



ORIGINAL ARTICLE



Investigations of kanuka and manuka essential oils for *in vitro* treatment of disease and cellular inflammation caused by infectious microorganisms

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Background: Diseases caused by infectious and inflammatory microorganisms are among the most common and most severe nosocomial diseases worldwide. Therefore, developing effective agents for treating these illnesses is critical. In this study, essential oils from two tea tree species, kanuka (*Kunzea ericoides*) and manuka (*Leptospermum scoparium*), were evaluated for use in treating diseases and inflammation caused by microorganism infection.

Methods: Isolates of clinically common bacteria and fungi were obtained from American Type Culture Collection and from Kaohsiung Veterans General Hospital. Minimum inhibitory concentrations for *Trichosporon mucoides*, *Malassezia furfur*, *Candida albicans*, and *Candida tropicalis* were determined by the broth microdilution method with Sabouraud dextrose broth. The

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antibacterial susceptibility of *Staphylococcus aureus*, *Streptococcus sobrinus*, *Streptococcus mutans*, and *Escherichia coli* were determined by the broth microdilution method. A human acute monocytic leukemia cell line (THP-1) was cultured to test the effects of the essential oils on the release of the two inflammatory cytokines, tumor necrosis factor- α and interleukin-4.

Results: Multiple analyses of microorganism growth confirmed that both essential oils significantly inhibited four fungi and the four bacteria. The potent fungicidal properties of the oils were confirmed by minimum inhibitory concentrations ranging from 0.78% to 3.13%. The oils also showed excellent bactericidal qualities with 100% inhibition of the examined bacteria. In THP-1 cells, both oils lowered tumor necrosis factor- α released after lipopolysaccharide stimulation. Finally, the antimicrobial and anti-inflammatory effects of the oils were obtained without adversely affecting the immune system.

Conclusion: These results indicate that the potent antimicroorganism and anti-inflammation properties of kanuka and manuka essential oils make them strong candidates for use in treating infections and immune-related disease. The data confirm the potential use of kanuka and manuka extracts as pharmaceutical antibiotics, medical cosmetology agents, and food supplements.

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Introduction

Kanuka (*Kunzea ericoides*) and manuka (*Leptospermum scoparium*) tea trees are small trees or shrubs distributed throughout New Zealand in widely varying climates, altitudes, and population densities. Early New Zealand records report the use of the bark, leaves, sap, and seed capsules of these plants in beverage supplements and in pharmaceutical and medicinal preparations.¹ Kanuka and manuka are commonly known as “tea trees” because many early New Zealand settlers made tea from the leaves.² Accumulating evidence of the unique biofunctional activities and medicinal properties of these oils has recently generated renewed interest in their potential commercial applications.³ Traditional medical applications of the white leaf gums of kanuka and manuka include internal or external sedatives, fever reducers, and cough suppressants. A decoction of the seed capsules can also be used to treat inflammation. A liquid form of the decoction alleviates dysentery, diarrhea, and colic pain. The capsules or leaves could also be chewed to relieve dysentery.⁴ A decoction formed by boiling the leaves and bark has proven effective for treating breast inflammation, back stiffness, and eye problems. A poultice formed from pounded capsules of the plants was used to dry open wounds or running sores and to treat scald and burn injuries. Finally, the liquid could be used as a mouthwash or gargle to treat mouth and throat sores.¹

A troubling global public health issue that has emerged in recent decades is hospital-acquired infections or nosocomial illnesses caused by microorganisms.⁵ Pathogenic fungi and bacteria are able to survive for extended periods on human superficial skin, mucosa, or environmental surfaces, and have been implicated in infectious outbreaks in hospitals, medical facilities, and institutions in many countries.^{6,7} As the use of broad-spectrum antibiotics increases, the most alarming characteristic of these microorganisms is their apparent resistance to almost all commercially available antimicrobial drugs. As a result, many of these microorganisms are now classified as highly

