

Kawakawa tea Intake and its anti-diabetic and anti-inflammatory effects: An integrative microRNA and mRNA analysis

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Abstract

Purpose

Piper excelsum (kawakawa), a vascular plant endemic to Aotearoa, New Zealand, is of cultural and medicinal importance to Māori. Presence of biologically active compounds in kawakawa may underpin its putative beneficial health properties, particularly its anti-inflammatory effects. However, no human studies on its anti-inflammatory effects exist.

Method

Blood samples from a crossover two-arm randomised controlled trial to assess the impact of ingesting kawakawa tea (4g leaves/250 mL hot-water) on postprandial plasma abundances of microRNAs (miRNA) involved in metabolic-related pathways and their respective gene and protein targets in healthy human volunteers (n = 26; Age; 33.6 ± 1.9 (yr) and BMI; 22.5 ± 0.4 (kg/m²)) compared to water control, were used for the analysis.

Results

Nine miRNAs showed significantly different abundances following the two interventions. The abundance of hsa-miR-17-5p, -21-5p, -320a-5p, let-7g-5p, -16-5p, -122-5p, and -144-3p was upregulated in response to kawakawa intake compared to water and the expression of hsa-miR-221-3p and -223-3p was downregulated in kawakawa intake compared to water over a 120 min postprandial period. *In-silico* analysis indicated miRNA enrichment in metabolic-related pathways, such as apoptosis, cytokine signaling, MAPK signaling, and MTOR pathways. Furthermore, the postprandial expression of *IL-8* (p = 0.03), *IL-6* (p = 0.01) and *PPAR-γ* was significantly reduced following kawakawa consumption compared to water control. While as IL-6 cytokine was significantly increased at 120 min in the kawakawa intervention only compared to baseline.

Conclusion

These findings highlight the interconnected nature of metabolic regulation pathways and highlight the need for further intervention studies to explore the chronic anti-inflammatory effects of kawakawa.

Clinical Trial Registration: The clinical trial is registered with Australian New Zealand Clinical Trials Registry (*ACTRN12621000311853*).

1. Introduction

Kawakawa, also known as *Piper excelsum* G. Forst (or synonymously as *Macropiper excelsum* (G. Forst.) Miq.), is a native plant of New Zealand (NZ) that holds both cultural and medicinal (Rongoā Māori) importance for Māori, the indigenous people of Aotearoa NZ[1, 2]. The leaves of the kawakawa plant are known to contain a diverse range of bioactive compounds, including but not limited to lignans ((+)-

excelsin and (+) diyangambin); amides (piperine, dihydropiperlonguminine, pellitorine, and fagaramide); phenylpropanoids (myristicin and elemicin); flavone glycoside (vitexin); and terpenoids (α -pinene and camphene)[3]. *In-vitro* and *in-vivo* studies exploring the bioactive properties of these chemicals from a range of plant sources have reported numerous potential health effects, including anti-inflammatory and anti-diabetic activities[2]; their presence also in kawakawa could contribute to its therapeutic uses in rongoā Māori and underpin the development of novel food and health products.

Despite the widespread use of kawakawa, there is limited research on its effects on humans. Previously, the first human dietary intervention study involving kawakawa in our group showed the potential implications of drinking kawakawa tea for regulating pathways related to insulin sensitivity and glycemic control[4]. While there is existing evidence of the bioactive effects of kawakawa in animal and cell models[5–7] and its impact on insulin sensitivity in the human dietary intervention study⁴, the specific mechanisms underlying these effects in humans remain uncertain.

microRNAs (miRNAs) are evolutionarily conserved small noncoding-RNAs with widespread biological functions[8], mainly acting as negative regulators of post-transcriptional gene expression[9]. Circulatory miRNAs are known to play a critical role in cell-to-cell communication[10] and have been increasingly implicated as potential biomarkers of disease states, prognosis and progression for conditions including type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD)[11, 12] with evidence demonstrating that miRNAs regulate the expression of a wide range of genes and proteins involved in various metabolic pathways[13, 14] including the insulin signalling and inflammatory pathways.

Based on the functional role of miRNAs, we hypothesise that intake of kawakawa tea would result in changes in the abundance of miRNAs related to insulin signalling and inflammatory pathways. Furthermore, these altered miRNA levels would correspond with the dysregulated expression of genes and proteins with known metabolic regulatory functions. Therefore, the current study aimed to quantify the abundance of plasma miRNAs in response to an acute intake of kawakawa tea. Based on *in-silico* functional target analysis of the differentially regulated miRNAs, mRNA targets of these miRNAs were isolated and analysed from the peripheral blood mononuclear cells (PBMCs). The analysis of PBMC genes serves as a non-invasive surrogate for predicting molecular mechanisms in inaccessible tissues [15]. PBMC gene expression also demonstrates significant agreement (80%) with gene expression of other tissue types[16].

2. Methods

Study design and population

The plasma samples utilised in this study were archived from the previously conducted dietary intervention study (TOAST study), with ethical approval inclusive of these secondary analyses from the Health and Disabilities Ethics Committee (HDEC) Auckland, New Zealand (20/STH/236)[4]. The study was conducted according to the Declaration of Helsinki guidelines. All participants provided written

informed consent. The TOAST study was prospectively registered with the Australian New Zealand Clinical Trials Registry at www.anzctr.org.au: ACTRN12621000311853.

A total of 30 participants were recruited, however, one withdrew after the initial intervention, and three were excluded due to elevated fasting glucose levels. Consequently, the ultimate sample size was refined to 26, comprising 11 males and 15 females.

Interventions and sample collection

Participants were provided with a standardised evening meal and were asked to fast overnight (10-12hr) and arrive at the Clinical Research Unit, Liggins Institute, between 7:00 AM and 8:00 AM on each of the two intervention days. At visit 1, they were assigned to one of two interventions (as shown in Fig. 1) in a randomised sequence with a 48-hour gap between each intervention.

The interventions consisted of consuming, after a 10-minute fasting baseline measurement, either: i) 250ml of kawakawa tea (made by infusing 4g of dried kawakawa leaves from ŌKU Ltd, New Zealand, in 250ml of 70°C-80°C hot water for 10 minutes); or ii) drinking only 250ml of hot water (70°C-80°C).

After consuming the tea or hot water, participants were given a 10 min window (at the 30 min mark) to consume a high-carbohydrate breakfast comprising two slices of white bread (935kJ), 10g of strawberry jam (80kJ), and 250mL of rice milk (578kJ). Fasting (0 min) and postprandial (60 and 120 min) venous blood samples were collected at each visit, as described below.

Sample collection

Fasting (time = 0 min) and postprandial (time = 60 and 120 min) venous blood samples were collected in EDTA-coated vacutainer tubes from each participant at each visit and immediately stored on ice before processing. Plasma was separated and aliquoted within 120 min of collection by centrifugation at $1,900 \times g$ for 15 min at 4°C and was then immediately stored at - 80°C until further analysis. PBMCs were extracted from whole blood samples following the Ficoll-paque method of extraction[17].

