Kawakawa (*Piper excelsum*): human phytochemical metabolism and functional activity

Ramya Jayaprakash

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Abstract

Several endemic plant species have a history of medical and therapeutic use amongst Māori in Aotearoa New Zealand, of which *Piper excelsum* (kawakawa) is of particular importance. Kawakawa is considered Taonga by Māori and has deep cultural significance. There is an interest amongst Māori emergent food and beverage businesses to explore health-promoting and therapeutic benefits of kawakawa in value-added food products. Despite its versatile use, there have not been any human intervention studies that have sought to evaluate the possible biological and therapeutic properties of kawakawa. In this thesis, I integrated LC-MS/MS based metabolomic analysis with molecular networking and multivariate analysis to investigate the kawakawa phytochemistry in aqueous extracts, representative of kawakawa tea. Sixty-five compounds were identified from eight leaf sources including phenylpropanoids, lignans, flavonoids, alkaloids and amides and provided novel insights into the complexity and functionality of kawakawa leaf chemistry. Eight of these compounds were absolutely quantified and provided information regarding their toxicity. I conducted the first human study to examine the bioavailability of these kawakawa compounds in healthy human volunteers following acute ingestion of kawakawa tea and confirmed the results by analysing samples from a second independent study. This enabled the provisional identification of twenty-six urinary metabolites and seven plasma metabolites associated with kawakawa tea consumption. This study provided information regarding biomarkers for kawakawa consumption and to further explore their underlying therapeutic properties. Subsequently, I conducted a human study to explore the effects of kawakawa complex mixture on resting and post-prandial energy metabolism using indirect calorimetry. This study revealed that kawakawa may influence whole-body energy metabolism mediated by carbohydrate oxidation and may have the ability to control satiety and appetite. Finally, I examined the functional activity of two of the kawakawa compounds, pellitorin and myristicin to determine their effects on expression levels of selected genes in HepG2 cell lines. This study revealed that pellitorin may regulate oxidative stress response and drug detoxification via Nrf-2 mediated transcription. The findings of these studies could serve as a basis for future investigation into the health-promoting properties of kawakawa leaf chemicals and their incorporation into the development of functional foods.
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My research with kawakawa was undertaken in partnership with Wakatū Incorporation, a values-driven, hapū-owned organisation that represents over 4,000 descendants of the original Māori landowners of Te Tauihu (the top of the South Island). I would like to express my sincere gratitude to the research team at Wakatū Incorporation; it is through their guidance and support that I was able to access kawakawa and contribute to the ongoing understanding of the therapeutic benefits of this indigenous plant. Throughout my research, I was mindful that kawakawa is revered by Māori as a taonga (treasure) and of the importance of ensuring that my work was conducted with due respect to the principles enshrined in Te Tiriti o Waitangi.

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17β - HSD - 17β- hydroxysteroid dehydrogenase
3T3 - L1 adipocytes - mouse fibroblast cells
ACTB - Actin Beta
ACTRN - Australian New Zealand Clinical Trial Registration Number
ADME - absorption, distribution, metabolism and excretion
AGC - Automatic gain control
AI - artificial intelligence
AIF - All Ions Fragmentation
AKRC1 - Aldo-Keto Reductase
AMPK - Adenosine monophosphate activated protein kinase (catalytic subunit - PRKAA1)
ANOVA - Analysis of variance
AP-1 - activator protein 1
AR - androgen receptor
ATP - adenosine triphosphate
AUC - area-under-the-curve
BAT - brown adipose tissue
Bax - B-cell lymphoma 2 (Bcl-2) associated X- protein
Bcl2- B-cell lymphoma 2
BMI - body mass index
BOKA-T - Bioavailability of Kawakawa Tea metabolites in human volunteers
C/EBP – CCAAT/enhancer-binding protein
Caco-2 - Human colorectal adenocarcinoma cells
CAMs- cellular adhesion molecules
CAT - catalase
c-FOS - Fos Proto-Oncogene, AP-1 Transcription Factor Subunit
CHO Ox - carbohydrate oxidation
CNS - central nervous system
COX- cyclooxygenases
COX-2 - cyclooxygenase 2
CPK-MB - creatine phosphokinase -MB
CPT1 - Carnitine palmitoyltransferase-1 (lipid transfer proteins)
CRU - clinical research unit
Ct - cycle threshold
CV - coefficient of variation
DDA - Data Dependent Acquisition mode
DMSO - Dimethyl Sulphoxide
DNA - deoxyribonucleic acid
DPPH - 2,2-diphenyl-1-picrylhydrazyl
EDTA - Ethylenediaminetetraacetic acid
EE - energy expenditure
ENaC - Epithelial sodium channel
EO - essential oil
ERK - extracellular signal-regulated kinase
Fab - 4 - fragment antigen binding
FAM - 6-carboxyfluorescein
FAS - fatty acid synthase
Fat Ox - fat oxidation
FBS - fetal bovine serum
FNDC5/irisin - fibronectin type III domain-containing protein 5
GAPDH - Glyceraldehyde 3-phosphate dehydrogenase
GC- MS - gas chromatography coupled with mass spectrometry
GCSC - Glutamate-Cysteine Ligase Catalytic Subunit
GM-CSF- granulocyte-macrophage colony stimulating factor
GPx - glutathione peroxide
GR – antioxidant enzymes
GST - Glutathione S-transferase
GSTM3 - Glutathione S-transferase M3
HAMA - Hamilton anxiety
HepG2 - hepatocellular carcinoma
HKG - housekeeping gene
HMG - CoA - β-Hydroxy β-methylglutaryl-CoA
HMGB1 - High mobility group box 1
HO-1 - Heme oxygenase -1
HPRT1 - Hypoxanthine phosphoribosyltransferase 1
hsCRP- high sensitivity C-reactive protein
HT 29 - human colorectal adenocarcinoma cell
IC50 - inhibitory concentration
ICC - intraclass correlation coefficient
iDXA - Dual-energy X-ray Absorptiometry
IKK- IκB kinase
IL – interleukins
IR- insulin resistance
IS – internal standard
JAK2- Janus kinase 2
Keap1 - Kelch-like-ECH-associated protein
KT - Kawakawa tea
LC-MS/MS - Liquid chromatography with tandem mass spectrometry
LDH - lactate dehydrogenase
LIF-leukemia inhibitory factor
LnCaP cell- androgen-sensitive human prostate adenocarcinoma cell
LnCaP+ cell - analogue of LnCaP cells that overexpresses aldo-keto reductace1C3 (AKR1C3)
LOX- lipoxygenase
LBTB4 - Leucotriene B4
MAPK - mitogen-activated protein kinase
MB-231 - human breast cancer cell line  
MCP - monocyte chemotactic protein  
MDA - malondialdehyde  
MDA-MB-231 – human breast adenocarcinoma cell  
MDP - methylenedioxyphenyl  
MeOH - methanol  
MIP - Macrophage inflammatory protein  
MMDA - 3-Methoxy 4,5-Methoxydioxamphetamine  
nmHg - millimeters of mercury  
MPO - Myeloperoxidase  
MYR - myristicin  
NAC - N-acetyl cysteine  
NADP - Nicotinamide adenine dinucleotide phosphate  
NAFLD – Nonalcoholic fatty liver disease  
NCE - normalised collision energy  
NFATc1 - nuclear factor of activated T cells 1  
NF-κ B - Nuclear factor kappa B  
NMR spectroscopy – nuclear magnetic resonance spectroscopy  
NO - nitric oxide  
NQO1 - NAD(P)H:quinone oxidoreductase  
Nrf2 - nuclear factor erythroid 2-related factor 2  
O/N - overnight  
P450 - human liver cytochrome P450 enzymes and its members (CYP3A4)  
PAF - Platelet-Activating Factor (1-O-hexadecyl-2-acetyl- sn-glyceryl-3-phosphorylcholine)  
PBK - Physiologically based kinetic  
PBS - phosphate buffer saline  
PC3 – human prostate cancer cell  
PCR - polymerase chain reaction  
PEL - pellitorin  
Peroxisome proliferator-activated receptor alpha (PPAR-α)  
PGE2 - prostaglandins 2  
pH - potential of hydrogen  
p-JNK - phosphorylated Jun N-terminal kinase  
p-p38 - Phospho-p38  
PPAR α - peroxisome proliferator-activated receptor alpha  
PPARγ - peroxisome proliferator-activated receptor gamma  
QC - Quality control  
REE – resting energy expenditure  
REML - Restricted maximum likelihood  
RIN - RNA integrity number  
RM ANOVA - repeated measures analysis of variance  
RPM - revolutions per minute  
RNA - ribonucleic acid
ROS/RNS - reactive oxygen species / reactive nitrogen species
RPMI 1640 Medium - Roswell Park Memorial Institute 1640 Medium
RQ - Respiratory quotient
RT - Retention time
Runx2 - Runx-t-related transcription factor 2
SCD1 - stearoyl-CoA desaturase 1 inhibitor
SEM - standard error of the mean
SFN - D-sulforaphane
SGOT - serum glutamic-oxaloacetic transaminase
SIRT 1 - Sirutin 1
SOD - superoxide dismutase
SREBP1 – Sterol regulatory element binding protein-1
STAT3 – signal transducer and activator of transcription 3
STR - Short tandem repeat
SULTs - sulfotransferase enzymes
T2DM - Type 2 diabetes mellitus
TBP - TATA-Box Binding protein
TB - Tuberculosis
TNFα – Tumor necrosis factor – α.
TOAST - Impact of acute Kawakawa Tea ingestion on postprandial glucose metabolism in healthy human volunteers
TRPA1 - Transient receptor potential cation channel subfamily A member 1
TRPV1 - Transient receptor potential cation channel subfamily V member 1
UCP 1 - uncoupling proteins 1
UGTs - UDP - glucuronosyltransferases
VAS - visual analog scale
VCO₂ - CO₂ production in mL/min
VO₂ - O₂ consumption in mL/min
Chapter 1 Introduction

This thesis is concerned with the plant secondary metabolism in *Piper excelsum* G.Forst (‘kawakawa’) and the possible role of secondary (or ‘specialized’) metabolites in contributing to the putative therapeutic properties of kawakawa.

Plant secondary metabolism refers to the production of a diverse range of chemical compounds that are not directly involved in the primary processes of plant growth and development. Plants have evolved mechanisms that involve the synthesis and accumulation of these secondary metabolites to address biotic (herbivory and pathogen defense, attraction of pollinators and symbiotic interaction with microbes) and abiotic (drought, salinity, light, pH, soil and temperature conditions) environmental challenges. The biological activity of some of these metabolites is thought to underpin the therapeutic and medicinal use of plants that is common amongst all traditional and contemporary societies. Scientific investigation of a small number of these plants has led to the development of pharmaceuticals that play a pivotal role in current medical practice. For example, extracts from *Salix* sp. have been used in traditional medicine to relieve pain, and scientific investigation of *Salix* extracts led to the development of aspirin which has anti-inflammatory, analgesic and possible anti-cancer properties, and is the most widely used drug in the world.

An important genus in traditional medicine is *Piper*. This introduction initially describes the traditional use of selected *Piper* species and aspects of their secondary metabolism. It continues with a summary of *Piper excelsum*, its traditional therapeutic use and current knowledge of its secondary metabolism. It then provides a brief discussion of the major experimental approaches that were used to explore the chemistry of kawakawa and its biological activity in subsequent experimental chapters. The introduction concludes with a summary of the aims and objectives of my research programme.
Figure 1.1 Piper spp. and their principal bioactive compounds

1.1 Piper species

Piper which encompasses 2,000 species is the largest genus within Piperaceae and is known for its extensive source of bioactive metabolites. Piper species are greatly diversified and distributed throughout pan-tropical regions of America (The Andes, Southern Mexico, Atlantic and Amazon forests of Brazil) and southeast Asia. Generally, all Piper plants grow well in low-lying areas of tropical forests avoiding higher altitudes, steeper slopes and closed canopy areas. Some of the common morpho-anatomical features of Piper plants include oblique digitate, peltate, alternately arranged leaves, umbrella-like bracts, diminutive flowers with spikelet inflorescence, single collateral vascular bundle and no perianth. Piper fruits are black, red, or green with prominent style and variable in shapes-drupaceous, globose (elliptical, obovoid, oval, or flask-shaped) and berry-like. These aromatic fruits are the commonly used part of the plant as spices. Piper plants were generally used by humans for brewing drinks, as fish bait and for medicinal purposes due to their sedative and anxiolytic properties. For centuries, certain Piper plant parts (leaves, roots and fruits) were used for inducing stimulating and mild-narcotic effects. Two common Piper species used for narcotic experiences in
occidental and oriental countries include *P. methysticum* and *P. betle* respectively. A summary of human studies carried out with some of these *Piper* plant extracts and their phytochemicals are listed (Table 1.1).

### 1.1.1 Botanical characteristics, traditional uses and phytochemistry of some *Piper* species

#### 1.1.1.1 *Piper methysticum*

*Piper methysticum* (kava plant or intoxicating pepper), is a perennial shrub growing up to 1-4m, heart-shaped alternate leaves, rarely producing berry-like fruits with one seed. It is native to the Polynesian islands and has been used by the native people of the Pacific Islands for more than 3000 years. The origin of this plant species remains unclear since it was taken eastward to the Pacific Islands by the native people during their sea voyages.

Kava is the traditional beverage prepared from *P. methysticum* roots (also known as awa, yaqona, sakau, seka, malok, malogu) used for ceremonial and medicinal purposes. *P. methysticum* extracts were used as a muscle relaxant, and pain reliever, to enhance unique tranquilizing experiences, genito-urinary tract infections, convulsions, inflammations, arthritis and rheumatism, gall bladder complaints, sore throat, bronchitis, insect bites and stings.

Phytochemical analysis of 63 samples (different plant parts) of different *P. methysticum* cultivars has been reported. Large variance in kavalactone content in different plant parts was observed decreasing progressively from roots to leaves (smaller roots with higher kavalactones than larger roots and peelings of the bark with higher kavalactone content than the stump) and also varied among the different cultivars grown in the same environmental conditions (soil and climate). Two flavanones and six kavalactones were isolated from root extracts namely, 5-hydroxy-4’,7-dimethoxyflavanone and mettucinol (5,7-dihydroxy-4’-methoxy-6,8-dimethylflavanone), 5,6-dehydro-kavain, kavain, yagonin, 7,8-dihydrokavain, dihydromethysticin and methysticin. Studies have reported more than 40 chemical constituents from *P. methysticum* root extracts including kavalactones, chalcones, steroids, alkaloids, cinnamic acid derivatives, fatty acids, flavanones, conjugated diene ketones, alcohols. Among these different classes of phytochemicals, kavalactones present in
abundance includes methysticin, dihydromethysticin, kavain, dihydrokavain, yangonin, demethoxyyangonin and tetrahydroyangonin contributing to its biological properties 23.

1.1.1.2 Piper nigrum

*Piper nigrum* (black pepper) is a perennial aromatic climber with simple, alternate, oval-shaped leaves, unisexual or bisexual flowers, drupe inflorescence and berry-like fruits, growing up to 50-60 cm in tropical and sub-tropical regions of the world 24. Originating in the tropical forests of the Western Ghats in South India, there are around 38 cultivars of black pepper in India. Recently, 10 important cultivars of black pepper in Malaysia and the general botanical characteristics of black pepper were documented 25. Black peppercorn (ripened dried fruits) is a major export crop of many tropical countries including, India, Brazil, Malaysia, Indonesia and Vietnam and is often referred to as the “King of spices” 26. Black pepper was first cultivated in the southern parts of India. Subsequently, other parts of the world have started their cultivation, especially in south and south-eastern Asia, with Vietnam now being the highest producer of pepper in the world 27. White and black pepper are produced from the same source but require different harvesting and processing time 24, 28.

Since ancient times, black pepper has been extensively used in culinary practices as food spices and traditional Ayurvedic medicine for treating fever, menstrual disorders, ear-nose-throat infections, gastrointestinal discomforts, skin diseases, fungal and bacterial infections, jaundice, snakebite, rheumatism, antispasmodic, blood purifier, antipyretic, carminative, appetite stimulator and for better digestion 10, 24, 29. In addition to that, black pepper is also used in perfumery, as an insecticide and biopesticide 26. Due to its attributed physiological properties, black pepper bears great commercial, medicinal and economic potential 24, 27, 30.

Phytochemical analysis of *P. nigrum* had shown the presence of a wide range of volatile and pungent compounds, including alkaloids, oleoresin volatile oils and other compounds like phenols, flavonoids, amides, steroids, lignans, and terpenes 31, 32. More than 50 phytochemicals were isolated from *P. nigrum* (seeds, fruits and leaves) including terpenes and phenylpropanoids- myrcene, β-caryophyllene, limonene, β-pinene, sabinene, terpinene-4-ol, α-terpinene, 1,8-cineole, δ-carene, α-pinene, α-thujene, δ-3-carene, gurjunene, β-phellandrene, α-phellandrene, d-limonene, m-cymene, β-cymene, β-linalool, α-copaene, δ-elemene, β-elemene, α-murrolene, β- bisabolene, β-εudesmol, elemol, δ-cadinene, β-selinene, β-
humulene, δ-cadinol, safrole and eugenol; alkaloids- piperine, chavicine, piperettine, pellitorine, piperyline, piperanine; piperamides and lignan- hinokinin \(^{24,33}\). Important phytochemicals of \(P.\text{nigrum}\) seeds contributing to the active pungent potency and pepper oleoresin responsible for the strong aroma include piperine and piperamides \(^{32,34}\). Piperine is the most potent bioactive compound with various therapeutic effects \(^{29}\). Several reviews of the literature have been published compiling the phytochemical composition, therapeutic potentials and mode of action of \(P.\text{nigrum}\) and its principal component piperine \(^{24,32,33}\).

1.1.1.3 Piper longum

\(Piper longum\) (long pepper) is a perennial shrub, with 5-9 cm long leaves, glabrous, unisexual flowers (2-7cm long male spikes and 1.25cm-2cm long female spikes), yellowish-orange, ovoid, berry like-fruits and it is native to the Indo-Malaya region \(^{35}\). It is referred to as ‘pippali’ (in Sanskrit), ‘pibo’ (in China) and long pepper (in Europe).

Traditionally, long pepper is used in culinary practices (as spice and seasoning) and for treating several health ailments. The most commonly used plant parts include roots, seeds and immature spikes and it is one of the principal ingredients listed in Ayurvedic medicine known for its bioavailability-enhancing properties \(^{36}\). The dried spikes of long pepper are used as thermogenic, emmenagogue, and expectorant, to treat cardiovascular disorders, respiratory discomforts (cough, cold, asthma and bronchitis), chronic malaria, tumors, reproductive infections, gastro-intestinal discomforts and allergies \(^{35,37}\).

Phytochemical analysis by mass spectrometric techniques revealed the presence of more than 40 phytochemicals in \(P.\text{longum}\) leaf, fruit and seed extracts including terpenes – caryophyllene, \(\alpha\)-pinene, limonene, linalool, copaene, pentadecane, thujene, zingiberene, p-cymene, bisabolene; alkaloids - piperine, piperlongumine or piplartine, guineensine, pellitorine, pipercabamide and trace amounts of lignans- sylvatin, sesamin, diaeudesmin dihydrostigmasterol and piplasterol; flavonoids-quercetin, kaempferol, apigenin, catechin and steroids \(^{37-39}\). Piperine is the principal active constituent of \(P.longum\). Bio-assay-directed fractionation of \(P.longum\) reported prenylated phenolic compounds including bakuchiol, bavachin, and isobava-chalcone \(^{40}\). Recently, 16 N-alkylamides were isolated including piperedone, fragaramide, cis-fagaramide, pipercide, and (2E and 4E, 15Z)-N-isobutyl-eicosa-2,4,15-trienamide \(^{41}\).
**1.1.1.4 Piper cubeba**

*Piper cubeba* (tailed pepper or cubeb) is a perennial climber growing up to 5-15m high, identified with ovate, glabrous and venated leaves; unisexual flowers arranged in scaly spikes and sub-globose, reticulate pericarp fruits elongated into a straight stalk. It is grown in Indonesian islands and mostly cultivated for its berries and essential oils. Fruits of *P. cubeba* were commonly used in traditional medicine.

*P. cubeba* seeds are used as powder, decoction and as an essential oil in Unani medicine for the treatment of respiratory problems, stomach ailments, reproductive illness and for wound healing and are listed as an important medicine for cancer treatment in Chinese and Moroccan traditional medicine practices.

Phytochemical analysis of dried *P. cubeba* berries indicated the presence of terpenes, alkaloids and lignans. Lignans isolated from *P. cubeba* include cubebin, hinokinin, clusin, haplomyrfolin, dihydroclusin and dihydrocubebin. H1-Nuclear magnetic resonance (NMR) coupled with multivariate data analysis putatively identified 12 metabolites from *P. cubeba* extracts including, cubebin, ρ-cymene, piperidine, cubebol, dihydrocubebin, hinokinin, dihydroclusin, D-germacrene, ledol, magnosalin, cubenin and yatein. Major components of *P. cubeba* fruit essential oils reported includes terpinen-4-ol, β-pinene, sabinene, cubebol, cubebene, germacrene, β-caryophyllene, α-copaene, γ-elemene, eucalyptol, nerolidol, bicyclosesquiphellandrene and camphor.

**1.1.1.5 Piper betle**

*Piper betle*, (betel) is an evergreen, perennial creeper growing up to 1m in height, identified with heart-shaped simple and alternate leaves, dioecious and female plant rarely produces flower or fruit. It is commonly called as “Golden heart of nature” for its cultural, traditional and nutritional significance.

It is a well-known Ayurvedic medicine used for treating skin ailments, stomach discomforts and urinary infections. *P. betle* leaves were normally used as the wrapper of areca nuts augmenting psychoactive experiences, and enhancing digestion; leaves poultice or decoctions are used as carminative, earache, cure inflammations, cold, cough and respiratory discomforts.
constipation, galactagogues during breastfeeding and in the treatment of skin infections, boils and wound healing \textsuperscript{50}.

