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Effect of Storage Temperature and Time on Lyophilized Water Bark Extract's Biological Activity

Jillian Schneider
University of Arkansas, Fayetteville

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Biological Engineering Program

Biological and Agricultural Engineering Department

College of Engineering

University of Arkansas

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Abstract

Sweetgum bark extract has been known to show biological activities such as antimicrobial and antioxidant capabilities. The storage capacity of the extract, however, was unknown and previously thought to diminish over time. However, upon experimentation, the freeze-dried sweetgum bark extract showed no signs that storage time or storage temperature had any significant effect on the biological activities. There was no significant difference across storage temperature over time in the experiment (ANOVA RM, $P \geq 0.05$). Therefore, the antioxidant capabilities of the sweetgum extract were not affected by the storage time or temperature treatments studied in this work. For the antimicrobial experiment, there was no significant difference across storage temperature over time in the experiment (ANOVA RM, $P < 0.95$) and there were no different homogeneous groups. However, concentration had significance in the experiment (ANOVA RM, $P < 0.01$ and Figure 3) showing that the zone of inhibition decreased with decreasing extract concentration. Overall, storage time and temperature did not have a negative effect on the biological activities of freeze-dried sweetgum bark extract.

Introduction

Sweetgum (*Liquidambar styraciflua* L.) is a native tree species that grows in the understory of managed Arkansas pine forests, which must be removed prior to pine harvesting. Once harvested, the sweetgum understory is transformed to thermal energy via combustion of the biomass. However, combustion is not the only use for sweetgum understory, as multiple studies have reported that sweetgum extractives can be used medicinally, e.g. to help reduce pain (El-Readi et al., 2013) and treat the flu (Enrich et al., 2008; Martin et al., 2010).

Sweetgum bark extracts have displayed antioxidant and antimicrobial capabilities (Djioleu, 2016). Unfortunately, many extraction methods involve the use of organic solvents, such as methanol, making subsequent biomass combustion operations hazardous. Yet, as shown by Carrier and Clausen (2008), water can be used as a solvent to create the extract which then allows the biomass to still be used in the bio-refining process. As such, there has been research into the application bark extract using water and its applications, which was shown to display antioxidant and antimicrobial properties (Djioleu, 2016).

The antioxidant characteristics of the natural extracts, however, do not appear to be completely shelf stable such that the biological properties were noted to decrease as a function of time (Samad et al. 2016). In addition, the sweetgum bark extract had been suspected of decreases in biological activities (i.e. antioxidant and antimicrobial) based on observed effects noticed by Dr. Kala Rajan in 2016 during her own research (personal communication, 4/15/16). Not knowing the shelf-life of the water-based sweetgum extract limits its applications because the uncertainty of the effectiveness at the time of use. For example, the extract would then never be used as sterilizing agent in a hospital or food industry, because the bacteria must be killed which may not occur if the extract loses its efficacy.

Therefore, the goal of this project was to determine if the antimicrobial and antioxidant capabilities of the sweetgum extract remain viable over a period of 9 months. The main objective was to

determine if the freeze-dried extract began to lose its effectiveness and if so at what time intervals and if storage temperature (22°C, 4°C, or -20°C) affected the efficacy during storage. I hypothesized that the sample stored at -22°C will maintain the effectiveness of the extract for the longest amount of time but will still show decreasing effectiveness after being stored for 9 months based on the work performed by Dr. Kala Rajan and Dr. Angele Djioleu.

The research will determine shelf life of the water extract allowing industries to determine potential costs or benefits associated with switching from the traditional chemical extraction methods. This is because a company would be able to identify how much of the extract would be needed and when to buy new extract. In addition, researchers would benefit with knowledge of the shelf life so that accurate test results can be obtained when using the extract.

Methods

Preparation of Water Extract

Finely ground bark from a mature sweetgum tree was extracted using 85°C de-ionized water based on Carrier and Clausen (2008) . The water, ground bark mixture was placed in a 2 L Parr reactor to maintain a constant temperature while being agitated for 60 min to ensure proper extraction. The slurry was filtered, via vacuum filtration, through a Whatman N° 1 filter paper and a Buchner funnel. The final liquid filtrate was then freeze dried and separated into three batches consisting of four individual portions of 0.8 g each and stored in small plastic sealable bottles at 22°C, 4°C, and -20°C until the time of testing for efficacy.

The antioxidant and antimicrobial properties were tested at 45, 115, and 206 days from initial storage to determine efficacy at 22°C, 4°C, and -20°C storage temperatures. The antioxidant capabilities were tested using the thiobarbituric acid reactive substance (TBARS) assay and the antimicrobial capacity was tested through disc diffusion assay on *Staphylococcus aureus* (*S. aureus*) streaked agar plates Adams

et al. (2014) and Djioleu (2016). For each sample period, a portion of each batch was reconstituted to a concentration of 150 g L^{-1}. The experiments were carried out in duplicate for each time period for each storage temperature.

