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Article

Leptospermum scoparium (Mānuka) and *Cryptomeria japonica* (Sugi) leaf essential oil seasonal chemical variation and their effect on antimicrobial activity

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Abstract: This study evaluated the antimicrobial activity of *Leptospermum scoparium* (Mānuka) and *Cryptomeria japonica* (Sugi) essential oils and assessed the effect of seasonal chemical variation on the oils' antimicrobial efficacies. Plate based assays were conducted to elucidate the oils' spectrum of *in vitro* antimicrobial activity and to determine the oils' minimum inhibitory concentrations (MIC) as a measure of antimicrobial efficacy. Gas chromatography – mass spectrometry was adopted to chemically profile oils distilled in different seasons. The resultant compositional information in conjunction with MIC data was used to evaluate the effect of seasonal variation on the oils' antimicrobial efficacy. Both Mānuka and Sugi essential oils were active against all classes of target microorganisms. However, limited activity was observed against Gram-negative bacteria. The oils displayed consistent chemotypic characteristics regardless of the time of distillation. Nonetheless, there were quantitative differences in compound abundance in both essential oils. Significant differences in the MIC of Sugi essential oil was observed against target microorganisms as a result of seasonal variation in constituent abundances while Mānuka essential oil's antimicrobial efficacy was unaffected. This study demonstrates that seasonal chemical variation is an important quality assurance parameter to consider for future application of essential oils as antimicrobial agents in consumer products.

Keywords: *Leptospermum scoparium*; *Cryptomeria japonica*; Mānuka; Sugi; Essential oils; Natural products; Secondary metabolites; Antimicrobials; Gas chromatography – Mass spectrometry

1. Introduction

Natural products derived from plants and microorganisms are important sources of bioactive compounds with potential applications in many industries. Currently, much effort is focused on the discovery of pharmaceutically relevant natural products that are typically active against human pathogens with low minimum inhibitory concentrations (MICs) or those useful for curing human diseases. However, there is a significant opportunity for application of bioactive natural products beyond the medical industry. For example, there is growth in the use of essential oils (EO) in aromatherapy and an increasing effort within the agrochemical industry to replace existing biocidal agents with "greener" alternatives [1–3].

Essential oils are a complex mixture of low-molecular-weight metabolites extracted from various plant tissues (e.g. leaves, branches, wood, and bark). Essential oil metabolites belong to a range of chemical classes mostly consisting of terpenoids, alcohols, aldehydes, and ketones. Typically, a few oil constituents are at high concentrations representing the majority of the oil proportion while many more are present in very low or trace amounts [3,4]. They are often referred to as secondary metabolites, which serve a variety of biological functions to control and regulate their immediate environment (e.g. attract pollinators, repel predators, plant communication, inhibit seed germination, etc.). Beyond biological functions, EOs possess other useful bioactive properties with potential

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applications in nutritional, pharmaceutical, cosmetic and health industries to name a few. Given the growing interest in research and application of EOs, there is a need to evaluate variation in oil chemistry especially in commercial sectors that require quality assurance for consistent product performance.

Leptospermum scoparium, commonly known as Mānuka, is an indigenous New Zealand plant of the myrtaceae family. The antibacterial and antifungal properties of Mānuka essential oil – often associated with its triketone content, have garnered international interest in its potential application in consumer products [5]. *Cryptomeria japonica*, commonly known as Japanese Sugi pine, is a monotypic genus of pinophyta indigenous to Japan. They are often planted around farms to create shelter lines, which increase pasture productivity by creating favorable microclimates for crop and stock [6]. While Sugi essential oil possess both antibacterial and antifungal properties, there are limited applications that take advantage of its bioactive properties [7,8]. In addition, due to the soft and low-density nature of Sugi wood, the plant is considered a decorative timber (i.e. used for cladding) rather than structural, thereby further restricting its use [9]. The limited range of applications for Sugi plants in New Zealand coupled with an abundance in supply makes the use of Sugi EOs as an antimicrobial product an attractive option for diversifying and adding value to an existing commodity.

Therefore, the purpose of this study was to first profile the bioactive properties of Mānuka and Sugi leaf EOs against common environmental microorganisms and correlate that with variation in oil composition of different production batches. Qualitative and quantitative differences in oil composition were assessed via gas chromatography – mass spectrometry. The oil samples were sourced from a single plantation site (Katikati region, New Zealand), distilled during different months of the year to evaluate batch variation during EO production. In addition, agar dilution assays were conducted to determine whether compositional variation affected the oil's antimicrobial efficacy. This study will serve as a preliminary examination to track the compositional variation of Mānuka and Sugi EOs produced by oil suppliers, and the data generated can be used to establish quality assurance parameters in the future for consumer products that exploit their antimicrobial properties.

2. Results

2.1. Antimicrobial activity

The essential oils were screened against common environmental microorganisms to evaluate their spectrum of *in vitro* antimicrobial activity via disk diffusion assays. Based on the presence of inhibition zones, Mānuka and Sugi EOs exhibited a broad spectrum of antimicrobial activity (**Table 1**). Neither of the EOs however was able to inhibit the growth of *Pseudomonas aeruginosa* nor *Aspergillus niger* and *Aspergillus oryzae*. Notably, while inactive against mycelial growth of *Aspergillus niger*, Mānuka EO inhibited its sporulation (**Figure 1**). Such anti-sporulating phenomena by essential oils were previously reported in literature [10]. The minimum inhibitory concentrations (MICs of each EO were obtained as a quantitative measure of antimicrobial efficacy via agar dilution assays. In general, the MIC values ranged from: 2.5 mg/mL to neat oil concentration against bacteria, 2.5 to 5 mg/mL against yeast, and 2.5 to 10 mg/mL against filamentous fungi (**Table 1**).

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Table 1. Minimum inhibitory concentrations of *Cryptomeria japonica* and *Leptospermum scoparium* leaf essential oils against environmental microorganisms determined via agar dilution assays.

		Minimum Inhibitory Concentration (mg/mL)	
Class	**Organism**	**MEO**	**SEO**
Bacteria	*Pseudomonas aeruginosa*	Not Active	Not Active
	Escherichia coli	Not Active	> 10
	Staphylococcus aureus	2.5	5
	Enterococcus faecalis	5	10
	Micrococcus luteus	2.5	5
Yeast	*Candida albicans*	5	5
	Saccharomyces cerevisiae	2.5	5
	Cryptococcus albidus	2.5	5
Filamentous fungi	*Aspergillus oryzae*	Not Active	Not Active
	Aspergillus niger	Not Active	Not Active
	Mucor plumbeus	2.5	5
	Rhizopus stolonifer	5	2.5
	Sclerotinia sclerotiorum	10	10

MEO: *Leptospermum scoparium* leaf essential oil, SEO: *Cryptomeria japonica* leaf essential oil

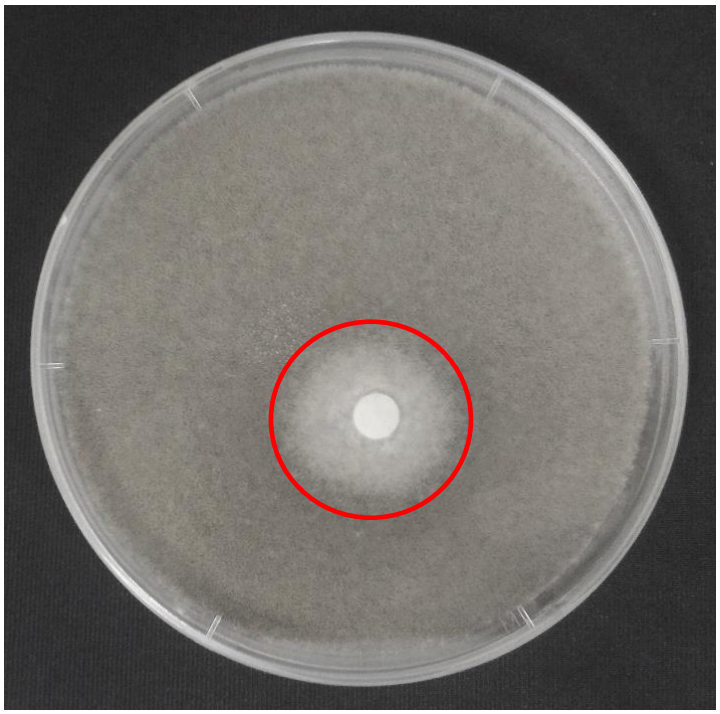

Figure 1. Disk diffusion assay demonstrating the anti-sporulating effect (highlighted in the red circle) of *Leptospermum scoparium* leaf essential oil on *Aspergillus niger* grown on potato dextrose agar in contrast to the outer pigmented region of sporulation.

