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The Cytotoxic and Antimicrobial Properties of Pine Essential Oils: A Characterization and Comparison

Richard Sakul
University of Arkansas, Fayetteville

The Cytotoxic and Antimicrobial Properties of Pine Essential Oils: A Characterization and Comparison

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By

Richard David Sakul
Hendrix College
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University of Arkansas

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_____
Dr. Danielle Julie Carrier
Thesis Director

_____
Dr. Edgar C. Clausen
Committee Member

_____
Dr. Thomas A. Costello
Committee Member

ABSTRACT

In the forestry industry, the pine tree species are important because of their durable timber and fast growth. In Arkansas, trees such as the loblolly pine compose almost a third of the timberland, seven million acres. In addition to the lignocellulosic biomass, pine bark and needles potentially have industrial importance as a waste stream from which high value (e.g., pharmaceutical, cosmetics) chemicals could be extracted, which could potentially increase the profit margin of forestry operations. In this research, the possibility that pine needles harvested from industry processed pine tree residues could be used as an antibacterial or cytotoxic chemical agent in order to provide an added value co-product for the lumber industry was investigated. *Pinus taeda* (loblolly pine tree) forestry residue and *Pinus echinata* (shortleaf pine tree) leaf essential oils were both effective cytotoxic agents against the Caco-2 cell line (heterogeneous human epithelial colorectal adenocarcinoma). The *P. taeda* caused complete cell culture death in 24 hours at the lowest concentration used, 0.15%, while the *P. echinata* essential oil was effective at 0.33%, reaching complete cell death at 1.25%. Both essential oils were tested against and showed some effectiveness against cocktails of the four bacterial species: *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella enterica*. Both these properties indicate that essential oils of both the *P. taeda* (loblolly pine tree) needle residue and *P. echinata* (shortleaf pine tree) needles have the potential to provide added value to the forestry industry, provided that their cytotoxic properties are further examined.

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DEDICATION

To Jay Sakul, Carissa Sakul, and Kevin Sakul, for their support and love.

TABLE OF CONTENTS

1. INTRODUCTION

Pine is an important lignocellulosic biomass species in the southeast United States. These trees, such as the loblolly pine, are a set of important species in Arkansas, which has approximately eighteen million acres in timberland, of which 29% is pine. Most research attention has focused on the loblolly pines' ability to produce timber because the species makes up a significant fraction of the United States industrial roundwood production (Smith et al., 2009). It is suspected that pine bark and needles could have industrial importance. During harvest, the pine trees are processed on site using a delimber implement that removes pine needles. This results in the generation of a waste stream from which valuable components could be extracted. By extracting useful components from pine needles or bark, the profitability of forestry operations could be increased. The essential oils of pine needles are reported to contain over 60 different chemicals; some of the components are: α pinene, β pinene, 3-carene, limonene, and terpineol (Kurose et al., 2007).

In a previous report, essential oil prepared from Arkansas pine needles was shown to contain: α-pinene (0.52-1.02 mg/g), β-pinene (0.04-0.67 mg/g), limonene (0.00-0.06 mg/g), terpineol (0.01-0.18 mg/g), and (-) caryophyllene (0.02-0.52 mg/g) (Adams et al., 2014). These chemicals already find uses in scent-based industries, one example being the perfume industry (Fakhari et al., 2005). Moreover, prior research has shown that that pine needle essential oil has some antimicrobial and anti-fungal properties. These pine-derived essential oils inhibit the growth of bacterial pathogens, including *Staphylococcus aureus* (Keun-young et al., 2000). Along with numerous other species, *S. aureus* is a bacterial pathogen that can cause many problems, such as skin infections (Archer, 1998). While not a serious threat unless untreated, these *S. aureus* infections may become life-threatening due to the development of strains that are

resistant to methicillin and other ß-lactam antibiotics; these *S. aureus* are termed methicillin resistant *Staphylococcus aureus* (MRSA) strains (Traber et al., 2008). A report from the World Health Organization (WHO) has stated that the amount of bacteria with resistance to antibiotics is steadily increasing and, as such, scientific research for determination of substances that have antimicrobial activity against MRSA strains is very important (World Health Organization, 2014).

The goal of this research was to demonstrate whether pine needles harvested from processed pine tree residues could be used as an antibacterial or cytotoxic chemical in order to provide value-added product for the lumber industry. It is important to note that extracting the value-added chemicals from residue is an important dimension of this work, as this unit operation could be integrated into existing forestry operations.

2. LITERATURE REVIEW

2.1. Essential Oils

Essential oils are aromatic oils obtained from plants in a variety of ways. Expression or fermentation of the plant material can be used to recover essential oils, but one of the most common commercial methods of obtaining essential oils is through steam distillation (Burt, 2004). Steam distillation consists of a simple distillation, where the volatile chemicals, in a liquid or liquid-solid mixture containing water, are heated. The volatiles are carried away with the steam vapor to a separate chamber where the vapor and volatiles condense, allowing for separation of the condensed steam and volatiles (King, 2014). Historically, commercial use of essential oils is centered in flavors and fragrances industries (Van de Braak & Leijten, 1999), such as the clove tree (*Eugenia caryophyllus*), which has uses in the food industry (Jirovetz et al., 2006). The lavender plant, *Lavandula angustifolia*, has commonly used essential oils in the fragrance industry (Fakhari et al., 2005).

Essential oils are known for other properties other than fragrance, including anti-cancer effects. The half minimal inhibitory concentration (IC_{50}) of essential oils of the *X. frutescens* (embira) were 33.9 µg/mL, 24.6 µg/mL, and 40.0 µg/mL for OVCAR-8 (ovarian adenocarcinoma), NCI-H358M (bronchoalveolar lung carcinoma) and PC-3M (metastatic prostate carcinoma) human tumor cell lines (Ferraz et al., 2013). Essential oils have also been shown to have antibiotic effects, such as the *Synsepalum dulcificum* (miracle berry) leaf essential oil, which had effects against six Gram-positive bacteria (*Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus albus*, *Staphylococcus aureus*, *Micrococcus tetragenus*, and *Micrococcus luteus*) and one Gram-negative bacterium (*Escherichia coli*). The respective zone of inhibitions were 17.50 ± 0.8 mm, 6.02 ± 0.7 mm, 5.14 ± 1.2 mm, 4.21 ± 1.0 mm, 16.92 ± 0.7 mm, 3.43 ± 0.6 mm,

and 14.60 ± 1.0 mm (Lu et al., 2014). These characteristics are possibly related to the function of these compounds in plants (Mahmoud & Croteau, 2002).

2.2. Antibacterial Properties of Essential Oils

The antibacterial property of essential oils is a well-catalogued occurrence. Essential oils extracted from species such as the *Eucalyptus chapmaniana* (Bogong gum tree) or the *Xylopia parviflora* (African striped pepper) have shown antimicrobial properties against various species of bacteria, including the more common *E. coli* or *Listeria monocytogenes* (Nadjib et al., 2014; Woguem et al., 2014). Furthermore, some research has shown that essential oils have some antimicrobial activity against what are the increasingly common antibacterial resistant bacteria, such as methicillin resistant *S. aureus* (MRSA) (World Health Organization, 2014). In a study using the essential oils from the pine needles from a single clone of young loblolly pine (*Pinus taeda*), the results showed that there was antimicrobial activity against the *S. aureus* strains with a zone of inhibition from 1 mm to 2 mm (Adams et al., 2014). The essential oil of *P. halepensis*, a plant in the same genus as both *P. taeda* (loblolly pine tree) and *P. echinata* (shortleaf pine tree), shared similar chemical composition, including α-pinene, β-pinene, 3-carene, and limonene (Dob et al., 2005; Fekih et al., 2014). This essential oil showed antimicrobial zones of inhibition against *L. monocytogenes* (10 mm), *Klebsiella pneumoniae* (10 mm), *E. faecalis* (9 mm) and *Acinetobacter baumanii* (9.5 mm).

In addition to pine, other essential oil preparations have been reported to inhibit microbial growth. As stated Chao et al. (2008) tested various commercially available water-distilled essential oils; preparations were evaluated for their antimicrobial actions against MRSA. Results showed that essential oils from lemongrass (*Cymbopogon citratus*), lemon myrtle (*Backhousia citriodora*), mountain savory (*Satureja montana*), cinnamon (*Cinnamomum verum*), and melissa

(*Melissa officinalis*) inhibited MRSA growth, with their zones of inhibition respectively reaching values greater than 8.3 cm, 6.5 cm, 6.25 cm, 6.0 cm, and 6.0 cm (Chao et al., 2008). Pepeljnjak et al. (2005) suggested that essential oil from dried Juniper berries (*Juniperus communis L.*) inhibited growth of various gram-positive bacteria, including *S. aureus* ATCC 6538 and *S. epidermidis,* and select gram-negative bacteria, such as *Salmonella enteritidis*. The resulting zones of inhibition were, respectively, 1.1 cm, 1.3 cm, and 0.8 cm (Pepeljnjak et al., 2005). As stated, the essential oil of the miracle berry can inhibit the growth of various bacterial species, including *S. aureus* and *E. coli* (Lu et al., 2014). Other plant species, such as *Mentha spicata*, *Pelargonium graveolens*, and *Rosmarinus officinalis*, have shown similar antimicrobial properties against a variety of bacteria species, including *E. coli* and *Streptococcus equinus*. These essential oils share some similar chemical components with *P. taeda* and *P. echinata*, such as α-pinene and terpineol (El Asbahani et al., 2015). Essential oils have also shown antimicrobial activity against *Salmonella enterica* and *L. monocytogenes*. Commercially available essential oils, such as for *Citrus* × *limon* (lemon) and *Origanum vulgare* (oregano), were tested against multiple strains of both *S. enterica* and *L. monocytogenes*, and showed effective activity (Mazzarrino et al., 2015).

Concerning the composition, most essential oils are composed of over 60 different constituents, making it difficult to identify the primary contributors to the antimicrobial effects of the essential oils. Major constituents often determine a significant portion of the efficacy of a particular essential oil, though other constituents can provide synergistic effects, despite low concentration in the essential oil (Jayasena & Jo, 2013). There is potential for the use of essential oils in food preservation, including meats (Jayasena & Jo, 2013). Components of pine essential oils have previously been reported to inhibit microbial growth. Specifically, α-pinene and β-

pinene have been previously reported to display antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) strains. Another attribute that lends to essential oils being used as an antimicrobial agent is that essential oils are likely not carcinogenic, as despite their ability to kill cells, they do not cause mutations in living cells (Bakkali et al., 2008). This antimicrobial effect can be applied against various common pathogens, both for humans and animals (Bakkali et al., 2008).

2.3. Anticancer Properties of Essential Oils

Determining whether the essential oils extracted from *Pinus taeda* (loblolly pine tree) needle residue and *Pinus echinata* (shortleaf pine tree) needles have cytotoxic effects could enhance the added value of loblolly pine and shortleaf pine forestry residue. Cytotoxicity is a property often sought out in chemicals, as chemicals with strong cell toxicity effects can potentially be used as treatment for cancer. Plants, often a good source of potential medicines, can have chemicals that are useful as medical treatments or form the basis for similar research (Bunel et al., 2014). One common method of determining cell viability is through an assay that measures continued metabolic activity. Such studies have focused on tetrazolium-based assays, which are widely used as cell viability tests. These assays are based on the tetrazolium salts for which they are named. The tetrazolium salts can be reduced by living cells, transforming the chemical into a formazan derivative. Formazan derivatives have coloration, which allow for spectrophotometric measurements. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is commonly used as the tetrazolium salt. When reduced, MTT becomes formazan, which displays a purple coloration. If the tested chemical/substance (i.e. in this case pine forestry scrap/needle essential oils) is toxic to cell cultures, the cell cultures will be unable to process the MTT compound, resulting in no color change.

