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Evaluating the effects of kānuka essential oil (*Kunzea ericoides*) grown in different locations in New Zealand on two pathogenic dermatophytes: An *in vitro* study

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Abstract
Kānuka (*Kunzea ericoides* (A. rich) Joy Thomps.), an endemic tree throughout New Zealand (NZ), has demonstrated anti-fungal, anti-bacterial effects, antispasmodic and anti-inflammatory actions. We tested commercially available kānuka oils from various NZ locations against two pathogenic fungi *Trichophyton rubrum* and *Microsporum canis*. All the oils reduced radial mycelial extension in a dose dependent manner. The most effective oils, sourced from Great Barrier Island (K2) and East Coast North Island (K3), gave IC_{50} values of 0.67 (K2), 0.7 (K3) and 2.7 (K2, K3) (v/v) for *M. canis* and *T rubrum* respectively, the least effective was from Coromandel (K4) with IC_{50} values of 6.3% and 16.8% respectively. At 1% (v/v), K4 significantly increased growth of *T. rubrum*. Significant variations were observed in (α)-pinene (60.12%-74.34%) and linalool (1.93%-4.45%). Our results suggest that the geographical location of the kānuka plants can affect the α-pinene and linalool constituents and thus the efficacy of the oils towards dermatophyte fungi.

Keywords: *Alpha*-pinene, antifungal, dermatophytes, essential oil, Kānuka oil

1. Introduction
Pathogenic dermatophytes, such as *Trichophyton rubrum and Microsporum canis* have been implicated in human fungal infections, ranging from the merely troublesome to potentially life threatening infections in immunocompromised patients [1]. *T. rubrum*, the most prolific, is the main causative agent of common superficial skin infections such as athlete's foot (tinea pedis), nail infections (onychomycosis), jock itch (tinea cruris) and ringworm (tinea corporis) [2]. Conditions such as tinea pedis can increase susceptibility to secondary bacterial skin infections and disfigured nails from onchymosis can affect shoe fit and gait, which can cause problems especially for people with diabetes [3]. *M. canis*, a fungus which primarily affects cats, can also become pathogenic in humans, particularly affecting the scalp and contributing to hair loss [4]. The presence of dermatophytes in the population is growing due to both a higher number of immune-compromised people and the use of communal swimming pools, gyms and health clubs, which all increase the ease of spread. These are typically treated with topical azole-containing creams [3].
Kānuka (*Kunzea ericoides* (A. Rich) Joy Thomps.) is an endemic tree that grows widely throughout New Zealand. Interest in the potential therapeutic effects of kānuka oil has grown since the 1990s when it first became widely commercially available. There is a long history of the plant being used in traditional Māori healing, along with the related Mānuka tree [5]. Kānuka oil has been found to be effective *in vitro* against a number of microorganisms including fungi and bacteria [6, 7], as well as having antispasmodic actions *in vitr* [8]. Chen *et al.* [6] compared the effects of commercially obtained Kānuka and Mānuka oils against six common human pathogens (*Trichosporon mucoides*, *Candida tropicalis*, *Streptococcus aureus*, *Streptococcus mutans*, *Streptococcus sabrinus* and *Escherichia coli*) obtained from a laboratory and two isolates obtained from hospital in-patients (*Malassezia furfur* and *Candida albicans*). Minimum inhibitory concentration (MIC) of commercially obtained kānuka and mānuka oil were examined. The authors found that kānuka oil had lower minimum inhibitory concentrations (MIC) than manuka, with the most potency observed against *M. furfur* and *T. mucoides* with a MIC of 0.78% v/v solution [6]. Unfortunately, no chemical analyses or source of the oils was provided, despite previous work highlighting the chemical diversity of kānuka oils that is dependent on the location from which the oils are sourced.

It is likely that this diversity will influence clinical effects [9-15].

