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Switchgrass Water Extracts: Extraction, Separation and Biological Activity of Rutin and Quercitrin

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Switchgrass (*Panicum virgatum* L.) has recently received significant attention as a possible feedstock for the production of liquid fuels such as ethanol. In addition, switchgrass may also be a source of valuable co-products, such as antioxidants, and our laboratory recently reported that switchgrass contains policosanols and α-tocopherol. Motivation for this work began when a switchgrass sample was extracted with water at 50 °C and was then tested for low-density lipoprotein (LDL) oxidation inhibition activity using the Thiobarbituric Acid Reactive Substances (TBARS) assay. The TBARS results showed that the switchgrass water extracts inhibited LDL oxidation by as much as 70% in comparison to the control. Liquid chromatography coupled with mass spectrometry (LC-MS) and high performance liquid chromatography (HPLC) were used to identify the compounds that were responsible for LDL oxidation inhibition activity as flavonoids: quercitrin (quercetin-3-*O*-rhamnoside) and rutin (quercetin-3-*O*-rutinoside). To maximize flavonoid concentrations, switchgrass was then extracted with water and 60% methanol at different temperatures. The 60% methanol treatment resulted in higher rutin and quercitrin yields when compared to water-only extraction; however, the use of this solvent would not be practical with current biorefinery technology. Centrifugal partition chromatography (CPC) was then used to purify rutin and quercitrin from the switchgrass water extract, which were then tested via the TBARS assay and shown to exhibit lipid peroxidation inhibition activity similar to that obtained with pure flavonoid standards. This is the first report on the presence of rutin and quercitrin in switchgrass. The results support the extraction of viable coproducts from switchgrass prior to conversion to liquid fuel.

KEYWORDS: Switchgrass; water extraction; flavonoids; TBARS; CPC; cellulosic ethanol

INTRODUCTION

The depletion of fossil fuels and increased concerns about carbon dioxide in the atmosphere have led to further development and deployment of sustainable energy production, including the use of biomass for energy production. The term, cellulosic ethanol, describes the conversion of nonfood biomass to ethanol. Cellulosic ethanol implies the production of ethanol from a wide variety of feedstocks including: agricultural plant wastes (i.e., cornstover, cereal straws, and sugar cane baggasse) and energy crops (i.e., woody crops and switchgrass) (*1*). The efficacy of the cellulosic biomass, via a biochemical conversion to ethanol, largely depends on the feedstock characteristics, pretreatment processes, fermentation technology, and scale-up. The use of water, or an acidified aqueous solution, as the extraction solvent allows for the straightfoward addition of a phytochemical recovery stream before the pretreatment process. This extra step would result in the addition of a value-added stream to the overall process (*2*).

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Switchgrass (*Panicum virgatum* L.) has been targeted as a possible source of biomass for conversion to liquid fuel. One way of making switchgrass, or any other biomass source, a more marketable and profitable crop is to find a product that can be extracted from the biomass prior to conversion. In this case, the product of interest belongs to a class of plant secondary metabolites called flavonoids, which are known for their antioxidant activity (*3*). In this study, the flavonoids recovered from switchgrass were rutin and quercitrin. Both rutin (quercetin-3-*O*-rutinoside) and quercitrin (quercetin-3-*O*-rhamnoside) share the aglycon, quercetin, which is the most naturally abundant flavonoid and is found in a variety of fruits and vegetables (*4*−*6*). Rutin has been found to hamper bone resorption in rats (*7*), possess antiangiogenic effects (*8*), and has found a use as an antioxidant (*9*). Quercetrin has been used as an antibacterial agent (*10*, *11*) and has been found to inhibit oxidation of low-density lipoproteins (*12*). Fabjan et al. found tartary buckwheat (*Fagopyrum tataricu* Gaertn.) to be a reliable source of rutin, quercetrin, and quercetin (*13*). Thus, switchgrass could be a source of valuable phytochemicals and also serve as a feedstock for cellulosic ethanol production. This research describes the

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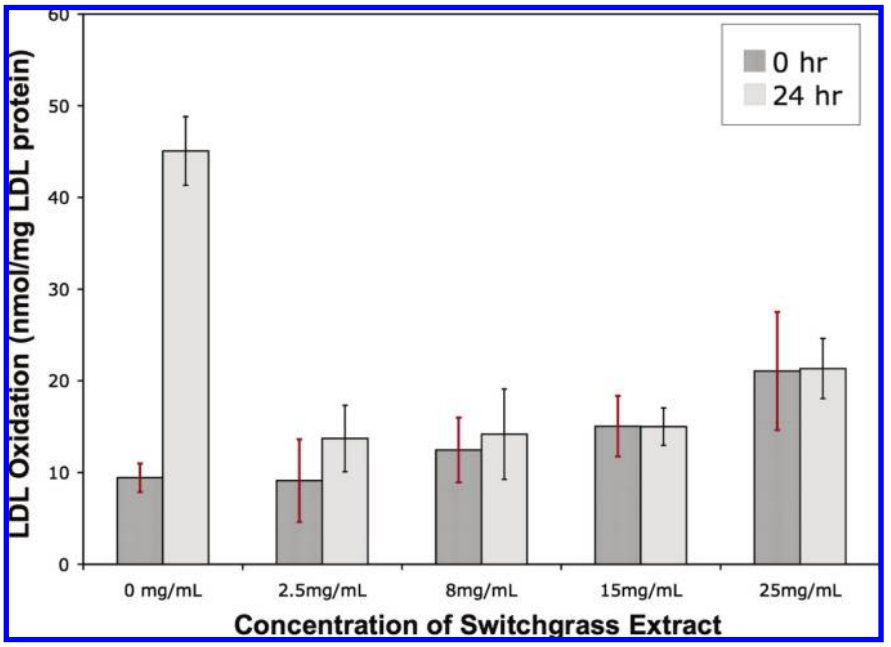

Figure 1. TBARS results of a crude switchgrass water extract. The decrease in oxidation levels as compared to the positive control, which is pure DMSO, confirms the presence of antioxidant compounds in the crude extract.

preparation of water-based and methanol/water-based switchgrass extracts, the separation and quantification of flavonoids and an assessment of biological activity as measured by the potential to inhibit the oxidation of low-density lipoproteins (LDL).