Thiobarbituric acid reactive substances (TBARS) Assay for Antioxidant Capacity

Antioxidant capacity of the bark extract was tracked over a 9-month period and determined using copper induced TBARS assay on human low-density lipoproteins (LDL). The method was performed similarly as described by Uppugundla et al. (2009). LDL was dialyzed using dialysis tubes in EDTA-free TRIS (pH 7.4) buffer for 34 hr at 4°C. Exactly 10 µL of the diluted (conc. 9.4 g L^{-1}) reconstituted bark extract (control wells received 10 µL of TRIS Buffer), 100 µL of dialyzed LDL, and 10 µM of copper sulfate were pipetted into the wells of a 96-well-assay plate. Immediately after pipetting, half of the wells or "zero hour wells" received 10 µL of butylated hydroxytoluene (BHT) to stop oxidation reaction, and then the plates were incubated at 37°C for 24 hrs.

After the 24 hr incubation period at 37°C, the "24 hr" wells received 10 µL of BHT. Then, 50 µL of 50% trichloroacetic acid (TCA) and 75 µL of 1.3% thiobarbituric acid (TBA) were then added to all wells and incubated for 40 min at 60°C. After incubation, absorbance of the plates was read in a microplate reader (BioTek, Winooski, VT) at 600 nm and 530 nm.

The antioxidant capabilities were determined from the difference (i.e., delta) of the two absorbance readings. An oxidation value was then determined using a best fit equation developed from the 1,1,3,3-tetraethoxypropane (TEP) Standard Curve. The equation was determined for each testing period (see Appendix Figure 4 - 7) using Microsoft Excel. The difference between the blank and the measured value was then used to estimate the actual oxidation value. Where the higher the value meant, more oxidation had occurred. Analysis of variance (ANOVA) with repeated measures (RM) across

treatment concentration, storage temperature, and time was used to evaluate differences (STATISTIX version 10 software, Tallahassee, Florida).

Antimicrobial Plating

Antimicrobial activity was monitored and tracked over the 9-month period using prepared agar plates and the disc diffusion assay in a similar process as Adams et al. (2014) and Djioleu (2016). Culture of *S. aureus* (see appendix on biological safety procedures) was grown for at least 48 hr before beginning the assay. The plates were inoculated with *S. aureus* from the culture via the streaking method using a sterile cotton-tipped applicator. The inoculated plates were then allowed to dry before applying the 6 mm diameter blank sterile paper disks using sterile forceps. The discs were gently pressed to ensure contact with the agar. The discs then had bark extract applied so that it can be absorbed into the paper; the extract was tested at four different concentrations 150 g L^{-1}, 75 g L^{-1}, 37.5 g L^{-1}, and 18.75 g L^{-1}. After incubating in an inverted position for 24 hr the plates were removed and the zones of inhibitions were measured using a ruler (Appendix Figure 8A).

The *S. aureus* diameter of the ring of growth inhibition was measured for each concentration at all three storage temperatures for every data collection period. The data was then examined using two different methods. The first was determining the minimum concentration that would inhibit bacterial growth for each data collection period. The second method was utilizing the ANOVA RM to evaluate the effect of storage and temperature in antimicrobial activity.

The minimum inhibition concentration (MIC) needed to prevent bacterial growth was determined by graphing the measured rings of inhibition versus the known concentration and creating a best fit equation. The MIC was determined by using 0.008 m as the independent variable in the best fit equation for each time-period. A ring of 0.008 m was used because it was determined to be the smallest

measurement that would show inhibition without being smaller than the 6 mm disc that held the extract. The MIC was then evaluated using ANOVA to evaluate the effects of storage and time.

Results

Antioxidation

The absorbance readings of the zero hour wells versus the 24-hour wells showed that oxidation reactions did occur during the incubation period in the 24-hour wells. This is indicative in the positive delta (24-hour well mean less the zero hour well mean) values (Table 1) because higher absorbance readings (i.e. darker colors) equate to more oxidation. In addition, when compared to the 24-hour control wells, the 24-hour test wells have smaller delta absorbance reading values indicating that oxidation was prevented by the presence of the bark extract (conc. 9.4 g L^{-1}). This is true for all test periods except the 115 days which could be attributable to random error since the 206 days continues the pattern shown at 45 days.

The initial oxidation value was 7.32 nM based on one measurement before storage (Table 2). The oxidation capacity varied over time across storage temperatures, ranging from <5 after 45 days of storage at 20°C to over 17 after 216 days of storage at 4°C. There was no significant effect of storage temperature (ANOVA RM, P=0.19), storage time (P=0.29) or the interaction of temperature and time (P=0.60) in this experiment.

The means of the oxidation values increased numerically with time based on Figure 1. However, the mean for both 45 days and 115 days of storage are less than the zero-day storage oxidation value implying that storage of freeze dried sweetgum extract did not change antioxidant capacity. The oxidation value only increased slightly past the initial value for the 206 days of storage except for the 4°C oxidation value (17.81 nM). The quantity of 17.81 nM seems abnormally high based on the previous tests, and may be due to an error that was not noticed. The causes the error for 206 (Figure 1) to be very large; potentially

affecting quality control of the bark extract. Overall, the sweetgum extract does not seem to be affected by the time or temperature in these experiments over 206 days of storage.