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2.2. Essential oil metabolite profile

2.2.1. Sugi essential oil composition

Around 200 volatile metabolites were identified from the GC-MS analysis of Sugi essential oils using the National Institute of Standards and Technology (NIST) 2017 mass spectral library. The top six most abundant compounds constituted approximately 50% of total oil composition while the majority were detected at low levels. No single metabolite represented more than 13% in total reference ion peak area of all metabolites identified (i.e. total oil composition). The six most abundant metabolites in Sugi oil distilled in May were α-pinene (12.41%), p-menth-2-ene (11.22%), γ-terpinene (9.28%), and β -myrcene (7.48%), 3-carene (6.95%) and α-thujene (6.92%). By contrast, α-pinene (12.09%), p-cymene (10.52%), γ-terpinene (9.95%), p-menth-2-ene (9.22%), terpinen-4-ol (6.64%) and D-limonene (6.42%) were the major constituents for the oil distilled in August. The top 50 most abundant metabolites detected in two different batches of Sugi leaf EO and their batch variation are presented in **Table 2**. The complete list of all compounds identified in Sugi leaf EO via GC-MS is provided as **Supplementary Material 1**.

Table 2. Top 50 most abundant *Cryptomeria japonica* leaf essential oil components detected via gas chromatography – mass spectrometry. Compound identification based of the National Institute of Standards and Technology (NIST) 2017 mass spectral library

Compound	Average Peak Area (%)	
	May	August
α-Pinene	12.41 ± 0.22	12.09 ± 0.05
p-Menth-2-ene	11.22 ± 0.15	9.22 ± 0.03
γ-Terpinene	9.28 ± 0.06	9.95 ± 0.04
β-Myrcene	7.48 ± 0.06	6.19 ± 0.12
3-Carene	6.95 ± 0.05	5.22 ± 0.04
α-Thujene	6.92 ± 0.16	4.80 ± 0.17
2- Carene	5.97 ± 0.03	5.33 ± 0.03
D-Limonene	5.02 ± 0.04	6.42 ± 0.08
Terpinen-4-ol	4.22 ± 0.04	6.64 ± 0.05
Camphene	2.96 ± 0.05	3.15 ± 0.02
Isoterpinolene	2.72 ± 0.03	2.90 ± 0.02
Cyclofenchene	2.01 ± 0.02	1.98 ± 0.01
p-Cymene	1.49 ± 0.02	10.52 ± 0.05
10-epi-Elemol	1.43 ± 0.02	0.68 ± 0.03
Bornyl acetate	1.27 ± 0.02	1.26 ± 0.02
δ-Cadinene	1.22 ± 0.03	0.71 ± 0.04
δ-Selinene, (+)-	0.95 ± 0.01	0.45 ± 0.02
γ-Eudesmol	0.91 ± 0.03	0.80 ± 0.05
α-Eudesmol	0.90 ± 0.02	0.52 ± 0.03
α-Patchoulene	0.82 ± 0.01	0.39 ± 0.02
Rosifoliol	0.76 ± 0.02	0.45 ± 0.03
α-Tricyclene	0.72 ± 0.03	0.70 ± 0.02
Kaur-16-ene	0.71 ± 0.02	0.56 ± 0.03
Cedrol	0.63 ± 0.02	0.37 ± 0.01
Linalool	0.50 ± 0.00	0.78 ± 0.01
(E)-4,8-Dimethylnona-1,3,7-triene	0.47 ± 0.01	0.11 ± 0.00
p-Camphorene	0.47 ± 0.02	0.38 ± 0.02
β-Selinene	0.41 ± 0.01	0.19 ± 0.01

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δ-Selinene	0.34 ± 0.01	0.21 ± 0.01
γ-Cadinene	0.33 ± 0.00	0.12 ± 0.00
Linalyl acetate	0.33 ± 0.00	0.12 ± 0.00
α-Muurolene	0.33 ± 0.01	0.16 ± 0.00
isoledene	0.31 ± 0.01	0.13 ± 0.01
Cyperene	0.31 ± 0.01	0.19 ± 0.01
Phyllocladene	0.31 ± 0.01	0.12 ± 0.01
Fenchene	0.28 ± 0.01	0.22 ± 0.01
Rimuen	0.28 ± 0.01	0.17 ± 0.01
3-Octanol, acetate	0.25 ± 0.01	0.11 ± 0.00
(1S)-1,3,3-trimethylnorbornan-2-ol	0.25 ± 0.01	0.57 ± 0.00
Cadina-1(6),4-diene	0.24 ± 0.01	0.09 ± 0.00
τ-Cadinol	0.23 ± 0.01	0.09 ± 0.00
Biformene	0.22 ± 0.00	0.01 ± 0.00
(-)-Germacrene D	0.22 ± 0.00	0.14 ± 0.01
(E)-Sabinyl acetate	0.22 ± 0.01	0.12 ± 0.00
β-Ocimene	0.20 ± 0.00	0.06 ± 0.00
Santrolina triene	0.19 ± 0.00	0.44 ± 0.01
1-Octen-3-yl-acetate	0.19 ± 0.01	0.11 ± 0.00
3,6-Octadienal, 3,7-dimethyl-	0.15 ± 0.00	0.12 ± 0.00
(Z)-p-Menth-2-en-1-ol	0.15 ± 0.00	0.17 ± 0.00
α-Cadinol	0.15 ± 0.01	0.07 ± 0.00

The average of 5 technical sample replicates ± standard deviation is reported for % Peak Area values. Individual peak abundance is expressed as a relative percentage of total peak area of all compounds detected.

Principal Component Analysis (PCA) using the top 50 most abundant compounds detected by the GC-MS analysis of Sugi oil shows that the two batches of EO are distinctly different in terms of the composition of volatile metabolites (**Figure 2**). A clear separation of Sugi EO samples according to the month of distillation was evidenced by the first principal component (PC1), which accounted for 98.5% of total variance in the samples. Out of 198 identified metabolites in Sugi EO, 185 showed significantly different relative concentration between the two batches analyzed by GC-MS ($p < 0.05$). The top 20 metabolite features in Sugi EO that best described differences between months of distillation as identified by PLS-DA is outlined in **Figure 3**. A large proportion of class discriminating features were present at higher levels in Sugi oil collected in August. Apart from p-cymene, all compounds represented less than 0.5% of proportional peak area in each sample. Fold difference of the important features ranged from 2.9-fold (γ-cadinene) to 171-fold (2-methyl-2-cyclohexen-1-ol).

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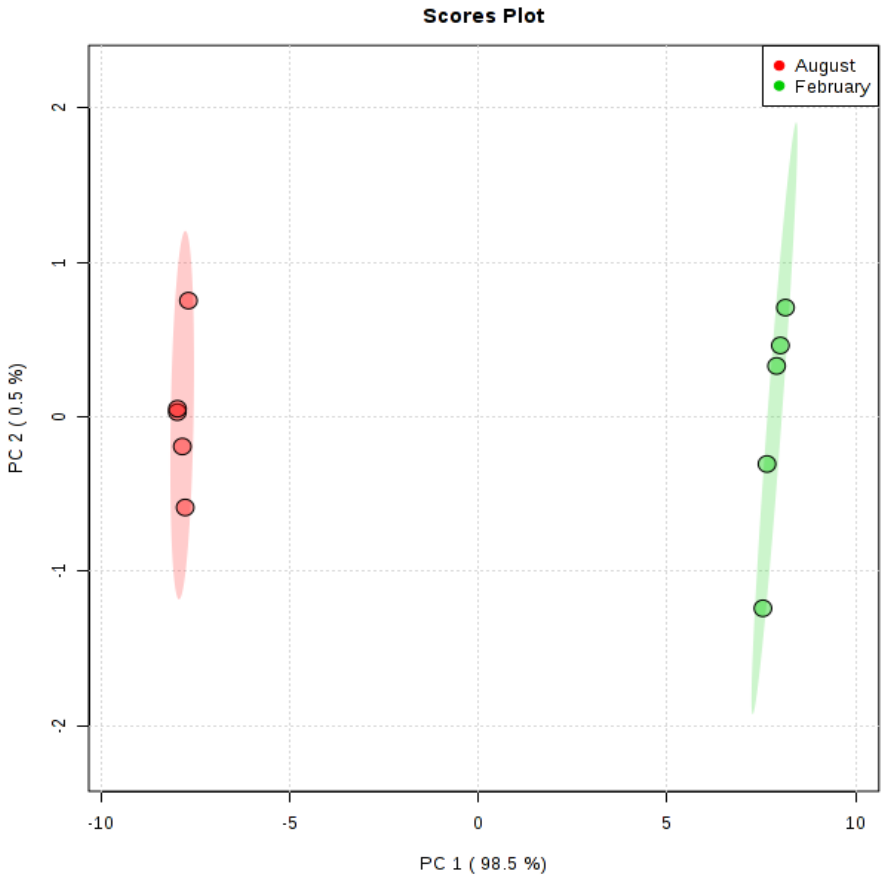