MATERIALS AND METHODS

Plant Materials. Cave-in-Rock switchgrass (*Panicum virgatum* L.) from the early fall harvest (September−October, 2006), similar to that used by Ravindranath et al. (*14*), was used in this study. The switchgrass plot was established from seed in 2001 and grown using dryland cultivation practices at the University of Arkansas Agricultural Research and Extension Center (Fayetteville, AR; Captina silt loam soil). *Albizia julibrissin* (also termed mimosa) plant material was identical to that used by Lau et al. (*15*). Both *P. virgatum* and *A. julibrissin* samples used in this study were lyophilized and pulverized in a household coffee grinder to an average particle diameter of 4 mm determined according to Standard ASAE S319.3 (*16*) prior to extraction.

Chemicals. HPLC grade methanol and acetonitrile were used for the HPLC analysis. Analytical grade hydrochloric acid, sulfuric acid, hexane, dimethylsulfoxide (DMSO), chloroform and formic acid were used. Octocosanol, triacontanol, tris, 2-thiobarbituric acid (TBA), 1,1,3,3-tetraethoxypropane (TEP), copper sulfate, and butylated hydroxytoluene (BHT) were obtained from Sigma (St. Louis, MO). Phenol was manufactured by EMD Chemicals (Darmstadt, Germany). Sodium chloride and trichloroacetic acid (TCA) were manufactured by Mallinckrodt Baker (Phillipsburg, NJ). *N*-Methyl-*n*-trifluoroacetamide (MSTFA) was purchased from Pierce (Rockford, IL). Sodium hydroxide was obtained from Fisher Scientific (Milford, MA). Quercitrin was acquired from Indofine Chemical Company Inc. (Somerville, NJ). Rutin was purchased from Alfa Aesar (Ward Hill, MA). α-Tocopherol was obtained from TCI-America (Portland, OR).

Extraction of Plant Material with Water. Two grams of a ground switchgrass sample were blended with 30 mL of water at 1000 rpm for 5 min in an Ultra Turrax T18 blender (IKA Works, Inc., Wilmington, NC). The blended mixture was combined with an additional 30 mL of water and then heated in a water bath (Thermo Electron, Milford, MA) at 50, 70, 80, or 90 °C for 20 min. The extracts were filtered with Whatman No.1 filter paper (Florham Park, NJ) under reduced pressure to remove the solids. The filtered extracts were stored at −20 °C for 24 h and then immersed in liquid nitrogen prior to lyophilization (Labconco Freezone, Kansas City, MO). The freeze-dried extracts were stored at 4 °C for subsequent analysis.

Extraction of Plant Material with 60% Methanol. The methanol extract was prepared in the same manner as the water extract except for using 30 mL of an aqueous 60% methanol solution during blending and then the addition of another 30 mL of 60% methanol solution prior to heating. The extracts were prepared in the same manner as the water extracts, except that methanol cannot be lyophilized. A Yamato RE 200 vacuum rotary evaporator (Akashi, Japan) was used to remove the methanol portion of the filtered extract. The remaining water was then frozen, lyophilized and stored in an identical manner as the water extract.

Identification of Phytochemicals. Both water and 60% methanol extracts were analyzed by liquid chromatography coupled with mass spectrometry (LC-MS) (*15*). Previously published results from our laboratory, using *A. julibrissin* foliage that was extracted with both water and 60% methanol at 50 °C and analyzed in a similar fashion, were used as the basis of comparison to detect the presence of flavonoids (*14*). The extraction of flavonoids from *A. julibrissin* was performed in parallel with the switchgrass extraction to aid in compound identification. A Hewlett-Packard 1100 series high-performance liquid chromatograph (HPLC) (Foster City, CA) with photodiode array (PDA) coupled to a mass spectrometer was used for analysis. The extract was filtered with a 0.45 μM syringe filter (National Scientific, Rockwood, TN) and then injected into a Waters Symmetry C_{18} (150 mm × 4.6 mm) column (Waters, Milford, MA) equipped with a 25 μL sample loop. The eluent consisted of 0.1% formic acid in water (Solvent A) and methanol (Solvent B). The flow rate was set at 0.55 mL/min and the gradient was initiated with 98:2 solvent A: solvent B and linearly decreased to 40% solvent A over 60 min. The sample was detected at 360 nm using the photodiode array (PDA) detector. The MS was operated in the positive ionization mode and ions were formed using electrospray ionization (ESI). The temperature of the drying gas (N_2) was set to 300 °C and flowed at 10 mL/min. The nebulizing pressure (N_2) was maintained at 2.1×10^5 Pa (30 psi).