Biochemical analysis

Fasting and postprandial glucose (nmol/L) analysis was performed on the Hitachi 902 autoanalyser (Hitachi High Technologies Corporation, Japan) using an enzymatic colorimetric assay[18][18] (Roche Diagnostics Ltd., Mannheim, Germany). Plasma Fasting plasma and postprandial insulin (mU/L) and triglycerides (mmol/L) were measured using a Cobas Modular P800 autoanalyser using the microparticle enzyme immunoassay (Roche Diagnostics, Auckland, New Zealand). The area under the curve (AUC) of blood glucose and insulin concentration was calculated using the trapezoid method. The Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), an index used to determine if a patient has insulin resistance[19], was subsequently calculated according to the following equation:

$$\text{HOMA-IR} = \text{fasting insulin (mU/L)} \times \text{fasting glucose (nmol/L)} / 22.5$$

Plasma and PBMC RNA isolation

Briefly, 250 µL plasma was used for total RNA extraction (including miRNAs) using a previously described protocol by Ramzan *et al.* [17]. A fixed plasma volume was utilised to minimise extraction variation between different samples and time points [20].

Total RNA was isolated from approximately 2.5×10^6 PBMCs collected at each time point using the AllPrep® DNA/RNA/miRNA Universal Kit (QIAGEN, Germany), following the manufacturer's protocol [21].

cDNA Synthesis and circulating miRNA quantitative PCR (qPCR)

2 µL of total RNA was used as an input for the cDNA synthesis reaction using TaqMan™ Advanced miRNA cDNA Synthesis Kit (Catalogue number: A28007, Applied Biosystems, USA), according to the manufacturer's recommendations. For quantification of circulatory miRNA abundances using qPCR analysis, custom human miRNA assays of hsa-miR-15a-5p, hsa-miR-16-5p, hsa-miR-17-5p, hsa-miR-21-3p, hsa-miR-21-5p, hsa-miR-122-5p, hsa-miR-144-5p, hsa-miR-320a, let-7g-5p, hsa-miR-221-3p, hsa-miR-222-3p and hsa-miR-223-3p were used (TaqMan MicroRNA Assays, Applied Biosystems, USA). Quantification was performed on a Quant Studio™ 12 Flex Real-Time PCR System (Thermo Fisher Scientific, USA), and the detected cycle threshold (Ct) of ≤ 35 was set for the miRNA analysis.

For the normalisation of abundant data, a geometric mean of three endogenous miRNAs (hsa-miR-423-5p[22, 23], hsa-miR-451a-5p[24], and hsa-miR-191-5p[22, 23]) was used[25]. Haemolysis of all samples was monitored by comparing miR-451a abundance (a highly expressed miRNA in red blood cells) with miR-23a-3p abundance (a miRNA unaffected by haemolysis)[26]. The resulting ΔCt (miR-23a-3p - miR-451a) was used to measure the degree of haemolysis; samples with an ΔCt of > 7 were excluded from further analysis. The abundance of miRNAs was measured using the $2^{-\Delta Ct}$ method [27].

In-silico target analysis

A miRNA-target gene network was built using miRNet (<https://www.mirnet.ca/>) by including the differentially abundant miRNAs[28]. All sets of genes targeted by the miRNAs were identified and were subsequently used for the prediction of pathways targeted by these miRNAs. Functional annotation of the dysregulated miRNA and the identification of miRNA-target gene-controlled pathways were determined via the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway based on the hypergeometric tests with p values ≤ 0.05 adjusted for false discovery rate (FDR).

qPCR gene expression analysis

Input RNA of 350 µg was used for cDNA synthesis using the SuperScript IV VILO Master Mix (Thermo Fisher USA, Cat no. 11756050). Quantification of gene expression (mRNA) was performed by qPCR on a LightCycler 480 II (Roche Applied Science, Germany) using TaqMan Universal Master Mix II (Thermo Fisher, USA, Cat no. 4440043). Genes quantified (Table 2.1) included peroxisome proliferator-activated receptor gamma (*PPAR-γ*), Fas Cell Surface Death Receptor (*FAS*), C-reactive protein (*CRP*), cluster of

differentiation 36 (*CD36*) and AMP-activated kinase (*AMPK*) and pro-inflammatory cytokines (tumour necrosis factor-alpha (*TNF-α*), interleukin-2 (*IL-2*), interleukin-6 (*IL-6*) and interleukin-8 (*IL-8*)). Primers for qPCR were designed using BLAST software[29]. For normalisation of the PCR data, the geometric mean[30] of three human reference genes[31, 32], valosin-containing protein (*VCP*), charged multivesicular body protein 2A (*CHMP2A*), and chromosome 1 open reading frame 43 (*C1orf43*), was used. The relative expression of mRNA was measured using the $2^{(\Delta Ct)}$ method [27].

Table 2.1
Forward and reverse primer nucleotide sequences (5'-3') for target genes, primer length, GC% and melting temperatures of each target primer.

Target Primer	Primer Sequence (5'-3')	Length	GC%	T _m
<i>IL-2</i>	Forward: CTCAAACCTCTGGAGGAAGTGC	22	54.5	60.8
	Reverse : CAATGGTTGCTGTCTCATCAG	22	50.0	60.1
<i>IL-6</i>	Forward : GCCCACCGGGAACGAAAGAGA	21	61.9	64.6
	Reverse : ACCGAAGGCGCTTGTGGAGA	20	60.0	64.2
<i>IL-8</i>	Forward: AGAGCTCTGTCTGGACCCCA	20	60.0	62.1
	Reverse: TTCTCAGCCCTCTTCAAAAACCTTC	24	41.6	59.4
<i>FAS</i>	Forward: GTTGGTGGACCCGCTCAGTA	20	60.0	61.8
	Reverse: TAGGAGGGTCCAGATGCCCA	20	60.0	61.9
<i>TNF-α</i>	Forward: AACCTCAGACGCCACATCC	20	60.0	62.1
	Reverse : TGGAGCCGTGGGTCTAGTATG	20	60.0	61.6
<i>PPAR-γ</i>	Forward : TGGTGACCAGAAGCCTGCAT	20	55.0	61.7
	Reverse : CCCAAAGTTGGTGGGCCAGA	20	60.0	62.6
<i>AMPK</i>	Forward: ATCATCACCTGACTCGGCCC	20	60.0	61.6
	Reverse : TATGGCGTGCCCTTGGTGTT	20	55.0	62.4

GC%, guanine-cytosine percentage ; *T_m*, melting temperature; *IL-2*, interleukin-2; *IL-6*, interleukin-6; *IL-8*, interleukin-8; *FAS*, FAS cell surface death receptor; *TNF-α*, tumor necrosis factor-alpha; *PPAR-γ*, peroxisome proliferator activated receptor-gamma; *AMPK*, AMP-activated kinase

Protein quantification- Enzyme-linked immunoassay (ELISA)

An immunoassay procedure was conducted to analyse the protein concentrations of plasma inflammatory cytokines, including IL-1β, IL-2, IL-6, IL-8 and TNF-α, using the MILLIPLEX Human Cytokine/ Chemokine/ Growth Factor Panel A Magnetic Bead Panel kit (Cat no. HCYTA-60K-PX48).

