock costs can account for up to half of the cost of producing sugar from cellulosic biomass and up to 75% of the cost of ethanol (*1*).

To offset the feedstock cost, valuable phytochemical streams could be extracted prior, during, or after cellulosic conversion. This concept fits into the biobased biorefinery as previously described by Regauskas et al. (*2*). In line with a biobased biorefinery, reports are showing that it is possible to extract high-value phytochemicals from biomass. For example, organic solvent-extracted lignin samples with antioxidant activity can be obtained from *Poplus nigra* (*3*). Forestry waste products were extracted with acetone, and the corresponding extracts were evaluated in terms of their antioxidant and radical-scavenging capacities (*4*). *Pinus sylvestris* L. forestry waste, more specifically the bark, has yielded phenolic compounds that displayed anti-inflammatory properties (*5*). These and others illustrate that health beneficial phytochemicals could be extracted from cellulosic biomass prior to energy conversion.

In addition to forestry waste, cellulosic biomass could be obtained from energy crops such as *Albizia julibrissin*. Also known in the United States as mimosa, *A. julibrissin* is a legume and is one of several energy crops being tested in the Auburn

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Table 1. Hyperoside and Quercitrin Concentrations of Methanol and Water Extracts of *A. julibrissin* Bark, Flower, Foliage, Whole Plant, and Wood[a]

	60% methanol 50 °C	60%methanol 85 °C	water 50 °C	water 85 °C
	Hyperoside (mg/g)			
bark	0.09 ± 0.02 e	0.12 ± 0.08 e	0.07 ± 0.03 e	0.08 ± 0.04 e
flower	1.05 ± 0.03 bcde	1.08 ± 0.65 bcde	0.72 ± 0.08 de	0.86 ± 0.33 cde
foliage	2.50 ± 0.57 ab	2.75 ± 1.19 a	2.23 ± 0.86 abc	1.93 ± 0.28 abcd
whole plant	2.08 ± 1.04 abcd	2.82 ± 1.38 a	1.51 ± 0.41 abcde	1.90 ± 0.48 abcd
wood	0.02 ± 0.01 e	0.02 ± 0.02 e	0.02 ± 0.01 e	0.01 ± 0.01 e
	Quercitrin (mg/g)			
bark	0.13 ± 0.02 d	0.16 ± 0.10 d	0.10 ± 0.05 d	0.10 ± 0.04 d
flower	7.41 ± 1.74 a	6.83 ± 3.55 ab	5.08 ± 0.74 abc	5.37 ± 1.68 abc
foliage	9.23 ± 2.69 a	9.44 ± 4.00 a	8.13 ± 3.87 a	6.61 ± 0.59 ab
whole plant	1.76 ± 0.61 cd	2.34 ± 1.06 bcd	0.91 ± 0.47 cd	1.54 ± 0.17 cd
wood	0.01 ± 0.01 d	0.02 ± 0.01 d	0.01 ± 0.01 d	0.02 ± 0.02 d

[a] Hyperoside means not followed by the same letter are significantly different. Quercitrin means not followed by the same letter are significantly different.

University energy crop research program. *A. julibrissin* shows an annual yield of 18 dry tons acre^{-1} (42.8 Mg ha^{-1} yr^{-1}) when harvested from four harvests per year and an average total biomass yield of 15.7 dry tons acre^{-1} (37.3 Mg ha^{-1} yr^{-1}) when harvested once per year over (*6*). These yield studies were obtained over a 4 year period (*6*). In addition to being a source of biomass, *A. julibrissin* has been recorded in the Chinese Pharmacopoeia for the treatment of pain and wounds (*7*). *A. julibrissin* biomass is reported to contain the julibrissoide saponins J_5, J_8, J_{12}, and J_{13} (*7*); the flavonoids luteolin-7-*O*-neohesperidoside (*8*), hyperoside, and quercitrin (*9*); and the lignans syringaresinol and medioresinol (*8*). The presence of high-value phytochemicals combined with its high biomass yield lends *A. julibrissin* as an interesting cellulosic feedstock for the biorefinery concept.

