Characterization and Variation of Essential Oil from *Pinus taeda* and Antimicrobial Effects against Antibiotic-Resistant and -Susceptible *Staphylococcus aureus*

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Abstract

Essential oils from forestry by-products, such as pine needles, have antimicrobial effects against pathogenic bacteria. Pine needle essential oils inhibit growth of *Staphylococcus aureus,* which is the causative agent for numerous human infections, ranging from superficial skin infections to deep abscesses and infections that are more serious. Crude essential oils from pine needles were proposed as a topical antimicrobial agent against both susceptible and methicillin-resistant strains of *S. aureus*. A Clevenger apparatus was used to extract essential oil from needles from a single clone of young loblolly pine (*Pinus taeda* L.). By gas chromatography–mass spectrometry analysis, it was determined that the major components of the oil were α-pinene (0.52 to 1.02 mg g^{-1}), β-pinene (0.04 to 0.67 mg g^{-1}), limonene (0.00 to 0.06 mg g^{-1}), terpineol (0.01 to 0.18 mg g^{-1}), and (–)-caryophyllene (0.02 to 0.52 mg g^{-1}), with quantities depending on sampling dates. Results demonstrated that the essential oils had antimicrobial activity against four *S. aureus* strains.

Hydrodistillation of pine needles from various pine species yields essential oils (EO), with the major components being α-pinene, sabinene, myrcene, β-caryophyllene, aromadendrene, and α-humulene in *Pinus halepensis* Mill. (Dob et al. 2005) and α-pinene, β-pinene, 3-carene, estragole, α-carinol, limonene, β-phellandrene, τ-cardinol, τ-muurolol, myrcene, and α-terpineol in small ponderosa pines (*Pinus ponderosa* Lawson; Kelkar et al. 2006). EO derived from hydrodistillation of pine needles are reported to have antimicrobial activity against various bacterial pathogens such as gram-positive *Staphylococcus aureus* and *Bacillus subtilis,* but not against gram-negative *Escherichia coli, Salmonella* Typhi, or *Enterobacter aerogenes* (Zafar et al. 2010). *S. aureus* is the causative agent for numerous human infections, ranging from superficial skin and wound infections to deep abscesses, food poisoning, and more serious infections, such as septicemia, urinary tract infections, osteomyelitis, and endocarditis (Archer 1998). These may become more serious and life threatening due to *S. aureus* strains that are resistant to methicillin and other β-lactam antibiotics, termed methicillin-resistant *S. aureus* (MRSA) strains (Novick 2008).

The state of Arkansas has approximately 18 million acres in timberland, of which 29 percent is pine. Loblolly pine (*Pinus taeda* L.) is both the dominant overall species and the pine species most harvested for timber, at 423.6 million ft^3/y (Rosson and Rose 2005). Usually loblolly pine is grown in

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Forest Prod. J. 64(5/6):161–165.
doi:10.13073/FPJ-D-14-00018

managed forests, located mainly in counties west of the Bayou Bartholomew to the western border of the state, then north to the Ouachita Mountains. Therefore, potential use of residue in the form of pine needles from standard forestry practices is plentiful and available for EO extraction. The objective of this study was to prepare EO from pine needles, to analyze and quantify major components as a function of sampling dates, and to determine whether these EO deter MRSA strain growth.

Materials and Methods

Plant material

Pine needles of juvenile, second growing season, loblolly pine trees were obtained from an established plot at the University of Arkansas at Monticello Teaching and Research Forest in Drew County, Arkansas (34°03′83″N, 92°22′22″W). The site previously had a 55-year-old stand of pine trees. The tree stand was destroyed during a tornado, and the site was cleared and planted with juvenile pine clones in January 2012. Mean annual precipitation in this area is 1,138 to 1,600 mm, and average temperatures recorded on the site for March, April, May, June, July, August, and September were 15.6°C, 20.0°C, 24.0°C, 26.6°C, 26.2°C, 18.8°C, and 21.7°C, respectively. Soil was predominantly a Calloway silt loam.