Statistical analysis

The expression data were evaluated for normality using the Shapiro-Wilk test. The differences in the abundance of circulatory miRNA, PBMC genes, AUC_{glucose} , AUC_{insulin} , and AUC_{TG} in relation to the acute dose of the meal were measured using a mixed effects model with time as a repeated factor and group as a between-subject factor, followed by Holm-Sidak multiple comparison corrections. Samples with an expression of more than 3 times the interquartile range were treated as outliers and were subsequently removed from further analysis[33]. Data are shown as means $\pm$ SEM unless otherwise stated. Analyses were conducted using SPSS version 25.0 (SPSS Inc., USA), and graphs were constructed using GraphPad prism-9 (GraphPad Software, USA). Statistical significance was set at $p \leq 0.05$.

3. Results

Study population characteristics

The baseline characteristics of the study participants are summarised in Table 3.1. The study population included self-reported healthy male (n = 11) and female (n = 15) participants aged 18–45 year and within the BMI range of 18–25 kg/m². The male and female participants differed in body weight ($p = 0.003$) at baseline (fasted state) but not in BMI

Table 3.1
Baseline clinical and demographic characteristics of the study participants.

Variables	Male	Female	p-value	All
Number of participants	11	15		26
Age (y)	34.2 $\pm$ 2.0	32.3 $\pm$ 1.4	0.55	33.6 $\pm$ 1.9
Body weight (kg)	66.5 $\pm$ 2.0	58.8 $\pm$ 1.8	>0.01	67.9 $\pm$ 2.0
BMI (kg/m ²)	22.1 $\pm$ 0.4	22.3 $\pm$ 0.4	0.63	22.5 $\pm$ 0.4
Glucose (mmol/L)	5.1 $\pm$ 0.07	4.9 $\pm$ 0.1	0.16	6.1 $\pm$ 0.1
Triglycerides (mmol/L)	0.9 $\pm$ 0.1	0.9 $\pm$ 0.1	0.70	0.9 $\pm$ 0.04
Insulin (mU/L)	47.9 $\pm$ 6.2	55.6 $\pm$ 6.0	0.47	52.5 $\pm$ 4.3
HOMA-IR	16.1 $\pm$ 2.4	17.8 $\pm$ 2.4	0.38	17.3 $\pm$ 1.7

Data presented as mean $\pm$ SEM. Significance level $p \leq 0.05$ (highlighted in bold). The stated p-value is from comparing males and females (average baseline of both visits) using a one-way ANOVA test. BMI - body mass index; BP- blood pressure; HOMA-IR -homeostasis model assessment-estimated insulin resistance; ANOVA - analysis of variance.

Baseline abundance of targeted circulatory miRNAs

The circulatory abundance of targeted miRNAs and the endogenous controls at baseline are summarised in Table 3.2. The baseline abundance did not differ between the two study interventions (kawakawa tea and water).

Table 3.2
Baseline abundance of targeted circulatory miRNAs and endogenous controls between study interventions.

microRNA	Kawakawa	Water	p-value
hsa-miR-15a-5p	0.62 ± 0.08	0.45 ± 0.05	0.07
hsa-miR-16-5p	2.27 ± 0.50	2.24 ± 0.46	0.95
hsa-miR-17-5p	1.72 ± 0.41	1.00 ± 0.19	0.11
hsa-miR-21-3p	0.02 ± 0.01	0.01 ± 0.00	0.24
hsa-miR-21-5p	0.01 ± 0.00	0.04 ± 0.00	0.10
hsa-miR-122-5p	0.04 ± 0.01	0.07 ± 0.01	0.13
hsa-miR-144-3p	1.11 ± 0.28	1.12 ± 0.17	0.96
hsa-miR-221-3p	0.68 ± 0.18	0.36 ± 0.10	0.13
hsa-miR-223-3p	2.02 ± 0.56	1.68 ± 0.46	0.64
hsa-miR-222-3p	0.08 ± 0.03	0.06 ± 0.01	0.39
hsa-miR-320a	0.88 ± 0.18	0.84 ± 0.08	0.25
let-7g-5p	0.13 ± 0.03	0.12 ± 0.01	0.22
Endogenous control			
hsa-miR-451a-5p	15.9 ± 14.9	13.1 ± 15.2	0.11
hsa-miR-191-5p	0.5 ± 0.08	0.5 ± 0.1	0.18
hsa-miR-423-5p	0.5 ± 0.06	0.4 ± 0.05	0.14

Data presented as mean ± SEM. Significance level ≤ 0.05. The stated p-value is from comparing study interventions at baseline using a one-way ANOVA test. K- kawakawa; W-water; ANOVA-analysis of variance

Postprandial abundance of circulatory miRNAs

The postprandial abundance of circulatory miRNAs following kawakawa tea differed from the water control. Of the 12 analysed, 9 miRNAs showed significantly different abundances following the two interventions (Fig. 3.1). A timepoint (0, 60 and 120min) effect was observed in hsa-miR-17-5p (p = 0.03), hsa-miR-21-5p (p = 0.01), hsa-miR-320a-5p (p = 0.03) and let-7g-5p (p = 0.001). An intervention(kawakawa

and water) effect was observed in hsa-miR-16-5p ($p = 0.03$), hsa-miR-122-5p ($p = 0.01$), hsa-miR-144-3p ($p = 0.01$), hsa-miR-221-3p ($p < 0.01$), and hsa-miR-223-3p ($p = 0.01$). The expression of hsa-miR-17-5p, hsa-miR-21-5p, hsa-miR-320a-5p, let-7g-5p, hsa-miR-16-5p, hsa-miR-122-5p, and hsa-miR-144-3p was upregulated in response to kawakawa intake compared to water, and the expression of hsa-miR-221-3p and hsa-miR-223-3p was downregulated in kawakawa intake compared to water. The circulatory abundance of hsa-miR-15a-5p, hsa-miR-222-3p, and hsa-miR-21-3p remained unaltered in response to either of the interventions and were excluded from further analysis.

Correlation analysis

A Pearson's correlation analysis was performed to ascertain the relationships between the differentially abundant miRNAs and biochemical biomarkers of metabolic health (Fig. 3.2). There was a weak, negative correlation between hsa-miR-17-5p and each of HOMA-IR and plasma insulin ($r = -0.01$, $p = 0.02$ and $r = -0.18$, $p = 0.03$, respectively). A weak, positive correlation was observed between hsa-miR-21-5p and plasma insulin ($r = 0.10$, $p = 0.04$), and hsa-miR-144-3p showed a weak, negative correlation with plasma triglycerides ($r = -0.18$, $p = 0.04$). However, no statistically significant correlation was observed between glucose and abundance of the other altered miRNAs.

Prediction of downstream mRNAs

Target gene prediction analysis demonstrated 4277 genes (both strong and weak interactions) as being putatively regulated by the differentially abundant miRNAs; hsa-miR-16-5p, hsa-miR-17-5p, hsa-miR-21-5p, hsa-miR-122-5p, hsa-miR-144-3p, hsa-miR-221-3p, hsa-miR-223-3p, hsa-miR-320a-5p, and let-7g-5p (Fig. 3.3).