Alpha-pinene (α-pinene), the principle constituent in Kānuka oil [16] has been found to have antifungal activity [17, 18]. Based on the limited evaluation of kānuka oils from different locations in and their potential differing effects against fungi, we present a study detailing the effects of kānuka oils sourced from different locations in New Zealand on two common pathogenic dermatophytes *(T. rubrum and M. canis)*. We also present a summary of the detailed chemical analyses of the oils to determine which constituents in the oils may have the anti-fungal effects [14].

2. Materials and Methods

Kānuka oil was obtained from four commercial producers around New Zealand. These producers were chosen based on them: harvesting and distilling their own essential oil in sufficient quantities for retail sale; having at least 100mL of oil immediately available for the study; having undertaken sufficient in house quality control measures to ensure correct kānuka identification; and having previously had their oil independently analyzed to determine quality. The locations of these producers are shown in Table 1 <insert table 1 here>, representing key commercial locations in New Zealand, along with technical production notes as supplied by the distillers. Five oils (which we have designated K1 – K5) were obtained, as one of the four growers noticed a color variation in oils distilled at the same time from trees with older branches. Each of the suppliers confirmed the oil had been distilled within the last year either via steam or vacuum distilled in large commercial quantities (> one liter oil obtained per distillation). The oils were chemically analyzed independently at Flinders Cook Technical Services (FCTS) (http://www.flinderscook.co.nz/) [19].

2.1 Oil preparation

Each oil sample was initially decanted into smaller bottles and diluted to concentrations (v/v) of between 1% and 40% with fractionated coconut oil (FCO) purchased from Pure Nature Ltd (www.purenature.co.nz). FCO was selected as the diluent as it needed to act as a solubiliser for the essential oil, minimize evaporation and be skin safe for potential future clinical studies [20]. Other possible diluents of glycerol and Tween 80 (polysorbate) were rejected due to their possible antifungal effects [21, 22].

2.2 Fungal cultures

Stock cultures of *M. canis* and *T. rubrum* were maintained on PDA agar plates. For experimental purposes, a 5 mm cork borer was used to take a mycelial plug from the growing edge of a stock culture. The plug was then transferred to the centre of a 60 mm diameter Petri dish that contained 5 mL of solidified PDA agar media. Prior to inoculation 50 mL of oil was added at the required concentration and this was spread smoothly over the surface of the agar using a sterilized glass spreader. Plates were incubated at 21ºC for seven days prior to imaging.

2.3 Imaging of mycelia

For imaging, the plates were placed on a black circular cardboard background to enhance contrast and photographed with a Nikon D90 camera with an AF Micro-Nikkor 60mm f/2.8D lens. Images were then downloaded and analyzed using ImageJ software (www.imagej.net) as described previously [23]. Each image was converted to 8-bit greyscale and the contrast brightness and contrast were optimized such that the maximum extent of the mycelium was clearly visible. A ruler was used to calibrate the measurement tool in ImageJ. Four lines were drawn at 90º angles to one another over the mycelia and their respective lengths (from the edge of the inoculation plug to the furthest extent of growth) were determined. The mean of the four lines was taken as the average radial extension of the plate. Plates with just FCO added were used as controls. For each oil concentration between 3 – 6 replicates were used and data are presented as mean radial extension of those plates ± SEM. IC_{50} values for each oil were calculated using a non-linear fit of a plot of inhibitor concentration versus response (3 parameters) in Graph Pad Prism (www.graphpad.com).

2.4 Statistical analysis

Treatment means were compared using one-way ANOVA and Tukey HSD tests in Prism. Data sets with unequal variances (as determined by Bartlett's test) were logarithmically transformed prior to analysis. Data are presented as the mean ± SEM. Sample sizes were between 3 – 7 experimental plates.