An important factor to effectively and economically extract phytochemicals from energy crops is the ability to easily couple their extraction to existing pretreatment technologies. Phytochemical extraction with organic solvents complicates the pretreatment step because organic solvents can interfere with the subsequent enzymatic conversion. By extracting the target phytochemical with water or under aqueous acidified conditions, a simple unit operation can be added to the existing biomass conversion technology, ultimately adding value to the energy crop. For example, in the case of *A. julibrissin* foliage, the use of 50 °C water for hyperoside and quercitrin extraction was comparable to using 60% methanol at 50 °C (*10*). Thus, for certain phytochemicals, a slip stream from the cellulosic biomass could be extracted prior to the energy conversion step.

This described research examines *A. julibrissin* as a potential crop for use in biobased biorefineries and the water-based extraction of specific components. Specific research goals included comparing the extract yields of flavonoids, hyperoside, and quercitrin from *A. julibrissin* and evaluating their potential as low-density lipoprotein (LDL) oxidation inhibitors.

MATERIALS AND METHODS

Plant Material. Samples of 1 year old *A. julibrissin* foliage, flowers, and whole plant were harvested by hand in August 2004, at Auburn University (Auburn, AL). The foliage included the petiole, branches, and leaflets of the entire compound leaf. The "flower" sample was strictly composed of the flowers. The whole plant sample was composed of all the components of the tree. All samples were placed in a forced air oven at 65 °C within 1 h of harvesting, were dried to constant weight, were ground in a household coffee grinder to a 0.32 mm particle size (*11*), and were stored in sealed plastic bags at room temperature. Voucher specimens were deposited at the Ralph E. Martin Department of Chemical Engineering, University of Arkansas (Fayetteville, AR).

Standards and Chemicals. Hyperoside, quercitrin, and rutin were purchased from Indofine Chemical Company, Inc (Somerville, NJ). Glutamine, sodium pyruvate, penicillin/streptomycin, mercaptoethanol, and gentamycin were purchased from Cellgro (Herndon, VA), and fetal bovine serum (FBS) was obtained from Invitrogen (Carlsbad, CA). High-performance liquid chromatography (HPLC) grade acetonitrile and methanol were obtained from EMD Chemicals Inc. (Gibbstown, NJ); formic acid was purchased from EM Science (Gibbstown, NJ); and H_2SO_4, NaOH, and Triton-X 100 were purchased from VWR International (West Chester, PA). 2-Thiobarbituric acid (TBA), 1,1,3,3-tetraethoxypropane (TEP), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), and butylated hydroxytoluene (BHT) were acquired from Sigma (St. Louis, MO). Dimethylsulfoxide (DMSO) and trichloroacetic acid (TCA) were purchased from Mallinckrodt Baker (Phillipsburg, NJ). Dulbecco's modification of Eagle's medium (DMEM) was purchased from American Type Cell Culture (ATCC) (Manassas, VA).

Extraction of Polyphenols. Two grams of dried foliage, flowers, or whole plant was extracted with 60 mL of water or 60% methanol at 50 °C or 85 °C by shaking the mixture in 280 mL amber bottles in a Precision shaking water bath (Winchester, VA) at 150 rpm. All extractions were performed at least in triplicate. To separate the supernatant from the solids, the resulting mixture of solvent and solids was filtered through cheesecloth and then was centrifuged at 10 000 rpm for 30 min in an induction drive centrifuge (Beckman Coulter, Fullerton, CA). The crude extracts were collected and stored at 4 °C for subsequent analysis.

Chromatographic Conditions. Aliquots (1 mL) of the extracts were dried under vacuum using a SpeedVac Plus (Savant Instruments, Holbrook, NY) without heat. After drying, the samples were redissolved in 1 mL of methanol and were filtered through a 0.45 μm syringe filter (VWR International, West Chester, PA). One hundred microliters of each sample was combined with 50 μL of rutin, which served as an internal standard.