A review study has concisely listed the phytochemical constituents of \textit{P.betle} and the mechanisms involved in various pharmacological activities \textsuperscript{51}. More than 46 phytochemicals were isolated from \textit{P.betle} leaves through different extraction methods \textsuperscript{49}. Methanolic extraction of \textit{P.betle} leaves contains higher quantities of hydroxychavicol, eugenol and β-caryophyllene \textsuperscript{52}. GC-MS analysis of hydro-distilled oil obtained from seven varieties of betel leaves identified diverse groups of phytochemicals including terpenes (mono- and sesquiterpenes) and phenylpropanoids \textsuperscript{53}. Two new phytochemicals, 1-n-dodecanoyloxyresorcinol and desmethylenesqualenyl deoxy-cepharadione-A were identified using NMR spectroscopic analysis \textsuperscript{54}. Ultra-sound extraction followed by GC-MS analysis reported the occurrence of phenolic compounds including hydroxychavicol, eugenol, isoeugenol and 4-allyl-1,2-diacetoxybenzene \textsuperscript{55}. LC-MS and NMR spectroscopic studies have identified the presence of phenolic compounds in Ethyl acetate extracts of \textit{P.betle} namely, tachioside, Pinoresinol, 4-O-β-D-glucopyranoside and 3,4-dihydroxyallylbenezene \textsuperscript{56}. \textit{P.betle} essential oil (EO) derived from fresh leaves and cured leaves revealed the presence of terpenes and phenylpropanoids- chavibetol, chavibetol acetate, myrcene, sabinene, 1,4-cineole, eugenol, limonene, piperitol, camphene, α-pinene, estragole linalool, α-copene, anethole, chavicol and β-caryophyllene; flavonoids – quercetin and luteolin along with allypyroctechol, piperbetol, β-sitosterol, hydroxychavicol, eugenol methyl ether and 4 chromanol \textsuperscript{49,57}. 
Table 1.1 An overview of human studies performed on phytochemicals or herbal formulations prepared from Piper plants and their pharmacological effects

<table>
<thead>
<tr>
<th>Phytochemicals formulation</th>
<th>Oral supplementation dose and duration</th>
<th>Study design</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcuminoids (curcumin, demethoxycurcumin, bisdemethoxycurcumin) plus piperine</td>
<td>daily dose of 10mg/day piperine with 1g curcuminoid for eight weeks</td>
<td>Randomised double-blind, placebo-controlled trial, 117 participants with metabolic syndrome</td>
<td>Piperine-curcuminoid combination produced anti-inflammatory and antioxidant effects by enhancing superoxide dismutase and reduced MDA and C-reactive protein levels (^5^8)</td>
</tr>
<tr>
<td>Curcuminoids (curcumin, demethoxycurcumin, bisdemethoxycurcumin) plus piperine</td>
<td>1000mg/day curcuminoids with 10mg piperine for eight weeks</td>
<td>Randomised, double-blinded, placebo-controlled trial, 118 participants with type 2 diabetes mellitus (T2DM)</td>
<td>Curcumin supplementation modulated adipokines in T2DM, decreased leptin: adiponectin ratio, increased adiponectin, leptin and TNF-α levels (^5^9); modified lipid profile in T2DM patients (^6^0)</td>
</tr>
<tr>
<td>Curcuminoids (curcumin, demethoxycurcumin, bisdemethoxycurcumin) plus piperine</td>
<td>500mg/day curcuminoids with 5mg/day piperine for 12 weeks</td>
<td>Randomised, double-blinded, parallel, placebo-controlled trial, 70 participants with ultrasound determined NAFLD</td>
<td>Beneficial effect observed in treating NAFLD severity (^6^1)</td>
</tr>
<tr>
<td>Curcuminoids (curcumin, demethoxycurcumin, bisdemethoxycurcumin) plus piperine</td>
<td>500mg/day curcuminoids with 5mg/day piperine for 3 months</td>
<td>Randomised, double-blinded, placebo-controlled trial, 100 participants with type 2 diabetes mellitus (T2DM)</td>
<td>Curcuminoids plus piperine supplementation modulated glycaemic and hepatic parameters by reducing glucose levels in serum, lowering alanine aminotransferase and aspartate aminotransferase (^6^2)</td>
</tr>
<tr>
<td>Treatment</td>
<td>Dose/Details</td>
<td>Study Design</td>
<td>Findings</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>------------------------------------------------------------------------------</td>
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<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Curcuminoids (curcumin, demethoxycurcumin, bisdemethoxycurcumin) plus piperine</td>
<td>1500mg/day curcuminoids with 15mg piperine for 4 weeks pre-treatment with 20mg piperine or placebo for 6 days and 200mg nevirapine on the 7th day.</td>
<td>Randomised double-blind, placebo-controlled trial, 89 participants with systemic oxidative stress and sulphur mustard-induced chronic pulmonary complications</td>
<td>Piperine supplementation enhanced the bioavailability of curcumin providing antioxidant defense by reducing MDA and increasing GSH and catalase.</td>
</tr>
<tr>
<td>Nevirapine with piperine</td>
<td>7th day. pre-treatment with 15mg piperine or placebo for 3 days and 10mg midazolam on the 4th day.</td>
<td>Randomised, crossover, placebo-controlled trial, 8 healthy male participants</td>
<td>Enhanced bioavailability of nevirapine when supplemented with piperine.</td>
</tr>
<tr>
<td>Midazolam with piperine</td>
<td>90mg and 120mg coenzyme Q10 administered in two different treatments: 1. single-dose study with placebo or 5mg piperine; 2. 14 and 21 days with placebo or 5mg piperine.</td>
<td>Randomised, crossover, placebo-controlled trial, 8 healthy male participants</td>
<td>Supplementation of piperine increased the half-life of midazolam and decreased clearance of midazolam which might be contributed to the inhibiting CYP3A4 enzyme activity of piperine.</td>
</tr>
<tr>
<td>Q10 coenzyme with piperine</td>
<td>5mg piperine.</td>
<td>Randomised double-blind, placebo-controlled trial, 6 healthy participants</td>
<td>Co-administration of piperine enhanced the Q10 coenzyme levels in plasma which could be contributed to its thermogenic properties.</td>
</tr>
<tr>
<td>Piperine with theophylline and propranolol</td>
<td>single dose of 40 mg propranolol or 150 mg theophylline alone or combined.</td>
<td>Crossover study, 6 healthy participants</td>
<td>Piperine supplementation enhanced bioavailability of theophylline and propranolol in the systemic circulation.</td>
</tr>
<tr>
<td>Herbal formulations with <em>P. nigrum</em>, ginger and turmeric extracts</td>
<td>Piperine (3.75 mg), gingerol (7.5 mg) and curcumin (300 mg) capsules taken twice a day for 4 weeks</td>
<td>Double-blind, two arm parallel group, randomized controlled trial, 60 osteoarthritis patients (grade 2-3).</td>
<td>improved PGE2 levels in chronic knee arthritis similar to Naproxen drug (^{68})</td>
</tr>
<tr>
<td>---</td>
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</tr>
<tr>
<td><em>P. methysticum</em> extracts</td>
<td><em>P. methysticum</em> extract equivalent to 20mg kavalactones/day (low dose) and 200mg (high dose)</td>
<td>Randomised double-blind trial, elderly participants with anxiety problems (36 in low dose and 33 in high dose)</td>
<td>no adverse events occurred and high-dose groups reported significantly higher HAMA (Hamilton anxiety) score and physical and psychological manifestation scores on day 28 (^{69})</td>
</tr>
<tr>
<td><em>P. longum</em> powder (pippali churna)</td>
<td>4g <em>P. longum</em> powder for three weeks</td>
<td>Randomized controlled trial with 21 patients in group A and 12 patients in group B having chronic respiratory problems (kaphaja kasa)</td>
<td>no adverse drug reactions observed (^{70})</td>
</tr>
<tr>
<td>LOWAT (L110903F containing <em>Dolichos biflorus</em> and <em>Piper betle</em> extracts)</td>
<td>300 mg capsule with herbal extracts of <em>Dolichos biflorus</em> and <em>Piper betle</em> extracts in the ratio 2:3 consumed for 8 weeks</td>
<td>Randomized double-blinded, placebo-controlled trial in 50 human subjects</td>
<td>significant increase in serum adiponectin, decrease in serum ghrelin with mild adverse events (^{71})</td>
</tr>
</tbody>
</table>
1.2 Piper excelsum (kawakawa)

Piper excelsum G. Forst (synonym Macropiper excelsum (G.Forst) Miq.) is a small tree or understory shrub endemic to Aotearoa New Zealand. It is a highly valued plant (taonga) by the Māori. It is popularly known as ‘kawakawa’ alluding to its bitter taste. It is widely distributed in the lowland forests all over the North Island and in the northern half of the South Island. It is a dicotyledonous shrub or small tree (1-3m in height) with glossy aromatic heart-shaped green or yellowish-green leaves that can grow upto 5-10 cm long and 6-2 cm wide, with palmate venation, and has oppositely arranged petioles with sheathing base\textsuperscript{72}. It has a flexuous erect stem, diminutive flowers loosely arranged on spikes growing upto 2.5 - 7.5cm long; solitary inflorescence with peltate bracts; drupaceous, aromatic, glabrous and yellowish-orange berry-like ripe infructescence (Figure 1.2)\textsuperscript{72,73}. It sustains conspicuous and substantial herbivory from Cleora scriptaria, a geometrid moth and leaves are generally observed with holes\textsuperscript{74}. Seed dispersal is by native New Zealand birds such as kerurū and Tui and introduced European birds such as blackbirds feeding on ripened berries. Propagation can be achieved by hardwood cuttings and growing from seeds\textsuperscript{75}.

\textbf{Figure 1.2} Piper excelsum (kawakawa)


1.2.1 Cultural and traditional significance

Culturally, kawakawa is used to welcome guests, launch a new canoe, new house opening, as a lucky charm for women of reproductive age and has significant spiritual importance as is worn as a mourning symbol in funerals.

Kawakawa has been used by Māori for culinary, cosmetic and medicinal purposes. The leaves, fruits and bark are used to treat various ailments including skin, respiratory and stomach ailments (Table 1.2). Kawakawa is included as part of the diet with the leaves used for the preparation of herbal tea and Ti-toki liqueur, and fruits as a source of spice. Kawakawa leaves were burnt and the smoke was used as an insecticide to protect crop fields.

<table>
<thead>
<tr>
<th>Table 1.2 Ethnomedicinal uses of kawakawa</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plant parts</strong></td>
</tr>
<tr>
<td>Leaves</td>
</tr>
<tr>
<td>Bark</td>
</tr>
<tr>
<td>Berries</td>
</tr>
<tr>
<td>Roots</td>
</tr>
</tbody>
</table>

1.2.2 Commercial uses

Dried kawakawa leaves are commercially sold as food and beverage products, including for tea, sauces, and marinades and as a spice for their peppery taste with reported rejuvenating and stimulating properties. Additionally, it is increasingly used for the manufacturing of NZ-cosmetic products including skin creams, serums, aromatic oils, bath salts, soap and shampoo.
bars and herb salts, with alleged anti-inflammatory, anti-microbial and for treatment of skin ailments⁷³,⁷⁷.

1.3 *P. excelsum* (kawakawa) leaf chemistry and biological properties

Kawakawa has a complex leaf chemistry but there exists a significant gap in the literature with regards to their potential biological and functional effects on human health. To date, very few studies attempted to explore the kawakawa leaf chemicals and their biological effects (Table 1.3).

Two previous studies reported the chemical composition of dried and fresh kawakawa leaves. Obst et al.⁷³ identified 24 features by NMR and LC-MS/MS analysis of kawakawa ethanolic extracts including amides, flavonoid glucosides, phenylpropanoids and lignans. Butts et al.⁸⁰ reported 21 features in LC-MS and NMR analysis of aqueous and ethanolic extracts of both commercially available dried leaves and fresh leaves from the East Cape regions of Te Ika-a-Māui, the North Island of New Zealand. Neither study reported absolute quantitative results. The characterization and quantification of bioactive compounds in kawakawa extracts have been explored using LC-MS/MS analysis⁷³,⁸⁰,⁸¹. However, no human studies have been sought to explore the bioavailability and functional activities of kawakawa phytochemicals. Limited number of studies have begun to understand the metabolism of some of the phytochemicals⁸²–⁸⁶.
Table 1.3 Bioactive kawakawa compounds and their biological properties

<table>
<thead>
<tr>
<th>Kawakawa plant parts</th>
<th>Extraction</th>
<th>Chemical compounds</th>
<th>Research outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>ethyl acetate, methanol and petrol</td>
<td>lignans: diayangambin, excelsin, epiexcelsin, demethoxyexcelsin, eudesmin, epieudesmin, diaeudesmin, sesangolin</td>
<td>reported new lignans 87</td>
</tr>
<tr>
<td>Roots</td>
<td>ether-acetone (1:1)</td>
<td>juvadecene</td>
<td>inducing juvenile hormonal activity in milkweed bug nymphs 88</td>
</tr>
<tr>
<td>Leaves</td>
<td>steam distillation</td>
<td>myristicin, isomyristicin (oxidised to myristicinic acid), dicyclic sesquiterpenes; α-pinene, camphene, α-phellandrene, aromadendrene, γ-cadinene, palmitic acid</td>
<td>essential oil extraction and exploring its chemical modifications 77</td>
</tr>
<tr>
<td>Dried leaves</td>
<td>hot water and ethanol</td>
<td>diayangambin, lignan isomers, myristicin, elemicin, piperchabamide, amides, vitexin and isovitexin glycosides</td>
<td>safety risk assessment of tea consumption 80</td>
</tr>
<tr>
<td>Leaves</td>
<td>hot water</td>
<td>bioactive constituents not characterised</td>
<td>anti-inflammatory, antioxidant and anti-cancer properties by inhibiting NO production and expression of IL-6 and TNF-α 89</td>
</tr>
<tr>
<td>Leaves</td>
<td>ethanolic extract</td>
<td>myristicin, diayangambin</td>
<td>no cytotoxic activities reported 90</td>
</tr>
<tr>
<td>Fruits</td>
<td>ethanolic extract</td>
<td>diayangambin, traces of myristicin, piperchabamide A, piperideide, piperine, piperanine, piperdardine, fragaramide and other amides</td>
<td>amides exhibited cytotoxic activities against MDA-MB-231 cells, PC3 and HT29 cells 90</td>
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</tbody>
</table>
Leaves ethanolic extract diayangambin, episesartemin, sesartemin, diosesartemin, myristicin, elemicin, fragaramide, kalecide, piperine, trans-pellitorine, amides influenced uptake of intestinal nutrient in Caco-2 cells 73

Leaves ethanolic extract not listed downregulated PPARγ in LnCaP cells and upregulated gene expression levels of PPARγ and AR in LnCaP+ cells 91

1.3.1 Phenylpropanoids

Phenylpropanoids are biosynthesized by plants via shikimate pathway from the enzymatic reactions of amino acid phenylalanine and tyrosine 92. They are characterised by a phenylpropene backbone, an aromatic phenyl ring attached to a propene group. Major phenylpropanoids identified in kawakawa leaf extracts are myristicin and elemicin 73,77. Myristicin (Methoxysafrole) and elemicin is a methylenedioxyphenyl (MDP)-containing alkylbenzene compound naturally occurring in plants and the principal aromatic constituent of the nutmeg seed, dill, parsley and mace volatile oil 93. Other plant sources include carrot, anise, fennel, celery, black pepper, cinnamon, wild ginger and lemon grass 94–96.

1.3.1.1 Biological properties

Myristicin and elemicin isolated from other plant sources were shown to have several biological activities, including anti-inflammatory, analgesic, antimicrobial, hepatoprotective and anti-oxidant properties (Figure 1.3) 95,97–104. Elemicin rich- *Cymbopogan khasianus* (Khasi lemongrass) essential oil exhibited biological activities including antioxidant, anti-inflammatory and anti-cholinesterase activity 102,104.

Some of the kawakawa phytochemicals extracted from other plant sources were shown to possess various biological properties including anti-inflammatory, cancer preventive, anti-proliferative, neuroprotective, antioxidant, antimicrobial and insecticidal properties.
Myristicin from nutmeg extracts mediated inflammatory response by down-regulating the interleukins (IL-6, IL-10), chemokines (MCP-1, MCP-3, GM-CSF, MIP-1A,1B), LIF, calcium and NO production by reducing intracellular calcium levels. Several studies have shown the ability of myristicin to inhibit PGE2 production to mediate inflammatory response. Elemicin from *Asiasarum sieboldii* extracts displayed anti-allergic properties by inhibiting LOX enzymes.

Myristicin and elemicin are classified as deliriant due to the hallucinogenic and empathogenic experiences upon nutmeg consumption. There were several clinical incidents reported on nutmeg abuse. The psychoactive ability of nutmeg was possibly contributed by the conversion of phenylpropanoids (myristicin, safrole and elemicin) into amphetamine-like drugs, MMDA (3-Methoxy 4,5-Methoxydioxyamphetamine).

Aqueous extracts of nutmeg and myristicin displayed antioxidant potential through free radical scavenging. inducing antioxidant enzymes (catalase, SOD, GPx, GR) modulating oxidative stress and regulating lipid peroxidation. Elemicin isolated from nutmeg and parsnip-leaved hogweed revealed significant antioxidant activity in lipid peroxidase, catalase and DPPH assays.

Myristicin isolated from nutmeg seeds (*M. fragrans*) exhibited influential anti-bacterial activity against all tested gram-positive bacteria, *Staphylococcus aureus, Bacillus subtilis, Mycobacterium luteus* and gram-negative bacteria, *Pseudomonas aeruginosa* and *Escherichia coli*. The presence of aromatic ring and unsaturated side chains with functional groups (-COOH, -NH2, -SH, -COOR) in the cinnamic acid derivatives was possibly responsible for the antibacterial activities. Myristicin extracted from shoots and essential oils of *Piper sarmentosum* potentially inhibited rice pathogens. Aromatic components (myristicin and elemicin) of *Ferula heuffelii* essential oil exhibited antimicrobial activity (against *Candida albicans, Micrococcus luteus, Micrococcus flavus* and *Bacillus subtilis*), spasmylytic inhibiting spontaneous contraction of rat ileum and antiradical activity displaying substantial scavenging of DPPH radical. Essential oils of aromatic medicinal plants (at higher concentrations) denature cytoplasmic proteins of microbial cells by integrating with the cell membrane.
1.3.2 Alkaloids

Alkaloids are plant metabolites that contain at least one nitrogen atom in a heterocyclic ring (true alkaloids) or the aliphatic chain (proto alkaloids)\(^\text{120}\). They are biosynthesized by a series of enzymatic reactions from amino acid precursors including phenylalanine, lysine, tryptophan, tyrosine and ornithine via shikimate, malonate and mevalonate pathway\(^\text{121}\). Piperidine alkaloids identified in kawakawa leaves include piperine, piperchabamide and piperdardine\(^\text{73}\). Other plant sources include *P. nigrum* and *P. longum*; chingchengenamide in *Zanthoxylum piperitum* (Japanese pepper) and antiepilepsirine was found to occur in *Piper capense*\(^\text{122,123}\).

1.3.2.1 Biological properties

Alkaloids isolated from other plant sources were shown to have biological activities, including anti-inflammatory, cancer-protective, neuroprotective and anti-oxidant properties (Figure 1.4)\(^\text{124,125}\). Biological properties of piperine are studied extensively and widely used for various therapeutic applications\(^\text{32,33,126}\). Piperine analogues extracted from kawakawa fruits including...
piperdardine, piperchabamide, piperlongumine exhibited cytotoxicity against HT 29 cells (IC$_{50}$ = 14 μM)⁹⁰. Other activities of piperidine alkaloids include anti-obesity effect via AMPK signaling pathway and alterations in lipid metabolism by activating CPT-1, UCPs ¹²⁷. Piperine isolated from *P. nigrum* inhibited adipogenesis in 3T3-L1 adipocytes by antagonizing PPAR-γ activity ¹²⁸. Piperine derivatives isolated from *P. nigrum* produced an anti-diabetic effect and also attenuated cardiac fibrosis as a PPAR-γ agonist ¹²⁹,¹³⁰. 3T3-L1 adipocyte cells treated with the piperine-epigallocatechin gallate complex synergistically reduced adiponectin levels. This lipid-lowering and anti-adipogenic activity was exerted through down-regulation of PPAR-γ, SREBP-1c, FAS, Fab-4, HMG-CoA and C/EMP-α and up-regulation of UCP-1 gene expression ¹³¹.

**Figure 1.4 Other plant sources and biological properties of alkaloids**

1.3.3 **Amides**

Amides are naturally occurring compounds consisting of an acyl group (R-C=O) linked by a single bond with a nitrogen atom ¹³². They are typically formed by enzymatic reactions facilitating carboxylic acid groups (hydroxycinnamic acid, straight chain fatty acids or phenolic acids) condensation with amines ¹³³. Amides present in kawakawa leaves include fagaramide, pellitorin, kalecide, and achilleamide ⁷³.
1.3.3.1 Biological properties

Pellitorin was found to occur in other plant sources including black pepper, betle leaves, hill pepper, P. retrofractum (Javanese long pepper), Asarum canadense (wild ginger) and were shown to exhibit anti-inflammatory, anti-thrombotic, anti-cancer, anti-fungal, anti-bacterial and insecticidal properties (Figure 1.5) \textsuperscript{124,134–140}. Fagaramide was identified in Zanthoxylum \textit{spp.} and were shown to possess anti-inflammatory and insecticidal properties (Figure 1.5) \textsuperscript{141,142}. Molecular docking and \textit{in vitro} study indicated the possibility of the involvement of hydrogen bonds and carbonyl O\textsubscript{20} atoms in eliciting anti-inflammatory potentials of fagaramide \textsuperscript{143}.

Pellitorin is structurally related to capsaicin and nonivamide without the vanillin group. Trigeminal active compounds treatment on differentiated Caco-2 cells demonstrated the reduction of free fatty acid uptake and increased acetyl-coA synthase activity but not via TRPV1 or ENaC activation \textsuperscript{135}. Trans-pellitorine from \textit{P. nigrum} reduced lipid accumulation through increased miRNA mmu-let-7b expression due to reduced PPAR-\textgreek{y} levels via TRPA1 and TRPV1 channels in 3T3-L1 cells. This study suggested that trans-pellitorin did not directly activate TRPV1 or TRPA1 and also more than one signaling pathway might be involved in elucidating the anti-adipogenic effect \textsuperscript{144}.

Few other studies have shown the anti-obesity effect of alkyl amides mediated by short-chain fatty acids reducing food intake, regulating gut microbiota and fat storage regardless of activation of TRPV1 \textsuperscript{145}. Pellitorin exhibited alpha glucosidase-1 enzyme inhibitory potential with IC\textsubscript{50} value of 34.39 ± 0.97 \textsuperscript{146}. It was also seen that vanillin does not exhibit this property indicating that presence of alkyl chain with amide moiety plays an important role in reducing fatty acid uptake. A similar study was conducted by Lieder et al. \textsuperscript{147} on 24 natural and synthetic Homovanillic acid esters to explore the impact of different structural moieties as intestinal fatty acid uptake inhibitors in differentiated Caco-2 cells. The presence of a branched fatty acid side chain was found as one of the important structural motifs enacting inhibitory potential related to fatty acid uptake in Caco-2 enterocytes. Other observations were increased activity of 1-
methylated compounds, no significant changes in inhibitory potential upon introducing double bonds and increased activity with an absence of methoxy group at the vanillyl residue\textsuperscript{147}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.5}
\caption{Other plant sources and biological properties of amides}
\end{figure}

\subsection*{1.3.4 Lignans}

Lignans are characterized by the presence of two phenylpropene units linked by a C-C bond or a C-O-C bond distinguished by the level of oxidation, substitution pattern and carbon framework\textsuperscript{148}. Some of the lignans occurring in kawakawa leaves include diayangambin, excelsin, epiexcelsin, demethoxyexcelsin, eudesmin, epieudesmin, diaeudesmin and lirioresinol-c dimethyl ether\textsuperscript{87,149,150}. Yangambin and its isomers were found to occur in \textit{Octea fasciculata} exhibiting anti-inflammatory and cardio-protective properties (\textbf{Figure 1.6})\textsuperscript{151–155}. 
Flavonoids are biosynthesized by a series of chemical reactions catalysed by enzymes including chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase, flavonoid 3-hydroxylase and dihydroflavonol 4-reductase in plants. Vitexin, isovitexin and orientin isomers and glycosides are found to occur in kawakawa leaves. They were observed to possess anti-inflammatory, cardio-protective, anti-cancer and neuroprotective properties. Vitexin effectively treated UV-induced adverse skin reactions by inhibiting free radical production. Vitexin and isovitexin is a phytoconstituent of many plants. To name a few, Passiflora sp. (passionflower), Vigna radiata (mung bean), Vitex agnus-castus L. (chaste tree), Fagopyrum esculentum (buckwheat), Crataegus monogyna (hawthorn) and Bambusa vulgaris (bamboo).

Other studies

In addition to these phytochemicals present in leaf extracts, kawakawa leaf essential oil was found to rich in myristicin (~40%) along with elemicin, caryophyllene, 4-terpinenyl acetate.
and α-Zingibirene, azulenes, palmitic acid, camphene, γ-cadenene, α-pinene, α-pellandrene, n-hexyl acetate, unidentified ketones and esters. Some of the kawakawa chemicals including piperchabamide, fagaramide, pellitorin and yangambin displayed insecticidal properties such as antiprotozoal, anti-proliferative and anti-leishmanial against *Leishmania* species.

**Figure 1.7 Other plant sources and biological properties of flavonoids**

### 1.4 Kawakawa phytochemical metabolism

Assessment of absorption, distribution, metabolism and excretion (ADME) of small molecules like kawakawa phytochemicals in humans is essential for identifying biomarkers of its consumption and to evaluate its efficacy, toxicity and functional activities. Metabolism of these compounds follows two phases mainly in the liver, but also in the kidney, intestine, and lungs or through gut microbiota biotransformation for easier elimination from the body by making it more hydrophilic and less toxic. Phase 1 metabolism involves the modification of these chemicals through oxidation, reduction, dealkylation or hydrolysis reactions mediated by P450 (CYPs) enzymes. These metabolites undergo phase 2 metabolism via acetylation.
(acetyl enzymes), conjugation with glucuronic acid, sulfate or glutathione groups mediated by UGT, SULT and GST enzymes, respectively\(^{184}\).

To date, there is limited literature available on human metabolism of phytochemicals and their metabolic conversion. Only a limited number of animal or in vitro studies have been carried out elucidating metabolic pathways of very few kawakawa phytochemicals including, myristicin, elemicin, piperine and vitexin. Phenylpropanoids, myristicin and elemicin were converted into amphetamine derivatives\(^{111}\). However, metabolomic analysis of urine samples from humans and rats after consuming nutmeg indicated that elemicin and myristicin majorly underwent metabolism by hydroxylation/dihydroxylation, demethylenation and o-demethylation but were not converted into amphetamine derivatives\(^{185}\). In vitro and in vivo studies in rats and human liver microsomes yielded myristicin metabolites, myristicin epoxide, 1-hydroxy myristicin and its NAC adducts, 2-dihydroxy-4-methoxy-5-allyl-benzene and its GSH conjugates\(^{186–188}\). The major metabolite, 5-allyl-1-methoxy-2,3-dihydrobenzene was observed to be mediated by P450 CYP3A4 and 1A2 enzymes in the human liver and its sulfate and glucuronide conjugates were observed in rat urine\(^{189,190}\). Depletion of GSTs by myristicin treatment indicated its chemopreventive potential via GST detoxification\(^{191}\). Physiologically based kinetic (PBK) modeling of bioactivation of myristicin and elemicin was studied in rat and human liver microsomes\(^{82,83}\). Likewise, piperine is reported to be metabolized via methylenedioxy cyclic ring fission, piperidine ring-opening, sulfate and glucuronide conjugation in rats\(^{86,192,193}\). Vitexin metabolism was observed to occur through glucuronide and sulfate conjugation and majorly through gut microbiota biotransformation converting into phenylacetic acid and propanoic acid\(^{85,194}\). Generally, lignans are found to be metabolized by intestinal flora into enterolactone and enterodiol followed by glucuronide and sulfate conjugation\(^{195}\). Demethylation of yangambin was observed during synthesis and could be a plausible way of its metabolism\(^{196}\). However, human metabolism of yangambin is yet to be explored. Likewise, human metabolism of other amides and alkaloids is yet to be discovered.

This review clearly indicates that there is a limited knowledge available on human metabolism of kawakawa phytochemicals and highlights the need to further explore kawakawa leaf
chemistry and human phytochemical metabolism to better understand its therapeutic potential for its use in the development of functional foods.

1.5 Instruments and techniques used in this research

This research was mainly approached using LC-MS/MS analysis to characterise the kawakawa chemical space and to identify human metabolites. Indirect calorimetry was used to evaluate a whole-body response to a complex mixture of kawakawa compounds. Finally, cell-based assays and TaqMan gene expression analysis were carried out to determine the functional activities of kawakawa compounds.

