Terpenoid Concentrations and Antimicrobial Characteristics of Tree Resins in the *Pinaceae Family*

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ABSTRACT

The incorporation of plants into medicine is a phenomenon that spans across human history and various cultures. For example, Native Americans commonly used tree resins in a variety of medicines. The purpose of this study was to evaluate the antimicrobial properties of the Pinaceae family by analyzing the chemical concentrations of terpenes. Resin samples were collected from *Abies balsamea, Pinus resinosa* and *Picea glauca*, and applied to cultures of *E. coli*. If resin in the *Pinaceae* family has antimicrobial properties, the *E. coli* population growth should be minimal. This experiment provides inconclusive results pertaining to the antimicrobial effectiveness of resins but includes terpene contents for *A. balsamea, P. resinosa* and *P. glauca* that may prove useful for future research. Our results suggested a need to further investigate how various concentrations of monoterpenes, diterpenes and sesquiterpenes correlate with the antimicrobial characteristics of tree resins.

INTRODUCTION

Tree resin has held a strong significance in ethnobotanical history; globally, cultures found tree resin to be useful in practices of incense and medicines (Pennacchio, 2010). While trees share their gifts and virtues with humanity, they also produce resin as a defense mechanism

(Savluchinske-Feio, 2006). When a tree is damaged, resin is secreted to repair and to seal off wounds from potential intruders. Similarly, humans use resin as a healing substance because of its antimicrobial properties that give it medicinal value (Vilanova, 2014). Much of our understanding of plant pathology is limited due to a lack of professionals within the field. As a consequence, aspects of antimicrobial responses in trees remain poorly understood (Pearce, 1996). Our project aims to better understand and appreciate the antimicrobial qualities that trees in the *Pinaceae* family exhibit.

This experiment focuses on *Pinaceae* tree resin due to its abundance and variety around Pellston, Michigan. The resin of Balsam fir (*Abies balsamea*) has been utilized by Native Americans for its medicinal value (Coté, 2016). More specifically, *A. balsamea* resin is commonly applied to bug bites to reduce swelling and itching. These qualities may be due to antimicrobial terpenoids, such as ⍺-Pinene, commonly found in the resin (Coté, 2016). The other three trees chosen are white spruce *(Picea glauca)*, eastern hemlock (*Tsuga canadensis)*, and red pine *(Pinus resinosa),* all in the family *Pinaceae.* There are studies involving the antimicrobial properties of resin, commonly attributed to the qualitative concentration of ⍺-Pinene, of *A. balsamea* resin. Dissimilarly, there is a lack of information detailing the presence of antimicrobial compounds in the other resins of the other *Pinaceae* trees observed.

In this paper, terpenoid contents are analyzed because terpenoids are commonly identified as defense compounds that combat pathogens and herbivores (Ashour et al., 2010). Terpenoids are grouped together by their universal characteric of being made up of C_3 units into isopentinoid structures. Differentiations between monoterpenes, sesquiterpenes, and diterpenes

arise from the varying composition of isoprenoid units. Monoterpenes, sesquiterpenes, and diterpenes are each composed of two, three, and four isoprenoid units (Ashour et al., 2010).

The premise of this experiment initially revolved around an investigation on one monoterpene, α-Pinene, and how its relative concentrations affect its antimicrobial quality. Upon further chemical analysis of the resins, varying relative concentrations of diterpenes indicated a potential effect on antimicrobial properties. If the relative concentrations of monoterpenes, specifically α-Pinene, and diterpenes are high, the antimicrobial effectiveness should increase.

MATERIALS AND METHODS

All resin collections were made in Reese's Swamp along the border of the Burt and Monroe townships in Michigan as to limit the variance of soil ecology and habitat effects on resin compositions. Coordinates for the *A. balsamea*, *P. resinosa*, *P. glauca* and *T. canadensis* were (45.548393, -84.683215), (45.549284, -84.684234), (45.549918, -84.685057), and (45.544527, -84.682022) respectively.