antibiotic-resistant.^{3,8} Therefore, the search for new therapeutic modalities has increased requirements in natural medicinal therapy. *Malassezia furfur* is a lipophilic yeast that lives on the normal skin flora of many animals, including humans. Because the growth of this fungus requires a fat source, it is most common in areas with many sebaceous glands, including the scalp, face, and upper body. Opportunistic infections involving some species of *M. furfur* may cause hypopigmentation in the trunk and other locations in humans.⁹ *Trichosporon mucoides* is usually found in soil and water. Although it can contaminate human skin, its effects in otherwise healthy individuals are usually either harmless or are limited to superficial skin and nail infections. In immunocompromised patients or in patients currently undergoing immunosuppressive therapy, however, an opportunistic infection with *Trichosporon* can be lethal.¹⁰ Another genus of yeast is *Candida*, in which the *Candida albicans* and *Candida tropicalis* species are common and easily recognized medical yeast pathogens because they are normal constituents of the human flora.^{11,12} The most common species of *Staphylococcus* is *Staphylococcus aureus*, which causes staphylococcal infections and is frequently found in the human respiratory tract and on skin surfaces. The emergence of antibiotic-resistant forms of pathogenic *S. aureus* (e.g., methicillin-resistant *S. aureus*) is a worldwide problem in clinical medicine.¹³ Another species in the *Streptococcus* genus is *Streptococcus sobrinus*, which is a spherically-shaped anaerobic and Gram-positive bacterium. They grow in pairs or chains, and they are not motile and do not form spores. *Streptococcus mutans* is a facultatively anaerobic, Gram-positive, coccus-shaped bacterium commonly found in the human oral cavity and is a significant contributor to tooth decay. The most intensively studied prokaryotic model organism is *Escherichia coli*, a Gram-negative, rod-shaped bacterium commonly found in the lower intestine of warm-blooded organisms. This bacterium is easily and inexpensively grown in a laboratory setting and has been studied intensively in the past half century.¹⁴

The human immune system can be defined as a mechanism for protecting the biochemistry of an organism against sickness, including viruses, microorganisms, worms, allergies, inflammation, and cancer. An important component of the immune system is the T-helper (Th) cell, which initiates and regulates immune responses.^{15,16} The four major subtypes of Th cells in humans are: Th1, which regulates inflammatory responses to infections; Th2, which modulates allergic responses; T regulatory cells, which have a pivotal role in immune suppression; and Th17, which is associated with autoimmunity. The inflammatory responses of Th1 are mainly triggered by cytokines from monocytes/macrophages. When monocytes polarize to macrophages, cells recognize parasitological antigens and secrete proinflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interleukins (ILs).¹⁷ TNF- α and IL-6 released from monocytes/macrophages then encounter and activate antigen-specific natural killer T cells in a process known as the Th1 response. By contrast, in response to allergen exposure, macrophages alternatively secrete Th2 cytokines, such as IL-4, and activate Th2-mediated allergic responses.^{18–20} Given the negative feedback between Th1 and Th2 (i.e., activation of one inactivates the other), an anti-inflammatory agent might also exhibit an increased but parallel allergic effect.²¹ Lipopolysaccharide (LPS) from bacteria is a model compound with a well-known role in inflammatory effectiveness, whereas TNF- α initiates Th1-mediated inflammatory responses, and IL-4 inhibits inflammatory responses and initiates allergic responses. This study detected both of these major cytokines, TNF- α and IL-4, which were generated by a macrophage cell line, human acute monocytic leukemia cell line (THP-1).

The antimicrobial and anti-inflammatory characteristics of plant extracts have been studied and applied for thousands of years.^{19,22} The use of plant-derived constituents as preservatives, cosmetics, and pharmaceuticals have recently attracted increased interest. The present work analyzed two essential oil extracts to identify their antimicrobial activities in four fungi and four bacteria. The releases of inflammatory factors TNF- α and IL-4 in THP-1 cells were also examined. The potent anti-microbial and anti-inflammatory properties observed in these oils confirm their strong potential use as medicinal pharmaceuticals.

Materials and methods

Reagents and oil samples

Dimethyl sulfoxide (DMSO) and Luria–Bertani broth were purchased from Sigma-Aldrich Chemical Inc. (St Louis, MO, USA). Sabouraud dextrose (SD) agar and SD broth were purchased from Creative Media Products, Ltd. (Wugu Shiang, Taiwan, R.O.C.). Dulbecco modified Eagle's medium, fetal bovine serum, penicillin, and streptomycin were purchased from Life Technologies Co., Ltd. (Gibco, Grand Island, NY, USA). XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] assay kit was purchased from Roche Diagnostics (Mannheim, Germany). Kanuka and manuka essential oils (100%) were provided by a local company (YO-HOON Trading Co, Ltd, Kaohsiung, Taiwan). Both essential oils were extracted from the leaves

of plants by steam distillation. All buffers and other reagents were of the highest purity commercially available.