The magnitude of the genes targeted by a single miRNA is proportional to the node's size. For example, hsa-miR-16-5p and hsa-miR-17-5p target and share the highest number of genes within the network; hsa-miR-16-5p alone targets approximately 1557 genes and hsa-miR-17-5p targets 1181 genes, as shown in **Table 3. 3**.

Table 3.3
List of microRNAs and the total number of genes targeted individually.

microRNA	Total number of target genes
hsa-mir-16-5p	1557*
hsa-mir-17-5p	1181*
hsa-mir-21-5p	612
hsa-mir-221-3p	368
hsa-mir-223-3p	98
let-7g-5p	341
hsa-mir-122-5p	610
hsa-mir-144-3p	211
hsa-mir-320a	584

The hypergeometric algorithm-based functional enrichment analysis, with a false discovery rate (FDR) of $p < 0.05$, identified that the target genes of the nine differentially expressed miRNAs are associated with several inflammatory and related pathways, including apoptosis, cytokine signalling, MAPK signalling, and MTOR pathways (Fig. 3.4). Modifications in these pathways have been previously described as associated with regulating metabolic homeostasis [34–36]. Additionally, pathways related to insulin signalling, T2DM, p53, and cancer signalling were highlighted, indicating these miRNAs' potential role in these chronic diseases.

PBMC gene expression

There were no significant interactions between kawakawa tea and water intake for IL-2, AMPK, TNF- α and CD36 genes (Table 3.4). However, significant intervention and time-point interactions were observed for *IL-6* ($p = 0.04$), *PPAR- γ* ($p = 0.02$) and *FAS* ($p = 0.05$). A time-point change for *PPAR- γ* ($p = 0.03$) and *IL-8* ($p = 0.01$) was also observed.

Table 3.4
P-values obtained from a 2-way repeated measures ANOVA test of IL-2, IL-6, IL-8, TNF- α , PPAR- γ , AMPK and FAS gene expression.

2-way ANOVA P-value			
	Intervention	Time-point	Interaction
IL-2	0.07	0.12	0.52
IL-6	0.13	0.15	0.04
IL-8	0.24	0.01	0.41
CD36	0.22	0.11	0.77
TNF- α	0.53	0.42	0.42
PPAR- γ	0.35	0.03	0.02
AMPK	0.25	0.16	0.55
FAS	0.07	0.05	0.05

Intervention (kawakawa tea or water); time-point (0, 60, 120 min); IL-2, interleukin-2; IL-6, interleukin-6; IL-8, interleukin-8; CD36, cluster of differentiation 36; TNF- α , tumour necrosis factor-alpha; PPAR- γ , peroxisome proliferator-activated receptor-gamma; AMPK, AMP-activated protein kinase; FAS, fas cell surface death receptor. Values of significance ($p < 0.05$) are presented in bold.

A significant difference was observed in the kawakawa intervention over the postprandial period following the multiple comparison tests. *IL-8* and *IL-6* demonstrated statistically significant changes in their relative gene expression between baseline and 120 min following kawakawa tea consumption. *PPAR- γ* expression was significantly decreased at 60 min post kawakawa tea consumption. *CRP* was too lowly expressed to be identified in the current sample set (Fig. 3.5).

Plasma Cytokines

Plasma levels of inflammatory cytokines before and after each intervention over the period of 120 minutes are shown in Fig. 3.6. TNF- α and IL-8 concentrations were not different between the kawakawa intervention and the water control. However, IL-6 concentration was significantly increased at 120 min in the kawakawa intervention compared to baseline. The abundance of both IL-1 β and IL-2 were below the detectable threshold levels.

4. Discussion

The present study aimed to investigate the effects of kawakawa tea (4g dried leaves in 250 mL hot water) consumption, compared to control (hot water), on the postprandial plasma abundances of

miRNAs involved in metabolic-related pathways and their respective gene and protein targets. As hypothesised, the current study observed a differential abundance of nine miRNAs (hsa-miR-16-5p, hsa-miR-17-5p, hsa-miR-21-5p, hsa-miR-122-5p, hsa-miR-144-3p, hsa-miR-221-3p, hsa-miR-223-3p, hsa-miR-320a-5p, and let-7g- 5p), three mRNAs (*IL-6*, *IL-8* and *PPAR-γ*) and a cytokine (IL-6) following acute consumption of kawakawa tea prior to a high-glycemic meal over the postprandial period of 120 minutes. Target gene prediction analysis revealed that these miRNAs play a role in altering the transcriptional regulation of complex cardio-metabolic diseases, as evidenced by their involvement in pathways such as insulin signalling, T2DM, p53, and cancer signalling. To our knowledge, the present study is the first time a human dietary intervention with kawakawa tea has provided insights into the mechanisms underlying its anti-diabetic and anti-inflammatory effects.

The influence of diet on regulatory networks modulated by miRNAs is a growing field of scientific research. It is known that diet-modulated miRNA profiles affect the risk of developing diseases[21, 37, 38]. Previous studies have shown a link between the consumption of bioactive compounds derived from diet and circulating miRNA levels, indicating that diet manipulation can represent an effective strategy to modify the risk of chronic disease development[39–42]. In this study, the immediate consumption of kawakawa tea before a high-glycemic meal influenced the expression of nine miRNAs. These miRNAs have previously demonstrated dysregulated profiles in individuals with metabolic disorders such as prediabetes, T2DM, MetS, and obesity. Notably, hsa-miR-16-5p, found at reduced levels in Brazilian women with MetS [43], has also shown a positive correlation with insulin sensitivity in non-diabetic, weight-stable individuals. Furthermore, hsa-miR-17-5p, hsa-miR-21-5p and hsa-miR-320a are shown to have decreased abundance in metabolically dysregulated individuals compared to healthy counterparts [44]. The increased abundance of these miRNAs observed on intake of kawakawa tea shed light on the potential health benefits associated with acute kawakawa tea consumption.

While kawakawa tea intake was found to upregulate the abundance of 7 miRNAs compared to water, the study also observed a decrease in the abundance of two miRNAs (hsa-miR-221-3p and hsa-miR-223-3p). This is interesting as hsa-miR-221-3p shows increased plasma abundance in individuals with obesity, insulin resistance¹⁵⁰ and MetS¹⁵¹. On the other hand, the decreased abundance of hsa-miR-223-3p could be attributed to the decreased expression of *PPARγ*. *PPARγ* is identified as a direct regulator of this miRNA in adipose tissue[45]. Given this study's findings of reduced abundance of both *PPARγ* and hsa-miR-223-3p following kawakawa tea intake, it remains to be clarified whether kawakawa tea has a modulatory impact on inflammation and related pathways by influencing the hsa-miR-223-3p/*PPARγ* axis.