3. Results and Discussion

3.1 Effects of the oils on the radial extension of mycelia

The effects of the different oils on radial extension of *M. canis* and *T. rubrum* mycelia are shown in Fig 1 and the respective IC_{50} values are shown in Table 2. Representative images of mycelia in K1 and K4 are shown in Fig 2. <insert fig 2 here> Of the two species, *M. canis* showed greater radial extension, extending 15.4 + 0.1 mm over 8 days, compared to 8.3 + 0.1 mm for *T. rubrum*. Initial preliminary tests were carried out to find the effective range of oil concentrations for determination of IC_{50} values, the range of oil concentrations that were tested were 1 – 10% (v/v) for K1 and K2, 1 – 20% (v/v) for K3 and 1 – 40% (v/v) for K4 and K5. There was no evidence of increased levels of branching in the presence of the oils, thus radial extension was taken to be an accurate determinant of the effect of the oils on fungal growth rates. Other mycelial characteristics, such as the appearance of the hyphae at the mycelial margin and red pigment production in *T. rubrum* did not appear to be affected by the oils.

For both species, the oils significantly reduced radial extension in a dose- dependent manner. As shown in Table 2 <Insert table 2 here>, the most effective oils were K2 and K3 (IC_{50} values of 0.67 and 2.7% (v/v) (K2) and 0.7 and 2.7% (v/v) (K3)) for *M. canis* and *T. rubrum* respectively and the least effective oil was K4 (IC_{50} values of 6.3 and 16.8% (v/v) for *M. canis* and *T. rubrum* respectively). For *T. rubrum*, K4 significantly increased growth at 1% (v/v) and had no effect at 5% (v/v), relative to the control. Four of the oils (K2, K3, K4 and K5) were more effective against *M. canis* than *T. rubrum* with lower IC_{50} values, while K1 was more effective against *T. rubrum.*

3.2 Chemical Analysis of the Kānuka oils

A summary of the ten most common constituents in each of the kānuka oils is shown in Table 1. The key constituent in all samples was α-pinene, which ranged from 60% in K3 through to ~74% in K2 and K4, which is consistent with other studies [15]. Other key constituents displayed much wider ranges between the different oils. The concentrations of *o*-cymene ranged from 0.44% (K4) to 3.95% (K5), of viridiflorene from 0.15% (K2) to 2.95% (K3) and viridiflorol from 1.06 (K2) to 4.11 (K2). For K1 and K2, sourced from the same location, but from different aged branches, the constituents were

broadly similar in concentration, although there was much higher levels of viridiflorene, viridiflorol and ledol in the younger branches and lower levels of *β*-selinene. K1 and K2 were the only oils that contained *β*-selinene. It was also notable that K4 contained low levels of *o*-cymene (0.44%), but much higher levels of linalool (4.45%) compared to the other oils.

3.3 Discussion

Each of the Kānuka oil samples were from commercially available steam distilled oil, from different locations around New Zealand. Some chemical variation is to be expected and the findings of the analyses is consistent with other studies on Kānuka oil from different regions [24]. Whilst the assumption, based on other studies, is that oils high in *Alpha*-pinene would exert the most antifungal effects, this was not the results of our study. K4, from the Coromandel area, had the second highest *Alpha*-pinene at 74.08%, but was the least effective oil on both organisms until a 20% dilution was tested. The most effective oil, K2, distilled from older branches, did have the highest α-pinene content at 74.35%. K2 also had higher *o*-cymene and *β*-selinene percentages compared to K4 so possibly there is a synergy occurring which may intensify the anti-fungal effects of the α-pinene. The only other major difference that K4 had from the other oils was a higher linalool content, at 4.45%, which is around twice the amount in the other oils. Linalool in isolation has been found to be effective against both *M. canis* [25] and *T. rubrum* [26], so the lowest antifungal effect in an oil with higher linalool and high α-pinene cannot be explained by these two constituents alone. Linalool is one of the most researched essential oil constituents as it is present in many plant species. There are different enantiomers of linalool which can also potentially affect biological activity [27]. Linalool enantiomers were not identified in any of the analyses conducted for this study, so again it is not possible to identify if this factor played any part in the observed effects. The main constituent that separates K2 as the most effective oi is *β*-selinene, present at 1.62%. This constituent is also present in some other essentials oil found to exert antifungal activity, however it is unclear if the effect is due to *β*- selinene [28]. There is limited information on the biological effects of *β*-selinene as a sole chemical [29]. Our observations suggest that it could potentially be a ratio of multiple oil constituents, which makes oils K1-K3 more effective than K4 and K5. Alternatively, it could be that oils K1-K3 have other minor constituents, which work in synergy with α-pinene to increase the antifungal activity.