Figure 2. Principal Component Analysis Scores plot of *Cryptomeria japonica* essential oils collected at different months of the year showing the first two principal components (PC1 and PC2); 5 samples from August and 5 samples from February. Red = Oil distilled in August 2017; Green = Oil distilled in February 2017. Explained variance is shown in brackets. Figure generated from "Metaboanalyst 4.0" [11].

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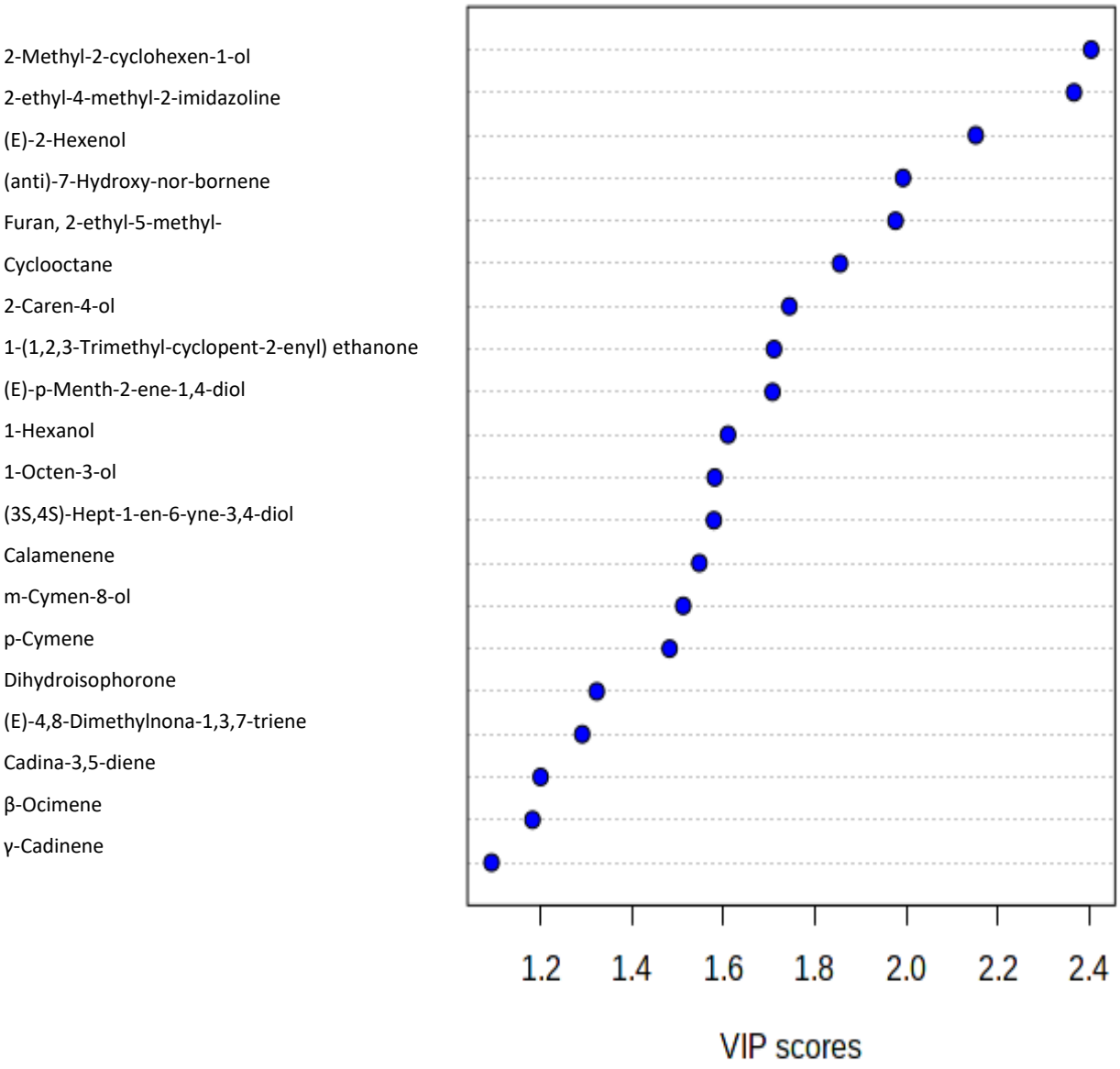

Figure 3. Top 20 important metabolite features in *Cryptomeria japonica* leaf essential oil determined by PLS-DA. Variable Importance in Projection (VIP) score was used to rank feature importance. Figure generated from "Metaboanalyst 4.0" [11].

2.2.2. Mānuka essential oil composition

GC-MS analysis of Mānuka leaf essential oils lead to identification of 157 compounds. No single compound represented more than 17% of total oil composition. Across all three months of distillation, the top six most abundant compounds constituted approximately 50% of total oil composition. For oil samples distilled in February, calamenene (15.14%), α-pinene (12.79%), β-pinene (11.72%), cadina-3,5-diene (4.36%), β-myrcene (3.69%) and caryophyllene (2.92%) were the six most abundant metabolites in order of proportion. Similarly, major constituents in Mānuka oil distillated in May were calamenene (16.37%), α-pinene (11.58%), cadina-3,5-diene (5.15%), β-myrcene (4.92%), β-pinene (4.22%) and Cadine-1,4-diene (3.29%). August distillates however, showed a notably different profile of major constituents compared to February and May, which consisted of β-pinene (15.80%), α-pinene (15.71%), calamenene (13.12%), eucalyptol (4.25%), p-cymene (3.71%) and linalool (3.02%). The top 50 most abundant compounds detected in Mānuka leaf essential oil and their batch variation is

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presented in **Table 3**. The complete list of all compounds identified in Mānuka leaf essential oils by GC-MS is provided as **Supplementary Material 2**.

Table 3. Top 50 most abundant *Leptospermum scoparium* leaf essential oil components detected via Gas chromatography – Mass spectrometry. Compound identification based of the National Institute of Standards and Technology (NIST) 2017 mass spectral library