Sap Collection

A. balsamea resin was collected by inserting a knife into the uppermost part of the bark blisters. Then the lip of a glass vial was pressed against the blisters at a position slightly lower than the knife puncture. The pressure between the glass vial and punctured blisters allowed for the discharge of resin. The same tree was punctured multiple times to provide an adequate amount of resin for GC-MS, and for inoculating tryptic soy agar, or TSA, plates. Collected resin was then capped to prevent its solidification.

P. glauca, *T. canadensis*, and *P. resinosa* resin was collected by cutting into the cambium of the trees. A rubber mallet was used to strike a machete into the outer bark. These incisions were made at a forty-five degree angle, in relation to the direction of the tree trunk, in the shape of a "v;" each incision was approximately six inches long (Hunter, 2019). Resin was collected by tying a glass vial with an elastic band to the southernmost tip of the v-shaped incision on each tree. In addition, tinfoil was shaped into a funnel to direct the flow of resin into the test tube. Test tubes were then collected 24 hours after setup and capped to prevent resin solidification.

Inoculating the *E. coli*

Once all sap samples were collected, three TSA plates were streaked with a flame sterilized inoculating loop: the loop was lightly run on the *E. coli* culture and then streaked along a TSA agar plate, rotated 90 degrees, streaked once more, rotated 45 degrees, and then streaked a final time. The newly streaked plate was then marked into halves with permanent marker; one half acted as a control and the other half received an application of one of the *Pinaceae* saps by inoculation loop streaking. A different loop was used for each plate and was sterilized before and after every step. Sterilization was executed by dipping loops into 70% ethyl alcohol followed by holding the loops over a flame for five seconds. All three plates were then left in an incubation chamber for 24 hours.

Creating Streak Plates

24 hours allowed for the growth of bacteria in the incubation chamber. After 24 hours an inoculating loop was used to swab the resin-treated regions of each agar. A clean agar was then swabbed and allowed to incubate for 24 hours. A quarter of the plate was streaked and the loop was sterilized. The loop was then streaked twice into the first quarter and used to streak the full

surface of the following quarter. This process was repeated until all quarters of the three plates were fully streaked, each successive quarter having a diluted concentration of the initially streaked substances. The plates were then labeled and incubated for 24 hours.

GC-MS

Gas Chromatography-Mass Spectrometry, or GC-MS, was used to determine the relative abundances of monoterpenes, sesquiterpenes, and diterpenes in *Pinaceae* resin and to synthesize this information with how each resin performed in our experimental trials.

RESULTS

The results from GC-MS analysis reveal the qualitative amounts of each terpene in the samples. Figure I shows the chart of the relative concentration of four large peaks for monoterpenes on the left, small peaks in the middle for sesquiterpenes, and many large peaks on the right for diterpenes. The plates display *E. coli* growth on resin-treated plate. The GC-MS analysis and plate for *T. canadensis* was not included because little to no sap was collected.

GC-MS Analysis of Monoterpenes

GC-MS analysis showed *P. resinosa* had the highest percentage of Monoterpenes (69.77%), *A. balsamea* had the second highest (51.82%) and *P. glauca* had the lowest (35.38%).

GC-MS Analysis of Sesquiterpenes

GC-MS analysis showed *A. balsamea* had the highest percentage of Sesquiterpenes (3.67%), *P. glauca* had the second highest (1.69%), and *P. resinosa* had the lowest (1.29%).

GC-MS Analysis of Diterpenes

GC-MS analysis showed *P. glauca* to have the highest percentage of Diterpenes (62.92%), *A. balsamea* had the second highest (44.5%) and *P. resinosa* had the lowest (28.93%).

Microbiological analysis

The streak plate with *P. resinosa* showed the first two quadrants with *E. coli* and minimum growth on the last two quadrants (Figure II)

The streak plate with *P. glauca* grew *E. coli* in all four quadrants (Figure III).