3.4 Tables and Figures

Table 1: Location and Analysis Summary of Kanuka Oils distilled around New Zealand

	CAS No.	**K1**	**K2**	**K3**	**K4**	**K5**
Geographical location		**GBI**	**GBI**	**ECNI**	**CNI**	**A(A)I**
GPS location		S 36"12.972" E 175"20.305"	S 36"12.800" E 175"20.112	37°34'51"S 178°11'45"E	36°44'56.4"S 175°43'42.6"E	N/A
Date Distilled		04 Aug 20 Young branches	1 Aug 20 4 year old branches	15 Dec 19 Young branches	Aug 20 Young branches	20219 N/A
Method of Distillation		Steam	Steam	Steam	Vacuum	Steam
Physical Properties						
Specific gravity (SG)		0.8833	0.8779	0.8838	0.8741	0.8725
Refractive index (RI)		1.4712	1.4701	1.4728	1.4683	1.4682
Eucalyptol	4470-82-6	5.95	5.58	5.51	6.6	4.34
Linalool	78-70-6	2.49	2.57	1.81	4.45	1.93
Alpha terpineol	98-55-5	1.39	0.94	1.06	1	0.8
Viridiflorene	21747-46-6	1.39	0.15	2.95	0.61	0.95
Cis calamenene	72937-55-4	1.72	1.38	1.9	0.92	1.21
Spathulenol	1139-30-6	1.35	0.64	0.94	0.75	0.5
Viridiflorol	552-2-3	4.11	1.06	3.5	2.32	1.3
Ledol	577-27-5	0.99	0.35	0.87	0.64	0.34

Key to Table 1
GBI=Great Barrier Island (www.barriergold.co.nz)
ECNI = east Coast North Island (www.manukaessentials.com)
CNI-Coromandel North Island (www.kanukaoilnz.com)
Arapaoa (Arapawa) Island (www.mshop.co.nz)
(Analysis conducted by www.flinderscook.co.nz)