1.5.1 Phytochemical analyses and biomarkers discovery using LC-MS/MS-based approaches

With recent advancements in analytical techniques, LC coupled with tandem mass spectrometry is considered a powerful tool used for the identification, quantification, and metabolic characterization of small molecules, and the systematic evaluation of their potential metabolic pathways. This analysis is performed in two ways namely, untargeted and targeted metabolomic analysis. Untargeted metabolomic analysis is a comprehensive data-driven approach essential for biomarker discovery, global metabolite profiling and hypothesis generation. Data analysis approaches used for untargeted analysis includes raw data acquisition in LC-MS and with the help of computational tools for data conversion, import, feature detection or peak resolution, screening for biomarkers, identification and interpretation. Targeted analysis is used to identify and accurately quantify targeted chemicals. Data analysis approached for targeted analysis include raw data acquisition, referential database generation, identification of target metabolites, normalization and quantification and biochemical interpretation.

In these analyses, data collection is performed in all-ion fragmentation (AIF), data-independent acquisition (DIA) and data-dependent acquisition (DDA) mode using tandem mass spectrometry (MS/MS or MSn). All ions are fragmented without any precursor ion isolation in AIF mode, well-suited for discovering unknown compounds and understanding
complex mixtures \(^{204}\). In DIA mode, fragmentation data for all ions within a specified mass range is collected simultaneously allowing for retrospective data analysis but more challenging to interpret than DDA data \(^{205}\). DIA and AIF are valuable for capturing a wide range of metabolites in complex mixtures, suitable for untargeted analyses and discovery studies \(^{202}\). Pre-defined criteria are set and precursor ions are selected typically based on the abundance of ions in a given mass range in DDA mode \(^{205}\). DDA is more selective and could miss low-abundant or unknown compounds that were not specifically targeted for fragmentation \(^{204}\). DDA is more focused and can be useful for targeted and quantitative analyses of known targets \(^{205}\). Various software applications such as NIST MS search, MS-DIAL, MS-FINDER, GNPS, Mass frontier, XCMS\(^2\), OpenMS, SIRIUS, etc. are used for the MS/MS search and to annotate/identify these small molecules \(^{204}\).

Despite the challenges prevailing, this method has significantly benefitted data mining extensively to uncover the key metabolites and for clinical biomarkers discovery \(^{206}\). Furthermore, a combination of fingerprint and footprint metabolomics enhanced the comprehensive analysis for biomarker discovery providing information for further pathological investigation \(^{207,208}\).

\[\text{Figure 1.8 Metabolomic analysis}\]
1.5.2 Indirect calorimetry to assess physiological responses of kawakawa chemicals in humans

Indirect calorimetry is considered as the gold standard technique to determine and monitor energy expenditure by indirectly measuring respiratory gas exchanges using a metabolic monitor connected with an analyser (mask or ventilation hood) (Figure 1.9). This works on the principle that energy production in the metabolic process is proportional to the volume of oxygen consumed and the volume of carbon dioxide produced. The respiratory quotient (RQ) is calculated from these measurements which is the ratio of the volume of carbon dioxide produced to the volume of oxygen consumed. For instance, an RQ value of 0.7 indicates that fat is the fuel source utilized, whereas an RQ value of 1.0 indicates that carbohydrates were utilized for energy production. This method is used to measure energy expenditure during various activities such as resting, exercise, and food digestion and to assess resting metabolic rate during nutritional interventions. This technique is widely used in clinical and research settings for the evaluation of nutrient requirements of critically ill-patients, assessment of metabolic disorders, global nutritional assessment estimating endogenous utilisation of carbohydrates, protein and lipids, for suggesting weight loss programs and determining energy requirement of athletes.

Figure 1.9 Metabolic monitor for Indirect calorimetry
(adapted from https://www.cosmed.com/en/products/indirect-calorimetry/q-nrg)
Indirect calorimetry could be potentially used to explore the thermogenic potential of phytochemicals. For example, bioactive phytochemicals such as capsaicin, caffeine, epigallocatechin-3-gallate, resveratrol and curcumin have been shown to potentially influence the stimulation of the postprandial thermogenic cascade by inducing thermogenesis and substrate oxidation \(214-219\). Similarly, kawakawa is reported to contain some of the pharmacologically active compounds, demonstrated to influence pathways related to thermogenesis \(73,80,90\). Piperine and its analogues were shown to influence the sympathetic nervous system, increase energy expenditure and fat oxidation, and alter intestinal fat and glucose absorption \(73\). Trans-pellitorin, an analogue of capsaicin and TRPV1 agonist may inhibit adipogenesis, induce satiety, activate brown adipose tissue, and modulate intestinal hormones and the microbiome \(73\).

1.5.3 \textit{In vitro} assessment of functional activities of kawakawa compounds

\textit{In vitro} techniques used to evaluate the functional activities include Cell viability assays, enzymatic assays and fluorescence-based gene expression analysis to understand the functional activities of individuals kawakawa compounds. Cell-based assays were used to understand cell viability, metabolic activity and to assess their toxicity. Based on the alarm blue assay principle, reduction of resazurin to resorufin is directly proportional to the metabolic activity and determine the number of viable cells \(220\). Enzyme based assays were used to evaluate the cytotoxicity of chemical compounds. Lactate dehydrogenase (LDH) assay works on the principle of converting lactate to pyruvate with subsequent reduction of NAD+ to NADH which is indicative of extent of cell death or cytotoxicity in response to phytochemical exposure \(221\). TaqMan gene expression analysis is the sensitive and specific method to detect and quantify nucleic acids which can be used to determine the changes in gene expression levels after chemical exposure \(222\). TaqMan probes are designed for specific genes which bind to the target DNA sequence eliciting fluorescence that can be measured in real-time. The main advantage of \textit{in vitro} assessment is that it enables us to manipulate different variables under controlled conditions to explore specific biological processes. However, it is limited by translating these findings to \textit{in vivo} conditions which requires further validation using \textit{in vivo} experiments and
clinical studies. Despite this limitations, *in vitro* assessment is a cost-effective and efficient method to identify therapeutic potential and to better understand their underlying mechanisms.

1.6 Summary

This review indicates that despite considerable mātauranga Māori of the therapeutic benefits of kawakawa, there is a significant gap in the research and the necessity for a better understanding of the pharmacology of kawakawa leaves. Limited research using cellular and/or animal models has confirmed that aqueous extracts are safe for consumption. Knowledge of the metabolic effects of biologically active compounds is often derived from their study in isolation in in vitro and model systems. However additive and synergistic effects between these compounds are likely. Kawakawa is used locally in both traditional Māori tonics and commercially manufactured foods and beverages. Therefore, this research will potentially help to identify any positive metabolic effects of consuming kawakawa and their underlying mechanisms, provide information on beverage formulation and will assist the development of functional food products by our commercial collaborators, Wakatū Incorporation. This research provides an opportunity to grow the mana of the partners, develop investment in Tikanga, develop the NZ economy, and enhance public health.

1.7 Thesis aims and objectives

The principal aim of this thesis is to investigate the phytochemical profile of kawakawa leaves and to characterise human phytochemical metabolism. Further, I aimed to understand the whole body’s physiological responses to human resting metabolic rate and substrate utilisation after consuming kawakawa-containing beverages. Lastly, I used cell-based assays to explore the functional activities of kawakawa compounds and their underlying molecular mechanisms (Figure 1.10).
My specific objectives were,

- To explore the kawakawa leaf chemistry using LC-MS/MS-based metabolomic approaches (Chapter 2).
- To investigate the bioavailability and metabolism of kawakawa leaf chemicals by humans (Chapter 3).
- To understand the impact of kawakawa-based beverages on human energy metabolism using indirect calorimetry (Chapter 4).
- To understand the functional activity of selected kawakawa compounds using TaqMan gene expression analysis (Chapter 5).
Chapter 2 Exploring *Piper excelsum* (kawakawa) chemical space

Abstract

The chemical profiles of *Piper excelsum* (kawakawa) leaves were analysed through targeted and untargeted LC-MS/MS. The phytochemical profile was obtained for both aqueous extracts, representative of kawakawa tea, and methanolic extracts. Sixty-five compounds were identified from eight leaf sources including phenylpropanoids, lignans, flavonoids, alkaloids and amides. Eight of these compounds were absolutely quantified. The chemical content varied significantly by leaf source, with two commercially available sources of dried kawakawa leaves being relatively high in phenylpropanoids and flavonoids compared to field-collected fresh samples that were richer in amides, alkaloids and lignans. The concentrations of pharmacologically active metabolites ingested from traditional consumption of kawakawa leaf as an aqueous infusion, or from novel use as a seasoning, are well below documented toxicity thresholds.

2.1 Introduction

It is of interest to investigate the kawakawa chemical space to identify pharmacologically active compounds that may be responsible for potentially beneficial effects on human health. This study reports a comprehensive analysis of the chemical compounds extracted from both commercially available dried kawakawa leaves, and from fresh leaves sourced from different geographic locations in Aotearoa New Zealand, and at different times of the year. Mass spectrometry-based molecular networking was employed to visualise the complexity of the kawakawa leaf chemistry and several of the most abundant compounds were quantified to inform regulatory approval for extending the use of kawakawa leaf as a food product.

2.2 Materials and methods

2.2.1 Solvents and chemicals

Authentic standards were obtained as follows: myristicin, pellitorin and fagaramide (Sigma-Aldrich, Castle Hill, Australia); elemicin, apiol, piperine and vitexin (Bio-strategy, Auckland, New Zealand); yangambin (ALB technology, Kowloon, Hong Kong); piperlongumine (AK Scientific, California, USA) and podophyllotoxin (AFT Pharmaceuticals, Auckland, New Zealand). Acetonitrile (Optima LC-MS grade), formic acid, and ammonium formate (Thermo...
Fisher Scientific, Auckland, New Zealand). Type 1 water was generated from a Millipore unit (Merck Millipore, Auckland, New Zealand).

2.2.2 Preparation of standards
A mix containing myristicin (1040 μM), yangambin (1120 μM), elemicin (48 μM), vitexin (23 μM), pellitorin (45 μM), fagaramide (40 μM), dopamine (65 μM), trigonelline (73 μM) and the podophyllotoxin internal standard [IS] (12 μM) was prepared and serially diluted. Accurate mass-to-charge ratio (m/z), retention time and MS/MS spectra were added to an in-house MS/MS library based on the 2020 NIST library (National Institute of Standards & Technology, Gaithersburg, Maryland).

2.2.3 Kawakawa leaf collection
Fresh kawakawa leaves were harvested by Wakatū Incorporation in 2020 and 2021 from sites in Te Tauihu, the Northern region of Te Wai Pounamu, the South Island of Aotearoa New Zealand (Table 2.1). Samples from Mohua, Golden Bay were collected in October 2020, from Whangamoa in early and late January, early and late March and April 2021, as well as from Arapaoa Island and Pōhara in late 2021. Fresh leaves were washed in cold water, frozen at -20°C, lyophilised, and ground to powder. Commercially available, dried kawakawa leaves harvested from unspecified locations in Te Ika A Māui, Aotearoa New Zealand’s North Island, were acquired in 2020 from ŌKU NZ Native Herbal Products Ltd and Phytomed Medicinal Herbs Ltd. 2.4. The drying process utilised for the commercially sourced leaves was understood to be via sun/air drying but full details were not provided.

2.3 Extraction and sample preparation for LC-MS/MS
2.3.1 Aqueous extraction
All leaf samples were finely ground in liquid nitrogen. A 10 mg mass of leaf powder was weighed into a 10 mL amber glass headspace vial, a 10 mL volume of preheated Type 1 water containing IS (12 μM) was added and the vial was capped and maintained at 80°C for 10 min. Vials were allowed to cool to room temperature and the particulate material was pelleted by centrifugation at 10,000g for 5 min at 4 °C. A volume of supernatant was transferred to a polypropylene autosampler vial and capped for LC-MS analysis. All extractions and analyses were conducted in triplicates.
2.3.2 Solvent extraction

Ultrasound-assisted methanol extraction was carried out in triplicate in 4 mL amber glass vials. Masses of 100 mg of the ŌKU commercial sample, and the Arapaoa and Pōhara fresh samples were ground as above and extracted with 2 mL of 80% MeOH ultrasonication for 30 min in an ultrasonic bath (Soniclean 80T, Soniclean Pty Ltd., Adelaide, Australia) on the highest power setting. The extract was centrifuged at 5,000g for 5min, and 1.5 mL of supernatant was collected. Another 1.5 mL of 80% methanol was added, and the pellet was re-extracted and centrifuged. This re-extraction was repeated until three volumes of supernatant were combined (4.5 mL). Extracts were dried in a SpeedVac centrifugal concentrator (Savant SC250EXP; Thermo Scientific, Hampton, USA) coupled with a refrigerated vapour trap (Savant RVT5105, Thermo Scientific, Hampton, USA) and the dry residue massed and resuspended in 10 mL 80% MeOH. A fourth extraction volume was obtained and analysed directly to assess extraction completeness.

Table 2.1 Kawakawa leaf sources

<table>
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<th>Label</th>
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<th>Provenance</th>
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</table>
AFT2 Late January 2021 Te Tauihu aqueous fresh
AFT3 Early March 2021 Te Tauihu aqueous fresh
AFT4 Late March 2021 Te Tauihu aqueous fresh
AFT5 April 2021 Te Tauihu aqueous fresh
AFM October 2020 Mohua, Golden Bay aqueous fresh

2.3.3 LC-MS/MS instrument and conditions
The liquid chromatograph comprised an Accela 1250 pump (Thermo Fisher Scientific, CA, USA), a Thermo TriPlus autosampler (Thermo Fisher Scientific, Hampton, USA), a HotDog5090 column oven and a Phenomenex Kinetex biphenyl analytical column (150 mm × 2.1 mm × 1.7 μm) held at 30 °C. Flow was 0.3 mL/min and the injection volume was 5 μL. Mobile phase was MilliQ with 5 mM ammonium formate and 0.1% formic acid (A) and 100% acetonitrile with 0.1% formic acid (B). The mass spectrometer was a Q-Exactive (Thermo Scientific, Dreieich, Germany) with a heated electrospray ionisation [HESI] source at 425°C. Source voltage was 3.5 kV, S-lens RF level 50, heated capillary temperature 263°C, AGC target 1e6 and resolution 17,500. Mass spectra were acquired in positive polarity and centroid mode.

Data for qualitative analysis was acquired in Data Dependent Acquisition mode [DDA]. The gradient was 0-100% B in 14 min, held for 1 min, and then re-equilibrated to 0% B from 15 to 20 min. Scan range was m/z 50-700 with normalised collision energy (NCE) of 30eV.

For comparative analysis of aqueous leaf extracts, spectral data were collected using All Ions Fragmentation [AIF]. The full scan range was from m/z 75 to 850, followed by two AIF scans from m/z 56.7 to 750 with 10 and 35 eV NCE, respectively. The gradient started at 0% B, ramped to 95% B at 16 min, held at 95% B until 18 min, ramped to 0% at 20 min, and then re-equilibrated at 0% B from 20 to 25.5 min.
MS conditions for quantitative analysis were the same as for qualitative analysis. The gradient was 0% B for 1 min, then 0-95% B at 13 min, held for 1 minute, then re-equilibrated at 0% B from 14 to 20 min. The same method was used for both methanolic and aqueous extracts.

2.4 Data processing, visualisation and statistical analysis

Data were collected and processed using Thermo Xcalibur software (version 4.2.47). Data were normalised to IS (podophyllotoxin). MetaboAnalyst (version 5.0) was used to create clustergrams to visualise the variation in metabolite abundance between different sample types. Absolute quantitative data were analysed using one-way ANOVA followed by a two-stage step-up method of Benjamini, Krieger and Yekutieli for multiple pairwise comparisons using GraphPad Prism version 9.1.2 (GraphPad, California, USA).

Chromatographically resolved features with MS/MS data from the aqueous extracts were annotated using a combination of chemoinformatics and manual inspection. Features were annotated using spectral matching in MS-DIAL v4.7 and an in-house MS/MS library based on the 2020 NIST library. A molecular network was exported from MS-DIAL with a mass tolerance of 0.025 Da and a 75% similarity threshold. Features were also manually annotated by comparison with the existing literature on the chemical space of the Piper genus. Adducts and other duplicates were filtered out, as were features not directly connected to an annotated node. The MS/MS spectra were loaded into MS-FINDER v3.52 for computational formula predictions, fragment annotations and structural elucidation. Final identities were assigned based on the above information, together with manual inspection of the MS/MS spectra to identify fragments and structural moieties common to multiple features and classes of compound. Cytoscape v3.9.0 was used to visualise the final network.

2.5 Results

2.5.1 Qualitative metabolic profiling of kawakawa aqueous extracts

Using MS/MS spectra from eleven authentic standards, trigonelline, dopamine, vitexin, fagaramide, yangambin, elemicin, piperine, myristicin and pellitorin were identified to Level 1 of the Metabolomics Standards Initiative [MSI] guidelines. A further 262 features were annotated by spectral matching to reference spectra using MS-DIAL with scores > 0.85. Compounds previously identified in P. excelsum and other Piper spp. were well represented, including phenylpropanoids, alkaloids, amides, flavonoids and lignans.
Examples include dihydropiperlongumine \textsuperscript{34}, myristicin \textsuperscript{45}, vitexin \textsuperscript{7}, vitexin-O-glucoside \textsuperscript{4}, cinnamoyl piperidine \textsuperscript{26} and cinnamoyl piperideine \textsuperscript{16} \textsuperscript{80}, dimethoxycinnamoyl piperidine \textsuperscript{22} \textsuperscript{230} and piperanine \textsuperscript{42}. Features annotated as apiole and piperlongumine\textsuperscript{80} did not match the retention time and mass spectra of those standards.

Molecular networking was used to filter the chemical space by reducing the 1,453 MS/MS spectra of chromatographically resolved features to a size that could be manually scrutinised. Nodes not directly linked to one already annotated were excluded. Adducts and other artifacts were manually filtered, and 154 features were processed using MS-FINDER to generate orthogonal annotations. Identities were assigned to a total of 65 features, including isomers.

Scrutiny of the MS/MS spectra of amides revealed patterns of fragmentation corresponding to the moieties on either side of the amide nitrogen. For example, the methylenedioxyccinnamoyl fragment of fagaramide yields an intense ion at m/z 175.0395, whilst the isobutyl fragment is m/z 57.0699 (Figure 2.1). The same pattern of fragmentation was observed with piperine and piperlongumine. This pattern was combined with the information from spectral matching and in silico structural elucidation using MS-FINDER to assign amide identities to 46 features in the data (Table 2.2). An annotated chromatogram of aqueous extracts indicating the peaks of the compounds identified/annotated in Table 2 is included (Appendix A). The amides could be divided into groups based on their structure, being based on either cinnamoyl, methylenedioxyccinnamoyl or fatty acid moieties. These were combined with a variety of amines such as piperidine, piperideine, pyrrolidine and isobutylamine. The cinnamoyl ring was sometimes modified with hydroxyl and methoxy groups, and cyclic amines on cinnamoyl amides sometimes exhibited hydroxyl or ketone groups. The hydrocarbon chain that links the phenyl ring of the cinnamoyl moiety with the amide varied in length and saturation.

\textbf{Table 2.2 Putatively identified compounds in kawakawa leaf extracts}

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<tr>
<th>ID</th>
<th>Annotations [empirical observations]</th>
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<th>Mass accuracy (ppm)</th>
<th>major MS/MS ions</th>
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<td>Rf value</td>
<td>Log P</td>
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Chemoinformatics allowed us to annotate several other features such as the catecholamine neurotransmitter dopamine, several flavonol glucosides and two lignans besides the yangambin isomers (Table 2.2). Often, chromatographically resolved peaks with very similar MS1 and MS2 spectra were observed. For example, authentic standard for trans-fagaramide coeluted and spectrally matched with the feature at 8.40 min so the one at 8.15, which was also a close spectral match, was assumed to be cis-fagaramide. Yangambin 32, was only observed in ACO extracts, at low abundance. Two other features which appeared a few seconds later in all other kawakawa extracts with similar MS2 spectra and much greater abundance were annotated as epiyangambin by spectral matching. The [M+NH4]+ adduct for these features was twice that of the [M+H]+ adduct, whereas for yangambin it was <10% (Figure 2.2). No reference spectra could be found for the third yangambin isomer, diayangambin, and an authentic standard was not available to purchase. Diayangambin was identified in kawakawa by Russell & Fenemore [5] so, it was assumed that the first and most abundant peak of these two was that, and the last one was epiyangambin.
Multiple features were annotated as dimethoxycinnamoyl piperidine 22, cinnamoyl piperidine 26, piperlonguminine 28, among others (Table 2.2). To visualise the kawakawa chemical space the annotated features are shown as a molecular network in Figure 2.3. Features with m/z 150.0553 and m/z 279.1222 were observed to be abundant in the kawakawa extracts but no identity could be robustly assigned to them. For clarity, only features that could be annotated with confidence are visualised.

Alkaloids and amides comprised the largest cluster in the network (Figure 2.3A). Cinnamoyl or methylenedioxyccinnamoyl moieties combined with piperidine were the most commonly observed and connected structures in the network and occupy the centre. A subcluster can be seen at the top of the network, populated with compounds whose amide groups carry hydroxyl or ketone groups, or that have unsaturated bonds. Molecules on the mid-to-lower-left mostly contain methylenedioxybenzole rings with longer hydrocarbon backbones than those on the right. Fatty acid amides mostly are localised to the right edge of the network. Compounds in the middle of the network, that are based on a cinnamoyl moiety, are methoxylated to varying degrees.

Flavonoids comprised the second-largest cluster (Figure 2.3B), including vitexin and its glucosides. Dopamine 3 was clustered together with another catecholamine, salsolinol 1, and methyldopamine 10. Methyldopamine was also connected to the aromatic amino acid, tryptophan 15. The phenylpropanoids myristicin 44 and elemicin 37, the lignan magnolin 33, three amides, and the alkaloid trigonelline 2 did not cluster with any other components.

### 2.5.2 Differences in kawakawa chemistry

The differences between the chemical profiles of the eight aqueous kawakawa extracts were visualised using clustermaps to visualise unsupervised hierarchical clustering analysis (Figure 2.4). Features in the extracts from commercial leaf sources, ACO and ACP, clustered together in the clustermap indicating similarities in their phytochemical profile, particularly in their phenylpropanoid content. Features in extracts of fresh sample extracts also clustered together and were observed to be particularly rich in amides, except for AFT4. The AFT3, 4 and 5 extracts were relatively rich in lignans. AFM extracts seem to be exceptionally rich in both phenylpropanoids and alkaloids. This latter sample is unique both in the geographic location (Mohúa, Golden Bay) and season (late October, i.e., springtime versus summer/autumn) in which it was collected.
Figure 2.1MS/MS fragmentation patterns of amides based on methylenedioxycinnamic acid.

The top row are amides with isobutylamine moieties, the middle row piperidine and the bottom one pyrrolidine. The left hand plots have a methylenedioxycinnamoyl. Under collision induced dissociation the amide bond breaks, yielding prominent ions for the two parts of the molecule. These distinct fragmentation patterns allow the structures of this class of compound to be inferred.
Figure 2. Chromatogram of three yangambin isomers and their MS and MS/MS spectra in positive ionization mode.

Yangambin is at 10.45 min, diayangambin is the largest peak at 10.68 min, and epiyangambin is at 10.76 min. A) Extracted ion chromatogram of yangambin [M+H]⁺ at m/z 447.2013. B) MS spectra of three yangambin isomers showing difference in the relative abundance of the [M+H]⁺ and [M+NH₄]⁺ adducts. C) MS/MS spectra of three yangambin isomers showing no significant difference. N.B. The retention times here differ from those in Table 2 as they were acquired under slightly different conditions.
Figure 2.3 continued..
Figure 2.3 Molecular networking of LC-MS/MS features in kawakawa extracts.

Nodes are unique features resolved by retention time or m/z. Numbers correspond to Table 2.2. Node borders are coloured by retention time from yellow to purple. Edge width indicates similarity between the compounds existing within same cluster (dot product 0.75-1). A) The main cluster contains features that were either identified as or annotated as amides. B) Features not connected to the main network of amides. Minor clusters include flavonoids, lignans and several amides. Six nodes were orphans, including several amides not connected to the main network, a lignan, phenylpropanoids and the unique compound trigonelline.
Figure 2.4 (i) Clustergram of normalised (to IS) and auto scaled peak areas from LC-MS/MS analysis of aqueous extracts. (ii) Clustergram derived from the area ratio of the phytochemicals estimated from the LC-MS/MS analysis of triplicate methanolic extracts of three types of kawakawa.

(i) The first letter of the sample label indicates aqueous extraction (A), the second letter commercial dried leaves (C) or fresh leaves (F), and the third the source as listed in Table 1. Te Tauihu samples were collected on multiple occasions throughout 2021 so a number is added to index this.

(ii) The first letter of the sample label indicates methanolic, the second letter commercial dried leaves (C) or fresh leaves (F), and the third the source (Table 1). In both the Clustergram, each row represents a resolved feature (Table 2) and each column represents one triplicate analysis from the eight different leaf sources. Heat scale units are standard deviations from the mean of all peaks for that feature. Hierarchical clustering on each axis demonstrates similarity of features (y axis) and samples (x axis).
2.5.3 Comparison of aqueous and methanolic kawakawa extract

Comparison of the quantitative data for the aqueous and methanolic extracts of the commercial ACO kawakawa show that methanolic extraction was more effective for most compounds, as would be expected. The exceptions were myristicin and elemicin, which were slightly elevated in aqueous extracts.