Biomass was harvested by excising needles at the base of the needle fascicle on the main lateral limbs. Needles were transferred to a portable cooler in the field and subsequently stored in 4°C in the laboratory until shipping on ice to the Department of Biological and Agricultural Engineering in Fayetteville, Arkansas. Biomass collections occurred on March 18 to 19, April 18 to 19, May 15 to 16, June 17 to 18, July 15 to 16, August 15 to 16, and September 16 to 17, 2013. While the site contained several genotypes, only needles from CellFor Variety 1 (germplasm owned by Arborgen) were used, to ensure that genetic effects would not confound any trends in environmental or temporal changes.

Standards

For reference standards we used α-pinene, 99 percent pure, 0.85 g mL^{-1} density (Acros Organic, Somerville, New Jersey); β-pinene, 97 percent pure, 0.86 g mL^{-1} density (Alpha Aesar, Ward Hill, Massachusetts); terpineol, 90 percent pure, with a density of 0.93 g mL^{-1} (Spectrum Chemical Manufacturing Corp., New Brunswick, New Jersey); limonene, 97 percent pure, 0.842 g mL^{-1}; and (–)-*trans*-caryophyllene, 98.5 percent pure, 0.902 g mL^{-1} (Sigma Aldrich, Milwaukee, Wisconsin).

Extraction

Needle moisture content was determined using a MB45 Moisture Analyzer (Ohaus Corporation, Pine Brook, New Jersey). Essential oils were extracted from the needles by hydrodistillation using a Clevenger-type apparatus (Pyrex, Corning Life Sciences, Kennebunk, Maine) as described by Ennajar et al. (2011). Needles that were extracted were harvested from the trees in mid-April, mid-May, mid-June, mid-July, and mid-September. Briefly, 50 g of fresh pine needles, cut to approximately 0.5 cm in length, were added to a 500-mL round-bottom flask containing 200 mL of 20 percent (wt/wt) aqueous NaCl in order to increase the boiling temperature of the water. A flask was set up with a Clevenger apparatus and heated to boiling point of water with an 85-V setting on the Reostat (Variac Transformer, Cleveland, Ohio) for 4 hours. Afterward, EO were decanted into 2-mL amber vials and stored in the dark at room temperature.

Chemical analysis

The gas chromatography–mass spectrometry (GC-MS) analysis of standards (diluted in hexane up to 10^{-5}) and samples (diluted in hexane up to 10^{-3}) were carried out on a Varian 320-MS triple quad mass spectrometer with an electroionization (EI) source coupled to a Varian 450-GC gas chromatograph (Bruker Daltonics, Billerica, Massachusetts) with a CombiPal autosampler. The column used was a Phenomenex Zebron ZB-5HT Inferno capillary GC column (30-m length, 0.25-mm inside diameter, 0.25 μm; Torrance, California). Working conditions included a split method (ratio, 1:10), with an injector temperature of 240°C and a source temperature of 200°C; oven temperature program of 50°C held for 3 minutes, 50°C to 200°C at 10°C min^{-1}, and held for 5 minutes, as modified from Ennajar et al. (2011). Standard dilutions of the reference compounds α-pinene, β-pinene, D-limonene, (–)-*trans*-caryophyllene, and terpineol, respectively, were included in each GC-MS run, and calibration curves were used for calculation of the stated standards.

