Inhibition of enzymes important for Alzheimer’s disease by antioxidant extracts prepared from 15 New Zealand medicinal trees and bushes

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ABSTRACT
Alzheimer’s disease is characterised by progressive mental deterioration, related to ageing and senility. Thirty methanol and ethyl acetate extracts from 15 native New Zealand trees and bushes were produced and tested for the inhibition of three enzymes related to neurotransmission: acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and beta-secretase (BACE). In addition the IC50 for the antioxidant potential of the extracts was determined. Weinmannia racemosa and Kunzea ericoides were effective inhibitors of AChE-BChE and beta-secretase, respectively. The IC50 for W. racemosa extracts against AChE and BChE ranged between 8.09 μg/mL and 37.07 μg/mL. The ethyl acetate extract of Schefflera digitata was also an effective inhibitor of BChE (IC50 = 25.38 μg/mL). K. ericoides IC50 for beta-secretase were 29.05 μg/mL and 36.40 μg/mL. The highest radical scavenging activity (RSA) was detected in the methanol extract of Aristotelia serrata (IC50 = 2.34 μg/mL), followed by both extracts of Hebe stricta and W. racemosa, and last the methanol extracts of K. ericoides and Pomaderris kumeraho, which presented higher RSA than the reference antioxidant (IC50 < 12.0 μg/mL). W. racemosa showed inhibitory activity against AChE and BChE enzymes and antioxidant activity, which suggests these extracts may have potential for application in patients suffering from Alzheimer’s disease and other dementias.

Introduction
Alzheimer’s disease (AD) is a type of dementia which affects nearly 50 million people worldwide (Gaudreault and Mousseau 2019). It is estimated that 3% of people aged 65–74 years are affected, while 47.2% of patients over 85 years old are affected. Based on statistics and demographic trends, it is likely that the number of AD patients will exponentially increase to an estimated 114 million by 2050 (Adewusi and Steenkamp 2011; Gaudreault and Mousseau 2019). The actual cause of AD is still unclear. Most researchers believed that AD is caused by more than a single factor. Two of the main factors cited include the presence of dense extracellular deposits of amyloid and phosphorylated tau...
protein that form neurofibrillary tangles (Babic 1999). ‘Cholinergic hypothesis’ is another explanation for the progression of AD, the main approach to the treatment of AD. It is believed that cognitive ability is directly related to the level of acetylcholine (ACh) in the brain (Talesa 2001). The loss of ACh is reduced by simulating the cholinergic receptors or prolonging the availability of ACh release. This is achieved by inhibiting ACh hydrolysis caused by acetylcholinesterase (AChE) enzyme (Babic 1999). The AChE inhibitors remain the mainstay in the treatment of AD.

Another brain enzyme called butyrylcholinesterase (BChE) is also believed to contribute to AD progression, especially in the later stages of the disease. The level of AChE may decline by up to 85% in the later stages of the disease, while BChE becomes the predominant cholinesterase in brain (Orhan et al. 2011). BChE is approximately 10% of the total cholinesterase enzyme in a normal human brain and the increase of this enzyme or its activity may also cause the formation of senile plaques (Schelterns and Feldman 2003). The inhibition of BChE activity by BChE inhibitors may retard this process. These inhibitors are especially useful to treat moderate or advanced stages of AD, and can be used as a specific BChE inhibitor or for dual inhibition functions to also reduce the hydrolysis of the brain ACh (Tappayuthpijarn et al. 2012).

As of now, there are yet to develop therapeutic interventions to completely cure AD or to reverse the disease’s progression. Most existing treatments treat AD symptomatically and provide temporary relief for AD patients. Current therapies have been shown to increase the quality of life of AD patients, such as improving their mood, increasing their social interaction, and diminishing memory loss and confusion (Herrmann et al. 2011). According to the Alzheimer’s Association (2020), there are five approved medications, three of which are cholinesterase inhibitors (donepezil, galanthamine, rivastagmine) usually prescribed for early to moderate stages. The other 2 are N-methyl-D-aspartate (NMDA), a receptor antagonist (memantine), and a drug that combines memantine and donepezil, both are prescribed for moderate-to-severe cases. New approaches in AD treatment are being developed. Among them is one based on β-secretase inhibitors. This type of treatment is thought to be an ideal therapeutic target by blocking the production of β-amyloid protein (a major component of the amyloid plaque), which is believed to play an early and crucial role in all cases of AD (Schelterns and Feldman 2003).

Oxidative stress has been known to be the main cause of many diseases. This includes several age-related diseases such as diabetes, autoimmune and central nervous system-type diseases (Khansari et al. 2009). Oxidative damage is thought to be associated with the two main pathological hallmarks of AD (senile plaques and neurofibrillary tangles). Tayeb et al. (2012) emphasised the importance of antioxidants such as Vitamins E and C as possible therapeutic approaches for dementia.

Phytomedicines have emerged as an alternative or complement to standard approved drugs in the pharmaceutical field, as they cause fewer side effects, and present multi-functional properties being procholinergic, antioxidant, anti-amyloid, and anti-inflammatory. In addition, phytomedicines are less toxic and can have multiple synergistic effects (Anekonda and Reddy 2005), such as enhanced cognitive and cholinergic functions.