Bacterial and fungal species

Six microorganism strains used in this study were purchased from American Type Culture Collection (ATCC), included *T. mucooides* (ATCC 204094), *C. tropicalis* (ATCC 9968), *S. aureus* (ATCC 29213), *S. mutans* (ATCC 25175), *S. sobrinus* (ATCC 33478), and *E. coli* (ATCC 35218). Clinical isolates of other microorganisms, *M. furfur* and *C. albicans*, were provided by Kaohsiung Veterans General Hospital, a 1400-bed tertiary referral medical center in Taiwan. The THP-1 cell line used in this study, which was purchased from ATCC (TIB-202), was derived from the peripheral blood of a 1-year-old human male with acute monocytic leukemia.

Miniaturized broth dilution susceptibility test of anti-fungal activity

The SD broth was used to examine the susceptibilities of the minimum inhibitory concentration (MIC) of kanuka and manuka essential oils by the microdilution method in sterile 96-well microtiter plates covered with lids. For analyzing oils, this method provides better reproducibility compared to the agar plate diffusion and dilution techniques used to study fungi.^{5,20,22,23} The MIC values of each strain were determined by dissolving the testing samples in DMSO at various concentrations with two-fold serial dilutions in broth. DMSO was used as a blank vehicle control solvent, and untreated microorganisms were used as negative controls. To ensure that DMSO did not affect the assays, DMSO concentrations used in the experiment were 5% lower than those in controls. Comparisons showed no significant difference between samples with and without DMSO.

Briefly, after incubation at 35°C for 2–7 days, *M. furfur*, *T. mucooides*, *C. tropicalis*, and *C. albicans* colonies from the SD agar plates were mixed with sterile water and then uniformly rotated. The fungal suspension was adjusted to an approximate turbidity of 1×10^6 – 5×10^6 CFU/mL (0.5 McFarland suspension, VITEK Special DR100 Colorimeter 52-1210; Hach Company, Loveland, CO, USA). Varying fold dilutions of the fungal suspension in sterile water (1×10^4 – 5×10^4 CFU/mL) were then added to 0.05 mL SD broth. After adding 0.01 mL of fungal solution to each well of a 96-well microtiter plate, the final fungi concentrations were 1×10^2 – 5×10^2 CFU/well. Addition of 0.05 mL kanuka or manuka essential oil then obtained concentrations ranging from 0.01% to 12.5%. After incubation in darkness for 2–7 days at 35°C, the MIC values of the two oils were established for each of the four fungi.²⁴ All experiments were performed in triplicate with the blank vehicle control, negative control, and experimental control groups.

Determination of antibacterial properties

The antimicrobial properties of the kanuka and manuka essential oils were investigated using previously described methods.^{5,13} Briefly, 10^5 CFU/mL microbial suspensions of four bacteria, *S. aureus*, *S. mutans*, *S. sobrinus*, and *E. coli*, were incubated in each well at 37°C for 24 hours. The

microbial bacteria were then harvested in normal saline and adjusted to McFarland 0.5 (1.5×10^8 CFU/mL). One milliliter of each bacterial sample suspension was centrifuged at 470g for 5 minutes and then treated with 0.3 mL of an essential oil to obtain a final concentration of 10% v/v. Reactions were then compared at 25°C at intervals of 5 seconds, 30 seconds, 60 seconds, 180 seconds, 300 seconds, and 900 seconds. A well containing DMSO was used as the growth blank vehicle control; a well containing medium only was used as the negative control; and all the other wells contained the experimental groups treated with essential oils. A test was considered valid if the well for the growth control group was positive and those for other groups were negative. After the specified reaction times, the bacteria were centrifuged for 2 minutes, washed once, and then suspended in sterile saline water. A 10^4 -fold dilution of the bacterial suspension (100 μ L), was then plated on blood agar plates. After a 24-hour incubation period, the bactericidal effects of the essential oils were determined in each sample by measuring bacterial growth in culture.²¹ The inhibition of bacterial growth was then measured by comparison with normal growth observed in microbes not treated with the samples.

