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Antimicrobial effects of pine essential oil against Listeria monocytogenes

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Antimicrobial Effects of Pine Essential Oil against *Listeria monocytogenes*

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1.0 Abstract

Short leaf and loblolly pine were harvested in May 2014 and June 2014, respectively, at the University of Arkansas Monticello in Monticello, Arkansas. Short leaf and loblolly essential oils were prepared by hydrodistillation, using a Clevenger apparatus. Essential oils were extracted at separate times and showed different concentrations of the components α-pinene, β- pinene, 3-carene, limonene, and terpineol. Pine essential oil samples were tested through disc diffusion assay for antimicrobial activity against *Listeria monocytogenes*. This was done by placing ten μL of each essential oil sample on a paper disc on an agar plate that was inoculated with *L. monocytogenes* at a concentration of 3.7×10^8 cells. Four of the five oils tested showed antimicrobial activity against *L. monocytogenes* and a liquid byproduct of hydrodistillation did not show any activity.

2.0 Introduction

Listeria monocytogenes is a microorganism that causes the foodborne illness in humans, listeriosis (Farber, 1991). *L. monocytogenes* is a facultative anaerobe that is able to grow in an environment of 1 to 45 ℃ and at a pH of 4.5 to 9.6 (Albrecht, 2005). Common food groups that are susceptible to foodborne *L. monocytogenes* include dairy products, meat products, and uncooked vegetables and fruits (Albrecht, 2005).

Listeriosis most commonly affects those with compromised immune systems, such as the elderly and pregnant women. The bacterium causes infections in parts of the human body, like the central nervous system. In pregnant women, a listeriosis infection may develop in the fetus and cause a miscarriage. Mortality in affected individuals may occur, though percentages of incidence vary based on the original cause of the compromised immune system (Farber, 1991).

Several types of treatments have been found to inhibit growth of *L. monocytogenes*. Streptomycin, penicillin, and gentamicin are all known examples of antibiotics that work to inhibit growth of *L. monocytogenes*. Some antibiotic resistant strains of *L. monocytogenes* have been identified (Charpentier, 1999). Essentials oils have also been shown to inhibit growth of *L. monocytogenes*. An essential oil is a volatile oil extracted from plants (Benchaar, 2008). Research conducted at South Bank University in London and Scottish Agricultural College in Auchincruive found that several essential oils, such as lemon verbena, basil, clove leaf, and cinnamon leaf, showed activity in inhibiting growth of *L. monocytogenes* (Lis-Balchin, 1997).

The laboratory at the University of Arkansas where my research was conducted has previous experience in pine essential oils (Adams, 2014). Their research showed pine essential oil to inhibit growth of *Staphylococcus aureus*. The laboratory generated their pine oil from loblolly pine harvested in Monticello, AR through hydrodistillation in a Clevenger apparatus. Major components of the essential oil

were tested through GC-MS and identified as α-pinene, β- pinene, 3-carene, limonene, and terpineol. Through disk diffusion assay, the essential oil produced by the laboratory inhibited growth of *S. aureus* (Adams, 2014).

Due to the fact that pine essential oil inhibited growth of *S. aureus* and that essential oils displayed anti *L. monocytogenes* activity, pine essential oil produced through hydrodistillation was tested for possible inhibitory effects on *L. monocytogenes.* The increase of *L. monocytogenes* resistance to antibiotics would also be important to any positive results from the experiments.

3.0 Methods

3.1 Distillation of Pine Essential Oil

Short leaf and loblolly pine needles were harvested at the Teaching and Research Forest for the University of Arkansas Monticello in Monticello, Arkansas in May and June 2014, respectively. Pine essential oil was obtained through hydrodistillation. A Clevenger apparatus was used in the process, as described in the report on pine essential oil effects on *S. aureus* (Adams, 2014). Boiling chips were placed in a 2000 mL round bottom flask, along with 160 g of NaCl (VWR, Radnor, PA) dissolved in 800 mL of distilled water. Then 200 g of chopped pine needles were added to the flask and mixed by shaking. The flask was assembled in the Clevenger apparatus and the pine needle and water mixture was held at its boiling point for four hours. This is shown in Figure 1. After the four hour boiling period, oil was extracted from the apparatus, stored in a small amber vial, and held at 4 °C. Each batch of oil obtained through hydrodistillation was treated as a separate sample based on the batch in which it was produced.

