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**The Effect of Water Extracted Silibinin on Reactive Oxygen Species (ROS)
Production of Macrophages**

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Introduction

The world is actively looking for technology to produce sustainable liquid fuels to replace our reliance on petroleum-based fuels. Biomass can be converted either through the thermochemical or saccharification platforms into fuels such as ethanol or butanol. In addition to converting the biomass into liquid fuels, valuable phytochemicals can be extracted prior, during, or after the conversion. Extracting useful phytochemicals, as a part of the overall conversion of biomass to fuels, is included in the concept of the biobased biorefinery. However, the key to effectively and economically extract phytochemicals from biomass is the ability to couple the extraction to the energy conversion steps; that is, extraction with either dilute acid or water. Phytochemical extraction with organic solvents cannot be easily coupled with an energy conversion process because the solvents must be removed before proceeding to energy conversion, thereby complicating and increasing the cost of the process. By extracting the phytochemicals with water or dilute acid, a simple unit operation can be added to the existing biomass conversion technology, ultimately adding value to the biomass.

Examples of phytochemicals that can successively be extracted from a water process are flavonoids (1) from *Albizia julibrissin* and flavonolignans from *Silybum marianum* (milk thistle) (2). Both flavonoids and flavonolignans have some value, as they inhibit low density lipoprotein (LDL) oxidization, which is a culprit in many inflammation based diseases (3).

Milk thistle- extracted silibinin is of particular interest because of its high activity in oxidized LDL inhibition (4) and can serve as an interesting model compound. Preliminary results are showing that solvent-extracted silibinin is displaying activity in

decreasing the production of reactive oxygen species (ROS). Evaluating the effect of silibinin on the production of ROS is important because there is a relationship between exaggerated ROS production and inflammatory diseases such as atherosclerosis, diabetes and even cancer. Identifying water-extracted phytochemicals that can mitigate ROS production could provide a relatively low cost solution to inflammatory diseases prevention.

This project is centered on evaluating the effect of pressurized hot water-extracted silibinin on ROS production of activated macrophages. Specifically, this work is aimed at determining if silibinin extracted at 120°C would produce degradation compounds that would hinder or enhance ROS production. This undergraduate thesis is under the umbrella of an on-going postdoctoral project aimed at identifying water-extracted high value compounds, adding value to the overall biomass-energy conversion process.

Materials and Methods

Materials:

All tissue culture media and reagents were obtained from commercial sources unless otherwise specified. Roswell Park Memorial Institute (RPMI-1640) growth medium was obtained from Hyclone (Logan, UT). Fetal bovine serum (FBS) from (Atlanta Biologicals, Lawrenceville, GA), and 2',7'-dichlorohydrofluorescein diacetate (DCFDA) (Molecular Probes, Eugene, OR). RAW 264.7 murine macrophage (MΦ) cultures were maintained in RPMI growth medium, both modified with 5% fetal bovine serum, 1% antibiotic/antimycotic, 1% L-glutamine, and 1% penicillin-streptomycin-neomycin (PSN). Cell culture additives (antibiotic/antimycotic, sodium pyruvate, L-glutamine, amphoterecin B) were obtained from Sigma Chemical Co. (St. Louis, MO).

Lipopolysaccharide (LPS) from *Escherichia coli* was purchased from Sigma Chemical Co. (St. Louis, MO). Silibinin is sold as a mixture of its diastereoisomers and was obtained from Sigma Chemical Co. (St. Louis, MO).

Silibinin Water Extraction:

Five mg of silibinin (SBN) was extracted with 200 ml of de-ionized water. A stainless steel vessel (# 452HC3) from Parr Instrument Company (Moline, IA) contains SBN and water. Extraction parameters included: temperature at 120°C, pressure at 411 kPa (pressurized using nitrogen), and an agitation rate of 150 rpm. SBN was added at time zero, which occurred when the water reached 120°C (4). Water extractions lasted 30 min. Water extracts were dried with a SpeedVac (Savant Instruments, Inc., Farmingdale, NY) and were reconstituted in methanol for High-Pressure Liquid Chromatography with UV Detector (HPLC-UV) analysis. Extract 1 was produced on February 21, 2008, while extract 2 was produced on February 26, 2008.