The HPLC analysis was conducted on a Waters 2695 Separations Module, equipped with a Model 2996 photodiode array detector and controlled with Empower software. A 10 μL sample was injected onto a Symmetry C_{18} (150 mm × 4.6 mm) column (Waters, Milford, MA). The gradient consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The run was initiated with 85:15 solvent A:solvent B, which was maintained for 5 min, followed by a linear increase to 80:20 over 20 min. The gradient was then increased to 20:80 over 1 min and then was increased to 10:90 over 10 min. Finally, the HPLC gradient was returned to 85:15 over 1 min, where it was maintained for 3 min. This process was followed by a re-equilibration of the column at the final operating condition for 10 min. The HPLC flow rate was 0.75 mL/min, and the column temperature was 30 °C. Each of the compounds was detected by the photodiode array detector at 360 nm. The authenticity of the peaks was compared to reference standards. Calibration curves were setup with concentrations ranging from 0.10 mg/mL to 0.40 mg/mL.

Preparation of DMSO Extracts. The concentrations of hyperoside and quercitrin in the extracts were determined by HPLC and were used together for extract standardization. Once the extract concentration was determined, the volume of extract needed to prepare 0, 2.5, 5, 10, and 20 μM preparations was calculated, pipetted, and evaporated to dryness. The dry extracts were then resolubilized in DMSO.

Oxidized-LDL (oxLDL) Generation. LDL (Biomedical Technoligies Inc., Stroughton, MA) was first dialyzed against EDTA-free TRIS buffer (pH 7.4) for approximately 24 h. $CuSO_4$-induced oxLDL generation experiments were performed in flat-bottomed, 96-well Costar (VWR International, West Chester, PA) plates using 100 μL of 220 μg/mL LDL incubated with 10 μL of 55 μM $CuSO_4$ in the presence or absence of 10 μL *A. julibrissin* extracts for 24 h. The reaction was stopped by the addition of 10 μL of 1 mM BHT.

TBARS Measurement. The extent of oxidation of LDL was determined by thiobarbituric acid reactive substances (TBARS) mea-