XTT cell viability assay and the inflammation evaluation

The THP-1 cells were grown in Dulbecco modified Eagle's medium with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin and maintained in 75 cm² culture dishes at 37°C in a humidified chamber with 5% CO₂ in air. The cells were routinely passed at a ratio of 1:2 at least every 4 days until the day of experiment as described previously.¹³ On the day prior to the experiment, all cells were washed, and all culture media were replenished. Immune responses produced by treatments with kanuka and manuka essential oils were then detected. The commercially available XTT cell proliferation assay kit (Roche Diagnostics) was used according to the manufacturer instructions. The THP-1 cells were seeded into 96-well plates (2×10^5 cells/mL) with or without kanuka/manuka oil treatments with 0%, 0.1%, 0.5%, 1%, 5%, and 10% essential oils. After 48 hours, XTT reagents were added into each well, and colorimetric formazan products were generated by incubation at 37°C for 4 hours. Absorbance of formazan product was measured at a wavelength of 450 nm with a reference wavelength of 630 nm. Results are expressed as relative cell viability normalized with untreated control.

The THP-1 human monocyte/macrophage cell line is widely used to assess immune regulation, and several cell-type-specific activation cytokines in THP-1 have been identified. Specifically, this study selected TNF- α and IL-4 as monocyte/macrophage triggering cytokine markers. LPS (0.2 μ g/mL) was used as the positive control for measuring inflammation, and selected target cytokines were analyzed with commercially available enzyme-linked immunosorbent assay kits (Quantikine; R&D System, Minneapolis, MN, USA) according to manufacturer instructions. After 48 hours of essential oil treatments, cultured THP-1 cells were centrifuged at 1000g for 10

minutes to obtain cell-free supernatants for use in measuring cytokine secretion.¹³

Statistical analysis

The data are expressed as the mean of values obtained in three experiments. Statistical comparisons were performed by Student *t* test for paired values.

Results

Comparisons of antifungal properties of essential oils

As described above, the literature shows growing evidence of the antimicrobial activities of natural products.⁵ Kanuka and manuka extracts are known to kill various human pathogens, including oral and gastrointestinal pathogens.^{25,26} The fungicidal assay in this study analyzed kanuka and manuka essential oils extracted from New Zealand tea trees. A broth micro-dilution assay was used to measure MIC values and to investigate the effects of the oils in terms of increased or decreased fungal growth. Table 1 demonstrates the assay results for the various concentrations of oils used in the experiment (0.01%, 0.02%, 0.05%, 0.1%, 0.2%, 0.39%, 0.78%, 1.56%, 3.13%, 6.25%, and 12.5%). The most potent effects of kanuka essential oil were observed in *M. furfur* and *T. mucooides* (MIC = 0.78%). In terms of inhibiting effects on fungal growth, kanuka oil was superior to manuka oil (MIC = 1.56%). Additionally, the MIC values of these two strains were two- to four-fold higher than those observed in the analyses of *Candida* species. Although tests of the other two strains showed higher MIC values, fungal growth was still suppressed.

Antibacterial effects of essential oils

The next experiment compared the antibacterial effects of the kanuka and manuka essential oils. At low concentration (10%), both oils were highly effective for inhibiting the growth of *S. aureus*, *S. mutans*, *S. sobrinus*, and *E. coli*. The antibacterial assay also illustrated that both essential oils effectively inhibited both Gram-negative and Gram-

Table 1 The minimum inhibitory concentration levels of the essential oils to four fungal species. The measured values (% v/v solution) obtained by visual inspection of *in vitro* inhibiting effects of kanuka and manuka essential oils on fungal growth (*n* = 3)

	Minimum inhibitory concentration (%)			
	<i>M. furfur</i>	<i>T. mucooides</i>	<i>C. albicans</i>	<i>C. tropicalis</i>
Kanuka essential oil	0.78	0.78	3.13	3.13
Manuka essential oil	1.56	1.56	3.13	3.13

positive bacteria. Specifically, for each microbe, whether Gram-negative or Gram-positive, growth was 100% inhibited by exposures of 5 seconds, 30 seconds, 60 seconds, 180 seconds, 300 seconds, and 900 seconds. The antibacterial effects of the oils still remained potent after long incubation times (data not shown). The data confirm that, in addition to their suppressive effects on bacterial growth, both oils have strong inhibiting effects. Additionally, the remarkable biofunctions of the kanuka and manuka tea tree oil samples analyzed within this study included both anti-fungal and antibacterial activities.