HPLC-UV Analysis:

The SBN content was characterized by HPLC-UV analysis using a Waters system (Milford, MA) composed of an Alliance 2690 separations module and a 996 Photodiode Array, controlled with Millennium³² chromatography software. Analysis of silibinin was obtained using a Symmetry® (Waters, Milford, MA) C₁₈ pre-column placed in series with a Symmetry® (Waters, Milford, MA) C₁₈ column (150 mm x 4.6 mm, 5 µm), both at 40°C. A 10 µL sample volume was injected. Solvent A consisted of 20:80 methanol:water, while solvent B was 80:20 methanol:water and followed the gradient described by Wallace *et al* (2003) (5). The flow rate was 0.75 mL/min, and silibinin was

monitored at 290 nm. Calibration curves were prepared with SBN in concentrations ranging from 1 mg/mL to 0.06 mg/mL. (3)

SBN aliquots were then evaporated to dryness with nitrogen and reconstituted in ethanol to total 300 μ M SBN in the well. The 300 μ M solutions were titrated to test a dose response.

Cell Culture:

Cells were cultured in serum-free RPMI for 24h. 20,000 cells per well are seeded in a 96-well plate. Oxidized low density lipoprotein (oxLDL) induced-respiratory bursts are detected with 10 μ M 2',7'-dichlorohydrofluorescein diacetate (DCFDA). 12.5 μ L of the molecular probe DCFDA was added to all wells and allowed to incubate for 15 min at 37°C. 12.5 μ L of the indicated concentrations of the extracts were added to the wells prior to the addition of 12.5 μ L lipopolysaccharide (LPS), which delivered 5 μ g/mL LPS to the cells. Appropriate wells containing only cells with LPS and DCFDA, but without SBN, were used as controls to indicate the maximum amount of ROS formation in the cells. Wells containing medium without cells, but with DCFDA, were used as blanks, and the corresponding background absorbance was subtracted from all other well readings.

Plate Analysis:

The respiratory burst activities of murine macrophage RAW 264.7 cells were quantified by the changes in fluorescence due to DCFDA oxidation products. Fluorescence was monitored in 10 min intervals for a total of 7 reads in a microplate reader (BioTek, Winooksi, VT) at excitation/emission wavelengths of 480/530 nm.

Statistical analysis:

All tests were run in triplicates for each experimental condition and each experiment was repeated 2 times, once using extract 1 and once using extract 2. The data were analyzed using JMP software (SAS Institute, Cary, SC) by one-way analysis of variance (ANOVA), and significant differences among treatments were detected using the least square means Student's *t* test with a significance level of $p < 0.05$. Data are presented as means +/- standard deviations.

Results and Discussion

Figure 1 presents a dose response of 120°C water extracted SBN ethanol preparations on reactive oxygen species (ROS) formation in murine macrophage RAW 264.7 cells. Extract 1 was used to produce the results in Figure 1 and preparation was based solely on SBN quantification. Although data was collected throughout the 0-120 minute cycle, data presented in Figure 1 is based on data that was collected at the 10 minute time point. Wells containing only cells with LPS and DCFDA (without SBN) were used as controls to indicate the maximum amount of ROS formation. The results presented in Figure 1 shows that the addition of 12.5 μ L of ethanol inhibited 80% of ROS production as compared to the control, while a 300 μ M SBN water extract ethanol preparation showed 55% ROS inhibition. These results show that water extracted SBN preparations do, indeed, inhibit the generation of ROS.

