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Mātauranga-guided screening of New Zealand native plants reveals flavonoids from kānuka (*Kunzea robusta*) with anti-*Phytophthora* activity

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ABSTRACT

Kauri is an ecologically important and culturally treasured tree species in Aotearoa New Zealand. It is under threat from the pathogenic oomycete *Phytophthora agathidicida*, which causes kauri dieback disease. We hypothesised that mātauranga Māori (Māori knowledge) of kauri forest health could be used to identify native plants that produce anti-*Phytophthora* compounds. We tested this hypothesis by using knowledge descended from Te Whare Wananga o Ngāpuhi to select and screen four native plants for activity against *P. agathidicida* and also *P. cinnamomi* (a broad host-range pathogen). Extracts of kānuka (*Kunzea robusta*) were active against various life cycle stages. Bioassay-directed isolation led to three flavanones, previously unreported from New Zealand *Kunzea*, as the main bioactives. These compounds have not previously been reported as having anti-*Phytophthora* activities. They inhibited *P. agathidicida* zoospore germination with IC_{50} values of 1.4–6.5 µg/mL, making them the most potent inhibitors reported against this stage of the life cycle. The three flavanones also inhibited zoospore motility at 2.5–5.0 µg/mL, and showed some inhibition of mycelial growth at 100 µg/mL. They were generally less active against *P. cinnamomi*. Overall, the results from this study emphasise the value of using mātauranga Māori in the response to kauri dieback.

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Introduction

Kauri (*Agathis australis*) is an important endemic species in New Zealand. It is a foundation species that has a profound influence on the surrounding soil, canopy, and biodiversity (Waipara et al. 2013; Wyse et al. 2013; Wyse et al. 2014). Kauri is also one of the longest-lived species known, with individual trees reported to be more than 1500 years old

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(Steward and Beveridge 2010). Massive trees with heights of up to 60 m and trunk diameters exceeding 7 m have been recorded (Ecroyd 1982; Steward and Beveridge 2010). However, land clearance and a century of unregulated logging (*circa* 1830–1930) has dramatically reduced the area of virgin primary kauri forest in New Zealand to less than 1% of that at the time of European settlement (Steward and Beveridge 2010). Kauri trees are now also threatened by the pathogen *Phytophthora agathidicida*, which causes kauri dieback disease (Beever et al. 2009; Weir et al. 2015).

P. agathidicida is a member of the oomycete genus *Phytophthora*, other members of which cause diseases in thousands of economically and ecologically important plants worldwide. Often referred to as 'fungus-like', *Phytophthora* are actually more closely related to diatoms and brown algae (Baldauf et al. 2000). Practically speaking, this means *Phytophthora* are unaffected by most agrichemical fungicides. *Phytophthora* lack many of the common fungicide targets, such as the ergosterol biosynthesis pathway and chitin-based cell walls (Judelson and Blanco 2005; Oliver and Hewitt 2014). *Phytophthora* also have several life cycle stages that are not found in most true fungi. In addition to a mycelial growth phase (similar to fungi), *P. agathidicida* produces two key types of spores: oospores and zoospores (Weir et al. 2015). Oospores are non-motile 'survival' spores, which are generally known to persist in plant tissues or the surrounding soil for years after the host plant dies (Collins et al. 2012; Crone et al. 2013). Zoospores are motile 'dispersal' spores. They are key to the epidemic spread of disease, facilitating host-to-host transmission (Carlile 1985; Tyler 2002). Once a zoospore locates a host plant, it encysts and initiates infection (Judelson and Blanco 2005).

When a kauri tree is infected by *P. agathidicida*, several symptoms typically occur including root and collar rot, trunk lesions, canopy thinning, and ultimately tree death (Beever et al. 2009; Bellgard et al. 2016). Trees of all ages are susceptible. Therefore this disease poses a significant threat to the long-term survival of kauri (Beever et al. 2009; Waipara et al. 2013).

Globally, there are few anti-oomycete formulations available for controlling *Phytophthora* diseases. Phosphite is the only treatment currently being used to control *P. agathidicida* (Horner 2013). It is primarily applied via injections into the trunks of trees that are already showing visible signs of infection. Furthermore, the use of existing agrichemicals, including phosphite, is jeopardised by increasing anti-microbial resistance around the world (Parra and Ristaino 2001; Dobrowolski et al. 2008; Gisi and Sierotzki 2008; Miao et al. 2016). There is an urgent need to discover and develop novel compounds that target the growth, survival and dispersal of *Phytophthora* in general, and of *P. agathidicida* in the particular case of controlling kauri dieback.

Plants are rich sources of known and potential anti-microbial compounds (Bennett and Wallsgrove 1994; Cowan 1999; Abreu et al. 2012; Pusztahelyi et al. 2015). In this study, we have begun to explore the anti-*Phytophthora* potential of New Zealand native plants. There are over 2,300 vascular plants native to New Zealand (de Lange et al. 2018) and ~80% of these are endemic. Many are already known to be useful in rongoā (indigenous plant-based medicine practices). Therefore, we hypothesised that mātauranga Māori (Māori knowledge) could be used to select native plants that were most likely to produce anti-*Phytophthora* compounds.