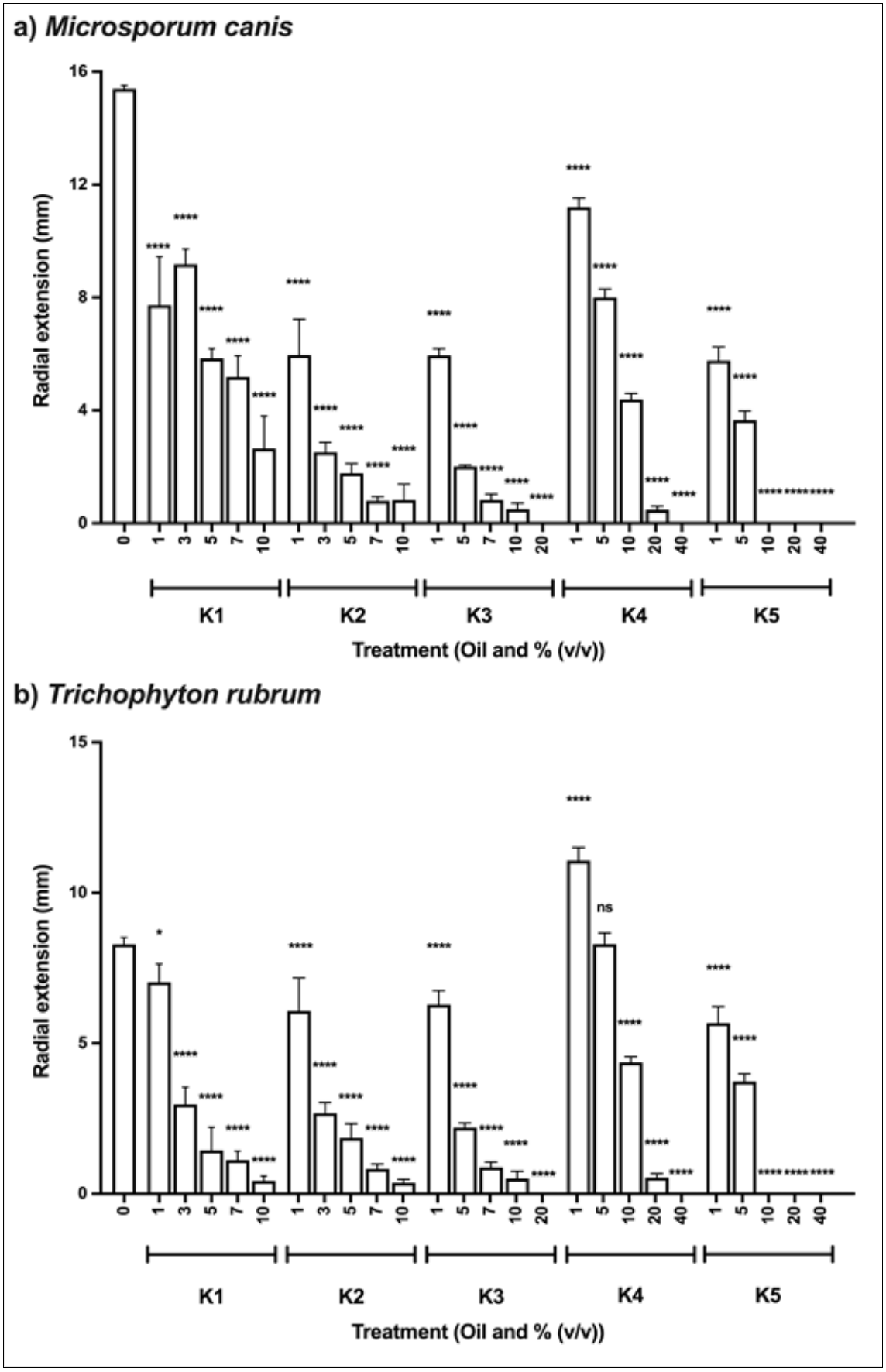

Fig 1: The effect of the kanuka oils on radial expansion of *Microsporum canis* and *Trychophyton rubrum.* Each of the oils reduced radial extension rates in a dose dependent manner. Control plates were treated with fractionated coconut oil. Data are presented as mean + SEM. Based on ANOVA and Tukey tests significant differences relative to the control are indicated as **** $P<0.0001$ and * $P<0.05$.