Despite the abundant biodiversity of New Zealand, only a handful of its endemic plants have been commercially exploited. New Zealand constitutes a distinctive botanic region, with almost 1500 species of higher plants that are endemic to the country (Brooker and Cooper 1961). A previous study revealed the inhibitory potential of extracts obtained
from several New Zealand plants against multiple enzymes of clinical significance (Kellam et al. 1992). Despite this, very few pharmacological studies have been conducted on native plants of New Zealand, especially on those related to the treatment of AD.

In this study, 15 small trees and bushes which are traditionally used in New Zealand by Māori for treatment of different ailments (Riley, 1994) were investigated for their potential role in the development of therapeutic agents for AD. Two different extracts prepared with methanol and ethyl acetate from selected plants were screened for the inhibition of AChE, BChE and beta-secretase. In addition the antioxidant potential of the extracts was also investigated using the DPPH radical scavenging activity (RSA) method and expressed in terms of IC$_{50}$ concentration.

**Materials and methods**

**Plants and preparation of extracts**

Table 1 shows the scientific and common names of the 15 plants (bushes and small trees endemic to New Zealand) selected for this study, as there is prior empirical knowledge from Māori people of the traditional plants uses for mental/memory-related issues. The following species and parts were tested: *Weinmannia racemosa* (bark), *Kunzea ericoides* (leaves), *Pseudowintera colorata* (leaves), *Leptospermum scoparium* (leaves), *Schefflera digitata* (leaves), *Myrsine australis* (leaves), *Aristotelia serrata* (leaves), *Pomaderris kumeraho* (leaves), *Acaena microphylla* (leaves), *Phormium tenax* (rhizomes), *Geniostoma ligustrifolium* (leaves), *Melicytus ramiflorus* (leaves), *Hebe stricta* (leaves), *Macropiper excelsium* (leaves), *Coprosma robusta* (leaves). Plant materials harvested during late Spring/Summer in 2013 were supplied and identified by Fred Allen, an expert with a profound

| Scientific name                  | Local name          | Part of plant used | Type of plant | Scientist                  |
|----------------------------------|---------------------|--------------------|---------------|----------------------------|
| *Weinmannia racemosa*            | kāmahi              | bark               | tree          | Linnaeus C. von f., 1782   |
| *Kunzea ericoides*               | kānuka              | leaves             | tree          | Richard A, 1832            |
| *Pseudowintera colorata*         | horopito, pepper tree | leaves             | small tree    | Raoul E, 1844              |
| *Leptospermum scoparium*         | mānuka, tea tree    | leaves             | tree          | Forster J. and Forster G., 1776 |
| *Schefflera digitata*            | patete, umbrella tree, seven finger | leaves | tree | Forster J. and Forster G., 1776 |
| *Myrsine australis*              | matipo              | leaves             | shrub or small tree | Richard A, 1832 |
| *Aristotelia serrata*            | makomako            | leaves             | small tree    | Oliver W.R.B., 1921        |
| *Pomaderris kumeraho*            | kumarahou           | leaves             | shrub         | Cunningham A., 1839       |
| *Acaena microphylla*             | piripiri            | leaves             | small herb    | Hooker J.D., 1853         |
| *Phormium tenax*                 | harakeke, flax      | rhizomes           | evergreen perennial plant | Forster J. and Forster G., 1776 |
| *Geniostoma ligustrifolium*      | hangehange          | leaves             | shrub         | Cunningham A., 1838       |
| *Melicytus ramiflorus*           | māhoe               | leaves             | small tree    | Forster J. and Forster G., 1776 |
| *Hebe stricta*                   | koromiko            | leaves             | herb          | Jussieu A.L., 1789        |
| *Macropiper excelsium*           | kawakawa            | leaves             | small tree    | Forster G., 1786          |
| *Coprosma robusta*               | karamu              | leaves             | bushy shrub   | Raoul E, 1844             |

*Information extracted from the internet: Māori Plant Use, [https://maoriplantuse.landcareresearch.co.nz](https://maoriplantuse.landcareresearch.co.nz)
knowledge of New Zealand native plants/trees/bushes, from Kiwi Plants Ltd (Member of Te Paepae Matua mo Te Rongoa, Nga Ringa Whakahaere o te iwi Māori, and Natural Products New Zealand). The plant samples, purchased as dried flakes at moisture content lower than 5%, were further dried in the dark inside an oven at 30°C–40°C for 48 h to remove possible moisture. Then, the dried materials were ground before being vacuum-sealed and stored in a dark condition at room temperature prior to extraction.

Soxhlet extraction uses higher temperature than maceration which can affect the phytochemical composition of plant extracts leading to decomposition of many secondary metabolites as well as the formation of artefacts. However, Soxhlet was the method chosen to prepare the extracts due to the following: higher extraction yield, ease of extraction with less time required for extraction, no filtration required after extraction, the ability to pre-evaporate for preparing the dried crude extract, preliminary results showing higher inhibition of enzymes by some of the extracts prepared using Soxhlet extraction compared to maceration at room temperature. Ten grams of finely ground sample was extracted with 125 mL of either methanol or ethyl acetate using a Soxhlet extractor (Extraction System B-811, BÜCHI Labortechnik AG, Flawil, Switzerland). Based on preliminary works, the optimised period for extraction of 10 g ground sample is 12 cycles (40–60 min). The ideal extraction temperature for each solvent was set according to their boiling points. The solution was filtered and concentrated by using a rotary evaporator (Rotavapor R-215, BÜCHI Labortechnik AG, Flawil, Switzerland). The concentrated extracts were further evaporated to dryness via air-drying at ambient temperature for 24 h. All residues were stored in the dark at −70°C prior to analysis.