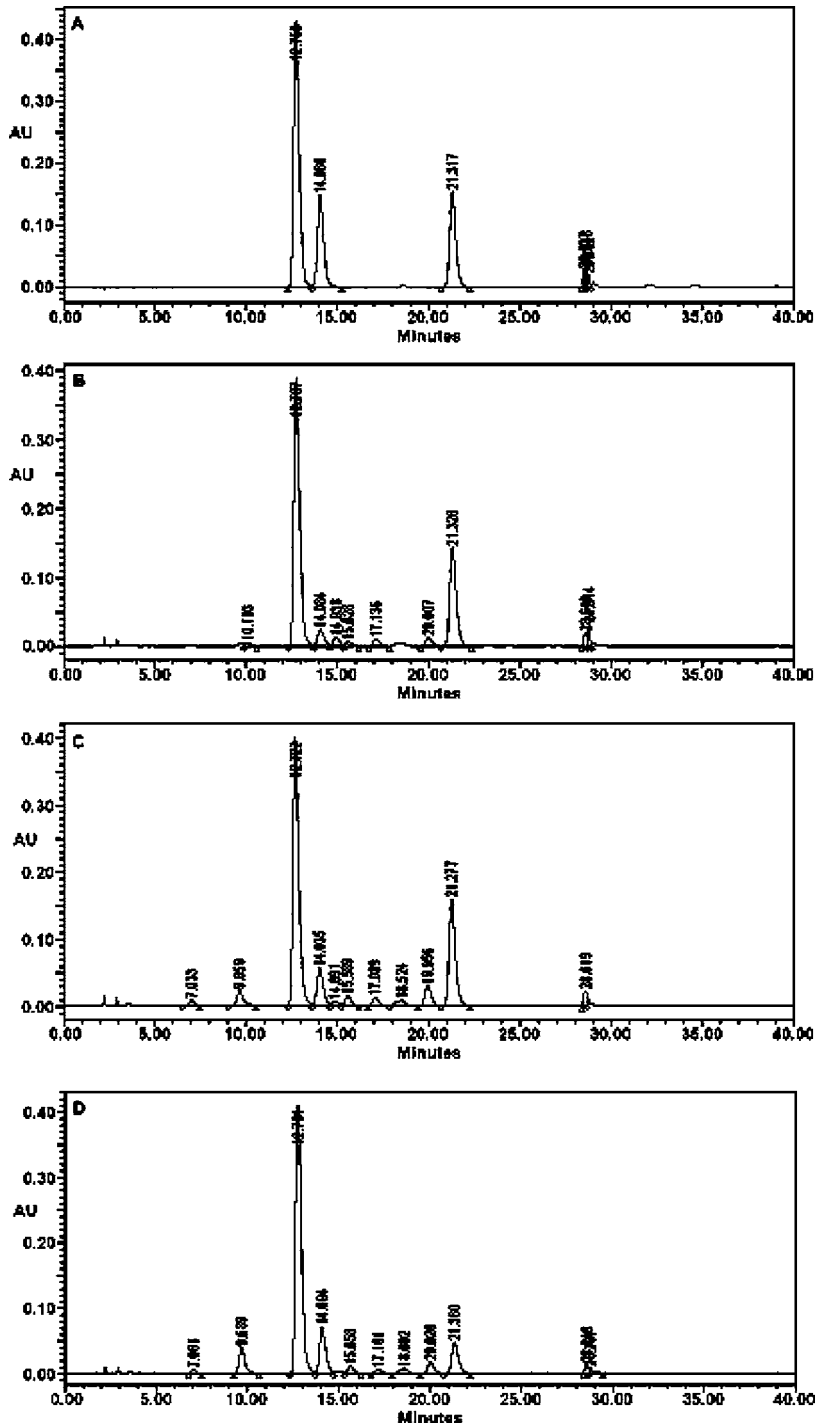

Figure 1. Chromatograms of (**A**) rutin, hyperoside, and quercitrin, (**B**) *A. julibrissin* flower water extract, (**C**) *A. julibrissin* foliage water extract, and (**D**) whole *A. julibrissin* water extracts. The general retention times of rutin, hyperoside, and quercitrin are 12.8, 14.1, and 21.3, respectively.

surement as described by Wallin et al. (*12*). A standard curve was prepared with TEP in the presence of 10 μL of 1 mM BHT. Absorbances at 530 and 590 nm were taken in a microplate reader (BioTek, Winooksi, VT), and the difference in absorbance between the two wavelengths was calculated.

Monocyte Adhesion Assay. Native LDL (nLDL) (200 mg/L) was incubated with Cu^{++} (5 μM) in the presence of *A. julibrissin* extracts for 24 h at 37 °C. LDL or oxLDL protein (at 5 μg/mL in 20 mM borate buffer/10 mM EDTA pH 8.5) was coated onto an ELISA plate (50 μL/well) for 16 h at 4 °C. Adhesion of fluorescent-labeled U937 cells to the protein-coated ELISA plate was performed to determine if *A. julibrissin* could effectively inhibit generation of the oxidized neoepitope in oxLDL recognized by scavenger receptors as described earlier (*13*). Fluorescent intensity was measured before and after inversion using a BioTek Synergy plate reader with a 485 nm excitation/530 nm emission filter. The percentage of cells adhered was calculated by the formula (fluorescence postinversion $\times$ 100)/fluorescence of preinversion.

MTT Assay. The assay was essentially performed as described by Mossman (*14*). Vero 76 cells were purchased from ATCC and were cultured in DMEM supplemented with 10% FBS. Each microplate well received 1×10^5 cells in DMEM, and 10 μL of the test extracts was dissolved in DMSO. The negative control consisted of 10 μL Triton-X 100 and 10 μL DMSO, while the positive control consisted only of 10 μL of DMSO. Plates were incubated for 18 h at 37 °C and with 5% CO_2. After incubation, 10 μL MTT (prepared as specified by the manufacturer) was added to each well and was incubated for an additional 2 h before adding 100 μL MTT solubilization solution. The background absorbance at 690 nm was subtracted from the measured