SBN was extracted at 120°C for 30 minutes on two occasions. The traces of both extractions are shown in Figure 2. SBN is a mixture of two diastereoisomers (5), with silybin A and silybin B eluting at 24 and 25 minutes, respectively. The peak eluting at 18 minutes is not identified at this point in time. The percentages of SBN remaining in the extracts after the extraction process were determined as described by equation [1].

$$\frac{SBN_{post-extraction}}{SBN_{pre-extraction}} * 100. \quad [1]$$

The percentages of SBN remaining in the extracts were determined as the summation of both silybin A and silybin B. As expected from the inspection of the HPLC-UV chromatogram, extract 2 resulted in an extract that contained more SBN. Calculations are presented in Table 1. It is important to note that the extract was analyzed with a UV detector set at 290 nm. It is possible that the extract could contain compounds that are not detected at this wavelength, explaining the 79.3 and 54.3% of degradation products calculated in extract 1 and 2, respectively. Nonetheless, the effect of degradation product(s), such as the compound eluting at 18 minutes, on ROS generation should be examined.

Extracts 1 and 2 were standardized on silybin A and B quantification; however extract 1 preparations contained a higher concentration of the compound eluting at 18 minutes than those prepared from extract 2. Each extract was diluted to 0, 9, 18, 39, 75, 150, and 300 μ M doses. However, the amounts of degradation products within this stock could not be standardized. Each of these doses was then tested for absorbance and the smallest dose that showed a significant decrease in the amount of ROS produced by the macrophages was recorded. Figure 3 presents side by side the dose of extract 1 and 2 that was necessary to produce a significant decrease ($p < 0.05$) in ROS generation as compared to that of the control in murine macrophage RAW 264.7 cells. At the 10 minute time point, 39 and 9 μ M of extract 1 and 2, respectively resulted in a significant decrease ($p < 0.05$) in ROS generation. Interestingly, extract 1 contained a higher proportion of the unknown peak (the peak eluting at 18 minutes) than that of extract 2. From the differences in Figure 3, it could be stipulated that the peak generated at 18 minutes is less

bioactive in the ROS assay than the SBN diastereoisomers. If this is the case, then the compounds that can be obtained during pressurized hot water extraction should be carefully examined in terms of the desired bioactivity.

Conclusion

Water extracted SBN preparations in ethanol did reduce the concentration of ROS production of murine macrophage RAW 264.7 cells. SBN water extracts were prepared twice. For both extracts, the percentages of SBN and degradation products were determined. Although the extracts were prepared using identical methodology, extract 1 displayed a higher proportion of the unknown compound eluting at 18 minutes. ROS production of murine macrophage RAW 264.7 with extract 1 and 2 was examined. Only a 9 μ M preparation of extract 2 was necessary to produce a significant difference in ROS expression as compared to the control. It could be inferred that the peak generated at 18 minutes is less bioactive than the SBN diastereoisomers. Work is currently under way to characterize the peak eluting at 18 minutes.

References

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Figures and Tables

	% Silibinin	% Degradation Products	Amount of Silibinin in Sample (mg)
Extract 1	20.7	79.3	1.035
Extract 2	45.7	54.3	2.285

Table 1. Calculations of recovered silibinin (SBN) and potential degradation productions (calculated through a mass balance) of 120°C-extracted SBN.