Compound	Average Peak Area (%)		
	February	May	August
Calamenene	15.14 ± 0.41	16.37 ± 0.19	13.12 ± 0.36
α-Pinene	12.79 ± 0.19	11.58 ± 0.25	15.71 ± 0.14
β-Pinene	11.72 ± 0.05	4.22 ± 0.05	15.80 ± 0.04
Cadina-3,5-diene	4.36 ± 0.16	5.15 ± 0.04	1.75 ± 0.09
β-Myrcene	3.69 ± 0.35	4.92 ± 0.03	2.90 ± 0.32
Caryophyllene	2.92 ± 0.05	3.15 ± 0.02	2.11 ± 0.02
Cadine-1,4-diene	2.78 ± 0.08	3.29 ± 0.06	1.52 ± 0.04
Cadina-1(6),4-diene	2.68 ± 0.08	3.04 ± 0.10	0.87 ± 0.01
p-Cymene	2.49 ± 0.06	2.02 ± 0.02	3.71 ± 0.06
α-Cubebene	2.48 ± 0.01	2.61 ± 0.03	1.77 ± 0.01
Eucalyptol	2.44 ± 0.04	1.40 ± 0.02	4.25 ± 0.02
Linalool	2.19 ± 0.07	1.52 ± 0.04	3.02 ± 0.08
Copaene	1.92 ± 0.03	2.33 ± 0.03	1.43 ± 0.02
δ-Cadinene	1.79 ± 0.05	2.12 ± 0.05	1.05 ± 0.04
β-Guaiene	1.72 ± 0.04	2.26 ± 0.02	1.12 ± 0.02
γ-Terpinene	1.52 ± 0.05	1.07 ± 0.03	1.94 ± 0.07
D-Limonene	1.42 ± 0.07	0.90 ± 0.02	2.30 ± 0.06
β-Selinene	1.34 ± 0.03	1.99 ± 0.04	0.98 ± 0.02
(+)-Eremorphilene	1.23 ± 0.02	1.81 ± 0.03	0.91 ± 0.01
Zonarene	1.23 ± 0.02	1.54 ± 0.03	0.50 ± 0.01
α-Humulene	1.21 ± 0.03	1.35 ± 0.02	1.14 ± 0.01
α-Amorphene	1.20 ± 0.03	1.47 ± 0.01	0.79 ± 0.01
α-Calacorene	1.13 ± 0.03	1.50 ± 0.02	0.69 ± 0.02
α-Eudesmol	1.12 ± 0.05	1.65 ± 0.02	1.86 ± 0.07
(Z)-1H-Indene,1-ethylideneoctahydro-7a-methyl	0.94 ± 0.04	1.28 ± 0.07	1.57 ± 0.06
Guaia-6,9-diene	0.91 ± 0.02	1.20 ± 0.01	0.55 ± 0.01
α-Selinene	0.90 ± 0.03	1.23 ± 0.03	0.65 ± 0.01
4,11-selinadiene	0.87 ± 0.03	1.47 ± 0.02	0.52 ± 0.01
α-Thujene	0.85 ± 0.03	0.60 ± 0.01	1.33 ± 0.03

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Ylangene	0.82 ± 0.01	1.05 ± 0.03	0.39 ± 0.00
Myrtenyl acetate	0.73 ± 0.01	0.46 ± 0.01	2.66 ± 0.02
γ-Eudesmol	0.61 ± 0.02	0.88 ± 0.00	1.03 ± 0.04
Leptospermone	0.61 ± 0.03	0.85 ± 0.02	0.26 ± 0.00
β-Elemene	0.50 ± 0.01	0.87 ± 0.02	0.42 ± 0.01
β-Maaliene	0.49 ± 0.01	0.48 ± 0.01	0.20 ± 0.00
(1S)-1,3,3-trimethylnorbornan-2-ol	0.45 ± 0.00	0.31 ± 0.01	0.75 ± 0.02
α-Terpinolene	0.39 ± 0.02	0.25 ± 0.00	0.48 ± 0.03
(Z)-Undec-6-en-2-one	0.38 ± 0.01	0.50 ± 0.01	0.23 ± 0.01
Isoterpinolene	0.37 ± 0.01	0.23 ± 0.02	0.48 ± 0.01
α-Dehydro-ar-himachalene	0.36 ± 0.01	0.41 ± 0.00	0.37 ± 0.01
γ-Cadinene	0.31 ± 0.01	0.37 ± 0.01	0.14 ± 0.00
Terpinen-4-ol	0.29 ± 0.01	0.23 ± 0.01	0.47 ± 0.02
(E)-β-Ocymene	0.28 ± 0.01	0.49 ± 0.02	0.13 ± 0.01
Di epi-Cubenol	0.24 ± 0.01	0.31 ± 0.01	0.15 ± 0.00
Caryophyllene oxide	0.22 ± 0.00	0.25 ± 0.01	0.16 ± 0.00
(Z)-3-Hexenyl Acetate	0.21 ± 0.01	0.18 ± 0.00	0.08 ± 0.01
Aromandendrene	0.20 ± 0.01	0.20 ± 0.00	0.09 ± 0.00
α-Farnesene	0.19 ± 0.01	0.25 ± 0.01	0.09 ± 0.00
β-Sabinene	0.18 ± 0.01	0.10 ± 0.00	0.29 ± 0.01
Flavesone	0.17 ± 0.00	0.29 ± 0.01	0.08 ± 0.00

The average of 5 technical sample replicates ± standard deviation is reported for % peak area values. Individual peak abundance is expressed as a relative percentage of total peak area of all compounds detected.

Principal Component Analysis (PCA) using the 50 most abundant compounds detected GC-MS analysis of Mānuka oils. The first three principal components explained 99.3% of total variance. Three distinct groupings of data points according to the month of distillation were observed showing the EO produced in August was the most different in composition of volatile metabolites among the three batches analysed (**Figure 4**). Samples from February and May batches clustered together along PC1 that explained 87.6% of total variance. Samples from February and May could be differentiated based on their metabolite profile by PC2, which explained 11.4% of total variance in the data. Lastly, PC3 explained the differences between replicate samples analyzed by GC-MS, which accounted for only 0.3% of total variance in the data. Significant differences ($p < 0.05$) in relative abundance was found for all metabolites identified by GC-MS of different batches of Mānuka oil samples as determined by a one-way ANOVA and Tukey's HSD post hoc test.

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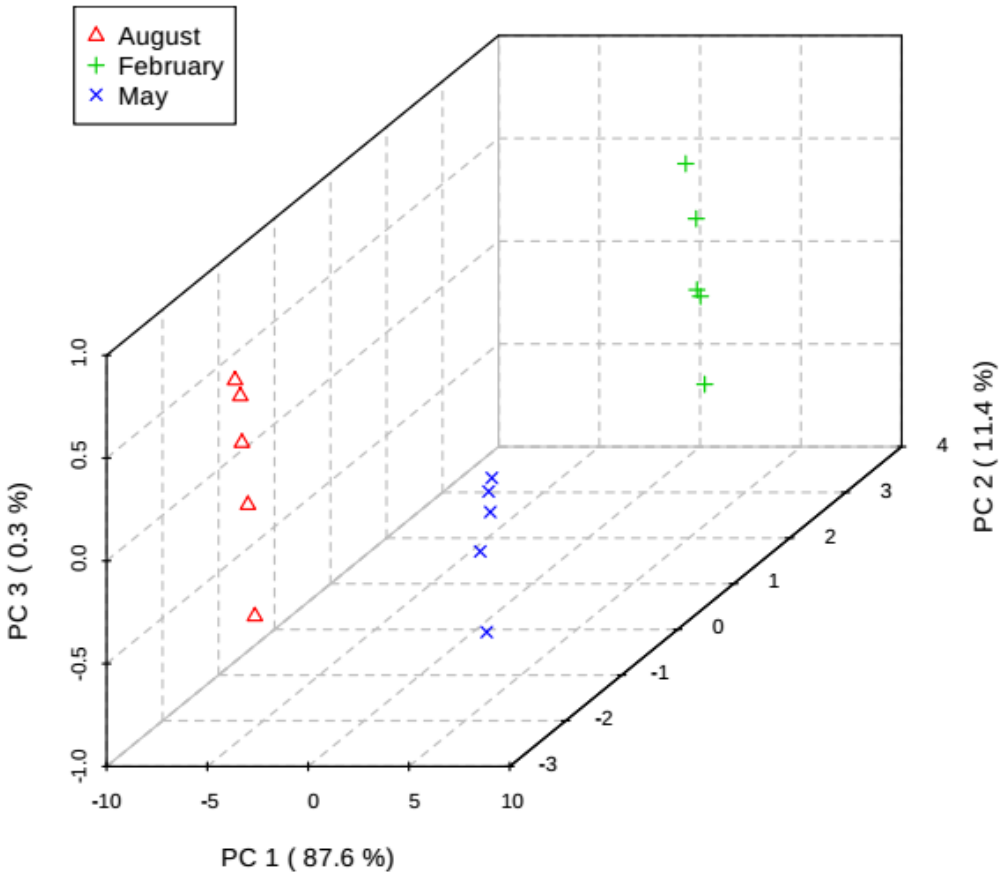

Figure 4. Three-dimensional Principal Component Analysis Scores plot of *Leptospermum scoparium* essential oils collected at different months of the year showing the first three principal components: Red = Oil distilled in August 2017; Green = Oil distilled in February 2017; Blue = Oil distilled in May 2017. Explained variance is shown in brackets. Figure generated from "Metaboanalyst 4.0" [11].

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The top 20 metabolite features in Mānuka EO that best describe differences between months of distillation identified by PLS-DA is outlined in **Figure 5**. The levels of most important features of Mānuka oil distilled in February were in between their relative abundance in samples obtained in August and May. By contrast to Sugi oil, PLS-DA revealed features of Mānuka oil that constitute a higher relative proportion of abundance where approximately 25% of important features were greater than 1% in relative proportion.