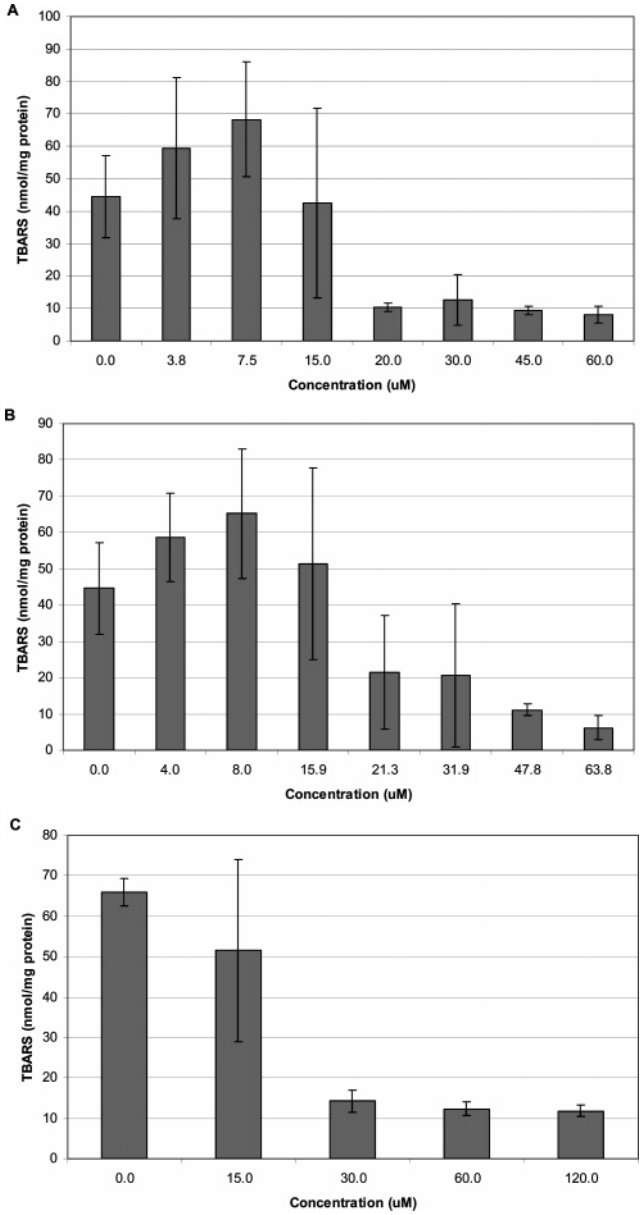

Figure 2. Protective effect of pure polyphenols on LDL oxidization. (**A**) Hyperoside, (**B**) quercitrin, (**C**) combination of hyperoside and quercitrin.

absorbance at 570 nm. The percent cellular health was calculated as % health = (sample absorbance − positive control)/(positive control − negative control).

Data Analysis. All experiments were repeated at least twice, from which standard deviations were calculated. A statistical analysis was performed with JMP software (SAS Institute, Cary, SC) using one-way ANOVA with means separations tested by Tukey analysis set at $p < 0.05$.

RESULTS AND DISCUSSION

Concentration of Polyphenols. Methanolic *A. julibrissin* foliage extracts are known to contain hyperoside and quercitrin (*9*); however, no reports are available on the distribution of these components in the various plant parts. Hence, ground *A. julibrissin* foliage, bark, flowers, and whole plant were extracted with water or 60% methanol at either 50 °C or 85 °C and were analyzed for their hyperoside and quercitrin content (**Table 1**). **Figure 1** presents HPLC traces of the standards as well as the water extracts of the foliage, flowers, and whole plant obtained at 50 °C. The hyperoside concentrations in the extracts ranged