The streak plate with *A. balsamea* grew, over 24 hours, *E. coli* in the first two quadrants with possible contamination in the third and fourth (Figure IV).

The streak plate with *A. balsamea* grew, over 48 hours, *E. coli* in all four quadrants (Figure V)

DISCUSSION

Our project sought to better understand the usage and effectiveness of *Pinaceae* resin as an antimicrobial substance. The reasons listed below highlight the mistakes that aided in the uncertainty as illustrated in the agar plates.

Sap collection

T. canadensis resin was not tested on *E. coli* because too little resin was collected. Also, the team strived to direct minimal damage to the trees by executing minimal lacerations to each individual organism that was collected from.

The workspace provided for this project was the Reichard building, which is not a sterile room nor a fully functioning microbiological laboratory. This certainly could have led to having

some potential contamination in our work and made it difficult to execute the procedure correctly.

Effect of *A. balsamea, P. glauca and P. resinosa* on *E. coli*

E. coli grew significantly in the presence of *A. balsamea* resin over a forty-eight hour time period but the results of this initial plating have been deemed inconclusive due to lab errors (Figure V). The first error was waiting forty-eight hours instead of the intended hours to check on *E. coli* growth. The second error made was not sterilizing the inoculating loop between each quarter streak. To make up for errors, a new streak plate was treated with *E. coli* and *A. balsamea* resin. The *A. balsamea*, *P. resinosa* and *P. glauca* plates, incubated for 24 hours, reveal *E. coli* growth and inconclusive results on terpene levels and antimicrobial quality.

It was anticipated that *A. balsamea* resin would be the most effective based on previous work revealing its antimicrobial properties. While *A. balsamea* resin ranked only second for monoterpenes, it ranked first in both sesquiterpenes and diterpenes. These have been found to have the most antimicrobial properties for Gram-positive bacteria whereas monoterpenes are most active against fungi (Himejima, 1992). The initial design of this experiment included the use of *S. aureus*, a Gram-positive bacteria, instead of *E. coli*, a Gram-negative bacteria. However, *S. aureus* was an unavailable option to work with in this project. A study done in Canada tested the antibacterial properties of *A. balsamea* on *E. coli* and *S. aureus* which showed the resin to be inactive against *E. coli* but active against *S. aureus* (Coté, 2016). If *A. balsamea*, which is similar to other trees in *Pinaceae*, is inactive in fighting *E. coli*, then better results would have been observed with the usage of *S. aureus*, revealing more information on the connection between terpene levels and antimicrobial effectiveness.

GC-MS Data Interpretation

Originally, this study looked at relative concentrations of α-Pinene through GC-MS and to use this as grounds to investigate whether or not these concentrations correlated with how well the resin of interest can kill *E. coli*. Due to the lack of useful qualitative microbiological data, this paper instead focuses on a generalized assay of the terpenoid contents so that the differences in concentrations could provide a foundation for future, better-executed experiments. Our results were inconclusive with multiple plates being incorrectly inoculated, making true comparisons impossible to achieve.

In progressing versions of the same experiment, it would be more effective to use *S. aureus* or another Gram-positive bacteria instead of *E. coli.* A larger spread of resin could be applied to the agar in order to avoid contamination picked up from surrounding, untreated bacteria by flakes of solidified resin. Future iterations of the experiment would require proper setting -- a sterile microbiology lab with proper facilities -- and a properly sterile collection method, especially concerning the machete, knife, resin gutters, glass vials and so on. If the experiment were to be redone using the analytical data obtained and the proper microbiological procedures, it is quite likely that more useful data will be obtained.