2.5.4 Absolute quantitative analysis of aqueous and methanolic extracts

LC-MS was used to absolutely quantify myristicin, elemicin, fagaramide, diayangambin, vitexin and pellitorin in aqueous extracts of the ACO and AFT1 kawakawa samples (Figure 2.5A). Compared to AFT1 samples, ACO samples had significantly higher levels of myristicin (p < 0.01) and elemicin (p < 0.001). Mean (± SD) myristicin concentration in ACO was 5.28 ±1.2 μmol per gram of dry weight leaf powder [μmol g⁻¹] compared to 0.88 ±0.1 μmol g⁻¹ in AFT1. Elemicin concentration in the commercial sample was 9.34 ±0.04 μmol g⁻¹, 178% higher than that of the fresh samples, which was 5.26 ±0.20 μmol g⁻¹. In contrast, the ACO samples had significantly lower levels of fagaramide (p < 0.01) and pellitorine (p < 0.001); mean pellitorin concentration of AFT1 was 43.2 ±2.0 μmol g⁻¹, or > 4,000% of the ACO concentration of 0.99 ±0.00 μmol g⁻¹.

The same compounds were quantified, together with yangambin, dopamine and trigonelline, in the methanolic extracts (MCO, MFA, and MFP; Figure 2.5B). There were no significant differences in the concentration of myristicin and elemicin amongst any of the samples. MCO samples had significantly lower levels of fagaramide, yangambin, dopamine and pellitorin than either of the fresh leaf samples, MFA and MFP (p < 0.001), and significantly higher levels of vitexin and trigonelline than fresh leaf samples, MFA (p<0.001) and MFP (p < 0.01). There were no significant differences in all the phytochemical concentrations between MFA and MPO samples.
Figure 2.5 Absolute quantification of kawakawa phytochemicals obtained from A) aqueous extraction of ŌKU leaf powder (ACO) and fresh leaves from the Nelson region (AFT1), and B) methanol extraction of ŌKU leaf powder (MCO) and fresh leaf powders collected from Arapaoa Island (MFA) and Pōhara (MFP). Values are mean ± SD (n=3).
2.6 Discussion

This investigation of the kawakawa leaf chemical space significantly builds on the work of Obst et al. \textsuperscript{73} and Butts et al. \textsuperscript{80}. The identities of eight compounds in kawakawa were confirmed and assigned identities to 57 others with confidence. The variation in commercial processed kawakawa leaf chemistry and in fresh leaves harvested over several geographic locations and seasons were explored. Finally, the absolute quantification of eight of the major kawakawa leaf compounds enabling us to make inferences about the risk and benefits associated with kawakawa consumption was provided. These results provide novel insights into the complexity and functionality of kawakawa leaf chemistry and that of the \textit{Piper} genus.

The 65 features, including isomers, to which identities were robustly assigned has greatly increased the understanding of the kawakawa chemical space. These features include several that have been reported in other members of the Piperaceae, but not in \textit{P. excelsum}. Many lack common names, so it was identified here using the names of closely related structures and the differences between them. So, an analogue of ilepcimide with a saturated hydrocarbon backbone, instead of the mono-unsaturated chain, is described as “saturated ilepcimide”. The kawakawa chemical space is dominated by amides whose structures vary quite systematically. They mostly consist of a cinnamoyl, methylendoxyccinnamoyl or a piperoyl moiety and an amine bonded by an amide. From the synthesis of piperine it was hypothesised that these structures likely form through the action of piperine synthase (piperoyl-CoA:piperidine piperoyl transferase), or the related enzyme, piperamide synthase \textsuperscript{232}. These enzymes condense an organic acid thioesterified to Coenzyme A with an amine. Piperine synthase favours piperic acid-CoA and piperidine as substrates. However, it can also utilise isobutylamine, yielding piperlongumine 28 and pyrrolidine, resulting in piperyline. This enzyme is one of two closely related enzymes described in \textit{P. nigrum} (black pepper) \textsuperscript{232}. The second, piperamide synthase, is reported to have a broader range of substrates, catalysing the conjunction of many different organic acids, cyclic and linear, to diverse amines. For example, it is involved in the synthesis of fatty acid amides reported in several Piperaceae \textsuperscript{233,234}, such as pellitorin \textsuperscript{48}. Pellitorin, the amide of 2,4-decadienoic acid and isobutylamine, deserves special mention as it is one of the most abundant compounds in kawakawa. Pellitorin is a potent agonist of the TRPV1 receptor.
and is reported to induce a numbing or tingling sensation upon consumption. It is likely responsible, in part, for the spicy flavour of kawakawa. Besides pellitorin, other features were also annotated as the piperidine amide of decadienoic acid, neopellitorin B 49, and the pyrrolidine amide, sarmentine 46. This diversity of form is common to many of the other amides identified. Piperidine, pyrrolidine and isobutylamine forms of several other compounds were observed including cinnamic acid, methylenedioxyacinnamic acid, and piperic acid, as well as some longer chain piperinoids such as piperardine 45 and piperolein A 47. This pattern suggests either that piperamide synthase dominates amide synthesis in *P. excelsum*, or that the synthesis of the piperic acid-CoA precursor is greatly reduced.

Cyclic amine moieties of the cinnamoyl amides often exhibited functionalisation, such as unsaturated carbon bonds, or oxidations in the form of hydroxyl or ketone groups. Notably, these functionalisations were not observed in any other amides. It may be that, despite a common synthetic pathway for all the amides, the cinnamoyl ones are further metabolised following amide formation. Or they may be products of a distinct synthetic pathway or locus.

Hydroxyls and methoxy groups were also observed on the aromatic rings of cinnamoyl moieties. This is likely to be produced by similar synthetic processes to two other major kawakawa metabolites, myristicin 44 and elemicin 37. Interestingly these two compounds both feature triple modifications to the aromatic ring: three methoxy groups in the case of elemicin; a methylenedioxy bridge and a methoxy group for myristicin. Piperic acid is synthesised in *P. nigrum* by the enzyme CYP719A37, which forms the methylenedioxy bridge. This enzyme has considerable selectivity for vanilloid precursors with an aliphatic chain length of five carbons or more. The majority of the amides observed here exhibited three carbons in the aliphatic chain, so another variant of this enzyme is likely responsible for this transformation in *P. excelsum*. The biosynthesis of myristicin does not seem to have been elucidated so it is not clear whether this putative enzyme is also responsible for forming the methylenedioxy bridge in myristicin. No amides with three modifications to the phenyl ring were observed in kawakawa. This, together with the abundance of unmodified cinnamoyl amides, indicates that the synthetic pathways of the alkenylbenzenes and amides diverge early in the phenylpropanoid pathways, as described in Vassao et al. 236.
Another unusual compound reported here is the anticonvulsant compound ilepcimide. Ilepcimide is prescribed as a medicine to epileptics in China, hence its synonym of antiepilepsirine. The compound is a truncated analogue of piperine and appears to be one of the most abundant compounds in some of the samples analysed here. The phenylpropyl amides awaine, cinnamoyl piperidine and cinnamoyl piperideine were also in the top ten most abundant features in the relative quantification data but are relatively obscure. There is little information about their pharmacology or health effects associated with their consumption in the literature. It has been reported that cinnamoylpyrrolidine, a closely related compound that was also identified in the kawakawa extracts at a much lower abundance than the piperidine and piperideine analogues, has been shown to have greater anti-inflammatory activity than aspirin in cellular assays of platelet aggregation.

Several fatty amides were identified in the current work, including remarkable concentrations of pellitorin. This amide of decadienoic acid and isobutylamine has been shown to obstruct the interaction between proteins that regulate pro-inflammatory cell-adhesion. It can reduce the uptake of glucose and fatty acids by human epithelial Caco-2 cells, and has been shown to reduce lipid accumulation at nanomolar concentrations. Pellitorin has been shown to be a potent agonist of TRPV1, as well as the Transient Receptor Potential Ankyrin 1 receptor. Pellitorin has a numbing effect and may contribute to the analgesic effect of kawakawa that make it useful for toothache in Rongoā Māori.

The semi-quantitative analyses reported in this study (Table 2.2, Figure 2.4) improve upon those reported previously in the confidence of our compound identification and the quantity of features identified. For example, based on our metabolite search with the characteristic fragmentation ion, features with m/z 232.1337 reported as an amide and m/z 230.1181 as piperchabamide by were annotated as awaine and cinnamoyl piperidine, respectively. Several other features which previously were identified no further than “amides” were annotated as dimethoxycinnamoyl piperidine and piperanine. Using chromatographic deconvolution a feature with the same m/z and fragmentation pattern as myristicin, but considerably greater in abundance and with a different retention time, was demonstrated to be an artifact from in-source fragmentation of the lignan, magnolin, and not the myristicin isomer as reported earlier (Figure 2.4). The absence of apiole and piperlongumine was verified by reference standard analysis.
The presence of the catecholamine neurotransmitter, dopamine, was reported for the first time in the Piperaceae. Dopamine has been reported in several other food plants and is suggested to function as a herbivore deterrent in algae. Dopamine is present in kawakawa at concentrations of several hundred µmol g\(^{-1}\). The abundance of dopamine receptors in the gut provides a mechanism by which consumption of kawakawa tea soothes upset stomachs and other gastrointestinal complaints in Rongoā Māori. It should not be surprising that the chemical profile of commercial, dried kawakawa leaves was much different to that of fresh leaves. This could be due to disparity in the post-harvesting processing, genetic differences between kawakawa genotypes, or other biotic and abiotic factors (or a combination thereof). It was unexpected, however, that the chemical profiles of leaves from different sources and seasons also varied as substantially as they did. The locations from which fresh kawakawa were sampled for this analysis were selected based on traditional harvest, pragmatic, and commercial considerations but the populations are located closely enough to experience gene flow, and their ecotypes were not observed to differ radically. Herbivore damage can cause plants to raise concentrations of secondary metabolites as a defence. However, field and laboratory experiments have found no effect of prior looper caterpillar (Cleora scriptaria) herbivory upon the palatability of kawakawa leaves to subsequent herbivores. It has not been ascertained whether herbivory influences the flavour or pharmacology of kawakawa leaves.

Regulatory approval in Aotearoa New Zealand for the sale of food products containing kawakawa currently is restricted to dried leaf herbal infusion (similar to ‘tea’) or dried leaf as a component of a seasoning. It is important to understand the absolute concentration of compounds within these sample types that have previously been associated with biological activity and toxicity. The concentrations of metabolites that were quantified would appear to be well below what could be speculated as toxic for humans. For example, it has been estimated that 31 - 36 µmol kg\(^{-1}\) day\(^{-1}\) of myristicin could cause psychopharmacological effects in humans. This equates to a dose of 2,170-2,520 µmol for a 70 kg adult. In rats, elemicin was observed to induce toxicity through metabolic bioactivation upon administration of 2,404 µmol kg\(^{-1}\) of elemicin. Using the conversion factor from rats to humans supplied by, this equates to >27,000 µmol for a 70 kg human adult. A mass of 1 g of the kawakawa leaves studied here infused in a 250 mL volume of hot water will release up to 5.5 µmol of myristicin and 9.0 µmol of elemicin. A gram of dried leaf powder as seasoning would contain up to 8.0
µmol elemicin and 7.1 µmol myristicin. These values are all <1% of concentrations associated with toxicity.

Comparisons with real world consumption of food containing elemicin and myristicin, such as carrots, are also reassuring [41]. Adult carrot consumption in Aotearoa New Zealand is estimated at 14.5 g d⁻¹ [42]. Using literature values for the elemicin and myristicin content of carrots, daily intake of elemicin were estimated as 68 µmol and myristicin as 7 µmol. These amounts were greater than, or similar to intake from the estimated values for kawakawa so its consumption could be very unlikely to be hazardous.

A No Observable Adverse Effect Level [NOAEL] of 22.3 µmol kg⁻¹ day⁻¹ of pellitorin 48, or 1,561 µmol for a 70 kg person, was reported from an in vivo animal study 248. Kawakawa tea would provide 173 µmol and a gram of kawakawa leaf powder up to 43 µmol. The data exists on the risks associated with consuming plant material containing vitexin 7 and yangambin 32 also suggests that kawakawa presents little risk 151,249. The pharmacology of ilepcimide 25 is not well documented but experimental doses of 38 µmol kg⁻¹ day⁻¹ have been prescribed to children, with no reported adverse effects 250. As it was not feasible to acquire an authentic standard for ilepcimide, fagaramide 24 was used as a proxy for ilepcimide quantification. Fagaramide is an almost identical compound, differing in its isobutylamine moiety instead of ilepcimide’s piperidine ring (Figure 2.1). Under electrospray ionisation they will have similar response factors, the four isomers elute within 33 seconds of each other, and they appear in similar relative abundance. The concentration of ilepcimide isomers were also estimated in kawakawa to be 5.7 µmol g⁻¹. This was less than 1% of the dose for a 20 kg child, so could be unlikely to be hazardous.

There is commercial interest in incorporating kawakawa leaf powder directly into food products. It is not known how bioavailable the metabolites in such material are. Of significance, the fatty amide, pellitorin, does not exert the same pungency as capsaicin or piperine when consumed orally, although it does have a remarkable tingling and numbing effect 251. This may make kawakawa more appealing as a dietary supplement to effect TRPV1 activation than capsaicin- or piperine-rich ingredients.
2.7 Limitations

This study has used cutting edge analytical techniques to explore the chemical space of kawakawa. However, there were some limitations in the sampling and coverage. Samples were collected for analysis in a somewhat ad hoc fashion, and not systematically. Our data does not cover the entire range of seasons. The sample sets for methanolic and aqueous extractions only marginally overlapped, limiting the comparisons that could be made between the two sample sets. The compounds which could be absolutely quantified include several of the features that exhibited the highest ion counts in the relative quantification analysis but there are other features which may be present at even higher concentrations. These include compounds which are known to be pharmacologically active, such as ilepcimide. Future studies should focus on synthesising such compounds and quantifying them absolutely in kawakawa. There were some technical limitations in the data collection and the techniques used. Our data did not include integration of GC-MS for additional identification of compounds, in particular, terpenes. However, preliminary analysis carried out with GC-MS and evidence from previous reports suggested a limited number of terpenes in kawakawa leaf extracts\textsuperscript{73,77,80}. Likewise, vitexin and its glucosides, often identified in negative mode, were detected in positive mode and analysis of data collected in negative ionisation mode did not provide any additional identification in the study\textsuperscript{252}. Hence, further analysis were carried out only in positive ionisation mode.

2.8 Conclusion

We report the chemical profile of methanolic and aqueous extracts of leaves of kawakawa, a plant endemic to Aotearoa New Zealand and of great cultural importance to Māori. LC-MS/MS-based metabolic profiling combined with molecular networking and multivariate analysis reveals geographical and seasonal variation in kawakawa chemistry. With its rich phytochemical diversity, and as a candidate for functional food and ingredient applications, further exploration of the bioavailability and biological properties of kawakawa could elucidate its health effects and the mechanism underlying its beneficial effects upon inflammation and glycaemic regulation. Phytochemical concentrations in kawakawa are unlikely to be hazardous for human consumers when consumed as an aqueous infusion or as a dry leaf powder.
Chapter 3 Bioavailability and metabolism of *Piper excelsum* (kawakawa) leaf chemicals in humans

Abstract

Comprehensive phytochemical profile of kawakawa leaf extracts has been explored in Chapter 2. In this chapter, I investigate the bioavailability and metabolism of kawakawa leaf chemicals in humans after ingestion of kawakawa tea. Six healthy male volunteers in a pilot study (BOKA-T) and thirty healthy volunteers (15 male and 15 female) in a follow-up study (TOAST) were administered a hot water infusion of dried kawakawa leaves. Urine and plasma samples were obtained for up to 24 h and analysed by non-targeted LC-MS/MS for metabolites derived from compounds within the KT. Twenty-six urinary metabolites were significantly associated with KT consumption in both BOKA-T and TOAST. Thirteen of these were provisionally identified as human metabolites of specific compounds in KT, eight further metabolites were identified as being derived from specific compounds in KT but without resolution of chemical structure, and five were of unknown origin. Seven of the 26 metabolites were also present in plasma. Kawakawa tea was well tolerated by the participants after an overnight fast. Analyses of urine and plasma indicates that several kawakawa compounds are bioavailable and modified by phase 1 and 2 metabolism. These metabolites may provide biomarkers for kawakawa consumption and insight regarding the basis of reported therapeutic properties of kawakawa. Further studies are required to elucidate the pathways for their biotransformation and to better understand the physiological responses and mode of action of kawakawa leaf compounds in humans.

3.1 Introduction

A comprehensive chemical analysis of kawakawa leaf extracts with the use of targeted and untargeted LC-MS/MS metabolomic analysis revealed the presence of nearly sixty kawakawa leaf compounds of diverse chemical classes including phenylpropanoids (myristicin and elemicin), amides (piperine, pellitorin and fagaramide), alkaloids (trigonelline), lignans (diayangambin), catecholamines (dopamine and trigonelline) and flavonoids (vitexin) (Chapter 2) (Figure 3.1). Two studies have demonstrated the safety of kawakawa tea (KT) consumption
in animals \textsuperscript{80} and in human subjects \textsuperscript{253}, and there is some evidence that acute ingestion of KT may modulate post-prandial glucose metabolism \textsuperscript{253}.

![Figure 3.1 Major P. excelsum (kawakawa) leaf compounds](image)

Cellular and animal studies have shown the biological activities of some of the compounds identified in kawakawa leaves \textsuperscript{73,89}. Elucidating the metabolism and mode of action of these dietary-derived plant metabolites in humans is required to understand the putative therapeutic effects of kawakawa and to inform its possible use in functional food products. Moreover, the identification of urinary metabolites may provide biomarkers of consumption that will be useful in clinical trials. This study aimed to provisionally identify human metabolites derived from kawakawa following the ingestion of a hot water extract of dried, commercially available kawakawa leaves through untargeted LC-MS/MS analyses.

### 3.2 Experimental

### 3.3 Human subjects and sample collection

Analyses were undertaken on plasma and urine samples from two previously described acute human intervention studies of similar design \textsuperscript{253} (Figure 3.2). BOKA-T
[ACTRN12620000629932] was a three-arm randomized crossover study in which participants consumed in a random order 250 mL of a hot water extract of 4 g of dried kawakawa leaves (sourced from ŌKU Ltd, Hamilton, New Zealand), a 1 g extract of dried kawakawa leaves or 250 mL of hot water. TOAST [ACTRN12621000311853] was a two-arm randomized crossover study in which 15 males and 15 females consumed in a random order 250 mL of a hot water extract of 4 g of dried kawakawa leaves or 250 mL hot water. Both studies were approved by New Zealand Health and Disabilities Ethics Committees, and all participants provided written informed consent ((BOKA-T (20/CEN/69) and TOAST (20/STH/236)).

**Figure 3.2 Study design of BOKA-T and TOAST**

Blood samples were collected at baseline (t = 0 min) and at 30, 45, 60, 90, 120, 180 min and at 24 h for BOKA-T and at 30, 45, 60, 90, 120 and 180 min for TOAST studies. Plasma was then separated within 2 h of collection at 15,000 × g for 15 min at 4°C. Spot urine samples were collected in both studies at baseline (t = 0 min). Subsequently, bulk urine samples were
collected in BOKA-T within the time intervals of 0-2, 2-4, 4-5 and 5-24 h, and for the interval 0-3 h in TOAST. Plasma and urine samples were aliquoted and stored at -80°C until further analysis.

3.4 Dosage information

The concentrations of the major kawakawa-derived compounds in the dried kawakawa leaves (from aqueous extractions and methanol extraction for which the standards were available), as published previously 81, are shown (Table 3.1).

Table 3.1 Estimated quantity of each of the major kawakawa compounds

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Composition (μmoles per g of dried kawakawa leaf powder)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>aqueous extracts&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>myristicin</td>
<td>5.3 ± 1.20</td>
</tr>
<tr>
<td>elemicin</td>
<td>9.3 ± 0.04</td>
</tr>
<tr>
<td>fagaramide</td>
<td>Not detected</td>
</tr>
<tr>
<td>yangambin</td>
<td>Not detected</td>
</tr>
<tr>
<td>diayangambin</td>
<td>3.0 ± 0.7</td>
</tr>
<tr>
<td>vitexin</td>
<td>0.22 ± 0.001</td>
</tr>
<tr>
<td>dopamine</td>
<td>Not measured</td>
</tr>
<tr>
<td>pellitorin</td>
<td>0.99 ± 0.001</td>
</tr>
<tr>
<td>trigonelline</td>
<td>Not measured</td>
</tr>
</tbody>
</table>

<sup>a</sup> values are represented as mean ± SEM (n = 3)

3.5 Solvents and chemicals

Authentic standards were obtained as follows: myristicin, pellitorin and fagaramide (Sigma-Aldrich, Castle Hill, Australia); elemicin, apiole, piperine and vitexin (Bio-Strategy, Auckland, New Zealand); yangambin (ALB Technology, Kowloon, Hong Kong); piperlongumine (AK
Scientific, California, USA); podophyllotoxin (AFT Pharmaceuticals, Auckland, New Zealand); buspirone (Axon Medchem, VA, USA). Other solvents, acetonitrile (Optima LC-MS grade), formic acid, and ammonium formate (Thermo Fisher Scientific, Auckland, New Zealand) were obtained. Type 1 water was generated from a Millipore unit (Merck Millipore, Auckland, New Zealand).

3.6 Preparation of standards and quality control samples

A mix containing myristicin (1040 μM), yangambin (1120 μM), elemicin (48 μM), vitexin (23 μM), pellitorin (45 μM), fagaramide (40 μM), dopamine (65 μM) and trigonelline (73 μM) with an internal standard (12 μM) (IS) was prepared and serially diluted. Buspirone was used as the IS when analysing urine samples from BOKA-T. It was replaced with podophyllotoxin as the IS when analysing plasma samples from BOKA-T and urine and plasma samples from TOAST as podophyllotoxin provided improved chromatographic resolution. Accurate mass-to-charge ratio (m/z), retention time and MS/MS spectra of the standards were added to an in-house MS/MS library based on the 2020 NIST library. Quality control (QC) standards for BOKA-T and TOAST plasma and urine analyses were prepared in each case by pooling the baseline plasma or urine samples from all of the respective study individuals.

3.7 Sample preparation for LC-MS/MS

Plasma and urine samples were thawed on ice before extraction. The samples were gently vortexed and 100 μL added to 400 μL ice-cold methanol (with 12 μM IS) in microfuge tubes. After centrifugation at 10,000g for 20 min to remove precipitated proteins, the supernatant was transferred to a 96-well plate and dried under nitrogen flow at room temperature (around 25°C). The residue was reconstituted in 200 μL 10% acetonitrile (BOKA-T urine samples) or 200 μL 40% MeOH (BOKA T plasma, TOAST urine and plasma samples) and prepared for LC-MS/MS analysis.
3.8 Instrumentation

The liquid chromatographic system comprised an Accela 1250 pump (Thermo Fisher Scientific, CA, USA), a Thermo TriPlus autosampler (Thermo Fisher Scientific, Hampton, USA), a HotDog 5090 column oven and a Phenomenex Kinetex biphenyl analytical column (150 mm × 2.1 mm × 1.7 μm) held at 30 °C. Flow was 0.3 mL/min and the injection volume was 5 μL. The mobile phase was MilliQ with 5 mM ammonium formate and 0.1% formic acid (solvent phase A), 100% acetonitrile with 0.1% formic acid (solvent phase B) and 100% isopropanol. The mass spectrometer was a Q-Exactive (Thermo Scientific, Dreieich, Germany) with a heated electrospray ionisation source. The electrospray (ESI) ion source voltage was 3.5 kV, S-lens RF level 50, heated capillary temperature 263°C, HESI temperature 425°C, AGC target 3e6 and resolution 17,500. Mass spectra were acquired in positive polarity and centroid mode.

Spectral data were acquired in All Ions Fragmentation [AIF]. For BOKA T urine analysis, the full scan range was from 50 to 750 m/z with 0 and 30 eV NCE for the AIF scans. The gradient started at 0% B, ramped to 30% B at 2 min, ramped to 100% B at 13 min, held at 100% B until 17 min, ramped to 0% B at 17.5 min and then re-equilibrated at 0% B from 18.5 to 22 min. For BOKA T plasma analysis, the full scan range was from m/z 75 to 850 with 0eV and 56.7 to 750 m/z with 10 and 35 eV NCE for the AIF scans. The gradient started at 0% B, ramped to 95% B at 2 min, ramped to 15% B and 80% C at 18 min, ramped to 95% B at 25 min, ramped to 0% B at 25.50 min, and then re-equilibrated at 0% B from 25.50 to 30 min. For TOAST urine analysis, the full scan range was from m/z 75 to 750 and 56.7 to 750 m/z with 0eV with 10 and 35 eV NCE for the AIF scans. The gradient started at 0% B, ramped to 30% B at 2 min, ramped to 100% B at 13 min, ramped to 100% B at 17 min, ramped to 0% B at 19 min and then re-equilibrated to 0% B from 19 to 23.50 min. For TOAST plasma analysis, the full scan range was from m/z 75 to 850 with 0eV and 56.7 to 750 m/z with 10 eV and 35 eV NCE from 0 to 15 min; the full scan range was from m/z 75 to 1000 with 0eV, m/z 66.7 to 850 m/z with 10 eV and m/z 56.7 to 850.5 with 35 eV NCE from 20 to 30 min; the full scan range was from m/z 350 to 1200 with 0eV and 80 to 1200 m/z with 10 eV and 35 eV NCE from 20 to 30 min for the AIF scans. The gradient started at 0% B, ramped to 95% B at 2 min, ramped to 15% B
and 80% C at 18 min, ramped to 95% B at 25 min, ramped to 0% B at 25.50 min, and then re-equilibrated at 0% B from 25.50 to 30 min.