During the preliminary literature search on research articles regarding MTT assays conducted using essential oils, approximately 200 articles were identified. Interestingly, many of the essential oils that were tested tended to share at least part of their chemical compositions with the essential oil of shortleaf pine and loblolly pine, despite not being a member of the same genus. One example is the silver fir (*Abies alba*), which also contains α-pinene, β-pinene, 3-carene, and limonene as major components in the essential oil. The essential oil of the silver fir was tested on human fibroblast (CCD-986SK) cells in an MTT assay and affected cell viability at a concentration of 5% (Yang et al., 2009). The essential oil of *Myrica rubra* (Chinese bayberry), was tested against the human adenocarcinoma cell lines HCT8, SW620, SW480, HT29 and Caco-2. The *Myrica rubra* essential oil preparation showed antiproliferative effects against these cell lines, affecting the Caco-2, HCT8, and HT29 beginning at 30 µL/mL. This essential oil contained some overlapping constituents, such as α-pinene and limonene (Langhasova et al., 2014). In addition, the component α-pinene has been shown to have some cytotoxicity effects against N2a neuroblastoma cells (Aydin et al., 2013).

Relatively few of these research articles used essential oil extracted from pine tree species as the primary chemical to be tested against cell lines. However there were some results with pine tree species from the literature, which are summarized below. Essential oils of *P. wallichaina* at the concentration 100 µg/mL were tested on A549 cell line (lung), C6 cell line (glioma), T47D cell line (breast), MCF cell line (breast) and TH-1 cell line (colon); all the cancer cell lines had their activity reduced (Qadir & Shah, 2014). The essential oil of *P. koraiensis*, a plant species in the same genus as the loblolly pine tree and the shortleaf pine tree, was tested against HepG2 (human hepatocarcinoma), displaying cytotoxic effect at concentrations of 200 µg/mL (Kim et al., 2012). The essential oils of the *P. roxburghii*, another pine tree in the same

genus, were tested against the human cell line MCF-7, which is a breast adenocarcinoma cell line. Both the essential oils extracted from *P. roxburghii* needles and bark displayed cytotoxic activity at 100 µg/mL (Satyal et al., 2013).

Pine needle essential oils are used on various cell lines and the outcomes for the assay are generally positive. In one study, the cell line Caco-2, a human colon adenocarcinoma line, was used and demonstrated some interesting properties, being a cell line that spontaneously differentiates, similarly to a mature enterocyte (Chantret et al., 1988). One of the interesting properties of the cell line is its relative sensitivity to chemicals. When Langhasova et al. (2014) used the cell line in their essential oil cytotoxicity study, they found that, of the five human adenocarcinoma cell lines tested, Caco-2 was one of the more sensitive lines (Langhasova et al., 2014). From the literature review, there were no cell lines that are consistently tested by every group nor were there a consistent number of cell lines tested. However, most of the published research was centered on the effects of an essential oil on cancer cell lines. Essential oils of *P. wallichaina* has been tested on the A549 (lung), C6 (glioma), T47D (breast), MCF (breast) and TH-1(colon) (Qadir & Shah, 2014). *Eucalyptus benthamii* adult leaf essential oil was test against multiple cell lines for the concentration that caused 50% cell death on the HeLa cell line (cervical cancer), IC50 was at 110.02 ± 2.89 µg/mL, on the Jurkat cell line (T leukemia cells) at 54.96 ± 5.80 µg/mL, and on the J774A.1 cell line (murine macrophage tumor), the IC50 concentration was at 252.55 ± 1.91 µg/mL, indicating that cell lines respond differently to the same preparation (Doll-Boscardin et al., 2012). The essential oil of *Pinus roxburghii* was tested against the MCF-7 (breast) cell line (Satyal et al., 2013). While there was some overlap between these studies in the cell lines chosen, they seem to be only driven by convenience.

In a similar manner to the choice of cell line, there is no hard and fast rule to the

concentration of tested doses. Most of the research articles tested concentrations from as low as 3 µg/mL to as high as 300 µg/mL. Some articles reported the testing of the studied essential oil at concentrations of 100 µg/mL (Qadir & Shah, 2014). Aydin et al. (2013) tested the effects of α-pinene, a primary constituent of many essential oils, tested concentrations from 10 mg/L up to 400 mg/L on N2a neuroblastoma cells. Cell proliferation decreased when the cells were exposed to concentrations of 400 mg/L (Aydin et al., 2013). Based on the articles found during the literature search, the effects of cytotoxicity generally began around 100 µg/mL and reached full effect by 300 µg/mL. At the upper end of essential oil concentrations (i.e. 300 µg/mL), the essential oils exhibited at least antiproliferative effects on the cells. Increasing past that concentration resulted in significant activity against the cells. However, the MTT assay began to show positive results, at the earliest, at 50 µg/mL.

2.4. Conclusion

In summary, the essential oils of various trees in the *Pinus* genus have cytotoxic and antimicrobial effects. These essential oils are typically extracted through steam distillation and could potentially be used for medicinal purposes. Concerning cytotoxic and anticancer properties of the essential oils, most showed at effectiveness against a wide range of cell lines. As for antimicrobial properties, the essential oils showed at minimum prohibitive effects against common bacterial strains and depending on the strain, the essential oils would be potent. For usage in medical situations, the desirable properties are dependent on the desired usage. If the essential oils were to be used as a sanitizer, it would be desirable for the essential oils to only have a strong antibacterial effect. If it were to be used as a chemotherapy agent, it would be desirable for the essential for the essential oil to have both cytotoxic and antimicrobial effects. However, the essential oils used in the literature were typically the essential oils that were

extracted from biomass in good condition, namely clean specifically plucked needles. Additionally, the pine trees studied are not all used commonly in the United States lumber market. In this study, the pine trees studied are the *P. taeda* (loblolly pine tree) and *P. echinata* (shortleaf pine tree).

3. OBJECTIVES

The goal of this project was to demonstrate whether the essential oil prepared from *Pinus taeda* (loblolly pine tree) needle residue and *Pinus echinata* (shortleaf pine tree) needles can have added value through antimicrobial or cytotoxic effects. In addition, this work compared the quality of the oils in terms of effects. The specific objectives were to:

1) Determine the composition of various selected constituents in *P. taeda* residue essential oil and the *P. echinata* essential oil.
2) Investigate the antimicrobial effects of the loblolly pine needle residue essential oil and the shortleaf pine needle essential oil on four bacterial species: *Listeria monocytogenes, Escherichia coli, Staphylococcus aureus,* and *Salmonella enterica*.
3) Using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide based (MTT) assay, investigate the cytotoxic effects of loblolly residue and shortleaf pine essential oil on the human colon carcinoma cell line Caco-2.

4. MATERIALS AND METHODS

4.1. Biomass Description

The short leaf and loblolly pine needles were harvested at the Teaching and Research Forest of the University of Arkansas at Monticello located in Drew County, Arkansas (34º03’83”N, 92º22’22”W) in May and June 2014. The short leaf pine needle biomass was harvested by excising needles from intact branches. Needles were transferred to a portable cooler in the field and stored in approximately 4.8º C in the laboratory until shipping on ice to the Department of Biological and Agricultural Engineering in Fayetteville, Arkansas.

The loblolly pine needles were collected from pine forestry residue on the forest floor in an established plot at the University of Arkansas at Monticello Teaching and Research Forest. They were also transferred to a portable cooler in the field and stored in approximately 4.8º C in the laboratory until shipping on ice to the Department of Biological and Agricultural Engineering in Fayetteville, Arkansas.

4.2. Extraction of Essential Oil

The essential oils of the short leaf pine needles and the loblolly pine tree forest residue (which consisted primarily of needles) were conducted using a hydrodistillation process. During hydrodistillation, the material was heated in a water-based solution, allowing the volatile elements to be carried away with the steam. The steam was then condensed in a condensing chamber, where the volatile elements were separated from the water. For this study, a Clevenger apparatus (Pyrex, Corning Life Sciences, Kennebunk, ME) was used as the condensing unit. A 2000 mL round bottomed flask was used as the container for the water-based solution. On average, 160 g of NaCl (VWR, Radnor, PA) was dissolved in 800 mL of distilled water and poured into the flask, in order to increase the boiling point. Then, 200 g of chopped pine needles

and boiling chips (VWR, Radnor, PA) 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. Upon completion of the boiling period, the oil was extracted from the apparatus, stored in a small amber vial 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. The labeling scheme for each sample was based on the date that the sample was extracted and the Clevenger unit used for extraction.

4.3. Characterization of Essential Oil

The essential oil was characterized using a gas chromatography (GC) unit coupled to a flame ionization detector. Specifically the analysis was carried out on a Varian 3800 GC (Bruker Daltonics, Billerica, MA) with a HTA-AS300 autosampler (HTA S.R.L., Brescia, Italy). The column used was an Agilent DB-5MS GC column (30 m length, 0.25 mm i.d., 0.25 um; Agilent Technologies, Santa Clara, CA). Conditions included a splitless method, with an injector temperature of 240 °C and a source temperature of 200 °C, an oven temperature program with an initial temperature of 50 °C, held for 3 minutes; increasing the temperature from 50°C to 200°C at 10°C per minute and then held for 5 minutes. This procedure was adapted from Ennajar et al. (2011). Essentially, analysis of essential oil components were carried out as described in Adams et al. (2014), where five components of the essential oil were characterized.

These components were identified as α pinene, β pinene, 3-carene, limonene, and terpineol. The pine essential oils used in the experiment were from short leaf pine needles extracted on November 17, 2014, November 19, 2014, December 8, 2014, and February 2, 2015 and from loblolly pine forest residue, distilled on December 1, 2014. Due to the nature of the essential oil extraction and yield, these samples were the only samples that produced sufficient

essential oil volume to allow for appropriate testing. Calibration curves for α-pinene, β- pinene, 3-carene, limonene, and terpineol were prepared using the GC instrument and method previously and are shown in figure 1. Standards for α-pinene, β-pinene, 3-carene, limonene, and terpineol were purchased from VWR (Radnor, PA).