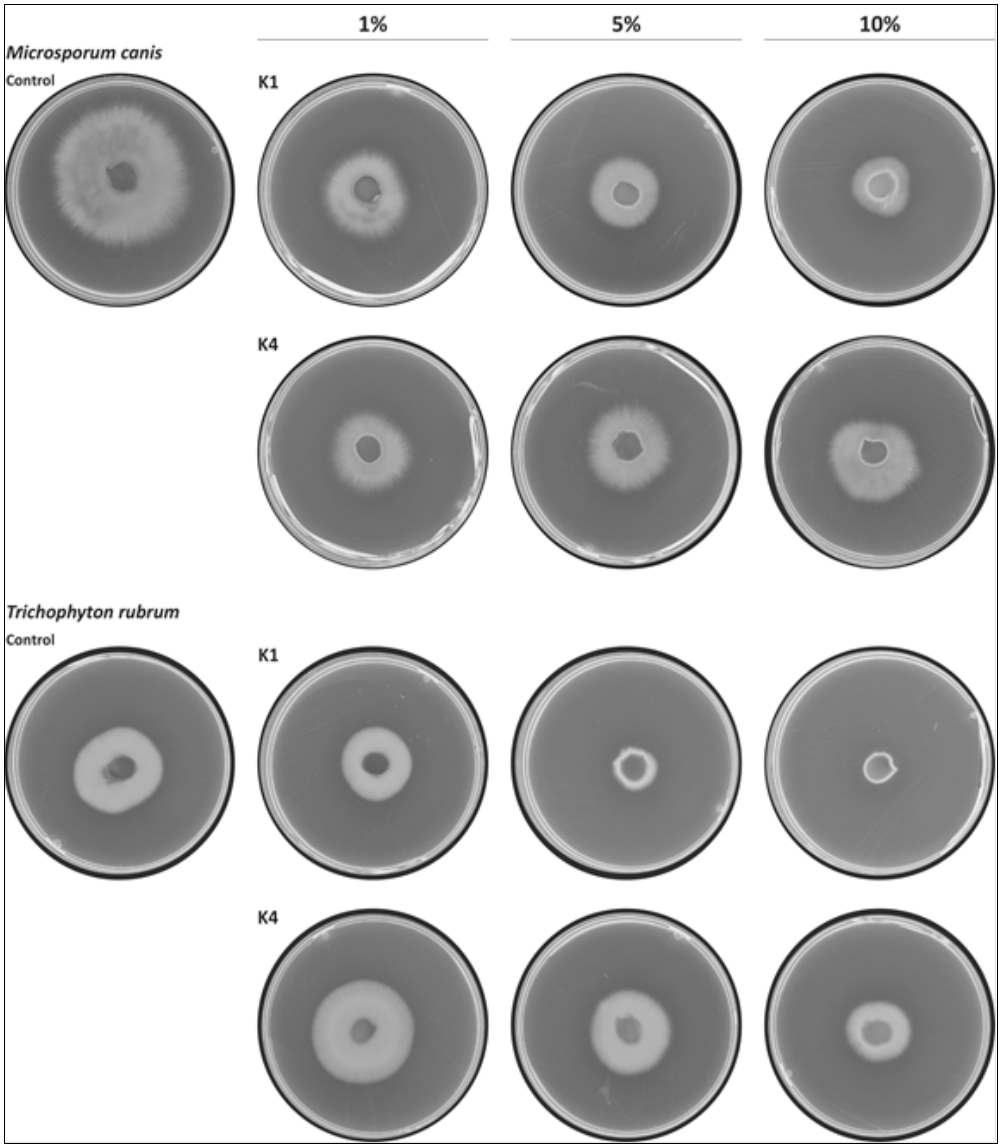

Fig 2: Representative images of mycelia of Microsporum canis and Trychophyton rubrum grown in the presence of kanuka oils K1 and K4. Images were photographed and converted to 8 bit images using Fiji. No adjustments have been made to brightness or contrast in the presented images. Control plates were treated with fractionated coconut oil.

4. Conclusions

This study has shown that all the kānuka oils tested exerted some degree of antifungal activity across repeated testing at various dilutions up to 40%. However, the antifungal properties of the kānuka oils depends on the oil composition, which varied depending on the geographical location of the parent tree. Therefore, it is essential to consider the location of the parent tree when choosing a particular kānuka oil as a potential treatment for dermatophytes infections.

The results obtained through this series of studies found that whilst all the Kānuka oils had effects against both *T. rubrum* and *M. canis*, one oil from Great Barrier Island (K2) was significantly more potent at the lowest concentration that the rest. The oils were tested in concentrations of up to 40%, whereas most essential oils tend to be applied to the skin at 5% or less. Therefore, a higher effect at a lower concentration is an important safety as well as cost consideration.

An interesting observation was made during the range finding testing, where plates were left for two weeks, where a red pigment was produced after two weeks of growth *T. rubrum*. The pigmentation of *T. rubrum* is highly dependent on the media pH [30]. At a more acidic media pH, *T. rubrum* has a yellow pigmentation, which was observed in the first two weeks of growth in the initial range finding studies. As the media becomes more alkaline the pigmentation of *T. rubrum* changed to a red color, which occurred after two weeks of growth [30]. This pigmentation change could suggest that *T. rubrum* is using the kānuka oil to produce a secondary metabolite, causing the pH of the media to rise. This effect was not observed in the later plates, which were measured after seven days of growth, and the plates were then destroyed.