3.9 Data processing and statistical analysis

Data were collected and processed using Thermo Xcalibur software (version 4.2.47). The LC-MS/MS data were processed using MS-DIAL v4.70 to identify and quantify features $^{225,254}$. Correlation-Based Deconvolution was used to extract MS/MS spectra, which were putatively identified with the use of an in-house spectral library based on the 2020 NIST high-resolution accurate mass library $^{255-258}$. The peak areas of compounds within urine samples were normalised to that of creatinine and IS, and those in the plasma samples normalised to IS. Quantitative data was used to identify features whose abundance was correlated with the kinetics of uptake, metabolism and excretion.

To identify possible KT-derived metabolites in urine, the following procedure was followed. Treatment groups (hot water, 1g and 4g/250mL KT) were separated into three different files. The various sampling points were arranged in different orders in line with the assumptions about how metabolites were up taken, yielding a negative correlation. 0 h samples would be the lowest, so it was be placed after the 24 h samples. Considering that those metabolites taken up directly in the small intestine would appear in the plasma quickly, whereas kawakawa compounds that are not taken up in the small intestine might be metabolised in the gut, leading to delays of hours before they could be evident in the plasma or urine. Additionally, secondary metabolism in the liver or kidneys may be delayed even further. For each treatment, linear regression analyses were undertaken with each of the MS features for the time series of the BOKA-T urine samples obtained following consumption of 1g/250 mL KT and 4g/250 mL KT with the exclusion of the features in hot water treatment followed by ranking by decreasing $R^2$ values. Features that had $R^2$ values $> 0.3$ were selected in 1g and 4g/250 mL KT consumption. These selected features were validated to be associated with kawakawa consumption by comparison of baseline and 0-3 h urine samples from TOAST by ANOVA. Significantly greater features ($p < 0.05$) in 0-3 h urine samples compared to baseline were submitted into MS Finder for identification. Further analyses of the selected features was undertaken in BOKA-T...
plasma samples from the 1g and 4g/250 mL intervention. Similarly, the plasma features identified in BOKA-T were validated in TOAST plasma samples.

3.10 Results and discussion

3.10.1 Data quality assessment

QC samples were injected after every 10 samples during the LC-MS/MS run to assess the quality of metabolomics data exclusive of RT drifts and mass calibration. The mass accuracy and retention time (RT) shift of the IS were found to be below 5 ppm and 6 seconds, respectively. Urine data was normalized to creatinine levels to account for individual variations throughout the intervention period based on hydration and diet \(^{259}\). Each dataset of urine and plasma were normalised to the corresponding internal standards used. IS spiking resulted in >80% IS recovery in all of our analyses.

3.10.2 Metabolite profiling and identification of urinary and plasma metabolites associated with KT consumption

More than 15,000 LC-MS features were quantified by untargeted analyses of urine samples. In the BOKA-T 4g/250 mL time series urine samples, 61 features had a R\(^2\) value > 0.3 suggesting an association with kawakawa consumption. Forty-five of these features were also significantly greater in 0-3 h urine samples in TOAST compared to baseline (p < 0.05). The mass spectra of these 45 features were extracted from MS-DIAL and submitted into MS-Finder. Of these, fifteen features were discarded as they did not have potentially resolvable spectra after the deconvolution process. The remaining thirty features were sorted based on RT. Four pairs had the same RT but with a m/z differences indicating they were [M+NH\(_4\)]\(^+\) and [M+Na\(^+\)] adducts. Of the twenty-six features/metabolites, putative identities were assigned to thirteen that were consistent with being derived from compounds in KT. A further eight metabolites had characteristic ions of compounds within KT but for which an identification of the human metabolite was not possible. No identities were assigned to five further features (Table 3.2, Figures 3.6 - 3.27).
Analysis of data collected in negative ionization mode for BOKA T urine indicated the presence of ~300 features and did not show any experimental effect (Appendix B). Therefore, all these analyses were carried out on the data collected in positive ionization mode. Blood samples were collected only for 5 h (BOKA-T) wherein gut microbial metabolites released after this time period could have been missed. However, urine samples collected for up to 24h could have captured gut microbial metabolites to some extent.

In Both BOKA-T and TOAST, the twenty-six metabolites were also found to be significantly greater in urine in participants who had consumed 1g/250 mL KT except for metabolites K10, K20 and K22. Seven metabolites were also found to be significantly greater in plasma following 4g/250mL KT consumption compared to baseline. There was no/minimal changes observed in all these metabolites following water consumption compared to KT consumption (Table 3.2, Figures 3.6 - 3.27). This metabolite identification is confirmed in two treatment groups in BOKA T and in the second (TOAST) study and therefore indicates that the statistical analysis is robust.

While the statistical analyses were robust, there were two anomalies in the data sets. Firstly, two of the twenty-six metabolites, K1 and K9, were present in baseline spot samples in the intervention with 4g/250 mL KT at higher levels than expected in the TOAST study if these metabolites were only derived from the KT intervention (Figures 3.6, 3.14). The apparent presence of these metabolites was due to urine from a single individual. Likewise, in BOKA-T, metabolites K16 (fagaramide) and K17 (hydroxy saturated fagaramide) were observed in 24 h plasma samples of five individuals at higher levels than within plasma collected at earlier times points (Figures 3.21, 3.22). In both BOKA-T and TOAST, participants were requested not to consume any dietary spices for 24 h before the first intervention and within the 48 h washout period between the two arms of BOKA-T and in the one-week washout periods between the three arms of TOAST. Despite this instruction and with no reported protocol breaches, it is possible that these anomalies may have arisen from the consumption of food that contains compounds that are also found in kawakawa prior to the study or within the washout periods. In this context, it is worth noting that fagaramide occurs in black pepper which is included in many processed foods which may not have been apparent to study participants.
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a) Retention time (RT) (min) indicated here is from BOKA-T urine analysis; calculated mass errors for all of the putatively identified features were less than 5 ppm; b) features observed in BOKA-T and TOAST plasma samples; c) features observed in BOKA-T and TOAST urine samples
**Elemicin metabolites:** A total of six metabolites (K1-K6) were associated with elemicin in human urine and plasma samples from both BOKA-T and TOAST (Table 3.2). The putative metabolic pathway of elemicin is summarized in Figure 3.3. These results indicated that phase 1 metabolism of elemicin involves hydroxylation, demethylation, reduction and carboxylation. Phase 2 metabolism involves conjugation with glucuronic acid and sulphate.

Metabolite K1 was putatively identified as hydroxy elemicin glucuronide (Figure 3.3). It was present in both plasma and urine samples as the [M+ NH₄]⁺ adduct at m/z 418.1335 and the [M+Na]⁺ adduct at m/z 423.0882. The loss of m/z 176 to generate fragment ions at m/z 193.0851 and 225.0746 is indicative of a glucuronide metabolite. 1-hydroxy elemicin has been reported previously to be a human metabolite of elemicin ²⁴⁴,²⁶⁰ (Figure 3.6).

Metabolite K2 was putatively identified as trimethoxycinnamic acid formed by carboxylation of elemicin (Figure 3.3). It was detected in both plasma and urine samples as the [M+H]⁺ adduct at m/z 239.0906 yielding fragment ions at m/z 149.0591 and 221.0788, which have been reported previously as diagnostic ions of trimethoxycinnamic acid ²⁴⁴. This identification is in agreement with the previously reported major metabolite of α-asarone, which is structurally similar to elemicin, differentiated only by the arrangement of the double bond on the aliphatic chain ²⁶¹,²⁶². (Figure 3.7).

Metabolite K3 was tentatively identified as trimethoxycinnamic acid glucuronide (Figure 3.3). It was only detected in urine samples as the [M+NH₄]⁺ adduct at m/z 432.1486. The product ions at m/z 221.0789 and 239.0905 following a loss of m/z 176 were similar to those for K2 and indicative of a glucuronide metabolite (Figure 3.8).

Metabolite K4 was putatively identified as hydroxy trimethoxycinnamic acid (Figure 3.3). It was detected only in urine samples as the [M+H]⁺ adduct at m/z 257.1008. The fragment ion at m/z 239.0893 would result from loss of an hydroxyl group and the presence of other product ions at m/z 193.0846 and 211.0945 was similar to previously reported elemicin metabolites ²⁶⁰ (Figure 3.9).
Urinary metabolite K5 was putatively identified as the [M+H]⁺ adduct of trimethoxy dihydrocinnamic acid (Figure 3.3) at m/z 241.1076. The fragment ions at m/z 193.0846 and 195.1013 were similar to previous reports (Figure 3.10).

Metabolite K6 was putatively identified as the [M+ NH₄]⁺ adduct of demethylated elemicin glucuronide (Figure 3.3). It was observed, in both urine and plasma samples, at m/z 388.1587 and with a characteristic fragment ion at m/z 195.1015 corresponding to demethylated elemicin and with a typical neutral loss of m/z 176 diagnostic of a glucuronide (Figure 3.11).

Figure 3.3 Proposed metabolic pathway of elemicin in human

Myristicin metabolites: A total of three metabolites (K7-K9) were putatively identified as being derived from myristicin. A further five metabolites (K10-K14) were likely to have been derived from myristicin but without resolution of their structure. All were observed in urine from samples from both BOKA-T and TOAST, and K7 also in plasma samples from the two studies (Table 3.2). The proposed metabolic pathway of myristicin metabolism is depicted in Figure 3.4.
Metabolite K7 was putatively identified as hydroxymyristicin glucuronide (Figure 3.4). It was detected in both plasma and urine samples as the [M+ NH₄]⁺ and [M+Na]⁺ adducts at m/z 404.1533 and 409.1096. The product ions at m/z 161.0622 and 193.0856 would indicate it is a myristicin derivative. The loss of m/z 176 is indicative of a glucuronide metabolite ⁸²,¹⁸⁸. (Figure 3.12).

Metabolite K8 was tentatively identified as allyl dihydroxyanisole glucuronide (Figure 3.4). It was detected only in urine samples as the [M+H]⁺ ion at m/z 357.1164. It was assumed to be derived from allyl dihydroxy anisole by demethylenation producing product ions at m/z 149.0591 and 181.0851 and a typical neutral loss of m/z 176 indicative of a glucuronide conjugate ¹⁸⁸. 5-allyl-2,3-dihydroxyanisole is one of the major myristicin metabolites of myristicin biotransformed by the CYP3A4 enzyme in human liver microsomes ¹⁹⁰. (Figure 3.13).

Metabolite K9 was putatively identified as allyl dihydroxyanisole sulphate (Figure 3.4). It was detected as the [M+H]⁺ at m/z 261.0432 with major MS/MS ion at m/z 181.0857 suggesting it to be a myristicin derivative, allyl dihydro anisole ¹⁸⁸. The typical neutral loss of m/z 80 is diagnostic for a sulphate conjugate. It was assumed to be formed as a result of CYP450 enzyme breaking the methylene dioxy ring (demethylenation) followed by the addition of sulphate at the hydroxyl site ¹⁸⁸,²⁶³ (Figure 3.14).

Metabolites K10, K11, K12, K13 and K14 are possibly derived from myristicin as they all have one or more of the characteristic fragment ions of myristicin at m/z 149.0227, 151.0748, 153.0539, 181.0857, 193.085 and 209.1164 ¹⁸⁸ (Table 3.2, Figure 3.15 - 3.19).
Figure 3.4 Proposed metabolic pathway of myristicin in humans.

**Diayangambin metabolite:** Metabolite K15 was tentatively identified as demethylated diayangambin sulphate (Figure 3.5A). It was detected only in urine as the [M+NH₄]⁺ adduct at m/z 530.1671, and contained the fragment ions of yangambin at m/z 181.0855 and 219.1005 [81]. The fragment ion at m/z 433.1838 suggests that one of the methoxy groups of diayangambin is demethylated and m/z 450.2102 indicates the neutral loss of m/z 80, indicative of a sulphated metabolite. (Figure 3.20).

**Fagaramide metabolites:** Metabolite K16 was identified as fagaramide. This metabolite was present in plasma and urine samples as the [M+H]⁺ ion at m/z 248.1272, similar to that observed in kawakawa leaf extracts [81]. It had the characteristic MS/MS fragment ions of fagaramide at m/z 175.0736 and 247.1325 [81]. The [M+NH₄]⁺ adduct of this metabolite was observed also, at m/z 265.1536 (Figure 3.21).

Metabolite K17 was tentatively identified as hydroxyl saturated fagaramide (Figure 3.5B). It was detected in both plasma and urine as [M+H]⁺ adduct of 266.1376 and with diagnostic fragment ions of saturated fagaramide at m/z 177.0535 and 248.1275 [81] (Figure 3.22).

Metabolite K18 may also be derived from fagaramide as it has fragment ions at m/z 98.0347 and 248.1271 (Table 3.2), but no further annotation was possible. (Figure 3.23).
**Dopamine metabolites:** Metabolite K19 was putatively identified as dopamine sulphate (Figure 3.5C). It was detected in both plasma and urine samples as the [M+H] + adduct at m/z 234.0416. It has characteristic fragment ions of dopamine at m/z 137.0595 and 154.0855 and a typical neutral loss of m/z 80 indicative of a sulphate conjugate.\(^{81,264}\) (Figure 3.24).

Metabolites K20 and K21 are likely to be derived from dopamine metabolites but not further annotation was possible. K20 was detected only in urine samples at m/z 336.0887 with characteristic fragment ions of dopamine at m/z 137.0595 and 154.0855 \(^{81}\) (Figure 3.25). Similarly, K21 was detected in urine at m/z 271.9979 with the characteristic ion at m/z 137.0595 \(^{81}\) (Figure 3.26).

No identities were assigned to other metabolites K22-26 (Table 3.2) Their kinetic profile observed at various time points in BOKA-T and TOAST are shown (Figure 3.27).
Figure 3.5 Metabolites of A) diayangambin in urine samples B) fagaramide in urine samples and C) dopamine in plasma and urine samples.
Figure 3.6 Metabolite K1, detected at m/z 418.1335 and identified as hydroxy elemicin glucuronide in A) plasma and B) urine samples of BOKA-T and C) plasma and D) urine samples of TOAST (n = 30). Y-values are represented as mean ± SEM.
Figure 3.7 Metabolite K2 identified as trimethoxycinnamic acid at m/z 239.0906 in A) plasma and B) urine samples of BOKA-T (n = 6) and C) plasma and D) urine samples of TOAST (n = 30); Y-values are represented as mean ± SEM.

Figure 3.8 Urinary metabolite K3 detected at m/z 432.1481 identified as trimethoxycinnamic acid glucuronide in A) BOKA-T (n = 6) and B) TOAST (n = 30) samples. Y-values are represented as mean ± SEM.

Figure 3.9 Urinary metabolite K4 detected at m/z 257.1008 identified as hydroxy trimethoxycinnamic acid in A) BOKA-T (n = 6) and B) TOAST (n = 30) samples. Y-values are represented as mean ± SEM.
Figure 3.10 Urinary metabolite K5 detected as trimethoxy dihydrocinnamic acid at m/z 241.1057 in A) BOKA-T (n = 6) and B) TOAST (n = 30) samples. Y-values are represented as mean ± SEM.

Figure 3.11 Metabolite K6 detected at m/z 388.1587, identified as demethylated elemicin glucuronide in A) plasma and B) urine samples of BOKA-T (n = 6) and C) plasma and D) urine samples of TOAST (n = 30). Y-values are represented as mean ± SEM.
Figure 3.12 Metabolite K7 detected at m/z 404.1533 identified as \([M+\text{NH}_4]^+\) of hydroxymyristicin glucuronide in A) plasma and B) urine samples of BOKA-T (n = 6) and C) plasma and D) urine samples of TOAST (n = 30). Y-values are represented as mean ± SEM.

Figure 3.13 Urinary metabolite K8 detected at m/z 357.1164 identified as allyl dihydroxyanisole glucuronide in A) BOKA-T (n = 6) and B) TOAST (n = 30) samples. Y-values are represented as mean ± SEM.
Figure 3.14 Urinary metabolite K9 detected at m/z 261.0419 identified as allyl dihydroxyanisole sulphate in A) BOKA-T (n = 6) and B) TOAST (n = 30) samples. Y-values are represented as mean ± SEM.

Figure 3.15 Urinary metabolite K10 detected at m/z 278.0699 in A) BOKA-T (n = 6) and B) TOAST (n = 30) samples. Y-values are represented as mean ± SEM.
Figure 3.16 Urinary metabolite K11 detected at m/z 454.0995 in A) BOKA-T (n = 6) and B) TOAST (n = 30) samples. Y-values are represented as mean ± SEM.

Figure 3.17 Urinary metabolite K12 detected at m/z 550.1304, in A) BOKA-T (n = 6) and B) TOAST (n = 30) samples. Y-values are represented as mean ± SEM.
Figure 3.18 Urinary metabolite K13 detected at m/z 277.0671, in A) BOKA-T (n = 6) and B) TOAST (n = 30) samples. Y-values are represented as mean ± SEM.

Figure 3.19 Urinary metabolite, K14 detected at m/z 280.1644, in A) BOKA-T (n = 6) and B) TOAST (n = 30) samples. Y-values are represented as mean ± SEM.

Figure 3.20 Urinary metabolite K15 detected at m/z 530.1671 and identified as demethylated diayangambin sulphate in A) BOKA-T (n = 6) and B) TOAST (n = 30) samples. Y-values are represented as mean ± SEM.
Figure 3.21 Metabolite K16, fagaramide detected at m/z 248.1272 in A) plasma and B) urine samples of BOKA-T (n = 6) and C) plasma and D) urine samples of TOAST (n = 30). Y-values are represented as mean ± SEM.
Figure 3.22 Metabolite K17 detected at m/z 266.1376 in A) plasma and B) urine samples of BOKA-T (n = 6) and C) plasma and D) urine samples of TOAST (n = 30), putatively identified as hydroxy saturated fagaramide. Y-values are represented as mean ± SEM.

Figure 3.23 Urinary metabolite K18 detected at m/z 286.0825 in A) BOKA-T (n = 6) and B) TOAST (n = 30) samples. Y-values are represented as mean ± SEM.

Figure 3.24 Metabolite K19 identified as [M+H]+ of dopamine sulphate at m/z 234.0416 in A) plasma and B) urine samples of BOKA-T (n=6) and C) plasma and D) urine samples TOAST (n=30); Y-values are represented as mean ± SEM.
Figure 3.25 Urinary metabolite K20 detected at m/z 336.0887 in A) BOKA-T (n = 6) and B) TOAST (n = 30) samples. Y-values are represented as mean ± SEM.

Figure 3.26 Urinary metabolite K21 detected at m/z 271.9979 in A) BOKA-T (n = 6) and B) TOAST (n = 30) samples. Y-values are represented as mean ± SEM.
Figure 3.27 continued..
Figure 3.27 Unidentified urinary metabolites (i) K22 detected at m/z 302.1042, (ii) K23 detected at m/z 546.1129, (iii) K24 detected at m/z 280.1532, (iv) K25 detected at m/z m/z 596.2315 and (v) K26 detected at m/z 610.2103 in A) BOKA-T (n = 6) and B) TOAST (n = 30) samples. Y-values are represented as mean ± SEM.

Untargeted LC-MS/MS analysis enabled the provisional identification of human metabolites derived from kawakawa leaf compounds. Three of the metabolites that were derived from myristicin, and seven derived from elemicin, are consistent with the previously reported metabolism of these compounds in animal models and modelling of their bioactivation.\(^{82,244,260}\) They were likely to be metabolised through hydroxylation, reduction and demethylenation followed by glucuronide conjugation. Dopamine sulfate has previously been reported to be the major dopamine metabolite circulating in human plasma.\(^{265}\) To our knowledge, the present study is the first to report human metabolites derived from fagaramide or diayangambin.

The untargeted LC-MS/MS analyses did not identify any metabolites that were likely to have been derived from vitexin or pellitorin in either plasma or urine, despite both being prominent
in kawakawa. Phytochemical analysis (Chapter 2) indicated lower concentration of pellitorin in commercially available kawakawa leaf source (ŌKU) which could possibly due to its degradation by drying at increased temperatures\textsuperscript{266}. The processing technique involved in commercial kawakawa leaf source (ŌKU) is not clearly known and the influence of drying process in phytochemical variation is yet to be explored. Challenges in detecting vitexin metabolites could likely be due to the difficulties in extracting highly water soluble metabolites in organic solvents and limitations of the conventional reverse phase columns to detect small polar molecules due to lack of retention and ion suppression problems\textsuperscript{267}.

3.11 Limitations

The major limitation of this study is the lack of chemical standards available for confirmation of the putative metabolites derived from kawakawa leaf compounds, and therefore the interpretation of mass spectra remains provisional. Stricter control of diet prior to future KT interventions will minimise potential interference from other dietary components, in particular black pepper and other spices.

3.12 Conclusion

Our study reports the presence of metabolites derived from kawakawa leaf compounds in plasma and urine following a single ingestion of KT. These metabolites are indicative of which leaf chemicals are bioavailable and indicate possible routes of metabolism prior to excretion. These results provide some insights regarding potential compounds in kawakawa that may underlie its reported therapeutic properties and provide biomarkers for kawakawa consumption for use in future research projects.
Chapter 4 Impact of *Piper excelsum* (kawakawa) containing beverage on resting and postprandial energy metabolism in healthy adults

Abstract

In light of the appearance of circulating kawakawa metabolites in plasma and urine following ingestion of kawakawa tea (Chapter 3), this study aims to assess the acute impact of consuming kawakawa leaf suspension on resting energy expenditure (REE), substrate utilisation and other biochemical changes before and after consumption of a standardised breakfast meal. The impact of combined ingestion with other potentially functional compounds, in the form of a mixed beverage, was also explored. REE and respiratory quotient (RQ; an index of substrate utilisation) were assessed in 18 healthy men and women (18-45y) by ventilated-hood indirect calorimetry at baseline and following ingestion of 250mL of either water (W), kawakawa leaf suspension (K), a mixed beverage (containing kiwifruit and ginger extracts, turmeric and lemon juice) (B), or a mixed beverage + kawakawa (B+K), in a randomised cross-over design. 30 minutes after ingestion, a standardised breakfast was given, and monitoring continued for a further 2.5h. The results indicated that ingestion of kawakawa leaf suspension did not have any acute effects on REE, RQ and substrate utilisation compared to water treatment. Immediate increase in REE, RQ and carbohydrate oxidation and decrease in fat oxidation observed after consuming beverages compared to consuming water or kawakawa leaf suspension. Comparing the post-breakfast induced REE changes in kawakawa suspension and water treatments, there was a borderline increase (p ≤ 0.05) in REE (between t = 70 and 80 min; t = 150 and 160 min) and carbohydrate oxidation (p ≤ 0.05) at similar time points (between t = 70 and 80 min; at t = 140 min). There was no difference observed between the post-breakfast induced REE changes in mixed beverages and mixed beverages with kawakawa. There was no effect of kawakawa observed on postprandial RQ, fat oxidation, glucose, insulin and triglycerides levels. There was a similarity observed between kawakawa leaf suspension and beverage consumption regarding the feeling of hunger, fullness and desire to eat. This study indicated that consuming kawakawa leaf suspension (K) may have the ability to influence resting energy expenditure possibly mediated by carbohydrate metabolism. Despite its lower caloric contents compared to beverages, kawakawa leaf suspension appears to have potential satiety-controlling properties. Further studies with a larger population and longer-term interventions are required.
4.1 Introduction

In Chapter 2, the chemical profile of kawakawa was investigated, and subsequently, the human metabolism and excretion of compounds derived from kawakawa were described in Chapter 3. These analyses identified the presence of several compounds and metabolites shown to have effects on energy metabolism. For example: dopamine \(^{268}\), the sympathomimetic compounds, myristicin \(^{106}\) and piperine \(^{269}\), vitexin, an activator of AMPK \(^{85}\) and, elemicin, a stearoyl-CoA desaturase 1 (SCD1) inhibitor \(^{244}\).

Acute ingestion of kawakawa tea has the potential to influence postprandial glycaemic and insulinaemic response \(^{253}\). However, whether the presence of these compounds is sufficient to induce an acute, measurable increase in either metabolic rate or substrate oxidative profile has not yet been examined. Therefore, in this chapter, I explore whether consuming kawakawa leaf suspension, which has a complex mixture of chemicals, has any acute physiological effects on energy expenditure (EE) and substrate oxidation. Furthermore, individual chemical compounds are often studied in isolation with the use of, for example, animal and cell models. While this approach generates useful data (and is indeed used in Chapter 5), it may prevent an understanding of potential additive and synergistic effects between these compounds. For instance, piperine is a well-known bioenhancer recognized to increase intestinal absorption and the bioavailability of a large number of micronutrients and drugs \(^{270}\). Hence it is necessary to consider the metabolic effects of this complex mixture of kawakawa compounds on both fasting energy metabolism, and metabolic response to any subsequent food ingestion. As such, in this chapter, I examine the acute effects of kawakawa on energy metabolism before and after consumption of a standardised breakfast meal, using a human randomized controlled intervention.