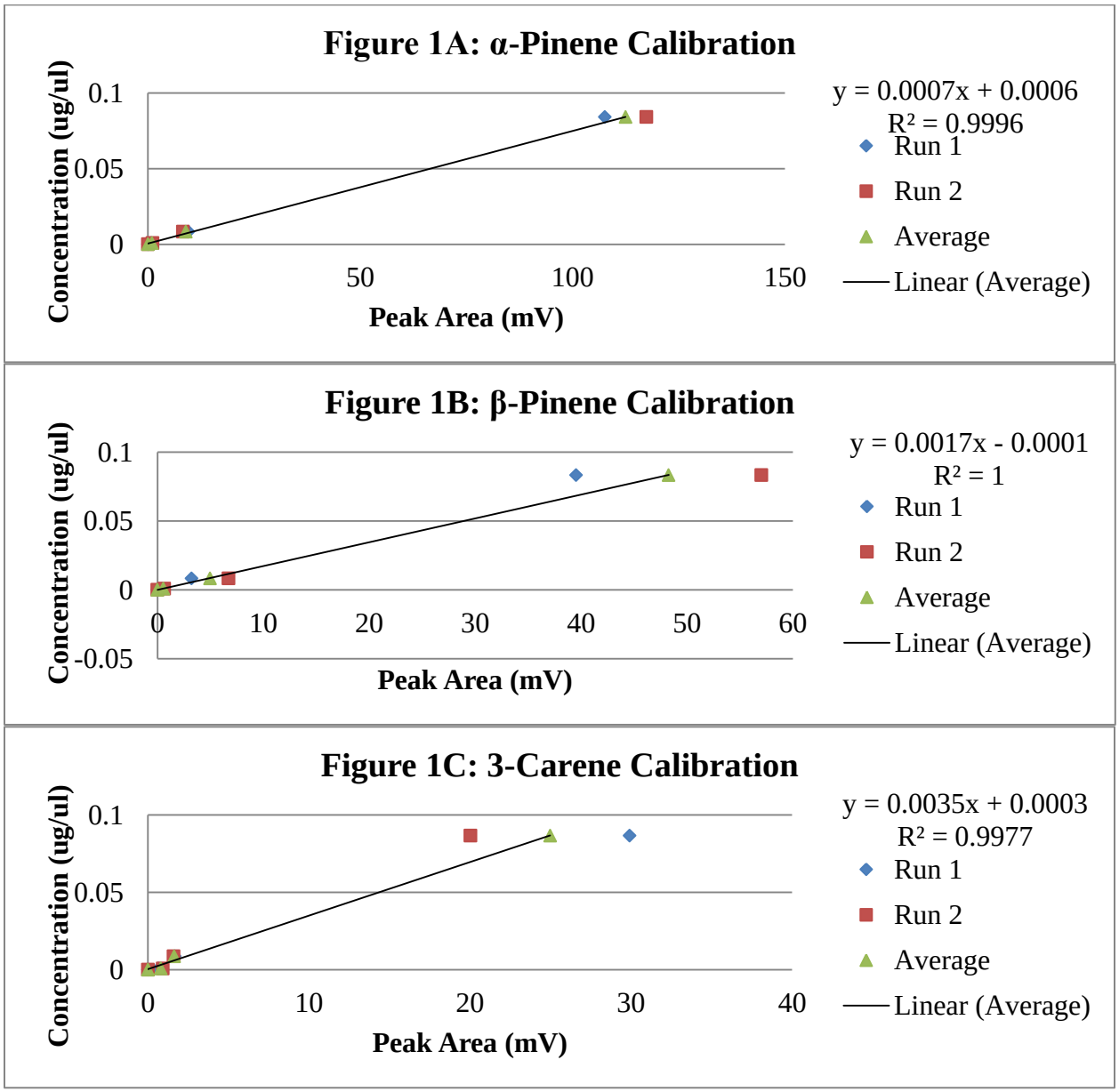
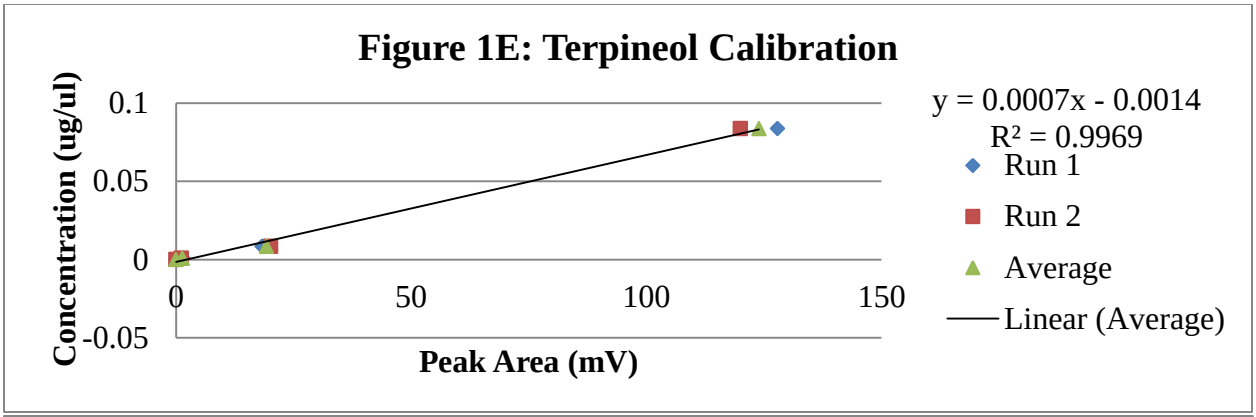


Figure 1: Calibration curves of α-pinene, β-pinene, terpineol, 3-carene, and limonene for a Varian 3800 GC using an Agilent DB-5MS GC column. Figure 1A, 1B, 1C, 1D, and 1E present calibrations of α-pinene, β-pinene, 3-carene, limonene, and terpineol, respectively, found in the samples.



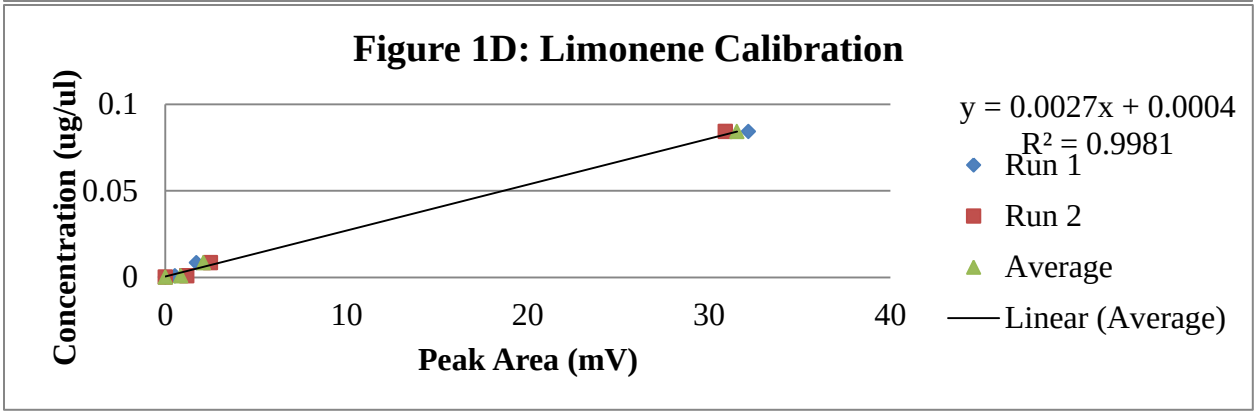


Figure 1 (cont'd).

4.4. Human Cell Culture

Caco-2 cells, a human epithelial colorectal adenocarcinoma (cancer) cell line, were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, and 2% antibacterial antimycotic solution. The media components and reagents used to create the media were obtained from GibcoR (Life Technologies, Carlsbad, CA). Cell cultures were maintained under sterile conditions and incubated at 37°C in a humidified 5% CO_2 atmosphere in a CO_2 incubator symphony 6.5 W (VWR symphony™, VWR International LLC, Radnor, PA).

4.5. Cytotoxicity Assessment

The assay used to determine the cytotoxicity of the essential oils was the 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a colorimetric assay for assessing cell viability. This analysis of cell viability was carried out on a methodology described by Kwak et al. (2006). The processes of the MTT assay are based on enzymes present inside living/viable cells. In this assay, the Caco-2 cell line was grown in a flask and incubated in 20% FBS media. In order to ensure that the cells had grown properly, the flask was inspected, and when the cell coverage of the flask was at least 80% covered and contained a minimum of 10^6 cell count, the assay proceeded. In general, 100 µl of Dulbeco's modified Eagles medium (DMEM) containing 20% FBS were seeded in the wells of a 96 well plate (Corning®, Radnor, PA) and incubated at 37°C and 5% CO_2. Usually, these plates were placed in an incubator overnight; however, if the cells were growing slowly, additional time was given. The media was then aspirated from the wells, one well at a time. To the aspirated well, 99 µL of fresh 20% FBS DMEM media were added. For testing cytotoxicity, 1 µL of each of experimental treatments was added to wells. The treatments consisted of 5.0% essential oil, 2.5% essential oil, 1.25% essential oil, 0.625% essential oil, 0.313% essential oil, 0.15% essential oil, 0% essential oil, positive control (20% FBS DMEM), and negative control (Triton X-100 (Sigma-Aldrich, St. Louis, MO)). The pine essential oils used in the experiment were from short leaf pine needles distilled on November 17, 2014, November 19, 2014, December 8, 2014, and February 2, 2015 and from loblolly pine forest residue, distilled on December 1, 2014.

These essential oil samples were labeled according to the date of extraction and the letter assigned to the Clevenger apparatus in which it was extracted. The 96 well plates (Greiner Bio-One, Monroe, NC) were covered and incubated for 24 hours. After the 24 hours incubation period, 10 µL of 10% MTT solution (VWR MTT assay kit, VWR International LLC, Radnor, PA) were added to each well and incubated for an additional 4 hours. Then, 100 µL of MTT

solubilization solution (10% Triton X-100 (Sigma-Aldrich, St. Louis, MO) and 0.1 N HCl (VWR International LLC, Radnor, PA) in anhydrous isopropanol (VWR International LLC, Radnor, PA) were added to each well in order to stop the reaction. The absorbance was then read at 570 nm with a reference wavelength of 690 nm using a plate reader (Synergy HT Multi-Mode Microplate Reader, BioTek Instruments, Inc., Winooski, VT). The 570 used as that the absorbance wavelength of the converted MTT, while the reference wavelength is used to measure the background absorbance.

After all absorbance readings were obtained, the data were corrected for the background absorbance of the control media. In the experiment, the "cell + media" (c+m) wells contained only cells and media and were used as a positive control, depicting uninhibited cell growth. The "cells + media + Triton X-100" (c+m+tx) wells were the cells mixed with media and surfactant to kill the cells, functioning as a negative control. These two controls essentially functioned as the maximum and minimum cell growth possible for the cells.

4.6. Bacterial Cultures

Four species of bacteria were tested in this project. These bacteria were *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella enterica*. Four *L. monocytogenes* strains were combined to form the cocktail that was used for testing the *Listeria* species: Li 1/2a V7, Li 4ab MURRAYB, Li 4b IOS, and Li ATCC33090. Four *E. coli* strains were used to form the cocktail that was used for testing: Ec ATCC11775**,** Ec O157.H7, Ec Dah Fung, and Ec ATCC25922. One strain of *S. aureus* was used for testing: Sa S41. Four *S. enterica* strains were used to form the cocktail that was used for testing: S ATCC43845, S ATCC8326, S ATCC8387, and S USDA1769NR.

To construct the bacterial cocktail mixture, the strains were mixed together in equal parts.

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 24 hours; the system was maintained at approximately 37ºC. The shaker was set at 100 rpm. After being passed twice as described above, 1 mL of each strain was added together to create a cocktail, which would be tested against the pine essential oil samples. As described earlier, the essential oils that were tested were from short leaf pine needles distilled on November 17, 2014, November 19, 2014, December 8, 2014, and February 2, 2015 and from loblolly pine forest residue, distilled on December 1, 2014. The experiments followed the University of Arkansas Institutional BioSafety Committee protocol #15015.

4.7. Growth Inhibition Assays

For the growth inhibition assay, each bacteria cocktail was inoculated onto two Petri plates (HiMedia Laboratories, Kennett Square, PA) using a cotton swab. Every iteration of this assay contained a total of eight different plates. Four 6 mm paper discs (BD, Sparks, MD) were then placed onto each plate, making sure the discs were spaced properly. Three of the paper discs were inoculated with 10 μL of selected essential oil samples and one disc was inoculated with 10 μL of 10% antibacterial streptomycin (Alfa Aesar, Haverhill, MA); all discs were placed on the plates. The essential oil samples that were tested were from short leaf pine needles distilled on November 17, 2014, November 19, 2014, December 8, 2014, and February 2, 2015 and from loblolly pine forest residue, distilled on December 1, 2014. The experiments followed the University of Arkansas Institutional BioSafety Committee protocols. After the discs were imbibed with essential oils or antibacterials, the Petri dishes were stored in an incubator (VWR, Radnor, PA) for approximately 24 hours; the system was maintained at approximately 37°C.