CONCLUSION

The antimicrobial effectiveness of *A. balsamea*, *P. resinosa*, and *P. glauca* resin is somewhat inconclusive. As highlighted above, *E. coli* still grew in the presence of *A. balsamea*, *P. resinosa*, and *P. glauca* resin but the methods used to inoculate plates could have led to unintended bacterial growth. A redesign of this experiment might include better inoculation

techniques, the use of a Gram-positive bacteria, and a control plate to compare resin-treated *E. coli* and untreated *E.coli*. Otherwise, the terpene content of *A. balsamea*, *P. resinosa*, and *P. glauca* resin could be useful indexes of *Pinaceae* resin oriented experimentation.

FIGURES AND TABLES

Figure I: GC-MS chart for *P. glauca*, *P. resinosa*, and *A. balsamea*

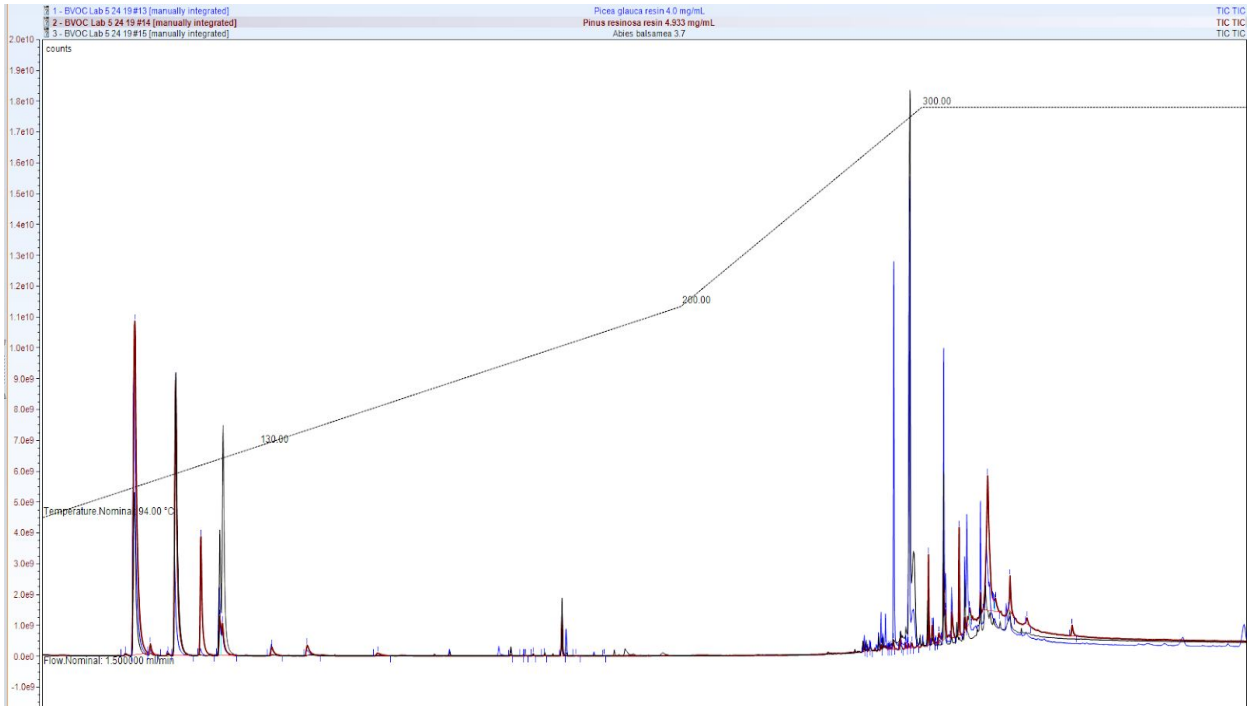


Figure II: Streak plate with *P. resinosa*

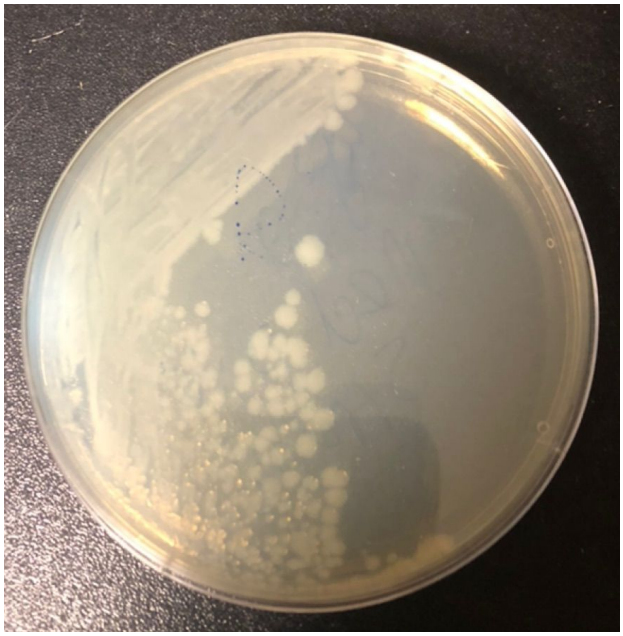


Figure III: Streak plate with *P. glauca*

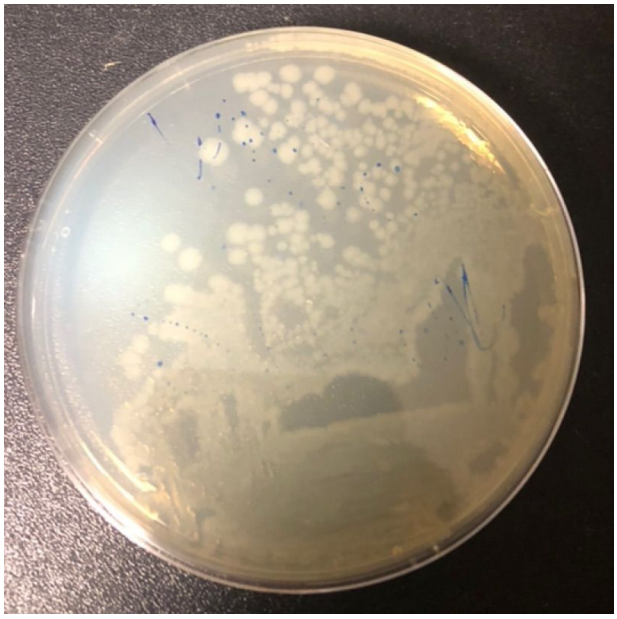
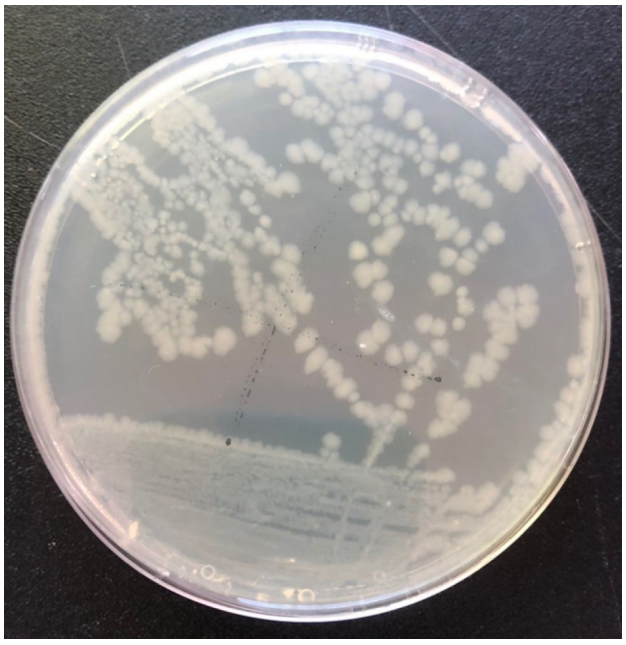


Figure IV: 24 hour streak plate with *A. balsamea*

Figure V: Twenty-four hour streak plate with *A. Balsamea*



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