Quantitative Analysis. The identity and concentrations of the compounds in the switchgrass extracts were determined using the HPLC method described by Vaughn et al. (*17*). Two milligrams of each extract were dissolved in 1 mL of methanol and analyzed via HPLC to determine which condition (temperature, extraction solvent) afforded the most abundance of rutin and quercetrin. Exactly 20 μL of the sample was injected onto a Symmetry C_{18} (150 mm × 4.6 mm) column. The eluent consisted of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). The run was initiated with 85:15 solvent A/solvent B, which was maintained for 5 min, followed by a linear decrease in A to 80:20 over 20 min. The gradient was then increased in B to 20:80 over 1 min, to 10:90 over 10 min, and then returned to the initial condition over

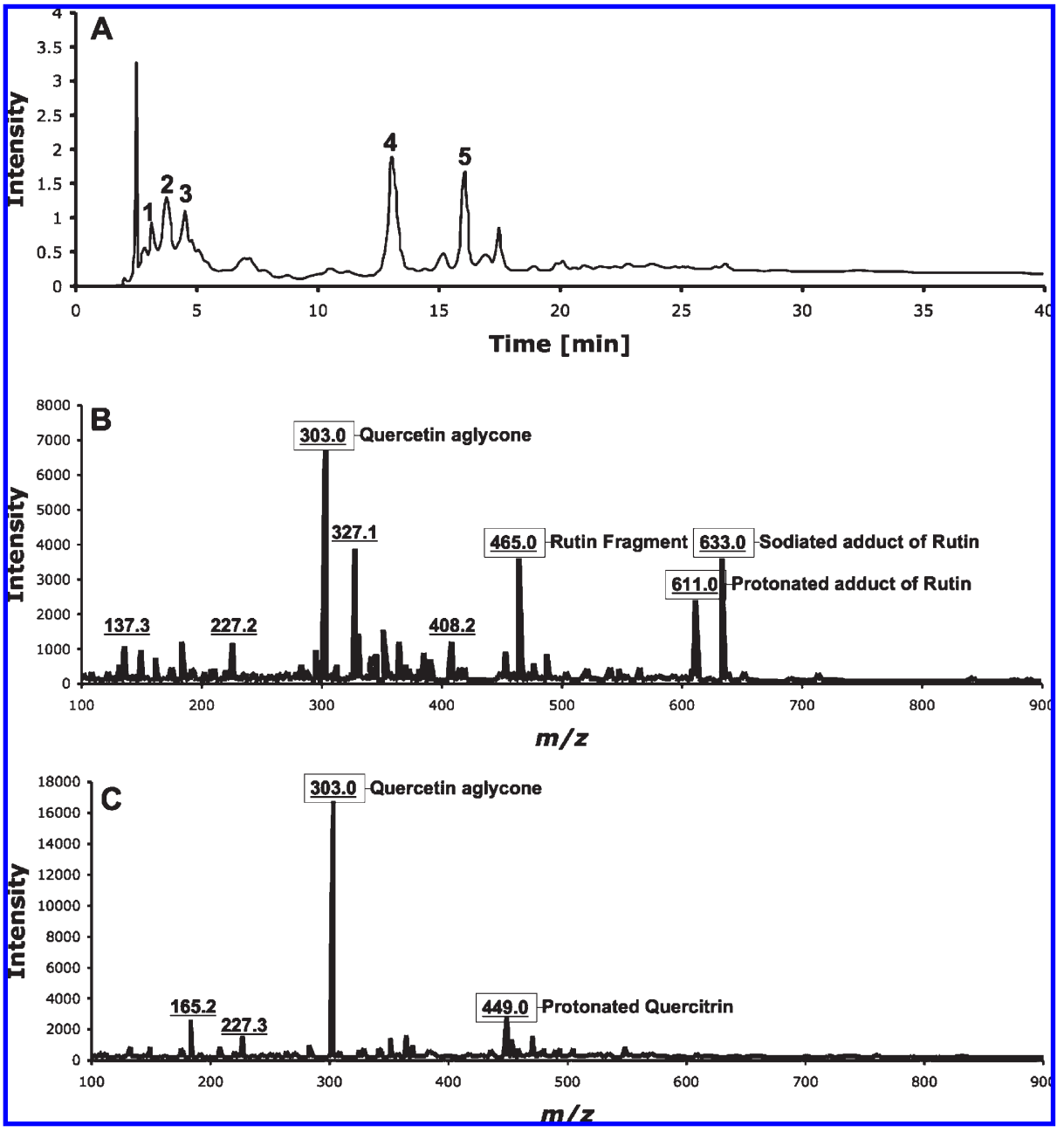

Figure 2. Switchgrass extracted with 50 °C water and analyzed by LC-MS. (**A**) LC trace of switchgrass water extract at 360 nm. (**B**) MS spectra of 4th peak in the LC trace of part A, with major peaks at *m*/*z* 303, 465, 611, and 633, which indicate that the compound is rutin. (**C**) MS spectra of 5th peak in the LC trace in part A, with predominant signals at *m*/*z* 303 and 449, which indicate the compound is quercitrin.

1 min where it was maintained for 3 min. This was followed by re-equilibration of the column at the initial operating condition for an additional 10 min. The HPLC flow rate was 0.75 mL/min, and the column temperature was 30 °C throughout the run. The compounds, rutin and quercitrin, were best detected at 360 nm by the PDA. A standard curve prepared with reference compounds was used to estimate the concentration of quercitrin and rutin concentrations in the extracts.