After the approximate 24-hour incubation period, zones of inhibition, an area around the paper disc where there is no bacterial growth, were measured around each disc. The measurement was of the thickness of the concentric zone of inhibition that extended around the disk.

4.8. Statistical Analysis

For the statistical analysis of the concentration of the chemicals in each essential oil sample, a single factor analysis of variance (ANOVA) was performed in R-3.2.5 (a statistical computing program) to determine if there were any significant differences in chemical composition. If there was a significant difference, several multiple comparisons tests (Tukey's Test, Fisher's Least Significant Difference, and Duncan's Multiple Range Test) were performed further elucidate the differences between the levels of the concentration. For the antibacterial assay, a single factor ANOVA was performed in R-3.2.5 (a statistical computing program) to determine if the concentrations of essential oils had any significant effect on the antibacterial properties. If there was a significant effect, several multiple comparisons tests (Tukey's Test, Fisher's Least Significant Difference, and Duncan's Multiple Range Test) were performed further elucidate the differences between the levels of the concentration. Similarly, for the statistical analysis of the results of the MTT assay, a two factor ANOVA in R-3.2.5 was used to determine if there were any significant differences between different samples or concentration between oil samples and the resulting cytotoxic properties. If there was any significant difference, then Tukey's Test, Fisher's Least Significant Difference, and Duncan's Multiple Range Test were performed to further elucidate the specific differences. Significance was established for $P < 0.05$.

5. RESULTS AND DISCUSSION

5.1. Description and Analysis of Essential Oil Samples

The overall essential oil yield of biomass was measured after extraction using steam distillation. It was determined that the shortleaf pine needle biomass had the highest essential oil yield per mass content, averaging at 0.2 mL/g dry weight, while the loblolly pine forestry residue had a numerically lower essential oil yield per mass, averaging at 0.0025 mL/g dry weight. These concentrations were somewhat similar to the range reported by Kurose et al. (2007). This group measured essential oil yields from the needles of nine species in the *Pinus* genus (*P. koraiensis*, *P. merkusii*, *P. palustris*, *P. parviflora*, *P. petula*, *P. ponderosa*, *P. pumila*, *P. rigida* and *P. rudis*). The highest yield was 0.0233 mL/g dry weight from the needles of *P. pumila*, while the lowest yield was 0.008 mL/g dry weight from the needles of *P. rigida* (Kurose et al., 2007).

The composition of the essential oils that were produced from loblolly pine needle forest residue and shortleaf pine needles were characterized, as shown in tables 1 and 2. Each sample of the short leaf pine biomass was characterized due to the inherent variability between separate extractions of the biomass. Each extraction was considered a unique sample, with a designation including the date of extraction. The extractions were further designated A or B, depending on which Clevenger apparatus was used to perform the extraction. These extractions from biomass are in contrast to the essential oil samples often used in literature, which were frequently purchased or created from biomass from several sources. There are several potential causes for this variation in composition. One possible source of variation is the storage time of the biomaterial from which the essential oil was produced. While the extraction and analysis methodology was the same for each sample, each sample was extracted on different dates. Another potential source of variation in chemical composition and concentrations was seasonal

variation, which had an effect on the essential oil present in the biological materials reported by Adams et al., 2014.

Table 1: Loblolly pine tree needle residue essential oil characterization data. Means and standard deviations are based on N = 2.

Loblolly Pine Tree Forestry Residue Essential Oil Characterization					
	alpha-pinene	**beta-pinene**	**3-carene**	**limonene**	**terpineol**
Sample[a]	**ug/g**	**ug/g**	**ug/g**	**ug/g**	**ug/g**
LL 12-1-14 A	0.88±1.02	6.51±6.90	0.26±0.15	33.93±24.46	2.22±1.60

[a] The sample designations include the date of extraction and a letter designating the distillation apparatus used. LL designates that this sample was extracted from loblolly pine forestry residue biomass.

Table 2: Shortleaf pine tree needle essential oil characterization data. Means and standard deviations are based on N = 2.

Short Leaf Pine Tree Needle Essential Oil Characterization					
	alpha-pinene	**beta-pinene**	**3-carene**	**limonene**	**terpineol**
Sample[a]	**ug/g**	**ug/g**	**ug/g**	**ug/g**	**ug/g**
SL 11-17-14 B	0.78±0.14	1.10±0.14	0.33±0.04	7.62±0.73	0.03±0.02
SL 11-19-14 B	0.61±0.22	0.88±0.32	0.24±0.02	6.28±2.19	0.02±0.01
SL 12-8-14 A	1.25±0.32	1.50±0.35	0.60±0.12	10.10±1.79	0.69±0.05
SL 2-2-15 B	4.04±1.57	3.08±1.02	0.81±0.20	30.27±7.55	0.86±0.02

[a] The sample designations include the date of extraction and a letter designating the distillation apparatus used. LL designates that this sample was extracted from loblolly pine forestry residue biomass.

Pine essential oils were obtained through a hydrodistillation method, as described in Adams et al. (2014). Through GC-FID analysis, α-pinene, β-pinene, terpineol, 3-carene, and limonene were identified. These chemicals were also reported by others as constituents of other essential oils. El Asbahani et al. (2015) conducted a compositional analysis of nine different species of plants (*Mentha piperita*, *Mentha pulegium*, *Mentha spicata*, *Pelargonium graveolens*, *Rosmarinus officinalis*, *Thymus leptobotrys*, *Thymus pallidus*, *Thymus satureioides* and *Citrus limon*) in the Souss-Massa region of Morocco and determined that the essential oils of the various species contained α-pinene, β-pinene, 3-carene, and terpineol (El Asbahani et al., 2015). These components of essential oils are not only present in herbaceous plants, but also in other

woody tissue plants. The essential oil of *Pinus halepensis*, which can be found in Algeria, was also reported to contain 3-carene in addition to α-pinene, β-pinene, 3-carene, limonene, and terpineol (Dob et al., 2005).

In figure 2, the five components, α-pinene, β-pinene, 3-carene, limonene, and terpineol were quantified. These components are commonly found in various plant essential oils. As a product produced from biological material, each essential oil sample is significantly different from the others, with a high level of variations between the concentrations of components. Each sample produced from each extraction operation was to be considered as a unique sample. A single factor analysis of variance (ANOVA) was used to determine if there were differences between concentrations of each of the constituents of the samples.

With respect to α-pinene, from the results of the single factor ANOVA, shown in table 3, to determine if there was any significant differences between samples, it can be concluded there were significant differences ($P < 0.05$) between samples on concentration of α-pinene. Several multiple comparison analyses were used to further elucidate the differences between the samples, with the results shown in table 4. The primary difference between the samples was that sample SL 2-2-15 B contained more α-pinene than the other samples, and there were other varying degrees of impact as shown in the table. The α-pinene concentrations of the shortleaf pine essential oils were compared to those of *Pinus peuce* Grisebach (Balkan pine), and were determined to be lower, indicating composition variability (Koukos et al., 2000). The essential oil of herbaceous leafy plants, such as *Glossogyne tenuifolia*, also contained α-pinene and β-pinene. The α-pinene levels of the *G. tenuifolia*, compared to those of *P. taeda*, contained 20.3 μg/g more α-pinene, which corresponded to a numerical 2409% higher value. The β-pinene levels were also higher, containing 52.2 ± 5.2 μg/g, which corresponded to a numerical 800% β-

pinene increase as compared to *P. taeda* forestry residue essential oil (Yang et al., 2014). Compared to the *P. echinata* essential sample average, the *G. tenuifolia* essential oil contained more α-pinene and β-pinene, numerically around 1000% more and 2750% more, respectively (Yang et al., 2014). From the results of the single factor ANOVAs for the concentrations of β–pinene, limonene, and terpineol (respectively shown in tables 5, 6, and 7), it can be concluded that there were no significant differences ($P < 0.05$) between samples on concentrations of those three chemicals.

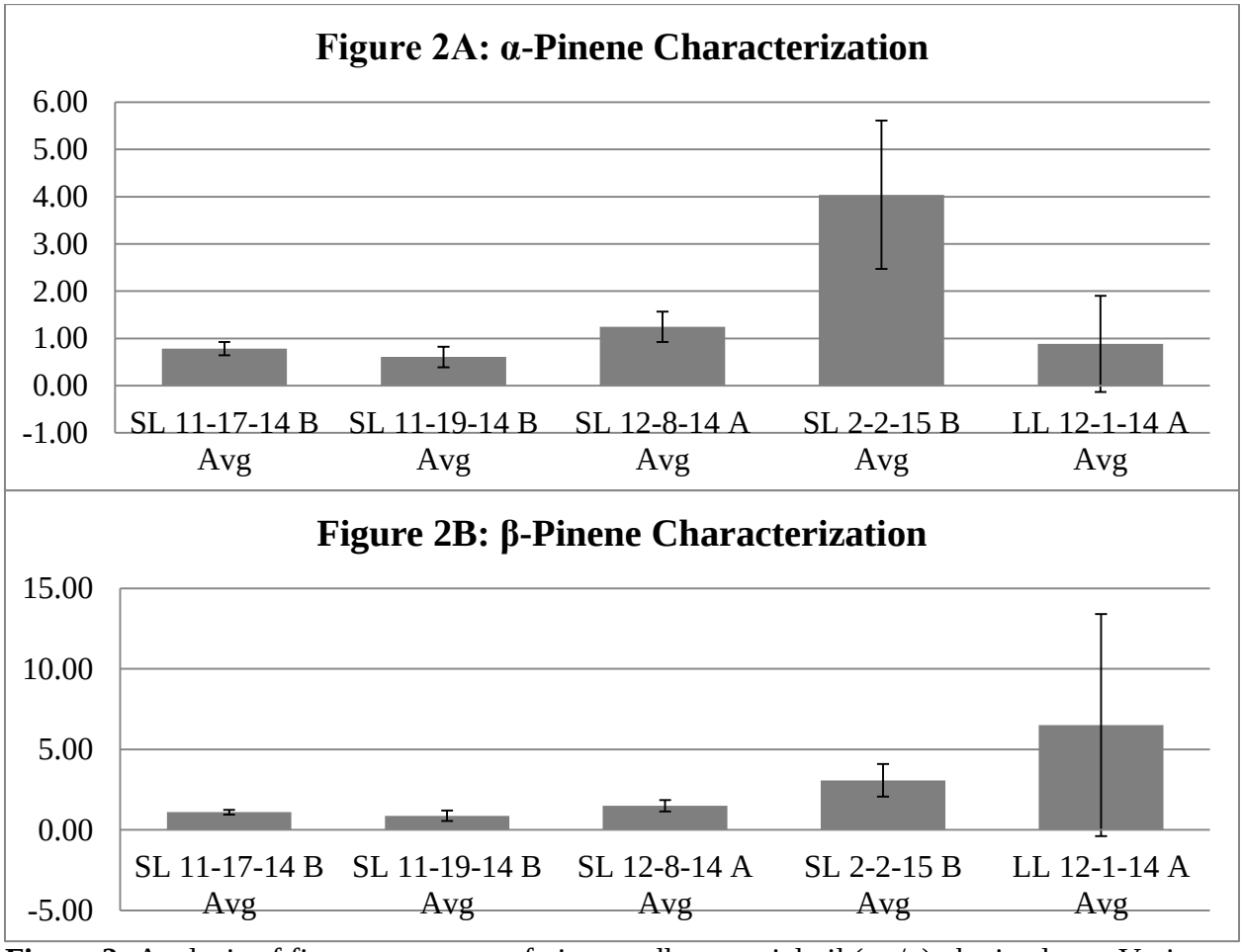


Figure 2: Analysis of five components of pine needle essential oil (µg/g) obtained on a Varian 3800 gas chromatograph (GC) equipped with an Agilent DB-5MS GC column. Figure 2A, 2B, 2C, 2D, and 2E present concentrations of α-pinene, β-pinene, 3-carene, limonene, and terpineol, respectively, found in the samples. Means and standard deviations are based on N = 2.