This study has significantly contributed to the limited extant knowledge related to the antifungal properties of kānuka by identifying chemical variations from commercially produced Kānuka oil grown around New Zealand. This chemical diversity relates to a difference in effect on the two fungi tested. Consumers ideally should appreciate that there are differences and to know where their oil has been obtained from, or have access to a current analysis to determine if the profile of the oil matches the expected usage. In addition, our studies suggest that both minor constituent variations are just as important as the major constituents are and more needs to understood about both synergistic and antagonist actions within an essential oil.

Given that Kānuka oil has not shown any evidence of toxicity and easily penetrates the dermal layer [13] it is promising potential alternative natural solution for the treatment of

common skin and nail infections caused by dermatophyte fungi. This is particularly important because many fungal species are becoming resistant to current synthetic treatments [31]. Further, *in vivo* human investigations related to the antifungal properties of kānuka oil is likely to be beneficial.

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6. References

1. Nettles NE, Nichols LS, McGuinn KB, Pipeling M.R, Scheel PJ Jr, Merz WG. Successful Treatment of Trichosporon mucoides Infection with Fluconazole in a Heart and Kidney Transplant Recipient. Clin. Infect. Dis 2003;36(4):e63-e66
2. DermNet NZ. Tinea Corporis, https://dermnetnz.org/topics/tinea-corporis/, 20 October 2020.
3. Garber G. An overview of fungal infections. Drugs 2001;61(1):1-12. Doi: 10.2165/00003495-200161001-00001
4. DermNet NZ. Tinea Capitis, https://dermnetnz.org/topics/tinea-capitis/, 20 October 2020
5. Riley M. Maōri Healing and Herbal New Zealand Ethnobotanical Source. Paraparaumu, NZ; Viking Sevenseas N.Z. Ltd 1994.
6. Chen C-C, Yan S-H, Yen M-Y, Wu P-F, Liao W-T, Huang T-S. Investigations of kanuka and manuka essential oils for *in vitro* treatment of disease and cellular inflammation caused by infectious microorganisms. J. of Microbiology, Immunology and Infection 2016;49(1):104-111.
7. Lis-Balchin M, Hart SL, Deans SG. Pharmacological and antimicrobial studies on different tea-tree oils (Melaleuca alternifolia, *Leptospermum scoparium* or Manuka and *Kunzea ericoides* or Kanuka), originating in Australia and New Zealand. Phyto. Research 2001;4(8):623-629.
8. Lis-Balchin MH, Hart SL. An Investigation of the Actions of the Essential oils of Manuka (*Leptospermum scoparium*) and Kanuka (*Kunzea ericoides*), Myrtaceae on Guinea-pig Smooth Muscle. J. of Pharmacy and Pharmacology 1998:50(7):809-811.
9. De Lange PJ, Murray BG. Chromosome numbers in Kunzea (Myrtaceae). Australian J of Botany 2004;52(5):609-617.
10. De Lange PJ. A revision of the New Zealand *Kunzea ericoides* (Myrtaceae) complex. PhytoKeys 2014;(40):1.
11. Chagné D, Thrimawithana A, Bowatte D, Hilario E, Crowhurst R, Smallfield B *et al*. Simple sequence repeat (SSR) markers for New Zealand mānuka (*Leptospermum scoparium*) and transferability to kānuka (*Kunzea* spp.): New Zealand J. of Crop and Horticultural Science 2017:45(3):216-222.
12. Maddocks-Jennings W, Wilkinson J, Shillington, D, Cavanagh H. A fresh look at manuka and kanuka essential oils from New Zealand. Int. J of Aromatherapy 2005:15(3):141-146