Anti-inflammatory properties of kanuka and manuka essential oils

After confirming the antimicrobial effects of kanuka and manuka essential oils, this study further investigated other effects such as suppression or inhibition of inflammatory reactions. Fig. 1 demonstrates that, after 48-hour exposure to test concentrations of kanuka and manuka essential oils ranging from 0.1% to 10%, the viabilities of THP-1 cells exceeded 100%. This reveals that the oils have no major toxic side effects on THP-1 cells. In the absence of LPS stimulation, 48-hour treatments with kanuka essential oil

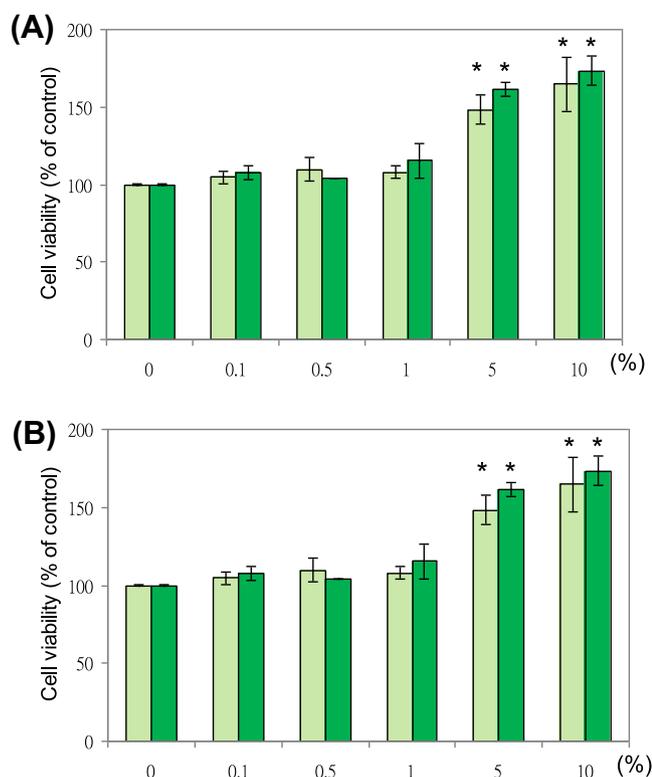


Figure 1. XTT assay results for THP-1 cells after 48 hours treatment with 0%, 0.1%, 0.5%, 1%, 5%, and 10% (A) kanuka and (B) manuka essential oils with dark green color, respectively; LPS (0.2 $\mu\text{g}/\text{mL}$) was used as the positive control for measuring inflammation reactions. * $p < 0.05$ versus untreated vehicle control group; mean \pm standard deviation. LPS = lipopolysaccharide; XTT = 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide; THP-1 = human acute monocytic leukemia cell line.

concentrations ranging from 0.1% to 10% did not significantly change the release of cytokines, TNF- α , or IL-4 from cultured THP-1 cells (Fig. 2). The bacterial endotoxin LPS (0.2 $\mu\text{g}/\text{mL}$) elicits a strong inflammatory response in THP-1 cells. Cotreatment with LPS and 0.1–10% kanuka oil during cell culture was performed to display the effects on THP-1 cytokine secretion. Kanuka essential oil treatment significantly reduced the LPS-induced TNF- α release from THP-1 cells, but did not affect IL-4. Similarly, manuka essential oil treatment had an anti-inflammatory effect on LPS-induced release of TNF- α , but with no influence on IL-4 (Fig. 3).

Discussion

Although some essential oils such as tea tree oils are known to have antimicrobial effects, clinical evidence of their efficacy for treating bacterial, fungal, or viral infections is limited. Kanuka is a large tree or shrub abundant throughout New Zealand and manuka is distinguishable by its relatively larger leaves and flowers, but smaller overall