Antimicrobial analysis

To determine the antimicrobial activity of the extracted oils, a disc diffusion assay was used. Three strains of methicillin-resistant *S. aureus* (COL, N315, and a clinical isolate kindly provided by Dr. Dave Gilmore at Arkansas State University, Jonesboro) and one vancomycin intermediate resistant *S. aureus* strain (13136 p–m+; Sabath et al. 1974, Brown and Reynolds 1980, Kuroda et al. 2001) were maintained on tryptic soy agar (TSA; Becton Dickinson, Franklin Lakes, New Jersey). For each assay, one colony from each respective *S. aureus* strain was added to 5 mL of tryptic soy broth (TSB; Becton Dickinson) and incubated at 37°C for 24 hours with shaking at 150 rpm. Inocula were prepared by diluting the 24-hour culture in sterile TSB to achieve a final concentration of 10^6 colony-forming units (CFU) per mL. For the disc diffusion assay, petri dishes containing TSA were inoculated with 100 μL of the respective *S. aureus* inoculum. Sterile, 6-mm blank paper discs (Becton Dickinson) were aseptically placed on the TSA plate, and 10 μL of extracted oil was added to each disc and allowed to absorb for 30 minutes. A blank paper disc with no inoculum was added as a negative control for each strain of *S. aureus* because undiluted extracted oils were used as opposed to diluting them in a solution or buffer. The TSA plates were inverted and incubated at 37°C for 24 hours. The growth inhibition zone (IZ) diameters were measured and recorded. All experiments were repeated in duplicate.

Statistical analysis

Essential oil compound concentrations, or percentages, were analyzed with analysis of variance and Dunnett's control test for multiple comparisons in JMP 10 (SAS Institute, Cary, North Carolina). Significance was established at $P < 0.05$.

Results and Discussion

Loblolly pine needles, harvested in mid-April, mid-May, mid-June, mid-July, and mid-September, were extracted. Pine needle EO, extracted by hydrodistillation, were analyzed by GC-MS; a representative chromatogram is presented in Figure 1. Retention times of α-pinene, β-pinene, D-limonene, terpineol, and (–)-caryophyllene were 7.1, 7.95, 8.86, 11.63, and 14.9 minutes, respectively (Figure 1). Identified compounds and their corresponding percentages, detected through the MS library, for mid-April, May, June, July, and September EO extracts are presented in Table 1. The total percent content of α-pinene, β-pinene, D-limonene, terpineol, and (–)-caryophyllene, added together, in EO for April, May, June, July, and September samples were 67.3, 67.7, 69.4, 67.5, and 75.5 percent, respectively (Table 1). Park and Lee (2011) reported the percentages of compounds in EO from *Pinus densiflora* and *Pinus thunbergii,* grown in South Korea. Of the 30 listed compounds, the following had the highest percentages: α-pinene (20.58% to 10.91%), camphene (22.38% to 2.86%), β-pinene (6.73% to 3.96%), β-myrcene (1.09% to 2.51), δ-3-carene (4.36% to 16.77%), α-terpinene (0.9% to 4.22%), β-phellandrene (0.00% to 13.36%), limonene (20.16% to 0.00%), terpineol (0.33% to 5.35%), and bornyl acetate (9.79% to 0.68%). Caryophyllene was not included in the list reported by Park and Lee (Park and Lee 2011). In *P. ponderosa,* percentages that were above 1 percent, as reported by Kelkar et al. (2006), included β-pinene (45.7%), α-pinene (10.2%), 3-carene (8.4%), estragole or methyl chavicol (8.0%), α-cadinol (2.7%), limonene combined with β-phellandrene (2.4%), τ-muurolol (2.0%), myrcene (1.4%), and α-terpineol (1.4%). Dob et al. (2005) reported that EO extracted by hydrodistillation of *P. halepensis* needles contained 41 major compounds, including β-caryophyllene (40.31%), α-humulene (7.92%), aromadendrene (7.1%), myrcene (3.07%), and α-pinene (1.23%). Koukos et al. (2000), listed α-pinene (23.07%), β-pinene (22.00%), citronellol (13.42%), bornyl acetate (9.76%), β-phellandrene (6.78%), camphene (5.52%), and β-caryophyllene (3.05%) as extracted compounds from pine needles (*Pinus peuce* Grisebach) using hydrodistillation. Results presented in Table 1 indicate that the EO composition of needles from *P. taeda* in our research have similarities to data from other pine species.