**Chemicals**

Methanol (megh3041) and ethyl acetate (etjhH078) were of chromatography grade and obtained from ECP (ECP laboratory research and chemicals, New Zealand). Phosphate buffer powder (KH₂PO₄) (P7994), potassium hydroxide (KOH) (P1767), 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB) (D218200), sodium hydrogen carbonate (NaHCO₃) (S5761), acetylthiocholine iodide substrate (ATChI) (A5751), butyrylthiocholine iodine (BTchI) (20820), acetylcholinesterase from human erythrocytes (AChE) (C0663), butyrylcholinesterase human serum (BChE) (B4186), dimethyl sulfoxide (DMSO) (41641), donepezil (D6821), eserine (E8375), quercetin (Q4951), ascorbic acid (PHR1008) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (D9132) were purchased from Sigma-Aldrich (NZ). Beta-secretase (BACE1) assay kit from PanVera Co. (USA) (P2985) was used to test the inhibition of BACE1. Donepezil is an approved Alzheimer medicine with potent AChE enzyme inhibition capacity and was used as a control for AChE inhibition. Eserine (phystogmine), quercetin, and ascorbic acid were used as positive controls for BChE inhibition, beta-secretase inhibition and antioxidant activity assays, respectively. Deionised water was used for all experiments.

**Determination of enzyme inhibition and IC₅₀ concentrations**

**Acetylcholinesterase and butyrylcholinesterase inhibition assays**

The assay for measuring AChE activity was measured by the microplate assay using Ellman’s colorimetric method and modified by Rauter et al. (2007). Buffers were prepared
as follows: 0.1 M phosphate buffer was freshly before each analysis (136.1 mg of KH2PO4 in 10 mL water, adjusted at pH 8.0 with KOH); 0.01 M DTNB solution (3.96 mg DTNB in 1 mL water containing 1.5 mg sodium hydrogen carbonate). 0.022 M ATchI solution (6.4 mg ATchI in 1 mL water); 1.32 Unit/mL AChE solution (4.4 mg of AChE enzyme (10 μL, 1.02041 U) in 1.0 mL buffer at pH 8.0.

For the enzymatic analysis, dry plant extracts in solid or paste form produced were initially dissolved in DMSO and diluted in distilled water to a concentration of 44 mg/mL to test a final extract concentration of 1000 μg/mL. Donepezil is an approved drug that inhibits AChE and was used as control. The compound was initially dissolved in DMSO and diluted in distilled water to 44 mg/mL to give a final test concentration of 1000 μg/mL. A blank test without plant extract demonstrated no inhibition of DMSO at the highest concentration used (12.5%).

The assay was achieved by adding 5 μL of plant sample extract, 200 μL phosphate buffer, 5 μL of AChE enzyme, and 5 μL DTNB reagent in a 96-well microplate kept for 15 min at 30°C. Then, 5 μL of ATchI substrate solution was added to the mixture to start the enzymatic reaction. Absorbances were determined using a microplate reader operated under controlled temperature (EnSpire Multimode Plate Reader) at 405 nm for every 45 s, 6 times consecutively at a controlled temperature of 30°C. For each extract the experiments were done in triplicate and the enzyme inhibition was calculated using equation 1:

\[
\text{Inhibition (\%)} = 100 - \left[ \left( \frac{V_{\text{extract}}}{V_{\text{max}}} \right) \times 100 \right] 
\]

where \(V_{\text{extract}}\) is the rate of change in the absorbance of the test containing plant extract (\(\Delta\text{abs}/\Delta\text{time}\)) and \(V_{\text{max}}\) is the maximum rate of change in the absorbance of the blank sample without any inhibitor.

Regarding BChE, buffer and solutions were freshly prepared as explained for AChE before each experiment. 0.44 U/mL BChE solution (2.9762 mg BChE enzyme was dissolved in 6.746 mL buffer at pH 8.0); 0.022 M BTchI solution (7.0 mg BTchI was dissolved in 1 mL water). The solid or paste plant extracts were dissolved with DMSO followed by distilled water to 44 mg/mL, to obtain a final concentration of 1000 μg/mL. BChE inhibition assay was similar to AChE and absorbances were also measured in the same microplate reader. A solution consisted of 200 μL buffer, 5 μL BChE enzyme, 5 μL DTNB and 5 μL plant extracts at the concentration of 44 mg/mL were mixed and kept for 15 min at 30°C in a temperature controlled water bath. Subsequently, 5 μL of BTchI substrate solution was added to the mixture to initiate the enzymatic reaction. Absorbances were taken at 410 nm using the microplate reader at 45 s intervals 13 times under regulated temperature of 30°C. The experiments were run in triplicate. The absorbances were used to calculate enzymatic inhibition using equation 1. Eserine is an approved drug used as a control for BChE inhibition and was tested at a final concentration of 10 μg/mL.