Table 1: Zero hour wells vs 24 hour wells: Comparison between control and test W=wells via delta absorbance reading (nm) for each storage temperature

Storage Time (Days)	45			115			206		
Temperature	Zero Hour Mean (nm)	24 Hour Mean (nm)	Delta (nm)	Zero Hour Mean (nm)	24 Hour Mean (nm)	Delta (nm)	Zero Hour Mean (nm)	24 Hour Mean (nm)	Delta (nm)
Control	0.07	0.16	0.09	0.11	0.12	0.01	0.11	0.40	0.29
-20°C	0.06	0.07	0.01	0.02	0.07	0.05	0.08	0.10	0.02
4°C	0.05	0.07	0.02	0.02	0.09	0.08	0.08	0.15	0.07
22°C	0.06	0.08	0.02	0.06	0.07	0.01	0.10	0.12	0.02

Table 2: Average Oxidation Values (nM) Days out from initial measurement date by storage temperature

	Storage Time (days)			
Storage Temperature	0	45	115	206
	Oxidation Value (TBARS present) nM			
-20°C	7.32	4.77	6.07	7.84
4°C		5.32	5.87	17.81
22°C		5.03	4.65	7.62

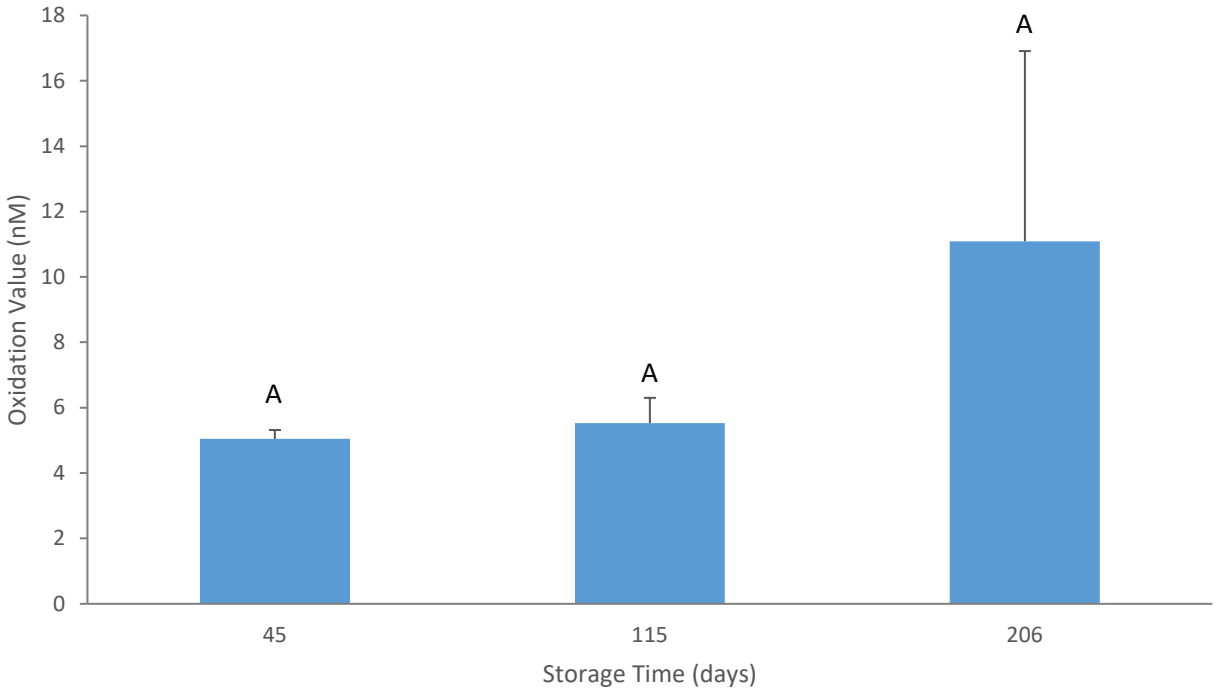

Figure 1: Average oxidation values (nM) versus storage time (days) with error bars and ANOVA homogeneous groups. Error bars: 45 days +0.27, 115 days +0.77, 206 days + 5.82

Antimicrobial Plating

The initial minimum inhibition concentration of microbial growth was 39 g L^{-1} based on one measurement before the freeze-dried material was stored at the three storage temperatures (Table 3). The minimum inhibition concentration for the sweetgum extract varied over time ranging, from 50 g L^{-1} after 115 days of storage at 4°C to 72 g L^{-1} after 45 days at both 4°C and 22°C. There were no obvious effects of storage temperature (ANOVA, P=0.93) that would have impacted the ability of the sweetgum extract to prevent growth which is supported by the minimum inhibition concentration (Table 3, Figure 2). However, the storage time did have considerable influence (P=0.04) but there was no pattern i.e., no consistent decrease over time.

A similar result occurred for the zone of inhibition caused by the antimicrobial activity (Table 4). It varied over time for each concentration and storage temperature, for example 150 g L^{-1} at 22°C ranged from 0.014 m to 0.020 m during the testing period. This occurred across all the various storage temperatures and concentrations. There was no significant difference in antimicrobial activities across storage temperature (ANOVA RM, P=0.78), storage time (P=0.12), or the interaction of temperature and time (P=0.95) in the experiment. However, the concentration of the extract had a major influence on the antimicrobial activity, (P<0.01, Figure 3) as well as the interaction of time, temperature, and concentration (P=0.03). There were four different homogeneous groups for concentration (Figure 3) where the mean zone of inhibition ranged from 0.016 m at 150 g L^{-1} to 0.000 m at 18.7 g L^{-1}.