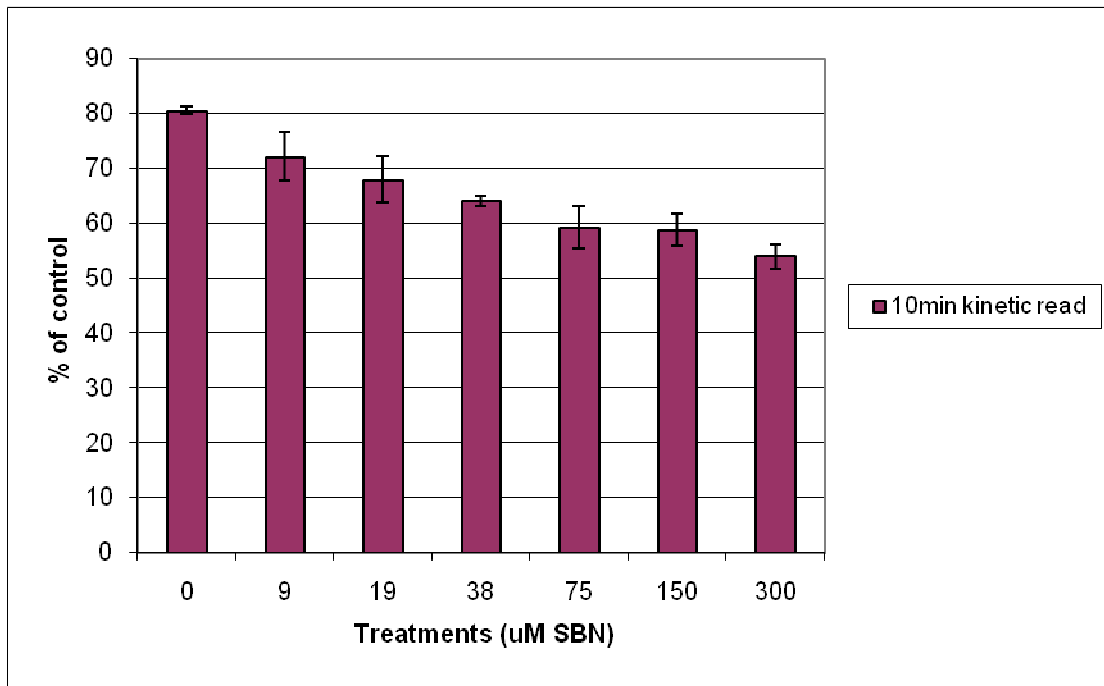
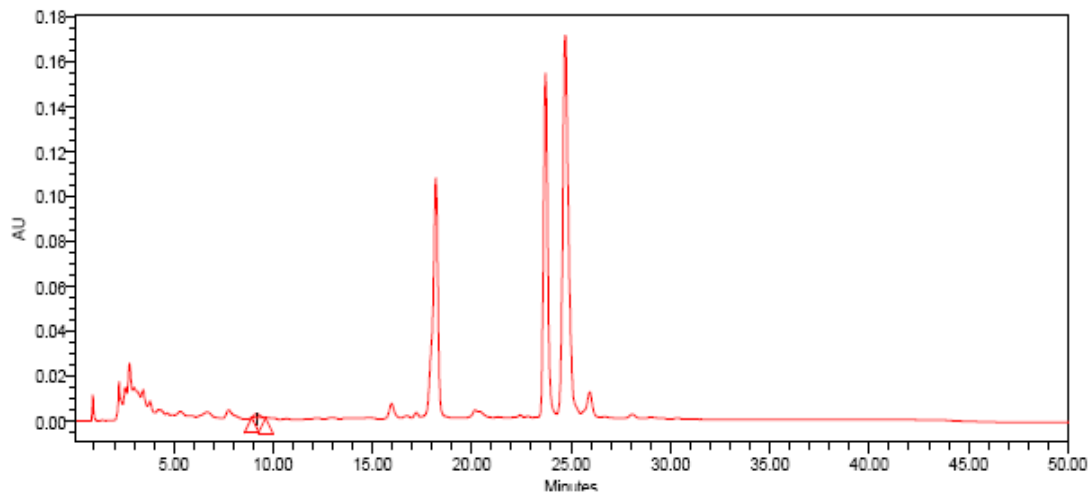


Figure 1: Dose response of water extracted SBN at 120°C, which was standardized based on silybin A and silybin B quantification. Extract 1 was used to prepare these results.

A.



B.