**Beta-secretase inhibition assay**

The beta-secretase inhibition procedure was carried out in accordance to the method provided by the manufacturer (PanVera Co., USA) with modifications (Lee et al. 2005). The beta-secretase assay kit allows for the detection of beta secretase activity in biological samples. The procedures involved in this assay were performed in a 96 well plates.
Buffers were freshly prepared before each experiment, the preparation are as follows: 1.0 Unit/mL (500 μM) of BACE1 enzyme – (50 mM Tris at pH 7.5, 10% glycerol) was diluted with BACE1 assay buffer (50 mM sodium acetate at pH 4.5) to produce 3X working solution. A total of minimum of 4.0 mL of this enzyme solution was prepared.

A 750 nM BACE1 substrate – The initial concentration of BACE1 substrate (Rh-EVNLD-AEFK-Quendcher in 50 mM ammonium bicarbonate) was 75 μM (300X). A 40 μL of BACE1 substrate (75 μM) was added to 3960 μL of BACE1 assay buffer (50 mM sodium acetate at pH 4.5) to prepare a 750 mM stock (3X). The substrate was stored in an amber container and was added directly to the wells.

Test sample – 10 μL of plant extract sample and quercetin (the control) were initially dissolved in DMSO and further dissolved in BACE1 assay buffer (50 mM sodium acetate at pH 4.5). The concentrations were 2 mg/mL for plant extract and 0.02 mg/mL for quercetin, to produce final concentration for the BACE1 assay of 500 μg/mL and 5 μg/mL, respectively. The total DMSO in the solution is less than 12.5% which is within the value specified by the manufacturer. The experiments were done in triplicate.

For the beta-secretase inhibition assay, 10 μL BACE1 substrate (75 μM) was added to 10 μL of extract sample and mixed gently. Later, 10 μL of BACE1 enzyme (1 U/ml) was added to the mixture to start the reaction. The whole arrangement was incubated for 60 min at room temperature in dark condition. The reaction is stopped by adding 10 μL stop buffer solution. The mixture was allowed for excitation at 545 nm and reading was recorded at 585 nm emissions. The inhibition ratio is calculated by the following equation (2):

\[
\text{BACE} \, (\%) = \left[1 - \left(\frac{S - S_0}{B - B_0}\right)\right] \times 100
\]

where \(S\) is the fluorescence of the tested samples (enzyme, sample solution and substrate) after 60 min of incubation, \(S_0\) is the fluorescence of the tested samples at time 0. \(B\) is the fluorescence of the blank sample without inhibitor extract/compound (enzyme, assay buffer and substrate) after 60 min of incubation, and \(B_0\) is the fluorescence of the blank at time 0.

**IC\(_{50}\) concentrations of most active extracts**

The IC\(_{50}\) (in μg/ml) is the plant extract concentration that inhibits 50% of enzyme activity. The IC\(_{50}\) concentrations were determined for the most inhibitory extracts (and the controls) by performing a nonlinear regression of the enzyme inhibition (%) against the log (extract concentration) plot, using GraphPad Prism 6 trial version (GraphPad Software Inc, La Jolla, CA, USA). The results for IC\(_{50}\) were reported as mean and 95% confidence interval. At least four different concentrations (×3 replicates) were tested and used for the regression.

**Determination of extracts’ antioxidant DPPH radical scavenging activity and IC\(_{50}\) concentrations**

The antioxidant activity of plant extracts was determined using free-radical scavenging effect on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical with slight modification.
Methanol DPPH solution (100 μM) was prepared a day before analysis, to ensure a fully dissolved solution with a stable wavelength measurement. An aliquot of 10 μL plant extract was mixed with 190 μL of DPPH solution in a clear 96-well microplate. The mixture was shaken vigorously before being kept in dark at room temperature for 40 min. All the test solutions were measured at 517 nm using a microplate reader (EnSpire Multimode Plate Reader). The results were shown as percentage of DPPH radical scavenging activity (RSA), and the values represented the radical scavenging capacity of the extracts. The experiments were run in triplicate. The percentage of DPPH activity is calculated as follows:

$$\text{DPPH RSA} (\%) = \left( \frac{\text{Abs}^{\text{blank}} - \text{Abs}^{\text{extract}}}{\text{Abs}^{\text{blank}}} \right) \times 100\% \quad (3)$$

The $\text{Abs}^{\text{blank}}$ is the absorbance of DPPH solution without plant extract while $\text{Abs}^{\text{extract}}$ is the absorbance of the sample containing the extract, which was registered after 40 min. Ascorbic acid was used as a standard antioxidant for DPPH RSA. Then, the $\text{IC}_{50}$ values (in μg/mL) were estimated from the RSA results obtained for four different extract concentrations, as $\text{IC}_{50}$ is the plant extract concentration (or ascorbic acid, control) which causes 50% DPPH RSA. For each plant extract, the $\text{IC}_{50}$ value was determined through a nonlinear regression of the plot RSA (%) against the log (extract concentration) using GraphPad Prism six trial version (GraphPad Software Inc, La Jolla, CA, USA). The results for $\text{IC}_{50}$ were reported as mean and 95% confidence interval.