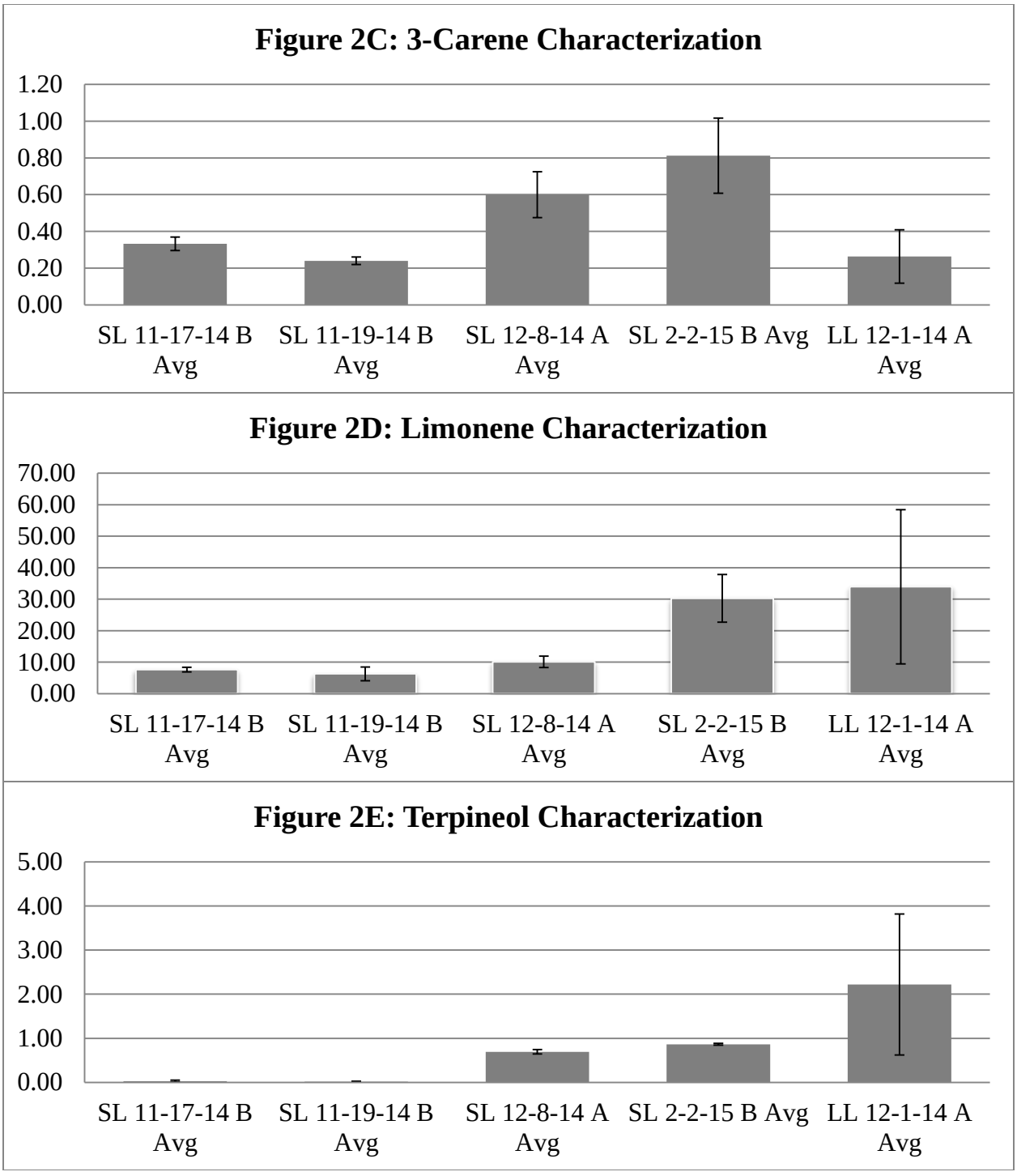


Figure 2 (continued).

Table 3. Results of the single factor ANOVA for the concentration of α-pinene. The dependent variable was the concentration of α-pinene. The independent variable was the sample used during testing.

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Sample	4	17.091	4.273	5.216	2.88E-02
Residuals	7	5.734	0.819		

Table 4. Results of multiple means comparison tests for the concentration of α-pinene. This use of multiple means comparison tests is to mitigate the biases of the specific tests. The dependent variable was the concentration of α-pinene. The independent variable was the sample used during testing.

Tukey's T-Test			**Fisher's Least Significant Difference Test**			**Duncan's Multiple Range Test**		
Treatment	**Means**	**M**	**Treatment**	**Means**	**M**	**Treatment**	**Means**	**M**
SL 2-2-15 B	4.0400	A	SL 2-2-15 B	4.0400	A	SL 2-2-15 B	4.0400	A
SL 12-8-14 B	1.2450	AB	SL 12-8-14 B	1.2450	A	SL 12-8-14 B	1.2450	B
LL 12-1-14 A	0.8825	B	LL 12-1-14 A	0.8825	A	LL 12-1-14 A	0.8825	B
SL 11-17-14 A	0.7800	B	SL 11-17-14 A	0.7800	A	SL 11-17-14 A	0.7800	B
SL 11-19-14 B	0.6050	B	SL 11-19-14 B	0.6050	A	SL 11-19-14 B	0.6050	B

Table 5. Results of the single factor ANOVA for the concentration of β-pinene. The dependent variable was the concentration of β-pinene. The independent variable was the sample used during testing.

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Sample	4	17.091	4.273	5.216	2.88E-02
Residuals	7	5.734	0.819		

Table 6. Results of the single factor ANOVA for the concentration of limonene. The dependent variable was the concentration of limonene. The independent variable was the sample used during testing.

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Sample	4	17.091	4.273	5.216	2.88E-02
Residuals	7	5.734	0.819		

Table 7. Results of the single factor ANOVA for the concentration of limonene. The dependent variable was the concentration of limonene. The independent variable was the sample used during testing.

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Sample	4	9.998	2.499	2.276	1.61E-01
Residuals	7	7.689	1.098		

With respect to 3-carene, from the results of the single factor ANOVA, shown in table 8, to determine if there was any significant differences between samples, it can be concluded there were significant differences ($P < 0.05$) between samples on concentration of 3-carene. Several multiple comparison analyses were used to further elucidate the significant differences between the sample choices, with the results shown in table 9. The primary differences found were that sample SL 2-2-15 B had significantly higher amounts of 3-carene, followed sample SL 12-8-14. Other varying degrees of impact are shown in the table. In contrast to the situation with the α-pinene values, the 3-carene content of the shortleaf pine essential oil was higher when compared to that of the Balkan pine (*Pinus peuce* Grisebach) (Koukos et al., 2000).

Table 8. Results of the single factor ANOVA for the concentration of 3-carene. The dependent variable was the concentration of 3-carene. The independent variable was the sample used during testing.

	Df	**Sum Sq**	**Mean Sq**	**F Value**	**Pr (>F)**
Sample	4	17.091	4.273	5.216	2.88E-02
Residuals	7	5.734	0.819		

Table 9. Results of multiple means comparison tests for the concentration of 3-carene. This use of multiple means comparison tests is to mitigate the biases of the specific tests. The dependent variable was the concentration of 3-carene. The independent variable was the sample used during testing.

Tukey's T-Test			**Fisher's Least Significant Difference Test**			**Duncan's Multiple Range Test**		
Treatment	**Means**	**M**	**Treatment**	**Means**	**M**	**Treatment**	**Means**	**M**
SL 2-2-15 B	0.8150	A	SL 2-2-15 B	0.8150	A	SL 2-2-15 B	0.8150	A
SL 12-8-14 B	0.6000	AB	SL 12-8-14 B	0.6000	AB	SL 12-8-14 B	0.6000	AB
LL 12-1-14 A	0.3350	B	LL 12-1-14 A	0.3350	AB	LL 12-1-14 A	0.3350	BC
SL 11-17-14 A	0.2650	B	SL 11-17-14 A	0.2650	B	SL 11-17-14 A	0.2650	C
SL 11-19-14 B	0.2400	B	SL 11-19-14 B	0.2400	B	SL 11-19-14 B	0.2400	C

Sample LL 12-1-14 A had concentrations that were not significantly different from the other samples. This is especially interesting as the shortleaf pine needle biomass used for this project were not forestry residue or undergrowth, but carefully gathered directly from the pine

trees, while the loblolly pine tree needle residue biomass was taken after the host trees were debarked. Loblolly pine forestry residue consisted of biomass gathered after a normal forestry operation, while the shortleaf pine material was carefully collected and harvested. This fact could possibly be useful for the production of value-added phytochemicals.

5.2. Bacterial Inhibition Potential of Essential Oils

Using the disc diffusion assay, the *P. taeda* (loblolly pine) forestry residue essential oil and the multiple samples of *P. echinata* (shortleaf pine) essential oil were tested for their potential to inhibit the growth of four bacterial species. Essential oils inhibited growth of *S. aureus*, as shown in table 10. Against *S. aureus* bacteria, the average zone of inhibition for the shortleaf pine needle essential oils was smaller than those from loblolly pine needle residue; the average zone of inhibition of the shortleaf pine needle essential oil was 0.45 cm smaller than those from the essential oil of loblolly pine. Unfortunately, both pine essential oils did not perform as well as the 10% streptomycin positive control.

Table 10. Discs were imbibed with tested essential oils; incremental radii (in cm) of the zones of inhibition against the four selected strains of bacteria are presented. The zone of inhibition was measured along a radius from the edge of the disc to the outer edge of the inhibition zone. The zone of inhibition represents the zone in which bacteria are unable to grow and is correlated to the strength of the antibacterial effect. Thus, a smaller value for the zone of inhibition means that there is less antibacterial effect from the chemical treatment. Means and standard deviations are based on N = 2. 10% streptomycin was used a positive control and deionized water was used as a negative control.

	Salmonella enterica	***Staphylococcus aureus***	***Escherichia coli***	***Listeria monocytogenes***
Sample Name	**Zone of Inhibition (cm)**			
LL 12-1-14 A	1.82±0.16	1.91±0.29	2.07±0.09	1.55±0.22
SL 11-19-14 B	0.00±0.00	0.86±0.04	0.00±0.00	0.00±0.00
SL 12-8-14 A	1.03±0.23	1.26±0.02	0.95±0.09	0.44±0.63
SL 11-17-14 B	0.00±0.00	1.21±0.20	0.00±0.00	0.00±0.00
SL 2-2-15 B	0.00±0.00	1.23±0.23	0.00±0.00	0.00±0.00
DI Water	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
10% streptomycin	2.63±0.37	2.21±0.21	2.39±0.47	2.57±0.37

A single factor analysis of variance (ANOVA) was used to determine if there were differences between samples on the zone of inhibition for each bacterial species. From the results of the single factor ANOVA to determine if there was any significant effect on *S. aureus*, as shown in table 11, it can be concluded there were significant differences ($P < 0.05$) between samples on the size of the zone of inhibition. Several multiple comparison analyses were used to further elucidate the significant differences between the sample choices. Shown in table 12, Tukey's T-test (TT) and Duncan's Multiple Range Test (DMR) shows that the sample LL 12-1-14 A is comparably in effect to the positive control, 10% streptomycin. Fisher's Least Significant Difference Test (LSD) considered sample LL 12-1-14 A to be the second most effective. There were other varying degrees of impact as shown in the table.