The antimicrobial activities (minimum inhibition concentration and zone of inhibition) of the sweetgum extract were not affected by time or temperature. Yet, the antimicrobial activities are clearly affected by concentration especially the zone of inhibition where lower concentrations resulted in smaller zones of inhibition or even none.

Table 3: S. aureus average minimum inhibition concentration (g L^{-1}) by measurement date and storage temperature

	Storage Time (days)			
Storage Temperature	0	45	115	206
	Concentration in g L^{-1}			
-20°C	39	70	64	65
4°C		72	50	71
22°C		72	53	70

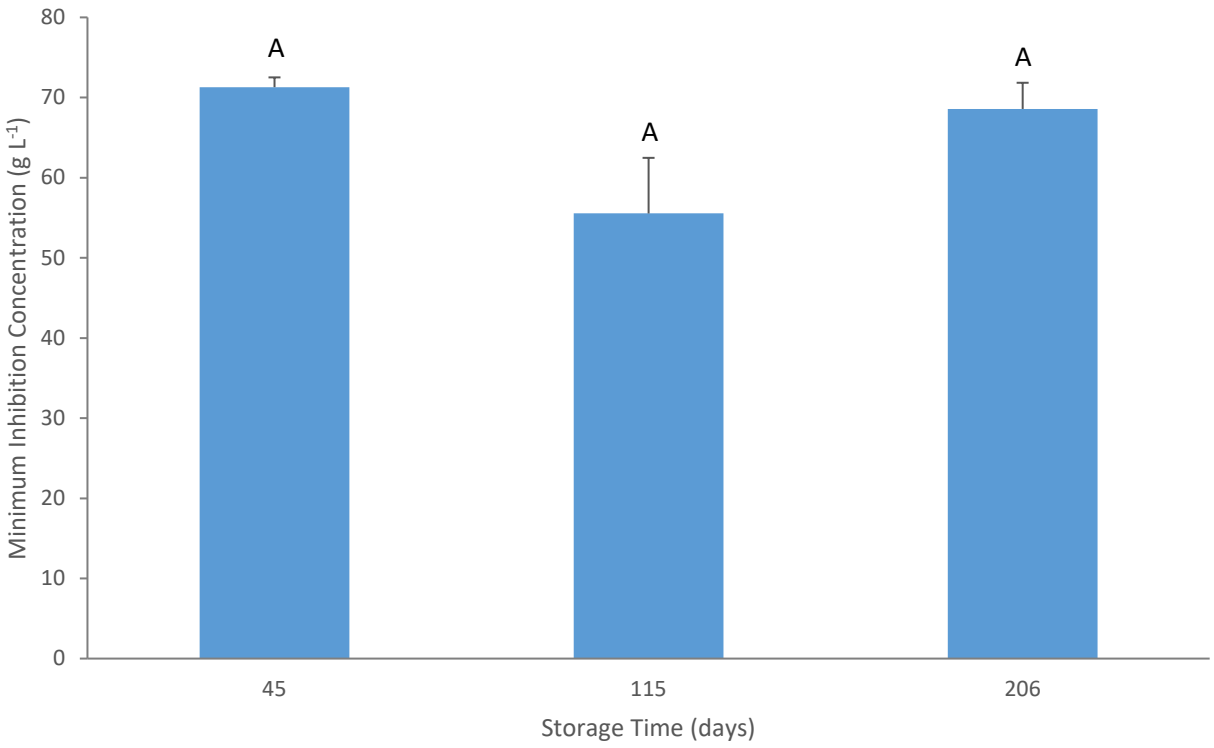

Figure 2: Average minimum inhibition concentration (g L^{-1}) versus storage time (days) for all storage temperatures with error bars and ANOVA homogenous groups

Table 4: Zone of inhibition (m) for each temperature and concentration with average of two replicate tests

Storage Time (Days)		0	45			115			206		
Conc.	**Temperature Replicate**	**Initial (m)**	**A (m)**	**B (m)**	**Mean (m)**	**A (m)**	**B (m)**	**Mean (m)**	**A (m)**	**B (m)**	**Mean (m)**
150 g L^{-1}	-20°C	0.018	0.015	0.016	0.016	0.020	0.018	0.019	0.016	0.017	0.017
	4°C		0.014	0.015	0.015	0.018	0.018	0.018	0.017	0.016	0.017
	22°C		0.016	0.014	0.015	0.017	0.017	0.017	0.016	0.016	0.016
75 g L^{-1}	-20°C	0.014	0.011	0.011	0.011	0.012	0.014	0.013	0.010	0.000	0.005
	4°C		0.013	0.009	0.011	0.013	0.012	0.013	0.010	0.009	0.010
	22°C		0.013	0.000	0.007	0.017	0.013	0.015	0.018	0.017	0.018
37.5 g L^{-1}	-20°C	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.007	0.000	0.004
	4°C		0.000	0.000	0.000	0.007	0.000	0.004	0.000	0.000	0.000
	22°C		0.006	0.000	0.003	0.000	0.008	0.004	0.000	0.000	0.000
18.7 g L^{-1}	-20°C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	4°C		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	22°C		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