A significant reduction in the expression of two pro-inflammatory genes (*IL-6* and *IL-8*) after consuming kawakawa tea demonstrates potential anti-inflammatory effects[46]. Unexpectedly, reduced gene expression did not correspond to changes in protein levels, defying the anticipated decrease in protein expression following decreased gene expression [47]. Despite this, an increase in IL-6 cytokine levels was observed following kawakawa tea intake. This rise in IL-6 may be attributed to its pleiotropic nature, allowing it to exhibit both pro- and anti-inflammatory functions. For example, during acute immune

responses, IL-6 can decrease pro-inflammatory cytokines like TNF- α without reducing the production of anti-inflammatory cytokines such as IL-1Ra[48]. Although not measured in our study, the consumption of kawakawa might impact the generation of pro-inflammatory cytokines differently in various tissues, such as skeletal muscles, and PBMCs may not accurately represent these tissue-specific alterations [49]. For example, Starkie *et al.* [50] observed increased plasma IL-6 concentrations immediately and at 2 hours after prolonged running, suggesting skeletal muscle as a potential source of circulating IL-6 rather than monocytes. This is corroborated by Ostrowski *et al.* [51], who found *IL-6* expression in the skeletal muscle but not in PBMCs of individuals engaged in prolonged running.

Although evidence strongly implicates hsa-miR-15a-5p[17], hsa-miR-222-3p[52], and hsa-miR-21-5p[53] in insulin signalling pathways, this present study did not observe changes in their levels in response to kawakawa tea or water. Changes in miRNA expression due to dietary modifications depend on various factors, such as the duration of exposure, gender, and health status. For example, studies have shown that the impact of dietary interventions on miRNA expression profiles can vary based on how long an individual has been exposed to specific dietary changes. Additionally, the response may differ between genders, highlighting the need for gender-specific considerations in understanding miRNA regulation through diet. Moreover, the health status of individuals plays a crucial role in shaping miRNA responses to dietary modulation. Individuals with different health conditions may exhibit distinct miRNA expression patterns in response to dietary changes. For instance, the miRNA expression profile in individuals with metabolic disorders like T2DM or obesity may differ from that in healthy individuals when subjected to the same dietary interventions.

The present study also did not observe any significant changes in the expression of the *CRP* gene throughout the postprandial period. Although CRP is considered an important marker of inflammation, it is usually expressed in response to chronic inflammatory-related disorders such as diverse infections, rheumatoid arthritis and CVD [54]. Since all the participants were self-reported to be healthy,, it is likely that CRP was not expressed due to an absence of prior inflammation in these individuals. It has also been shown that expression of CRP usually occurs within the initial 4–24 hours following stimuli [55]. Owing that for this study, gene expression was measured at 60 and 120 minutes, it's conceivable that *CRP* might not have been expressed within this short duration. However, it's crucial to note that these observations remain associative with no clear mechanistic understanding of how kawakawa influences *CRP* and inflammation.

Limitations and Further Research

The consumption of kawakawa presents promising potential for anti-inflammatory properties through the modulation of gene and miRNA expressions. However, it's crucial to acknowledge several limitations in this current study. Sexual dimorphism and ethnicity significantly affect the abundance of many miRNAs in the plasma [56, 57]. However, this study was not powered enough to understand the sexual dimorphism of these changes. This initial human dietary intervention trial with kawakawa, conducted as a pilot with healthy participants, establishes fundamental insights for shaping future research questions.

To improve the generalisability and translatability of these findings, forthcoming investigations should involve individuals with diverse metabolic statuses. This study also undertook a limited and targeted qPCR-based analysis of both miRNA and mRNA, with the latter performed only in circulatory PBMCs. Although these cells have been widely used as proxy tissue to understand whole-body metabolic status, they are not always appropriate surrogates [58]. Lastly, there isn't a universally agreed-upon minimal threshold for profiling plasma miRNA abundance [59]. This lack of consensus underscores that plasma miRNA abundance may not consistently serve as an appropriate biomarker when distinguishing between biological significance and experimental noise. Thus, interpreting the biological significance of minor changes, as seen in the current study, remains challenging. Consequently, integrating high-throughput small RNA sequencing strategies²¹ and larger population cohorts in subsequent analysis would provide a more comprehensive evaluation of the biological significance of the global regulation of non-coding and coding RNA transcripts.

Conclusion

This study demonstrated that consuming kawakawa tea before a high-glycemic meal influenced the circulatory levels of specific miRNAs. The target genes of these miRNAs primarily participate in pathways related to inflammation and insulin signalling. These findings contribute to advancing our understanding of the molecular mechanisms underlying the positive health effects associated with kawakawa tea consumption.

Declarations

Consent for publication

Not Applicable

Availability of data and material

The data generated and analysed during this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare no competing interests.

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Authors' contributions

FR, MF, JMC, CP and RM designed TOAST. FR and RJ conducted and coordinated the dietary intervention study. ST and JG conducted the laboratory experiments. ST and JG conducted the statistical analysis. FR wrote the paper. All authors provided content and feedback on the manuscript. FR has primary responsibility for the final content of the manuscript.