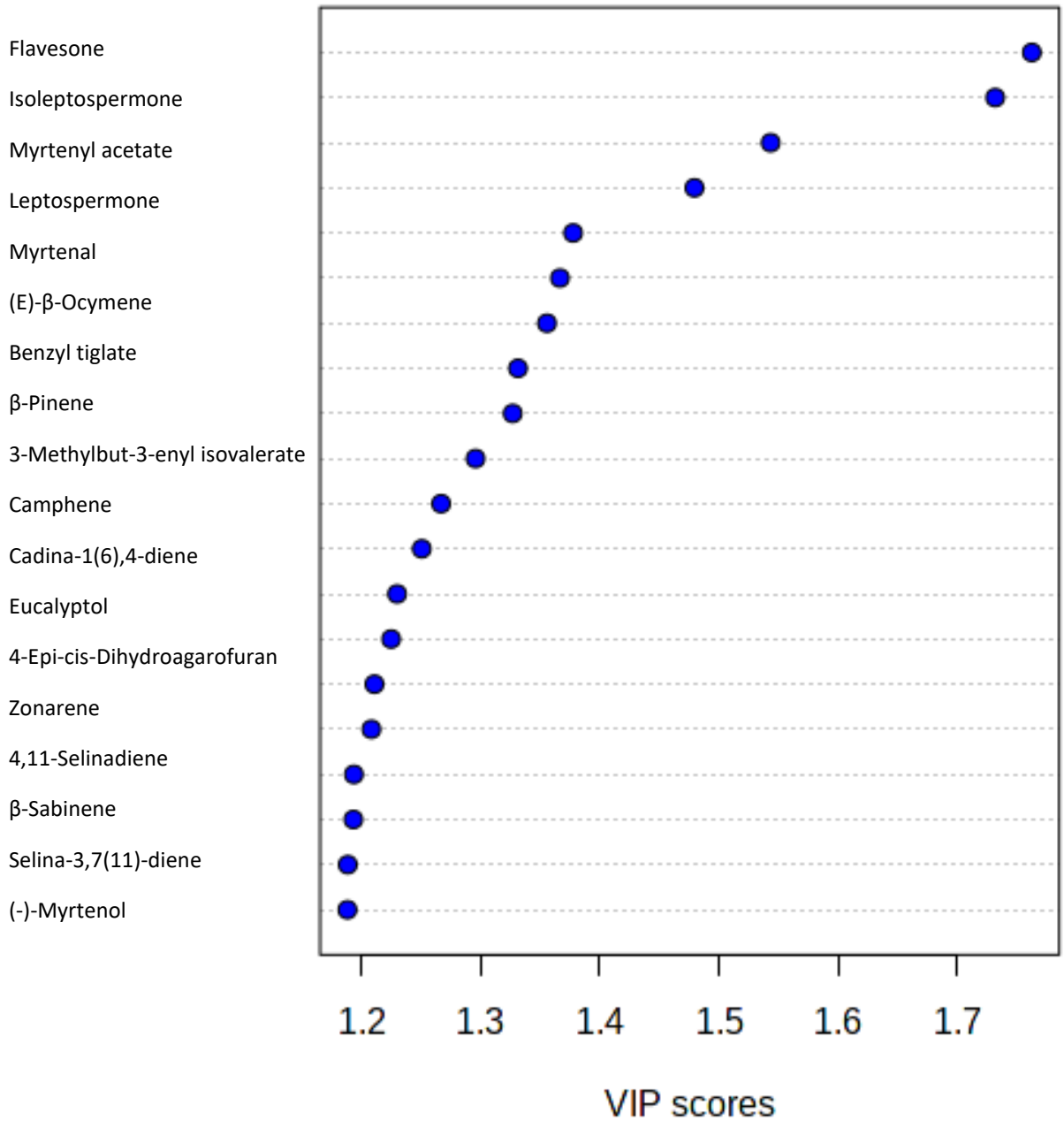

Figure 5. Top 20 important metabolite features in *Leptospermum scoparium* leaf essential oil determined by PLS-DA. Variable Importance in Projection (VIP) score was used to rank feature importance. Boxes on the right indicate relative abundance of features in each group. Figure generated from "Metaboanalyst 4.0" [11].

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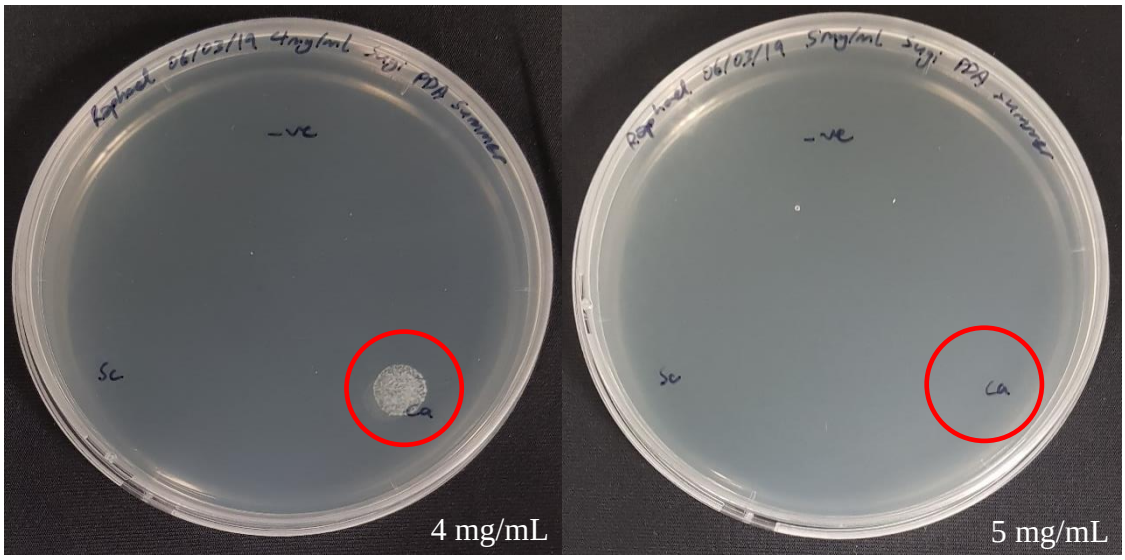
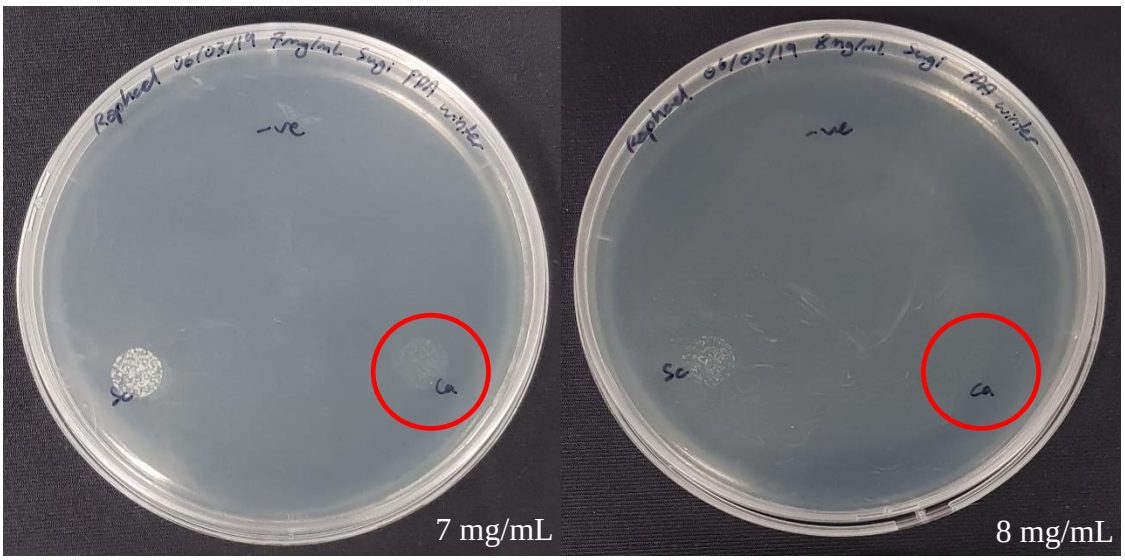
2.3. Effect of batch variation on antimicrobial activity

Agar dilution assays were performed using Mānuka and Sugi essential oils obtained throughout the year to assess whether oils distilled in different months ("batches") affect the oils' minimum inhibitory concentration (MIC). *Staphyloccocus aureus*, *Micrococcus luteus*, *Candida albicans* and *Saccharomyces cerevisiae* were used as model target organisms.