Table 11. Results of the single factor ANOVA for the antibacterial activity assay for the bacterial species *S. aureus*. The dependent variable was the size of the zone of inhibition (cm). The independent variable was the sample used during testing.

	Df	**Sum Sq**	**Mean Sq**	**F Value**	**Pr (>F)**
Sample	6	11.066	1.844	64.510	6.23E-08
Residuals	11	0.315	0.029		

Table 12. Results of multiple means comparison tests for the effect of samples on the zone of inhibition for the bacterial species *S. aureus*. This use of multiple means comparison tests is to mitigate the biases of the specific tests. The dependent variable was the size of the zone of inhibition (cm). The independent variables were the samples and controls used during testing.

Tukey's T-Test			**Fisher's Least Significant Difference Test**			**Duncan's Multiple Range Test**		
Treatment	**Means**	**M**	**Treatment**	**Means**	**M**	**Treatment**	**Means**	**M**
10% strep	2.2098	A	10% strep	2.2098	A	10% strep	2.2098	A
LL 12-1-14 A	1.9050	A	LL 12-1-14 A	1.9050	AB	LL 12-1-14 A	1.9050	A
SL 12-8-14 B	1.2573	B	SL 12-8-14 B	1.2573	BC	SL 12-8-14 B	1.2573	B
SL 2-2-15 B	1.2319	B	SL 2-2-15 B	1.2319	C	SL 2-2-15 B	1.2319	B
SL 11-17-14 A	1.2065	B	SL 11-17-14 A	1.2065	C	SL 11-17-14 A	1.2065	BC
SL 11-19-14 B	0.8636	B	SL 11-19-14 B	0.8636	C	SL 11-19-14 B	0.8636	C
DI Water	0.0000	C	DI Water	0.0000	D	DI Water	0.0000	D

The *P. taeda* (loblolly pine) essential oil sample, LL 12-1-14 A, and the *P. echinata* (shortleaf pine) essential oil samples, SL 11-19-14 A and SL 12-8-14 A, were shown to be effective against the *Escherichia coli* strain cocktails. Samples SL 11-19-14 B, 11-17-14 A, and SL 2-2-15 B did not display inhibitory effects against *E. coli* cocktails. The loblolly pine needle residue essential oil was more effective against this bacterial strain than the two shortleaf essential oil samples, as its zone of inhibition was numerically 100% larger than that of either *P. echinata* essential samples SL 11-19-14 A and SL 12-8-14 A. In all cases, the zones of inhibition of the pine essential oil experiments, shown in table 10, were generally smaller than those of the 10% streptomycin positive control zone of inhibition, indicating that the essential extracted is not as effective as the streptomycin.

From the results of the single factor ANOVA for *E. coli*, as shown in table 13, it can be concluded that sample choice had a significant effect ($P < 0.05$) on the size of the zone of inhibition. Several multiple comparison analyses were used to further elucidate the significant differences between the sample choices. Shown in table 14, the Tukey's T-test, Fisher's Least Significant Differences (LSD) Test, and Duncan's Multiple Range Test (DMR) shows that the sample LL 12-1-14 A is comparably in effect to the positive control, 10% streptomycin. Samples SL 2-2-15 B, 11-17-14 A, and SL 11-19-14 B were considered as effective as the negative control an inhibiting the growth of *E. coli*.

Table 13 Results of the single factor ANOVA for the antibacterial activity assay for the bacterial species *E. coli*. The dependent variable was the size of the zone of inhibition (cm). The independent variable was the sample used during testing.

	Df	**Sum Sq**	**Mean Sq**	**F Value**	**Pr (>F)**
Sample	6	19.753	3.292	53.800	1.61E-07
Residuals	11	0.842	0.061		

Table 14. Results of multiple means comparison tests for the effect of samples on the zone of inhibition for the bacterial species *E. coli*. This use of multiple means comparison tests is to mitigate the biases of the specific tests. The dependent variable was the size of the zone of inhibition (cm). The independent variables were the samples used during testing.

Tukey's T-Test			**Fisher's Least Significant Difference Test**			**Duncan's Multiple Range Test**		
Treatment	**Means**	**M**	**Treatment**	**Means**	**M**	**Treatment**	**Means**	**M**
10% strep	2.5654	A	10% strep	2.5654	A	10% strep	2.5654	A
LL 12-1-14 A	1.5494	A	LL 12-1-14 A	1.5494	A	LL 12-1-14 A	1.5494	A
SL 12-8-14 B	0.4445	B	12-8-14 B	0.4445	B	SL 12-8-14 B	0.4445	B
SL 2-2-15 B	0.0000	C	SL 2-2-15 B	0.0000	BC	SL 2-2-15 B	0.0000	C
SL 11-17-14 A	0.0000	C	11-17-14 A	0.0000	BC	SL 11-17-14 A	0.0000	C
SL 11-19-14 B	0.0000	C	SL 11-19-14 B	0.0000	BC	SL 11-19-14 B	0.0000	C
DI Water	0.0000	C	DI Water	0.0000	C	DI Water	0.0000	C

For the potential growth inhibition effects against *L. monocytogenes*, the loblolly pine forestry residue essential oil sample, LL 12-1-14 A, and the shortleaf pine essential oil samples, SL 11-19-14 A, and SL 12-8-14 A were shown to be effective against the strain cocktails. The loblolly pine needle residue essential oil was more effective against these bacteria than the two shortleaf essential oil samples, with the loblolly essential oil sample having a zone of inhibition at least 0.50 cm larger. Samples SL 11-19-14 B, 11-17-14 A, and SL 2-2-15 B did not have any inhibitory effects against *L. monocytogenes* strain cocktails. However, the zones of inhibition of the pine essential oil experiments, as shown in table 3, were generally smaller than those of the streptomycin zone of inhibition. The results of pine essential oil inhibiting growth of *L. monocytogenes* are consistent with previously reported results.

From the results of the single factor ANOVA to determine if there was any significant effect on *L. monocytogenes*, as shown in table 15, it can be concluded that sample choice had a significant effect ($P < 0.05$) on the size of the zone of inhibition. Several multiple comparison analyses were used to further elucidate the significant differences between the sample choices. The samples had much the same effect on *L. monocytogenes* as they did on *E. coli*. Shown in table 16, the three means comparisons tests found that none of the essential oils were comparable

to the positive control. The shortleaf pine essential oil samples have an effect comparable to that of the negative control and could have been considered essentially nonfunctional as antibacterial agent.

Table 15 Results of the single factor ANOVA for the antibacterial activity assay for the bacterial species *L. monocytogenes*. The dependent variable was the size of the zone of inhibition (cm). The independent variable was the sample used during testing.

	Df	**Sum Sq**	**Mean Sq**	**F Value**	**Pr (>F)**
Sample	6	20.241	3.374	44.090	4.61E-07
Residuals	11	0.842	0.077		

Table 16. Results of multiple means comparison tests for the effect of samples on the zone of inhibition for the bacterial species *L. monocytogenes*. This use of multiple means comparison tests is to mitigate the biases of the specific tests. The dependent variable was the size of the zone of inhibition (cm). The independent variables were the samples used during testing.

Tukey's T-Test			**Fisher's Least Significant Difference Test**			**Duncan's Multiple Range Test**		
Treatment	**Means**	**M**	**Treatment**	**Means**	**M**	**Treatment**	**Means**	**M**
10% strep	2.5654	A	10% strep	2.5654	A	10% strep	2.5654	A
LL 12-1-14 A	1.5494	B	LL 12-1-14 A	1.5494	B	LL 12-1-14 A	1.5494	B
SL 12-8-14 B	0.4445	C	SL 12-8-14 B	0.4445	C	SL 12-8-14 B	0.4445	C
SL 2-2-15 B	0.0000	C	SL 2-2-15 B	0.0000	C	SL 2-2-15 B	0.0000	C
SL 11-17-14 A	0.0000	C	SL 11-17-14 A	0.0000	C	SL 11-17-14 A	0.0000	C
SL 11-19-14 B	0.0000	C	SL 11-19-14 B	0.0000	C	SL 11-19-14 B	0.0000	C
DI Water	0.0000	C	DI Water	0.0000	C	DI Water	0.0000	C

The loblolly forestry residue sample, LL 12-1-14 A, and the shortleaf pine samples, SL 11-19-14 A and SL 12-8-14 A, were shown to be effective against the *S. enterica* strain cocktails. The loblolly pine needle residue essential oil was more effective against these bacteria than the two shortleaf essential oil samples. Interestingly, samples SL 11-19-14 B, 11-17-14 A, and SL 2-2-15 B did not display any inhibitory effects against the *Salmonella* strain cocktails. This is likely due to a difference between chemical compositions of these specific samples. As for other microbial strains, the zones of inhibition of the pine essential oil trials, shown in table 10, were generally smaller than those of the streptomycin zone of inhibition, which indicated that the essential oils were not as effective at bacterial growth inhibition. The results of both pine

essential oils inhibiting growth of *S. enterica* are consistent with other research on the topic.

From the results of the single factor ANOVA to determine if there was any significant effect on *S. enterica*, as shown in table 17, it can be concluded that sample choice had a significant effect ($P < 0.05$) on the size of the zone of inhibition. Several multiple comparison analyses were used to further elucidate the significant differences between the sample choices. Shown in table 18, the positive control, 10% streptomycin, was found to be the most effective at inhibiting the growth of *S. enterica*. Samples SL 2-2-15 B, 11-17-14 A, and SL 11-19-14 B were found to be comparable in effect to the negative, indicting complete lack of inhibitory properties against *S. enterica*. There were other varying degrees of impact, as shown in the table.

Table 17. Results of the single factor ANOVA for the antibacterial activity assay for the bacterial species *S. enterica*. The dependent variable was the size of the zone of inhibition (cm). The independent variable was the sample used during testing.

	Df	**Sum Sq**	**Mean Sq**	**F Value**	**Pr (>F)**
Sample	6	21.768	3.628	82.920	1.64E-08
Residuals	11	0.481	0.044		

Table 18. Results of multiple means comparison tests for the effect of samples on the zone of inhibition for the bacterial species *S. enterica*. This use of multiple means comparison tests is to mitigate the biases of the specific tests. The dependent variable was the size of the zone of inhibition (cm). The independent variables were the samples used during testing.