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Figures

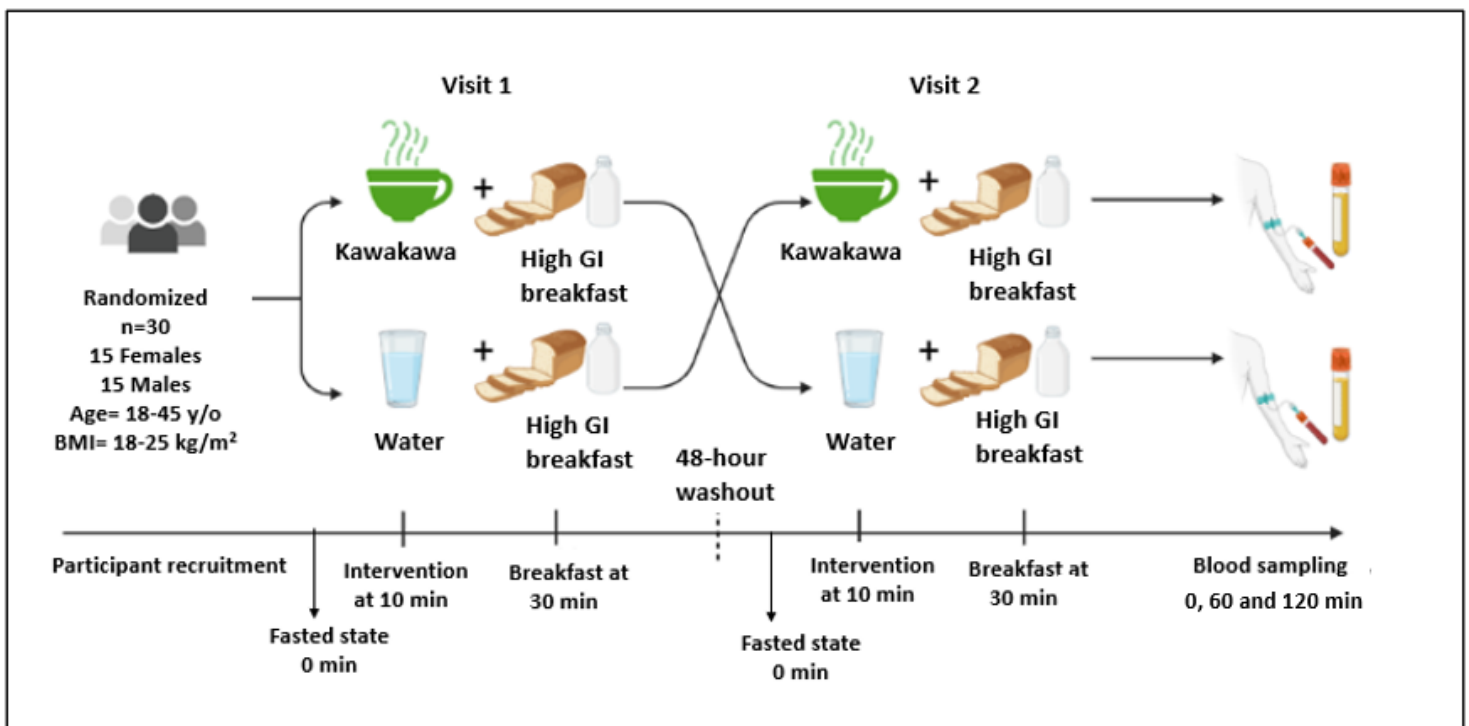


Figure 1

Overview of TOAST study design. n-number; y/o- year old; BMI- body mass index; min- minutes; GI- glycaemic index

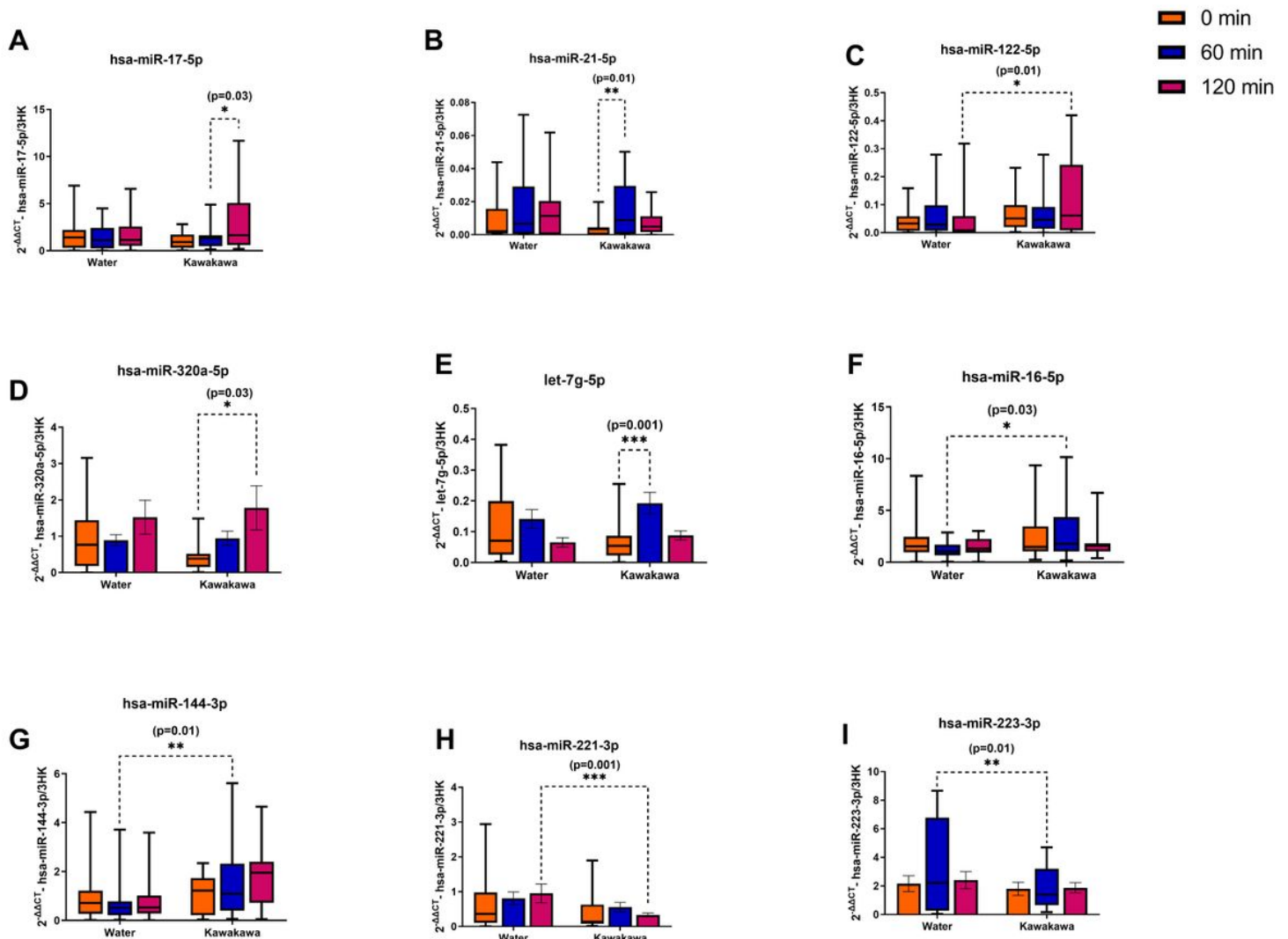


Figure 2

Figure 3.1: Postprandial abundance of circulatory miRNAs

*Different colours represent time points at fasting (0 min, Orange colour), at 60 min (Blue colour) and at 120 min (Red colour).; *indicates $p \leq 0.05$, ** indicate $p \leq 0.01$, *** indicate $p \leq 0.001$*