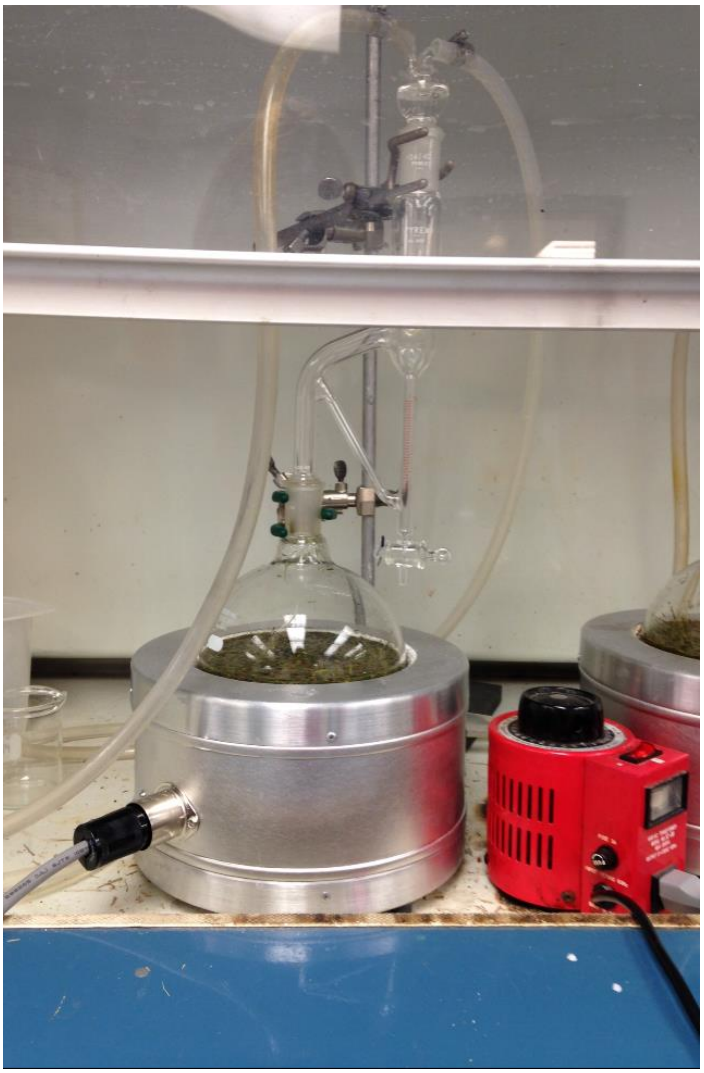

Figure 1. The Clevenger apparatus used to extract the pine essential oil through hydrodistillation.

The pine essential oils used in the experiment are from short leaf pine needles distilled on November 17, 2014, November 19, 2014, December 8, 2014, and February 2, 2015. Also included in the experiment was loblolly pine needles, distilled on December 1, 2014, and the liquid removed from the Clevenger apparatus along with oil extraction from short leaf needles, distilled on January 26, 2015. Table 1 highlights the naming of the six oil samples. The produced essential oils were

characterized by gas chromatography (GC), as described in the report on pine essential oil effects on *S. aureus* (Adams, 2014). GC quantification of components of the extracted oils was done in the context of Mr. Richard Sakul's MS work that is currently in progress.

Table 1. Reference Name of the Oil Samples used in the Experiment.

Date Extracted	Type	Reference Name
January 26, 2015	Short Leaf Liquid	1
December 1, 2014	Loblolly Oil	2
November 17, 2014	Short Leaf Oil	3
November 19, 2014	Short Leaf Oil	4
December 8, 2014	Short Leaf Oil	5
February 2, 2015	Short Leaf Oil	6

3.2 Bacterium Cultures

Four strains of *Listeria monocytogenes* were obtained from the Food Science department at the University of Arkansas. The strains include Li 118 6b V22 (SLCC 5639) VICAM, Lm 100 4a V4 (ATCC 19114) VICAM, Lm 127 4c V6(ATCC 19116) VICAM, and Lm 106 6a V14(ATCC 35897) VICAM. A culture of each strain was individually passed twice by adding 1 mL of the inoculum to 9 mL of Bacto 0.6% yeast extract tryptic soy broth (BD, Franklin Lakes, NJ) and vortexed. The mixture was placed on an orbital shaker that was located within an incubator (VWR, Radnor, PA) for approximately 20 hours at 37 ℃. The temperature of 37 ℃ is a known condition where *L. monocytogenes* will grow (Albrecht, 2005). The shaker was set at 100 rpm. After being passed twice in the process described, 1 mL of each strain was added together to create a cocktail, which was tested against the pine essential oil samples. The inoculation level of the cocktail was

determined to be 3.7 x 10^8 by counting the cells at different concentrations. The experiments followed the University of Arkansas Institutional BioSafety Committee protocol #15007.