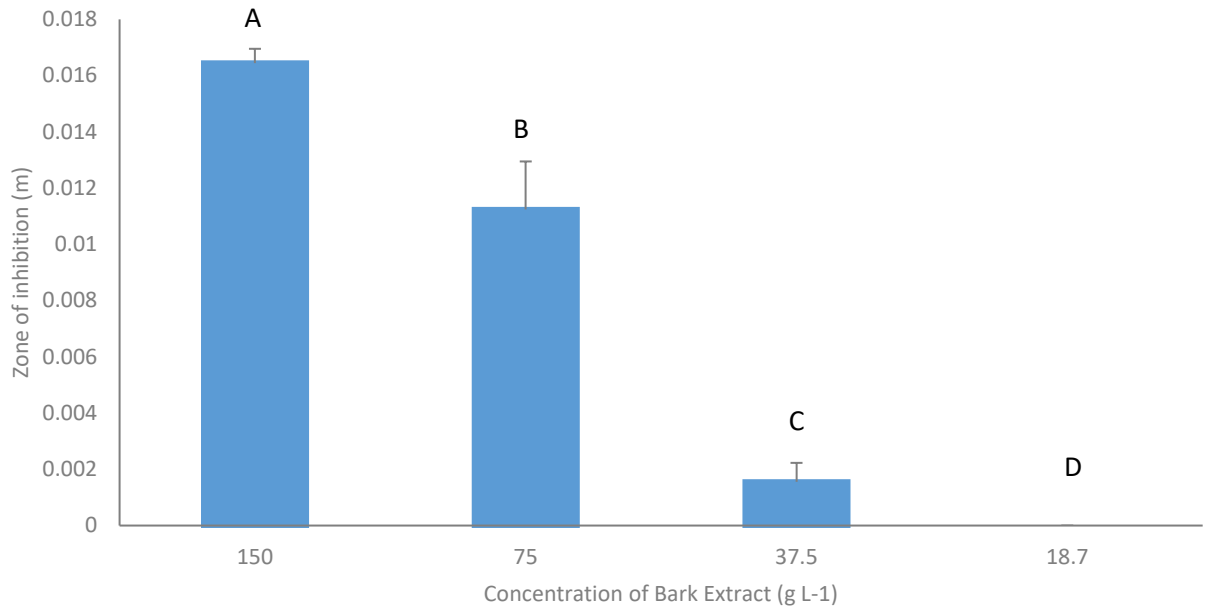

Figure 3: ANOVA groups and average zone of inhibition for each concentration across all temperatures and time with error bars

Discussion and Future Opportunities

The results were different than what was expected. It was hypothesized that the biological activities of the sweetgum bark extract would decrease with time up to 9 months across all storage temperatures with the 22°C storage temperature showing the greatest decrease. It is very possible that the unexpected result was due to the change in the storage preparation, freeze drying the extract, before long term storage. This opens the availability to repeat the test where the variable is freeze drying before storage to determine if freeze drying allowed for better preservation of the extract.

The results of the *S. aureus* inhibition initially showed an increase in the minimum inhibition concentration, but it then varied over time leveling off after 115 days of storage. This shows that both a shorter and longer testing period could be used to create further tests. The shorter test could be used to pinpoint more accurately when the extract's antimicrobial capabilities begin to decline. The longer test would determine if the variability that occurred in the last three test dates were random, if the minimum inhibition concentration would eventually level out at a certain point between the two extremes, or if the MIC would decrease even further with more time. However, no matter the result of potential future experiments, the results did show that a concentration of 150 g L^{-1} could be used to effectively eliminate all *S. aureus* for a 206-day period.

In a similar disc diffusion assay, performed by Dr. Angele Djioleu, the antimicrobial activity of the sweetgum bark extract was recorded to have a zone of inhibition of 15 mm at a concentration of 160 g L^{-1} (Djioleu, 2016). Another study found that water extracted sweetgum bark at a concentration of 150 g L^{-1} had a zone of inhibition of 13 mm against *S. aureus* (Rajan et al., 2017). These zones of inhibition are less than the measured values (14 mm – 20 mm) for this experiment at the 150 g L^{-1} concentration. However, the values obtained in this experiment range at most by 5 mm.

In comparison to other research that looked at antioxidant capabilities the oxidation of the LDL was completely prevented at a concentration of 12.5 g L^{-1} and had a little over 21 nmol mg^{-1} protein oxidized at an extract concentration of 6.25 g L^{-1} (Hurd, 2012). This experiment used 9.4 g L^{-1} and had oxidized protein levels that fall within the range established by Hurd. The results of the TBARS did show some initial decrease but then increased with time back up but the effect of temperature and storage time was not significant in this study. This could have been due to many factors such as incomplete mixing of the sample before storing resulting in an uneven distribution of the antioxidant agents in the extract but is most likely due to random variability in the biological agent effectiveness. However, there was only one initial measurement which limited the ability to statistically compare the effects of initial storage.