**Results and discussion**

**Enzyme inhibition by the New Zealand plant extracts**

The AChE, BChE and beta-secretase (BACE1) inhibitions of 30 trees/bushes extracts are summarised in Table 2. The detection limit of this method is about 5%, the enzyme inhibition variability/error, and so <5% indicates no inhibition or residual inhibition. The methanol and ethyl acetate extracts of 15 species were analysed at 1000 μg/mL for AChE and BChE, and at a concentration of 500 μg/mL for BACE1. Extracts from three plants were very active against the three enzyme systems and seem promising in terms of Alzheimer’s disease application: *Weinmannia racemosa*, Kunzea ericoides and *Pseudowintera colorata* with inhibitions above 50% for all enzymes tested. *W. racemosa* and *K. ericoides* presented higher inhibitions above 90% for AChE-BChE and BACE1, respectively. The bark decoction of *W. racemosa* was traditionally used to treat abdominal and thoracic pains (Brooker et al. 1987). It contains up to 20% of tannins and some traces of catechin (Cambie 1976). Tannic acid, which is a form of tannin has been shown to have strong inhibition against both AChE and BChE (Patil et al. 2019; Türkan et al. 2019). The $\text{IC}_{50}$ concentrations of the most active extracts were also determined (presented in Table 3). A low $\text{IC}_{50}$ is desirable as it indicates a lower extract concentration for 50% enzyme inhibition.

With respect to AChE, 12 species inhibited the enzyme at 1000 μg/mL. The most active one was *W. racemosa* (86%–90% inhibition at 1000 μg/mL) which was comparable to the control donepezil at a concentration of 10 μg/mL. Then follows Leptospermum scoparium (75%–88%), Schefflera digitata (69%–84%), *K. ericoides* (65–77%) and methanol extract of
Table 2. Inhibition (%) of three enzymes related to Alzheimer’s disease by methanol and ethyl acetate extracts of 15 New Zealand native trees/bushes: 1000 μg/mL of extract for AChE (acetylcholinesterase) and butyrylcholinesterase (BChE), and 500 μg/mL of extract for beta-secretase (BACE1)*.

| Plant species                          | Extract concentration | AChE (μg/mL) | BChE (μg/mL) | BACE1 (μg/mL) |
|----------------------------------------|-----------------------|--------------|--------------|--------------|
| Weinmannia racemosa (bark)             | Methanol              | 90.36 ± 2.09 | 97.96 ± 1.58 | 56.90 ± 3.83 |
|                                        | Ethyl acetate         | 85.76 ± 7.94 | 100.00 ± 0.00 | 38.85 ± 8.30 |
| Kunzea ericoides (leaves)              | Methanol              | 73.32 ± 4.93 | 74.23 ± 0.85 | 95.86 ± 3.29 |
|                                        | Ethyl acetate         | 64.97 ± 3.75 | 82.13 ± 3.08 | 90.59 ± 1.05 |
| Pseudowintera colorata (leaves)        | Methanol              | 75.49 ± 2.13 | <5           | 43.77 ± 1.96 |
|                                        | Ethyl acetate         | 60.29 ± 5.02 | 61.67 ± 5.93 | 62.59 ± 5.94 |
| Leptospermum scoparium (leaves)        | Methanol              | 88.15 ± 1.12 | 22.64 ± 5.94 | 27.67 ± 1.39 |
|                                        | Ethyl acetate         | 75.49 ± 2.13 | <5           | 43.77 ± 1.96 |
| Schefflera digitata (leaves)           | Methanol              | 68.67 ± 1.14 | 71.42 ± 0.59 | 37.16 ± 0.71 |
|                                        | Ethyl acetate         | 84.41 ± 5.71 | 88.32 ± 5.59 | <5           |
| Myrsine australis (leaves)             | Methanol              | 51.26 ± 0.64 | 57.60 ± 1.94 | 16.17 ± 1.34 |
|                                        | Ethyl acetate         | 59.06 ± 6.60 | 71.18 ± 5.48 | 8.76 ± 4.55  |
| Aristotelia serrata (leaves)           | Methanol              | 50.23 ± 1.95 | 98.42 ± 0.06 | 8.33 ± 3.27  |
|                                        | Ethyl acetate         | 36.05 ± 4.95 | 94.35 ± 0.12 | 14.82 ± 4.81 |
| Pomaderris kumeraho (leaves)           | Methanol              | 73.11 ± 3.14 | 63.73 ± 1.76 | 28.42 ± 2.03 |
|                                        | Ethyl acetate         | 14.95 ± 3.48 | 38.64 ± 3.28 | 15.68 ± 3.26 |
| Acaena microphylla (leaves)            | Methanol              | 31.51 ± 4.21 | 10.64 ± 3.37 | 4.19 ± 3.74  |
|                                        | Ethyl acetate         | 55.69 ± 3.98 | 95.36 ± 0.24 | 18.54 ± 6.63 |
| Phormium tenax (rhizomes)              | Methanol              | 28.82 ± 1.53 | 14.90 ± 1.73 | 32.34 ± 3.89 |
|                                        | Ethyl acetate         | 46.83 ± 2.96 | <5           | 31.44 ± 3.08 |
| Geniostoma ligustrifolium (leaves)     | Methanol              | <5           | 29.06 ± 4.21 | 45.55 ± 3.55 |
|                                        | Ethyl acetate         | <5           | 31.64 ± 4.35 | 19.81 ± 6.50 |
| Melicytus ramiflorus (leaves)          | Methanol              | <5           | <5           | <5           |
|                                        | Ethyl acetate         | <5           | 49.14 ± 6.00 | <5           |
| Hebe stricta (leaves)                  | Methanol              | <5           | 20.59 ± 2.92 | <5           |
|                                        | Ethyl acetate         | <5           | <5           | <5           |
| Macropiper excelsum (leaves)           | Methanol              | 16.82 ± 7.62 | <5           | 2.87 ± 3.95  |
|                                        | Ethyl acetate         | 33.77 ± 2.82 | <5           | 37.27 ± 4.68 |
| Coprosma robusta (leaves)              | Methanol              | 22.05 ± 6.76 | <5           | <5           |
|                                        | Ethyl acetate         | 46.09 ± 5.06 | <5           | 24.71 ± 3.32 |
| Donepezila 10 μg/mL                    |                       | 92.03 ± 0.33 | -            | -            |
| Eserine 10 μg/mL                       |                       | -            | 89.38 ± 6.06 | -            |
| Quercetin 5 μg/mL                      |                       | -            | 38.73 ± 2.71 | -            |