3.3 Disc Diffusion

A Mueller Hinton Agar No. 2 nutrient agar plate (HiMedia Laboratories, Kennett Square, PA) was inoculated with the *L. monocytogenes* cocktail using a cotton swab. Six blank 6 mm paper discs (BD, Franklin Lakes, NJ) were then placed onto each plate with equal spacing around each disc. Ten μL of each of the six pine essential oil samples was placed onto an individual disc on the plate. The process was done in triplicate for each experiment and three experiments were performed.

Control experiments were conducted by inoculating the nutrient agar plate with the *L. monocytogenes* cocktail, as previously described. Two blank paper discs were placed on the plate. Ten μL of water was added to one disc as the negative control, while 10 μL of the antibiotic streptomycin (Sigma, St. Louis, MO) was added to the other disc as the positive control. Water is known to not inhibit *L. monocytogenes* growth and streptomycin is known to be a strong inhibitor of *L. monocytogenes* growth (Charpentier, 1999).

3.4 Incubation and Measurements

The three experiments and the control experiment plates were placed in an incubator at 37 ℃ for 48 hours. After that time, zones of inhibition were measured around each disc. The zone of inhibition was measured as the diameter of the area around the disc where bacterial growth is not present. The growth of *L. monocytogenes* was observed as a cloudy appearance over the agar plate, while no growth appeared as a clear area around the paper discs.

4.0 Results

The components of each type of oil were identified using gas chromatography (GC). The liquid was excluded from this analysis because of its composition. Preliminary results show each essential oil sample to contain α-pinene, β-pinene, 3-carene, limonene, and terpineol, but at different concentrations. Composition of the essential oils is currently being confirmed by GC mass spectrometry. Final compositional analysis of the essential oils is therefore not reported in this thesis.

The average zones of inhibition for each type of oil are as shown below in Table 2. The control experiments are shown in Table 3. Photos of antimicrobial growth on several of the disc diffusion plates are shown in the figures in Appendix A.

Table 2. Pine Essential Oil Zones of Inhibition against *L. monocytogenes* Cocktail.

Oil Samples						
	(1) Liquid 1.26	(2) Loblolly 12.1	(3) Short Leaf 11.17	(4) Short Leaf 11.19	(5) Short Leaf 12.8	(6) Short Leaf 2.2
Average Size (cm)	0	0.20±0.45	0	0.70±0.64	1.06±0.09	0.68±0.66

Table 3. Control Experiment Zones of Inhibition against *L. monocytogenes* Cocktail.

Control Experiments		
	Water (Negative Control)	Streptomycin (Positive Control)
Average Size (cm)	0	3

5.0 Discussion

Pine essential oil exhibited activity against *L. monocytogenes* in the loblolly oil sample and three short leaf oil samples. The zones of inhibition of the pine essential oil experiments, shown in Table 2, were smaller than that of the streptomycin zone of inhibition, which was 3 cm. The zones of inhibition for four of the pine essential oil samples were larger than the negative control experiment, water.

The results of pine essential oil inhibiting growth of *L. monocytogenes* are consistent with other research on the topic. A study on reducing bacteria in a cattle feedlot showed that using pine oil to treat the pens of cattle would significantly decrease *L. monocytogenes* growth in the cattle manure removed from the pens (Wells, 2014). Research on how different essential oils affect the growth of *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica* also reported that essential pine oil showed activity against *L. monocytogenes*. Pine oil was not shown to be the most effective oil on the bactericide of *L. monocytogenes* out of the 96 essential oils tested, ranking in the bottom 57 oils in the overall effectiveness of reducing the four types of bacteria (Friedman, 2002).

In future work, essential oil composition, determined by GC and GC mass spectrometry analysis could shed light as to a potential relationship between presences of components with the ensuing zones of inhibition.

6.0 Conclusion

Four of the five tested essential pine oil samples showed antimicrobial activity against *L. monocytogenes*. The essential oil samples contained α-pinene, β- pinene, 3-carene, limonene, and terpineol but at different concentrations. No relationship between *L. monocytogenes* growth inhibition and compound concentrations could be made at this time.

Further experiments are necessary to confirm that pine essential oil inhibits the growth of *L. monocytogenes* in a variety of oil extractions, since one oil sample did not show any antimicrobial activity. The liquid extracted from the Clevenger apparatus could be excluded from the experiments in the

future since it showed no activity and does not have the same composition as the essential oils. The results show promising data that could potentially be applied to the food industry to inhibit growth of *L. monocytogenes* and reduce foodborne illness.