Purification of Phytochemicals. Centrifugal partition chromatography (CPC) (FCPC, Kromaton, Angers, France) was used to purify the rutin and quercitrin from the extraction solution. The solvent system was comprised of ethyl acetate/ethanol/water (2:1:2, v/v/v). The solvent system was chosen based on previously published use in separating quercetin glycosides (*18*) and the partition coefficients of the compounds. The partition coefficient, *K*, was determined with reference standards using the shake flask method (*19*). The partition coefficient was determined using the areas under the peaks on the HPLC chromatogram for the compound of interest in the relevant phase. For example, the partition coefficient for rutin was calculated by dividing the area of the rutin peak in the upper phase by the area of the rutin peak in the lower phase. The partition coefficient for rutin was calculated to be 0.74 and the partition coefficient for quercitrin was 2.20. The calculated coefficients were adequate for CPC separation, since they were within in the acceptable range ($0.5 < K < 2.5$) (*20*).

The solvent system for CPC was prepared by combining the proper volume ratio of solvents in a separatory funnel, was well mixed and then allowed to settle for at least 30 min prior to use. The solvents were then separated and the 200 mL CPC rotor was filled with the lighter organic phase. The aqueous phase was used as the mobile phase and was introduced at 3 mL/min in the system using a Waters 510 adjustable solvent delivery pump (Millford, MA), once the rotor had been filled with two times the capacity (400 mL). The mobile phase displaced 104 mL of stationary phase before equilibrium in the rotor was attained. The CPC was connected to a variable single wavelength UV−vis absorbance monitor (VUV 24, Reflect Scientific, Orem, UT) set to 360 nm.

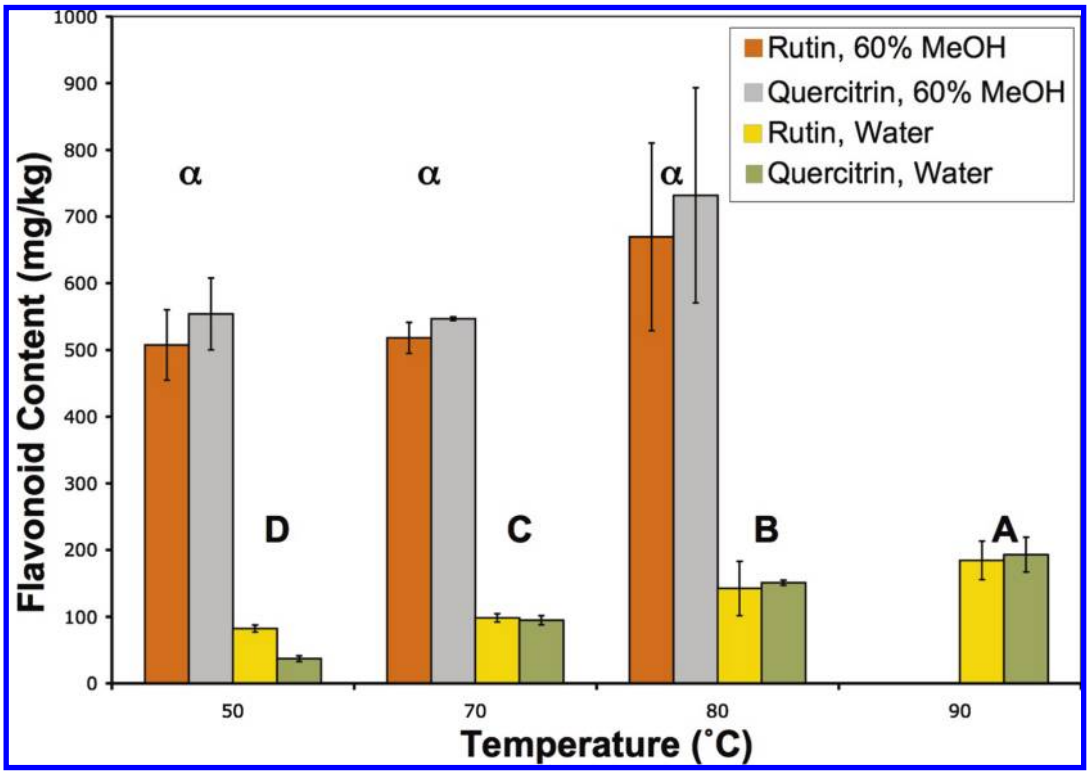

Figure 3. Quantity of flavonoid recovered for both water and 60% methanol extracts at different temperatures. Statistical analysis was performed using ANOVA, student *t*-test and least squared on JMP. Columns that share the same letter are not significantly different from each other at the $P \leq 0.05$ level: the 60% methanol extracts are designated using Greek letters while the water extracts use English letters.

The sample to be analyzed was prepared by adding 6 mL of aqueous phase and 4 mL of organic phase to approximately 600 mg of freeze-dried switchgrass water extract. The sample was then filtered through a 1 μM syringe filter (Whatman, Florham Park, NJ). The sample was injected after hydrodynamic equilibrium of the two phases in the rotor was achieved. The rotor spun at 1100 rpm and the total separation time was 150 min. Fractions were collected every 2 min, adding up to 6 mL/tube. Each tube was dried under reduced pressure in a Speedvac (Savant, Farmingdale, NY). The fractions were then reconstituted with 1 mL of methanol and each tube was analyzed for purity using HPLC.