There were variations among compound percentages as a function of sampling date (Table 1). However, percentages of α-pinene, terpineol, and (–)-caryophyllene remained constant as a function of sampling date. Throughout the sampling dates, proportions of β-pinene and D-limonene significantly increased, while that of α-cadinol significantly decreased. Overall, although not dramatic, EO composition does change as a function of sampling date (Table 1). Previous reports also presented variations of percentages of compounds as a function of sampling date: *Eucalyptus* species (de Oliveira et al. 2008, El Zalabani et al. 2008, Sefidkon et al. 2010), *Zanthoxylum clava-herculis* (Eiter et al. 2010), *Citrus aurantium* L. (Boussaada and Chemli 2007), *Juniperus excelsa* M.B. (Salehi and Mirza 2006), *Juniperus phoenicea* L. (Ennajar et al. 2011), *Liriodendron tulipifera* (Miller et al. 2009), and *Juniperus scopulorum* (Cupressaceae; Powell and Adams 1973, Adams 2012). Specifically, Papadopoulou and Koukos (1996) reported that hydrodistillation of needles from Balkan pine (*P. peuce*) grown in northern Greece exhibited percentage variations of EO when the needles were harvested in the autumn compared with spring. Park and Lee (2011) suggested that variations in EO extraction yields of *P. densiflora* and *P. thunbergii* as a function of sampling dates were due to a range of factors, including environmental, tree species, soil types, and distillation techniques.

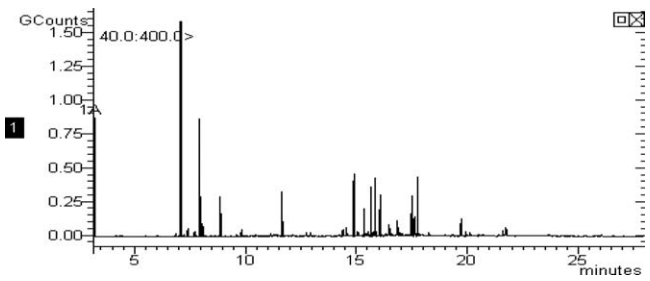

Figure 1.—Gas chromatography–mass spectrometry chromatogram of volatile oil recovered from Arborgen clone needles from June 2013 harvest. Retention times of α-pinene, β-pinene, α-limonene, terpineol, and caryophyllene were 7.1, 8.0, 8.9, 11.6, and 14.9 min, respectively.

Recovery, quantified in milligrams per gram, of α-pinene, β-pinene, α-limonene, terpineol, and (–)-*trans*-caryophyllene was monitored in April, May, June, July, and September 2013 samples (Table 2). From April to September, α-pinene and β-pinene decreased as a function of time, with September samples containing the lowest concentrations. However, although α-pinene, β-pinene, and (–)-*trans*-caryophyllene concentrations were considerably lower, limonene and terpineol concentrations were almost nondetectable in July and September samples. The highest concentration of EO (milligrams per gram) occurred in the spring month of April (Table 2). Similar outcomes were noted in EO from the leaves of *Taxodium distichum* as reported by Adams (2012) where the quantity of oils increased in April to May and decreased soon after leading into summer and fall. These results, although from a different tree species than pine, are interestingly comparable since the geographical locations have similar latitudes, with Waco, Texas, at 31.55°N and Monticello, Arkansas, at 33.62°N.