The hypothesis was incorrect. Despite that, the experiment showed there is some variability in the biological activities that was uncontrollable i.e., the unpredictable variance between each test period. This could present problems for the product being commercialized, because there would still be uncertainty of the effectiveness of the extract due to the unpredictability. However, the experiment did prove that the extract has a longer shelf life than previously thought (personal communication, 4/15/16) with active biological agents that prevent oxidation and growth of *S. aureus*. Going forward, an extensive test that breaks down variability in the extract for both the oxidation and the inhibition abilities would need to be tested to market the product. This experiment would determine a range of effectiveness and therefore establish a minimum concentration which would guarantee a known result i.e., eliminating all *S. aureus* that the 150 g L^{-1} extract encounters.

The results also showed that storage temperature had negligible effect on the biological activities of the freeze-dried extract. This is promising because it allows the extract to be stored wherever a consumer prefers without decreasing the effectiveness of the extract. This helps increase its marketability as it is cheaper, easier, and more convenient to store a product at room temperature than in the freezer. Further testing should be performed if the sweetgum bark extract were to be created into a

commercialized product, but the results of this experiment are promising because biological activities of the extract do not diminish in a 9-month period.

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Appendix

Biological Safety Procedures for *Staphylococcus aureus* (BioSafety Manual)

The *S. aureus* must be carefully contained, handled, and stored to prevent contamination. Containment during use involved a BSC with UV light, high Efficiency Particulate Air (HEPA) filters, and glass shielding only raised 4 inches when *S. aureus* was present. The *S. aureus* when mixing had to have the test tube lid tightly secured to prevent any pathogens escaping via airborne method. After use of bacteria the space had to be properly wiped down with an appropriate disinfectant. When not in use the *S. aureus* was to be locked in the storage refrigerator with proper identification labels. Any contaminated waste was to be properly disposed of as a biological hazard. Protective clothing and gear was to be used and removed/disposed of properly before leaving the room.

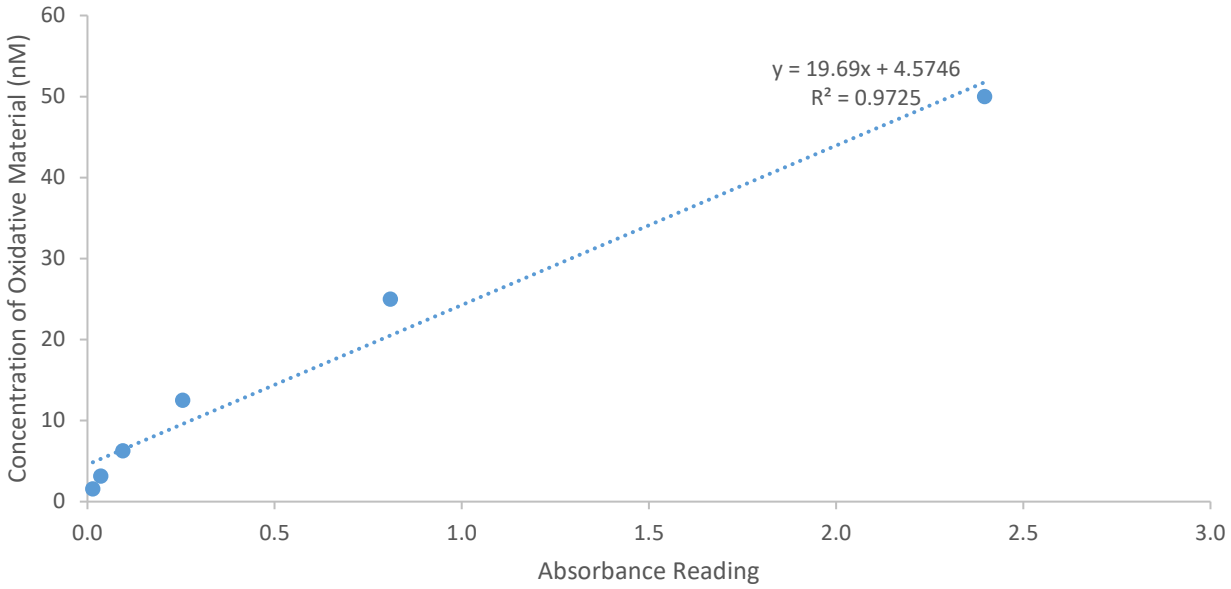


Figure 4A: May TEP standard curve with best fit equation to determine oxidation value based on readings