Here we report the screening of four plants that were selected based on mātauranga Māori for potential activity against *P. agathidicida*. In order to assess whether activity

was specific to *P. agathidicida*, or generalisable to other *Phytophthora*, a second species (*P. cinnamomi*) was also tested. *P. cinnamomi* is a broad host-range pathogen capable of infecting thousands of different plant species worldwide (Kamoun et al. 2015; Hardham and Blackman 2018). In New Zealand, *P. cinnamomi* is found across native ecosystems, exotic forests, nurseries and both agricultural and horticultural settings (Scott and Williams 2014). *P. cinnamomi* has also been linked to ill-thrift of kauri (Waipara et al. 2013). However its overall impact in New Zealand remains poorly understood.

The four plants screened for anti-*Phytophthora* activities were harvested from Waima, New Zealand. Root and leaf extracts of each plant were screened *in vitro* for inhibition of three key steps of the *Phytophthora* lifecycle process: zoospore motility, zoospore germination, and mycelial growth. The results of this screening, as well as identification and further characterisation of three compounds purified from kānuka (*Kunzea robusta*), are reported.

Materials and methods

Selection of plants for screening

Mātauranga Māori was used as the basis for selection of four endemic plants for anti-*Phytophthora* screening. The knowledge used to select these plants descends from Te Whare Wananga o Ngāpuhi (the sacred house of learning of Ngāpuhi). More information on the history of this mātauranga can be found in the Supplemental Information.

Intact plant samples of kānuka (*K. robusta* de Lange et Toelken, family Myrtaceae, voucher code 180508_04), karamū (*Coprosma robusta* Raoul, Rubiaceae, 180508_05), kawakawa (*Piper excelsum* Forst. f., Piperaceae, 180508_01) and nīkau *(Rhopalostylis sapida* Wendl. et Drude, Arecaceae, 180508_02) were collected from the riparian margin of a tributary of the Waima River, Waima, New Zealand in May 2018. We note that *Kunzea robusta* is often referred to as kānuka; however, native speakers of Ngāpuhi dialect refer to this plant as mānuka – as its flowers and timber are white or mā. In order to avoid confusion, the name kānuka is used throughout this manuscript to describe *Kunzea robusta*. Other Māori/common names of plants species referred to in this paper are consistent with Ngāpuhi dialect.

One sample of each plant species was collected at the first harvest. Three additional kānuka plants were collected from the same location in July 2018 (180720_03, 04, 06). Botanical voucher samples were prepared for each plant and are available from the Plant & Food Research, University of Otago herbarium collection (see codes above). The identification of the kānuka as *K. robusta* was made by P. de Lange.

Preparation of crude extracts

Crude root and leaf extracts were prepared as follows: finely ground dried plant material (2 g) was shaken overnight with 95% ethanol (20 mL), then filtered to give extracts. Sub samples were taken and dried overnight in a SpeedVac (Thermo Scientific, Waltham, MA) at ambient temperature. Crude extracts were prepared by resuspending the dried samples in ethanol at approximately 10–20 mg/mL (*i.e.* the highest concentrations possible, as determined by solubilities of the dried extracts). The crude extract concentrations

used in biological assays were 100-fold dilutions of these solutions. The final ethanol concentration in the biological assays was therefore 1% (v/v); this concentration of ethanol was shown to be non-inhibitory in the assays used.

Phytophthora isolates and culture conditions

P. agathidicida isolate NZFS 3770 and *P. cinnamomi* isolate NZFS 3910 (both provided by Scion, Rotorua, New Zealand) were routinely cultured at 22°C in darkness on clarified 20% V8 juice agar (cV8-agar). Detailed instructions for the preparation of this and other media used are provided in the Supplemental Information.

For zoospore production, 10 agar plugs (6 mm diameter) were removed from the edge of an actively growing mycelial mat and then transferred to a petri dish containing 15 mL of a 1:10 dilution of cV8 broth (*P. cinnamomi*) or carrot broth (*P. agathidicida*). These were grown for ~30 h at 25°C. Broth was then removed and replaced with 15 mL of Chen-Zentmyer salt solution (for *P. cinnamomi*) or 5% (w/v) sterile soil extract (for *P. agathidicida*). Dishes were incubated at room temperature for 45 min, then the solutions removed and replaced with fresh salt solution/soil extract. This was repeated after another 45 min and the dishes were then incubated overnight at room temperature under light. The following morning, zoospore release was induced by removing the liquid and washing each dish three times with sterile Milli-Q water that had been cooled to 4°C. Each wash was for 20 min, with the first two at room temperature and the final wash at 4°C. Wash volumes were 15 mL per dish for the first two washes, and 10 mL for the final wash. Following the final wash, dishes were returned to room temperature for 30–90 min until sufficient numbers of zoospores had been released. Zoospore densities were in the range of 10^3–10^4 per mL.

Zoospore motility assays

Zoospore motility assays were conducted essentially as described previously (Lawrence et al. 2017). Briefly, motility assays were conducted in 24-well plates. Each well contained 1 mL of a zoospore suspension, and crude extracts were added to a final concentration of ~100–200 µg/mL. Negative control wells contained 1% (v/v) ethanol. Wells were observed at 40 × magnification using an inverted microscope every 5 min for the first 30 min, then at 60 min and every 1 h thereafter. The observation time point at which no zoospores were moving in the well was recorded. The kānuka root and leaf extracts were retested at a range of concentrations (0–100 µg/mL for root extract; 0–200 µg/mL for leaf extract) under the same conditions as above. All assays were performed in duplicate.