*There is a high variability when working with enzymes and it is difficult to measure any inhibition below 5%, as the error is of that magnitude. Inhibition values are mean ± standard deviation of triplicate experiments.

**Control donepezil and eserine are approved drugs used as medicines for Alzheimer's disease which inhibit AChE and BChE, respectively. Quercetin is an inhibitor of BACE1. The inhibition values are included to indicate results of reference compounds. As references/controls are pure chemicals, inhibitions are expected to be much higher than in a plant extract.

Pomaderris kumeraho (73%). L. scoparium commonly known as manuka is already a bush of already great commercial importance in the New Zealand honey production and exports. Regarding IC$_{50}$ determinations, the AChE IC$_{50}$ concentrations of W. racemosa bark extract were 8.09 μg/mL for the methanol extract and 11.25 μg/mL for the ethyl acetate extract. The methanol extract of L. scoparium presented an IC$_{50}$ concentration of 65.25 μg/mL, notable for a crude extract. Geniostoma ligustrifolium, Melicytus ramiflorus and Hebe stricta had no inhibition effect over AChE enzyme. The inhibitory potential of L. scoparium extracts may be attributed to their terpenoid compounds, which was identified from the leaves of L. scoparium (Häberlein and Tschiersch 1994). In a study involving marine products, terpenoid compounds have been shown to have partial inhibition of AChE enzyme (Beedessee et al. 2013). In addition, manuka (or tea tree), the common name for L. scoparium, is already a commercial culture abundant
Table 3. Acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and beta-secretase (BACE1) IC$_{50}$ concentrations and lower/upper limits of 95% confidence intervals for the methanol and the ethyl acetate extracts of selected New Zealand bushes/trees*.

| Plant species               | Extract    | AChE   | 95% confidence interval | BChE   | 95% confidence interval | Beta-secretase | 95% confidence interval |
|-----------------------------|------------|--------|-------------------------|--------|-------------------------|----------------|-------------------------|
|                             |            | IC$_{50}$ (μg/mL) | Lower limit | Upper limit | IC$_{50}$ (μg/mL) | Lower limit | Upper limit | IC$_{50}$ (μg/mL) | Lower limit | Upper limit |
| *Weinmannia racemosa* (bark) | Methanol   | 8.094  | 7.129 | 9.190         | 37.07  | 33.48 | 41.04         | ND          |
|                             | Ethyl acetate | 11.25 | 8.21  | 15.41         | 24.88  | 23.25 | 26.62         | ND          |
| *Kunzea ericoides* (leaves) | Methanol   | 215.4  | 171.7 | 270.2         | 361.6  | 320.3 | 408.2         | 29.05        | 26.00 | 32.46         |
|                             | Ethyl acetate | 687.2 | 575.0 | 821.4         | 464.9  | 375.2 | 576.2         | 36.40        | 29.80 | 44.46         |
| *Leptospermum scoparium* (leaves) | Methanol | 65.25  | 54.97 | 77.45         | ND     | ND    | ND            | ND          |
|                             | Ethyl acetate | 218.5 | 200.1 | 238.6         | ND     | ND    | ND            | ND          |
| *Scheflera digitata* (leaves) | Methanol   | 439.8  | 401.9 | 481.4         | 168.0  | 135.0 | 209.0         | ND          |
|                             | Ethyl acetate | 270.5 | 227.7 | 321.4         | 25.38  | 18.09 | 35.59         | ND          |
| *Aristotelia serrata* (leaves) | Methanol   | ND     | ND    | ND            | 24.50  | 19.68 | 30.49         | ND          |
|                             | Ethyl acetate | ND    | ND    | ND            | 67.88  | 63.66 | 72.38         | ND          |
| *Donepezil* (standard)      |            | 0.7570 | 0.6317 | 0.9071         | ND     | ND    | ND            | ND          |
| *Eserine* (standard)        |            | ND     | ND    | ND            | 0.9438 | 0.8573 | 1.039         | ND          |
| *Quercetin* (standard)      |            | ND     | ND    | ND            | 5.691  | 4.530 | 7.150         | ND          |