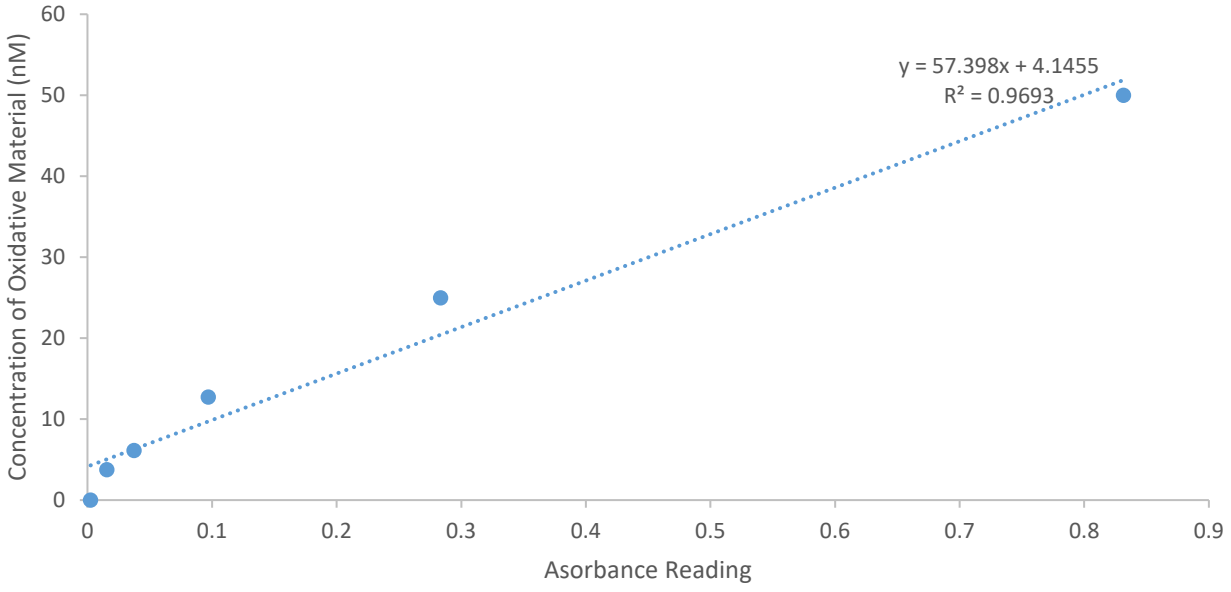


Figure 5A: July TEP standard curve with best fit equation to determine oxidation value based on readings

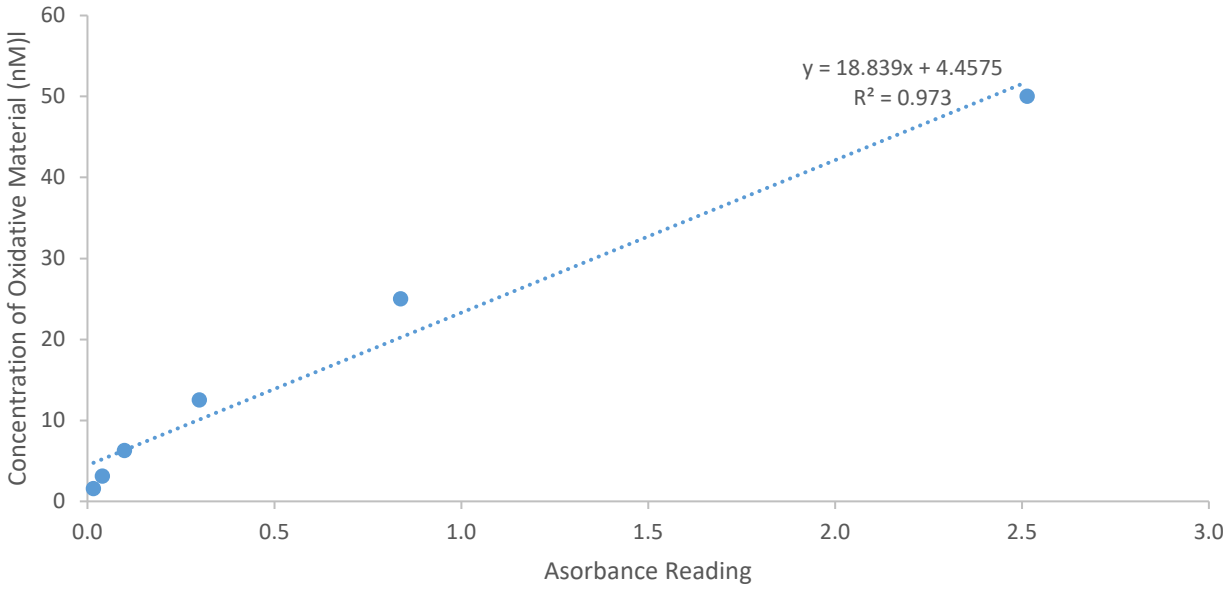


Figure 6A: September TEP standard curve with best fit equation to determine oxidation value based on readings