Appendix A: Figures

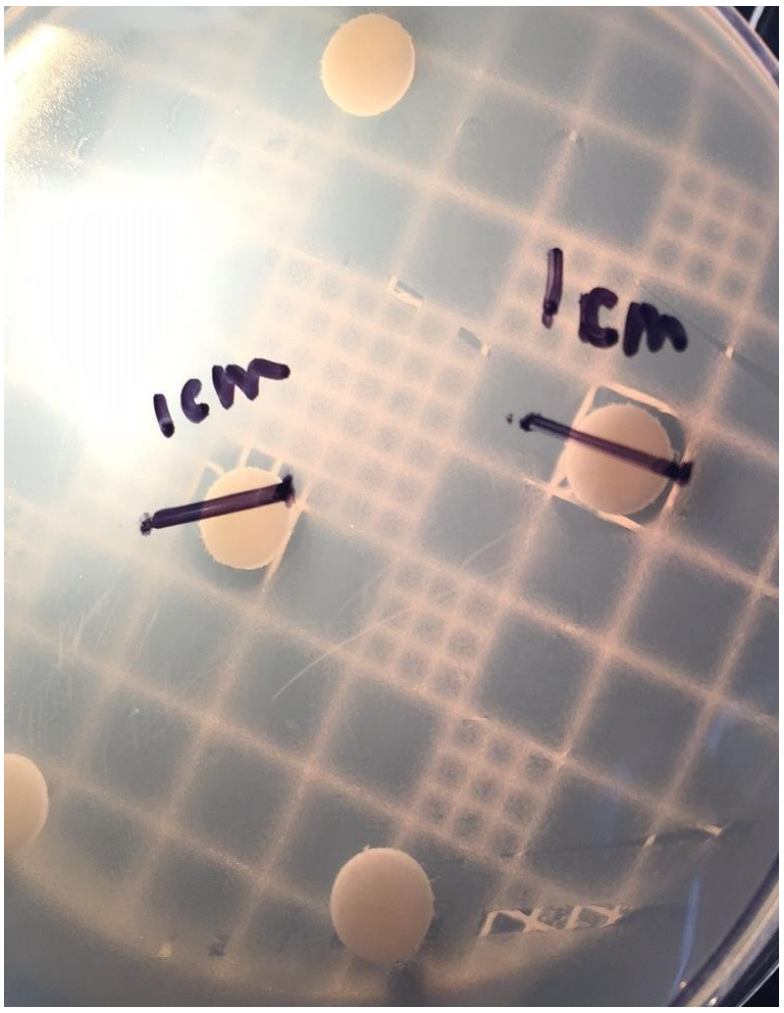

Figure 2. Zones of inhibition showing 1 cm for oil (5) and 1 cm for (6).

Figure 3. Zones of inhibition showing 1.2 cm for oil (4) and 1.2 cm for (5).