Zoospore germination assays

Zoospore suspensions (50 µL) were added to 1 mL cornmeal agar wells amended with either crude extracts (~100–200 µg/mL) or pure compounds. Negative control wells contained 1% (v/v) ethanol. The plates were incubated at 25°C overnight and 25 zoospores were counted per well. Zoospores with germ tubes greater than twice the spore diameter in length were considered germinated. Germination inhibition rates are reported as the percentage of inhibition relative to the number of germinated spores in the negative (ethanol only) control. All assays were performed in duplicate.

Mycelial growth inhibition assays

Assays were performed in 24-well plates. A 2 mm diameter plug was taken from the edge of an actively growing mycelial mat and placed in the centre of each well. Wells contained cornmeal agar amended with extracts at 100–200 µg/mL. Negative control wells contained 1% ethanol. Plates were incubated at 25°C for ~24 h (depending on growth rate), and mat diameters were measured (two perpendicular measurements were averaged for each well). Growth inhibition was calculated by dividing the treatment mat diameter by the negative control mat diameter. Following this initial screen, extracts that showed mycelial growth inhibition (kānuka root and leaf) were tested again at a range of concentrations (0–100 µg/mL for root extract; 0–200 µg/mL for leaf extract), in duplicate, under the same conditions as above.

Extract fractionation and active compound preparation

Kānuka dried leaf sample was finely ground and extracted overnight by shaking with 95% ethanol, 5% H_2O (1:10 leaf mass to solvent volume) then filtered to give an extract, which was stored at – 20°C. Extract (930 mg) was coated onto 1 g of Reversed-Phase (RP) C18 (Aldrich octadecyl-functionalised silica gel) by rotary evaporating at 30°C; then applied to a 5 g C18 Isolute SPE cartridge preconditioned with ethanol (EtOH), then 1:1 EtOH:H_2O, then H_2O (10 mL of each). Elution with 2 × 10 mL each of H_2O, then 1:4 EtOH: H_2O, 1:1 EtOH: H_2O, 4:1 EtOH: H_2O, EtOH, and ethyl acetate (EtOAc) gave twelve 10 mL fractions. Subsamples (200 µL) were vacuum-dried, resuspended in 96% ethanol, and tested against *P. cinnamomi* and *P. agathidicida* in zoospore germination and mycelial growth assays, as described above, to identify the active fractions.

A subsample (28 mg) of active RP fraction 7, eluted from the first RP column with 4:1 EtOH: H_2O, was subjected to preparative RP-liquid chromatography (LC) using an Agilent HP1260, controlled with Agilent OpenLab, at 30°C on a C18 column (Phenomenex Luna ODS(3) 5 µm 100 Å 250 × 10 mm) with a 25 × 4 mm C18 guard column. Peaks were detected at 206 nm. The mobile phase was 60% acetonitrile (MeCN) and 40% H_2O, both with 0.1% formic acid, with a flow rate of 5 mL/min. Six fractions were collected and compounds in fraction 4 (compound **1**, about 8 min, 5 mg) and fraction 5 (compound **2**, about 11 min, 5 mg) were identified by comparing nuclear magnetic resonance (NMR) spectra with published data. A later peak at about 15 min was collected from preparative RP-LC of active fraction 8, identified as compound **3** (<1 mg).

Compound characterisation and bio-activity assays

Analytical RP-LC

Analytical reversed phase RP-LC was carried out using an Agilent HP1260 controlled with Agilent OpenLab, at 30°C on a C18 column (Phenomenex Luna ODS(3) 5 µm 100 Å 150 × 3 mm) with a 2 × 4 mm C18 guard column. Peaks were detected at 206 and 295 nm. The mobile phase was MeCN in H_2O, both with 0.1% formic acid, using a linear gradient starting at 10% MeCN:H_2O to 100% MeCN over 40 min, returning to 10% MeCN:H_2O over 5 min with a 5 min equilibration time, with a flow rate of 0.5 mL/min.

Gas chromatography–mass spectrometry (GC–MS)

Pure compounds were dissolved in chloroform to 0.4 mg/mL and analysed by GC–MS. Analyses were performed on an Agilent 7890A gas chromatograph with a CTC Analytics PAL system autosampler and an Agilent 5975C inert XL MSD with triple axis detector (under the control of Enhanced MassHunter software). The injector (260°C) was split 1:5. Injections (1 μL) were made into a 30-m Agilent HP5-ms column with a 0.25 mm internal diameter and 0.25 μm film. The carrier gas used was hydrogen with a flow of 1.5 mL/min. The flow was split using deactivated silica columns between the MS detector (MSD, split arm 0.5 mL × 0.1 mm ID) and the flame ionisation detector (FID) (2 mL × 0.18 mm ID). The oven was heated from 50°C to 175°C at 5°C/min then to 300°C at 20°C/min and held for 8.75 min. Detection was by total ion current mass spectrometry (the MS transfer line was held at 270°C, the MS source was held at 230°C and the MS quad held at 150°C) over the mass range 35–600 Da and FID. A series of n-alkanes was injected separately and used to calculate the retention indices (RIs) of the pure compounds.