4.2 Materials and methods

4.2.1 Sample size calculation, participant recruitment and screening

There were no prior studies available looking at the thermic effect of kawakawa and this was a feasibility study. Based on the a-priori sample size calculation specific to the intervention, the required sample size was estimated as the number of participants required to detect a “physiologically meaningful” increase in metabolic rate (the primary variable) of 5%, a type I
error (α) of 0.05 and a desired power (1-β) of 0.90, and assuming a within-participant variability in resting metabolic rate of 0.04 kcal/min. This calculation suggested a sample size of 7 participants per group, however, we aim to increase recruitment to 10 per group to maintain statistical power in the event of participant drop-outs. As lean mass is a key determinant of resting metabolic rate and to account for potential sex differences in the thermic effect of food, we recruited 10 men and 10 women for this study, evenly divided between normal- and overweight (by body mass index); i.e., a total of 20 participants. Owing to the repeated-measures, cross-over design and a low level of intra-individual variability relative to inter-individual variability in indirect calorimetry studies \(^{27}\), this comparatively small sample size will still provide sufficient statistical power to detect a physiologically meaningful effect on metabolic rate.

Eighteen healthy men (n=9) and women (n=9) of different ethnicity, aged 18-45 years, participated in this Tūhauora study (Figure 4.1). Participants were in the BMI range of 18-30 kg/m\(^2\) (healthy to overweight). This study was approved by the Central Health and Disability Ethics Committee (21NTB119) and registered with the Australian New Zealand Clinical Trials Registry at www.anzctr.org.au (accessed on 14 January 2023) (ACTRN12622000275763). This study was conducted according to the Declaration of Helsinki guidelines and all participants provided written informed consent.

Volunteers were invited to participate in this study through mail advertisements. Interested participants visited the Liggins Clinical Research Unit (CRU) to complete a screening questionnaire regarding their lifestyle and medical history, to confirm eligibility, and to familiarize themselves with the instrumentation and study procedure, after which they signed the informed consent. Participants had their height and weight measured, with an average of two measurements taken. Body weights were assessed using a digital weighing scale (Wedderburn WM206, Taiwan) and height was measured using a stadiometer (Holtain Ltd., Crymych, Dyfed, UK) without shoes, to the nearest 0.1 cm and 0.1 kg, respectively. Body composition was assessed using Dual-energy X-ray Absorptiometry (iDXA, GE-Lunar, Madison, USA) at the CRU, according to the manufacturer’s instructions. iDXA is based on the 3-compartment model of body composition and uses two x-ray energies to estimate body fat mass, lean mass, and bone mineral mass. Participants were allowed to relax on the bed for 5 min prior to measurement and lie recumbent on the open scanner bed for ~10 min. Individuals
with any current or pre-existing gastrointestinal (i.e. celiac, Crohn’s, colitis, etc.), cardiovascular or metabolic disease, currently taking any medications, dietary or herbal supplements, or claustrophobia were excluded from this study. Participants were weight-stable (+/- 1.5 %) across the intervention period.

Participants were advised to abstain from intense exercise, caffeinated or herbal drinks, spices and alcohol in the 24 h prior to each intervention visit. Participants were provided with a standardised meal (two servings; Energy: 1342 kJ; Protein: 62.4 %en; Carbohydrate: 42.8 %en; Fat: 94.6 %en) for dinner before each intervention visit to reduce variability in the fasting substrate oxidation profile. Each participant completed four intervention visits with a washout period of at least 48 h.

---

![Figure 4.1 Tāhauora study design](image)
4.2.2 Test Beverage and standardized breakfast

Four test beverages were used in this study differing in the presence (kawakawa leaf suspension (K), base beverage formulations + kawakawa (B+K)) or absence of kawakawa (water (W), base beverage formulations (B)), as shown (Table 4.1). On each intervention day, the required beverage was prepared by mixing 14 g of beverage powder (B or (B+K containing 1g kawakawa leaf powder)), 1 g kawakawa leaf powder (K) with 250 mL room temperature water or 250 mL room temperature water (W). The dry powder used to prepare B contained a mix of kiwifruit and ginger extracts, turmeric and lemon juice. The K powder consisted of finely ground freeze-dried kawakawa leaf. 250 mL of room temperature water was used as a control. All beverages were served at room temperature in an opaque cup.

<table>
<thead>
<tr>
<th>Beverage and breakfast composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kJ per serve)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Kawakawa leaf powder (K)</td>
</tr>
<tr>
<td>Base beverage (B)</td>
</tr>
<tr>
<td>Base beverage + kawakawa (B+K)</td>
</tr>
</tbody>
</table>

Table 4.1Beverage and breakfast composition

CHO: carbohydrate

The standardized breakfast given consisted of 2 slices of white bread toast, honey (15g) and butter (9g). Energy: 1215 kJ; Protein: 8.6 %en; Carbohydrate: 63.8 %en; Fat 27.5 %en.

4.2.3 Intervention visits and cardio-metabolic measurements

On the intervention day, participants arrived at the clinical research unit at 8.30 AM following a 12 h overnight fast. Participants voided their bladder, providing 0 h urine samples. Following this, the participant’s weight was recorded. Waist circumference was measured at the level of
the belly button using a non-extensible measuring tape, following an international standardized procedure \textsuperscript{272}. Seated blood pressure (BP; mmHg) and heart rate (beats per minute) was recorded using a digital Critikon Dinamap Sphygmomanometer (GE Healthcare, Shanghai, China) at regular intervals (t = 0, 60, 120, 180 min). A BD Insyte cannula (Beckton Dickinson Suspension Therapy Systems, Utah, USA) was inserted into the median cubital vein. The participant was then seated comfortably in a bed adapted for calorimetric monitoring. Resting EE (REE) and respiratory quotient (RQ) were assessed using a ventilated hood system (Cosmed QNRG, Sydney, Australia). Metabolic measurements were conducted for at least 30 min, until stabilization of EE, after 15 min of rest. Stabilization was assessed by comparing measured REE with that predicted according to the Harris-Benedict equation \textsuperscript{273}, with no consistent upward or downward trend apparent. The ventilated hood was then removed while the participant ingested one of the four beverages described above, within a 10 min period. The order in which the beverages were given was randomized, and all participants were blinded to this order. The ventilated hood was replaced and metabolic measurements were continued for 30 min. Standardized breakfast was then given, to be consumed within 10 min, and the ventilated hood was replaced and measurements continued for a further 150 min. Participants were requested to breathe normally and watch a calm movie or a documentary, such that they remained relaxed throughout the measurement period \textsuperscript{274}. Measurements were conducted in a room with centralized air-conditioning and the temperature (20 - 22 °C) was controlled by an automated thermostat, which remained stable during the measurement period. Gas (5% CO$_2$, 16% O$_2$ and N$_2$) and volume (3 L) calibrations of the metabolic cart were conducted fortnightly, according to the manufacturer’s instructions. REE was calculated using adjusted Weir equation (i) \textsuperscript{275},

$$REE = 1.44 \times (VO_2 \times 3.94) + (VCO_2 \times 1.11) \tag{i}$$

where, REE = resting energy expenditure in kcal/day; $\dot{VO}_2$ = $O_2$ consumption in mL/min; $\dot{VCO}_2$ = $CO_2$ production in mL/min.

Respiratory quotient (RQ) was calculated \textsuperscript{276} as,

$$RQ = \frac{(Volume \ of \ CO_2 \ produced)}{(Volume \ of \ O_2 \ consumed)} \tag{ii}$$
Carbohydrate oxidation (CHO Ox) and fat oxidation (Fat Ox) were calculated using equations (iii) and (iv), assuming the urinary nitrogen excretion rate to be negligible \(^{277-279}\),

\[
CHO \text{ Ox. } (g/min) = 4.55 VCO_2 - 3.25 VO_2 \quad (iii)
\]

\[
Fat \text{ Ox. } (g/min) = 1.67 VO_2 - 1.67 VCO_2 \quad (iv)
\]

Delta (\(\Delta\)) values were calculated for each parameter by subtracting the baseline values from the post-beverage and post-breakfast measurements.

4.2.4 Visual Analog Scales

Visual analog scale (VAS) scores of hunger, satiety, desire to eat, thirst, comfort and nausea were recorded throughout the measurement period according to the methodology of Blundell et al \(^{280,281}\). In brief, participants were asked to make a hand-written mark on a 10 cm line representing a continuum between two anchors, as shown in **Figure 4.2**. VAS scores were collected at baseline and 15, 30, 60, 90, 120 and 180 min post-beverage ingestion.
4.3 Samples collection and biochemical analysis

Fasting and postprandial venous blood samples (~ 10 mL) were collected in EDTA-coated vacutainer tubes over the 3 h period at 15, 30, 60, 90, 120 and 180 min post-beverage ingestion. Plasma was stored on ice upon collection and separated at 1500 × g for 15 min at 4°C within 2 h, aliquoted and immediately stored at -80°C until further analysis.
Plasma glucose was measured by enzymatic colorimetric assay (Hitachi 902 autoanalyser, Hitachi High Technologies Corporation, Japan) according to the manufacturer’s instructions, using commercially available reagents. Microparticle enzyme immunoassay (Cobas Elecsys®, 2010; Roche Diagnostics, Basel, Switzerland) using Cobas Modular P800 (Roche Diagnostics, Auckland, New Zealand) was used to measure plasma insulin levels. Plasma triglycerides and cholesterol were measured using Cobas Modular P800 (Roche Diagnostics, Auckland, New Zealand).

4.4 Metabolomic analysis of beverage samples

Representative beverage samples were analysed in triplicate, using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Quantitative analysis was performed in data-dependent acquisition (DDA) mode with similar mass spectrometric conditions. The elution gradient was 20 - 40% B at 7 min, 80% B at 8 min and held for 1 min then back to 20% B from 9 to 12 min. Some of the major phytochemicals of kawakawa leaf and the beverage formulations for which standards were available, such as myristicin, elemicin, yangambin, pellitorin, fagaramide, dopamine, trigonelline, curcumin and gingerol, were quantified.

4.5 Data and statistical analysis

Indirect calorimetry values were recorded every 30 s and averaged in 5 min intervals across the baseline, 30 min post-beverage and 2h 30 min post-meal periods. Intra- and inter-individual variability was calculated for the baseline values of each participant between four treatments, and between participants for each treatment, respectively, for all the measured variables by estimating the CV%.

Data are presented either as mean absolute change from baseline (95 % confidence interval) or absolute values (95 % confidence interval) and analysed using a mixed models approach to repeated measures for a cross-over design. Fixed effects of time, group and order of presentation of the interventions and the random effect of participants nested within the order of presentation were modelled using REML (Restricted maximum likelihood) using SAS (v9.4, SAS Institute Inc, Cary NC) and a group by time interaction main effect explored. Models were fitted with repeated time effects assuming an unstructured covariance. Using this model post hoc comparison of the group means sliced through each time point was performed and the overall pairwise error rate was preserved at 5% by using a false discovery rate protected P for
these comparisons. Where the adjusted P for a between-group difference sliced at any time point was <0.05, between groups differences were sought using Tukey’s procedure. Order of presentation was included to enable testing for significant carry-over effects.

The total area under the curve analysis (AUC) of all the measured variables was calculated using the trapezoid method, including the positive and negative peak areas, using GraphPad Prism version 9.1.2 (GraphPad, CA, USA). Two-way repeated measures ANOVA were performed using SPSS statistics 27.0 (SPSS Inc., Chicago, IL, USA) for total AUC changes, to evaluate the effect of kawakawa and the base beverage. Additionally, iAUC for glucose and insulin levels were calculated using the values normalised to baseline values (t =0 min) to determine the postprandial responses. Two-way repeated measures ANOVA were performed using SPSS statistics 27.0 (SPSS Inc., Chicago, IL, USA) for total AUC changes to evaluate the effect of kawakawa and beverage.

Variability in the postprandial responses was assessed using intraclass correlation coefficient (ICC) estimates. ICC values were calculated using SPSS statistics 27.0 (SPSS Inc., Chicago, IL, USA) based on a single measurement, absolute agreement and one-way random model for the AUC values obtained from the postprandial responses of all measured variables.

Two-way repeated measures ANOVA followed by a two-stage step-up method of Benjamini, Krieger and Yekutieli were performed to determine the difference between the VAS scores post-intervention.

LC-MS/MS analysis of the beverage samples is presented as mean ± SEM. These data were analysed using two-way ANOVA followed by a two-stage step-up method of Benjamini, Krieger and Yekutieli for multiple pairwise comparisons using GraphPad Prism version 9.1.2 (GraphPad, CA, USA). The analysis of variance was considered statistically significant at P < 0.05.
4.6 Results

4.6.1 Participant characteristics and baseline values

The clinical and demographic characteristics of the participants are summarized in Table 4.2.

Table 4.2 Clinical and demographic characteristics of the participants

<table>
<thead>
<tr>
<th>Variable</th>
<th>Male</th>
<th>Female</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number (n)</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>33.20 ± 0.31</td>
<td>28.75 ± 0.48</td>
<td>0.73</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>79.13 ± 0.62</td>
<td>64.5 ± 0.34</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>24.49 ± 0.28</td>
<td>26.36 ± 0.06</td>
<td>0.75</td>
</tr>
<tr>
<td>Fat-free mass (kg)</td>
<td>44.33 ± 2.11</td>
<td>36.39 ± 1.33</td>
<td>0.30</td>
</tr>
<tr>
<td>Fat mass (g)</td>
<td>26.73 ± 1.94</td>
<td>23.98 ± 1.09</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Data are represented as mean ± SEM; BMI: body mass index; ANOVA was performed to compare the difference between males and females.

There were no statistical differences in baseline values of any of the experimental parameters at baseline between treatment visits (Table 4.3).

Table 4.3 Mean of baseline values

<table>
<thead>
<tr>
<th>Variables</th>
<th>W</th>
<th>K</th>
<th>B</th>
<th>B+K</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RQ</td>
<td>0.77 ± 0.03</td>
<td>0.76 ± 0.03</td>
<td>0.77 ± 0.04</td>
<td>0.78 ± 0.03</td>
<td>0.43</td>
</tr>
<tr>
<td>REE (kJ/min)</td>
<td>4.45 ± 0.79</td>
<td>4.43 ± 0.77</td>
<td>4.39 ± 0.74</td>
<td>4.38 ± 0.72</td>
<td>0.99</td>
</tr>
<tr>
<td>CHO Ox. (g/min)</td>
<td>0.07 ± 0.02</td>
<td>0.05 ± 0.80</td>
<td>0.07 ± 0.05</td>
<td>0.07 ± 0.03</td>
<td>0.25</td>
</tr>
<tr>
<td>Fat Ox. (g/min)</td>
<td>0.08 ± 0.01</td>
<td>0.08 ± 0.02</td>
<td>0.08 ± 0.02</td>
<td>0.08 ± 0.02</td>
<td>0.68</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.35 ± 0.28</td>
<td>5.28 ± 0.38</td>
<td>5.33 ± 0.41</td>
<td>5.33 ± 0.41</td>
<td>0.94</td>
</tr>
<tr>
<td>Insulin (uU/mL)</td>
<td>7.76 ± 3.42</td>
<td>7.01 ±3.12</td>
<td>7.31 ±2.83</td>
<td>7.53 ±2.72</td>
<td>0.90</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.12 ±0.45</td>
<td>1.20 ±0.67</td>
<td>1.17 ±0.55</td>
<td>1.27 ±0.50</td>
<td>0.88</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>4.21 ±0.86</td>
<td>4.12 ±0.84</td>
<td>4.20 ±0.82</td>
<td>4.17 ±0.84</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SEM; W - water, K - kawakawa leaf suspension; B - base beverage and B+K - base beverage + kawakawa; RQ - respiratory quotient, REE - resting energy expenditure; CHO Ox. - carbohydrate oxidation; Fat Ox. - fat oxidation. ANOVA was performed to compare means of baseline values of each treatment for each parameter.
Intra-individual variability was calculated as mean CV% of each participant between intervention visits (n = 4) and inter-individual variability was calculated as CV% between all participants within each treatment (n = 18) for each variable (Table 4.4). The results indicated intra-individual variability is lesser than inter-individual variability, i.e., variability between participants was greater than variability within. The reliability of measurements was assessed using ICC values as poor (< 0.50), moderate (between 0.5 and 0.75), good (between 0.75 and 0.90) and excellent (> 0.9) based on the guidelines suggested by Koo and Li. 

Table 4.4 Intra- and inter-individual variance of baseline values and Intraclass correlation coefficient of postprandial responses across four intervention visits

<table>
<thead>
<tr>
<th>Variables</th>
<th>Intra-individual variance (% CV_intra)</th>
<th>Inter-individual variance (% CV_inter)</th>
<th>ICC for postprandial responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>REE (kJ/min)</td>
<td>0.008-0.03</td>
<td>16.5-17.8</td>
<td>0.063*</td>
</tr>
<tr>
<td>RQ</td>
<td>0.01-0.03</td>
<td>4.2-5.6</td>
<td>0.326*</td>
</tr>
<tr>
<td>CHO Ox. (g/min)</td>
<td>0.1-1.6</td>
<td>40.1-71.9</td>
<td>0.088*</td>
</tr>
<tr>
<td>Fat Ox. (g/min)</td>
<td>0.03-0.2</td>
<td>23.5-28.8</td>
<td>0.125*</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>1.5-4.1</td>
<td>5.2-7.7</td>
<td>0.851#</td>
</tr>
<tr>
<td>Insulin (uU/L)</td>
<td>3.3-28.6</td>
<td>36.1-44.4</td>
<td>0.615**</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>2.4-21.9</td>
<td>39.5-55.9</td>
<td>0.956**</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>1.1-8.0</td>
<td>19.6-20.5</td>
<td>0.965**</td>
</tr>
</tbody>
</table>

RQ - respiratory quotient, REE - resting energy expenditure; CHO Ox. - carbohydrate oxidation; Fat Ox. - fat oxidation; CV - coefficient of variation, ICC - intraclass correlation coefficient. * Poor reliability; ** moderate reliability; # good reliability and ## excellent reliability.

4.6.2 Postprandial responses of REE, RQ, CHO and fat oxidation before and after consuming standardised breakfast meal

On analyzing the ΔREE data, the results of one participant (female) were visually clearly distinct during the B+K condition, compared to their responses during the other conditions, and those of all other study participants, under all conditions (Figure 4.3). Due to a lack of clarity for excluding that participant on technical or physiological grounds, delta changes were plotted for the whole data set, and AUC analysis was carried out both including and excluding that participant.
Figure 4.3 Considerations for outlier participant

A) Participant 1 is representative of the rest of the population and B) Participant 2 is observed to be an outlier after ingestion of water (W), kawakawa leaf suspension (K), base beverage (B) and base beverage + kawakawa (B+K). The dotted lines (30-45 min) represent the hood-off time when the breakfast meal was given.

The effect of kawakawa on REE changes before and after consuming breakfast meals compared to water treatment was assessed by performing statistical analysis on the respective ΔREE values. There was no difference observed before consuming a breakfast meal. Post-breakfast ingestion indicated a borderline impact of kawakawa leaf suspension with an increase on ΔREE at t = 70 min (p = 0.0531) and between 150 min and 160 min (p = 0.054, 0.048, 0.048) and at 170 min (p = 0.04) compared to water consumption (Figure 4.4A). Total AUC analysis for REE indicated there was a significant difference in the effect of kawakawa (without K (W, B) compared with K (K, B+K)) including the outlier (p = 0.04) and a borderline effect on REE when excluding the outlier (p = 0.07) (Figure 4.4B, C).

Fasting RQ values were between 0.7 and 0.75 over all four treatment visits (Figure 4.4D). Following water (W) and kawakawa leaf suspension (K) treatments (i.e., in the absence of the base beverage, B), there was an increase in RQ from 0.7 to 0.85 starting from 60 min after breakfast meal, and remaining at 0.85 from 90 min to 180 min post-breakfast meal consumption. However, there was no effect of kawakawa versus water on RQ observed in any of the statistical and AUC (ΔRQ) analyses (p = 0.19, including outlier participant; p = 0.18, excluding outlier participant (Figure 4.4E, F)). Additionally, there was an evident increase in RQ from 0.75 to 0.85 after base beverage consumption (B and B+K treatments), with no apparent changes induced post-meal consumption.
Similarly, there was a rise in CHO oxidation from 60 min to 180 min when kawakawa leaf suspension or water was consumed prior to the breakfast meal. There was a borderline effect of kawakawa on ΔCHO oxidation between 70 and 80 min (p < 0.05) and at 140 min (p = .057) compared to water treatment post-breakfast meal (Figure 4.4G). Statistical analysis and AUC (ΔRQ) analysis indicated no effect of kawakawa (p = 0.28, including outlier participant; p = 0.36, excluding outlier participant) (Figure 4.4H, I). There was an immediate increase in CHO utilization after consuming the base beverage (B and B+K treatments), with no further increases observed post-meal.

On the other hand, there were no changes in fat oxidation following the consumption of kawakawa leaf suspension (K) and water (W) prior to the meal. There was a decline in fat oxidation post-breakfast meal after consuming kawakawa and water but the effect of kawakawa was not significant on fat oxidation neither before nor after breakfast consumption (Figure 4.4J). Similarly, there was no effect of kawakawa (p = 0.27, including outlier participant; p = 0.34 excluding outlier participant) (Figure 4.4K, L) observed in AUC analysis. There was an immediate decline in fat oxidation observed after following consumption of the base beverage (B and B+K), with no further changes in fat oxidation induced post-meal.
Figure 4.4 Changes in A) resting energy expenditure (\(\Delta EE, \text{kJ/min}\)), D) respiratory quotient (RQ), G) carbohydrate oxidation (\(\Delta \text{CHO Ox.}, \text{g/min}\)), J) fat oxidation (\(\Delta \text{Fat Ox.}, \text{g/min}\)), their respective AUC (i.e. over 180 min) analysis including the outlier (B, E, H, K) and respective AUC analysis excluding the outlier (C, F, I, L) after ingestion of water (W), kawakawa leaf suspension (K), base beverage (B) and base beverage + kawakawa (B+K). Values are presented as mean ±SEM. The dotted lines (30-45 min) represent the hood-off time when the breakfast meal was given. Fixed effects of time and groups were assessed for the delta changes of each parameter using REML and * indicates \(p \leq 0.05\). Two-way repeated measures ANOVA statistical analysis was performed for treatment groups (without K (W, B) versus with K (K, B+K)). One-way RM ANOVA was performed for the treatment groups (W versus K and B versus B+K).
4.6.3 Postprandial response of blood parameters

There was an increase in blood glucose post-beverage ingestion in all four beverage conditions from 0 -30 min and but there were no changes after breakfast consumption observed during any of the four treatments (Figure 4.5A). Statistical analysis indicated that there were group effects in post-prandial glucose levels at t = 15, 30, 120 and 180 min. However, post hoc analyses indicated there were significant changes between all other conditions (W versus B, K versus B, W versus B+K, K versus. B+K) but no difference in W versus K and B versus B+K conditions. Incremental area-under-the-curve (iAUC) analysis indicated no significant effect of kawakawa (p ≤ 0.9) (Figure 4.5B, C) in the post-prandial glucose responses.

A similar statistical analysis conducted on insulin changes post beverage and breakfast consumption indicated that there was an overall group effect at t = 15 and 30 min for insulin changes. However, post hoc analyses indicated there were no significant changes in W versus K conditions (Figure 4.5D). iAUC analysis of insulin changes indicated no effect of kawakawa (p =0.9) (Figure 4.5E, F).

Statistical analysis indicated no changes in triglycerides in any of the treatment groups. iAUC analysis of triglycerides indicated there was no effect of kawakawa (p = 0.19) (Figure 4.5G, H, I).
Figure 4.5 Changes in plasma A) glucose D) insulin levels, and G) triglycerides levels and their respective iAUC analysis including the outlier (B, E, H) and excluding the outlier (B, F, I), after ingestion of water (W), kawakawa (K), base beverage (B) and base beverage + kawakawa (B+K) and consuming standardized breakfast meal (t = 30 min). Values are presented as mean ± SEM of plasma concentrations. Two-way repeated-measures ANOVA was used to assess the effect of kawakawa on post-prandial glucose, insulin and triglycerides changes using iAUC values.
4.6.4 VAS scores

VAS-rated hunger, fullness, desire to eat, thirst level, comfort and nausea are shown in Figure 4.6. There were decreased feelings of hunger and desire to eat, and increased feelings of fullness reported after consumption of each intervention (K, B and B+K) compared to the water control, but no differences in any of these parameters were observed post-breakfast meal (Figure 4.6A, B, C). However, there were decreased levels of comfort (Figure 4.6E) and increased levels of nausea reported throughout the study during these interventions as compared to the W condition (Figure 4.6F). No changes in thirst level were observed at any time or under any condition (Figure 4.6D).
Figure 4.6 VAS scores for A) hunger B) fullness C) desire to eat D) thirst E) comfort and F) nausea at over 180 min ($t=0$ - 180 min) after ingestion of water (W), kawakawa leaf suspension (K), base beverage (B) and base beverage + kawakawa (B+K). The dotted lines indicate breakfast meal consumption (at $t = 30$ min). Two-way ANOVA followed by a two-stage step-up method of Benjamini, Krieger and Yekuteli was performed (* indicates $p < 0.001$ between $W$ versus $K$, $W$ versus $B$, $W$ versus $B+K$; # indicates $p < 0.01$ between $W$ versus $K$).
4.6.5 Haemodynamic measurements

There were no differences noted in any of the haemodynamic measurements, BP (systolic (mmHg) and BP diastolic (mmHg)) and heart rate (bpm) at over 180 min (t = 0 - 180 min) after the interventions (Figure 4.7).