EO from June pine needles were tested for inhibiting the growth of *S. aureus* strains 13136, N315, COL, and ASU 56. *P. taeda* EO was most effective against strain 13136—an intermediate MRSA strain—while exhibiting no effect on MRSA strain ASU 56. Zafar et al. (2010) reported that EO from *Pinus roxburghii* obtained by hydrodistillation had antimicrobial activity against *S. aureus* and *B. subtilis,* but not against *E. coli, Salmonella* Typhi, or *E. aerogenes*. The major composition of the EO reported by Zafar et al. (2010) was α-pinene 29.3 percent, caryophyllene 21.9 percent, 3-carene 14.2 percent, and α-terpineol 4.5 percent. Testing showed that some of the standard components of pine oil, α-pinene and β-pinene, were effective against various strains of *Staphylococcus*: *S. aureus, S. epidermidis, S. pyogenes,* and *S. pneumonia* (Leite et al. 2007). Results presented in Tables 1 and 2 indicate that EO prepared in this work had similar composition, thus supporting biological activity.

The components α-pinene and β-pinene have been previously reported to display antimicrobial activity against MRSA strains. Chao et al. (2008) tested various commercially available water-distilled EO preparations for their

Table 1.—Analysis of compounds from hydrodistillation of pine needles from mid-April, mid-May, mid-June, mid-July, and mid-September 2013 harvest of the Arborgen clone.[a]

Compound	RT (min)	Avg. (%)				
		Mid-Apr	Mid-May	Mid-Jun	Mid-Jul	Mid-Sep
α-Pinene	7.1	41.51 ± 0.23	37.30 ± 1.68	36.61 ± 2.14	40.30 ± 1.54	42.48 ± 1.03
Camphene	7.4	1.15 ± 0.09	*	*	*	0.74 ± 0.49
β-Pinene	7.95	13.31± 0.34	12.65 ± 0.53	14.32 ± 0.84	11.96 ± 0.46	14.70 ± 0.22
β-Myrcene	8.08	1.24 ± 0.07	1.05 ± 0.93	0.97 ± 0.75	*	1.18 ± 0.07
D-Limonene	8.86	1.55 ± 0.26	2.97 ± 0.84	4.34 ± 0.25	1.83 ± 0.08	4.90 ± 1.38
β-Phellandrene	8.89	*	2.03 ± 0.11	1.64 ± 0.13	1.69 ± 0.12	1.75 ± 0.39
Terpineol	11.63	2.78 ± 2.25	4.49 ± 0.39	7.14 ± 0.72	3.52 ± 0.72	6.16 ± 1.26
Caryophyllene	14.9	8.09 ± 0.78	10.23 ± 0.56	7.64 ± 0.36	9.88 ± 0.56	7.24 ± 1.02
α-Caryophyllene	15.32	1.48 ± 0.41	0.84 ± 1.45	1.96 ± 0.23	2.31 ± 0.10	1.69 ± 0.20
Germacrene D	15.65	3.00 ± 1.45	6.85 ± 0.36	4.57 ± 0.25	5.19 ± 0.24	1.80 ± 0.64
Bicyclogermacrene	15.84	3.72 ± 1.22	7.42 ± 0.38	6.16 ± 0.31	6.77 ± 0.25	1.81 ± 0.79
β-Caryophyllene oxide	16.95	2.55 ± 0.06	*	*	*	1.99 ± 0.55
τ-Muurolol	17.56	4.30 ± 0.31	4.95 ± 0.30	3.57 ± 0.31	3.88 ± 0.32	2.36 ± 0.12
α-Cadinol	17.77	5.87 ± 0.53	6.39 ± 0.33	5.95 ± 0.39	5.12 ± 0.38	2.85 ± 0.21
Total (%)		90.55	97.17	94.87	92.45	91.65

[a] Retention times and percentages reflect the chromatograph of the gas chromatography–mass spectrometry run. Percentages listed are the total percentages of essential oils extracted. Compounds below the range for integration are indicated with an asterisk.

Table 2.—α-Pinene, β-pinene, limonene, terpineol, and caryophyllene content in essential oils of pine needles from Arborgen clones, grown at the University of Arkansas, Monticello.