Oxidation of Low-Density Lipoproteins. The biological activity of the purified switchgrass extracts was evaluated with copper mediated thiobarbituric acid reactive substances (TBARS) assay, as outlined by Wallin et al. (*21*). Essentially, the assay was performed as described by Vaughn et al. (*17*). Human low-density lipoprotein (LDL) (Biomedical Technologies Inc., Stroughton, MA) was dialyzed using dialysis tubes (10 000 molecular weight cutoff, Pierce, Rockford, IL) in EDTA-free TRIS (pH 7.4) buffer for 24 h at 4 °C. The purified switchgrass extract samples were prepared in DMSO such that their final concentration in the wells ranged from 9 to 150 μM. Exactly 100 μL of LDL, 10 μL of 55 μM copper sulfate, and 10 μL purified test compounds were pipetted into the wells in a 96 well assay plate (Becton Dickinson, Franklin Lanes, NJ). Exactly 10 μL BHT (1 mM) was added to the 0 h wells and the plate was incubated at 37 °C for 24 h in a water bath. After the 24 h incubation, 10 μL of BHT was added to the remaining wells to stop the activity. Exactly 50 μL of 50% (w/v) TCA and 75 μL of 1.3% (w/v) TBA were then added to all the wells, and the plate was incubated at 60 °C for 40 min in a water bath. The amount of TBARS formed is the difference between the 0 h and the 24 h wells and is proportional to the oxidation of LDL. This difference was measured using a microplate reader (BioTek, Winooksi, VT) at 530 and 590 nm. A standard curve prepared with TEP was used to estimate the amount of malondialdehyde (MDA) formed. Results were expressed as nanomoles of MDA per milligram of LDL protein as described by Vaughn et al. (*17*).

Statistical Analysis. All of the data were obtained in triplicate and subjected to statistical analysis using ANOVA, student *t*-test, and least squared fit on JMP (SAS Institute, Cary, NC). The significance was determined at the $P < 0.05$ level.

RESULTS AND DISCUSSION

Crude Switchgrass Extract with Antioxidant Activity. In switchgrass extraction studies with hexane, Ravindranath et al. (*14*) reported that Cave-in-Rock switchgrass contained approximately 150−200 mg/kg of policosanols and 300−450 mg/kg of α-tocopherol. Although policosanols are valuable compounds, the design and implementation of a hexane-based extraction process would be challenging in a biochemical based biorefinery setting, where aqueous-based pretreatment and hydrolysis are the rule. In an aim to design a process to extract coproducts from switchgrass prior to energy conversion, early fall Cave-in-rock switchgrass was extracted with hot water and tested for antioxidant activity using the *in vitro* TBARS assay (*21*). The results from this assay showed that crude switchgrass water extracts in DMSO inhibited LDL oxidation by as much as 70%, when compared to a control of pure DMSO as shown by **Figure 1**. Results presented in **Figure 1** show that the crude switchgrass extract at 25 mg/mL did not inhibit as much LDL oxidation as at 2.5 mg/mL; this can possibly be attributed to the fact that the TBARS assay is based on colorimetric readings which may render the detection of darker wells problematic. However, these results indicated that switchgrass water extracts contained unidentified compounds that exhibited antioxidant properties. Because the extracts were water-based, the compounds responsible for the antioxidant activity must be hydrophilic. Therefore, they were not policosanols or α-tocopherol, which are lipophilic, and further scrutiny was necessary to identify the inhibiting compounds.

Identification of Phytochemicals. To determine which compounds were responsible for the LDL oxidation inhibition, LC