NMR spectroscopy

NMR spectra were recorded for D6-acetone solutions at 25°C on a Varian instrument: ^{1}H at 500 MHz, ^{13}C at 125 MHz. ^{1}H NMR spectra of crude extracts were recorded for $CDCl_3$ solutions at 25°C on a Varian instrument at 400 MHz.

5,7-Dihydroxy-6-methylflavanone (strobopinin) 1

CAS Registry Number (RN) 11023-71-5; analytical RP-LC retention 26.67 min; GC Retention Index (RI) 2576; +ve EI-MS *m/z* (relative intensity %): 270 (M, 100), 193 (75), 166 (100), 138 (95); −ve ESI-MS *m/z*: 269.0820 (M−H, calc for $C_{16}H_{13}O_4$ 269.0819; ^{1}H and ^{13}C NMR) data matching (Mayer 1990) allowing for different solvent; $[\alpha]_D$ ($CHCl_3$, 0.02 mg/mL) −13.5°.

5,7-Dihydroxy-6,8-dimethylflavanone 2

RN 56297-79-1; RP-LC 28.67 min; GC RI 2592; +ve EI-MS *m/z* (%): 284 (M, 95), 207 (40), 180 (80), 152 (100); -ve ESI-MS *m/z*: 283.0981 (M−H, calc for $C_{17}H_{15}O_4$ 283.0976; ^{1}H and ^{13}C NMR) data matching (Mustafa et al. 2005); $[\alpha]_D$ ($CHCl_3$, 0.02 mg/mL) −12.0°.

5-Hydroxy-7-methoxy-6-methylflavanone 3

RN 55743-20-9; RP-LC 32.5 min; GC RI 2532; +ve EI-MS *m/z* (%): 284 (M, 100), 207 (90), 180 (100), 152 (80); ^{1}H NMR data matching reference sample (Plant & Food Research unpublished).

Compound bio-activity assays

Zoospore germination, chemotaxis and motility, and mycelial growth assays were carried out in duplicate as previously described. No motility assays were done for 5-hydroxy-7-methoxy-6-methylflavanone (**3**) due to lack of material.

Intraspecific variation in compound abundance

Variation in compound abundance among individual kānuka plants was assessed using leaf and root extracts prepared from three plants collected in Waima, New Zealand, in

August 2018. Extraction was done as described above. The concentrations of the three active compounds in each extract were determined by RP-LC.

Results

Selection of plants for screening

From the perspective of Ngāpuhi mātauranga, regenerating native forest of the type found in the northern kauri rainforest, broadly speaking, comes in three waves. The first wave are plants that help to secure, cleanse and prepare the soil for the next generations to follow. The second wave are plants which typically become the second story in the canopy. These plants, typically fruiting plants, bring the fertility and the conditions for high biodiversity. The third wave includes plants that are long lived, such as kauri. Third-wave plants bring stability and relative permanency. Kauri stand above all other plants as the great protector.

Based on this knowledge, four known first wave plants were selected for study: kānuka, karamū, kawakawa and nīkau. While these are all established rongoā (medicine) plants, they are also known (according to the mātauranga of Ngāpuhi) as essential parts of a natural process to establish and maintain the health of kauri forests. Therefore, this knowledge comes from and belongs to mana whenua (the iwi or hapū which hold customary rights and authority over land and taonga in an area).

Plants offered by mana whenua for testing were selected based on this traditional knowledge. While additional first wave plants are known, these are outside the scope of the present study.

Screening crude extracts for potential anti-Phytophthora activities

Crude extracts of kānuka, karamū, kawakawa and nīkau leaves and roots were tested for their ability to inhibit zoospore motility, zoospore germination, and mycelial growth of *P. agathidicida* and *P. cinnamomi*.

To assess the ability of the crude extracts to abolish zoospore motility, extracts were added to suspensions of swimming zoospores, and the motility of the spores was monitored over time (Table 1). The negative controls (1% ethanol) remained motile for >6 h. As shown in Table 1, most of the crude extracts tested had no impact on zoospore motility, with the zoospores remaining motile over the full length of the experiment (6 h). However, karamū root extract caused immediate motility loss for *P. agathidicida*

Table 1. Effect of crude extracts on zoospore motility. The length of time zoospores remained motile after the addition of crude extract is shown .

Extract	*P. agathidicida*	*P. cinnamomi*
Kānuka leaf	<5 min	<5 min
Kānuka root	<5 min	<5 min
Karamū leaf	>6 h	>6 h
Karamū root	<5 min	<4 h
Kawakawa leaf	>6 h	>6 h
Kawakawa root	>6 h	>6 h
Nīkau leaf	>6 h	>6 h
Nīkau root	>6 h	>6 h

zoospores and a modest reduction in motility time for *P. cinnamomi* zoospores. Most notably, the kānuka leaf and root extracts caused immediate loss of zoospore motility for both species of *Phytophthora* (Table 1).

The crude leaf and root extracts of kānuka also completely eliminated germination of *P. agathidicida* zoospores (Figure 1A); in these assays, zoospore lysis was observed (data not shown). In contrast, no lysis or appreciable inhibition of *P. agathidicida* zoospore germination was observed with either the root or leaf extracts of karamū, kawakawa or nīkau (Figure 1A). For *P. cinnamomi* (Figure 1B), kānuka leaf, but not kānuka root, substantially reduced zoospore germination. The other leaf and root extracts had only a moderate impact on *P. cinnamomi* germination rates (*i.e.* <50% inhibition) (Figure 1B).