![Figure 4.7](image.png)

**Figure 4.7** Haemodynamic measurements. A) BP systolic (mmHg), B) BP diastolic (mmHg) and C) heart rate (bpm) at over 180 min (t = 0 - 180 min) after ingestion of water (W), kawakawa leaf suspension (K), base beverage (B) and base beverage + kawakawa (B+K). The dotted lines indicate breakfast intervention (at t = 30 min). Two-way ANOVA followed by a two-stage step-up method of Benjamini, Krieger and Yekuteli was performed.

4.6.6 Phytochemical quantification of kawakawa and beverage samples

Targeted LC-MS/MS analysis was performed to quantify eight major phytochemicals present in methanol extracts of kawakawa leaf powder (K) and the base beverage powders (B and B+K), including myristicin, elemicin, fagaramide, yangambin, diayangambin, dopamine,pellitorin, and trigonelline, along with major phytochemicals of turmeric (curcumin) and ginger.
It was observed that B samples had significantly lower levels of phytochemicals compared to K and B+K samples ($p<0.001$). There were changes observed in the concentration levels of dopamine ($p = 0.008$) and pellitorin ($p < 0.001$) between K and B+K samples. Curcumin and gingerol were not identified in K samples and there were no significant differences in the levels of curcumin and gingerol between B and B+K samples (Figure 4.8).

![Figure 4.8 Absolute quantification of kawakawa phytochemicals obtained from methanol extraction of kawakawa leaf (K), base beverage (B) and base beverage + kawakawa (B+K) powders. Values are presented as mean ± SEM ($n=3$). Significant differences between the mean of K and B+K samples were determined using two-way ANOVA followed by a two-stage step-up procedure of Benjamini, Krieger and Yekutieli for multiple pairwise comparisons.]

### 4.7 Discussion

The present study is the first to investigate the potential acute effects of kawakawa on whole-body energy metabolism. Kawakawa leaf suspension, in isolation or combination with a mixed beverage, was studied for its potential to influence resting energy expenditure before and after the consumption of a standardised breakfast meal. Overall, the kawakawa-containing beverage conditions were shown to elicit a small increase in energy expenditure, due to apparent increases in rates of carbohydrate oxidation. However, this effect remained constant (i.e., was
not amplified) following consumption of the meal, potentially due to the meal's relatively high carbohydrate content - meaning that carbohydrate oxidation may have been approaching its maximum rate. Additionally, despite demonstrating the presence of several compounds known to elicit effects on the sympathetic nervous system and pathways of lipid and carbohydrate oxidation, no evidence was found of a synergistic effect of kawakawa and the components of the base beverage at the whole-body level. However, the poor reliability of measurements, as assessed by ICC for REE and CHO Ox indicates that the study may have been underpowered leading to detect such differences.

Despite no significant alterations in RQ and fat oxidation rates observed due to kawakawa ingestion in the present study, cellular and animal studies conducted on some of its phytochemicals in isolation indicate potential influence on related metabolic pathways. For example, piperine promoted beneficial metabolism during acute endurance exercise in healthy mice \(^\text{283}\), and attenuated insulin resistance after 10 successive weeks of piperine treatment in obese mice \(^\text{284}\). Similarly, Trans-cinnamaldehyde stimulated mitochondrial biogenesis via induction of PPARβ/δ and PGC-1α expressions, enhancing GLUT4 expression \(^\text{285}\) in cellular models. In vitro studies and a 3-week intervention in obese mice have demonstrated cinnamon extract improves insulin sensitivity and influences carbohydrate metabolism mediated via PPAR γ induction \(^\text{286}\). Lipid-modifying effects were observed after 8 weeks of combined ingestion of curcuminoids and piperine in patients diagnosed with metabolic syndrome (dyslipidemia) \(^\text{62}\). Preliminary cell-based studies indicated that pellitorin has the potential to modulate lipid metabolism via regulating PPARγ and is indirectly influenced by TRPV1 activation \(^\text{144}\). It is, therefore, possible that the lack of observed effect in the present study may have arisen due to the small sample size, or indeed compensatory mechanisms that may be in operation to suppress the translation of changes at the cellular or organ level to those at the whole-body level – although this hypothesis remains to be explored.

Trigeminally active compounds identified in kawakawa including pellitorin and other piper amides were shown to modulate nutrient and fatty acid uptake in cellular models \(^\text{73}\). The only published human study on the acute metabolic effects of ingestion of kawakawa tea reported modulations in insulin sensitivity after 4g/250 mL kawakawa tea consumption \(^\text{253}\). However, in this study, there was no significant effect of kawakawa leaf suspension observed on postprandial glucose and insulin. This apparent discrepancy may be due to the matrix in which
the kawakawa compounds were consumed in these studies, as tea and leaf suspension respectively. Enzymatic digestion and absorption of a complex mixture of kawakawa chemicals ingested as leaf suspension is possibly delayed contrary to the phytochemicals in aqueous extracts, which may be more readily absorbed by the body. Indeed, previous studies have shown that the mode of delivery may influence the bioavailability and bioactivity of these compounds. Phytochemical analyses for beverage samples indicated differences in some of the phytochemicals in B+K compared to K which could be possibly due to difficulties in factorizing gradient profiles when dealing with complex beverage mixtures.

Despite the low caloric content of kawakawa leaf suspension, there were similar responses reported in terms of subjective feelings of post-consumption hunger, and desire to eat and greater feelings of fullness as compared to those following the base beverage consumption. This indicates a novel, potentially beneficial effect of kawakawa compounds in terms of appetite control. This post-ingestive satiety effect could be associated with the presence of fatty acid amides or fibrous contents, slow energy release, low digestible energy required or interference with any hormones, such as ghrelin and leptin, regulating appetite. This is the first study to report such effects, and therefore further studies are warranted to investigate these properties.

4.8 Conclusion

This study is the first to explore the beneficial potential of kawakawa on energy metabolism, appetite and satiety, before and after the consumption of a standardised breakfast meal. Whilst the results here indicate a potentially positive effect on these parameters, large variability in response was observed, and the sample size was consequently too small to draw firm conclusions. Likewise, discrepancies with regards to kawakawa’s influence on postprandial glycaemia as compared with previous literature warrant further investigation, particularly into the effect that mode of delivery may have. Further studies are therefore needed to better understand the mechanisms by which these kawakawa compounds may influence pathways of whole-body energy metabolism, what effect kawakawa may have on postprandial response to meals of differing nutrient composition, and importantly, the potential effect of longer-term kawakawa ingestion in healthy and metabolically at-risk individuals alike.
Chapter 5 Pellitorin, a fatty acid amide identified in *Piper excelsum* (kawakawa) leaves influences PPARγ and HO-1 gene expression in HepG2 cells

Abstract

Pellitorin (PEL) is one of the most abundant fatty acid amides in *Piper excelsum* leaves. The present study explored whether pellitorin could modify the expression of selective genes in HepG2 cells that are involved in the regulation of inflammatory pathways, metabolism and antioxidant response. Three different experiments were undertaken with different exposure to pellitorine, 0.3% DMSO and 20 μM D-sulforaphane (SFN) as negative and positive controls, respectively. In addition to that, the functional activities of myristicin, an abundant kawakawa phenylpropanoid were explored. There were no significant changes observed in any of the gene expressions after myristicin (MYR) treatment. Exposure of HepG2 cells to 10 μM and 20 μM pellitorin for 24 h significantly suppressed PPARγ expression levels, similar to that observed with SFN treatment. Additionally, PEL upregulated HO-1 gene expression levels at 6 h and downregulated at 24 h, again similar to SFN treatment. The similarity in response between pellitorin and sulforaphane suggests that it may act by inducing Nrf-2 mediated transcription.

5.1 Introduction

Research studies on exploring kawakawa phytochemistry revealed the presence of several compounds of diverse classes (Chapter 2). Some of these compounds could be metabolized via phase 1 and phase 2 human metabolism (Chapter 3). Acute ingestion of kawakawa tea was shown to modulate glucose and insulin metabolism \(^{253}\) and may also influence energy expenditure and respiratory quotient (Chapter 4). To complement these studies exploring the physiological effects of chemically complex kawakawa leaf extracts, in this chapter, I investigate the functional activity of two individual components of that complex mix, pellitorin and myristicin, with the use of the hepatic human cell line, HepG2.

Pellitorin is one of the several kawakawa compounds that possess chemesthetic properties and have been shown to stimulate trigeminal nerves \(^{73,81,292}\). Pellitorin has been shown to exhibit anti-inflammatory potential by inducing Nrf2 activation \(^{293}\), to attenuate PPARγ expression and the indirect modulation of TRPV1 receptor channel\(^{144}\). Moreover, it has been reported to be
able to permeate the gut mucosa and blood-brain barrier in rodents. Myristicin has also been shown to upregulate the Nrf2/HO-1 axis in rats.

As PEL and MYR have been reported to induce Nrf2-mediated transcription, it was decided to use sulforaphane (SFN), as a potent activator of Nrf2 transcription as a positive control. This study aims to investigate the influence of PEL and MYR expression of selected genes involved in inflammatory pathways, insulin sensitivity, glucose and lipid metabolism and immune functions in HepG2 cells.

5.2 Materials and methods

5.2.1 Chemicals and reagents

Authentic standards of pellitorin (PEL), myristicin (MYR) and D-sulforaphane (SFN) were purchased (Sigma-Aldrich, Castle Hill, Australia). Other reagents and chemicals obtained were as follows: Dimethyl Sulfoxide 100 mL, molecular biology grade, suitable for cell culture (DMSO) (Thermo Fisher, Auckland, New Zealand); MG-FBS0820-500ML fetal bovine serum (FBS) (Medi-ray, Auckland, New Zealand), phosphate buffer saline (PBS), Type 1 water was generated from the Millipore unit (Merck Millipore, Auckland, New Zealand); Penicillin (100 U/mL), streptomycin (100 μg/mL), Glutamax, Trypsin-EDTA (0.25%), phenol red, RPMI 1640 Medium (Life Technologies, Auckland, New Zealand). Trypan Blue solution, 0.4%, liquid, sterile-filtered, suitable for cell culture (Sigma-Aldrich, Castle Hill, Australia); MycoAlert Mycoplasma Detection Kit, 50 Tests (Alphatech Systems Ltd., Auckland, New Zealand); CyQUANT LDH cytotoxicity assay kit (200 assays) (Thermo Fisher, Auckland, New Zealand).

5.2.2 Preparation of chemical compounds for cytotoxicity studies

The stock solutions of chemical compounds were prepared as follows: pellitorin (10 g/L), D-sulforaphane (10 g/L) and myristicin (10 g/L) in 0.3% DMSO. The working solutions of 100 μM of these chemicals were prepared from which the required concentrations (pellitorin 1, 5, 10 and 20 μM; myristicin 10 μM; SFN 20 μM) were prepared accordingly on the experiment days.
5.2.3 Cell culture and treatment

HepG2 cells (available from ATCC) were cultured in RPMI (Thermo Fisher Scientific, Auckland, New Zealand) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Auckland, New Zealand), 5% penicillin/streptomycin and GlutaMAX (Life Technologies, Auckland, New Zealand). Cells were maintained in 75 cm² flasks at 37°C, 5% CO₂, split twice each week at ~80% confluence using PBS to wash, trypsin treatment to detach the cells and used for the experiments between passages 19 and 25. On every occasion, cells were centrifuged (1000 RPM, 5min, room temperature) and suspended in fresh media. Only monolayers with at least ~80% confluency were used for all the experiments. Mycoplasma contamination was routinely tested using MycoAlert Mycoplasma Detection Kit (cat# CAMLT07-418, Alphatech Systems Ltd., Auckland, New Zealand), according to the manufacturer’s protocol.

5.2.4 Short tandem repeat (STR) profiling

STR analysis is a rapid and sensitive technique applied to confirm their identity ensuring the accuracy and reliability of cell lines used. The genetic background of the cell lines was investigated using the amplification method of short tandem repeats. Cultured HepG2 cells were trypsinized and genomic DNA was extracted using prepGEM kit, according to the manufacturer’s tissue culture protocol. An extracted DNA sample was then sent to DNA diagnostics, IGENZ Ltd (Auckland, New Zealand) for STR analysis.

5.2.5 Cell viability and cytotoxicity assessments

Alamar blue cell proliferation assay was used to optimize FBS concentration, DMSO concentration and to assess the toxic effect of PEL on HepG2 cells. Cells (1 × 10⁵ cells/mL) were seeded in 96-well plates and incubated overnight (O/N) to obtain 70 - 80% confluence. All these experiments were carried out in six independent replicates in each plate, and at least three independent experiments.

To optimize the FBS concentration, cells (1 × 10² cells/mL) were seeded onto a 96-well plate, incubated O/N and FBS concentrations ranging from 0% - 10% (0, 0.31, 1.25, 2.5, 5 and 10%) were added to the adherent cells followed by incubation for 2, 4 and 6 days. Similarly, to
optimize the DMSO concentration, O/N seeded cells (1 × 10^5 cells/mL) on 96-well plates were treated with different DMSO concentrations ranging from 0 to 4% (0, 0.06, 0.12, 0.25, 0.5, 1 and 4 %) for 2, 4 and 5 days. To determine the toxic effect of PEL, cells were exposed to different doses of PEL ranging from 0 to 135 μM (0, 1, 2, 4, 8, 17, 34, 67 and 135 μM) for 3 and 5 days and 0.3% DMSO as a control to eliminate the cytotoxic effects exerted by the vehicle. Every concentration of pellitorin and DMSO was tested in six independent replicates in each plate, and at least three independent experiments.

Cell viability was determined by adding 5 μL resazurin sodium salt (0.5 mg/mL) to each well followed by 2 h incubation at 37°C with 5% CO₂. Based on the principle of changing from an oxidised form (resazurin – blue colour) to a reduced form (resorufin – red colour) 220, fluorescence intensity measurements were recorded using a Tecan Infinite M1000 Pro automated plate reader system with an excitation wavelength of 560 nm and an emission wavelength of 590 nm.

PEL cytotoxicity on HepG2 cells was assessed by lactate dehydrogenase (LDH) assay. LDH is used as a marker of cell death and LDH assay is based on the principle of the catalytic action of LDH (released from damaged cells into culture medium) in converting lactate to pyruvate via reducing NAD+ into NADH, which is further oxidized by diaphorase leading to conversion of tetrazolium salt to formazan product (red) 220. LDH assay using CyQUANT™ LDH cytotoxicity assay kit (Cat# C20300, Invitrogen, Thermo Fisher Scientific, Auckland, New Zealand) as per the manufacturer’s protocol. Firstly, cell number was optimized by performing an LDH assay for different concentrations of cells ranging from (1 × 10^2) to (1 × 10^10) cells/mL and a cell concentration of 1 × 10^8 cells/mL was determined for PEL exposure. A 100 μL of cells (1 × 10^8 cells/mL) were seeded onto 96-well plates O/N followed by PEL (100, 50, 5, 1 and 0 μM) treatment for 1h and 24 h. Two controls were included, spontaneous LDH release (0 μM wells i.e. 0.3% DMSO wells) to correct for spontaneous LDH release and a maximum LDH release (lysis buffer wells) to estimate the maximum LDH activity at maximal cell death. On the LDH assay day, 10 μL of the respective solutions (water/lysis buffer) were added into the wells and incubated for 45 min at 37°C. Afterward, 50 μL of culture supernatant was added to a new 96-well plate and an equal volume of reaction mixture was added and incubated for 30 min at room temperature. Followed by adding 50 μL stop solution and reading the
absorbance at 490 nm and 680 nm on an automated plate reader. For analysis, absorbance at 680 nm was subtracted to correct for optical imperfections. Every concentration of pellitorin was tested in triplicates on each plate, and at least four independent experiments were carried out.

5.2.6 Cell culture exposure experiments for TaqMan gene expression studies

Three independent experiments were carried out for the TaqMan gene expression analysis. For all these experiments, HepG2 cells were exposed to PEL or other compounds (MYR and SFN) after seeding the cells O/N onto a transparent, flat-bottomed 6-well plate (Sigma-Aldrich, Castle Hill, Australia) by adding 2 mL of cell suspension (6 x 10^5 cells/mL) per well. Following this, TaqMan gene expression analysis was carried out.

**Exposure experiment 1:** In this experiment, PEL concentrations at 1 and 5 μM were selected from the cell viability and cytotoxicity assay results. HepG2 cells were treated with PEL (1 and 5 μM) and 0.3% DMSO as control, in triplicates and incubated for 24 h at 37°C, 5% CO₂ to evaluate the gene expression changes of the target genes: TRPV1, TRPA1, AMPK, PPARγ, UCP1, SIRT 1, FAS along with housekeeping genes: ACTB, TBP and GAPDH.

**Exposure experiment 2:** PEL concentration (10 μM) based on the phytochemical analyses and previous studies reported gene expression changes at this concentration. Cells were treated with PEL (10 μM), MYR (10 μM) and 0.3% DMSO as control (0 μM) in triplicates and incubated for 6 h and 24 h at 37°C and 5% CO₂. SFN (20 μM) was used as the positive control for the target genes. The target genes assessed in this TaqMan gene expression analysis were, TNF-α, HO-1, NQO1 and AKRC1 in addition to the gene panel used in the previous experiment, TRPV1, AMPK, PPARγ, UCP1, SIRT 1, FAS and housekeeping genes: ACTB, HPRT1 and GAPDH.

**Exposure experiment 3:** Cells were treated with PEL (10 and 20 μM) and 0.3% DMSO (0 μM) in triplicates and incubated for 6 h, 24 and 30 h at 37°C, 5% CO₂. SFN (20 μM) was used as the positive control. PPARα was included in the test panel, in addition to the target genes used in the previous experiment: TRPV1, AMPK, PPARγ, HO-1 and housekeeping genes: ACTB, HPRT1 and GAPDH.
5.2.7 Reverse transcriptase – quantitative PCR (RT-qPCR)

After each cell exposure treatment, total cellular RNA was extracted using Purelink™ RNA Mini kit (cat # 12183025), according to the manufacturer’s instructions. RNA concentration was quantified using NanoPhotometer® N60 (Implen, Munich, Germany). The absorbance ratio used were 260/280 and 230/260, with ratios ≥ 1.8 considered acceptable purity. RNA integrity (RIN) was determined using an Agilent Bioanalyzer RNA 6000 Nano kit on Agilent 2100 bioanalyzer (Agilent Technologies, Auckland, New Zealand), according to the manufacturer’s protocol. First stranded cDNA was synthesized using RNA (2μg) with the SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme (cat# 11766500) (Invitrogen, Auckland, New Zealand) and Real-time PCR was performed as per manufacturer’s instructions on the Mastercycler X50s (Eppendorf, Hamburg, Germany).

5.2.8 TaqMan gene expression analysis

TaqMan real-time polymerase chain reaction (PCR) was performed on an ABI Prism 7900HT Sequence Detector (Applied Biosystems, CA, USA) to determine the transcript abundance based on the manufacturer’s protocol (Thermo Fisher Scientific, Auckland, New Zealand). A standard real-time PCR protocol with the following run conditions on a Quant Studio 6 Flex real-time PCR system (Applied Biosystems™, Thermo Fisher Scientific, Waltham, USA): 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s, 60 °C for 1 min or 50 °C for 2 min, 95 °C for 20 s, 95 °C for 3 s, 60 °C for 30 s (40 cycles) was used for all the reactions using different TaqMan primers in all three experiments carried out. All cDNA samples were run in duplicate in 384-well plates using 2μL cDNA with a total reaction volume of 10 mL for experiments 1 and 2, whereas, all cDNA samples were run in 5 replicates in Experiment 3. Predesigned TaqMan Gene Expression Assay (FAM) and TaqMan™ gene expression master mix were obtained for all the target and reference genes (Table 5.1) (Thermo Fisher, Auckland, New Zealand). For the standard curve, serial dilutions (1:10, 1:100, 1:1000 and 1:10000) of cDNA were prepared from 2.5 μg RNA from the sample set. The standard curve of each target gene was extrapolated and used to calculate the amplification efficiency (i),

\[ E = 10^{-\frac{1}{\text{slope}}}(i) \]
Relative gene expression ratio was determined using the Pfaffl method\textsuperscript{300–303} with the housekeeping genes (HKG): ACTB, GAPDH and TBP/HPRT as internal controls. The geometric mean of Ct values of the three selected reference genes was calculated for each sample and used for target analysis. Gene expression ratio was calculated by using the equation (ii)\textsuperscript{300},

\[
\text{Gene expression ratio} = \frac{(E_{\text{target}})^{\Delta Ct_{\text{target}}(\text{control-sample})}}{(E_{\text{HKG}})^{\Delta Ct_{\text{HKG}}(\text{control-sample})}} \quad (ii)
\]

Data were normalized to the appropriate HKG as an internal control and calibrated against the average Ct of the control samples for the relative quantitative comparison.

\textit{Table 5.1 Predesigned TaqMan probes}

<table>
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<th>Assay ID</th>
<th>Gene</th>
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<tr>
<td>Hs02786624_g1</td>
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<tr>
<td>Hs04230636_sH</td>
<td>Aldo-Keto Reductase (AKRC1)</td>
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<tr>
<td>Hs01562315_m1</td>
<td>AMP – activated protein kinase (AMPK) (catalytic subunit - PRKAA1)</td>
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<tr>
<td>Hs00236330_m1</td>
<td>Fatty acid synthase (FAS)</td>
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<td>Hs02786624_g1</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)</td>
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<td>Hs01110250_m1</td>
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</table>
5.2.9 Data processing and statistical analysis

For the alamar blue and LDH assays, data are represented as mean ± standard error of the mean (SEM) of three independent experiments performed in six independent replicates. Statistical analysis and nonlinear regression analysis of the resulting values were analysed using GraphPad Prism software version 9.1.2. (GraphPad, California, USA). The concentration required to cause a 50% reduction (IC₅₀) was calculated from each dose-response curve obtained in each independent experiment and the mean ± SEM was provided. Half-maximal inhibitory concentration (IC₅₀) values of pellitorin were determined by fitting a sigmoidal dose-response model “log (pellitorin concentration) versus response-variable slope (four parameters)”. Significant differences among the groups treated with different concentrations of PEL in LDH assay were determined using two-way repeated measures analysis of variance (RM ANOVA, p < 0.05) followed by posthoc analyses (Sidak) as necessary. Significant differences among the groups treated with different concentrations of the chemical compounds (PEL, MYR, SFN) and the control group (0.3% DMSO) in TaqMan gene expression assays were determined using two-way repeated measures analysis of variance (RM ANOVA, p<0.05) with a two-stage step-up method of Benjamini, Krieger and Yekutieli for multiple pairwise comparisons ²²⁴ using GraphPad Prism version 9.1.2 (GraphPad, California, USA).

5.3 Results

5.3.1 Cell line identification by short tandem repeat (STR) profiling and Mycoplasma testing

The STR analysis results obtained using Applied Biosystems AmpFSTR® Identifiler™ on an Applied Biosystems SeqStudio™ Genetic Analyser performed by DNA diagnostics (Igenz, Auckland, New Zealand) were submitted to https://celldive.dsmz.de/str/search. This STR profile search authenticated HepG2 cell lines. The cell line tested negative for mycoplasma contamination.

5.3.2 Cell viability and cytotoxicity assessments

The cytotoxic effects associated with PEL towards HepG2 cells were assessed using the Alamar Blue assay. Cell viability was determined after 3 and 5 days of different concentrations of PEL treatment. Exposure to PEL at the highest concentration of 100 μM initiated the
apoptotic process from Day 1 as it cells started to form rounded cells. After 3 days of exposure, PEL concentration of 33 μM and higher started to induce cell death with maximum cell death at 134 μM, whereas, the concentration of 1 – 16 μM did not affect cell viability. The inhibitory concentration that decreased cell viability by 50% (IC₅₀) was calculated to be 75.2 ± 0.36 μM after 3 days of PEL exposure (Figure 5.1A).

For the LDH assay, it was observed that after PEL treatment on HepG2 cells for 1h and 24 h, A490 nm values significantly decreased and LDH activity markedly increased at 100 μM (Figure 5.1B), indicating at the highest concentration of PEL (100 μM) inhibits proliferation and survival of HepG2 cells. Similarly, at 24 h, PEL concentration at 1, 5 and 50 μM showed increased A490 nm values and lower LDH release. Results indicated that 24 h exposure to PEL concentration at 1 and 5 μM could interfere with the metabolic activity of cultured HepG2 cells without damaging or causing cell death.

![Figure 5.1 Cell viability and cytotoxic assessment of pellitorin on HepG2 cells A) Alamar blue assay and inhibitory dose-response curves of pellitorin with IC₅₀ values of 71.6 μM calculated for 3 days pellitorin treatment. B) LDH cytotoxicity assessment after treating the HepG2 cells for 1h and 24 h. values are expressed as mean ± SEM (n = 3). Data analysis was performed in GraphPad prism using repeated measure ANOVA and posthoc analysis (Sidak). p-values are indicated on the graph.]

5.3.3 RNA purification and RIN assessment

RNA purity was assessed using NanoPhotometer® N60 at the absorbance ratios 260/230 and 280/260. The results indicated that the ratios were ≥ 1.9 indicating the RNA samples were pure without any other contamination. Additionally, RIN (RNA integrity number) performed by the bioanalyzer yielded RIN numbers for all the samples which were > 9.0 (Figure 5.2)
suggesting that RNA remained intact after PEL treatment and not denatured by the RNA purification process.  

Figure 5.2 Electrophoresis gel run and electropherogram for HepG2 cells treated with 0.3% DMSO and PEL (1 and 5 μM).