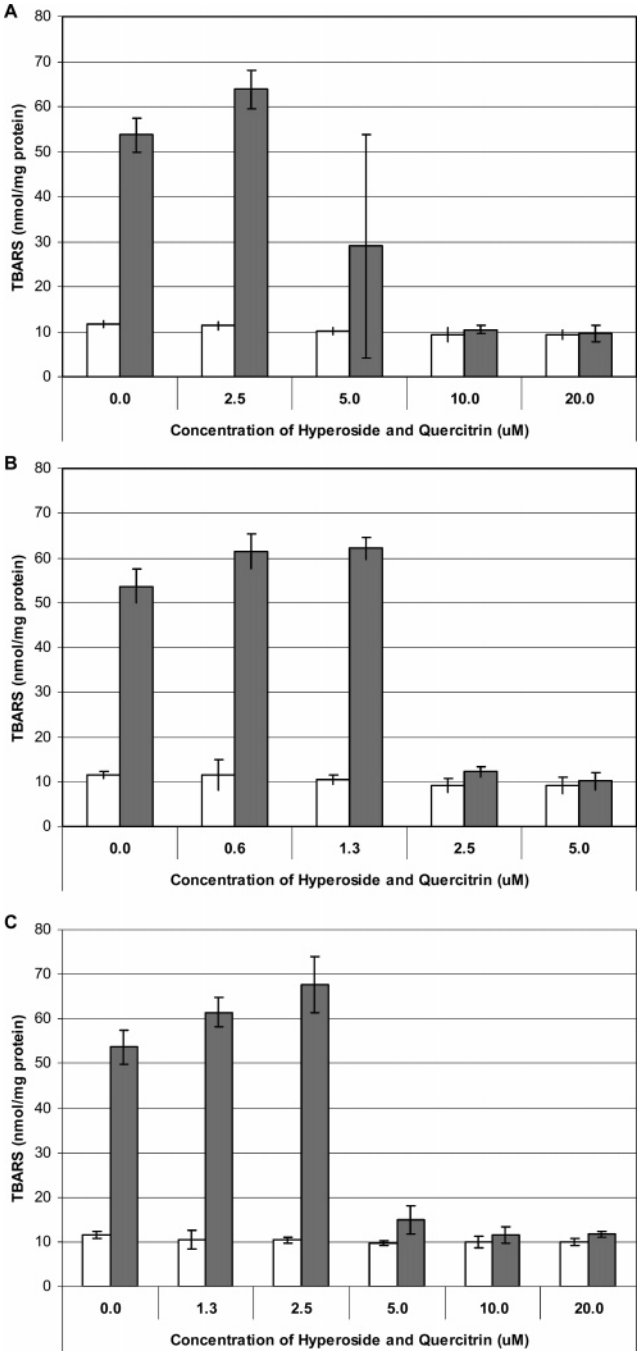

Figure 3. Protective effect of *A. julibrissin* biomass on LDL oxidization: (**A**) flowers, (**B**) foliage, and (**C**) whole extracts; white bars, 0 h; black bars, 24 h.

from a low of 0.01 mg/g for *A. julibrissin* wood extracted in water at 85 °C to 2.82 mg/g for the whole plant extract in 60% methanol at 85 °C. The highest concentrations of hyperoside were obtained when extracting ground foliage or whole plant with 60% methanol at 85 °C, with no statistically significant differences between the two. However, at all four extraction conditions (60% methanol at 50 °C, 60% methanol at 85 °C, water at 50 °C, water at 85 °C), *A. julibrissin* wood yielded the lowest concentrations of hyperoside.

The quercitrin concentration ranged from a low of 0.01 mg/g for *A. julibrissin* wood extracted in water at 50 °C to 9.44 mg/g for foliage extracted with 60% methanol at 85 °C. The highest quercitrin concentrations were obtained when extracting *A. julibrissin* foliage and flowers. Independent of the solvent and temperature combinations used, the extraction of wood yielded