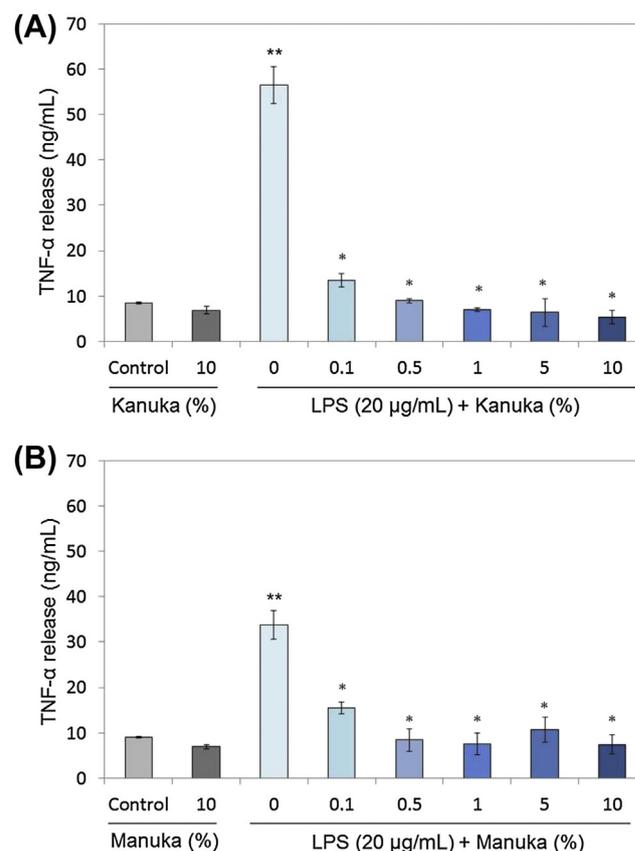


Figure 2. Enzyme-linked immunosorbent assay results for tumor necrosis factor (TNF- α) cytokine released from THP-1 cells after stimulation with LPS (20 $\mu\text{g}/\text{mL}$) and 48 hours treatment with 0%, 0.1%, 0.5%, 1%, 5%, and 10% (A) kanuka and (B) manuka essential oils; ** $p < 0.05$ versus untreated vehicle control group; * $p < 0.05$ versus group treated with 0.1–10% of the two oils after LPS incubation; mean \pm standard deviation. LPS = lipopolysaccharide; THP-1 = human acute monocytic leukemia cell line.

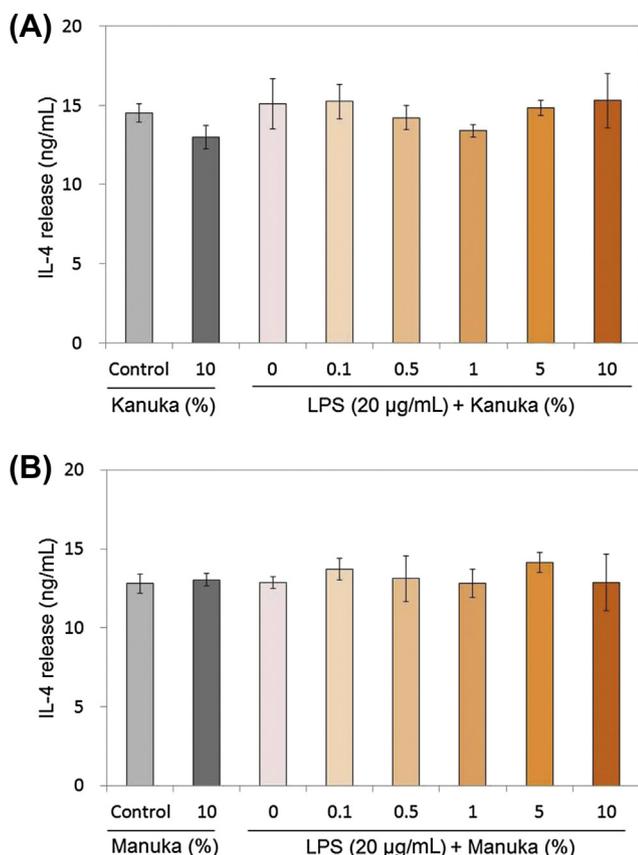


Figure 3. Enzyme-linked immunosorbent assay results for IL-4 cytokines released from THP-1 cells after stimulation with LPS (20 µg/mL) and 48 hours treatment with 0%, 0.1%, 0.5%, 1%, 5%, and 10% of (A) kanuka and (B) manuka essential oils versus untreated control samples. IL = interleukin; THP-1 = human acute monocytic leukemia cell line.