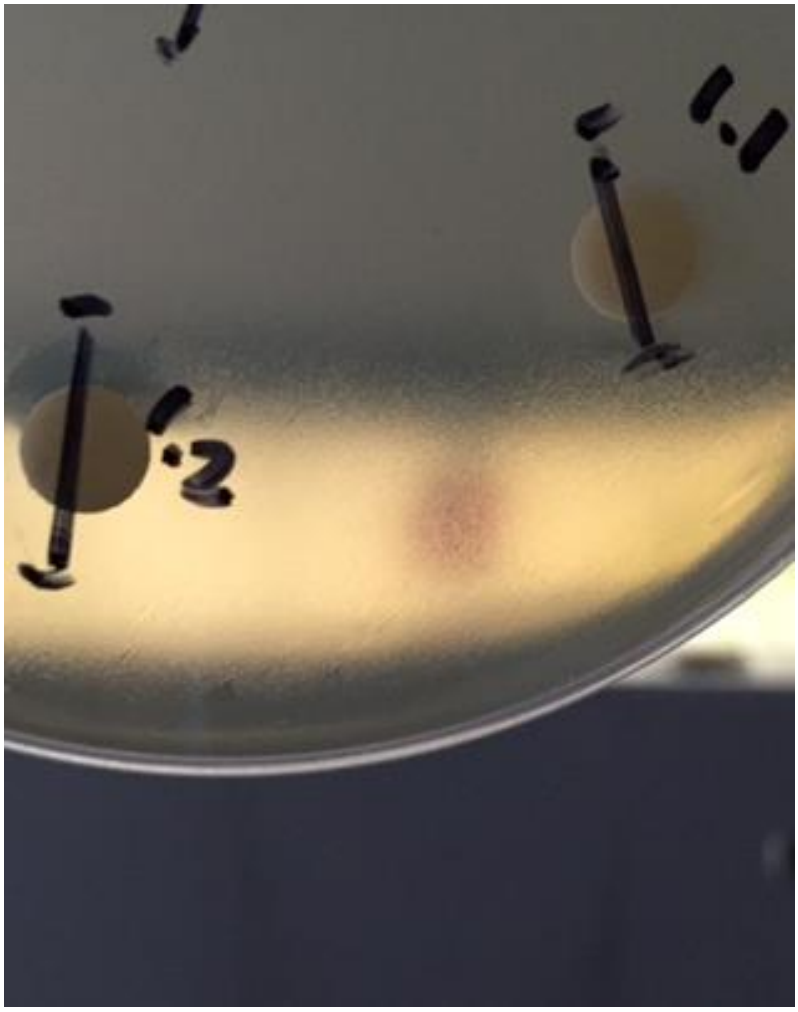

Figure 4. Zones of inhibition showing 1.2 cm for (4) and 1.1 cm for (5).

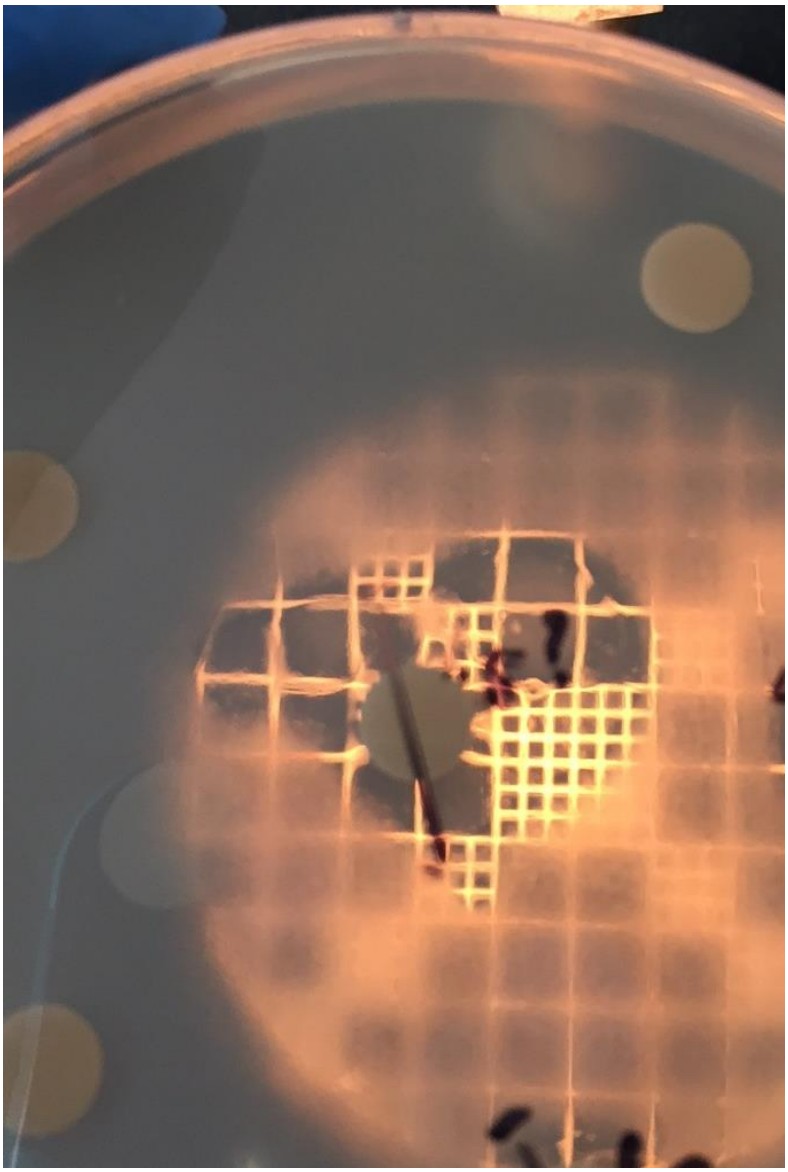

Figure 5. Zone of inhibition showing 1.5 cm for oil (6).

Appendix B: References

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