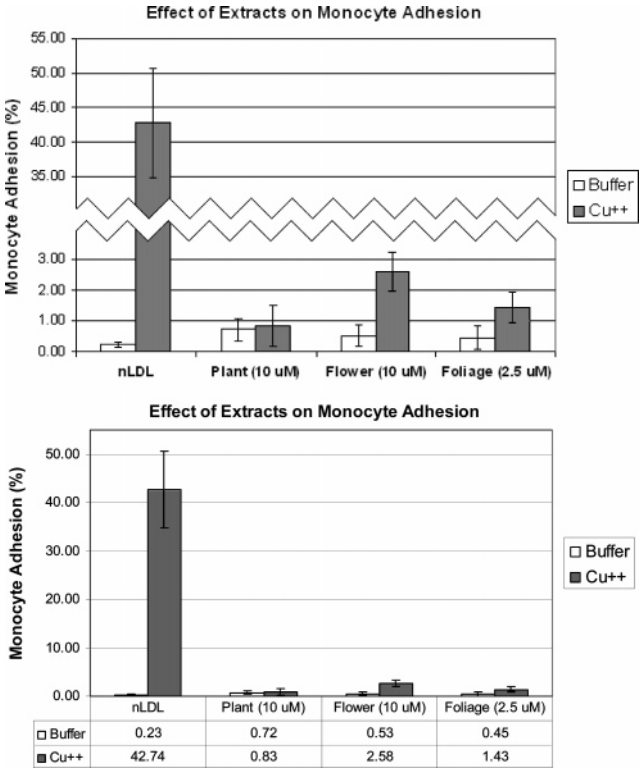

Figure 4. Monocyte adhesion assay.

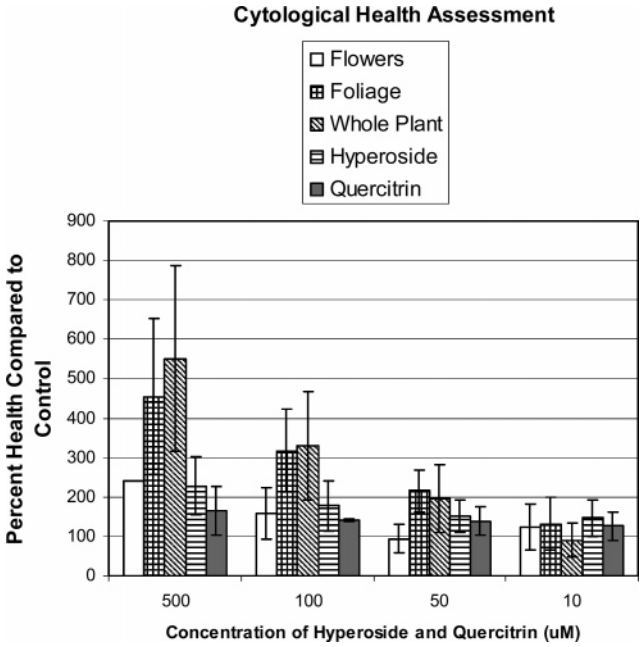

Figure 5. MTT assay performed with plant extracts and reference compounds.

the lowest quercitrin concentrations. The presence of hyperoside and quercitrin has previously been reported by Kaneta et al. (*15*) in unspecified *A. julibrissin* biomass. The presence of quercitrin was reported by Li et al. (*16*) and Kang et al. (*17*) in flowers of *A. julibrissin* but not in the foliage.

Although the highest concentrations of hyperoside and quercitrin were obtained when extracting the biomass with 60% methanol at 85 °C, the use of 50 °C water yielded comparable flavonoid concentrations. Hence, the use of 50 °C water could be contemplated as an alternative extraction solvent. In addition, the production of slip streams prior to energy production could be integrated into a biorefinery (*2*). To develop an embedded slip stream in a biomass-to-biofuels process, the extraction of flavonoids from *A. julibrissin* should be conducted with water, eliminating the need for organic solvent removal prior to the cellulosic pretreatment step. The results presented in **Table 1** show that extracting the flavonoids with 50 °C water results in yields that are similar to those obtained with 60% methanol, indicating that it would be possible to couple value-added recovery to energy conversion.