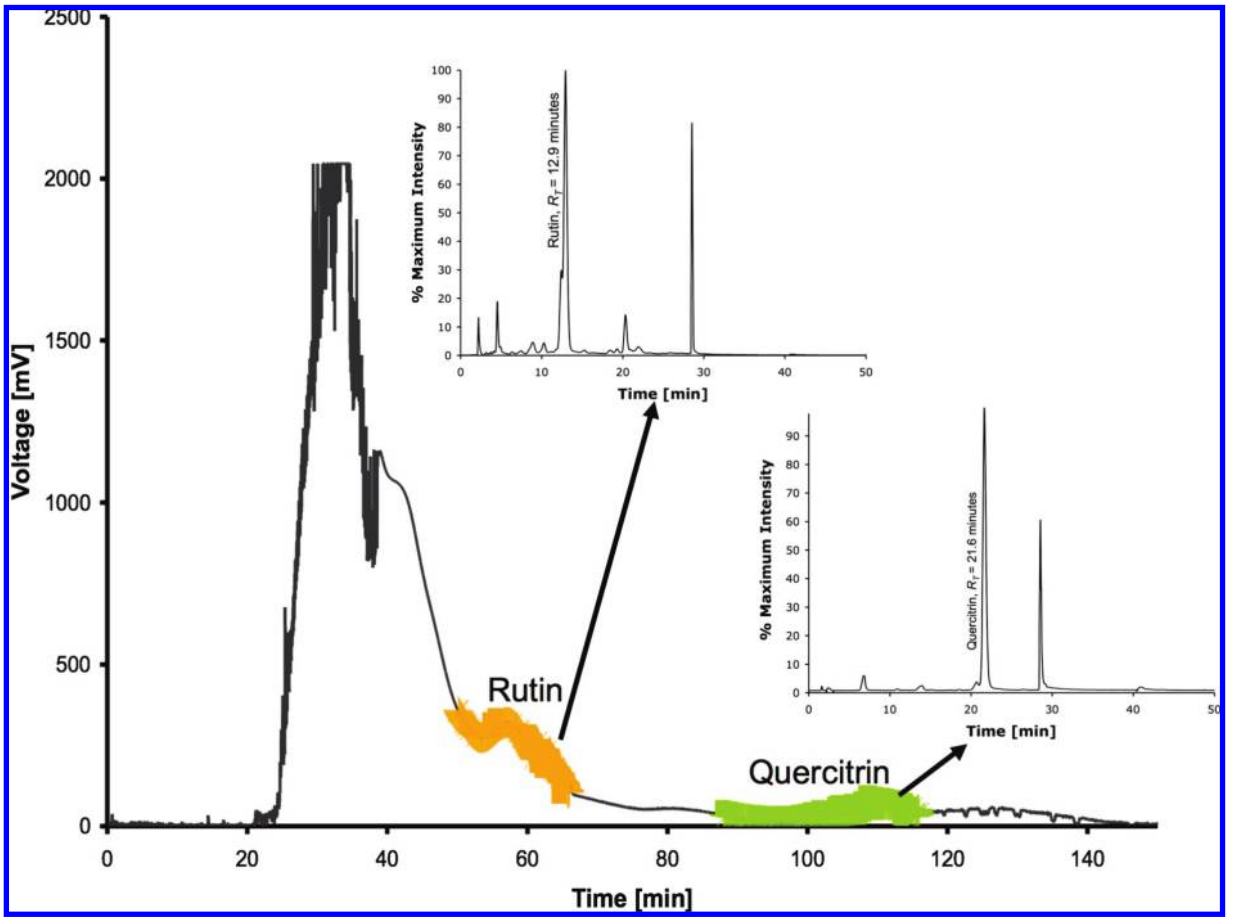

Figure 4. Resulting CPC chromatogram with rutin and quercetrin peaks indicated. Peak contents were verified via HPLC and the rutin and quercetrin fraction HPLC traces are shown inset alongside the CPC trace.

coupled with an electron spray ionization mass spectrometer, operated in the positive ionization mode, was used to analyze the crude switchgrass water extracts. **Figure 2** displays the results from the LC-MS analysis of a water extract performed at 50 °C: part **A** is the LC trace, part **B** shows the corresponding mass spectrum (MS) of peak 4 with significant ions at *m*/*z* 303, 465, and 611, and part **C** displays the mass spectrum of peak 5 with significant ions at *m*/*z* 303 and 449.

The *m*/*z* 303 ion is consistent with a quercetin aglycone fragment ion, which previous work in our laboratory indicated was also present in *A. julibrissin* (*17*). Flavonoids are ubiquitously present in plants and are known to be antioxidants that inhibit LDL oxidation (*12*). Polar solvents, such as methanol or water, can be used to extract flavonoids. Therefore, it was suspected that crude switchgrass water extracts would likely contain flavonoids. Using the assignment of *m*/*z* 303 as the quercetin aglycone, the peaks at *m*/*z* 611 and *m*/*z* 633 in **Figure 2B** were consistent with protonated and sodiated adducts of rutin. The peak at *m*/*z* 465 is most likely another fragment of rutin in which one of the two sugar units attached to the aglycone has been cleaved. In **Figure 2C**, the peak at *m*/*z* 449 was consistent with protonated quercitrin. LC/MS results were confirmed with analysis of quercitrin and rutin reference compounds. To further confirm compound identification, *A. julibrissin* and *P. virgatum* biomass were simultaneously extracted with methanol and analyzed by HPLC as described by Lau et al. (*15*). Through these manipulations and cochromatograph experiments using reference standards, peak 5 in **Figure 2A** was identified as quercitrin and peak number 4 was indeed rutin.

Quantification of Phytochemicals. To determine the best extraction conditions that resulted in the maximum quercitrin and rutin recovery, switchgrass was extracted with water at temperatures ranging from 50 to 90 °C and 60% methanol at temperatures ranging from 50 to 80 °C. Each of the extracts was analyzed using the HPLC method described above. The results are summarized in **Figure 3**. Quercitrin and rutin were extracted at higher yields with a 60% methanol solution than water at every condition.

With 60% methanol, the rutin yields were in the range of 502 and 620 mg/kg and the quercitrin yields varied between 554 and 732 mg/kg. However, the student *t*-test and least-squares fit analysis showed these differences were not statistically significant over the given temperature range. In the water extracts, the results did show a statistically significant increase in the amount of flavonoid extracted as a function of temperature. The extracts at 50 °C produced the lowest yields, with 82 mg/kg for rutin and 37 mg/kg for quercitrin. At 90 °C, the yields increased to 186 mg/kg for rutin and 193 mg/kg for quercitrin. It should be noted that the quercitrin to rutin recovery ratio did not vary with extraction temperature for the 60% methanol extracts.

Because one of the goals of this project was to design a coproduct extraction process that could seamlessly fit into an anticipated biochemical conversion platform, 90 °C water was used for further analysis instead of any of the 60% methanol extracts, even though the latter enabled the recovery of higher flavonoid concentrations.