Sugi oil distilled in February showed greater antibacterial activity against *S. aureus* compared to oil distilled in August (MIC: 5 mg/mL vs 6 mg/mL respectively) (**Figure 6**). Likewise, the antifungal activity of Sugi oils against yeasts were greater for oil collected in February than in August (MIC: 5 mg/mL vs 8 mg/mL against *C. albicans* respectively) (**Figure 6**). These findings demonstrate that time of distillation during the year affects the antimicrobial properties of Sugi oil. Furthermore, the extent of change in the oil's antibacterial and antifungal properties − according to time of distillation − vary as evident by the differential increases in MICs against *S. aureus* versus *C. albicans* (1 mg/mL versus 3 mg/mL respectively) between February and August samples.



(a)



(b)

Figure 6. Assessment of Minimum Inhibitory Concentrations (MIC) of *Cryptomeria japonica* essential oils obtained in February 2018 (a) and August 2018 (b) determined by agar dilution assay against *Candida albicans*: February 2018 MIC = 5 mg/mL; August 2018 MIC = 8 mg/mL.

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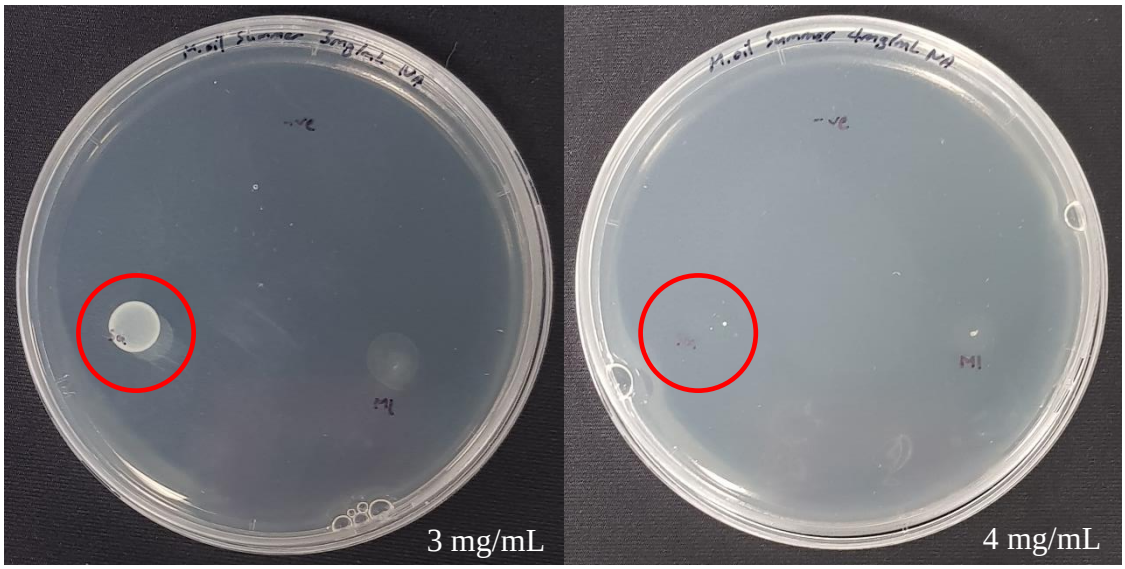
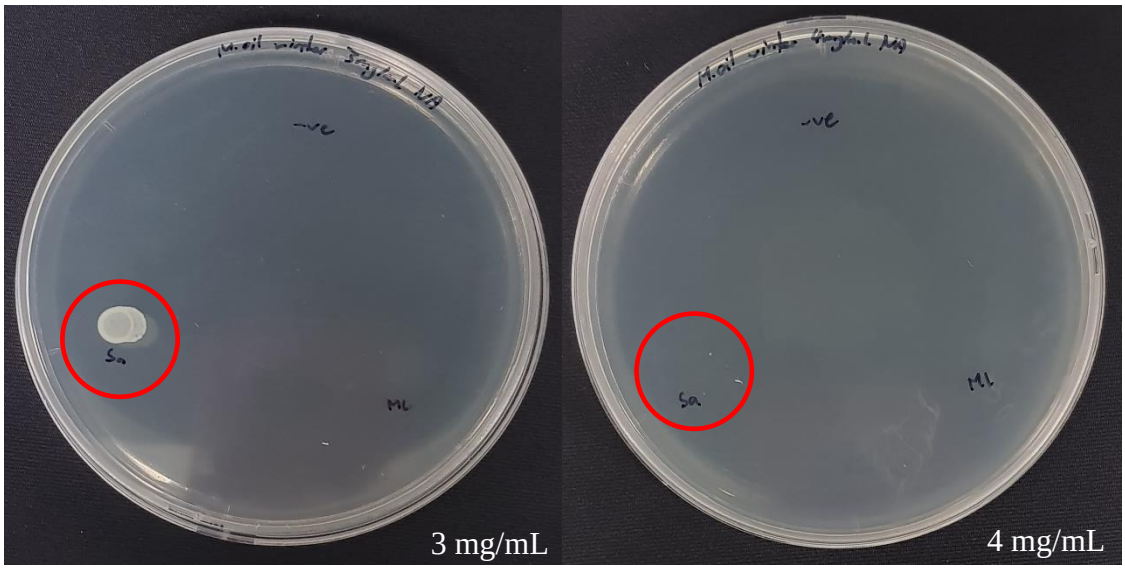
Only Mānuka oil distilled in May and August were used to assess changes in the oil's antimicrobial properties. Samples from May and August were selected as samples collected in these months presented the greatest compositional difference. Comparison of antimicrobial efficacy of Mānuka oil distilled in May and August revealed no difference in the MIC against *S. aureus* (MIC: 4 mg/mL) as shown in **Figure 7**. Likewise, no difference in the MIC was observed against the target yeast *S. cerevisiae* (MIC: 5 mg/mL) regardless of when the oil was collected (**Figure 7**). Together, these findings suggest that changes in the chemical profile of Mānuka oils was not significant enough to alter the oil's antimicrobial properties.



(a)



(b)

Figure 7. Assessment of Minimum Inhibitory Concentrations (MIC) of *Leptospermum soparium* essential oils obtained in May 2017 (row A) and August 2017 (row B) determined by agar dilution assay against *Staphylococcus aureus*: May 2017 MIC = 4 mg/mL; August 2017 MIC = 4 mg/mL.

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3. Discussion

Plant essential oils often exhibit broad spectrum antimicrobial activity owing to their chemical complexity. Essential oils are composed of several chemical classes such as terpenes, aliphatic, and aromatic compounds comprising of up to 300 different components of which 20 to 60 may be bioactive [12,13]. In addition, the mechanism of antimicrobial activity can be specific or non-specific (e.g. intracellular molecule specific interaction or interaction with biomembranes, respectively) and may also be a result of synergistic effects between multiple constituents. Accordingly, both Sugi and Mānuka EOs displayed broad spectrum antimicrobial activity against target microorganisms in this study. Sugi EO was active against all classes of microorganisms tested while Mānuka EO was ineffective against Gram-negative bacteria. These results are in agreement with existing data on the bioactive properties of Mānuka and Sugi oils [7,14,15]. However, there are conflicting reports in the literature on Mānuka and Sugi EOs' effectiveness against Gram-negative bacteria [16,17]. The likely reasons for the discrepancy in antimicrobial activity spectrum can be attributed to variation in EO composition due to the plant's genotype, chemotype, geographical origin, temporal differences in oil collection, and strain specificity of outer membrane composition in Gram-negative bacteria [18,19]. Lipopolysaccharides present on the outer membrane of Gram-negative bacteria are known to act as a permeability barrier against hydrophobic molecules, providing natural resistance to hydrophobic antimicrobial compounds [20]. Notably, *P. aeruginosa* was unaffected by both EOs - likely due to the organism's intrinsic antimicrobial resistance machinery including constitutive or inducible expression of efflux pumps and the aforementioned low permeability of the outer membrane widely reported in literature [21,22]. Since EOs are primarily composed of hydrophobic compounds with trace quantities of hydrophilic/semi-hydrophilic constituents, it is expected that EOs are less effective against Gram-negative bacteria than Gram-positive bacteria. Nonetheless, the outer membrane is not completely impermeable to hydrophobic molecules where limited transport is possible via protein channels such as porins [21]. Thus, at sufficiently high concentrations, EOs may exert antimicrobial effects as observed in the bioactivity test of Sugi EO against *E. coli* which displayed a high MIC (> 10 mg/mL) relative to efficacies observed against other target microorganisms (2.5 mg/mL ~ 10mg/mL) in this study.