In mycelial growth assays, no inhibition was observed with the karamū, kawakawa or nīkau extracts (data not shown). The crude leaf and root extracts of kānuka showed slight inhibition of mycelial growth for both species (Figure 2). The greatest inhibition observed was with kānuka leaf extract, which resulted in ~40% inhibition of *P. agathidicida* mycelial growth (Figure 2A).

Overall, the crude kānuka extracts showed the highest activity in all assays performed and kānuka leaf extract was generally more effective than kānuka root. Based on these results, we selected the kānuka leaf extract for further characterisation.

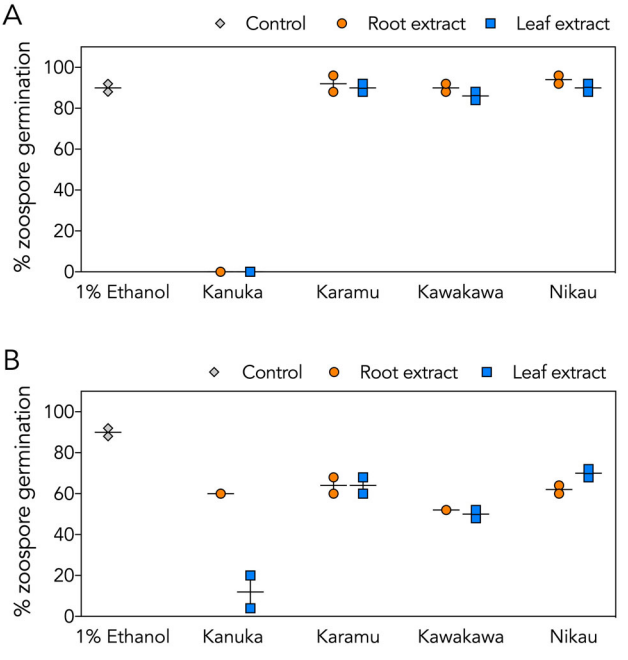

Figure 1. Effects of crude root and leaf extracts on zoospore germination. **A,** *P. agathidicida* zoospore germination. **B,** *P. cinnamomi* zoospore germination. Overall, kānuka leaf extract was the most effective at inhibiting zoospore germination of both species tested. Results from root extracts are shown as orange circles, leaf extracts are shown as blue squares, and the negative control (1% (v/v) ethanol) as grey diamonds. Vertical lines indicate the range, and horizontal lines indicate the mean from two independent assays.

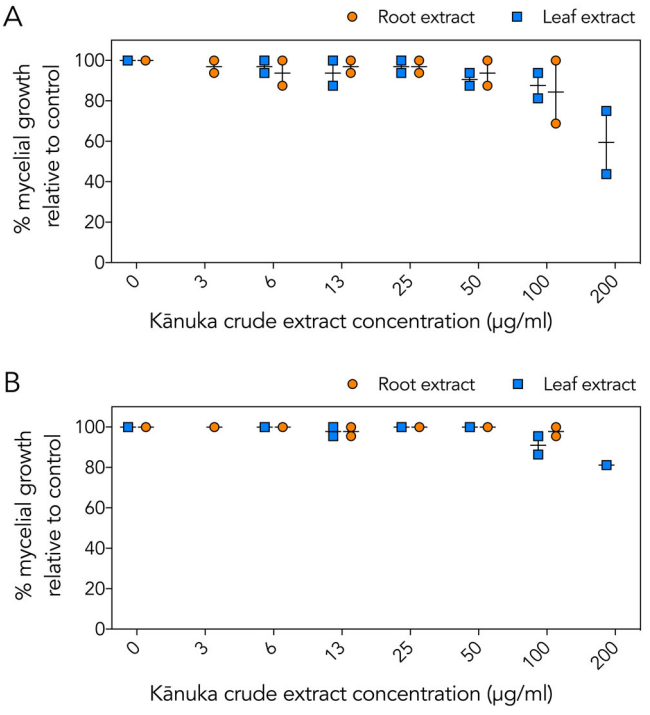

Figure 2. Effect of crude kānuka root and leaf extracts on mycelial growth. **A,** *P. agathidicida* mycelial growth. **B,** *P. cinnamomi* mycelial growth. Growth is reported as a percentage relative to the growth of the negative controls (1% (v/v) ethanol). Results from root extracts are shown as orange circles; leaf extracts are shown as blue squares. Vertical lines indicate the range, and horizontal lines indicate the mean from two independent assays.

Isolation and identification of kānuka anti-Phytophthora compounds

Bioactivity-directed fractionation was used to isolate the active compounds from the kānuka leaf extract. The extract was subjected to a rapid RP fractionation, which separates the molecules based on polarity (Blunt et al. 1987). Twelve fractions were generated, and these fractions were tested for bioactivity (Figure 3). To simplify the screening workflow, we focused on the inhibition of *P. agathidicida* mycelial growth and zoospore germination as our primary bioactivity screens.

In mycelial growth assays, three of the medium polarity fractions (fractions 7, 8, and 9) inhibited growth (Figure 3A). Fraction 7 displayed by far the highest level of activity, inhibiting mycelial growth by ~75%. Fractions 7, 8 and 9 also strongly inhibited zoospore germination (Figure 3B).