Tukey's T-Test			**Fisher's Least Significant Difference Test**			**Duncan's Multiple Range Test**		
Treatment	**Means**	**M**	**Treatment**	**Means**	**M**	**Treatment**	**Means**	**M**
10% strep	2.6289	A	10% strep	2.6289	A	10% strep	2.6289	A
LL 12-1-14 A	1.8161	B	LL 12-1-14 A	1.8161	B	LL 12-1-14 A	1.8161	B
SL 12-8-14 B	1.0287	C	SL 12-8-14 B	1.0287	B	SL 12-8-14 B	1.0287	C
SL 2-2-15 B	0.0000	D	SL 2-2-15 B	0.0000	C	SL 2-2-15 B	0.0000	D
SL 11-17-14 A	0.0000	D	SL 11-17-14 A	0.0000	C	SL 11-17-14 A	0.0000	D
SL 11-19-14 B	0.0000	D	SL 11-19-14 B	0.0000	C	SL 11-19-14 B	0.0000	D
DI Water	0.0000	D	DI Water	0.0000	C	DI Water	0.0000	D

Adams et al. (2014) previously demonstrated that the *P. taeda* essential oil, extracted from carefully harvested needles, displayed antimicrobial activity against two strains of MR *S. aureus*, creating a zone of inhibition of 1.0 ± 0.0 mm against the strain NC315 and 1.25 ± 0.35

mm against the strain COL (Adams et al., 2014). *P. halepensis* essential oil did not display any antimicrobial activity against *S. aureus* (Fekih et al., 2014), which indicated that the inhibitory effect against *S. aureus* was not consistent across all pine essential oils. The essential oils of the *P. taeda* Research on how different commercially available essential oils affect the growth of *E. coli* reported that the essential oils of many common cooking ingredients, such as oregano, thyme, cinnamon, palmarosa, bay leaf, clove bud, lemon grass, and allspice, showed antimicrobial activity against *E. coli* (Friedman et al., 2002). Commercially available grape seed essential and miscellaneous pine bark essential oil showed antimicrobial activity against *E. coli*, though the commercially available grape seed essential oils had more activity, as compared to the extracted miscellaneous pine bark essential oil (Ahn et al., 2007).

Research on how different essential oils affect the growth of *L. monocytogenes* illustrated that the commercially purchased essential oils of gardenia, cedarwood, bay leaf, clove bud, oregano, cinnamon, allspice, thyme, and patchouli, exhibited antimicrobial activity against *L. monocytogenes* (Friedman et al., 2002). *P. halepensis* essential oil showed antimicrobial activity against *L. monocytogenes* (Fekih et al., 2014). The essential oil of *Satureja horvatii*, an herb related to rosemary, displayed antimicrobial properties against *L. monocytogenes* growth in pork meat (Bukvički et al., 2014). Of the essential oils available for commercial purpose, at least the oils of thyme, oregano, cinnamon, clove bud, allspice, bay leaf, palmarosa, and marjoram were able to inhibit the growth of *S. enterica* (Friedman et al., 2002). The inhibitory effects of essential oil of the leaves of *Laurus nobilis*, an evergreen tree, demonstrated some inhibitory effect against *Salmonella typhimurium*, a bacterial species in the same genus as *S. enterica*. The *L. nobilis* essential oil shares some of the components as that of the *P. taeda* and *P. echinata* essential oil, such as α-pinene, β-pinene, and terpineol (Nehir et al., 2014). From these multiple

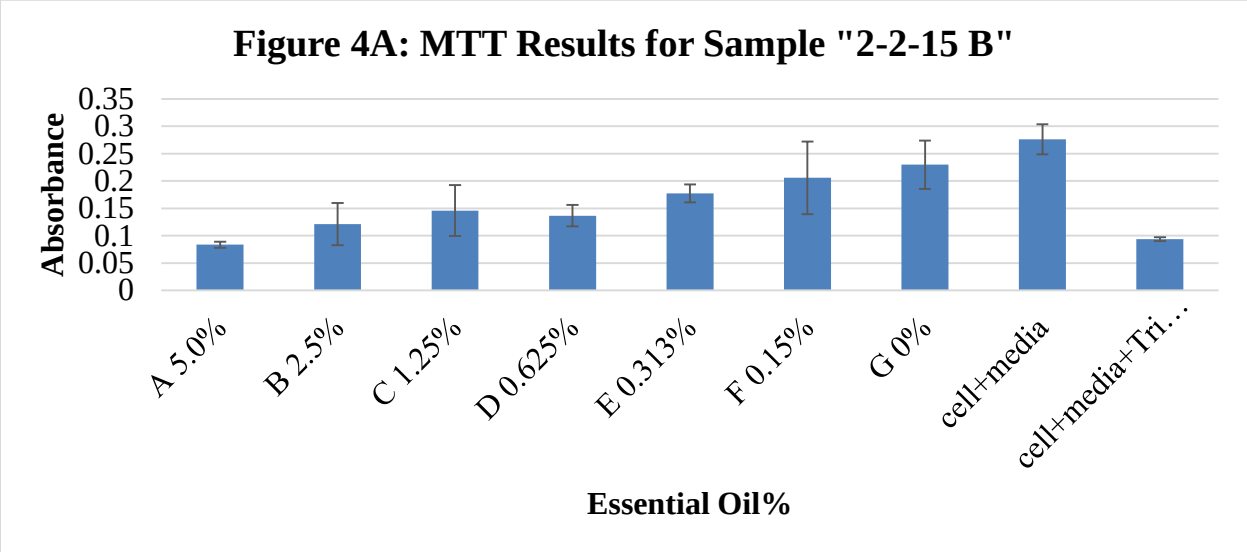
comparisons tests, it is shown that the 10% streptomycin exhibits the most antibacterial activity of the treatments, though the loblolly forestry residue could be considered to be of a similar, though slightly lesser effect. From these results, it can be concluded that choice of essential oil was important.

5.3. Cytotoxicity Assay

A standard protocol to test for cytotoxicity is the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. To interpret the outcome of the assay, the greater the intensity of the purple coloration that is present in the cell culture after the initial steps, the more cells that survived. The cell line that was selected in this study was Caco-2, which often stated to be relatively sensitive to chemicals (Langhasova et al., 2014). Figure 4 shows the effects that the essential oils had on the cells Caco-2, as determined by the MTT assay. If chemical/substance is shown to have cytotoxic effects, there is potential for the chemical/substance to be used as treatment for cancer, provided it is not also carcinogenic (Bunel et al., 2014).

As the cell cultures were only exposed to at 0.5 µL of the samples, the results point to the potency of the two separate biomasses. The shortleaf pine needle essential oil samples were shown to have an increasing effect on the Caco-2 cells; in the 0.15% solution, where the essential oil is only present in a minor amount, there was already a noticeable decline in the amount of living cells. This trend continued as a function of increasing essential oil concentrations. These results are in agreement with studies performed on cell lines using essential oils. Kwak et al. (2006) showed that the essential oil of *P. densiflora* exhibited cytotoxic properties against numerous tumor cell lines: MCF-7 (human breast adenocarcinoma), SNU-638 (human gastric carcinoma), and HL-60 (human leukemia cells) (Kwak et al., 2006). In stark contrast to the shortleaf pine needle essential oil, the loblolly needle residue was toxic to the Caco-2 cells at the

lowest concentration. Similarly, the *Myrica rubra* essential had a low concentration before reaching inhibitory concentrations (Langhasova et al., 2014). This is likely due to the differences in the essential oil compositions.



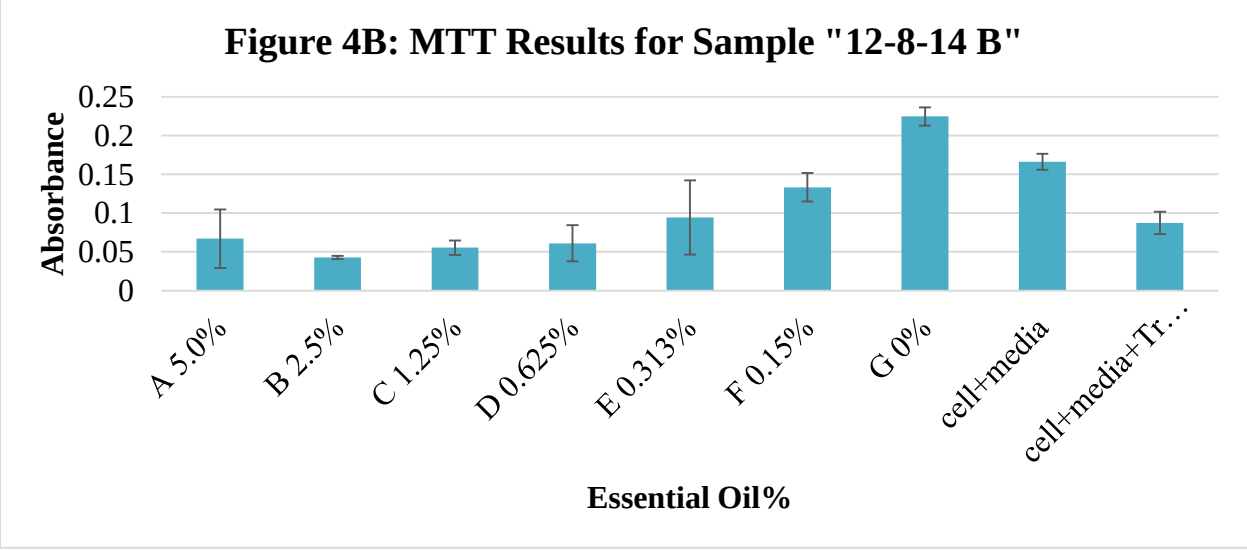
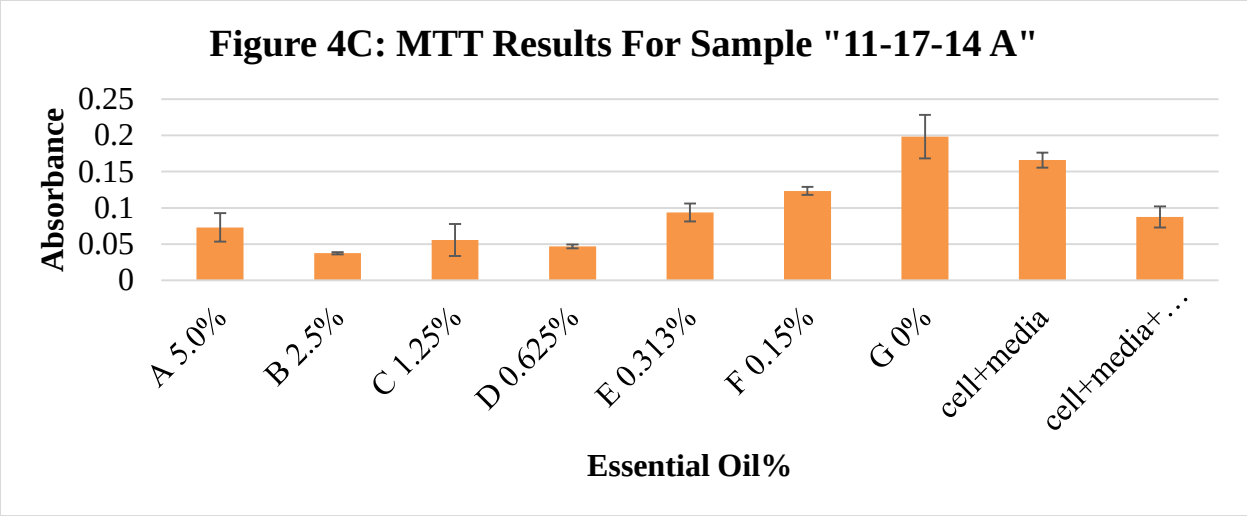
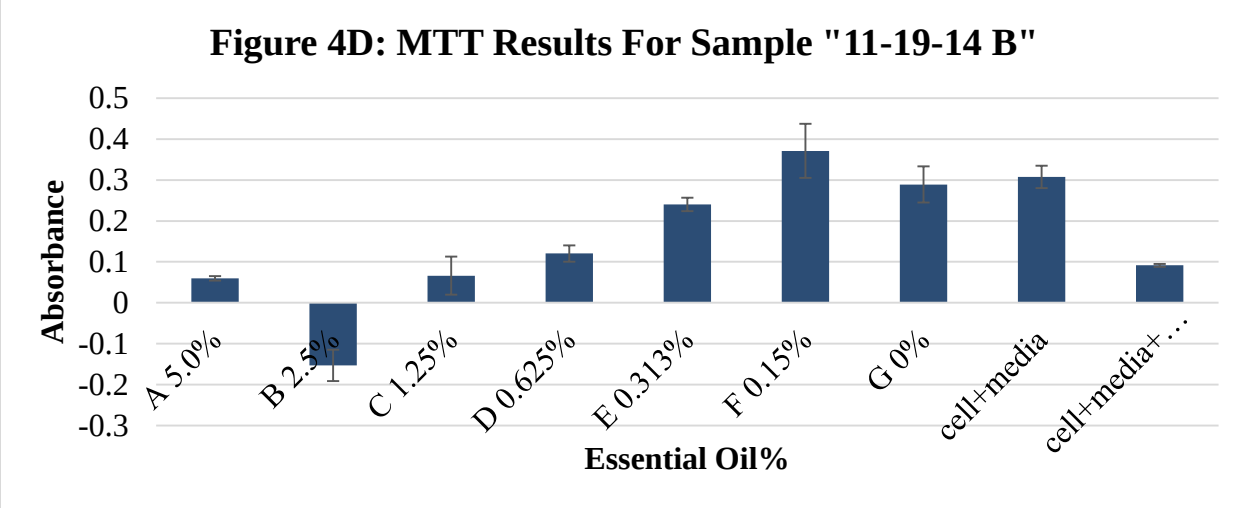


Figure 4. Average absorbance of each well for each sample. Each well contained essential oil samples in varying concentrations. The positive control was "Media" and the negative control was "Triton X". Bars represent absorbances after incubation of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) with Caco-2 cells. Concerning the absorbance, a low absorbance means that the more cells in that cell culture are no longer living or viable. A high absorbance means that cells are able to process the MTT and are thus viable living cells.