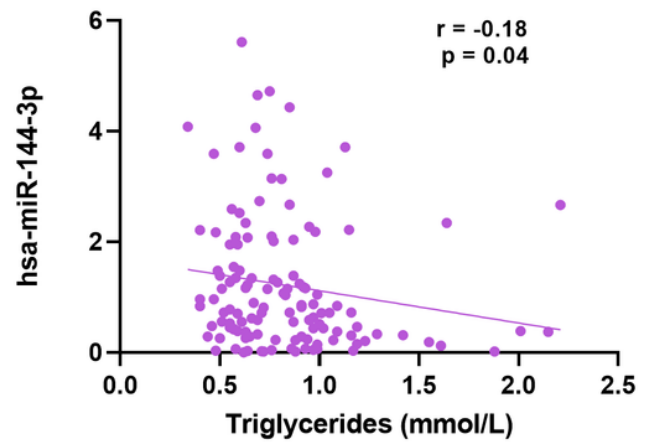
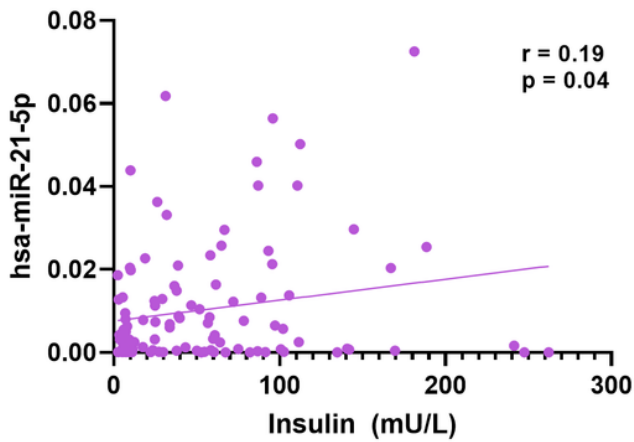
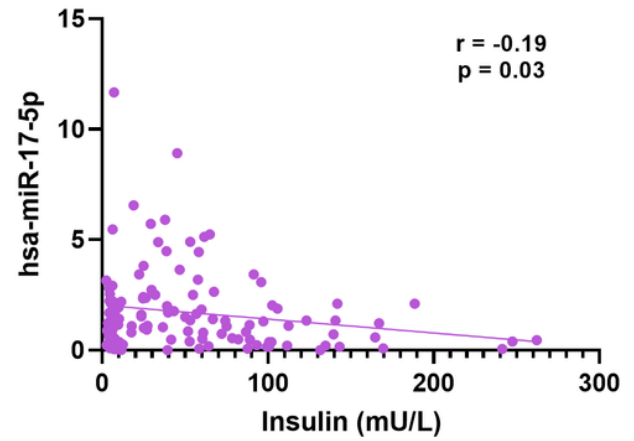
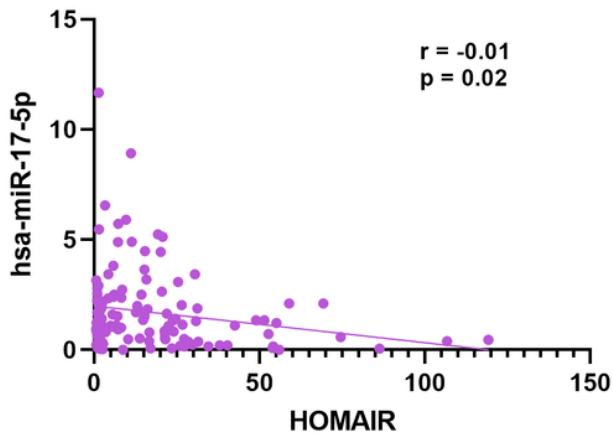


Figure 3

Figure 3.2: Pearson's correlation plots.

The biochemical parameters are plotted on the X-axis, and the relative microRNA expression ($2^{-\Delta\Delta C_t}$) is on the y-axis. r-correlation coefficient; p-significance value.

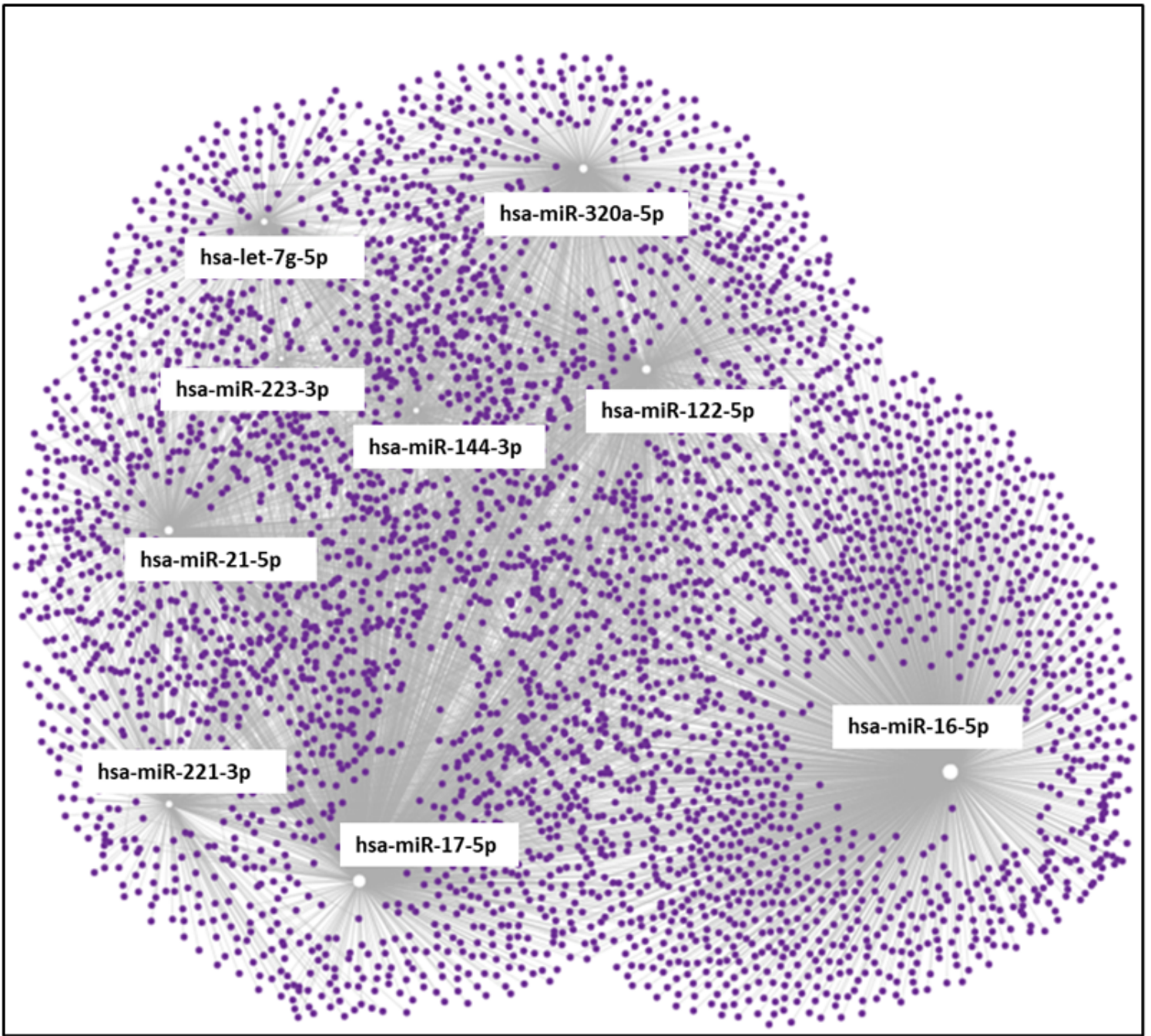


Figure 4

Figure 3.3: Functional enrichment analysis

Network visualisation of the significant circulatory miRNA (White boxes) and their respective gene targets (purple dots).