The main bioactive compounds in these fractions were purified by RP LC, and identified by high-resolution electrospray ionisation mass spectrometry and NMR spectroscopy. The three active compounds purified from the kānuka leaf extract were identified as flavanones by characteristic H2-$H_2$3 spin system in their ^{1}H NMR spectra, which also showed unsubstituted B-rings (Figure 4). NMR spectra showed that **1** was 5,7-dihydroxy-6-methylflavanone, **2** was 5,7-dihydroxy-6,8-dimethylflavanone, and **3** was 5-hydroxy-7-methoxy-6-methylflavanone. We could not find any previous reports of anti-*Phytophthora* activity for these flavanones.

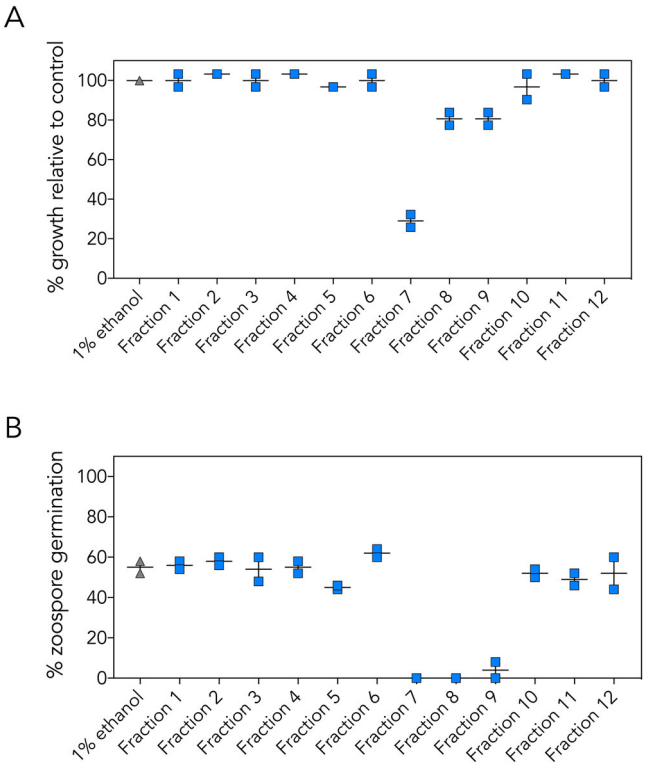

Figure 3. Bioactivity of kānuka leaf extract fractions. Fractions were tested for inhibition of *P. agathidicida* **A** mycelial growth and **B** zoospore germination. 1% (v/v) ethanol was used as the negative control (grey diamonds). Vertical lines indicate the range, and horizontal lines indicate the mean from two independent assays.

Quantification of the anti-Phytophthora activities of isolated kānuka flavanones

The three isolated compounds were tested for their ability to inhibit mycelial growth and zoospore germination of both *Phytophthora* species. Zoospore motility assays were also conducted with compounds **1** and **2**, but not with the small amount of **3** isolated.

Consistent with our observations for the crude extracts (Figure 2), the purified flavanone compounds did not inhibit mycelial growth of *P. cinnamomi*, and were only weakly inhibitory to mycelial growth of *P. agathidicida* (Supplementary Figure 2).

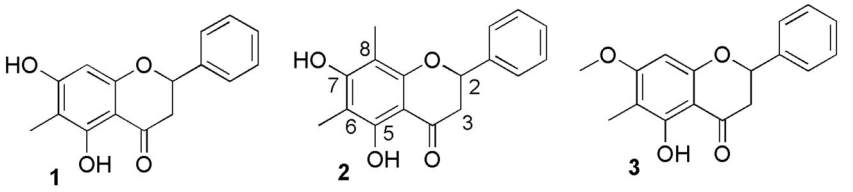

Figure 4. Anti-*Phytophthora* flavanones isolated from kānuka leaves: 1 = 5,7-dihydroxy-6-methylflavanone; 2 = 5,7-dihydroxy-6,8-dimethylflavanone; 3 = 5-hydroxy-7-methoxy-6-methylflavanone.

Table 2. IC_{50} values for inhibition of zoospore germination by compounds **1**–**3**.[a]

Compound	*P. agathidicida*	*P. cinnamomi*
1 = 5,7-dihydroxy-6-methylflavanone	1.4 µg/mL (1.2–1.5)	3.1 µg/mL (2.9–3.3)
2 = 5,7-dihydroxy-6,8-dimethylflavanone	6.5 µg/mL (4.0–17)	90 µg/mL (54–470)
3 = 5-hydroxy-7-methoxy-6 methylflavanone	3.8 µg/mL (3.1–4.8)	10 µg/mL (6.7–18)

Numbers in parentheses are 95% confidence intervals.

Table 3. Minimum concentration of the compounds **1**–**3** that resulted in immediate loss of zoospore motility.

Compound	*P. agathidicida*	*P. cinnamomi*
1 = 5,7-dihydroxy-6-methylflavanone	2.5 µg/mL	2.5 µg/mL
2 = 5,7-dihydroxy-6,8-dimethylflavanone	5.0 µg/mL	1.25 µg/mL
3 = 5-hydroxy-7-methoxy-6-methylflavanone	Not tested	Not tested

Table 4. Concentrations of flavanones **1**–**3** in leaf extracts of four individual kānuka plants.