*The IC$_{50}$ is the concentration of plant extract (μg/mL) which inhibited 50% of enzyme activity, and was estimated by non-linear regression of the curve enzyme inhibition vs. log concentration of extract. The values in italic are the 95% confidence interval lower and upper limits. Lower IC$_{50}$ values indicate more inhibition capacity of the extract. Donepezil, eserine and quercetin are standard compounds inhibitory of AChE, BChE and beta-secretase, respectively; ND = not determined.
across New Zealand and mostly cultivated for the manuka honey production, known worldwide by its medicinal properties.

Regarding BChE, all the extracts demonstrate some level of BChE inhibitory activity at 1000 µg/mL except for *Macropiper excelsum* and *Coprosma robusta*. The inconsistency between AChE and BChE inhibition suggest that these extracts may react with the enzymes in a different way (Orhan et al. 2004). Similar to AChE the most powerful plant for BChE inhibition was *W. racemosa* (98%–100% inhibition at 1000 µg/mL, IC\textsubscript{50} = 24.88 µg/mL–37.07 µg/mL) and *Aristotelia serrata* (94%–98% inhibition at 1000 µg/mL, IC\textsubscript{50, methanol} = 24.50 µg/mL). The extract of *A. serrata* had been previously demonstrated to contain major component of aristoteline, with some trace of aristotelinone and serretoline, a type of alkaloids (Bick et al. 1980). Since most of the AChE inhibitors are known to contain nitrogen (Orhan et al. 2004), the high BChE inhibitory activity in this extract may be related to its rich alkaloidal content. Following in BChE inhibition was *S. digitata* (71%–88%, IC\textsubscript{50, ethyl acetate} = 25.38 µg/mL) and *K. ericoides* (74%–82%), *Myrsine australis* (58%–71%). Curiously, the ethyl acetate extract of *Acaena microphylla* presented 95% inhibition while the methanol extract of the same plant only showed 11% inhibition. One possible explanation is that at a concentration of 1000 µg/mL, some of the extracts behave inconsistently due to their nature such as solubility in solvent and final test solution. Due to this, enzymatic testing at a lower concentration (eg 500 µg/mL) would be preferable in this case.

For beta-secretase (BACE1) *K. ericoides*, locally known as kanuka was found to be very inhibitory (91%–96% inhibition at 500 µg/mL) presenting IC\textsubscript{50} values of 29.05 µg/mL for the methanol extract and 36.40 µg/mL for the ethyl acetate extract. A previous study has reported a major component of glycoside flavonoids presence in this plant (Wyatt et al. 2005), and might be the component responsible for the inhibition of BACE1. Flavonoids were shown to directly inhibit BACE1, in particular four flavonols (myricetin, quer cetin, kaempherol and morin) and one flavone compound (apigenin) (Sathy et al. 2012). *W. racemosa* and *P. colorata* presented less inhibition (57%–63%) of BACE1. All the other plants presented some degree of inhibition, except *M. ramiflorus* and *H. stricta* with no inhibition (<5%).

**Antioxidant activity**

The antioxidant activity of methanol and ethyl acetate extracts at several concentrations was assessed by DPPH test. This method measures the hydrogen atom or electron donor capacity of the extracts by scavenging the stable free radical of DPPH, and result is the percentage DPPH radical scavenging activity (RSA, %) (Equation 3). Table 4 shows the results expressed in terms of IC\textsubscript{50}, the plant extract concentrations (µg/mL) that causes 50% of RSA. A low IC\textsubscript{50} value is preferable as it represents good RSA, thus good antioxidant activity. The $R^2$ for the regression lines of RSA response vs extract concentration (µg/mL) for all the plant extracts are between 72%–99%, and the IC\textsubscript{50} extract concentrations were estimated from the dose–response lines. The results presented in Table 4 show that all the plant extracts analysed contained good levels of antioxidant capacity. The highest antioxidant activity was identified on methanol extract of *A. serrata* (2.34 µg/mL). In total, seven extracts exhibited greater inhibition than ascorbic acid, a standard antioxidant used as a control (12.01 µg/mL). These include both extracts...
of *H. stricta* and *W. racemosa*, and the methanol extracts of *A. serrata*, *K. ericoides*, *P. kumeraho*. The excellent antioxidant potential in *A. serrata* may be explained by the presence of the main alkaloids, aristoteline, and other minor indole alkaloids such as aristotelinone and serratoline (Bick et al. 1980). Several chemical compounds have been identified from the roots, stems, and leaves of makomako, the common name of *A. serrata*. These include the major alkaloid, aristoteline, and other alkaloids such as makomakine, makonine, aristoserratine, and aristoserratenine (Saxton 2009). Flavonoids are another source of natural antioxidants. This type of phenolic compounds has been identified from the leaf extracts of *K. ericoides* by Wyatt et al. (2005), which identified traces of aglycone and glycoside flavonoids. In a different study conducted with the extracts of *L. scoparium*, flavonoids and triterpenoids, known antioxidant compounds, were detected. Triterpenoids are considered as nonvolatile terpenoids, the largest group of plant chemicals that range between 15000 and 20000 compounds. A previous study by Liu et al. (2010) found that a triterpenoid compound possessed antioxidant activity after a positive result of this particular compound on ABTS radical test.