Purification of Phytochemicals. Quercitrin and rutin were purified from the crude switchgrass 90 °C water extracts by CPC and then used in a TBARS assay to assess their individual antioxidant activity. The solvent system, ethyl acetate/ethanol/water (2:1:2; v/v/v), was effective in separating a mixture of

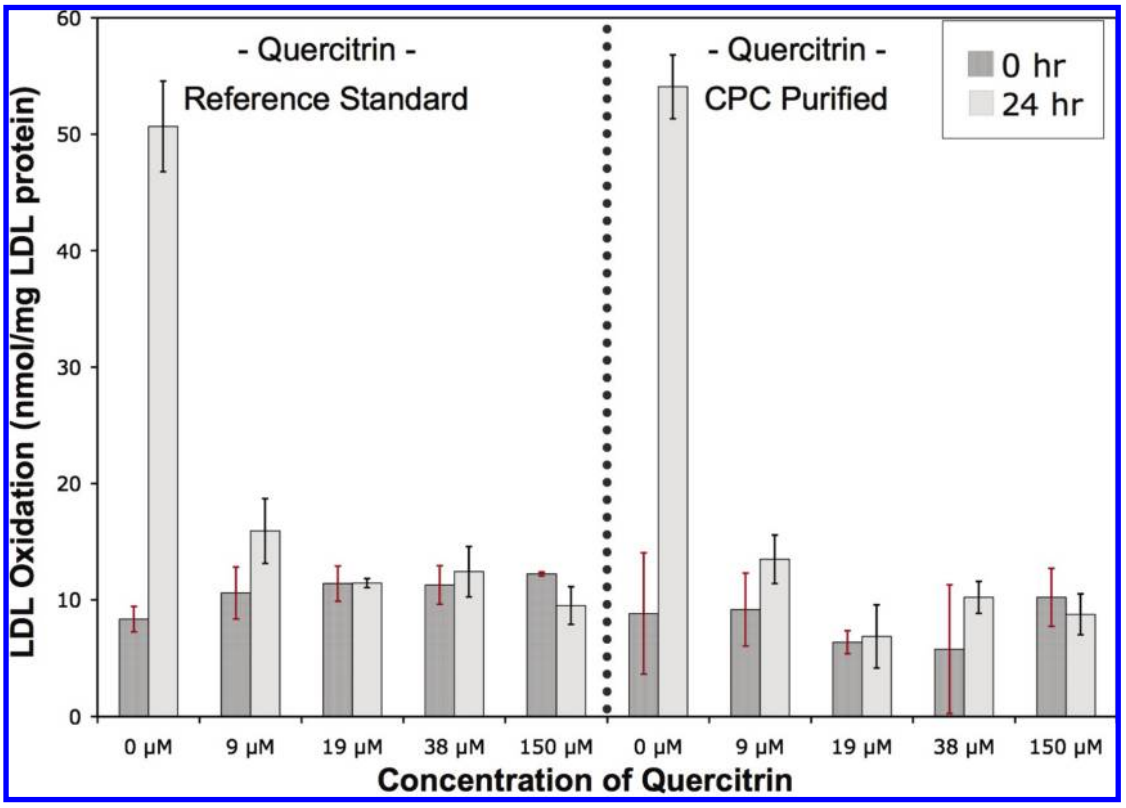

Figure 5. TBARS formation with various concentrations of quercitrin standard and quercitrin obtained from combined fractions of a CPC separation. Statistical analysis on both the standard and the purified quercitrin show that only the 0 μM well, the control, showed significant difference from wells containing varying amounts of quercitrin.

hyperoside, quercitrin and rutin (*18*). As determined with preliminary testing using reference standards, rutin had a retention time (R_T) of 50 to 65 min, while quercitrin eluted between 88 and 115 min. The fractions obtained from the CPC experiments were analyzed by HPLC for their purity. The CPC separation was performed two more times, with similar results, and the fractions were combined based on contents determined during initial HPLC analysis. **Figure 4** shows the chromatogram of the final CPC run with the location of rutin and quercetrin elution indicated. CPC fractions of rutin and quercitrin were combined and quantified on HPLC for use in biological activity assessment.

The HPLC trace of the combined CPC fractions of rutin and quercitrin showed distinct peaks with retention times of 12.9 and 21.6 min, respectively, and are inset in **Figure 4**. From the HPLC analysis, the purity of the flavonoid compounds fractionated on CPC was determined to be 61.4% for the rutin fraction and 76.7% for the quercitrin fraction.

Antioxidant Activity. The antioxidant activity of quercitrin and rutin were analyzed with Cu^{2+} induced LDL oxidation using the *in vitro* TBARS assay (*21*, *22*). **Figure 5** displays the results of the TBARS assay obtained with reference standards of quercitrin and, for direct comparison, the fractions of quercitrin that were purified with CPC, with each tested in concentrations of 150, 38, 19, and 9 μM. **Figure 6** displays the results for rutin, with the standard and the CPC fraction purified in the same manner as **Figure 5**. The fractions of quercitrin and rutin from the CPC were not 100% pure, so the concentrations used in the TBARS assay were made up to the specified concentrations based on the quantified amounts from HPLC and there may have been more compounds present in the well while performing the assay. It should be noted that unlike quercitrin, the rutin standard at concentration as low as 9 μM did not inhibit LDL oxidation, indicating less biological activity in this *in vitro* biological assay. All other flavonoid preparations showed significant reduction in the TBARS formation. Quercitrin and rutin, as purified by CPC in concentrations as low as 9 μM, showed significant reduction in the TBARS formation by as much as 70%, thus indicating that both quercitrin and rutin had inhibitory effect on LDL oxidation reaction.