	Compound (mg/g of foliage dry weight)		
Kānuka plant samples	Compound **1**	Compound **2**	Compound **3**
(a) Hokianga May 2018	10.5	4.3	6.6
(b) Hokianga Aug 2018	1.3	4.8	0.5
(c) Hokianga Aug 2018	5.4	5.3	4.2
(d) Hokianga Aug 2018	3.1	3.3	1.2

Of the compounds tested, **1** showed the strongest effect on *P. agathidicida* mycelial growth, reducing growth by approximately 50% at the highest concentration tested (100 µg/mL).

All three compounds were strongly inhibitory to zoospore germination, especially for *P. agathidicida*. The half maximal inhibitory concentration (IC_{50}) for each compound was calculated using dose–response curves (Supplementary Figure 1). The calculated IC_{50} values for all compounds are presented in Table 2. Compound **1** was particularly effective at inhibiting *P. agathidicida* zoospore germination, with an IC_{50} value of 1.4 µg/mL. The IC_{50} values for compounds **1** and **3** were approximately twice as high in *P. cinnamomi*, while compound **2** was relatively ineffective against this species, with an IC_{50} around 90 µg/mL.

Compounds **1** and **2** also inhibited zoospore motility in both *Phytophthora* species. The minimum concentration causing immediate loss of motility was ≤5 µg/mL for both compounds (Table 3).

Preliminary assessment of plant-to-plant variation in abundance of compounds 1–3

Quantitative RP-LC analysis revealed that the active compounds were much more concentrated in leaf extracts than in root extracts (where they were barely detectable).

The yield of the bioactive compounds (**1**–**3**) varied within an order of magnitude between individual kānuka plants that were harvested from the same area but at different times of the year (Table 4).

Discussion

Mātauranga-guided biodiscovery

The hit rate in this study, with one out of four rongoā plants showing strong anti-*Phytophthora* activity, supports the use of mātauranga Māori to focus screening efforts. For comparison, this hit rate is much higher than our previous study, in which we screened over 100 commercially-available antimicrobial compounds and identified only eight that showed activity against both *P. agathidicida and P. cinnamomi* (Lawrence et al. 2017). These results are also consistent with the other larger international studies that demonstrate the predictive power of traditional knowledge systems in bioprospecting efforts (Saslis-Lagoudakis et al. 2012; Buenz et al. 2018).

The most active extracts obtained by the preparation methods used in this study were from kānuka (*Kunzea robusta*). We note that other preparation methods (in particular, methods based on rongoā knowledge) may yield different results. Overall, the kānuka leaves contained higher levels of active compounds than roots, therefore we predict that the flavanones are localised within 'oil glands', as they are in mānuka (Killeen et al. 2015). The main bioactives in kānuka leaves (flavanones **1** and **2**) exerted strong inhibitory effects on zoospore motility in both *P. agathidicida* and *P. cinnamomi* (Table 2). Compounds **1**–**3** all inhibited the germination of *P. agathidicida* zoospores with IC_{50} values in the range 1.4–6.5 μg/mL (Table 3). The most efficacious chemical in our previous screen was $CuCl_2$, which showed an IC_{50} of ∼5 μg/mL (Lawrence et al. 2017). Thus, the kānuka-derived compound **1** (IC_{50} = 1.4 μg/mL) is the most potent inhibitor of *P. agathidicida* zoospore germination reported to date.

Conversely, compounds **1**–**3** were all relatively ineffective at inhibiting mycelial growth when tested individually. At the highest concentration tested (100 μg/mL), compound **1** inhibited the growth of *P. agathidicida* mycelia by ∼50% (Supplementary Figure 2). In contrast, $CuCl_2$ was ∼15-fold more effective (IC_{50} = 6.8 μg/mL) (Lawrence et al. 2017) while the commercially available agrichemical phosphite is 25-fold more effective (IC_{50} = 4.0 μg/mL) (Horner 2013). The biochemical basis of this specificity towards zoospore germination and motility, but not mycelial growth, remains to be elucidated for compounds **1**–**3**.

Kānuka chemistry

Kānuka is mostly used as a name for trees and shrubs of the genus *Kunzea*, but there are many similarities in form and habitat between *Kunzea* and *Leptospermum scoparium*, (most commonly called mānuka). Furthermore, ten *Kunzea* species have been described in Aotearoa New Zealand (de Lange 2014). The Waima kānuka selected for this study have been botanically identified as *K. robusta*.

Kānuka is commonly found in kauri forests, and it is an effective nurse tree for young kauri (Lloyd 1960). Kānuka is also well-known to rongoā practitioners for its medicinal properties. For example, the bark of kānuka was traditionally used for treating diarrhoea and dysentery (Best 1905). This has led to a number of previous studies to isolate bioactive compounds from the species. Compounds reported previously from kānuka (*i.e. Kunzea* from Aotearoa New Zealand) include phloroglucinols (Bloor 1992) and terpenes (Perry et al. 1997; Porter and Wilkins 1999). Kānuka essential oils and/or purified compounds

have activity against bacteria (Lis-Balchin et al. 2000; Chen et al. 2016; Prosser et al. 2016), viruses (Bloor 1992) and fungi (Chen et al. 2016). However, we are the first to report anti-*Phytophthora* activity of kānuka, and this also seems to be the first report of flavanones from a New Zealand species of *Kunzea*.