In most cases, radical scavenger activity is found to be higher in the methanol extracts than in the ethyl acetate extracts. This is particularly distinctive in *A. microphylla* which produced an extract with a relatively low IC$_{50}$ value of 17.96 μg/mL using methanol, as compared to 253.3 μg/mL when extracted with ethyl acetate. The same effect can also be noted in the extracts of *M. excelsum*, *L. scoparium*, *P. colorata*, and *G. ligustrifolium*. This result may suggest that all the methanol extracts probably contain more flavonones, and flavonoids (Liu et al. 2000; Pinelo et al. 2005; Adewusi and Steenkamp 2011).

### Conclusions

Among the 15 native New Zealand plants analysed in this study, several plants showed inhibition of AChE, BChE, beta-secretase, and antioxidant activity. *W. racemosa* bark

### Table 4. Antioxidant activity (IC$_{50}$) of the methanol and ethyl acetate extracts of selected New Zealand trees/bushes*.

| Plant species                        | Methanol extract (μg/ml) | Ethyl acetate extract (μg/ml) |
|--------------------------------------|--------------------------|------------------------------|
| *Weinmannia racemosa* (bark)         | 5.607 (4.601–6.833)      | 5.866 (4.848–7.098)          |
| *Aristotelia serrata* (leaves)       | 2.345 (0.7931–6.935)     | 14.81 (10.57–20.76)          |
| *Hebe stricta* (leaves)              | 8.263 (6.486–10.53)      | 9.914 (6.109–16.09)          |
| *Kunzea ericoides* (leaves)          | 9.579 (6.702–13.69)      | 29.59 (27.38–31.99)          |
| *Pomaderris kumeraho* (leaves)       | 9.672 (7.974–11.73)      | 31.81 (28.85–35.07)          |
| *Schefflera digitata* (leaves)       | 24.67 (21.97–27.70)      | 40.18 (38.56–41.86)          |
| *Leptospermum scoparium* (leaves)     | 17.32 (16.27–18.43)      | 72.15 (66.27–78.54)          |
| *Myrsine australis* (leaves)         | 48.63 (47.04–50.27)      | 50.95 (48.64–53.38)          |
| *Phormium tenax* (rhizomas)          | 55.18 (49.39–61.65)      | 33.30 (27.82–39.86)          |
| *Pseudowintera colorata* (leaves)     | 38.78 (36.31–41.41)      | 94.6 (85.33–104.9)           |
| *Acaena microphylla* (leaves)        | 17.96 (14.03–22.99)      | 253.3 (170.7–375.9)          |
| *Macropiper excelsum* (leaves)       | 79.46 (75.04–84.14)      | 119.3 (109.0–130.6)          |
| *Coprosma robusta* (leaves)          | 120.4 (108.8–133.3)      | 138.5 (123.5–155.3)          |
| *Melicytus ramiflorus* (leaves)      | 310.9 (243.1–397.7)      | 178.6 (160.3–199.0)          |
| *Geniostoma ligustrifolium* (leaves)  | 128.4 (120.3–137.1)      | 680.4 (459.0–1009)           |
| *Ascorbic acid* (control)            | 12.01 (10.23–14.11)      |                              |

*IC$_{50}$ is the concentration of plant extract (μg/ml) needed to reduce the DPPH scavenging activity to 50%. The values in parenthesis are the 95% confidence interval lower and upper limits. Lower IC$_{50}$ values indicate more antioxidant capacity of the extract. Ascorbic acid is a standard antioxidant used as a control.*
extracts showed effective inhibition of AChE, BChE, and also antioxidant activity. The leaf extracts of *L. scoparium* (manuka) and *K. ericoides* (kanuka) were effective inhibitors of AChE (IC$_{50}$ = 65.25 μg/mL) and beta-secretase (IC$_{50}$ = 29.05 μg/mL), respectively, which may add to the commercial value of these plants in New Zealand. A limitation of the study is that although most extracts were sourced from the Wellington region in late 2013, the exact time and place of collection of the commercially supplied extracts is unknown and voucher specimens were not retained so it is unclear whether these findings will apply to extracts from the same plants throughout NZ or only in certain regions. Therefore, another interesting topic for further research is to compare the enzyme inhibition of different plant batches from the same location, and to compare enzyme inhibition of plant batches from different locations in New Zealand. Regarding RSA, both extracts of *Hebe stricta* and *W. racemosa*, and the methanol extracts of *Aristotelia serrata*, *K. ericoides* and *P. kumeraho* showed IC$_{50}$ concentrations lower than the reference ascorbic acid (<12.0 μg/mL).

In conclusion, a few native New Zealand medicinal plants have the potential to be used as alternative treatments or as a complement to usual therapeutics for Alzheimer’s disease.

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