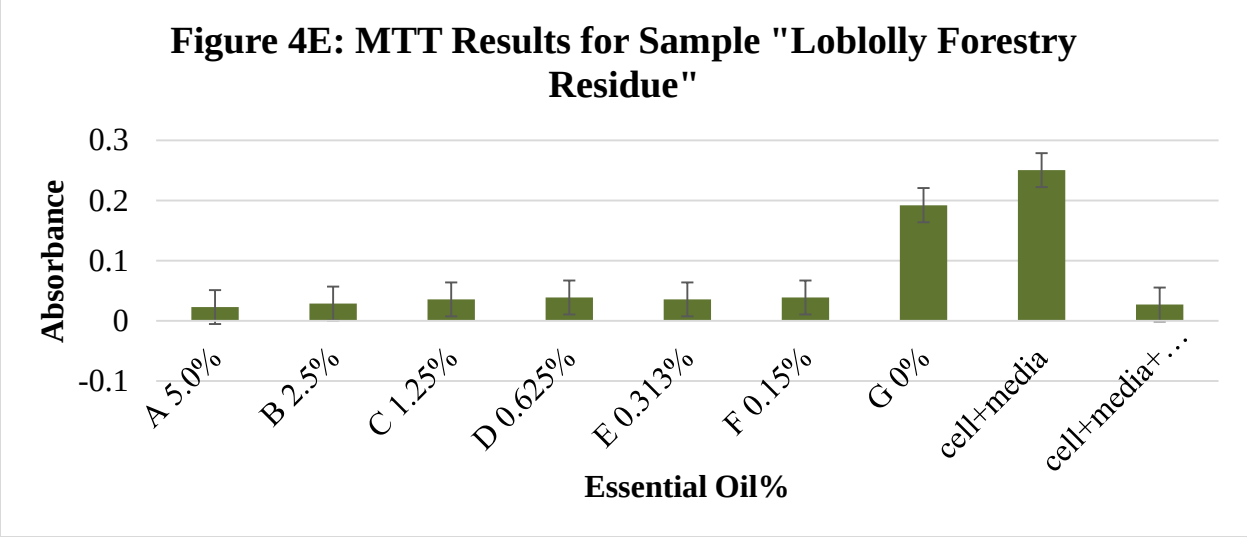


Figure 4 (continued).

For the MTT assay for cell viability, a two way analysis of variance (ANOVA) was used to determine if the sample used or the concentration tested had any significant effect on the cell viability. From the results of the two-factor ANOVA, as shown in table 20, it can be concluded there were significant differences ($P < 0.05$) between both samples and concentrations. Several multiple comparison analyses were used to further elucidate the significant differences between the sample choices and their effects on cell viability. As shown in table 21, the multiple means comparisons tests found that sample L 12-1-14 A had the most effect on the cell viability. The shortleaf pine needle samples, having varying degrees of effect, were not be as effective as the loblolly forestry residue sample, though there was some variation depending on the sample.

Table 20. Results of the two-factor ANOVA for the MTT assay. The dependent variable was the absorbance at 690 nm of the Caco-2 cell culture.

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Sample	4	0.05307	0.01327	3.55600	0.01650
Concentration	8	0.23683	0.02960	7.93400	7.90E-06
Residuals	40	0.11940	0.00373		

Table 21. Results of multiple means comparison tests for the effect of samples on the Caco-2 cell viability. This use of multiple means comparison tests is to mitigate the biases of the specific tests.

Tukey's T-Test			**Fisher's Least Significant Difference Test**			**Duncan's Multiple Range Test**		
Sample	**Means**	**M**	**Sample**	**Means**	**M**	**Sample**	**Means**	**M**
SL 2-2-15 B	0.1634	A	SL 2-2-15 B	0.1634	A	SL 2-2-15 B	0.1634	A
SL 11-19-14 B	0.1546	AB	SL 11-19-14 B	0.1546	AB	SL 11-19-14 B	0.1546	AB
SL 12-8-14 B	0.1035	AB	SL 12-8-14 B	0.1035	AB	SL 12-8-14 B	0.1035	ABC
SL 11-17-14 A	0.0980	AB	SL 11-17-14 A	0.0980	AB	SL 11-17-14 A	0.0980	BC
LL 12-1-14 A	0.0746	B	LL 12-1-14 A	0.0746	B	LL 12-1-14 A	0.0746	C

In order to determine the significant differences between the effects on the concentration on cell viability, several multiple comparison analyses were used. The results are shown in table 22. The multiple comparisons test found 0% concentration had the least effect on the cell

viability and was comparable to the negative control, Media. Concentrations 0.15%, 0.625%, and 5.0% were found to have a cytotoxic effect comparable to that of the positive control, Triton X, but was not considered as have the largest effect on cell viability. The group that caused the largest effect on cell viability was group C (B 2.5% concentration), which is interesting as this was not the highest concentration tested on the cell cultures. This may be due to the essential oil sample having miscibility issues at the highest concentration.

Table 22. Results of multiple means comparison tests for the effect of concentration on the Caco-2 cell viability. This use of multiple means comparison tests is to mitigate the biases of the specific tests.

Tukey's T-Test			**Fisher's Least Significant Difference Test**			**Duncan's Multiple Range Test**		
Sample Conc.	**Means**	**M**	**Sample Conc.**	**Means**	**M**	**Sample Conc.**	**Means**	**M**
Media	0.2332	A	Media	0.2332	A	Media	0.2332	A
G 0%	0.2269	A	G 0%	0.2269	A	G 0%	0.2269	A
F 0.15%	0.1746	AB	F 0.15%	0.1746	AB	F 0.15%	0.1746	AB
E 0.313%	0.1283	ABC	E 0.313%	0.1283	ABC	E 0.313%	0.1283	BC
D 0.625%	0.0808	BC	D 0.625%	0.0808	BC	D 0.625%	0.0808	CD
Triton X	0.0774	BC	Triton X	0.0774	BC	Triton X	0.0774	CD
C 1.25%	0.0717	BC	C 1.25%	0.0717	BC	C 1.25%	0.0717	CD
A 5.0%	0.0612	BC	A 5.0%	0.0612	BC	A 5.0%	0.0612	CD
B 2.5%	0.0153	C	B 2.5%	0.0153	C	B 2.5%	0.0153	D

It was expected that the sample choice and concentration had an impact on the viability of the cells, as the literature has shown that both variables can have an effect. As the sample choice was found to exhibit an effect on cell viability, it can be concluded that the choice of essential oil is an important factor. Similarly, concentration was also found to exhibit an effect on cell viability, leading to the conclusion that the concentration is an important factor. Concerning the use of essential oils, it is important to take into consideration the essential oil toxicity and the various factors related to it. From the study conducted on the essential oils extracted from the *P. taeda* and *P. echinata* biomass, it has been shown that the essential oils have a toxic effect on the

Caco-2 cell line, and from the literature, it was shown that essential oils extracted from a variety of plants had cytotoxic effects on a wide variety of cell lines. As essential oils have little to no mutagenicity, the essential oils are primarily devoid of carcinogenicity (Bakkali et al., 2008), meaning that the *Pinus* essential oils could function as a chemotherapy agent or as an antimicrobial agent. Considering these properties of the *Pinus* essential oils, there is the potential for its use in a hospital setting, in roles such as a disinfectant.

6. CONCLUSION

The added value potential of the essential oil of *Pinus taeda* (loblolly pine tree) residue and *Pinus echinata* (shortleaf pine tree) needles was investigated in this project. The potential added value was to be determined from the medicinal properties of the essential oil extracts of the *P. taeda* (loblolly pine tree) needle residue and *P. echinata* (shortleaf pine tree) needles for medicinal purposes, primarily as a cytotoxic agent capable of antimicrobial or antitumor effects. The essential oils of both the *P. taeda* (loblolly pine tree) needle residue and *P. echinata* (shortleaf pine tree) needles proved to have varying degrees of effectiveness in both these regards.

The *P. taeda* (loblolly pine tree) forestry residue essential oil was an effective cytotoxic agent. Against the Caco-2 cell line (heterogeneous human epithelial colorectal adenocarcinoma), the *P. taeda* essential oil was more cytotoxic, as compared to *P. echinata* essential oil, reaching complete cell culture death at the lowest concentration used, 0.15%. The *P. echinata* essential oil also caused cell death, with effects starting at 0.33%, reaching full effect at 1.25%. As for the antibacterial effectiveness of the essential oils, both showed some degree against cocktails of the four bacterial species: *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella enterica*. Though effective, neither had as strong antibacterial effect as the positive control, 10% streptomycin. It can be concluded from the statistical analysis that the loblolly forestry residue essential oil has the potential to be a strong antibacterial as the diluted streptomycin. However, cell culture death and microorganism inhibition properties indicate that either biomaterial could be processed for potential added value. The loblolly pine tree forestry residue especially shows good potential, considering that the source for the biomaterial used in this study was a waste stream that directly resulted from forestry operations.

Future work should investigate the efficacy of the *P. taeda* residue and *P. echinata* needles against other cancer cell lines and bacterial strains. Additionally, it would be important to individually investigate the constituents of these essential oils and to perhaps determine strong synergistic effects. As the method of storage potential is an issue, a future study on determining proper pine needle storage and its ensuing effect on essential oil quality should be determined.

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8. BIOSAFETY APPROVAL LETTER

UNIVERSITY OF ARKANSAS

Office of Research Compliance

March 16, 2015

MEMORANDUM

TO: Dr. Danielle Julie Carrier

FROM: W. Roy Penney
Institutional BioSafety Committee

RE: IBC Protocol Approval

IBC Protocol #: 15015

Protocol Title: "Testing pine essential oil and sweetgum extract for toxicity using the MTT assay"

Approved Project Period: Start Date: March 12, 2015
Expiration Date: March 11, 2018

The Institutional Biosafety Committee (IBC) has approved Protocol 15015, "Testing pine essential oil and sweetgum extract for toxicity using the MTT assay" You may begin your study.

If further modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572
Fax: 479-575-3846 • http://vpred.uark.edu/199.php