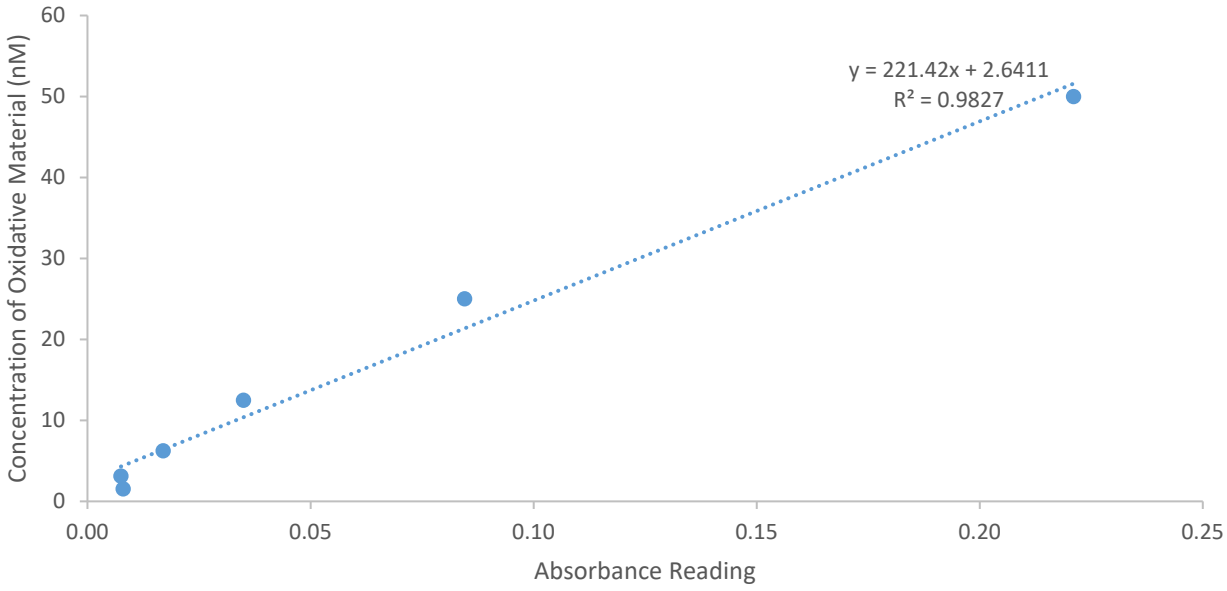
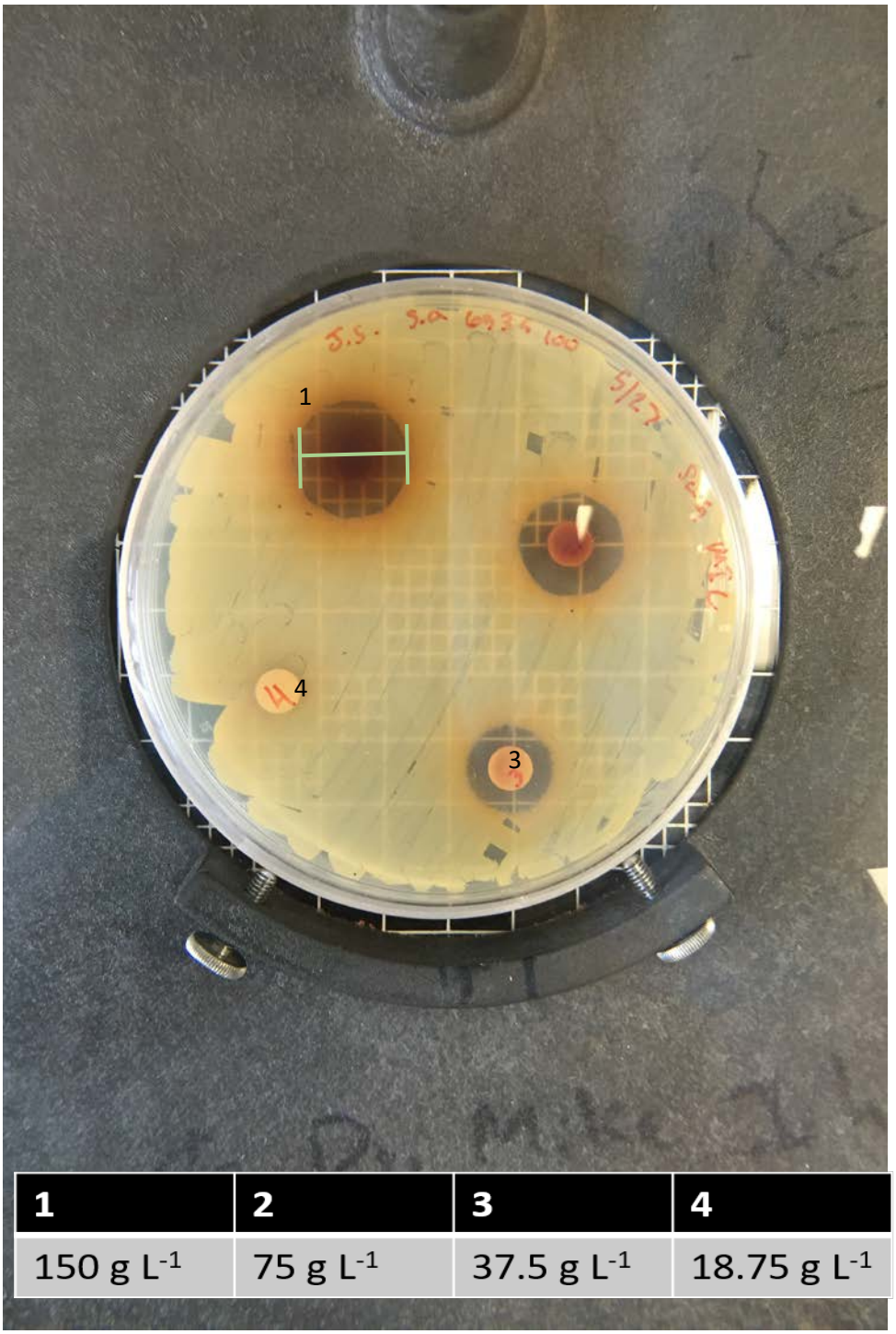


Figure 7A: December TEP standard curve with best fit equation to determine oxidation value based on readings



1	2	3	4
150 g L^{-1}	75 g L^{-1}	37.5 g L^{-1}	18.75 g L^{-1}

Figure 8A: S. aureus plating with four discs showing zone of inhibition for each concertation and example measurement bar