Compositional analyses of Sugi EOs via GC-MS revealed around 200 compounds identified by the NIST 2017 mass spectral library. Changes in EO composition between different months investigated in this study represents a cultivar level chemistry as batches of oil distillates were pooled together in their respective months of collection. Upon review of literature, chemical profiles of Sugi leaf EO could be categorized into five distinct chemotypes with qualitative and quantitative differences in oil composition: 1) α-Pinene chemotype, 2) Elemol chemotype, 3) Eudesmol chemotype, 4) Eucalyptol chemotype and 5) 16-Kaurene chemotype [23,24]. The Sugi oils used in the present study corresponded to the α-Pinene chemotype with very low levels of 16-Kaurene which was also reported in Japan, Reunion Island, and Portugal [23,25]. Regardless of when the EO was distilled, qualitative chemical composition of Sugi EO remained characteristic of the α-Pinene chemotype. This was expected as chemotypic differences in EOs are mostly under genetic control [26].

For minor components (< 1% total peak area) present in Sugi EO, the largest fold difference in relative proportions between February and August was up to 8-fold. The fold-difference of major constituents (> 1% total peak area) between February and August however, fluctuated an average of 1.2-fold with the exception of p-cymene (Fold difference = 7). In accordance with the assessment of fold-changes, the most discriminatory compounds identified from PLS-DA of Sugi oil composition were trace components except for the terpenoids (E)-4,8-dimethylnona-1,3,7-triene (DMNT) and p-cymene. Terpenoids play a significant role in biochemical processes associated with primary cell metabolism including maintenance of membrane dynamics and photosynthesis. Therefore, environmental conditions such as seasonal differences are expected to affect terpene content in EOs including p-cymene [27]. DMNT is a homoterpene typically produced in plants for plant-plant communication as a response to herbivory. The level of DMNT was around four times greater in

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February than in August. This reflects its role in secondary metabolism which is expectedly greater in warmer seasons as observed in this study [28].

It is well-established that different seasons of the year can affect both the abundance of oil components and overall EO composition of many plants [27,29,30]. Therefore, it is reasonable to expect a logical relationship between the oil's chemical composition and antimicrobial efficacy. Considerably large differences in MICs of Sugi oil were observed between samples collected in February versus August against *S. aureus* and *S. cerevisiae* (MIC difference of 1 mg/mL and 3 mg/mL, respectively). Correlation analysis was performed between MICs and the Sugi GC-MS profile data (**Supplementary material 3**) which revealed 65 oil components with strong correlation coefficients (Coefficient > 0.75) - 59 of which were minor components (< 1% total peak area). Notably, the major component p-cymene showed significant difference in relative proportion between seasons. Whether this compound is the primary driver of the difference in MICs of Sugi oil is unclear due to the complexity of EO composition. Moreover, p-cymene is reported to augment the effects of other bioactive metabolites rather than exhibiting a direct antimicrobial effect which could amplify the antimicrobial effects of minor constituents via synergistic interaction [31]. As most major components of Sugi oil (>1% proportion) have a relatively low fold difference between batches compared to low abundance compounds, and that most strongly correlated metabolites were minor constituents, it may be argued that minor constituents are responsible for the observed discrepancy in MIC between seasons and possibly acting synergistically. Similar findings were reported where substantial differences in insecticidal activity was associated with relatively stable levels of major constituents but high fold-changes in levels of trace components in Sugi EO [32].

Four broad chemotypes of Mānuka are widely described in literature with distinct geographical grouping: 1) Triketone rich (i.e. leptospermone, isoleptospermone, flavesone, and grandifolone); 2) Linalool and Eudesmol rich; 3) Pinene rich and 4) Triketone, Linalool and Eudesmol deficient chemotypes. These chemotypes have demonstrated vastly different antimicrobial efficacies – the most prominent being the Triketone chemotype from the East Cape of New Zealand owing to its uniquely high levels (approximately 20%) of β-Triketones (e.g. Manex™) [33,34]. The most prominent components in the EO of *L. scoparium* in this study were α- and β-pinene – monoterpenes known to affect membrane integrity, respiration and ion transport processes in microorganisms [35]. This pinene-rich chemotype is typical of mature Mānuka plants as reported by Porter 1998 [36]. Alongside the pinenes, calamenene was present at high levels across all three months of oil collection. In addition, three major β-triketones (i.e. leptospermone, isoleptospermone, and flavesone) and one minor triketone (i.e. grandifolone) were detected but present at very low levels (i.e. < 1%). These key constituents reflect the chemotype characteristic of Mānuka from the Otaio and Woodstock, Canterbury region [34]. Furthermore, the results presented in this study correspond to both compositional and geographical data reported by Douglas et al 2004 according to ten Mānuka chemotypes determined via cluster analysis of Mānuka oils collected from 87 plantation sites throughout New Zealand [33]. Although differences in the relative peak area were evident between months of distillation, the overall chemotype was characteristic of the α-Pinene chemotype.

PCA of Mānuka oil data revealed leaf distillate collected in August was more dissimilar to those obtained in February and May (i.e.; across the first principal component) with the greatest difference observed to be between August and May. Specifically, PLS-DA revealed that all three major Mānuka β-triketones were among the most discriminatory variables - although present at very small levels, along with myrtenyl acetate and myrtenal. Oil distilled in May showed the highest level of β-triketones while August contained the lowest (i.e. β-triketone level fold difference > 3 in May). By contrast, levels of myrtenal and myrtenyl acetate were 4- and 5-fold higher respectively in August than in May. Across all major oil components identified (i.e.; peak area > 1%), maximum fold-difference between the three months was no greater than 3.7-fold.

Although chemical analysis of Mānuka oils revealed a maximum 6-fold variation in compound abundance, there was no detectable difference in MIC determined by agar dilution assays between May and August distillates against bacteria and yeast (MIC = 5 mg/mL). This finding suggests that either 1) the extent of chemical variation is not large enough to reduce the MIC of Mānuka oil by 1mg/mL or 2) the antimicrobial activity of Mānuka oil is mediated by components minimally affected

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by batch variation. High importance is often placed on triketone enriched Mānuka oil (e.g. Manex™) in literature due to their superior antibacterial activity [36]. However, the low triketone content (i.e.; < 1% peak area) coupled with large seasonal variation with no change in the MIC of Mānuka oil indicates other classes of compounds (e.g.; terpenes and terpenoids) are more likely to be responsible for the antimicrobial activity observed in agar dilution assays in this study. In support of this, literature indicates that Mānuka oils with a density less than 0.92 g/mL are unlikely to have significant antimicrobial activity associated with β-triketones [14].

4. Materials and Methods

4.1 Culture media

All fungal organisms used in this study were maintained on potato dextrose agar (PDA) (Difco) containing potato starch (4 g/L), dextrose (20 g/L), and agar (15 g/L) adjusted to pH 5.5. All bacterial organisms were maintained on nutrient agar (NA) containing peptone (5 g/L), yeast extract (3 g/L), sodium chloride (5 g/L), and agar (15 g/L) adjusted to pH 6.8.

4.2 Target organisms

Target bacteria and fungi used for the assessment of the essential oils' spectrum of bioactivity and efficacy were *Escherichia coli* (W3110), *Pseudomonas aeruginosa* (type strain SVB-B9), *Staphylococcus aureus* (type strain SVB-B13), *Enterococcus faecalis* (type strain NCTC 775), *Micrococcus luteus* (type strain SVB-B32), *Candida albicans* (type strain SC5314), *Saccharomyces cerevisiae* (CEN.PK113.7D), *Cryptococcus albidus* (type strain SVB-Y13), *Aspergillus niger* (type strain ICMP 17511), *Aspergillus oryzae* (type strain ICMP 1281), *Mucor plumbeus* (type strain ICMP 12920), *Rhizopus stolonifer* (type strain ICMP 13555), and *Sclerotinia sclerotiorum* (type strain ICMP 13844). Filamentous fungi and yeast were grown at 25 °C and 28°C, respectively. All bacteria were grown at 28°C.