13. Maddocks W. Kanuka: essential oil monograph. Aromatherapy Today 2019;76:12-16.
14. Maddocks W. Diversity in the essential oil of New Zealand grown Kānuka, *Kunzea ericoides* (A. Rich) Joy Thomps. Am J of Essent Oils and Natural Products 2019:(1);32-38.
15. Maddocks W. Kanuka (*Kunzea ericoides*) essential oil: A fresh look. The Int. J of Prof. Aromatherapy 2019, 8(1).
16. Porter NG, Wilkins AL. Chemical, physical and antimicrobial properties of essential oils of *Leptospermum scoparium* and *Kunzea ericoides*. Phytochemistry 1999;50(3):407-415.
17. Rivas da Silva AC *et al*. Biological activities of α-pinene and β-pinene enantiomers. Molecules 2012;17(6):6305-16.
18. Miron D, Battisti F, Silva FK, Lana AD, Pippi B, Casanova B *et al*. Antifungal activity and mechanism of action of monoterpenes against dermatophytes and yeasts. Revista Brasileira de farmacognosia 2014:24(6):660-667.
19. FlindersCook Technical Services. University of Canterbury Technical Report: O/No: 685738. New Zealand 2020.
20. Gomez AM, Amat Bou M, Gonzalez Cortés MV, Segura Navas L, Moreno Palanques MA, Bartolomé B. Coconut anaphylaxis: Case report and review. Allergol Immunopathol (Madr) 2015;43(2):219-20.
21. Inouye S, Tsuruoka T, Uchida K, Yamaguchi H. Effect of sealing and Tween 80 on the antifungal susceptibility testing of essential oils. Micro. and Imm 2001;45(3):201-208.
22. Lind H, Broberg A, Jacobsson K, Jonsson H, Schnürer J. Glycerol enhances the antifungal activity of dairy propionibacteria. Int. J of Micro 2010;2010:430873. https://doi.org/10.1155/2010/430873
23. Lundy SD, Payne RJ, Giles KR, Garrill A. Heavy metals have different effects on mycelial morphology of Achlya bisexualis as determined by fractal geometry. FEMS microbiology letters 2001;201(2):259-263.
24. Essien SO, Baroutian S, Dell K, Young B. Value-added potential of New Zealand mānuka and kānuka products: A review. Ind. Crops and Products 2019;130:198-20.
25. Silva KV, Lima MI, Cardos GN, Santos AS, Silva GS, Pereira FO. Inibitory effects of linalool on fungal pathogenicity of clinical isolates of Microsporum canis and Microsporum gypseum. Mycoses 2017;60(6):387-393.
26. De Oliveira Lima M, De Medeiros AA, Silva KS, Cardoso GE, De Oliveira Lima E, De Oliveira Pereira F. Investigation of the antifungal potential of linalool against clinical isolates of fluconazole resistant Trichophyton rubrum. J de mycologie medicale 2017;27(2):195-202.
27. Aprotosoaie AC, Hăncianu M, Costache I-I, Miron A. Linalool: a review on a key odorant molecule with valuable biological properties. Flav. and Frag. J 2014;29(4):193-219.
28. Simić A, Soković M, Ristić S, Grujić-Jovanović J, Vukojević P, Marin D. The chemical composition of some Lauraceae essential oils and their antifungal activities. Phytotherapy Research: An Int. J. Devoted to Pharm. and Tox. Eval. of Nat. Prod. Derivatives 2004;18(9):713-717.
29. National Center for Biotechnology Information. Pub Chem Compound Summary for CID 442393, beta-Selinene. n.d. https://pubchem.ncbi.nlm.nih.gov/compound/442393. 09 June 2021
30. Blechert O, Zheng H, Zang X, Wang Q, Liu W. Influence of the cultivation medium and pH on the pigmentation of Trichophyton rubrum. PloS one 2019;14(9):e0222333.
31. Monk BC, Goffeau A. Outwitting multidrug resistance to antifungals. Science 2008;321(5887):367-369.