The comparison study demonstrates that flavonoids purified from a switchgrass water extract possess similar activity as that of the reference compounds. In fact, the flavonoid fractions that were extracted with water and purified via CPC showed better inhibition of LDL oxidation. This can possibly be attributed to the additional HPLC peak, at 28.5 min, observed in each of the quercitrin and rutin fractions (see insets in **Figure 4**). This peak has not yet been identified, but may be a compound present in switchgrass that has an additive effect on the inhibition of LDL oxidation along with quercitrin and rutin. The unidentified peaks may be degradation products of quercitrin and rutin with very similar partition coefficients to the flavonoids themselves. This would explain why they did not separate from their root product during the CPC purification.

Vaughn et al. showed that hyperoside and quercitrin reference standards inhibited LDL oxidation at concentrations of 30 and 48 μM, respectively, while *A. julibrissin* whole plant extracts, showed antioxidant activity at concentrations >5 μM (*17*). Wagner et al. (*6*) used the TBARS assay to show that quercitrin reduced the lipid peroxidation of rat brain homogenates induced by quinolinic acid and sodium nitroprusside at a concentration of 20 μM.

The bioactivity of rutin in reducing the oxidative damage, determined using TBARS, was shown by Pereira et al. (*23*). Additionally, rutin reduced the lipid peroxidation in rats at a concentration of 10 μM (*24*). Yokozawa et al. identified the antioxidant activities of 41 types of flavonoids using TBARS (*25*).

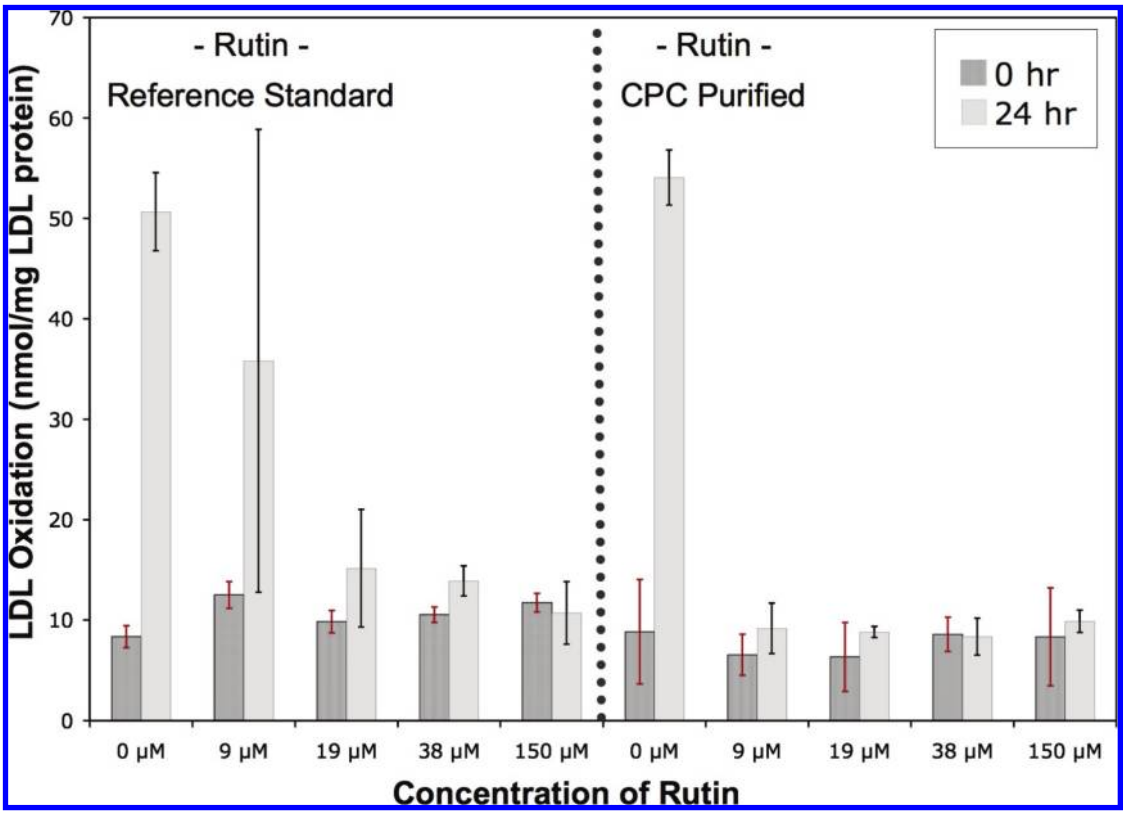

Figure 6. TBARS formation with various concentrations of rutin standard and rutin obtained from combined fractions of a CPC separation. Statistical analysis on both the standard and the purified rutin show that only the 0 μM well, the control, showed significant difference from wells containing differing amounts of rutin.

Interestingly, both quercitrin and rutin reduced the lipid peroxidation in rat liver homogenates induced by hydrogen peroxide at concentrations of 12 and 22 μM, respectively. However, rutin had no effect on lipid peroxidation induced by Fe^{2+} and H_2O_2 (*25*).

The chemical structure of the flavonoids plays a vital role in determining its antioxidant activity and the presence of a sugar at C-3 reduces the antioxidant activity of the flavonoid (*25*).

In conclusion, this study demonstrated that water extracts from switchgrass possess LDL oxidation inhibition activity due to the presence of flavonoids, namely, quercitrin and rutin. Because these flavonoids can be extracted from switchgrass with hot water, this extraction step could be performed prior to existing pressurized water or dilute acid pretreatment, which are critical unit operations in the biochemical conversion of feedstocks to biofuels (*26*). The production of a phytochemical-rich slip-stream in a biorefinery could enhance its economic viability with auxiliary revenues. These streams could find use in human foods, food products, or in animal feeds as antioxidants.

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