4.3 Mānuka and Sugi source

Both Mānuka and Sugi essential oils used in this study were provided by GoodSport (Katikati, New Zealand; 37°39'02.9"S, 175°56'56.1"E). Both essential oils originate from plants originally sourced from the Coromandel region (North Island, New Zealand) and grown in the Katikati region. Three batches of Mānuka leaf biomass were harvested, and steam distilled on 21st February 2017, 20th May 2017, and 29th August 2017 from 2-year-old plants. The specific gravity of resulting Mānuka essential oils was 0.9188, 0.9325, and 0.9021, respectively. Two batches of Sugi leaf biomass were harvested, and steam distilled on 6th February 2018 and 19th August 2018 from mature Sugi trees approximately 40 years old. The specific gravity of resulting Sugi oils was 0.9037 and 0.8877 respectively. Determination of relative density followed the ISO 279:1998 reference method.

4.3.1 Essential oil extraction

Plant essential oils were obtained through steam distillation of Sugi or Mānuka plant biomass. The biomass used for distillation comprised plant leaves (approximately 95% by weight) and twigs no greater than 5mm in diameter. Approximately 130 kg of plant biomass was placed in a 330 L capacity stainless steel basket and steam distilled in a 665 L distilling pot over 4 to 4.5 hours at 120°C. A 2 m long straight tube 250 mm in diameter was used to condense the resulting steam via heat exchange with water at ambient temperature. The oils obtained were filter sterilized using a 0.22 um solvent resistant filter and stored in a non-corrosive metal vessel. The headspace of the vessel was flushed with nitrogen to prevent autooxidation and stored at -80°C to minimize the loss of volatile compounds.

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4.4 Disk diffusion assay

As a qualitative approach to assess the candidates' spectra of activity, disk diffusion assays were performed following the EUCAST antimicrobial susceptibility test protocol with some modifications [37]. For filamentous fungal targets, mycelial plugs of 8 mm in diameter were used. For each essential oil, 15 µL of the neat oil was loaded onto a 6 mm sterile paper disk and placed on target inoculated agar plates. The plates were then incubated for 24 hours at 25 °C for filamentous fungi and 28 °C for yeast and bacterial targets. Positive activity was assessed using the presence of an inhibition zone around the paper disks through visual inspection of plates held 30 cm from the naked eye.

4.5 Agar dilution assay

Agar dilution assays were performed following the CLSI protocol with some modifications to determine MICs of the two plant essential oils [38]. Agar dilution was adopted as a preferred method of evaluating *in vitro* efficacy due to the limited solubility of essential oils in aqueous solutions used in broth microdilution assays. Agar incorporated with Mānuka or Sugi oils were prepared to final concentrations of 10 mg/mL, 5 mg/mL, 2.5 mg/mL and 1 mg/mL in NA and PDA for initial activity spectrum screening. To compare the antimicrobial efficacy of oils collected from different batches, Mānuka or Sugi oil incorporate agar were prepared separately to final concentrations of 1 - 10 mg/mL in 1 mg/mL increments. Ethanol and Tween80 was also added to the agar mixture at concentrations of 3% v/v and 0.5% v/v respectively after filter sterilization (0.22 µm; solvent resistant) to emulsify the essential oil. Agar without essential oils but containing 3% (v/v) ethanol and 0.5% Tween80 was also prepared as the positive growth control. For filamentous fungi targets, mycelial plugs were transferred from fresh cultures and placed evenly around the agar. The yeast and bacterial target plates were incubated (28 °C) while filamentous fungi target plates were incubated at 25 °C. Plates were incubated until target growth in positive control plates was visible to the naked eye from a distance of 30 cm. The MIC was defined as the concentration of essential oil at which no target growth was visible to the naked eye.

4.6 Essential oil sample preparation for chemical analysis

Mānuka and Sugi essential oils were diluted in n-Hexane to a final concentration of 5% (v/v) for compositional analysis via Gas chromatography – Mass spectrometry. 4-Decanol (347 mg/L) was added to each sample for analysis as an internal standard for data normalization. All samples were analysed via GC-MS in quintuplicates

4.7 Gas chromatography – Mass spectrometry

Gas chromatography – Mass spectrometry (GC-MS) was used for relative quantification and identification of Mānuka and Sugi essential oil components. The instrument was an Agilent 7890B GC coupled to a 5977A inert MS with a split/splitless inlet. The column used was a fused silica ZB-1701, 30 m long, 250 µm (internal diameter), 0.15 µm stationary phase (86% dimethylpolysiloxane, 14% cyanopropylphenyl, Phenomenex). One microlitre of the diluted essential oil sample was injected directly to the GC system using an Agilent autosampler into a glass split/splitless 4 mm (internal diameter) straight inlet liner packed with deactivated glass wool (Supelco). The inlet temperature was fixed at 220 °C. Samples were injected in split mode (ratio 35:1) using ultra-high purity grade Helium as carrier gas at a column flow rate of 1 mL/min. The GC oven temperature was set at 60 °C and ramped at a rate of 3 °C/min to a final temperature of 246 °C and held for 15 min. The transfer line to the mass selective detector was maintained at 250 °C, the ion source at 250 °C, and quadrupole at 150 °C. The ion source was operated in electron impact ionization mode at 70 eV. Compounds were detected using mass spectra acquired in scan mode in the range of 33 to 330 m/z.

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4.8 Data processing

Raw GC-MS data generated from sample analysis was processed through the software Automated Mass spectral Deconvolution and Identification System (AMDIS) for deconvolution. Identification of compounds was based on the National Institute of Standards and Technology (NIST) 2017 mass spectral library, only considering those with a match quality above 80%. Compounds were considered as tentatively identified (putative identification). An in-house R package was used for automated integration of reference ion peak area. Each identification was individually screened, and manual retention time correction and subsequent re-integration was performed where required. The resulting peak abundance values were normalised by the internal standard 4-decanol followed by normalisation by the respective specific gravity of oil samples. Generalised log transformation was first applied to the data followed by Pareto scaling to make features within the data more comparable prior to applying analytical methods. Student's t-test and a one-way analysis of variance (ANOVA) in conjunction with Tukey's honestly significant difference (HSD) post hoc test was applied (for 2 and 3 group comparisons respectively) to elucidate whether the relative proportions of compounds detected in Sugi or Mānuka essential oils was significantly different between months of distillation. Principal Component Analysis (PCA) and Partial Least Squares – Discriminant Analysis (PLS-DA) was conducted on the R Project (Ver 3.2.2) using the 'prcomp' and 'pls' package, respectively. The web-based analytical platform "Metaboanalyst 4.0" [11] was used for data visualisation where stated.

5. Conclusions

Through qualitative and quantitative approaches, this study characterized the spectrum of antimicrobial activity and efficacies of Mānuka and Sugi essential oils collected in different times of the year from a single plantation site in Katikati, New Zealand. Seasonal chemical variation in EO composition has implications on their antimicrobial properties. However, as demonstrated in this study, differences in the chemical profile may or may not (Sugi and Mānuka, respectively) directly translate to alteration in the oil's antimicrobial efficacy. Therefore, reliable quality assurance parameters must be established to ensure consistent performance of the EOs when considering the use of EOs in commercial antimicrobial products. Besides factors commonly attributed to chemical variation of essential oils in literature (e.g. chemotype, plant age and seasonal differences), other endogenous and exogenous variables should be considered when explaining discrepancies in oil composition. For example, subtle difference in the type of biomass distilled such as leaf versus leaf with branches were shown to greatly influence essential oil composition [8]. In addition, some monoterpenes are reported to undergo spontaneous chemical conversion due to natural processes (i.e. aging) as well as extraction processes such as steam distillation [39–41]. Establishing whether variation in oil composition is strictly due to abiotic factors or arise from other sources will require further repeated chemical analyses of oils obtained between and within seasons over multiple years with standardised oil production methods.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Total composition of Sugi leaf essential oils obtained via steam distillation in February and August analyzed through gas chromatography – mass spectrometry., Table S2: Total composition of Mānuka leaf essential oils obtained via steam distillation in February, May and August analyzed through gas chromatography – mass spectrometry., Table S3: Result of Pearson's correlation analysis of minimum inhibitory concentration and proportion of Sugi oil constituents identified by Gas chromatography – Mass spectrometry..

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