size. The Maori people and early New Zealand settlers used both plants in traditional medicinal preparations.^{1,27} Maddocks-Jennings et al²⁷ reported verification suggesting that a gargle or mouthwash containing the essential oils of kanuka and manuka extracts could delay the development of mucositis and reduce associated health problems. According to these lecture reports, both essential oils were with low irritations to human skin, functions on improve oral infection, benefits in ease-breath, and help in cooling down user's body.^{1,28} Commercial applications of tea tree essential oils for their antimicrobial and antifungal properties are in soaps, creams, mouthwashes, toothpastes, and other preparations. Internal and external use of these oils by herbalists and aroma therapists has as well increased in the past twenty years.^{4,11}

The recent emergence of new strains of bacteria and fungi warrants intensive study of therapeutic agents for their control and eradication.^{29,30} Due to the widespread use of tea tree oils for body massage and aromatherapy, *in vivo* studies have been performed to investigate their pharmacological and antimicrobial activities in guinea pig ileum, rat uterus, rat skeletal muscle, chick biventer muscle, and rat phrenic nerve diaphragm.⁴ Studies of manuka honey produced by New Zealand tea trees

suggested that, whereas the antibacterial properties of honey products are derived from hydrogen peroxide, those of manuka oil are derived through other mechanisms.^{31,32} Our data indicate that both oils might possess various properties to inhibit microorganism growth. Unique manuka factor is currently the global standard for identifying and measuring the antibacterial strength of manuka in specific strains of bacteria.¹¹ The antibacterial activity of manuka honey is now standardized in terms of phenol concentration equivalent, which is expressed as a unique manuka factor value.^{30,33} However, further studies are needed to identify the antimicroorganism mechanisms of these two target essential oils.

Our data specify that neither oil treatment had stimulatory effects on cytokine release in untreated THP-1 macrophages. Interestingly, both oils showed strong inhibiting effects on inflammation induced by LPS treatment. Because TNF- α release from monocytes/macrophages regulates Th1-mediated inflammatory responses, short-term nontoxic dosages of the two essential oils may be effective for treating inflammation. For example, the oils may be effective for treating lesions caused by insect bites or for repairing infectious wounds. Both essential oils reduced the TNF- α production (Fig. 2) and did not cause cytotoxicity to THP-1. It is possible that essential oil may directly interact with the toll-like receptor 4, which is an LPS receptor. However, we did not have enough evidence for this interaction within the present study. We cannot rule out the possibility for the involvement of the toll-like receptor 4 competition with essential oils, and this needs to be further investigated in the future. Therefore, we focused on the changes of inflammatory outcome, the TNF- α marker. The oil treatments did not significantly affect IL-4 release, which suggests that, in addition to the anti-inflammatory properties, the oils have potential applications as anti-allergic agents. Because of their anti-inflammatory properties and their absence of adverse allergic reactions resulting from cytokine release, kanuka and manuka oils may also be effective in human epidermal-related products.

Aromatherapy is an alternative medicine practice in which volatile plant materials, known as essential oils, and other aromatic compounds are used to improve the emotional state, cognitive function, or physical health.⁴ However, the literature on the efficacy of aromatherapies for treating medical conditions, especially studies that have applied a rigorous methodology, is very limited. Nevertheless, some data indicate that essential oils might have therapeutic applications.¹⁵ Essential oils are occasionally used to describe fragrant oils extracted from plant materials by any solvent-based extraction methods. Aromatherapists and other practitioners need to understand that the clinical use of kanuka or manuka oils for the varying compositions of the extracts.² Another issue that arises when new pharmaceutical applications of plant components are introduced is their modulating effects when used in combined therapy with commercial antibiotics or other anti-inflammatory medicines. Although some studies have reported synergistic effects of natural plant extracts and antibiotics when used in combined therapy,⁵ further study is needed to identify the efficacy of essential oils for inhibiting microbial growth and inflammation

and particularly the chemical and taxonomic properties of kanuka and manuka. Given the enormous potential of the clinical applications of essential oils and the growing evidence of their antimicrobial and anti-inflammatory effects, continuing study of potential medical applications of these and other locally produced extracts is needed. The promising findings of this study warrant further evaluation of the current model for use in objectively measuring a contaminated wound environment and for assessing the mechanisms of medicinal applications of kanuka and manuka oils. However, long-term tests of the toxicity and ion-specific effects of these oils are needed to verify their safety and effectiveness for clinical use as wound healing agents and for aromatherapy.

Conflicts of interest

No contributing author has a conflict of interest in the publication of this study.

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