2013 harvest	Avg. (mg g^{-1})					Total
	α-Pinene	β-Pinene	Limonene	Terpineol	Caryophyllene	
Mid-Apr	1.02 ± 0.91	0.39 ± 0.36	0.03 ± 0.02	0.11 ± 0.09	0.29 ± 0.29	1.84
Mid-May	0.49 ± 0.01	0.33 ± 0.01	0.05 ± 0.00	0.05 ± 0.01	0.31 ± 0.11	1.23
Mid-Jun	0.46 ± 0.06	0.42 ± 0.02	0.06 ± 0.00	0.10 ± 0.01	0.41 ± 0.10	1.45
Mid-Jul	0.08 ± 0.01	0.06 ± 0.01	0.00 ± 0.00	0.01 ± 0.00	0.39 ± 0.06	0.54
Mid-Sep	0.05 ± 0.02	0.04 ± 0.02	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.13

antimicrobial actions against MRSA and reported that products from lemongrass (*Cymbopogon citratus*), lemon myrtle (*Backhousia citriodora*), mountain savory (*Satureja montana*), cinnamon (*Cinnamomum verum*), and melissa (*Melissa officinalis*) inhibited MRSA growth. Moreover, Pepeljnjak et al. (2005) suggested that EO from dried Juniper berries (*Juniperus communis* L.) inhibited growth of various gram-positive bacteria, including *S. aureus* ATCC 6538; *S. epidermidis*; select gram-negative bacteria, such as *Salmonella* Enteritidis and *E. coli*; select yeasts and yeast-like fungi, *Candida, Cryptococcus, Geotrichum,* and *Hansenula* species; as well as reported *Dermatophytes, Microsporum,* and *Trichophyton* species. Interestingly, the major components of *J. communis* EO were α-pinene (29.17%), β-pinene (17.84%), sabinene (13.55%), limonene (5.52%), and myrcene (0.33%), which has similarities to the composition of *P. taeda* EO presented in Table 1.

Table 3.—Growth inhibition of pine needle essential oils against three methicillin-resistant Staphylococcus aureus *strains and one intermediate strain (13136).*

S. aureus strain	Avg. IZ in mid-Jun (mm)[a]
13136	2 ± 0.0
N315	1 ± 0.0
COL	1.25 ± 0.35
ASU 56	0 ± 0.0

[a] IZ = zone of inhibition.

In the present study, we were able to limit confounding issues such as genetic variability by using a single clone and were able to control environmental conditions by growing the clones within a specific area, enabling conclusions to be drawn solely on seasonal variations. Results presented in Table 2 indicate that EO yields decreased as a function of season, being the highest in April needles. Albeit minimal, results presented in Table 3 show that EO inhibited the growth of some MRSA strains. In addition, minimal inhibitory concentrations (MIC) were not provided in this work because the volumes of EO produced were too small to carry out the necessary experiments; however, this will be investigated in future work.

Conclusions

Future work should test spring-produced EO for its inhibition potential, possibly leading to the production of larger inhibition zones. Barring pine needle collection logistics, EO could be produced from loblolly pine needles and could serve as an additional revenue stream to existing forestry operations. Most likely, spring would be the desirable time to harvest and process the pine needles because highest yields were obtained with this material. Thus, current southeastern US forestry infrastructure could

foster the production of EO for natural antimicrobial purposes, supplementing existing forestry operations.

Acknowledgments

This work would have not been possible without the support from the Arkansas Biosciences Institute, Division of Agriculture. The authors acknowledge support from the Department of Biological and Agricultural Engineering and Food Sciences at the University of Arkansas. The authors also acknowledge the Plant Powered Production (P3) Center. P3 is funded through the RII: Arkansas ASSET Initiatives (AR EPSCoR) I (EPS-0701890) and II (EPS-1003970) by the National Science Foundation and the Arkansas Science and Technology Center. The authors would like to thank Shakuntala Sharma, graduate student, for collecting needle samples and Dr. Chantal Bergeron for scientific guidance. Lastly, the authors wish to thank Dr. Jack Lay of the Arkansas Statewide Mass Spectrometry Facility, where the GS/MS research was performed.

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