5.3.4 Gene expression changes after PEL, SFN and MYR treatment on HepG2 cells

5.3.4.1 Experiment 1

The relative gene expression of the selective target genes (AMPK, TRPV1, TRPA1, PPARγ, SIRT1, FAS and UCP1) were studied after PEL (1 μM and 5 μM) treatment in comparison to 0.3% DMSO treatment as control (0 μM) after 24 h. The mRNA expression levels of the target genes were normalised against three housekeeping genes ACTB, TBP and GAPDH and plotted as individual values in the graph (Figure 5.3). There was no significant difference in any of
the gene expression levels after treating with 1 and 5 μM PEL. It was observed that the TRPA1 gene was not expressed in HepG2 cells.

Figure 5.3(Experiment 1). Relative gene expression levels of the selected target genes after 24 h PEL (1 and 5 μM) treatment. Statistical significance was determined using two-way ANOVA followed by a two-stage step-up method of Benjamini, Krieger and Yekutieli for multiple pairwise comparisons. p-values are indicated on the graph.

5.3.4.2 Experiment 2

To further elucidate the effect of PEL at an increased concentration (10 μM), relative gene expression levels quantified obtained after treating HepG2 cells for 6 h and 24 h with PEL (10 μM) and MYR (10 μM) in comparison to 0.3% DMSO treatment (0 μM) as control and SFN (20 μM) as a positive control. The mRNA expression levels of the target genes (AMPK, TRPV1, TRPA1, PPARγ, SIRT1, FAS and UCP1, TNF-α, IL-6, HO-1, AKRC1 and NQO1) were normalised against three housekeeping genes ACTB, HPRT1 and GAPDH and plotted as individual values in the graphs (Figure 5.4). It was observed that the IL-6 gene was not expressed in HepG2 cells.

After 6 h, there was no difference observed in gene expression levels with MYR (10 μM) and PEL (10 μM) treatment (Figure 5.4A, B). SFN (20 μM) increased the expression levels of AKRC1, NQO1, HO-1, SIRT1 and FAS and decreased PPARγ, TNFα, UCP1 and SIRT1 expression levels compared to DMSO control samples (Figure 5.4C).

After 24 h, there was no difference observed in gene expression levels with MYR (10 μM) (Figure 5.4D). PEL (10 μM) treatment increased the expression of AMPK and decreased HO-1, TRPV1, FAS and PPARγ expression levels compared to DMSO control (Figure 5.4E). With SFN (20 μM) treatment, there were increased levels of expression observed for AMPK,
AKR1C1, NQO1 and FAS and decreased expression levels of TRPV1, PPARγ, TNFα and UCP1 except that HO-1 and SIRT 1 level returned to baseline (no statistical difference) (Figure 5.4F).

5.3.4.3 Experiment 3

To confirm the results in Experiment 2, the relative gene expression of the selective target genes which were significantly induced (PPARγ, AMPK, TRPV1 and HO-1 and PPARα) were studied after PEL (10 μM and 20 μM) treatment and with SFN (20 μM) as positive control and 0.3% DMSO treatment and a negative control after 6 h, 24 h and 30 h (n = 5).

After 6 h, there was no statistical significance in the expression of any of the genes except upregulation of HO-1 with PEL (10 μM) treatment (Figure 5.5A). PEL (20 μM) exposure increased HO-1 expression compared to DMSO control (Figure 5.5B). With SFN (20 μM) treatment, expression levels of PPARγ were lower and HO-1 was higher compared to DMSO control (Figure 5.5C).

After 24 h PEL (10 μM) treatment, the expression levels of PPARγ were lower and AMPK was greater than DMSO control (Figure 5.5D). Treatment with PEL (20 μM) decreased PPARγ levels (Figure 5.5E) and with SFN (20 μM) exposure, expression levels of PPARγ, PPARα and TRPV1 were decreased compared to DMSO control(Figure 5.5F).

After 30 h, there was no difference in expression between the treated and control with PEL (10 μM) (Figure 5.5G). Lower expression levels of HO-1 were observed after 30 h PEL (20 μM) exposure (Figure 5.5H). With SFN (20 μM) treatment, lower levels of PPARα and TRPV1 expression and higher levels of AMPK expression was observed (Figure 5.5I).
Figure 5.4 (Experiment 2). Relative gene expression levels of the selected target genes after 6h MYR (10 μM), PEL (10 μM) and SFN (20 μM) (A, B, C) and after at 24 h MYR (10 μM), PEL (10 μM) and SFN (20 μM) treatment (D, E, F) compared with DMSO controls at the respective time points. Statistical significance was determined using two-way ANOVA followed by two-stage step-up method of Benjamini, Krieger and Yekutieli for multiple pairwise comparisons. p-values are indicated on the graphs.
Figure 5.5 (Experiment 3). Relative gene expression levels of the selected target genes after PEL (10 μM) at A) 6 h, B) 24 h and C) 30 h; PEL (20 μM) at D) 6h, E) 24 h and F) 30 h; SFN (20 μM) at G) 6h, H) 24 h and I) 30 h. Statistical significance was determined using two-way ANOVA followed by a two-stage step-up method of Benjamini, Krieger and Yekutieli for multiple pairwise comparisons. p-values are indicated on the graphs.
5.4 Discussion

To date, the potential biological activities of kawakawa compounds and their underlying molecular mechanisms remain unclear. This study aimed to characterize the changes in the expression of selected genes in HepG2 cells following exposure to two kawakawa compounds, pellitorine and myristicin. Sulforaphane, a known inducer of Nrf2-mediated transcription[^305] was used as a positive control.

In Experiments 2 and 3, PEL and SFN treatment both increased HO-1 and AMPK gene expression and decreased PPARγ expression levels. No changes in gene expression were observed following MYR treatment. The similarities in the activity of PEL and SFN may suggest that they may both be affecting gene expression via Nrf2-mediated transcription. In response to oxidative stress and inflammation, the sulfhydryl group of SFN interferes with Kelch-like-ECH-associated protein (Keap1) triggering a cascade of events involved in repressing Nrf-2 signaling in the cytosol targeting Nrf2 degradation[^306]. Dissociated Nrf2 translocate into the nucleus and bind with antioxidant response element (ARE) initiating enzymatic reactions (phase II and antioxidant proteins) and stimulating the expression of Nrf2 genes including HO-1, NQO1, AKRC-1, GSTs, SOD, ferritin and catalases[^307] ([Figure 5.6](#)). This, in turn, interferes with other metabolic pathways inducing the expression of downstream genes such as PPAR, FAS, AMPK and TRPV1. Studies have shown that SFN treatment can reduce oxidative stress, misfolded protein accumulation, reversal of age-related immunity deficiency, and cardiac and muscular dysfunction[^307,308]. For example, SFN treatment upregulated Nrf-2 antioxidant genes, HO-1, NQO1 and GSTM3 and also significantly decreased lipid peroxidation and fat accumulation in a mouse model of hepatic fibrosis induced by ethanol plus carbon tetrachloride[^309]. SFN treatment was observed to modulate the expression of genes involved in lipid metabolism (FAS, PPARγ, SREBP-1c, C/EBP-α, and AP2) and lipolysis (UCP-1 and CPT-1)[^310]. SFN upregulated HO-1 gene expression at 24 h exerting anti-inflammatory properties via activating Nrf2/HO-1 pathway[^311]. They were also shown to induce weight loss and improve metabolic function by modulating leptin action and activating the Nrf2-signaling pathway[^312].
Figure 5.6 Effect of sulforaphane (SFN) on Nrf2 transcriptional activation. In the cytosol, SFN interacts with Kelch-like-ECH-associated protein (Keap1) and free SFN is translocated to the nucleus stimulating the expression of a gene involved in phase II enzymes and antioxidant defenses (HO-1, NQO1 and AKRC-1) and further influence on the downstream gene involved in inflammation, glucose and lipid metabolism.

Additionally, SFN was shown to reduce the expression of TRPV1 in experiment 3 (p < 0.001, Figure 5.4F) and there was an indication that PEL may also reduce the expression of TRPV1 although the results had a lower level of significance (p < 0.09, Figure 5.4D). Unlike HO-1, TRPV1 has not been previously recognized as being regulated by nrf2, although some association has been noted previously. Modulation of TRPV1 may reduce pro-inflammatory processes which are important in several chronic diseases. A previous study reported that trans-pellitorin indirectly modulated TRPV1 and TRPA1 gene expression levels to reduce lipid accumulation.

However, in contrast, different results were observed regarding the effect of PEL and SFN on FAS gene expression levels. PEL treatment decreased FAS expression levels (p < 0.04, Figure
5.4E) whereas SFN increased FAS expression levels (p < 0.01, Figure 5.4F). Previous studies have shown that SFN may increase FAS $^{315,316}$ and also decrease FAS expression $^{317,318}$ depending upon the extent of exposure of the experimental system to SFN.

The similarity between PEL and SFN in regulating gene expressions of target genes involved in inflammation and metabolism (PPARγ, AMPK and TRPV1) and in particular HO-1 gene expression suggests that PEL may regulate oxidative stress response and drug detoxification via Nrf-2 mediated transcription.

5.5 Conclusion

Kawakawa compounds, with their diverse chemical class, might possess potential biological properties beneficial to human health. This study explored the functional activities and the underlying molecular mechanism of one of its abundant compounds, pellitorin and myristicin in comparison with the D-sulforaphane mode of action. Pellitorin treatment on HepG2 cells decreased PPARγ and increased HO-1 and AMPK gene expression levels similar to SFN treatment. These results indicate PEL could interfere with hepatoprotective and antioxidant defense mechanisms in association with the Nrf2 signaling pathway and possibly modulate inflammation and lipid metabolism. Further, in vitro and clinical studies are warranted to better understand its possible health implications and to be successfully implemented in the development of functional foods.
Chapter 6 General Discussion

Several endemic plant species have a history of medical and therapeutic use amongst Māori in Aotearoa New Zealand, foremost of which is *Piper excelsum* (kawakawa). The historical and current use of kawakawa amongst Māori and recent interests in the development of plant-based functional foods has led to the scientific investigation of kawakawa leaf chemistry. However, there remains a significant gap in the scientific literature with only three studies (using cellular and animal models) investigated the biological effects of kawakawa. No human intervention study has been reported to evaluate the biological effects and therapeutic properties of kawakawa. The primary aim of my research was to characterize the kawakawa phytochemistry and to explore human metabolism.

6.1 Main findings

In Chapter Two, I investigated the phytochemical composition of kawakawa leaf chemicals in aqueous and methanolic extracts obtained from commercially available dried kawakawa leaves and fresh leaves sourced from different geographical regions in Aotearoa New Zealand. A previous study on kawakawa aqueous/solvent extracts identified 24 features by NMR and LC-MS/MS. By integrating LC-MS/MS based metabolomics with molecular networking and multivariate analysis, I have demonstrated the complexity and diversity of the kawakawa chemical space. These analyses supplemented the previous two studies with putative identification of 57 chemical compounds including isomers of diverse chemical classes. Furthermore, eight of the kawakawa compounds were absolutely quantified. This is essential to determine the possibilities of risk and benefits associated with kawakawa consumption.

Although a complete understanding of geographical or processing conditions has not been possible, the study provided an insight into phytochemical variations amongst samples commercially available, collected at different times of the year and from different localities. Studies have shown that phytochemical diversity and the number of secondary metabolites accumulate in plants are invariably dependent on the geography, seasonal variations, climate, sun exposure and growing conditions.
The study also indicated that leaf materials processed with different techniques may alter phytochemical content. Processing methods such as drying at high temperatures and freezing could alter the nature of phytochemicals. Studies have shown that drying techniques at increased temperatures have significantly influenced the phytoconstituents, flavor profile and Maillard reaction compared to other thermal (infrared, hot-air and pulsed-vacuum drying) and non-thermal (freeze-drying) techniques. There is a possibility of enzymatic degradation and changes in pigments altering physicochemical properties of phytochemicals by thermal processing. Whereas, freeze-drying method largely preserved phytochemical content. It was also observed that processing techniques may interfere with the bioaccessibility and chemical interaction of dietary phytochemicals.

In Chapter three, I investigated the bioavailability of kawakawa tea in human healthy volunteers after acute ingestion of different doses of kawakawa tea in two different human studies. I identified 26 urinary compounds provisionally associated with kawakawa tea consumption and explored the possible routes of metabolism of kawakawa compounds. This is the first human study investigating the bioavailability of kawakawa compounds. These findings provided evidence that at least some of the kawakawa compounds are absorbed into the human systemic circulation, are modified via phase I and phase II metabolism, and are excreted in the urine. These urinary metabolites may provide insights into identifying biomarkers for kawakawa consumption as well as indicate which kawakawa compounds may underlie reported therapeutic properties (Table 1.2).

Understanding the bioavailability and metabolism of dietary phytochemicals is essential to identify their role in promoting health and in disease prevention. I proposed the metabolic pathway of elemicin following acute ingestion of kawakawa tea based on the identification of elemicin metabolites which underwent carboxylation, reduction and/or hydroxylation followed by glucuronide conjugation (Figure 3.3). In this study, trimethoxy cinnamic acid was observed as the major elemicin metabolite followed by hydroxylation and glucuronide conjugation. Previous studies in rats indicated that major urinary metabolite of elemicin was 3-(3,4,5-trimethoxyphenyl)propionic acid and its glycine conjugate produced through cinnamoyl
pathway, whereas, 3-(3,4,5-trimethoxyphenyl)propane-1,2-diol mediated by epoxide-diol pathway. Another study detected O-methylated derivative of elemicin in rats and dehydroxylated and/or O-demethylated elemicin metabolites in human urine.

Similarly, I proposed metabolic pathway of myristicin following acute ingestion of kawakawa tea based on the identification of myristicin metabolites which were dehydrogenated followed by glucuronidation and demethylenated followed by glucuronidation and sulphation (Figure 3.4). In this study, allyl dihydroxyanisole (5-allyl-1-methoxy-2,3-dihydroxybenzene) and its glucuronide and sulphate conjugate were observed (Figure 3.4). This agrees with previous studies which reported major myristicin metabolites in rats and human liver, 5-allyl-1-methoxy-2,3-dihydroxybenzene and 1-hydroxymyristicin. Additionally, studies have shown that myristicin was metabolised by hydroxylation in rats, whereas, dihydroxylation and demethylenation reactions were observed in human urine samples.

Toxicological evaluation studies indicated no myristicin toxicity in rats following acute ingestion of 10 mg/kg b.w. of nutmeg. Moreover, studies indicated that phenylpropanoids in nutmeg were not metabolized to amphetamine derivatives as they could not detect amphetamine derivatives in humans and rat urine samples. Physiologically based kinetic (PBK) modelling of elemicin and myristicin metabolism indicated that formation of toxic 1-sulfoxymetabolites were limited in human liver and rats and possibilities of risk associated with these metabolites. However, in this study, sulfoxymetabolites of elemicin and myristicin were not observed. As previously discussed in Chapter 2, the concentrations of these two compounds were considerably below estimated toxic levels of myristicin (2,170-2,520 µmol for a 70 kg adult) and elemicin (> 27,000 µmol for a 70 kg adult) for humans.

This is the first study to report the presence of dopamine in kawakawa leaf extracts (Figure 2.5). It has previously been recorded in several other plant species, including potato and banana. Dopamine sulphate was identified in urine after consuming kawakawa tea (Figure 3.5C, 3.24). Studies have reported the interaction of dopamine sulphate with the central nervous system acting as a vasopressor in the peripheral and central nervous system. Dopamine has also been
reported to influence glucose plasma regulation in rats\textsuperscript{331}. Dopamine content in kawakawa suggests it may contribute to its traditional therapeutic use, but further research is required.

Previous studies have shown that piperine and its analogues influence the sympathetic nervous system, increase energy expenditure (EE) and fat oxidation, and alter intestinal fat and glucose absorption\textsuperscript{73}. Likewise, \textit{Trans}-pellitorin, an analogue of capsaicin and a TRPV1 agonist may inhibit adipogenesis, induce satiety, activate brown adipose tissue, and modulate intestinal hormones and the microbiome\textsuperscript{73}. In chapter four, I investigated the impact of kawakawa leaf suspension on whole-body energy expenditure at resting state and post-breakfast meal consumption compared to water consumption. While there was only a borderline effect observed within our study of limited sample size, these findings suggest kawakawa compounds may have effects on whole-body energy metabolism, satiety and appetite.

Other dietary compounds or plant extracts such as catechins from green tea, caffeine, turmeric and black pepper have been shown to influence thermogenesis in humans and rodents. Some of these compounds were shown to have thermic response similar to kawakawa leaf suspension consumption. Black pepper containing beverage were observed to influence appetite without modulating postprandial glycaemia, gut and thyroid hormones\textsuperscript{332}. An acute ingestion of mustard, horseradish, black pepper and ginger did not have significant effects on diet-induced thermogenesis and appetite in younger adults under normal BMI range (18-25 kg/ m\textsuperscript{2})\textsuperscript{333}. The same study indicated that mustard bioactives may have thermogenic potential\textsuperscript{333}. In rats, caffeine ingestion enhanced caloric expenditure of physical activity in rats\textsuperscript{334}. In humans, caffeine consumption may influence EE promoting thermogenesis and potentially used in obesity treatment\textsuperscript{335}. In humans, acute ingestion of green tea rich in catechins and caffeine had thermogenic effects and promote fat oxidation\textsuperscript{336}. In high-fat-diet-fed-mice, curcumin was observed to improve EE mediated by FNDC5/irisin activation\textsuperscript{337}.

There are several factors which might influence EE in humans including age, weight, hormonal condition, and duration of exposure to dietary chemicals. For instance, consumption of black pepper did not have any effect on 24 h EE in overweight postmenopausal women\textsuperscript{338}. Longer
term green tea supplementation (for 12 weeks) in 60 Caucasian men and women (normal BMI 18-25 kg/m²; age 18-50 y) did not have any effect on fat absorption, resting energy expenditure, respiratory quotient and body composition. There was age-related thermic response observed as older women had blunted thermic response to caffeine compared to young women. Additionally, studies indicated the influence of brown adipose tissue activation (BAT) in altering EE. For instance, consuming capsinoids after 2h cold exposure (BAT-activation) increased EE in humans.

This study also provided information regarding possible effects of the mode of delivery of kawakawa. An acute metabolic effect of ingestion of kawakawa tea reported modulations in insulin sensitivity after 4g/250 mL kawakawa tea consumption but there were no significant changes observed in insulin levels when kawakawa leaf suspension was consumed (Chapter 4).

The bioavailability and functional activity of phytochemicals does not only depend on the quantity consumed, but also on the physical and chemical food matrix. Food matrix design plays an important role in improving the bioaccessibility of phytochemicals and interaction with other dietary phytochemicals. For instance, using excipient food emulsion, bioaccessibility of curcumin was shown to be improved in a simulated gastrointestinal tract model.

It is very important to evaluate the balance between the therapeutic potential and toxic side effects of phytochemicals. Elucidating the cellular pathways of dietary phytochemicals provide insights of how these compounds might affect physiological processes such as oxidative stress, inflammation and lipid metabolism. In chapter five, I demonstrated the cytotoxicity of pellitorin and the changes in expression level of selected genes in HepG2 cells after pellitorin treatment. Through comparison with sulforaphane, a known potent inducer of Nrf2-mediated transcription, it would appear that pellitorin may act by modulating redox status involving Nrf2-mediated transcription in a similar manner to sulforaphane. However, no effect of myristicin observed on the expression levels of selected genes.
The structure-activity relationship of pellitorin remains unclear. Structurally related alkamides with medium to long chain alkyl chains (16-20 carbon atoms) were shown to influence PPARγ activation. Understanding the size and shape, functional groups and stereochemistry of phytochemicals is essential as it influences the ability of phytochemicals to modulate receptor binding ability, chemical reactivity and its interaction with cellular pathways. The presence of functional groups (-OH), (-C=O) and (-C-O) may exert antioxidant and anti-inflammatory properties. For example, anti-inflammatory potentials of fagaramide could be activated by the hydrogen bonds and carbonyl O atoms. Inhibiting ability of glycosidase might be affected by the changes in the number and stereochemistry of hydroxy groups of indolizidine alkaloids.

Due to its complex leaf chemistry, the elucidation of the mechanistic basis of functional properties of kawakawa is challenging. Identification of human metabolites after kawakawa consumption indicated certain compounds were bioavailable which may assist further exploration of its functional properties. Consuming kawakawa leaf suspension was likely to have thermogenic potential and further studies with larger number of participants would help clarify its thermic responses. Additionally, functional activity of pellitorin to mediate antioxidant response via Nrf2-mediated transcription suggests further approaches to explore the traditional uses of kawakawa in treating digestive, respiratory and skin conditions.

This study provided evidence for the complex and diverse kawakawa leaf chemistry, kawakawa tea is palatable and kawakawa metabolites are bioavailable. This would provide insight into biomarkers of kawakawa consumption, explore its underlying molecular mechanism and its benefits for human health. Overall, the experimental results suggests that kawakawa phytochemicals are likely to have a spectrum of biological activity that might underpin its traditional uses and indicated further research areas to be explored that may aid its use within functional foods.
6.2 Limitations

The major limitation in the phytochemical profiling was the lack of pure chemicals for the level 1 identification of phytochemicals. This also limited the absolute quantification of the kawakawa compounds. Moreover, there is limited mass spectral information available in the existing database library for level 2 identification of these compounds. It is often complex, time-consuming and expensive to chemically synthesize these compounds. NMR spectroscopy could have been of value for structural elucidation, but it would have required the purification of a significant amount of the target compound that was beyond the scope of this research project.

This is the first human study of kawakawa consumption. Analyses were only undertaken in plasma and urine samples. The gut microbiota may likely have been of importance in the biotransformation of certain kawakawa metabolites in the lower gut prior to absorption. This may be of particular importance for vitexin and yangambin – these types of polyphenolic compounds are known to be transformed by the gut microbiota into smaller phenolic acids which are difficult to specifically identify in plasma. It would have also been useful to have analysed fecal samples for unmetabolized and unabsorbed kawakawa compounds. Plasma analyses are more challenging than the analyses of urine as the concentration of metabolites is lower and there may also be interaction with blood proteins that may reduce extraction. Similar to phytochemical analyses in chapter 2, the unavailability of pure chemical standards limits the precise identification of human metabolites. There were also technical difficulties in the analyses of polar metabolites and those metabolites which would be observed in negative ionization mode in LC-MS/MS analysis. Additionally, integration of GC-MS analysis would have enabled further identification of kawakawa compounds.

This is the first study to explore the whole-body energy metabolism of kawakawa compounds. It is useful to elucidate the beneficial influence of kawakawa compounds on resting and post-prandial whole-body energy metabolism, but the studies were limited due to sample size and the variation observed in the responses between individuals and the different interventions. The
indication of an effect of kawakawa on energy expenditure would suggest that further studies are warranted, which can be powered appropriately.

Cell based assays can provide important insights into the potential health benefits of these dietary compounds. However, it is important to note that these assays were conducted at *in vitro* conditions and may not reflect the complex interactions *in vivo*. Furthermore, estimating physiological concentrations of PEL which might undergo first pass metabolism or circulate in the human body is challenging. Further studies are needed to confirms its effects of these dietary phytochemicals on human health. My studies on the biological activity of individual compounds were restricted due to time availability. I was only able to explore the effects of pellitorine on gene expression in HepG2, and, to a lesser extent myristicin. Ideally, I would have sought to have used compounds from each of the different chemical groups and a range of cell lines.

### 6.3 Future directions

Chemical synthesis of kawakawa compounds with potential therapeutic potentials would help the absolute quantification of those compounds. Kawakawa leaves are generally seen with holes due to *Cleora scriptaria* herbivory. The role of this moth in altering the phytochemical composition is yet to be explored. Investigating the diversity in the chemical profile of kawakawa sources from different geographical locations and environments across New Zealand, including subspecies on off-shore islands would enhance the understanding of the influence of geographical and seasonal variations on phytochemical contents. Phytochemical analysis of herbarium material sourced from diverse locations may provide a comprehensive understanding of the phytochemical variation.

Longer-term intervention of kawakawa and in larger human studies including the analysis of fecal samples would provide broader insight into elucidating the metabolic pathways of kawakawa compounds. Confirming the identity of provisionally identified kawakawa metabolites with the help of chemically synthesized respective compounds would further support the exploration of its functional pathways. Implementation of functional metabolomics to understand the interactions between metabolites and other biological molecules such as
proteins and lipids would assist the understanding of underlying mechanisms of biological processes and provide insight into the role of metabolites and their contribution to cellular and organismal physiology \(^{350,351}\). Functional metabolomics integrated with other -omics applications such as genomics, transcriptomics and proteomics would provide a comprehensive analysis of disease management and develop new therapeutic strategies \(^{352,353}\). Next-generation sequencing (NGS) of cell lines to explore the effect of exposure to kawakawa compounds on gene expression \(^{354}\), implementation of bioinformatics to identify differentially expressed genes \(^{355}\) and metabolomics to understand the metabolism of these compounds would provide a comprehensive view of the molecular changes and to identify potential pathways and mechanisms involved in these effects \(^{206}\).
Chapter 7 Conclusion

This thesis examined the diversity and complexity of kawakawa phytochemistry, the bioavailability and metabolism of individual phytochemicals, the metabolic effects of consuming a kawakawa leaf suspension, and the biological activity of a small number of individual leaf compounds. Further research is required in each of these areas to gain a more comprehensive insight into the basis of the reported therapeutic activity of kawakawa as used in traditional practices in Aotearoa New Zealand.

I conclude by reiterating my gratitude to my Māori colleagues for allowing to undertake studies on this most important taonga species.
Appendix A  TIC Chromatogram of aqueous extracts of kawakawa numbered based on the features identified/annotated in Table 2.2
Appendix B Analysis of Boka-T urine data in negative ionization mode
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