Known sources and activities of identified anti-Phytophthora flavanones 1–3

The three active compounds identified in the present study are flavanones, a sub-group of the flavonoid natural products. Flavonoids are ubiquitous in plants and fulfil numerous important roles, including pigmentation, signalling and defence against pathogenic microbes (Havsteen 2002; Samanta et al. 2011). Some members of another flavonoid sub-group, the isoflavones, are known to be chemoattractive to *Phytopthora* spp. (Morris and Ward 1992; Tyler et al. 1996; Hua et al. 2008) and some have been shown to be inhibitory (Rivera-Vargas et al. 1993; Subramanian et al. 2005).

The three flavanones identified in this study have also been identified from other (non-kānuka) plant species. However, none of these compounds were previously known to have activity against *Phytophthora*, and this is the first time these compounds have been identified in *K. robusta*.

The most potent anti-*Phytophthora* compound we identified was 5,7-dihydroxy-6-methylflavanone (**1**, also known as strobopinin). It has previously been isolated from a number of other plants including *Pinus strobus* (Matsuura 1957) and *L. scoparium* (mānuka) (Mayer 1990). Strobopinin inhibits the growth of *Staphylococcus aureus* (a Gram-positive bacterium) (Massaro et al. 2014)and also has been reported to have anti-malarial activity (Boonphong et al. 2007).

5,7-Dihydroxy-6,8-dimethylflavanone (**2**) has been found previously in a range of plants, including pōhutukawa (*Metrosideros excelsa*, Myrtaceae) (Mustafa et al. 2005). It has been reported to have anti-bacterial (Massaro et al. 2014), antimalarial (Boonphong et al. 2007; Joseph et al. 2007) and antiviral (Dao et al. 2010) activities.

5-Hydroxy-7-methoxy-6-methylflavanone (**3**) has been found in other members of the family Myrtaceae, including *Heteropyxis natalensis* from South Africa (Maroyi 2019), *Syzygium samarangense* from Thailand (Srivarangkul et al. 2018), and *L. scoparium* from New Zealand (Mayer 1990; Häberlein and Tschiersch 1998). Antimicrobial properties of this compound are currently unknown, but it has been shown to inhibit dengue virus infectivity (Srivarangkul et al. 2018).

Future research and potential applications

In the present study, active flavanone concentrations varied ~10-fold among foliage extracts from four individual kānuka plants from Waima (Table 4). Geographic and seasonal variations in chemical composition and antimicrobial activity have been observed in some Myrtaceae (Häberlein and Tschiersch 1998; Demuner et al. 2011; Sá et al. 2012), but not in others (Demuner et al. 2011; Cascaes et al. 2015). Overall, more samples are needed to determine potential geographic and/or temporal differences in the production of compounds **1**–**3**.

Further research is also needed to determine the mechanisms of action of these compounds against *Phytophthora*. Flavonoid bactericidal activity generally occurs through

one of three mechanisms: (i) perforation of the plasma membrane; (ii) inhibition of energy metabolism; or (iii) inhibition of nucleic acid synthesis (Ahmad et al. 2015). Known antifungal mechanisms of action include membrane disruption, cell wall damage and inhibition of efflux pumps (Seleem et al. 2017). Their action in *Phytophthora* remains unknown, but may involve some of the same processes seen in fungi and bacteria.

In this study, the kānuka extracts and purified compounds were most effective against zoospore motility and germination, but less effective against the mycelial growth that occurs within infected trees. These results suggest that, while not useful for treating existing plant infections, kānuka could have potential applications in limiting zoospore-mediated spread of disease. Possible application methods include companion planting kānuka with host plants, and/or the development of sprays or soil drenches based on the purified compounds. However, any potential applications will require significantly more research. This includes studies of the plant-to-plant variation in flavanone production, the potential of the compounds to leach from roots or leaf matter into surrounding soils, and/or considerations around compound stability and formulation. Furthermore, we caution that while investigation into these (and myriad other) research questions continues, it remains absolutely critical to prevent spread of the disease into uncontaminated kauri forest.

Treaty of Waitangi obligations and Wai 262 obligations

A legal framework for the protection of indigenous knowledge remains elusive, since most provisions for intellectual property law have evolved out of a western view of knowledge as a commodity owned by individuals, not by communities. Under current New Zealand law, researchers can use traditional Māori knowledge without consent or acknowledgement. Similarly, scientific research of taonga plant species is legally allowed to take place without input or consent from mana whenua. The question remains as to how mana whenua can protect their indigenous intellectual property rights and gain economically from the development and implementation of such intellectual knowledge.

We believe that ethical collaboration between mātauranga Māori practitioners and scientists is key. We have specifically sought to frame our research within the principles of the Treaty of Waitangi (*i.e.* partnership, participation and protection). We support the recommendations from Ko Aotearoa Tēnei: Report on the Wai 262 Claim (Waitangi Tribunal 2011). We recognise that although indigenous plants may be valued for their practical benefits, they are not simply resources to be exploited. These plants are taonga to Māori, and therefore the right of mana whenua to practice kaitiakitanga (stewardship) should be acknowledged and respected.

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No potential conflict of interest was reported by the authors.

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