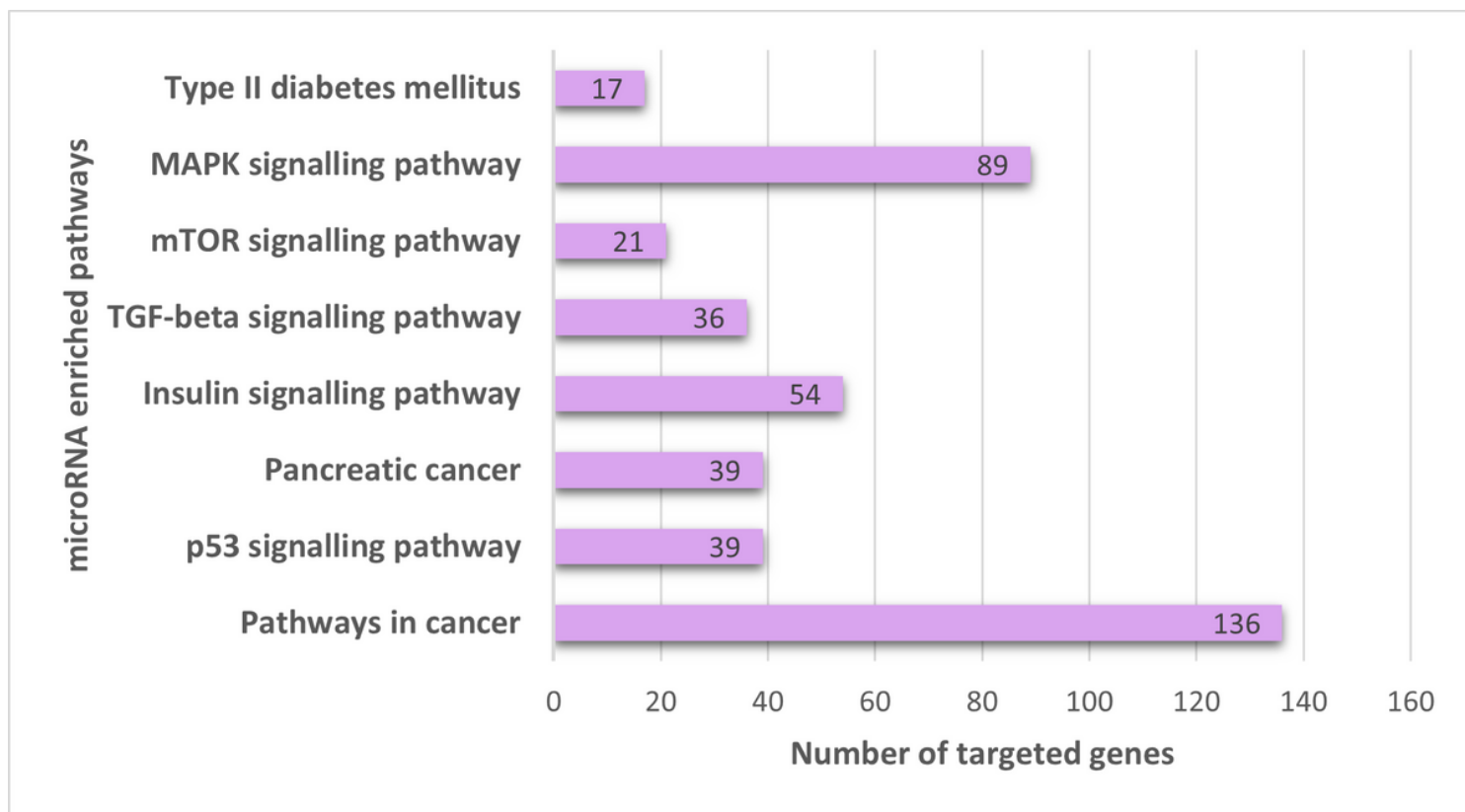


Figure 5

Figure 3.4.: miRNA-target gene network analysis.

KEGG pathway enrichment analysis of significant miRNA and their gene targets; numbers on bars indicate target gene numbers in the particular pathways.

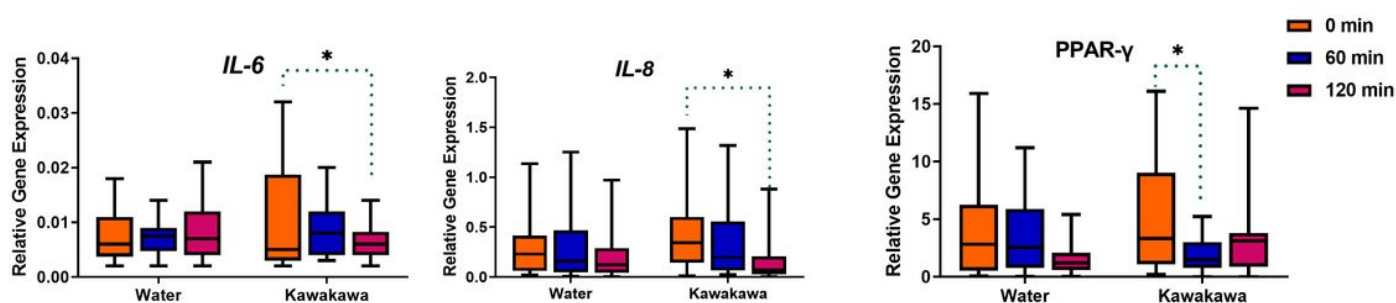


Figure 6

Figure 3.5: Relative gene expression of IL6, IL8 and PPAR-γ following water and kawakawa tea intake at baseline (pre), 60 min and 120 min.

*Different colours represent time points at fasting (0 min, Orange colour), at 60 min (Blue colour) and at 120 min (Red colour).; *indicates $p \leq 0.05$.*

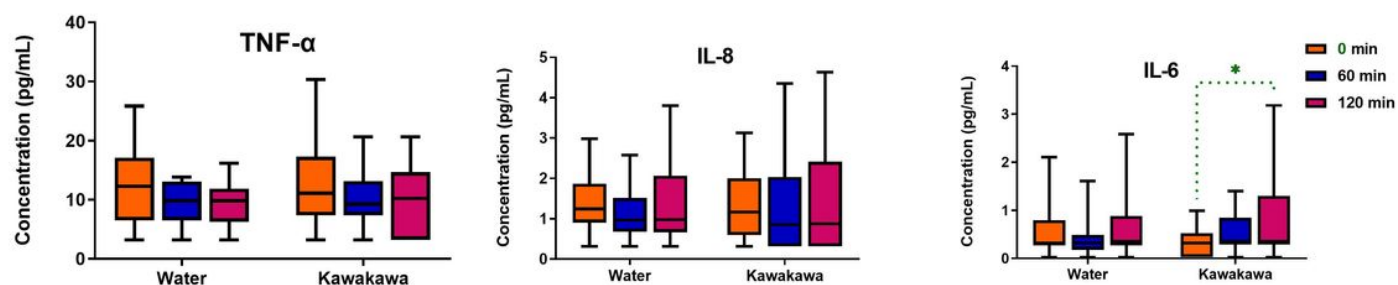


Figure 7

Figure 3.6: Comparison of inflammatory cytokines TNF- α , IL-8 and IL-6 following kawakawa tea intake and water at fasting (0 min), 60 min and 120 min.

*Different colours represent time points at fasting (0 min, Orange colour), at 60 min (Blue colour) and at 120 min (Red colour).; *indicates $p \leq 0.05$.*