LDL Oxidization. The oxidation of LDL was obtained by incubating LDL with $CuSO_4$. Copper (Cu^{++}) is part of the proposed mechanisms that are known to oxidize LDL (*18*, *19*). The validity of the experimental system was confirmed by observing the increased electrophoretic mobility, which confirms the oxidization of the LDL (results not shown). **Figure 2** presents the LDL oxidation as a function of pure standard concentration. Pure hyperoside inhibited LDL oxidation at concentrations of 20 μM and above, while quercitrin reduced LDL oxidation at concentrations of 48 μM and above. A preparation of combined hyperoside and quercitrin inhibited LDL oxidization at concentrations of 30 μM and above. LDL oxidation inhibition was also tested with water extracts prepared from *A. julibrissin* flowers, foliage, and whole plant biomass. Flower and whole plant water extracts significantly inhibited LDL oxidation at concentrations of 10 μM and 5 μM, respectively, as seen in **Figure 3**. Foliage water extracts significantly inhibited LDL oxidation at concentrations greater than or equal to 2.5 μM. Large standard deviations occur for those concentrations that show moderate oxLDL inhibition. However, at higher concentrations where oxLDL inhibition is at its maximum, the standard deviations are minimized. The literature indicates that hyperoside extracted from Crataegus flowers inhibited Cu^{++} LDL oxidation, where an ED_{50} of 1.96 μM was reported (*20*). In other research, apple extracts containing quercitrin and hyperoside inhibited lipid peroxidation (*21*). Likewise, a flavonoid-rich extract containing hyperoside and quercitrin was prepared from St-John's Wort and was tested for its antioxidant potential. Results showed that hyperoside and quercitrin inhibited lipid peroxidation (*22*).

Because the generation of TBARS is not very specific, an alternative method was sought for confirmation of the antioxidant properties. As atherosclerotic events are initiated by the macrophage-scavenger receptor mediated binding of oxidized LDL (oxLDL), the effect of *A. julibrissin* foliage, flowers, and whole plant water extracts on reducing scavenger receptor mediated binding to oxLDL was determined. Water extracts of flower, foliage, and whole plants were prepared at concentrations of 10, 2.5, and 10 μM, respectively. The results from the monocyte adhesion assay are shown in **Figure 4**. A 2.5 μM foliage water extract resulted in a reduction from 43% to only 1% of the observed monocyte adherence in Cu^{++} treatments and thus in the amount of oxLDL formed. Flower and whole plant extracts were also efficacious in reducing monocyte adherence. These results suggest that the *A. julibrissin* water extracts prevent in vitro LDL oxidation. Validation of this hypothesis should be examined in vivo and in clinical trials. However, if *A. julibrissin* water extracts prevented LDL oxidation, this could be a source of flavonoids. A similar idea has been proposed where flavonoid-rich extracts would be prepared from apple juice processing waste (*23*).

MTT Toxicity. To have commercial application, *A. julibrissin* water extracts should have minimal toxicity. The MTT assay described by Mossman (*14*) was conducted with monkey kidney-derived cells (Vero 76) to perform a preliminary assessment of

the toxicity of *A. julibrissin* water extracts. Aside from the 50 μM flower extract and 10 μM whole plant extract, cell viability was greater than the control. The results presented in **Figure 5** indicate that the extracts were not cytotoxic to the in vitro cultured cells. In fact, most of the data indicated that improved cellular health was observed when cells were exposed to the extracts. In the literature, aqueous broccoli, onion, carrot, and licorice in water extracts also showed a protective effect, and even increased cellular proliferation, of Vero 76 when incubated with various *N*-nitroso compounds (*24*). However, caution must be exerted in interpreting increased viability because the plant extracts were shown to interact with MTT formazan formation by way of increasing absorbance (*25*).

In summary, the above results show that *A. julibrissin*, a potential lignocellulosic energy crop, could also be a source of flavonoid extracts. Hence, *A. julibrissin* biomass could be extracted with 50 °C water prior to the pretreatment step, which could be carried out with either hot water or aqueous acidified solutions. Inserting an extraction step in the energy conversion processing scheme could yield a stream rich in hyperoside and quercitrin. These flavonoid streams could be used eventually in human foods or in animal feeds as antioxidants. Preliminary toxicity tests indicate that these water extracts are devoid of toxic factors.

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