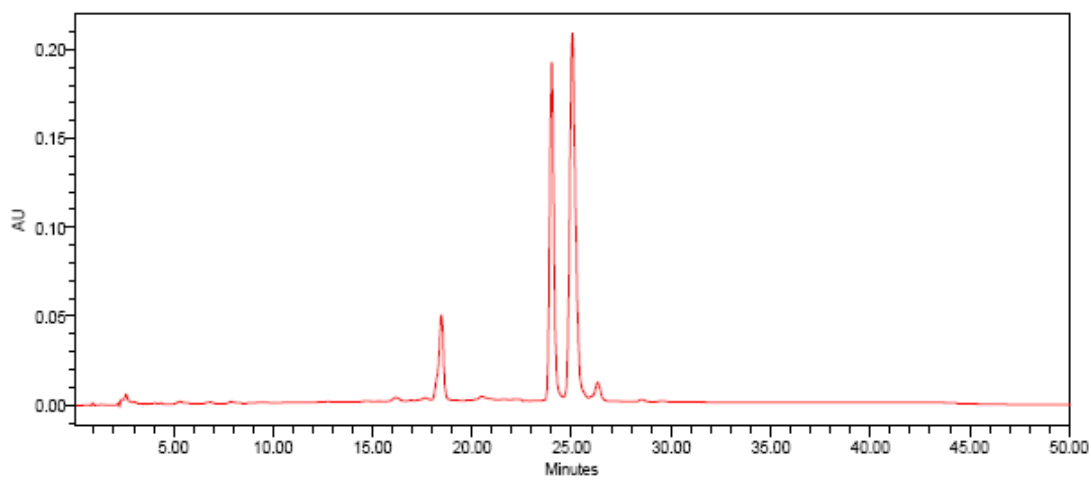


Figure 2: HPLC-UV chromatograms of 5 mg of silibinin (SBN) extracted in 120°C water. The peaks eluting at 24 minutes and 25 minutes are silybin A and silybin B, respectively. The peak eluting at 18 minutes is unknown at this time. A. Extract 1 (2/21/2008). B. Extract 2 (2/26/2008).

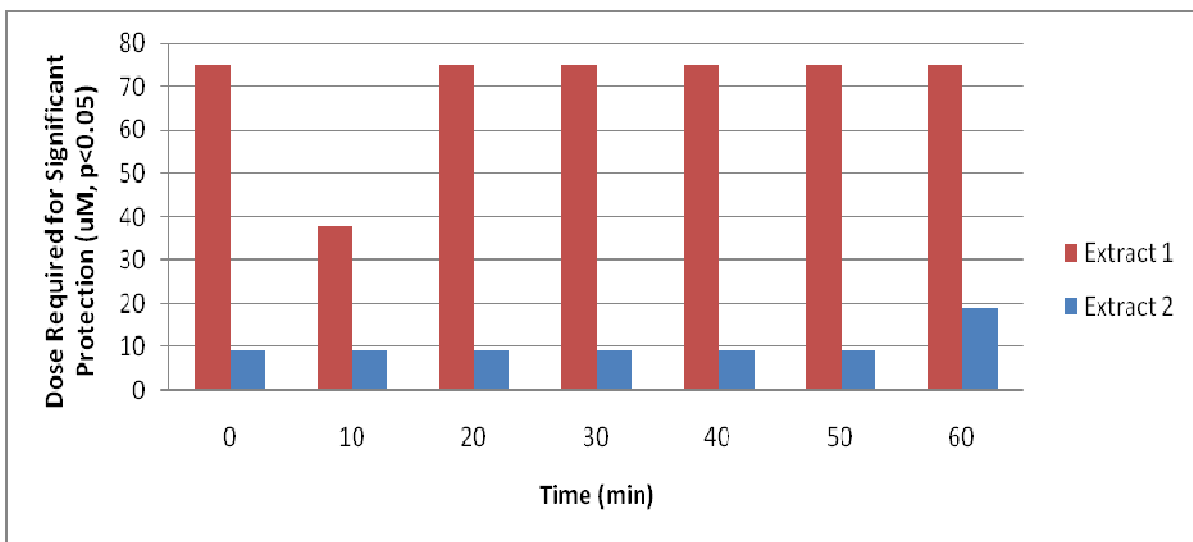


Figure 3: Dose required of extracts 1 and 2 for significant differences in ROS productions as compared to that of the control.